










Steps of cryopreservation of coffee seeds: physiological responses and antioxidant systems

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ABSTRACT: The cryopreservation of plant germplasm at ultralow temperatures is an alternative technique for the long-term storage of seeds of the genus *Coffea sp.* However, for this technique to be successful, cell integrity must be maintained at all stages of the process on the basis of scientific research. The present study investigated validated cryopreservation protocols for *Coffea arabica* L. seeds and evaluate the effects on the physiological and biochemical characteristics of the seeds at each stage of the process. Seeds were dried on silica gel or with saturated saline solution, precooled or not in a biofreezer, immersed in nitrogen, and reheated in a water bath. After each of these steps, the physiological and biochemical quality of the seeds was determined. Pre-cooling is a step that can be dispensed with in the cryopreservation of *Coffea arabica* seeds, direct immersion in liquid nitrogen being more indicated. *Coffea arabica* L. seeds tolerate cryopreservation after rapid drying in silica gel up to water contents of 17 or 20% (wb), with greater survival at 17%. The enzyme activities of catalase, polyphenol oxidase and peroxidase are indicators of the quality of *C. arabica* L. seeds subjected to cryopreservation.

Key words: *Coffea arabica* L, drying, precooling, cryopreservation, isoenzymes.

Etapas da criopreservação de sementes de café: respostas fisiológicas e de sistemas antioxidantes

RESUMO: A criopreservação de germoplasma vegetal em temperaturas ultrabaixas é uma alternativa para o armazenamento em longo prazo de sementes do gênero *Coffea sp.* Entretanto, para que essa técnica apresente sucesso, a realização de pesquisas que garantam a manutenção da integridade celular em todas as etapas do processo é de fundamental importância. O objetivo com o presente trabalho foi investigar protocolos de criopreservação validados para sementes de *Coffea arabica* L., avaliando separadamente, os efeitos sobre as características fisiológicas e bioquímicas das sementes, em cada etapa do processo. As sementes foram secadas em sílica gel ou em solução salina saturada, submetidas ou não ao pré-resfriamento em biocongelador, em seguida imersas no nitrogênio e reaquecidas em banho-maria. Após cada uma dessas etapas, a qualidade fisiológica e bioquímica das sementes foi determinada. O pré-resfriamento é uma etapa que pode ser dispensada na criopreservação de sementes de *Coffea arabica*, sendo mais indicada a imersão direta em nitrogênio líquido. Sementes de *Coffea arabica* L. toleram a criopreservação após secagem rápida em sílica gel até teores de água de 17 ou 20% (wb), com maior sobrevivência a 17%. A atividade das enzimas catalase, polifenoloxidase e peroxidase são indicadoras da qualidade de sementes de *Coffea arabica* L. submetidas à criopreservação.

Palavras-chave: *Coffea arabica* L, secagem, pré-resfriamento, criopreservação, isoenzimas.

INTRODUCTION

Coffee is of great importance because it is the second most traded commodity in the world. It belongs to the genus *Coffea*, and two species are globally important, *Coffea arabica* and *Coffea canephora*. Due to the great economic importance of coffee, the conservation of the genetic diversity of *Coffea sp.* is essential (BERJAK & PAMMENTER, 2014).

The low longevity of coffee seeds is a limiting factor for long-term germplasm maintenance and endangers existing genetic variability. Because of their intermediate to recalcitrant behavior (ELLIS et al., 1990), seeds of the genus *Coffea* are not preserved in

conventional seed banks at -18 °C to -20 °C due to their sensitivity to desiccation and cooling (PAMMENTER & BERJAK, 2014; COELHO et al., 2017b).

To ensure the safe storage of coffee genetic resources, research has been conducted to improve low-temperature storage techniques for intermediate or recalcitrant seeds, such as cryopreservation, to avoid the formation of ice crystals inside the plant cells. Drying, cooling and rewarming are the main steps that influence the success of cryopreservation (ENGELMANN, 2011; BERJAK & PAMMENTER, 2014; FIGUEIREDO et al., 2021).

In cryopreservation, the water content of the seeds is a crucial factor to ensure survival

during storage in liquid nitrogen because water, when frozen, expands and forms sharp ice crystals that can perforate the internal structures, causing cell death (ENGELMANN, 2011). However, drying, which is a mandatory step for the removal of water prior to freezing, can cause stress in seeds that are sensitive to desiccation, causing damage to cellular metabolism due to the substantial production of free radicals (BERJAK & PAMMENTER, 2013).

The classic cryopreservation method, one of the most widely used methods for seed preservation, involves controlled cooling of the sample, while the modern method consists of direct immersion in liquid nitrogen (ENGELMANN, 2011); both of these techniques are performed after partial dehydration of the seeds. However, cell damage related to the drying and precooling steps can occur in these methods (PAMMENTER & BERJAK, 2014), and there are no conclusive studies on which method is the safest to cryopreserve *Coffea sp.* seeds or on the behavior of the antioxidant systems under cell damage due to freezing.

Coffee cryopreservation techniques have been studied to develop safe protocols for use in typical coffee seed/germplasm banks (DUSSERT et al. 1998, 2001, 2003, 2012; DUSSERT & ENGELMANN, 2006; FIGUEIREDO et al., 2017; COELHO et al. 2017a, 2017b and 2019; FIGUEIREDO et al., 2021). However, due to the wide variety of existing cultivars, in addition to diverging results, there is still much to be studied.

Considering the strategic need for the long-term conservation of coffee plant genetic resources and the lack of detailed studies of each stage of the cryopreservation process, the objective of the present study was to investigate different protocols for cryopreservation of *C. arabica* L. seeds described in the literature and to evaluate the effects on the physiological and biochemical characteristics of the seeds at each stage of the process, i.e., drying, cooling and rewarming after immersion in liquid nitrogen.

MATERIALS AND METHODS

Physiologically mature fruits of the species *Coffea arabica* L., cultivar Catuaí amarelo IAC 62, were harvested from crops at the Fazenda Experimental Procafé, in Varginha, southern Minas Gerais. The fruits were selectively harvested from the middle parts of the middle branches of the plants,

and after harvesting, they were again selected for uniformity of the maturation stage. After mechanical stripping, the seeds were demucilaged by fermentation in water for 24 hours at room temperature and then predried in the shade to remove surface moisture. To ensure uniformity in size, the seeds were sieved, and only those retained in circular sieves no. 18/64 to 20/64 were used.

After processing, the seeds were subjected to determination of the initial water content by the oven method at 105 °C for 24 hours (BRASIL, 2009), with two replicates of 10 seeds for each experimental plot. Results were expressed as percentages based on the wet weight of the seeds.

The seeds were used to investigate the effects at each stage of previously validated protocols for the cryopreservation of *C. arabica* L., i.e., drying, precooling and immersion in liquid nitrogen. The steps investigated in each protocol are described in Table 1.

Drying of Coffea arabica L. seeds

Initially, the seeds were pre-dried in the shade on sieves to remove surface water. After this operation, the initial water content of the seeds (43% wb) was determined, as well as the physiological quality. Then, the seeds were subjected to two drying methods, using silica gel or saturated saline solution, until reaching moisture contents of 35, 20, 17 and 12% (wet basis, wb).

For silica drying, the seeds were placed in a single layer on metal screens inside gerbox-type acrylic boxes containing 60 mg of activated silica gel below the screens. The boxes containing the seeds were hermetically sealed and kept in *biochemical oxygen demand* (BOD) chambers at 25 °C in the absence of light. The water loss during drying was monitored by continuous weighing on a scale with a precision of 0.0001 grams until the samples reached the water levels mentioned above.

For drying with saturated saline solutions, two salts were used, ammonium sulfate at 81% relative humidity or sodium chloride at 75% relative humidity, in the same types of hermetic containers described above at 25 °C. The seeds were placed in a single layer on metal screens above the saturated saline solutions in gerbox-type acrylic boxes. The water loss during drying by hygroscopic equilibrium was monitored until the samples reached the same water levels as those obtained in the faster drying method with silica gel. After drying, the seeds were subjected to physiological and biochemical evaluations.

Table 1 - Description of the steps of the investigated protocols for cryopreservation of *Coffea arabica* L.

Protocol	Stages of cryopreservation			
	Drying	Precooling	Immersion in liquid nitrogen	Rewarming
1*	Silica gel to 20% wb	In a biofreezer at 1 °C/min until reaching -40 °C	In aluminum foil envelopes	water bath, 40±1 °C/2 min
2*	Silica gel to 17% wb			
3	Silica gel to 20% wb	Without precooling (direct immersion)	In aluminum foil envelopes	water bath, 40±1 °C/2 min
4	Silica gel to 17% wb			
5*	Saturated (NH ₄) ₂ SO ₄ solution to 17% wb	In a biofreezer at 1 °C/min until reaching -50 °C	In cryotubes	water bath, 40±1 °C/2 min
6	Saturated NaCl solution to 17% wb	Without precooling (direct immersion)	In cryotubes	water bath, 40±1 °C/2 min

*Samples from protocols 1, 2 and 5, which included a precooling step, were evaluated before and after immersion in liquid nitrogen. Protocols 1 to 4: (Figueiredo et al., 2021; Coelho et al., 2017a and 2017b). Protocol 5: (Dussert et al. (1997; 1998; 2000); Dussert and Engelmann, (2006)). Protocol 6: Eira et al. (2005).

Precooling in biofreezer

For evaluation of the effects of precooling, seeds previously dried on silica gel until reaching a moisture content of 20% wb (protocol 1) or 17% wb (protocol 2) and seeds dried with saturated ammonium sulfate saline solution (protocol 5) were subjected to pre-cooling, which is an optional preparatory step before immersion in liquid nitrogen. Seeds that had been dried on silica gel were placed in trifoliate aluminum foil packages, and those that had been dried with saturated ammonium sulfate saline solution were packed in cryotubes, according to each protocol (Table 1). Pre-cooling was performed in an Icecube 14S-B *biofreezer* with SY-LAB *software* (Minitub of Brazil[®]) at a rate of -1 °C min⁻¹ to -40 °C or -50 °C, as summarized in Table 1. To assess the physiological and biochemical effects of pre-cooling, the seeds of all treatments at this stage were reheated in a water bath at 40 °C for two minutes (DUSSERT et al., 1998) or on a laboratory bench at an average temperature of 25 °C.

Immersion in liquid nitrogen, rewarming and resumption of growth

To evaluate the effects of immersion in liquid nitrogen, the seeds from each protocol (dried with silica gel or saturated saline solution, precooled or not, as described in Table 1), were directly immersed in liquid nitrogen; this process corresponded to a cooling rate on the order of 200 °C min⁻¹ (DUSSERT et al., 2001). After 36 hours in the cryotank, the seeds were quickly removed and reheated in direct contact with water in a water bath at 40 °C ± 1 °C for two minutes (DUSSERT et al., 1998). After these

procedures, the seeds were subjected to physiological and biochemical evaluations.

Physiological evaluations

The germination test was performed with four replicates of 50 seeds, which were sown on germination paper moistened with distilled water at two and a half times the weight of the dry paper. Acrylic plates were used for sowing, and the rolls with the seeds were placed in a germinator set at 30 °C in the presence of light, according to BRASIL (2009), with modifications. In the germination test, the percentage of normal seedlings at 30 days was determined (BRASIL, 2009), as well as the percentage of root protrusion at 15 days, the percentage of strong normal seedlings, the seedlings that presented hypocotyls of three centimeters or more, the percentage of seedlings with expanded cotyledon leaves at 45 days after sowing, and, at the end of the test, the root and shoot dry mass of normal seedlings after drying in a forced air oven at 60 °C for five days. The dry mass was determined on a scale with a precision of 0.0001 grams and expressed in mg/seedling.

The electrical conductivity test was performed for all treatments with four replicates of 12 seeds after the drying and precooling steps and four replicates of 25 seeds after the removal of liquid nitrogen, according to the methodology of KRZYZANOWSKY et al. (1991) with modifications. The seeds were weighed to two decimal places and then kept in containers containing 18.7 ml or 37.5 ml of distilled and deionized water according to the protocol used (precooling or cryopreservation,

respectively). After 24 hours of soaking at 25 °C, the electrical conductivity was measured with a conductivity meter (MS TECNOPON®, model mCA 150), and the results were expressed in $\mu\text{S cm}^{-1} \text{g}^{-1}$ of seeds.

Biochemical analyzes

The expression of isoenzymes in electrophoresis gels was determined after each step described above. The seeds without parchment were ground in a refrigerated mill at 4 °C at 22,500 RPM in the presence of polyvinylpyrrolidone (PVP) and stored at -80 °C until analysis. For the extraction of enzymes, 0.2 M Tris HCl buffer (pH 8.0 + 0.1% v/v mercaptoethanol) was used at a ratio of 320 μL per 100 mg of seed powder. The material was vortexed and kept in the refrigerator for one hour, followed by centrifugation at 14,000 RPM for 60 minutes at 4 °C.

The electrophoretic run occurred in a 7.5% polyacrylamide gel system (separator gel) with 4.5% concentrator gel. The gel/electrode system used was Tris-glycine pH 8.9. A total of 40 μL of the sample supernatant was applied to the gel, and the electrophoretic run was performed at 150 V for five hours. After the run, the gels were developed for the enzymes superoxide dismutase (SOD), catalase (CAT), peroxidase (PO), and polyphenol oxidase (PPO) (ALFENAS et al., 2006), and the expression of isoenzymes in the gels was visually analyzed and interpreted.

The analyses were performed in a completely randomized design in a double factorial scheme for the precooling step (two types of drying x two rewarming methods). The drying and nitrogen immersion stages, composed of seven and six treatments, respectively, were analyzed with four replicates. Analysis of variance was used, and the means were grouped by the *Tukey test* at 5% probability (FERREIRA, 2014).

RESULTS AND DISCUSSION

Results of physiological evaluations

Evaluation after seed drying

Seeds dried to different water contents (Table 2) were evaluated with germination and electrical conductivity tests. According to the analysis of variance of the physiological data, there were no significant differences in the percentage of root protrusion ($P < 0.05$). The other variables were affected by the different moisture levels tested; the wet seeds with 43% water content exhibited high

physiological quality, with 91% normal seedlings in the germination test.

In general, the seeds dried to 17% moisture did not suffer a loss of physiological quality with drying, as indicated by the high percentage of germination in these treatments, which ranged from 94.5 to 82.5%. Seeds dried to 17% in NaCl obtained similar or even higher germination and vigor results (CL and ADM) than wet seeds. However, lower percentages of normal seedlings, cotyledonary leaves and dry mass were observed in the driest seeds with 12% moisture (Table 2).

A reduction in seed vigor was observed after the drying treatments, according to the variables strong normal seedlings, root dry mass and electrical conductivity (Table 2).

Seeds dried to 12% and 17% displayed a reduction in vigor when compared to seeds with 43% moisture, except for those dried with saturated NaCl saline solution, which maintained vigor after drying. The seeds dried in $(\text{NH}_4)_2\text{SO}_4$ saline solution exhibited a reduction in vigor, presumably caused by the longer drying time than those in the other protocols, which possibly caused damage to the seeds (Table 2).

In the electrical conductivity test, the greatest exudate leaching was observed in the seeds that were dried to 12% ($39.5 \mu\text{S cm}^{-1} \text{g}^{-1}$), followed by the seeds dried to 17% and 20%, both in the fast drying process on silica gel and the slower process. As the seeds lost water, there was an increase in leaching according to the electrical conductivity test (Table 2).

COELHO et al. (2015) reported that seeds sensitive to desiccation can survive lower water contents if subjected to faster drying because there is not enough time for damage to accumulate. Other authors have identified the rapid drying of coffee seeds as the best method to achieve lower water contents without loss of viability (DUSSERT & ENGELMANN, 2006; COELHO et al., 2015). Conversely, fast drying has greater potential for damage to the endosperm than to the embryos (COELHO et al., 2015) and is not recommended when seeds are dried at intermediate levels and/or stored for long periods (ABREU et al., 2014).

According to these results, drying, which is a prerequisite step for successful cryopreservation, can negatively affect the initial seed quality, which will influence seed survival after immersion in liquid nitrogen. Therefore, a method should be found that minimizes the deleterious effects of water withdrawal

Table 2 - Results of the physiological evaluation of *Coffea arabica* L. seeds after drying to different water contents by different methods. Root protrusion (RP); normal seedlings (NS); normal-strength seedlings (NSS); seedlings with expanded cotyledonary leaves (CL); dry mass of aerial parts (ADM); root dry mass (RDM); electrical conductivity (EC).

Water content	Drying method	RP %	NS %	NSS %	CL %	ADM (mg)	RDM (mg)	EC ($\mu\text{S cm}^{-1} \text{g}^{-1}$)
43%	Shade	93.5 a	91.0 ab	44.0 a	86.5 bc	51.5 a	09.0 a	20.8 a
35%	Silica	96.0 a	91.0 ab	44.0 a	90.5 a	54.0 a	08.2 ab	24.6 ab
20%	Silica	97.5 a	91.5 ab	26.0 abc	87.5 bc	50.0 ab	08.2 ab	32.0 c
17%	Silica	94.5 a	82.5 bc	14.5 c	71.5 bc	41.5 bc	05.5 c	32.8 cd
0	(NH ₄) ₂ SO ₄	95.5 a	88.5 abc	21.0 bc	75.5 abc	41.4 bc	07.0 bc	32.2 c
17%	NaCl	96.0 a	94.5 a	35.5 ab	90.5 a	52.8 a	08.2 ab	28.7 bc
12%	Silica	93.5 a	80.0 c	18.0 bc	62.5 c	38.6 c	05.8 c	39.5 d
CV (%)		3.30	5.11	29.16	9.39	8.23	8.51	10.20

Means followed by the same letter do not differ significantly from each other by the Tukey test at the level of 5% probability.

from the seeds to increase the success of the cryopreservation technique.

Evaluation of precooled coffee seeds in a biofreezer

In this step, seeds from protocols 1, 2, and 5, which included precooling, were evaluated after biofreezer treatment, after drying and before immersion in liquid nitrogen (Table 1). This procedure, as well as the rewarming temperature, influenced the physiological quality of the seeds. In general, seeds with 17% moisture that had been pre-cooled after drying with saturated (NH₄)₂SO₄ saline solution obtained lower average values at the two rewarming temperatures for the evaluated parameters, except for root protrusion and electrical conductivity, indicating loss of seed quality. Seeds dried rapidly in silica gel obtained better results than those dried with saturated saline solution for the two rewarming temperatures tested (Table 3).

The electrical conductivity results showed that the seeds that had been subjected to rapid drying to 20% and precooled exhibited higher leaching of exudates when reheated at room temperature (average of 25 °C) than when reheated in a water bath at 40 °C for two minutes, indicating that the former treatment is more detrimental to the physiological quality of the seeds (Table 3).

The rewarming temperature did not significantly affect the parameters of the seeds dried to 20% (wb) on silica gel, with overall means of 94.5% protrusion, 86.5% normal seedlings, 18.75% normal-strength seedlings, 83% cotyledonary leaves, 40% aerial part dry weight and 7.7% root dry weight (Table 3).

In general, seeds dried on silica gel showed better physiological quality after precooling and rewarming than seeds dried with saturated saline

Table 3 - Physiological evaluation of *Coffea arabica* L. seeds dried to 20 or 17%, precooled in a biofreezer and reheated at 25 °C or in a water bath at 40 °C. Root protrusion (RP); normal seedlings (NS); normal-strength seedlings (NSS); seedlings with expanded cotyledonary leaves (CL); dry mass of aerial parts (ADM); root dry mass (RDM); electrical conductivity (EC).

Protocol	-----RP %-----		-----NS %-----		-----NSS %-----		-----CL %-----	
	25 °C	40 °C	25 °C	40 °C	25 °C	40 °C	25 °C	40 °C
1. Silica gel to 20%	95.0 Aa	94.0 Aa	87.0 Aa	86.0 Aa	20.0 Aa	17.5 Aa	83.0 Aa	82.5 Aa
2. Silica gel to 17%	90.5 ABa	93.0 Aa	89.0 Aa	85.5 Aa	19.0 Aa	14.5 Aa	82.5 Aa	86.0 Aa
5. (NH ₄) ₂ SO ₄ to 17%	87.5 Ba	87.2 Aa	44.6 Ba	52.5 Ba	11.2 Aa	11.2 Aa	40.0 Ba	47.0 Ba
CV (%)	7.82		29.32		62.85		33.07	
Protocol	-----ADM (mg)-----		-----RDM (mg)-----		-----CE ($\mu\text{S cm}^{-1} \text{g}^{-1}$)-----			
	25 °C	40 °C	25 °C	40 °C	25 °C	40 °C		
1. Silica gel to 20%	40.9 Aa	39.3 Aa	8.3 Aa	7.0 ABa	64.8 Ab	58.8 Aa		
2. Silica gel to 17%	41.5 Aa	40.8 Aa	8.3Aa	9.1 Aa	63.2 ABa	63.2 Aa		
5. (NH ₄) ₂ SO ₄ to 17%	19.3 Ba	23.4 Ba	4.0 Ba	4.9 Ba	58.0 Ba	60.4 Aa		
CV (%)	34.18		33.55		9.16			

Means followed by the same uppercase letter in a column and lowercase letter in a row for each parameter do not differ by Tukey's test at the 5% probability level.

solution. In addition, the vigor test demonstrated that the rewarming temperature did not influence the quality of the seeds, and the type of drying was the most relevant factor at this stage (Table 3).

Rewarming can be performed slowly at room temperature or quickly using a water bath at 40 °C (DUSSERT et al., 1998). The rewarming method used depends on the type of material being cryopreserved. Typical seeds can be reheated slowly at room temperature without any apparent harmful effects (TOWILL, 2002). Cotton seeds that had been exposed to liquid nitrogen for different periods up to 90 days were reheated at room temperature and evaluated for germination and vigor, and no loss in quality was observed (ROCHA et al., 2009). In another study, tobacco seeds were cryopreserved in liquid nitrogen and then reheated in a water bath or at room temperature, and no loss in physiological quality was observed with either rewarming method (LOPES et al., 2018).

According to PANIS et al. (2005), rapid rewarming reduces the possibility of recrystallization of intracellular ice, thus leading to greater recovery and survival of materials cooled in liquid nitrogen. In addition to the cryopreservation process, the rewarming method should be considered because the faster rewarming proceeds, the better the preservation of seed physiological characteristics, according to JAGANATHAN & LIU (2014).

Evaluation at the end of seed cryopreservation

The seeds were evaluated at the end of cryopreservation via the different protocols in this study. For evaluation, seeds from all protocols (1 to 6) were removed from the liquid nitrogen and reheated in a water bath at 40 °C for two minutes. Significant differences were observed for all variables tested, and the coffee seeds subjected to slow drying with saturated saline solution (protocols 5 and 6) had the lowest means, indicating lower quality (Table 4).

In contrast to previous studies (FIGUEIREDO et al., 2021; COELHO et al., 2017a), the seeds cryopreserved after drying with saturated $(\text{NH}_4)_2\text{SO}_4$ saline solution to 17% wb did not survive immersion in liquid nitrogen (Table 4). Since the seeds exhibited no damage from desiccation, with 90% germination after drying (Table 2), this low survival after exposure to liquid nitrogen may be because of damage in the rewarming step after the removal of liquid nitrogen.

COELHO et al. (2019) observed that slow drying is harmful when drying coffee seeds for cryopreservation. For all parameters evaluated in this study, fast drying to 20% wb (0.25 g g⁻¹) was

better than slow drying. These results demonstrated the importance of seed drying rate to successful cryopreservation.

Some studies related to the cryopreservation steps, i.e., drying, cooling and rewarming, of seeds of the genus *Coffea* report inconsistent results regarding the evaluated parameters (DUSSERT & ENGELMANN, 2006; COELHO et al., 2015, COELHO et al., 2019; FIGUEIREDO et al., 2017). In these studies, the cryopreservation of *C. arabica* L. seeds after drying with saturated saline solution maintained seed quality at a high level.

Factors such as initial seed quality, drying conditions, cooling rate and thawing method are important for the survival of species post-cryopreservation. In the present study, high initial seed quality was observed, as well as high quality after drying; however, when the seeds were dried more slowly with saturated saline solution and immersed in liquid nitrogen, they did not survive.

In general, the results of this study suggested that rapid drying in silica gel is ideal for the cryopreservation of *C. arabica* L. Furthermore, the seed pre-cooling method, direct immersion or slow freezing with the use of the biofreezer, did not influence the final quality when seeds were dried on silica gel to a moisture content of 17 or 20%. Hence, the crucial factor for the successful cryopreservation of *C. arabica* L. seeds is the drying step, and care must be taken in rewarming the seeds after immersion in liquid nitrogen.

Results of the analyzes of antioxidant systems

The changes in the process of seed preparation for cryo-preservation with respect to drying were evaluated by the electrophoresis gel expression of CAT, SOD, PPO and PO isoenzymes (Figure 1). Only three drying treatments were used for this step (43, 35 and 12% moisture), which were the treatments with the greatest discrepancy in the physiological analyses.

CAT (Figure 1A) showed lower activity in wet seeds with 43% and 35% water content. When the seeds were dried to 12%, there was an increase in the activity of this enzyme. Because CAT acts by removing free radicals, such as hydrogen peroxide (H_2O_2), which is decomposed into water (H_2O) and oxygen (O_2) (GARG & MANCHANDA, 2009), lower CAT expression may indicate less damage from drying due to the lower amount of reactive oxygen species (ROS).

The stress caused to seeds by drying results in an increase in the production of ROS and

Table 4 - Physiological evaluation of *Coffea arabica* L. seeds subjected to different cryopreservation protocols. Root protrusion (RP); normal seedlings (NS); normal-strength seedlings (NSS); seedlings with expanded cotyledonary leaves (CL); electrical conductivity (EC).

Protocol	Water content/drying method	-----Precooling-----	--RP %--	--NS %--	-NSS %-	-CL %-	EC ($\mu\text{S cm}^{-1} \text{g}^{-1}$)
1	20% silica	-1 °C min ⁻¹ to -40 °C	91.0 A	81.5 A	12.0 A	74.0 A	27.8 A
2	17% silica	-1 °C min ⁻¹ to -40 °C	93.5 A	80.0 A	10.5 A	73.0 A	29.0 A
3	20% silica	Direct immersion	91.5 A	75.0 A	22.0 A	72.0 A	26.3 A
4	17% silica	Direct immersion	93.0 A	76.5 A	08.0 BC	70.5 A	29.0 A
5	17%(NH ₄) ₂ SO ₄	-1 °C min ⁻¹ to -50 °C	21.0 B	04.0 B	01.0 C	03.0 B	28.1 A
6	17% (NaCl)	Direct immersion	0.0 C	0.0 B	0.0 C	0.0 B	30.4 A
CV			5.82	11.65	40.87	15.07	7.62

Means followed by the same letter do not differ significantly from each other by Tukey's test at 5% probability.

stimulates the generation of hydrogen peroxide. This process most likely stimulated increased expression of the isoenzymatic CAT complex during drying, explaining the higher activity in the driest seeds (Figure 1A). These results do not agreed with those of BRANDÃO JÚNIOR et al. (2002), who found a decrease in CAT activity in coffee seeds with lower physiological performance resulting from desiccation.

Regarding the expression of the SOD isoenzyme (Figure 1B), the opposite behavior to that of CAT was observed: higher expression was observed in wet seeds at 43% and 35%, with the appearance of another band. SOD is considered the first enzyme to act in the antioxidant system of cells and performs dismutation of the superoxide radical (O_2^-), resulting in hydrogen peroxide (H_2O_2) (HENDRY, 1993).

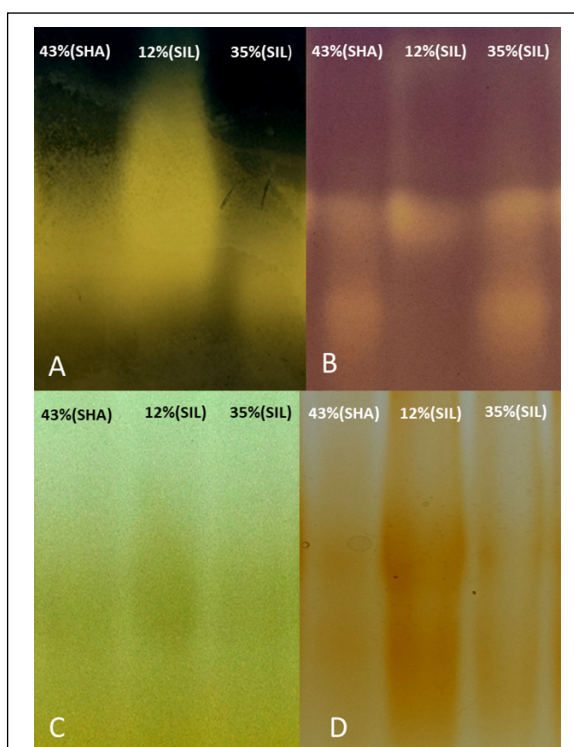


Figure 1 - Electrophoretic patterns of the enzymes catalase (A), superoxide dismutase (B), polyphenoloxidase (C) and peroxidase (D) in *Coffea arabica* L. seeds dried to different water contents, 43, 35 and 12% bu. Drying in shade (SHA) or drying in silica gel (SIL).

Higher PPO activity was observed in seeds with a moisture content of 12% wb than in seeds with 43 and 35% wb, similar to the CAT expression results (Figure 1C). PPO is bound to cell membranes and is activated and released when these structures rupture, which explains the higher activity in the driest seeds with 12% moisture. PIMENTA & VILELA (2003) reported that low PPO activity is related to lower-quality coffee.

The PO expression results were similar to those of CAT and PPO activity, in which seeds dried to 12% wb moisture, with lower physiological quality, showed higher activity of this enzyme (Figure 1D).

Figure 2 shows the enzyme expression results after pre-cooling of the coffee seeds in a biofreezer and rewarming at a temperature of 25 or 40 °C. Regarding CAT, the samples that were dried with saturated saline solution showed lower expression after being pre-cooled, regardless of the rewarming temperature (Figure 2A). It is noteworthy that this protocol (5) showed the lowest physiological quality in the germination test (Table 3).

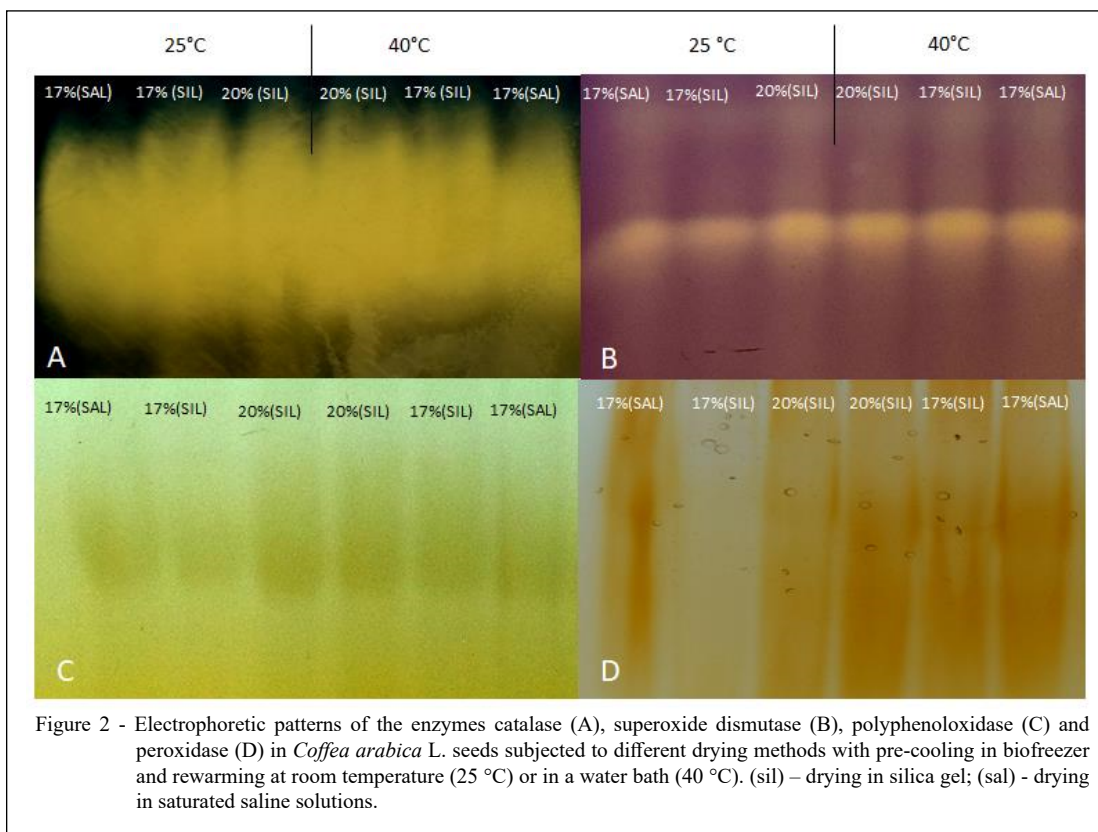
Lower SOD and PPO activities were observed in the seeds that were pre-cooled after

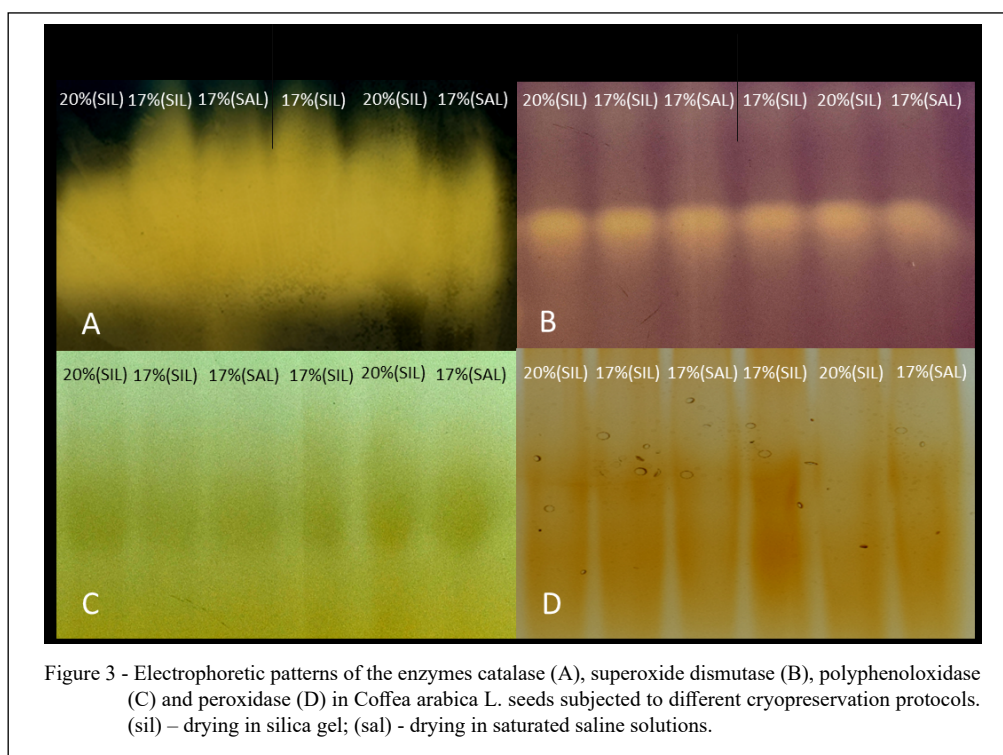
drying on silica gel to 17% wb and reheated at 25 °C (Figure 2B and 2C), which may indicate a possible reduction in free radicals due to less damage caused in this type of drying.

Regarding PO activity (Figure 2D), lower activity was observed in the seeds reheated at 25 °C than in those reheated at 40 °C. In addition, very low expression of this enzyme was observed in seeds that were dried to 17% on silica gel, pre-cooled and reheated at room temperature at 25 °C, as occurred for SOD and PPO.

SOD, CAT and PO act by removing free radicals, also known as “scavengers”. These enzymes are involved in an antioxidative response by neutralizing the toxic oxygen in cells formed during stress conditions (WINSTON, 1990); such stress conditions include the drying and cooling steps, which can cause damage to the seeds. Stressful situations, such as the severe removal of water from cells and the abrupt lowering of temperature, induce oxidative processes and the production of free radicals, which are highly reactive (HENDRY, 1993).

Among the samples that were pre-cooled and cryopreserved (Figure 3), higher CAT





activity was observed in the seeds that were dried to 17% (wb) on silica gel for both the precooling and direct immersion protocols. These treatments showed high seed quality after the removal of liquid nitrogen (Table 3). Among the samples that were directly immersed in the cryotank, lower activity was observed in the seeds subjected to slow drying (Figure 3A).

Small differences were observed in the expression profiles of SOD isoenzymes (Figure 3B), which demonstrated that this enzyme may not be a good biochemical marker for the study of cryo-preservation protocols in coffee seeds (Figure 3B), corroborating the results found by COELHO et al. (2019).

The PPO activity was higher in the seeds that underwent direct immersion than in the pre-cooled samples. In general, no differences were observed in the physiological quality of seeds subjected to different cryopreservation methods. However, a decrease in viability occurred when the seeds were dried with saturated saline solution before immersion in liquid nitrogen (Table 3). Therefore, the small increase in PPO expression when seeds were cryopreserved by direct immersion in liquid nitrogen may have occurred due to greater damage to the membrane system, which was not reflected in the results of the physiological tests.

PPO is an important enzyme protecting against tissue damage caused by physical damage, and a reduction in the activity of this enzyme is related to the integrity of the membrane system. In addition, this enzyme has been used as an indicator of coffee beverage quality (LORENZETTI et al., 2018).

PO expression was similar among the evaluated treatments, except in the seeds dried on silica gel to 17% moisture and immersed directly in liquid nitrogen without the precooling step in a biofreezer, which provided higher expression of this enzyme (Figure 3D).

COELHO et al. (2015) studied the biochemical changes in coffee seeds subjected to drying on silica gel and drying with saturated saline solution and concluded that the enzymatic profile of coffee seeds is affected by the water content of the seeds and the drying rate.

Regarding the CAT, SOD and PO enzymatic systems, COELHO et al. (2017) observed that the bands of all these enzymes varied in quantity and intensity depending on the drying method, seed water content and storage temperature, which together influence seed deterioration.

Normally, ROS act in plant cells as signaling responses, and the occurrence of stress leads to an increase in ROS generation, causing an imbalance in cellular homeostasis and possibly leading to oxidative

stress. Thus, an increase in the activity of antioxidant enzymes is necessary to neutralize the deleterious effect of ROS (SILVEIRA et al., 2015).

From all the evaluations performed in the present study, better physiological and biochemical results were observed in *C. arabica* L. seeds subjected to cryopreservation protocols 3 and 4, i.e., drying in silica gel to 17 or 20% (wb), followed by direct immersion in liquid nitrogen and rewarming in a water bath at 40 ± 1 °C for two minutes. This is because in these protocols, there is no need for a pre-cooling step; this reduces the cost of the process and simplifies the technique by eliminating one step.

This study elucidated some obstacles regarding the long-term conservation of the species *C. arabica* L. and contributes to the knowledge on the storage of coffee seeds. These findings are of great value for studies related to desiccation tolerance and the improvement of cryopreservation techniques of this species.

CONCLUSION

Pre-cooling is a step that can be dispensed in the cryopreservation of *Coffea arabica* seeds, direct immersion in liquid nitrogen being more indicated. *Coffea arabica* L. seeds tolerate cryopreservation after rapid drying in silica gel up to water contents of 17 or 20% (wb), with greater survival at 17%. The enzyme activities of CAT, PPO and PO are indicators of the quality of *C. arabica* L. seeds subjected to cryopreservation.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflicts of interest. The funding institutions played a role in the study design; in data collection, analysis, or interpretation; in the writing of the manuscript; or in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

ACS performed the experiment and wrote the manuscript; SDVFR coordinated and supervised the experiment

and co-worked the manuscript; MCC, MAF, CCP, SVBC, ALOV and DSP co-worked the experiment; DSP co-worked the statistical analyses; ACS, SDVFR and SVBC reviewed and approved the final version of the work.

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