

MICROPROPAGATION OF *Spilanthes acmella* MURR. FROM NODAL SEGMENT AND APICAL SHOOT TIP CULTURES

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ABSTRACT

Spilanthes acmella Murr. was successfully micropropagated using nodal segments and apical shoot tips. The explants were cultured on MS medium supplemented with different concentrations of BAP for shoot initiation. All the concentrations of BAP alone induced shoot regeneration with varying frequency. High regeneration frequency was observed at 2 mg/L concentration of BAP in apical shoot tips (70%) and nodal segment (60%). The regenerated shoots were multiplied on MS medium with different concentrations of BAP alone and in combination with NAA and IAA. Highest frequency of multiple shoot induction (90%) was observed at 2.0 mg/L BAP + 1.0 mg/L IAA with maximum number of shoots 25 and 40 after first and second subculture. The highest shoot length (4.8 cm) was observed at 2.0 mg/L BAP + 1.0 mg/L NAA with 80% shoot multiplication. The regenerated shoots were transferred onto rooting media different concentrations of IBA and NAA. All the concentrations of IBA and NAA produced roots with varying frequencies. High percentage of rooting (90%) was observed for 1.0 mg/L IBA and NAA at 1.0 mg/L also showed good percentage of rooting (80%). Rooted plantlets were hardened and established in pots with 100% survival rate.

KEYWORDS: *Spilanthes acmella*, Micropropagation, Nodal Segments, Apical Shoot Tips, Multiple Shoots.

Medicinal plants are an important source of a large number of important chemicals with impressive biological activities like antimicrobial, antibiotics, insecticidal and valuable pharmacological products. *Spilanthes acmella* Murr. (Family Asteraceae) is an important medicinal plant traditionally used as a remedy for toothache and in the treatment of many ailments like flu, cough, psoriasis, rheumatism, fever, tuberculosis, stammering in children etc. (Sahu et.al.,2011). *S.acmella* has been well documented for its uses as antimalarial, antibacterial, antifungal, larvicidal, anti-inflammatory and immunomodulating properties (Sharma & Shahzad, 2013). It is an erect herb with beautiful flowers on head inflorescence. The active chemical component is spilanthol, an alkamide which is present in roots and all aerial parts of the plant (Sahu et.al., 2011). The plant has been found to produce important secondary metabolites like spilanthol,scopoletin,myrecene, α amyryn, β amyryn etc (Prachayasittikul et.al., 2013). The plant extract has great industrial demand for its use in pharmaceutical, cosmetic and toothpaste industry.

Because of its wider applications for commercial use, the plant is quickly getting depleted from its natural habitat. The plant is not meeting the industrial demand due to less commercial cultivation. The dried plant parts like flowers and roots are being sold at a market rate of Rs.2000-3000/kg. The plant extracts like the pure spilanthol standard obtained from this plant is being sold for a very high price in the international market. Hence, the present study has been undertaken to standardize a micropropagation protocol which can be utilized for large scale cultivation of

S.acmella. Although several workers have studied the *in vitro* regeneration of *S.acmella* through hypocotyls, leaf, axillary buds and nodal explants, but the percentage of shoots regenerated was very low (Saritha et.al.,2012; Niratker et.al.,2014; Haw & Keng, 2003; Singh & Chaturvedi, 2010) . There are limited studies so far on *in vitro* regeneration of *S.acmella* with apical shoot tip explants (Sharma & Shehzad, 2013) but the regenerated multiple shoots exhibited retarded growth. The present paper reports the micropropagation of *S.acmella* using apical shoot tip and nodal explants with higher frequency of multiple shoot regeneration.

MATERIALS AND METHODS

Establishment of *Spilanthes acmella* Plants

The seeds of *S.acmella* were procured from Medicinal and Aromatic Plants Research Station, Rajendranagar, Hyderabad and maintained in the Botanical garden in Osmania University College for Women, Koti, Hyderabad. Nodal explants and apical shoot tips were collected from the field grown plants and used for standardization of micropropagation protocols.

Sterilization of Explants

The nodal segments and apical shoot tip explants were thoroughly washed with running tap water and treated with 1% bavistin for 20 minutes, followed by three rinses with distilled water. They were washed with tween twenty for 2 minutes and rinsed thrice with sterile water. Then the explants were rinsed with 70% alcohol for 1 minute followed by distilled water washing twice. The explants were then treated

with 0.1% (w/v) mercuric chloride for 3 minutes under aseptic conditions. After this explants were then thoroughly washed 4 to 5 times with sterilized double distilled water to remove the traces of mercuric chloride.

Media Preparation and Culture Conditions

MS basal medium (Murashige & Skoog, 1962) supplemented with plant growth regulators and gelled with 3% (w/v) sucrose and 0.8% agar was used throughout the study. The pH of medium was adjusted to 5.8 prior to autoclaving for 20 minutes at 121°C and 15 psi. The cultures were maintained at 25 ± 2°C under 16/8 light/dark cycle with the light intensity of 3000 lux. Different plant growth regulators like Benzyl amino purine (BAP), Naphthalene acetic acid (NAA), Indole-3-acetic acid (IAA), Indole-3-Butyric acid (IBA) were employed for regeneration and root induction studies.

Shoot Induction from Nodal and Apical Bud

The nodal segments of about 1cm length were taken and their extreme portions were cut and taken for inoculation. A little portion of the apical shoot tips was cut from below prior to inoculation. All the sterilized explants were inoculated on MS medium fortified with different concentrations of BAP (0.5-2.0 mg/L). The explants were observed daily for recording time taken in bud break and percent bud break. The initial data for percent response was recorded after 28 days of culture.

Shoot Multiplication

When the regenerated shoots from apical shoot tip and auxiliary shoots from nodal explants attained a length of 2-3cm, they were excised and inoculated onto ½ MS media. The media was supplemented with various combinations and concentrations of BAP, IAA and NAA to determine best hormonal concentration for multiplication and growth. For each explant, two subculture cycles were performed with 15 days interval and the data were recorded during sub culture and after 30 days of second culture.

Rooting

In vitro regenerated shoots from nodal explants and apical buds with 2-3 cm height were excised and transferred to rooting medium consisting of MS medium supplemented with different concentrations (0.5 mg/L to 3.0 mg/L) of IBA and NAA.

Hardening and Acclimatization

The *in vitro* developed plantlets were removed from the rooting medium and washed thoroughly with

sterile water to remove agar. The plantlets were then transferred into small cups containing sterilized soil and sand mixture (3:1). They were kept in moistened heat chamber. After 15 days, the plants were transferred to bigger polythene bags in greenhouse and were maintained under natural conditions of day length, temperature and humidity.

RESULTS

Explant Response

The nodal and apical shoot tip explants couldn't produce any multiple shoots on MS basal medium that served as a control. Only a single shoot emerged from the nodal segment and the shoot tip got elongated to produce a single shoot on MS medium. All the concentrations of BAP alone and in combination with other hormones induced shoot regeneration with varying frequency of plant regeneration. Shoot initiation from nodal explants and apical meristem was observed within one week of inoculation and later they developed to individual shoots (Fig1 A,B).



Figure 1: Multiple shoots from (A). Nodal segments and (B). Apical shoot tips of *S.acmella* on MS medium with BAP 2mg/L.

Among all the concentrations of BAP tested, high frequency of plant regeneration i.e 60% was observed in the medium supplemented with 2.0 mg/L BAP with nodal segment explants. For the nodal explants, 0.5 and 1.0 mg/L BAP concentration gave 50 % of regeneration and it was highest at 2.0 mg/L concentration i.e. 60%. The regeneration percentage reduced to 50% and 40% on further increase in the concentration of BAP to 3 and 4 mg/L respectively (Table1).

Table1: Effect of different concentration of BAP on regeneration from apical and nodal explants of *S.acmella*

Explant	Concentration of BAP (mg/L)	No. of explants inoculated	No. of explants responded	Response %	No. Of days for bud break	No. Of shoots per explant	Shoot length (cm) Mean \pm SE
Nodal segments	Control	10	2	20	14	1	1.4 \pm 0.05
	0.5	10	5	50	7	2	2.5 \pm 0.14
	1.0	10	5	50	7	2	2.8 \pm 0.07
	2.0	10	6	60	5	2	3.2 \pm 0.10
	3.0	10	5	50	9	2	3.0 \pm 0.10
	4.0	10	4	40	10	2	2.9 \pm 0.08
Apical Shoot tips	Control	10	2	20	10	1	2.4 \pm 0.10
	0.5	10	6	60	7	4	3.0 \pm 0.10
	1.0	10	7	70	8	4	3.2 \pm 0.05
	2.0	10	7	70	8	4	3.5 \pm 0.05
	3.0	10	6	60	8	4	2.5 \pm 0.10
	4.0	10	5	50	10	2	2.3 \pm 0.06

Values are \pm SE of 5 replicates

Plant regeneration after 4 weeks in culture

For the apical shoot tip explants, high frequency of plant regeneration of 70% was observed in the medium supplemented with 2.0 mg/L BAP. The 50% regeneration was observed at 0.5 mg/L BAP and it increased to 70% at 1.0 and 2.0 mg/L concentrations of BAP. Thereafter, it decreased to 60% and 50% on further increase in BAP to 3.0 and 4.0 mg/L respectively (Table 1).

Though with BAP the frequency of regeneration was good, the height of multiple shoots was observed to be less with the highest being 3.5 for apical shoot tip explants and 3.2 for nodal explants at 2 mg/L concentration. The effect of different concentrations of BAP on regeneration from apical and nodal explants of *S.acmella* is shown in Table 1.

Shoot Proliferation

Though cytokinin BAP alone is capable of inducing shoots, the combinations of BAP with NAA and IAA were tried to improve the plant regeneration in an attempt to increase the number and length of multiple shoots. Multiple shoots initiated and proliferated on all the concentrations and combinations of BAP, NAA and IAA tested.

The frequency of multiple shoot induction on BAP from nodal explants ranged from 50 to 70% with good number of 5 to 8 shoots after first subculture and 8 to 15 shoots after second sub culture with shoot lengths (2.0-3.6 cm). The frequency of multiple shoot induction on BAP from apical shoot tips ranged from 70 to 90% with high number of shoots 8 to 15 after first subculture

and 20 to 35 shoots after second subculture (Tables 2 & 3) (Fig 2A-2D).





Figure 2: Shoot proliferation and multiplication, A. On MS medium with BAP 2mg/L after first subculture B. On MS medium with BAP 2mg/L after second subculture C. On MS medium with BAP2mg/L and IAA 1mg/L D. On MS medium with BAP 2 mg/L and NAA 1mg/L.

Table 2: Effects of different plant growth regulators on shoot multiplication from nodal explants of *S.acmella*

Nodal explants						
Concentration of hormones	No. Of explants inoculated	No. of explants responded	Response %	No. of shoots after I subculture	No.of shoots after II subculture	Length of shoots
Control	10	5	50	1.0	1.0	2.0±0.10
0.5 BAP	10	7	70	6	12	2.8±0.10
1.0 BAP	10	7	70	6	14	3.2±0.10
1.5 BAP	10	8	70	8	16	3.5±0.10
2.0 BAP	10	8	80	8	20	3.8±0.01
2.5 BAP	10	7	70	6	8	3.6±0.02
3.0BAP	10	6	60	5	11	3.0±0.00
2.0BAP+0.5NAA	10	7	70	8	18	4.2±0.10
2.0BAP+1.0NAA	10	8	80	10	20	4.4±0.10
2.0BAP+2.0NAA	10	6	60	6	15	4.0±0.10
2.0BAP+0.5IAA	10	7	70	9	20	3.9±0.00
2.0 BAP+1.0IAA	10	9	90	10	25	4.0±0.00
2.0 BAP+2.0IAA	10	8	80	8	18	4.1±0.10

Values are ± SE of 5 replicates

Among the different combinations of BAP and NAA for nodal explants, the combination of 2 mg/L BAP + 1 mg/L NAA gave the highest regeneration percentage (80%) and the length of shoots was also observed to be 4.4 cm at this concentration. The combination of 2 mg/L BAP + 0.5 NAA gave 70% regeneration with 4.2 cm of shoots. The frequency of shoot regeneration reduced to 60 % on combination of 2.0 mg/L BAP and 0.5 mg/L NAA and the shoot length was also less (4cm).

Among the different combinations of BAP and IAA for apical shoot tips, 2mg/l BAP with 1 mg/L IAA gave the highest frequency i.e 90% of regeneration and the shoot length was 4.2 cm at this combination. The combination of BAP (2mg/L) with 1 mg/L NAA produced longest shoots of 4.8 cm with 80 % multiple shoot induction.

The effects of different plant growth regulators on shoot multiplication from nodal and apical shoot tip explants of *S.acmella* is shown in Table3 and Table 4.

Rooting and Acclimatization

The induction of roots was observed in some cases in the BAP multiplication medium itself ranging from concentration 0.5 to 1.0 mg/L without transfer into a separate rooting medium (Fig 3A). However, these roots were very thin, short and very less in number. MS full strength and half strength media produced 20 and 25 % of rooting respectively but the roots were observed to be short, thin and less (5 to 6) in number. Many roots of good quality were produced by inoculating the shoots on different concentrations of IBA and NAA (Fig 3B, 3C).



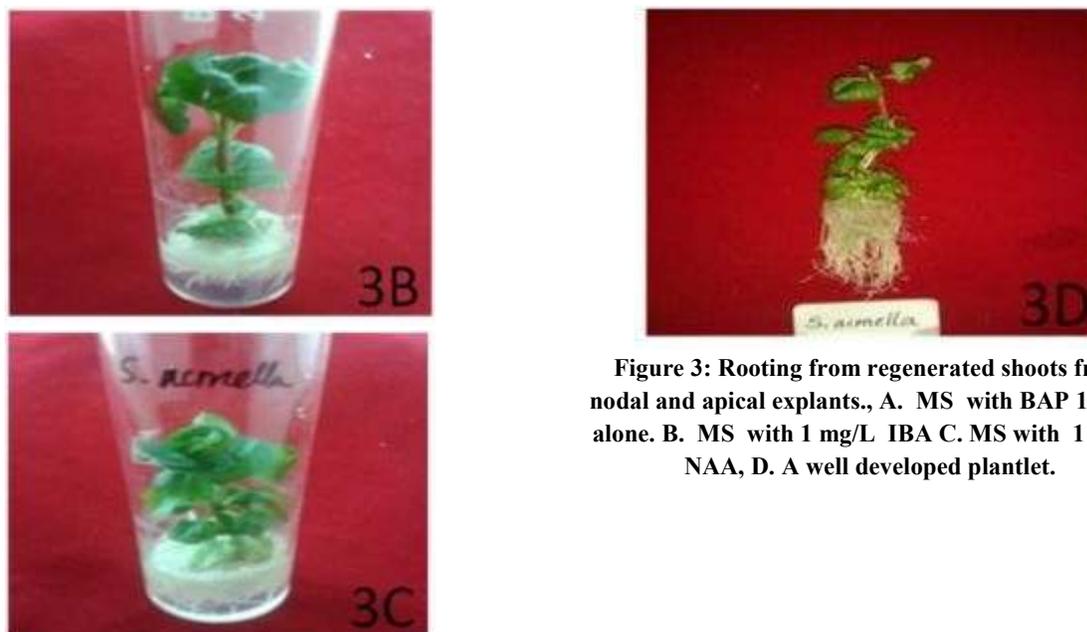


Figure 3: Rooting from regenerated shoots from nodal and apical explants., A. MS with BAP 1 mg/L alone. B. MS with 1 mg/L IBA C. MS with 1 mg/L NAA, D. A well developed plantlet.

Table 3: Effects of different hormones on multiplication of shoots induced from the apical shoot tips of *S.acmella*

Apical shoot tip explants						
Concentration of hormones	No. of explants inoculated	No. of explants responded	% of response	No. of shoots after I subculture	No. of shoots after II subculture	Length of shoots
Control	10	5	50	1.0	1.0	2.0±0.09
0.5 BAP	10	8	80	8	20	3.0±0.06
1.0 BAP	10	9	90	8	22	3.4±0.02
1.5 BAP	10	9	90	10	25	3.6±0.10
2.0 BAP	10	9	90	15	35	3.8±0.10
2.5 BAP	10	8	80	12	28	3.4±0.10
3.0BAP	10	7	70	8	20	3.0±0.00
2.0BAP+0.5NAA	10	8	80	8	30	4.6±0.05
2.0BAP+1.0NAA	10	8	80	10	38	4.8±0.02
2.0BAP+2.0NAA	10	7	70	8	18	4.0±0.10
2.0BAP+0.5IAA	10	7	70	10	25	4.0±0.00
2.0 BAP+1.0IAA	10	9	90	14	40	4.2±0.10
2.0 BAP+2.0IAA	10	8	80	10	22	4.0±0.05

Values are ± SE of 5 replicates

Among the different concentrations of IBA, highest rooting percentage (90%) was observed with 1.0 mg/L IBA. The roots were short, thin in nature at this concentration with an average height of 2.5 cm long. The IBA at 0.5 mg/L IBA gave 70 % rooting which are short and thin around 2.5 cm in length. The concentration 2.0 mg/L IBA gave 85% rooting and the roots were short, healthy with an average height of 3.5 cm. The percentage of rooting was observed to be reduced to 60% at the concentration of 3.0 mg/L IBA and the root length was also less (3.0 cm) at this concentration (Table 4).

Among the different concentrations of NAA, highest rooting percentage (80%) was observed with the concentration 1.0 mg/L NAA and the roots produced were long and thin in nature and were 4.5 cm long. The concentration 0.5 mg/L NAA gave 60 % rooting and produced long and thin roots of 4.2cm length. The concentration 2.0 mg/l NAA gave 70% rooting and the roots were short and healthy with average height of 4.0cm. The percentage of rooting was observed to be reduced to 50% at 3.0 mg/L NAA and the root length was also less (3.8 cm) at this concentration. The Rooting from *in vitro* regenerated shoots of *S.acmella*

on different concentrations of IBA and NAA is shown in Table 4.

Table 4: Rooting from *in vitro* regenerated shoots of *S.acmella*.

S No	Media and Conc. of Hormones (mg/L)	No. of shoots inoculated	No. of shoots with rooting responded	Rooting %	Length of roots (cm)	Nature of roots
1	MS Full Strength	20	4	20	1.4±0.01	Short,very thin
2	MS half Strength	20	5	25	1.2±0.10	Short,very thin
3	MS + 0.5 IBA	20	14	70	2.5±0.05	Short,thin
4	MS +1.0 IBA	20	18	90	2.8±0.07	Short, thin
5	MS +2.0 IBA	20	17	85	3.5±0.10	Short,healthy
6	MS +3.0 IBA	20	12	60	3.0±0.10	Short,healthy
7	MS +0.5 NAA	20	12	60	4.2±0.10	Long,thin
8	MS +1.0 NAA	20	16	80	4.5±0.05	Long,thin
9	MS +2.0 NAA	20	14	70	4.0±0.10	Long,thin
10	MS +3.0 NAA	20	10	50	3.8±0.10	Long,thin

Values are ± SE of 5 replicates

Root growth after 4 weeks in culture

The *in vitro* rooted plants from nodal explants and apical bud were successfully hardened and survived well with 100% survival rate (Fig 4A, 4B).

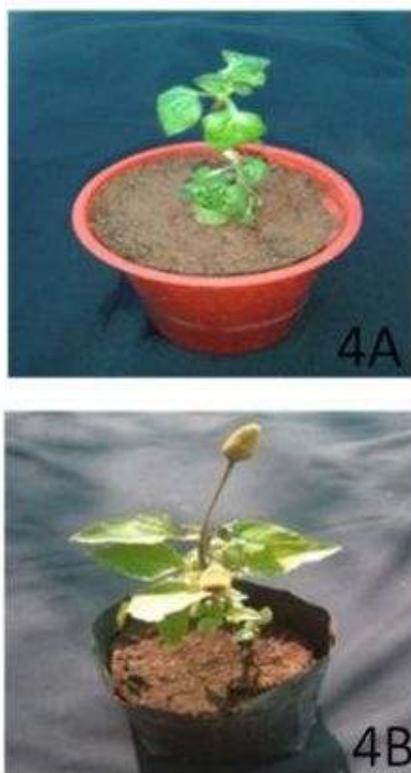


Figure 4: Acclimatization of regenerated plantlets of *S.acmella*. (A).Primary hardening (B). A hardened plant flowering.

In the present study, as many as 8-10 multiple shoots were produced from single explants and multiplied further by sub culturing for two cycles. Through this procedure, 60 to 80 plants were produced from single explants within a short period of 10 to 12 weeks, which can be exploited for mass production of planting material for commercial propagation.

DISCUSSION

In the present study, all the concentrations of BAP alone and in combination with other hormones induced shoot regeneration. The shoot induction in a medium containing cytokinin alone indicates that there are sufficient levels of endogenous auxins or its capability of *de novo* synthesis (Goyal et al., 2010). The combinations of BAP with NAA and IAA gave good results of regeneration by increasing the number and length of multiple shoots. The stimulatory effect of BAP and NAA in multiplication and elongation of shoots has also been reported in various plants like *Vernonia cineria*, *Flaveria trinervia*, *Tagectus erecta*, *Xanthium strumarium* (Seetharam et al., 2007). The induction of roots in the BAP multiplication medium itself ranging from concentration 0.5 to 1.0 mg/L without transfer into a separate rooting medium is in accordance with the previous studies on *Acmella radicans* and *S.acmella* (Yadav K & Singh N.; 2012).

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