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ORIGINAL PAPER

An evaluation of genetic fidelity of encapsulated microshoots of the medicinal plant: *Cineraria maritima* following six months of storage

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Abstract Regrowth of encapsulated microshoots, using alginate encapsulation, of *Cineraria maritima* reached 82.35% following 6 months of storage. Amongst developing plantlets, 33.33% exhibited formation of multiple shoots at the onset of regrowth and 11.76% demonstrated simultaneous formation of shoots and roots. Healthy root formation was observed in plantlets following 2 weeks of their transfer to half-strength Murashige and Skoog medium containing 1.0 mg l^{-1} α-naphthalene acetic acid. Plants were transplanted to the greenhouse in three batches with 90% frequency of survival. Molecular analysis of randomly selected plants from each batch was conducted using 20 random amplified polymorphic DNA (RAPD) markers. Of 20 primers tested, 14 produced amplification products, and a total of 69 bands with an average of 4.93 bands per primer were observed. Of these 69 scorable bands, only 20% of bands were polymorphic. Cluster analysis of the RAPD profiles revealed an average similarity coefficient of 0.944 thus confirming molecular stability of plants derived from encapsulated microshoots following 6 months of storage.

Keywords *Cineraria maritima* · Germplasm conservation · RAPD analysis · Encapsulated microshoots

Abbreviations

BA	Benzyladenine
MS	Murashige and Skoog (1962) medium
NAA	α-Naphthalene acetic acid
UPGMA	Unweighted pair grouping method with arithmetic-mean
RAPD	Randomly amplified polymorphic DNA

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Introduction

Establishment of gene banks for ex situ conservation of plant germplasm in the form of field gene banks, seed gene banks, in vitro collections, and cryogenically preserved tissues is a common practice (Withers 1983; Rao 2004; Borner 2006). Alginate encapsulation provides a viable approach for in vitro germplasm conservation as it combines the advantages of clonal multiplication with those of seed propagation and storage (Standardi and Piccioni 1998; Ara et al. 2000).

Potential advantages of synthetic seeds include their designation as "genetically identical materials", ease of handling and transportation, along with increased efficiency of in vitro propagation in terms of space, time, labor and over-all cost (Nyende et al. 2003). Although synthetic seeds have been widely utilized for micro propagation and conservation of various medicinal plant species (Ara et al. 2000; Mandal et al. 2000; Anand and Bansal 2002; Nyende et al. 2003; Manjkhola et al.2005; Singh et al.2006a, b; Narula et al. 2007; Faisal and Anis 2007; Ray and Bhattacharyaa 2008; Lata et al. 2009), the genetic stability of synthetic seed-derived plantlets remains relatively unknown with the exception of a recent report on *Ananus comosus* (L.) Merr (Gangopadhyay et al. 2005). In contrast, the impact of cryogenic conservation, via encapsulation/dehydration or vitrification methods, of plant germplasm on observed genetic variability has attracted more attention

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(Chaudhary et al. 1989; Chandel and Chaudhary 1995; Ashmore 1997). The genetic fidelity of plants recovered from cryogenic storage has been assessed in a wide range of plant systems (Häggman et al. 1998; Aronen et al. 1999; Hirai and Sakai 2000; Scocchi et al. 2004; Bekheet et al. 2007). The increasing utilization of synthetic seeds for germplasm conservation and propagation necessitates assessment of genetic stability of conserved propagules following their conversion (Dehmer 2005).

Cineraria maritima L. (Asteraceae), commonly known as 'Dusty Miller' or 'Silver dust', is an important annual exotic medicinal herb cultivated in Europe and in certain regions of the United States. This plant is used in preparation of homeopathic medicines, particularly for treating cataract and corneal opacity (Vikramaditya and Joshi 1971). Limited availability of this plant in India drives import of prepared drug formulations from other countries. An efficient multiplication protocol suitable for commercial use has been previously developed (Banerjee et al. 2004) to keep pace with the growing demand for this herb in pharmaceutical markets.

In recent years, systematic sampling of germplasm and analysis of their molecular status through the use of DNA marker technology, such as random amplified polymorphic DNA (RAPD) (Jokipii et al. 2004), has become common practice (Borner 2006; Guo et al. 2006; Mandal et al. 2007). In this study, conservation of the germplasm of *C. maritima* using synthetic seed technology is undertaken to ensure steady supply of quality plants. Moreover, the genetic profiles of conserved propagules following their conversion from encapsulated microshoots are assessed using RAPD analysis.

Materials and methods

Plant material

In vitro-grown proliferating shoot cultures of *C. maritima* (designated as "M") were used as sources of explants. Shoot-tips and nodal stem segments were dissected (3–5 mm), and used as explants for encapsulation.

Encapsulation

The gelling mixture, consisting of half-strength Murashige and Skoog (MS) (1962) medium, 3% sodium alginate (Sigma, St. Louis, MO, USA), 1.5% sucrose, and 3% calcium chloride solution ($CaCl_2$), was autoclaved at 120°C for 20 min under 15 lb pressure. Shoot-tips and nodal segments of *C. maritima* (M) were suspended in the sterile sodium alginate gelling mixture, and were dispensed dropwise into the $CaCl_2$ solution under continuous shaking. The resultant beads were allowed to remain in the $CaCl_2$ solution for 20–30 min to complete the ion-exchange reaction. The resultant encapsulated microshoots were subsequently washed thoroughly with sterilized distilled water, and stored in a moist-environment for 6 months at 25 ± 2°C. The moist condition was maintained by spraying encapsulated shoots with sterile distilled water (in the form of mist) at 15 days interval. The stored encapsulated microshoots were plated on a plant growth regulator (PGR)-free semi-solid MS basal medium for regrowth. Data on regrowth frequencies as well as number of plantlets with both shoots and roots were recorded after 2 and 4 weeks of culture, respectively. Developing shoots without roots were further transferred to half-strength MS medium containing 1.0 mg l^{-1} α-naphthalene acetic acid (NAA) for rhizogenesis.

The experiment was repeated three times, and healthy plants from each batch were transferred to the greenhouse. In vitro-grown *C. maritima* (M) mother plants were also established in the greenhouse and served as controls.

RAPD analysis

Four randomly-selected plants, following at least 2 months of growth in the greenhouse, from each of the three batches of 20 plants derived from encapsulated microshoots along with the mother control plant (M) were subjected to RAPD analysis. Young leaves (150 mg) were ground in liquid nitrogen, and DNA isolation was performed according to Edwards et al. (1991). DNA yield was determined using a Nanodrop spectrophotometer (Nanodrop®, ND—1000, Nanodrop Technologies, Wilmington, Delaware, USA).

A set of 20 decamer RAPD primers (OPJ 1–20) (Operon Tech., Alameda, CA, USA) were used for DNA amplification. Amplification was performed using 25 µL PCR mixture of consisting of 2.5 µL 10× buffer (GeNei™, Bangalore, India), 1.0 µL dNTP (10 µM: 2.5 µM each of the dNTPs viz. dCTP, dATP, dGTP, and dTTP) (GeNei™), 0.2 µL *Taq* polymerase (GeNei™), 1.0 µL DNA (approximate 25 ng/µL), 1.0 µL primer (5 pM), and 19.2 µL MiliQ water. Amplifications were carried out in an *i*-cycler™ Thermal Cycler (model 4.006, Bio-Rad, Hercules, California, USA) with an initial denaturation of DNA at 94°C for 5 min, followed by 1 min denaturation at 94°C, 1 min annealing at 35°C, and 2 min extension at 72°C. The reaction continued for 45 cycles followed by a final extension of 3 min at 72°C.

PCR products were loaded with 5 µL of bromophenol blue, and separated on 1.2% agarose gel (Himedia, Mumbai, India) in 1.0% TAE buffer (40 mM Tris acetate, pH 8.2; 1 mM EDTA) using a Minipack-250 electrophoresis system (GeNei™,) at 50 V for 3 h. A 1 Kb λ DNA ladder, double-digested with *Eco*RI and *Hin*dIII, was used a

molecular standard. Gels were documented using a Image Master VDS, Thermal Imaging System, FTI- 500 (Amersham Pharmacia Biotech., Piscataway, New Jersey, USA).

Data scoring and analysis

Only clear and reproducible bands on gels were scored, based on presence and absence. Two independent amplification reactions were performed with all RAPD primers. Data were subjected to the NTSys PC version 2.02j (www.exetersoftware.com) statistical analysis package, and cluster analysis was conducted. Similarity estimates were calculated (Nei and Li 1979), and cluster analysis was carried out using unweighted pair grouping method with arithmetic mean (UPGMA).

Results and discussion

After 6 months of storage at 25 ± 2°C in moist condition, the encapsulated microshoots of *C. maritima* were capable of regrowth within 2 weeks following culture (Fig. 1a–e). The frequency of regrowth was 82% after 4 weeks of culture on PGR-free MS medium (Fig. 1b). This observation substantiate earlier report involving *Vanilla* species where encapsulated shoot-buds and protocorms demonstrated 80% germination after 10 months of storage in sterile water (Divakaran et al. 2006). Amongst the responding encapsulated microshoots of *C. maritima*, 33% exhibited formation of multiple shoots at the onset of regrowth (Fig. 1c); while, 28% revealed single shoot formation per encapsulated microshoot (Fig. 2). The emergence of a single or multiple shoots from one encapsulated explant indicates the presence of one or more than one primordia on the explant, which has also been noted earlier in other medicinal plant species (Mandal et al. 2000; Lata et al. 2009). In case of *C. maritima*, ~9% of growing shoots exhibited some form of abnormal phenotype, including yellowing of leaves, vitrification, or callusing at basal end and these were discarded. Moreover, around 11% of developing shoots demonstrated simultaneous formation of roots (Fig. 1e). For those non-rooted shoots, root

Fig. 1 Encapsulated microshoots of *C. maritima* and its response. **a** Calcium alginate beads formed by encapsulation of explants using 3% sodium alginate and 3% $CaCl_2.2H_2O$; **b** shoot formation from plated encapsulated microshoots on MS medium after 4 weeks of plating; **c** multiple shoot formation from encapsulated microshoots on MS medium; **d** single shoot formation from encapsulated microshoots on MS medium; **e** simultaneous formation of shoots and root from encapsulated microshoots on MS medium; **f** root induction in encapsulated microshoots derived shoots grown on rooting medium after 3 weeks of culture; and **g** healthy plants derived from encapsulated microshoots growing in the greenhouse

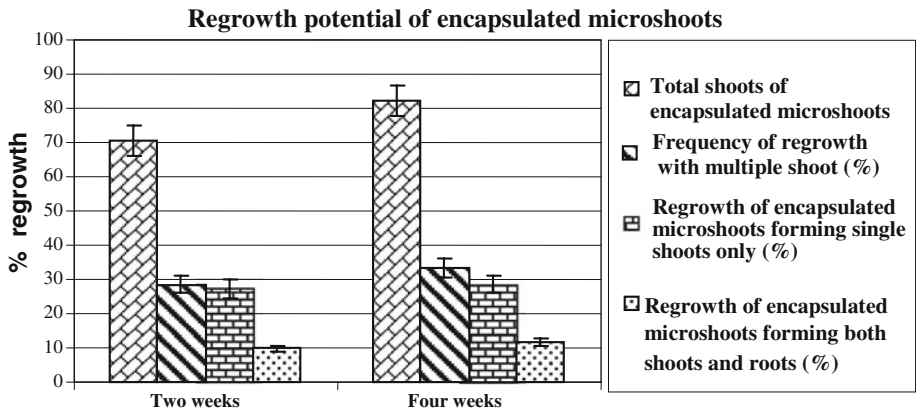

Fig. 2 Frequency of regrowth of encapsulated microshoots of *C. maritima*

formation was induced after 2–3 weeks following transfer to root induction medium (Fig. 1f). It is pertinent to mention here that, conservation of germplasm through encapsulation method has been practiced relating to numerous medicinal plant species, such as *Ocimum* species (Mandal et al. 2000), *Adhatoda vasica* (Anand and Bansal 2002), *Phyllanthus amarus* (Singh et al.2006a), *Withnia somnifera* (Singh et al. 2006b), *Dioscorea bulbifera* (Narula et al. 2007), *Tylophora indica* (Faisal and Anis 2007), *Rauvolfia serpentina* (Ray and Bhattacharyaa 2008), *Cannabis sativa* (Lata et al. 2009), etc., but unlike the present study, regrowth of encapsulated explant into complete plantlets has, by and large, been analyzed on PGR supplemented media. Moreover, this study reports successful storage of the encapsulated microshoots of *C. maritima* at 25 ± 2°C in contrast to several of the above cited reports where storage has been carried out at 4°C (Mandal et al. 2000; Singh et al.2006a, b; Narula et al. 2007; Faisal and Anis 2007). Both these aspects give preferentiality to the present study for developing a cost-effective conservation strategy with minimum complexities for maintenance of low temperature. Additionally, the periods of conservation in most of the aforementioned cases are relatively shorter than that reported in the current investigation, which undoubtedly gives a boost to the basic conservation approach.

In *C. maritima*, a total of 90% plant survival was obtained when these were acclimatized and transferred to the greenhouse (Fig. 1g). This observation is in agreement with several earlier findings (Singh et al. 2006a, b; Faisal and Anis 2007).

Of the 20 primers tested, 14 produced clear and scorable amplification products during repetitions (Table 1; Fig. 3). Amongst these 14 amplified primers, seven primers produced monomorphic patterns; while, the rest generated polymorphic bands. The number of bands for each primer varied from 2 (OPJ-12 & 18) to 9 (OPJ-08) and ranged in size from 200 bp to 2.0 kb (Fig. 3). All amplified primers produced a total of 69 bands with an average of 4.93 bands per primer (Table 1; Fig. 3). Out of the 69 scorable bands, 55 were monomorphic (79.71%); while, only 14 were

Table 1 RAPD primers amplified in *Cineraria maritima*

Primer	Primer sequences	No. of bands scored	No. of polymorphic bands	% Polymorphic bands
OPJ-01	CCCGGCATAA	8	2	25
OPJ-04	CCGAACACGG	5	0	0
OPJ-05	CTCCATGGGG	6	1	16.6
OPJ-06	TCGTTCCGCA	5	0	0
OPJ-08	CATACCGTGG	9	3	33.3
OPJ-09	TGAGCCTCAC	5	0	0
OPJ-10	AAGCCCGAGG	5	3	60.0
OPJ-11	ACTCCTGCGA	3	0	0
OPJ-12	GTCCCGTGGT	2	1	50
OPJ-15	TGTAGCAGGG	3	0	0
OPJ-17	ACGCCAGTTC	6	2	33.3
OPJ-18	TGGTCGCAGA	2	0	0
OPJ-19	CACAGGCGGA	4	0	0
OPJ-20	GTGTCGCGAG	6	2	33.3
Total		69	14	20.29

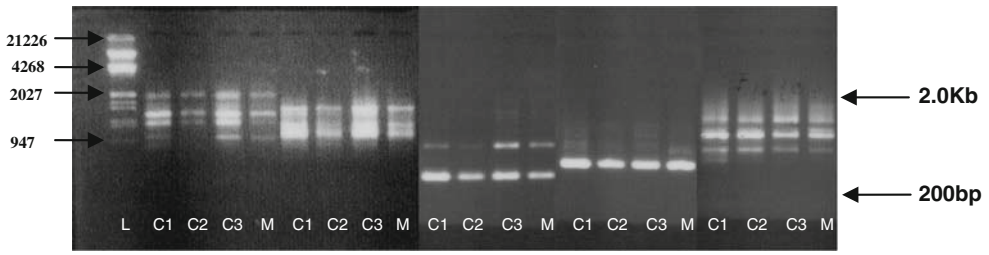

Fig. 3 RAPD amplification profiles with primers OPJ- 17, 15, 18, 19 and 20; *Lane 1* is molecular marker (λ marker double digested with *Eco*RI and *Hin*dIII); *M*, Mother plant; *C1–C3*, plants derived from regrowth of encapsulated microshoots of *C. maritima* and representing the three different batches of cultures

polymorphic (20.29%; Table 1; Fig. 3). Lower frequencies of polymorphic banding patterns have also been reported to occur in *Abies cephalonica* (Aronen et al. 1999) and *Melia azedarach* (Scocchi et al. 2004) during cryopreservation.

A similarity matrix calculated from the RAPD results was subsequently subjected to cluster analysis using the UPGMA clustering method. Three groups were observed from the UPGMA phenogram based on RAPD profile similarities, clustered at similarity coefficients of 0.932–0.955 with a mean value of 0.944. These data indicated that plants derived from encapsulated microshoots resembled the parental control genetic profiles, based on RAPD profiles, after 6 months of storage.

The present findings substantiate earlier reports of the genetic stability of synthetic seed derived-plants of *Ananus comosus* (L.) Merr, tested after short periods (45 days) of storage (Gangopadhyay et al. 2005). Furthermore, results obtained in this study were similar to those previously reported in cryopreserved tissues of various plant species (Häggman et al. 1998; Hirai and Sakai 2000; Dixit et al. 2003; Jokipii et al. 2004; Liu et al. 2004; Scocchi et al. 2004; Bekheet et al. 2007). It is also interesting to note that the encapsulation technique has been efficiently utilized for cryopreservation of stable genotypes of *Solanum tuberosum* L. (Hirai and Sakai 2000) and *Melia azedarach* L. var. *gigantea* (Scocchi et al. 2004). These results further support the feasibility of this cost effective and simple germplasm conservation approach for *C. maritima*.

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