IN VITRO FLOWERING OF SHOOTS REGENERATED FROM CULTURED NODAL EXPLANTS OF *Spilanthes acmella* MURR. - AN ORNAMENTAL CUM MEDICINAL HERB

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Abstract. An efficient protocol for *in vitro* flowering of *Spilanthes acmella* Murr., a medicinally valuable plant, has been developed. Multiple shoot formation of up to 4 shoots was obtained on Murashige and Skoog (MS) medium supplemented with BAP (1.0 mg/l). Regenerated shoots were subcultured cultured on MS medium containing various concentrations of BAP alone or in combination with IAA. Presence of BAP in the culture medium was observed to be absolutely essential for induction of flower. Maximum percentage (50 %) of flower induction occurred when regenerated shoots were cultured on MS medium supplemented with BAP (2.0 mg/l) + IAA (0.5 mg/l) under photoperiod of 16/8 h (light/dark cycle).The 3-week intervals for three consecutive subcultures on this medium were efficient for flower induction. The regenerated shoots rooted best on 1/2 MS medium containing IBA (1.0 mg/l). Rooted plantlets were hardened and established in pots with 70% survival rate.

Keywords: multiple shoots, *in vitro* flowering, *Spilanthes acmella,* photoperiod, subculture time

Abbreviations: BAP - Benzyl amino purine; **2,4-D -** 2,4-Dichlorophenoxyacetic acid; **MS -** Murashige and Skoog (1962); **BM** - Basal medium; **NAA -** *a*-naphthalene acetic acid; **IBA -** Indole-3-butyric acid; **IAA -** Indole acetic acid.

INTRODUCTION

Spilanthes acmella Murr. (Asteraceae), commonly known as Toothache plant, is an important ornamental cum medicinal plant widely distributed to tropical and subtropical region of the world. It is an endangered plant species [25]. It is well documented for its uses as antibacterial, antifungal and antimalarial activity [1, 22, 24].The extract of this genus has been used as folk medicine to cure severe toothache, affections of throat and gums, stomatitis, paralysis of tongue, and psoriasis [4]. The antimicrobial activity of this species is due to presence of a highly valuable biologically active compound spilanthol [13].

The transition from vegetative state to reproductive development in plants is of great interest to botanist but is still poorly understood [14]. *In vitro* culture provides an ideal experimental system to study molecular mechanisms of flowering [18]. Flowering is considered to be a complex process regulated by a combination of environmental and genetic factors. *In vitro* flowering has been reported in a number of plant species e.g. *Oscimum basilicum, Panax ginseng, Withania somnifera, Rauvolfia tetraphylla, Anthem graveolens* [3, 10, 28, 29, 37]. In many plants, *in vitro* flowering was normally achieved by the application of exogenous hormones to the culture medium [10]. Some studies have focused on a variety of factors involved in the *in vitro* flowering such as the level and interaction of exo- and endogenous phytohormones, sugars, minerals, phenolics [33, 34], subculture time, length of photoperiod etc. during *in vitro* culture [32].

Due to over-exploitation and lack of vegetative propagation methods, this valuable plant species has declined fast and is listed as endangered [25]. The development of efficient plant tissue culture procedures for *in vitro* flowering in this plant is important for the application of these technologies for improvement. To the best of our knowledge, literature availability on *in vitro* flowering in this plant is very scanty and the percentage of seed germination and viability is also very low. The objective of this study was to identify media suitable for *in vitro* flower formation in *S. acmella.*

MATERIALS AND METHODS

Plant materials

Nodal explants were excised from the plants growing in Polyhouse of Botany Department, Kurukshetra University, Kurukshetra, India. The explants were washed with liquid detergent under running tap water to remove dust particles. The explants were then treated with 0.1% (w/v) mercuric chloride for 3-5 minutes under aseptic conditions. After this these explants were then thoroughly washed 4-5 times with sterilized double distilled water to remove the traces of mercuric chloride.

Medium preparation

The nodal explants were trimmed to 1cm long pieces and were cultured on MS medium [20] containing 30 g/l sucrose and 8 g /l agar supplemented with BAP at 1.0 mg/l [39]. The pH of all media was adjusted to 5.8 with 1 N NaOH or 1 N HCl prior to autoclaving.

Culture conditions

20 ml of medium was dispensed into individual 25x150 mm glass culture tubes and finally autoclaved at 1.05 kg/cm^2, 121 °C for 20 min. Cultures were maintained at a temperature of 25±2ºC with a 16/8 h light/dark photoperiod under an illumination of 20 µmol m^{-2} s^{-1} photosynthetic photon flux intensity provided by cool- white fluorescent light.

After 4 weeks of initial culture, the regenerated nodal explants were aseptically sub-cultured to fresh MS medium containing 30 g/l sucrose and 8 g /l agar

supplemented with various concentrations of BAP alone or in combination with IAA. To examine the subculture time, explants were sub cultured to fresh medium after every 3 weeks. After this period the plants were transferred to fresh medium. Different durations of photoperiod, 3 light/dark cycles i.e., 14/10, 16/8, and 18/6 h were also tested to find out their optimum level for *in vitro* flowering.

To establish root proliferation, the *in vitro* raised shoots (2.5-4.0 cm long) from shoot multiplication cultures were excised and transferred to cultural tubes containing half strength MS medium fortified with IBA (1.0 mg/l). When adequate rooted shoots were obtained, the plantlets were transferred to pots containing sterilized soil and sand mixture (3:1) for 2 weeks for hardening.

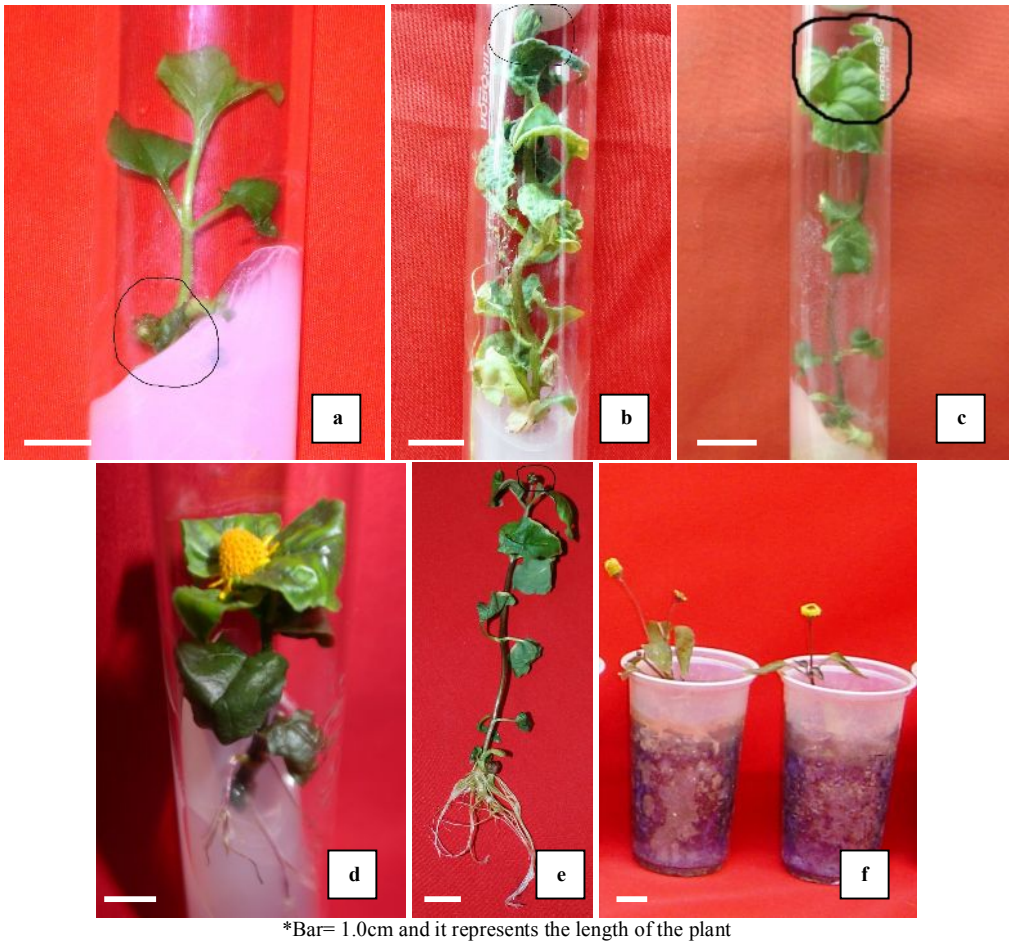
Statistical analyses

For the above experiments, 30 tubes were inoculated for each treatment. The results are expressed as a mean ± SE of three independent experiments. The data were analyzed statistically using one-way analysis of variance (ANOVA) and the significant differences between means were assessed by Duncan's multiple range test at $P \leq 0.05$. All statistical analyses were performed with Statistical Package for Social Sciences (SPSS, version 11.5).

RESULTS

Results obtained from our previous study [39] revealed that, after 4 weeks of initial culture, nodal explants cultured on MS medium with BAP (1.0 mg/l) developed maximum number of multiple shoots. So, we used above protocol for producing multiple shoots. It suggests that bud formation in this plant required cytokinins. Therefore MS medium containing various concentrations of BAP alone and in combination with IAA (0.5 mg/l) was considered as optimal for shoot proliferation and the shoots with green expanded leaves and single main stem regenerated *in vitro* were further multiplied on the same medium. We routinely used this protocol for multiplication of shoots used in the successive subcultures, after every three weeks.

Regenerants sub cultured on the media containing BAP either alone and in combination with IAA initiates flowering (Fig 1a;c). BAP in both cases strongly affected the flower bud initiation. Maximum percentage of flowering (50%) was obtained on MS medium containing BAP (2.0 mg/l) + IAA (0.5 mg/l) after 7 weeks of culture (Table 1) (Fig 1b). The flowers produced *in vitro* appeared morphologically normal, yellowish gold in color. One to two flower buds were produced for each *in vitro* cultured plant.



*Bar= 1.0cm and it represents the length of the plant

Fig. 1. *In vitro* flowering of *Spilanthes acmella* through cultured nodal explants. **(a)** Floral bud induction on MS medium + BAP (2.0 mg/l); **(b)** Floral bud induction on MS medium + BAP (2.0 mg/l) + IAA (0.5 mg/l); **(c)** Floral bud induction on MS medium with 3.0 mg/l BAP + 0.5 mg/l IAA; **(d)** Fully blommed flower under *in vitro* condition; **(e)** Regenerated plantlet with flower bud; **(f)** Hardening.

Sugars are necessary carbon source for reliable induction and development of flowers. Addition of sugar to the medium is necessary for induction of floral stimulus. In the present investigation, flower bud differentiation was observed by keeping the sucrose concentration at 30 g/l.

Length of photoperiod, recorded after 9 weeks of culture, showed no significant effect on shoot multiplication but did influence flowering *in vitro*. The percentage of flowering was 50% indicating that the flowering stimulus did occur. In the present study, it was observed that the exposure of plantlets to light (16/8 h photoperiod) was most effective for flowering which showed highest cultures producing *in vitro* flower buds (Table 2). So, it was observed that under 16/8 and 18/6 h light/dark cycles induced flower buds attained full bloom (Fig 1d).

Table 1. *In vitro* flowering responses of cultured nodal explants of *Spilanthes acmella* Murr. on MS medium supplemented with various growth regulators under photoperiod of 16/8 (light/dark cycle).

Auxins/ cytokinins (mg/l)	Concentration of plant growth regulators (mg/l)	Weeks in culture	Flowering (%)	No. of flowers/explants (Mean ± SE)
BAP	0.5	09	20.0 ± 0.40c	1.0 ± 0.00d
	1.0	08	33.3 ± 0.48b	1.20 ± 0.42c
	2.0	07	43.3 ± 0.50a	1.38 ± 0.50bc
	3.0	07	36.6 ± 0.49b	1.25 ± 0.45bc
BAP+IAA	1.0 + 0.5	09	23.3 ± 0.43c	1.1 ± 0.37c
	2.0 + 0.5	07	50.0 ± 0.50a	1.8 ± 0.41a
	3.0 + 0.5	07	46.6 ± 0.50a	1.5 ± 0.51b
LSD ($P \leq 0.05$)			0.3	0.2427
ANOVA ($F_{6,14}$)			3.653**	1.732**

Data shown are Mean ± SE of thirty replicates.
Mean value followed by different alphabet/s within a colum do not differ significantly over one other at P≤0.05 lead by Duncan's Multiple Range Test.
** Significant at $p \leq 0.05$

Table 2. Effect of different photoperiod on *in vitro* flowering in *Spilanthes acmella* Murr. recorded after 9 weeks of culture on MS medium containing 2.0 mg/l BAP + 0.5 mg/l IAA.

Photoperiod (light/dark)	Explants flower *in vitro* (%)
14/10	30 ± 0.47b
16/8	50.0 ± 0.50a
18/6	46.6 ± 0.50a
LSD ($P \leq 0.05$)	0.484
ANOVA ($F_{2,6}$)	3.292**

Data shown are Mean ± SE of thirty replicates.
Mean value followed by different alphabet/s within a colum do not differ significantly over one other at P≤0.05 lead by Duncan's Multiple Range Test.
** Significant at $p \leq 0.05$

Subculturing was carried out to maintain the cultures and for flower induction. After the 3-week-intervals for three consecutive subcultures, the media supplemented with BAP (2.0 mg/l) + IAA (0.5 mg/l) showed 50% *in vitro* flowering (Table 3). Earlier the flower buds were small but normal. It seems that a period of nine weeks in culture was appropriate for flowering in the present study.

Table 3. Effect of subculture time on *in vitro* flowering in *Spilanthes acmella* Murr. on MS medium containing 2.0 mg/l BAP + 0.5 mg/l IAA under photoperiod of 16/8 (light/dark cycle).

Subculture period (weeks)	Explants flower *in vitro* (%)
3	20.0 ± 0.40b
6	43.3 ± 0.50a
9	50.0 ± 0.50a
LSD ($P \leq 0.05$)	0.489
ANOVA ($F_{2,6}$)	3.570**

Data shown are Mean ± SE of thirty replicates.
Mean value followed by different alphabet/s within a colum do not differ significantly over one other at P≤0.05 lead by Duncan's Multiple Range Test.
** Significant at $p \leq 0.05$

To establish complete plants, regenerated shoots were excised and transferred to half strength MS medium containing IBA (1.0 mg/l) for rooting [39]. Roots that developed on this medium were long and thin (Fig. 1e). Three weeks of rooting was adequate before transplanting to pots containing sterilized soil and sand mixture (3:1). *In vitro*- derived plants did not display any phenotypic variation during subsequent vegetative development (Fig. 1f).

DISCUSSION

Results obtained from our previous study [39] revealed that, after 4 weeks of initial culture, nodal explants cultured on MS medium with BAP (1.0 mg/l) developed maximum number of multiple shoots. Effectiveness of BAP in inducing bud break was also observed in many other plant species [8, 15, 16, 40]. Cytokinin is a common requirement for *in vitro* flowering [30]. A number of studies report the use of cytokinins for *in vitro* flowering in a number of species like *Murraya paniculata* [11], *Fortunella hindsii* [12], *Gentiana triflora* [42], *Pharbitis nil* [7] and *Ammi majus* [21]. Earlier studies have indicate the beneficial effect of BAP on the induction of *in vitro* flowering for other plants such as *Withania somnifera, Rauvolfia tetraphylla, Anethem graveolens* [3, 10, 29]. BAP was found to be playing an important role not only as a growth regulator, but also as a factor regulating floral organ formation of regenerated plantlets [17]. Not only BAP, effect of other cytokinins on *in vitro* flowering have also been noted by Meeks-Wagner *et al.* [19], who showed that in *Nicotiana tabacum*, Kn promoted flower formation. In our study, BAP have used alone or in combination with IAA for the induction of *in vitro*

flowering and it may be playing a major role in floral bud formation and maturation. The combined effect of auxin and cytokinin on *in vitro* flower induction was also reported in a number of previous studies [2, 38]. Similarly, Taylor *et al.* [35] reported that phytohormones affected flowering by mediating growth changes within the apical meristem and that cytokinins, in particular, played a key role in the initiation of mitosis and the regulation of cell division and organ formation.

Sucrose is known to be the main carbon source of choice for *in vitro* flower culture studies [26]. Sucrose availability in aerial parts of the plant promotes flowering in *Arabidopsis thaliana* [27]. Sucrose and cytokinins interact with each other for floral induction in *Sinapis alba* by moving between shoot and root [9].

The most predictable factor in plants to time their reproduction is light period or day length [5]. Importance of photoperiod for *in vitro* flowering has been frequently demonstrated in *C. nobilis* Lour × *C. deliciosa* Tenora, which only flowered at 16-h photoperiod [31]. The effects of photoperiod on vegetative and reproductive development were also investigated in *Psygmorchis pusilla* [36]. Therefore day length plays a crucial role in flower induction both *in vivo* and *in vitro* possibly due to altered photosynthetic turnover on flowering [23]. Cultures maintained under dark did not produce flower buds. This is in agreement with earlier observations [23, 32].

The age of mother plants influenced flower induction [6, 12] therefore, it is possible that the explants used in this study were at the transition phase. Wang *et al*. [38] stated that subculture time before flower induction could substantially affect *in vitro* flowering.

In tissue culture, *in vitro* flowering serves as an important tool for many reasons. One of the most important ones being to shorten the life cycles of plants, other aims includes studying flower induction and initiation, and floral development. Controlling the environment and media components enables the manipulation of different variables that affect these processes [42]. So, this technique is of practical importance and can also serve for mass production of specific organs with unique compounds for pharmaceutical, nutritional and other uses.

Acknowledgements. The authors are grateful to University Grants Commission, New Delhi for financial assistance and to Kurukshetra University, Kurukshetra for providing laboratory facilities to carry out the work.

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Received: 23 March 2011
Accepted: 3 May 2011
Published Online: 6 May 2011
Analele Universităţii din Oradea – Fascicula Biologie
http://www.bioresearch.ro/revistaen.html
Print-ISSN: 1224-5119
e-ISSN: 1844-7589
CD-ISSN: 1842-6433