J. Appl. Hort., 5(2):65-68, July-December, 2003

Micropropagation of *Spilanthes acmella* L., a bio-insecticide plant, through proliferation of multiple shoots

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Abstract

Spilanthes acmella L. was successfully micropropagated using axillary buds as explants. The aseptic axillary buds formed multiple shoots within five weeks when cultured on Murashige and Skoog (MS) medium supplemented with 2.0 mg/l N^6-Bezyl adenine (BA). The addition of Indole-3-butyric acid (IBA) had no significant effect on the multiple shoots formation of this plant. MS medium supplemented with 0.5 mg/l BA was sufficient for the proliferation of rooted multiple shoots of *S. acmella* L. First subculturing of the *in vitro* individual shoots in the same proliferation medium could double the formation of multiple shoots.

Key words: Axillary buds, insecticidal properties, micrpropagation, mass propagation, N^6-Bezyl adenine (BA), Indole-3-butyric acid (IBA), *Spilanthes acmella*

Introduction

Malaysia being a tropical country supports many plants that contain useful secondary metabolites in its rainforest. More than 2000 plant species with insecticidal properties have been documented (Broussalis *et al.,* 1999). The secondary metabolites produced by these plants is used as a defence mechanism against pest (Luthria *et al.*, 1993). Plants from the Compositae family have been reported to contain useful insecticidal compounds. The genus *Spilanthes* consists of 42 known species and several insecticidal compounds have been reported in *Spilanthes mauritiana, S. alba, S. ocymyfolia, S. oleracea* and *S. acmella* (Cook, 1996; Jondiko, 1986; Krishnaswamy *et al.,* 1975; Borrges-del-Castillo *et al.*, 1984; Burkill, 1966; Ramsewak *et al.*, 1999).

Spilanthes acmella has been well documented for its uses as spices, antiseptic, anti-bacterial, anti-fungal, anti-malarial and as remedy for toothache, flu, cough, rabies diseases and tuberculosis (Burkill, 1966; Oliver-Bever, 1986; Di Stasi *et al.*, 1994; Akah and Ekekwe, 1995; Singh, 1995; Storey and Salem, 1997; Ramsewak *et al.*, 1999). Prasad and Seenayya (2000) reported that *S. acmella* also possessed excellent anti-microbial activities against red halophillic cocci from salt cured fish. There are reports that *S. acmella* contains alkaloids that have the potential to act as an insecticide (Krishnaswamy *et al.*, 1975; Borges-del-Castillo *et al.*, 1984) and were found to be able to control *Aedes aegypti* in Kenya (Jondiko, 1986).

To the best of our knowledge, no report is available on *in vitro* propagation of *S. acmella*. This study was carried out to investigate the possibility of micropropagating *S. acmella* for the mass production of *in vitro* plantlets that can be subsequently used as plant source for the production of bio-insecticide compounds.

Materials and methods

Establishment of aseptic explant: *Spilanthes acmella* growing wild in the field was collected and planted at the herbal garden of Plant Tissue and Cell Culture Laboratory, Universiti Sains Malaysia, Penang, Malaysia. Axillary buds of the plant were used as explants and were first washed with detergent and rinsed under running tap water for 30 minutes. They were then dipped into 70% alcohol for one minute before surface sterilizing, using mercury chloride ($HgCl_2$) 0.08% (w/v) for 5 minutes. After rinsing three times with sterile distilled water, the explants were again sterilized with Clorox® 15% (w/v) for 15 minutes. They were then again rinsed three times with sterile distilled water. The explants were dried using sterile filter paper before inoculating into basic MS culture medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose. The pH of the medium was adjusted to 5.7 –5.8, followed by addition of 7.5 g l^{-1} bacto agar before autoclaving at 121 °C and 1.06 kg/cm^2 for 13 minutes using autoclave Fuji EAC-4000D. All the cultures were incubated at 25 ± 2 °C under 24–h photoperiod provided by white fluorescent light with a light intensity of 32.5 mE m^{-2} s^{-1}. The aseptic shoots that were obtained after two weeks of cultures were used as the source of explants for the subsequent experiments.

Induction of multiple shoots formation: The aseptic axillary buds were cultured on MS medium supplemented with 0.0, 2.0, 4.0, 6.0, 8.0 and 10.0 mgl^{-1} of BA and 0.0, 2.0, 4.0, 6.0, 8.0 and 10.0 mgl^{-1} of IBA using a factorial experimental design. The pH of the culture media was adjusted to 5.7 – 5.8 with 0.1 M NaOH or 0.1 M HCl prior to autoclaving at 121 °C and 1.06 kg/cm^2 for 13 minutes using autoclave Fuji EAC-4000D. Thirty explants were used for each medium treatment and the experiment was repeated three times. The cultures were incubated at 25 ± 2 °C under continuous illumination with cool white fluorescent tubes at an intensity of 32.5 mE m^{-2} s^{-1}. Responses of the cultures were observed and recorded over a period of five weeks. The shoot length and number of shoots formed were recorded. Explant that produced more than two shoots was considered as multiple shoots.

Selection of best medium for mass propagation: Based on the results obtained from the study stated in the above section, it was found that only BA was needed to be supplemented into the MS medium for the induction of multiple shoots formation. To study the effect of reduced BA concentration (0.5 – 2.5 mgl^{-1}) on multiple shoots formation of *S. acmella*, the separated individual *in vitro* shoots of *S. acmella* were cultured on MS medium supplemented with 0.5, 1.0, 1.5, 2.0 and 2.5 mgl^{-1} BA. Thirty

explants were used for each medium treatment and the experiment was repeated three times. The number and length of shoots formed were recorded after five weeks of culture.

Data Analysis: Data were analyzed by Analysis of Variance (ANOVA) and comparison of means by Duncan's Multiple Range Test at p=0.05 using SAS programme.

Results and discussion

Within 5 weeks, an average of 2.3 to 2.6 shoots were formed from each axillary bud of *Spilanthes acmella* when they were cultured on MS supplemented with 2.0, 4.0, 6.0 and 8.0 mg l^{-1} of BA. The addition of IBA, as low as 2 mg l^{-1}, into MS medium containing BA, however, did not show significant influence on multiple shoots formation from the axillary bud explants (Table 1). This observation suggested that the induction of multiple shoots formation of *S. acmella* depended only on the presence of BA in the culture medium. The axillary buds cultured on basic MS medium without any growth regulator produced single shoot with complete root system (Fig. 1). All the multiple shoots formed in MS media supplemented with BA of 2.0, 4.0, 6.0, 8.0 mg/l formed small clusters, and accompanied with swelling and callus growth without any root system (Fig. 2). 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 10.0 mg/l BA induced callus formation and resulted in the eventual death of explants.

Table 1. Number of shoots formed from the axillary buds of *Spilanthes acmella* when cultured on MS medium supplemented with various concentration of BA and IBA within five weeks

MS +		Number of shoot / bud (± SE)
BA (mg l^{-1})	IBA (mg l^{-1})	
0.0	0.0	1.1 ± 0.0 f
2.0		2.5 ± 0.0 a
4.0		2.4 ± 0.1 a
6.0		2.6 ± 0.1 ab
8.0		2.3 ± 0.1 b
10.0		1.1 ± 0.1ef
0.0	2.0	1.8 ± 0.0 c
2.0		1.7 ± 0.1 cd
4.0		1.8 ± 0.1 cd
6.0		1.8 ± 0.1 c
8.0		1.3 ± 0.1 ef
10.0		0 g
0.0	4.0	1.4 ± 0.1 de
2.0		1.8 ± 0.1 c
4.0 – 10.0	6.0	0 g
0.0 – 10.0	8.0	0 g
0.0 – 10.0	10.0	0 g

Mean values followed by the same alphabet are not significantly different using DMRT (p=0.05)

The axillary buds cultured on MS medium supplemented with high concentration of IBA (6-10 mg l^{-1}) formed callus with hairy roots without any shoot formation. Axillary buds cultured on

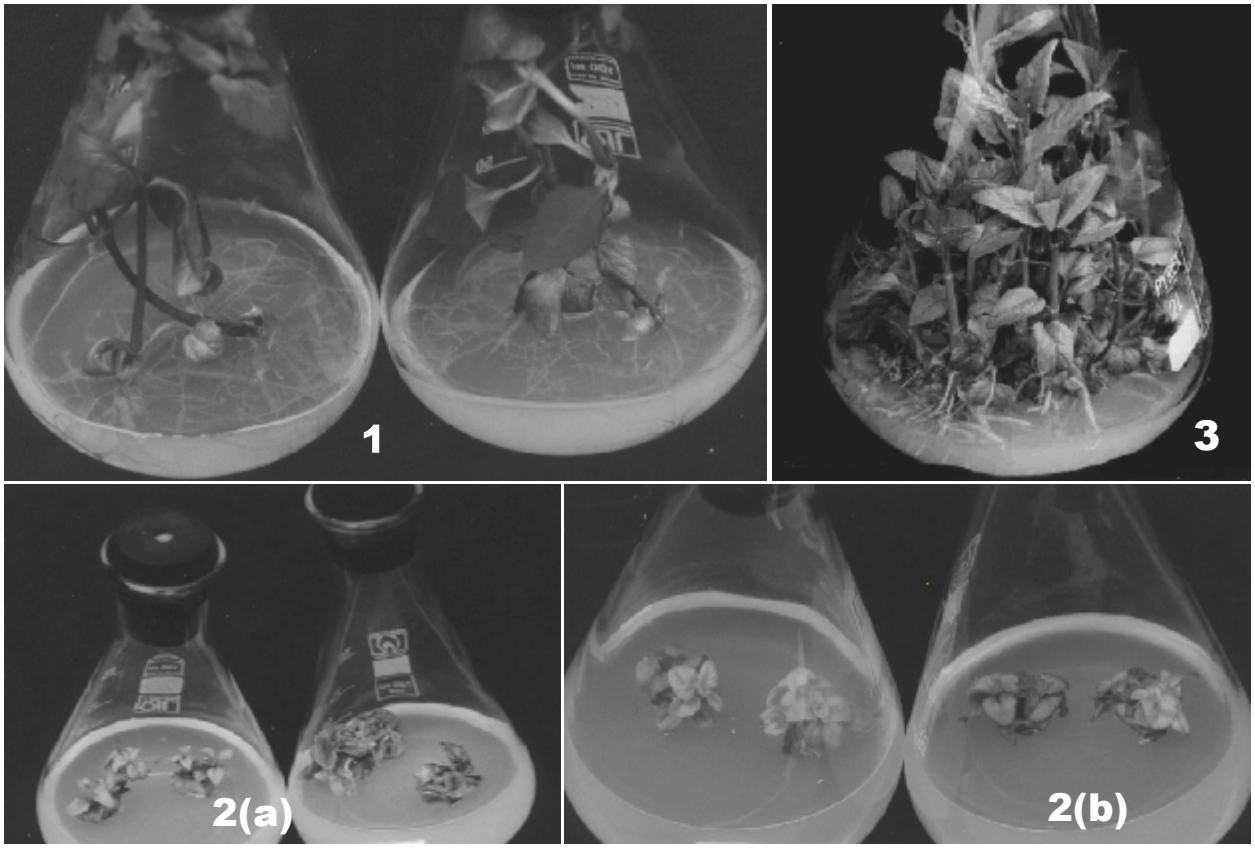

Fig. 1. Axillary bud of *S. acmella* formed single shoot with complete root system in MS basic medium without growth regulators

Fig. 2. Small cluster of Multiple shoots formed from axillary buds of *S. acmella* when cultured in MS medium supplemented with BA after 5 weeks (a) MS + BA 2.0 mg l^{-1}; (b) MS + BA 4.0 mg l^{-1}

Fig 3. Multiple shoots formation of *S. acmella* axillary buds when first subcultured in MS media supplemented with BA 0.5 mg l^{-1} in five weeks

MS medium supplemented with combination of BA higher than 4 mg l^{-1} and IBA higher than 6 mg l^{-1} also did not induce any shoot formation. These explants formed callus or became necrotic. According to Èellárová and Kimáková (1999), higher concentration of BA could induce the formation of callus tissue that also caused the chromosomal instability of the regenerated plants. Culture medium containing higher concentration of BA also reported to increase abnormal shoot production in the axillary buds of papaya explants (Chan and Teo, 1993) and *Tectona grandis* (Goswami *et al.*, 1999).

More multiple shoots were formed when the explant were cultured in MS medium supplemented with low concentration of BA (0.5 – 2.5 mg l^{-1}). However, the number of shoots formed within this range of BA concentration was not significantly different. Small shorter shoots were formed as the concentration of BA increased in the culture medium. Result indicated that culture medium MS + 0.5 mg l^{-1} of BA was sufficient to produce multiple shoots culture of *S. acmella* and an average of 3.4 shoots were formed from each axillary bud in this medium within five weeks (Table 2). Èellárová and Kimáková (1999) reported that BA was found to be most effective for induction of multiple shoot formation when the concentration was not more than 1.0 mg l^{-1}. Su *et al.* (2000) reported that MS medium added with low concentrations of BA produced normal shoots and roots for *Typhonium flagelliforme*. The same phenomena was observed for *S. acmella* and all its *in vitro* multiple shoots were normal with complete root system when low concentration of BA was added into the culture medium.

Table 2. Number of shoots formed and shoot length of *S. acmella* when cultured in MS medium supplemented with low concentration of BA (0.5 – 2.5 mg) within five weeks

MS + BA (mg l^{-1})	Number of shoots / bud	Shoot length / shoot (cm ± SE)
0.5	3.4 ± 0.1 a	3.6 ± 0.1 ab
1.0	2.8 ± 0.1 a	4.0 ± 0.2 a
1.5	2.8 ± 0.1 a	4.1 ± 0.2 a
2.0	2.7 ± 0.1 a	2.5 ± 0.1 b
2.5	2.8 ± 0.1 a	2.8 ± 0.1 ab

Means within the same column followed by the same alphabet are not significantly different using DMRT, $p = 0.05$.

Table 3. Number of shoots formed and shoot length of *S. acmella* when cultured in MS medium supplemented with low concentration of BA (0.5 – 2.5 mg l^{-1}) after first sub-culture

MS + BA (mg l^{-1})	Number of shoots / bud	Shoot length / shoot (cm ± SE)
0.5	6.5 ± 1.0 a	5.3 ± 1.0 a
1.0	5.5 ± 1.0 a	4.4 ± 1.1 b
1.5	6.5 ± 2.1 a	4.2 ± 1.0 b
2.0	6.6 ± 1.7 a	3.0 ± 0.6 c
2.5	6.6 ± 1.2 a	3.3 ± 1.0 c

Means within the same column followed by the same alphabet are not significantly different using DMRT, $p = 0.05$.

Number of shoots formed increased about two fold after the first sub-culturing of the separated individual shoots. The shoot height was still affected by the concentration of BA present in the culture medium. The shoot height was found to be reduced as the concentration of BA increased in the culture medium (Table 3). Healthy, normal multiple shoots with complete root system were produced in MS supplemented with 0.5 mg l^{-1} BA after the first subcuturing (Fig. 3). This indicated that repeated sub-culturing could produce a large amount of *in vitro* plantlets of *S. acmella* for future use.

An *in vitro* propagation of *S. acmella* could be achieved by culturing the aseptic axillary bud explants on MS medium supplemented with 0.5 mg l^{-1} BA. Subculture the separated individual shoots on the same medium could induce twice the number of multiple shoots with complete root system.

Acknowledgement

We thank Universiti Sains Malaysia, Penang, Malaysia for the research facilities and The Penang State Government for permission to use *S. acmella* for research.

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