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Artificial Seeds for Propagation and Preservation of *Spilanthes acmella* (L.) Murr., a Threatened Pesticidal Plant Species

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ABSTRACT

Synthetic seeds (synseeds) offer several advantages, easy handling, storage, reduced size of propagules and transportability. Germplasm can be effectively stored in the form of synseeds. Nodal segments obtained from *in vitro* raised seedlings of *Spilanthes acmella* were encapsulated in calcium alginate beads. The best gel complexion was achieved using 4% sodium alginate and 100 mM calcium chloride. The maximum frequency (87.8 ± 1.15%) of conversion of encapsulated nodal segments into plantlets was obtained on Murashige and Skoog (MS) medium containing 1.0 µM 6-benzyl adenine (BA) and 0.5 µM α-naphthalene acetic acid (NAA) after 6 weeks of culture. Encapsulated nodal segments stored at 4°C for 1-8 weeks also showed successful conversion with variable percent in successive weeks of transfer, followed by development into complete plantlets when returned to regeneration medium. Conversion of encapsulated nodal segments into plantlets also occurred when the calcium alginate beads were sown directly into Soilrite™ moistened with quarter-strength MS salts. Plants regenerated from encapsulated nodal segments were successfully hardened, acclimatized and established in soil, with a success rate of 90%.

Keywords: alginate bead, encapsulation, nodal segment, Soilrite™, synseed

INTRODUCTION

Long-term storage of plants *in vitro*, without regular subcultures, allows one to rationalize production of nuclear stocks and maintain gene collections as storage in liquid nitrogen (Withers and Engelmann 1999) cannot be applied to all genotypes.

Synthetic seed (synseed) technology offers an efficient means for germplasm storage and preservation at low temperature but above 0°C (Lisek and Likowska 2004) of medicinally important plant species especially those which are at high risk of extinction. Encapsulation of vegetative propagules has become a potentially cost-effective clonal propagation system and can be used as an alternative to synthetic seeds derived from somatic embryos (Sarkar and Naik 1997; Standardi and Piccioni 1998; Adriani *et al*. 2000; Sharma *et al*. 2009). In addition, these encapsulated vegetative propagules can also be used for germplasm conservation of elite plant species and exchange of axenic plant materials between laboratories (Hasan and Takagi 1995; Singh *et al*. 2009). Despite these advantages, there are only a few reports on encapsulation of vegetative propagules (Sharma *et al*. 2009; Singh *et al*. 2009).

Spilanthes acmella (L.) Murr. belongs to the family Asteraceae, a threatened medicinal plant grown in tropics and subtropics. *S. acmella* has been well documented for its antibacterial, antifungal and antimalarial activity. Traditionally, the plant is used in the treatment of toothache, flue, cough and tuberculosis (Haw and Keng 2003). The antimicrobial activity is mainly due to the presence of an alkaloid spilanthol (*N*-isobutyl-2,6,8-decartrienamide) (Khadir *et al*. 1989; Saritha *et al*. 2002). The hexane extract of dried flower buds of *S. acmella* contains bioactive *N*-isobutylamide, which is effective against *Aedes aegypti* and *Helicoverpa zea* neonate larvae (Ramsewak *et al*. 1999). The plant also been reported for its larvicidal activity against *Culex quinquefasciatus* (Amer and Mehlhorn 2006).

Over the past few years, considerable efforts have been made for *in vitro* plant regeneration of this threatened plant species using various explants (Saritha *et al*. 2002; Haw and Keng 2003; Deka and Kalita 2005; Saritha *et al*. 2008). Though there is a single report on synseed production using shoot tips of *S. acmella* (Singh *et al*. 2009), considerable optimization is still needed to the protocol of the *S. acmella* synseed system before it can be considered to be routine for long-term *in vitro* conservation. Thus, we conducted research to fulfill the following objectives: (1) to develop a standard protocol for encapsulation of nodal segments; (2) to evaluate the morphogenic response of encapsulated nodal segments in various treatments; (3) to test the ability of encapsulated nodal segments to retain their viability following storage at a low temperature (4°C) for different durations; (4) to study the effect of different planting substrates on conversion of encapsulated nodal segments into complete plantlets.

MATERIALS AND METHODS

Sterilization of plant material and explant preparation

The inflorescences of *S. acmella* were procured from the Medicinal Plant Nursery of Tamnaar, District Raigarh, State Chhattisgarh, India. The plant species was identified by the taxonomist of our department and a specimen was deposited in the departments' herbarium (No. 31301). Healthy seeds were isolated mechanically from mature inflorescences and washed under running tap water for 30 min to remove any adherent particles. The seeds were kept in 1% (w/v) Bavistin (Carbendazim Powder, BASF India Ltd.), a broad spectrum fungicide, for 20 min and then washed in 5% (v/v) Teepol (Qualigens, India), a liquid detergent, for 15 min. The treated seeds were agitated in sterilized double distilled water (DDW) to remove the chemical inhibitors to germination. The seeds were surface sterilized with 70% (v/v) ethanol and 2-3 drops (v/v) of Tween-20 (Qualigens) for 30 s, followed by immersion in an aqueous solution of 0.1% (w/v) $HgCl_2$ (Qualigens) for 3 min under

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the sterile condition. Finally, the seeds were washed 5-6 times with sterilized DDW to remove all traces of sterilant. The surface sterilized seeds were inoculated aseptically in culture tubes (25 × 150 mm, Borosil) containing half-strength MS medium supplemented with gibberellic acid (GA_3) at 0.5 µM. All plant growth regulators (PGRs) were purchased from Sigma-Aldrich, Germany. Nodal segments (2-3 from the terminal bud, 3-4 mm) from axenic seedlings (3-weeks old) were excised and used as explants for encapsulation.

Encapsulation

For encapsulation, the protocol developed by Faisal and Anis (2007) was followed. Sodium alginate from two sources (Central Drug House, India; Loba Chemie, India) was prepared in the range of 1, 2, 3, 4 and 5% (w/v), whereas calcium chloride (Qualigens) solution was prepared in the range of 25, 50, 75, 100, 150 and 200 mM in either liquid MS medium or distilled water. Both the gel matrix and the complexing agent were autoclaved at 121°C for 20 min, after adjusting the pH to 5.8 ± 0.05 using 1 N NaOH and 1 N HCl. Encapsulation was achieved first by mixing the nodal segments in the alginate solution and then by dropping them into calcium chloride solutions. The droplets containing explants were left for 20-25 min to achieve polymerization of sodium alginate. The drops set as isodiameteric beads (6-7 mm). Beads were collected and rinsed 3 times with sterilized distilled water to remove excess calcium chloride.

Planting media and culture conditions

For conversion into complete plantlets under *in vitro* conditions, encapsulated nodal segments were transferred to MS medium supplemented with or without BA (1.0 µM) + NAA or indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) at 0.5 µM. The germination media solidified with 0.8% (w/v) agar and pH was adjusted to 5.8 ± 0.05 with 1 N NaOH and 1 N HCl before being autoclaved at 121°C and 1.02 kg cm^{-2} for 20 min. All the cultures were maintained at 25 ± 2°C under a 16-h photoperiod with a photosynthetic photon flux density of 50 µmol m^{-2} s^{-1}, provided by cool-white fluorescent lamps (Philips, India).

Effect of different storage durations on conversion of encapsulated and non-encapsulated nodal segments into plantlets

Encapsulated and non-encapsulated nodal segments were stored in sterilized beakers (moist with DDW) sealed with two layers of Parafilm at 4°C. Five different low temperature storage times (1, 2, 4, 6 or 8 weeks) were evaluated for their effect on regeneration. After each period of storage, encapsulated nodal segments were cultured on MS medium supplemented with BA (1.0 µM) + NAA (0.5 µM) for conversion into plantlets.

Effect of different planting substrates on conversion of encapsulated nodal segments into plantlets

Non-stored encapsulated nodal segments were also sown directly in Soilrite™ moistened with tap water or quarter-strength MS salts, Soilrite™ and soil mixture (1: 1) moistened with tap water or quarter-strength MS salts for *ex vitro* conversion into plantlets.

Acclimatization

Plantlets with a well-developed shoot and roots were removed from the culture medium, washed gently under running tap water and transferred to thermocol cups (expanded polystyrene) containing Soilrite™ under diffuse light (50 µmol m^{-2} s^{-1}) with a 16-h photoperiod. Potted plants were covered with a transparent polythene membrane to ensure high humidity (90%). After 1 month, the survived plantlets were transferred to pots containing normal garden soil and maintained in a greenhouse at 30 ± 5°C and 80% relative humidity.

Data analysis

All the experiments were repeated three-times and 10 replicates were employed for each treatment. The percentage response for the conversion of encapsulated nodal segments into complete plantlets was recorded after 6 weeks of culture. The conversion frequency was calculated on the basis of differentiation into roots and shoots. The data were analyzed statistically using SPSS version 12 (SPSS Inc., Chicago, IL, USA) and significant differences between mean were assessed by Tukey's test at 5% probability. The results were expressed as the mean ± standard error (SE) of 3 experiments.

RESULTS AND DISCUSSION

Encapsulation

Of the two different commercial sources of alginate used, the best results with respect to synseed formation were obtained with the product from Central Drug House. Bead formation was influenced by the different concentrations and combination of sodium alginate (1, 2, 3, 4, and 5%) and calcium chloride (25, 50, 75, 100 and 200 mM). The coating must not only be mild enough to protect the explant and allow germination, but it must be sufficiently durable to resist manipulation up to planting. An optimal ion exchange between Na^{+} and Ca^{++}, producing firm, clear, isodiameteric beads, was achieved using a 4% solution of sodium alginate and 100 mM $CaCl_2 \cdot 2H_2O$ (**Fig. 1A**). Higher concentration of sodium alginate (5%) or $CaCl_2 \cdot 2H_2O$ (200 mM) were found to be unsuitable because the resulting beads were too hard and caused a delay in germination, white beads formed using lower concentration of sodium alginate (1, 2 and 3%) or $CaCl_2 \cdot 2H_2O$ (25 and 50 mM) were too fragile to handle (**Tables 1, 2**). Sodium alginate preparation at lower concentrations was unsuitable, probably because of a reduction in their gelling ability after exposure to high temperature during autoclaving (Larkin *et al*. 1998). The influence of the commercial source from which alginate was used, as well as its concentration on capsule quality and plant retrieval, has been reported earlier (Ghosh and Sen 1994; Castillo *et al*. 1998; Mendal *et al*. 2000).

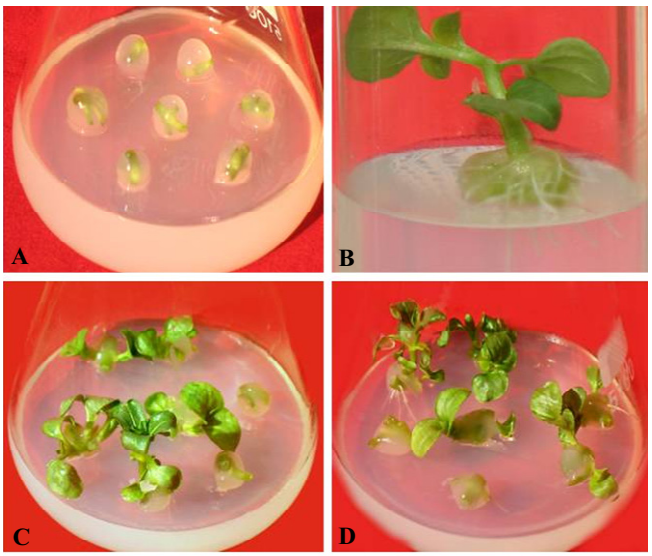

Fig. 1 Plant recovery from encapsulated nodal segments. (**A**) Calcium alginate beads formed by the encapsulation of nodal segments using 4% sodium alginate and 100 mM $CaCl_2 \cdot 2H_2O$; (**B, C**) Encapsulated nodal segments showing regeneration of complete plantlet (shoot and root development) on MS + BA (1.0 µM) + NAA (0.5 µM) after 4 weeks of culture; (**D**) Emergence of shoots on MS + BA (1.0 µM) + NAA (0.5 µM) from encapsulated nodal segments placed after 4 weeks of storage at 4°C, 6 weeks old culture.

Table 1 Effect of sodium alginate concentration on conversion of encapsulated nodal segments after 6 weeks of culture on MS medium.

Sodium alginate (% w/v)	Conversion response (%) into plantlets
1.0	Fragile beads
2.0	Fragile beads
3.0	73.4 ± 1.88 a (but soft to handle)
4.0	74.8 ± 1.46 a
5.0	45.6 ± 1.69 b

Different concentrations of sodium alginate and 100 mM $CaCl_2 \cdot 2H_2O$ were added to MS medium. Values represent mean ± SE. Mean followed by a different lower-case letter are significantly different at 5% probability using Tukey's test.

Table 2 Effect of calcium chloride concentration on conversion of encapsulated nodal segments after 6 weeks of culture on MS medium.

Calcium chloride (mM)	Conversion response (%) into plantlets
25	Fragile beads
50	Fragile beads
75	65.4 ± 2.03 b
100	74.8 ± 1.46 a
200	39.0 ± 2.44 c

Different concentrations of $CaCl_2 \cdot 2H_2O$ and 4% (w/v) sodium alginate were added to MS medium. Values represent mean ± SE. Mean followed by a different lower-case letter are significantly different at 5% probability using Tukey's test.

Table 3 Effect of different media on conversion of encapsulated nodal segments after 6 weeks of culture.

Medium + PGR (µM)	Conversion response (%) into plantlets
MS	74.8 ± 1.46 b
MS + BA (1.0) + NAA (0.5)	87.8 ± 1.15 a
MS + BA (1.0) + IAA (0.5)	80.2 ± 2.83 b
MS + BA (1.0) + IBA (0.5)	73.4 ± 1.02 b

Values represent mean ± SE. Mean followed by a different lower-case letter are significantly different at 5% probability using Tukey's test.

Table 4 Effect of different storage durations on conversion of encapsulated and non-encapsulated nodal segments into plantlets.

Storage duration (weeks)	Conversion of encapsulated nodal segments into plantlets (%) (encapsulation matrix prepared in MS basal medium)	Conversion of encapsulated nodal segments into plantlets (%) (encapsulation matrix prepared in distilled water)	Conversion of non-encapsulated nodal segments into plantlets (%)
0	87.8 ± 1.15 a	27.0 ± 1.22 a	99.0 ± 1.00 a
1	82.8 ± 1.15 ab	15.0 ± 1.58 b	73.0 ± 2.00 b
2	76.6 ± 1.88 bc	4.0 ± 0.31 c	51.0 ± 3.31 c
4	73.6 ± 1.56 b	0.0 ± 0.00 d	28.0 ± 2.54 d
6	58.0 ± 2.00 d	0.0 ± 0.00 d	16.0 ± 1.87 e
8	46.0 ± 1.87 e	0.0 ± 0.00 d	9.2 ± 0.37 e

Values represent mean ± SE. Mean followed by a different lower-case letter are significantly different at 5% probability using Tukey's test.

Table 5 Effect of different planting substrates on conversion of encapsulated nodal segments into plantlets.

Substrates	Conversion of encapsulated nodal segments into plantlets (%)
Soilrite + 1/4-MS salts	63.0 ± 2.00 a
Soilrite + tap water	51.2 ± 0.96 b
Soilrite + soil + 1/4-MS salts	46.6 ± 1.88 b
Soilrite + soil + tap water	26.8 ± 1.93 c
Soil + 1/4-MS salts	15.6 ± 2.31 d
Soil + tap water	00.0 ± 0.00 e

Sodium alginate (4% w/v) and 100 mM $CaCl_2 \cdot 2H_2O$ were added to MS medium. Values represent mean ± SE. Mean followed by a different lower-case letter are significantly different at 5% probability using Tukey's test.

Effect of different media on conversion of encapsulated nodal segments

Nodal segments encapsulated in 4% (w/v) sodium alginate and 100 mM $CaCl_2 \cdot 2H_2O$ exhibited shoot re-growth after 2-3 weeks of culture on MS media supplemented with BA (1.0 µM) + NAA or IAA or IBA (0.5 µM) (**Table 3**). The maximum percentage conversion of encapsulated explants into plantlets (87.8 ± 1.15%) was recorded on MS medium supplemented with BA (1.0 µM) + NAA (0.5 µM) after 6 weeks of culture (**Fig**. **1B, 1C**).

Effect of different storage durations on conversion of encapsulated and non-encapsulated nodal segments into plantlets

After 4 weeks of storage at 4°C, the percentage conversion of non-encapsulated nodal segments into complete plantlets was 28.0 ± 2.54%, after 6 weeks of culture on MS medium supplemented with BA (1.0 µM) + NAA (0.5 µM) while in case of encapsulated nodal segments (encapsulation in MS basal medium) percentage conversion into complete plantlets was 73.6 ± 1.56%, after 6 weeks of culture on same medium (**Table 4, Fig. 1D**). Retrieval of plants after storage was feasible only when the encapsulating matrix was enriched with MS nutrients which served as an artificial endosperm, thereby providing nutrients for re-growth (Bapat and Rao 1962; Nieves *et al*. 1998). Antonietta *et al*. (1999) reported that a synthetic endosperm should contain nutrients and a carbon source for germination and conversion. The use of distilled water in making sodium alginate and chloride failed to support sprouting, perhaps because of poor nutrition after 2 weeks of storage. According to Redenbaugh (1987) the capsule gel can potentially serve as a reservoir for nutrients that may aid the survival and rapid growth of the vegetative propagules. The percentage of recovery of plantlets from encapsulated nodal segments decreased as the period of storage increased beyond 4 weeks (**Table 4**). The decline in the conversion response could be attributed to inhibition of tissue respiration by the alginate matrix, or a loss of moisture due to partial desiccation during storage (Danso and Ford Lloyd 2003).

Effect of different planting substrates on conversion of encapsulated nodal segments into plantlets

Among the various planting substrates tested for conversion of encapsulated nodal segments into plantlets under *ex vitro* condition, Soilrite™ moistened with quarter-strength MS salts was found to be the most suitable planting substrate (**Table 5**).

Acclimatization

Plantlets with well-developed shoots and roots (**Fig. 2A**) were transferred to thermocol cups containing sterile Soilrite™ and covered with a polythene membrane (**Fig. 2B**). After 1 month, they were transferred to pot containing normal garden soil and maintained in a greenhouse. Approximately 90% of plantlets were established in these pots.

Fig. 2 (**A**) Complete plantlet of *S. acmella* recovered from encapsulated nodal segment; (**B**) An acclimatized plantlet of *S. acmella* in Soilrite™.

CONCLUSION

A practicable protocol has been established for synseed formation in *S. acmella* using nodal segments, storage at low temperature and with subsequent growth on various planting media. Although Singh *et al*. (2009) utilized shoot tips for encapsulation, we used nodal segments which were more numerous in the donor plant, making the process more effective and productive. In addition, recovery of complete plantlets from encapsulated nodal segments in Soilrite™ moistened with quarter-strength MS salts shows that this method could be useful in developing a cost-effective propagation system for *S. acmella*. The present results offer a feasible preservation technique to store disease-free *S. acmella* germplasm because storage is not only beneficial for long-term usage but also prevents any somaclonal variation that may ensure the long-term maintenance of the non-embryogenic tissue (Makowczyńska and Andrzejewska-Golec 2006). Such work could be useful for production of synseeds in which somatic embryogenesis is not reported or somatic embryos failed to germinate into complete plantlets.

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