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PLANT TISSUE CULTURE

In vitro micropropagation, flowering, and tuberization of *Ceropegia maculata* Bedd.—an endemic plant of Southern Western Ghats

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Abstract

Ceropegia maculata Bedd. is an endemic plant of Southern Western Ghats, Tamil Nadu, India. It has important medicinal properties, edible tubers, and ornamental flowers. *In vitro* propagation protocol of this plant is required and is established by using nodal explants. Sterilized nodal explant was inoculated on Murashige and Skoog (MS) medium supplemented with various plant growth regulators (PGRs) and additives for *in vitro* shoot multiplication. Maximum shoot induction (86%) with an average of 2.43 shoots per explant was obtained on MS medium supplemented with 1.5 mg L^{-1} of N6-benzyl adenine (BA). The highest number of shoots (6.66) per explant was observed on MS medium containing combination of 1.5 mg L^{-1} BA and 0.5 mg L^{-1} indole-3-butyric acid (IBA). In this study, *in vitro* flowering (93.33% and 4.86 flowers per mature shoot) on MS medium plus 0.5 mg L^{-1} BA and tubers (95.33%) on MS medium plus combination with 2.0 mg L^{-1} BA with 0.5 mg L^{-1} naphthalene acetic acid (NAA) were observed. The highest number of roots (9.33) per shoot was recorded on half-strength MS medium supplemented with 0.5 mg L^{-1} IBA. The rooted plantlets were hardened with sand and coconut coir mixed with red soil 1:1:1 (w/w/w) ratio. Acclimatized plants were transferred to field and survival rate was 90%. For the first time, developed this protocol allows an efficient method for *in vitro* plant regeneration and conservation of this endangered species.

Keywords *Ceropegia maculata* · Node explant · Micropropagation · Rooting · Hardening

Introduction

Ceropegia maculata Bedd. is an ethnomedical plant which belongs to the family Apocynaceae. The genus *Ceropegia* comprising twiners, herbs, and occasionally subshrubs is distributed in tropical and subtropical regions of Asia, Africa, Australia, Malaysia, and the Canary and Pacific Islands (Nayar and Sastry 1988; Anonymous 1992). The plant names are universally called as the lantern flower, Christensen, parasol flower, parachute flower, bushman's pipe, the string of hearts, snake creeper, wine-glass vine, rosary vine, necklace vine flower, Chinese lantern, lantern plant, trap flowers, and pitfall trap flowers (Yadav 1996; Quattrocchi 2000). The six recognized centers of diversity of the genus are East Africa, Africa of the West, Southern Africa, the Indian subcontinent, the Arabian Peninsula, and Madagascar (Chavan *et al.* 2018). The maximum variety of flowers of the *Ceropegia* spp. is found in subtropical Africa on the eastern side of the African continent (Dyer 1983; Bruyns 2003; Bruyns *et al.* 2015). The Indian *Ceropegia* was first updated with 44 species, of which 28 were endemic; after 13 new additions, the genus now comprises 57 species, 3 varieties, and 2 subspecies in which 35 species are endemic to the Western Ghats (Karthikeyan *et al.* 2009). The Indian *Ceropegia* species are present in limited, inaccessible pockets of the Himalayas and the Western Ghats due to over exploitation for medicinal purposes. The edible sweet–sour leaves of *Ceropegia* are known to be digestive tonic. *Ceropegia* tubers are also edible as they contain starch, sugar, gum, albumin, carbohydrates, fats, and raw fiber (Mabberley 1987; Jain and Defillips 1991). Tubers of some *Ceropegia*

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contain 40–50% starch and act as coolant (Khare, 2007). Nutritional profile of *C. hirsuta* and *C. bulbosa* exhibited the presence of ash, protein, phosphorus, Cu, Mn, Fe, Zn, Vit-C, carbohydrates, protein, and fibber (Deshmukh and Rathod 2013). Cerpegin (1,1-dimethylfuro[3,4-C] pyridine-3,4(1H,5H)-dione), a pyridine type of alkaloid which is relatively rare in nature, has been isolated from the root tubers of *Ceropegia* (Adibatti *et al.* 1991; Phulwaria *et al.* 2013). The overall alkaloidal fractions have shown promising hepatoprotective, antipyretic, anti-ulcer, analgesic, stabilizing mast cells, tranquilizing, and hypotensive activities (Adibatti *et al.* 1991). The *Ceropegia* tubers were cooked to make curries by tribal women to promote fertility and vitality. In Ayurvedic medications, the *Ceropegia* tubers have been used to treat diarrhea and dysentery (Kirtikar and Basu 1935; Jain and Defillips 1991; Beena *et al.* 2003; Khare 2007).

Ceropegia maculata Bedd. is a perennial twiner with terete stems and protruding spots. Natural fruit setting of this species is very rare, while *in vitro* flowering was recorded by Nair *et al.* (2007). Earlier, Nayar and Sastry (1987) assessed this plant as endangered or possibly extinct, while it has been reported to be collected from few places of South India (Kambale and Yadav 2019). However, to our knowledge, only one article is available on rediscovery of *C. maculata* from Tirunelveli Hills of Tamil Nadu, and yet the data on the distribution and extent still needs to be studied (Rajasekar *et al.* 2018). Hence, studies on conservation measures of this rediscovered species have become very important. There are few reports available on *in vitro* micropropagation methods of *Ceropegia* species like *C. spiralis* (Chavan *et al.* 2011a), *C. attenuate* (Chavan *et al.* 2011b), *C. bulbosa* (Dhir and Shekhawat 2013, 2014; Shete 2014), *C. noorjahaniae* (Chavan *et al.* 2014a), *C. juncea* (Balakrishnan *et al.* 2015), *C. ensifolia* (Reddy *et al.* 2015), and *C. mahabalei* (Upadhye *et al.* 2015). *In vitro* micropropagation of *Ceropegia maculata* species is not studied yet and is required to establish *in vitro* plant regeneration protocol for this species; therefore, we aimed to establish an effective micropropagation protocol for rapid mass propagation, *in vitro* flowering, tuberization, and conservation of *C. maculata.*

Materials and Methods

Explant Source *C. maculata* plants were collected from Karaiyar dam of Inchikuzhi region from Tirunelveli District, Tamil Nadu, India. The collected specimens were maintained in earthen pots at the glasshouse in the Department of Botany, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India, and used as the source of explant. A portion of the plant was fixed for herbarium and submitted to the Botanical Survey of India, Regional Canter, Coimbatore, Tamil Nadu, for authentication (No.: BSI/SRC/5/23/2021/Tech/93).

Explant Preparation and Sterilization Nodal segments of ~ 2 cm long were excised from the glasshouse-maintained plants and washed under running tap water for 15 min. Then, the explants were surface-sterilized with 70% (v/v) ethanol for 30 s, followed by 0.1% (w/v) $HgCl_2$ for 3 min and rinsed in sterile distilled water each time for 3–4 times.

Basal Medium and Culture Conditions Murashige and Skoog (MS) medium (1962) fortified with 3% sucrose, meso-inositol (100 mg L^{-1}), and 0.8% agar was used as basal medium for *in vitro* shoot regeneration, flowering, tuberization, and rooting. The pH of the medium was adjusted to 5.8 ± 0.02 with 1 N HCl or NaOH prior to autoclaving at 120 °C for 20 min. All the cultures were incubated in the culture room at 25 ± 2 °C, under 16-h photoperiod with a light intensity of 60–70 µM m^{-2} s^{-1} supplied by cool white fluorescent lamps (Phillips, Mumbai, India), and relative humidity of 55–60%. All the chemicals used in the experiments were purchased from HiMedia®, Mumbai, India.

Shoot Bud Induction, Shoot Multiplication, and Elongation The sterilized nodal explants were cultured on MS medium supplemented with N6-benzyladenine (BA), thidiazuron (TDZ), and N6-(2-isopentyl) adenine (2iP) at various concentrations (0.5, 1.0, 1.5, 2.0, and 2.5 mg L^{-1}) for shoot bud induction. *In vitro* shoots were cultured on MS medium containing BA 1.5 mg L^{-1} supplemented with indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), and α-naphthalene acetic acid (NAA) at different concentrations (0.1, 0.5, 0.7, and 1.0 mg L^{-1}) for shoot multiplication.

MS medium containing BA (1.5 mg L^{-1}) and IBA (0.5 mg L^{-1}) and addition of 25, 50, and 100 mg L^{-1} of ascorbic acid (AA) and adenine sulfate (Ads) were used for shoot elongation.

***In Vitro* Rooting, Hardening, and Acclimatization** The elongated shoots (4–5 cm) excised from multiple shoot clump were further cultured on rooting medium consisting of half-strength MS medium (½ MS) supplemented with IAA, IBA, and NAA at different concentrations (0.1, 0.3, 0.5, 0.7, and 1.0 mg L^{-1}). Then, the rooted plantlets were removed from the culture tubes/flasks and rinsed with sterile distilled water to remove the adhering agar and introduced on paper cups (5 cm in diameter) containing sterile (autoclaved) sand and coconut coir mixed with red soil at 1:1:1 (w/w/w) ratio for hardening. The hardened plantlets were sprayed with one-fourth strength of MS basal (100 mL) salt solution devoid of sucrose and meso-inositol every 4 d, for 6 wk. Finally, the hardened plantlets were moved to a glasshouse condition.

***In Vitro* Flowering** The *in vitro* shoots (3–4 cm) from shoot multiplication medium were used for *in vitro* flowering. A full-strength MS medium with BA and TDZ at different concentrations (0.3, 0.5, 0.7, and 1.0 mg L^{-1}) was used to study their influence on *in vitro* flowering.

***In Vitro* Tuberization and Histology** The shoots (3–4 cm) obtained from shoot multiplication medium were cultured on full-strength MS medium, containing 3% reinforced sucrose (w/v) with various PGRs such as, BA (1.0, 1.5, 2.0, and 3.0 mg L^{-1}), NAA (1.0, 1.5, 2.0, and 3.0 mg L^{-1}), BA (2.0 mg L^{-1}) with IBA (0.5, 1.0, 1.5 mg L^{-1}), and NAA (0.5, 1.0, 1.5 mg L^{-1}) for *in vitro* tuber formation.tuberization with an average tuber diameter of 0.85tuberization with an average tuber diameter of 0.85

To confirm the tuber formation, the developing tubers as fresh and after dehydration were fine-sectioned using hand and microtome (Leica, Guragon, India), stained with iodine (I2/KI) and observed under light microscope (Labomed, Guragon, India) and confocal microscope (Carl Zeiss, model-LSM 710, Jena, Germany) following Ovecka *et al.* (2012).

Statistical Analysis Data on percentage of response, mean number, and length of shoot and root were recorded after 45 and 20 d of culture period, respectively. Frequency of flowering, number of inflorescence and flower, frequency of tuberization, tuber nature, and tuber diameter (mm) were recorded after 30 and 60 d of culture period, respectively. All the experiments were carried out in a completely randomized block design with triplicates. All the data were subjected to variance analysis, and the significance of differences was carried out between mean values using Duncan's multiple range test (DMRT) at $P < 0.05$ using SPSS software, version 22.0 (SPSS Inc., Armonk, NY). The results were expressed as the mean ± standard error (SE) of the triplicates.

Results and Discussion

Influence of Cytokinins on Shoot Induction and Multiplication Nodal explant cultured on MS basal medium supplemented with different cytokinins exhibited a variety of responses depending on the form and concentration of the substance used for shoot bud induction. Shoot induction response was recorded after 45 d on medium with BA 1.5 mg L^{-1} (Fig. 1*a*), TDZ 2.0 mg L^{-1}, and 2iP 1.5 mg L^{-1}, respectively. MS medium supplemented with BA 1.5 mg L^{-1} induced a maximum number of 2.43 shoots per nodal explant after 45 d of culture (Table 1). At this concentration, 86% of nodal explant cultures exhibited shoot induction and multiplication. Successful results on shoot production using BA have been reported in *Ceropegia* species such as *Ceropegia bulbosa* (Dhir and Shekhawat 2013), *C. noorjahaniae* (Chavan *et al.* 2014a), *C. barnesii* (Ananthan *et al.* 2018), and *C. mohanramii* (Adsul *et al.* 2019). BA was found to have beneficial impact at concentrations ≤ 2.0 mg L^{-1} on shoot multiplication and elongation, while TDZ showed similar response at ≥ 1.5 mg L^{-1}. The ability of BA on induction of cytokinin accumulation may be responsible for the effective *in vitro* shoot regeneration over TDZ or 2iP (Baskaran and Jayabalan 2007; Phulwaria *et al.* 2012). This may also be due to increased auxin aggregation and translocation (Chavan *et al.* 2013).

Synergistic Effect of Auxin on Shoot Multiplication The BA-exposed shoots were transferred to a secondary medium containing auxins for shoot multiplication after a 30-d incubation on basal MS medium. For the multiplication of *C. maculata* shoots, a lower concentration of IBA combined with BA 1.5 mg L^{-1} was most effective. At all concentrations, IBA outperformed other auxins (IAA and NAA) in terms of shoot multiplication. The maximum number of 6.66

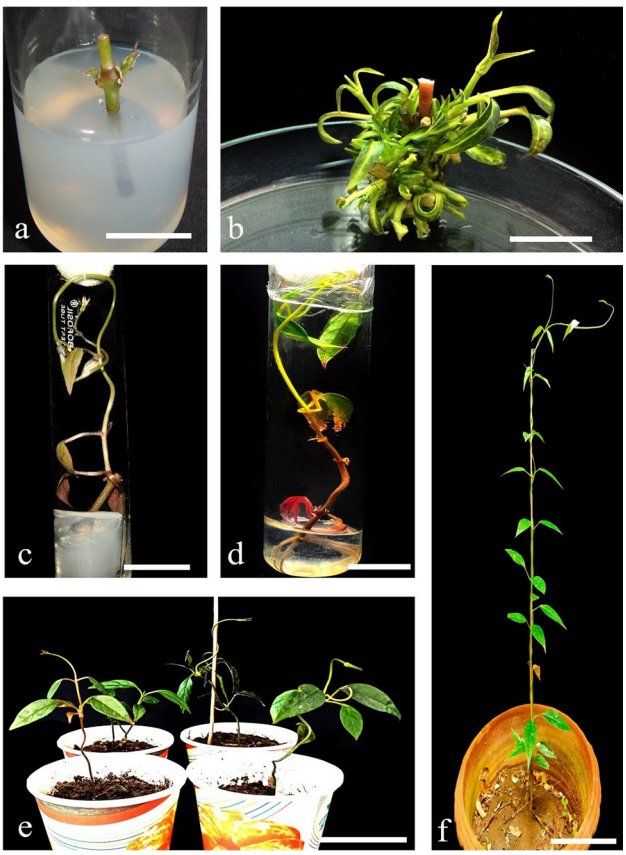

Figure 1. *In vitro* micropropagation of *C. maculata* Bedd. through axillary node explant. (*a*) Explant. (*b*) Shoot multiplication BA 1.5 mg L^{-1} + IBA 0.5 mg L^{-1}. (*c*) Shoot elongation on BA 1.5 mg L^{-1} + IBA 0.5 mg L^{-1} + ascorbic acid 50 mg L^{-1}. (*d*) Rooting on IBA 0.5 mg L^{-1}. (*e* and *f*) Hardened plants. *Bars*: (*a*) 1 cm; (*b–d*) 2 cm; (*e*) 2.5 cm; (*f*) 10 cm.

Table 1. Shoot bud induction from axillary bud explant of *C. maculata* on MS medium supplemented with cytokinins, after 45 d

Cytokinin (mg L^{-1})	Freq. of response (%)	Number of shoots per explant	Avg. shoot length (cm)
BA			
0.5	42.33 ± 0.88^{i}	1.36 ± 0.88^{ef}	1.73 ± 0.88^{f}
1.0	55.33 ± 0.88^{ef}	1.73 ± 0.88^{d}	2.06 ± 0.88^{de}
1.5	86.66 ± 0.88^{a}	2.43 ± 0.88^{a}	4.33 ± 0.88^{a}
2.0	74.33 ± 1.45^{b}	2.06 ± 0.88^{bc}	3.76 ± 0.88^{b}
2.5	57.66 ± 1.20^{de}	1.86 ± 0.12^{cd}	3.13 ± 0.88^{c}
TDZ			
0.5	46.33 ± 1.20^{h}	1.13 ± 0.88^{f}	1.83 ± 0.66^{ef}
1.0	53.33 ± 0.88^{f}	1.43 ± 0.12^{e}	1.63 ± 0.88^{fg}
1.5	56.66 ± 0.88^{de}	1.76 ± 0.88^{d}	2.23 ± 0.88^{d}
2.0	67.33 ± 1.20^{c}	2.26 ± 0.88^{ab}	2.26 ± 0.88^{d}
2.5	58.66 ± 1.11^{d}	2.03 ± 0.33^{bc}	2.33 ± 0.88^{d}
2iP			
0.5	34.66 ± 0.88^{j}	0.83 ± 0.66^{g}	1.13 ± 0.88^{i}
1.0	56.66 ± 0.88^{de}	1.73 ± 0.33^{d}	1.63 ± 0.88^{fg}
1.5	50.33 ± 0.66^{g}	1.43 ± 0.33^{e}	1.43 ± 0.88^{gh}
2.0	47.33 ± 0.88^{h}	1.33 ± 0.88^{ef}	1.36 ± 0.14^{ghi}
2.5	40.33 ± 0.33^{i}	1.86 ± 0.06^{cd}	1.26 ± 0.66^{hi}

Values represent means ± S.E. Values followed by the same *letter* are not significantly different at $p \leq 0.05$ according to DMRT

shoots was obtained from nodal explants, with 87% response on BA (1.5 mg L^{-1}) supplemented with IBA (0.5 mg L^{-1}) treatment (Table 2 and Fig. 1*b*). The potential of IBA along with BA on *in vitro* multiplication of shoots has been registered in *Ceropegia santapaui* (Chavan *et al.* 2014b), *C. candelabrum* (Beena *et al.* 2003), *C. noorjahaniae* (Chavan *et al.* 2014a), and *C. mohanramii* (Adsul *et al.* 2019).

Effect of Supplements on Shoot Elongation The shoots cultured on MS medium supplemented with BA (1.5 mg L^{-1}) and IBA (0.5 mg L^{-1}) were considered control for both adenine sulfate (Ads) and ascorbic acid (AA) supplementation. AA at 50 mg L^{-1} showed the best response in shoot elongation (13.6 cm) (Fig. 1*c*) with reduced frequency of 72.33%, and reduced shoot number (3.73) (Table 3). Due to the effective shoot elongation response, the number of shoots was reduced, compared to the previous experiments (Tables 1 and 2). *In vitro* shoot multiplication has been reported to be influenced by AA in *Eulophia ochrea* (Shriram *et al.* 2014) and *Litsea glutinosa* (Shah *et al.* 2013). Among the adenine sulfate concentration, 50 mg L^{-1} showed the best response in terms of shoot number (2.10) and frequency of response (54%) from nodal explant–derived shoots. The high efficiency of AA over other supplements on shoot regeneration has been noticed with *Ceropegia thwaitesii* (Muthukrishnan *et al.* 2012), *Hoya wightii* (Lakshmi *et al.* 2010), and

Table 2. Shoot multiplication of *C. maculata* on MS medium supplement with BA (1.5 mg L^{-1}) and auxins, after 45 d

PGRs (mg L^{-1})	Freq. of response (%)	Number of shoots per explant	Avg. shoot length (cm)
IBA			
0.1	45.66 ± 0.66^{d}	2.96 ± 0.66^{c}	3.73 ± 0.88^{c}
0.5	87.66 ± 0.88^{a}	6.66 ± 0.88^{a}	7.33 ± 0.12^{a}
0.7	65.66 ± 1.76^{b}	3.76 ± 0.88^{b}	4.66 ± 0.88^{b}
1.0	46.66 ± 1.45^{d}	2.86 ± 0.88^{c}	3.66 ± 0.12^{c}
NAA			
0.1	33.66 ± 0.88^{e}	1.93 ± 0.88^{d}	3.26 ± 0.12^{de}
0.5	46.33 ± 0.88^{d}	1.66 ± 0.88^{def}	3.46 ± 0.14^{cd}
0.7	54.66 ± 0.88^{c}	1.73 ± 0.18^{de}	3.66 ± 0.12^{c}
1.0	52.66 ± 1.20^{c}	1.53 ± 0.66^{efg}	3.23 ± 0.18^{def}
IAA			
0.1	24.66 ± 0.88^{g}	1.16 ± 0.66^{h}	2.93 ± 0.88^{efg}
0.5	28.66 ± 0.33^{f}	1.36 ± 0.88^{fgh}	2.90 ± 0.11^{fg}
0.7	$31.66 \pm 0.88e^{f}$	1.30 ± 0.11^{gh}	2.66 ± 0.88^{gh}
1.0	29.33 ± 0.88^{f}	1.26 ± 0.88^{gh}	2.53 ± 0.03^{h}

Values represent means ± S.E. Values followed by the same *letter* are not significantly different at $p \leq 0.05$ according to DMRT

Prosopis cineraria (Shekhawat *et al.* 1993). The addition of exogenous ascorbic acid to plant tissue is said to boost metabolic activity and speed up sugar release, allowing for better growth and development (George 1993). In plant tissue culture research, it is one-of-a-kind natural supplement.

***In Vitro* Rooting, Hardening, and Acclimatization** The *in vitro* derived shoots of *C. maculata* (up to 5 cm long) were transferred to rooting medium containing half-strength MS supplemented with auxins. Among the auxin, IBA at 0.5 mg L^{-1} showed the highest frequency of 87% (Fig. 1*d*) rooting

Table 3. Effect of additives on shoot multiplication of *C. maculata* on MS supplemented with BA (1.5 mg L^{-1}), IBA (0.5 mg L^{-1}), and additives, after 45 d

Additives (mg L^{-1})	Freq. of response (%)	Number of shoots per explant	Avg. shoot length (cm)
Ascorbic acids			
25	35.66 ± 1.45^{c}	2.16 ± 0.12^{c}	5.66 ± 0.12^{c}
50	72.33 ± 1.28^{a}	3.73 ± 0.88^{a}	13.6 ± 0.14^{a}
100	52.66 ± 1.32^{b}	3.06 ± 0.03^{b}	6.76 ± 0.13^{b}
Adenine sulfate			
25	54.33 ± 0.33^{b}	2.10 ± 0.05^{c}	4.53 ± 0.12^{d}
50	33.66 ± 0.88^{c}	1.96 ± 0.06^{c}	3.93 ± 0.88^{e}
100	30.66 ± 0.66^{c}	1.30 ± 0.15^{c}	3.26 ± 0.06^{f}

Values represent means ± S.E. Values followed by the same *letter* are not significantly different at $p \leq 0.05$ according to DMRT

response withpotential of IBA along an average of 9.33 roots per shoot (Table 4). All the roots are continued its linear growth without lateral roots. The effect of IBA at low concentration on *in vitro* rooting has been reported for *Ceropegia candelabrum* (Beena *et al.* 2003), *C. attenuates* (Chavan *et al.* 2011b), *C. thwaitesii* (Muthukrishnan *et al.* 2012), *C. barnesii* (Ananthan *et al.* 2018), and *C. mohanramii* (Adsul *et al.* 2019). In contrast, plantlets rooted in IAA and IBA reported to produce basal callus and unsuitable for acclimatization *Actinidia deliciosa* (Nasib *et al.* 2008). Hardening of *in vitro* rooted plantlets in paper cups containing a soil mixture grew well and was transferred to glasshouse conditions for acclimatization (Fig. 1*e*). The hardened plantlets were well acclimatized in the greenhouse condition in 30 d with a 90% survival rate (Fig. 1*f*).

***In Vitro* Flowering** The influence of cytokinin on *in vitro* flowering has been noticed in *Ceropegia* species such as *C. bulbosa*, *C. hirsute*, *C. lawii*, *C. maccannii*, *C. oculate* and *C. Sahyadri*, and *C. mohanramii* (Nair *et al.* 2007; Adsul *et al.* 2019). Similarly, in the present study, MS medium with BA and TDZ at low concentration showed a varied response in flower bud induction (Fig. 2*a*). Shoots cultured on BA 0.5 mg L^{-1} showed the highest response of 93% flower bud induction, with 4.86 flower buds per shoot (Fig. 2*b*), while BA at 2.0 mg L^{-1} with IAA reported to favor flowering in *C. mohanramii* (Adsul *et al.* 2019). Also, MS medium supplemented with 0.5 mg L^{-1} concentration of TDZ induced a maximum of 3.73 floral buds and showed a culture response rate of 85% (Table 5). The beneficial impact of TDZ on *in vitro* flowering has been observed in some *Ceropegia* species like *C. fantastica* (Chandore *et al.* 2010) and *C. bulbosa* (Britto *et al.* 2003). In the current study, blooming of flowers were observed in 20 d of culture period.

Table 4. Rooting of shoots of *C. maculata* on half-strength MS medium supplemented with auxins, after 20 d

Auxins (mg L^{-1})	Freq. of rooting (%)	Number of roots per shoot	Root length (cm)
IBA			
0.1	32.66 ± 1.20^{c}	3.53 ± 0.23^{cd}	1.43 ± 0.24^{bcd}
0.3	38.06 ± 1.43^{c}	4.26 ± 0.52^{c}	1.66 ± 0.12^{b}
0.5	87.66 ± 2.60^{a}	9.33 ± 0.32^{a}	3.26 ± 0.12^{a}
0.7	66.66 ± 1.97^{b}	6.86 ± 0.23^{b}	1.56 ± 0.17^{bcd}
1.0	57.33 ± 2.18^{b}	6.03 ± 0.73^{b}	1.43 ± 0.14^{bcd}
NAA			
0.1	0.00 ± 0.00^{d}	0.00 ± 0.00^{f}	0.00 ± 0.00^{e}
0.3	29.33 ± 1.67^{c}	1.66 ± 0.83^{e}	0.80 ± 0.41^{bcde}
0.5	36.33 ± 2.17^{c}	2.13 ± 0.38^{de}	1.13 ± 0.08^{bcd}
0.7	32.66 ± 1.45^{c}	2.23 ± 1.12^{de}	1.63 ± 0.82^{bc}
1.0	28.33 ± 1.20^{c}	2.96 ± 0.38^{cde}	0.73 ± 0.08de
IAA			
0.1	0.00 ± 0.00^{d}	0.00 ± 0.00^{f}	0.00 ± 0.00^{e}
0.3	0.00 ± 0.00^{d}	0.00 ± 0.00^{f}	0.00 ± 0.00^{e}
0.5	28.33 ± 1.70^{c}	2.16 ± 0.38^{de}	0.86 ± 0.08b^{cd}
0.7	22.33 ± 0.88^{c}	2.83 ± 0.40^{cde}	0.76 ± 0.08^{cde}
1.0	18.33 ± 1.20^{cd}	2.96 ± 0.08^{cde}	1.03 ± 0.08^{bcd}

Values represent means ± S.E. Values followed by the same *letter* are not significantly different at $p \leq 0.05$ according to DMRT

***In Vitro* Tuberization** Several factors have been reported to affect *in vitro* tuber formation, including medium type and strength; sucrose, auxin, and cytokinin concentrations; temperature; and photoperiod (Skoog and Miller 1957; Madec 1963; Hussey and Stacey 1984; Uranbey *et al.* 2010). Even the response to *in vitro* tuber induction differed depending on the species and explant type (Uranbey *et al.* 2010). The levels of auxin and cytokinin in the medium were investigated in this study. *In vitro* raised shoots cultured on medium containing BA, NAA, BA with IBA, or NAA produce only basal tubers at varied response and size in diameter (Table 6) which were morphologically similar to the previous reports in *C. media* (Pandey *et al.* 2021), *C. spiralis* and *C. pusilla* (Murthy *et al.* 2012), *C. ensifolia* (Reddy *et al.* 2015), and *C. spiralis* (Murthy *et al.* 2010). Of the

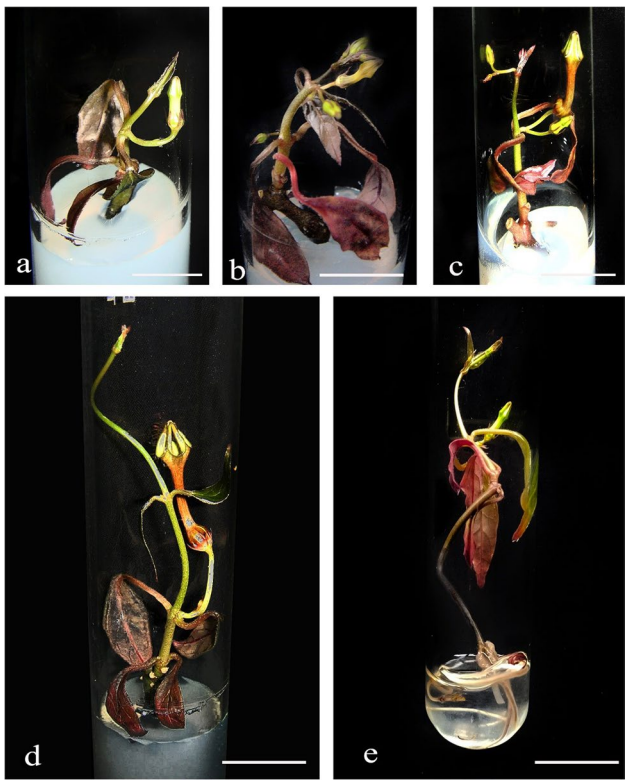

Figure 2. *In vitro* flowering of *C. maculata* Bedd. (*a*) Flower bud induction on BA 0.3 mg L^{-1}. (*b*) Multiple flower buds on BA 0.5 mg L^{-1}. (*c*) Inflorescence bearing flower buds. (*d*) Mature *in vitro* flower. (*e*) *In vitro* flower on rooted plantlet. *Bars*: (*a*) 1 cm; (*b–d*) 1.5 cm; (*e*) 2.0 cm.

treatments, MS medium supplemented with BA 2.0 mg L^{-1} and NAA 0.5 mg L^{-1} produced the highest average tuber diameter of 1.43 cm with a maximum response of 95.33% tuberization (Fig. 3*a*). While NAA at 3.0 mg L^{-1} had an overall frequency of 46.66% tuberization with an average tuber diameter of 0.85 cm (Fig. 3*b*). Skoog and Miller (1957) and Vanderhoef and Key (1968) stated that cytokinins are important for tuber formation and growth. This is expressed in the current research that BA induced higher percentage of *in vitro* tuber formation over the auxin NAA. The effect of cytokines on *in vitro* basal tuberization was noticed in other species of the family Asclepiadoideae, namely *C. jainii*, *C. bulbosa* var. *blbosa* and *C. bulbosa* var. *lushii* (Patil 1998), *C. pusilla* (Kalimuthu *et al.* 2014), *C. spiralis* (Murthy *et al.* 2012), *C. media* (Pandey *et al.* 2021), and *C. woodii* (Barakat *et al.* 2021). BA (2.0 mg L^{-1}) in combination with IBA at (0.5 mg L^{-1}) produced a maximum response of 78% basal tubers with an average diameter of 1.03 cm (Fig. 3*c*). A similar effect of NAA in combination with a cytokinin (BA) study on microtuber (Fig. 3*d*) formation was published in *Ceropegia pusilla* by Murthy *et al.* (2012). The impact of BA with IBA on basal tuber formation was recorded in *Ceropegia spiralis* by Murthy *et al.* (2012). In contrary, Mbanaso *et al.* (2007) recorded aerial tubers in *Dioscorea rotunda* on the nutrient-depleted medium. The histological sections of the developing *in vitro* tubers confirmed the

Table 5. *In vitro* flowering of *C. maculata* on MS medium supplemented with cytokinins, after 30 d of culture

Cytokinins (mg L^{-1})	Freq. of response (%)	Number of inflorescence	Number of flowers
TDZ			
0.3	72.66 ± 1.45^{cd}	1.63 ± 0.21^{c}	2.50 ± 0.17^{c}
0.5	85.66 ± 1.84^{ab}	2.06 ± 0.03^{b}	3.73 ± 0.08^{b}
0.7	66.66 ± 1.70^{cd}	0.73 ± 0.08^{e}	1.83 ± 0.06^{d}
1.0	61.33 ± 1.09^{d}	1.06 ± 0.03^{de}	2.46 ± 0.24^{c}
BA			
0.3	34.66 ± 1.17^{e}	0.73 ± 0.12^{e}	1.23 ± 0.03^{e}
0.5	93.33 ± 2.17^{a}	2.93 ± 0.06^{a}	4.86 ± 0.03^{a}
0.7	77.33 ± 1.45^{bc}	1.23 ± 0.12^{d}	2.36 ± 0.27^{c}
1.0	75.33 ± 0.33^{bc}	1.30 ± 0.05^{d}	2.66 ± 0.12^{c}

Values represent means ± S.E. Values followed by the same *letter* are not significantly different at $p \leq 0.05$ according to DMRT

Table 6. *In vitro* tuberization of *C. maculata* on MS medium supplement with BA, NAA, BA + IBA, and BA + NAA, after 60 d of culture

PGRs (mg L^{-1})	Freq. of response (%)	Nature of the tuber	Tuber diameter (cm)
BA			
1.0	42.66 ± 1.35^{h}	Basal tuber	0.91 ± 0.02^{cde}
1.5	52.66 ± 1.45^{f}	Basal tuber	1.05 ± 0.07^{bc}
2.0	67.66 ± 0.88^{d}	Basal tuber	1.20 ± 0.17^{b}
3.0	58.33 ± 0.88^{e}	Basal tuber	0.94 ± 0.01^{cd}
NAA			
1.0	31.33 ± 0.88^{j}	Basal tuber	0.65 ± 0.02^{f}
1.5	35.33 ± 0.88^{i}	Basal tuber	0.65 ± 0.02^{f}
2.0	40.66 ± 1.76^{h}	Basal tuber	0.74 ± 0.14^{ef}
3.0	46.66 ± 1.45^{g}	Basal tuber	0.85 ± 0.14^{de}
BA + IBA			
2.0 + 0.5	78.66 ± 1.20^{c}	Microtuber	1.03 ± 0.14^{bcd}
2.0 + 1.0	59.66 ± 1.76^{e}	Basal tuber	0.93 ± 0.03^{cd}
2.0 + 1.5	53.33 ± 1.20^{f}	Basal tuber	0.88 ± 0.01^{cde}
BA + NAA			
2.0 + 0.5	95.33 ± 0.88^{a}	Basal tuber	1.43 ± 0.03^{a}
2.0 + 1.0	84.33 ± 2.02^{b}	Basal tuber	1.02 ± 0.03^{cd}
2.0 + 1.5	77.33 ± 1.45^{c}	Basal tuber	0.87 ± 0.01^{cde}

Values represent means ± S.E. Values followed by the same *letter* are not significantly different at $p \leq 0.05$ according to DMRT

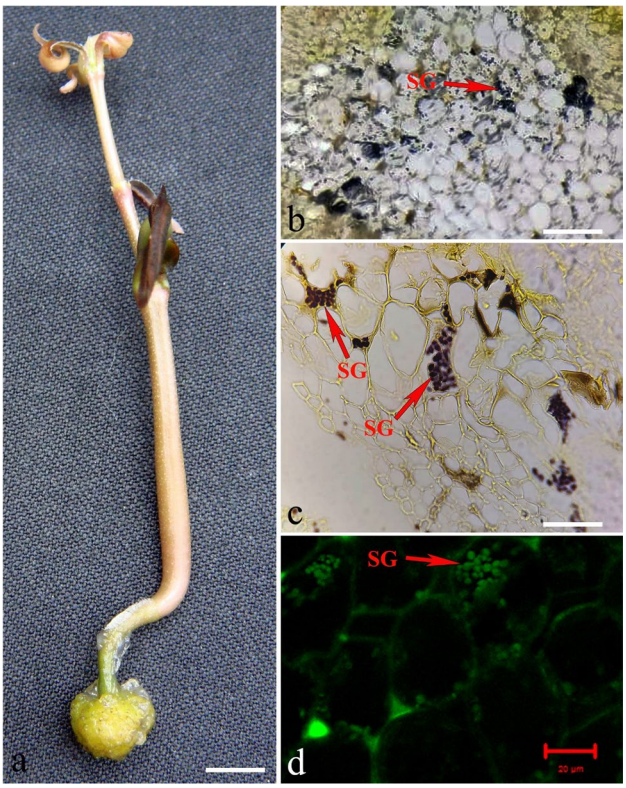

Figure 3. *In vitro* tuberization of *C. maculata* and histology. (*a*) Developing *in vitro* tuber on BA 2.0 mg L^{-1}. (*b*) Hand-made section of *in vitro* tuber with dark stained (*arrow*) starch granules (SG) (amyloplast) by diluted iodine (I2/KI), under light microscope. (*c*) Microtome section of developing *in vitro* tuber with pinkish-brown stained (*arrows*) starch granules (SG) (amyloplast) by iodine (I2/KI), under light microscope. (*d*) Starch granule-specific green fluorescence emission (*arrow*) detected using BA515/30 filter on confocal microscope. *Bars*: (*a*) 0.5 cm; (*b–d*) 100 µm, 40 µm, and 20 µm.

presence of starch granules (SG) (amyloplast) as dark to pinkish-brown, under a light microscope in their storage cells, by stained with iodine (I2/KI) (Fig. 3*b-c*). Confocal microscopic examination with 488 nm excitation also confirmed that the *in vitro* tuber contained storage starch by starch granule-specific green fluorescence emission detected using LSM488 filter (Fig. *3d*) (Ovecka *et al.* 2012). The starch granules were confirmed in bulbous plant, *Ledebouria ovatifolia*, by histological studies (Baskaran *et al.* 2016).

Conclusions

The present study describes effective protocols for micropropagation, *in vitro* flowering, and *in vitro* tuberization for *Ceropegia maculata*. Effective shoot production was achieved from nodal explants on MS medium supplemented with BA (1.5 mg L^{-1}) and IBA (0.5 mg L^{-1}). MS medium supplemented with BA (0.5 mg L^{-1}) influenced efficient *in vitro* flowering. Basal tubers were produced on BA (2.0 mg L^{-1}) with NAA (0.5 mg L^{-1}) combination. The tubers were confirmed with developing starch grains by using histological studies. *In vitro* shoots were best rooted on half-strength MS medium supplemented with IBA (0.5 mg L^{-1}). The rooted plantlets were successfully hardened and acclimatized with 90% survival rate. Developed protocol in this study is helpful for mass propagation and conservation of *C. maculata.*

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