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Research Note

Plant regeneration from alginate-encapsulated shoot tips of *Momordica dioica* for short term storage and germplasm exchange and distribution

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Abstract

An efficient protocol for regeneration of encapsulated shoot tip explants of spine gourd (*Momordica dioica* Roxb. ex. Willd) has been developed. Shoot tip explants excised from *in vivo* proliferated shoots were encapsulated in calcium alginate beads. A gelling matrix of 3% sodium alginate and $CaCl_2 \cdot 2H_2O$ was found most suitable for formation of ideal calcium alginate beads. Maximum response (100%) for conversion of encapsulated shoot tip explants into plantlets was obtained on 0.7% agar solidified full-strength MS medium containing 0.5 µM BAP. Encapsulated shoot tips could be stored at low temperature (4°C) up to 10 weeks with a survival frequency of 50%. Well developed regenerated plantlets were hardened, acclimatized and established in field with 90% survival frequency, where they grew well without any detectable variations. The present synthetic seed technology could be useful in large-scale propagation as well as short-term conservation, germplasm distribution and exchange of spine gourd.

Keywords: *Momordica dioica*, encapsulation, synthetic seeds, cold storage, hardening.
Abbreviations: BAP: 6-benzylaminopurine; $CaCl_2$: Calcium chloride; MS: Murashige and Skoog.

Introduction

Synthetic seed technology offers an efficient means for mass propagation of plant species irrespective of season, space, environmental factor and other hindering conditions. Encapsulation of vegetative propagules (axillary buds, shoot tips, nodal segments etc.) 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; Chand and Singh, 2004). 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 and pharmaceutical industries (Hasan and Takagi, 1995; Rai et al., 2008a; 2009). Alginate encapsulation of shoot tips along with cold preservation offers a strong possibility for germplasm storage and plant regeneration. It can provide a source of axenic plant material (Hasan and Takagi, 1995; Singh et al., 2006b) that can be used if stock plants or proliferation cultures become infested with bacteria, fungi, or arthropods (West et al., 2006). Successful cases of synthetic seed production and plantlet regeneration have been reported for a wide range of plants including cereals, vegetables, fruits, ornamentals, medicinal plants and forest trees (Redenbaugh et al., 1991; Castillo et al., 1998; Ara et al., 2000; Mandal et al., 2000; Singh et al., 2006a; Rai et al., 2009). Among several non-embryogenic propagules, shoot tip explants are more responsive than other explants because of greater mitotic activity in the meristem (Ballester et al., 1997). There are few reports on encapsulation of shoot tips obtained from *in vivo* raised plants (Singh et al., 2009; Verma et al., 2010). Spine gourd (*Momordica dioica* Roxb. ex. Willd) is a perennial, dioceous climbing creeper belonging to the family Cucurbitaceae. It is originated from the Indo-Malayan region and has been highly cultivated in India, China, Nepal, Bangladesh, Myanmar, Pakistan and Sri Lanka (Rakh and Chaudhari, 2010). Immature green fruits are cooked as vegetable and young leaves, flowers and roots are also consumed. Fruits contain high amounts of protein, calcium, phosphorous, iron, and highest amount of carotene amongst the cucurbitaceous vegetables (Bharathi et al., 2007). The plant was reported to exhibit anti-diabetic, anticancer, anti-fertility abortificiant, anti-inflammatory, antioxidant activity, jaundice and bleeding pile properties (Luo et al., 1998; Reddy et al., 2006; Deokule, 2006; Bawara et al., 2010). This popular vegetable has high demand in market but still remains underutilized and underexploited (Bharathi et al., 2007; Ali et al., 1991) due to vegetative mode of propagation and dioecious nature. The improvement of *M. dioica* through conventional breeding techniques has several limitations because of its dioecious nature and difficulty in seed germination (Rashid, 1976; Ali et al., 1991). Propagation by tuberous roots is limited due to the low multiplication rate (Mondal et al., 2006). During past years, considerable efforts have been made for *in vitro* plant regeneration of this important plant through direct organogenesis (Nabi et al., 2002; Thiruvengadam et al., 2006; Shekhawat et al., 2011). In our previous studies, we have reported plant regeneration from somatic embryogenesis of petiole derived callus (Thiruvengadam et al., 2007). To the best of our knowledge, there has been no report on synthetic seed production using either somatic embryos or vegetative propagules in spine gourd. Hence, this investigation is aimed to develop an efficient protocol for clonal propagation and method of germplasm exchange and distribution through

synthetic seeds in *M. dioica*, an important vegetable and medicinal plant.

Results and discussion

Encapsulation of shoot tips

A new procedure in synthetic seed technology was reported with the use of non-embryogenic plant propagules (Standardi and Piccioni, 1998). The main advantage of using vegetative propagules for the preparation of synthetic seeds would be in those cases where somatic embryogenesis is not well established or somatic embryos do not germinate into complete plantlets (Rai et al., 2009). In the present investigation, shoot tips excised from *in vivo* proliferated shoots were used as an explant for the development of synthetic seed in *M. dioica*. Shoot tips generally yield better response than other non-embryogenic vegetative propagules. The reason for such differences in response may be due to greater mitotic activity in shoot tips than in lateral buds, which are subjected to apical dominance (Verma et al., 2010). In such cases, synthetic seeds can be produced from shoot tips for cost-effective mass clonal propagation, potential long-term germplasm storage, and delivery of tissue-cultured plants. Encapsulation of shoot tips was affected by the concentration of sodium alginate and calcium alginate beads with entrapped shoot tips differed qualitatively with respect to texture, shape, and transparency. In our study 3.0% sodium alginate and 100 mM $CaCl_2 \cdot 2H_2O$ was found most suitable for formation of ideal calcium alginate beads (Fig. 1A) and subsequent conversion of encapsulated shoot tip explants into plantlets. Similar observations were also made in *Punica granatum* (Naik and Chand, 2006), *Phyllanthus amarus* (Singh et al., 2006a), and *Withania somnifera* (Singh et al., 2006b). Lower concentrations of sodium alginate [1-2% (w/v)] and $CaCl_2 \cdot 2H_2O$ (50 mM) resulted in beads without a defined shape and were too soft to handle, whereas at higher concentrations of sodium alginate (4-5%) or $CaCl_2 \cdot 2H_2O$ (150 mM), the beads were isodiametric but were hard enough to cause considerable delay in shoot emergence. A successful propagation system routed through encapsulation is based on significant evaluation of factors affecting the gel matrix and both sodium alginate and $CaCl_2 \cdot 2H_2O$ play an important role in complexation and capsule quality (Singh et al., 2006a).

Plantlet development from alginate encapsulated shoot tips

Shoots and roots emerged from the encapsulated shoot tips (Fig. 1B) by breaking the capsule wall within 1 week on MSB medium, however, conversion into complete plantlets (Fig. 1C) occurred after 4-5 weeks of culture. The conversion of encapsulated and non-encapsulated shoot tip explants into plantlets was achieved on MSF, MSH, MSB and MSK, however, conversion percent varied with different media composition. Maximum percentage response for conversion of encapsulated shoot tips into plantlets was achieved on MSB (Table 1; Fig. 1C). It was observed that encapsulated shoot tips exhibited significantly higher conversion than non-encapsulated shoot tips under the same culture conditions (Table 1). The use of double distilled water for preparing gel matrix reduced the shoot emergence as compared to liquid MS medium (data not shown). The reason for these results may be attributed to the protection provided by capsules as well as the presence of nutrients in the gel matrix, which apparently served as a nutrient bed around the propagules which facilitated growth and survival, and allowed to germinate (Redenbaugh et al., 1987; Ara et al., 1999). Plantlets regenerated from encapsulated shoot tips were successfully established under field conditions and about 90.0% of them survived.

Short-term storage of encapsulated shoot tips

Percentage response for the conversion of encapsulated and non-encapsulated shoot tips decreased gradually with increasing storage duration at 4°C (Fig. 2). Similarly, the conversion frequency of encapsulated shoot tips of *Phyllanthus amarus* also declined markedly following storage at low temperature (Singh et al., 2006a). Encapsulated shoot tips showed higher resistance to storage at 4°C than non-encapsulated shoot tips. After storage for 10 weeks, the percentage response for conversion of encapsulated shoot tips was 50%, whereas non-encapsulated shoot tips did not convert into plantlets (Fig. 2). It is assumed that decline in the conversion of encapsulated propagules stored at low temperatures may be due to the inhibited respiration of plant tissues because of the alginate cover (Redenbaugh et al., 1987; Naik and Chand, 2006). Similarly, the conversion of encapsulated nodal segments of *Punica granatum* also declined markedly following storage at low temperature (Naik and Chand, 2006). Alginate encapsulation is a technique that can be used for germplasm storage or for reducing the need of transferring and subculturing, out of season (West et al., 2006). Cold storage has the potential to reduce the cost of maintaining germplasm cultures because of the reduced need for manual labor due to less frequent subculturing (West et al., 2006). An important feature of the encapsulated vegetative propagules is their capability to retain viability after storage for a sufficient period required for exchange of germplasm between laboratories and extension centers (Rai et al., 2008b). Plantlets with six to eight fully expanded leaves and well-developed roots obtained from encapsulated shoot tip explants were successfully acclimatized in plastic pots containing garden soil (Fig. 1D). The percentage survival was recorded to be 90%. They were successfully transplanted into field conditions where they grew normally without any morphological variations. This is the first report of synthetic seed production in *M. dioica* using vegetative propagules as explants. The synthetic seed technology described in the present paper provides an alternative method of propagation of this important vegetable and medicinal plant. Successful plant retrieval from encapsulated shoot tip explants following at low temperature indicates that the method described in this paper could be potentially used to preserve desirable elite genotype of *M. dioica* over a short period. This could also facilitate transport of encapsulated shoot tip explants to laboratories and extension centers of distant places.

Materials and methods

Plant materials

Tubers of *Momordica dioica* Roxb. ex. Willd (one year old) were collected from Semmalai hills and the plants were raised in the Botanical Field Evaluation Garden at Tiruchirappalli, India. Shoot tip explants were collected and washed in running tap water for 5 min and surface sterilized with 70% (v/v) ethanol for 1min. Further, explants were treated with 1.0% (v/v) sodium hypochlorite solution for 10 min by occasional agitation. Finally, the explants were rinsed four times with sterile double distilled water, blotted dry and trimmed from both ends to about 0.6-0.7 mm.

Table 1. Effect of different planting media on conversion into plantlets of encapsulated and non-encapsulated shoot tips of *M. dioica*.

Planting media	Conversion of encapsulated shoot tip explants into plantlets (%)	Conversion of non- encapsulated shoot tip explants into plantlets (%)
MSH	88 ± 1.0cd	77 ± 0.5d
MSF	95 ± 0.5b	89 ± 0.5b
MSB	100 ± 00a	95 ± 00a
MSK	90 ± 1.0c	86 ± 0.4bc

MSH - MS half-strength medium, MSF - MS full-strength medium, MSB - MS medium containing 0.5 µM BAP, MSK - 1.0 µM Kinetin. Values are mean of three independent experiments. Data were recorded after 4 weeks of culture. Mean followed by the same letter in a column are not significantly different at P<0.05 according to Duncan's multiple range test.

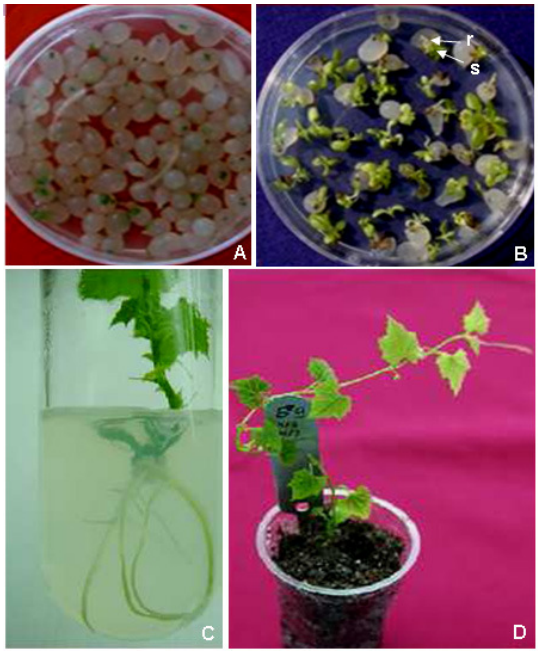

Fig 1. Plantlet regeneration from encapsulated shoot tips of *Momordica dioica***.**
(A) Shoot tips encapsulated in calcium alginate beads; (B) Shoot (s) and root (r) emergence from encapsulated shoot tips; (C) Conversion of encapsulated shoot tips into plantlet on full-strength MS medium containing 0.5 µM BAP. (D) Hardened plants of *M. dioica*. Arrows indicate emerging shoot (s) and root (r) from encapsulated shoot tips.

Encapsulation of shoot tips

For encapsulation, sodium alginate (Sigma, USA) was prepared in the range of 2.0, 3.0, 4.0, or 5.0% (w/v), whereas calcium chloride ($CaCl_2{\cdot}2H_2O$) solution was prepared in the range of 50, 100, or 150 mM (w/v) in either double-distilled water or liquid MS (Murashige and Skoog, 1962) medium without any plant growth regulator. Aliquots of alginate solution, each of which contained one shoot tip explant, were taken up with a sterile spatula and then gently dropped into 100 ml $CaCl_2{\cdot}2H_2O$ solution. The droplets containing shoot tip explants were held for 20-30 min in the $CaCl_2{\cdot}2H_2O$ solution to achieve polymerization of sodium alginate.

Germination of encapsulated shoot tips into plantlets

To evaluate the effect of medium on plantlet conversion, encapsulated shoot tips were cultured on different planting media; growth regulator free and 0.7% agar-solidified MS half-strength medium (MSH), MS full-strength medium (MSF), MS full-strength medium containing 0.5 µM BAP (MSB) or 1.0 µM Kinetin (MSK). Non-encapsulated shoot tips were used as control for all treatments. The pH of the medium was adjusted to 5.8 prior to autoclaving for 15 min at 121°C and 1.1 kg cm^{-2} pressure. Cultures were maintained at 25 ± 2°C with 16/8 h (light/dark) photoperiod at a photon flux of 50 µmol m^{-2} s^{-1} from white fluorescent tubes.

Short-term storage of encapsulated shoot tips

For cold preservation, encapsulated and non-encapsulated shoot tips were stored in cold room at 4°C for different duration (0, 2, 4, 6, 8 and 10 weeks) in full-strength liquid MS medium under dark condition. After each storage period, encapsulated and non-encapsulated shoot tips were transferred into growth regulator-free full-strength solid MS medium for the regeneration of plantlets.

Acclimatization and field transfer of plantlets

Well-developed plantlets regenerated from encapsulated shoot tips were transferred to plastic pots containing a mixture of sand and soil (3:1) moistened with tap water. Plantlets were covered with polyethylene bag to maintain high humidity and kept in culture room at 25± 2°C and a photon flux density of 50 µmol m^{-2} s^{-1} provided by white fluorescent tubes. After 2-3 weeks, the pots were transferred to sunlight, initially for a short time, and gradually the time

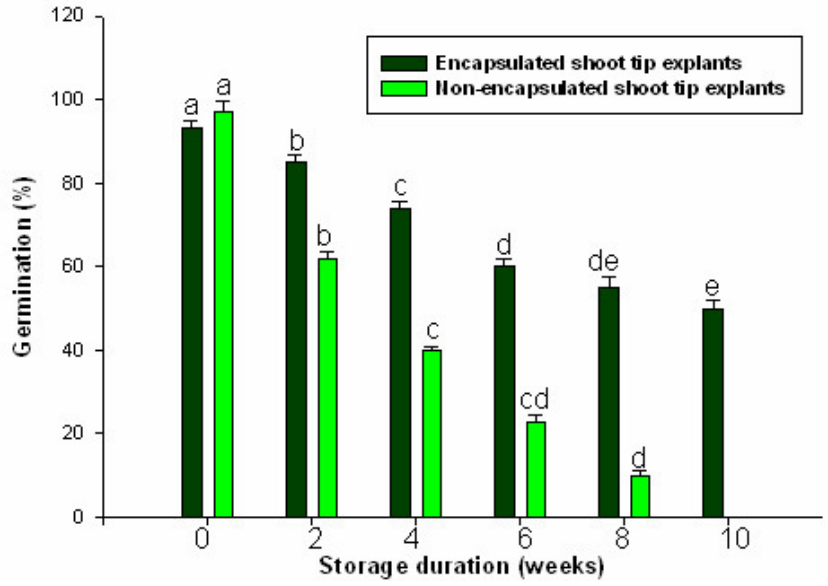

Fig 2. Plantlet conversion from encapsulated and non-encapsulated shoot tip explants of *M. dioica* after storage at low temperature (4°C) for different durations.
Values are mean of three independent experiments. Data were recorded after 4 weeks of culture. Mean followed by the same letter in a column are not significantly different at P<0.05 according to Duncan's multiple range test.

was increased. The survival rate was recorded after 30 days. Successfully established plantlets were subsequently transferred to field conditions.

Experimental design and data analysis

The frequency of plantlet conversion was calculated as the percentage of encapsulated shoot tips showing well-developed shoot and root out of total number of shoot tips encapsulated. For the above experiments, 24 replicates were used for each treatment and each experiment was repeated thrice. The mean standard error and one-way ANOVA were calculated using SPSS (version 10) software. The mean separations were carried out using Duncan's multiple range tests and significance was determined at $P < 0.05$.

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