British Biotechnology Journal
3(3): 405-415, 2013

SCIENCEDOMAIN *international*
www.sciencedomain.org

Efficient Micropropagation of *Spilanthes acmella* (L.) Murr.: A Threatened Medicinal Herb

Shiwali Sharma[1] and Anwar Shahzad[1*]

[1]Department of Botany, Plant Biotechnology Laboratory, Aligarh Muslim University, Aligarh-202002, India.

Authors' contributions

This work was carried out in collaboration between two authors. Author AS suggested the study and extended over all guidance during the experimentation. Author SS performed the experimental work, analyzed data and wrote the first draft of the manuscript. Both the authors read and approved the final manuscript.

Research Article

Received 4th March 2013
Accepted 13th June 2013
Published 29th June 2013

ABSTRACT

The present study describes an efficient and reproducible protocol for micropropagation of *S. acmella*. Shoot tips taken from 3 week-old aseptic seedlings were cultured on Murashige and Skoog (MS) semi-solid medium supplemented with different concentrations of TDZ. Among various concentrations, 0.25 µM TDZ was found to be optimum for shoot regeneration as it induced a maximum of 30.0 shoots per explant however with retarded growth (1.0 cm). Among different volumes of culture media, 15 ml liquid culture medium favored best response wherein a maximum of 80.2 shoots per explant with an average shoot length of 7.0 cm were induced after 6 week of subculturing. Successful *in vitro* rooting was induced on 2.5 µM NAA containing half-strength MS medium. Almost 96% rooted plants successfully transferred and acclimatized *ex vitro* under green house conditions. Morphological and physiological parameters compared with the *in vivo*-grown seedlings of the same age appeared to be 'normal' in respect to the fundamental characteristics examined.

Keywords: Asteraceae; in vitro proliferation; in vitro rooting; shoot tip; acclimatization.

**Corresponding author: Email: ashahzad.bt@amu.ac.in;*

ABBREVIATIONS

6-benzyladenine (BA); gibberellic acid (GA); indole-3-acetic acid (IAA); indole-3-butyric acid (IBA); 2-isopentyladenine (2iP); kinetin (Kn); α-naphthalene acetic acid (NAA); thidiazuron (TDZ).

1. INTRODUCTION

Spilanthes acmella (L.) Murr. belongs to the family Asteraceae, is a threatened medicinal herb [1]. It is native to the tropics of Brazil. *S. acmella* has been well documented for its uses as antimalarial [2], antibacterial [3], antifungal [4,5], larvicidal [6], anti-inflammatory [7,8] and immunomodulating properties [9]. In addition, *in vitro* results on antiobesity [10] and antioxidant [11] activities for *S. acmella* are encouraging. Medicinal activities are mainly due to the presence of an alkaloid spilanthol (*N*-isobutyl-2, 6, 8-decatrienamide) [12]. Recently, scopoletin has also been detected in *S. acmella* flower buds [13,14]. Spilanthol also showed anti-ageing activity by inhibiting contractions in subcutaneous muscles, notably those of face and can be used as an anti-wrinkle product. Many anti-ageing products are available in commercial market as registered brands (Gatuline®, SYN®-COLL, ChroNOline™) containing spilanthol. Such wide spectrum uses of spilanthol makes it a wonder drug for pharmaceutical sector.

Conventional propagation by seeds and cuttings has various constraints such as poor germination [2], rooting of seeds and infestations of plant by various pests [15], therefore *in vitro* plant regeneration is preferred. As the *in vivo* plants are vulnerable to pest [15], seedlings were used in the present study as the source of explant to reduce contamination and easy availability of explant throughout the year. Moreover, seedling explants are advantageous for transformation and micropropagation studies due to easy planning of experiments. During the last few years, considerable efforts have been made for *in vitro* plant regeneration of this threatened medicinal herb using semi-solid nutrient media supplemented with adenine derived cytokinins and auxins [2,14,16,17,18,19,20,21], but the results were not much promising for commercial production as the regeneration rate was less. In our recent report also [5], only 8 shoots per shoot tip were possible on BA containing medium. Thus in the present study, TDZ and liquid culture system were used to maximize the shoot regeneration in *S. acmella*. In liquid medium, the close contact of the tissue with the medium may stimulate and facilitate the uptake of nutrients and phytohromones, leading to better shoot and root growth [22]. The purpose of establishing a liquid culture system in the present study was to save labor and expenses by eliminating gelling agents which would consequently reduce the cost of micropropagated plantlets [22].

2. MATERIALS AND METHODS

2.1 Plant Materials, Explant Preparation

The inflorescences of *S. acmella* were procured from the Medicinal Plant Nursery of Tamnaar, District Raigarh, State Chhattisgarh (India). The plant species was identified by the taxonomist in the Department of Botany of Aligarh Muslim University (A.M.U.) and a specimen was deposited in the herbarium of the Department (Number 31301). The seeds were isolated mechanically from the dried inflorescence and washed under running tap water for 30 min to remove any adherent particle. The seeds were kept in 1% (w/v) Bavistin (Carbendazim Powder, BASF India Limited), a broad spectrum fungicide, for 20 min and

then washed in 5% (v/v) Teepol (Qualigens, India), a liquid detergent for 15 min. The treated seeds were agitated in sterilized double distilled water (DDW) to remove the chemical inhibitors for germination. The seeds were surface sterilized with 70% (v/v) ethanol and 2-3 drops (v/v) of Tween-20 (Qualigens) for 30 s, followed by immersion in an aqueous solution of 0.1% (w/v) $HgCl_2$ (Qualigens) for 3 min under the sterile condition. Finally, the seeds were washed 5-6 times with sterilized DDW to remove all traces of sterilants. The surface sterilized seeds were inoculated aseptically in culture tubes (25 × 150 mm, Borosil, India) containing half-strength MS medium supplemented with 0.5 µM gibberellic acid (GA). Shoot tips (1.0 cm) with a pair of leaf primordia were excised from 3 week-old aseptic seedlings and used as explants (5).

2.2 Multiple Shoot Induction on Semi-Solid Medium

The MS medium containing 3% (w/v) sucrose (Qualigens) with or without 0.8% (w/v) agar (Qualigens) was used throughout the experiment. All the plant growth regulators (PGRs) used in the present study were purchased from Duchefa, Netherlands. For the regeneration trials, varying concentrations of TDZ (0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 µM) were added to MS medium containing 0.8% agar. The medium without TDZ is treated as control. The pH of the culture medium was adjusted to 5.8 by 1 N NaOH or 1 N HCl before autoclaving at 121ºC and 103.5 kPa for 20 min.

2.3 Shoot Proliferation and Elongation on Liquid Medium

Basal regenerating tissues of shoot tip explant having maximum number of shoot buds regenerated on TDZ supplemented medium were excised from the mother explants and transferred to static liquid MS medium supplemented with BA (1.0 µM) for further enhancement of shoot multiplication rate. In order to identify the minimal amount of liquid medium required for optimum growth and multiplication so as to overcome the problems of their submergence and hyperhydricity, different volumes of liquid medium (10, 15, 20, 30 ml) were tested in 150 ml Erlenmeyer flask (Borosil). After 2 weeks of transference, shoot clusters were cut into four pieces and then each piece was sub-cultured to the fresh nutrient medium of respective volume. Shoots of four pieces were aggregated and then recorded as mean number of shoots per explant.

2.4 *In vitro* Rooting

For *in vitro* root induction, elongated shoots (greater than 3 cm) were excised from the proliferating cultures and transferred to half-strength MS medium supplemented with or without varying concentrations of different auxins like, NAA, IAA and IBA (1.0, 2.5 and 5.0 µM). Half-strength MS medium devoid of any auxin is treated as control treatment.

2.5 Culture Conditions

All the cultures were incubated in a culture room at 25 ± 2ºC under fluorescent tube light (16 h hotoperiod) with a photon flux density (PFD) of 30 µmol m^{-2} s^{-1} [5].

2.6 Acclimatization

The rooted shoots were taken out from the culture vessels after 4 week of root induction, delicately washed under running tap water to remove the adhering culture medium from the

roots and subsequently transferred to thermocol cups (expanded polystyrene) containing sterilized soilrite. Each cup was placed in a hardening chamber to ensure high humidity. After one month, hardened-off plantlets were transferred to pots containing normal garden soil and farmyard manure (2: 1) under field conditions [5].

2.7 Morphological and Physiological Study

Five *in vitro* regenerated plantlets (after 4 month of field transfer in pots containing garden soil and manure) and five seedling derived *in vivo* plants (4 month-old) were uprooted and compared for different morphological and physiological parameters such as plant height, fresh and dry mass of different plant parts (root, stem, leaf and flower head), number of flower heads per plant, chlorophyll & carotenoids content and Net Photosynthetic rate (P_N). For the determination of dry mass, tissues were dried in oven at 30℃ for 20 days. Leaf chlorophyll and carotenoids were extracted with 80% acetone and estimated spectrophotometrically (UV-1700 Pharma Spec, Shimadzu, Japan) [23, 24]. The P_N was measured on five upper, fully expanded and healthy leaves with the help of a portable photosynthetic system (LI-COR 6400, LI-COR Biosciences, Lincoln, NE, USA) at 900 µmol m^{-2} s^{-1} photo-synthetically active radiation between 11:00 a.m. to 12:00 noon [25].

2.8 Statistical Analysis

All the experiments were repeated thrice with 20 replicates for each treatment. The data were analyzed by one-way ANOVA using SPSS Version 12 (SPSS Inc., Chicago, USA). The significance of differences among means was analyzed using Tukey's test at 5 % level and data represented as mean ± standard error (SE).

3.1 RESULTS

3.1 Multiple Shoot Induction on Semi-Solid Medium

The explants cultured on MS basal medium (control) failed to induce multiple shoot buds even after 4 week of culture. On control treatment, all the shoot tip explants elongated into single shoot. Supplementation of TDZ (0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 µM) to MS nutrient medium stimulated the direct shoot bud induction from the basal cut end of the explants (Table 1). The explants swelling had been observed from their cut ends within one week of incubation (Fig. 1A). First appearance of buds was observed from swollen cut ends after 2 week of culture. Subsequently, frequency of shoot bud induction increased drastically. Among different concentrations, 0.25 µM TDZ was found optimum wherein a maximum of 30.0 shoots per explant were induced in 98% cultures after 4 week of incubation. On this treatment shoots were of rosette like due to poor inter-node elongation (1.0 cm) and each explant was transformed into a dense mass of profusely regenerating shoot buds. However, on lowering the concentration of TDZ (0.1 µM) regeneration frequency was reduced significantly (6.8 shoots per explant). On the highest concentration of TDZ (5.0 µM), the multiplication rate was least (2.8 shoots per explant) due to intense callogenesis.

To improve the shoot growth with enhanced multiplication, basal regenerating tissue of shoot tip explants regenerated on 0.25 µM TDZ were excised from the explant and transferred to different volumes of liquid nutrient media.

Table 1. Effect of different concentrations of TDZ on multiple shoot formation from shoot tip explants after 4 week of culture

TDZ (µM)	% Response	Mean number of shoots/explant	Mean shoot length (cm)
0.0 (control)	100.0 ± 0.0^{a}	1.0 ± 0.0^{f}	3.8 ± 0.1^{a}
0.1	84.0 ± 2.4^{b}	6.8 ± 0.6^{cd}	1.4 ± 0.1^{b}
0.25	98.0 ± 2.0^{a}	30.0 ± 0.3^{a}	1.0 ± 0.1^{bc}
0.5	82.0 ± 3.7^{b}	11.8 ± 0.9^{b}	1.0 ± 0.1^{bc}
1.0	70.0 ± 3.1^{c}	8.8 ± 0.6^{c}	1.0 ± 0.1^{bc}
2.5	48.0 ± 2.0^{d}	5.6 ± 0.4^{d}	0.8 ± 0.0^{c}
5.0	46.0 ± 2.4^{d}	2.8 ± 0.4^{e}	1.1 ± 0.1^{bc}

Values are mean ± SE (3 separate experiments each with 20 replicates). Mean values within the column with same superscript are not significantly different (P=5%; Tukey's Test).

3.2 Shoot Proliferation and Elongation on Liquid Medium

A rapid enhancement in shoot proliferation was noticed when regenerating basal clumps were transferred to liquid culture medium. New shoots were formed only through adventitious organogenesis of the regenerating shoot clumps there was no axillary or apical bud branching. When different volumes of liquid medium were compared, growth (in term of number of shoots and shoot length) was optimal in 15 ml liquid MS medium supplemented with 1.0 µM BA wherein shoot multiplication increased by 40.2 to 80.2 shoots per explant (2.7 fold increase) after 2 (Fig. 1B) and 6 week of transfer from TDZ containing medium thereafter shoots turned to yellowish and underwent hyperhydricity caused necrosis and did not improve regeneration efficiency further. Hyperhydricity in regenerating shoots after 8 weeks of sub-culturing might be due to the prolonged and direct contact to the liquid medium. Although, 10 ml liquid medium did not cause any hyperhydricity symptom even after 8 week of incubation (as 10 ml volume provided very little submergence to the regenerative tissue) but shoot proliferation was comparatively lower than 15 ml culture medium. A maximum of 55.8 shoots per explants with 5.3 cm of shoot length were noticed after 8 week of culture. Significant improvement in shoot number and shoot length on 15 ml culture medium than 10 ml was possibly due to the better availability of nutrients in 15 ml culture medium than 10 ml. While, on higher volumes (20 and 30 ml) hyperhydricity was early in appearance (after 4 and 2 week respectively) due to submergence of shoots, caused yellowing followed by highly vitrified shoots and considerable decline in the multiplication rate to 45.0 and 37.8 shoots per explant recorded after 8 weeks on 20 ml and 30 ml volume respectively (Table 2).

3.3 *In vitro* Rooting

Microshoots longer than 3 cm in length were dissected and transferred to rooting media containing half-strength MS medium with or without varying concentrations (1.0, 2.5 and 5.0 µM) of NAA, IBA and IAA. Although shoots cultured on the medium devoid of auxins induced roots but they were very thin and delicate. The root system was poor, devoid of secondary branching, suggesting the requirement of exogenous auxin supplementation for healthy root system. Supplementation with three concentrations of IBA and IAA did not give any encouraging result on *in vitro* rooting. Root initiation occurred after 2 week of transfer to rooting medium and proceeded with the formation of callus and little bulging at the bottom from where roots were produced.

Table 2. Effect of different volumes of liquid MS medium containing BA (1.0 µM) on shoot proliferation and shoot elongation

Period of culture (wk)	Volume of the liquid medium (ml) in 150 ml flask							
	10		15		20		30	
	Mean shoot no. /explant	Mean shoot length (cm)	Mean shoot no. / explant	Mean shoot length (cm)	Mean shoot no. / explant	Mean shoot length (cm)	Mean shoot no. / explant	Mean shoot length (cm)
2	37.4 ± 0.9[d]	2.8 ± 0.1[a]	40.2 ± 0.7[c]	3.9 ± 0.1[c]	35.8 ± 0.7[c]	3.3 ± 0.1[c]	32.4 ± 0.7[a]	2.3 ± 0.1[a]
4	45.0 ± 0.9[c]	3.7 ± 0.1[c]	65.6 ± 0.7[b]	5.8 ± 0.1[b]	43.6 ±0.7[b]	3.6 ± 0.1[b]	40.2 ± 0.7[a] (vitrified)	2.3 ± 0.1[a]
6	50.4 ± 0.7[b]	4.1 ± 0.1[b]	80.2 ± 0.8[a]	7.0 ± 0.1[a]	47.4 ± 0.9[a] (vitrified)	3.9 ± 0.0[a]	38.4 ± 0.9[a] (vitrified)	2.3 ± 0.1[a]
8	55.8 ± 0.6[a]	5.3 ± 0.1[a]	78.4 ± 0.8[a] (vitrified)	7.2 ± 0.1[a]	45.0 ± 0.3[ab] (vitrified)	3.8 ± 0.1[ab]	37.8 ± 0.7[a] (vitrified)	2.3 ± 0.1[a]

Values are mean ± SE (3 separate experiments each with 20 replicates). Mean values within the column with same superscript are not significantly different (P=5%; Tukey's Test).

Though mean root number was highest (32.8 roots per shoot) on IBA (2.5 µM) supplemented medium but the roots were stunted (3.2 cm) and superficially spread on the medium and did not show any further elongation. Among three auxins assessed, NAA proved to be the best wherein root initiation was possible just after 4-5 days of incubation and a maximum of 30.4 roots per shoot with an average length of 18.6 cm were induced after 4 week of incubation. Although number of roots per shoot on NAA (2.5 µM) enriched medium was fewer than IBA (2.5 µM) but the root system was much healthier and roots were longer than those in IBA supplemented medium (Fig. 1C, D, E; Table 3).

Table 3. Effect of different concentrations of auxins augmented to half-strength basal MS media on *in vitro* rooting after 4 week of transfer

Auxin (µM)	Mean number of roots/shoot	Mean root length (cm)	Callusing
Control	8.6 ± 0.4^{fg}	8.8 ± 0.6^{c}	No callusing
NAA (1.0)	20.2 ± 0.6^{bc}	13.4 ± 0.2^{b}	No callusing
NAA (2.5)	30.4 ± 0.5^{a}	18.6 ± 0.2^{a}	No callusing
NAA (5.0)	20.4 ± 0.5^{bc}	12.8 ± 0.4^{b}	Little callusing
IBA (1.0)	23.2 ± 1.0^{b}	5.5 ± 0.2^{d}	Little callusing
IBA (2.5)	32.8 ± 1.2^{a}	3.2 ± 0.2^{ef}	Little callusing
IBA (5.0)	16.8 ± 0.9^{cd}	2.2 ± 0.2^{g}	Moderate callusing
IAA (1.0)	11.6 ± 1.0^{ef}	4.1 ± 0.1^{e}	Little callusing
IAA (2.5)	14.4 ± 0.7^{de}	5.3 ± 0.1^{d}	Moderate callusing
IAA (5.0)	7.6 ± 0.7^{g}	2.9 ± 0.1^{fg}	Intense callusing

Values are mean ± SE (3 separate experiments each with 20 replicates). Mean values within the column with same superscript are not significantly different (P=5%; Tukey's Test).

The plantlets were successfully acclimatized in soilrite as described in the materials and method (Fig. 1F). After 4 week in soilrite, the plantlets were transferred to earthen pots where 96% survival percentage was noticed for those plantlets which were rooted on NAA containing nutrient medium. While, shoots rooted in IBA and IAA supplemented media showed 85% and 70% survival rate respectively. This may be due to improper development of root system in such cultures. In field conditions plantlets showed luxurious growth and exhibited normal morphology and flowering after 1 month of transfer (Fig. 1G, H).

3.4 Morphological and Physiological Study

The data on some morphological and physiological characters were given in Table 4. The micropropagated plants appeared morphologically uniform as compared to the seedling derived plants. However, slight variations in the morphology of micropropagated plantlets in terms of plant height, fresh and dry mass of root, stem, leaf and flower head and mean number of flower head per plant were recorded in comparison to the seedling derived plants. Leaf mass (fresh and dry), total chlorophyll and carotenoids content, chl a/b ratio and P_N were higher in micropropagated plantlets than seedling derived plants.

Table 4. Comparison of various morphological and physiological parameters of *in vitro* raised plantlets and seedling derived *in vivo* plants of *Spilanthes acmella*

Parameters	*In vitro* raised Plantlet	Seedling derived *in vivo* plant
Plant length (cm)	30.84 ± 0.61	31.98 ± 0.54
Root fresh mass (g plant^{-1})	2.42 ± 0.03	2.74 ± 0.08
Root dry mass (g plant^{-1})	0.20 ± 0.00	0.23 ± 0.01
Shoot fresh mass (g plant^{-1})	10.16 ± 0.51	10.82 ± 0.46
Shoot dry mass (g plant^{-1})	1.03 ± 0.05	1.24 ± 0.08
Leaf fresh mass (g plant^{-1})	6.16 ± 0.09	5.25 ± 0.04
Leaf dry mass (g plant^{-1})	0.51 ± 0.01	0.43 ± 0.03
Flower head fresh mass (g plant^{-1})	8.26 ± 0.12	8.54 ± 0.05
Flower head dry mass (g plant^{-1})	1.80 ± 0.01	1.92 ± 0.01
Number of flower head (plant^{-1})	9.40 ± 0.75	10.00 ± 0.31
Total chlorophyll content (mg g^{-1})	1.50 ± 0.03	1.25 ± 0.02
Chlorophyll a/b ratio	2.51 ± 0.01	2.40 ± 0.01
Carotenoid content (mg g^{-1})	0.29 ± 0.01	0.26 ± 0.00
Net Photosynthetic Rate (P_N)	8.19 ± 0.06	8.02 ± 0.04

Values are mean ± SE (each with five replicates).

Fig. 1. Shoot regeneration from shoot tip explants using TDZ (A) Adventitious bud induction from the basal cut end of the shoot tip explant on 0.25 µM TDZ after 1 week of culture; (B) Prolific shoot multiplication on 15 ml liquid MS medium supplemented with 1.0 µM BA after 2 week of transfer; (C) & (D) Elongated roots induced on half-strength basal MS Medium with 2.5 µM NAA after 4 week of transfer; (E) Stunted rooting on half-strength basal MS Medium with 2.5 µM IBA after 4 week of transfer; (F) An acclimatized plantlet in soilrite; (G) *In vitro* raised plantlet on transfer to garden soil; (H) A healthy twig of *S. acmella* showing flowering after 4 month of field transfer

4. DISCUSSION

In our previous study with adenine-based cytokinins, only 8 shoots per shoot tip was possible on 1.0 µM BA supplemented MS medium after 4 weeks of culture [5], therefore urea based cytokinin i.e., TDZ was employed for enhanced shoot regeneration through shoot tip explants. Among the concentrations, 0.25 µM TDZ was found optimum as induced a maximum of 30.0 shoots per explant. Similar response with the low concentration of TDZ has been reported by Banerjee et al. and He et al. in *Cineraria maritima* and *Hydrastis canadensis* respectively [26,27]. The possible reason for the higher activity of individual TDZ treatment might be its high stability due to its resistance to cytokinin oxidase and suggesting its substitutive activity for both auxin and cytokinin combination [28].

Although TDZ promoted higher frequency adventitious shoot regeneration but shoots were rosette like and failed to elongate. Therefore for further improvement in shoot growth with enhanced proliferation, regenerating tissue of shoot tip explants were excised and transferred to liquid culture medium supplemented with 1.0 µM BA. A highly significant increase in shoot number (40.2 to 80.2 shoots per explant) and shoot length (3.9 to 7.0 cm) was recorded when clump of TDZ regenerated shoots were transferred to 15 ml static liquid medium after 6 week. Rapid enhancement in shoot proliferation efficiency in liquid medium was possibly due to the close contact of tissue with the medium which might facilitate the uniform access and better utilization of nutrients and PGRs.

In vitro rooting was best on half-strength MS medium augmented with 2.5 µM NAA as it induced early and healthy rooting within 4-5 days of transfer. The stimulatory effect of NAA on root formation has been reported in many medicinal plants like *Carthamus tinctorius* [29], *Trichosanthes dioica* [30]. The results in the present study is in contrast to results reported by Dhar and Joshi (2005) in *Saussurea obvallata*, Mohapatra *et al*. (2008) in *Centella asiatica* and Saritha and Naidu (2008) in *S. acmella* where half-strength MS with IBA was proved to the best for *in vitro* root induction [31,32,18].

5. CONCLUSION

In conclusion, the present protocol reports the efficiency of urea based cytokinin i.e., TDZ over adenine based cytokinin i.e., BA. The implementation of liquid culture system for further shoot proliferation seems to have a good potential for maximization of shoot regeneration. Thus, the *in vitro* propagation protocol developed in the present study can be effectively utilized for maximization and domestication of this multipurpose species of *Spilanthes* and is expected to help replenish and sustain dwindling populations in the natural environment.

ACKNOWLEDGMENTS

Dr. Anwar Shahzad gratefully acknowledges the financial support provided by UGC and UP-CST in the form of research projects (vide no. 39-369/2010 SR and vide no. CST/D3836 respectively). Dr. Shiwali Sharma is also thankful to UGC, for the award of Dr. D.S. Kothari Post Doc Fellowship (1st April 2013) for providing research assistance.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Rao NK, Reddy RK. Threatened plants of Tirupati and its environs. Howarh. An Assessment of Threatened Plants of India. 1983;167-8.
2. Pandey V, Agrawal V. Efficient micropropagation protocol of *Spilanthes acmella* L. possessing strong antimalarial activity. *In Vitro* Cell Dev Biol-Plant 2009;45:491-99.
3. Prasad MM, Seenaya G. Effects of spices on growth of red halophilic cocci isolated from salt cured fish and solar salt. Food Res Int. 2000;33:793-98.
4. Sabitha AR, Suryanarayana UM. Antifungal potential of flower head extract of *Spilanthes acmella* Linn. African J Biomed Res 2006;9:67-9.
5. Sharma S, Shahzad A, Shahid M, Jahan N. An efficient *in vitro* production of shoots from shoot tips and antifungal activity of *Spilanthes acmella* (L.) Murr. Int J Plant Dev Biol. 2012;6:40-5.
6. Amer A, Mehlhorn H. Larvicidal effects of various essential oils against *Aedes*, *Anopheles*, and *Culex* larvae (Diptera, Culicidae). Parasitol Res. 2006;99:466-72.
7. Chakraborty A, Devi RKB, Rita S, Sharatchandra K, Singh TI. Preliminary studies on anti-inflammatory and analgesic activities of *Spilanthes acmella* in experimental animal models. Indian J Pharma. 2004;36:148-50.
8. Wu LC, Fan NC, Lin MH, Chu IR, Huang SJ, Hu CY, Han SY. Anti-inflammatory effect of Spilanthol from *Spilanthes acmella* on murine macrophage by down-regulating LPS-induced anti-inflammatory mediators. J Agric Food Chem. 2008;56:2341-9.
9. Savadi RV, Yadav R, Yadav N. Study on immunomodulatory activity of ethanolic extract of *Spilanthes acmella* Murr. leaves. Indian J Nat Prod Res. 2010;1:204-7.
10. Ekanem AP, Wang M, Simon JE, Moreno DA. Antiobesity properties of two African plants (*Afromomum meleguetta* and *Spilanthes acmella*) by pancreatic lipase inhibition. Phytotherapy Res. 2007;21:1253-5.
11. Tanwer BS, Choudhary R, Vijayvergia R. *In vitro* and *in vivo* comparative study of primary metabolites and antioxidant activity in *Spilanthes acmella* Murr. Int J Biotech Biochem. 2010;6:819-25.
12. Sharma G, Gupta V, Sharma S, Shrivastava B, Bairva R. Toothache plant *Spilanthes acmella* Murr.: a review. J Natural Conscious. 2010;1:135-42.
13. Prachayasittikul S, Suphapong S, Worachartcheewan A, Lawung R, Ruchirawat S, Prachayasittikul V. Bioactive metabolites from *Spilanthes acmella* Murr. Molecule 2009;14:850-67.
14. Singh M, Chaturvedi R. Improved clonal propagation of *Spilanthes acmella* Murr. for production of scopoletin. Plant Cell Tiss Organ Cult. 2010;103:243-53.
15. Chandra S, Sharma HP, Chandra R, Jha S. Medicinal herbs-*Spilanthes* species: a review. Pharmbit. 2007;15:17-22.
16. Saritha KV, Prakash E, Ramamurthy N, Naidu CV. Micropropagation of *Spilanthes acmella* Murr. Biol Plant. 2002;45:581-4.
17. Haw AB, Keng CL. Micropropagation of *Spilanthes acmella*, a bio-insecticide plant through proliferation of multiple shoots. J App Hortic. 2003;5:65-8.
18. Saritha KV, Naidu CV. Direct shoot regeneration from leaf explant of *Spilanthes acmella*. Biol Plant. 2008;52:334-8.
19. Deka P, Kalita MC. *In vitro* clonal propagation and organogenesis in *Spilanthes acmella* (L.) Murray: a herbal pesticidal plant of north-east India. J Plant Biochem Biotech. 2005;14:69-71.
20. Singh SK, Rai MK, Asthana P, Sahoo L. An improved micropropagation of *Spilanthes acmella* L. through transverse thin cell layer culture. Acta Physiol Plant. 2009;31:693-98.

21. Singh M, Chaturvedi R. Screening and quantification of an antiseptic alkylamide, spilanthol from *in vitro* cell and tissue cultures of *Spilanthes acmella* Murr. Industrail Crop Prod. 2012;36:321-28.
22. Sandal I, Bhattacharya A, Ahuja PS. An efficient liquid culture system for tea shoot proliferation. Plant Cell Tiss Organ Cult. 2001;65:75–80.
23. Mackinney G. Absorption of light by chlorophyll solution. J Biol Chem. 1941;140:315-22.
24. MacLachlan S, Zalik S. Plastid structure, chlorophyll concentration and free amino acid composition of chlorophyll mutant of barley. Can J Bot. 1963;41:1053-62.
25. Sahai A, Shahzad A. High frequency *in vitro* regeneration system for conservation of *Coleus forskohlii*: a threatened medicinal herb. Acta Physiol Plant. 2013;35:473-81.
26. Banerjee S, Tripathi J, Verma PC, Dwivedi PD, Khanuja SPS, Bagchi GD. Thidiazuron-induced high-frequency shoot proliferation in *Cineraria maritima* Linn. Curr Sci. 2004;87:1287-90.
27. He SS, Liu CZ. Plant regeneration of an endangered medicinal plant *Hydrastis canadensis* L. Sci Hort. 2007;113:82-86.
28. Huetteman CA, Preece JE. Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tiss Organ Cult. 1993;33:105-19.
29. Radhika K, Sujatha M, Nageshwer Rao T. Thidiazuron stimulates adventitious shoot regeneration in different safflower explants. Biol Plant. 2006;50:174-9.
30. Malek MA, Bari Miah MA, Al-Amin M, Khanam D, Khatun M. *In vitro* regeneration in pointed gourd. Bangladesh J Agri Res. 2007;32:461-71.
31. Dhar U, Joshi M. Efficient plant regeneration protocol through callus for *Saussurea obvallata* (DC.) Edgew. (Asteraceae): effect of explant type, age and plant growth regulators. Plant Cell Rep. 2005;24:195-200.
32. Mohapatra H, Barik DP, Rath SP. *In vitro* regeneration of medicinal plant *Centella asiatica*. Biol Plant. 2008;52:339-42.

© 2013 Sharma and Shahzad; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=217&id=11&aid=1595