Artificial Seed Technology

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Abstract

Artificial seeds are produced by encapsulating somatic embryos, shoot tips, or any other micropropagule which have the ability to convert into a plant in vitro or ex vitro. The need of artificial seed production was felt due to failed seed propagation in some crop species due to very small seed size, seed heterozygosity, reduced endosperm, no germination in the absence of seed–mycorrhizal association as in case of orchids and also time-consuming vegetative means of propagation in some seedless varieties of crops such as *Citrullus lanatus* and *vitis vinifera,* etc. Effective seed coating of micropropagules is done using different gelling agents such as alginate, agar, carrageenan, gellan gum, sodium pectate and carboxy methyl cellulose. However, sodium alginate has been documented as most frequently used gelling agent. The absence of seed coat and endosperm in somatic embryos necessitates the encapsulation matrix to be supplemented with nutrients and growth regulators such as 0.5 mg/L indoleacetic acid (IAA), 0.5 mg/L naphthalene acetic acid (NAA), 2 mg/L 6-benzyl aminopurine (BA), 2 mg/L Fe-EDTA and 30 g/L sucrose. In many plant species such as *Allium sativum*, *Ananas comosus*, *Dioscorea bulbifera*, *Cineraria maritima*, *Cucumis sativus,* etc. genetic stability of the plants derived from artificial seeds has also been examined with the help of biochemical and molecular markers and found them genetically consistent.

Keywords

Artificial seed · Encapsulation · Sodium alginate · Somatic embryos

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Abbreviations

BA Benzyl Aminopurine
IAA Indoleacetic Acid
NAA Naphthalene Acetic Acid

8.1 Introduction

Encapsulated somatic embryos (raised from tissue culture), shoot tips, embryonic calluses, axillary buds, or any other micropropagules which can be planted as seed and have the ability to develop in to a whole plant in vitro and ex vitro are called as artificial or synthetic seed (Capuano et al. 1998; Ara et al. 2000; Rihan et al. 2011). The concept of coating somatic embryos and using them with the same ease as a normal seed was first given by Murashige in the year 1977. He defined artificial seed as "an encapsulated single somatic embryo". Later on Gray et al. (1991) defined artificial seed as "a somatic embryo that is engineered for the practical use in commercial plant production". Initially artificial seed production was only confined to somatic embryos and therefore has been utilized in only those plant species in which successful production of somatic embryos could be well exhibited (Rihan et al. 2017). Later, with the report of shoot tip coating by Bapat et al. (1987) in *Morus indica*, the definition of artificial seed has been modified as "an encapsulated somatic embryo or in vitro raised other plant propagules which are capable to develop in to a plant when grown in vitro or ex vitro with the characteristic of prolonged storage" (Daud et al. 2008; Saiprasad 2001; Ara et al. 2000). The objective of producing artificial seed was to promote cost effective and large-scale multiplication of superior plant genotypes or commercially valuable plant species (Reddy et al. 2012; Saiprasad 2001). The need of technological interventions in vitro was felt so that the problems such as failed seed propagation in some crop species due to very small seed size, heterozygosity of seed, reduced endosperm, no germination in the absence of seed–mycorrhizal association as in case of orchids could be addressed. Also, the time-consuming vegetative means of propagation in some seedless varieties of crops such as *Citrullus lanatus* and *vitis vinifera,* etc. (Saiprasad 2001) could be supplemented to certain extent. The prevailing traditional breeding system in coniferous forest species is cumbersome due to their prolonged life cycle. Attainment of better progeny is not always possible because of the heterogeneous nature of coniferous forest species. Artificial seeds can play a very important role in cloning of these trees at reduced cost and time (Desai et al. 1997). Hybrid seed production by hand pollination in some vegetable crops like tomato and seedless watermelon is very labour intensive and therefore responsible for the increased seed cost. Similarly, vegetative means of propagation is also very time consuming. Presence of cleistogamous flowers in cotton and soyabean increases the production cost of hybrid seeds at commercial level since pollination is done by hand. Significant reduction in the

cost may be expected by developing synthetic seeds in such species by economizing labour and also, time and space constraints may also be dealt with (Chee and Cantliffe 1992; Tian and Brown 2000). Plants raised from artificial seeds have also been tested for their genetic stability in various plant species such as *Allium sativum*, *Ananas comosus*, *Dioscorea bulbifera*, *Cineraria maritima*, *Cucumis sativus,* etc., using biochemical and molecular tools and found them genetically consistent (Srivastava et al. 2009; Gangopadhyay et al. 2005; Narula et al. 2007; Tabassum et al. 2010; Bekheet 2006).

8.2 Advantages of Artificial Seed

Germplasm conservation through cryopreserving artificial seeds particularly in desiccation sensitive species like mango, cocoa, coconut, etc., utilization in hybrid seed production, i.e. use of artificial seed in propagating those plants which exhibit male or female sterility, multiplication of polyploid species, freedom from pathogens, easy handling during storage, transport feasibility, long term storage with no viability loss, and maintenance of the clonal nature of plants by using genetically identical somatic embryos, suitable medium to deliver the novel plant lines obtained through biotechnological means straight to the greenhouse or field, cost effective large scale propagation of superior plant varieties, etc. are some of the advantages of artificial or synthetic seed (Saiprasad 2001). Artificial seeds have been produced in various plant species including vegetable crops, fruit crops, medicinal plants, cereals, orchids, sugar crops, and forest trees (Siong et al. 2012; Masri et al. 2019; Shallal et al. 2020; Ismail et al. 2016; Rslan 2018; Bekheet 2006; Tsai and Saunders 1999; Bapat et al. 1987; Jain et al. 2018; Roy and Mandal 2008; Nieves et al. 2003).

8.3 Production of Artificial Seeds: The Prerequisites

8.3.1 Explants

The most commonly used explants are somatic embryos because they contain apical and basal meristem which gives rise to shoot and root (Ara et al. 2000), reproduction level is more; plants raised from somatic embryos are proficient and retain their regenerative capacity for a longer duration resulting in to uniform plant population (Leroy et al. 2000). Artificial seed production with the help of somatic embryos has been reported in *Gentiana kurroo* (Kotvi et al. 2016), *Daucus carota* (Kitto and Janick 1982), *Medicago sativa* (Gupta and Durzan 1987), *Vitis vinifera*, *Mangifera indica* (Ara et al. 1999), *Citrus reticulate* (Antonietta et al. 1999), *Saccharum spp.* hybrid (Nieves et al. 2003), *Oryza sativa* (Kumar et al. 2005), *Plumbago zeylanica* L. (Jain et al. 2018), etc.

Somatic embryos are the bipolar structures developed from somatic cells, instead of zygotes by means of somatic embryogenesis and thus used for clonal propagation

(Saiprasad 2001). There are two routes, i.e. direct and indirect, by which somatic embryogenesis can be induced in vitro. Formation of somatic embryos takes place at the side of an explant in direct embryogenesis, whereas in case of indirect way it takes place through the growth of an unorganized mass of cells called callus (Quiroz-Figueroa et al. 2006).

8.3.2 Shoot Tips, Axillary Buds, Internode Cuttings, Microshoots

Production of artificial seed has also been a success in other micropropagules used as explants. Some of the examples are coating of shoot tips (Aida et al. 2012; Masri et al. 2019; Ismail et al. 2016) and internode cutting of *Beta vulgaris* (Ismail et al. 2016), microshoots in *Saintpaulia ionantha* wendl. (Daud et al. 2008) and *Brassica oleracea* var. *botrytis* (Siong et al. 2012), axillary and apical buds of *Manihot esculenta* Crantz (Hegde et al. 2016), nodal segments and shoot tips of *Mimosa pudica* L. (Banu et al. 2014), nodal segments of *C. angustifolia* (Bukhari et al. 2014) and bulblets of *Allium sativum* L. (Bekheet 2006).

8.3.3 Encapsulation of Explants

Different gelling agents are used for the effective seed coating of micropropagules such as alginate, agar, carrageenan, gellan gum, sodium pectate and carboxy methyl cellulose. Based on the properties of being soluble at room temperature and to form hydrogel with calcium chloride, the sodium alginate has been recognized as most frequently used gelling agent (Bapat et al. 1987; Kikowska and Thiem 2011). Encapsulation of embryos, pro-embryos and embryo-like structures of androgenic origin in rice (Roy and Mandal 2008), cauliflower (Siong et al. 2012) and sugar beet was done using sodium alginate as gelling material. Tragacanth gum, carrageenan, polyox, agar, carboxy methylcellulose, guar gum, gelrite, sodium pectate ethyl cellulose and nitrocellulose, agarose, polyacrylamide, polyco 2133, alginate were examined for their suitable use in the synthetic seed production (Ara et al. 2000; Saiprasad 2001; Lambardi et al. 2006). Polyox and resins soluble in water have been found most appropriate for somatic embryos coating (Kitto and Janick 1982). Sodium alginate was found most suitable gelling agent in celery, cauliflower, alfalfa, and carrot (Redenbaugh et al. 1984; Redenbaugh et al. 1986). Encapsulation of explants is done using two types of solutions. One is polymeric solution, and the other is a solution which contains divalent metal ions. Polymeric solution when comes in contact with the solution containing divalent metal forms hydrogel due to the cross-linking reaction. Explants are dipped in the solution of sodium alginate followed by their dropwise placement into the calcium chloride solution for at least 30 min. When drops of sodium alginate touch the calcium chloride, ion exchange occurs between Na^+ with Ca^{2+} resulting into bead formation. Each bead represents the one explant. Beads so produced are then taken out from the solution of calcium chloride and washed two to three times using sterilized distilled water (Hegde et al.

2016; Banu et al. 2014; Kotvi et al. 2016). These artificial seeds are then transferred to the petri plates containing germination medium enriched with macro and micronutrients from MS medium with additional 30 g/L of sucrose and 7 g/L of agar agar. These plates are kept at 25 °C in complete dark in the culture room (Pond and Cameron 2003). Different combinations of sodium alginate and calcium chloride concentrations have been tested for making artificial or synthetic seeds to achieve best encapsulation efficiency in different plant species. The firmness, size, texture, and shape of the beads are the deciding factors in selecting most suitable combination of sodium alginate and calcium chloride concentrations for encapsulation. In general, the procedure in which mixing of explants is done with 3% concentration of sodium alginate followed by their exposure to 100 mM of calcium chloride has been used most widely. The firm and round beads were produced when encapsulation of nodes of in vitro derived cassava variety and microshoots of African violet (*Saintpaulia ionantha* Wendl.) was done using 3% (w/v) sodium alginate and 100 mM calcium chloride ($CaCl_2$. $2H_2O$) solutions (Hegde et al. 2016; Daud et al. 2008). However, germination frequency under in vitro condition was on the higher side when the encapsulations were made in combinations of 2% and 3% of sodium alginate and 75 mM and 100 mM of $CaCl_2.2H_2O$ in case of cassava (Hegde et al. 2016). In sugar beet, instead of calcium chloride 100 mM calcium nitrate was used as combining solution along with 4% sodium alginate for the encapsulation of microshoots (Masri et al. 2019). Successful encapsulation of somatic embryos in an important medicinal plant *Gentiana kurroo* has also been reported using the combination of 3% sodium alginate and 100 mM calcium chloride (Kotvi et al. 2016). The viability of artificial seed is very much dependent on the material of gel matrix used for encapsulating the plant material. Longer viability of artificial seed must be ensured if the coating material provides protection to explants, exhibits proficiency in inclusion of nutrients, facilitates the storage, handling, and germination of the artificial seed, is non-toxic and compatible with biological and chemical system (Khor and Loh 2005), and sodium alginate was found to be the most suitable seed coating material containing all these characteristics (Saiprasad 2001).

8.3.4 Artificial Endosperm

Unlike zygotic embryos, somatic embryos are devoid of protective seed coat and endosperm which necessitates the coating material to be supplemented with nutrients and growth regulators such as 0.5 mg/L indoleacetic acid (IAA), 0.5 mg/L naphthalene acetic acid (NAA), 2 mg/L 6-benzyl aminopurine (BA), 2 mg/L Fe-EDTA and 30 g/L sucrose, which acts like artificial endosperm (Murashige and Skoog 1962). These added nutrients and growth regulators in the encapsulation material contribute by enhancing germination efficiency and viability of the somatic embryos. It has been suggested that storage of artificial seeds at 4 °C may help to retain their viability for a longer duration, i.e. up to 6 months. Addition of fungicides, pesticides, antibiotics, and microorganism such as rhizobia into the coating material has also

been recommended to protect somatic embryos from desiccation and mechanical damage (Saiprasad 2001). Artificial seed production is considered successful only when the produced seeds are vigorous and their conversion efficiency is high. It has been reported that activated charcoal when added into the coating material, the vigour and conversion efficiency of coated somatic embryos were enhanced. When sodium alginate breaks up in the presence of charcoal, an increase in the respiration of somatic embryos occurs. Besides, activated charcoal withholds the nutrients within the gel matrix and also responsible for their moderate release to the growing somatic embryo (Saiprasad 2001).

8.4 Steps of Producing Artificial Seeds

Although the production of artificial seeds may vary among different species as per their type, need and economic viability, but in general, a process of making artificial seed has been outlined by Redenbaugh et al. (1987). The process includes (1) technological and commercial potentiality based crop selection, (2) establishment of species specific procedure for the development of somatic embryo, (3) protocol standardization for the clonal production system in order to obtain viable, mature embryos with the ability to convert in to normal plants, (4) self-regulated embryo production, (5) post-treatment, i.e. induction of quiescence in mature embryos, (6) embryo coating, (7) standardization of artificial endosperm, (8) extensive production of seeds, (9) streamlining the procedures required for plant growth in green house and field conditions, (10) pests and disease control, if any.

8.5 Types of Artificial Seeds

Artificial seeds can be categorized as (1) desiccated or hydrated (Ara et al. 2000; Bapat and Mhatre 2005) and (2) uncoated quiescent or uncoated non-quiescent (Grey 2003).

8.5.1 Desiccated or Hydrated Artificial Seeds

Such seeds are somatic embryos non-coated or coated with polyethylene glycol which are desiccated afterwards. Somatic embryos become quiescent on desiccation resulting into the hardening of protective cover. Handling of such seeds is easy if stored under unsophisticated conditions for a longer duration. On rehydration, protective hard cover softens, and somatic embryos resume growth. Drying is possible by two methods, i.e. rapid and slow. Rapid drying takes place by keeping seeds overnight in open petriplates, whereas slow drying of seeds is attained by reducing the relative humidity over a prolonged period under controlled condition. Such types of artificial seeds can be produced only when somatic embryos are desiccation tolerant (Sharma et al. 2013). Induced desiccation tolerance in somatic

embryos was achieved by using maturation medium of high osmotic potential (Sundararaj et al. 2010). The osmotic potential of the medium can be increased by incorporating different osmotic agents such as sucrose, mannitol, etc. or by increasing the strength of the gel. However, attainment of desiccation tolerant somatic embryos has also been reported when some stresses like low temperature or nutrient distress were applied (Pond and Cameron 2003). Somatic embryos coated in hydrogel are called as hydrated seeds, which can be produced in recalcitrant plant species (Ara et al. 2000). It has been reported that encapsulation is important for transferring the micropropagules to the field, provided the material to be used for encapsulation helps in promoting germination (Latif et al. 2007). Also, encapsulation can be seen as a best way for protecting, as well as converting tissue culture derived micropropagules into artificial or synthetic seeds (Redenbaugh 1993).

8.5.2 Uncoated Non-Quiescent or Uncoated Quiescent Artificial Seed

Non-quiescent somatic embryos can be used in crops being raised through micropropagation, whereas quiescent seeds can be stored as germplasm.

8.6 Production of Artificial Seeds in Sugar Beet

Sugar beet (*Beta vulgaris* L.) improvement is primarily done by conventional means but now modern techniques particularly genetic transformation have also been introduced in sugar beet breeding (Ivic et al. 2001). With reference to genetic transformation and tissue culture, sugar beet is considered as recalcitrant species (Elliott et al. 1996; Krens et al. 1996). Also, some superior genotypes of sugar beet have reportedly been propagated through shoots regenerated in vitro (Grieve et al. 1997; Zhong et al. 1993) but it was observed that explants selected from different genotypes showed regenerative variations (Saunders and Tsai 1999). Protocol to produce synthetic seed in sugar beet has been developed for prolonged storability, minimizing cost of production and to facilitate seed handling (Ghosh and Sen 1994).

In sugar beet, shoot tip coating in 4% sodium alginate followed by its multiplication in the medium containing 2 mg/L BA gave best results in terms of leaf and shoot count. However, maximum shoot length was obtained in a medium which contained kinetin @2 mg/L. There was a significant improvement in germination when MS medium along with 30 g/L sucrose was added to the encapsulated seeds. Effective root formation was achieved by using 2 mg/L NAA. Media was also supplemented with osmotic agents, mannitol or sorbitol to examine artificial seed storage. Increased plant survival was reported when media was supplemented with these osmotic agents at 0.5 M concentration. The revelation that sugar beet genotype Francesca was found better than the Toro genotype in reference to its suitability for encapsulation, also suggests that not all the genotypes of a particular species are suitable for developing artificial seeds. Molecular analysis showed that not only the

application of mannitol or sorbitol but also their interaction with the genotypes plays a crucial role in the storage of artificial seeds.

Masri et al. (2019) have reported their procedure as cost effective, time saving and suitable for long term conservation of sugar beet germplasm based on the recovery of plants resulted from the artificial seeds stored at 4 °C for a period of 2 months. 4% sodium alginate along with 100 mM $Ca(NO_3).24H_2O$ was used as gel matrix. Vitality of artificial seeds based on the germination was found better in the solution in which 1.3 BAP was added in addition to MS, 3% sucrose, 4% sodium alginate, 2% sorbitol and 2% mannitol.

Ismail et al. (2016) have suggested the solution containing sodium alginate @4%, 1.2% agar, 1.5 mg BA/L and 3% sucrose as best encapsulating solution for artificial seed production. According to them, encapsulation matrix should be enriched with MS, sucrose and BA in order to ensure the germination of artificial seeds. Based on the results of no germination of those artificial seeds (1) coated with only sodium alginate solution or/and (2) coated with sodium alginate and sucrose dissolved in water, they suggested that the coating of artificial seed with sodium alginate and sucrose dissolved in MS is required by the seed to germinate. However, artificial seed exhibited no change in germination frequency when coating was done with 4% sodium alginate dissolved in MS medium containing either 1.3 mg/L BA or 40 g/L sucrose. It has been reported that encapsulated shoot tips germinated earlier than the internode cutting in the sugar beet cultivar Frida, which emphasized that the nature of explants has an important role to play both in production and storability of the synthetic seeds. Shoot tip derived synthetic seeds remained viable for a longer duration as compared to synthetic seeds produced from internode and lost their viability after 2 months. Also, in *M. arvensis* when germination behaviour was compared between encapsulated shoot tip and nodal segment, the highest shoot formation was obtained from the artificial seeds developed from shoot tips (Islam and Bari 2012).

8.7 Future Prospects

Artificial seed production specifically with somatic embryos in comparison to other micropropagules as explants has been recommended in various studies as they possess both radical and plumule and can be coated in dried as well as in hydrated form (Kitto and Janick 1982). However, apical shoot buds/apical shoot tips have also been used successfully in producing synthetic seeds in many plant species such as *Actinidia deliciosa* (Kiwi fruit), *Arachis hypogaea* (Groundnut), *Brassica campestris* (Mustard), *Daucus carota* (Carrot), *Malus Pumila (Mill)* (Apple root stock M. 26), *Mangifera indica* L. (Mango cv. Amrapali), *Solanum melongena* (Egg plant), *Vitis Vinifera* (Grape), *Zingiber Officinale Pose* (Ginger), and Cucumber *(Cucumis sativus)* (Ara et al. 2000; Latif et al. 2007; Tabassum et al. 2010). The significant success in artificial seed production at commercial level can be achieved if rate of conversion of artificial seeds into vigorous plantlets will be high. Not only high conversion rate but also uniform transformation is also essential for making

their use for clonal plant propagation (Magray et al. 2017). The self-incompatible behaviour exhibited by most of the fruit species has limited their mode of propagation mainly to vegetative means. Germplasm conservation of these species in the form of artificial seeds in a small space through cryopreservation would be the best way to minimize the cost of maintaining field gene banks and also the risk of adverse environmental conditions on germplasm can be avoided (Towill 1988). Extensive hybrid seed production in cross pollinated species particularly in maize by traditional breeding method is time absorbing and resource exhausting process due to the maintenance of parental lines. Use of artificial seeds in hybrid seed production may help in the commercialization of new hybrids and superior genotypes can be propagated in less time and cost as the step of maintaining parental line would be eliminated. Also, artificial seed production may be an alternative of conserving those forest species in which vegetative propagation is not possible and which are in the verge of extinction due to increasing desertification (Desai et al. 1997).

8.7.1 Limitations Associated with Artificial Seed Production

Although use of somatic embryos in artificial seed production has been reported in various plant species (Sharma et al. 2013), some limitations have been encountered in terms of asynchrony in somatic embryos development, non-uniform maturity, reduced conversion efficiency, unsuitability for long term storage (Reddy et al. 2012), reduced viability and attainment of low plant recovery if stored at low temperature (Makowczynska and Andrzejewska-Golec 2006) which need to be addressed in order to make artificial seed production system more efficient. Other than these factors some of the problems enlisted in the applicability of artificial seed technology are (1) high cost production of somatic embryos at commercial level, (2) extra care is required to prevent the somatic embryos from mechanical injury, microbial attack, desiccation, etc., (3) insufficient oxygen and nutrients, if not supplied properly, may adversely affect the germination of seeds, (4) somaclonal variations, (5) artificial seeds cannot be implanted directly in the substrates like vermiculite and compost, etc. (Singh et al. 2020).

8.8 Conclusion

Successful production of artificial seed by way of encapsulating micropropagules has been reported in vegetables, medicinal plants, fruit crops, cereals, orchids, sugar crops and forest trees. Protocols were standardized to obtain best combination of gel matrix to produce artificial seeds with enhanced vigour and high conversion efficiency. Studies have also shown positive effect of growth regulators and nutrients on germination behaviour and viability of somatic embryos when applied in the encapsulation material. This technology has made possible the germplasm conservation in desiccation sensitive species. Multiplication of polyploid species, easy handling during storage, transport feasibility, cost effective propagation of superior plant

varieties are the advantages of artificial or synthetic seed. However, certain limitations such as asynchrony in somatic embryos development, non-uniform maturity, reduced conversion efficiency, etc. need to be resolved to enhance the efficiency and applicability of artificial seed technology in different plant species.

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