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PERSPECTIVE

THE ROLE OF ENCAPSULATION IN THE DEVELOPMENT OF PLANT CRYOPRESERVATION TECHNIQUES

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Abstract

Over the past 35 years, encapsulation technologies have played an important role in the development and diversification of cryopreservation techniques for cells and organized structures from numerous plant species. Encapsulation with calcium alginate was initially developed as a technological approach for the production of synthetic seeds. However, the coating of biological material with a hydrogel matrix subsequently promoted new applications that provided additional benefits to in vitro multiplication and preservation techniques. Alginate coatings can be complemented with growth regulators, antioxidants, and nanoparticles. These compounds have helped mitigate the toxic effects of cryoprotectants by regulating their penetration rate and have enabled the implementation of drastic desiccation treatments that would have otherwise been lethal. Furthermore, encapsulation facilitates the simultaneous handling of large quantities of samples, simplifying the ongoing handling required. Cryopreservation protocols based on encapsulation include Encapsulation-Dehydration, Encapsulation-Vitrification, V- and D-cryoplate methods. The objective of this review is to provide information on the impact of encapsulation of plant material on the advancement of cryogenic procedures and, consequently, on the understanding of tolerance of plant tissues to cryopreservation. In view of the advantages of encapsulation, it is considered that it will facilitate the long-term conservation of a greater number of plant species.

Keywords: calcium alginate beads; cryoplates; growth recovery; liquid nitrogen; vitrification-based procedures.

INTRODUCTION

Encapsulation of biological material is an useful strategy that offers practical advantages and involves the formation of a thin hydrogel matrix that covers cells or tissues without inhibiting their subsequent development. The synthetic capsules commonly used in plant biotechnology are produced through the chemical interaction of sodium alginate with calcium chloride and that reaction results in the formation of calcium alginate gel (1).

According to Standardi in 2012 (2), the concept of encapsulation was first proposed by Murashige in 1978, who suggested the use of this technology to protect embryos obtained in vitro and to facilitate their handling, transport and sowing (3). Later, encapsulation was expanded to

any meristematic plant tissue, which could be encapsulated with a synthetic nutritive coating, retaining its capacity to develop as a plant in in vitro or in vivo culture. Storage of encapsulated explants was then tested using various temperatures, including room temperature (15 - 20 °C) (4), 4 °C (5) and -196 °C after immersion of samples in liquid nitrogen (6).

For plant cryopreservation, i.e. the storage of cells and tissues at ultra-low temperature, usually that of liquid nitrogen (LN), encapsulation in calcium alginate has contributed to mitigate the detrimental effect of stressful treatments involved in the protocol. In addition, encapsulation has also broadened the array of available cryogenic techniques. The aim of this review is to provide information on the impact of encapsulation of plant material on the advancement of cryogenic procedures and, consequently, on the tolerance of encapsulated plant tissues to cryopreservation.

KEY PARAMETERS OF ENCAPSULATION-BASED METHODS FOR CRYOPRESERVATION

The use of encapsulation for cryopreserving plant germplasm was first reported in 1990 by the late Prof. Jean Dereuddre's working group in France. The publication that led to the development of a novel technique called Encapsulation-Dehydration (E-D) was successfully applied for cryopreserving potato shoot tips (7). Coincidentally, that year marked the beginning of the era of vitrification-based plant cryopreservation techniques, which are based on the principle of the direct transition of intracellular solutes from the liquid phase to an amorphous solid phase during rapid cooling of samples, that prevents the detrimental formation of ice crystals at low temperature (8). Among the vitrification-based procedures, E-D has probably been one of the most significant methods to date (9). This approach not only served as the starting point for the implementation of encapsulation in plant cryogenics but also led to the development of new alternative methods, such as Encapsulation-Vitrification (E-V) (10) and the so-called cryoplate procedures (Vitrification-Cryoplate [V-Cp] (11) and Dehydration-Cryoplate [D-Cp]) (12).

Encapsulation

For the encapsulation of biological material, the type of sodium alginate commonly used is low viscosity (13, 14). This aspect defines physical characteristics such as the viscosity and solubility of alginate and influences the rigidity of the polymerized form. The Ca^{2+} cation is the most widely used due to its low toxicity compared to other multivalent cations such as Ba^{2+} and Fe^{3+} (15).

The way encapsulation is used for plant cryopreservation defines the shape, volume and function of the gelled matrix. A spherical capsule can be formed by gradually dropping sodium alginate solution in a calcium chloride solution. The resulting capsule is typically 4-5 mm in diameter and can trap several explants within the bead, which, once encapsulated, are subsequently subjected to dehydration treatments and cooling in LN. This type of encapsulation is characteristic of the E-D and E-V techniques.

The other form of encapsulation involves the gelation of explants placed in small wells of prefabricated aluminum plates. In this case, the quantity of the gel layer is minimal and is delimited by the size of the well. Its main function is to fix the explants to the aluminum cryoplate and the adherent samples will then undergo the dehydration and cooling processes. This type of encapsulation is characteristic of the V-Cp and D-Cp techniques.

Calcium alginate encapsulation has been shown to have a positive impact both before and after sample cryopreservation. The benefits are associated, first, with physical protection during the freeze-thaw cycle and, second, with the culture conditions for recovery after cryopreservation (15).

Dehydration

In most cases, biological samples must be dehydrated to prevent the lethal formation of ice crystals at low temperature (13). Osmotic dehydration can remove large amounts of freezable water from cells, either by using a single chemical agent at high concentration or by combining several of them in specific formulations. The best-known mixtures are called plant vitrification solutions (PVS). However, given the high concentrations required and the composition of PVSs, cryoprotective treatments can exert a critical toxic effect, especially when working with tropical plant species, which are usually more sensitive (16).

Chemical toxicity of cryoprotectants can be mitigated by the surface coating provided by calcium alginate gel. Encapsulation itself slows down the penetration of cryoprotectants into

tissues and cell groups without the need to reduce the temperature to achieve the same effect during dehydration. In addition, the synthetic matrix also enables drastic desiccation treatments that can replace a severe osmotic dehydration treatment in cases of high sensitivity of the samples to any PVS. Encapsulated explants can be exposed for several hours to desiccation with silica gel or under the airflow of a laminar flow cabinet. However, without previous encapsulation, drastic physical evaporation would not be possible without killing the tissues due to over-dehydration (17).

Cooling and rewarming

After the cryoprotection stage, rapid cooling is essential to promote vitrification of the internal solutes and of the surrounding cryoprotective medium during the temperature decrease. Calcium alginate encapsulation allows successful cryopreservation of organized structures such as apices and somatic embryos using both rapid and ultrarapid cooling rates. Rapid cooling is achieved by plunging the cryovials containing alginate beads with the encapsulated samples in LN (E-D technique). Samples can also be rapidly cooled in cryovials with a small volume of PVS surrounding the beads. This latter procedure is characteristic of the E-V technique. Ultrarapid cooling is achieved by direct immersion in LN of samples encapsulated on aluminum cryoplates (using the V-Cp and D-Cp methods).

Similarly, rapid warming is required to prevent devitrification or recrystallization of internal solutes during the rewarming phase. With the E-D and E-V techniques, rapid warming is usually achieved by placing the cryovials with samples in a water-bath at +37°C for 2-3 min. With the V-Cp and D-Cp methods, ultrarapid rates of rewarming are achieved by transferring directly the cryoplates to an unloading solution supplemented with 1 M sucrose at room temperature.

Using the V-Cp method, the cooling and warming rates of Dalmatian chrysanthemum (*Tanacetum cinerariifolium*) shoot tips placed on the plates and measured with a platinum thermocouple, were approximately 79.5 °C s^{-1} and 75.4 °C s^{-1}, respectively (14).

Encapsulation can sometimes delay the conversion of explants into new shoots. This delay has been linked to the quality of the biological material subjected to cryopreservation, to the excess $CaCl_2$ salt remaining in the synthetic matrix and to the size of the beads (18). However, although the growth reactivation response is initially delayed, the encapsulated material generally overcomes this delay quickly if viability is not affected by cryopreservation (13).

Growth recovery

To remove the excess $CaCl_2$, which could interfere with regrowth and/or damage tissues, some authors have included a washing step after polymerization of the beads, either by using a liquid culture medium devoid of $CaCl_2$ (9) or sterile distilled water (19). Another important washing step is mandatory when samples have been osmotically dehydrated with PVS. In that case, encapsulated samples are usually washed using an unloading solution consisting of liquid medium supplemented with 1.2 M sucrose. The objective is to reduce the toxicity of the cryoprotectants, to avoid osmotic shock and to prepare the explant for regrowth after rewarming (20). When using the V-Cp procedure, the rewarming method by immersing the cryoplates in the 1 M sucrose solution serves a dual purpose: washing the PVS and rewarming the samples.

After rewarming, an additional factor to consider is whether it is necessary to extract the cryopreserved explants from the surrounding gel to ensure faster recovery. Explants can be manually removed from the synthetic matrix, thus facilitating their direct contact with the fresh culture medium, accelerating nutrient absorption and ensuring adequate tissue aeration. Examples of cryopreserved explants that required removal from alginate beads for recovery include oilseed microspore embryos (21) and grape shoot tips (22). When using the V-Cp and D-Cp methods, any gel that fails to detach from the explants during warming is usually carefully removed manually.

Another alternative for eliminating the synthetic coating is the use of sodium citrate to dissolve the calcium alginate matrix (23). Sodium citrate acts as a calcium sequestrant, allowing the release of biological material trapped in the gel. To our knowledge, the use of this method has not yet been reported for plant cryopreservation, but it was successfully used to rescue citrus embryos formed during in vitro culture of encapsulated protoplasts. It could represent an additional alternative to recover encapsulated and cryopreserved material without the risk of mechanical damage during manual extraction of the explants.

On the other hand, some authors have reported that the incorporation of certain

additives into the encapsulation medium could improve the response to cryopreservation and provide better conditions for recovery.

A first approach was to supplement alginate beads with higher sucrose concentrations, although beads usually contain sucrose at the level used in the normal culture medium (13). A higher concentration of sucrose (0.4 M) was added to alginate beads to cryopreserve wasabi (24) and strawberry (25) meristems using the E-D technique. Growth recovery was 40 % and 67 %, respectively. In the case of wasabi meristems, regrowth after cryopreservation was further increased (up to 90–100 %) by supplementing the alginate beads with 0.4 M sucrose + 2 M glycerol following the E-V procedure (26).

More recently, the combination of various types of nanoparticles (NPs) such as gold (AuNPs), silver (AgNPs) and zinc oxide (ZnONPs), with calcium alginate encapsulation, improved the ex vitro performance of plants derived from cryopreserved shoot tips (27). Nanoparticles can deliver and enhance the distribution of cryoprotective agents, particularly those to which the cell membrane is impermeable, such as trehalose (studies in mammalian cells), whose effectiveness is greater when it is found both inside and outside the cell (28). In addition, NPs may reduce the mechanical stress that ice produces by limiting the ice crystal growth during the freeze-thaw cycle. Another benefit is that NPs can stimulate the biosynthesis of secondary metabolites and the production of substances linked to defense mechanisms and antioxidant functions (29). Therefore, integrating NPs into the hydrogel may enhance survival and regrowth after cryopreservation. Kulus and Tymoszuk, 2021 reported that AuNPs added to calcium alginate beads improved recovery of cryopreserved shoot tips of *Lamprocapnos spectabilis* following an E-V protocol (30).

Oxidative stress represents another major problem affecting plant recovery after cryopreservation. However, the beneficial effects of melatonin (N-acetyl-5-methoxytryptamine) during cryopreservation have been demonstrated by improving the regeneration efficiency of ginseng (*Panax quinquefolius*) (31) and American elm (*Ulmus americana* L.) (32) shoot tips. The inclusion of melatonin in the calcium alginate coating also produced significant antioxidant action in reducing oxidative stress and contributed to plant regrowth of yam (*Dioscorea alata* and *D. cayenensis*) shoot tips after cryopreservation (33). The antioxidant effect of melatonin has been associated with its activity as a direct ROS scavenger, in addition to its signaling role in metabolic regulation (34, 35). The encapsulation of light- and oxygen-sensitive antioxidants in the calcium alginate matrix is a convenient alternative for prolonging their release to cells or tissues during cryopreservation.

Another suggested supplement is chitosan, a polyamine saccharide derived from chitin and a major component of crustacean cuticles (36). Chitosan has been proposed as potential additive to alginate beads due to its antimicrobial properties and antioxidant functions as oxidative molecule scavenger (37, 38).

The addition of activated charcoal to synthetic beads also demonstrated a beneficial effect. Activated charcoal promoted the growth of *Castanea sativa* embryonic axes after cryopreservation using an E-V protocol. The development period of the explants was shortened and their viability increased from 50% to 70% compared to those encapsulated without this additive (39).

Therefore, all these practical examples demonstrate that the incorporation of specific additives into the synthetic matrix can improve the effectiveness of the protocol used for the long-term conservation of plant germplasm.

Other important factors, such as a suitable recovery medium and control of light conditions, are also determining parameters for in vitro regrowth regardless of the cryopreservation technique used. The most comprehensive review of these critical aspects was recently published by Popova et al. (16).

Plant growth regulators (PGRs) are often essential components of the culture medium for the recovery of cryopreserved samples. The supplementary balance provided by PGRs depends on the type of biological material and the culture objectives: cell proliferation, morphogenesis or embryogenesis. Furthermore, the initial action of PGRs is known to promote first the recovery of survival and, subsequently, the morphogenic response of cryopreserved samples.

An example of PGRs added to the shoot tip recovery medium of four genotypes of *Malus* x *domestica* cryopreserved using the E-D and E-V methods was the combination of BA (0.5 mg L^{-1}) and IBA (0.05 mg L^{-1}) (40). In contrast, following the same cryogenic procedures (E-D and E-V), *Olea europea* somatic embryos did not require addition of PGRs for recovery (41).

On the other hand, control of physical conditions of incubation is also important. After cryopreservation, samples are always cultured in darkness for at least the first 7 d to prevent the accumulation of photooxidative effects, particularly reactive oxygen species (ROS), which are harmful and can be lethal. When samples are subsequently exposed to photoperiod, the intensity and spectrum of the illumination can be modulated. The most common condition is the use of cool white light provided by fluorescent lamps. However, it has been reported that a combination of red LED light with 10 % blue light doubled the regeneration percentages of five potato cultivars after cryopreservation (42). These results suggest that modifying the light spectrum during the regrowth phase may improve post-cryopreservation regeneration in other plant species.

BRIEF DESCRIPTION OF ENCAPSULATION-BASED CRYOGENIC PROCEDURES

Encapsulation-dehydration

This technique combines synthetic seed production and dehydration technologies (43, 7). This was the first procedure proposed to incorporate encapsulation of plant material in a cryopreservation protocol.

An E-D protocol comprises the following successive steps:

- Selection and isolation of plant material, preferably derived from in vitro cultures.
- Encapsulation: Preparation of a calcium-free liquid basal medium supplemented with 3 % (w/v) sodium alginate (low viscosity, 250 cps) and sucrose at the usual concentration of the culture medium. The alginate solution is mixed with the explants and gradually dropped in a previously prepared liquid medium containing a high concentration (0.1 M) of calcium chloride, using a cut-tip pipette. Gelation of the beads in the calcium solution takes 20 to 30 min, to ensure complete polymerization.
- Preculture: The beads containing the explants are precultured directly for 1 d in liquid medium with a high concentration of sucrose (usually 0.75 M) or by exposure to daily increasing concentrations of sucrose (from 0.50 to 1.25 M). Preculture treatment in agitated liquid medium can last from 1 to 7 d depending on the sucrose concentration and on the species.
- Desiccation: The beads are exposed to the air current of a laminar flow cabinet or to silica gel in hermetically sealed containers. In general, the bead water content that ensures the highest viability after cryopreservation is around 20 % (FW basis). It depends on the species and on the type of samples (size of explants and beads).
- Cooling: After dehydration, the beads are transferred directly to sterile polypropylene cryovials, which are rapidly immersed in LN. However, slow freezing before immersion in LN has also been used to achieve greater survival in specific cases (44, 45).
- Rewarming: Slow rewarming is usually performed at room temperature by removing the beads from the cryovials and exposing them to the air current of a laminar flow. Rapid warming is performed by placing the cryovials in a water bath at 37 °C for 2-3 min.
- Growth recovery: After cryopreservation, the encapsulated samples are usually placed on a medium with the same composition used for micropropagation of donor mother plants. Samples are kept in the dark for at least the first week of culture to prevent photooxidation damage and then transferred to standard light conditions. Conditions at this stage depend on the species and on the type of plant material. In some cases, it is necessary to remove the explants from the beads to speed their recovery.

Encapsulation-vitrification

E-V combines the encapsulation-dehydration and vitrification protocols. This procedure was first developed by Tannoury et al., in 1991 (10).

An E-V protocol comprises the following successive steps:

- The selection and encapsulation steps are the same as those described for the E-D technique.
- Loading treatment: The beads containing the explants are exposed to a loading solution containing 2 M glycerol and 0.4 M sucrose (46) for 20 min at room temperature.
- Dehydration: After loading, the encapsulated samples are exposed to a PVS solution at room temperature until the level of dehydration reached guarantees the highest possible viability after immersion in LN. The most used PVS are PVS2 solution [13.7%

(w/v) sucrose, 30.0% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) dimethylsulfoxide] (47) and PVS3 solution [50% (w/v) glycerol and 50% (w/v) sucrose] (48). Nevertheless, Kim et al., suggested other alternative combinations of PVS (49).

- Cooling: After dehydration, the beads are directly transferred into sterile polypropylene cryovials containing a minimal volume of fresh PVS and then the cryovials are rapidly immersed in LN.
- Rewarming: Rapid rewarming is always performed by removing the cryovials from LN and placing them in a water bath at +37 °C for 2-3 min.
- Washing of cryoprotectants: The PVS is eliminated at room temperature using an unloading solution composed of liquid culture medium supplemented with 1.2 M sucrose for 15 min (50).
- Growth recovery: This step is the same as described for E-D procedure.

Cryoplate techniques

The cryoplate methods were developed from 2011 onwards. First, the V-Cp cryoplate procedure by Yamamoto et al. in 2011 (14), which combines encapsulation-vitrification and droplet-vitrification; later, Niino et al. in 2013 (51) introduced the D-Cp technique, which combines encapsulation of samples and desiccation. D-Cp applies the same physical dehydration procedure as the E-D technique, thus eliminating the potential chemical stress caused by PVS solutions (52).

The main characteristics of the aluminium (99.5% purity) cryoplates used in both cryogenic methods are: length (37 mm) × width (7 mm) × thickness (0.5 mm). They are specifically designed to fit in 2 mL cryovials, and each one has 10 wells. There are four well sizes, which are suitable for different explant sizes. All cryoplates are custom manufactured by the Japanese company Taiyo Nippon Sanso Corp., Tokyo, Japan (53).

Vitrification cryoplate (V-Cp). The V-Cp protocol comprises the following successive steps:

- Selection and isolation of plant material, preferably originating from in vitro cultures.
- Encapsulation: the sodium alginate solution (2% w/v), prepared in calcium-free culture basal medium, with or without 0.4 M sucrose, is poured into the wells of the aluminium plate using a micropipette. The explants are placed one by one in the wells and the calcium chloride solution (0.1 M) dissolved in basal culture medium (with or without 0.4 M sucrose) is poured carefully over the section containing the tissues in alginate inside the wells. Complete polymerization is usually achieved after 15 min, and the calcium solution is then carefully removed from the cryoplates with a micropipette or by gently tapping the plates on filter paper for absorption.
- Loading treatment: The cryoplates holding the samples are exposed to a loading solution usually composed of 2 M glycerol and 1.0 M sucrose for 20 min at room temperature. The sucrose concentration of the loading solution can vary from 0.4 to 1.6 M (14).
- Dehydration: After loading, the cryoplates with samples are exposed to a PVS solution at room temperature until the level of dehydration is reached that guarantees the highest possible viability after immersion in liquid nitrogen. Vitrification during cooling will not cause additional viability loss beyond that produced during PVS dehydration under optimal conditions.
- Cooling: After performing the dehydration treatment, the cryoplates are rapidly immersed in LN. Direct contact of the samples with LN produces ultra-rapid cooling due to the high thermal conductivity of the aluminium plates and to the minimal layer of calcium alginate gel covering the explants.
- Rewarming: Rewarming of samples is performed by rapid transfer of the aluminum cryoplates in an unloading solution containing basal culture medium supplemented with 1 M sucrose for 15 min at room temperature.
- Growth recovery: After cryopreservation, the samples detached from the cryoplates during unloading are transferred, free of gel residue, to a culture medium with the same composition used for micropropagation of the donor mother plants. The samples are kept in the dark for at least the first week of culture before being exposed to standard light conditions. As previously described, the conditions at this stage depend on the species and type of plant material.

Dehydration-cryoplate (D-Cp). The D-Cp protocol comprises the following successive steps:

- The selection, encapsulation, loading treatment, cooling, rewarming and growth recovery are performed as previously described for V-Cp. However, after the loading treatment, dehydration is performed using any of the desiccation procedures implemented with the E-D technique.
- Dehydration: The cryoplates holding the samples are first placed on filter paper to remove the excess loading solution and then they are exposed to the airflow of a laminar flow cabinet. Optimal drying time depends on the laminar flow capacity and relative humidity at room temperature. Silica gel in airtight containers can also be used for this purpose. The optimal dehydration period in both cases is usually between 30 and 180 min (53).

SELECTED EXAMPLES OF ENCAPSULATION-BASED CRYOPRESERVATION PROCEDURES

Figure 1 is a graphic representation of all the previously described encapsulation-based cryopreservation techniques, which shows that the advances achieved not only allowed the direct immersion of biological material in LN, eliminating the need for programmable freezers, but also led to the diversification of protocols for different types of materials (cell suspensions, embryogenic tissues, shoot apices, seeds, etc.). Table 1 presents some representative cryopreservation studies using these four methods.

Cryopreservation of sugarcane (*Saccharum officinarum*) shoot tips is one of the most illustrative examples of the successful adaptation and application of different encapsulation-based techniques. E-D was the first procedure that allowed the survival and regeneration of cryopreserved shoot tips using two slightly different protocols (54) and (55) but following the same approach (13). Subsequently, two other encapsulation-based techniques, V-Cp (11) and D-Cp (12) were successfully applied to sugarcane apices. The average recovery of 15 sugarcane varieties cryopreserved using E-D was 62 %. The average recovery of 11 sugarcane varieties applying V-Cp was 70%, and the average recovery of the same 11 varieties using D-Cp was 52%. These results obtained in different laboratories of three countries (France and Cuba for E-D and Japan using both cryoplate methods), demonstrated the great adaptability of these cryogenic techniques to many sugarcane varieties with high efficiency (over 50 %). Furthermore, another significant finding was that the application of the optimized V-Cp protocol to shoot tips of two sugarcane cultivars allowed the elimination of sugarcane mosaic virus (SCMV) from infected plants, a process called cryotherapy (56).

On the other hand, Niino et al. in 2013 reported that D-Cp produced a significantly higher average regrowth than V-Cp (86.3% vs 52.5%, respectively), when cryopreserving shoot tips from the same 20 mat rush lines (51). A similar trend was observed when comparing both techniques for cryopreserving *Ullucus tuberosus* shoot tips (57). In both cases, physical evaporation (D-Cp) was less detrimental than osmotic dehydration (V-Cp) induced by PVS. However, when comparing the same methods to cryopreserve shoot tips of *Clinopodium odorum*, V-Cp was more effective than D-Cp (58).

Comparing V-Cp with D-V and slow freezing for cryopreserving mint (*Mentha* spp.) shoot tips showed that V-Cp was the most effective, especially when applied to a large group of species (59). V-Cp also resulted in higher regrowth (93 %) compared to D-V (80 %) and vitrification (68 %) for cryopreservation of *Stevia rebaudiana* shoot-tips (60, 61).

In our study of vegetative growth of *Vanilla planifolia* Jacks ex Andrews plants originating from cryopreserved shoot tips, after one year of growth under greenhouse conditions, D-Cp and D-V allowed greater ex vitro regrowth and revealed higher genetic integrity assessed with seven ISSR primers compared to V-Cp (62).

More recently, Halmagyi & Coste, (2025) reported that D-V and E-D were similarly effective for the cryopreservation of *Moehringia jankae* Griseb. ex Janka, an endangered European species. The highest regrowth was 83% with D-V and 85% with E-D (9).

Encapsulation-based cryopreservation procedures have also been employed for several economically important crops at the cryobank level: *Fragaria* spp. using E-D in the National

German Strawberry Genebank (63), sweet potato with E-V in the USDA-ARS National Laboratory for Genetic Resources in Fort Collins, USA (64), and *Solanum* spp. using both V- and D-Cp methods in NARO Genetic Resource Center in Japan (65). Therefore, the adaptability of encapsulation-based cryogenic protocols to manipulate large numbers of samples has also favoured their operational scaling in genebanks and complementarily supported the application of various cryopreservation techniques.

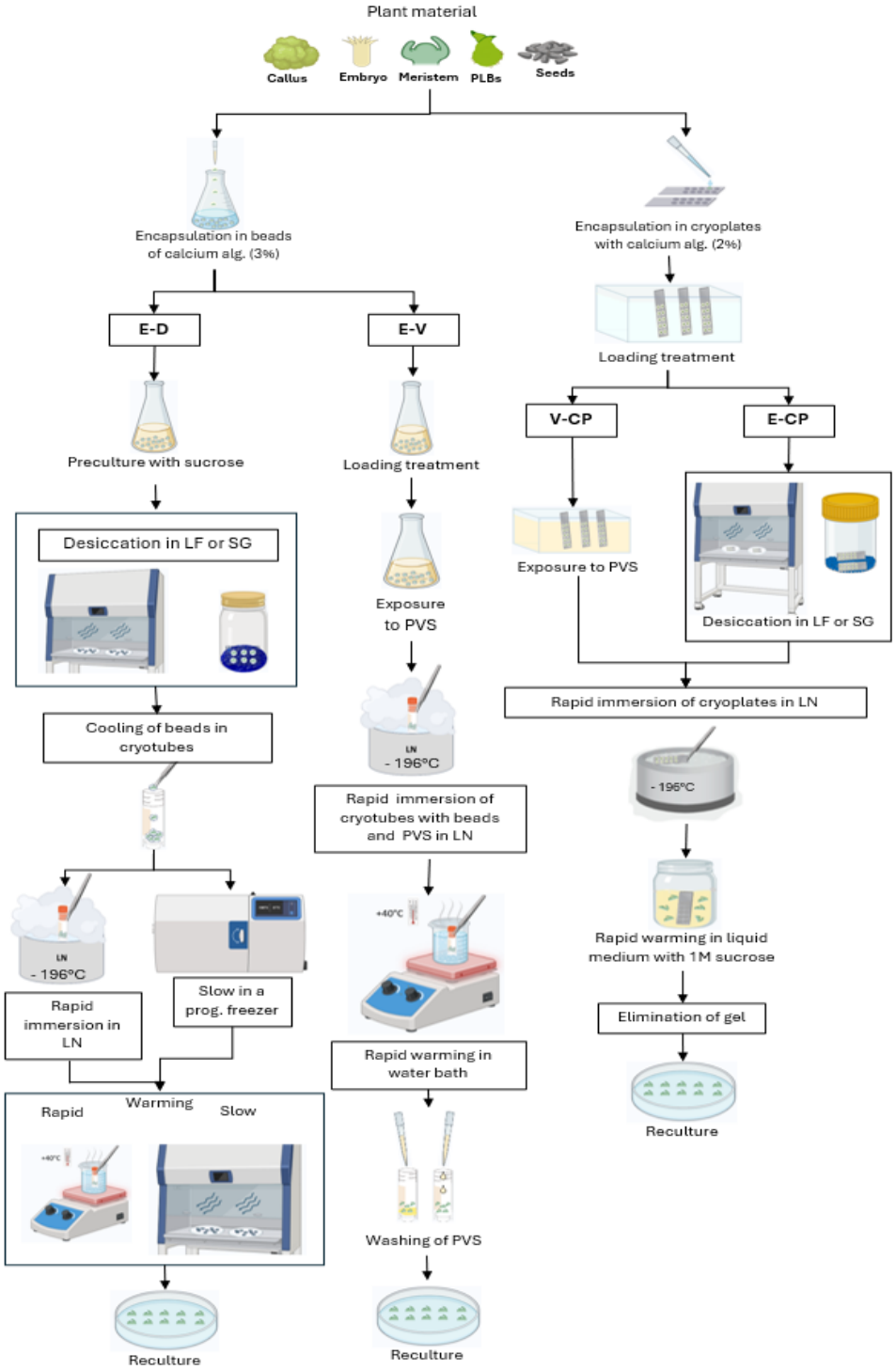

Figure 1. Graphical representation of encapsulation-based procedures for plant cryopreservation.

Table 1. Selected examples of encapsulation-based techniques used to cryopreserve different types of biological materials.

Species	Biological material	Technique	Ref
Medicago sativa L.	Cells	E-D	(67)
Vitis vinifera L.			(68)
Dioscorea bulbifera L.			(69)
Vitis spp.		E-V	(70)
Vitis vinífera			(71)
Vitis spp.			(72)
Phoenix dactylifera	Callus	E-D	(73)
Anemarrhena asphodeloides Bunge		E-V	(74)
Areca catechu L.		V-Cp	(75)
Arum palaestinum			(76)
Rubus idaeus L.	Shoot-tips	E-D	(77)
Saccharum spp.			(78)
Prunus armeniaca L.			(79)
Fragaria spp.			(80)
Ananas comosus (Stickm.) Merr.		E-V	(81)
Hladnikia pastinacifolia Rchb.			(82)
Lamprocapnos spectabilis L.			(27)
Papaveraceae Cultivars			(29)
Clinopodium odorum		V-Cp	(58)
Vanilla planifolia Jacks.			(83)
Saccharum spp. *h*ybrids			(56)
Stevia rebaudiana Bertoni			(84)
Prunus domestica L.			(85)
Vanilla planifolia Jacks.		D-Cp	(83)
Citrus spp.	Somatic embryos	E-D	(86)
Theobroma cacao L.			(87)
Quercus suber L.			(88)
Olea europea L.		E-V	(41)
Saccharum CP52-43			(89)
Petiveria alliacea		V-Cp	(90)
Agave tequilana Weber cultivar ‘Chato’			(91)
Phoenix dactylifera L.	Polyembryonic masses	D-Cp	(92)
Podophyllum hexandrum Royle	Zygotic embryos	V-Cp	(93)
Oncidium bifolium S.	Seeds	E-D	(94)
Lilium ledebourii'(baker) Bioss			(95)
Dendrobium cruentum Rchb. F.		V-Cp	(96)
Phalaenopsis bellina	PLBs	E-D	(97)
Cymbidium Twilight Moon ‘Day Light’			(98)
Dendrobium Bobby Messina			(99)
Dendrobium candidum Wall. ex Lindl.		E-V	(100)
Vanda lilacina Teijsm. & Binn.		D-Cp	(101)
Oncidium bifolium Sims.	Protocorms	E-D	(94)
Maesa lanceolata & *Medicago truncatula*	Roots	E-D	(102)
Rubia akane (Nakai)		E-V	(103)
Cocos nucifera L.	Plumules	E-D	(104)
Quercus petraea (Matt.) Liebl.		D-Cp	(105)
Chaetoceros mueller	Algae	E-V	(106)
Passiflora pohlii	Nodal segment		(107)
Juncus decipiens Nakai	Buds	V-Cp	(51)
Cannabis sativa L.			(84)
Diospyros kaki Thunb. ‘Saijo’	Dormant buds	D-Cp	(108)

EVOLUTION IN THE PUBLICATION OF SCIENTIFIC ARTICLES ON ENCAPSULATION-BASED CRYOGENIC TECHNIQUES

Technological advances are always supported by scientific publications that explain and describe the progress achieved in different laboratories and research groups around the world. A review based on Scopus, Google Research and Web of Science records produced a total of 379 articles on encapsulation-based cryopreservation techniques between 1990 and September 2025. The main centres of technological development and scientific dissemination of these procedures have so far been France and Japan. In Latin America, countries such as Cuba, Brazil and Argentina were the first to contribute to the publication of results from collaborative projects incorporating these technologies (66).

Figure 2 shows a bibliometric analysis of scientific articles on plant cryopreservation based on the use of encapsulation. The evolution of each cryogenic procedure from its beginnings to the present is represented. Overall, the number of publications showed an upward trend over the years for all techniques. The highest number of publications was recorded between 2011 and 2015 for E-D, between 2011 and 2020 for E-V, between 2021 and 2025 for V-Cp and between 2016 and 2020 for D-Cp, which maintained a similar publication level until September 2025.

Another important remark is that these publications demonstrated the high efficiency of all encapsulation-based protocols for over 100 species and their successful adaptation to various types of materials (see some examples in Table 1). In addition, they reflect relevant national and international collaborations between specialists from the five continents.

CONCLUSION

Encapsulation-based cryopreservation techniques offer numerous advantages, which can be summarized as follows:

i. Easy handling of explants throughout the procedure, as only calcium alginate beads or cryoplates holding large numbers of samples are manipulated.
ii. No risk of harming or losing explants during transfers throughout the cryopreservation protocol, as beads or cryoplates are manipulated instead of naked explants.
iii. Rapid cooling by direct immersion of samples in LN, which avoids the use of programmable freezers and results in the vitrification of intracellular solutes and extracellular medium during cooling.
iv. Rapid rewarming of samples, avoiding the possibility of devitrification and/or ice recrystallization during temperature increase.

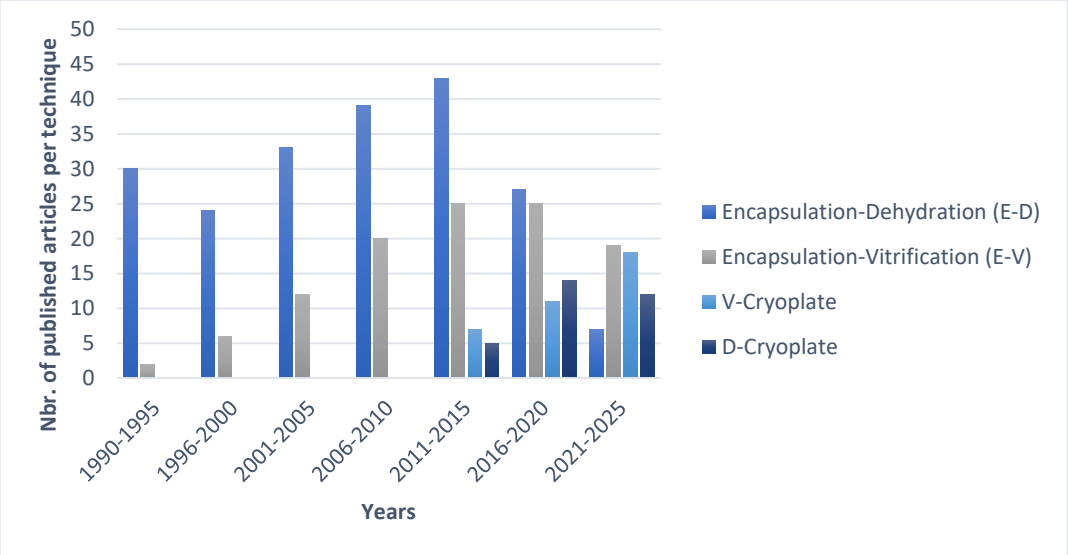

Figure 2. Evolution of publications on encasulation-based cryopreservation techniques for plant materials between 1995 and 2025.

v. High regrowth (%) after cryopreservation of large explants originating from tropical and/or subtropical species.
vi. Applicability of these techniques for the eradication of viruses by cryotherapy.

Consequently, and based on the advantages described above, the transfer of these efficient techniques to different laboratories and cryobanks around the world is straightforward and will expand their range of application. This should significantly contribute to increase the number of plant species which are safely stored for the long-term using cryopreservation. We expect that these advantages will be used to develop new cryopreservation protocols more suitable for cryobanks in the genebank system, in order to preserve larger numbers of plant genetic resources.

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