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# CONSERVATION OF PLANT GERMPLASM USING SYNTHETIC SEED TECHNOLOGY - REVIEW

**Ioana-Cătălina NICOLAE[1, 2], Oana VENAT[2], Adrian George PETICILĂ[1], Dorel HOZA[1]**

[1]Faculty of Horticulture, University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Mărăști Blvd, District 1, Bucharest, Romania
[2]Research Center for Studies of Food Quality and Agricultural Products, University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Mărăști Blvd, District 1, Bucharest, Romania

Corresponding author email: oana.venat@qlab.usamv.ro

***Abstract***

*Plants are an essential resource for the existence of our planet. They sustain the ecosystems, produce the oxygen that is mandatory for the life of the other species and they are a source of fibres, medicine, materials and, the most important, they are our main source of food. In recent years, plant species are more and more threatened with extinction, due to urbanization, the development of various industries and habitat destruction. In addition to direct anthropological factors, natural habitats are endangered by climatic changes, changes that result in natural disasters as floods, bush fires, extreme temperatures and irregular precipitations. Conserving the plant species in in situ conditions, botanical gardens or field conditions exposes the germplasm to the risk of loss due to all these climatic accidents, and in vitro conservation techniques, such as synthetic seeds can provide a valuable solution for the future of our plant resources.*

***Key words****: artificial seed, encapsulation, germplasm, in vitro, micropropagation.*

## INTRODUCTION

The Convention on Biological Diversity defines *in situ* conservation as the "conditions where genetic resources exist within ecosystems and natural habitats, and, in the case of domesticated or cultivated species, in the surroundings where they have developed their distinctive properties" and *ex-situ* conservation as "the conservation of components of biological diversity outside their natural habitats" (UNCED, 1992). *In vitro* conservation methods include conservation through slow growth, for short and medium-term storage and cryopreservation for long-term conservation (Engelmann, 1999). The slow growth conservation implies that the plant material is being conserved under growth-limiting conditions, such as lower temperature, lower light intensity, or by adding osmotic agents or growth retardants in the medium, with the aim to reduce the physiological processes and growth rate of the explants (Englemann, 1999). Trejgell et al. (2015) observed that the presence of ABA (in a concentration of 3.8 or 9.5 μM) in the conservation medium stimulated the survival and regeneration of *Senecio macrophyllus* shots conserved using slow growth storage technique. Benelli et al., 2022 listed some species stored through slow growth technique: *Castanea sativa* with 48 months storage time at 8°C, *Fragaria spp*. 15 to 18 months at 4°C, *Malus* spp. at 4°C from 18-20 to over 36 months with the addition of 2% mannitol and MS modified medium, *Pyrus* spp. at 4°C, from 12 to 48 months, depending on media formulation. Genebanks have a major role in germplasm conservation and ensure that genetic resources are protected and continuously available (FAO, 2014). The US Potato Genebank (Sturgeon Bay, Wisconsin) is holding the national collection of germplasm for *Solanum tuberosum*, a number of approximately 6000 accessions of 100 species of or related to *Solanum tuberosum,* one of the most important species of vegetables (Bamberg et al., 2016). Cultivars at the US Potato Genebank are cultured in small 20 mm x 150 mm glass tubes with 10 ml of medium and 3% D-sorbitol after being multiplied by axillary bud cutting. After a period of two weeks of establishment and growth at 20-22°C, they are moved into a chamber for long-term preservation, at 8-10°C, with lower light

intensity, where those plants can remain viable for 1-1.5 years (Bamberg et al., 2016).

Figure 1. Example of the growing chamber for in vitro conservation of *Solanum* clones at US Potato Gene Bank (source: Bamberg et al., 2016)

According to the website of the International Potato Center (CIP), headquartered in Lima, Peru, this genebank was founded in 1971 and after 45 years of continuous focus on germplasm collection to eliminate mixtures, atypical plants, and plants with virus symptoms, from 17.347 ascensions, with duplicate removal, the nowadays potato collection includes 4870 accessions with 4467 traditional landrace cultivars from 17 countries (mainly from the Andean region) and improved varieties. The entire collection is conserved by *in vitro* techniques and all distribution is made by tissue culture material. Plants are continually reevaluated to identify duplicates, verify identity, and maintain accurate morphological, taxonomic, and cytogenetic characterization. Potato is a species optimized for long-term cryopreservation, so there are over 450 new ascensions entered into this procedure over one year. Regarding the sweet potato germplasm collection, the International Potato Center (CIP) has one of the world's largest cultivated sweet potato gene banks, with over 5500 accessions. All material is *in vitro* maintained and the main CIP objective for this seventh most worldwide important food crop is to conserve the collection biodiversity and make it available for breeding research and training, as they mentioned on their public page.

Maintaining germplasm *in vitro* cultures is labor intensive, time-consuming and exposes the plant material to the risk of losing the germplasm due to contamination or increase of somaclonal variations, even in the case of using slowed/reduced growth conditions (Pannis et al, 2001). To overcome this, synthetic seed technology may be applied to conserve species on short and medium time and cryopreservation for long-term preservation. Synthetic seeds are represented by somatic embryos, axillary or terminal buds, nodal segments, cell aggregates, or other types of artificially encapsulated tissues that can be used for sowing and can transform into plants, and that can retain this ability even after short term and medium-term storage (Hussain et al., 2000; Micheli & Standardi, 2016; Magray et al., 2017). The concept of synthetic seed was first introduced in 1977, by Murashige. Initially, the term referred only to encapsulated somatic embryos (Murashige, 1977), but later, the concept was extended to non-embryogenic tissues as well (Bapat et al., 1987). The technology of synthetic seeds has many applications: it can be used to asexually propagate endangered species, and valuable genotypes of species that normally don't produce seeds (Qahtan et al., 2019). The technique can also be used for exchange between laboratories and institutions and short and medium conservation of germplasm (Standardi a Micheli, 2013). Long-term conservation of synthetic seeds can be achieved by cryopreserving the plant material. Otherwise, these can be stored for short and medium time in the fridge, at 2-8°C, depending on the species (Micheli & Standardi, 2016).

Synthetic seeds can be stored up to 90 days in the fridge, but the optimum storage period is dependent on the species, but generally, most species can be stored in the fridge, at 4-6°C (Qahtan et al., 2019). Some species can be stored at room temperature, in dark conditions (Standardi & Micheli, 2013). High humidity and low temperature are essential for storing the synthetic seeds (Mallikarjuna et al., 2016). In terms of plant genetic resources

conservation, cryopreservation is one of the safest methods. Once a cryopreservation method is established for each species, the germplasm can be stored for a very long period, without the need for further transfers or replanting (Bamberg et al., 2016). Even though the initial cost of storing the plant material in liquid nitrogen is high, the cost of culture maintenance in LN is lower than storing them as field cultures or as *in vitro* cultures (Bamberg et al., 2016).

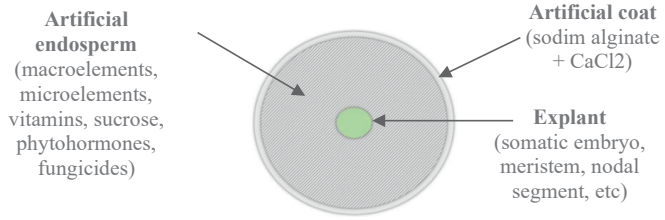


Figure 2. Structure of synthetic seeds

**Types of explants used for encapsulation: Somatic embryos**

The advantage of using somatic embryos for synthetic seeds is that these types of explants have a bipolar structure and can develop roots and shoots simultaneously, without the need for further auxin treatment (Hussain et al., 2000). The use of somatic embryos in the synthetic seed technology was studied in numerous species, such as *Rotula aquatica* (Chithra et al., 2005), *Oryza sativa* (Kumar et al., 2005), *Pinus patula* (Malabadi & Staden, 2005), *Pinus radiata* (Aquea et al., 2008), *Vanda tessellate* (Manokari et al., 2021), *Quercus suber* L. (Pintos et al., 2008), *Litchi chinensis* Sonn (Das et al., 2016). Malabadi and Staden (2005), obtained a regeneration rate of 89 % for encapsulated somatic embryos of *Pinus patula.* The synthetic seeds were conserved at 2°C, for 120 days and did not register a significant decrease regarding viability, compared to the embryos that were not encapsulated, where the viability decreased to 2%, after only 20 days of storage at 2°C.

Holobiuc and Catana (2012), developed a protocol for somatic embryogenesis and synthetic seed production for *Gentiana lutea,* a species regarded as critically endangered in Romania. Studies by Kamińska et al., 2017, indicate that synthetic seeds of *Taraxacum pieninicum*, a critically endangered species of the Asteraceae family, can be stored for up to 12 months at 4°C, with successful recovery and without any genetic variations. *Vanda tessellata*, an epiphytic orchid whose roots are valued in traditional medicine (Hossain, 2011; Shengji and Zhiwei, 2018) can be stored for 12 months at -4°C, and then regenerated on medium with 0.5 mg/L BAP (6-(benzylaminopurine), 0.5 mg/L KIN (kinetin) and 0.5 mg/L IAA (Indole-3-acetic acid) (Manokari et al., 2021).

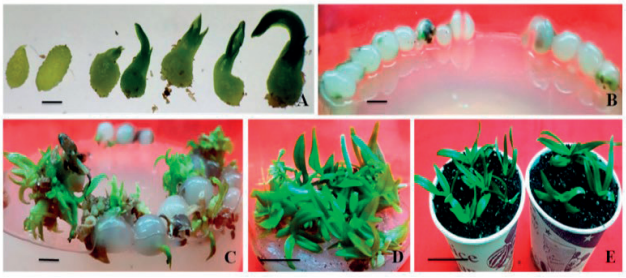


Figure 3. Regeneration of *Vanda tessellata* using encapsulated somatic embryos: A. various developmental stages of SE; B. inoculation and emergence of shoots; C. germination and emergence of shoots; D. multiplication and elongation; E. rooted plantlets (source: Manokari et al., 2021)

**Protocorm-like bodies (PLBs)**

Sarmah et al. (2010) successfully conserved encapsulated PLBs of *Vanda coerulea* for 100 days in cold storage, with a conversion rate of 94.9%. Encapsulated PLBs of *Flickingeria nodosa* can be stored for 3 months at 4°C with a regeneration rate of 95% (Nagananda et al., 2011).

**Meristematic tissues. Apical and axillary buds. Nodal segments**

Because of the limits that somatic embryos possess, in 1978, Bapat proposed the use of non-embryogenic tissues for encapsulation as synthetic seeds, especially in the case of species that are recalcitrant to somatic embryogenesis. Generally, these types of tissues are easier to obtain compared to somatic embryos, and the risks of somaclonal variations are highly reduced. In addition, the use of non-embryogenic tissues for synthetic seeds can be used for the vast majority of species (Standardi & Micheli, 2013).

Apical and axillary buds can easily regenerate plants from synthetic seeds, as long as these explants have a source of nutrients and if their the rooting process is not problematic. In many species, the regeneration of shoots and rizogenesis happen simultaneously (Gantait et al., 2015). In 1990, Bapat and Rao obtained *Morus indica* plantlets from synthetic seeds sown *in vivo*, without auxin treatments. Ganapathi et al., 1993, obtained 100% regeneration of *Musa* synthetic seeds sown on White's medium, without any treatment for rhizogenesis. In the case of using apical and axillar buds in species that have difficulties rooting, it is recommended that auxin treatments are applied to induce the process (Hussain et al., 2000). Capuano et al., 1998, obtained *Malus domestica* rootstock 'M26' plantlets from encapsulated apical and axillary buds using IBA in a concentration of 24.6 µM.

Shoot tips are frequently used as explants in the production of synthetic seeds. Explants can be relatively easily produced and these have the capacity to retain their viability and regeneration potential for a relatively long time (Piccioni & Standardi, 1995). Shoot tip encapsulation was studied in numerous species, such as *Solanum tuberosum* (Ghanbarali et al., 1995), *Ruta graveolens* (Ahmad et al., 2012), *Glochidion velutinum (*Mallikarjuna et al., 2016*), Helianthus annus* L. *(*Katouzi et al., 2011*), Vitis vinifera* (Benelli, 2016), *Begonia spp* (Sakhanokho et al., 2013), *Mentha arvensis* L. (Islam & Bari, 2012). Benelli (2016) encapsulated nodal segments and shoot tips of *Vitis vinifera* rootstock 'Kober 5BB' and after a storage period of 9 months at 4°C and dark conditions, the encapsulated nodal segments and shoot tips had regeneration rates of 55.6% and 83.3%, respectively. Katouzi et al. (2011) encapsulated shoot tips in alginate solutions containing different concentrations of salicylic acid and studied the viability of the explants after 90 days of cold storage. The addition of salicylic acid in the encapsulation matrix may increase the explant tolerance to cold temperatures during storage. The addition of 25 µM and 50 µM salicylic acid proved to have a beneficial effect on viability of the seeds and increased the viability rates to 48%, respectively 59%.

**Callus cells**

As a source of explant for synthetic seed production, callus cells are the least used The undifferentiated nature of these cells and the special conditions required for the differentiation of cells can be problematic when used for synthetic seeds (Gantait et al., 2015). Kim and Park (2007) regenerated *Allium sativum* plants from callus cells encapsulated in a solution of halved concentration MS salts, 1.5% sodium alginate, 3% sucrose, kinetin, NAA, and 50 mM $CaCl_2$. The encapsulated cells had a regeneration rate of 88%, after 40 days of storage at 4°C. Zych et al. (2005), successfully encapsulated differentiated callus cells of *Rhodiola kirilowiiI.* The percentage of developed shoots stored for 1-6 weeks at 4°C was registered between 95% and 100%.

***In vitro* conservation of *Solanum tuberosum***

Storing potato tubers requires growing them annually in nurseries, an operation that is time-consuming and exposes the vegetal material to pests, diseases, and climatic accidents (Roque-Borda, 2021). Conserving the germplasm in this species through true potato seeds is not possible, as this species is highly

heterozygous and the seeds produced are not true to type (Dodds et al., 1991).

Conservation using synthetic seed technology was achieved using nodal segments (Sarkar and Naik, 1998; Patel et al., 2000), axillary buds (Ghanbarali et al., 2016), shoot tips (Nyende et al., 2003; Patel et al., 2000) and somatic embryos (Maid et al., 2010; Fiegert et al., 2000), call (Patel et al., 2000).

Ghanbarali et al. (2016) concluded that the optimum beads were obtained using 3% sodium alginate and 1% $CaCl_2$ for the encapsulation matrix. Concentrations of Na-alginate lower than 3% produced capsules that were too fragile and difficult to handle, whereas at concentrations higher than 3%, capsules were too hard and the regrowth speed of the encapsulated buds was delayed. Nyende et al. (2003) investigated the encapsulation of *S. tuberosum* shoot tips in calcium alginate hollow beads. Sarkar and Naik, 1998, encapsulated *in vitro* derived nodal segments for potato propagule production, with direct sowing into the soil, in ex vitro conditions, with a 57% rate of survival in soil. The encapsulated segments were incubated for 3 days and treated with rooting powder at the time of planting.

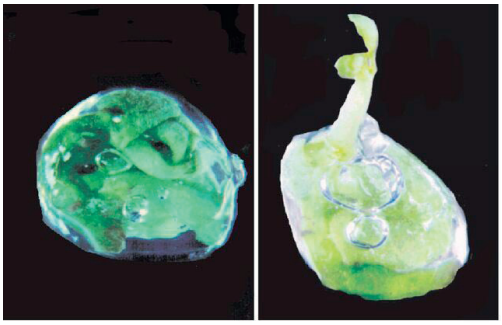

Figure 4. Synthetic seeds of *Solanum tuberosum;* left: before germination and right: after germination (Source: Bernand et al., 2002)

Nyende el al. (2003), concluded that shoot tips of *S. tuberosum* can be stored with a 100% regeneration rate for 180 days at 4°C and 10°C and at for 270 days at 4°C. Increasing the storage time to 360 days at 4°C, the recorded viability rates dropped between 70.8% and 51.5% depending on the cultivar that was encapsulated in the beads. It was also observed that shoot tips progressively turned brown during storage, faster at 10°C than at 4°C.

Ghanbarali et al. (2016) conserved *S. tuberosum* synthetic seeds for 90-120 days at 4°C without loss of viability, depending on the cultivar, using explants that were initially precultured on MS medium with 10-6 M 24-epibrassinolide for 2 days, which might improve the tolerance to cold temperatures. Preculture of the shoots in medium containing 10-6 M 24-epibrassinolide positively influenced the regrowth rate, speed, and shoot length for both the cultivars studied (‘Sante’ and ‘Agria’). 24-epibrassinolide (EBr) is a plant growth regulator from the brassinosteroids class and influences a range of processes of growth and development (Ghanbarali et al., 2016) and tolerance to different types of abiotic stresses: salt stress (Alam et al., 2019), high temperatures (Dhaubhadel et al., 1999; Singh and Shono, 2005; Pociecha et al., 2017).

The advantages of these type of seeds are that, like seeds obtained from sexual propagation, they are easy to store, as they don’t need a lot of space or special conditions, easy transportation, the possibility of using sowing equipment are the sterile environment in which they are stored provides protection from pests and diseases. Moreover, cryopreservation can be applied to increase the storage capacity of the encapsulated explants (Lambardi et al., 2006). Synthetic seeds, unlike orthodox seeds, don’t need a dormancy period for germinating and are genetically identical to the mother plant (Saxena et al., 2019).

## CONCLUSIONS

The risk of losing our biodiversity is increased nowadays, as more and more species are threatened with extinction, due to both climatic and anthropological factors, and conservation of plant germplasm is crucial. Traditional conservation in botanical gardens or field collections still exposes germplasm to threatening factors and *in vitro* methods become more and more reliable for plant conservation. But these methods generally come with a risk of somaclonal variations, and contamination, and they are time-consuming and manual labor is required, subcultures need to be reduced to a minimum. Synthetic seed technology combines the advantages of clonal

propagation with sexual propagation, as these are easy to store, to transport, sowing equipment can be used, they are protected against pests and diseases and they produce plants identical to the mother plant. Numerous studies have shown that synthetic seed technology can aid *in vitro* conservation of plant germplasm.

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