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Callus induction and plant regeneration from leaf explants of *Spilanthes acmella* Murr. : An endangered medicinal plant

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ABSTRACT

A callus induction and plant regeneration protocol was developed from leaf explants of the endemic Spilanthes acmella. Explants were cultured on Murashige and Skoog medium (MS) supplemented with different plant growth regulators (PGRs) [α-naphthaleneacetic acid (NAA), BAP and IBA, 2 4-dichlorophenoxyacetic acid (2, 4-D), Zeatin]. The combinations and concentrations of PGRs were shown significant variations for the frequency of callus formation, appearance of callus and the potential of callus differentiation. 2, 4-D (5mg/lit) alone while BAP 0.5mg/l with NAA 5.0mg/l have been found highly effective in callusing with quite good in texture and friable in nature 90% to 80% respectively along with single shoots. After the callus formation the formed callus were cultured on different combinations of BAP and NAA and BAP and Zeatin for multiple shoots and shoot elongation. MS medium containing 2.0 mg/l BAP and 1mg/l Zeatin was found to be the best medium for maximum in vitro response i.e., 93% shooting and multiple shoots (7.5)with maximum 8cm in length. Best rooting was achieved on half strength MS medium fortified with 1mg/l NAA respectively.

Key words: Growth regulators, Indirect organogenesis, *Spilanthes acmella*, Murrashige and Skoog

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INTRODUCTION

Tissue culture technique is being increasingly exploited for clonal propagation and *in-vitro* conservation of valuable indigenous germplasm that are threatened with extinction (Boro *et al;* 1998).Micropropagation method is specifically applicable to species in which clonal propagation is needed (Gamborg & Phillips, 1995). *Spilanthes acmella* has been administered as a traditional medicine for years to cure toothaches, stammering and stomatitis. Its diuretic, antibacterial, anti-inflammatory activities had also been studied previously. This plant is widely distributed in the tropical and sub-tropical regions including America, Africa, Srilanka and India. *S.acmella* is a genus of 60 species found in different parts of the world. In India, it is confined to South India, Chhattisgarh and Jharkhand (Anonymous, 1989).

The main active ingredient of this plant, spilanthol, stigmasteryl-3-o-b-D glucopyranoside and a mixture of triterpene provide a medicinal importance to this plant (Ramsewak et al.1999). Its leaves, flowers, roots are used as a medicine for toothache, antidiuretic and as a spices. The root of *S.acmella* has its commercial price in pharmaceutical market. Due to poor availability, possibilities of facing threats by this species, it is the need of time to increase the population size and ensure the greater biomass availability and conserve the species as well. This paper describes the indirect organogenesis strategies developed by using tissue culture technology for this species under the influence of different combinations and concentration of certain growth regulators.

MATERIALS AND METHODS

Collection of Plant material:
Young leaves of *Spilanthes acmella* from 6 months old plant were used as explants and were collected from the pot culture maintained in nursery of Energy Park, Raipur.

Sterilization of Explants & Preparation of culture:
The leaves were thoroughly washed with running tap water to remove all the dust particles adhere with 1% bavistin for 10 minutes, followed by three rinses with sterile double distilled water. To eliminate other contamination 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 3-5 minutes under aseptic conditions. After this explants were then thoroughly washed 4-5 times with sterilized double distilled water to remove the traces of mercuric chloride. (Bhojwani & Razdan, 1996).

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Callus Induction medium:
The leaf segments were inoculated on MS medium supplemented with various concentrations (0.5- 5.0 mg/l) of Auxins (NAA, 2, 4-D, IBA) and (0.5mg/l) of cytokines (BAP) in various combinations for callus induction. The cultures were incubated at a temperature of 25±2ºC and a photoperiod of 8 hrs light (intensity of 2000 lux) and 16 hrs of dark.

Visual observations like callus induction, growth of callus, number of days taken for shoot regeneration and number of shoots regenerated per explants were recorded regularly. A mean of 30 replicates was taken per treatments.

Shoot Induction and multiplication Medium:
The *in-vitro* regenerated shoots from callus of length of average (1-2cm) were excised and inoculated in different concentrations of BAP, NAA and Zeatin under aseptic conditions for multiple shoot development. MS medium supplemented with different combination and concentration of BAP, NAA and Zeatin was used for shoot induction, multiplication and elongation. The cultures were incubated at a temperature of 25±2ºC and a photoperiod of 16 hrs light (intensity of 2000 lux) and 8 hrs of dark

Rooting Of Elongated Shoots:
After proper shoot elongation, the plantlets were properly removed from medium and carefully washed with sterile double distilled water so as to remove any traces of medium on shoots. *In vitro* regenerated shoots (3-4cm) were excised and transferred onto half strength MS medium supplemented with different concentrations of auxins, NAA and IBA for rooting. After one month the complete plantlets were transferred in field for hardening.

Hardening of *in-vitro* raised plantlets:
The rooted plantlets were washed with 1% bavistin and transferred to humus rich soil: coco-pit filled polythene bags in the ratio 3:1 and were kept under moist chamber in green house for 25days (35-38ºC temperature and humidity at 90% RH). After 25 days the rooted plantlets were transplanted to single net shade house in polythene bags of traditional potting mixture containing soil: compost: coco pit in the ratio 1:1:1 for the secondary hardening.

RESULTS AND DISCUSSION

Callus formation was observed from leaf segment after 20-25 days of inoculation in MS medium supplemented with different combinations of 2,4-D,BAP and IBA (Table-1). The better response of explants for callus formation was observed in the MS medium supplemented with (0.5mg/l+ 3.0mg/l) BAP and NAA in combination, (1.5mg/l) 2, 4-D and (0.5mg/l+5.0mg/l) BAP and IBA in combination with 80% success rate. Arumugam *et al.*, (2009) explained that

the callus formation is depending upon the several factors including the culture, environment, nature of explants and hormonal and non-hormonal regulators which may act synergistically in determining the proper induction, proliferation and regeneration of callus into plantlets.(Paulsamy *et al.*, 2013).

Shoot initiation was more pronounced by the sub-culturing of callus on to the MS medium supplemented with Zeatin and 2,4-D in combination (1.0mg/l and 0.5mg/l) and (2.0mg/l+1.0mg/l) BAP and Zeatin, respectively. The higher amount of response (93%) was observed. Various studies had also showed that high concentration of cytokinin generally inhibited root formation of plants (Schraudolf and Reinert, 1959; George and Sherrington, 1984). MS medium supplemented with 10.0mg/l IBA induced callus formation and resulted in the eventual death after 3 weeks. The axillary buds cultured on MS medium supplemented with higher concentration of IBA (6-10mg/l) formed callus with hairy roots without any shoot formation. Axillary buds cultured on MS medium supplemented with combination of BAP higher than 4mg/l and IBA higher than 6mg/l also did not induce any shoo formation. These explants formed callus and became necrotic after few days (Haw and Keng, 2003). Higher concentration of BAP could induce the formation of callus tissue that also caused the chromosomal instability of the regenerated plants (Eellarova and Kimakova, 1999).The effect of different treatments quantified and data series of experiments were conducted and analyze statistically. Data were showed some prosperous effect, included in the table and repeated in mean ±S.E of 30 explants per treatment and repeated three times. Mean values were not significantly different more than one.

The efficiently developed and elongated shoots were observed to produce short thin roots in half strength MS medium supplemented with low concentration (0.5-1.0mg/l) of IBA and NAA. After one month of root development, 15 plantlets were transferred to green house for primary and secondary hardening. The survival was recorded as 100% in the transplantation. After one month the fully matured plantlets were transferred to open nursery for exposure to direct sunlight, showed 100% survival with flowering within next 20 days.

The *in-vitro* indirect organogenesis and elite plantlet production protocol establishment of a medicinal plant species, *Spilanthes acmella* from leaf explants by employing plant tissue culture technique is more useful to enhance the population and hence the biomass of this endangered species.

Callus growth measurements:

The growth of the leaf callus cultures was measured in terms of fresh weights (FW) as well as dry weight (DW). The explants were placed between the folds of blotting paper to remove excess moisture and the fresh weight was then determined. Dry weight was measured after drying the fresh materials in an oven at 60 ± 1°C for 48 hrs.

Table 1: Treatment combinations and callus induction percentage from leaf explants of species *S.acmella*

Sr.No	No. of explants Cultured	Concentrations of growth regulator (mg/l)	No. of for responsive explants for callus induction %	Morphogenic response of Callus	Weight of Callus (mg)	
		2,4-D			Fresh weight	Dry weight
1	30	0.5	80	Creamy, Friable	1790	154
2	30	1.0	68	White loose	1990	158
3	30	1.5	86	Greenish, Friable	2000	115
4	30	2.0	84	Creamy Friable	1980	148
		BAP+NAA				
1	25	0.5+1.0	60	White, Friable	2000	159
2	25	0.5+2.0	70	Creamy, Soft, SS	1990	142
3	25	0.5+3.0	80	white, Friable, SS	1980	146
		BAP + IBA				
4	30	0.5 + 1.0	60	White, Loose	1992	158
5	30	0.5 + 3.0	65	White, Friable, SS	1908	158
6	30	0.5 + 5.0	80	Greenish, Friable, SS	2100	112

Table 2: Effect of different concentration of growth regulators in shoot regeneration from *in-vitro* originated callus (after 25 days of sub culturing)

Sr.No	Treatments	Shoot Initiation %	Mean No of Shoots ±SE	Mean Length of shoot(mm)±SE
MS+ 2,4-D+ Zeatin				
1	0.5 mg/L + 0.5 mg/L Zeatin	80%	2.1 ± 1.7	1.65 ± 0.82
2	**0.5 mg/L + 1.0 mg/L Zeatin**	**88%**	**3.3 ± 1.0**	**2.64 ± 1.98**
3	0.5 mg/L + 2.0 mg/L Zeatin	73%	1.7 ± 1.6	0.72 ± 0.67
MS+ BAP+ Zeatin				
4	0.5 mg/L BAP + 0.5 mg/L Zeatin	78%	4.3 ± 1.4	2.10 ± 1.34
5	0.5 mg/L BAP + 1.0 mg/L Zeatin	88%	5.6 ± 2.1	3.59 ± 1.55
6	2.0 mg/L BAP + 0.5 mg/L Zeatin	89%	6.7 ± 6.2	4.30 ±1.67
7	**2.0 mg/L BAP +1.0 mg/lit Zeatin**	**93%**	**7.5 ± 2.1**	**5.40 ± 0.63**

Table 3: effect of different combinations of growth regulators in shoot elongation

Sr. No.	Growth regulators	Mean no. of shoots ± SE	Mean shoot length ± SE
MS + BAP			
1	1.0mg/L BAP	5.0 ± 1.5	5.2 ± 2.2
2	**2.0 mg/L BAP**	6.9 ± 1.3	7.8 ± 3.2
3	3.0 mg/L BAP	6.7 ± 3.2	6.8 ± 1.2
MS + BAP + NAA			
4	1.0 mg/l BAP +0.25 mg/L NAA	6.3 ± 2.1	4.0 ± 1.5
5	**2.0 mg/LBAP +0.5 mg/L NAA**	**6.5 ± 1.5**	**5.4 ± 1.8**
6	2.0 mg/LBAP + 1.0 mg/l NAA	7.4 ± 2.1	5.0 ± 1.4

Table 4: Effects of *in-vitro* rooting of elongated Shoots of *Spilanthes acmella* (After 15 days of inoculation)

Media used	Number of roots Mean ± SE	Length of Root (cm.) Mean ± SE	Morphogenic response of roots
½ MS	0.4 ± 0.48		Thin, short
½ MS+ 0.5 mg/l IBA	-	-	Thin, short
½ MS+1.0 mg/l IBA	**1.8 ± 0.78**		**Thin, lengthy**
½MS+0.5 mg/L. NAA	-	-	Thick, lengthy
½MS+1 mg/L. NAA	**1.2 ± 0.83**		**Thick, lengthy**

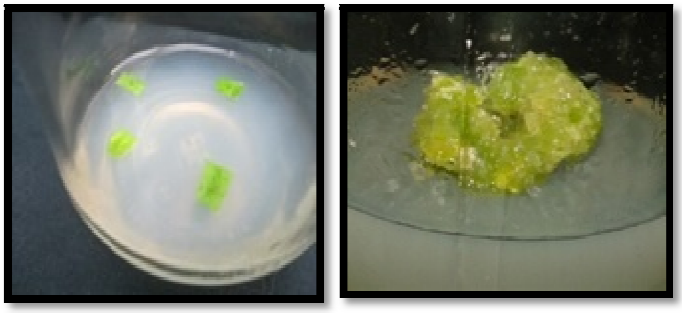

Fig. A.: Leaf of *S.acmella* formed callus in MS medium supplemented with BAP (0.5mg/l)+IBA (5.0mg/l) , after 3 weeks of inoculation

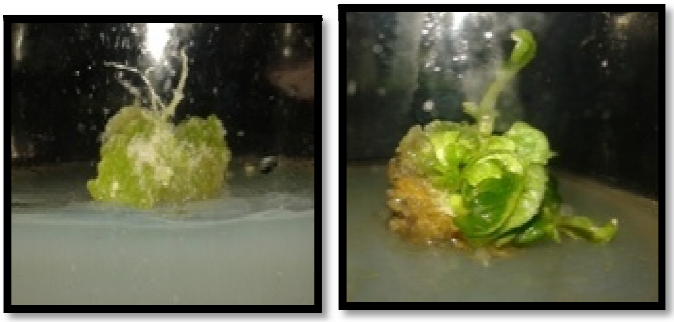

Fig. B.: Small cluster of multiple shoots formed from callus of *S.acmella* when subcultured in MS medium supplemented with BAP (2.0mg/l) + Zeatin (1.0mg/l) and 2,4-D, (0.5mg/L) + Zeatin (1.0mg/L)

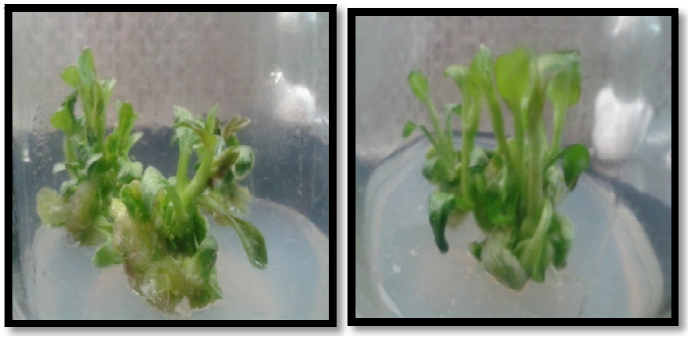

Fig. C: Elongation of shoots of *S.acmella* when callus originated shoots were transferred in MS medium with BAP (2.0 mg/l)

Fig. D: Initiation of roots in half strength MS medium supplemented with IBA (1.0mg/l) and NAA(1.0mg/l)

Fig. E: Secondary hardening in green house and flowering in plantlets of *S. acmella* in open Nursery

CONCLUSION

In the present study, a fruitful in direct organogenesis protocol was set up for Spilanthes acmella through callus formation, shoot regeneration and successful regeneration of new plantlets.The result of this study shows that tissue culture techniques can play an important role in clonal propagation of elite genotypes of *Spilanthes acmella*, which has diverse medicinal applications and eventually due to over exploitation and irregular concern this plant is facing local extinction. This protocol can be exploited for commercial propagation and conservation of potential rare medicinal plant resources.

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