# Physiological, biochemical, and ultrastructural aspects of *Coffea arabica* L. seeds under different cryopreservation protocols

## Aspectos fisiológicos, bioquímicos e ultraestruturais de sementes de *Coffea arabica* L. submetidas a diferentes protocolos de criopreservação

Madeleine Alves de Figueiredo[1] [ID], Sttela Dellyzete Veiga Franco da Rosa[2*] [ID], Marcela Andreotti Ricaldoni[1] [ID], Cristiane Carvalho Pereira[1] [ID], Stefânia Vilas Boas Coelho[1] [ID], Luciano Coutinho Silva[3] [ID]

[1]Universidade Federal de Lavras/UFLA, Departamento de Agricultura/DAG, Lavras, MG, Brasil
[2]Empresa Brasileira de Pesquisa Agropecuária/Embrapa, Embrapa Café, Brasília, DF, Brasil
[3]Universidade Federal da Paraíba/UFPB, Centro de Biotecnologia, João Pessoa, PB, Brasil
*Corresponding author: sttela.rosa@embrapa.br

## ABSTRACT

Cryopreservation is a technique that may potentially conserve the germplasm of species of the *Coffea* genus for an indeterminate time. The aim of this study was to evaluate the physiological, biochemical and ultrastructural characteristics of cryopreserved seeds of *Coffea arabica* L., cultivar Catucaí amarelo IAC 62, which was subjected to different protocols regarding dehydration, precooling, cooling, rewarming and cathode water use. According to each protocol, the seeds were subjected to fast or slow drying to moisture contents of 17 or 20% (wet basis), cooled in different ways, and then immersed in liquid nitrogen for 24 hours. Different rewarming times in a water bath were also used. Physiological, biochemical and ultrastructural analyses were performed on the seeds after the cryopreservation steps. Moisture content at a 17% wb is the key factor for the cryopreservation of *Coffea arabica* L. seeds, which have better physiological quality and better preserved cell structures. Precooling of coffee seeds before immersion in liquid nitrogen does not provide advantages compared to direct immersion. The rewarming times tested (2, 4, and 6 minutes) and cathode water use did not cause changes in the physiological and biochemical quality or in the cell structures of *Coffea arabica* L. cryopreserved seeds. The pattern of cell structure observed in all seeds indicates that the damage from cryopreservation is less drastic in the cells of the embryos than in those of the endosperm, with the latter less tolerant to the stresses of dehydration, precooling, and rewarming.

**Index terms**: Cathodic water; dehydration; long-term conservation; ultrafast cooling.

## RESUMO

A criopreservação é uma técnica que pode potencialmente conservar o germoplasma de espécies do gênero *Coffea* por tempo indeterminado. O objetivo neste estudo foi avaliar os aspectos fisiológicos, bioquímicos e ultraestruturais de sementes criopreservadas de *Coffea arabica* L., cultivar Catuaí amarelo IAC 62, submetidas a diferentes protocolos de desidratação, pré-resfriamento, resfriamento, reaquecimento e uso de água catódica. De acordo com cada protocolo as sementes foram submetidas à secagem rápida ou lenta, até 17 ou 20% de umidade (base úmida), resfriadas por diferentes maneiras e, em seguida, foram imersas em nitrogênio líquido por 24 horas. Diferentes tempos de reaquecimento em banho-maria também foram utilizados. Análises fisiológicas, bioquímicas e ultra-estruturais foram realizadas nas sementes após as etapas de criopreservação. O teor de umidade a 17% bu é o fator chave para a criopreservação de *Coffea arabica* L., umidade em que as sementes apresentam melhor qualidade fisiológica e estruturas celulares mais preservadas. O pré-resfriamento das sementes de café antes da imersão em nitrogênio líquido é dispensado por não apresentar vantagens em relação à imersão direta. Os tempos de reaquecimento testados (2, 4 e 6 minutos) e o uso de água catódica não causam alterações na qualidade fisiológica e bioquímica, bem como nas estruturas celulares das sementes criopreservadas de *Coffea arabica* L. O padrão de estrutura celular observado em todas as sementes indica que o dano da criopreservação é menos drástico nas células dos embriões do que nos endospermas, sendo este último menos tolerante aos estresses de desidratação, pré-resfriamento e reaquecimento.

**Termos para indexação:** Água catódica; desidratação; conservação a longo prazo; resfriamento ultra-rápido.

# INTRODUCTION

The genus *Coffea* currently has more than 100 described species, and among these, *Coffea arabica* L. is the most marketed worldwide (Davis et al., 2006). Brazil is the world leader in the production and export of coffee, and the conservation of the genetic diversity of the *Coffea* genus is fundamental. However, the *Coffea* genus cannot be conserved in conventional seed banks. Orthodox seeds have a water content near 5% and can be stored at low temperatures for long periods of time; however, recalcitrant seeds do not exhibit such characteristics and quickly degrade under these conditions (Roberts, 1973). *Coffea arabica* L. seeds partially tolerate water loss (approximately 10% to 13% moisture) and are sensitive to storage at temperatures below zero (°C); thus, they are considered intermediate seeds (Ellis; Hong; Roberts, 1990).

An alternative for seeds with this behavior to remain viable for a long period in a reliable manner is via storage at ultralow temperatures (-196 °C) in liquid nitrogen (LN). However, the chemical, physical, and physiological properties of different tissues under cryopreservation can affect the viability and genetic integrity of the seeds (Coelho; Gonçalves; Romano, 2020).

The formation of intracellular ice crystals is the main problem in the cryopreservation process, and it can be prevented by inducing vitrification of the sample using substances of high molecular weight that hinder the formation of these crystals. In this way, the potentially nonfreezable water that remains will be converted into a noncrystalline amorphous solid of high viscosity. In such cases, water molecules are impeded from organizing and initiating nucleation (Noor; Sulong; Reed, 2019). Another advantage is the metabolic quiescence achieved from formation of the vitreous state, which prevents oxidative reactions. However, the processes used for vitrification can cause damage to plant tissues sensitive to desiccation and cooling, such as the tissues of coffee seeds (Orjuela-Palacio et al., 2019).

The membrane system is easily damaged by ice crystal formation (Walters, 2015). In addition, the processes of cooling and rewarming can lead to enzyme denaturation and the generation of reactive oxygen species (ROS) (Berjak; Sershen; Pammenter, 2011). The increase in endogenous antioxidants or the supplementation of exogenous antioxidants can contribute to success in cryopreservation by reducing the oxidative damage promoted by reactive oxygen species (ROS). Cathode water has been reported to have antioxidant effects (Len; Koh; Tan, 2019) and great potential for improving responses to stresses arising from cryopreservation procedures in plant materials (Berjak; Sershen; Pammenter, 2011).

Many contradictory results related to the conservation of the *Coffea* genus have been observed in studies about cryopreservation regarding dehydration, cooling, and rewarming (Dussert; Engelmann, 2006; Abreu et al., 2014; Coelho; Rosa; Fernandes, 2017b; Figueiredo et al., 2017). The combination of the effects promoted by these steps may affect the final quality of the cryopreserved seeds. Thus, the aim of this study was to evaluate the physiological, biochemical and ultrastructural characteristics of cryopreserved *Coffea arabica* L. seeds subjected to different protocols regarding dehydration, cooling, precooling, rewarming and the use of cathode water.

# MATERIAL AND METHODS

### Cryopreservation protocol

Previously published cryopreservation protocols (Dussert et al., 1997; 1998; 2000; Dussert; Engelmann, 2006; Figueiredo et al., 2017; Coelho; Rosa; Fernandes, 2017b) were compared to new protocols based on combinations of two moisture contents and different drying procedures as well as precooling before immersion in LN and rewarming for different periods, as described in Table 1.

### Preparation of seeds for cryopreservation

Coffee fruits of cultivar Catuaí amarelo IAC 62 in the physiological maturity stage (cherry) were harvested on the Procafé Experimental Farm in Varginha/MG, Brazil, at an altitude of 980 m. This site has a subtropical highland climate (Cwb) according to the Köppen classification. The fruits were harvested in the physiological maturity stage (cherry) and fully washed mechanically. The mucilage was removed by fermentation in water for a period of 24 hours at ambient temperature, and then the seeds were surface-dried by placing them in a single layer on a screen in the shade for 2 hours.

The moisture content of the seeds was determined after physical dehydration in a laboratory oven, and then the initial physiological quality was evaluated via germination (Brasil, 2009), tetrazolium (Brasil, 2009), and electrical conductivity (Krzyzanowsky; França Neto; Henning, 1991 modified) tests.

In the germination test, the percentage of normal seedlings at 30 days after sowing was obtained and the percentage of radicle emergence at 15 days, strong normal seedlings at 30 days, seedlings with expanded cotyledonary leaves at 45 days, and seedling dry matter were evaluated.

**Table 1:** Description of the steps used in the cryopreservation protocols of *Coffea arabica* L seeds.

| CRYOPRESERVATION STEPS | | | | | |
|---|---|---|---|---|---|
| Protocols | Dehydration | Precooling | Packaging/immersion | Rewarming** | Reference |
| 1 to 3 | Silica gel to 20% wb | $-1\ °C\ min^{-1}$ to $-40\ °C$ | Aluminum trilaminate pouches | Protocol 1 - 2 min<br>Protocol 2 - 4 min<br>Protocol 3 - 6 min | Figueiredo et al. (2017) |
| 4 to 6 | Silica gel to 17% wb | | | Protocol 4 - 2 min<br>Protocol 5 - 4 min<br>Protocol 6 - 6 min | |
| 7 | Saturated $(NH_4)_2SO_4$ solution (81% RH) to 17% wb | $-1\ °C\ min^{-1}$ to $-50\ °C$ | Cryotubes | Water bath, 40±1 °C, for 2 min | Dussert et al. (1997; 1998; 2000); Dussert and Engelmann, (2006) |
| 8 | Saturated NaCl solution (75% RH) to 17% wb | Without precooling | Aluminum trilaminate pouches and direct immersion | | Eira et al. (2005) |

*840 seeds were placed in each package. ** Water bath at 40±1 °C.

For drying in silica gel, 170 seeds were placed on a metal screen in a single layer in sealed plastic boxes (with dimensions of 11× 11 × 3.5 cm) containing 60 grams of activated silica, and they were maintained in a Biochemical Oxygen Demand (B.O.D.) chamber at 25 °C in the absence of light. Loss of water during drying was monitored until the samples reached moisture contents of 20% and 17% wb, Protocols 1-6). The same containers were filled with 170 seeds and treated at the same temperature for dehydration in saturated $(NH_4)_2SO_4$ (Protocol 7) or NaCl (Protocol 8) salt solutions (65 g of salt in 15 mL of water). To avoid contact with the saturated solutions, the seeds were placed over screens, and water loss during dehydration was monitored until the samples reached a moisture content of 17% wb.

**Precooling procedure**

Precooling was carried out using 840 seeds in trilaminate aluminum foil packaging or in cryotubes, which were then cooled in a computer-controlled biofreezer (Icecube, 14S-B, SY-LAB software, Minitub do Brasil) at a rate of -1 °C min⁻¹ until a temperature of -40 °C or -50 °C (Table 1). After precooling, the packages or cryotubes containing the seeds were plunged in the LN. The seed samples of Protocol 8 were not subjected to slow cooling but were directly plunged in the LN after dehydration.

**Rewarming**

After 36 hours of immersion in LN, the trifoliate aluminum packaging containing the cryopreserved seeds was removed from the cryotank with the help of canisters and quickly opened. Then, the seeds were immediately rewarmed in a water bath at 40 °C±1 °C for different periods of time (2, 4, or 6 minutes) according to each protocol (Table 1).

**Cathode water treatment**

After rewarming, half of the seeds of each treatment underwent antioxidant treatment, which consisted of immersion in 250 mL of cathode water for 60 minutes in the presence of light and at ambient temperature. Cathode water was prepared according to the method described by Berjak, Sershen and Pammenter (2011), with modifications. One liter of the solution containing the electrolytes 0.5 mM $CaCl_2.2H_2O$ and 0.5 mM $MgCl_2.6H_2O$ was divided and placed in a horizontal tub specifically designed for electrophoresis run. The circuit was completed using an agar-based salt bridge containing potassium chloride, and the solution was electrolyzed by applying a potential difference of 60 V through a platinum electrode. Electrolysis was performed for one hour at ambient temperature, thus producing 500 mL of anode (oxidized) water with a pH near 3-4 and 500 mL of cathode (reduced) water with a pH near 11-12, which was used in the experiment.

Seeds treated or not in cathode water were subjected to one hour of superficial drying on paper towels and their parchments were manually removed. The moisture content was determined, and the samples were subjected to physiological, biochemical, and structural evaluations.

## Moisture content determination and physiological evaluation

The moisture content was determined by the laboratory oven method at 105 °C for 24 hours (Brasil, 2009), with two replications of 10 seeds for each treatment. The results are expressed as percentages based on the wet weight of the seeds.

For the germination test, four replications of 25 seeds were used for each treatment. Seeds were sown in rolls of germination paper moistened with distilled water (2.5 g $H_2O$. 1 $g^{-1}$ dry paper) and then placed in a seed germinator at 30 °C under light (Brasil, 2009). The percentage of radicle emergence at 15 days and the percentage of normal seedlings (presence of the primary root and at least two lateral roots) at 30 days after sowing were determined. The percentage of strong normal seedlings at 30 days (seedlings that exhibited hypocotyls of three centimeters or more) and the percentage of seedlings with expanded cotyledonary leaves at 45 days after sowing were also determined (França Neto; Krzyzanowski; Costa, 1999).

The root and shoot dry matter of the normal seedlings was determined at the end of the germination test. The material was dehydrated in a forced air circulation oven at 60 °C for 4 to 5 days until reaching constant weight and then weighed on an analytical balance with precision of 0.001 g (França Neto; Krzyzanowski; Costa, 1999).

The tetrazolium test was performed with four replications of 10 embryos extracted from the seeds after 36 hours of soaking in distilled water. After extraction, the embryos were maintained in a polyvinylpyrrolidone (PVP) antioxidant solution up to the time of immersion in 0.5% tetrazolium solution. The samples were maintained in dark containers at a temperature of 30 °C for three hours (Brasil, 2009). Analysis of the viability of the embryos was performed with the aid of a stereoscopic magnifier at 10× magnification according to the location and extent of the damage after a longitudinal cut in the embryonic axis (Brasil, 2009).

The mass electrical conductivity test was performed with four replications of 25 seeds according to the modified method of Krzyzanowsky, França Neto and Henning, (1991). The seeds were weighed to a precision of two decimal places and were then immersed in 37.5 mL of distilled water. At 24 hours after soaking at a temperature of 25 °C, electrical conductivity was determined in a conductivity meter, and the results were expressed in $\mu S$ $cm^{-1}$ $g^{-1}$.

## Biochemical analyses and electronic microscopy

To analyze the isoenzyme expression in electrophoresis gels, two replications of 50 seeds were performed, and they were ground and stored at a temperature of -86 °C. Seeds without parchment were ground in a refrigerated grinder at 4 °C and 22500 RPM along with PVP; and for enzyme extraction, 0.2 M Tris HCl buffer at pH 8.0 + (0.1% v.v β-mercaptoethanol) was used at a proportion of 320 μL per 100 mg of seed powder. The material was homogenized in a vortex and maintained in a refrigerator for one hour, followed by centrifugation at 14000 RPM for 60 minutes at 4 °C.

The electrophoretic run was performed in a system of polyacrylamide gels at 7.5% (resolving gel) and 4.5% (stacking gel). The gel/electrode system used was Tris-glycine pH 8.9. A quantity of 40 μL of the supernatant of the samples in the gel was applied, and the electrophoretic run was carried out at 150 V for 5 hours. At the end of the run, the gels were stained for the enzymes catalase, esterase, peroxidase, and ascorbate peroxidase (Alfenas et al., 2006).

For the electrophoretic analysis of the heat-resistant proteins, 100 mg of ground seed powder from each treatment, placed in 2 mL microtubes, was used. Extraction of the proteins was carried out by adding 1000 μL extraction buffer (50 mM Tris-HCl, pH=7.5; 500 mM NaCl; 5 mM MgCl2; and 1 mM PMSF). Subsequently, the microtubes were shaken in a vortex and centrifuged at 14000 RPM for 30 minutes at 4 °C. The supernatant was incubated in a water bath at 85 °C for 15 minutes and was once more centrifuged for 30 minutes at 14000 RPM. Then, 70 μL of the supernatant was placed in microtubes, to which 40 μL of the sample buffer (5 mL glycerol, 2.5 mL buffer solution of the stacking gel, and 2.5 mg bromophenol blue) was added, and distilled water to a final volume of 25 mL. Subsequently, they were placed in a boiling water bath for 5 minutes and 50 μL of each sample was placed in each channel of the polyacrylamide gel SDS-PAGE at 12.5% (resolving gel) and 6% (stacking gel).

The electrophoretic run was conducted with running buffer Tris-glycine + SDS pH 8.0 at 150 V for 4 hours. The gels were stained with Coomassie Blue (0.5 g Coomassie Blue R-250, 250 mL of ethanol, 50 mL of glacial acetic acid, and distilled water to a final volume

of 500 mL) for 12 hours and destained in 10% acetic acid solution and 5% ethanol, according to Alfenas et al. (2006).

The seeds of the most contrasting treatments (Protocols 1, 4, and 8) were immersed in modified Karnovsky fixative solution (25% glutaraldehyde, 10% formaldehyde in 0.2 M sodium cacodylate buffer, pH 7.2), pH 7.2, and placed in cold storage at 10 °C until preparation for scanning electron microscopy (SEM). For preparation, the materials (seeds and embryos) were washed in 0.05 M cacodylate buffer (three times for 10 minutes), postfixed in 1% osmium tetroxide for 1 hour and subsequently dehydrated in an increasing series of acetone concentrations (30, 50, 70, 90, and 100% three times). Final dehydration was performed in a critical point dryer (BAL-TEC CPD 030). The samples obtained were mounted on aluminum supports with double-sided carbon tape placed on a sheet of aluminum foil and then sputter coated with gold (BAL-TEC SCD 050) and observed under a LEO EVO 40XVP scanning electron microscope. Images were generated and registered digitally, with variable increases for each sample at 20 Kv and a distance from 7 to 8 mm.

### Experimental design and statistical analysis

The experiment was conducted in a completely randomized design in an $8 \times 2$ factorial arrangement consisting of eight cryopreservation protocols and two treatments (with and without immersion in cathode water), with four replications. The results were subjected to an analysis of variance, and mean values were compared by the Scott-Knott test at 5% probability through the statistical program SISVAR (Ferreira, 2014).

## RESULTS AND DISCUSSION

### Physiological evaluation prior cryopreservation

Analysis of newly collected and noncryopreserved seeds indicated good physiological quality, with germination of 93% and viability of 100%, and according to the other parameters evaluated, they had high vigor (Table 2). According to the standards of production and trade of coffee seeds, the minimum percentage of germination required for *Coffea arabica* L. is 70% (Brasil, 2012).

### Moisture content of cryopreserved seeds

After immersion in LN and subsequent rewarming, the moisture content of the samples of the different protocols, treated or not in cathode water, was determined,

and the values were compared to the moisture contents before cryopreservation (Table 3). An increase in the water content of the seeds was observed in all the studied cryopreservation protocols.

Seeds dehydrated in silica gel to 20% or 17% wb (Protocols 1 – 6) had a greater increase in moisture content than those dehydrated to 17% wb in saturated salt solutions (Protocols 7 and 8). The greatest increase in moisture content (8.8%) occurred in the seeds of Protocol 4 (dehydrated in silica gel to 17%, cooled at the rate of -1 °C min$^{-1}$ to -40 °C, and rewarmed for 2 minutes) that were not immersed in cathode water. The smallest increase (1.7%) was observed in the seeds of Protocols 7 and 8, which were immersed in cathode water after rewarming. These differences in seed moisture contents are due to variations in protocols and exposure times.

### Physiological evaluation

Significant interactions were not observed among the different cryopreservation protocols and the use of cathode water regarding the physiological quality of the seeds. However, a significant difference was observed among the cryopreservation protocols (Figure 1).

Seeds that exhibited greater vigor were those under Protocols 4 – 8 (Figure 1A). In these protocols, the seeds were dehydrated, cooled, and rewarmed in different ways, but they all shared the same moisture content (≈17%). Moreover, regarding the normal seedlings at 30 days after rewarming, the best results (89% and 94%) were observed with Protocols 7 and 8, respectively (Figure 1B). Again, the common factor between these two protocols is the moisture content (≈17%) (Tables 1 and 3), which indicates that the dehydration procedure in saturated saline solutions and the cooling procedures are also as important as the moisture content. In addition, similar to the newly collected seeds (Table 2), samples subjected to Protocols 7 and 8 exhibited a high percentage of germination after cryopreservation. Conversely, the lowest physiological viability occurred on Protocols 1 – 3, which all had higher moisture content (≈20%) (Figure 1).

Regarding the percentage of strong normal seedlings (Figure 1C), the best result was obtained in Protocol 7, in which seeds were dehydrated in saturated $(NH_4)_2SO_4$ solution and subjected to controlled cooling. In contrast, dehydration in NaCl (Protocol 8) followed by direct immersion in LN exhibited a mean value similar to seeds with moisture of 20% wb (Protocols 1 – 3).

**Table 2:** Mean values of moisture content (θ), radicle protrusion (RE), normal seedlings (NS), seedlings with expanded cotyledonary leaves (CL), root dry matter (RDM), shoot dry matter (SDM), viable embryos in tetrazolium salt (VE), and electrical conductivity (EC) of newly collected seeds of *Coffea arabica* L.

|  | Θ (% wb) | RE (%) | NS (%) | CL (%) | RDM (g) | SDM (g) | VE (%) | EC (µS cm$^{-1}$g$^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| Seeds | 38 | 97 | 93 | 92 | 0.231 | 1.104 | 100 | 10.53 |

**Table 3:** Moisture content in wet basis (wb%) of coffee seeds before cryopreservation (θi), after cryopreserved seeds that were subjected or not to treatment with cathode water (CW) (θf), and the increase in moisture content after rewarming (IMC - %).

| Protocol | θi (% wb) | CW Treatment | θf (% wb) | IMC (% wb) | Rewarming* |
|---|---|---|---|---|---|
| 1 | | + | 25.7 | 5.2 | 2 min |
| | | - | 28.2 | 7.7 | |
| 2 | 20.5 | + | 26.1 | 5.6 | 4 min |
| | | - | 28.3 | 7.8 | |
| 3 | | + | 25.7 | 5.2 | 6 min |
| | | - | 28.6 | 8.1 | |
| 4 | | + | 22.9 | 5.8 | 2 min |
| | | - | 25.9 | 8.8 | |
| 5 | 17.1 | + | 22.1 | 5.0 | 4 min |
| | | - | 24.5 | 7.4 | |
| 6 | | + | 23.5 | 6.4 | 6 min |
| | | - | 24.8 | 7.7 | |
| 7 | 17.4 | + | 19.1 | 1.7 | 2 min |
| | | - | 19.4 | 2.0 | |
| 8 | 17.5 | + | 19.2 | 1.7 | 2 min |
| | | - | 20.4 | 2.9 | |

*Water bath at 40±1 °C.

Higher percentages of seedlings with expanded cotyledonary leaves (Figure 1D) were obtained in the protocols with seed moisture contents of 17% wb, especially Protocols 7 and 8, after dehydration in saturated salt solutions. The root dry matter (Figure 1E) of the seeds dehydrated in NaCl to 17% wb and directly immersed in LN (Protocol 8) stood out in relation to the other protocols. In the same way, a greater shoot dry matter weight (Figure 1F) was also obtained in seeds dehydrated in saturated salt solutions in relation to seeds dehydrated in silica gel. According to these variables that indicate vigor, lower physiological quality was found when seeds were dehydrated to 20% wb. The viability of the embryos in the tetrazolium test (Figure 1G) remained high in the protocols in which seeds had a moisture content of 17% wb.

Electrical conductivity (Figure 1H), in turn, was greater in seeds under Protocols 3 and 7. In Protocol 3, the seeds had a moisture content of 20% wb (silica gel dehydration) and were subjected to precooling to -40 °C, while in Protocol 7, the seeds had a moisture content of 17% wb (dehydrated in saturated $(NH_4)_2SO_4$ solution) and were subjected to precooling to -50 °C. This finding may indicate that precooling promoted damage to seed tissues, thus leading to a higher electrical conductivity. Nevertheless, the electrical conductivity values were low.

The rewarming process has been reported as a key factor that determines the tolerance of coffee seeds to exposure in LN (Dussert; Engelmann, 2006). In general, except for the electrical conductivity parameter (Figure 1H), the different rewarming times (2, 4, or 6 minutes) promoted
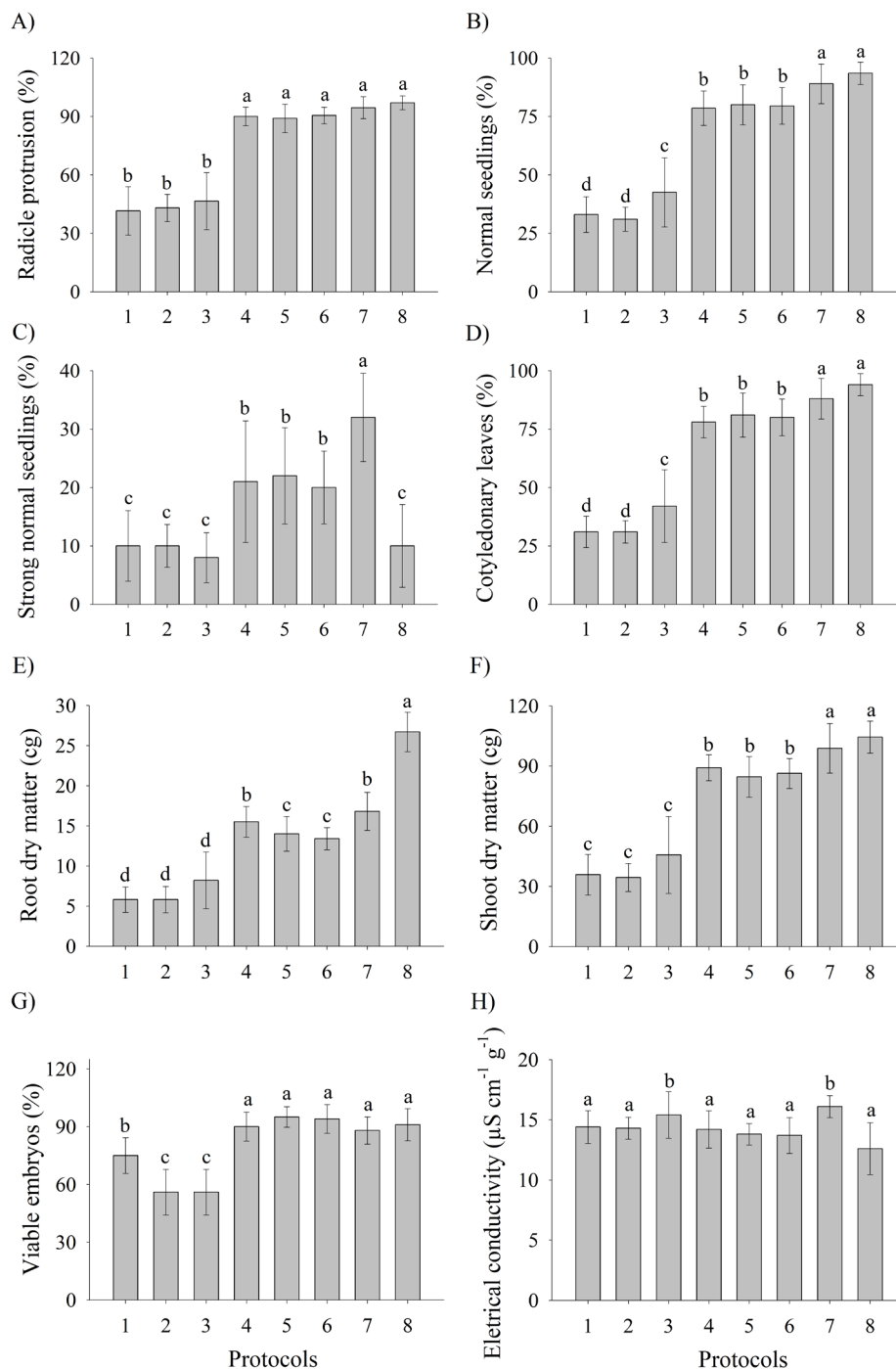
**Figure 1:** Results of evaluations of the physiological quality of *Coffea arabica* L. seeds under different cryopreservation protocols. Protocols 1, 2, and 3: dehydration in silica gel to 20% wb, cooling at the rate of -1 °C min^{-1} to -40 °C, and rewarming at 40±1 °C for 2, 4, or 6 minutes, respectively. Protocols 4, 5, and 6: dehydration in silica gel to 17% wb, cooling at the rate of -1 °C min^{-1} to -40 °C, and rewarming at 40±1 °C for 2, 4, or 6 minutes, respectively. Protocol 7: dehydration in saturated $(NH_4)_2SO_4$ solution to 17% wb, cooling at the rate of -1 °C min^{-1} to -50°C, and rewarming at 40±1 °C for 2 min. Protocol 8: dehydration in saturated NaCl solution to 17% wb, without controlled cooling, and rewarming at 40±1 °C for 2 minutes.

a significant difference in all variables tested when comparing the same rewarming time versus the moisture content. Seeds dehydrated until 17% wb always had better results than those dehydrated until 20% wb for the same rewarming time (Figure 1 A – G). Thus, the shorter time of 2 minutes would be most recommended to rewarm cryopreserved coffee seeds, and it is also recommended in the studies of Dussert et al. (1998) on procedures for the cryopreservation of coffee seeds.

For germination, which corresponds to the percentage of normal seedlings, the seeds that stood out were those under slower dehydration in saturated salt solutions. Dussert and Engelmann (2006) also observed that tolerance to LN exposure was greater when coffee seeds were dehydrated in saturated salt solution (81% RH). *Citrus* seeds show behavior similar to coffee seeds and also had a higher germination percentage after cryopreservation when dehydrated in saturated salt solutions with equilibrium relative humidity from 64% to 85% (Graiver; Califano; Zaritzky, 2011).

The viability of embryos of cryopreserved seeds (Figure 1G) was also higher when seeds were previously dehydrated to 17% wb. Desiccation of zygotic embryos of coconut also increases viability after cryopreservation as long as rapid dehydration, cooling, and rewarming are used (Sisunandar et al., 2010).

The main factor that has the greatest effect on cryopreservation of *Coffea arabica* L. seeds is moisture, which is more important than the dehydration rate, cooling or precooling procedures, and rewarming. A small variation in coffee seed moisture content can affect tolerance to cryopreservation. Dussert and Engelmann (2006) affirmed that an absolute lower limit of moisture content occurs and not an interval below which coffee seeds do not survive cryopreservation, regardless of the drying rate. In a study on cryopreservation of seeds of different cultivars of *Coffea arabica* L., the better performance of seeds dehydrated in silica gel compared to saturated salt solutions was confirmed (Coelho; Rosa; Fernandes, 2017b).

### Biochemical and ultrastructural evaluation

For the physiological quality parameters, the electrophoretic profiles of isoenzymes display no significant difference regarding immersion in cathode water. However, a difference was observed among cryopreservation protocols, with an increase in catalase activity observed in the seeds with 17% moisture content (wb), especially in Protocol 7 (Figure 2A).

In all the protocols, high ascorbate peroxidase activity was observed with high band intensity (Figure 2B).

Varghese and Naithani (2008) also observed high activity of the ascorbate peroxidase enzyme in cryopreserved *Azadirachta indica* A. Juss. seeds. This enzyme is more responsible for fine modulation of ROS for signaling than for removal of the excess of these radicals during oxidative stress (Maruta et al., 2016), which explains the high activity of ascorbate peroxidase in the seeds, indicating that for all the protocols, free radicals were produced during the cryopreservation process. In contrast, in dehydrated samples of Chinese fan palm (*Livistona chinensis*) embryos, ascorbate peroxidase activity declined after cooling (Wen et al., 2012).

Esterase activity also increased in seeds with 17% moisture content (wb) but was highest in Protocols 4 and 7 that were not subjected to cathode water. In seeds with a moisture content of 17% (wb), an increase was observed in the intensity of peroxidase enzyme bands except for seeds under Protocol 8 (Figure 2C). The high activity of esterase in more dehydrated seeds was also observed by Coelho et al. (2015) in coffee seeds, regardless of the drying method used. Esterease indicates deterioration in seeds. However, in the present study, the increase in its expression in more dehydrated seeds did not coincide with low physiological quality.

The low activity of peroxidase in seeds under Protocol 8 (Figure 1D) may be related to the fact that they were not precooled but directly immersed in LN after dehydration. In addition to its antioxidant function, peroxidase acts in the formation of the cell wall, thus contributing to its structural integrity (Pandey et al., 2017). Therefore, this protocol may have benefitted the seeds, which might not have undergone damage related to the formation of extracellular ice crystals during precooling, which can also be confirmed by their good physiological quality (Figure 1 – Protocol 8) and lower degradation observed in the structure of the cells (Figure 3 C, F and I) under this treatment.

For Protocols 1, 2, and 3, the low activity of peroxidase in the seeds can be explained by the lower damage brought about by slower drying in the saturated salt solution. Nevertheless, the worst physiological quality was observed in these seeds, which may be related to the greater moisture content present during precooling and could have caused the damage by the formation of extracellular ice crystals. Thus, except for the seeds that were not precooled, the seeds dehydrated to 17% wb underwent damage due to both dehydration and cooling methods. Abreu et al. (2014) and Coelho et al. (2015) also found that rapid and slow dehydration of coffee seeds of some treatments increases the activity of this enzyme.
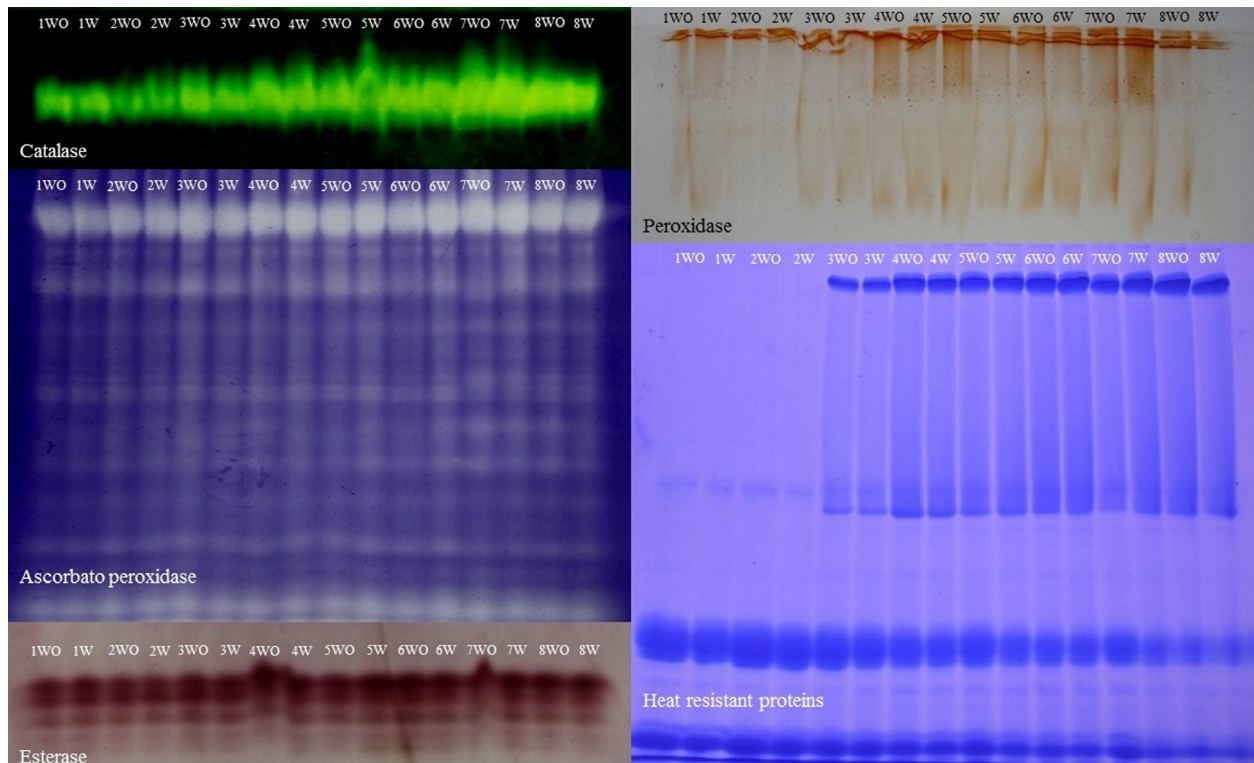
**Figure 2:** Electrophoretic pattern of isoenzymes in *Coffea arabica* L. seeds under different cryopreservation protocols. Protocols 1, 2, and 3: dehydrated in silica gel to 20% wb, cooling at the rate of -1 °C min$^{-1}$ to -40 °C, and rewarming at 40±1 °C for 2, 4, and 6 minutes, respectively. Protocols 4, 5, and 6: dehydrated in silica gel to 17% wb, cooling at the rate of -1 °C min$^{-1}$ to -40 °C, and rewarming at 40±1 °C for 2, 4, and 6 minutes, respectively. Protocol 7: dehydrated in saturated $(NH_4)_2SO_4$ solution to 17% wb, cooling at the rate of -1 °C min$^{-1}$ to -50 °C, and rewarming at 40±1 °C for 2 minutes. Protocol 8: dehydrated in saturated NaCl solution to 17%, without controlled cooling, and rewarming at 40±1 °C for 2 minutes. Evaluations performed without (WO) and with (W) treatment in cathode water after rewarming.

An analysis of all the electrophoretic profiles shows that the cathode water exhibited significantly positive results only for catalase (Figure 2A), which indicates that cathode water generally did not have an antioxidant effect on coffee seeds that underwent cryopreservation in the manner carried out in this study. Similarly, the results of the tests performed to verify physiological quality confirm this premise (Figure 1).

In relation to heat-resistant proteins, except for Protocols 1 and 2, strong band profiles were observed, especially in Protocol 3, in which the seeds with 20% wb were rewarmed for 6 minutes (Figure 2E). The accumulation of heat-resistant proteins is associated with the physiological quality of coffee seeds (Stavrinides et al., 2020). Abreu et al. (2014) also found that the band profile of heat-resistant proteins was more intense in coffee seeds that underwent slower dehydration. In addition, a reduction in moisture content led to the synthesis of these proteins. However, the present study shows that although the number of bands was greater in more dehydrated seeds, the intensity of the bands decreased according to the dehydration, precooling, and rewarming methods used. The immersion in cathode water also caused a decrease in the intensity of the bands in all the protocols studied (Figure 2).

The results of ultrastructural analyses in endosperm and embryo cells of coffee seeds under different cryopreservation protocols are shown in Figure 3. As the rewarming time in a water bath after cryopreservation did not affect seed physiological and biochemical quality, we decided to analyze the seeds subjected to the shorter rewarming time with different manners of dehydration and precooling procedures, i.e., Protocols 1, 4, and 8 (Table 1). The general structure of the embryo and endosperm cells of these protocols can be observed in Figure 3A.
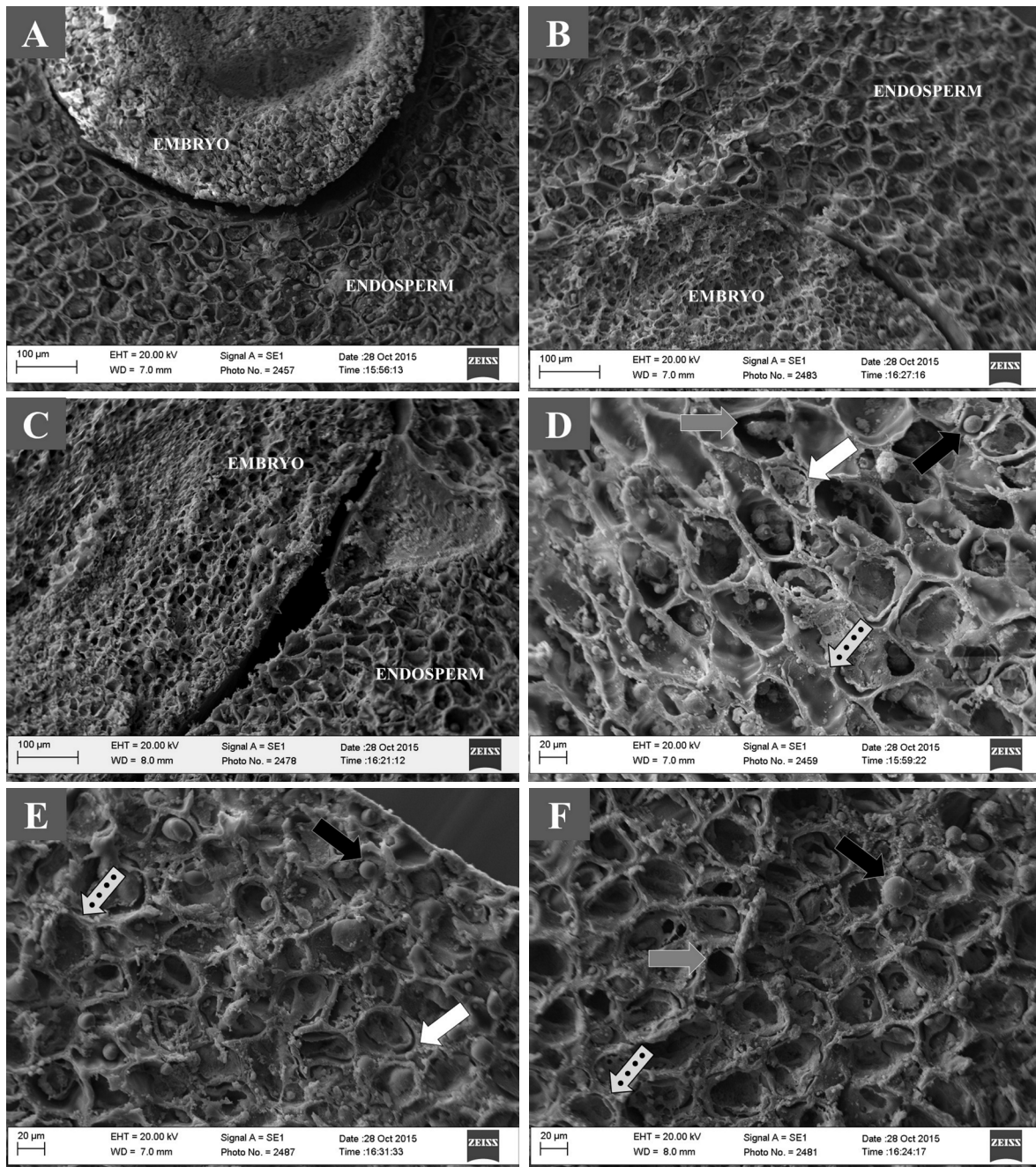
**Figure 3:** Scanning electron microscopy of *Coffea arabica* L. seeds under different cryopreservation protocols. Protocol 1: dehydration in silica gel to 20% wb, cooling at the rate of -1°C min$^{-1}$ to -40°C, and rewarming at 40±1°C for 2 minutes. (A, D, and G). Protocol 4: dehydration in silica gel to 17% wb, cooling at a rate of -1°C min$^{-1}$ to -40°C, and rewarming at 40±1°C for 2 minutes. (B, E, and H). Protocol 8: dehydration in saturated NaCl solution to 17% wb, without controlled cooling, and rewarming at 40±1°C for 2 minutes. (C, F, and I). These protocols did not include treatment with cathode water. Details of the embryo cells and of the endosperm cells (A, B, and C); only of the endosperm cells (D, E, and F); and only of the embryo cells (G, H, and I). Details of contracted cells (white arrows), oil drops (black arrows), empty cell spaces (gray arrows), and filled intercellular spaces (arrows with black circles).
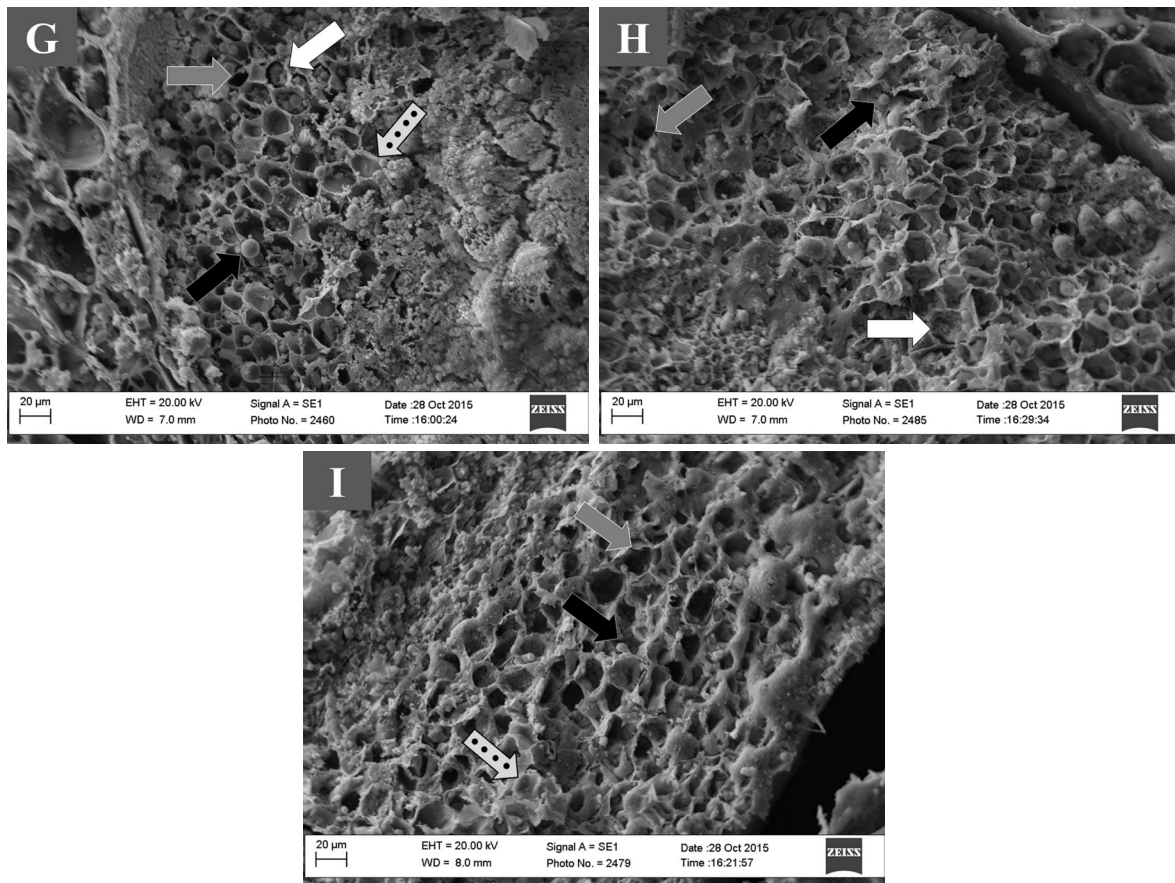
Continue...

**Figure 3:** Continuation.

The integrity of the cell membrane of the samples coming from Protocol 1 (Figure 3A) exhibited greater damage compared to the seeds of Protocols 4 (Figure 3B) and 8 (Figure 3C). The greater quantity of oil drops present in the embryos than in the endosperm confirms this more pronounced degradation.

For the endosperm cells from Protocol 1 (Figure 3D), the cells were more turgid. However, the integrity of the plasmatic membrane of some of these cells was compromised, resulting in leakage of the cytoplasm. In relation to Protocols 4 and 8 (Figures 3E and 3F, respectively), retraction in the cell wall and a reduction in cell content were observed, although the seeds remained intact. In these cells, less degradation of the cell plasmatic membrane occurs, and consequently, a smaller quantity of oil drops is observed. However, as was observed for the seed physiological quality (Figure 1), it was not possible to detect a clear difference in the cell structure of the endosperm between these last two

protocols despite the different forms of dehydration and cooling.

The cell structures of the embryos in the three protocols remained similar (Figure 3G, 3H, and 3I). In all electron micrographs, contracted cells (white arrow), oil drops (black arrow), turgid and intact cells (gray arrow), and filled intercellular spaces (arrows with black circles) were observed.

The cell structure of endosperm under Protocol 1 (Figure 3D) suffered greater damage through the formation of ice crystals during precooling because the moisture content present in these seeds was greater. In the studies of Saath et al. (2010), coffee seeds dehydrated to moisture contents from 30%-20% wb that showed compromised quality also exhibited damage to the cell structure of the endosperm.

Nevertheless, in the endosperm cells under Protocols 4 (Figure 3E) and 8 (Figure 3F), less structural damage was noted. These samples were more dehydrated,

providing greater tolerance to cryopreservation. Saath et al. (2010) also found a reduction in the cell volume of endosperm cells of coffee seeds as the moisture content was reduced.

Borém, Marques and Alves (2008) observed drops of oil spread across the surface of the endosperm tissues of coffee seeds that had the plasmatic membrane of the cells ruptured due to greater deterioration through an increase in drying temperature. In the present study, an abundance oil drops were observed in the electron micrographs of the cells of the endosperms of the seeds in Protocol 1 also confirmed the greater deterioration caused by this treatment compared to the others.

The structures of the embryo cells of seeds under the different protocols maintained similar patterns (Figure 3G-I). This result may indicate that the damage from cryopreservation is less drastic in the cells of the embryos than in those of the endosperm, the latter being more tolerant to the stresses of dehydration, precooling, and rewarming. These results corroborate those of other authors who performed studies on dehydration and cryopreservation of coffee seeds (Dussert; Engelmann, 2006; Coelho et al., 2015).

The dehydration rate and the moisture content as well as the use of precooling had significant effects on the physiological, biochemical, and structural quality of coffee seeds. Coelho et al. (2015) also observed an effect of the dehydration rate and moisture content on the physiological quality of coffee seeds when subjected to below-zero temperatures.

All the evaluations in the different protocols showed better physiological, biochemical, and ultrastructural results in the seeds subjected to Protocols 7 and 8. Although these two protocols are statistically equal regarding physiological performance, it should be emphasized that in Protocol 8, the seeds were directly plunged into LN. Thus, without the use of a computer-controlled biofreezer, the cryopreservation in Protocol 8 is simpler and more economical. For other species that are also sensitive to dehydration and cooling, direct immersion in LN is the best option. Michalak, Plitta-Michalak and Chmielarz (2015) observed that cherry (*Prunus avium* L.) seeds tolerate cryopreservation when they are dehydrated in a range of moisture contents from 17%-20% wb and are directly immersed in LN.

## CONCLUSIONS

Moisture content at 17% wb is the key factor for the cryopreservation of *Coffea arabica* L. seeds,

which have better physiological quality and better preserved cell structures. Precooling of *Coffea arabica* L. seeds before immersion in liquid nitrogen is not recommended because it does not provide advantages compared to direct immersion. The rewarming times tested (2, 4, and 6 minutes) and the use of cathode water did not cause changes in the physiological and biochemical quality or in the cell structures of *Coffea arabica* L. cryopreserved seeds. The pattern of cell structure observed in all seeds indicates that the damage from cryopreservation is less drastic in the cells of the embryos than in those of the endosperm, the latter being less tolerant to the stresses of dehydration, precooling, and rewarming.

## ACKNOWLEDGMENTS

## REFERENCES

ABREU, L. A. S. et al. Behavior of coffee seeds to desiccation tolerance and storage. **Journal of Seed Science**, 36(4):399-406, 2014.

ALFENAS, A. C. et al. **Eletroforese e marcadores bioquímicos em plantas e microorganismos**. 2. ed. Viçosa: Editora UFV, 2006. 627p.

BERJAK, P.; SERSHEN, B. V.; PAMMENTER, N. W. Cathodic amelioration of the adverse effects of oxidative stress accompanying procedures necessary for cryopreservation of embryonic axes of recalcitrant-seeded species. **Seed Science Research**, 21(3):187-203, 2011.

BORÉM, F. M.; MARQUES, E. R.; ALVES, E. Ultrastructural analysis of drying damage in parchment *Arabica coffee* endosperm cells. **Biosystems Engineering**, 99(1):62-66, 2008.

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento - Secretaria de Defesa Agropecuária. **Regras para análise de sementes**. Brasília: MAPA/ACS, 2009. 398p.

BRASIL. Instrução Normativa n. 35 de 29 de novembro de 2012. **Estabelecer as normas para a produção e comercialização de material de propagação de cafeeiro (*Coffea arabica* L. e *Coffea canephora* Pierre ex A. Froehner) e os seus padrões, com validade em todo o território nacional, visando à garantia de sua identidade e qualidade**. Brasília, 29 de novembro de 2012. Número 232, Seção 1, p.11. 2012.

COELHO, N.; GONÇALVES, S.; ROMANO, A. Endemic plant species conservation: Biotechnological approaches. **Plants**, 9(3):345, 2020.

COELHO, S. V. B. et al. Physiological and biochemical changes in coffee seed dried in silica gel and saturated saline solutions. **Pesquisa Agropecuária Brasileira**, 50(6):483-491, 2015.

COELHO, S. V. B.; ROSA, S. D. V. F.; FERNANDES, J. S. Cryopreservation of coffee seeds: A simplified method. **Seed Science & Technology**, 45(3):1-12, 2017b.

DAVIS, A. P. et al. An annotated taxonomic conspectus of genus Coffea (Rubiaceae). **Botanical Journal of the Linnean Society,** 152(4):465-512, 2006.

DUSSERT, S.; ENGELMANN, F. New determinants for tolerance of coffee (*Coffea arabica* L.) seeds to liquid nitrogen exposure. **CryoLetters**, 27(3):169-178, 2006.

DUSSERT, S. et al. Beneficial effect of post-thawing osmoconditioning on the recovery of cryopreserved coffee (*Coffea arabica* L.) seeds. **CryoLetters**, 21(1):47-52, 2000.

DUSSERT, S. et al. Cryopreservation of coffee (*Coffea arabica* L.) seeds: Importance of the precooling temperature. **CryoLetters**, 18:269-276, 1997.

DUSSERT, S. et al. Cryopreservation of seed of four coffee species (*Coffea arabica, C. costatifructa, C. racemosa* and *C. sessiliflora*): Importance of water content and cooling rate. **Seed Science Research**, 8(1):9-15, 1998.

EIRA, M. T. S. et al. Banco de sementes de café em criopreservação: Experiência inédita do Brasil. **Circular Técnica**, 42:1-3, 2005.

ELLIS, R. H.; HONG, T. D.; ROBERTS, E. H. An intermediate category of seed storage behaviour?: I. Coffee. **Journal of Experimental Botany,** 41(9):1167-1174, 1990.

FERREIRA, D. F. Sisvar: A Guide for its Bootstrap procedures in multiple comparisons. **Ciência e Agrotecnologia**, 38(2):109-112, 2014.

FIGUEIREDO, M. A. et al. Exploratory studies for cryopreservation of *Coffea arabica* L. seeds. **Journal of Seed Science**, 39(2):150-158, 2017.

FRANÇA NETO, J. B.; KRZYZANOWSKI, F. C.; COSTA, N. P. Metodologia do teste de tetrazólio em sementes de soja. In: KRZYZANOWSKI, F. C.; VIEIRA, R. D.; FRANÇA NETO, J. B. **Vigor de sementes:** Conceitos e testes. Londrina: ABRATES. Cap 8.5, p.1-27, 1999.

GRAIVER, N.; CALIFANO, A.; ZARITZKY, N. Partial dehydration and cryopreservation of *Citrus* seeds. **Journal of the Science of Food and Agriculture**, 91(14):2544-2550, 2011.

KRZYZANOWSKY, F. C.; FRANÇA NETO, J. B.; HENNING, A. A. Relatos dos testes de vigor disponíveis para as grandes culturas. **Informativo ABRATES**, 1(2):15-50. 1991.

LEN, J. S.; KOH, W. S. D.; TAN, S. X. The roles of reactive oxygen species and antioxidants in cryopreservation. **Bioscience Reports**, 39(8):1-25, 2019.

MARUTA, T. et al. Diversity and evolution of ascorbate peroxidase functions in chloroplasts: More than just a classical antioxidant enzyme? **Plant and Cell Physiology**, 57(7):1377-1386, 2016.

MICHALAK, M.; PLITTA-MICHALAK, B. P.; CHMIELARZ, P. A new insight in desiccation tolerance and cryopreservation of mazzard cherry (*Prunus avium* L.) seeds. **Central European Journal of Biology**, 10(1):354-364, 2015.

NOOR, N. M.; SULONG, N.; REED, B. M. Cryopreservation of shoot tips of recalcitrant and tropical species: Advances and strategies. **Cryobiology,** 87:1-14, 2019.

ORJUELA-PALACIO, J. M. et al. Effect of the desiccation tolerance and cryopreservation methods on the viability of *Citrus limon* L. Burm cv. Eureka seeds. **Cryobiology**, 89:51-59, 2019.

PANDEY, V. P. et al. A comprehensive review on function and application of plant peroxidases. **Biochemistry & Analytical Biochemistry**, 6(1):1-16, 2017.

ROBERTS, E. H. Predicting the storage life of seeds. **Seed Science and Tecnology**, 1(4):499-514, 1973.

SAATH, R. et al. Scanning electron microscopy of the endosperm of coffee (*Coffea arabica* L.) during the drying process. **Ciência e Agrotecnologia**, 34(1):196-203, 2010.

SISUNANDAR, R. A. et al. Cryopreservation of coconut (*Cocos nucifera* L.) zygotic embryos does not induce morphological, cytological or molecular changes in recovered seedlings. **Planta**, 232(2):435-447, 2010.

STAVRINIDES, A. K. et al. Seed comparative genomics in three coffee species identify desiccation tolerance mechanisms in intermediate seeds. **Journal of Experimental Botany**, 71(4):1418-1433, 2020.

VARGHESE, B.; NAITHANI, S. C. Oxidative metabolism-related changes in cryogenically stored neem (*Azadirachta indica* A. Juss) seeds. **Journal of Plant Physiology**, 165(7):755-765, 2008.

WALTERS, C. Orthodoxy, recalcitrance and in-between: Describing variation in seed storage characteristics using threshold responses to water loss. **Planta**, 242(2):397-406, 2015.

WEN, B. et al. Cytological and physiological changes in recalcitrant Chinese fan palm (*Livistona chinensis*) embryos during cryopreservation. **Protoplasma**, 249(2):323-335, 2012.