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Exploratory studies for cryopreservation of *Coffea arabica* L. seeds [1]

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ABSTRACT – Cryopreservation is a viable option for conservation of coffee germplasm. However, for this technique to be completely successful, it is of fundamental importance to carry out studies that ensure maintenance of cell integrity before and after immersion in liquid nitrogen (LN). Therefore, the aim in this study was to investigate the water content, cooling rate, and final temperature most suitable for cryopreservation of *Coffea arabica* L. seeds. The seeds were dried by silica gel to water contents of 5, 10, 15, 20, 30 and 40 % wb, subjected to slow cooling treatments at speeds of -1, -3 and -5 °C min.-1 to final temperatures of -40, -50 and -60 °C and then directly immersed in LN. After storage, seeds were rewarmed at 40 °C for two minutes. The survival rate and viability of the seeds and embryos were evaluated by the tetrazolium and germination tests. Results of the tetrazolium test indicate that embryos excised from cryopreserved seeds are less sensitive to cryopreservation than whole seeds are. The water content of 20% wb and the use of zygotic embryos led to the highest survival rate of the coffee seeds, depending on the cooling rate and the final temperature of precooling.

Index terms: silica gel, water content, slow cooling, liquid nitrogen, coffee.

Estudos exploratórios para a criopreservação de sementes de *Coffea arabica* L.

RESUMO – A criopreservação é uma opção viável para a conservação de germoplasma de café. Entretanto, para que essa técnica apresente total sucesso é de fundamental importância realizar pesquisas que garantam a manutenção da integridade celular antes e após a imersão em NL. Portanto, o objetivo neste estudo foi investigar o teor de água, velocidade e temperatura final de resfriamento mais adequados para criopreservar sementes de *Coffea arabica* L. As sementes foram secadas em sílica gel até teores de água de 5, 10, 15, 20, 30 e 40% bu, submetidas a tratamentos de resfriamento lento nas velocidades -1, -3 e -5 ºC min^{-1} até às temperaturas finais de -40, -50 e -60 ºC e diretamente imersas em NL. Após armazenamento as sementes foram reaquecidas por dois minutos à 40 ºC. A sobrevivência e a viabilidade de sementes e embriões foram avaliadas por meio do teste de tetrazólio e de germinação. Por meio do teste de tetrazólio, observa-se que embriões excisados das sementes criopreservadas são menos sensíveis à criopreservação quando comparados às sementes inteiras. O teor de água de 20% bu permite maior sobrevivência das sementes de *Coffea arabica* L., bem como de embriões zigóticos após a criopreservação, dependendo das velocidades e temperaturas finais de pré-resfriamento.

Termos para indexação: sílica gel, teor de água, resfriamento lento, nitrogênio líquido, café.

Introduction

Safety, the need for little space and the lowest cost, as well as other advantages offered by the cryopreservation of genetic resources of coffee in relation to other methods of *ex situ* conservation (literally means, "off-site conservation") (Dulloo et al., 2009) has promoted research and advances of this technique for the preservation of this species. However, many questions about the behavior of these intermediate seeds, partially tolerant to desiccation and low temperatures (Ellis et al., 1990) are still researched by the scientific community in order to elucidate the obstacles that hamper their cryopreservation success.

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According to Dekkers et al. (2015) seed longevity is closely related to its tolerance to desiccation.

Orthodox seed cryopreservation is done after dehydration followed by storage in liquid nitrogen (LN) (Panis and Lambardi, 2006; Kaviani, 2011). However, this procedure must be used with caution in *Coffea* spp and research related to the storage of germplasm of this genus/species at ultra-low temperature (-196 °C) has been the subject of investigations for more than 20 years. Within the genus *Coffea*, species are found with different degrees of desiccation tolerance. The species *C. liberica* is less tolerant and *C. racemosa* is the most tolerant to water loss (Dussert et al., 1999). There is also variation to dehydration tolerance within *Coffea* spp. (Dussert et al., 1998). It has already been demonstrated that for *C. arabica* cv. Caturra the range of moisture in which seeds could be cryopreserved is very narrow. However, failure to obtain normal seedlings after cryopreservation of coffee seeds submitted to different precooling rates and temperatures prior to immersion in LN is reported (Dussert et al., 1997; 1998).

The dehydration procedure to which a lot of coffee seeds is subjected is linked to the success of the cryopreservation itself, as well as to the practicality of the application of this long-term preservation method. Coffee seeds were successfully cryopreserved, forming normal seedlings (11 to 67% viability, depending on the lot used) after slow dehydration in saline solutions (Dussert and Engelmann, 2006). However, it has recently been reported that slow dehydration (in saline solutions) is more damaging than rapid dehydration (on silica gel) for coffee beans and it takes almost twice as long to reach the desired moisture (Coelho et al., 2015).

For tropical and subtropical recalcitrant species, classical cryopreservation, consisting of the stages of desiccation, slow cooling, followed by a rapid immersion of the samples in liquid nitrogen, has been shown to be quite effective (Engelmann, 2011; Berjak and Pammenter, 2014). However, the stress caused to the cells by desiccation induced by slow cooling, in response to changes in water properties, may compromise their integrity and cause changes in seed viability (Walters et al., 2008).

Seeds sensitive to desiccation usually present three types of damage. In mechanical damage related to desiccation, the membrane system is adversely affected and there may be a collapse of vacuoles and macromolecular structures. As for physiological damages, linked to the metabolism due to osmotic shocks, they occur during desiccation or hydrated storage. Such damage harms the membrane, protein and DNA system by forming free radicals and toxic byproducts. Finally, after a storage time at high temperatures, there may be damage by macromolecular denaturation with formation of many dilated vacuoles (Pammenter and Berjak, 2000; Umarani et al., 2015). According to Farrant et al. (1997) the sensitivity to desiccation is directly proportional to the degree of vacuolization.

According to Coelho et al. (2015), the water content remaining in the coffee seeds after drying and the speed with which it occurs can affect the seeds physiological quality. These authors have stated that these seeds tolerate greater loss of water when submitted to rapid drying. Air desiccants such as silica gel reduce relative humidity to less than 20% in a short time, providing rapid drying by creating a water gradient between the sample and the surrounding medium (Walters et al., 2008). Recalcitrant seeds can also undergo mechanical damage if dried at very low water contents and physiological damage if the water content is high at the time of storage, these being the possible reasons for the conflicting results in the research carried out until then (Umarani et al., 2015). For example, seeds of *Citrus paradisi* Macfad dried on silica gel have shown better viability after cryopreservation compared to those dried in saline solutions (Zhang et al., 2014).

Slow cooling causes intracellular water to move into the extracellular medium through a gradient of water potential, allowing the cellular content to become more concentrated. This concentration lowers the intracellular freezing point and consequently may allow ice crystals to form only outside the cell to a certain temperature below freezing (Pammenter and Berjak, 2014). As with drying, the speed and final temperature at which slow cooling occurs can also cause mechanical and physiological damages. High concentrations of electrolytes can damage cells if they are dehydrated through a very slow cooling rate. Another possible cell damage can also occur if water is not efficiently drained into the extracellular medium due to a high cooling rate. Such an event leads to the formation of intracellular ice crystals, which are usually lethal (Mazur, 2004). As the cells dehydrate as the temperature decreases, setting the final cooling temperature may also be critical to prevent damage from occurring (Pammenter and Berjak, 2014).

The manner in which classical cryopreservation steps are conducted may contribute to the reduction of cryopreserved seed damage. In this sense, the objectives in this study were to investigate the most adequate water contents, the final precooling rates and temperatures less harmful for cryopreserved seeds of *Coffea arabica* L., cultivar Catuaí Amarelo IAC 62.

Materials and Methods

Plant material

Fruits at a physiological maturity stage (cherry) of the species *Coffea arabica* L., cv. Catuaí Amarelo IAC 62 have been harvested in crops on the "Fazenda Experimental de Varginha"– Fundação PROCAFÉ (Programa Integrado de Apoio

à Tecnologia Cafeeira) and transported to Universidade Federal de Lavras (UFLA), where they were mechanically pulped. The seeds were then fermented in water for a period of 24 hours at room temperature and thereafter arranged in a single layer on a screen and kept under shade to be superficially dried.

After the processing, seeds were submitted to determination of the initial water content and an evaluation of the initial physiological quality through germination tests and a viability test in tetrazolium salt (described below) at Central Seed Laboratory – UFLA.

Procedures for cryopreservation of coffee seeds

Preliminary tests of slow drying (salt saturated solutions) and fast drying (silica gel) of coffee seeds were performed. As fast drying provided better physiological results (Coelho et al., 2015), this drying method was chosen in this experiment.

Drying was performed by placing the seeds, with the initial water content determined, in hermetic containers containing 60 grams of activated silica gel, which were kept in B.O.D (*Biochemical Oxygen Demand*) chambers regulated at 25 ºC in the absence of light. Care was taken so that the seeds were not in direct contact with the drying agent, placing them on a stainless steel screen with small holes, and the loss of water during drying was monitored until the samples reached the following water contents of interest: 40, 30, 20, 15, 10 and 5% wb.

As the coffee seed samples reached the water contents of interest, one part of these samples was used to perform tetrazolium and germination tests and the other part was used to make up the cooling tests described below.

For the cool test, 170 seeds for each water content (40, 30, 20, 15, 10 and 5%) were packed in trifoliate aluminum packs and placed in a bio-freezer (Icecube, model 14S-B, software SY-LAB – Minitub do Brasil), through which different cooling curves were programmed, varying the speeds (-1, -3 and -5 ºC min^{-1}.) until the final temperatures (-40, -50 and -60 ºC) were reached.

According to a methodology by Dussert et al. (1998), with modifications, after completing the cooling curves, the packages containing the seeds were immersed directly in liquid nitrogen (LN) at -196 °C. After 36 hours of storage they were withdrawn and directly reheated in water (water bath) for 2 minutes at 40 ± 1 ºC. Seeds were then superficially dried on paper towel and their parchments manually removed to be submitted to physiological evaluations by germination and tetrazolium tests.

Determining the seed water content

Determination of the water content was carried out using the oven method at 105 °C during 24 hours (Brasil, 2009), with two replicates of 10 seeds. Results were expressed as a percentage based on the seeds dry weight.

Determination of survival and viability of coffee seeds and embryos

Germination test: Carried out with four replicates of 25 seeds for each treatment, seeded on Germitest® paper towel moistened with distilled water in the amount of 2.5 times the dry paper weight. Germination rolls were conditioned in a germinator regulated at 30 ºC in the presence of light (Brasil, 2009). Percentages of radicle protrusion at 15 days and normal seedlings at 30 days after sowing were determined, being computed as normal seedlings those presenting main root and at least two lateral roots. In the germination test were also determined: percentage of strong normal seedlings, being computed those presenting a hypocotyledonous stem measuring at least 3 centimeters, and percentage of seedlings with cotyledonary leaves expanded at 45 days after sowing.

Seedlings dry matter: Seedling dry matter was performed at the end of the germination test in normal seedlings. For this, the shoots were separated from the roots with the aid of a scalpel and the plant material was placed in paper bags and submitted to drying in a forced circulation air oven at 60 ºC for 4 to 5 days or until reaching constant weight. The dry matter was determined on a precision scale.

Tetrazolium test: Carried out with four replicates of 10 seeds. The seeds were soaked in water for 36 hours for the extraction of the embryos. The embryos extracted, kept in a polyvinylpyrrolidone (PVP) antioxidant solution, were washed in running water, immersed in a 0.5% tetrazolium solution using dark vials and stored at 30 ºC temperature for 3 hours (Brasil, 2009; Clemente et al., 2011). Embryo viability analysis was performed using a stereoscopic magnifying glass with a 10-fold increase for better visualization of the embryos internal (central cylinder) and external structures. For this, a longitudinal cut was made in the embryos, which were classified as viable and nonviable according to the location and extent of the damage (Brasil, 2009).

Experimental design

The experimental design was completely randomized in a 6x3x3 factorial scheme (coffee seeds with six water contents, three speeds and three final cooling temperatures) with four replications. Results were submitted to analysis of variance, qualitative averages were compared by the Scott-Knott test, and quantitative data were submitted to regression at 5% probability (Ferreira, 2011).

Results and Discussion

Figure 1 shows the drying curve for coffee seeds submitted to rapid drying on silica gel until reaching different water contents. Silica gel retains the water molecules on the surface of its pores by means of physical adsorption (José et al., 2009). However, it is observed that the average drying rate decreases as the seed water content decreases. This happens because in drier seeds the water becomes strongly attached to the colloidal surfaces of the macromolecules, making it difficult for water to escape (Marcos-Filho, 2005).

In the analysis of variance of the data, a significant triple interaction of the factors studied was noticed. Table 1 shows the results of the interaction of the factors and their effects on the germination and tetrazolium test variables in embryos after cryopreservation of the seeds.

For the variables that comprised germination tests such as root protrusion (RP), normal seedling formation (NS) and strong normal seedling formation (SNS), only seeds with water content of 20% wb presented significant results at all cooling rates and final temperatures (Table 1). This result indicates that this water content is the most suitable for the cryopreservation of *C. arabica* L. cultivar Catuaí Amarelo IAC 62. It has already been reported that for seeds sensitive to dehydration, water content between 10 and 20% is indicated for cryopreservation (Engelmann, 2011). However, our results demonstrate that for coffee seeds this humidity limit can be much narrower. A survival rate was observed for the seeds, for RP observed and even for NS formation, although without significant difference, with water content of 15% wb. However, both RP and NS in the humidity of 15% wb are very low in relation to those of 20% wb. Graiver et al. (2011) stated that drying *Citrus* seeds, also sensitive to desiccation, to a limiting water content that prevents intracellular ice formation is essential for their survival to cryopreservation.

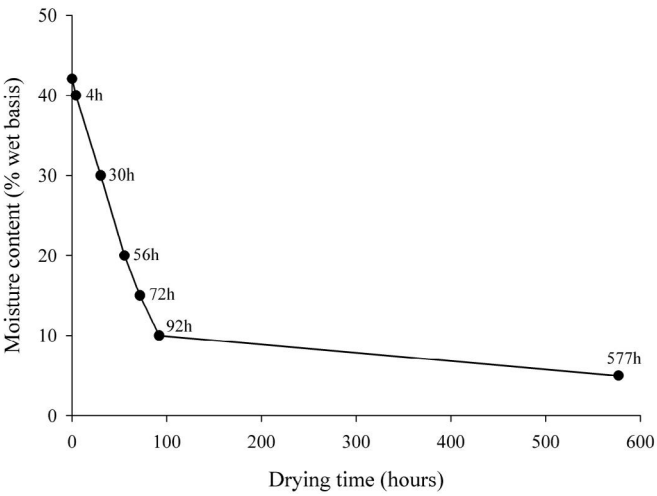

Figure 1. Drying curve of *Coffea arabica* L. seeds subjected to rapid drying on silica gel.

Slow cooling allows freezing extracellular water (Mazur, 2004), leading to drainage of the intracellular water. Therefore, coffee seeds with water contents below 20% may have suffered damage due to excess desiccation (Vertucci, 1993; Vertucci and Farrant, 1995; Pammenter and Berjak, 2000; Pammenter and Berjak, 2014; Umarani et al., 2015). For seeds with water contents above 20% wb, the situation is reversed since, in addition to extracellular freezing, the high water content inside the cell allows intracellular crystallization (Mazur, 2004). Such an event is considered lethal, causing irreversible damage to the cellular structure if the intracellular crystallization is intense (Benson, 2008). Zhang et al. (2014) observed that the higher the water content in seeds of *Citrus paradisi* Macfad. The more sensitive these are at subzero temperature. According to Pritchard (2007), water content present in the seeds is the factor that most affects their cryopreservation.

Failure to form NS after cryopreservation of *C. arabica* seeds dehydrated on silica gel has already been reported (Dussert et al., 1998). On the other hand, the use of slow drying in saline solutions of *C. arabica,* cv. Caturra, followed by slow precooling before immersion in LN, provided maximum viability of the cryopreserved seeds with formation of NS in 67% (Dussert and Engelmann, 2006). Although not using a bio-freezer to monitor the cooling, Dussert and Engelmann (2006) observed that precooling in an ultra-freezer (-80 °C) before immersion in LN favors the increase of the tolerance of coffee seeds to cryopreservation. Our study has also demonstrated high viability with 85% of RP, 79% of NS and 33% of SNS, and this parameter has not been evaluated in previous studies. These results, in addition to the high percentage (77%) of cotyledonary leaf development (CL), and better results for root dry matter (RDM) and shoot dry matter (SDM), were obtained by dehydration of the seeds on silica gel to a moisture content of 20% wb, followed by slow cooling at a rate of -1 °C min^{-1}. until the final temperature of -40 °C (Table 2) before immersion in LN. The great advantage of the method used here, besides the positive results in seed cryopreservation, is the ease of the dehydration process on silica gel, when compared to dehydration in saline solutions. In addition, it has already demonstrated that rapid dehydration for coffee seeds is less damaging than the slow one (Coelho et al., 2015).

The large number of null values (nonviable seeds) present in the results (Table 1) may have been the cause of the high

Table 1. Effects of the final cooling temperatures at each cooling rate and water content of the seeds on the percentages of radicle protrusion (RP), normal seedlings (NS), strong normal seedlings (SNS), seedlings with expanded cotyledonary leaves (CL), root dry matter (RDM), shoots dry matter (SDM) and viable embryos in tetrazolium salt (VE) of cryopreserved coffee seeds.

Water content (% wb wet basis)	Speed (ºC/min.)	Final temperature (ºC)	RP (%)	NS (%)	SNS (%)	CL (%)	RDM (cg)	SDM (cg)	VE (%)
5	-1	-40	0 A	0 A	0 A	0 A	0 A	0 A	50 A
		-50	0 A	0 A	0 A	0 A	0 A	0 A	43 A
		-60	0 A	0 A	0 A	0 A	0 A	0 A	48 A
	-3	-40	0 A	0 A	0 A	0 A	0 A	0 A	40 A
		-50	0 A	0 A	0 A	0 A	0 A	0 A	38 A
		-60	0 A	0 A	0 A	0 A	0 A	0 A	30 A
	-5	-40	0 A	0 A	0 A	0 A	0 A	0 A	40 B
		-50	0 A	0 A	0 A	0 A	0 A	0 A	50 B
		-60	0 A	0 A	0 A	0 A	0 A	0 A	68 A
10	-1	-40	0 A	0 A	0 A	0 A	0 A	0 A	73 A
		-50	0 A	0 A	0 A	0 A	0 A	0 A	85 A
		-60	0 A	0 A	0 A	0 A	0 A	0 A	83 A
	-3	-40	0 A	0 A	0 A	0 A	0 A	0 A	85 A
		-50	0 A	0 A	0 A	0 A	0 A	0 A	80 A
		-60	0 A	0 A	0 A	0 A	0 A	0 A	63 B
	-5	-40	0 A	0 A	0 A	0 A	0 A	0 A	68 B
		-50	0 A	0 A	0 A	0 A	0 A	0 A	60 B
		-60	0 A	0 A	0 A	0 A	0 A	0 A	85 A
15	-1	-40	0 A	0 A	0 A	0 A	0.0 A	0.0 A	90 A
		-50	3 A	1 A	0 A	1 A	0.2 A	1.1 A	70 B
		-60	0 A	0 A	0 A	0 A	0.0 A	0.0 A	58 B
	-3	-40	0 B	0 A	0 A	0 A	0.0 A	0.0 A	88 A
		-50	4 A	0 A	0 A	1 A	0.3 A	1.0 A	80 A
		-60	5 A	3 A	0 A	1 A	0.3 A	1.8 A	90 A
	-5	-40	0 B	0 A	0 A	0 A	0.0 A	0.0 A	73 A
		-50	4 A	1 A	0 A	1 A	0.2 A	1.1 A	83 A
		-60	7 A	3 A	3 A	0 A	0.0 A	0.0 A	85 A
20	-1	-40	85 A	79 A	33 A	77 A	19.6 A	103.7 A	75 A
		-50	37 C	28 B	16 B	25 B	6.1 B	33.0 B	70 A
		-60	51 B	28 B	13 B	25 B	6.3 B	33.0 B	80 A
	-3	-40	83 A	71 A	16 A	65 A	15.2 A	77.5 A	70 A
		-50	8 C	7 C	4 B	7 C	1.5 C	8.8 C	40 B
		-60	55 B	37 B	8 B	36 B	8.0 B	41.1 B	73 A
	-5	-40	61 A	49 A	19 A	47 A	12.5 A	67.3 A	80 B
		-50	9 C	3 C	3 B	3 C	0.6 C	3.8 C	58 C
		-60	55 B	39 B	19 A	35 B	8.9 B	44.5 B	95 A
30	-1	-40	0 A	0 A	0 A	0 A	0.0 A	0.0 A	0 A
		-50	0 A	0 A	0 A	0 A	0.0 A	0.0 A	0 A
		-60	0 A	0 A	0 A	0 A	0.0 A	0.0 A	0 A
	-3	-40	0 A	0 A	0 A	0 A	0.0 A	0.0 A	0 A
		-50	0 A	0 A	0 A	0 A	0.0 A	0.0 A	0 A
		-60	0 A	0 A	0 A	0 A	0.0 A	0.0 A	0 A
	-5	-40	0 A	0 A	0 A	0 A	0.0 A	0.0 A	0 A
		-50	0 A	0 A	0 A	0 A	0.0 A	0.0 A	0 A
		-60	0 A	0 A	0 A	0 A	0.0 A	0.0 A	0 A
40	-1	-40	0 A	0 A	0 A	0 A	0.0 A	0.0 A	0 A
		-50	0 A	0 A	0 A	0 A	0.0 A	0.0 A	0 A
		-60	0 A	0 A	0 A	0 A	0.0 A	0.0 A	0 A
	-3	-40	0 A	0 A	0 A	0 A	0.0 A	0.0 A	0 A
		-50	0 A	0 A	0 A	0 A	0.0 A	0.0 A	0 A
		-60	0 A	0 A	0 A	0 A	0.0 A	0.0 A	0 A
	-5	-40	0 A	0 A	0 A	0 A	0.0 A	0.0 A	0 A
		-50	0 A	0 A	0 A	0 A	0.0 A	0.0 A	0 A
		-60	0 A	0 A	0 A	0 A	0.0 A	0.0 A	0 A
CV (%)			29.99	41.09	101.02	43.33	42.36	44.17	20.61

Means followed by the same letter in the column do not differ statistically by the Scott-Knott test at 5% probability.

Table 2. Effects of the cooling rate and final temperature on the results of the germination test of *Coffea arabica* L. seeds cryopreserved with water content of 20% (wb).

Variables	Speed	Final temperature		
		-40 ºC	-50 ºC	-60 ºC
Radicle protrusion (%)	-1	85 Aa	37 Ac	51 Ab
	-3	83 Aa	8 Bc	55 Ab
	-5	61 Ba	9 Bb	55 Aa
	CV (%)		10.04	
Normal seedling (%)	-1	79 Aa	28 Ab	28 Bb
	-3	71 Ba	7 Bc	37 Ab
	-5	49 Ca	3 Bc	39 Ab
	CV (%)		13.31	
Strong normal (%)	-1	33 Aa	16 Ab	13 Bb
	-3	16 Ba	4 Bb	8 Cb
	-5	19 Ba	3 Bb	19 Aa
	CV (%)		33.24	
Cotyledon leaf (%)	-1	77 Aa	25 Ab	25 Bb
	-3	65 Ba	7 Bc	36 Ab
	-5	47 Ca	3 Bc	35 Ab
	CV (%)		14.26	
Root dry matter (cg)	-1	19.6 Aa	6.1 Ab	6.3 Bb
	-3	15.2 Ba	1.5 Bc	8.0 Ab
	-5	12.5 Ca	0.6 Bc	8.9 Ab
	CV (%)		14.02	
Shoot dry matter (cg)	-1	103.7 Aa	33.0 Ab	33.0 Ab
	-3	77.5 Ba	8.8 Bc	41.1 Ab
	-5	67.3 Ca	3.8 Bc	44.5 Ab
	CV (%)		14.67	

Means followed by the same lowercase letter in the line and uppercase letter in the column do not differ statistically by the Scott-Knott test at 5% probability.

coefficient of variation of the statistical analysis. Therefore, another analysis was applied only to the data obtained with seeds of 20% wb in order to verify with more clarity the interference of speed and final cooling temperature in these seeds (Table 2).

Root protrusion was higher in coffee seeds cooled at speeds of -1 or -3 °C min.$^{-1}$ until the final temperature of -40 °C with maximum value of 85% (Table 2). According to Kulus and Zalewska (2014), the choices of final speed and temperature at which ornamental plant material is subjected to cooling must be adjusted according to species and is generally between a rate of 0.1 and 1.0 °C min.$^{-1}$ until the temperature of - 40 °C. Cold can cause cell damage in some plant species, even before the formation of ice crystals, or even by simple desiccation of the cytoplasm caused by cooling (Trigiano and Gray, 2011). This fact justifies the positive results obtained at the - 40 °C comparing to the others (-50 and -60 °C) tested in the present study (Tables 1 and 2).

For the variable percentage of normal seedlings, the best results were found when the coffee seeds were cooled at a rate of -1 ºC min.$^{-1}$ until the final temperature of -40 ºC (Table 2). It is noticed that better seed vigor was also observed when they were submitted to these same conditions. In this situation, although dehydration occurs more slowly, the higher temperature does not allow the formation of ice crystals in the intracellular environment, benefiting the maintenance of cell integrity. Although not using a bio-freezer to monitor the cooling, Dussert and Engelmann (2006) observed that precooling in an ultra-freezer (-80 °C) before immersion in LN favors the increase of the tolerance of coffee seeds to cryopreservation.

For all variables analyzed, the final temperature of -50 ºC was less efficient, resulting in lower viability and vigor of coffee seeds, especially when submitted to -3 and -5 °C min.$^{-1}$ (Table 2). Dussert et al. (1997) observed that the cooling rate of -1 °C min.$^{-1}$ to -50 °C provided better viability of coffee seeds, but few developed in normal seedlings.

As for the final precooling temperature of -60 °C, the result was contrary to the temperature of -50 °C in the cooling speeds of -3 and -5 °C min.$^{-1}$, which gave better results for RP and NS (Table 2). Increasing the cooling rate reduces the intracellular water outlet but, on the other hand, lower final temperature provides longer time for intracellular dehydration. Thus it may prevent the formation of ice crystals within the cell (Pammenter and Berjak, 2014), resulting in less damage and greater seed viability.

The results of the tetrazolium test in the embryos (Table 1) also showed triple interaction of factors in studies. Embryo viability was observed in only four water contents tested, and at 30 and 40% content the viability was zero. Pukacki and Juszczyk (2015) have observed significant damage in the embryonic axis tissues of two *Acer* species when these exceeded 30% water content and were cooled to -40 °C or cryopreserved.

By means of the tetrazolium test, the lower sensitivity of embryos excised from cryopreserved seeds with different water contents in relation to whole seeds, which were only viable with water content of 20% (Table 1), can be observed. Dussert and Engelmann (2006) and Coelho et al. (2015) also observed greater viability of embryos in relation to seeds in studies about cryopreservation and drying with coffee, respectively.

As performed for whole seeds, another statistical analysis was performed only for the treatments that presented embryo survival. The percentage of viable embryos varied according to water content, speed and final cooling temperature at which the seeds were submitted. For the temperature of -40 °C (Figure 2A), the maximum viability of the embryos was

91% when the seeds presented approximately 14% wb and were cooled at a rate of -3 °C min.$^{-1}$. Also at this temperature, a linear increase in embryo viability was observed when the seeds were cooled at -5 °C min.$^{-1}$, with maximum value of 84% in seeds with water content of 20% wb. The highest viability of embryos (84%) found for the final temperature of -50 °C (Figure 2B) was also observed when the seeds were cooled at -3 °C min.$^{-1}$ but at a lower water content, around 12,5% wb. As for the temperature of -60 °C (Figure 2C), there was a linear increase when the seeds were cooled at -5 °C min.$^{-1}$, with a maximum percentage of 96% of viable embryos when the seeds had water content of 20% wb. Sisunandar et al. (2010) observed that desiccation increases the viability of cryopreserved coconut zygotic embryos.

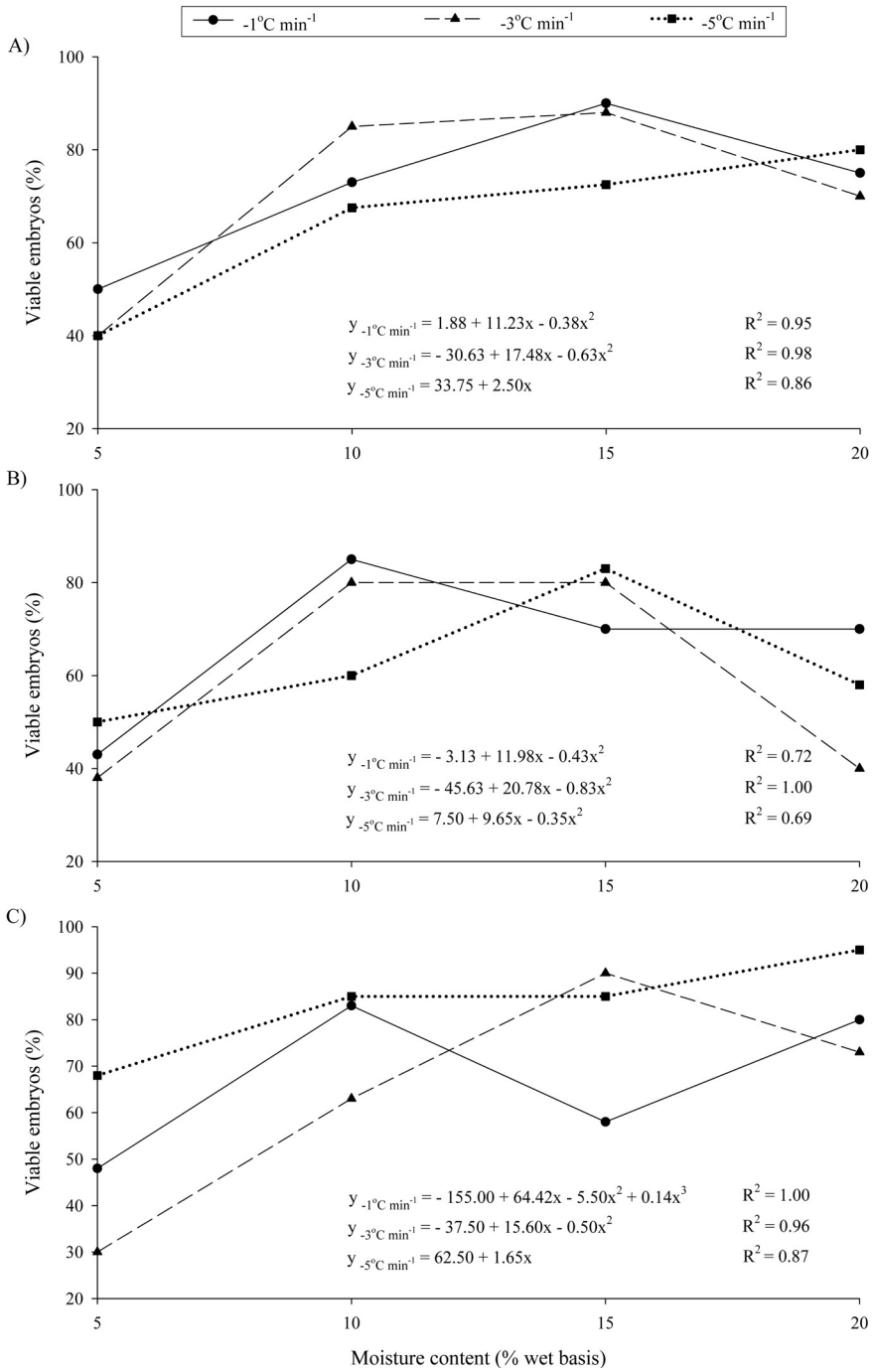

Figure 2. Percentage of viable embryos by the tetrazolium test, excised from cryopreserved coffee seeds dried on silica gel at 5, 10, 15 and 20% wb, cooled to rates of -1, -3 and -5 °C min.$^{-1}$ to the final temperatures of -40 °C (A), -50 °C (B) and -60 °C (C).

It is observed that, regardless of the water content present, at the temperatures of -40 and -50 °C cooling the seeds at a faster rate (-5 °C min.$^{-1}$) is detrimental to the embryos viability and that this result is the reverse when the final temperature is lower, that is, high embryo viability is observed in seeds cooled at a faster rate (-3 or -5 °C min.$^{-1}$) until lower final temperature (-60 °C).

Embryos are made up of tissues more tolerant to dehydration compared to seeds as a whole (Sisunandar et al., 2010; Engelmann, 2011). In general, good percentages of embryo viability were obtained in the water content range between 10% and 20% wb. Pukacki and Juszczyk (2015) observed high viability, after cryopreservation, of embryonic axes of recalcitrant seeds of *Acer pseudoplatanus* dried at water content between 15 and 20%. However, cryopreservation of zygotic embryos requires greater commitment from researchers compared to seeds. It was demonstrated in this work that by using a simpler methodology of silica gel dehydration together with slow cooling stage, high rates of viability of coffee seeds can be obtained after cryopreservation, making possible long term storage of this type of material.

Conclusions

The water content of 20% (wb) allows higher seed survival as well as the formation of the highest percentages of normal and strong normal seedlings of *Coffea arabica* L. cultivar Catuaí Amarelo IAC 62 after cryopreservation when cooled to a -1 °C min.$^{-1}$ until the final temperature of -40 °C before immersion in LN.

The tetrazolium test indicates that zygotic embryos exhibit high viability when cooled at speeds greater than -1 °C min.$^{-1}$ until the final temperature of -60 °C before immersion in LN.

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