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Research Article

IN VITRO FLOWERING FOR THE PRODUCTION OF BIOACTIVE ALKAMIDES IN *ACMELLA CILIATA* (KUNTH) CASS.: A PROMISING HERB IN PHARMACEUTICALS

Sugathakumari Neethu Mohan[1], Achuthan Sudarsanan Hemantha kumar*[2], Thankappan Suvarna Preetha[1]

[1]*Plant Tissue Culture Laboratory, Department of Botany, University College, Thiruvananthapuram, Research Centre, University of Kerala, Kerala, India*

[2]*Biotechnology and Bioinformatics Division, Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Thiruvananthapuram, Kerala, India*

**Corresponding author: preethahemanth@yahoo.com*

ABSTRACT

Acmella ciliata (Kunth) Cass. (Synonym: *Spilanthes ciliata* Kunth), a herbaceous medicinal plant of Family Asteraceae commonly known by the name 'Toothache plant' is used as a spice and as a traditional medicine for the treatment of toothaches. The plant has got various pharmacological properties due to the presence of phytochemicals like flavanoids, alkaloids, coumarins, tannins, etc. and the most important among them is an isobutylamide 'spilanthol' that has got anaesthetic and various other properties. As per earlier reports, highest spilanthol content was present in flower heads of this plant genus. We in this study, focus on the induction of *in vitro* flowers so as to formulate a system for spilanthol production which have a high demand in pharmaceutical industries. The *in vitro* plants raised in Murashige and Skoog (MS) medium containing appropriate phytohormones were subcultured to MS medium containing 6-Benzyl adenine (BA) (1.0, 2.0, 3.0 mgl^{-1}), Kinetin (1.0, 2.0, 3.0 mgl^{-1}) in combination with Silver nitrate ($AgNO_3$) (5.0, 6.0, 7.0 mgl^{-1}) and with 0.5 mgl^{-1} Indole 3-acetic acid (IBA). Other treatments like coconut water and Giberellic acid (GA_3) at different concentrations were also tested. Among the various treatments, *in vitro* flowers were formed in MS medium containing 1.0 mgl^{-1} Kinetin, 5.0 mgl^{-1} $AgNO_3$ in combination with 0.5 mgl^{-1} IBA with 65.21 response percentage with 8/16 light to dark hours period. This is a stepping stone for the bioproduction of spilanthol that would get accomplished with the ongoing research for elucidation of the compound which may become useful in phytopharma industry.

Keywords: *Acmella ciliata, In vitro* flower induction, Photoperiod, Subculture duration, Sucrose, BA.

1. INTRODUCTION

Acmella ciliata (Kunth) Cass. (Family Asteraceae) is an ornamental cum medicinal plant that possesses diverse bioactive properties and has immense utilization in medicine, health care, cosmetics and as health supplements. Among other phyo-constituents, the most abundant isolates of this plant species are lipid alkamides especially, the spilanthol (N-Isobutyl-2(E),6(Z),8(E)-decatrienamide), the key component and responsible for most of its pharmaceutical applicability especially the antimalarial property [1] along with other bioactive metabolites *viz*. phenolic, flavanoid, coumarin and triterpenoid compounds. Additionally, spilanthol has efficient analgesic, anti-inflammatory and antimicrobial properties [2]. It is a high value bioactive molecule and at present there are about twenty three pharmaceutical companies both in Indian and International scenario that are manufacturing the spilanthol containing products. Roots, leaves, stems and flower heads of *Acmella*/ *Spilanthes* species, possess both aliphatic and aromatic alkamides [3, 4]. Current supply of spilanthol from *Spilanthes*/ *Acmella* plant population existing at present is insufficient to meet the increasing demand by these sectors. The development of efficient *in vitro* system is necessary for scaling up of biomass production, *in vitro* flowering as well as *in vitro* synthesis of 'spilanthol' and a study regarding this aspect is not attempted yet in this plant as well as in other *Acmella* species.

Flowering is a complex physiological process in the life of a plant, which includes switch from vegetative stage to reproductive stage of growth regulated by both internal and external factors and studies regarding *in*

vitro flowering is rare in Asteraceae with a few examples in *Artemesia annua* [5], *Spilanthes acmella* [6, 7] and *Gerbera jamesonii* [8]. Very recently, the performance of chicory (*Cichorium intybus*) and lettuce plants (*Lactuca sativa*) exhibiting phase changes to flowering during *in vitro* culture was scientifically validated [9]. Many researchers had critically analysed 'florigen', the hormone which help in flowering and its isolation until now, is still a mystery. Alterations in physicochemical factors which attribute for *in vitro* flowering mechanism have been experimentally analysed by some authors. The different factors affecting the flowering include day length, vernalization, ambient temperature, irradiance and water/mineral availability, etc. Previous studies suggest that *in vitro* flowering has been promoted by cytokinins at optimum concentrations. Apart from auxins and cytokinins, gibberllins also influenced *in vitro* flowering responses. *In vitro* flowering experiments were conducted not only in dicots, but also in monocots like maize, bamboo, orchids, palms, etc. Production of flowers under *in vitro* conditions was studied well and effectively explained in medicinal and aromatic plant species also. However, there are no reports yet in *Acmella* species regarding the induction of flowers *in vitro*. We here report for the first time the *in vitro* flowering response in *Acmella ciliata*.

2. MATERIAL AND METHODS

2.1. Plant Material

The plant material for the present study *Acmella ciliata* (Kunth.) Cass. were collected from the natural habitat in Aruvikkara, Thiruvananthapuram, Kerala, India and maintained in the green house of Department of Botany, University College, Thiruvananthapuram, Kerala, India. The herbarium samples were authenticated by the experts in Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, Kerala, India (Voucher Nos. TBGT 32710-32711).

2.2. Shoot culture establishment

Shoot cuttings bearing shoot tip and nodal segments (first, second, third and fourth nodes) taken from *A. ciliata* plants growing in Botany Department green-house, the leaves were removed and the explants were cut into appropriate sizes. They were washed in running tap water for 30 minutes to remove all the dust particles followed by washing with detergent solution (Labolene) (0.5%) (v/v) for 20 minutes and washed again in running tap water to remove the detergent remaining. Then the explants were rinsed 5-7 times with distilled water and subsequent surface sterilization procedures were done inside the laminar airflow chamber, where the explants were surface sterilized in 0.1% (w/v) $HgCl_2$ for 5-10 minutes depending upon the hardness of the tissues. After rinsing 3-4 times with sterile distilled water they were cut into appropriate sizes and the nodal segments (0.8-1.0 cm) were inoculated vertically into the nutrient medium *i.e.,* Murashige and Skoog (MS) medium [10] supplemented with 0.5-2.0 mgl^{-1} BA in culture tubes to initiate the shoot cultures.

The *in vitro* shoots raised in all the phytohormone combinations were transferred to the fresh medium of the same combination for 10 subsequent subculture passages at an interval of 4 weeks each, for further multiplication. The subculture passages were carried out in the medium which exhibited better shoot initiation as well as multiplication in *A. ciliata*.

2.3. *In vitro* flowering in *A. ciliata*

For inducing *in vitro* flowering in *A. ciliata*, the *in vitro*-raised shoots were subjected to the following treatments:

(i) MS supplemented medium with different phyto-hormones *viz*. Kinetin/ BA (1.0, 2.0 and 3.0 mgl^{-1}) in combination with 0.5 mgl^{-1} IBA along with 5.0, 6.0 and 7.0 mgl^{-1} $AgNO_3$

(ii) MS medium augmented with 1.0 mgl^{-1} BA, 0.5 mgl^{-1} IBA, 5.0 mgl^{-1} $AgNO_3$ along with 1.0, 2.5, 5.0, 10.0 and 20.0% (v/v) Coconut Water (CW)

(iii) MS medium fortified with 1.0 mgl^{-1} Kinetin, 0.5 mgl^{-1} IBA along with 0.5, 1.0 and 2.0 mgl^{-1} GA_3

(iv) MS medium supplemented with 0.5 mgl^{-1} BA along with different concentrations of sucrose (1-12%) (w/v)

(v) The effect of photoperiod on *in vitro* flowering was also conducted by culturing the plant in appropriate plant growth regulators and maintained in different photoperiods such as 8/16, 10/14, 16/8 and 14/10 light to dark period in the above mentioned treatments.

(vi) Moreover, the role of subculture passages in induction of *in vitro* flowers was also noticed by culturing the *in vitro* shoots for 1-10 subculture passages (each lasted for 4 weeks) in all these treatments.

2.4. Statistical analysis

Each *in vitro* culture experiment consisted of 10 replicates with one explant per culture vessel. All experiments were repeated three times. The data were

statistically analyzed by Analysis of Variance (ANOVA) and the means were compared by Duncan's multiple range test ($p \leq 0.05$) using the computer software SPSS/ PC + version 4.0 (SPSS Inc., Chicago, USA).

3. RESULTS AND DISCUSSION

In vitro flowering is a novel technique which helps to understand the physiological activities of the flowering phenomenon and it provides a platform for carrying out modern plant breeding techniques thereby improving the plant variety through selective breeding and helps in raising new improved varieties. To investigate the effect of various parameters that induced *in vitro* flowering responses in *A. ciliata*, treatments with different plant growth regulators, other growth influencing factors, various concentrations of sucrose, coconut water and photoperiodic responses have been analyzed. In the present study, *in vitro* flowers were formed in *A. ciliata* during the 8th subculture period when the shoots were transferred to MS medium fortified with 1.0 mgl^{-1} Kinetin and 0.5 mgl^{-1} IBA along with 5.0 mgl^{-1} $AgNO_3$ after 2 months of inoculation comprising 15 days dark period.

3.1. Effect of $AgNO_3$ and Plant growth regulators

Shoots treated with only PGRs exhibited only shoot multiplication rather than flowering. Among all the phytohormones and $AgNO_3$ treated, MS medium supplemented with 1.0 mgl^{-1} Kinetin and 0.5 mgl^{-1} IBA along with 5.0 mgl^{-1} $AgNO_3$ produced 1-2 flowers from the *in vitro* shoots after 8 weeks (Table 1, Fig.1). Here, the flowering was noticed during the eighth subculture period. An average of 1-2 flowers were produced from the *in vitro* shoots which lasted for 10 days. *In vitro* flower induction was reported in *Spilanthes acmella* in MS medium supplemented with 2.0 mgl^{-1} BA and 0.5 mgl^{-1} IAA which evoked 70% flowering response after 7 weeks of culture producing 1-2 flower buds for each *in vitro* cultured plant [7]. It was previously reported in *Nicotiana tabacum* that not only BA, other cytokinins *viz*. Kinetin also promoted *in vitro* flowering [11] which supports the present study. In *Rotula aquatica* medium with $AgNO_3$ at different concentrations (5.87-17.7 µM) induced *in vitro* flowers after 45 days of culture [12]; whereas the synergy of auxin (NAA) and $AgNO_3$ was superior for the induction of flowers *in vitro* thereby suggesting that the synergistic effect of plant growth regulators (auxin IBA and cytokinin Kinetin) coupled with $AgNO_3$ treatment as well as the exposure to dark period might have shifted the photomorphogenic responses to stimulate flower induction *in vitro* in *A. ciliata* also. The presence of cytokinins in the medium stimulated flowering of *in vitro* shoots in *Capsicum annum* [13]. The effect of $AgNO_3$ as a potent flower inducer was already proven [14]; [15] in Coffee and *Capsicum frutescens* respectively. Flower induction (12 *in vitro* flowers per culture) was noticed in *Solanum nigrum* in MS medium supplemented with 2.0 mgl^{-1} BA, 1.0 mgl^{-1} Kinetin and 0.5 mgl^{-1} IAA along with 6.0 mgl^{-1} $AgNO_3$ [16] which is in agreement with the present finding.

In all the flower induction treatments, the plantlets showed vigorous multiplication but their morphological response was different from that of normal *in vitro* multiplication. In MS medium fortified with BA (0.5, 1.0 and 2.0 mgl^{-1}) combined with 0.5 mgl^{-1} IBA and $AgNO_3$ at different concentrations (5.0, 6.0 and 7.0 mgl^{-1}), the plantlets exhibited rosette growth pattern with short, thick stem having large, dark green, crowded well expanded leaves and the shoots were also vigorous as well as healthy. In MS medium supplemented with GA_3 (0.5, 1.0 and 2.0 mgl^{-1}) along with 0.5 mgl^{-1} IBA and 1.0 mgl^{-1} Kinetin combination, the shoots produced were thin, elongated and unhealthy with small, pale green coloured leaves. Also, they were vastly growing.

Fig. 1: *In vitro* flowering in *A. ciliata*

3.2. Effect of coconut water

The present study has analysed the effect of coconut water in different concentrations *viz*. 1.0, 2.5, 5.0, 10 and 20% (v/v) augmented to MS medium along with 1.0 mgl^{-1} BA, 0.5 mgl^{-1} IBA and 5.0 mgl^{-1} $AgNO_3$ in

evoking the flowering response in *A. ciliata*. Here, the different treatments induced only multiplication of shoots (Table 2). Among the different concentrations of CW tested, maximum 2.38±0.54 shoots were produced in 10% CW along with other PGRs such as 1.0 mgl^{-1} BA, 0.5 mgl^{-1} IBA and 5.0 mgl^{-1} $AgNO_3$.

3.3. Effect of sucrose

In order to investigate the effect of C:N ratio in flower induction, the present study has analysed different sucrose concentrations supplemented to MS medium aided with 0.5 mgl^{-1} BA, but, no *in vitro* flowering occurred, and the shoots exhibited differential shoot multiplication responses at varying sucrose concentrations wherein 3% (w/v) sucrose was optimum (4.42±0.40 shoots). MS medium supplemented with 4% (w/v) sucrose also showed multiple shoot production (3.56±0.37 shoots) and the shoots were healthy with large, dark green leaves. Reduced shoot multiplication frequency was noticed as the sucrose concentrations were increased further (Fig. 2) (Table 3). However, *in vitro* flower induction was noticed in *Gerbera* (Family Asteraceae) when full strength MS medium was supplemented with 5.0 mgl^{-1} GA_3 and 8% (w/v) sucrose [8] and sucrose alone has no significant effect on flowering and showed increased shoot and root growth as the sucrose concentration increased. In contrast, in *Withania somnifera* MS medium when augmented with BA (1.5 mgl^{-1}) and IAA (0.3 mgl^{-1}) along with 4% (w/v) sucrose resulted in 67% *in vitro* flowering having 9 flowers per shoot and 4 fruits per shoot in elongated shoots [17]. Also, sucrose has an indispensable role in *in vitro* flowering of *Trichodesma indicum* and MS medium augmented with 30 gl^{-1} sucrose aided with BA (1.33 mM) and GA_3 (1.44 mM) succeeded in the production of *in vitro* flowers at highest percentage (93.2%) [18]. In addition, *in vitro* flower induction was possible in *Guizotia abyssinica* (Family Asteraceae) in MS medium augmented with 0.1 mgl^{-1} BA and 0.1 mgl^{-1} GA_3 which exhibited 70% flowering response [19]. However, in the study presented here, GA_3 as well as sucrose treatment could not accomplish the production of *in vitro* flowering in *A. ciliata*, and the shoots produced upon GA_3 treatment were elongated and unhealthy. In short day plants, giberrellins are generally ineffective or may have only limited effects on flowering. In the present study, *in vitro* flower induction in *A. ciliata*, which prefer short days for flowering, was achieved under non-inductive conditions by GA_3 similar to the findings established in *Gerbera jamesonii* [8].

Table 1: Effect of $AgNO_3$ and Plant growth regulators on *in vitro* flowering response in *A. ciliata*

Treatments					Shoot induction %	Response %	Response	Mean number of shoots
BA	Kinetin	IBA	GA_3	$AgNO_3$				
1.0	-	0.5	-	5.0	91.24^{a}	---	Shoot Multiplication	2.64 ± 0.15^{a}
1.0	-	0.5	-	6.0	85.37^{e}	---	”	2.52 ± 0.24^{ab}
1.0	-	0.5	-	7.0	82.16^{f}	---	”	1.87 ± 0.63^{h}
2.0	-	0.5	-	5.0	88.28^{c}	---	”	2.14 ± 0.52^{f}
2.0	-	0.5	-	6.0	79.51^{i}	---	”	2.32 ± 0.37^{cd}
^{a}2.0	-	0.5	-	7.0	68.47^{m}	---	”	2.01 ± 0.62^{g}
3.0	-	0.5	-	5.0	65.23^{n}	---	”	1.84 ± 0.36^{h}
3.0	-	0.5	-	6.0	73.48^{l}	---	”	1.67 ± 0.48^{j}
3.0	-	0.5	-	7.0	78.62^{j}	---	”	1.25 ± 0.34^{l}
-	1.0	0.5	-	5.0	91.58	65.21	*In vitro* flowers formed**	2.42 ± 0.42^{bc}
-	1.0	0.5	-	6.0	87.34^{d}	---	Shoot multiplication	2.47 ± 0.63^{bc}
-	1.0	0.5	-	7.0	89.56^{b}	---	”	2.61 ± 0.48^{a}
-	2.0	0.5	-	5.0	88.47^{b}	---	”	2.15 ± 0.54^{f}
-	2.0	0.5	-	6.0	90.35^{a}	---	”	2.05 ± 0.61^{g}
-	2.0	0.5	-	7.0	80.73^{h}	---	”	2.35 ± 0.42^{cd}
-	1.0	0.5	0.5	-	77.49^{k}	---	”	1.75 ± 0.38^{i}
-	1.0	0.5	1.0	-	85.26^{e}	---	”	2.17 ± 0.64^{f}
-	1.0	0.5	2.0	-	89.34^{b}	---	”	2.07 ± 0.61^{g}

*--- No in vitro flowering; ** 1-2 flowers from the shoots*

Data represents mean±SE values of ten replicates repeated thrice, recorded after 4 weeks of culture. The mean values followed by the same letter in the superscript in a column do not differ significantly based on ANOVA and t-test at $p\leq0.05$.

Table 2: Effect of coconut water on *in vitro* flowering response in *A. ciliata*

Treatments							Shoot induction %	Response %	Response	Mean number of shoots
BA	Kinetin	IBA	GA_3	$AgNO_3$	CW (%)	Sucrose (%)				
1.0	-	0.5	-	5.0	1.0	3	90.71[a]	---	SM	1.42±0.37[cd]
1.0	-	0.5	-	5.0	2.5	3	88.53[b]	---	”	1.73±0.63[c]
1.0	-	0.5	-	5.0	5.0	3	85.36[e]	---	”	2.24±0.42[ab]
1.0	-	0.5	-	5.0	10	3	81.67[g]	---	”	2.38±0.54[a]
1.0	-	0.5	-	5.0	20	3	91.42[a]	---	”	2.12±0.62[ab]

--- No in vitro flowering; Data represents mean±SE values of ten replicates repeated thrice, recorded after 4 weeks of culture. The mean values followed by the same letter in the superscript in a column do not differ significantly based on ANOVA and t-test at p≤0.05.

Table 3: Effect of sucrose on *in vitro* flowering response in *A. ciliata*

Treatments						Shoot induction %	Response %	Response	Mean number of shoots
BA	Kinetin	IBA	GA_3	$AgNO_3$	Sucrose (%)				
-	1.0	0.5	-	5.0	3	91.58[a]	65.21	*In vitro* flowers formed**	2.42±0.42
0.5	-	-	-	-	1	82.48	---	*SM*	1.28±0.13
0.5	-	-	-	-	2	75.34	---	”	1.23±0.13
0.5	-	-	-	-	3	96.47	---	”	4.42±0.40
0.5	-	-	-	-	4	95.71	---	”	3.56±0.37
0.5	-	-	-	-	5	89.32	---	”	1.67±0.33
0.5	-	-	-	-	6	78.56	---	”	1.58±0.16
0.5	-	-	-	-	7	66.38	---	”	1.27±0.13
0.5	-	-	-	-	8	85.42	---	”	1.53±0.16
0.5	-	-	-	-	9	87.38	---	”	1.14±0.10
0.5	-	-	-	-	10	91.24	---	”	1.26±0.13
0.5	-	-	-	-	11	75..64	---	”	1.24±0.13
0.5	-	-	-	-	12	82.45	---	”	1.04±0.00

*--- No in vitro flowering; ** 1-2 flowers from the shoots Data represents mean±SE values of ten replicates repeated thrice, recorded after 4 weeks of culture. The mean values followed by the same letter in the superscript in a column do not differ significantly based on ANOVA and t-test at p≤0.05.*

Fig. 2: Morphogenic response of shoots of *A. ciliata* in different sucrose concentrations (1-12% sucrose From left to right)

3.4. Effect of photoperiod

Among the different photoperiods tested, *in vitro* flowering response was noticed after 2 months of inoculation after 8th subculture in 16 hours dark period at 25±2°C temperature (Table 4). Regarding the effect of photoperiod, when MS medium fortified with 1.0 mgl^{-1} Kinetin along with 0.5 mgl^{-1} IBA and 5.0 mgl^{-1} $AgNO_3$ and given 8/16 light and dark period resulted in 65.14% *in vitro* flower production and also 10/14 light/dark period has given off *in vitro* flowers comparatively in low percentage (42.75%). As the light hours increased to 14 and 18 hours, no *in vitro* flowering response was noticed even after 2 months of culture maintenance. Thus in *A. ciliata, in vitro* flowering occurred at a photoperiod having high percent of darkness and low exposure (8 hours) of light conditions. In contrast, in *S. acmella in vitro* flowering was achieved efficiently after 9 weeks of culture and the exposure of plantlets to 16/8 hour (light/dark) photoperiod [7]. The influencing effect of photoperiod along with other stimulating factors on *in vitro* flower induction is well adjudicated in experiments conducted in other plant species. For example, in *Rosa hybrida* flower induction occurred when the regenerated shoots were transferred to MS medium having hormone combinations *viz*. 13.3 mM BA and 9.3 mM Kinetin under the photoperiod of 12/12 (light/dark cycle) and efficient flower induction was noticed in 3 weeks interval for two consecutive times/ subculture passages on the same medium [20]. Experiments on *in vitro* flower induction was conducted in *Torenia fournieri* [21] and found that 60 days older shoots produced higher percentage of *in vitro* flowers when they were cultured in hormone-free ¼MS media supplemented with 60 gl^{-1} sucrose along with 1.0 gl^{-1} activated charcoal under 10 hour light period and 14 hour dark photoperiod. All these findings substantiates the role of photoperiod in flowering responses in plant species which is very particular in short day as well as long day plant categories.

Table 4: Effect of different photoperiod on *in vitro* flowering

Photoperiod (Light/Dark)	*In vitro* flowering %
8/16	65.21[a]
10/14	42.75[b]
14/10	NIL
18/6	NIL

Data represents mean values ± SE of 10 replicates repeated thrice, recorded after 4 weeks of culture. The mean values followed by the same letter in the superscript in a column do not differ significantly based on ANOVA and t-test at $p \leq 0.05$.

3.5. Effect of subculture duration

In the present study, *in vitro* flowers were formed in *A. ciliata* during the 8th subculture period when the shoots were transferred to MS medium fortified with 1.0 mgl^{-1} Kinetin and 0.5 mgl^{-1} IBA along with 5.0 mgl^{-1} $AgNO_3$ after 2 months of inoculation comprising 16 days dark period (Table 5). An average of 1-2 flowers were produced from the *in vitro* shoots which lasted for 10 days.

The subculture time and light period played an important role in *in vitro* flowering of *A. ciliata*. Subculturing of plantlets was done after every 4 weeks, 6 weeks and 8 weeks duration. Among these, subculturing of plantlets after 8 weeks showed *in vitro* flowering response (75.28 %). In *A. ciliata*, *in vitro* flowering was achieved during the 8th subculture period. In concomitant with this, in *Leptinella nana* produced vigorous growth of *in vitro* plantlet with prolific flowering after 16 months of inoculation at every 3 weeks interval subculture [22].

The schematic representation of different parameters standardized for inducing flowers *in vitro* in *A. ciliata* is shown in Fig. 3. Although 65.21% *in vitro* flowering was achieved in *A. ciliata*, a more refined system for *in vitro* flower induction needs to be elucidated that can be extended further for the bioproduction of bioactive alkamides from the *in vitro* flower heads in the species.

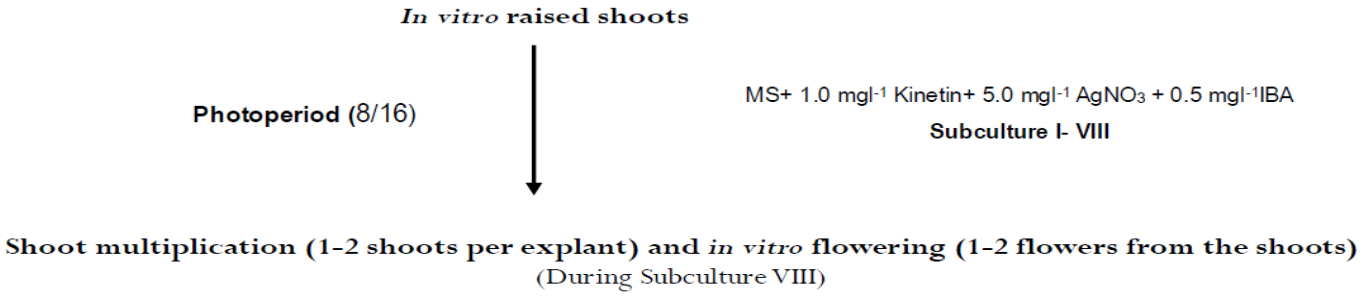

Fig. 3: Schematic representation of the Protocol for *in vitro* flowering in *A. ciliata*

Table 5: Effect of subculture duration on *in vitro* flowering in *A. ciliate*

Subculture period (weeks)	*In vitro* flowering %
4	NIL
6	NIL
8	65.21
10	NIL

4. CONCLUSION

This paper describes the influence of various parameters which influence the induction of *in vitro* flowering in *A. ciliata*. The shoots cultured in MS medium along with kinetin, IBA and $AgNO_3$ succeeded in producing *in vitro* flowers after the eighth subculture with a photoperiod of 8/16 light to dark hours. The study here offers a stepping stone for the enhanced production of spilanthol, a wonder compound as the flower head contains highest spilanthol content. Large scale production of *in vitro* flowers would benefit the ongoing research for elucidation of compound spilanthol which may later be useful for phytopharma industry.

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