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Cryopreservation of C*offea canephora* Pierre ex A. Froehner seeds: importance of drying rate and moisture content

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Abstract

Seeds of the *Coffea canephora* species are considered more recalcitrant than those of the *Coffea arabica* species. They tolerate partial dehydration and they cannot be stored in conventional seed banks at -18ºC because they are sensitive to freezing temperatures. Cryopreservation is a reliable method for storing seeds with these characteristics for long periods. However, preliminary studies are necessary to determine ideal storage conditions. The aim of this study was to investigate the ideal physical and physiological conditions for cryopreservation of *Coffea canephora* seeds to reduce seed mortality caused by the formation of intracellular ice crystals and to avoid cell damage caused by excessive desiccation. Seeds were subjected to rapid drying in silica gel and slow drying in saturated NaCl solution to moisture contents of 0.20, 0.25, and 0.28 $g.g^{-1}$ (dry basis), followed by direct immersion in liquid nitrogen for rapid freezing. Physiological and biochemical analyses were performed to evaluate seed quality before and after cryopreservation. Rapid drying of *Coffea canephora* seeds to values near 0.20 $g.g^{-1}$ (db) does not cause reduction in physiological quality. Moisture content of 0.25 $g.g^{-1}$ brings about greater survival of *Coffea canephora* seeds after cryopreservation. Drying rate affects the physiological quality of cryopreserved *Coffea canephora* seeds, and rapid drying in silica gel is more favorable than slow drying in a saturated NaCl solution. The activity of the enzymes catalase, esterase, glutamic oxaloacetic transaminase, and polyphenol oxidase are indicators of seed quality for *Coffea canephora* seeds subjected to cryopreservation.

Keywords: antioxidant enzymes; drying in silica gel; ice crystals; physiological quality; rapid drying; recalcitrant seeds.
Abbreviations: *C. canephora*_*Coffea canephora* Pierre ex A. Froehner; db_dry basis.

Introduction

Seeds of *Coffea canephora* Pierre ex A. Froehner are characterized by high metabolic activity and sensitivity to desiccation and to cooling (Pammenter and Berjak, 2014), which are two major factors that create difficulty in storing recalcitrant seeds as compared to orthodox seeds. This makes it impossible to conserve the plant material of *Coffea canephora* Pierre in conventional germplasm banks.

While conventional storage of seeds tolerant to desiccation is carried out in storage chambers from 18 to 20 ºC below zero, the only viable alternative for *ex-situ* conservation of recalcitrant species is cryopreservation (Engelmann, 2011), which consists of storage of seeds in liquid nitrogen at -196 ºC, or in its vapor phase at -150 ºC (Walters, 2015). Under these conditions, cell division and biochemical and metabolic activities are drastically reduced, and the seeds can be stored for indeterminate periods of time, without changes in physiological quality (Benson, 2008).

The main focus in the cryopreservation technique is avoiding the formation of ice crystals in the intracellular medium, which is lethal for cells if the moisture content is not adequate for the species. The ice crystals occur because water, upon freezing, expands and forms sharp structures that damage cell walls, leading to leakage of cell content (Wesley-Smith et al., 2014). Drying rate and moisture content thus become essential for success of the cryopreservation of seeds most sensitive to desiccation, especially considering the narrow range of moisture tolerated by seeds during cryopreservation (Kaviani, 2011).

The physiological performance of seeds after drying depends on the rate at which water is removed (Rosa et al., 2005; José et al., 2011; Coelho et al., 2015). According to Pammenter and Berjak (2014), seeds sensitive to desiccation can survive the lower moisture contents if subjected to more rapid drying, due to the insufficient length of the time period for damage to accumulate during the process.

Among current techniques of cryopreservation is rapid cooling, mainly used for seeds and zygotic embryos (Wesley-Smith et al., 2014), which consists of dehydration of plant material to an ideal moisture content and subsequent direct immersion in liquid nitrogen (Engelmann, 2011). During this

procedure, the water present in the cells changes to the vitreous state, which is desirable to prevent crystallization. The vitreous state in this case occurs due to the rapid rate of freezing, together with the high viscosity of the intracellular solutions (Kaviani, 2011; Wesley-Smith et al., 2014). To achieve the desired level of viscosity that can transform a fluid to a vitreous state, seeds need to have an ideal and regulated moisture content, which can be achieved by means of drying.

Tolerance to cryopreservation of diverse species of the *Coffea* genus, including *C. canephora,* was investigated by Eira et al. (1999) and Dussert et al. (2001). Eira et al. (1999) observed that *C. canephora* seeds survive storage at temperatures of -10 and -20 ºC for thirty days, but do not survive at a temperature of -150 ºC. Dussert et al. (2001) studied cryopreservation through rapid cooling and slow cooling in *C. canephora* seeds; however, he did not achieve seed survival after thawing.

The stress caused to seeds subjected to drying and freezing conditions can cause ionic imbalance in the cells, reduction in energy available for metabolic processes, production and accumulation of free radicals, changes in the permeability of the cell membrane, denaturation of proteins, and induction of cell death (Baust, 2007). Evaluation of the activity of enzymes involved in some metabolic pathways can assist interpretation of physiological results, as well as clarify which processes are involved in tolerance to drying and to freezing.

Success in cryopreservation consists of determining the ideal conditions of drying rate, ideal moisture content, and freezing rate, as well as the interaction of these factors. Considering the importance of preserving the genetic resources of coffee and the lack of an ideal protocol for seed cryopreservation, the aim of this study was to investigate the physical and physiological conditions ideal for cryopreservation of *Coffea canephora* Pìerre seeds.

Results and discussion

Drying and physiological analyses of non-cryopreserved seeds

Figure 1 shows the drying curves of coffee seeds that underwent rapid drying in silica gel and slow drying in saturated NaCl solution until reaching moisture contents of 0.20, 0.25, and 0.28 $g.g^{-1}$.

When subjected to rapid drying in silica gel, the seeds with initial moisture content of 0.70 $g.g^{-1}$ took 61, 71, and 93 hours to reach the moisture contents of 0.28, 0.25, and 0.20 $g.g^{-1}$, respectively, with a mean drying rate of 0.54 $\%.h^{-1}$. In slow drying, in saturated NaCl solution, seeds took 165, 189, and 260 hours to reach the same moisture contents, with a mean drying rate of 0.19 $\%.h^{-1}$. Drying in silica gel was 2.8 times faster than drying in saturated NaCl solution.

In analysis of variance of the results of physiological evaluations of dry, non-cryopreserved seeds, a significant effect was found only for the drying rate factor for all the response variables studied (Table 1), except for viable embryos.

According to the results of the germination test (Table 1) after rapid drying in silica gel, the seeds exhibited better physiological quality than that of the seeds from slow drying in NaCl solution. There was also no reduction in the percentage of germination of seeds during the rapid drying process, and the moist seeds (control) had a mean equal to that of dry seeds.

When the zygotic embryos were removed from the dry seeds and evaluated in the tetrazolium test (Table 1), no difference was observed between the viability of seeds under rapid drying and under slow drying; the results were statistically similar.

The results of vigor tests of dry seeds without cryopreservation (Table 1) show that under rapid drying in silica gel, seeds exhibit better results than under slow drying, indicating that rapid drying was better for preserving the physiological qualities of *Coffea canephora* seeds, corroborating the results of the germination test.

These results show that, in general, the drying rate influences seed viability and vigor. *Coffea canephora* seeds dried rapidly have higher physiological quality than those dried slowly.

Rosa et al. (2005) studied the effect of drying rate on the physiological quality of *Coffea canephora* Pierre seeds and observed that immediately after drying, only the effect of final moisture content of the seeds was significant, and the rate at which moisture was removed was not relevant. It should be noted that the drying conditions examined by Rosa et al. (2005) were different than those of the present study. In addition, these authors studied moisture contents that were quite different, from 51 to 15 %; and as *Coffea canephora* Pierre seeds are considered recalcitrant, the final moisture level influenced seed physiological quality.

In the present study, statistical differences were not observed among the moisture contents of 0.20, 0.25, and 0.28 $g.g^{-1}$. Since these values were so near each other, they did not influence seed physiological quality. Furthermore, the study of Rosa et al. (2005) showed that germination of *Coffea canephora* seeds decreased as the seeds lost moisture; however, that was not observed in the present study.

Coelho et al., (2015) studied the effects of drying rate on the quality of *Coffea arabica* L. seeds and observed that in rapid drying, seeds tolerate desiccation at lower moisture contents. In contrast, slow drying was more efficient when the seeds were dried to higher moisture contents.

According to Berjak and Pammenter (2008), recalcitrant seeds can survive at a lower moisture content when dried rapidly. This is because when drying is instantaneous, there is rapid progression through the intervals of intermediate moisture content, in which damage related to aqueous metabolism occurs, with ionic imbalance and accumulation of free radicals (Pammenter and Berjak, 2014). In other words, if seeds rapidly pass through this intermediate moisture content, there is not sufficient time for damage to accumulate to lethal levels.

Physiological analyses of cryopreserved seeds

In analysis of variance of the results of the physiological evaluations after cryopreservation of the seeds, a significant interaction was found for the two factors studied, drying rate and moisture content, for all the response variables studied (Figure 2).

In the present study in which seeds were stored in liquid nitrogen, the results of the germination test (Figure 2A)

show that the moisture content most recommended for cryopreservation of *Coffea canephora* seeds is 0.25 $g.g^{-1}$, and rapid drying in silica gel is ideal for reaching this content. Seeds dried rapidly to 0.20 $g.g^{-1}$ exhibited some survival, but it was a very low survival rate compared to seeds with moisture of 0.25 $g.g^{-1}$. The moisture content of 0.28 $g.g^{-1}$ is highly injurious for cryopreservation of *C. canephora* seeds, regardless of the drying rate.

The results of the vigor tests (Figure 2, B-E) show the same tendency as the results of the germination test, i.e., the best moisture content for cryopreserving *Coffea canephora* Pierre seeds is 0.25 $g.g^{-1}$, dried in silica gel. Moisture contents above or below this value cause drastic reduction in the physiological quality of cryopreserved seeds, which may indicate that there is a specific moisture level ideal for storing seeds in liquid nitrogen and not a range of moisture contents.

These results also suggest that ice crystals formed under the moisture contents studied, especially moisture content above 0.25 $g.g^{-1}$, since the dry seeds before immersion in liquid nitrogen had a survival rate greater than 80%. Although the ideal state of hydration of the seeds before exposure to liquid nitrogen would be that all of them had only non-freezable water, in practice, this is not achieved. Variation in moisture among the seeds during and after drying interferes in the amount of freezable water that remains in the cells at the time of cryopreservation (Berjak and Pammenter, 2013), and this may be the reason for which only 43% of the seeds germinated after immersion in liquid nitrogen.

In relation to the drying rates investigated, slow drying is harmful when the aim is drying coffee seeds for cryopreservation (Figure 2). In all the tests performed, rapid drying to 0.25 $g.g^{-1}$ was better than slow drying (Figure 2) when seed quality is evaluated after cryopreservation. From these results, the importance of the drying rate to which seeds are subjected is evident; it is crucial for success in the cryopreservation technique.

Figueiredo et al. (2017) studied cryopreservation protocols of *Coffea arabica* L. seeds, another species of the *Coffea* genus with characteristics similar to *Coffea canephora*, and observed that the moisture of 20% wb (which corresponds to 0.25 $g.g^{-1}$ db) leads to greater survival of seeds and also of zygotic embryos after cryopreservation.

Dussert et al. (2001) studied the tolerance of nine species of *Coffea* to cryopreservation and observed that, regardless of the moisture content and freezing rate, *Coffea canephora* seeds do not germinate after immersion in liquid nitrogen. However, the authors concluded that the damage occurs mainly in the endosperms of the seeds since the embryos survive upon being extracted from these cryopreserved seeds.

Moreover, in the present study, embryos removed from the cryopreserved seeds had a higher survival rate compared to the results of the germination test (Figure 2, A and F), especially at the moisture contents of 0.20 and 0.28 $g.g^{-1}$, which were lethal for the coffee seeds, similar to the results found by Dussert et al. (2001). Embryos of coffee seeds dried slowly to 0.28 $g.g^{-1}$ did not survive, showing this to be the worst treatment among those investigated in this study for cryopreservation of *Coffea canephora* seeds.

Factors such as initial quality of the seeds, drying conditions, freezing rate, and thawing are important for survival of the species after cryopreservation. In the present study, there was high initial quality of the seeds, as well as high quality after drying, indicating that the freezing and reheating steps are the obstacles to achieving high survival of cryopreserved seeds.

In general, the results found in this study suggest that the rapid drying rate in silica gel is ideal for cryopreservation of *Coffea canephora* seeds. However, the uniformity with which drying is carried out, as well as the cryopreservation technique used, require greater study.

Analyses of cryopreserved seeds

The isoenzymatic systems of *Coffea canephora* seeds subjected to cryopreservation revealed and quantified for catalase (CAT), superoxide dismutase (SOD), esterase (EST), glutamic oxaloacetic transaminase (GOT), malate dehydrogenase (MDH), and polyphenol oxidase (PPO) are represented in Figures 3, 4, and 5.

As seed moisture decreases, the enzymatic activity of catalase increases (Figure 3A), especially in slow drying. The treatments involving slow drying before cryopreservation exhibited lower catalase activity compared to the rapid drying treatments. There was very low expression of the catalase enzyme in the cryopreserved seeds at 0.28 $g.g^{-1}$ after slow drying, and this treatment led to the worst physiological quality among the treatments tested in the present study.

Catalase is involved in removal of hydrogen peroxides (H_2O_2) from the cells, and its greater activity may be related to reduction in mechanisms for prevention of oxidative damage (Caverzan, Casassola and Brammer, 2016). These results corroborate those of Brandão Júnior et al. (2002), who observed a reduction in catalase activity in coffee seeds that had lower physiological performance resulting from desiccation. The stress undergone by seeds due to drying and cryopreservation causes an increase in production of reactive oxygen species and stimulates the generation of hydrogen peroxide. This process probably stimulated the increase of the isoenzymatic complex of the catalase enzyme over the drying period, explaining the greater activity in drier seeds (Figure 3A).

The enzyme superoxide dismutase (SOD) is considered the first to act in the antioxidant system of the cells, carrying out dismutation of the superoxide radical (O_2^-), resulting in hydrogen peroxide (H_2O_2) (Das and Roychoudhury, 2014). In expression of this enzyme (Figure 3B), the cryopreservation protocol of *Coffea canephora* seeds that resulted in seeds with greater viability and vigor (i.e., seeds dried to 0.25 $g.g^{-1}$ in silica gel and then immersion in liquid nitrogen) exhibited the lowest activity of this enzyme, which may indicate low activity of free radicals in this treatment. In general, there was greater expression of this enzyme in the treatments dried in saturated NaCl solution compared to drying in silica gel within a given moisture content, indicating greater deterioration of the seeds in these NaCl treatments.

There was an increase in expression of the enzyme esterase as the moisture content of the seeds decreased (Figure 4C), and the treatments of seeds cryopreserved with 0.20 $g.g^{-1}$ of moisture had the greatest intensity of the bands. Furthermore, seeds that were dried slowly and cryopreserved at 0.28 $g.g^{-1}$ of moisture had very low expression of esterase, and that treatment exhibited the

Table 1. Mean values of germination, strong normal seedlings, seedlings with expanded cotyledonary leaves, root dry matter, hypocotyl dry matter, and viable embryos of non-cryopreserved *Coffea canephora* Pierre seeds, under two drying methods, rapid drying in silica gel and slow drying in saturated NaCl solution.

Variables	Drying		Control	CV (%)
	Silica gel	NaCl		
Germination (%)	88 A	81 B	88	7.51
Strong normal seedlings (%)	22 A	15 B	18	18.38
Expanded cotyledonary leaves (%)	43 A	30 B	38	13.03
Root dry matter (mg)	66.08 A	43.08 B	98.50	17.46
Hypocotyl dry matter (mg)	450.11 A	310.98 B	386.00	16.68
Viable embryos (%)	86 A	80 A	90	13.86

Means followed by the same uppercase letter in the line do not differ from each other by the *Scott-Knott* test at the level of 5% probability.

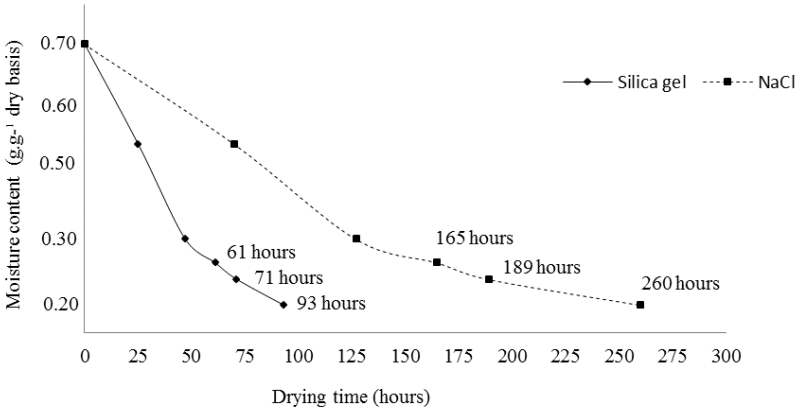

Fig 1. Drying curve of *Coffea canephora* Pierre seeds under rapid drying in silica gel and slow drying in saturated NaCl solution up to moisture contents of 0.20, 0.25, and 0.28 g.g^{-1}.

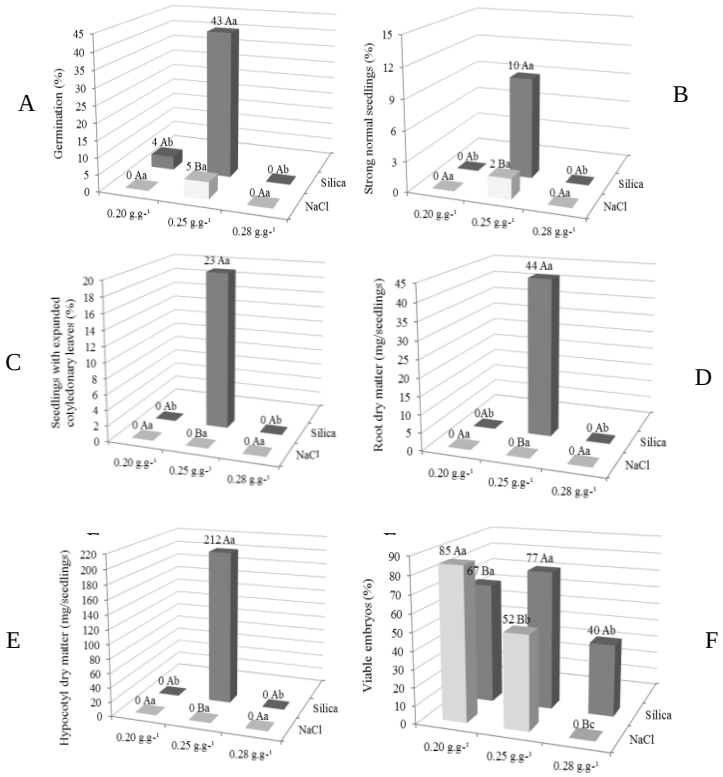

Fig 2. Mean percentage of germination (A), of strong normal seedlings (B), and of seedlings with expanded cotyledonary leaves (C); mean weight of root dry matter (D) and of hypocotyl dry matter (E); and viability of embryos by the tetrazolium test (F) of *Coffea canephora* Pierre seeds cryopreserved after rapid drying in silica gel and slow drying in saturated NaCl solution up to moisture contents of 0.20, 0.25, and 0.28 g.g^{-1}. Mean values followed by the same letter do not differ from each other by the Scott-Knott test at 5% probability. Uppercase letters compare the drying rates within the same moisture content. Lowercase letters compare the moisture contents within the same drying rate.

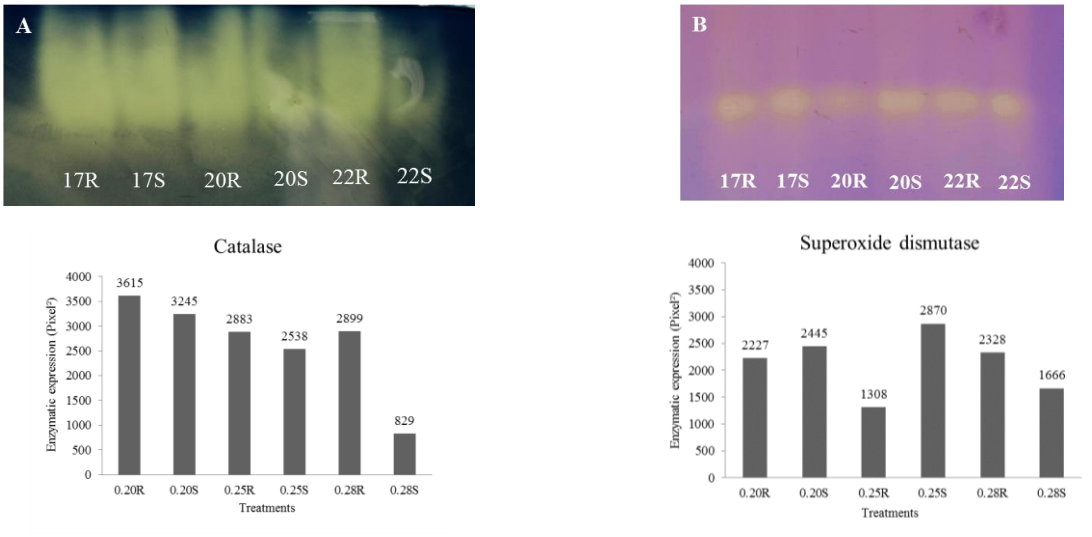

Fig 3. Electrophoretic profile and densitometric quantification for the enzymes catalase (A) and superoxide dismutase (B) in *Coffea canephora* Pierre seeds cryopreserved by fast cooling after rapid drying (R) in silica gel and slow drying (S) in saturated NaCl solution to moisture contents of 0.20, 0.25, and 0.28 g.g^{-1}

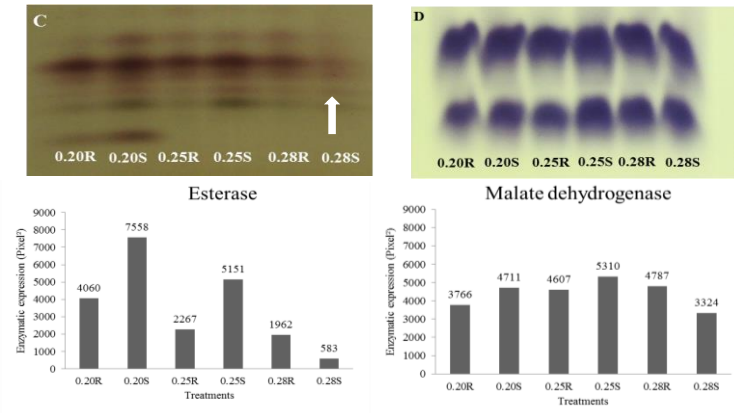

Fig 4. Electrophoretic profile and densitometric quantification for the enzymes esterase (C) and malate dehydrogenase (D) in *Coffea canephora* Pierre. seeds cryopreserved by fast cooling after rapid drying (R) in silica gel and slow drying (S) in saturated NaCl solution to moisture contents of 0.20, 0.25, and 0.28 g.g^{-1}

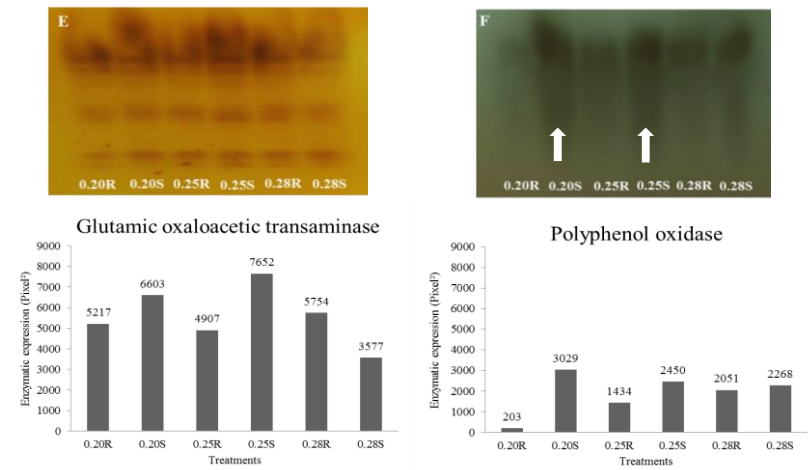

Fig 5. Electrophoretic profile and densitometric quantification for the enzymes glutamic oxaloacetic transaminase (E) and polyphenol oxidase (F) in *Coffea canephora* Pierre seeds cryopreserved by fast cooling after rapid drying (R) in silica gel and slow drying (S) in saturated NaCl solution to moisture contents of 0.20, 0.25, and 0.28 g.g-1.
worst physiological quality. The esterase enzyme

participates in hydrolysis reactions of esters and may also act on membrane phospholipids (Borrelli and Trono, 2015). Brandão Júnior et al. (2002) observed greater expression of this enzyme in samples taken from dried coffee seeds.

Little change was observed in relation to expression of the enzyme malate dehydrogenase among the treatments studied (Figure 4D). Nevertheless, greater activity occurred in seeds dried in NaCl solution compared to seeds that underwent rapid drying before cryopreservation, except for the moisture content of 0.28 $g.g^{-1}$. This greater activity may be related to the high rate of seed respiration and, consequently, greater deterioration (Catão et al., 2016). The enzyme MDH performs an important function in the Krebs cycle, catalyzing the conversion of malate to oxaloacetate, producing NADH, which is a fundamental product in production of ATP and of intermediary compounds essential to cell operation (Williams and O'Neill, 2018).

The enzymes glutamic oxaloacetic transaminase (Figure 5E) and polyphenol oxidase (Figure 5F) exhibited similar expression. For these enzymes, the drying rate had a bigger effect on the activity than the final moisture content of the seeds. Thus, the seeds dried slowly had greater expression of these enzymes than the seeds dried rapidly, regardless of the moisture content of the seeds.

Glutamic oxaloacetic transaminase acts in oxidation of amino acids and supplies energy for the Krebs cycle or for reduction of α-ketoglutarate, directed to biosynthesis of new amino acids for embryo growth (Yang et al., 2014). Thus, stresses applied to seeds can act directly or indirectly on this enzyme, thus affecting the resumption of embryo growth (Figure 2F) and, consequently, seed germination (Figure 2A).

The enzyme polyphenol oxidase acts on the phenolic compounds of the seeds, which function as antioxidants and in protection of aldehydes, such as chlorogenic and caffeic acids. These enzymes are bound to cell membranes, and when there is damage in the membranes, these enzymes are released and activated and can react with intra and extracellular phenolic substrates and transform them into quinones (Araji et al., 2014). This explains the high activity of this enzyme in the slow drying treatments and provides evidence of the damage caused to cell membranes and consequent reduction in physiological quality.

In general, the seeds dried slowly to 0.28 $g.g^{-1}$ and cryopreserved showed very low or nearly null expression for most of the enzymatic complexes studied. The seeds were dead since they had null values in the germination and tetrazolium tests, explaining the low or null activity (Figure 2, 3, 4, and 5).

In this study, physical, physiological, and biochemical analyses were carried out regarding cryopreservation of coffee seeds, and this allowed detection of some transformations that occurred during this process. This study clarifies some obstacles in relation to long-term cryopreservation of the *Coffea canephora* species that had not yet been studied, and also contributes to the pool of information in respect to coffee seed storage and longevity. This is highly useful for studies related to tolerance to desiccation for the purpose of refining cryopreservation techniques.

Materials and methods

Biological material and processing of fruits

Fruit of the *Coffea canephora* Pierre species, cultivar Apoatã, were collected in fields of the Varginha Experimental Farm - Fundação Procafé (Programa Integrado de Apoio à Tecnologia Cafeeira) – in the 2014/2015 crop season. The fruit in the cherry maturation stage was selectively collected from the middle branches of the plants and in the middle parts of these branches. After harvest, the fruit was selected to standardize the maturation stage, and then it was mechanically pulped. The seeds were demucilaged by fermentation in water for 48 hours at ambient temperature and then pre-dried in the shade to remove surface moisture. To standardize size, seeds with parchments retained in circular screen sieves no. 18/64 and 20/64 were used, and those of larger or smaller size were discarded.

The initial moisture content of the seeds was determined by the laboratory oven method at 105ºC (Brasil, 2009); initial physiological quality was evaluated by the germination test (Brasil, 2009) and viability of embryos by the tetrazolium test (Clemente et al., 2011).

Drying and cryopreservation of seeds

The seeds were dried to the moisture contents of 0.20, 0.25, and 0.28 $g.g^{-1}$ (dry basis). For rapid drying, the seeds were placed in a single layer over metal screens in *gerbox* acrylic boxes, containing 80 mg of activated silica gel within the boxes below the screens. As drying proceeded, the silica was exchanged daily, at an established time. For slow drying, the seeds were placed the same way in boxes containing saturated NaCl solution (75% relative humidity at 25ºC); this solution was able to maintain internal relative humidity stable. The saturated salt solution was made using 65 mg of NaCl for 20 ml of distilled water for each gerbox. The containers were sealed and kept in B.O.D. type chambers at a constant temperature of 25°C. Water loss during drying was monitored by continual weighings on a balance with precision of 0.001 g until the seeds reached the desired moisture levels.

After the seeds reached the desired moisture levels and before immersion in liquid nitrogen, physiological tests were performed to evaluate the quality of the seeds before cryopreservation.

The dry seeds were then placed in three-layer laminated aluminum packaging and directly immersed in a tank containing liquid nitrogen, which provides ultra-rapid cooling at the rate of approximately -200°C/minute (Dussert et al., 2001). After 24 hours, the packages containing the seeds were removed, and the seeds were thawed in a water bath at 40°C for 2 minutes (Dussert et al., 1998). For the thawing process, the seeds were quickly removed from their respective packages and immersed directly in the water bath. After that, they were dried with paper toweling to remove surface water, and their parchments were removed manually for physiological and biochemical evaluation.

The following seeds were used for physiological evaluations: control seeds, i.e., moist seeds that had not passed through any treatment; seeds dried to the moisture contents of 0.20, 0.25, and 0.28 $g.g^{-1}$, without immersion in liquid nitrogen; and cryopreserved seeds.

Physiological analyzes

The germination test was carried out with four replications of 25 seeds for each treatment. The seeds were sown in sheets of germination paper and moistened with water in the amount of 2.5 times the weight of the dry paper. The seeds were kept in a germinator at a constant temperature of 30ºC, and the percentage of normal seedlings was evaluated after 30 days, following the directives of the RAS (Brasil, 2009). In the germination test, determination was also made of the percentage of strong normal seedlings at 30 days (seedlings were regarded as strong if they had a hypocotyl arch of three centimeters or more), percentage of seedlings with expanded cotyledonary leaves at 45 days after sowing, and seedling dry matter.

For determination of seedling dry matter, the hypocotyl-radicle axes of the normal seedlings were isolated, placed in paper bags, and dried in an air circulation laboratory oven at 60ºC for 5 days. After this period, the dry matter of the roots and above ground parts of the seedlings was determined, and the results were expressed in milligrams per seedling.

The tetrazolium test was conducted using four replications of 10 seeds, which were soaked in distilled water for a period of 48 hours at 30ºC (Clemente et al., 2011). After soaking, the embryos were removed with the aid of a scalpel, avoiding damage. Embryos were stained by immersing them in a 0.5% tetrazolium solution in the absence of light for a period of 3 hours at 30ºC. They were then evaluated and the results were expressed in percentage of viable embryos.

Biochemical analyses

For biochemical analyses, only the cryopreserved seeds were used. The seeds were macerated in liquid nitrogen in the presence of polyvinylpyrrolidone and the samples were stored at a temperature of -86ºC (deep frozen) up to the time of analyses. The methodology proposed by Alfenas (2006) was used for extraction, electrophoretic run, and revelation of the isoenzymes catalase (CAT), superoxide dismutase (SOD), esterase (EST), malate dehydrogenase (MDH), glutamic oxaloacetic transaminase (GOT), and polyphenol oxidase (PPO).

For evaluation of the activity of the enzymes CAT, SOD, EST, MDH, and GOT, interpretation of the results was based on qualitative analysis of the electrophoresis gels, taking into consideration the presence/absence and the intensity of each one of the electrophoretic bands in each isoenzymatic system evaluated. For quantitative analysis of the bands, the ImageJ (Rasband, 1997) image analysis software was used, measured in pixel². Each digital band of the enzymes was calculate by defining the number of pixels in the rows and columns direction and these pixel values were used for graphical representation of activity of the enzymes.

Experimental design and statistical analysis

The experimental design was completely randomized in a 3 x 2 factorial arrangement, with three moisture contents, 0.20, 0.25, and 0.28 $g.g^{-1}$ (db), and two drying rates, rapid and slow, with four replications. The results of the physiological tests were subjected to analysis of variance through the SISVAR statistical program (Ferreira, 2014), and the mean values were compared by the Scott-Knott test at the level of 5% probability.

Conclusion

The moisture content of 0.25 $g.g^{-1}$ (db) provides for a greater survival rate of *Coffea canephora* seeds after cryopreservation by direct immersion. The drying rate affects the quality of cryopreserved *Coffea canephora* seeds; rapid drying in silica gel is more favorable than slow drying in saturated NaCl solution for achieving the moisture content of 0.25 $g.g^{-1}$ (db). Rapid drying of *Coffea canephora* seeds to values near 0.20 $g.g^{-1}$ (db) does not cause reduction in physiological quality. The activity of the enzymes catalase, esterase, glutamic oxaloacetic transaminase, and polyphenol oxidase are indicators of the quality of *Coffea canephora* seeds subjected to cryopreservation.

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References

Alfenas AC (2006) Eletroforese e marcadores bioquímicos em plantas e microorganismos. 2 ed. Viçosa: Ed. UFV, 627 p.

Araji S, Grammer TA, Gertzen R, Anderson SD, Mikulic-Petkovsek M, Veberic R, … Escobar MA (2014) Novel roles for the polyphenol oxidase enzyme in secondary metabolism and the regulation of cell death in walnut. Plant Physiol. *164*(3): 1191–1203.

Baust JM (2007) Properties of cells and tissues influencing preservation outcome: molecular basis of preservation induced cell death. In: Baust JG, Baust JM (ed) Advanced in biopreservation. New York: CRC Press, Taylor & Francis Group, cap 3, p. 63-87.

Benson EE (2008) Cryopreservation theory. In: Reed BM. Plant cryopreservation: a practical guide. New York: Springer. p 15-32.

Berjak P, Pammenter NW (2008) From Avicennia to Zizania: seed recalcitrance in perspective. Ann Bot. 101(2): 213–228.

Berjak P, Pammenter NW (2013) Implications of the lack of desiccation tolerance in

recalcitrant seeds. Front plant sci. 4(478).

Brandão Júnior DS, Carvalho MLM de, Vieira M das GGC (1999) Variações eletroforéticas de proteínas e isoenzimas relativas à deterioração de sementes de milho envelhecidas artificialmente. Rev Bras Sementes. 21(1):114-121.

Bandão Júnior DS, Vieira M das GGC, Hilhost HWM (2002) Aquisição da tolerância à dessecação nos diferentes estádios de desenvolvimento de sementes de cafeeiro (*Coffea arabica* L.). Ciênc Agrotec. 26(4): 673-681.

Brasil (2009) Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. Regras para análise de sementes. Brasília, DF: MAPA: SDA, 395 p.

Borrelli GM, Trono D (2015). Recombinant lipases and phospholipases and their use as biocatalysts for industrial applications. Int J Mol Sci. 16(9):20774-20840.

Catão HCR, Gomes LAA, Guimarães RM, Fonseca PHF, Caixeta F, Morodin JC (2016) Physiological and isozyme alterations in lettuce seeds under different conditions and storage periods. J Seed Sci. 38(4): 305-313.

Caverzan A, Casassola A, Brammer SP (2016) Antioxidant responses of wheat plants under stress. Genet Mol Biol. *39*(1), 1–6.

Clemente ACS, Carvalho MLM, Guimarães RM, Zeviani WM (2011) Preparo das sementes de café para a avaliação da viabilidade pelo teste de tetrazólio. Rev Bras Sementes, 33(1): 38-44.

Coelho SVB, Figueiredo MA, Clemente ACS, Coelho LFS, Rosa SDVF (2015) Alterações fisiológicas e bioquímicas em sementes de café secas em sílica gel e soluções salinas saturadas. Pesq Agropec Bras. 50(6): 483-491.

Das K, Roychoudhury A (2014) Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Front Env Sci. 2(53).

Dussert S, Chabrillange N, Rocquelin G, Engelmann F, Lopez M, Hamon S (2001) Tolerance of coffee (*Coffea* spp.) seeds to ultra-low temperature exposure in relation to calorimetric properties of tissue water, lipid composition, and cooling procedure. Physiol Plant. 112(4): 495-504.

Dussert S, Chabrillange N, Engelmann F, Anthony F, Hamon S (1998) Cryopreservation of seed of four coffee species (*Coffea arabica, C. costatifructa, C. racemosa* and *C. sessiliflora*): importance of water content and cooling rate. Seed Sci Res. 8(1): 9-15.

Eira MTS, Walters C, Caldas LS, Fazuoli LC, Sampaio JB, Dias MCLL (1999) Tolerance of *Coffea* spp. seeds to desiccation and low temperature. Rev Bras Fisiol Veg. 11(2): 97-105.

Engelmann F (2011) Use of biotechnologies for the conservation of plant biodiversity. In Vitro Cell Dev Biol Plant. 47(1): 17-25.

Ferreira DF (2014) Sisvar: A guide for its bootstrap procedures in multiple comparisons. Ciênc Agrotec. 38(2): 109-112.

Figueiredo MA, Coelho SVB, Rosa SDVF, Vilela AL, Silva LC (2017) Exploratory studies for cryopreservation of *Coffea arabica* L. seeds. J Seed Sci. 39(2):150-158.

José AC, Silva EA, Davide AC, Melo AJS, Toorop PE (2011) Effects of drying rate and storage time on *Magnolia ovate* Spreng. seed viability. Seed Sci Technol. 39(2): 425-434.

Kaviani B (2011) Conservation of plant genetic resources by cryopreservation. Aust J Crop Sci. 5(6): 778–800.

Pammenter NW, Berjak P (2014) Physiology of desiccation-sensitive (recalcitrant) seeds and the implications for cryopreservation. Int J Plant Sci. 175(1): 21-28.

Rosa SDVF, Brandão Júnior D da S, Von Pinho EVR, Veiga AD, Silva LHC (2005) Effects of different drying rates on the physiological quality of *Coffea canephora* Pierre seeds. Braz J Plant Physiol. 17(2): 199-205.

Walters C (2015) Orthodoxy, recalcitrance and in-between: describing variation in seed storage characteristics using threshold responses to water loss. New York: Springer.

Wesley-Smith J, Berjak P, Pammenter NW, Walters C (2014) Intracellular ice and cell survival in cryo-exposed embryonic axes of recalcitrant seeds of *Acer saccharinum*: an ultrastructural study of factors affecting cell and ice structures. Ann Bot. 113: 695–709.

Williams NC, O'Neill LAJ (2018) A role for the krebs cycle intermediate citrate in metabolic reprogramming in innate immunity and inflammation. Front Immunol. 9:141.

Yang C, Ko B, Hensley CT, Jiang L, Wasti AT, Kim J, DeBerardinis R J (2014) Glutamine oxidation maintains the TCA cycle and cell survival during impaired mitochondrial pyruvate transport. Mol Cell. *56*(3): 414–424.