

Cryopreservation of coffee seeds: a simplified method

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Abstract

Coffee seeds are sensitive to desiccation and to storage. Advances in the technique of cryopreservation of these seeds have been achieved in recent years, and the aim of this study was to evaluate *Coffea arabica* seeds cryopreserved through direct immersion in liquid nitrogen after rapid and slow drying. Seeds of the cultivars 'Arara', 'Catiguá', 'Catuaí Amarelo' and 'Mundo Novo' underwent rapid and slow drying to 20% moisture content (dry basis); they were then immersed in liquid nitrogen for 24 hours and after that, reheated in a water bath. *C. arabica* seeds have better physiological quality after slow drying, but rapid drying is better for cryopreservation of these seeds. The seeds of the cultivars investigated have different levels of tolerance, but all can be cryopreserved; 'Catuaí Amarelo' is the most tolerant and 'Arara' the most sensitive to cryopreservation, regardless of the drying speed. The activity of the enzymes catalase, peroxidase and esterase increased after drying and after cryopreservation. Rapid drying in silica gel to 20% moisture content, followed by direct immersion in liquid nitrogen, allows cryopreservation of coffee seeds in a fast, simple and economical manner.

Introduction

Conservation of plant genetic resources is an established and recognised priority worldwide because genetic variability of species is fundamental for development of new cultivars (Pilatti *et al.*, 2011). Conservation of *Coffea arabica* L. germplasm is of great socioeconomic importance in Brazil because Brazil is the world's largest producer and exporter of this commodity (Abic, 2016). Due to the intermediate or recalcitrant nature of *Coffea* seeds, they are conserved through *ex situ* germplasm banks in field plantings. This methodology, however, involves high maintenance cost; moreover, plants conserved in the field are vulnerable to pests, diseases and climatic factors (Eira *et al.*, 2005; Dussert *et al.*, 2012).

Ex situ conservation through cryopreservation is the most promising technique for preservation of plant genetic resources for species that produce recalcitrant or intermediate seeds (Dussert *et al.*, 2012; Berjak and Pammenter, 2014). It is a method of conserving

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biological material in liquid nitrogen at -196°C , or in its vapour phase at -150°C after controlled dehydration (Berjak and Pammenter, 2014). This procedure prevents metabolic reactions, ensuring viability in storage for an indeterminate period of time without loss in physiological quality (Engelmann, 2011).

Drying seeds to an ideal seed moisture content before cryopreservation is fundamental for success because this avoids formation of ice crystals in the intracellular space (Kaviani, 2011). The ideal moisture content is necessary for vitrification whereby water becomes a non-crystalline, supersaturated, amorphous and metastable solid, thus impeding rupture of cells by ice crystals (Engelmann, 2011). However, in intermediate species such as *Coffea arabica* L., the rate of water removal can bring about irreversible damage to cells and compromise seed viability (Kohoma *et al.*, 2006). Dussert *et al.* (1998, 2002) and Dussert and Engelmann (2006) showed that the moisture range within which *C. arabica* seeds can be cryopreserved and then show high viability ($> 80\%$) after thawing is very narrow, between 0.20 and 0.22 g $\text{H}_2\text{O g}^{-1}$ dry weight. However, the rate at which water is removed from the seeds to achieve the percentage of moisture adequate for cryopreservation has not yet been clarified. According to Coelho *et al.* (2015), the drying rate and the residual water content in the seeds are essential factors and they must be considered when studying protocols for conservation of coffee seeds.

Exposure of seeds to stress conditions, such as cryopreservation, can promote physiological and biochemical changes. Free radicals are the main causes of damage to the membrane system and to macromolecules, causing change in metabolic processes, ionic imbalance, and induction of cell death through apoptosis (Engelmann, 2011). Reactive-oxygen-species-removing enzymes, such as catalase, superoxide dismutase and peroxidase, among others, can mitigate or reverse damage caused by these stresses, which makes biochemical studies important for elucidating the effect of cell damage caused by cryopreservation (Berjak, 2006; Dussert and Engelmann, 2006).

In recent years, significant advances have been achieved in relation to use of the cryopreservation for coffee seeds (Dussert *et al.*, 1997, 1998, 2000, 2003, 2012; Eira *et al.*, 2005; Dussert and Engelmann, 2006). However, the best results from cryopreservation were obtained after drying seeds in saturated saline solutions. Hence it is important to try and simplify this technique and to study the tolerance to cryopreservation of seeds of the main coffee cultivars currently cultivated in Brazil.

Given the importance of preserving coffee genetic resources and the absence of studies on the effect of drying rate on seed cryopreservation, the aim of this study was to evaluate seeds from different cultivars of *C. arabica* after cryopreservation by direct immersion in liquid nitrogen after rapid and slow drying.

Materials and methods

Seeds of four commercial cultivars of *C. arabica*, 'Arara', 'Catiguá', 'Catuaí Amarelo' and 'Mundo Novo', from the 2014/2015 crop season were used. The fruits were harvested on the Procafé Experimental Farm in Varginha, Minas Gerais, at an altitude of 980 m a.s.l., with a subtropical highland climate (Cwb), according to the Köppen classification.

Coffee fruits in the cherry stage were selectively harvested from plants of the coffee cultivars (grown under the same climate and management practices) from the middle part of the middle branches. After harvest, the fruits were selected to standardise the stage of maturity, and they were mechanically pulped. Mucilage was removed from the seeds by fermentation in water for 24 hours (fully washed) and they were pre-dried in the shade for removal of surface moisture. Seed size was standardised by taking seeds from circular mesh sieves no. 18/64 and 20/64; seeds of bigger or smaller size were discarded.

After hulling, the seeds were dried to 20% moisture content (dry basis) because this is the moisture content recommended for cryopreservation of coffee seeds according to previous studies (Dussert *et al.*, 1997, 1998, 2000, 2003, 2012; Eira *et al.*, 2005; Dussert and Engelmann, 2006). Two types of drying, rapid and slow, were used. The two types of drying were performed in a hermetic environment on screens in acrylic boxes, 'gerbox type', that were sealed with PVC film to prevent the ingress of atmospheric air.

For rapid drying, the seeds were arranged in a single layer on metal screens in the boxes; the boxes contained activated colour-indicator silica gel below the screen. During drying, the silica was exchanged before it changed colour. For slow drying, the seeds were arranged in the same manner, but in boxes containing saturated NaCl solution (75% relative humidity). The containers were sealed and kept in B.O.D. incubators at 25°C. Water loss during drying was monitored by repeatedly weighing on a precision balance with 0.001 g readability until the seeds reached the desired moisture content.

The dry seeds, with 20% moisture content were then placed in tri-laminate aluminum packages and directly immersed in a tank containing liquid nitrogen (ultra-rapid freezing). After 24 hours of cryopreservation, the packages containing the seeds were removed and the seeds were thawed in a water bath at 40°C for two minutes (Dussert *et al.*, 1998). In the thawing process, the seeds were quickly removed from their respective packages and immersed directly in the water bath. After that, they were dried in paper towels and their parchments were manually removed. The seeds then underwent physiological and biochemical evaluation.

The moisture content of the seeds was determined by drying samples in a laboratory oven at 105°C for 24 hours (Brasil, 2009); the results are expressed as percentages based on the dry weight of the seeds. The physiological quality of the seeds was evaluated by the percentage of normal seedlings, of seedlings with expanded cotyledonary leaves, of embryo viability in the tetrazolium test and by seedling dry matter.

The germination test was conducted with four replications of 25 seeds between sheets of paper, moistened with water at 2.5 times the weight of the dry paper. The seeds were kept in a seed germinator at 30°C, and the percentage of normal seedlings was evaluated after 30 days (Brasil, 2009). Forty-five days after the beginning of the germination test, the number of seedlings with expanded cotyledonary leaves was counted, and the results were expressed in percentage.

Seedling dry matter was also determined 45 days after the start of the germination test, when the hypocotyl-radicle axes of the normal seedlings were isolated, placed in paper bags and dried in an air-circulation laboratory oven at 60°C for five days. After this period, the root and shoot dry matter of the seedlings was determined, and the results were expressed in milligrams per seedling.

In the tetrazolium test, four replications of 10 seeds were used, which were soaked in distilled water for 48 hours at 30°C (Clemente *et al.*, 2011). After soaking, the embryos were removed with a scalpel to prevent damage to them. They were immersed in a tetrazolium solution at 0.5% in the absence of light for three hours, at which time the staining pattern was evaluated. The results are expressed as percentage of viable embryos.

For biochemical analyses, the seeds were macerated in liquid nitrogen in the presence of polyvinylpyrrolidone and the samples were stored at -86°C (deep-freezing) until the analysis. The methodology proposed by Alfenas (2006) was used for extraction, electrophoresis and determination of catalase (CAT), peroxidase (PO) and esterase (EST).

A completely randomised experimental design was used in a 2 × 4 factorial arrangement, consisting of two types of drying (rapid and slow) and four cultivars, with four replications. The results of the physiological tests were subjected to ANOVA using SISVAR (Ferreira, 2011). The means were compared by the Scott-Knott test at 5% probability.

Results

Results of evaluation after drying of seeds

Seeds of coffee cultivars 'Arara', 'Catiguá', 'Catuaí Amarelo' and 'Mundo Novo' had an initial moisture content of 59, 61, 54 and 59% (dry basis), respectively, and germination of 69, 83, 75 and 75 %, respectively. Seeds that were dried rapidly over silica gel took 49 hours to reach 20% moisture; seeds that were slowly dried over NaCl took 162 hours to reach the same moisture content. The drying rate decreased as the seeds lost water, but mean drying rate was 0.775% hour⁻¹ with rapid drying and 0.234% hour⁻¹ with slow drying.

Analysis of variance of the results of the evaluations after drying showed significant interaction effects of drying rate and cultivar for all the response variables (figure 1). The results of evaluation of normal seedlings and seed vigour (figure 1A-D) showed that slower drying led to better results compared with rapid drying for 'Arara', 'Catuaí Amarelo' and 'Mundo Novo'. For 'Catiguá', the drying rate did not affect germination and seed vigour. 'Mundo Novo' was more tolerant to desiccation than the other cultivars when seeds were dried slowly.

The tetrazolium test did not identify differences depending on the drying rate for the four cultivars (figure 1E). Seeds of 'Arara' that were dried in silica gel had a high percentage of viable embryos, even though they had low physiological performance in the germination test.

For all four cultivars, undried seeds had the lowest catalase activity (figure 2A). When the coffee seeds were dried, at both the more rapid and slower rate, an increase in expression of this enzyme was observed. Similarly, greater intensity of the esterase bands was observed for the dry seeds, especially for 'Catuaí Amarelo' and 'Mundo Novo' (figure 2B). For 'Arara' and 'Catiguá', there was also an increase in activity after drying the seeds, however, without apparent differences in expression between seeds that were subjected to rapid or slow drying. The cultivars 'Catuaí Amarelo' and 'Mundo Novo' had greater expression in seeds dried more rapidly.

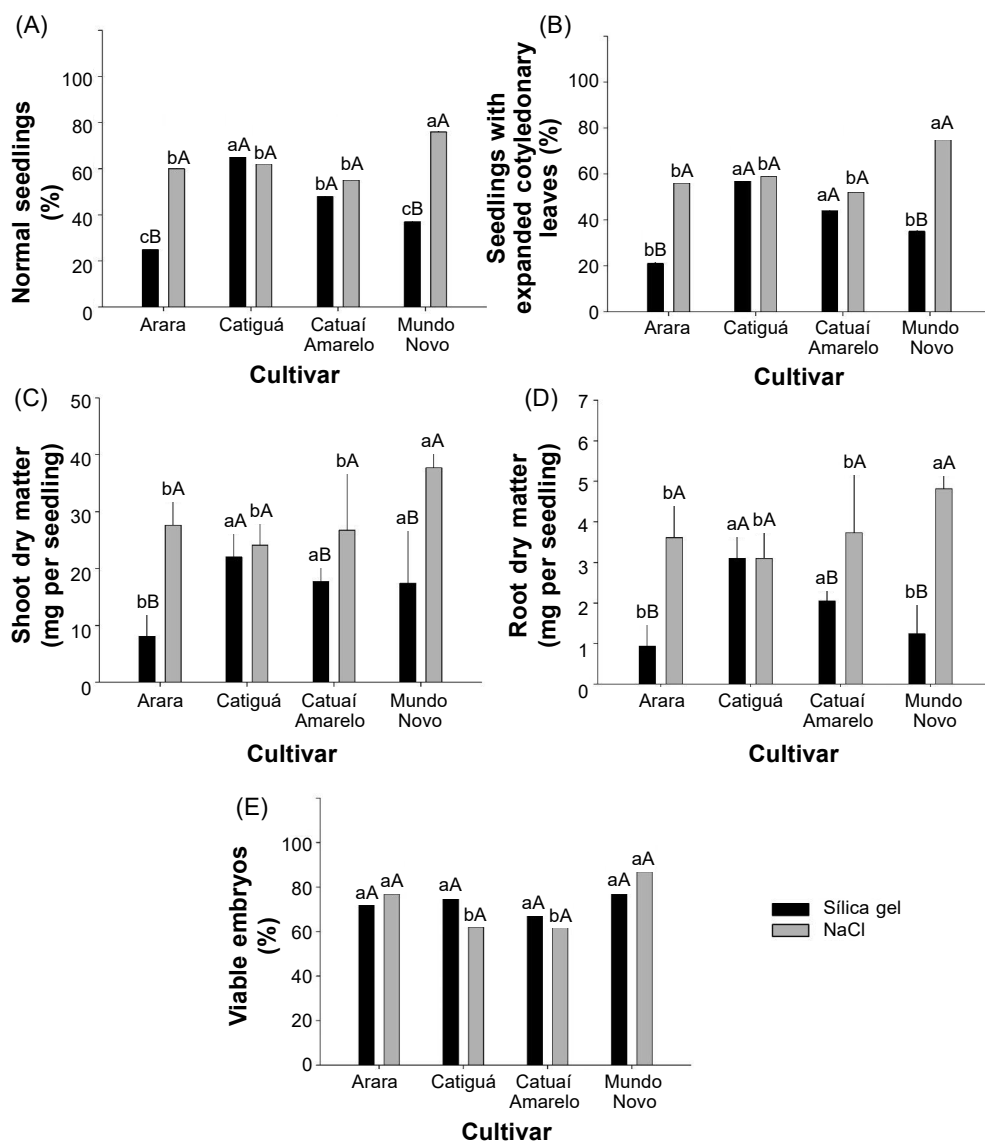


Figure 1. Mean percentage of normal seedlings (A) and of seedlings with expanded cotyledonary leaves (B), mean weight of shoot dry matter (C) and root dry matter (D) in the germination test, and embryo viability in the tetrazolium test (E) of seeds from four cultivars of *Coffea arabica* after drying with silica gel and or saturated saline solution. Uppercase letters compare the type of drying within the same cultivar; lowercase letters compare the cultivars within the same type of drying.

There was an increase in levels of peroxidase upon drying; moist seeds had very low or no activity compared with dry seeds (figure 2C). Of the four cultivars studied, ‘Catuaí Amarelo’ and ‘Mundo Novo’ had lower expression of this enzyme after slow drying. The cultivars ‘Arara’ and ‘Catiguá’ had greater expression after fast drying.

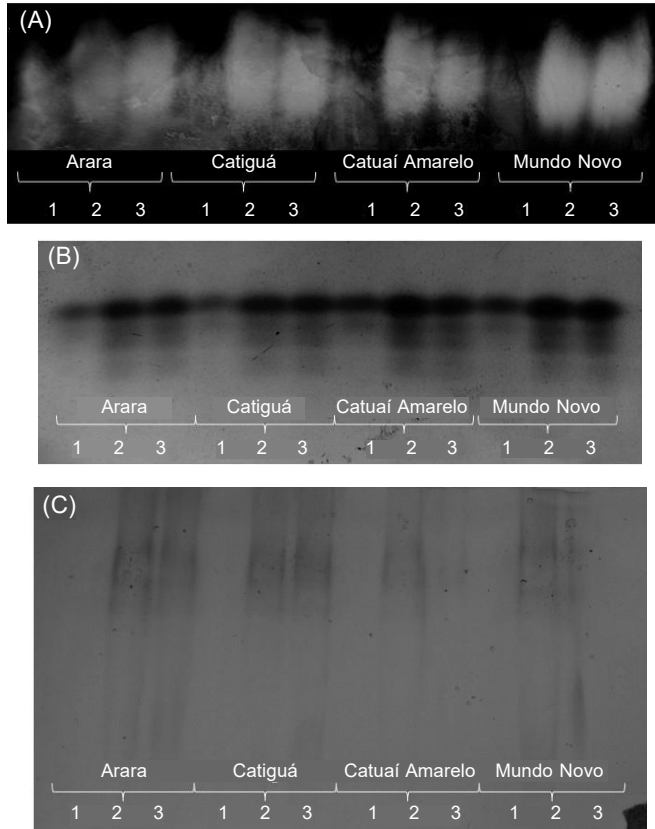


Figure 2. Levels of catalase (A), esterase (B) and peroxidase (C) in seeds of four commercial cultivars of *Coffea arabica* before and after drying determined by electrophoresis. Lanes labeled 1 = control (moist seeds, without drying); lanes labeled 2 = seeds dried quickly over silica gel; lanes labeled 3 = seeds dried slowly over saturated NaCl solution. [Colour version of this figure available in the online publication.]

Results of evaluation after cryopreservation of seeds

Analysis of variance of the data after cryopreservation showed significant interaction effects of drying rate and cultivar for all the response variables (figure 3). In contrast to the effects of drying alone on seed quality, rapid drying in silica gel was more favourable for cryopreservation than slow drying, except for the cultivar ‘Catuaí Amarelo’, for which there was no difference between the slow- and fast-dried seeds (figure 3A-D). Seeds of ‘Catuaí Amarelo’ dried in silica gel had greater tolerance to cryopreservation, regardless of the manner of drying. Seeds of ‘Arara’ were most sensitive to the effects of cryopreservation, based on the results of the germination test (figure 3A).

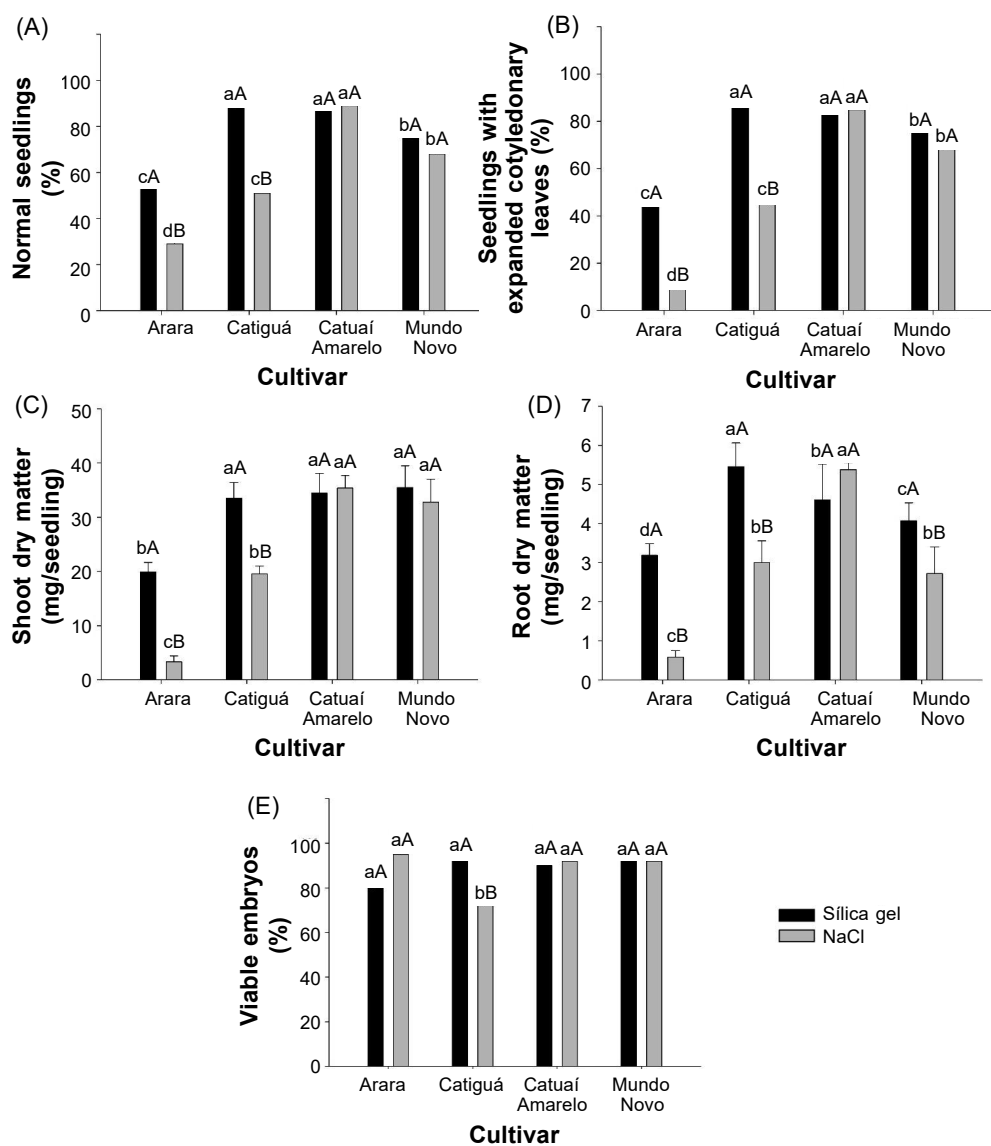


Figure 3. Mean percentage of normal seedlings (A) and of seedlings with expanded cotyledonary leaves (B), mean weight of shoot dry matter (C) and root dry matter (D) in the germination test, and embryo viability in the tetrazolium test (E) of seeds from four commercial cultivars of *Coffea arabica* after drying with silica gel or saturated NaCl solution and cryopreservation. Uppercase letters compare the type of drying within the same cultivar; lowercase letters compare the cultivars within the same type of drying.

In the tetrazolium test, in which the viability of coffee embryos is evaluated (figure 3E), no significant differences were found depending on the method of drying or on cultivar, except seeds of ‘Catiguá’ that were dried slowly, had relatively low embryo viability. The results of the tetrazolium test also showed greater physiological quality of the coffee embryos compared with the physiological quality of the seeds for all the cultivars studied, regardless of the type of drying.

The electrophoretic profile of CAT showed, for all cultivars, that the undried seeds (control) had low activity compared with the dried and cryopreserved seeds (figure 4A). Esterase had a result similar to that of CAT, in which expression increased after cryopreservation of the seeds, for all the cultivars (figure 4B). Greater band intensity in the slowly dried and cryopreserved seeds was observed for ‘Arara’. The cultivars ‘Catuaí Amarelo’ and ‘Mundo Novo’ had a result opposite to that of the cultivar ‘Arara’, in which rapidly dried and cryopreserved seeds had greater expression of this enzyme.

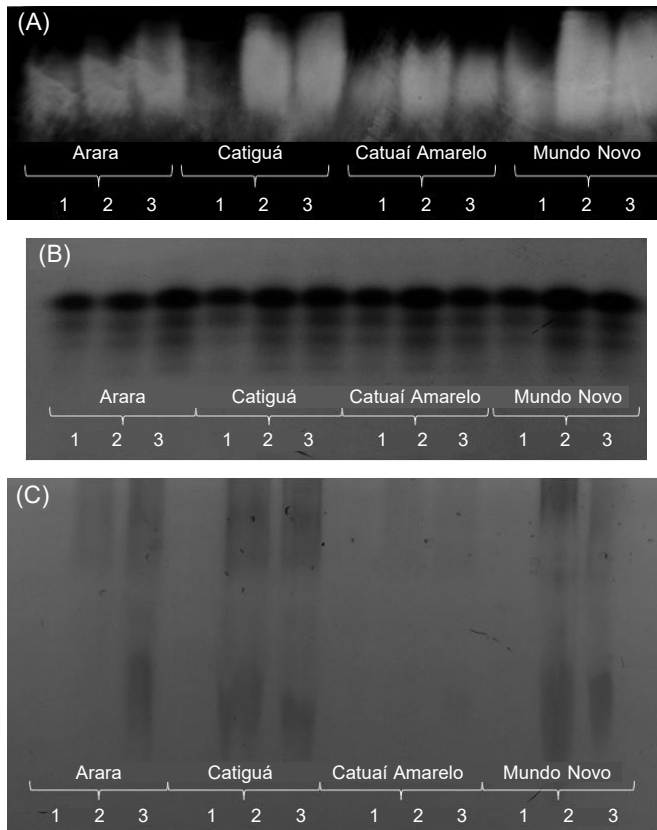


Figure 4. Levels of catalase (A), esterase (B) and peroxidase (C) in seeds of four commercial cultivars of *Coffea arabica* after cryopreservation determined by electrophoresis. Lanes labeled 1 = control (moist seeds, without drying); lanes labeled 2 = seeds dried quickly over silica gel and immersed in liquid nitrogen; lanes labeled 3 = seeds dried slowly over saturated NaCl solution and immersed in liquid nitrogen. [Colour version of this figure available in the online publication.]

Seeds of the control treatment of all cultivars did not show any PO activity (figure 4C). The cryopreserved seeds of 'Catuaí Amarelo' also did not show expression of PO. The cryopreserved seeds of 'Catigua' did not show different expression of PO in seeds dry in silica gel and saturated NaCl solution.

Discussion

The results of this study show that *C. arabica* seeds have superior physiological quality after slow drying, but rapid drying is recommended for cryopreservation of the seeds of this species (figures 1 and 3). José *et al.* (2011) studied the best method for drying seeds of *Magnolia ovata* Spreng. (a species with intermediate seed behaviour, like coffee seeds) and observed that germination is reduced more by rapid drying than by slow drying for the same moisture content, corroborating the results found in this study. However, according to Walters *et al.* (2008), when dehydration is faster, there is not enough time for accumulation of the damage that occur during drying, that is, the tissue are at moisture contents in which degradation processes can occur for a shorter time and before damage can accumulate to harmful levels.

Zhang *et al.* (2014) investigated the effects of cryopreservation on the physiological quality of seeds of *Citrus paradisi* Macfad., another species with intermediate seeds, and also found that seeds dried in silica gel had greater viability after exposure to liquid nitrogen compared with those dried in saturated saline solutions. That result is similar to the result of the present study, in which rapid drying in silica gel is better than slower drying in saturated saline solution (75% relative humidity) for cryopreservation of coffee seeds (figure 3). According to Pammenter and Berjak (2014), slow drying induces the loss of viability at higher moisture contents, whereas seeds dried more rapidly can survive the lower moisture content, which is sufficient to allow vitrification of the residual intracellular solution and hence prevent the formation of ice crystals.

Based on the results of the present study with different cultivars, coffee seeds can be cryopreserved but have different levels of tolerance to immersion in liquid nitrogen; 'Catuaí Amarelo' is the most tolerant and 'Arara' is the most sensitive to immersion in liquid nitrogen, regardless of the drying rate of the seeds (figure 3). Due to the narrow genetic base of cultivars of *C. arabica*, there is a great deal of similarity between parental lines, and, in many cases, genotypic and phenotypic discrimination is difficult. Nevertheless, the seeds of the cultivars investigated in this study had different responses to the drying and cryopreservation process. It is noteworthy that in this study the results of the germination test performed on the seeds after drying were inferior to the results in the seeds after cryopreservation (figures 1 and 3). These results indicate that cryopreservation acted in a beneficial manner in coffee seeds that had lower physiological quality before immersion in liquid nitrogen. The causes of this response should be investigated further, but there are signs that the beneficial effects occurred in the endosperm of the coffee seeds since the embryos isolated from those seeds had high viability in the tetrazolium test (figure 1E).

The results of the tetrazolium test also showed greater physiological quality of the coffee embryos compared with the physiological quality of the cryopreserved seeds, for all the cultivars studied (figure 3). This indicates that the endosperms are more sensitive than the embryos to damage that occurred during drying and immersion in liquid nitrogen. The embryos isolated from seed lots that had low percentages of normal seedlings due to the damage from drying and immersion in liquid nitrogen showed high percentages of viability in the tetrazolium test.

Rapid drying to 20% moisture content (dry basis), followed by direct immersion in liquid nitrogen and later reheating in a water bath for two minutes allowed effective cryopreservation of coffee seeds, and this protocol is faster and simpler than the protocols used in other studies. Dussert *et al.* (2001) found that slow cooling at $1^{\circ}\text{C minute}^{-1}$ to -40°C in a biofreezer is better than direct immersion in liquid nitrogen for coffee seeds dried using a saturated saline solution of $(\text{NH}_4)_2\text{SO}_4$ (81% RH) to $0.20 \text{ gH}_2\text{O g}^{-1}$ dry weight, the same moisture content used in this study. Eira *et al.* (1999) found different levels of tolerance among four *C. arabica* cultivars to exposure to -150°C after drying in an NaCl saturated saline solution, to moisture content of around $0.20 \text{ gH}_2\text{O g}^{-1}$ dry weight, rather placing the seeds directly in contact with the vapor phase of the liquid nitrogen.

The activity of CAT, PO and EST increased with drying and upon cryopreservation of the *C. arabica* seeds. Catalase is a redox isoenzyme and acts in removal of free radicals during oxidative stresses (Berjak, 2006). Its greater activity may be related to greater intensity of damage that occurs during seed drying, indicating that drying causes an increase in free radicals in cell metabolism. This requires the expression and action of these enzymes, preparing the defense mechanism in seeds and protecting them against damage to tissues.

Lower activity of CAT in coffee seeds that did not undergo physiological stress was also found by Santos *et al.* (2014) and Abreu *et al.* (2014), confirming that loss of seed viability during the drying and cryopreservation is followed by an increase in the levels of reactive oxygen species (ROS) due to the physiological stress that occurred.

Esterase is also important as an indicator of seed deterioration (Nakada *et al.*, 2010). According to Russel *et al.* (2011), this enzyme is part of the different plant metabolic processes because it acts in ester hydrolysis and lipid metabolism. In spite of the EST enzyme having an important catalytic function in cell disintoxication, its activity may indicate seed deterioration (Russel *et al.*, 2011).

The enzymatic system of peroxidase also plays a fundamental role in the cleaning mechanism of ROS because it uses hydrogen peroxide as an electron acceptor (Taveira *et al.*, 2012). In the *C. arabica* seeds in this study, changes were observed in the profiles of PO, with an increase in the expression of the enzyme during the drying process (figure 2D). Moist seeds did not show activity or showed very low activity compared with seeds that underwent drying, indicating that this enzyme is activated when the tissues pass through the stress of water loss. Among the four cultivars studied, 'Catuaí Amarelo' and 'Mundo Novo', which had the lowest expression of PO after slow drying, also had the best physiological quality. According to Abreu *et al.* (2014), in moist seeds, the enzymatic mechanisms of repair against free radicals such as CAT and PO are not activated, which explains the absence of expression of PO in the seeds that did not undergo drying.

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