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An Efficient Microcloning of *Spilanthes Ciliata* Kunth. For Conservation and Sustainable Utilization

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Abstract: *Spilanthes ciliata Kunth. (Family: Asteraceae) has got immense pharmacological applications because of the presence of various phytochemicals. Lack of vegetative propagation and poor seed germination capacity has made a gap between the need and supply of this plant. The present study focussed on the development of a reliable and an efficient micropropagation method to solve this problem. MS medium supplemented with different plant growth regulators were used for the shoot multiplication. MS medium along with 1.0 mgl^{-1} BA showed better response and further subculture resulted in scaling up of in vitro shoots on successive passages. In vitro rooting response was checked using half and full strength MS basal liquid medium and MS liquid medium supplemented with 0.5 mgl^{-1}IBA. Better rooting response was noticed in medium containing 0.5 mgl^{-1}IBA. The rooted plantlets were hardened and established in pots with 80% survival rate and were genetically uniform in the ISSR banding pattern. The established protocol can be utilized for conservation and sustainable utilization of S. ciliata for bioactive metabolite production.*

Keywords: *Spilanthes ciliata, microcloning, de novo shoots, ISSR markers, conservation, sustainable utilization*

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I. Introduction

Spilanthes ciliata Kunth (Family Asteraceae) is a medicinal cum ornamental plant widely distributed to tropical and sub tropical regions of the world [1]. Around 60 species of genus *Spilanthes* have been reported from different regions of the world. *Spilanthes* is well documented for its uses as a spice, antiseptic and antimicrobial agent, a remedy for tooth ache, cough, and throat and gum infections [2]. Extracts of this plant proved to possess anti-nociceptive activity against continuous inflammatory pain and anti-hyperalgesic activity, possibly by inhibiting prostaglandin synthesis. Various phytochemicals like alkaloids, glycosides, flavanoids, tannins, anthraquinones, saponins and cardiac glycosides have been reported in this plant. Among these, spilanthol, an alkamide is the pharmaceutically important phytochemical which account for most of its pharmacological applications.

S. ciliata is conventionally propagated through seeds which lose its viability within a short period of time. Moreover, propagation by seeds is also undesirable because of the highly heterozygous nature of the plant due to protandry, which prevents self pollination. Because of poor seed germination capacity and over exploitation of local population as well as pharmaceutical companies, there is a gap between demand and supply of this plant. Moreover, there is no previous report on *in vitro* propagation of *S. ciliata*. So the present study is a novel approach aimed on the development of an efficient micropropagation method in *S. ciliata* for large scale production of *in vitro* plantlets followed by genetic fidelity assessment of the microclones using ISSR markers for further isolation of secondary metabolite production from this industrially important medicinal species.

II. Materials And Methods

2.1 Plant material

Spilanthes ciliata kunth. (Syn. *Acmella ciliata* (kunth) Cass.) (Herbarium Voucher Nos. TBGT 32710-32711) collected from Aruvikkara, Thiruvananthapuram, Kerala, India maintained in the green house of Department of Botany, University College, Thiruvananthapuram, Kerala, India served as the source of explants for the present study.

2.2 Surface Sterilization

Explants like shoot tips and nodal segments (first, second, third and fourth) were taken and washed under running tap water to remove the dust particles, followed by washing with detergent solution, 0.5% (w/v) Labolene for 20 minutes and washed again in running tap water to remove the traces of detergent. Then, the explants were rinsed several times in distilled water before being treated with 0.1% (w/v) $HgCl_2$ at various time intervals (5-10 minutes) and subsequently rinsed 2-3 times with sterile distilled water.

2.3 Shoot culture establishment

Murashige and Skoog (MS) medium [3] supplemented with various plant growth regulators like BA/Kinetin (0.5, 1.0 and 2.0 mgl^{-1}) and NAA/ IAA (0.1 mgl^{-1}) either alone or in combination were checked for *in vitro* shoot production and multiplication. Individual shoots were transferred to fresh medium of the same composition that used for shoot multiplication for four subsequent subculture passages at an interval of 4 weeks each, for enhancing the multiplication.

2.4 Direct *de novo* shoot organogenesis

In vitro-derived leaf explants were cultured in MS medium supplemented with 1.0 mgl^{-1} BA along with 0.5 mgl^{-1} Kinetin as well as 0.5 mgl^{-1} BA in combination with 1.0 and 2.0 mgl^{-1} IAA or IBA for inducing direct *de novo* regeneration of shoots. Such shoots were also transferred to fresh medium of the same composition for increasing the multiplication frequency.

2.5 Rooting, acclimatization and field transfer

The elongated shoots (5-7 cm) obtained during shoot multiplication were suspended on filter paper bridges in MS basal (R1) and ½ MS (R2) as well as MS liquid medium containing 0.5 mgl^{-1} IBA (R3). Liquid medium was used for this experiment. The *in vitro* shoots producing roots were then slowly removed from the culture tubes and gently washed in tap water in order to remove the traces of minerals. Then it is transferred to cups containing garden soil and sand (in the ratio 3:1) covered with a polythene bag to maintain humidity and after 7-10 days some holes were made in it. After 2 weeks, these plants were transferred to a green house.

2.6 Genetic uniformity analysis by ISSR markers

The genomic DNA from total ten samples, of which one sample collected from mother plant and nine *in vitro*-derived plants, were isolated using cetyltrimethylammonium bromide (CTAB) method [4]. ISSR assay was carried out in 25 µL reaction mixture containing 0.2 mM dNTP's, 10 mM Tris-HCL,1.5 mM $MgCl_2$, 50 mM KCl, 0.1%Triton X-100, 1.0 U Taq DNA polymerase (Finzymes, Helsinki, Finland),15 pmol primers (`IDT, Coralville, USA) and 50 ng of genomic DNA. The amplification was performed in a thermal cycler (Eppendorf ESP-S). After the initial cycle of 2 min at 93 °C, 2 min at 50 to 55 °C*, and 2 min at 72 °C .A total of 39 cycles of 1 min at 93 °C, 1 min at 50 to 55 °C and 1 min at 72 °C were performed. The last cycle was performed by 10 min extension at 72 °C. Amplified products where resolved in 1.40% agarose gel (1xTBE) followed by EtBr staining.

*annealing temperature of the primers ranges from 50 to 55 °C for the different primers used in this study.

III. Results

3.1 Surface sterilization and Explant selection

Explants like shoot tips and nodal segments (first, second, third, fourth) were inoculated in MS medium supplemented with appropriate plant growth regulators. When the nodal segments were inoculated in MS medium containing 0.5 mgl^{-1} BA, the explant started bud breaking after fifth day of inoculation and produced shoots with maximum 80.14 % response. Maximum response percentage was obtained when the explants were surface sterilized with 0.1% $HgCl_2$ for 10 minutes (TABLE 1). As the time interval was reduced to 5 and 7 minutes, the contamination percentage became higher. The first nodal segments give better response when compared to other explant types. So, for further experiments, first nodal segments have been selected for shoot multiplication.

TABLE1: Standardization Of $Hgcl_2$ Exposure Period During Surface Sterilization In Different Explants Of *S. ciliata*

Explant type	$HgCl_2$ exposure period (minutes)	Response percentage
Shoot tips	5	21.23^g
	7	42.56^c
	10	76.43^b
Node I	5	35.03^d
	7	42.79^c
	10	80.14^a

Node II	5	23.41^{g}
	7	30.19^{e}
	10	31.42^{e}
Node III	5	32.13^{e}
	7	35.42^{d}
	10	41.52^{c}
Node IV	5	30.12^{e}
	7	28.43^{f}
	10	32.64^{e}

Data represents mean 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 $p \leq 0.05$

3.2 Shoot initiation

The first nodal segment inoculated in MS medium augmented with 0.5 mgl^{-1} BA produced 2.7±0.26 shoots (Fig 1a) which upon subsequent subculture on different plant growth hormones produced multiple shoots. In almost all treatments, more than one shoots have been produced. Significant differences were observed in the number of shoots per explant, length of shoots and the number of nodes per shoot among various concentration and combination of PGRs (TABLE 2).

3.2.1 Effect of individual concentration of cytokinins

Different concentrations of BA and Kinetin facilitated the differentiation of shoot buds. MS medium when fortified with 1.0 mgl^{-1} BA, a maximum of 4.4±0.40 shoots were produced with a mean length of 6.0 ±0.83 cm and 3.43±0.22 nodes per shoot (TABLE 2). When the concentration of BA was increased to 2.0 mgl^{-1}, mean number of shoots decreased to 2.1±0.28 and it also showed the formation of basal callus. Basal callus formation was obtained invariably in all the concentration of BA and the amount of callusing increased as the concentration of plant hormones increased. MS medium when aided with different concentration of Kinetin resulted in single-to-double shoot formation only (Fig 1b). But the plantlets thus produced were vigorous with thick stem and broad leaves and almost 93% axillary shoots produced long roots. The axillary shoots produced roots in almost all concentrations of Kinetin and its frequency was higher in lower concentration (0.5 mgl^{-1}). MS medium along with 0.5 mgl^{-1} Kinetin produced 2.8±0.38 shoots. Maximum shoot length (9.0 ±0.62) was recorded in MS medium fortified with 1.0 mgl^{-1} Kinetin.

3.2.2 Effect of combination of cytokinins

Combination of two cytokinins *viz*. BA and Kinetin also effected multiple shoot bud induction similar to that of either BA or Kinetin supplemented individually. However, number of shoots produced was lesser than that of 1.0 mgl^{-1} BA which showed maximum response when employed individually. But the rate of multiplication was more than that of different concentration of Kinetin. Healthy vigorous shoots and broad, green leaves were the characteristic features noticed (Fig 1c). When BA and Kinetin were used in combination, most of them showed basal callusing with prominent roots. Maximum 3.2±0.39 shoots were obtained in MS medium supplemented with 2.0 mgl^{-1} BA along with 0.5 mgl^{-1} Kinetin (TABLE 2).

3.2.3 Effect of combination of cytokinins and auxins

Cytokinin-auxin combination showed similar results on shoot production as that of treatment with different cytokinin combination and concentrations. Maximum (3.1 ± 0.27) shoots having 6.9±0.45 cm mean length and 3.07 ±0.20 nodes per shoots were obtained in MS medium supplemented with 1.0 mgl^{-1} BA and 0.1 mgl^{-1} NAA. The plantlets formed were having long healthy multiple shoots with elongated roots and very little basal callus. Mean length of shoots and mean number of shoots were higher in MS medium augmented 1.0 mgl^{-1} BA and 0.1 mgl^{-1} NAA combination (TABLE 2).

TABLE 2: Morphogenic response of *S.ciliata* during shoot culture initiation

Plant Growth Regulators (mgl^{-1})				Mean number of Shoots	Mean length (cm)	Nodes per shoot
BA	Kinetin	NAA	IAA			
0.5	-	-	-	2.7±0.26^{c}	4.4±0.56^{f}	2.9±0.45^{d}
1.0	-	-	-	4.4±0.40^{a}	6.0±0.83^{c}	3.4±0.22^{c}
2.0	-	-	-	2.1±0.28^{e}	1.5±0.60^{k}	1.7±1.30^{g}
-	0.5	-	-	2.8±0.38^{c}	4.6±0.77^{f}	2.7±0.16^{e}
-	1.0	-		1.2±0.13^{g}	9.0±0.62^{a}	3.9±0.23^{b}
-	2.0	-	-	1.8±0.25^{f}	4.9±0.79^{e}	4.7±1.9^{a}
0.5	0.5	-	-	2.4±0.27^{d}	3.6±0.49^{g}	2.5±0.25^{f}
1.0	0.5	-	-	2.4±0.22^{d}	3.2±0.44^{h}	2.4±0.35^{f}
2.0	0.5	-	-	3.2±0.39^{b}	4.8±0.83^{e}	2.5±0.33^{f}
0.5	-	0.1	-	2.1±0.28^{e}	5.2±1.09^{d}	2.2±0.38

1.0	-	0.1	-	3.1±0.28[b]	6.9±0.45[b]	3.1±0.19[d]
2.0	-	0.1	-	2.4±0.16[d]	5.8±0.47[c]	3.0±0.23[d]
0.5	-	-	0.1	2.5±0.30[d]	3.1±0.30[i]	1.8±0.15[g]
1.0	-	-	0.1	2.4±0.48[d]	5.3±0.53[d]	2.7±0.27[e]
2.0	-	-	0.1	1.8±0.28[f]	2.8±0.28[j]	2.4±0.14[f]

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≤0.05.

3.3 Shoot multiplication through subculture passages

Single shoots isolated from the established shoot cultures when transferred to the respective medium for further subculture passages and as the subculture passages went on, two fold increase in the frequency of multiplication was noticed (TABLE 3). MS medium fortified with 1.0 mgl^{-1} BA in subsequent subculture passages produced a mean number of 7.0±0.66, 11.2±0.23, 20.7 ±0.52, 37.4±0.23 shoots per explant during first, second, third and fourth subculture passages respectively (Fig 1d & e) (TABLE 3). This was the highest multiplication frequency among the various hormonal composition and concentration tested in the present study. However, the rate of multiplication was comparatively lesser in BA-NAA combination compared to the two fold scale up in BA-IAA (TABLE 3).

TABLE 3: Scaling up of Shoot multiplication in *S. ciliata* during subculture passages

Plant Growth Regulators (mgl^{-1})				Mean number of Shoots			
BA	Kinetin	NAA	IAA	Subculture I	Subculture II	Subculture III	Subculture IV
0.5	-	-	-	4.5±0.72[c]	8.2±0.51[c]	15.4±0.78[b]	25.8±0.48[b]
1.0	-	-	-	7.0±0.66[a]	11.2±0.23[a]	20.7±0.52[a]	37.4±0.23[a]
2.0	-	-	-	4.0±0.32[d]	7.4±0.14[d]	13.1±0.42[c]	21.1±0.58[c]
-	0.5	-	-	3.1±0.42[f]	5.1±0.47[f]	9.1±0.82[e]	15.7±0.16[e]
-	1.0	-	-	2.1±0.37[h]	2.8±0.72[i]	5.6±0.49[g]	9.8±0.42[g]
-	2.0	-	-	5.2±0.14[b]	9.4±0.38[b]	13±0.67[c]	16.1±0.31[e]
0.5	0.5	-	-	3.2±0.59[f]	5.1±0.24[f]	10.1±0.18[d]	17.2±0.90[d]
1.0	0.5	-	-	2.3±0.67[g]	4.4±0.17[h]	7.5±0.64[f]	12.4±0.72[f]
2.0	0.5	-	-	4.1±0.43[d]	7.5±0.57[d]	13.1±0.42[c]	21.5±0.18[c]
0.5	-	0.1	-	2.1±0.73[h]	4.2±0.21[h]	6.0±0.90[g]	10.4±0.72[g]
1.0	-	0.1	-	2.4±0.52[g]	4.6±0.78[g]	7.1±0.31[f]	11.5±0.84[f]
2.0	-	0.1	-	1.5±0.34[i]	2.8±0.49[i]	4.3±0.79[h]	8.2±0.57[h]
0.5	-	-	0.1	3.2±0.42[f]	5.5±0.81[e]	11.2±0.71[d]	20.3±0.49[c]
1.0	-	-	0.1	3.8±0.42[e]	7.2±0.52[d]	12.5±0.62[c]	21.8±0.54[c]
2.0	-	-	0.1	3.0±0.66[f]	5.1±0.47[f]	9.6±0.38[e]	15.3±0.21[e]

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≤0.05.

3.4 *De novo* shoot organogenesis

Direct *de novo* regeneration of shoots was observed from the *in vitro*-derived leaf explants in MS medium supplemented with 1.0 mgl^{-1} BA along with 0.5 mgl^{-1} Kinetin (Fig 1f) as well as 0.5 mgl^{-1} BA in combination with 1.0 and 2.0 mgl^{-1} IAA or IBA (Fig 1g). Short but healthy shoots were formed from the leaf explants. All the shoots were produced from the proximal region of the *in vitro* leaf and no direct shoot organogenesis was observed from the distal part.

TABLE 4: Direct *de novo* shoot organogenesis in *S. ciliata*

Plant Growth Regulators (mgl^{-1})				Mean number of shoots
BA	Kinetin	IAA	IBA	
0.5	0.5	-	-	0.0 ±0.00
1	0.5	-	-	2.1±0.45[d]
2	0.5	-	-	2.5±0.24[c]
0.5	-	0.5	-	0.0 ±0.00
0.5	-	1	-	3.1±0.64[b]
0.5	-	2	-	3.4±0.70[a]
2	-	0.1	-	2.1±0.29[d]
0.5	-	-	0.5	0.0 ±0.00
0.5	-	-	1	2.2±0.16[d]
0.5	-	-	2	3.1±0.37[b]

Data represents mean values ± SE of 10 replicates repeated thrice. 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

3.5 *In vitro* rooting

In vitro shoots of *S. ciliata* transferred to a rooting medium comprising different hormonal and salt composition *viz.* full strength MS basal liquid medium, half strength MS liquid medium and MS liquid medium fortified with 0.5 mgl^{-1} IBA for plantlet development produced roots. Better response was observed in MS liquid medium augmented with IBA. A mean number of 25.1±0.26 small roots with hairs were produced in this treatment; whereas, a mean number of 10.7±0.64 white long roots were produced on MS basal medium (Fig 1h) (TABLE 5).

TABLE 5: Rooting response in *S.ciliata*

PGR Concentration (mgl^{-1})	Number of roots	Length of roots (cm)	Colour of roots	Nature of roots
MS basal medium (R1)	10.7±0.64^{b}	12.4±0.31^{a}	White	Long, thin slender roots
½ MS medium (R2)	4.2±0.43^{c}	10.1±0.42^{b}	Lightly green and white	Long, thin tapering roots
MS+IBA(0.5) (R3)	25.1±0.26^{a}	0.5±0.18^{c}	White	Numerous roots with very thin and short hairs

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 & t-test at p≤0.05

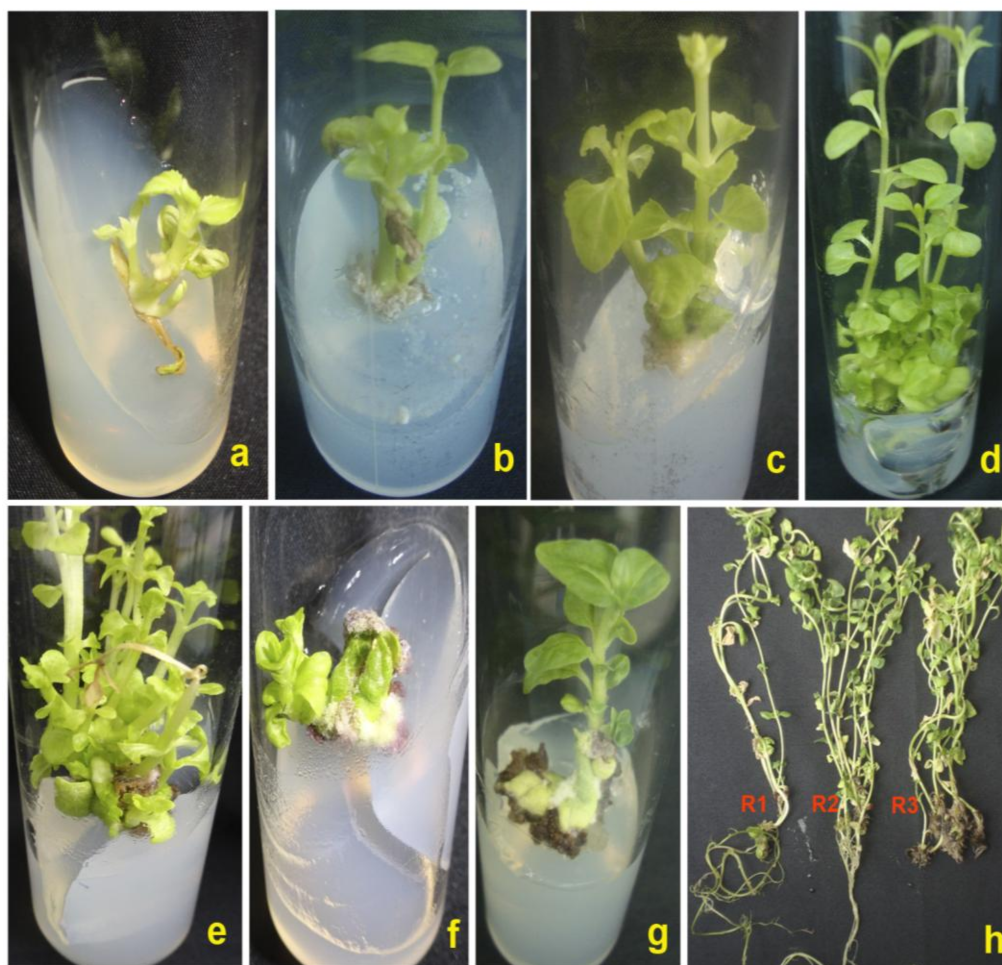

Fig 1: Microcloning of *S. ciliata*. a) Shoot initiation in MS medium augmented with 0.5 mgl^{-1} BA. b) Shoot initiation in MS medium along with 0.5 mgl^{-1} Kinetin. c) Multiple shoot induction in MS medium supplemented with 2.0 mgl^{-1} BA along with 0.5 mgl^{-1} Kinetin. d&e) Scaling up of shoots in MS medium fortified with 1.0 mgl^{-1} BA. f&g) Direct *de novo* regeneration of shoots in MS medium supplemented with 1.0 mgl^{-1} BA along with 0.5 mgl^{-1} Kinetin and 0.5 mgl^{-1} BA in combination with 1.0 mgl^{-1} IAA respectively. h) *In vitro* rooting of *S. ciliata*

3.6 Acclimatization /Hardening and field transfer

The rooted plantlets were removed from culture vessels and washed under running tap water. Then the plantlets were transferred to a pot having 3:1 garden soil and sand and covered with polythene bag. The hardening procedure produced 70-80% plantlet establishment. These were slowly transferred to a green house where they survived at 80% efficiency.

3.7 Genetic uniformity of the plantlets

The microcloned plantlets exhibited uniform banding pattern irrespective of the ISSR primers confirming their genetic fidelity (Fig. 2).

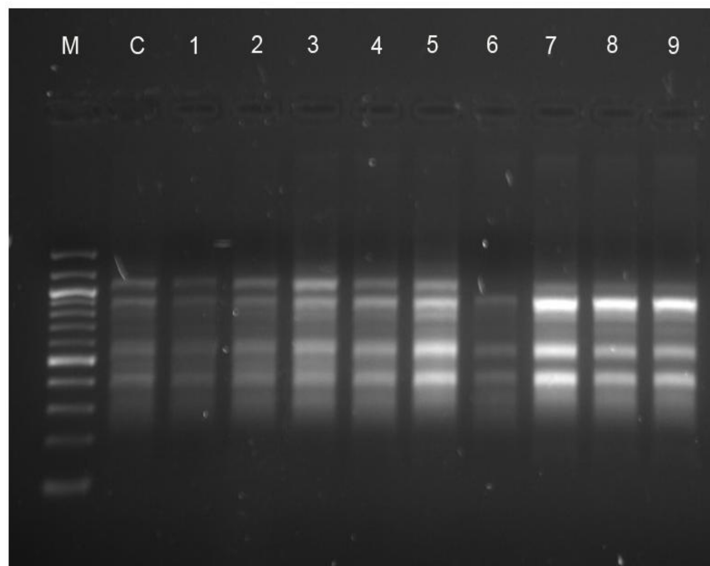

Figure 2: ISSR banding pattern of the microclones of *S. ciliata*. (*M* Marker, *C* Mother plant, *lanes 1-9* microclones)

IV. Discussion

Medicinal plants are an important health and economic component of the floras in developed as well as developing countries. Increasing world – wide interest in herbal remedies, expanding reliance of local health care of traditional remedies and a renewed interest in the development of pharmaceuticals from plant sources have greatly increased the trade in medicinal plant materials. With the increasing worldwide demand for plant-derived medicines, there has been a simultaneous increase in the demand for raw materials. However, the increasing human and livestock populations have affected the status of wild plants, particularly those used for making herbal medicines. *Spilanthes ciliata* is one such medicinal plant with immense pharmacological applications and due to this demanding feature; local populations as well as pharmaceutical companies are over-exploiting this plant to an extent that there is a gap between requirement and supply of it. Hence the present study focussed on the rapid micropropagation and multiplication of this medicinally important plant as a part of standardizing an efficient *in vitro* shoot culture establishment for further experimental procedures for secondary metabolite elucidation.

4.1 Shoot culture establishment

4.1.**1 Surface sterilization**

The plants growing *in vivo* harbour a large number of microorganisms primarily on their surfaces and in some cases, they are concealed within the explants. The rapid production of pathogen-free plants is a fundamental goal of the plant tissue culture process and an aseptic technique is critical for the success of plant cell, tissue and organ culture. To initiate an aseptic culture, certain disinfectants like sodium hypochlorite, mercuric chloride, ethanol, fungicides and antibiotics were generally used. Subsequently, these surface sterilants were washed off several times with sterile distilled water. The choice of surface sterilant depends upon the age, type of tissue and its hardness. The young nodes of *S. ciliata* washed with 0.5% (w/v) labolene for 10 minutes and then in 0.1% (w/v) $HgCl_2$ for 10 minutes produced maximum shoot initiation with 80% response percentage. In contrast, exposure of explants to 0.1% (w/v) $HgCl_2$ for 5-6 minutes has produced 80% of the aseptic cultures and maximum number of shoots; while the exposure at higher time intervals proved lethal for the plant in *S. acmella* [5, 6, 7]. Also the use of 0.08% (w/v) $HgCl_2$ for 5 minutes and then with 15% (w/v) Clorox treatment showed maximum response percentage in *S. acmella* [8].

4.2 Shoot initiation and multiplication

For shoot initiation, 0.5 mgl^{-1} BA was found to be effective. Maximum numbers of 4.4 ±0.40 shoots were produced in *S. ciliata* in MS medium fortified with 1.0 mgl^{-1} BA. This is in corroboration with the earlier findings in *S. acmella* which reported maximum shoot initiation within 3-4 days in the medium containing BA alone [9]. Increase in the concentration of BA resulted in the formation of basal callus. Higher concentration of BA could induce the formation of callus tissue that also caused the chromosomal instability of the regenerated plants. More multiple shoots were formed in *Hypericum perforatum,* when the explants were cultured in MS medium supplemented with low concentration of BA (0.5-2.5 mgl^{-1}) [10] is in agreement with our finding. BA was found to be most effective for the induction of multiple shoot formation when the concentration was not more than 1.0 mgl^{-1}.

In the present study, MS medium supplemented with 0.5 mgl^{-1} Kinetin produced 2.8±0.38 shoots and they were healthy and vigorous. Similar results reported in *S. acmella* [6] and other medicinal species *viz. Adhatoda beddomei* [11], *Dendrocalamus strictus* [12] and *Peganum harmala* [13]. A combined effect of cytokinins namely BA and Kinetin also produced healthy shoots with roots in *S. ciliata*, while cytokinin and auxin combination evoked no significant response when compared to all other treatments. However, maximum multiplication (7.0 shoots) per explants was reported in *S. acmella* [14] and *S. mauritiana* [15] in MS medium supplemented with BA and NAA.

4.3 Shoot multiplication through subculture passages

Subculture of initiated shoots of *S. ciliata* in fresh medium of the same composition resulted in two fold increase in the multiplication rate. Number of shoots went on increasing as the subculture passages goes on. Similarly in *S. acmella,* the number of shoots formed increased about two fold after the first subculturing of the separated individual shoots and the shoot height found to be reduced as the concentration of BA increased in the culture medium. Complete root system formed along with healthy multiple shoots in MS medium containing 0.5 mgl^{-1} BA after the first subculture indicated that repeated subculturing could produce a large amount of *in vitro* plantlets of *S. acmella* for future use [7], in agreement with our findings.

4.4 *De novo* shoot regeneration

In *S. ciliata, de novo* shoot regeneration was noticed in MS medium augmented with combination of cytokinins and also in cytokinin auxin combination. Maximum *de novo* shoot formation occurred in MS medium having BA and IAA supplementation. Similarly adventitious shoots were successfully regenerated from the leaf explants in MS medium containing 1.0 mgl^{-1} BA and 0.1 mgl^{-1} NAA in *S. acmella* [16]. In contrast with this in *S. acmella,* cytokinin alone induced shoot formation as these explants contain sufficient level of endogenous auxins or capable of its *de novo* synthesis, which induced shoot formation [17].

4.5 *In Vitro* Rooting

For the development of a perfect plantlet, the elongated shoots have to be transferred to a rooting medium having different hormonal and salt composition from the shoot multiplication medium. However, the nutritive medium for rooting, vary from tissue to tissue as well as species to species. Efficiently developed and elongated shoots produced short thin roots in half strength MS medium supplemented with low concentration (0.5-1.0 mgl^{-1}) of BA and NAA in callus regenerated plants of *S. acmella* [18]. MS half strength medium supplemented with 1.0 mgl^{-1} of IBA proved best in root formation in *S. acmella* in earlier study [6]. Among all the treatments, good response of rooting was observed in *S. ciliata* in medium containing 0.5 mgl^{-1} IBA as similar to the reports in *S. acmella* [19].

4.6 Acclimatization And Field Transfer

Most of the species grown under *in vitro* conditions require proper acclimatization process in order to ensure maximum survival rate and vigorous growth upon field transfer. The regenerated plantlets with sufficient roots were taken out of the culture vessels, excess minerals were washed off and then as per the hardening procedure described were planted in to pot containing 3: 1 garden soil and sand, subsequently transferred to a green house survived at 80% efficiency. This was similar to the report in *S. acmella* [5, 6] only exception with our result is the sprinkling of half strength MS medium to the pot on alternate days before transferring to the field. The regenerated plants were morphologically and genetically identical as they exhibited uniform banding pattern in ISSR analysis

V. Conclusion

The present study revealed a reproducible and reliable microcloning procedure for *in vitro* large scale production of *Spilanthes ciliata,* a high demanding medicinal plant and the genetic uniformity analysis of the microclones *via* ISSR markers for the first time. These findings can be further extended for the standardization

of a procedure for the *in vitro* production of the bioactive compound spilanthol for benefiting the pharmaceutical applications.

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