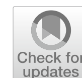
Journal of Plant Growth Regulation (2021) 40:1139–1151
https://doi.org/10.1007/s00344-020-10173-6



Combinatorial Approach Through In Vitro Regeneration and Phytochemical Profiling of *Ceropegia media* (Huber) Ans.: A Potential Way Forward in the Conservation of an Endangered Medicinal Plant from the Western Ghats in India

Meena Pandey[1,5] · Bhushan B. Dholakia[1,4] · Ramesha H. Jayaramaiah[1,3] · Sachin A. Punekar[2] · Ashok P. Giri[1]

Received: 9 July 2019 / Accepted: 12 May 2020 / Published online: 22 June 2020
© Springer Science+Business Media, LLC, part of Springer Nature 2020

Abstract

Ceropegia media is an endemic and endangered plant as its propagation through seeds is unreliable due to low germination, slow growth and seedling decay under natural conditions. Also, tubers of this plant are edible serving as carbohydrate source with medicinal values leading to severe population decline in the natural habitat. To provide a sustainable solution, an efficient in vitro propagation protocol along with phytochemical profiling was developed for *C. media*. Callus cultures were induced from seedling and wild leaf tissues using the most effective Murashige and Skoog's (MS) medium with 2,4-dichlorophenoxyacetic acid (2,4-D; 2 μM) and sucrose (3%). Somatic embryos were acquired on MS medium with 1 μM 6-Benzylaminopurine (BAP) and 1 μM 2,4-D. Conversion into plantlets was attained only from tissue culture-derived seedling leaf (TCDSL) explant. Further, in vitro tuberization was achieved from TCDSL callus with BAP and Naphthalene acetic acid (NAA). $AgNO_3$ as an elicitor had a positive effect on both fresh and dry weights of callus. Successful acclimatization (58%) was attained after two months resulting in normal phenotype in pots. Further, metabolite profiles of ten different tissues from wild and in vitro plants were compared. Total 82 compounds comprising alkaloids, fatty acids, fatty acid ester, steroids, terpenes and hydrocarbons were identified. Overall, results suggested enhanced production of selected metabolites

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00344-020-10173-6) contains supplementary material, which is available to authorized users.

✉ Meena Pandey
dr.meenajoshi@gmail.com

✉ Bhushan B. Dholakia
bhushan1410@yahoo.com

✉ Ashok P. Giri
ap.giri@ncl.res.in

[1] Plant Molecular Biology Unit, Division of Biochemical Sciences, CSIR-National Chemical Laboratory, Dr. Homi Bhabha Road, Pune, Maharashtra 411008, India

[2] Biospheres, Eshwari, 52/403, Lakshminagar, Parvati, Pune, Maharashtra 411009, India

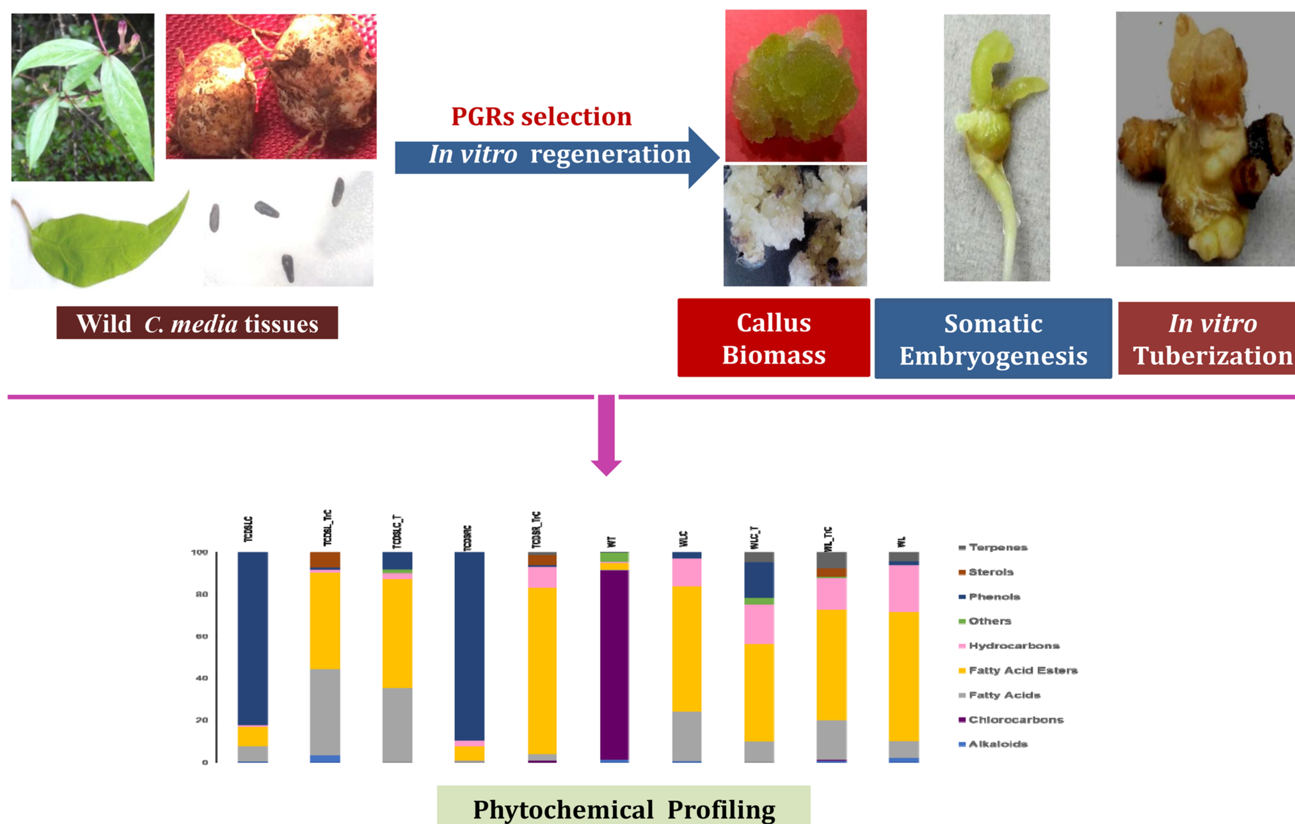
[3] Hawkesbury Institute for the Environment, Western Sydney University, Penrith, NSW 2751, Australia

[4] Indian Institute of Science Education and Research-Pune, Dr. Homi Bhabha Road, Pune, Maharashtra 411008, India

[5] D Y Patil International University, D Y Patil Educational Complex, Sector 29, Nigdi Pradhikaran, Akurdi, Pune, Maharashtra 411044, India

with in vitro propagation and $AgNO_3$, alleviating the problem of unavailability of planting materials. Thus, the current study might offer potential ways for the conservation of such RED enlisted species as *C. media*.

Graphic Abstract



Keywords *Ceropegia media* · GC–MS · Microtuberization · Secondary metabolites · Silver nitrate · Somatic embryogenesis

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
$AgNO_3$	Silver nitrate
ANOVA	Analysis of variance
BAP	6-Benzylaminopurine
BSI	Botanical Survey of India
ESEM	Environmental scanning electron microscopy
GC-FID	Gas chromatography-flame ionization detector
GC–MS	Gas chromatography-mass spectrometry
IAA	Indole-3-acetic acid
Kin	Kinetin
MS	Murashige and Skoog's medium
NAA	Naphthalene acetic acid
PGR	Plant growth regulator
PLS-DA	Partial least square-discriminant analysis

Introduction

Ceropegia media (Huber) Ansari (family: Apocynaceae APG III 2009) is a perennial twining tuberous herb, which is endemic and endangered in India (Jagtap and Singh 1999; Mishra and Singh 2001; Punekar 2015). It is found at higher elevations (500 to 1500 msl) in the Western Ghats of India. Regeneration of this species occurs through perennial tubers, which sprout only during the rainy season and develops into a single plant. Besides, propagation through seeds is unreliable due to low germination rate, slow growth and seedling decay under natural conditions. Also, the tubers (boiled or roasted) of this plant are edible and consumed by local people and shepherds as a rich source of carbohydrates (Nikam and Savanth 2007; Punekar 2015). Additionally, alkaloids extracted from tubers revealed anti-cancer, anti-diarrheal, anti-inflammatory, anti-diabetic activities, and cure urinary disorders (Awoyinka et al. 2007; Monika

et al. 2012). Several reports have also indicated that the consumption of tuber from *Ceropegia* species by tribal women improved their fertility and vigor; and also used to cure of urinary bladder stones, as the antidote for snake bites, diarrhoea, and dysentery (Khare 2007; Swarnkar and Katewa 2008; Duraisamy and Subramaniam 2010). Hence, there is a severe decline in their population in natural habitats (Mishra and Singh 2001; Murthy and Kondamudi 2011) and there is an urgent need to find ways for the conservation of this fast demising plant species. Although most of the species of *Ceropegia* have significant importance as a source of ornamental flowers, edible tubers and their implications in traditional medicine practices, the vegetative propagation techniques hinder the mass propagation and so their commercialization (Chavan et al. 2018). Lately, the genus has appealed the attention for conservation, since many of the *Ceropegia* species are listed in the rare & endangered (RED) data book of Indian medicinal plants (Srinivasarao et al. 2010). Plant tissue culture can provide an option not only for the conservation of wild resources but may influence the ability to produce high-quality drugs. Its different procedures have been extensively studied to boost the production of plant metabolites, which may impart the medicinal properties (Savithramma et al. 2011). It could also justify the commercial growth of exhausted natural resources and thus, may lead to the conservation and sustainable utilization. The callus is one of the utmost vital stages in the in vitro propagation that initiate the explants to form parenchymal cells and thus, are exploited as the source of secondary metabolites without reaching mature stage (Belal et al. 2008). Microtubers are ideal for the germplasm collection as they are less fragile and superior for long-term storage (Hoque et al. 1996). Moreover, they can be yielded throughout the year and would be useful over the seasonal seeds (Kanwal et al. 2006). Furthermore, elicitation by various stimuli enhances the production of secondary metabolites and is routinely used in the plant tissue culture. Elicitors can affect the expression of genes for the biosynthesis of metabolites. Overall, this stimulates plant's antioxidant-based protection, possibly through an increase in metabolite levels (Zhao et al. 2005). These bioactive compounds can accumulate in different tissues and cells. Thus, different in vitro approaches have been extensively studied to improve the production of phytochemicals. Therefore, metabolite profiling of tissue-cultured plants could be valuable for the production of active compounds on a large scale, avoiding exploitation of the natural resources and utilization of whole plants. Also, providing genetically uniform clones for sustainable use through tissue culture and consequently saving the natural wealth of such endangered species is the paramount task. Hence, the present study reports callus induction, somatic embryogenesis and tuberization for *C. media.* The influence of $AgNO_3$ on the growth of *C. media* callus was also investigated. Further, gas chromatography-mass spectrometry (GC–MS)-based comparative phytochemical profiling was explored to see if metabolites from various tissues between wild and tissue-cultured plants matched. Thus, the use of later might offer some protection to the wild plant and serve as an alternative for the production of key metabolites conserving natural resource of *C. media*. The present investigation has indicated that tissue culture-derived seedling leaf (TCDSL) explant as the possible best source for the successful regeneration of *C. media* and also enhanced production of some key metabolites with in vitro propagation and $AgNO_3$ treatment.

Materials and Methods

Plant Material, Explants Preparation, and Establishment of Cultures for Callus Induction

The wild tubers (WT), leaves (WL) and follicles of *C. media* population were collected from the natural habitat (Sinhagad fort area; altitude 1127 m asl; latitude 17° 43′ N and longitude 73° 48′ E) from Pune District of Maharashtra State, India. The necessary permissions were obtained from the respective forest departments for the above-said location to collect the samples. *C. media* plants were identified by the experts and herbarium vouchers (SAP-MP 120814) were deposited at the Botanical Survey of India (BSI), Western Regional Centre, Pune. For seedling explants, follicles with seeds were surface-sterilized by washing thoroughly under tap water, then rinsed with 95% ethanol for 30 s followed by washing twice with autoclaved double-distilled water (DDW). It was then immersed in 1.5% sodium hypochlorite, with 2% (v/v) Tween-20 solution for 10 min followed by rinsing it five times with autoclaved DDW. After sterilization, follicles were dissected longitudinally along the sutures to get the seeds. Finally, seeds were cultured on hormone-free (HF) MS (Murashige and Skoog 1962) medium containing 3% sucrose and 0.7% agar for germination. Tissue culture-derived seedling leaf (hereafter, TCDSL) and root (TCDSR) explants were obtained from the 5-week-old axenic seedling, cultured on various media. Also, WL segments procured from natural habitat were washed under tap water, and later with a detergent, teepol (5%, v/v) for 5 min. After washing with autoclaved DDW, they were surface sterilized with mercuric chloride (0.5%, w/v) solution for 10 min, and again washed thoroughly with autoclaved DDW. An outer margin of the WL was removed and was cut into appropriate sizes (leaf 1 cm^2). All the explants of WL, TCDSL and TCDSR were subjected to the same in vitro pre-condition. For callus induction, all the explants were cultured on MS medium with 6-benzylaminopurine (BAP; 0, 1, and 2 μM), and 2,4-dichlorophenoxyacetic acid (2,4-D; 0, 1, 2, and 4 μM), while one set on HF-MS media kept as

the control. Three replicates per treatment with five explants in each were studied and observations were recorded after 8 weeks.

Somatic Embryogenesis and Environmental Scanning Electron Microscopy

For inducing somatic embryogenesis, the calli obtained from MS medium supplemented with 2 µM 2,4-D (the treatment that showed best callus frequency) from all the three explants (namely wild leaf calli-WLC; tissue culture-derived seedling leaf calli-TCDSLC and tissue culture-derived seedling root calli-TCDSRC) were excised into small pieces (100 ± 50 mg fresh weight per piece). Later, they were transferred on BAP and 2,4-D, (1 and 2 µM, respectively) media. For these, three replicates per treatment with four explants each were used. The percentage and number of somatic embryos (SEs) per culture were calculated after 8 weeks. All the explants at cotyledonary stage embryos were separated and transferred to the medium containing different levels of BAP (0, 1, and 2 µM) for the plantlet production. The cultures were regularly observed under a stereomicroscope (Leica S8 APO, Wetzlar, Germany). SEs were also observed by environmental scanning electron microscopy (ESEM) (Pandey et al. 2017).

Standardization of Induction Media for the In Vitro Tuberization of Explants

In vitro tuberization was carried out by culturing (2 µM; 2,4-D) calli derived from all the explants and transferred to MS medium supplemented with Naphthalene acetic acid (NAA), Indole-3-acetic acid (IAA) and Kinetin (Kin) at various concentrations (1, 2, and 4 µM each of them), BAP (1 and 2 µM) and 7% sucrose, while one set on HF-MS medium was utilized as a control. The microtubers were observed after 6 weeks of incubation. Besides, different explants cultures were also used to compare percentage microtuberization on 7% sucrose.

Acclimatization and Transplantation to Soil

The plantlets, each about 6–8 cm in height with 10–12 leaves and well-developed roots from TCDSL were removed from agar medium, washed in tap water to remove the culture medium, and transferred to pots (8.5 × 7.0 cm) containing a mixture of soil, peat moss and sand (1:1:1). The pots were placed into glass chambers at 25 ± 2 °C under a 12 h (each light/ dark) photoperiod for 30 days. To reduce the relative humidity inside the chambers, the covers were gradually opened after the 2nd week and completely removed by 30 days after transplanting. Plant survival was evaluated after a period of two months.

Elicitor Treatment

Different concentration of $AgNO_3$ was used as an elicitor to assess the inhibition of browning and increment of callus growth. $AgNO_3$ was filter-sterilized through a 0.45 µM bacteria-proof filter (Millipore, Merck, India) and added into sterile media to avoid any cross-contamination. The 2,4-D (2 µM)-incubated cultures from WLC, TCDSLC and TCDSRC explants were treated with different concentrations (10, 20, and 30 µM) of $AgNO_3$, while calli without elicitor were used as the control. The cultures were maintained under the same conditions as described in the following section. After 6 weeks, calli from all the tissues were extracted for phytochemical analysis. One g of calli tissues were weighed, and placed in 15 mL falcon tubes that were later stored in − 80 °C deep freezer until further use.

In Vitro Culture Conditions and Statistical Analysis

The pH of all the media was adjusted to 5.6–5.8 ± 0.2 before the addition of agar and later they were sterilized with an autoclave (under 1.5 Pa for 15 min). The tissue culture room was maintained in a 16/8 h (light/dark) photoperiod with 50 µmol m^{-2} s^{-1} provided by cool-white fluorescent light and at 25 ± 2 °C and 55 ± 5% relative humidity. These culture conditions were maintained during the entire experiments. Observations were made at specific intervals for all the experiments and were repeated thrice. The data were summarized as a mean ± standard error and arranged in factorial basis on the randomized block design. The effect of different treatments was quantified and the level of significance was determined by Analysis of Variance (ANOVA) with GraphPad PRISM Software (GraphPad Software, Inc., San Diego, CA, USA). Means were evaluated using least significance difference (LSD) at $P < 0.05$.

Phytochemical Extraction, Interpretation of Mass Spectra and Data Analysis

All the tissues from wild samples were frozen in liquid nitrogen immediately after plucking from the natural habitats and stored at − 80 °C until further use. Tissues from wild and in vitro generated callus from 2,4-D (2 µM)-incubated cultures from WLC, TCDSLC and TCDSRC explants (8 weeks) were utilized for metabolite profiling. Phytochemical extractions and their analysis on GC–MS and GC–FID were carried out as described previously (Pandey et al. 2017). Interpretation of the mass spectra of GC–MS was performed using the NIST2011 database (NIST Ver. 2.0 of 2011) supplied with the instrument. Metabolites were identified by comparing the retention times and fragmentation patterns with that of standards or with the spectral data obtained from with NIST 2011 and Wiley 10th edition

mass spectral libraries. Three biological samples with three technical replicates for each sample were used for all the tissues. Multivariate and statistical analyses were performed as described earlier (Pandey et al. 2017).

Results

In vitro Seed Germination, Callogenic, and Somatic Embryogenic Capacity Depend on Tissue Types and PGR Composition

Plant organs of *C. media* were identified and collected from the natural habitat at the flowering stage (Suppl. Fig. S1a, b). Different tissues such as wild tuber, wild leaf and follicles with seeds as explants (Suppl. Fig. S1c–e) were utilized in the present study. After incubation on HF-MS medium, seeds became swollen and germinated within 2 weeks. The effect of plant growth regulators (PGRs; auxins and cytokines) on the callogenic and somatic embryogenic capacity of WL, TCDSL and TCDSR explants were evaluated. Explants that were cultured on HF-MS medium (without PGRs) showed no sign of growth after 8 weeks. However, in the presence of 2,4-D (2 µM), TCDSL explants indicated significantly high callus frequency (100%) ($P<0.05$) with the highest callus weight (3.9 g/explant), followed by 80% callusing in WL (2.0 g/explant). TCDSR had the least callus frequency (60%) (Fig. 1a). Furthermore, an increase in tissue growth was observed at 5th week. Development of SEs was compared in all the tissues after 8 weeks in the SE induction medium. TCDSLC incorporated with 1 µM each of BAP and 2,4-D resulted in a significantly high percentage (100%) ($P<0.05$) of SEs and also the highest number of SEs (22.7 SE/explant), while WL showed 66% frequency of SE and 9 SE/explant (Fig. 1b). However, some cultures stopped proliferating and also indicated rapid phenolic oxidation after 12 weeks; and subsequently, they died.

Stereomicroscopic observation revealed significant variation in callus morphology in all the three explants (Fig. 2a–c). The first morphological change was observed in all the explants after 2 weeks. After induction, a small amount of unorganized tissue growth around the explants was noticeable typically between 3 and 4 weeks. The callus appearance was friable, compact and creamy white in texture with 2,4-D-treated explants as compared to those from 2,4-D and BA combinations that led to nodular embryogenic and slimy callus (Fig. 2d). The SE response in TCDSL was very competent and initial changes were observed between 1st and 2nd weeks, with a thickening in the edges of the explants and cell proliferation. This quickly increased between 3rd and 4th weeks, generating cell proliferating mass, which then started to form various embryonic stages (Fig. 2e–i). The development of various

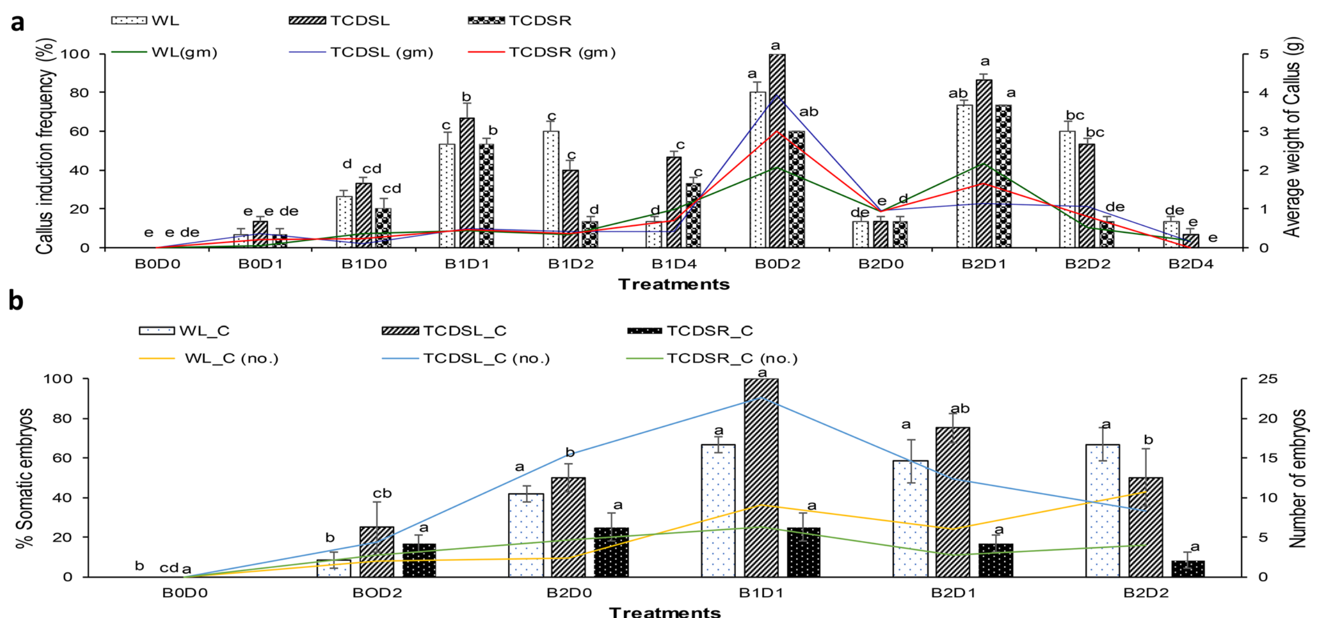


Fig. 1 Effect of different plant growth regulators on callus induction and somatic embryogenesis of *C. media*: **a** callus induction frequency, average weight (in g) of callus, represents mean values. Data recorded after 8 weeks culture, BAP: 2,4-D (µM); B0D0 (Control); B0D1 (0:1); B1D0 (1:0); B1D1 (1:1); B1D2 (1:2); B1D4 (1:4); B0D2 (0:2); B2D0 (2:0); B2D1 (2:1); B2D2 (2:2); B2D4 (2:4). **b** Percentage somatic embryos and number of embryos using various explants BA: 2,4-D (µM); B0D0 (Control); BOD2 (0:2); B2D0 (2:0); B1D1 (1:1); B2D1(2:1); B2D2 (2:2). Vertical bars are standard errors. Similar pattern bars with same lower-case letters are non-significant according to LSD ($P<0.05$)

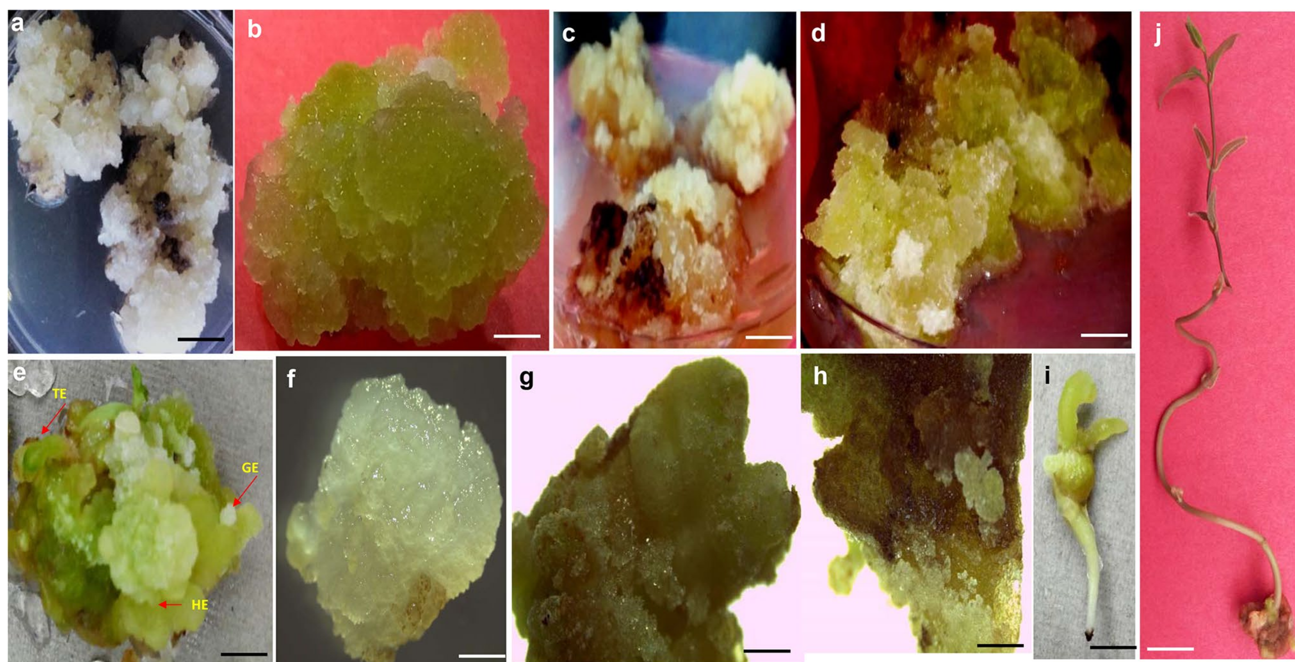


Fig. 2 Plant regeneration via somatic embryogenesis in *C. media* (**a**–**c**); morphological view of callus from three utilized explants WL, TCDSL, TCDSR in basal MS medium supplemented with 2 µM 2,4-D, and 30 g/L sucrose (**d**–**e**); embryogenic callus initiation and various stages globular embryo (GE), heart-shaped embryo (HE), torpedo stage embryo (TE) grouped together in the MS medium in combination with 1 µM each of BAP and 2,4-D (**f**–**h**); isolated stages from globular, heart to torpedo stages (**i**); cotyledonary embryos with shoot and root ends and advanced stages of somatic embryos (**j**) germinated somatic embryo. *Scale bar*- 5 mm (**a**, **c**), 1 mm (**b**, **f**–**h**), 2 mm (**d**–**e**, **i**), and 1 cm (**j**)

embryogenic stages was predominant from the 6th week onwards. At the 8th week complete plants were regenerated (Fig. 2j).

Competent and Rapidly Growing Somatic Embryos Recognized in TCDSLC

For closer evaluation, the SE developmental stages were observed weekly by ESEM (Fig. 3). Subsequently, numerous swollen cell cultures were formed on the surface of the TCDSL explants after 2nd week in SE medium containing BA with 2,4-D (Fig. 3a, b). Embryogenic calli (EC) were detected after the 3rd week and developed into globular embryos (GE) and heart-shaped embryos (HE) (Fig. 3c, d), which showed demarcation from a group of periclinal cells. Spherical cells were covered with a membranous layer. Isodiametric cells and elongated tubular cells were visible (Fig. 3e, f). Higher magnification revealed the smooth surface of callus, covered with some torn parts of membranous layers. Later, the WLC developed into HE and torpedo-shaped embryo (TE) with vascular tissue that finally resulting in mature SEs. Like TCDSL, similar growth was also observed in WLC (Fig. 3g–q). Different embryo stages during plant regeneration were identified by ESEM.

In Vitro Tuber Induction with Different PGRs and Plantlet Regeneration on the Potting Mixture for Acclimatization

In vitro callus derived from medium containing 2,4-D (2 µM) showed microtuberization of *C. media* after 4 to 5 weeks of incubation in all the explants. Tissue culture-derived seedling leaf calli induced tuber (TCDSLC_T) indicated significantly higher (83%) ($P < 0.05$) in vitro tuberization and a maximum weight (2.5 g/explant) when treated with 2 µM each of BAP and NAA along with 7% sucrose within 6 weeks and produced fibrous roots directly all over the tuber (Fig. 4a–d, Suppl. Fig. S2). On the other side, tissue culture-derived seedling root calli induced tuber (TCDSRC_T) showed lowest tuberization (27%; 0.89 g/explant) response and produced morphologically diverse brown tuber without root in the same medium (Fig. 4e). Growth progressions were also observed through ESEM in all the explants (Fig. 4f, g) indicating that the cells were embedded with starch granules. The callus growing on the MS medium with 1 µM NAA and 7% sucrose exhibited nodular callus first from the calli of TCDSL and produce 1–2 fibrous roots. On the other hand, IAA containing all MS media led to callusing and subsequent adventitious roots, while the use of Kinetin resulted

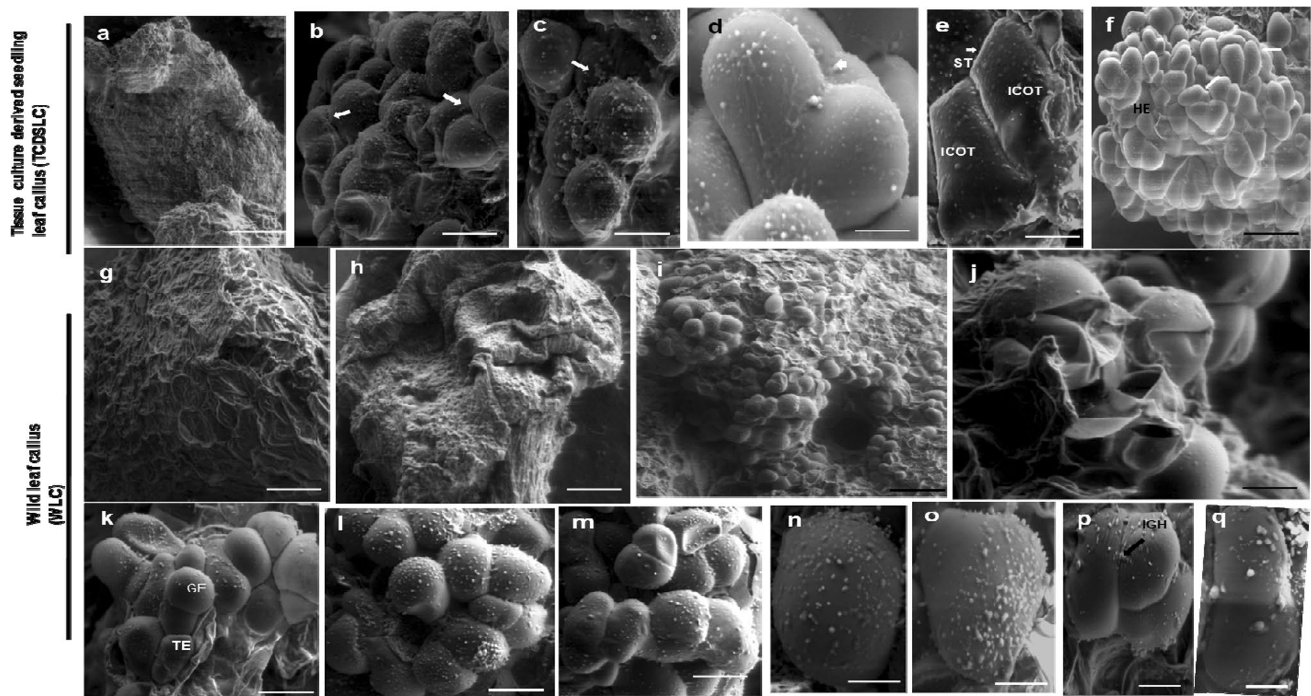


Fig. 3 Environmental scanning electron microscopy (ESEM) images (**a**–**f**) of somatic embryos from TCDSLC of *C. media*: **a** swollen cell clusters 2 weeks; **b** active mitotic transitions (arrows) 4 weeks of culture; **c** globular somatic embryos (GEs), shown by an arrow; **d** heart-shaped somatic embryos (HE); **e** elongated, torpedo-shaped embryos with two incipient cotyledons (ICOT) and a clasping distinct shoot tip (ST); (**f**) multiple meristematic zones along with secondary somatic embryos developed on primary somatic embryos. Images (**g**–**q**) of somatic embryos from WLC of *C. media* (**g**, **h**) symmetric non-fasciated structures are consistently observed; (**i**, **j**) embryogenic structures originated on the surface of WLC; (**k**–**m**) various embryo stages marked with the arrows (GE—globular embryos, TE—torpedo embryo; (**n**) isolated globular-shaped somatic embryos with glandular hair (**o**, **p**) isolated pre-heart and heart-shaped somatic embryos with a distinct notch at the top (arrow) and initials of glandular hair (IGH); **q** torpedo-shaped embryos with glandular hair. *Scale bar*-500 µm (**a**), 300 µm (**i**), 100 µm (**c, f**–**h**), 50 µm (**b, j**–**m**), 30 µm (**e**), and 20 µm (**d, n**–**q**)

in the browning and inhibition of callus growth. Due to this, tubers produced with the PGRs other than BAP and NAA were excluded from further study. The frequency of tuber formation decreased with the increased concentrations beyond 2 µM of BAP and NAA. Lower than 2 µM BAP resulted in less sprouting while higher concentrations almost stopped the growth.

Regeneration frequency varied with various explants and the combination of different PGRs in the media. Complete plants were developed only from the TCDSL-derived cotyledonary stage embryos on MS basal media along with 2 µM BAP and 3% sucrose (Fig. 4h, i). Plantlets were transferred to the acclimatization medium, containing soil, peat moss and sand in the ratio (1:1:1). Following their growth, an acclimatization rate of 58% was found two months after the transfer of plantlets to *ex vitro* conditions. Plants showed a normal phenotype (Fig. 4j) like a wild plant, which established a method to propagate *C. media* through somatic embryogenesis, tuberization and demonstrated the ability of the seedling leaf to induce complete regeneration.

$AgNO_3$ Facilitated Improvement in Growth and Inhibited Browning of the Callus

As an elicitor can affect callus growth by modulating gene/metabolite expressions, the various concentrations of $AgNO_3$ was utilized. The stimulatory effect of $AgNO_3$ (10 to 30 µM) on callus proliferation was prominent (Table 1). Fresh and dry weights of WLC and TDSLC were significantly affected by $AgNO_3$. In term of callus biomass, 20 µM $AgNO_3$ [with a combination of MS medium + 2 µM 2,4-D] showed maximum positive effect with significantly ($P < 0.05$) high fresh weight (10.56 ± 1.80 g) and dry weight (2.8 ± 0.4 g) of wild leaf $AgNO_3$-treated callus (WL_TrC). This was followed by tissue-cultured derived seedling leaf $AgNO_3$-treated callus (TCDSL_TrC) and tissue-cultured derived seedling root $AgNO_3$-treated callus (TCDSR_TrC) with the increase in fresh and dry weights compared to their respective controls at the same elicitor level (Table 1). Also, the explants indicated significant ($P < 0.05$) improvement over the control. Further, we also compared the metabolites among

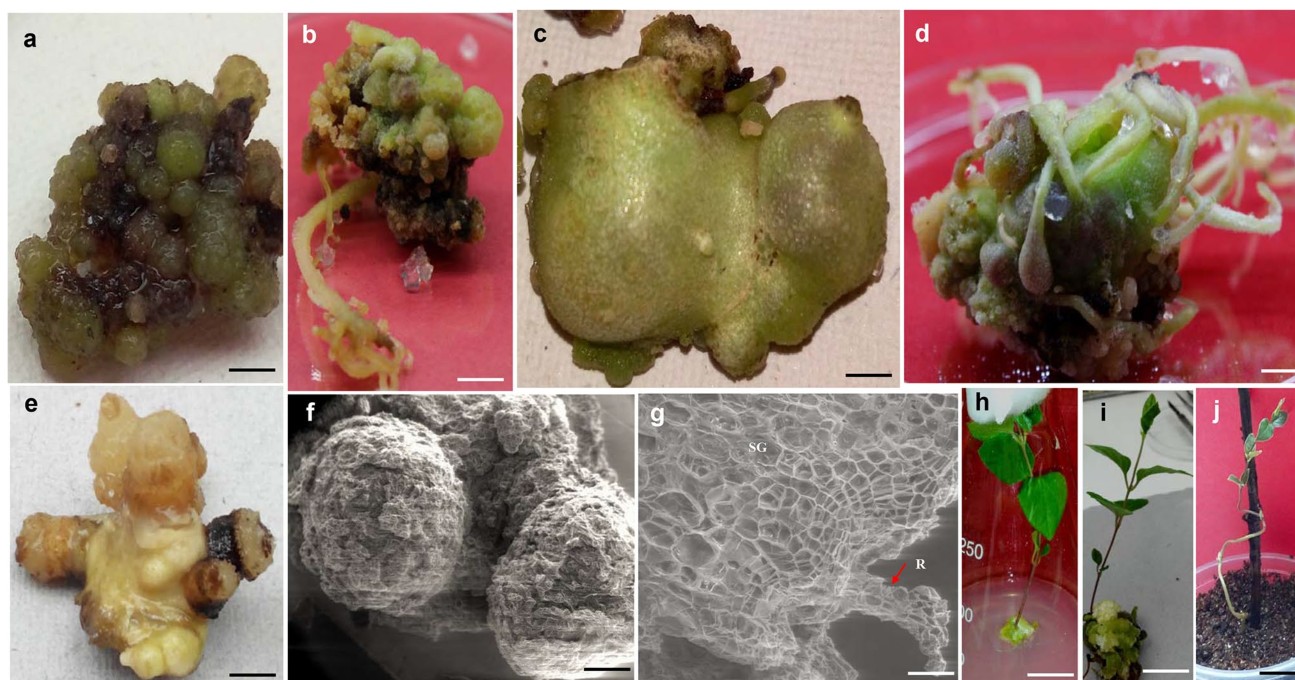


Fig. 4 In vitro tuberization of *C. media* (**a**–**d**); microtubers initiation along with root in the medium containing of various concentration of plant growth regulators from TCDSLC; (**e**) in vitro microtuber in MS medium supplemented with 2 µM BAP and 1 µM NAA from TCDSR_T; (**f**) ESEM image showing initiation of microtubers from TCDSLC; (**g**) microtuber showing connectivity with the root (R) tissue with abundant starch granules (SG); (**h**) embryos transferred in shoot induction medium showed germination in only 2 µM BAP from TCDSL explants; (**i**) plant with well-developed shoot and root; (**j**) fully developed plant with well-developed strong shoot and root system transfer in soil: peat moss: sand (1:1:1). *Scale bar*- 3 mm (**a**, **b**, **c**, **d**, **e**), 500 µm (**f**), 200 µm (**g**), 20 mm (**h**), 10 mm (**i**), and 50 mm (**j**)

Table 1 Influence of different concentrations of $AgNO_3$ on fresh and dry weights of *C. media* callus culture

$AgNO_3$[1] (µM)	Callus fresh weight (g/jar)*			Callus dry weight (g/jar)*			Remarks
	WL_TrC	TCDSL_TrC	TCDSR_TrC	WL_TrC	TCDSL_TrC	TCDSR_TrC	
0	2.03 ± 0.20^c	3.33 ± 0.75^c	3.16 ± 0.81^b	0.13 ± 0.01^b	0.16 ± 0.007^c	$0.15 \pm .004^b$	C+
10	5.56 ± 0.58^b	6.30 ± 0.70^b	3.76 ± 0.66^b	0.21 ± 0.03^b	1.13 ± 0.66^b	0.11 ± 0.09^b	FC++
20	10.56 ± 1.80^a	8.53 ± 0.11^a	6.4 ± 1.96^a	2.8 ± 0.4^a	2.3 ± 0.43^a	1.17 ± 0.45^a	FC+++
30	4.46 ± 0.80^b	5.7 ± 0.30^b	3.5 ± 0.25^b	0.19 ± 0.07^b	0.31 ± 0.007^c	0.17 ± 0.02^b	C+

WL_TrC- $AgNO_3$-treated wild leaf callus; TCDSL_TrC- $AgNO_3$-treated tissue culture raised seedling leaf callus; TCDSR_TrC- $AgNO_3$-treated tissue culture raised seedling root callus

C callus, *FC* friable callus

+—Slight callus, ++—Moderate callus, +++—Massive callus

*-Mean values in each column followed by the same lower- case letters did not differ significantly (LSD < 0.05);

[1]Values represent mean weight of callus ± SD (g) data recorded after 6 weeks in culture and replicated thrice

wild and in vitro tissues through GC–MS, to see if $AgNO_3$ influenced the phytochemical profiles of *C. media* in vitro tissues. Effect of $AgNO_3$ treatment on callus showed variation in metabolic profiling (Fig. 5; Suppl. Fig. S3) as tissues yielded phytosterols such as cholest-4-en-3-one, stigmasterol, cholesta-4,6-dien-3-ol, cholesta-3,5-dien-7-one, cholesta-4,6-dien-3-ol, benzoate, (3β) as seen from WL_TrC. Three metabolites were common in TCDSL_TrC and TCDSR_TrC. $AgNO_3$ (20 µM) enhanced the growth of callus, with enhanced sterol levels compared to the control tissues. Among the various metabolites, levels of 9,12-Octadecadienoic acid (Z, Z)-, methyl ester (33%), n-Hexadecanoic acid (39%), and Octadecanoic acid, 9,10-dichloro-, methyl ester (2.7%) were higher in $AgNO_3$-treated tissues than in their respective controls (Fig. 5).

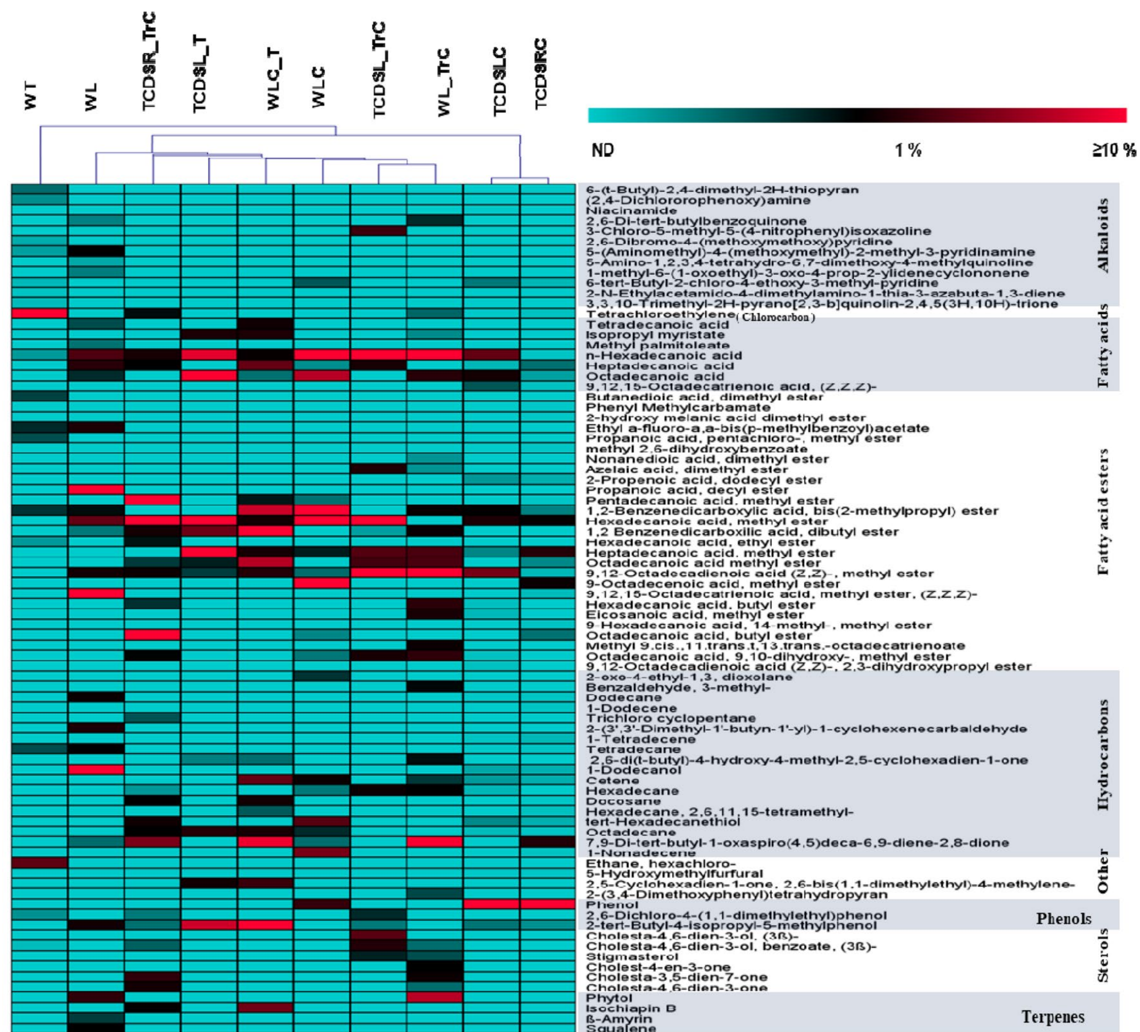


Fig. 5 Heat-map showing the shared metabolites among the tissues in relative percentage. Metabolites identified were grouped into different class of compounds. Hierarchical clustering analysis (HCA) above the heat map representing the chemotypic relationship between the tissues based on their shared metabolites

Phytochemical Profiling Revealed the Presence of Important Metabolites in Wild and In Vitro Tissues

As *C. media* belongs to highly threatened & endangered plants, comparative phytochemical profiling was performed to explore if in vitro tissues have similar metabolites and thus, may offer some respite to the wild plants. GC–MS analysis revealed the presence of a total of 82 metabolites in ten different tissues of *C. media*. These metabolites include alkaloids, fatty acids, fatty acid ester, sterols, phenols, terpenes, hydrocarbons and others (Fig. S3). The chemical composition represented the relative metabolite diversity in each tissue. Nearly all the tissues were particularly rich in fatty acid esters and fatty acids except TCDSLC and TCDSRC that had more phenols. Total 12 alkaloids were identified from various tissues, particularly in the cases of WT and WL (Suppl. Table S1; Fig. S3). The partial least square-discriminant analysis (PLS-DA) score plot based on the identified metabolites indicated the clear separation of natural tissue, i.e., WT and WL as a separate cluster, while in vitro tissues were distributed into two other groups (Suppl. Fig. S4). Samples of WLC_T, WL_TrC and WLC were grouped based on their tissue origin. Consequently, all the seedling originated tissues were placed together. The sources of individual metabolites were clearly articulated in the loadings plot of PLS-DA (Suppl. Fig. S5). This revealed individual metabolites contributing to the separation of different groups as observed in the score plot.

Comparative metabolite profiles of all ten different tissues visualized through the heat-map showed the distribution of all the metabolites across various tissues (Fig. 5). This indicated presence of a higher number (36) of secondary metabolites in TCDSRC and WL_TrC (28). Conversely, TCDSLC_T had the least number (12) of metabolites.

Interestingly, squalene was only present in the WL (1.06%) along with terpene, (β-amyrin) (0.7%). 2-tert-butyl-4-isopropyl-5-methylphenol was identified in all the tissues except WT, WLC and WL_ TrC with highest concentrations for in vitro tuber WLC_T (16.77%). On the other side, phytol was only detected from two tissues, i.e., WL (2.6%) and WL_TrC (7.6%), indicating that the concentration of phytol was probably enhanced after the $AgNO_3$ treatment (Suppl. Figs. S3, S4). Several fatty acids such as octadecanoic acid, pentanoic acid were also detected in our study. α-linolenic acid or 9,12,15-Octadecatrienoic acid (Z,Z,Z) was present only in TCDSLC (0.57%) and TCDSRC (0.04%). Some other compounds such as amines and chlorocarbons (tetrachloroethylene, TCE) were also identified in the extract of WT. In vitro tuber tissues showed high amounts of octadecanoic acid (TCDSLC_T; 24%), heptadecanoic acid (WLC_T 4.6%) and heptadecanoic acid methyl ester (TCDSL_ T; 33.3%).

Discussion

The present findings highlight an effective, prolific in vitro regeneration system for the endangered plant *C. media* via an indirect process involving somatic embryogenesis, tuberization using wild leaf and axenic seedling explants. All the stages were optimized using various PGRs. All the explants displayed differential developmental capabilities. This protocol appeared to be strongly dependent on the explant types and PGRs used. Many PGRs have been used in tissue culture and their effects vary depending on the species and explant types (You et al. 2011; Parimalan et al. 2011). In the present study, neither callogenesis nor somatic embryogenesis occurred on MS medium devoid of PGRs. Two μM 2,4-D was the best PGR for callusing and suggested that the presence of 2,4-D is essential for callus induction in *C. media*. A key function of 2,4-D, during in vitro responses has been widely reported (Stanišić et al. 2015; Ruffoni and Bassolino 2016). Tissue-cultured derived seedling explant was ideal as compared to the wild leaf and root explants. Only a few reports are available on in vitro plant regeneration and somatic embryogenesis in *Ceropegia* genus, namely *C. candelabrum* (Beena and Martin 2003; Beena et al. 2003); *C. spiralis* and *C. pusilla* (Murthy et al. 2010, 2012a); *C. mahabali*, *C. media* (Nikam et al. 2012); and *C. karulensis* (Pandey et al. 2017). We observed that a combination of 2,4-D and BAP resulted in enhanced SEs. However, there are no such reports on SE and ESEM on *C. media* till date to the best of our knowledge. The conservation strategies and microtuberization studies have been reported from other *Ceropegia* such as *C. lawii, C. maccannii, C. oculata, C. sahyadrica, C. bulbosa, C. spiralis*, *C. pusilla, C. evansi*, *C. panchganiensis* and *C. juncea* (Goyal and Bhadauria 2006; Pandit et al. 2008; Murthy et al. 2012b; Chavan et al. 2013a, b; Binish 2018). Recently, Chavan et al. (2018) have suggested that microtuber can be an ideal approach over the natural seeds for the conservation of *Ceropegia*. This is due to root tubers, which are store-house of starch, sugars, albuminoids and valuable bioactive compounds. The current investigation also reported the occurrence of microtubers in callus cultures of *C. media* and the induction of a tuber organogenesis pathway from the callus cells using NAA and BAP. The present findings have suggested that 2 μM BAP can be more effective for the conversion of the somatic embryo into plantlets. The advantage of BAP in shoot multiplication has been also reported in *C. bulbosa* and *C. intermedia* (Britto et al. 2003; Karuppusamy et al. 2009).

This is probably the first study that has offered novel insights in secondary metabolite compositions among the wild and in vitro tissues of *C. media*. The use of appropriate conditions in plant tissues-culture is essential for the efficiency and natural potency of phyto-compounds. Likewise, potency is also dependent on the presence of the required quantity and quality of secondary metabolites in a raw drug (Vinoth et al. 2011). Accumulation of secondary metabolites may vary in different tissues of some therapeutic species (Ramesha et al. 2008; Shetty et al. 2014). Therefore, the selection of a suitable species and specific organs for the induction of in vitro calli, cells, or organs are essential. Previously such similarities and differences in metabolites were identified of plant species from the same genus *C. karulensis* (Pandey et al. 2017). However, a detailed study would be required to unravel the exact sites for biochemical pathways leading to the secondary metabolite synthesis (Farnham et al. 2004). Our study also revealed differential levels of secondary metabolites between wild and in vitro tissues of *C. media*. Large quantities of fatty acids may possess antimicrobial and antifungal potential (Carolina et al. 2011), and can also impart tolerance to abiotic and biotic stresses (Upchurch 2008). The previous study (Si et al. 2006) has revealed that 2-tert-butyl-5-methylphenol exhibited high antimicrobial activity. The anti-oxidative competence of phenolic compounds is improved when t-butyl groups are located at positions 2, 4 and 6 of the aromatic ring (Kumar and Pandey 2013) and were identified in all the tissues except WLC and WLC_TrC in the current study. Various saturated and unsaturated fatty acid (e.g., pentadecanoic acid, hexadecanoic acid and methyl tetradecanoate) from *Excoecaria agallocha* showed antifungal activity (Agoramoorthy et al. 2007) and these were also found in the *C. media* tissue. They are non-toxic and are known for their cardiotonic, insecticidal and antimicrobial properties (Okwu 2001). β-Sitosterol has been identified as an anti-inflammatory, apoptosis-inducing, chemopreventive, hypo-cholesterolemic, angiogenic, anti-oxidative

and anti-diabetic agent (Berger et al. 2004). It also exhibits anti-mutagenic activity against tetracycline and was also present in the present tissue samples. Squalene and β-Amyrin, compounds have been reported to have anti-tumour and anti-inflammatory activities (Villaseñor et al. 2002; Ghosh et al. 2011; Saeidnia et al. 2014). It is also an essential precursor for the synthesis of phytosterols such as stigmasterol, campesterol, sitosterol, as well as a precursor for triterpenoids such as lupeol, amyrin and betulin. Phenols are the secondary metabolites, which have been suggested as antioxidant agents (Nithya et al. 2016). Effect of phytosterols has been studied on cholesterol metabolism and MAP kinase in MDA-MB-231 human breast cancer cells (Atif et al. 2003). We observed increased phytol concentration in $AgNO_3$-treated callus. Phytol is found to be effective as an antioxidant, and antinociceptive agent (Ginty et al. 2010; Santos et al. 2013). Thus, the addition of $AgNO_3$ as an elicitor might play an important role in reducing phenol leaching and enhancing callus growth. It is an ethylene inhibitor and revealed the significant role in callus cultures (Kumar et al. 2009). It also improved plant regeneration (Dang and Wei 2009; Steinitz et al. 2010). Giridhar et al. (2003) established direct somatic embryogenesis from hypocotyl explants of *Coffea arabica* and *C. canephora*. However till now, $AgNO_3$ has never been used as an elicitor in *Ceropegia* species and thus, present study for the first time explored the effect of $AgNO_3$ on callus proliferation, growth and metabolite profiling to the best of our knowledge. Besides, *C. media* plant showed metabolites richness with various types of phytosterols and their enhancement upon $AgNO_3$ treatment. Thus, plant tissue culture develops environmental friendly substitutes for the production of metabolites from RED enlisted species such as *C. media* when natural source is limited or chemical syntheses is unviable.

Conclusions

A protocol has been developed for in vitro propagation of *C. media* along with the optimum PGR, explant type and metabolic profiles for various tissues. Findings suggested that TCDSL could be more suited for callus induction and somatic embryogenesis than the other explants; and in vitro tuberization could be directly commenced from the callus. Also, 20 µM of $AgNO_3$ could be optimum to improve the callus production and reduce browning. Moreover, key secondary metabolites were identified from the wild and in vitro tissues of *C. media*. Thus, this efficient protocol might offer a better strategy for the conservation and sustainable utilization of *C. media*. Further, understanding of the tissue-specific metabolites might potentially aid advance studies for the sustainable production of plant for therapeutic and commercial applications.

Acknowledgements Financial assistance to MP from Department of Science and Technology, Government of India, New Delhi (Project No. SB/YS/LS-266/2013), is gratefully acknowledged.

Author contributions APG and MP conceived and planned the work; APG supervised the work. SP Identified the species. All the authors were involved in all field work. MP conducted in vitro study, analyzed the data, while MP and RHJ performed GC–MS experiments and analyzed the data. MP prepared figures and initial draft of the manuscript with some inputs from BBD. Subsequently, BBD and APG suggested changes and edited entire manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

References

Agoramoorthy G, Chandrasekaran M, Venkatesalu V, Hsu MJ (2007) Antibacterial and antifungal activities of fatty acid methyl esters of the blind-your-eye mangroove from India. Braz J Microbiol 38:739–742

Iii APG (2009) An update of the angiosperm phylogeny group classification for the orders and families of flowering plants. Bot J Linn Soc 161:105–121

Atif BA, Heinric W, Carol SF (2003) Effect of phytosterols on cholesterol metabolism and MAP kinase in MDA-MB-231 human breast cancer cells. J Nutr Biochem 14:111–119

Awoyinka AO, Balogun IO, Ogunnowo AA (2007) Phytochemical screening and *in vitro* bioactivity of *Cnidoscolus aconitiifolus* (Euphorbiaceae). J Med Plant Res 1:63–65

Beena MR, Martin KP, Kirti PB, Hariharan M (2003) Rapid *in vitro* propagation of medicinally important *Ceropegia candelabrum*. Plant Cell Tissue Organ Cult 72:285–289

Beena MR, Martin KP (2003) *In vitro* propagation of the rare medicinal plant *Ceropegia candelabrum* L. through somatic embryogenesis. In Vitro Cell Dev Biol Plant 39:510–513

Belal AH, Deeb EI, Shehata MD (2008) Indirect somatic embryogenesis of five date palm cultivars in *in vitro*. Proceeding of the 3rd conference on date palm. EI-Arish Egypt

Berger A, Jones PJ, Abumweis SS (2004) Plant sterols: factors affecting their efficacy and safety as functional food ingredients. Lipids Health Dis 3:1–19

Binish T (2018) Micropropagation of traditional medicinal plant *Ceropegia juncea*. Ann Plant Sci 7:1992–1996

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

Carolina HP, Johan L, Kock F, Vuyisile ST (2011) Antifungal free fatty acids: a review. In: Méndez Vilas A (ed) Science against microbial pathogens: communicating current research and technological advances. Formatex Research Center, Badajoz, pp 61–71

Chavan JJ, Gaikwad NB, Dixit GB, Yadav SR, Bapat VA (2018) Biotechnological interventions for propagation, conservation and improvement of 'Lantern Flowers' (*Ceropegia* spp.). South Afr J Bot 144:192–216

Chavan JJ, Gaikwad NB, Yadav SR (2013a) High multiplication frequency and genetic stability analysis of *Ceropegia panchganiensis*, a threatened ornamental plant of Western Ghats: conservation implications. Sci Hortic 161:134–142

Chavan JJ, Gaikwad NB, Kshirsagar PR, Umdale SD, Bhat KV, Dixit GB, Yadav SR (2013b) Highly efficient *in vitro* proliferation and genetic stability analysis of micro propagated *Ceropegia evansii* by RAPD and ISSR markers: a critically endangered plant of Western Ghats. Plant Biosyst 149:442–450

Dang W, Wei ZM (2009) High frequency plant regeneration from the cotyledonary node of common bean. Biol Plant 53:312–316

Duraisamy S, Subramaniam P (2010) Phonological observation and population dynamics of six uncommon medicinal plants in the grasslands of Nilgiris, Western Ghats India. Maejo Int J Sci Technol 4:185–192

Farnham M, Wilson P, Stephenson K, Fahey J (2004) Genetic and environmental effects on glucosinolate content and chemoprotective potency of broccoli. Plant Breed 123:60–65

Ghosh T, Maity TK, Singh J (2011) Evaluation of antitumor activity of stigmasterol, a constituent isolated from *Bacopa monnieri* Linn aerial parts against Ehrlich Ascites Carcinoma in mice. Oriental Pharm Exp Med 11:41–49

Ginty Mc D (2010) Fragrance material review on phytol. Food Chem Toxicol 48:559–563

Giridhar P, Indu EP, RamuVD RGA (2003) Effect of silver nitrate on *in vitro* shoot growth of Coffee. Trop Sci 43:144–146

Goyal D, Bhadauria S (2006) *In vitro* propagation of *Ceropegia bulbosa* using nodal segments. Indian J Biotechnol 5:565–567

Hoque MI, Mila MB, Khan MDS, Sarkar RH (1996) Shoot regeneration and *in vitro* microtuber formation in potato (*Solanum tuberosum* L) Bangladesh. J Bot 25:87–93

Jagtap AP, Singh NP (1999) Asclepiadaceae and periplocaceae, in fascicles of Flora of India. Botanical Survey of India, Kolkatta, p 24

Kanwal A, Ali A, Shoaib K (2006) *In vitro* microtuberization of potato (*Solanum tuberosum* L.) cultivar Kuroda—a new variety in Pakistan. Int J Agric Biol 8:337–340

Karuppusamy S, Kiranmai C, Aruna V, Pullaiah T (2009) *In vitro* conservation of *Ceropegia intermedia*—an endemic plant of South India. Afr J Biotechnol 8:4052–4057

Khare CP (2007) Indian medicinal plants: an illustrated dictionary. Springer, New York, pp 139–140

Kumar S, Pandey AK (2013) Chemistry and biological activities of flavonoids: an overview. Sci World J. https://doi.org/10.1155/2013/162750

Kumar V, Parvatam G, Ravishankar GA (2009) $AgNO_3$: a potential regulator of ethylene activity and plant growth modulator. Electron J Biotechnol 12:8–9

Mishra DK, Singh NP (2001) Endemic and threatened flowering plants of Maharashtra. Botanical Survey of India, Kolkata, p 411

Monika J, Anil B, Aakanksha B, Priyanka P (2012) Isolation, characterization and *in vitro* antiurolithiatic activity of cerpegin alkaloid from *Ceropegia bulbosa* var. lushii root. Int J Drug Dev Res 4:154–160

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

Murthy KSR, Kondamudi R, Karuppusamy S (2012a) Microtuberization of *Ceropegia spiralis* Wight and *Ceropegia pusilla* Wt. and Arn. Afr J Plant Sci 6:321–327

Murthy KSR, Kondamudi R, Pullaiah T (2012b) High frequency somatic embryogenesis in *Ceropegia spiralis* Wight- An endemic and endangered medicinal plant. Indian J Biotechnol 9:414–418

Murthy KSR, Kondamudi R (2011) Rapid shoot regeneration from thin cell layer explants of an endangered Asclepiad *Ceropegia spiralis*. Plant Tissue Cult Biotechnol 21:63–73

Murthy KSR, Kondamudi R, Vijayalakshmi V (2010) Micropropagation of an endangered medicinal plant *Ceropegia spiralis* L. J Agric Technol 6:179–191

Nikam TD, Patil JD, Ahire ML, Ghane SG, Nitnaware KM, Naikwadi VB (2012) Axillary multiplication of *Ceropegia mahabalei* Hemadri and Ansari and *Ceropegia media* (Huber) Ansari: critically endangered ethnomedicinal herbs of Western Ghats, Maharashtra state of India. Int J Plant Dev Biol 6:27–33

Nikam TD, Savanth RS (2007) Callus culture and micropropagation of *Ceropegia sahyadrica* Ans. et Kulk: an edible starchy tuberous rare asclepiad. Indian J Plant Physiol 12:108–114

Nithya PY, JelastinKala SM, Mohan VR (2016) Evaluation of total phenolics, flavonoid contents and *in-vitro* antioxidant properties of *Catharanthus pusillus* (Apocynaceae). IJPSR 7:3021–3027

Okwu DE (2001) Evaluation of the chemical composition of indigenous spices and flavoring agent. Global J Pure Appl Sci 7:455–459

Pandey M, Jayaramaiah RH, Dholakia BB, Punekar SA, Giri AP (2017) A viable alternative *in vitro* system and comparative metabolite profiling of different tissues for the conservation of *Ceropegia karulensis*. Plant Cell Tissue Organ Cult 131:391–405

Pandit SS, Aneesh KN, Naik DD (2008) Towards conservation of threatened *Ceropegia* species endemic to a biodiversity hot spot *in vitro* microtuber production and proliferation, a novel strategy. J For Sci 24:79–88

Parimalan R, Venugopalan A, Giridhar P, Ravishankar GA (2011) Somatic embryogenesis and *Agrobacterium*-mediated transformation in *Bixaorellana* L. Plant Cell Tissue Organ Cult 105:317–328

Punekar SA (2015) Molecular systematics, phylogeny and ecology of *Ceropegia* L. (Apocynaceae-Asclepiadoideae) in India. Report submitted to Science & Engineering Research Board, Department of Science and Technology Delhi under Start-Up Research Grant

Ramesha BT, Amna T, Ravikanth G, Gunaga RP, Vasudeva R, Ganeshaiah KN, Uma Shaanker R, Khajuria RK, Puri SC, Qazi GN (2008) Prospecting for camptothecines form *Nothapody tesnimmoniana* in the Western Ghats, South India: identification of high-yielding sources of Camptothecin and new families of camptothecines. J Chromatogr Sci 46:362–368

Ruffoni B, Bassolino L (2016) Somatic embryogenesis in Lisianthus (*Eustomarus sellianum* Griseb.). In: Maria AG, Maurizio L (eds) In vitro embryogenesis in higher plants. Springer, New York, pp 359–370

Saeidnia S, Manayi A, Gohari AR, Abdollahi M (2014) The story of beta-sitosterol-a review. Eur J Med Plants 4:590–609

Santos CCMP, Salvadori MS, Mota VG, Costa LM, Almeida AACO (2013) Antinociceptive and antioxidant activities of phytol *in vivo* and *in vitro* models. Neurosci J, 1–9

Savithramma N, Rao L, Beenaprabha M (2011) Phytochemical studies of *Dysophylla myosuroides* (Roth.) Benth. In. wall. and *Talinum cuneifolium* (Vahl.) Willd. Res J Phytochem 5:163–169

Shetty MR, Harisha GA, Jayanth Y, Kumar AH (2014) Production of secondary metabolites from *in vitro* cultures of *Rauwolfia serpentina* (L.) Benth. Int J Sci Technol Res 2:844–852

Si W, Gong J, Tsao R, Zhou T, Yu H, Poppe C, Johnson R, Du Z (2006) Antimicrobial activity of essential oils and structurally related synthetic food additives towards selected pathogenic and beneficial gut bacteria. J Appl Microbiol 100:296–305

Srinivasarao C, Soni A, Dulloo ME, Naithani SC (2010) Overcoming physiological dormancy in *Ceropegia odorata* seeds, an endangered rare species, with GA3, H_2O_2 and KNO_3. Seed Sci Technol 38:341–347

Stanišić M, Raspor M, Ninković S, Milošević S, Ćalić D, Bohanec B, Trifunović M, Petrić M, Subotić A, Jevremović S (2015) Clonal fidelity of *Iris sibirica* plants regenerated by somatic embryogenesis and organogenesis in leaf-base culture- RAPD and flow cytometer analyses. S Afr J Bot 96:42–52

Steinitz B, Barr N, Tabib Y, Vaknin Y, Bernstein N (2010) Control of *in vitro* rooting and plant development in *Corymbia maculate* by silver nitrate, silver thiosulfate and thiosulfate ion. Plant Cell Rep 29:1315–1323

Swarnkar S, Katewa SS (2008) Ethnobotanical observation on tuberous plants from tribal area of Rajasthan (India). Ethnobotanical Leaflets 12:647–666

Upchurch RG (2008) Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. Biotechnol Lett 30:967–977

Villaseñor IM, Angelada J, Canlas AP, Echegoyen D (2002) Bioactivity studies on β-sitosterol and its glucoside. Phytother Res 16:417–421

Vinoth S, Kanna RP, Gurusaravanan P, Jayabalan N (2011) Evaluation of phytochemical, antimicrobial and GC-MS analysis of extracts of *Indigo feratrita* L.F. spp. Subulata (Vahl ex poir). Int J Agric Res 6:358–367

You CR, Fan TJ, Gong XQ, Bian FH, Liang LK, Qu FN (2011) A high-frequency cyclic secondary somatic embryogenesis system for *Cyclamen persicum* Mill. Plant Cell Tissue Organ Cult 107:233–242

Zhao J, Davis LC, Verpoorte R (2005) Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol Adv 23:283–333

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.