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IN-VITRO REGENERATION, FLOWERING & GC-MS ANALYSIS IN CALLUS OF LINDERNIA MADAYIPARENSE - AN ENDEMIC PLANT TO MADAYIPARA, KERALA, INDIA

Mottukkan RAJILA, Arumugam ARUNPRASATH*

PG & Research Department of Botany, PSG College of Arts & Science, Coimbatore, Tamil Nadu, India

Abstract

In the current study, in-vitro micropropagation technique of the endemic plant Lindernia madayiparense which is an emergent aquatic or amphibious plant belongs to the family Linderniaceae newly reported from Madayipara, Kerala. The explant such as nodes and internodes subjected to different combination and concentration of plant growth hormones such 6-Benzylaminopurine (BAP), Indole-3-acetic acid (NAA), Indole-3-butyric acid (IBA), and Ggibberellic acid (GA3) in addition to MS medium. The brown friable callus initiated in 0.5mg/L NAA& BAP with the highest percentage (79.25±0.43) of callus induction was observed. From results, the nodal segments in MS medium along with 0.5mg BAP + 0.5mg NAA shows the maximum number of shoot induction (5.35±0.28) and also in-vitro flowering was observed and maintained in the same medium after 4 weeks of culture. The well developed shoot was transferred to the rooting medium (MS) with various concentration of NAA and IAA. The rooting medium with 1mg of NAA shows efficient root induction and then the well-rooted plantlets are subjected to acclimatized and established in the suitable field about 90 percentage of survival. The in-vitro callus from culture is subjected to GCMS analysis and 5 compounds are obtained with maximum peak values.

Keywords*: Endemic; Friable callus; GC-MS analysis; In-vitro propagation; Lindernia, 6-Benzylaminopurine.*

Introduction

The biodiversity ecosystem is responsible for a variety of plants and animals. *Lindernia madayiparense* is a newly reported endemic plant belongs to the family Linderniaceae from Madayipara, Kannur district, Kerala, India which has rich biodiversity ecosystem. During a recent floristic exploration in the Madayipara hillock area, the scientists collected an interesting specimen belonging to the Genus Lindernia in 2012 and named as *L. madayiparense* after the place where it collected [1]. *L. madayiparense* is an aquatic or amphibious annual herb located in pond sides. Flowering and fruiting occur in August-December. The plant shows distinct characteristics such as dimorphic leaves in water as well as in soil habitat. The stems are spongy and leaves are whorled in water whereas the stem is not spongy and leaves are dentate in soil habitat.

Plant tissue culture technology provides a wide range of applications in micropropagation, conservation, and improvement of the natural levels of important active constituents and to attain pharmaceutical industry demands and decrease the exploitation of harvesting of natural forest resources [2]. Now a day the usage of plant products are increased in medical and other fields. Hence the awareness in the study of a biochemical compounds

* Corresponding author: arunprasath@psgcas.ac.in

found in plants also increased. The GC-MS analysis is a method for qualitative and quantitative investigation of active components in the plant.

Materials and methods

Lindernia madayiparense was collected from Madayipara, Kannur district, Kerala. Healthy explants like nodal region was selected and sterilized by standard methods. Sterilized explants were inoculated on medium for micropropagation.

Culture medium, Initiation and acclimatization

The media used in this study were based on Murashige and Skoog (MS) basal medium [3] added with different concentration and the combination of growth regulators such as BAP, NAA, IBA, GA_3 to study their response on multiplication of shoot and flower production. In addition to BAP and NAA, IAA is also used to study the response to the production of the root. All the plant growth regulators are mixed with mg/L proportion. After this the initiation, acclimatization and transplantation of plantlets were carried out. The percentage of explant responding for multiple shoot induction, root initiation, number of shoot and roots produced, average number of inflorescence per explant were also recorded after 30 days of culture.

In acclimatization the well-developed plantlets were removed from the culture bottles and washed with tap water to remove trace of agar and dipped in fungicide for a few minutes. Then the plantlets were planted on to net pot contains a different type of potting media. After 20 days of step-wise hardening processes the pots were watered at two days interval under shade house condition. After 60 days, the frequency of survival was calculated.

GC-MS analysis

GC-MS analysis of the ethanol extract of in vitro callus of *L. madayiparense* was performed using Shimadzu Japan gas chromatography QP2010. The mass spectrum was so equipped with a computer fed mass spectra bank. German Hermlez233M-Z centrifuge used.

Results and discussion

Indirect organogenesis

The combination of BAP, GA3, IBA, and NAA induce brown friable callus from the explant of *L. madayiparense*. The initiation of callus cut ends after 10 days of culture. The highest percentage (79.25±0.43) of callus induction was observed in MS medium with BAP (0.5 mg), NAA (0.5mg) (Fig. 1A and B). The regeneration of shoot induction was observed after 10-20 (Table 1). The callus transmitted to the fresh MS medium containing BAP (0.5mg), NAA (0.5mg) during the subculture the highest number of shoot developed (4.86±0.74) and shoots length (3.5±0.42).

Table 1. Effect of MS medium and different concentrations of BAP, NAA, GA_3 and IBA on callogenesis and shoot formation from stem of *L. madayiparense*

S. no	Plant growth regulators (mg/L)			% of roots formed from shoot n subculture	Average no. of roots emerged from shoot	Average length of roots per shoot (cm)
	NAA	BAP	IAA			
1.	0.2	0.2		42.20±0.15	3.60±0.08	0.83±0.10
2.	0.5	0.2		36.05±0.60	1.80±0.32	0.51±0.01
3.	0.5	0.5		58.00±0.38	4.20±0.60	0.90±0.54
4.	1.0	0.5		89.35±0.38	7.80±0.43	1.70±0.22
5.			0.2	29.28±0.50	1.58±0.03	0.60±0.58
6.			0.5	57.60±0.49	2.32±0.57	0.80±0.22
7.			1.0	74.65±0.98	4.53±0.26	1.20±0.41

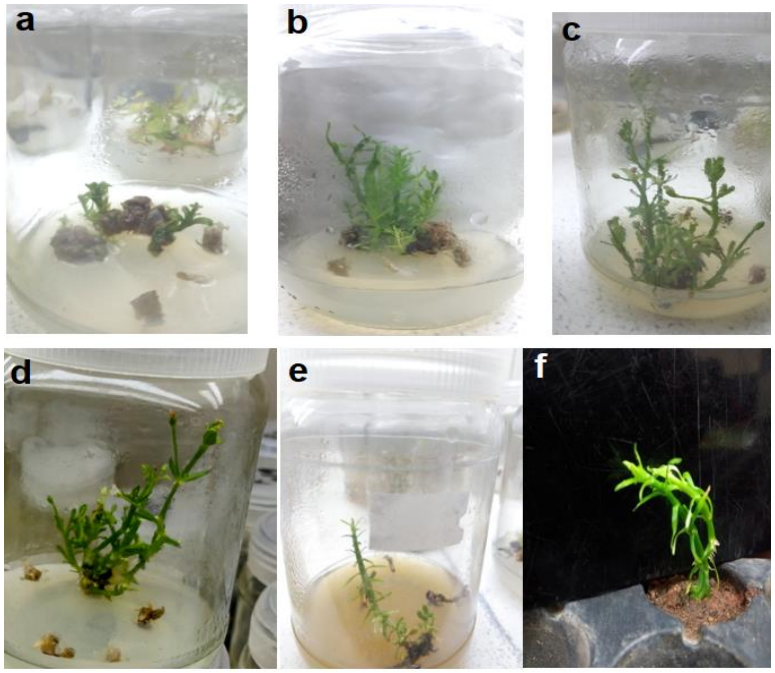


Figure 1. Initiation of callus in medium containing 0.5mg/ L NAA and BAP(A, B), *In-vitro* shooting and flowering in MS medium with 0.5mg/L BAP & NAA (C, D), *In-vitro* rooting in MS medium with 1mg/L NAA and 0.5mg/L BAP (E), Acclimatization of *in-vitro* rooted plant into hardening medium (F)

Direct organogenesis

The nodal explants were inoculated in MS medium with different PGRs. The initiation of shoots observed on fourth week of culture (Table 2).

Table 2. Effect of plant growth regulators added to MS medium on in-vitro regeneration and Flowering of *L. madayiparense*

S. no	Plant growth regulators (mg/L)				Days taken for initiation of shoot	Average number of shoots from explants	Mean shoot length (cm)	Average number of leafs per explant on culture	Days taken for flowering	% of flower response	Average no. of flowers
	IBA	GA_3	NAA	BAP							
1	0.5	0.5			29	1.8±0.44	1.5±0.26	6±0.58	_	_	_
2	1.5	1.5			23	2±0.32	4.8±0.11	9.25±0.74	_	_	_
3	2.5	2.5			20	2.8±1.85	5.44±1.25	10.03±0.65	_	_	_
4	3.5	3.5			16	4.98±1.20	7.50±0.77	15±1.42	24	34.07±0.30	1.23±0.21
5			0.5	0.5	20	5.35±0.28	12.32±0.20	15.5±0.42	27	79.03±0.30	3.07±0.15
6			1.5	1.5	25	2.50±0.11	4.26±0.74	9.20±1.7	_	_	_
7			2.5	2.5	23	2.01±0.06	5.36±0.38	6.85±0.08	_	_	_
8			3.5	3.5	18	1.48±0.55	3.25±1.2	7.25±1.3	_	_	_

The multiplication rate and shoot formation observed in high while using BAP and NAA. The maximum number of shoot per explant (5.35±0.28), shoot length (12.32±0.20cm) and number of leaves per explant (15.5±0.42) was observed on MS medium with BAP (0.5mg) and NAA (0.5mg) (Fig. 1CF) followed by the second highest response was observed in combination of GA3 (3.5mg), and NAA (3.5mg), with number of shoots per explant (4.98±1.20), shoot length (7.50±0.77cm) and number of leaves per explant (15±1.42) respectively.

In-vitro flowering

The flowering of the in-vitro cultured shoot was observed after fourth week of culture at same concentration of NAA 0.5µg and BAP 0.5µg. The induction of flowering takes 23-28

days after shooting (Table 2 and Fig. 1D). The average of three flowers per explant arises and the flowering percentage was highly observed as (79.03±0.30), with the average number of flower (3.07±0.15).

***In-vitro* rooting**

The raised shoots were transferred to the rooting medium. The shoots were treated with different combinations and concentration of MS medium +NAA (Table 3). The current work shown that the significant influence of the different concentration of NAA on root development in the shoot (Fig. 1E). The percentage of rooting was highest (89.35±0.38) in MS medium with NAA (1mg) with root numbers (7.8±0.43) and length (1.7±0.22cm) followed by second highest percentage (74.65±0.98) with root numbers (4.53±0.26) and root length (1.2±0.41cm) was recoeded in MS medium containing IAA (1mg)

Acclimatization

Well rooted plantlets are separated and changed to garden pots filled with autoclaved red soil, Vermicompost and sand (1:1:1) and covered with plastic bags to keep up high humidity (Table 4). Well grown plants were consequently transferred to large grow pots and maintained in green house. Finally the hardened plantlets were shifted to the field (Fig. 1F).

Table 3. Effect of plant growth regulators added to MS medium on in-vitro rooting of *L. madayiparense*

S. No	Plant growth regulators(mg/L)				Days taken for initiation of callus	% callus from stem on subculture	Average number of shoots from callus on subculture	Mean shoot length(cm)
	IBA	GA_3	NAA	BAP				
1.	0.5	0.5			-	-	-	-
2.	1.5	1.5			23	32.2±0.32	0.78±0.11	0.65±0.74
3.	2.5	2.5			20	39.6±1.85	0.94±1.25	0.33±0.5
4.	3.5	3.5			10	64.98±1.20	2.50±0.77	1.23±1.42
5.			0.5	0.5	12	79.25±0.43	4.86±0.74	3.5±0.42
6.			1.5	1.5	16	62.58±0.11	2.26±0.74	1.20±0.7
7.			2.5	2.5	22	55.1±0.26	1.36±0.38	0.85±0.08

Table 4. Effect of substrates on the acclimatization of plantlets regenerated multiple shoots of *L. madayiparense*

S. No	Planting substrates	No. of plants transferred	No. of plants survived	Survival (%)
1.	Vermicompost	10	6	74.06±0.72
2.	Red soil + Vermicompost	10	4	60.02±0.56
3.	Red soil + Sand + Decomposed coir waste	10	5	70.08±0.24
4.	Hardening media (decomposed coir waste: garden soil: vermiculture)	10	7	90.09±0.25

GCMS analysis

The callus extract of *L. madayiparense* (ethanol extract) was evaluated by GC-MS. The occurrence of active principles confirmed by comparing with standard mass spectra of NIST and Willey library. In the GC-MS study on *L. madayiparense* callus showed five compounds in ethanol extract. The active constituents with their retention time (RT), molecular formula (MF), molecular weight (MW), peak area(%), structure, medicinal uses in ethanol extract of *L. madayiparense* callus are presented in (Table 5 and Fig. 2) respectively. Chromatograms of ethanol extract are shown correspondingly.

The five compounds which are found in ethanolic extract such as Methyl 14-methylpentadecanoate (5.976), Methyl oleate (48.145), 18-Nonadecen-1-ol(32.446), 1-Octadecyne (9.111), Cis-9,10-Epoxyoctadecan-1-ol (4.322).

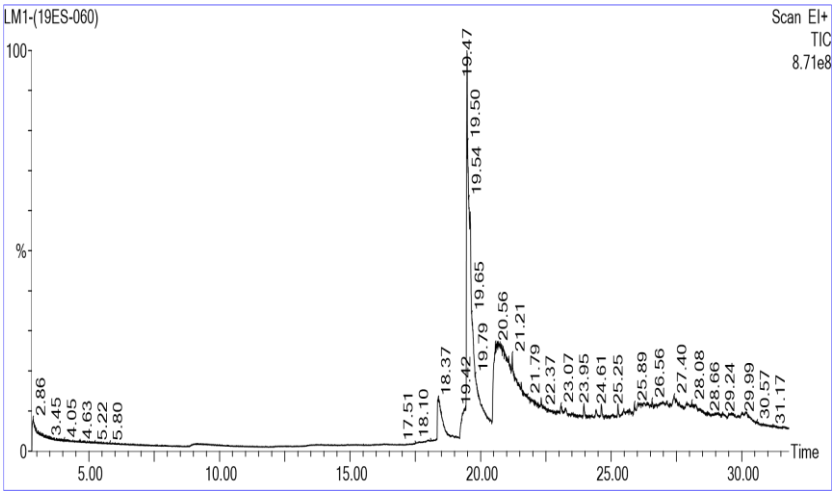
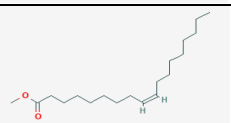
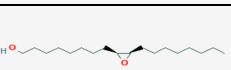


Figure 2. Graphical representation of GC-MS analysis

Table 5. GC-MS analysis on in-vitro callus of *Lindernia madayiparense*

	RT	Name of compound	Formula	Molecular Weight	Peak value	Structure	Medicinal uses
1	18.385	Methyl 14-methylpentadecanoate	$C_{17}H_{34}O_2$	270.457 g/mol	5.976		Cardiovascular system antiviral,dermatological problem [4]
2	19.470	Methyl oleate	$C_{19}H_{36}O_2$	296.495 g/mol	48.145		Antifungal and antioxidant activity Flavouring agent [4]
3	20.661	18-Nonadecen-1-ol	$C_{19}H_{38}O$	282.512 g/mol	32.446		Antiasthmatics, Bronchodilators, Drug for dermatological problem [4]
4	21.206	1-Octadecyne	$C_{18}H_{34}$	250.47 g/mol	9.111		Neurological problems , antiasthmetics ,antidepressant [4]
5	21.546	Cis-9,10-Epoxyoctadecan-1-ol	$C_{18}H_{36}O_2$	284.484 g/mol	4.322		Antimicrobial [5]

In present study, initiation of callus and the subsequent multiplication was observed. The brown friable callus was regenerated from the cut ends of the stem of *L. madayiparense* in MS medium with 0.5mg BAP & NAA. According to study of [6] MS medium mixed with 10.7 µM NAA and 2.2µM BAP showed 90 percent repeatability to provoke callus with 7th day callus induction, similarly, concentrations of NAA singly or in combination with BAP and Kn supported callus induction in *Strobilanthus flaccidifolius* [7]. At a concentration of 6µM BA, ~75% of explants responded positively [8]. *N. Ahmad, et al* [9] also reported the multiple shoot initiation from the surface of callus in *Ruta graveolens* when MS nutrients added with BAP, KN, IAA, IBA and NAA in various concentrations and combinations. Similarly, in the combination of MS medium fortified with 5.0mg BAP and 0.5 mg NAA showed 60% of shoot induction from callus of internode than petiole and leaf callus in *Orthosiphon stamineus* [10].

And also the in vitro shoot regeneration percentage of *Lindernia madayiparense* were determined by culturing nodal parts on full potency MS medium mixed with BAP and NAA with a concentration of 0.5mg showed the largest percentage of shoot regeneration. BAP was

reported to be the most suitable medium in the culture establishment in *Myriophyllum* sp. [10] and *Spilanthes acmella*[11]. *A.B. Haw and C.L. Keng* [12] reported that out of two cytokinins, BAP was found to be more suitable than Kn. As BAP resulted in a quicker and better response than the latter while, the addition of NAA (0.2mg) proved synergistic [12]. In *Ceropegia bulbosa*, from Asclepiadaceae nodal explants show the best response in the combination of BAP and NAA [13]. Similarly, the same combination was reported in *Hydrocotyl conferta* [14] and *Adhathoda vasica* [15].

In the same concentration of BAP and NAA in-vitro flowering was recorded after 4 weeks of inoculation. Three flowers from explant are observed in the culture. Arifullah Mohammed [16] reported that in *Andrographis lineata* (Nees) flower initiation and fruit development occur in medium mixed with BAP and NAA. Flower development occurs after 2 months of culture. The elongated shoot was placed in full MS medium supplemented with various concentration of NAA for root induction. Of the different concentration of NAA inducing root a concentration of NAA (1mg/L) support maximum root initiation.

In the present study, 5 compounds have been reported in ethanolic extract of *in-vitro* callus of *L. madayiparense* by GCMS analysis. The compound present in callus are Methyl oleate (48.145), 18-Nonadecen-1-ol (32.446), 1-Octadecyne (9.111), Methyl 14methylpentadecanoate (5.976), Cis-9,10-Epoxyoctadecan-1-ol (4.322). Methyl oleate shows antifungal and antioxidant activity [17] and also act as a flavouring agent. 18-Nonadecen-1-ol act as antiasthmatics and bronchodilators and also used as a drug in dermatological problems. 1-Octadecyne treats the neurological problem and act as an antidepressant. Methyl 14methylpentadecanoate is used as a drug to cure cardiovascular as well as dermatological problems. Cis-9, 10-Epoxyoctadecan-1-ol has antimicrobial activity [5]. Similarly in the study conducted by *Cheong et al*, [18] reviewed that the GC-MS analysis on methanol callus of *Strobilanthes crispus* extract revealed the presence 12 main compounds, with the presence of beta-humulene (26.22) at the most, 4,22 - Stigmastadiene-3-one (9.08%), Stigmast-4-en-3-one(5.56%), stigmasterol (1.86%) and others. *B.E. Cheong et al*, [19] studied that the major compounds revealed in the callus extracts of *Biophytum sensitivum.*

Conclusion

In-vitro propagation methods help in mass multiplication of plant species, which have restrictions of conventional propagation. The present study has provided an efficient methodology for plant micropropagation through nodal culture of the endemic plant *L. madayiparense.* The spectrum analysis provides the information of compound in the plant which helpful for further studies in future.

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