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An Efficient *in Vitro* Production of Shoots from Shoot Tips and Antifungal Activity of *Spilanthes acmella* (L.) Murr.

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ABSTRACT

An efficient method for the propagation of *Spilanthes acmella* (L.) Murr. through shoot tips (collected from 3 week-old axenic seedlings) has been successfully developed. This protocol can be employed on a commercial scale for the production of spilanthol. Among the cytokinins, 6-benzyladenine (BA), kinetin (Kn) and 2-isopentenylaminopurine (2-iP) were tested. Murashige and Skoog (MS) medium supplemented with 1.0 µM BA was optimum for inducing bud break. When auxin was augmented with an optimal cytokinin concentration, the regeneration efficiency of explants was enhanced. The maximum response (96%) with highest number of shoots per explant (i.e., 33.0) was possible on MS medium containing 1.0 µM BA and 0.1 µM α-naphthaleneacetic acid (NAA). Small shoots (3.0-4.0 cm) were rooted *in vitro* with half-strength MS medium containing 2.5 µM NAA, forming a maximum of 32.2 roots/shoot. The well developed micropropagated plants were successfully acclimatized within 4 weeks in Soilrite™ and planted *ex vitro* in garden soil, farmyard soil and sand (2: 1: 1), where they grew well without any apparent morphological variation from the parent plant. Moreover, the phytomedicinal effect of various plant tissues was also evaluated against human pathogenic fungi. The alcoholic extracts of *in vitro* plant were more effective than *in vivo* plant materials. Maximum inhibition zone (MIZ) was noticed against *Candida krusei* followed by *Candida albicans*, *Aspergillus fumigatus* and *Aspergillus niger*. Among different explants, flower heads showed best response against *C. krusei* as highest of 12.3 and 12.0 cm MIZs were noticed for *in vitro* and *in vivo* source, respectively.

Keywords: Asteraceae, fungicidal, micropropagation, spilanthol

INTRODUCTION

Spilanthes acmella (L.) Murr., which belongs to the Asteraceae family, is a threatened medicinal plant (Rao and Reddy 1983) and is commonly known as 'Akarkara'. It is native to the tropics of Brazil and mainly found in tropical regions throughout the world. The flower heads are chewed to relieve toothache and affection of throat and gums and paralysis of tongue (Sharma *et al.* 2010).

An alkaloid named spilanthol has been obtained from the dry flowers (Prachayasittikul *et al*. 2009). It is pungent and possesses a strong sialogogic action, stimulating the salivary glands to increase flow of saliva and consequently promoting digestion (Jayaweera 1981). The plant has also shown antibacterial (Fabry *et al.* 1996, 1998; Prasad and Seenaya 2000), antifungal (Sabitha and Suryanarayana 2006), larvicidal (Ramsewak *et al.* 1999; Saraf and Dixit 2002; Amer and Mehlhorn 2006), antimalarial (Pandey *et al.* 2007), anti-inflammatory (Chakraborty *et al.* 2004; Wu *et al*. 2008) and immunomodulating properties (Savadi *et al.* 2010). In addition, *in vitro* results on antiobesity for *S. acmella* are encouraging (Ekanem *et al*. 2007). Recently, scopolectin has also been detected in *S. acmella* flower buds (Prachayasittikul *et al*. 2009; Singh and Chaturvedi 2010). Indiscriminate collection of *S. acmella* has led to rapid depletion of wild populations resulting to its listing as a threatened medicinal plant in India (Rao and Reddy 1983).

Conventional vegetative propagation by stem cuttings is arduous and inadequate to meet the needs for ayurvedic drug preparations. Rapid large-scale propagation is a prerequisite to meet the pharmaceutical needs and for effective conservation of this valuable medicinal plant. In recent years, there has been an increased interest for *in vitro* plant tissue culture which offers a viable tool for mass multiplication and germplasm conservation of rare, endangered, aromatic, medicinal and ornamental plants (Anis *et al*. 2009). This technology could be a cost-effective mean for mass production of elite plant materials throughout the year, without any seasonal constraints. To the best of our knowledge, although reports are available for micropropagation of *S. acmella* using various explants (Saritha *et al*. 2002; Haw and Keng 2003; Deka and Kalita 2005; Saritha and Naidu 2008; Pandey and Agrawal 2009; Singh and Chaturvedi 2010), no report is available using shoot tip explants. Moreover, previous results are not satisfactory due to low regeneration rate. Therefore, there is an urgent need to develop a refined protocol for large scale propagation of this valuable medicinal herb.

In recent years, plant-derived antibiotic research became a hot topic in medicinal science. Though plant species cure microbial infections and yield antimicrobial compounds in nature, their clinical uses are limited due to limitation of existing compound and lack of available clinical studies. There is indeed an urgent need to redouble the efforts both in academia and industry to search or develop new agents so that plant derived compounds can be used as novel strategies to cope with diagnosis and treatment of infectious diseases (Shahid *et al*. 2009a, 2009b). There is a single report on antifungal activity of *S. acmella* against human pathogenic fungi using *in vivo* grown flower heads (Sabitha *et al*. 2006), but no report is available on comparative antifungal analysis between *in vivo*- and *in vitro*-derived vari-

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ous plant parts. Nowadays, various studies have been undertaken for screening the biological activities of *in vitro* raised calli in various plant species. In this regard, Tanwer *et al*. (2010) reported antioxidant activity of *in vitro* leaf derived calli of *S. acmella*. They also compared various metabolite contents in different plant parts and found maximum sugar concentration in callus tissues, which was associated with an effective antifungal activity. Thus, the present study reports a comparative evaluation of antifungal activity among various *in vivo* and *in vitro* raised plant parts and calli of *S. acmella* along with an efficient and reproducible protocol for *in vitro* conservation of this threatened species.

MATERIALS AND METHODS

Micropropagation study

1. Establishment of axenic seedlings

The inflorescences of *S. acmella* were procured from the Medicinal Plant Nursery of Tamnaar, District Raigarh, State Chhattisgarh, India. The plant species was identified by the taxonomists of the Department of Botany, A.M.U. and a specimen was deposited in the Departments' herbarium (Number 31301). Healthy seeds were isolated mechanically from mature inflorescence and washed under running tap water for 30 min to remove any adherent particles. The seeds were kept in 1% (w/v) Bavistin (Carbendazim powder, BASF India Ltd.), a broad spectrum fungicide, for 20 min and then washed in 5% (v/v) Teepol (Qualigens, India), a liquid detergent for 15 min. The treated seeds were agitated in sterilized double distilled water (DDW) to remove the chemical inhibitors to germination. The seeds were surface sterilized with 70% (v/v) ethanol and 2-3 drops (v/v) of Tween-20 (Qualigens) for 30 s, followed by immersion in an aqueous solution of 0.1% (w/v) $HgCl_2$ (Qualigens) for 3 min under the sterile condition. Finally, the seeds were washed 5-6 times with sterilized DDW to remove all traces of sterilant. The surface sterilized seeds were inoculated aseptically in culture tubes (25 × 150 mm, Borosil) containing half-strength Murashige and Skoog (MS) medium supplemented with gibberellic acid (GA_3) at 0.5 µM.

2. Culture medium and conditions

The MS medium augmented with 3% (w/v) sucrose (Hi-media, Mumbai, India) and 0.8% (w/v) agar (Hi-media) was used throughout the experiment. The pH of the medium was adjusted to 5.8 by 1 N NaOH or 1 N HCl before adding agar followed by autoclaving at 121°C and 1.06 kg cm^{-2} for 15 min. The cultures were incubated at 25 ± 2°C, 16-h photoperiod with 50 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) provided by cool white fluorescent tubes (40 W; Philips, India) and 55 ± 5% relative humidity.

3. Shoot induction and proliferation

Two sets of experiment were carried out on shoot induction from shoot tip explants. In the first experiment, shoot tips (1.0-1.5 cm excised from 3 weeks old seedlings) were cultured on MS medium augmented with various concentrations (0.1, 0.5, 1.0, 2.5 and 5.0 µM) of cytokinins such as 6-benzyladenine (BA), kinetin (Kn) and 2-isopentenylaminopurine (2-iP). MS medium lacking plant growth regulators (PGRs) served as the control. In the second experiment different concentrations (0.1, 0.5 and 1.0 µM) of three auxins (α-naphthalene acetic acid (NAA), indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA)) were added to an optimal cytokinin (1.0 µM BA) to see the synergistic effect of cytokinin-auxin combination on shoot regeneration. All PGRs were purchased from Duchefa, The Netherlands.

4. Root induction in shoots

The *in vitro* raised shoots (3.0-4.0 cm) with fully expanded leaves were transferred to full- and half-strength MS medium supplemented with auxins IBA, NAA and IAA at 1.0, 2.5 and 5.0 µM. Full-strength MS medium without growth regulators was used as control.

5. Acclimatization

The rooted plantlets were removed from the culture medium and washed the roots gently under running tap water to remove adhering medium. Roots were immersed in 1% (w/v) Bavistin for half an hour, then plantlets were transferred to pots containing autoclaved Soilrite™ and irrigated with tap water as required. They were covered with transparent glass bottles to ensure high humidity (90%) for initial 2 weeks and then removed gradually. After 4 weeks, acclimatized plantlets were transferred to pots filled with garden soil, farmyard soil and sand (2: 1: 1). The potted plantlets were initially maintained under culture room conditions (5 weeks) and then transferred to normal laboratory conditions (4 weeks). Afterwards, the plantlets were transplanted to net-house and kept under shade for further growth and development.

Antifungal activity

1. Plant parts used

Leaves, stem segments and young flower heads, collected from a 4 month-old *in vivo* parent plant and 4 month-old *in vitro* plant grown in the Medicinal Plant Nursery of Tamnaar and the Department of Botany, A.M.U. respectively, were used to compare their antifungal activities.

2. In vitro culture of nodal explants for callus induction

Nodal segments were used for *in vitro* callus induction. The explants were sterilized according to above described method and inoculated aseptically on MS medium supplemented with 7.5 µM thidiazuron (TDZ) alone and 5.0 µM TDZ with 1.0 µM BA for 4 weeks.

3. Preparation of plant extract

Both the aqueous and alcoholic extracts were tested for antifungal activity. The extracts were obtained according to the method of Singh and Singh (2000) with some modifications as described below. Fresh leaves, stem segments and flower heads (15.0 g each) from both sources (*in vivo* and *in vitro*) were surface sterilized in 70% ethyl alcohol for 1 min and then washed 3 times with sterilized DDW. Besides, the calli of nodal explants were aseptically removed from the culture tubes and all the plant materials, including calli, were grounded with a sterilized pestle and mortar in 150 ml sterilized DDW. The homogenized tissues were centrifuged at 5,000 rpm for 15 min and supernatants were treated as the aqueous extracts. Similarly, the alcoholic extracts were prepared using an equal volume of absolute ethanol in place of DDW.

4. Antifungal test

Clinical isolates of fungi were obtained from the Department of Microbiology, Jawaharlal Nehru Medical College and Hospital, A.M.U., Aligarh. The fungal strains used were *Candida albicans*, *Candida krusei*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus* and *Fusarium* species. They were grown at 25 ± 2°C and maintained on Sabourand's Dextrose Agar (SDA) slants. Antifungal testing was performed according to NCCLS (1997) (document M27-A for yeasts) and NCCLS (2002) (document M38-A for filamentous fungi). Stock fungal solutions were thawed and immediately suspended in Brain Heart Infusion broth supplemented with 2-3 drops of Tween-20 (Qualigens). An inoculums size of 2 × 10^5 yeast cells or fungal spores was used for inoculating SDA plates. Two sets of SDA, each containing two plates, were lawn cultured with fungal suspensions. Seven wells were made in each plate in a diameter of 5 mm using a sterile borer. The 20 µl plant extracts (alcoholic as well as aqueous extracts) were poured in the wells of respective plates. Sterilized DDW and ethanol served as negative controls in the aqueous and alcoholic plates, respectively, whereas the antifungal drug, voriconazole was used as a positive control. The reference voriconazole powder was obtained from Pfizer and used at 0.1 µg/µl. The plates were kept upright for 5-10 min until the solution diffused into the medium and then inoculated aerobically at 25 ± 2°C in a biological oxygen demand

Table 1 Effect of different concentration of growth regulators on shoot number and shoot length using shoot tip explants after 4 weeks of culture.

PGR (µM)	% Response	Mean no. of shoots/explant	Mean shoot length (cm)	Frequency of callogenesis
BA (0.1)	34.0 ± 2.44 cde	3.6 ± 0.24 cd	2.5 ± 0.36 ef	-
BA (0.5)	38.0 ± 2.00 cd	4.4 ± 0.40 bc	2.7 ± 0.50 def	-
BA (1.0)	66.0 ± 2.44 a	8.0 ± 0.31 a	3.9 ± 0.30 bcd	-
BA (2.5)	60.0 ± 3.16 a	5.8 ± 0.37 b	4.7 ± 0.28 ab	+
BA (5.0)	60.0 ± 3.16 a	4.0 ± 0.31 cd	4.2 ± 0.15 abc	+ +
Kn (0.1)	32.0 ± 2.00 de	2.0 ± 0.31 e	3.0 ± 0.17 cde	-
Kn (0.5)	46.0 ± 2.44 bc	2.6 ± 0.40 de	4.0 ± 0.17 abcd	-
Kn (1.0)	64.0 ± 2.44 a	3.6 ± 0.40 cd	4.5 ± 0.29 ab	+
Kn (2.5)	62.0 ± 2.00 a	1.6 ± 0.24 e	5.3 ± 0.27 a	+ +
Kn (5.0)	56.0 ± 4.00 ab	1.6 ± 0.24 e	4.4 ± 0.20 ab	+ +
2-iP (0.1)	22.0 ± 2.00 e	1.2 ± 0.20 e	1.9 ± 0.21 efg	-
2-iP (0.5)	32.0 ± 2.00 de	1.4 ± 0.24 e	2.1 ± 0.12 efg	+
2-iP (1.0)	44.0 ± 4.00 bcd	1.6 ± 0.24 e	2.3 ± 0.18 efg	+ +
2-iP (2.5)	54.0 ± 2.44 ab	1.4 ± 0.24 e	1.6 ± 0.13 fg	+ +
2-iP (5.0)	44.0 ± 2.44 bcd	1.2 ± 0.20 e	1.2 ± 0.10 g	+ + +

Data represents Mean ± SE of 20 replicates. Mean value followed by the same alphabets are not significantly different according to Tukey's Test at 5% probability.
-, +, + +, + + +, indicate no, slight, moderate, intense callusing respectively.

Table 2 Effect of different combination of auxin (NAA, IBA and IAA) with 1.0 µM BA on shoot number and shoot length using shoot tip explants after 4 weeks of culture.

PGR (µM)	% Response	Mean no. of shoots/explant	Mean shoot length (cm)	Frequency of callogenesis
NAA (0.1)	96.0 ± 2.44 a	33.0 ± 1.09 a	5.2 ± 0.09 a	-
NAA (0.5)	92.0 ± 2.00 ab	28.2 ± 0.66 b	5.5 ± 0.17 a	+
NAA (1.0)	82.0 ± 2.00 bc	23.2 ± 0.86 c	5.6 ± 0.22 a	+ + +
IBA (0.1)	92.0 ± 3.74 ab	12.8 ± 0.73 d	6.1 ± 0.11 a	+ +
IBA (0.5)	72.0 ± 3.74 cd	15.0 ± 0.31 d	5.6 ± 0.28 a	+ + +
IBA (1.0)	62.0 ± 3.74 de	13.6 ± 0.50 d	5.9 ± 0.13 a	+ + +
IAA (0.1)	76.0 ± 2.44 c	8.0 ± 0.31 e	5.5 ± 0.41 a	+ +
IAA (0.5)	56.0 ± 2.44 ef	2.6 ± 0.40 f	2.1 ± 0.10 b	+ + +
IAA (1.0)	48.0 ± 2.00 f	1.0 ± 0.31 f	1.4 ± 0.13 b	+ + +

Data represents Mean ± SE of 20 replicates. Mean value followed by the same alphabets are not significantly different according to Tukey's Test at 5% probability.
-, +, + +, + + +, indicate no, slight, moderate, intense callusing respectively.

(BOD) incubator for 48-72 h.

Statistical analysis

The data were examined after 4 weeks for shoot and root induction while MIZs were measured after 2-3 days of incubation. Each treatment consisted of 20 replicates and single explant was cultured per test tube. All the experiments were repeated in triplicate. The data were analyzed statistically using SPSS version 12 (SPSS Inc., Chicago, IL, USA) and significant differences between mean were assessed by Tukey's test at 5% probability. The results were expressed as the mean ± standard error (SE).

RESULTS AND DISCUSSION

Micropropagation study

1. Shoot induction and proliferation

The seeds began to germinate after 4-5 days of inoculation on half-strength MS medium supplemented with 0.5 µM GA. Shoot tips derived from these aseptic seedlings (3 week-old) were used for determining their ability to induce multiple shoots (**Fig. 1A**). On PGR-free MS basal medium the explants failed to induce shoot regeneration, while on cytokinin-augmented media shoot regeneration occurred. The percentage response, mean number of shoots/explant and mean shoot length varied considerably with the type of PGR used at different concentrations (**Table 1**). The explants showed the first response by initially enlarging with the formation of new leaves within 1 week of incubation (**Fig. 1B**). Among the cytokinins tested, 1.0 µM BA was optimal as it induced a maximum of 8.0 shoots/explant and a mean shoot length of 3.9 cm in 66% of cultures after 4 weeks (**Fig. 1C**). A linear increase was achieved with an increase in cytokinin concentration from 0.1-1.0 µM for BA and Kn and from 0.1-2.5 for 2-iP with respect to regeneration efficiency. By further increasing the concentration beyond the optimal level, the regeneration capacity was suppressed considerably due to basal callusing (yellowish-green to white). Proliferation of callus is always disadvantageous and suppressive for direct shoot organogenesis as it has already been advocated by Cellárová and Kimáková (1999), whose study showed a higher concentration of BA (> 1.0 mg l^{-1}) induced the formation of callus tissue and caused a chromosomal instability in the regenerated plants. By increasing the BA concentration beyond the optimal level, a gradual reduction in regeneration efficiency has also been reported for several medicinal plants including *With a-nia somnifera* (Sen and Sharma 1991), strawberry (Indhra and Dhar 2000) and *Artemisia vulgaris* (Sujatha and Ranjitha 2007).

When Kn or 2-iP were used as the sole cytokinin, no significant response was observed. The superiority of BA over Kn and 2-iP for shoot induction has also been reported for several medicinal and aromatic plant species such as *Ocimum basilicum* (Begum *et al*. 2002), *Picrorhiza kurroa* (Chandra *et al*. 2004), *Coleus blumei* (Rani *et al*. 2006) and *Stevia rebaudiana* (Mousumi 2008).

The addition of a low concentration of auxin to an optimal concentration of cytokinin (1.0 µM BA) exhibited a positive effect on the induction of multiple shoots due to synergism between cytokinin and auxin (**Table 2**). Among the auxins, NAA at 0.1 µM significantly enhanced the regeneration capacity of explants. The maximum response (96%) with the highest number of shoots/explant (i.e., 33.0) was possible on MS medium containing 1.0 µM BA and 0.1 µM NAA after 4 weeks of incubation (**Fig. 1D, 1E**). Higher concentration of NAA i.e., 0.5 and 1.0 µM reduced the regeneration potential due to high frequency callogenesis from the base of shoots. However, the incorporation of IBA or IAA with BA did not show better response than 1.0 µM BA with 0.1 µM NAA combination and only 15.0 and 8.0 shoots/explant were induced on 1.0 µM BA with 0.5 µM IBA and 1.0 µM BA with 0.5 µM IAA supplemented MS media, respectively. The results are in accordance with earlier findings of several scientists in which the addition of a low level of NAA with cytokinin promoted shoot prolifera-

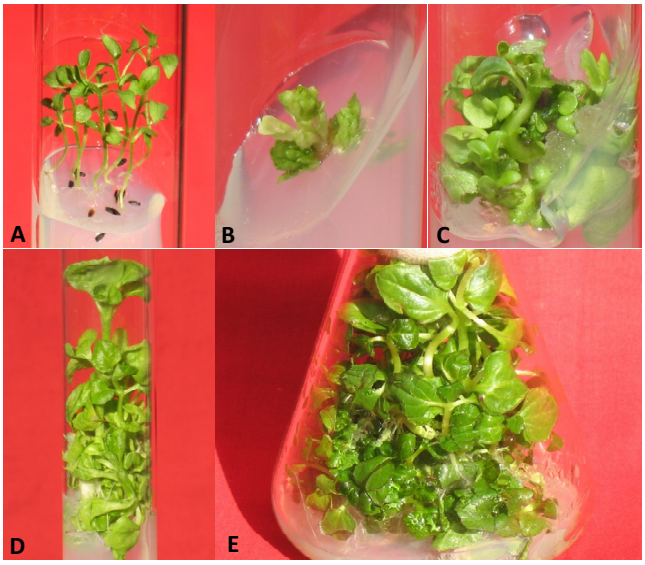

Fig. 1 (**A**) Aseptic seedlings grown on half-strength MS medium containing GA (0.7 µM) after 3 weeks of germination; (**B**) Direct shoot bud induction from shoot tip explant on MS medium containing BA (1.0 µM) after 2 weeks of culture; (**C**) Multiple shoot regeneration from shoot tip on MS medium containing BA (1.0 µM) after 4 weeks of culture; (**D, E**) Proliferation and elongation of shoots on MS medium containing BA (1.0 µM) + NAA (0.1 µM) after 3 and 4 weeks of culture.

tion as reported in *Petasites hybridus* on 17.6 µM BA plus 0.54 µM NAA (Wildi *et al*. 1998), *Hybanthus enneaspermus* on 8.8 µM BA plus 2.6 µM NAA (Prakash *et al*. 1999) and *Trichosanthes cucumerina* on 1.0 mg l^{-1} BA plus 0.1 mg l^{-1} NAA (Devendra *et al*. 2008).

Thus, our findings concluded that the BA and NAA combination was critical for inducing maximum number of shoots in *S. acmella*. In contrast to our results, in the same plant species Haw and Keng (2003) reported greater shoot formation on BA-supplemented MS medium than cytokinin-auxin combination. This intricacy might be because of the origin of explants as Haw and Keng (2003) used *in vivo* grown explants of *S. acmella*.

Table 3 Effect of nutrient strength and different auxin (IBA, NAA and IAA) concentration on *in vitro* rooting after 4 weeks of culture.

PGR (µM)	Mean no. of roots/shoot	Mean length of root (cm)	Frequency of callogenesis
MS	9.2 ± 0.37 gh	6.8 ± 0.33 ij	-
MS + IBA (1.0)	10.4 ± 0.50 gh	8.1 ± 0.12 hi	+ +
MS + IBA (2.5)	18.2 ± 1.06 de	10.3 ± 0.46 fg	+ + +
MS + IBA (5.0)	10.2 ± 0.58 h	5.5 ± 0.19 j	+ + +
MS + NAA (1.0)	21.8 ± 1.06 cd	11.2 ± 0.35 ef	+
MS + NAA (2.5)	18.0 ± 1.51 de	9.2 ± 0.14 gh	+
MS + NAA (5.0)	14.8 ± 0.86 efg	9.4 ± 0.22 gh	+ + +
MS + IAA (1.0)	10.0 ± 0.70 h	12.0 ± 0.35 e	+ +
MS + IAA (2.5)	9.6 ± 0.67 h	8.5 ± 0.11 h	+ +
MS + IAA (5.0)	8.2 ± 0.66 h	9.0 ± 0.16 gh	+ +
½ MS	11.0 ± 0.44 fgh	6.5 ± 0.16 ij	-
½ MS + IBA (1.0)	18.4 ± 1.07 de	13.7 ± 0.25 cd	+
½ MS + IBA (2.5)	24.4 ± 0.50 c	16.5 ± 0.50 ab	+ +
½ MS + IBA (5.0)	14.8 ± 0.96 efg	11.5 ± 0.47 ef	+ +
½ MS + NAA (1.0)	37.2 ± 1.15 a	17.9 ± 0.48 a	-
½ MS + NAA (2.5)	32.2 ± 0.70 b	15.2 ± 0.33 bc	-
½ MS + NAA (5.0)	23.2 ± 0.86 c	12.8 ± 0.25 de	-
½ MS + IAA (1.0)	15.0 ± 1.00 ef	14.3 ± 0.19 cd	-
½ MS + IAA (2.5)	20.0 ± 0.70 cd	14.5 ± 0.35 c	+ +
½ MS + IAA (5.0)	12.2 ± 0.96 fgh	9.6 ± 0.25 gh	+ +

Data represents Mean ± SE of 20 replicates. Mean value followed by the same alphabets are not significantly different according to Tukey's test at 5% probability.
-, +, + +, + + +, indicate no, slight, moderate, intense callusing respectively.

Fig. 2 (**A**) Root induction from *in vitro* raised shoot on half-strength MS medium supplemented with NAA (1.0 µM) after 2 weeks of culture; (**B**) An expanded view of well developed root system after 4 weeks of culture; (**C**) Acclimatized micropropagated plantlet of *S. acmella* in Soilrite™ after 4 weeks; (**D**) An *in vitro* regenerated plant of *S. acmella* showing flowering in field conditions.

2. Root induction in shoots

In vitro raised shoots rooted successfully on full- and half-strength MS media either omitted or supplemented with auxin within 1-2 weeks of incubation (**Table 3**). The best rooting response was obtained on half-strength MS medium augmented with 1.0 µM NAA, where shoots produced a mean of 37.2 roots, each measuring 17.9 cm after a period of 4 weeks of incubation (**Fig. 2A, 2B**). The roots formed with this treatment were directly linked with the shoot base. Optimum rooting response using half-strength MS with NAA has also been reported for several plants including *Cephaelis ipecacuanha* (Jha and Jha 1989), *Scoparia dulcis* (Rashid *et al*. 2009) and *Swertia chirata* (Balaraju *et al*. 2009). However, callogenesis at the base of regenerated shoots on full-strength MS medium with NAA led to the formation of thin and delicate roots. This type of rooting was undesirable for successful transplantation of the plantlets (Shahzad *et al*. 2007). On the other hand, IBA and IAA favored profuse and moderate callusing on full- and half-strength MS media, respectively.

Acclimatization

Since the plantlets are grown in protective culture conditions, it becomes imperative to make them autotrophic before transplantation to field conditions. After successful acclimatization, there was no detectable variation among the hardened plantlets with respect to morphological and growth characteristics. All the micropropagated plantlets were free from external defects (**Fig. 2C, 2D**).

Antifungal activity

The results related to antifungal activity of the aqueous and alcoholic extracts of various plant tissues including calli, against respective fungi are shown in **Table 4**. The sterilized DDW did not show any zone of inhibition, whereas the ethanolic control showed a zone of inhibition in a range

Table 4 Comparative antifungal activity of alcoholic extracts of *in vitro* derived explants and calli with extract of *in vivo* derived explants*.

Alcoholic extract	Zone of inhibition (mm) ± SE					
	Candida albicans	*Candida krusei*	*Aspergillus niger*	*Aspergillus flavus*	*Aspergillus fumigatus*	*Fusarium* species
Callus						
C_1	11.3 ± 0.66 bc	10.0 ± 0.57 bcd	7.3 ± 0.33 b	8.3 ± 0.33 b	8.6 ± 0.33bc	0.0 ± 0.00 c
C_2	8.3 ± 0.33 cd	10.3 ± 0.33 bcd	8.3 ± 0.33 ab	8.0 ± 0.33 b	8.3 ± 0.33c	0.0 ± 0.00 c
Stem						
In vitro	10.3 ± 0.88 b	9.3 ± 0.33 cd	0.0 ± 0.00 d	9.0 ± 0.57 ab	0.0 ± 0.00e	0.0 ± 0.00 c
In vivo	9.3 ± 0.33 bc	10.3 ± 0.33 bcd	0.0 ± 0.00 d	0.0 ± 0.00 d	0.0 ± 0.00 e	0.0 ± 0.00 c
Leaf						
In vitro	0.0 ± 0.00 e	8.0 ± 0.57 d	0.0 ± 0.00 d	0.0 ± 0.00 d	8.6 ± 0.66 bc	0.0 ± 0.00 c
In vivo	0.0 ± 0.00 e	11.3 ± 0.66 abc	0.0 ± 0.00 d	0.0 ± 0.00 d	8.3 ± 0.33 c	0.0 ± 0.00 c
Flower head						
In vitro	12.6 ± 0.6 bc	12.3 ± 0.33 ab	9.0 ± 0.57 a	9.3 ± 0.33 ab	11.3 ± 0.33 a	0.0 ± 0.00 c
In vivo	0.0 ± 0.00 e	12.0 ± 1.00 ab	9.0 ± 0.57 a	0.0 ± 0.00 d	11.0 ± 0.57 ab	0.0 ± 0.00 c
Positive control	18.0 ± 0.00 a	13.0 ± 0.00 a	9.0 ± 0.00 a	10.0 ± 0.00 a	9.5 ± 0.00 abc	9.0 ± 0.00 a
Negative control						
DDW	0.0 ± 0.00 e	0.0 ± 0.00 f	0.0 ± 0.00 d	0.0 ± 0.00 d	0.0 ± 0.00 e	0.0 ± 0.00 c
Alcohol	4.0 ± 0.00 de	3.8 ± 0.00 e	3.3 ± 0.00 c	3.6 ± 0.00 c	3.6 ± 0.00 d	4.0 ± 0.00 b

Diameter of zone of inhibition is a mean ± SE. Mean value followed by the same alphabets are not significantly different according to Tukey's Test at 5% probability.
C_1 = callus grown on MS + TDZ (5.0 µM) + BA (1.0 µM)
C_2 = callus grown on MS + TDZ (7.5 µM)
- = No zone of inhibition
*Aqueous extract did not response against any fungal strain tested, so only the data presented here based on the alcoholic extracts.

of 3.3-4.0 mm. The positive control (variconazole) showed the zone of inhibition in a range of 9-18 mm against fungi. The aqueous extracts were not effective against all the fungal strains tested whereas the ethanolic extracts exhibited varied responses depending on the strain. MIZ was found for *C. krusei* followed by *C. albicans*, *A. fumigatus* and *A. niger*. None of the extracts showed antifungal activity against *Fusarium* species. Among different explants, flower heads showed best response against *C. krusei* as 12.3 and 12.0 cm MIZ was noticed for *in vitro* and *in vivo* source, respectively. In the present study both *in vivo*- and *in vitro*-derived extracts showed a similar trend of antifungal activities against all strains except *A. flavus* wherein only *in vitro* raised explants and calli exhibited antifungal capacity. This shows the presence of some antifungal compounds that might be produced under *in vitro* conditions, but were either absent or present in very low quantities in *in vivo* grown plants, which were unable to show the same antifungal activity.

CONCLUSION

To conclude, the present communication describes an efficient and effective regeneration system for *S. acmella* using shoot tip explants. The protocol outlined above offers a potential system for conservation of this threatened medicinal plant and would facilitate its use in future for genetic engineering strategies. Furthermore, because of very high frequency of regeneration and establishment of plant under natural conditions, the propagule production cost could be reduced considerably and would be of industrial importance. Based on the preliminary study towards antifungal activity, it is thus suggested that the alcoholic extracts of this plant including from calli may be used in phytotherapy as an antifungal agent. As the extracts from *in vitro* raised plant parts and calli showed significant antifungal activity, *in vitro* cultivation of the tissues may be used to obtain phytotherapeutic compounds, especially, at places where the plants cannot be grown because of the adverse atmospheric conditions. It will also lead to use of *in vitro* raised cells for herbal antifungal drug formulation which would certainly be helpful to save this threatened species by minimizing the pressure of collection of plants from its natural habitat. In future, the bioactive compounds responsible for antimicrobial activity could further be enhanced by nutritional and hormonal manipulations of the cultivation medium.

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