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ORIGINAL PAPER

Evaluation of nutrient uptake and physical parameters on cell biomass growth and production of spilanthol in suspension cultures of *Spilanthes acmella* Murr.

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Abstract *Spilanthes acmella* Murr. has a plethora of highly valuable biologically active compounds and has been listed as one of the important medicinal plants of the world. However, no perceptible biotechnological advances have been made for this genus to exploit or enhance its utility. To nullify the effect of seasonal variations, the present report is the first attempt to establish in vitro cell suspension cultures and to evaluate the production of spilanthol from them, a key component of the plant responsible for most of its pharmaceutical activities. The study examined the biomass growth in relation to the consumption of major nutrients and sucrose, agitation speed and dynamic change in pH. Results indicated that the consumption of phosphate resulted in the onset of decline phase in cultures. Spilanthol production was observed to be growth associated and maximum production occurred on the 15th day. Among the carbon sources, the highest production of spilanthol as 91.4 μg g^{-1} DW was recorded in the medium supplemented with sucrose, followed by glucose which produced 56.8 μg g^{-1} DW of spilanthol. Spilanthol could not be detected in fructose containing medium. Maximum viable cultures were obtained at a rotation speed of cells at 120 rpm. This study signifies the utility of *Spilanthes* suspension cultures for biosynthesis and constant production of spilanthol, throughout the year. The results of present study are useful for further scale-up process.

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Keywords Agitation speed · Batch kinetics · Cell viability · Spilanthol · *Spilanthes* · Suspension cultures

Abbreviations

BAP	N^6-Benzylaminopurine
2,4-D	2,4-Dichlorophenoxyacetic acid
IAA	Indole-3-acetic acid
Kinetin	N^6-furfuryladenine
NAA	α-Naphthaleneacetic acid
pCPA	para-Chlorophenoxyacetic acid
TDZ	Thidiazuron

Introduction

Plants are able to synthesize an extensive array of secondary metabolites, which are used by man since antiquity for a number of applications, such as pharmaceuticals, biopesticides, flavours, fragrance, colours and food additives. Currently, the bulk of these industrial products are collected from plants growing in the wild or from field-cultivated sources. The utilization of massive quantities of whole plant parts raised a major concern that it can diminish local plant populations and erode genetic diversity. Moreover, the plants growing in wild undergo various climatic and environmental fluxes that lead to change in their chemical profile. Plant cell cultures have been viewed as promising alternatives to whole plant extraction for obtaining valuable chemicals, irrespective of seasons and regions, with facilitated yield and consistent production. Particularly, the cell suspension cultures offer a condensed biosynthetic cycle to study growth and production kinetics within a short cultivation time (about 2–4 weeks) with an

added advantage of tunability that can help to implement optimal conditions for the production of a number of high value medicinal compounds in good quantities [1].

Spilanthes acmella Murr. or "Akarkara", of the family Asteraceae, is used in folk medicines since ancient times to cure severe toothache, infections of throat and gums, stomatitis, paralysis of tongue and psoriasis [2]. It is well documented for its antiseptic, analgesic, antioxidant, antimalarial, immune-modulatory and diuretic properties [3–9]. Phytochemical studies revealed that the genus contains bioactive alkylamides, phenolics, coumarin and triterpenoids which account for its use in traditional medicines all over the world, since long [8]. Of these, the most active principle is an antiseptic alkylamide, (2E, 6Z, 8E)-deca-2,6,8-trienoic acid *N*-isobutyl amide, commonly known as spilanthol [10]. Spilanthol has immense applications in pharmaceuticals, food, health and body care products.

In spite of being a plant of potential medicinal interest, till date, no reports are available on the dynamics of biosynthetic capacity of *S. acmella* cells in suspension cultures. Therefore, it seemed appropriate to undertake the studies on this aspect. The present investigation is focussed on the establishment of cell suspension cultures from superior cell lines and development of optimal operating conditions in shake flask suspension cultures for biomass growth and spilanthol production. Dynamic changes of parameters, such as pH, wet and dry cell concentrations, consumption of major nutrients, carbon source and agitation speeds, were investigated to understand the culture characteristics of suspended cells and their ultimate effect on spilanthol production.

Materials and methods

Plant material and establishment of cell suspension culture

Healthy leaves of *Spilanthes* were collected from the campus of Indian Institute of Technology, Guwahati. Leaves were washed with 1% (v/v) Savlon® (Johnson and Johnson, India) for 20 min, followed by three rinses in SDW. The remaining steps were carried out inside a laminar-air-flow cabinet (Saveer Biotech, India). The leaves were surface-sterilized with 0.1% mercuric chloride solution ($HgCl_2$) for 10 min, followed by three rinses with SDW. Leaf-disc explants of 5 mm size were prepared by punching the leaves with a cork-borer prior to implantation on Murashige and Skoog (MS) [11] medium containing 3% sucrose and 0.8% agar (HiMedia Laboratories, India).

MS basal medium supplemented with varying combinations and concentrations of auxins and cytokinins, including 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), para-chlorophenoxyacetic acid (pCPA), N^6-benzylaminopurine (BAP), thidiazuron (TDZ) and N^6-furfuryladenine (Kinetin) was used to induce callusing from the explants.

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. 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 (1,000–2,000 lux). After callus induction, the cell biomass was multiplied constantly by inoculating 0.1 g of calli onto fresh medium of parental composition at every 5-week intervals.

Suspension cultures were initiated in Erlenmeyer flasks of 250 mL capacity, containing 50 mL of liquid medium and inoculated with 0.1 g of fresh calli. The cultures were incubated in an orbital shaker under shaking conditions at 25 ± 2 °C in dark. The cell biomass was subcultured on fresh liquid medium, regularly, at every 15th day.

Determination of cell growth, residual nitrate, phosphate and sucrose

To establish the kinetics of cell growth and nutrient uptake, cells were harvested from liquid medium at an interval of 3 days, washed with distilled water and filtered under vacuum. Subsequently, its fresh weight was taken on high-precision analytical balance (Sartorius, India). Thereafter, the cells were dried in oven at 30 ± 2 °C until constant weight was achieved. The drying temperature was kept low to prevent decomposition of thermolabile compounds. The pH and residual nutrients (phosphate, nitrate and sugar) of the suspension cultures were monitored at every third day. Phosphate was estimated by the standard calibration curve made from dihydrogen sodium phosphate (NaH_2PO_4); to 0.5 mL of standard or sample solution, 4 mL of reagent [acetone (CH_3COCH_3), sulphuric acid (H_2SO_4) 2.5 M and ammonium molybdate tetrahydrate ($(NH_4)_2MoO_4{\cdot}4H_2O$) 10 mM, mixed in the ratio of 2:1:1] were added. After thoroughly mixing the solutions, 0.4 mL of 1 M citric acid was added and absorbance was taken at 355 nm. Similarly, for nitrate estimation, standard curve was made from 0.01 N stock solution of potassium nitrate (KNO_3), preserved in chloroform. After acidification of samples with hydrochloric acid, absorbance was recorded at 275 nm in a UV–visible spectrophotometer (Cary 100, Netherlands). For sucrose estimation, anthrone reagent was prepared as described by Trevelyan and Harrison [12] by dissolving 0.2 g of anthrone in 100 mL of H_2SO_4. The reagent was allowed to stand for 30–40 min, and then to 1 mL of standard or sample solution, 5 mL of anthrone reagent was added. After incubating the reaction mixture in boiling water bath for 5 min, absorbance was taken at 620 nm. Standard curve was made by glucose.

Carbon source

Three carbon sources, glucose, fructose and sucrose were tested at 3% concentration to understand the growth and production profile of cells in suspension cultures. The *Spilanthes* cells were inoculated such that each 250 mL Erlenmeyer flasks containing 50 mL of medium had 0.1 g of the cells. The cell biomass was harvested at the end of the growing phase of 15 days to analyse dry cell weight and metabolite content.

Agitation speed

Effect of agitation speeds was evaluated on fresh and dry weight of cells and their viability, at the end of each passage. Callus cells weighing approximately 0.1 g were harvested at the end of growth period and re-inoculated in 50 mL of fresh medium of the same composition. The cultures were incubated in shaking conditions at 60, 90, 120, 150 and 180 rpm, under darkness, for a period of 24 days and their fresh and dry weights were recorded. The viability of cells under each condition was checked with 1% FDA solution.

Preparation of plant extracts

To prepare samples, dried powdered cell mass was soaked in methanol (analytical grade, Merck, India) for 12 h. The methanolic samples were then centrifuged in a high-speed refrigerated centrifuge (Sigma 4K15C, Osterode Am Harz, Germany) at 5,000 rpm for 10 min. The supernatant was transferred into 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 rotary evaporator (Buchi Rotavapor R-200, Japan) at 40 °C. The methanolic fraction, thus obtained, was redissolved in HPLC grade methanol, filtered through a 0.22 μm nylon membrane filter prior to analysis and aliquots of 20 μL of clean solution were injected into HPLC system.

Estimation of spilanthol

Quantitative estimation of spilanthol was carried out on Varian Prostar HPLC system (Varian, USA) consisting of Ultraviolet (UV) detector, a prostar binary pump and a 20-μL injection loop. Hypersil BDS RP-18 column (Thermo, USA) of dimensions 4.6 × 250 mm was used with acetonitrile:water (93:7) as mobile phase at a flow rate of 0.5 mL min^{-1}. The eluted samples were detected at 237 nm. Spilanthol peaks obtained in HPLC were identified by electrospray ionization mass spectra and by comparing with published data. As there was no commercially available standard for spilanthol, it was tentatively quantified on the basis of another compound, dodeca-2(E), 4(E)-dienoic acid isobutylamide (Chromadex, USA), which is structurally similar to spilanthol. Both the compounds have isobutylamide group and long carbon chain.

Stock solutions (1,000 μg mL^{-1}) of dodeca-2(E), 4(E)-dienoic acid isobutylamide, 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 five levels of external standards obtained by serial dilutions of stock solutions at a concentration range of 250–15 μg mL^{-1}. Each concentration of standard was filtered through a 0.22-μm nylon membrane filter (Millipore, USA) before HPLC analysis. Method linearity was demonstrated by determining a calibration curve, injecting standard at different concentrations and calculating the regression coefficient (r^2). Slope equation obtained was used to calculate the amount of the spilanthol in unknown samples. Spilanthol content was reported as μg g^{-1} DW of sample.

Mass spectroscopy

MS detection of spilanthol was carried out on Water quadrapole Tof Premier mass spectrometry with micro channel plate detector (Waters, USA). The analysis was done with an ESI probe source in positive mode and with collision energy of 3 V. The cell entrance and exit voltage were set at 2 and −10 V, respectively. For spilanthol identification, all peaks that appeared in HPLC were collected, concentrated and than redissolved in methanol prior to analysis. Confirmation of the spilanthol peak was done by comparing the mass spectra of samples and that reported in published literature.

Statistical analysis

For batch kinetic studies, all experiments were done in triplicate to check the reproducibility of the results. The investigated parameters were analysed using analysis of variance (ANOVA) and significance determined at $P < 0.05$. The data were analysed statistically using SPSS (version 16) software and significant differences among the mean values were assessed on the basis of the Duncan's multiple range test. Results are represented as mean ± standard deviation. The specific growth rate (μ) was calculated by

$$\mu = \ln (MT_2 - MT_1)/T_2 - T_1; T_2 > T_1$$

where, MT_2 and MT_1 are biomasses at the different time points (T_1 and T_2), respectively.

Results and discussion

Establishment of cell lines

Leaf disc explants of 5 mm size were cultured on a range of media to induce callus. Of all the various growth regulator treatments, MS medium supplemented with BAP (5.0 μM), 2,4-D (1.0 μM) and NAA (1.0 μM) induced maximum callus formation. On this medium, after 2 weeks, friable, light brown calli began to develop from leaf-disc explants in 100% cultures. Although callus proliferation increased with subsequent subcultures but remained brown in colour throughout two passages at 5-week intervals, by the 11th week, vigorously growing light-green, fresh and friable callus was obtained, which remained unorganized (dedifferentiated). The calli were multiplied and maintained on the fresh medium of same parental composition at every 5-week interval.

After 4–5 passages of subcultures, healthy, friable and soft calli, maintained on responding semi-solid medium, were utilized to establish suspension cultures. The same growth regulator combination, MS + BAP (5.0 μM) + 2,4-D (1.0 μM) + NAA (1.0 μM), which was used for callus culture, worked for cell suspension cultures as well. However, the cell growth was faster in liquid medium than in semi-solid medium. It took only 18 days for the cell cultures to complete the growth cycle in comparison with calli on semisolid medium which needed at least 5 weeks to attain maximum growth. This may be because of facilitated nutrient uptake in liquid medium [13].

Kinetics of cell growth and spilanthol production

The kinetic profiles of cell growth are represented by fresh and dry cell weight, and medium pH in *Spilanthes* suspension cultures are presented in Fig. 1. It was observed that *Spilanthes* suspension culture moved onto the exponential growth phase after 6 days of lag phase. The biomass increased till 18th day following which the death phase started (Fig. 1), which may be due to consumption of the nutrients and lack of oxygen in the medium. During death phase fresh and dry cell weight decreased dramatically, but the pH of the medium continued to increase. There is an increase in the pH from 6th day till 21st day and after which the medium pH was stable till the last day of cultivation (24th day). The increase in the pH may be attributed to the release of intracellular substances into the medium. Under the experimental conditions, with the formula mentioned in materials and methods, the maximum specific growth rate (μ) was 0.279 day^{-1} during the exponential phase.

The presence of spilanthol was observed in our initial studies with callus cultures (data not shown). Extension of the same protocol on extraction and HPLC analysis was performed with the cell biomass obtained from suspension cultures. Since spilanthol standard was commercially not available, HPLC and then mass spectroscopic analysis were performed for the identification of spilanthol in samples. Acetonitrile and water at the ratio of 93:7 as the mobile phase was found to be appropriate for satisfactory separation of compounds at a flow rate of 0.5 mL min^{-1}. For spilanthol characterization, all peaks eluted from HPLC were collected, concentrated and analysed by mass spectrometry. Samples were analysed in both positive and negative electrospray ionization mode, but the sensitivity and reproducibility of the dominant ions in the positive electrospray ionization mode were better than in negative ionization mode. Therefore, all HPLC peaks were analysed in positive mode. Spilanthol was identified by its fragmentation profile which was further confirmed with literature data. It has been observed that the peak eluted at 7.34 ± 0.12 min in HPLC has characteristic fragmentation pattern of spilanthol. Figure 2 shows the mass spectrum of spilanthol compound. The spectrum has a base peak at *m*/*z* 222 corresponding to the protonated $[M + H]^+$ molecular ion. The ion at *m*/*z* 244 was generated due to sodium ion adduct formation $[M + Na]^+$. The characteristic fragment at *m*/*z* 149 was formed due to the dissociation of the C–N bond. It is an acyllium ion fragment, which indicates the amount of carbon atoms in the alkyl chain. This fragment is formed by the loss of isobutyl amine group $[MH\text{-}C_4H_{11}N]^+$. The mechanism involved in the acyllium ion formation is charge-remote homolytic cleavage that yields a resonant distonic radical cation, which subsequently undergoes hydrogen rearrangement. Another fragment seen at the *m*/*z* 123 can be attributed to the $[MH\text{-}C_5H_9NO]^+$. The loss of a fragment with specific *m*/*z* 99 from the protonated $[M + H]^+$ molecular ion (*m*/*z* 222) confirmed that spilanthol contains isobutylamide group. The samples analysed, each having a peak at approximately 7.34 ± 0.12 min, were all found to contain the fragments diagnostic of spilanthol. In samples, spilanthol was tentatively quantified on the basis of another alkylamide, dodeca-2(E), 4(E)-dienoic acid isobutylamide. Like spilanthol, this compound also has isobutyl amide group and long carbon chain. In mass spectra, this amide forms acyllium ion at the *m*/*z* 179 (Table 1).

For spilanthol estimation, the cells harvested at every 3rd day from liquid suspension cultures revealed that the spilanthol production started at the end of the lag phase (6th day). During exponential phase spilanthol content increased and was found to be growth associated and showed an increase with the increase in biomass until 15th day. Thereafter, spilanthol production declined rapidly and dramatically due to nutrient depletion and cell death. The same set of experiment was repeated three times and, every time, the similar trend on spilanthol production was observed. Extracellular spilanthol, in the medium, was not

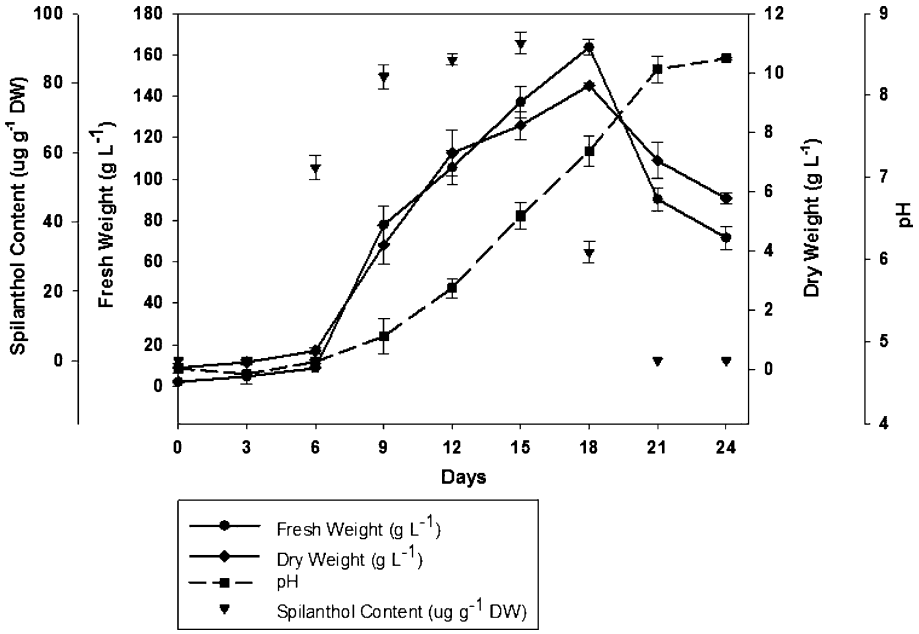

Fig. 1 Kinetics of cell growth and spilanthol production in cell suspension cultures of *Spilanthes acmella* Murr.

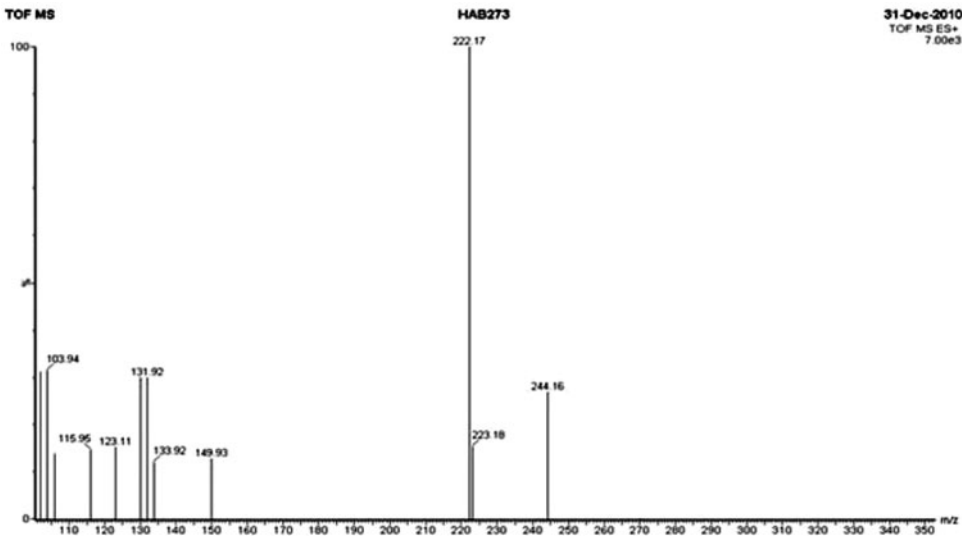

Fig. 2 Positive electrospray ionization mass spectra of spilanthol, purified from HPLC fraction of crude extract obtained from cell biomass

Table 1 Fragments generated in MS analysis of spilanthol and dodeca-2(E), 4(E)-dienoic acid isobutylamide

S. No.	Compound	MH^+	Fragments	References
1	Spilanthol	222	166, 149[a], 123, 121, 81	Boonen et al. [14]
2	Dodeca-2(E), 4(E)-dienoic acid isobutylamide	252	196, 179[a], 161, 119, 95	Cech et al. [15]

[a] Acyllium fragment formed by dissociation of the C–N bond to lose the entire amine functional group ($MH^+ - 73$)

detected at any stage during the course of cultivation and kinetic studies. This suggests that spilanthol was synthesized only by viable cells and degraded rapidly upon cell death and lysis. Similar to the present study, growth-associated synthesis of secondary metabolites has been observed in case of *Lantana camara* [16] and *Panax quinquefolium* [17].

The kinetics of spilanthol production was based on the Luedeking–Piret model [18]. According to this model, the product formation rate (r_P) depends on both the instantaneous biomass concentration and the growth rate in a linear manner which is given by following equation:

$$r_p = dP/dt = \alpha\, dx/dt + \beta x$$

where α and β are the product formation constants. In the present case, due to absence of stationary phase, βx term is zero and spilanthol production rate (r_p) is proportional to

the growth rate. In this case, the rate of spilanthol formation can be given by the following equation:

$$r_p = dP/dt = \alpha\, dx/dt$$

where α is a constant term. In suspension culture, major spilanthol production occurs in exponential phase which represents growth-associated product formation as deduced from Fig. 1.

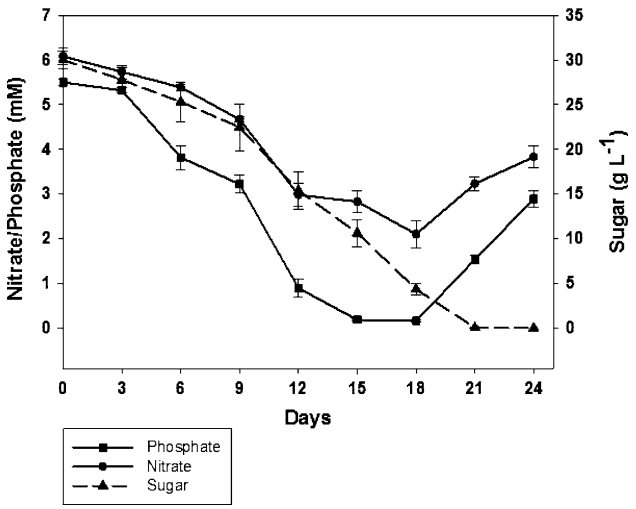

Fig. 3 Kinetics of nutrient uptake in cell suspension cultures of *Spilanthes acmella* Murr.

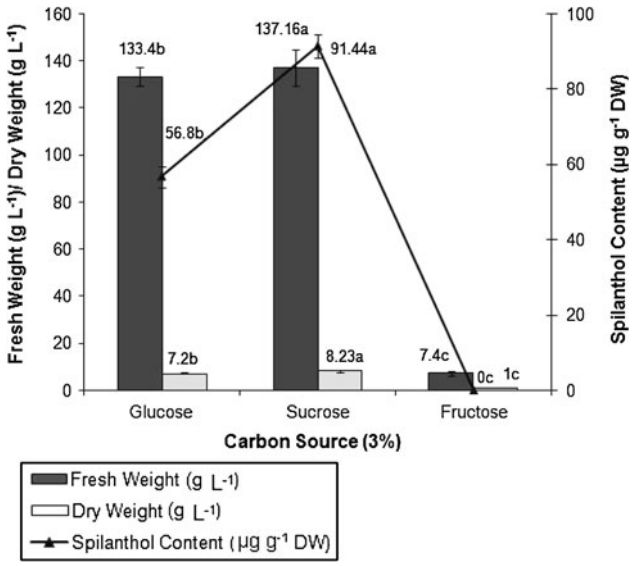

Fig. 4 Effect of different carbon sources on cell growth and production profile of spilanthol, in cell suspension cultures of *Spilanthes acmella* Murr., after 15 days. For fresh weight, dry weight and spilanthol content, mean values sharing the same letter do not differ significantly ($p < 0.05$) according to Duncan's multiple range test

Nutrient uptake kinetics

Typical profiles of the consumption of substrates (phosphate, nitrate and sucrose) during suspension culture are shown in Fig. 3. Sucrose uptake occurred at a relatively slower rate and it was consumed completely from the medium by the cells in 21 days of culture. In contrast to sucrose, it was invariably observed that the medium phosphate was almost completely consumed by the 15th day of culture. Thereafter, its concentration in the medium increased from 18th day onwards till the end of cultivation, which might be due to significant cell death and lysis under stress conditions that resulted in the leakage of phosphate from the cells into the medium. In comparison with phosphate, uptake of nitrate was observed at a slower rate,

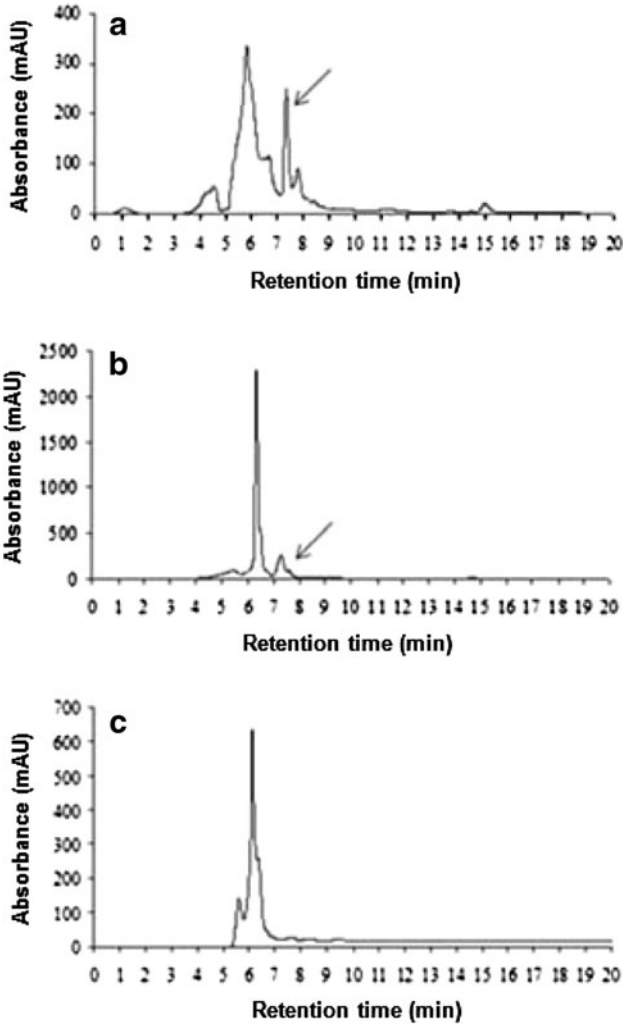

Fig. 5 Effect of carbon source on spilanthol content. HPLC chromatograms representing the presence/absence of spilanthol (*arrow marked*) in cultures grown on: **a** 3% sucrose containing medium. **b** 3% glucose containing medium. **c** 3% fructose containing medium. Note the absence of spilanthol peak

in the present study. It was consumed by cells till 18th day after which its concentration has increased. Therefore, it may be speculated that complete disappearance of phosphate from the medium resulted in the onset of decline phase in *spilanthes* suspension culture and this was the major limiting nutrients for cell growth. Similar type of kinetic profiles, where phosphate is consumed faster than the nitrate, has been observed by many workers in different plant species [16, 19–21].

Effect of carbon sources on biomass accumulation and spilanthol production

In the present study, the effect of glucose, fructose and sucrose were evaluated on cell biomass growth and metabolite production. The type of carbon source bears profound effect on quantity of metabolite produced. Sucrose was found to be the ideal carbohydrate source for the cell growth, which yielded the biomass of 8.2 g L^{-1} DW, followed by glucose which accumulated the biomass 7.2 g L^{-1} DW. The lowest accumulation of biomass was recorded in the medium supplemented with fructose which yielded about 1.0 g L^{-1} DW. The highest production of spilanthol as 91.4 μg g^{-1} DW was recorded in the medium supplemented with sucrose followed by glucose, which produced 56.8 μg g^{-1} DW. Spilanthol could not be detected in fructose-containing medium (Figs. 4, 5).

It has been previously reported that growth, development and chemical profile of in vitro cell cultures were highly dependent on type and concentration of carbohydrates used in the medium [22, 23]. Among several carbohydrates, sucrose tends to be the most efficacious carbon and energy source for cultured plant cells, especially with respect to secondary metabolite production. Sucrose introduced externally to plant cell suspensions is usually rapidly hydrolyzed to glucose and fructose that are then taken up by a passive or active transport process, depending on the species. This hydrolysis appears to be catalysed by a wall-bound or extracellular acid invertase [24]. The beneficial effect of sucrose over other carbon sources for metabolite production has been previously reported in many species [25, 26]. In addition to serving as carbon and energy sources, sugars also affect the osmotic pressure of the medium [27], which stimulates mitochondrial activity and, hence, production of energy required for metabolite synthesis [28]. In the combined presence of glucose and fructose, on hydrolysis of sucrose, cells exhibit preference for glucose whereas fructose is utilized only after glucose is depleted from the medium [29]. Thus, the presence of sucrose facilitated the constant availability of utilizable

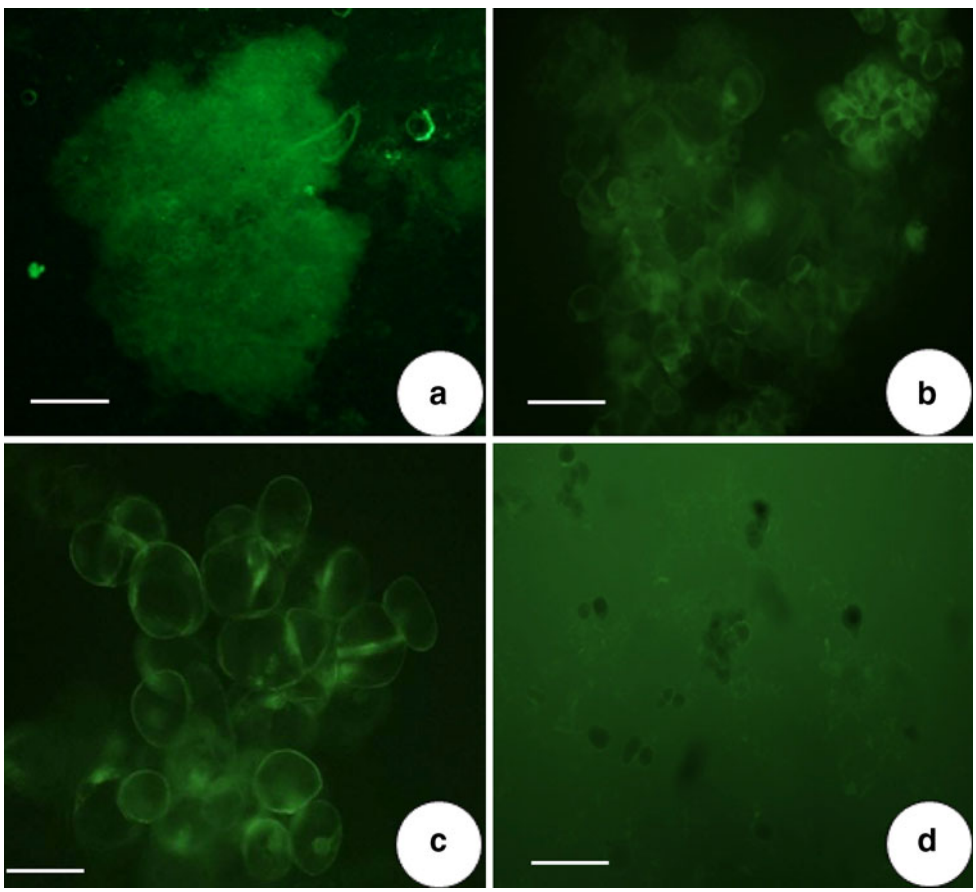

Fig. 6 Effect of agitation speed on growth and viability of cells. Three-week-old cells stained with 1% fluorescein diacetate solution. **a** Cellular clump at 60 rpm, showing aggregate of compact cells (*bar* 2.5 mm). **b** Cellular clump at 90 rpm, showing aggregate with loosely attached cells (*bar* 2.5 mm). **c** The cultures maintained at 120 rpm in the cell suspension, showing individual, live and healthy, fluorescent green-stained cells with intact wall (*bar* 9.3 mm). **d** Same at 180 rpm, showing dead (*dark bodies*) and sheared cells (*bar* 9.3 mm)

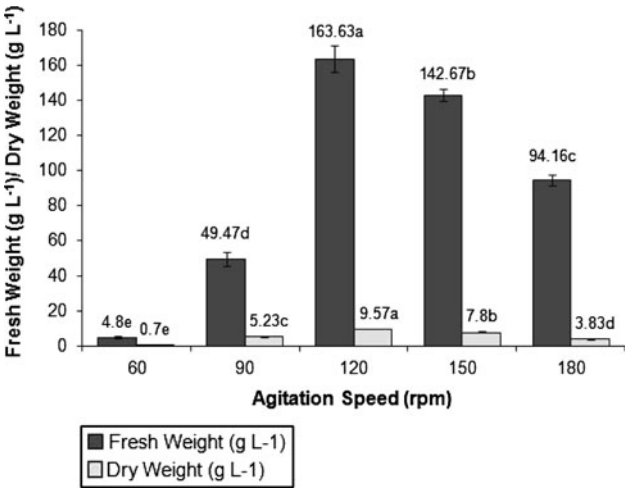

Fig. 7 Effect of different agitation speeds on fresh and dry weights of cells in suspension cultures of *Spilanthes acmella* Murr., after18 days. For fresh and dry weights, mean values sharing the *same letter* do not differ significantly ($p < 0.05$) according to Duncan's multiple range test

forms of carbohydrates, if glucose or fructose, applied individually, in the medium, resulted in starvation-like situation due to faster consumption of glucose within few days by the cells or a very slow uptake of fructose by the cells in the initial growth phase.

Effect of agitation speed on cell survival and viability

Speed of agitation directly affects the growth and viability of cells in culture due to aeration and agitation. The viability profile of *Spilanthes* cells at different agitation speeds is shown in Fig. 6. Similar to many other plant species, *Spilanthes* cells were also found to be highly sensitive to increase in agitation speed. The maximum fresh weight (163.63 g L^{-1}) (Fig. 7) and maximum viability (Fig. 6) were observed at 120 rpm. At higher rpm (150–180), the biomass and viability profile was highly unsatisfactory. At lower rpm (60–90), the cells died due to aggregation and clumping. At 60 rpm, cells were aggregated into hard clump while at 90 rpm cells were loosely attached in the clump. Only the cells at the outermost layer of the aggregate were alive and fluoresce green when stained with fluorescein diacetate (FDA). At higher rpm (150–180) cells died due to rupturing and shear effect.

Agitation speed is a very important parameter for establishment of plant cell suspension cultures. It is mainly responsible for mixing the plant cells in the medium and, thus, to facilitate homogeneous nutrient uptake and also for providing a sufficient O_2 and CO_2 supply. However, agitation and aeration cause hydrodynamic stress on the cells, which can be due to the physical characteristics of the suspended cells, such as their size, the presence of thick cellulose-based cell wall and the existence of large vacuoles [30]. The cells subjected to these shear forces show many physiological and morphological changes, such as aggregate size and shape, cell integrity and viability and finally, biomass accumulation and secondary metabolism. In this perspective, viability test by a dye, FDA, is important in determining viability of cells in suspension cultures. FDA is a non-fluorescing, non-polar dye that freely permeates through the plasma membrane. Within the living cells, the molecule is cleaved by esterase activity to fluorescein which is unable to pass through the cell membrane of live cells while it leaches out from the dead cells. Hence, only the live, intact cells take up the stain and fluoresce green.

Conclusions

This is the first study on establishment of cell suspension cultures of *Spilanthes*. An interesting finding of this study is the identification and quantification of spilanthol, an antiseptic alkylamide, from in vitro sources whereby cells in suspension produced 91.4 μg g^{-1} DW of spilanthol. The synthesis of spilanthol was found to be growth associated. Phosphate was observed as growth limiting nutrient seeing that its complete consumption leads to the onset of death phase of cells in the cultures. Among the carbon sources, sucrose supported maximum biomass and spilanthol production. The results of the present study, thus, form a backdrop for future studies related to large-scale production of spilanthol in bioreactors.

References

1. Dörnenburg H, Knorr D (1995) Strategies for the improvement of secondary metabolite production in plant cell cultures. Enzym Microb Technol 17:674–684
2. Anonymous (1989) The wealth of India: a dictionary of Indian raw materials and industrial products. Council of Scientific and Industrial Research, New Delhi, vol 10, pp 11–12
3. Jondiko IJO (1986) A mosquito larvicide in *Spilanthes mauritiana*. Phytochemistry 25:2289–2290
4. Ramsewak RS, Erickson AJ, Nair MG (1999) Bioactive N-isobutylamides from the flower buds of *Spilanthes acmella*. Phytochemistry 51:729–732
5. Pandey V, Agrawal V (2009) Efficient micropropagation protocol of *Spilanthes acmella* L. possessing strong antimalarial activity. In Vitro Cell Dev Biol Plant 45:491–499
6. Guiotto P, Woelkart K, Grabnar I, Voinovich D, Perissutti B, Invernizzi S, Granzotto M, Bauer R (2008) Pharmacokinetics and immunomodulatory effects of phytotherapeutic lozenges (bonbons) with *Echinacea purpurea* extract. Phytomedicine 15:547–554
7. Matthias A, Connellan P, Thompson D, Bone KM, Lehmann RP (2008) Acute immune-modulatory effects of bioavailable Echinacea alkamides. Planta Med 74:1008–1009

8. Prachayasittikul S, Suphapong S, Worachartcheewan A, Lawung R, Ruchirawat S, Prachayasittikul V (2009) Bioactive metabolites from *Spilanthes acmella* Murr. Molecules 14:850–867
9. Adler RJ (2006) Compositions for the acute and/or long term treatment of periodontal diseases using herb extracts. Chem Abstr 145:14791. Eur Patent WO 2006059196
10. Khadir HA, Zakaria MB, Ketchil AA, Azirum MS (1989) Toxicity and electrophysiological effects of *Spilanthes acmella* Murr. extracts on *Periplaneta americana* L. Pesticide Sci 25:329–335
11. Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 15:473–497
12. Trevelyan WE, Harrison JS (1952) Studies on yeast metabolism 1. Fractionation and microdetermination of cell carbohydrates. Biochem J 50:298–303
13. Nigra HM, Alvarez MA, Giulietti AM (1990) Effect of carbon and nitrogen sources on growth and solasodine production in batch suspension cultures of *Solanum eleagnifolium* Cav. Plant Cell Tiss Org 21:55–60
14. Boonen J, Baert B, Roche N, Burvenich C, De Spiegeleer B (2010) Transdermal behaviour of the *N*-alkylamide spilanthol (affinin) from *Spilanthes acmella* (Compositae) extracts. J Ethnopharmacol 127:77–84
15. Cech NB, Tutor K, Doty BA, Spelman K, Sasagawa M, Raner GM, Wenner CA (2006) Liver enzyme-mediated oxidation of *Echinacea purpurea* alkylamides: production of novel metabolites and changes in immunomodulatory activity. Planta Med 72:1372–1377
16. Srivastava P, Sisodia V, Chaturvedi R (2011) Effect of culture conditions on synthesis of triterpenoids in suspension cultures of *Lantana camara* L. Bioproc Biosyst Eng 34:75–80
17. Kochan E, Chmiel A (2011) Dynamics of ginsenoside biosynthesis in suspension culture of *Panax quinquefolium*. Acta Physiol Plant 33:911–915
18. Luedeking R, Piret EL (1959) A kinetic study of the lactic acid fermentation: batch process at controlled pH. J Biochem Microbiol Technol Eng 1:393–431
19. Zhang Y-H, Zhong J-J, Yu J-T (1996) Enhancement of ginseng saponin production in suspension cultures of *Panax notoginseng*: manipulation of medium sucrose. J Biotechnol 51:49–56
20. Fett-Neto AG, Zhang WY, Dicosmo F (2004) Kinetics of taxol production, growth and nutrient uptake in cell suspensions of *Taxus cuspidate*. Biotechnol Bioeng 44:205–210
21. Prakash G, Srivastava AK (2007) Azadirachtin production in stirred tank reactors by *Azadirachta indica* suspension culture. Process Biochem 42:93–97
22. Rolland F, Baena-Gonzalez E, Sheen J (2006) Sugar sensing and signalling in plants conserved and novel mechanisms. Ann Rev Plant Biol 57:675–709
23. Wang Y, Weathers PJ (2007) Sugars proportionately affect artemisinin production. Plant Cell Rep 26:1073–1081
24. Masuda H, Takahashi T, Sugawara S (1988) Acid and alkaline invertases in suspension cultures of sugar beet cells. Plant Physiol 86:312–317
25. Bondarev N, Reshetnyak O, Nosov A (2003) Effects of nutrient medium composition on development of *Stevia rebaudiana* shoots cultivated in the roller bioreactor and their production of steviol glycosides. Plant Sci 165:845–850
26. Nagella P, Murthy HN (2010) Establishment of cell suspension cultures of *Withania somnifera* for the production of with anolide A. Bioresour Technol 101:6735–6739
27. Su WW (1995) Bioprocessing technology for plant cell suspension cultures. Appl Biochem Biotech 50:189–230
28. Bonga JM, Von Aderkas P (1992) In vitro culture of trees. Kluwer Academic Publishers, Boston
29. Kretzschmar FS, JrCJF Oliveira, Braga MR (2007) Differential sugar uptake by cell suspension cultures of *Rudgea jasminoides*, a tropical woody Rubiaceae. In Vitro Cell Dev Biol Plant 43:71–78
30. Chattopadhyay S, Farkya S, Srivastava AK, Bisaria VS (2002) Bioprocess considerations for production of secondary metabolites by plant cell suspension cultures. Biotechnol Bioproc E 7:138–149