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Towards Conservation of Threatened *Ceropegia* Species Endemic to a Biodiversity Hotspot: *In Vitro* Microtuber Production and Proliferation, a Novel Strategy

Sagar Subhash Pandit[1,3]*, Aneeshkumar Nair[1], and Dhiraj Dilip Naik[2]

[1]*Plant Molecular Biology Unit, Division of Biochemical Sciences, National Chemical Laboratory, Dr. Homi Bhabha Road, Pune-411008, MS, INDIA*

[2]*Department of Biology, West Virginia University, 53 Campus Drive, P.O. Box 6057, Morgantown, WV-26506, USA*

[3]*Max Planck Institute for Chemical Ecology, Department of Molecular Ecology, Beutenberg Campus, Hans-Knöll-Str. 8, 07745-Jena, Germany (Present address)*

ABSTRACT : Twenty-eight of 44 Indian *Ceropegia* species are endemic and their survival is threatened. As a step towards conservation, we implied *in vitro* methods for the sustainable propagule production in *C. hirsuta*, *C. lawii*, *C. maccannii*, *C. oculata* and *C. sahyadrica*. Effects of explant, growth regulators, sucrose and photoperiod were studied. High frequency microtuber production was achieved with the seedling-apical buds, grown on MS medium containing 4-6 mg l^{-1} BAP, 3-8% (w/v) sucrose, under continuous illumination. Each microtuber, when subcultured proliferated to form a cluster of secondary microtubers. Every primary and secondary microtuber bore at least one shoot-bud and a root primordium. Each tuber (formed with any of the significantly effective treatments) weighed more than 500 mg, enough to plant directly in non-sterilized soils. Microtubers could be produced and proliferated round the year. Proliferation could be solely attributed to *in vitro* procedures as these plants bear solitary tubers *in vivo*. Microtubers could be sprouted *in vitro* to prepare ready to pot plantlets. As, this novel method succeeded for all five species, though they belong to different eco-physiological backgrounds, we recommend its implementation in the conservation programs for a broader range of *Ceropegia* species, supported by other integrated strategies.

Keywords : Asclepiadaceae, Endangered species, *Ex situ* conservation, Microtuberization, Western ghats

Abbreviations : 2,4-D: 2,4 dichlorophenoxyacetic acid, BAP: N^6 benzyl amino purine, KN: Kinetin, NAA: Naphthaleneacetic acid, IAA: Indoleacetic acid, IBA: Indolebutyric acid, PGR: plant growth regulator

INTRODUCTION

Ceropegia (Asclepiadaceae) is a genus of tuberous plants (climbers, erect herbs and subshrubs) that comprises about 200 species. Most of the Indian species of *Ceropegia* are the relict endemics to the different eco-geographical zones of the two biodiversity hotspots, the East-Western Himalayas and the Western ghats of India. With 28 out of total 44 species (Ansari, 1984) *Ceropegia* dominates the list of Indian endemic plants. Northern ranges of Western ghats (Maharashtra state) (Fig. 1), harbor considerable diversity of *Ceropegia* species with 16 species being strictly endemic to this region.

Tubers of *Ceropegia* are edible (Mabberley, 1987) and contain starch, sugars, gum, albuminoids, and a medicinally important alkaloid ceropegin (Anonymous,1980; Jain and Defilips, 1991). Overexploitation of *Ceropegia* species for these tubers by the human and various animals, abreast to the factors such as endemicity, small and localized populations, and severe anthropogenic pressures on the forest-land have caused their decline in the wild; influence of human activities is intense especially in the Northern zone of Western ghats which has been a subject of rapid urbanization in the recent past because of its proximity to

* Corresponding author: (E-mail) spandit@ice.mpg.de

the international and economic hub-city, Mumbai (Fig. 1).

Natural propagation of these plants is not at a pace to overcome the exploitation and destruction. Propagation from seeds is held back by low germination and survival rates due to the inept environmental conditions. As a result their populations are squeezing at an alarming rate. Such conditions have also thinned the possibilities of *in situ* conservation of these plants. Thus, *Ceropegia* species in this region call for salvaging attempts before the *coup de grâce* is struck.

In vivo vegetative propagation methods through stem cuttings that are well established for many of the American and European *Ceropegia* species (McNew, 2002; Hodgkiss, 2007; Reynolds, 2006), can not be employed for the Indian species, as they do not respond to such practices, probably because of their specialized adaptations to respective microclimatic pockets. Consequently, to empower these taxa, conservation measures supported by *in vitro* methods have been asked for (Patil, 1998; Walter and Gillett, 1998; Beena et al., 2003). On the brighter side, these plants have responded promisingly to the *in vitro* experiments, signifying the utility of such technology for them (Patil, 1998; Beena et al., 2003; Britto et al., 2003; Aneesh Kumar et al., 2007). However, diligent approach to meet the propagation requirements for reintroduction of these plants is still awaited.

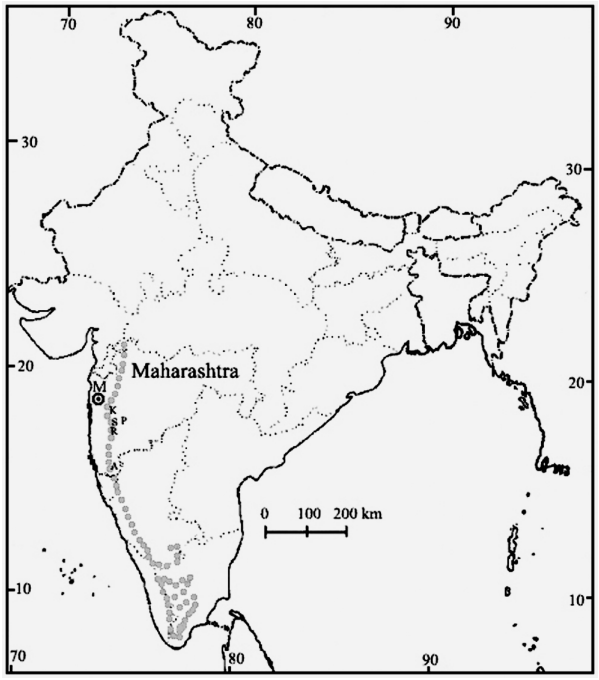

Fig. 1. Map of India showing the crest line (grey dotted) of Western Ghats, Mumbai (M), the capital of Maharashtra state and collection localities (from north to south) Khandala (K), Pune (P), Sinhagad (S), Rajgad (R) and Amboli (A) for *C. oculata*, *C. hirsuta*, *C. maccannii*, *C. lawii*, and *C. sahyadrica*, respectively.

As the *in vivo* tubers, because of their solitary nature do not ensure the multiplication of plants, *in vitro* microtuberization would be an ideal strategy for these plants if microtubers can be yielded throughout the year and would be advantageous over the seasonal seeds (Fay, 1992). 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 is a proven savior strategy in case of potato (Kwiatkowski et al., 1988; Gopal et al., 2004) and yam (Malaurie et al., 1998) germplasms whereas in *Zingiber in vitro* rhizome formation has helped in conservation (Tyagi et al., 2006). Accordingly, in the present experimentation, we attempted '*in vitro* tuberization' in five species of *Ceropegia C. hirsuta* Wt. *et* Arn, *C. lawii* Hook, *C. maccannii* Ansari, *C. oculata* Hook and *C. sahyadrica* Ansari *et* Kulkarni. *C. hirsuta* is a widely distributed species whereas other four occur restrictedly in the pockets of Western ghats, particularly in the state of Maharashtra (Fig. 1) and are threatened (Table 1) (Ansari, 1984; Walter and Gillett, 1998).

MATERIALS AND METHODS

Plant materials of *C. hirsuta*, *C. lawii*, *C. maccannii*, *C. oculata* and *C. sahyadrica* were collected from different localities in the Northern-Western ghats (Maharashtra, India) such as Pune, Rajgad, Sinhagad, Khandala and Amboli, respectively (Fig. 1; Table 1).

The *in vitro* cultures were established using the seeds derived from mature follicles. Follicles were surface sterilized, dissected to obtain seeds which were allowed to germinate *in vitro* as descried by Aneesh Kumar et al.

Table 1. Occurrence information and IUCN Red list status of *Ceropegia* spp used in the present study (Ansari, 1984; Walter and Gillett, 1998).

Name of the species	Habit	Collection locality	Distribution	Distribution status	Threat status
C. hirsuta	Climbing herb	Pune	India	Wide	Data deficient
C. lawii	Erect herb	Rajgad	Maharashtra	Endemic	Endangered
C. maccannii	Erect herb	Sinhagad	Maharashtra	Endemic	Rare
C. oculata	Climbing herb	Khandala	Maharashtra	Endemic	Rare
C. sahyadrica	Erect herb	Amboli	Maharashtra	Endemic	Rare

(2007). Explants were either derived directly from the *in vitro* raised seedlings or from the *in vitro* established plantlets regenerated from the seedlings. Explants used from the seedling were root, cotyledon, hypocotyl, axillary and apical buds. Similarly, axillary and apical buds from the regenerated shoots were also used as explants. All the explants were inoculated with standard as well as inverted orientation. Primary microtuber clusters obtained in all the media compositions were dissected and sub-cultured on fresh medium of same respective composition; resultants were also tested with all the condition sets that were tried for the primary induction of tubers and were subjected to 20 sub-cultures in the same respective medium every 15 days.

All explants were tested in MS medium (Murashige and Skoog, 1962) with the combinations of BAP, KN (both the PGRs 0.0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 mg l^{-1}), IAA, NAA, IBA and 2, 4-D (all 0.0, 0.5, 1.0, 1.5, 2.0 and 3.0 mg l^{-1}), sucrose (3.0, 4.0, 5.0, 6.0, 7.0 and 8.0%, w/v), photoperiod (8 h, 16 h and uninterrupted till harvesting) at 40 μmol m^{-2} s^{-1} and incubation (1 week to three months). Basal medium and culture conditions used for *in vitro* tuberization were same throughout the experiment unless specified otherwise. Medium pH was adjusted to 5.8 using 0.1 N NaOH. Agar agar (0.8%, w/v) was added for gelling. Media were autoclaved at 121℃ and 15 psi for 20 minutes.

Number and weight of microtubers produced by each responsive explant were recorded after every 5 days. All the experiments were repeated thrice using a minimum of 30 explants. Data were analyzed by one-way and two-way ANOVA. Percentages were subjected to arcsine transformation. Significant differences between the means were assessed using Duncan's multiple range test (Duncan, 1955) at $P \leq 0.05$.

RESULTS

Morphology of microtubers

Microtubers induced with all the variations in explant type, growth regulator type and concentrations, sucrose concentration and photoperiod were morphologically similar. One to six microtubers were formed in a cluster at the base of responsive explants (Fig. 2a, b); these *de novo* formed (primary) microtubers with initial explant trimmed, when sub-cultured on the same respective media, they proliferated to form a cluster of new secondary tubers (Fig. 2c). Primary and secondary microtubers were morphologically alike. Primary microtubers were initially green; at about 10 days of incubation, their outer layer started turning brown and the inner cell mass turned opaque white; these features resembled those of *in vivo* tubers. Microtubers generated in all culture condition combinations showed presence of at least one shoot bud and a root primordium. In all five species, tubers weighed more than 500 mg after 45 days of incubation.

Effect of explant

In preliminary experiments, among the various explants tested, we found that only apical buds of *in vitro* germinated seedlings successfully produced microtubers (Fig. 2a, b). After 20 days of transfer to the tuberization medium, seedling-apical buds showed bud break and subsequent elongation of shoots. Microtubers were formed at the base

of each aerial shoot without the formation of any intermediary callus after 30 days. Axillary buds from the same seedlings could not bear tubers. Apical and axillary buds from the regenerated shoots also could not develop tubers; instead, they developed shoots bearing floral buds and subsequently formed flowers (Aneesh Kumar et al., 2007). All the explants including the apical bud could not produce microtubers when inoculated with reversed polarity (data not shown).

Though the apical bud was absolutely necessary for microtuberization, the primary microtubers produced by these buds served as the best explant for generation and multiplication of tubers *in vitro*. Primary microtubers when separated and sub-cultured on the same medium, a cluster of new secondary tubers started forming around each of them after 15 days (Fig. 2c); this way, proliferation continued up to 45 days. After 45 to 55 days of incubation, multiplication congested and later microtubers sprouted. Each secondary microtuber, when separated and sub-cultured on the same medium every 15 days, continued proliferation with the same rate even up to 20 subcultures. Primary or secondary microtuber clusters when transferred to fresh media without separating the units, could not multiply further.

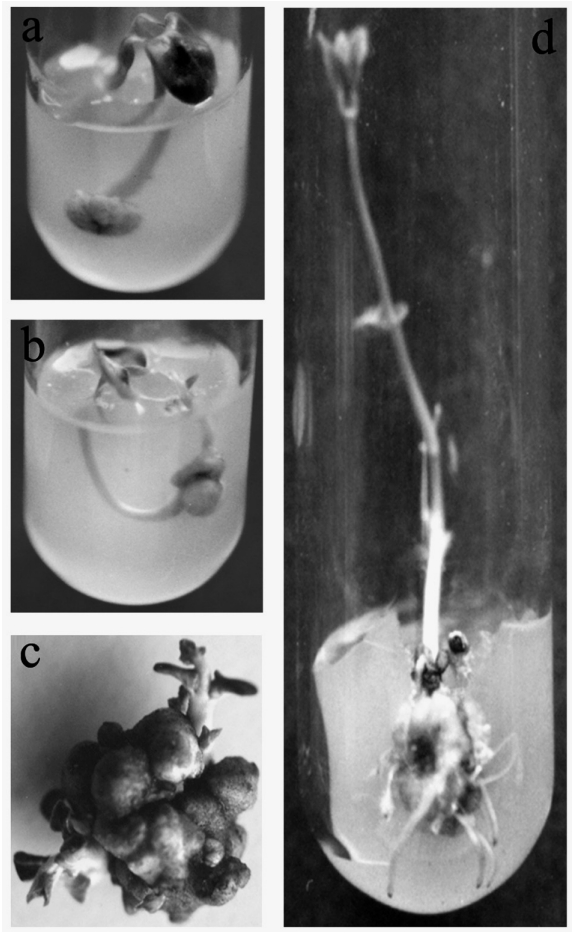

Fig. 2. a. Tuber formation at the base of the seedling-apical bud explant of *C. hirsuta*; **b.** multiple tuber formation in *C. lawii*; explant; **c.** secondary tuber clusters of *C. sahyadrica*; **d.** germinated tuber with well developed multiple shoots and roots of *C. maccannii*.

Effect of PGRs on microtuberization

Among the auxins (NAA, IAA, IBA and 2, 4-D) and cytokinins (BAP and KN) tested only BAP showed significant effect on tuber induction (Table 2). MS medium with IBA and IAA showed rare incidences of tuber formation along with callusing and subsequent adventitious rooting, while the use of KN resulted in multiple shoot formation and tuberization in rare events. Due to this inconsistency, tubers formed by the influence of PGRs other than BAP were not used in the further experiments and are also not mentioned in any further results. We also observed significant differences between PGR treatment effect. On the medium containing BAP (2-6 mg l^{-1}), tubers developed within a period of 25 to 40 days. At higher concentrations of BAP (4-6 mg l^{-1}) tuberogenesis was early (25-30 days) and vigorous (Table 3). The frequency of tuber formation increased with the increasing concentration of BAP (Table 3). The highest percentage of tuber producing shoots was obtained on MS medium supplemented with 6 mg l^{-1} BAP. Concentrations of BAP above 6 mg l^{-1} caused early (5-10 days) necrosis in the explants and could not induce tuberization.

All primary microtubers proliferated in all the concentrations of BAP between 2 and 6 mg l^{-1} (Table 4). BAP concentrations below 2 mg l^{-1} resulted in less sprouting whereas the concentrations above 6 mg l^{-1} almost stopped the proliferation and induced malformations in microtuber clusters. Similar to primary induction, microtuber proliferation was and mean fresh weight of tubers was also highest

Table 2. Effect of different growth regulators and their altered concentrations on frequency of primary microtuber formation and microtuber fresh weight in seedling-apical bud cultures of *Ceropegia* spp. in MS media with 4% (w/v) sucrose and under continuous illumination, recorded after 30 days of incubation.

Growth regulators	Microtuber formation (%) at varied concentrations (mg l^{-1}) of each growth regulator			Microtuber fresh weight (mg) at varied concentrations (mg l^{-1}) of each growth regulator		
	1.25	2.5	5.0	1.25	2.5	5.0
IAA	2.3±0.1a	2.2±0.6a	3.0±0.3a	0.1±0.03a	0.2±0.02a	0.2±0.03a
NAA	0	0	0	0	0	0
IBA	1.3±0.3a	2.4±0.3a	3.4±0.4a	0.2±0.02a	0.2±0.02a	0.3±0.01a
2,4-D	0	0	0	0	0	0
BAP	38.9±0.8a	88±0.8b	100±0.8b	0.4±0.04a	0.6±0.05b	0.8±0.06b
Kinetin	3.0±0.6a	3.2±0.4a	5.5±0.2a	0.2±0.05a	0.4±0.03a	0.4±0.03a

BAP, at all its concentrations, showed significant effect on microtuber formation and microtuber fresh weight as compared to that of all other growth regulators and therefore, this comparison is not suffixed by any statistical denotations. For the effect of different concentrations of each growth regulator, significance is denoted by alphabets where, values within a row followed by a similar letter were not significantly different according to one way ANOVA ($F [5, 5.67] = 22.19$, $P \leq 0.05$).

Table 3. Effect of BAP and photoperiod on microtuberization in seedling-apical bud cultures of *Ceropegia* spp. in MS media with 4% (w/v) sucrose, recorded after 30 days of incubation.

Species name	BAP concentration (mg l^{-1})	Percentage of shoots forming tubers		Mean fresh weight of primary tuber (g) (Mean ± SE)	
		16 h photoperiod	24 h photoperiod	16 h photoperiod	24 h photoperiod
C. hirsuta	2.0	24.8a	36.8ab	0.9±0.04a	1.3±0.03a
	4.0	96.4c	100.0c	1.8±0.05a	2.2±0.08ab
	6.0	100c	100.0c	2.6±0.09b	3.1±0.03b
C. lawii	2.0	42.8b	48.3b	1.4±0.3a	1.6±0.04a
	4.0	94.8c	100.0c	2.9±0.3b	3.3±0.05b
	6.0	100.0c	100.0c	6.3±0.4c	6.9±0.05c
C. maccannii	2.0	21.5a	34.6ab	0.8±0.05a	1.5±0.03a
	4.0	49.5b	58.3b	0.9±0.07a	2.3±0.03ab
	6.0	68.9b	74.5bc	1.7±0.03a	2.9±0.05b
C. oculata	2.0	15.6a	24.1a	1.4±0.03a	1.9±0.03a
	4.0	44.8b	56.9b	2.0±0.09ab	2.8±0.02b
	6.0	72.3bc	83.6c	3.4±0.03b	3.9±0.08b
C. sahyadrica	2.0	35.8ab	54.3b	1.3±0.5a	1.9±0.05a
	4.0	96.8c	100.0c	3.8±0.9b	4.2±0.06b
	6.0	100.0c	100.0c	6.3±0.2c	6.8±0.05c

Significance of the effect of each parameter was tested by Duncan's multiple range test where, means followed by the same letter are not significantly different from each other ($F [1, 324] = 19.27$, $P \leq 0.05$).

Table 4. Proliferation of the microtubers in *Ceropegia* spp. at 6 mg l^{-1} BAP, 4% (w/v) sucrose and continuous illumination.

Name of the species	No. of secondary tubers per primary tuber explant (Mean ± SE)			Fresh weight of secondary tuber (g) (Mean ± SE)		
	15 days	30 days	45 days	15 days	30 days	45 days
C. hirsuta	2.0±0.9a	6.8±0.3a	14.2±1.6a	2.9±0.6a	14.6±1.6a	34.2±2.6a
C. lawii	4.5±1.3ab	15.0±1.6b	31.3±1.9b	6.4±1.3b	32.1±2.4b	75.2±3.5b
C. maccannii	1.7±0.2a	5.8±0.8a	12.1±0.9a	2.5±0.4a	12.4±1.4a	29.1±2.1a
C. oculata	5.3±1.6b	5.4±0.6a	11.2±1.2a	2.6±0.9a	11.5±1.2a	27.0±1.9a
C. sahyadrica	4.3±0.9ab	14.3±1.3b	29.9±1.6b	6.1±0.7b	30.7±1.6b	71.8±3.4b

For all five species, at every 15 days' interval, microtuber multiplication was significant and therefore, this comparison is not suffixed by statistical denotations. Significance of species-specific response is shown by alphabetical denotations where, means within a column followed by the same letter are not significantly different from each other according to one way ANOVA ($F [4, 5.86] = 20.36$, $P \leq 0.05$).

in 6 mg l^{-1} BAP. In none of the examined species, PGRs other than BAP could induce microtuber proliferation.

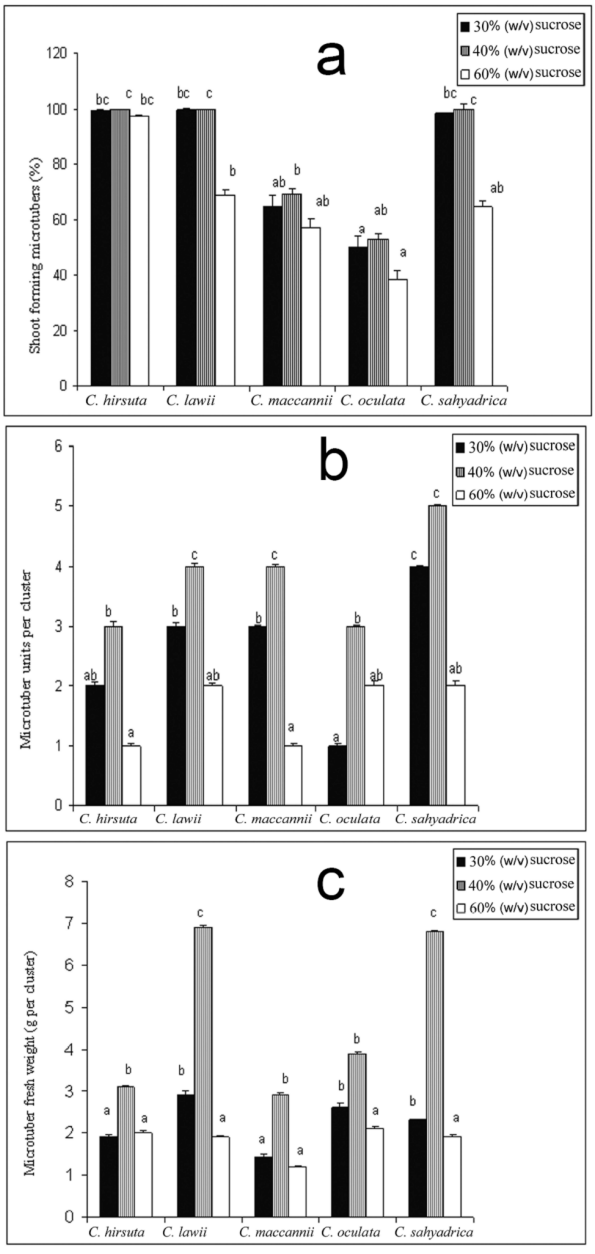

Fig. 3. Influence of sucrose treatment on primary microtuberization in *Ceropegia* spp. (in MS with 6 mg l^{-1} BAP, continuous illumination and recorded after 30 days). **a.** Percent shoots forming microtubers ($F_{[2,\ 252]} = 5.39$, $P \leq 0.05$); **b.** Number of primary microtuber units per culture vessel ($F_{[2,\ 252]} = 6.22$, $P \leq 0.05$); **c.** Fresh weight of microtuber cluster (g) ($F_{[2,\ 252]} = 5.00$, $P \leq 0.05$). For each species, bars with same letters are not significantly different at $P \leq 0.05$.

Effect of sucrose on tuberization

All the concentrations of sucrose (3-8%, w/v) coupled with the optimum concentration of BAP (6 mg l^{-1}) allowed microtuberization, whereas sucrose alone failed to induce tuberogenesis. A significant increase was observed in number of responding explants (Fig. 3a), number of tubers per explant (Fig. 3b) and fresh weight of the tubers (Fig. 3c) at 4% (w/v) sucrose and values of all the parameters significantly decreased above and below 4% (w/v) sucrose.

Use of 4% (w/v) sucrose also accelerated the proliferation of microtubers. This rate drastically lowered down above 6% (w/v) sucrose. Sucrose concentrations of and above 8% (w/v) (with or without BAP) were detrimental for the cultures. Sucrose effectively influenced the fresh weight of secondary tubers; in all the species maximum tuber weight was gained when 4-5% (w/v) sucrose was supplied in the medium. Trends of primary as well as secondary tuberization in response to sucrose were similar in all five species.

Effect of day length on tuberization

Primary microtuber formation was observed only when the cultures were incubated at the day length of 16 h or more (Table 3). Highest rate was recorded at continuous illumination and the light intensity of 40 μmol m^{-2} s^{-1}. Photoperiod was found to be critical only in case of primary tuberization; however, though at lower rate, tuber proliferation occurred even at 8 h day lengths. Primary as well as secondary tubers formed with continuous illumination were large, more in number and their fresh weight was higher than that of the ones produced at 16 h day lengths (Table 3). All five species responded similarly to the variation in photoperiod.

Species specific response

The set of conditions under which maximum tuberization was achieved was same for all five species (6 mg l^{-1} BAP, 40 g l^{-1} sucrose and continuous illumination) (Table 3) (Fig. 3a). In all five species, tuber proliferation pro-

gressed with a significant rate when noted every 15 days' interval (Table 4). Variation by species was observed in the number of tuber forming shoots, mean weight of primary tubers and the number of primary tubers per explant (Table 3) (Fig. 3b). With 4 and 6 mg l^{-1} BAP, all the explants of *C. hirsuta*, *C. lawii* and *C. sahyadrica* developed tubers however, in *C. hirsuta*, mean tuber weight (g) was far lower (3.1±0.03) than that of *C. lawii* (6.9±0.05) and *C. sahyadrica* (6.8±0.05). Highest mean fresh weight of tuber was recorded in *C. lawii* (6.9±0.05) closely followed by that recorded in *C. sahyadrica* (6.8± 0.05) (Fig. 3c). The tuber forming capacities of *C. oculata* and *C. maccannii* were significantly lower than the other three species; multiplication rate and tuber size were also low in these two species (Table 4).

Sprouting of tubers

Primary as well as secondary microtubers sprouted after 45 days of incubation in all BAP and sucrose concentrations. Quick sprouting (within 10 days) was observed in all the microtubers when transferred to MS media with 1-1.5 mg^{-1} BAP and illuminated for 8 or 16 h. With continuous illumination, this sprouting period could be delayed up to 30 days. Shoot bud of each tuber developed a clump of shootlets and roots developed from the primordia (Fig. 2d). These *in vitro* germinated microtubers were ready for potting and appeared like the miniaturized *in vivo* plants. Sprouting ability of primary and secondary microtubers was the same. These results were invariable for all five species.

DISCUSSION

This article describes *in vitro* microtuberization in five different species of *Ceropegia*, first time for this set of species. This work reveals various novelties about the *in vitro* tuberization, especially in *Ceropegia*. This phenomenon has been reported previously in *C. bulbosa* var *bulbosa*, *C. bulbosa* var *lushii* and *C. jainii* (Patil, 1998; Britto et al., 2003). However, these two protocols could not significantly produce microtubers in the current set of species. Instead, the experimentation carried out for inducing the microtuberization resulted in a protocol that succeeded appreciably for all the considered species.

Patil (1998) and Britto et al. (2003) have suggested the use of regenerated shoots as explants for *in vitro* tuberization in *Ceropegia jainii* and *C. bulbosa*. Other reports on successful *in vitro* tuberization of potato (Hussey and Stacey, 1981; 1984; Rosell et al., 1987; Garner and Blake, 1989; Estrada et al., 1986; Anjum and Villiers, 1997; Gopal et al., 1998) and yam (Forsyth and Staden, 1984; Ng, 1988; Jean and Cappadocia, 1992)] have demonstrated a variety of explants such as apical buds, axillary buds, nodal segments, stolons. Though the effect of explant type and polarity in microtuberization has been significant, numerous reports on potato (Goodwin and Adisarwanto, 1980; Goodwin et al., 1980; Amirouche et al., 1985; Khuri an Moorby, 1996) and yam (Ng, 1988; Mantell and Hugo, 1989; Kohmura et al., 1995) support the fact that various parts of these plants retain the regenerative ability to form microtubers. However, in the current set of *Ceropegia* spp., seedling-apical buds were the only successful explants whereas, the regenerated shoot derived explants developed flowering (Aneesh Kumar et al., 2007). These results clearly point out that the physiological age of the explants is the critical factor in *Ceropegia* and for microtuberization and juvenility of the explants is the most important consideration. Secondly, continuous and direct proliferation of microtubers is the most prominent discovery of this experimentation and is unique to *Ceropegia*. All the species used in the present work bear solitary tubers *in vivo*, therefore secondary tuber cluster formation can be solely attributed to the *in vitro* attempts. Irrespective of the critical explant considerations, such proliferation provides an easiest, most rapid and industrious means of desired propagule multiplication and a handy tool for conservation initiatives.

Among all the PGRs, we found BAP as the most influential one for the tuber induction in *Ceropegia*. Our results are in agreement with those reported by Palmer and Smith (1969), Smith and Palmer (1970), Hussey and

Stacey (1984), NG (1988), Mantell and Hugo (1989), Jean and Cappadocia (1992), Gopal et al. (1998) Villafranca et al. (1998) and Britto et al. (2003), where cytokinin accumulation proved to be one of the major tuber inductive factors in various plants. In most of the reports, KN has proved to induce tuberogenesis while the role of BAP has been accounted prominently by Hussey and Stacey (1984), Anjum and Villiers (1997) and Gopal et al. (1998). However the combined treatment of KN and BAP was successful for the tuberogenesis in *C. bulbosa* and *C. jainii* (Patil, 1998).

In BAP induced tubers, weight gained at 4% (w/v) sucrose suggests that the source of storable food can significantly influence the quality of tubers. This synergistic effect of BAP and sucrose can be explained by the proven interactions between the carbon-rich nutrients and endogenous or exogenous growth substances (Anker, 1974; Krul and Colclasure, 1977; Kochba et al., 1982) and those between sucrose and the regulatory element of tuberization (Perl et al., 1991). Most of the reports (Forsyth and Staden, 1984; Ng, 1988; Perl et al., 1991; Khuri and Moorby, 1996; Sudhir and Mukundan, 2002) advocated 8% (w/v) sucrose for tuberization; however, in *Ceropegia*, 4% (w/v) sucrose was optimum for tuber induction. Our results are congruent with those from the previous studies in *Ceropegia* (Patil, 1998; Britto et al., 2003). These results in addition to those about the sensitivity of cultures to higher amounts of sucrose suggest that the sucrose requirement is plant species specific and elevated sucrose levels may not be the universal factor for tuber induction. In addition to BAP and sucrose, other chemical factors like gibberellins (in potato) (Amador et al., 2001: Martínez-García et al., 2001), jasmonic acid (in yam) (Koda and Kikuta, 1991; Jasik and Mantell, 2000), potassium salts (in *Pinellia ternata*) (Xue et al., 2006) and Nitrogen (in *Xanthosoma sagittifolium*) (Omokolo et al., 2003) have also been reported to induce microtubers. Thus the prerequisites for tuberization vary by species and the information that piles up with each study, with its collective inference helps to design the strategies for the untamed and awkward plant species.

Physical factors such as day length, light intensity, temperature have also been shown to have significant effect on tuberization. Among these, photoperiod has been the most influential factor in various plants [Amador et al., 2001 (potato); Martínez-García et al., 2001 (Potato); Omokolo et al., 2003 (cocoyam); Allemann and Hammes 2006 (Livingstone potato); Rodríguez-Falcón et al., 2006 (potato)]. Most of these reports recommend long day lengths for tuber induction. *Ceropegia* seedling-apical bud explants also responded to the longer photoperiods with tuberization; reciprocally, tubers showed quick shoot bud sprouting at shorter day lengths. These responses may be correlated to the *in vivo* tendency of *Ceropegia* where these plants germinate, grow and photosynthesize accumulate to enough food material during the long days and at the onset of short days they shed their aerial shoots and the tubers resume dormancy. It also indicates the endogenous control of tuberization prior to that of the exogenous factors.

Fay (1992; 1994) recommended seed as a source of explant for the conservation of endangered species as it can be obtained in sufficient quantity and helps maintaining wide genetic base; indeed, *Ceropegia* spp. of the Indian region are so rare that obtaining enough explant material for the *in vitro* experiments is barely possible. Therefore, in the present work, we implemented Fay's notion (1992; 1994) for the successful propagation of endemic and threatened *Ceropegia* spp. Seeds being delicate, possibility of explant damage during the surface sterilization was nullified by using the entire follicles as a starting material. Considered the low success of *in vivo* germination, these experiments put forth a successful example of rescue for five species of *Ceropegia*. *In vitro* development of plantlets by microtuber germination confirmed the self-sufficiency of this protocol. Regardless of the fact that the species tested in present experiment belong to different microhabitats and differ significantly in their habit as well as eco-physiology, the optimum conditions for tuberization are same in all species; secondly, this optimized set of conditions also enabled proliferation with a significant rate in all five species. It proves that the current protocol

is comprehensive and can be applied to a wide range of *Ceropegia* species; we recommend its implementation in the conservation programs.

It has been demonstrated that *Ceropegia* plants respond positively to *in vitro* procedures (Patil, 1998; Beena et al., 2003; Britto et al., 2003; Aneesh Kumar et al., 2007). Their reintroduction remains the next challenge especially, in the regions where the anthropogenic pressures remain ever increasing. Method of tuber proliferation is novel and has encouraging potential to boost the existing conservation and reintroduction strategies for this endangered tuberous taxon. To ensure the sustainability of conservation programs, these attempts need to be supported with the other options available in the integrated conservation strategies (Falk, 1990). In this effort, strategy of microtuberization undoubtedly provides economic and effortless option and eliminates the need for support of other expensive and resource demanding biotechnological tools such as synthetic seeds and cryopreservation. For germplasm collections, they are convenient propagules to maintain which also can be obtained at a required scale. Thus, economy of this strategy allows us to concentrate for *ex situ* backup and reintroduction efforts. These plants have awkward eco-physiology that substantially affects their success in the wild; therefore conservation measures should also consider habitat management backed with awareness campaigning as a pivotal step. Awareness campaigning can be included with the domestication efforts for these plants by exploiting their ornamental attributes. Such an exercise may also balance the economic aspects of propagation and conservation.

In conclusion, we have described a comprehensive protocol for the microtuberization for threatened *Ceropegia* spp. A novel phenomenon of microtuber proliferation is also demonstrated. This protocol is economic for the industrious production and consequently, conservation of the tuberous *Ceropegia* spp. However, for effective reintroduction of these plants, these *in vitro* efforts must be backed by the habitat conservation and other appropriate ex vitro strategies.

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