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A reliable rapid protocol for characterization of *in vitro* totipotency in *Spilanthes oleracea*

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Abstract

***Spilanthes oleracea* is an important medicinal herb and is also called 'Toothache Plant' or 'Eye Ball Plant'. It is used to prepare herbal formulation to cure many diseases. Its property to heal the dental wounds can be exploited as an alternative to synthetic medicines used presently. An efficient protocol for *in vitro* shoot multiplication of *Spilanthes oleracea* has been developed from axillary bud explants. Nodal segments, from young plants, were taken as explants; shoot multiplication was induced on slightly modified Murashige and Skoog's (MS) medium supplemented with 6- Benzyl amino purine, (BAP, 0.5 ppm) and Naphthalene Acetic Acid, (NAA, 0.1 ppm) and BAP (0.5 ppm) + Indole 3-Acetic Acid, (IAA, 0.3 ppm). Shoot proliferation could be induced using different combinations of BAP, IAA and NAA. Shoots were further multiplied through continued subculture of nodal segments with sprouted shoots. Micro-shoots were rooted in the basal medium supplemented with NAA (1.71 µM) alone and BAP (0.44 µM) + NAA (1.0 µM) concentration.) Survival of i*n vitro* grown plantlets 2 months after transplantation in the pots, containing equal parts of sand and top soil, was found to be 97 per cent.**

Keywords: *Axillary buds, Conservation, Micropropagation, Multiple shoots, Plant growth substances Spilanthes oleracea*

Introduction

Spilanthes oleracea Linn (Asteraceae) is a herbaceous, tropical/tender perennial with a growing height of 12-18 in. (30-45 cm); its bloom color is red, bright yellow and blooming time is mid summer/late summer, aromatic and blooms repeatedly (Akah and Ekekwe, 1995). *Spilanthes oleracea* is very beautiful, and can be grown as an annual in most climates. It has striking cone-like flowers, much smaller than *Echinacea*. There are no flower petals, but golden buds with a rust-red center, which look like an eyeball (Raju and Raju, 1996).

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This plant is called Toothache plant because we can chew on the fresh or dried flower, or take the extract to help deaden pain from a tooth until we can visit the dentist. It is not only topically anesthetic for gums and teeth, but it is also bacteriostatic, helping to fight tooth decay. Plant parts used to cure ailments like throat pain, constipation and other diseases (Holetz, *et al*., 2002; Kala, 2005). Leaves are pretty potent and cause tingling or numbing of the gums when chewed, which depends on the age of plant. It is supposed that leaves are strongest near the time of flowering.

Tissue culture is now being commonly used for clonal propagation of a large number of horticulture plants, medicinal plants and also for forest trees (Murashige, 1974). Rare and endangered plants as well as medicinally important plants are being conserved and exploited by using *in vitro* techniques all over the world (Ang and Chan, 2003; Baskaran and Jayabalan, 2005). Here, authors have tried to standardize the protocol for optimum

production of this medicinal herb in *in vitro* conditions.

Materials and Methods:

Young plants of *Spilanthes oleracea* were collected from villages of Azamgarh Dstrict, Uttar Pradesh, India and were grown in the green house of Society of Pollution and Environment Conservation Scientists (SPECS). Nodal explants with axillary buds were taken from young & healthy plants for culture initiation (Goel *et al*., 2009).

Explants were first washed in running tap water. Afterwards the explants were placed in a beaker containing water; 3-4 drops of Tween-20 were added and the beaker was shaken lightly for 3-4 minutes, followed by thorough washing with tap water (x 4) and double distilled water (x 4). Surface disinfection was carried out with 0.1% $HgCl_2$ (w/v) for 4 minutes followed by several washings with sterilized distilled water (Gamborg and Phillips, 1995, Goswami *et al*., 1999, Singh *et al*., 2010). The explants were allowed to dry in laminar hood for 20 minutes to remove surface water, and the nodal segments were then inoculated under aseptic conditions on agar solidified Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962; Murashige, 1974) with slight modifications (Manganese sulphate dihydrate was used in place of Manganese sulphate tetrahydrate) and concentration of $Na_2EDTA.2H_2O$ was 30.5mg/l in place of 37.25 mg/l.). The medium was supplemented with usual salts and vitamins and 3.0% sucrose (w/v; Hi- Media), 100mg/litre myo-inositol (E. Merck) and 0.8% agar (w/v; Difco-Bacto, Becton Dickinson U.S.A.).

Media were supplemented with various concentrations of BAP (6-benzylamino purine) alone and in combinations with NAA (α-naphthalene acetic acid) and IAA (Indole-3- Acetic acid). The pH of the media was adjusted to 5.8 before the addition of agar and autoclaved at 121^0C with 1.5 kg lb/cm^2 for 20 minutes. The cultures were kept at 25$\pm$ 2^0C under illumination with white fluorescent tubes (50 μM $m^{-2}s^{-1}$) at 78% relative humidity. They were maintained under light for 14 hours followed by 10 hours dark period. Each treatment had 5 replicates and the experiments were repeated 3 times. Sprouting of axillary buds was seen on nodal segments after 15-25 days of culture (Figure 3 A). These buds, with part of the growing nodal segments, were subcultured on modified medium supplemented with BAP (1.30-4.40 μM) + IAA (1.40-2.30 μM) or NAA (0.44-1.33μM) for further shoot multiplication. Nodal explants (0.7-0.9 cm) from the axenic shoots were recultured on agar solidified medium containing different concentrations of BAP, NAA and IAA. Gibberellic Acid, (GA_3, 0.15-3.10 μM) for elongation of shoots (Fig 4 & 5); the shoots attained height of 2.3$\pm$0.10 cm. Roots were then induced in these shoots measuring 2-3 cm long by transferring to MS medium supplemented with different combinations of IAA, and NAA. The roots were initiated in rooting medium as well as in basal medium. The roots produced in basal medium were thin and short.

Eight weeks old plantlets were transferred to pots containing sterilized soil and sand (1:1), covered with polythene bags with perforations, for 10 days and the pots were kept below 25$\pm$2°C, for acclimatization. These were then transferred to green house, after removing polythene covers, for hardening (Saritha *et al*., 2003).

Results and Discussion

Best induction of multiple shoot formation from nodal explants occurred on medium containing BAP (4.84 μM) and IAA (2.60 μM) - Figure 3 A. After that the cultures were transferred to the medium that favored multiple shoot regeneration. Amongst different combinations of plant growth substances used, maximum shoot regeneration per explant was found to take place with BAP (4.00 μM) and NAA (2.80 μM) - (Figure 1 & Figure 3 B) with about 25 shoots in 40 days. Use of BAP (2.22 μM) with IAA (1.50 μM) also gave multiple shoots (15 shoots per explant after 40 days). In contrast, the number of shoots formed in control cultures was only 2$\pm$ 0.5; shoot induction was late as well as shoots formed were less viable (survival only 10%). Further it was found that the slight increase in sucrose concentration was able to increase the shot multiplication rate (Figure 1) while higher concentrations of BAP inhibited the shoot multiplication rate as well as induction of shoots (Figure 2). Root initiation was tried with combinations of BAP, IAA and NAA, but best root growth was promoted by BAP (0.40 μM) used with IAA (1.75 μM; Figure 3 C) and with IAA alone (1.75 μM). Plants transferred after acclimatization in green house (Figure 3 D), showed 93% survival capacity.

Table 1. Effect of Plant Growth Substances on shoot multiplication from cultured nodal explants in *Spilanthes oleracea* (Values are means ± SE of five replicates per treatment)

S.No.	Plant Growth Substances (conc. in µM)	25 Days No. of shoots	25 Days Average length of shoots (mm)	45 Days No. of shoots	45 Days Average length of shoots (mm)
1.	Control	0	-	0	-
2.	BAP (2.20)	4	1.5 ±0.3	7	16 ±0.9
3.	BAP(2.20)+NAA (2.20)	9	1.7 ±0.4	15	12 ±0.8
4.	BAP (2.20)+ IAA (1.80)	10	3.0 ±0.7	18	20 ±1.3
5.	BAP (3.40)+ NAA (2.80)	11	3.5 ±0.9	20	25±1.8
6.	BAP (3.30)+ NAA (2.40) + GA_3 (0.28)	12	4.9 ±1.2	22	35 ±1.4
7.	BAP (4.50)+ NAA (2.80)	5	1.8 ±0.2	9	22 ±0.9

*Only those combinations are shown that produced optimum results.

Table 2. Effect of Plant Growth Substances on rooting of *in vitro* raised microshoots (Values are means ± SE of five replicates per treatment)

S.No.	Plant Growth Substances (conc. in µM)	25 Days No. of roots	25 Days Average length of roots (mm)	45 Days No. of roots	45 Days Average length of roots (mm)
1.	Control	0	-	0	-
2.	IAA 1.70	3	1.7 +0.8	5	6 +0.8
3.	NAA 1.70	5	1.8+0.4	5	13 +1.3
4.	BAP (0.40)+ NAA(1.70)	9	3.9+0.8	16	20+2.4
5.	BAP (0.80)+ NAA(1.70)	4	2.5+0.3	9	9 +0.6
6.	BAP (0.40)+ IAA(0.80)	7	1.8+0.2	11	10 +0.9

*Only those combinations are shown that produced optimum results.

Table 3: Survival of plantlets under *ex vitro* conditions (Values are means ± SE of five replicates repeated thrice)

Group No.	No. of plantlets produced & transferred to pots	No. of surviving plants after 60 days	Survival percent (%)
1	45	42	93.33
2	37	33	89.12
3	42	41	97.62
4	53	49	92.45
5	35	33	94.29
		Average survival % 93.362	

It was observed in the present investigations that multiple plant regeneration from nodal explants of *Spilanthes oleracea* could be induced on slightly modified MS medium. Plant multiplication rate was dependent on appropriate combinations of plant growth substances (PGSs). Higher concentrations of PGSs, especially BAP was found to inhibit shoot multiplication. The current work provides preliminary information and methodology for rapid propagation of this valuable plant from nodal explants that might help in the improvement of conservation methods.

Conclusion

Spilanthes oleracea is widely used in many traditional medicines prescribed under different systems of medicine. *Spilanthes* species have long been used as traditional medicine for local anesthetic, antibacterial (Sabitha Rani and Suryanarayana Murty, 2005), antiviral, antihypertensive, larvicidal (Pandey *et al*., 2007) and diuretic actions. The whole plant leaves and roots are used for a variety of purposes in many herbal medicines (Ley *et al*., 2006). For example, the leaves are used to cure throat infections (Chandra Prakash Kala, 2005) and for the treatment

of ulcers (Chauhan *et al.*, 2003). It is shown that the plant is being used traditionally in treatment of several respiratory diseases. It is, therefore, important to maintain a balance between its use and conservation status. Many researchers have paid attention in this direction.

Propagation and conservation of some pharmaceutically important *Spilanthes species* was attempted using tissue culture technique (Ang and Chan, 2003). Suspension cultures widely used for the *in vitro* production of secondary metabolites using large and small scale fermenters, proved the importance of tissue culture technology (Curtin, 1983). In the present study, direct shoot multiplication was preferred for generating true-to-type plants than callus regeneration. This study supported the rapid multiplication of this useful medicinal plant by *in vitro* conditions.

In vitro mass propagation of *Spilanthes oleracea* reported here may provide some help in this direction. The protocol developed is easy and reproducible through which its mass multiplication can be attempted at commercial level.

It is less time taking and the survival rate of *in vitro* grown plants was also found to be more than 97%, a considerable improvement over earlier studies (Karthikeyan, *et al.*, 2007; Babeet *et al.*, 2010).

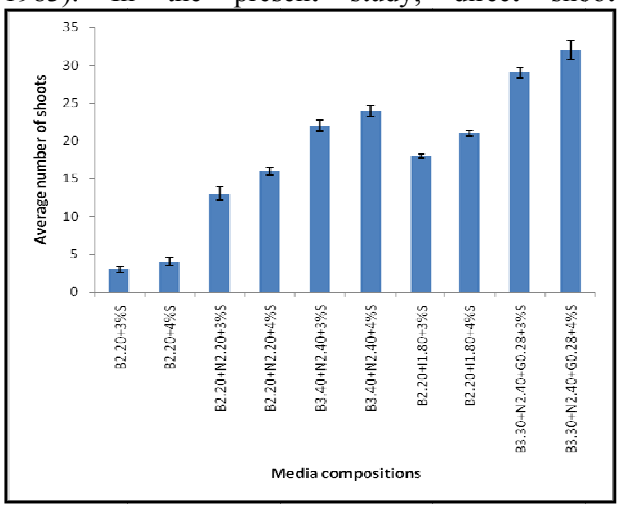

Figure 1. Frequency of multiple shoot regeneration with varying concentrations of sucrose.
B= BAP, N= NAA, I= IAA, G= GA_3 and S = Sucrose; Error bar indicates standard deviations.

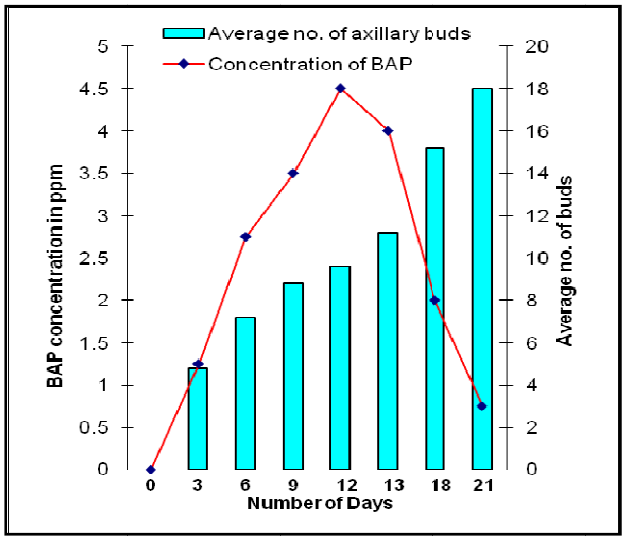

Figure 2. Effect of increasing BAP concentration on axillary bud proliferation in *Spilanthes oleracea*

Figure 3

Figure 3: *In vitro* propagation of *Spilanthes oleracea*.
(A) Origin of multiple shoots from nodes, after 3 weeks on MS medium supplemented with BAP (4.84 µM) and IAA (2.60 µM).
(B) Mass multiplication of shoots after 4 weeks on the MS medium supplemented with BAP (4.00 µM) and NAA (2.80 µM).
(C) Development of healthy and viable roots on medium supplemented with BAP (0.40 µM) used with IAA (1.75 µM after 18 days.
(D) A 6 week old plant ready to be planted in pots; the survival of such plantlets, 4 weeks after plantation, was found to be more than 93%.

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References

Akah, P.A. and Ekekwe, R.K., 1995. Etnopharmacology of some Asteraceae family used in Nigerian traditional medicine. ***Fitoterapia***, 66: 351-355.

Ang Boon Haw and Chan Lai Keng, 2003. Micropropagation of *Spilanthes acmella* L., a bio-insecticide plant, through proliferation of multiple shoots. ***J. Appl. Hort***., 5(2): 65-68.

Babeet, S.T., Choudhary, R. and Vijayvergia, R., 2010. *In Vitro* and *in vivo* comparative study of primary metabolites and antioxidant activity in *Spilanthes acmella* Murr., ***International Journal of Biotechnology and Biochemistry,*** 6(5): 819–825.

Baskaran, P. and Jayabalan, N., 2005. An efficient Micropropagation system for *Eclipta alba*m a valuable medicinal herb. ***In vitro Cell. Dev. Biol. Plant***., **41**: 532-539.

Chandra Prakash Kala, 2005. Ethnomedicinal botany of the Apatani in the Eastern Himalayan region of India. ***Journal of Ethnobiology and Ethnomedicine***, 1: 11

Chauhan, D.K., Agrawal, B.P., 2003. *Spilanthes acmella* Murr. A potential herb for the treatment of recurrent aphthous ulcers and ulcerative stomatitis. ***National Seminar on New Millenium Strategies for Quality, Safety & GMPs of Herbal Drugs/Products***, NBRI, Lucknow, p. 79, November 11-13.

Curtin, N.E., 1983. Harvesting profitable products from plant tissue culture. ***Biotechnology***, 1: 649-657.

Gamborg, O.L. and Phillips, G.C., 1995. Sterile Techniques, Ed OL Gamborg and G.C. Phillips, Plant Cell, Tissue and Organ Culture, Fundamental Methods. Chapter 3, p.35-42, Springer-Verlag Berlin, Heidelberg, New York.

Goel, N., Singh, N. and Saini, R., 2009. Efficient *in vitro* multiplication of Syrian Rue (*Peganum harmala* L.) using 6- benzylaminopurine pre-conditioned seedling explants. ***Nature and Science***, **7**: 129-134.

Goswami, H., Chan, L.K. and Teo, C.K.H., 1999. *In vitro* shoot multiplication of *Tectona grandis*. ***Journal of Bioscience***, 10: 47-54.

Holetz, F.B., Pessini, G.L., Sanches, N.R., Cortez, D.A., Nakamura, C.V. and Filho, B.P., 2002. Screening of some plants used in the Brazilian folk medicine for the treatment of infectious diseases. ***Mem. Inst. Oswaldo Cruz.***, 97 (7): 1027–1031.

Karthikeyan, K., Chandran, C., Kulothungan, S., 2007. Rapid regeneration of *Phyllanthus niruri* L. from shoot tip and nodal explants. ***Ind. J. Appl. Pure Biol***., **22**: 337- 342.

Ley, J.P., Blings, M., Krammer, G., Reinders, G., Schmidt, C.O. and Bertram, H.J., 2006. Isolation and synthesis of acmellonate, a new unsaturated long chain 2-ketol ester from *Spilanthes acmella*. ***Nat. Prod. Res.***, 20 (9): 798–804.

Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. ***Physiol. Pl***., 15: 473.

Murashige, T., 1974. Plant propagation through tissue culture. ***Annual Review***, ***Plant Physiology***, 25: 136.

Pandey, V., Agrawal, V., Raghavendra, K. and Dash, A.P., 2007. Strong larvicidal activity of three species of *Spilanthes* (Akarkara) against malaria (*Anopheles stephensi* Liston, *Anopheles culicifacies*, species C) and filaria vector (*Culex quinquefasciatus* Say). ***Parasitol. Res***., 102: 171-174.

Raju, C.P. & Raju, R.V., 1996. Some rare and interesting Asteraceous taxa from the forests of Andhra Pradesh. **India.** ***J. Econ. Tax. Bot***., 20: 261- 265.

Sabitha R.A. and Suryanarayana M.U., 2005. Evaluation of antimicrobial activity of *Spilanthes acmella* flower head extract. ***Journal of Natural Remedies***. 5/2: 170-171.

Saritha, K.V., Parkash, E., Swamy, P.M. and Naidu, C.V., 2003. Indirect shoot regeneration from leaf explants of *Spilanthes acmella*. ***J. Plant Biol***., **30**: 31-36.

Singh N., Kaur, A. and Yadav, K., 2010. A reliable *in vitro* protocol for rapid mass propagation of *Sapindus mukorossi* Gaertn. ***Nature and Science***, 8(10): 41-47.