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Plant regeneration from alginate-encapsulated somatic embryos of *Dalbergia sissoo* Roxb.

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A method has been developed for plant regeneration by encapsulation of somatic embryos obtained from callus cultures derived from semi-mature cotyledon explants of *Dalbergia sissoo* Roxb. (family Fabaceae). Embryogenic callus was developed from cotyledon pieces on Murashige and Skoog (1962) medium supplemented with 9.04 μ*M* 2,4-dichlorophenoxyacetic acid and 0.46 μ*M* kinetin. The somatic embryos were induced from embryogenic callus on hormone free ½-MS medium with 2% sucrose. Cotyledonary stage somatic embryos were encapsulated using sodium alginate (2.5%) and calcium chloride (75 m*M*) as gelling matrix. The highest frequency (43.3%) for conversion of encapsulated somatic embryos into plantlets was achieved on ½-MS medium with 2% sucrose. Plantlets with well developed shoots and roots were established in pots containing autoclaved mixture of peat moss and soil. Under *ex vitro* conditions, the conversion of encapsulated somatic embryos into plantlets was also achieved when these were directly sown in autoclaved peat moss moistened with ½-MS0 medium.

Keywords: *Dalbergia sissoo*, encapsulation, legume tree, somatic embryogenesis, synthetic seeds

Introduction

Dalbergia sissoo Roxb., commonly known as sissoo, is a deciduous multipurpose tree found in the Himalayas and adjacent valleys of North India, Pakistan and Nepal[1]. It is one of the most valued timber-yielding trees of Indian subcontinent and used for house construction, ornamental wood work, fuel and charcoal, but disappearing rapidly due to its indiscriminate removal. The main difficulties in the propagation of *D. sissoo* are poor seed germination, death of young seedlings, and susceptiblity to wilt disease[1].

One of the alternative methods adopted in recent years is to use biotechnological approach like synthetic seed technology for improving the propagation of tree species. Synthetic seeds, consisting of tissue culture derived somatic embryos encapsulated in a protective coating, have many advantages such as ease of handling, high scale-up production and low cost of production[2]. Encapsulation may provide physical protection to the somatic embryos, carry nutrients, growth regulators, antibiotics, fungicides required during germination and guard against pathogens. Synthetic seed technology provides the only means realistically amenable to the extensive scale-up required for the commercial production of elite clones[3]. As the technique for somatic embryogenesis has improved, the possibility of encapsulation of somatic embryos for synthetic seeds production has been explored by several workers. Somatic embryos have been successfully encapsulated for preparation of synthetic seeds in several plant species such as sandalwood[4], *Citrus reticulata*[5], *Theobroma cacao*[6], *Hopea parviflora*[7], *Paulownia elongata*[8], *Nothofagus alpina*[9], and *Pinus radiata*[10]. Vegetative propagules have also been used for encapsulation in calcium alginate beads in some plants species[11-16]. Although synthetic seed technology seems promising for propagation of a number of plant species, but practical implementation of this technology is constrained due to the following reasons: (i) asynchronous development of somatic embryos, (ii) improper maturation of somatic embryos that limit the germination and conversion into normal plants, and (iii) lack of dormancy and stress tolerance

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in somatic embryos that results in decrease in storage efficiency of synthetic seeds.

Singh and Chand[17] reported efficient plant regeneration from somatic embryos in *D. sissoo*. Somatic embryos developed from cotyledon explants of *D. sissoo* germinate and convert into normal plant rapidly more or less similar to true seeds. There is no report on utilizing somatic embryos to produce synthetic seeds in *D. sissoo*. The development of synthetic seeds from somatic embryos could offer a tool for mass propagation of *D. sissoo.* In the present study, investigations were made to retain the viability of encapsulated somatic embryos after storage and to convert them into plantlets.

Materials and Methods

Plant Material

Green pods of *D. sissoo* (40-50 d after anthesis) were collected from trees growing in the campus of Devi Ahilya University, Khandwa Road, Indore, India. The pods were washed under running tap water for 30 min and then treated with tween-20 solution (10 drops/100 mL distilled water, v/v) for 15 min, and rinsed with distilled water, followed by savlon antiseptic solution (Johnson & Johnson, USA; active ingredients-chlorhexidinde gluconate and cetrimide) treatment (1.0 mL/100 mL distilled water, v/v for 15 min, and washed 3-4 times with distilled water. Further sterilization treatments were done under a laminar-flow chamber. The pods were treated with 70% ethanol for 1 min and washed with sterile distilled water and then surface sterilized with 0.1% (w/v) freshly prepared aqueous mercuric chloride for 20 min followed by 4-5 times washing with sterile distilled water. After surface sterilization, seeds were isolated from the pods and cotyledons without embryogenic axis were excised and used as explants. Cotyledon pieces (5×5 mm) were aseptically cultured on Murashige and Skoog[18] medium supplemented with 9.04 μ*M* 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.46 μ*M* kinetin (Kn) for callus induction. For induction of somatic embryos, callus clumps (250 ±25 mg) were cultured on ½-MS medium with 2% sucrose without any plant growth regulators. The *p*H of the medium was adjusted to 5.76± 0.02 with 0.1 N NaOH or 0.1 N HCl solution prior to adding 0.8% (w/v) agar-agar. The medium was steam-sterilized at 121°C at 15 psi for 15 min. The cultures were maintained under a 16/8 h light/dark photoperiod, at 25±2°C. A light intensity of 50 μmol $m^{-2}s^{-1}$ was provided by cool-white, fluorescent tubes (Philips, India). Cultures with developing somatic embryos were transferred to a fresh medium at intervals of 4 wks for multiplication and maturation of somatic embryos.

Encapsulation of Somatic Embryos

For proper maturation, cotyledonary stage somatic embryos were transferred to ½-MS0 medium [½-MS0 - half-strength Murashige and Skoog (1962) medium without plant growth regulator] with 10% sucrose for 2 wks prior to encapsulation. Cotyledonary stage somatic embryos were immersed in 1.5, 2.5, 3.0, 3.5, 4.0 and 5% sodium alginate solution prepared in ½-MS0 medium with 2% sucrose and *p*H was adjusted at 5.76±0.02. Somatic embryos, mixed well in sodium alginate solution, were dropped individually with a pipette (inside diameter 8 mm) into 25, 50, 75, and 100 m*M* calcium chloride solution prepared in ½ -MS0 medium with 2% sucrose and left for 20 min. Later, calcium chloride solution was decanted and the encapsulated somatic embryos were washed 4 times with sterile distilled water.

Conversion of Encapsulated Somatic Embryos into Plantlets

In one experiment, non-encapsulated and encapsulated somatic embryos were cultured on ½-MS0 medium with 2% sucrose, MS0 medium [MS0-Murashige and Skoog (1962) medium without plant growth regulator], ½-MS medium supplemented with 0.26 μ*M* ∝-naphthaleneacetic acid and 2.22 μ*M* 6-benzylaminopurine or water agar for conversion into plantlets for 3 wks while in other experiment, encapsulated and non-encapsulated somatic embryos were stored in sterile Petri dishes at 4°C for 15, 30 and 45 d before transfer to the conversion media. Non-stored and stored encapsulated somatic embryos were also planted on peat moss moistened with ½-MS0 medium enriched with 2% sucrose or tap water, peat moss and soil (1:1) moistened with ½-MS0 medium with 2% sucrose or tap water, soil moistened with ½-MS0 medium with 2% sucrose or tap water for *ex vitro* conversion into plantlets.

Transfer of Plantlets to Pots

Plantlets were removed from the culture medium and transferred to ½-and ¼-liquid MS0 medium for 10 d and then to plastic pots containing autoclaved peat moss and soil mixture (1:1). Pots were covered with transparent polythene bags poked with small pores for 7 d to maintain high humidity and kept in the culture room at 25±2°C and 50 μmol $m^{-2}s^{-1}$ light intensity provided by white fluorescent tubes.

After 3 wks, the plantlets were transferred to larger pots containing peat moss, soil and compost (1:1:1) mixture and kept outdoor under full sun.

Statistical Analysis

Percentage response for conversion of non-encapsulated and encapsulated (stored and non-stored) somatic embryos was recorded after 4 wks of culture. Conversion frequency was calculated on the basis of differentiation into roots and shoots. Each treatment consisted of 20 replicates and experiment was repeated three times. Standard error of the mean value was calculated. Least significant difference (LSD) at $P<0.05$ level was applied to determine the significant difference among the mean values.

Results

Embryogenic Callus Formation and Development of Somatic Embryos

Embryogenic callus was induced after 25 d of culture from cotyledon pieces on MS medium supplemented with 9.04 µ*M* 2,4-D and 0.46 µ*M* Kn. Embryogenic callus clumps were transferred to the same medium for one more passage in order to achieve multiplication. Somatic embryogenesis was achieved from embryogenic callus clumps (250±25 mg) on 1/2-MS0 medium after 6 wks of culture. Somatic embryos were multiplied and matured on the same somatic embryos induction medium in subsequent subcultures. Somatic embryos enlarged into distinct bipolar structures and passed through each of the typical developmental stages: globular, heart, torpedo and cotyledonary stage. A large number of somatic embryos, mostly cotyledonary stage, developed on the surface of embryogenic callus clumps on ½-MS0 medium. Transfer of somatic embryos to ½-MS0 medium with 10% sucrose for 2 wks prior to encapsulation, significantly enhanced the conversion of encapsulated somatic embryos into plantlets.

Influence of Sodium Alginate and Calcium chloride on Beads Formation

Cotyledonary stage somatic embryos were picked up from the callus cultures derived from semi-mature cotyledons (Fig. 1a) and encapsulated to get synthetic seeds (Fig. 1b). A 2.5% sodium alginate and 75 m*M* calcium chloride was found most suitable for encapsulation and conversion of somatic embryos into complete plantlets (Fig. 2). Beads formed using lower concentrations of sodium alginate (1-1.5%) and calcium chloride (25-50 m*M*) were too fragile to handle. At higher concentration of sodium alginate (4-5%), beads were isodiametric and hard to cause considerable delay in conversion into plantlets

Conversion of Encapsulated and Non-encapsulated Somatic Embryos into Plantlets

Non-encapsulated and encapsulated somatic embryos were converted into plantlets on ½-MS0 medium with 2% sucrose, ½-MS medium supplemented with 0.26 µ*M* NAA and 2.22 µ*M* BAP, MS0 medium without any growth regulators and water agar. Maximum percentage response for conversion of non-encapsulated and encapsulated somatic embryos was 72.3 and 43.3%, respectively on ½-MS medium with 2% sucrose, after 20 d of culture (Fig. 3). Percentage response for conversion into plantlets decreased gradually after storage of

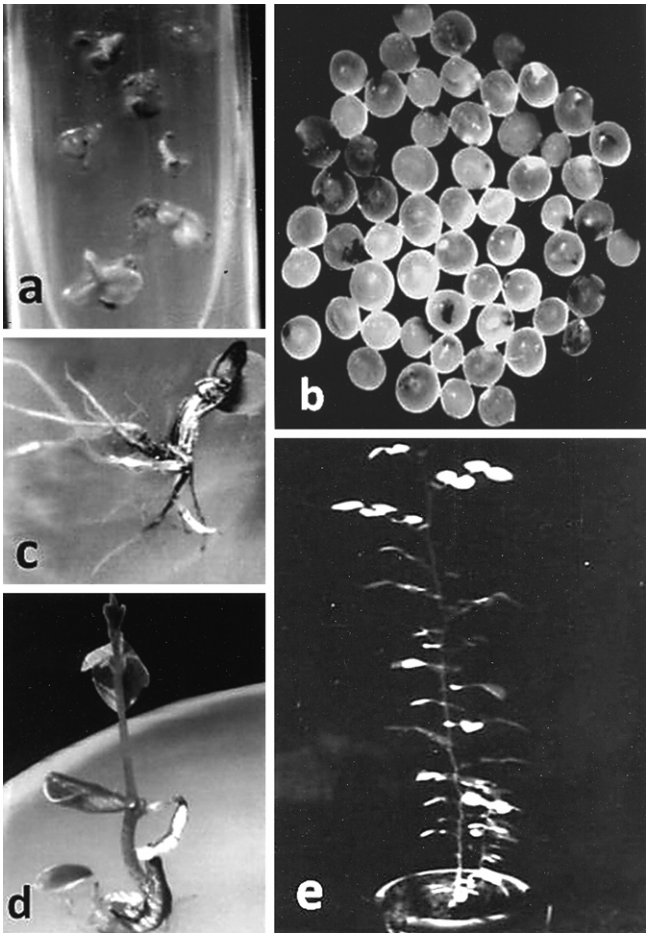

Fig. 1—Plant regeneration from alginate-encapsulated somatic embryos of *D. sissoo* Roxb.: a. Isolated somatic embryos used for encapsulation in calcium alginate; b. Calcium alginate beads formed by the encapsulation of somatic embryos using 2.5% sodium alginate and 75 m*M* $CaCl_2.2H_2O$; c & d. Conversion of encapsulated somatic embryos into plantlet on ½-MS0 medium with 2% sucrose; & e. Transfer of plantlets to pots.

encapsulated as well as non-encapsulated somatic embryos at 4°C. Upon storage at 15, 30, and 45 d duration, conversion of encapsulated somatic embryos were 29.4, 21.4 and 16.1% on ½-MS medium with 2% sucrose, respectively (Fig. 4). Non-encapsulated somatic embryos upon storage at 30 and 45 d duration at 4°C failed to germinate. Addition of ½-MS nutrients with 2% sucrose in gel matrix was found essential for conversion of stored and non-stored encapsulated somatic embryos into plantlets. Among various media tested, ½-MS medium with 2% sucrose was found most effective for conversion of stored and non-stored encapsulated somatic embryos into plantlets (Fig. 4). Germination of encapsulated somatic embryos occurred after 15-20 d by appearance of shoots and roots piercing out of the beads (Figs 1c & d). Plants with well developed shoot and roots were transferred to pots (Fig. 1e). About 88% plants were established in pots.

Non-stored and stored encapsulated somatic embryos were also germinated under *ex vitro* conditions on peat moss moistened with ½-MS0 medium or tap water and peat moss and soil mixture (1:1) moistened with ½-MS0 or tap water, soil moistened with ½-MS0 medium or tap water. Among the various planting substrates tested for conversion of encapsulated somatic embryos into plantlets under *ex vitro* conditions, peat moss moistened with ½-MS0 medium proved to be better and about 22.2% non-stored encapsulated somatic embryos and 14.2% stored encapsulated somatic embryos (30 d of storage at 4°C) were converted into plantlets (Fig. 5).

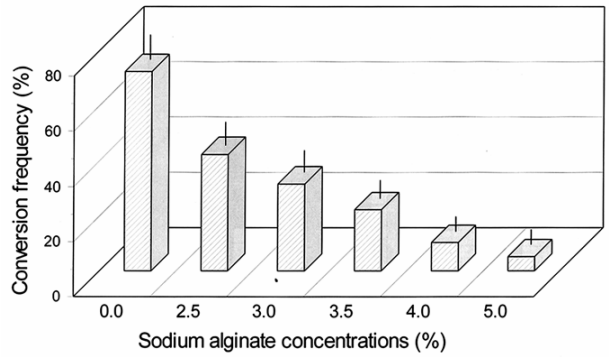

Fig. 2—Effect of different concentrations (2.5-5%) of sodium alginate on conversion of encapsulated somatic embryos. Non-encapsulated somatic embryos were regarded as control. LSD at 5% level= 9.13. Vertical bar represents standard error of the mean.

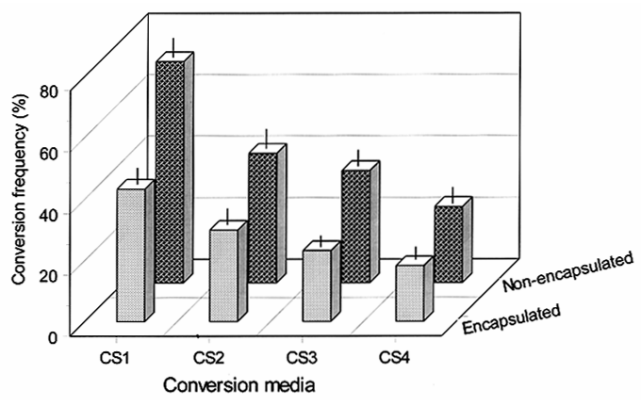

Fig. 3—Effect of different media (CS1: 1/2-MS0 + 2% sucrose, CS2: MS0, CS3: DW, CS4: ½-MS + 0.26 µ*M* NAA + 2.22 µ*M* BAP) on conversion of encapsulated and non-encapsulated somatic embryos into plantlets. LSD at 5% level, for conversion frequency of encapsulated and non-encapsulated somatic embryos= 14.88 and 15.61, respectively. Vertical bar represents standard error of the mean.

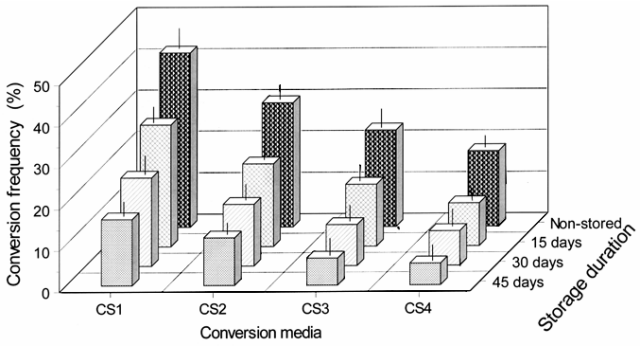

Fig. 4—Effect of different storage durations (0-45 d) on conversion of encapsulated somatic embryos on various conversion media (CS1: ½-MS0+ 2% sucrose, CS2: MS0, CS3: DW, CS4: ½-MS + 0.26 µ*M* NAA + 2.22 µ*M* BAP). LSD at 5% level, for conversion frequency of non-stored, 15, 30 and 45 d stored encapsulated somatic embryos= 15.96, 17.30, 11.04 and 9.41, respectively. Vertical bar represents standard error of the mean.

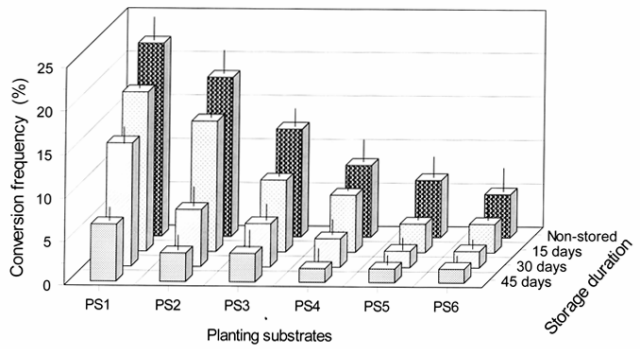

Fig. 5—Effect of different planting substrates (PS1= peat moss moistened with ½-MS0 medium, PS2= peat moss moistened with tap water, PS3= peat moss and soil mixture moistened with ½-MS0 medium, PS4= peat moss and soil mixture (1:1) moistened with tap water, PS5= soil moistened with ½-MS0 medium, PS6 = soil moistened with tap water) on conversion of non-stored and stored (15, 30, and 45 d) encapsulated somatic embryos. LSD at 5% level for conversion frequency of non-stored, 15, 30 and 45 d stored encapsulated somatic embryos= 8.64, 7.84, 5.93 and 5.13, respectively. Vertical bar represents standard error of the mean.

Discussion

Synthetic seeds, consisting of somatic embryos encapsulated in a protective coating, have been recognised as a powerful technique for mass propagation of elite plant species. Encapsulated somatic embryos can be sown under *in vitro* or *ex vitro* conditions for producing uniform clones. In view of the low cost of production and subsequent propagation, this technique is considered as an effective alternative method for propagation.

A protocol has been developed for *in vitro* plant regeneration through somatic embryogenesis in *D. sissoo* by Singh and Chand[19]. But, plant regeneration could not be achieved from non-encapsulated somatic embryos upon storage at 4°C. High quality and matured cotyledonary stage somatic embryos of *D. sissoo* could be used effectively for synthetic seed production. Synthetic seeds developed by encapsulation of somatic embryos of *D. sissoo* can be stored up to 45 d. Stored and non-stored synthetic seeds can also be germinated under *in vitro* as well as *ex vitro* conditions. Synthetic seed technology can provide effective means realistically amenable to the extensive scale-up commercial production of elite clones of *D. sissoo.*

In the present study, cotyledonary stage somatic embryos were found suitable for encapsulation in calcium alginate beads as they were excellent plant material for synthetic seed production besides possessing a great potential to germinate into plantlets. Transfer of somatic embryos on ½-MS0 medium with 10% sucrose for 2 wks prior to encapsulation significantly enhanced the conversion of encapsulated somatic embryos into normal plantlets. High sucrose concentration in the maturation medium improved somatic embryo production in black spruce (*Picea mariana*) and white spruce (*P. glauca*)[20,21]. The presence of sucrose might serve as a signal for the synthesis of storage proteins, resulting in improved quality and germinability of somatic embryos. Sucrose might act as a regulatory factor in addition to serving as a source of carbon, energy and as an osmoticum during the maturation of somatic embryos.

Present study revealed that encapsulation of somatic embryos is influenced by the concentration of sodium alginate and calcium chloride. At higher concentrations of sodium alginate (4-5%), beads became harder and inhibited germination. Castillo *et al*[22] reported that 2.5% sodium alginate was optimum for maximum germination (77.5%) of *Carica papaya* synthetic seeds. Kersulec *et al*[23] reported that respiration and germination rates decreased with an increase in sodium alginate concentration. Bapat and Rao[4] observed that embryos of sandalwood encapsulated in 2 and 4% sodium alginate germinated with less frequency. Lower concentration of calcium chloride (25 m*M*) not only prolonged the ion exchange duration but also affected quality of beads.

Our study revealed that upon storage at 4°C, germination and subsequent conversion of encapsulated somatic embryos into plantlets markedly decreased in *D. sissoo*. Bazinet *et al*[24] reported in *Daucus carota* that plant regeneration rate after storage is reduced by loss of viability caused by mechanical constraints or diffusional limitation. Storage of encapsulated somatic embryos using an alginate encapsulation has been attempted in only a few species with various degree of success[25]. Less than 5% of *Santalum album* encapsulated embryos germinated following storage at 4°C for 45 d[4] and only 8.33% of *Asparagus cooperi* somatic embryos germinated following 90 d of storage at 2°C[26]. It is thought that the decline in the germination frequency observed among encapsulated propagules stored at low temperature may be due to inhibition of respiration of plant tissues by alginate[27]. In the present investigation, it was observed that addition of ½-MS nutrients with 2% sucrose in gel matrix was essential for conversion of stored and non-stored encapsulated somatic embryos into plantlets. Castillo *et al*[22] found that the addition of ½-MS salts and 2% sucrose in the encapsulation gel matrix enhances the germination and conversion frequency in *C. papaya*.

We have observed in our experiments that non-encapsulated somatic embryos exhibited higher conversion frequency than encapsulated embryos. Non-encapsulated somatic embryos are directly in contact with the medium as opposed to encapsulated somatic embryos. Ghosh and Sen[26] have also reported that conversion frequency was higher for non-encapsulated somatic embryos than encapsulated somatic embryos in *A. cooperi*.

The aim of the present investigation was to develop synthetic seeds by encapsulating somatic embryos and to use them for mass clonal propagation and *ex vitro* conversion of encapsulated somatic embryos into plantlets in *D. sissoo*. Conversion of encapsulated somatic embryos into plantlets in peat moss and soil mixture moistened with ½-MS nutrients or tap water indicated that this method could be useful

in developing cost effective propagation system for *D. sissoo*. Preserving the viability of encapsulated somatic embryos even after 30 and 45 d of storage at 4°C offers the possibility of using this method for germplasm conservation of this important tree species. The encapsulated somatic embryos could be conventionally packed and transported to distant places while maintaining high viability.

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