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Regeneration of Shoots from Callus of *Ceropegia pusilla* Wight and ARN

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Authors' contributions

This work was carried out in collaboration between the three authors. Author KK designed the study, performed the statistical analysis, and made the final draft of the manuscript. Authors RP and TS managed the literature searches, carried out the study under the supervision of author KK and wrote the protocol. All authors read and approved the final manuscript.

Others Article

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ABSTRACT

This study was undertaken to evaluate the most suitable concentration of plant growth regulators and suitable explants for callus induction and subsequent organogenesis of an endangered medicinal plant *Ceropegia pusilla.* The best performance of callus induction and morphogenesis was found on MS medium supplemented with 6- benzylaminopurine (BA) and naphthalene acetic acid (NAA), from node and internode the maximum account of callus initiation was recorded on MS medium supplemented with BA+NAA (2.22µM + 5.37 µM) and the maximum % of callus induced on subculture is 5.47 ± 0.68. Rooting was best achieved on MS medium augmented with IBA (2.46µM). The maximum number of roots (4.72 ± 0.66 cm) and root length (4.8 ± 0.49 cm) were recorded). Plantlets regenerated *in vitro* with well-developed shoot and roots were successfully acclimatized in pots containing a mixture of decomposed coir waste, perlite and compost 1: 1:1 ratio and grown in a shade house with 81 ± 3.16 percent survival rates.

Keywords: Callus; Ceropegia pusilla; multiple shoots; organogenesis; in vitro propagation.

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1. INTRODUCTION

Ceropegia (Asclepiadaceae) is an old world tropical genus of climbers, herbs and rarely sub shrubs distributed in tropical and subtropical Asia, Africa, Australia, Malaysia and in the Canary and Pacific Islands [1]. The genus *Ceropegia* represented by about 200 species in the world, of which 55 *Ceropegia* species found in India [2]. Twenty eight species of *Ceropegia* are endemic to the peninsular India [3-4]. The existence of the *Ceropegia* species has become restricted to remote pockets in the Himalayas and the Western Ghats, two biodiversity hot spots. Unfortunatly, the *Ceropegia* genus has now been added to the list of Indian endangered plants [5]. *Ceropegia pusilla* is an annual herb grown widely in South India and is in the endangered category [6-7]. The alkaloid, ceropegin is present in the root tubers [8-9]. The root tubers also contain starch, sugars, gum, albuminoids, fats, crude fiber and pyridine alkaloid is used in many traditional Indian Ayurvedic drug preparations that are active against ulcers, inflammation etc., [10]. The tubers are a kind of energy source, and among other things they are used to suppress fatigue.

Ceropegia pusilla is an ephemeral species that appears with the first rain shower of South West monsoon. Propagation of *C. pusilla* through seed is limited by low viability and germination rate, scanty and delayed rooting of seedlings. Seed-derived progenies are not true-to type because of cross-pollination. Vegetative propagation by root tubers is onerous, and is too low to meet the commercial needs. Natural populations of these plants is declining because of increase in demand in the pharmaceutical market. 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. Therefore, there is an urgent need to conserve this species *ex situ* through *in vitro* methods such as micropropagation [11]. However, there are very few reports on *C. pusilla* micropropagation [12-13]. The present *in vitro* micropropagation study was under taken to develop a method for multiplication through morphogenic callus of this endangered species.

2. MATERIALS AND METHODS

2.1 Plant Material and Surface Disinfestation

Ceropegia pusilla were collected from foot the hills of Ooty, Nilgiri Dt., Tamilnadu, (India), and authenticated by the Botanical Survey of India (BSI / SRC/ 5/23/2012-13/ tech 1268) Coimbatore (Fig. 1A).The plants were grown in earthen pots in the shade house at Government Arts College, Coimbatore. The explants such as node, internode and leaves were collected from the garden grown plants and washed with running tap water for 15 min. The explants were then cut (1-2 cm) separately and washed with Tween 20 detergent solution (5% v/v) for 5 min. After through washing, the surface disinfestation of explants were carried out by rinsing in 70% ethanol for 30 sec and finally treated with mercuric chloride (0.12 % w /v) ($HgCl_2$) for 3 min followed by and rinsing in with sterile distilled water three to four times to remove trace of toxic chemicals.

2.2 Culture Medium and Culture Conditions

A culture medium containing Murashige and Shoog (MS) [14] salts supplemented with macro elements, micro elements and 3% w /v sucrose (Hi Media, India) and solidified with agar 0.8 % (Hi Media, India) was used as basal medium along with plant growth regulators. The pH of the medium was adjusted to 5.8 with 1N NaOH or 1N HCl. The media were steam

sterilized in an autoclave under 15 psi and 121ºC for 20 min. All the cultures were incubated under 50µ mol m^{-2} s^{-1} light provided by cool-white fluorescent lamps for 16 h photo period at temperature 24 ± 2ºC.

2.3 Callus Initiation and Shoot Proliferation

Node, internode, and leaf explants from field- grown plants were used as primary explants. The explants were cultured on MS medium supplemented with 2.22- 13.32µM 6 benzyl aminopurine (BA) plus 5.37 µM naphthalene acetic acid (NAA). Twenty explants were used for each treatment. Days for callus induction, percentage of explants responding for callus induction, morphology of callus and shoot formation were recorded. In the subsequent sub cultures, the callus and other parts obtained *in vitro* cultures were harvested and used as explants. Sub culturing was carried out at the regular interval of 15-20 days.

2.4 Rooting of *in vitro* Multiple Shoots

Shoots 4-5 cm in height were separated and individual shoots were transferred to MS medium containing different concentrations of IBA (2.46µM-14.76µM). The cultures were incubated under 16h photoperiod for root development. The rooting percentage, mean root number and mean root length were measured.

2.5 Acclimatization and Transplantation of Plantlets

The rooted plantlets were removed from the culture bottles and washed with sterilized distilled water to remove traces of agar. The plantlets were planted in to pots containg potting media and survivability rate was determined after 20 days of hardening. Hardened plants were transferred to pots containing a mixture of red soil, sand and compost (1:1:1 ratio). The pots were watered for every two day intervals and maintained under shade house condition. After 60 days, the frequency of survival was calculated.

2.6 Statistical Analysis

Data were measured after 30-40 days for shoot multiplication and rooting respectively, Mean value with the same superscript were not significantly different (P=0.05%) according to Duncan's multiple range test [15].

3. RESULTS AND DISCUSSION

The combination of BA and NAA induced an excellent amount of callus from the node and internodes of *C. pusilla* and the morphology of the callus was green, friable, and nodular in nature. There was a wide range of variation on percentage of callus induction (0 to100). The caulogenic effect of BA, along with NAA observed in the present study was in consonance with other reports [12, 16]. Best growth of callus however occurred on MS + (2.22µM) BA + (5.37µM) NAA. The other concentrations of BA was also effective, but not at the level of previous combination. Similar observation was reported in *Tylophora indica* [16] and *C. pusilla* from the cell layer explants [12] that BA+NAA were basically involved at the development of callus. Callus started at the cut ends or along the entire surface after 8 days of culture, and after 2 to 3 weeks the entire segment turned into a mass of green, soft, and friable callus (Fig. 1B). The young node and internode derived callus was highly viable, whereas the callus derived from the leaf explant was soft and could not be maintained

beyond a second or third sub culture (data not shown) similar to observations also found in *Tylophora indica* [17] and *Ceropegia jainii, C. bulbosa* var. *bulbosa* [18]. In *Ceropegia candelabrum* [19] and Decalepis *hamiltonii* [20] callus was produced from leaf and internodal explants. Later, same callus has produced somatic embryos too. Whereas in Hemidesmus *indicu,* [21] embryogenic callus obtained from roots and leaves. The optimum callus was observed on MS medium fortified with 13.32 µM BA + 0.45 µM 2.4-D, for *Ceropegia sahyadrica* [22].

The combination of BA with NAA had the organogenic ability for certain extent. The stem explants are cultured on the medium supplemented with BA and NAA and produced an excellent callus and few shoots; the callus is very competent and friable in nature. The regeneration of shoot primordia on the callus was observed (Table 1). On the same medium containing BA+NAA (2.22µM + 5.37 µM) or when calli was transferred to fresh medium 17.25±1.58 shoots were induced from explant within 20-25 days the shoots elongated and grew and developed many leaves (Fig. 1C & 1D). The shoots proliferation effects of BA+NAA observed is in consonance with other reports [12] but the number of shoot production is very low (4) with compare to the present study.

The *in vitro* regenerated shoots were transferred to MS medium supplemented with IBA (Table 2) for root induction. After one week the root formation occurred on the basal cut portion of the shoots. The presence of IBA at low concentration in MS medium was found to be more effective for rooting (Fig. 1E) and the best rooting was achieved in MS medium supplemented with 2.46µM IBA, fairly good root number (4.72±0.66) and root length per shoots (4.8±0.49) (Table 2). The success of IBA in promoting efficient root induction has been reported in *C. pusilla* [12-13].

Plantlets with well-developed roots were successfully acclimatized inside the shade house with fogger system in selected planting substrates (Table 3) for two weeks (Fig. 1F). 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 (Decomposed coir waste: Perlite: Compost 1:1:1) 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.

PLATE-1

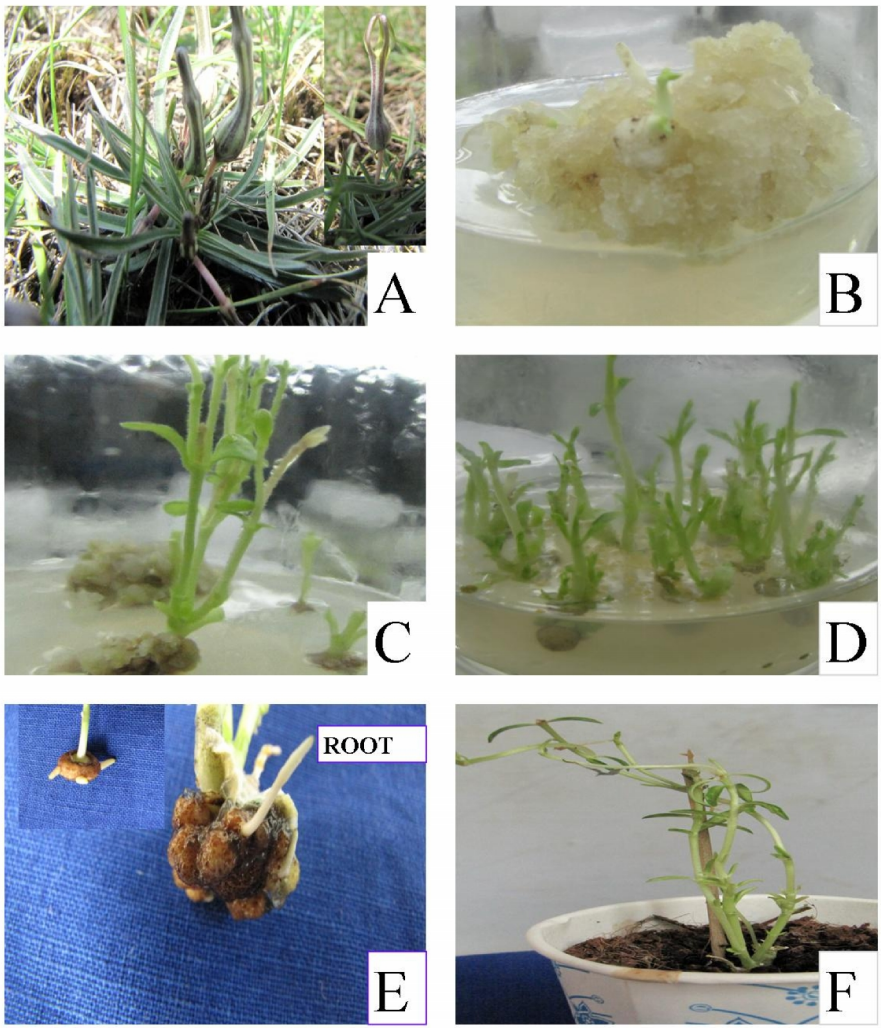

Fig. 1. *In vitro* propagation through nodal callus and multiple shoot proliferation of *C. pusilla*

A- Wild Plant with flower
B-Callus initiation from nodal explants cultured on MS +BA +NAA (0.5+0.1).
C -Shoot initiation from callus on MS +BA +NAA (0.5+0.1).
D -Multiple Shoot formation on MS +BA +NAA (0.5+0.1).
E-Shoot along with tuber root formation on MS +BA +NAA (0.5+0.1).
F- Acclimatized plantlet after five weeks.

Table 1. Effect of different concentrations of BA and NAA on callus induction and shoot formation of *Ceropegia pusilla*

MS medium BA+NAA µM	% node and internode producing callus Mean ± SE	Nature of the callus	Days to callus induction	% callus forming shoots Mean ± SE
Basal medium	-	-	-	-
2.22+5.37	20.0 ± 1.5[e]	Green friable	17	5.47 ± 0.68[e]
4.44+5.37	50.0 ± 4.78[d]	Green friable	15	14.58 ± 1.45[d]
6.66+5.37	80 ± 6.12[c]	Green friable	11	40.50 ± 3.17[c]
8.88+5.37	100 ± 0[a]	Green friable	7	90.95 ± 7.93[a]
11.10+5.37	90.0 ± 5.26[b]	Friable and dark green	8	75.80 ± 9.15[b]
13.32+5.37	75.0 ± 6.38[c]	Friable and dark green	7	40.50 ± 2.63[c]

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

Table 2. Effect of MS Medium with IBA on root formation of *Ceropegia pusilla.*

IBA µM	Response %	Mean number of root per shoot ± SE	Mean root length (cm) ± SE
2.46	90-95	4.72 ± 0.66[a]	4.8 ± 0.49[a]
4.92	75-80	2.28 ± 0.37[b]	4.0 ± 0.26[b]
7.38	30-40	1.60 ± 0.45[c]	3.2 ± 0.31[c]
9.84	28-30	1.56 ± 0.33[c]	2.5 ± 0.18[d]
12.30	18-19	1.75 ± 0.41[c]	2.1 ± 0.13[d]
14.76	-	-	-
Basal medium	-	-	-

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

Table 3. Evaluation of planting substrates for acclimatization of *in vitro* plantlets of *Ceropegia pusilla*

Planting substrates	No. of plants transferred	No. of plants survived Mean ± SE	Survival (%) Mean ± SE
Garden soil	20	10 ± 0.89[b]	50 ± 1.66[d]
Vermiculite	20	11 ± 1.23[b]	55 ± 1.79[c]
Decomposed coir waste	20	15 ± 1.54[a]	74 ± 2.47[b]
Harding media (Decomposed coir waste: Perlite: Compost 1:1:1)	20	16 ± 1.38[a]	81 ± 3.16[a]

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

4. CONCLUSION

In conclusion, micropropagation protocol for a pharmaceutically important endemic medicinal plant, *C. pusilla* were developed. This offers a potential system for conservation, improvement, and mass multiplication of *C. pusilla* from callus, thereby minimizing the pressure on wild populations of this valuable species.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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