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BRIEF COMMUNICATION

Storage and conversion of *Eclipta alba* synseeds and RAPD analysis of the converted plantlets

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Abstract

The encapsulated shoot tips and nodal segments of *Eclipta alba* were stored at 4, 12 and 20 °C under irradiance of 1.5 μmol m^{-2} s^{-1} and high conversion was observed in synseeds stored at 4 °C for 8 weeks. Duration of storage was extended up to 12 weeks by decreasing sucrose concentration in the alginate matrix from 3 to 1 or 2 % and conversion frequency was 71.2 - 76.1 %. Synseed-derived plantlets survived by 100 % in *ex vitro* conditions. RAPD analysis revealed uniform amplification profile in donor and synseed derived plantlets.

Additional key words: alginate matrix, encapsulated explants, *ex vitro* transfer, genetic uniformity, sucrose.

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Eclipta alba is a common herb of tropical, subtropical and temperate climate. In current years, the plant has been harvested in huge quantities from the wild to meet up the pharmaceutical demand. For this use, cultivation of *Eclipta alba* is preferred to the collection, however, systematic cultivation of this plant is not adopted till date. Moreover, the seeds are very small, wind dispersed (Neff and Baldwin 2005) and fast germinating (within less than 5 d of shedding; Ferreira *et al*. 2001).

Synthetic seed (synseed) has become an efficient tool that facilitates conservation as well as mass propagation of elite plant species by encapsulating somatic embryo or meristem tissue. Inclusion of meristematic tissues instead of somatic embryos hastened widespread exploitation of this technology in recent years. Encapsulation of meristematic tissues of *E. alba* for developing alternate germplasm source is an attractive option in the above mentioned situation. Although continuous production of *E. alba* can be achieved through micropropagation (Ray and Bhattacharya 2008) its high growth rate limits storage and maintenance under *in vitro* condition as frequent subcultures are required. Encapsulation provides two-fold advantages, synseeds offer short term conservation of germplasms; besides, germinating synseeds can provide readily available tissue source for easy mass propagation where each synseeds can virtually act as zygotic seeds giving rise to plantlets. Finally, synseed-generated plantlets with their genetic fidelity confirmed through RAPD analysis ensured raising of genetically homogenous plant population.

The aim of present research was optimization of storage period and conversion frequency of *E. alba* synseeds by changing physico-chemical condition of storage. Suitable storage temperature was selected in the first step followed by optimization of sucrose concentration in the beads. The study also included experimentation on *ex vitro* survival of synseed-derived plantlets and checking their genetic fidelity by RAPD analysis.

In vitro culture of *Eclipta alba* (L.) Hassk. was established according to Ray and Bhattacharya (2008). Axenic shoot tips and nodal segments were dissected from *in vitro* plantlets and were used as explants for encapsulation. Explants mixed with 3 % sodium alginate were suspended drop wise in 1.11 % $CaCl_2$ solution so that each drop contained single explant. They were kept for 30 - 45 min in magnetic stirrer for complete polymerization of alginate. The beads, hence formed, were washed thrice with sterile distilled water, soaked in filter paper. Finally, encapsulated propagules were transferred

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Abbreviations: c.f. - conversion frequency; n-synseed - encapsulated nodal segment; PCR - polymerase chain reaction; RAPD - random amplified polymorphic DNA; s-synseed - encapsulated shoot tip.

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to culture vessels containing MS with 3 % sucrose without growth hormones and maintained under different storage conditions. Entire process of encapsulation was carried out in sterile conditions.

Synseeds were incubated under low irradiance of 1.5 μmol m^{-2} s^{-1} and different temperatures (4, 12 and 20 °C). Synseeds stored at 4 °C were also subjected to the lower sucrose concentrations (0, 0.5, 1 and 2 %) in their alginate coating instead of standard 3 %.

Stored synseeds from either set were taken out at 2 weeks interval to standard culture condition (20 ± 2 °C and 18-h photoperiod with irradiance of 30 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps). The conversion frequency (c.f.) was noted and was expressed as percentage of synseeds performed both shoot and root formation. Synseed-derived plantlets were transferred to *ex vitro* condition to small plastic pots containing *Soilrite*, *Soilrite* + loamy soil or loamy soil. Finally, well grown plantlets were transplanted to soil in earthen pots and grown under natural environment.

Random amplified polymorphic DNA (RAPD) analysis was carried out to test genetic fidelity of donor and 14 randomly selected synseed derived plants after four weeks of their transfer to soil. For each sample, DNA was extracted from 200 mg of fresh leaves according to Doyle and Doyle 1987. It was purified with RNAaseA and used as template. RAPD analysis were performed using thirty decamer primers [twenty from *Operon Technologies* (Alameda, CA, USA; OPB11-20, OPC 1, 2, 4-6, 9, 11, 15, 18, 20) and ten self designed primers from *Bangalore Genei* (Bengaluru, Karnataka, India; BG1-10)]. Polymerase chain reaction (PCR) reaction mixture (0.025 cm^3) consisted of 50 ng template DNA, 1 unit of Taq polymerase (*IDT*), 1× buffer, 250 μM of each dNTP, 2.5 mM $MgCl_2$ and 50 ng primer. The amplification was carried out in a DNA thermal cycler (*Gene Amp PCR System 2400*, *Perkin Elmer*, CA, USA) in the following way: initial 3 min at 94 °C, then 35 cycles of 1 min at 94 °C, 1 min at 35 °C and 2 min at 72 °C. An additional cycle of 10 min at 72 °C was used for final extension. The amplified products were electrophoresed in 1.5 % agarose gels, the gels were stained with 0.5 μg cm^{-3} ethidium bromide, checked under UV radiation and photographed in gel documentation system (*Gel-doc 1000*, *Bio-Rad*, Hercules, USA). The molecular masses of the bands were estimated by *Quantity One 1-D Analysis Software* (version *4.6.2*, *Bio-Rad*).

Each treatment consisted of 10 replicates and the experiment was repeated three times. All data were subjected to analysis of variance (*ANOVA*) and comparisons of means were made with least significant difference test at the 5 % level of probability.

Growth of encapsulated explants (*i.e.* both n- and s-synseeds; Fig. 1*A*) resumed within 5 - 8 d after taking out from either of the storage conditions and it required no hormonal treatment. Elongation of shoot meristems mostly preceded root initiation during conversion and formation of both roots and shoots from converting synseeds mimicked germination of zygotic embryos (Fig. 1*B*). High rate of conversion (94 - 100 %) was noted after second and fourth weeks of storage. However, the synseeds incubated at 12 °C and 20 °C could not be stored beyond 4 weeks due to random shoot formation even while in storage. Similar to this phenomenon, the limited storage potential of encapsulated shoot tips was observed earlier in cassava (Danso and Ford-Lloyd 2003) and it was attributed to the rapid resumption of metabolic activities in encapsulated explants. On the other hand, synseeds at 4 °C could be stored to eight weeks with high c.f. (82.6 - 85.4 %) and hence this temperature was chosen as optimum for further experiments. Low temperature above freezing (around 4 °C) was described suitable for storage and subsequent high conversion of synthetic seeds of *Ananas comosus* (Gangopadhyay *et al*. 2005) and *Morus indica* (Kavyashree *et al*. 2006), *etc*. Hence, for extending storage period of synseeds more than 8 weeks, sucrose concentration of the gel matrix was reduced from 3 to 0, 0.5, 1 or 2 %.

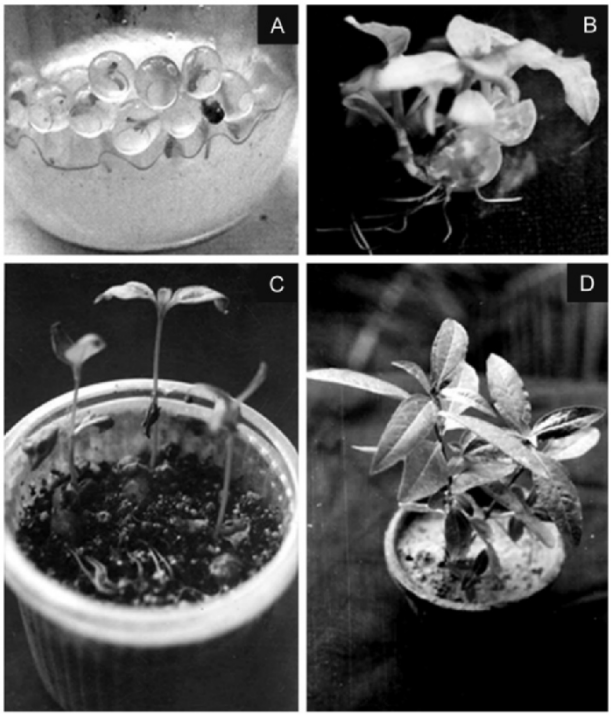

Fig. 1. *Eclipta alba* synseeds at different developmental stages: *A* - synseeds under storage, *B* - sprouting of synseeds with simultaneous rooting, *C* - transplantation of plantlets into *Soilrite* in plastic pots, *D* - full grown plant in earthen pot.

Synseeds with 1 or 2 % sucrose could be stored for 18 weeks under 4 °C, however high c.f. (71 - 76 %) was noted only up to 12 weeks of storage (Table 1). Storage extended to 18 weeks caused delayed conversion, loss of viability of majority of explants, and subsequent drastic fall in c.f.. Whereas, the synseeds with 0 and 0.5 % sucrose remained green (viable) in storage to 8 weeks, while only a small fraction converted after 4^{th} and 8^{th} weeks of storage, respectively. After 8 weeks, the

Table 1. Effect of sucrose concentration in the alginate matrix on conversion frequencies [%] of *E. alba* s-synseeds and n-synseeds stored for 4 to 18 weeks at 4 °C. Means ± SE, * - non-viable synseeds, ** - random conversion during storage.

Storage [week]	0 %		0.5 %		1 %		2 %		3 %	
	s-synseeds	n-synseeds	s-synseeds	n-synseeds	s-synseeds	n-synseeds	s-synseeds	n-synseeds	s-synseeds	n-synseeds
4	34.8±0.70	33.2±0.34	45.4±1.04	43.9±0.87	92.9±1.67	90.4±2.54	93.5±2.02	90.6±2.66	94.3±1.15	93.6±1.15
8	16.9±0.09	13.3±0.67	25.5±0.5	23.8±0.67	83.2±1.85	81.1±1.73	84.1±2.37	82.6±1.51	85.4±5.00	82.6±4.00
12	-*	-*	-*	-*	76.1±1.73	71.2±2.42	75.7±1.56	74.6±2.66	-**	-**
15	-	-	-	-	44.3±1.33	43.1±1.79	41.7±1.15	39.7±1.56	-	-
18	-	-	-	-	13.9±1.09	9.0±0.29	16.9±1.09	13.4±0.81	-	-

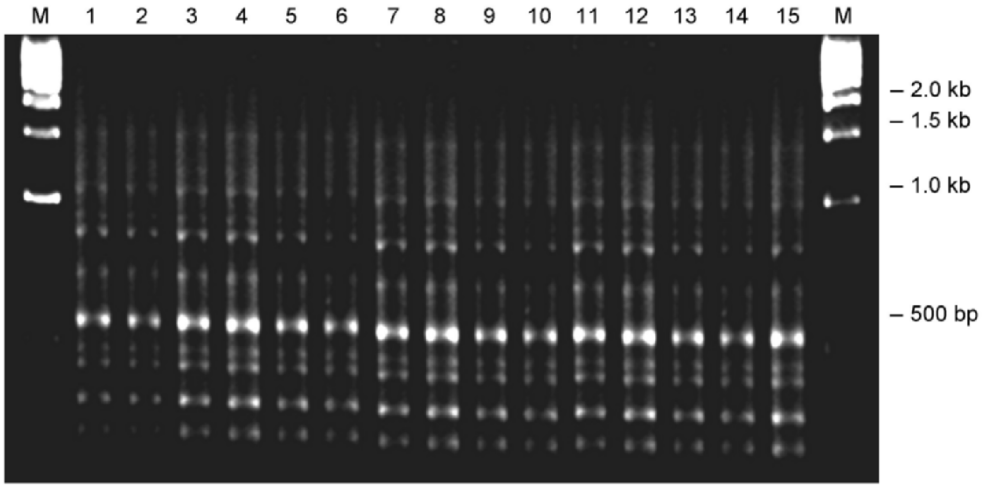

Fig. 2. DNA amplification obtained with primers OPC-9. *Lane* 1 - donor plant, *lanes* 2 to 15 - synseed derived plants and *lane* M - 500 bp DNA ladder.

synseeds gradually turned white and became non-viable. It suggested that sucrose concentration less than 1 % might not be suitable. Similarly, Tsvetkov *et al*. (2006) revealed the effect of alginate matrix composition on regrowth of encapsulated apical microcuttings of aspen. They showed that sucrose has pronounced effect on regrowth and synseeds without sucrose displayed significantly worse regrowth. Since sucrose provides nutrients to the growing tissue, absence of it or even reduction in concentration restricted the conversion, leading to non-viability at later stage. Role of sucrose in synseed conversion was also described by Adriani *et al*. (2003) who demonstrated that sucrose availability can be a limiting factor in conversion of *Actinidia* synseeds. The decline in the conversion as a consequence of prolonged storage was earlier reported in *Phyllanthus amarus* (Singh *et al*. 2006) and this may be due to inhibited respiration of plant tissues encapsulated by alginate matrix (Kavyashree *et al*. 2006) that resulted in delayed conversion and loss of viability. The present experiment explicitly indicated that sucrose concentration lower than 3 %, *i.e.* 1 or 2 % had an impact in enhancing storage potential of synseeds upto a certain period beyond which synseeds lost their viability.

Emerging plantlets were kept at standard culture condition for 15 - 20 d for further development of shoots and roots. Production of roots at the time of conversion avoided exogenous root induction treatment and enabled direct transfer of plantlets into the hardening medium. The well developed plantlets were able to resist the initial shock of transplantation to the sterilised *Soilrite* (when the empty beads were still attached with it; Fig 1*C*), *Soilrite* + loamy soil and finally to loamy soil. After 15 - 20 d of transplantation almost 100 % *ex vitro* survival was noted. This behaviour more or less resembled the direct planting of sugarcane artificial seeds in a greenhouse in controlled condition (Nieves *et al*. 2003). After one month, the plants became vigour showing normal growth (Fig. 1*D*) and looked morphologically similar with donor. Synthetic seed production by encapsulating somatic embryos has been highly favoured since somatic embryos possessing both shoot and root primordia are usually able to form complete plants without any pretreatment. Meristematic tissues encapsulated in our experiments provided the similar sort of advantage where synseeds behaved almost like zygotic seeds giving rise to both roots and shoots directly.

In RAPD analysis, nine primers out of thirty produced consistently reproducible bands with a total of 48 bands

and on average 5.6 bands per primer. The molecular mass of the bands ranged from 300 bp to 2.7 kb. A representative figure shows RAPD assay with primer OPC9 revealing bands of 320 bp to 1.48 kb (Fig. 2). The primers produced monomorphic band pattern across all 14 randomly selected synseed-derived plants and the donor.

Plant tissue culture condition represents a physiological stress that is characterized by disruption of normal developmental controls (Cassells and Curry 2001). It can ultimately lead to various kinds of aberrations at the nucleotide sequence level. However, it is well documented that direct regeneration from organized tissues like meristems, rather than callus lowers the chances of genetic integrity loss. Moreover, callus culture can generate abnormal embryos as reported by Wang *et al*. (2010). However, *in vitro* storage of explants perhaps did not affect the genetic integrity at this stage, however, it needs further confirmation. Very few scientists assessed genetic stability of *in vitro* conserved plantlets or synseed derived plantlets in particular. Hao and Deng (2004) reported analysis of genetically stable apple plants through amplified fragment length polymorphism (AFLP) and methylation sensitive amplified polymorphism (MSAP). More relevant to our study is the encapsulation of *Ananas comosus* by Gangopadhyay *et al*. (2005), where they raised uniform population of pineapple plants confirming their genetic similarity by both RAPD and inter simple sequence repeats (ISSR).

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