

## MICROPROPAGATION OF AN ENDANGERED AND ENDEMIC MEDICINAL PLANT *CAYRATIA PEDATA* VAR. *GLABRA*

SHARMILA S\*, RAMYA EK, MOWNIKA S, DHIVYA SM

Department of Botany, Vellalar College for Women (Autonomous), Erode, Tamil Nadu, India. Email: drsharmilas@yahoo.com

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### ABSTRACT

**Objective:** The objective of the present study was to develop standardization protocol for the successful *in vitro* mass propagation of *Cayratia pedata* var. *glabra* through leaf and stem explants, since it is a rare, endangered, and endemic medicinal plant using biotechnological involvements and to conserve this endangered species.

**Methods:** The application of biotechnological principles for the establishment of micropropagation under *in vitro* conditions has been studied by following the methods. The explants, namely, leaf and stem harvested from *in vivo* plants were thoroughly washed and properly sterilized with sterilizants. The explants were transferred to Murashige and Skoog (MS) medium supplemented with growth regulators 6-benzyladenine (BAP) and naphthalene acetic acid (NAA) in the concentration range of 0.5–3.0 mg/l which were tested for callus induction and morphogenesis. The elongated shoots were transferred to MS medium supplemented with NAA at different concentrations for root induction.

**Results:** The explants collected from the field (shola) were treated in different sterilizants with various concentrations at different time for sterilization. Among the various combinations tried, the Teepol treatment for 10 min followed by bavistin 20 min, antibiotics, namely, ampicillin and rifampicin for 20 min, 70% alcohol for 30 s, and 0.12 % HgCl<sub>2</sub> for 3 min was found to be effective. The explants were cultured in MS medium supplemented with various concentrations of BAP and NAA. The results noted that an increase in the concentration of BAP concomitantly reduced the frequency of callus formation. The maximum callusing frequency and more number of shoot formation was observed in the lower concentration of BAP (0.5 mg/l) in combination with NAA (0.2 mg/l). The callus obtained from all the above combinations was sub-cultured on MS medium with same combinations of BAP and NAA. The maximum frequency of root formation in leaf callus was 85% and 75% in stem callus and both were achieved on MS medium with NAA (1 mg/l) after 2 weeks.

**Conclusions:** The current investigation provides a competent *in vitro* propagation method for *C. pedata* var. *glabra* which could be commercialized for developing identical plants with high-quality mass multiplication rate and for better conservation of the germplasm. Both the methods described here are well suited for the mass multiplication of this critically endangered and endemic climber species.

**Keywords:** *Cayratia pedata*, Murashige and Skoog, Micropropagation, Callus, Endangered.

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### INTRODUCTION

In recent days, *in vitro* plant regeneration techniques or biotechnological applications have been used as an alternative technique and are remarkably benevolent in conserving rare [1,2], endangered, threatened [3,4], and endemic medicinal plants with high prudent significance [5]. According to Engelmann [6], a typical culture environment can be effectively utilized for short to medium term *in vitro* conservation of plant germplasm, through increasing intervals between subcultures in slow growing species. The documentation of ethnopharmacobotanical information of herbaceous plants in Thiashola (Thiashola in Tamil), Manjoor, Nilgiris South Division, Western Ghats, was undertaken with proper permission from the Principal Chief Conservator of Forests, Chennai, and the District Forest Officer, Ooty under Section 28 (j) of Wildlife Protection Act, 1972. Based on the conducted investigation, the species *Cayratia pedata* (Lam.) Gagnep. var. *glabra* Gamble was selected for the plant regeneration biotechnological research works, since this species is listed in red data book as per the IUCN red list categories, which has a wider use for different ailments among the tribes of Thiashola due to its enriched medicinal value [7-9]. *C. pedata* var. *glabra* belongs to the family Vitaceae is a weak climber, commonly known as “Kattu Pirandai” is one such endemic and endangered species in Thaisolai, Nilgiris South Division, Western Ghats, and scrambling over the hedges and trees. It is used as medicine in Siddha, Ayurveda, and Folk medicine for treating diarrhea, refrigerant, hysteria, astringent, and ulcers. A decoction is administered in uterine and other flukes.

Tissue culture technique is now oriented toward the commercial concept of large scale production of medicinally active bioactive compounds in important medicinal plants. The potentiality of the plant cell can be enhanced for the production of useful secondary metabolites by applying various *in vitro* techniques. It is imperative that, viable strategies have to be taken to conserve the surviving population; at least, the critically important medicinal species from further loss [10,11]. Since the species *C. pedata* var. *glabra* is rare and endangered due to ecological anthropological pressure, it is need to be conserved. As there is no strong micropropagation studies recorded on this medicinally potent climber, the present work was undertaken to lay down *in vitro* micropropagation as a conservation strategy to produce large scale plantlets within a short time without any genetic variation.

The findings of the current study can be useful to progress and surge further scientific investigation on the aerial parts of this species to compare the different sterilizants, phytohormones, explants, and populations for micro propagation and *in vitro* conservation of selected species, and to determine the optimal method for the production of a greater number of plants. This protocol will serve in the future as a base to establish an *in vitro* genetic bank for germplasm preservation, to improve the bioactive value of these species and to isolate the genes responsible for drought resistance.

**METHODS**

**Source of plant material**

Aerial plant of *C. pedata* var. *glabra* (Map 1 and 2) (Figs. 1 and 2) was collected from Thiashola, Manjoor, Nilgiris South Division, Western Ghats before that we got proper permission from the Principal Chief Conservator of Forests, Chennai, and the District Forest Officer, Ooty, under Section 28 (i) of Wildlife Protection Act, 1972, in the month of October and the voucher herbarium specimen was processed followed by standard methods [12]. The collected plants were identified with the help of the existing Floras [13-15] and compared with type specimens available in the herbarium of Botanical Survey of India, Southern Circle, TNAU Campus, Coimbatore (No. BSI/SRC/5/23/2010-2011/Tech. 1300), Tamil Nadu. All the preserved specimens were deposited for future reference at the Department of Botany, Vellalar College for Women (Autonomous), Erode, Tamil Nadu, India, with their accession number (VCW/BH/Acc. No.). The explants were collected from 2 to 3 years old plants from Thiashola, Manjoor, Nilgiris South Division, Western Ghats.

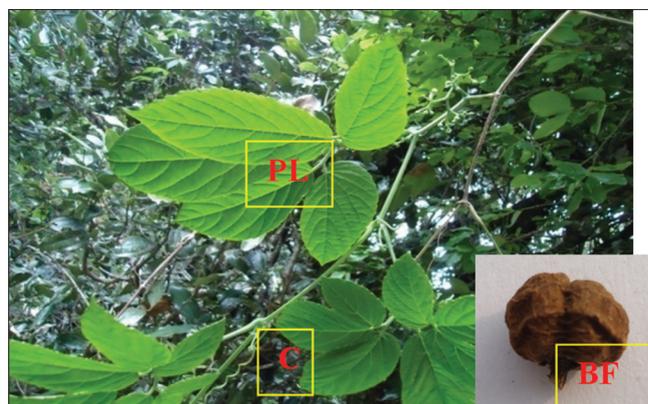
**Explants selection and mode of sterilization**

The explants, namely, leaf and stem were harvested from *in vivo* healthy plants, then thoroughly washed with running water and kept under running water for 20 min. After that, the explants were washed thoroughly with Teepol solution for 5-10 min and treated with 10% (w/v) of bavistin-methyl-3-benzimidazole carbamate solution and ampicillin and rifampicin for 15-20 min. Finally, the explants were subsequently surface sterilized with 70% alcohol for 30-60 s and 0.1% (w/v) mercuric chloride solution for 3-7 min. and washed for

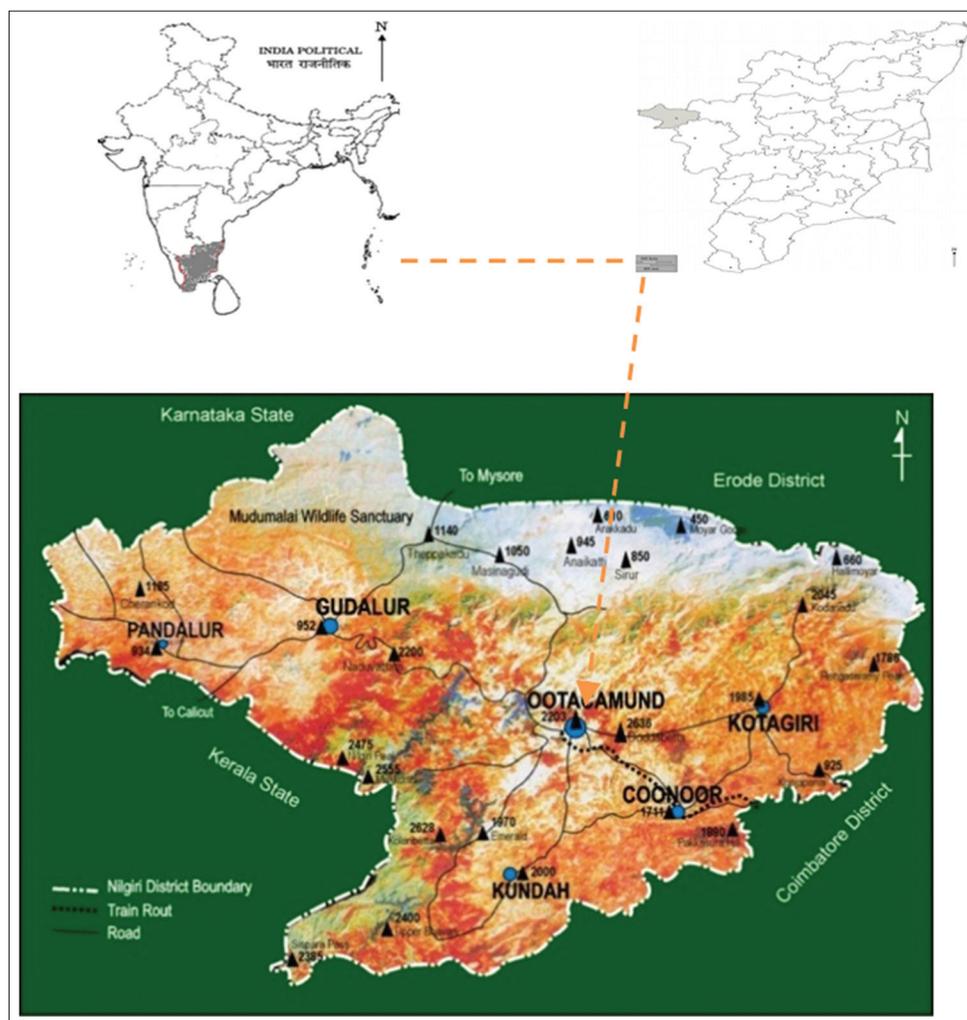
3-4 times in sterile distilled water. The surface sterilized explants were trimmed gently with the help of sterile surgical blade (Lister No: 10) and aseptically inoculated onto pre-cooled autoclaved medium.

**Culture media employed and their composition**

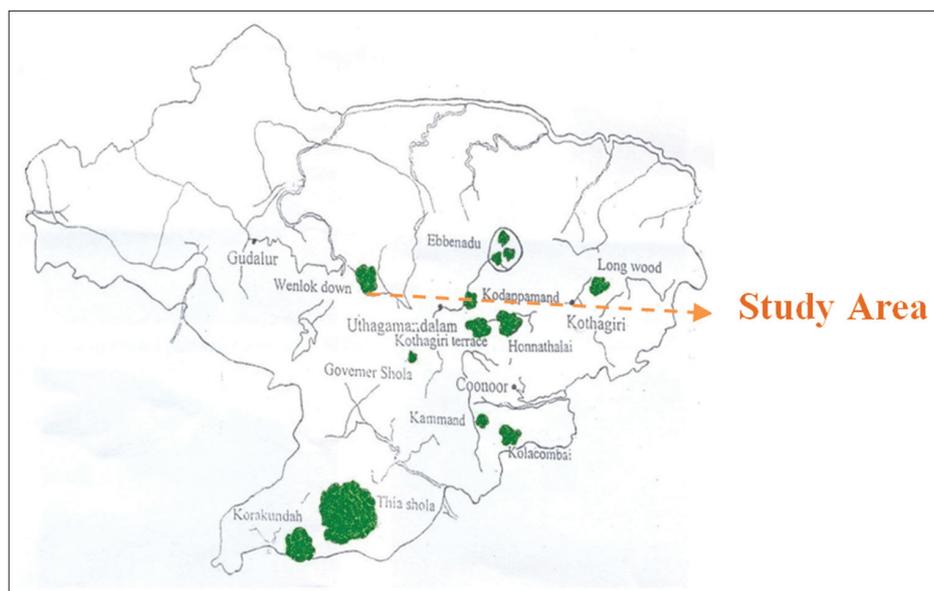
Murashige and Skoog (MS) [16] basal medium full strength was employed in the present study. The composition of the medium is given below:



**Fig. 1: The flowering twig and enlarged fruiting twig of the ecologically weaker, economically important species *Cayratia pedata* var. *glabra*. PL: Pedate leaf; C: Climber; BF: Bilobed fruit**



**Map 1: The Nilgiri district satellite map showing important places with altitude in meter**



Map 2: Location of the study shola



Fig. 2: Origin point of the study shola

#### Preparation of the medium

For the preparation of medium analytical reagents of "HiMedia" grade chemicals, "Borosil" glasswares and double distilled water were used. The nutrient medium basically consists of inorganic nutrients, carbon source, vitamins, iron source, amino acids, and natural supplements (optional). The chemicals were weighed accurately in electronic weighing balance (Shimadzu AY 220). All the stock solutions were prepared and stored in well-stoppered sterilized bottles and preserved in a refrigerator at 4°C. Specific quantity of the stock solutions of the chemicals and growth regulators were pipetted onto a 1 l beaker. Required sucrose and other organic supplements and complex additives (optional) were added. The final volume was made up with distilled water and the pH was adjusted to 5.8–5.9 with either 0.1 N NaOH or 0.1 N HCl using a pH meter (Systronics).

To the above said media 0.8% (w/v) agar (extra pure gelling point 32–35°C, HiMedia, Bombay) or Phytigel (HiMedia, Bombay) was added, melted in water bath and the medium was dispensed into 150 ml bottles (30 ml of medium). The bottles after covering with a polyethylene cap were autoclaved at 1.06 kg pressure/sq cm for about 20 min at 121°C. The autoclaved medium in the culture bottles was cooled and allowed to solidify and it was stored in dark for future use. The inoculations were done after 4 days to ensure that the bottles were free from contamination.

#### Growth regulators, growth adjuvant, and their preparation

Two important groups of growth regulators such as auxins and cytokinins were used in the experiments. All the growth regulators are stored at 4°C until use.

#### Auxins and their preparation

Auxin, namely,  $\alpha$ -naphthalene acetic acid (NAA) was used in these experiments. The stock solution was prepared by dissolving 10 mg of auxin individually in 1 ml of ethanol. Then, the volume was made up to 100 ml with sterile distilled water. The required volume of auxins was added to the nutrient media, before autoclaving and was used as different concentrations preparations.

#### Cytokinins and their preparation

The stock solution was prepared by dissolving 10 mg of 6-benzyladenine (BAP) in 1 ml of 0.1 N hydrochloric acid (HCl) and the volume was made up to 100 ml by adding sterile distilled water. The required volume of cytokinin was added to the nutrient media, before autoclaving and was used in different concentrations.

#### Culture conditions

All the cultures were maintained in the culture room at a temperature of 25±SS2°C and relative humidity of 65–70%. The cultures were kept under white light at intensity of 3000 Lux provided from white fluorescent lamps (Philips, India) with 14 h photoperiodic duration.

#### In vitro studies

The present investigations on *in vitro* propagation of *C. pedata* var. *glabra* were carried out in the tissue culture laboratory of the Department of Botany, Vellalar College for Women, Erode, India. *In vitro* techniques such as micropropagation, callus induction and regeneration were attempted.

#### Callus induction

The explants, namely, leaf and stem collected from Thiashola were used as primary explants. Explants measuring about 0.4–0.6 cm length were cultured on MS medium supplemented with growth regulators BAP and NAA. Twenty-five explants were used for each culture. Each experiment was repeated twice. MS medium enriched with BAP and NAA in the concentration range of 0.5–3.0 mg/l was tested for callus induction and morphogenesis. Sub-culturing was carried out at the regular interval of 20–30 days. The *in vitro* developed microshoots were cut into segments (0.4–0.6 cm in length) each. These were sub-cultured on MS medium containing various growth regulators, namely, BAP and NAA in different combinations. The percentage of callus induction of 6-week-old cultures was calculated. Calli were sub-cultured regularly at an interval of 3 weeks. MS medium enriched with BAP in the concentration of 0.5–3.0 mg/l was tested for regeneration.

### Callus frequency

The percentage of callusing was recorded at the end of 5<sup>th</sup> week. The frequency of callus induction was calculated as shown below and was represented as percentage:

$$\text{Frequency of response (\%)} = \frac{\text{Number of explants responded}}{\text{Total number of explants cultured}} \times 100$$

### Rooting of in vitro multiplied shoots

The elongated shoots were transferred to MS medium supplemented with NAA at different concentrations for root induction.

### Statistical analysis

For *in vitro* micropropagation studies, the values are expressed as Duncan's multiple range test.

## RESULTS AND DISCUSSION

### Explants sterilization

The explants collected from the field (shola) were treated in different sterilants with various concentrations at different time for sterilization

(Table 1). Among the various combinations tried, the Teepol treatment for 10 min followed by Bavistin 20 min, antibiotics, namely, ampicillin and rifampicin for 20 min, 70% alcohol for 30 s, and 0.12% HgCl<sub>2</sub> for 3 min was found to be effective (Table 1) by having no dried explants and no contamination. The other treatment in Teepol for 10 min followed by Tween 20 for 10 min, Domestos for 10 min, Bavistin for 20 min, antibiotics for 20 min, 70% alcohol for 30 s, and 0.12% of HgCl<sub>2</sub> for 3 min was however occupied next position to the previous treatment in terms of the degree of survivability of explants and contamination level (Table 1). However, here the response was 90% and the dried buds were around 10%. Drying of explants is due to higher concentration as well as too many sterilants in the particular combinations. The explants responded along with surface contamination heavily to the 0.1% concentration, which proved that the concentration and the exposure timing were not adequate to eliminate the surface contaminants of the explants. This was in corroboration with the work of Thrope and Patel [17]. The importance of the choice of explants, which served as the inoculum for axenic culture, is well documented in the classical compilation of George and Sherrington [18].

### Callusing and multiple shoot induction

Explants such as leaf and stem were employed for the induction of callus. The explants were cultured in MS medium supplemented with

**Table 1: Effect of different sterilization treatments in initiation (*in vivo* explants of *Cayratia pedata* var. *glabra*)**

S. no.	Mercuric chloride		Teepol (min)	Tween 20 (min)	Domestos (min)	Bavistin (min)	Ampicillin and rifampicin antibiotics (min)	70% alcohol (s)	No. of explants	No. of explants responded	No. of explants dried	No. of explants contaminated
	%	Time (min)										
1.	0.12	3	10	-	-	20	20	30	20	20	-	-
		5	20	-	-	30	30	45	20	10	10	-
		7	30	-	-	40	40	60	20	5	15	-
2.	0.12	3	10	10	-	20	20	30	20	15	5	-
		5	20	20	-	30	30	45	20	7	13	-
		7	30	30	-	40	40	60	20	5	15	-
3.	0.12	3	10	10	10	20	20	30	20	18	2	-
		5	20	20	20	30	30	45	20	9	11	-
		7	30	30	30	40	40	60	20	5	15	-
4.	0.1	3	10	-	-	20	20	30	20	6	2	12
		5	20	-	-	30	30	45	20	4	9	7
		7	30	-	-	40	40	60	20	3	14	3
5.	0.1	3	10	10	-	20	20	30	20	7	2	11
		5	20	20	-	30	30	45	20	6	11	3
		7	30	30	-	40	40	60	20	5	13	2
6.	0.1	3	10	10	10	20	20	30	20	9	2	9
		5	20	20	20	30	30	45	20	5	9	6
		7	30	30	30	40	40	60	20	4	12	4

**Table 2: Effect of MS medium and growth regulators on callus induction and shoot formation (*in vivo* explants of *Cayratia pedata* var. *glabra*)**

S. no.	MS medium+growth regulators mg/l		Leaf		Stem	
	BAP	NAA	% of leaf producing callus <sup>#</sup>	% of callus forming shoots <sup>#</sup>	% of stem producing callus <sup>#</sup>	% of callus forming shoots <sup>#</sup>
1.	0.5	0.1	79.5 <sup>d</sup>	20.0 <sup>d</sup>	80.0 <sup>c</sup>	22.0 <sup>d</sup>
2.	1.0	0.1	90.0 <sup>b</sup>	15.0 <sup>e</sup>	85.0 <sup>b</sup>	15.0 <sup>e</sup>
3.	1.5	0.1	90.5 <sup>b</sup>	10.0 <sup>f</sup>	87.5 <sup>a</sup>	9.0 <sup>f</sup>
4.	2.0	0.1	72.5 <sup>e</sup>	2.0 <sup>e</sup>	65.5 <sup>e</sup>	2.0 <sup>g</sup>
5.	2.5	0.1	30.5 <sup>j</sup>	-	20.0 <sup>j</sup>	-
6.	3.0	0.1	25.5 <sup>k</sup>	-	18.5 <sup>k</sup>	-
7.	0.5	0.2	95.0 <sup>a</sup>	90.0 <sup>a</sup>	80.0 <sup>c</sup>	75.0 <sup>a</sup>
8.	1.0	0.2	85.5 <sup>c</sup>	60.0 <sup>b</sup>	70.0 <sup>d</sup>	55.0 <sup>b</sup>
9.	1.5	0.2	63.5 <sup>f</sup>	25.0 <sup>c</sup>	60.5 <sup>f</sup>	25.0 <sup>c</sup>
10.	2.0	0.2	60.5 <sup>g</sup>	5.0 <sup>g</sup>	58.5 <sup>g</sup>	3.0 <sup>g</sup>
11.	2.5	0.2	45.0 <sup>h</sup>	-	40.5 <sup>h</sup>	-
12.	3.0	0.2	40.0 <sup>i</sup>	-	35.0 <sup>i</sup>	-

<sup>#</sup>Values are means of three replicates. Means followed by common superscript letter(s) are not significant at p<0.05 level. MS: Murashige and Skoog, NAA: Naphthalene acetic acid, BAP: 6-benzyladenine

Table 3: Effect of the growth regulator on rooting of leaf and stem callus

S. no.	MS medium+Growth regulator (NAA mg/l)	% of rooting	
		Leaf callus #	Stem callus #
1.	1.0	85 <sup>a</sup>	75 <sup>a</sup>
2.	1.5	65 <sup>b</sup>	60 <sup>b</sup>
3.	2.0	50 <sup>c</sup>	50 <sup>c</sup>
4.	2.5	45 <sup>d</sup>	45 <sup>d</sup>
5.	3.0	30 <sup>e</sup>	30 <sup>e</sup>
6.	3.5	25 <sup>f</sup>	25 <sup>f</sup>

# Values are means of three replicates. Means followed by common superscript letter(s) are not significant at  $p < 0.05$  level. MS: Murashige and skoog, NAA: Naphthalene acetic acid

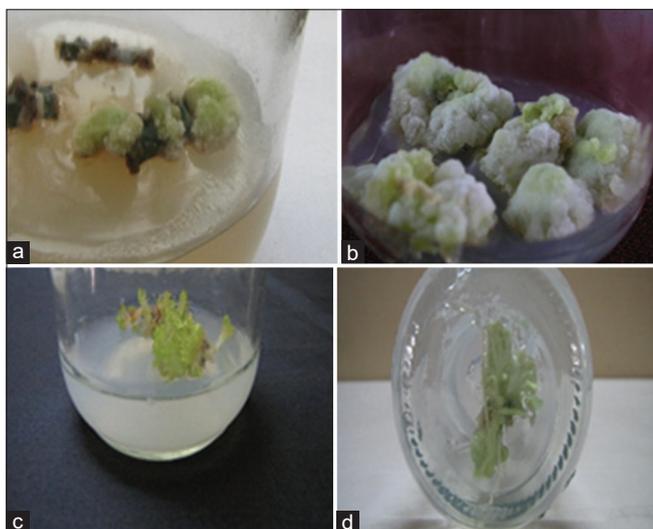


Fig. 3: *In vitro* culture of *Cayratia pedata* var. *glabra* through leaf explants. a - Initiation of callus from leaf explants; b - well developed callus from leaf explants; c - induction of shoots from the leaf derived callus; d - rhizogenesis from leaf derived callus

various concentrations of BAP and NAA (Table 2). It has been noted that an increase in the concentration of BAP concomitantly reduced the frequency of callus formation. The morphological features of callus developed from leaf and stem explants were varied widely in the present study. The callus induced from the leaves was friable, soft, and yellowish in color and grow faster than the other explant (Fig. 3). Paulsamy [19] reported the callus formation in leaf disks for a shola species *Berberis tinctoria*. In *Berberis trifoliata*, 0.5 mg/l BAP and NAA actively initiate the callus formation [20].

The maximum callusing frequency and more number of shoot formation were observed in the lower concentration of BAP (0.5 mg/l) in combination with NAA (0.2 mg/l). The callus obtained from all the above combinations were sub-cultured on MS medium with same combinations of BAP and NAA. Chalupa [21] stated out that the cytokinin, benzylaminopurine (BAP) is the hormone induced the morphogenesis effectively in dicots. However, Ranganatha et al. [22] explained direct regeneration of shoots obtained in 3.0 mg/l 2iP alone and in combination with 0.1 mg/l IAA and/or 1.0 mg/l BAP with average shoot length being  $6.5 \pm 0.17$ – $8.0 \pm 0.92$  cm and percentage response was between 68% and 75%. The regenerated axillary shoots were excised and transferred to MS medium with different concentrations of NAA (Table 3). The maximum frequency of root formation in leaf callus was 85% and 75% in stem callus and both were achieved on MS medium with NAA (1 mg/l) after 2 weeks.

## CONCLUSIONS

The success of micropropagation and *in vitro* conservation of *C. pedata* var. *glabra* plant depends on the optimal selection of the explants

and on the efficiency of the sterilization method. The present study concluded that the effective *in vitro* regeneration of the study plant on the basal medium is standardized with the hormone concentration 0.5 mg/l of BAP and 0.2 mg/l NAA for shoot formation and 0.5 mg/l NAA for root initiation. The massive production of plantlets through this method will be helpful to meet the demand of this bioresource at one side. The most important step for the establishment of an *in vitro* culture protocol for these species is the initiation of aseptic proliferative cultures and to choose the optimal nutrient media. After this step, the multiplied material could be maintained *in vitro*, cryopreserved or used in restoration programs. This high regeneration system would provide an effective strategy for the conservation and proliferation of these medicinal plant species on large scale for commercial purpose.

## AUTHORS' CONTRIBUTION

S. Sharmila, working as an Assistant Professor in the Department of Botany, Vellalar College for Women (Autonomous), Tamil Nadu, designed and performed the experiments. E.K. Ramya, Ph.D. Research Scholar in Botany carried out the statistical analysis and review process. S. Mownika, Ph.D. Research Scholar in Botany performed to separate the tables, figures in category. All authors read and approved the final manuscript.

## CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest concerning this article.

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