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Full Length Research Paper

Synthetic seed technology for encapsulation and regrowth of *in vitro*-derived *Acacia* hyrid shoot and axillary buds

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In this study, various concentrations of sodium alginate solutions and calcium chloride solutions were tested in order to optimize the size, shape and texture of alginate synthetic seeds or beads for *Acacia* hybrid bud-sprouting. The shoot buds and axillary buds from *in vitro Acacia* hybrids, as explants were encapsulated with 2 to 5% sodium alginate (w/v) in the Murashige and Skoog (MS) free of calcium salt solution solvent and exposed to 25 to 100 mM calcium chloride solution ($CaCl_2.2H_2O$). Rounded beads were observed by the encapsulation with alginate 3% and exposed to 75 to 100 mM $CaCl_2.2H_2O$ combinations and; the encapsulation with alginate 4 to 5% and exposed to any $CaCl_2.2H_2O$ combinations. The produced synthetic seeds were then tested on the plantlets regeneration ability. The germination rate was within 73.3 to 100% in the duration of 6 to 20 days. It showed that encapsulation at any alginate concentrations and exposed to any of the $CaCl_2.2H_2O$ concentrations, gave high germination frequency. These plantlets could then be used as the source of explants for the subsequent experiments. The synthetic seeds have the possibility of being an alternative planting material meant for forestry sector in the future, especially for the highly demanded species.

Key word: *Acacia* hybrid, synthetic seeds, encapsulation, alternative planting material.

INTRODUCTION

The idea of synthetic seeds or artificial seeds was first conceived by Murashige in 1977. Initially, the development of synthetic seeds had been restricted to encapsulation of somatic embryos in a protective jelly. It had been considered that the induction of somatic embryogenesis (SE) and/or pollen embryogenesis which genetically differs from zygotic embryogenesis is the prerequisite for the preparation of synthetic seeds.

Synthetic seed refers to encapsulated explants such as shoot tips, axillary buds and somatic embryos in cryoprotectant material like hydrogel, alginate gel, ethylene glycol, dimethylsulfoxide (DMSO) and others that can be developed into a plant. The coating protects the explants from mechanical damage during handling and allows germination and conversion to occur without inducing undesirable variations (Harikrishna and Ong 2002). They behave like true seeds and sprout into seedlings under suitable conditions.

Its potential advantages include stabilities during handling, potential for long term storage without losing viability, transportation and planting directly from *in vitro* to field conditions and higher scale at a low cost production (Ghosh and Sen, 1994).

Germplasm conservation has become necessary for future sustainable harvesting systems, and as a means of maintaining species diversity to prevent genetic erosion. The propagation and conservation of this species traditionally take place by seeds. The alginate encapsulation technique and cryogenic procedures may be reliable methods for long-term storage of plant genetic resources without apparent risk of genetic instability using minimum space and with lower labour and maintenance costs.

The species, *Acacia* hybrid, is a cross between *Acacia mangium* and *Acacia auriculiformis* that are two tropical acacias natural to Australia, Papua New Guinea and

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Table 1. Formation of synthetic seeds by the encapsulation with 2 to 5% sodium alginate solution and 25 to 100 mM $CaCl_2.2H_2O$.

Alginate / $CaCl_2$ (mM)	2%	3%	4%	5%
25	Fragile	Tailed	Rounded	Rounded
50	Fragile	Rounded	Rounded	Rounded
75	Fragile	Rounded	Rounded	Rounded and hard
100	Fragile	Rounded	Rounded	Rounded and hard
Mean ± S.E	2.17 ± 0.4^b	3.33 ± 0.1^a	4.00 ± 0.0^a	4.00 ± 0.0^a

Means followed by the same letter are not significantly different at P = 0.05.

Indonesia. Both have potential for timber and pulp production. The hybrids tend to grow vigorously, have better form than *A. auriculiformis* and have lighter branching than *A. mangium* which is self-prune (Rufelds and Lapongan, 1986). It has a slightly higher wood density, is good for producing chipwood, pulp, paper, medium density fiber board, oriented-strand board, and for general construction and furniture. Seed collected from *Acacia* hybrid trees yields highly variable and poorly performing offspring and are not commonly used in regeneration programs. Propagation and conservation by vegetative means are desirable for better preservation of true-to-type genetic characteristics.

To our knowledge, there is no published report on the production of synthetic seed from encapsulated vegetative parts such as shoot buds and axillary buds of *Acacia* hybrid. Thus, we would like to report a method for encapsulation of vegetative parts to produce ideal beads for this species. Therefore, the aim of this study was to determine the optimum concentration of encapsulation matrix (sodium alginate solution and calcium chloride solution) to optimize the size, shape and texture of alginate beads for bud-sprouting. The produced synthetic seeds were then tested on the plantlets regeneration ability.

MATERIALS AND METHODS

Shoot buds and axillary buds from *in vitro Acacia* hybrids plantlets cultured on MS medium (Murashige and Skoog, 1962) were used as explants. The plant materials were excised and only small amount of expanded leaf primordial were retained when the size of the explant was 2 to 3 mm.

Preparation of encapsulation matrix

For encapsulation purposes, 2, 3, 4 and 5% sodium alginate (w/v) in the MS free of calcium salt solution solvent were tested. For complexation (an ion exchange reaction between Na^+ and Ca^+ resulting in the formation of insoluble calcium alginate), different concentrations of calcium chloride solutions ($CaCl_2$ $2H_2O$: 25, 50, 75 and 100 mM) were prepared in liquid MS medium containing the same adjuncts as in the sodium alginate solution (Mathur et al., 1989).

Formation of beads

The explants were transferred to the sodium alginate solution. Explants in the alginate solutions were pipetted using a Pasteur pipette with tip cut off individually and dropwise into the calcium solution and maintained for at least 30 min to polymerize the beads. When sodium alginate drops come in contact with calcium chloride solution, surface complexion begins and firm round beads are formed; each bead contains one explant. The beads were recovered by decanting the $CaCl_2$ $2H_2O$ and blotted dry on filter paper.

Culture medium and condition

The encapsulated explants or the beads were cultured on the basal medium which consisted of MS mineral salts and vitamins supplemented with 3% sucrose and 0.3% gelrite agar. The pH was adjusted to 5.8 before sterilization by autoclaving at 121°C for 15 min. All cultures were maintained in the culture room at 26 ± 1°C under a 16 h photoperiod with a photon flux density of about 35 $\mu molm^{-2}s^{-1}$ provided by cool white fluorescent lamps to observe the germination ability of the beads.

Statistical analysis

For the formation of beads experiments, 240 explants were used and each experiment was repeated thrice. The SAS procedure was used to perform an ANOVA to test for statistical significance. Means were separated using Student-Newman-Keuls test (P = 0.05) when *F-test* were determined to be significant.

RESULTS AND DISCUSSION

Encapsulation

With the complexing timing fixed at 30 min, the assessment of various concentrations of sodium alginate (2 to 5% w/v) and calcium chloride (25 to 100 mM) for the formation of beads procedure is presented in Table 1. Different shape and texture of beads were observed and they were classified as fragile, tailed or rounded. The size of the bead is controlled by varying the inner diameter of the Pasteur pipette.

The analysis of variance showed that there was a significant difference in the shape of the beads when

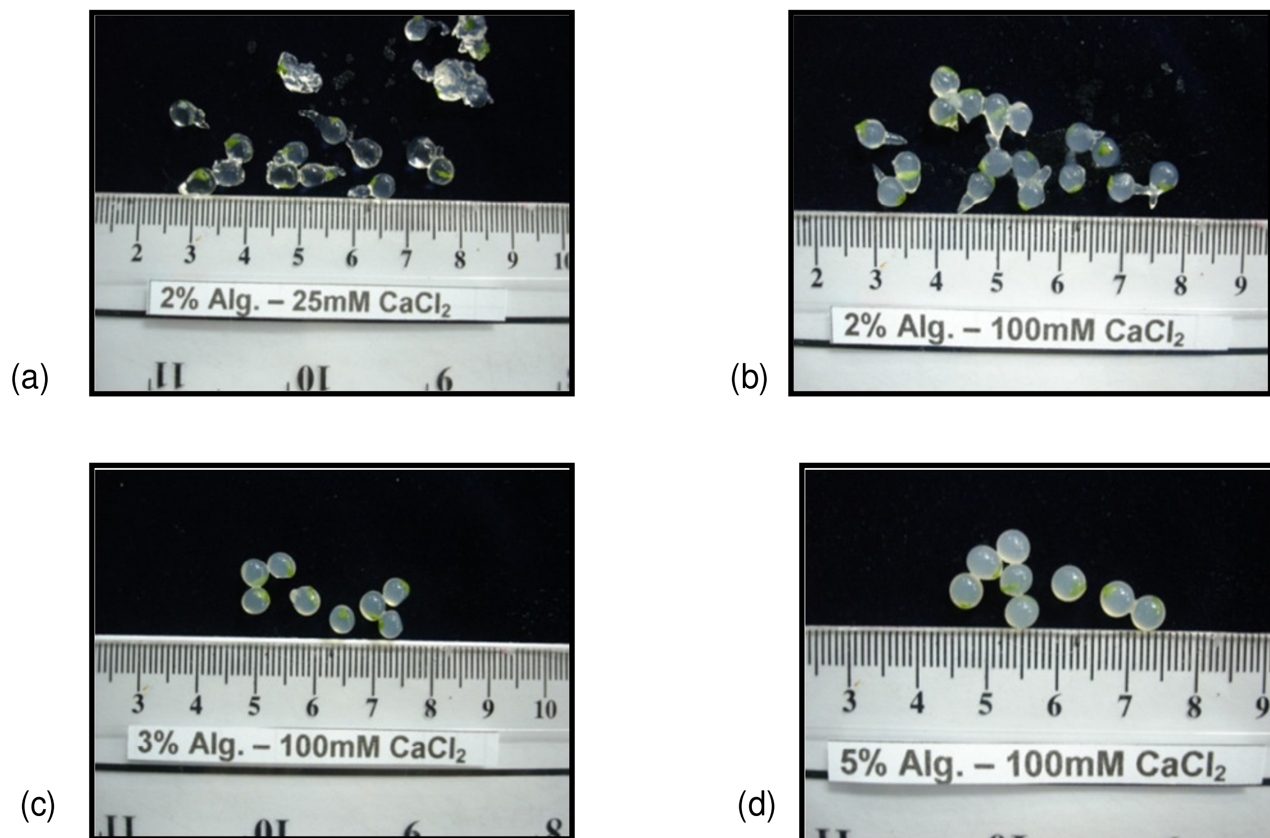

Figure 1. Synthetic seeds with (a) fragile beads; (b) tailed beads; (c) rounded beads and (d) rounded and hard beads.

using alginate 2% and 3 to 5% (Table 1). It was observed that the shoot buds and axillary buds encapsulated with 2% alginate and exposed to 25 to 50 mM $CaCl_2.2H_2O$, resulted in the formation of fragile beads (Figure 1a). The beads were not firm and strong enough to facilitate transfer with forceps to the culture medium. The encapsulation with alginate 2% and exposed to 75 to 100 mM; and the encapsulation with alginate 3% and exposed to 25 to 50 mM $CaCl_2.2H_2O$, produced tailed beads (Figure 1b), while the rest of the combinations gave round, firm, clear and uniform beads (Figure 1c) when encapsulated with alginate 3 to 5% and exposed to 75 to 100 mM $CaCl_2.2H_2O$. However, the alginate 5% with 75 to 100 mM $CaCl_2.2H_2O$ combinations produced hard texture beads (Figure 1d). The exposure of at least 30 min to complexing agent ($CaCl_2.2H_2O$) was required to achieve complete gelation (Ipekci and Gozukirmizi, 2003). However, both alginate and calcium concentration play a role in complexing time and capsule hardness.

This study showed that in encapsulation with a high concentration of sodium alginate (3 to 5%), uniform and sufficiently firm beads were formed. Sodium alginate was used as the encapsulating agent due to its solubility at room temperature and its ability to form completely permeable gel with $CaCl_2.2H_2O$ (Datta et al., 1999).

Survival and germination of the beads

In this study, germination was determined when the expanded leaves of the explants appeared and break the gel (Machii, 1992). We found that encapsulation at any alginate concentrations and exposure to any of the $CaCl_2.2H_2O$ concentrations, could give high germination frequency. The beads started to germinate after the second day of the culture medium. Generally, the germination rate for all combinations was within 73.3 and 100% in the duration of 6 to 20 days. However, the lowest germination rate observed was 40 to 46.6% due to the fungal contaminations. This study observed that the encapsulation with 3% sodium alginate polymerized in 100 mM $CaCl_2.2H_2O$ was the optimal concentration to produce synthetic seeds. Similar reports were given by Daud et al. (2008), Geetha et al. (2009) and Sarmah et al. (2010).

According to Redenbaugh et al. (1991), the capsule gel can potentially serve as reservoir for nutrients that may aid the survival and speed the growth of the explants. We also found that the beads formed at high concentration of sodium alginate (4 to 5%) were harder and Daud et al. (2008) believed that this probably suppressed the ability of shoots and roots to emerge. The concentration of the

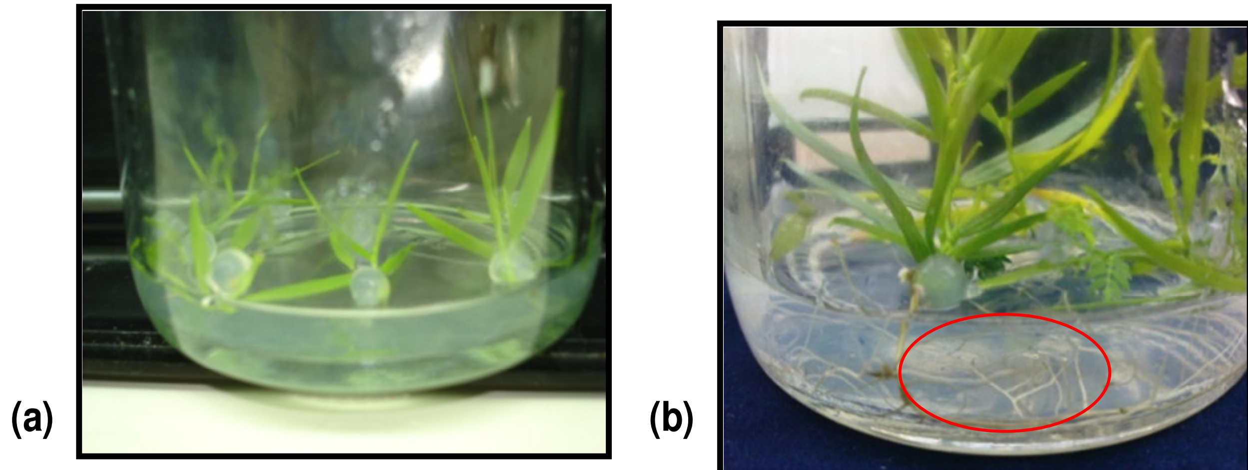

Figure 2. (a) The germinated synthetic seeds and (b) the rooted synthetic seeds.

complexing agent, $CaCl_2$, also affected the frequency of the conversion of encapsulated explants.

Plantlets regeneration

Some of the germinated encapsulated beads showed varied responses of shoot and root initiation when roots developed without being treated with rooting medium. Plantlets formation was observed once the shoots elongated and roots began to emerge (Figure 2). It took about 50 to 60 days (from the time the beads were cultured to the medium), respectively, for it to become a complete plantlet. On subculture in fresh medium, the plantlets developed well-grown shoots and roots. These plantlets could then be used as the source of explants for the subsequent experiments.

The encapsulation of shoot buds and axillary buds has resulted in scaling up the micropropagation technique for *Acacia* hybrid. With the respectively high germination rate, the encapsulation allows mass propagation as well as having a potential for automation of the whole production process. This technique has been successful for some species, example sandalwood (Bapat et al., 1988), *Valeriana wallichii* (Mathur et al., 1989), *Guazuma crinita* (Maruyama et al., 1998) and *Paulownia elongata* (Ipekci and Gozukirmizi, 2003).

Conclusion

Synthetic seeds have the potential to be used as a substitute when plants fail to produce seeds naturally, or when they produce only a small number of fertile seeds. Therefore, the synthetic seeds production idea is to prepare a simple, inexpensive delivery unit of tissue culture propagated plants and a method for direct sowing of encapsulated material in the field. They have the possibility of being alternative planting material meant for forestry sector in the future, especially for the highly demanded species.

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