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ORIGINAL PAPER

Improved clonal propagation of *Spilanthes acmella* Murr. for production of scopoletin

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Abstract A reproducible protocol for clonal propagation of *Spilanthes acmella* has been established. Routinely, the cultures were established in spring (January–April) season because of the highest aseptic culture establishment and high frequency shoot proliferation. Incorporation of 5 μM N^6-benzyladenine (BA) to Murashige and Skoog (MS) basal medium showed 100% bud-break and promoted multiple shoot proliferation in cultures. Interestingly, a higher concentration of BA (7–15 μM) promoted stunted shoots with pale leaves while a lower concentration (1–3 μM) resulted in shoots with long internodes and excessive adventitious root proliferation from all over their surface. For recurrent shoot multiplication, single node segments from in vitro-developed shoots were excised and cultured on MS + BA (5 μM) medium where 20.3-fold shoot multiplication was achieved every 5 weeks. Finally, these shoots were successfully rooted on half-strength MS medium (major salts reduced to half-strength) with 50 g l^{-1} sucrose, with a frequency of 100%. Transplantation survival of micropropagated plants was 88.9%. Additionally, accumulation of scopoletin, a phytoalexin, was revealed for the first time in the uninfected leaves of *Spilanthes*. Further, the quantitative estimation by HPLC with a fluorescence detector showed that the amounts of scopoletin content (0.10 μg g^{-1} DW) in the leaves of micropropagated plants are comparable to those of field-grown mother plants. The study thus signifies the effectiveness of in vitro methodology for true-to-type plant regeneration of *Spilanthes* and their later utility for biosynthesis and constant production of scopoletin throughout the year.

Keywords Analysis · Optimization · Micropropagation · Scopoletin · *Spilanthes acmella* L

Abbreviations

BA	N^6-benzyladenine
Kinetin	N^6-furfurylaminopurine
2-iP	Isopentenyl adenine
NAA	α-naphthaleneacetic acid
GA_3	Gibberellic acid

Introduction

Spilanthes acmella Murr., a perennial herb (Family Asteraceae), is renowned for its medicinal and insecticidal properties. It is widely grown in the tropics and subtropics, and can be found in damp pastures, at swamp margins, on rocks near the sea and as a weed of roadsides. It has been well documented for its anti-inflammatory, antibacterial, antimicrobial and antifungal properties (Saritha et al. 2002; Wu et al. 2008; Prachayasittikul et al. 2009). Flowers and leaves of the plant have a pungent taste and have been used as a spice for appetizers and as folk medicine for stammering, toothache, stomatitis and throat complaints (Nakatani and Nagashima 1992; Ramsewak et al. 1999). The genus contains a wide array of compounds with a diverse range of bioactivity. One such compound, scopoletin (6-methoxy-7-hydroxycoumarin), has attracted the most attention because of its use in cardiovascular disease, and antitumor and antithyroid treatment. In addition to this,

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scopoletin also possesses antioxidant, antimicrobial, anti-inflammatory, antipyretic and hepatoprotective properties. It is one of the major phytoalexins reported in tobacco plants (Taguchi et al. 2001). Its production is mainly seen upon pathogenic infection and is considered as an important defense mechanism against bacteria and fungi (Smith 1996). Recently, scopoletin has also been detected in *Spilanthes* flower buds (Prachayasittikul et al. 2009), but quantitative analysis has not so far been done.

S. acmella is conventionally propagated through seeds which lose their viability within a short period of time. Dependence on season and slow germination rates are some of the other major limiting factors in conventional propagation (Pati et al. 2006; Dobránszki and da Silva 2010). Moreover, propagation by seeds is also undesirable because of the highly heterozygous nature of the plant due to protandry, which prevents self-pollination (Reddy et al. 2004). Many small, bright-colored flowers are aggregated into a capitulum (flower head) that make them attractive to insects, thus paving the way for entomophily. The genetic variation due to insect pollination may result into high heterogeneity in quality and quantity of the chemical make-up of the plant.

In order to produce high value secondary metabolites such as scopoletin, a constant source of plant material is required which could be utilized as a ready stock to meet the demand of the pharmaceutical industries for the production of bioactive compounds. In this context, in vitro approaches have proved handy in establishing plants that are genetically uniform and enriched in selected characters. It has revolutionized the commercial business through its enormous multiplicative capacity in a relatively short span of time, production of healthy and disease-free plants, and the ability to generate large-scale plant material round the year. Using this technology, a large number of plants could be cloned from a single parent plant on an annual basis. Such a method has considerable implications as it would reduce the pressure on the natural population and, thus, may prevent the plant from becoming endangered. Additionally, it may also help to steer clear of contaminated pharmaceutical raw material.

During the past years, a few efforts were made for in vitro propagation of this important plant through organogenesis (Saritha et al. 2002; Haw and Keng 2003; Saritha and Naidu 2008; Pandey and Agrawal 2009; Singh et al. 2009). However, the conditions for micropropagation were not optimized in either of these studies. Furthermore, in the majority of the studies, organogenesis occurred via adventitious mode which requires de novo differentiation of shoot-buds and, thus, may cause a change in ploidy due to chimera formation.In contrast, clonal propagation by axillary shoot proliferation favors more uniformly diploid plants where the bud present a priori on the initial explant grows into a multinodal shoot. Thus, it promotes a faster rate of multiplication with least genotypic changes.

In the present study, we report (1) an efficient system of in vitro clonal propagation for ex situ conservation of the rare medicinal plant *S. acmella*, and (2) chemical evaluation of the regenerated plants for accumulation of a bioactive compound, scopoletin. A detailed description is provided of in vitro clonal propagation protocol including explant collection, in vitro shoot multiplication, rooting and hardening. Since the major goal of our work is to develop a true-to-type plant that can be utilized as a stock for scopoletin production, a comparative analysis on scopoletin content present in the leaves of micropropagated *Spilanthes* plants in relation to their mother plant is performed here. To the best of our knowledge, this is the first report on this aspect and HPLC analysis of scopoletin in *Spilanthes acmella* Murr.

Materials and methods

Plant material and establishment of aseptic cultures

Nodal segments, bearing two opposite axillary buds, were collected from plants growing in the campus of the Indian Institute of Technology-Guwahati at monthly intervals to establish in vitro cultures. After removing the leaves, nodal segments were thoroughly washed in 1% (v/v) solution of savlon (Johnson-Johnson, India) with two drops of tween-20 for 20 min followed by rinsing with sterile distilled water (SDW). All subsequent operations were carried out inside a laminar air-flow cabinet (Saveer Biotech, India). The cuttings were given a quick rinse (30 s) in 90% ethanol before surface sterilizing with 0.1% (w/v) mercuric chloride solution for 6 min. Each treatment was followed by repeated washing (three washings) in SDW. The cuttings were slightly trimmed at both ends to expose the fresh tissue, leaving behind 1.5–2.0 cm of internodal portions on either side of the node, and planted on different nutrient media.

Axillary shoot proliferation and culture conditions

Sterilized nodal explants with two opposite axillary buds were cultured on MS (Murashige and Skoog 1962) basal medium for bud-break to occur. Initially, basal medium was examined with three carbon sources viz. sucrose, glucose or maltose. Subsequently, modified MS media with major inorganic salts either reduced to half-strength (½ MS) or increased to double-strength (2 MS) were also tested. The medium was variously supplemented with cytokinins, N^6-benzyladenine (BA), N^6-furfurylaminopurine (Kinetin) or 2-isopentenyladenine (2-iP), individually,

at a fixed concentration of 3 μM or BA in combination with an auxin, α-naphthaleneacetic acid (NAA at 1 or 5 μM) or Gibberellic acid (GA_3 at 1 μM) to evaluate their effect on in vitro shoot proliferation.

The pH of the media was adjusted to 5.8 with 0.1 N NaOH or 0.1 N HCL before autoclaving at 1.06 kg cm^{-2} and 121°C for 15 min. Unless mentioned otherwise, all media contained 30 g l^{-1} sucrose and were solidified with 0.8% agar (Hi Media Laboratories, India). The cultures were maintained at 25 ± 2°C temperature with 50–60% relative humidity and a 16/8 h (light/dark) photoperiod provided with diffuse light (1,600 lux). Observations were taken at weekly intervals. Final observation on the number of shoots per axillary bud (on nodal explant), number of nodes per shoot, shoot length and percent axillary-bud proliferation were recorded at week 5 from total aseptic nodal segment cultures. The rate of shoot multiplication was determined as the average number of shoots per axillary buds (on nodal explant) × the average number of axillary buds per in vitro shoots, at the end of a multiplication cycle (growth period).

Rooting and transplantation

For in vitro rooting, terminal 3-cm-long portions of 5-week-old in vitro-developed shoots were excised, given an oblique cut at the base to increase the surface area of absorption for the nutrients from the medium and cultured on MS and ½ MS (major salts reduced to half-strength) basal medium containing 10, 30 and 50 g l^{-1} sucrose. Excised shoots were cultured in 25 × 150 mm culture tube (Borosil, India) containing 20 ml of medium. Four-week-old rooted shoots were washed to remove the agar, transferred to the plastic pots (5 cm) containing a mixture of autoclaved vermiculite, perlite and garden soil (4:1:4) and maintained at standard culture room conditions. The plants were acclimatized by covering the pots with polythene bags to maintain high humidity for 6–7 days and irrigated with major salt solution of MS medium. After 7 days, 3–4 small holes were made in the bag and plantlets were irrigated as before but at frequent intervals. After 25 days, the polythene bags were removed and the acclimatized plants were transferred to a shaded area under natural conditions in the month of February when the temperature range was 20–25°C and photoperiod was 12/12 h (light/dark).

Anatomical studies

Fresh specimens of nodal segments were taken from in vitro grown shoots. The sections, passing through the nodes, were cut manually with the help of Gillette razor blades. After staining with 0.1% acridine orange for 5 min, the sections were observed under Nikon Fluorescence Microscope.

Statistical analysis

For each experiment, 24 replicates were raised and each experiment was repeated thrice. Standard error of the mean was calculated and is represented as bars in the graph. All the investigated parameters were analyzed using analysis of variance (ANOVA) and significance was determined at $P < 0.05$. The data was analyzed statistically using SPSS v.16 software and significant differences among the mean values were assessed on the basis of the least significant difference (LSD). Percentages have been transformed using arcsine transformation before statistical analysis. Student's *t* test was used to analyze scopoletin content.

Preparation of standard solution

Scopoletin standard was procured from Sigma–Aldrich. A stock solution of scopoletin (1,000 μg ml^{-1}) was prepared by dissolving 5 mg of the compound in 5 ml of HPLC grade methanol. The solution was then stored at −20°C. Quantification was carried out using 5 levels of external standards obtained by serial dilutions of stock solutions at a concentration range of 0.110–0.006 μg ml^{-1}. Each concentration of standard was filtered through a 0.20-μm membrane filter before HPLC analysis and run at least thrice to check the repeatability and precision of results.

Preparation of sample solution

To prepare samples, 10 g dried powdered leaf samples from in vivo- and in vitro-grown *Spilanthes acmella* plants were soaked, separately, in methanol for 12 h. The methanolic samples were then centrifuged in a high speed refrigerated centrifuge (Sigma 4K15C, Osterode Am Harz, Germany) at 5,000*g* for 10 min. The supernatant was transferred into a new tube and the residue was re-extracted thrice with 10 ml methanol. Thereafter, the residue was discarded and the supernatant was pooled, filtered and evaporated to dryness in a rotatory evaporator (Buchi Rotavapor R-200, Japan) at 40°C. The methanolic fraction thus obtained was redissolved in HPLC grade methanol, filtered through a 0.20-μm membrane filter prior to analysis and aliquots of 20 μl of clean solution were injected into HPLC system.

Chromatographic conditions

High performance liquid chromatography was conducted using Varian Prostar HPLC system (Varian, USA) consisting of Ultraviolet (UV) and Fluorescence (FL) detector,

a prostar binary pump, a 20-µl injection loop, and Hypersil BDS RP-18 column (Thermo, USA) of dimensions 4.6 × 250 mm. The mobile phase used was 50% A (50% methanol + 50% Acetonitrile + 0.1% phosphoric acid) and 50% B (Milli Q water + 0.1% phosphoric acid) with a flow rate of 0.8 ml min^{-1}. The eluted samples were detected by UV detector at 346 nm. FL detector was used with an excitation wavelength at 385 nm and an emission wavelength at 445 nm. The chromatographic peaks of the analytes were confirmed by comparing their retention time with those of the scopoletin standard and by spiking samples with standard scopoletin.

Linearity of developed method was checked by running the standard compound at five different concentrations, ranging from 0.110 µg ml^{-1} to 0.006 µg ml^{-1}. Calibration curve was constructed by plotting the peak area (y) against concentration in µg ml^{-1} of standard solutions (x). The standard equation obtained from the curve was used for quantification of the scopoletin in the unknown samples. Scopoletin content was reported as, µg g^{-1} DW of the sample.

Precision of developed assay was evaluated by running same concentration of standard compounds at least four times on the same day (intraday) and twice at 1-day intervals (interday). The values were calculated in terms of relative standard deviation (RSD).

HPLC grade methanol, analytical grade methanol, phosphoric acid and acetonitrile, used for analysis, were purchased from Merck, India. Purified water, used for HPLC analysis was obtained from Milli Q system.

Results

Effects of season on culture establishment

The extent of contamination as well as bud-break was highly dependent on the season. The cultures initiated during spring season (January–April) showed best response not only in terms of the frequency of bud-break but also in the vigor of the shoots; significantly ($P < 0.05$) higher aseptic culture establishment (87.4%) and bud-break (64%) was observed during this season with the least contamination rate (12.5%). Around 23% of the aseptic cultures did not respond and turned brown after 2 weeks of inoculation in the medium. Since, summer (May–August) is the period that concurs with the rainy season in the north-east India, 79% of cultures raised in these months were prone to infection (Fig. 1). By winter, the shoots become old and it was difficult to break the dormant state of the buds. Therefore, routinely, the cultures were raised in January–April because of the higher number of aseptic culture establishment and best shoot growth recorded in the season.

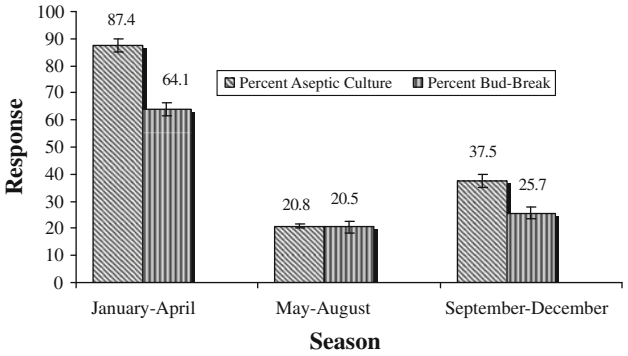

Fig. 1 Effect of season on percent aseptic culture establishment and bud-break response from nodal explants of *Spilanthes acmella* cultured on MS basal medium. Values are mean of three independent experiments. Data were recorded after 5 weeks of culture. LSD at 5% for percent aseptic culture = 5.90, and for percent bud-break = 4.67; *df* 6

Effect of carbon source on shoot proliferation

For shoot induction from axillary buds, three carbon sources, sucrose, glucose and maltose were evaluated at a fixed concentration of 30 g l^{-1}. The incorporation of maltose or glucose to the MS medium was observed to be unsatisfactory. The growth of the shoots, number of nodes per shoot and the rate of shoot multiplication were significantly higher ($P < 0.05$) on sucrose than on the other two carbon sources (Table 1). Consequently, in all subsequent experiments, only sucrose was used as carbon source. On sucrose-containing medium, 64% cultures showed bud-break with 6.4-fold shoot multiplication, every 5 weeks.

Effect of major inorganic salts on shoot proliferation

Shoot proliferation from nodal segments of *S. acmella* varied considerably at different major inorganic salt concentrations. Double-strength of major salts (2MS) inhibited shoot induction completely (Table 2); only rooting was noticed at the base of the explant. Results on shoot induction in half (½MS) and full-strength of major salts (MS) were significantly ($P < 0.05$) different where 60 and 64% cultures, respectively, showed bud-break. MS basal medium also supported multiple shoot proliferation (1.6 shoots/axillary bud) with highest rate of shoot multiplication as 6.4 axillary buds/explant every 5 weeks (Table 2). Therefore, for all subsequent experiments, MS basal medium was selected with full strength of major inorganic salts.

Effect of growth regulators on shoot proliferation

Nodal explants of *S. acmella* bearing two opposite axillary buds were cultured on MS basal medium or basal medium

Table 1 Effect of carbon source on shoot proliferation from nodal segments of *Spilanthes acmella*

Carbon source (3%)	Percent bud-break	Shoot length (cm)	Number of shoots per axillary bud	Number of nodes[a] per shoot	Rate of shoot multiplication[b]
Glucose	41.6	1.1	1.2	1.0	2.4
Maltose	59.3	1.5	1.3	1.0	2.6
Sucrose	64.1	3.5	1.6	2.0	6.4
5% LSD 6 *df*	0.84	0.18	0.18	0.30	0.40

Growth period; 5 weeks; Control: MS medium with 3% sucrose

Values are mean of three independent experiments. Least significant difference (LSD) was used to compare means

df Degrees of freedom

[a] Each node bears 2 opposite axillary buds

[b] Unit for rate of shoot multiplication is number of axillary buds/explant per multiplication cycle

Table 2 Effect of major inorganic salt concentration on shoot proliferation from nodal segments of *Spilanthes acmella*

Strength of medium	Percent bud-break	Shoot length (cm)	Number of shoots per axillary bud	Number of nodes[a] per shoot	Rate of shoot multiplication[b]
Half	60.0	3.5	1.0	1.5	3.0
Full	64.1	3.5	1.6	2.0	6.4
Double	0.0	0.0	0.0	0.0	0.0
5% LSD	1.58	0.26	0.11	0.17	0.64
6 d.f.					

Growth period: 5 weeks; Control: MS medium with full strength of inorganic salts

Values are mean of three independent experiments. Least significant difference (LSD) was used to compare means.

df Degrees of freedom

[a] Each node bears 2 opposite axillary buds

[b] Unit for rate of shoot multiplication is number of axillary buds/explant per multiplication cycle

supplemented with BA, Kinetin or 2-iP at 3 μM concentration. Although on basal medium, the frequency of bud-break was appreciable (64%), and incorporation of BA to the basal medium had further improved the incidence of bud-break and promoted multiple shoot formation on the nodes (2 shoots/axillary bud). While BA favored 100% shoot-bud induction from axillary buds, only 20% response was observed on Kinetin-supplemented medium, and 2-iP was noted as being inhibitory for axillary bud proliferation. The addition of a low concentration of GA_3 (1 μM) to the BA-supplemented medium promoted multiple shoot formation in 70% of the cultures but the shoots remained weak and stunted. On the other hand, a single shoot with long internodes was developed from axillary buds, in 100% cultures, when NAA (1 or 5 μM) was added to BA-containing medium. Irrespective of the treatments, excessive adventitious root proliferation was observed either at the base of the explant or all over the surface of the in vitro-developed shoot.

Since the shoot multiplication rate was higher with the addition of BA, it was further evaluated at a concentration range of (1–15 μM) to promote shoot proliferation with no adventitious roots. The rate of shoot multiplication and length of shoots varied with the concentration of the BA (Fig. 2). At its optimum level (5 μM), 20.3-fold shoot multiplication occurred every 5 weeks with no adventitious root formation. On MS + 5 μM BA, bud-break occurred within a week and an average of 2.9 shoots per axillary bud were formed within 4 weeks in 100% cultures. The shoots grew well and attained a length of 4.9 cm with 3.5 nodes (or 7.0 axillary buds) per shoot, after 5 weeks (Fig. 3a). There was a marked decline in the growth of shoots and the rate of shoot multiplication with the increasing concentration of BA; the node was crowned with several newly formed small shoots difficult to count. BA at 15 μM resulted in stunted shoots with pale leaves (Fig. 3b). Interestingly, lower concentration of BA (1–3 μM) supported multiple shoot formation with long internodes. Moreover, the incidence of excessive adventitious root proliferation was observed from all over the surface of the in vitro-developed shoots and the explants as well, at all lower concentrations of BA (Fig. 3c). The hand sections of the nodal region of the shoots in culture revealed the presence of a ring of lignified xylem cells which fluoresce bright yellow when stained with acridine orange. Multiple (2–3 numbers) adventitious roots (arrow) were originated

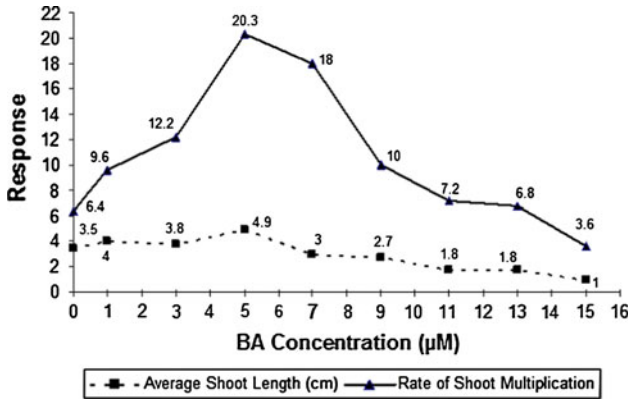

Fig. 2 Effect of various concentrations of BA on shoot proliferation from nodal segments of *Spilanthes acmella* Murr. Growth period: 5 weeks; Control: MS basal medium. Values are mean of three independent experiments. Rate of shoot multiplication is calculated as number of axillary buds/explant per multiplication cycle LSD at 5%, for average shoot length = 0.27, and for rate of shoot multiplication = 1.36; *df* 18

from these lignified tissues (Fig. 3d, e); in Fig. 3d, an axillary bud can also be viewed at one side.

Thus, MS + 5 μM BA proved optimum for shoot multiplication. At the end of the passage, each shoot was cut into single node segments and planted on the fresh medium of the same composition. Each node again produced multinodal, multiple shoots after 5 weeks. Shoot multiplication rate in various subculture (S) passages, $S_1 = 20.2$, $S_2 = 20.3$, $S_3 = 20.4$, $S_4 = 20.6$, $S_5 = 20.6$, $S_6 = 20.5$, $S_7 = 20.5$, $S_8 = 20.5$, $S_9 = 20.6$, $S_{10} = 20.6$, was >20-fold from S_1 to S_{10} on MS + 5 μM BA. Since every time the explants were taken from freshly formed in vitro shoots, we have therefore not observed any significant ($P < 0.05$) difference (variation) in the results.

Rooting and transplantation

Terminal 3-cm-long portions of shoots from 5-week-old cultures on MS + 5 μM BA were used for rooting. The remaining portions of the shoots were cut into single node segments and utilized for further multiplication. MS basal medium was tested at full- and half (½ MS)-strengths of the major inorganic salts. All the media were supplemented with 0, 10, 30, and 50 g l^{-1} sucrose. Although MS medium supported direct rooting at the base of shoot, it did not promote the growth of the shoot at all tested concentrations of sucrose. Half MS was distinctly better than full MS basal medium, in terms of length of the shoot, percent rooting, number of roots per shoot and number of laterals present on the roots. The rooting was positively correlated with the sucrose concentration in the medium. There were significant increases ($P < 0.05$) in the percent rooting and the number of roots per shoot with increase in sucrose concentration (Table 3). On ½ MS + 50 g l^{-1} sucrose, which proved to be the best rooting medium, 100% shoots formed more than 35 roots directly from the basal cut end of the shoots (Fig. 3f). On this medium, roots appeared after 2 weeks and maximum response was observed after 4 weeks. Some of the roots had developed laterals.

Following the protocol described under materials and methods, rooted shoots from ½ MS + 50 g l^{-1} sucrose were transferred out of culture. The plantlets were acclimatized successfully with 88.9% survival rate (Fig. 3g). During in vitro hardening, shoots elongated, leaves turned greener, and their lamina expanded. Consequently, the plants seemed much healthier and grew more vigorously after in vitro hardening.

Standard curve analysis

The calibration curve for scopoletin showed good linearity with high reproducibility and accuracy at all five tested concentrations (0.110–0.006 μg ml^{-1}). Regression analysis of the calibration curve data points showed excellent correlation coefficient (R^2) of 0.9792. The linear regression equation for standard scopoletin was: $y = 2.0964\ x + 0.0153$, where x was the concentration of standard scopoletin (μg ml^{-1}) and y was the total peak area. The equation thus generated from the curve by external standard method was used to calculate the amount of compound present in crude samples. Presence of compound was reconfirmed with the use of internal standard by co-injecting it with the crude extract. A very distinct and clear separation of compound can be seen (Fig. 4a–d).

The precision of the developed method, as mentioned in "Chromatographic conditions" in "Materials and methods", was evaluated by measuring intra- and inter-day variability in terms of relative standard deviation. The standard samples, at the same concentrations, were analyzed at least four times within the same day and the RSD value obtained was 0.7%. Similarly, for inter-day variability, the same concentrations of the three standards were run at least twice at 1-day intervals and the values for these calculated to be 1.3%. Under the HPLC conditions described above, standard scopoletin was separated within 10 min.

Quantification of the scopoletin

By following the protocol as described in "Materials and methods", the methanol extract was analyzed quantitatively by HPLC for the presence of scopoletin. Samples were monitored by UV and FL detectors connected successively. Scopoletin could be detected by both UV and FL detectors, but the sensitivity of scopoletin by FL

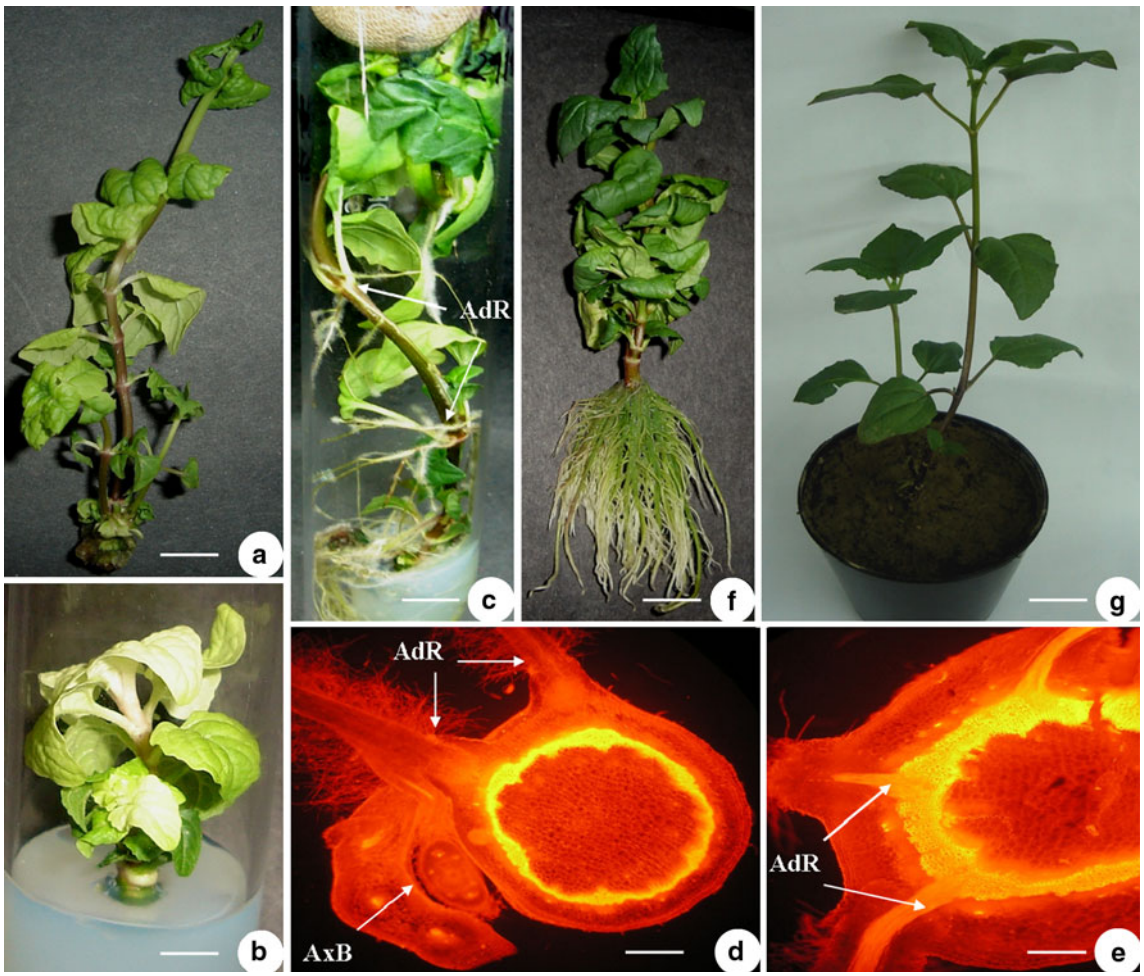

Fig. 3 In vitro clonal propagation of *Spilanthes acmella* by axillary shoots proliferation. **a** A nodal segment on MS + 5 μM BA, showing long, multinodal shoots, after 5 weeks of culture initiation (*bar* 1.37 cm). **b** A nodal segment on MS + 15 μM BA, after 8 weeks of culture initiation, showing stunted shoots with pale leaves (*bar* 0.73 cm). **c** A nodal segment on MS + 3 μM BA, after 8 weeks of culture initiation, showing a shoot with long internodes and with adventitious roots all over its surface (*bar* 1.1 cm). **d** An anatomical section of nodal segment from c, stained with acridine orange, showing differentiation of adventitious roots (*AdR*) and position of axillary bud (*AxB*) (*bar* = 0.5 mm). **e** Same as d, showing origin of adventitious root differentiation (*AdR*) at different points (*bar* 0.5 mm). **f** A shoot from (**a**), rooted on ½ MS + 50 g l^{-1} sucrose. Roots have developed directly from the basal cut end (*bar* 1.27 cm). **g** A hardened micropropagated plant, 1 month after transfer to soil (*bar* 2.73 cm)

Table 3 Effect of different sucrose concentrations on in vitro rooting of the excised shoots cultured on ½ MS basal medium

Sucrose (g l^{-1})	Percent response	Number of roots per shoot
0	20.9	3.1
10	76.3	14.1
30	88.9	23.3
50	100	35.1
5% LSD 8 *df*	2.11	1.59

Growth period: 4 weeks

Values are mean of three independent experiments. Least significant difference (LSD) was used to compare means.

df Degrees of freedom

detector was much higher than that of UV detector. Thus, in all samples, scopoletin was examined by HPLC-FL detector by comparing its retention time with that of an authentic standard. The single peak of scopoletin standard was obtained at the retention time of 5.1 ± 0.4 min (Fig. 4a, arrow). When methanolic extracts of leaves, obtained from in vitro and field grown plants, were analysed, separately, they showed peak (Fig. 4b, c, arrow) at the retention time corresponding to that of the standard scopoletin. Presence of scopoletin was further confirmed by spiking (addition) of in vitro leaf extract with that of the standard (Fig. 4d, arrow). The standard equation generated through the calibration curve demonstrated that scopoletin production from in vitro regenerated shoots was

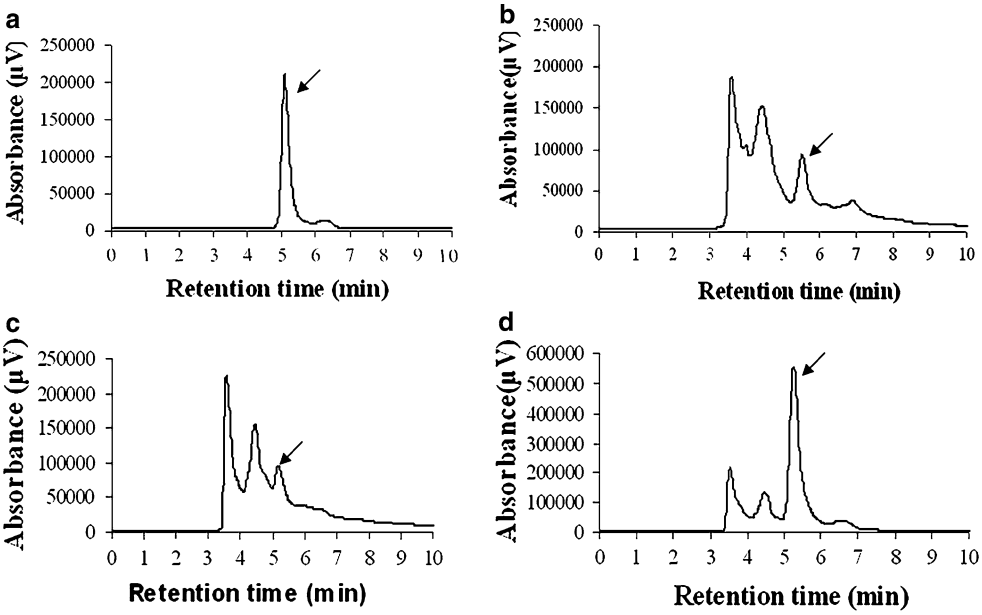

Fig. 4 Chromatograms of scopoletin analysis **a** HPLC chromatogram of standard scopoletin showing single peak of scopoletin (*arrow*) **b** HPLC chromatogram showing presence of scopoletin (*arrow*) in in vitro leaf extract of *Spilanthes* **c** HPLC chromatogram showing presence of scopoletin (*arrow*) in vivo leaf extract of *Spilanthes* **d** HPLC chromatogram of in vitro leaf extract, spiked with standard scopoletin. Sample scopoletin and standard scopoletin co-eluted at the same retention time (*arrow*)

comparable to those from field-grown mother plants. Leaves of in vitro-grown plants accumulated 0.104 ± 0.03 µg g^{-1} DW of scopoletin while those of field-grown plants contained 0.101 ± 0.04 µg g^{-1} DW of scopoletin. Statistical analysis confirmed that these values are not significantly different ($P < 0.05$).

Discussion

The most important technique in micropropagation is meristem proliferation where the nodal segments bearing pre-existing axillary buds are cultured to regenerate multiple shoots without any intervening callus phase. This would relinquish dependency on seeds whose availability is always doubtful. A few reports are available on micropropagation of *S. acmella*. A majority of these reports (Saritha et al. 2002; Haw and Keng 2003; Saritha and Naidu 2008; Pandey and Agrawal 2009; Singh et al. 2009) deal with in vitro plant regeneration by adventitious shoot proliferation from seedling explants, leaf or hypocotyls or thin cell layer sections, which may run the risk of change in ploidy due to de novo differentiation of shoot-buds resulting in chimera formation. Later, Haw and Keng (2003) attempted clonal propagation by axillary shoot proliferation but the study lacked crucial information on percent culture response, the rate of proliferation in recurrent cycles of shoot multiplication, frequency of rooting, etc., and transplantation was not attempted. The present study gives a systematic report on a highly reproducible and recurrent method of clonal propagation whereby detailed description on in vitro shoot multiplication, rooting and hardening are described. Using this method, 20-fold shoot multiplication every 5 weeks was achieved strictly through axillary-bud proliferation. Rooting and transplantation survivals achieved in the present work are 100 and 88.9%, respectively.

Contamination of *S. acmella* nodal explants was a major problem during initiation of cultures under in vitro conditions. The extent of contamination was strongly influenced by the season during which the material was collected. The cultures initiated during January–April showed significantly ($P < 0.05$) higher (64%) bud-break than those raised in other times of year. The reason for the difference in response may be due to high meristematic activity and fresh shoot growth in spring season from January to April.Later in the year, shoots became old and, thus, make it difficult to break the dormant state of the buds. Similar results of seasonal effects on culture establishment have been reported in mulberry (Chitra and Padmaja 2002), neem (Chaturvedi et al. 2004), litchi (Kumar et al. 2006) and lotus (Shou et al. 2008). The thickness and maturity of the explant with increasing distance from the apex of the shoot not only determine their survival in culture but also contribute to their effective performance in respect of proliferation (Arora et al. 2010). This could be the reason for a significant difference (23%) between percent aseptic cultures (87.4%) and percent bud-break (64.1%). Arora et al. (2010) noticed a gradual increase in percent explants showing bud-break with the increasing distance (maturity) of node up to a certain level from apex of the shoot. Hsia and Korban (1996) concluded that nodal stem segments having larger diameters and a better nutrient translocation from the donor mother plant, hence developed larger buds, which sprouted sooner when placed in vitro in case of *Rosa* sp.

Several species require a moderate salt concentration for their initial survival in cultures (Bhojwani and Razdan 1996; Chaturvedi et al. 2004). Davies (1980) worked on the effect of different nutrient salt concentrations on multiplication of various cultivars of rose and found that unchanged MS salts proved to be the best. Bressan et al. (1982) noticed a negative influence of low salt concentration on vigor and viability of shoots. These are in agreement with the present report where normal strength of major salts proved significantly ($P < 0.05$) better than half- and double-strength of major salts for establishment and multiplication of shoots in nodal segment cultures.

In cultured plant tissues, a continuous supply of carbohydrate from the medium is essential, as they are needed for growth and organized development of the plant and are necessary as a source of energy and carbon skeletons for biosynthetic process. In the present study, sucrose (3%) supported the significantly ($P < 0.05$) highest rate of shoot multiplication than glucose or maltose in nodal segment cultures. Sucrose is the most commonly used carbohydrate for plant tissue cultures, and most culture media have it as the sole carbohydrate source. One explanation for the favorable effect of sucrose is that it is easily recognized and hydrolyzed by cell wall-bound invertase into more efficiently utilizable forms of sugars, glucose and fructose which are incorporated into the cells. Moreover, glucose derived from sucrose hydrolysis is more accessible to the cultured tissues than glucose derived by maltose hydrolysis, due to a rapid sucrose hydrolysis but a slow maltose hydrolysis in the media (Ślesak et al. 2004). The starvation effect of maltose due to slow hydrolysis and/or a slow uptake may lead to a drop in available carbohydrates within the cell, which may create a signal to reorient the developmental programmes (Blanc et al. 2002; Krogstrup et al. 2005). The positive effect of sucrose may be related to its increasing the osmotic pressure of the medium, which stimulates mitochondrial activity and, hence, production of energy required for shoot initiation (Bonga and Von Aderkas 1992). Furthermore, in the present study, glucose by itself was not promotory for bud-break and shoot length, whereas combined presence of glucose and fructose, derived through sucrose hydrolysis, was noted as being essential for the highest percentage of bud-break and shoot length. In the combined presence of glucose and fructose, cells exhibit a preference for glucose whereas fructose is utilized as a substrate only after glucose is depleted from the medium (Kretzschmar et al. 2007). Thus, the presence of sucrose felicitated the constant availability of utilizable forms of carbohydrates in the medium, throughout the culture period. Consequently, initial high flux of glucose might have promoted bud-break and shoot development, whereas fructose enhanced shoot length later.

In vitro shoot proliferation and multiplication are largely based on media formulation containing cytokinins as a major plant growth regulator. In the present study, the maximum promotive effect on percent bud-break and rate of shoot multiplication were observed with BA as compared to 2-iP or Kinetin. The promotive role of BA on shoot proliferation has been previously documented in *Schinopsis balansae* (Sansberro et al. 2003), *Holarrhena antidysenterica* (Kumar et al. 2005), *Eclipta alba (*Ray and Bhattacharya 2008), *Nelumbo nucifera* (Shou et al. 2008), and *Centaurea ultreiae* (Mallón et al. 2010). In the present work, 6.4-fold shoot multiplication was obtained in 64% cultures on hormone-free medium. Addition of BA has further enhanced the percent culture response (100%) with the highest rate of shoot multiplication (>20 fold) at its optimum concentration of 5 μM. While the effect of Kinetin on bud-break was significantly lower ($P < 0.05$), 2-iP proved inhibitory. Differences in the activity of cytokinins can be explained by their various translocation rates to meristematic regions and metabolic processes, in which the cytokinins may be degraded and conjugated with physiologically inert compounds, like sugars or amino acids (Kaminek 1992).

The lower concentrations of BA (1–3 μM) in the present study resulted in shoots with long internodes and with excessive adventitious root proliferation. However, when exposed to a high concentration of BA (7–15 μM), the shoots formed were short and stunted. These changes in morphogenic responses could be attributed to the differences in BA uptake and metabolism that lead to the formation of other compounds with different degrees of hormonal activity.

The hand sections of the nodal region of the shoots, grown at lower concentrations of BA (1–3 μM), revealed the presence of a band of vascular bundles with associated 2–3 numbers of adventitious roots. The sections were when stained with acridine orange (a cationic dye) confirmed the occurrence of a ring of lignified xylem tissues which fluoresce bright yellow. Although acridine orange has pronounced affinity to nucleic acid; being a cationic dye, it has strong affinity to acid materials as well as lignin (Drnovšek and Perdih 2005; Li and Reeve 2005). It can also accumulate in vacuoles, the acidic compartments of cells, by ion trap mechanism, and gives orange fluorescence (Oparka 1991). Acridine orange is a metachromatic fluorescent dye and the amount of metachromasia or spectral shifts depend on the concentration of dye, ratio of binding sites to dye molecules, pH, staining duration and chemical and physiological properties of plant samples (Bertalanffy 1963; Robbins and Marcus 1963). Deoxyribonucleic acid (DNA) intercalate acridine orange and fluoresces green while ribonucleic acid (RNA) electrostatically bind to it and fluoresces orange red due to

spectral shift (Myc et al. 1992). RNA being less polymerized offers more sites for binding acridine orange and hence accumulates a higher concentration, but DNA also fluoresces red if depolymerized. Acridine orange can also be utilized to distinguish between quiescent and activated proliferating cells due to quantitative differences in RNA content (Bertalanffy 1963). In the present study, the majority of the cells in the section took on orange colour which may be due to vacuolar accumulation of acridine orange. The lignified xylem cells fluoresced bright yellow due to weak dye–dye coupling of neighboring dye molecules. Moreover, the differential staining of cambium cells due to higher RNA content cannot be ruled out.

Terminal 3-cm-long portions of shoots from 5-week-old cultures on MS + 5 μM BA were used for rooting. On ½ MS + 50 g l^{-1} sucrose, 100% shoots formed an average of 35 roots directly from the basal cut end of the shoot. Similar to this study, Serres et al. (1990) observed that the percentage of rooting and number of roots per shoot is positively correlated with sucrose concentrations in chestnut microcuttings. High sucrose concentration increases the osmotic pressure of the medium, which in turn stimulates the mitochondria to generate more energy to facilitate rooting (Bonga and Von Aderkas 1992). Almost 88.9% plants survived transplantation.

Phytoalexins are common secondary metabolites, found in many plant species, whose production is seen upon infection and are considered as an important defense mechanism. Among them, scopoletin, 6-methoxy-7-hydroxy-coumarin, is one of the major phytoalexins. Scopoletin production has been induced in several plant species upon infection by different pathogens (Matros and Mock 2004; Kai et al. 2006; Lerat et al. 2009). This is the first report on quantification of scopoletin from the leaves of in vitro and field-grown plants of *S. acmella.* The study revealed that even the uninfected leaves of *Spilanthes* could accumulate the scopoletin.

A novel HPLC method with a fluorescence detector was developed for the quantitative estimation of scopoletin in *S. acmella* Murr., which can detect even a low concentration of scopoletin and could be easily implemented in routine practice. Interestingly, the scopoletin content of micropropagated plants was comparable to the mother plant (0.01 μg g^{-1} DW of leaves). The advantage of clonal propagation in horticulture and forestry is well known for producing 'true-to-type' plants. However, to the best of our knowledge, this is the first report comparing the scopoletin content in the tissue-cultured plants of *Spilanthes* produced from plants with known scopoletin content. These findings open up the possibility of producing *Spilanthes* plants with the desired metabolite content which will help the pharmaceutical industry to achieve better output by using superior quality raw materials.

Conclusions

The present study provides an efficient system of in vitro clonal propagation of *S. acmella*. The protocol, thus developed, could be utilized to raise disease-free plant material. Using this method, it is estimated that over 2×10^{10} plants can be produced year round without any seasonal constraints. Such a rate of shoot multiplication could not be achieved by any traditional method of vegetative propagation. These in vitro plantlets could serve not only as a constant source of uniform plantlets for mass propagation but also as a ready stock to meet the demand of the pharmaceutical industry for the production of bioactive compounds.

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