

## Cryopreservation in *Coffea canephora* Pierre seeds: Slow and fast cooling

### Criopreservação em sementes de *Coffea canephora* Pierre: Resfriamento lento e rápido

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#### ABSTRACT

Coffee is one of the main *agricultural commodities* in the country, and it is important to conservation of plant material for breeding programs. Cryopreservation is a promising alternative for preserving in the long-term the germplasm of species considered recalcitrant. However, studies should be performed to achieve maximum survival of seedlings after immersion in liquid nitrogen. The objective of this work was to find a cryopreservation protocol for storing seeds of *Coffea canephora*, studying two methods of cryopreservation, slow and fast cooling. Seeds were subjected to drying in silica gel up to the water content of 0.25 g g<sup>-1</sup>. In the first experiment, dried seeds were subjected to treatments of slow cooling at speeds of -1 °C min<sup>-1</sup>, -3 °C min<sup>-1</sup> and -5 °C min<sup>-1</sup> until the end temperatures of -40 °C, -50 °C and -60 °C, by means of a bio freezer and subsequently immersed in liquid nitrogen. In the second experiment, the best result was selected of the first experiment and compared with the rapid cooling, in which dried seeds, with 0.25 g g<sup>-1</sup> of water content, were immersed directly into liquid nitrogen. Physiological and biochemical alterations occurring in the seeds after cryopreservation were evaluated. *Coffea canephora* seeds respond better to cryopreservation by rapid cooling, when compared to slow cooling. Drying, one of the cryopreservation steps does not affect the viability of *Coffea canephora* Pierre seeds, when these seeds are dried to 0.25 g g<sup>-1</sup> of water content. Catalase and esterase enzymes are good biochemical markers for cryopreserved coffee seeds and their activity is greater in larger seed physiological quality.

**Index terms:** Drying in silica gel; recalcitrant seeds; ice crystals; isoenzymes; physiological quality.

#### RESUMO

O café é umas das principais *commodities* agrícolas do país, sendo importante a conservação do material vegetal para os programas de melhoramento genético. A criopreservação é uma alternativa promissora para conservar em longo prazo o germoplasma de espécies consideradas recalcitrantes. Entretanto, estudos devem ser conduzidos para alcançar a máxima sobrevivência de plântulas após imersão em nitrogênio líquido. Objetivou-se neste trabalho estabelecer um protocolo de criopreservação para armazenar sementes de *Coffea canephora* Pierre, aplicando-se dois métodos de criopreservação, resfriamento lento e rápido. As sementes foram submetidas à secagem em sílica gel, até o teor de água de 0.25 g g<sup>-1</sup>. No primeiro experimento, as sementes secas foram submetidas a tratamentos de resfriamento lento nas velocidades -1 °C min<sup>-1</sup>, -3 °C min<sup>-1</sup> e -5 °C min<sup>-1</sup> até as temperaturas finais de -40 °C, -50 °C, e -60 °C, por meio de um biocongelador, e posteriormente imersas em nitrogênio líquido. No segundo experimento, foi selecionado o melhor resultado do primeiro experimento e comparado com o resfriamento rápido, em que as sementes secas, com 0.25 g g<sup>-1</sup> de umidade, foram imersas diretamente em nitrogênio líquido. As alterações fisiológicas e bioquímicas ocorridas nas sementes após criopreservação foram avaliadas. As sementes de *Coffea canephora* respondem melhor a criopreservação por resfriamento rápido quando comparado ao resfriamento lento. A secagem das sementes de *Coffea canephora* até o teor de água de 0.25 g g<sup>-1</sup> não prejudica a viabilidade das sementes. As enzimas catalase e esterase são bons marcadores bioquímicos para sementes de café criopreservadas e sua atividade é maior nas sementes de maior qualidade fisiológica.

**Termos para indexação:** Secagem em sílica gel; sementes recalcitrantes; cristais de gelo; isoenzimas; qualidade fisiológica.

#### INTRODUCTION

The genus *Coffea* has currently more than 100 described species, and among these, *Coffea arabica* L. and *Coffea canephora* Pierre are the mainly commercialized species worldwide (Ico, 2017). Seeds of *Coffea arabica*

L. are partially tolerant to desiccation and storage, being therefore considered as intermediate behavior (Eira et al., 1999; Ellis; Hong; Roberts, 1990). *Coffea canephora* Pierre seeds, considered more recalcitrant than *Coffea arabica* L., are characterized by being propagated with high water content, do not tolerate dehydration at appropriate

levels for storage of conventional seeds in seed banks in temperatures of -18 to -20 °C and are metabolically active, which makes it difficult to conserve the species plant germplasm (Berjak; Pammenter, 2013).

To ensure the safe coffee genetic resource, researchers have sought technique improvement for seed conservation with recalcitrance characteristics, as the coffee, in liquid nitrogen (-196 °C) or in its vase a vapor steam, at -150 °C (Berjak; Pammenter, 2014; Coelho et al., 2017; Dussert et al., 2012; Figueiredo et al., 2017). Cryopreservation is a viable option for long term conservation of plant material for these species (Engelmann, 2011), and the storage can be carried out through an unlimited period of time, without occurring cell divisions and metabolic changes (Wesley-Smith et al., 2014).

Among the current methods of cryopreservation, two are used for seeds. The classic method consists of plant material slow cooling up to a certain temperature, followed by rapid immersion in liquid nitrogen (Engelmann, 2011). The most modern method is based on vitrification, in which the material is immersed directly into liquid nitrogen, after a controlled dehydration (Walters et al., 2013). According to Berjak and Pammenter (2014), for recalcitrant tropical/subtropical species, better results are observed when using classic cryopreservation, using the slow cooling.

The major bottleneck to be overcome in cryopreservation protocols are damages associated to the formation of intracellular ice crystals, once that they affect cells integrity (Wesley-Smith et al., 2014). Seeds water content is the most important factor, because it is crucial to seeds survival after soaking in liquid nitrogen (Lynch et al., 2016). Seed viability loss will occur if the water content is high enough to cause the formation of intracellular ice, or low enough to cause physical stress to the cells, once they are species susceptible to desiccation (Pammenter; Berjak, 2014).

As cells dehydrate as temperature decreases, setting the cooling speed, as well as the pre-cooling temperature may be crucial to prevent damage occurring in seeds (Kaviani, 2011). In contrast, fast cooling induces vitrification, which could be beneficial to seeds.

The seed exposure to stress conditions, such as cryopreservation, promotes physiological and biochemical changes in cells, which produces free radicals, which are the main cause of damage to the membrane system and macromolecules (Engelmann, 2011). Antioxidant isoenzymes such as catalase, superoxide dismutase and peroxidase, among others, may decrease or reverse the damage caused by these stresses, which makes biochemical studies important for elucidating the effect of cell damage caused by cryopreservation (Berjak; Pammenter, 2013;

Dussert; Engelmann, 2006). In addition, the biochemical study using isoenzymes also helps in understanding the physiological results, serving as a complement to understand the effects of cryopreservation on plant cells.

Until now, there is not a safe and efficient methodology for the conservation of *Coffea canephora* Pierre seeds in the long term, and cryopreservation work involving species are obsolete (Dussert et al., 2001; Eira et al., 1999). In face of the economic importance of the species and the need to conserve plant germplasm, the objective of this work was to find a safe and efficient cryopreservation protocol for storing seeds of *Coffea canephora* Pierre, studying two methods of cryopreservation, slow and fast cooling.

## MATERIAL AND METHODS

### Biological material and processing of fruits

Fruits in cherry ripening stage, of *Coffea canephora* Pierre species, cultivar Apoatã, were selectively harvested in crops of an Experimental Farm of the Foundation Procafé (Integrated Program of Support for the Coffee Technology), in the city of Varginha, MG. After the harvest, the fruits were transported to the Universidade Federal de Lavras, where they were again selected to standardize the maturation stage, pulped mechanically and seeds had their mucilage removed by fermentation in water for a period of 48 hours at room temperature (approximately 25 °C). Subsequently, seeds were arranged in a single layer on screen and kept in the shade, for the removal of surface water.

To determine the degree of seed initial water content, the method of studying at 105 °C for 24 hours (Brasil, 2009) and evaluation of the physiological quality was performed by means of germination test (Brasil, 2009) and embryo viability by tetrazolium test (Clemente et al., 2011).

### Seed drying

Initially, preliminary tests were performed to determine the best drying speed, as well as the ideal water content for cryopreservation of seeds of *Coffea canephora*. According to these tests (results not shown), the fastest drying, in silica gel, and the water content after seed drying, 0.25 g g<sup>-1</sup> (dry basis) were the treatments that provided best results.

Seeds were then dried in single layer on metal screens inside acrylic boxes of gerbox type containing 80 mg of activated silica gel in its interior and below the screen. Silica gel was changed daily, at the same time, for water withdrawal capacity regeneration. Containers were sealed and kept in B.O.D type chambers, at a constant

temperature of 25 °C. Water loss during drying process was monitored by continuous weighing in precision scale of 0.001 g, until seeds reached the water content of interest. Seeds with initial water content of 0.72 g g<sup>-1</sup> were exposed to the silica gel over a period of 55 hours to reach the desired water content of 0.25 g g<sup>-1</sup>

### Slow cooling

Testing slow cooling (classic cryopreservation), seeds with water content of 0.25 g g<sup>-1</sup> were wrapped in aluminum trifoliate paper and placed in bio freezer (Icecube, Model 398S-B, software SY-LAB - Minitub do Brazil), by means of which different curves of cooling were programmed, varying speed (-1 °C min<sup>-1</sup>; -3 °C min<sup>-1</sup>; -5 °C min<sup>-1</sup>) and final pre-cooling temperatures (-40 °C; -50 °C and -60 °C). After cooling, seeds were removed from bio freezer and immersed directly into tank containing liquid nitrogen (-196 °C), where they remained for 24 hours. For thawing, packages containing seeds were removed quickly from liquid nitrogen, and immersed in water bath, during 2 minutes at 40 °C, according to methodology proposed by Dussert et al. (1998). Seeds were then dried superficially in paper towel, having their endocarps (parchment) manually removed for completion of physiological and biochemical evaluations.

### Fast cooling

It was also tested the cryopreservation by direct immersion methodology, or fast cooling. For this reason, seeds with water content of 0.25 g g<sup>-1</sup> were wrapped in

aluminum trifoliate paper and immersed directly into tank containing liquid nitrogen, which constitutes a cooling speed of approximately 200 °C min<sup>-1</sup> (Dussert et al., 2001). Seeds remained in the cryotank for 24 hours, and the sample thawing was also carried out by immersion in water bath, during 2 minutes at 40 °C (Dussert et al., 1998).

Therefore, the following treatments were investigated in this study.

### Physiological analyzes

Seeds subjected to treatments described in Table 1 were subjected to physiological evaluation, by means of the germination test.

Germination test was performed with four replicates of 25 seeds for each treatment, sown in sheets of germination paper, germitest type, moistened with water in a quantity equivalent to 2.5 times the weight of the dry paper. Seeds were kept in a germinator at a constant temperature of 30 °C, and percentage of normal seedlings were evaluated after 30 days, according to the Regras para Análise de Sementes (RAS) requirements (Brasil, 2009). In germination test it was also determined the 15 days radicle protrusion percentage, seedlings with expanded cotyledonary leaves percentage and seedling dry matter, at 45 days after sowing.

At 45 days of germination test, dry matter of seedlings was also evaluated, when the hypocotyl-radicle axis of normal seedlings were isolated, packed in paper bags and dried in air circulation oven at 60 °C for 5 days. After this period, dry mass of roots and aerial parts of

**Table 1:** Treatment identification and procedures description used for *Coffea canephora* Pierre seed cryopreservation.

Number	Treatment	Drying	Pre cooling	
			Speed	Final temperature
1	Control	Without drying (0.72 g g <sup>-1</sup> )	-	-
2		Drying in silica gel until 0.25 g g <sup>-1</sup>	-	-
3	Fast cooling*	Silica gel until 0.25 g g <sup>-1</sup>	Direct immersion in nitrogen	
4				-40 °C
5			-1 °C/minute	-50 °C
6			-60 °C	
7	Slow cooling**	Drying in silica gel until 0.25 g g <sup>-1</sup>		-40 °C
8			-3 °C/minute	-50 °C
9				-60 °C
10				-40 °C
11			-5 °C/minute	-50 °C
12			-60 °C	

\* Direct immersion in liquid nitrogen; \*\* Pre-cooling in bio freezer.

seedlings were determined, and results were expressed in milligrams per seedling.

In tetrazolium test four replications of 10 seeds were used, which were soaked in distilled water for a period of 48 hours, at 30 °C (Clemente et al., 2011). After soaking, embryos were removed with the aid of a scalpel, avoiding damage to them. For staining, embryos were immersed in tetrazolium solution at 0.5%, in the absence of light, for a period of 3 hours, at 30 °C, when they were evaluated, being results expressed in percentage of viable embryos.

### Biochemical analyzes

For biochemical analyzes, seeds from the treatments described in Table 1 were macerated in liquid nitrogen, in the presence of polyvinyl pyrrolidone and samples stored at room temperature -86 °C (deep-freezer) until the time of completion of the electrophoretic analysis of isoenzymes. Methodology proposed by Alfenas (2006) was used for extraction, electrophoretic run and revelation of isoenzymes catalase (CAT), superoxide dismutase (SOD), peroxidase (PO), and esterase (EST).

### Experimental design and statistical analysis

For the effect of final speed and temperature of pre-cooling study (first experiment), factorial 3 x 3 was used, being three cooling speeds and three final cooling temperatures, with four replications

In the second experiment, cryopreservation protocols of slow and fast cooling were compared to control and seeds dried up to 0.25 g g<sup>-1</sup>, in a completely randomized design, with four replications.

Data from both experiments were subjected to analysis of variance in the statistical program SISVAR (Ferreira, 2014), and averages were compared by the Scott-Knott test at 5% probability.

## RESULTS AND DISCUSSION

### Slow Cooling

In Figure 1, results of physiological tests performed in *Coffea canephora* Pierre seeds dried up to 0.25 g g<sup>-1</sup> water content, in silica gel, and subsequently subjected to cryopreservation protocol for slow cooling are presented.

According to germination test results (Figure 1A), it was observed that the slower cooling rates, i.e., -1 °C/min and -3 °C/min, were highly harmful to coffee seed physiological quality, regardless of the final temperature. It is observed that in cooling rate of -3 °C/min, seeds

only survive when submitted to pre-cooled until final temperature of -60 °C before immersion in liquid nitrogen. Among used cooling rates, the one which provided some survival after cryopreservation was the fastest, i.e., -5 °C/min, until the final temperature of -40 °C, being that seeds of this treatment showed 20% of normal seedlings in the germination test (Figure 1A).

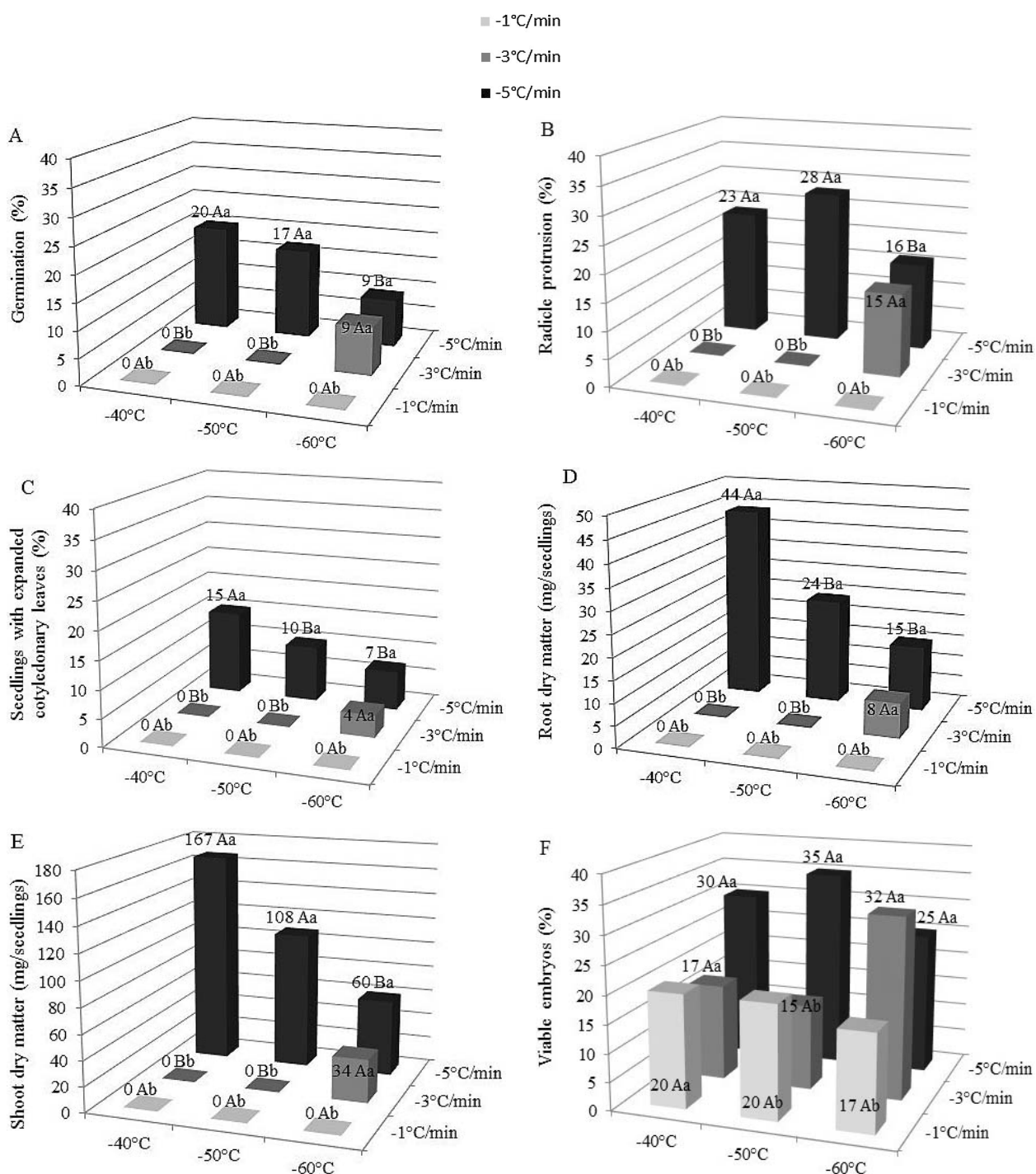
According to root vigor protrusion test results, seedling with expanded cotyledonary leave and root and hypocotyl dry matter (Figure 1, B-E), it was also observed that the slower cooling rate, -1 °C/minute, was extremely harmful to seeds of *Coffea canephora*. The fastest cooling speed, i.e., -5 °C/minute combined with the final temperature of pre-freezing temperatures of -40 °C, was the one that gave better results in evaluations, these results were also found in germination test (Figure 1A).

According to Berjak and Pammenter (2008) the choice of final speed and freezing temperature must be adjusted according to the species and, generally, is between a rate of 0.5-10 °C min.<sup>-1</sup> up to the temperature of about -40 °C. In the present study, the fastest freezing rate, i.e., -5 °C/min was the one that provided some survival of *Coffea canephora* Pierre seeds, compared to the slowest rate of -1 °C/min. This result gives evidence that a speed higher than -5 °C/min may be more appropriate for this species seeds cryopreservation. Since the temperature of nucleation of ultra-pure water is approximately -40 °C (Morris; Acton, 2013), temperatures below this value can be harmful to cells, which justifies the best result for this temperature found in this work.

Cryopreservation method based on slow cooling is one of the best methods used in seeds, which consists of drying stages, plant tissue cooling at a controlled rate until a temperature of pre-freezing, followed by immersion in liquid nitrogen and subsequent rewarming (Figueiredo et al., 2017). In this method, water molecule crystallization is initiated in the extracellular spaces, which causes a difference in water potential, resulting in migration of water molecules from the intra to the extracellular medium (Mazur, 2004; Engelmann, 2004). If cooling rate is slow enough and suitable for species in question, water loss will result in cellular dehydration and the intracellular solute concentrate will remain thawed (Mazur, 2004), which is desired in cryopreservation protocols.

It should be noted with these results the importance of finding a cryopreservation protocol that is appropriate for each species, since there are differences mainly among seed size. Applied cooling rate may not be as slow or as fast as expected depending on the studied species, which will interfere in germination results after immersion in liquid nitrogen.





**Figure 1:** Influence of final speed and temperature of pre-cooling in the average results of germination (A), radicle protrusion (B), seedlings with expanded cotyledonary leaves (C), root dry matter (D), shoot dry matter (E) and viable embryos in the tetrazolium test (F), in seeds of *Coffea canephora* Pierre, after cryopreservation by slow cooling. Averages followed by the same letters do not differ among themselves, by the Scott-Knott at 5% of probability. Uppercase compare cooling speeds within the same pre-cooling temperature. Lowercases compare the pre-cooling speeds within the same cooling temperature.

Through the tetrazolium test (Figure 1F) it was possible to observe higher survival rates of embryos removed from seeds cooled more quickly, i.e.,  $-5\text{ }^{\circ}\text{C}/\text{min}$  and  $-3\text{ }^{\circ}\text{C}/\text{min}$ , like that found in germination test. Survival of seed embryos is observed which were cooled slowly to  $-1\text{ }^{\circ}\text{C}/\text{min}$ , while the result of the seeds germination was 0%. These results may indicate a greater sensitivity of tissues from the endosperm to drying and cooling stress, since embryos presented coloring in tetrazolium test, while in seed germination test, there was no occurrence of normal seedlings, corroborating the results found by Coelho et al. (2015 and 2017) and Dussert et al. (2001).

According to Dussert et al. (1998), the occurrence of major damage to endosperms compared to damage to the embryo during cryopreservation prevents the supply of nutrients necessary for embryonic axis growth, thus compromising germination and consequently, seedling formation.

In general, for all variable response analyzed in the present, although no treatment has resulted in good physiological performance, faster speed of cooling,  $-5\text{ }^{\circ}\text{C}/\text{min}$ , was the most efficient, resulting in seeds with higher values of germination and vigor, especially when they were pre-cooled until the final temperature  $-40\text{ }^{\circ}\text{C}$ . Unlikely the results found in this study, Dussert et al. (1997) observed that cooling speed of  $-1\text{ }^{\circ}\text{C min}^{-1}$  until a temperature of  $-50\text{ }^{\circ}\text{C}$  provided better viability of seeds of *Coffea arabica* L., however, only 30% developed in normal seedlings. Likewise, Figueiredo et al. (2017) in studies on cryopreservation protocols for *Coffea arabica* L. seeds, observed that pre-freezing temperatures up to  $-40\text{ }^{\circ}\text{C}$  to  $-1\text{ }^{\circ}\text{C}/\text{min}$  provided better results, with 79% of normal seedlings. It should be emphasized that there are differences among species of the genus *Coffea*, being *Coffea canephora* Pierre seeds more sensitive to salinity than those of *Coffea arabica* L. (Eira et al., 2005; Rosa et al., 2005).

### Comparison between slow and fast cooling

Figure 2 shows physiological results of *Coffea canephora* seeds subjected to cryopreservation protocols by slow and fast cooling. From the results of the previous experiment, the best result of final speed and pre-cooling temperature was selected (Figure 1), i.e.,  $-5\text{ }^{\circ}\text{C}/\text{minute}$  up to  $-40\text{ }^{\circ}\text{C}$  before immersion in liquid nitrogen. This treatment has been designated as the method of cryopreservation by slow cooling and was compared to the cryopreservation method by fast cooling, in which seeds were dried in silica gel until  $0.25\text{ g g}^{-1}$  and immersed directly into liquid nitrogen. In Figure 2, seed physiological results are

presented corresponding to these treatments, in addition to the results of wet seeds (not subjected to drying), with  $0.72\text{ g g}^{-1}$ , and seeds were dried in silica gel until  $0.25\text{ g g}^{-1}$ .

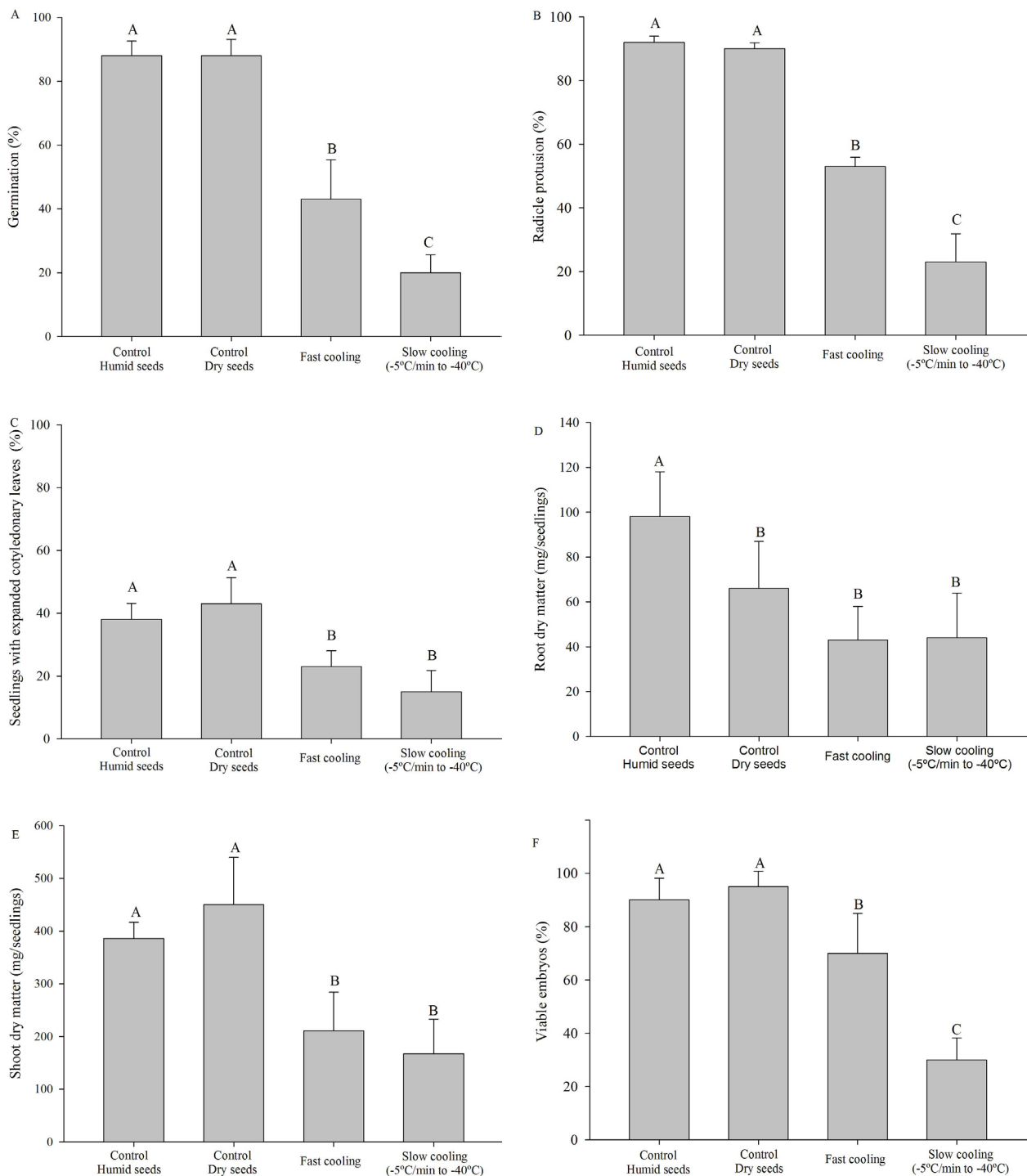
It was observed that, in general, drying up to  $0.25\text{ g g}^{-1}$  does not affect the physiological quality of *Coffea canephora* Pierre seeds (Figure 2). However, there was a decrease in vigor of these seeds, as observed in root dry mass, which value was statistically lower than the wet seeds, not subjected to drying. This sensitivity of *Coffea canephora* seeds was also observed by Rosa et al. (2005), who studying the effect of drying speed on the physiological quality of *Coffea canephora* Pierre seeds, observed that the reduction of seed water content causes a reduction in the values of germination and vigor, regardless of the used drying speed.

According to Figure 2, it is also observed that the two cryopreservation protocols, either by slow cooling or by direct immersion (rapid cooling) are harmful to *Coffea canephora* seeds. However, the cryopreservation protocol for fast freezing provides higher survival rates, with results of 43% of normal seedlings when compared to the seeds cryopreserved by slow freezing. This greater survival was confirmed in all analyzed response variables.

The use of fast freezing can prevent the formation of ice crystals, by means of vitrification, due to high speed of cooling used, around  $-200\text{ }^{\circ}\text{C}/\text{minute}$  (Dussert et al., 2001). Under these conditions, freezing water remaining in cells is transformed into an amorphous solid, non-crystallizable and with high viscosity, preventing the water molecules from becoming ice crystals (Engelmann, 2011).

In general, recalcitrant species seeds are large, and this size prevents the cooling from being fast enough to induce vitrification, which interferes with the success in cryopreservation (Berjak; Pammenter, 2013), this is a possible explanation for the seed low germination after rapid cooling. Still according to Berjak and Pammenter (2013), for recalcitrant seeds, it is necessary that the drying, cooling and rewarming be fast, in order to prevent the crystallization and recrystallization in intercellular spaces.

One of the advantages of using the fast cooling to cryopreserve seeds consists in the fact of not being necessary to use specific equipment for performing pre-cooling, which increases the cost of the procedure, in addition to being more laborious (Dussert et al., 2012). In addition to simplifying and making the process more economical, in the fast cooling, through seed direct immersion in liquid nitrogen, pre-cooling is eliminated, reducing also the handling and possibilities of stresses to seeds. In seeds of *Coffea arabica* L., the simplification of cryopreservation protocol, with seed direct immersion in liquid nitrogen resulted in better outcomes, according to studies from Coelho et al. (2017).



**Figure 2:** Average germination data (A), radicle protrusion (B), seedlings with expanded cotyledonary leaves (C), root dry matter (D), shoot dry matter (E) and viable embryos by the tetrazolium test (F), of wet *Coffea canephora* Pierre seeds, with  $0.72 \text{ g g}^{-1}$  (Control- Humid seeds) and dried with  $0.25 \text{ g g}^{-1}$  (Control-dry seeds) (dry basis), and subjected to the cryopreservation protocols for fast and slow cooling. Averages followed by the same letters do not differ among themselves, by the Scott-Knott at 5% of probability.

On the other hand, Dussert et al. (1998) observed that *Coffea arabica* seeds, only the slow cooling to  $-2\text{ }^{\circ}\text{C}/\text{min}$  up to  $-50\text{ }^{\circ}\text{C}$  resulted in normal seedlings, in a study on the fast and slow cooling in the cryopreservation of four species from the genus *Coffea*. Dussert et al. (2001) studying seed tolerance of nine species of *Coffea* to exposure to liquid nitrogen, observed that for *Coffea canephora*, no normal seedlings were formed after seeds cryopreservation, regardless of the used freezing rate. Dussert and Engelmann (2006) verified that performing a pre-cooling before immersion in liquid nitrogen favors the tolerance increase of coffee seeds to cryopreservation. However, according to Berjak et al. (1989) and Wesley-Smith et al. (1992), fast drying followed by fast freezing can be used for cryopreservation of various species sensitive to desiccation.

### Biochemical evaluation

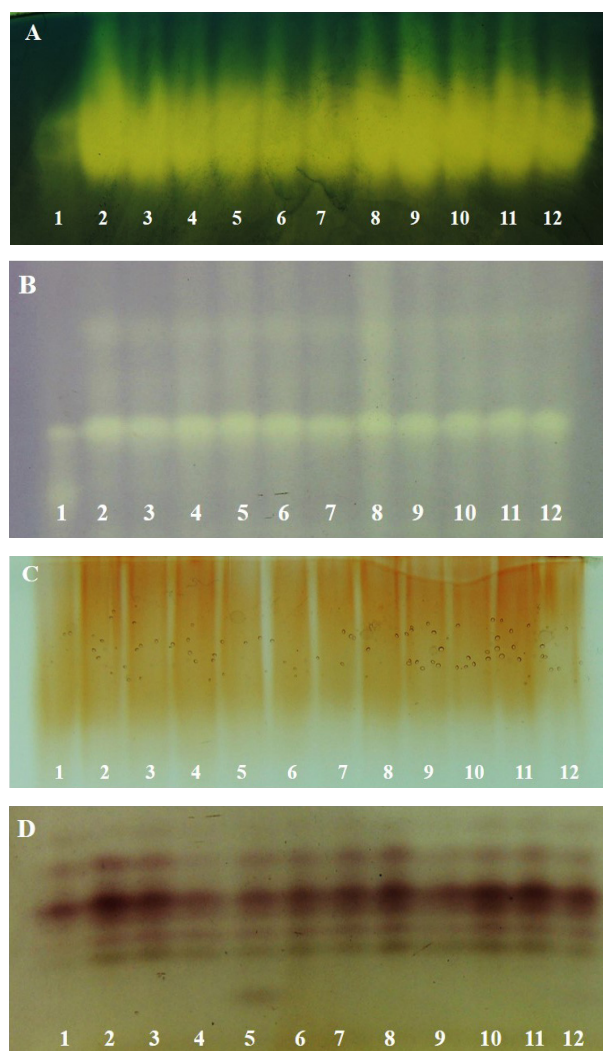
Biochemical changes were evaluated by means of the isoenzyme electrophoretic patterns analysis (Figure 3), of all treatments studied in this work.

Superoxide dismutase, catalase and peroxidase enzymes are known to act by removing free radicals, also known as “scavengers”. These enzymes are involved in an antioxidative response to neutralize the toxic oxygen in cells, formed during stresses conditions (Kurutas, 2016), and among these is the drying and cooling, which can cause damage to seeds. Stress situations, such as severe removal of water from cells and abrupt drop in temperature, induce oxidative processes and production of free radicals, which are highly reactive (Das; Roychoudhury, 2014).

Catalase enzyme (Figure 3A) showed higher activity in treatments 2, 9, 10 and 11, i.e., in the dried seeds up to  $0.25\text{ g g}^{-1}$  without cryopreservation, and seeds that were cryopreserved at cooling speeds of  $-5\text{ }^{\circ}\text{C}/\text{minute}$ . It is observed that these treatments are those that presented greater seeds survival, thus indicating that catalase activity increases in treatments of better physiological performance. Similar result was found by Brandão Júnior, Carvalho and Vieira (1999), that higher catalase activity may be related to greater viability of seeds. As catalase acts by removing free radicals, such as hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), decomposing it into water ( $\text{H}_2\text{O}$ ) and oxygen ( $\text{O}_2$ ) (Caverzan; Casassola; Brammer, 2016), a greater expression of this can be indicative of better physiological quality of seeds, with minor damage originated from cryopreservation.

Superoxide dismutase (SOD) is the first enzyme to act on antioxidant system, performing the dismutation of superoxide radical ( $\text{O}_2\text{-}\bullet$ ) (Das; Roychoudhury, 2014). According to Figure 3B, small differences are observed in the profile of expression of this enzyme, except in treatment

1, in which lower activity is observed, characterized by the seeds that did not undergo drying and cryopreservation stress. The result is similar to that found in the expression of the enzyme superoxide dismutase was also observed for the peroxidase (Figure 3 C). For these enzymes, the effect of different protocols for cryopreservation in coffee seeds were insignificantly, not being observed difference among electrophoretic activities. It is noted that superoxide dismutase and peroxidase enzyme systems are not good biochemical markers for the study of cryopreservation protocols in *C. canephora* seeds.



**Figure 3:** Electrophoretic profile of the isoenzymes catalase (A), superoxide dismutase (B), peroxidase (C) and esterase (D) of *Coffea canephora* Pierre seeds subjected to different protocols for cryopreservation. Treatments are described in Table 1.



The esterase (Figure 3D) acts on ester hydrolysis, which may act on membrane phospholipids in seeds (Coelho et al., 2015). In this study, higher activity of this enzyme was observed in treatments 2, 10 and 12, i.e., those who presented higher values in the germination test. According to Nakada et al. (2010), esterase is indicative of deterioration in seeds, however, in this study, increasing its expression does not coincide with low physiological quality.

In all studied enzymes, it is observed that treatment 1, characterized by wet seeds, expression is very small, or almost non-existent in some enzymes. This happens because in hydrated cells, water acts as a “shock absorber” between radicals and target macromolecules, thus reducing damage.

Analyzing all evaluations performed in this study, better physiological and biochemical results were seen in seeds subjected to cryopreservation protocols 3, i.e., drying in silica gel until 0.25 g g<sup>-1</sup> and direct immersion in liquid nitrogen, and reheating at 40 ± 1 °C for 2 minutes. Despite low germination percentage achieved with cryopreservation of *C. canephora* seeds (40%), there was an improvement in relation to previous studies. Despite of being obsolete, studies involving conservation of *C. canephora* seeds were not satisfactory and seedlings survival was 0% after cryopreservation (Dussert et al., 2001; Eira et al., 1999).

In other recalcitrant species, cryopreservation by fast cooling is also the best option. Michalak, Plitta-Michalak and Chmielarz (2015) observed that cherry seeds tolerate cryopreservation when they are dried at a range of water content between 0.20 and 0.25 g g<sup>-1</sup> (BS) and are immersed directly into liquid nitrogen.

## CONCLUSIONS

*Coffea canephora* seeds respond better to cryopreservation by rapid cooling, i.e. direct immersion in liquid nitrogen, when compared to slow cooling. Drying, one of the cryopreservation steps does not affect the viability of *Coffea canephora* Pierre seeds, when these seeds are dried to 0.25 g g<sup>-1</sup> of water content. Catalase and esterase enzymes are good biochemical markers for cryopreserved coffee seeds and their activity is greater in larger seed physiological quality.

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