Acta Physiol Plant (2009) 31:693–698
DOI 10.1007/s11738-009-0280-9

ORIGINAL PAPER

An improved micropropagation of *Spilanthes acmella* L. through transverse thin cell layer culture

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Received: 6 September 2008 / Revised: 30 October 2008 / Accepted: 20 January 2009 / Published online: 12 February 2009

Abstract An efficient and improved in vitro propagation system for *Spilanthes acmella* L. using transverse thin cell layer (tTCL) culture system was established. The frequency of shoot regeneration from tTCL nodal segments was affected by concentrations of plant growth regulators and orientation of the explant. MS (Murashige and Skoog in Physiol Plant 15:473–497, 1962) medium with 5.0 mg dm^{-3} BAP was optimal for shoot regeneration. Upon this medium, the explant inoculated in the upright orientation exhibited a high frequency of shoot regeneration (about 97%), and the highest number of shoots (31.5) per explant. The intact node (1.0–1.5 cm) cultured on the same medium had significantly lower shoot multiplication ability with only 4.5 shoots per responsive explant. As compared to BAP alone, the combination of BAP and Kin or NAA did not have positive effects on shoot multiplication from tTCL nodal segments. Rooting of shoots was achieved on growth regulator free full-strength MS medium. Plantlets were transplanted into soil with 90–100% survival rate.

Communicated by E. Lojkowska.

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Keywords In vitro propagation · Organogenesis · Plant growth regulators · *Spilanthes acmella* · Thin cell layer explant

Abbreviations

BAP	6-Benzylaminopurine
Kin	Kinetin
MS	Murashige and Skoog (1962)
NAA	α-Naphthalene acetic acid
PGRs	Plant growth regulators
tTCL	Transverse thin cell layer

Introduction

Spilanthes acmella L. (syn. *Acmella oleracea* L.), commonly known as Toothache plant, is an important medicinal plant of the family *Asteraceae* and is widely distributed to tropical and subtropical region of the world. The plants have shown anti-inflammatory, antibacterial, and antifungal properties. The antimicrobial activity of this species is due to presence of a highly valuable biologically active compound spilanthol (Khadir et al. 1989; Saritha et al. 2002). Traditionally, this plant is also used in treatment of gum diseases. The hexane extract of dried flower buds of *S. acmella* contains bioactive *N*-isobutylamides, which are effective against *Aedes aegypti* and *Helicoverpa zea* neonate larvae (Ramsewak et al. 1999). The plant has also been reported for its uses as larvicidal activity against *Culex quinquefasciatus* (Amer and Mehlhorn 2006).

With the worldwide increasing demand for plant-derived medicines, there has been a concomitant increase in the demand for raw material. However, the increasing human and livestock populations have affected the status of

wild plants, particularly those used in herbal medicine (Hu et al. 2005). Plant tissue culture is a useful tool for the conservation and rapid propagation of medicinally important and endangered plants (Baskaran and Jayabalan 2008). Since first report by Tran Thanh Van (1973), thin cell layers (TCLs) of plant tissues such as shoots, stem nodes, hypocotyls, etc. have been successfully used as explants for plant regeneration in several plant species such as *Lilium longiflorum* (Bui et al. 1999), *Lupinus* species (Mulin and Bellio-Spataru 2000), *Oryza sativa* L. (Nhut et al. 2000), *Spinacia oleracea* (Le'guillon et al. 2003) or *Brassica napus* (Ghnaya et al. 2008). The TCL system, consists of explants of a small size (0.1–5 mm) excised from different plant organs either longitudinally or transversally (Teixeira da Silva 2003; Rout et al. 2006), has been applied to shoot regeneration and somatic embryogenesis (Nhut et al. 2000; Zhao et al. 2007), and was proved to be more efficient than other conventional in vitro culture methods with regard to the total output of plantlets (Lakshmanan et al. 1995).

Due to medicinal importance and pesticidal activity, *S. acmella* has increased its demand. Hence, there is need for development of an efficient regeneration system in *S. acmella*. The plant is conventionally propagated through seed and grows generally in moist and wet places. Due to this habit it is vulnerable to pest and diseases. In order to stop using contaminated pharmaceutical raw materials and fulfill the demands of pharmaceutical industries, it is necessary to develop an efficient method of propagation of *S. acmella* by a suitable agrotechnology. During past years, considerable efforts have been made for in vitro plant regeneration of this important medicinally important and herbal pesticidal plant through organogenesis (Saritha et al. 2002; Haw and Keng 2003; Deka and Kalita 2005; Saritha and Naidu 2008). So, in order to obtain rapid and efficient plant regeneration with a high frequency, the transverse thin cell layer (tTCL) culture method was exploited for mass propagation of *S. acmella*.

Materials and methods

Explant source, surface sterilization, and preparation of explants

Nodal segments of *S. acmella* obtained from field grown plants were used as explant source. Nodal segments were washed thoroughly for 30 min under running tap water, and treated for 5–10 min with 2% (v/v) cetrimide (disinfectant; ICI India) and two to three drops of Tween-20 (Hi-media, India). This was followed by washing under tap water for removal of detergents and disinfectants. Subsequently, nodal segments were immersed in 70% ethanol for 30 s and surface-disinfected with an aqueous solution of 0.05% (w/v) freshly prepared mercuric chloride for 3–5 min and washed three to four times with sterile distilled water. The stem segments with nodes were transversely sliced into pieces of about 1–4 mm in thickness, and the slices from the nodes were used as tTCL explants for plant regeneration.

Culture media and culture conditions

For shoot proliferation, the tTCL nodal segments were inoculated on MS (Murashige and Skoog 1962) medium containing 3% (w/v) sucrose and different concentrations (0–6.0 mg dm^{-3}) of BAP or Kin (Sigma Chemical Co., St Louis, MO, USA). To study the interactive effect of BAP and Kin or NAA, tTCL were cultured on MS medium supplemented with BAP (5.0 mg dm^{-3}) and different concentrations (1.0, 3.0 or 5.0 mg dm^{-3}) of Kin or NAA. Regenerated shoots on different multiplication media were excised and subcultured on growth regulator free full-strength MS medium for the shoot elongation and root induction. The media were solidified with 0.8% (w/v) agar (Merck India Ltd, Mumbai, India). The pH was adjusted to 5.8 ± 0.02 with 0.1 N NaOH or 0.01 N HCl prior to autoclaving at 121°C for 15 min at 1.1 kg cm^{-2} pressure. All cultures were incubated at 24 ± 2°C under a 16-h photoperiod, with the light intensity of 40 μmol m^{-2} s^{-1} provided by cool white, fluorescent tubes (Philips, India).

Comparative study of tTCL and intact nodal segments for shoot multiplication

Surface sterilized intact nodal segments (1.0–1.2 cm) and tTCL nodal segments (1–4 mm) were cultured on MS medium supplemented with BAP (0–5.0 mg dm^{-3}) for the study of their comparative responses for shoot multiplication.

Influence of orientation of explants on shoot multiplication

To evaluate the effect of orientation of explants on shoot multiplication, the explants were cultured in two orientations: upright (with the basal, i.e., proximal end cut surface touching on the medium) and inverted (the apical, i.e., distal end cut surface touching on the medium).

Acclimatization of regenerated plantlets

Rooted shoots with four to five fully expanded leaves, retrieved from TCL nodal segments were transferred to plastic pots containing a 1:1 (w/w) mixture of sand and soil. Plantlets were covered with polyethylene bag to

maintain high humidity and irrigated with tap water. Pots with plantlets were kept at 25 ± 1°C in artificial light (irradiance of 50 μmol m^{-2} s^{-1}) provided by cool white fluorescent tubes for 3–4 weeks and then the pots were transferred to direct sunlight. After 2–3 months, all plants were transferred to garden soil under field condition.

Data analysis

For the above experiments, 24 replicates were used for each treatment and each experiment was repeated thrice. The data was analyzed statistically using SPSS (ver 10) software. The mean separations were carried out using Duncan's multiple range tests and paired sample *T* test and significance was determined at $P < 0.05$.

Results and discussion

Influence of PGRs on shoot multiplication from tTCL nodal segments

Shoot buds were induced on MS medium with or without any plant growth regulators within 2–3 weeks from tTCL nodal segments. However, when growth regulators were absent, only one shoot developed. Multiple shoots were induced from tTCL nodal segments inoculated on media with different PGRs. Irrespective of PGRs, the adventitious shoot buds emerged on the edge of the explants within 2 weeks of culture (Fig. 1a), afterward arose from the basal region of the explants and finally the regenerated shoot and adventitious buds grew into a clump (Fig. 1b).

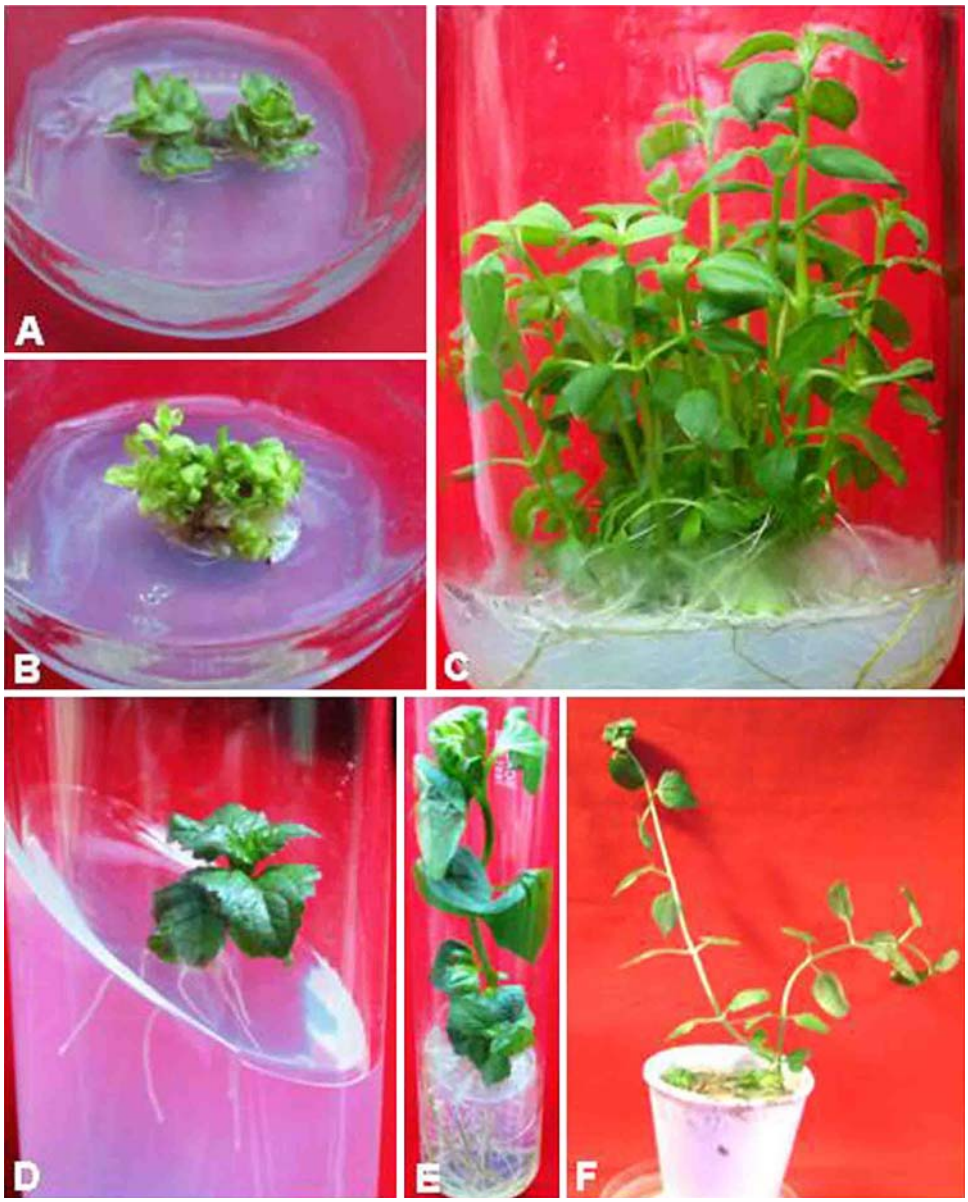

Fig. 1 Plant regeneration from tTCL nodal segments of *S. acmella*. **a** Adventitious shoot buds emerging from the edge of the explants. **b** Adventitious shoot buds developing into a clump. **c** Shoot multiplication from tTCL nodal segments on 0.8% agar-solidified full-strength MS medium + BAP (5.0 mg dm^{-3}). **d** Rooting of microshoot after 1 week of subculture on growth regulator free MS medium from shoot multiplication medium. **e** Rooting after 4 weeks on growth regulator free MS medium. **f** Well-hardened plant of *S. acmella* and branching of shoot after hardening in soil

The tTCL nodal segments cultured on media containing BAP alone had better potential for multiple shoot induction than kinetin alone (Fig. 2). Medium supplemented with BAP (5.0 mg dm^{-3}) was most effective for shoot multiplication from tTCL nodal segments (Figs. 1c, 2). The percent response for shoot regeneration was about 95% and the average number of adventitious buds per responsive explant was 31.5 on medium containing 5.0 mg dm^{-3} BAP (Fig. 2). This number appears to be the higher as compared to all other previous reports on the same plant species (Saritha et al. 2002; Haw and Keng 2003; Deka and Kalita 2005; Saritha and Naidu 2008). In the trials that aimed at investigating the interactive effect of BAP and Kin or NAA, the number of shoots produced per explant was higher in the medium containing 5.0 mg dm^{-3} BAP alone and inclusion of Kin (1.0–5.0 mg dm^{-3}) or NAA (1.0–5.0 mg dm^{-3}) in medium did not have positive effects on shoot multiplication. As compared to 5.0 mg dm^{-3} BAP (31.5 shoots) alone, the number of shoots per responsive explant significantly ($P < 0.05$) reduced on media containing the combination of 5.0 mg dm^{-3} BAP and different concentrations of Kin (15–20 shoots) or NAA (3–12 shoots) (Table 1). Effectiveness of BAP in axillary shoot regeneration was reported in a number of other medicinal plant species (Agarwal et al. 2005; Ray and Bhattacharya 2008; Siddique and Anis 2008). Superiority of BAP for shoot induction may be attributed to the ability of plant tissues to metabolize BAP more readily than other synthetic growth regulators or to the ability of BAP to induce production of natural hormones such as zeatin within the tissue (Malik et al. 2005).

Comparative study of tTCL and intact nodal segments for shoot multiplication

As compared to intact node (1–1.5 cm), tTCL nodal segments had great shoot multiplication ability when cultured on medium containing different concentrations of BAP (0–5.0 mg dm^{-3}). On medium containing BAP (5.0 mg dm^{-3}), tTCL nodal segments were induce to produce about 31.5 shoots per responsive explant, whereas, at same concentration of BAP, intact nodal segments were produced significantly less number (about 4.5 shoots per responsive explant) of shoots (Table 2).

Dramatic mass propagation of multiple shoot from the tTCL explants (0.1–5 mm) are caused by homogenous cell nature of explant tissue (Rout et al. 2006). Furthermore, due to thin layer of explants, availability of growth substances is easy for each and every cells of the homogenous tissue. Due to uniformly exposer of reduced cell number tTCL explants get easily affected the morphogenic responses of explants by the external environments (Rout et al. 2006). TCL technology has also been effectively used in the micropropagation of vegetable, leguminous, and medicinal plants, including *Amaranthus edulis* (amaranth), *Beta vulgaris* (sugar beet), *B. napus* (oilseed rape), *Lupinus* spp. (lupin), *Panax ginseng* (ginseng), and *Phaseolus vulgaris* (common bean) (Nhut et al. 2003).

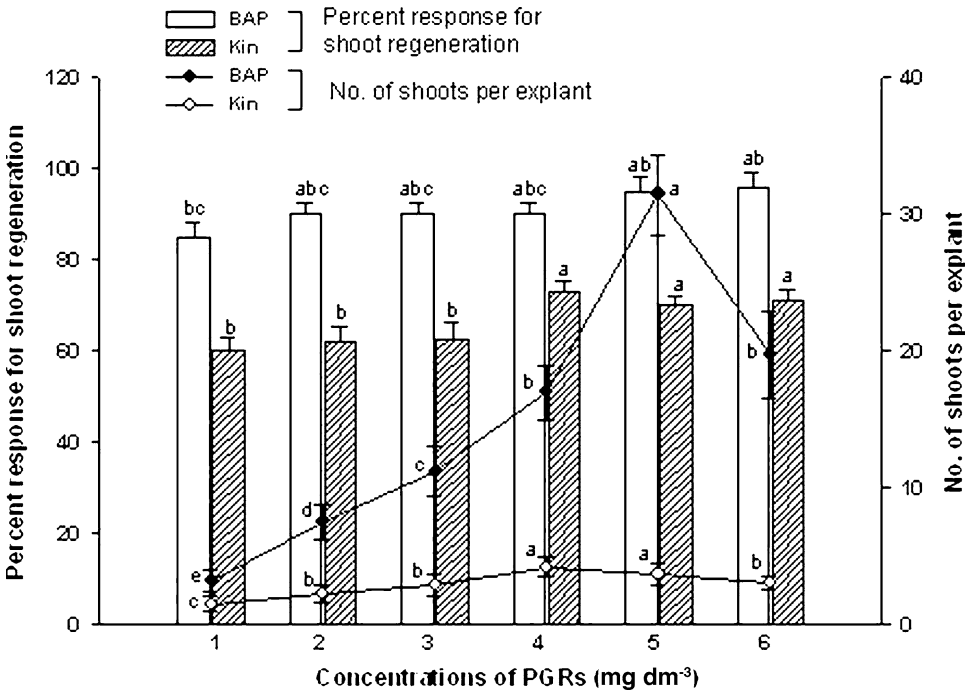

Fig. 2 Effect of BAP and Kin on shoot proliferation and multiplication from tTCL nodal segments of *S. acmella*. Values are mean ± SE of three independent experiments. Data was recorded after 4 weeks of culture on shoot multiplication medium. Mean values sharing the same *letter* do not differ significantly ($P < 0.05$) according to Duncan's multiple range test

Table 1 Interactive effect of BAP with Kin or NAA on shoot proliferation and multiplication from tTCL nodal segments

Concentration of PGRs (mg dm^{-3})			Shoot regeneration (%)	Number of shoots per responsive explant
BAP	Kin	NAA		
5.0	0.0	0.0	95a	31.5a
5.0	1.0	0.0	80b	19.0b
5.0	3.0	0.0	90ab	20.5b
5.0	5.0	0.0	90ab	15.0bc
5.0	0.0	1.00	60c	12.0cd
5.0	0.0	3.00	54c	6.5de
5.0	0.0	5.00	50c	3.0e

Medium: 0.8% agar-solidified full-strength MS medium

Values are means of three independent experiments. Data was recorded after 4 weeks of culture on shoot multiplication medium. Mean values followed by the different letter under different treatments within a column are significantly different from each other at $P < 0.05$ (Duncan's multiple range test)

Table 2 Responses of the intact nodal segments and thin cell layer culture of the nodal segments

Concentration of BAP (mg dm^{-3})	Number of shoots per explants	
	Intact node (1–1.5 cm)	tTCL nodal segments (2–4 mm)
0.0	1.0c	1.0e
1.0	1.7c	3.2e
2.0	2.9b	7.5d
3.0	3.2b	11.2c
4.00	3.8ab	17.0b
5.00	4.5a	31.5a

Medium: 0.8% agar-solidified full-strength MS medium

Values are the means of three independent experiments. Data was recorded after weeks of culture on shoot multiplication medium. Mean values followed by the different letter under different treatments within a column are significantly different from each other at $P < 0.05$ (Duncan's multiple range test)

Influence of orientation of explants on shoot multiplication

Efficiency of shoot regeneration was also affected by the orientation of tTCL nodal segments. As compared to inverted orientation, tTCL incubated in an upright orientation on the medium showed significantly higher percent shoot regeneration ($df = 2$, $t = 45.033$, $P < 0.0001$) and number of shoots per responsive explant ($df = 2$, $t = 13.306$, $P < 0.006$). The shoot regeneration for the tTCL upright orientation was about 97% and the average number of adventitious buds per responsive explant was about 31.5 whereas for the inverted orientation of the explant it was only 45% with the average shoot number per responsive explant of 11.9 (Table 3). Similarly in the case of *Dendrobium candidum*, upright oriented tTCL nodal explant gave better response than inverted explants (Zhao et al. 2007). Morphogenesis is determined by the nutrients and growth factors in the somatic cells and recently it was found that the accumulation of macronutrients was very significantly affected by the pattern of ontogenetic development (Zhao et al. 2007). The effect of orientation of explants shoot regeneration may be due to appearance of a polarity in somatic cells caused by nutrients and growth regulators (Saini and Jaiwal 2002).

Table 3 Influence of orientation of tTCL nodal segments on shoot multiplication

Explant orientation on the medium	Percentage of explants with shoots (%)	Number of shoots per responsive explant
Upright	97.2a	31.5a
Inverted	45.0b	11.9b

Medium: 0.8% agar-solidified full-strength MS medium + BAP (5.0 mg dm^{-3})

Values are the means of three independent experiments. Data was recorded after weeks of culture on shoot multiplication medium. Mean values followed by the different letter under different treatments within a column are significantly different from each other at $P < 0.05$ (according to paired sample T test)

Rooting of shoots and acclimatization

Regenerated shoots with four to six leaves were rooted on the growth regulator free MS medium after 1 week of subculture from shoot multiplication media (Fig. 1d). The percentage of rooting was 100% and 15–20 roots per shoot induced after 4 weeks on root induction medium (Fig. 1e). The rooted plantlets were successfully acclimatized and established in soil (Fig. 1f) with a survival frequency of 90–100%. All the established plants were apparently uniform and did not show any detectable variation.

To conclude, the success of rapid and direct shoot regeneration from tTCL explants opens an efficient way to mass propagation of *S. acmella*. Such small explants are relatively uniform, very sensitive and react faster than traditional explants to the applied treatments (Le'guillon et al. 2003). The improved method that we have established for plant regeneration in *S. acmella* through tTCL could be applied for efficient genetic transformation assays and pharmaceutical purposes.

Acknowledgments The author (S. K. S.) thanks the Head, Center for Energy at Indian Institute of Technology Guwahati and North Eastern Development Finance Corporation Ltd (NEDFL) for providing the necessary facilities. Financial assistance provided by Council of Scientific and Industrial Research (CSIR), New Delhi, to the authors (M. K. R. and P. A.) are gratefully acknowledged.

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