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S. Mohan Jain *Editor*

Protocols for In Vitro Cultures and Secondary Metabolite Analysis of Aromatic and Medicinal Plants

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Preface

It is well known that plants consistently synthesize, accumulate, and use a bewildering range of secondary metabolites as a part of their overall defense strategy. Many of these metabolites have been used around the world as medicines for various human health problems. In recent years the quest for quality of life and a common belief that plants are "natural and therefore safe" have paved the way for a wider acceptance of plant-based medicines worldwide. International trade in medicinal plants has become a major force in the global economy, and the demand is increasing in both developed and developing countries. Thus, the continued rise in consumer demand for plant-based medicines and the expanding world population have resulted in the indiscriminate harvest of wild species of medicinal plants. As well, a reduction of natural habitats for medicinal plants has placed many wild species in danger of extinction. The impact of rapid climate changes may also have an adverse effect on wild plant species leading to the loss of useful genetic material. Most medicinal plants are harvested from the wild, and the traditional agricultural and horticultural practices have not been developed even for most commonly used medicinal plant species.

The quality and consistency of the products are most challenging issues facing the plant-based medicines. The production of medicinal metabolites in plants is affected by plant genotype, cultivation, harvesting, processing, and distribution. Medicinal plant preparations may also be contaminated with microbes and soil contaminants such as heavy metals, herbicides, pesticides, and other agricultural chemicals which can cause qualitative and quantitative changes in the levels of medicinal metabolites. The widespread occurrence of chemical variability and compromised quality of medicinal plants remain the major factors in inconsistent results of clinical trials of plant-based medicines. New regulations are currently being developed internationally to ensure consistency, safety, and efficacy of plant-based medicines as well as how they are developed, manufactured, and marketed. Clearly, there is an imminent need for the development of new technologies and production approaches to improve the overall strategy of medicinal plant production to comply with upcoming legal regulations.

In vitro cell culture and controlled environment production systems offer an excellent opportunity for the selection and seasonal independent propagation of elite lines with specific, consistent levels of medicinal metabolites with minimum contamination. Additionally, the plant materials produced by in vitro techniques allow efficient application of the emerging analytical methods. The impact of these techniques perhaps greatest in the improvement of medicinal plants since the resulting genetic diversity may open avenues for the discovery of new medicinal metabolites and treatments. This book provides a detailed step-by-step description of protocols for the establishment of in vitro cultures of important medicinal plants, their mass multiplication in controlled environment, and stepwise secondary metabolite analysis. In addition many of these protocols will provide a basis for much needed efforts of in vitro germplasm conservation or cryopreservation of medicinal plant species at the brink of extinction as well as to protect them from the adverse impact of rapid climatic changes. This book will certainly appeal to graduate and postgraduate students, researchers, biotechnologists, industry, and the Government agencies and could be used as a textbook.

This book contains 30 book chapters dealing with in vitro propagation of medicinal plants. Each manuscript has been peer reviewed and revised accordingly.

We greatly appreciate all reviewers who have contributed their time to review manuscripts that certainly helped in improving the quality of contributory manuscripts. Our sincere thanks are due to the Humana Publishers for giving us the opportunity to edit this book.

Helsinki, Finland *S. Mohan Jain*

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Chapter 1

Protocols for In Vitro Mass Multiplication and Analysis of Medicinally Important Phenolics of a Salep Orchid, *Satyrium nepalense* D.Don ("Salam Mishri")

Shashi B. Babbar and Deepak K. Singh

Abstract

Satyrium nepalense is a rare and threatened medicinal orchid, populations of which in its native habitats are dwindling because of indiscriminate collections and habitat destruction, thus necessitating the development of methods for its in situ and ex situ conservation. Because of non-endospermous nature of the seeds and the immature embryos at seed dispersal stage, orchids cannot be seed-propagated as other plants. Micropropagation, using plant tissue culture techniques, offers an effective method for the multiplication of orchids. In this chapter, a five-step efficient reproducible protocol for large-scale in vitro multiplication of *Satyrium nepalense* is described. The first step involves asymbiotic germination of seeds isolated from immature green pods and cultured on Mitra's medium (M) gelled with 0.8 % agar and supplemented with 2 % sucrose and 1 % peptone (hereafter referred to as basal medium, BM). On this medium, seeds start germinating after a week of culture. Protocorms developed from the seeds are sub-cultured on BM fortified with 4 μM kinetin (Kn) after 8 weeks, for shoot differentiation and multiplication. The shoots developed on Kn-supplemented medium are transferred to BM alone for their elongation for the same period. The elongated shoots are transferred to the rooting medium, comprising BM supplemented with 0.5 or 1.0 μM indole-3-butyric acid, for further 8 weeks. The regenerated plantlets are transferred to a potting mix of sand and vermiculite (1:1) for acclimatization. The tubers and leaves excised from both in vitro-developed plants and those from their native habitats are analyzed and compared for the contents and concentration of medicinally important phenolics using high-performance liquid chromatography (HPLC), details of which are provided in this chapter.

Key words Asymbiotic seed germination, High-performance liquid chromatography, Medicinal orchid, Micropropagation, Phenolics, *Satyrium nepalense*, Shoot multiplication

1 Introduction

Satyrium nepalense (Fig. 1a), a terrestrial orchid, is a threatened medicinal herb, which is also considered as Salep orchid or "Salam Mishri" [1–3], a name also used for other orchids, such as *Dactylorhiza hatagirea* Syn. *Orchis latifolia* [4], *Eulophia dabia* [1], and *E. nuda* [5]. The plant is a stout aromatic terrestrial attaining a height of 25–60 cm. It is usually found at the higher altitudes ranging from 2400 to 5000 m [6]. The native people of

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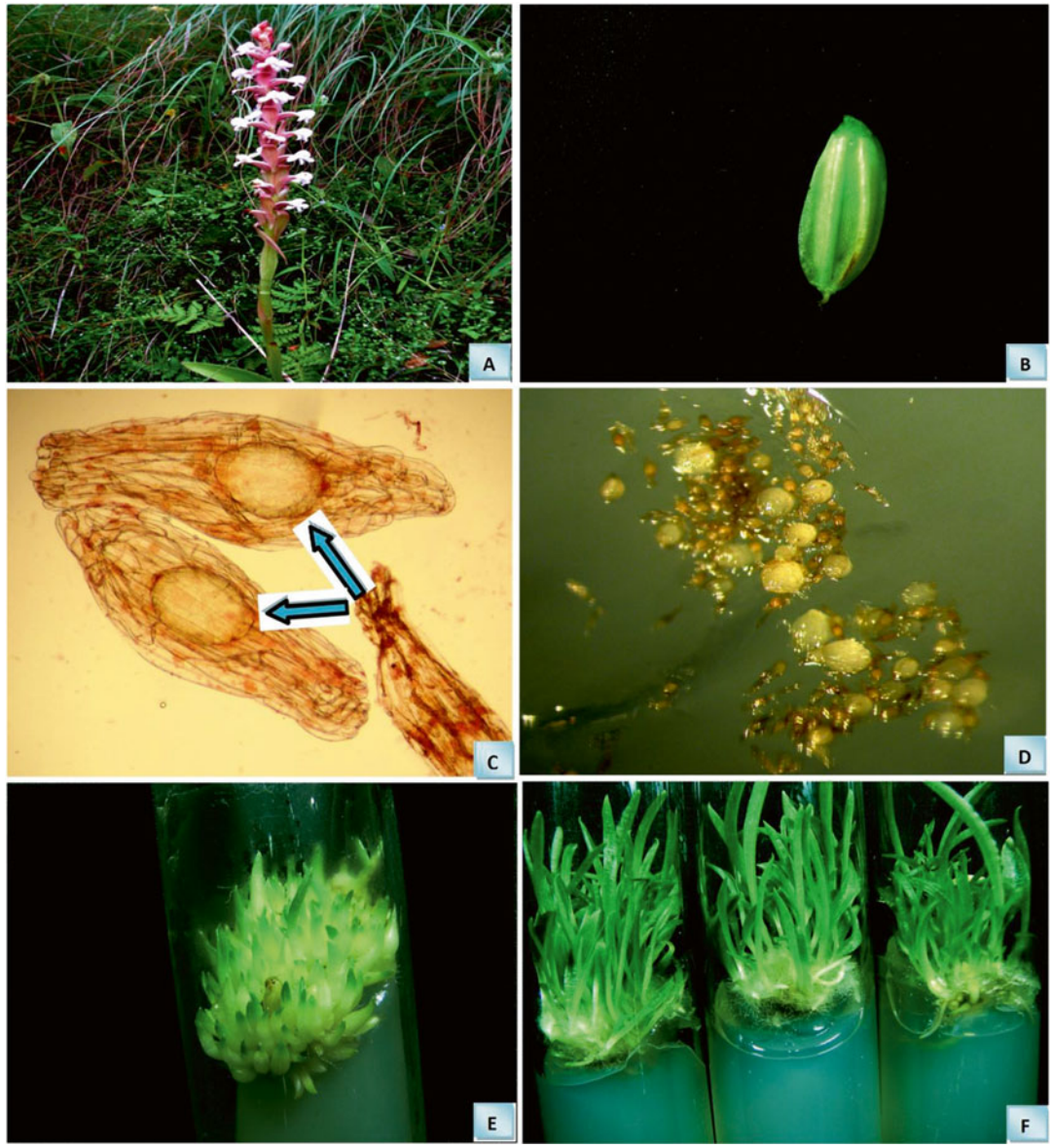

Fig. 1 In vitro multiplication of *Satyrium nepalense* -seeds to shoots. (**a**) A mature plant in its natural habitat. (**b**) A capsule pod. (**c**) Acetocarmine-stained seeds to show the stage of embryo at the time of culture (*arrow*). (**d**) PLBs developed from the seeds germinated on BM, after 10 days of culture. (**e**) Initiation of leaves on PLBs on BM after 60 days of culture. (**f**) Multiple shoots from the sub-cultured PLBs after 60 days of culture on BM + 4 μM Kn

upper Nilgiris correlates flower (twin spur) of *S. nepalense* with "bullock's horns" [7]. Decoctions of stems, roots, and tubers of the plant have been prescribed in various contagious ailments and also as dietary supplement since ancient times [8]. It is also used as a food and energizing tonic, and in diarrhea, dysentery, fever, and malaria [2, 7–10]. The methanolic extract of *S. nepalense* tubers has been demonstrated to have antimicrobial properties [6]. The plant is assumed to have aphrodisiac properties [11].

Phenolics are secondary metabolites abundant in edible and nonedible plants with a variety of roles in plant growth, development, and defense mechanisms [12, 13]. Many of these have proved to be beneficial for human health due to their antioxidant properties [14]. In addition to having antioxidant properties [15], phenolics also act as metal chelators [16–18], antimicrobial agents [19], clarifying agents [20], antimutagen, and anticarcinogens [21–23]. Extensive collections of *S. nepalense* due to its therapeutic properties and destruction of its habitats have led to decrease in its numbers in wild [24]. To avoid the possibility of its extinction in future, there is an urgent need for formulating conservation strategies involving domestication of the species by standardizing methods for its large-scale multiplication and developing agro-techniques for its cultivation to the extent fulfilling the need of pharmaceutical industries. Orchids cannot be propagated through seeds like other crops because of their peculiar characteristics. The seeds of orchids are microscopic; lack endosperms, cotyledons, and root initials; and require fungal infection for germination and initial growth [25]. In nature out of thousands to millions of microscopic seeds produced by a single pod of orchids, only 2.3 % of them germinate [26]. Multiplication by means of vegetative propagation is extremely slow and time consuming [27]. Its slow growing properties hardly fulfill the need of people, market, and various pharmaceutical companies [28]. In vitro methodology circumvents these difficulties by reducing the length of time needed for germination as well as large-scale multiplication [27]. The development of a defined nutrient medium by Knudson in 1922 [29] for asymbiotic seed germination of orchids revolutionized the hybridization and commercial propagation of orchids [30, 31], however, predominantly for ornamental orchids. In this chapter, an efficient protocol for the mass multiplication of *S. nepalense* is described. A detailed method for isolation of some important phenolics from the roots and leaves of both in vitro and in vivo plants and their estimation by high-performance liquid chromatography (HPLC) is also documented. Micropropagation of this orchid has also been reported earlier by Mahendran and Bai [7].

2 Materials

2.1 Plant Material

1. Immature (green) capsules of the *Satyrium nepalense* (Fig. 1b), collected from the wild (*see* **Note 1**).

2.2 Surface Sterilization of Capsules

1. Two drops each of Tween-20 and Teepol in 50 ml distilled water.
2. 70 % Ethanol.
3. Sterilized distilled water (SDW, *see* **Note 2**).

2.3 Germination of Seeds

1. Rimless test tubes (25 mm × 150 mm).
2. Cotton plugs (non-adsorbent cotton wrapped in cheese cloth).

2.4 Shoot Differentiation and Multiplication

1. BM + 4 μM kinetin (Kn), pH 5.6 (*see* **Note 3**).
2, 3. Same as 1 and 2 under Subheading 2.3

2.5 Elongation of Shoots

1. BM alone.
2, 3. Same as 1 and 2 under Subheading 2.3

2.6 Rooting of In Vitro-Regenerated Shoots

1. BM + 0.5 or 1.0 μM indole-3-butyric acid (IBA), pH 5.6 (*see* **Note 3**).
2, 3. Same as 1 and 2 under Subheading 2.3

2.7 Acclimatization of Plantlets

1. Sterilized vermiculite and sand (1:1, w/w).
2. Plastic pots (diameter 12 cm).
3. BM without sucrose and agar.
4. 0.1 % Bavistin (a fungicide) prepared in distilled water.
5. Transparent polythene bags.

2.8 Incubation of Cultures

1. Culture trolleys with shelves, each fitted with cool daylight fluorescent tubes providing an irradiance of 63 μmol/m^2/s in 16-h photoperiod. The trolleys are housed in culture room maintained at 25 ± 2 °C.

2.9 HPLC Analysis of Phenolics

1. Oven dried leaves and tubers.
2. Liquid nitrogen.
3. HPLC-grade acetonitrile.
4. 50 mM Ammonium acetate, pH 3.5, adjusted with acetic acid, to be used as one of the mobile phases.
5. HPLC-grade water.
6. HPLC-grade glacial acetic acid.
7. Stock solutions (1 mg/ml, prepared in acetonitrile:water, 1:1) of standards of gallic acid, syringic acid, *p*-hydroxybenzoic acid (HBA), and caffeic acid and store in freezer at 4 °C (*see* **Note 4**).

3 Methods

3.1 Surface Sterilization of Capsules

1. Treat green capsules with the solution of Tween-20 and Teepol for 3 min and then rinse the capsules thrice with sterilized distilled water.
2. In a laminar flow cabinet, sterilize the capsules with 0.25 % mercuric chloride for 8 min. Thereafter, rinse them thoroughly (5–6 times) with SDW.
3. Dip the capsules in 70 % alcohol for 30 s.
4. Flame the alcohol-treated capsules.

3.2 Raising Seed Cultures

1. Transfer the capsules, treated as described above, to a sterilized Petri plate. Give longitudinal slit to the capsules and scoop out the seeds (*see* **Note 5**).
2. Inoculate around 9–10 seeds (Fig. 1c) per culture tube containing 20 ml of BM. BM comprises Mitra's (M, Mitra et al. 1976) medium (Table 1) containing 2 % sucrose and gelled with 0.8 % agar and supplemented with 0.1 % peptone, pH of the medium adjusted to 5.6 by addition of 0.1 N NaOH or 0.1 N HCl, before autoclaving at 121 °C and 105 kPa for 15 min.

Table 1
Composition of Mitra's medium (Mitra et al. 1976)

Constituents	mg/L
Major inorganic nutrients	
KNO_3	180.0
$MgSO_4 \cdot 2H_2O$	250.0
KH_2PO_4	150.0
$(NH_4)_2SO_4$	100.0
$CaNO_3 \cdot 4H_2O$	200.0
Minor inorganic nutrients	
KI	0.03
H_3BO_3	0.06
$ZnSO_4 \cdot 7H_2O$	0.05
$Na_2MoO_4 \cdot 2H_2O$	0.05
$CuSO_4 \cdot 5H_2O$	0.05
$Co(NO_3)_2 \cdot 6H_2O$	0.05
$MnCl_2$	0.42
Iron source	
$FeSO_4 \cdot 7H_2O$	27.8
$Na_2EDTA \cdot 2H_2O$	37.3
Vitamins	
Thiamine HCl	0.3
Pyridoxine HCl	0.3
Nicotinic acid	1.25
Riboflavin	0.05
Biotin	0.05
Folic acid	0.3

3. Incubate the cultures at 25 ± 2 °C for 6 weeks in the dark. Within 6 weeks, protocorm-like bodies (PLBs) with rhizoids at the basal regions develop from the seeds (Fig. 1d). Transfer the cultures to illuminated conditions (63 μmol/m^2/s, 16-h photoperiod) after 6 weeks.

3.3 Shoot Development, Multiplication, and Elongation

1. After 8 weeks of initiation of seed cultures, transfer the PLBs (three per tube, each containing 20 ml of the culture medium) having rhizoids at the basal region to BM + 4 μM Kn for shoot development and multiplication. Incubate the cultures in illuminated conditions for 2 months during which multiple shoots develop from each PLB (Fig. 1e, f).
2. Separate the individual shoots (0.5–1.0 cm) from the clumps and transfer (two shoots per culture) to BM alone and incubate the culture for 2 months under lighted conditions (*see* **Note 6**).

3.4 Rooting of In Vitro-Regenerated Shoots

1. Sub-culture vertically the 3–4 cm long shoots having two expanded leaves on BM + 0.5 or 1.0 μM IBA (two per tube, each containing 20 ml culture medium). In 2 months of incubation, the shoots develop 3–4 thick adventitious roots (Fig. 2b). Now the plantlets are ready for the transfer from the culture tubes.

3.5 Acclimatization of the Regenerated Plants

1. Remove plantlets (5–6 cm long, Fig. 2c) from the culture tubes and wash them thoroughly under running tap water and later with lukewarm water to remove any adhering agar (*see* **Note 7**).
2. After washing, plant the in vitro-raised plantlets individually in the sterilized potting mix of sand and vermiculite (1:1) contained in perforated plastic pots (12 cm diameter, Fig. 2d). Cover the plantlets with porous transparent poly bags and keep the pots in culture room for 2 weeks.
3. After transplanting, spray the plants with 0.1 % bavistin (a fungicide) and mist irrigate with liquid BM on alternate days for 2 weeks. During the third week, spray bavistin only if required and irrigate with liquid BM alternating with water alone. Porous poly bags were removed gradually.
4. Shift the potted plants to the laboratory conditions and maintain them there for 1 week by watering them with tap water on alternate days.
5. After 4 weeks of acclimatization, plants can be transferred to the field under their native conditions.

3.6 Biochemical Profiling of Phenolics by HPLC

1. Dry in vitro and in vivo leaves and tubers for 48 h in an oven maintained at 50 °C.
2. Pulverize the plant material in liquid nitrogen.

3.6.1 Preparation of Plant Extracts

3. Take 100 mg powder of each sample and incubate individually for 12 h in 5 ml solution of acetonitrile and water (1:1, v/v)

Fig. 2 In vitro multiplication of *Satyrium nepalense* shoots to plants. (**a**) Elongated shoots 60 days after transfer to BM. (**b**) Rooted shoots (plantlets), 60 days after transfer to BM + 0.5 μM IBA. (**c**) Plantlets ready for transfer to pots. (**d**) Plantlets transferred to potting mix (sand and vermiculite (1:1)) for hardening

contained in 50 ml Falcon tubes on a rotary shaker run at 200 rpm and maintained at 25 °C.

4. Centrifuge the extracts at 10,000 × *g* for 20 min.
5. Filter the supernatant through Millipore filters (size: 0.22 μm) and store the filtrates at 4 °C till further use.

3.6.2 Analysis and Estimation of Phenolics

1. The profile of phenolics can be analyzed and the concentrations of individual phenolic acids can be estimated by HPLC.
2. The protocol described here employs "Waters" chromatographic system consisting of dual 515 pump, C18 Colum (250 mm × 4.6 mm, 5 μm), and 2998 photodiode array detector. Estimations are done using gradient elution system.
3. Sonicate 50 mM acidic ammonium acetate for 2 min. Wash the column (C-18) with both mobile phases, comprising ammonium acetate and acetonitrile, till a stable base line is observed.
4. The mobile phases used are ammonium acetate and acetonitrile. Set the initial and final conditions for ammonium acetate at 90 % for 0–20 min, so that both mobile phases return to the original condition in last 5 min. Monitor the eluent at 270 nm.

5. Plot the calibration curve by using 50–500 μg/ml of each standard. Prepare the required concentrations of each standard by diluting the stock solutions with acetonitrile and water (1:1).
6. Inject 20 μl of each sample and to have reproducible estimates repeat twice for each sample.

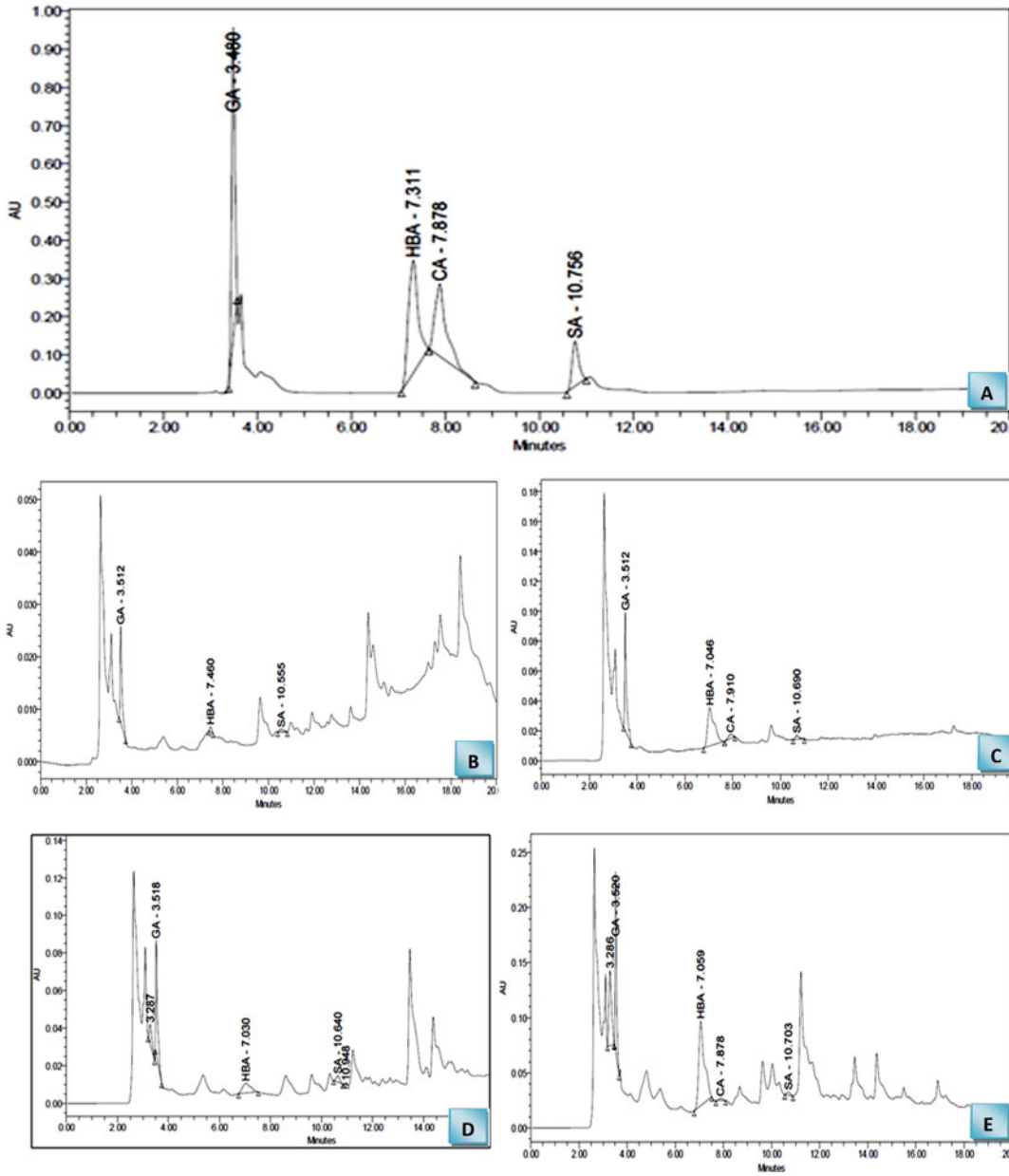

Fig. 3 Biochemical profiling of *Satyrium nepalense* for medicinally important phenolic acids, gallic acid (GA), *p*-hydroxybenzoic acid (HBA), syringic acid (SA), and caffeic acid (CA) by HPLC. (**a**) HPLC profile of the standards to show their retention time. (**b**–**e**) HPLC profiles of the four phenolic acids in in vivo tubers (**b**), in vivo leaves (**c**), in vitro tubers (**d**), and in vitro leaves (**e**)

7. Confirm the presence of particular phenolic acids by comparing the retention times of the resolved peaks in chromatograms of the plant extract with those of the standards (Fig. 3a). This can be further confirmed by "spiking" the samples with standard solutions of the phenolic acids. Use "Empower" or equivalent software to estimate the concentration of individual phenolic acids (Fig. 3b–e).

4 Notes

1. *Satyrium nepalense* is a widely distributed plant. It grows at higher altitudes (1300–1400 m) and flowers from July to September. Capsules can be collected from September onwards.
2. Sterilize all culture media, potting mixture, distilled water, glassware, and instruments by autoclaving at 103 kPa at 121 °C for 15 min.
3. For preparing the stock solution of Kn, first dissolve its weighed amount in few drops of 1 N NaOH and then raise the volume to the desired level with distilled water. Likewise, IBA is first dissolved in ethanol before addition of distilled water to make up the desired volume.
4. Prepare stock solution of each standard, gallic acid (GA), syringic acid (SA), HBA, and caffeic acid (CA), by individually dissolving 10 mg of each in 10 ml of solvent containing equal volume of distilled water and acetonitrile. Store stock solutions at 4 °C.
5. The capsules dipped in 70 % alcohol are flamed only as long as the alcohol is burning. Thereafter, these are transferred to a sterilized Petri plate. The longitudinal slit is given to the capsules with a flame-sterilized and cooled scalpel only after the surface of the capsules becomes totally dry. For scooping out the seeds from the slit capsules, use a narrow tapered spatula with minimum possible thickness at the tapered end. It has to be flame sterilized and cooled by dipping in sterilized distilled water each time the seeds are picked to be inoculated.
6. Injury to shoots should be avoided while separating them from the clumps for transfer to BM for elongation. The shoots injured while separating should be discarded. While planting the shoots on BM, the leaves should not touch the medium.
7. For transfer to the potting mix from the culture tubes, the plantlets are to be pulled out gently to avoid injury.

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Chapter 2

In Vitro Culture and Phytochemical Analysis of *Passiflora tenuifila* Killip and *Passiflora setacea* DC (Passifloraceae)

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Abstract

We have developed reproducible micropropagation, callus culture, phytochemical, and antioxidant analysis protocols for the wild passion fruit species *P. tenuifila*, and *P. setacea*, native to the Brazilian endangered biomes Atlantic Forest, Cerrado, and Caatinga, by using seeds and explants from seedlings and adult plants. Genotype and explant origin-linked differences are visible amongst the Passiflora species concerning callus production, total phenolics, and antioxidant activity. The protocols developed for screening phytochemicals and antioxidants in *P. tenuifila* and *P. setacea* callus extracts have shown their potential for phenolic production and antioxidant activity. The high level of phenolic compounds seems to account for the antioxidant activity of methanolic extracts of *P. tenuifila* derived from 45-day-old immature seed callus. The methanolic extracts of callus derived from *P. setacea* seedling leaf node and cotyledonary node explants have shown the highest antioxidant activity despite their lower content of phenolics, as compared to cotyledon callus extracts. The optimized micropropagation and callus culture protocols have great potential to use cell culture techniques for further vegetative propagation, in vitro germplasm conservation, and secondary metabolite production using biotic and abiotic elicitors.

Key words *Passiflora tenuifila*, *Passiflora setacea*, Micropropagation, Callus, Total phenolics, Antioxidant activity

1 Introduction

Passiflora setacea and Passiflora tenuifila are endemic to the endangered Brazilian biome Cerrado, Caatinga, and the Atlantic Forest. Their fruits have great potential for commercial use by nutritional, pharmaceutical, and cosmetic industries due to the high content of phenolic compounds which are antioxidants, especially *P. tenuifila* [1]. *P. tenuifila* is a potential source of resistant genes for genetic improvement as it is resistant to the pathogens *Xanthomonas axonopodis* pv. passiflorae and *Cladosporium herbarum*. The main constraints for plant production of several *Passiflora* species as *P. setacea*

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(occurring in Caatinga, Cerrado, Atlantic Forest) and *P. tenuifila* (occurring in Cerrado and Atlantic Forest) are the low seed germination rate, slow seedling emergence, and lack of efficient seed conservation protocols [2–4]. In vitro propagation techniques have been established for several *Passiflora* species such as *P. alata*, *P. caerulea*, *P. cincinnata*, *P. edulis*, *P. setacea*, *P. foetida*, and *P. suberosa*. These techniques have been used for the rapid multiplication of healthy and disease-free elite plants, source of fruits, and medicinal products as the antioxidants and other biologically active compounds [5–7]. However, plant tissue culture systems for secondary metabolite and antioxidant assessment have been established for *P. quadrangularis* [5] and *P. alata* Curtis [8, 9]. The protocols described herein are based on efficient and reproducible methods for *P. tenuifila* and *P. setacea* shoot culture initiation, callus culture establishment, and evaluation of callus phenolic content and antioxidant activity. We have optimized in vitro callus culture systems of *P. tenuifila* and *P. setacea* according to the antioxidant activity [10]. In vitro culture and antioxidant responses are genotype, explant type, and callus age dependent. The phytochemical and antioxidant screening carried out on *P. tenuifila* and *P. setacea* callus extracts demonstrates potent antioxidant activity.

2 Materials

2.1 Plant Material

1. Collect seeds from stock mother plants of *P. tenuifila* and *P. setacea* from the germplasm collection maintained at the experimental station, Embrapa Cerrados, Empresa Brasileira de Pesquisa Agropecuária (Embrapa), Unidade Planaltina (Planaltina, DF, Brazil).

2.2 Culture Media

1. Murashige and Skoog [11] powder basal medium (MS), plant cell culture tested (Table 1).
2. 59 mM Sucrose grade I, plant cell culture tested.
3. 88.5 mM D-(+)-glucose, plant cell culture tested.
4. 88.5 mM D-(−)-fructose, plant cell culture tested.
5. 2 g/L Phytagel, plant cell culture tested.
6. Plant growth regulators for plant cell culture: gibberellic acid (GA_3), indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D).

2.3 Extraction and Estimation of Total Phenolics

1. Folin-Ciocalteu reagent.
2. Gallic acid (*see* **Notes 1** and **2**).
3. 20 % (w/v) sodium carbonate solution: Prepare a stock solution and store at 4 °C.

Table 1
Murashige and Skoog basal medium composition

Component	Concentration (mL/L)
Ammonium nitrate	1650.0
Boric acid	6.2
Calcium chloride (anhydrous)[a]	332.2
Cobalt chloride·$6H_2O$	0.025
Cupric sulfate·$5H_2O$	0.025
Ethylenediaminetetracetic acid (disodium salt)·$2H_2O$	37.26
Ferrous sulfate·$7H_2O$	27.8
Magnesium sulfate[b]	180.7
Manganese sulfate	16.9
Molybdic acid (sodium salt)·$2H_2O$	0.25
Potassium iodide	0.83
Potassium nitrate	1900.0
Potassium phosphate monobasic	170.0
Zinc sulfate·$7H_2O$	8.6
Glycine (free base)	2.0
Myoinositol	100.0
Nicotinic acid (free acid)	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1

[a]Original formula contains calcium chloride dihydrate at 440.0 mg/L
[b]Original formula contains magnesium sulfate heptahydrate at 370 mg/L

4. 80 % (v/v) methanol, high-performance liquid chromatography (HPLC) grade.
5. Ultraviolet-visible (UV-Vis) microplate reader.

2.4 Free Radical Scavenging Effect of 2,2-Diphenyl-1-picrylhydrazyl

1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) reagent (*see* **Note 3**).
2. 80 % (v/v/) methanol, high-performance liquid chromatography (HPLC) grade.
3. 3,5-Di-*tert*-4-butylhydroxytoluene analytical standard (BHT).
4. Ultraviolet-visible (UV-Vis) microplate reader.

3 Methods

3.1 Preparation of Culture Medium

1. Prepare standard aqueous solutions of plant growth regulators (*see* Subheading 2.2, **item 6**) by adding two drops of 1 M NaOH.
2. Prepare appropriate culture medium by adding the recommended concentration of the carbon source (*see* Tables 2, 3, 4, 5, 6, 7, and 8) together with plant growth regulators (*see* **Notes 4**, **5**, and **9**).
3. Adjust medium pH to 5.8 with 1 M NaOH before adding 2 g/L Phytagel. Dispense into 25 × 150 mm glass culture tubes (8 mL/tube) and autoclave at 121 °C for 18 min.

3.2 Culture Condition

1. Seal vessels with transparent polypropylene film (76 × 76 mm) and maintain the cultures at 25 °C, 70 % relative humidity (RH) under a 16-h photoperiod, photosynthetic photon flux of 20–25 μmol/m²/s supplied by fluorescent light tubes. In all protocols use these culture conditions.

Table 2
Composition of culture media used for shoot culture initiation and callus induction from different explant types of *Passiflora tenuifila*

In vitro process	Explant type	Explant origin	Culture medium	Carbon source	PGR	Induction (%)[a]	Callus texture
Shoot culture	Shoot tips	Ex vitro-germinated seedling	MS	59 mM sucrose	None	100	–
		Adult plant	MS	59 mM sucrose	None	100	–
Callus culture	Immature seeds	Developing fruits	MS	59 mM sucrose	2.5 μM 2,4-D	60	Friable
	Shoot tips	Adult plant	MS	59 mM sucrose	1.25–5 μM NAA	92	Friable
		Ex vitro-germinated seedling	MS	59 mM sucrose	1.25–5 μM NAA	100	Friable
	Stem segments	Shoot cultures from adult plant	MS	88.5 mM sucrose	2.5 μM NAA	100	Friable
		Shoot cultures from seedling	MS	88.5 mM sucrose	1.25 μM NAA or 2.5 μM 2,4-D	100	Friable
		Shoot cultures from seedling	MS	88.5 mM glucose	2.5 μM NAA	100	Friable
		Shoot cultures from seedling	MS	88.5 mM fructose	2.5 μM NAA	100	Friable

Note: Basic MS basal medium containing 2 mg/L Phytagel
[a]Values are means of 3 replicates of 15 explants each per treatment. The photosynthetic photon flux at culture level was 20–25 μmol/m²/s

Table 3
Composition of culture media used for in vitro shoot culture growth from seedling and adult plant shoot tips of *Passiflora tenuifila*

Explant origin	Culture medium	Carbon source (mM)	PGR type	PGR (μM)	Shoot induction (%)[x]	Rooting (%)[x]	No. of shoots [y]	Shoot length (cm)[y]	No. of leaf nodes[y]
Seedling	MS	59 mM sucrose	IBA	0	40	20 a	1 a	7.8 a	7 a
	MS	59 mM sucrose		1.25	100	50 b	1.3 a	11.8 b	19.5 b
	MS	59 mM sucrose		2.5	100	40 b	1.8 a	8.9 a	15.8 b
	MS	59 mM sucrose		5	100	40 b	1.2 a	7.5 a	15.2 b
Adult plant	MS	59 mM sucrose	GA_3	0	100	0	2.2 a	8.5 ab	7.2 a
	MS	59 mM sucrose		1.25	100	0	1.6 a	13.8 b	10.8 b
	MS	59 mM sucrose		2.5	100	0	2.2 a	5.3 a	11.6 b
	MS	59 mM sucrose		5	100	40 b	2 a	4.5 a	10.2 b

Note: Basic MS basal medium containing 2 mg/L Phytagel. The photosynthetic photon flux at culture level was 20–25 μmol/m^2/s

[x]Values are proportions of ten replicates per treatment

[y]Values ($n = 15$) within a single column followed by the same letter were not significantly different according to the Tukey test ($p \leq 0.05$)

Table 4
Composition of culture media used for in vitro biomass production of *P. tenuifila* callus derived from different explant types cultured in light

Explant origin	Culture medium	Carbon source type	Carbon source (mM)	PGR type	PGR (μM)	Callus FW (mg)[x]	Callus DW (mg)[x]
Immature seeds	MS	Sucrose	88.5	2,4-D	2.5	1383.8 ± 187.2 c	99.4 ± 7.5 c
Stem segments	MS	Sucrose	88.5	2,4-D	2.5	1021 ± 208.7 a	91.1 ± 11.19 b
Stem segments	MS	Sucrose	88.5	NAA	1.25	924.8 ± 185.3 a	80 ± 7.9 b b
Stem segments	MS	Glucose	88.5	NAA	2.5	1063.5 ± 109.7 b	55.4 ± 2.6 a
Stem segments	MS	Fructose	88.5	NAA	2.5	1082.4 ± 134.8 b	56.6 ± 3.8 a

Note: Basic MS basal medium containing 2 g/L Phytagel. The photosynthetic photon flux at culture level was 20–25 μmol/m^2/s

FW fresh weight, *DW* dry weight

[x]Values ($n = 10$) within a single column followed by the same letter were not significantly different according to the Tukey test ($p \leq 0.05$)

Table 5
Composition of culture media used for in vitro biomass production, total phenolics, and percentage of DPPH* inhibition of *P. tenuifila* callus extracts derived from different explant types cultured in light

Explant type	Culture medium	Carbon source type	Carbon source (mM)	PGR type	PGR (μM)	Culture period (days)	Total phenolics (μg GAE/g DW)[x]	DPPH* inhibition (%)[x]
Immature seeds	MS	Sucrose	88.5	2,4-D	2.5	45	20.41 ± 0.06 c	84 ± 0.60 c
Stem segments	MS	Sucrose	88.5	2,4-D	2.5	80	14.07 ± 0.03 a	21.72 ± 0.38 a
Stem segments	MS	Fructose	88.5	NAA	2.5	80	13.92 ± 0.03 a	31.83 ± 0.88 b
Stem segments	MS	Sucrose	88.5	2,4-D	2.5	80	17.28 ± 0.03 b	22.27 ± 0.25 a

Note: Basic MS basal medium containing 2 g/L Phytagel. The photosynthetic photon flux at culture level was 20–25 μmol/m²/s
GAE gallic acid equivalent, *DW* dry weight
[x]Values ($n = 5$) within a single column followed by the same letter were not significantly different according to the Tukey test ($p \leq 0.05$)

Table 6
Composition of culture media used for shoot culture initiation and callus induction of *Passiflora setacea*

In vitro process	Explant type	Explant origin	Culture medium	Sucrose (mM)	2,4-D (μM)	Induction (%)[x]	Callus texture
Shoot culture initiation	Shoot tips	Seedling (ex vitro)	MS	59	0	100	–
Callus culture initiation	Root	Seedling (in vitro)	MS	59	5	100	Friable
	Hypocotyl	Seedling (in vitro)	MS	59	5	100	Friable
	Cotyledonary node	Seedling (in vitro)	MS	59	2.5	100	Compact
	Leaf node	Seedling (in vitro)	MS	59	2.5	100	Friable
	Cotyledon	Seedling (in vitro)	MS	59	5	100	Compact

Note: Basic MS basal medium containing 2 mg/L Phytagel
[x]Values are means of three replicates of ten explants each per treatment. The photosynthetic photon flux at culture level was 20–25 μmol/m²/s

Table 7
Composition of culture media used for in vitro shoot growth from shoot tip culture of *P. setacea*

Culture medium	Carbon source type	Carbon source (mM)	IBA (μM)	Shoot induction (%)[x]	Rooting (%)[x]	No. of shoots[y]	Shoot length (cm)[y]	No. of leaf nodes[y]
MS	Sucrose	59	0	100	0	1.4 ab	5.4 b	12.6 b
MS	Sucrose	59	1.25	100	60	1.6 b	8.5 c	17.8 c
MS	Sucrose	59	2.5	100	0	1 a	6.7 b	10 b
MS	sucrose	59	5	100	0	1.2 a	4.2 a	5.8 a

Note: Basic MS basal medium containing 2 mg/L Phytagel. The photosynthetic photon flux at culture level was 20–25 μmol/m^2/s

[x]Values are proportions of ten replicates per treatment

[y]Values ($n = 15$) within a single column followed by the same letter were not significantly different according to the Tukey test ($p \leq 0.05$)

Table 8
Composition of culture media used for in vitro biomass production, total phenolics, and percentage of DPPH* inhibition of *P. setacea* callus extracts derived from different seedling explant types cultured in light

Explant type	Culture medium	Sucrose (mM)	2,4-D (μM)	Culture period (days)	Callus FW (mg)[x]	Callus DW (mg)[x]	Total phenolics (μg GAE/g DW)[y]	DPPH* inhibition (%)[y]
Root	MS	88.5	2.5	45	1822.0 ± 271.0 c	82.8 ± 6.7 c	8.47 ± 0.02 a	55.51 ± 2.00 a
Cotyledonary node	MS	88.5	2.5	45	1347.0 ± 204.0 b	64.4 ± 8.1 a	12.34 ± 0.02 b	77.66 ± 0.90 c
Hypocotyl	MS	88.5	2.5	45	1044.8 ± 163.0 ab	61.4 ± 10.4 a	10.11 ± 0.01 b	63.84 ± 1.89 b
Leaf node	MS	88.5	2.5	45	1107.6 ± 200.8 b	61.4 ± 10.4 a	11.10 ± 0.03 b	88.17 ± 0.50 d
Cotyledon	MS	88.5	2.5	45	960.0 ± 207.3 a	70.2 ± 10.4 b	17.26 ± 0.04 c	52.50 ± 1.17 a

Note: Basic MS basal medium containing 2 g/L Phytagel. The photosynthetic photon flux at culture level was 20–25 μmol/m^2/s

GAE gallic acid equivalent, *FW* fresh weight, *DW* dry weight

[x]Values ($n = 10$) within a single column followed by the same letter were not significantly different according to the Tukey test ($p \leq 0.05$)

[y]Values ($n = 5$) within a single column followed by the same letter were not significantly different according to the Tukey test ($p \leq 0.05$)

3.3 Establishment of Shoot Cultures of P. tenuifila

3.3.1 Ex Vitro Seed Germination

1. Collect the seeds from fresh mature fruits of *P. tenuifila* immediately after harvesting.
2. Remove the seed arils.
3. Immerse *P. tenuifila* seeds completely in 2 mL 2.5 % (w/v) gibberellic acid aqueous solution at 25 °C for 5 days (*see* **Note 5**).
4. Remove *P. tenuifila* seeds from the GA_3 aqueous solution and sow in 50 mL plastic cups containing 28 g soil. Add 8 mL distilled water.
5. Place plastic cups in 23.5 cm × 16.9 cm × 10 cm polystyrene trays.
6. Maintain the trays either at 25 °C or in the greenhouse with a natural photoperiod at 16 °C during the night and 40 °C during the day (*see* **Note 5**).

3.3.2 Surface Sterilization of Seedling and Adult Plant Shoot Tips

1. Excise shoot tips, 3–4 cm in length comprising one apical bud and two axillary buds, from 45- to 60-day-old seedlings (*see* Fig. 1a) or greenhouse-grown 15-month-old adult *P. tenuifila* plants.
2. Rinse shoot tips in 100 mL tap water, containing 2–3 drops of commercial detergent.
3. Wash four times with distilled water.
4. Surface sterilize shoot tips of *P. tenuifila* seedlings for 1 min 30 s in 70 % (v/v) alcohol, rinse for 3 min in sterile distilled water, and immerse in commercial bleach (2.5 % active chlorine) with 2–3 drops of Tween 20 for 2 min 30 s.
5. Surface sterilize shoot tips of *P. tenuifila* adult plants for 3 min in 70 % (v/v) alcohol, rinse for 3 min in sterile distilled water, and immerse in commercial bleach (2.5 % active chlorine) with 2–3 drops of Tween 20 for 4 min.
6. Rinse the shoot tips three times each for 10 min in sterile distilled water.

3.3.3 Shoot Initiation and Multiplication

1. Place surface-sterilized shoot tips of *P. tenuifila* vertically on the MS basal medium containing 59 mM sucrose and 2 g/L Phytagel (*see* **Note 5**, Fig. 1b).
2. Promote in vitro shoot elongation by transferring the 30-day-old in vitro shoots to MS basal medium with 59 mM sucrose, 2 g/L Phytagel, and either 1.25 μM IBA (*P. tenuifila* seedling shoot tips) or 1.25 μM GA_3 (*P. tenuifila* adult plant shoot tips) (*see* **Note 7**) (Fig. 1c).
3. Evaluate the percentage of shoot formation, number of shoots, shoot length, and number of nodes initiated per explant after 12 weeks. The results for shoot induction and growth of *P. tenuifila* are shown in Tables 2 and 3.

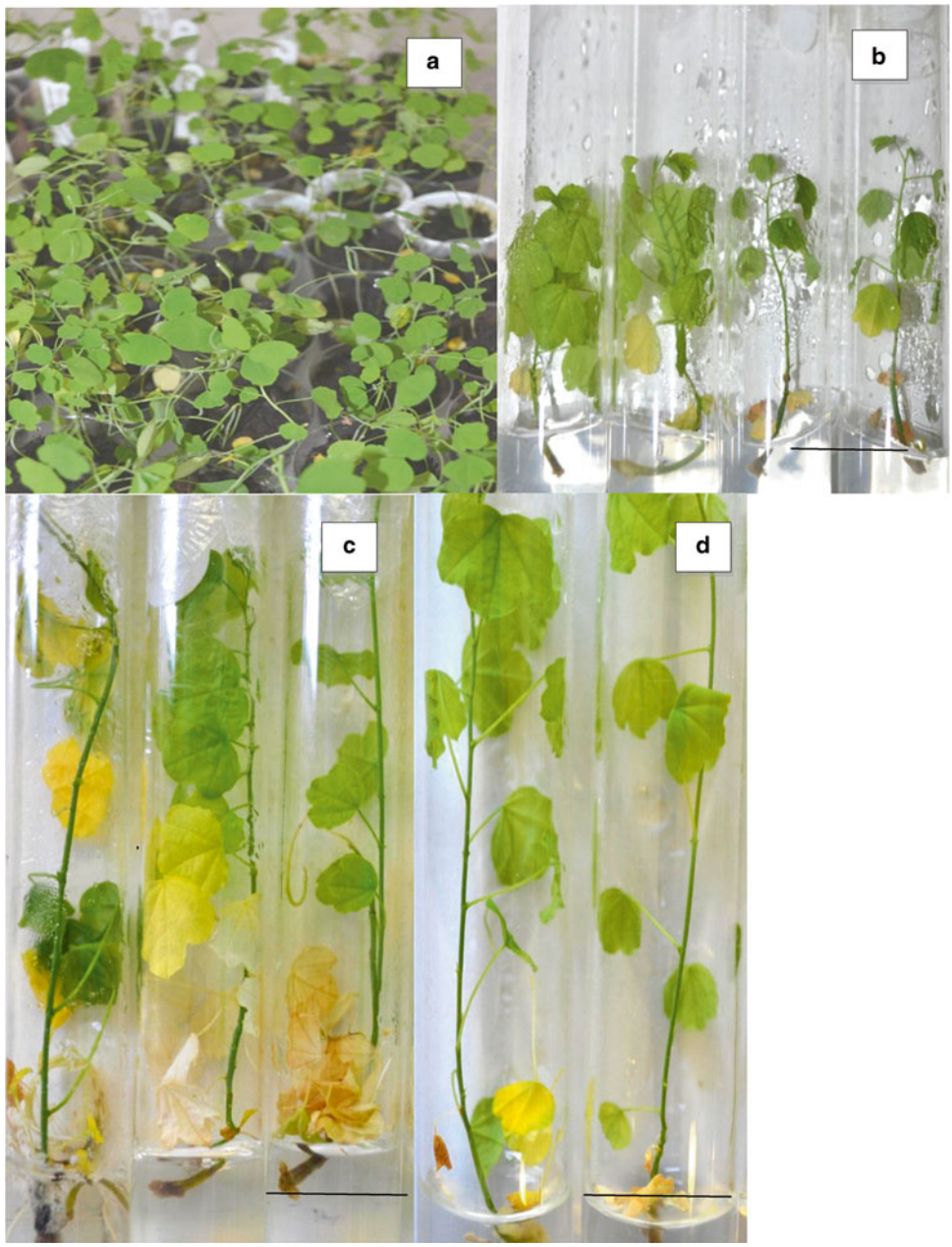

Fig. 1 Ex vitro-germinated seedlings of *Passiflora tenuifila* (**a**). In vitro shoot cultures originated from seedling shoot tip explants of *Passiflora tenuifila* cultured on MS basal medium with 2 mg/L Phytagel, 59 mM sucrose (**b**); 59 mM sucrose and 1.25 μM GA_3 (**c**) after 90 days; 88.5 mM glucose and 1.25 μM IBA (**d**) after 45 days. Bars = 25 mm

4. For shoot multiplication and shoot culture stock maintenance remove 2 cm long shoot tips and single-leaf node explants (ca. 1.0 cm long) from the 8-week-old *P. tenuifila* plantlets originated from the shoot tips cultured according to the procedures 1–2 (*see* **Note 8**).

5. Place explants vertically on the MS basal medium supplemented with 88.5 mM glucose, 2 g/L Phytagel, and 1.25 μM IBA. The results for in vitro shoot growth of *P. tenuifila* are shown in Fig. 1d (*see* **Note 8**).
6. Subculture after every 60-day culture cycle.

3.4 Establishment of Callus Cultures

3.4.1 Establishment of Callus Cultures from Immature Seeds

1. Harvest immature fruits (4-week-old after anthesis) from *P. tenuifila* greenhouse-grown plants.
2. Rinse fruits in 100 mL tap water containing 2–3 drops of commercial detergent.
3. Surface sterilize fruits for 5 min in commercial alcohol in the flow cabinet.
4. Remove the seeds and place them on MS basal medium amended with 59 mM sucrose, 2 g/L Phytagel, and 2.5 μM 2,4-D (*see* **Notes 9** and **10**).
5. After 45 days evaluate the callus induction. Example results of callus induction are shown in Table 2, Fig. 2a.
6. Subculture callus (ca. 50 mg fresh weight) every 30-day cycle to MS basal medium containing 88.5 mM sucrose, 2 g/L Phytagel, and 2.5 μM 2,4-D (*see* **Note 11**).
7. After 45 days evaluate callus fresh and dry mass. The results for callus growth are shown in Table 4, Fig. 2b (*see* **Notes 10** and **11**).

3.4.2 Establishment of Callus Cultures from Seedling and Adult Plant Shoot Tips

1. Surface sterilize shoot tips of *P. tenuifila* following the procedures described in Subheading 3.3.2.
2. Place explants vertically on the MS basal medium supplemented with 59 mM sucrose, 2 g/L Phytagel, and 1.25 μM NAA (*see* **Note 9**).
3. Evaluate the percentage of callus induction and fresh and dry mass after 45 days. The results for callus induction are shown in Table 2, Fig. 2c–e (*see* **Notes 10** and **11**).

3.4.3 Establishment of Callus Cultures from Stem Segments of In Vitro-Formed Shoots

1. Remove stem segments (2–3 mm in length) from 60-day-old shoot cultures of *P. tenuifila* established according to the procedures described in Subheading 3.3.2 (Fig. 1).
2. Place the explants on MS basal medium containing 88.5 mM of either sucrose, glucose, or fructose, 2 g/L Phytagel, and either 1.25–2.5 μM NAA or 2,4-D (*see* **Note 9**).
3. After 45 days, evaluate the percentage of callus induction, fresh mass, and dry mass. The results for callus induction and growth are shown in Tables 2 and 4, Fig. 2f (*see* **Notes 10** and **11**).

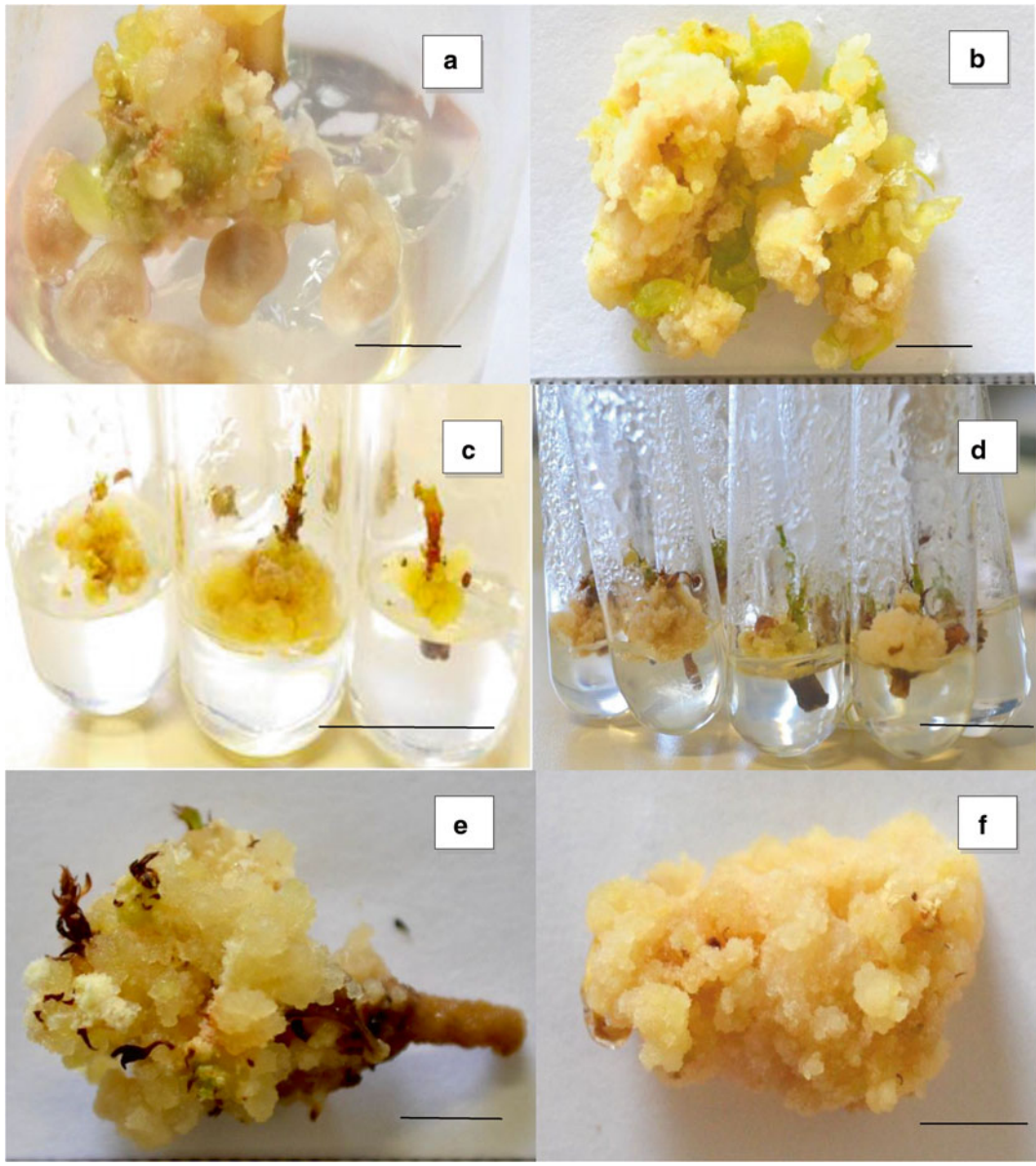

Fig. 2 Callus originated from immature seeds (**a**, **b**), from shoot tips of seedlings (**c**), adult plants (**d**, **e**), and stem segment explants of in vitro-formed shoots (**f**) of *Passiflora tenuifila* cultured on MS basal medium with 59 mM sucrose, 2 mg/L Phytagel, and 2.5 μM 2,4-D (**a**, **b**); 2.5 μM NAA (**c**–**e**); or 88.5 mM sucrose and 2.5 μM 2,4-D (**f**), after 50-day culture. Bars = 5 mm (**a**, **b**, **e**, **f**). Bars = 25 mm (**c**, **d**)

3.5 Establishment of Shoot Cultures of P. setacea

3.5.1 Ex Vitro Seed Germination

1. Collect the seeds from fresh mature fruits of *P. setacea* immediately after harvesting.
2. Remove the seed arils.
3. Sow *P. setacea* seeds in 50 mL plastic cups containing 28 g soil. Add 8 mL distilled water.
4. Place plastic cups in 23.5 cm × 16.9 cm × 10 cm polystyrene trays.

5. Keep the trays in the greenhouse with natural photoperiod and temperatures of 16 °C during the night and 40 °C during the day (*see* **Note 6**).

3.5.2 Shoot Initiation and Multiplication

1. Surface sterilize ex vitro-grown seedling shoot tips of *P. setacea* (*see* Subheading 3.3.2, **steps 1–4**; Fig. 3a).
2. Place surface-sterilized shoot tips of *P. setacea* vertically on the MS basal medium supplemented with 59 mM sucrose and 2 g/L Phytagel (*see* **Note 5**).
3. Evaluate the percentage of shoot formation, number of shoots, shoot length, and number of nodes initiated per explant after 12 weeks. The results of shoot induction and growth are shown in Tables 6 and 7, Fig. 3b (*see* **Note 12**).
4. For shoot elongation, multiplication and maintenance of shoot culture stocks of *P. setacea* follow the procedures described in Subheading 3.3.3, **steps 4–6** (*see* **Note 8**). The results for in vitro shoot growth of *P. setacea* are shown in Fig. 3c (*see* **Note 8**).

3.6 Establishment of Callus Cultures

3.6.1 Seed Surface Sterilization and In Vitro Seed Germination of *P. setacea*

1. Rinse seeds of selected *P. setacea* plants in 100 mL tap water with 2–3 drops of commercial detergent, and wash four times with distilled water.
2. Surface sterilize seeds for 10 min in commercial bleach (2.5 % active chlorine). Add 2–3 drops of Tween 20.
3. Rinse three times for 10 min in sterile distilled water and culture the seeds on MS basal medium supplemented with 59 mM sucrose and 2 g/L Phytagel (*see* **Note 6**).

3.6.2 Establishment of Callus Cultures from Axenic Seedling Explants

1. Remove segments (1 cm long) of either root, hypocotyl, epicotyl, cotyledonary node, or leaf node obtained from 60-day-old aseptically grown seedlings of *P. setacea* (Fig. 3d).
2. Place the explants horizontally on MS basal medium with 88.5 mM sucrose, 2 g/L Phytagel, and 2.5 μM 2,4-D (*see* **Note 13**).
3. After 45 days, take callus fresh and dry weight. The results of callus induction are shown in Table 6 (*see* **Notes 11** and **12**).
4. Subculture callus at 30-day interval by following the procedures described in Subheading 3.4.1, **step 6**. The results for callus growth are shown in Table 8, Fig. 4 (*see* **Notes 10** and **11**).

3.7 Phytochemical Analysis and Antioxidant DPPH Test

3.7.1 Preparation of Callus Extract for Estimation of Total Phenolic Compounds

1. Grind 1 g fresh callus material in 10 mL 80 % (v/v) methanol in water and leave the extract for 1 h in the darkness.
2. Centrifuge contents at 11.76×*g* for 5 min. The supernatant solution is filtered under vacuum into a volumetric flask and the filtrate is saved.

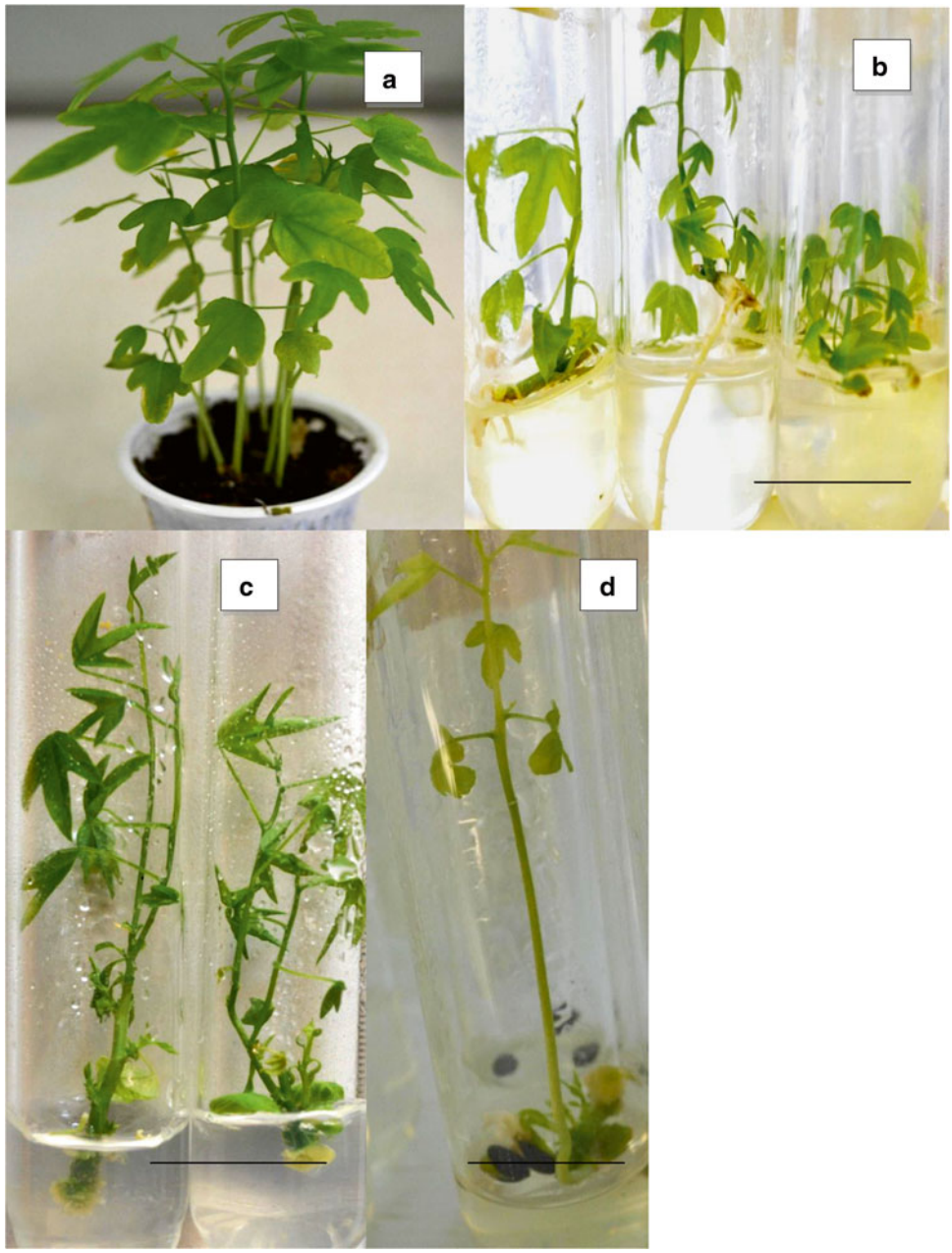

Fig. 3 Ex vitro-germinated seedlings of *Passiflora setacea* (**a**). In vitro shoots of *Passiflora setacea* originated from seedling shoot tip explants cultured on MS basal medium with 2 mg/L Phytagel and either 59 mM sucrose (**b**, **c**) or 88.5 mM glucose μM IBA (**d**) after 45 days. In vitro-germinated seedling of *Passiflora setacea*. Bars = 25 mm

3.7.2 Estimation of Total Phenolics Contents in the Callus Extract

1. Mix 40 μL methanolic extract with 3.16 mL deionized water and add 200 μL 10 % Folin-Ciocalteu reagent.

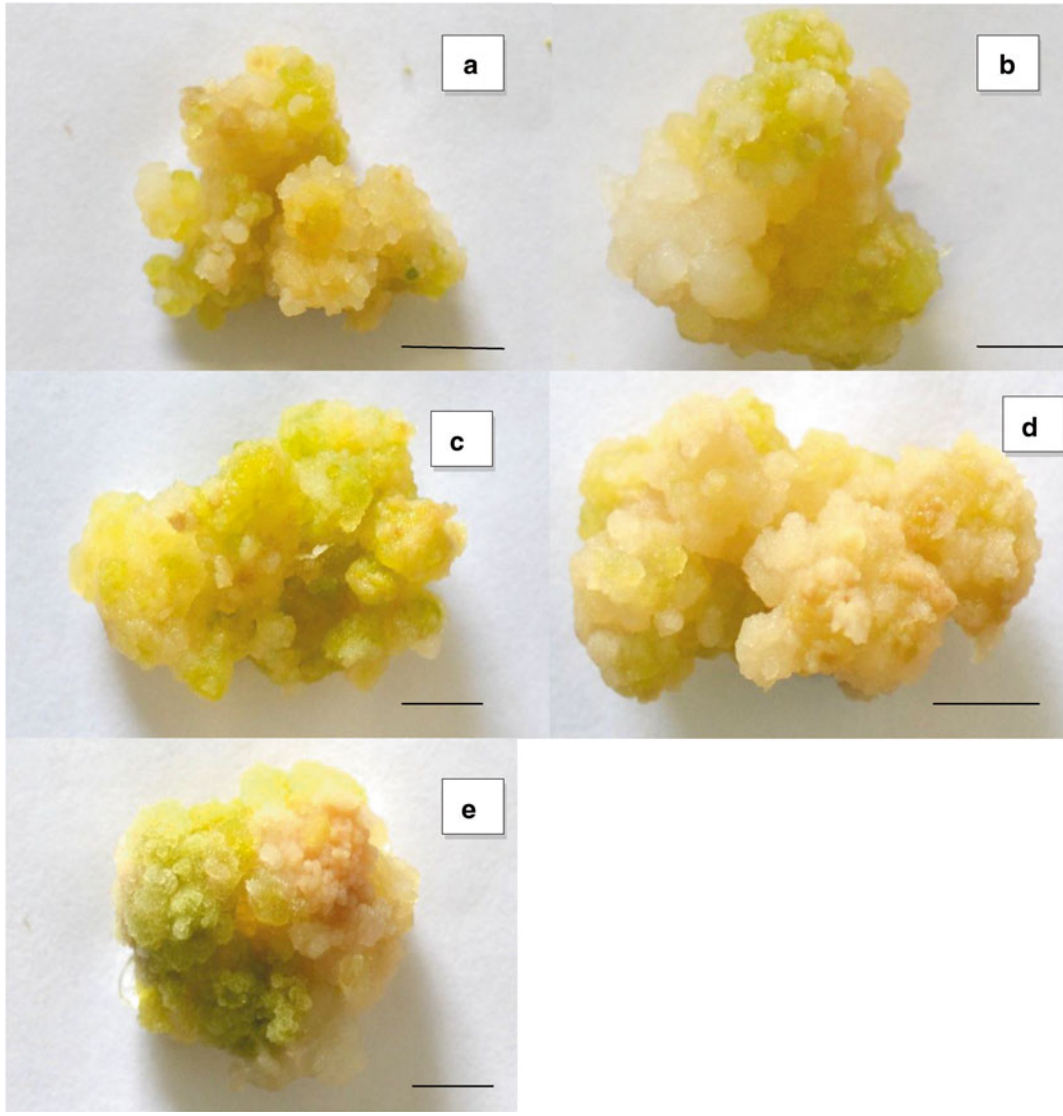

Fig. 4 Callus originated from roots (**a**), hypocotyl (**b**), cotyledonary node (**c**), leaf node (**d**), cotyledon (**e**) of in vitro-cultured seedlings of *Passiflora setacea* cultured on MS basal medium with 88.5 mM sucrose, 2 mg/L Phytagel, and 2.5 μM 2,4-D, after 45-day culture. Bars = 5 mm

2. After 6 min the reaction is neutralized with 600 μL 20 % sodium carbonate solution. The color will develop after incubation for 2 h at the room temperature in the darkness.
3. 300 μL of each sample and control are transferred to 96-well microplate and the absorbance is detected at 760 nm on a UV-visible microplate reader. The measurements are compared to the standard curve for gallic acid (0–1000 μg/mL) (*see* **Notes 1** and **2**).

4. The results for total phenolics are expressed as microgram of gallic acid equivalent per gram of dry callus. The results of total phenolic contents in callus of *P. tenuifila* (Table 5) and *P. setacea* (Table 8) are shown (*see* **Note 14**).

3.7.3 Free Radical Scavenging Effect on 2,2-Diphenyl-1-picrylhydrazyl

1. Mix samples of 10 μL methanolic extract with 290 μL 0.05 mM methanolic solution of DPPH in a 96-well microplate (*see* **Note 3**).
2. A DPPH blank sample, without extract, containing 10 μL 80 % methanol and 290 μL DPPH solutions is prepared and assayed as control. The positive control is the DPPH solution plus *tert*-butylhydroxytoluene (BHT).
3. After incubation in the darkness at room temperature for 2 h, the absorbance of the reaction mixture is measured at 515 nm using a UV-visible microplate reader.
4. The percentage decrease in the absorbance at 515 nm is recorded for each sample and the percentage of quenching of the DPPH* radical is calculated on the basis of the observed decrease of the radical according to the formula DPPH* inhibition percentage $= [(A_{\mathrm{DPPH}} - A_{\mathrm{Extr}})/A_{\mathrm{DPPH}}] \times 100$ where A_{DPPH} is the absorbance value of the DPPH blank sample (control) and A_{Extr} is the absorbance value in the presence of the extract. The results for the percentage of DPPH scavenged of callus extracts of *P. tenuifila* (Table 5) and *P. setacea* (Table 8) are shown (*see* **Note 15**).

4 Notes

1. Prepare a stock solution of gallic acid by dissolving 100 mg gallic acid in 100 mL deionized distilled water. Firstly dissolve the reagent in 1 mL 80 % methanol and increase the volume to 100 mL by adding deionized distilled water. Store this stock solution in an amber glass flask in a refrigerator to use as fresh working standards. Stock solution should be maintained at room temperature before use. Analyze total phenolics in the callus extract spectrophotometrically by using Folin-Ciocalteu reagent [12].
2. Prepare working standards of 50–1000 μg/mL standard gallic acid solution. Total phenolics is expressed as microgram of gallic acid equivalent per gram of dry extract (μg GAE/g) using a standard curve (0–1000 μg/mL) of gallic acid.
3. Prepare fresh 0.05 mM DPPH* stock solution in 80 % methanol (w/v) and store in the darkness at 4 °C in a flask, covered with aluminum foil. DPPH radical has been widely used to evaluate the free radical scavenging capacity (antioxidant activity) of plant and microbial extracts [13, 14].

4. The media and plant growth regulator solutions are prepared in double-distilled water. Adjustment of pH is done before the addition of Phytagel. Store all the stock solutions at 4 °C until use.
5. To optimize the *Passiflora tenuifila* and *P. setacea* shoot and callus culture protocols, determine the most appropriate concentration of sucrose, glucose, IBA, GA_3, NAA, and 2,4-D. The optimum culture conditions for shoot culture initiation and growth are shown in tables (*see* Tables 2, 3, 6, and 7).
6. *Passiflora tenuifila* seeds fail to germinate in vitro. Seeds can be germinated in soil either at 25 °C or in the greenhouse (natural day light, minimum temperature 16 °C, maximum temperature 40 °C), after immersion for 5 days in 2.5 % GA_3 aqueous solution. The germination rates of the GA_3-treated seeds are 80–90 % within 20 days and mechanical scarification was unnecessary. We have successfully modified the originally proposed *P. alata* seed germination method [7].
7. *P. tenuifila* in vitro shoots are produced in 90–100 % shoot tips originated either from seedling or adult plant. IBA (for seedling shoot tip) and GA_3 (for adult plant shoot tip) are effective promoters of in vitro shoot elongation and growth (*see* Table 3). The multiplication rates varied from 10 to 20 propagules per culture cycle depending on the shoot tip origin. Rooting is achieved in 40–50 % in vitro shoots cultured in IBA-containing medium. After 8 weeks of culture, in vitro shoots formed highest rooting rate (*see* Table 3).
8. *P. tenuifila* and *P. setacea* shoot culture stocks are successfully maintained by subculturing shoot tips and leaf node segments on MS basal medium containing 88.5 mM glucose, 1.25 μM IBA, and 2 g/L Phytagel for 24 months without decreasing the shoot proliferation capacity (*see* Figs. 1 and 3) (8-week subculture time).
9. The best culture conditions for *P. tenuifila* callus induction (Table 2) and growth (Table 4) are shown. NAA is effective for callus induction from shoot tips while 2,4-D is effective for callus induction from immature seeds.
10. Consistent biomass (fresh and dry mass) is produced by the callus culture systems developed for *P. tenuifila* and maximum callus dry mass is obtained from immature seed-derived callus after ca. 45-day subculture cycle (*see* Table 5). *P. setacea* callus growth is affected by explant type and consistent callus biomass (fresh and dry mass) is produced by all callus culture systems developed for *P. setacea*. Callus derived from root and cotyledon segments explants shows the highest biomass accumulation (*see* Table 8). Maximum callus biomass is obtained after a 30–45-day subculture cycle.
11. The subculture of *P. tenuifila* callus cultures originated from in vitro-formed shoot stem segments and from shoot tips, either

from seedling or adult plants, is not as effective as the subculture of immature seed-derived callus. The maintenance of *P. tenuifila* immature seed callus and *P. setacea* seedling explant callus culture stocks is carried out successfully for at least 18 months through periodic subculture (30-day time) on MS basal medium supplemented with 88.5 mM sucrose, 2.5 μM 2,4-D, and 2 mg/L Phytagel (*see* Tables 4 and 8).

12. *P. setacea* in vitro shoots are produced in 100 % explants, originated from seedling shoot tips. Addition of 1.25 μM IBA efficiently promotes in vitro shoot growth (number of shoots, shoot length, and number of nodes/micro shot) and rooting (*see* Table 7). The multiplication rate is ca. 15–18 propagules per culture cycle. Rooting is achieved in 60 % of the in vitro shoots. Approximately 8-week time is required to achieve the highest rooting rate from *P. setacea* in vitro shoots (*see* Table 7).
13. The best culture conditions for *P. setacea* callus induction (Table 6) and growth (Table 8) are shown. Maximum callus induction rate (100 %) was achieved from all explant types.
14. The levels of total phenolic compounds in *P. tenuifila* callus extracts are shown in Table 5. Callus age and explant-type linked differences are observed. The analysis shows that phenolic compound contents are higher in 45-day-old callus originated from immature seeds of *P. tenuifila*. The levels of total phenolic compounds in *P. setacea* callus extracts are shown in Table 8. The phytochemical analysis of callus extracts reveals explant-type linked differences and suggests that phenolic compound contents are superior in callus originated from cotyledon segments.
15. The antioxidant activity of *P. tenuifila* callus extract is explant type and callus age dependent. The highest antioxidant activity is observed in callus originated from immature seeds which show the highest levels of total phenolics (*see* Table 5). The DPPH* test of *P. setacea* callus extracts also reveals explant-type linked differences and the highest antioxidant activity is detected in leaf node callus. Therefore, the maximum level of antioxidant activity is not dependent on the highest content of phenolic compounds (*see* Table 8).

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Chapter 3

Shoot Tip Meristem Cryopreservation of *Hypericum* Species

Katarína Bruňáková and Eva Čellárová

Abstract

Based on our long-standing experience with in vitro culture of *Hypericum perforatum*, a clonal multiplication system and vitrification-based cryopreservation protocols have been applied to several *Hypericum* species: *H. humifusum* L., *H. annulatum* Moris, *H. tomentosum* L., *H. tetrapterum* Fries, *H. pulchrum* L., and *H. rumeliacum* Boiss. The shoot tips were cryopreserved using a uniform procedure that includes pretreatment with abscisic acid (ABA), PVS3 cryoprotection, and direct immersion into the liquid nitrogen (LN). The freezing-tolerant *Hypericum* species were pre-exposed to the cold acclimation conditions performed by a 7-day exposure to 4 °C. The content of naphtodianthrones (hypericins) including hypericin, pseudohypericin, and their protoforms was quantified by HPLC. Ploidy of plants was determined by both flow cytometry of leaf tissue and chromosome counts of root tip meristematic cells. We have shown that the post-thaw recovery rate of the shoot tips, pretreated with 0.076 μM ABA for 7 days at room temperature, led to the post-cryogenic survival from 5 % in *H. tomentosum* to 21 % in *H. annulatum*. As compared to the untreated (control) plants, the content of hypericins in plants regenerated after cryopreservation remained unchanged or decreased in *H. perforatum*, *H. humifusum*, *H. annulatum*, *H. tomentosum*, *H. tetrapterum*, and *H. rumeliacum*. However, the pre-exposition of the freezing-tolerant *H. perforatum* to cold acclimation prior to excision of the shoot tips has improved the post-thaw recovery to 45 % and resulted in threefold increase of the total hypericin content.

Key words Micropropagation, Vitrification, LN, Ploidy, Flow cytometry, HPLC, Naphtodianthrones, Emodin

1 Introduction

The remarkably diverse genus *Hypericum* (Hypericaceae) comprises nearly 500 species inhabiting all temperate regions worldwide and higher altitudes of the tropical mountains [1]. Due to a long history of the use in herbal medicine, especially for the treatment of wounds, burns, and psychological disorders, *Hypericum perforatum* L. (St. John's wort) is the best studied and commercially used species. Among several classes of bioactive secondary metabolites including naphtodianthrones hypericin and pseudohypericin (hypericins), phloroglucinol derivatives hyperforin and adhyperforin, flavonol glycosides rutin and hyperosid, biflavonoids,

S. Mohan Jain (ed.), *Protocols for In Vitro Cultures and Secondary Metabolite Analysis of Aromatic and Medicinal Plants, Second Edition*, Methods in Molecular Biology, vol. 1391, DOI 10.1007/978-1-4939-3332-7_3,

and essential oils [2], hypericins attract a special interest, namely due to anticancer [3], antiviral [4], antimicrobial [5], and anti-inflammatory activities [6].

Although the biosynthesis of hypericin is not fully understood, the experimental evidence for a polyketide synthase-mediated pathway to emodin, which is considered to be the precursor of hypericin, has been established [7]. In *herba Hyperici*, the naphtodianthrones exist in various forms, including the protopigments: under the exposure to a visible light, the protohypericin and protopseudohypericin are converted into hypericin and pseudohypericin, respectively [8]. The content of these compounds in *H. perforatum* is low, ranging from 0.05 to 0.30 % [9]. Within the species, the amount of naphtodianthrones varies, depending on the genotype, developmental stage, and plant organs, as well as the environmental conditions, e.g., the geographic area, seasonal variation, altitude, light conditions, nitrogen supplementation, water and carbon availability, light intensity, and biotic stressors, such as the metal contamination, pathogens, and herbivores [10].

To study the plant-environment interactions, an efficient in vitro clonal multiplication system has been established for *H. perforatum* [11]. Among the genetic components of the variability, both somaclonal variation and ploidy level were shown to impact on hypericin quantity in the model plant *H. perforatum* [12, 13].

To preserve the original qualities of the authentic plant individuals, the cryopreservation is often used for a long-term storage. Although *H. perforatum* should be considered as a model representative of the genus, there are many species attracting the attention due to their unique phytochemical profiles. A uniform cryoconservation protocol comprising the ABA pretreatment, PVS3 cryoprotection, and direct immersion of the isolated shoot tips into the LN has been applied to several other *Hypericum* species of different origin and distribution [14]. It was found that the genetically predetermined freezing tolerance of the species may function as an indicator for a successful cryopreservation. As shown by our recent observation, the pre-exposition of the intact plants of the freezing-tolerant *H. perforatum* to the cold acclimation, performed by a 7-day exposure to 4 °C prior to the PVS3 cryoprotection of the excised shoot tips, has improved the recovery rate nearly threefold, reaching the level of 45 % (Bruňáková, unpubl.).

In addition, a possible stimulatory effect of a "cold shock," a subfreezing temperature of –4 °C, for the elicitation of the naphtodianthrone biosynthesis in *H. perforatum* has been found [15]. The application of the cold pre-exposure with or without the treatment of the whole plants with high nonphysiological concentration of ABA during the pre-culture period resulted in a substantial, almost threefold increase in the hypericin content recorded in *H. perforatum* and *H. rumeliacum* (Bruňáková, unpubl.).

2 Materials

The experimental plant material includes *Hypericum perforatum* L., *H. humifusum* L., *H. annulatum* Moris, *H. tomentosum* L., *H. tetrapterum* Fries, *H. pulchrum* L., and *H. rumeliacum* Boiss.

2.1 Initiation and Long-Term Storage of Hypericum spp.

2.1.1 Culture Media and Cryoprotectant Mixtures

1. The basal solid MS medium contains Murashige-Skoog's salt mixture [16], Gamborg's B5 vitamins [17], supplemented with sucrose, myoinositol, and glycine.
2. Plant growth regulators—6-benzyladenine (BA), abscisic acid (ABA).
3. Loading solution (LS) [18].
4. PVS3 cryoprotectant mixture [18].
5. Syringe filter with 0.2 μM pore size.
6. Magnetic stirrer.

2.1.2 Initiation and Micropropagation of Hypericum spp.

1. Silver nitrate ($AgNO_3$).
2. Sterile double-distilled water.
3. Test tube with polypropylene or glass stopper.
4. Culture vessels.
5. Sterile Petri dishes, tweezers.
6. Laminar flow box.

2.1.3 Cryopreservation of Hypericum spp.

1. Sterile 25, 50, and 100 mL Erlenmeyer flasks, cryovials, Petri dishes, and filter paper.
2. Grinded ice.
3. Thermostatic cabinet with an adjustable temperature in the range from 22 to 4 °C, 16/8-h (day/night) photoperiod, and 35 μmol/m^2/s of PAR.
4. Orbital shaker.
5. Water bath with a lift-up Makrolon cover.
6. Liquid nitrogen (LN) stored in a cryogenic Dewar flask.

2.2 Chromosome Counts and Flow Cytometry Screen of Ploidy

2.2.1 Cytogenetic Technique for Chromosome Number Determination

1. 8-Hydroxyquinoline.
2. Ethanol, glacial acetic acid, hydrogen chloride (HCl).
3. Giemsa solution.
4. Synthetic resin—Solacryl.
5. 25 mL Erlenmeyer flask, beakers.
6. Microscopic slides, cover slips, cellophane.
7. Water bath.
8. Light microscope (Olympus BX51) equipped with a digital camera.

2.2.2 Flow Cytometry for Ploidy Determination

1. General-purpose buffer (GPB) [19].
2. Propidium iodide (PI).
3. RNase.
4. Syringe membrane filter with 0.22 μm pore size, 42 μm nylon filters, tubes, Petri dishes.
5. Grinded ice.
6. Flow cytometer CyFlow ML equipped with a green laser (532 nm, 150 mV), and FloMAX 2.7 software.

2.3 Determination of Hypericins in Hypericum spp.

2.3.1 Chemicals and Standards

1. HPLC-grade methanol and acetone, Pro-UV ethanol.
2. Acetonitrile (ACN).
3. Trifluoroacetic acid (TFA).
4. Hypericin and emodin standards.

2.3.2 Material and Equipment

1. Forced-air oven.
2. Plastic storage tubes with a cap, 2 mL Eppendorf tubes.
3. Milli-Q Reference Water Purification System.
4. Tissue-lyser.
5. Sonicator water bath.
6. Agilent 1260 HPLC system equipped with a DAD detector. The stationary phase represents a reversed-phase nonpolar Poroshell C18 column (3.0×50 mm i.d., 2.7 μm particle size) at 40 °C.

3 Methods

3.1 Initiation and Long-Term Storage of Hypericum spp.

3.1.1 Culture Media and Cryoprotectant Mixture Preparation

1. Preparation of 1 L MS basal medium: Weigh 4.4 g Murashige-Skoog's salt mixture [16] containing Gamborg's B5 vitamins [17], 30 g sucrose, 100 mg myoinositol, and 2 mg glycine. Dissolve in 500 mL double-distilled water by adding in an Erlenmeyer flask, using a magnetic stirrer.
2. Add 6 g agar in 400 mL double-distilled water and boil in a microwave.
3. Add hot agar solution to the medium and raise the volume to 1 L by adding double-distilled water.
4. Adjust pH to 5.6 with NaOH and HCl before autoclaving at 121 °C and 105 kPa for 15 min.
5. Prepare 1 L MS medium containing 2.2 μM BA by adding 0.5 mg stock solution (*see* **Note 1**) before autoclaving.
6. Prepare MS liquid medium containing filter-sterilized ABA by adding filter-sterilized ABA stock solution to the autoclaved medium cooled at 40 °C (*see* **Note 2**).

7. Prepare loading solution (LS) containing 2.0 M glycerol and 0.4 M sucrose [18] by adding 184.2 g glycerol and 106.9 g sucrose to the liquid MS basal medium. The pH is adjusted to 5.8 before autoclaving.
8. Prepare 100 mL PVS3 cryoprotectant mixture [18] by adding 50 g sucrose and 50 g glycerol into 100 mL Erlenmeyer flask and raise volume up to 100 mL with double-distilled water. Stir this mixture to dissolve at 90–100 °C on a magnetic stirrer and autoclave.

3.1.2 Initiation and Micropropagation of Hypericum spp.

1. Place *Hypericum* seeds, free of dust and solid particles, into the test tube filled with 1 % (w/v) $AgNO_3$ solution.
2. Seal test tubes with the stopper and agitate gently by tipping over for 15 min.
3. Remove $AgNO_3$ solution and rinse seeds three to four times with the sterile distilled water.
4. Place sterile seeds onto the surface of solid basal MS medium for germination.
5. After 4 weeks of germination, cut shoots into pieces containing 4–6 foliage leaves.
6. Transfer shoot cuttings to solid basal MS medium containing 2.2 μM BA. After 4 weeks, multiple shoots with minimal callus are formed [11] (*see* **Note 3**).
7. For rooting, culture the regenerated shoots or shoot cuttings on the solid basal MS medium without any plant growth regulators [11].
8. Keep all cultures at standard conditions as follows: temperature 22 ± 2 °C, 40 % relative humidity, 16/8-h (day/night) photoperiod, and 35 μmol/m²/s PAR (photosynthetically active radiation).
9. Subculture shoots at 28-day interval.

3.1.3 Cryopreservation

Shoot tips, 5–10 mm long, consisting of one apical and one pair of axillary meristematic domes with surrounding leaf primordia, are excised from the *Hypericum* donor plants, growing in vitro on the solid basal MS media (*see* **Note 4**) at the standard growing conditions.

Pretreatment

The isolated shoot tips are put into the 50 mL Erlenmeyer flasks filled with 10 mL liquid MS medium containing 2.2 μM BA (*see* **Note 4**) and 0.076 μM ABA and pre-cultured for 10 days on the orbital shaker at 90 rpm at 40 % relative humidity, 16/8-h (day/night) photoperiod, and 35 μmol/m²/s of PAR (*see* **Notes 5** and **6**).

Cryopreservation Procedure and Cooling

1. The excised shoot tips (10–12 per sample) are put into 25 mL Erlenmeyer flask filled with 10 mL LS and are continuously shaken at 120 rpm for 20 min at the room temperature.
2. For subsequent dehydration, shoot tips are placed into 1.8 mL cryovials (10–12 per one cryovial), filled with 1 mL PVS3, and equilibrated on ice for 180 min (*see* **Note 7**).
3. Immediately, the cryovials are plunged directly into the LN and stored for at least 24 h (*see* **Note 8**).

Post-cryogenic Conditions

1. The cryovials are thawed rapidly in a water bath at 42 °C for 2 min.
2. To remove the vitrification solution, the shoot tips are transferred from the cryovials to 25 mL Erlenmeyer flasks filled with 10 mL liquid MS medium containing 1.2 M sucrose (*see* **Note 9**) and shaken for 20 min at 90 rpm at laboratory temperature.
3. Subsequently, the shoot tips are transferred to a sterile Petri dish and carefully rinsed with MS liquid medium followed by moderate drying on the filter paper moistened with the MS medium (*see* **Note 10**).
4. The shoot tips are transferred onto a semisolid regeneration MS medium containing 2.2 μM BA and cultured for 14 days in the darkness, then at a 10 μmol/m^2/s of PAR for 7 days, followed by the exposure to 35 μmol/m^2/s of PAR.
5. The shoot tip recovery rate is observed continually during 12-week interval after the rewarming of the shoot tips (Fig. 1). The explants with regenerating shoots are scored. The recovery rate is expressed in % of the total number of the cryopreserved shoots.

3.2 Chromosome Counts and Flow Cytometry Screen of Ploidy in Several Hypericum Species

3.2.1 Cytogenetic Technique for Chromosome Number Determination

1. *Sample preparation*. At least five root tips per plant containing the mitotically active meristematic tissues are used (*see* **Note 11**). When plants are growing on the solid media, the medium residues are gently removed by rinsing the excised root tips in the water (*see* **Note 12**).
2. *Pretreatment*. To increase the proportion of cells at metaphase phase, the chemical pretreatment is used: the root tips, 5–10 mm, are cut with a razor into 25 mL Erlenmeyer flask filled with 10 mL 2 mM 8-hydroxyquinoline (*see* **Note 13**) and kept for 4 h at room temperature. The pretreated root tips are rinsed under a tap water for 10 min and subsequently washed with distilled water.
3. *Fixation*. To preserve the natural state of the cells and tissue constituents, a mixture of 96 % ethanol and glacial acetic acid, ratio 3:1, is used. The objects are fixed for 16 h at room temperature. Until the next processing, the material, stored in 70 % ethanol, can be kept in the refrigerator.
4. *Washing*. To elute the fixative and soften the tissue, the fixed material is rinsed two to three times in a glass beaker filled with the distilled water at room temperature.

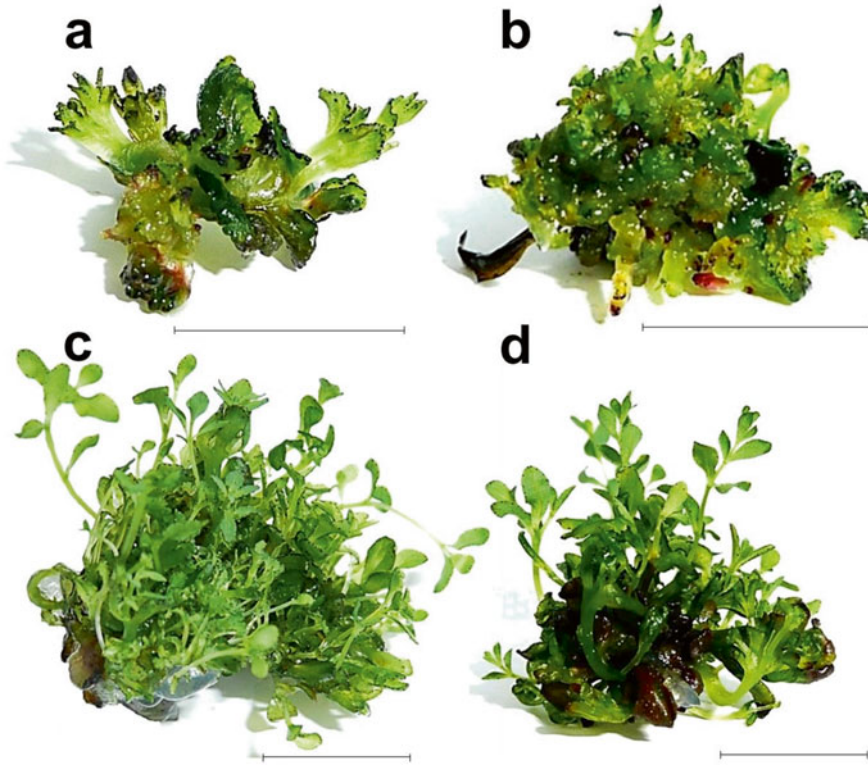

Fig. 1 The initiation of *Hypericum perforatum* multiple shoot formation from the cryopreserved shoot tips regenerated on the MS media containing 2.2 μM BA for 28 days (**a**, **b**) and after a 28-day subculture on the basal MS media (**c**, **d**). Shoot tips were excised from plants pre-cultivated in liquid basal MS media supplemented with 76 μM ABA (**a**, **c**) or cold acclimated for 7 days at 4 °C without ABA pretreatment (**b**, **d**). Bars represent 10 mm. Photo: K. Bruňáková

5. *Acid digestion*. To separate the cells in the tissue, pectic middle lamella must be destroyed. The root tips are incubated for 6 min in 1 M HCl in the water bath at 60 °C. The hydrolysis is completed in the cold 1 M HCl followed by rinsing of the root tips two to three times in the distilled water.
6. *Slide preparation*. The root tips are cut in a drop of 45 % acetic acid (v/v) and the slides are prepared by a squash and smear technique using the cellophane [20] (*see* **Note 14**).
7. *Staining*. After stripping of the cellophane, the slides are stained with Giemsa solution (*see* **Note 15**) for 30 min in the dark, air-dried, and mounted in a synthetic resin before being covered with a cover slip.
8. The mitotic metaphases are observed and captured under ×1000 magnification. The chromosome number is determined by counting of the metaphase chromosomes (Fig. 2). At least five root tips per plant are scored for the chromosome counts.

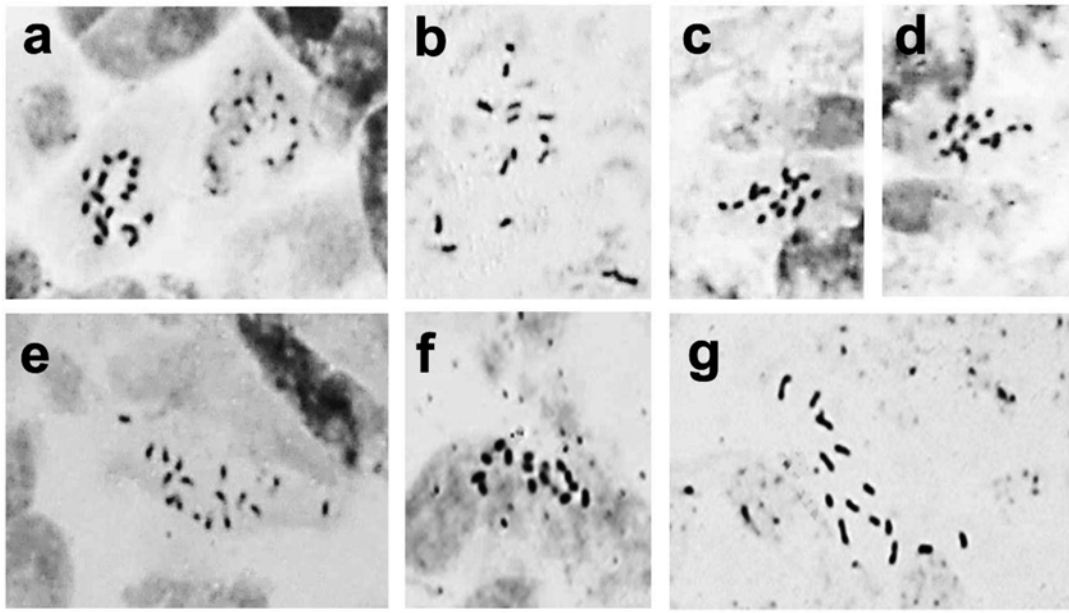

Fig. 2 Metaphase plates of root tip meristematic cells of *Hypericum* representatives including (**a**) *H. perforatum* ($2n = 2x = 16$), (**b**) *H. humifusum* ($2n = 2x = 16$), (**c**) *H. annulatum* ($2n = 2x = 16$), (**d**) *H. pulchrum* ($2n = 2x = 18$), (**e**) *H. tomentosum* ($2n = 2x = 16$), (**f**) *H. tetrapterum* ($2n = 2x = 16$), and (**g**) *H. rumeliacum* ($2n = 2x = 16$). Photo: J. Koperdáková, M. Skyba, Z. Lacková

3.2.2 Flow Cytometry to the Determination of Ploidy

Preparation of the nuclear suspensions:

1. Five to ten young leaves per plant, equivalent to 40–50 mg, are excised and placed into a 60 mm Petri dish (*see* **Note 16**).
2. 1 mL GPB buffer (*see* **Note 17**) is added and the tissue is chopped using a new razor blade for approximately 60 s (≈100 chops per sample) to homogenize the tissues and release the nuclei.
3. The leaf tissues from both the sample and the internal reference DNA standard (*see* **Note 18**) should be chopped simultaneously.
4. To remove the large debris, the resulting homogenate is filtered through a 42 μm nylon filter into a tube and subsequently the nuclear suspension is stained with 50 μL PI stock solution (*see* **Note 19**).
5. For preventing the staining of a double-stranded RNA, 50 μL RNase stock solution (*see* **Note 20**) is added to the sample immediately.
6. The samples are incubated on ice and analyzed within 10 min.

Flow cytometry:

1. Green argon laser at 532 nm wavelength is used as a light source.
2. Optical long-pass filter is used to selectively transmit emission wavelength of PI.

3. The fluorescence intensity of PI is detected and projected on a linear scale.
4. The flow of at least 5000 nuclei is measured in each sample.
5. The ploidy level (*see* **Note 21**) is calculated according to the formula *the ploidy of the sample = the mean fluorescence intensity of the sample/the mean fluorescence intensity of the reference sample*.

3.3 Determination of Hypericins in Hypericum spp.

1. To ensure the representative specimen for analytical testing, all the plants should be of the same ploidy level (*see* **Note 22**).
2. Each sample contains shoots from at least four cultivation vessels.

3.3.1 Sample Preparation

3. Plant material is left to dry naturally at room temperature. The drying is finished in a forced-air oven at 40 °C for 2 h (*see* **Note 23**).
4. Dried plant material is homogenized at a tissue-lyser (QIAGEN, Germany). Before further processing, the pulverized sample is transferred to a plastic tube of an appropriate size, closed, and stored at 4 °C in the darkness.

3.3.2 Extraction Procedure

1. Weigh 50 mg of each samples of the dried and homogenized plant material in 2 mL Eppendorf tubes, suspend in 1.5 mL ethanol:methanol:acetone (1:1:1, by volume) mixture, and sonicate for 60 min at 25 °C (*see* **Note 24**).
2. Samples are centrifuged at 21,250 × *g* and 20 °C for 30 min.
3. The supernatant is transferred into the dark HPLC vials for subsequent analyses (*see* **Note 25**).

3.3.3 Analytical HPLC Method

1. Six-step solvent gradient is applied over a 17-min period at a flow rate of 1.3 mL/min (*see* **Note 26**). The mobile phase is consisted of phase A (10 % (v/v) ACN; pH = 2.7) (*see* **Note 27**) and phase B (100 % ACN) is started from 80:20 (A:B) to 20:80 in 8.5 min, then is changed to 0:100 in 1 min, held at this composition for 6 min, returned to 80:20 in 1.2 min, and held for 0.3 min. The total run time is 17 min and the equilibration time between runs is 3 min giving a total analysis time of 20 min.
2. The calibration curves are prepared from a methanol dilution series of the reference standard solutions (*see* **Note 28**): seven different dilutions in the range from 0.1 to 200 μg/mL of hypericin and from 0.1 to 50 μg/mL of emodin are prepared. The same volume (5 μL) of each dilution is injected in a triplicate and averaged. The calibration curve is constructed by plotting the peak area versus the corresponding concentration of the standard.
3. The injection volume of the sample is 5 μL.
4. The identification of hypericins and emodin is performed by an HPLC-DAD analysis and comparing the retention times of the

peaks in the extracts with those of the reference standards. To avoid any misidentification, the co-chromatography of the sample with the small amounts of standards is used. The purity of the peaks is checked by comparing the absorption spectra of each peak with those of the reference compounds (*see* **Note 29**; Fig. 3e).

5. The peaks for hypericin, pseudohypericin, and their protoforms are detected at 590 nm (Fig. 3a–d); the identification of emodin is performed at 440 nm.
6. The content of "total hypericins" and emodin (μg/mg DW) in the samples is calculated according to the calibration curve of the respective standards (*see* **Note 30**).

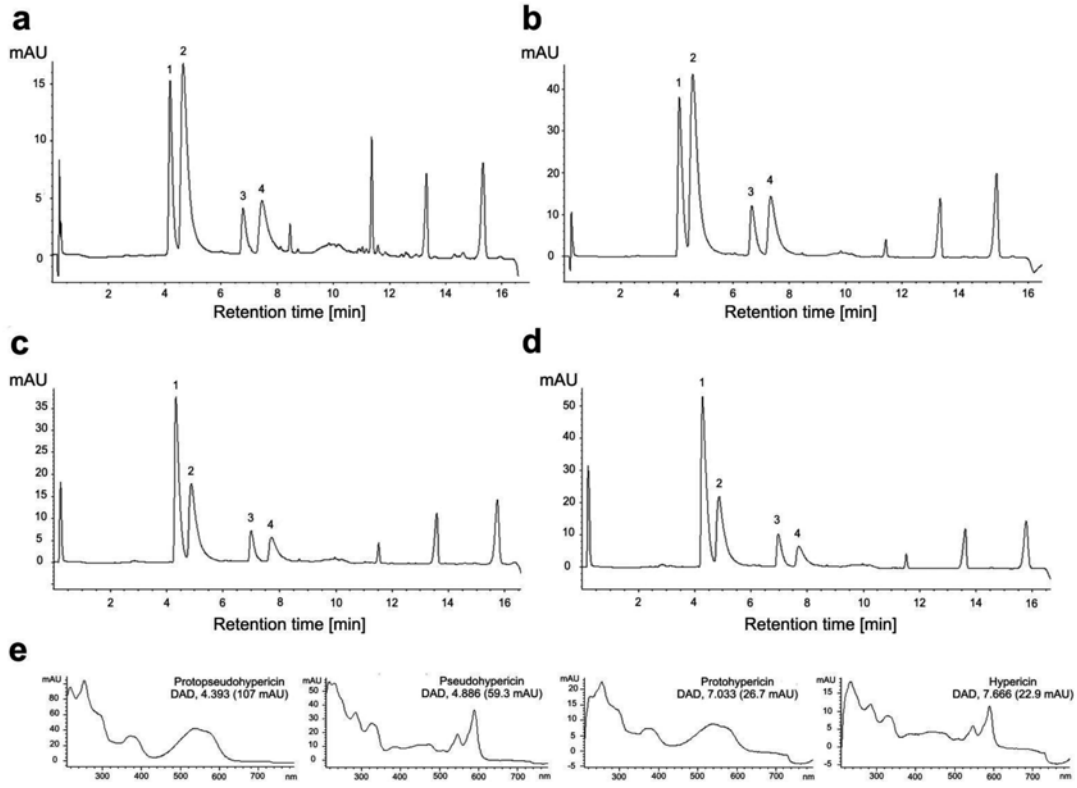

Fig. 3 The elicitation effect of the cryogenic procedure on the naphtodianthrone content in two representatives of the genus *Hypericum* after cryopreservation—the HPLC chromatograms of *H. perforatum* (**a**, **b**) and *H. rumeliacum* (**c**, **d**) extracts obtained from the untreated (control) in vitro plants (**a**, **c**) and shoots regenerated from the shoot tip meristems excised from plants, which were pre-cultured in the liquid media supplemented with 76 μM ABA for a 7-day period followed by a 7-day exposure to 4 °C; the isolated shoot tips were dehydrated in LS and PVS3, and cryopreserved by a direct immersion to LN. The naphtodianthrones protopseudohypericin (*1*), pseudohypericin (*2*), protohypericin (*3*), and hypericin (*4*) were detected at 590 nm. Hypericins are well distinguished by their characteristic UV spectra (**e**) at the retention times of 4.2 min for protopseudohypericin, 4.6 min for pseudohypericin, 6.8 min for protohypericin, and 7.4 min for hypericin

4 Notes

1. Preparation of 2.2 μM BA stock solution: Dissolve 5 mg BA in 1 mL 1 N NaOH, raise volume up to 5 mL with distilled water in 5 mL volumetric flask, and keep in the refrigerator.
2. As an exogenous source of ABA, the racemic (±)-ABA which is a 1:1 mixture of (+) or S-ABA (natural form) and (−) or R-ABA (unnatural or analogue form) is used. The 76 μM stock solution is prepared by dissolving 20 mg ABA in 1 mL 1 N NaOH or 1 N KOH in a 5 mL volumetric flask and raise volume up to 5 mL by adding distilled water and filter-sterilize. To dissolve ABA in NaOH or KOH, several minutes of vigorous shaking are required. Storing of the aqueous ABA for more than 1 day is not recommended. For the preparation of 1 L liquid MS medium containing 0.076 and 76 μM ABA, add 5 μL and 5 mL of the stock solutions. ABA is also soluble in the organic solvents, e.g., methanol, ethanol, acetone, aether, and chloroform. The solubility of ABA in these solvents is approximately 20 mg/mL. However, the use of ethanol as a solvent in the ABA stock solution decreased freezing tolerance [21].
3. The efficient micropropagation system established for *H. perforatum* [11] is applicable to some extent or with modification to other *Hypericum* species involved in this study.
4. Alternatively, the clusters of multiple shoots regenerating from the explants cultured on the solid MS media supplemented with 2.2 μM BA can be used as the source of the shoot tips. Considering the fact that the long-term exposure of plants to BA negatively impairs the regeneration capability of cryopreserved *H. perforatum* meristems, the shoots originated from the clusters should be cultivated on the basal solid MS media for at least 14 days before the excision of the apices [22]. While the long-term culture of plants on media supplemented with BA may cause delay of the protective effect of ABA used as a cryoprotectant that is reflected by a decreased rate of regeneration of cryopreserved *H. perforatum* meristems, the presence of the growth regulator in the media during the short-term pretreatment of the excised shoot tips prior to cryopreservation in LN is necessary for the improvement of the recovery rate [22].
5. For the improvement of freezing tolerance, both method used and ABA concentration should be considered. ABA applied to roots is easily absorbed and translocated [23]. In *H. perforatum* and *H. canariense*, no increase in the freezing tolerance was seen after 7-day cultivation in the liquid basal MS media supplemented with 0.076 μM ABA at 22 ± 2 °C (Bruňáková, unpubl.). Alternatively, the intact plants with well-differentiated roots are transferred into 100 mL Erlenmeyer flasks containing

30 mL liquid basal MS media supplemented with 76 μM ABA and cultivated for 7 days at 90 rpm at 40 % relative humidity, 16/8-h (day/night) photoperiod, and 35 μmol/m²/s of PAR (Fig. 1a, c).

6. As an alternative to ABA pre-conditioning, cold acclimation can be used for freezing-tolerant *Hypericum* species. The sealed culture vessels with 14- to 21-day-old plantlets, cultured on basal MS media, are transferred to the cooling chamber with temperature adjusted to 4 °C for 7 days, 40 % relative humidity, 16/8-h (day/night) photoperiod, and 35 μmol/m²/s of PAR [14] (Fig. 1b, d). The exposure of plants to gradually decreasing temperature up to 4 °C during 21 days at the rate of 1 °C/day is also applicable (Fig. 1b, d). As compared with the post-thaw recovery of the shoot tips that were precultured in the liquid MS media supplemented with 0.076 μM ABA at room temperature during a 7-day period, a 7-day exposure of the intact plants of the freezing-tolerant *H. perforatum* to 4 °C at the standard light conditions resulted in a threefold increase in the recovery rate (Bruňáková, unpubl.). Based on our preliminary observation, the combination of the pretreatment of the whole plants in the liquid media amended with 76 μM ABA at room temperature, followed by the cold acclimation, could improve the post-cryogenic recovery of the freezing-tolerant *Hypericum* species (Bruňáková, unpubl.).
7. Based on the differential scanning calorimetry (DSC) of *H. perforatum* shoot tips, 180 min of PVS3 exposure time was optimal to accomplish the highest regeneration rate within 59.3–71.3 % [24].
8. Besides, on vaporization, LN displaces oxygen and may cause asphyxiation. Therefore, the cryogenic liquid must be handled and stored in well-aired areas. The vapors must not be inhaled.
9. MS medium containing 1.2 M sucrose is prepared by adding 380.8 g sucrose to the basal MS medium. The pH of liquid MS medium is adjusted to 5.6 before autoclaving.
10. Meristems can easily dry and damage by the airflow in the laminar flow box. Therefore, they should be handled as rapidly as possible. In addition, the rapid temperature changes during the process of freezing and re-warming result in producing reactive oxygen species (ROS). To increase the post-cryogenic survival, the photooxidative stress accompanying the cryopreservation procedure should be minimized. Therefore, switching off the light in the flow box when manipulating the cryo-samples immediately after thawing is recommended.
11. To prepare the slides of metaphase chromosomes of *Hypericum* spp., the procedure developed for a karyotype study in *H. perforatum*, published by [25], is used.

12. The root tips must be excised from the actively growing and healthy plants without any signs of wilting or any other damage. The mitotic index is the highest when root tips are isolated in the morning. Prior to processing, the excised root tips should be held in an ice water.
13. The aqueous solution of 8-hydroxyquinoline can be stored at room temperature for at least 1 year [26]. The chemical pretreatment of the plant tissue must be used with caution as its action is to arrest cell division in the living tissue.
14. Cover root tip, size 1–2 mm, in a drop of acetic acid with a wet square of cellophane (20 × 20 mm), soaked in distilled water. The specimen is taped gently with a rubber tube stopper and a tube is rolled across the cellophane. The extent of squashing should be controlled by observing under a microscope. The slide is blotted with a filter paper. The piece of cellophane can be carefully removed by a moderate warming of slides.
15. The Giemsa solution is diluted at a ratio 1:9 (v/v) with a phosphate buffer. 100 mL phosphate buffer consists of 61.2 mL solution A and 38.8 mL solution B with a pH adjusted to 6.8. To prepare 100 mL aqueous A and B solutions, dissolve 2.4 g $Na_2HPO_4 \cdot 12H_2O$ and 1.0 g KH_2PO_4, respectively. The solution remains stable for several months, stored in the refrigerator.
16. The procedure for isolating the nuclei was adopted from that published by [19].
17. The isolation buffer, a general-purpose buffer (GPB) prepared according to [19], consists of 0.5 mM spermine · 4HCl, 30 mM sodium citrate, 20 mM 4-morpholine propane sulfonate (MOPS), 80 mM KCl, 20 mM NaCl, and 0.5 % (v/v) Triton X-100, pH adjusted to 7.0.
18. As an internal standard, the young leaves of the in vitro-cultured diploid *H. perforatum* L. can be used. The ploidy level of the reference *H. perforatum* L. plant ($2n = 2x = 16$) was verified cytogenetically: Five root tips were studied and at least ten meristematic cells in the metaphase per root tip were scored for the chromosome number.
19. The stock solution containing 100 μg/mL PI is prepared by dissolving 1 mg PI in 10 mL distilled water, filtered through a membrane filter with 0.22 μm pore size, and stored at −20 °C.
20. The stock solution containing 1 mg/mL RNase is prepared by dissolving 10 mg RNase in 10 mL distilled water, filtered through a 0.22 μm membrane filter, incubated at 90 °C for 15 min, and stored at −20 °C.
21. The negative correlation between the ploidy and the content of naphtodianthrones seems to be due to an increasing

biomass in polyploids but genetically conserved number of multicellular nodules, which are the hypericin-accumulating structures in the leaves, flowers, and stems regardless of ploidy [12]. Therefore, the determination of the chromosome number followed by the flow cytometric evaluation of the ploidy level should be an inevitable part of the experimental design aimed at the assessment of the secondary metabolite production in the genus *Hypericum*.

22. All the plants analyzed for the content of hypericins were diploid: $2n = 2x = 14$ or for *H. rumeliacum*, $2n = 2x = 16$ for *H. perforatum*, *H. humifusum*, *H. annulatum*, *H. tomentosum*, and *H. tetrapterum*, and $2n = 2x = 18$ for *H. pulchrum* (Fig. 2).
23. The lyophilization is also a suitable method for drying the plant material.
24. The extraction procedure is performed according to [27].
25. Hypericin is a lipophilic molecule sparingly soluble in nonpolar organic solvents, aqueous solutions, and oil. The unstable behavior of hypericin and pseudohypericin that are sensitive to oxidation is caused by the exposure to the light and air. Therefore, isolation and handling of the extracts should be carried out as fast as possible. It is recommended storing the extracts at most for 2 h before analyses. The extracts should be kept in the dark.
26. The HPLC analytical method is performed according to *(30)* with minor modifications.
27. The pH of the mobile phase consisted of 10 % (v/v) ACN and is equilibrated to pH = 2.7 using 25 % trifluoroacetic acid (TFA).
28. Add 1 mg hypericin powder to the 5 mL volumetric flask and raise the volume to 5 mL with methanol preparing the hypericin stock solution. Similarly, the stock solution containing 50 μg/mL emodin is prepared by dissolving 1 mg emodin in 20 mL methanol. Both standard solutions should be stored at 4 °C in the dark.
29. Hypericins are well distinguished by their characteristic UV spectra [27] at the retention time 4.2 min for protopseudohypericin, 4.6 min for pseudohypericin, 6.8 min for protohypericin, 7.4 min for hypericin, and 4.5 min for emodin. The modification of the HPLC method used for analyzing naphtodianthrones in *H. perforatum* separates hypericin from pseudohypericin; the chromatograms also show the fully resolved peaks for protopseudohypericin and protohypericin (Fig. 3). The method is applicable to assess the hypericin content in untreated (control) plants as well as regenerants after cryopreservation in all *Hypericum* species used in this study. The

nearly threefold increase in hypericins in shoots regenerated from the cryopreserved shoot tip meristems in *H. perforatum* (Fig. 3a, b) and *H. rumeliacum* (Fig. 3c, d) is shown.

30. As pseudohypericin presents the same UV spectrum as hypericin, this compound can be quantified according to the hypericin calibration curve which is also suitable for the assessment of the amount of protopseudohypericin and protohypericin [27].

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Chapter 4

Protocols for Biotechnological Interventions in Improvement of Vanilla (*Vanilla planifolia* Andrews.)

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Abstract

Vanilla (*Vanilla planifolia* Andrews (*syn. V. fragrans* Salisb.), a native of Central America, is the primary source of natural vanillin and plays a major role in the global economy. The gene pool of vanilla is threatened by deforestation and overcollection that has resulted in disappearance of natural habitats and wild species. Continuous vegetative propagation and lack of natural seed set and sufficient variations in the gene pool hamper crop improvement programs. In vitro techniques, one of the key tools of plant biotechnology, can be employed for overcoming specific problems, viz. production of disease-free clones, inducing somaclonal variations, developing hybrids, gene pool conservation, incorporating desired traits by distant hybridization, genetic engineering, etc. However, realization of these objectives necessitates standardization of protocols. This chapter describes the various protocols optimized for crop improvement in *Vanilla* species.

Key words Artificial seeds, Cryopreservation, Embryo rescue, In vitro conservation, Micropropagation, Plant regeneration, Seed culture, Somaclones, Vanilla

Abbreviations

BA	Benzyl adenine
IBA	Indole-3-butyric acid
Kin	Kinetin
NAA	α-Naphthalene acetic acid

1 Introduction

[*Vanilla planifolia* Andrews (*syn. V. fragrans* Salisb.)], the fermented and cured fruit of the orchid *V. planifolia* used extensively in the flavor industry, is an important crop in the flavor industry and a commercially important orchid spice. It is also used in perfumery and to some extent in medicine as a nerve stimulant. The fragrance and flavor of vanilla beans are mainly due to vanillin.

S. Mohan Jain (ed.), *Protocols for In Vitro Cultures and Secondary Metabolite Analysis of Aromatic and Medicinal Plants, Second Edition*, Methods in Molecular Biology, vol. 1391, DOI 10.1007/978-1-4939-3332-7_4,

The highly purified vanillin is widely used as a chemical intermediate in the synthesis of numerous pharmaceutical products [1]. Vanilla is by gram weight the world's most valuable spice crop and is widely employed as a flavor ingredient.

The vanilla fruit is special for many reasons, including that it is one of the few fruit crops that combines a natural image with a high socioeconomic value due to its traditional and sustainable mode of production and process. Various biotechnological approaches like micropropagation, somaclonal variation, in vitro conservation, synseed technology, protoplast technology, production of flavor and coloring components, and development of novel transgenics have great significance in conservation, utilization, and increasing the production and productivity of spices. Breeding of improved and new vanilla cultivars with desirable traits like producing higher quality fruits consistently that are resistant to biotic (fungi, viruses) and abiotic stress (drought and heat) will significantly increase the income that small-holder vanilla farmers can receive for their harvest. These changes are critical for survival of vanilla cultivation and the industry, especially with the threat of a *Fusarium* pandemic that is destroying vanilla vines and jeopardizing world vanilla production, and in the face of the accelerated threat of global warming that has already affected the timing of *Vanilla planifolia* flowering and impacted successful fertilization [2].

Although cultivated throughout the tropics, its natural populations in South Mexico—the most critical sources of novel genetic diversity—are on the verge of disappearance [3]. The rapid destruction of vanilla natural habitats has compelled to search for alternate methods of conservation, multiplication, and genetic variability. In addition, a great threat of losing vanilla plantations is looming due to common diseases of fungal origin, viz. foot rot and wilting caused by *Phytophthora meadii*, *Fusarium oxysporum*, *Calospora vanillae*, and *Sclerotium* rot, and lack of genetic diversity in the available gene pool [4].

Continuous vegetative propagation, lack of natural seed set and sufficient variations, diseases, and absence of disease-tolerant species in the gene pool hamper crop improvement programs. In vitro culture is an important tool of plant biotechnology, which can be employed for overcoming specific problems, viz. production of disease-free clones, inducing somaclonal variations, developing hybrids, gene pool conservation, in vitro gene banks, incorporating desired traits into cultivated vanilla, etc. These objectives can be accomplished with the standardization of simple protocols. This chapter describes the various protocols optimized for crop improvement in *Vanilla* species [3, 5]. Cryopreservation of pollen will help to overcome the availability of viable pollen due to asynchronous flowering for interspecific hybridization while conserving the haploid gene pool. This protocol describes an efficient plant regeneration system, a prerequisite to exploiting somaclonal

variation, in vitro selection, development of novel genotypes through transgenic pathway, etc., which was extended to micropropagate diverse Vanilla species overcoming the species/clonal specific nature of various protocols.

2 Materials

2.1 In Vitro Propagation

2.1.1 Explant Materials

Appropriate tissues are chosen as explants, from field-grown vines of different *Vanilla* species, viz. *Vanilla planifolia* (Fig. 1), *V. andamanica*, *V. aphylla*, and *V. pilifera*, for achieving desired objectives. Nodal segments and shoot tips for micropropagation, pollen for hybridization, seeds for generating segregating progenies, and interspecific hybrids were chosen.

2.2 Glassware

Borosilicate conical flasks (500 mL), glass bottles (250 mL), culture tubes (22 cm × 3.5 cm), and 250 mL conical flasks.

2.3 Growth Regulators

Auxins—α-naphthalene acetic acid (NAA), indole-3-butyric acid (IBA) 0–3.0 mg/L; cytokinins—6-benzylaminopurine (BA) and 6-furfurylamino purine (kinetin) (Sigma Chemicals) 0–3.0 mg/L.

2.4 Gelling Agents

Bacteriological grade agar, 7.0 g/L.

2.5 Culture Medium

1. Murashige and Skoog (MS) [6] basal medium (Table 1).
2. Stocks solutions for macronutrients, micronutrients, vitamins, amino acids, and plant growth regulators (Table 1).
3. Sucrose 20–30 g/L.
4. Adjust pH to 5.8 before adding agar, melt agar in microwave oven before adding to the medium, and autoclave the media at 121 °C at 16 psi for 20 min.

Fig. 1 Vanilla vine with pods

Table 1
Composition and stock concentration of Murashige and Skoog (MS) medium

Sl. No.	Ingredients		Std mg/L	Actual quantity per liter of stock solution (g)	Volume of stock solution per liter of medium
1.	Macroelements				
	Ammonium nitrate	NH_4NO_3	1650	33	50 mL/L
	Potassium nitrate	KNO_3	1900	38	
	Magnesium sulfate	$MgSO_4$	370	7.4	
	Potassium dihydrogen ortho phosphate	KH_2PO_4	170	3.4	
	Calcium chloride	$CaCl_2$	330	6.6	
2.	Microelements				
	Potassium iodide	KI	0.83	0.0415	10 mL/L
	Boric acid	H_3BO_3	6.2	0.318	
	Manganese sulfate	$MnSO_4$	22.3	1.115	
	Zinc sulfate	$ZnSO_4$	8.6	0.43	
	Sodium molybdate	Na_2MoO_4	0.25	0.125	
	Copper sulfate	$CuSo_4$	0.025	0.000125	
	Cobalt chloride	$CoCl_2$	0.025	0.000125	
3.	Vitamins				
	Nicotinic acid	$C_6H_5NO_2$	0.5	0.025	10 mL/L
	Pyridoxine HCl	$C_8H_{12}NO_3HCL$	0.5	0.025	
	Thiamine	$C_{12}H_{17}ClN4O_5$	0.1	0.005	
	Glycine	$C_2H_5NO_2$	2.0	0.1	
4.	Iron source				
	Iron sulfate	$FeSO_4$	27.8	1.3952	10 mL/L
	Sodium EDTA	Na_2EDTA	37.3	1.865	
5.	Meso inositol	$C_6H_{12}O_6$	100	5	10 mL/L

Dissolve each constituent separately before mixing to the final stock

2.6 Incubation Conditions

Incubate cultures at 22 ± 2 °C and with 14-h photoperiod, 35 μmol/m^2/s, light provided by "Philips" cool white fluorescent tubes.

2.7 In Vitro and Cryopreservation

1. *In vitro conservation*: Use in vitro-regenerated shoot buds as propagules for conservation by slow growth induction.
2. *Production of synthetic seeds*: Use in vitro-regenerated shoot buds, protocorms, and callus for encapsulation. Sodium alginate and calcium chloride for preparation of synthetic seeds.
3. *Long-term storage by cryopreservation*: Synseeds containing miniaturized shoot tips, protocorms, pollen, and liquid nitrogen.

3 Methods

3.1 In Vitro Seed Germination and Embryo Rescue [4, 7, 8]

1. Collect 4–7-month-old mature pods, and surface sterilize under laminar airflow by dipping in 95 % ethanol and flaming it.
2. Split open capsules to remove seeds and transfer them to sterilized culture medium.
3. Both solid and liquid media can be used for seed germination and culture; MS-solidified medium supplemented with 0.5 mg/L kinetin, 20 g/L sucrose; and continuously rotate liquid cultures on a rotary shaker at 200 rpm.
4. Transfer germinating seeds to multiplication medium, MS medium supplemented with 1 mg/L BA and 0.5 mg/L IBA (Fig. 2).

3.2 Micropropagation and Multiplication of True-To-Type Plants [4, 9]

1. Healthy vines of different species are identified as mother plants for source material collection, from which shoot tips and nodal segments are utilized for all experiments. In the authors' laboratory, *V. planifolia*, *V. aphylla*, *V. pilifera*, and *V. andamanica* vines were included.
2. Explants should be washed and cleaned with a dilute detergent solution, e.g., teepol (100 mL/L), and then transferred to the laminar flow chamber for surface sterilization with 1 g/L mercuric chloride for 5–7 min.
3. A fresh basal cut is made after thorough washing with sterilized double-distilled water (to remove any traces of mercuric chloride), before inoculation into MS medium supplemented with 1 mg/L BA and 0.5 mg/L IBA.
4. Subcultures to the fresh culture medium of same composition every 20-day interval should be done to replenish exhausted nutrients. Incubate cultures at 22 ± 2 °C and with a photoperiod of 14 h and an irradiance of 35 μmol/m²/s, provided by "Philips" cool white fluorescent tubes.
5. An initial period of 5–7 months is needed for culture establishment, bud initiation, and multiple shoot induction in the explants before they start multiplying in a 1:12 ratio (Fig. 3).

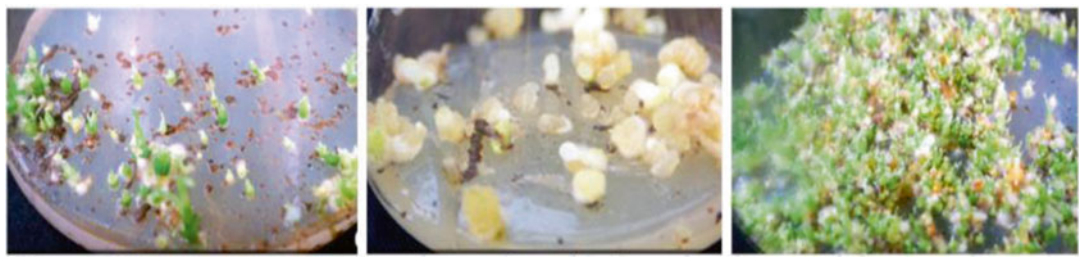

Fig. 2 Initiation of germination from minute black seeds, indirect and direct seed germination

Fig. 3 Induction of multiple shoots and subsequent proliferation

Fig. 4 Root induction from the base as well as from the nodes

3.3 In Vitro Rooting and Production of Plantlets

1. Vanilla is known to produce roots in all nodes and in vitro behavior is no different; hence a specific stage or medium is not required for in vitro rooting in vanilla (Fig. 4).
2. Separate 2.0 cm long shoots from the multiplying clusters, individually to MS medium devoid of plant growth regulators. The rooting initiates from the third day of shoot culture.
3. Each well-rooted shoot (as mentioned below) can be transferred for next cycle of multiplication or planted out to the field.
4. For next cycle of shoot multiplication, re-culture 2 cm long shoots or single nodes by cutting shoots having 2–3 nodes.

3.4 Callus Induction and Creating Variability

1. Any explants, derived from in vitro shoots, roots, or protocorms, can be utilized for callus induction.
2. Inclusion of auxin and cytokinin in the basal MS medium is desirable for dedifferentiation of explants into callus. MS medium containing 1 mg/L BA and 0.5 mg/L NAA is ideal for all the species.

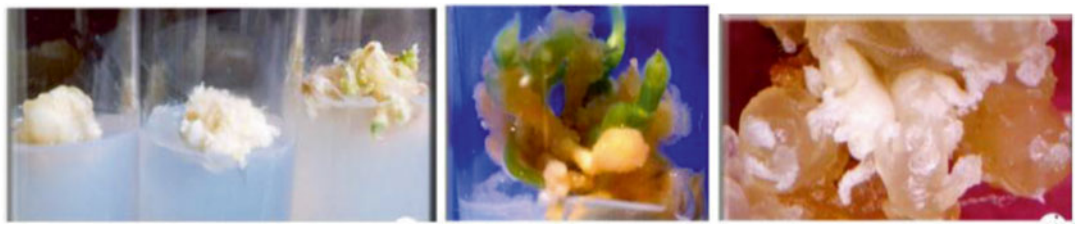

Fig. 5 Different stages in plant regeneration from callus

3. Some of the explants, especially seeds and protocorms, need an initial incubation in the dark for 2–5 days for callus induction.
4. Once initiated, subculture callus cultures to fresh culture medium of same composition at every 3-week interval.

3.5 Plantlet Regeneration via Direct and Indirect Pathways [4]

1. Transfer proliferating callus to MS medium supplemented with 1 mg/L BA and 0.5 mg/L IBA, for re-differentiation. Subculture callus onto the fresh same culture medium at 3-week interval.
2. Callus re-differentiates into protocorms and shoots with roots in about 3–6-month time (Fig. 5).
3. Subculture callus on the same proliferation medium for maintaining callus and utilize later for regeneration or isolation of protoplasts. The well-developed shoots with roots are transferred ex vitro, for hardening and planting out.

3.6 Artificial Seed Production [4]

1. Propagules, viz. in vitro-developed protocorms or shoot buds (0.5–1.0 cm), are dropped in a gel matrix of MS medium containing 4 % sodium alginate (4 % w/v, Sigma).
2. Pipette out the gel matrix containing explants into calcium chloride ($CaCl_2{\cdot}2H_2O$) solution (1.036 g/150 mL) using a Pasteur pipette with a cut end for easy passage of propagules, such that each drop that transforms into a bead contains one explant.
3. Leave explants in $CaCl_2{\cdot}2H_2O$ solution for 30–40 min, shaking on a gyratory shaker (100 rpm) for proper bead formation.
4. The beads are recovered by decanting $CaCl_2$ solution and wash them in sterile water two times (Fig. 6).
5. The artificial/synthetic seeds or "synseeds" thus produced can be utilized for germplasm conservation and as propagules with more than 80 % germination rate at 22 ± 2 °C.
6. Whenever needed transfer synthetic seeds for germination in multiplication medium (MS medium amended with 1 mg/L BA and 0.5 mg/L IBA).

Fig. 6 Synthetic seed production

3.7 Hardening and Planting Out

1. In vitro plantlets with well-developed roots are washed under running water to remove the traces of nutrient medium on the roots. Care should be taken to prevent any damage to roots while cleaning roots, but remove all medium to prevent microbial growth.
2. Dip them in 0.3 % Dithane-M45 for 5–10 min and transplant in poly bags containing a mixture of garden soil, sand, and vermiculite in equal proportions (1:1:1).
3. Keep the transplanted plantlets in a humid chamber (relative humidity 70–80 % and light intensity 25–30 μmol/m^2/s) for 3–4 weeks for hardening and establishment. Keep hardened plants in the nursery for 1 year and finally transfer to the field.

3.8 In Vitro Conservation of Vanilla Genetic Resources [9, 10]

3.8.1 Medium-Term In Vitro Conservation by Slow Growth

1. Induce slow growth in vanilla shoots by reducing the carbon source, reducing basal media concentration, adding mannitol, and minimizing evaporation loss to increase subculture intervals substantially.
2. Transfer in vitro-grown vanilla shoots (2 cm long) to MS medium containing 15 g/L sucrose and mannitol, respectively, without plant growth regulators.
3. Seal the culture vessels with polypropylene caps or parafilm to minimize evaporation and maintain them at 22 ± 2 °C, 12-h photoperiod, with reduced light intensity of 30 μmol/m^2/s.
4. These shoot cultures with minimal growth can be maintained in vitro for 7–10 years with yearly subculture on the fresh medium (Fig. 7).
5. For normal growth, transfer plantlets in MS medium containing 1.0 mg/L BA and 0.5 mg/L IBA, 22 ± 2 °C and light intensity of 35 μmol/m^2/s.

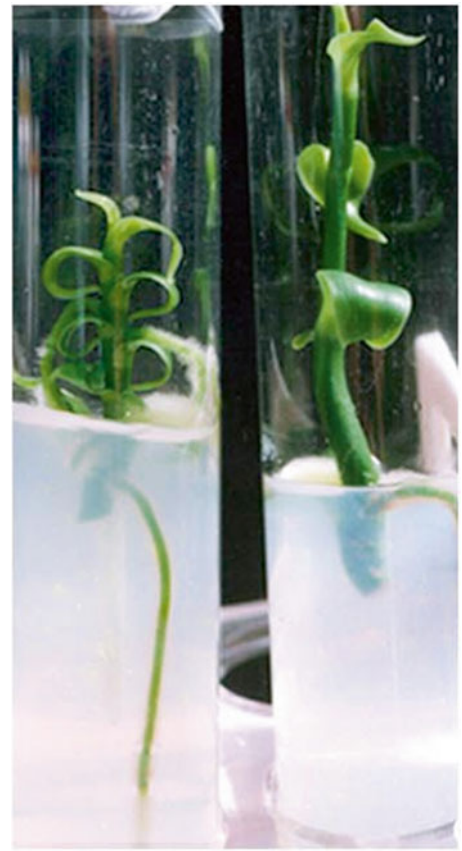

Fig. 7 In vitro conservation by slow growth. (The shorter shoots indicate minimal growth with root system while the longer shoots show growth in normal medium requiring frequent subcultures)

3.8.2 Long-Term Storage of Encapsulated Shoot Tips and Pollen by Cryopreservation

Pretreatment and Dehydration

1. Choose uniform-sized synthetic seeds (4–5 mm diameter) for cryopreservation.
2. Pre-culture synseeds on MS liquid medium amended with increasing sucrose concentrations (0.1, 0.3, 0.5, 0.7, and 1.0 M) for 5 days, with 1 day for each concentration, and then dehydrate in a laminar airflow chamber at room temperature by spreading on a sterilized filter paper for 1–10 h.
3. Germination tests were conducted to determine optimum drying time.

Cryopreservation

1. Transfer ten dehydrated beads per 2.0 mL cryo vials.
2. Rapid freezing is done by plunging the cryo vials directly in the liquid nitrogen.
3. Samples can be stored in liquid nitrogen for 10 years.

Thawing, Post-culture, and Regeneration of the Whole Plant

1. Cryopreserved samples are retrieved by immersing vials for 5–10 min directly in a water bath at 40 °C.
2. The synseeds are cultured in a 90 mm petri dish containing 25 mL solidified MS medium containing 30 g/L sucrose, 1 mg/L BAP, and 0.5 mg/L IBA in the darkness initially for 7 days and then provided with a light intensity of 3000 lux for shoots to grow. The recovery rate is 50–70 %.
3. After 2 weeks, shoot buds that emerge out from the synthetic seed coat are subcultured on the fresh MS basal medium for proper growth of the shoot and root system and finally plant them for hardening and field transfer.

3.9 Pollen Cryopreservation for Utilization in Interspecific Hybridization between Asynchronously Flowering Species [11, 12]

1. Collect pollinia on the previous day of flower opening.
2. Culture pollen on Brewbaker and Kwack (BK) medium [11] and different sucrose concentrations (5–15 %) to assess germination and 10 % sucrose provided the most appropriate osmotic potential for rapid pollen germination for the species of *Vanilla* studied (Fig. 8). Germination counts of pollen are made after 18–24 h and incubation at 25 °C in the dark.
3. Cryostorage of pollen: Pollinia were subjected to different pretreatments, like desiccation in laminar airflow cabinet for 5–15 min and desiccation combined with chemical cryoprotection with 5 and 10 % of dimethyl sulfoxide (DMSO).
4. Transfer the pretreated pollen samples to cryovials and plunge into liquid nitrogen (LN_2).

Fig. 8 The different Vanilla species included in the study

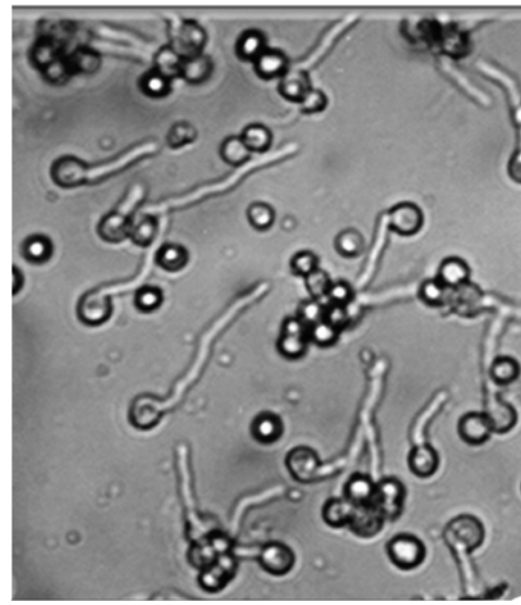

Fig. 9 Pollen germination in *V. planifolia*

5. Thaw the cryopreserved pollen by rapid thawing process by dipping the cryovial in 40 °C water bath for 30 min after cryogenic storage.
6. Viability of fresh and cryopreserved pollen samples is assessed by germination in vitro, by culturing in Brewbaker's medium (Fig. 9).
7. Flowers, of the desired female parent, are emasculated. Cryopreserved pollen of another species, after thawing, is applied on the receptive stigma. Pollinated flowers and fruit set are marked (Fig. 10).
8. Harvest the fruits after 2–4 months and culture the seeds in vitro, to prevent any embryo abortion.

3.10 Isolation of Protoplasts for Somatic Hybridization [13]

1. Digest leaf tissue with a mixture of macerozyme and cellulose, using one-step method of enzyme digestion.
2. Prepare enzyme solution in cell protoplast washing (CPW) medium.
3. Add mannitol to maintain the osmotic strength of cytoplasm and to prevent plasmolysis or bursting of the protoplasts.
4. Incubate leaves in CPW medium with mannitol for pre-plasmolysis.
5. Immerse 1 g of mechanically macerated leaves from in vitro-cultured plants in 10 mL isolation medium and incubate in the darkness for up to 16 h.

Fig. 10 Successful interspecific hybridization

6. After digestion, filter the enzyme solution containing protoplasts through a stainless steel mesh (60 mesh size, Sigma) to remove larger particles of undigested tissues and cell clumps.
7. Observe sample under inverted microscope to confirm enzymatic digestion and release of protoplasts.
8. Distribute filtrate into sterilized, screw-capped centrifuge tubes and centrifuge in Beckman tabletop centrifuge for 10 min at 700 rpm.
9. Remove the supernatant enzyme solution using a Pasteur pipette without disturbing the pellet. Suspend the pellet (containing protoplasts) in CPW medium.
10. Repeat centrifugation and resuspension in fresh medium three times so as to wash the protoplasts and remove traces of enzyme solution, and then resuspend the pellet in 1 mL CPW medium (Table 2) and layer on top of 9 mL floatation medium, to centrifuge at 700 rpm for 10 min.
11. Collect live protoplasts at the interphase, with a Pasteur pipette.
12. Add two droplets of protoplast suspension each from *Vanilla planifolia* and *V. andamanica* to another droplet of PEG solution (Table 3), and allow to mix at room temperature for 30 min.
13. Maintain as droplet cultures on glass slides and periodically observe for fusion of protoplasts (Fig. 11), cell wall regeneration, and cell division.

Table 2
Composition of media used for protoplast isolation and culture in vanilla

Components (mg/L)	CPW medium (mg/L)	Floating media (mg/L)	Protoplast culture media (mg/L)	
			I	II
NH_4NO_3	–	–	1650	1650
KNO_3	101.0	101.0	1900	1900
$CaCl_2{\cdot}2H_2O$	1480.0	1480.0	440	440
$MgSO_4{\cdot}7H_2O$	246.0	246.0	370	370
KH_2PO_4	27.2	27.2	170	170
KI	0.16	0.16	0.83	0.83
H_3BO_3	–	–	6.2	6.2
$MnSO_4{\cdot}4H_2O$	–	–	22.3	22.3
$ZnSO_4{\cdot}7H_2O$	–	–	8.7	8.7
$Na_2MoO_4{\cdot}2H_2O$	–	–	0.25	0.25
$CuSO_4{\cdot}5H_2O$	0.025	0.025	0.025	0.025
$CoCl_2{\cdot}6H_2O$	–	–	0.025	0.025
$FeSO_4{\cdot}7H_2O$	–	–	27.8	27.8
$Na_2EDTA{\cdot}2H_2O$	–	–	37.3	37.3
Myoinositol	–	–	100.0	100.0
Nicotinic acid	–	–	0.5	0.5
Thiamine HCl	–	–	0.5	0.5
Pyridoxine HCl	–	–	0.5	0.5
Glycine	–	–	2.0	2.0
Sucrose	–	21 %	2 %	3 %
Mannitol	7–10 %	–	7 %	4 %
Gibberellic acid	–	–	0.5	0.5
BA	–	–	0.5	1.0
NAA	–	–	0.5	1.0
2,4-D	–	–	0.5	–

3.11 Isolation of DNA for Molecular Profiling [14]

1. Isolation of DNA: The CTAB method [13] was used for the isolation of genomic DNA from vanilla leaf tissue.
2. Prepare the stock solutions and buffers for DNA isolation following [14].
3. Develop RAPD profiles as per the method suggested by Williams et al. [15].

Table 3
Composition of PEG solution[a] for protoplast fusion

Constituents	Molar concentration (mM)	g/L
NaCl	140	8.18
KCl	5 0	37
Na_2HPO_4	0.75	0.11
Glucose	5	0.90
$CaCl_2 \cdot 2H_2O$	125	18.40
PEG (MW 4000)		400.00

[a]pH was adjusted to 5.8

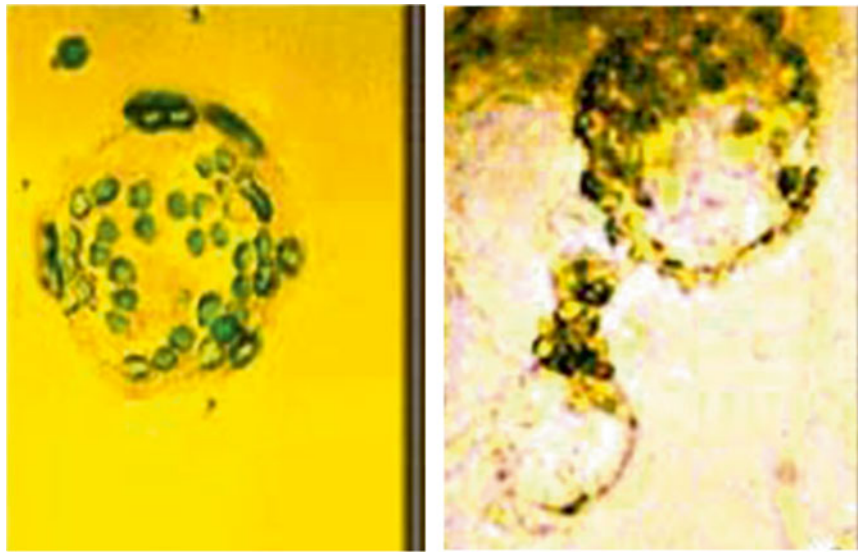

Fig. 11 Somatic hybridization between different species of *Vanilla*

4. The dNTPs, Taq polymerases, and other chemicals were procured from Sigma Aldrich, Germany.
5. Sixteen arbitrary primers were used for PCR reaction. Each primer contained at least 60–70 % GC content and no self-complementary ends.
6. PCR amplification reaction volumes were 25 mL PCR, each containing the reagents added sequentially to 25 mL of assay buffer—1, 150 mM dNTPs, 2 mM $MgCl_2$, 1 U of Taq DNA polymerase, 40 ng genomic DNA, and 10 pM primer.
7. Amplification employed a programmable thermal cycler (with cycling regimes of an initial denaturation temperature of

94 °C for 5 min, followed by 33 repeats of a PCR core cycle of 94 °C for 1 min, 40 °C for 1 min, and 72 °C for 1 min, followed by a final extension cycle of 72 °C for 15 min.

8. Resolve amplicons electrophoretically alongside a 1 kb ladder, on a 2 % agarose gel stained with 0.5 mg/mL ethidium bromide in a 1 × TAE buffer. Equal volumes of the completed reaction mix were loaded and run at 90 V for 3 h in an OWL separating system.
9. The completed gel is documented with the help of GelDoc 1000 documentation system (Fig. 12). Test 20 arbitrary 10-mer primers and give 9 polymorphic and scorable amplified products, and use for RAPD profiling, following the protocol of Williams et al. [15].
10. Carry out duplicate amplification to confirm reliability of the bands. Amplified products are listed as discrete character states in a present (1) or absent (0) matrix.
11. Relationships among genotypes are evaluated with a phonetic cluster analysis using the unweighted pair grouping mathematical average (UPGMA) analysis and plotted in a phenogram using NTSYS pc version 2.0j [16]. Consolidate polymorphic loci, exhibited by the different genotypes, in a chart to study the expressed loci of each genotype. Perform Bootstrap analysis using WinBoot [17] with 1000 repetitive sampling using Dice's coefficient, of the data to compute bootstrap P values.

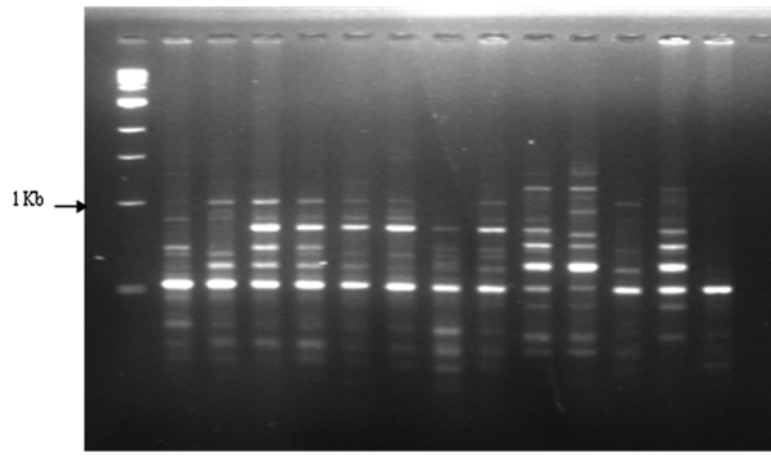

Fig. 12 RAPD profiles of interspecific hybrids of vanilla using OPERON primer OPB20

4 Notes

1. While flaming pods, the process can be repeated to avoid contamination and care should be taken to avoid any damage to seeds that may have an adverse affect on seed viability.
2. Contaminated liquid culture should be completely discarded and autoclave it before throwing away. Hence for embryo rescue and in case of interspecific hybridization solid medium helps in saving unaffected seeds.
3. Most vanilla stocks are infected with one or more viruses. Therefore, use virus diagnostic kits (ELISA/RT-PCR) to detect virus-infected stock plants.
4. Make sure that the stock plant material is completely virus free.
5. Seeds germinate directly into plantlets in the medium supplemented with BA (0.5 mg/L) alone, without any intervening callus phase; hence this medium was selected for the germination of vanilla seeds.
6. Germinating seeds, axillary buds, and shoots produced multiple shoots when cultured on MS medium supplemented with BA (1 mg/L) and IBA (0.5 mg/L).
7. Indirect plant regeneration via callus was induced in MS medium fortified with NAA (0.5 mg/L) and BA (1.0 mg/L) in which 75 % of the cultures developed callus.
8. When full-strength MS medium containing 30 g/L sucrose is used for in vitro conservation experiments, shoot cultures grow rapidly and within 180 days culture vessels are full of shoots; medium is exhausted; and shoot cultures dry up. For preventing this condition, modify culture medium with reduced sucrose concentration (10–15 mg/L) and half-strength nutrients, and add mannitol (10–15 mg/L). This change would result in reduced shoot growth rate (2–3 cm in 360 days) and highest survival rate of at least 80 %.
9. In vitro-regenerated shoot buds, protocorms, and regenerating calli could be encapsulated in 4 % sodium alginate and stored in sterile water for 10 months, at 22 ± 2 °C. When cultured on MS medium supplemented with BA (1 mg/L) and IBA (0.5 mg/L), the synthetic seeds germinated (80 %) after 2 weeks.
10. The pollen cryostorage described is under Indian conditions, the moisture content of pollinia may vary in different climatic conditions; hence it should be standardized before proceeding for actual use in hybridization experiments.
11. Among the different treatments tried all the pollen samples survived freezing with maximum percentage of germination in pollen desiccated for 5 min in the air current of laminar flow and cryoprotected with 5 % DMSO.

12. The experiments conducted are in *Vanilla planifolia* and few of its related species (Fig. 7).
13. Protoplasts were successfully isolated from the two species when incubated in an enzyme solution containing macerozyme R10 (0.5 %) and cellulase Onozuka R10 (2 %) for 8 h at 30 °C in the dark. The protoplast yield was 2.5×10^5 per gram and 1.0×10^5 g of leaf tissue and protoplast viability were 72 % and 55 % in *V. planifolia* and *V. andamanica*, respectively.
14. The molecular profiles indicate variability among the seedlings and species. Paired affinity indices were calculated to quantify the variability which could be utilized for crop improvement programs.

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Chapter 5

Assessment of In Vitro Biological Activities of Anthocyanins-Rich Plant Species Based on *Plinia cauliflora* Study Model

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Abstract

Plinia cauliflora (jaboticaba) is a native fruit tree from Brazilian rainforest widely used in popular medicine to prevent diarrhea, asthma, and infections. Studies have shown that the major therapeutic potential of jaboticaba fruits is on its peel, a rich source of anthocyanins. These secondary metabolites have well-known antioxidant and anti-inflammatory activities and have been claimed to be effective to treat diabetes, cancer, cardiovascular diseases, and stroke. This chapter describes a series of methodologies to evaluate important in vitro biological activities like cytotoxicity, proliferation, and migration of a hydroalcoholic extract of jaboticaba peel on mouse fibroblast L929 line. Assays to assess total phenolic, flavonoid, and anthocyanin contents and antioxidant activities are described as well.

Key words *Plinia cauliflora*, Phytochemistry, Phenolic compounds, Total flavonoids, Anthocyanins, Biological activities, DPPH assay, MTT assay, Scratch assay, EdU assay

1 Introduction

The phytochemical analysis aims to study the chemical constituents of plant species. When there is no chemical study on the species of interest, the preliminary phytochemical analysis can indicate the relevant groups of secondary metabolites in the sample material. However, if the interest is restricted to a specific class of compounds or substances responsible for a certain biological activity, research should be directed to the structural elucidation and quantification of those compounds, as well as their isolation [1].

Although thousands of compounds from plants and microorganisms have already been determined, natural sources of secondary metabolites appear to be inexhaustible to medicinal chemistry [2].

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The *Plinia cauliflora* species is a native fruit to Brazil, belonging to the family Myrtaceae, popularly known as jaboticaba or jaboticaba-açu. It has been found in the Atlantic Rainforest, especially in the southern and southeastern regions in Brazil [3]. The fruits are usually consumed *in natura*, as liqueurs, wines, and jellies [4]. Chemically, jaboticaba is known as a source of tannins, vitamins [5], and organic acids in the following order of amounts: ellagic acid > citric acid > succinic acid > malic acid > oxalic acid > acetic acid [6].

There are many studies evaluating the biological activities of extracts from different parts of the jaboticaba plant, e.g., leaves, stem, bark, and seeds. The jaboticaba bark extract has shown astringent properties and is widely used in the treatment of diarrhea, skin irritation, hemoptysis, and asthma, and gargled for chronic inflammation of the tonsils [7–9]. Recent studies performed in rats in a diet-induced obesity model showed that the intake of jaboticaba bark extract decreased oxidative stress in tissues, reduced circulating saturated lipids [10], increased HDL cholesterol, and improved insulin resistance [11, 12]. On the other hand, jaboticaba peel extract showed antiproliferative effects against leukemic cells and prostate cancer cells [13].

Among the biological assays currently used to evaluate complex matrices such as plant extracts, cytotoxicity and cell migration and proliferation have been frequently adopted. For example, fibroblast migration and proliferation are important steps in the wound healing process, mainly for extracellular matrix formation [14]. In vitro-positive results in tests evaluating these parameters are evidence that the substance is effective to treat in vivo healing problems.

In this context, this chapter describes a series of protocols for the chemical analysis of the hydroalcoholic extracts of jaboticaba fruit peels focusing on the determination of the total contents of phenolic, flavonoid, and anthocyanin compounds. Besides, biological activity assays regarding the cytotoxicity and the cell migration and proliferation are also described using the mouse fibroblast L929 cell line treated with hydroalcoholic extracts of jaboticaba. For that, the MTT, scratch, and EdU assays were performed.

2 Materials

2.1 Collection and Preparation of Biomass Samples

1. Jaboticaba (*Plinia cauliflora*) fruits were collected during the harvest season (spring, 2014) in Guaxupé county, Minas Gerais state (southeastern Brazil), at a backyard format planting system.
2. Pulper.
3. Distilled water.
4. Lyophilizer.
5. Grinder.

2.2 Extraction Process

1. Ethanol P.A.
2. Distilled water.
3. 1 % Hydrochloric acid solution (v/v).
4. 50 % Ethanol solution (pH 3.6).
5. pH meter.
6. Analytical balance.
7. Microwave oven.
8. Cellulose membranes—pore size 14 μm ∅.
9. Vacuum filtration system.

2.3 Analysis of Phenolic Compounds

1. Ethanol P.A.
2. Methanol P.A.
3. Distilled water.
4. 95 % Methanol solution (v/v).
5. 95 % Ethanol solution (v/v).
6. 50 % Ethanol solution (pH 3.6—v/v).
7. 5 % Sodium carbonate solution (m/v).
8. 1 % Hydrochloric acid solution (v/v).
9. Folin-Ciocalteu phenol reagent.
10. Gallic acid standard.
11. Vortex.
12. Ultraviolet–visible (UV–Vis) spectrophotometer.

2.4 Analysis of Total Flavonoids

1. Ethanol P.A.
2. Methanol P.A.
3. Distilled water.
4. 50 % Ethanol solution (pH 3.6—v/v).
5. 1 % Hydrochloric acid solution (v/v).
6. 2 % Aluminum chloride solution in methanol P.A. (m/v).
7. Quercetin standard.
8. Vortex.
9. Ultraviolet–visible (UV–Vis) spectrophotometer.

2.5 Analysis of Anthocyanins

1. Distilled water.
2. 0.025 mol/L Potassium chloride buffer solution (pH = 1.0).
3. 0.4 mol/L Sodium acetate buffer (pH = 4.5).
4. Hydrochloric acid P.A.
5. 1 % Hydrochloric acid solution (v/v).
6. Volumetric flasks (1 L).

7. Beakers (50 mL).
8. pH meter.
9. Ultraviolet–visible (UV–Vis) spectrophotometer.

2.6 Antioxidant Activity by DPPH Assay

1. 2, 2-Diphenyl-2-picryl hydrazyl (DPPH).
2. Methanol P.A.
3. Distilled water.
4. 80 % Methanol solution (v/v).
5. Volumetric flasks.
6. 96-well plates.
7. Vortex.
8. Ultraviolet–visible (UV–Vis) spectrophotometer.

2.7 MTT Assay

1. Mouse fibroblast L929 cell line.
2. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS—v/v), 1 % penicillin/streptomycin (m/v), 1 % l-glutamine (m/v), 1 % HEPES (*N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid)) solution (m/v), 4.5 g glucose, 1.5 g sodium bicarbonate ($NaHCO_3$).
3. 96-Well plates.
4. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide—stock solution (5 mg/mL) in phosphate-buffered saline (PBS, pH 7.2–7.6).
5. Dimethylsulfoxide (DMSO).

2.8 Scratch Assay

1. Mouse fibroblast L929 cell line.
2. DMEM supplemented with 10 % FBS (v/v), 1 % penicillin/streptomycin (m/v), 1 % l-glutamine (m/v), 1 % HEPES solution (m/v), 4.5 g glucose, 1.5 g sodium bicarbonate ($NaHCO_3$).
3. 24-Well plates.
4. 13 mm Circular cover slips.
5. Pipette tips.
6. PBS (pH 7.2–7.6).

2.9 Proliferation Assay

1. Mouse fibroblast L929 cell line.
2. DMEM Gibco) supplemented with 10 % FBS (v/v), 1 % penicillin/streptomycin (m/v), 1 % l-glutamine (m/v), 1 % HEPES solution (m/v), 4.5 g glucose, 1.5 g sodium bicarbonate ($NaHCO_3$).

3. 24-Well plates.
4. PBS (pH 7.2–7.6).
5. 4 % Paraformaldehyde in PBS (m/v).
6. 0.5 % Triton® X-100 in PBS (v/v).
7. 1 % Bovine serum albumin (BSA) in PBS (pH 7.4) (m/v).
8. 0.1 % Tween 20 in PBS (v/v).
9. DMSO.
10. 13 mm Circular cover slips.
11. Deionized water.
12. EdU: Prepare stock solution (10 mM) by adding 2 mL DMSO or water to EdU and then mix well. Store at ≤–20 °C.
13. Alexa fluor azide: Prepare working solution by adding 70 μL DMSO to Alexa fluor azide and then mix well. Store working solution at ≤–20 °C.
14. Click-iT EdU reaction buffer. Dilute one volume from Click-iT EdU reaction buffer to 1:10 with deionized water. Store diluted solution at 2–6 °C.
15. Click-iT EdU buffer additive. Prepare solution before use diluting the volume that will be used in tests of Click-iT EdU buffer additive 1:10 with deionized water.
16. 4,6-Diamidino-2-phenylindole (DAPI) solution: Stock solution 1 mg/mL in PBS.

3 Methods

3.1 Collection and Preparation of Samples

1. Fruit peels are considered as the residual biomass after the pulps have been collected by using a pulper apparatus and distilled water (3 V). Store the samples at –20 °C.
2. Fruit peels are lyophilized.
3. Dried biomass is ground by a grinder into jaboticaba peel flour.
4. The compounds of interest are then extracted from the jaboticaba peel flour (*see* **Note 1**).

3.2 Extraction Process

1. 1 g (dry weight) jaboticaba peel flour is added to 9 mL 50 % ethanol solution (pH 3.6) (*see* **Note 2**).
2. The mixture is microwaved, i.e., three pulses of 5 s with 60-s interval, to extract the compounds of interest.
3. The hydroalcoholic extract is recovered by filtration on cellulose membranes under vacuum.

3.3 Analysis of Phenolic Compounds [15]

1. 1 mL Supernatant from the extraction process is diluted in 5 mL 95 % methanol solution (*see* **Note 3**).
2. The diluted sample (1 mL) is transferred to a test tube and added 1 mL 95 % ethanol solution, 5 mL distilled water, and 0.5 mL Folin-Ciocalteu phenol reagent.
3. Mix and incubate for 7 min.
4. After incubation, 1 mL 5 % sodium carbonate solution is added, mixed well, and kept in the darkness for 1 h.
5. Prepare a blank solution (*see* **Note 4**).
6. The samples are vortexed and the absorbance is measured at 725 nm using a UV–Vis spectrophotometer.
7. The quantification of the total phenolic compounds is carried out using an external standard curve of gallic acid (0–100 μg/mL) and the amount values are expressed as μg of gallic acid equivalents per gram (dry weight) of jaboticaba biomass.

3.3.1 External Gallic Acid Standard Curve

1. Add 10 mg gallic acid in 10 mL 50 % ethanol solution (pH 3.6), i.e., the *gallic acid solution 1* (1 mg gallic acid/mL).
2. Dilute 1 mL *gallic acid solution 1* in 10 mL 50 % ethanol solution (pH 3.6), i.e., the *gallic acid solution 2* (0.1 mg gallic acid/mL).
3. Prepare the following solutions of gallic acid according to the concentrations described in Table 1 from the *gallic acid solution 2* (*see* **Note 3**).
4. 1 mL of each gallic acid solution (*see* Table 1 for the concentrations) is transferred to a test tube and add 1 mL 95 % ethanol solution, 5 mL distilled water, and 0.5 mL Folin-Ciocalteu phenol reagent.

Table 1
Preparation of the gallic acid external standard curve

Concentrations of gallic acid solutions	50 % Ethanol solution (pH 3.6) (μL)	Gallic acid solution 2 (μL)
Blank	1000	0
10 μg/mL	900	100
20 μg/mL	800	200
40 μg/mL	600	400
60 μg/mL	400	600
80 μg/mL	200	800
100 μg/mL	0	1000

Table 2
Concentrations of gallic acid solutions and values of the arithmetic average of the corresponding absorbance to build a standard curve

Concentrations of gallic acid solutions	Absorbance average values
Blank	
10 μg/mL	
20 μg/mL	
40 μg/mL	
60 μg/mL	
80 μg/mL	
100 μg/mL	

5. Mix and incubate for 7 min.
6. After incubation period, 1 mL 5 % sodium carbonate solution is added, mixed well, and kept in the darkness for 1 h.
7. Prepare a blank solution (*see* **Note 4**).
8. The samples are mixed and the absorbance is measured at 725 nm using a UV–Vis spectrophotometer.
9. Calculations of the total content of phenolic compounds.
   9.1 In a spreadsheet of *Excel*® software, make a table (*see* Table 2). In the left column (*x*-axis), enter the values of the respective concentrations of the gallic acid solutions. In the right column (*y*-axis), enter the values of the arithmetic average of the corresponding absorbances.
   9.2 Select the two columns with your mouse and in the top flap of *Excel*® software choose the *Insert*, and then *XY graph (scatter)*. A graph will be generated.
   9.3 In one of the graph points, click once with the mouse right button and select *add line trend*. A new window will be opened.
   9.4 Select the following options: *set intersection*, *display equation on chart*, and *display R-squared value on the graph* (*see* **Note 5**).

3.4 Analysis of Flavonoid Contents [16]

1. 0.5 mL Supernatant from the *extraction process* is added to 2.5 mL ethanol P.A. and 0.5 mL 2 % aluminum chloride solution (*see* **Notes 3** and **6**).
2. Mix well and incubate for 1 h.
3. Prepare a blank solution (*see* **Note 7**).

4. The absorbance is measured at 420 nm using a UV–Vis spectrophotometer.
5. The quantification of total flavonoids is carried out using the external quercetin standard curve (0–200 μg/mL), as described in Subheading 3.3.1, and the content values are expressed as μg of quercetin equivalents per gram dry weight of jaboticaba biomass.

3.5 Analysis of Anthocyanins: Differential pH Measurement [17, 18]

Solution 1–0.025 mol/L hydrochloric acid solution (pH = 1.0) (*see* **Note 8**).

1. Dilute 1.86 g potassium chloride in 980 mL distilled water.
2. Measure the pH and add hydrochloric acid P.A. in drops until pH = 1.0.
3. Raise the volume to 1 L (use volumetric flask) with distilled water.

Solution 2–0.4 mol/L acetate buffer solution (pH = 4.5) (*see* **Note 9**).

1. Dilute 54.43 g sodium acetate in 960 mL distilled water.
2. Measure the pH and add 1 % hydrochloric acid solution (v/v) in drops until pH = 4.5.
3. Raise the volume to 1 L (use volumetric flask) with distilled water.

Assay

1. Add 1 mL supernatant from the *extraction process* in each beaker (*see* **Note 3**).
2. Add 50 mL solution 1 in each beaker.
3. Add 50 mL solution 2 in each beaker.
4. Mix thoroughly the beakers' contents and let them stand for 30 min, protected from light, at room temperature.
5. Measure the absorbance at 510 nm and 700 nm using a UV–Vis spectrophotometer.
6. Calculate anthocyanin contents (mg/L) using the formula

$$\left[\left(\text{Abs } 510 - \text{Abs } 700\right)_{s1} - \left(\text{Abs } 510 - \text{Abs } 700\right)_{s2}\right] \times \text{MW} \times D \times 1000 / \varepsilon,$$

where

Abs: absorbance

S1: Solution 1

S2: Solution 2

MW (molecular weight): cyanidin-3-glucoside = 449.2 g/mol.

ε (molar extinction coefficient): cyanidin-3-glucoside = 26,900 M/cm.

D: dilution factor = 1:50.

3.6 Antioxidant Activity by the DPPH Assay [19] (See Note 10)

1. Dissolve 20 g DPPH in 5 mL methanol P.A. This is the *DPPH solution 1* (*see* **Note 11**).
2. 1 mL *DPPH solution 1* is diluted in 99 mL 80 % methanol solution. This is the *DPPH solution 2*.
3. Check the absorbance of *DPPH solution 2* using a microplate reader. It should be around 0.5 and 0.6 (*see* **Note 12**).
4. Pipette 290 µL *DPPH solution 2* and 10 µL supernatant from *extraction process* using a 96-well microplate (*see* **Note 3**).
5. Mix and incubate for 30 min.
6. After incubation, the absorbance is measured three consecutive times at 531 nm using a UV–Vis microplate reader. The final absorbance is an average of the three consecutive readings.
7. The results are calculated according to the formula below and expressed as inhibition percentage (%):

$$\text{Inhibition}\,(\%) = \frac{\left(\text{Abs}_{\text{DPPH}} - \text{Abs}_{\text{Sample}}\right) \times 100}{\text{Abs}_{\text{DPPH}}}$$

where

$\text{Abs}_{\text{sample}}$ = absorbance of the sample

Abs_{DPPH} = absorbance of *DPPH solution 2*

3.7 MTT Assay

1. Mouse fibroblast L929 cells are cultured in DMEM under controlled conditions, at 37 °C, in a humidified 5 % CO_2 atmosphere.
2. Fibroblast L929 cells are added in a 96-well plate at a density of 5×10^3 cells per well and incubate at 37 °C in a humidified 5 % CO_2 atmosphere overnight.
3. After incubation, DMEM should be replaced by medium culture containing 1, 5, 10, 25, 50, and 100 µg/mL jaboticaba hydroalcoholic extract (concentrations based on yield—*see* **Note 13**), except in control treatment where the culture medium should be replaced by fresh DMEM (*see* **Note 14**).
4. Incubate cells for 48 h, at 37 °C, in a humidified 5 % CO_2 atmosphere.
5. After this period, replace all the culture medium by 100 µL fresh DMEM.
6. Add 10 µL MTT solution per well and incubate the cultures in the dark for at least 3 h, at 37 °C, in a humidified 5 % CO_2 atmosphere. A negative control without cells with 100 µL DMEM and 10 µL MTT solution is required. Protect the plates from light (*see* **Note 15**).

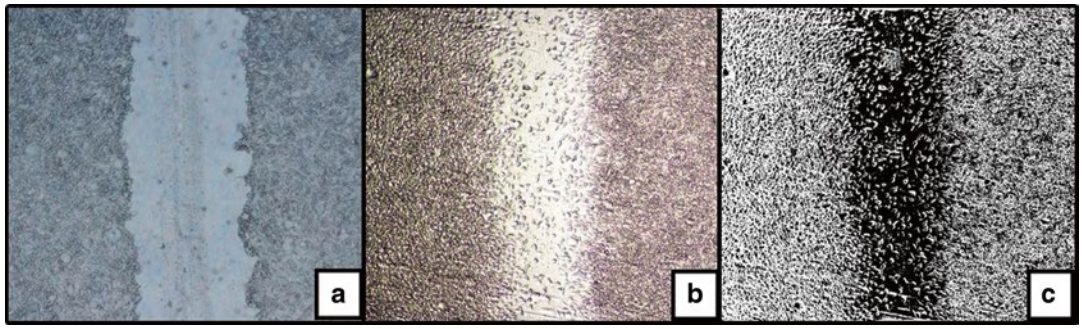

Fig. 1 Scratch assay images immediately after the wounding (**a**) and after 12 h (**b**). Image B processed with *ImageJ®* software, after **step 9.2** (**c**)

7. Subsequently, remove 85 µL culture medium, add 50 µL DMSO onto each well, and incubate for more 10 min, at 37 °C, in a humidified 5 % CO_2 atmosphere.
8. Homogenize carefully to dissolve formazan crystals.
9. Measure the absorbance on an ELISA plate reader with wavelength 540 nm.

3.8 Scratch Assay [20]

1. Mouse fibroblast L929 cells should be cultured as described in MTT assay.
2. After hygienization (*see* **Note 16**), put cover slips at the bottom of each well in a 24-well plate and seed L929 cells above it at a density of 5×10^5 cells/well (*see* **Note 17**).
3. Allow cells to grow for 24 h, at 37 °C, in a humidified 5 % CO_2 atmosphere.
4. After incubation, DMEM is completely removed and the adherent cell layers are scratched with a yellow pipette tip (*see* **Note 18**).
5. Wash (1×) the culture with PBS to remove cell debris (*see* **Note 19**).
6. Add DMEM with 1, 5, 10, 25, 50, and 100 µg/mL jaboticaba hydroalcoholic extract (concentrations based on yield), except in control treatment where only fresh DMEM should be added in the wells.
7. Incubate at 37 °C in a humidified 5 % CO_2 atmosphere for 12 h.
8. Digital images (Fig. 1) shall be captured from the wounded cultures at 0 h (right after to scratch - time 0) and 12 h (*see* **Note 20**).
9. Process images on *ImageJ®* software.
   9.1 Open *ImageJ®* and the file that will be analyzed (*File* → *Open* → Select your file).

9.2 Go to *Process→ Binary→ Convert to mask* followed by *Image→ Adjust→ Threshold→ Black and White* (B&W). The background must be black (click in "*Dark Background*").

9.3 Select the area that will be measured by clicking on *Edit→ Selection→ Create selection* (the selected area becomes yellow).

9.4 Finally, to find out the area without cells in pixels go to *Analyse→ Measure*.

9.5 Decrease scratch area rate can be calculated using the following steps:

First, the percentage of samples area without cells must be calculated using the formula:

Sample area without cells 12 h (in pixels) × 100/ Time 0 area without cells (in pixels)

Subsequently, subtract the result of the formula above from 100 (%).

These results area expressed as decrease scratch area rate after 12 hours related to time 0 (%).

3.9 Proliferation Assay

1. Mouse fibroblast L929 cells should be cultured as described in MTT assay.
2. Plate mouse fibroblast L929 cells on cover slips (*see* **Note 16**) at a density of 2×10^4 cells/well and then allow them to recover overnight, at 37 °C, in a humidified 5 % CO_2 atmosphere.
3. Add 1 mL/well DMEM with 1, 5, 10, 25, 50, and 100 μg/mL jaboticaba hydroalcoholic extract (concentrations based on yield), except in control treatment where only fresh DMEM should be placed on the wells.
4. Incubate the cells for 48 h, at 37 °C, in a humidified 5 % CO_2 atmosphere.
5. After this period, add 1 μL/well EdU solution (10 mM) and incubate for 4 h, at 37 °C, in a humidified 5 % CO_2 atmosphere.
6. Remove the culture medium and fix cells with 4 % paraformaldehyde (1 mL/well) for 15 min at room temperature.
7. Remove the paraformaldehyde and wash the cells with 0.1 % Tween 20 (1 mL/well), followed by 1 % BSA (1 mL/well).
8. Discard the previous solution, add 0.5 % Triton® X-100 (1 mL/well), and then incubate at room temperature for 20 min (*see* **Note 21**).
9. Wash the cells with 0.1 % Tween 20 (1 mL/well), followed by 1 % BSA (1 mL/well).
10. Prepare Click-iT reaction cocktail adding the reagents in the order listed in Table 3. Use this solution within 15 min of preparation.

Table 3
Composition of the Click-iT reaction cocktail (*see* Notes 22 and 23)

Components	Volume (μL)
Click-iT EdU reaction buffer	43
$CuSO_4$	2
Alexa fluor azide	0.12
Click-iT EdU buffer additive	5
Total volume per well	≅50

11. Remove BSA solution and add 50 μL Click-iT reaction cocktail above each cover slip.
12. Incubate the plate for 30 min at room temperature, protected from light.
13. Remove the Click-iT reaction cocktail, and then wash each well once with 1 % BSA (1 mL/well), followed by PBS (1 mL/well).
14. Remove PBS and add 1 mL of PBS plus 1 μL DAPI per well.
15. Incubate for 30 s at room temperature, protected from light.
16. Observe and take pictures from the cells in a fluorescence microscope (green filter for Alexa fluor 488 and blue filter for DAPI—Fig. 2). All cell nuclei will become blue with DAPI (using blue filter) and nuclei of cells in proliferation stage (during DNA replication) will become green with EdU (using green filter) (*see* **Note 24**).
17. Take the same picture twice: one using the green filter and the other the blue one (do not move the stage). Repeat this procedure 3 times per well and use these results to calculate an average.
18. Processing images from proliferation assay on *ImageJ*® software.
    18.1 Repeat **steps 9.1** from scratch assay.
    18.2 Click in Process followed by Subtract background. A new window will be opened. In a box called "Rolling ball radius," put a number. Choose a number that improves the image for your specific analysis. Lower numbers increase the contrast of the background in the image.
    18.3 Go to *Image→ Adjust→ Threshold→ Black and White* (B&W).
    18.4 Separate adjacent cells: *Process→ Binary→ Convert to mask* and *Process→ Binary→ Watershed*.

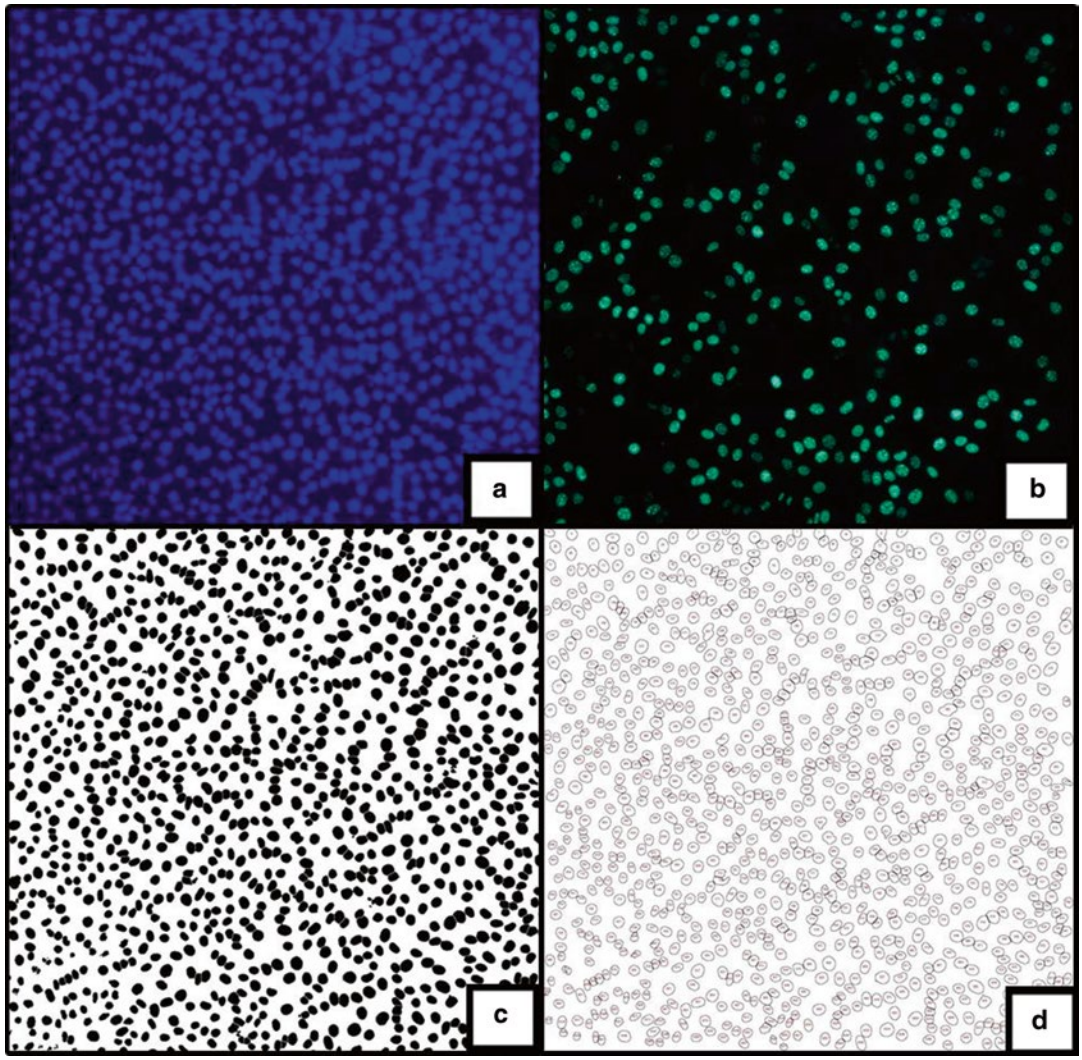

Fig. 2 Cell proliferation assay images. Fluorescence microscopy for DAPI (**a**) and EdU (**b**) treatments. Assessment of the total number of cells was performed using the *ImageJ®* software. Image after **step 18.2** (**c**). After selecting the option "*Show outline*" and clicking *ok*, a new image will be opened (**d**) showing all the outline of cells that were counted by the program

18.5 Finally, *Analyse → Analyse particle*. In a new window, fulfill the box *"Size"* with a lower and higher area of the cells in each image (*see* **Note 25**). *"Circularity"* box is related to the circle shape, where *one* is a perfect circle and *zero* is a totally irregular circle. Choose "*Show outline"* and *ok*. A new window with the number and area of cells will be opened.

18.6 The percentage of proliferative cells for each treatment is calculated using the formula:

Number of cells counted with EdU × 100/ Number of cells counted with DAPI

4 Notes

1. Jaboticaba peel flour is stored in freezer at –20 °C.
2. Adjust the pH of the extraction solution (50 % ethanol) using 1 % HCl solution (v/v).
3. All the procedures need to be carried out in the absence of light and in triplicate.
4. The blank solution is prepared replacing the sample by extraction solvent, i.e., 50 % ethanol solution (pH 3.6), and adding 1 mL 95 % ethanol solution, 5 mL distilled water and 0.5 mL Folin-Ciocalteu phenol reagent. A blank solution is used to remove the color of the reagent set.
5. By building a graph using *Excel*® software, you can select the following options:
   - *Set intersection*: The line that connects the points on the graph will pass through the origin.
   - *Display equation on the graph*: The equation of a straight line is usually written this way: $y = a.x + b$. The b variable is the y intercept, the point where the straight line crosses the y-axis and it should be adjusted to zero so that the line passes through the origin (0, 0). With the adjusted equation (i.e., $y = a.x$) you can now use the arithmetic average absorbance value for y and find the matching concentration value for x from the samples. The absorbance values of the samples must be inside the absorbance range of the standard curve. If the value is above, it is necessary to dilute the sample and do the procedure again.
   - *Display R-squared value on the graph*: R-squared value means how linear is your standard curve. The closer to 1 is the R-squared value, the greater is the linearity.
6. 2 % Aluminum chloride solution is prepared using methanol P.A.
7. Prepare blank solution replacing the sample by the extraction solvent, i.e., 50 % ethanol solution (pH 3.6), and add 2.5 mL ethanol P.A. and 0.5 mL 2 % aluminum chloride solution. Use blank solution for removing reagent set color.
8. 0.025 mol/L Hydrochloric acid solution (pH = 1.0) is stable at room temperature. It is necessary to measure the pH before use.
9. 0.4 mol/L Acetate buffer solution (pH = 4.5) is stable at room temperature. Take the pH before use.
10. The 2, 2-diphenyl-2-picryl hydrazyl (DPPH) assay is a chemical method applied to determine the antioxidant capacity of a compound to scavenge free radicals. The reduction of DPPH is monitored by the decrease in absorbance during the reaction.

11. Use volumetric flask to prepare the *DPPH solution 1* and the vortex to stir up the solution until completely solubilized.
12. Use volumetric flask to prepare the *DPPH solution 2* and the vortex to stir up until full dilution. The absorbance of *DPPH solution 2* should be around 0.5 and 0.6 to confirm that the dilution procedure of the stock solution (*DPPH solution 1*) was performed correctly.
13. Yield calculation: Put 1 mL extract on Petri dishes (accurately weighed) and weigh. Put them in an oven at 45 °C. When the Petri dishes with the extract reach a constant weight, calculate yield by subtracting the weight values of extract-containing dishes from the control (empty dishes). It is suggested to perform the calculation in triplicate, expressing the yield value (mg dry extract/mL).
14. For verification that ethanol in jaboticaba hydroalcoholic extract does not impair cells, test only similar concentration of ethanol as used in the MTT assay. The ethanol final concentration should be kept below 0.1 % per well [21].
15. MTT is photosensitive and it is important to protect plates from the light after adding to the culture medium.
16. For an aseptic manipulation, the cover slips should be placed in a 24-well plate with 1 mL 70 % ethanol/well. Leave the plate under UV light for 20 min, followed by washing the cover slips in wells (3×) with sterile PBS.
17. Use and separate different cover slips for each treatment (control, 1, 5, 10, 25, 50, and 100 μg/mL) and time (0 and 12 h). Once the picture has been taken, the cover slip must be discarded to avoid culture contamination.
18. A glass slide could be used above the well to make a straight line.
19. It is important to use PBS with calcium and magnesium to keep cells attached on cover slips.
20. Remove cover slips from the plate and put them on a glass slide with the cell surface upward. Take photographs from scratch in 40× magnification using a built-in camera in the microscope.
21. Click-iT EdU buffer additive can be prepared during this incubation. This reagent must be fresh.
22. Multiply these values by the number of wells that will be used in the experiment.
23. Use diluted Click-iT EdU reaction buffer and Click-iT EdU buffer additive for Click-iT reaction cocktail preparation.
24. Remove cover slips from the plate and put them on a glass slide with the cell surface downward. Put a drop of PBS between the glass slide and the cover slip. Take a picture in 100× magnification.

25. Choose the smallest and the biggest cells and outline these cells using the circular tool on *ImageJ*® and go to *Analyse* → *Measure* to know the total area. Fulfill the box *"Size"* with these values.

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Chapter 6

Biotechnological Approaches for Biomass and Cardenolide Production in *Digitalis purpurea* L.

Naivy Pérez-Alonso, Borys Chong-Pérez, Alina Capote, Anabel Pérez, André Gerth, Geert Angenon, and Elio Jiménez

Abstract

Digitalis purpurea L. is one of the main economically viable sources of cardenolides (cardiac glycosides) for the pharmaceutical industry. Nevertheless, production of cardenolides in plants grown by traditional agriculture is not always an efficient process and can be affected by biotic and abiotic factors. This chapter provides two biotechnology strategies for biomass and cardenolide production in *D. purpurea*. Firstly, we report biomass production using a temporary immersion system (TIS), combined with cardenolide extraction and quantification. Secondly, an efficient protocol for genetic transformation via *Agrobacterium tumefaciens* is provided. These strategies can be used independently or combined in order to increase the content of cardiac glycosides in *D. purpurea* and to unravel biosynthetic pathways associated to cardiac glycoside production.

Key words Cardenolides, Foxglove, Genetic transformation, Temporary immersion system, Secondary metabolites

1 Introduction

Digitalis purpurea L. (foxglove) is an important medicinal plant belonging to the family *Plantaginaceae*. Leaves of *D. purpurea* have been used as medicine for many centuries because of the presence of cardenolides, which can increase the force of systolic contractions and regulate heart rhythms in humans. Nowadays plants are still the sole source for commercial acquisition of cardenolide and only *D. purpurea* and *D. lanata* are of economic interest [1].

The contents of cardiotonic glycosides in *Digitalis* spp. obtained through traditional agriculture are generally low and strongly affected by climate, soil conditions, and genotype (reviewed in ref. [1]). For these reasons, many biotechnological strategies have been developed to enhance the production of biomass and valuable cardenolides from *Digitalis*. Earlier studies demonstrated the use of cell and tissue cultures of members of the

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Digitalis genus for cardenolide production, such as suspension cultures [2], embryogenic cell cultures [3], as well as root [4] or shoot cultures [5]. However, cardenolide content in such cultures is generally low.

Large-scale tissue culture has been considered as an alternative to the traditional methods of culture for the production of biochemicals. Bioreactors are designed for intensive culture and afford maximal opportunity for monitoring and controlling microenvironmental conditions [6]. However, traditional bioreactors are usually quite expensive and complex as regards cleaning, sterilization, inoculation, and harvesting [7, 8]. In order to reduce the costs, increase simplicity of handling, and improve growth and physiological state of the plant material, bioreactors have been designed based on the principle of temporary immersion in liquid medium [9, 10]. Because such temporary immersion systems (TIS) are suitable for large-scale culture of plant organs, they also represent an attractive alternative for production of plant secondary metabolites [11–13].

Genetic engineering is another alternative that could be used to improve cardenolide production both in vitro and in vivo. This tool can be used to unravel the metabolic pathway involved in cardenolide production. Moreover, through genetic engineering breeders can overexpress genes implicated in cardenolide biosynthesis or silence genes belonging to competitive pathways, resulting in the increase of cardiotonic glycosides.

This chapter describes two biotechnological approaches in *Digitalis purpurea*, firstly on the production of biomass and cardenolides employing TIS. The second part describes a detailed reproducible protocol for efficient *Agrobacterium*-mediated transformation, providing a fast and reliable tool for metabolic engineering of cardenolide production and genetic improvement of this species.

2 Material

2.1 Biomass and Cardenolide Production in Temporary Immersion Systems

2.1.1 Plant Material and Surface Sterilization

1. Seeds of *D. purpurea* cv. Berggold as source of explants.
2. 70 % (v/v) ethanol.
3. Sodium hypochlorite (NaOCl) with 5 % of active Cl.
4. Graduated cylinders (1000 mL).
5. 50 mL Sterile tube or similar container for disinfection procedure.
6. Flasks with sterile distilled or deionized water for rinses.
7. Tissue culture facilities and tools (laminar flow cabinet, scalpel, forceps, tool sterilizer such as vertical autoclave and glass-bead sterilizer, culture room) and personal protective equipment (i.e., laboratory coat).

Table 1
Murashige and Skoog (MS) basal medium with vitamins and growth regulators

Compounds		mg/L
Macronutrients		
Calcium chloride	$CaCl_2$	332.02
Potassium dihydrogen phosphate	KH_2PO_4	170.00
Potassium nitrate	KNO_3	1900.00
Magnesium sulfate	$MgSO_4$	180.54
Ammonium nitrate	NH_4NO_3	1650.00
Micronutrients		
Cobalt chloride	$CoCl_2 \cdot 6H_2O$	0.025
Cupric sulfate	$CuSO_4 \cdot 5H_2O$	0.025
Iron sodium EDTA	FeNaEDTA	36.70
Boric acid	H_3BO_3	6.20
Potassium iodide	KI	0.83
Manganese sulfate	$MnSO_4 \cdot H_2O$	16.90
Sodium molybdate	$Na_2MoO_4 \cdot 2H_2O$	0.25
Zinc sulfate	$ZnSO_4 \cdot 7H_2O$	8.60
Vitamins		
Myoinositol		100
Thiamine HCl		1.0
Growth regulators (use for shoot multiplication)		
6-Benzylaminopurine	6-BAP	1.0
Indole-3-acetic acid	IAA	0.1

Add sucrose 3 % (w/v). Adjust the pH of the medium to 5.7 with 0.5 N KOH before autoclaving at 1.2 kg/cm^2 and 121 °C for 20 min. Add Gelrite 3.0 g/L for semisolid medium

2.1.2 *Culture Medium*

1. Medium based on Murashige and Skoog (MS) salts [14]; see medium formulations in Table 1.
2. Sucrose.
3. Growth regulators: Indole-3-acetic acid (IAA) and 6-benzylaminopurine (6-BAP).
4. Gelrite.
5. Potassium hydroxide (KOH) (0.5 N).
6. Hydrochloric acid (HCl) (0.5 N).

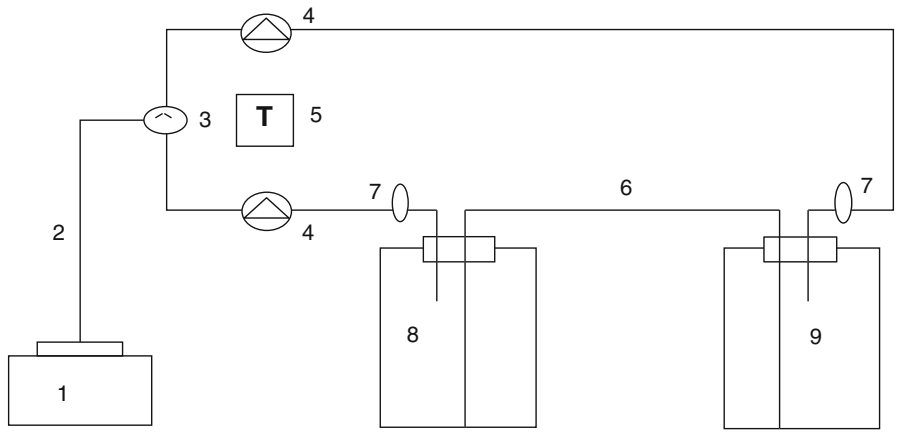

Fig. 1 Diagram of the temporary immersion system showing the different components: (**1**) Air compressor; (**2**) reinforced PVC tubing (ID 10 mm); (**3**) pressure regulation station; (**4**) three-way solenoid electrovalve; (**5**) programmable timer; (**6**) autoclavable silicone tubing (ID 6 mm); (**7**) sterilizable filter (0.22 μm, Midisart 2000, Sartorius AG); (**8**) culture flask; (**9**) medium reservoir vessel

7. Vessels for plant tissue culture: 500 mL Polycarbonate containers.
8. Tissue culture facilities and tools (technical and analytical balances, magnetic stirrer, pH meter, microwave oven or hot plate, refrigerator and −20 °C freezer, autoclave) and personal protective equipment (i.e., laboratory coat).

2.1.3 Temporary Immersion Systems

1. TIS units of 1 L, comprising glass or plastic vessels with all components, are employed (see diagram in Fig. 1).
2. Individual shoots (1.5–2.0 cm long).
3. Tissue culture facilities and tools (laminar flow cabinet, scalpel, forceps, tool sterilizer such as vertical autoclave and glass-bead sterilizer, culture room) and personal protective equipment (i.e., laboratory coat, latex gloves).

2.1.4 Determination of Cardenolide Content in Shoots Cultured in TIS

Extraction Protocol

1. Lyophilizer.
2. Mortars and pestles.
3. Balance.
4. Ultrasonic bath.
5. Micropipettes.
6. Centrifuge.
7. Rotary evaporator.
8. Latex gloves.
9. 500 mL Separatory funnels.
10. Funnels.

11. Flasks with caps.
12. 50 and 500 mL evaporating flasks.
13. 250 mL Erlenmeyer flasks.
14. 100 and 500 mL graduated cylinders.
15. Whatman No. 1 filter paper.
16. 50 mL Falcon tubes.
17. Wash bottle with bi-distilled water.
18. 70 % (v/v) ethanol.
19. $Pb(CH_2COOH)_2{\cdot}2H_2O$ (15 % w/v).
20. $Na_2HPO_4{\cdot}2H_2O$ (10 % w/v).
21. Chloroform/isopropanol (3:2 v/v).
22. Absolute ethanol for HPLC analysis.

HPLC Analysis

1. HPLC, Agilent 1100 equipped with vacuum degasser, quaternary pump, auto sampler, diode array detector, and an Inertsil ODS-3 column (150×4.6 mm; 5 μm).
2. Acetonitrile/water (25:75 v/v).
3. Authentic standards, HPLC-grade digoxin and digitoxin for calibration.

2.2 Agrobacterium tumefaciens-Mediated Genetic Transformation

2.2.1 Equipment, Instruments, and Solutions

1. Micropipette and sterile 1.0 mL and 200 μL pipet tips.
2. Sterile 9 cm Petri dishes.
3. Sterile 9 cm diameter filter paper disks to remove excess *Agrobacterium*.
4. Parafilm® to seal Petri dishes.
5. Sterile 250 mL Erlenmeyer flasks.
6. Sterile conical centrifuge tubes.
7. 50 mL Glass test tubes.
8. Wire loop.
9. Tissue culture chamber.
10. Flasks with bi-distilled or deionized water.
11. 70 % (v/v) ethanol.
12. Orbital shaker.
13. Centrifuge.
14. Spectrophotometer.
15. Tissue culture facilities and tools (pH meter, vertical laminar flow cabinet, scalpel, forceps, tool sterilizer such as vertical autoclave and glass-bead sterilizer, culture room) and personal protective equipment (i.e., laboratory coat, latex gloves).

2.2.2 Plant Material

1. Leaf segments (1.0 cm^2 adaxial surface to the medium) from in vitro plants (second to seventh subculture).

2.2.3 Culture Medium for Bacterial Culture, Cocultivation, and Regeneration of Transformants

1. Antibiotics: Spectinomycin, streptomycin, rifampicin, cefotaxime, timentin, geneticin (G-418).
2. Acetosyringone (4-acetyl-2,6-dimethoxyphenol).
3. Luria Broth (LB) medium and YEP liquid medium for bacterial culture.
4. MS basal medium (Table 1) for inoculation medium, semisolid callus induction medium (CIM), and semisolid regeneration medium.

2.2.4 Agrobacterium tumefaciens Strain and Binary Vector

1. *Agrobacterium* strain C58C1Rif^R containing the helper plasmid pMP90 [15].
2. Binary vector pTJK136 [16] that contains an *aminoglycoside adenyltransferase* marker gene (*aadA*), which confers bacterial resistance to spectinomycin/streptomycin. Moreover a T-DNA with *P35S-uidAint-nos* (35S RNA promoter of the cauliflower mosaic virus, *β-glucuronidase* gene with potato *st-ls1* intron, *nopaline synthase* gene terminator) and *Pnos-nptII-ocs* (*nopaline synthase* gene promoter, *neomycin phosphotransferase* gene, *octopine synthase* gene terminator) as chimeric plant screenable and selectable marker genes, respectively (Fig. 3a).

2.3 Histochemical β-Glucuronidase Assays

1. X-Gluc solution for histochemical analysis [17]: 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) in 0.1 mM phosphate buffer (pH 7.0) containing 10 mM ethylene diamine tetraacetic acid (EDTA), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 0.1 % Triton X-100. Store at −20 °C.
2. 70 % (v/v) ethanol.
3. Micropipette and sterile 1.0 mL pipet tips.
4. Sterile conical centrifuge tubes.
5. Sterile scalpels and forceps.
6. Incubator.

2.4 Molecular Analysis

2.4.1 DNA Isolation from Transformed Tissue

1. Sterile mortars and pestles.
2. Liquid nitrogen.
3. Eppendorf tubes.
4. Micropipettes and tips.
5. Gloves.
6. Extraction buffer: 10 mM Tris–HCl pH 8.0, 1.4 M NaCl pH 8.0, 20 mM ethylene diamine tetraacetic acid (EDTA), 4 % (w/v) cetyltrimethylammonium bromide (CTAB), 2 % (w/v)

polyvinylpyrrolidone (PVP), 10 mM β-mercaptoethanol. Store at room temperature.

7. TAE buffer (50×): Tris (242 g), glacial acetic acid (57.1 mL), EDTA (0.5 M, pH 8.0, 100 mL), adjust pH to 8.0 and make up volume.
8. Agarose molecular grade.
9. TE buffer (10×): 100 mM Tris, 10 mM EDTA, pH 8.0 (with HCl).
10. 24:1 Chloroform-isoamyl alcohol.
11. 70 % (v/v) ethanol.
12. RNase solution.
13. Sodium acetate 3 M.
14. Water bath (set at 55 °C).
15. Vortexer.
16. Fume hood.
17. Spectrophotometer.

2.4.2 Polymerase Chain Reaction

1. For the polymerase chain reaction (PCR) mix: Sterile bi-distilled water, PCR buffer mix, dNTPs, forward primer, reverse primer, Taq DNA polymerase, $MgCl_2$ solution, DNA template.
2. Agarose molecular grade.
3. 200 µL PCR tubes.
4. Crushed ice, ice bucket.
5. Micropipettes and tips.
6. Gloves.
7. Thermal cycler.

2.4.3 Southern Hybridization

1. *Sac*II, store enzyme at –20 °C.
2. Agarose molecular grade.
3. Nylon membrane: Hybond N+ (GE Healthcare).
4. Labeled probe: PCR amplification of the 488 bp *nptII* gene fragment is labeled with DIG-dUTP using a DIG-High Prime DNA Labeling and Detection Kit.
5. Micropipettes and tips.
6. X-ray film, cassette to hold X-ray film, X-ray film developer, clingfilm.
7. Gloves.
8. Others: Materials for Southern hybridization analysis according to standard procedures [18].

3 Methods

3.1 Biomass and Cardenolide Production in Temporary Immersion Systems

3.1.1 Plant Material, Surface Sterilization, and Culture Conditions

1. Add distilled or deionized water up to ½ the final medium volume in a beaker and mix each component of MS basal medium and supplements (Table 1). MS basal medium is supplemented with 30 g/L sucrose for germination phase. For multiplication phase, this medium is also supplemented with vitamins and growth regulators (Table 1) and named MMS. Growth regulators are added to the culture medium prior to sterilization (*see* **Note 1**). Mix the solution until all components are dissolved. Bring to the final volume with distilled or deionized water. Adjust pH to 5.8 with 0.5 N KOH or 0.5 N HCl.
2. Add 3.0 g/L Gelrite, heat until gelling agent is fully dissolved before autoclaving at 1.2 kg/cm^2 and 121 °C for 20 min or add Vitrofural® as chemical sterilization (*see* **Note 2**), and dispense into autoclavable containers. Use 70 mL in each 500 mL culture vessel for germination and multiplication phases. Store the medium in a clean and dark area at 4 °C. Use within 2 weeks.
3. Sterilize all instruments and the laminar flow cabinet before use.
4. In the laminar flow cabinet, place the seeds (Fig. 2a) in tubes with an aqueous solution of sodium hypochlorite (NaOCl) with 5 % of active Cl and shake during 5 min.
5. Rinse seeds three times with sterile distilled or deionized water (*see* **Note 3**).
6. Culture sterile seeds on an MS basal medium and incubate at 27 ± 2 °C under 16-h photoperiod and 125–150 μmol/m^2/s photosynthetic photon flux supplied by cool white fluorescent lamps (*see* **Note 4**).
7. Grow seedlings (Fig. 2b) on MMS culture medium until they have attained 2–4 cm length under the same culture conditions as of **step 6**. Leaves and roots are eliminated. The plantlets (Fig. 2c) are subcultured every 28 days. Divide shoots individually, cut shoots approximately in 1.5–2.0 cm length (Fig. 2d), and use for multiplication on semisolid medium and TIS. Discard any contaminated shoots.

3.1.2 Temporary Immersion Systems

1. Prepare TIS units (Fig. 2e) and connect all components as shown in Fig. 1.
2. Prior to sterilization, add to each TIS 250 mL MMS supplemented as described (Subheading 3.1.1, **step 1**) without Gelrite (*see* **Note 5**).

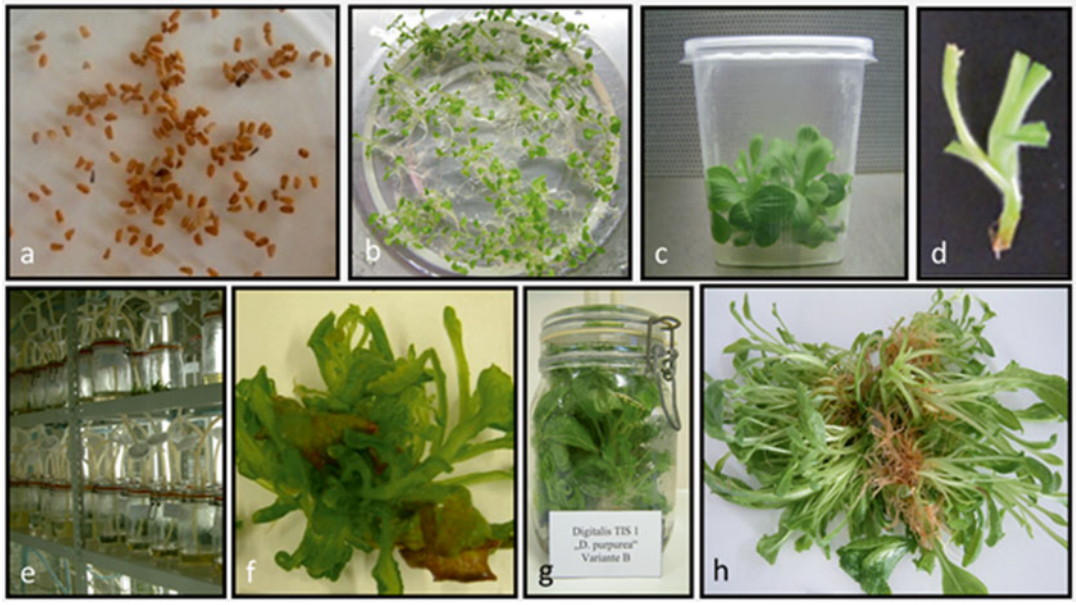

Fig. 2 In vitro propagation of *Digitalis purpurea*, (**a**) seeds as explant source, disinfected with 5 % NaOCl during 5 min, (**b**) seedlings after 15 days growing on MMS culture medium supplemented with 1.0 mg/L 6-BAP and 0.1 mg/L IAA, (**c**) in vitro plants ready for subculturing after 28 days on MMS culture medium, (**d**) 1.5–2.0 cm long individual shoots, (**e**) temporary immersion system used for shoot multiplication, (**f**) hyperhydric shoots, (**g**) plantlets growing on TIS under 2 min of immersion every 4 h, (**h**) harvested biomass from TIS after 28 days

3. Check functioning of TIS before inoculation. Program the timer for some parameters like time and immersion frequency (2 min every 4 h) (*see* **Note 6**).
4. Inoculate 12 individual shoots (1.5–2.0 cm long) per TIS in a laminar flow cabinet.
5. Reconnect TIS to the installation in a culture room at 27 ± 2 °C under 16-h photoperiod and 125–150 μmol/m^2/s photosynthetic photon flux supplied by cool white fluorescent lamps.
6. Collect biomass after 28 days (Fig. 2g, h), rinse with distilled water, and thereafter blot dry on filter paper.
7. Suggested parameters to evaluate: number of hyperhydric shoots, water content (*see* **Note 7**), length and the number of shoots per TIS, and fresh and dry weights (g) produced per TIS, which represent biomass production (*see* **Notes 8** and **9**).

3.1.3 Cardenolide Content

1. Freeze-dry biomass in a lyophilizer (*see* **Note 10**).
2. Finely grind dried in vitro shoots in a mortar.
3. Extract powdered plant material sample (1.5 g) with 15 mL ethanol (70 %, v/v) in an ultrasonic bath at 70 °C for 15 min.
4. After cooling to room temperature add 25 mL bi-distilled water.

5. Add 10 mL lead acetate (15 %) and divide extract in two 50 mL tubes. Rinse flask twice with bi-distilled water.
6. Centrifuge at 3000 × *g* for 5 min.
7. Filter samples using Whatman No. 1 filter paper and rinse filter twice with bi-distilled water in Erlenmeyer flask.
8. Add 12 mL Na_2HPO_4 (10 %), and dispense the extract in four tubes.
9. Centrifuge at 3000 × *g* for 5 min.
10. Filter samples using Whatman No. 1 filter paper and pass extract to a separatory funnel.
11. Add 30 mL chloroform/isopropanol (3/2) and mix.
12. Extract the organic phase to 500 mL evaporating flask (first extraction).
13. Add 20 mL chloroform/isopropanol (3/2) to extract in separatory funnel and mix. Extract the organic phase and repeat again, and mix fractions (*see* **Note 11**).
14. Evaporate under reduced pressure using a rotary evaporator equipped with a water bath held at 40 °C to approximately 1.0 mL, then rinse evaporating flask several times with warm ethanol, transfer the extract to a 50 mL evaporating flask, and evaporate again.
15. Dissolve solid residue with 1.0 mL warm ethanol (ultrasonic bath) and transfer to a vial (1.5 mL) for HPLC analysis.
16. Store prepared samples at 4 °C.
17. Prepare a calibration curve by injecting standards of several known concentrations, digoxin (0–256 μg/mL) and digitoxin (0–625 μg/mL). Plot peak area against concentration.
18. Inject 10 μL of sample in Agilent 1100 HPLC system.
19. Use acetonitrile/water (25/75; v/v) mixture as eluent at 1.5 mL/min flow rate. Carry out measurements at 40 °C (*see* **Note 12**).
20. Detect glycosides at 220 nm wavelength. Compare UV spectra with authentic commercially available standards and identify digitoxin and digoxin on the basis of their retention time.

3.2 Agrobacterium tumefaciens-Mediated Genetic Transformation

3.2.1 Solutions and Culture Media

1. Spectinomycin: Prepare stock solution in bi-distilled water by adding 100 mg/mL. Filter sterilize and store in 1.0 mL aliquots at −20 °C.
2. Streptomycin: Prepare stock solution in bi-distilled water by adding 300 mg/mL. Filter sterilize and store in 1.0 mL aliquots at −20 °C.
3. Rifampicin: Prepare stock solution in bi-distilled water by adding 50 mg/mL. Filter sterilize and store in 1.0 mL aliquots at −20 °C.

4. Timentin (a 15:1 mixture of ticarcillin and clavulanic acid): Prepare stock solution in bi-distilled water by adding 200 mg/mL. Filter sterilize and store in 1.0 mL aliquots at −20 °C.
5. Geneticin (G-418): Prepare stock solution in bi-distilled water by adding 50 mg/mL. Filter sterilize and store in 1.0 mL aliquots at −20 °C. Light sensitive, consequently geneticin-containing medium should be stored in the dark.
6. Cefotaxime: Prepare stock solution in bi-distilled water by adding 500 mg/mL. Filter sterilize and store in 1.0 mL aliquots at −20 °C.
7. Acetosyringone (AS): Prepare stock solution in dimethylsulfoxide at 500 mM (e.g., 0.098 g in 1.0 mL); there is no need to sterilize but solution should always be prepared freshly in a laminar flow.
8. Luria Broth (LB) medium: Dissolve 10 g tryptone, 10 g of yeast extract, and 10 g NaCl in 900 mL of bi-distilled water. Make the volume up to 1.0 L with bi-distilled water. Adjust the pH to 7.5 and add 15 g/L of agar. Supplement with the appropriate antibiotics (100 mg/L spectinomycin and 300 mg/L streptomycin) after cooling the autoclaved medium to 50–55 °C. Dispense 25 mL in sterile 9 cm diameter Petri dishes.
9. YEP liquid medium: Dissolve 5 g/L NaCl, 10 g/L peptone, and 10 g/L yeast extract in bi-distilled water. Adjust pH to 7.5. Supplement with the appropriate antibiotics (100 mg/L spectinomycin and 300 mg/L streptomycin) after cooling the autoclaved medium to 50–55 °C. Dispense 3.0 mL into 15 mL culture tubes and 50 mL into sterile 250 mL Erlenmeyer flasks, according to the scale of the experiment.
10. Inoculation medium: Dissolve 100 % MS salts (Table 1), 20 g/L sucrose, 1.98 g/L D(+)-glucose, and 3.9 g/L 2-[N-morpholino]ethane sulfonic acid (MES) in bi-distilled water and adjust pH to 5.5 before autoclaving. Supplement with 200 μM AS just before inoculation.
11. Semisolid callus induction medium (CIM): Dissolve 100 % MS salts (Table 1), 4.0 mg/L thiamine HCl, 100 mg/L myoinositol, 30 g/L sucrose, and 3.0 g/L Gelrite in bi-distilled water. Adjust pH to 5.8 before autoclaving.
12. Semisolid regeneration medium: Dissolve 100 % MS salts (Table 1), 0.57 μM IAA, 4.4 μM BAP, 30 g/L sucrose, and 2.5 g/L Gelrite. Adjust pH to 5.7 before autoclaving.

3.2.2 Growth of Agrobacterium Cultures and Cocultivation

Isolation of Single Bacterial Colonies from a Glycerol Stock

1. Using a 200 μL pipet tip, scratch a small amount from the surface of the frozen glycerol bacterial stock (*see* **Note 13**) and drop onto the surface of the selective semisolid LB plate. Place back the frozen bacterial stock immediately into the freezer to avoid thawing.
2. Using a flamed and cooled wire loop, streak cells across the selective semisolid LB plate to spread bacteria.

3. Incubate the inoculated selective LB plate upside-down at 28 °C until single colonies are visible.

Inoculation with *Agrobacterium* and Cocultivation

1. Select 2–3 isolated single colonies from the plate using sterile pipet tips.
2. Inoculate one colony per 15 mL tube containing YEP medium supplemented with 100 mg/L rifampicin, 100 mg/L spectinomycin, and 300 mg/L streptomycin.
3. Grow *Agrobacterium* cultures overnight on an incubating shaker set at 200 rpm and 28 °C. Approximate OD_{600nm} at harvest: 1.5–2.0.
4. Collect 50 μL of grown culture and inoculate 250 mL Erlenmeyer flask containing YEP medium supplemented with 100 mg/L spectinomycin and 300 mg/L streptomycin.
5. Grow *Agrobacterium* culture overnight on an incubating shaker set at 200 rpm and 28 °C. Approximate OD_{600nm} at harvest: 1.2–1.5.
6. Pellet bacterial cells by centrifugation for 10 min at 3200 × *g*.
7. Discard supernatant and gently wash the pellet once with inoculation medium described above (Subheading 3.2.1, **step 10**).
8. Discard washing medium and gently resuspend the pellet in inoculation medium. Measure the $OD_{600\ nm}$ and adjust with the above medium to 0.7 units (*see* **Note 14**).
9. Place 30–40 leaf discs (1 cm^2) in capped 50 mL disposable polypropylene conical centrifuge tube containing 30–40 mL of *Agrobacterium* suspension (*see* **Note 15**, Fig. 3b).
10. Incubate tubes containing *Agrobacterium* suspension and leaf segments at room temperature for 15 min with gentle manual agitation every 2 min.
11. Take away leaf segments from *Agrobacterium* suspension and remove excess bacteria from explants by blotting them on sterile filter paper using sterile forceps (*see* **Note 16**).
12. Transfer leaf segments (adaxial surface to the medium, five segments per plate) to cocultivation on CIM (Subheading 3.2.1, **step 11**) supplemented with 200 μM AS. Incubate plates in the dark at 21 °C for 5 days.

3.2.3 Histochemical β-Glucuronidase Assays

Transient GUS expression is determined in leaf segments inoculated with *Agrobacterium* strain.

1. After cocultivation, dip untransformed control and transgenic leaves into X-Gluc solution and incubate at 37 °C in the dark for 6 h or overnight.
2. After incubation, remove X-Gluc solution, add the same volume of 70 % (v/v) ethanol, and incubate overnight to

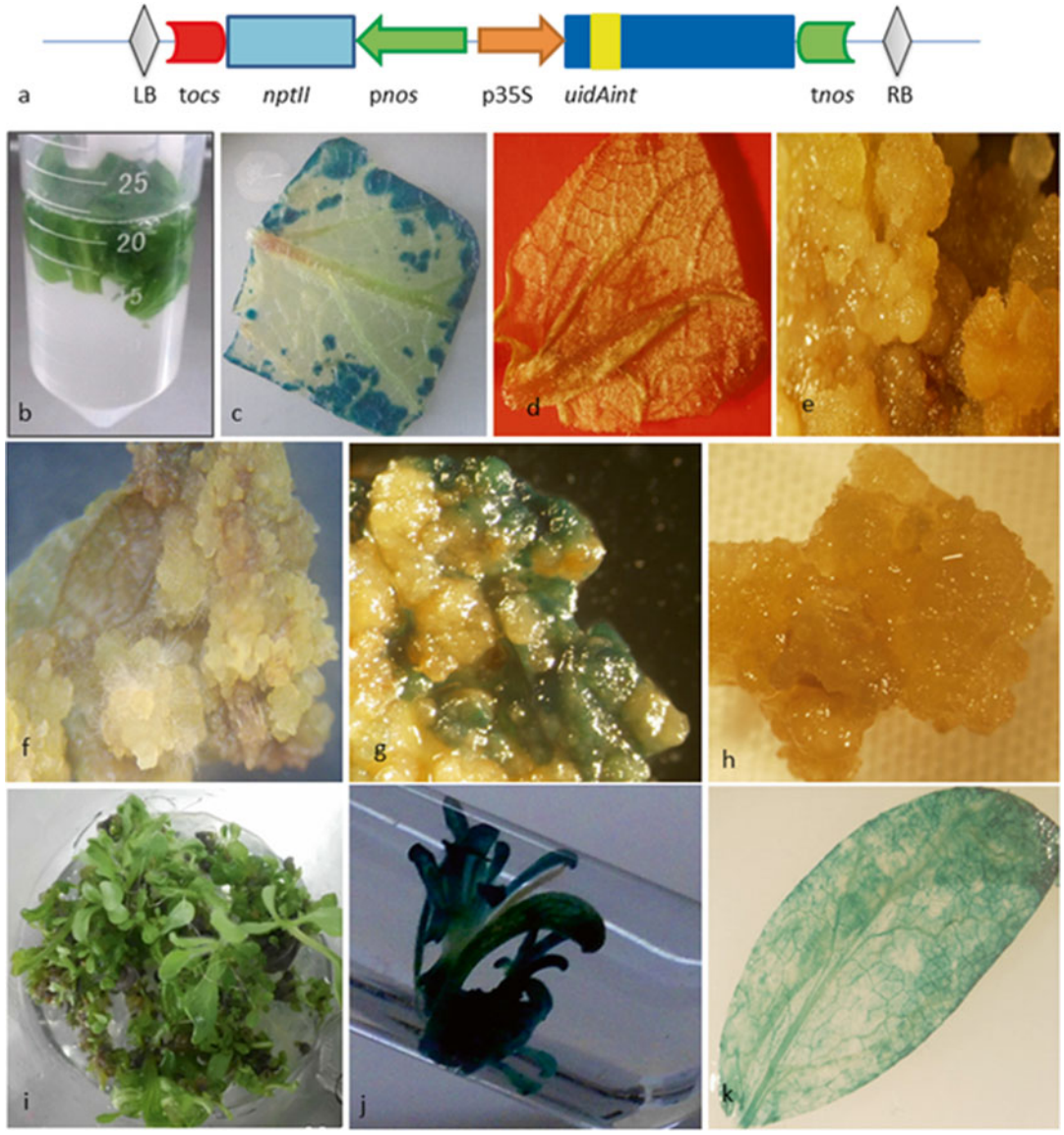

Fig. 3 *Agrobacterium tumefaciens*-mediated genetic transformation of *Digitalis purpurea* L. (**a**) T-DNA region of a pTJK136 [16], LB and RB, left border and right border, respectively; P35S, cauliflower mosaic virus 35S RNA promoter; *uidA*-intron, intron-interrupted *β-glucuronidase* gene; P*nos* and T*nos*, *nopaline synthase* gene promoter and polyadenylation signal; *nptII*, *neomycin phosphotransferase* gene; T*ocs*, octopine synthase gene polyadenylation signal, (**b**) inoculated leaf segments in tubes containing *Agrobacterium* suspension, (**c**) transient GUS expression in a leaf inoculated with C58C1Rif^{R} (pMP90) (pTJK136), (**d**) untransformed leaf, (**e**) callus formation from untransformed leaf on CIM without antibiotics, (**f**) callus formation from transformed leaf segment on CIM with 70 mg/L G-418, (**g**) stable GUS expression in transformed callus, (**h**) callus from untransformed segments without GUS-positive spots, (**i**) regenerated plants from transformed segments. Stable GUS expression in transformed (**j**) plantlets and (**k**) leaf

remove chlorophyll and other pigments prior to visual analysis and photographing. Note blue coloration indicating transient expression of the *uidA* gene (Fig. 3c, d).

3.2.4 Selection and Regeneration of Transformants

1. Transfer inoculated leaf segments into 50 mL centrifugation tubes; rinse twice with 30–40 mL sterile liquid CIM containing 200 mg/L timentin and 500 mg/L cefotaxime.
2. Blot leaf segments dry on sterile filter paper.
3. Place leaf segments onto CIM containing 70 mg/L G-418 as selection agent and 200 mg/L timentin to inhibit *Agrobacterium* growth (*see* **Note 17**).
4. Incubate selection plates for 8–12 weeks at 27 ± 2 °C in the dark with subculture to fresh selective plates every 2 weeks until calli form (Fig. 3e, f).
5. Perform histochemical β-glucuronidase assay of calli as described above (Subheading 3.2.3, Fig. 3g). Non-transgenic calli are simultaneously stained in the same way (Fig. 3h).
6. Transfer the individual putatively transformed callus induced on selection medium to 9 cm Petri dishes containing regeneration medium described above (Subheading 3.2.1, **step 12**) and keep in a growth chamber at 27 ± 2 °C under 16-h photoperiod and 70 μmol/m²/s photosynthetic photon flux supplied by cool white fluorescent lamps.
7. Transfer differentiated shoots individually (Fig. 3i) to sterile 50 mL glass test tubes containing MMS medium.
8. Stable GUS expression in regenerated plants is assessed as described above (Subheading 3.2.3, Fig. 3j, k). Non-transgenic regenerated plants are simultaneously stained in the same way.

3.3 Molecular Analysis

Total DNA is extracted following the protocol described by Khayat et al. [19] with minor modifications.

3.3.1 DNA Isolation from Transformed Tissue

1. Take 1 g leaf tissue from a transformed *Digitalis* plant. Grind tissue to fine powder in a mortar with liquid nitrogen.
2. Add 10 mL of extraction buffer (Subheading 2.4.1, **step 6**) per gram of tissue and shake vigorously using vortexer (*see* **Note 18**).
3. Incubate the mixture at 55 °C for 30 min with occasional inversion.
4. Cool the mixture on ice for 5 min.
5. Centrifuge at 3200 × *g*, for 5 min at 4 °C. Transfer the supernatant to a new tube.
6. Remove RNA by adding RNase to a final concentration of 200 μg/mL and incubate at 37 °C for 15 min.

Table 2
Sequence of the primers used for *nptII* and *uidA* gene fragment amplification by PCR

Gene	Sequence		
	Forward	Reverse	(bp)
nptII	5′ATGATTGAACAAGATGGA TTGCACGC 3′	5′TGATGCTCTTCGTCC AGATCATC 3′	488
uidA	5′AACGGCAAGAAAAAGCAGTC 3′	5′ GAGCGTCGCAGAACATTACA3′	1031

7. Add an equal volume of chloroform-isoamyl alcohol (24:1) to the above mixture and separate the two phases by centrifuging at 12,857 × *g* for 10 min at 4 °C.
8. Transfer the upper aqueous phase to a fresh tube.
9. Add an equal volume of isopropanol and mix gently by inversion. Incubate for 60 min at −80 °C or overnight at −20 °C.
10. Pellet genomic DNA by centrifugation at 18,500 × *g* for 15 min at 4 °C.
11. Wash the pellet once with 1.0 mL 70 % (v/v) ethanol.
12. Centrifuge at 18,500 × *g* for 15 min at 4 °C and discard ethanol.
13. Vacuum dry pellet for 5 min and dissolve in 30 μL of TE buffer (1×).
14. Store at −20 °C.
15. Check DNA by agarose gel electrophoresis and quantify 1:10 dilutions of DNA in spectrophotometer at 260/230 and 260/280 nm. DNA can be used for either PCR or Southern hybridization analysis.

3.3.2 PCR

PCR is performed using genomic DNA of each plant as a target. pTJK136 plasmid isolated from *Escherichia coli* DH5α-pTJK136 strain is used as positive control. Bacterial culture is grown overnight on an incubating shaker set at 200 rpm and 37 °C. Plasmid DNA is isolated using Purification kit Wizard plus SV Minipreps.

The primer sequences for analyzing tissues transformed with *nptII* and *uidA* genes are given in Table 2.

1. Fill out a PCR worksheet with the sample identifiers of all samples to be used.
2. Label PCR tubes with sample identifier numbers from the PCR worksheet.
3. Place PCR tubes in the freezer rack to keep cold.

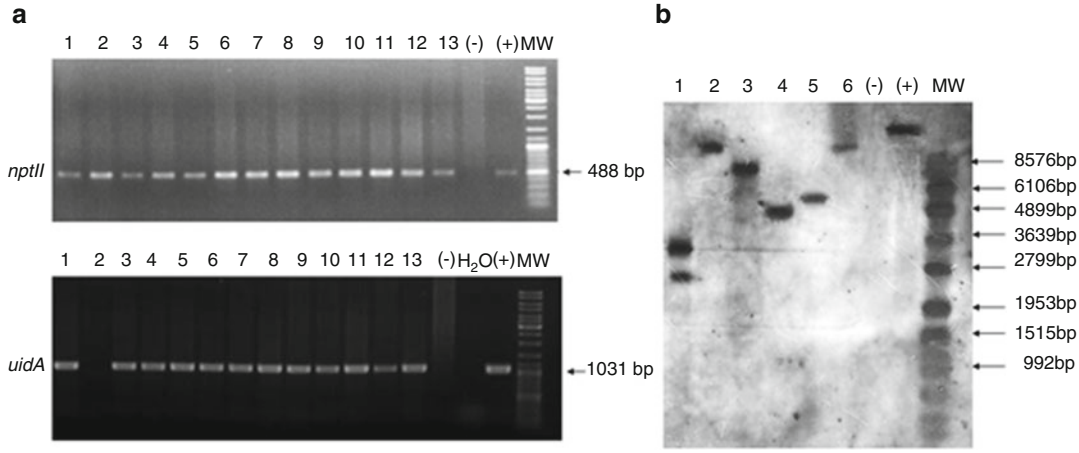

Fig. 4 Molecular analysis of putative transformed *Digitalis purpurea* L. plantlets. (**a**) PCR analysis for the *nptII* and *uidA* genes in transgenic tissues, *lane 1–13* genomic DNA from putative transgenic lines obtained with C58C1Rif^{R} (pMP90) (pTJK136), (–) untransformed control, (+) pTJK136 plasmid control, MW molecular weight marker Gene Ruler™ DNA Ladder Mix (Fermentas). (**b**) Southern hybridization of putative transformed *D. purpurea* L. PCR-positive plantlets, *lanes 1–6* genomic DNA from putative transgenic lines obtained with C58C1Rif^{R} (pMP90) (pTJK136), (–) untransformed plant, (+) pTJK136 plasmid control, MW digoxigenin-labeled DNA molecular weight marker VII (Roche)

4. Calculate the amount of each component needed for the total number of samples taking into account that PCR amplification reactions are carried out in 25 µL of total volume (*see* **Note 19**).
5. Close caps of all tubes firmly and place the tubes into the thermal cycler.
6. Start the program (denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 or 60 s, with a final extension at 72 °C for 10 min).
7. After the PCR is complete, analyze amplified fragments by electrophoresis at 100 V for 1 h on 1.0 % Tris-acetate-EDTA agarose gel followed by staining in ethidium bromide (5 µg/mL) and detection and photography under UV illumination. An *nptII*- and *uidA*-specific band of 488 bp and 1031 bp, respectively, is observed in the transformed plants while being absent in the untransformed control plants and water (Fig. 4a, *see* **Note 20**).

3.3.3 Southern Hybridization

The integration of the T-DNAs of randomly selected PCR-positive lines is analyzed by Southern blot hybridization (*see* Fig. 4b).

1. Digest 20 µg of genomic DNA with *Sac*II.
2. Separate digested genomic DNA samples on a 0.8 % Tris-acetate-EDTA agarose gel (w/v) run at 25 V for 12 h.

3. Blot DNA to a Hybond-N+ nylon membrane (RPN203B; GE Healthcare) by upward capillarity forces using 20× SSC buffer (3 M NaCl, 0.3 M sodium citrate, pH 7.0) overnight at room temperature.
4. Label the 488-bp *nptII* gene fragment, amplified from plasmid pTJK136 with the same primers mentioned above (Table 2), with DIG-dUTP using a DIG-High Prime DNA Labeling and Detection Kit.
5. Perform hybridization, washing, and detection according to the manufacturer's instructions.
6. Expose membrane to X-ray film in the presence of an intensifying screen for 30 min at room temperature.

4 Notes

1. BAP: Stock solution at 1 mM. Dissolve 22.52 mg BAP in a few drops of 1 M sodium hydroxide, dilute to 100 mL with distilled or deionized water, and store at 4 °C. IAA: Stock solution at 1 mM. Dissolve 8.76 mg IAA in a few drops of 1 M sodium hydroxide, dilute to 50 mL with distilled or deionized water, and store at 4 °C.
2. It is possible to use chemical sterilization with Vitrofural® (2-bromo-5-(2-bromo-2-nitrovinyl)-furan) 114 mg/L. Firstly, boil full medium to dissolve Gelrite (3.0 g/L), then dissolve this compound into 1/10 part of hot medium (~90 °C), and immediately add to the rest boiled medium. This compound is used by adding in culture vessels for seed germination and shoot multiplication on semisolid medium. All the culture vessels used in chemical sterilization with Vitrofural® are previously rinsed in sodium hypochlorite solution at 0.05 % (v/v). Store the medium in a clean area under dark conditions at least 3 days before use. Vitrofural® has therapeutic effect [20] and has allowed the substitution of the autoclave in the sterilization of culture medium used for the micropropagation of banana, plantain, potato, and sugarcane [21, 22]. However, this antimicrobial compound has never been used in *Digitalis* culture. For shoot multiplication in TIS, the use of Vitrofural® is not convenient taking into account that TIS has several components and that it is very difficult to completely sterilize all of them. On the other hand, previous studies have shown that *Digitalis* leaves are very sensitive to direct contact with Vitrofural® when liquid medium is used (unpublished data).
3. Be careful, *Digitalis* seeds are very small and it is necessary to use a filter when they are rinsed. It is possible to obtain less than 1 % of contamination with this simple disinfection protocol.

4. After 15 days, seed germination rate is more than 80 %. In a tropical location it is possible to incubate seeds and seedlings under natural light conditions, photoperiod 13/11 h and 20–45 μmol/m²/s on average.
5. Employ physical sterilization for TIS, and autoclave at 1.2 kg/cm² and 121 °C for 30 min. Prepare TIS carefully before autoclaving; all components are rinsed in sodium hypochlorite solution at 0.05 % (v/v).
6. Duration and frequency of immersions affect nutrient supply, composition of the internal atmosphere in the culture vessel, and occurrence of hyperhydricity [9]. The latter phenomenon concerns morphological, anatomical, and physiological disorders. Shoots are categorized as hyperhydric shoots according to their external appearance [23–25] (Fig. 2f shows the morphology of hyperhydric shoots). Hyperhydric shoots appear turgid, watery at their surface, and hypolignified, in some cases less green and easily breakable. In *D. purpurea*, the best biomass production (values for fresh (106.2 g) and dry weight (5.82 g) per 1 L flask) and lowest percentage of hyperhydricity were obtained using 2 min immersion every 4 h [26].
7. Dry weight is recorded after the biomass is dried to constant weight at 60 °C. The water content is an important variable to evaluate because it shows the shoot quality and allows determining favorable conditions to avoid hyperhydricity. Water content (WC) is calculated as [27]

$$\mathrm{WC}\% = \frac{(\mathrm{FW} - \mathrm{DW})}{\mathrm{FW}} \times 100$$

8. Since cardenolide biosynthesis is basically dependent on morphological differentiation [28], there are attempts to use organ culture, which is easily produced in TIS. The protocol described for *D. purpurea* biomass production in TIS is also applicable for biomass and cardenolide production in *D. lanata* shoots. Yields are different because of genotype influence. The most important reason for the efficacy of TIS is that they combine ventilation of the plant tissues and intermittent contact between the entire surface of the tissue and the liquid medium [9], which results in increased growth rates and biomass production.
9. Elicitation is one of the most effective methods to enhance the production of several secondary metabolites from medicinal plants [29]. Our results suggest that elicitation of *Digitalis* shoots cultivated in TIS or semisolid medium could influence the competition between biomass and secondary metabolite production [30, 31]. Elicitors as Chitoplant® and Silioplant® are effective to increase cardenolide content in shoots of *D. purpurea* and *D. lanata*. In *D. lanata*, the highest accumulation of lanatoside C was achieved with Chitoplant® (0.1 g/L),

which resulted in 316 μg/g-DW, and with Silioplant® (0.01 g/L) giving 310 μg/g-DW; this accounted for a 2.2-fold increase in lanatoside C content compared to non-elicited shoot cultures in TIS [30]. The optimization of elicitor treatment may improve TIS performance. Also, SilioPlant® (0.01 g/L) did not affect biomass production and at the same time increased 3.6-fold and 6.9-fold the digoxin and digitoxin content, respectively, in *D. purpurea* shoots cultured in semisolid medium [31]. Elicitors are dissolved in distilled or deionized water and added to the culture medium before sterilization. A set of control cultures without elicitors could be included.

10. Alternatively, dry plant material in an incubator at 60 °C during 36 h until constant weight. This drying method is less convenient due to the loss of some organic compounds during the process, although not in significant amounts.
11. Due to the minor contents of pharmaceutical agents we extracted only twice with the 20 mL mixture. If there are high contents, the extraction could be repeated. In order to eliminate water, Na_2SO_4 could be added, mix it by hand, centrifuge at $11{,}500 \times g$ for 1 min, and pass the organic phase to new flask. The solution should be clear.
12. The gradient elution profile is $4 \rightarrow 34$ min, A (acetonitrile) = 25 %; $34 \rightarrow 45$ min, A = 37 %; $45 \rightarrow 60$ min, A = 50 %; $60 \rightarrow 65$ min, A = 25 %.
13. Bacterial stocks are maintained in 20 % (v/v) glycerol-containing growing medium with appropriate antibiotics and stored at −80 °C.
14. More dense bacterial cultures may cause overgrowth during cocultivation.
15. Include extra control samples for the transformation and selection process and for assessing transient reporter gene expression. In order to control the selection process place untransformed leaf segments on antibiotic-free CIM as well as on a medium supplemented with both geneticin and timentin. Finally, include several control samples, which will not be infected with *Agrobacterium* and are cultured on nonselective medium to assess the callus formation and regeneration capacity and to provide untransformed control plants for later analyses.
16. Sterilize paper towels by wrapping in aluminum foil and autoclaving for 20 min at 121 °C.
17. The selection agent used will depend on the selectable marker gene (SMG) present on the T-DNA of the binary vector. Only those plant cells containing the T-DNA harboring the SMG will survive in the presence of the selection agent to which the

marker provides resistance. The *nptII* gene is one of the most commonly used selectable markers; it confers resistance to kanamycin, geneticin (G-418), and paromomycin. For callus induction in *D. purpurea* geneticin can be used as selective agent at 70 mg/L. Geneticin is light sensitive, so store and incubate selective CIM in the dark. The timentin is incorporated into the medium to kill the *Agrobacterium*. At a concentration of 200 mg/L, timentin is effective in eradicating several *Agrobacterium* strains (e.g., C58C1Rif^R (pMP90), LBA4404, EHA101, EHA105) without evident damage to plant cells.

18. Add β-mercaptoethanol in fume hood just before use.
19. PCR amplification reactions contain 200 ng genomic DNA, 0.5 μM of each primer, 1.5 mM $MgCl_2$, 200 mM dNTP, 1X Taq polymerase reaction buffer, and 0.25 U Taq polymerase. Prepare a master mix without template DNA in a single tube, which can then be aliquoted into individual tubes (number of samples +1, components are provided in excess of the required amount to minimize the possibility of pipetting errors and to save time). Be sure to mix the PCR master mix well before aliquoting it into the sample tubes. Template DNA and primers are added to individual samples. Include a negative control (PCR tube with water instead of genomic DNA) to check contamination. Prepare each sample twice (one for each pair of primers used for both genes: *nptII* and *uidA*).
20. A few non-transgenic cells are protected from the selection agent by the high number of calli obtained per leaf segment and escapes occurred. With this protocol, only four GUS-negative lines obtained with strain C58C1Rif^R (pMP90) showed neither *uidA* nor *nptII* genes (4.6 % escapes). Perhaps the increase of selection pressure (e.g., by applying an antibiotic gradient) could prevent this problem. As observed in Fig. 4a, one GUS-negative line (lane 2) did not contain the *uidA* gene but the *nptII* gene was present. These results indicate the integration of a truncated T-DNA. All transgenic plants analyzed that were positive for the presence of the *uidA* gene in the PCR assay also showed constitutive GUS expression in leaves, indicating the presence of a full functional transgene.

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Chapter 7

In Vitro Regeneration of Endangered Medicinal Plant *Heliotropium kotschyi* (Ramram)

Manal Ahmed Sadeq, Malabika Roy Pathak, Ahmed Ali Salih, Mohammed Abido, and Asma Abahussain

Abstract

Heliotropium kotschyi (Ramram) is an important endangered medicinal plant distributed in the Kingdom of Bahrain. Plant tissue culture technique is applied for ex situ conservation study. Nodal stem segments are cultured in modified MS media supplemented with various combination and concentration of plant growth regulators (PGRs). Plants are regenerated via shoot organogenesis from the nodal meristems. Plants are regenerated in three different steps: initial shoot development, shoot multiplication, and rooting. After 4 weeks of culture, 100 % explants respond to shoot initiation on the medium containing 8.88 μM BAP and 5.71 μM IAA. The highest frequency of shoot regeneration is observed in the same media after second subculture of shoots. The highest rooting frequency is observed in the presence of 2.85 μM IAA. After root development, the plantlets are transferred to pots filled with soil and 60 % of plants survived after 45 days. This plant regeneration protocol is of great value for rapid desert plant propagation program.

Key words Endangered, Ex situ conservation, *Heliotropium kotschyi*, Organogenesis, Plant regeneration

1 Introduction

Desert plants in dry ecosystems owe their importance to a long history of evolution and adaptation in dry and hot deserts. They are important as a source of good gene pool in food chain, extreme environmental adaptability, herbal medicine for human health, etc. Also, they prevent soil erosion for maintaining soil fertility. In Bahrain, 81 plant species are indigenous and reported to be used in traditional herbal medicine [1]. The importance of medicinal plants both in drug research and genetic biodiversity conservation is now well recognized.

Heliotropium kotschyi (local name Ramram) belongs to Boraginaceae family, and is an endangered, important medicinal

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plant distributed in Bahrain [2]. The plant is in general used as an antidote for snake venom; it is used so either by drinking the water extract of leaves or by applying leaf paste on the snake bite [3]. Global worry about the loss of valuable plant genetic resources has stimulated many advanced programs to conserve genetic diversity using either in situ or ex situ conservation strategies [4]. The application of in vitro culture as an important conservation tool of the rare, threatened, and endangered plants has gained a huge drive in the last two decades and is considered as one of the greatly applicable program of ex situ conservation strategy [5–8]. Different techniques of plant tissue culture are used for propagation, rapid proliferation, preservation, and storage of several medicinally important endangered and threatened plants [8, 9]. Considering the multipurpose importance of endangered desert plants, the biotechnological tool of plant tissue culture is applied to multiply them rapidly for conservation purposes [2, 9]. Here, we describe a highly efficient in vitro plant regeneration protocol for *H. kotschyi*.

2 Materials

2.1 Explant

1. Collection of apical shoots of 1-year-old Ramram (*Heliotropium kotschyi*) plants (Fig. 1a) from Al-Areen Wildlife Park in the Kingdom of Bahrain (*see* **Note 1**)

2.2 Chemicals

1. Murashige and Skoog (MS) medium [10] (*see* **Note 2**).
2. Stock solutions of vitamins (*see* **Note 3**).
3. Stock solutions of plant growth regulators (*see* **Note 4**).
4. 1 % (v/v) Lux solution (Soap).
5. 0.5 % mercuric chloride solution.
6. Tween 20.
7. 0.1 % Copral solution.
8. 50 % (v/v) Clorox solution.
9. 70 % (v/v) ethanol.
10. 0.1 M hydrochloric acid (HCl).
11. 0.1 M sodium hydroxide (NaOH).
12. Casein hydrolysate.
13. Sucrose.
14. Agar.

2.3 Equipment

1. Balance.
2. Measuring cylinder.

Fig. 1 Stages of micropropagation of *Heliotropium kotschyi*. Field-grown plants (**a**); stem segment for surface sterilization (**b**); explants in media 1 after 4 weeks (**c**); explants show shoot initiation response in media 4 after 2 weeks (**d**); initiated shoots in media 4 after 4 weeks (**e**); multiplication of initially developed shoot in first transferred media after 4 weeks (**f**); multiplication of shoots in second transferred media after 4 weeks (**g**); micro-shoots developing roots (**h**); plantlet growing in pots containing soil (**i**)

3. Magenta vessels.
4. pH meter.
5. Autoclave.
6. Millipore water, autoclaved.
7. Laminar flow air cabinet.
8. Refrigerator.
9. Plant culture room.

3 Methods

3.1 Preparation of Culture Media

1. Dissolve 4.4 g MS powder in 800 ml autoclaved water and supplement with 0.3 % casein hydrolysate, 3 % sucrose, 0.1 % nicotinic acid, 0.1 % pyridoxine–HCl, 1 % thiamine–HCl. Adjust pH of the medium to 5.8 by adding NaOH, make up the volume to 1 L, solidify by adding 0.9 % agar, and autoclave at 121 °C, for 20 min at 15 psi.
2. Add different concentrations and combinations of filter-sterilized plant growth regulators (PGRs) such as 6-benzylaminopurine (BAP), kinetin (KI), 3-indoleacetic acid (IAA), 1-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA) in the autoclaved modified MS media for culture initiation, shoot multiplication, and rooting (Tables 1 and 2).

3.2 Explant Sterilization

1. Use apical shoots of *H. kotschyi* (Fig. 1b) for surface sterilization (*see* **Note 5**).

3.3 Culture Initiation and Plant Regeneration

1. Culture initiation experiments follow completely randomized design (CRD) with three replicates and 3–5 explants per replication.
2. Transfer surface-sterilized nodal stem segments to culture initiation media containing various concentrations and combinations of PGRs (Table 1).
3. Incubate cultures in culture room for 4 weeks to see the initial response. The culture room should provide 40–50 μmol/m^2 s^{-1} fluorescent light intensity for 16 h photoperiod, 24 °C± 2 °C temperature, and 70–80 % relative humidity.
4. MS media without any PGRs show no shoot initiation response (Fig. 1c).
5. The nodal explants start shoot initiation in media containing PGRs within 2–4 weeks of culture initiation (Fig. 1d).
6. Initially developed shoots show good growth after 4 weeks of culture and 100 % explants respond to initiate shoot development in the presence of 8.88 μM BAP with 5.71 μM IAA after 4 weeks (Fig. 2) (*see* **Note 6**).
7. A comparison of shoot initiation frequencies in different PGRs combinations and concentrations is shown in (Table 3) (*see* **Note 7**). The highest shoot initiation frequency has been noticed in the presence of 8.88 μM BAP with 5.71 μM IAA (Fig. 1e).
8. Transfer initially developed shoots to different multiplication media (Table 4) and observe the best performance in subculture media containing 8.88 μM BAP with 5.71 μM IAA (Fig. 1f) (*see* **Note 8**).

Table 1
Concentration and combination of PGRs in modified MS media for culture initiation and multiplication

Name of media	Concentration of PGRs (μM)
1	Medium without hormone
2	4.44 μM BAP + 2.85 μM IAA
3	6.66 μM BAP + 2.85 μM IAA
4	8.88 μM BAP + 5.71 μM IAA
5	13.3 μM BAP + 5.71 μM IAA
6	4.44 μM BAP + 2.68 μM NAA
7	6.66 μM BAP + 2.68 μM NAA
8	8.88 μM BAP + 5.37 μM NAA
9	13.3 μM BAP + 5.37 μM NAA
10	4.65 μM KI + 2.85 μM IAA
11	6.97 μM KI + 2.85 μM IAA
12	9.29 μM KI + 5.71 μM IAA
13	13.9 μM KI + 5.71 μM IAA
14	4.65 μM KI + 2.68 μM NAA
15	6.97 μM KI + 2.68 μM NAA
16	9.29 μM KI + 5.37 μM NAA
17	13.9 μM KI + 5.37 μM NAA
18	0.89 μM BAP
19	2.22 μM BAP
20	4.44 μM BAP
21	8.88 μM BAP

Table 2
Concentration and combination of PGRs in modified MS media for subculture and rooting

Name of media	Concentration of PGRs (μM)
22	8.88 μM BAP + 1.14 μM IAA
26	1 % charcoal + 7.4 μM IBA
27	1 % charcoal + 9.84 μM IBA
28	0.93 μM KI + 5.71 μM IAA
30	2.85 μM IAA

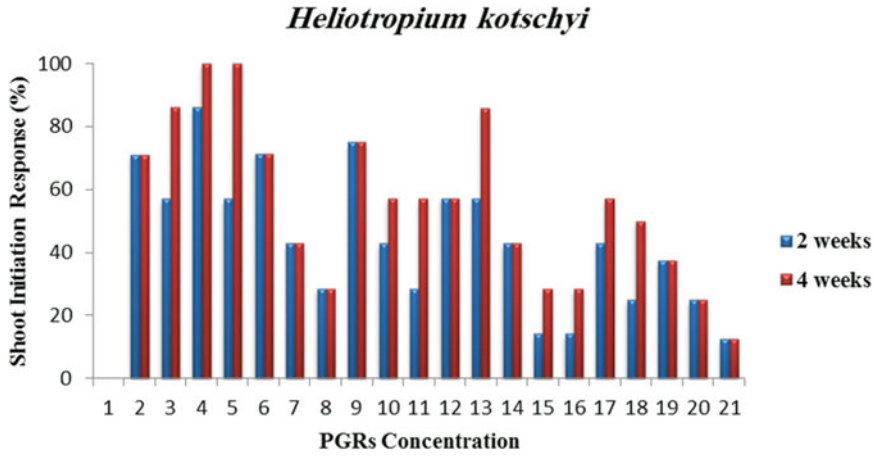

Fig. 2 Effect of various plant growth regulators supplemented to modified MS medium on in vitro shoot initiation from nodal segments of explants of *H. kotschyi* after 2 and 4 weeks of culture. PGR concentration in Table 1

Table 3
Effect of various plant growth regulators supplemented to modified MS media on in vitro shoot initiation response from shoot apex and nodal explants of *H. kotschyi* after 4 weeks of culture. Results are means of shoots developed per explant. Means followed by the same letter are not significantly different at $P \leq 0.05$

Name of media	PGR concentrations (µM)	Mean
4	8.88 µM BAP + 5.71 µM IAA	10.66 A
5	13.3 µM BAP + 5.71 µM IAA	8 B
3	6.66 µM BAP + 2.85 µM IAA	5.33 C
13	13.9 µM KI + 5.71 µM IAA	4 D
10	4.65 µM KI + 2.85 µM IAA	3.66 D E
14	4.65 µM KI + 2.68 µM NAA	3.66 D E
9	13.3 µM BAP + 5.37 µM NAA	3 D E F
11	6.97 µM KI + 2.85 µM IAA	3 D E F
12	9.29 µM KI + 5.71 µM IAA	3 D E F
2	4.44 µM BAP + 2.85 µM IAA	2.66 E F G
19	2.22 µM BAP	2.66 E F G
6	4.44 µM BAP + 2.68 µM NAA	2 F G
15	6.97 µM KI + 2.68 µM NAA	2 F G
17	13.9 µM KI + 5.37 µM NAA	2 F G
20	4.44 µM BAP	2 F G

continued

Table 3
(continued)

Name of media	PGR concentrations (μM)	Mean
7	6.66 μM BAP + 2.68 μM NAA	1.66 G
18	0.89 μM BAP	1.66 G
8	8.88 μM BAP + 5.37 μM NAA	1 H
16	9.29 μM KI + 5.37 μM NAA	1 H I
21	8.88 μM BAP	1 H I
1	Medium without PGRs	0 I

Table 4
Effect of BAP, KI, IAA, IBA, and NAA on percentage of shoot multiplication frequency after first transfer of *H. kotschyi*. PGR concentration in Tables 1 and 2

Name of media	Percentage of proliferated shoot	Shoot multiplication frequency
1	0	0
3 → 4	100	15
4 → 4	92.3	12.07
5 → 4	75	2.5
6 → 4	87.5	8.1
7 → 4	33.3	3.33
7 → 28	100	12.5
8 → 4	50	7.5
9 → 4	100	5
20 → 22	100	7.6

9. Calculate plant regeneration capacity of *H. kotschyi* based on shoot initiation frequency of explants and multiplication frequency of initially developed shoots after first and second transfer (Fig. 3) (*see* **Note 9**).
10. Transfer newly formed micro-shoots, 1–2 cm long to rooting media containing 2.85 μM IAA (Fig. 1h) (*see* **Note 10**).
11. Transfer plantlets to pots containing soil (Fig. 1i) (*see* **Note 11**).

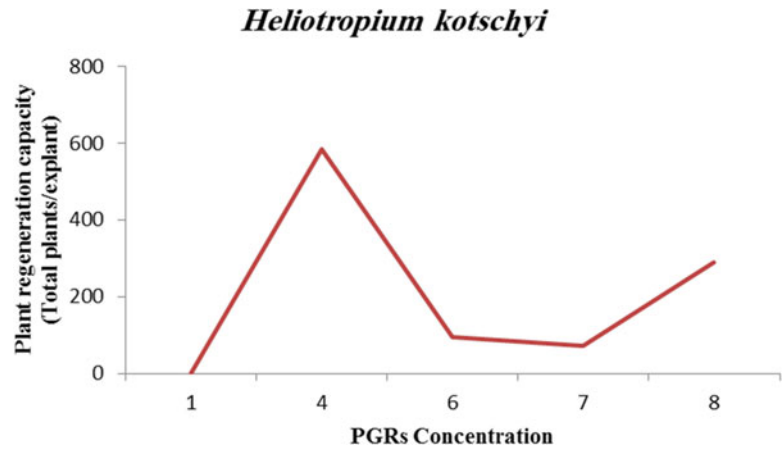

Fig. 3 Plant regeneration capacity of *H. kotschyi* after 2nd transfer in media 1 while initial shoots develop from culture of nodal explants in different media [1, 4, 6–8] and their multiplication in media 4. PGR concentration in Table 1

4 Notes

1. Collect apical shoots (12–15 cm long) from 1-year-old actively growing field-grown plants for experiments. Cut them into pieces and store in plastic bags at 4 °C for future use.
2. Use MS powder for all experiments.
3. Make stock solutions of vitamins: 1 mg/ml nicotinic acid, 1 mg/ml pyridoxine–HCl, and 10 mg/ml thiamine–HCl, then filter-sterilize with sterile Acrodisc 0.45 μm, and store in a refrigerator (4 °C) until future use.
4. For the preparation of PGR stock solutions at 2 mg/ml concentration, weigh 100 mg IAA, NAA, IBA, KI, BAP, separately, dissolve in 2 ml 100 mM NaOH solution, add autoclaved water to raise the volume to 50 ml, then filter-sterilize using sterile Acrodisc 0.45 μm. Store them at −20 °C for future use and keep at 4 °C for routine use.
5. Surface-sterilize the stem segments by using subsequent mixtures of 1 % Lux solution (soap solution), 0.5 % mercuric chloride with few drops of Tween 20, 0.1 % Copral, 50 % (v/v) Clorox (containing 2.625 % hypochlorite), 70 % ethanol. Then slice the stem segments into smaller segments (1–1.5 cm), each containing one node, for use as explants for culture initiation.
6. The nodal explants initiate highest 100 % direct multiple shoot formation on the modified MS medium containing 8.88 μM BAP with 5.71 μM IAA after 4 weeks of culture.

7. The results show significant differences ($P \leq 0.05$ level) by ANOVA according to Duncan's multiple range test (DMRT) using JMP (version 9) statistical software. After 4 weeks of culture, the highest shoot initiation frequency is 10.6 on MS medium supplemented with 8.88 μM BAP and 5.71 μM IAA.
8. 100 % shoot proliferation in association with the highest shoot multiplication frequency of initially developed shoots is observed during subculture in media containing 8.88 μM BAP with 5.71 μM IAA.
9. The highest plant regeneration capacity is observed while explants are cultured subsequently in media with higher concentration of BAP (8.88 μM) and lower concentration of IAA (5.71 μM) than other combinations and concentrations of PGRs. Similarly, several studies report that higher concentrations of BAP with lower concentrations of IAA induce multiple shoots in *Salvia africana-lutea* L. [11], *Melissa officinalis* L. [12]. The stages of in vitro plantlet regeneration of *H. kotschyi* are shown in Fig. 1.
10. Gently remove culture media sticking to roots by washing with autoclaved distilled water and transfer rooted plantlets (Fig. 1h) to plastic pots containing autoclaved compost soil (1:1 mixture of peat substrate and potting soil); and keep them in a small transparent covered chamber to maintain humidity. The plants acclimatize in room conditions at 25 °C ± 3 °C, 16/8 h photoperiod and by regular watering at 3 days interval.
11. Expose gradually well-developed rooted plantlets (Fig. 1i) to normal growing conditions and 60 % plantlets survive after 45 days.
12. This is the first report on the micropropagation of this endangered plant in the Kingdom of Bahrain.
13. The establishment of in vitro culture and efficient plant regeneration protocol is a crucial initial step of ex situ conservation strategy.

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Chapter 8

Micropropagation and Biomass Production of True-to-Type *Stevia rebaudiana* Bertoni

Arpan R. Modi, Vikas Sharma, Ghanshyam Patil, Amritpal S. Singh, N. Subhash, and Nitish Kumar

Abstract

Here we describe an efficient micropropagation protocol for *Stevia rebaudiana* Bertoni. We present experiments carried out to optimize the suitable media for in vitro shoot multiplication and root induction and to study the effect of culture vessel on shoot multiplication. Among all different media tested for in vitro shoot multiplication, hormone-free liquid medium is most suitable. The highest number of nodes per shoot (5.4) and length of shoot (4.76 cm) at 4 weeks after subculturing are observed when single node explants are placed on modified MS medium supplemented with 1 % sucrose and 0.7 % agar. The highest response of multiplication rate (9.56) is observed on half strength of macroelement of MS with full strength of microelement of MS and 170 mg/l KH_2PO_4, and 185 mg/l $MgSO_4$ in plastic growth container. Further, RAPD marker analysis of in vitro-raised plants maintained their clonal fidelity and true-to-type without showing any somaclonal variation.

Key words Culture vessel, Genetic fidelity, Liquid culture, Micropropagation, RAPD, *Stevia rebaudiana*

1 Introduction

Stevia, *Stevia rebaudiana* Bertoni, is a small shrub of family Asteraceae, and valuable to the industry and pharmacy. Its leaves contain a glycoside called stevioside, which has been used as a natural sweetener for a long time [1]. The stevioside is reported to be over 200–300 times sweeter than sucrose [2]. This sweetener, being non-carbohydrate in nature, does not induce tooth decay, can be consumed safely by diabetic patients, and can also be used in low-caloric diets to reduce human body weight. Conventional propagation by seeds is restricted due to the poor seed viability coupled with poor germination rate [3]. Moreover, the crop is cross-pollinated, and to raise an elite uniform plant population with high stevioside content is not possible. Thus, conventional

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propagation through seeds do not produce true-to-type plants. Vegetative propagation by rooted stem cuttings is being impractical and inadequate to meet the demand of the industry. Thereby there is a need for a reliable and efficient micropropagation method for the large-scale production of true-to-type plants and genetic improvement of the species using genetic engineering techniques.

Micropropagation of *S. rebaudiana* with in vitro techniques has been reported [4–7]. For developing an efficient micropropagation protocol, we evaluated various factors affecting in vitro multiplication of *S. rebaudiana* viz., strength of Murashige and Skoog (MS) salt, culture vessel type, and media consistency (solid or liquid). Considering the suitability of liquid culture systems in scaling up of production by automation through the use of bioreactors and reduced cost of media due to elimination of solidifying agent and comparative analysis of in vitro multiplication on both solid and liquid media, Modified MS salt medium [8] proved effective, and could also eliminate the cost of the macroelements to half. In addition, scaling up of any micropropagation protocol is severely hindered by somaclonal variation. Somaclonal variation occurs mostly in response to the stresses imposed on the plants under in vitro conditions [9]. Hence, a stringent quality check in terms of genetic similarity of the tissue culture raised plants becomes mandatory. Randomly amplified polymorphic DNA (RAPD) based detection of genetic polymorphism [10, 11] has been found successful application in describing somaclonal variability in micropropagated individuals of several plants species [12–18]. This book chapter describes a simple and efficient in vitro micropropagation method for mass propagation of true-to-type plants and to evaluate the genetic stability of micropropagated *S. rebaudiana* plants using the RAPD marker.

2 Materials

2.1 Culture Establishment in Liquid MS Medium and Micropropagation

1. Axillary nodes of Stevia plants are collected from the nursery.
2. Autoclaved deionized water.
3. 1 mg/ml cefotaxime.
4. 0.5 mg/ml kanamycin.
5. 1 mg/ml Bavistin.
6. Stock solution of MS salts [19]: Nutrient salts and vitamins stock solutions (Table 1).
7. 0.2 mg/ml 6-benzylaminopurine (BAP).
8. 0.2 mg/ml kinetin.
9. 0.2 mg/ml indole-3-butyric acid (IBA).
10. 0.1 % mercuric chloride.

Table 1
Required amount of components of MS medium for stock preparation

Category	Chemical	Required concentration (mg/l)	Weight for stock (mg/l)	Stock concentration
Macroelements	NH_4NO_3	1650	33,000	20×
	KNO_3	1900	38,000	
	$CaCl_2{\cdot}2H_2O$	440	8800	
	$MgSO_4{\cdot}2H_2O$	370	7400	
	KH_2PO_4	170	3400	
Minor elements	KI	0.83	166	200×
	H_3BO_3	6.2	1240	
	$MnSO_4{\cdot}2H_2O$	22.3	4460	
	$ZnSO_4{\cdot}7H_2O$	8.6	1720	
	$Na_2.MoO_4{\cdot}2H_2O$	0.25	50	
	$CuSO_4{\cdot}5H_2O$	0.025	5	
	$CoCl_2{\cdot}6H_2O$	0.025	5	
Iron source	$FeSO_4{\cdot}7H_2O$	27.8	5560	200×
	$Na_2EDTA{\cdot}2H_2O$	37.3	7460	
Vitamins	Myo-inositol	100	20,000	200×
	Nicotinic acid	0.5	100	
	Pyridoxine–HCl	2	100	
	Thiamine–HCl	2	100	
	Glycine	2	400	
Carbon source	Sucrose	30,000	Use directly	–

2.2 DNA Isolation

1. Tris–HCl 1 M (pH 8).
2. Sodium chloride (5 M).
3. EDTA (0.5 M).
4. Magnetic stirrer.
5. Deionized water.
6. Cetyl trimethyl ammonium bromide (CTAB) (2 %).
7. ß-mercaptoethanol (1 %).
8. Polyvinyl pyrrolidone (PVP) (1 %).
9. Sodium acetate (3 M).
10. TBE (Tris–borate–EDTA) electrophoresis buffer.
11. Ethanol (70 %).
12. Ethidium bromide (10 mg/ml).

2.3 PCR Amplification

1. DNA 4 ng/μl.
2. Taq DNA polymerase 0.1 U/μl.
3. dNTP mix 0.2 nmol
4. PCR buffer containing 1.5 mM of $MgCl_2$.
5. Primer 0.4 pmol.

2.4 Gel Electrophoresis

1. Agarose gel (1.8 %).
2. 100 ml TBE buffer (pH 8.0).
3. Ethidium bromide (10 mg/ml).

3 Methods

Prepare all solutions using ultrapure water deionized (prepared by purifying Milli-Q water purification system) and molecular grade reagents. Prepare and store all reagents at 4 °C temperature.

3.1 Preparation of Stock Solutions

1. Cefotaxime (1 mg/ml): 1000 mg cefotaxime powder in 1 L autoclaved deionized water.
2. Kanamycin (0.5 mg/ml): Add 500 mg kanamycin in 1 L autoclaved deionized water.
3. Bavistin (1 mg/ml): Add 1000 mg Bavistin in 1 L autoclaved deionized water.
4. BAP (0.2 mg/ml): Add 20 mg BAP in 100 ml autoclaved deionized water (*see* **Note 1**).
5. Kinetin (0.2 mg/ml): Add 20 mg kinetin in 100 ml autoclaved deionized water (*see* **Note 1**).
6. IBA (0.2 mg/ml): Add 20 mg IBA in 100 ml autoclaved deionized water (*see* **Note 1**).
7. Mercuric chloride (0.1 %): Add 100 mg mercuric chloride in 1 L autoclaved deionized water. Utmost precaution should be taken while handling this chemical (*see* **Note 2**).
8. Tris–HCl 1 M (pH 8): Add 12.11 g Tris base in 100 ml autoclaved deionized water (*see* **Note 3**).
9. Sodium chloride (5 M): Add 146.1 g NaCl in 500 ml autoclaved deionized water (*see* **Note 4**).
10. EDTA (0.5 M): Add 181.6 g of $Na_2EDTA{\cdot}2H_2O$ to about 1000 ml of autoclaved deionized water (*see* **Note 5**).
11. Sodium acetate (3 M): Add 2.461 g of sodium acetate in 10 ml autoclaved deionized water.
12. Ethanol (70 %): Add 30 ml autoclaved deionized water in 70 ml of ethanol.

13. TBE (Tris–borate–EDTA) electrophoresis buffer (1 L): Add 108 g Tris base (890 mM), 55 g boric acid (890 mM), and 40 ml (0.5 M EDTA, pH 8.0) in 960 ml autoclaved deionized water.

3.2 Culture Establishment on Liquid MS Medium and Micropropagation

1. Surface-sterilize explants with various sterilizing agents (*see* **Note 6**) as mentioned in Table 2.
2. Prepare shoot induction and proliferation MS media: Both liquid and solid medium containing full and half strength of MS salts for the comparative performance. These media contain 2 mg/l kinetin and 1 mg/l BAP. Moreover, recommended hormone-free modified MS mediums in solid and liquid condition are also taken (Table 3).
3. Culture aseptic nodal segments vertically on full, half and modified MS medium amended with half strength of macroelements and full strength of microelement and 170 mg/l KH_2PO_4, and 185 mg/l $MgSO_4$; and supplemented with or without 1 mg/l BAP and 2 mg/l kinetin for the emergence of shoots from auxiliary buds and shoot proliferation (Table 3).
4. Mass multiplication of shoots using different culture vessel types: For the increased biomass production, proliferated further multiply in different culture vessel type, viz., Flask, Bottle, and Big sized container (2 L capacities) and growth obtained is shown in Fig. 1 (Table 4).
5. Root induction and hardening: Root induction is carried out in liquid medium (Table 5) containing 0.1 mg/l IBA, 100 mg/l charcoal and both with full and half strength of MS salts (Fig. 2). Micropropagated plants further hardened on soil–coco peat (1:1) mixture and then transferred to polythene bags containing soil as a medium.

Table 2
Surface sterilization treatment for the establishment of axenic cultures

Treatment	Concentrations	Time
Tween-20	2 drops	1 min
Autoclaved deionized water	–	5 min
Cefotaxime	1 mg/ml	5 min
Kanamycin	0.5 mg/ml	5 min
Bavistin	1 mg/ml	10 min
Autoclaved deionized water	–	5 min
Mercuric chloride	0.1 %	8 min
Autoclaved deionized water (5 washes)	–	5 min

Table 3
Shoot induction media and their response on shoot growth

Treatment	Length of shoot (cm)	No. of nodes per shoot
½ MS + 2 mg/l Kn + 1 mg/l BAP + 3 % Sucrose + 0.7 % agar	3.46 ± 0.11	3.6 ± 0.55
½ MS + 2 mg/l Kn + 1 mg/l BAP + 3 % sucrose	3.22 ± 0.08	2.6 ± 0.89
MS + 2 mg/l Kn + 1 mg/l BAP + 3 % sucrose + 0.7 % Agar	3.92 ± 0.24	4.4 ± 1.52
MS + 2 mg/l Kn + 1 mg/l BAP + 3 % sucrose	3.70 ± 0.14	4.6 ± 0.89
Modified MS + 1 % sucrose + 0.7 % agar	4.76 ± 0.32	5.4 ± 1.14
Modified MS + 1 % sucrose	5.50 ± 0.27	7.2 ± 1.1

Modified MS = Half strength of macroelement of MS + full strength of microelement of MS + 170 mg/l KH_2PO_4 + 185 mg/l $MgSO_4$ (Source: [21])

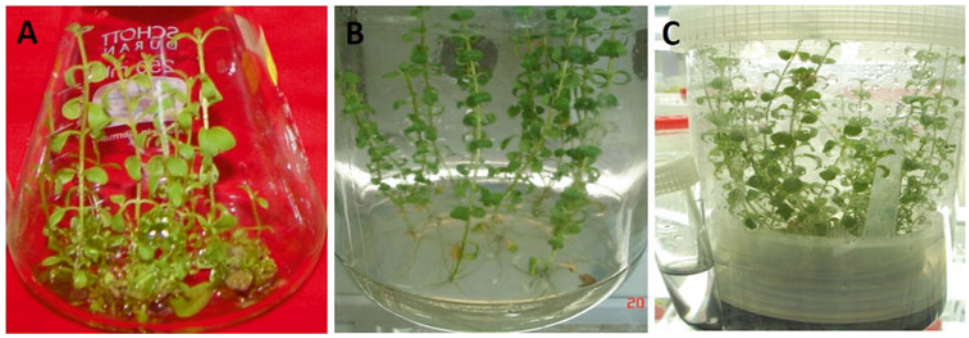

Fig. 1 Shoot elongation in different culture vessel types: (**a**) flask culture; (**b**) glass bottle culture; and (**c**) culture in big sized container (2 L capacity). (Source: [21])

Table 4
Shoot multiplication using different types of culture vessels containing modified MS medium and 1 % sucrose

Culture vessel	Media consistency	Multiplication rate per node
Glass jar	Solid	6.16 ± 0.94
Glass jar	Liquid	6.45 ± 1.04
Glass flask	Liquid	5.96 ± 0.84
Plastic growth container	Liquid	9.56 ± 1.08

Modified MS = Half strength of macroelement of MS + full strength of microelement of MS+ 170 mg/l KH_2PO_4+ 185 mg/l $MgSO_4$ (Source: [21])

Table 5
Observations for rooting seen under various root-inducing medium

Media code	Percentage of rooting (%)	Average no. of roots	Length of longest root (cm)	Length of shortest root (cm)	Average length of root (cm)	Shoot height (cm)	No. of leaves
RI 1	33.3	4	1.0	0.3	0.5	5.75	23.1
RI 2	66.6	3	0.7	0.2	0.5	6.91	19.6
RI 3	87.5	20	1.2	0.3	0.7	7.81	22.5
RI 4	88.8	19	5.0	0.3	2.7	8.86	20.1
RI 5	100	13	4.3	0.3	3.3	9.52	28
RI 6	100	25	5.0	0.3	3.9	7.7	22.2

RI 1: MS + 0.1 mg/ml IBA; RI 2: ½ MS + 0.1 mg/ml IBA; RI 3: MS + 0.1 mg/ml IBA + 0.1 mg/ml charcoal; RI 4: ½ MS + 0.1 mg/ml IBA + 0.1 mg/ml charcoal; RI 5: MS + 0.1 mg/ml charcoal; RI 6: ½ MS + 0.1 mg/ml charcoal

Fig. 2 Root induction in micropropagation of Stevia in different liquid medium. RI 1: MS + 0.1 mg/ml IBA; RI 2: ½ MS + 0.1 mg/ml IBA; RI 3: MS + 0.1 mg/ml IBA + 0.1 mg/ml charcoal; RI 4: ½ MS + 0.1 mg/ml IBA + 0.1 mg/ml charcoal; RI 5: MS + 0.1 mg/ml charcoal; RI 6: ½ MS + 0.1 mg/ml charcoal

3.3 DNA Extraction

DNA isolation is carried out using the method described in Ref. [20] with minor modifications.

1. Take 0.2 g of leaf tissue in liquid nitrogen and crush it to make a fine powder of tissue.
2. Add 1 ml of CTAB extraction buffer (Table 6) prepared freshly and keep the sample in water bath at 65 °C for 1 h and mix gently in between at an interval of 15 min.
3. Keep the sample at room temperature for 10 min.

Table 6
Components used in CTAB extraction buffer with their final concentrations

Component	Final concentration	Aliquot (10 ml)
Tris (1 M)	0.1 M	1.0 ml
EDTA (0.5 M)	0.04 M	0.8 ml
CTAB	2 %	200 mg
PVP	1 %	100 mg
ß-mercaptoethanol	1 %	0.1 ml
NaCl	1.5 M	3.0 ml
Autoclaved deionized water	–	5.1 ml

4. Centrifuge the sample at 2,000 × *g* for 5 min.
5. To the supernatant, add 1 ml of chloroform–isoamyl alcohol (24:1) in supernatant and mix vigorously.
6. Centrifuge the sample at 11,000 × *g* for 8 min at 4 °C.
7. Take aqueous phase (*see* **Note 7**) and add double volume of absolute alcohol containing 0.3 M (final concentration) of sodium acetate.
8. Incubate at −20 °C for 4 h to overnight.
9. Centrifuge at 16,000 × *g* for 15 min at 4 °C.
10. Discard supernatant and wash the pellet with 80 % ethanol at 7,000 × *g* for 5 min at 4 °C.
11. Discard supernatant and add 0.2 ml of 1× TE buffer.
12. Quantify the DNA from sample and make the final concentration of DNA as 20 ng/μl and use it as a working solution of DNA.

3.4 Polymerase Chain Reaction and Gel Electrophoresis

1. To 20 ng/μl DNA put a PCR reaction with different series of RAPD primers.
2. Screen primers showing more than six bands (*see* **Note 8**).
3. Add PCR component as mentioned in Table 7 and put a PCR with the cycling condition mentioned in Table 8.
4. Gel electrophoresis is performed in horizontal position on agarose matrix. Prepare 1.8 % agarose gel in TBE buffer and add 3 μl EtBr per 100 ml gel. Pour the gel in gel caster and put combs in it. Load the samples along with 100 bp DNA ladder (Fig. 3).

Table 7
PCR components and their concentrations and aliquots for a reaction of 25 µl

Component	Working concentration	Aliquot
DNA template	20 ng/µl	5.0 µl
Taq DNA polymerase	5 U/µl	0.5 µl
PCR Buffer	10×	2.5 µl
dNTP Mix	10 mM	0.5 µl
Primer	10 pmol	2.0 µl

Table 8
Steps of PCR showing temperature, time, and cycling condition

Step	Temperature	Time (MM:SS)	Cycle
Initial denaturation	94 °C	05:00	1
Denaturation	94 °C	01:00	40
Annealing	37 °C	01:00	
Extension	72 °C	02:00	
Final extension	72 °C	07:00	1
Hold	4 °C	–	–

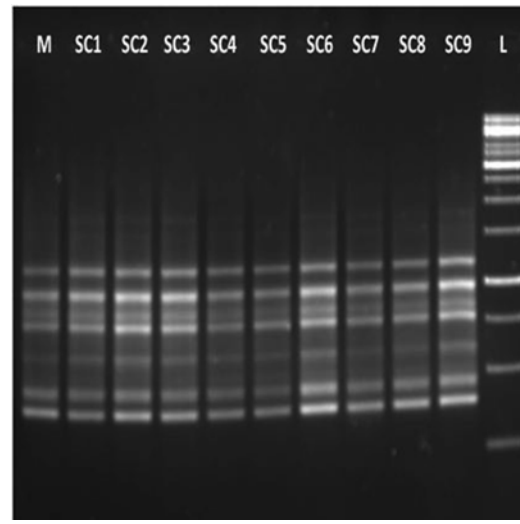

Fig. 3 RAPD amplification by primer OPE-4 (5′GTGACATGCC3′) of micropropagated plants of *Stevia rebaudiana. M* mother plant, *SC1 to SC9* tissue culture raised clones of Stevia, *L* 100 base pairs DNA ladder. (Source: [21])

4 Notes

1. BAP, Kinetin, and IBA are hard to dissolve in water, so they are first dissolved in 0.1 N NaOH and then final volume is raised to 100 ml with distilled water.
2. Mercuric chloride is hard to dissolve in water because of its larger granule size. Take mercuric chloride powder in a dry mortar and crush it vigorously using pestle into fine powder.
3. For the preparation of Tris–HCl buffer, take 12.11 g of commercially available Tris buffer powder and dissolve in 90 ml autoclaved deionized water. Adjust the pH of the solution to 8.0 and make the final volume up to 100 ml with autoclaved deionized water.
4. It is difficult to dissolve high concentration of NaCl in water. Dissolve it in small amount at one time. Weigh 146.1 g NaCl, take small quantity in a beaker, and add 300 ml autoclaved deionized water and dissolve it by shaking or stirring on a magnetic stirrer, and likewise dissolve the entire amount.
5. For the preparation of 0.5 M solution, take 181.6 g of $Na_2EDTA{\cdot}2H_2O$ and dissolve in 800 ml of autoclaved deionized water. While stirring with a magnetic stirrer, adjust the pH of solution by pellets of NaOH (approximately 20 g of NaOH is required) and make the final volume up to 1000 ml with autoclaved deionized water.
6. Sterilizing agents such as fungicides, bactericides, and mercuric chloride are dissolved in autoclaved deionized water under aseptic condition in laminar air hood while pre-sterilizing treatment such as Tween 20 wash may be carried out without aseptic condition.
7. Carefully take upper aqueous phase using cut-end tips so that no DNA can be disturbed and shearing after DNA extraction can be prevented.
8. While screening primers, choose showing six or more bright bands. If primer is showing more number of lighter bands, eliminate the primer to avoid any problem during analysis.

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Chapter 9

Panax ginseng Adventitious Root Suspension Culture: Protocol for Biomass Production and Analysis of Ginsenosides by High Pressure Liquid Chromatography

Hosakatte Niranjana Murthy and Kee Yoeup Paek

Abstract

Panax ginseng C.A. Meyer (Korean ginseng) is a popular herbal medicine. It has been used in Chinese and Oriental medicines since thousands of years. Ginseng products are generally used as a tonic and an adaptogen to resist the adverse influence of a wide range of physical, chemical and biological factors, and to restore homeostasis. Ginsenosides or ginseng saponins are the principal active ingredients of ginseng. Since ginseng cultivation process is very slow and needs specific environment for field cultivation, cell and tissue cultures are sought as alternatives for the production of ginseng biomass and bioactive compounds. In this chapter, we focus on methods of induction of adventitious roots from ginseng roots, establishment of adventitious root suspension cultures using bioreactors, procedures for processing of adventitious roots, and analysis of ginsenosides by high pressure liquid chromatography.

Key words Adventitious roots, Bioreactors, Ginsenoside, Herbal medicine, *Panax ginseng*, Saponin

1 Introduction

Panax ginseng C.A. Meyer (Korean ginseng, Araliaceae) is the most valued medicinal plant used in Chinese and Oriental medicine. It is used as a general tonic and shows various pharmacological effects including antiaging, antidiabetic, anticancerous, antihepatitis, antioxidant, anti-inflammatory, and immune-stimulatory activities [1]. Ginseng is also an important medicine for neurodegenerative disorders [2], cardiovascular diseases [3], and liver disorders [4]. Nowadays, ginseng has gained popularity as a functional food, nutraceutical, and dietary supplement [5, 6]. Ginsenosides or ginseng saponins are the principal active ingredients of ginseng and more than 38 ginsenosides have been recognized [1, 7]. Ginsenosides consist of a ganone steroid nucleus with 17 carbon atoms arranged in four rings. The characteristic biological response of each ginsenoside is attributed to the differences in the type,

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position and number of sugar moieties attached to glycosidic bond at C-3 and C-6. Based on the structural differences, they are classified into three groups: the panaxadiol group (e.g., Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rh2, Rh1), the panaxatriol group (e.g., Re, Rf, Rg1, Rg2, Rh1), and oleonic acid group (e.g., Ro). Each one of these ginsenosides is having different pharmacological effects [1].

The production of ginseng by field cultivation is a slow process and needs 4–6 years from planting to root harvesting stage. Further, its production is hampered by environmental factors, pests and diseases, and ginsenoside content varies depending on the age of plant, season of harvest, and the extraction method [8, 9]. Therefore, in vitro cell and organ cultures have become alternatives for the production of ginseng biomass and ginsenosides [10]. Recently, bioreactor technology has become an excellent tool for large-scale cultivation of plant cells and organs. These systems control readily various important process parameters such as mixing, aeration, temperature, pH [11, 12]. Additionally, there is possibility for year round production of biomass with reduced cost and time. Recently, adventitious roots have been induced from *Panax ginseng* roots and successfully cultured for the production of ginsenosides [10, 12]. This chapter describes relevant protocols for the induction of adventitious roots, establishment of suspension cultures for biomass and metabolites production, analysis of genetic stability of adventitious roots maintained in suspension cultures through flow cytometry, processing of adventitious root biomass and isolation and quantification of ginsenosides by high pressure chromatography.

2 Materials

2.1 Induction of Adventitious Roots from Root Explants and Establishment of Suspension Cultures

1. *Panax ginseng* C.A. Meyer roots collected from the wild (Mt. Odaesan, Gangwon province, Republic of Korea).
2. Murashige and Skoog (MS) [13] medium stock solutions (MS stock I, II, III and IV) (Table 1). Store in the freezer or cold room at 4 °C (*see* **Note 1**).
3. Stock solutions (100 μM) of 2,4-dichlorophenoxy acetic acid (2,4-D), indole butyric acid (IBA), and kinetin and store in the freezer at −20 °C (*see* **Note 2**).
4. Fresh solutions of methyl jasmonate (150 μM MJ) (*see* **Note 3**).
5. Gelrite.
6. 5-L and 20-L balloon type bubble bioreactors; 500-L and 1000-L balloon type bubble bioreactors, BTBB (*see* **Note 4**).
7. Polytetrafluoroethylene (PTFE) air filters: 0.20 μM PTFE membrane filters.

Table 1
Chemical composition of MS medium[a]

Chemical constituents	Concentration (mg/L)	Volume per liter (mL)
Major inorganic nutrients		
NH_4NO_3	33,000	50
KNO_3	38,000	
$CaCl_2{\cdot}2H_2O$	8800	
$MgSO_4{\cdot}2H_2O$	7400	
KH_2PO_4	3400	
Minor inorganic nutrients		
KI	166	5
H_3BO_3	1240	
$MnSO_4{\cdot}2H_2O$	4460	
$ZnSO_4{\cdot}7H_2O$	1720	
$Na_2.MoO_4{\cdot}2H_2O$	50	
$CuSO_4{\cdot}5H_2O$	5	
$CoCl_2{\cdot}6H_2O$	5	
Iron source		
$FeSO_4{\cdot}7H_2O$	5560	5
$Na_2EDTA{\cdot}2H_2O$	7460	
Organic supplements		
myo-Inositol	20,000	5
Nicotinic acid	100	
Pyridoxine·HCl	100	
Thiamine·HCl	100	
Glycine	400	
Carbon source		
Sucrose	As per the experiment	

[a]After dissolving all the stock solutions in enough deionized water make it up to 1 L, adjust the pH to 5.8 (add 2 g/L Gelrite or 0.8 g/L agar if semisolid medium) and autoclave for 25 min at 120 °C

2.2 Flow Cytometry

1. Flow cytometer equipped with UV excitation lamp.
2. Extraction buffer and 4,6-diamidino-2-phenylindole (DAPI).
3. Nuclei which are extracted in buffer and stained with DAPI.
4. Nylon meshed sieves (50 μM pore size).

2.3 Drying of Ginseng Adventitious Roots and Extraction of Ginsenosides

1. Air dryer.
2. Heat reflux extraction unit.
3. 80 % ethanol, high pressure liquid chromatography (HPLC) grade.

2.4 Estimation of Total Phenols

1. 2 N Folin–Ciocalteu reagent. Store at 4 °C.
2. 1000 ppm Gallic acid. Store at 4 °C in the dark (*see* **Note 5**).
3. 20 % sodium carbonate stock solution. Store at 4 °C.
4. 80 % methanol, high pressure liquid chromatography (HPLC) grade.

2.5 Estimation of Total Flavonoids

1. 1000 ppm (+) catechin stock solution and store at 4 °C in the dark.
2. 10 % Aluminum chloride solution. Store stock solution at 4 °C.
3. 5 % Sodium nitrate solution. Store stock solution at 4 °C.

2.6 Scavenging Effect on 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical (Scavenging Activity of Natural Antioxidants)

1. 2,2′-diphenyl-1-picrylhydrazyl (DPPH) (*see* **Note 6**).
2. Ultraviolet (UV)–visible spectrophotometer.

2.7 HPLC Analysis of Ginsenosides

1. HPLC-grade acetonitrile (*see* **Note 7**).
2. HPLC-grade water (*see* **Note 7**).
3. Standard Rb and Rg ginsenosides.

3 Methods

3.1 Induction of Adventitious Roots from Root Explants

1. Wash roots collected from the wild thoroughly (Fig. 1a) in tap water.
2. Immerse roots in 70 % ethanol for 30 s, and surface sterilize in 20 % sodium hypochlorite solution containing few drops of Tween 30 detergent for 30 min (*see* **Note 8**).
3. Rinse 3× in cold, sterile water for 5 min.
4. Slice roots into 0.3 cm^3 pieces under laminar flow bench and culture individually on a semisolid MS medium supplemented with 30 g/L sucrose, 4.5 μM 2,4-D, and 0.46 μM kinetin, pH 5.8.
5. Incubate cultures at 25 ± 2 °C for 4 weeks in the dark. Within 8 weeks callus will develop from the root explants (Fig. 1b). Subculture the calli to fresh medium at 6-weeks interval.
6. Culture calli on semisolid MS medium containing 30 g/L sucrose and 24.6 μM IBA.

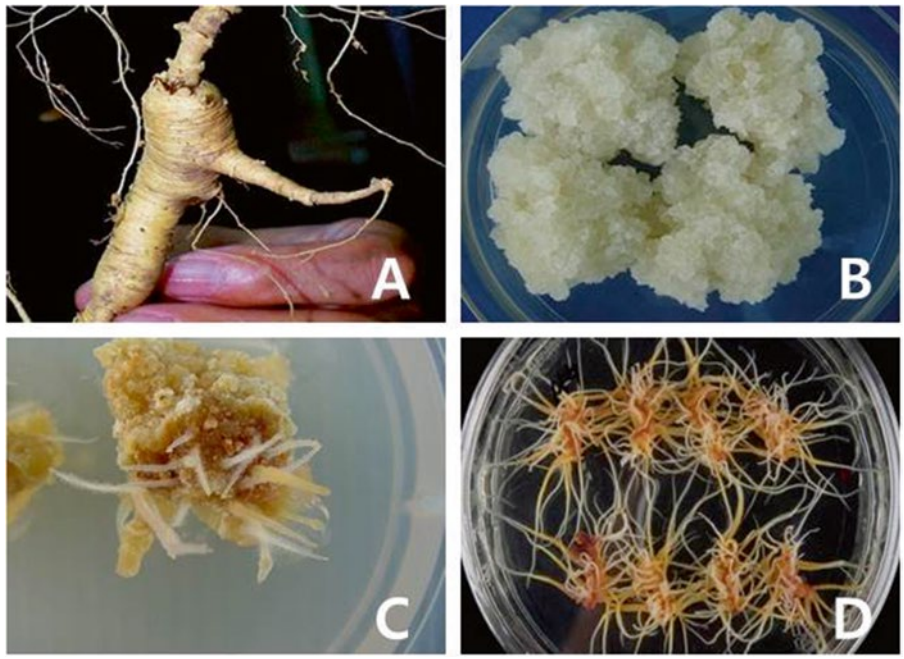

Fig. 1 Wild ginseng root used for induction of adventitious roots (**a**), Callus developed from ginseng roots on MS medium supplemented with 30 g/L sucrose, 4.5 μM 2,4-D, and 0.46 μM kinetin (**b**), Adventitious roots developed from callus on MS medium supplement with 30 g/L sucrose, 24.6 μM IBA (**c**), Proliferation of adventitious roots

7. Incubate cultures at 25 ± 2 °C for 4 weeks in the dark. Adventitious roots will develop from the calli within 4 weeks (Fig. 1c). Subculture the adventitious roots to the fresh medium at 4-weeks interval.
8. The adventitious roots proliferate on the MS medium amended with 30 g/L sucrose and 24.6 μM IBA. Use actively growing adventitious roots as explants for further experiments (Fig. 1d).

3.2 Proliferation of Adventitious Roots in Liquid Medium

1. Collect actively growing root lines from the semisolid cultures, cut them into 2-cm long segments and inoculate 5 g/L fresh biomass into a 250-mL flask containing 100 mL MS liquid medium supplemented with 30 g/L sucrose and 24.6 μM IBA (*see* **Note 9**).
2. Incubate cultures in dark at 25 ± 2 °C and shake at 100 rpm. Maintain cultures by regular subculturing at 4 weeks interval.

3.3 Suspension Cultures for the Production of Ginsenosides

1. Collect roots in the exponential phase or stationary phase and initiate suspension cultures for the production of ginsenosides.
2. Inoculate 5 g/L fresh 2-cm long root biomass in a 500-mL Erlenmeyer flask containing 200 mL MS medium supplemented with 50 g/L sucrose and 24.6 μM IBA.

Fig. 2 Adventitious root cultures in 20 L balloon type bubble bioreactors

3. Incubate cultures in the dark at 25 °C on a rotary shaker at 100 rpm. At these conditions, adventitious roots grow and multiply at faster rate.
4. After 7 weeks of culture, assess the growth of adventitious roots in terms of fresh weight, dry weight, growth ratio and amounts of phenols, flavonoids and ginsenosides.

3.4 Establishment of Suspension Cultures for the Production of Ginsenosides in a Bioreactor

1. Culture 5 g/L adventitious roots in 5 L or 20 L (Fig. 2) or 500 L or 1000 L (Fig. 3a) capacity balloon type bubble bioreactors containing MS medium amended with 50 g/L sucrose and 24.6 μM IBA.
2. Maintain bioreactor cultures in the dark at 20 °C. Aerate cultures, air flow of 0.1 vvm (air volume/culture volume/min). This favors profuse growth and multiplication of adventitious roots (Fig. 3b) (*see* **Note 10**).
3. Supplement bioreactor cultures with 100 μM methyl jasmonate (MJ) after 40 days of culture. Maintain the cultures for another 10 days after MJ treatment.
4. After 50 days of culture, assess growth of adventitious roots in terms of fresh weight, dry weight, growth ratio and amount of phenols, flavonoids and ginsenosides.

3.5 Flow Cytometry

Flow cytometry assessment of ginseng adventitious roots is carried out after completion of 10 cultivation cycles. The stepwise methodology for flow cytometry analysis [14] of ginseng adventitious roots can be carried out as follows.

1. Use Partec PAS II flow cytometer, equipped with a mercury HBO 100-W lamp, a dichroic mirror (TK 420) and a built-in program for the data analysis.

Fig. 3 500 L and 1000 L balloon type commercial bioreactors (**a**), adventitious root biomass in 1000 L bioreactor (**b**)

2. Take roots of mother plant (wild Korean ginseng) and ginseng adventitious roots and chop a 10 mm root tissue in 400 μL nuclei extraction buffer diluted with 1600 μL staining buffer.
3. Sieve nuclei suspension through a 50-μm mesh and transfer into 2.5 mL eppendorf tubes.
4. For each measurement, count a minimum of 2500 nuclei. Take observations as a linear scale on a real-time graph with nuclei size (intensity of the epifluorescence emitted) on abscissa, and count the number of nuclei on ordinates.
5. Prior to use, calibrate cytometer with barley leaves (*Hordeum vulgare* L.; Fig. 4a) a standard [15] (*see* **Note 11**).
6. Compare the peak of ginseng adventitious roots (Fig. 4b) with mother plant (wild ginseng root, Fig. 4c).

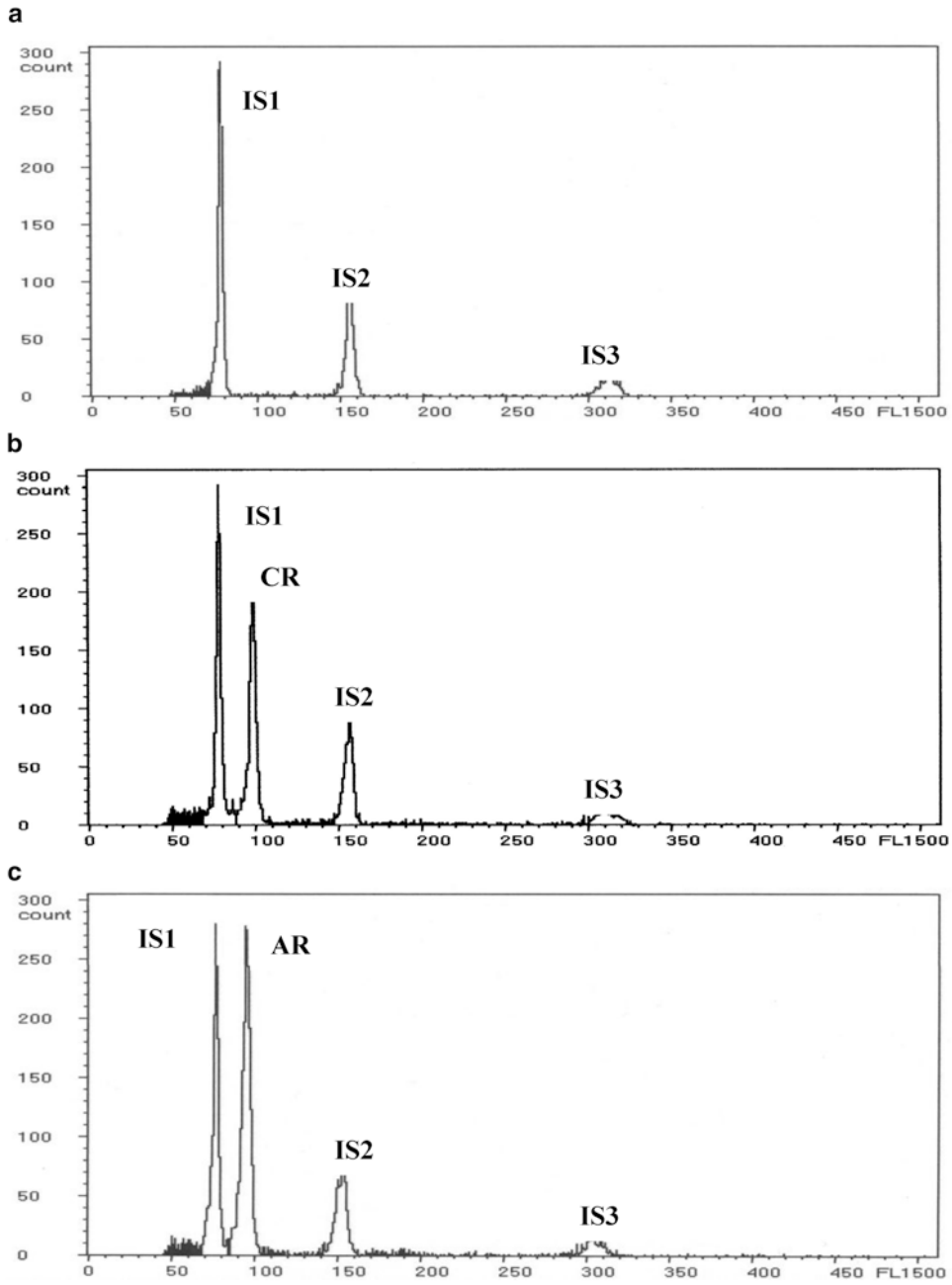

Fig. 4 Typical flow cytometry profile of *Hordeum vulgare* cv. Sultan (used for calibration of instrument) (**a**), Profile of cultivated ginseng (*Panax ginseng* C.A. Meyer) (**b**), Profile of ginseng adventitious roots (**c**; four peaks as identical to mother plant)

3.6 Estimation of Root Biomass

1. Filter cultures through a stainless steel sieve of pore size 40 mesh (420 μm) to separate the roots from the culture medium.
2. Wash roots thoroughly in sterile water and blot excessive surface water. Root biomass produced in bioreactors is shown in Fig. 5a.

Fig. 5 Ginseng adventitious roots harvested from bioreactors (**a**), Drying of ginseng adventitious roots in forced air dryer (**b**)

3. Record dry weight after drying of roots at 50 °C for 10 h in a forced air unit (*see* Subheading 3.7 for details) to attain a constant weight.
4. Growth ratio is determined by using the formula: GR = harvested dry biomass (g) – inoculated dry biomass (g)/inoculated dry biomass (g).

3.7 Drying of Adventitious Roots and Preparation of Root Extract for Analyzing Bioactive Compounds

1. Adventitious roots are dried in forced air-drying unit (Fig. 5b) at 50 °C for 10 h. The dried roots can be stored at room temperature (20–35 °C) for a longer duration without deterioration [16].
2. Take 2 g dried roots and carry out extraction at 80 °C for 6 h using 40 mL 70 % ethanol (Rotary evaporator) [17].
3. Filter the extract through double layers of Whatman no. 1 filter paper.
4. Re-extract the residue as in **steps 1** and **2** and make up the final volume to 100 mL using 70 % ethanol.

3.8 Estimation of Total Phenol Content in Adventitious Root Extract

The amount of total phenols in adventitious root extract is analyzed spectrophotometrically by using Folin–Ciocalteu reagent.

1. Mix 100 μL ethanolic extract with 2.5 mL deionized water and add 0.1 mL 2-N Folin–Ciocalteu reagent. Mix the contents well and allow to stand for 6 min.
2. After 6 min, add 0.15 mL 20 % sodium carbonate solution. After incubation at room temperature for 30 min the purple color will develop.
3. Working standards of 20, 50, 100, 150, and 200 ppm of gallic acid are used for plotting standard calibration curve.
4. The absorbance of solutions is detected at 760 nm on a UV–visible spectrophotometer. The measurements are compared with the standard curve for gallic acid. The results are expressed as mg of gallic acid equivalent per gram of dry roots.

3.9 Estimation of Total Flavonoid Contents in Adventitious Root Extract

The amount of total flavonoids in adventitious root extracts analyzed spectrophotometrically by aluminum chloride method (*see* **Note 12**).

1. Mix 0.25 mL ethanolic root extract and a (+) catechin standard solution with 1.25 mL deionized water. Add 75 μL 5 % sodium nitrate solution and allow it to stand for 6 min.
2. After 6 min, add 0.15 mL 10 % aluminum chloride solution and allow the mixture to stand for 5 min and then add 0.5 mL 1 M sodium hydroxide and mix the contents thoroughly.
3. Measure the absorbance immediately at 510 nm on a spectrophotometer. The results should be expressed as mg of (+) catechin equivalents per gram of dry roots.

3.10 Scavenging Effect on 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical

1. For the analysis of antioxidants, mix 0.5-mL aliquots of each extract with 300 μL 1 mM methanolic solution of DPPH in a 4 mL cuvette. Make the total volume to 3.0 mL by adding methanol (*see* **Note 13**).
2. After incubation in the dark at room temperature for 15 min, assay the reaction mixture at 517 nm using UV–visible spectrophotometer (*see* **Note 13**).
3. For eliminating the interference with the DPPH reaction by extract pigments, assay the blanks of the extracts using 300 μL methanol. Prepare and assay a DPPH blank sample containing 2.7 mL methanol and 300 μL DPPH daily. All experiments should be carried out in duplicate and should be repeated at least 2 times.
4. Record the percentage decrease in absorbance at 517 nm for each concentration and calculate percentage of quenching of the DPPH radical on the basis of the observed decrease of the radical. The inhibition percentage can be calculated according to the formula:

$$\text{Inhibition percentage} = \left[\left(A_{\text{DPPH}} - A_{\text{Extr}} \right) / A_{\text{DPPH}} \right] \times 100$$

where A_{DPPH} is the absorbance value of the DPPH blank sample and A_{Extr} is evaluated as the difference between the absorbance value of the test solution and that of its blank. Curves showing inhibition percentage/μL of extract are used to find the concentrations at which 50 % radical scavenging occurred (EC_{50}).

3.11 HPLC Analysis of Ginsenosides

1. Carry out extraction of ginsenosides as described in the Subheading 3.7. Filter all the extracts through a 0.45 PTEE filter into an HPLC vial and cap the vial.
2. Initially wash/run the chromatography system consisting of vacuum degasser, quaternary pump, auto sampler, thermostated column compartment, and diode array detector (DAD) with water twice.
3. Analyze ginsenosides with XTerra RP 18 column, particle size 3.0 μm, 150 mm × 3 mm. The mobile phase should be (a) water and (b) acetonitrile. The ginsenosides fractions are separated by gradient elution as follows: initial 25 % acetonitrile for 10 min; 37 % acetonitrile for last 25 min, at a flow rate of 1.2 mL/min. The detector monitors the eluent at 203 nm. Adjust the column temperature to 30 °C. and inject 20 μL sample. Three injections of each sample can be used.
4. Peaks are identified on the basis of their retention time values and UV spectra in comparison with the standard (Fig. 6). Peak identity can also be confirmed by spiking the extracts with pure standards (*see* **Note 14**).

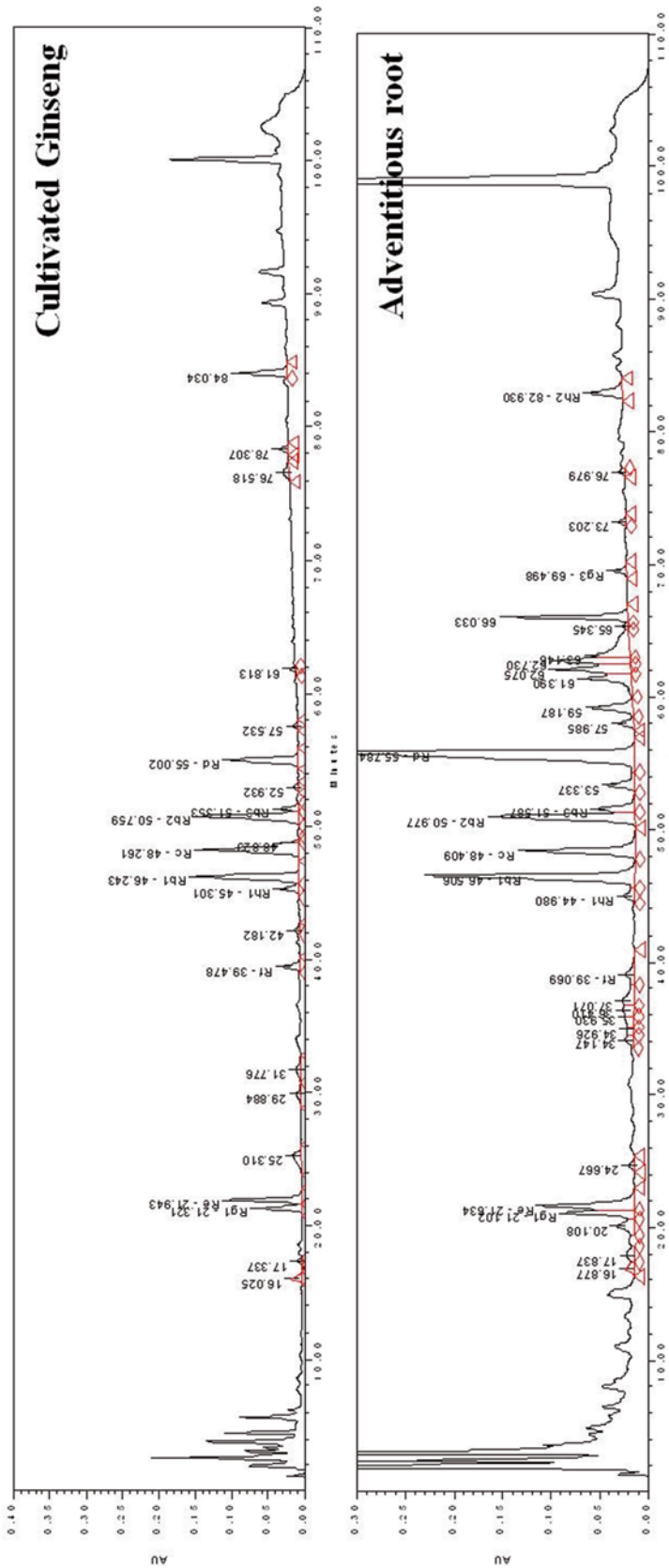

Fig. 6 HPLC profiles of standard ginsenosides (**a**), adventitious roots (**b**)

5. Eluted experimental samples were detected by photo diode array detector coupled to the HPLC system, by comparing the UV spectra of each peak with those of authentic reference samples.
6. Prepare stock standard solutions of each ginsenoside as follows: 2.0 mg of each compound accurately weighed and placed into a 5-mL volumetric flask. Then dilute stock solutions with methanol to yield a series of standard solutions with different concentrations for linearity validation.

4 Notes

1. The most efficient way of preparing MS medium (Table 1) is to prepare stock solutions of major inorganic nutrients, minor inorganic nutrients, iron source, vitamins, and individual growth regulators. The vitamins are stored in small batches at −20 °C and other stock solutions at 4 °C. Do not store inorganic stock solutions more than 1 month. It is advisable to prepare 100 μL stocks of growth regulators fresh for each batch of media. Any changes in the concentration of stock solutions due to precipitation can seriously affect growth of the cultures. Alternatively, stock solutions of macroelements, microelements, vitamins are commercially available.
2. Add filter-sterilized IBA to the culture medium after autoclaving and cooling to below 40 °C.
3. Methyl jasmonate should be filter-sterilized before adding to the medium. Methyl jasmonate as an elicitor boosts the accumulation of ginsenosides.
4. With small scale bioreactors (5 and 20 L capacity), silicon tubes connected to incoming air filters, and outgoing air filters should be chipped air tight and sealing of the lid should be checked thoroughly before and after autoclaving. Air filters should be of high quality, 0.20 μm PTEE membrane filters, and should be changed after using 3–4 times.
5. Gallic acid is prepared by weighing 1005 mg gallic acid powder and by dissolving it in 1 L deionized distilled water. The addition of 1 mL ethanol will help in the dissolution. Store stock solution in an amber glass container in a refrigerator.
6. Before use, prepare fresh stock solution (1 mM methanolic solution) of DPPH and should be stored in the dark at 4 °C in an amber-colored bottle (exposure to light should be avoided).
7. Use all solvents of HPLC grade; solvents and solutions to be analyzed should be filtered through 0.45 μm polytetrafluoroethylene (PTFE) filters before use.

8. Sometimes explants cultured on the medium are prone to infection and in such cases stringent surface sterilization of explants is needed; the explants should be sterilized for 10–15 min by using 0.1 % mercuric chloride. Subsequently the explants should be washed thoroughly with sterilized distilled water and are cultured on the nutrient medium.
9. Use actively growing roots with root meristem during the initiation of first batch of cultures. Such root explants grow well and proliferate quickly. After one to two subcultures, adventitious roots are randomly cut into 2-cm long explants, and roots with or without root tips can proliferate successfully.
10. Adventitious root biomass accumulation in bioreactor cultures is dependent on physical factors, such as culture conditions other than chemical composition of the medium. For example, inoculum density, aeration volume, light–dark photoperiod, and temperature conditions have to be developed over several series of experiments. Alteration of these parameters could severely affect the biomass production and accumulation of secondary metabolites.
11. Carry out initial flow cytometric measurements with flow of solution, i.e., buffer and DAPI dye solution with nuclei at lower level (~2 μL/s). If the flow of solutions is too high, peaks in the histograms tend to become wider and hence accuracy decreases. DAPI specifically binding to the adenine and thymine bases of DNA is excited under UV (at 372) and emits (at 456 nm) fluorescence that is proportional to the relative DNA content per nucleus.
12. The principle of aluminum chloride method is as follows: Aluminum chloride forms acid stable complex with C-4 keto group and either the C-3 or C-5 hydroxy group of flavones and flavonols [18].
13. A fresh DPPH solution is prepared before use, store in the dark at 4 °C in a bottle covered with aluminum foil. The DPPH radical has widely been used to evaluate the free radical scavenging activity of natural antioxidants [19]. Purple colored DPPH solution turns yellow product after being reduced by an antioxidant, DPPH + antioxidant → Yellow non-radical product.
14. Standard ginsenosides can be procured from reputed chemical suppliers.

Acknowledgements

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Chapter 10

Immobilization of *Rubia tinctorum* L. Suspension Cultures and Biomass Production

Pınar Nartop

Abstract

Plants are natural sources of valuable secondary metabolites used as pharmaceuticals, agrochemicals, flavors, fragrances, colors, biopesticides, and food additives. There is an increasing demand to obtain these metabolites through more productive plant tissue applications and cell culture methods due to the importance of secondary metabolites.

Immobilization of plant cells is a method used in plant cell cultures to induce secondary metabolite production. In this method, plant cells are fixed in or on a supporting material or matrix such as agar, agarose, calcium alginate, glass, or polyurethane foam. In the present study, three natural lignocellulosic materials, loofah sponge, and the long fibers of sisal and jute, were used to immobilize suspended *R. tinctorum* cells.

Key words Immobilization, Plant cells, Suspension culture, Biomass, Lignocellulosic matrices, Loofah, Sisal, Jute, Secondary metabolite production

1 Introduction

The immobilization of plant cells is one of the secondary metabolite-inducing techniques used in cell cultures of many plant species. This technique comprises the fixation of cells onto a solid support, a membrane or into a solid matrix to increase the stability of the cells (Fig. 1). Most reports have indicated that immobilized cultures may induce the yields of secondary metabolite production 16 times more in comparison to cell suspension cultures. Due to the secretion of metabolites to the culture medium, the cells are not only disturbed but also may carry on secondary metabolite production [1, 2].

Plant cells are often in the form of aggregates; they both cling to each other and surfaces as well; therefore, agitation is needed for plant cell culturing. On the other hand, agitation is detrimental to plant cells because of their low tolerance to shear stress. Furthermore, cell-to-cell contact is another important

S. Mohan Jain (ed.), *Protocols for In Vitro Cultures and Secondary Metabolite Analysis of Aromatic and Medicinal Plants, Second Edition*, Methods in Molecular Biology, vol. 1391, DOI 10.1007/978-1-4939-3332-7_10,

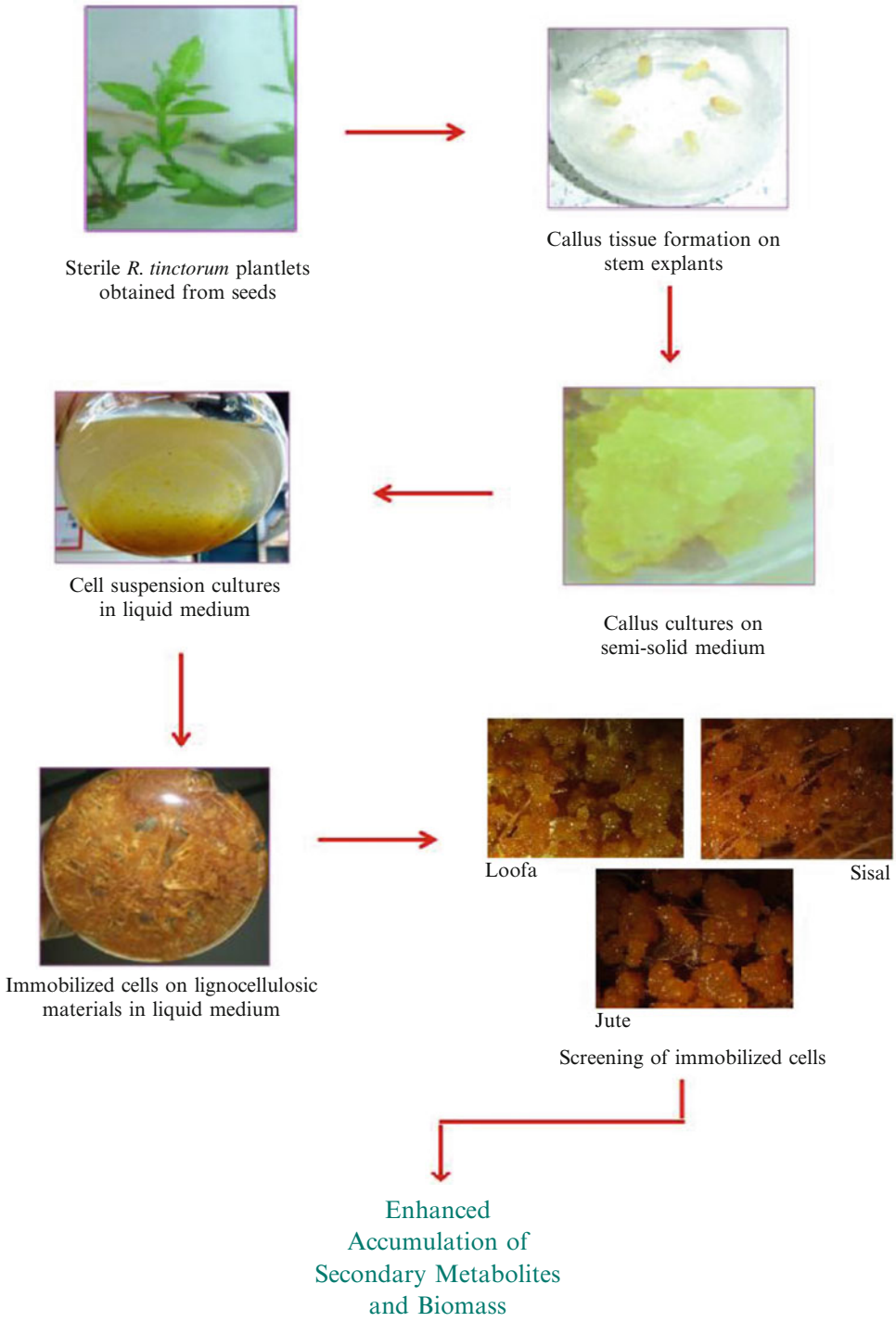

Fig. 1 Steps of immobilization procedure from plantlets to immobilized cells and enhanced accumulation of secondary metabolites and biomass

point in cell metabolism which allows metabolites to move from one cell to another. Secondary metabolite production is usually higher in slow growing or non-growing cultures [3, 4]. The immobilization of plant cells addresses all of these factors and may be the best approach for increasing secondary metabolite production. This technique offers the potential for dealing with the limitations of secondary metabolite production in liquid plant cell cultures.

In order to immobilize cells, ideally grown suspension cultures at exponential phase must be obtained firstly. The suspension cultures are started from callus or protoplast cultures of the plant species of interest (Fig. 1).

Although there is no ideal immobilization matrix for plant cell cultures, cell carriers should have some features in order to ease the production of biomass and secondary metabolites. The most common matrices used for plant cell immobilization are calcium alginate [1] and reticulate polyurethane foam [2]. Besides these matrices, agar and agarose [5], polyester [6] glass [7] fibers, hybrid sol–gel matrices [7], loofah sponge, jute and sisal fibers [8] have also been utilized for immobilization.

We used lignocellulosic materials (loofah, sisal, and jute) in order to immobilize suspended cells of *Rubia tinctorum* L. (Fig. 2). Immobilizing cells from *R. tinctorum* L. on lignocellulosic materials provided around three times higher biomass production in comparison to suspension cultures. Alizarin and purpurin contents of immobilized cells were 6 and 23 times higher in inoculated cells, respectively. In this chapter, the methods for establishing an ideally grown suspension culture and immobilization of suspended cells on lignocellulosic materials are discussed.

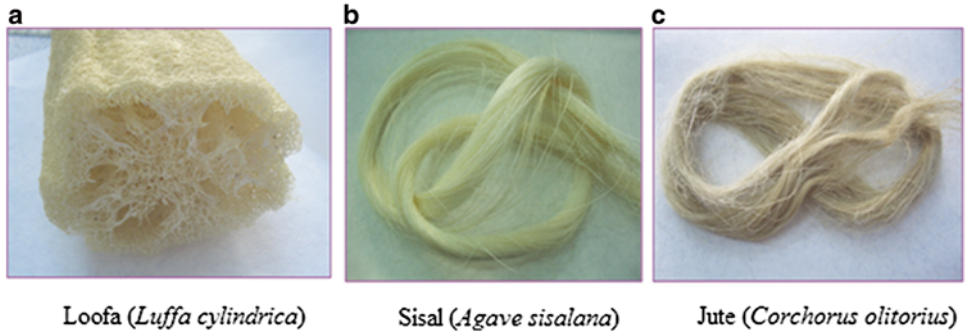

Fig. 2 Natural lignocellulosic materials used for immobilization of plant cell cultures

2 Materials

2.1 Laboratory Materials and Equipment

1. Laminar flow cabinets.
2. Bunsen burner.
3. Dissecting forceps and scalpel.
4. Beakers.
5. Cylinders.
6. pH-meter.
7. Analytical balance.
8. Stirrer with hot plate.
9. Culture vessels containing semisolid media.
10. 250 ml Erlenmeyer flasks for use as culture vessels for liquid medium.
11. 250 or 500 ml bottles for storage of liquid cultures.
12. Orbital shaker.
13. A digital camera fitted on a compact stereomicroscope.
14. Autoclave.
15. Incubator at 170 °C.
16. Autoclavable 30 mesh sieve.
17. Filter paper.
18. Sterile petri dishes to weigh callus on an analytical balance in laminar flow cabinet (sterile aluminum foil can also be used instead of petri dishes).
19. Funnel.
20. Distilled water sterilized by autoclaving (121 °C for 30 min at a pressure of 1.2 kg/cm^2).

2.2 Plant Material

1. Internode parts of shoots, which were grown from the shoot tips of clonally micropropagated plantlets in micropropagation medium (Table 1).

2.3 Immobilization Materials

1. Loofah (mature and dried fibrous fruit of *Luffa cylindrica*) (Fig. 2a).
2. Sisal (fibers of *Agave sisalana* leaves) (Fig. 2b).
3. Jute (fibers of *Corchorus olitorius* leaves) (Fig. 2c).

2.4 Stock Solutions, Semisolid and Liquid Media

1. Stock solutions (1 mg/100 ml) of 2,4-dichlorophenoxy acetic acid (2,4-D), indole-3-butyric acid (IBA), and kinetin and store in the freezer at 4 °C.
2. Stock solutions of Murashige and Skoog (MS) medium [9] (Table 2).

Table 1
Contents of micropropagation, callus initiation (CI), and liquid CI (L-CI) media

	Media composition				
Medium name	**Basal medium**	**Growth regulators**	**Sucrose**	**Agar**	**pH**
Micropropagation medium	MS	0.5 mg/l IBA	3 %	0.7 %	5.8
Callus initiation medium (CI)	MS	0.1 mg/l 2,4-D 0.5 mg/l BA 0.5 mg/l Kinetin	3 %	0.7 %	5.8
Liquid CI medium (L-CI)	MS	0.1 mg/l 2,4-D 0.5 mg/l BA 0.5 mg/l Kinetin	3 %	–	5.8

Table 2
Composition of MS medium

MS medium[a]			
Stock solutions	**g/l**	**g/l for 100 ml stock solution**[b]	**Stock code**
KI	0.00083	0.0166	MS1
$MgSO_4 \cdot 7H_2O$	0.37	7.4	MS2
$MnSO_4 \cdot 4H_2O$	0.0223	0.446	
$ZnSO_4 \cdot 7H_2O$	0.0086	0.172	
$CuSO_4 \cdot 5H_2O$	2.5E-05	0.0005	
KH_2PO_4	0.17	3.4	MS3
$CaCl_2 \cdot 2H_2O$	0.44	8.8	MS4
H_3BO_3	0.0062	0.124	MS5
$Na_2MoO_4 \cdot 2H_2O$	0.00025	0.005	
$CoCl_2 \cdot 6H_2O$	2.5E-05	0.0005	
$Na_2 \cdot EDTA$	0.0373	0.746	MS6
$FeSO_4 \cdot 7H_2O$	0.0278	0.556	
Nicotinic acid	0.0005	0.01	MS7
Pyridoxine·HCl	0.0005	0.01	
Thiamine·HCl	0.0001	0.002	
Glycine	0.002	0.04	MS8
NH_4NO_3	1.65	No stock solution	–
KNO_3	1.9	No stock solution	–

[a]Dissolve all the stock solutions, NH_4NO_3, and KNO_3 in enough deionized water and make it up to 1 L. Adjust the pH to 5.8 (if necessary, add agar at this step) and autoclave for 15 min at 121 °C at a pressure of 1.2 kg/cm
[b]Use 5 ml stock solution for 1 L medium

3. *Micropropagation medium (semisolid MS medium)*: MS components, 0.5 mg/l IBA, 3 % sucrose, 0.7 % agar, pH 5.8 (Table 1).
4. *Callus initiation medium (CI) (semisolid MS medium)*: MS components, 0.1 mg/l 2,4-D, 0.5 mg/l BA, 0.5 mg/l kinetin, 3 % sucrose, 0.7 % agar, pH 5.8 (Table 1).
5. *Liquid CI medium (L-CI)*: MS components, 0.1 mg/l 2,4-D, 0.5 mg/l BA, 0.5 mg/l kinetin, 3 % sucrose, pH 5.8 (Table 1) (*see* **Notes 1** and **2**).

2.5 HPLC Analyses of Samples

1. Ultrasonic device.
2. SpeedVac concentrator.
3. Thermo Hypersil C-18 100 μm × 2.1 μm × 3 μm column.
4. HPLC-grade trifluoroacetic acid (TFA).
5. HPLC-grade acetonitrile.
6. HPLC-grade methanol.
7. HPLC-grade alizarin and purpurin (reference compounds).
8. HPLC-Grade HCl.
9. HPLC-Grade Na_2CO_3.
10. HPLC-grade ethyl acetate.

3 Methods

3.1 Stability Tests of Immobilization Materials

1. Place pieces (approximately 2 g DW) of each immobilization material (loofah sponge and fibers of sisal and jute) into glass beaker (*see* **Note 3**).
2. In the first stability test, autoclave samples three times for 15 min at 121 °C at 1.2 kg/cm^2. There should be no change in physical characteristics after the last autoclaving (*see* **Note 4**).
3. In the second stability test, put samples into incubator and subject them at 170 °C for 60 min three times. Avoid any change in physical characteristics except a little browning after the last incubation (*see* **Note 4**).

3.2 Preparation of Immobilization Materials

1. Cut loofah sponge transversely in 1 cm sections (approximately 2 g DW). Place two grams (DW) of sisal and jute fibers and loofah sponge into 250 ml flasks such that they fill the bottom area (*see* **Note 3**).
2. Wash fibers under tap water for 15 min. Soak them in boiling water for 30 min and wash them again three times with 200 ml distilled water. Close flask's mouth with cotton and cover it with aluminum foil (*see* **Note 5**).

3. After the washing process, place fibers into an incubator for drying and sterilization for 60 min at 170 °C. Soak them with sterile distilled water (approximately 5–10 ml) before addition of suspended cells (*see* **Note 6**).

3.3 Screening of Immobilized Cells on Natural Lignocellulose Materials

1. Use a digital camera fitted on a compact stereomicroscope and take images of immobilized cells on immobilization material.

3.4 Immobilization of R. tinctorum L. Cell Suspension Cultures

1. Cut internodes of shoots grown from the shoot tips of clonally micropropagated plantlets, in 1 cm sections and place them into CI medium (Fig. 1). Incubate them at 26 ± 1 °C in the darkness (*see* **Note 7**).
2. After 4 weeks, subculture calli grown on internode explants to fresh CI medium for producing more callus biomass. Incubate them at 26 ± 1 °C in the darkness (*see* **Note 8**).
3. Subculture callus cultures at least four times at 2-week interval to obtain friable callus. This step will facilitate in obtaining suspended cells in the liquid medium (*see* **Note 9**).
4. At the end of the fourth week of callus culturing, weigh 2 g (fw) callus for each flask and inoculate into 50 ml L-CI medium in 250 ml flasks in laminar flow cabinet under sterile conditions (*see* **Note 10**).
5. Four weeks later, filter liquid medium with cell clumps and suspended cells through a 30 mesh sieve. Dilute 25 ml filtrate (consisting of only suspended cells, not clumps) (8×10^5 living cells/ml) to 50 ml with L-CI medium and subculture four times at 14-day interval (*see* **Note 11**).
6. Adjust 25 ml of these suspension cultures to 50 ml, 75 ml, and 100 ml with L-CI medium (inoculation ratio 1/2, 1/3, and 1/4, respectively) and pour onto the sterilized immobilization materials in 250 ml flasks with two replicates (Fig. 3). Cultivate immobilized cells on an orbital shaker at 100 rpm and 26 ± 1 °C in the darkness (*see* **Note 12**).

3.5 Determination of Fresh and Dry Weights and Relative Dry Weight Ratios

1. At the fourth week of the immobilized cells culturing, filter the biomass through a funnel and normal filter paper. Remove cell clumps from the immobilization materials and measure the total fresh weights (g) of biomass for each flask (*see* **Note 13**).
2. After freeze-drying, calculate dry weights (g) and relative dry weight ratios (dry weight/fresh weight) (*see* **Note 14**).

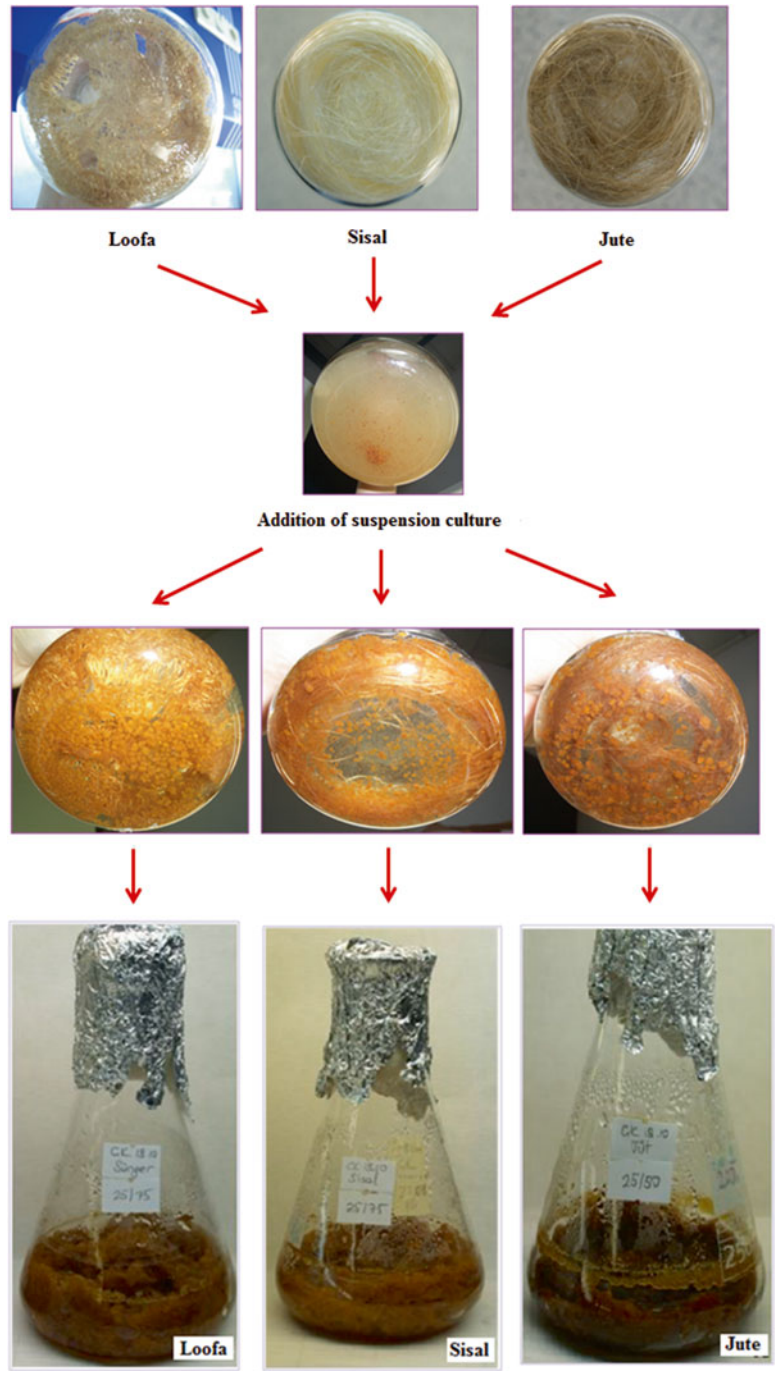

Fig. 3 Immobilization of *Rubia tinctorum* L. suspension cultures on lignocellulosic materials

3.6 HPLC Analysis of Samples

1. Extract the samples three times for 10 min with 5 ml methanol. Filter each extract, combine and evaporate them to dryness under vacuum at 60 °C (*see* **Note 15**).
2. Hydrolyse the residues by refluxing with 10 ml 5 % HCl for 1 h. Neutralize the samples with 1 M Na_2CO_3 and extract them four times with 10 ml ethyl acetate. Combine each extract and evaporate them to dryness under vacuum at 60 °C (*see* **Note 16**).
3. Dissolve all samples with 10 ml methanol and analyze them with Thermo Hypersil C-18 100 μm × 2.1 μm × 3 μm column. The mobile phase should be 0.01 % TFA in water (solvent A) and acetonitrile (solvent B) with gradient elution of 27 % B for 2.38 min; from 27 % B to 50 % B in 4.83 min; to 55 % B in 2.46 min; hold at 95 % B for 1.2 min and finally return to initial condition (27 % B) for 1.2 min respectively (*see* **Note 16**).
4. The flow rate during analysis should be 750 μl/min and inject 5 μl for each sample. The detector monitors the fluent at 250 nm.
5. Establish calibration curves by dissolving 10 mg reference compounds (alizarin and purpurin) with 100 ml HPLC grade methanol. Construct the calibration curves by plotting the peak areas of alizarin and purpurin versus their concentrations (*see* **Note 17**).

4 Notes

1. Nutrient medium should be autoclaved for 15 min at 121 °C at a pressure of 1.2 kg/cm^2 and then should be stored in darkness at last for 1 month. Liquid media should be shacked before use.
2. Adjust pH of the media to 5.8 with 0.5 M NaOH and 0.5 M HCl. Instead of NaOH, solutions like KOH can be used to adjust pH. If the volume of the medium is low (e.g., 100–200 ml), try to use solutions at lower concentrations (0.1–0.5 M).
3. Immobilization material should cover bottom of the flask as much as possible to obtain homogeneity in cultures.
4. Stability tests should be done prior to study. Once the tests are performed, there is no need to repeat them again before every experiment.
5. Among the other factors, cotton and aluminum foil are the most important factors to keep the culture sterile during culture period. Use tight cotton plug in flask without any space. Aluminum foil should cover the neck of flask completely (Fig. 3).

6. It is very important to soak immobilization materials with 5–10 ml distilled water before addition of suspended cells. In failing to do so, they will absorb nutrient medium and the volume of culture medium will be decreased.
7. Explants can be cut smaller than 1 cm up to 2 mm. If the desired metabolite is synthesized in light, then the cell culture should be incubated in light conditions.
8. Subculturing period is a tentative parameter for callus cultures. For fast growing callus, the subculture period should be shorter. In contrast, some callus types grow more rapidly and need to be subcultured more frequently (e.g., every 7–10 days).
9. In order to enhance the quality of cell cultures, subculture callus tissues at least four times before transferring to the liquid medium. The number of subcultures of friable callus can be reduced.
10. Biomass amount for inoculation should be optimized prior to the study. Optimum callus biomass amount for inoculation varies depending on the plant species. Some callus types adapt to liquid medium much easier than the others. It is very important to shorten the lag phase. If the culture enters to log phase quickly, this means the culture is in a good condition and the cells have started to divide. Callus biomass amount for inoculation can be reduced to 0.5 g/50 ml liquid medium in these cases. However, in some cases, 2 g callus is not enough to initiate cell culture otherwise up to 3–4 g callus is used to initiate suspension cultures. Conditioned medium can be a solution in these situations. The liquid medium in which cells are grown can be diluted (1/10 or 1/5) with fresh medium and can be used as a conditioned medium. This application will help to shorten the lag phase.
11. Use sieves with different mesh sizes (30–100) for obtaining suspended cells. If sieve mesh size is smaller, suspension culture will have smaller suspended cells/aggregates. However, cell suspension will contain lower number of cells per ml that would take longer lag phase. Therefore, sieve mesh size is amongst the most important factors for determining the quality of cell suspension culture and should be optimized prior to the study.
12. Reduce cell inoculums of fast growing cells to 1/10. Lag phase duration can also be used as a parameter here.
13. Before weighing, wash biomass with 50 ml distilled water to remove culture medium leftovers at the filtering step. Fresh and dry weights of filter papers should also be determined prior to filtration.

14. Freeze-drying is not necessary to determine dry weight. Incubation in an oven at 50–60 °C or air-drying can also be used. However, if the desired metabolite is unstable, freeze-drying is the most suitable method.
15. Powder the samples before extraction.
16. Use HPLC grade chemicals and solutions.
17. Prepare calibration curves by serially diluting the stock solutions of alizarin and purpurin twofold with methanol (100 mg/ml; 50 mg/ml; 25 mg/ml; 12.5 mg/ml; 6.25 mg/ml; 3.125 mg/ml).

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Chapter 11

In Vitro Propagation and Conservation of *Bacopa monnieri* L.

Neelam Sharma, Rakesh Singh, and Ruchira Pandey

Abstract

Bacopa monnieri L. (common name brahmi) is a traditional and renowned Indian medicinal plant with high commercial value for its memory revitalizer potential. Demand for this herb has further escalated due to popularization of various brahmi-based drugs coupled with reported anticancer property. Insufficient seed availability and problems associated with seed propagation including short seed viability are the major constraints of seed conservation in the gene banks. In vitro clonal propagation, a prerequisite for in vitro conservation by enhanced axillary branching was standardized. We have developed a simple, single step protocol for in vitro establishment, propagation and medium-term conservation of *B. monnieri*. Single node explants, cultured on Murashige and Skoog's medium supplemented with BA (0.2 mg/L), exhibited shoot proliferation without callus formation. Rooting was achieved on the same medium. The in vitro raised plants were successfully transferred to soil with ~80 % survival. On the same medium, shoots could also be conserved for 12 months with high survival and genetic stability was maintained as revealed by molecular markers. The protocol optimized in the present study has been applied for culture establishment, shoot multiplication and medium-term conservation of several *Bacopa* germplasm, procured from different agro-ecological regions of India.

Key words *Bacopa monnieri*, Brahmi, Clonal propagation, Genetic stability, ISSR, In vitro conservation, In Vitro Genebank, Medicinal plant, RAPD

1 Introduction

Bacopa monnieri L. (synonym *B. monniera*), commonly known as "brahmi," "jalanimba," or "the thinking man's herb" is an ancient and important "Medhyarasayana" drug in the traditional system of Indian medicine—the Ayurveda. In India, it grows in damp areas up to 1320 m. It forms an important ingredient of a number of Ayurvedic preparations, such as "*Brahmighrit*," "*Brahmirasayana*," "*Sarasvatarisht*," and "*Brahmivati*." The whole plant is used as a drug to cure epilepsy, relieve mental stress, improve intelligence and memory power, and anxiety neurosis [1, 2]. Besides having anti-inflammatory, analgesic, and antipyretic

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properties, the plant is known to also have anticancer and antioxidant properties. The saponins—bacoside A and B have been indicated for nervine tonic properties. Additionally, it has a good market especially for Brahmi oil due to its high medicinal value. The demand for *Bacopa* has further escalated owing to popularization of brahmi-based drugs like "*Mentat*," "*Memory Plus*," and "*Memory Perfect*" in the Indian and global market, and the recent report of anticancer activity of the herb extracts using *Sarcosoma* cell culture. Lack of concerted efforts regarding its cultivation/improvement, coupled with high demand of this medicinal herb by pharmaceutical companies has led to collection of material from wild population. A large amount of plant material is required for drug extraction primarily due to drug content of the plant being very low (~0.2 %). Further, the plant requires very specific growing conditions. Thus conservation of this precious herb needs immediate attention to ensure its availability and sustainable utilization now, and in future [3].

Despite the medicinal potential and high commercial value and realization of the need for conservation, there are no reports regarding conservation of this precious herb in the natural habitat. Application of seed conservation methods is constrained due to insufficient seed availability, difficult seed propagation and short seed viability. Thus, use of in vitro techniques, increasingly applied for rapid clonal propagation and conservation of valuable and threatened germplasm of medicinal importance with varying degrees of success [4, 5], appeared to be a viable conservation strategy for this plant. Standardization of efficient in vitro propagation protocol is an essential prerequisite for application of in vitro conservation. We have developed a method for in vitro clonal multiplication and medium-term conservation of *Bacopa* [2, 6].

There are few published reports on regeneration of *Bacopa* from various explants through axillary bud break, adventitious shoot formation, somatic embryogenesis and/or callus regeneration [2, 7–10]. For conservation, enhanced axillary branching is the preferred mode of regeneration as it ensures genetic stability of the conserved germplasm. However, genetic stability of the conserved cultures needs to be tested, while developing new protocol for conservation, using available molecular tools. This chapter describes various steps of a reliable in vitro clonal propagation and conservation protocol developed in our laboratory [2, 6]. This protocol would be of great commercial uses and for large-scale production of high-quality planting material and/or secondary metabolite production as also for conservation of this valuable medicinal species.

2 Materials

2.1 Plant Material

Healthy young shoots of *Bacopa* plants maintained in pots in the net house for culture initiation and young leaves for genetic stability studies.

2.2 Media Preparation

1. Equipment: Magnetic stirrer cum hot plate, Weighing balances, Analytical balance to weigh from 0.1 mg up to a few grams, Top pan balance from few to hundreds of grams, Microwave, Gas stove, pH meter, Micropipettes, Autoclave, Refrigerator.
2. Chemicals/reagents: Salts required for Murashige and Skoog's (MS) [11] medium (*see* Table 1) and distilled/reverse osmosis (RO) water.
   4.0, 7.0 and 9.2 pH buffers and 1 N NaOH/HCl for setting pH of medium, ethanol.
3. Glassware/plasticware: Reagent bottles, conical flasks (250, 1000 ml capacity), beakers, measuring cylinders (varying capacities), culture tubes (25 × 150 mm), culture tube enclosures (cotton plug/plastic caps/screw caps), glass rods.

2.3 Surface Sterilization and Inoculation

1. Equipment: Laminar air flow, Bead sterilizer.
   Tissue culture tool kit comprising 8″ to 12″ rust proof stainless steel forceps, surgical scalpels with supply of removable sterile surgical blades (straight and sharp), tissue paper, cotton wool, etc.
2. Chemicals/reagents: 0.01–0.1 % Mercuric chloride ($HgCl_2$), teepol, 80 % ethanol, sterile distilled water, culture medium.
3. Glassware/plasticware: Conical flasks (100, 250 ml capacity), beakers (250 ml capacity), sterile petri dishes.

2.4 Hardening and Transfer Out

1. Chemicals/reagents: Water, bavistin, ½ strength MS (liquid without sucrose).
2. Glassware/plasticware: Large petri dishes, forceps 12″, brush to remove agar, plastic and earthen pots, polythene bags, potting mix, soil.

2.5 In Vitro Conservation

1. Equipment: Same as listed in Subheadings 2.2, **item 1** and 2.3, **item 1**, culture incubation chambers at different low temperatures (4, 10, 15, 20, 25 °C), stereo microscope.
2. Chemicals/reagents: Culture medium (*see* Subheading 2.2, **item 2**), 80 % ethanol, mannitol, alginic acid (low viscosity), calcium chloride ($CaCl_2$).
3. Glassware/plasticware: Conical flasks (100 ml capacity), beakers (250 ml capacity), sterile petri dishes, cryovials.

Table 1
Media composition for Murashige and Skoog's [11] for stock preparation

Designated stock	Compound	Formula	Concentration (mg/L)	Quantity for stock solution (g/20 L)	Volume of solution (ml)	Volume required for 1 L
	Major					
A (Macro 1)	Ammonium nitrate	NH_4NO_3	1650	33	1000	50
	Potassium nitrate	KNO_3	1900	38		
	Magnesium sulfate	$MgSO_4{\cdot}7H_2O$	370	7.4		
	Potassium dihydrogen phosphate	KH_2PO_4	170	3.4		
B (Macro 2)	Calcium chloride	$CaCl_2{\cdot}2H_2O$	440	8.8	1000	50
C	*Minor*					
	Manganese sulfate	$MnSO_4{\cdot}4H_2O$	22.3	0.446	200	10
	Zinc sulfate	$ZnSO_4{\cdot}7H_2O$	8.6	0.172		
	Boric acid	H_3BO_3	6.2	0.124		
	Potassium iodide	KI	0.83	0.0166		
	Sodium molybdate	$Na_2MoO_4{\cdot}2H_2O$	0.25	0.005		
	Copper sulfate	$CuSO_4{\cdot}5H_2O$	0.025	0.0005		
	Cobalt chloride	$CoCl_2{\cdot}6H_2O$	0.025	0.0005		
D	Iron					
	Ferrous sulfate	$FeSO_4{\cdot}7H_2O$	27.8	0.556	200	10
	EDTA disodium	$Na_2EDTA{\cdot}2H_2O$	37.2	0.744		
E	Vitamins					
	Nicotinic acid		0.5	0.01	20	1
	Pyridoxine hydrochloride		0.5	0.01		
	Thiamine hydrochloride		0.1	0.002		
	Glycine		2	0.04		
F	Meso-inositol		100	2	200	10
	Sucrose		30 g/L			
	Agar		8 g/L			

2.6 Genetic Stability Assessment

2.6.1 DNA Isolation

1. Equipment: High speed centrifuge, Microfuge, Autopipettes (2–20 µl, 20–200 µl, 200–1000 µl), Water bath, –20 °C Deep freezer, Refrigerator, Freeze-drier (optional), Fume hood.
2. Chemicals/reagents: CTAB (Cetyl Trimethyl Ammonium Bromide) Buffer [1.4 M NaCl, 100 mM Tris, 20 mM EDTA (Ethylene diamine tetra acetic acid), 0.2 % β-Mercaptoethanol, 1.5 % CTAB, 1 % PVP (Polyvinyl pyrrolidone)], Isopropanol, Saturated phenol, NaOAc (Sodium acetate), Chloroform–iso-amyl alcohol (24:1) mixture, 10:1 TE (10 mM Tris 1 mM EDTA), RNase A (10 mg/ml), 70 % ethanol.
3. Glassware/plasticware: Conical flasks, beakers, centrifuge tubes (50 ml), Eppendorf tubes, mortar and pestle, liquid nitrogen.

2.6.2 Randomly Amplified Polymorphic DNA (RAPD) Analysis

1. Equipment: DNA Thermocycler, Microfuge, Autopipettes of range: 2–20 µl, 20–200 µl, 200–1000 µl, DNA electrophoresis unit with power supply, –20 °C Deep Freezer, Refrigerator, Laminar air flow.
2. Chemicals/reagents: 50× TAE, pH 8.0 (2 M Tris–acetate, pH 8.0, 0.05 M EDTA, pH 8.0); 10× Loading Buffer (Bromophenol Blue: 0.25 % Xylene Cyanol FF: 0.25 % Glycerol: 50 % TAE: 1×); Ethidium bromide stock 10 mg/ml ethidium bromide in double distilled water; AmpliTaq DNA Polymerase, $MgCl_2$, Primers, Reaction buffer.
3. Glassware/plasticware: conical flasks, beakers, PCR tubes.

2.7 Intersimple Sequence Repeats (ISSR) Analysis

1. Equipment: Same as **item 1** in Subheading 2.6.2.
2. Chemicals/reagents: Same as **item 2** in Subheading 2.6.2.

3 Methods

3.1 Media Preparation

Media is generally prepared by diluting concentrated stock solutions.

3.1.1 Stock Solution Preparation

Prepare and store separate stock solutions for macronutrients, micronutrients, iron and vitamins (Tables 1 and 2, **Note 1**). Weigh and dissolve each medium component in a small quantity of water and make up the final volume by adding distilled water.

1. For preparation of iron stock, dissolve $Na_2EDTA{\cdot}2H_2O$ in 100 ml hot water. Add $FeSO_4{\cdot}7H_2O$ and shake well till iron gets properly dissolved and forms a clear light yellow solution. Add distilled water to make final volume.
2. Stock of plant growth regulator is prepared by dissolving BAP (a cytokinin) in a minimum volume of 0.5 N NaOH or NAA (an auxin) in 0.5 N NaOH/ethanol and then making up the volume with distilled water.

Table 2
Recommended storage duration of stock solutions (*see* Note 1)

Stock	Storage period (Months)	Storage temperature (°C)
A Macro 1	6	4
B Macro 2	6	4
C Micro	6	–20
D Iron	6	4
E Vitamins	2	–20
F Myo-inositol	2	–20
G Auxin/cytokinin	1	4

3.1.2 Preparation and Sterilization of Media

Protocol to make 1 L MS medium (*see* **Note 4**).

1. Pour 500 ml distilled water in a 1-L flask with a magnetic stirring bar.
2. Add 30 g sucrose and dissolve.
3. Add required stock solutions in the order (*see* Table 1).
4. Add aliquot of auxin stock/cytokinin stock solution (as per requirement).
5. Adjust the pH to 5.8 using pH meter and 1 N NaOH or HCl, with constant stirring.
6. After adjusting the pH, makeup the volume to 1 L using a graduated cylinder or graduated beaker, pour back in to flask; add 8.0 g agar and keep for melting.
7. Dispense about 15–20 ml medium per culture tube and cap the tube (*see* **Note 5**).
8. Autoclave at 121 °C with a pressure of 15 psi (1.06 kg/cm^2), for 15 min (*see* **Notes 2–4**).
9. Take out from autoclave and allow the medium to cool at room temperature. For preparing slants, keep the tubes tilted (at an angle of 45°) during cooling.

The powdered medium is dissolved in distilled water (10 % less than the final volume), and after adding sugar and other supplements, the final volume is made up with distilled water (*see* **Note 5**).

3.2 Explant Preparation

1. Collect healthy young shoots with three to four nodes, from plants maintained in the net house (Fig. 1a), in a beaker containing distilled water.
2. Remove leaves and wash in 1 % (v/v) Labolene detergent for 10 min on a magnetic stirrer.
3. Wash these thoroughly under running tap water for 2 h.

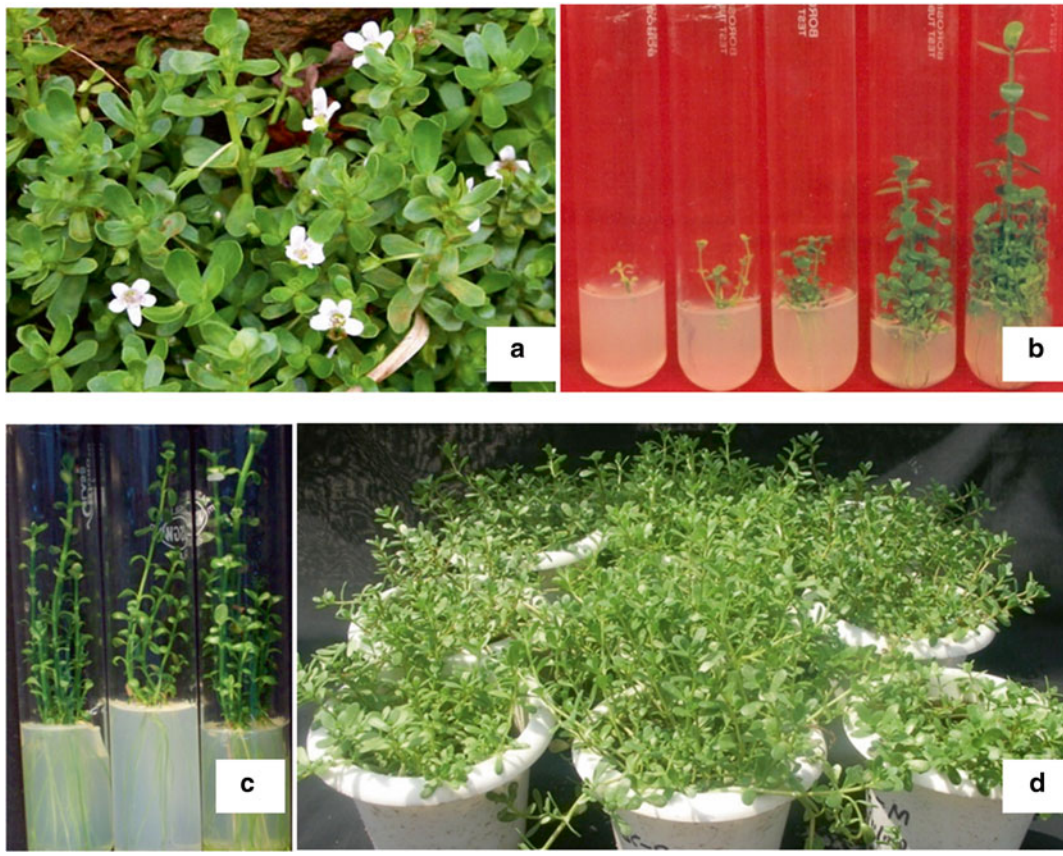

Fig. 1 (**a**–**d**) In vitro propagation of *Bacopa monnieri*. (**a**) Source of explants; (**b**) In vitro establishment and shoot multiplication on MS + 0.2 mg/L BAP (L–R); (**c**) 8-week-old cultures of some accessions showing multiple shoots and roots on MS + 0.2 mg/L BAP; (**d**) Plantlets established in pots

3.3 Surface Sterilization and Establishment of In Vitro Cultures

1. Disinfect the surface of the laminar flow with 75 % alcohol after UV light exposure for 15 min.
2. Arrange surface sterilized culture containers and tools on the bench.
3. Dip forceps/scalpels in ethanol followed by flaming after each operation.
4. Sterilize the stem segments with 0.1 % (w/v) mercuric chloride for 10 min under laminar air flow followed by rinsing (5 times) with sterile de-ionized water (*see* **Notes 6** and **7**).
5. Using sterilized forceps, transfer the explants into a sterile container and rinse 2–3 times with sterile water.
6. Transfer the segment to a sterile petri plate and with the help of a scalpel (with sterile blade), cut the edge (exposed ends) of the explants.
7. Trim single node segments to ca. 0.8 cm and culture on MS medium supplemented with 0.2 mg/L BAP.

8. Inoculate single explant in each culture tube and incubate at 25 ± 2 °C under 16/8 h photoperiod (30 μmol/m²/s) light/dark provided by cool white fluorescent lamps (*see* **Notes 8** and **9**).

3.4 Shoot Multiplication and Rooting

For shoot multiplication contamination-free cultures are subcultured every 4–6 weeks on shoot multiplication medium (Fig. 1b) (*see* **Notes 10** and **11**).

1. Gently take the cultures out of culture tube and dissect nodal explants to required size, on a petri plate using sterile surgical blade and forceps.
2. Using aseptic forceps, transfer the explants vertically onto shoot multiplication medium.
3. Hold the opening of the tube in the flame for few seconds, and then cap it.
4. Label appropriately using a standardized format and seal with Para film.
5. Maintain cultures at 25 ± 2 °C, 16/8 h photoperiod (30 μmol/m²/s) light/dark provided by cool white fluorescent tubes under controlled relative humidity of 50 to 60 %.
6. Monitor the cultures periodically for contamination, hyperhydricity or growth abnormalities (after each subculture cycle).

On shoot multiplication medium 100 % cultures form roots hence avoid separate rooting medium (Fig. 1b, c).

3.5 Acclimatization and Transfer Out

1. Transfer the rooted shoots (after 8 weeks of culture) to pots for hardening.
2. Gently remove well-rooted plantlets from the culture tubes, keeping the roots intact.
3. Wash off the adhering agar from roots very carefully with a brush, under running tap water.
4. Moisten the sterile vermiculite in plastic pots (7.5 cm diameter), transfer plantlets to pots and water lightly.
5. Cover with perforated polythene bags to maintain high humidity (~90 %).
6. Water the plants every 3 days with half-strength MS salt solution (pH 5.8) for 3 weeks.
7. Remove the polythene bags after 3 weeks and transfer the plants to earthen pots (20 cm diameter) containing garden soil, after 4–6 weeks and allow the plants to adjust to ambient conditions (Fig. 1d) (*see* **Notes 12** and **13**).

3.6 In Vitro Conservation: Slow Growth

1. Use cultures multiplied for three passages as source of explants
2. Transfer single nodal segment (0.8 cm) from 8-week-old cultures to glass tubes (25 × 150 mm) containing 15 ml of shoot multiplication medium solidified with 0.8 % agar.

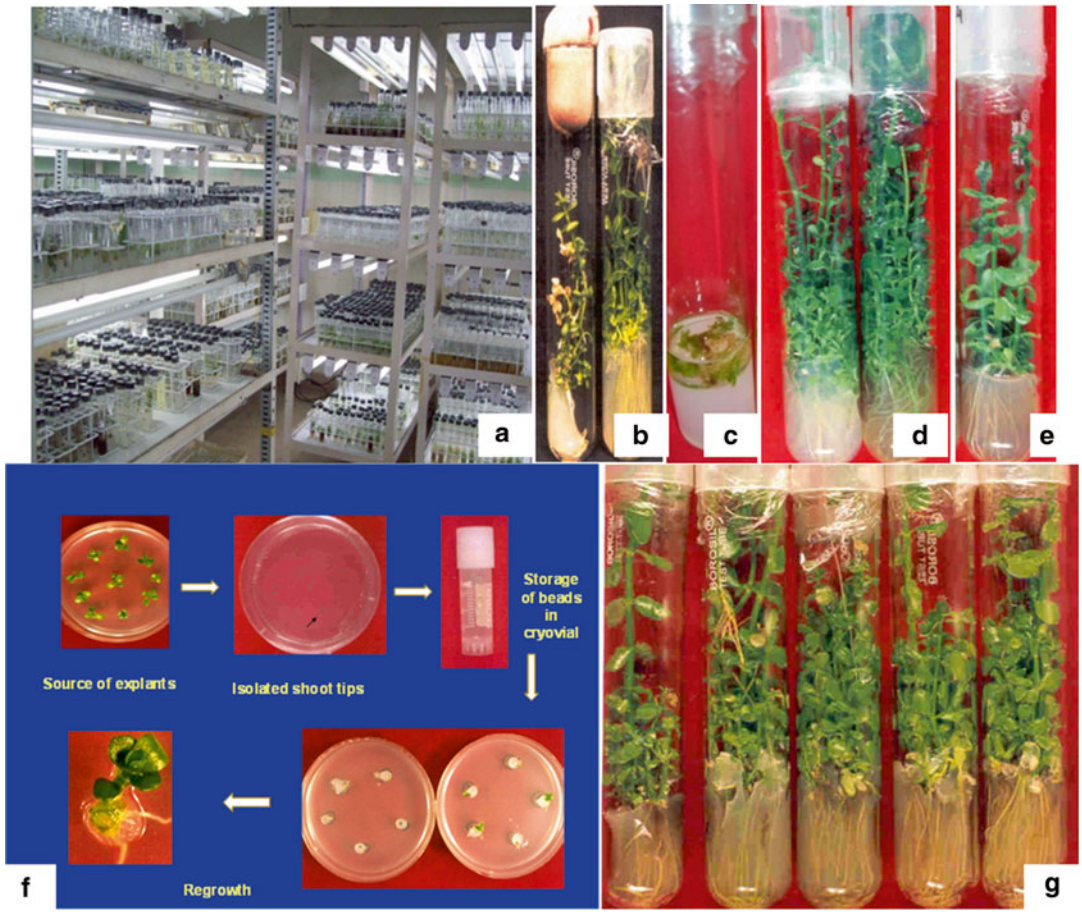

Fig. 2 (**a**–**g**) In vitro conservation of *Bacopa monnieri* using slow growth strategies. (**a**) A view of the In Vitro Genebank at NBPGR, India conserving multi-crops including *Bacopa*. Effect of various slow growth strategies (**b**–**f**). (**b**) Effect of culture tube enclosure; (**c**) Effect of mineral oil; (**d**) Effect of minimal media; (**e**) Regrowth of conserved cultures; (**f**) Storage of encapsulated shoot tips in a cryovial without nutrient medium; (**g**) Conserved shoot cultures of various accessions after 12 months on MS + 0.2 mg/L BAP

3. Incubate all the cultures in culture room for 10 days to detect and eliminate contamination (Fig. 2a).
4. To standardize the most suitable strategy for conservation of germplasm in a gene bank, the following slow growth strategies (*see* **Note 14**) (Fig. 2a–f) are tested:
   (a) To study effect of enclosures, close the tubes with either cotton plugs or polypropylene caps. Maintain cultures in the culture room.
   (b) For low temperature storage, transfer healthy cultures to low temperature, 10, 15, and 20 °C to select optimum responding temperature (*see* **Note 15**).
   (c) To test the effect of media modifications, various osmotica (sucrose, mannitol) are included in the media. Maintain cultures under culture room conditions.

(d) To test the effect of mineral oil, culture single node in each culture tube, and using a micropipette, pour ca. 3 ml mineral oil (10 mm depth) (autoclaved separately and cooled to 25 °C) over the explants to cover the explants completely. Maintain cultures in the culture room.

(e) To test the effect of storing encapsulated shoot tips in vials without any nutrient medium,

- Collect apical shoot tips from the shoot culture of the same subculture age (8 weeks after last subculture).
- Using fine forceps and scalpel dissect out shoot tips (1–2 mm) under the stereomicroscope, in a laminar air flow cabinet. The explants should contain the apical meristem dome along with one to three non-expanded leaf primordia.
- Make encapsulated shoot tips (referred as beads) by using 3 % sodium alginate as gel matrix and 100 mM calcium chloride ($CaCl_2 \cdot H_2O$) for complexation. Suspend excised shoot tips for ~20 min in sodium alginate (low viscosity alginic acid in liquid MS medium without calcium, pH 5.7). Explant along with sodium alginate are sucked (aspirated) into a sterile disposable Pasteur pipette and dropped one by one into calcium chloride solution. Allow beads to stand in the solution for 30 min with gentle shaking, for polymerization. Then take them out by decanting off calcium chloride solution, rinsing with sterile water followed by surface drying on sterile filter paper.
- Transfer five uniform beads with one explant each, in a cryovial without any medium, and maintain them under culture room conditions.

5. Periodically assess survival of cultures by visual examination and/or by the ability of explant to resume growth on fresh medium (Figs. 2 and 3) (*see* **Notes 15–20**).
6. For testing regrowth of conserved beads, transfer the beads onto shoot multiplication medium periodically after 2 weeks and score for emergence of shoots.

3.7 Genetic Stability Assessment

3.7.1 Preparation of Extraction Buffers

1. Add calculated amount of CTAB, NaCl, EDTA, Tris–HCl, and Polyvinyl pyrrolidone PVP in 200 ml of water and make the final volume to 1 L.
2. Adjust pH to 8.0 with HCl and autoclave before use.
3. Warm at 60 °C in a water bath for 30 min.
4. Add 0.2 % β-mercaptoethanol to the extraction buffer under fume hood

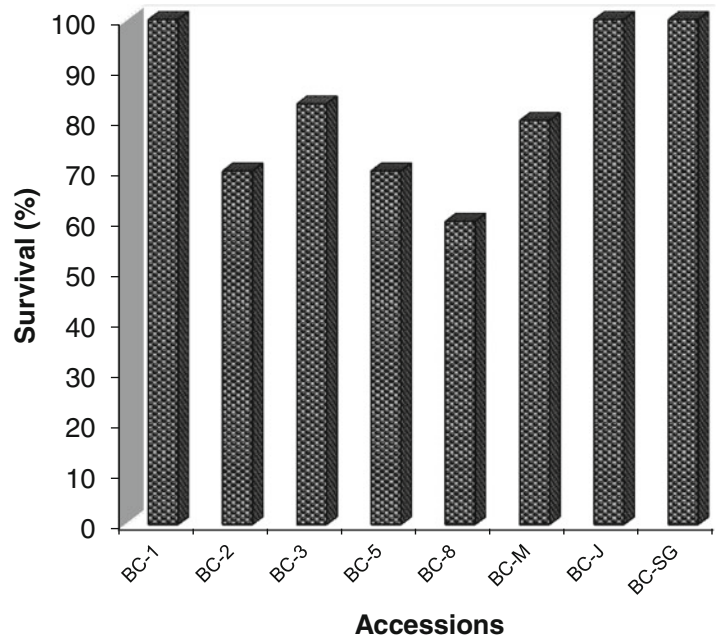

Fig. 3 Survival of some accessions of *Bacopa monnieri* after conservation for 12 months on MS + 0.2 mg/L BA, under culture room conditions (*see* **Note 21**)

3.7.2 Plant DNA Isolation and Purification

Method for Total genomic DNA extraction [12] (*see* **Note 21**):

1. Weigh 5 g of clean young leaf tissue and grind to fine powder with a pestle and mortar after freezing in liquid nitrogen.
2. Transfer to 50 ml centrifuge tube with 20 ml CTAB buffer maintained at 60 °C in a water bath.
3. Mix vigorously or vortex. Incubate at 60 °C for one hour. Mix intermittently.
4. Add 20 ml chloroform: isoamyl alcohol. Mix gently by inverting for 5 min.
5. Spin at 34,541 × *g* for 10 min with SS34 rotor in Sorval RC-5C centrifuge at 25 °C Transfer aqueous phase to a fresh centrifuge tube.
6. Add equal amount of isopropanol and let the DNA to settle down for 20 min.
7. Spool out the DNA or spin at 17,210 × *g* for 15 min and drain liquid phase.
8. Add 0.5 ml 70 % ethanol. Mix gently and incubate for 30 min.
9. Spin at 17,210 × *g* for 15 min and decant and repeat the 70 % ethanol treatment.
10. Decant off and dry the pellet under vacuum. Dissolve DNA in minimum volume of 10:1 TE buffer.

11. Add RNAse (0.2 ml) and incubate at 37 °C for 1 h. Add proteinase (0.2 ml) and incubate at 37 °C for 1 h (*see* **Note 22**).
12. Add equal volume of phenol–chloroform (1:1), mix properly for at least 2 min and spin for 5 min.
13. Take out the DNA supernatant and after this perform two chloroform: isoamyl alcohol extractions as before. Spin at 17,210 × *g* for 15 min after each extraction.
14. Precipitate DNA by adding 1/10 volume of 3 M NaOAc and 2.5 times of the total volume chilled ethanol.
15. Mix and spool out the DNA. Remove extra salts by two washings with 70 % ethanol.
16. Dry under vacuum. Add minimum volume of TE (10:1).
17. Dissolve at room temperature. Store frozen at –20 °C.

3.7.3 Randomly Amplified Polymorphic DNA (RAPD) Analysis (See Note 23)

1. Switch on the thermocycler at least 15 min earlier.
2. Pipette out accurately using appropriate autopipettes into sterile 0.5 ml microtubes the reagents in the following order:

   dd H_2O: 13.80 µl

   10× Reaction buffer: 2.50 µl

   25 mM $MgCl_2$: 2.50 µl

   10 µM Primer: 1.00 µl

   10 mM mix of dNTPs: 2.50 µl

   Taq DNA polymerase (5 U/µl): 0.2 µl

   Template DNA (10 ng/µl): 2.50 µl

   Total reaction volume: 25.00 µl
3. Mix by repeated pipetting.
4. Spin down the contents (2–5 s).
5. Place the tubes firmly in the wells.
6. Start thermocycler.

   The temperature cycling conditions are:

   Step 1. Denature at 94 °C for 4 min Step

   Step 2. Denature at 94 °C for 1 min Step

   Step 3. Annealing at 37 °C for 1 min Step

   Step 4. Polymerization at 72 °C for 2 min

   Step 5. Repeat from step 2–4: 39 times

   Step 6. Extended polymerization at 72 °C for 8 min

   Step 7. Refrigerate.
7. At the end of the run take out the tubes and add 2.5 µl 10× loading dye. Spin for 2–5 s. Store at 4 °C till electrophoresis.

8. Agarose Gel Electrophoresis: The amplified products in RAPD analysis are usually smaller than 4 kb size. Hence, they are separated by electrophoresis in 1.4–1.8 % agarose gels and visualized by staining with ethidium bromide and viewing under UV light.
   (a) Prepare the gel tray by taping the open ends. Place the comb and level the tray.
   (b) Boil and prepare 1.4 % agarose gel in 1× TAE buffer. Cool to 60 °C. Pour into gel tray avoiding air bubbles. Allow setting for 30–40 min.
   (c) Remove the adhesive tapes. Place in electrophoresis tank filled with 1× TAE buffer. Remove the comb carefully. Pour 1× TAE buffer till the gel is fully immersed.
   (d) Load the samples carefully. Take care to load suitable DNA size markers (about 200 ng). Connect the leads and start electrophoresis run at constant 60 V.
   (e) Stop the run when bromophenol blue dye has traveled less than 2/3 the length of gel.
   (f) Stain in 0.5–1 μg/ml ethidium bromide in distilled water for 30–40 min. Wash briefly in distilled water (*see* **Note 24**).
   (g) View under UV light. Photograph the gel (*see* **Note 24**).
   (h) Score the amplified products across the lanes comparing their respective molecular weight (Fig. 4). The data can be analyzed statistically using suitable packages (*see* **Note 25**).

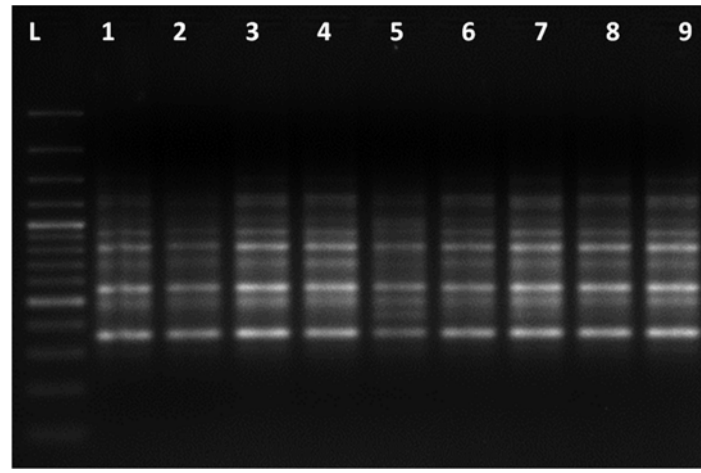

Fig. 4 Representative RAPD profiles (primer OPC-15) generated from culture (1–4) and ex vitro transferred plants (5–9) of *Bacopa monnieri*, *L*—denotes GeneRuler™ 100 bp DNA ladder

3.7.4 Intersimple Sequence Repeats (ISSR) Analysis (See Note 26)

1. Switch on the thermocycler at least 15 min earlier (Table 2).
2. Pipette out accurately using appropriate autopipettes into sterile 0.5 ml microtubes the reagents in the following order:

   H_2O: 14.30 µl

   10× Reaction buffer: 2.50 µl

   25 mM $MgCl_2$: 1.00 µl

   5 µM Primer: 3.00 µl

   2.5 mM mix of dNTPs: 2.00 µl

   Taq DNA polymerase (5 U/µl): 0.20 µl

   Template DNA (10 ng/µl): 2.00 µl

   Total reaction volume: 25.00 µl
3. Mix by repeated pipetting.
4. Spin down the contents (2–5 s).
5. Place the tubes firmly in the wells.
6. Start thermocycler.

   The following general thermocycling steps are followed

   Step 1: Initial denaturation at 94 °C for 5.0 min

   Step 2: Denaturation at 94 °C for 1.0 min

   Step 3: Primer annealing at 52–59 °C (depending upon primer) for 45 s

   Step 4: Primer extension at 72 °C for 2.0 min

   Step 5: Go to step 2, 39 times

   Step 6: Final extension at 72 °C for 7 min

   Step 7: 4 °C for ever
7. After the run is completed turn off the machine and remove the samples. Add 2.5 µl gel loading dye in each tubes and carry out electrophoresis or store samples at 4 °C until electrophoresis.
8. Agarose gel electrophoresis and analysis (Fig. 5): same as RAPD.

4 Notes

1. Store iron stock, preferably in an amber-colored bottle, at 4–10 °C in a refrigerator. However, prolonged storage of auxins is not recommended due to photo-oxidative degradation.
2. Media are generally sterilized by autoclaving at 121 °C with a pressure of 15 (1.06 kg/m^2). The time required for sterilization is dependent on the quantity of medium and type of container. A slow exhaust follows sterilization, as quick decompression will cause the medium to boil off the vessels.

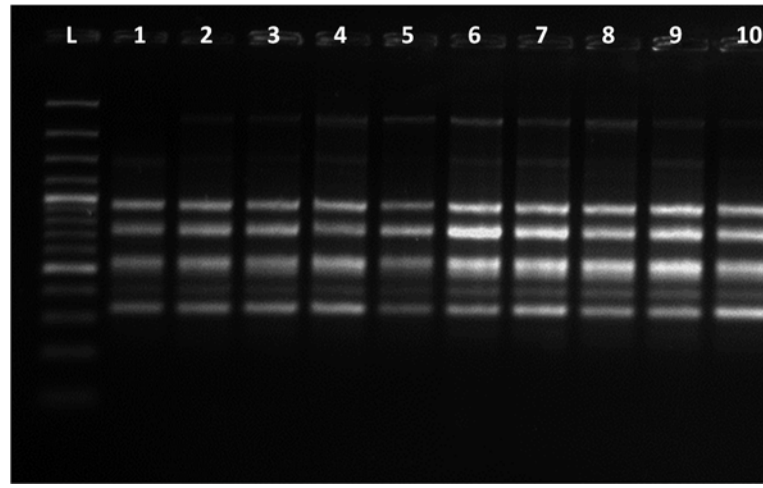

Fig. 5 Representative ISSR profiles (primer UBC-808) generated from culture (1–5) and ex vitro transferred plants (6–10) of *Bacopa monnieri*, *L*—denotes GeneRuler™ 100 bp DNA ladder

3. Media constituents (such as antibiotics) subject to modification by heat can be added to the autoclaved medium following filter-sterilization (passing through pre-autoclaved bacteria-proof membrane filtration unit).
4. Contamination can occur in the stock solutions of vitamins and growth regulators as well as in the media which are inadequately sterilized.
5. When using ready-made powdered media, it is essential that the powder is completely dissolved. Any turbidity of the solution indicates that one or more of the ingredients is not dissolved, and thus not available to the plant cells.
6. Explants are sterilized normally by treating with 3–5 % sodium hypochlorite or 0.05–0.1 % mercuric chloride solution for 5–15 min. When working with explants of a new genotype, it is better to run preliminary screening tests to determine the exposure time tolerated by explants.
7. If the explants turn brown or white, they are over-sterilized and the cells are dead. It is recommended to reduce the length of time or the concentration of sterilizing agent. In contrast, if the surface sterilization treatment does not yield clean cultures then the procedure needs to be more stringent: increase time or concentration of the sterilant.
8. It is recommended to check inoculated cultures for contamination every 2–4 days for at least 10 days. Contaminated cultures should be immediately removed and autoclaved before washing.
9. The standardized protocol for surface sterilization resulted in 60–100 % aseptic culture establishment at initiation stage.

However, minor time adjustment may be required in some genotypes. Inclusion of 0.1 % streptomycin was beneficial in some accessions exhibiting bacterial contamination during culture initiation.

10. Propagation methods may require modification depending upon the genotypes/accessions/diverse collections to be conserved, in the Genebank.
11. Amongst various combinations of plant growth regulators tested, MS supplemented with 0.2 mg/L was most suitable (*see* [2]). The described protocol has been adopted for in vitro establishment and propagation of more than 20 accessions (genotypes) collected from different agro-ecological regions of India. Though there was significant variation with respect to rate of multiplication, a minimum of 11 shoots/explants were obtained in the least responding accession [2].
12. Usually the most critical step of in vitro propagation is the establishment of plantlets in the soil. As the plantlets are propagated in vitro under high relative humidity with little or no photosynthesis, the acclimatization during the transition from culture to soil conditions must be gradual. Watering the plants is very critical as too little water may lead to permanent wilt, while too much water may lead to decay.
13. This protocol has the potential application for the conservation of germplasm using slow growth and cryopreservation techniques [2, 6, 14] and also for bulking up material for secondary metabolite production.
14. Slow growth strategy is based on optimizing subculture duration without risking germplasm loss and genetic stability through stressful treatments. It is important to note that genotype response to growth limiting treatments can be highly variable.
15. Various methods such as change of enclosure, media modification, low temperature incubation etc. have been employed to achieve slow growth in a large number of crops. Amongst several slow growth strategies and different type of explants experimented upon in *Bacopa*, subculture duration could be enhanced to 14 months with mannitol, 12 months with sucrose, 15 months with minimal media, and 18–24 months with mineral oil overlay, using polypropylene caps as closures, under culture room conditions. Incubation of cultures at low temperature (4 and 10 °C) was not successful.
16. Amongst various storage temperatures, the optimum temperature at which encapsulated shoot tips, conserved in a cryovial without nutrient medium, remained viable for 6 months was 25 °C. On transfer to medium for regrowth, thus conserved shoot tips, developed into normal shoots. This methodology has the potential for exchange of germplasm, as being free of

nutrient medium, problems related to melting of agar and contamination are minimized.

17. This simple single step protocol is suitable for the propagation and conservation of a total of 22 accessions of *Bacopa* germplasm, on MS + 0.2 mg/L BAP. They have been maintained in the In Vitro Genebank at NBPGR, India, for more than 10 years with subculture duration of 10–16 months, depending upon the accession.
18. One of the limitations of germplasm maintenance in the In Vitro Genebank, especially in the tropical region, has been the contamination of cultures during various stages. Under ideal situation it is best to discard contaminated cultures and start fresh from mother plants. However, in case of non availability of mother plant, as an emergency measure, cultures can be rescued following resterilization of explants using 0.05 or 0.1 % (w/v) mercuric chloride + 0.1 % (w/v) streptomycin for 5–8 min.
19. In the In Vitro Genebank, periodic monitoring of cultures for survival, chlorosis, hyperhydricity and contamination must be done. Subculture of stored cultures should be carried out when 30 % cultures are dead/dried/contaminated. It is recommended not to subculture all cultures of an accession at one go (on the same day) to rule out the possibility of losing the precious germplasm due to contamination/ technical/equipment error/mislabeling.
20. Monitoring genetic stability is an integral part of any in vitro conservation program. In *Bacopa*, no morphological, molecular or biochemical variation was detected in selected accessions using a few strategies [6]. Hence, the described in vitro conservation strategy can be safely implemented for medium-term conservation of *Bacopa* germplasm, especially the elite lines till effective long-term conservation protocols are available [13–16].
21. The isolation of DNA from plant cells essentially involves two steps—(1) The lysis of plant cells and solubilisation of DNA and (2) Enzymatic or chemical methods to remove contaminating protein, RNA and other macromolecules. Isolation and purification of DNA is an important step because pure and high molecular weight DNA is essential for the reliability of DNA for molecular biology techniques. Any impurities in the genomic DNA can inhibit enzymatic reactions, electrophoresis and quantification and in turn their downstream applications. For plant cells with a rigid cell wall, the disruption of cells usually requires that the tissue is ground using a pestle and mortar after thoroughly freezing them in liquid nitrogen. The powdered plant tissue is then transferred to an extraction buffer that contains detergent to disrupt the membranes. The extraction buffer also contains a reducing agent (β-mercaptoethanol) and a chelating agent (ethylenediamine tetra acetic acid, EDTA). This helps to

inactivate nucleases that are released from the plant cell which can cause serious degradation of the genomic DNA.

22. Dissolve RNase A in 10 mM Tris–Cl, pH 7.5, 15 mM NaCl. Heat at 100 °C for 15 min. Cool to room temperature. Store as aliquots at −20 °C.
23. The RAPD technique is based on the polymerase chain reaction (PCR). A DNA sequence is exponentially amplified with the help of arbitrary primers and a thermostable DNA polymerase. The reaction involves repeated cycles, each consisting of a denaturation, a primer annealing and an elongation step. In the first step the DNA is made single stranded by increasing the temperature up to 94 °C (denaturing step) in the second step, lower the temperature to about 37 °C results in prime annealing to their target sequences on the template DNA (annealing step). For the third step temperature is chosen where the activity of the thermostable polymerase is optimal, i.e., usually 72 °C. The polymerase now extends the 3′ ends of the DNA-primer hybrids towards the other primer-binding site. Since this happens at both primer-annealing sites on both the DNA strands, the target fragment is completely replicated. Repeating these three step cycles 35–45 times results in the exponential amplification of the target between the 5′ ends of the two primer binding sites. Amplification products are separated by gel electrophoresis and visualized by ethidium bromide staining (Fig. 4).
24. Ethidium bromide is a mutagen and a probable carcinogen. Wear gloves when working with ethidium bromide solutions. Also use care not to contaminate the work area with the solution. UV light is damaging and must be used with caution. UV light causes burns and can damage the eyes.
25. For genetic stability assessment, for each marker system, score the strong amplified fragments for their presence (1), absence (0) and missing data (9). Compute genetic similarity values based on Jaccard's coefficient using NTSYS-PC version 2.11a. Perform cluster analyses based on similarity matrices for combined scores of RAPD and ISSR using unweighted pair group method with arithmetical averages (UPGMA)—a method used for constructing phylogenetic tree using similarity matrix.
26. ISSRs are semi arbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite. Amplification in the presence of non-anchored primers also has been called microsatellite-primed PCR, or MP-PCR. Such amplification does not require genome sequence information and leads to multi-locus and highly polymorphous patterns. Each band corresponds to a DNA sequence delimited by two inverted microsatellites (Fig. 5). Like RAPDs, ISSRs markers are quick and easy to handle, but they seem to have the reproducibility of SSR markers because of the longer length of their primers. ISSR technique differs from the RAPD in the sense

that here longer primer sequences are used and hence the annealing temperature is also higher which together provide more stringency to the technique. Consequent to higher stringent conditions, ISSR markers are more reproducible as compared to the RAPD markers.

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Chapter 12

Establishment, Culture, and Scale-up of *Brugmansia candida* Hairy Roots for the Production of Tropane Alkaloids

Alejandra Beatriz Cardillo, Julián Rodriguez Talou, and Ana María Giulietti

Abstract

Brugmansia candida (syn. *Datura candida*) is a South American native plant that produces tropane alkaloids. Hyoscyamine, 6β-hydroxyhyoscyamine (anisodamine), and scopolamine are the most important ones due to their anticholinergic activity. These bioactive compounds have been historically and widely applied in medicine and their demand is continuous. Their chemical synthesis is costly and complex, and thereby, these alkaloids are industrially produced from natural producer plants. The production of these secondary metabolites by plant in vitro cultures such as hairy roots presents certain advantages over the natural source and chemical synthesis. It is well known that hairy roots produced by *Agrobacterium rhizogenes* infection are fast-growing cultures, genetically stable and able to grow in hormone-free media. Additionally, recent progress achieved in the scaling up of hairy root cultures makes this technology an attractive tool for industrial processes. This chapter is focused on the methods for the induction and establishment of *B. candida* hairy roots. In addition, the scaling up of hairy root cultures in bioreactors and tropane alkaloid analysis is discussed.

Key words *Brugmansia candida*, *Agrobacterium rhizogenes*, Hairy root cultures, Stirred tank bioreactor, Hyoscyamine, Scopolamine, 6β-hydroxyhyoscyamine

1 Introduction

Hyoscyamine, 6β-hydroxyhyoscyamine (also named anisodamine) and scopolamine are tropane alkaloids produced by *Solanaceous* plants [1, 2]. These compounds have a long history of application in medicine according to their anticholinergic activity [3].

In addition, 6β-hydroxyhyoscyamine is useful in medicine for the treatment of microvascular diseases, glomerulonephritis, rheumatoid arthritis, gastrointestinal colic, hemorrhagic necrotic enteritis, and eclampsia as well as for the control of toxic and septic shock and organophosphate poisoning [4–8].

According to the well-established use of these compounds in medicine, their demand is continuous [9–12]. For this reason,

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much effort has been invested in the development of cost-effective strategies for their production.

Nowadays, tropane alkaloids are industrially produced by the isolation from plants belonging to the Solanaceae family including, *Hyoscyamus niger* L., *Anisodus tanguticus*, several *Datura* species, *Scopolia tangutica* Maxim, and *Atropa belladonna* [13–15]. In addition, tropane alkaloids can be isolated from *Brugmansia candida* (syn. *Datura candida*), a South American native plant [16, 17].

Although tropane alkaloids can be chemically synthesized, the chemical synthesis is costly and time consuming [18]. Thus, the isolation of tropane alkaloids from natural sources such as plants grown in greenhouses is still the strategy of choice [14].

The application of in vitro cultures, such as micropropagated plants or hairy roots, is becoming a very interesting system for large-scale secondary metabolite production. This strategy guarantees a stable and uniform year-round supply, independent of the weather and soil conditions [13, 19–21].

Additionally, the recent progress achieved for the scaling up of hairy root processes makes this technology an attractive tool for industrial processes [22, 23]. As mentioned above, the production of tropane alkaloid in bioreactors, instead of their isolation from plants is an interesting strategy because it guarantees that the process will be carried out under defined and controlled conditions, thus preventing or reducing the variations in the product quality and alkaloid yield [24].

This chapter describes the protocols related to the induction and establishment of hairy root cultures of *B. candida* plants. In addition, it describes the methodologies for the analysis of the hairy roots produced, the scaling up of the cultures in a modified stirred tank bioreactor, and the analysis of the secondary metabolites.

2 Materials

2.1 Sterilization of B. candida Seeds and Seedling Growth

1. *Brugmansia candida* seeds collected from the Jardín Botánico of Buenos Aires, Argentina.
2. Seed sterilization solutions: 70 % (v/v) ethanol, 10 % (v/v) hypochlorite solution, sterile deionized water.
3. Gibberellic acid 0.1 % solution prepared with deionized water and sterilized by filtration trough a 0.22 μm pore membrane.
4. Gamborg B5/2 medium (half concentration of mineral salts), supplemented with sucrose 20 g/L and agar 8 g/L [25] (*see* **Note 1**) (Table 1).
5. Agar, petri dishes, Erlenmeyer flasks, and culture flasks.

Table 1
Chemical composition of the stock solutions of Gamborg B5 medium

10× major inorganic nutrient solution	*Stock volume: 1000 mL*
NaH_2PO_4	1.3 g
KNO_3	25 g
$(NH_4)_2SO_4$	1.34 g
$MgSO_4$	1.22 g
Cl_2Ca	1.14 g
1000× minor inorganic nutrient solution	*Stock volume: 100 mL*
$MnSO_4 \cdot H_2O$	1000 mg
H_3BO_3	300 mg
$CoCl_2 \cdot 6H_2O$	2.5 mg
$CuSO_4 \cdot 5H_2O$	2.5 mg
$Na_2MoO_4 \cdot 2H_2O$	25 mg
$ZnSO_4 \cdot 7H_2O$	200 mg
100× Fe-EDTA solution	*Stock volume: 200 mL*
$FeSO_4 \cdot 7H_2O$	556 mg
Na_2EDTA	746 mg
2000× KI	*Stock volume: 100 mL*
KI	150 mg
1000× vitamins solution	*Stock volume: 100 mL*
Thiamine·HCl	1 g
Pyridoxine	0.1 g
Nicotinic acid	0.1 g

2.2 Agrobacterium rhizogenes: Growth and Maintenance

1. *Agrobacterium rhizogenes* LBA 9402 culture or cryopreserved glycerol stock.
2. Yeast mannitol broth (YMB): 0.4 g/L yeast extract, 10 g/L mannitol, 0.5 g/L K_2HPO_4, 0.2 g/L $MgSO_4 \cdot 2H_2O$, 0.1 g/L NaCl, pH 7.0. For solid YMB medium add 15 g/L agar. Autoclave at 1 atm, 121 °C for 20 min.
3. Antibiotic stock solution: 100 mg/mL rifampicin.
4. Glycerol, cryovials.

2.3 Induction and Establishment of B. candida Hairy Roots

1. Petri dishes containing: (1) 8 g/L agar-water, (2) Gamborg B5/2 supplemented with 1 g/L ampicillin and (3) Gamborg B5/2 without ampicillin.
2. Scalpel, ampicillin.

2.4 Verification of the Transgenic Nature of B. candida Hairy Roots

1. CTAB extraction buffer: 100 mM Tris–HCl (pH 7.5), 700 mM NaCl, 50 mM EDTA (pH 8), 1 % CTAB, 140 mM β-mercaptoethanol (*see* **Note 2**).
2. Chloroform–isoamyl alcohol (24:1 v/v), isopropyl alcohol, ethanol absolute, 70 % ethanol.
3. RNase 10 mg/mL.
4. TE buffer: 10 mM Tris–HCl (pH 7.5), 1 mM EDTA (pH 8).
5. PCR reagents, oligonucleotide primers (Table 2), dNTPs, agarose, ethidium bromide.
6. Mortar and pestle, liquid N_2, conical tubes, PCR tubes, water bath, thermocycler, electrophoresis equipment.

2.5 Hairy Root Culture in a 1.5-L Modified Stirred Tank Bioreactor

1. Stirred tank bioreactor. Plastic mesh. Gamborg B5/2 medium (*see* Subheading 2.1).

2.6 Tropane Alkaloids Extraction and HPLC Analysis

1. Chloroform, 0.2 M H_2SO_4, 1 N NaOH, methanol–water (50:50v/v), gaseous N_2, hyoscyamine, 6β-hydroxyhyoscyamine, and scopolamine standards.
2. HPLC Shimadzu LC-20AT system equipped with an UV–Vis detector, column oven.
3. Li-ChroCART 125-4 column with LiChrospher 60 RP-select B (Merck), particle size 5 μm.
4. Mobile phase: octanesulfonic acid (0.01 M, pH 3)–methanol (65:35 v/v).
5. Conical tubes, amber vials, 0.45 μm nylon membrane.

Table 2
Primers used for the verification of the transgenic nature of hairy roots by PCR

Gene	Primers	Amplicon size (bp)
rolB	5′-AGTTCAAGTCGGCTTTAGGC-3′	770
	5′-TCCACGATTTCAACCAGTAG-3′	
rolC	5′-TAACATGGCTGAAGACGACC-3′	534
	5′-AAACTTGCACTCGCCATGCC-3′	
aux1	5′-TTCGAAGGAAGCTTGTCAGAA-3′	350
	5′-CTTAAATCCGTGTGACCATAG-3′	
virD	5′-ATGTCGCAAGGCAGTAAGCCC-3′	438
	5′-GGAGTCTTTCAGCATGGAGCAA-3′	

3 Methods

3.1 Sterilization of B. candida Seeds and Seedling Growth

1. Hydrate *B. candida* seeds for 3–5 days under tap water.
2. After hydration remove the brown seed coat (Fig. 1).
3. Surface-sterilize the seeds by immersing them into a 70 % (v/v) ethanol solution for 30 s. Transfer them immediately to a 10 % (v/v) hypochlorite solution in a closed sterile flask.
4. Incubate the material for 30 min at room temperature, stirring the flask every 10 min.
5. Rinse the seeds with abundant sterile water for 5 min. Repeat this step 4–5 times.
6. Incubate the sterilized seeds in 0.1 % gibberellic acid at 24 ± 1 °C for 16 h in order to facilitate and accelerate the germination of *B. candida* seeds.
7. Transfer the seeds to petri dishes containing Gamborg B5/2 medium (4–5 seeds/petri dish) and incubate them until germination at 24 ± 1 °C, under a 16 h photoperiod using cool fluorescent lamps at a light intensity of approximately 90 μmol/m²/s (PAR).
8. Check the plates the subsequent days to discard any contamination. If contamination is found, transfer the uncontaminated seeds to fresh medium.
9. Subculture the germinated seeds to fresh Gamborg B5/2 medium for seedling growth. Incubate the culture flasks in the same conditions as described above for 4–6 weeks.

3.2 Agrobacterium rhizogenes Growth for Hairy Root Induction

1. Inoculate a plate or 5 mL of YMB medium supplemented with 100 μg/mL rifampicin for growth of *A. rhizogenes* LBA 9402 (*see* **Note 3**). The inocula can be done using a single colony previously grown on YMB plates or from a stock sample cryopreserved with glycerol.
2. Incubate the plates or flasks for 24–48 h at 28 °C, 180 rpm.

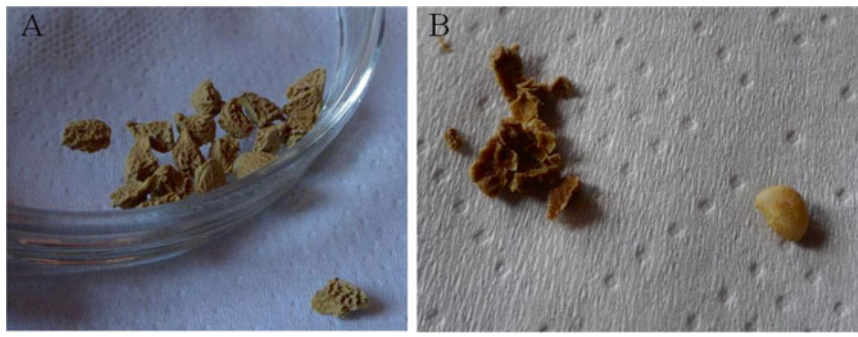

Fig. 1 (**a**) *B. candida* seeds, (**b**) Peeled seed

3. Subculture the bacteria in YMB-rifampicin plates and incubate them for 24 h at 28 °C for *Agrobacterium*-mediated transformation (*see* **Note 4**).

3.3 A. rhizogenes Maintenance

1. *Agrobacterium* strains can be maintained by continuous subculturing on YMB-rifampicin plates or by preparing glycerol stocks.
2. For cryopreservation, grow the bacteria in liquid YMB medium supplemented with the antibiotic for 24–48 h at 28 °C, 180 rpm.
3. Mix 800 μL of the grown culture with 300 μL of glycerol. Mix the tubes thoroughly by vortexing and store immediately at −80 °C.

3.4 Induction and Establishment of Hairy Roots by Agrobacterium rhizogenes-Mediated Transformation

1. 4–6 week old seedlings are used for hairy root production (Fig. 2a). The youngest leaves are harvested in order to provide explants for *Agrobacterium* transformation (*see* **Note 5**).
2. Under laminar flow, cut the leaves into small pieces (1 cm^2 approximately) and make incisions especially in the major veins by using a scalpel blade (*see* **Note 6**).
3. Coat the sectioned surface of the explants with the 24–48 h *Agrobacterium* culture. Scrape the surface of the plant tissue with a scalpel saturated with the bacteria.
4. Transfer the infected explants to agar-water plates (2–3 explants/plate). Place explants adaxial side down (*see* **Note 7**).
5. Incubate the infected explants in darkness at 24 ± 1 °C for 3 days.

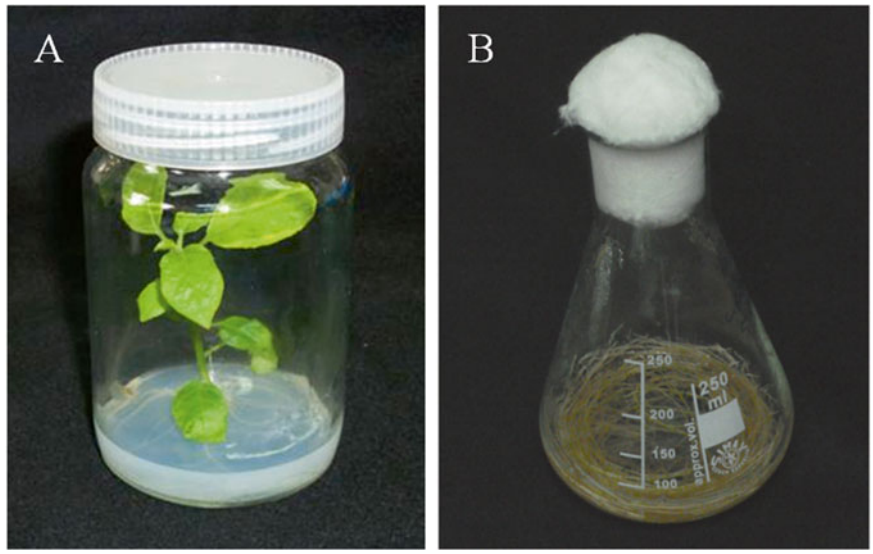

Fig. 2 *B. candida* (**a**) In vitro plant (**b**) Hairy root culture

6. Transfer the explants to Gamborg B5/2 medium supplemented with 1 g/L ampicillin to inhibit *A. rhizogenes* growth.
7. Incubate the infected explants at 24 ± 1 °C under a 16-h photoperiod using cool fluorescent lamps at light intensity of 90 μmol/m^2/s (PAR).
8. Subculture explants at a 20-day interval. Repeat this step at least three times maintaining 1 g/L ampicillin to eliminate *Agrobacterium* and then gradually reduce the concentration of the antibiotic.
9. Aseptically excise the root tips that appear at the infection sites and transfer to individual culture flasks containing Gamborg B5/2 medium supplemented with ampicillin (Fig. 3). After three to four passages, roots should be cleared of *Agrobacterium*. It is frequently observed that *A. rhizogenes* remains associated to the hairy root for a long time.
10. Subculture roots into liquid Gamborg B5/2 medium (Fig. 2b). If the bacteria are eliminated remove ampicillin from the culture medium.

3.5 Verification of the Transgenic Nature of B. candida Hairy Roots

3.5.1 DNA Isolation from Hairy Root Cultures

1. Grind the hairy roots (0.2–0.3 g) to a fine powder with liquid N_2 (*see* **Note 8**).
2. Add 2 mL CTAB buffer and transfer to a 15-mL conical tube. Incubate at 65 °C for 1 h 30 min.
3. Remove the sample from the water bath and let it cool.
4. Add 3 mL of chloroform–isoamyl alcohol (24:1) and mix by inversion for 10 min.
5. Centrifuge at 1,500 × *g* for 10 min at room temperature (*see* **Note 9**).

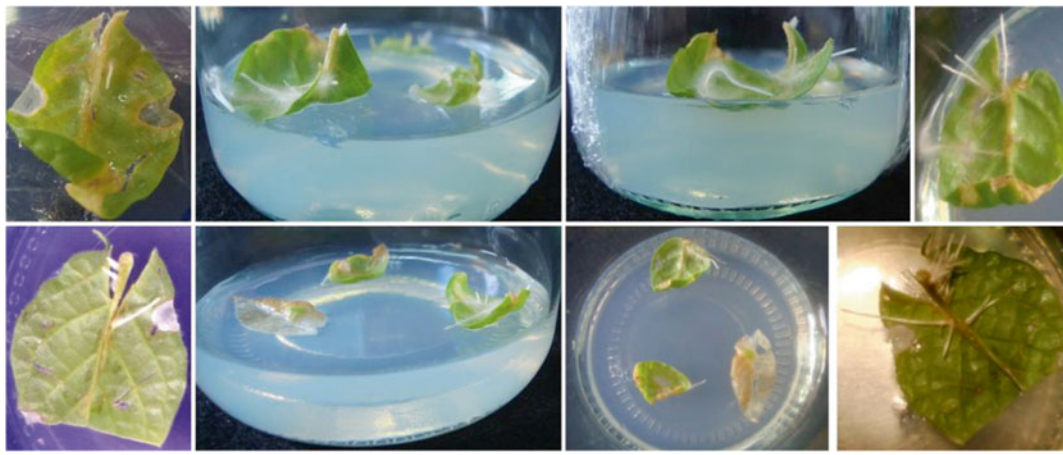

Fig. 3 *B. candida* root tips growing at the infection sites after *Agrobacterium*-mediated transformation

6. Transfer the aqueous phase to a new tube and repeat **steps 4–6**.
7. Add 10 µL of 10 mg/mL RNase, mix and incubate at room temperature for 30 min.
8. Add 3 mL of isopropyl alcohol, mix and incubate for 10 min at room temperature.
9. Centrifuge at 10,000 rpm for 10 min and discard the supernatant carefully.
10. Add 200 µL absolute ethanol and transfer to a 1.5-mL microcentrifuge tube. Rinse the sample for 1 min and centrifuge.
11. Discard the supernatant carefully; add 200 µL 70 % ethanol. Rinse the sample for 5 min and centrifuge.
12. Air-dry the DNA pellet and dissolve with 500 µL TE buffer. Store DNA at −20 °C.

3.5.2 PCR Analysis of rolB, rolC, auxI, and virD Genes

1. For PCR reactions, use 5 µL of the template (*see* Subheading 3.5.1) in a 25-µL reaction containing 5 µL of 5× PCR Buffer, 1 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM dNTPs, 0.5 µL of each 10 µM primer (Table 2), 0.6 U of Taq polymerase, and sterile water to a final volume of 25 µL.
2. Add all reaction components on ice. Gently mix the reaction and collect all liquid to the bottom of the tube by a quick spin if necessary. Transfer the reactions to the thermocycler.
3. PCR conditions used for *rolB*, *rolC*, *auxI*, and *virD* amplification are: initial denaturation at 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 1 min and a final extension at 72 °C for 5 min (*see* **Note 10**).
4. Visualize the results by gel electrophoresis in 1 % agarose gels stained with ethidium bromide.

3.6 Hairy Root Culture in a 1.5-L Modified Stirred Tank Bioreactor

1. Inoculate 250-mL Erlenmeyer flasks containing 50 mL of Gamborg B5/2 medium with 0.2–0.3 g fresh weight (FW) hairy roots.
2. Incubate hairy root cultures at 24 ± 1 °C, 100 rpm under a 16 h photoperiod for 15–20 days. These cultures are used as inocula for the bioreactor.
3. Place a plastic mesh forming a zigzag arrangement around the baffles in order to increase the surface area where the roots could be trapped (Fig. 4c) (*see* **Note 11**).
4. For a 1.5-L bioreactor capacity, fill the vessel with 1–1.2 L of Gamborg B5/2 culture medium.
5. Inoculate the bioreactor with 10 g FW of 15- to 20-days-old hairy roots previously grown in Erlenmeyer flasks as described above (*see* **Note 12**).

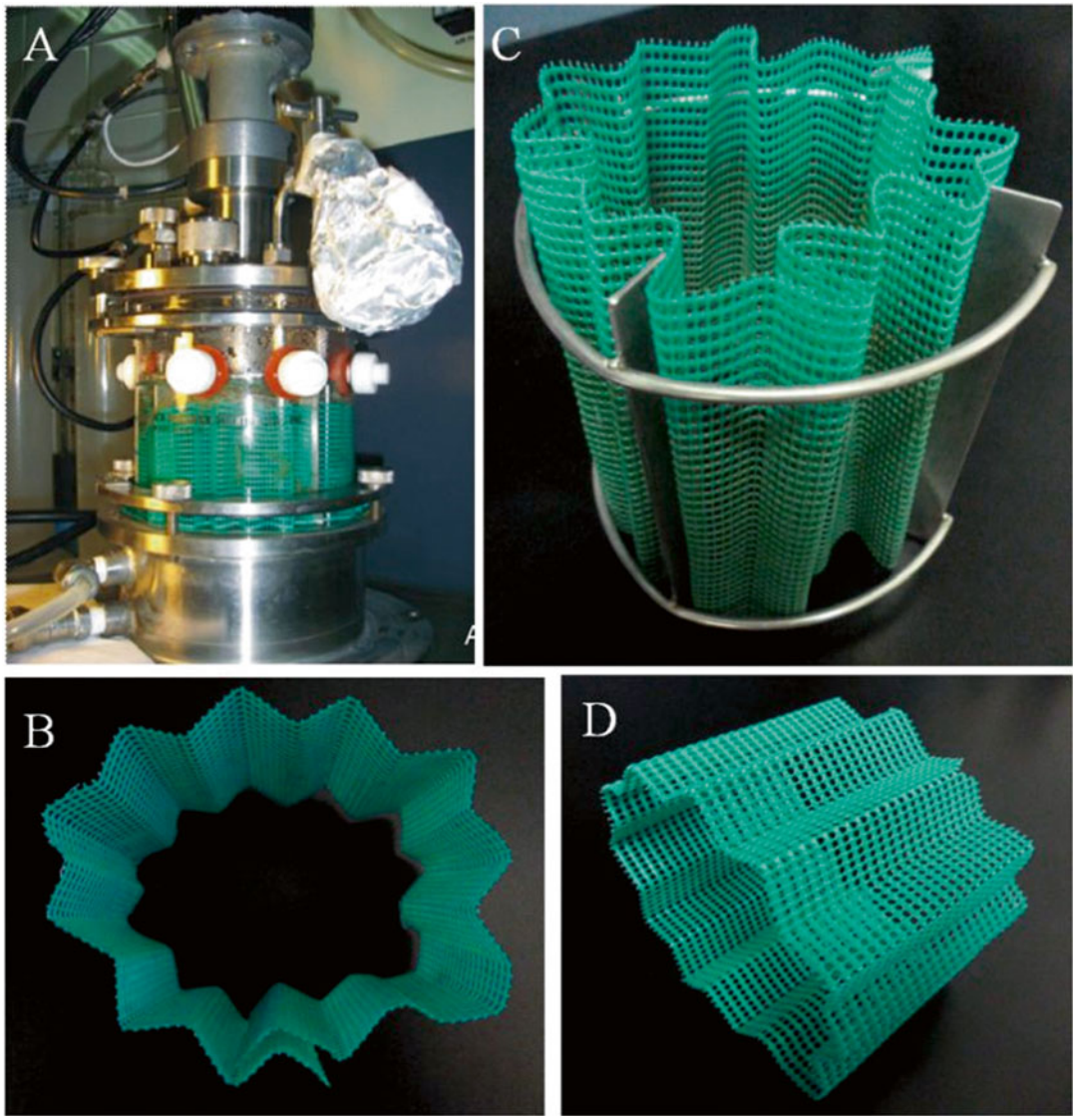

Fig. 4 (**a**) Bioreactor configuration, (**b**) top view (**c**, **d**) lateral view of the plastic mesh arrangement

6. Maintain the airflow rate at 0.5 vvm and the temperature of the bioreactor vessel at 24 ± 2 °C.
7. Harvest the roots 20 days after inoculation (Fig. 5) and analyze the samples to estimate FW, growth index (GI) and content of tropane alkaloids (*see* **Note 13**).
8. To estimate FW, vacuum-filter hairy roots, dry between two sheets of filter paper, and weigh.
9. Calculate the GI using the formula: $(W_f - W_i)/W_i$, where W_f is the final FW at 20 days, and W_i is the initial FW of the culture.

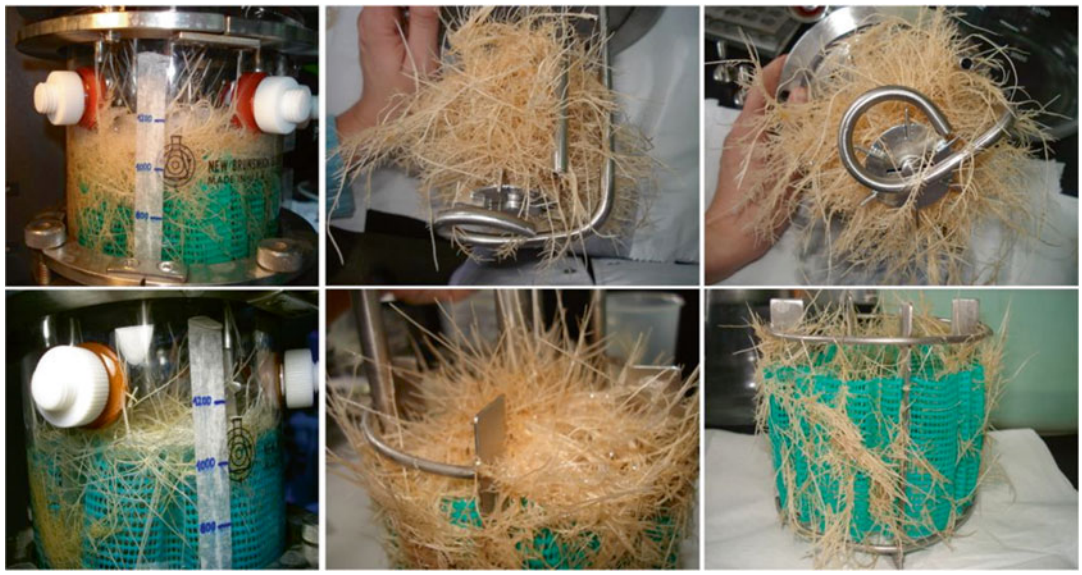

Fig. 5 *B. candida* hairy root cultures at the day of harvest of the modified stirred tank bioreactor

3.7 Hairy Root Processing and Organic Extraction of Tropane Alkaloids

1. Grind hairy root samples to a fine powder with liquid N_2.
2. Incubate the powder (0.3–0.8 g FW) for 2 h in 5 mL 0.2 M H_2SO_4 on an orbital shaker at 100 rpm.
3. Filter the mixture and alkalinize the aqueous phase up to pH 12 with 1 N NaOH (*see* **Note 14**).
4. Extract the alkaloids from the alkalinized aqueous phase with 5 mL chloroform. Agitate samples by vortexing for 2 min (*see* **Note 15**).
5. Transfer the organic phase to an amber vial.
6. Repeat **steps 4** and **5**.
7. Evaporate the organic phase under gaseous N_2.
8. Dissolve the residue in methanol–water (50:50 v/v) and filter through a 0.45-μm-pore nylon membrane.

3.8 HPLC Analysis of Tropane Alkaloids

1. Analyze and quantify tropane alkaloids with a Li-ChroCART 125-4 column with LiChrospher 60 RP-select B (Merck), particle size 5 μm.
2. Elute the alkaloids isocratically using as mobile phase octanesulfonic acid (0.01 M, pH 3)–methanol (65:35 v/v) at a flow rate of 1 mL/min (solvent quality for HPLC). Detection is carried out at 220 nm.
3. Adjust the column temperature to 40 °C using a column oven and inject 20-μL samples.
4. Identify peaks by comparing the retention times of sample peaks with standards (Fig. 6).

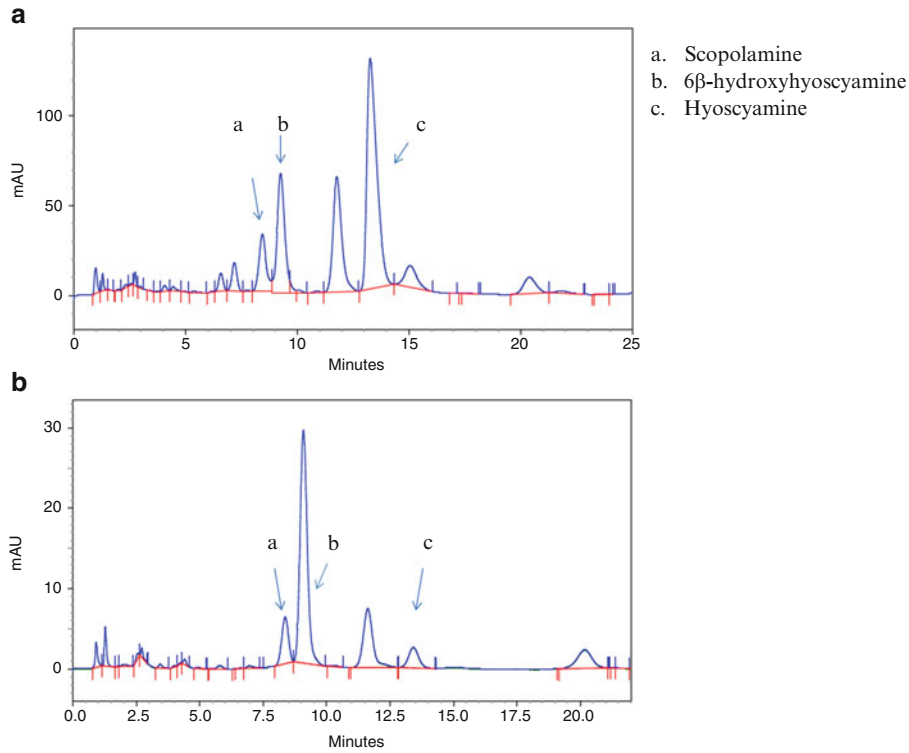

Fig. 6 Typical chromatograms of the organic extracts. (**a**) Alkaloid profile of hairy root samples (**b**) Alkaloid profile of culture media samples

5. For the standard curve: prepare stock standard solutions of hyoscyamine, 6β-hydroxyhyoscyamine or scopolamine in methanol–water (50:50 v/v) in a final concentration of 1000 ppm.
6. Dilute the stock solution with methanol–water (50:50 v/v) to prepare a series of standard solutions of 500, 250, 125, 50, and 12.5 ppm.

4 Notes

1. For the preparation of Gamborg B5/2 medium, add 50 mL 10× Major inorganic nutrient solution, 0.5 mL 1000× Minor inorganic nutrient solution, 5 mL 100× Fe-EDTA solution, 0.25 mL of 2000× KI solution, 100 mg/L myo-inositol, 1 mL of 1000× vitamins, water to 1000 mL final volume. For the composition of stock solutions *see* Table 1. Adjust pH to 5.5–5.6 and autoclave at 1 atm, 121 °C for 20 min.
2. Add β-mercaptoethanol to CTAB buffer the day of DNA extraction. Once added, the half-life of the buffer is just 2–3 days. It is preferable to use it within the day.

3. *B. candida* can be successfully transformed using not only *A. rhizogenes* LBA 9402 but also *A. rhizogenes* 15834 and *A. tumefaciens* C58C1 strains. For simplicity, the protocol described mentions only *A. rhizogenes* LBA 9402.
4. *Agrobacterium* mediated-transformation of *B. candida* plants can be done using solid or liquid cultures of the bacteria. However, the methodology is more efficient when the transformation is carried out with solid cultures.
5. The top young leaves are chosen as explants for transformation because the lower ones and those obtained from flowering plants have inferior transformation efficiency.
6. *B. candida* hairy roots can also be initiated from the stem of in vitro plants by wounding the organ with a scalpel saturated with the bacteria (Fig. 2a). However, the rate of adventitious roots generated by this approach is higher than that obtained using leaves as explants for transformation.
7. Ensure that the infected area of the plant tissue does not contact the agar surface to prevent the excessive growth of *A. rhizogenes* over the explants and culture medium. This complicates the elimination of the bacteria.
8. Keep the ground tissue frozen in order to improve DNA recovery
9. The CTAB-DNA complex precipitates under 15 °C. Avoid using refrigerated centrifuges.
10. The transgenic nature of *B. candida* hairy roots can be verified by PCR analysis for amplification of the *rolB*, *rolC*, and *auxI* genes. In addition, the *virD* gene can be analyzed by PCR to confirm the elimination of the bacteria from plant tissues. Hairy roots grow fast and vigorously in media without the addition of growth regulators. This is a good indication of the transgenic nature of the roots. However, the characteristic phenotype of hairy roots is caused by integration and expression of the *aux* and *rol* genes from the root-inducing (Ri) plasmid in plant cells infected by *A. rhizogenes* [26, 27]. *Vir* genes are not transferred to the plant genome. The amplification of *virD* indicates the presence of the bacteria associated to the tissue under analysis. For this purpose, it is necessary to ensure the elimination of the bacteria from the samples to avoid false positive results.
11. The system includes a plastic mesh to facilitate the root arrangement inside the vessel and its aeration by a stainless steel sparger located below the turbine (Fig. 4).
12. In order to obtain a uniform distribution of roots within the vessel, the culture is initially agitated at 50 rpm for 10 min after the inoculation process.

13. *B. candida* hairy root culture in the modified stirred tank bioreactor is finished on day 20 when the root biomass reaches the maximum capacity of the vessel (Fig. 5).
14. This step is critical. It is important to work quickly in order to minimize the hydrolysis of tropane alkaloids after alkalinization of samples.
15. Continuous vortexing for 2 min improves the transfer of the alkaloids to the organic phase.

Acknowledgements

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Chapter 13

Micropropagation, Acclimatization, and Greenhouse Culture of *Veratrum californicum*

Sarah A. White, Jeffrey Adelberg, Jacqueline Naylor-Adelberg, David A. Mann, Ju Yeon Song, and Youping Sun

Abstract

Micropropagation and production of *Veratrum californicum* is most successful when using a premixed Murishage and Skoog basal medium with vitamins and a 5-week subculture cycle at 16 °C for multiplication. These culture conditions provide the best percent survival after acclimatization in the greenhouse. However, clone response to temperature and light quality within culture conditions varies. Micropropagated plants have mass and morphology similar to 2- or 3-year-old seedlings. Acclimatized plantlets can then be grown in the greenhouse using sub-irrigation (ebb and flood) to maintain substrate volumetric water content > 44 %. Growth cycle in the greenhouse must be about 100 days, followed by dormancy for 5 months at 5 °C.

Key words *Veratrum californicum*, Cyclopamine, Dormancy, Low-temperature propagation, Chilling, Ebb-and-flood irrigation

1 Introduction

Veratrum californicum produces cyclopamine, a compound with therapeutic potential [1, 2]. Micropropagation of this rhizomatous, perennial species, which is adapted to alpine wet meadows, is challenging due to its slow growth rate and the genotypic diversity of wild propagative material [3]. Fixing genetic diversity is desirable for therapeutic purposes, as some of the clonal lines produce greater concentration of cyclopamine. However, micropropagation tends to minimize genetic diversity via selection of clonal lines that adapt well to culture conditions and quickly multiply. Therefore one could inadvertently lose desirable clonal lines through simple propagative selection. Validation of the micropropagation protocol included acclimatizing hundreds of plantlets in the greenhouse. Determination of culture and acclimatization conditions was key components of developing a propagation system for this difficult species.

S. Mohan Jain (ed.), *Protocols for In Vitro Cultures and Secondary Metabolite Analysis of Aromatic and Medicinal Plants, Second Edition*, Methods in Molecular Biology, vol. 1391, DOI 10.1007/978-1-4939-3332-7_13,

The micropropagation and greenhouse production methodologies detailed below encompass five years of research with *V. californicum*. Plants collected from the wild were stored for up to 6 months at 5 °C, disinfected, initiated (stage I), subcultured (stage II), stably multiplied (stage III), and then transferred to the greenhouse for acclimatization and assessment of survival ratios (stage IV; Fig. 1). Over 1250 plantlets (35 %) of those transferred from laboratory survived transfer to the greenhouse [3]. For plantlets grown using the methods detailed below survival ratios increased to 55 %.

2 Materials

When water is mentioned below, we are referring to double-distilled, deionized (DDI) water unless another water source is specifically stated.

2.1 Agar and Nutrient Support

The pH of solutions is adjusted using 1 M NaOH.

1. Stirring hot plate.
2. Magnetic spin bar.
3. 1 M NaOH solution.
4. 10 mM benzyladenine (BA) stock.
5. Fixed speed vortex mixer (single tube).
6. 1 mM 1-naphthaleneacetic acid (NAA) stock.
7. Tri-scale culture medium (*see* **Note 1**, Tables 1 and 2).
8. Root-promoting culture medium (*see* **Note 1**, Tables 1 and 2).
9. Dispensing peristaltic pump.
10. Tray sized to hold vessels.
11. GA-7 (6 cm × 6 cm × 10 cm), polycarbonate vessel with lid.
12. Parafilm M or polyethylene vessel sealing film.
13. Autoclave.

2.2 Tissue Handling

1. Sterilization solution I: Tween 80 (Table 3).
2. Sterilization solution II: Ethanol (EtOH) solution (Table 3).
3. Sterilization solution III: 50 % bleach solution (Table 3).
4. Sterilization solution IV: 10 % bleach solution (Table 3).
5. 70 % EtOH solution (140 proof, laboratory grade).
6. Spray bottle (300 mL).
7. Alcohol burner, with wick and 95 % ethanol.
8. Utensils: scalpel and forceps.
9. Glass test tube (34 mL capacity).

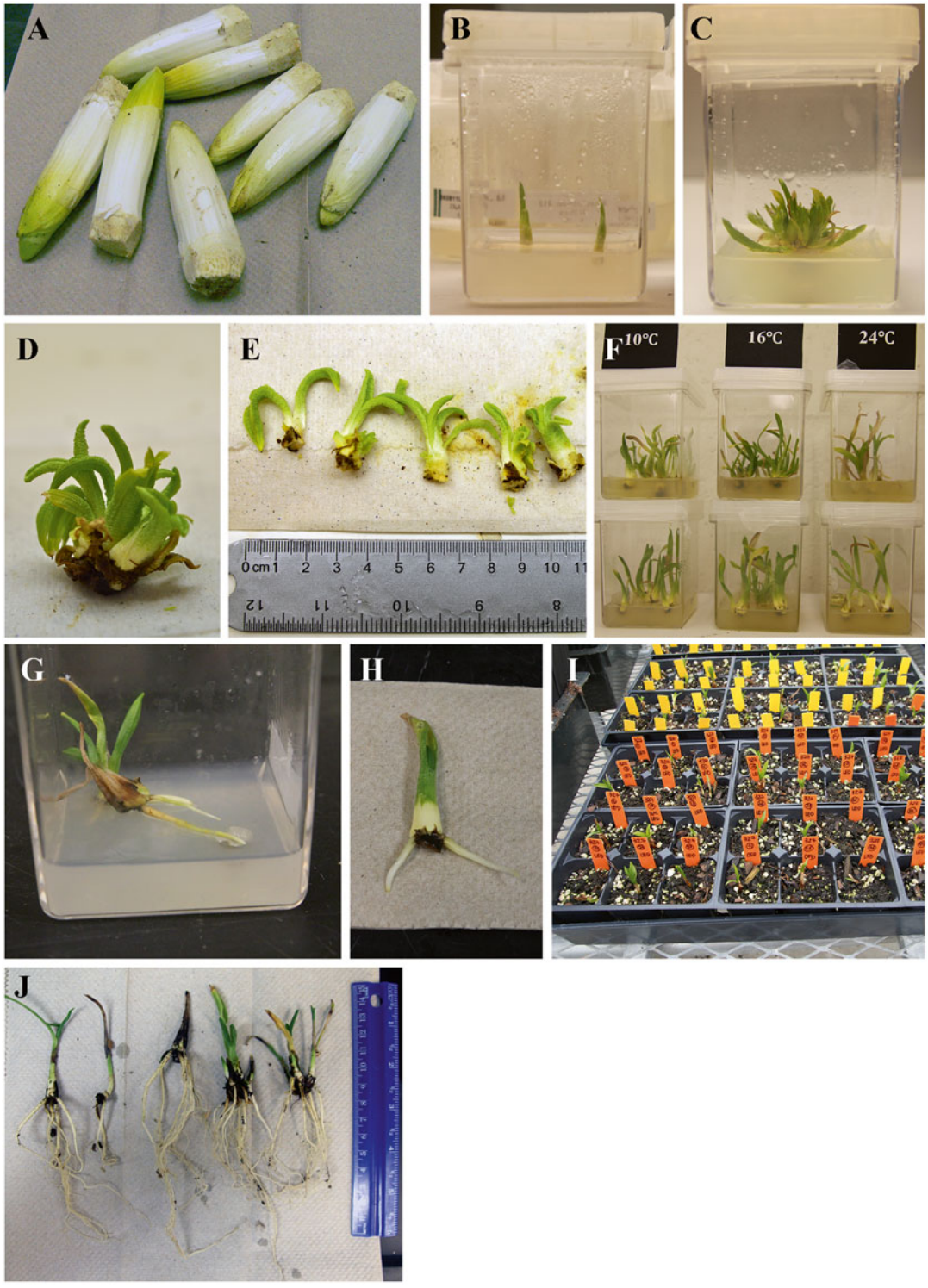

Fig. 1 Stages of micropropagation: (**a**) explant tri-scale for initiation, (**b**) shoot induction, (**c**) shoot proliferation and (**d**) shoots (**e**) divided for (**f**) stable multiplication, (**g**) rooting of plantlets, (**h**) plantlet with agar washed from roots prior to (**i**) planting in 72 cell tray and acclimatization, and (**j**) plantlet after growth in the greenhouse

Table 1
Mineral components and vitamins within the Murishage and Skoog (MS) Basal Medium with Vitamins as detailed by supplier and combined into concentrated stock powder, from which nutrient solutions are prepared as detailed by Murishage and Skoog [5]

Chemical composition	mg/L
Ammonium nitrate	1650
Boric acid	6.2
Calcium chloride, anhydrous	332.2
Cobalt chloride·$6H_2O$	0.025
Cupric sulfate·$5H_2O$	0.025
$Na_2EDTA{\cdot}2H_2O$	37.26
Ferrous sulfate·$7H_2O$	27.8
Magnesium sulfate, anhydrous	180.7
Manganese sulfate·H_2O	16.9
Molybdic acid (sodium salt)·$2H_2O$	0.25
Potassium iodide	0.83
Potassium nitrate	1900
Potassium phosphate, monobasic	170
Zinc sulfate·$7H_2O$	8.6
Glycine (free base)	2
myo-Inositol	100
Nicotinic acid (free acid)	0.5
Pyridoxine·HCl	0.5
Thiamine·HCl	0.1

10. Tri-fold paper toweling.
11. Laminar flow hood, positive pressure room (*see* **Note 2**).

2.3 Micro-propagation Conditions

1. Positive pressure culture room (*see* **Note 2**). Temperature maintained at 24 °C.
2. Cool white fluorescent (ambient) light: 20 μmol/m^2 s photosynthetic photon flux density (PPFD).
3. Low temperature chambers with light emitting diode (LED) light source: 20 μmol/m^2/s monochromatic red (peak at 660 nm), 20 μmol/m^2/s blue (peak at 480 nm), 20 μmol/m^2/s red–blue (1:1), and 40 μmol/m^2/s red–blue (3:1, Fig. 2).

Table 2
Specific components of tri-scale culture medium and root-promoting culture medium[a]

Culture media	Components	Mass/volume
Tri-scale	MS Basal Medium with Vitamins	4.43 g
	Sucrose	30 g
	Micropropagation Type II Agar	7 g
	10 mM BA stock	2.3 mL
Root-promoting	MS Basal Medium with Vitamins	4.43 g
	Sucrose	30 g
	Micropropagation Type II Agar	7 g
	1 mM NAA stock	5.0 mL

[a]2-L glass cylinder or flask (*see* **Note 1**), dispense 1-L water and add MS basal medium with vitamins, sucrose, agar, and growth regulator, then adjust pH. Melt agar on stirring hot plate and dispense prior to boiling

Table 3
Sterilization solutions for sanitizing tissues[a]

Sterilization solution	Components	Mass/volume
I	Tween 80	2 drops
	DI (deionized) water	200 mL
II	95 % EtOH (190 proof, laboratory grade)	as needed
III	Bleach (5.25 % sodium hypochlorite, NaOCl)	50 mL
	Water	50 mL
IV	Bleach	10 mL
	Water	90 mL

4. Shelving systems with fluorescent or LED lights 30 cm from surface of containers.

2.4 Greenhouse Conditions

1. Potting substrate Fafard 3B mix: 45 % Canadian sphagnum peat moss, 25 % processed pine bark, 15 % perlite, 15 % vermiculite, starter nutrients (40–230 mg/L N; 5–30 mg/L P; 40–200 mg/L K, Ca, and S; 25–80 mg/L Mg), wetting agent, dolomitic limestone.
2. 300 mg/L Subdue® [25.1 % Metalaxyl: *N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl) alanine methyl ester, 74.9 % inert ingredients; 7.6-L (#3) plastic container].
3. 72 cell tray (53.3 cm × 27 cm × 5.7 cm) with clear-plastic dome lid.

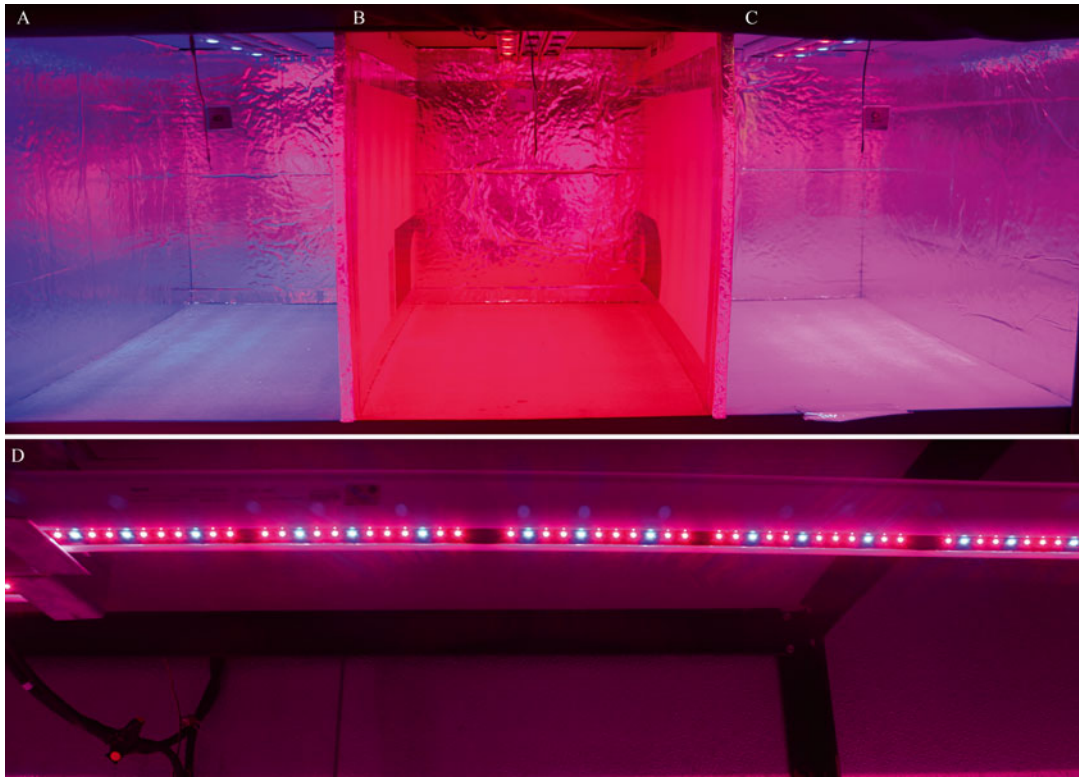

Fig. 2 Light emitting diodes in controlled environment room providing desired light qualities for culturing *Veratrum californicum* plantlets. 20 μmol/m²/s monochromatic blue (**a**), 20 μmol/m²/s red (**b**), 20 μmol/m²/s red–blue (**c**), and 40 μmol/m²/s red–blue (**d**)

4. Controlled environment room (CER) with relative humidity and temperature control.
5. Mist-bed. Greenhouse bench outfitted with misting emitters to mist water over plants at regimented intervals.
6. Square trays (60 cm × 60 cm) that are 10 cm deep.
7. LI-90 Quantum light sensor.
8. 1000-W metal halide lamp.
9. Soil moisture sensors.
10. Dayton® utility pump.
11. Orbit® signature timer control.
12. Honeywell drain valve.
13. 190-L tank.
14. Drip irrigation line and tubing with emitters (3.78 L/min).

3 Methods

3.1 Preparation of Agar and Nutrient Support Recipes

1. Prepare tri-scale or root promoting culture media in 2-L glass beaker. Place beaker on a hot plate and stir with a magnetic spin bar while heating until all compounds are dissolved.
2. Dispense 50 mL aliquots of solution into individual vessels using the peristaltic pump, place lids on the vessels. Prime the peristaltic pump with manufacturer recommended volume of solution prior to dispensing measured aliquots of solution (*see* **Note 3**).
3. Autoclave vessels filled with culture medium for 25 min at 15–20 atm and 121 °C.
4. Transfer sterilized vessels to positive pressure culture room (*see* **Note 2**), and transfer plantlets into the containers under laminar flow hood to minimize contamination. Seal vessels with Parafilm or polyethylene sealing film.

3.2 Plant Handling Prior to Initiation of Micropropagation

1. *Veratrum californicum* rhizomes with attached bulbs were collected in Heber Valley and Bolger Canyon, Utah and shipped overnight to the greenhouse.
2. Rhizomes with attached bulbs were immediately sorted and potted into #3 plastic containers with Fafard 3B substrate (*see* **Note 4**) and drenched with Subdue® to limit potential for root rot.
3. Containers with plants were then placed in a CER at 10 °C and 70 % relative humidity (RH) for 2 weeks, and then CER temperature was reduced to 5 °C with a RH of 65 % and rhizomes with attached bulbs were chilled up to 6 months.

3.3 Micropropagation: Stage I—Initiation via Tri-scale

1. Remove containers with dormant bulbs from the CER. Remove bulb and rhizome from the container. Excise the bulb from the rhizome and roots, and wash with tap water to remove soilless substrate (*see* **Note 5**, Fig. 3a). Remove sheath and several layers of the outer scales.
2. Soak bulbs in Sterilization Solution I, stir (on a stir-plate with a magnetic spin bar) for 10 min, and rinse with DDI water three times (Fig. 3b).
3. Surface-sterilize bulbs by soaking in Sterilization Solution II for 2 min (Fig. 3c).
4. Transfer bulbs to Sterilization Solution III for 2 min and rinse in sterile DDI water (Fig. 2d).
5. Peel outer scales from bulb and crosscut into wedge-shaped pieces.
6. Sterilize utensils between cuts using the flame burner as needed. Hood surfaces should be sterilized using spray bottle filled with 70 % EtOH between each bulb cutting event, and

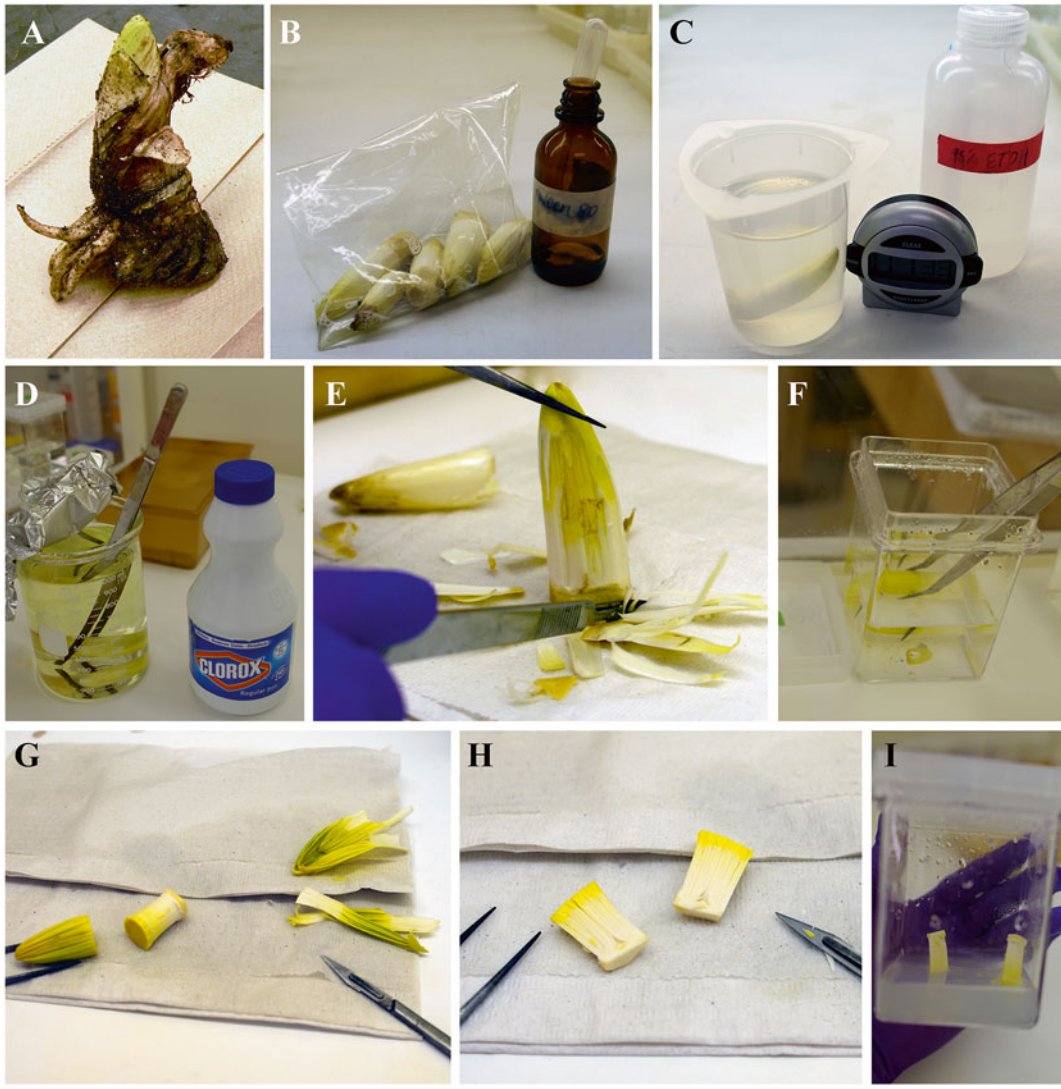

Fig. 3 Bulbs (**a**) were detached from rhizomes, soaked in distilled water with Tween 80 (**b**, Sterilization Solution I), rinsed and placed in 95 % ethanol (**c**, Sterilization Solution II), and then transferred to 50 % commercial bleach solution (**d**, Sterilization Solution III). Outer layers were peeled off (**e**) and bulb was placed in 10 % bleach solution (**f**, Sterilization Solution IV), the tissue wedge (**g**) was then sliced into sections leaving leaf scales connected to basal plate (**h**) and inserted into a vessel for shoot induction (**i**)

utensils sterilized again by placing them in test-tube with 95 % EtOH and flaming.

7. After cutting pieces into wedges, soak in Sterilization Solution 4 (10 % bleach) for 10 min, and then rinse in sterile DDI water (Fig. 3e).
8. In laminar-flow hood, further split the sterilized tissue wedges (Fig. 3f) into tri-scale sections by slicing (scalpel) tissue, leave

a minimum of three leaf scales connected through the basal plate (*see* **Note 6**, Fig. 3g).

9. Place each tri-scale explant (clone) in GA7 Vessel filled with tri-scale culture medium.
10. Incubate explants in the dark at 16 °C for 4–5 weeks to facilitate adaptation to culture conditions. Discard contaminated explants and transfer only "clean" explants (*see* **Note 7**).
11. After a 4–5 week interval, trim necrotic tissues from clean explants, if necessary. Transfer explants to fresh tri-scale culture medium and place under light in plant growth chamber at 16 °C (*see* **Note 8**). Adjust light intensity, regardless of source, to between 20 and 40 μmol/m^2/s (*see* **Note 9**) with a 16 h light–8 h dark cycle.
12. Subculture cycle duration will range from 4 to 6 weeks, depending upon clone [3]. Shoots form 5–6 weeks after transfer, depending upon clone (*see* **Note 10**).
13. Calculate multiplication ratios by counting the number of shoots per vessel at the conclusion of each subculture cycle and dividing by the initial number of shoots when explant placed within a new vessel.
14. Once stable (Stage-II) multiplication rates are attained, transfer plantlets to GA7 vessels containing 50 mL root-promoting culture medium and place in low temperature chamber at 16 °C. Light intensity for rooting is 40 μmol/m^2/s provided by red–blue LED light, with a 16 h light–8 h dark cycle (Fig. 2d).
15. Once roots have formed, generally after 5 weeks in the root-promoting culture medium, remove plants from the culture medium, wash the agar sticking to the roots under running tap water, and transfer plantlets into 72 cell plastic trays.
16. Transfers for acclimatization or growth in the greenhouse should be carried out during fall or winter, when average day temperature is below 22 °C (*see* **Note 11**)

3.4 Acclimatization of Plantlets and Greenhouse Production

1. Water plants in 36 cell tray (*see* **Note 12**), cover with a plastic dome, and place in a mist-bed for 10 days.
2. After 10 days, remove the plastic domes from the trays. Leave the trays in the mist bed for an additional 20 days.
3. Transfer well-acclimatized plants (*see* **Note 13**) to a non-misted bench.
4. Use an ebb-and-flood irrigation system to irrigate plantlets (*see* **Note 14**), and maintain average soil moisture above 44 % to promote the healthiest growth (*see* **Note 15**).

5. Light levels in the greenhouse should be > 100 W/m^2, supply supplemental light (1000-W metal halide lamp), as needed. Monitor light levels with a quantum sensor, and log light level readings over time, provide supplemental light when needed.
6. When leaves senesce, let plants dry, treat with Subdue fungicide, and place in CER.

4 Notes

1. MS medium prepared within a container that is at least 0.2-L larger than desired final volume, as medium must be stirred and agar melted prior to distribution in culture containers. For 1-L water and add 4.43 g Murishage and Skoog (MS, Table 1) basal medium with vitamins. Weigh 30 g sucrose and transfer to cylinder. Weigh 7 g agar (Micropropagation Type II) and transfer to cylinder. Add 2.3 mL 10 mM BA stock (23 μM BA final concentration). Melt agar on stirring hot plate and dispense prior to boiling.
2. A positive pressure culture room maintains a flow of air out of the room, protecting the plantlets from possible fungal or bacterial pathogens that could otherwise contaminate the cultures.
3. Prior to dispensing growth medium solutions, we prime our peristaltic pump with at least 300 mL solution, and also periodically calibrate the unit to ensure accuracy of volumes dispensed into tissue culture vessels.
4. *Veratrum* rhizomes with attached bulbs should be planted high within containers, with 1/3 to 1/2 of bulb above the substrate surface, similar to *Amaryllis* container production, to minimize potential for rot [1].
5. Handling of rhizomes and bulbs prior should occur in an area separate from culture rooms, where cross-contamination of other cultures is not a concern. When handling the propagative materials, the soil was washed from all roots in the head house of the greenhouse, and relatively clean material were then transferred to a laboratory for subsequent sterilization procedures.
6. A section of the basal plate remained attached to each of the pieces of the wedge as it was sliced. Each tri-scale consisted of a minimum of three-scales in each explant piece [4].
7. Contamination occurred if microbial or fungal growth within the vessel was supported on sugar-containing medium, despite efforts to sterilize the tissue. Contamination rates averaged 78 % for first transfer and 42 % for the second transfer from

greenhouse grown *V. californicum* bulbs, while bulbs initiated into culture, directly after harvest with no chilling interval, averaged 4 % contamination for the first transfer and 12 % for the second transfer.

8. Three different culture temperatures 10, 16, and 24 °C were evaluated. 16 °C was the ideal temperature for proper plant acclimatization and growth in the greenhouse. *Veratrum californicum* is unique and requires low temperature during multiplication to affect apparent quality in subsequent culture cycles or upon acclimatization in the greenhouse.
9. Light quality during initiation and multiplication stage did not affect all clones in a similar manner, thus we cannot recommend a single light quality as "best" as clones reacted in a differential manner [3].
10. Genotypic differences of clonal lines initiated resulted in staggered subculture cycles, with some cycling and needing transfer after only 4 weeks, with others needing transfer only after 6 weeks.
11. Because *V. californicum* is a high-alpine plant species, it is adapted to cooler day temperatures (15–20 °C) and a dip in night temperature to 5 or 10 °C. Thus, when transfers to the greenhouse for acclimatization and growth occur, schedule timing so that a minimum of 100 days are available before average day temperatures excessively (+5 °C) exceed 20 °C and while minimum night temperatures still dip to 15 °C.
12. When watering in plantlets, be sure that the substrate is watered evenly and that minimal "gouging" of the surface occurs. A water wand or other tool that allows the water to be applied as a spray, in a relatively even manner over the substrate surface, is the best way to water these delicate plantlets.
13. Acclimatized plantlets are those with green leaves and a fleshy base (primary rhizome) that is turgid after 6 weeks in the greenhouse.
14. We developed an automated ebb-and-flood watering system based on time intervals. Water began to fill the trays, in which plants were placed, at 1800 in the evening and drained out at 0600 the following day. These time intervals were chosen to minimize potential for algal growth in recycled irrigation solutions. The ebb-and-flood system consisted of the square tray on the greenhouse bench, fitted with a drain valve (Fig. 4). The irrigation system cycled on at 1800, and water from the sub-irrigation tank (190-L tank) was pumped into the tray for 5 min, until the water level in the tray was approximately 2.8 cm deep. At 0600 the next day, the drain valve opened and

Fig. 4 Overview of ebb and flood sub-irrigation system. Square trays (**a**) which held either propagative stock plants or micropropagated seedlings. Drip lines (**b**) dispensed solutions from the stock tank (**c**) on a regulated basis. The Honeywell motorized valve (**d**) closes and opens at the drain valve (**e**) on a timed basis as regulated by the controller (**f**). The Dayton pump (**g**) was powered on by the power start relay (**h**) when the controller sent the signal

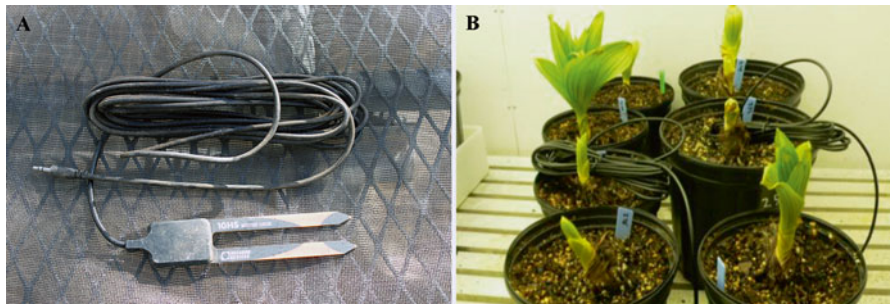

Fig. 5 Decagon 10-HS soil moisture sensors (**a**) inserted into #3 containers (**b**) to monitor soil moisture in situ

the solution drained back into the sub-irrigation tank. This flood duration, permitted the substrate to become saturated with water, and provided enough water to the plants for growth the following day.

15. We used Decagon 10-HS soil-moisture sensors, inserted into soilless substrate within the trays, to monitor soil moisture (Fig. 5). We determined that an ebb-and-flood event on a daily basis maintained the soil moisture more consistently around 44 %, which was preferred by *V. californicum* [2].

Acknowledgement

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Chapter 14

In Vitro Propagation of *Withania somnifera* (L.) Dunal

Pritika Singh, Rupam Guleri, and Pratap Kumar Pati

Abstract

Withania somnifera (L.) Dunal known as Ashwagandha is commonly used in traditional Indian medicine system. It possesses immense therapeutic value against a large number of ailments such as mental diseases, asthma, inflammation, arthritis, rheumatism, tuberculosis, and a variety of other diseases including cancer. The therapeutic potential of *W. somnifera* is due to the presence of secondary metabolites mainly, tropane alkaloids and withanolides (steroidal lactones). The growing realization of commercial value of the plant has initiated a new demand for in vitro propagation of elite chemotypes of *Withania*. Micropropagation which is an important tool for rapid multiplication requires optimization of number of factors such as nutrient medium, status of medium (solid and liquid), type of explant, and plant growth regulators. Similarly, an efficient and reproducible in vitro regeneration system which is a prerequisite for the development of genetic transformation protocol requires precise manipulation of various intrinsic and extrinsic factors.

Key words *Withania somnifera*, In vitro propagation, Shoot multiplication, Liquid culture medium, Shoot regeneration, Rooting, Secondary metabolites

1 Introduction

In recent years there has been renewed interest in natural medicines that are obtained from plant parts or plant extracts. Herbal medicines have attracted global attention due to their safety and efficacy. At present about 75–80 % of the population in developing countries depends on herbal medicines for primary health care [1]. *Withania somnifera* (L.) Dunal is highly reputed in the Indian traditional medicine systems of Ayurveda and Unani [2]. It is commonly known as Ashwagandha and belongs to the family Solanaceae. It also appears in World Health Organization (WHO) monographs on selected medicinal plants [3]. The plant is widely distributed in Indian subcontinent as well as in Asia, Africa, Mediterranean region, and Middle East [4]. *W. somnifera* is well known for its antistress, anti-inflammatory, antitumor, antibacterial, antioxidative, analgesic, antiarthritic, anticoagulant, immunomodulatory, cardioprotective, and neuroprotective properties [2, 5, 6]. The pharmacological properties are mainly attributed to the presence of secondary

S. Mohan Jain (ed.), *Protocols for In Vitro Cultures and Secondary Metabolite Analysis of Aromatic and Medicinal Plants, Second Edition*, Methods in Molecular Biology, vol. 1391, DOI 10.1007/978-1-4939-3332-7_14,

metabolites such as withanolides (withaferin A, withanone, withanolide A, and withanolide D) and tropane alkaloids (isopelletierine, anaferine, tropine, pseudotropine) [7].

Biotechnological tools are important to select, multiply, conserve, and engineer the critical genotypes of *W. somnifera*. In vitro propagation of plants holds tremendous potential for the rapid production of high-quality plant-based medicines. Developing an efficient in vitro propagation system would ensure steady supply of uniform, healthy plant material throughout the year for the health sector and would also be instrumental in understanding basic physiological aspects of secondary metabolite production, accumulation, and transport. The present chapter describes various protocols for in vitro propagation and regeneration of *W. somnifera* from different explants. We also report for the first time a reproducible protocol for somatic embryogenesis in *W. somnifera*.

2 Materials

2.1 Micropropagation of W. somnifera

1. Seeds and nodal explants of *W. somnifera* collected from the field.
2. 0.4 % (v/v) sodium hypochlorite solution.
3. 70 % ethanol.
4. Stock solutions of Murashige and Skoog (MS) [8] medium (MS stock I, II, III, and IV) (Table 1). Store at 4 °C (*see* **Notes 1** and **2**).
5. Sucrose.
6. Stock solutions (5 mM) 6-benzylaminopurine (BAP), kinetin (KN), thidiazuron (TDZ), 1-naphthalene acetic acid (NAA), indole acetic acid (IAA), and indole butyric acid (IBA) (Table 2). Store the stock solutions at 4 °C (*see* **Note 3**).
7. 1 N NaOH and 1 N HCl.
8. Agar.
9. Beakers, measuring cylinders, Erlenmeyer flasks (250 ml), culture tubes (8.9″ × 1″), glass jars (350 ml), and petri dishes (90 mm).
10. Micropipettes (2–20 μl, 20–200 μl, 100–1000 μl).

2.1.1 Hardening of Plantlets

1. In vitro raised microshoots.
2. Sterile mix of sand and soil (1:1).
3. Earthen pots.

2.2 In Vitro Regeneration of W. somnifera

1. Seeds from field grown plants and leaf explant from in vitro raised plants.
2. Stock solutions (5 mM) of 6-benzylaminopurine (BAP), 2,4-dichlorophenoxy acetic acid (2,4-D), and indole acetic acid (IAA) (Table 2). Store at 4 °C (*see* **Note 3**).

Table 1
Preparation of MS stock solutions and MS medium

Constituents		Concentration (mg/l)	Concentration of stock solution (mg/l)	Volume per liter (ml)
Macroelements stock-I	NH_4NO_3	1650	33,000	50
	KNO_3	1900	38,000	
	$CaCl_2{\cdot}2H_2O$	440	8800	
	$MgSO_4{\cdot}7H_2O$	370	7400	
	KH_2PO_4	170	3400	
Microelements stock-II	KI	0.83	166	5
	H_3BO_3	6.2	1240	
	$MnSO_4{\cdot}4H_2O$	22.3	4460	
	$ZnSO_4{\cdot}7H_2O$	8.6	1720	
	$Na_2MoO_4{\cdot}2H_2O$	0.25	50	
	$CuSO_4{\cdot}5H_2O$	0.025	5	
	$CoCl_2{\cdot}6H_2O$	0.025	5	
Iron stock stock-III	$FeSO_4{\cdot}7H_2O$	27.85	5560	5
	Na_2EDTA	37.25	7460	
Vitamins stock-IV	Meso inositol	100.0	20,000	5
	Glycine	2.0	400	
	Nicotinic acid	0.5	100	
	Pyridoxine–HCl	0.5	100	
	Thiamine–HCl	0.1	20	

Table 2
Preparation and storage of various plant growth regulators used for in vitro propagation and regeneration of *W. somnifera*

		Preparation and storage		
Growth regulator	Molecular weight	Solvent	Diluent	Storage
2,4 D	221.0	Ethanol/1 N NaOH	Water	0–4 °C
IAA	175.2	Ethanol/1 N NaOH	Water	0–4 °C
IBA	203.2	Ethanol/1 N NaOH	Water	0–4 °C
NAA	186.2	1 N NaOH	Water	0–4 °C
BAP	225.3	1 N NaOH	Water	0–4 °C
TDZ	220.2	DMSO	Water	0–4 °C
KN	215.2	1 N NaOH	Water	0–4 °C

2.3 Histological Evidence of In Vitro Regeneration in W. somnifera

1. Fixative: FAA (formaldehyde–acetic acid–50 % alcohol) (1:1:18).
2. Rectified alcohol.
3. *tert*-butyl alcohol (TBA).
4. Paraffin wax.
5. Xylene.
6. Gelatine (1 % (w/v) solution in distilled water).
7. Safranin stain (1 % (w/v) solution in distilled water).
8. Fast green stain (0.25 % (w/v) solution in 50 % clove oil prepared in absolute ethanol).
9. Clove oil.
10. DPX mountant.
11. Rotary microtome.
12. Slide warmer.

3 Methods

3.1 Micropropagation

3.1.1 Preparation of MS Medium

1. Mix the MS stock solutions as mentioned in Table 1. Add sucrose at a final concentration of 3 % (w/v).
2. Add the desired PGR in the required amount and adjust pH of the medium to 5.8 using either 1 N HCl or 1 N NaOH.
3. Prepare solid medium by adding agar (0.8 % w/v), whereas use no gelling agent for making liquid medium.
4. Sterilize the medium by autoclaving it for 20 min at 1.1 kg/cm^2 pressure and 121 °C.

3.1.2 Initiation of Aseptic Cultures of W. somnifera

1. Wash thoroughly the explants (seeds and nodal segments) collected from the field with Tween 20 solution. Treat the explants with 70 % ethanol for 5 min.
2. Perform the surface sterilization of explants with 0.4 % (v/v) sodium hypochlorite solution containing two to three drops of Tween 20 for 20–30 min (*see* **Note 4**) and wash properly 3–4 times with sterile double distilled water till the traces of Tween 20 are removed completely.
3. Cut the exposed ends from both the sides of nodes and inoculate the segments in tubes containing solid basal MS medium and 3 % sucrose (w/v), pH 5.8 (*see* **Notes 5** and **6**).
4. For seed germination, give a small incision to seeds and inoculate them in petri dishes containing MS basal medium and 1 % sucrose (w/v), pH 5.8.
5. Incubate the cultures in culture room at 25 °C and photon flux density (PFD) of 40 μmol/m^2/s provided by cool, white fluorescent lamps. Maintain the photoperiod of 16 h in 24 h light–dark cycle.

6. After 4 weeks of culture, seeds start to germinate and multiple shoots form from the nodal segments. Use actively growing shoots and seedlings for further experimentation.

3.1.3 Shoot Proliferation

1. Excise the in vitro growing shoots and inoculate them in flasks containing MS medium supplemented with different concentrations of cytokinins, viz. BAP (0–15 μM), KN (0–15 μM), and TDZ (0–15 μM). Proliferate the shoots both in solid (0.8 % agar) and static liquid culture (Fig. 1) (*see* **Notes 7** and **8**).

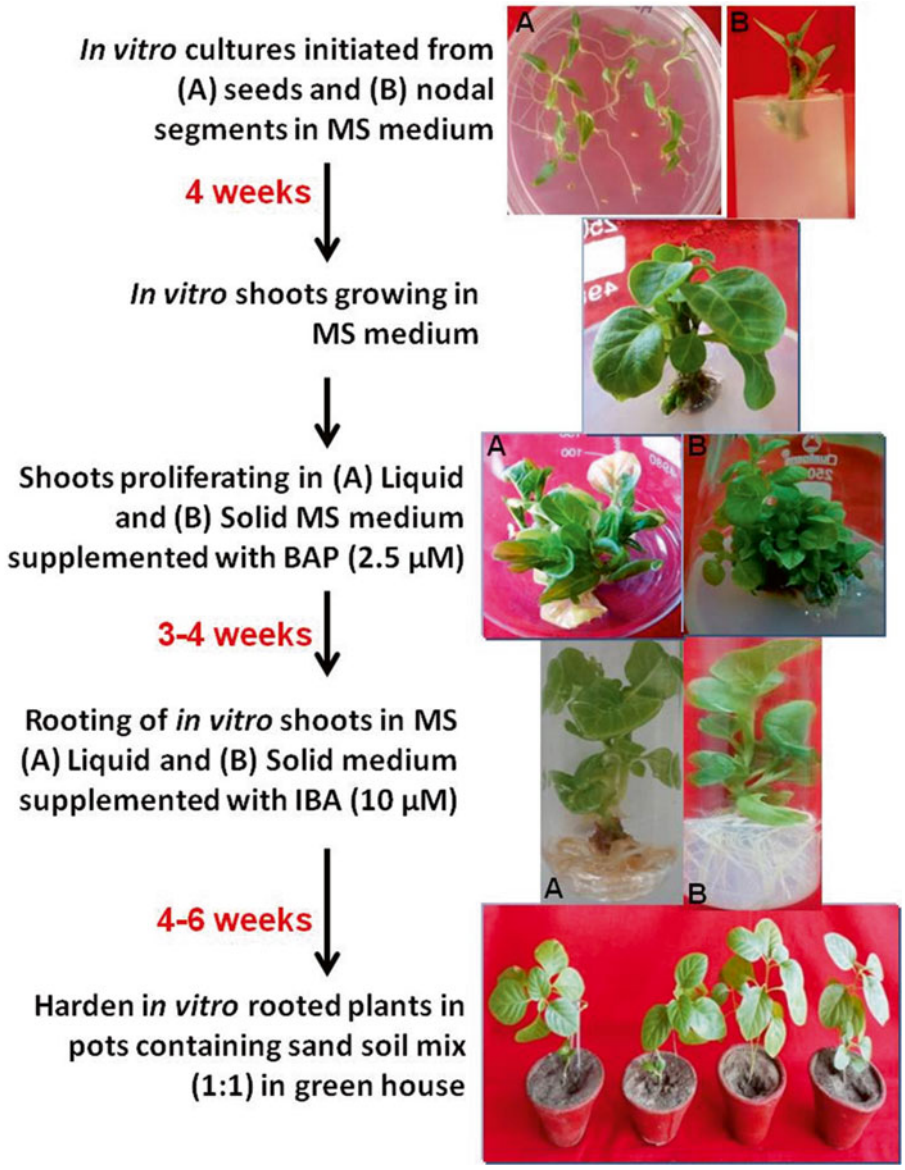

Fig. 1 Schematic representation of micropropagation protocol of *W. somnifera*

2. Incubate the cultures for 4 weeks at 25 °C, photon flux density (PFD) of 40 μmol/m²/s, and photoperiod of 16 h in 24 h light–dark cycle.

3.1.4 Rooting of Shoots

1. Excise the shoots of 2–4 cm length and inoculate them in culture tubes containing agar-gelled and liquid MS medium supplemented with different concentrations of auxins, viz. IBA (0–15 μM), IAA (0–15 μM), and NAA (0–15 μM) (Fig. 1).
2. Incubate the cultures for 4 weeks at 25 °C and photon flux density (PFD) of 40 μmol/m²/s. Maintain the photoperiod of 16 h in 24 h light–dark cycle.

3.1.5 Hardening of Microshoots

1. Take out the plantlets from the culture tubes and wash them properly so that agar is completely removed.
2. Transfer the plantlets to earthen pots containing sterile mixture of sand and soil (1:1).
3. Cover the pots with fluorescent sheets and maintain them in greenhouse under controlled light and humidity conditions for acclimatization (*see* **Note 9**).
4. Once they acclimatize remove the florescent sheets.

3.2 Regeneration of W. somnifera

3.2.1 Direct Regeneration from Leaf Explant

1. Excise the young leaves from in vitro growing shoots and inoculate them in tubes containing MS medium supplemented with different concentrations of BAP (0–15 μM) and 3 % (w/v) sucrose.
2. Incubate the cultures at 25 °C in white fluorescent light (40 μmol/m²/s) with 16 h day length for 4 weeks.
3. After 4 weeks of culture multiple shoots start to emerge from the petiole and base of leaves (Fig. 2).
4. Proliferate the shoots in flasks containing MS medium and BAP (2.5 μM).
5. For in vitro rooting, inoculate the shoots formed above in culture tubes containing MS medium supplemented with IBA (10 μM) (Fig. 2).
6. After 4 weeks of culture harden the in vitro raised plantlets in earthen pots containing sand soil mix (1:1) as described in Subheading 3.1.5.

3.2.2 Indirect Regeneration from Leaf Explant

1. Cut the young leaves from in vitro growing shoots and inoculate them in tubes containing MS medium supplemented with different concentrations of BAP (0–15 μM).
2. Callus and shoot buds start to form from cut edge of leaves after 4 weeks of culture. Subculture the callus in the same medium for further proliferation (Fig. 3).
3. Incubate the cultures at 25 °C in white fluorescent light (40 μmol/m²/s) with 16 h day length for 4–6 weeks. Observe the shoot bud formation under the stereomicroscope.

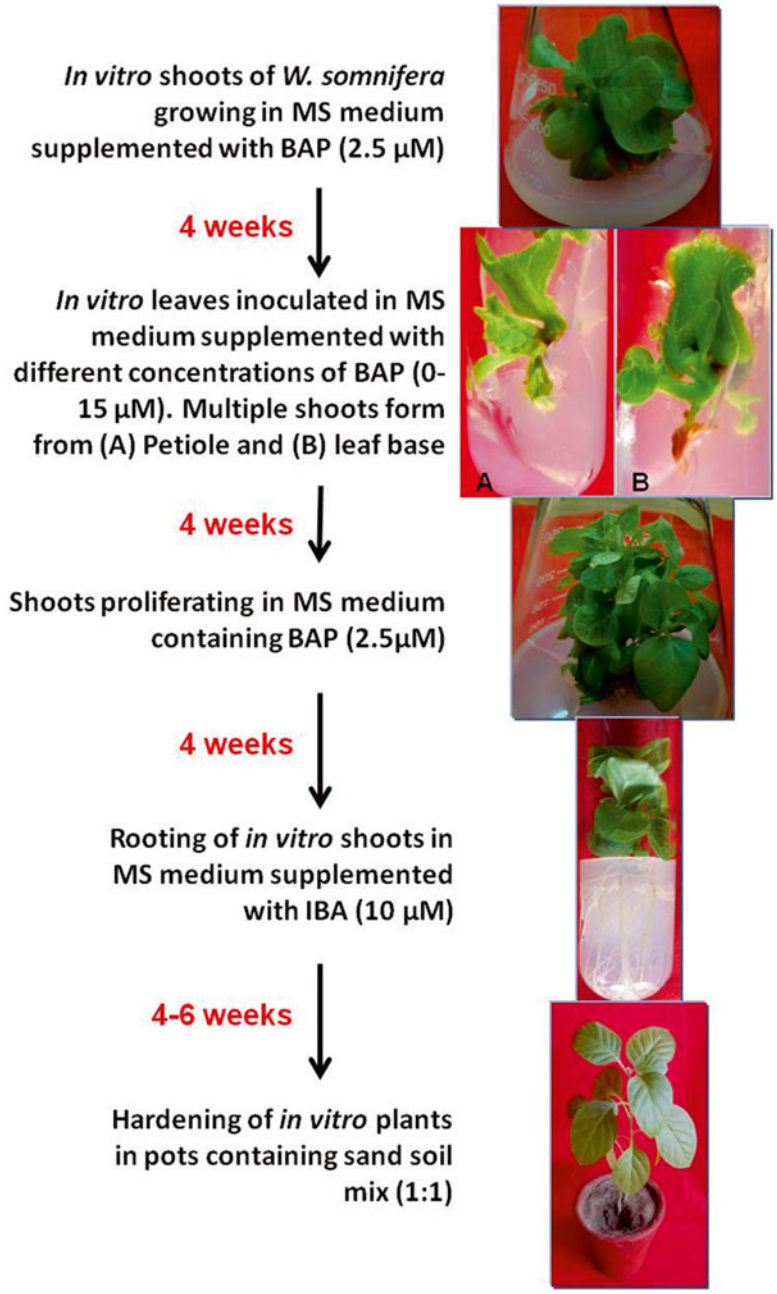

Fig. 2 Schematic representation of protocol for direct regeneration from in vitro leaves of *W. somnifera*

4. To further proliferate shoots emerging from the shoot buds, inoculate the shoot buds in flasks containing MS medium and BAP (2.5 µM).
5. For in vitro rooting, inoculate the shoots in culture tubes containing MS medium and IBA (10 µM). Harden the in vitro raised plantlets after 4 weeks of culture in earthen pots containing sand

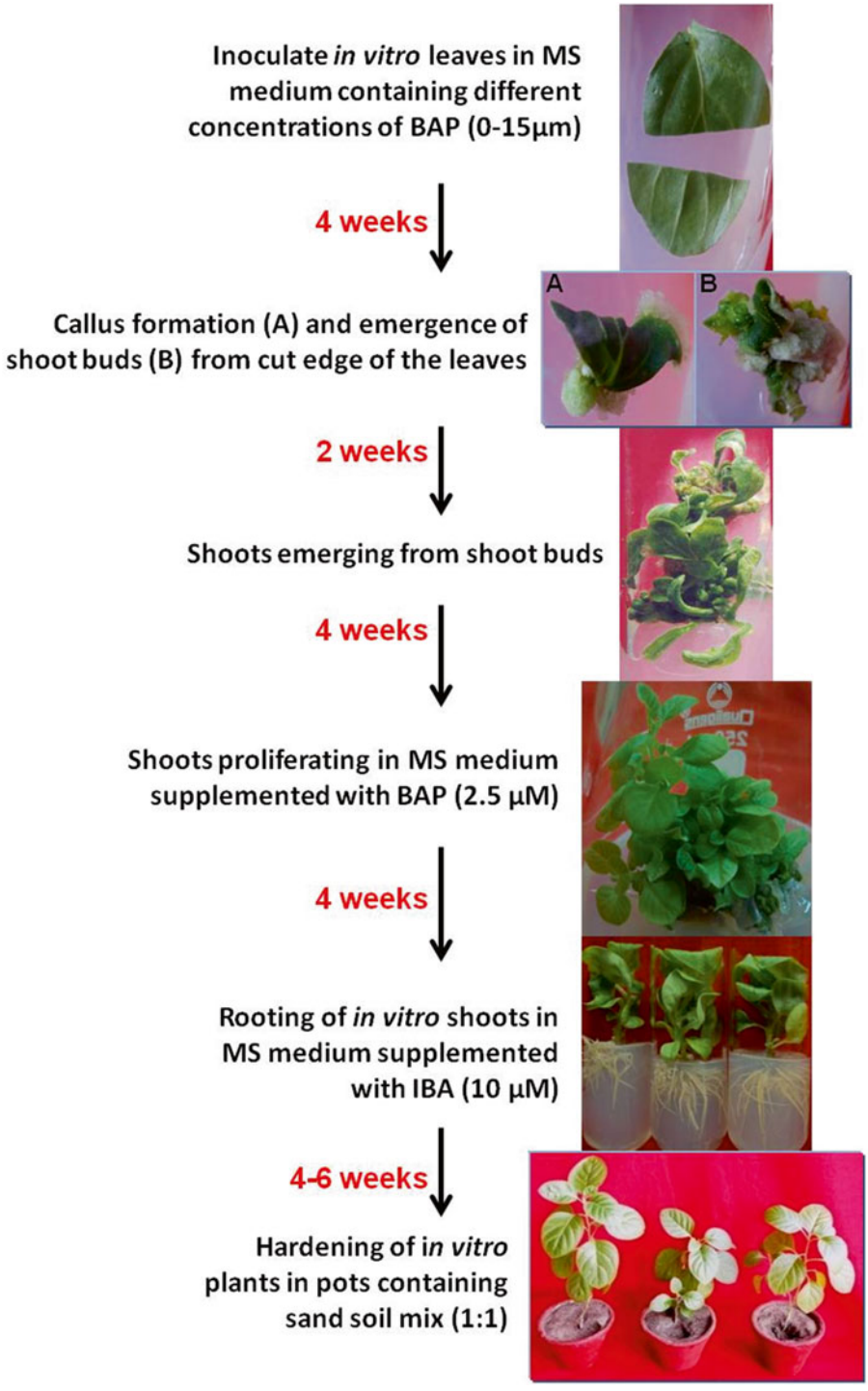

Fig. 3 Schematic representation of protocol for indirect regeneration from in vitro leaves of *W. somnifera*

soil mix (1:1). Once the plants acclimatize they are shifted to greenhouse.

3.2.3 Somatic Embryogenesis from Seed Derived Tissue of W. somnifera

1. Inoculate sterilized seeds in petri dishes containing MS medium supplemented with different concentrations of 2,4-D (0–15 μM) and sucrose (1–9 % w/v).
2. After 2–3 weeks of culture, the seeds germinate and cotyledonary leaves begin to appear.
3. Embryogeneic calli start to form after 21 days from cotyledonary leaves in same medium.
4. Somatic embryos begin to emerge from the calli after 6 weeks of culture (Fig. 4). For further proliferation transfer the embryos in MS medium supplemented with different concentrations of 2,4-D (2.5 μM) and 3 % (w/v) of sucrose.
5. Somatic embryos regenerate to form shoots and roots in MS basal medium without any PGR after two subcultures each at an interval of 4 weeks.

3.2.4 Histological Evidence for Regeneration in W. somnifera

In order to study the structure and origin of regenerated structures, histological studies are carried out as follows [9].

Fixation and Dehydration of Samples

1. Fix the sample in FAA (formaldehyde–acetic acid–50 % alcohol) (1:1:18) for 1 week.
2. Transfer the material to 70 % alcohol overnight.
3. Carry out the sequential dehydration in TBA (*t*-butyl alcohol) as described in Table 3 (*see* **Note 10**). Keep the tissue in each grade for 2–3 h except for grade "c" where it is left overnight.

Waxing

1. Keep the glass vials containing material in pure TBA in dry oven pre-set at 60 °C for 15–20 min to equilibrate TBA with oven temperature.
2. Add a few flakes of solid paraffin wax to the vials after every 15–20 min and caps of the vials should be left open. Ensure that sample is never left dry.
3. Carry the whole procedure for 48–72 h till wax infiltrates completely in the sample and all the TBA evaporates (*see* **Note 11**).

Block Preparation and Section Cutting

1. Melt fresh wax at 60 °C and prepare rectangular or square blocks using a holder (*see* **Note 12**). Place the sample carefully in the block in correct orientation.
2. Allow the block to solidify overnight and cut 10–12 μm sections with the help of a rotary microtome.

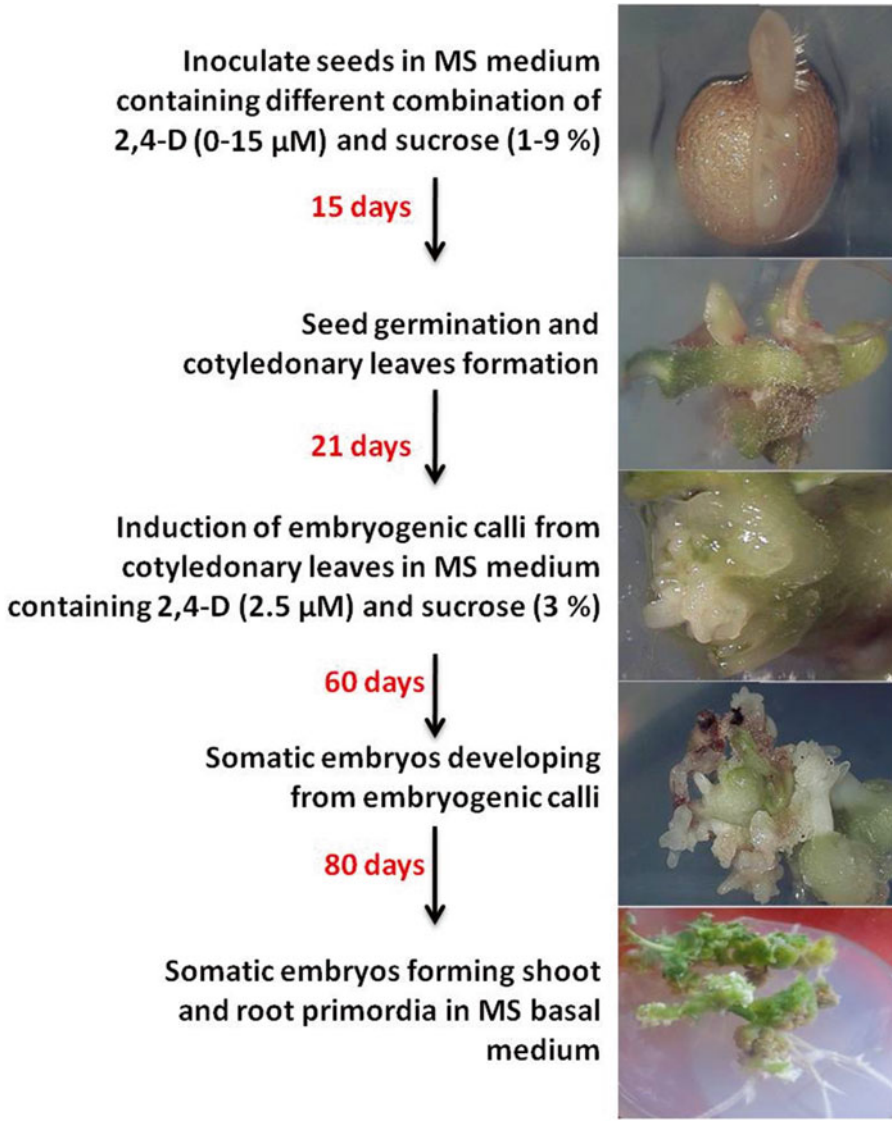

Fig. 4 Schematic representation of protocol for somatic embryogenesis from seeds of *W. somnifera*

Table 3
Different gradations for dehydration of tissue for histological preparations

Gradations	Rectified alcohol (ml)	TBA (ml)
a	30	20
b	50	20
c	50	35
d	45	55
e	25	75
f	–	100

Mounting and Stretching of Ribbon

1. Clean the glass slides thoroughly by dipping them in xylene overnight and dry them the next day.
2. Coat 1 % gelatine solution on the slides and allow them to dry for 15–20 min (*see* **Note 13**).
3. After cutting the ribbon, put two to three drops of water on the slides and place the ribbon containing the sections on it.
4. Stretch the ribbon by warming the slide on a slide warmer maintained at 60 °C (*see* **Note 14**). Keep the slides overnight in a dust-free environment.

Dewaxing and Staining

1. Put the glass slides in Coplin jars containing xylene for 1–2 h. Subsequently pass the slides through different gradations of xylene and ethanol (Table 4).

Table 4
Staining procedure for histological samples

a	75 ml xylene	25 ml ethanol
b	50 ml xylene	50 ml ethanol
c	25 ml xylene	75 ml ethanol
d	–	Rectified alcohol
e	25 ml water	75 ml ethanol
f	50 ml water	50 ml ethanol
g	75 ml water	25 ml ethanol
h	1 % (w/v) Safranin (6–24 h)	
i	75 ml water	25 ml ethanol
j	50 ml water	50 ml ethanol
k	25 ml water	75 ml ethanol
l	–	Rectified alcohol
m	–	Rectified alcohol
n	–	Rectified alcohol
o	Clove oil in 25 % ethanol	
p	Clove oil in 50 % ethanol	
q	Fast green (prepared in 50 % clove oil)	
r	Clove oil in 50 % xylene	
s	Clove oil in 25 % xylene	
t	Xylene (30 min)	
u	Xylene (30 min)	

2. Keep the slides in each grade for 2–3 min unless otherwise mentioned (*see* **Note 15**).
3. Mount the slides with DPX mountant and observe under the compound microscope (*see* **Notes 16** and **17**).

4 Notes

1. Prepare MS medium stocks by carefully weighing each component. MS stock III should be prepared and stored in amber bottle as it is light sensitive. pH of the medium should be set at 5.8.
2. 100 ml medium is poured in 250 ml flasks for preparing solid medium and 20 ml for liquid medium. 20–25 ml medium is poured in the tubes.
3. Prepare the PGR stocks by dissolving it in solvent first as mentioned in Table 2 and then make up the volume with distilled water.
4. Tween 20 added in sterilizing agents acts as a surfactant and helps in removing the surface contaminants.
5. Overheating of the medium should be avoided as it leads to degradation of PGRs.
6. All the glassware and instruments should be properly sterilized to avoid any contamination.
7. Volume of the liquid medium should be optimized to reduce the hyperhydricity of shoots.
8. Prolonged subculture of shoots in BAP medium leads to vitrification of shoots and yellowing of leaves. Hence, in order to reduce the residual effect of BAP, shoots are subcultured in low concentration of auxin (IAA 0.5 μM) followed by subculture in basal MS medium before transferring them to proliferation medium.
9. Ensure that during hardening process water content of plants is maintained. Plants should not be allowed to dry.
10. Volume of each grade should be made up to 100 ml. Keep the tissue in grade "c" overnight.
11. Slow penetration of wax in the tissue should be carried out. TBA should be removed completely.
12. While performing histology wax should not be reheated often as it destroys the wax's properties.
13. Gelatine should be allowed to dry completely otherwise the sections may be lost during staining.
14. Do not overheat the slides to stretch the ribbon as it would lead to melting of wax and destruction of sample.

15. Keep the sections in each grade during staining for 2–3 min. The sections should be kept in safranin stain for 6–24 h. Filter the safranin stain using Whatman filter paper to remove the granular particles.
16. While transferring the slides from one grade to another the solution should be added slowly so that sections do not wash away.
17. The sections should not be allowed to dry.

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Chapter 15

In Vitro Propagation of Sambong (*Blumea balsamifera* Linn.)

Thelma L. Soriano and Evangelina C. Cangao

Abstract

Terminal shoot tips of sambong (*Blumea balsamifera* Linn.) are cultured to initiate and regenerate shoots on Murashige and Skoog (MS) medium containing 1.0 mg/L benzyl adenine (BA). After 1 month, shoots, usually 4.5 cm long are separated and subcultured for multiplication. Regenerated shoots, about 6 cm long are rooted on MS medium supplemented with 1.0 mg/L naphthalene acetic acid (NAA). Exposure of shoots to high humidity for the first 2 weeks and equal proportion (1:1:1) of sterile sand, compost, and coir dust as potting mix favors the development of whole sambong plants. Young shoots from in vitro-derived sambong plants could also be used for propagation.

Key words Acclimatization, Explant, In vitro, In vivo, Shoot tip, Stolon, Subculture, Transplantation

1 Introduction

Sambong (*Blumea balsamifera* Linn.), a coarse and strong aromatic herb belongs to the family Compositae. It is one of the ten herbs that have been screened and approved by the Department of Health (DOH), Philippines, for the treatment of certain health disorders [1]. Different forms and mode of preparation of sambong leaves are used for the treatment of unopened wound, colds, back pains, rheumatism, chronic pus, discharge of the eye, diarrhea, stomach pains, hypertension, and uterine discharge and even to eliminate intestinal worms [2]. Sambong is also known as a diuretic and is used in cases of hypertension and mild to moderate congestive heart failure. A 250 mg tablet in foil strip which was formulated by DOH from powdered sambong leaves is now being promoted to cure urinary tract pain and burn sensations [3].

In Philippines, sambong is not commercially cultivated. Sambong plant cannot be produced rapidly because it is conventionally propagated from root cuttings and stolons (with three or more leaves) taken from the sides of the main plant. The rising demand for sambong as a herbal medicine calls for increased production of this plant. Interest in Philippine medicinal plant

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industry has been revived and plant species such as sambong are used as key ingredients. The resurgence of public interest in plant-based medicine, coupled with the rapid growth of the pharmaceutical industry has brought about the need for the increased production of herbal medicine.

In vitro culture is an alternative method for propagation and is widely used for the commercial cultivation of a large number of plant species, including many medicinal plants [4]. In Philippines, multiplication of a large number of medicinal plants especially sambong through in vitro culture offers a possibility to solve the poor natural propagation by means of root cuttings and stolons. So far, a method for rapid propagation of sambong has not been reported.

This study aimed to develop sambong in vitro culture protocol for mass propagation of planting materials and develop potting out technique.

2 Materials

2.1 Initiation and Regeneration of Cultures

1. Terminal shoots of sambong (*Blumea balsamifera*) collected from Bureau of Plant Industry San Andres, Malate, Manila (*see* **Note 1**).
2. Murashige and Skoog [5] medium (Table 1) (*see* **Note 2**).
3. Stock solutions of 100 ppm benzyl adenine (BA) and naphthalene acetic acid (NAA) (*see* **Note 3**).
4. 0.1 M hydrochloric acid (HCl) or 0.1 M potassium hydroxide (KOH) solutions.
5. pH meter.
6. Autoclave.
7. Sterile water.
8. Clorox (disinfectant).
9. Tween 20.
10. Sterilized paper.
11. Laminar flow air bench (*see* **Note 4**).
12. Plant growth room provided with fluorescent lights, light intensity 20 μmol/m^2/s, 8 h photoperiod daily, 24–25 °C.
13. Culture bottles (8 oz bottle jars with plastic cap).

2.2 Plantlet Acclimatization

1. Benches in the greenhouse.
2. Soap.
3. Fungicide (benlate) at 5 g/gallon.
4. Transparent plastic containers (175 mm × 145 mm × 60 mm).

Table 1
Stock solutions for preparation of Murashige and Skoog's (MS) medium[a]

Chemical constituents	Concentration
Macro salt content	mg/L
KNO_3	1900
NH_4NO_3	1650
$CaCl_2 \cdot 2H_2O$	440
$MgSO_4 \cdot 2H_2O$	370
KH_2PO_4	170
Micro salt content	mg/L
KI	0.83
H_3BO_3	6.2
$MnSO_4 \cdot 2H_2O$	22.3
$ZnSO_4 \cdot 7H_2O$	8.6
$Na_2.MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
Iron source	mg/L
$FeSO_4 \cdot 7H_2O$	27.8
$Na_2EDTA \cdot 2H_2O$	37.3
Vitamins	mg/100 ml
Nicotinic acid	50
Pyridoxine·HCl	50
Thiamine·HCl	10
Glycine	200
Organic supplements	mg/L
myo-Inositol	100

[a]After dissolving all the stock solutions in 1 L distilled water, adjust the pH to 5.8 by adding solutions of either hydrochloric acid (HCl) or potassium hydroxide (KOH) solution and add 20 g/L sugar and 7 g/L agar and autoclave at 120 °C at 15 psi for 15 min

5. A 1:1:1 (v/v) of potting mix of sand, coir dust, and compost.
6. Barbecue stick.
7. Masking tape.
8. Pot (8.89 cm wide and 11.43 cm tall having five drain holes).

3 Methods

3.1 Initiation and Regeneration of Cultures

1. Wash shoots of sambong (Fig. 1) thoroughly with soapy water (*see* **Note 5**).
2. Disinfect/sterilize shoots with 15 % Clorox mixed with two to three drops of Tween 20 for 10 min followed by three times rinsing with sterile water.
3. Excise shoots on a sterilized paper under the laminar flow air bench.
4. Strip off side leaflets to obtain a small shoot tip portion (Fig. 2) (*see* **Note 6**).
5. Initiate cultures in Murashige and Skoog medium (Table 1) containing 1.0 mg/L benzyl adenine (BA) (Fig. 3).
6. Subculture in MS medium supplemented with 1.0 mg/L BA for multiplication culture (Figs. 4 and 5) and 1.0 mg/L naphthalene acetic acid (NAA) for plantlet regeneration (Fig. 6) (*see* **Note 7**).

Fig. 1 Terminal shoot as a source of explant of sambong (*Blumea balsamifera* L.)

Fig. 2 Excised shoot tip of sambong for initial culture

Fig. 3 Sambong shoot tip cultured in Murashige and Skoog medium

Fig. 4 Regenerated sambong (*Blumea balsamifera* L.) shoots in MS medium

Fig. 5 Sambong (*Blumea balsamifera* L.) shoots ready for subculture

Fig. 6 Adventitious shoot proliferation on medium with 1.0 mg/L BA

Fig. 7 Growth of sambong shoots after 1 month of culture

7. Transfer cultures under light conditions in plant growth room provided with fluorescent light tubes, 20 μmol/m²/s, 8 h photoperiod daily, 24–25 °C, until rooting.
8. One month after inoculation, subculture shoots or split into five groups for shoot multiplication.
9. Subculture multiple shoots in four to six cycles in preparation for potting out. Multiply shoot cultures and regenerate plantlets using MS media containing 1.0 mg/L BA (optimized medium) and 1.0 mg/L NAA, respectively (Fig. 7) (*see* **Note 8**).

Fig. 8 Sambong cultures transferred to greenhouse for acclimatization

3.2 Plantlet Acclimatization

1. Place the plantlets in jars on benches inside the greenhouse for a period of 1 week (Fig. 8) (*see* **Note 9**).
2. Pull out gently the plantlets and wash off the agar particles adhering to the root section (Fig. 9) (*see* **Note 10**).
3. Treat with fungicide (Benlate) at 5 g/gallon the in vitro developed plantlets prior to potting in transparent plastic containers (175 mm × 145 mm × 60 mm) containing a potting mix (1:1:1) of equal proportion of sand, coir dust, and compost.
4. Carefully set up the young and tender plantlets in plastic containers containing a potting mix of sand, coir dust, and compost, which has been previously dibbled by a barbecue stick, and firmly press the soil around the stem (Fig. 10) (*see* **Note 11**).
5. Enclose the plastic containers with another plastic of the same size and seal with a masking tape (Fig. 11).
6. After growing 2 weeks in enclosed plastic containers, allow the plants to acclimatize by loosening the plastic cover around the plastic container to allow greater air circulation (*see* **Note 12**).
7. Three weeks after acclimatization (Fig. 12), transfer plants to individual pots containing an equal potting mix (1:1:1) of garden soil, compost, and coir dust on the greenhouse bench (Fig. 13) (*see* **Note 13**).

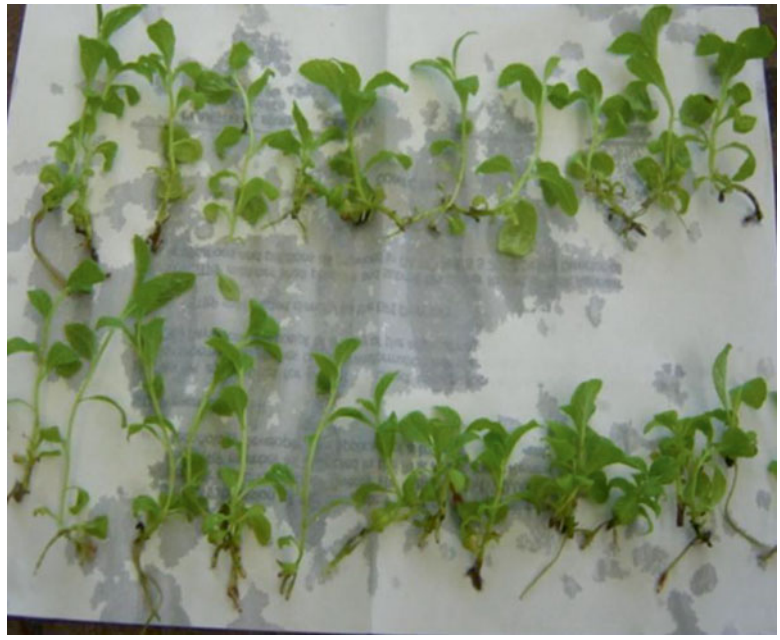

Fig. 9 Newly removed sambong plantlets from jars ready for potting out

Fig. 10 Tissue-cultured sambong plantlets in potting media

Fig. 11 Sambong tissue-cultured plants enclosed in a plastic container

Fig. 12 Sambong plants after 2 weeks in the potting medium (**a**) basal parts with developed roots, (**b**) terminal parts

Fig. 13 Individually potted plants ready for field planting

Fig. 14 Conventionally propagated sambong plants from in vitro derived shoot cuttings

3.3 Conventional Method Using Shoot Cuttings from In vitro Derived Plants (See Note 14)

1. Collect very young shoots, 10–12 cm long from in vitro derived sambong plants.
2. Place in a medium containing a combination (1:1) of moist compost and garden soil for rooting.
3. The environment in the greenhouse should be humid by wetting down the floors with gravel and sand.
4. Rooting of in vitro derived sambong plants takes 1 month (Fig. 14).
5. After 2 months in the potting medium, transfer the in vitro derived sambong plants to the field.

4 Notes

1. Terminal shoots, about 8–10 cm length, in their active vegetative growth stage were obtained from healthy vigorous sambong plants, grown in the experimental station, Bureau of Plant Industry, San Andres Malate Manila.
2. Dissolve chemicals one by one in doubled distilled water for preparing stock solutions of macro salts, micro salts, iron, vitamins, and organic supplements; store them at low temperature (5 °C) in dark bottles. Use appropriate amount of stock solutions and raise desirable volume with distilled water. Prolonged storage of media should be avoided.
3. To prepare 1 mg/ml stock solutions of NAA and BA, dissolve 100 mg of each plant growth regulator by adding 2–5 ml KOH in 100 ml volumetric flask and store in dark bottles at low temperature (5 °C).

4. Before washing with soapy water, some of the side leaves covering the terminal shoots are stripped off to keep 5.0–7.0 cm shoot length and excise further up to 1–1.5 cm long.
5. Do not pinch or squash the delicate shoot with forceps and scalpel.
6. After 1 month, 3–6 cm long shoots are separated and subcultured in a fresh MS medium for multiplication and plantlet regeneration.
7. Subculture shoot cultures in fresh MS medium at a 4-week interval.
8. Regenerated shoots, about 4–8 cm tall are rooted on MS medium supplemented with 1.0 mg/L naphthalene acetic acid (NAA).
9. The 8 oz bottle jars containing five plantlets are transferred to the greenhouse under the shade provided either with two to three layers of agricultural net or with plastic sheets for a period of 1 week.
10. Transfer in vitro plantlets with five to eight leaflets into soil by removing agar adhering to the roots by keeping under mild running water for 10 min. This step is carried out to prevent infection of molds.
11. Thirty young, tender, and 2-month-old plantlets are transplanted in a plastic container containing a potting mix of garden soil, compost, and coir dust, and kept in the greenhouse. After transplantation, water the plantlets regularly.
12. Plastic covers of the culture vessels are loosened to allow greater air circulation and to adjust to a more natural environmental condition.
13. Individual potted plants in the greenhouse are watered by misting every day.
14. Shoots, 10–12 cm long, taken from in vitro-derived sambong plants. growing in the greenhouse, are divided into two parts (terminal and basal), about 5.5–6.0 cm long, comprising three to five leaflets on each part and potted in vivo in a potting mix of moist compost and garden soil in a transparent plastic container for 2 months. It is important that each part has intimate contact with the potting mix, and soil should be firmly pressed around the stem. Water the transplanted young shoots every alternate day.

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Chapter 16

Production of Gymnemic Acid from Cell Suspension Cultures of *Gymnema sylvestre*

Praveen Nagella, Vijayalaxmi S. Dandin, and Hosakatte Niranjana Murthy

Abstract

Gymnema sylvestre R. Br. is a popular herbal medicine. It has been used in ayurvedic system of medicine for thousands of years. It is popularly called as "Gur-mar" for its distinctive property of temporarily destroying the taste of sweetness and is used in the treatment of diabetes. The leaves of gymnema possess antidiabetic, antimicrobial, anti-hypercholesterolemic, anti-sweetener, anti-inflammatory, and hepatoprotective properties and have traditional uses in the treatment of asthma, eye complaints, and snake bite. The leaves contain triterpene saponins such as gymnemic acid which is an active ingredient of *Gymnema*. Since the cultivation of *G. sylvestre* is a very slow process and the content of gymnemic acid depends on the environmental factors, cell suspension culture is sought as an alternative means for the production of *Gymnema* biomass and to enhance the gymnemic acid content. In this chapter, the methods employed for the induction of callus and subsequent establishment of cell suspension cultures for the production of biomass and analysis of gymnemic acid using high performance liquid chromatography are described.

Key words Antioxidant activity, Cell suspension cultures, *Gymnema sylvestre*, Gymnemagenin, Gymnemic acid, Herbal medicine, Triterpenoid saponin

1 Introduction

Gymnema sylvestre R.Br., a woody climber of the Asclepiadaceae family, has been known for many years for its medicinal value and it has a key place in ayurvedic medicine. It is popularly called as "Gurmar" for its distinctive property of temporarily destroying the taste of sweetness and is used in the treatment of diabetes [1]. It possesses potent antidiabetic properties and has traditional use in the treatment of asthma, eye complaints, and snake bite. It also possesses antimicrobial, anti-hypercholesterolemic, and hepatoprotective properties [2]. The leaf extract of this plant is used as stomachic, stimulant, laxative, diuretic, anti-sweetener [3], and possesses antiviral and anti-inflammatory [4] activities. The leaves of the species contain triterpene saponins such as gymnemic acid, deacyl gymnemic acid, gymnemagenin [5, 6], 23-hydroxylnogispinogenin, and

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gymnestrogenin [7, 8] belonging to the oleanane and dammarene classes, the former being the gymnemic acid and gymnemasaponins, and the latter gymnemaside. The aglycone of gymnemic acid is known as gymnemagenin.

The antidiabetic components of the plant are identified as a group of closely related gymnemic acids following their successful isolation and purification from the leaves [9]. Recently, gymnemic acid formulations have been found to be useful against obesity [1]. In folk medicine, these compounds are obtained as extracts or infusions from *G. sylvestre* wild or cultivated plants, causing a drastic decrease in this plant population. The production of gymnema by field cultivation is a very slow process and needs 4–6 years from planting to the harvesting stage. Further, its production is hindered by environmental factors, pests, and diseases, and the quantity of gymnemic acid, the active principle in *Gymnema* leaves, is, however, variable among accessions from different ecoclimatic regions [10]. A way to mitigate this problem is by developing plant tissue culture techniques for this species, since plant cell/organ cultures are not limited by environmental, ecological, and climatic conditions, and thus, cells/organs can be preferred to proliferate at higher growth rates than whole plant in cultivation [11]. Therefore, in vitro cell suspension cultures have become an alternative source for the production of Gymnema biomass and gymnemic acid. Additionally, there is possibility for year round production of biomass with reduced cost and time. In the present chapter, the relevant methods employed for the induction of callus from leaf explants of in vitro grown *G. sylvestre* seedlings using Murashige and Skoog (MS) medium supplemented with various growth regulators and subsequent proliferation of the callus in the MS medium are described, and the establishment of cell suspension cultures for the production of biomass and analysis of gymnemic acid using high performance liquid chromatography are discussed.

2 Materials

2.1 Induction of Callus from Leaf Explants of In Vitro Grown Seedlings and Establishment of Suspension Cultures

1. *Gymnema sylvestre* R. Br. seeds were collected from the botanical garden of Karnatak University campus, Dharwad, Karnataka, India (Fig. 1a).
2. Murashige and Skoog (MS) [12] medium stock solutions (MS stock I, II, III, and IV) (Table 1). Store in the cold room or in the refrigerator at 4 °C (*see* **Note 1**).
3. Stock solutions (10 mg/10 ml) of 2,4-dichlorophenoxy acetic acid (2,4-D) and kinetin. Store in the deep freezer at −20 °C.
4. Agar-agar to be used at 8 g/L.

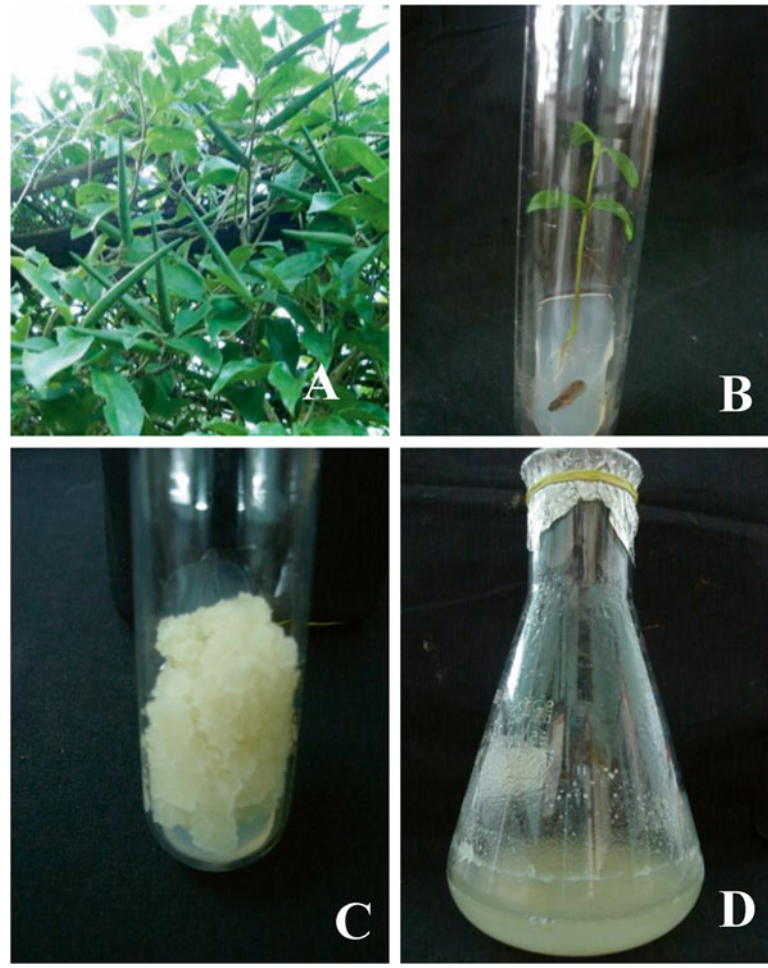

Fig. 1 Habit of the *Gymnema sylvestre* plant with fruits (**a**), 4-week-old in vitro grown seedling (**b**), callus developed from gymnema leaf on MS medium supplemented with 30 g/L sucrose, 2.0 mg/L 2,4-D, and 0.1 mg/L kinetin (**c**), proliferation of cell suspension in liquid medium (**d**)

2.2 Drying of Gymnema Cell Suspension Culture and Extraction of Gymnemic Acid

1. Hot air oven.
2. Heat reflux extraction unit.
3. Extraction solvent (1:1 volume of methanol–water).
4. Potassium hydroxide (11 %).
5. Concentrated HCl.
6. Nylon filter (0.45 μm).

2.3 Estimation of Total Phenols

1. 0.2 N Folin–Ciocalteu (FC) reagent. Store at 4 °C.
2. 1000 ppm gallic acid stock solution. Store at 4 °C in dark condition (*see* **Note 2**).
3. 15 % sodium carbonate solution. Store at 4 °C.
4. 80 % methanol, HPLC grade.

Table 1
Chemical composition of MS medium[a]

Chemical constituents	Concentration (mg/L)	Volume per liter (ml)
Major inorganic nutrients		
NH_4NO_3	33,000	50
KNO_3	38,000	
$CaCl_2{\cdot}2H_2O$	8800	
$MgSO_4{\cdot}2H_2O$	7400	
KH_2PO_4	3400	
Minor inorganic nutrients		
KI	166	5
H_3BO_3	1240	
$MnSO_4{\cdot}2H_2O$	4460	
$ZnSO_4{\cdot}7H_2O$	1720	
$Na_2.MoO_4{\cdot}2H_2O$	50	
$CuSO_4{\cdot}5H_2O$	5	
$CoCl_2{\cdot}6H_2O$	5	
Iron source		
$FeSO_4{\cdot}7H_2O$	5560	5
$Na_2EDTA{\cdot}2H_2O$	7460	
Organic supplements		
myo-Inositol	20,000	5
Nicotinic acid	100	
Pyridoxine·HCl	100	
Thiamine·HCl	100	
Glycine	400	
Carbon source		
Sucrose	30 g/L	

[a]Dissolve all the stock solutions of appropriate volume in distilled water and make it up to 1 L, adjust the pH to 5.8 (add 8 g/L agar for semisolid medium) and autoclave for 20 min at 121 °C

2.4 Estimation of Total Flavonoids

1. 1000 ppm quercetin stock solution. Store at 4 °C in the dark condition (*see* **Note 3**).
2. 10 % aluminum chloride solution. Store at 4 °C.
3. 1 M potassium acetate solution. Store at 4 °C.

2.5 Radical Scavenging Effect on 2,2-Diphenyl-1-Picrylhydrazyl (DPPH)

1. 2,2′-diphenyl-1-picrylhydrazyl (DPPH).
2. Ultraviolet (UV)–visible spectrophotometer.

2.6 Total Reducing Power Ability

1. 200 mM sodium phosphate buffer (pH 6.6).
2. 1 % potassium ferricyanide.
3. 10 % trichloroacetic acid.

4. 0.1 % ferric chloride.
5. Deionized water.
6. 1000 mg/L vitamin C.
7. Ultraviolet (UV)–visible spectrophotometer.

2.7 Phosphomolybdenum Activity

1. Reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate).
2. Ultraviolet (UV)–visible spectrophotometer.

2.8 HPLC Analysis of Gymnemagenin

1. HPLC-grade acetonitrile (*see* **Note 4**).
2. HPLC-grade water (*see* **Note 4**).
3. Standard gymnemagenin.

3 Methods

3.1 Induction of Callus from Leaf Explants

1. Wash *Gymnema sylvestre* seeds thoroughly under running tap water for 30 min followed by several rinses in distilled water (5×).
2. Surface-sterilize seeds in 2 % sodium hypochlorite solution containing few drops of Tween 20 detergent for 20 min.
3. Carry out the final step of sterilization in a horizontal laminar air flow chamber by rinsing the seeds in sterile distilled water (2×), followed by 0.5 % mercuric chloride solution for 5 min.
4. Finally rinse seeds several times (5×) in sterile distilled water.
5. Inoculate seeds onto MS basal medium (Murashige and Skoog [12]) for germination.
6. Inoculate leaf explants from 4-week-old germinated seedling (Fig. 1b) on MS medium containing 30 g/L sucrose and medium supplemented with 2.0 mg/L 2,4-D and 0.1 mg/L kinetin (KN), pH 5.8.
7. Incubate the cultures at 25±2 °C, with a 16 h photoperiod (40 mmol/m^2/s) provided with 40-W white fluorescent lamps. Within 4 weeks callus will develop from the leaf explants (Fig. 1c).
8. Culture the callus obtained on semisolid MS medium containing 30 g/L sucrose, 2.0 mg/L 2,4-D, and 0.1 mg/L kinetin (KN), pH 5.8 for proliferation.
9. Transfer the cultures to a fresh medium at 2-week intervals. Use actively growing cell line as explants for further experiments.

3.2 Proliferation of Cell Suspension in the Liquid Medium

1. Inoculate actively growing friable cell lines from the semisolid cultures (5 g/L fresh biomass) into a 250 ml Erlenmeyer flask containing 50 ml MS liquid medium supplemented with 30 g/L sucrose, 2.0 mg/L 2,4-D, and 0.1 mg/L kinetin (KN) (Fig. 1d).
2. Incubate cultures at 16-h photoperiod of cool-white fluorescent light (40 μmol/m^2/s) and 25 ± 2 °C and agitate at 110 rpm. Maintain cultures by regular subculturing at 2-week intervals.

3.3 Cell Suspension Cultures for the Production of Gymnemic Acid

1. Collect the cells at exponential growth phase and initiate the suspension cultures for the production of gymnemic acid.
2. Inoculate 5 g/L fresh cell biomass in a 250 ml Erlenmeyer flask containing 50 ml MS medium supplemented with 30 g/L sucrose, 2.0 mg/L 2,4-D, and 0.1 mg/L kinetin (KN).
3. Incubate cultures at 16-h photoperiod of cool-white fluorescent light (40 μmol/m^2/s) and 25 ± 2 °C and agitate at 110 rpm on a rotary shaker. The cells at these conditions will grow and multiply at a faster rate.
4. After 4 weeks of culture, assess the growth of cell suspensions in terms of fresh weight, dry weight, growth ratio, and amounts of phenolics, flavonoids, and gymnemic acid content.

3.4 Estimation of Cell Biomass

1. Filter the cell suspensions through a stainless steel sieve of pore size 0.45 μm to separate cells from the culture medium.
2. Wash the cells thoroughly in sterile water and blot excessive surface water. Record the fresh weight of cells.
3. Record the dry weight after drying the cells at 50 °C for 24 h in a hot air oven to attain a constant weight.
4. Growth ratio can be determined by using the formula: GR = harvested dry biomass (g) – inoculated dry biomass (g)/ inoculated dry biomass (g).

3.5 Preparation of Cell Extract for Analyzing Bioactive Compounds

1. Dry the cells in a hot air oven unit at 50 °C for 24 h. The dried cells can be stored at room temperature (20–35 °C) for a longer duration in the vacuum desiccator.
2. Take 2 g dried powder and perform the extraction at 40 °C for 6 h using 25 ml of 80 % methanol (rotary shaker).
3. Filter the extract through a double layer of Whatman No. 42 filter paper.
4. Re-extract the residue as in **steps 2** and **3** and mix the extracts.
5. Evaporate the solvent using vacuum rotary evaporator.
6. To the residue add 10 ml of 80 % methanol.

3.6 Estimation of Total Phenolic Content in Cell Culture Extract

The amount of total phenol extracted from cell culture extract is analyzed spectrophotometrically by using Folin–Ciocalteu reagent.

1. Mix 500 μl methanolic extract with 2.5 ml 0.2 M freshly prepared Folin–Ciocalteu reagent. Mix the contents well and allow to stand for 6 min.
2. After 6 min, add 2.0 ml 20 % sodium carbonate solution. After incubation at room temperature for 2 h the purple color will develop.
3. Working standards of 20, 40, 60, 80, and 100 ppm of gallic acid are used to plot the standard calibration graph.
4. The absorbance of test solutions is detected at 760 nm on UV–visible spectrophotometer. The readings are compared with the standard graph for gallic acid. The results are expressed as mg of gallic acid equivalent per 100 g dry weight. All the experiments should be carried out in triplicates and each assay should be repeated at least 2 times.

3.7 Estimation of Total Flavonoid Content in Cell Culture Extract

The amount of total flavonoid present in the cell culture extract can be analyzed spectrophotometrically by following the aluminum chloride method.

1. Mix 500 μl methanolic extract with 0.1 ml of 10 % aluminum chloride, 0.1 ml of 1 M potassium acetate, and 4.3 ml of deionized water and incubate at room temperature for 30 min.
2. After incubation, the absorbance of the test solutions is measured on a UV–visible spectrophotometer at 430 nm.
3. Working standards of 20, 40, 60, 80, and 100 ppm of quercetin are used to plot the standard calibration graph.
4. The readings are compared with the standard graph for quercetin. The results are expressed as mg of quercetin equivalent per 100 g dry weight. All the experiments should be carried out in triplicates and each assay should be repeated at least 2 times.

3.8 Radical Scavenging Activity on 2,2-Diphenyl-1-Picrylhydrazyl (DPPH)

1. For the analysis of the antioxidant activity, mix 100 μl aliquots of the extract and make up the volume to 1.0 ml with methanol.
2. To this sample solution add 2.0 ml of 0.06 M methanolic solution of DPPH.
3. Incubate the sample solutions in dark at room temperature for 30 min and assay the reaction mixture at 517 nm using UV–visible spectrophotometer.
4. For eliminating the interference with the DPPH reaction by extract, a blank sample has to be assayed. For the blank (negative control), 1.0 ml of methanol has to be taken, to which add

2.0 ml of 0.06 M methanolic solution of DPPH and incubate as mentioned above. Carry out all the experiments in triplicate and each assay should be repeated at least 2 times.

5. Record the decrease in percentage of absorbance at 517 nm for the extracts and calculate the percentage of radical scavenging activity of DPPH on the basis of observed decrease in the radical. The inhibition percentage or radical scavenging activity can be calculated by using the following formula:

$$\%\text{Inhibition or }\%\text{Radical scavenging activity} = \left[\left(\text{Ab} - \text{As}\right) / \text{Ab}\right] \times 100$$

where Ab is the absorbance of blank (has the highest value), As is the absorbance of sample (has the lowest value). Curves showing inhibition percentage/μl of the extract are used to determine the concentration at which 50 % radical scavenging occurs (IC_{50}).

3.9 Total Reducing Power Ability

1. Mix 100 μl aliquots of the extract and make up the volume to 1.0 ml with methanol.
2. Then, add 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6), and 2.5 ml of 1 % potassium ferricyanide and incubate at 50 °C for 20 min.
3. After incubation, add 2.5 ml of 10 % trichloroacetic acid and centrifuge at $2000 \times g$ for 10 min.
4. Mix the upper layer in each tube (2.5 ml) with 2.5 ml of deionized water and 0.5 ml of 0.1 % ferric chloride.
5. Measure the absorbance at 700 nm against a blank. Carry out all the experiments in triplicate and repeat each assay at least 2 times.
6. The reducing power increases with the increase in absorbance. The total reducing power of the extract is compared to vitamin C as a positive control and the results are expressed as vitamin C equivalent (mM).

3.10 Phosphomolybdenum Activity

1. Mix 100 μl aliquots of the extract combined with 1 ml of the reagent solution.
2. Cap the tubes tightly and incubate in boiling water bath at 95 °C for 90 min.
3. After 90 min, cool the tubes to room temperature.
4. Prepare a blank solution using 1 ml of reagent solution instead of sample.
5. Measure the absorbance of the sample and the blank at 693 nm. Carry out all the experiments in triplicate and repeat each assay at least 2 times.

3.11 HPLC Analysis of Gymnemic Acid

1. Weigh 500 mg dried sample powder and add 50 ml extraction solvent (1:1 volume of methanol–water) and 10 ml of 11 % potassium hydroxide solution in a 500 ml round bottom flask. Reflux the mixture for an hour; add 9 ml HCl and reflux again for 1 h. Cool the mixture to room temperature, filter the extract through 0.45 μm nylon filter and make the volume to 100 ml with extraction solvent and use the clean supernatant for HPLC analysis.
2. Initially wash the chromatographic equipment consisting of degasser, pump, auto sampler, thermostated column compartment, and diode array detector (DAD) with HPLC grade water twice.
3. Carry out the analysis of the gymnemic acid using C18 (5 μm) column. Use acetonitrile–water (80:20) as the mobile phase. The column temperature should be maintained at 27 °C. The injection volume should be 20 μl. The detection of the eluent is measured at 210 nm. Maintain a running time of 30 min and a flow rate of 1 ml/min. For analysis, use three separate injections of each sample.
4. The gymnemagenin can be identified on the basis of the retention time values and the absorbance of the UV spectra in comparison with the standard gymnemagenin (Fig. 2). The confirmation of the peak can also be performed by spiking the extract with the pure standard gymnemagenin.
5. Eluted samples can be detected with a variable dual wavelength detector coupled to the HPLC system, by comparing the UV spectra of the peak with those of authentic reference samples.
6. Prepare standard stock solutions of gymnemagenin as follows: weigh 10.0 mg standard gymnemagenin accurately and dissolve in 10 ml of HPLC grade methanol in 10 ml volumetric flask. Then, dilute stock solutions with methanol to prepare a series of standard solutions with concentrations of 25, 50, 100, and 150 μg/ml for linearity validation.

   The conversion of gymnemagenin to gymnemic acid is done using the formula:

   Molecular weight conversion of gymnemagenin to gymnemic acid (809.0/506.7) = conversion.

4 Notes

1. Prepare stock solutions of major, minor inorganic nutrients, iron source, vitamins, and individual plant growth regulators (Table 1). Store stock solutions at 4 °C, except vitamins which is stored at −20 °C. Avoid storage of the stock solutions for longer durations (not more than 2 months) as they may get

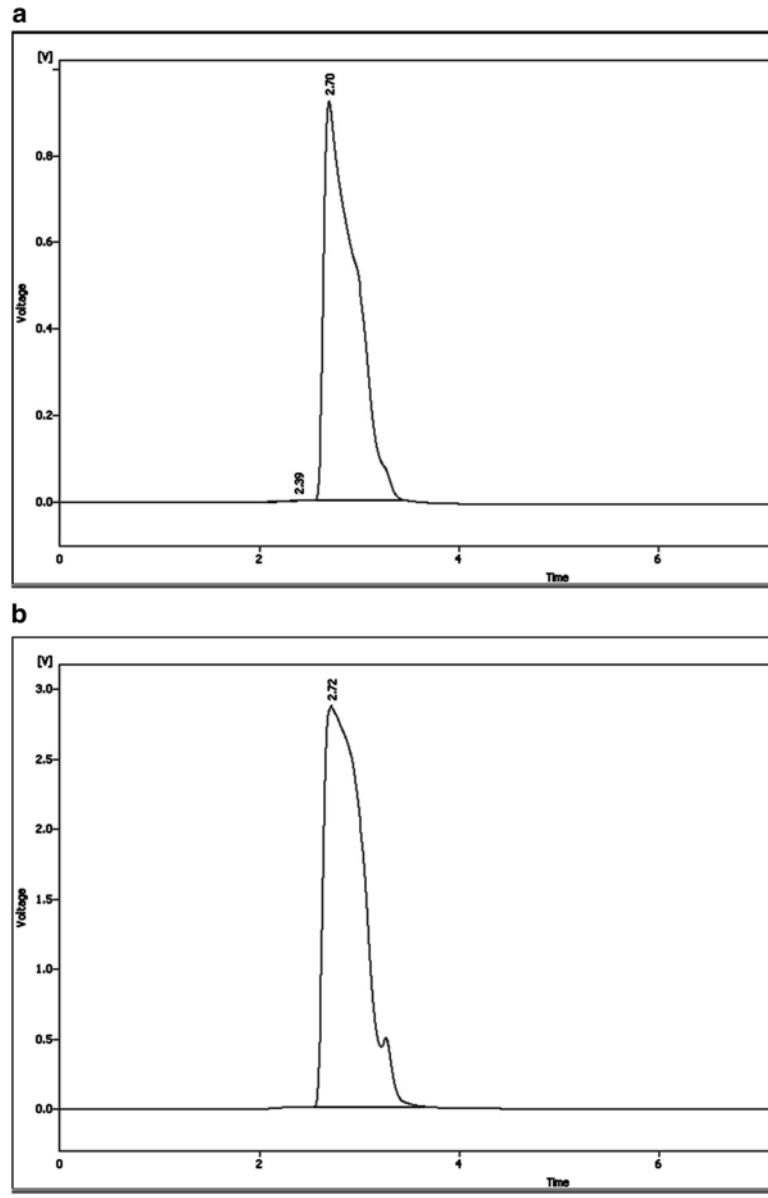

Fig. 2 HPLC profiles of standard gymnemagenin (**a**), cell suspension culture (**b**)

contaminated or precipitated. Prepare fresh plant growth regulator solutions every time. Any color change in the stock solutions may be due to precipitation which can seriously affect the growth of cultures. Alternatively, stock solutions of macro-elements, micro-elements, vitamins, and the ready-to-use MS medium are commercially available.

2. Standard gallic acid is prepared by weighing 1000 mg gallic acid powder and by dissolving it in 1 L distilled water. The addition of 1 ml DMSO or ethanol will readily dissolve the

compound. Store the stock solution in an amber-colored bottle at 4 °C.

3. Quercetin is prepared by weighing 1000 mg quercetin powder and dissolving in 1 L deionized distilled water. Add 1 ml DMSO or ethanol to make the quercetin powder to dissolve readily. Store the stock solution in an amber-colored bottle at 4 °C.
4. Prepare fresh solution of 0.06 M methanolic solution of DPPH and store in darkness at 4 °C in an amber-colored bottle (DPPH reacts with light). All the solutions should be prepared freshly and stored in the refrigerator at 4 °C. All the solvents used for the HPLC analysis should be of HPLC grade and filtered through 0.45 μm polytetrafluoroethylene (PTFE) filters before use.

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Chapter 17

Scale-Up of *Agrobacterium rhizogenes*-Mediated Hairy Root Cultures of *Rauwolfia serpentina*: A Persuasive Approach for Stable Reserpine Production

Shakti Mehrotra, Vikas Srivastava, Manoj K. Goel, and Arun K. Kukreja

Abstract

Roots of *Rauwolfia serpentina*, also known as "Sarpagandha" possess high pharmaceutical value due to the presence of reserpine and other medicinally important terpene indole alkaloids. Ever increasing commercial demand of *R. serpentina* roots is the major reason behind the unsystematic harvesting and fast decline of the species from its natural environment. Considering *Agrobacterium rhizogenes*-mediated hairy root cultures as an alternative source for the production of plant-based secondary metabolites, the present optimized protocol offers a commercially feasible method for the production of reserpine, the most potent alkaloid from *R. serpentina* roots. This end-to-end protocol presents the establishment of hairy root culture from the leaf explants of *R. serpentina* through the infection of *A. rhizogenes* strain A4 in liquid B5 culture medium and its up-scaling in a 5 L bench top, mechanically agitated bioreactor. The transformed nature of roots was confirmed through PCR-based *rol* A gene amplification in genomic DNA of putative hairy roots. The extraction and quantification of reserpine in bioreactor grown roots has been done using monolithic reverse phase high-performance liquid chromatography (HPLC).

Key words Bioreactor, Hairy roots, HPLC, *Rauwolfia serpentina*, Reserpine, Up-scaling

1 Introduction

Rauwolfia serpentina, a distinguished member of family Apocynaceae, has drawn special attention all over the world because of its high medicinal importance. The plant is known to produce medicinally important terpenoid indole alkaloids (TIAs) such as reserpine, ajmalicine, ajmaline, serpentine, vomiline, and yohimbine. [1]. Among other TIAs, reserpine, a 3,4,5-trimethyl benzoic acid ester of reserpic acid, is the most potent alkaloid with remarkable antihypertensive properties. The alkaloids are concentrated mostly in the plant roots, reported to yield about 85–90 % of the total alkaloid content of the whole plant [2]. Presence of these

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TIAs contributes to the medicinal properties of *Rauwolfia* roots which are strong antihypertensive, laxative, anthelmintic, and diuretic in nature. The root extract is present as a major ingredient in a variety of commercially available drugs that are used for the treatment of hypertension, high blood pressure, mental illness, and problems related to central nervous system (CNS). The crude extract is also used in sedatives, aphrodisiac and antispasmodic medicines. Further, hypoglycemic and hypolipidemic activities are also reported from the root extract of other species of *Rauwolfia* [3]. The only source for the procurement of *Rauwolfia* roots for medicinal purposes is the natural reserves which due to unsystematic exploitation are fast declining. The International Union for the Conservation of Nature and Natural Resources (IUCN) enlisted this species among those endangered plants for which cultivation and conservation are prioritized through conventional and alternative methods in India (NMPB; www.nmpb.nic.in).

The *Agrobacterium rhizogenes*-mediated hairy root cultures are known as an alternative source for the production of plant-based secondary metabolites under laboratory conditions [4]. Regular demand of *R. serpentina* roots for commercial medicinal uses and limited supply from natural resource have laid the background for the establishment and use of *A. rhizogenes*-mediated hairy root cultures (HRCs) for TIA production [5, 6]. The hairy root cultures of various species of *Rauwolfia* represent a rich repository of a range of alkaloids as they produce noticeable amounts of these TIAs [6]. These easily manageable hairy root cultures can be a promising source for the production of reserpine and other valuable alkaloids. In the development and progress of techniques for commercial production of hairy root-based secondary metabolites, the up-scale culture of roots in bioreactors is the definitive step. Keeping the pharmaceutical importance of reserpine in mind, the present protocol was developed to establish *Agrobacterium rhizogenes*-mediated *R. serpentina* hairy root cultures for high reserpine biosynthesis and up-scaling of these hairy root cultures in a bench top, mechanically agitated 5 L capacity bioreactor. Before up-scaling in bioreactor, the transformed nature of hairy root line was confirmed through PCR based *rol* A gene amplification. The accumulation of reserpine was detected by high performance liquid chromatography.

2 Materials

2.1 Media Preparation

1. Stock solutions of Gamborg's B5 and Murashige and Skoog (MS) medium (*see* Table 1) (*see* **Notes 1** and **2**).
2. Agar.
3. Beakers, measuring cylinders, and glass rods (*see* **Note 3**).

Table 1
Components of MS and B5 growth medium

Designated stock	Constituents of stock	Concentration (mg/L)	
		MS	B5
A	NH_4NO_3	1650	–
	$(NH_4)_2SO_4$	–	134
B	KNO_3	1900	2500
	$MgSO_4{\cdot}7H_2O$	370	250
	$MnSO_4{\cdot}4H_2O$	22.3	10
C	$ZnSO_4{\cdot}7H_2O$	8.6	2
	$CuSO_4{\cdot}5H_2O$	0.025	0.025
	$CaCl_2{\cdot}H_2O$	440	–
	KH_2PO_4	170	150
D	NaH_2PO_4	–	3
	H_3BO_3	6.2	0.75
	KI	0.83	0.25
	$Na_2MoO_4{\cdot}2H_2O$	0.25	0.025
	$CoCl_2{\cdot}6H_2O$	0.025	–
F	$FeSO_4{\cdot}7H_2O$	27.85	27.85
	Na_2EDTA	37.35	37.35
	Thiamine HCl	0.1	10
	Pyridoxine HCl	0.5	10
G	Nicotinic acid	0.5	1
	Glycine	2	–
	Folic acid	–	–
	Biotin	–	–

4. 0.1–10 ml pipettes and/or 0.5–100 µl micropipettes.
5. 1 N NaOH/1 N HCl.
6. 250 ml narrow-neck Erlenmeyer flasks.

2.2 Bacterial Stock Culture and Suspension

1. *Agrobacterium rhizogenes* strain A4 (pRA4).
2. Yeast Mannitol Broth (YMB) and Yeast Mannitol Agar (YMA) [7] (Table 2 *see* **Note 4**).
3. Disposable petri plates (90 mm).

Table 2
Composition of YMA/YMB

Constituents	Concentration (g/L)
KH_2PO_4	0.5
$MgSO_4 \cdot 7H_2O$	2
NaCl	0.1
Mannitol	10
Yeast extract	0.4
Agar-agar	1.50 %

4. Streaking loops.
5. Sample tubes with screw top (10 ml;) (*see* **Note 5**).

2.3 Infection, Co-cultivation, and Establishment of Hairy Root Cultures

1. Culture medium (semisolid MS in petri plates and liquid B5 in culture flasks).
2. In vitro maintained 6–8-week-old stock multiple shoot cultures of *R. serpentina* [8] (*see* **Note 6**).
3. Disposable syringes (1 ml).
4. Sterile distilled water.
5. Autoclaved scissors and 8–12″ rust-proof stainless steel forceps.
6. Sporidex antibiotic (Ranbaxy; India).

2.4 Confirmation of Transformed Nature of Hairy Roots

2.4.1 Isolation and Quantification of Genomic DNA of Normal and Putative Hairy Roots

1. Mortar and pestle.
2. Liquid nitrogen.
3. Autoclaved eppendorf tubes.
4. Buffers (Table 3, *see* **Notes 7** and **8**).
5. Chloroform–isoamyl alcohol (24:1; *v*/*v*).
6. 5 M NaCl in water.
7. Isopropanol (Propane-2-ol).
8. Desiccators with vacuum pump.
9. Milli-Q water.
10. Ethidium bromide stock (1 %) in water.
11. Agarose.
12. 6× loading dye (*see* **Note 9**).

2.4.2 Polymerase Chain Reaction (PCR) for Gene Amplification

1. PCR tubes (*see* **Note 10**).
2. Template DNA.
3. PCR master mixture (2×, Fermentas) (*see* **Note 11**).

Table 3
Composition of buffers

Extraction buffer	High salt TE	TAE buffer (50×; 1 L)
1.4 M NaCl	1 M NaCl	0.5 M EDTA (100 ml)
100 mM Tris–HCl	10 mM Tris–HCl	Tris base 242 g
20 mM EDTA	1 mM EDTA	57.1 ml Glacial acetic acid
2.5 % CTAB		
0.2 % β-mercaptoethanol		
1 % PVP		

4. Gene specific (*rol A*) primer(s). In the present study, to confirm the transformed nature of roots, *rol* A amplification is performed. However, for this purpose any of the *rol* genes (A, B, C) or even T_L sequence (left border sequence from bacterial plasmid which is essential for hairy root induction) from the bacterial origin can be amplified in host genome with their respective primers. Primer sequence for *rol* A:

   Forward 5′-GGAATTAGCCGGACTAAACG-3′ and

   Reverse 5′-CCGGCGTGGAAATGAATCG-3′
5. Marker DNA (Quick load 100 bp, NEB)
6. Milli-Q water.
7. Ice bath.
8. Thermal power PCR cycler (iCycler).

2.5 Up-Scaling of Hairy Roots in Bioreactor

1. Bioreactor setup (in the present protocol, model Bio flow 3000, M/s New Brunswick Scientific, USA; modified air lift with top driven mechanical agitation through marine blade impeller is used). The reactor setup is also fitted with probes for dissolved oxygen (DO), pH, and temperature.
2. 4–6-week-old stock hairy root cultures maintained in shake flasks [6] (*see* **Note 12**).
3. Gamborg's Liquid B5 culture medium.
4. Ethanol for surface sterilization.
5. Sterile forceps.

2.6 Chemical Analysis

2.6.1 Extraction of Alkaloids and High-Performance Liquid Chromatography for Reserpine

1. Dried root samples.
2. Chloroform–methanol, 3:1 (*v*/*v*).
3. Distilled water.
4. Hydrochloric acid.
5. Rotavapor R-144 (Buchi).
6. RP-18e Chromolith HPLC Columns (4.6×100 mm).

7. Glacial acetic acid.
8. Di-sodium-di-hydrogen orthophosphate.
9. HPLC-grade acetonitrile (Merck; Darmstadt, Germany)
10. HPLC-grade water (Sigma-Aldrich, USA).
11. Standard reserpine (Sigma-Aldrich, USA).

3 Methods

3.1 Preparation of Medium

1. Use MS and B5 stock solutions to prepare the culture medium (*see* **Notes 1** and **2**; Table 1)
2. For semisolid medium melt the calculated amount of agar separately. Normally 7.5–8 % agar is used in the protocol or unless otherwise stated.
3. Mix the required volume of stock solutions, Sucrose (30 g/l) and myoinositol (0.1 g/l) medium. In the case of semisolid medium add the aforementioned to the molten agar by stirring. In the case of liquid B5 medium do not add agar. Make the final volume of culture medium as per requirement.
4. Adjust the medium pH at 5.86 ± 0.02 with the help of 1 N NaCl/1 N HCl.
5. Dispense the medium in appropriate culture vessels. Use cotton plugs to seal the vessels.
6. Sterilize the medium at 121 °C at 15 lb pressure for 15–20 min.
7. Pour lukewarm semisolid MS medium in petri plates and allow it to solidify under laminar air flow.
8. Store the culture medium at 25 °C.

3.2 Maintenance of Stock Bacterial Culture and Preparation of Bacterial Suspension

1. Prepare the stock bacterial culture by streaking single cell bacterial colonies on semisolid YMA and incubate the cultures at 28 ± 2 °C. New bacterial colonies develop within 48 h incubation. Store the cultures at 4 °C and maintain the stock cultures by regular subculturing on to the same medium at every 6–8 week interval (*see* **Note 13**).
2. Take 5 ml liquid YMB medium in sterile sample tubes.
3. Inoculate the medium with single cell colonies from stock bacterial cultures with the help of sterile streaking loops.
4. Incubate the culture at 28 ± 2 °C; 75–80 rpm on a rotary shaker for 48 h (*see* Fig. 1a).

3.3 Infection and Co-cultivation of Leaf Explants

1. Excise juvenile (3–5-week-old) leaf explants from in vitro maintained shoot cultures of *R. serpentina* under the laminar flow.

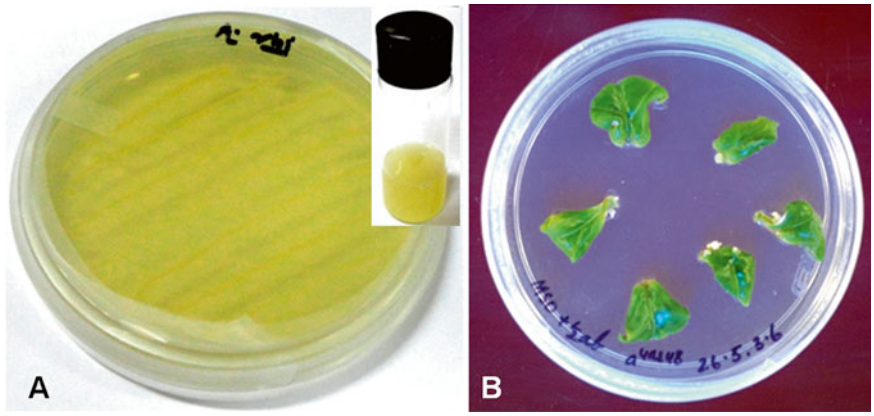

Fig. 1 Stock bacterial culture (**a**; *inset* 48-h-old suspension culture); Co-cultivation in leaf explants (**b**)

2. Prick the leaf explants with sterile needle dipped in 48 h-old bacterial suspension (*see* **Note 14**).
3. Leaf explants pricked with the sterile needle dipped in sterile water can be treated as control.
4. Place the infected explants on MS medium in petri plates for co-cultivation.
5. Incubate the petri plates in 40 μmol/m^2/s light intensity at 25 ± 2 °C.
6. Check the excessive growth of bacteria by transferring the explants on MS medium containing antibiotic (Sporidex, 1 mg/ml culture medium; *see* **Note 15**) after every 2–3 days till the vestige bacterial growth disappears (*see* Fig. 1b).

3.4 Disinfection and Establishment of Root Cultures

1. Within 12 days of infection roots emerge at infection sites of leaf explants. Single root emerging from an infection site can be considered as an individual putative hairy root line (*see* Fig. 2a).
2. Excise the individual putative hairy roots (≥1 cm) emerging from the leaves.
3. Inoculate individual root lines separately on B5 semisolid medium containing antibiotic.
4. Incubate the petri plates in light at 25 ± 2 °C (*see* **Note 16**).
5. Upon growth, transfer the root lines individually (approximately 200 mg fresh tissue weight) in 250 ml Erlenmeyer flasks containing 30 ml liquid B5 growth medium. Incubate the liquid cultures on a rotary shaker at 60 rpm; 25 ± 2 °C in continuous light (*see* Fig. 2b).
6. Maintain the root cultures in shake flasks through regular subculturing at every 6-week interval in the same medium and similar culture conditions.

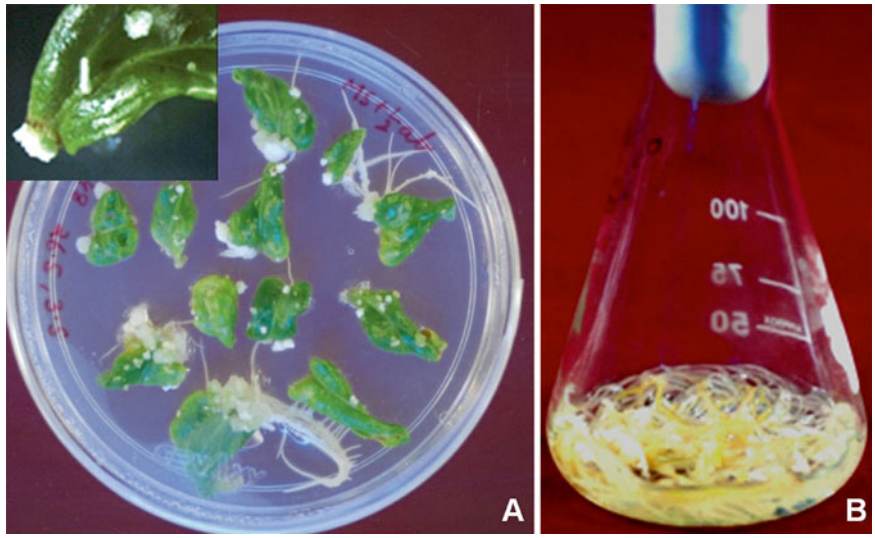

Fig. 2 Emergence of roots at infection sites of leaf explants (**a**; *inset* emergence of single root line); axenic root culture in liquid B5 medium (**b**)

3.5 Confirmation of Transformed Nature of Roots

3.5.1 Isolation of Genomic DNA from Different Root Lines

1. Isolation of genomic DNA is done by modified CTAB method [9].
2. Prepare the extraction buffer by mixing calculated amounts of cetyl trimethyl ammonium bromide (CTAB), 5 M NaCl, ethylene diamine tetraacetic acid (EDTA; pH 8.0), Tris–HCl (pH 8.0), and polyvinyl pyrrolidone (PVP) (*see* Table 3).
3. Make the final volume by adding sterile distilled water and warm the extraction buffer at 60 °C in water bath for 30 min.
4. Add β-mercaptoethanol to the extraction buffer prior to use (*see* **Note 17**).
5. Grind approximately 0.2–0.5 g fresh root tissue of putative root lines (six in present case) along with normal roots grown in vitro (control) to a fine powder in liquid nitrogen.
6. Immediately transfer the powdered tissue to sterile eppendorf tubes (1.5 ml micro centrifuge tubes) containing pre-warmed (55–60 °C) 1 ml extraction buffer (*see* **Note 18**). Inversely mix or gently shake the mixture to form slurry.
7. Incubate them at 60–65 °C in a water bath for 1 h for lysis of plant cell. Mix regularly at every 20 min interval.
8. Add equal volumes of chloroform–isoamyl alcohol (24:1) and gently mix by inversion for 10 min to form an emulsion.
9. Centrifuge the tubes for 10 min at 10,000×*g* at 25 °C.
10. Pipette out the upper aqueous layer (~800 μl) containing mostly nucleic acid.

11. Add 5 M NaCl (300 µl) solution and propanol-2-ol (0.6 volume of the total solution). Gently mix by inversion and allow this mixture to stand for 1–2 h at room temperature.
12. Centrifuge for 10 min at 10,000 rpm at 25 °C.
13. Discard the supernatant and wash the pellet with 80 % ethanol by centrifugation for 5 min at 10,000×*g* at 25 °C. Discard the supernatant and dry the pellet under vacuum for 1–2 min to remove the traces of alcohol.
14. Dissolve the pellet in high salt TE (400 µl) buffer (*see* Table 3). It may take some time to dissolve.
15. Add RNAse A (5 µl) and incubate at 37 °C in a water bath for 30 min.
16. Extract with equal volume chloroform–isoamyl alcohol (24:1) to remove the remaining proteins and other impurities by gentle inversion and centrifugation for 10 min at 10,000×*g* at 25 °C.
17. Transfer the upper aqueous layer to sterile eppendorf tube and add double volume of ice cold ethanol. Incubate at –20 °C for 1–2 h for the precipitation of DNA.
18. Centrifuge this mixture at 10,000×*g* for 10 min at 4 °C. Discard the supernatant and wash the pellet with 80 % ethanol at 10,000×*g* for 5 min at 25 °C.
19. After vacuum drying dissolve the pellet in 50 µl Milli-Q water and store at –20 °C for further use.

3.5.2 Quantification of DNA and PCR Amplification

1. The DNA yields can be measured either by spectrophotometer (at 260 nm) or by agarose gel (0.8 %) electrophoresis and its visualization under transilluminator (Preparation of agarose gel is described below. For loading of gel *see* **Note 19**; Fig. 3a).
2. To use appropriate amount of sample DNA and ease of pipetting, the DNA dilutions are required. Normally, single PCR reaction mix requires 20–25 ng of DNA. The dilution of DNA should be made with sterile Milli-Q water in such a way that 1 µl should contain approximately 20–25 ng of DNA.
3. Polymerase chain reaction: Prepare the PCR reaction mixture by adding sample DNA (1 µl), forward and reverse *rol* A gene primers (1 µl each from 10 pM stock), PCR master mix (12.5 µl). Make up the volume to 25 µl by adding Milli-Q water (*see* **Note 20**).
4. Prepare the PCR reaction mixture on ice bath.
5. Spin the PCR tube containing PCR mix for few seconds for proper mixing.
6. Place the tubes in thermal cycler for amplification. Set the conditions for amplification as:

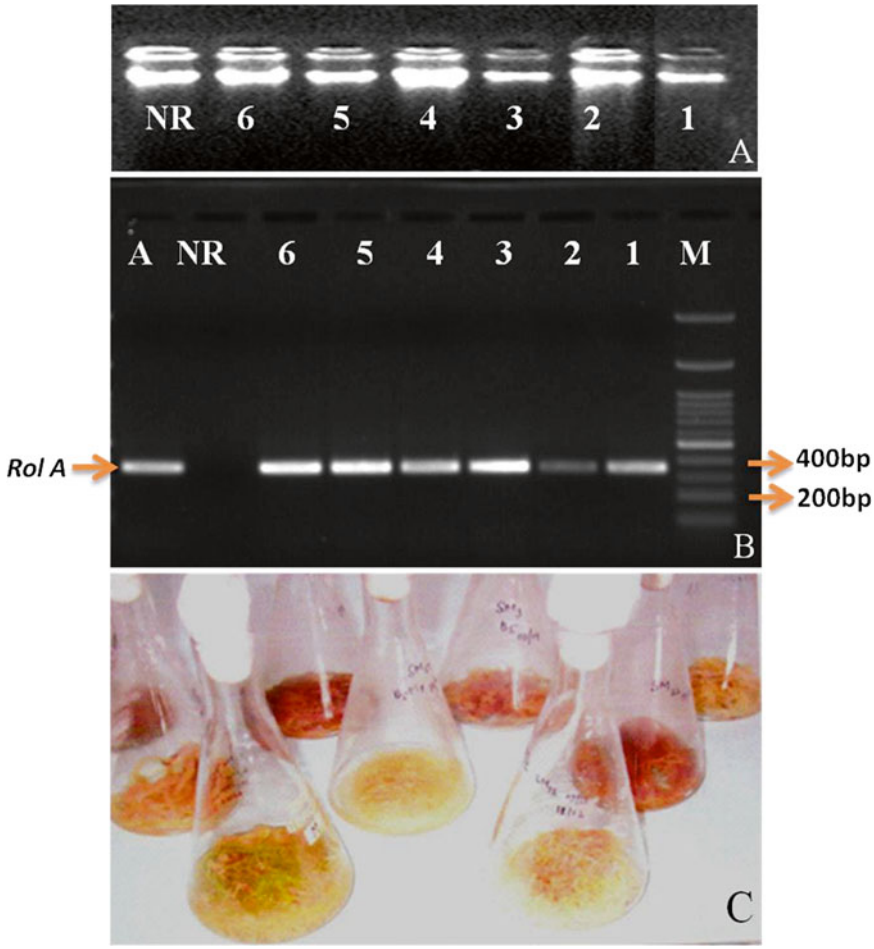

Fig. 3 Quantification of DNA obtained from normal roots and putative hairy root lines 1–6 (**a**); amplification of *Rol* A gene in genomic DNA of *A. rhizogenes* strain A4 (**b**), normal roots (NR) and hairy root lines 1–6. M = marker DNA; hairy root cultures of *R. serpentina* (**c**)

(a) Initial denaturation at 94 °C for 5 min;

(b) 35 cycles each consisting of a denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1 min, amplification at 72 °C for 2 min;

(c) Final extension at 72 °C for 5 min followed by storage of reaction at 4 °C for infinite period.

7. Gel electrophoresis: To prepare agarose gel (0.8–1.2 %), add measured quantity of agarose powder to the required volume of 1× TAE buffer. Boil and gently swirl the solution till agarose get completely melts. Allow it to cool to 45–50 °C. Add EtBr when the temperature of gel remains lukewarm (*see* **Notes 21** and **22**).

8. In a pre-prepared gel casting tray fitted with desired comb, pour the molten agarose gently by avoiding any bubble formation. Allow the gel to solidify. As the gel solidifies carefully remove the comb and submerge the gel tray in the gel reservoir containing 1× TAE buffer.
9. Load the amplified DNA on 1.2 % agarose gel stained with 0.5 μg/ml EtBr in 1× TAE buffer (*see* **Note 23**).
10. Turn on the power supply. Take note of polarities (red-to-red, black-to-black) as the DNA in gel runs from negative to positive end (*see* **Note 24**). Run the loaded gel for 2 h and visualize the bands on a gel documentation system (*see* Fig. 3b).

3.6 Up-Scaling of Hairy Roots in Bioreactor

3.6.1 Bioreactor Setup and Culture

1. The bioreactor used for the present study is of 5 L capacity, modified air lift type. The thick glass culture vessel consists of top driven mechanical agitation through marine blade impeller.
2. The culture vessel is fitted with probes for dissolved oxygen (DO), pH, and temperature to control and optimize the respective culture conditions.
3. In this protocol, an autoclavable nylon mesh (pore size 200 μm) is manually fabricated in the center of the vessel that divides the culture vessel into two halves. In upper half of the vessel, mesh provides anchorage surface to the inoculated and growing tissue and rescues them from getting sunk in the medium during growth phase. The lower half of the vessel consists of a single sparger and a marine blade impeller provided for aeration and agitation respectively (*see* Fig. 4).
4. A hydrophobic membrane filter (Whatman, USA; 0.22 μm) is used to pass the influent air from sparger into the medium.
5. All the parts of the reactors are thoroughly washed and then surface-sterilized with ethyl alcohol prior to assembling the unit and lubricated with silicon grease to make the unit airtight.
6. Pour the liquid B5 medium supplemented with 3 % *w/v* sucrose into the assembled reactor culture vessel.
7. In present protocol, the complete air tight unit filled with growth medium (2.5 L; pH adjusted to 5.86 ± 0.02) is sterilized by autoclaving at 120 °C and 15 lbs pressure for 15 min.
8. The sterile culture vessel is inoculated on a clean laminar bench provided with HEPA filters with 5–6-week-old hairy root cultures (*see* **Note 25**). A fast growing hairy root line (whose transformed nature has been confirmed through *rol* gene amplification) is selected for the cultivation in reactor.
9. The impeller speed is maintained at 75 rpm throughout the culture duration. The experiment is conducted at 25 ± 2 °C in continuous light provided externally by compact fluorescent lamps.

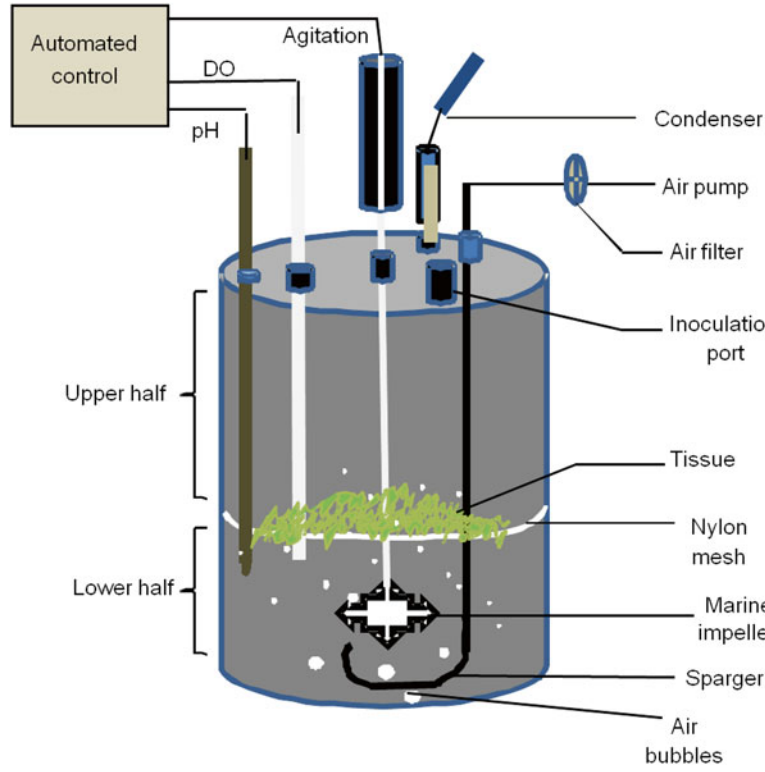

Fig. 4 Diagrammatic presentation of reactor configuration

10. The bioreactor is operated for 11 weeks. During this culture period rapid growth of roots resulted in the formation of root clumps (*see* Fig. 5a; [10]). This has led to the depletion of dissolved oxygen in the medium and an increased oxygen demand. Therefore, the air supply is increased from 0.25 to 1.0 L/min.

3.6.2 Harvesting of Roots

1. Harvest the cultured roots from the reactor vessel (*see* Fig. 5b). Carefully separate the thick clumps of roots from the baffle assembly. Wash the roots in running tap water to remove the traces of medium.
2. Dry the roots on filter paper towel to remove excess water.
3. Dry the harvested roots in hot air oven at 50 °C.

3.7 Chemical Analysis

3.7.1 Extraction of Indole Alkaloids and HPLC Analysis

1. Grind 1.0 g oven-dried *R. serpentina* hairy roots into fine powder.
2. Extract the root powder thrice with (3 × 10 ml) chloroform and methanol (3:1) over night at room temperature.
3. Pool the extracts and dry under vacuum, 417 bars at 40 °C in a Rotavapor (R-144) (Buchi).

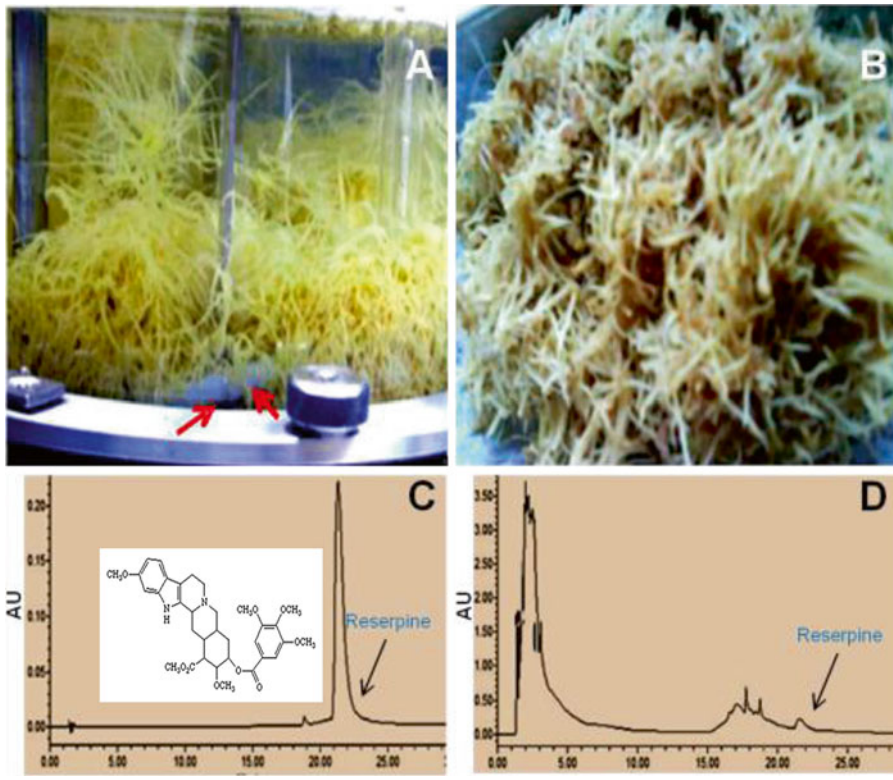

Fig. 5 Growth performance of *R. serpentina* hairy root culture in bioreactor. Dense root growth during culture (**a**), *arrows* indicate the position of nylon mesh; Hairy root biomass harvested after 11 weeks of culture (**b**); HPLC chromatogram of standard reserpine (**c**) and in hairy roots grown in bioreactor (**d**)

4. Redissolve the dried extract in small amount of chloroform and methanol (3:1) and transfer to small glass tube and allow the solvent to evaporate and dry in desiccators and store in refrigerator at 4 °C. This extract can be used for quantitative analysis of reserpine and other indole alkaloids through HPLC.
5. Before analysis redissolve the dried extract in acidic methanol i.e., methanol:HCl—98:2 (*v*/*v*) through ultrasonication. Centrifuge the dissolved extract (equivalent to 1 g/ml on tissue dry weight basis) at 10,000 rpm for 30 min.
6. Stock solution of standard reserpine: dissolve 1 mg standard reserpine in 1 ml of methanol.
7. Filter the sample extract and standard reserpine solutions through HPLC millipore filter paper (0.45 mm).
8. Analyze the samples through an analytical HPLC system. In present method, the HPLC system consists of a LC-20 AD solvent delivery pump, a DGU-20A 5 degasser, a CTO-20A column oven, a 10AF auto sampler, and a SPD-M 20A photodiode array detector.

9. Quantitative estimation of reserpine is done using reversed-phase HPLC gradient method with photodiode array (PDA) detection method.
10. A gradient program (pump A acetonitrile and pump B 0.01 M phosphate buffer containing 0.5 % glacial acetic acid; pH 3.5) is used. The samples are run at a flow rate of 1.0 ml/min at 26 ± 2 °C. A chromolith RP-18e HPLC column, 4.6 × 100 mm, is used for all the analyses.
11. Data acquisition is performed on Lab Solution 3.21 at a wavelength of 254 nm.
12. The reserpine identity in the sample run is confirmed by R_f comparison. The area under respective peak is recorded and used for percent content of the alkaloid in the *R. serpentina* hairy root sample (*see* Fig. 5c, d).

4 Notes

1. Store the stock solutions of basal growth medium in refrigerator. Avoid contamination in stock solutions while using. Always prepare iron stock by dissolving $FeSO_4$ and Na EDTA separately to overcome problem of iron insolubility. Na EDTA is used to get iron in chelated form (Na Fe–ethylene-diamine tetraacetic acid).
2. Prepare the stock solutions of the growth medium using the following equation:

   $N_1V_1 = N_2V_2$

   Where: N_1 = concentration of stock solution

   N_2 = required concentration of the solution in the medium

   V_1 = volume of the stock solution needed

   V_2 = final volume of the medium
3. All glassware should be cleaned with a liquid detergent and thoroughly washed with tap water. Rinse the glassware with double distilled water and dry in hot air oven at 150 °C for 2 h before use.
4. Alternatively, this can be prepared by the Yeast Mannitol Agar/Broth (YMA/YMB) salt mixture which is also commercially available. Adjust the medium's pH at 7.0.
5. As an alternative to sample tubes, test tubes can also be used to prepare bacterial suspension.
6. Multiple shoot cultures of *R. serpentina* can be established and maintained easily in hormone-supplemented MS medium under standard in vitro conditions [8].
7. Add water to make the required volume of 1 × TAE buffer. Maintain the buffer at pH 8.0.

8. Use autoclaved bottles to store the TE and TAE buffers to avoid any contamination during DNA isolation and gel electrophoresis.
9. Prepare 6× loading dye by mixing glycerol (30 % in water), 0.25 % bromophenol blue, and 0.25 % xylene cyanol. Store the dye at –20 °C.
10. Autoclave the PCR tubes prior to use to avoid any contamination during the procedure as contamination at this stage may affect the results.
11. Commercially available PCR master mix is a 2× concentrated mixture of *Taq* DNA polymerase, dNTPs, and all other components required for PCR, except DNA template and primers. Use of readymade PCR master mix reduces contamination and handling errors during pipetting. Individual components can also be mixed separately to prepare PCR reaction mix with Milli-Q water.
12. Morphological variations can be seen in different hairy root clones of *R. serpentina* [10]. Any visibly healthy (free from callus and browning) and fast growing hairy root line can be selected for up-scaling.
13. Careful subculturing and maintenance of bacterial stock culture is required as these cultures get easily contaminated which ultimately lead to the loss of original culture. Ensure the maintenance of original bacterial culture by cross-checking the presence of *rol* gene through PCR.
14. While pricking the leaf explants care should be taken. A slight break in epidermal layer of leaf surface would be sufficient for bacterial cells to infect and transform wounded host cells. Little extra pressure during pricking can result in the death of leaf cells which ultimately leads to an unsuccessful exercise.
15. Dissolve the powder content of a 500 mg Sporidex capsule in 10 ml distilled water (50 mg antibiotic in 1 ml water). Dissolve it and filter through Whatman filter paper. Filter-sterilize the antibiotic solution under laminar flow with the help of syringe filters before mixing in the medium. Store the sterile antibiotic solution in sterile tubes in refrigerator.
16. After the establishment of axenic root cultures, gradually remove the antibiotic from the culture medium.
17. β-mercaptoethanol is added to the extraction buffer just before use. Care should be taken while using the chemical as it is considered toxic.
18. Avoid delay in transferring the powdered tissue to the extraction buffer. Do not let thawing of tissue.
19. Loading of gel for DNA quantification: Mix well the DNA sample (2 μl), loading 6× dye (2 μl), and Milli-Q water (8 μl). Load the 12 μl mixture in the wells of gel carefully.

20. Alternatively, the PCR reaction mix can be prepared by adding individual components. For this, add sample DNA (1 μl), 10× Reaction buffer (2.5 μl), 10 mM dNTPs (1 μl, i.e., 0.25 μl each dNTP), reverse and forward primers (1 μl of 10 pM stock), and *Taq* DNA polymerase (0.2–0.3 μl). Make up the volume to 25 μl by adding Milli-Q water.
21. There can be a significant loss of solution volume due to evaporation of water while melting the agarose. This can cause change in the concentration of agarose as well as will increase the concentration of buffer. The simplest way to get rid of this problem is to replenish the amount of water lost by evaporation.
22. The final concentration of ethidium bromide in gel should be 0.5 μg/ml.
23. Avoid contact of body parts with β-mercaptoethanol and EtBr. These chemicals are highly mutagenic and carcinogenic.
24. Check the gel regularly to prevent the samples from running off the gel.
25. Before inoculating the reactor vessel switch on the UV light for 30 min. and surface-sterilize the laminar bench with alcohol. Wipe the head plate of reactor with alcohol and inoculation port should also be heated to avoid contamination while inoculation. Care should also be taken while aseptic transfer of root tissue as any contact with inoculation port can damage the tissue due to heat.

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Chapter 18

In Vitro Shoot Cultures and Analysis of Steroidal Lactones in *Withania coagulans* (Stocks) Dunal

Rohit Jain, Sumita Kachhwaha, and S.L. Kothari

Abstract

Withania coagulans (Stocks) Dunal (Solanaceae), also known as 'Panir Bandh' is an important medicinal plant that is extensively used as a home remedy for several diseases in the Indian subcontinent. The plant possesses specific steroidal lactones known as withanolides which show high level of pharmaceutical activity against a broad spectrum of microorganisms. Natural propagation of the plant occurs through *See*d but due to unisexual nature of the flowers; chances of *See*d setting are very limited and the plant is on the verge of extinction because of overexploitation and reproductive failure. Plant tissue culture techniques offer opportunities for ex situ conservation and mass multiplication of endangered plant species through micropropagation and also enhancement of in vitro biosynthesis of bioactive compounds. In this chapter we present protocols for the mass multiplication of *W. coagulans*, assessment of clonal fidelity by RAPD, and estimation of bioactive compounds (withanolides) by thin layer chromatography (TLC) and reverse phase HPLC developed in our laboratory.

Key words *Withania coagulans*, Micropropagation, Withanolides, Clonal fidelity, RAPD, HPLC

1 Introduction

W. coagulans (Stocks) Dunal (synonym: *Puneeria coagulans* Stocks), commonly known as Indian rennet, is distributed in the warmer and drier parts of India [1]. The plant is native of the Asia-temperate (Western Asia: Afghanistan) and Asia-tropical regions. It is a rare and endangered medicinal plant restricted to the Northern part of Indian subcontinent and observed only twice in Rajasthan in the vegetative state only [2]. The plant has a long tapering light brown root, it is surmounted by a knotty crown from which spring several shrubby, flexose round branches, 1–5 ft in length (*see* Fig. 1). The whole plant is covered with small branched and pointed white hairs, which give it a hoary appearance. The odor is pungent and disagreeable like horse's urine. The chromosome number is $2n = 48$.

S. Mohan Jain (ed.), *Protocols for In Vitro Cultures and Secondary Metabolite Analysis of Aromatic and Medicinal Plants, Second Edition*, Methods in Molecular Biology, vol. 1391, DOI 10.1007/978-1-4939-3332-7_18,

Fig. 1 *Withania coagulans* plant growing in field

The plant has diverse pharmacological properties, including anti-inflammatory, anticancer, chemoprotective, hepatoprotective, immunomodulatory, antifungal and antibacterial, hypoglycemic, hypolipidemic, antihyperglycemic, cardiovascular, and central nervous system related activities [3–5]. *W. coagulans* is commercially important also because of its ability to coagulate milk due to the presence of an enzyme in the fruits of the plant. The concentrated enzyme can successfully be used in place of animal rennet to prepare soft cheese like cheddar. The essential oil of the plant is found to be active against *Micrococcus pyogenes* var. *aureus* and *Escherichia coli* [6]. This plant is well recognized in the native system of medicine for the treatment of ulcers, rheumatism, dropsy, consumption, sensile debility, asthma, biliousness, strangury, dyspepsia, flatulent colic, and other intestinal infections. In the Indian subcontinent, the fruits are used as blood purifier and the twigs of the plant are chewed for cleaning teeth [7].

The chemistry of *W. coagulans* has been comprehensively studied and several groups of chemical constituents such as steroidal lactones, alkaloids, flavonoids, and tannin have been identified, extracted, and isolated [8, 9]. The major chemical constituents of the plant, withanolides, are mainly localized in leaves and roots [9]. The withanolides are a group of naturally occurring C-28 steroidal lactones built on an intact or rearranged ergostane framework, in which C-22 and C-26 are appropriately oxidized to form a six-membered lactone ring. The basic structure is designated as the withanolide skeleton (*see* Fig. 2) [10–12].

The withanolide skeleton is a 22-hydroxyergostan-26-oic acid-26, 22-lactone structure. There are many structural alternatives of withanolides with modifications either of the carbocyclic skeleton or the side chain and these have been described as modified withanolides or ergostane type steroids related to withanolides. The characteristic feature of withanolides and ergostane type steroids is one C8 or C9-side chain with a lactone (either six-membered or five-membered) or lactol ring, fused with the carbocyclic part of

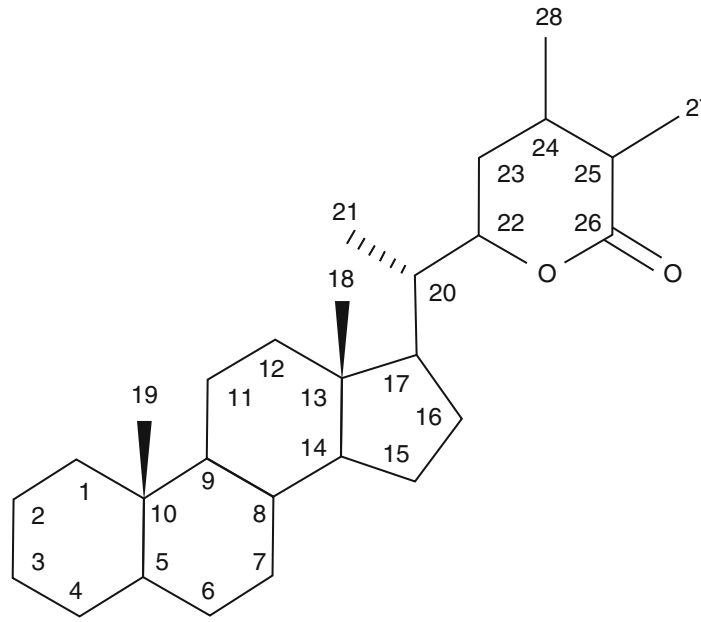

Fig. 2 Basic withanolide skeleton

the molecule through a carbon–carbon bond or through an oxygen bridge. Appropriate oxygen substituents may lead to formation of new bonds, aromatization of rings, and many other kinds of rearrangements resulting in compounds with novel structures [11, 13, 14].

According to Mishra et al. [15] *W. coagulans* is phytochemically unique in predominantly producing the neuroactive metabolite withanolide A in aerial parts of the plant, implying relatively easier and economical harvest of the withanolide. Naturally, *W. coagulans* propagates through seeds but seed setting is limited due to unisexual nature of flowers. Reproductive failure and overexploitation have rendered the species highly vulnerable to complete extinction [16]. Therefore, there is an urgent need of ex situ conservation of this important medicinal plant through micropropagation techniques.

2 Materials

2.1 Media Preparation: (See Notes 1–3)

1. Stock solutions of Murashige and Skoog (MS) medium (Table 1).
2. Agar.
3. Beakers, measuring cylinder.
4. 1 N HCl/1 N NaOH.
5. Stock solutions of different plant growth regulators.
6. Sucrose.

Table 1
Composition of MS basal medium

Nutrient		Weight in stock solution (mg)	Final concentration in medium	
			mg/L	mM
A	NH_4NO_3	33,000	1650	20.6
B	KNO_3	38,000	1900	18.8
C	KH_2PO_4	6800	170	1.25
	KI	33.2	0.83	5×10^{-3}
	H_3BO_3	248	6.2	0.1
D	$Na_2MoO_4 \cdot 2H_2O$	50	0.25	1.03×10^{-3}
	$CoCl_2 \cdot 6H_2O$	5	0.025	0.11×10^{-3}
E	$CaCl_2 \cdot 2H_2O$	17,600	440	2.99
F	$MgSO_4 \cdot 7H_2O$	14,800	370	1.5
	$MnSO_4 \cdot H_2O$	676	16.9	0.1
	$ZnSO_4 \cdot 7H_2O$	344	8.6	29.91×10^{-3}
G	$CuSO_4 \cdot 5H_2O$	5	0.025	0.1×10^{-3}
H	$FeSO_4 \cdot 7H_2O$	1114	27.8	0.1
	Na_2EDTA	1490	37.3	0.1
I	Thiamine–HCl	10	0.1	0.3×10^{-3}
	Pyridoxine–HCl	50	0.5	2.43×10^{-3}
	Nicotinic acid	50	0.5	4.06×10^{-3}
	Glycine	200	2	26.64×10^{-3}
J	Myo-inositol	2000	100	0.56

2.2 Surface Sterilization

1. "Extran" liquid detergent.
2. Mercuric chloride.
3. Distilled water.

2.3 Analysis of Clonal Fidelity by RAPD

2.3.1 Genomic DNA Isolation

1. Leaf samples: 2 g fresh leaves.
2. Cetyl trimethyl ammonium bromide (CTAB) extraction buffer (100 ml): 2 % (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris–HCl pH 8.0 (*see* **Note 4**).
3. Tris EDTA (TE) buffer (100 ml): 10 mM Tris–HCl (pH 8.0), 1 mM EDTA (pH 8.0) (*see* **Note 5**).
4. Ice-cold isopropanol.

5. Chloroform–isoamyl alcohol (24:1 v/v).
6. Sodium acetate (3.0 M) pH 5.2 (Adjust pH with glacial acetic acid).
7. Ethanol (100 % and 70 %).
8. RNAse A: 10 mg/ml (*see* **Note 6**).
9. Milli-Q Water.

2.3.2 PCR for DNA Amplification

1. Template DNA.
2. PCR tubes (*see* **Note 7**).
3. dNTP mix.
4. RAPD primer.
5. Taq DNA Polymerase.
6. Taq Buffer solution (with $MgCl_2$).
7. Milli-Q Water.

2.3.3 Gel Electrophoresis

1. Gel electrophoresis setup unit.
2. Agarose (1.2 % w/v) (*see* **Note 8**).
3. TAE buffer (50×) (*see* **Note 9**).
4. Ethidium bromide (1 % w/v in Milli-Q water) (*see* **Note 10**).
5. Gel loading dye (6×) (*see* **Note 11**).

2.4 Analysis of Secondary Metabolites (Withanolides) by TLC and HPLC

2.4.1 Secondary Metabolite Extraction

1. Plant material (shoot buds and leaves, 2 g).
2. Mortar and pestle.
3. Methanol (25 % (v/v) in water).
4. Separatory funnel.
5. *n*-hexane.
6. Chloroform.

2.4.2 Thin Layer Chromatography

1. TLC plates (precoated Silica Gel G-60 plates).
2. Solvent system (*see* **Note 12**).
3. Anisaldehyde reagent (*see* **Note 13**).
4. Standard withanolides.

2.4.3 HPLC Analysis

1. Methanol (HPLC grade).
2. Deionized water (HPLC grade).
3. Acetic acid (HPLC grade).
4. Reverse-phase (RP) column (Eclipse XDB c-18, particle size 1.8 μm, 4.5 mm × 250 mm).
5. Authentic withanolide standards.

3 Methods

3.1 Micropropagation of W. coagulans

Basal medium used in the present study is MS (Murashige and Skoog, 1962) medium [17].

3.1.1 Preparation of Medium

1. Prepare stock solutions of various inorganic and organic nutrients of MS medium (Table 1). To prepare stock solutions of growth regulators, dissolve auxins (IAA, IBA, PAA, NAA) in few drops of absolute alcohol and cytokinins (BA, Kn) in few drops of 1 N HCl, and make up the final volume by adding distilled water (20 mg/100 ml) (*see* **Note 1**).
2. Prepare the medium by mixing all the mineral nutrients, sucrose (3 %, w/v), and growth regulators in appropriate quantities (*see* **Notes 2** and **3**).
3. Adjust the pH to 5.8 by 1 N HCl/NaOH.
4. Weigh appropriate amount of agar (0.9 %) and mix in the medium followed by heating in a microwave oven until the agar is dissolved (*see* **Note 8**).
5. Stir the medium and dispense approximately 40 ml or 20 ml into 100 ml Erlenmeyer flask or culture tubes, respectively.
6. Plug the culture vessels with non-absorbent cotton and wrap with paper prior to autoclaving at 121 °C and 1.06 kg/cm^2 pressure for 20 min.
7. Use the autoclaved medium after solidification.

3.1.2 Aseptic Manipulations

1. Autoclave all other accessories such as conical flasks, measuring cylinders, reagent bottles, Petri plates, forceps, and scalpel blades.
2. Measured volume of distilled water required for surface sterilization of field explants should also be sterilized by autoclaving.
3. Clean the laminar air flow cabinet with spirit and put all the required accessories along with the culture vessels containing medium and distilled water.
4. Irradiate the cabinet with UV rays for 25–30 min.
5. After irradiation open the cabinet and start the surface sterilization process under a continuous airflow (*see* **Note 14**).

3.1.3 Surface Sterilization, Explant Culture, and Incubation

1. Excise the explants from mature plants and wash with liquid detergent "Extran" (5 %, v/v) for 5–10 min and rinse 4–5 times thoroughly with sterile distilled water.
2. For surface sterilization dip the explants in mercuric chloride (0.1 %, w/v) solution in laminar airflow cabinet for 3 min and wash with distilled water 3–4 times.

3. Inoculate the explants in the medium with the help of forceps and scalpel on a spirit lamp/burner in the cabinet (*see* **Note 15**).
4. Place the nodal segments having axillary buds and shoot tips with apical meristem separately in the culture vessel containing shoot induction medium (*see* **Note 2a**) in such a way that the cut end of the explant remains in contact with the medium.
5. Inoculate the horizontally excised leaf explants of appropriate size (2–3 cm long), with petiolar end abaxially on the shoot induction medium (*see* **Note 2b**).
6. Incubate the cultures in the growth chamber at 26 ± 1 °C under 16/8 h photoperiod with 25 μmol/m^2/s photosynthetic photon flux density provided by white fluorescent tubes.

3.1.4 Subculture of Shoot Buds

1. Within 3 weeks of incubation bud break occurs from the nodal segments and shoot tips (*see* Fig. 3a, b).
2. Transfer the primary shoots to fresh medium after 4–5 weeks of culture incubation.
3. Excise the shoot buds induced from the explant into groups each having 3–4 shoot buds.
4. Inoculate these clusters on the first stage shoot proliferation medium (*see* **Note 2c**).
5. Within 3–4 weeks the multiplication of shoot buds occurs (*see* Fig. 3c). Transfer the developed shoots on second stage shoot proliferation medium for further growth and elongation of shoots (*see* **Note 2d**).
6. Within 2 weeks of culture elongation occurs in the shoots (*see* Fig. 3d).

3.1.5 Rooting, Hardening, and Field Transfer of Plantlets

1. Excise and inoculate the elongated shoots (>3 cm) on first step rooting medium (R1) for pulse treatment for 7 days (*see* **Note 3a**).
2. Transfer the shoots on second step rooting medium (R2) (*see* **Note 3b**).
3. The roots emerge from the shoot after 3 weeks of culture (*see* Fig. 3e).
4. Carefully take out the regenerated plantlets with well-developed shoot and root systems and wash thoroughly with tap water to remove agar clinging to roots.
5. Prepare earthen pots containing mixture of garden soil and vermiculite (1:1) and transfer the plantlets in the pots followed by transfer to green house at 26 ± 1 °C temperature and 85 % humidity.
6. After acclimatization transfer the plants to field conditions (*see* Fig. 3f).

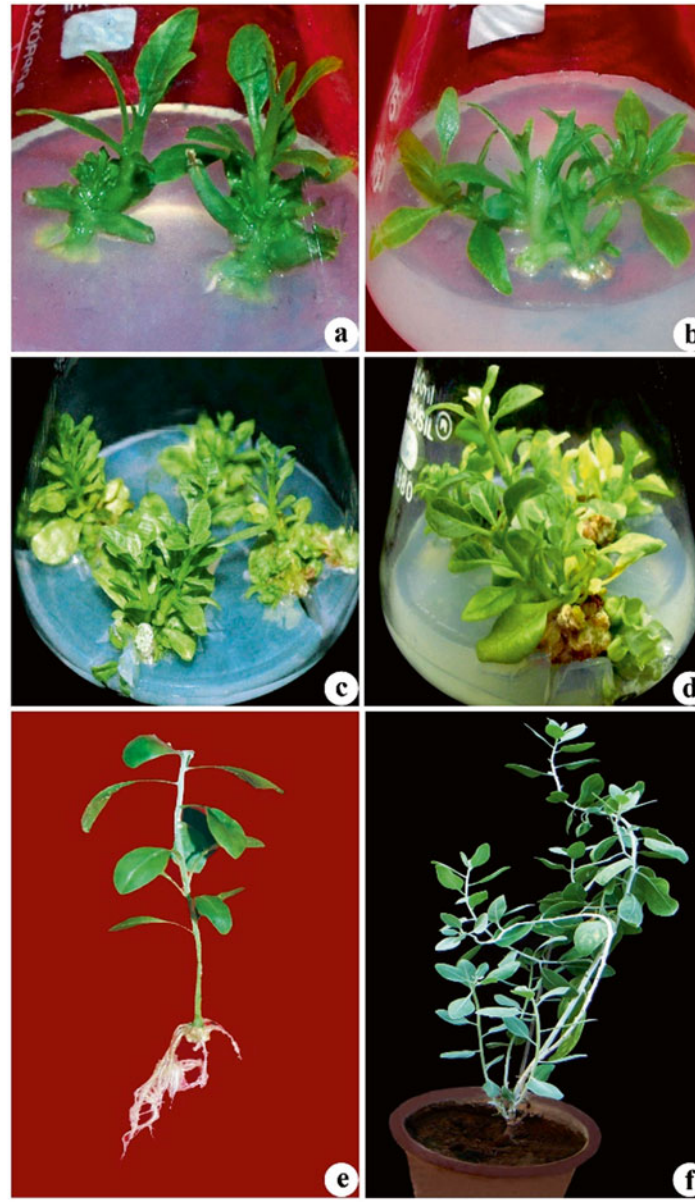

Fig. 3 Morphogenic response of *Withania coagulans* cultured on MS medium supplemented with various growth regulators. (**a**) Bud break from nodal explant, (**b**) Bud break from shoot tip explant, (**c**) Multiplication response of shoot buds, (**d**) Elongation of shoot buds, (**e**) Rooting response of in vitro-regenerated shoots, (**f**) Field-transferred plant

3.2 Analysis of Clonal Fidelity by RAPD

3.2.1 Genomic DNA Isolation from Mother Plant and Tissue Culture Raised Plantlets

CTAB method of Doyle and Doyle [18] as described below is used to isolate genomic DNA.

1. Collect young leaf samples from at least 15–20 tissue culture raised plants growing in the field condition.
2. Grind 100 mg of frozen plant material to fine powder with mortar and pestle using liquid nitrogen.
3. Transfer the powder immediately to 5 ml of pre-warmed (65 °C) CTAB extraction buffer and 2 μl RNase A.
4. Incubate the mixture for 1 h at 65 °C in a water bath and mix gently after every 10 min.
5. Add equal volume of chloroform–isoamyl alcohol (24:1, v/v) to the mixture and centrifuge the resultant slurry at 12,000 rpm (18,500 × *g*) at 4 °C for 10 min.
6. Separate the upper aqueous phase after centrifugation and transfer to test tubes.
7. Add 2 μl RNase A to each tube at this step and incubate at room temperature for 30 min.
8. To each test tube, add 0.6 volume of ice-cold isopropanol to the aqueous phase followed by gentle but thorough mixing by inverting the tubes several times. At this stage, the DNA-CTAB complex is precipitated out. Centrifuge the precipitate containing DNA by centrifugation at 6000 rpm at 4 °C for 10 min.
9. Wash the pellet with 70 % ethanol by gentle agitation followed by centrifugation (10 min, 5000 rpm, 4 °C).
10. Discard the ethanol and allow to air-dry the pellet obtained after centrifugation and add 100 μl of TE buffer and leave overnight for dissolving the DNA.
11. The concentration of purified DNA in the solution is estimated on 0.8 % (w/v) agarose gel electrophoresis by comparing with uncut lambda DNA of known concentration.

3.2.2 Molecular Analysis Using RAPD

1. PCR amplification: Prepare the PCR reaction mixture on ice bath in 25 μl volume by mixing all the components given in Table 2.
2. Spin the PCR tube containing reaction mixture for few seconds and place the tubes in the thermal cycler for PCR amplification. Set the PCR amplification conditions as follows:
   Initial denaturation at 94 °C for 4 min followed by 40 cycles of 94 °C for 45 s, 37 °C for 45 s and 72 °C for 2 min, and a final extension at 72 °C for 10 min.
3. Gel electrophoresis: Prepare the 1.2 % (w/v) agarose gel (100 ml) using l× TAE gel running buffer.

Table 2
Composition of PCR reaction mixture

Components	Volume
Taq buffer (with $MgCl_2$)	2.5 µl
dNTP mix	0.5 µl
DNA	1 µl (25 ng/µl)
Primer	2 µl
Taq polymerase	0.25 µl (3 Units/µl)
Molecular water	18.75 µl

4. Mark the height of the level of buffer with a mark on the outside of the flask. Boil the solution in microwave to dissolve the agarose completely; swirl flask occasionally to mix. Check the height of the liquid level in the flask. If necessary, add sterile distilled water to bring the liquid back to the original level.
5. Allow the dissolved agarose to cool to about 45–50 °C and add 2–3 µl EtBr solution (1 mg/ml) and mix.
6. Pour the agarose into a gel casting tray after sealing properly the ends and placing appropriate comb to make the gel wells. Allow the gel to solidify. This can take 15–20 min, depending on the agarose concentration.
7. Once the gel is solidified, remove the comb from the gel. To aid in removing the comb, pour a small amount of 1× TAE running buffer around the comb before loosening the comb (*see* **Note 16**).
8. Transfer the gel on the gel tray to the gel tank. Add 1× TAE running buffer to immerse the gel completely (*see* **Note 9**).
9. Prepare DNA samples in eppendorf tubes to be loaded on the gel. Add 1.0 µl of gel loading buffer for every 5 µl of DNA sample. Mix the sample and loading buffer completely.
10. Carefully load the samples in the gel wells. In the very first well of the gel load the appropriate DNA molecular marker (0.1 Kb or 1.0 Kb). Record which samples are loaded in which lane.
11. Connect the apparatus to the power pack and allow electrophoresis to proceed at 5 V/cm of the gel.
12. When tracing dye reaches the end of the gel, turn-off the power, remove the gel from the casting tray, and visualize the bands on gel documentation system.
13. Analyze the RAPD banding pattern for monomorphic and polymorphic bands present in the gel and the size of the bands by comparing with the 0.1 Kb and 1 Kb DNA molecular weight marker (*see* Fig. 4a, b).

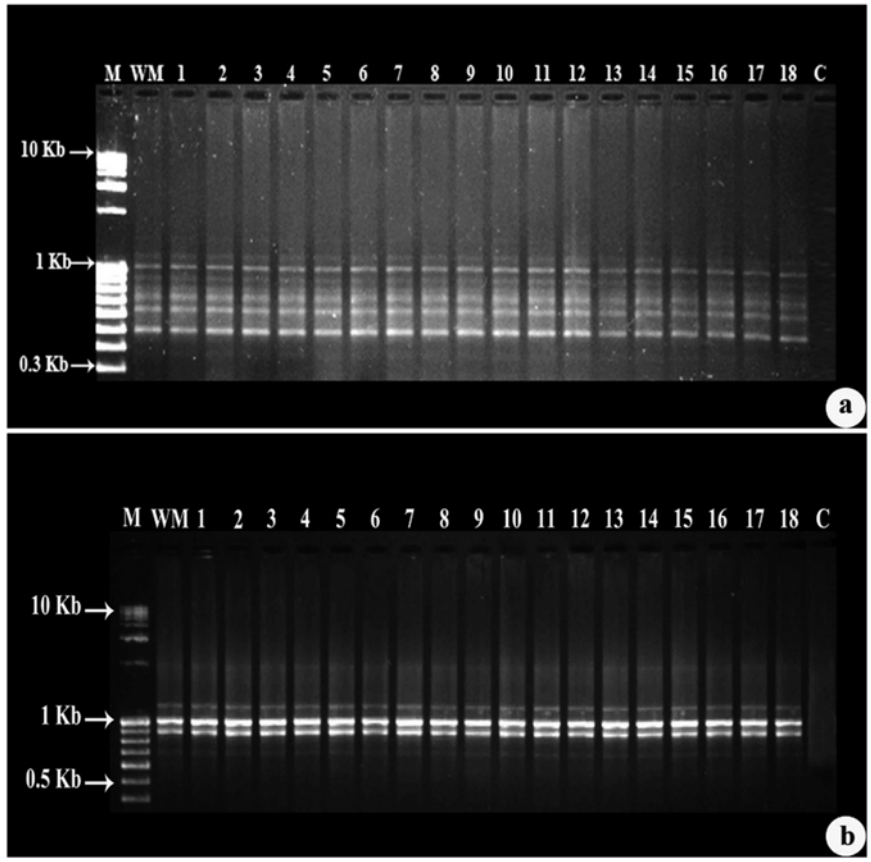

Fig. 4 Agarose gel electrophoresis of RAPD fragments. (**a**) Banding profile amplified by RAPD primer OPT-8, (**b**) Banding profile amplified by RAPD primer OPF-6

3.3 Estimation of Secondary Metabolites by TLC and HPLC

3.3.1 Extraction of Withanolides from W. coagulans

1. Extract 2 g freshly harvested plant material from *W. coagulans* three times with 20 ml extraction system containing 25 % methanol (v/v, in distilled water) in Erlenmeyer flask on a platform shaker at 30–40 rpm for 1 h in each extraction (*see* **Note 17**).
2. Filter the solvent composition and recover the extract. Pool all the three filtrates and put in the separating funnel for liquid–liquid partition chromatography.
3. At first, treat the extract with equal amount of n-hexane in the separating funnel to remove the pigments and fatty acids (*see* **Note 18**).
4. Discard the lower *n*-hexane layer and repeat the process three times.
5. Treat the defatted and depigmented extract with chloroform (equal volume, three times) to recover withanolides in the chloroform layer.
6. Pool the chloroform fractions of each extract and allow to dry at room temperature.

7. Dissolve the residue in known volume (1 ml) of HPLC grade methanol and filter through a 0.45 μm membrane filter prior to TLC and HPLC.

3.3.2 Qualitative Analysis of Withanolides by TLC

Qualitative withanolide profiling is done through TLC as described by Jain et al. [19].

1. Fill the chromatography chamber with the solvent system and cover the chamber with glass lid for saturation (*see* **Note 12**).
2. Meanwhile load 10 μl sample on the preactivated silica gel G-60 plate along with the authenticated withanolide standards (*see* **Note 19**).
3. Place the plate in the saturated chamber and allow the solvent to run. Remove the plate after completion of the solvent run on the plate and air-dry the plate.
4. Develop the plate by spraying anisaldehyde reagent followed by heating at 110 °C (*see* **Note 13**).
5. Mark the spots visible after development with reference to the standard withanolides.

3.3.3 Quanitative Analysis of Withanolides by HPLC

Quanitative withanolide profiling was done through HPLC as described by Jain et al. [19].

1. In the present study the quantification of extracts is analyzed through an analytical HPLC system having solvent reservoir bottles, solvent delivery pumps, degasser, autosampler, column oven, reverse phase column (Eclipse XDB c-18, particle size 1.8 μm, 4.5 mm × 250 mm), and UV-Diode Array Detector.
2. A gradient program with HPLC grade water (pump A) and HPLC grade methanol (pump B) each containing 0.1 % acetic acid is used (*see* **Note 20**).
3. Set the solvent gradient as A:B, 60:40 to 25:75, 0 to 45 min; 10:90, 45 to 60 min at a flow rate of 0.6 ml/min with a reference wavelength of 227 nm.
4. Load the sample vial in the autosampler tray and set the injection volume to 10 μl in the program.
5. Set the column temperature at 27 °C during the run.
6. Use the authenticated withanolide standards to ascertain their discrete resolution from each other under these conditions.
7. Data acquisition is done on the Agilent Chemstation 2.0. For computation of withanolide concentration in the samples prepare a calibration curve of concentration versus detector response (peak area) and regression equation ($Y = mX + C$) for each standard separately, using different concentrations (50–1000 ng/μl) of standard solution (1.0 mg/ml) in HPLC grade methanol.

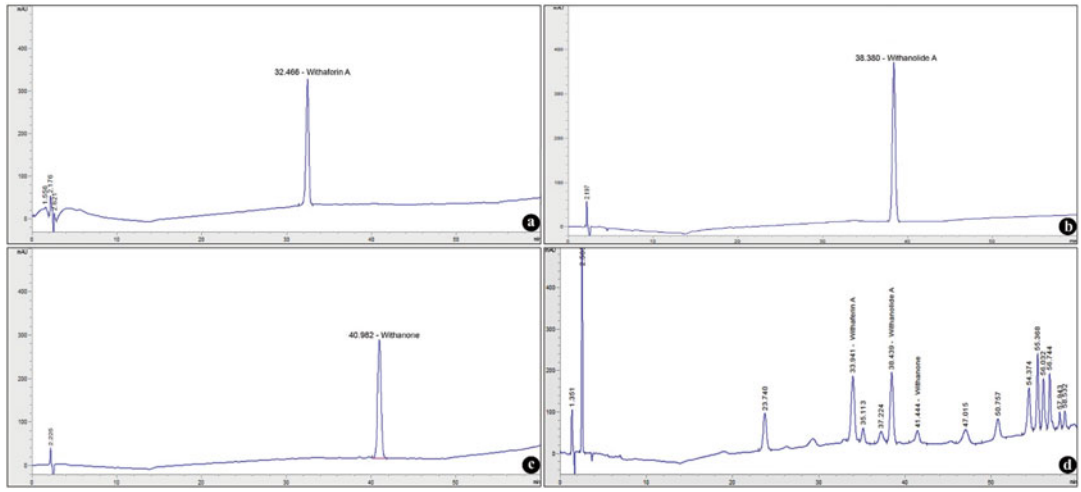

Fig. 5 HPLC chromatogram of shoot cultures of *W. coagulans*. (**a**) Standard withaferin A, (**b**) Standard withanolide A, (**c**) Standard withanone, (**d**) Chromatogram of extract isolated from shoot cultures of *W. coagulans*

8. Calculate the concentration of different withanolides present in the extract by using the detector response of HPLC chromatogram for each withanolide peak at the same retention time as in the standard withanolide chromatogram and the regression equation (*see* Fig. 5).

4 Notes

1. Label all the stocks and store in refrigerator. Use stock solutions within a week of preparation. Mix thermostable chemicals with other ingredients of the media before autoclaving, while filter-sterilized thermolabile chemicals are added to the autoclaved media in the laminar air flow cabinet.
2. Use following concentrations and combinations of PGRs in the medium for shoot bud induction and proliferation.
   (a) Shoot induction medium for shoot tip and nodal segments: N-6-benzyladenine (BA) 2.2 μM in the MS medium.
   (b) Adventitious shoot induction medium for leaf explant: Combination of BA 22.2 μM and kinetin (Kn) 2.3 μM in MS medium.
   (c) First stage shoot proliferation medium: Combination of BA 2.2 μM and Kn 2.3 μM in MS medium.
   (d) Second stage shoot proliferation medium: Combination of BA 2.2 μM, Kn 2.3 μM, and phloroglucinol (PG) 3.9 μM in MS medium.

3. Use following concentrations and combinations of PGRs in the medium for rooting.
   (a) First step rooting medium (R1): choline chloride (CC) 71.6 μM, PG 3.9 μM in half-strength MS medium.
   (b) Second step rooting medium (R2): indole-3-butyric acid (IBA) 1.2 μM, phenylacetic acid (PAA) 3.6 μM, CC (71.6 μM), and PG (3.9 μM) in half-strength MS medium.
4. Autoclave Tris–HCl, NaCl, and EDTA. CTAB should be added after autoclaving and extraction buffer should be pre-heated (65 °C) before using.
5. Dissolve and make up to 100 ml with distilled water, autoclave and store at 4 °C.
6. Dissolve RNase A in TE and boil it for 15 min at 100 °C to destroy DNase and store at –20 °C.
7. Autoclave the PCR tubes to avoid any contamination during the reaction.
8. When using the microwave, be sure to use cotton gloves (temperature proof) to pick up hot flasks. Do not swirl a flask to mix the contents of the flask until the flask has cooled briefly. Point the mouth of the flask away from you. Swirling an overheated flask may cause the liquid inside to "boil out" of the hot flask.
9. To prepare 50× TAE, mix Tris 242 g, glacial acetic acid 57.1 ml, 0.5 M EDTA pH 8.0 in 100 ml distilled water. Make up the volume to 1000 ml and autoclave. Add 2 ml of 50× TAE in 98 ml of distilled water to prepare 1× gel running buffer.
10. Always wear nitrile gloves while handling EtBr as it is carcinogenic.
11. Mix 30 % (v/v) glycerol in water and add 0.25 % (w/v) bromophenol blue and 0.25 % (w/v) xylene cyanol. Store the dye at –20 °C.
12. Prepare the solvent system by mixing chloroform, ethyl acetate, methanol, and toluene (74:4:8:30; v/v) in a chromatography chamber and cover the chamber with lid for saturation.
13. 250 μl anisaldehyde in a mixture of 20 ml acetone, 80 ml water, and 10 ml 60 % perchloric acid.
14. Make sure that the UV light is turned off before opening the cabinet.
15. The accessories should be properly heated on the spirit lamp and hands should also be properly wiped with spirit. Special care must be taken to avoid the tissue being touched by hot forceps/scalpels as it reduces the viability of the tissue.
16. When removing the comb, do not disturb the wells since deformed wells may affect the proper mobility of the DNA.

17. Filter the extract and collect the filtrate in a separate flask. Extract again with fresh extraction system (25 % methanol (v/v, in water).
18. Shake vigorously the separating funnel containing extract and n-hexane and remove the vapors by inverting the funnel and opening the tap.
19. Let the sample dot dry after each drop of extract is added. The drying keeps the pigment dot from spreading out too much.
20. All the solvents to be used for HPLC must be filtered by the 0.45 μm membrane filter to avoid any contamination during the run.

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Chapter 19

In Vitro Propagation of *Cannabis sativa* L. and Evaluation of Regenerated Plants for Genetic Fidelity and Cannabinoids Content for Quality Assurance

Hemant Lata, Suman Chandra, Ikhlas A. Khan, and Mahmoud A. ElSohly

Abstract

Cannabis sativa L. (Marijuana; Cannabaceae), one of the oldest medicinal plants in the world, has been used throughout history for fiber, food, as well as for its psychoactive properties. The dioecious and allogamous nature of *C. sativa* is the major constraint to maintain the consistency in chemical profile and overall efficacy if grown from seed. Therefore, the present optimized in vitro propagation protocol of the selected elite germplasm via direct organogenesis and quality assurance protocols using genetic and chemical profiling provide an ideal pathway for ensuring the efficacy of micropropagated *Cannabis sativa* germplasm. A high frequency shoot organogenesis of *C. sativa* was obtained from nodal segments in 0.5 μM thidiazuron medium and 95 % in vitro rhizogenesis is obtained on half-strength MS medium supplemented with 500 mg/L activated charcoal and 2.5 μM indole-3-butyric acid. Inter Simple Sequence Repeats (ISSR) and Gas Chromatography-Flame Ionization Detection (GC-FID) are successfully used to monitor the genetic stability in micropropagated plants up to 30 passages in culture and hardened in soil for 8 months.

Key words *Cannabis sativa* L., Micropropagation, Nodal explants, Genetic fidelity, ISSR markers, GC-FID

1 Introduction

Cannabis sativa L. is an open pollinated crop belonging to the family Cannabaceae. It is an important medicinal plant well known for its pharmacologic and therapeutic potency that contains a unique class of terpenophenolic compounds (the cannabinoids), which accumulates mainly in the glandular trichomes of the female plant [1]. At present, 104 cannabinoids have been identified from this plant but most of the biological and pharmacological activities are attributed to the psychoactive compound, Δ^9-tetrahydrocannabinol (THC) [2]. Medicinally, THC possesses analgesic, anti-inflammatory, appetite stimulant, and antiemetic properties, making this cannabinoid a very promising drug for therapeutic purposes [3].

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Due to the dioecious (male and female flowers on different plants) and allogamous (cross fertilization) nature of *C. sativa*, it is difficult to maintain the consistency in its chemical profile and cannabinoid content if grown from seeds. Since the seeds of *C. sativa* are highly heterozygous and are thus of limited interest for the conservation of particular genotypes, the use of plant tissue culture techniques offer advantages for multiplication and large-scale production of true-to-type *Cannabis* plants. Several medicinal plants such as *Rauvolfia tetraphylla* [4]; *Picrorhiza kurroa* [5]; *Withania somnifera* [6]; *Pterocarpus marsupium* [7]; *Acacia sinuata* [8]; *Cimicifuga racemosa* [9], and *Veronica anagallis-aquatica* [10] have been multiplied through various strategies of micropropagation. In addition to being an alternative means of plant improvement, micropropagation techniques can also potentially overcome many factors limiting traditional approaches to *C. sativa* improvement [11]. Research on in vitro regeneration of Cannabis has resulted in several protocols [12–16]; however, considerable variation has been reported in the response of cultures and in the morphogenic pathway. Since our goal is to develop a secure and stable *in vitro* clonal repository of elite medicinal plants germplasm that will assure future availability of desirable pharmacological active chemotypes, the present protocol has been developed covering the following objectives: (a) propagation of the quality plant material that will allow large-scale clonal production, and (b) comparison of micropropagated plants with the mother plant for consistency in terms of genetic and chemical profiles. This approach of propagation of *C. sativa* would provide a rapid and reliable system for the production of large number of genetically uniform disease-free plantlets and would be a promising tool for commercial level propagation of high-yielding elite varieties for the pharmaceutical use.

2 Materials

2.1 Equipment and Apparatus

1. Autoclaves.
2. Laminar flow hoods (*see* **Notes 1** and **2**).
3. Water demineralizer.
4. Water distilling unit.
5. Refrigerator.
6. Analytical balance to weigh from 0.1 or 0.01 mg to a few grams.
7. pH meter.
8. Stirrer with hot plate.
9. Water bath.
10. Vacuum pump.
11. Filter units, 0.22 μm.

Table 1
Plant growth regulators used

Plant growth regulators	Solubility	Sterilization	Storage
Auxin			
Indole-3-butyric acid (IBA)	1 N NaOH	Filter sterilize	4 °C
Cytokinin			
Thidiazuron (TDZ)	1 N NaOH	Filter sterilize	4 °C

2.2 Media Preparation

1. Murashige and Skoog (MS) Basal Salts Mixture.
2. Phytohormones (*see* Table 1).
3. pH buffers, 4.0, 7.0, and 10.0.
4. 1 N NaOH.
5. 1 N HCl.
6. Sterile beakers, 250 ml and 1000 ml (*see* **Note 3**).
7. Measuring cylinders 100, 250, and 1000 ml.
8. Micropipettes 20–1000 μl.

2.3 Sterilization and Establishment of In Vitro Cultures

1. 0.1 % Tween 20.
2. 0.5 % NaOCl (15 % w/v bleach).
3. Sterile distilled water.
4. Stainless steel forceps 8–12″.
5. Razor blade # 10 and scalpel.
6. Sterile petri dishes, 100 × 25 mm.
7. Culture sealant, Parafilm.
8. Culture vessels, baby food jars with Magenta B caps (4 cm diameter × 9.5 cm high).
9. Thermocol cups.
10. Coco Natural growth medium.
11. Sterile potting mix—Fertilome (Canna Continental).
12. Plastic pots 2″, 5″, 10″.
13. Incubator/tissue culture room with light and temperature control.

2.4 Quality Assurance of Micropropagated Plants: Assessing Genetic Fidelity

2.4.1 Isolation of Plant Genomic DNA

1. Eppendorf tubes 2 ml.
2. Mixer Mill MM 2000.
3. DNeasy plant mini kit.
4. NanoDrop 1000 spectrophotometer.
5. Liquid nitrogen.

Table 2
Nucleotides sequences of primers used for ISSR analysis

Primer	Sequence
UBC 807	5′-AGAGAGAGAGAGAGAGT-3′
UBC 808	5′-AGAGAGAGAGAGAGAGC-3′
UBC 811	5′-GAGAGAGAGAGAGAGAC-3′
UBC 812	5′-GAGAGAGAGAGAGAGAC-3′
UBC 817	5′-CACACACACACACACAA-3′
UBC 826	5′-ACACACACACACACACC-3′
UBC 834	5′-AGAGAGAGAGAGAGAGYT-3′
(GGC)6W	5′-GGCGGCGGCGGCGGCGGCW-3′
(AAG)6Y	5′-AAGAAGAAGAAGAAGAAGY-3′
(GGAT)4H	5′-GGATGGATGGATGGATH-3′
(GGGGT)3M	5′-GGGGTGGGGTGGGGTM-3′
UBC 836	5′-AGAGAGAGAGAGAGAGYA-3′
UBC 842	5′-GAGAGAGAGAGAGAGAYG-3′
UBC 845	5′-CTCTCTCTCTCTCTCTRG-3′

6. 5 mg/ml ethidium bromide (EB) stock in water.
7. Agarose.
8. 6× loading dye.

2.4.2 PCR Amplification

1. Template DNA.
2. Taq DNA polymerase.
3. Deoxyribonucleoside triphosphates (dNTPs) mix (dATP; dTTP and dGTP; dCTP).
4. 10× polymerase buffer.
5. 1.5 mm $MgCl_2$.
6. Primers (*see* Table 2).
7. Polymerase chain reaction (PCR) tubes.
8. Milli-Q water.
9. Ice.
10. DNA ladder 1 Kb plus.
11. 2 % TAE agarose gel.
12. Bio-Rad gel imaging system.

2.4.3 Assessment of the Cannabinoids Profile and Content

1. Chloroform.
2. Methanol.
3. 4-androstene-3,17-dione.
4. Erlenmeyer flask 25 ml.
5. Metal sieve No. 14 (opening 0.0555 in.).
6. Screw cap vial, 3.7 ml.
7. GC vials 2 ml.
8. Pipettes 5–10 ml.
9. Micropipettes.

2.4.4 Gas Chromatographic Analysis

1. Column: DB-1, 15 m × 0.25 mm, with 0.25 μm film thickness.
2. Helium gas as carrier gas.
3. Varian CP-3380 Gas chromatograph equipped with a Varian CP-8400 automatic liquid sampler.

3 Methods

Different steps involved in the micropropagation of *Cannabis sativa* through direct organogenesis are shown in Figs. 1 and 2.

3.1 Media Preparation

1. Prepare the Murashige and Skoog's medium [17] as per the directions on the bottle.
2. Add 3 % (w/v) sucrose. Make the final volume of culture medium as required. Set the pH 5.7.
3. Add 0.8 % (w/v) type E agar.
4. Autoclave the media at 121 °C at 105 kPa for 30–45 min (*see* **Notes 4, 5** and **6**).
5. Cool the medium and swirl the solution as it cools.
6. Add Murashige and Skoog vitamins.
7. For shoot multiplication semisolid medium (SM), add Thidiazuron (TDZ) at a concentration ranging from 0.05 to 9.0 μM.
8. For root induction, semisolid medium (RI), add the auxin indole-3-butyric acid (IBA) at 2.5–5.0 μM concentration supplemented with 500 mg/L activated charcoal.
9. Dispense the sterile medium (25 ml) in glass culture vessel (4 cm diameter × 9.5 cm height baby food jars with magenta B caps).

3.2 Explant Preparation

1. Prepare the explants by cutting 1.0–1.5 cm long nodal segments containing at least one axillary bud with the help of a sharp blade.
2. Thoroughly wash the explants in continuous flow of tap water for 1 h, followed by soaking in solution of 0.1 % Tween 20, then rinsing in tap water for 10 min.

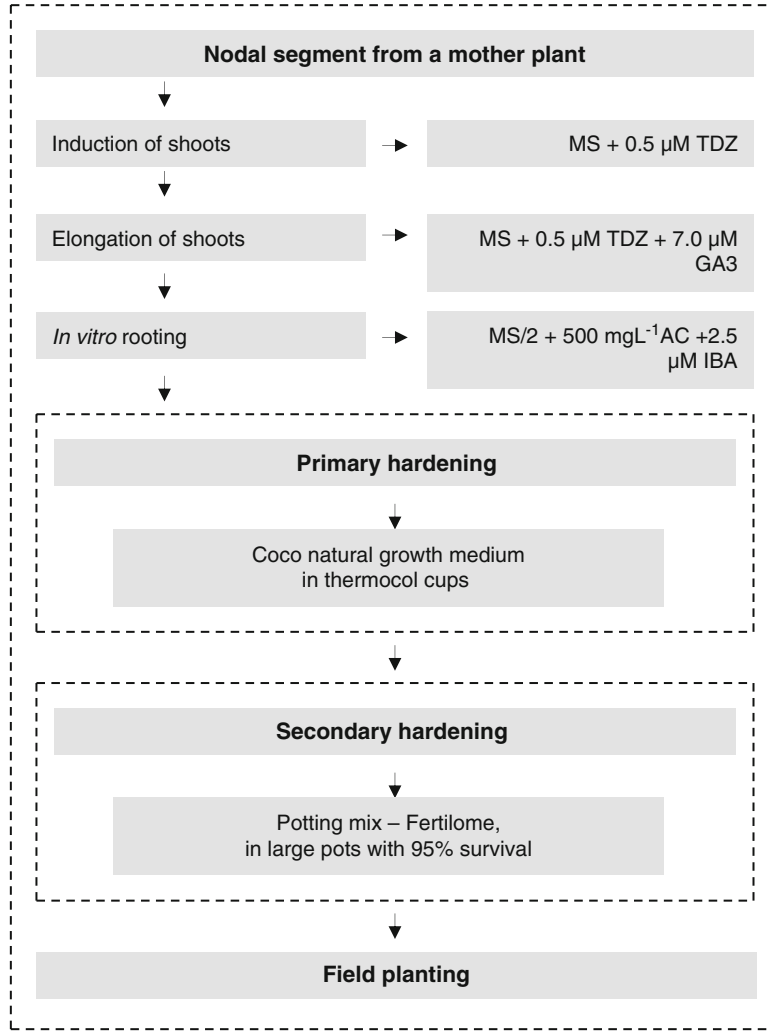

Fig. 1 Schematic diagram of in vitro propagation of *Cannabis sativa*

3.3 Surface Sterilization and Establishment of In Vitro Cultures

1. Sterilize the explants surface using 0.5 % NaOCl (15 % v/v bleach) and 0.1 % Tween 20 for 20 min under a laminar hood.
2. Wash the explants in sterile distilled water by rinsing 3 times for 5 min each.
3. Soak the explants in 0.1 % mercuric chloride for 2 min, followed by distilled water (*see* Subheading 4) (*see* **Note 7**).
4. Slice off the exposed end slightly using a sterilized blade prior to inoculation on the culture medium (*see* **Note 8**).
5. Inoculate single nodal explants in the culture vessel containing the SM media and incubate in the culture room at 25 ± 2 °C with 16-h photoperiod under fluorescent light with a photon flux of 52 μmol/m^2/s and 60 % relative humidity.

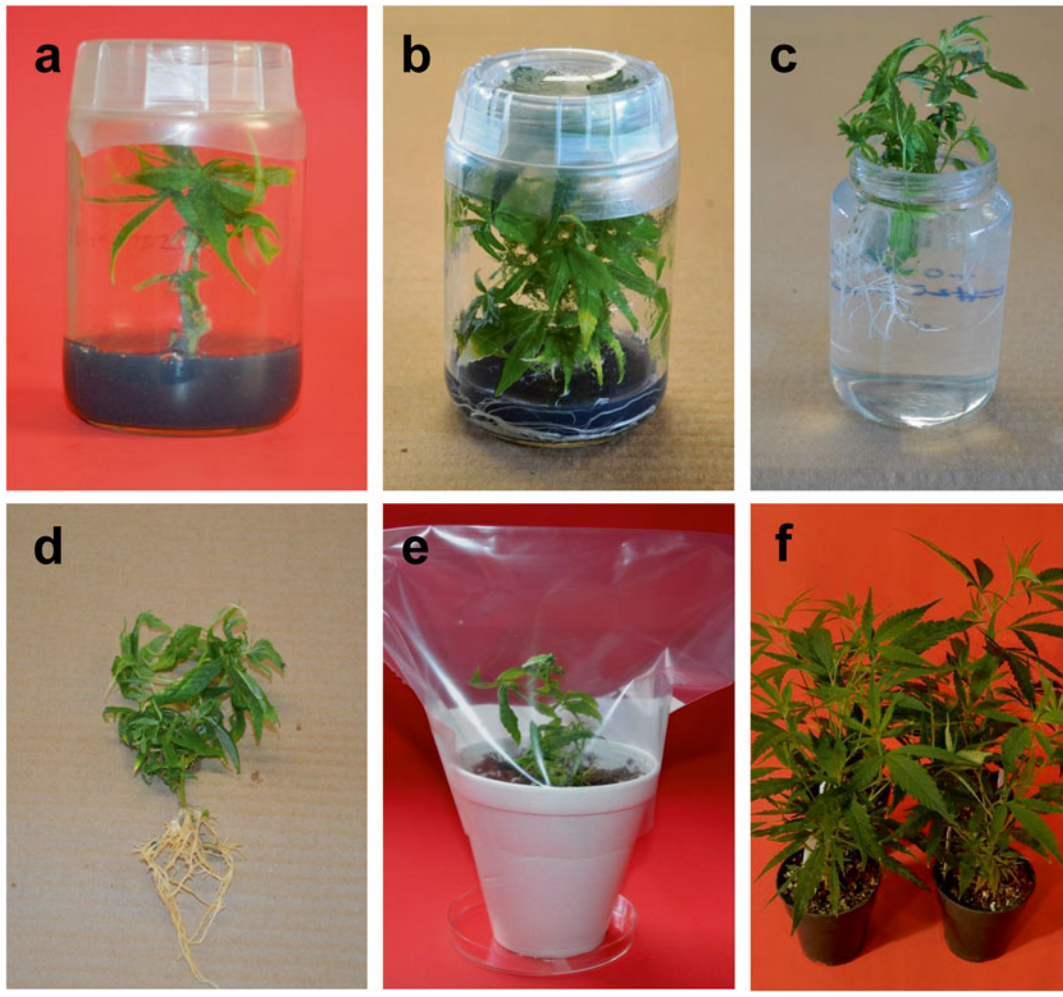

Fig. 2 Micropropagation of *Cannabis sativa*. (**a**) Shoot formation on MS + 0.5 μM TDZ (**b**) Rooting on ½ MS medium supplemented with 500 mg/L activated charcoal and 2.5 μM IBA (**c**, **d**) Well-rooted plantlet (**e**) A well-rooted plant at soil acclimatization stage (**f**) Fully acclimatized *Cannabis sativa* plants

6. Observe the cultures weekly. Note any morphological changes and growth responses in the cultured tissue. Record the following data:
   (a) The time of emergence of shoots from axillary buds.
   (b) The frequency of axillary branches per responding culture.
   (c) The number of shoots per culture.
7. After in vitro establishment, these cultures can be multiplied by inoculating 2–3 nodal segments per vessel. The culture may be used as a source of inocula for further multiplication.

3.4 Root Induction

1. Transfer isolated or two shoots to semisolid root induction medium.
2. Observe the cultures weekly for root initiation. Record the following data:
   (a) The time of root emergence.
   (b) Frequency of shoot developing from roots derived from each explants.
   (c) The number of roots per shoot.
3. Carefully remove the in vitro-developed plantlets (Fig. 2) after 6 weeks and clean the roots very gently removing agar using tap water (*see* **Note 9**).

3.5 Acclimatization

1. Wash the rooted plantlets thoroughly in running water to remove all traces of medium.
2. Preincubate the plantlets in Coco Natural growth medium in Thermocol cups.
3. Cover the cups with polyethylene bags to maintain humidity and keep in the indoor growth room for at least 1 week (Fig. 2).
4. Transfer the in vitro-hardened plantlets in large pots containing sterile potting mix Fertilome. Keep the plantlets under similar environmental conditions maintaining 16-h photoperiod, 25–30 °C temperature, and 60 % relative humidity.
5. After 30–40 days as new leaves start appearing and the plants appear robust, transfer the plants to the field conditions (*see* **Note 10**).

3.6 Quality Assurance of Micropropagated Plants: Assessing Genetic Fidelity

3.6.1 Isolation of Plant Genomic DNA

Total genomic DNA is extracted using a DNeasy Plant Mini Kit.

Protocol for DNA Extraction using DNeasy Plant Mini Kit

1. Finely powder the plant sample.
2. Weigh 20 mg (0.02 g) in a preweighed 2 ml sterile tube. Immediately put the tube on ice (crushed). Powder sample in a mixer mill (30 s., amplitude max = 100).
3. Add 600 μl API buffer into powder sample tube. Vortex upside and down (both sides) 15 s. Add 4 μl RNase A and vortex for 20–30 s.
4. Incubate the mixture for 10 min at 65 °C. Mix 2 or 3 times during incubation by inverting tubes.
5. Add 195 μl AP2 Buffer to the lysate and vortex for 20–30 s. Incubate on ice for 5 min.
6. Centrifuge the lysate for 5 min at 18,000 × *g*.
7. Pipette the lysate into QIA shredder mini spin column (purple/lilac) placed in a 2 ml collection tube. Centrifuge for 2 min at 14,000 rpm.

8. Transfer the flow-through from the previous step into a new 2 ml tube without disturbing the cell debris pellet. (At this point check the amount of lysate obtained after centrifugation).
9. For 500 µl lysate obtained, add 750 µl AP3 Buffer. Mix by pipetting.
10. Use the white DNeasy Mini Spin column for this step. Pipette 650 µl of the mixture (may include any precipitate), into the DNeasy Mini Spin column placed in a 2 ml collection tube. Centrifuge for 1 min at 800 rpm. Discard the flow-through. Reuse the collection tube for the next step.
11. Repeat the previous step with the remaining sample. Discard the flow-through and collection tube.
12. Place the DNeasy Mini Spin column into a new 2 ml collection tube.
13. Add 500 µl AW Buffer. Centrifuge for 1 min at 800 rpm. Discard the flow-through and reuse the collection tube for the next step.
14. Add 500 µl AW Buffer to DNeasy Mini Spin columns. Centrifuge for 2 min at 14,000 rpm (to dry the membrane). Discard the flow-through and reuse the collection tube.
15. Add 500 µl AW Buffer to DNeasy Mini Spin columns. Centrifuge for 2 min at 14,000 rpm (to dry the membrane). Discard the flow-through and reuse the collection tube.
16. Transfer the DNeasy Mini Spin column to a 1.5 ml microcentrifuge tube (let the tube be open). Pipette 50 µl AE Buffer onto the DNeasy membrane. Incubate for 5 min at room temperature (15–25 °C), centrifuge for 1 min at 8000 rpm to elute.
17. Again pipette 50 µl AE Buffer to the membrane. Incubate for 5 min at room temperature. Centrifuge for 1 min at 8000 rpm to elute.
18. DNA is obtained (about 100 µl) in an eppendorf tube. Store on ice and switch to –80 °C.

3.6.2 Quantification of DNA

1. The DNA can be quantified by running on 1 % agarose gel and checking the absorbance at 260 nm (*see* **Note 11**).
2. Agarose gel: Mix 2 ml 50× TAE Buffer to a final volume of 100 ml. Add 1 g agarose, boil in a microwave, and cool to 50–60 °C. Carefully add EB. Seal the free ends of gel tray, fix the combs, and dispense the molten gel. Allow it to solidify. Remove the comb and put the gel tray in the gel reservoir containing 1× TAE Buffer. Make sure the gel is fully submerged (*see* **Note 12**).

3. Mix the DNA sample, loading 6× dye, and Milli-Q water (1 + 2 + 9 μl) by repeated pipetting. Load in the wells of gel carefully.
4. Close the lid of the gel reservoir and turn on the power supply. The gel runs from the negative pole (black) towards positive pole (red). Check after few minutes if the gel is running. Check the gel regularly to prevent the samples from running off the gel (*see* **Note 13**).
5. For PCR, 20 ng amount of DNA is sufficient per reaction and therefore dilution of DNA should be made with sterile Milli-Q water in such a way that 1 μl contains approximately 20–25 ng of DNA (*see* **Notes 14** and **15**).

3.6.3 PCR for DNA Amplification

1. Amplification reaction is performed using MJ Research PTC-225 gradient cycler.
2. PCR is carried out in a total volume of 25 μl for each reaction in 0.2 ml PCR tube.
3. Set up the PCR reaction mixture using 14 primers (*see* Table 2), based on the criterion of the generation of distinct bands that are reproducible between samples. Each PCR reaction contains 0.1 μM of each primer, 1 unit Platinum Taq DNA polymerase, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 20 ng template DNA, and PCR buffer (*see* **Note 16**).
4. Taq polymerase should be added at the end.
5. All this should be carried out in ice.
6. Transfer this reaction mixture in PCR tube and spin it for few seconds for uniform mixing.
7. Carry out the PCR in thermal cycler using the following conditions:
   (a) Initial denaturation at 94 °C for 3 min.
   (b) Followed by 45 cycles each consisting of a denaturation step at 94 °C for 30 s, primer annealing step at 50 °C for 30 s, and amplification at 72 °C for 3 min.
   (c) Final extension at 72 °C for 7 min followed by arresting the reaction at 4 °C for infinite period.
8. Load the amplified DNA on 2 % agarose gel in 1× TAE Buffer stained with 0.5 μg/ml EB. Scan the gel with Bio-Rad Gel Imaging System and analyze with Quantity One Analysis Software Version 4.3.0 (*see* **Notes 17** and **18**).
9. Run the amplified products on the gel with molecular size standard 1 kb plus DNA ladder.
10. Score only the well separated bands in size range of 0.1–3.0 kb as present or absent for ISSR markers.

3.7 Quality Assurance of Micropropagated Plants: Cannabinoids profile and Content

3.7.1 Harvesting

1. Take the biomass samples from apical segments (flowering buds) of the mother plant, in vitro-propagated and vegetatively propagated plants in triplicates.
2. Dry the samples at 120 °F in oven overnight and individually manicure by hand.

3.7.2 Extraction

1. Manicure all the samples in a 14 mesh (0.0555 in. opening) metal sieve to remove seeds and stems.
2. Extract the powdered/sieved material (triplicated samples 0.1 g sample each) with 3 ml of internal standard/extracting solution (100 mg of 4-androstene-3, 17-dione + 10 ml chloroform + 90 ml ethanol) at room temperature for 1 h.
3. Withdraw the extracts into disposable transfer pipettes through cotton plugs for filtration and transfer into GC vials, cap the vials, and place on auto sampler. This extract can be used for quantitative analysis of the cannabinoids through GC/FID.

3.7.3 Gas Chromatography-Flame Ionization Detection (GC-FID)

1. Quantitative estimation of cannabinoids is carried out by gas chromatography analyses following Ross et al. [18] using Varian CP-3380 gas chromatograph, equipped with Varian CP-8400 automatic liquid sampler, a capillary injector, and a dual flame ionization detector (*see* **Note 19**).
2. The column used is 15 mm × 0.25 mm DB-1, 0.25 μ film
3. Data is recorded using a Dell OptiPlex GX1 computer and Varian Star workstation software (version 6).
4. Helium is used as a carrier gas and detector makeup gas, with an upstream indicating moisture trap and a downstream indicating oxygen trap.
5. The following parameters are used:
   (a) Air—30 psi (400 μl/min).
   (b) Hydrogen—30 psi (30 ml/min).
   (c) Column head pressure—14 psi (1.0 ml/min).
   (d) Split flow rate—50 ml/min.
   (e) Split ratio—50:1.
   (f) Septum purge flow rate—5 ml/min.
   (g) Makeup gas pressure—20 psi (20 ml/min).
   (h) Injector temperature—240 °C.
   (i) Detector temperature—260 °C.
   (j) Initial oven temperature—170 °C.
   (k) Initial temperature hold time—1 min.
   (l) Temperature rate—10 °C/min.

(m) Final oven temperature—250 °C and final temperature hold time—3 min.

6. The concentration of a specific cannabinoid is calculated as follows:

$$\text{Cannabinoid}(\%) = \{\text{GC area}(\text{cannabinoid}) / \text{GC area}(\text{ISTD})\} \times \{\text{Volume}(\text{ISTD}) / \text{Amount}(\text{sample})\} \times 100$$

4 Notes

1. It is very important to work on a surface that is free of dust and other potential contaminants.
2. Regularly check the air flow gauge of laminar air flow chamber. Switch on the UV light for 30 min. Before starting any activity in the laminar air flow bench, wipe the surface of the laminar air bench with alcohol frequently during any aseptic operation. Fumigate the entire transfer room.
3. Before using, soak all the glassware overnight in Clorox solution and powdered detergent. Thoroughly wash with tap water to remove the last trace of detergent. Finally, rinse glassware with double distilled demineralized water and dry in hot air oven at 150 °C for 2 h.
4. While autoclaving, allow sufficient air volume in the flask to prevent the media from boiling over.
5. Make sure that the volume of the autoclaved medium plus the volume of the added filter-sterilized compound sum to the final volume of the prepared medium.
6. The standard conditions for autoclaving media are 121 °C at 105 kPa for 20 min. For larger volumes the time should be increased: for 500 ml, 30 min; for 1000 ml, 40 min. Prior testing of the 40-min autoclaving is recommended, unless sugars are autoclaved separately.
7. Avoid contact of body parts with $HgCl_2$, β-mercaptoethanol, and EB as all these chemicals are highly mutagenic, carcinogenic, and hazardous. As a precaution, wear gloves while handling and dispose of the chemicals properly!
8. Possible source of danger exists if a person, after flaming an instrument reinserts the hot instrument into the alcohol dip. *Caution*: Ethanol is flammable! One should be very careful.
9. In vitro roots are delicate, so do not let them break.
10. Disposal of the cannabis plant material should be done strictly following DEA regulations.
11. Alternatively, DNA yield may also be checked spectrophotometrically.

12. While preparing the agarose gel, take care that the final volume should never be reduced due to evaporation during boiling. Take care: do not entrap any air bubble.
13. Clean the gel tray, gel reservoir, combs, and other materials with ethanol properly before and after use.
14. While loading the sample, do not let the sample spill out. Wear gloves during the entire operation and prevent contamination.
15. Pay attention to the quality of DNA-band when there are many fragments.
16. To minimize the error and for convenience mix all the dNTPs in equal amounts and make a stock in advance, and then take 1 μl for each PCR reaction.
17. If the DNA does not dissolve at room temperature or at 37 °C, incubate at 65 °C for 1 h.
18. Insufficient denaturing of the target genomic DNA in the initial cycle is a common cause of PCR reaction failure. The incubation time for primer extension at 72 °C varies according to the length of the target DNA sequence to be amplified. For most purposes, 2 min is usually sufficient.
19. The method used for the analysis should be precise, accurate, robust, and validated. The general steps of chromatography should be followed. Carefully record all the observations of the experiment.

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Chapter 20

Transcript Quantification of Genes Involved in Steviol Glycoside Biosynthesis in *Stevia rebaudiana* Bertoni by Real-Time Polymerase Chain Reaction (RT-PCR)

Arpan Modi, Nitish Kumar, and Subhash Narayanan

Abstract

Stevia (*Stevia rebaudiana* Bertoni) is a medicinal plant having sweet, diterpenoid glycosides known as steviol glycosides which are 200–300 times sweeter than sucrose (0.4 % solution). They are synthesized mainly in the leaves via plastid localized 2-C-methyl-D-erythrose-4-phosphate pathway (MEP pathway). Fifteen genes are involved in the formation of these glycosides. In the present protocol, a method for the quantification of transcripts of these genes is shown. The work involves RNA extraction and cDNA preparation, and therefore, procedures for the confirmation of DNA-free cDNA preparation have also been illustrated. Moreover, details of plant treatments are not mentioned as this protocol may apply to relative gene expression profile in any medicinal plant with any treatment. The treatments are numbered as T0 (Control), T1, T2, T3, and T4.

Key words Real-time PCR, Transcript quantification, Secondary metabolism, Stevia, Stevioside, Steviol glycosides

1 Introduction

Steviol glycosides (SG) are found predominantly in *Stevia rebaudiana*, *Stevia phlebophylla*, and *Rubus suavissimus* (a Chinese sweet tea) [1]. They are low caloric sweeteners. Major steviol glycosides are stevioside, rebaudioside, steviolmonoside, steviolbioside, and duclosides. These steviol glycosides along with other secondary metabolites like alkaloids, phenols, and flavonoids in *S. rebaudiana* make such plants suitable for medicinal use and food additives [2].

Steviol glycosides share a common pathway with gibberellic acid (GA_3) as they are terpenoids and both are derived from 2-C-methyl-D-erythrose-4-phosphate (MEP) pathway operated within plastids and as a result these glycosides are synthesized abundantly in the leaves and in very minute quantities are found in other organs of the plant [3]. The concentrations of steviol glycosides are 10,000 times higher than gibberellic acid which shows the

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dedication of this plant towards SGs in view of MEP pathway. Major steviol glycosides are steviolmonoside, steviolbioside, stevioside, and rebaudioside-A among which stevioside is most abundant and has 143 times higher sweetening power than normal sugar whereas rebaudioside-A is up to 320 times sweeter but lower in concentration as compared to stevioside [1, 4]. All terpenoids are derived from the five-carbon precursor isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). According to carbon numbers 5, 10, 15, 20, 30, and 40 they are called hemiterpenes, monoterpenes, sesquiterpenes, diterpenes, triterpenes, and tetraterpenes, respectively. The most abundant plant pigment after chlorophyll is carotenoid which is a tetraterpene.

In 1950s, mevalonic acid pathway for the production of isoprenoids in yeast and animals was reported but few years later, MVA independent pathway, called MEP pathway was detected in bacteria and plants. Efforts were made to upregulate the genes of MEP pathway in plants so that respective metabolites can be enhanced [5, 6]. Biosynthesis of steviol glycoside pathway (MEP pathway) highlights many secondary metabolites some of which are commercially and biologically important to the plants. Moreover, microbes can be utilized in the enhancement and production of natural compounds. Strategies to achieve this goal include increasing the precursor supply, overexpressing or increasing the efficiency of respective enzymes, altering the regulation of gene expression, reducing flux towards unwanted by-products or competing pathways, and reconstituting the entire pathway in a heterologous host [7]. In view of cloning and characterization of the genes involved in MEP pathway, cloning of full length cDNA of seven genes of the pathway has been reported [8]. The present scenario implies that the elucidation of secondary metabolite pathway is carried out by evaluating upregulation and downregulation by measuring transcript levels of genes involved, quantitatively [9] and one of the techniques, through which such quantification may be possible in a precise, reproducible, and reliable manner, is an application of real-time PCR.

Therefore, in the present protocol, we describe the method for the relative transcript quantification of candidate genes involved in stevioside biosynthesis pathway. In the present protocol, detailed information about plant treatments is not given and the treatments are mentioned only as T1, T2, T3, and T4.

2 Materials

2.1 Plant Growth

1. Tissue culture-raised uniform plants (Fig. 1) are cultivated in a 1:1 mixture of soil–vermicompost [10] (*see* **Note 1**).
2. Control as well as treated plants should be uniform and sampling should be done from the same stage from the same axial positioned leaf of control and treated plants [8] (*see* **Note 2**).

Fig. 1 Tissue culture-raised uniform plantlets of Stevia after secondary hardening

2.2 In Silico Procedures

2.2.1 Primer Design for Endogenous Control Selection

Three endogenous controls are selected for the relative gene expression study, viz., *actin*, *ubiquitin*, and *glyceraldehyde-3-phosphate dehydrogenase*. Primer blast tool is used to design primers for three endogenous control as well as other target genes. Gene sequence for the primer design of *actin* (*act*) has been reported and has been obtained from NCBI website. For the *ubiquitin* (*ubq*) and *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*), gene sequence from model organism is taken.

2.2.2 Primer Design for Candidate Genes

The list of all the 15 candidate genes with their accession numbers is mentioned in Table 1. Gene sequence and ESTs of the candidate genes are reported in the public domain of NCBI. Reported sequences of ESTs in Stevia are taken for the primer design.

2.2.3 Primer Design for Confirmation of DNA

Intronic region of reported *actin* sequence in Stevia is taken to design primers for checking DNA contamination in extracted sample of total RNA.

2.3 RNA Extraction

The following chemicals and consumables are required for RNA extraction:

1. DEPC-treated water (*see* **Note 3**).
2. Extraction buffer (*see* **Note 4**).
3. Chloroform.
4. Isopropanol.
5. Ethanol.
6. Chloroform-treated, autoclaved vials (1.5 and 2.0 ml) and tips (10, 100 and 1000 μl) (*see* **Note 5**).

2.4 cDNA Preparation

From the total RNA, first strand cDNA synthesis is carried out from the Fermentas First Strand cDNA synthesis kit according to

Table 1
Details of the genes of the steviol biosynthetic pathway, their accession numbers and percentage identity/similarity with others

Gene	Accession number	Reported as	Percentage identity/similarity with other accession numbers	References
DXS	AJ429232	Characterized	74/86 to Q38854	[13]
DXR	AJ429233	Characterized	79/89 to AAF73140	[13]
CMS	DQ269452	Putative	82/91 to NP_565286.1	–
CMK	DQ269453	Putative	70/80 to NP_180261.1	–
MCS	DQ631427	Putative	77/87 to NP_850971	–
HDS	DQ768749	Putative	82/92 to AAM19840	–
HDR	DQ269451	Putative	79/89 to AAN87171	–
GGDPS	DQ432013	Putative	63/80 to P34802	–
CDPS	AF034545	Characterized	53/69 to NP_192187	[1]
KS	AF097310	Characterized	52/69 to AAC39443	[1]
KO	AY364317	Characterized	60/79 to NP_197962	[14]
KAH	–	Characterized	52/72 to NP_188087.1	Unpublished
UGT85C2	AY345978	Characterized	47/67 to NP_173652.1	[15]
UGT74G1	AY345982	Characterized	44/63 to NP_973682.1	[15]
UGT76G1	AY345974	Characterized	45/62 to NP_196207.1	[15]

manufacturer's instruction. Kit components and other materials are as follows:

1. Template RNA.
2. DEPC-treated water.
3. Reverse transcriptase.
4. dNTPs.
5. Buffer.
6. $MgCl_2$.
7. Oligo $d(T)_{18}$ primers.
8. RiboLock RNase inhibitor.

2.5 Real-Time PCR

Real-time PCR is performed using SYBR Green chemistry and relative gene expression is studied. The following chemicals are used while performing real-time PCR run.

1. Fast SYBR Green Master Mix.
2. cDNA template.
3. Gene specific primers.

4. Nuclease-free water.
5. Fast plate.
6. Plate seal.

2.6 DNA Extraction

Following chemicals and consumables are required for RNA extraction.

1. Autoclaved water.
2. Extraction buffer (*see* **Note 6**).
3. Chloroform.
4. Isoamyl alcohol.
5. Absolute alcohol.
6. 3 M sodium acetate.
7. 0.3× TE Buffer.
8. Autoclaved tips (10, 100, and 1000 μl) and vials (1.5 and 2.0 ml).

3 Method

3.1 Primer Design

For the sequences that are not reported (*ubq* and *gapdh*), sequence of the respective gene from model organism is taken in primer blast tool of NCBI. BLAST is done against reported ESTs of Stevia. The parameters for the BLAST are mentioned (*see* **Note 7**).

For the *act* and other candidate genes, primers are designed directly from the primer blast tool with the parameters mentioned (*see* **Note 8**).

Primers are supplied to make the stock solution 100 pmol with 0.3× TE buffer and for the PCR master mix preparation it is further diluted to 10 pmol and used (1.0 μl each for DNA confirmation and 0.2 μl each for real-time experiments in 25.0 μl and 20 μl systems, respectively).

3.2 RNA Extraction

Total RNA extraction from various medicinal plants has been reported [11], and here, RNA isolation is carried out using the same protocol with minor modifications as described below:

1. Take 200 mg leaf tissue and grind it with mortar and pestle in liquid nitrogen (*see* **Note 9**).
2. Under chilled conditions, add 3.0 ml extraction buffer and grind further till the liquid state appears.
3. Allow the homogenate to stand for 10 min (*see* **Note 10**).
4. Add 400 μl chloroform, mix gently, and allow it to stand for 10 min.
5. Centrifuge the sample at 17,949 × *g* for 10 min at 4 °C.

6. Take the upper aqueous phase and add 0.6th volume of isopropanol to it.
7. Keep the sample at 4 °C for 10 min.
8. Centrifuge the sample at 17,949 × *g* for 10 min at 4 °C.
9. Discard the supernatant and wash the pellet with 500 μl of 70 % ethanol.
10. Centrifuge the sample at 6,797 × *g* for 2 min at 4 °C.
11. Discard the supernatant, air-dry the pellet, and resuspend it in 30–50 μl DEPC-treated water (*see* **Note 11**).

3.3 cDNA Preparation

1. Take 0.1–5 μg total RNA and 1.0 μl primer (Oligo $d(T)_{18}$) in a sterile tube.
2. Make the volume of the reaction up to 11.0 μl with nuclease-free water.
3. Incubate the mixture at 65 °C for 5 min and chill on ice (*see* **Note 12**).
4. Add 4.0 μl 5× reaction buffer, 2.0 μl 10 mM dNTP mix, and 1.0 μl RNase inhibitor in it.
5. Incubate at 37 °C for 5 min.
6. Add 40 units of M-MuLV reverse transcriptase and incubate the reaction mixture at 37 °C for 60 min.
7. Terminate the reaction by incubating it at 70 °C for 5 min.
8. Chill on ice.
9. Dilute the sample to 100 μl with nuclease-free water (*see* **Note 13**).

3.4 DNA Extraction and Primer Validation

DNA is extracted according to the method widely used [12] with minor modifications as described below:

1. Take 200 mg leaf sample and crush it in liquid nitrogen using mortar and pestle.
2. Take the sample powder in 2.0 ml autoclaved vial and add 1 ml of pre-warmed extraction buffer in the sample.
3. Keep the sample at 65 °C for 60–90 min with occasional mixing.
4. Centrifuge the sample at 2,655 × *g* for 5 min at 4 °C and take the aqueous phase.
5. Add equal volume of chloroform–isoamyl alcohol (24:1) to the sample (800 μl approx.).
6. Centrifuge the sample at 10,000 rpm for 8 min at 4 °C and take 600 μl of aqueous phase from it.
7. Transfer the aqueous phase in 2.0 ml autoclaved vial and add equal volume of chloroform–isoamyl alcohol (24:1) to it.

8. Centrifuge the sample at 10,621 × *g* for 8 min at 4 °C and take 500 µl of the aqueous phase from it.
9. Transfer the aqueous phase in 1.5 ml autoclaved vial and add 1.0 ml absolute alcohol containing 10 % 3 M sodium acetate to it.
10. Keep the sample at −20 °C for 3 h.
11. Centrifuge the sample at 15,294 × *g* for 15 min at 4 °C.
12. Wash the pellet with 80 % ethanol (at 8000 rpm for 10 min 4 °C).
13. Air-dry the pellet and resuspend it in 100 µl of 0.3× TE buffer.
14. Quantify the DNA and make the working solution of 20 ng/µl for PCR.

3.5 Primer Validation through Melt Curve

1. Take cDNA of the control sample and run it in real-time PCR using all the primers.
2. Select melt curve analysis in the study and add PCR steps before the melt curve.
3. Adjust cycling condition as mentioned (*see* **Note 14**).
4. Analyze the melt curve (Fig. 2).

3.6 Confirmation of Absence of DNA

1. DNA, extracted from the leaf sample, was taken to put PCR reaction with the primer synthesized from intronic region with the cycling condition as mentioned (*see* **Note 15**).
2. Take all the treated as well as control sample template and perform PCR reaction using the primer designed from intronic region and gene specific primer (*actin*). Thus, this reaction is a multiplexing.
3. Cycling conditions are the same as mentioned above (*see* **Note 15**).
4. Run the product on an agarose gel.
5. Analyze the gel image (Fig. 3).

3.7 Relative Gene Expression through Real-Time PCR

1. Put one plate for the selection of endogenous control.
2. Note down threshold cycle for each and every treatment and gene.
3. Calculate standard deviation.
4. Select the gene having the least standard deviation value.
5. Put the qPCR reaction with selected endogenous control and all the target genes with control and treated samples. Cycling condition is mentioned (*see* **Note 16**).
6. Save data in .txt or .xls format and analyze the file in Data Assist tool (Fig. 4) (*see* **Note 17**). The resulting data will be seen as fold change in expression of genes of treated plants as compared to untreated plants (control).

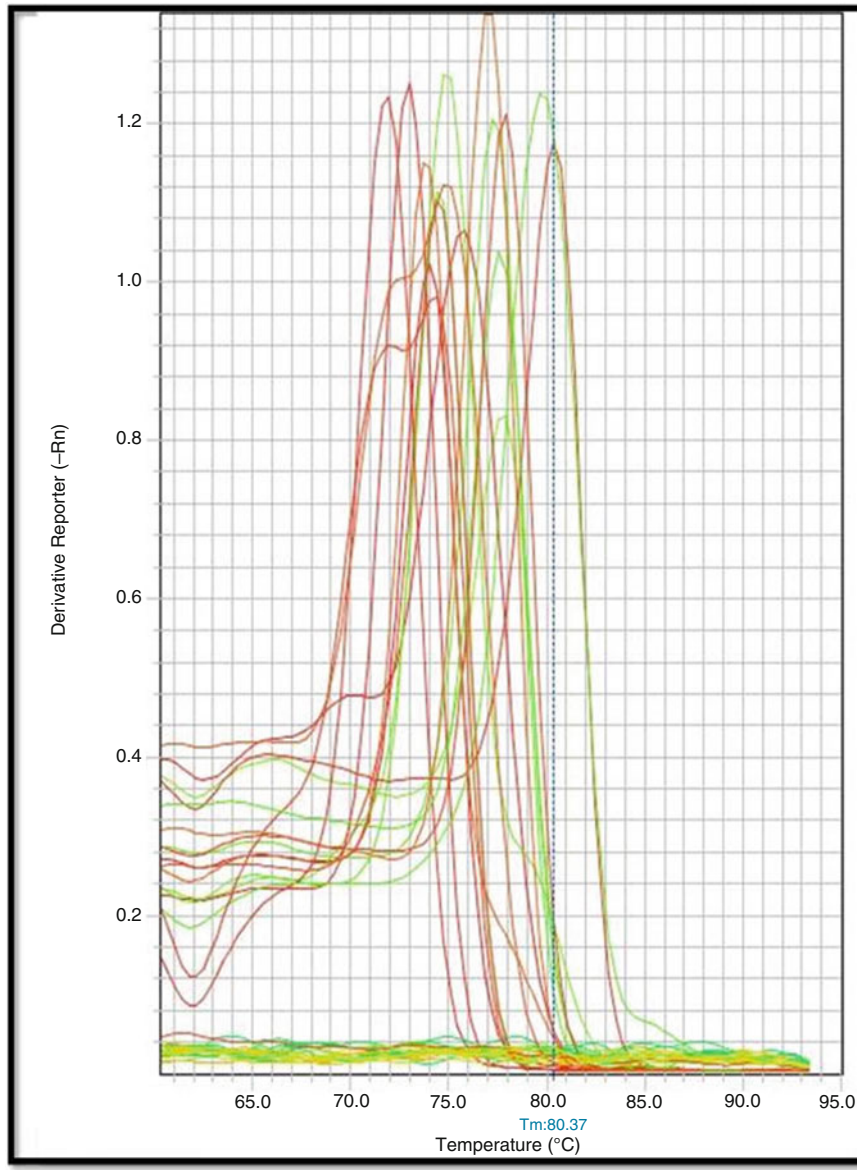


Fig. 2 Validation of designed primers through melt curve analysis. Peaks having different Tm values represent different gene products involved in steviol glycoside biosynthetic pathway

4 Notes

1. Uniformity of the plants is very critical condition in gene expression analyses. Plants, like Stevia, are greatly influenced by external environmental conditions. Care should be taken in maintenance of the plants until any treatment is given to them.
2. Axial position of leaf on the plant also matters in terms of secondary metabolite synthesis. In Stevia, the stevioside content

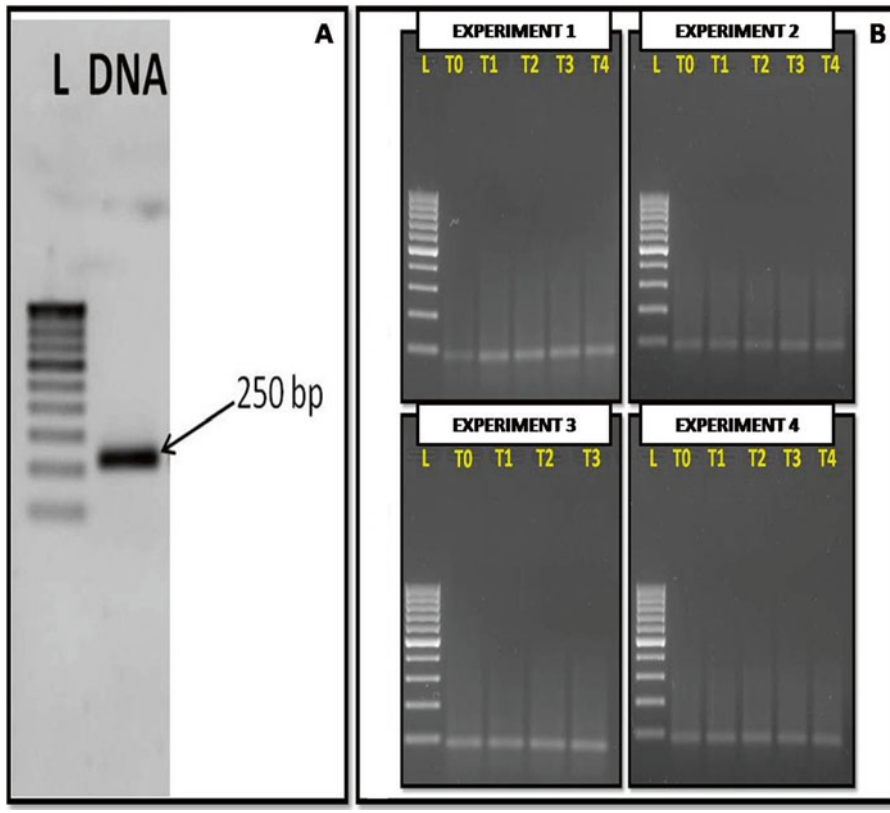


Fig. 3 Validation of DNA amplifying marker by normal PCR in (**a**) in which 250 bp product represents the presence of DNA and the same is not found in different experiments (**b**). Here, 100 bp band is a product of *actin* gene which represents success of PCR

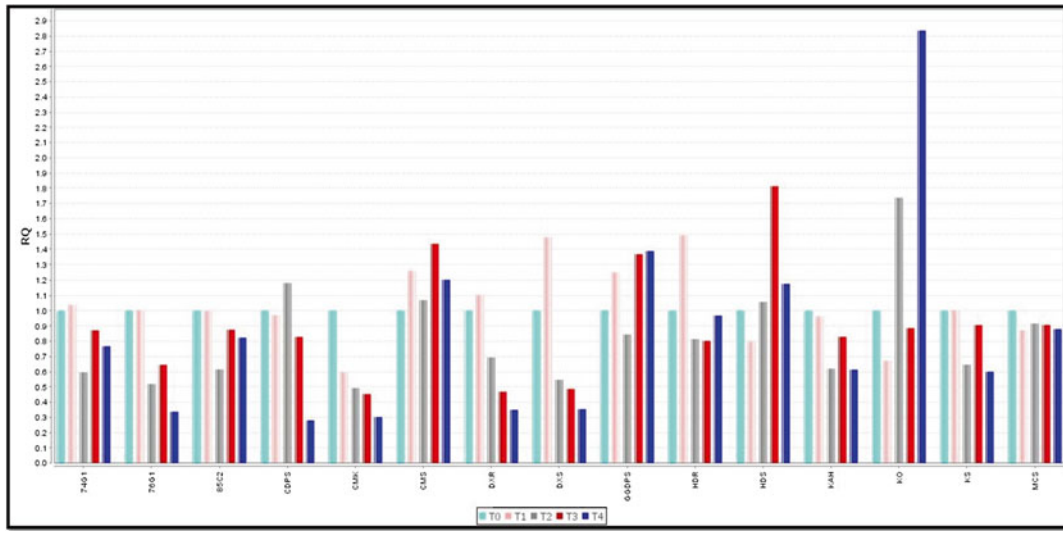


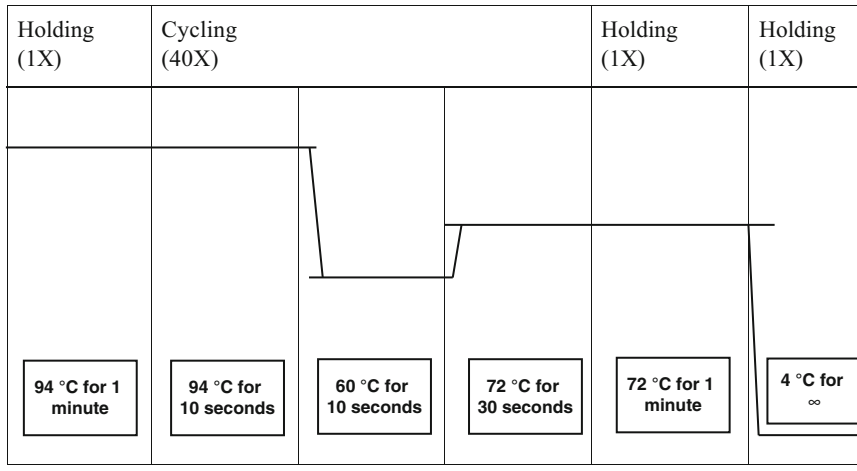
Fig. 4 Relative quantification (RQ) plot of all the genes in all the treatments of one of the experiments conducted. Here, T0 represents control (untreated) plants and T1–T4 represent treatments

varies significantly from leaf to leaf. Thus, while sampling, they should be taken from the same axial position from all the treatments.

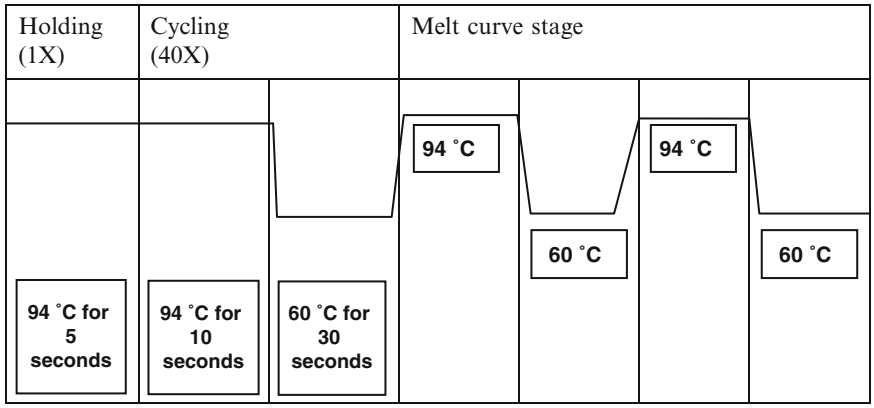
3. Diethylpyrocarbonate (DEPC) is a potent inhibitor of RNase. In RNA extraction and cDNA preparation experiments, water should be treated with DEPC, for which, 0.5 ml of commercially available DEPC is taken and added in 500 ml distilled water. It is kept at 37 °C for 2 h and then the water is autoclaved. Keep the bottle cap slightly open so that evaporated carbon dioxide and ethanol from DEPC on heating can be released. No sweet smell of ethanol should be there after autoclaving.
4. Extraction buffer is composed of 200 μl 0.5 M EDTA, 8.7 ml 10 mM Tris saturated phenol, 1.0 ml 3 M sodium acetate, and 100 μl 10 % sodium dodecyl sulfate in 10 ml buffer solution.
5. Chloroform is a potent denaturing agent of all the proteins and enzymes. To make the tubes and tips DNase, RNase, and protease free, they are washed gently with chloroform and dried completely in oven. No residue of chloroform should remain in tubes and tips, otherwise it may also hinder the downstream processes. Completely dried consumables are then autoclaved and may be used.
6. Extraction buffer (10 ml) for DNA isolation is composed of 300 mg cetyl trimethyl ammonium bromide (CTAB), 100 mg polyvinylpyrrolidone (PVP), 3 ml 5 M NaCl, 0.8 ml 0.5 M EDTA, 1.0 ml 1 M Tris, 0.1 ml of β-mercaptoethanol and the final volume is adjusted to 10.0 ml with autoclaved water.
7. Sequences of some endogenous control are not reported, and thus, sequences from the reported model organisms are queried with the available ESTs of our organism of interest. Sequence information of *ubq and gapdh* is obtained from Arabidopsis and BLAST is performed with default parameters except database and organism input. Database is set with EST from other organisms and organism is set as *S. rebaudiana*. To this query, megablast is performed.
8. Those genes whose sequence information is available are used to design primers directly from primer blast tool. Accession number or gene sequence is put on query sequence information and melting temperature is set at 60 °C with ±1 °C variation. Product range is set from 70 to 130 bp and other parameters remain the same as mentioned in default page. Select the primer which has the least 3′ self complement ability.
9. Mortar and pestle are also treated with chloroform and autoclaved as tips and tubes. In a chilled condition 200 mg leaf sample is crushed in liquid nitrogen and after making fine powder of the sample 2 ml extraction buffer is added in it which will freeze at a time. Break the frozen buffer and make fine

powder of the buffer and eventually allow the slurry to attain liquid state. Add 1 ml DEPC-treated water, collect the sample with 1 ml tip, and distribute it in two vials.

10. Homogenate is kept in ice for 10 min in horizontal position. In this position crushed sample will have maximum contact with extraction buffer which helps to increase the final product.
11. While drying the vials, take remaining ethanol left at bottom of the tube by tips carefully without disturbing the RNA pellet.
12. RNA sample contains GC-rich sequences or secondary structure and thus the sample is put at this temperature. Moreover, the samples should not be incubated more than 5 min at this temperature, otherwise RNA may get degraded.
13. Dilution of sample depends on transcript quantity of the target gene. Usually five times dilution is sufficient. In a separate tube five times dilution is made and taking that cDNA as a template, end point PCR is performed; if a faint band appears, then dilution may be reduced accordingly as higher dilution may result in higher C_T value in real-time PCR.
14. In Applied Biosystems 7500 Fast Real-Time PCR, select melt curve mode for the run which contains only melt curve stages as default steps. Add PCR steps before melt curve. PCR parameters are the same as that for relative gene expression analysis. Since it is a melt curve analysis, data is recorded only in the melt curve stage and during PCR, only temperature plot will be visible.
15. In a sample containing cDNA, if DNA is present, then it may be amplified by primers designed from intronic region. Here, gene sequence from *actin* is selected and from the intronic region primers are designed as mentioned previously except the product size is from 200 to 250 base pairs. To the cDNA sample, PCR is performed using this primer and absence of product determines confirmation of DNA-free cDNA samples. Cycling conditions are as follows:



16. Cycling conditions for primer validation and relative gene expression are given below:



17. After completion of real-time PCR run, save all data in .txt format and open Data Assist tool. Select new study and add description of the study. Select the saved .txt formatted file and click OK. In sample design menu (the upper left corner of the window), all the treatments will be displayed from which any unwanted treatment may be omitted. The target as well as endogenous control genes will be seen in the upper middle part of the window. Here also any uninterested gene may be omitted. Select the endogenous control having the least standard deviation value as selected control from the drop-down menu. In the analysis setting menu (upper right corner), choose endogenous control as a normalization method and reference sample. Click on perform analysis. At the bottom of the window graphical results will be shown from which click on RQ plot. Select "RQ vs. Target" as plot type and "Linear" as graph type. The next window will be as same as Fig. 4.

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Chapter 21

In Vitro Propagation and Conservation of *Withania somnifera* (Dunal) L.

Nigar Fatima, Naseem Ahmad, and Mohammad Anis

Abstract

Plant tissue culture offers several techniques for rapid clonal propagation, germplasm conservation, regeneration of genetically manipulated superior clones, production of phyto-constituents, and ex vitro conservation of valuable phytodiversity. An improved and efficient micropropagation protocol for *Withania somnifera* (L.), a drug-producing medicinal plant, using juvenile explants (nodal explants) has been developed. Highest multiplication and subsequent elongation of shoots is observed on MS medium containing BA and NAA. The regenerated microshoots roots best on ½ MS medium containing NAA, established in earthen pots containing garden soil and are maintained in the greenhouse with 95 % survival rate. Genetic uniformity of micropropagated plants is confirmed by PCR-based DNA fingerprinting techniques, viz., RAPD and ISSR. No variation is observed in DNA fingerprinting patterns among the micropropagated plants, which are similar to that of the donor plant illustrating their genetic uniformity.

Key words Aseptic seedlings, Clones, Conservation, Clonal fidelity, Juvenile explants, DNA fingerprinting, Micropropagation, Genetic stability, Genetic diversity, PCR amplifications, Stratification, Germplasm conservation, Phytohormones, Soilrite, Phytodiversity

1 Introduction

Withania somnifera (L.) Dunal (Solanaceae), commonly known as "Ashwagandha" and "Winter Cherry," is widely distributed throughout the drier and subtropical parts of India [1]. The genus has high medicinal value and is extensively used in Ayurvedic formulations as "Rasayana" [2]. Its roots and leaves are used in numerous medical preparations, for their anti-inflammatory [3], antimicrobial [4], antitumor and radio-sensitizing [5, 6], immunological [7], and antioxidant properties [8], besides promoting vigor and stamina [9]. Ashwaganda's pharmacological activity has been attributed to two main metabolites, viz., withaniferin A and withanolide. The herb has been identified by National Medicinal Plant Board of India as one of the 32 selected priority medicinal plants, which are in great demand in the domestic and international

S. Mohan Jain (ed.), *Protocols for In Vitro Cultures and Secondary Metabolite Analysis of Aromatic and Medicinal Plants, Second Edition*, Methods in Molecular Biology, vol. 1391, DOI 10.1007/978-1-4939-3332-7_21,

markets [10]. A long time gap between planting and harvesting, excessive exploitation of natural resources, non-availability of procedures for synthetic production of withanolides, and ever-increasing demand–supply ratio are reasons enough to apply modern bio-techniques for the plant.

Ashwagandha propagates through seeds; moreover, the plant does not possess the natural ability for vegetative propagation [11]. However, the commercial exploitation for the production of pharmaceuticals and conventional propagation is hampered due to its poor seed viability [12]. In addition, low germination rate restricts its propagation through seeds even after stratification [13], making the long term seed storage futile [14]. Moreover, the genetic diversity of the species in India is now getting degraded [15] at an alarming rate. Lack of proper cultivation, ruinous harvesting practices for the production of medicines, loss of habitats, and the illegal, indiscriminate collection of the plant material from its natural habitat pose a serious threat to its existence in the wild. Owing to the immense importance of the plant, several researchers have reported in vitro regeneration in *W. somnifera* using various explants [16–22].

The present study describes an efficient and rapid propagation protocol [23] and evaluation of clonal fidelity of micropropagated plantlets through molecular techniques in *W. somnifera* [24].

2 Materials

2.1 Media Preparation for Plant Cell Culture

1. The formulation of Murashige and Skoog's (MS) salts (*see* Table 1) [25].
2. Double distilled water.
3. Stock solutions of micronutrients, macronutrients, iron, and vitamins (*see* **Notes 1**).
4. Sucrose.
5. Agar.

2.2 Plant Growth Regulators

1. Phytohormones (*see* Table 2).

2.3 Plant Materials

1. Seeds procured from mature fruits of *W. somnifera*.

2.3.1 Surface Sterilization of Seeds

1. 5 % Labolene.
2. 0.1 % $HgCl_2$.

2.3.2 Inoculation

1. Laminar air flow hood.
2. Sterilized forceps, scalpels, sterile blades, burner, petri plates, glass bead sterilizer (Dent-eq, BS 1000), 70 % ethanol, mercuric chloride.

Table 1
Nutritional components of Murashige and Skoog (MS) medium

Components	Compositions (mg/L)	Stocks solution (mg/L)
Macronutrients		(20×)
$MgSO_4 \cdot 7H_2O$	370	7440
KH_2PO_4	170	3400
KNO_3	1900	38,000
NH_4NO_3	1650	33,000
$CaCl_2 \cdot 2H_2O$	440	8800
$NaH_2PO_4 \cdot H_2O$	–	–
$(NH_4)_2 \cdot SO_4$	–	–
Micronutrients		(200×)
H_3BO_3	6.2	1240
$MnSO_4 \cdot 4H_2O$	22.3	4460
$MnSO_4 \cdot H_2O$	–	–
$ZnSO_4 \cdot 7H_2O$	8.6	1720
$Na_2MoO_4 \cdot 2H_2O$	0.25	50
$CuSO_4 \cdot 5H_2O$	0.025	5.0
$CoCl_2 \cdot 6H_2O$	0.025	5.0
Kl	0.83	166
Stock solution III		(100×)
$FeSO_4 \cdot 7H_2O$	27.8	2780
$Na_2EDTA \cdot 2H_2O$	37.3	3730
Organic supplements		(100×)
Thiamine HCl	0.5	50
Pyridoxine HCl	0.5	50
Nicotinic acid	0.5	50
Myo-inositol	100	10,000
Others		
Glycine	2.0	200
Sucrose (g)	3 %	30

Table 2
Details of plant growth regulators

Plant growth regulators	Molecular weight	Solubility	Sterilization	Storage
Auxin				
α-napthalene acetic acid (NAA)	186.21 g/mol	1 N NaOH	*Co-autoclave*	4 ° C
Cytokinin				
6-Benzyladenine (BA)	225.25 g/mol	1 N NaOH	*Co-autoclave*	4 ° C

2.3.3 Establishment of Seedlings

1. Half-strength MS medium with 3 % sucrose, 0.8 % agar, pH 5.8.

2.3.4 Culture of Explants

1. MS medium with BA and NAA.

2.4 In Vitro Rooting

1. Half-strength MS medium containing NAA.

2.5 Acclimatization and Field Transfer of Rooted Plantlets

1. Sterilized Soilrite, garden soil, vermicompost.
2. Diluted MS salts, Thermacol cups, transparent polythene bags.

2.6 Genetic Fidelity Evaluation

1. Leaves collected from ten randomly selected micropropagated plantlets and mother plants are used for genetic homogeneity analysis.

2.6.1 Plant Genomic DNA Isolation

1. Genomic DNA.

2.6.2 Reagents

1. *DNA extraction buffer*: 2.5 % cetyl trimethyl ammonium bromide (CTAB), 0.5 M ethylenediaminetetraacetic acid (EDTA) 5 M NaCl, 0.5 M Tris–HCl, pH 8.0.
2. 5× TBE Buffer: 1 M Tris–HCl, 0.5 M EDTA, mQ water.
3. Isopropanol.
4. Gel loading dye (6×): 30 mg bromophenol blue dye, 80 % glycerol, 30 mg xylene-cyanol, 300 μl EDTA.

2.6.3 PCR Amplification

1. Genomic DNA.
2. *Primers*: RAPD (OPB and OPC Kit) and ISSR.
3. *PCR amplification mixture* (20 μl): 10× buffer (2 μl), 25 mM $MgCl_2$ (1.2 μl), 10 mM dNTPs (0.4 μl), 2 μM primers (ISSR/RAPD), 3 Unit Taq DNA polymerase (0.2 μl), and 40 ng template DNA.
4. Agarose.

5. Ethidium bromide.
6. 6× dye (bromophenol blue dye).
7. *Marker*: λ DNA/EcoR1 + HindIII.
8. Milli-Q water.

2.7 Glassware

1. 25 × 150 mm rimless culture tubes, 250 ml wide-mouth Erlenmeyer flasks, beakers, measuring cylinders, pipettes (0.1–10 ml), glass rods, petri dishes, conical flasks.
2. PCR tubes, eppendorf tubes.

3 Methods

3.1 Media Preparation

1. To avoid precipitation, store stock solutions at 4 °C.
2. MS stock is prepared as categorized (*see* Table 1) (*see* **Notes 1** and **2**).
3. Mix properly the required volume of stock solutions, sucrose, and agar to make the final volume of culture medium as required. To prepare cytokinin stock solution, weigh the hormone and dissolve it in a few drops of NaOH (1 N) and make up the final volume with double distilled water.
4. Prepare auxin stocks solution by dissolving amount of auxin in minimum amount of ethanol and raise to required volume by double distilled water.
5. Adjust pH of the medium to 5.8 with 1 N NaOH and 1 HCl, before dispensing the media into culture vessels.
6. Pour 20 ml culture medium into test tubes, 50 ml into culture flasks, and 80 ml into glass bottles (*see* **Note 3**).
7. Use cotton plugs to cap test tubes and flasks and transparent polypropylene lids bearing a plugged with non-absorbent cotton.
8. Autoclave the media at 121 °C and 1.05 kg/cm^2 pressure for 15 min.

3.2 In Vitro Regeneration from Juvenile Explants

1. Collect mature and healthy seeds from 6-month-old *W. somnifera* plant.
2. Wash the seeds thoroughly under running tap water for 30 min, treat with 5 % Labolene for 20 min followed by a thorough washing under running tap water for 15 min.
3. Surface-sterilize the seeds with 0.1 % $HgCl_2$ solution for 5 min in the laminar flow cabinet (*see* **Notes 4** and **5**), Rinse thrice with sterile distilled water to remove traces of the $HgCl_2$.
4. Culture disinfected seeds on half-strength MS basal medium (3 % sucrose and 0.8 % agar).

5. Excise nodal segments from 15-day-old aseptic seedlings and culture on MS medium containing BA with 3 % sucrose and 0.8 % agar for multiple shoot induction (*see* Fig. 1a).

3.3 Shoot Regeneration and Multiplication

1. Highest multiplication and subsequent elongation of shoots is achieved on BA and NAA (*see* Fig. 1b).
2. Establish proliferating shoot cultures by repeatedly subculturing onto the fresh culture medium at a 3-week interval (*see* **Note 8**).

3.4 Rooting

1. Transfer shoots (3–4 cm long) into half-strength MS augmented with NAA (*see* Fig. 1c).

3.5 Acclimatization and Hardening

1. Remove rooted plantlets from the rooting medium and wash thoroughly under running tap water to remove all traces of agar attached to it (*see* **Note 9**).
2. Acclimatize plantlets in Soilrite inside the growth room substrate for 4 weeks and establish in garden soil (*see* Fig. 1d).

3.6 Assessment of Clonal Fidelity of Tissue Culture-Raised Plants

1. Isolate total genomic DNA from fresh leaf tissues of micropropagated plantlets and mother plant using modified cetyl trimethyl ammonium bromide (CTAB) method [26].
2. Freeze 1 g fresh leaf tissues using liquid nitrogen and grind the frozen samples thoroughly into fine powder using chilled mortar and pestle.
3. Add 3 ml pre-warmed (65 °C) CTAB extraction buffer with 0.2 % (v/v) β-mercaptoethanol to the homogenized plant samples.
4. Mix thoroughly and incubate the samples at 65 °C in water bath for 1 h with occasional shaking. Add equal volume of chloroform–isoamylalcohol (24:1) to samples and mix well to avoid any protein contamination, debris, and interphase materials. Centrifuge (Hettich) the sample at 15,000 rpm (32953 × *g*) at 4 °C for 10 min.
5. Transfer the supernatant to eppendorf tube containing 170 μl sodium chloride solution and double volume of chilled isopropanol (to precipitate the DNA). Mix the samples well and incubate at room temperature for 30 min.
6. Resuspend the pellet in 300 μl (TE) buffer and 3 μl RNAse and incubate at 65 °C in a water bath for 30 min.
7. Pellet the precipitated DNA by centrifugation at 10,000 rpm for 10 min at 4 °C.
8. Decant the supernatant and air-dry the pellet properly.
9. Dissolve the pellet in 100 μL Milli-Q water.
10. Store the DNA at −20 °C.

Fig. 1 (**a**) Multiple shoot induction from nodal explants on MS medium amended with BA (2.5 μM), 8 weeks; (**b**) Multiplication and subsequent proliferation of shoots on MS medium amended with BA (2.5 μM) + NAA (0.5 μM), 8 weeks; (**c**) Rooting of in vitro microshoots on ½ MS + NAA (0.5 μM), 4 weeks; (**d**) Well-established hardened plant in pot containing soil, 2 months old

3.6.1 Quantification of Genomic DNA

1. Quantify the DNA by running the DNA on 1 % agarose gel. Measure the optical density at 260 nm using nanophotometer.
2. Use RAPD or ISSR primers for screening (*see* Tables 3 and 4).
3. Perform PCR amplifications using thermocycler.

3.6.2 PCR Assay

1. Prepare the reaction buffer (3 μl of 10× buffer): 0.75 μl $MgCl_2$ (25 mM), 0.75 μl dNTPs (10 mM each of dATP, dGTP, dTTP, and dCTP), 1.5 μl primers (RAPD/ISSR), 0.15 μl Taq DNA polymerase, and 18.85 μl mQ water.

Table 3
Nucleotide sequences of randomly amplified polymorphic DNA primers (RAPD)

S. no.	Kit B		Kit C	
	Primers	Sequence (5′–3′)	Primers	Sequence (5′–3′)
1	OPB01	GTTTCGCTCG	OPC01	TTCGAGCCAG
2	OPB02	TGATCCCTGG	OPC02	GTGAGGCGTC
3	OPB03	CATCCCCCTG	OPC03	GGGGGTCTTT
4	OPB04	GGACTGGAGT	OPC04	CCGCATCTAC
5	OPB05	TGCGCCCTTC	OPC05	GATGACCGCC
6	OPB06	TGCTCTGCCC	OPC06	GAACGGACTC
7	OPB07	GGTGACGCAG	OPC07	GTCCCGACGA
8	OPB08	GTCCACACGG	OPC08	TGGACCGGTG
9	OPB09	TGGGGGACTC	OPC09	CTCACCGTCC
10	OPB10	CTGCTGGGAC	OPC10	TGTCTGGGTG
11	OPB11	GTAGACCCGT	OPC11	AAAGCTGCGG
12	OPB12	CCTTGACGCA	OPC12	TGTCATCCCC
13	OPB13	TTCCCCCGCT	OPC13	AAGCCTCGTC
14	OPB14	TCCGCTCTGG	OPC14	TGCGTGCTTG
15	OPB15	GGAGGGTGTT	OPC15	GACGGATCAG
16	OPB16	TTTGCCCGGA	OPC16	CACACTCCAG
17	OPB17	AGGGAACGAG	OPC17	TTCCCCCCAG
18	OPB18	CCACAGCAGT	OPC18	TGAGTGGGTG
19	OPB19	ACCCCCGAAG	OPC19	GTTGCCAGCC
20	OPB20	GGACCCTTAC	OPC20	ACTTCGCCC

2. Set the DNA amplification program of 45 cycles: (1) initial denaturation at 94 °C for 5 min, (2) annealing at 35 °C for 1 min and elongation at 72 °C for 1 min, (3) extension at 72 °C for 10 min.
3. Fraction DNA amplification products by electrophoresis in 0.8 % (w/v) agarose gels and stain with 4 μl ethidium bromide in (100 ml) 1× TBE buffer (pH 8.0).

3.6.3 Agarose Gel Electrophoresis

1. Warm agarose solution and allow melting resulting in a clear solution; cool at around 60 °C and add 4 μl ethidium bromide to it.

Table 4
Nucleotide sequences of Inter Simple Sequence Repeat (ISSR) primers

S. no.	Name of primers	Primers sequences (5′–3′)
1	UBC-801	$(AT)_8T$
2	UBC-811	$(GA)_8C$
3	UBC-825	$(AC)_8T$
4	UBC-827	$(AC)_8G$
5	UBC-834	$(AG)_8YT$
6	UBC-841	$(GA)_8YC$
7	UBC-855	$(AC)_8YT$
8	UBC-866	$(CTC)_6$
9	UBC-868	$(GAA)_6$
10	UBC-880	$(GGGGT)_3G$
11	UBC-889	$DBDA(CA)_6C$
12	UBC-891	$HVHT\ (GT)_6G$
13	UBC-900	ACTTCCCCACAGGTTAACAC

2. Seal the gel casting tray with adhesive tape and arrange the gel mold with the comb placed in a manner such that it does not touch the base of the mold (*see* **Note 10**).
3. Pour carefully the molten agarose into the mold, avoiding formation any air bubble.
4. After solidification, remove the comb and sealing tape carefully and place the gel in the electrophoresis tank, and properly fill up with TBE buffer.
5. Load the first well of the gel (lane M) with 10 μl λ 1 kb ladder.
6. Add 5 μl loading dye to each PCR amplification product and load the entire volume into the well.
7. Run the gel at 50 V for 2 h.
8. Visualize and photographs of gel using gel documentation system (*see* Figs. 2a, b and 3a, b).

3.6.4 Data Scoring and Analysis

1. Only distinct, reproducible, and well-resolved fragments are taken in the analysis.

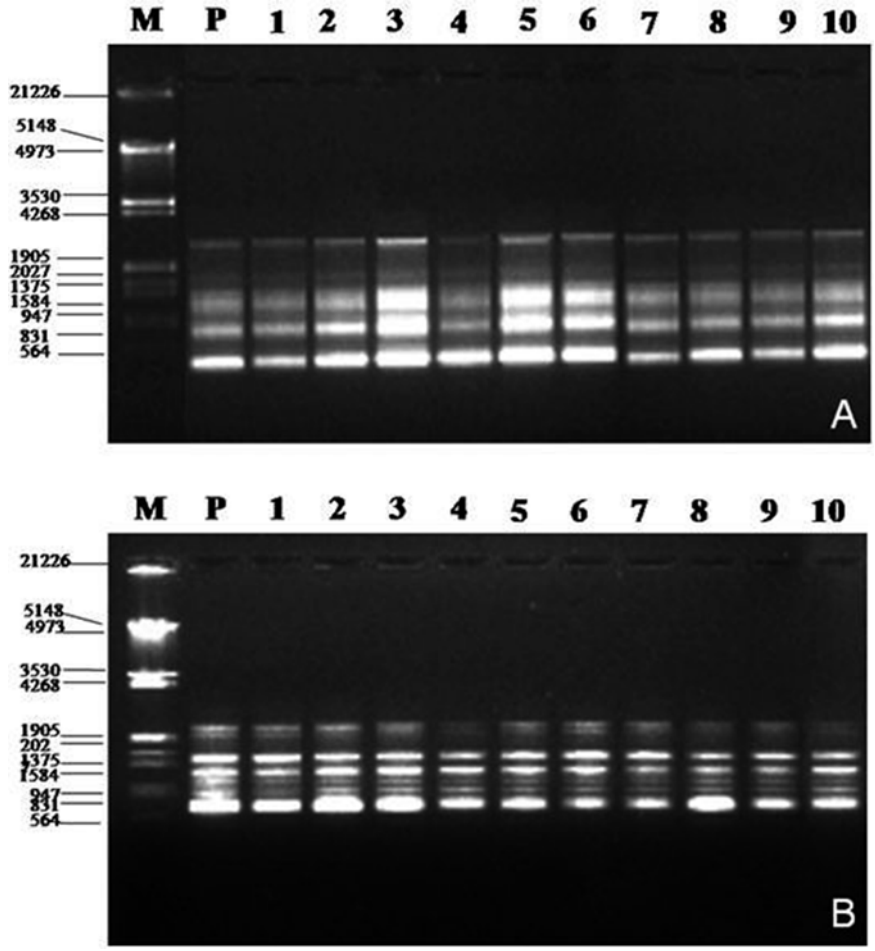

Fig. 2 Profile of polymerase chain reaction (PCR) amplification products from lane 1–10 micropropagated plants of *W. somnifera* using randomly amplified polymorphic DNA (RAPD) primers. (**a**) primer OPB 04 (**b**) primer OPC 08

4 Notes

1. Sterilization of glassware, culture media, and instruments should be carried out by autoclaving at 121 °C and 1.05 kg/cm^2 pressure.
2. Calibrate the pH meter before taking the medium pH. Adjust pH of the medium using 1 N NaOH or 1 N HCl.
3. Dipense 20 ml medium in 25 mm × 150 mm culture tubes and close them with the cotton plugs made with double layered muslin cloths stuffed with non-absorbent cotton.
4. Regularly check the air flow gauge of laminar air flow chamber. Switch on the UV light for 15 min before starting inocula-

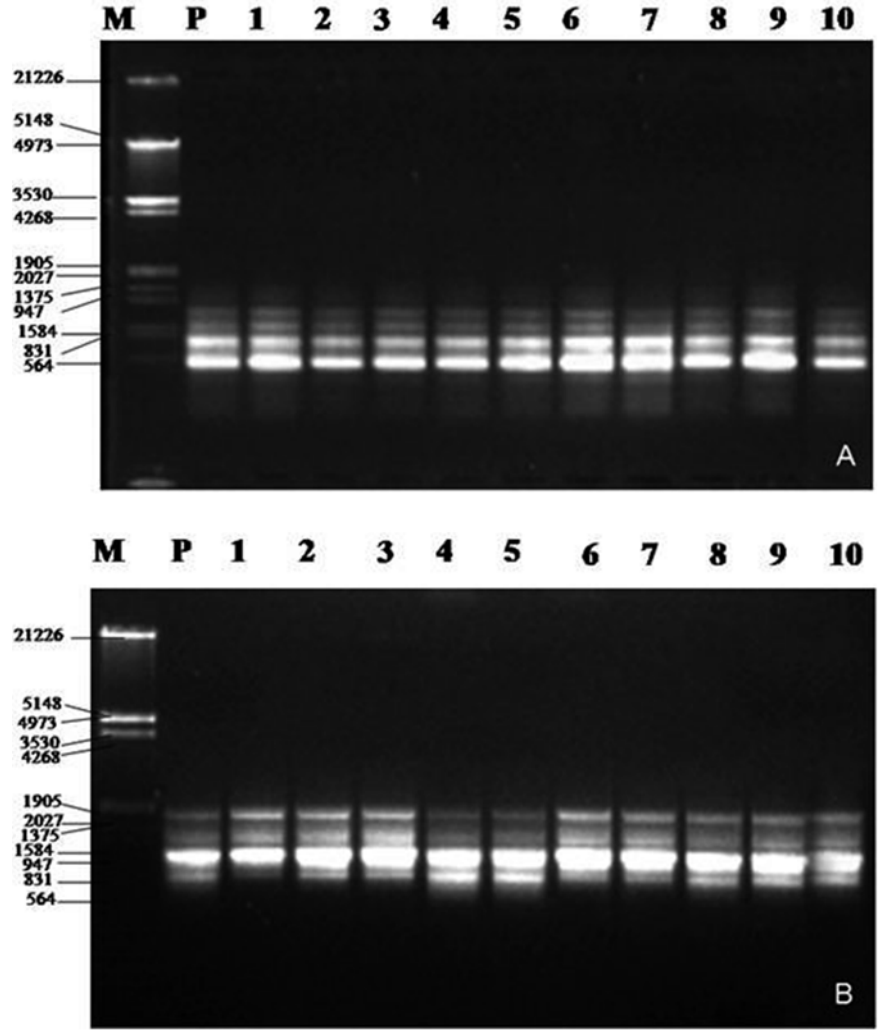

Fig. 3 A profile of polymerase chain reaction (PCR) amplification products from lane 1–10 micropropagated plants using Inter Simple Sequence Repeat (ISSR) primer (**a**) UBC 866; (**b**) primer UBC 891. M = Marker (DNA/EcoR1 + HindIII indicated in bp); P = Donor plant; Lane 1–10 = Micropropagated plants

tion/subculturing activity in the laminar air flow hood. The bench is wiped with alcohol frequently during any aseptic operations.

5. Precautions should be taken while using chemicals like $HgCl_2$, β-mercaptoethanol, ethidium bromide as they are highly carcinogenic, mutagenic, and hazardous.
6. Ethanol is inflammable. Therefore, one should be very careful while flaming an instrument.
7. Ultraviolet (UV) irradiation poses serious health risk.

8. Subculturing of shoots is carried out at a regular interval of 3 weeks. During subculturing the larger sized shoot clusters obtained at the end of each multiple cycle are further divided into smaller clusters.
9. The in vitro regenerated roots are very soft and delicate in nature. Therefore, physical handling should be done with utmost care.
10. Clean the gel tray, combs, and other materials properly before and after use to prevent contamination. Wear gloves during the entire operation.

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Chapter 22

Construction of Hypericin Gland-Specific cDNA Library via Suppression Subtractive Hybridization

Rupesh Kumar Singh, Weina Hou, and Gregory Franklin

Abstract

Hypericin, an important determinant of the pharmacological properties of the genus Hypericum, is considered as a major molecule for drug development. However, biosynthesis and accumulation of hypericin is not well understood. Identification of genes differentially expressed in tissues with and without hypericin accumulation is a useful strategy to elucidate the mechanisms underlying the development of the dark glands and hypericin biosynthesis. Suppression Subtractive Hybridization (SSH) is a unique method for PCR-based amplification of specific cDNA fragments that differ between a control (driver) and experimental (tester) transcriptome. This technique relies on the removal of dsDNA formed by hybridization between a control and test sample, thus eliminating cDNAs of similar abundance, and retaining differentially expressed or variable in sequence cDNAs. In our laboratory we applied this method to identify the genes involved in the development of dark glands and accumulation of hypericin in *Hypericum perforatum*. Here we describe the complete procedure for the construction of hypericin gland-specific subtracted cDNA library.

Key words *Hypericum perforatum*, Suppression subtractive hybridization, Dark glands, Hypericin biosynthesis, Polymerase chain reaction, Gene sequencing

1 Introduction

Hypericum perforatum is an important medicinal plant used worldwide to treat several ailments [1]. Many of the pharmaceutical properties of this species have been attributed to hypericin, a natural product structurally belonging to the chemical class of naphthodianthrones. Hypericin exhibits anticancer, anti-HIV, and neurorestorative characteristics while having reduced side effects, making it as an emerging multifunctional lead drug molecule in new therapies [2, 3]. Especially, its photoexcitation properties are under intensive investigation as a molecule for fluorescent diagnosis and photodynamic therapy (PDT) in the treatment of a variety of tumors [4]. In spite of several applications

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in the pharmaceutical industry, hypericin biosynthesis is poorly understood, mainly due to the lack of information about the genes involved in this pathway.

Hypericin is produced only in Hypericum species (e.g., *H. perforatum*) that contain characteristic dark glands (also known as hypericin glands). These glands are mainly distributed in the aerial parts of the plants such as leaves, stem, and flowers. Accumulation of hypericin in the dark glands is considered as a mechanism to avoid the potential toxicity of this light-sensitive compound to plant tissues [5]. A clear positive correlation between the presence of dark glands and hypericin accumulation is evidenced by our own [6, 7] and other studies [8, 9]. For instance, *H. perforatum* cell suspension cultures do not possess dark glands and cannot synthesize or accumulate hypericin [6]. Similarly, *H. perforatum* green organogenic nodules [10] and the calli derived from its thin sections do not have dark glands or hypericin synthesis in the initial stages of development [11]. Moreover, *Hypericum* species with and without dark glands, namely, *H. perforatum*, *H. undulatum* and *H. androsaemum*, *H. heterophyllum*, *H. scabrum* etc., synthesize and do not synthesize hypericin, respectively [1, 12], establishing a clear correlation between the presence of dark glands and hypericin synthesis. This hypothesis is further emphasized by the presence of HpPKS1 and HpPKS2, the two genes known to be involved in hypericin biosynthesis, in the dark glands [13].

Taking advantage of this strong correlation between the presence of dark glands and hypericin accumulation, we performed subtraction between cDNAs of tissues with and without hypericin glands to construct a hypericin gland-specific cDNA library. This would help us to understand the complex phenomenon of dark gland formation and hypericin biosynthesis. Here, we describe the protocol for the construction of dark gland-specific cDNA library via SSH technique.

2 Materials

2.1 In Vitro Seed Germination

1. *H. perforatum* (cv. Helos) seeds.
2. Tween 20 (Sigma, USA).
3. Sterile water.
4. 70 % ethyl alcohol.
5. 1.5 % active chlorine.
6. Sterile filter paper.
7. Water–agar medium.

2.2 RNA Isolation and mRNA Purification

1. Plant material: The tissues for RNA extraction can be obtained as described in Subheading 3.2.
2. RNA extraction buffer: 2 % CTAB, 2 % PVP, 25 mM EDTA pH 8.0, 100 mM Tris–HCl, pH 8.0, 2 M NaCl, 0.5 g/L spermidine, 2 % β-mercaptoethanol (βMe). Prepare in RNase-free water and store at 4 °C.
3. 3.0 M sodium acetate: adjust to pH 5.5, autoclave, and store at 4 °C.
4. 24:1 mixture of chloroform and isoamyl alcohol (Sigma-Aldrich, USA).
5. Isopropanol.
6. TE buffer pH 8.0, autoclave and store at 4 °C.
7. 8 M LiCl, autoclave and store at 4 °C.
8. 96 % ethyl alcohol.
9. RNase-free water.
10. RiboMinus™ Plant Kit for RNA-Seq (Invitrogen, USA).

2.3 cDNA Synthesis

1. 2 μg Poly A+ RNA from tester and driver.
2. 10 μM cDNA synthesis primer.
3. 100 units/μL SMARTScribe™ Reverse Transcriptase (Clontech, USA).
4. 3 U/μL T4 DNA Polymerase (Clontech, USA).
5. 5× first strand buffer: 250 mM Tris–HCl, pH 8.3, 30 mM $MgCl_2$, 375 mM KCl.
6. 5× second strand buffer: 500 mM KCl, 50 mM ammonium sulfate, 25 mM $MgCl_2$, 0.75 mM ß-NAD, 100 mM Tris–HCl, pH 7.5, 0.25 mM BSA.
7. 20× Second Strand Enzyme Cocktail: 6 U/μL DNA polymerase I, 0.25 U/μL RNase H, 1.2 U/μL *E. coli* DNA ligase.
8. 10 mM dNTP mix.
9. 20 mM DTT (dithiothreitol).
10. Sterile distilled water.
11. EDTA–glycogen mix: 0.2 M EDTA, 1 mg/mL glycogen (Clontech, USA).
12. 24:1 mixture of chloroform and isoamyl alcohol (Sigma-Aldrich, USA).
13. 4 M ammonium acetate.
14. 100 % ethyl alcohol.
15. 70 % ethyl alcohol.

2.4 cDNA Subtraction

1. Tester and driver cDNA.
2. PCR-Select™ cDNA Subtraction Kit (Clontech, USA).
3. 400 U/μL T4 DNA ligase with 3 mM ATP (Clontech, USA).
4. 5× DNA Ligation Buffer: 250 mM Tris–HCl, pH 7.8, 50 mM $MgCl_2$, 10 mM DTT, 0.25 mM BSA.
5. 10 μM adaptor 1.
6. 10 μM adaptor 2R.
7. 10 U/μL RsaI restriction enzyme (Clontech, USA).
8. 10× RsaI restriction buffer: 100 mM Bis-Tris Propane-HCl, pH 7.0, 100 mM $MgCl_2$, 1 mM DTT.
9. 4× hybridization buffer (Clontech, USA).
10. Dilution buffer: 20 mM HEPES, pH 8.3, 50 mM NaCl, 0.2 mM EDTA, pH 8.0.
11. 10 μM PCR primer 1—5′CTAATACGACTCACTATAGGGC3′.
12. 10 μM Nested PCR primer 1—5′TCGAGCGGCCGCCCGGGCAGGT3′.
13. 10 μM Nested PCR primer 2R—5′AGCGTGGTCGCGGCCGAGGT3′.
14. 10 mM dNTP mix.
15. 20× EDTA–glycogen mix: 0.2 M EDTA, 1 mg/mL glycogen.
16. 4 M ammonium acetate.
17. Taq DNA polymerase (Fermentas, USA).
18. QIAquick PCR Purification kit (QIAGEN, Germany).

2.5 Cloning of Subtracted cDNA

1. pGEM-T Easy Vector system (Promega, USA).
2. DH5 α *E. coli* strain (Invitrogen, USA).
3. 100 mM $CaCl_2$ solution, autoclave and store at 4 °C.
4. 50 mg/mL ampicillin (Calbiochem, USA).
5. 0.2 M IPTG, filter-sterilize and store at –20 °C.
6. 20 mg/mL X-Gal, filter-sterilize and store at –20 °C.
7. 10 U/μL T4 DNA ligase (Fermentas, USA).
8. 10× ligation buffer—pH 7.8: 400 mM Tris–HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP.
9. LB medium solidified with 1.5 % agar.

2.6 Screening of Subtracted cDNA Library

1. *E. coli* transformed with T vector containing subtracted cDNA fragments.
2. PCR components: universal 10 μM M13 F and M13 R primers, 5 U/μL Taq DNA polymerase (Fermentas, USA), 10 mM dNTP mix, 10× PCR buffer.
3. Thermocycler (Mastercycler gradient®, Eppendorf, Germany).

3 Methods

3.1 In Vitro Seed Germination

1. Take approximately 50–100 *H. perforatum* seeds in an Eppendorf tube.
2. Add 1 mL sterile water and a drop of Tween 20.
3. Mix well and keep in dark at 4 °C overnight.
4. Discard the solution and rinse twice with sterile distilled water (*see* **Note 1**).
5. Decontaminate the seeds with 1 mL 70 % (v/v) ethyl alcohol for 60 s followed by commercial bleach containing 1.5 % (v/v) active chlorine for 3 min (*see* **Note 2**).
6. Wash the seeds three times in sterile distilled water and blot-dry on a sterile filter paper.
7. Gently transfer the disinfected seeds onto WA medium.
8. Incubate the cultures in culture room at 25 °C under photoperiodic condition for germination.

3.2 Collection of Tissues with and Without Hypericin Glands

1. After 2 weeks of germination, seedlings would have a pair of cotyledonary leaves without hypericin glands and a pair of primary leaves with hypericin glands (Fig. 1).

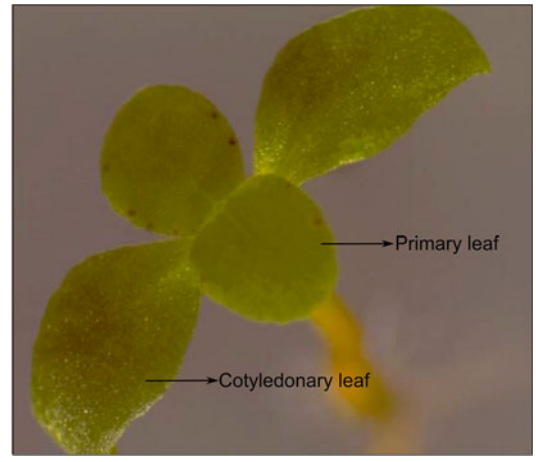

Fig. 1 *H. perforatum* seedling germinated on water–agar medium showing cotyledonary leaves without dark glands and primary leaves with dark glands

2. Transfer the seedlings aseptically to a sterile petri dish containing sterile distilled water.
3. Excise the cotyledonary and primary leaves with scalpel blade and collect into separate Falcon tubes placed in liquid nitrogen.
4. Freeze the tissues in liquid nitrogen and store at −80 °C until RNA extraction.

3.3 Total RNA Isolation (High Quality)

1. Grind 0.5 g of frozen tissue from each sample (with and without glands) into fine powder in liquid nitrogen with a sterile mortar and pestle (*see* **Note 3**).
2. Collect the ground tissues into 15 mL Falcon tubes.
3. Warm RNA extraction buffer to 60 °C and add 3 mL to each tube (*see* **Note 4**).
4. Vigorously vortex the tubes to mix the samples.
5. Incubate the tubes at 60 °C for 10 min in a water bath.
6. Cool down to room temperature and add 3 mL chloroform–isoamyl alcohol (24:1).
7. Vigorously vortex the tubes to mix the samples.
8. Centrifuge the tubes at 12,000 × *g* for 10 min at 4 °C.
9. Carefully transfer the top aqueous phase into new tubes.
10. Add ½ volume of ice-cold ethyl alcohol 96 % (v/v) and mix well by gentle inversion.
11. Incubate the tubes on ice for 30 min to precipitate polysaccharides.
12. Centrifuge the tubes at 10,000 × *g* for 10 min at 4 °C.
13. Collect the supernatant to new tubes and add equal volume of 8 M LiCl.
14. Incubate the tubes at −20 °C overnight for RNA precipitation (*see* **Note 5**).
15. Centrifuge the tubes at 12,000 × *g* for 30 min at 4 °C to pellet precipitated RNA.
16. Discard the supernatant and wash the pellets with 5 mL of 96 % (v/v) ice-cold ethyl alcohol.
17. Dry and dissolve the pellet in 50 μL of TE buffer (*see* **Note 6**).
18. Check for RNA quantity and purity in a NanoDrop (*see* **Note 7**).
19. Resolve 1–2 μL sample to check the RNA quality and integrity on 1.2 % (w/v) agarose gel (Fig. 2).

3.4 mRNA Purification

1. We have used RiboMinus™ Plant Kit for RNA-Seq for mRNA purification from total RNA (*see* **Note 8**).
2. Transfer 10 μg of total RNA isolated from tissues with and without glands to different Eppendorf tubes and make up the volume to 10 μL using RNase-free water.

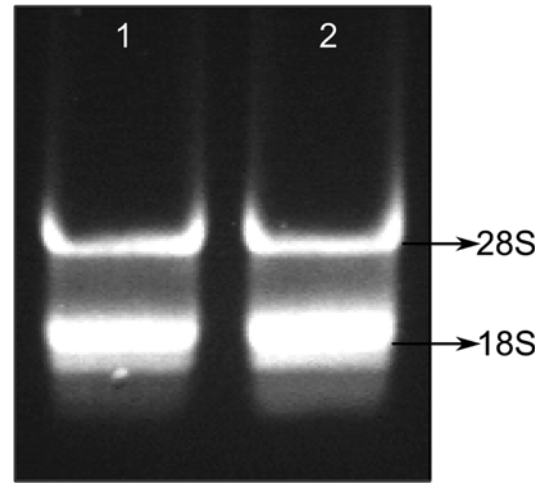

Fig. 2 Total RNA isolated from leaf tissues resolved on 1.2 % agarose gel. Lane 1. Total RNA from leaf tissues having hypericin glands and Lane 2. Total RNA from leaf tissues without hypericin glands

3. Incubate the tubes at 60 °C for 3 min.
4. Add 100 μL hybridization buffer pre warmed to 37 °C and 10 μL RiboMinus probe—both supplied with the RiboMinus kit (*see* **Note 9**).
5. Incubate the tube at 70 °C for 5 min in a hot water bath to disrupt all the secondary structures of RNA (*see* **Note 10**).
6. Cool down the tube to 37 °C (*see* **Note 11**).
7. Add 200 μL of streptavidin RiboMinus™ Magnetic Beads previously suspended in hybridization buffer (*see* **Note 12**).
8. Incubate the tube at 37 °C for 15 min in water bath (*see* **Note 13**).
9. Place the tube on magnetic separator to pellet the magnetic beads to the bottom (*see* **Note 14**).
10. Collect the supernatant containing mRNA.
11. Repeat the **steps 7–10** with 250 μL of streptavidin RiboMinus™ Magnetic Beads to purify mRNA further.
12. Transfer the final supernatant to a new tube.
13. Add 1 μL of 20 μg/μL glycogen, 30 μL of 3 M sodium acetate, and 750 μL of 96 % ethyl alcohol.
14. Mix by simple inversion and incubate at −80 °C for 30 min to precipitate mRNA.
15. Centrifuge the tube at 12,000 × *g* for 15 min at 4 °C to pellet down mRNA.
16. Discard the supernatant and wash the pellet with 500 μL ice-cold 70 % ethyl alcohol.

17. Dry the pellet and dissolve mRNA in 5 μL of nuclease-free water.
18. Check the concentration and purity of mRNA in a NanoDrop (*see* **Note 15**).
19. Now, the mRNA (poly A^+ RNA) is ready for cDNA synthesis (*see* **Note 16**).

3.5 First Strand cDNA Synthesis

1. Take 2 μg of poly A^+ RNA from each sample in separate PCR tubes and make up their volumes to 4 μL (*see* **Note 17**).
2. Add 1 μL of 10 μM cDNA synthesis primer and incubate the tubes at 70 °C for 5 min in a thermocycler.
3. Cool on ice for 5 min and centrifuge the tubes briefly to bring the samples to the bottom.
4. Add the following components to each tube (*see* **Note 18**):

5× first strand buffer	2 μL
10 mM dNTP mix	1 μL
20 mM DTT	1 μL
SMARTScribe™ reverse transcriptase	1 μL

5. Incubate the tubes at 42 °C in a thermocycler.
6. After 2 h, terminate the reaction by snap cooling the tubes on ice.
7. Proceed to second strand cDNA synthesis.

3.6 Second Strand cDNA Synthesis

1. To each first strand cDNA synthesis reaction tube, add the following components to reach the final volume of 80 μL:

5× second strand buffer	16 μL
10 mM dNTP mix	1.6 μL
20× second strand enzyme cocktail	4 μL
Sterile distilled water	48.4 μL

2. Incubate the tubes at 16 °C in a thermocycler for 2 h.
3. Add 1 μL T4 DNA Polymerase to the tubes and continue the incubation at 16 °C for 1 h.
4. Add 4 μL of EDTA–glycogen mix to terminate the reaction.
5. Transfer the reaction mixtures to Eppendorf tubes, add equal volume of chloroform–isoamyl alcohol (24:1) and mix gently by pipetting up and down (*see* **Note 19**).
6. Centrifuge the tubes at 12,000 × *g* for 10 min at room temperature.

7. Collect the top aqueous layer in a new tube, add 100 μL of chloroform–isoamyl alcohol (24:1) and mix (*see* **Note 19**).
8. Centrifuge the tubes at 12,000 × *g* for 10 min at room temperature and collect the upper aqueous layer in a new tube.
9. Add 25 μL 4 M ammonium acetate, 187.5 μL 100 % ethyl alcohol and mix gently to precipitate cDNA.
10. Pellet down the cDNA by centrifugation at 14,000 × *g* for 10 min at room temperature.
11. Discard the supernatant and wash the pellet with 100 μL of 70 % ethyl alcohol.
12. Air-dry and dissolve the pellet in 50 μL sterile distilled water.
13. To ensure the quality and quantity, check the cDNA in a NanoDrop (*see* **Note 20**).

3.7 Subtraction of cDNA

3.7.1 RsaI Digestion

1. Digest tester and driver cDNA with RsaI restriction enzyme to generate shorter blunt ended ds cDNA fragments.
2. Set up the reaction for each tester and driver as below in PCR tubes.

cDNA	43.5 μL
10× RsaI restriction buffer	5 μL
10 U/μL RsaI restriction enzyme	1.5 μL

3. Mix the components by pipetting up and down.
4. Incubate the tubes at 37 °C for 2 h in a thermocycler.
5. Terminate the reaction by adding 2.5 μL of EDTA–glycogen mix to the tubes.
6. Add 50 μL chloroform–isoamyl alcohol (24:1) and mix thoroughly by pipetting up and down (*see* **Note 19**).
7. Centrifuge the tubes at 12,000 × *g* for 10 min at room temperature.
8. Collect the top aqueous layer in fresh tubes and repeat the **steps 6** and **7** once again.
9. Carefully transfer the upper aqueous layer to sterile Eppendorf tubes.
10. Add 25 μL of 4 M ammonium acetate, 187.5 μL of 100 % ethyl alcohol and mix gently to precipitate.
11. Pellet down the digested cDNA by centrifugation at 14,000 × *g* for 10 min at room temperature.
12. Remove the supernatant and wash the pellet with 70 % ethyl alcohol.
13. Air-dry and dissolve the pellet in 5.5 μL of sterile water (*see* **Note 21**).

14. To ensure the quality and quantity, check the digested cDNA in a NanoDrop (*see* **Note 22**).
15. Store the RsaI-digested cDNA at –20 °C.

3.7.2 Adaptor Ligation of Tester cDNA

1. Dilute 1 μL RsaI-digested tester cDNA to 4 μL using sterile distilled water.
2. Aliquot 2 μL of the diluted tester cDNA in 2 PCR tubes (Tube 1 and Tube 2) for ligating adaptors 1 and 2R respectively.
3. Set up the reaction in Tube 1 and Tube 2 as below (*see* **Note 18**):

	Tube 1	Tube 2
Diluted tester cDNA	2 μL	2 μL
10 μM Adaptor 1	2 μL	–
10 μM Adaptor 2R	–	2 μL
5× Ligation buffer	2 μL	2 μL
400 U/μL T4 DNA ligase	1 μL	1 μL
Sterile water	3 μL	3 μL

4. Incubate the tubes at 16 °C in a thermocycler for 12 h.
5. Add 1 μL of EDTA–glycogen mix to the tubes to terminate the reaction.
6. Inactivate the enzyme by heating the tubes to 72 °C for 5 min in thermocycler.
7. Now adaptor ligated tester cDNAs are ready and can be stored at –20 °C (*see* **Note 23**).

3.7.3 First Hybridization

1. RsaI-digested cDNA from tissues without hypericin glands (as described in Subheading 3.7.1) is used as driver.
2. In the first hybridization, an excess of driver cDNA is hybridized with tester cDNA ligated to adaptor 1 and 2R in two reactions.
3. Set up first hybridization reaction in two PCR tubes (Tube 1 and Tube 2) as described below:

	Tube 1	Tube 2
RsaI-digested Driver cDNA	1.5 μL	1.5 μL
Tester cDNA ligated to adaptor 1	1.5 μL	–
Tester cDNA ligated to adaptor 2R	–	1.5 μL
4× hybridization buffer	1 μL	1 μL
Final volume	4.0 μL	4.0 μL

4. Incubate the tubes in a thermocycler for 2 min at 98 °C for denaturation followed by 6–12 h at 68 °C for annealing (*see* **Note 24**).
5. Now the first hybridization is over and the samples are ready for second hybridization.

3.7.4 Second Hybridization

1. Prepare more driver cDNA to be used in the second hybridization by mixing the following in a PCR tube:

RsaI-digested driver cDNA	1 μL
4× hybridization buffer	1 μL
Sterile water	2 μL

2. Heat at 98 °C for 2 min for denaturation of driver cDNA.
3. Mix the two first hybridization samples at 68 °C, add the denatured driver cDNA, and mix by pipetting up and down (*see* **Note 25**).
4. Continue incubation at 68 °C, overnight.
5. After overnight incubation, add 200 μL of dilution buffer to the tube and continue incubation at 68 °C for further 5 min (*see* **Note 26**).
6. Store the subtracted product at –20 °C.

3.7.5 First Nested PCR

1. Prepare reactions in 7 PCR tubes, each with the following components:

Subtracted product	1 μL
10 mM dNTP mix	0.5 μL
10 μM PCR primer 1	1 μL
10× PCR buffer	2.5 μL
Taq DNA polymerase	1 U

2. Adjust the final volume of reaction mixture to 25 μL using sterile distilled water. Mix well by pipetting up and down gently.
3. Perform the PCR reaction: 5 min at 75 °C for end filling, followed by 27 cycles of denaturation (94 °C, 30 s), annealing (66 °C, 30 s), and extension (72 °C, 2 min) in the thermocycler (*see* **Note 27**).
4. Resolve 8 μL of PCR product in 2 % (w/v) agarose gel electrophoresis. This will give a very faint smear and some bands in between (Fig. 3).

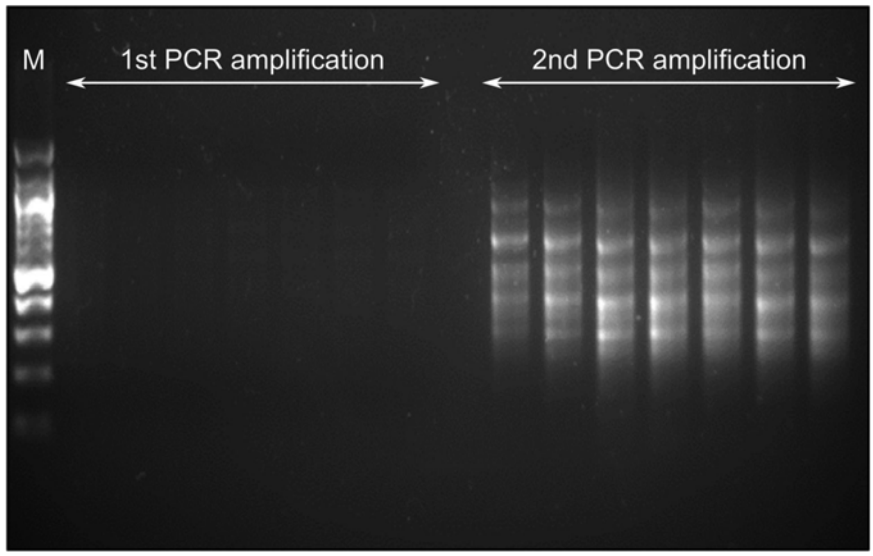

Fig. 3 First and second PCR amplification of subtracted cDNA. M—marker-TriDye™ 100 bp DNA Ladder

3.7.6 Second PCR Amplification

1. Prepare template for second PCR amplification by diluting 3 μL of PCR product from each first amplification reaction to 30 μL with sterile distilled water (*see* **Note 28**).
2. Set up second PCR reaction in 7 PCR tubes as described below (*see* **Note 18**):

Diluted product from 1st PCR	1 μL
10× PCR buffer	2.5 μL
10 mM dNTP mix	0.5 μL
10 μM nested PCR primer 1	1 μL
10 μM nested PCR primer 2R	1 μL
Taq DNA polymerase	1 U

3. Adjust the final volume of reaction mixture to 25 μL using sterile distilled water.
4. Perform PCR amplification: 30 cycles of denaturation (94 °C, 30 s), annealing (66 °C, 30 s), and extension (72 °C, 2 min) in the thermocycler.
5. Purify the subtracted PCR product using QIAquick PCR Purification kit.
6. Resolve 8 μL of PCR product in 2 % (w/v) agarose gel electrophoresis (*see* **Note 29**).
7. Here the subtracted cDNA is ready for cloning (Fig. 3).

3.8 Construction of Subtracted cDNA Library

3.8.1 Ligation of Subtracted Product

1. Mix the following components in a sterile tube (*see* **Note 18**).

Purified subtracted PCR product	100 ng
10× T4 DNA ligase buffer	1 μL
pGEMT easy cloning vector	250 ng
10 U/μL T4 DNA ligase enzyme	1 μL

2. Adjust the reaction volume to 10 μL with sterile distilled water.
3. Incubate at 16 °C for 24 h in a thermocycler for ligation.

3.8.2 Transformation of E. coli

1. Thaw an Eppendorf tube containing 200 μL of competent *E. coli* cells on ice.
2. Add 5 μL of the ligation product to the bacterial suspension and mix gently by pipetting up and down.
3. Incubate the tube on ice for 30 min.
4. After incubation, give a brief heat shock to the cells in a water bath at 42 °C for 90 s.
5. Place the tube on ice for 5 min.
6. Add 800 μL LB medium and incubate the culture at 37 °C with shaking at 100–150 rpm/min for 1 h.
7. Spread 50 μL aliquots of bacterial suspension on LB agar plates prepared for blue–white colony screening (*see* **Note 30**).
8. Incubate the plates at 37 °C. Blue and white colonies normally appear after overnight incubation (Fig. 4).

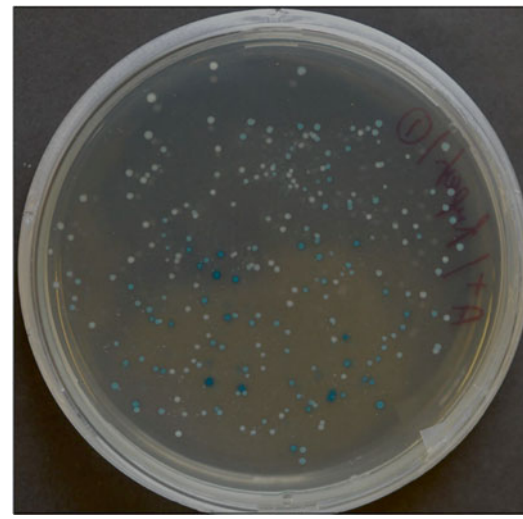

Fig. 4 Blue and white colonies on LB agar plates containing IPTG and X-Gal after 24 h incubation at 37 °C

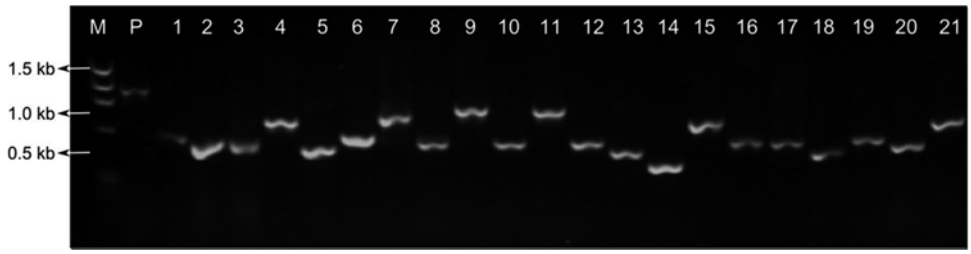

Fig. 5 Colony PCR of white colonies showing varying amplicon size. M—marker-TriDye™ 100 bp DNA Ladder, P—positive control and lanes 1–21 are bacterial colonies

3.8.3 Screening of Library by Colony PCR

1. Prepare a master mix cocktail sufficient for the desired number of reactions, each containing the following components and enough water to bring the volume to 50 µL (*see* **Note 18**).

M13 F	1 µL
M13 R	1 µL
dNTPs	1 µL
Taq buffer	5 µL
Taq polymerase	0.2 µL

2. Carefully pick the white colonies using toothpick and transfer to the PCR tubes containing 50 µL PCR reaction mixture.
3. Mix well by pipetting up and down gently.
4. Amplify the inserts with a hot start at 94 °C for 3 min, followed by 35 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 30 s) and extension (72 °C, 1.5 min), with a final extension of 5 min at 72 °C in the thermocycler.
5. Resolve 4 µL PCR product on 0.8 % agarose gel to roughly estimate the insert size (Fig. 5).
6. Store the remaining PCR product at −20 °C which may be used for sequencing.

3.9 Sequencing and Sequence Analysis

1. Choose clones having equal to or more than 500 bp amplicon size (*see* **Note 31**).
2. Purify PCR product using QIAquick PCR Purification kit.
3. Purified PCR product can be directly sequenced (*see* **Note 32**).
4. We have sequenced the PCR products using M13 F universal primer in an ABI Prism automated DNA sequencer by single pass reading.

3.10 Hypothetical Functional Annotation of Genes

1. Clean up vector sequence and other impurities such as adaptor and primer sequences using VecScreen (http://www.ncbi.nlm.nih.gov/tools/vecscreen/).

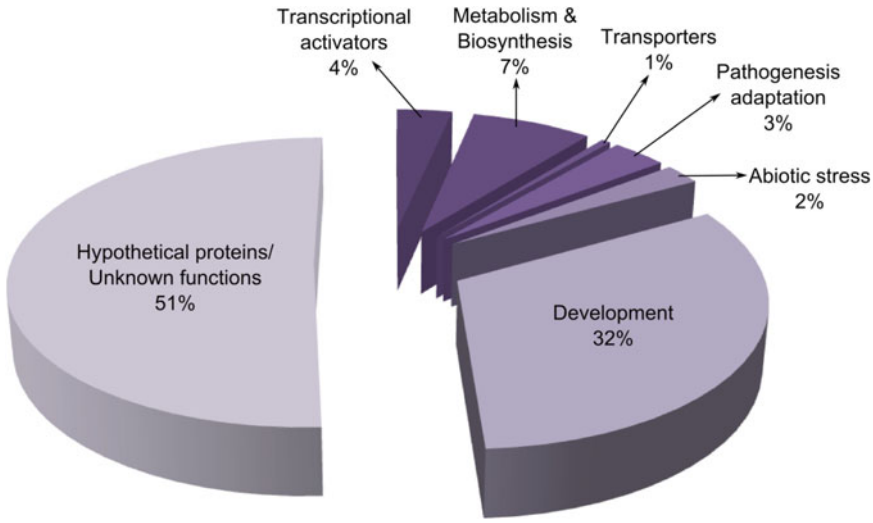

Fig. 6 Pie chart depicting functional classification of expressed transcripts identified from the hypericin gland-specific subtracted library

2. Edited sequences are then analyzed by BLASTx and BLASTn to assign putative functions on the basis of sequence similarity to genes or proteins of known function in GenBank (www.ncbi.nlm.nih.in).
3. Assemble the sequences into clusters based on the presence of overlapping, identical or similar sequences.
4. In our case, a total of 300 ESTs were analyzed in GenBank and their putative functions are assigned.
5. Some sequences do not give any homology with the available database and these are treated as unknown/novel genes.
6. All these ESTs with significant homology with previously reported genes and others with unknown functions are summarized in a pie diagram (Fig. 6).

4 Notes

1. **Steps 4–7** should be carried out inside the laminar airflow chamber.
2. Avoid skin contact to bleach. Commercial bleach can vary in active chlorine content. Make sure to check the product label and adjust dilution to obtain a 1.5 % (v/v) final concentration of active chlorine.
3. Before use, mortar and pestle must be wiped with 70 % ethyl alcohol, cleaned properly with RNAse-free water, and dried in hot air oven overnight to avoid contamination.

4. Ensure to add 2 % βMe to the pre-warmed buffer. After adding βMe, all the steps should be performed inside fume hood chamber to avoid spillage in air.
5. Alternatively, RNA can be precipitated by incubating the tubes at −80 °C for 4 h.
6. Once dissolved, take 1–2 μL from each sample in another tube for quantification. Rest of the samples must be frozen quickly in liquid nitrogen and stored immediately at −80 °C to avoid degradation.
7. For our samples, the ratio of absorbance at 260/280 nm was 2.1, which represent an excellent quality of RNA. Generally, the concentration is about 2 μg/μL. When RNA concentration is too high, it is better to dilute the sample and then calculate the quantity accordingly.
8. For this method of mRNA purification, the total RNA concentration must be more than 1 μg/μL. Although other methods of mRNA purification are possible, using this method would result in mRNA devoid of rRNA contamination, which is good for downstream sequencing.
9. RiboMinus probe is specific for conserved regions of the large cytosolic and chloroplast rRNA transcripts. Each probe is single stranded and contains 3′ LNA™ (Locked Nucleic Acid) monomers incorporated at specific locations. The incorporation of LNA into the oligonucleotide probe increases the rRNA-probe stability.
10. This would facilitate the binding of RiboMinus probe to cytosolic and chloroplast rRNA transcripts.
11. Do not cool down by keeping the tubes on ice. Changing the temperature of the water bath to 37 °C without disturbing the tubes would allow the samples to cool gradually.
12. Follow the manufacturer's protocol for the preparation of beads suspended in hybridization buffer.
13. As the 5′ end of each RiboMinus probe is conjugated to biotin, rRNA/probe complexes bind to streptavidin RiboMinus™ Magnetic Beads. To facilitate binding of more rRNA/probe complexes to the beads, incubation time may be increased to 20 min and the tubes can be gently tapped every 5 min during incubation.
14. Large cytosolic and chloroplast rRNA transcripts bound with RiboMinus probes are now precipitated with magnetic beads.
15. For our preparation, the 260/280 ratio is 2.0 and the concentration is 500 ng/μL.
16. Proceed to cDNA synthesis immediately because mRNA may degrade very fast. Therefore, it is always recommended to convert mRNA into cDNA before storing.

17. It is important to maintain the volume of 4 μL. If needed, use RNase-free water to make up the volume.
18. Enzyme should be added finally. After adding enzyme, mix the reaction mixture gently by pipetting up and down and bring the solution to the bottom by brief centrifugation.
19. This should be performed inside the fume hood chamber to avoid spillage in air.
20. For our preparation, the 260/280 ratio was 1.8 and the concentration was 35 ng/μL for each sample.
21. Make sure that no trace of ethyl alcohol is left in the tube.
22. For our preparation, the 260/280 ratio was 1.8 and the concentration was 250 ng/μL for each sample.
23. If proceeding to hybridization immediately, the tubes can be maintained on ice.
24. Annealing results in tester–tester homohybrid, tester–driver heterohybrid, double-stranded driver, single-stranded driver, and single-stranded tester in both reactions. Due to the second order of hybridization kinetics, generating homohybrid and heterohybrid cDNAs is faster for more abundant molecules than annealing of the less abundant cDNAs that remain single-stranded.
25. Mixing of first hybridization samples with denatured drivers should be very quick. Under these conditions, only single stranded type cDNAs are able to reassociate and form tester–tester homohybrids, tester–driver heterohybrids, and new tester–tester heterohybrids. The later hybrids are double-stranded tracer molecules with different single-stranded ends, one of which corresponds to adaptor 1 and another to adaptor 2R.
26. Here the subtraction process is over and the subtracted product has the genes specific to hypericin glands.
27. After filling in the ends, the differentially expressed tester sequences will have different primer binding at 5′ and 3′ ends
28. Store the rest of the PCR product at −20 °C for future use.
29. Show a clear smear with some bands in between.
30. Prepare LB medium with 1.5 % agar. Once the medium reached about 60 °C, add 50 mg/L ampicillin and pour 20 mL medium in each petri dish. Once solidified, spread 10 μL of IPTG and 40 μL of X-Gal on the surface of the medium. IPTG and X-Gal should be spread at least 10 min before plating bacteria so that they will be absorbed properly by the medium.
31. Inserts of 300–500 bp size are good for sequencing. Since amplification with M13 universal primer includes approximately 150 bp from the vector sequence, the clones having more than 500 bp amplicon must carry 250 bp from the cDNA.
32. Alternatively plasmids can be isolated and sequenced.

Acknowledgment

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Chapter 23

In Vitro and Cryopreservation Techniques for Conservation of Snow Mountain Garlic

Ritu Mahajan

Abstract

Garlic is an important medicinal herb of culinary value by imparting its flavors and odors to the food. Allicin, a notable flavonoid in garlic, is a powerful antibiotic and antifungal compound. Due to poor bioavailability, garlic is of limited use for oral human consumption. Being sexually sterile, propagation of garlic is done by individual cloves from a bulb which increases the chances of transfer of viral diseases. In this chapter, an efficient and improved regeneration protocol for explant establishment and shoot multiplication under in vitro conditions is described. A high rate of shoot multiplication is obtained on MS medium supplemented with 0.5 mg/l BAP, 1.0 mg/l KN, and 2.0 mg/l GA_3. Addition of 1.0 mg/l NAA to MS medium resulted in rooting at the shoot bases. A detailed method for encapsulation of explant in sodium alginate beads and their cryopreservation using encapsulation-dehydration is also described.

Key words Garlic, In vitro, Micropropagation, Conservation, Cryopreservation, Encapsulation

1 Introduction

Garlic (*Allium sativum*), belonging to family Alliaceae, is a potential herb of nutraceutical value and is widely cultivated for its medicinal importance, which is due to the presence of number of secondary metabolites [1]. It is used to enhance the flavor of foods and also to prevent age-related problems like cardiovascular and Alzheimer's disease [2]. The plant has antibiotic, antiaging, antitumor, and antiantherosclerosis value, besides having antioxidant, antifungal, and antibacterial properties [3, 4]. The antimicrobial property is due to the presence of allicin (allyl 2-propene thiosulfinate) which is formed by the action of allinase enzyme [5, 6]. Besides being rich in phenolics and flavonoids, garlic also contains some sulfur-containing compounds such as alliin, S-allylcysteine, and diallylsulfide [7, 8].

Snow Mountain Garlic (also called as Kashmiri garlic), an important herb, is found in the snow-covered mountains of the Himalayas at high altitudes (6000 ft). It can survive at extremely

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low temperatures of −10 °C. In ancient times, this garlic was used by the mountaineers to raise their energy levels and also to detoxify their body in extreme cold weather conditions. It is generally consumed by local people as a remedy for rheumatoid arthritis.

Garlic is sexually sterile, so the conventional method of multiplication is through vegetative propagation which is less efficient as the rate of the propagation is low (approx. 5–10 %). The method is labor intensive as it takes many years to produce sufficient number of seed bulbs for the cultivation of a new variety [9]. This necessitates establishment of a protocol for in vitro propagation for shoot induction and proliferation. This method is an important tool in conservation as it produces a large number of genetically alike plants that can be cultivated from only one single plant stock [10, 11]. Ex situ conservation techniques like cryopreservation is a safe strategy for long-term preservation so as to avoid chilling injury and dehydration injury to the plants that remain viable for a short time and also for plant species that are endangered and threatened [12–14].

2 Materials

2.1 Plant Material

Cloves of snow mountain garlic are collected from Kashmir Valley and used for all the experiments (Fig. 1).

2.2 Equipments and Apparatus

1. Hot plate.
2. PH meter.
3. Autoclave.
4. Laminar flow chamber.

Fig. 1 Cloves of snow mountain garlic

5. Water distillation unit.
6. Water Millipore system.
7. Refrigerator.
8. Deep freezer (−20 °C).
9. Water bath.
10. Cryocan (50 L).
11. Analytical weighing balance (milligram to few grams).
12. Incubator shaker.

2.3 Preparation of Reagents

Prepare all the reagents of analytical grade at room temperature using autoclaved double distilled water and store them in the refrigerator (4 °C).

2.3.1 Media Preparation

1. MS media (*see* ref. 15), (*see* Table 1) (*see* **Notes 1** and **2**).
2. Stock solutions of BAP (benzyl amino purine), kinetin (6-furfuryl amino purine), NAA (naphthalene acetic acid), GA_3 (gibberellic acid).
3. Autoclaved flasks (250 ml, 500 ml, and 1000 ml).
4. Autoclaved reagent bottles (100 ml, 250 ml, and 500 ml).
5. Measuring cylinders (100 ml, 500 ml, and 1000 ml).
6. Micropipettes (0.5–100 μl, 20–1000 μl).
7. Sterilized microtips.
8. Cotton plugs.
9. Buffers (pH 4.0, 7.0, 9.0).
10. 1.0 N NaOH/1.0 N HCl.
11. Glass rod.
12. Lab markers.

2.3.2 Explant Preparation and In Vitro Multiplication

1. Stainless steel sterile forceps (7 ″ and 11″).
2. Scalpel handle (No. 3; 5 in.).
3. Sterile surgical blades (11 No.).
4. Sterile petri plates (100 × 20 mm).
5. Autoclaved flasks 250 ml.
6. Autoclaved beakers (100 ml).
7. Autoclaved culture tubes (20 × 150 mm).
8. 0.2 % Tween 20.
9. 0.5 % mercuric chloride.
10. 70 % ethanol.
11. $KMnO_4$ 1 ppm.

Table 1
Composition of Murashige and Skoog medium [15]

Macrosalts	(mg/lit)
$(NH_4)NO_3$	1650
KNO_3	1900
$CaCl_2 \cdot 2H_2O$	440
$MgSO_4 \cdot 7H_2O$	370
KH_2PO_4	170
Microsalts	
$FeSO_4 \cdot 7H_2O$	27.8
$Na_2EDTA \cdot 2H_2O$	37.2
$MnSO_4 \cdot 4H_2O$	22.3
$ZnSO_4 \cdot 7H_2O$	8.6
$H_3BO_3 \cdot 7H_2O$	6.2
KI	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CuSO_2 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
Vitamins	
Glycine	2.0
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Myo-inositol	100
Sucrose	30,000
Agar	7 %

12. Disinfectant for hand sanitization.
13. Thermocol cups.
14. Test tube stand.
15. Plastic pots 5″.

2.3.3 Cryopreservation Components

1. Liquid MS media.
2. 3 % sodium alginate (w/v) in liquid MS media at pH 5.7 (*see* **Note 3**).
3. Shoot tips from 8-week-old in vitro-grown snow mountain garlic plants.

4. 100 mM $CaCl_2$.
5. 0.4 M sucrose.
6. Recovery media (MS medium without growth regulators).
7. Autoclaved forceps and scalpel handle with sterilized blade.
8. Cryovials (2.0 ml).
9. Cryostrips.
10. Cryogloves.
11. Sterile petri plates (60 × 15 mm).
12. Liquid nitrogen.
13. 70 % ethanol.
14. Parafilm.
15. Whatman No. 1 filter paper.
16. Thermometer.
17. Timer.
18. Double distilled water.
19. Cryobox (4 °C).

3 Methods

3.1 MS Medium for Micropropagation and Organogenesis

1. Mix all the stock solutions (macro nutrients, micronutrients, Fe-EDTA, and vitamins) in 600 ml distilled water in 1 L flask (*see* **Note 1**; Table 1).
2. Add 100 mg myoinositol and 30 gm sucrose in the flask. Stir with a glass rod.
3. Make the final volume to 1 L with distilled water.
4. Adjust the pH to 5.8 by adding 0.1 N HCl or 0.1 N NaOH.
5. Add 7 % agar, mix well, and boil the solution till it becomes homogeneous.
6. For liquid MS medium agar is not added.
7. For shoot multiplication, add BAP (0.1 mg/l, 0.5 mg/l, and 1.0 mg/l) and kinetin (0.1 mg/l, 0.5 mg/l, and 1.0 mg/l) to solid MS medium. The concentration of GA_3 is kept constant (2.0 mg/l).
8. For root induction add NAA (0.1 mg/l, 0.5 mg/l, and 1.0 mg/l) to solid MS medium.
9. Dispense the medium in flask and test tubes, seal them with cotton plugs, and autoclave (*see* **Note 2**).
10. After autoclaving store the culture vessels (flasks and test tubes) at 25 °C in dust-free closed cabinet.

3.2 Preparation of Explant

1. Wipe the floor of the laminar flow chamber with ethanol, keep all the glassware (culture vessels, flasks, petri plates, and beakers), forceps, and scalpel with surgical blades inside the chamber, and switch on the UV light for 15–20 min.
2. Remove the outer, dry, papery bulb scales of the healthy cloves using forceps.
3. Wash the cloves with two to three drops of Tween 20 in 100 ml distilled water for 10–15 min and then rinse them twice for 5–10 min with distilled water in laminar flow chamber.
4. Surface-sterilize cloves by washing them in 70 % ethanol for 20–30 sec, $KMnO_4$ (1 ppm for 2 min) and then with 0.5 % $HgCl_2$ for 3 min, containing two drops of Tween 20 per 100 ml in an autoclaved beaker. Again wash the cloves three times for 2 min in autoclaved distilled water.

3.3 Establishment of Aseptic Cultures

1. Excise the shoot bases of the cloves aseptically inside a laminar flow chamber, with sterile surgical blades (*see* **Note 4**).
2. Inoculate the explants on basal MS media (without growth hormones) in the flasks for their initial establishment (Fig. 2a, b).
3. Incubate the cultures in the flasks at 25 ± 2 °C and 50–60 μmol/m^2/s (light intensity) for 3–4 weeks and monitor them for the presence of any contamination.

3.4 Shoot Multiplication

1. After the initial aseptic establishment of cultures, transfer the shoot cultures on to MS medium containing 0.5 mg/l BAP, 1.0 mg/l KN, and 2.0 mg/l GA3 (*see* **Note 5**).
2. After 4 weeks shoot multiplication will occur (Fig. 3a, b). Record the data. Maintain the shoot cultures by regular subculturing at an interval of every 2–3 weeks. Take every possible care to prevent any further contamination.

3.5 Rooting and Acclimatization

1. Separate the shoots (7–8 cm) and culture them singly on the MS medium containing 1.0 mg/l NAA in the test tubes for root induction (Fig. 4a).
2. After 4 weeks, take out the plants having well developed roots (Fig. 4b) and wash them gently with running tap water so as to remove all the media sticking to them.
3. Place plants on the cotton, dipped in autoclaved distilled water, on a tray and keep them in the culture room for 1 week at 25 ± 2 °C.
4. Transfer the rooted plantlets in the pots containing autoclaved sand and soil in the ratio 1:3 and cover the plants with glass jars so that around 60–70 % humidity is maintained (*see* **Note 6**).
5. Keep the plants in the glasshouse and after 2 weeks remove the jars. Allow the plants to grow further for 7–8 weeks in the glasshouse and subsequently transfer them to the field.

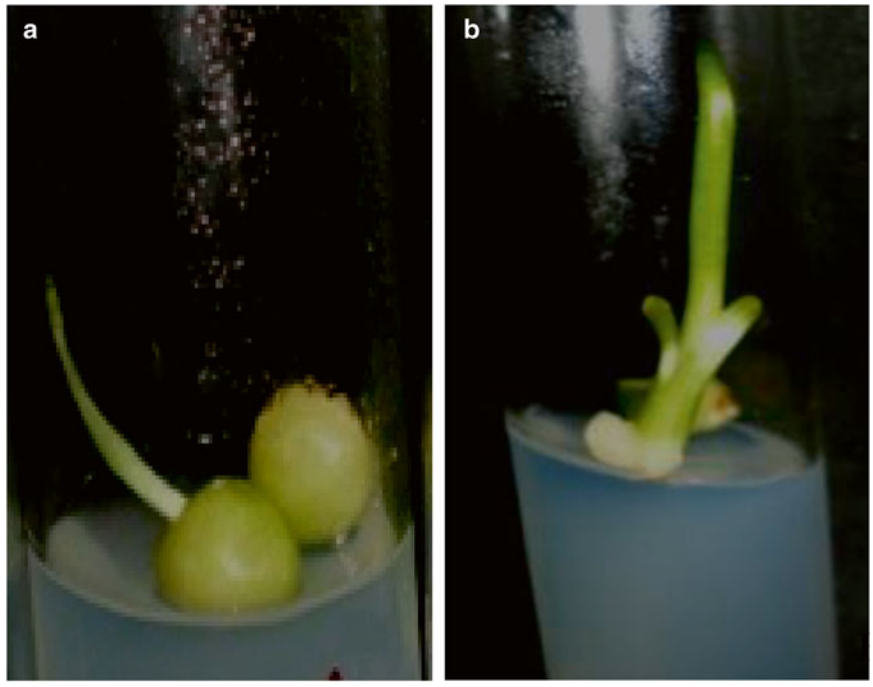

Fig. 2 (**a**) Shoot initiation from the explant inoculated on basal MS medium. (**b**) Shoot elongation on MS medium supplemented with growth hormones

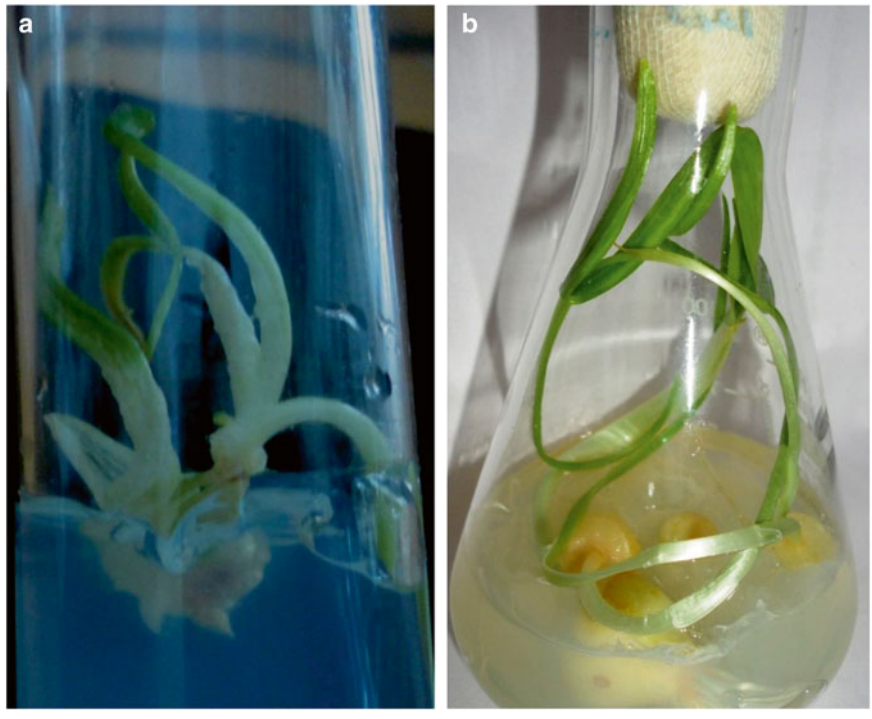

Fig. 3 Shoot multiplication in (**a**) MS medium supplemented with 0.1 mg/l BAP, 0.1 mg/l KN (**b**) MS medium supplemented with 0.5 mg/l BAP, 1.0 mg/l KN, and 2.0 mg/l GA3

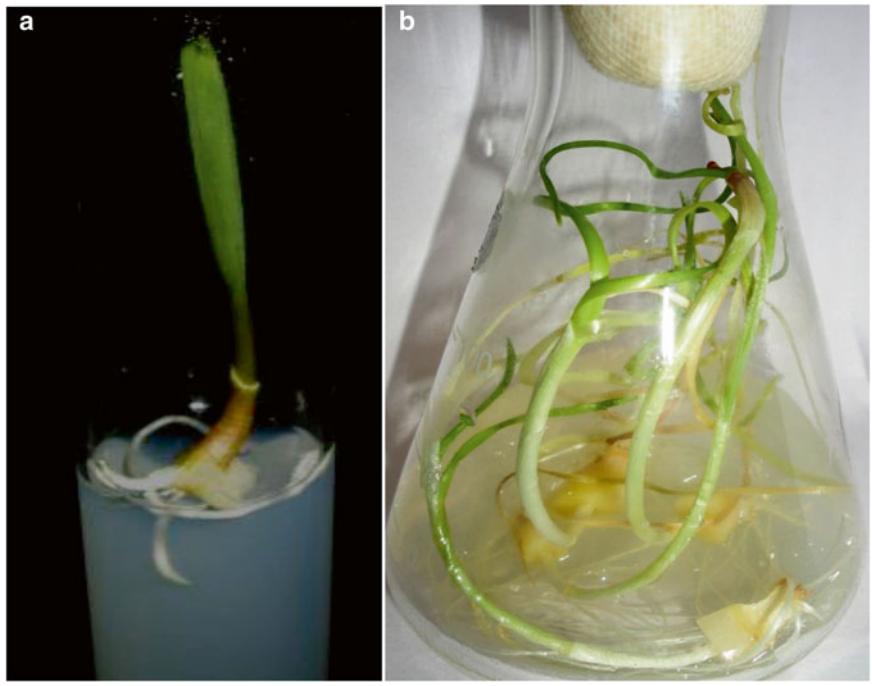

Fig. 4 (**a**) Root initiation from the shoots on MS medium supplemented with 1.0 mg/l (**b**) Well developed roots after 4 weeks

3.6 Encapsulation-Dehydration

1. Excise the shoot tips from 8-week-old in vitro-grown plants of snow mountain garlic in laminar flow chamber.
2. Encapsulate the explants (shoot tips) into alginate beads in 3 % sodium alginate (w/v) in liquid MS media at pH 5.7 (*see* Fig. 4a, *see* **Note 7**).
3. Allow the beads to polymerize in MS medium for 30 min with 100 mM $CaCl_2$ solution. Pretreat the beads with liquid MS medium containing 0.4 M sucrose solution for 48 h in a rotary shaker (50 rpm) at 25 °C (*see* **Note 8**).
4. Blot the beads dry on sterile Whatman's filter paper and desiccate them for 6 h (approx. 20 % moisture content) in a glass petri plate under laminar flow (0.6 m/s) at 24–25 °C and 35 ± 2 % relative humidity (*see* **Note 9**).
5. Place the dried beads into 1.2 ml autoclaved cryovials (10 beads per cryovial). Plunge the cryovials directly into liquid nitrogen (*see* **Note 10**).
6. Thaw the cryovials at 45 °C in distilled water maintained at 25 °C for 1 min in a water bath (*see* **Note 11**). Shake the cryovials vigorously during thawing for 1.5 min so as to prevent crystal formation.

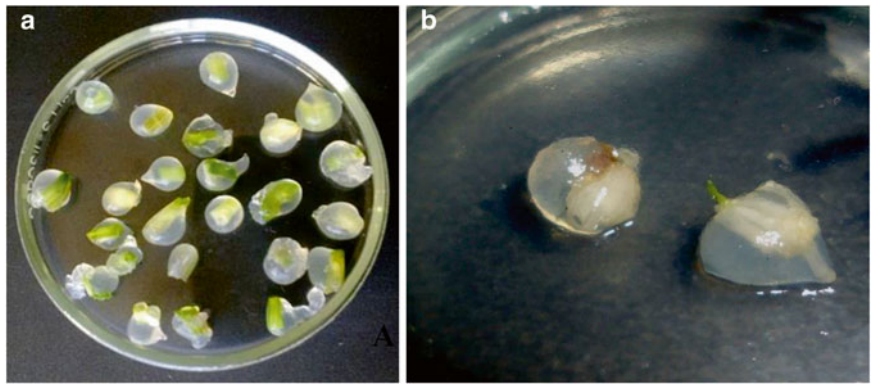

Fig. 5 (**a**) Explants encapsulated in alginate beads (**b**) Shoots emerging from encapsulated explant in the petri plate containing MS media with 0.5 mg/l BAP and 0.5 mg/l KN

7. Wipe the cryovials to remove excess water and then wipe them with 70 % ethanol.
8. Transfer the cryopreserved beads in liquid MS medium and rehydrate them for 5 min.
9. Transfer the beads onto recovery medium in petri plates.
10. Keep the petri plates in diffused light 36 μmol/m^2s for 5 days at 25 ± 2 °C and then transfer them to normal light conditions (*see* **Note 12**).
11. Check the petri plates for the greening of explants after 2 weeks of post culture.
12. After 3 weeks transfer the beads with green explants to flasks containing MS media supplemented with BAP (0.5 mg/l) and KN (0.5 mg/l) (Fig. 5b).
13. Subculture the elongated shoots for shoot multiplication on MS medium containing 0.5 mg/l BAP and 1.0 mg/l kinetin.

4 Notes

1. MS media is most commonly used as solid, semisolid, and liquid. Prepare stock solutions of all macronutrients, micronutrients, Fe-EDTA, and vitamins in reagent bottles (250 ml) and store at 4 °C. Similarly prepare the stock solutions of plant growth regulators (stock 1 mg/L) in 100 ml reagent bottles and store at 4 °C. Some medium components such as amino acids and vitamins are heat labile and get destroyed on autoclaving, so sterilize them by filtration through filter membranes (0.22–0.45 μm).
2. Autoclaving is highly effective and the commonly used method for sterilization of culture medium, glassware, distilled water, and

tissue culture tools. The intense heat generated from steam kills all bacteria, viruses, fungal spores by hydrolyzing their proteins.

3. Both 3 % sodium alginate and 100 mM $CaCl_2$ are most suitable for bead formation. When sodium alginate is added to $CaCl_2$ solution, the calcium ions cross-link the polymers of alginate. This cross-linking creates a soft solid gel bead. Beads formed using lower concentration of sodium alginate (1.5 %) and 50 mM $CaCl_2$ are too fragile to handle, while beads formed from a high concentration of sodium alginate (5.0 %) are too hard for conversion of explants to plantlets.
4. A totipotent explant should be taken for in vitro culture which can be from any part of the plant including portions of shoots, leaves, stems, flowers, roots, and single, undifferentiated cells, as the aerial parts are highly contaminated with soil microflora. In present work both shoot disk, which is full of meristematic tissue, and whole garlic clove are used as explants.
5. Different concentrations and combinations of plant growth regulators are used in plant tissue culture for both shoot initiation and shoot multiplication. In our experiment we get best results for shoot multiplication when the MS media is supplemented with 0.5 mg/l BAP, 1.0 mg/l KN, and 2.0 mg/l GA_3 (*see* ref. 16).
6. When shoots or plantlets are transplanted from culture room to the greenhouse conditions, they may desiccate or wilt rapidly and can die due to the change in environment. So the rotted plants are covered with the jar so as to maintain the humidity (*see* Ref. 17).
7. Suspend the explants in alginate solution and then pick a single explant along with some alginate solution using a sterile pipette and slowly dispense it in 100 mM $CaCl_2$ solution. The explant gets encapsulated in alginate beads. Allow the beads to polymerize for 30 min and dispense them in hormone-free liquid MS medium containing 0.4 M sucrose.
8. The beads are treated with different concentrations of sucrose solution prior to cryopreservation so that its accumulation will increase the stability of cell membrane during severe dehydration conditions (*see* ref. 18). In our experiment, the highest survival rate is obtained by treating the beads with 0.4 M sucrose solution (*see* ref. 16).
9. The beads are dehydrated in the air current of laminar flow or the beads are kept in air-tight container containing silica gel or in a stream of compressed air (*see* ref. 19) so as to remove the excess of water from the tissues. The cells have to be sufficiently dehydrated as too much dehydration increases the toxicity as internal solutes become concentrated (*see* ref. 20).
10. Rapid cooling of cryovials in the liquid nitrogen results in the formation of microcrystals (from internal solutes of the tissue

or cell) that maintains the integrity and osmotic balance of the tissues (*see* ref. 21).

11. Rapid thawing is necessary so as to avoid the re-crystallization of the solutes present internally and which can damage the cell membranes (*see* ref. 22).
12. The petri plates containing beads are kept in the darkness or diffused light for 5–7 days after cryopreservation for preventing photo-oxidation of the explants which could be harmful to the plant material (*see* ref. 23).

Acknowledgement

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Chapter 24

In Vitro Callus Induction and Plant Regeneration from Stem Explants of *Ceropegia noorjahaniae*, a Critically Endangered Medicinal Herb

Jaykumar J. Chavan and Mahendra L. Ahire

Abstract

An efficient protocol has been developed for in vitro regeneration of a large number of plantlets of *Ceropegia noorjahaniae* Ansari via indirect organogenesis from stem explants excised from in vitro-germinated seedlings. The callus was efficiently induced from the stem explants using Murashige and Skoog (MS) medium supplemented with auxins and their combinations. The highest number of shoots (16.0 ± 0.2) and shoot length (5.5 ± 0.1 cm) was achieved when the callus was subcultured to MS medium supplemented with 6-benzylaminopurine, BAP (2.0 mg/l) and indole-3-acetic acid, IAA (0.2 mg/l). The in vitro-developed shoots were rooted well in half-strength MS medium supplemented with 1.0 mg/l of indole-3-butyric acid (IBA) and 0.3 mg/l of α-naphthalene acetic acid (NAA). The plantlets were successfully hardened with 82 % survival rate. This is the first report on the regeneration of plants through indirect shoot organogenesis from stem derived calli of *C. noorjahaniae*.

Key words Acclimatization, *C. noorjahaniae*, Callus culture, Critically endangered, Medicinal herb, Organogenesis

1 Introduction

Ceropegia L. (Apocynaceae, APG III-2009) is one of the largest genera with more than 220 species distributed in tropical and subtropical regions of the world [1]. The maximum diversity of *Ceropegia* occurs in South Africa followed by Kenya, Madagascar, and Indian subcontinent [2]. In India, there seems to be two major distribution zones for this genus, viz., the Himalayan region and the Western Ghats, two mega-biodiversity centers of the world. The species of *Ceropegia* as a whole are under threat, owing to either destructive collection or habitat degradation. Most of the *Ceropegia* species have morphologically unique flowers and hence few of them are cultivated in Europe and the USA [3, 4]. Tubers of Ceropegias are storehouse of starch, sugars, gum, albuminoids, fats, crude fiber, and other valuable phytoconstituents.

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The starchy tubers are a good source of nutritive tonic [5]. The pharmacological importance of the genus is also due to the presence of a pyridine alkaloid, cerpegin which has several pharmacological properties [6].

Ceropegia noorjahaniae Ansari is one of endemic and critically endangered species of the Western Ghats, India. The plant is well known for its edible tubers and ornamental flowers. A typical habitat of this plant is found along the slopes of Western Ghats in well-drained rocky-gravelly soil above 1000 m altitude. Being endemic, scarcity of pollinators and poor seed setting are the chief causes in its declining numbers, leading to a continuous reduction of its natural population [7]. High frequency multiplication through plant tissue culture technique would minimize the damage to remnant populations. There are several reports on direct shoot organogenesis in *Ceropegia* species (including *C. noorjahaniae*) using nodal explants [8]. However, very little work has been carried out on indirect shoot organogenesis of *Ceropegia* spp. In this context, an efficient indirect shoot organogenesis and plant regeneration system has been developed using stem explants of in vitro-germinated seedlings of *C. noorjahaniae*.

2 Material

2.1 Plant Material

1. The mature follicles of *C. noorjahaniae* are collected directly from the plants growing along the hilly slopes in the northern Western Ghats.

2.2 In Vitro Seed Germination

1. Fresh Tween 20 solution (1 % Tween 20) (*see* **Note 1**).
2. Fresh mercuric chloride solution (0.1 % $HgCl_2$) (*see* **Note 2**).
3. Murashige and Skoog (MS) [9] medium stock solutions (Table 1). Store in the freezer or cold room at 4 °C (*see* **Note 3**).
4. Agar (plant tissue culture grade).

2.3 Callus Induction and Shoot Regeneration

1. Stock solutions (10 mg/100 ml) of 6-benzylaminopurine (BAP), 2,4-dichlorophenoxy acetic acid (2,4-D), indole butyric acid (IBA), and NAA (α-naphthaleneacetic acid) (*see* **Note 4**) and store in the freezer at −20 °C.

2.4 Rooting and Acclimatization

1. One plastic pots (4.5 cm high).
2. Coco peat (*see* **Note 5**).
3. Fine-sieved sterilized sand (*see* **Note 6**).
4. Polythene bags (15 × 8 cm) filled with garden soil (*see* **Note 7**).

Table 1
Chemical composition of Murashige and Skoog's basal medium for plant tissue culture

Sr. no	Constituents	MS medium (mg/l)	Stock (mg/100 ml)	Stock required for 1 L
I	*Macronutrient stock*			
	NH_4NO_3	1650	16,500	10 ml
	KNO_3	1900	19,000	
	$MgSO_4{\cdot}7H_2O$	370	3700	
	KH_2PO_4	170	1700	
II	*CaCl2 stock*			
	$CaCl_2{\cdot}2H_2O$	440	4400	10 ml
III	*KI stock*			
	KI	0.83	83	10 ml
IV	*Micronutrient stock*			
	H_3BO_3	6.2	620	1 ml
	$MnSO_4{\cdot}4H_2O$	22.3	2230	
	$ZnSO_4{\cdot}7H_2O$	8.6	860	
	$CuSO_4{\cdot}2H_2O$	0.025	2.5	
	$CoCl_2{\cdot}6H_2O$	0.025	2.5	
V	*Na2MoO4 stock*			
	Na_2MoO_4	0.25	25	1 ml
VI	*Iron stock*			
	Na_2EDTA	37.6	752	10 ml
	$FeSO_4{\cdot}7H_2O$	27.8	556	
VII	*Vitamins + Amino acids stock*			
	Glycine	2.0	40	5 ml
	Nicotinic acid	0.5	10	
	Pyridoxine HCl	0.5	10	
	Thiamine HCl	0.1	4	

3 Methods

3.1 Plant Material and Seed Germination

1. Collect mature follicles of *C. noorjahaniae* and separate seeds from the follicles.
2. Air-dry the seeds and store in paper bags at 23–25 °C and 30–50 % relative humidity (RH).
3. Place the seeds in 100 ml flask, wash with liquid detergent (1 %, Tween 20) for 10 min with constant shaking followed by repeated rinsing with distilled water.
4. Treat the seeds with 0.1 % $HgCl_2$ for 5 min followed by rinsing thrice with sterilized distilled water (*see* **Note 8**).
5. Transfer individual seeds in culture tubes containing phytohormone-free Murashige and Skoog (MS) [9] medium (Fig. 1a).

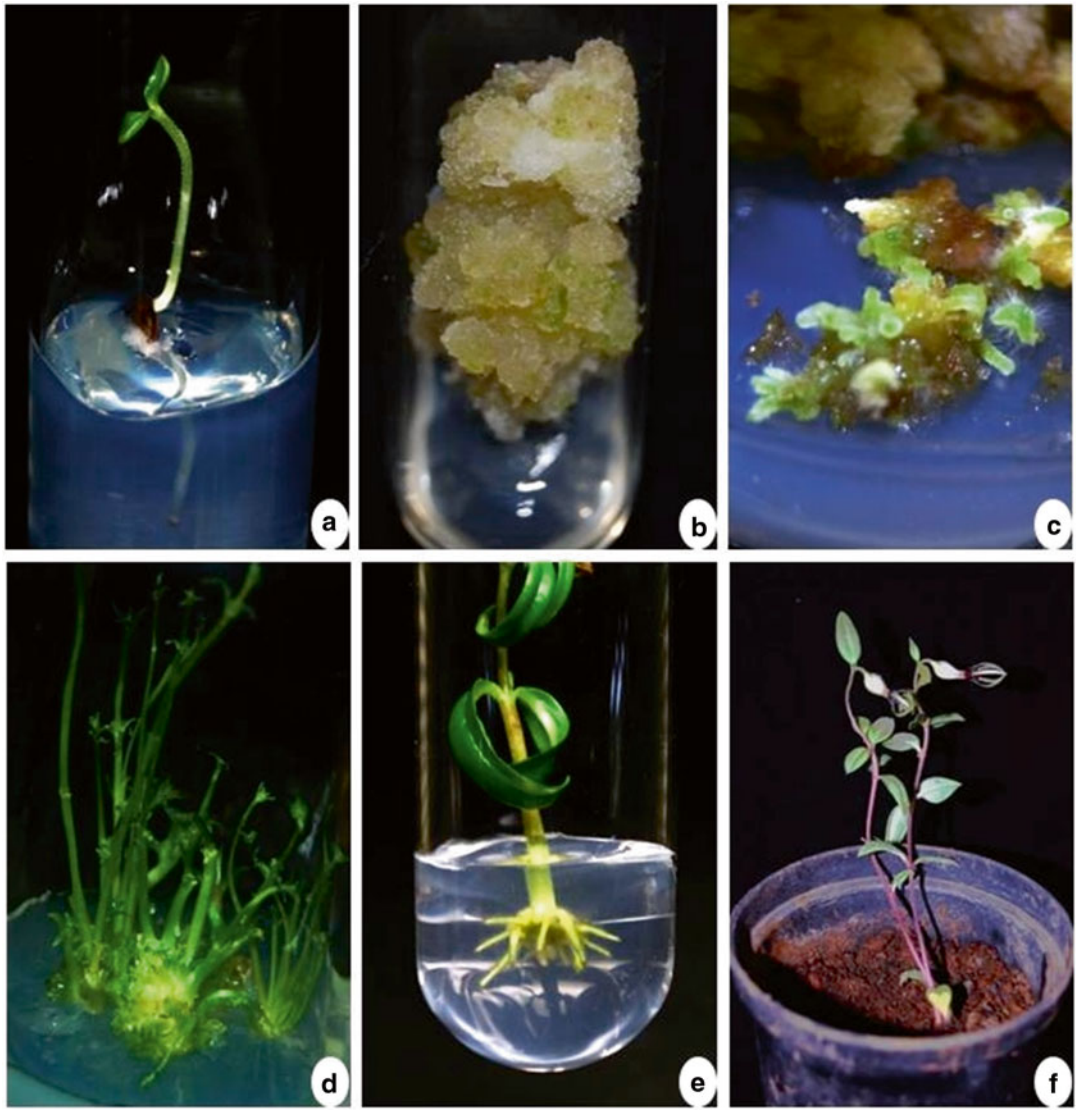

Fig. 1 Callus induction and plant regeneration in *C. noorjahaniae*; (**a**) In vitro seed germination (1/2 MS basal medium); (**b**) callus induction and proliferation from internode explants on MS + 2,4-D (2.0 mg/l); (**c**, **d**) shoot induction and multiplication on MS + BAP (2.0 mg/l) + IBA (0.2 mg/l); (**e**) in vitro rooting on ½ MS + IBA (1.0 mg/l) + NAA (0.3 mg/l) and (**f**) hardened plants

3.2 Callus Induction and Proliferation

1. Use stem segments (internodes) from in vitro-grown shoots as a source of explant for callus induction and proliferation.
2. Cut explants into appropriate sizes (1 cm) and culture on MS medium with 2.0 mg/l 2,4-D.
3. Subculture calli at 4-week interval on to the same or other medium for further proliferation (Fig. 1b; Table 2).

Table 2
Effect of various concentrations and combinations of 2,4-D and BAP on callus induction from stem explants of *C. noorjahaniae*

2,4-D (mg/l)	BAP (mg/l)	Callus induction frequency (%)	Remark
0.0	0.0	0.0	No callus induction
0.5	0.0	55	Cream yellow, compact
1.0	0.0	60	Cream yellow, compact
1.5	0.0	75	White, friable
2.0	0.0	80	Yellowish green, friable
2.5	0.0	60	Brownish, compact
3.0	0.0	53	Brownish, compact
2.0	0.2	70	Yellowish, soft
2.0	0.4	75	Pale yellow, soft
2.0	0.6	50	Pale yellow, semihard
2.0	0.8	50	Pale yellow, semihard

3.3 Adventitious Shoot Induction and Multiplication

1. To evaluate the effect of plant growth regulators (PGRs) on shoot regeneration, transfer callus (approx. 200 mg), developed on optimum proliferation medium (2.0 mg/l 2,4-D), to MS medium containing different concentrations of BAP (2.0 mg/l) in combination with IBA (0.2 mg/l; *see* **Note 9**; Fig. 1c, d) and maintain the cultures at 25 ± 2 °C with a 16 h photoperiod and 35 μmol/m²/s light intensity from cool fluorescent tubes (Philips, India).
2. Record observations on shoot induction frequency, mean number of shoots per culture, and mean shoot length (Table 3).

3.4 In Vitro Rooting

1. Separate well-developed shoots (4–6 cm in height) and transfer for rooting on MS basal medium containing auxins 1.0 mg/l IBA in combination with 0.3 mg/l NAA or medium lacking growth regulators (Fig. 1e).
2. Record observations on root induction frequency, mean number of roots per shoot, and mean root length (Table 4).

3.5 Hardening and Field Transfer of In Vitro-Regenerated Plantlets

1. Remove shoots with well-developed roots from culture tubes, wash under running tap water and transfer to small plastic pots containing sterile sand and coco peat (1:1; Fig. 1f).
2. After 35 days, transfer the hardened plantlets to the poly-bags (10 × 15 cm) containing garden soil and grow in the glasshouse

Table 3
Effects of various concentrations and combinations of BAP and IBA on shoot regeneration from stem-derived callus of *C. noorjahaniae*

Sr. no.	BAP (mg/l)	IBA (mg/l)	Shoot induction frequency (%)	No. of shoots per culture	Length of shoot (cm)
1.	0.0	0.0	0.0	0.0	0.0
2.	0.5	0.0	33	1.2 ± 0.2*	2.3 ± 0.4**
3.	1.0	0.0	45	3.3 ± 0.7**	3.0 ± 0.7**
4.	1.5	0.0	45	4.0 ± 0.4**	3.0 ± 0.1**
5.	2.0	0.0	65	7.4 ± 0.1**	3.2 ± 0.7**
6.	2.5	0.0	50	7.0 ± 0.1**	2.8 ± 0.3**
7.	2.0	0.1	68	11.3 ± 0.3**	4.7 ± 0.4**
8.	2.0	0.2	78	16 ± 0.2**	5.5 ± 0.1**
9.	2.0	0.3	75	7.3 ± 0.4**	5.0 ± 0.1**
10.	2.0	0.4	58	4.5 ± 0.1**	5.2 ± 0.3**
11.	2.0	0.5	55	4.6 ± 0.5**	4.3 ± 0.3**

Values represent mean ± standard error of 20 replicates per treatment in three repeated experiments. The values are significantly different at *$P < 0.05$ and **$P < 0.01$ level when compared by Dunnett multiple comparisons test

Table 4
Effect of auxins on in vitro root induction in microshoots obtained from indirect regeneration of *C. noorjahaniae*

PGRs (mg/l)		Root induction frequency (%)	Number of roots/shoot	Length of root (cm)
IBA	NAA			
0.0	0.0	0.0	0.0	0.0
0.5	0.0	60	2.2 ± 0.2**	0.7 ± 0.3^{ns}
1.0	0.0	68	4.0 ± 0.3**	1.6 ± 0.6*
1.5	0.0	71	3.7 ± 0.5**	2.0 ± 0.1**
2.0	0.0	69	3.1 ± 0.1**	2.3 ± 0.5**
2.5	0.0	63	2.8 ± 0.1**	1.9 ± 0.1**
1.0	0.1	65	2.6 ± 0.4**	3.2 ± 0.4**
1.0	0.2	60	6.3 ± 0.1**	3.7 ± 0.4**
1.0	0.3	75	11.2 ± 0.4**	3.4 ± 0.3**
1.0	0.4	73	6.3 ± 0.3**	1.2 ± 0.3*
1.0	0.5	70	5.8 ± 0.2**	1.1 ± 0.4*

Values represent mean ± standard error of 20 replicates per treatment in three repeated experiments. The values are significantly different at *ns*—nonsignificant, *$P < 0.05$ and **$P < 0.01$ level when compared by Dunnett multiple comparisons test

conditions (humidity 65 %; temperature 32 °C; photoperiod 16 h with 50 μmol/m^2/s).

3. The hardened plantlets are shifted to the nursery and eventually to the field.

4 Notes

1. Measure 1 ml Tween 20 and mix in 50 ml sterilized distilled water and raise the final volume to 100 ml with sterilized distilled water.
2. Weigh 100 mg $HgCl_2$ and dissolve in 50 ml sterilized distilled water. Make the final volume to 100 ml with sterilized distilled water.
3. For the preparation of MS medium, prepare stock solutions of major inorganic nutrients, minor inorganic nutrients, sodium molybdate, iron source, vitamins, and amino acids (Table 1). The vitamins and amino acids stock solution is stored in small batches at −20 °C and other stock solutions at 4 °C. Do not store inorganic stock solutions more than 1 month as it leads to precipitation which affects the growth of culture. Nowadays ready-mix MS medium is commercially available in different combinations, like MS with sucrose and agar; MS without agar; and MS with $CaCl_2$, sucrose, and agar.

   For preparation of 1000 ml MS medium, desired quantities of nutrient ingredient stocks of MS media are added in a conical flask containing 400–500 ml DW in the following sequence: Macronutrients: calcium chloride; potassium iodide; Micronutrients: sodium molybdate; iron stock; Vitamins. Myo-inositol (100 mg/l) and sucrose (3 % w/v) are weighed separately at the time of media preparation and dissolved in it. When required, concentrations of plant growth regulators are added before sterilization (or otherwise specified). Make the final volume of the medium to 1000 ml with DW. Adjust pH of the medium to 5.8 with 0.1 N NaOH and 0.1 N HCl. Add 8 g agar (0.8 %) in the medium. Keep the flasks in microwave oven to digest the agar. When agar is uniformly homogenized, 15 ± 2 ml of the medium is poured in each culture tube. Plug the culture tubes with non-absorbent cotton and sterilize at 121 °C for 15 min.
4. 6-benzylaminopurine (BAP), 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA): Weigh 10 mg of plant growth regulator (BA, 2,4-D, IBA, and NAA) separately and dissolve in 2 ml 1 N NaOH and gradually dilute to 100 ml with sterilized distilled water in a volumetric flask separately and store at 4 °C.

5. Commercially coco peat is available in blocks; cut the coco peat into small pieces and ground it into a fine powder and sterilize the coco peat in an autoclave at 121 °C for 20 min.
6. Locally available sand is sieved through the 600 MCIS mesh and this sieved sand is sterilized in an autoclave at 121 °C for 20 min.
7. Fill the polythene bags with garden soil and farmyard manure. The garden soil and farmyard manure is mixed in 1:1 proportion and the mixture is filled in the polythene bags and watered with tap water.
8. Seeds cultured on the medium carry seed-borne fungi and bacteria. These seeds are prone to infection and in such cases surface sterilization of seeds is necessary; such seeds should be washed thoroughly in water and then surface sterilization is carried for 10–15 min by using 0.1 % mercuric chloride. After sterilization wash the seeds thoroughly with sterilized distilled water followed by drying on sterile tissue paper or blotting paper and culture on the nutrient medium. All these procedures should be carried out under aseptic conditions.
9. Add desired quantity of filter-sterilized IBA from stock solution to the culture medium after autoclaving and cooling to below 40 °C.

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Chapter 25

Somatic Embryogenesis of Date Palm (*Phoenix dactylifera* L.) Through Cell Suspension Culture

Poornananda M. Naik and Jameel M. Al-Khayri

Abstract

Date palm (*Phoenix dactylifera* L.) is the oldest and most economically important plant species distributed in the hot arid regions of the world. Propagation of date palm by seeds produces heterogeneous offspring with inferior field performance and poor fruit quality. Traditionally, date palm is propagated by offshoots, but this method is inefficient for mass propagation because of limited availability of offshoots. Plant regeneration through tissue culture is able to provide technologies for the large-scale propagation of healthy true-to-type plants. The most commonly used technology approach is somatic embryogenesis which presents a great potential for the rapid propagation and genetic resource preservation of this species. Significant progress has been made in the development and optimization of this regeneration pathway through the establishment of embryogenic suspension cultures. This chapter focuses on the methods employed for the induction of callus from shoot tip explants, establishment of cell suspension culture, and subsequent somatic embryogenesis and plant regeneration.

Key words Callus, Cell suspension culture, In vitro regeneration, *Phoenix dactylifera*, Somatic embryogenesis

1 Introduction

Date palm (*Phoenix dactylifera* L.) is a dioecious fruit tree native to the hot arid regions of the world, mainly grown in the Middle East and North Africa. Since ancient times this majestic plant has been recognized as the *tree of life* because of its integration into human settlement, well-being, and food security in hot and barren parts of the world, where only a few plant species can flourish [1]. Dates represent a high energy source besides being rich in dietary fibers [2, 3]. Their carbohydrate content range is 44–88 %, while proteins represent 2.3–5.6 % and fats 0.2–9.3 % [4]. Date fruits were found to be rich in phenolics. The phenolics present in dates are known to exhibit various pharmacological activities including antiaging, anticancerous, antioxidant, antiviral, and antimicrobial properties, making them a remedy for certain

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diseases and prevention of chronic inflammations. They also prevent oxidative damages caused as a result of phagocytosis activity of invasive pathogens and pests by lymphocytes [5]. Phenolics are known to reduce blood pressure and have antithrombotic and anti-inflammatory effects [6, 7]. In addition, phenolics inhibit α-amylase and α-glucosidase activities behind the postprandial increase in blood glucose level, which is often manifested in type-II diabetes [5, 8, 9]. Conventionally, this plant is propagated from offshoots and their availability is often limited. However, it produces only about 20 offshoots during the first 10–15 years of the tree life, but plant regeneration through tissue culture is able to provide technologies for the large-scale propagation of healthy true-to-type plants. Plant tissue culture is also an essential tool for plant breeding programs [10] and the conservation of plant genetic resources [11].

Research in date palm tissue culture has received increasing interest, resulting in plant regeneration protocols for a number of commercial date palm cultivars. The literature indicated that plant regeneration of date palm was achieved via somatic embryogenesis depending upon the genotype and the composition of the culture medium. Date palm somatic embryogenesis was improved using biotin [12], abscisic acid (ABA) supplement [13, 14], and coconut water additive [15, 16]. Moreover, the stimulation of direct somatic embryo regeneration from shoot tip explants using N-phenyl N′-1,2,3-thiadiazol-5-ylurea (TDZ) was achieved by Sidky and Zaid [17]. Somatic embryogenesis, which leads to embryonic differentiation from somatic cells and not from fertilized ovules, is often employed because of its numerous advantages [18]. Embryogenic cells are used for the isolation of protoplasts with a highly regeneration capacity [19], genetic engineering [20], and the cryopreservation of plant genetic resources [21].

Cell suspension culture and somatic embryo genesis provide a number of avenues for the date palm genetic improvement based on mutagenesis and in vitro selection studies [22–26]. For the date palm, Al-Khayri [27] successfully determined the suspension growth curve, optimum plating efficiency, and influence of liquid medium on somatic embryogenesis.

The protocol explained here is based on induction of callus from shoot tip explants and somatic embryo genesis via cell suspension culture which has been shown to be widely applicable to numerous commercially important date palm cultivars [12, 15, 27–30]. Procedures including explant preparation, callus induction, cell suspension culture, somatic embryo development, plant formation, and acclimatization are described.

2 Materials

2.1 Explant and Media Preparation

1. Date palm offshoots.
2. Murashige and Skoog (MS) [31] medium stock solutions (MS stock I, II, III and IV) (Table 1). Store in the freezer or cold room at 4 °C (*see* **Note 1**).
3. Beakers, measuring cylinders, and glass rods (*see* **Note 2**).
4. 0.1–10 mL pipettes and/or 0.5–100 μL micropipettes.

Table 1
Chemical composition of the modified MS medium (After dissolving all the stock solutions in the deionized water make up the volume to 1 L, adjust the pH of the medium to 5.8 with 0.1 N HCl/0.1 N NaOH and add 7 g/L agar-agar and autoclave the media for 20 min at 121 °C)

Chemical constituents	Concentration (mg/L)	Volume per liter (mL)
Major inorganic nutrients		
NH_4NO_3	33,000	50
KNO_3	38,000	
$CaCl_2{\cdot}2H_2O$	8800	
$MgSO_4{\cdot}2H_2O$	7400	
KH_2PO_4	3400	
$NaH_2PO_4{\cdot}H_2O$	3400	
Minor inorganic nutrients		
KI	166	5
H_3BO_3	1240	
$MnSO_4{\cdot}2H_2O$	4460	
$ZnSO_4{\cdot}7H_2O$	1720	
$Na_2.MoO_4{\cdot}2H_2O$	50	
$CuSO_4{\cdot}5H_2O$	5	
$CoCl_2{\cdot}6H_2O$	5	
Iron source		
$FeSO_4{\cdot}7H_2O$	5560	5
$Na_2EDTA{\cdot}2H_2O$	7460	
Organic supplements		
myo-Inositol	25,000	5
Glutamine	40,000	
Nicotinic acid	200	
Pyridoxine·HCl	200	
Thiamine·HCl	5000	
Glycine	400	
Carbon source		
Sucrose	30 g/L	

5. 1 N NaOH/1 N HCl.
6. Activated charcoal, used at 1.5 g/L.
7. Agar-agar, used at 7 g/L.

2.2 Induction of Callus from Shoot Tip Explants and Establishment of Cell Suspension Cultures

1. Stock solutions of 2,4-dichlorophenoxyacetic acid (2,4-D) (10 mg/L), 2-isopentenyladenine (2iP) (1 mg/L), and naphthalene acetic acid (NAA) (1 mg/L) are prepared and stored in the freezer at −20 °C.

2.3 Packed Cell Volume (PCV)

1. Graduated centrifuge tube.
2. Centrifuge machine.

2.4 Plating Efficiency (PE)

1. Hemocytometer.
2. Petri dishes.
3. Illuminated colony counter.

2.5 Somatic Embryo Development

1. Hormone-free MS medium.
2. Rotary shaker.

2.6 Plant Formation

1. Embryo.

2.7 Hardening and Acclimatization

1. Peat.
2. Vermiculite.
3. Sand.
4. Dehydrated cow manure.

3 Methods

3.1 Explant Preparation

1. 3–4-year-old date palm offshoots are isolated from mother trees and the outer leaves removed, exposing the shoot tip regions which are excised and immediately placed in a chilled antioxidant solution consisting of ascorbic acid and citric acid, 150 mg/L each, to prevent oxidation-induced browning.
2. Shoot tip tissues of about 8 cm long are surface-sterilized in 70 % ethanol for 1 min followed by 15 min in 1.6 % (w/v) sodium hypochlorite solution (30 % v/v Clorox, commercial bleach) containing three drops of Tween 20 per 100 mL solution.
3. The tissue is rinsed 4 times in sterilized distilled water for 5 min.
4. The tissue surrounding the shoot tips is removed until the shoot tip terminal is exposed, about 1 cm long, which is excised and sectioned longitudinally into 6–12 small sections under the laminar flow bench/clean bench (Fig. 1a).

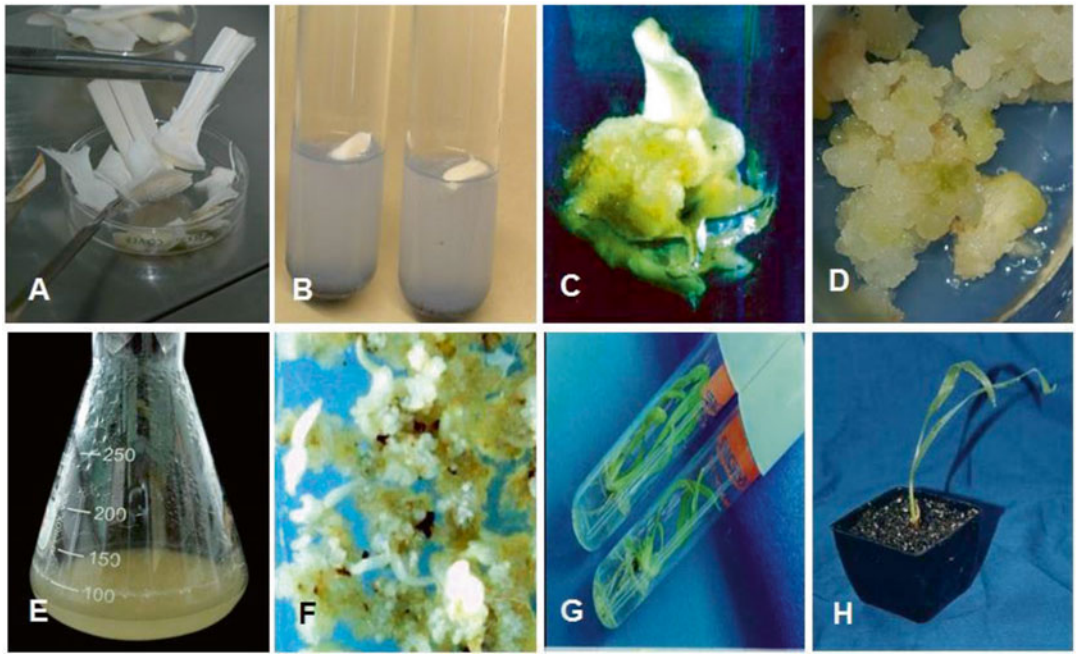

Fig. 1 Stages of date palm cell suspension culture and embryogenesis: (**a**) Explant preparation, (**b**) Culture initiation, (**c**) Callus induction, (**d**) Callus proliferation, (**e**) Cell suspension culture, (**f**) Embryo formation, (**g**) Rooting, (**h**) Transplanted plant. (Source: Part figures (**c**), (**f**), (**g**), (**h**) are taken from Al-Khayri JM (2012) Date palm *Phoenix dactylifera* L. In: Jain SM, Gupta PK (eds) Protocols of somatic embryogenesis in woody plants. Springer, Berlin, pp 309–318)

3.2 Induction of Callus from Shoot Tip Explants

1. These explants are placed on the surface of semisolid MS medium supplemented with 30 g/L sucrose, 100 mg/L 2,4-D, 3 mg/L 2iP, and 1.5 g/L activated charcoal, pH 5.8 for culture initiation (Fig. 1b; Table 2).
2. Incubate cultures at 24 °C ± 3 °C for 12 weeks in the dark. Within 8–10 weeks callus will develop from the shoot tip explants.
3. Culture the resultant callus on semisolid MS medium containing 30 g/L sucrose, 6 mg/L 2iP, 10 mg/L NAA, and 1.5 g/L activated charcoal, pH 5.8 for culture proliferation (Fig. 1c, d; Table 2).
4. Transfer cultures to a fresh medium at 3-week intervals. Use actively growing cell line as explants for the further experiments.

3.3 Proliferation of Cell Suspension in the Liquid Medium

1. Collect actively growing friable cell lines from the semisolid cultures and inoculate 1 g fresh biomass into a 250 mL Erlenmeyer flask containing 50 mL MS liquid medium supplemented with 30 g/L sucrose, 1.5 mg/L 2iP, and 10 mg/L NAA (*see* Table 2). Incubate cultures at 16-h photoperiod of

Table 2
Hormonal and activated charcoal supplements to the culture medium used for date palm callus induction and cell suspension

Media additives	Culture phase		
	Culture initiation	Callus proliferation	Cell suspension
2,4-Dichlorophenoxyacetic acid (2,4-D)	100 mg/L	–	–
2-Isopentenyladenine (2iP)	3 mg/L	6 mg/L	1.5 mg/L
Naphthalene acetic acid (NAA)	–	10 mg/L	10 mg/L
Activated charcoal	1.5 g/L	1.5 g/L	–

cool-white florescent light, 40 μmol/m²/s, and 23 ± 2 °C and agitated at 150 rpm. Maintain cultures by regular subculturing at 2-week interval (Fig. 1e).

3.3.1 Packed Cell Volume (PCV)

1. To estimate the PCV, place 5 mL cell suspension in a sterile graduated centrifuge tube and centrifuge at 2000 × *g* for 5 min.
2. Record the packed cell volume as percentage cell mass of the total centrifuged volume and then the samples are returned to the original cultures.
3. Measurements are taken weekly for 12 weeks. Plot the PCV values in relation to time, to construct a growth curve reflecting various phases of cell growth (Fig. 2).

3.3.2 Plating Efficiency (PE)

1. Dilute cell suspension concentration to give initial cell density of 100, 500, 1000, 5000, 10,000, 50,000, and 100,000 cells/mL with hemocytometer.
2. Mix samples of cell suspensions with melted agar medium after letting the autoclaved medium to cool down to 30–35 °C.
3. The medium used is similar to the cell suspension medium, but contains 7 g/L agar, and is dispensed in 15 × 100 mm petri dishes, at 20 mL per dish (*see* **Note 3**).
4. Mix the cell suspension and molten medium and evenly spread in the plate to solidify, forming a fixed thin layer of cells.
5. To assess the recovery potential of cell suspension to form cell colonies in relation to the initial cell concentration, count the colonies using an illuminated colony counter (Figs. 3 and 4; *see* **Note 4**).

3.4 Somatic Embryo Development

1. Induce cell suspension cultures together with friable callus to undergo somatic embryogenesis by transferring them to a hormone-free medium.

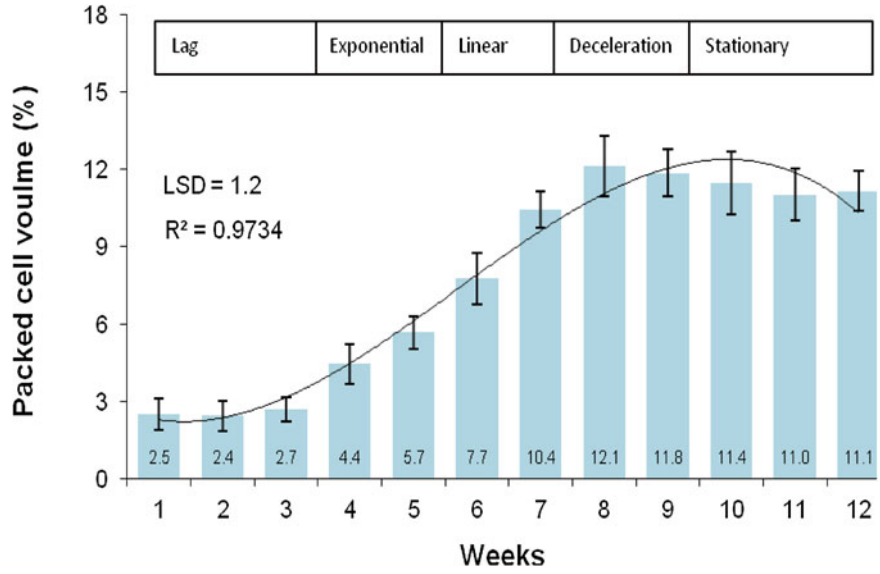

Fig. 2 The cell growth of date palm suspension culture, showing PCV in relation to time as it pertains to each of the growth phases (lag phase, exponential phase, linear phase, progressive deceleration phase, and stationary phase). (Source: This figure is taken from Al-Khayri JM (2012) Determination of the date palm cell suspension growth curve, optimum plating efficiency, and influence of liquid medium on somatic embryogenesis. Emir J Food Agric 24(5): 444–455)

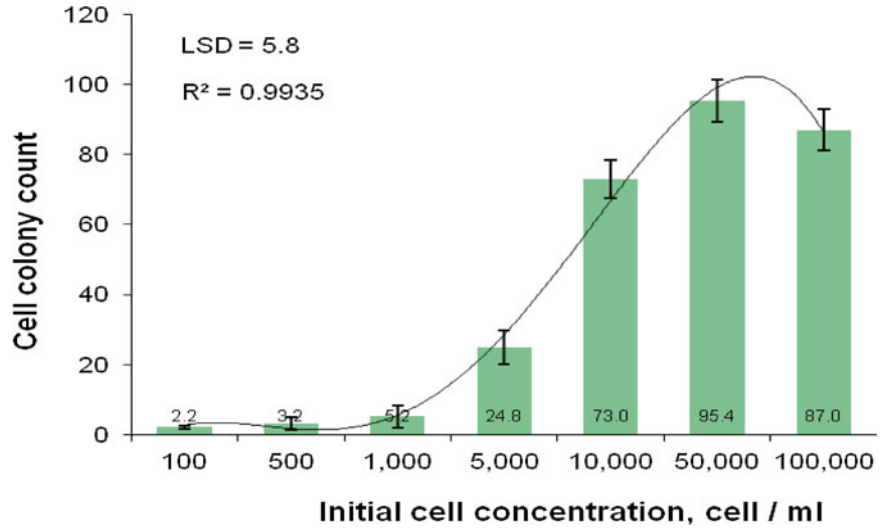

Fig. 3 The cell growth of date palm suspension culture, expressed in the number of colonies, recovered after plating on a semisolid medium at various cell densities. (Source: This figure is taken from Al-Khayri JM (2012) Determination of the date palm cell suspension growth curve, optimum plating efficiency, and influence of liquid medium on somatic embryogenesis. Emir J Food Agric 24(5): 444–455)

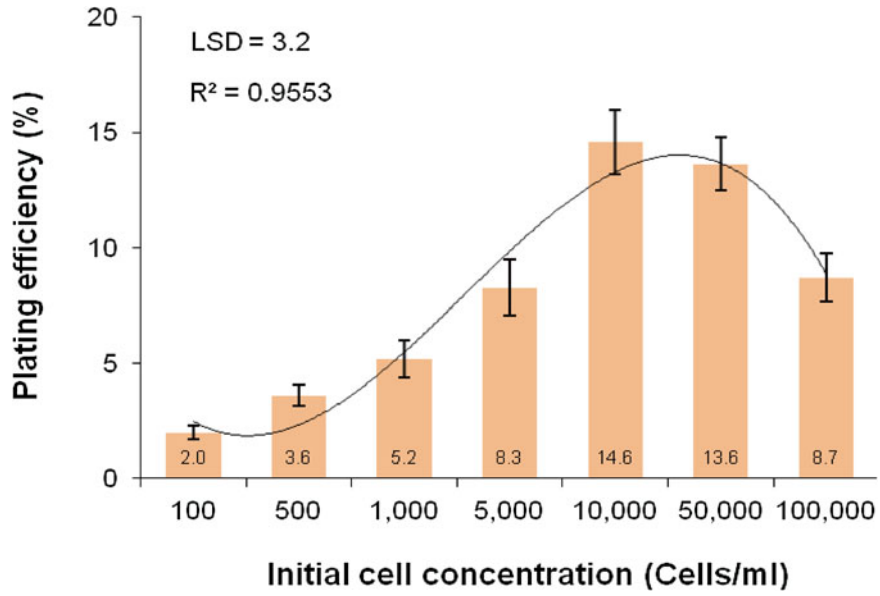

Fig. 4 The cell growth of date palm suspension culture, expressed in plating efficiency, recovered after plating on a semisolid medium at various cell densities. (Source: This figure is taken from Al-Khayri JM (2012) Determination of the date palm cell suspension growth curve, optimum plating efficiency, and influence of liquid medium on somatic embryogenesis. Emir J Food Agric 24: 444–455)

2. MS liquid medium supplemented with 30 g/L sucrose
3. Incubate cultures at 16-h photoperiod of cool-white florescent light (40 μmol/m²/s) and 23 ± 2 °C and agitate at 150 rpm. Maintain cultures by regular subculturing at 2-week intervals up to 12 weeks (Fig. 1f).

3.5 Plant Formation

1. To accelerate the complete plant formation, transfer mature or germinating embryos to rooting medium (Fig. 1g; *see* **Note 5**).

3.6 Hardening and Acclimatization

1. Remove the plantlets nearly 5 cm in length with shoot and root from culture tubes and gently rinse under a slow stream of water to remove residual agar media from the root region.
2. Plantlets are transferred to pots with peat–vermiculite mixture of 2:1 ratio in Styrofoam cups of uniform size (Fig. 1h).
3. The plantlets are initially covered with polythene bag to maintain high humidity to prevent the plants from dehydration and are maintained in the plant growth room.
4. Perforate the polythene bags and gradually remove them in a span of 3 weeks.
5. The hardened plants are transferred to potting mixtures consisting of 1:1:1 peat, sand, and dehydrated cow manure in pots (12 in.) and are maintained in the greenhouse under natural light at 27 ± 2 °C and 50–60 % relative humidity.

4 Notes

1. The most efficient way of preparing MS medium (Table 1) is to prepare stock solutions of major, minor inorganic nutrients, iron source, vitamins, and separate growth hormones. The stock solutions are stored at 4 °C except for the vitamins which is stored in small batches at −20 °C. Do not store the stock solutions for longer periods (not more than 2–3 months). It is always recommended to prepare the growth regulators fresh for each batch of media. Any color changes in the stock solutions may be due to precipitation, which can seriously affect the growth of cultures. Alternatively, stock solutions of macroelements, microelements, vitamins, and the ready-to-use MS medium are commercially available.
2. All the glassware should be cleaned with a liquid detergent and be thoroughly washed with tap water. Rinse the glassware with double distilled water and dry in hot air oven at 150 °C for 2 h before use.
3. Autoclave the petri dishes prior to use to avoid any contamination during the procedure as contamination at this stage may affect the results.
4. The plating efficiency is determined using the following equation:

$$\mathrm{PE} = \left(\text{final number of colonies per plate} \,/\, \text{initial number of cellular units per plate}\right) \times 100.$$

5. Half-strength MS medium supplemented with 0.2 mg/L NAA and 0.7 g/L agar.

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Chapter 26

Protocols for Improvement of Black Pepper (*Piper nigrum* L.) Utilizing Biotechnological Tools

K. Nirmal Babu, Minoo Divakaran, G. Yamuna, P.N. Ravindran, and K.V. Peter

Abstract

Black pepper, *Piper nigrum* L., the "King of spices" is the most widely used spice growing in the South-Western region of India. The humid tropical evergreen forest bordering the Malabar Coast (Western Ghats is one of the hot spot areas of plant bio-diversity on earth) is its center of origin and diversity. However, the crop faces constraints like rampant fungal and viral diseases, lack of disease free planting material, hence biotechnological tools can be utilized to address these problems and strides have been made successfully. The standardization of micropropagation, somatic embryogenesis, in vitro conservation, protoplast isolation, and genetic transformation protocols are described here. The protocols could be utilized to achieve similar goals in the related species of *Piper* too.

Key words Artificial/synthetic seeds, Cryopreservation, Embryo culture, Genetic transformation, In vitro conservation, Micropropagation, Plant regeneration, Somatic embryogenesis, Suspension cultures

Abbreviations

BA	Benzyl adenine
IBA	Indole-3-butyric acid
Kin	Kinetin
MS	Murashige and Skoog
NAA	α-naphthalene acetic acid
SH	Schenk and Hildebrandt
TDZ	Thiaduzuron
WPM	Woody plant medium

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1 Introduction

Black pepper, *Piper nigrum* L., is the most important spice of the world accounting for 40 % of export earnings from spices. India is the major producer and exporter of this spice and being the native home of black pepper, collection, cataloguing, and conservation of the precious genetic resources of this crop [1], development of resistant varieties are the major thrust areas in improvement of black pepper.

Molecular markers have been used in resolving complex phylogenetic problems in pepper and many reports on molecular characterization of Piper are available [2, 3]. Conserving and fingerprinting the genetic diversity in pepper and development of resistant varieties to "*Phytophthora* foot rot" are few areas that seek immediate solutions. In the past few years protocols utilizing biotechnological methods for commercial propagation, protoplast culture, genetic transformation and molecular profiling, conserving the genetic resources in in vitro and cryo banks have been developed [2, 4–6]. Genetic stability of micropropagated plants of *Piper longum* was validated using RAPD markers [3]. Similarly RAPD profiles for many cultivars of black pepper for identification of varieties have been studied [12]. The ability to manipulate and study plant tissues has led to the appreciation of role played by plant biotechnology in improving a crop species. Black pepper is no exception and the protocols developed for addressing crop-specific problems are reported.

2 Materials

2.1 Plant Material

Shoot tips, leaf segments, and nodal segments of *Piper nigrum* (Fig. 1) for all experiments and seeds for somatic embryogenesis.

Fig. 1 Black pepper vine

2.2 Surface Sterilization of Capsules

1. Diluted detergent, viz., two to three drops of Tween 20 in 50 ml distilled water.
2. Copper oxychloride as fungicide.
3. 0.1 % mercuric chloride.
4. 70 % ethanol.
5. Sterilized double distilled water.

2.3 Culture Vessels

1. Borosil test tubes and conical flasks.
2. Closures made of non-adsorbent cotton in gauze cloth.

2.4 Chemicals

Murashige and Skoog [7] medium (Table 1) for all in vitro culture methods.

2.5 Hardening of Plantlets

1. Potting mixture composed of sterilized vermiculite, garden soil, and sand (1:1:1) in equal proportions.
2. Micro-cups, polythene bags, and pots.

2.6 Molecular Profiling

2.6.1 Genomic DNA Isolation

1. 2× extraction buffer: (2 % cetyltrimethylammonium bromide (CTAB), 100 mM Tris–HCl, pH 8, 20 mM ethylenediaminetetraacetic acid (EDTA), pH 8, 1.4 M NaCl, 1 % polyvinylpolypyrrolidone (PVPP)).
2. Chloroform–isoamyl alcohol (24:1).
3. 100 % ethanol or isopropanol, 70 % alcohol.
4. TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8).
5. RNAse A (10 mg/ml).
6. 50× Tris–Acetate–EDTA (TAE) buffer (pH 8).
7. Agarose.
8. Ethidium bromide (10 mg/ml).
9. 6× loading dye (30 % glycerol, 5 mM EDTA, 0.15 % bromophenol blue, 0.15 % xylene cyanol).
10. 1000 bp DNA ladder.

2.6.2 For RAPD PCR

1. Taq DNA polymerase with 10× buffer.
2. 10 mM dNTPs: 10 mM each of dATP, dCTP, dGTP, and dTTP.
3. 25 mM $MgCl_2$.
4. 10 μM primers.

3 Methods

3.1 Micropropagation

Micropropagation protocol is used to multiply adequate planting material of black pepper (Fig. 2).

Table 1
Composition of tissue culture media (mg/l) used in the study

	Murashige and Skoog	Schenk and Hildebrandt	Woody plant medium
NH_4NO_3	1650	–	400.00
$(NH_4)_2\ SO_4$	–	–	–
$NH_4H_2PO_4$	–	300	–
$CaCl_2{\cdot}2H_2O$	440	200	96.00
$Ca(NO_3)_2{\cdot}4H_2O$	–	–	556.00
$MgSO_4{\cdot}7H_2O$	370	400	370.00
KCl	–	–	–
KNO_3	1900	2500	–
KH_2PO_4	170	–	170.00
K_2SO_4	–	–	990.00
$NaH_2PO_4H_2O$	–	–	–
Na_2SO_4	–	–	–
$CoCl_2{\cdot}6H_2O$	0.025	0.1	–
$CuSO_4{\cdot}5H_2O$	0.025	0.2	0.25
$FeSO_4{\cdot}7H_2O$	27.85	15.0	27.80
Na_2-EDTA	37.25	20.0	37.30
$MnSO_4{\cdot}4H_2O$	22.3	10.0	22.30
KI	0.83	1.0	–
$Na_2MoO_4{\cdot}2H_2O$	0.25	0.1	0.25
$ZnSO_4{\cdot}7H_2O$	8.6	1.0	8.60
H_3BO_3	6.2	5.0	6.2
Inositol	100	1000	100
Nicotinic acid	0.5	5.0	0.50
Pyridoxine–HCl	0.5	0.5	0.50
Thiamine–HCl	0.1	5.0	1.0
Glycine	2.0	–	2.00

3.1.1 Preparation of Culture Media

Murashige and Skoog (MS) basal medium is used for culture. Prepare separate stocks for macronutrients, micronutrients, vitamins, amino acids, and plant growth regulators separately.

1. Mix the stocks as mentioned, add 30 g/l sucrose, dissolve well, and make up to 1 L.
2. Adjust the pH to 5.8 and add 6 g/l agar.

Fig. 2 Production of multiple shoots in vitro

3. Transfer it to culture tubes and flasks in the required quantity and autoclave at 121 °C for 20 min.

3.1.2 Establishment of In Vitro Culture

1. Explants (shoot tips) from hygienically maintained source plants are collected into conical flask containing a fungicide solution of copper oxychloride with a dilute detergent (*see* **Notes 1–3**).
2. Surface-sterilize the explants with 0.1 % mercuric chloride ($HgCl_2$) in laminar flow hood and wash with three changes of sterile double distilled water.
3. Dry them on sterile filter papers and cut to the required size.
4. Inoculate in the culture tubes containing nutrient media and incubate them in growth room at 25 ± 2 °C with 16 h photoperiod, light intensity 33.8 μmol/m^2 s.
5. MS medium fortified with BA (0.5 mg/l for bud break, and 3 mg/l for multiple shoots) and hormone free MS with or without charcoal (2 %) for rooting is standardized as the ideal medium (Fig. 3).
6. Transfer the well-developed plants to a potting mixture and maintain at high humidity (>85 %) either in the nursery or hardening facility for 15–30 days and slowly bring them to open conditions.

3.2 Somatic Embryogenesis from Mature Seeds

1. Mature and ripe seeds of black pepper are squeezed so as to remove the pericarp, followed by thorough washing under running tap water.
2. Surface-sterilize the seeds inside a laminar flow by treating with 0.1 % mercuric chloride for 10 min, followed by rinsing with three to four changes of sterile double distilled water.
3. Inoculate the seeds on solid SH [8] basal medium (Table 1) with 30 g/l sucrose, gelled with 0.8 % agar.

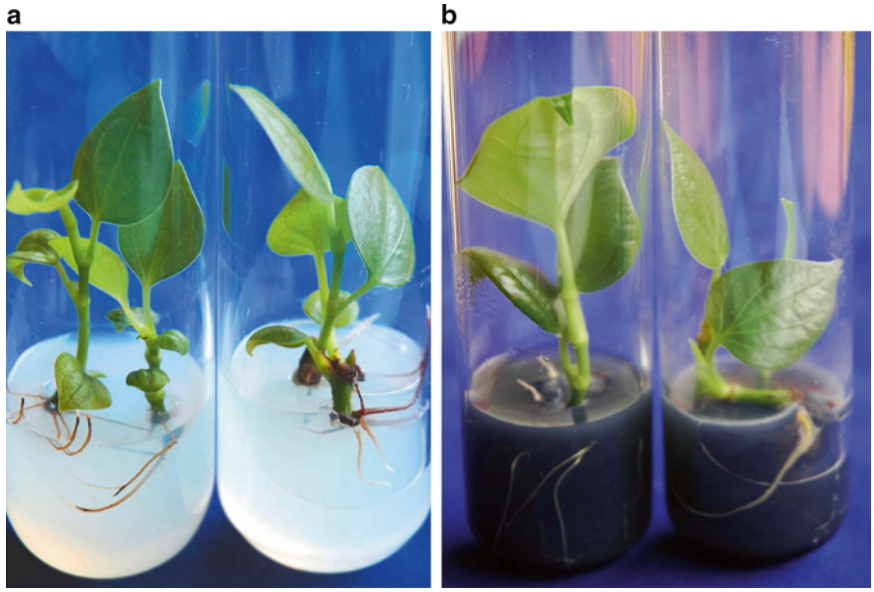

Fig. 3 (**a**, **b**) In vitro cultures of black pepper (activated charcoal is incorporated in the medium, whenever phenolics exudation into the medium is observed)

Fig. 4 Somatic embryogenesis in black pepper

4. Incubate the cultures under complete darkness at 25 ± 1 °C, and observe the cultures for germination at weekly intervals.
5. After germination of majority of seeds (30–40 days), subculture them with micropylar region in contact with fresh medium.
6. Maintain the cultures and regularly observe for somatic embryogenesis from the micropylar ring tissue (usually occurs between 60 and 120 days after culture initiation in 10–25 % seeds).
7. Once somatic embryo induction is observed, allow five to ten embryos to form on the explants (Fig. 4).

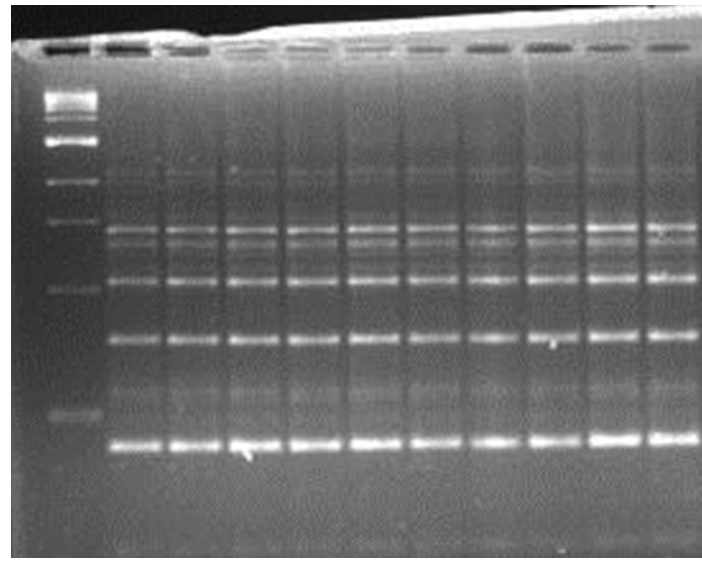

Fig. 5 RAPD profiles of plantlets developed through somatic embryogenesis

8. Transfer the explants with somatic embryos to the medium of same composition for proliferation combined with maturation or to SH medium supplemented with 15 g/l sucrose for more proliferation (Culture conditions remain the same).
9. Germinate the somatic embryos in agitating liquid SH medium containing 30 g/l sucrose at 110 rpm in the dark.
10. After complete formation of plants allow them to grow under light in the same flask (with medium replenishment once in 15 days) or transfer to individual tubes containing static liquid medium for better growth [9, 10].
11. After the formation of at least four leaves keep the cultures outside the culture room for 10 days
12. Transfer plants to 350 ml plastic cups filled with autoclaved river sand and cover with polythene bags having a few holes, till the emergence of fresh leaf growth.
13. Molecular profiles of these plants show absolute genetic uniformity (Fig. 5).

3.2.1 Somatic Embryogenesis from Leaf Explants

1. Prepare culture medium supplemented with BA (0.05–1 mg/l) and TDZ (0.05–1 mg/l).
2. Select very young leaves (preferably the freshly opened leaf of 1–2 cm size) from established aseptic cultures.
3. Culture the leaf explants in the medium prepared above and keep them in the dark for 15–20 days.
4. Keep the petiole of the leaf intact and in contact with the medium.
5. Cultures with callus growth are eliminated. Observe for direct somatic embryos from leaves, which occur only in 3–5 % cultures.

6. Transfer the leaf explants with somatic embryos very carefully to medium of same composition.
7. Observe for proliferation of somatic embryos by budding (*see* **Note 4**).
8. Culture bigger and mature embryos on solidified growth regulator free medium for development of plantlets.

3.3 Establishment of Cell Suspension Cultures

1. Supplement the basal MS medium with 2,4-D (0.5 mg/l) and BA.
2. Select leaf/stem/root explants from established aseptic cultures. Explants from aseptically germinated seedlings and embryos can also be used.
3. Culture the explants in the prepared medium and keep them in the dark for 15–20 days. Periodically observe for in vitro responses.
4. Cut explants into smaller pieces and callus growth from the cut ends would be observed. The majority of callus developed will be hard which can be transferred to regeneration medium for organogenesis and plant regeneration.
5. Separate friable and loose callus from the hard callus and put into the liquid medium of the same composition and agitate in orbital shakers at 100–150 rpm to establish suspension cultures.
6. Use these suspension cultures for radiation/mutation/in vitro selection studies.
7. Plate the selected calli on solidified regeneration medium for development of hard callus and plant regeneration. Addition of TDZ to the culture medium will enhance plant regeneration and sometimes somatic embryogenesis.
8. Transfer the hard callus into regeneration medium for plant development. Observe cultures periodically under the microscope for growth and differentiation and transfer to the suitable medium.

3.4 Production of Synthetic Seeds

1. 3 % sodium alginate is incorporated into MS medium as the encapsulation matrix.
2. The explants are mixed into the above medium, dropped into calcium chloride solution (1.036 g in 100 ml) and rotated gently for bead formation.
3. The solution is decanted and beads or Synseeds thus retrieved can be stored up to 9 months in sterile water with over 80 % viability (Fig. 6).

3.5 Protoplast Isolation

1. *Source tissue*: Leaves/callus is cut into small pieces to ensure proper enzymatic digestion, as it is difficult to peel off the epidermis.

Fig. 6 Recovery and growth of cryopreserved "synthetic seeds" of *Piper*

Table 2
Composition of enzyme solutions (ES) for isolation of protoplasts[a]

Code	Mannitol (%)	Macerozyme R-10 (%)	Hemicellulase (%)	Onozuka cellulase R-10 (%)
ES-1	10	0.5	–	1
ES-2	9	0.5	–	1
ES-3	8	0.5	–	1
ES-4	7	0.5	–	1
ES-5	6	0.5	–	1
ES-6	5	0.5	–	1
ES-7	10	0.5	0.5	2
ES-8	9	0.5	0.5	2
ES-9	8	0.5	0.5	2
ES-10	7	0.5	0.5	2
ES-11	6	0.5	0.5	2
ES-12	5	0.5	0.5	2
ES-13	10	1	–	3
ES-14	9	1	–	3
ES-15	8	1	–	3
ES-16	7	1	–	3
ES-17	6	1	–	3
ES-18	5	1	–	3

[a]Enzyme solutions are prepared in CPW medium

2. *Enzymatic digestion*: Enzyme digestion method is used to release the protoplasts by digesting the tissue with a mixture of macerozyme and cellulase (Table 2). Prepare enzyme mixture in Cell Protoplast Washing (CPW) medium (Table 3). The enzymes are filter-sterilized using Milli-Q filtration unit.

Table 3
Composition of media used for protoplast isolation and culture

Components	Cell protoplast washing (CPW) medium (mg/l)	Floating media (mg/l)	Protoplast culture media (mg/l)	
			I	II
NH_4NO_3	–	–	1650	1650
KNO3	101	101	1900	1900
$CaCl_2{\cdot}2H_2O$	1480	1480	440	440
$MgSO_4{\cdot}7H_2O$	246	246	370	370
KH_2PO_4	27.2	27.2	170	170
KI	0.16	0.16	0.83	0.83
H_3BO_3	–	–	6.2	6.2
$MnSO_4{\cdot}4H_2O$	–	–	22.3	22.3
$ZnSO_4{\cdot}7H_2O$	–	–	8.7	8.7
$Na_2MoO_4{\cdot}2H_2O$	–	–	0.25	0.25
$CuSO_4{\cdot}5H_2O$	0.025	0.025	0.025	0.025
$CoCl_2{\cdot}6H_2O$	–	–	0.025	0.025
$FeSO_4{\cdot}7H_2O$	–	–	27.8	27.8
$Na_2EDTA{\cdot}2H_2O$	–	–	37.3	37.3
Myo-inositol	–	–	100	100
Nicotinic acid	–	–	0.5	0.5
Thiamine HCl	–	–	0.5	0.5
Pyridoxine HCl	–	–	0.5	0.5
Glycine	–	–	2	2
Sucrose	–	21 %	2 %	3 %
Mannitol	7–10 %	–	7 %	4 %
Gibberellic acid	–	–	0.5	0.5
BA	–	–	0.5	1
NAA	–	–	0.5	1
2,4-D	–	–	0.5	–

3. *Osmoticum*: Osmotic pressure of the medium is adjusted by addition of mannitol at different concentrations (8 %, 9 %, and 10 %) (*see* **Note 5**).
4. *Incubation conditions for protoplast isolation*: One gram mechanically macerated leaves from in vitro cultured plants is immersed in 10 ml isolation medium for pre-plasmolysis and incubated in the dark for 16 h.

Table 4
Composition of PEG solution[a] for protoplast fusion

Constituents	Molar conc.	g/l
NaCl	140 mM	8.18
KCl	5 mM	0.37
Na_2HPO_4	0.75 mM	0.11
Glucose	5 mM	0.90
$CaCl_2 \cdot 2H_2O$	125 mM	18.40
PEG (MW 4000)		400.00

[a]pH is adjusted to 5.8

5. *Protoplast purification*: A combination of filtration, centrifugation, washing, and floatation centrifugation is used to purify the protoplasts (*see* **Notes 6** and 7). Observation under inverted microscope (Leica) confirms enzymatic digestion and release of protoplasts.
6. *PEG-mediated hybridization/fusion*: Two droplets of protoplast suspension from the two species to be fused are added to another droplet of PEG solution (Table 4), and allowed to mix at room temperature for 30 min. They are maintained as droplet cultures on glass slides and observed periodically for fusion of protoplasts.
7. *Protoplast culture*: Culture protoplasts initially in the liquid medium in petri dishes and incubate in the dark at 25 °C. Fresh culture medium with 3 % sucrose and 7 % mannitol is added after every 7 days to replenish the nutrients. The samples are periodically observed for cell wall regeneration and cell division. After 40–60 days of culture they are plated on 0.25 % agarose medium and monitored for microcallus formation and further development.

3.6 Molecular Marker Profiling to Assess the Genetic Diversity

In this protocol, fresh and healthy leaves from in vitro conserved plants maintained in the in vitro gene bank are used for DNA isolation.

3.6.1 Isolation of DNA

3.6.2 DNA Extraction Procedure

CTAB method [11] is used for the isolation of genomic DNA. Stock solutions and buffers are prepared as in Tables 5 and 6.

1. Grind 2 g leaf tissue in the liquid nitrogen to a fine powder using prechilled mortar and pestle.
2. Transfer the powder to 50 ml oak ridge containing 10 ml preheated CTAB + β mercaptoethanol.

Table 5
Composition of various stock solutions for DNA isolation

Solutions	Method of preparation
1 M Tris–HCL (pH 8.0) 500 ml	60.55 g Tris base + 300 ml distilled water Adjust pH to 8, make up the volume to 500 ml. Dispense into reagent bottles and sterilize by autoclaving
0.5 M EDTA pH 8.0	93.05 g EDTA-disodium salt + 300 ml water Adjust pH to 8, make up volume to 500 ml. Dispense into reagent bottles and autoclave
5 M NaCl 500 ml	146.1 g NaCl + 200 ml water. Adjust the final volume to 500 ml. Dispense into reagent bottles and autoclave
3 M sodium acetate (pH 5.2) 250 ml	61.523 g anhydrous sodium acetate + 200 ml water. Adjust the pH to 5.2 with glacial acetic acid (99–100 %). Autoclave
Ethidium bromide 10 mg/ml, 100 ml	1 g ethidium bromide + 100 ml distilled water. Dissolve on magnetic stirrer. Dispense into amber colored reagent bottle and store at 4 °C
70 % ethanol, 500 ml	360 ml ethanol + 140 ml distilled water. Store at 4 °C
Chloroform–isoamyl alcohol (24:1), 500 ml	Measure 450 ml chloroform and 20 ml isoamyl alcohol. Mixed and stored in room temperature
1 M $MgCl_2$, 100 ml	Weigh 20.33 g $MgCl_2$, dissolve in double distilled water, make up to 100 ml, autoclave

Table 6
Composition of various buffers used for DNA isolation

	Buffer	Method of preparation
1	CTAB Extraction Buffer: for 1 L 100 mM Tris–HCl (pH 8.0) 20 mM EDTA (pH 8.0) 1.4 M NaCl 2 % CTAB (w/v) Merck 0.2 % β-mercaptoethanol (v/v)-Merck	Measure 100 ml Tris (1 M), 280 ml NaCl, 40 ml EDTA (0.5 M). Mix with about 400 ml of hot distilled water, add 20 g CTAB to this. Adjust final volume to 1 L. Dispense into reagent bottles and autoclave. Just before use, add 0.2 % β-mercaptoethanol
2	TE (0.1 mM) buffer: for 100 ml 100 mM Tris–HCl (pH 8.0) 0.1 mM EDTA (pH 8.0)	Take 1 ml Tris–HCl (1 M), 20 ml EDTA (0.5 M). Mix with 99 ml sterile distilled water taken in a reagent bottle, mix thoroughly, autoclave
3	TAE buffer 10×: for 1 L	Weigh 48.4 g of Tris base; add 20 ml EDTA (0.5 M); 11.42 ml glacial acetic acid and around 150 ml distilled water. Dissolve the salt and adjust volume to 1 L. Autoclave
4	Gel loading buffer (6×): for 100 ml 0.25 % bromophenol blue 30 % glycerol	Dissolve 0.25 g BPB in 99 ml 30 % glycerol. Keep on magnetic stirrer for several hours to get the dye completely dissolved. Dispense into reagent bottles and keep in 4 °C

3. Incubate the sample at 65 °C for 30–60 min with occasional mixing by gentle shaking.
4. Add equal volume of chloroform–isoamyl alcohol (24:1). Spin at 38,638 × *g*, for 15 min, at 4 °C Transfer the aqueous phase to fresh tubes with cut tips.
5. Add 2/3 volume of ice-cold isopropanol and mix by gentle shakings.
6. Incubate at 4 °C for half an hour.
7. Centrifuge at 38,638 × *g*, for 15 min, at 4 °C.
8. Discard supernatant, add 70 % ethanol, and wash the precipitate by gentle swirling for 3–4 min.
9. Spin at 26,832 × *g*, for 10 min at 4 °C. Pour off supernatant, invert the tubes for 15 min to drain off excess alcohol, and vacuum-dry the pellet.
10. To the dried pellet, add 500 µl TE to redissolve the DNA and transfer the solution to 1 ml sterile microfuge tubes.

3.6.3 Purification Procedure

1. Add 2 µl DNase-free RNase (10 mg /ml) and incubate at 37 °C for 1 h.
2. Extract with equal volume of phenol–chloroform–isoamyl alcohol (25:24:1), by spinning at 26,832 × *g* and transferring aqueous phase to fresh tubes.
3. Extract twice with equal volume of chloroform–isoamyl alcohol (24:1), by spinning at 26,832 × *g* and transferring aqueous phase to fresh tubes.
4. Add 1/10th volume of 3 M sodium acetate (pH 5.2) and 0.8 volumes of ice-cold isopropanol and mix by gentle shaking to precipitate the DNA.
5. Centrifuge at 26,832 × *g* for 5 min.
6. Decant supernatant carefully. Wash the pellet with 70 % cold ethanol.
7. Air-dry the pellet and dissolve in 250 µl TE (pH 8).

3.6.4 Quality Analysis of DNA

1. Prepare 0.8 % agarose gel in 1× TAE buffer, add ethidium bromide (10 mg/ml), and cast the gel.
2. Load 3 µl DNA sample mixed with 2 µl gel loading buffer (6×).
3. The gel is run at 60 V, for 2 h.
4. Visualize the image under UV Transilluminator in Bio-Rad Gel Doc 1000 system and take a printout.

3.6.5 PCR Amplification

1. Amplify 20–50 ng of genomic DNA in a reaction mix containing 1.0 U *Taq* DNA polymerase, 1 µM primer, 1.5–2.0 mM $MgCl_2$, and 0.125 mM each of dNTPs, and 1× *Taq* DNA polymerase buffer.

2. The amplification profile consists of an initial denaturation of 3 min at 94 °C followed by 35–40 cycles of denaturation for 1 min at 94 °C, annealing for 37 °C for 1 min, and extension at 72 °C for 2 min and final extension for 6 min at 72 °C.

3.6.6 Gel Electrophoresis

1. The amplified RAPD products are separated by horizontal electrophoresis in 1.5 % (w/v) agarose gel, with 1× TAE buffer, stained with ethidium bromide (0.5 μg/ml), and analyzed under ultraviolet (UV) light.
2. The length of the DNA fragments is estimated by comparison with DNA ladder.
3. Variability is then scored as the presence or absence of a specific amplification product. Each gel is analyzed by scoring the present (1) or absent (0) polymorphic bands in individual lanes.
4. The binary matrix is transformed into similarity matrix using Dice similarity (NTSYS-PC 2.01; Numerical Taxonomy System of Multivariate Programs).

3.7 Genetic Transformation

1. Cut off the healthy young leaves from a sterile plant in the laminar flow hood and place them in a petri dish with sterile water. Place 3 Whatman 1 filter papers cut in circles (sterile) in a petri dish. Wet them with sterile water and place two leaves over the filter paper. Cut out disks (about 5 mm diameter) using a cork borer. Using a bacterial loop, transfer each disk from water to a piece of dry Whatman 1 paper. Blot the disk dry and then place it inverted on MS medium. Incubate for two days in a tissue culture room.
   (a) 2 ml culture of the *Agrobacterium tumefaciens* strain (pBZ 100) (Kan 50/μml, 28 °C) (*see* **Notes 8** and **9**).
2. Day 2: Inoculate 20 ml YEP culture with 0.1, 0.2, 0.4, and 0.6 ml of the starting culture containing *A. tumefaciens*, in separate flasks.
3. Monitor the cultures by measuring the O.D. at 600 nm. Use a culture that has an optical density value of 1 at 600 nm. Transfer 5 ml culture to a sterile petri dish. Using a bacterial loop, transfer the leaf disks to *Agrobacterium* suspension. Leave for two minutes and then blot dry on a Whatman 1 paper. Place the leaf disks on MS medium (Table 1) and incubate for 2 more days.
4. Transfer the leaf disks on to the selection medium containing kanamycin in which only plant cells transformed with neomycin phosphotransferase would grow. This medium also contains carbenicillin, which would kill *Agrobacterium* (Fig. 7). Among the antibiotics concentrations tried, tolerance and survival is better in 25–100 mg/ml [12].
5. Callus appears around the edges of the leaf disks. The leaf disk can be selected on the antibiotic-amended MS medium with

Fig. 7 Regeneration from ***Agrobacterium*** treated tissues of black pepper in selection medium

2,4-D 1 mg/l, NAA 1 mg/l, sucrose 30 g/l, 8 g/l agar, kanamycin (<100 mg/l), and carbenicillin (<100 mg/l). Regeneration of the transformed cells can be carried out subsequently in the shoot multiplication (WPM supplemented with 3 mg/l BA and 1 mg/l Kin) and rooting in basal WPM media (*see* **Note 10**). After the development of roots, the plants are transferred to moist vermiculite in pots and transferred to growth chambers and subsequently to the greenhouse.

3.8 In Vitro Conservation [13]

3.8.1 In Vitro Storage by Minimal Growth

1. Shoot tips (2–3 cm long) are collected from 3-year-old plants, thoroughly washed in running tap water and then wiped with 70 % ethanol.
2. The explants are surface-sterilized in 0.1 % mercuric chloride for 3 min, followed by rinsing in sterile distilled water three times.
3. Shoot tips are cultured on the WPM supplemented with 3 mg/l BA + 1 mg/l Kin for the induction of multiple shoots [6, 8, 10]. This is used as the base material for further studies.
4. The cultures could be stored up to 360 days with 80 % survival in half WPM (Woody Plant Medium) medium supplemented with 15 g/l each of sucrose and mannitol in sealed culture vessels and the growth rate was also reduced.
5. For encapsulation of axenic shoot tips (1–2 mm) different concentrations of sodium alginate are tried and 4 % is the optimum concentration. Encapsulated shoot tips are cryopreserved using vitrification technique.
6. In encapsulation-vitrification method, the encapsulated shoot tips are pre-cultured on MS medium supplemented with 0.3 M, 0.5 M, and 0.7 M sucrose (pH 5.8) for three days followed by dehydration with PVS2 (containing 30 % (w/v) glycerol, 15 % (w/v) ethylene glycol, and 15 % (w/v) DMSO mixed in MS medium containing 0.4 M sucrose) solution (100 %) at pH 5.8, and 0 °C for 3 h. After dehydration, the

beads (ten beads containing shoot tips), are immersed in 0.8 ml PVS2 solution in a 1.5 ml cryo-tube, and are frozen rapidly by direct immersion into liquid nitrogen (−196 °C) and kept for 1 hour.

7. After 1 hour the samples are thawed at 40 °C for 90 s in a water bath. PVS2 solution is decanted and the beads are washed thrice with 1.2 M sucrose (2 min/washing).
8. For recovery, the encapsulated shoot tips are re-cultured on medium containing 0.5 mgl^{-1} BAP and kept in the dark for 2 weeks under a 16 h light–8 h dark photoperiod.
9. Subsequent subculture is done every 20 days. Six-month-old plants are hardened.

3.8.2 Cryopreservation of Zygotic Embryos

1. Embryos are extracted from the surface-sterilized seeds and placed on Murashige and Skoog medium with 2 M glycerol + 0.4 M sucrose in the dark at 22 ± 2 °C for 3 days before being used for freezing experiments.
2. Zygotic embryos are cryopreserved using the vitrification technique. Zygotic embryos are incubated for 3 h at 0 °C in a loading solution containing ice cold PVS2 vitrification solution.
3. Zygotic embryos are frozen rapidly by direct immersion in liquid nitrogen after direct treatment with full-strength vitrification solution. About ten embryos can be immersed in 1 ml PVS2 solution/1.8 ml cryotube.
4. After storage at −196 °C the embryos could be retrieved by thawing. This is done by plunging the cryotubes for 90 s in a water bath at 40 °C.
5. The PVS2 solution is eliminated by washing the embryos twice with medium containing 1.2 M sucrose.
6. Embryos are then transferred on MS basal media with 0.1 M sucrose. Survival is measured by assessing the percentage of embryos having germinated [3]. Around 80 % recovery of cryopreserved embryos can be obtained (*see* **Note 11**).

4 Notes

1. Endogenous bacterial contamination is a major problem affecting black pepper cultures. Initial inoculation of explant containing antibiotics will help in reducing the bacterial contamination. But these cultures could not be retained in antibiotic containing medium as the antibiotics will affect further growth and multiplication, and hence they must be shifted to fresh culture medium without antibiotics within one week.
2. Initial reduction of sucrose to 15 g/l will help to reduce the growth of bacterial contamination. These initial aseptic

cultures will be the source explants (leaves, shoot tips, nodal segments, and roots) for callus induction, plant regeneration, establishment of embryogenic suspension cultures, and genetic transformation experiments.

3. The micro shoots, embryos derived from aseptic cultures will be the starting materials for all cryopreservation experiments. This will increase the efficiency of cryopreservation and recovery of cryopreserved propagules.
4. Transfer the somatic embryos to plant growth regulator free liquid medium and agitate on orbital shaker at 100 rpm for production of large number of somatic embryos. These operations in the dark enhance adventive embryogenesis and maturation of embryos.
5. During protoplast isolation osmotic strengths of cytoplasm and the isolation medium need to be balanced, to prevent plasmolysis or bursting of the protoplasts.
6. After digestion, the enzyme solution containing protoplasts is filtered through a stainless steel mesh (60 mesh size from Sigma) to remove larger particles of undigested tissues and cell clumps.
7. The filtrate is distributed into sterilized screw capped centrifuge tubes and centrifuged in Beckman tabletop centrifuge for 10 min at 700 rpm. The protoplasts form a pellet at the bottom of the tube. The supernatant enzyme solution is removed using a Pasteur pipette without disturbing the pellet. The pellet is suspended in Cell Protoplast Washing (CPW) medium. Centrifugation and resuspension in fresh medium is repeated three times so as to wash the protoplasts and remove traces of enzyme solution. After washing, the pellet of protoplasts is resuspended in 1 ml CPW medium and layered on top of 9 ml floatation medium (Table 4) and centrifuged at 700 rpm for 10 min. The live protoplasts form a band at the interphase, which is collected with a Pasteur pipette and transferred to culture medium.
8. Black pepper leaf tissues could be transformed using *Agrobacterium tumefaciens*. The construct used contained gene for osmotin, a PR protein known to induce *Phytophthora* resistance, and kanamycin as antibiotic selection marker [10]. Leaf explants from both juvenile as well as mature tissues and zygotic embryos are used as explants. Among the different variables tested, while performing transformation, viz., co-cultivation period, pre-culture of explants, temperature, and pH, co-cultivation of 48 h is efficient. Use leaf explants from both juvenile as well as mature tissues and zygotic embryos as explants for transformation.
9. The treated tissues are transferred in the selection medium (WPM with BAP 1 mg/l + Kinetin 1 mg/l + Cefotaxime 250 mg/l + Kanamycin 50 mg/l). Putative transgenics shoots are regenerated from leaf disks treated with *Agrobacterium* containing osmotin.

10. MS [7] and WPM [14] medium can be used for in vitro cultures in *Piper* species.
11. Black pepper cultures could be stored under minimal growth conditions up to 360 days in half WPM or MS medium supplemented with 15 g/l each of sucrose and mannitol in sealed culture vessels. The cultures could be retrieved with 80 % survival. The conservation of genetic resources in in vitro gene bank utilizing medium and long -term cryopreservation methods has led to development of a conservation strategy (Fig. 8), which can be used as a safe alternative to germplasm conservation.

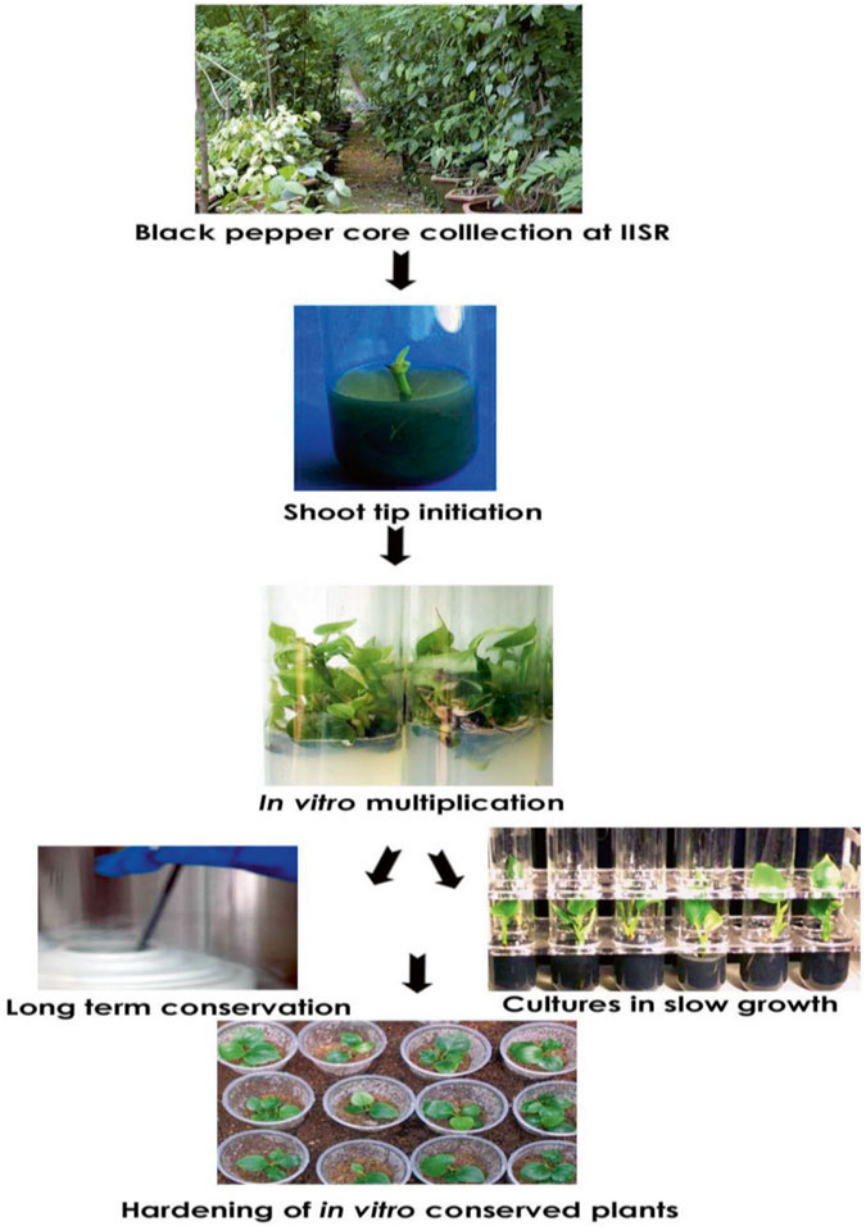

Fig. 8 Schematic representation of the in vitro conservation strategy adopted in black pepper

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Chapter 27

Protocols for In Vitro Propagation, Conservation, Synthetic Seed Production, Microrhizome Production, and Molecular Profiling in Turmeric (*Curcuma longa* L.)

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Abstract

Turmeric is a rhizomatous herbaceous perennial but cultivated as annual, belonging to the family Zingiberaceae. It is a native of India and South East Asia. The tuberous rhizomes or underground stems of turmeric are used from antiquity as condiments, a dye and as an aromatic stimulant in several medicines. Turmeric is an important crop in India and it is used as a spice, food preservative, coloring agent, cosmetic as well as for its medicinal properties. Propagation is done vegetatively with rhizome bits as seed materials. It is plagued by rhizome rot diseases most of which are mainly spread through infected seed rhizomes. Micropropagation will help in production of disease-free seed. Sexual reproduction is rare in turmeric, making recombinant breeding very difficult. In vitro technology can thus become the preferred choice and it can be utilized for multiplication, conservation of genetic resources, generating variability, gene transfer, molecular tagging, and their utility in crop improvement.

Key words Conservation, Cryopreservation, Embryo rescue, In vitro conservation, Micropropagation, Microrhizome production, Molecular profiling, Plant regeneration, Somaclonal variation, Synthetic seeds, Turmeric

Abbreviations

BA	Benzyl adenine
IBA	Indole-3-butyric acid
Kin	Kinetin
NAA	α-naphthalene acetic acid
MS	Murashige and Skoog

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1 Introduction

Turmeric (*Curcuma longa* L.) is an important spice crop belonging to the family Zingiberaceae and known for its varied medicinal properties (Fig. 1). Turmeric is a native of India and South East Asia and is used since ancient times as a spice and a dye and cultivated extensively in China, India, Indonesia, and Thailand and throughout the tropics, including tropical regions of Africa and America. It is extensively used in Chinese, Ayurvedic, Unani, and Siddha systems of medicine and as a home remedy for various ailments. The coloring principle of turmeric "curcumin" is responsible for many medicinal properties [1] and used in cheese, spices, mustard, cereals, pickles, potato flakes, soups, ice creams, yogurts and also in pharmacy, confectionery, and food industries. Recent research is focused on turmeric's antioxidant, hepatoprotective, anti-inflammatory, anticarcinogenic, and antimicrobial properties, in addition to its use in cardiovascular disease and gastrointestinal disorders. India is the world's largest producer, consumer, and exporter of turmeric with an annual production of about 9,86,690 tonnes from an area of 1,94,330 ha during 2012–2013.

Turmeric rarely sets seeds, hampering recombination breeding. Conventionally propagated through rhizomes, its cultivated

Fig. 1 Turmeric plant

types exhibit much morphological variations probably due to accumulated vegetative mutations. The past few years have seen the solutions of many breeding bottlenecks being solved by the use of biotechnological tools. Somaclonal variation, anther and protoplast culture, rDNA techniques, etc. are successfully utilized for production of disease-free planting material, conservation and improvement of genetic resources. Protocols for various techniques have been standardized and are summarized below. Morphological and molecular variations among micropropagated and callus-regenerated plants were studied and variations were found in both but with higher percentage of variation in callus-regenerated somaclones [2, 3]. In vitro plants developed through microrhizome exhibited least amount of variations. The study inferred that this is due to the accumulated vegetative mutations (mosaic) in turmeric. The genetic fidelity studies of turmeric germplasm conserved in in vitro gene bank using RAPD profiling showed their genetic integrity [4].

2 Materials

2.1 Explant Materials

Vegetative bud explants from rhizomes.

2.2 Glassware

Borosilicate conical flasks (500 ml), glass bottles (250 ml), culture tubes (22 cm × 3.5 cm), and 250 ml conical flasks, culture vessels, tubes, bottles, and flasks. Cotton plugs (made of non-absorbent cotton covered with cheese cloth), aluminum foil, or polypropylene caps.

2.3 Growth Regulators

Auxins—α-naphthalene acetic acid (NAA), indole-3-butyric acid (IBA) 0–1.0 mg/l;

Cytokinins—6-benzylaminopurine (BA) and 6-furfurylamino purine (kinetin) 0–3.0 mg/l.

2.4 Gelling Agents

Bacteriological grade agar, 7–8.0 g/l.

2.5 Culture Medium

- Murashige and Skoog (MS) basal medium (Table 1).
- Stock solutions for macronutrients, micronutrients, vitamins, vamino acids, and plant growth regulators (Table 2).
- Sucrose—30 g/l for multiplication and 90 g/l for microrhizome induction.

2.6 Culture Establishment

Rhizomes from potted plants.

2.7 Culture Initiation

MS medium supplemented with 3 % sucrose and 0.5 mg/l Kinetin.

Table 1
Composition of Murashige and Skoog[a] basal medium

Composition		Concentration (mg/l)
Macronutrients		
Ammonium nitrate	NH_4NO_3	1650.00
Potassium nitrate	KNO_3	1900.00
Calcium chloride	$CaCl_2{\cdot}2H_2O$	440.00
Potassium orthophosphate	KH_2PO_4	170.00
Magnesium sulfate	$MgSO_4.7H_2O$	370.00
Micronutrients	Na_2EDTA	37.30
Sodium EDTA	$FeSO_4{\cdot}7H_2O$	27.80
Ferrous sulfate	H_3BO_3	6.20
Boric acid	$MnSO_4.4H_2O$	22.30
Manganese sulfate	KI	0.83
Potassium iodide	$ZnSO_4{\cdot}7H_2O$	8.60
Zinc sulfate	$Na_2MoO_4{\cdot}2H_2O$	0.25
Sodium molybdate	$CuSO_4{\cdot}5H_2O$	0.025
Copper sulfate	$CoCl_2{\cdot}6H_2O$	0.025
Cobalt chloride		
Vitamins		
Myo-inositol	$C_6H_{12}O_6$	100.00
Thiamine HCl	$C_{12}H_{17}CIN_4OS{\cdot}HCl$	0.10
Nicotinic acid	$C_6H_5NO_2$	0.50
Pyridoxine HCl	$C_8H_{11}NO_3{\cdot}HCl$	0.50
Amino acid		
Glycine	$C_2H_5NO_2$	2.00

[a]Murashige and Skoog [8]

2.8 In Vitro Conservation

In vitro regenerated shoot buds.

2.9 Production of Synthetic Seeds

In vitro regenerated shoot buds, protocorms, and callus; sodium alginate, and calcium chloride.

2.10 Long-Term Storage by Cryopreservation

Synseeds containing miniaturized shoot tips, pollen are used as explants, and are immersed in liquid nitrogen.

Table 2
Details of various stock solutions for MS medium

Stock	Composition	Stock strength	Quantity[a]
A	Macronutrients	×20	50 ml
	NH_4NO_3		
	KNO_3		
	$CaCl_2 \cdot 2H_2O$[b]		
	KH_2PO_4		
	$MgSO_4 \cdot 7H_2O$		
B	Micronutrients	×100	10 ml
	H_3BO_3		
	$MnSO_4 \cdot 4H_2O$		
	KI		
	$ZnSO_4 \cdot 7H_2O$		
	$Na_2MoO_4 \cdot 2H_2O$		
	$CuSO_4 \cdot 5H_2O$[a]		
	$CoCl_2 \cdot 6H_2O$[a]		
C	Micronutrients	×100	10 ml
	Na_2EDTA[a]		
	$FeSO_4 \cdot 7H_2O$[a]		
D	Vitamins	×100	10 ml
	Thiamine HCl		
	Nicotinic acid		
	Pyridoxine HCl		
E	Amino acid	×100	10 ml
	Glycine		
F	Myo-inositol	×100	10 ml
	Growth regulators		
	2,4-D	50 mg/200 ml	
	NAA	50 mg/200 ml	
	BA	50 mg/200 ml	
	Kinetin	50 mg/200 ml	

[a]For preparation of 1 L medium. Make up the remaining is double distilled water up to 900 ml adjust the pH and add the remaining water to 1000 ml
[b]Dissolved separately before mixing in the final stock

3 Methods

3.1 Micropropagation and Multiplication of True-to-Type Plants

3.1.1 Establishment of Cultures in Turmeric (Fig. 2)

1. Newly sprouting buds are used as a source of explants for the culture.
2. Wash the explants under running tap water for 15 min followed by detergent solution (Tween 20) for 10–15 min.
3. Surface-sterilize using copper oxychloride, Bavistin (10 min each), 70 % ethanol (30–40 s) and wash with sterile distilled water for 3–4 min to remove the remnants of the fungicide.
4. Before starting inoculation, clean/treat the laminar flow with ethyl alcohol (70 %) for 30–60 s, wash the explants again in sterile double distilled water followed by treatment with 0.1 % $HgCl_2$ for 3–5 min and rinse with sterile distilled water for 3–4 times.
5. Cut the sterilized buds into small pieces suitable for inoculation and dissect the sprouts and remove the outer leaf sheaths under aseptic condition.
6. Inoculate the explants in test tubes containing half-strength MS media supplemented with 3 mg/l BA and 1 mg/l NAA.

3.1.2 Multiplication of Contamination-Free Cultures in Turmeric (Fig. 3)

1. Use 1-week-old, contamination-free cultures for multiplication. Explants are taken out carefully under sterile conditions and transferred into multiplication medium MS medium containing 5 mg/l BA and 1.0 mg/l NAA.
2. Use 7- to 8-week-old cultures for subculturing and for further multiplication. 20–25 plants can be harvested from 8-week-old culture.

3.2 Direct Regeneration of Plantlets from Immature Inflorescence

1. Collect immature, 1- to 10-day-old inflorescence (during flowering season) and sterilize.
2. Remove the outer bracts under aseptic condition and transfer remaining inflorescence to culture medium.

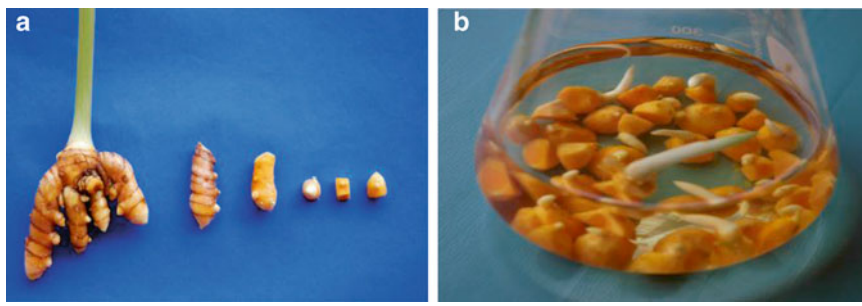


Fig. 2 (**a**, **b**) Explant sources of turmeric

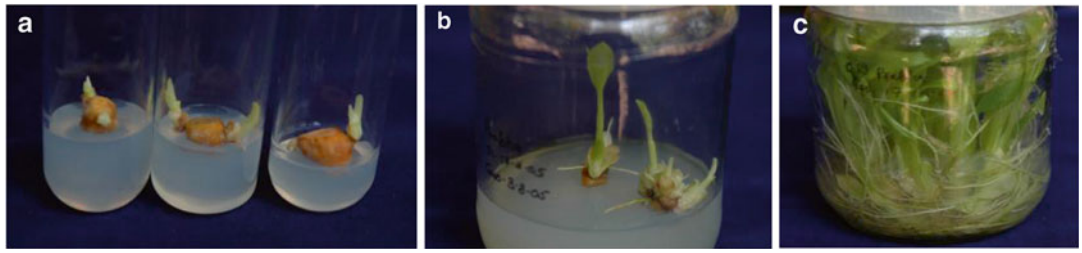


Fig. 3 (**a–c**) Stages of culture establishment and in vitro multiplication in turmeric

Fig. 4 (**a–d**) Stages in microrhizome induction and in vitro rhizome formation in turmeric

3. Use MS medium amended with 10 mg/l BA and 0.2 mg/l 2,4-D.
4. MS liquid medium having 1 mg/l NAA enhances rooting.

3.3 In Vitro Rooting and Production of Plantlets

1. Turmeric cultures do not require separate culture medium for rooting. Once the shoots become around 3–4 cm they automatically root in the media used.
2. MS liquid medium with 1 mg/l NAA can be used to improve rooting before transplanting to the soil.

3.4 Microrhizome Induction (Fig. 4)

Technology for microrhizome development in turmeric was standardized [5].

1. Microrhizome formation will be noticed in MS medium supplemented with 90 g sucrose after 90–100 days of inoculation and reaches maturity by 120 days.
2. Plant out microrhizomes directly in micro-cups or in the field for normal rhizome development.

3.5 Callus Culture, Plant Regeneration from Callus, and Inducing Variability (Fig. 5)

Technology for plant regeneration from various tissues in turmeric is available, and used to exploit somaclonal variation for increasing variability in this crop [6].

1. Wash new buds under running tap water followed by washing with 0.1 % $HgCl_2$ for 5–10 min. Rinse the explants 3–4 times to wash away the traces of $HgCl_2$.

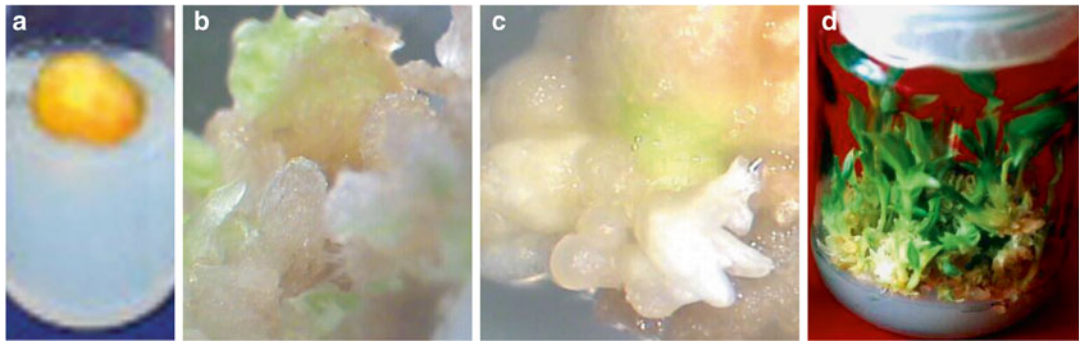


Fig. 5 Different stages of plant regeneration in turmeric, (**a**) Explant, (**b**) organogenesis from callus, (**c**) embryogenesis, (**d**) well-developed plantlets

2. Remove the outer leaf primordial under aseptic condition and place a small segment of bud on MS media supplemented with 13.6 μm 2,4-D and 16 μm NAA for callus induction.
3. Subculture 2-month-old callus on MS medium containing 0.2 mg/l 2,4-D and 10 mg/l BAP for organogenesis and plant regeneration.

3.6 Hardening and Planting Out

1. Take out healthy and well-rooted in vitro plantlets from the culture media and wash carefully to remove the traces of culture medium.
2. Transfer to polythene bags containing garden soil, sand, and farmyard manure in equal proportions and keep in humid chamber.
3. Maintain 90–100 % relative humidity for 20–30 days for hardening and establishment.

3.7 Encapsulation and Synthetic Seed Production

1. Suspend in vitro grown shoots/somatic embryos in MS basal medium supplemented with 4 % (w/v) Na alginate, 2 M glycerol, and 0.4 M sucrose.
2. Drop the mixture containing micro-shoots, with a sterile pipette into 0.1 M $CaCl_2$ solution containing 2 M glycerol and 0.4 M sucrose and leave for 20 min to form beads 4 mm in diameter, each bead containing at least one shoot.
3. Store the beads in sterile containers for 6–8 months [7].
4. Transfer to MS medium amended with 1 mg/l BAP + 0.5 mg/l NAA for regrowth.

3.8 Conservation of Genetic Resources

3.8.1 Medium Term In Vitro Conservation by Slow Growth

In Vitro Storage by Minimal Growth (Fig. 6)

1. Use vegetative bud explants and establish cultures in MS [8] basal medium containing 0.5 mg/l kinetin.
2. Multiply established shoot cultures on MS medium supplemented with 0.5 mg/l NAA and 1.0 mg/l BAP with an average of 8 shoots per culture in 90 days of culture.
3. The in vitro developed shoots are maintained on half-strength MS medium amended with 15 g/l each of sucrose and mannitol in sealed culture tubes up to 12 months with 80 % survival.
4. Maintain cultures the same medium with yearly subculture up to 7 years.
5. The miniaturized in vitro stored cultures can be recovered to normal size by transferring to the multiplication medium (MS + 1 mg/l BAP + 0.5 mg/l NAA), thereby resuming growth and multiplying normally in 3 weeks of culture. Multiple shoots and roots are produced in the same medium.
6. Transfer the rooted plantlets to nursery cups and maintain at 90–100 % relative humidity for 20–30 days for hardening and establishment with 80–90 % survival [9, 10].

3.8.2 Long-Term Storage of Encapsulated Shoot Tips by Cryopreservation

Cryopreservation (Fig. 7)

1. Initiate the rhizomes excised from field-grown plants on MS medium supplemented with 0.5 mg/l kinetin, 20 g/l sucrose, 0.7 % agar in tissue culture tubes at 22 ± 20 °C under 278 cd for a period of 2 weeks. Adjust pH 5.8 of the medium prior to autoclaving at 121 °C for 15 min. Multiply the plantlets obtained through initiation culture in MS medium containing BAP (1.0 mg/l) and NAA (0.5 mg/l). Excise apical shoot tips with two leaf primordial (about 1–2 mm in size) for cryopreservation.
2. For osmoprotection suspend the excised shoot tips in 0.3 M sucrose for a period of 3 days in the dark.
3. Dehydrate the osmoprotected shoot tips with ice-cold PVS2 solution at 0 °C for 3 h. PVS2 solution contains 30 % (w/v)

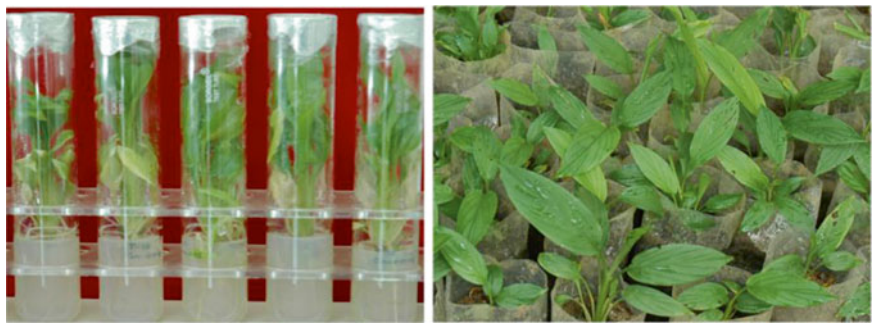
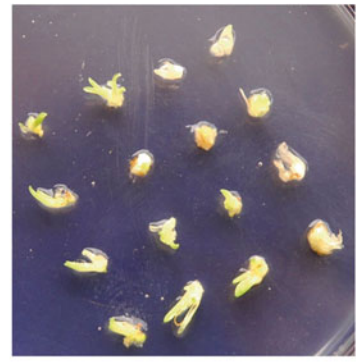


Fig. 6 In vitro storage by minimal growth in turmeric

In vitro cultures of turmeric

Shoot tip selection



Preculturing on 0.1 to 0.7 sucrose for 3 days

LN storage

Shoot tips with fresh PVS2

Dehydration with PVS2 at 0°C for 3 hrs.

Thawing

Shoot tip Recovery

Development after recovery

Fig. 7 Various stages in cryopreservation of turmeric

glycerol, 15 % (w/v) ethylene glycol, and 15 %(w/v) DMSO in MS medium supplemented with 0.4 M sucrose (pH 5.8). After dehydration, suspend 10 shoot tips in 1 ml PVS2 solution in a 1.8 ml cryo-tube and then plunge into liquid nitrogen for 1 h.

Thawing and Regrowth

1. Thaw by rapidly immersing the cryotubes in water bath at 40 °C for 1 min
2. For encapsulation-vitrification method, PVS2 is drained off from the vitrified samples, and replaced with 1.2 M sucrose solution twice at 10 min intervals.
3. Propagules after thawing are placed on MS + BA (1 mg/l) and NAA (0.5 mg/l) medium in petri dishes and maintained at the same culture conditions as stock explants.
4. Around 80 % recovery of cryopreserved shoots can be obtained [11].

3.9 Molecular Profiling [11, 12]

3.9.1 Isolation of DNA

There are various protocols for isolation of DNA from plant tissues. For turmeric CTAB method was used [13]. The protocol used for extraction of DNA from turmeric leaf tissues is as follows (Tables 3 and 4)

1. Grind 5 g young leaves in the liquid nitrogen with a mortar and pestle and add 25 ml preheated (65 °C) CTAB buffer. Add 0.2 % β-mercaptoethanol prior to use.
2. Incubate at 60 °C for 30 min.
3. Extract with equal volume of chloroform–isoamyl alcohol (24:1) at 26,832 × *g* for 10 min at room temperature.
4. Take the aqueous phase and add 2/3rd volume of ice-cold isopropanol.
5. Incubate at −20 °C for 2 h and centrifuge at 26,832 × *g* for 15 min at 4 °C.
6. Discard the supernatant and invert the tube on paper towel for few minutes.
7. Dissolve the pellet and add 1.5 ml TE buffer. Store at room temperature.
8. Add 10 µg/ml RNase A and incubate at 37 °C for 30 min.
9. Add equal volume of Tris saturated phenol, mix it well and centrifuge at 26,832 × *g* for 10 min.
10. To the aqueous phase, add equal volume of phenol–chloroform–isoamyl alcohol (25:24:1), shake and centrifuge at 26,832 × *g* for 10 min.
11. Take the aqueous phase and add equal volume of chloroform–isoamyl alcohol (24:1), shake and centrifuge at 26,832 × *g* for 10 min.

Table 3
Composition of various stock solutions for DNA isolation

Solutions	Method of preparation
1 M Tris–HCl (pH 8.0) 500 ml	Dissolve 60.55 g Tris base (Sigma) in 300 ml distilled water. Adjust pH to 8 by adding concentrated HCl. Adjust volume to 500 ml. Dispense into reagent bottles and sterilize by autoclaving
0.5 M EDTA pH 8.0	Dissolve 93.05 g f EDTA-disodium salt (sigma) in 300 ml water. Adjust pH to 8 by adding NaOH pellets. Adjust volume to 500 ml. Dispense into reagent bottles and autoclave
5 M NaCl 500 ml	Weigh 146.1 g NaCl (Merck) add 200 ml water and mix well. When the salts get completely dissolved, adjust the final volume to 500 ml. Dispense into reagent bottles and autoclave
3 M Sodium acetate (pH 5.2) 250 ml	Dissolve 61.523 g anhydrous sodium acetate (Qualigens) in 200 ml water and mix well. When dissolved completely adjust the pH of the solution to 5.2 with glacial acetic acid (99–100 %). Autoclave
Ethidium bromide 10 mg/ml, 100 ml	Add 1 g ethidium bromide to 100 ml distilled water. Keep on magnetic stirrer to ensure that the dye has dissolved completely. Dispense into amber colored reagent bottle and store at 4 °C
70 % ethanol, 500 ml	Take 360 ml ethanol; mix with 140 ml distilled water. Dispense into reagent bottle and store at 4 °C
Chloroform–isoamyl alcohol (24:1), 500 ml	Measure 450 ml chloroform and 20 ml isoamyl alcohol. Mixed and stored in room temperature
1 M $MgCl_2$, 100 ml RNase A (10 mg/ml)	Weigh 20.33 g $MgCl_2$, dissolve in double distilled water, make up to 100 ml, autoclave. Make up 10 mg/ml RNase in distilled water. Boil for 10 min to destroy DNase. Divide into 1 ml aliquots and store at –20 °C

12. To the aqueous phase add one-tenth volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol and incubate at –20 °C for 1 h or at –70 °C for 30 min
13. Centrifuge at 26832 × *g* for 10 min and wash the pellet in 70 % ethanol (26,832 × *g* for 5 min).
14. Air-dry the pellet and dissolve in 1.5 ml TE and estimate the yield.
15. Estimate DNA amount using Scanning Shimadzu Spectrophotometer. DNA shows a clear absorbance peak at 260 nm and value of 1.0 OD_{260} is calculated equivalent to 50 µg/ml. DNA solution is considered to be pure if the value of OD_{260}:OD_{280} is 1.8. The DNA is visualized on (0.8 %) agarose gel for its quality and stored at –20 °C.

3.9.2 Molecular Profiling Using ISSR (Fig. 8) and SSR Primers

1. Perform amplification in a total volume of 25 µl including 2.5 µl 10× Taq DNA polymerase Buffer, 50 ng DNA, 0.15 mM dNTP, 1.5 mM $MgCl_2$, 0.4 µM primers, and 1U Taq DNA polymerase primers.

Table 4
Composition of various buffers used for DNA isolation

	Buffer	Method of preparation
1	CTAB Extraction Buffer: for 1 L 100 mM Tris–HCl (pH 8.0) 20 mM EDTA (pH 8.0) 1.4 M NaCl 2 % CTAB (w/v) Merck 0.2 % β-mercaptoethanol (v/v)-Merck	Measure 100 ml Tris (1 M), 280 ml NaCl, 40 ml EDTA (0.5 M). Mix with about 400 ml of hot distilled water, add 20 g CTAB to this. Adjust final volume to 1 L. Dispense into reagent bottles and autoclave. Just before use, add 0.2 % β-mercaptoethanol
2	TE (0.1 mM) buffer…for 100 ml 100 mM Tris–HCl (pH 8.0) 0.1 mM EDTA (pH 8.0)	Take 1 ml Tris–HCl (1 M), 20 ml EDTA (0.5 M). Mix with 99 ml sterile distilled water taken in a reagent bottle, mix thoroughly, autoclave
3	TAE buffer 10×: for 1 L	Weigh 48.4 g Tris base; add 20 ml EDTA (0.5 M); 11.42 ml glacial acetic acid and around 150 ml double distilled water. Dissolve the salt and adjust volume to 1 L. Autoclave
4	10× DNA loading dye	Ficoll (Type 400) 25 % (w/v), bromophenol blue 0.4 % (w/v) and xylene cyanol FF 0.4 % (w/v). Dissolve 0.25 g of BPB in 99 ml 30 % glycerol. Keep on magnetic stirrer for several hours to get the dye completely dissolved. Dispense into reagent bottles and keep in 4 °C

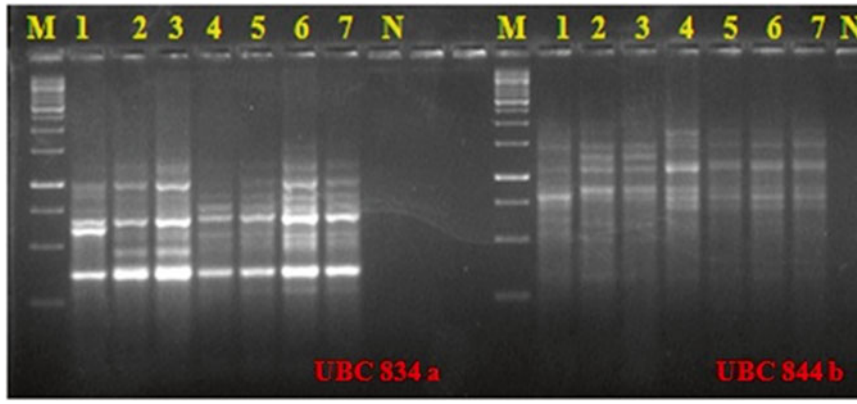


Fig. 8 ISSR profiling of released varieties of turmeric (Lanes 1–7 represent turmeric varieties Suvarna, Suguna, Sudharsana, Prabha, Prathiba, Alleppey Supreme, and Kedaram) (**a**) Using the primer UBC 834a 5′-AGAGAGAG AGAGAGAGCT-3′ and (**b**) Using primer 815 (5′-CTC TCT CTC TCT CTC TG-3′)

2. employ Amplification in a programmable thermal cycler with cycling regimes of an initial denaturation at 94 °C for 5 min, followed by a 33 repeats of a PCR core cycle of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, followed by a final extension cycle of 72 °C for 10 min.

Table 5
ISSR primers for DNA profiling of turmeric varieties

Sl no	Primer name	Sequence
1	UBC 810	5′-GAG AGA GAG AGA GAG AT-3′
2	UBC 811	5′-GAG AGA GAG AGA GAG AC-3′
3	UBC 812	5′-GAG AGA GAG AGA GAG AA-3′
4	UBC 815	5′-CTC TCT CTC TCT CTC TG-3′
5	UBC 834a	5′-AGAGAGAGAGAGAGAGCT-3′
6	UBC 835a	5′-AGAGAGAGAGAGAGAGCC-3′
7	UBC 844b	5′-CTCTCTCTCTCTCTCTGC-3′
8	UBC 850a	5′-GTGTGTGTGTGTGTGTCC-3′
9	UBC 864	5′-ATGATGATGATGATGATG-3′

3. Use the ISSR Primers listed in Table 5.
4. Resolve amplicons electrophoretically alongside a 1 kb ladder, on a 1.5–2 % agarose gel stained with 0.5 mg/ml ethidium bromide in a 1× TAE buffer (pH 8.0), and run at 90 V for 3 h in an electrophoresis unit.
5. Carry out duplicate amplification to confirm reliability of the bands. Amplified products are listed as discrete character states in a present (1) or absent (0) matrix.
6. Relationship among genotypes is evaluated using the Unweight Pair Grouping Mathematical Average (UPGMA) analysis and plotted to produce a dendrogram using NTSyS software.

4 Notes

1. The explant source and stage are the most important criteria for establishing contamination-free cultures with good in vitro responses.
2. Disease- and virus-free source material must be used and the individual plants are also checked to be free from symptoms.
3. Plant the rhizome bits in the greenhouse under protected conditions with 4–5 in. of sand on top and periodically (once in 20 days) spray/drench 0.3 % copper oxychloride/Bavistin/Dithane M-45 to minimize contamination.
4. Since explants from underground are used, there will be a high chance of contamination. To minimize the costs only small amounts (5 ml) of half-strength MS media are used.

5. Fungal and bacterial contamination appears within 5–10 days and the contaminated cultures are discarded.
6. An initial period of 25–30 days is required for the explants to accustom to the nutrient medium before exhibiting the first signs of growth and bud break.
7. The shoot tips show signs of growth in 3–4 weeks and within 5–6 weeks axillary shoot buds also emerge.
8. After activation of rhizome buds, the newly sprouted buds are excised and transferred to fresh media.
9. MS medium [8] is best suited for turmeric. Adjust the pH to 5.8 before adding agar. Agar is melted to ensure uniform distribution in the medium, which is autoclaved at 121 °C at 16 psi for 20 min.
10. For microrhizome induction, use sufficiently grown plants under in vitro conditions. Under sterile conditions separate the well-grown plants and use them for the induction of microrhizome. Trim the roots and shoot before transferring into microrhizome induction media.

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Chapter 28

Protocols for In Vitro Propagation, Conservation, Synthetic Seed Production, Embryo Rescue, Microrhizome Production, Molecular Profiling, and Genetic Transformation in Ginger (*Zingiber officinale* Roscoe.)

K. Nirmal Babu, K. Samsudeen, Minoo Divakaran, Geetha S. Pillai, V. Sumathi, K. Praveen, P.N. Ravindran, and K.V. Peter

Abstract

Ginger is a rhizomatous plant that belongs to the family Zingiberaceae. It is a herbaceous perennial but cultivated as annual, with crop duration of 7–10 months. Ginger is native to India and Tropical South Asia. The tuberous rhizomes or underground stems of ginger are used as condiment, an aromatic stimulant, and food preservative as well as in traditional medicine. Ginger is propagated vegetatively with rhizome bits as seed material. Cultivation of ginger is plagued by rhizome rot diseases, most of which are mainly spread through infected seed rhizomes. Micropropagation will help in production of disease-free planting material. Sexual reproduction is absent in ginger, making recombinant breeding very impossible. In vitro technology can thus become the preferred choice as it can be utilized for multiplication, conservation of genetic resources, generating variability, gene transfer, molecular tagging, and their utility in crop improvement of these crops.

Key words Anther culture, Artificial/synthetic seeds, Cryopreservation, Embryo rescue, In vitro conservation, Micropropagation, Molecular profiling, Plant regeneration, Protoplast isolation, Somaclonal variation, Somatic embryogenesis, Transgenics, Ginger, *Zingiber officinale*

Abbreviations

BA	Benzyl adenine
IBA	Indole-3-butyric acid
Kin	Kinetin
NAA	α-Naphthalene acetic acid
MS	Murashige and Skoog

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1 Introduction

Ginger, one of the oldest and most important spices, is being cultivated in Tropical Asia for over 3000 years and is the underground rhizome of *Zingiber officinale* Rosc (Fig. 1a) belonging to the family Zingiberaceae [1]. Ginger is an essential part of Indian food system and is known for its aromatic as well as medicinal properties. India is a leading producer of ginger in the world and during 2012–2013 the country produced 6, 69,350 tons of the spice from an area of 1, 34,430 ha. Ginger is widely used as a spice in various forms: fresh ginger (green), dried ginger or dry powdered ginger and preserved ginger, ginger paste, ginger oleoresin, ginger oil, etc. Fresh ginger is widely used in cooking to make ginger products and drinks in South-East Asia. Ground dried ginger and preserved ginger are used worldwide for domestic culinary purposes and also in flavoring of processed foods, especially in bakery and confectionary. Ginger oleoresin which has the flavor, aroma, and pungency of the spices is used for flavoring and also in pharmaceuticals. The essential oil from ginger, which has the aroma and flavoring but lacks pungency, is used in flavoring beverages, in cosmetics, confectionary, perfumes, and pharmaceuticals. Ginger is used in medicines

Fig. 1 Ginger

especially as carminative, stimulant of the gastrointestinal tracts, rubefacient, diaphoretic, diuretic, anti-inflammatory, antiemetic, sialagogic emmenagogue, abortifacient, and vermifuge [2].

Lack of seed set in ginger leads to narrow genetic base hampering crop improvement programs and vegetative propagation by rhizomes results in spread of diseases like rhizome rot (caused by *Pythium aphanidermatum*) and bacterial wilt (caused by *Ralstonia solanacearum*). The in vitro techniques and protocols standardized and described below can help solve these major bottlenecks in breeding and cultivation

2 Materials

2.1 Explant Materials

Vegetative bud explants from rhizomes.

2.2 Glassware

Borosilicate conical flasks (500 ml), glass bottles (250 ml), culture tubes (22 cm × 3.5 cm), and 250 ml conical flasks, culture vessels, tubes, bottles and flasks, cotton plugs (made of non-absorbent cotton covered with cheese cloth), aluminum foil, or polypropylene caps.

2.3 Growth Regulators

Auxins—α-naphthalene acetic acid (NAA), indole-3-butyric acid (IBA) 0–1.0 mg/l;

Cytokinins—6-Benzylaminopurine (BA) and 6-furfurylamino purine (kinetin) (Sigma Chemicals) 0–3.0 mg/l.

2.4 Gelling Agents

Bacteriological grade agar, 7–8.0 g/l.

2.5 Culture Medium

- Murashige and Skoog (MS) [3] basal medium (Tables 1 and 2).
- Stock solutions for macronutrients, micronutrients, vitamins, amino acids, and plant growth regulators (Table 2).
- Sucrose—30 g/l for multiplication and 90 g/l for microrhizome induction.

2.6 Culture Establishment

Healthy rhizomes, collected from potted plants.

2.7 Equipment

Laminar airflow, autoclave, MilliQ filter sterilization unit, Leica inverted microscope, PCR machine, electrophoresis unit, refrigerated centrifuge, He-driven particle delivery system (PDS).

Table 1
Composition of Murashige and Skoog[a] basal medium

Composition		Concentration (mg/l)
Macronutrients		
Ammonium nitrate	NH_4NO_3	1650.00
Potassium nitrate	KNO_3	1900.00
Calcium chloride	$CaCl_2{\cdot}2H_2O$	440.00
Potassium orthophosphate	KH_2PO_4	170.00
Magnesium sulfate	$MgSO_4{\cdot}7H_2O$	370.00
Micronutrients		
Sodium EDTA	Na_2EDTA	37.30
Ferrous sulfate	$FeSO_4{\cdot}7H_2O$	27.80
Boric acid	H_3BO_3	6.20
Manganese sulfate	$MnSO_4{\cdot}4H_2O$	22.30
Potassium iodide	KI	0.83
Zinc sulfate	$ZnSO_4{\cdot}7H_2O$	8.60
Sodium molybdate	$Na_2MoO_4{\cdot}2H_2O$	0.25
Copper sulfate	$CuSO_4{\cdot}5H_2O$	0.025
Cobalt chloride	$CoCl_2{\cdot}6H_2O$	0.025
Vitamins		
Myoinositol	$C_6H_{12}O_6$	100.00
Thiamine HCl	$C_{12}H_{17}ClN_4OS{\cdot}HCl$	0.10
Nicotinic acid	$C_6H_5NO_2$	0.50
Pyridoxine HCl	$C_8H_{11}NO_3{\cdot}HCl$	0.50
Amino acid		
Glycine	$C_2H_5NO_2$	2.00

[a]Murashige and Skoog, 1962

3 Methods

3.1 Micro-propagation

3.1.1 Establishment of Cultures in Ginger (Fig. 2)

1. Rhizomes with buds/sprouts can be used as explants.
2. Select the rhizome cuttings or newly sprouting buds. In the laminar airflow hood, wash the explants thoroughly with sterile double-distilled water and dry on sterile filter paper.
3. Wash the explants under running tap water for 15 min followed by detergent solution (Tween 20) for 10–15 min.
4. Surface sterilize using copper oxychloride, bavistin (10 min each), and 70 % ethanol (30–40 s) and wash with sterile distilled water for 3–4 min to remove the remnants of the fungicide.
5. Before inoculation, the laminar flow should be cleaned with ethyl alcohol (70 %) for 30–60 s.

Table 2
Details of various stock solutions for MS medium

Stock	Composition	Stock strength	Quantity[a]
A	Macronutrients NH_4NO_3 KNO_3 $CaCl_2 \cdot 2H_2O$[b] KH_2PO_4 $MgSO_4 \cdot 7H_2O$	×20	50 ml
B	Micronutrients H_3BO_3 $MnSO_4 \cdot 4H_2O$ KI $ZnSO_4 \cdot 7H_2O$ $Na_2MoO_4 \cdot 2H_2O$ $CuSO_4 \cdot 5H_2O$[b] $CoCl_2 \cdot 6H_2O$[b]	×100	10 ml
C	Micronutrients Na_2EDTA[b] $FeSO_4 \cdot 7H_2O$[b]	×100	10 ml
D	Vitamins Thiamine HCl Nicotinic acid Pyridoxine HCl	×100	10 ml
E	Amino acid Glycine	×100	10 ml
F	Myoinositol Growth regulators 2,4-D NAA BA Kinetin	×100 50 mg/200 ml 50 mg/200 ml 50 mg/200 ml 50 mg/200 ml	10 ml

[a]For preparation of 1 l medium. Make up the remaining in double-distilled water up to 900 ml, adjust the pH, and add the remaining water to 1000 ml
[b]Dissolved separately before mixing in the final stock

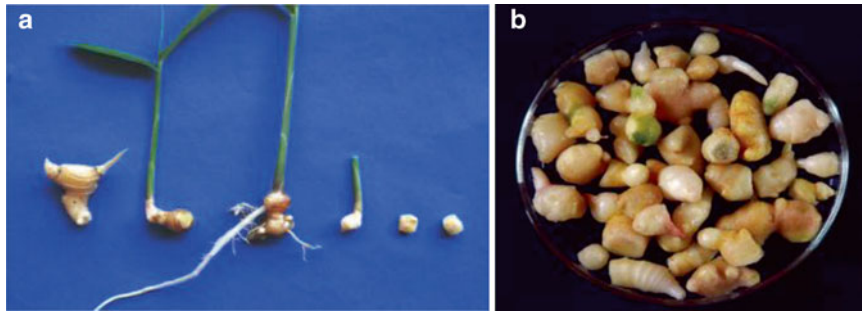

Fig. 2 Explant sources of ginger

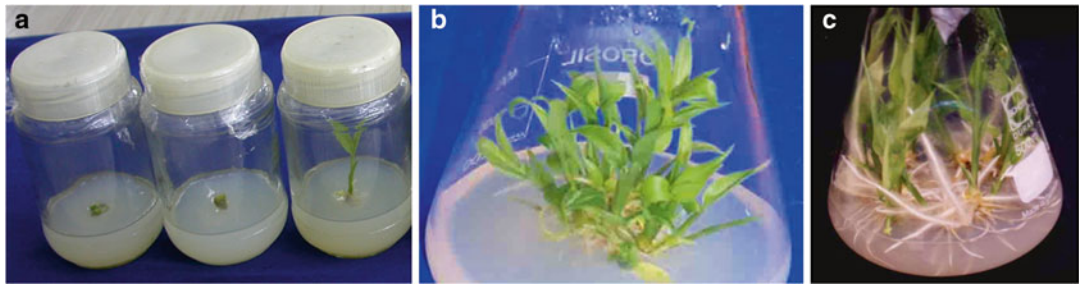

Fig. 3 Stages of culture establishment and in vitro multiplication—ginger (**a**–**c**)

6. In the laminar airflow hood, wash the explants again in sterile double-distilled water followed by treatment with 0.1 % $HgCl_2$ for 3–5 min and rinse with sterile distilled water 3–4 times.
7. Sterilized buds can be cut to required size usually 1 cm with 2–3 dormant buds or 3–4 mm with growing vegetative bud and inoculate to the initiation medium.
8. Inoculate the explants in test tubes containing half-strength MS media supplemented with 3 mg/l BA and 1 mg/l NAA. Adjust pH to 5.8 before adding agar melt to ensure uniform distribution in the medium, and autoclave the media at 121 °C at 16 psi for 20 min.

3.1.2 Multiplication (Fig. 3)

1. One-week-old, contamination-free, cultures can be used for multiplication. Explants taken out carefully under sterile conditions transfer into multiplication MS medium supplemented with 5 mg/l BA and 1.0 mg/l NAA.
2. Use 7–8-week-old cultures for sub-culturing and for further multiplication. 20–25 plants can be harvested from 8-week-old culture [4].

3.2 Direct Regeneration of Plantlets from Immature Inflorescence (Fig. 7a)

1. Collect immature, 1–10-day-old inflorescence (during flowering season) and sterilize.
2. Remove the outer bracts under aseptic condition and transfer remaining inflorescence to culture medium.
3. Use MS medium supplemented with 10 mg/l BA and 0.2 mg/l 2,4-D.
4. Use of MS liquid medium with 1 mg/l NAA enhances rooting [5].

3.3 In Vitro Rooting and Production of Plantlets (Fig. 3)

1. Ginger cultures do not require separate culture medium for rooting.
2. Shoots 3–4 cm long automatically root in the media used.
3. Use MS liquid medium containing 1 mg/l NAA to improve rooting before transplanting to the soil.

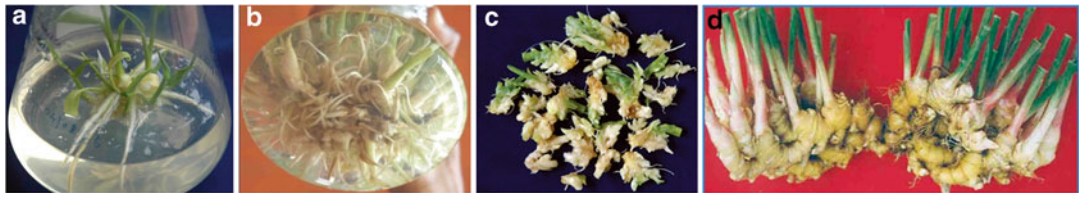

Fig. 4 Stages in microrhizome induction and in vitro rhizome formation in ginger; (**a**) In vitro development of plantlets; (**b**) induction of microrhizomes, in vitro; (**c**) in vitro rhizomes ready for planting; (**d**) shoot development and microrhizome proliferation

3.4 Microrhizome Induction (Fig. 4)

1. In vitro-developed plantlets (4–5 cm) with well-formed roots are used for microrhizome induction.
2. Separate the well-grown plants under sterile condition and use. Roots and shoots should be trimmed before transferring into microrhizome induction media.
3. Microrhizome formation will be noticed in MS medium supplemented with 90 g sucrose after 90–100 days of inoculation and reaches maturity in 120 days.
4. These microrhizomes can be directly planted out in bags or in the field for normal rhizome development.

3.5 Callus Culture and Inducing Variability

3.5.1 Plant Regeneration from Leaf-Derived Callus [4]

1. Wash the explants under running tap water followed by washing with 0.1 % $HgCl_2$ for 5–10 min. Rinse the explants 3–4 times to wash away the traces of $HgCl_2$.
2. Outer leaf sheaths can be removed under aseptic condition and place a small segment of innermost leaf tissue on MS media supplemented with 2,4-D and NAA. Callus is formed in the medium containing 0.2 mg/l 2,4-D and 0.5 mg/l NAA.
3. Two-month-old callus can be sub-cultured in MS medium containing 0.2 mg/l 2,4-D and 10 mg/l BAP for organogenesis and plant regeneration.

3.5.2 Plant Regeneration from Ovary-Derived Callus (Fig. 5)

1. Collect 7–15-day-old inflorescence for inoculation.
2. Disinfect by washing with 5 % teepol solution for 20 min followed by sterile water. Surface sterilization can be done with 0.1 % $HgCl_2$ solution for 5–7 min and rinse 4–5 times with sterile water.
3. Excise ovaries from individual flowers under aseptic condition and culture in MS medium containing 30 g/l bacteriological grade agar supplemented with 0.2 mg/l 2,4-D and 10 mg/l BAP for inducing somatic embryogenic cultures [6].
4. Culture somatic embryoids in MS medium containing 1 mg/l NAA for further growth and development of plantlets.

3.5.3 Plant Regeneration from Anther-Derived Callus Cultures (Fig. 6)

1. Collect the inflorescence, wash under running tap water, and keep at 0 °C for 1–9 days for cold treatment.

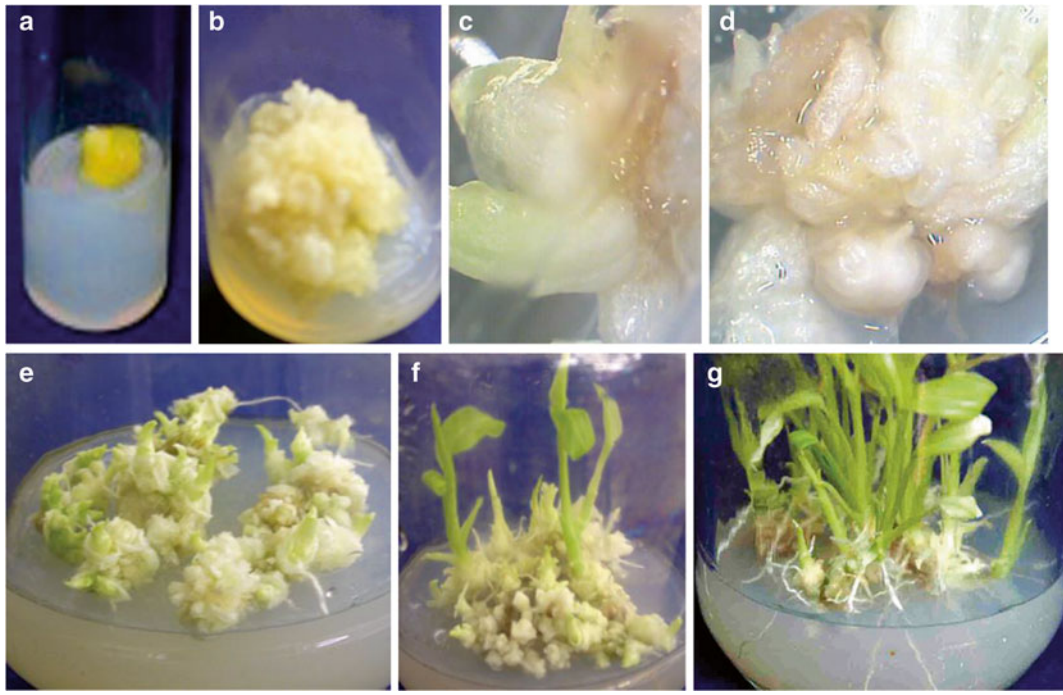

Fig. 5 Different stages of plant regeneration in ginger, (**a**) explant, (**b**) callus, (**c**) organogenesis, (**d**) embryogenesis, (**f**) and (**g**) repeated budding after organogenesis from ovary-derived callus cultures and plant development from them and (**g**) well-developed plantlets

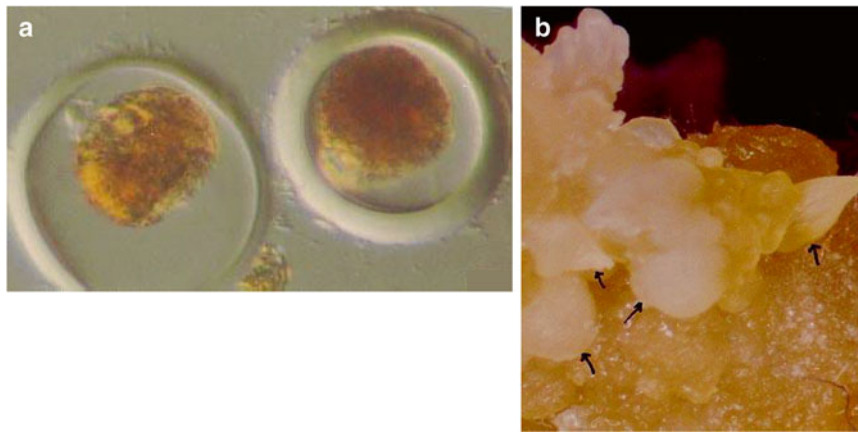

Fig. 6 Uni-nucleate pollen (**a**) from ginger and plant regeneration (**b**) from anther-derived callus

2. Individual flowers are excised from inflorescence and surface sterilize with 0.1 % mercuric chloride for 10 min followed by 3–4 times washing with sterile water.
3. For organogenesis and plant regeneration, dissect flowers and anthers with uninucleate microspores (determined by

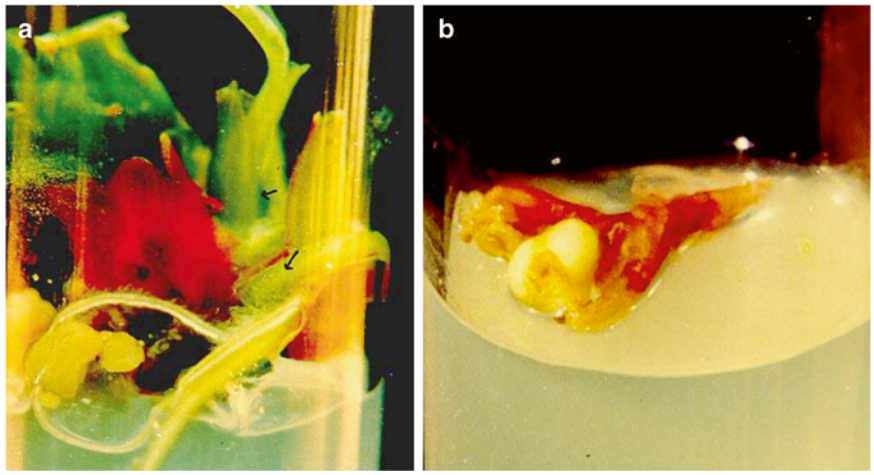

Fig. 7 (**a**) Inflorescence culture and plant development and (**b**) embryo rescue and fruit development in ginger

acetocarmine staining, where a distinct nucleus was visible) could be inoculated in MS medium supplemented with 0.2 mg/l 2,4-D and 10 mg/l BAP [7, 8].

3.6 Hardening and Planting Out

1. Take out healthy and well-rooted in vitro plantlets, and wash carefully to remove the traces of culture medium.
2. Transfer to polythene bags containing garden soil, sand, and farmyard manure in equal proportions and keep in humid chamber.
3. 90–100 % relative humidity should be maintained for 20–30 days for hardening and establishment.

3.7 Embryo Rescue and Fruit Development in Ginger (Fig. 7b) [5, 6]

1. Select aseptic flowers from preculture inflorescences.
2. Keep the single flowers along with ovary, stigma, and the anther around the style without damage on MS medium supplemented with BA.
3. Disrupt the anther so that pollen will come out of it and assist the pollen so that it reaches the stigma.
4. In about 15–30 days the ovary will show the signs of growth and fruit development.
5. Dissect the seeds from the mature fruit and culture them in the same medium for plantlet development.
6. This technique can be very useful to assist recombination breeding in ginger.

3.8 Encapsulation and Synthetic Seed Production

1. Use in vitro-developed shoots/embryoids 1–3 mm in size consisting of the apical dome for developing synseeds (Fig. 8).

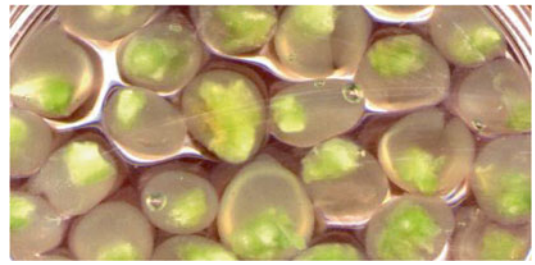

Fig. 8 Synthetic seeds (encapsulated micro shoots/somatic embryos) of ginger

2. Suspend in vitro-grown shoots/somatic embryos in MS basal medium supplemented with 4 % (w/v) Na alginate, 2 M glycerol, and 0.4 M sucrose.
3. Drop the mixture containing micro-shoots, with a sterile pipette into 0.1 M $CaCl_2$ solution containing 2 M glycerol and 0.4 M sucrose, and leave for 20 min to form beads about 4 mm in diameter, each bead containing at least one shoot.
4. Store the beads in sterile containers for 6–8 months.
5. Transfer to MS medium with 1 mg/l BAP + 0.5 mg/l NAA for regrowth [9].

3.9 Conservation of Genetic Resources

3.9.1 Medium-Term In Vitro Conservation by Slow Growth (Fig. 9)

1. Use vegetative bud explants to establish cultures in MS basal medium amended with 0.5 mg/l kinetin.
2. Multiply shoot cultures on MS medium supplemented with 0.5 mg/l NAA and 1.0 mg/l BAP with an average of eight shoots per culture in about 90 days of culture.
3. Maintain in vitro shoots on half-strength MS medium supplemented with 15 g/l each of sucrose and mannitol in sealed culture tubes which induces slow growth and they can be stored for up to 12 months with 80 % survival.
4. The cultures can thus be maintained on the same medium with yearly subculture for up to 7 years.
5. The miniaturized in vitro-stored cultures can be recovered to normal size by transferring to the multiplication medium (MS + 1 mg/l BAP + 0.5 mg/l NAA), resume growth, and multiply normally in 3 weeks of culture. Multiple shoots and roots are produced in the same medium.
6. Transfer rooted plantlets to the nursery cups and maintain at 90–100 % relative humidity for 20–30 days for hardening and with 80–90 % survival.

3.9.2 Long-Term Storage of Encapsulated Shoot Tips by Cryopreservation

In vitro-maintained shoot buds (0.5–1.0 mm in length) of ginger consisting of the apical dome with 3–4 leaf primordial are used.

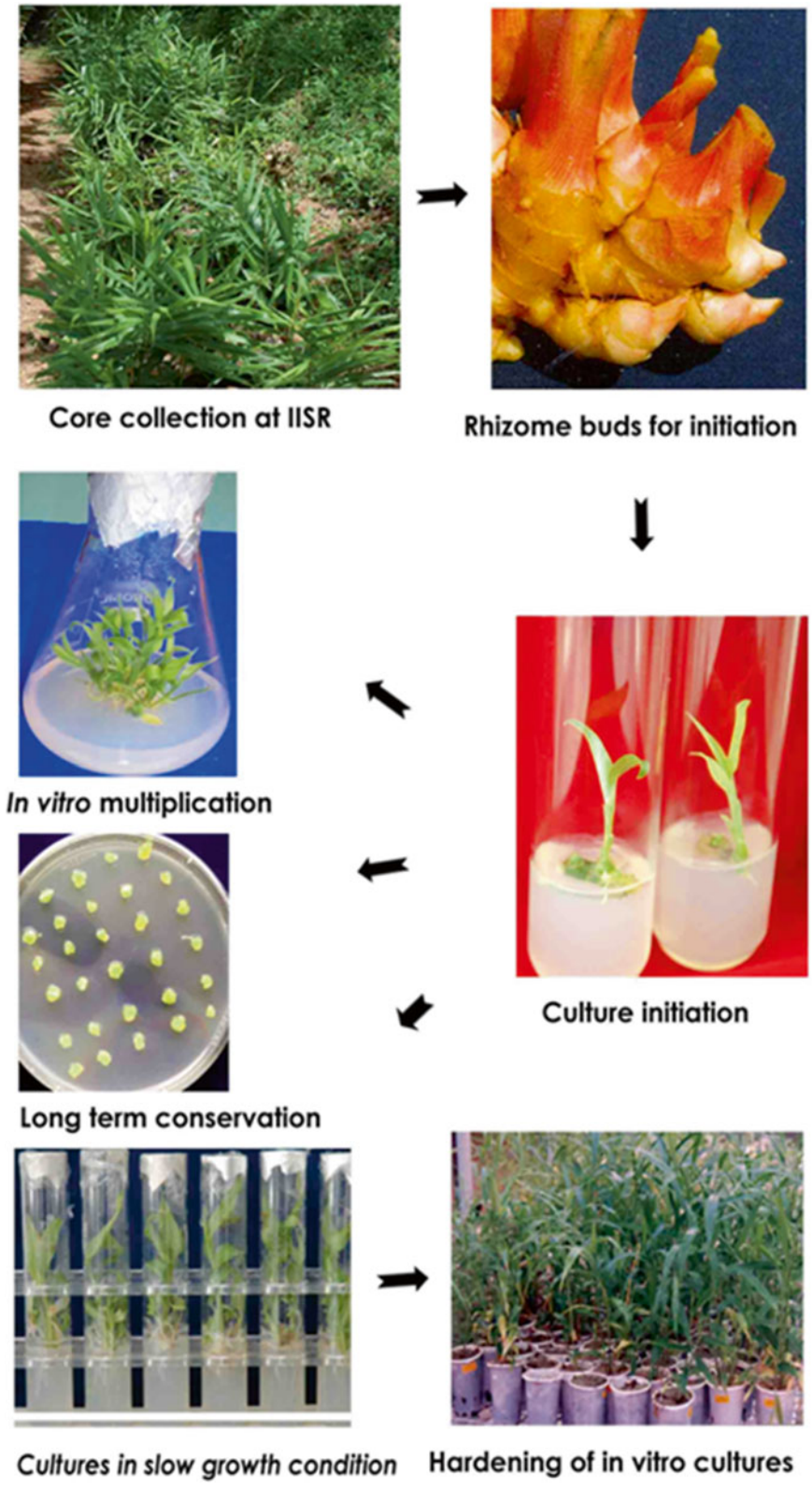

Fig. 9 Different stages of conservation of ginger genetic resources through in vitro and cryoconservation

Pretreatment and Dehydration

1. Preculture the encapsulated shoots—stepwise—on MS medium enriched with different concentrations of sucrose, 0.3, 0.5, 0.75, and 1.0 M, for 4 days with 1 day on each.
2. Place the precultured beads on sterile fitter paper in petri dishes (diameter 90 mm) and dehydrate by air-drying on a flow bench (at room temperature and humidity) for 0–10 h to determine the optimal dehydration time.
3. Transfer the dehydrated beads into a 2 ml cryovial (ten beads per tube) and directly immerse in the liquid nitrogen for 24 h.
4. The beads can be subjected to thawing by rapidly immersing in a water bath at 40 °C for 1 min.

Cryopreservation

Vitrification procedure (Fig. 10).

1. Shoot tips (0.5–1.0 mm) are precultured in 0.3 M sucrose for 72 h and then immersed for 20 min at room temperature in a series of five cryo-protectant mixtures containing 5 % DMSO; 10 % DMSO; 5 % glycerol; 10 % glycerol; or 5 % DMSO + 5 % glycerol.
2. Further, treat the shoots with loading solution (2 M glycerol + 0.4 M sucrose) for 20 min at 25 °C.
3. Pretreated and osmoprotected shoots can be dehydrated in 20 ml PVS2 in a 50 ml Erlenmeyer flask on a rotary shaker (60 rpm) at 25 °C for 0–60 min.
4. After dehydration shoots can be suspended in 1 ml PVS2 in cryotubes and plunged into LN for 24 h.

Thawing and Regrowth

1. Thawing can be done by rapidly immersing the cryotubes in a water bath at 40 °C for 1 min for all three treatments.
2. For encapsulation-vitrification- and vitrification-treated samples, PVS2 solution is drained from the cryotubes and replaced twice with 1.2 M sucrose solution at 10-min interval.
3. Place thawed propagules directly on MS medium supplemented with BA (1 mg/l) and NAA (0.5 mg/l) in petri dishes, maintain initially for 2 days in the darkness, and transfer to light with intensity of 33.8 μmol/m^2/s^2.
4. Around 80 % recovery of cryopreserved shoots can be obtained [10–13].

3.10 Protoplast Culture in Ginger

Technology for isolation and culture of ginger and turmeric protoplasts is available [13].

3.10.1 Isolation of Protoplasts (Tables 3 and 4)

1. Mesophyll tissues of in vitro-derived leaves or actively growing callus tissue from 5- to 7-day-old suspension culture can be used for protoplast isolation.
2. The lower surface of the leaves is punctured for enzymatic digestion.

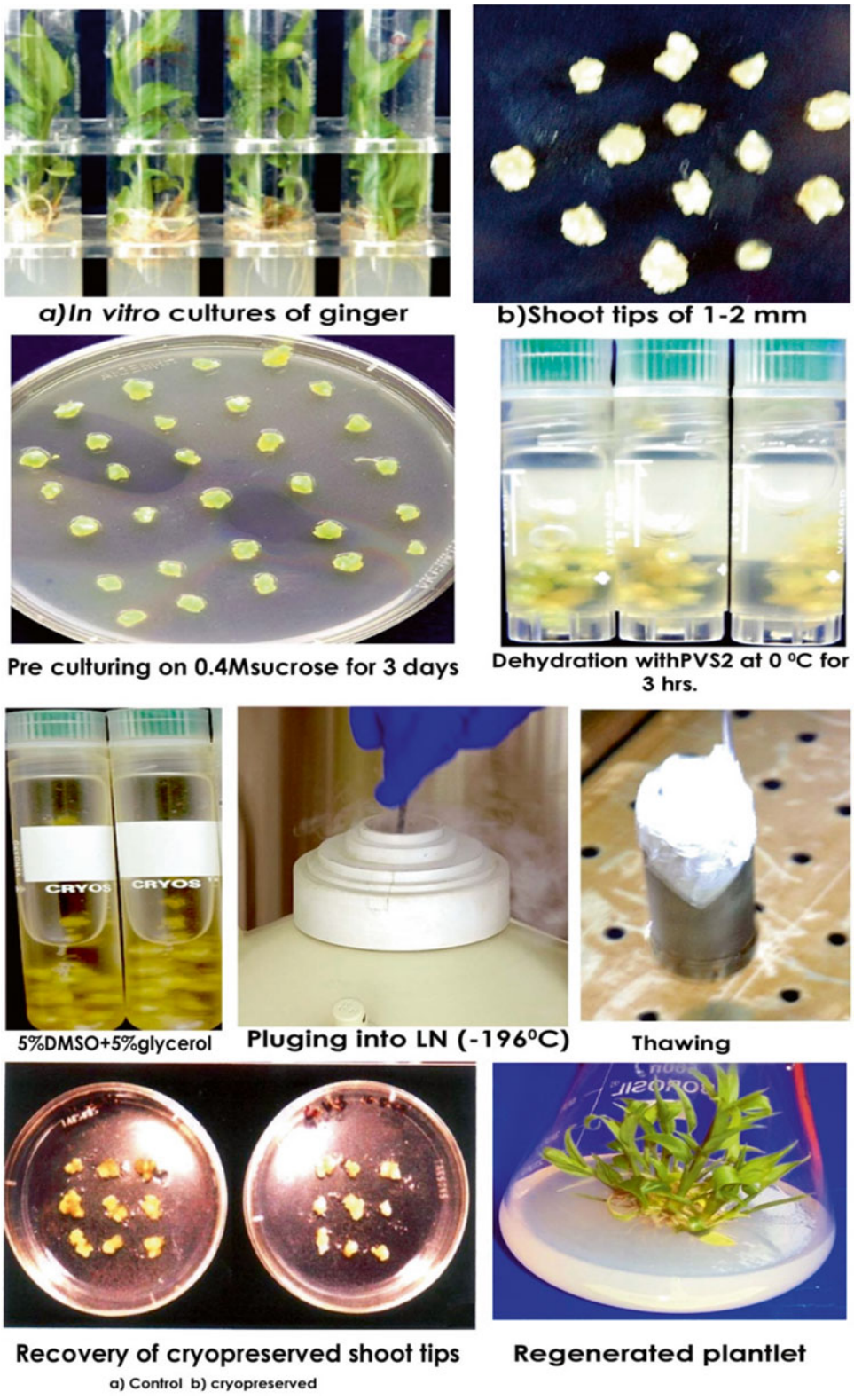

Fig. 10 Different stages in cryopreservation of ginger micro shoots through vitrification

Table 3
Composition of enzyme solutions (ES) for isolation of protoplasts[a]

Code	Mannitol (%)	Macerozyme R-10 (%)	Hemi-cellulase (%)	Onozuka cellulase R-10 (%)
ES-1	10	0.5	–	1.0
ES-2	9	0.5	–	1.0
ES-3	8	0.5	–	1.0
ES-4	7	0.5	–	1.0
ES-5	6	0.5	–	1.0
ES-6	5	0.5	–	1.0
ES-7	10	0.5	0.5	2.0
ES-8	9	0.5	0.5	2.0
ES-9	8	0.5	0.5	2.0
ES-10	7	0.5	0.5	2.0
ES-11	6	0.5	0.5	2.0
ES-12	5	0.5	0.5	2.0
ES-13	10	1.0	–	3.0
ES-14	9	1.0	–	3.0
ES-15	8	1.0	–	3.0
ES-16	7	1.0	–	3.0
ES-17	6	1.0	–	3.0
ES-18	5	1.0	–	3.0

[a]Enzyme solutions were prepared in CPW medium

3. Suspension cultures are centrifuged and use 1 g pelleted callus for enzymatic digestion.
4. Mixture of macerozyme, cellulase, and Onozuka 10 at different combinations is used.
5. Enzyme solution is prepared in CPW medium with osmoticum (9 % mannitol).
6. Induce pre-plasmolysis by incubating tissues in the above medium.
7. Immerse 1 g each of mechanically macerated in vitro leaves and calli in 10 ml each of the enzyme solution and incubate in the dark.
8. Incubate leaf tissues for 16 h (10 h at 15 °C followed by 6 h at 30 °C) and callus for 18 h (10 h at 15 °C followed by 8 h at 30 °C) with gentle shaking at 53 rpm.

Table 4
Composition of media used for protoplast isolation and culture in ginger

Components	Cell protoplast washing (CPW) medium (mg/l)	Floating media (mg/l)	Protoplast culture media (mg/l)	
			I	II
NH_4NO_3	–	–	1650	1650
KNO_3	101.0	101.0	1900	1900
$CaCl_2{\cdot}2H_2O$	1480.0	1480.0	440	440
$MgSO_4{\cdot}7H_2O$	246.0	246.0	370	370
KH_2PO_4	27.2	27.2	170	170
KI	0.16	0.16	0.83	0.83
H_3BO_3	–	–	6.2	6.2
$MnSO_4{\cdot}4H_2O$	–	–	22.3	22.3
$ZnSO_4{\cdot}7H_2O$	–	–	8.7	8.7
$Na_2MoO_4{\cdot}2H_2O$	–	–	0.25	0.25
$CuSO_4{\cdot}5H_2O$	0.025	0.025	0.025	0.025
$CoCl_2{\cdot}6H_2O$	–	–	0.025	0.025
$FeSO_4{\cdot}7H_2O$	–	–	27.8	27.8
$Na_2EDTA{\cdot}2H_2O$	–	–	37.3	37.3
Myoinositol	–	–	100.0	100.0
Nicotinic acid	–	–	0.5	0.5
Thiamine HCl	–	–	0.5	0.5
Pyridoxine HCl	–	–	0.5	0.5
Glycine	–	–	2.0	2.0
Sucrose	–	21 %	2 %	3 %
Mannitol	7–10 %	–	7 %	4 %
Gibberellic acid	–	–	0.5	0.5
BA	–	–	0.5	1.0
NAA	–	–	0.5	1.0
2,4-D	–	–	0.5	–

9. Purification is done with a combination of filtration, centrifugation, washing, and floating centrifugation.
10. Undigested tissues and cell clumps should be filtered and cleaned.
11. Separate the released protoplasts by centrifugation at 700 rpm for 10 min and remove supernatant carefully without disturbing the pelleted protoplasts.
12. Pellet can be suspended in CPW medium and repeat the centrifugation three times to remove the traces of enzyme solution.
13. After washing, suspend protoplasts in 1 ml CPW medium and layer it on 9 ml floating medium (Table 4) and centrifuge at 700 rpm for 10 min. The protoplasts will form a layer at the interphase.

3.10.2 Protoplast Culture (Fig. 11)

1. Isolated protoplasts can be cultured in the liquid medium in petri dishes.
2. Five drops of the culture medium containing protoplast can be incubated in the dark at 25 °C in well-sealed petri plates.
3. Fresh culture medium can be poured periodically to avoid nutrient depletion and observe for cell wall regeneration and cell division, under Leica inverted microscope.
4. 40–60-day-old culture can be plated on 0.25 % agarose medium for further development and micro callus formation.

3.11 Molecular Profiling

CTAB method was used [14]. The protocol *is* used for DNA extraction from leaf tissues (*see* Tables 5 and 6).

3.11.1 Isolation of DNA

1. Grind 5 g young leaves in liquid nitrogen with a mortar and pestle and add 25 ml preheated (65 °C) CTAB buffer. Add 0.2 % β-mercaptoethanol prior to use.
2. Incubate at 60 °C for 30 min.
3. Extract with equal volume of chloroform:isoamyl alcohol (24:1) at 26,832 × *g* for 10 min at room temperature.
4. Take the aqueous phase and add 2/3rd volume of ice-cold isopropanol.
5. Incubate at −20 °C for 2 h and centrifuge at 26,832 × *g* for 15 min at 4 °C.
6. Discard the supernatant and invert the tube on paper towel for few minutes.
7. Dissolve the pellet and add 1.5 ml of TE buffer. Store at room temperature.
8. Add 10 μg/ml of RNase A and incubate at 37 °C for 30 min.
9. Equal volume of Tris-saturated phenol is added, mix well, and centrifuge at 26,832 × *g* for 10 min.

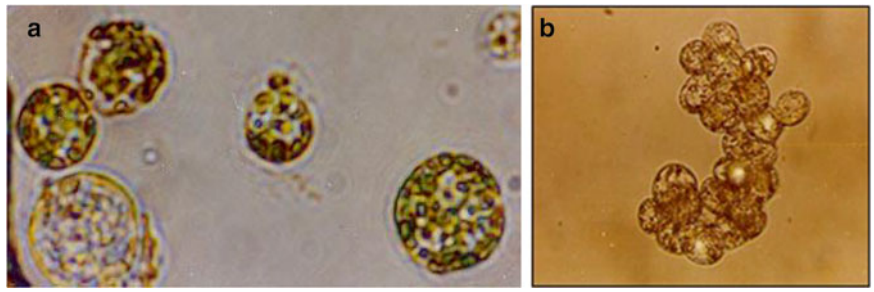

Fig. 11 Protoplasts in ginger (**a**) and micro calli (**b**) derived from the protoplasts

Table 5
Composition of various stock solutions for DNA isolation [16]

Solutions	Method of preparation
1 M Tris (pH 8.0) 500 ml	Dissolve 60.55 g Tris base (Sigma) in 300 ml distilled water. Adjust pH to 8 by adding concentrated HCl. Adjust volume to 500 ml. Dispense to reagent bottles and sterilize by autoclaving
0.5 M EDTA pH 8.0	Dissolve 93.05 g of EDTA-disodium salt (sigma) in 300 ml of water. Adjust pH to 8 by adding NaOH pellets. Adjust volume to 500 ml. Dispense into reagent bottles and autoclave
5 M NaCl 500 ml	Weigh 146.1 g NaCl (Merck), add 200 ml of water, and mix well. When the salts get completely dissolved, adjust the final volume to 500 ml. Dispense into reagent bottles and autoclave
3 M Sodium acetate (pH 5.2) 250 ml	Dissolve 61.523 g of anhydrous sodium acetate (Qualigens) in 200 ml of water and mix well. When dissolved completely adjust the pH of the solution to 5.2 with glacial acetic acid (99–100 %). Autoclave
Ethidium bromide 10 mg/ml, 100 ml	Add 1 g. ethidium bromide to 100 ml of distilled water. Keep on magnetic stirrer to ensure that the dye has dissolved completely. Dispense to amber-colored reagent bottle and store at 4 °C
70 % ethanol, 500 ml	Take 360 ml of ethanol; mix with 140 ml of distilled water. Dispense to reagent bottle and store at 4 °C
Chloroform:isoamyl alcohol (24:1), 500 ml	Measure 450 ml of chloroform and 20 ml of isoamyl alcohol. Mixed and stored in room temperature
1 M $MgCl_2$, 100 ml	Weigh 20.33 g of $MgCl_2$, dissolve in double-distilled water, make up to 100 ml, autoclave
RNase A (10 mg/ml)	Make up 10 mg/ml RNase in distilled water. Boil for 10 min to destroy DNase. Divide into 1 ml aliquots and store at −20 °C

10. To the aqueous phase add equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), shake, and centrifuge at 26,832 × *g* for 10 min.
11. Take the aqueous phase and add equal volume of chloroform:isoamyl alcohol (24:1), shake, and centrifuge at 26,832 × *g* for 10 min.

Table 6
Composition of various buffers used for DNA isolation [16]

	Buffer	Method of preparation
1	CTAB extraction buffer: for 1 l 100 mM Tris–HCl (pH 8.0) 20 mM EDTA (pH 8.0) 1.4 M NaCl 2% CTAB (w/v) Merck 0.2% B-mercapto ethanol (v/v)-Merck	Measure 100 ml Tris (1 M), 280 ml of NaCl, 40 ml of EDTA (0.5 M). Mix with about 400 ml of hot distilled water, add 20 g of CTAB to this. Adjust final volume to 1 l. Dispense to reagent bottles and autoclave. Just before use, add 0.2 % β-mercaptoethanol
2	TE (0.1 mM) buffer: for 100 ml 100 mM Tris–HCl (pH 8.0) 0.1 mM EDTA (pH 8.0)	Take 1 ml of Tris–HCl (1 M), 20 ml of EDTA (0.5 M). Mix with 99 ml of sterile distilled water taken in a reagent bottle, mix thoroughly, autoclave
3	TAE buffer 10×: for 1 l	Weigh 48.4 g of Tris base; add 20 ml of EDTA (0.5 M); 11.42 ml of glacial acetic acid and around 150 ml d.water. Dissolve the salt and adjust volume to 1 l. Autoclave
4	10× DNA loading dye	Ficoll (Type 400) 25 % (w/v), bromophenol blue 0.4 % (w/v), and xylene cyanol FF 0.4 % (w/v). Dissolve 0.25 g of BPB in 99 ml of 30 % glycerol. Keep on magnetic stirrer for several hours to get the dye completely dissolved. Dispense to reagent bottles and keep at 4 °C

12. To the aqueous phase add one-tenth volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol and incubate at –20 °C for 1 h or at –70 °C for 30 min.
13. Centrifuge at 26,832 × *g* for 10 min and wash the pellet in 70 % ethanol (26,832 × *g* for 5 min).
14. Air-dry the pellet and dissolve in 1.5 ml TE and estimate the yield.
15. Estimate DNA amount using Scanning Shimadzu Spectrophotometer. DNA shows a clear absorbance peak at 260 nm and value of 1.0 OD_{260} is calculated equivalent to 50 µg/ml. DNA is considered to be pure if the value of OD_{260}:OD_{280} is 1.8. DNA is visualized on (0.8 %) agarose gel for its quality and stored at –20 °C.

3.11.2 Molecular Profiling Using ISSR and SSR Primers

1. Amplification is carried out in a total volume of 25 µl including 2.5 µl × Taq DNA polymerase buffer, 50 ng DNA, 0.15 mM dNTP, 1.5 mM $MgCl_2$, 0.4 µM primers, and 1 U Taq DNA polymerase primers. Amplification employs a programmable thermal cycler (with cycling regimes of an initial denaturation temperature of 94 °C for 5 min, followed by 33 repeats of a PCR

core cycle of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, followed by a final extension cycle of 72 °C for 10 min).

2. Use the ISSR primers listed in Table 7.
3. Resolve amplicons electrophoretically alongside a 1 kb ladder, on a 1.5–2 % agarose gel stained with 0.5 mg/ml 1× ethidium bromide in a 1× TAE buffer(pH 8.0), and run at 90 V for 3 h in an electrophoresis (Fig. 12).

Table 7
ISSR and SSR primers for DNA profiling of ginger varieties

Sl no	Primer name	Sequence
ISSR		
1	UBC 807	5′-AGAGAGAGAGAGAGAGT-3′
2	UBC 808	5′-AGAGAGAGAGAGAGAGC-3′
3	UBC 810	5′-GAG AGA GAG AGA GAG AT-3′
4	UBC 811	5′-GAG AGA GAG AGA GAG AC-3′
5	UBC 815	5′-CTC TCT CTC TCT CTC TG-3′
6	C 1	5′-AGCACACACACACACAC
7	C 10	5′-ACCTCCTGCAGATTCGTGTC-3′
8	UBC 840a	5′-GAGAGAGAGAGAGAGACT-3′
9	UBC 841 a	5′-GAGAGAGAGAGAGAGACC-3′
10	UBC 844b	5′-CTCTCTCTCTCTCTCTGC-3′
SSR		
11	GB-ZOM-040	F 5′-AGGGGGCAGTGGAGAG-3′ R 5′-ACGTTCCTGCACTTGACG-3′
12	GB-ZOM-103	F 5′-GCTGCGGACTAAATGCTG-3′ R 5′-ACGCTAGGGAACAGGGAG-3′

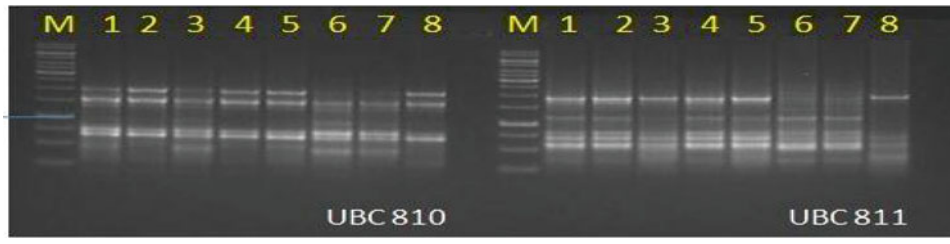

Fig. 12 ISSR profiling of high-yielding varieties of ginger using primer UBC 810 and UBC 811. *Lane 1* represents ginger varieties Varada, *lane 2*: Rejatha, *lane 3*: Mahima, *lane 4*: Suprabha, *lane 5*: Suruchi, *lane 6*: Athira, *lane 7*: Karthika, and *lane 8*: OCP 1222

4. Carry out duplicate amplification to confirm reliability of the bands. Amplified products are listed as discrete character states in a present (1) or absent (0) matrix.
5. Relationships among genotypes are evaluated with a phonetic cluster analysis using the unweighted pair grouping mathematical average (UPGMA) analysis and plotted in a phenogram using many available software based on suitability.

3.12 Genetic Transformation in Ginger (Fig. 13)

A simple approach [2, 11] for transient expression of β-glucuronidase (GUS) gene in ginger embryogenic callus with "BioRad" helium-driven PDS-1000/He system is presented here.

3.12.1 Transient Gene Expression Studies Using Helium-Driven PDS-1000/He Biolistic System

3.12.2 Preparation of Embryogenic Calli Culture

1. Transfer freshly growing embryogenic calli to petri dishes, as thin layers, with MS medium containing 0.25–0.75 % mannitol, 0.25 % phytagel, or 0.9 % agarose and devoid of plant growth regulators.
2. Incubate cultures in the darkness for 12 h before subjecting to particle bombardment.

3.12.3 Preparation of Plasmid DNA

1. Use plasmid vector pHAC 25(*25*) containing ubiquitin-β-glucuronidase (Ubi-GUS).
2. Isolate ubiquitin-phosphinothricin-acetyl transferase (Ubi-BAR) from *E. coli* XL1-Blue using alkaline lysis method (*22*) followed by ethanol precipitation.
3. The GUS gene in this construct is expressed from the maize ubiquitin promoter.
4. The concentration of DNA is quantified by Hitachi U 2000 Spectrophotometer.

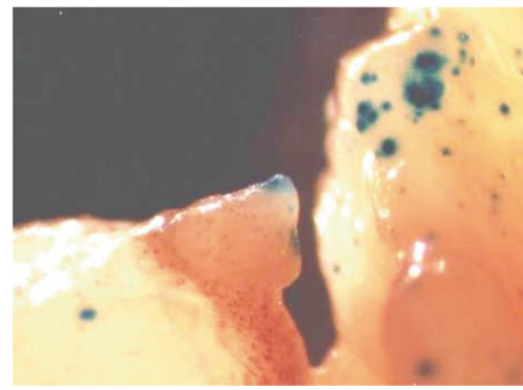

Fig. 13 Transient expression of GUS in embryogenic callus of ginger

3.12.4 Microcarrier (Gold/Tungsten Particles) Preparation

For 120 bombardments use 500 μg gold particles per bombardment.

1. In a 1.5 ml microfuge tube, weigh out 60 mg microparticles.
2. Add 1 ml 70 % ethanol, freshly prepared.
3. Vortex on a platform vortexer for 3–5 min.
4. Incubate for 15 min.
5. Pellet the micro-particles by spinning for 5 s in a microfuge.
6. Remove the liquid and discard.
7. Add 1 ml sterile water. Vortex for 1 min.
8. Allow the particles to settle for 1 min. Pellet the micro-particles by spinning for 2 s in a microfuge. Remove the liquid and discard (repeat it three times).
9. Add sterile 50 % glycerol to bring the micro-particle concentration to 60 mg/ml (assume no loss during preparation).
10. Store the micro-particles at room temperature for up to 2 weeks.

3.12.5 Coating DNA onto Microcarriers

The following procedure can be adopted for six bombardments, if fewer bombardments are needed; prepare enough microcarriers by reducing all volumes as required. When removing aliquots of microcarriers, it is important to vortex the tube containing the microcarriers continuously in order to maximize uniform sampling.

1. Vortex the microcarriers prepared in 50 % glycerol (60 mg/ml) for 5 min on a platform vortexer to resuspend and disrupt agglomerated particles.
2. Remove 50 μl (3 mg) microcarriers to a 1.5 ml microfuge tube.
3. While vortexing vigorously, add in order:

   5 μl DNA (1 μg/μl).

   50 μl $CaCl_2$ (2.5 M).

   20 μl spermidine (0.1 M).
4. Continue vortexing for 2–3 min.
5. Allow the microcarriers to settle for 1 min.
6. Pellet the microcarriers by spinning for 2 s in a microfuge.
7. Remove the liquid and discard.
8. Add 140 μl of 70 % ethanol without disturbing the pellet.
9. Remove the liquid and discard.
10. Add 140 μl 100 % ethanol without disturbing the pellet.
11. Remove the liquid and discard.
12. Add 48 μl 100 % ethanol.

13. Gently resuspend the pellet by tapping the side of the tube several times, and then by vortexing at low speed for 2–3 s.
14. Remove 6 µl aliquots of microcarriers and transfer them to the center of the macrocarrier. An effort is made to remove equal amounts (500 µg) of microcarriers each time and to spread them evenly over the central 1 cm of the microcarrier using the pipette tip. Desiccate immediately.

3.12.6 Sterilization of Macrocarriers

1. Sterilize macrocarriers, rupture discs, and stopping screens in 70 % alcohol.
2. Sterilize macrocarriers and dry in the laminar airflow hood before spreading the microcarriers over them.

3.12.7 Biolistic Transformation

1. Bombard the embryogenic callus cultures with DNA coated with 1 µm gold particles.
2. Use 900–1100 psi rupture disc to regulate the helium pressure, at the target distance of 6–9 cm.
3. The following parameters may be kept constant:

Chamber vacuum	28″ Hg (0.06 atm)
Gap distance	¼″
Macrocarrier travel distance	8 mm (stopping screen at middle level)
Microcarriers/ bombardment	500 µg
DNA/bombardment	833 ng

4. Seal the petri plates containing bombarded calli with parafilm and heal by incubating them at 30 °C in the dark for 24 h.
5. Test a portion of the bombarded calli for GUS expression assay.
6. For plant regeneration, subculture the remaining calli into fresh MS medium supplemented with 1.0 mg/l BAP, and 0.5 mg/l NAA, at 22 ± 2 °C, 12-h photoperiod of 33.8 µmol/m^2/s^2 [15].

3.12.8 GUS Expression Assay

1. The location of GUS expression is visualized using a histochemical reaction [16].
2. The cells are analyzed for GUS expression, 24–48 h after bombardment.
3. For GUS analysis, the bombarded calli are transferred to plates containing 2 ml GUS assay buffer (0.5 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid, 0.1 M sodium phosphate buffer, pH 7.0, 0.5 mM potassium ferrocyanide and 0.5 mM potassium ferricyanide, 0.1 % Triton X 100).
4. Incubate at 37 °C for 24 h and wash in 70 % alcohol.

5. Blue color sectors are observed under the stereomicroscope after 24 h and evaluate GUS expression.
6. Count the number of spots per cm^2 for estimating the transformation efficiency.

4 Notes

1. Explant source and stage is the most important step for establishing contamination-free cultures with good in vitro responses.
2. The explants are to be collected from disease- and virus-free plantations and the individual plants are also checked to be free from symptoms.
3. Plant the rhizome bits in the greenhouse under protected conditions with 4–5 in. of sand on top and periodically (once in 20 days) spray/drench copper oxychloride/bavistin/dithane M-45 at 0.3 % to minimize the contamination.
4. Select the newly sprouting buds and use as a source of explant for the culture. Collect the explants from field directly into 0.3 % copper oxy chloride/bavistin solution and keep for 10–15 min before surface sterilization.
5. Since explants from underground are used, still chances of contamination remain high. Use 5 ml half-strength MS medium supplemented with 3 % sucrose and 0.5 mg/l kinetin and gelled with 0.67 % bacteriological grade agar.
6. Fungal and bacterial contamination appears within 5–10 days and the contaminated cultures are discarded.

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Chapter 29

Hairy Root Cultures of *Gymnema sylvestre* R. Br. to Produce Gymnemic Acid

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Abstract

Gymnema sylvestre R. Br. (Asclepiadaceae) is an endangered species extensively used in the management of diabetes, obesity, and treatment of various diseases. Uncontrolled exploitation to meet the increasing demand and low seed viability hastens the disappearance of the plant from its natural habitat. Hairy root culture provides a suitable alternative for the enhanced production of active principles. The current protocol provides the optimized culture conditions for the establishment of hairy root cultures and elicitation studies and also confirmation of stable integration of *A. rhizogenes* plasmid T-DNA into host genetic material by PCR and RT-PCR. Furthermore, it also discusses the suitable methods for the extraction procedures, and qualitative and quantitative analysis of gymnemic acid by HPTLC and HPLC.

Key words *Gymnema sylvestre*, Gymnemic acid, *Agrobacterium rhizogenes*, Hairy root cultures, Elicitors, HPLC

1 Introduction

Gymnema sylvestre R.Br. (Asclepiadaceae) is a valuable and multipurpose medicinal plant. It is a slow-growing, perennial woody climber of tropical and subtropical region. The plant is popularly known as "gur-mur" or "madhunashini" for its distinctive property of temporarily destroying the taste of sweetness. *G. sylvestre* is used in the treatment of asthma, eye complaints, inflammations, and snake bites and also acts as feeding deterrent. In the management of diabetes mellitus *G. sylvestre* brings about glucose homeostasis through repair and regeneration of pancreatic β-cells or repair and stimulation of enzymes responsible for uptake of glucose and utilization [1, 2], stimulation of insulin release [3, 4], and finally increased glucose tolerance [5]. The medicinal property of *G. sylvestre* is due to the presence of a group of triterpenoid saponins known as "gymnemic acid" and gymnemosides, which fall under

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oleanane and dammarene type of saponins, respectively [6, 7], that have been investigated by various groups [8–10].

G. sylvestre is fast disappearing from the wild habitat, and had become endangered not only because of its overexploitation to meet the requirements of demand, but also owing to low seed viability, slow rate of germination, and poor rooting ability which hamper the conventional propagation. Plant tissue cultures provide promising alternatives for the enhanced production of secondary metabolites. There have been many strategies in the published literature for the product enhancement in plant cell and tissue cultures. Establishment of hairy root cultures has been one of the strategies for the secondary metabolite production and for their overproduction hairy roots are elicited with various elicitors. Hairy root culture is produced by infecting the explants with *A. rhizogenes* and nurturing on growth regulator-free medium, exploiting the advantage of naturally occurring hair root disease in dicotyledons for the production of metabolites. Their ease of maintenance, short doubling time, higher genetic stability, and autotrophicity to plant growth regulators make them advantageous over cell suspension cultures. This chapter focuses on recent advances in the optimization of culture conditions for successful establishment of callus, regeneration cultures, and hairy root cultures and their elicitation for the production of "gymnemic acid" and also describes the validated methods for the qualitative and quantitative analysis of gymnemic acid.

2 Materials

2.1 Explants for In Vitro Culture Establishment

The meristematic tissue like apical buds, hypocotyls, meristematic leaf tissue, and cotyledonary leaf segments of 3.0–4.0 cm is used for the callus induction. Cotyledons and leaves (5 × 5 mm explants) are used for the establishment of hairy root cultures.

2.2 Reagents for Surface Sterilization

1. 2 % Tween-20 (v/v) or 1 % laboline (v/v) and 0.06–0.08 % cetrimide.
2. 0.05 % H_2O_2 (v/v) or 0.1 % $HgCl_2$ (w/v).
3. 0.01 % (w/v) citric acid or 0.01 % ascorbic acid (w/v) and 0.01 % PVP (w/v).
4. Double-distilled water.

2.3 Culture Medium

1. Murashige and Skoog (MS) medium: Macronutrients, micronutrients, iron source, and vitamins are prepared as respective stock solutions [11] and stored at 4 °C (Table 1).
2. Prepare (1 mg/ml) stock solutions of NAA, IAA, IBA, picloram, and 2,4--D as auxins, and BAP and kinetin as cytokinins, and store at 4 °C.

Table 1
Composition of MS (Murashige and Skoog, 1962) medium

Constituents	Concentration (mg/l)	
	Actually required	Concentrated (×)
Stock solution—I (macronutrients) (10×)		
$MgSO_4 \cdot 7H_2O$	370	3700
KH_2PO_4	170	1700
KNO_3	1900	19,000
NH_4NO_3	1650	16,500
$CaCl_2 \cdot 2H_2O$	440	4400
Stock solution—II (micronutrients) (100×)		
H_3BO_3	6.2	620
$MnSO_4 \cdot 4H_2O$	22.3	2230
$ZnSO_4 \cdot 7H_2O$	8.6	860
$Na_2MoO_4 \cdot 2H_2O$	0.25	25
$CuSO_4 \cdot 5H_2O$	0.025	2.5
$CoCl_2 \cdot 6H_2O$	0.025	2.5
KI	0.83	83
Stock solution—III (iron source) (10×)		
$FeSO_4 \cdot 7H_2O$	27.8	278
$Na_2EDTA \cdot 2H_2O$	37.3	373
Stock solution—IV (vitamins and organic supplements (100×)		
Thiamine HCl	0.5	50
Pyridoxine HCl	0.5	50
Nicotinic acid	0.5	50
Myoinositol	100	10,000
Glycine	2.0	200

3. Overnight-grown culture of *Agrobacterium rhizogenes* strain KCTC 2703.
4. 100 mg/l Kanamycin sulfate and 400 mg/l cefotaxime in sterile distilled water (kanamycin sulfate and cefotaxime exclusively insoluble in organic solvents).

2.4 PCR

1. DNA isolation kit (Fermentas, GlenBurnie, MD, USA) and RNA isolation kit (Fermentas, Glen Burnie, MD, USA).
2. GeneAmp PCR System DNA thermal cycler.
3. Primers.
4. PCR master mix.
5. Distilled water.
6. 1.0 % Agarose.
7. Gel electrophoresis unit.

8. *Hind III*-digested λDNA.
9. 0.5 μg/ml of ethidium bromide (*see* **Note 1**).
10. Gel documentation system.

2.5 Elicitation Studies

1. Hairy root cultures.
2. Oleic acid and linolenic acid at respective concentrations.
3. 250 ml Conical flasks.
4. MS nutrient broth.

2.6 Extraction Procedures

2.6.1 Conventional Method

1. Homogenized tissue.
2. Reflux condenser.
3. Heating mantle.
4. 50 ml of 50 % ethanol (v/v).
5. 10 ml of 11 % KOH (w/v) in water and 9 ml of concentrated HCl.
6. 0.45 and 0.22 μm PVDF syringe-driven filters.

2.7 Phytochemical Screening

2.7.1 Thin-Layer Chromatography

1. Silica gel G60 F_{254} is coated onto the carrier glass plates with dimension of 20 × 10 cm.
2. Chloroform:methanol:acetic acid (5:1:1) as mobile phase.
3. Vanillin-sulfuric acid reagent, 1 % vanillin in ethanol containing 5 ml of acetic anhydride and sulfuric acid.

2.7.2 HPTLC

1. The pre-coated Silica gel 60 F_{254} layered (10 × 10 cm) on aluminum support sheets.
2. Toluene:ethyl acetate:methanol (2:7:1) as mobile phase.
3. Camag Linomat V (Switzerland) sample applicator.

2.7.3 HPLC

1. 0.45 and 0.22 μm PVDF syringe-driven filters.
2. HPLC-grade acetonitrile and Milli Q water.
3. 0.1 % Trifluoroacetic acid (v/v) HPLC grade.
4. Gymnemagenin standard.

3 Methods

3.1 Explant Surface Sterilization

1. Pre-soak the explants in distilled water for 2–3 h, and wash under running tap water for 10 min.
2. Wash with detergent 2 % Tween-20 (v/v), 0.06–0.08 % cetrimide (w/v), and fungicide (0.12 % w/v bavistin) for 3–4 min. Rinse explants with sterile distilled water 4–5 times with frequent shaking to remove the traces of detergent.

3. Treat the explants with 75 % ethanol for 30 s and rinse 3–4 times with sterile distilled water. Surface-sterilize the explants with 0.05 % H_2O_2 (v/v) and wash 3–4 times with sterile distilled water in a laminar airflow cabinet to remove traces of H_2O_2.
4. Finally wash the explants in filter-sterilized antioxidant solution containing ascorbic acid (0.01 % w/v), citric acid (0.01 %), and PVP (0.01 %) for about 3 min.
5. Surface-sterilized explants are used directly for inoculating onto basal growth medium.

3.2 Establishment of Callus Culture

1. Add the required proportions of MS medium stock solutions, 3 % sucrose, 0.8 % agar, and plant growth regulators like BAP (2 mg/l), NAA (2 mg/l), picloram (0.2 mg/l), and/or 2,4-D (0.5 mg/l) in sequence into glass beaker, and adjust pH to 5.6–5.8 with 0.1 N NaOH/0.1 N HCl before autoclaving (*see* **Note 2**).
2. Suspend agar (0.8 %) into the medium, subject for melting by heating, distribute into culture vessels, and autoclave at 121 °C/15 lbs pressure. Cool the vessels and transfer to laminar airflow cabinet.
3. By trimming the edges of surface-sterilized explants aseptically, directly inoculate onto culture medium supplemented with 2,4-D/picloram at required concentration (Fig. 1).
4. Observe after 15–20 days for the callus induction.

3.3 Establishment of Regeneration Culture

1. For regeneration inoculate the surface-sterilized explants by cutting at edges, onto MS medium enriched with 2 mg/l BAP, 0.5 mg/l kinetin, and 0.05 mg/l NAA (*see* **Note 3**).
2. After 15 days of incubation observe for the shoot induction (Fig. 2b).
3. Excise the individual shoot and transfer to rooting medium, i.e., MS half-strength medium fortified with 3 mg/l IBA and 0.05 % activated charcoal.
4. After 10–15 days observe for the root induction (Fig. 2c).

3.4 Establishment of Hairy Root Cultures

1. Use overnight-grown cultures of *A. rhizogenes* strain KCTC 2703 (at $OD_{600} = 1$) for infecting the explants (cotyledons/leaves/stem segments) for an incubation period of 30 min [12].
2. Blot the explants between sterile tissue paper layers, dry on Whatman No. 1 filter paper, and place on MS semisolid medium (0.8 % agar) without any growth regulators in petri dishes (*see* **Note 4**).

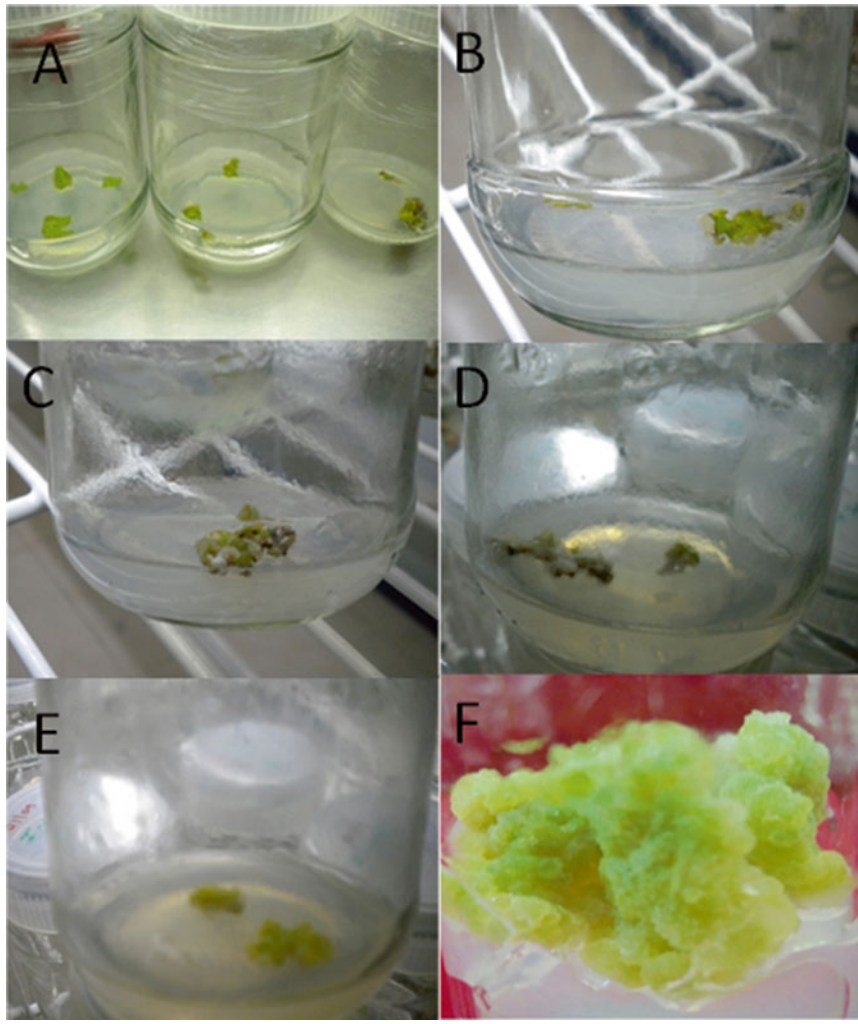

Fig. 1 Callus induction MS + 0.5 mg/l BP and 2 mg/l NAA (**a**), callus induction MS + 0.5 mg/l 2,4-D (**b**), callus induction MS + 0.2 mg/l picloram (**c**), callus induction MS + 0.5 mg/l BP (**d**), callus induction MS + 2mg/l NAA (**e**), callus induction MS + 0.5 mg/l 6-AP **+** 0.5 mg/l 2,4-D/0.2 mg/l picloram +2 mg/l NAA (**f**)

3. Maintain control set of explants inoculated in a similar way, but devoid of bacterial infection.
4. After 3 days of incubation, transfer the explants onto MS semi-solid medium (0.8 % agar) containing bacteriostatic antibiotics cefotaxime (400 mg/l) and kanamycin sulfate (100 mg/l) in a petri dish (*see* **Note 5**).
5. Subculture the explants for every 2 weeks onto fresh medium in petri dish with similar composition. Observe for the root protuberances developed in the explants after two subcultures (4 weeks) (Fig. 3).

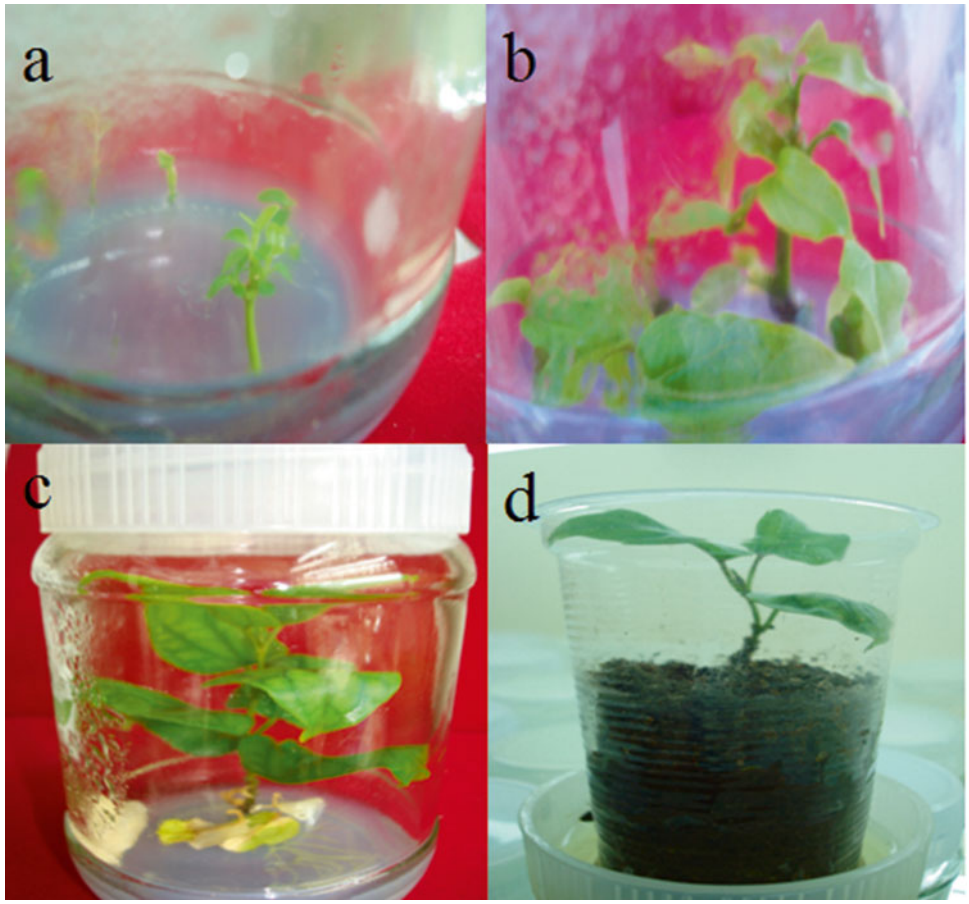

Fig. 2 Regeneration in nodal explants (**a**), multiple shoot induction in nodal explants (**b**), elongation of regenerated shoot (**c**), rooting and hardening (**d**)

6. Transfer 500 mg of developed roots into Erlenmeyer's flask containing 50 ml of MS liquid medium (with antibiotics) and maintain in orbital shaker (100 rpm) with successive subculturing for every 28 days of incubation period.
7. Gradually decrease the cefotaxime and kanamycin sulfate for each subculture and finally completely omit the cefotaxime after 8 weeks, but still maintain kanamycin sulfate [13].
8. Maintain the growth conditions of 16-h photoperiod and temperature 25 ± 2 °C throughout the culture period.

3.5 Characterization of Hairy Roots by PCR and RT-PCR

1. PCR and RT-PCR analysis of transformant DNA confirms the stable integration of the T-DNA into the host genetic material.
2. The conventional PCR is carried out in order to detect the presence of T-DNA in the genomic DNA of host cell, while RT-PCR (reverse transcriptase) is carried out to confirm the size of target DNA-amplified product.

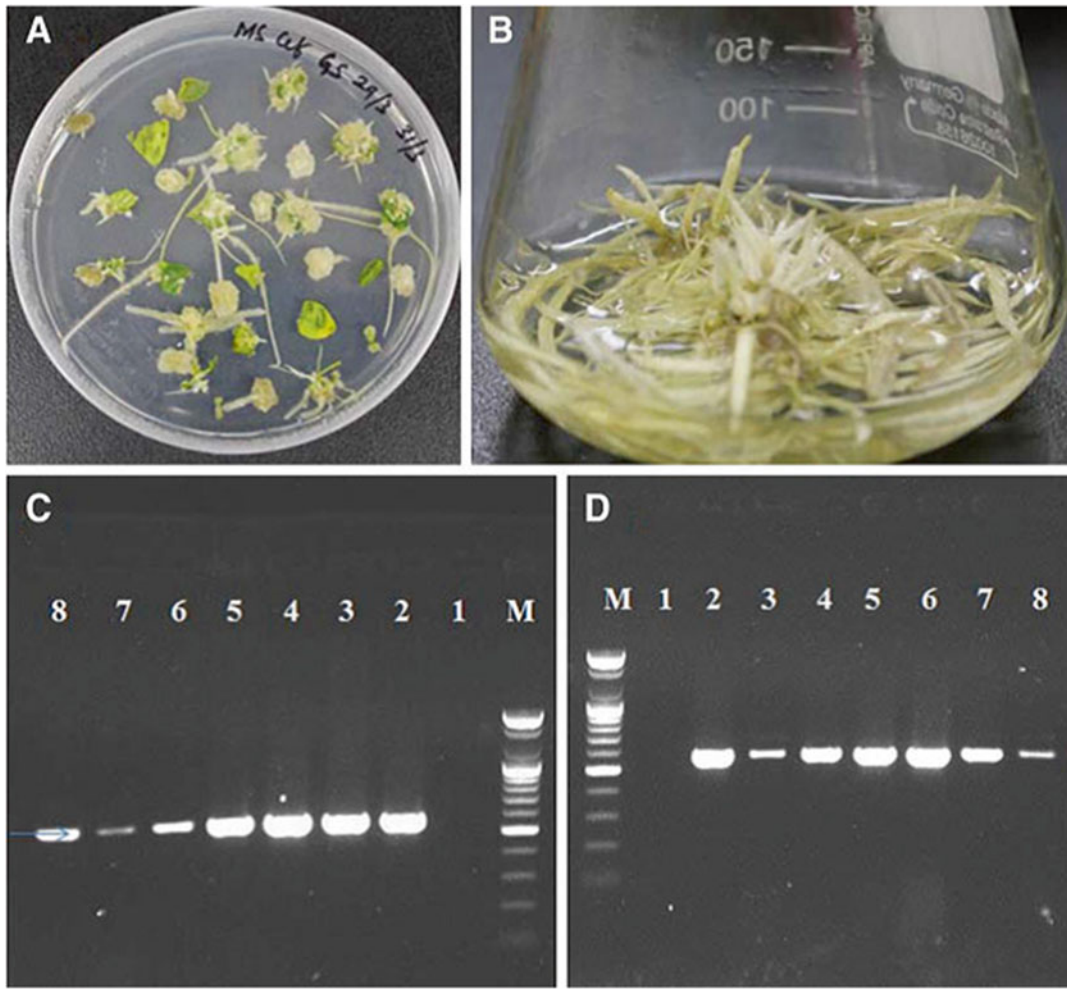

Fig. 3 Induction of hairy roots following inoculation of leaf explants of *G. sylvestre* with *A. rhizogenes* strain KCTC 2703 (**a**), *Agrobacterium*-induced hairy roots of *G. sylvestre* in MS-based liquid medium lacking growth regulators after 25 days of culture (**b**), PCR analysis of the rolC gene in the hairy root lines of *G. sylvestre*. *Lane M* marker, *lane 1* roots from a non-transformed plant (negative control), *lane 2* plasmid DNA (positive control), *lanes 3–8* root clones induced by *A. rhizogenes* (**c**), RT-PCR analysis of the rolC gene in the hairy root lines of *G. sylvestre*. *Lane M* marker, *lane 1* roots from a non-transformed plant (negative control), *lane 2* plasmid DNA (positive control), *lanes 3–8* root clones induced (**d**). *Primers for amplification of* rolC *gene by PCR* 5′-ATG-GCT-GAA-GAC-GAC-CTG-TGT-T-3′ and 5′-TTA-GCC-GAT-TGC-AAA-CTT-GCA-C-3′. Primers for *rolC* gene for RT-PCR 5′-ATGGCT-GAA-GAC-GAC-CTG-TGT-T-3′ and 5′-TTA-GCCGAT-TGC-AAA-CTT-GCA-C-3′

3. For the PCR reaction, isolate the genomic DNA by using plant genomic DNA isolation kit (Fermentas, GlenBurnie, MD, USA) following the manufacturer's instructions (*see* **Note 6**).
4. Polymerase chain reaction: Prepare the master mix by adding l µl of 1 Unit Taq polymerase, 2.5 µl of 100 nM dNTP, 1 µl of 20 pM primer, 1 µl of 20 ng/µl template DNA, and 2.5 µl of 10× reaction buffer; it was made up to 25 µl by sterile distilled H_2O.

5. Spin the reaction mixture for few seconds (30 s).
6. Set the following program for the amplification in the thermal cycler:
   (a) Denature for 4 min at 94 °C.
   (b) Annealing of primer for 1 min at 60 °C.
   (c) Amplification for 2 min at 72 °C.

   Final extension of the amplified DNA for 5 min at 72 °C.
7. Separate the DNA amplicon by agarose gel electrophoresis along with λDNA *Hind III*-digest marker, stain with ethidium bromide, and document in a gel documentation system.
8. Similarly, confirm the expression of gene insert in the host chromosomal DNA by RT-PCR analysis using a kit and following the manufacturer's instructions provided.
9. Isolate total RNA from selected fast-growing root clones of *G. sylvestre* using RNA isolation kit (Fermentas, Glen Burnie, MD, USA) (*see* **Note 6**).
10. Initially synthesize template strand from RNA isolate and amplify by using Fermentas Revert First Strand Complementary DNA (cDNA) Synthesis Kit (Fermentas, USA) following the manufacturer's instructions.
11. Separate the obtained DNA (cDNA) by agarose gel electrophoresis along with λDNA *Hind III*-digest marker, stain with ethidium bromide, determine the cDNA size, and document in a gel documentation system.

3.6 Elicitation of Hairy Root Cultures for Product Enhancement

1. Inoculate 500 mg of established hairy root cultures into 250 ml conical flask containing 50 ml of liquid MS medium without any growth regulators.
2. Add the elicitors like oleic acid and linolenic acid to the culture flasks at the respective concentrations on day 15 [14].
3. Observe the influence of different elicitors on growth rate and gymnemic acid production from day 20 onwards (*see* **Note 7**).
4. Maintain the culture conditions under continuous agitation at 100 rpm in an orbital shaker and at 25 ± 2 °C, with a 16-h photoperiod (40 μmol/m^2/s) as the root cultures grow and multiply at faster rate.
5. After 8 weeks of culture, assess the growth of root cultures in terms of fresh weight/dry mass, growth ratio, and total saponin content and also for the amount of gymnemic acid produced.
6. Determine the amount of gymnemic acid by HPLC analysis after deglycosylation (to obtain sapogenin "gymnemagenin") by treating with KOH and HCl, by comparing with retention time of the "gymnemagenin" standard (*see* Subheading 3.7).

7. Use molecular weight corrections for the determination of gymnemic acid produced in the form of gymnemagenin by using the formula (809.0/506.7) [15].

3.7 Extraction of Gymnemic acid

1. For the extraction of "gymnemic acid" follow the conventional method of extraction.
2. Dry the fresh mass of hairy roots at 50 °C and make into powder. Suspend 500 mg of this powder into 10 ml of 50 % ethanol (v/v) with frequent shaking at room temperature. Subject this extract for total saponin content determination and thin-layer chromatography (TLC) analysis.
3. Subject the saponin extract for deglycosylation (to convert gymnemic acid to gymnemagenin) for alkali and acid hydrolysis. To the ethanol extract, add 10 ml of 11 % KOH and heat in a boiling water bath under reflux for 1 h to carry out alkaline hydrolysis, and cool to room temperature. Further, add 1.8 ml of concentrated HCl and heat in a boiling water bath under reflux for 1 h to carry out acid hydrolysis.
4. After cooling to room temperature, adjust the pH to 7.5–8.5 with 1 N KOH and dilute the solution with 50 % ethanol (v/v) or methanol up to 100 ml, filter through 0.45 μm nylon filter, and use for the chromatography analysis [15].

3.8 Phytochemical Screening

3.8.1 Preparation of the Reference Standard

Dissolve 10 mg gymnemagenin into 10 ml of 50 % methanol to obtain 1 mg/ml stock solution.

3.8.2 Thin-Layer Chromatography

1. Separate analytes present in extract filtrate on 20 cm × 10 cm TLC plates with silica gel G60 F_{254} as stationary phase spread with a thickness of 0.25 and 2 mm for analytical and preparative purposes, respectively. Develop the chromatogram using chloroform:methanol:acetic acid (5:1:1) as mobile phase in a pre-saturated development tank (*see* **Notes 8** and **9**).
2. After air-drying detect the analytes on chromatogram by spraying with vanillin-sulfuric acid reagent followed by oven drying at 100 °C for 15 min. Observe for the "gymnemic acid/gymnemagenin (deglycosylated form)" that appears as purple-colored spots (Fig. 4).
3. Confirm the presence of gymnemagenin by comparing with reference standard Rf values.

3.8.3 HPTLC

1. Use commercially available HPTLC plates. Wash the plates with methanol and activate at 60 °C for 5 min in an oven and use for the chromatographic separation by using Camag Linomat V (Switzerland) sample applicator.

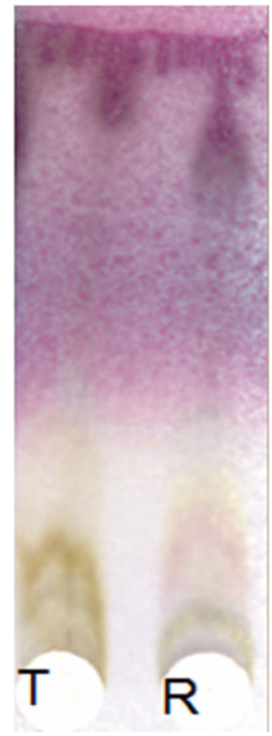

Fig. 4 TLC chromatogram of gymnemic acid. T: Callus extract; R: reference standard

2. Apply 10 µl of the extract aliquots and standard separately onto the HPTLC plate, develop up to 80 mm with mobile phase toluene:ethyl acetate:methanol (2:7:1), and detect by scanning UV reflectance mode at 210 nm.
3. Determine the amount of gymnemagenin in the sample with the help of calibration curve obtained for reference standard gymnemagenin (Fig. 5) [16].

3.8.4 HPLC

1. Inject 20 µl of the filtered extract with manual injector.
2. Use C18 column (4.6×250 mm 5 µm) and acetonitrile:water (1:3 ratio with 0.1 % trifluoroacetic acid) for HPLC separation and detect the separated analytes at 210 nm using diode array detector (DAD) (*see* **Note 10**). Maintain the temperature at 30 °C.
3. Determine the amount of gymnemic acid in the extract with the help of retention time of reference standard "gymnemagenin" and by interpretation from HPLC data in terms of area under the peaks of test sample and reference standard (Fig. 6).

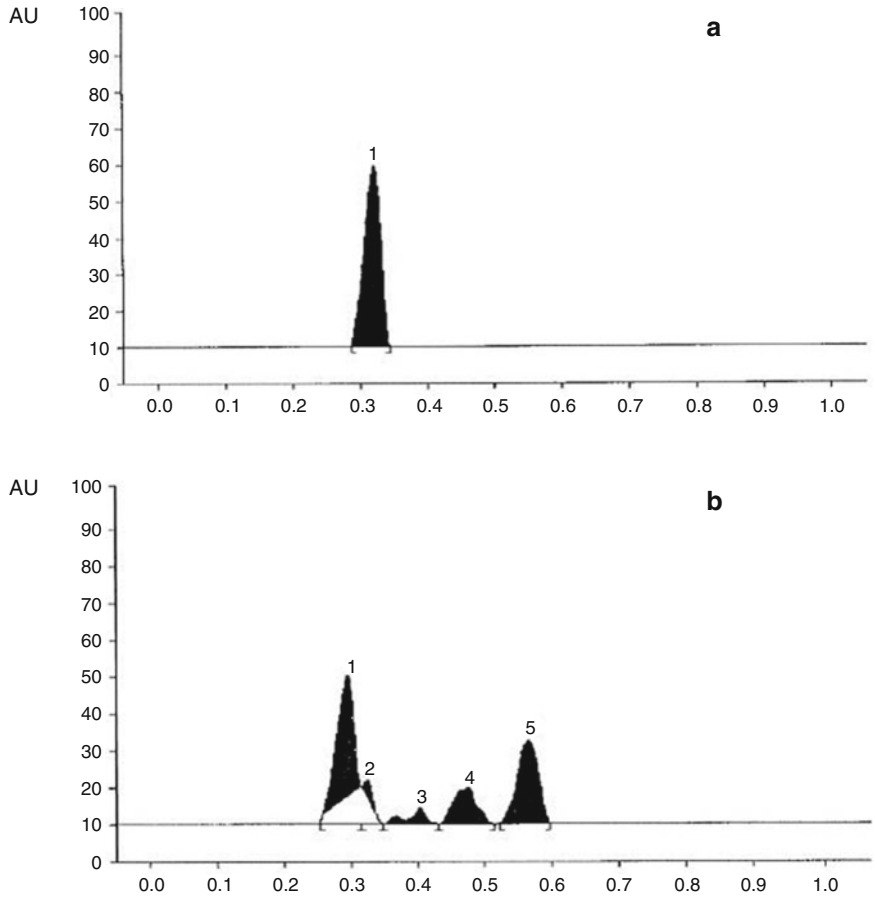

Fig. 5 HPTLC chromatograms of standard gymnemagenin (**a**), leaf extract of *Gymnema sylvestre* hydrolyzed according to the described method (**b**). Peak 1 = gymnemagenin

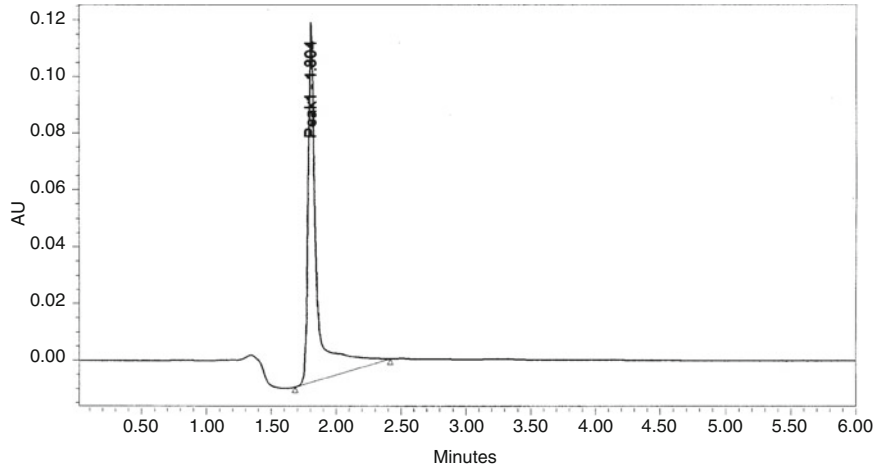

Fig. 6 HPLC chromatogram of gymnemagenin standard

4 Notes

1. Ethidium bromide is highly carcinogenic and should be handled using gloves.
2. Cytokinins and auxins at their varied concentrations differently influence the callus-inducing response (Table 2).
3. The ratios of growth regulators influence the morphogenesis of the explants into undifferentiated callus/shooting and rooting response. High cytokinin to auxin promotes the shooting (Table 3), while high auxin to cytokinin promotes the rooting in the explants (Table 4).
4. Hairy root cultures have advantages over other types of in vitro cultures, in having higher genetic stability and relatively fast growth rates compared to undifferentiated tissue (callus); further it is not required to supplement growth regulators in the culture medium.
5. Cefotaxime is an antibiotic used for the complete elimination of background *Agrobacterium* after cocultivation, while kanamycin sulfate is used in order to select the putative transformed hairy roots.
6. Conventionally DNA from the plant tissues is isolated by CTAB method [17]; alternatively total RNA is isolated by phenol:chloroform:isoamyl alcohol method [18].
7. The growth rate is determined as GR = (harvested dry weight-inoculated dry weight)/inoculated dry weight.
8. In order to remove water and any other contaminant materials on the adsorbent, the TLC plates are activated.
9. The saturation of the development tank is performed for the prevention of edge effect, i.e., where solvent front in the middle of TLC plate moves faster than the edge, which leads to distorted and irregular separation of the compounds.
10. Peak tailing is reduced at lower pH conditions, for which trifluoroacetic acid is added to the mobile phase, so as to obtain the better resolution and peak shape of the chromatogram. In addition to this, trifluoroacetic acid also has least UV cutoff (205 nm) and does not interfere with absorption of the light source.

Table 2
Effect of different auxins and cytokinins on leaf callus growth

Auxins (mg/l)					Cell biomass	
2,4-D	NAA	IBA	IAA	Picloram	FW	DW
0.2	–	–	–	–	10.64 ± 0.3	0.94 ± 0.02
0.5	–	–	–	–	12.83 ± 0.12	1.46 ± 0.14
1	–	–	–	–	11.14 ± 0.28	1.28 ± 0.11
2	–	–	–	–	10.8 ± 0.15	0.96 ± 0.01
5	–	–	–	–	09.76 ± 0.14	0.85 ± 0.01
10	–	–	–	–	08.12 ± 0.28	0.78 ± 0.01
–	0.2	–	–	–	07.73 ± 0.12	0.65 ± 0.006
–	0.5	–	–	–	07.56 ± 0.08	0.63 ± 0.015
–	1	–	–	–	07.72 ± 0.31	0.67 ± 0.006
–	2	–	–	–	08.22 ± 0.11	0.7 ± 0.005
–	5	–	–	–	08.08 ± 0.05	0.68 ± 0.017
–	10		–	–	07.36 ± 0.20	0.81 ± 0.008
–	–	0.2	–	–	05.32 ± 0.06	0.49 ± 0.01
–	–	0.5	–	–	05.82 ± 0.11	0.58 ± 0.01
–	–	1	–	–	05.62 ± 0.09	0.52 ± 0.01
–	–	2	–	–	05.05 ± 0.10	0.50 ± 0.01
–	–	5	–	–	04.71 ± 0.11	0.53 ± 0.008
–	–	10	–	–	04.13 ± 0.15	0.4 ± 0.005
–	–	–	0.2	–	04.43 ± 0.14	0.40 ± 0.01
–	–	–	0.5	–	04.92 ± 0.15	0.44 ± 0.01
–	–	–	1	–	05.33 ± 0.20	0.47 ± 0.017
–	–	–	2	–	05.86 ± 0.08	0.52 ± 0.01
–	–	–	5	–	05.16 ± 0.12	0.49 ± 0.008
–	–	–	10	–	04.42 ± 0.08	0.39 ± 0.008
–	–	–	–	0.02	05.43 ± 0.12	0.59 ± 0.005
–	–	–	–	0.05	06.03 ± 0.17	0.61 ± 0.006
–	–	–	–	0.10	8.5 ± 0.1	0.96 ± 0.005
–	–	–	–	0.20	7.03 ± 0.08	0.75 ± 0.008
–	–	–	–	0.50	6.63 ± 0.13	0.64 ± 0.006
–	–	–	–	1.00	7 ± 0.05	0.68 ± 0.008

(continued)

Table 2
(continued)

Cytokinins (mg/l)		Cell biomass	
BAP	**Kinetin**	**FW**	**DW**
0.2	–	4.58 ± 0.01	0.31 ± 0.01
0.5	–	5.23 ± 0.17	0.36 ± 0.01
1	–	5.53 ± 0.08	0.44 ± 0.02
2	–	6.96 ± 0.08	0.54 ± 0.01
5	–	6.12 ± 0.25	0.48 ± 0.008
10	–	5.68 ± 0.08	0.46 ± 0.01
–	0.2	4.56 ± 0.03	0.40 ± 0.08
–	0.5	4.83 ± 0.08	0.42 ± 0.01
–	1	4.26 ± 0.14	0.36 ± 0.01
–	2	4.46 ± 0.14	0.39 ± 0.01
–	5	3.92 ± 0.05	0.35 ± 0.01
–	10	3.56 ± 0.14	.33 ± 0.003

Table 3
Effect of cytokinins on morphogenesis in Gymnema sylvestre nodal explants

Cytokinin	Concentration (mg/l)	% of response	Days to bud sprout	No. of shoots per explant
	–	–	–	–
BAP	0.2	38.67 ± 0.88	20.75 ± 1.57	1.0 ± 0.23
	0.5	55.33 ± 2.20	20.33 ± 0.88	1.0 ± 0.32
	1	70.86 ± 1.85	18.86 ± 1.02	1.67 ± 0.33
	2	72.86 ± 1.85	15.67 ± 1.45	2.0 ± 0.24
	5	64.24 ± 1.0	15.56 ± 1.20	1.33 ± 0.33
	10	60.0 ± 2.08	12.00 ± 1.52	1.33 ± 0.67
Kinetin	0.2	46.21 ± 1.02	21.56 ± 0.97	1.30 ± 0.33
	0.5	45.33 ± 1.75	21.33 ± 1.25	1.0 ± 0.28
	1	36.55 ± 1.33	20.55 ± 0.88	1.0 ± 0.24
	5	35.67 ± 0.89	18.16 ± 1.95	1.0 ± 0.05
	10	30.33 ± 0.66	17.28 ± 1.45	1.0 ± 0.03
2-iP	0.2	29.85 ± 0.95	21.69 ± 1.70	1.0 ± 0.06
	0.5	37.67 ± 1.22	21.26 ± 1.02	1.0 ± 0.02
	1	40.33 ± 1.74	21.11 ± 1.17	1.0 ± 0.03
	2	35.86 ± 1.66	20.89 ± 1.33	1.0 ± 0.06
	5	20.75 ± 1.46	18.55 ± 1.67	1.0 ± 0.04
	10	20.46 ± 1.57	18.33 ± 1.25	1.0 ± 0.03

Table 4
Effect of different auxins on rooting

Auxin	Concentration (mg/l)	Rooted shoots (%)	Mean no. of roots/shoot	Mean root length (cm)	Plant survival (%)
NAA	1	25.61 ± 1.22	2.55 ± 0.55	0.81 ± 0.63	9.77 ± 1.56
	2	31.55 ± 1.02	4.13 ± 0.78	1.21 ± 0.49	20.11 ± 1.41
	3	60.23 ± 0.98	5.75 ± 1.11	2.35 ± 1.06	30.14 ± 1.71
	4	65.71 ± 0.88	7.21 ± 1.63	6.62 ± 1.22	33.88 ± 0.88
	5	57.42 ± 1.02	5.96 ± 1.45	4.52 ± 1.09	32.79 ± 1.06
IBA	1	34.71 ± 0.86	4.66 ± 1.21	1.15 ± 0.88	20.76 ± 1.02
	2	40.32 ± 1.02	9.74 ± 0.99	2.94 ± 0.95	35.31 ± 1.22
	3	71.95 ± 0.67	15.79 ± 0.56	8.26 ± 0.56	60.11 ± 0.95
	4	74.33 ± 1.11	15.12 ± 1.65	7.78 ± 1.02	52.46 ± 1.78
	5	75.51 ± 1.26	14.45 ± 1.21	7.31 ± 1.29	50.12 ± 1.56
IAA	1	22.62 ± 0.95	1.43 ± 0.88	0.52 ± 1.55	7.12 ± 0.55
	2	28.66 ± 1.22	3.66 ± 0.95	0.97 ± 0.86	9.99 ± 1.23
	3	45.23 ± 1.05	5.24 ± 1.09	3.54 ± 1.11	22.56 ± 1.45
	4	50.51 ± 1.45	5.54 ± 1.02	2.72 ± 1.02	30.15 ± 0.89
	5	52.92 ± 0.79	5.10 ± 1.56	1.43 ± 0.55	30.95 ± 1.22

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Chapter 30

In Vitro Mass Propagation of *Cymbopogon citratus* Stapf., a Medicinal Gramineae

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Abstract

Cymbopogon citratus (D.C.) Stapf. is a medicinal plant source of lemon grass oils with multiple uses in the pharmaceutical and food industry. Conventional propagation in semisolid culture medium has become a fast tool for mass propagation of lemon grass, but the production cost must be lower. A solution could be the application of in vitro propagation methods based on liquid culture advantages and automation. This chapter provides two efficient protocols for in vitro propagation via organogenesis and somatic embryogenesis of this medicinal plant. Firstly, we report the production of shoots using a temporary immersion system (TIS). Secondly, a protocol for somatic embryogenesis using semisolid culture for callus formation and multiplication, and liquid culture in a rotatory shaker and conventional bioreactors for the maintenance of embryogenic culture, is described. Well-developed plants can be achieved from both protocols. Here we provide a fast and efficient technology for mass propagation of this medicinal plant taking the advantage of liquid culture and automation.

Key words Lemon grass, Medicinal plant, Organogenesis, Somatic embryogenesis, Temporary immersion system

1 Introduction

Plant propagators have been developing cost-effective organogenesis and somatic embryogenesis methods for the in vitro propagation of different plant species using liquid culture medium [1]. Because of liquid culture advantages for commercial propagation of plants, different technologies have been developed for complete or semiautomation of the in vitro propagation process. Temporary immersion system (TIS) and conventional bioreactors have become efficient techniques for mass production of organs and somatic embryos in several medicinal plants [2–5].

Lemon grass [*Cymbopogon citratus* (D.C.) Stapf.] is a perennial and medicinal herb belonging to the Poaceae family. It has been widely cultivated in tropical and subtropical countries to produce lemon grass oil, which fundamentally contains citral,

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farnesol, nerol, citronellal, and myrcene. Citral is the essential oil component, located mainly in the leaves and primarily used to flavor food [6]. It is also known that the essential oil of this vegetable species is the chief component in the multiple medicinal uses that are popularly attributed to this plant due to its anti-inflammatory, anti-microbial action and anti-mutagenic properties [7–9].

Although the propagation of lemon grass has been conventionally carried out by suckering [10], this method is time consuming, and the rate of propagation is rather low. Rapid in vitro propagation via organogenesis and somatic embryogenesis using simple and conventional bioreactors could remedy this problem. In consequence, TIS have been applied for the production of not only biomass [11, 12] but also secondary plant metabolites [13]. In the in vitro biomass of *C. citratus* the presence of citrals is only detected in shoots, and majorly in TIS than semisolid culture, although the yield of the in vitro biomass is lower than plants on field condition [14].

This chapter describes a detailed reproducible protocol for the organogenesis and somatic embryogenesis of *Cymbopogon citratus* Stapf. using TIS and conventional bioreactors.

2 Material

2.1 Organ Culture

2.1.1 Surface Sterilization of Shoots and Culture Conditions

1. Last portion of the stem, comprising the apical shoots of the *C. citratus* (Fig. 1a), collected from field plants.
2. Sodium hypochlorite (NaOCl) with 3 % of active Cl.
3. Sterilized distilled or deionized water for rinses.
4. Tissue culture facilities and tools: Laminar flow cabinet, scalpel, forceps, tool sterilizer such as vertical autoclave and glass-bead sterilizer, culture room, and personal protective equipment (i.e., laboratory coat).

2.1.2 In Vitro Establishment

1. Liquid culture medium based on Murashige and Skoog (MS) salts [15] for in vitro establishment of apical shoot (named MS-I); see medium formulations in Table 1.
2. Growth regulators: 6-Benzylaminopurine (6-BAP).
3. Vessels for plant tissue culture: Test tubes (25 mm × 150 mm).
4. Filter paper as explant support (Fig. 1b, *see* **Note 1**).

Fig. 1 (continued) every 4 h, 3 weeks after culture on MS-I medium; (**h**) shoots growing in 10 L TIS under 3 min of immersions every 4 h, 4 weeks after culture; (**i**) multiple shoots from 10 L TIS; (**j**) rooted shoots (plantlets), 3 weeks after transfer to rooting medium MS-II (MS + without growth regulators); (**k**) plantlets during acclimatization ready for field plantation, 6 weeks after transfer to organic matter and zeolite (4:1, v/v)

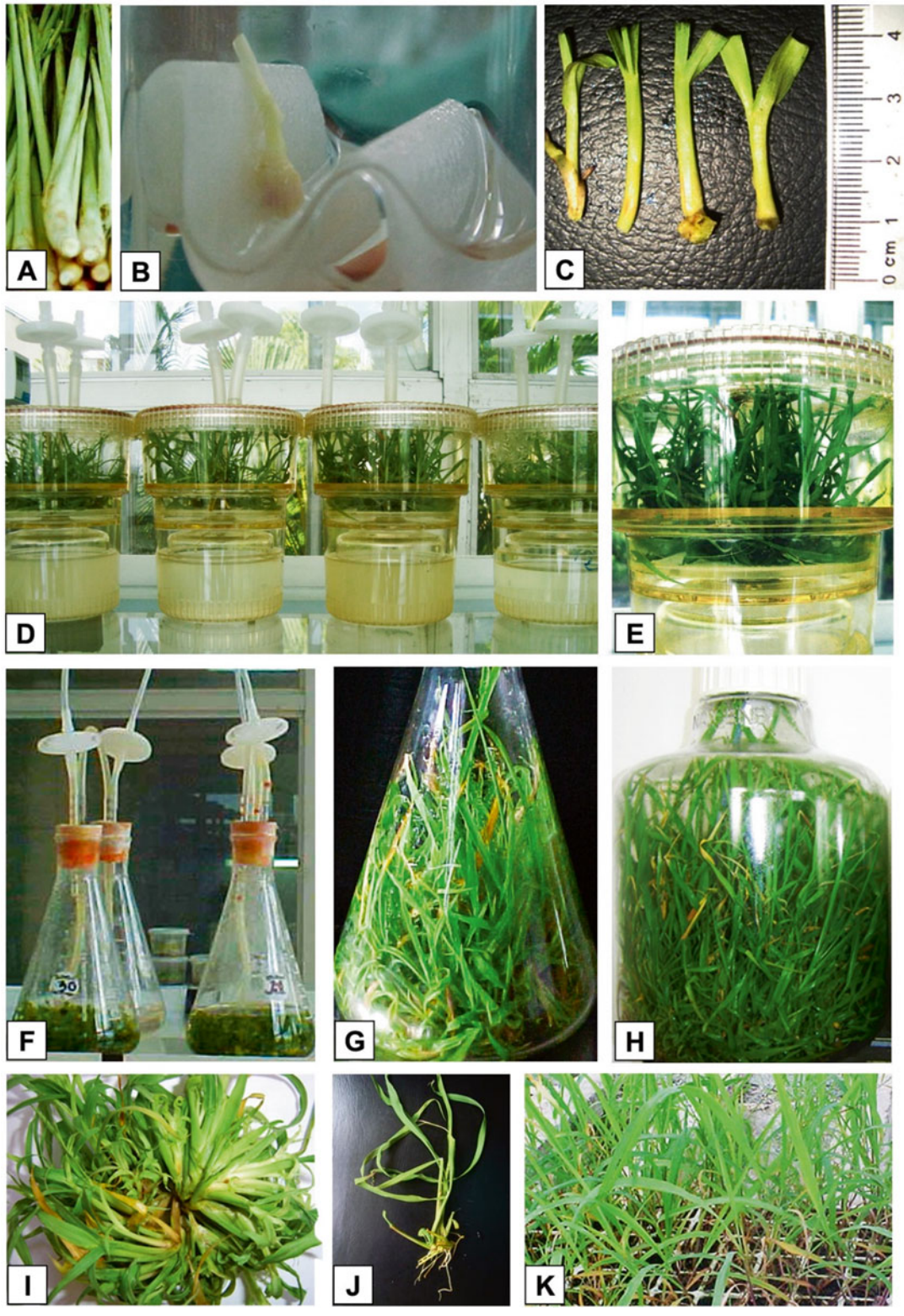

Fig. 1 In vitro propagation of *Cymbopogon citratus* in temporary immersion system, (**a**) the apical region of the stem from field plants (named spindle); (**b**) apical shoot culture in MS-I medium (MS + 0.89 μM BA); (**c**) 2.5–3.0 cm long individual shoots; (**d**, **e**) shoots growing in RITA under 1 min of immersion every 4 h, 3 weeks after culture on MS-I medium (MS + 0.89 μM BA); (**f**, **g**) shoots growing in 1 L TIS under 2 min of immersion

Table 1
The basal media used in tissue culture of *Cymbopogon citratus*

Component (mg/L)	MS-I	MS-II	CI	CM	IEC	DSE	GSE
$CaCl_2$	332.02	332.02	332.02	332.02	332.02	332.02	332.02
KH_2PO_4	170.00	170.00	170.00	170.00	170.00	170.00	170.00
KNO_3	1900.00	1900.00	1900.00	1900.00	1900.00	1900.00	1900.00
$MgSO_4$	180.54	180.54	180.54	180.54	180.54	180.54	180.54
NH_4NO_3	1650.00	1650.00	1650.00	1650.00	1650.00	1650.00	1650.00
$CoCl_2{\cdot}6H_2O$	0.025	0.025	0.025	0.025	0.025	0.025	0.025
$CuSO_4{\cdot}5H_2O$	0.025	0.025	0.025	0.025	0.025	0.025	0.025
$Na_2EDTA{\cdot}2H_2O$	36.70	36.70	36.70	36.70	36.70	36.70	36.70
H_3BO_3	6.20	6.20	6.20	6.20	6.20	6.20	6.20
KI	0.83	0.83	0.83	0.83	0.83	0.83	0.83
$MnSO_4{\cdot}H_2O$	16.90	16.90	16.90	16.90	16.90	16.90	16.90
$Na_2MoO_4{\cdot}2H_2O$	0.25	0.25	0.25	0.25	0.25	0.25	0.25
$ZnSO_4{\cdot}7H_2O$	8.60	8.60	8.60	8.60	8.60	8.60	8.60
Myoinositol	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Thiamine HCl	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Nicotinic acid	–	–	–	0.50	0.50	0.50	–
Pyridoxine HCl	–	–	–	0.50	0.50	0.50	–
Glycine	–	–	–	2.00	2.00	2.00	–
l-Arginine	–	–	–	–	50.00	50.00	–
l-Cysteine	–	–	–	–	10.00	10.00	–
Citric acid	–	–	–	–	50.00	–	–
Malt extract	–	–	–	–	50.00	100.00	–
Casein hydrolyzate	–	–	–	–	1000	200.00	–
Sucrose	30,000	40,000	20,000	40,000	40,000	80,000	20,000
6-BAP (μM)	0.890	–	–	–	–	–	–
2,4-D (μM)	–	–	22.52	22.52	13.57	4.52	–
Indole-3-acetic acid (μM)	–	–	–	7.30	–	–	–
Kinetin (μM)	–	–	–	4.10	–	–	–
Coconut milk (mL/L)	–	–	180.00	100.00	50.00	50.00	180.00
Agar	–	–	7000	8000	–	9000	7000

The basal media used for in vitro establishment and shoot multiplication (MS-I), shoot rooting (MS-II), callus initiation (CI), callus multiplication and induction of embryogenic culture (CM), maintenance of embryogenic culture (IEC), development of somatic embryos (DSE), and germination (GSE) based on modification of MS medium (Murashige and Skoog, 1962) proposed by Payán et al. [17] and Heinz and Mee [18]
Adjust the pH of the medium to 5.6 with 0.5 N KOH before autoclaving at 1.2 kg/cm² and 121 °C for 20 min

2.1.3 Shoot Multiplication in RITA and 1 L Temporary Immersion Systems

1. RITA units with all components.
2. TIS units of 1 L, comprising glass vessels (Erlenmeyers) with all components.
3. Individual shoots (2.5–3.0 cm long) (Fig. 1c).
4. Liquid culture medium similar to in vitro establishment of apical shoot (named MS-I); see medium formulations in Table 1.

2.1.4 Scale-Up of Shoot Multiplication in 10 L TIS

1. TIS units of 10 L, comprising plastic vessels with all components.
2. Individual shoots (2.5–3.0 cm long) (Fig. 1c, *see* **Note 2**).
3. Liquid culture medium for shoot multiplication similar to establishment medium (named MS-I); see medium formulations in Table 1.

2.1.5 Rooting of In Vitro Shoots

1. Liquid culture medium for shoot rooting (named MS-II); see medium formulations in Table 1.
2. Vessels for plant tissue culture: 250 mL glass flask.

2.1.6 Acclimatization of Plantlets

1. Compost and zeolite (4:1, v/v) (*see* **Note 3**).
2. Multipots of 70 hole (50 cm × 70 cm).

2.2 Somatic Embryogenesis

2.2.1 Callus Initiation

1. Leaf segment (0.5 cm) of the most internal leaves classified as A and B [16] of field plants of *C. citratus* as source of explants.
2. Semisolid culture medium based on modified [17] medium (named CI); see medium formulations in Table 1.
3. Growth regulators: 2,4-Dichlorophenoxyacetic acid (2,4-D).
4. Coconut milk.
5. Vessels for plant tissue culture: 250 mL glass flask.

2.2.2 Callus Multiplication and Induction of Embryogenic Culture

1. Semisolid culture medium based on modified [18] medium (named CM); see medium formulations in Table 1.
2. Growth regulators: 2,4-D, kinetin (Kin), indole-3-acetic acid (IAA).
3. Coconut milk.
4. Vessels for plant tissue culture: 250 mL glass flask.

2.2.3 Maintenance of Embryogenic Culture

1. Liquid culture medium based on modified MS medium [15] (named IEC); see medium formulations in Table 1.
2. Vessels for plant tissue culture: 100 mL Erlenmeyer flask.
3. Growth regulators: 2,4-D.
4. Coconut milk.
5. Rotatory shaker.

2.2.4 Proliferation of Embryogenic Culture in Bioreactors

1. Bioreactor unit, comprising 1.8 L glass vessels with controlled atmosphere.
2. Liquid culture medium IEC; see medium formulations in Table 1.
3. Growth regulators: 2,4-D.
4. Coconut milk.

2.2.5 Development of Somatic Embryos

1. Semisolid culture medium based on modified MS medium [15] (named DSE); see medium formulations in Table 1.
2. Growth regulators: 2,4-D.
3. Coconut milk.

2.2.6 Germination

1. Semisolid culture medium based on [16] medium free of growth regulators (named GSE); see medium formulations in Table 1.
2. Coconut milk.

3 Methods

3.1 Organ Culture

3.1.1 Surface Sterilization of Shoots and Culture Conditions

1. Sterilize all instruments and the laminar flow cabinet before use.
2. Cut from the field plants the last region of the stem comprising the apical meristem and the leaves that surround it (about 20 cm long) (Fig. 1a).
3. Remove external leaves of the explants giving a spindle form.
4. Reduce the size of the spindle from 20 to 5 cm conserving the apical meristem region (*see* **Note 4**).
5. In the laminar flow cabinet, place the reduced spindle in 350 mL flask with an aqueous solution of sodium hypochlorite (NaOCl) with 3 % of active Cl and shake during 15 min.
6. Rinse spindle three times with sterile distilled or deionized water.
7. Under aseptic conditions and with the help of a stereomicroscope, excise apical shoot of about 5 mm size.
8. Inoculate one apical shoot (Fig. 1b) per culture tube containing 10 mL liquid medium MS-I (Table 1). MS-I comprises basal MS medium [14] supplement with 30 g/L sucrose and 1.0 mg/L thiamine and 0.89 μM BA (*see* **Note 5**), pH of the medium adjusted to 5.6 by addition of 0.1 N NaOH or 0.1 N HCl, before autoclaving at 121 °C and 105 kPa for 15 min (*see* **Note 6**).

9. Incubate the explants at 28 ± 2 °C under natural light conditions, photoperiod 13/11 h and 20–45 μmol/m²/s (*see* **Note 7**).
10. After 3 weeks (*see* **Note 8**), singulate and cut shoots approximately in 2.5–3.0 cm length (Fig. 1c) for TIS inoculation. Discard any contaminated shoots.

3.1.2 Shoot Multiplication in RITA and 1 L TIS

1. Prepare RITA units (*see* **Note 9**).
2. Add to each RITA 230 mL liquid medium MS-I, supplemented as described (Table 1) (*see* **Note 10**).
3. After sterilization connect the RITA to the shelf for the temporary immersion. Program the timer for some parameters like time and immersion frequency (1 min every 4 h).
4. Check functioning of RITA for 3 days before inoculation. Discard any contamination.
5. Inoculate five individual shoots (2.5–3.0 cm long) per RITA in a laminar flow cabinet.
6. Reconnect RITA to the shelf for the temporary immersion, and incubate the culture for 3 weeks at 28 ± 2 °C under natural light conditions, photoperiod 13/11 h and 20–45 μmol/m²/s (*see* **Note 7**).
7. After 3 weeks of culture (Fig. 1d, e), harvest the biomass in a laminar flow cabinet. Produced shoots can be sub-cultivated again into RITA or into 1 and 10 L TIS.
8. Separate the individual shoots from the explants. Cut the shoots transversally eliminating apical dominance and reducing shoot size to 2.5–3.0 cm length, and transfer again into TIS.
9. For shoot multiplication in 1 L TIS, prepare TIS units (*see* **Note 9**) and connect all components.
10. Add to each 1 L TIS unit 500 mL MS-I liquid medium, supplemented as described in Table 1 (*see* **Note 11**).
11. Connect the TIS to the shelf for the temporary immersion. Program the timer for some parameters like time and immersion frequency (2 min every 4 h).
12. Same as **step 4**.
13. Inoculate 20 individual shoots (2.5–3.0 cm long) (Fig. 1c) per 1 L TIS (Fig. 1f) in a laminar flow cabinet.
14. Same as **step 6**.
15. The shoots grow quickly filling the flask in 3 weeks of culture (Fig. 1g). Averages of 12.3 shoots per explants with 5.25 cm of length are achieved. Shoots can be individualized and transferred to rooting phase or used for scale-up in 10 L TIS.
16. Individualize and cut shoots approximately in 2.5–3.0 cm length (Fig. 1c) for 10 L TIS inoculation. Discard any contaminated shoots.

3.1.3 Scale-Up of Shoot Multiplication in 10 L TIS

1. Prepare TIS units (*see* **Note 9**) and connect all components.
2. Add to each 10 L TIS unit a volume of 3000 mL liquid culture medium MS-I, supplemented as described in Table 1 (*see* **Note 12**).
3. Connect the TIS to the shelf for the temporary immersion. Program the timer for some parameters like time and immersion frequency (3 min every 4 h for 10 L TIS).
4. Same as **step 4** under Subheading 3.1.2.
5. Inoculate 20 individual shoots (2.5–3.0 cm long) (Fig. 1c) per 10 L TIS.
6. Same as **step 6** under Subheading 3.1.2.
7. After 4 weeks (Fig. 1h), collect the biomass and separate the individual shoots (more than 3 cm) from the clumps (Fig. 1i). Averages of 17.3 shoots/explants are achieved.

3.1.4 Rooting and Acclimatization of Plantlets

1. Reduce shoot size to 5 cm in length and leaf area to 50 %. Transfer six shoots into 250 mL culture flask, each containing 30 mL rooting liquid culture medium (named MS-II), and see medium formulation in Table 1. MS-II is similar to MS-I medium formulation, but without growth regulator. After 21 days of culture, the shoots grow fast and develop 3–4 adventitious roots (Fig. 1j).
2. Remove plantlets from the culture flask and wash them with water to remove rest of liquid culture medium. Now the plantlets are ready for the transfer to acclimatization.
3. After washing, plant in vitro plantlets individually and according to their length in the substrate mix of organic matter and zeolite (4:1, v/v) contained in perforated plastic multipot tray (50 cm × 70 cm, with 70 holes). Transfer the multipots in the greenhouse. During the first 2 weeks of acclimatization, reduce light intensity (50 %) by covering the plantlets with a plastic black mesh (Zaran). Keep the multipots under high humidity by watering during 30 s every 1 h.
4. In the third week, remove the Zaran and space watering frequency every 6 h. Keep this condition for 1 week more. Reduce watering frequency every 12 h the next 2 weeks. Well-developed and acclimated plants were achieved 45 days after ex vitro transfer (Fig. 1k).

3.2 Somatic Embryogenesis

3.2.1 Callus Initiation

1. Cut from the field-grown plants the last region of the stem comprising the apical meristem and the leaves that surround it (about 20 cm long) (Fig. 2a, b).

   2–6. Same as **steps 2–6** under Subheading 3.1.1 for plant material and disinfection procedure (*see* **Note 13**).

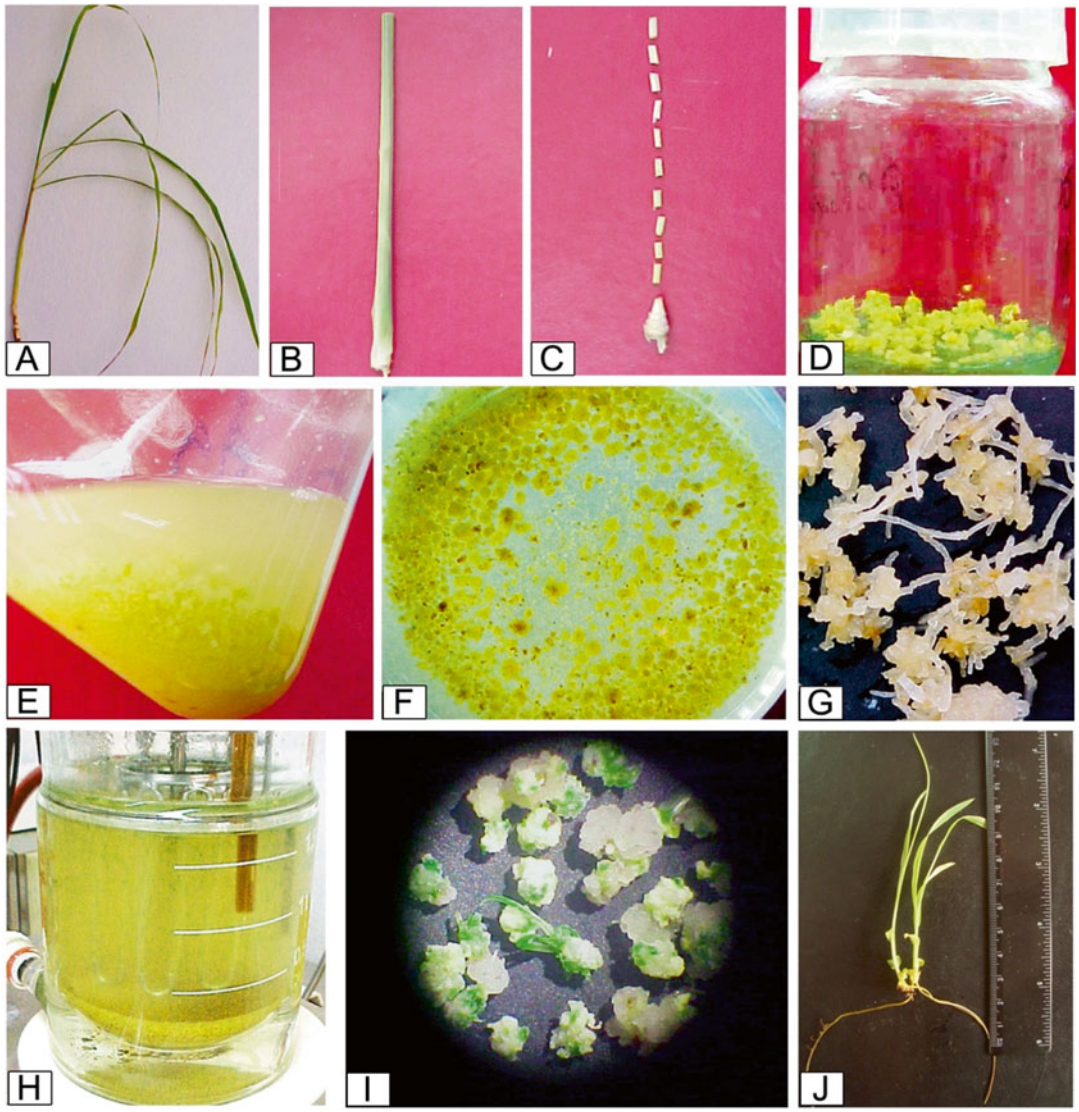

Fig. 2 Plant regeneration via somatic embryogenesis of *Cymbopogon citratus* in liquid culture, (**a**) apical branch from field of plants, (**b**) the apical region of the stem (about 20 cm long) from field plants (named spindle), (**c**) leaf segment (0.5 cm long) of the most internal leaves classified as A and B [16], (**d**) yellowish callus corresponding to embryogenic tissue from CM culture medium (MS + 13.57 μM 2,4-D + 4.1 μM kinetin + 7.3 μM IAA), (**e**) cell suspension obtained by transferring 2.0 g of yellowish callus into 50 mL of IEC culture medium (MS + 22.52 μM 2,4-D), (**f**) stable embryogenic cell suspension obtained 2 months after initiation in the presence of coconut milk, (**g**) roots growth in the cellular aggregates cultivated in liquid culture medium without cocconut milk, (**h**) 1.8 L bioreactor unit with embryogenic cultures on IEC culture medium, (**i**) germinated somatic embryos 1 week after culture in GSE medium (MS + 180 mL/L coconut milk and without regulator of growth), (**j**) plantlets from somatic embryos 4 weeks after culture on GSE medium

2. After the disinfection eliminate the external leaves in the laminar flow cabinet by keeping only the most internal leaves classified as A and B according to [16] for *Saccharum sp.* species. Discard apical meristem.
3. Excise leaf segments of 0.5 cm length from the A and B leaves (Fig. 2c). Inoculate vertically around five segments per 250 mL culture flask containing 25 mL CI medium (Table 1). Adjust pH of the medium to 5.6 by addition of 0.1 N NaOH or 0.1 N HCl, before autoclaving at 121 °C and 105 kPa for 20 min.
4. Incubate the cultures at 28 ± 2 °C for 4 weeks in the darkness. Within 4 weeks, callus develops from the leaf segments.

3.2.2 Callus Multiplication and Induction of Embryogenic Culture

1. Cut the callus into small fragments (around 5 mm size), discard watery and mucilaginous callus, and transfer the compact callus to the multiplication medium (CM) (Table 1). Adjust the pH of the medium to 5.6 by addition of 0.1 N NaOH or 0.1 N HCl, before autoclaving at 121 °C and 105 kPa for 20 min.
2. Distribute around 15–20 small callus fragments per 250 mL flask culture, each containing 25 mL of the culture medium. Incubate the cultures in dark conditions.
3. After 3 weeks, subculture the callus to fresh medium CM following the same procedure and culture conditions described in **step 1**. At this stage, select only the yellowish and friable callus (Fig. 2d).

3.2.3 Maintenance of Embryogenic Culture

1. Maintenance of embryogenic culture is more efficient by establishing cell suspension cultures. A cell suspension is obtained by transferring 2.0 g yellowish callus into 50 mL liquid culture medium for the induction of cell suspension culture (IEC) (Table 1). The resulting cell suspension cultures are cultured in 250 mL Erlenmeyer flasks at 26 °C ± 2 °C on a rotary shaker at 110 rpm.
2. After 2 weeks, add weekly 25 mL IEC medium during 4 weeks. Renew the 50 % of the culture medium every week for two more weeks. Stable embryogenic cell suspension cultures are obtained 2 months after initiation in the liquid medium (Fig. 2e). For subculturing in a 250 mL Erlenmeyer containing 50 mL liquid medium, aggregates are collected by physical separation using metallic sieve (375 μM pore size).
3. Maintenance cell suspension cultures by subculture at 8-week interval following the same procedure as described in **step 2**. Keep cell suspensions in the darkness. Coconut milk is indispensable during the entire process to the maintenance embryogenic cultures (Fig. 2f). In the absence of coconut milk, cells lose embryogenic competence and develop into root (Fig. 2g).

3.2.4 Proliferation of Embryogenic Culture in Bioreactors

1. For proliferation of embryogenic cultures, inoculate 60 g embryogenic masses from cell suspension into 1.8 L bioreactor (Fig. 2h) containing 1.8 L induction of embryogenic culture medium (IEC) (Table 1). For the control of the dissolved oxygen bioreactors are equipped with a unit to gas mixture (air, O_2, N_2, CO_2). Keep cultures at 26.0 ± 0.1 °C, and speed of agitation at 110 rpm, and for gassy mix fix the sparger flow at 1.43 L/h. Do not control pH. Keep the culture in the darkness for 9 days.
2. After 9 days the fresh and dry weight increases five and six times, respectively. Dry weight is recorded after the biomass is dried to constant weight at 60 °C.

3.2.5 Development of Somatic Embryos

1. Collect embryogenic cultures from the proliferation medium on 250 μm metallic sieve.
2. Rinse embryogenic cultures on metallic sieve with 50–100 mL liquid medium for embryo development (DSE) (Table 1).
3. Transfer 200 mg FW embryogenic culture from metallic sieve to 250 mL flask containing 30 mL DSE medium gelled with 7.0 g/L agar for development of somatic embryos. Keep culture under the darkness for 3 weeks.

3.2.6 Germination of Somatic Embryos

1. Transfer group of 2–3 green somatic embryos into semisolid germination medium (GSE). Distribute 15 groups into 250 mL flask containing 30 mL GSE medium (Table 1). Adjust the pH of the medium to 5.6 by adding 0.1 N NaOH or 0.1 N HCl, before autoclaving at 121 °C and 105 kPa for 20 min.
2. The somatic embryos turn green and hypocotyls emerge about 1 week after transfer to the germination medium (Fig. 2i), while root emergence occurs 1 week later. Because most of the somatic embryos remain together it is common to observe two or more hypocotyls emerging for somatic embryos germinated (Fig. 2j). More than 95 % germination is recorded at 4 weeks of culture.
3. After six weeks, an average of 80 plants are regenerated from initial 200 mg FW embryogenic cultures, placed development of somatic embryos (DSE) medium and subsequent germinated in GSE medium.

4 Notes

1. Filter paper as support avoids the complete immersion of shoots in the liquid medium.
2. Individual shoots could come from RITA or 1 L TIS.

3. Compost consisted of bovine manure with 8 months of decomposition.
4. Because of the hardness of the spindle, use a knife to reduce their size.
5. 6-BAP 1 mM stock solution: Dissolve 22.52 mg 6-BAP in a few drops of 1 M sodium hydroxide, dilute to 100 mL with distilled or deionized water, and store at 4 °C.
6. IAA 1 mM stock solution: Dissolve 8.76 mg IAA in a few drops of 1 M sodium hydroxide, dilute to 50 mL with distilled or deionized water, and store at 4 °C.
7. Before the liquid medium distribution in the culture tube, add filter paper bent in the form of M at the bottom of the culture tube. To discard contamination, conserve the medium in a clean and dark area at 4 °C at least for 3 days, but use within 2 weeks.
8. It is possible to cultivate in vitro at 16-h photoperiod and 125–150 μmol/m^2/s photosynthetic photon flux supplied by cool white fluorescent lamps.
9. During shoot culture establishment, the percent of shoot tips is more than 82 %, after 7 days. The apical shoots grow fast and develop several buds in the establishment. After 3 weeks the buds can be inoculated in TIS.
10. Prior RITA and TIS assembling, wash all pieces with deionized water. Connect all components according to the maker's instructions.
11. For preventing wetting of filters, protect them during sterilization by covering with aluminum foil on the filter inlet. To avoid the internal pressure in the containers, loosen the top of RITA and after sterilization seal it before removing from the autoclave.
12. Prior to the sterilization, distribute the half of medium (1500 mL) for each vessels. To discard contamination, store medium in darkness at 4 °C at least for 3 days.
13. For somatic embryogenesis induction, reduce the spindle size from 20 to 15 cm.

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