Journal of Plant Biology, June 2007, 50(3) : 374-377

SHORT COMMUNICATION

High-Frequency *in Vitro* Flowering in Six Species of *Ceropegia*

Aneesh Kumar Nair[1], Naik Dhiraj Dilip[2], and Pandit Sagar Subhash[1]*

[1]Plant Molecular Biology Unit, Division of Biochemical Sciences, National Chemical Laboratory, Pune- 411008, Maharashtra, India

[2]Department of Biology, West Virginia University, Morgantown, WV 26506, USA

High-frequency *in vitro* flowering is reported here from *in vitro* regenerated shoots of *in vitro*-raised seedlings of rare and endemic *Ceropegia lawii*, *Ceropegia maccannii*, *Ceropegia oculata*, and *Ceropegia sahyadrica*, as well as the widely distributed *Ceropegia bulbosa* var. *bulbosa* and *Ceropegia hirsuta*. In our first set of experiments, the MS medium contained 87 mM sucrose and was supplemented with varying concentrations of BAP (4.4 to 26.6 μM). For the second set of trials, varying concentrations of sucrose (87 to 233 mM) were tested in MS media containing a constant 4.4 μM BAP. Sub-cultured apical as well as axillary buds flowered with similar frequencies after 30 d of incubation. For all six species, the highest percentage of flowering shoots was obtained with either 26.6 μM BAP or 175 mM sucrose. Although smaller in size, their *in vitro* flowers were morphologically comparable with *in vivo*-derived flowers. Variations among species were noted for the number of flower buds per shoot and the percentage of flower formation. Because all six species showed similar responses in both experiments, we can suggest that this protocol is applicable across the wide range of *Ceropegia* species.

Keywords: Asclepiadaceae, BAP, biodiversity hotspot, rare and endemic, sucrose

Ceropegia L. (Asclepiadaceae) is a genus of climbers and erect herbs that are distributed in tropical and subtropical Asia, Africa, and Australia (Anonymous, 1992). The flowers of almost all *Ceropegia* species are morphologically unique, having a long corolla tube at the distal end, which dissects out into five corolla lobes that are connate at their apices, thus creating five small windows. These colorful flowers form an attractive cage for insects. Amateur botanists and collectors have always been fascinated with this exotic appearance, and many species are thus used as ornamentals in Europe and the United States (McNew, 2002; Hodgkiss, 2004; Reynolds, 2006). Although none of the Indian *Ceropegia* species has yet been domesticated for this purpose, plants of the Indian subcontinent are under threat because of either habitat degradation or overutilization of their tubers. Moreover, many species of *Ceropegia* from peninsular India have been designated as rare (Nayar and Sastry, 1988; Walter and Gillet, 1998) and their existence has become restricted to remote pockets in the Himalayas and the Western Ghats, two biodiversity hotspots. Regrettably, the *Ceropegia* genus has now been added to the list of Indian endangered plants (Botanical Survey of India, 2002). For its conservation, large scale propagation, especially *in vitro*, has long been encouraged (Walter and Gillet, 1998). Moreover, the reproductive physiology of these endemic species must be studied in order to design efficient breeding and conservation strategies. To the best of our knowledge, most Indian species remain to be investigated in this regard. Therefore, we describe our research on *in vitro* flowering for six species of *Ceropegia*. Except for *C. bulbosa*, which has been previously published, this is believed to be the first report of success using such an approach with this genus.

*Corresponding author; fax +91-20-25902648
e-mail ss.pandit@ncl.res.in

MATERIALS AND METHODS

During November and December (2003, 2004, and 2005), we collected the mature follicles of rare and endemic *Ceropegia lawii* Hook., *C. maccannii* Ansari., *C. oculata* Hook., and *C. sahyadrica* Ansari et Kulkarni, as well as the widely distributed *C. bulbosa* Roxb. var. *bulbosa*, and *C. hirsuta* Wt et Arn. These were gathered from various regions in the Western Ghats of India. The follicles were initially held under running tap water for 10 min, and then soaked for 20 min in 100 mL of tap water containing 2 to 4 drops of Tween-20 before being washed twice with sterile distilled water. The tissues were then treated with 70% (v/v) ethanol for 1 min, followed by rinsing with sterile distilled water. Afterward, they were surface-sterilized with 0.1% (w/v) aqueous mercuric chloride (freshly prepared) for 5 min. Finally, these materials were washed thrice with sterile distilled water prior to inoculation under laminar airflow. The follicles were cut longitudinally along their sutures to obtain the seeds, which were then inoculated on 15 mL MS (Murashige and Scoog, 1962) media containing 87 mM sucrose (HiMedia, India) and solidified with agar (0.8%, w/v) (HiMedia) for germination. The media pH was adjusted to 5.8 ± 0.02 before autoclaving at 15 lb and 121°C for 20 min. These *in vitro* germinated seeds were used as the explant source. Axillary as well as apical buds from two-week-old seedlings were inoculated on MS media containing 4.4 μM BAP. They were also sub-cultured on media of the same composition. These culture tubes were incubated at 25 ± 2°C and 70% relative humidity under a 16-h photoperiod provided by cool white fluorescent tubes (40 μmol m^{-2} s^{-1}; Phillips, India). For all experiments, the cultures grown on MS basal media only with 87 mM sucrose served as our control. In the first set of trials, an MS medium containing 87 mM sucrose was supplemented with one of six concentrations of BAP (4.4, 8.8, 13.3, 17.7, 22.2, and 26.6 μM). In MS medium containing a constant 4.4 μM BAP, various concentrations of sucrose (87, 116, 146, 175, 204, and 233 mM) were used for the second set of experiments. All experiments were performed three

times, with 30 explants per treatment. The percentage of cultures showing successful floral induction was subjected to analysis of variance (ANOVA), and significant differences among means were assessed by Duncan's multiple range tests, at the level of p= 0.05 (Duncan, 1955).

RESULTS

Seeds from all six *Ceropegia* species germinated within 14 d. Axillary and apical bud segments from two-week-old *in vitro*-grown seedlings showed bud break on Day 3 when cultured in a medium supplemented with BAP. The conversion of shoot buds into shoots was seen after 7 d of culture. These sub-cultured shoots gave rise to multiple new, vigorously growing adventitious shoots after 15 d of incubation (data not shown). Their apical and axillary buds were then inoculated on MS media with 87 mM sucrose and 4.4 to 26.6 μM BAP, where they flowered profusely after 30 d (Table 1). However, the more immediate response (Days 5 to 15) by these buds was shoot regeneration and flowering occurred only after the incubation for more than four weeks on the same media. Each inflorescence consisted of four to five flower buds. Apical and axillary buds flowered with similar frequency. For all six species, the number of flower-producing shoots was greatest on the MS medium containing 26.6 μM BAP (Table 1). The levels above that were associated with leaf fall and shoot tip necrosis. Higher frequencies of flowering were also obtained with all six species when the apical and axillary buds from regenerated shoots were trans-

Table 1. Effect of BAP on *in vitro* floral bud formation in *Ceropegia* species cultured on MS media containing 175 mM sucrose. Means within a column followed by the same letter are not significantly different from each other, based on Duncan's multiple range test at 5% probability level.

Species	BAP (μM)	Percent explants with floral buds	Number of floral buds per explant
C. bulbosa var. *bulbosa*	4.4	3.6 ± 1.57[a]	1.0 ± 0.21[a]
	8.8	5.4 ± 0.58[a]	1.3 ± 0.12[a]
	13.3	16.5 ± 0.58[a]	1.6 ± 0.43[a]
	17.7	19.2 ± 3.06[b]	1.5 ± 0.21[a]
	22.2	26.1 ± 1.00[b]	1.7 ± 0.30[b]
	26.6	29.7 ± 2.08[d]	1.2 ± 0.11[a]
C. hirsuta	4.4	9.5 ± 2.52[a]	1.0 ± 0.50[a]
	8.8	13.8 ± 1.53[a]	1.3 ± 0.81[a]
	13.3	27.9 ± 0.58[b]	2.5 ± 0.82[c]
	17.7	34.6 ± 1.53[c]	2.6 ± 0.53[c]
	22.2	49.6 ± 1.53[d]	3.1 ± 0.80[c]
	26.6	56.3 ± 1.53[e]	2.6 ± 0.51[c]
C. lawii	4.4	12.5 ± 0.58[a]	1.8 ± 0.30[b]
	8.8	16.8 ± 1.53[a]	1.9 ± 0.52[b]
	13.3	32.5 ± 0.58[c]	2.1 ± 0.81[b]
	17.7	41.8 ± 2.52[d]	3.4 ± 0.50[c]
	22.2	55.0 ± 1.00[d]	3.6 ± 0.32[c]
	26.6	69.8 ± 2.52[e]	2.5 ± 0.71[b]
C. maccannii	4.4	8.9 ± 1.53[a]	1.0 ± 0.50[a]
	8.8	10.5 ± 2.00[a]	1.3 ± 0.81[a]
	13.3	16.8 ± 1.00[b]	1.7 ± 0.90[a]
	17.7	21.9 ± 1.53[b]	1.9 ± 0.31[b]
	22.2	31.6 ± 1.52[c]	1.9 ± 0.50[b]
	26.6	34.5 ± 1.52[d]	1.1 ± 0.61[a]
C. oculata	4.4	5.8 ± 1.00[a]	1.0 ± 0.80[a]
	8.8	13.9 ± 2.08[a]	1.2 ± 0.51[a]
	13.3	15.6 ± 3.06[a]	1.7 ± 0.30[b]
	17.7	24.0 ± 1.00[b]	2.3 ± 0.91[b]
	22.2	35.6 ± 1.73[c]	2.9 ± 0.50[c]
	26.6	44.9 ± 1.53[d]	2.1 ± 0.21[c]
C. sahyadrica	4.4	10.3 ± 1.53[a]	1.0 ± 0.30[a]
	8.8	19.0 ± 2.00[b]	1.5 ± 0.51[a]
	13.3	27.3 ± 1.00[b]	1.9 ± 0.60[b]
	17.7	39.6 ± 2.52[c]	2.1 ± 0.51[c]
	22.2	52.9 ± 1.52[c]	2.4 ± 0.80[c]
	26.6	61.8 ± 1.53[d]	1.6 ± 0.31[a]

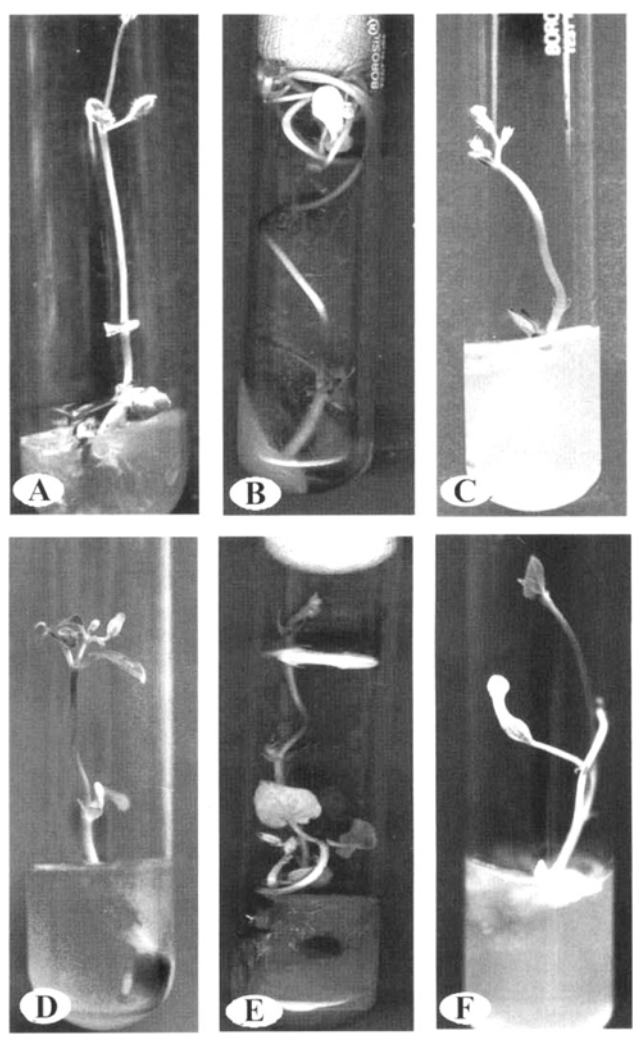

Figure 1. *In vitro* floral bud formation from *in vitro* regenerated shoots of *Ceropegia* species (**A**, *C. bulbosa* var. *bulbosa*; **B**, *C. hirsuta*; **C**, *C. lawii*; **D**, *C. maccannii*; **E**, *C. oculata*; **F**, *C. sahyadrica*) after 30 d of culture on MS medium containing 4.4 μM BAP and 175 mM sucrose.

Table 2. Effect of sucrose concentration on flower induction in shoot explants from six *Ceropegia* species cultured on MS media containing 4.4 μM BAP. Means within a column followed by the same letter are not significantly different from each other, based on Duncan's multiple range test at 5% probability level.

Species	Sucrose (mM)	Percent explants with floral buds	Number of floral buds per explant
C. bulbosa var. *bulbosa*	87	3.6 ± 1.67[a]	1.0 ± 0.10[a]
	116	15.6 ± 0.58[a]	1.0 ± 0.10[a]
	146	19.6 ± 1.15[b]	1.6 ± 0.58[a]
	175	28.6 ± 3.51[b]	2.0 ± 0.10[b]
	204	14.0 ± 1.00[b]	1.0 ± 0.10[a]
	233	12.6 ± 1.53[b]	1.3 ± 0.58[a]
C. hirsuta	87	9.3 ± 2.52[a]	1.0 ± 0.10[a]
	116	31.6 ± 1.53[b]	1.6 ± 0.58[a]
	146	64.3 ± 0.58[c]	2.6 ± 0.58[b]
	175	95.6 ± 0.58[d]	6.6 ± 0.58[c]
	204	28.6 ± 1.53[b]	1.6 ± 0.58[a]
	233	26.6 ± 0.58[b]	1.3 ± 0.58[a]
C. lawii	87	12.3 ± 0.58[a]	1.6 ± 0.58[a]
	116	28.0 ± 1.00[b]	1.3 ± 0.58[a]
	146	51.3 ± 3.06[c]	2.3 ± 0.58[b]
	175	98.6 ± 1.53[d]	5.3 ± 0.58[c]
	204	27.6 ± 0.53[b]	1.6 ± 0.58[a]
	233	25.0 ± 1.73[b]	1.3 ± 0.58[a]
C. maccannii	87	8.6 ± 1.53[a]	1.3 ± 0.58[a]
	116	18.0 ± 1.00[b]	1.3 ± 0.58[a]
	146	41.3 ± 0.58[c]	1.6 ± 0.58[a]
	175	95.6 ± 1.53[d]	2.6 ± 0.58[b]
	204	15.6 ± 1.53[a]	1.3 ± 0.58[a]
	233	14.3 ± 0.58[a]	1.0 ± 0.10[a]
C. oculata	87	5.0 ± 1.00[a]	1.0 ± 0.58[a]
	116	14.6 ± 1.53[b]	1.3 ± 0.58[a]
	146	18.6 ± 1.53[b]	1.6 ± 0.58[a]
	175	49.0 ± 1.00[c]	3.0 ± 1.00[b]
	204	15.3 ± 1.15[b]	1.3 ± 0.58[a]
	233	13.3 ± 0.58[b]	1.0 ± 0.10[a]
C. sahyadrica	87	11.6 ± 1.53[a]	1.0 ± 0.10[a]
	116	25.3 ± 2.08[b]	1.3 ± 0.58[a]
	146	48.0 ± 2.00[c]	3.0 ± 1.00[b]
	175	99.6 ± 0.58[d]	8.0 ± 1.00[c]
	204	35.6 ± 4.04[b]	1.3 ± 0.58[a]
	233	33.3 ± 1.15[b]	1.3 ± 0.58[a]

ferred to MS media containing 4.4 μM BAP (constant) and varying concentrations of sucrose (87 to 233 mM) (Table 2; Fig. 1). The percentage of flowering shoots and the number of flowers per explant were enhanced as the sugar concentration increased, and the most flowering shoots were obtained at 175 mM sucrose, above this concentration their frequency decreased (Table 2). This precocious flowering continued for up to six weeks.

For all treatments, responses varied by species with regard to the frequency of *in vitro* flower formation as well as the number of flowers produced per explant (Tables 1, 2). The highest percentage of flowering shoots in the BAP-varying experiment was observed in *C. lawii* (69.8%), followed by *C. sahyadrica* (61.8%), *C. hirsuta* (56.3%), *C. oculata* (44.9%), *C. maccannii* (34.5%), and *C. bulbosa* Roxb. var. *bulbosa* (29.7%). Similarly, the highest percentage of flowering shoots in the sucrose-varying experiment was recorded for *C. sahyadrica* (99.6%), closely followed by *C. lawii* (98.6%), *C. hirsuta* (95.6%), and *C. maccannii* (95.6%), with the low success rate for *C. oculata* (49.0%) and *C. bulbosa* Roxb. var. *bulbosa* (28.6%). This could have been due to fluctuations in the floral signal receptivity of the shoot buds of those species. Both BAP and sucrose induced morphologically normal *in vitro* flowers in all six species, but these were smaller and paler in color than the *in vivo* flowers. This might have resulted from the miniaturization of shoots during *in vitro* culturing. Floral development also appeared normal, and *in vitro* flowering with either BAP or sucrose could be obtained in any season of the year (data not shown). It should be noted that neither floral fertility nor pollen viability were tested in this current set of experiments.

DISCUSSION

Many factors elicit *in vitro* flowering, e.g., high temperatures in *Oncidium pusillum* (Livingston, 1962), TDZ in *Bambusa edulis* (Lin and Chang, 1998; Lin et al., 2006), proline in *Saccharum officinarum* (Virupakshi et al., 2002), or shock caused by the sudden deprivation of growth regulators in *Cucumis sativus* (Rajasekaran et al., 1983). In several higher plants, cytokinins also can promote this transition to the reproductive stage *in vitro*, either alone (Harada, 1966; Tran Thanh, 1977) or in combination with auxins (Saritha and Naidu, 2007). BAP is especially useful for inducing *in vitro* flowering in bamboo (Nadgauda et al., 1990) or the development of shoot tips in bitter melon (Wang et al., 2001). In the regenerated shoots of *Perilla frutescens*, BAP with ammonium nitrate promotes such flowering (Zhang, 2007). Likewise, a combination of BAP and GA_3 influences *in vitro* flowering in *C. bulbosa* var. *bulbosa* (Britto et al., 2003), while in *C. jainii*, application of both BAP and spermine induces floral production (Patil, 1998). In our experiments as well, BAP stimulated flowering in all six species, thus proving to be a key component for the *in vitro* flowering in genus *Ceropegia*. Increasing the concentration of sucrose in the culture medium also stimulates flowering in the organ segments of *Cichorium* (Harada, 1966), *Nicotiana* (Tran Thanh, 1977), and *Torenia* (Tanimoto and Harada, 1981). Furthermore, the addition of 87 to 233 mM sucrose to the media leads to flower formation in *Fortunella hindsii* (Jumin and Nito, 1996).

Although being short day species, BAP or sucrose made all six species flower *in vitro*, round the year, this suggests the strong influence of both the factors in *Ceropegia*. Moreover, induction of flowering in all six species of *Ceropegia* in the present experiment indicated marginally superior effect of sucrose to that of BAP. These species belong to different micro-niches; still respond to similar culture conditions, suggesting that the present protocol can be comprehensively

used across the range of *Ceropegia* species.

The Indian species of this genus possess ornamental attributes that can be preserved through domestication. This can be achieved if researchers are able to overcome major production barriers, e.g., seasonality, and improve other commercial values. *In vitro* studies will direct us toward a standardized, industrious protocol that will allow us to introduce a new flower in the bouquet.

ACKNOWLEDGEMENTS

The authors thank the late Dr. GM Bansude as well as Dr. HV Ghate (Modern College, Pune 411005, India) and Dr. VS Gupta (National Chemical Laboratory, India) for their laboratory support and continuous encouragement during this work.

Received January 6, 2007; accepted April 11, 2007.

LITERATURE CITED

Anonymous (1992) *Ceropegia* Linn. (Asclepiadaceae), The wealth of India, Vol 3. CSIR, New Delhi, pp 448-449

Botanical Survey of India (2002) Studies on Rare and Endangered Species. http://www.envfor.nic.in/bsi/research.html

Britto SJ, Natarajan E, Arockiasamy DI (2003) *In vitro* flowering and shoot multiplication from nodal explants of *Ceropegia bulbosa* Roxb. var. *bulbosa*. Taiwania 48: 106-111

Duncan DB (1955) Multiple range and multiple F-tests. Biometrics 11: 1-42

Harada H (1966) Effects of photoperiod on the formation of flower buds by flower stalk sections of *Cichorium intybus* in tissue culture. Bot Mag Tokyo 79: 119-123

Hodgkiss RJ (2004) http://www.succulent-plant.com/ceropg.html

Jumin HB, Nito N (1996) *In vitro* flowering of *Fortunella hindsii* (Champ.). Plant Cell Rep 15: 484-488

Lin CS, Chang WC (1998) Micropropagation of *Bambusa edulis* through nodal explants of field grown culms and flowering of regenerated plantlets. Plant Cell Rep 17: 617-620

Lin CS, Tseng MC, Hong PI, Chang WC (2006) Albino inflorescence proliferation of *Dendrocalamus latiflorus*. In Vitro Cell Dev Biol Plant 42: 331-335

Livingston R (1962) *Oncidium pusillum* blooming in flask! Amer Orchid Soc Bull 31: 1007

McNew R (2002) http://www.shoalcreeksucculents.com.ceropegias.html

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15: 473-497

Nadgauda RS, Parasharami VA, Mascarenhas AF (1990) Precocious flowering and seeding behaviour in tissue cultured bamboos. Nature 344: 335-336

Nayar MP, Sastry ARK (1988) Red Data Book of Indian Plants, Vol 2. Botanical Survey of India, Calcutta, pp 39-60

Patil VM (1998) Micropropagation studies in *Ceropegia* spp. In Vitro Cell Dev Biol Plant 34: 240-243

Rajasekaran K, Mullins MG, Nair Y (1983) Flower formation *in vitro* by hypocotyl explants of cucumber (*Cucumis sativus* L.). Ann Bot 52: 417-420

Reynolds S (2006) http://www.sagereynolds.com/cero/clist.com

Saritha KV, Naidu CV (2007) *In vitro* flowering of *Withania somnifera* Dunal: An important antitumor medicinal plant. Plant Sci 172: 847-851

Tanimoto S, Harada H (1981) Effects of IAA, zeatin, ammonium nitrate and sucrose on the initiation and development of floral buds in *Torenia* stem segments cultured *in vitro*. Plant Cell Physiol 22: 1553-1560

Tran Thanh van M (1977) Regulation of morphogenesis, *In* W Barz, E Reinhard, MH Zenk, eds, Plant Tissue Culture and Its Biotechnological Application. Springer Verlag, Berlin, pp 367-385

Virupakshi S, Manjunath BR, Naik GR (2002) *In vitro* flower induction in callus from a juvenile explant of sugarcane, *Saccharum officinarum* L. var. CoC 671. Curr Sci 83: 1195-1197

Walter KS, Gillet HJ (1998) 1997 IUCN Red List of Threatened Plants. Compiled by the World Conservation Monitoring Centre, IUCN The World Conservation Union, Gland, Switzerland, Cambridge, pp 63-65

Wang S, Tang L, Chen F (2001) *In vitro* flowering of bitter melon. Plant Cell Rep 20: 393-397

Zhang T (2007) *In vitro* flowering of *Perilla frutescens*. In Vitro Cell Dev Biol Plant 43: 91-94