Agriculture and Food Sciences Research
Vol. 12, No. 2, 123-130, 2025
ISSN(E) 2411-6653/ ISSN(P) 2518-0193
DOI: 10.20448/aesr.v12i2.7621

Cryopreservation and its effects in coffee-tree (*Coffea* spp.) culture

Yanelis Castilla Valdés[1]
María Esther González Vega[2]

[1,2]*Department of Plant Genetics and Breeding, National Institute of Agricultural Sciences, Mayabeque, Cuba.*
[1]*Email: yanelis@inca.edu.cu*
[2]*Email: esther@inca.edu.cu*

(✉ Corresponding Author)

Abstract

Coffee is one of the most commercialized beverages worldwide. It is obtained from the fruits of the coffee plant (*Coffea spp*), a genus that is difficult to conserve because its seeds tolerate a certain amount of desiccation but become sensitive to the low temperatures used in conventional germplasm banks. Hence, the importance of using other methods, such as cryopreservation, which allows for storage over extended periods. During this process, tissues are subjected to different treatments and methods that can cause cryogenic damage and stress. To determine their occurrence and rule out the appearance of genetic variability, various analyses are performed, ranging from morphological to epigenetic. The present bibliographic review was conducted to identify the main research on cryopreservation and its effects on coffee cultivation. The analyses applied to evaluate cryogenic damage were studied at the morphological, histological, physiological, and biochemical levels. The effects detected represent tissue responses to stress caused by this long-term preservation technique. Molecular studies have not been reported so far; thus, it is necessary to continue deepening these analyses to achieve their complementarity, in order to corroborate the genetic stability of the coffee plants recovered from cryopreservation.

Keywords: Conservation, Cryodamage, Genetic stability, In vitro culture, ROS, Stress, Vitrification.

Citation | Valdés, Y. C., & Vega, M. E. G. (2025). Cryopreservation and its effects in coffee-tree (Coffea spp.) culture. *Agriculture and Food Sciences Research*, *12*(2), 123–130. 10.20448/aesr.v12i2.7621
History:
Received: 15 September 2025
Revised: 20 October 2025
Accepted: 27 October 2025
Published: 31 October 2025

Publisher: Asian Online Journal Publishing Group

Funding: This study received no specific financial support.
Institutional Review Board Statement: Not applicable.
Transparency: The authors confirm that the manuscript is an honest, accurate, and transparent account of the study; that no vital features of the study have been omitted; and that any discrepancies from the study as planned have been explained. This study followed all ethical practices during writing.
Competing Interests: The authors declare that they have no competing interests.
Authors' Contributions: Both authors contributed equally to the conception and design of the study. Both authors have read and agreed to the published version of the manuscript.

Contents

Contribution of this paper to the literature
This study consolidates, for the first time in the literature, research conducted on the effects of cryopreservation on coffee cultivation. It provides new insights into the methods used to detect genetic stability after long-term preservation of this genus and enhances understanding of the causes of cryogenic damage.

1. Introduction

Coffee represents one of the tropical agricultural products of higher importance, contributing to the economy of about 80 coffee grain producer countries. Its cultivation currently faces numerous challenges, with climate change being one of the most relevant, as it influences biotic and abiotic factors that affect its phytogenetic resources [1, 2]. Although 130 species of *Coffea* spp. are currently known [3], world production is based mainly on two species: *Coffea arabica* L. and *C. canephora* Pierre [4].

In view of their characteristics during conventional storage, coffee seeds have been classified as intermediate, since they tolerate a certain level of desiccation but do not survive total desiccation or the combined effects of desiccation and low temperatures [5]. For this reason, other *ex situ* conservation methods are applied to achieve their preservation for longer periods of time, such as cryopreservation.

Cryopreservation is a process in which cells or tissues are preserved by freezing at ultra-low temperatures, below zero degrees, such as -196°C (boiling point of liquid nitrogen). At these low temperatures, metabolic processes are slowed down [6], including biochemical reactions that can cause aging and cell death, so that material maintained under these conditions can theoretically be stored for prolonged periods [7]. This is the method of choice to ensure long-term storage of germplasm of unorthodox vegetatively propagated seeded plant species or important cell lines [8].

The different cryopreservation methods for long-term preservation of germplasm *in vitro* comprise numerous stages, ranging from tissue culture, pre-growth or dehydration, cryoprotection, freezing, rewarming, material recovery, and regeneration. Consequently, tissues are exposed to physical, chemical, and physiological stresses, which can result in cryodamage. Successful cryopreservation is often evaluated by the survival of plant tissues and their ability to regenerate whole plants [9].

Several cryogenic studies have been conducted to preserve coffee plant genotypes using various methods over extended periods. However, many of these studies are limited to reporting only the germination results or the viability of cryopreserved explants, without further analysis or long-term assessment [10-13], while others refer to morphological, histological, biochemical, or physiological aspects of the seeds, embryos, or seedlings recovered after the application of this technique [14–26]. The present bibliographic review was conducted to identify the main research conducted on cryopreservation and its effects on coffee cultivation.

2. Cryopreservation

Cryopreservation, or the storage of biological material at the temperature of liquid nitrogen (-196 °C), is an alternative to *ex situ* germplasm conservation, which makes it possible to maintain plant genetic resources in the long term in a safe and cost-effective manner [27].

Liquid nitrogen is "relatively inexpensive, chemically inert, readily accessible, and independent of electricity" [28]. At this temperature, metabolic processes are slowed down so that plant material can be stored for very long periods of time [5].

In addition, cryopreservation eliminates the need for regular renewal of the collection, which reduces the risk of genetic erosion caused by pests, diseases, climatic conditions, contamination, and genetic variations [29]. It therefore represents a safe and cost-effective option for the long-term preservation of all categories of species that are difficult to preserve by other methods [5].

Among the main cryopreservation methods are: the conventional protocols or classical method (slow freezing), encapsulation-dehydration, pre-culture-desiccation and desiccation, vitrification, and derived protocols (droplet-vitrification, encapsulation-vitrification, and cryo-plate) [30], and the more recently developed cryo-mesh [31]).

Classical method: The classical method is based on slow freezing of the explants (0.1 to 2 °C min^{-1}) usually down to -40 °C, and then their rapid immersion in liquid nitrogen. Dehydration of the samples is achieved during the temperature decrease, although solutions with cryoprotective properties or a physical dehydration step can also be used previously. Disadvantages of this method include the complexity of the operation, the use of equipment that controls the cooling rate (programmable freezers), and the formation of ice crystals in the extracellular solutions, which can cause loss of water from the cells in the so-called freeze-dehydration process [32].

Encapsulation-dehydration: This technique has the advantage of being relatively simple, since it facilitates handling a large number of explants enclosed in calcium alginate capsules at the same time and uses only sucrose as an osmotic agent. It replaces the use of expensive and sophisticated programmable freezing equipment with rapid immersion in liquid nitrogen. Return to normal culture temperature is usually accomplished by exposing the encapsulated material to flowing air in a laminar flow hood, instead of using a 40 °C water bath [30].

Vitrification method: Vitrification protocols have the common characteristic that the critical step to achieve the survival of the cryopreserved material is the dehydration stage, not the freezing stage, as occurs in classical protocols. In these procedures, the removal at positive temperatures of most or all of the freezable water from the cells is promoted through osmotic dehydration using highly concentrated solutions [32]. Among these are the so-called Plant Vitrification Solutions or PVS. They are composed of penetrating and non-penetrating cryoprotective agents used to increase osmolarity in plant tissues and reduce the free water content of the cells [33].

To avoid phase transitions at ultra-low temperatures, without excessively reducing the water content of the cells, vitrification, or solidification of a liquid forming an amorphous or glassy structure, must be achieved [6, 34]. Thus, in the amorphous solid state, the molecules associate without acquiring the organized structure characteristic of crystallization [35].

Highly concentrated solutions sufficiently dehydrate the cells and cause the solute molecules to hinder the organization of water molecules that can form ice crystals. The ultra-fast cooling rate prevents sufficient time for ice crystals to form before all molecules are immobilized by the temperature of the liquid nitrogen. These two conditions allow the formation of the glassy state [34].

The most commonly used plant vitrification solutions are: PVS 2, composed of 30% glycerol +, 15% ethylene glycol +, 15% dimethyl sulfoxide +, and 0.4 M sucrose [36], and PVS 3, composed of 50% glycerol + 50% sucrose [37].

- Droplet-Vitrification. It is derived from the vitrification method and differs from it in the ultra-fast cooling and heating speeds achieved to treat the samples. Instead of using cryovials, the tissues are transferred to a reduced volume of a drop of the vitrification solution, placed on a small sheet of aluminum foil, in which they are directly immersed in liquid nitrogen [38].
- For rewarming, the foil with the samples is immersed directly in liquid culture medium supplemented with 1.2 M sucrose [39] at room temperature. The excellent thermal conductivity of the aluminum foil, combined with the small volume of cryoprotective solution in contact with the tissues, allows both cooling and heating to proceed at a very high rate [40].
- Encapsulation-Vitrification. In this method, the tissues are encapsulated in calcium alginate, similar to the procedure used in the encapsulation-dehydration technique. The loading and dehydration treatments with the PVS solution are performed as described for the vitrification method. The capsules are placed in cryovials with the vitrification solution and immersed in liquid nitrogen until heated in a water bath [30].
- Cryo-plate. In the cryo-plate method, the explants are also encapsulated, but they are immobilized in a thin layer of calcium alginate that gels on the surface of an aluminum plate [32]. This method has two variants: V cryo-plate and D cryo-plate. In the case of the V cryo-plate, the explants are subjected to the vitrification solution for dehydration, and then the same steps of the vitrification procedure are followed. In the D cryo-plate, dehydration is performed physically (with silica gel or in the laminar flow cabinet) and proceeds with the steps of the encapsulation-dehydration technique [41].

An advantage shared by the encapsulation-vitrification and cryo-plate methods is that they allow the handling of numerous explants immobilized in calcium alginate, unlike the vitrification method, which involves the direct handling of each tissue. It is also possible to shorten the total duration of the cryopreservation protocol compared with that required for encapsulation-dehydration [42].

- Cryo-mesh. It is a recently developed method [31]. In general, it resembles the V cryo-plate protocol, with the difference that the samples are placed in a stainless steel mesh that also allows high cooling and reheating rates.

Vitrification-based procedures are appropriate for complex organs such as apical meristems and embryos, which contain a wide variety of cell types with specific requirements. Moreover, being operationally simpler than classical methods, they have the potential for wider applicability, as they demand only minor modifications for different cell types [43].

Desiccation method: Equally important is the desiccation method, which is simple and very appropriate for cryopreserving seeds, and which, combined with a previous pre-culture in medium supplemented with sugars, results in the so-called pre-culture-drying protocol and can be effective for successfully cryopreserving zygotic and somatic embryos [44].

For seed cryopreservation, the pre-culture phase is omitted, which implies the use of a more simplified and shorter protocol. Desiccation can be achieved in different ways: by exposing the explants to the air current of a laminar flow cabinet; by placing them in hermetically sealed containers with silica gel; or by achieving controlled atmospheres with saturated saline solutions [30].

2.1. Differential Scanning Calorimetry

The most widely used technique, so far, to identify and quantify crystallization and glass transition events is differential scanning calorimetry (DSC or Differential Scanning Calorimetry) [45], which is a very valuable tool to optimize liquid nitrogen immersion conditions and to develop low-temperature protection strategies [46].

The fundamental principle of DSC is based on applying regulated temperature changes to the sample under study, while measuring the heat flow and temperature associated with its thermal transitions. The equipment detects temperature differences between a reference and the sample, and recalculates the differential heat flux [47].

This data is of great interest because it allows obtaining valuable information on endothermic, exothermic events, or changes in the heat capacity of the plant material. It can be used to determine the glass transition, ice nucleation temperature, melting, boiling, and crystallization time, which are important characteristics in cryopreservation processes. It is considered a very advantageous method and provides a relatively fast, real-time measurement of the thermal characteristics during the dehydration of the evaluated sample [47].

2.2. Cryodamage

Cryopreservation techniques for long-term preservation of germplasm *in vitro* comprise numerous steps, ranging from tissue culture, pre-growth or dehydration, cryoprotection, immersion in liquid nitrogen, rewarming, material recovery, and regeneration, all of which expose tissues to physical, chemical, and physiological stresses. Successful cryopreservation is often evaluated by the survival of plant tissues and their ability to regenerate whole plants [8].

When water is removed from the cells of dehydration-sensitive organisms, two main factors can explain the damage caused, according to some authors [48]: damage resulting from mechanical stresses that disrupt structural organelles at high moisture contents (greater than -5 MPa), and damage to macromolecular structures following more extreme desiccation.

Consistent with these approaches, recent research evidence indicates damage to cellular metabolic activity at an intermediate level of humidity. These cells can continue to respire but cannot eliminate the toxic metabolic products that accumulate [48].

This metabolic disorder that occurs in partially dehydrated cells can also cause the death of cells that remain partially hydrated for a certain period of time, which is the basis of the accelerated aging test [49].

Generally, seeds lose viability when they are kept at a moisture content where respiration is possible (-15 to -5 MPa), but repair processes are not efficient [50]. This may contribute to damage to the moist stored seeds or sensitive seeds that are slowly dried, as they are kept at a critical moisture level for a long period of time [48].

When biological material is exposed to decreased temperature, all kinds of processes can be affected; chemical reactions are delayed or stopped (thus metabolism as well), and membrane stability is affected. The formation of extracellular ice crystals causes tissue rupture and cell disconnection. During slow freezing, effects related to dehydration occur as the first extracellular ice crystals are formed, resulting in a concentrated solution surrounding the cells [6].

Since intracellular and extracellular fluids are in osmotic equilibrium, water leaves the cells, leading to freeze dehydration and, consequently, irreversible lethal plasmolysis. The formation of intracellular ice crystals during exposure to subzero temperatures causes penetration of the membrane and loss of its semipermeability, ultimately resulting in cell death [6].

2.2.1. Oxidative Stress

Oxidative stress refers to the state of imbalance between oxidation and antioxidation caused by the massive production of reactive oxygen species in cells under extreme conditions, such as low temperatures [51].

Management of oxidative stress is vital for the successful application of cryopreservation to plant tissues. Freezing damage induces the production of free radicals, mainly reactive oxygen species or ROS, which attack the lipid fraction of membranes [52]. The steps prior to storage in liquid nitrogen (conditioning and dehydration) can induce an excess of ROS, such as hydroxyl radical, superoxide, and hydrogen peroxide, the accumulation of which causes negative effects on plant tissues. Some of these effects may include lipid peroxidation, protein oxidation, and DNA (Deoxyribonucleic acid) damage. In some cases, programmed cell death (PCD) can be induced, which ultimately kills the cells after cryopreservation [53].

Enzymes that remove reactive oxygen species, such as catalase, superoxide dismutase, and peroxidase, among others, can mitigate or reverse the damage caused by oxidative stress, making biochemical studies important to elucidate the effect of cell damage caused by cryopreservation [54, 55]. In this regard, some results have been obtained in embryos of barley (*Hordeum vulgare* L.) [56] and the palm *Livistona chinensis* (Jacq.) R.Br. ex Mart. [57] and in castor bean (*Ricinus communis* L.) seeds [58].

2.3. Studies for the Determination of Stability and Genetic Variability in Cryopreservation of Plant Species

In vitro tissue culture is an integral part of cryopreservation protocols; it is currently used as a basis for the establishment and maintenance of plant species and during the post-culture process for the recovery of cryopreserved tissues [53].

Unlike animal cells, plant cells exhibit the phenomenon of cell totipotency, which is the ability of a cell to regenerate a whole plant [59]. When differentiated and fully organized plant tissues are introduced into in vitro culture and induced into the cell division phase by growth hormones, this can result in genetic changes, a phenomenon known as somaclonal variation [60].

Somaclonal variation can manifest as modifications in plant morphology, chromosome number, accumulation of genetic mutations, gene expression levels in RNA (ribonucleic acid), protein profiles, and molecular changes in DNA sequences [8].

Considering also the damage that can occur in DNA as a consequence of oxidative stress caused by cryopreservation [61] before using this technique routinely for the long-term maintenance of plant genetic resources, it is necessary to verify that the genetic stability of the cryopreserved material is not altered [62].

Different techniques can be applied for this purpose: phenotypic variation analysis (morphological and biometric studies), histological-cytological analysis (mechanisms, techniques, and cytological studies), biochemical analysis (metabolite, enzyme, or protein analysis), molecular genetic analysis (Genome structure, DNA hybridization, PCR- Polymerase Chain Reaction, RAPD- Random Amplified Polymorphic DNA, SSR- Simple Sequence Repeats, AFLP- Amplified Fragment Length Polymorphisms), epigenetics (chromatin analysis, DNA methylation studies, DNA hybridization, and methylation-specific PCR) [8].

2.4. Cryopreservation Studies in Coffee Cultivation

2.4.1. Encapsulation-Dehydration Method

Among the first approaches to the subject of the effects of cryopreservation in coffee cultivation is a study on the histo-cytology of apices of the species *C. racemosa* and *C. sessiliflora*, subjected to the encapsulation-dehydration method. A survival rate of 38% was obtained for *C. sessiliflora*, whose apices required a pre-growth of 3 to 10 days in Murashige and Skoog [63] liquid medium with 0.75 M sucrose, and 4.5 hours of dehydration in a laminar flow cabinet. Likewise, *C. racemosa* apices demanded a progressive increase in sucrose concentration from 0.5 to 1 M, and a dehydration period of 6 hours, to reach 27% survival. After the dehydration phase, the cells of some apices were severely damaged. In these cells, membrane rupture, retracted cytoplasm, non-visible organelles and nucleoli, osmophilic granules towards the periphery of the plasmalemma, and the presence of plasmolysis were observed. However, cytological analysis 21 days after cryopreservation of the plant material showed that most of the meristematic cells recovered from immersion in liquid nitrogen. The apex was able to form leaf primordia, with little difference between the survival of dehydrated apices and those that were dehydrated and cryopreserved [14].

2.4.2. Desiccation Method/Classical Method

Some studies differ in the convenience of applying a slow desiccation treatment (saturated salt solutions) or a rapid desiccation (silica gel) to coffee seeds. According to some authors [64], balancing the moisture content under the relative humidity obtained with saturated salt solutions allows reaching the optimum moisture content for cryopreservation with little loss of viability, in a simple and reproducible manner. However, a minimum of 14 days is required for the seeds to reach equilibrium moisture content. Other researchers report that desiccation in silica gel is more beneficial, since, as it occurs more quickly, there is not enough time for the accumulation of damage that occurs during desiccation, and the tissues are at moisture contents in which, if there are degradation processes, these occur in a shorter time and before the damage can accumulate to high levels [18].

In the coffee tree culture, the desiccation method (rapid cooling by direct immersion in liquid nitrogen) was frequently compared with the classical method (slow cooling with the use of a programmable freezer) to evaluate the effect caused by the different cooling speeds on the survival of cryopreserved explants. Such is the case of a study led by researchers from IRD (Institut de Recherche pour le Développement), from France [15]. They

cryopreserved seeds of nine coffee species that differ in their sensitivity to desiccation, with the use of saturated saline solutions, to achieve slow dehydration until the moisture content reached equilibrium, depending on the type of salt. The species studied were classified into three groups, taking into account the germination obtained in relation to the cooling rate.

From later studies, the French research team suggested two strategies for routine cryopreservation in coffee germplasm banks [65]. Both methods coincide in the stage of drying the seeds using saturated saline solutions. However, they differ in that Strategy 1 involves slow cooling at -1 °C min^{-1} to -50 °C before immersion in liquid nitrogen, osmo-conditioning of the seeds, and germination under greenhouse conditions. This strategy has the advantage of not requiring *in vitro* tissue culture, but it necessitates a programmable freezer, which is not available in all laboratories. The second strategy involves direct immersion in liquid nitrogen and inoculation of the embryos *in vitro*, with acclimatization of the vitro plants before transfer to the greenhouse. This approach yields higher survival percentages and does not require a programmable freezer, although it takes more time due to the *in vitro* culture stage, which carries an additional risk of contamination. Nevertheless, it was the alternative chosen by CATIE (Centro Agronómico Tropical de Investigación y Enseñanza), from Costa Rica, to establish a coffee seed cryobank in 2002.

Brazilian researchers from the Federal University of Lavras, Embrapa Brazil, and other institutions have conducted extensive studies on the cryopreservation of different coffee genotypes by comparing the drying and cooling rates of the samples and analyzing their physiological, biochemical, and histological indicators.

In a study conducted in 2017 on Coffea arabica (cultivars Arara, Catiguá, Catuaí Amarelo, and Mundo Novo), seeds were subjected to rapid desiccation using silica gel and slow desiccation using a saturated NaCl solution, followed by immersion in liquid nitrogen for 24 hours. In general, rapid drying with silica gel to 20% moisture content resulted in higher percentages of normal seedlings, expanded cotyledonary leaves, and dry mass, as well as improved physiological indicators. The seeds of these cultivars showed different levels of tolerance to cryopreservation, with Catuaí Amarelo being the most tolerant and Arara the most sensitive, regardless of the desiccation rate [18].

The activity of catalase, peroxidase, and esterase enzymes increased after desiccation and exposure to ultra-low temperatures. Seeds at their initial moisture content showed no or very low activity compared to seeds subjected to desiccation, indicating that these enzymes are activated when tissues undergo the stress of water loss and ultra-low temperatures. Stress causes an increase in free radicals in cellular metabolism, which requires the expression and action of these enzymes, which induce defense mechanisms in seeds and protect them against cryogenic damage [18].

Additional research determined the effect of moisture content, different slow cooling rates, and final temperature on cryopreservation of Catuaí Amarelo IAC 62 seeds. Only seeds dried in silica gel up to a moisture content of 20% germinated, regardless of the cooling rate and final temperature. In general, when the temperature was lowered to -40 °C, the highest values of radicle protrusion, normal seedlings, strong normal seedlings, seedlings with expanded cotyledons, root dry mass, and shoot dry mass were obtained, with significant statistical differences compared to the rest of the treatments. The results of the tetrazolium test indicated that embryos extracted from seeds stored in liquid nitrogen were less sensitive to cryopreservation than whole seeds, and embryos showed high viability when subjected to rates of -3 or -5 °C min^{-1} up to the temperature of -60 °C, before declining to -196 °C [19].

However, a more in-depth study on this same cultivar determined that desiccation in saturated saline solutions also favored the quality of the recovered seedlings, although a moisture content of 17% was the main factor for seed preservation, regardless of the type of cryopreservation protocol used. Under these conditions, they maintained better physiological quality (radicle protrusion, normal seedlings, strong normal seedlings, cotyledonary leaves, root and aerial part dry matter, embryo viability) and better preserved cellular structures. Slow cooling before storage in liquid nitrogen did not provide advantages compared to direct immersion. In general, for catalases, esterases, and peroxidases, activity was higher in seeds that were dehydrated to 17% moisture content. The better physiological quality of the plants recovered from these treatments indicated that the higher activity of these enzymes favored overcoming desiccation stress. The pattern of cellular structure observed in all the seeds analyzed indicated that the effects of cryopreservation are less drastic in embryo cells than in endosperm cells, which are more sensitive to stresses caused by dehydration, pre-freezing, and rewarming [24]. More recent research has corroborated part of these results [26].

When comparing the cryopreservation of seeds (cv. Catuaí Amarelo IAC 62), with storage in a cold chamber (at 10 °C and 45% relative humidity) after drying in a stationary dryer, it was determined that dehydration in silica gel up to 17% of the moisture content, and preservation in liquid nitrogen, was the most effective method. Seedlings from seeds dried in silica gel and cryopreserved were vigorous and showed vegetative development (number of pairs of leaves, average height of the seedling, average diameter of the cap) similar to that of seedlings produced from seeds with 32% humidity and stored for six months in cold storage. Besides, cryopreservation only requires the liquid nitrogen supply for maintenance, which allows the reduction of storage space and the long-term preservation of germplasm [22].

The use of an osmo-conditioning treatment is essential to promote the proportion of seeds that develop into normal seedlings after exposure to liquid nitrogen. Its effect is associated with a reduction of membrane imbibition damage [65]. This was corroborated in zygotic embryos of Catuai Amarelo IAC 144 that were successfully cryopreserved using dehydration in silica gel for 60 min (23% moisture content), followed by osmotic rehydration using solutions with decreasing sucrose concentrations (from 1 M to 0.10 M), after rewarming. According to the TTC test, the embryos showed maximum viability (75%) and vigor (26%) with this treatment, among all those evaluated. The percentages of germination and normal seedlings reached 90% and 75%, respectively. During acclimatization, 100% survival of seedlings from cryopreserved zygotic embryos was obtained. For the morphological variables evaluated during this stage, statistical differences were observed in shoot length and root length between seedlings originating from dehydrated and cryopreserved embryos and those from fresh embryos, as well as from dehydrated and non-cryopreserved embryos. This could be due to osmotic rehydration, which is considered beneficial in seed germination and plant vigor of different crops under stress conditions. However, there were no phenotypic differences between treatments in leaf shape, color, or organ formation [17]. In cryopreservation of *C. canephora* seeds, the effectiveness of three slow cooling rates was also compared with direct

immersion in liquid nitrogen. Desiccation to 0.25 g g^{-1} of water content did not affect viability, and although both cooling rates (fast or slow) were somewhat harmful to seeds, seeds responded better to direct immersion in liquid nitrogen because higher percentages of germination, normal seedlings, and viability were obtained. Catalase and esterase enzymes proved to be good biochemical markers for cryopreserved seeds, and their activity was higher in those of higher physiological quality. On the other hand, for superoxide dismutase and peroxidase, the effect of the different cryopreservation treatments was insignificant, since no differences were observed between the electrophoretic activities, so in this case, they were not considered effective biochemical markers in the cryopreservation of seeds of this species [20].

A more exhaustive study on *C. canephora* compared two desiccation rates to determine the optimal physiological conditions of seeds after cryopreservation. For dried, non-cryopreserved seeds, a significant effect of desiccation speed was observed for all the variables studied, except for embryo viability: seeds dried quickly (in silica gel) exhibited higher physiological quality than those dried slowly (in saturated NaCl salt solution). No significant differences were observed between different moisture contents (0.20, 0.25, and 0.28 g g^{-1}). Conversely, for dried and cryopreserved seeds, a moisture content of 0.25 g g^{-1}, achieved by rapid drying, was most effective in obtaining the highest germination percentage (43%) and the best physiological characteristics. The activity of enzymes such as catalase, esterase, glutamic oxaloacetic transaminase, and polyphenol oxidase served as indicators of seed quality following cryopreservation [21].

A recent investigation evaluated the effect of moisture content and packaging types (mesh bags, Falcon tubes, and trilaminated aluminum foil envelopes) on the cryopreservation of *C. racemosa* and *C. liberica* var dewevrei seeds. It was determined that there was an interaction between the moisture content and the type of seed packaging. Desiccation of seeds to 20% and packaging in trifoliate aluminum foil envelopes was the most effective protocol among those studied, with survival rates of 79% for *C. racemosa* and 8% for *C. liberica*. In general, for *C. racemosa*, glutamic oxaloacetic transaminase, peroxidase, and esterase isoenzymes showed lower activity when seeds were cryopreserved at 20% or 18% moisture content and packed in mesh bags. In addition to the oxidative stress damage caused by the drying process, this type of packaging tended to increase stress due to the direct contact of liquid nitrogen with the endosperm. The opposite was observed with aluminum foil packaging, which was more effective for seed storage in terms of survival and physiological quality [25].

2.4.3. Vitrification Methods

Although vitrification-based methods are considered very successful due to their ease, reproducibility, and applicability to a wide range of species and explants [66], they are still little studied in coffee plants. Among the first investigations referring to the vitrification method in coffee plants were those carried out by Castilla, et al. [67] in *C. arabica* and *C. canephora* using vitrification solutions PVS 2 and PVS 3, which demonstrated the possibility of using this method successfully in both species.

In *C. arabica* cv. Catuaí Amarelo IAC 144, the vitrification method was used to develop a cryopreservation protocol for zygotic embryos, with dehydration in PVS 2 for different periods of time between 10 and 250 min and at two temperatures (0 and 25 °C). The selected treatment was dehydration in PVS 2 for 100 min at 0 °C. Under these conditions, the solution was not toxic, and 87% of normal seedling formation was achieved. In the histological analysis, it was determined that the cryopreserved embryos showed cells with characteristics very similar to those of the control treatment. However, in the protoderm and primary meristem, an increase in the number of plasmolyzed cells was observed. In this case, the plasmolysis detected was due to an excess in water efflux caused by the osmotic action of the cryoprotective solution. Rewarming directly in the recovery solution at 25 °C for 15 min favored the percentages of germination and formation of normal seedlings, although it did not differ statistically from the values obtained in embryos that were rewarmed for 1 min in a water bath [16].

For zygotic embryos of *C. arabica*, two cryopreservation methods based on vitrification were developed. In the encapsulation-vitrification method, embryos were encapsulated in alginate and dried in the laminar flow cabinet for 30 or 60 minutes prior to immersion in liquid nitrogen. In the droplet-vitrification method, dehydration in PVS 3 was evaluated for different periods of time (40, 50, 60 minutes). For the encapsulation-vitrification method, the maximum germination obtained was 83%, while the embryos cryopreserved by the droplet-vitrification method reached 100% germination, regardless of the time they remained in PVS 3. In the temporary immersion system, the seedlings reached a higher multiplication rate (4.2 shoots per explant) compared to the same culture medium in a semi-solid state (1.3 shoots per explant). The results of the root initiation experiments showed that 90% of the shoots developed roots in the liquid medium, and all shoots showed two to three primary roots with an average length of 5.5 cm at 6 weeks. Rooted seedlings transplanted to the greenhouse showed a survival rate of 95% after one month of growth under *ex vitro* conditions. DSC analysis confirmed the absence of phase transitions in zygotic embryos cryopreserved by the droplet-vitrification method, which reached 100% germination [23].

In general, the literature contains few reports and events that highlight genetic modifications related to cryopreservation, and if such modifications occur, the exact mechanisms and the nature of genetic instability have not been elucidated, considering the different stages involved in the process (*in vitro* culture-cryopreservation-regeneration) [68]. Thus, cryopreservation is considered a cost-effective alternative to *ex situ* germplasm conservation, which ensures the genetic stability of germplasm over time [34].

Considering that the most recent research on the storage of coffee seeds under conventional conditions reaffirms the increasing loss of longevity and the impossibility of obtaining normal plants after a period of two years [69], in our opinion, it confirms the need to employ cryopreservation as an alternative for the *ex situ* conservation of coffee germplasm. In fact, it is considered advisable to test other methods, such as cryo-plate and cryo-mesh in order to achieve new results, adapted to the conditions of different laboratories.

Based on all the benefits of this alternative for the preservation of plant genetic resources, the Global Plant Cryopreservation Initiative, jointly conceived by CGIAR (Consultative Group on International Agricultural Research) and the Global Crop Diversity Trust, has emerged in recent years. The initiative aims to create a sustainable system for conserving clonal crop collections and non-orthodox seeds to safeguard genetic diversity for future generations. Due to its global importance, the coffee plant is considered to be one of the five crops vital to the livelihoods of developing countries and is therefore included among the priority species of this project [70].

3. Conclusions

The desiccation method has been the most widely used for cryopreservation of coffee plants. The germination losses of the cryopreserved seeds or embryos are due to their sensitivity to desiccation, or to the speed of desiccation or cooling used in the different treatments. The effects detected at the morphological, histological, physiological, or biochemical levels represent tissue responses to the various types of stress that can be caused by these long-term preservation techniques. To our knowledge, to date, no molecular genetic studies have been carried out to corroborate the genetic stability during cryopreservation of explants of this genus.

References

[1] P. Bramel, S. Krishnan, D. Horna, B. Lainoff, and C. Montagnon, *Global conservation strategy for coffee genetic resources*. Bonn, Germany: Crop Trust, 2017.

[2] S. Krishnan *et al.*, "Vulnerability of coffee (Coffea spp.) genetic resources in the United States," *Genetic Resources and Crop Evolution*, vol. 68, no. 7, pp. 2691-2710, 2021. https://doi.org/10.1007/s10722-021-01217-1

[3] A. P. Davis and F. Rakotonasolo, "Six new species of coffee (Coffea) from northern Madagascar," *Kew Bulletin*, vol. 76, no. 3, pp. 497-511, 2021. https://doi.org/10.1007/s12225-021-09952-5

[4] R. H. Ellis, T. Hong, and E. Roberts, "An intermediate category of seed storage behaviour? I. Coffee," *Journal of Experimental Botany*, vol. 41, no. 9, pp. 1167-1174, 1990. https://doi.org/10.1093/jxb/41.9.1167

[5] D. Ballesteros and V. C. Pence, "Survival and death of seeds during liquid nitrogen storage: A case study on seeds with short lifespans," *CryoLetters*, vol. 38, no. 4, pp. 278-289, 2017.

[6] B. Panis, "Sixty years of plant cryopreservation: From freezing hardy mulberry twigs to establishing reference crop collections for future generations," *Acta Horticulturae*, vol. 1234, pp. 1-8, 2018.

[7] M. E. Martinez-Montero and K. Harding, *Cryobionomics: Evaluating the concept in plant cryopreservation, PlantOmics: the omics of plant science*. New Delhi: Springer. https://doi.org/10.1007/978-81-322-2172-2_23 2015, pp. 655-682.

[8] K. Harding, "Genetic integrity of cryopreserved plant cells: A review," *CryoLetters*, vol. 25, no. 1, pp. 3-22, 2004.

[9] A. Abdelnour, V. Villalobos, and F. Engelmann, "Cryopreservation of zygotic embryos of Coffea spp," *CryoLetters*, vol. 13, no. 5, pp. 297–302, 1992.

[10] T. Hatanaka, T. Yasuda, T. Yamaguchi, and A. Sakai, "Direct regrowth of encapsulated somatic embryos of coffee (Coffea canephora) after cooling in liquid nitrogen," *CryoLetters*, vol. 15, pp. 47–52, 1994.

[11] M. Martínez, M. T. González-Arnao, C. Urra, R. Rojas, and M. Cuba, "Preliminary studies on the cryopreservation of embryogenic callus and botanical seed of Coffea arabica variety 9722," *Cultivos Tropicales*, vol. 16, no. 3, pp. 74-76, 1995.

[12] M. Martínez, M. T. González-Arnao, C. Urra, R. Rojas, M. Cuba, and D. García, "Preliminary studies for the cryopreservation of zygotic embryos of Coffea arabica variety 9722," *Tropical Crops*, vol. 17, no. 1, pp. 79–81, 1996.

[13] N. Vásquez, K. Salazar, F. Anthony, N. Chabrillange, F. Engelmann, and S. Dussert, "Variability in response of seeds to liquid nitrogen exposure in wild coffee (Coffea arabica L.)," *Seed Science & Technology*, vol. 33, pp. 293–301, 2005. https://doi.org/10.15258/sst.2005.33.2.03

[14] S. Mari, F. Engelmann, N. Chabrillange, C. Huet, and N. Michaux-Ferrière, "Histo-cytological study of apices of coffee (Coffea racemosa and C. sessiliflora) in vitro plantlets during their cryopreservation using the encapsulation-dehydration technique," *CryoLetters*, vol. 16, no. 5, pp. 289–298, 1995.

[15] S. Dussert, N. Chabrillange, G. Rocquelin, F. Engelmann, M. Lopez, and S. Hamon, "Tolerance of coffee (Coffea spp.) seeds to ultra-low temperature exposure in relation to calorimetric properties of tissue water, lipid composition, and cooling procedure," *Physiologia Plantarum*, vol. 112, no. 4, pp. 495-504, 2001. https://doi.org/10.1034/j.1399-3054.2001.1120406.x

[16] De Freitas, R. T., Paiva, R., Sales, T. S., Da Silva, D. P. C., De Souza, A. C., Dos Reis, M. V., & Da Rosa, S. D. V. F., "Cryopreservation of Coffea arabica L. zygotic embryos by vitrification," *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, vol. 44, no. 2, pp. 445-451, 2016. https://doi.org/10.15835/nbha44210553

[17] M. d. S. Pinto, R. Paiva, D. P. C. d. Silva, P. A. A. Santos, R. T. d. Freitas, and L. C. Silva, "Cryopreservation of coffee zygotic embryos: Dehydration and osmotic rehydration," *Ciência e Agrotecnologia*, vol. 40, no. 4, pp. 380-389, 2016.

[18] S. Coelho, S. Rosa, and J. Fernandes, "Cryopreservation of coffee seeds: A simplified method," *Seed Science and Technology*, vol. 45, no. 3, pp. 638-649, 2017. https://doi.org/10.15258/sst.2017.45.3.15

[19] M. A. d. Figueiredo, S. V. B. Coelho, S. D. V. F. d. Rosa, A. L. Vilela, and L. C. Silva, "Exploratory studies for cryopreservation of Coffea arabica L. seeds," *Journal of Seed Science*, vol. 39, no. 02, pp. 150-158, 2017. https://doi.org/10.1590/2317-1545v39n2171123

[20] S. V. B. Coelho, S. D. V. F. d. Rosa, T. B. Fantazzini, J. L. Baute, and L. C. Silva, "Cryopreservation in Coffea canephora Pierre seeds: Slow and rapid cooling," *Ciência e Agrotecnologia*, vol. 42, no. 6, pp. 588-597, 2018. https://doi.org/10.1590/1413-70542018426017718

[21] S. V. B. Coelho, D. V. F. Sttelada Rosa, T. B. Fantazzini, and L. C. Silva, "Cryopreservation of'Coffea canephora'Pierre ex A. Froehner seeds: importance of drying rate and moisture content," *Australian Journal of Crop Science*, vol. 13, no. 8, pp. 1335-1342, 2019. https://doi.org/10.21475/ajcs.19.13.08.p1747

[22] M. A. Ricaldoni, S. D. V. F. da Rosa, M. A. de Figueiredo, M. A. de Figueiredo Carvalho, and T. B. Fantazzini, *Evaluation of development parameters of Coffea arabica L. seedlings originated from cryopreserved seeds. In X Symposium on Brazilian Coffee Research (Proceedings, Vitória, ES, Brazil)*. Brasília, DF, Brazil: Embrapa Coffee, 2019.

[23] V. Y. Castilla, M. R. Shukla, M. E. González Vega, and P. K. Saxena, "Improved conservation of coffee (Coffea arabica L.) germplasm via micropropagation and cryopreservation," *Agronomy*, vol. 11, no. 9, p. 1861, 2021. https://doi.org/10.3390/agronomy11091861

[24] M. A. Figueiredo, S. D. V. Rosa, M. A. Ricaldoni, C. Pereira, S. Coelho, and L. Silva, "Physiological, biochemical, and ultrastructural aspects of Coffea arabica L. seeds under different cryopreservation protocols," *Science and Agrotechnology*, vol. 45, p. e027020, 2021. https://doi.org/10.1590/1413-7054202145027020

[25] S. V. B. Coelho *et al.*, "Optimizing cryopreservation protocols for coffea racemosa and coffea liberica: Impact of moisture content and packaging on seed viability," *Agronomy*, vol. 14, no. 12, p. 3058, 2024. https://doi.org/10.3390/agronomy14123058

[26] A. C. d. Souza *et al.*, "Steps of cryopreservation of coffee seeds: Physiological responses and antioxidant systems," *Ciência Rural*, vol. 54, no. 2, p. e20220480, 2023. https://doi.org/10.1590/0103-8478cr20220480

[27] M. T. González-Arnao, Y. Martínez-Ocampo, and J. Molina-Torres, "To conserve plant genetic biodiversity," *Revista Ciencia*, vol. 60, no. 2, pp. 78–86, 2009.

[28] V. C. Pence *et al.*, "Cryobiotechnologies: Tools for expanding long-term ex situ conservation to all plant species," *Biological Conservation*, vol. 250, p. 108736, 2020. https://doi.org/10.1016/j.biocon.2020.108736

[29] E. Popova, M. Shukla, H. H. Kim, and P. K. Saxena, *Plant cryopreservation for biotechnology and breeding. In Advances in plant breeding strategies: breeding, biotechnology and molecular tools*. Cham: Springer International Publishing, 2015, pp. 63-93.

[30] M. T. González-Arnao and F. Engelmann, *Theoretical and practical considerations for the cryoconservation of plant germplasm. In M. T. González-Arnao & F. Engelmann (Eds.), Plant Cryoconservation in Latin America and the Caribbean (Chap. 4)*. IICA: San José, Costa Rica, 2013.

[31] B. Funnekotter, E. Bunn, and R. L. Mancera, "Cryo-mesh: A simple alternative cryopreservation protocol," *CryoLetters*, vol. 38, no. 2, pp. 155-159, 2017.

[32] C. A. Roque-Borda, D. Kulus, A. Vacaro de Souza, B. Kaviani, and E. F. Vicente, "Cryopreservation of agronomic plant germplasm using vitrification-based methods: An overview of selected case studies," *International Journal of Molecular Sciences*, vol. 22, no. 11, p. 6157, 2021. https://doi.org/10.3390/ijms22116157

[33] G. D. Elliott, S. Wang, and B. J. Fuller, "Cryoprotectants: A review of the actions and applications of cryoprotective solutes that modulate cell recovery from ultra-low temperatures," *Cryobiology*, vol. 76, pp. 74-91, 2017. https://doi.org/10.1016/j.cryobiol.2017.04.004

[34] B. Panis, M. Nagel, and I. van Den Houwe, "Challenges and prospects for the conservation of crop genetic resources in field genebanks, in in vitro collections and/or in liquid nitrogen," *Plants*, vol. 9, no. 12, p. 1634, 2020. https://doi.org/10.3390/plants9121634

[35] S. Bojic *et al.*, "Winter is coming: The future of cryopreservation," *BMC Biology*, vol. 19, no. 1, p. 56, 2021. https://doi.org/10.1186/s12915-021-00976-8

[36] J. Zamecnik, M. Faltus, and A. Bilavcik, "Vitrification solutions for plant cryopreservation: Modification and properties," *Plants*, vol. 10, no. 12, p. 2623, 2021. https://doi.org/10.3390/plants10122623

[37] N. R. Dolce, F. Hernández-Ramírez, and M. T. González-Arnao, "Cryopreservation of vanilla (Vanilla planifolia) root-tips: A new alternative for in vitro long-term storage of its germplasm," *Acta Horticulturae*, vol. 1234, pp. 203–210, 2019. https://doi.org/10.17660/actahortic.2019.1234.27

[38] B. Panis, B. Piette, and R. Swennen, "Droplet vitrification of apical meristems: A cryopreservation protocol applicable to all Musaceae," *Plant Science*, vol. 168, no. 1, pp. 45-55, 2005.

[39] Z. Liu, W.-L. Bi, M. R. Shukla, and P. K. Saxena, "In vitro technologies for American chestnut (Castanea dentata (Marshall) Borkh) conservation," *Plants*, vol. 11, no. 3, p. 464, 2022. https://doi.org/10.3390/plants11030464

[40] M. A. Torres, "Plant cryopreservation: Its application to the conservation of garlic germplasm (Allium sativum L.)," *Agrotecnia de Cuba*, vol. 48, pp. 1-13, 2024.

[41] B. Kaviani and D. Kulus, "Cryopreservation of endangered ornamental plants and fruit crops from tropical and subtropical regions," *Biology*, vol. 11, no. 6, p. 847, 2022. https://doi.org/10.3390/biology11060847

[42] S.-i. Yamamoto, T. Rafique, K. Fukui, K. Sekizawa, and T. Niino, "V-cryo-plate procedure as an effective protocol for cryobanks: case study of mint cryopreservation," *CryoLetters*, vol. 33, no. 1, pp. 12-23, 2012.

[43] N. Sánchez-Chiang and V. M. Jiménez, "In vitro conservation techniques for the establishment of germplasm banks in tropical crops," *Agronomía Mesoamericana*, vol. 21, no. 1, pp. 193-205, 2010.

[44] V. C. Pence, *Cryopreservation of recalcitrant seeds", in Biotechnology in Agriculture and Forestry 32, Cryopreservation of plant germplasm I, Y. P. S. Bajaj, Ed.* Heidelberg: Springer Berlin, 1995.

[45] C. W. Vertucci, "Effects of cooling rate on seeds exposed to liquid nitrogen temperatures," *Plant Physiology*, vol. 90, no. 4, pp. 1478-1485, 1989. https://doi.org/10.1104/pp.90.4.1478

[46] D. Dumet and E. Benson, *The use of physical and biochemical studies to elucidate and reduce cryopreservation-induced damage in hydrated/desiccated plant germplasm", in Cryopreservation of tropical plant germplasm. Current research progress and application, F. Engelmann, and H. Takagi, Eds.* Tsukuba-Rome: JIRCAS-IPGRI, 2000.

[47] S. D. Hammond, M. Faltus, and J. Zámečník, *Methods of thermal analysis as a tool to develop cryopreservation protocols of vegetatively propagated crops", in Cryopreservation-Current Advances and Evaluations, M. D. Quain, Ed.* London, UK: IntechOpen, 2019.

[48] C. Walters, N. Pammenter, P. Berjak, and J. Crane, "Desiccation damage, accelerated ageing and respiration in desiccation tolerant and sensitive seeds," *Seed Science Research*, vol. 11, no. 2, pp. 135-148, 2001. https://doi.org/10.1079/SSR200168

[49] J. C. Delouche and C. C. Baskin, "Accelerated aging techniques for predicting the relative storability of seed lots," *Seed Science & Technology*, vol. 1, pp. 427-452, 1973.

[50] C. W. Vertucci and J. M. Farrant, *Acquisition and loss of desiccation tolerance", in Seed Development and Germination, J. Kigel, G. Galili, Eds.* New York-Basel: Marcel Dekker Inc, 1995.

[51] S. Evangelista-Vargas and A. Santiani, "Detection of intracellular reactive oxygen species (superoxide anion and hydrogen peroxide) and lipid peroxidation during cryopreservation of alpaca spermatozoa," *Reproduction in Domestic Animals*, vol. 52, no. 5, pp. 819-824, 2017. https://doi.org/10.1111/rda.12984

[52] D. Kulus, "Managing plant genetic resources using low and ultra-low temperature storage: A case study of tomato," *Biodiversity and Conservation*, vol. 28, no. 5, pp. 1003-1027, 2019. https://doi.org/10.1007/s10531-019-01710-1

[53] L. Ren, M.-R. Wang, and Q.-C. Wang, "ROS-induced oxidative stress in plant cryopreservation: Occurrence and alleviation," *Planta*, vol. 254, no. 6, p. 124, 2021. https://doi.org/10.1007/s00425-021-03784-0

[54] P. Berjak, "Unifying perspectives of some mechanisms basic to desiccation tolerance across life forms," *Seed Science Research*, vol. 16, no. 1, pp. 1-15, 2006. https://doi.org/10.1079/SSR2005236

[55] S. Dussert and F. Engelmann, "New determinants for tolerance of coffee (Coffea arabica L.) seeds to liquid nitrogen exposure," *CryoLetters*, vol. 27, no. 3, pp. 169-178, 2006.

[56] M. A. Şipoş and D. Cachiţă-Cosma, "Peroxidases activity in the embryos of germinated barley cariopses, after grain cryopreservation," *Analele Universităţii din Oradea, Fascicula Biologie*, vol. 16, no. 1, pp. 129-131, 2009.

[57] B. Wen, C. Cai, R. Wang, S. Song, and J. Song, "Cytological and physiological changes in recalcitrant Chinese fan palm (Livistona chinensis) embryos during cryopreservation," *Protoplasma*, vol. 249, no. 2, pp. 323-335, 2012. https://doi.org/10.1007/s00709-011-0283-4

[58] A. Villalobos-Olivera *et al.*, "The role of antioxidants, peroxidase and superoxide dismutase, in ameliorating oxidative stress associated with cryopreservation of Tamarindus indica seeds," *Acta Physiologiae Plantarum*, vol. 45, no. 7, p. 86, 2023. https://doi.org/10.1007/s11738-023-03569-w

[59] G. Haberlandt, "Culture experiments with isolated plant cells,"sitzungsberg," *Proceedings of the Imperial Academy of Sciences, Mathematical and Natural Sciences Class*, vol. 111, pp. 69-92, 1902.

[60] P. J. Larkin and W. R. Scowcroft, "Somaclonal variation—a novel source of variability from cell cultures for plant improvement," *Theoretical and Applied Genetics*, vol. 60, no. 4, pp. 197-214, 1981. https://doi.org/10.1007/BF02342540

[61] X. Liu *et al.*, "The feasibility of antioxidants avoiding oxidative damages from reactive oxygen species in cryopreservation," *Frontiers in Chemistry*, vol. 9, p. 648684, 2021. https://doi.org/10.3389/fchem.2021.648684

[62] M.-R. Wang *et al.*, "Epigenetic and genetic integrity, metabolic stability, and field performance of cryopreserved plants," *Plants*, vol. 10, no. 9, p. 1889, 2021. https://doi.org/10.3390/plants10091889

[63] T. Murashige and F. Skoog, "A revised medium for rapid growth and bio assays with tobacco tissue cultures," *Physiologia Plantarum*, vol. 15, no. 3, pp. 473-497, 1962. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

[64] S. Dussert, N. Chabrillange, F. Engelmann, F. Anthony, and S. Hamon, "Cryopreservation of coffee (Coffea arabica L) seeds: Importance of the precooling temperature," *Cryo-Letters*, vol. 18, no. 5, pp. 269-276, 1997.

[65] S. Dussert, N. Vásquez, K. Salazar, F. Anthony, and F. Engelmann, *Cryopreservation of coffee genetic resources. In F. Engelmann, M. E. Dulloo, C. Astorga, S. Dussert, & F. Anthony (Eds.), Conserving coffee genetic resources: Complementary strategies for ex situ conservation of coffee (Coffea arabica L.) genetic resources: A case study in CATIE, Costa Rica.* Rome, Italy: Bioversity International, 2007.

[66] M. Nagel, V. Pence, D. Ballesteros, M. Lambardi, E. Popova, and B. Panis, "Plant cryopreservation: Principles, applications, and challenges of banking plant diversity at ultralow temperatures," *Annual Review of Plant Biology*, vol. 75, pp. 797–824, 2024. https://doi.org/10.1146/annurev-arplant-070623-103551

[67] Y. Castilla, M. E. Martínez-Montero, M. E. González, and F. Engelmann, "New results in the cryopreservation of two coffee species (Coffea spp.) using the vitrification method," in *Proceedings of the IX International Congress on Plant Biotechnology, Bioveg, Ciego de Ávila, Cuba*, 2013.

[68] C. Benelli, "Plant cryopreservation: A look at the present and the future," *Plants*, vol. 10, no. 12, p. 2744, 2021. https://doi.org/10.3390/plants10122744

[69] R. Alixandre *et al.*, "Physiological quality of seeds of arabic coffee cultivars stored for a period of two years," *Brazilian Journal of Biology*, vol. 84, p. e285879, 2024. https://doi.org/10.1590/1519-6984.285879

[70] S. Krishnan, C. Lusty, M. Engbers, B. Cate, and D. Ellis, "The global plant cryopreservation Initiative: Building capacity and ensuring long-term conservation of clonal and recalcitrant seed collections," *Acta Horticulturae*, vol. 1421, pp. 43-46, 2025.