# The *in vitro* culture of *Ceropegia* species, important medicinal and ornamental plants: a review

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**Abstract**

The genus *Ceropegia* of the Asclepiadaceae family encompasses a range of vines with medicinal and ornamental value. One way to clonally propagate such material is through *in vitro* culture. This review highlights the achievements made in *in vitro* culture and related biotechnologies of members of this genus. Details of protocols are provided and difficulties and hurdles that are yet to be overcome are pointed out. This short review provides a succinct synthesis of the literature on this topic and also a practical manual for researchers wishing to engage in new research involved with the *in vitro* culture of members of this genus. The range of studies conducted thus far is limited, and this review will serve as an impetus for new studies focusing on research themes that have not yet been explored.

**Key words:** *in vitro* regeneration, lantern flowers, plant growth regulator, vines.
**Abbreviations:** 2,4-D, 2,4-dichlorophenoxyacetic acid; 2iP, $N^6$-isopentenyladenine or 6-γ,γ-dimethylallylaminopurine; AC, activated charcoal; BA, $N^6$-benzyladenine; B5, Gamborg et al. (1968) medium; CIM, callus induction medium; DDW, double-distilled water; DSO, direct shoot organogenesis; FIM, flower induction medium; $HgCl_2$, mercury chloride; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; ISO, indirect shoot organogenesis; ISSR, inter-simple sequence repeat; Kin, kinetin ($N^6$-furfyladenine); MIM, microtuber induction medium; MS, Murashige and Skoog (1962) medium; NAA, α-naphthaleneacetic acid; NaOCl, sodium hypochlorite; NR, not reported; PGR, plant growth regulator; picloram, 4-amino-3,5,6-trichloropicolinic acid; PP, photoperiod; PPFD, photosynthetic photon flux density; RAPD, random amplified polymorphic DNA; RH, relative humidity; RIM, root induction medium; rpm, revolutions per minute; RTP, running tap water; SDW, sterile distilled water; SIM, shoot induction medium; TCL, thin cell layer; TDZ, thidiazuron (N-phenyl-N'- 1,2,3-thiadiazol-5-ylurea); tTCL, transverse thin cell layer

## The importance of *Ceropegia*

APG III (2009) lists 220 species within the genus *Ceropegia* (Asclepiadaceae family), commonly known as lantern flowers, which are mostly distributed in tropical and sub-tropical regions (Bruyns 2003). Despite the size of the genus, only a single study has used nuclear ribosomal internal transcribed spacer and three non-coding chloroplast DNA sequences, including intergenic spacers *trnT-L* and *trnL-F*, and *trnL* introns to study the phylogeny and evolutionary relationships among Indian *Ceropegia* and their allies (Surveswaran et al. 2009). Many species have medicinal value while several others have been domesticated as house plants and are used as ornamentals (Reynolds 2006; Chavan et al. 2011b). The highest species diversity occurs in South Africa, while India ranks fourth (Murthy et al. 2012b), with a heavy concentration (approx. 50 species) in Western Ghats (Karthikeyan et al. 2009), 28 of which are endemic to peninsular India. Unsurprisingly, there are a large number of conservation- and tissue culture-related studies emerging from India (Murthy et al. 2012b). For example, *C. candelabrum* (glabrous goglet flower) root tubers contain the alkaloid ceropegine, common to many *Ceropegia* spp. (Sukumar et al. 1995), starch, sugar, gum, albuminoids, fats and crude fiber of this species are used in Indian Ayurvedic medicine, as "soma", to treat diarrhea and dysentery (Beena et al. 2003, and references therein). *C. pusilla* also has a wide range of medicinal properties (Kalimuthu, Prabakaran 2013a, and references therein). In addition to their health, medicinal and ornamental value, *C. spiralis, C. panchganiensis* and *C. evansii* were shown to possess 1,1-diphenyl-2-picryl hydrazyl radical scavenging activity, ferric reducing antioxidant power and metal-chelating ability and to contain phenolic compounds such as gallic acid, vanillin, cathechol and ferulic acid (Chavan et al. 2013a). *C. santapaui* also possessed similar activities (Chavan et al. 2014). Other economically important characteristics have been described in more detail by Kalimuthu and Prabakaran (2013c). Muthukrishnan et al. (2013a) describe a factor-by-factor assessment of the micropropagation of *Ceropegia* spp. as well as an overview of ceropegin retrosynthesis.

## *Ceropegia in vitro*

There are two reasons for the surge in studies on *Ceropegia* spp.: (a) the listing of all species in India as being endangered (Murthy et al. 2012b); (b) the limited propagative capacity,

low seed set and viability (Yadav, Kamble 2006). Several studies reported on somatic embryogenesis, for example in *C. candelabrum* (Beena et al. 2003) in the presence of 4.52 µM 2,4-dichlorophenoxyacetic acid (2,4-D) in Murashige and Skoog (MS; 1962) medium, although no histological proof was provided or any photographic evidence of all four stages of somatic embryogenesis. Flowers induction *in vitro* was possible for several species: *C. bulbosa* (Britto et al. 2003), *C. attenuata* (Chavan et al. 2011b) and *C. pusilla* (Kalimuthu, Prabakaran 2013b). *In vitro* flowering of plants allows for the production of sterile floral tissues, useful for the study of reproductive developmental processes, regeneration from floral tissues and the use of floral organs for genetic transformation (Teixeira da Silva et al. 2014). Several studies used thin cell layers (Teixeira da Silva, Dobránszki 2013) to enhance shoot production (Table 1).

The few studies on the molecular verification of the stability of *in vitro* clonal material exist. Chavan et al. (2013b) used random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) markers to detect for somaclonal variation among tissue-cultured *C. panchganiensis* plants in comparison to mother plants, finding 1.31% variation (RAPD and ISSR combined), confirming similar values (0.81%) for similar previous studies on *C. spiralis* using RAPD and ISSR (Chavan et al. 2013c). The same two techniques were used by Chavan et al. (2014) on *C. santapaui* in which RAPD detected no variation while ISSR detected 1.2% polymorphism between plantlets derived from direct and indirect organogenic routes and mother plants. Similarly, Chavan et al. (2015) used RAPD and ISSR to test for polymorphism in tissue-cultured *C. evansii* and found no variability (0% polymorphism) among micropropagated plants from 759 bands generated. Dhir and Shekhawat (2013) found a higher level of polymorphism (7.8%) in RAPD banding between micropropagated and mother *C. bulbosa* var. *bulbosa* plants. The latter study was the only study to date to encapsulate nodal segments in calcium alginate (3.0% sodium alginate + 100 mM $CaCl_2$ $2H_2O$) beads, which could be stored at a low temperature (4 °C) for up to 60 days with 50.7% survival. Murthy et al. (2013) also devised encapsulation protocols for *C. pusilla* and *C. spirallis*. Encapsulation studies and the use of synthetic seeds and low-temperature storage are an important first step for establishing cryopreservation protocols for the long-term storage of material (Sharma et al. 2013). Other than these studies, no other molecular-based analyses exist for *Ceropegia* spp.

Most studies thus far on *Ceropegia* spp. have focused on the *in vitro* propagation, primarily with a concern for the conservation of endangered species. To achieve this goal, *in vitro* propagation has been the only biotechnological tool used thus far. Considering that only approximately one dozen species have been propagated thus far *in vitro,* it is expected that protocols for the remaining 2-3 dozen in India will emerge soon. Syntheses of the literature, such as for *Bauhinia* spp., another climbing ornamental (Teixeira da Silva 2013), provide an important platform for the advancement of *in vitro* (including micropropagation) studies in this genus.

**Acknowledgements**

The author thanks Prof. K. Sri Rama Murthy (Montessori Mahila Kalasala, India), Dr. Jaykumar J. Chavan (Yashavantrao Chavan Institute of Science, India), Prof. Gyan S. Shekhawat (Banasthali University, India), Mafatlal M. Kher (Sardar Patel University, India) and Dr. Judit Dobránszki (University of Debrecen, Hungary) for providing literature and/or for advice and comments on the *Ceropegia* literature. The author declares no conflicts of interest, financial, or other.

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**Table 3.** *In vitro* propagation and micropropagation of *Ceropegia* species. 2,4-D, 2,4-dichlorophenoxyacetic acid; 2iP, $N^6$-isopentenyladenine or 6-γ,γ-dimethylallylaminopurine; AC, activated charcoal; BA, $N^6$-benzyladenine [BA is used throughout even though BAP (6-benzylamino purine) may have been used in the original, according to Teixeira da Silva 2012b]; B5, Gamborg et al. (1968) medium; CIM, callus induction medium; DDW, double-distilled water; DSO, direct shoot organogenesis; FIM, flower induction medium; $HgCl_2$, mercury chloride; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; ISO, indirect shoot organogenesis; ISSR, inter-simple sequence repeat; Kin, kinetin ($N^6$-furfyladenine); MIM, microtuber induction medium; MS, Murashige and Skoog (1962) medium; NAA, α-naphthaleneacetic acid; NaOCl, sodium hypochlorite; NR, not reported; PGR, plant growth regulator; picloram, 4-amino-3,5,6-trichloropicolinic acid; PP, photoperiod; PPFD, photosynthetic photon flux density; RAPD, random amplified polymorphic DNA; RH, relative humidity; RIM, root induction medium; rpm, revolutions per minute; RTP, running tap water; SDW, sterile distilled water; SIM, shoot induction medium; TCL, thin cell layer; TDZ, thidiazuron (N-phenyl-N'- 1,2,3-thiadiazol-5-ylurea); tTCL, transverse thin cell layer

| Genus, species and/or cultivar | Explant used | Sterilization procedure | Culture medium, PGRs and additives | Culture conditions | Remarks, experimental outcome, acclimatization and variation | Reference |
|---|---|---|---|---|---|---|
| *C. jainii, C. bulbosa* Roxb. var. *bulbosa*, *C. bulbosa* var. *lushii* | Nodes (2–3 mm long) from 10–12 week-old plants. | Explants washed in water + 10% Tween 20. Rinse in RTW. 0.1% $HgCl_2$ for 5 min, and 3–4 rinses in SDW. | MS + 9.05 μM 2,4-D (CIM). ½ MS + 8.88 μM BA (SIM or 2.22 μM BA for multiple shoots). The latter + 0.25 μM spermine = FIM (*C. jainii*). ½ MS + 22.2 μM BA + 23.2 μM Kin (MIM). ¼ MS + 2.5 μM IBA (RIM). 3% sucrose; 0.2% Phytagel; pH 5.8. | 14-h PP, PPFD = 45 μmol $m^{-2}$ $s^{-1}$, 25 ± 2 °C | *In vitro* shoots dipped in 49.2 μM IBA for 15 s, then embedded in sterile sand. Maximum of 82% and 88% of explants formed shoots and microtubers, respectively. | Patil 1998 |
| *C. candelabrum* L. | Leaf and internode segments from young shoot tips of mature plants. | Segments were washed under RTW then in 5% extran (a detergent) for 5 min. After several washes in DDW, segments were surface sterilized with 0.5% $HgCl_2$ for 12–14 min and rinsed with 3 washes (5 min each) of sterile DDW. Leaves cut into 1 $cm^2$ explants, internode 1 cm) | MS + 2.26–9.05 μM 2,4-D alone or in combination with 2.22 μM BA or 2.32 μM Kin (SEIM). Carbon source NR; 0.8% agar; pH 5.8. For suspension culture, 100 mg of friable callus was transferred to 100-mL conical flasks with 25 mL of liquid SEIM. Suspension cultures were incubated in the dark on a rotary shaker at 120 rpm. | 16-h PP, PPFD NR, 25 ± 2 °C. Sub-cultured every 40 days. | A claim of somatic embryogenesis but without histological proof. 100 mg of callus induced > 500 somatic embryos. On PGR-free ¼ MS solid medium (maturation medium), 50% of somatic embryos converted to plantlets, 90% of which could survive in the field. | Beena, Martin 2003 |
| *C. candelabrum* | Axillary buds at nodes from the tender stems of mature plants. | Nodal segments were washed under RTW, then in 5% extran (a detergent) for 5 min. After several washes in DDW, segments were surface sterilized with 0.5% $HgCl_2$ for 10–14 min and rinsed with several washes of sterile DDW. | MS + 8.87 μM BA + 2.46 μM IBA (SIM and axillary shoot sub-culture). ½ MS + 0.49 μM IBA (RIM). 3% sucrose; 0.8% agar; pH 5.8. | 16-h PP, 25 μmol $m^{-2}$ $s^{-1}$, 25 ± 2 °C | 7.8 shoots/node or 10 shoots/node in sub-cultures (SIM). 6.9 roots/shoot (RIM). Rooted plantlets acclimatized in soilrite : sand (1:1), then to the field. | Beena et al. 2003 |

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**Table 1.** *continued*

| Genus, species and/or cultivar | Explant used | Sterilization procedure | Culture medium, PGRs and additives | Culture conditions | Remarks, experimental outcome, acclimatization and variation | Reference |
|---|---|---|---|---|---|---|
| *C. bulbosa* Roxb. var. *bulbosa* | Nodes from garden plants. | Young shoots (2 cm long) washed in RTW for 30 min, then one wash in SDW, 0.1% $HgCl_2$ for 4 min, and 3 rinses in SDW. | B5 + 3 mg/L BA + 0.05 mg/L NAA (SIM). 2 mg/L IBA (RIM). 0.5 mg/L BA+ 1 mg/L $GA_3$ (FIM). 0.05 mg/L Kin + 2 mg/L IBA (MIM). | 16-h PP, 3000 lux, 25 ± 2 °C | 12 shoots/node. 70% acclimatization possible. 76% of plants in FIM flowered, and 65% of those seeds were viable. | Britto et al. 2003 |
| *C. bulbosa* | Nodes from field-grown plants. | Stems placed in tap water with 4–5 drops Tween 20. 2–3 cm long nodal segments treated with 0.05–0.10% Bavistin (fungicide) for 10 min, then 0.05–0.10% streptomycin (antibiotic) for 10 min. Each time 3X rinses with SDW. 0.1–0.5% $HgCl_2$ for 2–3 min, and 3 rinses in SDW. | MS + 4.44 µM BA + 0.58 µM $GA_3$ + 0.27 µM NAA (SIM). MS + 11.42 µM IAA (RIM). 3% sucrose; 0.8% agar-agar; pH 5.8. | 16-h PP, 2000 lux, 25 ± 2 °C | 2.2. shoots/node. 2.8 roots/shoot. | Goyal, Bhadauria 2006 |
| *C. lawii* Hook., *C. maccannii* Ansari., *C. oculata* Hook., *C. sahyadrica* Ansari et Kulkarni, *C. bulbosa* Roxb. var. *bulbosa*, *C. hirsuta* Wt et Arn. | Apical buds and axillary buds from 2-week-old seedlings. | Follicles held under RTW for 10 min, soaked for 20 min in 100 mL of tap water + 2–4 drops Tween-20; washed 2X with SDW. 70% ethanol for 1 min, 1X rinse with SDW. 0.1% $HgCl_2$ for 5 min. Washed 3X with SDW. Follicles cut longitudinally along sutures to obtain the seeds under aseptic conditions. | MS + 87 mM sucrose (seed germination). MS + 26.67 µM BA or 175 mM sucrose (FIM). 0.8% agar-agar; pH 5.8. | 16-h PP, 40 µmol $m^{-2}$ $s^{-1}$, 25 ± 2 °C | Sub-cultured apical buds and axillary buds showed similar flowering frequencies (maximum of 1.7–3.6 across the 6 species; response to BA levels; 1.0–6.6 across the 6 species; response to sucrose levels), within 30 days. *In vitro* flowers were smaller than in planta counterparts. | Nair et al. 2007 |
| *C. sahyadrica* Ans. & Kulk. | Nodes from mature indehiscent follicle | Not available | MS + 1 mM 2,4-D + 5 mM BA (CIM). MS + 1 mM BA (SIM). MS + 6 mg/L spermidine + 5% sucrose (RIM). 3% sucrose. 0.8% agar. pH 5.8. | 16-h PP, 50–80 µmol $m^{-2}$ $s^{-1}$, 25 ± 2 °C | 6.1 shoots/explant | Nikam, Savant 2007 |

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**Table 1.** *continued*

| Genus, species and/or cultivar | Explant used | Sterilization procedure | Culture medium, PGRs and additives | Culture conditions | Remarks, experimental outcome, acclimatization and variation | Reference |
|---|---|---|---|---|---|---|
| *C. odorata* Hook, *C. maccannii* Ansari | Node, internode, leaves (up to node 6) | Wash in SDW. 0.1% $HgCl_2$ for 5-7 min. Washed 5X with SDW. | MS + 7.5 µM BA + 3% sucrose + 0.8% agar (SIM) ½ MS + 5% sucrose + 0.5 µM IBA (liquid; RIM) pH 5.5-6.0. | 16-h PP, 50–80 µmol $m^{-2}$ $s^{-1}$, 25 ± 2 °C | 5.2 or 6.1 shoots/explant (*C. maccannii, C. odorata*, respectively). Acclimatization in garden soil completely covered for 2 days, then natural conditions, resulting in about 80% survival. | Nikam et al. 2008a |
| *C. hirsuta* Wt & Arn. | Node, internode, leaves | Wash in SDW. 0.1% $HgCl_2$ for 4–5 min. Washed 5–6X with SDW. | MS + 7.5 µM BA (SIM). ½ MS (liquid) + 2 µM IAA (RIM). 3%, 5% sucrose (SIM, RIM); 0.8% agar; pH 5.8. | 9-h PP, 18–24 µmol $m^{-2}$ $s^{-1}$, 25 ± 2 °C | 5.7 shoots/node. Acclimatization reported, but not quantified. | Nikam et al. 2008b |
| *C. hirsuta, C. lawii, C. maccannii, C. oculata, C. sahyadrica* | Axillary and apical buds of seed-derived shoots | Protocol unclear. | MS + 6 mg/L BA (MIM). 4% sucrose; 0.8% agar-agar; pH 5.8. | 16-h or 24-h PP, 40 µmol $m^{-2}$ $s^{-1}$, temp. NR | Axillary and apical buds could not develop tubers but instead flowered *in vitro*. Secondary tuberization also possible. | Pandit et al. 2008 |
| *C. intermedia* | Axillary shoots from 1–1.5 cm long nodes. | Young shoots with 6 internodes from garden plants washed with RTW for 15 min. 4 cm nodes washed with Tween 20 (5% v/v) for 10–15 min, 3–4 rinses with SDW, 80% ethanol for 30 s then 0.1% $HgCl_2$ for 5 min. 4–5 washes with SDW. | MS + 6.66 µM BA (SIM). MS + 5.37 µM NAA (RIM). 2% sucrose; 0.8% agar-agar; pH 5.8. | 16-h PP, PPFD and temp. NR. | 5.51 shoots/node. 75% acclimatization survival in garden soil : forest humus (1:1). | Karup-pusamy et al. 2009 |
| *C. sahyadrica* | Nodes and internodes from garden-grown plants. | Stems washed in SDW; 0.1% $HgCl_2$ for 5 min; 4 rinses with SDW. | MS + 1.0 µM 2,4-D + 5.0 µM BA (CIM). MS + 7.5 µM Kin (SIM). 0.5-2.0 µM IAA or NAA (RIM). 0.8% agar; pH 5.8. | 16-h PP, 20–30 µmol $m^{-2}$ $s^{-1}$, 25 ± 2 °C | Nodes performed best: 8.3 shoots/explant. Highest cerpegin content (470 µg/g dry weight) from callus induced on CIM with 10.0 µM IAA instead of 2,4-D. | Nikam, Savant 2009 |
| *C. bulbosa* | Epicotyls from seedlings. | NR. | MS + 1.0 mg/L 2,4-D (CIM). MS + 1.0 mg/L BA + 0.1 mg/L NAA (SIM). ½ MS + 1.0 mg/L BA + 0.1 mg/L NAA + 0.1% AC (RIM). | NR. | *In vitro* shoots rooted *ex vitro* after dipping in 100 mg/L IBA. No evidence provided for *in vitro* growth, no evidence or data provided for claims of somatic embryogenesis and *in vitro* flowering. | Rathore, Shekhawat 2009; Rathore et al. 2010 |

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**Table 1.** *continued*

| Genus, species and/or cultivar | Explant used | Sterilization procedure | Culture medium, PGRs and additives | Culture conditions | Remarks, experimental outcome, acclimatization and variation | Reference |
|---|---|---|---|---|---|---|
| *C. fantastica* Sedgw. | Nodes (1 cm long). | Shoots from tubers were washed in RTW for 30 min., a wash in liquid soap (5%, v/v) for 10 min, DDW several times, 0.1% $HgCl_2$ for 7 min, 4–5 rinses in SDW. | MS + 1.5 mg/L BA (SIM). $CaCl_2$-free MS + 1 mg/L IBA (RIM). 3% (w/v) sucrose; 0.2% gelrite; pH 5.8. | 16-h PP, 25 µmol $m^{-2}$ $s^{-1}$, 25 ± 2 °C | 10.2 shoots/node. 65% acclimatization after potted in soil : coco-peat (1:1) and watered with sucrose- and PGR-free ½ MS. | Chandore et al. 2010 |
| *C. pusilla* Wight and Arn. | Node, internode, TCLs from 1-month-old plant. | Nodes washed in RTW. 0.3% fungicide + 0.3% bacteriocide, each for 10 min. Tween 20 (5% v/v) for 4 min. 0.1% $HgCl_2$ for 2 min. Several washes with SDW. | MS + 13.32 µM BA + 0.45 µM 2,4-D (CIM). MS + 22.7 µM TDZ (SIM). MS + 13.32 µM BA + 0.49-1.23 µM IBA (RIM). ½ MS + 0.02 mg/L IAA + 3% sucrose (FIM). Carbohydrate NR (CIM, SIM, RIM). 0.9% agar-agar; pH 5.7. | 16-h PP, 18-24 µmol $m^{-2}$ $s^{-1}$, 24 ± 2 °C | Best callus induction from TCLs. 37.54 shoots per callus clump. 60% of explants flowered with a maximum of 20 flowers/plantlet. | Kondamudi et al. 2010; Kondamudi, Murthy 2011 |
| *C. spiralis* | TCLs of nodes and internodes. | Same protocol as Kondamudi et al. 2010. | MS + 13.32 µM BA + 4.52 µM 2,4-D (CIM). MS + 13.32 µM BA + 0.54 µM NAA (SIM). ½ MS + 10.74 µM NAA (RIM). Carbohydrate NR. 0.9% agar-agar; pH 5.7. | 16-h PP, 18-24 µmol $m^{-2}$ $s^{-1}$, 24 ± 2 °C | 17.34 shoots/TCL. Plantlets obtained within 8 weeks. 90% survival after acclimatization. | Murthy et al. 2010a, 2010b, 2012; Murthy, Kondamudi 2011 |
| *C. spiralis* | Single nodes (1 cm) of greenhouse plants. | Stems with 5–6 nodes washed under RTW for 20 min. 0.5% (w/v) Bavistin (fungicide) for 5 min. 3 washes with SDW. 5% Tween 20 for 10 min. Several washes with SDW. 70% ethyl alcohol for 1 min and a wash with SDW. 0.1% $HgCl_2$ for 4 min; 3 rinses with SDW. | MS + 2.0 mg/L BA + 0.5 mg/L TDZ (SIM). 3% (w/v) sucrose; 0.2% gelrite; pH 5.8. | 16-h PP, 30 µmol $m^{-2}$ $s^{-1}$, 25 ± 2 °C | 10.2 shoots/node, increasing to 12.1/node on the 3rd subculture. Acclimatization on sterile soil, sand and coco peat (1:2:1), but not quantified. | Chavan et al. 2011a |

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**Table 1.** *continued*

| Genus, species and/or cultivar | Explant used | Sterilization procedure | Culture medium, PGRs and additives | Culture conditions | Remarks, experimental outcome, acclimatization and variation | Reference |
|---|---|---|---|---|---|---|
| *C. attenuata* Hook. | Single nodes of mature greenhouse plant. | Shoot segments defoliated and washed under RTW for 20 min, washed with detergent, Tween 20 (5% v/v) for 10 min, Bavistin (0.5% w/v) for 3 min then SDW. Under aseptic conditions, explants dipped in 70% (v/v) alcohol for 30 s and 0.1% (w/v) $HgCl_2$ for 5 min. Each treatment followed by 3–4 rinses with SDW. | MS + 13.31 µM BA (SIM); 4.14 µM picloram (FIM); ½ MS + 2.46 µM IBA (RIM). All media with 3% (w/v) sucrose and 0.2% gelrite. | 16-h PP, 33 µmol $m^{-2}$ $s^{-1}$, 25 ± 2 °C | On SIM, 12.9 shoots/explant. 100% flowering achieved on FIM within 4 weeks. 85% of in vitro rooted plantlets survived in garden soil watered with ½ MS. | Chavan et al. 2011b |
| *C. juncea* | Shoot tips, nodes and internodes from *in vitro* plantlets. | Seeds washed with two drops of 1% Tween-20 for 20 min with constant shaking. Rinse in RTW for 30 min and repeated rinses with Millipore water. Under aseptic conditions, seeds washed with SDW, 70% ethanol for 30 s, wash with SDW, 20% $HgCl_2$ for 6 min and 5 rinses 5 with SDW. | Sterilized seeds germinated on PGR-free ½ MS. MS + 8.87 µM BA + 4.54 µM TDZ (SIM). MS + 4.90 µM IBA + 1.27 µM NAA (RIM). 2% sucrose; 0.6% agar; pH 5.8. | 16-h PP, 60 µmol $m^{-2}$ $s^{-1}$, 25 ± 2 °C | 20.65 shoots/node.78% acclimatization survival in garden soil : forest humus (1:1). | Krishnareddy et al. 2011 |
| *C. elegans* | Nodes from greenhouse plants. | Young shoots with 6 internodes washed with RTW for 15 min. 4 cm nodes washed with 5% Tween 20 for 15 min then rinsed 3–4 times with SDW. 80% ethanol for 30 s, then 0.1% $HgCl_2$ for 6 min, 4–5 rinses with SDW. | MS + 23.20 µM Kin + 5.71 µM IAA (SIM). MS + 4.90 µM IBA (RIM). 3% sucrose; 0.8% agar; pH 5.8. | 16-h PP, 50 µmol $m^{-2}$ $s^{-1}$, 25 ± 2 °C | 7.11 shoots/node. 72% acclimatization survival in garden soil : forest humus (1:1). | Krishnareddy, Pullaiah 2012 |
| *C. thwaitesii* Hook. | Nodes (2 cm long). | Stems washed in RTW for 20–30 min; 70% ethanol for 30 s; rinsed with SDW; 0.1% $HgCl_2$ for 3 min; 3–5 washes with SDW. | MS + 13.94 µM Kin + 28.54 µM IAA (SIM). MS + 2.46 µM IBA (RIM). 3% sucrose; 0.7% agar; pH 5.8. | 16-h PP, 60 µmol $m^{-2}$ $s^{-1}$, 26 ± 2 °C | 3.28 and 6.42 shoots/node upon first subculture and subculture, respectively. Removing agar from medium improved shoot regeneration and increased chlorophyll content. 73.33% acclimatization in red soil + sand + coconut coir (1:1:1). | Muthukrishnan et al. 2012, 2013b |

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**Table 1.** *continued*

| Genus, species and/or cultivar | Explant used | Sterilization procedure | Culture medium, PGRs and additives | Culture conditions | Remarks, experimental outcome, acclimatization and variation | Reference |
|---|---|---|---|---|---|---|
| *C. mahabalei* Hemadri & Ansari and *C. media* (Huber) Ansari | Node, internode, leaves | RTW for 10 min. 0.1% Tween-20 for 5 min. 0.1% $HgCl_2$ for 5 min. Washed 8X with SDW. | MS + 5 µM BA (SIM). MS (liquid) + 1 µM NAA (RIM). 3%, 4% sucrose (SIM, RIM); 0.8% agar; pH 5.8. | 8-h PP, 40 µmol $m^{-2}$ $s^{-1}$, 25 ± 2 °C, 60 ± 10% RH. | Best regeneration from nodes with a sub-culture every 3 weeks (~10 shoots/explant). Acclimatization in garden soil : sand (1:1), max 200 µmol $m^{-2}$ $s^{-1}$, 80 ± 10% RH under a shaded net house for 2 weeks, then transferred to field conditions (18-32°C; max 400 µmol $m^{-2}$ $s^{-1}$, 55-80% RH). 88% survival reported. | Nikam et al. 2012 |
| *C. panchganiensis* Blatter and McCann | Single nodal explants from in vitro-derived shoots. | Vegetative cuttings washed under RTW for10 min. Shaken in freshly prepared 0.5% Bavistin (w/v) for 20 min, washed twice in SDW, then surface sterilized with 0.2% $HgCl_2$ for 6 min, then 3X rinses with SDW (5 min/rinse). Cuttings divided into single node explants | Development of axillary shoots from nodes: MS + 3% sucrose + 0.2% ClariGel + 4.44 µM BA; pH 5.8 MS + 13.31 µM BA + 2.69 µM NAA (SIM). 9.05 µM 2,4-D (CIM). ½ MS + 4.44 µM BA + 175 mM sucrose (FIM). ½ MS + 17.74 µM BA + 175 mM sucrose (MIM). ½ MS + 7.36 µM IBA (RIM). | 16-h PP, 50 µmol $m^{-2}$ $s^{-1}$, 25 ± 1 °C | On SIM, 13.2 shoots/explant. 95% of explants formed callus on CIM. 80% of explants on FIM formed in vitro flowers. 85% of explants formed microtubers on MIM. 96% of shoots rooted on RIM. From RAPD and ISSR analyses, 1.31% polymorphism (syn. somaclonal variation) was observed. 85% of *in vitro*-derived plantlets could be acclimatized in a mixture of soil, sand and coco peat (1:2:1) at 60-65% RH. | Chavan et al. 2013b |
| *C. bulbosa* var. *bulbosa* | Single nodal segments (1–1.5 cm) from mature plants. | Shoot segments with single nodes were rinsed under RTW for 45 min then washed with 2% cetrimide + 1–2 drops of Tween-20 + 1% NaOCl for 15 min, then RTW for 30 min. Surface sterilization with 70% ethanol for 30 s, then 0.05% $HgCl_2$ for 3 min, 5 rinses with SDW. | MS + 8.88 µM BA (SIM and axillary shoot sub-culture). ½ MS (auxin-free) (RIM). 3% sucrose; 0.8% agar; pH 5.8. | 16-h PP, 40 µmol $m^{-2}$ $s^{-1}$, 25 ± 2 °C, 50 ± 5% RH | 9.7 shoots/node on SIM (100% of cultures), although Kin, TDZ and NAA also induced shoot formation. On RIM, as many as 11 roots/shoot. Nodal segments encapsulated in calcium alginate beads could form plantlets within 4 weeks, which could then be fully acclimatized within a further 2 weeks. 7.8% polymorphism shown by RAPD. | Dhir, Shekhawat 2013 |

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**Table 1.** *continued*

| Genus, species and/or cultivar | Explant used | Sterilization procedure | Culture medium, PGRs and additives | Culture conditions | Remarks, experimental outcome, acclimatization and variation | Reference |
|---|---|---|---|---|---|---|
| *C. pusilla* | Nodes from field-grown plants. | Shoots with 5-6 nodes washed with RTW for 15 min. Nodes (1–2 cm) washed with Tween 20 (5% v/v) for 5 min and 3–4 rinses in SDW. 70% ethanol for 30 s, 0.12% (w/v) $HgCl_2$. 3–4 rinses with SDW. | MS + 11 µM BA + 5.35 µM NAA (SIM). MS + 2 mg/L BA + 1.0 g/L NAA (CIM). MS + 6.66 µM BA + 5.35 µM NAA (MIM). ½ MS + 4.44 µM BA + 2.46 µM IBA (FIM). 3% sucrose; pH 5.8. | 16-h PP, 50 µmol $m^{-2}$ $s^{-1}$, 25 ± 2 °C. | 3.5 shoots/explant. 90-95% of callus formed shoots. *In vitro* flowering induced within 32 days and microtubers in 90% of explants within 20-25 days. 81% of acclimatized plants survived in decomposed coir. | Kalimuthu, Prabakaran, 2013b; Prabakaran et al. 2013; Kalimuthu et al. 2014 |
| *C. bulbosa* | Seedling-derived epicotyls. | Seeds surface sterilized with 0.1% $HgCl_2$ for 4-5 min, 2–3 rinses with SDW. | PGR-free MS (seed germination). MS + 1 mg/L 2,4-D (CIM). MS + 1 mg/L 2,4-D + 0.5 mg/L BA (callus proliferation). MS + 1 mg/L BA + 0.1 mg/L NAA (SIM). | 12-h PP, 30-40 µmol $m^{-2}$ $s^{-1}$, 26 ± 2 °C | Callus-derived shoots. Maximum of 75% of explants formed white, friable callus. Shoots could be rooted *ex vitro* when dipped in 100 mg/L IBA for 3 min and acclimatized (100%) in organic manure, clay soil and sand (1:1:1). | Phulwaria et al. 2013 |
| *C. santapaui* Wadhwa and Ansari | Cotyledonary nodes and cotyledons from 15-day-old seedlings. | Seeds from mature plants were separated from follicles, washed and shade-dried for 3 days, surface sterilized with 0.1% $HgCl_2$ for 5 min under aseptic conditions and rinsed 2–3 times with SDW. Seeds were germinated on PGR-free MS medium. | MS + 2 mg/l 2iP (SIM – DSO). MS + 1.5 mg/L picloram (CIM – ISO). MS + 2.5 mg/L BA + 0.4 mg/L IBA (SIM – ISO). All media with 3% sucrose + 0.1 g/L *myo*-inositol + 0.2% ClariGel; pH 5.8. | 16-h PP, 50 µmol $m^{-2}$ $s^{-1}$, 25 ± 1 °C | 8.1 shoots/cotyledonary node in SIM – DSO. A maximum of 97.5% of cultures produced callus in CIM – ISO and 19.7 shoots/callus piece in SIM – ISO. Plants were acclimatized in the *in vitro* growth room under same light and temperature conditions, watering soil, sand and coco peat (1:2:1) with ½ MS. Two-week-old plantlets were transferred to a glasshouse (30 °C; 70% RH; 50 µm $m^{-2}$ $s^{-1}$). No variation was detected in DSO- and ISO-derived plantlets by RAPD, relative to mother plants, but ISSR detected 1.2% polymorphism. The choice of PGRs affected the *in vitro* secondary metabolites and antioxidant capacity of extracts. | Chavan et al. 2014 |

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**Table 1.** *continued*

| Genus, species and/or cultivar | Explant used | Sterilization procedure | Culture medium, PGRs and additives | Culture conditions | Remarks, experimental outcome, acclimatization and variation | Reference |
|---|---|---|---|---|---|---|
| *C. bulbosa* | tTCLs from young nodal segments (1-1.5 cm) from mature plants. | Nodal segments rinsed under RTW for 30 min then washed with 2% cetrimide + few drops of Tween-20 for 5–10 min, then 0.1% $HgCl_2$ for 3–5 min, then some rinses with SDW. | MS + 4.5 µM 2,4-D + 2.2 µM BA (CIM). MS + 8.88 µM BA (direct SIM). MS + 8.88 µM BA + 0.27 µM NAA (indirect SIM). ½ MS (auxin-free) (RIM). 3% sucrose; 0.8% agar; pH 5.8. | 16-h PP, PPFD NR, 25 ± 2 °C, 50 ± 5% RH | 95% of explants formed callus. 15.6 shoots/tTCL (direct route) or 22.2 (via callus route). On RIM, 10.9 roots/shoot. 89% survival after acclimatization in sterile vermiculite + soil (1:2). Variation among *in vitro*-derived plantlets using ISSR. | Dhir, Shekhawat 2014 |
| *C. evansii* | Nodal explants. | Nodal explants washed under RTW then 0.1% (w/v) Bavistin (fungicide) for 10–15 min. 0.1% $HgCl_2$ for 7 min; 3–4 rinses with SDW. | MS + 4 mg/L BA + 0.3 mg/L IAA (SIM). ½ MS + 1 mg/L IBA (RIM). Agar, carbohydrate concentration and pH NR. | 16-h PP, 40 µmol $m^{-2}$ $s^{-1}$, 25 ± 1 °C | 11.6 shoots/node and 85% shoot multiplication frequency. 92% of shoots rooted with 90% acclimatization on sterile soil, sand and coco peat (1:2:1). No genetic differences between *in vitro*-derived plantlets using RAPD and ISSR. | Chavan et al. 2015 |


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*Received 13 December 2014; received in revised form 26 January 2015; accepted 24 February 2015*