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Microtuberization of *Ceropegia pusilla* Wight and Arn. an Endangered Medicinal Plant

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Authors' contributions

All the authors have cordially supported to the work and preparation of manuscript. Authors KK and RP have designed the entire study and protocols with interpretations of the results and prepared the first draft of the manuscript. Author SJ managed the analyses of the study and computational work respectively. Author SP guided in the entire research and documented the final draft of the manuscript. All the authors have read and approved the final manuscript.

Research Article

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ABSTRACT

An efficient micropropagation (direct & indirect) and microtuberization protocol for an endemic and ethanomedicinal plant *Ceropegia pusilla* is described. The highest number of shoot initiation (3.50 ±0.34) and higher average shoot number in shoot subculture (15.40 ± 1.65) were recorded on MS medium supplemented with BAP+NAA (2.5+0.5 mg/l). The best performance of callus induction and morphogenesis was found on MS medium supplemented with BAP and NAA from stem. The callus initiation was recorded on MS medium supplemented with BAP+NAA (0.5 + 1.0 mg/l) and the higher percentage of callus forming shoots from callus subculture is determinate to be 90-95 ± 7.93. MS medium supplemented with various concentrations of cytokinins and auxins supported the induction of microtubers, *in vitro* flowering and roots. The microtubers and root formation occurred on the basal region of the shoots in MS medium containing BAP (1.5mg/l) and NAA (0.5mg/l). The higher average number of root length was (2.8 ± 0.68cm) also observed in the same concentration. The plantlets regenerated *in vitro* with well-developed shoots, microtubers and roots were successfully established in pots containing

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hardening media and grown in a shade house with 81± 3.16% survival rate.

Keywords: Microtubers; Ceropegia pusilla; basal tubers; medicinal plants.

ABBREVIATIONS

BAP 6- benzylaminopurine; 2,4-D 2,4-Dichlorophenoxyacetic acid; NAA a-Naphthaleneacetic acid; TDZ – Thidiazuron; MS - Murashige and Skoog.

1. INTRODUCTION

Ceropegia, is an old world tropical genus, includes about 200 species and distributed in tropical and sub-tropical Asia, Africa, Australia, Malaysia, and in the canary of Pacific Islands[1] In India, 50 species are present [2] out of which 28 are endemic to peninsular India [3] and among them *Ceropegia pusilla* Wt. and Arn. is an endemic and endangered plant, grows widely in hilly tracts of South India [4]. The tubers are edible and contain an alkaloid called ceropegin [5] and are used in Ayurvedic drug preparation that are active against many diseases especially diarrhea, dysentery and syphilis. The root tuber also contain starch and used as a nutritive tonic and blood purifier [6-7]. This plant is also used as an antidote for snake bite [8].

The vegetative propagation through stem cuttings of Indian *Ceropegia* species is not possible as they not have semi-hard wood [9]. However increasing habitat disturbances, over exploitation and slow reproduction and need of appropriate pollinator, limit population of this species [10]. The development of an efficient method for rapid clonal propagation is important to meet the pharmaceutical needs and for conservation of this valuable rare medicinal plant. The microtuberization studies in *Ceropegia* are as follows: Studies on microtuber production in *Ceropegia lawii*, *C. maccannii*, *C. oculata*, and *C. sahyadrica* were conducted by [9]; study on *C. bulbosa* was conducted by [11] study on *C. panchganiensis* was conducted by [12] and study on *C. pusilla* was reported by [13]. However, there are very few reports on *C. pusilla* microtuberization [14-15].

In vitro microtuberization would be an ideal strategy for this plant, if microtubers can be advantageous over the seasonal seeds [13]. Microtubers are easy to acclimatize and reintroduce in comparison with the other propagules. They are easy to store and are less vulnerable to transportation conditions, they also get established well in soil and thus are the choice of interest for international germplasm transfer [16]. *In vitro* rhizome formation has helped in conservation of *Zingiber* [17] where in potato; *in vitro* tuberization has proven savior strategy [18]. Therefore, the present study was under taken to develop a method for *in vitro* propagation and tuberization for this endangered species.

2. MATERIALS AND METHODS

2.1 Plant Material and Surface Disinfestation

Plant of *Ceropegia pusilla* was collected from Ooty, Nilgiri District., Tamilnadu (India), and authenticated by Botanical Survey of India (BSI/ SRC/ 5/23/2012-13/ tech, 1268) Coimbatore (Fig. 1A). The plants were grown in earthen pots in shade house at Government Arts College, Coimbatore. The shoot segments with five to six nodes were

collected from the plants grown in garden and washed with running tap water for 15 min. The nodes were cut (1-2 cm) separately and they were washed with Tween 20 detergent solution (5% v/v) for 5 min. Surface sterilization of explants was followed by rinsing with sterile distilled water 3 or 4 times to remove trace of detergent, rinsing in 70% ethanol for 30 seconds and finally treated with mercuric chloride (0-12% W /V) ($Hg\ Cl^2$) for 3 min duration.

2.2 Culture Medium and Culture Conditions

A culture medium containing MS [19] salt supplemented with macro elements, micro elements, iron, vitamins, amino acid and 3% sucrose (HiMedia, India) was used. The pH of the medium was adjusted to 5.8 by 1N NaOH or 1N HCl after adding the growth regulators. The media were steam sterilized in an autoclave under 15 psi and 121ºC for 20 min. All of the cultures were incubated under 50$\mu mol^{-2}S^{-1}$ light provided by cool while fluorescent lamp for a photo period of 16 h at 25±2ºC.

2.3 Shoot Initiation and Multiplication

Nodal explants from field grown plants were used as primary explants. Node explants measuring about 0.4-0.6 cm length were cultured on MS medium supplemented with various concentrations of growth regulators (BAP+NAA). Twenty five explants were used for each culture. Each experiment was repeated twice. The percent of explant responding for multiple shoot induction, shoot number per explant and total number of shoots per explant per sub culture were recorded after 40 days. In the subsequent sub-cultures, the nodes obtained *in vitro* produced shoots were harvested and used as explants to culture on the same medium. Sub-culturing was carried out at the regular interval of 15-20 days.

2.4 Callus Initiation and Shoot Proliferation

Node, internodes and leaf explants from field grown plants were used as primary explants. The explants were cultured on MS medium supplemented with various concentrations of growth regulators (BAP and NAA). Twenty explants were used for each culture. The percent of explants responding for callus induction shoot formation, nature of callus and number of days taken for callus induction were recorded after 40 days. In the subsequent subcultures, the callus and other parts obtained *in vitro* cultures were harvested and used as explants. Sub culturing was carried out at regular interval of 15-20 days.

2.5 *In vitro* Tuberization

The shoots from direct and indirect micropropagation were transferred to the same media for multiple shoot induction. The multiple shoots obtained from the subculture were transferred to the tuberization medium (MS + BAP and NAA). The tuberization percentage, diameter of the tuber, number of roots, tuber and length of the roots were recorded after 4 weeks. Tuberized plantlets were carefully removed from the culture bottle, washed with water and transferred to cup filled with hardening media (decomposed coir waste; perlite and compost 1:1:1 ratio) and hardened in the shade house.

2.6 *In vitro* Flowering

In vitro flowering was observed on the same tuberization medium (MS + BAP and NAA). Flowers were formed after 28 days of culture transferred to the rooting medium.

2.7 Acclimatization and Transplantation of Plantlets

The tuberized plantlets were removed from the culture bottles and washed with tap water to remove trace of agar. Then, the plantlets were planted onto net pot contains different type of potting media and survivability rate were determined after 20 days of hardening. Hardened plants were transferred to pot containing mixture of red soil, sand and compost (1:1:1 ratio). The pots were watered at two days interval under shade house condition. After 60 days, the frequency of survival percentage was calculated.

3. RESULTS AND DISCUSSION

The morphogenetic response of nodal explants to growth regulators (BAP andNAA) are summarized in Table1. The explants placed in the medium without growth regulators (Control) induced no shoots. The percentage of response varied with the concentrations of growth regulators used. Swelling of the dormant axillary bud occurred within twelve days of inoculation and then, differentiation into multiple shoots occurred after five weeks (Fig. 1C and 1E). Among the different combination of BAP and NAA tested, the effective growth occurred on MS medium with BAP (2.5mg/l) + NAA (0.5mg/l) is followed by MS+ BAP (3.0 mg/l) + NAA (0.5mg/l).

Table 1. Morphogenic response of Nodal explants of *Ceropegia pusilla* to different concentration of BAP and NAA on MS medium

S.No	MS medium BAP+NAA mg/l	Response %	Shoot number/ explant	Shoot number/ explants-subculture
1	0.5 + 0.5	20	0	0
2	1.0 + 0.5	40	1.0 ± 0.21^{d}	1.4 ± 0.27^{d}
3	1.5 + 0.5	60	1.65 ± 0.32^{d}	4.60 ± 0.85^{c}
4	2.0 + 0.5	75	2.20 ± 0.17^{c}	4.35 ± 0.78^{c}
5	**2.5 + 0.5**	**90**	**3.50 ± 0.34^{a}**	**15.40 ± 1.65^{a}**
6	3.0 + 0.5	70	2.75 ± 0.29^{b}	6.50 ± 1.05^{b}
7	Basal medium	-	--	-

Values are mean ± SD of six samples
Column Means followed by a common superscript are not significant at 5% by DMRT

Multiple shoot formation occurred on the same medium MS+BAP (2.5mg/l) + NAA (0.5 mg/l)) or when the shoot was transferred to fresh medium. 15-20 shoots were induced per explant within 25-30 days. The shoot elongation and growth were well and produced many leaves (Fig.1E). Nodal segments containing axillary buds have quiescent or active meristems depending upon the physiological stage of the plant. These buds have the potential to develop into complete plantlets. However, using micropropagation techniques, the rate of shoot multiplication greatly enhanced by nodal culture in a nutrient medium containing suitable combinations and concentrations of growth regulators.

In the present study, the stimulatory effect of BAP on bud break and multiple shoot formation in was similar to that reported earlier in *C.edulis* [20] and *Tylophora indica* [21]. In contrary, the TDZ induced multiple shoot formation *in C. pusilla* from callus [14]. *In vitro* BAP or cytokinins cause activation of meristem by removing constraints posed by anti-metabolites. Once this is done the meristem are conditioned, the anti-metabolites are diluted and shoots are amplified unabated even in the presence of low or no cytokinins [20].

Singular supplement of NAA at an optimal concentration (0.5 mg/l) had promotive influence on shoot development and multiple shoot formation. The synergic effect of BAP in combination with an auxin has been demonstrated in many medicinal plants from the Asclepiadaceae family such as *C. candelabrum* [22]; *C. edulis* [20] and *Hemidesmus indicus* [23]. In accordance with these reports, the present work also showed the effect of BAP in combination with NAA on shoot induction efficiency as well as a noticeable synergistic influence on multiple shoot formation. The effect of auxins in combination with cytokinin on multiple shoot formation was also reported earlier in *C.edulis* [20].

The combination of BAP and NAA induced greater amount of callus from the nodes and internodes of *C. pusilla* and the morphology of the callus was green and friable and nodular in nature. There was a wide range of variation on percentage of callus induction (0 to100) according to the concentration of hormones. The caulogenic effect of BAP along with NAA observed in the present study is in consonance with other reports [14,24,25]. Best growth of callus however occurred on MS+ BAP (0.5mg/l) +NAA (1.0mg/l). The other concentrations of BAP were also effective but not at the level of previous combination. Similar observation was reported in *Tylophora indica* [24] and *C. pusilla* from the cell layer explants [14]; which indicate that BAP+NAA are basically involved in the development of callus. Callusing started at the cut ends or along the entire surface after 8 days of culture and after 18 to 21 days the entire segment turned with a mass of green soft and friable callus (Fig. 1B). The young stem derived callus is highly viable, whereas the callus derived from the leaf bits was soft and could not maintain beyond the second or third subcultures. Similar observations were also made in *Tylophora indica* [26], *C.jainii,* and *C. bulbosa* var. *bulbosa* [27]. Contrary to this, in *C. candelabrum* [28], and *Decalepis hamiltonii* [29]; it was reported that callus was produced from the leaf and internodal explants. Later, same callus has produced somatic embryos too. Whereas**,** *H. indicus* obtained embryogenic callus from roots and leaves [30]. The optimum callusing was observed on the MS medium fortified with BAP13.32 mg/l +2.4-D 0.45 mg/l, as in case of *C. sahyadrica* [31].

The combination of BAP with NAA had the organogenic ability for certain extent. The stem explants are cultured on to the medium supplemented with BAP and NAA and produced higher amount of callus and few shoots; the callus is very competent and friable in nature. The regeneration of shoot primordia on the callus was observed clearly (Table 2). On the same medium containing BAP+NAA (0.5mg/l+1.0mg/l) or when calli was transferred to fresh medium 17.25±1.58 shoots were induced per explant within 20-25 days the shoots elongated and grew and developed many leaves. The shoot proliferation effects of BAP+NAA observed is in consonance with other reports [14], but the number of shoot production is very low (4) in comparison to the present study.

In vitro raised shoots of the *C. pusilla* were transferred onto the tuberization medium. There was a wide range of variation on percentage of tuberization according to the concentration of hormones. Of the different combinations and concentrations of BAP and NAA used, the percentage of tuberization was highest (90%) in MS medium supplemented with BAP(1.5mg/l) and NAA (0.5mg/l) (Table 3). Formation of higher sized tubers were observed

on MS medium supplemented with 3% sucrose and BAP (1.5mg/l) along with NAA (0.5mg/l) (2.79±0.56 cm) (Table 3, Fig. 1D and1H) which bears roots at their base. The other medium containing BAP (1.0mg/l), NAA (0.5) with 3% sucrose induced microbes with the diameter of 1.8±0.34 cm which bears roots.

Table 2. Effect of MS medium and different concentrations of BAP on callus inductions and shoot formation of *Ceropegia pusilla*

S. No.	MS medium BAP+NAA mg/1	% of stem producing callus	% of callus forming shoots	Nature of the callus	Days taken for callus induction
1	0.5+1.0	20.0 ± 1.5^{e}	5.47 ± 0.68^{e}	Green friable	17.25 ± 1.58^{a}
2	1.0+1.0	50.0 ± 4.78^{d}	14.58 ± 1.45^{d}	Green friable	15.83 ± 1.25^{b}
3	1.5+1.0	80 ± 6.12^{c}	40-50 ± 3.17^{c}	Green friable	11.50 ± 1.06^{c}
4	**2.0+1.0**	**100 ± 0^{a}**	**90-95 ± 7.93^{a}**	**Green friable**	**7.45 ± 0.83^{e}**
5	2.5+1.0	90.0 ± 5.26^{b}	75-80 ± 9.15^{b}	Friable and dark green	8.90 ± 1.14^{d}
6	3.0+1.0	75.0 ± 6.38^{c}	40-50 ± 2.63^{c}	Friable and dark green	7.0 ± 0.68^{e}
7	Basal medium	-	-	-	-

Values are mean ± SD of six samples
Column Means followed by a common superscript are not significant at 5% by DMRT

Table 3. Influence of plant growth regulators on *in vitro* tuberization in C.*pusilla*

S. No.	MS+BAP + NAA	*In vitro* tuberization percentage	Diameter of the tuber in cm	Number of roots / tuber	Root length
1	0.5 + 0.5	20	0.88 ± 0.13^{c}	1	1.5 ± 0.24^{c}
2	1.0 + 0.5	40	1.80 ± 0.34^{b}	2	2.0 ± 0.16^{b}
3	**1.5 + 0.5**	**90**	**2.79 ± 0.56^{a}**	**4**	**2.8 ± 0.68^{a}**
4	2.0 + 0.5	80	1.78 ± 0.27^{b}	2	2.1 ± 0.45^{b}
5	2.5 + 0.5	30	1.10 ± 0.22^{c}	1	1.0 ± 0.22^{d}
6	3.0 + 0.5	10	0.98 ± 0.18^{c}	0	0
7	0.5 + 1.0	20	1.10 ± 0.23^{c}	1	1.0 ± 0.17^{d}
8	1.0 + 1.0	30	1.20 ± 0.11^{c}	1	1.2 ±0.13^{cd}
9	1.5 + 1.0	35	1.80 ± 0.21^{b}	1	1.1 ± 0.08^{d}
10	2.0 + 1.0	20	1.10 ± 0.09^{c}	1	1.0 ± 0.11^{d}
11	2.5 + 1.0	-	0.88 ± 0.14^{c}	-	-
12	3.0 + 1.0	-	0.40 ± 0.05^{d}	-	-
13	MS Basal	-	0.30 ± 0.06^{d}	-	-

Values are mean ± SD of three samples in each group
Values not sharing the common superscript differ significantly (DMRT)

The *in vitro* tuberization in *C. pusilla* was good and the tubers are more or less uniform in their size with roots (Fig. 1F and 1I). The microtubers were induced within 20-25 days at temperature and photoperiods prevailed in the laboratory. All the tubers were white in colour, the anatomical studies through cross section of tuber is confirmed that it is a tuber not a callus because the presence of more storage tissues (Fig. 1J) As they enlarged the external surfaces of the tubers turned into brown colour. Many of the tubers originated in clusters called multiple tubers or secondary tubers (Fig. 1H and1I). The microtuber formation is governed by many factors like auxins, cytokinins, basal media type, strength and sucrose as

well as temperature and photoperiod etc. [32-34]. In the present study, auxin and cytokinin concentration, basal medium and sucrose concentration played main role in inducing microtubers.

Role of cytokinin and auxins was observed individually or in combination with auxins were reported to increase the frequency of in *vitro* tuberization in a number of yam species [35,34,13]. Revealed that BAP alone had significant effect on microtuber diameter and fresh weight. High concentrations of BAP and its role in the induction of microtubers were reported by [36].In accordance with these reports, the present work also exhibited similar effects of concentrations of BAP in combination with NAA on tuber formation efficiency. Cytokinin is not directly responsible for tuberization as reported by many workers. However, it plays key role in cell division, thus creating sink activity of the developing tubers [37]. A well-known fact is that cytokinin is synthesized in the roots and plays a vital role in cell divisions. The optimum temperature for the induction of microtubers was around 24 ± 2ºC and results are in accordance with [33,13]. *In vitro* flowering was observed on the same tuberization medium (MS + BAP + NAA). Flowers were formed after 28 days of culture transferred to the rooting medium. *C. spiralis* [37] *, C. pusilla* [14,37],have been reported to induce *in vitro* flowering in the above mentioned species, various concentrations and combinations of plant growth regulators were used.

Plantlets with well-developed tubers were successfully acclimatized inside the shade house with fogger system in selected planting substrates (Table4) for 14 days. Partially hardened plantlets then transferred to the poly bag containing red soil, sand and compost in the ratio of 1:1:1 for two weeks. Of the four different types of planting substrates examined, the percentage survival of the plantlet was highest (81±3.16%) in hardening media (Table4). In tissue culture, the period of transfer during the process of hardening from the *in vitro* to the *ex vitro* environment is the most important steps. In this period care was taken over the physical and other factors employed.

Table 4. Evaluation of different planting substrates for acclimatization of *in vitro* tuberized plantlets of *Ceropegia pusilla*

S.No	Planting substrates	No. of plants transferred	No. of plants survived	Survival (%)
1	Garden soil	20	10 ± 0.89^{b}	50 ± 1.66^{d}
2	Vermiculite	20	11 ± 1.23^{b}	55 ± 1.79^{c}
3	Decomposed coir waste	20	15 ± 1.54^{a}	74 ± 2.47^{b}
4	Harding media- decomposed coir	20	16 ± 1.38^{a}	81 ± 3.16^{a}

Values are mean ± SD of six samples

Column Means followed by a common superscript are not significant at 5% by DMRT

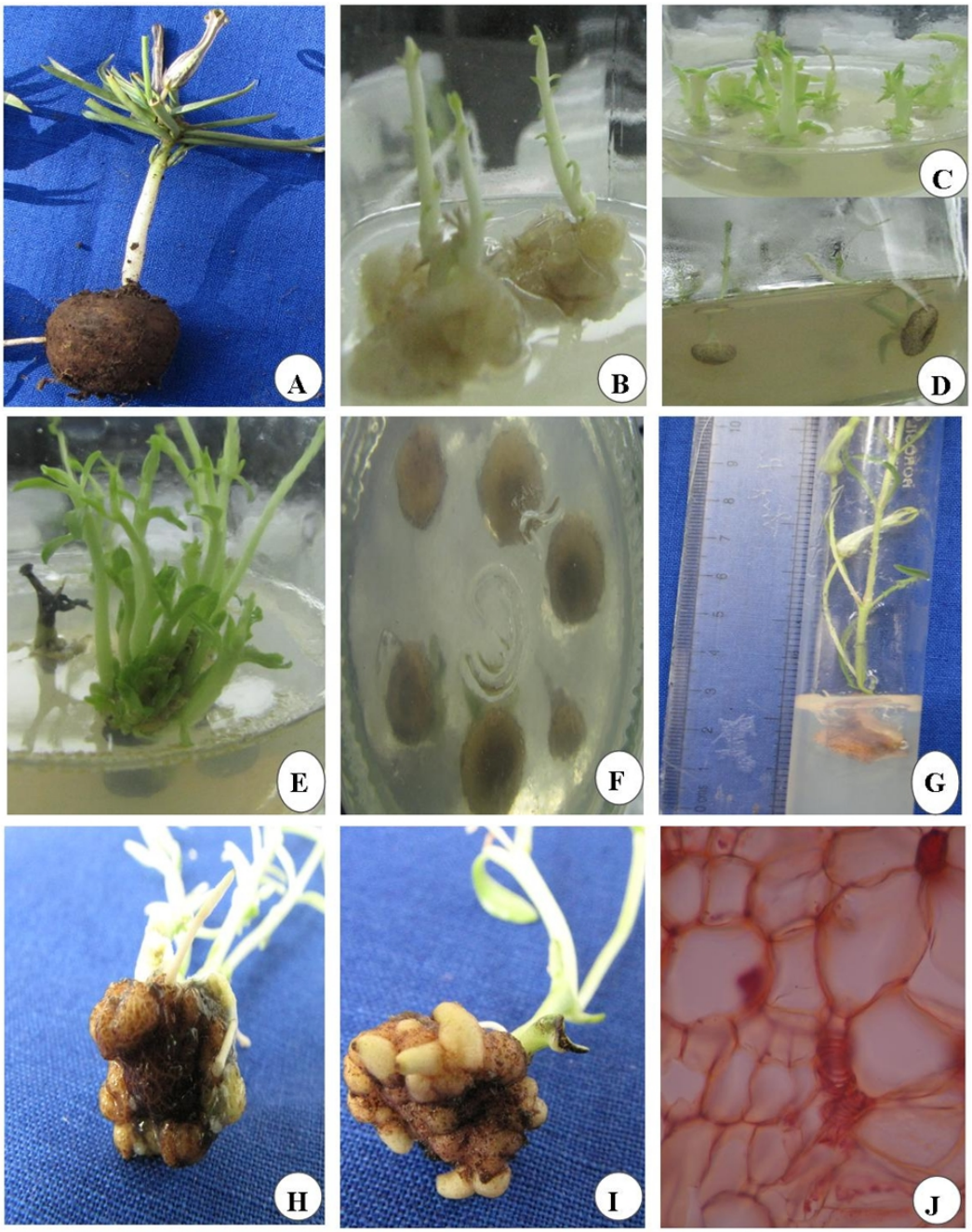

Fig. 1. *In vitro* cultures of *Ceropegia pusilla:* (A) habit (B) callus with shoot formation (C) multiple shoots initiation (D) tuber initiation from nodes (E) multiple shoots from nodal region (F) tuber formation (G) *in vitro* flowering (H) mature tubers (I) tuber with roots (J) anatomy of tuber

4. CONCLUSION

A direct, indirect micropropagation and basal tuberization initiation were developed for a pharmaceutically important medicinal plant, *C. pusilla*. This protocol offers a potential system for conservation, improvement and mass multiplication of *C. pusilla* from nodal explant. MS medium supplemented with BAP +NAA (2.5+0.5mg/l) is the best for shoot induction and proliferation. MS medium supplemented with BAP + NAA (0.5+1.0mg/l) is the best callus induction and morphogenesis. MS medium with BAP (1.5mg/l) and NAA (0.5 mg /l) is the best for microtuberization. Among the various potting substrate used the hardening media was the best in the survival percentage (81%). This can be employed with a propagation of endemic taxa *C. pusilla* and helps in conservation and domestication.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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