

In Vitro Studies on Shoot Regeneration through Various Explants and Alginate-Encapsulated Nodal Segments of *Spilanthes mauritiana* DC., an Endangered Medicinal Herb

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ABSTRACT

This study describes an improved, efficient protocol for adventitious shoot regeneration (through shoot tip and leaf explants) and conservation through synthetic seed (synseed) technology of *Spilanthes mauritiana*, an endangered medicinal herb. MS (Murashige and Skoog 1962) basal medium augmented with 1.0 and 2.5 μM BA was optimum for the induction of multiple shoots formation through shoot tip and leaf explants, respectively. Cytokinin and auxin combinations considerably enhanced the frequency of shoot induction. A maximum of 18.8 shoots/shoot tip were induced on MS basal medium supplemented with 1.0 μM 6-benzyl adenine (BA) and 0.5 μM indole-3-acetic acid (IAA); 15.0 shoots/leaf explants on MS with 2.5 μM BA and 0.5 μM IAA. Microshoots were best rooted on half-strength MS medium supplemented with 2.5 μM NAA. Synseeds, produced by encapsulating axillary buds in calcium alginate gel exhibited a critical response to nutrient concentration for conversion into complete plantlet. On half-strength MS basal medium germination percentage was maximum. A low temperature storage (4°C) experiment was also carried out to understand the explants' ability to revive physiological activity leading to plantlet development. Almost all the synseeds sprouted well and developed into plantlets when cultured on nutrient media after storage, up to three weeks of storage although subsequent storage reduced sprouting capability. Plants retrieved from rooting medium and synseeds were hardened off and successfully established in soil with a 90% survival rate and exhibited normal morphological and growth behavior when compared with *in vivo* grown plants.

Keywords: adventitious shoot regeneration, *in vitro*, microshoot, synseed

INTRODUCTION

Spilanthes mauritiana DC., a monogeneric-endangered herb belonging to the Asteraceae family, is a native of Eastern Africa and is used in the local pharmacopoeia to cure infections of the throat and mouth (Watt and Brayer-Brandwijk 1962) and as a remedy for stomach ache and diarrhea (Kokwaro 1976). Kamba tribes in Kenya chew the flower of *S. mauritiana* for the relief of toothache and treatment of pyorrhea (Watt and Brayer-Brandwijk 1962) and an infusion of the herb is used as febrifuge (Dalziel 1937). In India the plant has been used for kidney stones, and bladder and kidney infections (Dragendroff 1898). So far the only isolated active principle in *S. mauritiana* is an antiseptic alkaloid, spilanthol, present at a concentration of as much as 1.25% in the flower (Watt and Brayer-Brandwijk 1962). Researchers have shown preliminary antimicrobial activity in the crude extract from roots and flower heads of *S. mauritiana* (Fabry *et al.* 1996, 1998).

Conventional vegetative propagation by stem cuttings is arduous and inadequate to meet the need for Ayurvedic drug preparations. Therefore, tissue culture was selected as an alternative for large-scale commercial propagation of this plant. This technology could be a cost effective means of high volume production of the elite planting material throughout the year without any seasonal constraints.

Micropropagation and conservation of medicinal plants gained momentum in recent years to protect rapidly diminishing plant species because of over exploitation without replenishments in their habitat (Anis *et al.* 2009). To date, only brief reports are available on the regeneration of two species of *Spilanthes* such as *S. mauritiana* (Bais *et al.* 2002) through axillary buds and *S. acmella* (Saritha *et al.*

2002; Haw and Keng 2003; Pandey and Agrawal 2009) through hypocotyl, axillary bud and leaf explants, respectively. They reported few shoots/explants, insufficient to adopt the protocol for a large-scale plantation programme. Further, they mentioned that rooting is generally a very slow process in *S. mauritiana*. Thus, an efficient, rapid and practical protocol for mass propagation of this endangered herb is still lacking. As far as the literature is concerned, there is no report on the application of synthetic seed technology for this plant.

Thus, with a view to develop an efficient protocol for large scale propagation and conservation through encapsulation of axillary buds of *S. mauritiana*, the present investigation was carried out.

MATERIALS AND METHODS

Collection of plant material

The flower heads were procured from a 6 month-old plant of *Spilanthes mauritiana* grown in Chhattisgarh state, India. The plant species was identified by our department's taxonomist and the specimen was deposited in the departments' herbarium (No. 31398). The seeds were extracted from the flower heads and washed in running tap water for 30 min to remove any adherent particles. Thoroughly washed seeds were then immersed in 1% (w/v) Bavistin (Carbendazim Powder, BASF India Ltd.), a broad-spectrum fungicide, for 20 min and 5% (v/v) Teepol (Qualigens, India), a liquid detergent. The treated seeds were washed in distilled water to remove the chemical inhibitors to germination. The seeds were surface sterilized with 70% (v/v) ethanol and 2-3 drops of Tween-20 (Qualigens) for 30 s, followed by an aqueous solution of 0.1% (w/v) freshly prepared HgCl_2 under sterile conditions for 3-4 min.

Finally, the seeds were washed 5-6 times with sterile distilled water to remove all traces of sterilant. The surface sterilized seeds were inoculated aseptically in culture tubes (25 × 150 mm, Borosil) containing plant growth-regulator (PGR)-free half-strength MS medium (Murashige and Skoog 1962) with 3% (w/v) sucrose (Qualigens) and 0.8% (w/v) agar (Qualigens). Various explants viz., shoot tips, nodal segments and leaves were used as explants, taken from 8 weeks-old aseptic seedlings.

Establishment of explants and *in vitro* conditions

The explants were cultured on sterilized basal MS medium with or without PGRs (Sigma-Aldrich, Germany). The medium was supplemented with 3% (w/v) sucrose as an energy source and 0.8% (w/v) agar (for shoot induction) or 0.2% (w/v) phytigel (for root induction; Sigma-Aldrich, Germany) as a solidifying agent. The pH of the medium was adjusted to 5.8 with 1 N NaOH and 1 N HCl before adding the agar and phytigel. All media were sterilized at 121°C, 1.06 Kg cm⁻² for 20 min. The cultures were kept in the controlled environment of a culture room at 25 ± 2°C temperature, 16-h photoperiod with 50 μmoles m⁻² s⁻¹ photosynthetic photon flux density provided by cool white light (Philips, India) and 55 ± 5% relative humidity.

Treatments of single cytokinin as well as combinations with auxins for shoot regeneration

Various cytokinins viz., 6-benzyl adenine (BA), kinetin (Kn) and isopentenylaminopurine (2-iP) were tried individually at 0.5, 1.0, 2.5 and 5.0 μM as well as in combination with various auxins viz., α-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) at 0.1, 0.5 and 1.0 μM to obtain the most suitable treatment for the regeneration of shoots through various explants.

In vitro rooting

For *in vitro* root induction, microshoots (3.5-4.0 cm long) with 3-4 fully expanded leaves were transferred to half-strength basal MS medium with or without various auxins viz., NAA, IBA and IAA at 1.0, 2.5 and 5.0 μM.

Synseed production

One month-old *in vitro*-raised plantlets were used as the explant source and 2-3 nodal segments from the terminal bud (3.0-4.0 mm) were taken for synseed production. As the protocol developed by Faisal and Anis (2007) in *Tylophora indica* for production of synseed through nodal segments, sodium alginate (Loba Chemie, India) was added at 3% (w/v) to MS liquid medium containing 3% (w/v) sucrose. For complexation, 100 mM CaCl₂·2H₂O (Qualigens) solution was prepared using double distilled water (DDW). The pH of the gel was adjusted to 5.8 prior to autoclaving at 121°C and 1.06 kg cm⁻² for 20 min. Encapsulation was accomplished by mixing the nodal segments with sodium alginate and dropping these into 100 mM calcium chloride solution with a pipette. Calcium alginate capsules containing the nodal segments were retrieved from the solution after 20 min and rinsed twice with autoclaved DDW to remove traces of calcium chloride.

Low temperature storage

Synseeds were stored in sterilized beakers (moist with DDW) sealed with two layers of Para film at 4°C for up to 4 weeks.

Germination of synseeds

The frequency of synseed germination (shoot and root development) after successive weeks of storage was evaluated on MS medium of different nutrient strengths (full-strength MS, half-strength MS and quarter-strength MS) with 3% (w/v) sucrose and 0.8% (w/v) agar.

Acclimatization and establishment of plantlets

Rooted plantlets were removed from the medium, washed under running tap water to remove solidifying agent, planted in soilrite containing thermocol cups (made up of expanded polystyrene) and covered with glass bottles to retain high humidity (90%). The bottles were gradually removed and exposed to culture conditions for 4 weeks. Thereafter, hardened plants were transferred to pots containing autoclaved garden soil and green manure (2: 1) and maintained in a greenhouse under normal day length conditions.

Data analysis

The data were examined after 8 weeks for shoot induction while for root induction and germination of synseed after 4 weeks. Each treatment consisted of 10 replicates and the experiments were repeated in triplicate. Data were analyzed by one-way ANOVA calculated using SPSS 12.0 (SPSS Inc., Chicago, IL) software. Significant differences between means were assessed by Duncan's test at P < 0.05.

RESULTS AND DISCUSSION

In the present investigation, three explants viz. shoot tip, nodal segments and leaf explants were used for determining their ability to induce multiple shoots. Among the three types of explants, shoot tip and leaf explants were more responsive than nodal segments for multiple shoot induction. Nodal segments induced only 1-2 shoots through axillary bud elongation in all the initial planting media tested (data not shown). Therefore, only shoot tip and leaf explants were selected for further investigation to explore the possibility of inducing high frequency shoot organogenesis. Shoot tip and leaf explants remained green and fresh but failed to develop multiple shoots in PGR-free basal MS medium (control). The addition of PGRs induced multiple shoot formation from both explants depending on the concentration and combination.

Effect of single cytokinin on shoot induction

Among various cytokinins tested, 1.0 μM BA was optimal with an 88% response over Kn and 2-iP for shoot regeneration via shoot tip explants. On this medium the regeneration of shoot buds was noticed with the appearance of a green bulged outgrowth at the compressed nodal region of the explants after 1 week of incubation followed by emergence of leaf primordia. A maximum of 8.6 shoots/explant measuring 1.9 cm in length was recorded after 8 weeks of inoculation (Table 1, Fig. 1A).

The shoots from leaf explants differentiated mainly from the basal petiolar portion while the apex of the leaf did not show any shoot bud differentiation. Greenish-white,

Table 1 Effect of cytokinins on direct shoot organogenesis through shoot tip explants of *S. mauritiana* (8 weeks).

Cytokinins (μM)	% Response (Mean ^a ± SE)	№ of shoots/explant (Mean ^a ± SE)	Shoot length (cm) (Mean ^a ± SE)
MS (control)	0.0 ± 0.0 h	1.0 ± 0.0 g	4.1 ± 0.1 a
BA 0.5	50.0 ± 3.1 cd	4.2 ± 0.3 cde	2.3 ± 0.1 de
1.0	88.0 ± 3.7 a	8.6 ± 0.2 a	1.9 ± 0.0 ef
2.5	82.0 ± 3.7 ab	6.8 ± 0.3 b	1.3 ± 0.0 h
5.0	50.0 ± 3.1 cd	4.6 ± 0.5 cde	1.5 ± 0.1 gh
Kn 0.5	38.0 ± 3.7 e	3.4 ± 0.5 ef	2.4 ± 0.1 d
1.0	82.0 ± 3.7 ab	5.2 ± 0.3 c	2.6 ± 0.1 cd
2.5	72.0 ± 3.7 b	4.8 ± 0.3 cd	1.7 ± 0.0 fg
5.0	58.0 ± 3.7 c	3.6 ± 0.5 def	1.6 ± 0.0 gh
2-iP 0.5	20.0 ± 3.1 f	2.8 ± 0.3 f	2.4 ± 0.1 d
1.0	46.0 ± 4.0 de	3.4 ± 0.5 ef	2.9 ± 0.0 bc
2.5	26.0 ± 2.4 f	4.8 ± 0.3 cd	3.0 ± 0.0 b
5.0	10.0 ± 3.1 g	1.0 ± 0.3 g	2.8 ± 0.0 bc

^a mean ± Standard Error (SE) of 10 replicates, means with different letters within a column are significant at P < 0.05.

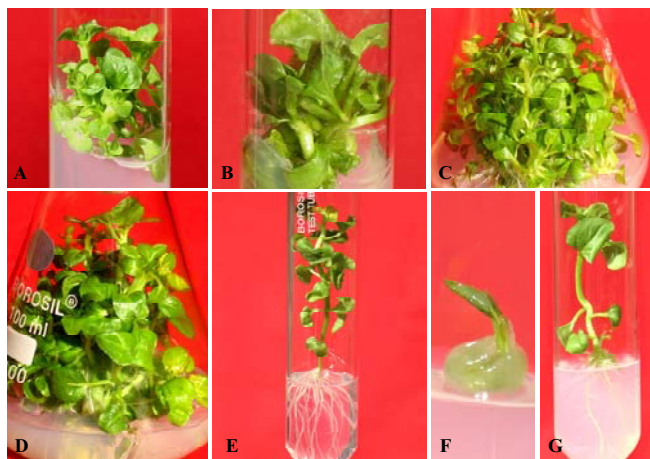


Fig. 1 Plant regeneration in *Spilanthes mauritiana*. (A) Multiple shoot regeneration from shoot tip explant on MS + BA (1.0 µM), after 8 weeks of culture; (B) Direct shoot bud induction from basal petiolar region of leaf on MS + BA (2.5 µM), after 8 weeks of culture; (C) Multiple shoot regeneration from shoot tip explant on MS medium + BA (1.0 µM) + IAA (0.5 µM), after 8 weeks of culture; (D) High frequency of direct shoot regeneration with basal callus from leaf explant on MS + BA (2.5 µM) + IAA (0.5 µM), after 2 weeks of culture; (E) Healthy root initiation and development in microshoot on half-strength MS + NAA (2.5 µM), after 4 weeks of transfer. (F) Emergence of axillary buds on half-strength MS from encapsulated node after 2 weeks of storage at 4°C, 3 days old culture; (G) An encapsulated nodal segment converted into complete plantlet after 2 weeks of culture.

Table 2 Effect of optimal concentration of BA (1.0 µM) in combination with various auxins on direct shoot organogenesis through shoot tip explants of *S. mauritiana* (8 weeks).

Auxins (µM)	% Response (Mean ^a ± SE)	N ^o of shoots/explant (Mean ^a ± SE)	Shoot length (cm) (Mean ^a ± SE)	Frequency ^b of callogenesis
NAA 0.1	70.0 ± 3.1 c	08.4 ± 0.3 de	4.4 ± 0.1 bc	-
0.5	86.0 ± 2.4 b	12.4 ± 0.5 b	4.2 ± 0.2 cd	-
1.0	66.0 ± 4.0 cd	07.6 ± 0.4 ef	5.1 ± 0.1 ab	+
IAA 0.1	84.0 ± 2.4 b	10.2 ± 0.4 c	3.9 ± 0.4 cde	-
0.5	96.0 ± 2.4 a	18.8 ± 0.3 a	2.8 ± 0.2 f	-
1.0	72.0 ± 3.7 c	09.0 ± 0.3 d	3.4 ± 0.1 ef	+
IBA 0.1	58.0 ± 3.7 d	06.6 ± 0.2 f	4.3 ± 0.2 cd	+
0.5	70.0 ± 3.1 c	10.6 ± 0.4 c	3.5 ± 0.1 def	+
1.0	60.0 ± 3.1 d	05.4 ± 0.5 g	5.3 ± 0.4 a	++

^a mean ± Standard Error (SE) of 10 replicates, means with different letters within the column are significant at P < 0.05.

^b -, +, ++, indicate no, slight, moderate callusing, respectively.

dense callus formation was also observed on the cut ends. This morphogenic ability, which increased from the tip towards the base of the leaf, was also observed in apple (Welander 1988) and *Tagetes erecta* (Misra and Datta 2001). Adventitious shoot regeneration through leaf explants was more significant in the presence of a petiole in the present study. However, according to Banerjee *et al.* (1999) leaf segments devoid of a petiole showed maximum sprouting in *Centella asiatica*. The frequency of shoot organogenesis increased linearly with BA up to 2.5 µM. BA at 2.5 µM was the optimum concentration as it induced a maximum of 7.6 shoots/explant 2.4 cm in length in 94% of cultures after 8 weeks of incubation (Table 2, Fig. 1B). The superiority of BA over the other cytokinins with respect to shoot bud production and proliferation of shoots has been reported for several medicinal and aromatic plant species such as *Ocimum basilicum* (Sahoo *et al.* 1997; Begum *et al.* 2002), *Psoralea corylifolia* (Jebakumar *et al.* 2000), *Boerhaavia diffusa* (Baig *et al.* 2004), *Clitoria ternatea* (Rout 2005), and *Mentha viridis* (Raja and Arockiasamy 2008).

By increasing or decreasing the concentration of BA beyond the optimal level, a gradual reduction in the number

of shoots was observed in both explant types. Chen *et al.* (1998, 2006) reported that a higher concentration of BA (0.25-2.0 mg l⁻¹) induced hyperhydric shoots while on Kn-supplemented media (0.1-0.8 mg l⁻¹) and in the control shoots were normal and non-hyperhydric in *Bupleurum kaoi* and *Dianthus caryophyllus*. However, in the present study satisfactory multiple shoot bud induction was also recorded with Kn-supplemented medium at a low concentration (1.0 µM) for shoot tips and a higher concentration (2.5 µM) for leaf explants. 2-iP (0.5-5.0 µM) was the least responsive in terms of percentage response and mean number of shoots/explant produced for both explant types.

Effect of cytokinin and auxin combination on shoot induction

The addition of a low concentration of auxin to the cytokinin (BA) exhibited a positive effect on the induction of multiple shoots from both explant types. Enhancement in the induction of shoots by the synergy of cytokinin and auxin observed in the present study has also been documented in *Mentha arvensis* (Shahzad *et al.* 2002), *Stevia rebaudiana* (Sivaram and Munkundan 2003), *Coleus blumei* (Rani *et al.* 2006) and *Ophiorrhiza prostrata* (Beegum *et al.* 2007).

Among all the cytokinin-auxin combinations tested, a maximum of 18.8 shoots were obtained at 1.0 µM BA with 0.5 µM IAA from shoot tip explants (Table 3, Fig. 1C), while through leaf explants a maximum of 15.0 shoots were induced on a medium comprising 2.5 µM BA with 0.5 µM IAA (Table 4, Fig. 1D). The above combinations were considered as the optimum PGR combinations for further shoot proliferation. The combination of BA and IAA has also proved to be more effective for shoot multiplication through

Table 3 Effect of cytokinins on direct shoot organogenesis through leaf explants of *S. mauritiana* (8 weeks).

Cytokinins (µM)	% Response (Mean ^a ± SE)	N ^o of shoots/explant (Mean ^a ± SE)	Shoot length (cm) (Mean ^a ± SE)
MS (control)	00.0 ± 0.0 g	0.0 ± 0.0 e	0.0 ± 0.0 g
BA 0.5	40.0 ± 3.1 d	2.0 ± 0.3 d	2.0 ± 0.0 bcd
1.0	46.0 ± 2.4 d	2.2 ± 0.3 d	2.1 ± 0.1 abc
2.5	94.0 ± 2.4 a	7.6 ± 0.5 a	2.4 ± 0.1 a
5.0	78.0 ± 4.8 b	4.8 ± 0.3 b	1.4 ± 0.1 e
Kn 0.5	30.0 ± 3.1 e	2.8 ± 0.3 cd	2.3 ± 0.2 ab
1.0	56.0 ± 2.4 c	2.8 ± 0.3 cd	1.7 ± 0.1 cde
2.5	78.0 ± 2.0 b	5.0 ± 0.3 b	2.1 ± 0.1 abc
5.0	40.0 ± 3.1 d	3.8 ± 0.3 c	2.1 ± 0.1 abc
2-iP 0.5	14.0 ± 2.4 f	2.2 ± 0.2 d	1.6 ± 0.1 de
1.0	16.0 ± 2.4 f	2.8 ± 0.3 cd	1.4 ± 0.1 e
2.5	48.0 ± 2.0 d	3.0 ± 0.3 d	1.8 ± 0.1 cde
5.0	26.0 ± 2.4 e	2.0 ± 0.3 d	1.0 ± 0.1 f

^a mean ± Standard Error (SE) of 10 replicates, means with different letters within the column are significant at P < 0.05.

Table 4 Effect of optimal concentration of BA (2.5 µM) in combination with various auxins on direct shoot organogenesis through leaf explants of *S. mauritiana* (8 weeks).

Auxins (µM)	% Response (Mean ^a ± SE)	N ^o of shoots/explant (Mean ^a ± SE)	Shoot length (cm) (Mean ^a ± SE)	Frequency ^b of callogenesis
NAA 0.1	88.0 ± 2.0 b	08.0 ± 0.3 d	3.1 ± 0.1 a	+
0.5	96.0 ± 2.4 a	10.6 ± 0.4 b	2.9 ± 0.0 a	++
1.0	82.0 ± 3.7 bc	05.8 ± 0.3 e	2.5 ± 0.1 b	++
IAA 0.1	96.0 ± 2.4 a	10.8 ± 0.3 b	1.8 ± 0.1 cd	+
0.5	96.0 ± 2.4 a	15.0 ± 0.3 a	1.8 ± 0.1 cd	+
1.0	80.0 ± 3.1 c	09.2 ± 0.3 c	2.1 ± 0.0 c	++
IBA 0.1	16.0 ± 2.4 d	02.6 ± 0.4 f	1.6 ± 0.0 d	++
0.5	0.0 ± 0.0 e	00.0 ± 0.0 g	0.0 ± 0.0 e	+++
1.0	0.0 ± 0.0 e	00.0 ± 0.0 g	0.0 ± 0.0 e	+++

^a mean ± Standard Error (SE) of 10 replicates, means with different letters within the column are significant at P < 0.05.

^b +, ++, +++, indicate slight, moderate, intense callusing, respectively.

Table 5 Effects of various auxins in half-strength MS medium on root induction in microshoots of *S. mauritiana* (8 weeks).

Auxins (μM)	No of roots/explant (Mean ^a \pm SE)	Root length (cm) (Mean ^a \pm SE)	Frequency ^b of callogenesis
½ MS (control)	08.0 \pm 0.3 d	4.2 \pm 0.2 cd	-
NAA 1.0	19.6 \pm 0.5 b	3.3 \pm 0.1 e	-
2.5	27.8 \pm 1.8 a	4.3 \pm 0.1 bc	-
5.0	20.4 \pm 1.2 b	3.8 \pm 0.1 cde	+
IAA 1.0	12.6 \pm 0.8 c	5.4 \pm 0.1 a	-
2.5	14.4 \pm 0.5 c	4.8 \pm 0.2 b	+
5.0	12.6 \pm 0.8 c	3.7 \pm 0.2 de	++
IBA 1.0	19.0 \pm 0.8 b	4.0 \pm 0.1 cd	+
2.5	17.6 \pm 0.9 b	3.9 \pm 0.1 cde	++
5.0	13.2 \pm 0.9 c	2.7 \pm 0.0 f	+++

^a mean \pm Standard Error (SE) of 10 replicates, means with different letters within the column are significant at $P < 0.05$.

^b -, +, ++, +++, indicate no, slight, moderate, intense callusing, respectively.

shoot tips in other plant species viz., *Thymus piperata* (Saez *et al.* 1994), *Adhatoda beddomei* (Sudha and Seeni 1994), *Catharanthus roseus* (Mitra *et al.* 1998) and through leaf explants in *Eryngium foetidum* (Arockiasamy *et al.* 1998), *Spilanthes acmella* (Saritha and Naidu 2008), *Epilobium angustifolium* (Turker *et al.* 2008).

The other BA and auxin (NAA or IBA) combination did not play any advantageous role on shoot induction in any type of explant, thus exhibiting a similar type of hormonal requirements for morphogenesis through both the explants types. However, the majority of reports emphasized on the use of BA with NAA for shoot organogenesis through shoot tip explants of *Trichosanthes dioica* (Debnath *et al.* 2000), *Morus alba* (Anis *et al.* 2003), *Coleus blumei* (Rani *et al.* 2006) and leaf explants of *Brassica* spp. (Dunwell 1981), *Coreopsis lanceolata* (Lee *et al.* 1994), and *Centella asiatica* (Mohapatra *et al.* 2008). Nevertheless, some studies advocated the use of BA and IBA for satisfactory results in *Bougainvillea glabra* (Sharma *et al.* 1981) through shoot tips and through leaf explants in *Hieracium aurantiacum* (Bicknell 1994), and *Ophiorrhiza prostrata* (Beegum *et al.* 2007).

In this study, less exogenous PGR supplementation was required for shoot tips than for leaf explants, because of the occurrence of higher endogenous hormonal concentrations which naturally required a smaller amount of exogenous

supply of hormones for organogenesis as described by Skoog and Miller (1957), who stated that a balanced cytokinin and auxin combination is necessary to initiate organogenesis.

Effect of auxin on *in vitro* root induction

Roots formed simultaneously on proliferation medium from microshoots derived from both shoot tip and leaf explants. These roots did not help in successful establishment of plantlets in the soil condition as they were thin and delicate. The spontaneous rooting observed in the explants cultured on medium without any PGRs is an indication that optimal endogenous levels of PGRs required for rooting are already present in the microshoots as reported by Catapan *et al.* (2001). Similarly, occasional spontaneous rooting also occurred in other members of Asteraceae as in *Tagetes erecta* (Misra and Datta 2001), *Dendranthema* (Teixeira da Silva 2003) and *Spilanthes acmella* (Saritha *et al.* 2002; Saritha and Naidu 2008).

Bais *et al.* (2002) reported that rooting was a very slow process for *S. mauritiana*. They did not observe rooting on basal MS medium. They used a two-step method for improving rooting, one for induction of roots on MS with IAA, while in the second step, roots were profusely multiplied by shaking them in liquid basal MS medium, but this was a time-consuming process. Therefore, to save time, we used half-strength MS with or without auxin not only to induce early rooting but also to improve the growth of roots. In the present study, early rooting was observed in all treatments (within 3–4 days of transfer to rooting medium). Half-strength MS medium fortified with NAA at 2.5 μM was best to induce and proliferate of roots more than IAA and IBA (Table 5, Fig. 1E). A similar response was also observed in various regeneration protocols for *Clitoria ternatea* (Kumar *et al.* 1993) and *Ophiorrhiza prostrata* (Beegum *et al.* 2007). In contrast to the present study, IBA induced the highest frequency of rhizogenesis in *Piper longum* (Soniya and Das 2002), *Tylophora indica* (Faisal and Anis 2003), *Ruta graveolens* (Faisal *et al.* 2006), *Carthamus tinctorius* (Sujatha and Kumar 2007) and *Clitoria ternatea* (Shahzad *et al.* 2007).

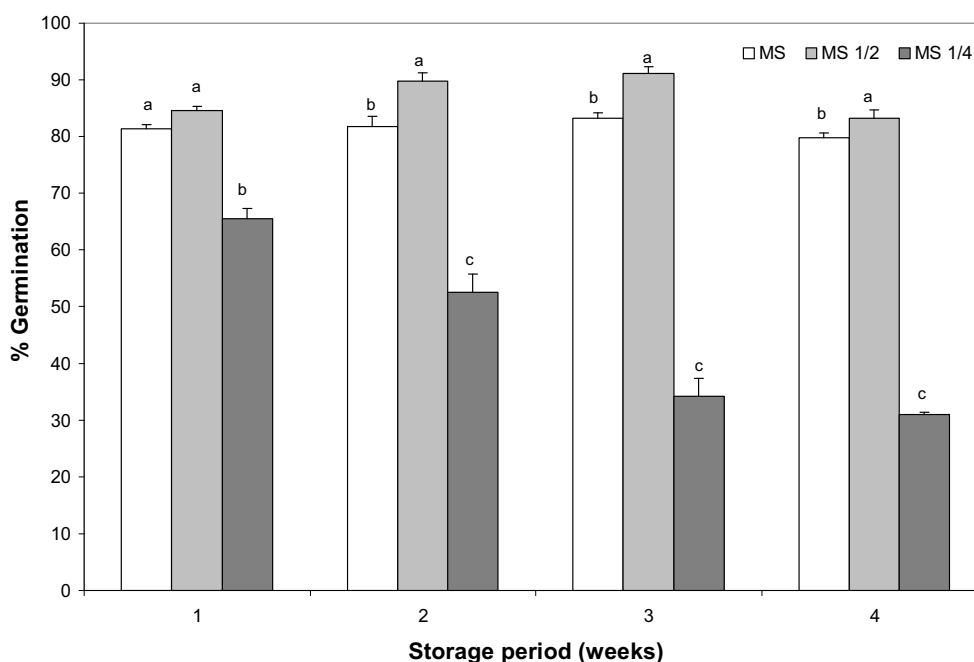


Fig. 2 Percentage of synseed germination (shoot and root formation) on different nutrient strengths (full-strength MS, half-strength MS and quarter-strength MS) after 1, 2, 3 and 4 week of low temperature storage at 4°C.

Effect of nutrient strength and period of storage on germination ability of synseed

In the present investigation nodal segments encapsulated in 3% (w/v) sodium alginate and 100 mM CaCl₂·2H₂O showed varied percentage of germination after storage at 4°C on varied MS salt strengths. The ability of synseeds to retain their viability and germination capacity was maximum on half-strength MS after 3 weeks of low temperature storage, where almost all synseeds sprouted successfully (Fig. 1F, 1G). Afterwards, a decline in the frequency of shoot emergence was observed with this treatment (Fig. 2).

The results of the present study are consistent with earlier findings in *Pyrus communis* (Nower et al. 2007) regarding percentage of synseeds germination, irrespective of medium strength (full-strength MS, half-strength MS). However, Naik and Chand (2006) evaluated the highest percentage sprouting (shoot development) in MS medium supplemented with 4.44 µM BA and 0.54 µM NAA and lowest in half-strength MS medium. The effect of different MS salt strengths and duration of germinated encapsulated buds of jackfruit (*Artocarpus heterophyllus*) when cultured on MS and half-strength MS resulted in the highest percentage conversion compared with other treatments (Badr-Elden 2005).

Since plantlets are grown in a protective regime of culture conditions, it becomes imperative to make them autotrophic prior to their transplantation for better acclimatization. After successful acclimatization, there was no detectable variation among hardened plants (data not shown) with respect to morphological and growth characteristics.

Thus, the present study provided a protocol for the conservation of an endangered medicinal herb through shoot tip, leaf and encapsulated nodal segments, which may lead to increased availability of the plant for its medicinal use and holds promise for genetic transformation to improve the quality of plants. Research is now in progress to enhance the frequency of plant retrieval from the encapsulated buds on natural planting substrates and to preserve them at low temperature for longer duration without markedly affecting viability.

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