BIOENCAPSULATION OF SOMATIC EMBRYOS IN WOODY PLANTS.

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1. INTRODUCTION

Application of synthetic seed technology in the field of micropropagtion, storage and transport has been well recognized in several agronomically important crops and woody species. Despite the spurt in synthetic seed research in the recent past, there is need for more studies mainly on the physiological and biochemical aspects of synthetic seeds, especially the factors affecting their germination and subsequent plant growth in the soil (Redenbaugh, 1990, 1993). Establishment of an efficient somatic embryogenesis system is a major prerequisite for a successful program on synthetic seeds. However, this has not been achieved very well in several important woody plants. Long life cycle, extended juvenility, poor and inconsistent seed yield and constraints in establishment of *in vitro* cultures are some of the major hurdles for woody plant species. It is always desirable to raise cultures from mature plants with known features and desired traits but explants from mature trees exhibit recalcitrance under aseptic conditions and therefore most studies on tree tissue culture use seedling parts. However in this, efficacy of progeny is not known.

The regeneration of plants in culture and their subsequent acclimatization and delivery to the field especially woody plants pose many problems and do not make tissue culture technology a viable proposition. The successful

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germination of encapsulated somatic embryos has initiated a new line of research on synthetic seed technology (Onishi *et al*., 1994, Rao *et al.,* 1998). The main thrust of research on synthetic seeds is on the direct sowing of encapsulated embryos under field condition. The concept of synthetic seeds or artificial seeds is based on the assumption that somatic embryos can be encapsulated and handled like a real seed for transport, storage and sowing. Alternatively, the encapsulation of *in vitro* derived propagules (buds, bulbs or any form of meristematic tissue) has ushered in a new era in synthetic seed research (Bapat *et al*., 1987, Piccioni and Standardi, 1995). The current broader definition of synthetic seeds hence is "an artificially encapsulated somatic embryo, shoot or any other mersitematic tissue which can develop into a plant under *in vitro* or *in vivo* conditions. Microbulbs, microtubers, rhizomes, corms, microcuttings, shoot apices, axillary buds, meristemoids, cell aggregates, clumps and primordia can be used as explants for the preparation of synthetic seeds. The concept of incorporating nutrients, biofertilizers, antibiotics or other essential additives to the matrix allowing easy handling, storing, shipping and planting makes the synthetic seed a unit of delivery for *in vitro* plants. The uniform and simultaneous production of somatic embryos and their germination after encapsulation could possibly minimize these disadvantages associated with natural seeds. Trees produce seeds only in certain months of the year whereas synthetic seeds can be made available throughout the year.

Success in the synthetic seed technology relies upon several major steps (Redenbaugh and Ruzin, 1989). These include establishment of efficient systems of somatic embryogenesis, synchronization of somatic embryos leading to plantlet regeneration, selection of non toxic encapsulating matrix, increased storage capability of synthetic seeds and then germination under *in vivo* conditions. These steps, however, are not easy to achieve in woody plants due to the inherent characters associated with trees.

2. PROTOCOL FOR SOMATIC EMBRYO ENCAPSULATION

2.1. Factors controlling somatic embryogenesis

Explants, generally young shoots, zygotic embryos or seedling parts are cultured in woody species after surface sterilization. Secretion of phenolic compounds detrimental to the culture is very common in majority of woody plants. Like herbaceous species, induction of embryos in woody plants is determined by a variety of factors such as age, proper developmental stage of explant, effective medium and other appropriate physical and chemical conditions (Stasolla and Yeung, 2003). Formulation of suitable medium for callus induction is genotype dependent. In several cases the callus induction medium may not support embryo induction. Hence, altered nutrient medium has to be used (Von Arnold *et al.,* 2002). Conversion of embryos to plants also may require nutrient medium changes

Among auxins, 2,4-D is widely used for the induction of callus although cytokinins in conjuction with auxins also stimulate callusing. Charcoal can also be a component in the medium mainly for reducing phenolic exudates. In an established embryogenic system, quantification of embryos is not easy due to lack of synchronous growth of the cultures. Techniques such as control of cell cycle, sieving of cultures, use of abscisic acid are available to achieve certain level of synchrony in cultures as well as useful to identify highly embryogenic cell lines among phenotypically similar plants. Several basal media, from relatively simple to more complicated have been employed but these cannot be generalized for any plant. It has been observed that embryogenic cells secrete proteins into the medium which stimulate to induce somatic embryogenesis in non embryogenic cells. Repetitive embryogeny for the continuous supply of embryos have been reported in woody plants essential for preparation of synthetic seeds continuously. Maturation of somatic embryos prior to encapsulation is essential for better germination (Stasolla and Yeung, 2003). Maturation and desiccation of somatic embryos has been carried out in woody plants for enhanced conversion of embryos to plants. Only morphologically mature embryos which have accumulated enough storage materials and have acquired desiccation tolerance develop into normal plants. The benefits of high sucrose, high nitrogen content or abscisic acid or drying of the tissue are well known. Compared to other herbaceous plants, much less work has been carried out on molecular mechanism of embryogenesis of woody plants, however, the utilization of methods such as cDNA

microarray, could lead to understanding of genes responsible for embryogenesis in woody genera. Characterization of gene expression during embryo development, maturation and germination has led to the identification of five distinct classes of developmentally regulated genes. Three sets of genes are presumed to influence the process of embryogenesis in woody species. First set expresses throughout the process, second set is involved during maturation and in late stages of development and the third set is responsible for conversion of embryos to plants. All these genes appear to express in a sequential pattern.

2.2. Encapsulation of somatic embryos

Germination of synthetic seeds depends upon the somatic embryos having functional shoot and root apices that can germinate like a zygotic embryo. For the encapsulation process, it is often necessary to encase the embryo in a matrix, which serves as a synthetic endosperm containing mineral nutrients, source of carbon, growth regulators and antimicrobial agents. The coating should be non toxic to the propagule, protect from mechanical damage during handling and allow its development and conversion to occur without any variation (Redenbaugh and Ruzin, 1989). Several agents including sodium alginate, sodium alginate with gelatin, potassium alginate, guar gum, agar, tragacanth gum, sodium pectate, carboxymethyl cellulose, carrageenan with locust bean gum, gerlite have been used for encapsulation (Redenbaugh *et al*., 1986, 1987). Amongst these, sodium alginate has been extensively used because it is less toxic and offers sufficient rigidity to the capsule allowing easy handling. In addition, polyethylene oxide homopolymers, acrylic copolymer, carboxyl methylized cold soluble swelling starch, synthetic trotachedral smectite, synthetic sodium magnesium lithium silicate, starch plus synthetic polymer of acrylamide and sodium crylate, anionic flocculant have been also used as coating agents in some studies (Janick *et al*., 1993). The calcium alginate capsule is generally wet and sticky causing problems in handling. Redenbaugh *et al*., (1987) tested various hydrophobic coatings and found Elvax polymer coating to be very effective for shoot emergence. Dupuis *et al*., (1994) used pharmaceutical type capsules as coating systems in which the capsule body was covered on its inner surface with watertight film

composed of polyvinylchloride (PVC), polyvinylacetate (PVA) and bentone as a thickener. This allowed a controlled and steady nutrient supply to the developing somatic embryos of carrot.

Somatic embryos have to be carefully isolated and blot dried on a filter paper prior to dipping for a few seconds in a mixture of sodium alginate gel (100ml) prepared in a basal medium preferably MS basal medium. The embryos are then picked up by a pair of forceps and dropped into a solution of $CaCl_2$.$2H_2O$ (1.036g / 150 ml). Each drop contains a single embryo. These are then kept in this solution for 40 to 50 minutes on a gyratory shaker (80rpm) in light (1000 lux). After the incubation period, the $CaCl_2$.$2H_2O$ solution is decanted, the beads are washed 3 to 4 times with hormone free MS medium. The alginate complexes in the presence of $CaCl_2$ forms a firm coating on the embryo. Such encapsulated embryos are then either cultured on the nutrient medium or stored at 10^oC in parafilm sealed petri dishes.

There are two types of synthetic seeds: hydrated and desiccated. Hydrated synthetic seeds consist of somatic embryos individually encapsulated in hydrogel such as calcium alginate. Use of polyoxyethylene glycol for encapsulation produces desiccated synthetic seeds. In this method, the coating mixture is allowed to dry for several hours on a Teflon surface in a sterile hood. The resultant wafers are then hydrated and placed on a medium for scoring embryo survival. Both these matrices have their own merits and demerits. Hydrated capsules are difficult to store because the embryo requires respiration. These capsules dry out unless kept in a humid environment or coated with a hydrophobic membrane. In case of dehydrated or desiccated capsules, the process of desiccation itself could cause damage to the embryo restricting survival.

2.3. Encapsulation of embryogenic cell suspension

For the encapsulation of embryogenic cell suspension, actively growing cells are selected and filtered through a nylon net (100 μ) prior to washing twice in hormone - free nutrient medium. Washed cells are then mixed with 2.5% sodium alginate in 1:1 proportion. The mixture is then pipetted and dropped into 50 ml of $CaCl_2$.$2H_2$ 0 (1.036g/150ml) prepared in

hormone free nutrient medium. Beads of 5-10mm formed by this technique are shaken in $CaCl_2$.$2H_20$ solution for 1 hour and washed twice with the culture medium. The beads are then suspended in 25ml medium (10 beads/flasks) and are shaken in continuous light (1000 lux) at 25 ± 2^0 C.

3. CASE STUDIES

3.1.Cocoa

Cacao is a major tropical crop, grown for its oil rich seeds which are the major source of cocoa solids and butter. Somatic embryogenesis has been reported from a number of tissues of cacao including zygotic embryos, nucellar tissue, young bud petals and leaves (Figueira and Janick, 1995). However, somatic embryos from zygotic embryos and nucellar tissue are mainly used for establishing cultures. Synthetic seeds, 4.5 to 5 mm were prepared from the excised embryos of mature seeds (approximately 120 days old pods) collected from ten year old cocoa trees by encapsulating the embryos in a medium containing 4% sodium alginate and complexed using75mM $CaCl_2$.$2H_20$ (Sudhakara *et al.* 2000). They were tested for their germination characteristics after a storage of 25 days at 10^oC and subsequently grown on either wet or dry cotton under aseptic conditions. Germination of synthetic seeds was 97.3% if tested immediately after encapsulation. This declined to 71% and 49% respectively, when stored in either wet or dry cotton medium for 25 days. Germination percentage of the seeds extracted from fresh pods was 90% and declined to 76% at the end of five days storage of pods at 27 ± 2^0 C. At the end of 10 days in storage, complete mortality was observed. A significant observation in this study was that the time taken for the initiation and completion of germination of root and shoot were shorter in the case of synthetic seeds compared to the normal seeds.

3.2. Camellia

Camellia is a genus of tropical or subtropical trees and shrubs whose leaves are used to prepare tea. In *Camella*, somatic embryogenesis has mainly been investigated in *C. sinensis, C. japonica and C. reticulata*. Cotyledons

are the main source of explants for establishing the cultures (Vieitez, 1995). In *C. japonica,* the establishment of somatic embryo cultures and subsequent plantlet regeneration was obtained on MS+BA(4.4µM)+IBA(0.4µM) and on MS+GA(14.4µM)+IAA(28.5µM) respectively (Janeiro *et al.,*1997).Somatic embryos of *Camellia japonica* were encapsulated using 3% sodium alginate and 0.1M Calcium chloride to produce synthetic seeds (Janeiro *et al*., 1997). Both germination and embryogenic capacity of the encapsulated embryos was investigated. The frequency of *in vitro* germination of artificial seeds was dependent on various nutrient additives to the encapsulation matrix. On a calcium free MS basal medium containing 3% sucrose, 14.4µM GA_3 and 28.5µM IAA, 63% plant recovery rate was obtained. This result was similar to that of non encapsulated embryos. This implies that encapsulation of somatic embryos did not negatively affect their embryogenic competence. The mean number of secondary embryos were significantly increased when the alginate beads were supplemented with growth regulators (4.44 µM BAP and 0.41µM IBA). Storage at 4^oC significantly reduced the survival and germination frequencies of both encapsulated and non encapsulated somatic embryos. However, the reduction was much greater for non encapsulated embryos. Plant recovery of encapsulated embryos was 40% and 30% following storage for 30 and 60 days respectively (Janeiro *et al*., 1997).

3.3. Mango

Mango (*Mangifera indica* L.) is an economically important fruit crop. Several groups working on tissue culture of mango have reported somatic embryogenesis and plantlet regeneration. Successful induction and subsequent regeneration of somatic embryos from nucellar explants of mango cv. Amrapali has been obtained (Ara *et al*., 1999). In this study, somatic embryos were induced on MS+ 2,4-D (4.5µM) + L glutamine (2.74mM) + sucrose (175mM) and plant regeneration was obtained on B5 medium. Somatic embryos were encapsulated in calcium alginate (2%) capsules prepared in liquid nutrient medium containing B5 macrosalts, MS microsalts and organics (quarter strength each). $CaCl_2$. $2H_20$ (100mM) solution was used for complexing. The encapsulated somatic embryos germinated on a medium containing B5 macrosalts(half strength), MS

microsalts (full strength), sucrose (87mM) and gibberellic acid (2.9µM). Encapsulated somatic embryos germinated at a higher rate than naked somatic embryos of the same size, on the same medium. The germination ability of encapsulated somatic embryos was increased when the medium was supplemented with full strength B5 macrosalts. Approximately 46% encapsulated embryos developed into plantlets. Incorporation of abscisic acid (0.004 or 0.02µM) did not enhance germination percentage. Instead, it delayed germination. Plants were successfully established in this study (Ara *et al*., 1999).

3.4 Sandalwood

Sandalwood is a major forest tree of India, well known for its oil and fragrant wood. Its seeds lose viability upon storage. Callus was raised from stem internodes of young shoots of a 20 year old sandalwood tree (*Santalum album* L) on MS medium containing sucrose (87.6mM) and 2,4-D (4.52 µM) . Subsequent transfer of the callus to MS basal medium containing IAA (2.85µM), BA (2.22µM) and sucrose (87.6mM) resulted in the development of a highly regenerative embryogenic callus which consisted of somatic embryos of all stages from globular to torpedo (Rao and Bapat, 1995).

Somatic embryos of late torpedo stage from several cultures were manually picked and mixed in 3% w/v sodium alginate. The mixture of somatic embryos and alginate was dropped in $CaCl_2$ $2H_20$ (1.036/150ml) solution and allowed to stand in this for 1h. After decanting off the $CaCl_2$ solution, the beads were washed with sterile water and were stored at 4^oC or cultured on nutrient medium.

The callus containing embryos of all stages was transferred to petridishes on a sterile filter paper and left to dry in the laminar air flow for 8 h at 28 ^0C in light. After this the petridishes containing the desiccated somatic embryos were sealed and kept in the dark at room temperature for various periods from 10 days to 30 days. Following this the tissue was cultured on fresh MS medium containing IAA (2.85µM) + BA (2.22 µM) + sucrose (87.6mM). Some desiccated embryos were encapsulated as mentioned

earlier in sodium alginate and their performance was compared to non-desiccated encapsulated ones. Both desiccated and non desiccated embryos showed revival of growth upon rehydration on White's medium (1954) and developed into plants. The tolerance to desiccation and regeneration of viable plantlets depended upon the pretreatment given to somatic embryos. Embryogenic callus subjected to dry state for 30 days showed revival of somatic embryogenesis upon transfer to fresh medium. Somatic embryos of sandalwood are therefore desiccation tolerant and an excellent material for preparation of synthetic seeds analogous to true botanic seeds. The viability of synthetic seeds too is observed to be better than normal sandalwood seeds (Bapat and Rao, 1988).

3.5. Eucalyptus

Eucalyptus trees are a significant source of fuel wood, timber, and raw material for the paper/pulp industry, honey, tannins and essential oils. *Eucalyptus citriodora* L. is grown extensively for its timber and essential oils (Muralidharan and Mascarenhas, 1995)..Induction of somatic embryos in *Eucalyptus* and their regeneration has been widely reported by several groups. Somatic embryogenesis from seeds has been established on MS+NAA (26.88µM) or on B5 +NAA (16.13µM). Germination of somatic embryos was obtained on B5 medium containing 2% sucrose. Studies have been done using a variety of matrices to encapsulate *Eucalyptus* embryos, storing the synthetic seeds at various different temperatures and testing their germination (Muralidharan and Mascarenhas, 1995). Sodium alginate (1 and 2 % w/v, complexed with 0.2% calcium nitrate solution), gerlite (0.2%) and agar (1 and 2% w/v) gelling at ambient temperatures were tested for encapsulation of isolated embryos. The use of sodium alginate gave the best results and formed a seed coat suitable to preserve the viability of isolated somatic embryos. Alginate (1%) formed a soft bead that eased handling of embryos with a forceps and that resulted in 30% germination of encapsulated embryos on a sterile medium, whereas all the other matrices were found to be too soft to handle.

A semi-automatic method has been adopted to encapsulate *Eucalyptus* somatic embryos. This method involves the use of a peristaltic pump to

control the rate of droplet formation of sodium alginate (1% w/v of Protanal LF, proton, Norway) and a magnetic stirrer to ensure uniform mixing of the calcium nitrate (0.2%) solution used for complexation. Individual embryos were picked with a pair of forceps and introduced automatically into the droplets, which were then allowed to fall into the stirred complexing agent. After 20min of complexation, all the beads were removed, washed with sterile water and stored in a test tube at room temperature or at 4^{o}C for varying period of time. Encapsulated embryos were then scored for germination capacity on a variety of media/substrates both under sterile as well as non-sterile conditions. Rate of germination of encapsulated embryos, stored at 25^{o}C (room temperature) declined on sterilized germination medium. After 1 day of storage, 38% of the embryos germinated and after 7 days storage only 28% germinated. No embryo germination was recorded when stored for 10 days. Embryos stored at 4^{0} C decreased their germination rate more rapidly and after 7 days none could germinate. Encapsulated embryos stored at room temperature were grown on a sterilized water agar medium, sterilized sand and non- sterilized soil. On sterilized water, he germination rate was 30% of which 98% of the plantlets survived. On sterile sand (irrigated with a solution of B_5 salts) germination was only 4%, but all the plantlets survived. On non- sterilized soil, none of the encapsulated embryos germinated, and were infested with microbial contamination (Muralidharan and Mascarenhas, 1995).

3.6. Aegle marmelos (L) CORR

Aegle marmelos is an important medicinal tree extensively planted for its fruits and roots, which are ingredients for Ayurvedic medicines. Seedling parts mainly cotyledons and hypocotyls have been used to establish cultures. Embryogenic cultures were established on MS+IAA (1.4µM) +BA (0.44µM) or on MS+ 2,4-D (1µM) +BA (0.88 µM)+glutamine (68.5µM) (Arumugam and Rao, 2000). Sodium alginate (O.5-5%) was complexed with $CaCl_2$ (2.5%) for making synthetic seeds (Arumugam and Rao, 2000). Amongst these, 3% sodium alginate was most suitable for encapsulation. The highest survival response was 70.4% and the percentage of synthetic seeds germinated was 45.5% on medium containing suitable growth regulators. At lower and higher concentrations of sodium alginate,

within 25 days of culture, 75% encapsulated embryos germinated when stored for 1-3 days at 4^0C. Storage at more days drastically inhibited the germination rate. The effect of storage of synthetic seeds on germination was statistically significant at 5% level. Various substrates were tested for germination on half strength solidified MS medium, moist cotton, and sterile soil moistened with half strength MS containing NAA (5.4μM) and BA(2.5μM).

On half strength MS medium, 60% seeds germinated within 30 days. Among the three substrates used, MS solid medium was most effective. Combinations of growth regulators (BA and NAA) were responsible for highest germination response. The germination response was poor on sterilized soil moistened with MS nutrients and on cotton moistened with growth regulators. The highest germination response (75.5%) was observed in BA (2.5μM)+ NAA(1μM) of which 23.4% germinated and 16.2% plantlets survived. The encapsulated embryos germinated within 15 days of culture. The alginate matrix ruptured, green leaves emerged and roots developed from encapsulated embryos. The plantlets obtained from encapsulated embryos were observed to be normal (Arumugam and Rao, 2000).

4. CONCLUSIONS AND FUTURE PROSPECT

Synthetic seed technology offers many useful advantages on a commercial scale for propagation of variety of crops. Key factors determining the success of somatic embryogenesis and synthetic seeds production, control the practical application of synthetic seed technology especially in trees. Induction of high quality somatic embryos followed by corresponding conversion of somatic embryos to plants is currently a major problem in woody species. The process of somatic embryogenesis and synthetic seeds is interlinked and depends on each other. The lack of synchrony of somatic embryos is the single most important hurdle to be overcome before advances leading to widespread commercialization of synthetic seeds can occur (Saiprasad, 2001). Non availability of well standardized protocol in several tree species and problems in developing an easy encapsulation procedure for making synthetic seeds are the other major hurdles in woody

plants. Germination of encapsulated embryos, contamination free of synthetic seeds and mechanical damage to seeds play a significant role once the embryos are encapsulated. Correct formulation of the medium in the coating complex helps to enhance the germination frequency of encapsulated embryos and requires elaborate studies. Automation of the technique depends upon all these factors and also upon imparting shelf life that is as long enough as a normal botanic seed and inherent tolerance against drying after sowing (Onishi *et al*., 1994). Use of encapsulation of somatic embryos in woody plants is limited because of lack of basic research and necessity for years of field testing to ensure clonal fidelity (Zimmermann, 1985). Finally, the issue of cost benefits needs to be addressed in each plant. Every plant has its own requirements and problems and therefore each has to be judged case wise. Nevertheless, the encapsulation technique using somatic embryos as propagules remains an attractive proposition, especially for tree species, which are known for loss of seed viability. The technique finds use for germplasm conservation of elite and unique species as well as products of wild hybridization and to be extinct species of tree crops.

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