Micropropagation of *Spilanthes acmella* Murr. – An Important Medicinal Plant

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Abstract: A rapid and efficient protocol for *in vitro* propagation of *Spilanthes acmella* Murr.-an endangered medicinal plant has been developed. Multiple shoots induced on Murashige and Skoog medium supplemented with various auxins and cytokinins individually and in various combinations. MS medium fortified with 1.0 mg/l BAP was found to be effective individually. The medium with 3.0 mg/l BAP + 1.0 mg/l IAA responded better as compared to other combinations. The *in vitro* raised shoots were excised and implanted on MS half strength medium fortified with NAA and IBA (0.5-3.0 mg/l) in an attempt to produce roots. The half strength medium supplemented with IBA (0.5-3.0 mg/l) developed roots after 15 days of implantation. The maximum frequency of roots obtained on 1.0 mg/l of IBA fortified medium. Most of the roots were long and healthy. The regenerated plantlets were successfully transferred to pots containing sterilized soil and sand mixture (3:1) and acclimatized with 70% survival rate in the field conditions. [Nature and Science 2010;8(9):5-11]. (ISSN: 1545-0740).

Key words: Multiple shoots, Nodal segments, *Spilanthes acmella*

1. Introduction

Spilanthes acmella Murr. (Family: Asteraceae) commonly known as Akarkara or toothache plant. This plant is widely distributed in the tropical and sub-tropical regions including America, North Australia, Africa, Malaya, Borneo, India and Sri Lanka (Jansen, 1981). In India, it is confined to South India, Chhatisgarh and Jharkhand (Anonymous, 1989).

The flowers and leaves of this plant have been used as traditional medicine for stammering, toothache, stomatitis and throat complaints. It has potent diuretic activity and the ability to dissolve urinary calculi. It exhibits antimalarial properties as well (Burkill, 1966; Singh, 1995; Ramsewak *et al.*, 1999; Pandey and Agarwal, 2009). Spilanthol, the most active antiseptic alkaloid extracted from this plant, is found effective at extremely low concentrations against blood parasites, and indeed is a poison to most invertebrates while remaining harmless to warm-blooded creatures. It is further recommended as a cure for dysentery and rheumatism, and to enhance the immune system. It exhibits general immunomodulator properties when used internally, boosting production of leukocytes and antiviral interferon, as well as promoting phagocytosis. It stimulates wound healing, protects the individual from cold and flu (Anonymous, 1989). The leaves are also used to treat bacterial and fungal skin diseases.

Due to these medicinal values, the plant is being over-exploited in recent years. In addition, the efficiency of reproduction is also found to be less due to its low seed germination and viability and lack of vegetative propagation methods. Thus the present study has been designed to develop a reliable and reproducible protocol of this important endangered plant which could be used for mass multiplication of this plant species to meet the increasing requirement of the pharmaceutical industry as well as for the conservation of germplasm.

2. Materials and Methods

Nodal segments (1.0-1.5cm) were excised from the plants growing in polyhouse of Botany Department, Kurukshetra University, Kurukshetra. All 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. The nodal segments were inoculated on MS medium supplemented with various concentrations (0.5-3.0 mg/l) of auxins (IAA, NAA, 2, 4-D and IBA) and cytokinins(BAP and Kn) alone and in various combinations for shoot regeneration and callus induction.

The cultures were incubated at a temperature of 25±2ºC and a photoperiod of

16hrs light (intensity of 2000 lux) and 8hrs of dark.

Visual observations like callus induction, growth of callus, number of days taken for bud break, percentage of bud break and number of shoots regenerated per explants were recorded regularly. A mean of 20 replicates was taken per treatments.

The *in vitro* developed single/multiple shoots (2.5 – 3.0 cm long) were excised and implanted in culture tubes containing full and half strength MS medium fortified with IBA and NAA under aseptic conditions for rooting.

After development of sufficient roots, the plantlets were gradually pulled out from the medium and immersed in water to remove agar-agar particles sticking to the root system by using a fine brush. These plantlets were planted in pots with sterilized soil and sand mixture (3:1). Each pot was covered with a polythene bag to maintain high humidity around the plants. The pots were supplied with MS (half strength) salt solution on alternate days. After about two weeks the polythene bags were removed for 3-4 hrs daily to expose the plants to the conditions of natural humidity. After about 4 weeks these plants were transferred to bigger pots and were maintained under natural conditions of day length and temperature.

3. Results and Discussion

3.1 Direct organogenesis

MS basal medium without growth regulators served as the control. This medium could produce only one shoot per explant. Similar observation were made by Paal *et al.* (1981) and Cavallini and Lupi (1987).

Supplementation of cytokinins gave better results than auxins in present investigation. The medium fortified with BAP supported much better results as compared to Kn in terms of period required for bud-break and number of shoots differentiated per explant. In case of BAP, maximum number of shoots (4.3) per explant was recorded in the medium fortified with 1.0 mg/1 and it decreased with any deviation from this concentration (Figure-1b). In case of Kn, maximum numbers of shoots (2.8) per explant was recorded in the medium fortified with 0.5 mg/1(Figure-1a). A similar result was also reported by Sudha and Seeni, (1994), Choudhary *et al.,* (2004), Goel *et al.* (2009) and Kumar and Singh (2009) in *Adhatoda beddomei, Dendrocalamus strictus, Peganum harmala* and *Prosopis cineraria* respectively. Earlier reports about the effectiveness of BAP has been reported by Thakur *et al.,* (2001) in *Alnus nepalensis,* Johonson *et al.,* (2002) in *Rhinacanthus nasutus* and Thind *et al.,* (2008) in *Aloe vera.* The per cent bud break was found to be eighty per cent in the media supplemented with 2.0 mg/1 of BAP and 3.0 mg/1 of Kn (Table-1.). Bud-break was not observed on the media supplemented with auxins(0.5, 1.0, 2.0 and 3.0 mg/l of NAA, IAA and 2, 4-D).

A combined effect of different cytokinins (BAP and kinetin) and auxins (IAA, NAA and 2, 4-D) in various combination was also studied. The medium with BAP (2.0 mg/l) + IAA (1.0mg/l) showed maximum (90%) bud break after 11.1 days of inoculation. Supplementation of NAA in place of IAA with kinetin did not make much difference. In case of Kn + NAA or IAA supplemented media, the medium with Kn (2.0 mg/l) + IAA (1.0 mg/l) showed eighty percent bud break after 12.5 days of inoculation with 2.3 shoots per explant (Table-1.). The combination of IAA with cytokinins (BAP or Kn) promoted shoot formation in various plant species as observed by Yasmeen and Rao (2005), Guo *et al.* (2007) and Jawahar *et al.,* (2008). The medium supplemented with BAP (3.0 mg/l) + IAA (1.0 mg/l) supported maximum number of shoots (4.0) per explant and responded best among all media tried in combinations (Figure-1c).

3.2 Callus formation

Simultaneously, callus formation was noticed in media fortified with various concentrations of auxins and cytokinins. Highest percentage of callus induction (80%) was observed in the media supplemented with 3.0 mg/l of 2, 4-D. Among cytokinins the medium supplemented with 3.0 mg/l BAP supported highest (70%) percent of callus induction. Among auxins, the media fortified with 2, 4-D (2.0 mg /1) showed better callus growth as compared to other combinations at all the three stages (Table-2.). Among cytokinins, callus growth was better in media containing 2.0 mg/1 BAP (Figure-1d). Callus so produced was greenish white and fragile. Young explants exhibited better response as these are physiologically and biochemically more active as well as they have less rigid cell wall (Mishra and Bhatnagar, 1995). The average number of days required for callus induction decreased with the increase in concentration of auxins and cytokinins. 2, 4-D was found to be better in terms of per cent callus induction as well as number of days required to induce callus while in *Fraxinus angustifolia,* BAP was found to be superior than 2, 4-D(Perez-Parson *et*

al., 1994). Similar observations have also been reported in *Asparagus officinalis* (Ha *et al.,* 2008) and *Elaeagnus angustifolia* (Zeng *et al.,* 2009). To achieve maximum callus induction, a defined auxin-cytokinin ratio was required, as also advocated by Latto *et al*., (2006) in *Chlorophytum arundinacem*, Junaid et al. (2007) in *Catharanthus roseus* and Burbulis *et al.,* (2008) in *Brassica napus.* A combined effect of cytokinins (BAP and Kn) and auxins (IAA, NAA and 2, 4-D) was also studied for callus formation. The media supplemented with BAP (2.0 mg/1) + 2, 4-D (1.0 mg/1) supported ninety per cent callus induction after 12 days of inoculation (Table-2.).

3.3 Rooting of *in vitro* regenerated shoots

Elongated and well developed regenerated shoots were aseptically excised and implanted on half strength MS medium supplemented with different auxins(IAA, NAA and IBA) for rooting. MS medium full and half strength supplemented with IAA failed to develop roots. MS half strength medium supplemented with 1.0 mg/1 of IBA proved best in terms of per cent root formation (Table-3, Figure-1e).

Table 1. Effect of cytokinins and auxins supplemented individually and in various combinations on nodal segments of *Spilanthes acmella.*

Auxins/ cytokinins (mg/l)	Concentration of growth regulators (mg/l)	%age of bud break	Number of days required for bud break	Number of shoots (Mean±SE)	Shoot Length (cm) (Mean ± SE)
Control	_	20	15.0 ± 0.70	1.0 ± 0.00	1.2 ± 0.48
BAP	0.5	50	11.5 ± 0.53	3.4 ± 0.53	2.5 ± 0.17
	1.0	60	11.3 ± 0.51	4.3 ± 0.08	2.8 ± 0.27
	2.0	80	11.2 ± 0.84	2.7 ± 0.70	3.2 ± 0.20
	3.0	70	10.7 ± 0.48	2.0 ± 0.57	2.0 ± 0.17
Kn	0.5	70	13.2 ± 0.75	2.8 ± 0.89	2.8 ± 0.12
	1.0	60	13.1 ± 0.64	2.6 ± 0.74	1.7 ± 0.38
	2.0	70	12.5 ± 0.53	2.2 ± 0.48	2.4 ± 0.13
	3.0	80	12.0 ± 0.53	1.8 ± 0.64	2.1 ± 0.30
MS+Kn+ IAA	1.0 +1.0	70	12.7 ± 0.95	2.4 ± 0.53	2.5 ± 0.15
	2.0 +1.0	80	12.5 ± 0.78	2.3 ± 0.74	2.2 ± 0.23
	3.0 +1.0	70	12.0 ± 0.10	2.7 ± 0.75	1.8 ± 0.36
MS+Kn+ NAA	1.0 +1.0	_	_	_	_
	2.0 +1.0	60	12.8 ± 0.75	2.1 ± 0.40	1.3 ± 0.17
	3.0 +1.0	60	12.5 ± 0.54	2.2 ± 0.44	1.9 ± 0.35
MS+BAP+ IAA	1.0 +1.0	70	11.0 ± 0.81	2.2 ± 0.48	1.3 ± 0.19
	2.0 +1.0	90	11.1 ± 0.69	2.5 ± 0.82	1.5 ± 0.25
	3.0 +1.0	80	10.7 ± 0.36	4.0 ± 0.28	1.9 ± 0.12
MS+BAP+ NAA	1.0 +1.0	70	11.2 ± 0.48	2.5 ± 0.54	1.4 ± 0.16
	2.0 +1.0	80	10.8 ± 0.35	2.6 ± 0.54	2.3 ± 0.28
	3.0 +1.0	80	10.7 ± 0.46	3.0 ± 0.74	2.1 ± 0.19

(–) No Response

*Data based on 20 explants per treatment and taken after 28 days of culture

Table 2. Effect of cytokinins and auxins supplemented individually and in various combinations on callus formation on internodal explants of *Spilanthess acmella.*

Media Composition	Concentration of growth regulators (mg/l)	No. of days required for callus induction	%age of callus induction	Nature of callus	Callus growth
MS control	–	–	–	–	–
MS + Kn	0.5	–	–	–	–
	1.0	–	–	–	–
	2.0	14	60	Greenish, white	C++
	3.0	12	50	Greenish, white	C+
MS+BAP	0.5	–	–	–	–
	1.0	12	30	Greenish, white	C+
	2.0	10	50	Greenish, white	C+++
	3.0	10	70	Greenish, white	C++
MS+ IAA	0.5	–	–	–	–
	1.0	14	40	Dark green	C+
	2.0	12	70	Dark green	C+
	3.0	10	60	Dark green	C++
MS+ NAA	0.5	–	–	–	–
	1.0	–	–	–	–
	2.0	–	–	–	–
	3.0	–	–	–	–
MS+ 2,4-D	0.5	12	60	Greenish yellow, fragile	C+
	1.0	10	70	Greenish yellow, fragile	C+
	2.0	08	70	Greenish yellow, fragile	C+++
	3.0	08	80	Greenish yellow, fragile	C++
MS+BAP+Kn	1.0+1.0	–	–	–	–
	2.0+1.0	14	70	Greenish white	C++
	3.0+1.0	14	70	Greenish white	C++
MS+BAP+ NAA	1.0+1.0	15	50	Dark Green, compact	C++
	2.0+1.0	14	80	Dark Green, compact	C++
	3.0+1.0	14	80	Dark Green, compact	C+++
MS+BAP+ IAA	1.0+1.0	–	–	–	–
	2.0+1.0	14	80	Dark Green	C+
	3.0+1.0	13	70	Dark Green	C++
MS+BAP+ 2,4-D	1.0+1.0	12	80	Greenish yellow, fragile	C++
	2.0+1.0	12	90	Greenish yellow, fragile	C+++
	3.0+1.0	11	80	Greenish yellow, fragile	C++

(–) No Response, (C+) Poor growth, (C++) Moderate growth, (C+++) Good growth.
*Data based on 20 explants per treatment and taken after 28 days of culture

Most of the roots were long and thin. Supplementation of IBA was also found to be effective on root development in *Artemisia judaica* (Liu *et al.*, 2003) and *Eclipta alba* (Bhaskaran and Jayabalan, 2005). The medium supplemented with 3.0 mg/l IBA was found most suitable for rooting because it regenerated roots in least time (15-16 days).

3.4 Acclimatization and transfer of plantlets to the soil

Complete regenerated plantlets with sufficient roots were taken out from the cultural tubes carefully and the medium sticked to roots was gently washed in water with the help of fine brush. The *in vitro* regenerated plantlets were then transplanted to small earthen pots containing sterilized soil and sand mixture (3:1) (Figure1-f). Each pot covered with polythene bags with small holes to maintain high humidity and kept them in the culture room to get acclimatized. The plantlets were initially irrigated with half strength (salts only) MS medium without sucrose on alternate days. The plantlets were exposed to 3-4 hours daily to the conditions for natural humidity after 10 days of transfer. After about 30 days the plants were transferred to bigger pots in greenhouse and were maintained under natural conditions of day length, temperature and humidity. Finally, the plants were transferred to the field conditions. Seventy per cent of the regenerants survived well.

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Table 3. Root formation on different media composition in *Spilanthes acmella.*

Media composition (mg/l)	Days required for root induction	% age of Root formation	Remarks
MS full strength without growth regulators	30	20	Long, Thin
MS half strength without growth regulators	–	–	–
MS half strength +0.5 mg/l IBA	22	70	Long, Thin
MS half strength +1.0 mg/l IBA	20	75	Long, Thin
MS half strength +2.0 mg/l IBA	16-18	50	Stout, Healthy
MS half strength +3.0 mg/l IBA	15-16	40	Stout, Healthy
MS half strength +0.5 mg/l NAA	24	40	Short, Healthy
MS half strength +1.0 mg/l NAA	22	50	Long, Thin
MS half strength +2.0 mg/l NAA	18-20	40	Long, Thin
MS half strength +3.0 mg/l NAA	20	20	Short, Healthy

(-) No Response
*Data based on 20 explants per treatment and taken after 28 days of culture

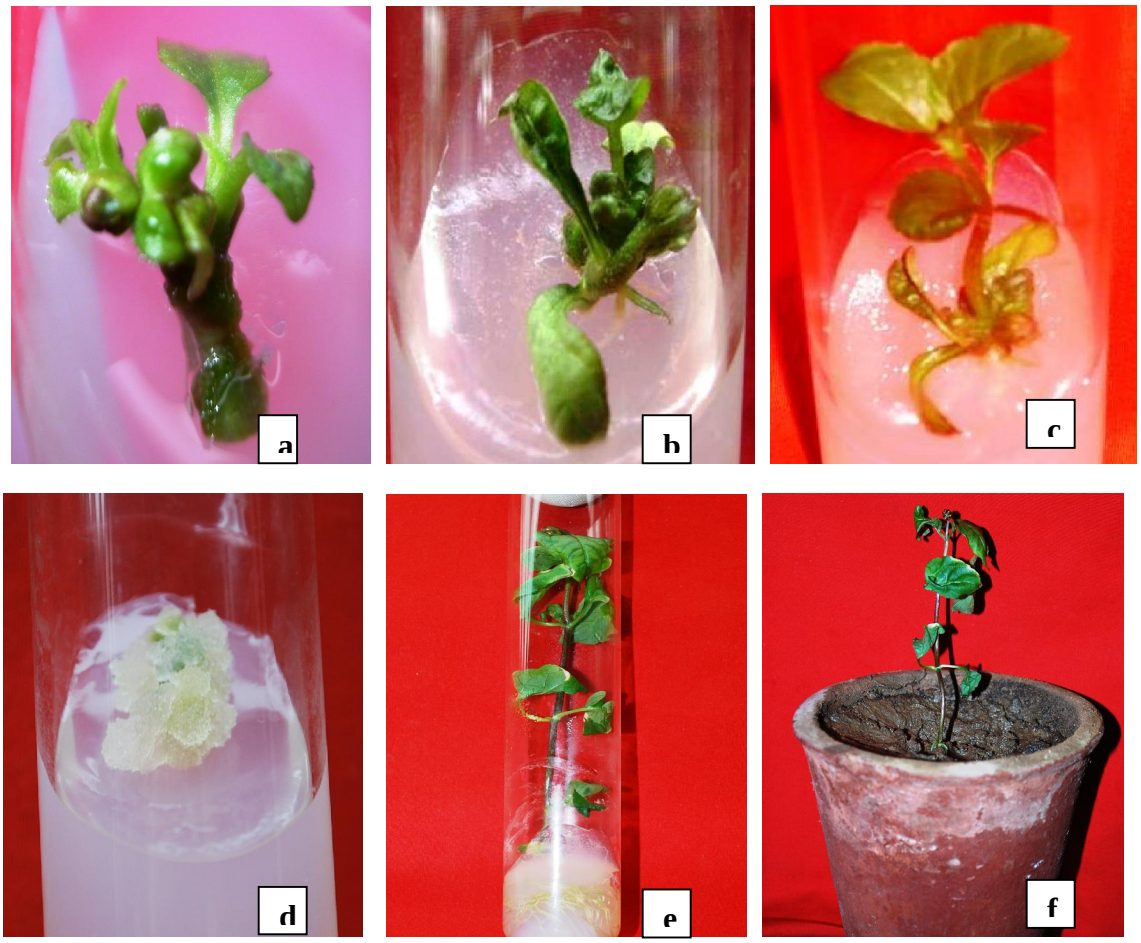

Figure 1(a-f): *in vitro* regeneration of *spilanthes acmella.* **a,** Shoot regeneration from nodal explant on MS medium + Kn (0.5mg/l) ; **b,** Regeneration of multiple shoots from nodal explant on MS medium + BAP (1.0mg/l); **c,** Regeneration of multiple shoots from nodal explant on BAP (3.0mg/l) + IAA (1.0mg/l); **d,** Callus growth on MS medium + BAP (2.0mg/l); **e,** Root formation from *in vitro* grown shoot on MS- medium half strength + IBA (1.0mg/l); **f,** Establishment of *in vitro* grown plantlets under natural conditions.

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