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Establishment of Direct Shoot Regeneration in *Spilanthes acmella* Murr. Using Leaf Explants - An *In vitro* Approach for Mass Propagation

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Key words: Direct regeneration, Leaf explants, *Spilanthes acmella*, *In vitro* propagation, Multiple shoots

Abstract

Direct shoot regeneration was obtained from leaf explants of *Spilanthes acmella* Murr. on MS medium supplemented with different combinations of plant growth regulators like BAP and in combination with NAA and IAA. Among all, 2.0 mg/l BAP produced 70% of shoot regeneration from leaf explants. The BAP at 2 mg/l BAP and 1 mg/l NAA gave the highest percentage of response (90%). The best treatment was observed at 2.0 mg/l BAP + 1.0 mg/l IAA with 80% of regeneration. The multiple shoots were sub cultured on the same media for further multiplication of shoots. Among the different combinations, 2 mg/l BAP + 1 mg/l NAA induced the highest number of multiple shoots i.e. 17 shoots in first subculture and 35 shoots in second subculture respectively. The BAP at 2 mg/l + 1 mg/l IAA induced the highest number of shoots i.e. 18 shoots in first subculture and 40 shoots in second subculture. Regenerated shoots developed efficent root system in half strength of MS medium with different concentrations of IBA and NAA. Among all, 1.0 mg/l IBA induced highest rooting percentage (90%) and 1.0 mg/l NAA induced 85% rooting. *In vitro* regenerated plantlets were established well in the field with 95% survival rate. This study appeared to be very useful for direct regeneration and mass propagation through *in vitro* culture of leaf explants.

Introduction

Plant tissue culture has emerged as a powerful tool for improvement of important and threatened medicinal plants. It involves the growth and propagation of plant cells, tissues or organs *in vitro*. Through tissue culture technology, uniform planting material can be supplied throughout the year without depending on seasons or environmental

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conditions. Through direct regeneration or micropropagation, many identical clones of plants can be attained in a short time and space. Thus, tissue culture is a proven technology for supply identical plants in large numbers for commercial propagation of this plant.

Spilanthes acmella Murr., commonly known as toothache plant is an important medicinal plant belonging to family Asteraceae. It has multiple pharmacological actions, which include antimicrobial, antipyretic, local anaesthetic, anticonvulsant, antioxidant, aphrodisiac, analgesic, antimicrobial, diuretic, anti-inflammatory effects etc. It has been reported to possess various biological activities like a antidiuretic, anti-inflammatory, antioxidant, immunomodulatory, hepatoprotective and anticancer. The plant has been found to produce important secondary metabolites like spilanthol, scopoletin, myrecene, α amyrin, β amyrin etc. The active chemical component is spilanthol, an alkamide which is present in roots and all aerial parts of the plant. Spilanthol has high industrial demand for its use in pharmaceutical, cosmetic and toothpaste industry (Rani et al. 2019).

S. acmella is quickly getting depleted from its natural habitat, because of its wider applications for commercial use. The plant is not meeting the industrial demand due to less commercial cultivation. The other major limiting factor in large scale propagation of *S. acmella* is low germination and viability of the seed (Pati et al. 2006, Dobranszki and Silva 2010). In this context, micropropagation can be effectively used for mass propagation of genetically uniform plants to meet the industrial demands as well as for conservation of *S.acmella.*

Materials and Methods

S. acmella leaves were collected from the field grown plants and surface sterilized by washing with running tap water, treating with 1% bavistin for 20 min, followed by three rinses with distilled water. Then explants were rinsed with 70% alcohol for 1 min followed by distilled water washing twice. The explants were then treated with 0.1% (w/v) mercuric chloride for 3-5 min followed by 4-5 washes with sterilized distilled water.

The leaf explants were cut into 1 cm size bits and cultured on MS medium supplemented with different combinations and concentrations of plant growth regulators (BAP alone, BAP and NAA and BAP and IAA in combination). The leaf explants were cultured such that their abaxial side stays in contact with the media. Multiple shoots were regenerated from the leaf explants within two weeks of culturing on all the hormonal concentrations tested. Regenerated shoots were sub-cultured at regular intervals and individual shoots were transferred to rooting media.

In vitro regenerated shoots were excised and transferred to rooting medium consisting of MS medium supplemented with different concentrations (0.5-3.0 mg/l) of IBA and NAA. The *in vitro* developed plantlets were removed from the rooting medium and washed thoroughly with sterile water to remove agar.

The plantlets with well-developed roots were transferred into small cups containing sterilized soil and sand mixture (3 : 1). They were kept in moistened heat chamber. After 15 days, the plants were transferred to bigger polythene bags in greenhouse and maintain under natural conditions of day length, temperature and humidity.

Results and Discussion

Multiple shoots were regenerated from the leaf explants within two weeks of culturing with varying frequencies on different hormonal concentrations tested (Table 1). Among the different concentrations of BAP alone tested, BAP at 2.0 mg/l concentration produced highest percentage of shoot regeneration (70%) with 10 shoots per explant (Fig. 1A). BAP at 0.5 mg/l gave 50% of regeneration with 6 shoots per explant. At 1.0 mg/l BAP, 60% regeneration was observed with 8 shoots per explants. The frequency of regeneration decreased to 60% on increasing the concentration of BAP to 3.0 mg/l and only 4 shoots were produced for each explant. The regeneration frequency further decreased to 50% on increasing the concentration of BAP to 4.0 mg/l and only 3 shoots were produced for each explant.

The days taken for bud break were also noted which were observed to be 15 days for 0.5 and 1.0 mg/l BAP. The BAP at 2.0 mg/l and 3.0 mg/l showed bud break after 12 and 16 days respectively. BAP at 4.0 mg/l showed bud break after 16 days of culture. Among all the hormonal concentrations tested, 2 mg/l BAP produced high frequency of plant regeneration (70%) from leaf explants.

Table 1. Direct shoot regeneration from leaf explants of *S. acmella* on different concentrations of BAP and various hormonal combinations.

Sl. No.	Concentration of plant growth regulators (mg/l)	No. of explants inoculated	No. of explants responded	Frequency of regeneration (%)	No. of shoots per explants	Shoot length (cm) Mean ± SE
1	0.5 BAP	10	6	50	6	3.5 ± 0.33
2	1.0 BAP	10	6	60	8	3.7 ± 0.32
3	2.0 BAP	10	7	70	10	4.0 ± 0.36
4	3.0 BAP	10	6	60	4	3.2 ± 0.34
5	4.0 BAP	10	5	50	3	3.0 ± 0.23
6	2.0 BAP + 0.5 NAA	10	8	80	2	4.2 ± 0.23
7	2.0 BAP +1.0 NAA	10	9	90	4	4.4 ± 0.33
8	2.0 BAP + 2.0 NAA	10	7	70	1	4.0 ± 0.34
9	2.0 BAP + 0.5 IAA	10	7	70	4	3.9 ± 0.45
10	2.0 BAP + 1.0 IAA	10	8	80	6	4.0 ± 0.25
11	2.0 BAP + 2.0 IAA	10	7	70	1	4.1 ± 0.28

Since the 2.0 mg/l BAP induced high percentage of shoot induction (70%), the same concentration in combination with other hormones like NAA and IAA was tested to enhance the frequency of regeneration (Table 1). The BAP with 2 mg/l BAP was tested with three different concentrations of NAA (0.5 to 2 mg/l). Among all, the combination of 2 mg/l BAP and 1 NAA gave the highest percentage of response (90%) and produced more number of shoots (4 shoots per explant) (Fig. 1B). The BAP at 2.0 mg/l along with different concentrations of IAA (0.5, 1.0 and 2 mg/l) were also tested for direct regeneration from leaf explants. Among these, the best treatment observed was the combination of 2 mg/l BAP and 1.0 mg/l IAA that gave the highest percentage of response (80%) with 6 shoots per explant (Fig. 1C). Among the different combinations of BAP with NAA and IAA, the regeneration frequency was highest (90%) at 2.0 mg/l BAP + 1.0 mg/l NAA. However, the number of regenerated shoots was less (only 4) and the shoots were observed to be very long and thin. So the combination of 2.0 mg/l BAP + 1.0 mg/l IAA that gave 80% regeneration with 6 shoots per explant was selected as the best combination.

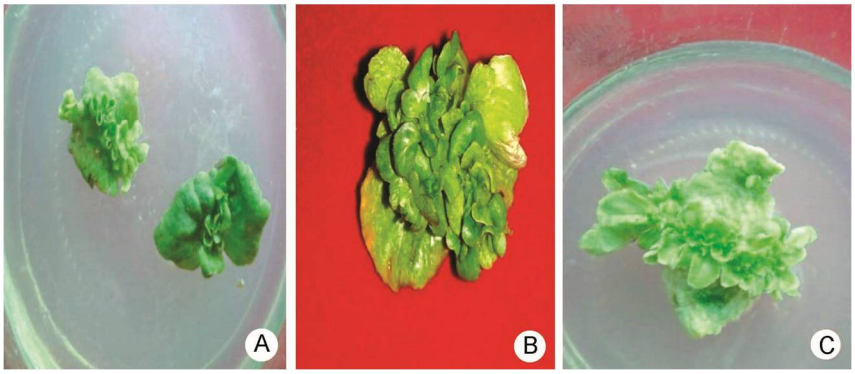

Fig. 1. Shoot induction on MS with: (A) 2 mg/l BAP, (B) 2 mg/l BAP + 1.0 mg/l NAA and (C) 2 mg/l BAP + 1.0 mg/l IAA

The multiple shoots were sub-cultured on the same media for further multiplication of shoots. Among the different combinations of BAP with NAA tested, 2 mg/l BAP + 1 mg/l NAA induced the highest number of shoots i.e. 17 shoots in first subculture (Fig. 2A) and 35 shoots in second subculture (Fig. 2B). In different combinations of BAP with IAA, BAP at 2 mg/l BAP + 1 mg/l IAA induced the highest number of shoots i.e. 18 shoots in first subculture and 40 shoots in second subculture.

The root induction plays a crucial role in tissue culture studies for successful transfer and establishment of regenerates. To inducing rooting, *in vitro* regenerated shoots from leaf explants with 2-3 cm height were excised and transferred to different rooting medium. Two media i.e MS and ½MS media without any hormonal supplementation were tested for root initiation. However, the rooting percentage was very low and low number of roots.

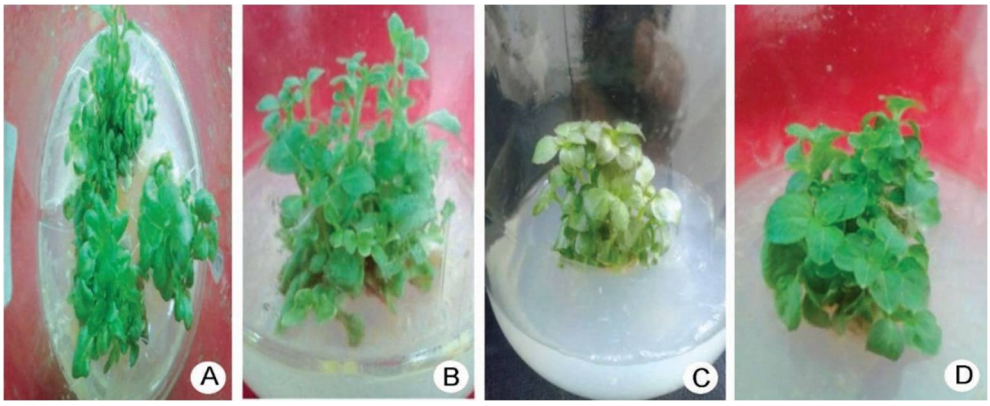

Fig. 2. Multiplication of shoots on MS with BAP and NAA: (A & B) First subculture (C &D) Second subculture.

To increase the percentage of rooting, half strength of MS medium supplemented with different concentrations of IBA and NAA (0.5-3.0 mg/l) were tested (Table 2). Among all, high percentage of rooting (90%) was observed in 1.0 mg/l IBA with long and thin roots of 2.8 cm length. Among the different concentrations of NAA, high percentage of rooting (85%) was observed at 1.0 mg/l with long and thin roots of 4.5 cm length (Fig. 3A). Among all media and concentration of hormones tested for rooting, ½MS strength media with 1.0 mg/l IBA gave 90% rooting and the same has been employed in all tissue culture studies. *In vitro* regenerated plantlets were transferred to polythene bags for primary hardening and latter established well in the field with 95% survival rate (Fig. 3B & C).

Table 2. Rooting from *in vitro* regenerated shoots of *S. acmella* on different concentrations of IBA and NAA.

Sl. No.	Media composition (mg/l)	No. of shoots inoculated	No. of shoots with rooting responded	Rooting (%)	Days required for root induction	Length of roots (cm) Mean ± SE	Nature of roots
1	MS Full Strength	20	4	20	25	1.4 ± 0.02	Short, very thin
2	MS half Strength	20	6	30	20	1.2 ± 0.10	Short, very thin
3	½MS + 0.5 IBA	20	14	70	20	2.5 ± 0.07	Long, thin
4	½MS + 1.0 IBA	20	18	90	15	2.8 ± 0.05	Long, thin
5	½MS + 2.0 IBA	20	16	80	18	3.5 ± 0.10	Thick, healthy
6	½MS + 3.0 IBA	20	12	60	16	3.0 ± 0.70	Thick, healthy
7	½MS + 0.5 NAA	10	7	70	23	4.2 ± 0.10	Long,thin
8	½MS + 1.0 NAA	10	8	85	20	4.5 ± 0.07	Long,thin
9	½MS + 2.0 NAA	10	6	60	18	4.0 ± 0.10	Long,thin
10	½MS + 3.0 NAA	10	5	50	20	3.8 ± 0.10	Long,thin

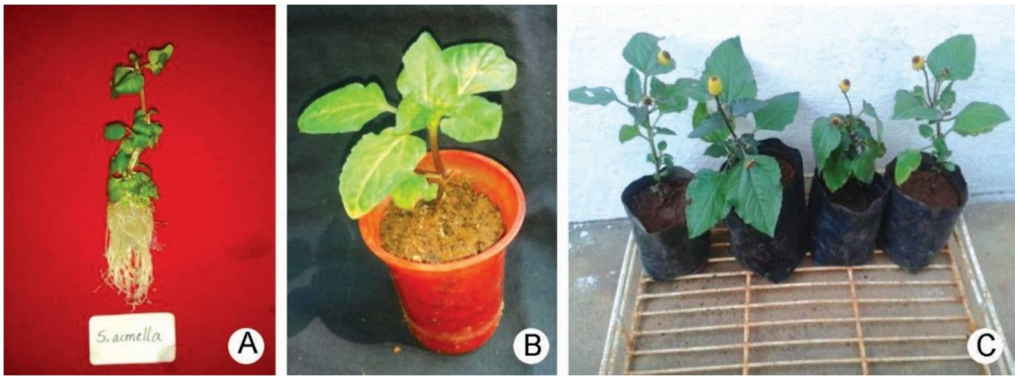

Fig. 3. (A) Rooting from regenerated shoots (B) Primary hardening (C) Secondary hardening.

From young leaves of *S. acmella,* multiple shoots were regenerated on MS supplemented with different concentrations of BAP alone and different combinations with NAA and IAA. Numerous shoot primordia developed within 2-3 weeks on the leaf lamina in the midrib regions without any callus phase. A similar observation with profound growth of shoot initials in the midrib region was observed in *Embelia ribes* (Raghu et al. 2006). The shoot clumps were separated from leaf explants and transferred onto different concentrations of BAP (0.5-4.0 mg/l). Among all, BAP at 2.0 mg/l produced highest percentage of shoot regeneration (70%). At high concentration of BAP (4.0 mg/l), regeneration frequency decreased to 50%. Earlier studies have shown that BAP at low concentration showed highest percentage (100%) of shoot organogenesis (Singh and Chaturvedi 2012).

To enhance the frequency of regeneration from leaf explants, 2.0 mg/l BAP in combination with other hormones like NAA and IAA was tested. Among all, the combination of 2.0 mg/l BAP with 1.0 mg/l NAA gave the highest percentage of response (90%). In earlier study in *S. acmella*, incorporation of NAA in the BAP containing medium showed 100% regeneration from leaf disc explants (Singh and Chaturvedi 2012). The BAP at 2.0 mg/l along with different concentrations of IAA (0.5, 1.0 and 2 mg/l) were tested for direct regeneration from leaf explants. Among these, the best treatment observed was the combination of 2 mg/l BAP and 1.0 mg/l IAA that gave the highest percentage of response (80%). Similarly, in an earlier study in *Withania somnifera*, shoots emerged directly from leaf explants on MS medium supplemented with IAA and BAP in combination (Anjali et al. 1996).

The production of roots was observed in some cases in the BAP multiplication medium itself (0.5-1.0 mg/l). Similarly spontaneous rooting from the shoots in the medium without any growth regulators was observed in other Asteraceae members like *Tagetus erecta* (Misra and Dutta 2001) and *Dendranthema* (Taixeira 2003). This indicates the presence of optimal endogenous levels of plant growth regulators in microshoots required for rooting.

To increase the percentage of rooting, half strength MS medium supplemented with different concentrations of IBA and NAA (0.5-3.0 mg/l) were tested. Among all, high percentage of rooting (90%) was observed in 1.0 mg/l IBA with long and thin root. Further increasing the concentration of IBA to 3.0 mg/l, the percentage of rooting decreased. The previous studies also reported that half strength MS medium containing different concentrations of IBA induced rooting in many plants including *Hemidesmus indicus* (Sreekumar et al. 2000), *Cunila galioides* (Fracaro and Echeverrigaray 2001), *Aloe polyphylla* (Abrie and Van Staden 2001) and *Gymnema elegans* (Komalavalli and Rao 1997).

Among NAA supplemented media, high percentage of rooting (85%) was observed at 1.0 mg/l with long and thin roots. Similarly, earlier studies on *S. acmella* have also shown induction of rooting from regenerated shoots in NAA supplemented media. (Sharma and Shahzad 2013).The *in vitro* regenerated plants well established in the field with 95% survival rate. Similar high survival rate was observed in the earlier studies on *S. acmella* (Joshi et al. 2015).

In this study, an efficient protocol was established for direct plant regeneration of *S. acmella* from the leaf explants. This investigation provides a rapid and reliable *in vitro* propagation regeneration protocol for *S. acmella.* The current research of direct regeneration from leaf explants of *S. acmella* can be utilized for production large number of identical plants. These multiple shoots can be exploited for mass propagation of *S. acmella* to supplement the ever increasing demand of industry. This study is very useful for direct regeneration and mass propagation through leaf explants *in vitro*.

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