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Micropropagation of endangered medicinal plant *Ceropegia candelabrum* L.

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Abstract
Several medicinal plants become endangered because of increasing biotic pressure on natural habitats and over exploitation. An efficient protocol was described for the rapid *In vitro* shoot multiplication and conservation of endangered, valuable and rare medicinal herb *Ceropegia candelabrum* Var. *candelabrum*. Nodal explants cultured on growth regulators free MS medium became necrotic and shown no sign of active growth. They were placed on full strength MS medium fortified with sucrose supplemented with different concentrations of two commonly used cytokinins (BAP/ KN (0- 2.5 mg/L) either alone or in combination with three auxins (NAA, IAA and IBA (0-2.5 mg/L). The initial rate of shoot regeneration from *Ceropegia candelabrum* was very low. Maximum shoot buds proliferated on MS medium containing 2mg/l of BAP and repeated sub culture on BAP (2mg/l) promoted further shoot multiplication. The best rooting was noticed in half strength MS basal medium containing 5% sucrose and 1.5mg/l of IBA. Sprouting of axillary buds and development of adventitious shoots at basal ends was seen (in 85% shoots) after two weeks of culture period. The regenerated plantlets were hardened and established in natural soil with 90% survival rate. The developed micropropagation protocol would help in *Ex-situ* conservation of this endangered species.

Keywords: *In vitro*, *Ceropegia candelabrum*, micropropagation

Introduction
Ceropegia candelabrum L., under the family Apocynaceae. The 'glabrous goglet flower' is an endangered herb. Root tubers contain the alkaloid "Ceropegine" [1] which is used in Indian Ayurvedic drug preparation [2]. The tuber of *Ceropegia candelabrum* is used as a remedy to cure headache in India [3]. The existing reports on Ceropegia species show that they were used as in traditional medical system. They also play a vital role in the Ayurvedic field. These Ceropegia species are placed under the categories of rare, endangered, vulnerable, extinct and threatened plants [4, 5]. According to All India Co-ordinate Project on Ethnobiology, about 7,500 wild plant species are used for medicinal purpose by the tribal communities and 950 are found to be new claims which are worthy of scientific scrutiny. Habitat degradation, low seed germination capability, loss of specific pollinators, indiscriminate collection and anthropogenic activities disturb the forest vegetation leading to the elimination of several valuble species. Alarming commercial over-exploitation of forest resources also leads to the genetic erosion of medicinal plants. To establish a standard micropropagation protocol for the conservation of *Ceropegia candelabrum* and re-introduction of the *in vitro* propagated plants in their natural habitat. So, far there was no report on tuberous root of *Ceropegia candelabrum* var. *candelabrum* in flora such as Flora of Eastern Ghates, Flora of the Presidency of Madras and the flora of the Tamil Nadu Carnatic.

Material and Methods
Collection of *Ceropegia candelabrum* L.
Ceropegia candelabrum plant is endemic in India and it was collected from Marthuvalmalai during rainy season and the collected plants were identified by using the using *Flora of Eastern Ghats* [6]. The collected plant parts such as node and shoot tips were micropropagated.

Morphological description
Ceropegia candelabrum L. glabrous perennial twiners, stems glabrous. Leaves variable, chartaceous, elliptic-ovate or lanceolate, 2-8 x 0.8-4 cm, glabrous, apex acutely apiculate, base rounded, acute or sub-cordate, Lateral veins 4-5 pairs, conspicuous beneath; petiole 1.2 cm long. Flowers many-flowered, lateral, umbelliform cymes; peduncle 2.8 cm long, pedicel

0.8 cm long; calyx 5-lobed; corolla green below, tips purplish, veins prominent, 4.5cm long, basal part inflated, cylindric, lobes ciliate within, corona biseriate, outer very small, inner processes spathulate (Plate -1). Follicles paired, 10-14 x 0.5-0.7 cm, cylindric, tapering at apex, glabrous; seeds 0.8 x 0.2 cm, ovate-oblong, margined with 2-2.5 cm long coma.

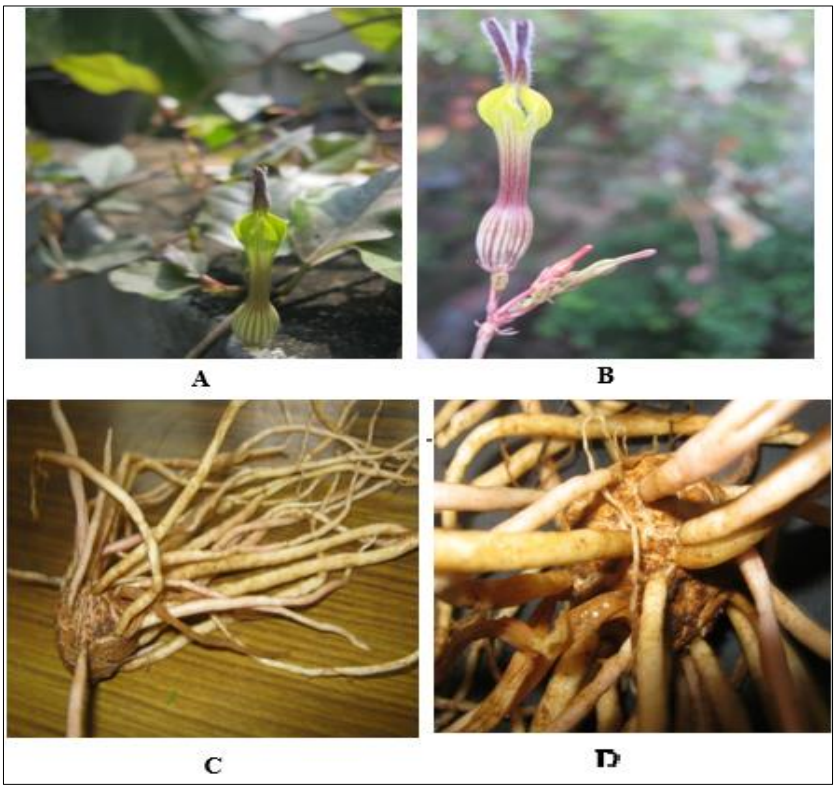

Plate 1: *Ceropegia candelabrum*

a. Habit
b. Flower
c. Tuberous roots
d. Tuberous roots – A portion enlarged

Result

Micropropagation of *Ceropegia candelabrum*

Effect of bap and KN on shoot proliferation from shoot tip and nodal explants of *Ceropegia candelabrum*

The basal MS medium supplemented with BAP (0.5-2.5 mg/L) and the results were summarized in table -1. Initially, a single shoot bud initiated from shoot tip but after 7 days, three to four axillary shoot buds were initiated at the base of the old ones. Various concentrations of BAP were tested and optimum number of shoot regeneration (4.5±1.04) was obtained with BAP (2.0mg/L) (Plate -2). Meanwhile, the medium showed maximum regeneration capacity by producing shoots within two weeks. MS medium supplemented with KN (1.5mg/L) showed 50% shoot sprouting frequency with the highest number 2.16±0.75 of shoots per explant with shoot length 3.50±0.54cm. BAP was found to be more effective than KN for shoot bud multiplication from nodal explants.

Table 1: *Ceropegia candelabrum*- Effect of various concentrations of BAP and KN on shoot induction and multiplication

BAP	KN	Response (%)	Number of mul.shoots/explants (Mean± SD)	Shoot length (cm) (Mean±SD)	Shoots with basal callus
0.5	0.5	40%	1.66 ± 0.81^{a}	4.03 ± 1.29^{bc}	_
1.0	1.0	55%	1.83 ± 0.98^{ab}	4.50 ± 1.87^{c}	_
1.5	1.5	60%	3.16 ± 0.75^{c}	4.33 ± 0.81^{bc}	_
2.0	2.0	73%	4.50 ± 1.04^{d}	4.83 ± 1.16^{ac}	_
2.5		45%	3.66 ± 0.51^{cd}	4.16 ± 1.16^{c}	_
		30%	1.16 ± 0.40^{ab}	2.66 ± 0.81^{a}	_
		46%	1.66 ± 0.81^{a}	3.50 ± 0.54^{ab}	_
		50%	2.16 ± 0.75^{bc}	3.50 ± 0.54^{ab}	_
		40%	1.33 ± 0.51^{ab}	3.66 ± 0.81^{b}	_

Values are the mean ± SD of six replicates, Different superscripts in the same column indicate significant differences within treatments (Tukey's HSD test, $P< 0.005$); (-) sign indicate no callusing

Combined effect of various concentration of BAP with NAA and IBA in shoot induction from nodal explants and shoot tip of *Ceropegia candelabrum.*

Shoot formation from shoot tip and nodal explants were cultured on MS medium supplemented with different concentration of cytokinin (BAP) and different concentration of auxin (NAA and IBA). Among the diverse concentrations BAP (1.0mg/L) with NAA (0.5mg/L) produced 2.16±0.75 number of shoots with a shoot length of 5.16±0.75cm, whereas BAP (1.0mg/L) with IBA (0.5mg/L) produced 2.16±0.75 number of shoots with a shoot length of 3.66±0.81cm. From this report it is concluded that NAA was

found to induce elongated shoots (Table -2). However, addition of BAP with IBA showed only limited growth. On the other hand, the medium containing BAP+NAA exhibited very low number of shoots (2 numbers), but they showed an excellent rate of growth (Plate 2-E).

Table 2: *Ceropegia candelabrum*- Combined effects of various concentrations of BAP with NAA and IBA in shoot induction from explants

BAP	NAA	IBA	Response (%)	Number of mul. shoots/explants (Mean± SD)	Shoot length (cm) (Mean±SD)	Shoots with basal callus
0.5	0.25	0.25	43%	1.50±0.54^{a}	5.16±0.75^{bcd}	+
1.0	0.5	0.5	70%	2.16±0.75^{abc}	5.16±0.75^{bcd}	+
1.5	0.75	0.75	40%	1.16±0.40^{ab}	5.16±0.40^{cd}	+++
2.0	0.1		80%	CP	CP	+++
0.5			50%	1.33±0.81^{a}	4.50±1.04^{bc}	_
1.0			60%	2.16±0.75^{b}	3.66±0.81^{b}	_
1.5			40%	1.16±0.40^{ab}	4.83±0.75^{acd}	_

Values are the mean ± SD of six replicates. Different superscripts in the same column indicate significant differences within treatments (Tukey's HSD test, P< 0.005); (-) sign indicate no callusing; (+) sign represent the intensity of callusing; CP- callus production.

Shoot multiplication of *Ceropegia candelabrum*

New shoots developed from cultures were cut into segments with single node on MS medium supplemented with BAP (2.0mg/L). The sprouting rate of axillary buds remained stable (around 90%) even in MS supplemented with BAP (2.0mg/L). The regenerated shoot buds were transferred to the fresh medium at the same concentration of BAP. Third to fourth sub-culture was required to generate sufficient number of shoot multiplications. The explants produced maximum number of shoots in the fourth sub-culture (Plate 2-D). The average number of shoots produced per explant was found to be 8.33±1.03. Further, increase in the concentration of BAP resulted in decreasing the rate of shoot regeneration ability. The *in vitro* raised shoots exhibited leaf and shoot tip abscission.

***Ceropegia candelabrum* - Effect of sub-culture of micro shoots on MS medium supplemented with 2.0mg/L BAP after 30 days interval.**

BAP (2.0mg/L)	Shoots/explants (mean ± SD)	Shoot length (mean ± SD cm)
1st	3.66±0.51	5.66±1.21
2nd	5.66±0.81	4.50±0.47
3rd	6.83±0.75	4.26±1.01
4th	8.33±1.03	4.11±0.78
5th	4.33±0.81	4.70±0.65

Each values represent mean value ± SD of experiment carried out with six replicate.

Enhancement of root induction from nodal and shoot tip explants of *Ceropegia candelabrum*

Rooting was induced on MS medium containing IAA, NAA and IBA. Proliferation of root was observed at the cut ends of the micro shoots within 15 days. Whereas, the shoots failed to induce roots or root initials in the absence of auxins (control). Elongated shoots were excised and cultured on Half-strength MS medium with 5% of sucrose, fortified with various concentrations and combinations of auxins IAA, NAA and IBA. Among them, the best rooting response (90%) was obtained in IBA (1.5mg/l) within 2 weeks of culture (Table -3) and (Plate 2-f). The number of roots per shoot was increased with increasing the concentrations of IBA 1.5mg/L and rooting percent was decreased when the IBA concentration was increased above 1.5mg/L. Among the tested three auxins 1.5mg/L IBA was the most effective for the root induction.

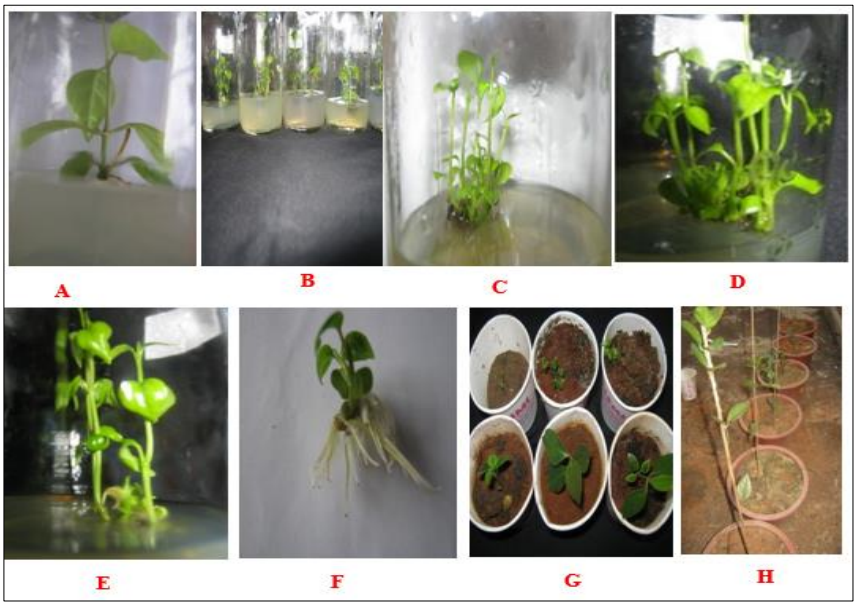

Plate 2*C*: *Candelabrum*- Shoot multiplication and Formations of roots

A. Shoot initiation from nodal explant on MS with BAP
B&C. Multiple shoot regeneration on MS medium supplemented with BAP (2.0 mg/L).
D. Maximum number of shoots in the fourth sub-culture on MS medium with BAP (2.0 mg/L).
E. Formation of 2-3 micro shoots from axillary bud portion on MS with BAP (1.0mg/L) + NAA (0.5mg/L).
F. Root on MS with IBA (1.5mg/L). **E** and **F**-*In vitro* propagated plantlets.

Table 3: *Ceropegia candelabrum* - Effect of different auxins on root induction from nodes and shoot tip explant on Half-strength MS medium

IBA	IAA	NAA	Concentration of sucrose (%)	Number of roots (Mean ± SD)	Average length of root per explants ± SD
			5%	1.83±0.75^{a}	4.08±1.02^{ab}
			5%	3.00±0.89b^{c}	4.11±1.13^{ab}
			5%	5.08±1.42d^{efg}	5.10±0.46^{ab}
			5%	7.41±1.62^{fg}	5.40±0.52^{b}
0.4	0.4		5%	4.25±1.54^{cd}	4.61±0.66^{b}
0.8	0.8	0.4	5%	1.66±0.51^{a}	3.55±0.52^{ab}
1.2	1.2	0.8	5%	2.33±0.51^{ab}	3.66±0.59^{ab}
1.5	1.5	1.2	5%	3.33±0.81^{abc}	3.21±0.39^{ab}
2.0	2.0		5%	3.66±0.51^{bc}	2.61±0.71^{a}
			5%	1.66±0.81^{a}	2.95±0.59^{a}
			5%	1.83±0.75^{ab}	5.25±0.59^{ab}
			5%	1.83±0.40^{ab}	5.55±0.47^{b}
			5%	0.75±0.40^{a}	3.66±0.51^{ab}

Values of last two columns represent mean ± SD of 7 replicates. Different superscripts in the same column indicate significant differences within trestments (Tukey's HSD test, P< 0.005).

Acclimatization of *Ceropegia candelabrum*

Regenerates of the sampling plants about 4-6 cm in height, with 8-10 leaves and well developed roots of 47 plantlets were transferred to pots containing hardening mixture of sterilized soil, coir waste and *Azolla* (1:1:1) at diffused light (16/8 h photo period) conditions. Afterwards 45 plantlets from *in vitro* conditions were transferred to pots containing different types of hardening materials like equal ratio of cow dung, autoclaved river sand and garden soil. The plantlets were covered with paper cup to maintain humidity. Further the survival rate was recorded after one month after it was transferred to pots. Among this, a combination of equal ratio of cow dung, autoclaved river sand and garden soil was found to be the most effective substrate for the acclimatization of *in vitro* regenerated plantlets with a survival rate of 85% Figure - 1). All plants had normal development and no morphological variations were noticed (Plate 2-H).

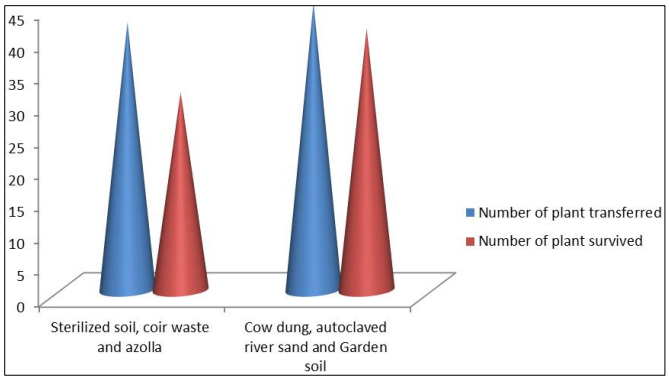

Fig 1: Effect of different potting mixture during acclimatization of *Ceropegia candelabrum*

Discussion and Confliction

In vitro techniques used for conserving wild and endemic species of *Ceropegia* by mass multiplication for subsequent reintroduction in their natural habitat [7]. Several reports were published on the *in vitro* studies of *Ceropegia* species i.e., *Ceropegia pusilla* var. *lushii* [7], *Ceropegia candelabrum* [2] and *Ceropegia intermedia* [8]. The shoot multiplication at an enhanced pace was achieved by subsequent cultures up to three to four cycles. In the present work it was noticed that the reproductive potential of shoot increases with the increase in the number of sub-cultures. Excision of the *in vitro* derived shoots and sub-culturing them on the same medium facilitated the development of 8.33 ± 1.03 micropropagated shoots in 2.0mg/L of BAP in *Ceropegia candelabrum.* All cultures formed some callus at the base from which new shoots emerged. The excision and culture of nodal explants from the primary culture as well as from the subsequent cultures, resulted in an increased shoot multiplication, as was reported in many other medicinal plants, such as *Gymnema sylvestre* [9], *Hemidesmus indicus* [10], *Decalepis arayalpathra* [11] and *Ceropegia intermedia* [8]. In second to third sub-culture shoots expressed callogenetic potential. Highest rooting percentage was obtained in half-strength MS medium with 5% of sucrose, fortified with various concentrations and combinations of auxins IAA, NAA and IBA in *Ceropegia candelabrum.* The similar results reported in *Ceropegia hirsuta* [12] *Ceropegia spiralis* [13]. In general, growth rate is considered as a function of sucrose concentration [14]. The present investigation has resulted in the development of a protocol which could be used for the large scale multiplication of *Ceropegia candelabrum* and offers a potential regeneration and multiplication system for improvement and conservation of this important endangered medicinal plant.

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