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Full Length Research Paper

Microtuberization of *Ceropegia spiralis* Wight and *Ceropegia pusilla* Wt. and Arn.

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The ability of *Ceropegia spiralis* Wight and *Ceropegia pusilla* Wight and Arn. to form microtubers *in vitro* was studied, to conserve these endemic and endangered species. Murashige and Skoog's (MS) medium supplemented with various concentrations of cytokinins and auxins supported induction of two types of microtubers, that is, basal and aerial tubers. The nodal explants of *C. spiralis* best tuberized on MS medium supplemented with 6-benzylaminopurine (BAP) 13.32 µM + Naphthalene Acetic Acid (NAA) 1.34 to 2.68 µM. The aerial tubers were observed on Indole-3-Butyric Acid (IBA) 49.2 µM + 6% sucrose and Indol-3-Acetic Acid (IAA) 11.54 µM + 3% sucrose augmented ½ MS media. In another case, single roots were tuberized on ½ MS medium supplemented with IAA 11.54 µM + 3% sucrose in *C. spiralis*. Whereas, in *C. pusilla*, the medium containing BAP 13.32µM + 2, 4-D 0.11 µM + 3% sucrose played major role in the induction of basal tubers. Strength of the medium, plant growth regulators (PGRs) concentration and additives played an effective role in the formation of microtubers. If these valuable ornamental and medicinal plants were conserved, we may add another flower to the bouquet and a new drug as well.

Key words: Aerial tubers, basal tubers, *Ceropegia spiralis, C. pusilla,* single root tubers, thin cell layers.

INTRODUCTION

Ceropegia L. is a genus of climbers, herbs and rarely sub shrubs distributed in tropical and sub tropical Asia, Africa, Australia, Malaysia and in the Canary of Pacific Islands (Bruyns, 2003). Almost 200 species have been reported throughout the world. In India, 50 species are present (Surveswaran et al., 2009), out of which 28 spp. are endemic to peninsular India (Ahmedulla and Nayar, 1986), among them, *Ceropegia spiralis* Wt. (Nimmatigadda) and *Ceropegia. pusilla* Wt. & Arn. (Churning stick) are the two endemic and endangered plants. *Ceropegia* tubers are edible and contain an alkaloid called "cerpegin" (Jain and Defillips, 1991). The tubers of *C. spiralis* and *C. pusilla* are used in Ayurvedic drug preparations that are active against many diseases especially diarrhea, dysentery and syphilis. Starchy tubers are used as a nutritive tonic and blood purifier (Prakash et al., 2008; Mabberley, 1987). Since the genus is endemic, more than 30 rare species were confined to Southern Peninsular, India. The Indian *Ceropegia* species cannot be propagated through vegetative stem cuttings (Pandit et al., 2008). As a result, micropropagation methods had emerged as an alternative strategy. The tissue culture studies in *Ceropegia* are as follows: studies on micro-propagation of *Ceropegia juncea*, *C. spiralis*, *Ceropegia intermedia*, *Ceropegia hirsute*, and *Ceropegia sahyadrica* were conducted by Krishnareddy (2011), Murthy et al. (2010), Karuppusamy et al. (2009), Nikam and Savanth (2007), and Nikam et al. (2008), respectively; study on induction of callus *C. pusilla* was conducted by Kondamudi and Murthy (2011); study on micro tuber production in *Ceropegia lawii*, *Ceropegia maccannii*, *C. oculata* and *C. sahyadrica* was conducted by Pandit et al. (2008); and study on Ceropegia bulbosa was conducted by Goyal and Badauria (2006)., *in vitro* production of *Ceropegia candelabrum* (Beena et al., 2003; Beena and Martin, 2003). However, delight approach

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to meet the propagation requirements for reintroduction of these plants is still looked for.

In vitro microtuberization would be an ideal strategy for those plants, if microtubers can be advantageous over the seasonal seeds. 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 fast in soil and thus are the choice of interest for international germplasm transfer (Malaurie et al., 1998). *In vitro* tuberization has proven savior strategy in case of potato (Gopal et al., 2004); whereas, in *Zingiber*, *in vitro* rhizome formation has helped in conservation (Tyagi et al., 2006). Therefore, this study aimed at *in vitro* tuberization in *C. spiralis* and *C. pusilla*.

MATERIALS AND METHODS

In vivo growing *C. spiralis* and *C. pusilla* were collected from Akashaganga of Tirumala hills and Sheveroy hills of Eastern Ghats, India, respectively. The voucher herbarium specimens were deposited in the Department of Biotechnology Herbarium (1671, 1809), Montessori Mahila Kalasala, Vijayawada, Andhra Pradesh, India. The apical and axillary buds were washed in running tap water followed by bavastene / cycloheximide 0.3% for 10 min and tween 20 (5% v/v) for 4 min. After repeated washes in double distilled water, the explants were washed with surface disinfectant $HgCl_2$ (0.1% w/v) for 2 min. The sterilized explants were then washed thoroughly with sterilized double distilled water. The explants were cut into appropriate sizes and cultured on Murashige and Skoog's medium (1962). Different plant growth regulators (PGRs) [6-benzylaminopurine (BAP), Kinetin (Kn), Thidiazuron (TDZ), 2,4-Dichlorophenoxyacetic acid (2, 4-D), Naphthalene Acetic Acid (NAA), Indol-3-Acetic Acid (IAA), Indole-3-Butyric Acid (IBA) and Ascorbic acid] at different concentrations either alone or in combinations were added to the media for the induction of tubers. The pH of the media was adjusted to 5.7 ± 2 with 1 N NaOH and 1 N HCl, and 0.9% (w/v) agar (gelling agent) was added. The media were sterilized in an autoclave at 121℃ and 15 lb/in^2 pressure for 20 min. The cultures were maintained at 20 to 22℃ under 16 hrs light and 8 hrs dark photoperiod with 30 to 40 $\mu Em^{-2} s^{-2}$ light intensity using fluorescent lights (Philips, India Ltd.,) and 80 to 90% relative humidity. All the plants were given a pretreatment in the medium containing auxins at various levels. So that, all the plants have become slightly bulged in their girth by accumulating starch, the bulged portions (aerial tubers) were cut into thin cell layers (TCLs) which responded on Murashige and Skoog's (MS) medium supplemented with cytokinins and auxins within a week.

When the explants failed to induce a specific response by the end of the first cycle, that particular hormonal combination was not considered suitable. Twenty cultures were used for each treatment and all experiments were repeated thrice. The response was observed weekly. Tuberization percentage was measured as number of explants responded / total number of explants used × 100. The data was analyzed statistically using one way analysis of variance (ANOVA), and the data means ± SD of at least three different experiments were represented and compared using Tukey-Kramer multiple comparisons test with the level of significance P = 0.05.

RESULTS

The *in vitro* raised shoots of *C. spiralis* (Figure 1A) were sub cultured on to the tuberization media. In *C. spiralis,* BAP 13.32 µM + NAA 2.68 µM resulted in 84% of tuberization (Table 1). Maximum size tubers were observed on MS medium supplemented with 3% sucrose and BAP 13.32 µm along with 1.34 or 2.68 µM NAA (2.00 ± 0.48 cm) (Table 1, Figure 1B). Whereas, in the medium containing IBA 49.2 µM, the plants were tuberized with the diameter of 1.54 ± 1.50 cm. On ½ MS medium supplemented with 3% sucrose and NAA 5.37 µM alone induced tubers with 1.47 ± 0.22 cm of diameter. Kn in combinations with auxins (IBA) played meager roles in the induction of *in vitro* tuberization in *C. spiralis*.

The responses were varied from one explant to the other viz. callusing, flowering and multiple shoot formation (Figure 1C).

Single root tuberization was also observed. Some of the roots were tuberized after rooting on ½ MS + 11.54 µM IAA with 3% sucrose (Figure 1D). In *C. spiralis*, 3% sucrose played a major role in the induction of microtubers *in vitro*; the increase or decrease in the sucrose concentrations was not suitable for the tuberization. BAP 13.32 µM favored *in vitro* tuberization, but increase or decrease in the BAP concentrations resulted in the failure of tuberization as the plant undergoes necrosis. Auxins played an important role in tuberization of *C. spiralis.* The internodal regions of plant were able to form aerial tubers (Figure 1E) on ½ MS media supplemented with 49.2 µM IBA and 6% sucrose and on ½ MS supplemented with 11.54 µM IAA and 3% sucrose (Table 1).

The *C. pusilla* shoots (Figure 1F) were transferred onto the tuberization medium. MS medium supplemented with 13.32 µM BAP + 0.11 µM 2,4-D with 3% sucrose was favorable for induction of microtubers which had 2.49 ± 0.01 cm diameter (Table 2). The other medium containing BAP 13.32 µm + NAA 0.53 µM with 3% sucrose induced microtubers with the diameter of 1.74 ± 0.01 cm which bears roots at their base (Figure 1G). Whereas, the medium supplemented with TDZ 0.90 µM with 3% sucrose helped the microtubers to enlarge up to 1.49 ± 0.01 cm in diameter. The combination of BAP, Kn, and TDZ along with ascorbic acid also favored the *in vitro* tuberization (Table 2). In addition to cytokinins, ascorbic acid was used as an additive to induce *in vitro* tubers in *C. pusilla.* More nutrient is needed for vegetative growth which may lead to the depletion of nutrients in the medium and triggers the tuberization mechanism.

The *in vitro* tuberization in *C. pusilla* was good when compared to *C. spiralis* because the tubers are more or less uniform in their size (Figure 1H). The microtubers were induced within a month at temperature and photoperiods prevailed in the laboratory.

Some of the microtubers are hairy in their nature and grew slowly, some reaching up to 100 mm long after 4 months. When the nutrients in the medium depleted along with sucrose, the plants undergo flowering or tuberization (basal/aerial) but not vegetative growth. So,

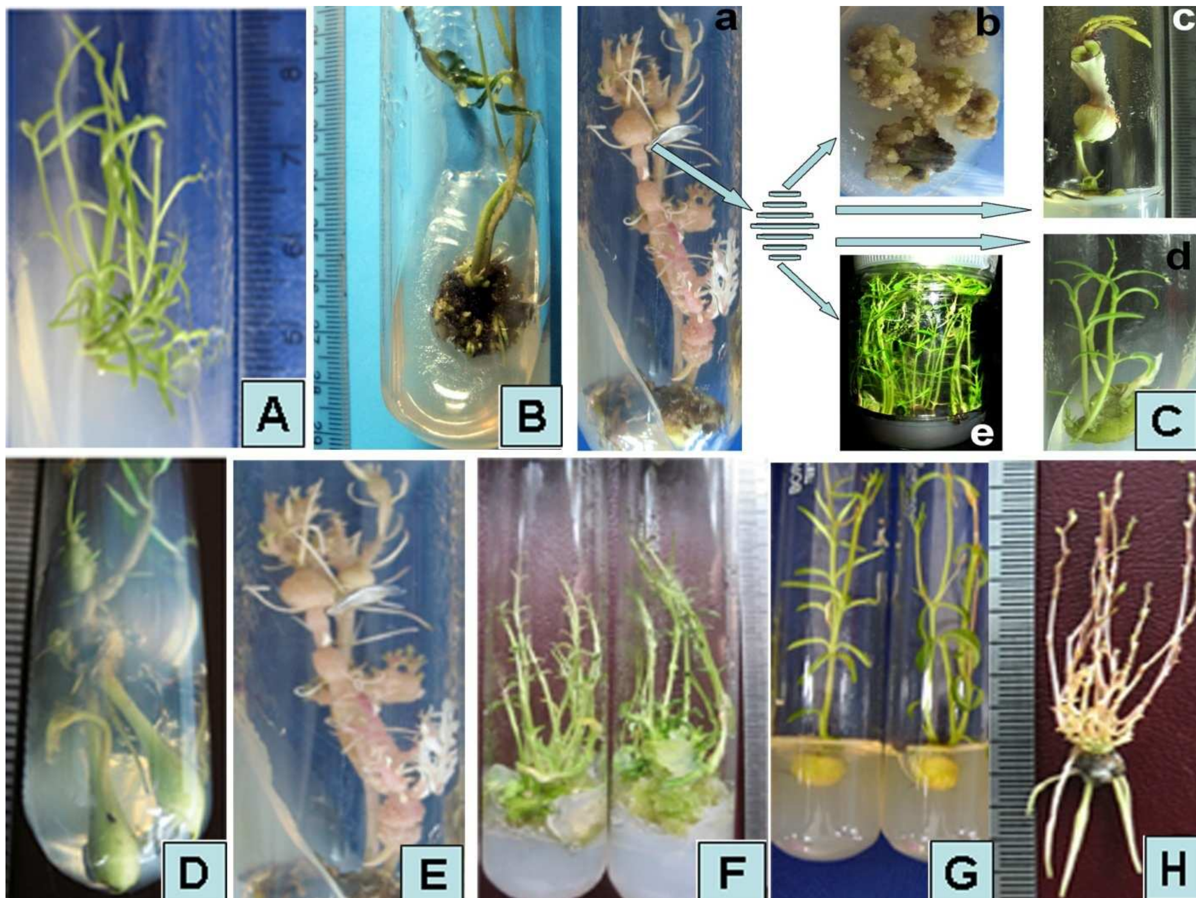

Figure 1. Micro tuberization- a conservative method in *Ceropegia spiralis* Wt. and *Ceropegia pusilla* Wt. & Arn. (A) Multiple shoots of *C. spiralis* on BAP 2.22 µM supplemented medium. (B) Underground tubers on MS + BAP 13.32 µM along with NAA 1.34 and 2.68 µM induced similar sized (2.00 ± 0.48 cm) microtubers in *Ceropegia spiralis*. (C) Illustration showing the deferent responses of an aerial tuber TCLs of *C. spiralis*; a. - A plant with aerial tubers, b. - An extensive callus from the aerial tuber TCLs, c. - *In vitro* flowering from the aerial tuber TCLs, d. - Shoots and Flower buds from the aerial tuber TCLs, e. - Induction of multiple shoots from the aerial tuber TCLs. (D) Single root tuberization on ½ MS + IAA 11.54 µM in *C. spiralis*. (E). Aerial tubers on ½ MS + IBA 49.2 µM with 6% sucrose and ½ MS + IAA 11.54 µM with 3% sucrose in *C. spiralis*. (F) Multiple shoots of *C. pusilla* on TDZ 22.7 µM supplemented medium. (G) Tubers bearing roots on MS medium supplemented with BAP 13.32 µM + NAA 0.53 µM in *C. pusilla*. (H) Underground tubers on MS+ BAP 13.32 µM +2, 4-D 0.11 µM induced similar sized (2.49 ± 0.01) microtubers in *C. pusilla*.

the basal and/or aerial tuberization was observed in *C. spiralis*.

The *in vitro* tubers of *C. spiralis* and *C. pusilla* were similar to that of *in vivo* tubers in their morphology. On the other hand, in *C. spiralis*, most of the tubers developed from the roots were spindle in their shape. All the tubers were white in colour. As they enlarged, the external surfaces of the tubers turned into brown colour. Some of the tubers originated in clusters called multiple tubers or secondary tubers.

DISCUSSION

The formation of microtubers is influenced by several factors like type and strength of medium, sucrose, auxins and cytokinins concentration as well as temperature and photoperiod etc. (Skoog and Miller, 1957; Madee, 1963; Hussey and Stacey, 1984; Le, 1999; Uranbey et al., 2004; Islam et al., 2008). The response of microtuber induction is also varied with the species and explant. The main factors under consideration in the present work are strength of the medium, sucrose concentration, and auxins and cytokinins levels in the medium.

The meristematic initials uninterruptedly respond as per the stimuli of PGR in culture medium and then the culture conditions. Therefore, it is clear that the response of aerial tuber's TCLs strongly suggests that the aerial tubers are far superior to any other explant in the plant body in rapid regeneration.

Table 1. Influence of medium strength, sucrose concentration and PGRs on *in vitro* tuberization in *C. spiralis.*

PGR in µM						Medium strength	Sucrose (%)	Diameter of the tuber (cm) Mean ± SD	*In vitro* tuberization percentage	Nature of the tubers
Kn	IBA	IAA	NAA	2-4,D	BAP					
	0.12					½	6	-	10	Single root
	49.2					Full	6	-	20	Aerial
	49.2					¼	6	-	10	Aerial
	49.2					½	6	1.54 ± 1.50[b]	70	Aerial
	4.92					½	3	-	10	Basal
	9.84					½	3	-	10	Basal
	49.2					½	3	-	10	Basal
		5.77				½	3	-	10	Basal
		11.54				½	3	-	10	Aerial / Single root
		46.16				½	3	0.50 ± 0.06[d]	33	Basal
			0.02			½	3	-	10	Single root
			5.37			½	3	1.47 ± 0.22[b]	70	Basal
			10.74			½	3	2.10 ± 0.28[a]	70	Basal
			53.7			½	3	-	10	Basal
4.56	4.92					½	3	0.89 ± 0.17[c]	20	Basal
9.12	9.84					½	3	1.44 ± 0.2[b]	60	Basal
13.68	0.49					Full	3	-	10	Single root
13.68		0.57				Full	3	-	10	Single root
	0.49				13.32	Full	3	-	10	Basal
	1.23				13.32	Full	3	-	10	Basal
	2.46				13.32	Full	3	-	10	Basal
	4.92				13.32	Full	3	-	10	Basal
		0.57			13.32	Full	3	0.54 ± 0.08[d]	50	Basal
		1.44			13.32	Full	3	0.63 ± 0.10[d]	60	Basal
		2.88			13.32	Full	3	0.74 ± 0.07[cd]	60	Basal
		5.77			13.32	Full	3	0.55 ± 0.07[d]	33	Basal
			0.53		13.32	Full	3	-	10	Basal
			1.34		13.32	Full	3	2.00 ± 0.48[a]	80	Basal
			2.68		13.32	Full	3	2.00 ± 0.48[a]	84	Basal
			5.37		13.32	Full	3	-	10	Basal
				0.45	13.32	Full	3	0.71 ± 0.14[cd]	50	Basal
				1.13	13.32	Full	3	0.74 ± 0.06[d]	50	Basal
				2.26	13.32	Full	3	0.80 ± 0.23[c]	50	Basal
				4.52	13.32	Full	3	0.53 ± 0.15[d]	33	Basal

* Data indicate mean ± standard deviation of the mean followed by the same letter was not significantly different by the Tukey-Kramer multiple comparison test at 0.05 probability. Ten replicates were used per treatment experiments and each practical was repeated at least thrice.

Table 2. Influence of PGRs on *in vitro* tuberization in *C. pusilla.*

PGR in µM							*In vitro* tuberization percentage	Diameter of the tuber in cm (mean ± SD)
Kn	BAP	TDZ	IBA	NAA	2,4-D	IAA		
		0.45					70	1.18 ± 0.11[b]
		1.36					60	1.17 ± 0.01[b]
		2.27					60	1.23 ± 0.01[b]
	0.44						20	0.49 ± 0.01[d]
	1.33						60	1.19 ± 0.01[b]
	2.22						-	-
0.45							-	-
1.36							20	0.49 ± 0.01[d]
2.28							33	0.59 ± 0.01[c]
0.45	0.44	0.45					60	1.22 ± 0.01[b]
		0.90					70	1.49 ± 0.01[b]
		2.72					60	0.99 ± 0.01[c]
		3.17					60	0.94 ± 0.01[c]
		3.63					60	1.04 ± 0.01[b]
		4.54					60	1.23 ± 0.01[b]
	13.32					5.77	60	0.99 ± 0.01[c]
	13.32				0.45		60	0.99 ± 0.01[c]
	13.32				0.11		70	2.49 ± 0.01[a]
	13.32			0.53			70	1.74 ± 0.01[a]
	13.32		2.46				50	0.49 ± 0.01[d]
	13.32		1.23				50	0.49 ± 0.01[d]

*Data indicate mean ± standard deviation of the mean followed by the same letter was not significantly different by the Tukey-Kramer multiple comparison test at 0.05 probability. Ten replicates were used per treatment experiments and each practical was repeated at least thrice.

The role of cytokinin and auxins combination in the tuber formation was observed by Badoni and Chauhan (2010) and Islam et al. (2008). The role of Kn in *in vitro* tuberization was best supported by Britto et al. (2003) and Patil (1998). The cytokinin alone, and in combination with auxins were reported to increase the frequency of *in vitro* tuberization in a number of yam species, with Kn resulting in the best tuberization (Uranbey, 2005). Hoque (2010) revealed that the higher concentrations of Kn had potentiality to produce heavier microtuber than lower concentrations. On the same line, Le (1999) revealed that BAP alone had significant effect on microtuber diameter and fresh weight as compared to its combination with Kn. The BAP and other cytokinins were found to stimulate the tuberization process (Hussey and Stacey, 1984). The increase or decrease in the sucrose concentrations did not support the tuberization which is in accordance with the findings of Patil (1998) and Ovano et al. (2007). Any deviation from the optimum BAP concentrations resulted in the failure of tuberization as the plant undergoes necrosis (Pandit et al., 2008). Zakaria et al. (2008) supported the high concentrations of BAP and its role in the induction of microtubers. Many workers strongly supported the apical bud as the most suitable explant for the tuberization when compared to node (Pandit et al., 2008; Britto et al., 2003; Patil, 1998).

Cytokinin is known to stimulate cell division (Skoog and Miller, 1957). There are indications that it inhibits cell elongation (Vanderhoef and Key, 1968), and is required for the tuber formation and development. Madec (1963) opined that the unknown tuberization stimulus could be a cytokinin-like substance. Although cytokinin is not directly responsible for tuberization as reported by many workers, without doubts it plays a key role in cell division, thus creating sink activity of the developing tuber. A known fact is that cytokinins are synthesized in the roots and plays a vital role in cell divisions. The auxins had the rooting activity. Both these activities resulted in the meager growth of the shoots and vice versa with roots. Hence, the roots will become more active when compared to shoots. The increased activity demands more amount of photosynthates/nutrients from the media etc. The basal and/or aerial tuberization was also observed in *Dioscorea rotundata* and *Dioscorea cayenensis* due to the depletion of nutrients in the medium (Mbanaso et al., 2007). From the sections of aerial parts, differential responses can be achieved in shorter period (Teixeira da Silva, 2003). The optimum temperature for the induction of microtubers was 20 to 22℃, and the results are in agreement wi th Uranbey et al. (2004). The addition of ascorbic acid (AA)

controls the basal callusing and improves multiple shoot forming ability (Ahuja et al., 1982; Richard et al., 1988; Sharma and Chandel, 1992; Komalavalli and Rao, 1997).

Conclusion

In conclusion, we have described phenomenon to multiply the endangered taxa through basal tubers and aerial tubers. The rapidity and the dependability of the tissue culture protocols always help in quick conservation of these ornamental and medicinal important endangered taxa. These efforts must be backed by the appropriate *ex vitro* conservational strategies. On the other hand, by adopting protocols like this, pharma companies can get the product as well as reduce the pressure on the wild population. We will reveal through this work, the truth of this claim, and that the application and success of TCL system is widespread. The possibilities of this tool for crop (ornamental and floricultural, endangered) improvement are endless, and go tightly hand-in-hand with molecular and genetic engineering tools. Moreover, this system provides a means of mass propagation of a species of interest.

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