RAPID PLANT REGENERATION FROM NODAL EXPLANTS OF *Spilanthes acmella* (L.) MURR. – AN ENDANGERED MEDICINAL PLANT

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Abstract. Excised nodal explants of *Spilanthes acmella* (L.) Murr., 'Toothache Plant' proliferate rapidly *in vitro* on MS medium containing 0.5- 2.0 mg/l of BAP. Rapid and prolific shoot proliferation occurred. Regenerated shoots vary considerably in size (3-10 cm long) and relative stage of development, with some (50%) producing adventitious roots without transferal to a separate rooting medium. With maximum possibility of adventitious roots induction was induced from middle order nodes (3rd to 5th node from apex) obtained from 3 months old *in vivo* plant on full-strength MS medium supplemented with 1.0 mg/l BAP under the photoperiod of 18-h. The possibility of adventitious roots induction directly from regenerated shoot was greatly influenced by the concentration of BAP, photoperiod, age of donor plant and nodal position on stem.

Keywords: *In vitro* propagation, *Spilanthes acmella*, Endangered, Nodal segments, Photoperiod.
Abbreviations: BAP – 6-Benzylaminopurine; **MS –** Murashige and Skoog (1962) basal medium.

INTRODUCTION

Spilanthes acmella (L.) Murr. (Asteraceae) is the well known as "Akarkara or Toothache plant", is an important endangered medicinal plant widely distributed in tropics and subtropics [21].

The plant has found applications in pharmaceuticals to treat mouth aliments, stammering, stomatitis, and throat complaints. Alkylamides from *Spilanthes* have demonstrated strong diuretic and insecticidal properties. The major pungent compound reported in *S. acmella* is alkaloid spilanthol (N-isobutyl-2,6,8-decatrienamide) [13].

With the increasing worldwide demand currently, the interest in utilization and conservation of medicinal plants is rapidly increasing, and mass micropropagation of plants via *in vitro* culture techniques has become an alternative approach to produce large-scale rapid plant multiplication to extract valuable chemical products rather than from plants grown and harvested in the field [10].

In vitro micropropagation technique has been proved to be employed in propagation of many of medicinal plant species [6, 25, 26, 30]. Micropropagation requires not only stable shoot multiplication, but also successful rooting of microcuttings. In fact, success of the most hazardous step of propagation, i.e. transfer and acclimatization of *in vitro* rooted microcuttings to an *ex vitro* environment, depends on the quality of the root system. Thus, research on adventitious root formation is highly important from the practical point of view.

In a previous studies [8, 11, 19, 23, 24, 27, 31], we found that *S. acmella* displays a good capacity for *in vitro* rooting, in the presence of auxin, mainly IBA in the medium. *In vitro* plants are generally considered susceptible to genetic changes due to culture stress [22]. So, the development of a direct plant regeneration system and assessment of clonal fidelity of the *in vitro* raised plants of S. *acmella* are crucial for successful commercial application of micropropagation protocol.

The purpose of this work was to acquire more information on the capacity of *in vitro* grown shoots to simultaneously regenerate adventitious roots and shoots by treatments of different endogenous and exogenous factors.

MATERIALS AND METHODS

Healthy nodal explants (1.0-1.5 cm) were excised from two- to four-month-old plants growing in poly house of Botany Department, Kurukshetra University, Kurukshetra. Nodal explants were divided into three groups based on to their position along the length of the branch of the in vivo plants (2, 3 and 4 month old): distal order (containing 1st - 2nd node from apex), middle order (containing 3rd - 5th node from apex) and basal order (containing 6th- 8th node from apex). 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 after trimming the ends were finally inoculated on MS medium [18] containing 30 g/l sucrose and 8 g /l agar supplemented with various concentrations (0.5 - 2.0 mg/l) of BAP alone. The pH of the medium was adjusted to 5.8 with 1N NaOH or 1N HCl prior to autoclaving at 121°C for 20 min.

The cultures were incubated at a temperature of 25±2ºC and a 16-h photoperiod (intensity of 4000 lux). Effect of different durations of photoperiod, 3 light/dark cycles i.e.,14/10, 16/8 and 18/6 h on the growth of plantlets produced was also tested.

When adequate number of *in vitro* raised adventitious roots from the surface of regenerated shoots penetrate deep in the basal media and reach the base of the culture tube, the plantlets were thoroughly washed to remove the adhering agar agar particles and transferred to pots containing sterilized soil and sand mixture (3:1) for acclimatization. The pots were covered with transparent polythene bags (to maintain humidity) with holes (to provide aeration) and kept in culture room initially for 15 days under the same light

and temperature conditions. Later these pots were kept in the greenhouse for acclimatization.

Each experiment consisted of 5 replicates with one explant per culture tube and was repeated twice. After 10 weeks in culture, the efficacy of rooting was assessed by the percentage and length of shoots producing roots in each culture container and by the extent of the adventitious root produced from each regenerated shoot.

Data were analyzed for significance using one-way analysis of variance (ANOVA) and the differences contrasted using a Duncan's multiple range test (DMRT) at $P \leq 0.05$. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS, version 11.5).

RESULTS

When nodal explants were transferred to BAP media (0.5-2.0 mg/l) good shoot multiplication and growth was obtained. After 4-5 weeks period, on media with a range of BAP concentrations, regenerated shoots continued to produce abundant adventitious roots without transferal to a separate root inducing medium, and grew rapidly.

The culture period for all the treatments was fixed to 2 months. The shoots regenerated were graded into three groups based on size and degree of adventitious root development. Smallest shoots with roots, shoot with moderate root system and larger shoots with vigorous adventitious roots (Fig. 1).

Figure 1. *In vitro* propagation of *S. acmella*: **(a)** larger shoots with abundant adventitious roots occurred simultaneously from *in vitro* regenerated shoots derived from middle order nodes of the 3-month old in vivo plants on MS + BAP (1.0 mg/l) under 18-h photoperiod; **(b)** larger shoots with moderate rooting derived from middle order nodes of the 4 -month old in vivo plants on MS + BAP (2.0 mg/l); **(c)** regenerated shoot with normal roots derived from 4th node of the 2-month old in vivo plants on MS + BAP (1.0 mg/l); **(d)** small shoot with a well developed root system derived from 5th node of the 4 -month old in vivo plants on MS + BAP (2.0 mg/l).
Bar = 1.0 cm and it represents the length of the plant

Significantly higher rooting was obtained from the shoots regenerated, when the nodal explants were directly transferred on MS medium supplemented with 1.0 mg/l BAP. Increasing the concentration of BAP, a little decrease in the length of regenerated shoots regeneration was observed, while root formation tended to decrease (Table 1).

Among the different age plants tested, explants from 3-month-old in vivo plants induced significantly highest shoot length with vigorous root formation (Fig. 1a; Table 1). A significant difference in the mean length of multiple shoot induction and the extent of adventitious rooting was recorded between the different positions of nodal explants on the plant.

Intriguing, no simultaneous root formation response occurred from first order nodal explants of 2 months old in vivo plants. The highest root regeneration response was obtained in explants located in the middle order nodal position of the 3-month old in vivo plants (Fig. 1a).

Root regeneration appeared to be negatively influenced by decreasing duration of photoperiod. In the BAP (1.0 mg/l) medium, simultaneous root production was lower in 14/10 h photoperiod but tended to increase as a consequence of longer duration of photoperiod up to 18/6 h (Table 2).

Table 1. Influence of age of the donor plant and relative position of nodes along the stem length of *S. acmella* cultured on MS medium with various concentrations of BAP on adventitious root proliferation and mean shoot length after 2 months of culture

Age of donor plant (months)	Position of the node of plant (from shoot apex)	Conc. of BAP (mg/l)	No. of days required for root induction	Mean shoot length (cm)	Adventatious rooting regeneration frequency (%)	Extent of rooting
2	Distal order	0.5	>60^{c}	7.55±0.27^{bc}	-	-
		1.0	>60^{c}	8.4±0.25^{ab}	-	-
		2.0	>60^{c}	6.63±0.43^{c}	-	-
	Middle order	0.5	>60^{c}	8.13±0.40^{ab}	-	-
		1.0	45.5±0.5^{b}	9.5±0.30^{a}	20^{b}	+
		2.0	48.0±0.0^{b}	7.8±0.00^{bc}	10^{b}	+
	Basal order	0.5	>60^{c}	7.42±0.34^{bc}	-	-
		1.0	44.5±0.0^{ab}	7.9±0.30^{b}	20^{b}	++
		2.0	48.5±0.0^{b}	6.85±0.25^{c}	20^{b}	+
3	Distal order	0.5	>60^{c}	7.74±0.42^{bc}	-	-
		1.0	46.5±0.0^{b}	8.55±0.25^{ab}	20^{b}	+
		2.0	52.5±0.0^{bc}	6.85±0.25^{c}	20^{b}	+
	Middle order	0.5	40.66±0.47^{a}	9.13±0.32^{a}	30^{ab}	++
		1.0	38.6±0.8^{a}	10.52±0.27^{a}	50^{a}	+++
		2.0	42.66±0.47^{ab}	7.6±0.32^{bc}	30^{ab}	++
	Basal order	0.5	42.5±0.0^{ab}	7.85±0.35^{bc}	20^{b}	+
		1.0	38.75±0.43^{a}	8.1±0.33^{ab}	40^{a}	++
		2.0	42.5±0.0^{ab}	6.3±0.3^{c}	20^{b}	++
4	Distal order	0.5	51.0±0.0^{bc}	6.8±0.0^{c}	10^{b}	+
		1.0	48.5±0.0^{b}	7.6±0.2^{bc}	20^{b}	+
		2.0	53.5±0.0^{bc}	6.2±0.2^{c}	20^{b}	+
	Middle order	0.5	42.66±0.47^{ab}	7.06±0.24^{bc}	30^{ab}	++
		1.0	39.75±0.43^{a}	8.4±0.28^{ab}	40^{a}	+++
		2.0	44.5±0.0^{ab}	6.4±0.2^{c}	20^{b}	++
	Basal order	0.5	43.5±0.0^{ab}	6.6±0.2^{c}	20^{b}	++
		1.0	42.66±0.47^{ab}	7.2±0.16^{bc}	30^{ab}	++
		2.0	46.5±0.0^{b}	5.95±0.05^{c}	20^{b}	+
LSD (p≤0.05)		3.844	5.604	26.413		
ANOVA ($F_{26,54}$)		4.417*	6.221*	8.978*		

The number of '+' sign donates extent of rooting,
+ = normal root formation, ++ = moderate root formation, +++ = vigorous root formation, - No root formation
Values represent mean ± standard error, n = 10.
Mean value followed by different alphabet/s within a column do not differ significantly over one other at p≤0.05 lead by Duncan's Multiple Range Test
*Significant at p≤0.05

Table 2. Effect of different photoperiods on *in vitro* adventitious root proliferation and mean shoot length from middle order nodal explants excised from 3 months old *S. acmella* cultured on MS medium containing 1.0 mg/l BAP after 70 days of culture

Photoperiod (light/dark)	Mean shoot length (cm)	Adventatious rooting regeneration frequency (%)	Extent of rooting
12/12	8.6±0.32^{b}	40^{b}	++
16/8	10.04±0.23^{a}	50^{a}	++
20/4	10.48±0.30^{a}	50^{a}	+++
LSD (P≤0.05)	1.795	1.8312	
ANOVA ($F_{2,6}$)	0.113*	0.160*	

The number of '+' sign donates extent of rooting,
++ = moderate root formation, +++ = vigorous root formation
Values represent mean ± standard error, n = 10.
Mean value followed by different alphabet/s within a column do not differ significantly over one other at P≤0.05 lead by Duncan's Multiple Range Test.
*Significant at p≤0.05

DISCUSSION

It is known that the regeneration of adventitious organs differs according a number of endogenous and exogenous factors such as genetic background, physiological influences, age and ontogenetic phase of mother plant, environment (light and temperature) and composition of the nutrient medium [16]. Root initiation involves the dedifferentiation of specific cells, leading to the formation of root meristems [17]. The rooting response is correlated with the production of particular flavonoids under the influence of the cytokinin in the shoot multiplication medium.

In particular, the auxin-cytokinin ratio appears to be the most important factor in channeling regeneration response towards a specific *in vitro* morphogenic process rather then another [4]. The promoter effect of BAP in inducing multiple shoots has been previously reported by many authors [3, 12, 28].

Endogenous cytokinins appear to have a role in adventitious root production [2]. The type and the amount of regenerated morphogenic structures may be related to the biological effectiveness of the growth regulators utilized. The concentration of growth regulator used to induce rooting and the duration of application can be critical for rooting response. It is possible that BAP is more effective in switching on endogenous cytokinin production, which in turn, may inhibit root initiation [29]. Cytokinins in the rooting medium itself are known to be inhibitory for rooting but beneficial for some fruit trees [1]. Similar results were also noted in *Ajuga reptans* [14].

De Klerk *et al*. [9] reported that the plants in which roots regenerate spontaneously, endogenous auxins produced at the shoot apex are transported basipetally to the cut surface and act as the trigger, removal of the apex reduces both the level of endogenous auxin in the basal portion of a cutting and the number of regenerated roots. Moreover, in these plants, application of exogenous auxin strongly increases the number of regenerated roots.

A factor that also played a primary role is the type of explants utilized. The age of the mother plant and the position of the explants used, are likely characterized by tissues at different cell differentiation stage [7]; thus, in such conditions, it is to be expected that the effects of a growth regulator treatment on channeling cell competence may differ according to the degree of cell differentiation, producing different regenerating responses. This would be in agreement with the results obtained by D'Onofrio and Morini [5]. This could be a kind of age-related response, because the position was related to the physiological age of the explants, and the closer to the apex the younger the explants [20].

The intensity, quality and extent of daily exposure of light are determining factors in plant tissue culture [31]. The light modulates the morphogenetic process which leads to the regeneration this plantlet [15].

Compared to a previous regeneration protocol for *S. acmella* where different concentrations and types of growth regulators were used, it is interesting to note that in the present work the obtained *in vitro* grown shoots possess the competence to simultaneously regenerate adventitious roots even when treated with the same growth regulator balance within 10 weeks treatment period only. This result further emphasizes the importance of growth regulator treatment on different cell differentiation stage carried by the in vivo explants used.

Micropropagation is therefore a valid technique for this important endangered medicinal plant and to plan the cycles for successful rooting and growth according to the market demand.

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