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BRIEF COMMUNICATION

# Direct shoot regeneration from leaf explants of *Spilanthes acmella*

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## Abstract

Multiple shoots of *Spilanthes acmella* Murr. were induced from nodal buds of *in vivo* and *in vitro* seedlings on Murashige and Skoog (MS) medium containing 1.0 mg $dm^{-3}$ 6-benzyladenine (BA) and 0.1 mg $dm^{-3}$ α-naphthalene-acetic acid (NAA). Adventitious shoots were successfully regenerated from the leaf explants derived from the above mentioned multiple shoots. The efficiency of shoot regeneration was tested in the MS medium containing BA, kinetin, or 2-isopentenyl adenine in combination with NAA, indole-3-acetic acid (IAA), or indole-3-butyric acid (IBA) and gibberellic acid. Maximum number of shoots per explant (20 ± 0.47) was recorded with 3.0 mg $dm^{-3}$ BA and 1.0 mg $dm^{-3}$ IAA. An anatomical study confirmed shoot regeneration *via* direct organogenesis. About 95 % of the *in vitro* shoots developed roots after transfer to half strength MS medium containing 1.0 mg $dm^{-3}$ IBA. 95 % of the plantlets were successfully acclimatized and established in soil. The transplanted plantlets showed normal flowering without any morphological variation.

*Additional key words*: growth regulators, medicinal plant, tissue culture.

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*Spilanthes acmella* is an acutely threatened plant species (Narayana Rao and Raja Reddy 1983). *S. acmella* (marati mogga) is one of the important medicinal plants belonging to the family *Asteraceae*. The active compound was found to be spilanthol (Ramsewak *et al.* 1999). It is a perennial herb grown in the tropics and subtropics. Direct regeneration from leaf, as another alternative step for clonal propagation and germplasm conservation, is a well established factor. Successful plant regeneration has been reported from leaf explants, *e.g.*, in Indian spinach (Mitra and Mukherjee 2001), *Plumbago* species (Das and Rout 2002), safflower (Radhika *et al.* 2006) and *Carthamus* species (Sujatha and Dinesh Kumar 2007). In view of the medicinal properties of *S. acmella*, its threatened nature and the increased demand for it in the pharmaceutical industry, there is a need for a large scale multiplication. In the present study, the direct shoot regeneration and histological studies from leaf explants of *S. acmella* have been studied. Direct regeneration of this plant species has not been reported so far.

*Spilanthes acmella* Murr. shoots 22 cm long with 5 nodes were collected from 15-week-old field grown plants raised from seeds. They were divided into shoot tips and nodes, each measuring about 1 - 4 cm in length. These served as explants for multiple shoot production. Explants were initially washed in running tap water for 15 min, and then rinsed in 10 % *Teepol* for 5 min followed by 0.1 % *Bavistin* for 5 min. The explants were thoroughly washed with sterile distilled water four times. Surface sterilization was carried out in 70 % ethanol for 45 s. Then the explants were treated with 0.05 % $HgCl_2$ for 4 min and 5 - 6 times rinses in sterile distilled water. The nodal and shoot tip explants were inoculated in the Murashige and Skoog (1962; MS) medium fortified with 1.0 mg $dm^{-3}$ 6-benzyladenine (BA) and 0.1 mg $dm^{-3}$ α-naphthalene acetic acid (NAA). The shoots initiated were used for further experiment.

Seeds were surface sterilized by 1 % *Teepol* for 15 min followed by immersion in 70 % ethanol for 1 min and in 0.1 % mercuric chloride for 10 min, and then rinsed thoroughly with sterile distilled water. Disinfected seeds were inoculated in the MS medium without growth hormones to raise aseptic seedlings. To test the efficacy of the leaf explant of *S. acmella* for direct shoot regeneration, leaf explants were used from aseptic seedling (aseptic leaf explant) and leaf explant from *in vitro* shoots developed from nodal bud of *in vivo* plants (mature leaf explant).

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*Abbreviations*: BA - 6-benzyladenine; $GA_3$ - gibberelic acid; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; 2-iP - 2-isopentenyl adenine (2-γ,γ-dimethylallyl aminopurine); Kn - kinetin; NAA - α-naphthalene acetic acid.

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The leaves were inoculated in the MS medium fortified with BA, 2-isopentenyl adenine (2-iP) or kinetin (Kn) single or BA + Kn or BA + Kn + gibberelic acid-($GA_3$ along with auxins indole-3-acetic acid (IAA) or NAA. The pH of the media was adjusted to 5.8 before autoclaving. All media were autoclaved at 1.06 kg $cm^{-2}$ and 121 ºC for 15 min. The cultures were incubated in growth room at temperature of 24 ± 2 °C, relative humidity 55 ± 5 %, and 16-h photoperiod at a photon flux density of 15 - 20 μmol $m^{-2}$ $s^{-1}$ (cool, fluorescent tubes). 20 replicate cultures were established and each experiment was repeated twice and the cultures were observed at regular intervals.

The number of shoots formed was recorded after two months of inoculation. The basal leaves of one to two months old cultures produced shoots directly without the callus phase. Such leaves were identified and isolated from the plantlets and transferred on to the same multiplication medium in which the plantlets were grown. The number of shoots formed was recorded two months after inoculation. Their histology was studied to scrutinize the development of shoot from leaf explant. Samples were fixed in a FAA solution (formaldehyde + glacial acetic acid + ethyl alcohol; 5:5:90; v/v). Following the fixation, tissues were dehydrated in an ethanolic graded series, and then embedded in paraffin as recommended by Johansen (1940). Serial sections of 5 - 10 μm in thickness were prepared. The sections were stained with safranin for 1 min and mounted on glass slides and then covered with cover slip with a drop of DPX mountout. The tissues were observed using a light microscope (*Nikon*, Tokyo, Japan).

The medium used for multiple shoot induction also induced roots but with interference of callus at the base. To reduce the callus formation at base, shoots of more than 3 - 4 cm length were separated from the clumps of multiple shoots and transferred to half strength MS medium supplemented with different concentrations of indole-3-butyric acid (IBA; 0.1 - 2.0 mg $dm^{-3}$) for rooting. Mean root length and root number were recorded on the $30^{th}$ day of the culture. After the development of roots the plantlets were removed carefully from the medium. They were washed thoroughly in gently running tap water to remove any traces of agar adhering to the plantlet. The cleaned plantlets were planted in plastic pots containing autoclaved *Vermiculite* and soil. The pots, covered with polythene bags to maintain high humidity, were kept in the greenhouse. The plantlets were acclimatized gradually by removing the polythene bags once in three days to expose them outside (for about 20 min). After acclimatization for one month, the plants were transferred to the field.

After 15 d of incubation the enlargement of most of the leaf explants was observed. Direct formation of shoots was observed after 45 d either in the midrib region or basal petiolar region or apical region of the leaf. Little cuts made with the scalpel on the explants resulted in the formation of callus at the cut regions. To avoid callus

Table 1. Effect of different plant growth regulators [mg $dm^{-3}$] added to MS medium on direct regeneration from mature leaves or leaves of *in vitro* grown plantlets of *Spilanthes acmella.* Observations after 4 weeks. Means ± SE of 20 independent determinations.

| | BA | Kn | 2-iP | $GA_3$ | NAA | IAA | IBA | Shoot number | Shoot length [cm] |
|---|---|---|---|---|---|---|---|---|---|
| Mature | 0.5 | - | - | - | 0.1 | - | - | 3 ± 0.30 | 0.25 ± 0.04 |
| leaves | 1.5 | - | - | - | 0.1 | - | - | 4 ± 0.70 | 0.92 ± 0.30 |
| | 2.0 | - | - | - | 0.1 | - | - | 6 ± 0.80 | 1.74 ± 0.50 |
| | 3.0 | - | - | - | 0.1 | - | - | 5 ± 0.05 | 1.25 ± 0.60 |
| | 3.0 | - | - | - | - | 1.0 | - | 5 ± 0.80 | 0.20 ± 0.07 |
| | 4.0 | - | - | - | - | 1.0 | - | 12 ± 0.60 | 0.50 ± 0.08 |
| | 0.2 | 0.2 | - | - | 0.2 | - | - | 2 ± 0.30 | 7.00 ± 0.50 |
| | 1.0 | 0.2 | - | - | 0.2 | - | - | 8± 0.20 | 0.47 ± 0.10 |
| | 2.0 | 0.2 | - | - | 0.2 | - | - | 10 ± 0.67 | 0.81 ± 0.20 |
| | 3.0 | 0.2 | - | - | 0.2 | - | - | 8 ± 0.42 | 0.68 ± 0.25 |
| | 4.0 | 0.2 | - | - | 0.2 | - | - | 6 ± 0.23 | 0.64 ± 0.20 |
| | 0.5 | 0.5 | - | 2.0 | - | - | - | 9 ± 0.07 | 5.23 ± 0.38 |
| | 0.5 | 0.5 | - | 3.0 | - | - | - | 5 ± 0.71 | 8.36 ± 0.80 |
| | - | - | 0.5 | - | - | 1.0 | - | 2 ±0.20 | 5.54 ± 0.40 |
| | - | - | 1.0 | - | - | 1.0 | - | 4 ± 0.21 | 3.12 ± 0.80 |
| | - | - | 2.0 | - | - | 1.0 | - | 3 ± 0.80 | 1.60 ± 0.80 |
| | - | - | 3.0 | - | - | 1.0 | - | 5 ± 0.60 | 1.92 ± 0.80 |
| | - | - | 4.0 | - | - | 1.0 | - | 2 ± 0.40 | 3.67 ± 0.40 |
| Plantlet | 0.5 | - | - | - | 0.5 | - | - | 12 ± 0.34 | 1.79 ± 0.09 |
| leaves | 1.0 | - | - | - | - | - | 0.1 | 17 ± 0.56 | 1.55 ± 0.10 |
| | 3.0 | - | - | - | - | 1.0 | - | 20 ± 0.47 | 1.42 ± 0.07 |
| | - | - | - | 2.0 | - | - | - | 10 ± 0.70 | 3.43 ± 0.20 |

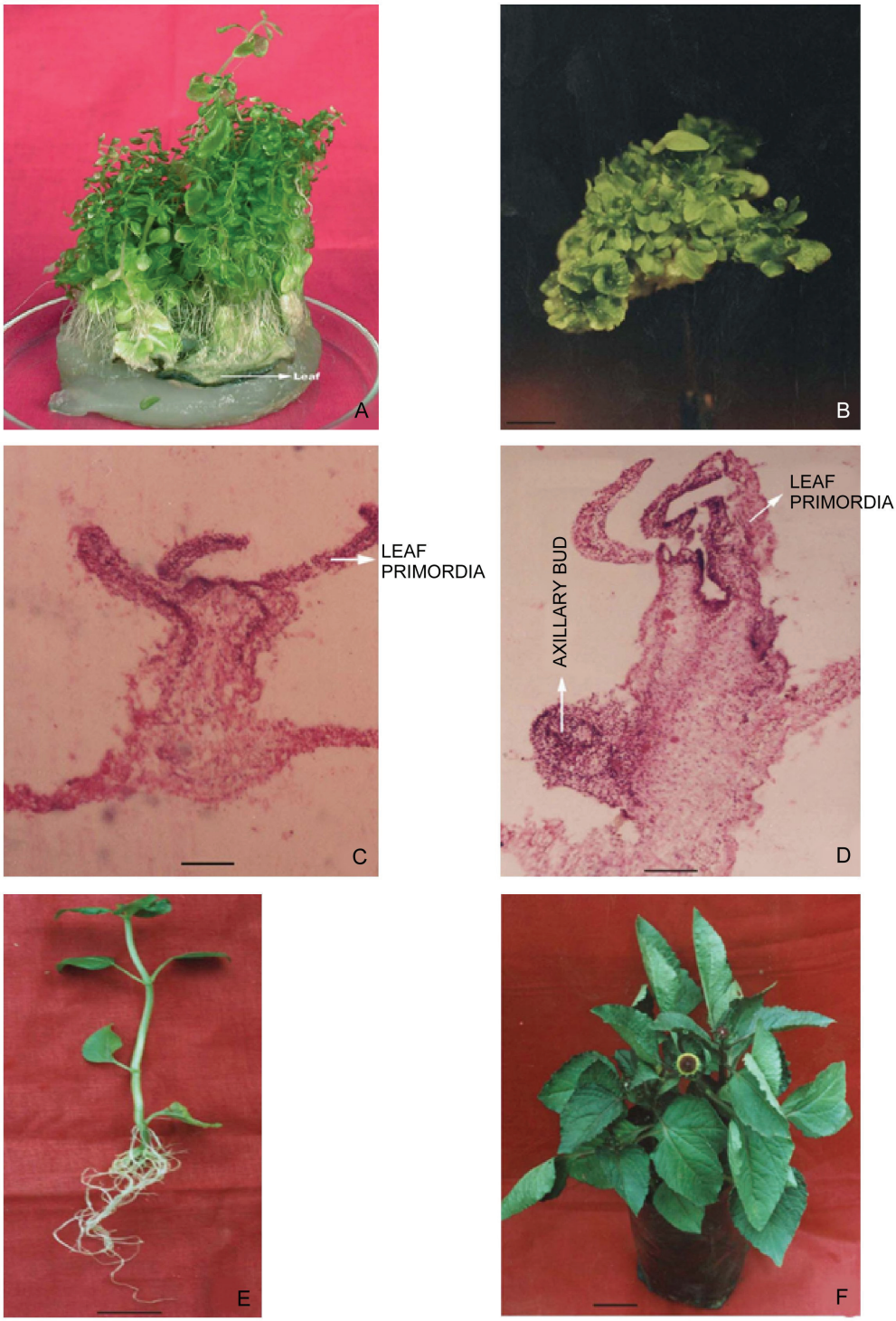


Fig. 1. *A* - Direct shoot formation from leaf explant of *Spilanthes acmella* on MS medium supplemented with 4.0 mg $dm^{-3}$ BA + 0.2 mg $dm^{-3}$ Kn + 0.2 mg $dm^{-3}$ NAA (*bar* = 0. 93 cm). *B* - Direct regeneration of shoots from leaf on MS basal medium supplemented with 3.0 mg $dm^{-3}$ BA + 1.0 mg $dm^{-3}$ IAA (telescopic shoots were observed) (*bar* = 0.7 cm). *C* - Longitudinal sections of leaf explant emerged shoot bud with leaf primordia (40×). *D* - longitudinal sections of the elongated shoots with axillary buds (40×), shoots consisting of dome and number of leaf primordia. *E* - Formation of roots from regenerated shoots derived from mature explants on MS medium supplemented with 1.0 mg $dm^{-3}$ IBA (*bar* = 2.0 cm). *F* - Transplanted plant showing flowering in polythene bag (*bar* = 0.5 cm)

formation whole leaf explants without petiole were inoculated. All the media induced shoot regeneration *via* direct organogenesis. The primordia always regenerated directly from the leaf without forming callus (Table 1).

The shoots were both microshoots (0.25 - 1.5 mm) and shoots (≥1.5 mm). The combination of BA (0.5 - 3.0 mg $dm^{-3}$) and NAA (0.1 mg $dm^{-3}$) induced shoot proliferation on the basal leaf of multiple shoot culture. The combination of BA (4.0 mg $dm^{-3}$) and IAA (1.0 mg $dm^{-3}$) induced the maximum number of shoots (12 ± 0.6) and the mean shoot length (0.5 ± 0.08 cm). Similar observations were also reported in *Plumbago* species (Das and Rout 2002). The combined effect of BA, Kn along with NAA was also studied. This combination also induced *de novo* shoot formation from leaf explants, but the hyperhydricity of shoots was observed in this combination (Fig. 1*A*). $GA_3$ promoted shoot elongation and suppressed vitrification as observed also Pereira *et al*. (2000). In the present study, foliar explants derived from aseptic seedlings showed high regeneration potential. Similar results were reported in *Acampe praemorosa* by Nayak *et al.* (1997).

The combination of 2-iP and IAA induced the minimum number of shoots. As the concentration of 2-iP increased the shoot length decreased. The shoots formed on 2-iP and IAA medium were stout, and anthocyanin pigmentation on their stems was observed. The directly regenerated shoots was subcultured on multiplication medium, and the number of shoots increased after subculturing. The combination of 0.5 mg $dm^{-3}$ BA, 0.5 mg $dm^{-3}$ Kn and 2.0 mg $dm^{-3}$ $GA_3$ was suitable for the organogenesis as it reduced vitrification. The combination 4.0 mg $dm^{-3}$ BA and 1.0 mg $dm^{-3}$ IAA was also considered for the organogenesis as this combination induced highest number of shoots from the mature leaf explant. When shoot cultures were grown in culture bottles with polypropylene caps, extensive axillary branching was noticed. The basal leaves of the axillary branches of *in vitro* grown shoots when they touched the medium shoot buds were induced from the dorsal side (abaxial). At this stage these leaves were isolated from the plantlet and transferred singly on the same multiplication medium in which the plantlet was developed. Besides BA, NAA, other growth regulators such as IBA, IAA, $GA_3$ were supplemented to the MS medium in different combinations and concentrations for direct regeneration from leaf in further experiments. The maximum number of shoots (20 ± 0.47) was observed in 3.0 mg $dm^{-3}$ BA + 1.0 mg $dm^{-3}$ IAA (Fig. 1*B*). The shoots raised on the medium supplemented with 2.0 mg $dm^{-3}$ $GA_3$ were very thick and their internodes were longer than in other shoots. From these experiments it was evident that the MS medium with 3.0 mg $dm^{-3}$ BA + 1.0 mg $dm^{-3}$ IAA could be the best for the production of shoots directly from aseptic seedling leaf. Histological studies indicated direct shoot regeneration from the leaf explant. The abaxial epidermal cells of the leaf increased in size and simultaneously hypodermal and epidermal cells were divided in several planes. The periphery of the explant showed meristemoids on the surface of the midrib, indicating the early stages of differentiation of the shoot. These meristemoids, by further cell division gave rise to small protrusions of tissue, which gradually became green and organized into a growing point similarly as described in *Pothomorphe umbellata* (Pereira *et al*. 2000). After 45 d of culture, the shoot bud development and its vascular connection with the explant tissue was observed (Fig. 1*C*). The axillary bud proliferation was also observed (Fig. 1*D*). The histological analysis suggested that there was a high frequency of direct morphogenesis from leaf explants. A number of leaf primordia were also noted. Similar observations were reported in Indian spinach (Mitra and Mukherjee 2001).

Table 2. Effect of different concentrations of IBA added to half strength MS medium on rooting of shoots raised from axillary bud and shoot tip in *S. acmella*. Observations after 4 weeks. Means ± SE of 20 independent determinations.

| IBA [mg $dm^{-3}$] | Root number [$shoot^{-1}$] | Root length [cm] | Callus |
|---|---|---|---|
| 0.1 | 3.5 ± 0.2 | 1.0 ± 0.2 | - |
| 0.5 | 4.0 ± 0.3 | 1.4 ± 0.4 | - |
| 1.0 | 10.0 ± 0.6 | 3.0 ± 0.2 | - |
| 1.5 | 8.0 ± 0.5 | 2.7 ± 0.1 | + |
| 2.0 | 3.0 ± 0.3 | 2.0 ± 0.3 | ++ |

In *S. acmela,* for root initiation, mineral nutrients and half-strength MS medium was used, as supported by the report of Monier and Ochatt (1995) and Saritha *et al.* (2002). Root induction was best in half strength MS basal medium with 1.0 mg $dm^{-3}$ of IBA. Occasionally rooting also occurred along with shoot multiplication in the presence of different auxin and cytokinin concentrations. The roots formed during the multiplication process were very thin and long. Leaving the culture bottles untouched for more than 8 weeks resulted in coiled and network of roots. Hence the formation of roots during the multiplication process was avoided by reducing the auxin concentration and a separate rooting was adopted. Roots were readily initiated from shoots within 2 weeks after their implantation in half strength MS medium with 1.0 mg $dm^{-3}$ IBA. This induced great frequency (95.5 %) of root induction, the maximum number (10.0 ± 0.6) of roots per shoot and the root length (3.0 ± 0.2 cm) (Fig. 1*E*, Table 2). Lateral root formation was observed 4 weeks after the inoculation. Basal callus interference was observed as the concentration of IBA increased. In *Rauvolfia tetraphylla* and *R. micranta* half strength MS medium supplemented with IBA 2.0 mg $dm^{-3}$ improved rooting efficiency (Patil and Jayanthi 1997). Pattanaik and Chand (1996) reported that the shoots of *Ocimum americanum* were rooted in half strength MS supplemented with 1.0 mg $dm^{-3}$ IBA.

Rooted shoots showed the maximum percentage of survival. After acclimatization for one month, the plantlets derived from mature explants were transferred to pots. About 95 % of survival rate was observed in *in vitro*

produced plants. The plants showed normal flowering within eight weeks without any morphological variations (Fig. 1*F*). Only approximately 60 % of the rooted plants of *Centella asiatica* survived in pots containing a 1:1:1 mixture of soil, sand and well rotted cow dung (Patra *et al*. 1998). Saxena *et al*. (1997) reported that rooted plantlets of *Psoralea corylifolia* were successfully transferred to a 1:1 mixture of soil and sand and 95 % of the regenerated plants survived in the greenhouse. In *Phyllanthus caroliniensis* 86 % of survival was observed by Captan *et al*. (2000).

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