ARTIFICIAL SEEDS

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1. Introduction

The idea that basic plant materials can be combined with a nutrient filled environment, preserved and subsequently be capable of revitalizing into a viable living plant connotes endless possibilities for a world where arable agricultural land is diminishing at an alarming rate annually. The case for artificial seeds is an attractive one. Better and clonal plants could be propagated similar to seeds; preservation of rare plant species extending biodiversity could be realised; and more consistent and synchronised harvesting of important agricultural crops would become a reality, among many other possibilities. The concept started inconspicuously, but slowly took hold until today the term artificial seed has matured to acceptance. This is the basis for this chapter, to examine some of the plant materials and the matrix materials used for the making of artificial seeds. No intention is made to provide a comprehensive review of the literature, but rather to briefly highlight some examples and discuss some of the issues related to the use of these materials.

2. Definitions of artificial seed

An artificial seed is often defined as a novel analogue to botanic seed consisting of a somatic embryo surrounded by an artificial coat [1]. This definition is primary based on the similarity of somatic embryo with zygotic embryo in morphology, physiology and biochemistry [2]. Redenbaugh et al. [1] pointed out that the developmental stage of even the most highly matured somatic embryo may at best be equal to that of a zygotic embryo that has reached the stage where it is capable of precocious germination. Such zygotic embryos are not fully developed and lack the vigour of mature embryos [1] as is the case in most artificial seeds containing somatic embryos encapsulated in an artificial seed coat. This implies that the somatic embryo encapsulated in an artificial seed coat is at most equivalent to an immature zygotic embryo, possibly at post-heart stage or early cotyledonary stage. In many plant species such as *Capsella bursa-pastoris* [3] an endosperm is still present in an immature seed-containing embryo of this stage; the endosperm tissue being reduced when the zygotic embryo reaches full maturity. Hence, if an artificial seed is to be defined as an analogue to botanic seed consisting of somatic

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embryo, the somatic embryo should be surrounded by not only an artificial seed coat but also an artificial endosperm.

Kamada [4] provided an alternative definition to an artificial seed. He defined an artificial seed as "comprises a capsule prepared by coating a cultured matter, a tissue piece or an organ which can grow into a plant body and nutrients with an artificial film". His artificial seed concept comprised of (a) "an external film for strengthening the seed" which possibly implies the seed coat, (b) "an internal film for encapsulating nutrients required for growth of the cultured matter and plant hormones for controlling germination", a layer that possibly simulates the endosperm tissue and (c) "a callus, and adventitious bud or an adventitious embryo which can grow into a plant body". This definition liberates the use of the term "artificial seed" from the requirement of a somatic embryo inside an encapsulated matrix to encompass any cultured materials that can develop into plant bodies whether directly or after further treatment.

Regardless of the definition interpretations, the two main components of an artificial seed remains, namely (1) a plant material that can grow into a plant body and (2) one or more matrix materials (typically polymers) used for the encapsulation of the plant material.

3. Plant materials used for encapsulation

3.1. SOMATIC EMBRYOS AND MICROSPORES-DERIVED EMBRYOS

Somatic embryogenesis is the process whereby either haploid or diploid cells develop into plants through characteristic embryological stages without fusion of the gametes [5]. The process may be direct, with embryogenic cells developing directly from explanted cells or indirect with an intermediate callus phase [6]. Somatic embryos can be produced clonally in large numbers and make them attractive materials for encapsulation. Artificial seeds, consisting of somatic embryos enclosed in a protective coating, have been proposed as a low cost, high volume propagation system [2,7]. The potential and usage of somatic embryos for the making of artificial seeds had been reviewed [1,8,9].

Redenbaugh [7] pointed out that one of the main problems in the use of somatic embryos for the making of artificial seeds is the regeneration of plants from somatic embryos. Although somatic embryogenesis has been achieved in many plant species, in most cases, only a few plants were produced. Unlike the zygotic embryos, many of these somatic embryos required further treatment before complete regeneration. For example, somatic embryos of *Elaeis quineensis* (oil palm) were required to be placed on different culture media for shoot and root regeneration [7]. Similarly, the secondary embryos of *Brassica napus* required transfer to a medium enriched with cytokinin for shoot regeneration and a plant growth regulator-free medium for root formation [10]. Such multiple steps requirement for "embryo conversion" remains one of the hurdles in the use of somatic embryos for field planting of artificial seeds. In addition, an ideal artificial seed made from somatic embryo of non-recalcitrant species should be able to be stored at room temperature under non-aseptic condition without precocious germination, like a natural seed. The somatic embryo, encapsulated in a matrix, should

remain dormant, until water is provided for simultaneous shoot and root formation under non-aseptic conditions. This would require further studies in the regulation of growth and development of the somatic embryos and the encapsulation procedure.

Similarly, direct somatic embryogenesis from microspores, either through anther cultures or pollen culture techniques, has been described for many plant species. As microspores are gametophytes, the embryos that derive from microspores are normally haploids. Plants regenerated through androgenesis are therefore mostly haploids and are useful materials in plant breeding programmes. One example for the encapsulation of microspores derived embryos is that of barley [11]. The embryos were encapsulated in calcium alginate beads. For germination, the artificial seeds were placed on a tissue culture medium, presumably under aseptic conditions. One interesting finding was that the percentages of germination in encapsulated embryos were found to be higher than the non-encapsulated controls. The advantage of encapsulation for germination was even more evident when the embryos were stored at low temperature in the dark for 6 months; about 38% of the encapsulated embryos germinated compared to no germination for non-encapsulated embryos. Direct encapsulation of microspore-derived embryos and regeneration will provide a short cut for the efficient production of haploid plants. However, some of the hurdles encountered in the use of microspore-derived embryos would be similar to those of the somatic embryos.

3.2. SHOOT BUDS AND SHOOT-TIPS

Apical and axial shoot buds were used for encapsulation of several plant species ranging from mustard [12], medicinal plant [13] to tree species [14]. Mathur *et al.* [13] encapsulated the apical and axial shoot buds of *Valeriana wallichii* in sodium alginate dissolved in MS medium and calculated that only 200 ml MS medium was required for 350 explants. On the other hand, nearly 4.5 L of MS medium is required for producing the same number of plants. Further, an Erlenmeyer flask of 250 ml capacity can accommodate only 5 non-encapsulated explants whereas the same space was sufficient for placing 30 beads. Hence, encapsulation of apical and axial shoot buds offers easy transportation of a large number of propagules in low bulk. Both apical and axial shoot buds are easy to regenerate into plants provided there is sufficient nutrient and rooting is not a problem. Again, how to desiccate or temporary suspend the growth of apical and axial buds until an appropriate time in an encapsulation matrix needs further investigation.

3.3. SEEDS

Seeds, with viable zygotic embryos are rarely used as plant material for encapsulation. Khor *et al.* [15] and Tan *et al.* [16] encapsulated orchid seeds in an attempt to make artificial seeds of orchids. The rationales for making artificial orchid seeds by encapsulating the zygotic seeds were based on some of the unique features of orchid seeds. Orchid seeds are minute and dust-like, ranging about 0.25-1.20 mm in length and 0.09-0.27 mm in width [17]. Each seed contains an embryo composed of 80-100 cells only [18]. Hence the seeds cannot be handled and propagated like other plant species [16]. The seeds are non-endospermic and the embryo is enclosed with only a membrane-like tissue. Orchid embryos are undifferentiated, and the embryos of most orchid species

do not have cotyledons [17]. The minuteness and lack of storage tissues make encapsulation of orchid seeds justifiable. Using a free-flowering tropical orchid species *Spathoglottis plicata* as a model plant, Khor *et al.* [15] and Tan *et al.* [16] developed a method to encapsulate the seeds with two coatings, one simulates the endosperm and the other simulate the seed coat (Figure 1). The method, using coacervation of alginate-chitosan, gave firm, round beads of about 4 mm in diameter, a size comparable to the seeds of many species such as Brassicas. The enlargement of "seed" size theoretically would enable the handling of seeds in an easier manner.

3.4. ORCHID PROTOCORMS AND PROTOCORM-LIKE BODIES

Singh [19] germinated seeds of *Spathoglottis plicata*, an orchid, into protocorms and then encapsulated the protocorms by using a single layer of calcium alginate. The encapsulated protocorms regenerated into complete plantlets in Vacin and Went medium [20]. The germination frequency was found to decrease after long-term storage at 4^{o}C. On the other hand, Khor *et al.* [15] used complex coacervation of alginate-chitosan and alginate-gelatin to develop a two-coat system for encapsulation of *Spathoglottis plicata* portocorms. About 83-100% of the protocorms were found to be viable 4 weeks after encapsulation and able to grow further.

Currently, orchid breeders germinate tens of thousands of seeds in a small container under aseptic conditions. The protocorms tend to become clusters of seedlings that need to be separated manually. Such process is tedious and labour intensive [15]. The development of an artificial seed system with individual seed or protocorm encapsulated in a proper matrix will obviously reduce the difficulty of seedling separation and planting.

Similarly, protocorm-like bodies could be used for encapsulation. In another orchid *Cymbidium giganteum*, the protocorm-like bodies were encapsulated in a single matrix of calcium alginate beads with Murashige and Skoog medium [21,22]. The conversion frequency *in vivo* was 64-88%. Protocorm-like-bodies are multiplied asexually and the artificial seeds made are theoretically clonal seeds, if there is no somaclonal variation among them.

3.5. MYCORRHIZAL FUNGI

Mycorrhiza has a symbiotic association between plant roots and certain soil fungi that play a key role in nutrient cycling in the ecosystem and also protects plants against environmental and cultural stress [23]. This association is usually a mutualistic symbiosis one because of the highly interdependent relationship established between both partners. The host plants normally receive nutrients *via* fungal mycelium while the heterotrophic fungus obtains carbon compounds from the host photosynthesis. Azcon-Anguilar and Barea suggested that the performance of artificial seeds might be greatly enhanced by ensuring a suitable mycorrhizal establishment at out planting.

Tan *et al.* [16] successfully attempted to infect seeds of *Spathoglottis plicata* encapsulated in a two-coat system with *Rhizoctonia*, an orchid mycorrhiza by just placing the encapsulated seeds on a PDA layer containing *Rhizoctonia* mycelium yielded higher percentage of infection (Figure 1, Method 1). About 84% of the encapsulated seeds were infected and the orchid mycorrhizal fungus established a

symbiotic relationship with the encapsulated seeds. The addition of sucrose or other nutrients were excluded and hence eliminate the potential of microbial contamination and provided further opportunity for further development of encapsulated materials grown under non-sterile conditions. Alternatively, the *Rhizoctonia* mycelium was encapsulated together with the seeds; about 39% of the encapsulated seeds were infected with the mycorrhiza when the mycelium was encapsulated with a potato-dextrose agar (PDA) block (Figure 1, Method 2).

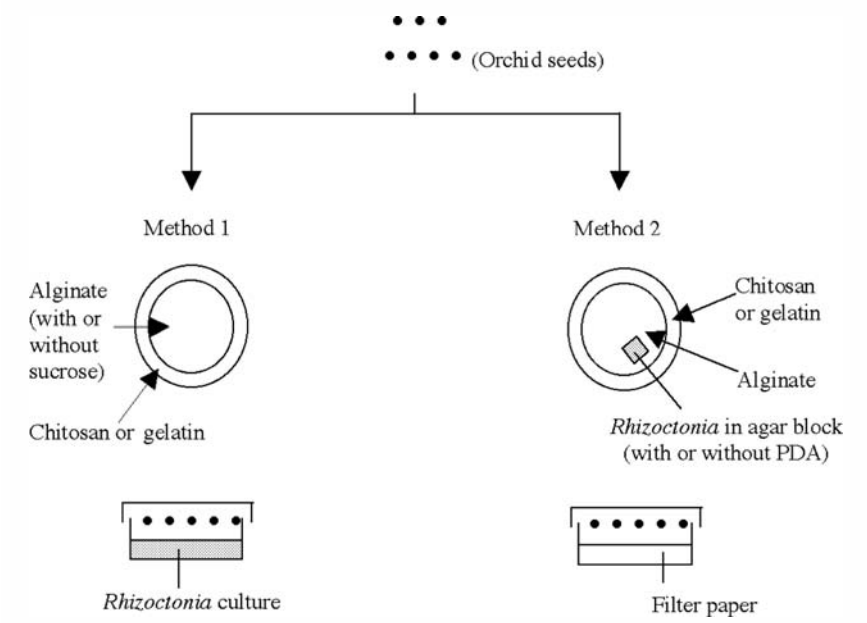

Figure 1. Methods for the infection of Spathoglottis plicata *seeds with* Rhizoctonia *in two-coat encapsulation systems [16].*

The interaction between mycorrhizal fungi and other artificial seed system have not been reported. Previous experience has shown that the earlier the infection of mycorrhizal fungi followed by a symbiotic relationship being established with the host plant at an appropriate stage, the greater the benefit [23]. The incorporation of mycorrhizal fungi also opens the door to developing a non-aseptic artificial seed system for certain plant species such as orchids [16]. Successful infection of protocorm-like-bodies of orchids in an artificial seed system will definitely advance the pragmatic use of the system in orchid micropropagation.

3.6. HAIRY ROOTS-DERIVED MATERIALS

Uozomi *et al.* [24] extended the concept of artificial seeds to include fragments of hairy roots. The infection of plants with *Agrobacterium rhizogenes* causes the transfer-DNA (T_L and T_R) contained in the plasmid to be inserted into the plant genome. Both T_L and

T_R have rhizogenic functions [25] and inoculation of plants with *A. rhizogenes* induces the formation of "hairy roots" that readily give rise to rapidly growing root cultures [26]. These hairy root cultures are considered as useful sources of enzymes [24] and secondary metabolites [25]. Both the root fragments and adventitious shoot primordia derived from horseradish (Armoracia rusticana) hairy roots were used for encapsulation [27]. An efficient method for the production of plantlets from horseradish hairy roots has been developed with the plantlets (0.4-4.0 mm) also being used for encapsulation by Nakashimada *et al.* [28].

4. Matrix material selection for artificial seeds

While the somatic embryo or plant material suitable for propagation into a plant is at the core of the artificial seed concept, the choice of the accompanying matrix materials used for combining with the biological material is equally vital. The matrix is responsible for the immediate surrounding of the plant material. Factors will include mediating the environment such as temperature and humidity to protect the biological material as well as providing a nutrient reservoir. Therefore, the role of matrix materials has an important bearing on the ultimate viability of the artificial seed. The concept of matrix materials has over the years developed into a rather sophisticated interplay that focuses on the germination viability of artificial seeds.

4.1. FLUID DRILLING

The idea of utilising a delivery system began primitively with the use of a fluid gel into which somatic embryos were mixed and sowed directly in the field [1]. The purpose of the gel was to better facilitate the manipulation of small plant materials, possibly in the millions, that was not readily managed because of their small size. This so called "fluid drilling" process was the first realization that a carrier was necessary for the bulk handling of embryos. Magnesium silicate clay and polymeric materials such as potassium starch acrylamide, copolymer of potassium acrylate and acrylamide, starch, and various cellulose-based materials have been tried as the gelling agents in this process [29]. For carrot somatic embryos, the survival rate for this method was a very low 4% and none in the absence of sucrose, attributed to somatic embryos succumbing to desiccation with this process leading to embryo death. In this process, there was really no attempt to encapsulate the biological material. But the realisation that handling and delivery techniques were necessary justifiably credits this work for laying the foundation for artificial seeds that followed.

4.2. POLYMERIC COATING

The next innovation in matrix development chronologically was the concept of a polymeric coating. Somatic embryos or callus were first pre-treated or "hardened" to improve survival rate with by exposure of the plant material to high sucrose concentrations and/or abscisic acid (ABA). Subsequently, commingling of the embryo suspension with water-soluble polyoxyethylene, polyox WSR-N750 [30] and dispensing onto non-adhering surfaces to permit drying under aseptic conditions, gave desiccated

artificial seed wafers. These wafers were readily stored and ready for planting. The survival rate for coated seeds with or without ABA for drying times of 5 or 6.5 hours ranged between 10 to 31%. The desiccation step alluded here is another step forward as it simulates what naturally occurs in nature when a seed develops. Presumably, the role of the polymer is as a covering for the plant material where the coverage is most likely sufficient but may not be complete.

4.3. HYDROGELS

What can be considered a true synthetic seed process is credited to Redenbaugh *et al.* who introduced hydrogels to produce artificial seeds. In the hydrogel approach, somatic embryos are mixed with a polymeric solution that when introduced drop-wise into a separate solution containing divalent metal ions, initiated a crosslinking reaction to form the hydrogel that in the process encapsulates the embryo. With this advancement, the true seed character of an embryo in a simulated endosperm was realised. The most common material used to generate this artificial endosperm is based on sodium alginate that in the presence of divalent calcium ions forms a hydrogel. The nature of the hydrogel being soft ensures that little pressure acts on the embryo therefore minimizing harm to the embryo. Nutrients and other materials such as bactericide and growth hormones for example could be included. Distinct beadlike artificial seeds of good quality could be obtained with an increased high rate of germination in the 80% and above range have been reported.

4.4. TWO-COAT SYSTEM

While the hydrogel systems can be considered as a worthy milestone, limitations of the system soon manifested. Hydrated capsules were tacky unless coated with an additional hydrophobic layer, the hydrogels rapidly dehydrate making storage difficult and their high porosity led to cell and nutrient leaching out. This situation is not difficult to comprehend if one considers that a true seed comprises not only the embryo and endosperm, but also the all-important seed coat.

Towards the resolution of the seed coat stipulation, *Tay et al.* introduced the *in situ* generation of an artificial seed complete with somatic embryo, artificial endosperm and the outermost layer constituting the seed coat [31]. In this process, an additional material – the polycationic chitosan polymer – interacts with the polyanionic alginate to form the "seed-coat" as depicted diagrammatically in Figure 2.

The resulting artificial seeds are less tacky to the touch, easy to handle and store as shown in Figures 3 and 4 using *Spathoglottis plicata* seeds as the plant material. With this final contribution, it can be stated that the puzzle of assembling of the artificial seed has been completed in all its conceptual assembly.

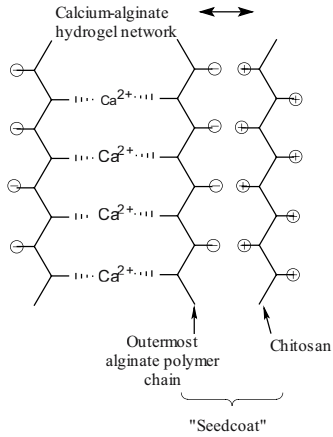

Figure 2. Diagrammatic representation of the two-coat system. The inner core of alginate polymer chains are crosslinked by Ca^{2+} ions forming the hydrogel network while the outermost alginate layer interacts with chitosan to form the polymer seed-coat [31].

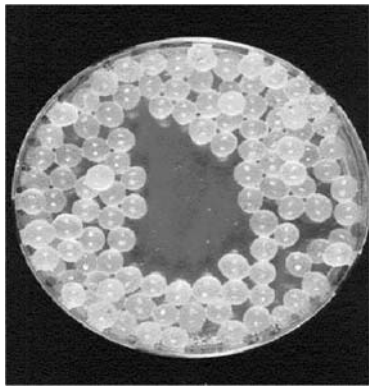

Figure 3. Collection of Spathoglottis plicata *artificial seeds obtained from the two-coat alginate-chitosan encapsulation system [31].*

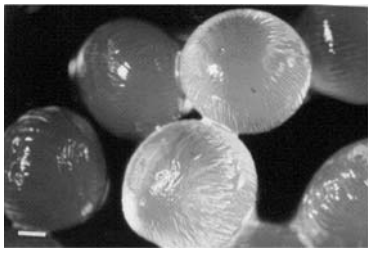

Figure 4. Close up view of Spathoglottis plicata *artificial seeds obtained from the two-coat alginate-chitosan encapsulation system showing the minute plant material in the matrix [31].*

4.5. TAKING STOCK

From the preceding survey, it is evident that the challenge in defining a suitable matrix, carrier or delivery system in artificial seed technology demands a set of requirements that was tricky to fulfil and garnered as the experience with trying various delivery methods grew. The evolved requirements can be conveniently summarised into five major considerations:

- The material must protect the embryo and/or other biological materials.
- The material must be capable of including nutrients and other growth and/or biological factors.
- The material must protect the formed artificial seed during storage and handling.
- The material must incorporate a mechanism for activating "germination".
- And of course the material must be non-toxic, compatible with the biological and chemical systems it comes into contact with and be available in a suitable and simple preparation for use.

In addition, several factors regarding the directions for developing a functional seed delivery system beyond those stated above become apparent:

- The material must have the capability to reversibly imbibe and discharge fluid, primarily water, to simulate desiccation and germination processes.
- The material must be water soluble in the first instance as a mode of introduction.
- The material should be preferably biodegradable.

The materials used to date revolve principally around both natural and synthetic polymers that in one way or another utilise the gelling effect of the polymers. Gelling is based on the ionic and/or polar interactions inherent in the polymers, a result of the individual properties of the functional groups of each monomer repeat unit. In some instances, the use of crosslinking agents are required to obtain the gel as is the case in alginates where calcium ions are used to pair up two anionic groups on the polymers (preferably from different chains) thereby generating the hydrogel. The number and/or extent of these interactions in turn determine the final capsule properties such as shape integrity, burst strength and fluid or media retention.

All these properties have direct bearing on the embryo's or plant material's survival and eventual germination. The parameters involved in determining the capsule properties included the concentration of polymer, the processing time that leads to "hardening" of the capsule and the functional groups distribution especially for natural polymers. It is interesting to note that to date there is no direct interaction *per se* of the polymeric gelling material with either the biological material or the growth and other culture media that is incorporated during the encapsulation process. In other words, a physical entrapment process is at work.

The two-coat system developed by Tay *et al.* presents a more sophisticated "barrier" effect that may be more efficient in retaining encapsulated material but nevertheless still has a physical basis for its action. This leaves a lot of scope to fine tune the encapsulation process where a more systematic consideration of the requirements in achieving the goals of developing a functional seed delivery system can lead to an

artificial seed hitherto not realised, provided of course, the cost of such a delivery system is not prohibitive.

5. Outlook

Redenbaugh [7] stated that the main objective for developing artificial seeds of crop plants is to produce "clonal seeds" at a cost comparable to true seeds. The objective is suitable if somatic embryos are used as plant materials for encapsulation. With the extension of the artificial seed concept to include encapsulation of other plant materials, the objective for the making of artificial seeds may be different. We would expect to see more reports on the encapsulation of transgenic plant materials, parasitic plants and the incorporation of various microorganisms in the matrix. We would also expect to see more innovative use of polymers and encapsulation procedures to satisfy the different objectives. Nevertheless, the requirement that the cost of producing artificial seeds should be comparable to that of true seeds remains valid and is a goal that may not be reached in the near future unless there are breakthroughs in the technology, materials used and manpower requirement.

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