Chapter 1

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

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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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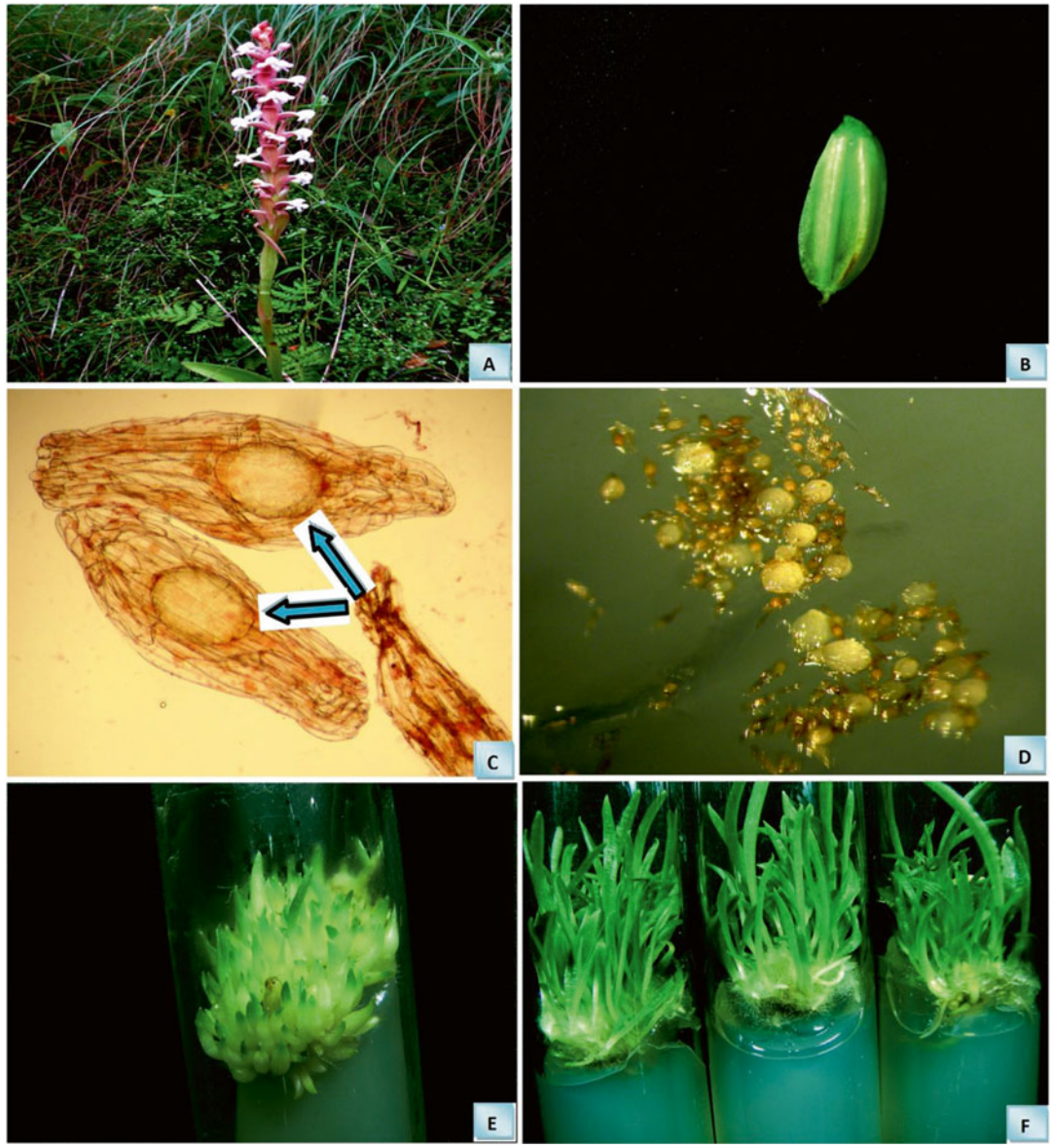

Fig. 1 In vitro multiplication of *Satyrium nepalense* -seeds to shoots. (**a**) A mature plant in its natural habitat. (**b**) A capsule pod. (**c**) Acetocarmine-stained seeds to show the stage of embryo at the time of culture (*arrow*). (**d**) PLBs developed from the seeds germinated on BM, after 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.

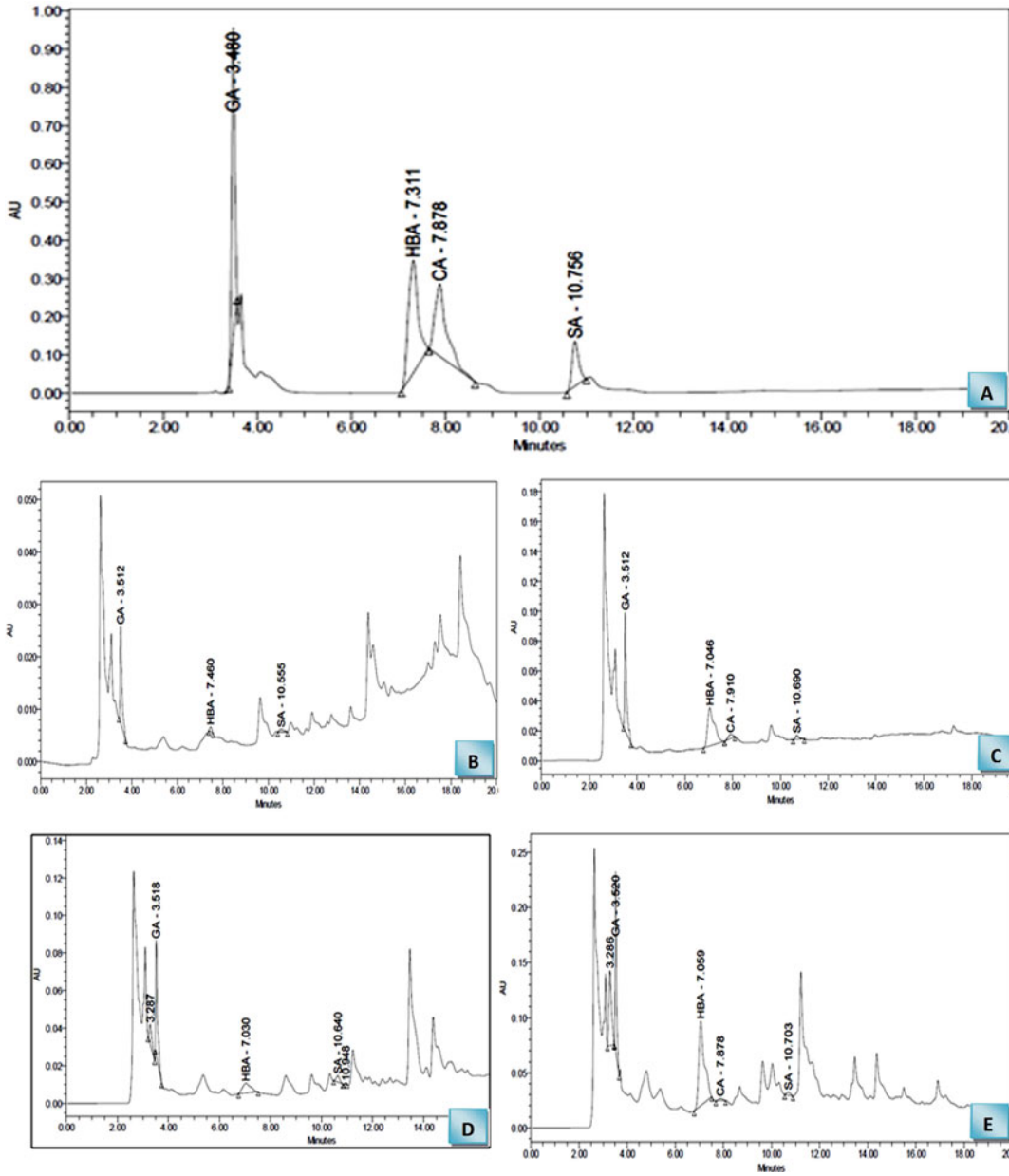

Fig. 3 Biochemical profiling of *Satyrium nepalense* for medicinally important phenolic acids, gallic acid (GA), *p*-hydroxybenzoic acid (HBA), syringic acid (SA), and caffeic acid (CA) by HPLC. (**a**) HPLC profile of the standards to show their retention time. (**b**–**e**) HPLC profiles of the four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