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Micropropagation of iron deficiency prevent plant *Ceropegia spiralis*

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Abstract
In vitro culture techniques will certainly go in a long way in the multiplication of endemic, endangered and rare species. The development of, an efficient protocol for rapid clonal propagation is important to meet the pharmaceutical needs and conservation of valuable *Ceropegia spiralis* medicinal plants. Micropropagation was carried out using nodal explants and reported that MS medium fortified with BAP & KN alone forms an effective hormonal combination for shoot induction. The number of shoots reduced on further increase in the concentration. Nodal explants from is *Ceropegia spiralis* cultured on MS medium fortified with BAP (1.25mg/L) had a better response of axillary buds. Maximum number of shoot regeneration resulted on MS medium supplemented with BAP 1.25mg/L+KN 1.0mg/L (5.85±1.21) shoots/explants with 80% response. The best rooting response was obtained in a medium containing NAA in 1.25mg/L concentration. In this concentration, 5.71 ± 0.75 roots had developed from each explants and nearly 72 rooted shoots are obtained within a period of 120 days.

Keywords: *In vitro, Ceropegia spiralis*, endangered

Introduction
The genus *Ceropegia* L. belongs to the sub-family Asclepiadoideae under the family Apocynaceae in APG III Classification. *Ceropegia spiralis* is a common condiment for various foods and beverages and it has a long history of being used as an important traditional medicinal herb for the treatment of stomach disorders. The root tubers of *Ceropegia spiralis* contain starch, sugar, gum, albuminoid, fats, crude fiber and many other valuable constituents used in several traditional Indian Ayurvedic drug preparations, which are particularly used for diarrhea and dysentery. The species analyzed contained remarkably high amount of Iron [1]. The starchy tubers are used as a nutritive tonic, blood purifier and tuber paste of *Ceropegia spiralis* is used for indigestion [2, 3, 4, 5, 6]. *Ceropegia* species are widely used by Kani tribes in treating stomach problems, skin diseases, vomiting, gastric disorders, diarrhea urinary tract disorders and dysentery. Monkey also ate *Ceropegia spiralis* tuber to cure various diseases. The importance given to *Ceropegia* species by Kanis clearly shows that it has certain effective and potential medicinal properties. *Ceropegia spiralis* is an anticancer agent and particularly effective for the treatment of colon cancer [7].
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 [8, 9]. Twenty eight species of *Ceropegia* are endemic to the Peninsular India [10, 11]. In most of the Indian *Ceropegia* species, the starchy tubers are prone to fungal infections; which lead to decay of tubers. Propagation from seeds is held back by low germination and survival rates. As a result their wild populations are diminishing at an alarming rate [12]. Large scale propagation is required for the effective conservation of this endangered species. Tissue culture techniques are the effective tools to conserve plants from extinction, homogenous production, maintain stable plant source produced them in large quantities and restore them to the natural habitats [13, 14, 15, 16, 17]. An efficient protocol was standardized for the micropropagation of *Ceropegia spiralis* by axillary shoot proliferation method using *in vivo* and *in vitro* nodal explants. Of the two different types of explants tested, *in vitro* nodal explant was found to be more successful.

Material and Methods
Collection of *Ceropegia spiralis* Wight
Ceropegia spiralis Wight is vulnerable and an endemic in India. It was collected from Mothiramali and Pechiparai hills in Kanyakumari District. The plants were collected during

rainy season with the help of Kani tribes. The collected plant was identified by using *Flora of Eastern Ghats* [17]. Juvenile shoots were obtained from mature plants of *Ceropegia spiralis* growing in pots and maintained in the Botanical Garden, Department of Botany, Nesamony Memorial Christian College, Marthandam, Kanyakumari District, Tamil Nadu. After the fourth week young shoots with nodes and internodes were collected from the potted plants and washed with running tap water for 30 minutes to remove the dust particles from the surface. Then these parts were micro propagated. Further the *in vitro* callus and *in vitro* plant parts were utilized for phytochemical analysis and hardened plant parts were reintroduced original place.

Morphological description

Ceropegia spiralis Wight. slender erect herb, tuberous stem glabrous. Leaves chartaceous, linear glabrous, 7-10 x 0.2-0.3 cm, apex and base acute. Flowers in 1-flowered cymes; corolla 4-5 cm long, tube sub-cylindric, hairy within above from ovate or orbicular base, generally spirally twisted; corona biseriate, outer corona bifid, deltoid lobes (Figure1.A). Follicles very slender, about 12.5 cm long (Figure 1.B).

Fig 1: A

Fig 1: B

Result

Micropropagation of *Ceropegia spiralis*

Nodal segments and shoot tips of *Ceropegia spiralis* cultured on MS medium supplemented with different concentration of BAP showed different response during primary establishment. The response of nodal explants treatment results are presented in Table 1. Out of these treatments, medium fortified with BAP (1.25mg/L) had a better response of axillary buds.

Table 1: Individual effects of various concentrations of BAP on shoot formation in mature nodes and young shoots explants of *Ceropegia spiralis* cultured on MS medium with 3% sucrose.

No	BAP (mg/L)	No. of bottles	Response of shoot tips (%)	Response of axillary buds (%)	Number of micro shoots /shoot tips (Mean ± SD)	No. of micro shoots/axillary buds (Mean ± SD)
1	0.25	10	40%	60%	1.28± 0.48	1.71± 0.48
2	0.5	10	50%	60%	1.42± 0.53	1.57± 0.53
3	0.75	10	50%	70%	1.57± 0.53	2.14± 0.37
4	1.0	10	60%	80%	1.85± 0.69	2.14± 0.37
5	1.25	10	80%	80%	2.57± 0.53	2.85± 0.69
6	1.5	10	80%	70%	1.57± 0.53	2.14± 0.69
7	1.75	10	60%	40%	1.28± 0.48	1.85± 0.37
8	2.0	10	50%	40%	1.14± 0.37	1.57± 0.53
9	2.25	10	40%	30%	1.14± 0.37	1.57± 0.53
10	2.5	10	40%	20%	1.14± 0.37	1.57± 0.53

Values of last two columns represent mean ± SD of seven replicates.

In this concentration of BAP (1.25mg/L) 2.57±0.53 micro shoots were produced in shoot tip explants. The response of axillary bud explants induction was investigated on MS medium containing various concentration of BAP resulted with 80% shoot sprouting frequency and highest shoot 2.85±0.69 were produced at the same concentration of BAP (1.25mg/L).

When the concentration of BAP was increased, the number of shoots reduced. The growth rate was varied from one to other explants even when they are inoculated on MS medium containing same concentration of different growth hormones. The performance of nodal segments is much better than the shoot tips.

Effect of different cytokinin (BAP and KN) on shoot proliferation of *Ceropegia spiralis*

Sub-cultured on the MS medium containing different concentrations of BAP in combination with other cytokinins KN were tested for shoot proliferation. BAP 1.25mg/L produced 86% of regeneration frequency with 5.71±1.11number of shoots per explants and showed the shoot length of 4.35±0.68cm, whereas KN 2.0mg/L exhibited 80% regeneration frequency and produced 2.42±0.53 shoots per explants with and average shoot length of 3.74±0.64cm.

MS medium containing optimal concentrations of BAP (1.25mg/L) in combination with other cytokinins with KN was also valuated for multiple shoot induction. With the addition of two cytokinins, Maximum number of shoot regeneration resulted on MS medium supplemented with BAP1.25mg/L+KN 1.0mg/L (5.85±1.21) shoots/explants with 80% response (Table 2& Figure 2).A callus was occasionally formed at the base of the explants retarding axillary bud formation and the subsequent growth of shoots. However, the rate of shoot multiplication can be greatly enhanced by performing axillary bud culture in a nutrient medium containing suitable cytokinin combinations.

Table 2: Combined effects of various concentrations of BAP and KN on multiple shoot regeneration of nodal and micro shoots tip explants of *Ceropegia spiralis* cultured on MS medium with 3% sucrose.

BAP	KN	Response (%)	Number of multiple shoots/explant (Mean±SD)	Average length of shoot/ explant ± SD (cm)	Shoots with basal callus
1.25		86%	5.71±1.11^{de}	4.35±0.68^{b}	+
1.5		76%	4.85±0.69^{cd}	3.97±0.76^{ab}	+
1.75		70%	3.57±0.78^{abc}	3.30±0.80^{ab}	+
	0.5	80%	1.57±0.53^{ab}	4.04±0.61^{ab}	_
	1.0	80%	1.71±0.48^{ab}	4.07±0.56^{ab}	_
	1.5	73%	2.14±0.37^{ab}	4.40±0.64^{ab}	_
	2.0	80%	2.42±0.53^{ab}	3.74±0.64^{ab}	+
	2.5	70%	1.42±0.53^{a}	3.78±0.14^{ab}	+
1.25	0.5	80%	4.28±1.38^{bd}	4.01±0.67^{ab}	+
1.25	1.0	80%	5.85±1.21^{e}	4.25±0.17^{b}	+
1.25	1.5	70%	4.57±0.53^{cd}	3.77±0.68^{ab}	+
1.25	2.0	70%	3.42±0.53^{bc}	3.88±0.42^{b}	_
1.25	2.5	76%	2.71±0.48^{b}	3.02±0.62^{ab}	_

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

Fig 2

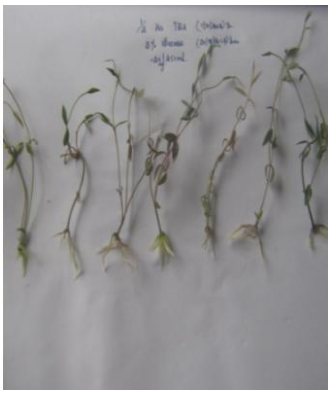

Fig 3

Fig 4

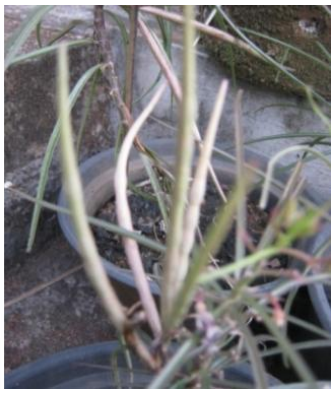

Fig 5

Effect of auxins on root induction of *Ceropegia spiralis*

The multiple shoots obtained from MS medium, shoots with 4 to 5 cm length were excised from shoot clumps and transferred to half strength or full strength MS rooting medium containing different composition and concentration of auxin (IAA, IBA, and NAA) alone for rooting and the plantlets were observed. There was no positive response on medium without regulators.

The usage of higher concentration of sucrose (5%), for root initiation and proliferation was noticed. Half strength MS medium supplemented with auxins at different concentrations showed varied effect on *in vitro* rooting. The nodal explants and shoot tips showed their rooting response within seven days. Highest rooting was achieved in cultures maintained on proliferation medium for four weeks in which sucrose or glucose was used in the rooting medium. Shoots older than four weeks lost their ability to root. Of the three auxins tested, IAA and IBA were equally effective in inducing roots. More or less similar results were obtained on the two auxins on the medium with 5% sucrose (Table 3).

A single root emerged after 12 days in the presence of IBA with one or two branches. Maximum of 3.57 ± 0.53 roots per shoot with 2.31 ± 0.74 cm root length were developed on IBA 1.0 mg/L (Figure 3). In IAA, the percentage of rooting and the number of roots formed were poor. In general, NAA was found to induce elongated roots. The best rooting response was obtained in a medium containing NAA in 1.25mg/L concentration. In this concentration, 5.71 ± 0.75 roots had developed from each explants (Figure 4).

Table 3: Effect of auxin on *in vitro* rooting in *Ceropegia spiralis*

IBA (mg/L)	NAA (mg/L)	IAA (mg/L)	Medium strength	Conc. of sucrose (%)	No. of roots (Mean±SD)	Average length of root per explant ± SD
0.5			½	5%	NR	NR
0.75			½	5%	1.57±0.53^{ab}	1.71±0.32^{a}
1.0			½	5%	3.57±0.53^{bc}	2.31±0.74^{ab}
1.25			½	5%	1.14±0.37^{a}	1.58±0.21^{a}
	0.5		½	5%	2.28±0.48^{ab}	2.12±0.33^{a}
	0.75		½	5%	2.71±0.48^{b}	3.12±0.30^{bc}
	1.0		½	5%	3.42±0.53^{abc}	3.75±0.93^{abc}
	1.25		½	5%	5.71±0.75^{d}	4.91±0.67^{c}
	1.5		½	5%	3.42±0.78^{abc}	3.62±0.72^{abc}
		0.75	½	5%	NR	NR
		1.0	½	5%	1.28±0.48^{ab}	1.74±0.26^{ab}

		1.25	½	5%	2.28±0.48^{ab}	2.61±0.21^{ab}
		1.5	½	5%	2.28±0.48^{ab}	3.17±0.41^{bc}
		1.75	½	5%	1.57±0.53^{ab}	3.05±0.82^{bc}

NR- no response; Values are the mean ± SD of seven replicates. Different superscripts in the same column indicate significant differences within trestments (Tukey's HSD test, P< 0.005).

Acclimatization and transplantation of plantlets

Root intact plantlets were taken out of the culture vessel, transferred to the portrays filled with two different types of hardening process. In the first part of transfer 10% of plants died and the remaining 90% of the field-transferred regenerates were successfully acclimatized to soil. Among the *in vitro* plantlets, 45% plants were hardened with a bio-fertilizer *Azolla* along with sterilized soil and coir waste (1:1:1) 45% plantlets with potting mixture of cow dung, autoclaved river sand and garden soil (1:1:1). In the present study, combination mixture of cow dung, autoclaved river sand and garden soil (1:1:1) was found to be the most effective substrate for the acclimatization of *in vitro* regenerated plantlets of *Ceropegia spiralis* which showed maximum survival of 42 percent with good plantlets growth as compared to other mixtures (Figure 5). Transfer of plantlets to the pots and field was accomplished.

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 [19]. There is an urgent need to understand the genetic architecture and evolutionary relationship of *Ceropegia* species to ensure their reestablishment in nature to grow in botanical, herbal gardens and other protective areas [20]. Several reports were published on the *in vitro* studies of *Ceropegia* species i.e., *Ceropegia jainii*, *Ceropegia pusilla* var. *lushii* [19], *Ceropegia candelabrum* [21], *Ceropegia intermedia* [22] *Ceropegia fantastica* [23], *Ceropegia spiralis* [24, 25].

The effectiveness of MS medium has been found to be more effective than other media for *in vitro* propagation of plants belonging to Apocynaceae by other investigators in *Ceropegia jainii*, *Ceropegia bulbosa* var. *bulbosa* and *Ceropegia bulbosa* var. *lushii* [19], *Ceropegia candelabrum* [21], *Ceropegia fantastica* [23], *Ceropegia spiralis* [26], *Caralluma adscendens* [27] and *Ceropegia thwaitesii* [28].

Superiority of BAP for shoot multiplication in Apocynaceae has been reported in many studies [29, 30, 31, 22]. The reason for the effectiveness of the BAP may lie in its ability to stimulate the plant tissue to metabolize the natural endogenous hormone or could induce the production of natural hormone system for the induction of shoot organogenesis [32].

Sucrose concentration acts as an enhancer of osmotic potential and also plays a vital role in root induction [28]. MS medium with 3% sucrose was used as a source of carbohydrate. The present study also carried out using MS media with slight modification. The sucrose concentration clearly enhances the growth and root induction of *Ceropegia spirali.* 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 spiralis.* The similar results reported in *Ceropegia hirsuta* [33], *Ceropegia spiralis* [34]. In general, growth rate is considered as a function of sucrose concentration [35].

This is a rapid, reproducible protocol for the conservation of this rare, medicinal, ornamental and threatened plant species. *In vitro* raised plants tubers possessed rich iron content and therefore it may be used as an effective medicine for anemia diseases like rapid or irregular heartbeat, pregnancy complications and delayed growth in infants and children.

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