**PALLOMA INDIARA CAPRONI MORAIS**

# ADVANCES IN THE CRYOPRESERVATION OF *COFFEA CANEPHORA* SEEDS

**LAVRAS - MG**
**2023**

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Tese apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós-Graduação em Agronomia/Fitotecnia, área de concentração em Produção Vegetal, para obtenção do título de Doutora.

Dra. Sttela Dellyzete Veiga Franco da Rosa

Orientadora

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# ADVANCES IN THE CRYOPRESERVATION OF *COFFEA CANEPHORA* SEEDS

# AVANÇOS NA CRIOPRESERVAÇÃO DE SEMENTES DE *COFFEA CANEPHORA*

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## RESUMO

Atualmente o germoplasma do cafeeiro é conservado em coleções de plantas vivas no campo *(ex situ)*, que além de demandar muito espaço e manutenção, há a chance de ocorrência de um desastre ambiental. Por isso, faz-se necessário o estudo de outras opções de conservação. A criopreservação já é uma opção para sementes ortodoxas, porém para sementes intermediárias e recalcitrantes são necessários mais estudos e os protocolos utilizados variam de acordo com a espécie. Até o momento, as sementes de *Coffea canephora* Pierre quando criopreservadas apresentam baixa sobrevivência e os maiores entraves da técnica são as etapas de resfriamento e aquecimento. Sendo assim faz-se necessário mais estudos com intuito de aumentar a sobrevivência após a criopreservação. Objetivou-se com este trabalho avaliar qual o melhor tempo e temperatura de aquecimento das sementes de *C. canephora* Pierre após serem submetidas às etapas de criopreservação, por meio de estudos fisiológicos, bioquímicos, ultraestruturais e moleculares. Para isso, foram realizados testes para melhor compreensão do que ocorre nas sementes durante as etapas da técnica. A pesquisa foi conduzida no Laboratório Central de Análise de Sementes, do Departamento de Agricultura da ESAL, UFLA, com sementes de *C. canephora* Pierre, cultivar Apoatã. Os frutos colhidos foram devidamente processados e as sementes foram secadas em sílica gel ou em secador estacionário até umidade de 20% (bu). Após a secagem, as sementes foram colocadas em saquinhos de filó e foram imersas diretamente em nitrogênio líquido. As sementes foram retiradas e aquecidas em banho-maria sob diferentes temperaturas (40, 45, 50, 55, 60, 70ºC) durante 1, 2 e 3 minutos. Foram realizados testes de germinação, de tetrazólio e de condutividade elétrica, além de avaliações bioquímicas, moleculares e de microscopia eletrônica de varredura. O aquecimento na temperatura de 70ºC é prejudicial e não deve ser recomendado para protocolos de criopreservação de sementes de *Coffea canephora* Pierre, independentemente do tempo de exposição. A secagem em sílica gel, visando a criopreservação, aumenta as chances de sucesso da técnica, quando comparado à secagem no secador, para sementes de *Coffea canephora* Pierre. A secagem em sílica gel até o teor de água de 20% (bu), seguido de imersão direta em nitrogênio líquido e aquecimento na temperatura de 50ºC, durante 2 minutos, em banho maria, garantiu maior sobrevivência das sementes.



**Palavras-chave**: Criogenia. Nitrogênio Líquido. Qualidade Fisiológica. Armazenamento. Conservação.

## ABSTRACT

Currently, coffee germplasm is conserved in collections of live plants in the field (*ex situ*), which requires a lot of space and maintenance, there is a chance of an environmental disaster occurring. Therefore, it is necessary to study other conservation options. Cryopreservation is already an option for orthodox seeds, but for intermediate and recalcitrant seeds further studies are needed and the protocols used vary according to the species. So far, *Coffea canephora* Pierre seeds, when cryopreserved, have low survival and the biggest obstacles to the technique are the cooling and warming stages. Therefore, further studies are needed in order to increase survival after cryopreservation. This study aimed to evaluate the best time and temperature for warming*C. canephora* Pierre seeds after being submitted to the cryopreservation stages, through physiological, biochemical, ultrastructural and molecular studies.For this, tests were carried out to better understand what happens in the seeds during the steps of the technique. The research was carried out at the Central Laboratory of Seed Analysis, of the Department of Agriculture of ESAL, UFLA, with seeds of *C. canephora* Pierre, cultivar Apoatã. The harvested fruits were mechanically hulledand the seeds were dried in silica gel or in a stationary dryer until a humidity of 20% (wb). After drying, the seeds were placed in mesh bags and immersed directly in liquid nitrogen.The seeds were removed and warmed in a water bath at different temperatures (40, 45, 50, 55, 60, 70ºC) for 1, 2 and 3 minutes. Germination, tetrazolium and electrical conductivity tests were carried out, in addition to biochemical, molecular and scanning electron microscopy evaluations. Warming at a temperature of 70ºC is harmful and should not be recommended for cryopreservation protocols for *Coffea canephora* Pierre seeds, regardless of the exposure time. Drying in silica gel, aiming at cryopreservation, increases the chances of success of the technique, when compared to drying in the dryer, for *Coffea canephora* Pierre seeds.Drying on silica gel to a water content of 20% (wb), followed by direct immersion in liquid nitrogen and warming at a temperature of 50ºC, for 2 minutes, in a water bath, ensured greater survival of the seeds.



**Keywords**: Cryogenics. Liquid nitrogen. Physiological Quality. Storage. Conservation.

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# 1 INTRODUCTION

The preservation of *Coffea* sp germplasm is essential, given the socio-economic and cultural importance that coffee represents for Brazil and other countries. Traditionally, *Coffea* sp is conserved *ex situ* through accessions in active banks, which are always subject to threats due to the narrowing and erosion of the genetic base and constant biotic and abiotic risks, which can compromise the genetic heritage in a short time.

On the other hand, preservation in conventional seed banks is not possible due to the recalcitrance characteristics of the *Coffea* spp. Therefore, an important technological demand for coffee farming is the medium-term conservation of seeds and the long-term conservation of germplasm, allowing respectively for propagation at a more appropriate time and the maintenance of the genetic diversity of coffee.

Cryopreservation of seeds in liquid nitrogen, at -196°C is an alternative that allows the safe conservation of these genetic resources for long periods. However, further research is needed as the chemical, physical, and physiological properties of different tissues to be cryopreserved must be considered, which can influence the viability and genetic integrity of the germplasm in question.

Much has been done in recent decades to improve cryoprotection and cooling processes. However, only recently have warming processes received greater attention from cryobiology researchers, as it has been discovered that the main bottleneck to the success of cryopreservation lies in the warming process, where non-uniform warming rates in the sample can cause damage, fractures, cracks, and even death in the seeds.

*Coffea* sp seeds do not tolerate storage under low humidity and sub-zero temperatures, presenting varied degrees of sensitivity. Among the commercial coffee species, *Coffea canephora* has a higher degree of recalcitrance than *Coffea arabica* L., which further complicates the safe storage of this species.

Therefore, considering the strategic requirement for the long-term conservation of coffee genetic resources, this proposal will certainly contribute to the sustainability of the coffee agribusiness in national and international scenarios. In addition, studies of the physiological, biochemical, ultrastructural, and gene expression changes that occur in coffee seeds with

exposure to liquid nitrogen will help elucidate the mechanisms involved in the tolerance to desiccation, cooling, and warming of these tissues subjected to cryopreservation.

# 2 THEORETICAL FRAMEWORK

## 2.1 The importance of the *Coffea*spp.

Coffee belongs to the Rubiaceae family, *Coffea* genus, and contains over 100 described species. It is native to the African continent (DAVIS et al., 2006). Despite the large diversity of species, only two of them are commercially cultivated: *Coffea arabica* L. and *Coffea canephora* Pierre, which practically represent the entirety of coffee production in the world (CARVALHO, 2008). The other species in the genus do not have commercial value but are used in breeding programs due to their genetic diversity (VAN DER VOSSEN; BERTRAND; CHARRIER, 2015).

The total area cultivated with coffee (*C. arabica* and *C. canephora*) in Brazil in 2023 amounts to 2.26 million hectares. Of this total, 355.5 thousand hectares are under formation and 1.9 million hectares are in production. Most of the national coffee production is distributed in the states of Minas Gerais, Espírito Santo, São Paulo, Bahia, Rondônia, and Paraná (CONAB, 2023).

In 2022, Brazil exported 39.8 million 60-kilo bags of coffee, which is 6.3% lower compared to the previous year. The export took the product to 145 countries, with the United States and Germany being the main destinations, accounting for 20.2% and 18.2%, respectively, followed by Italy with 9%, Belgium with 7.9%, and Japan with 4.8% (CONAB, 2023). In terms of value, coffee exports reached the highest value ever recorded in the historical series of the product, which started in 1997. Despite the lower quantity exported, the high price abroad ensured that coffee exports reached US$9.2 billion, representing a 45% increase compared to 2021 (CONAB, 2023).

Coffee is one of the most traditional crops in Brazilian (FAGAN et al., 2011) and of indisputable socioeconomic importance for the country, due to the foreign exchange generated by exports and the workforce employed in the different production stages (ARAUJO et al., 2008). Thus, it is fundamental to preserve the genetic diversity of this specie since the sustainability of coffee production depends on this diversity. For the development and release of new cultivars, the existence of germplasm banks is necessary, which are considered the primary source for any breeding program (NASS et al., 2008). Genetic diversity is essential for breeding programs to

seek genotypes that are more adapted, resistant to pests and diseases, tolerant to environmental stress, and capable of increasing productivity (DULLOO et al., 2009).

## 2.2 Characteristics of seed recalcitrance in the *Coffea* genus

Seeds are the most common form of conservation for cultivated species. However, the low longevity of coffee seeds is a limiting factor for the long-term conservation of its variability (DUSSERT et al., 2012), as *Coffea* seeds are recalcitrant and intermediate, making storage in conventional seed banks unviable (ELLIS; HONG; ROBERTS, 1990; ABDELNOUR-ESQUIVEL et al., 1992; DUSSERT et al., 1997).

*Ex situ* conservation is how *Coffea* are currently being conserved, meaning they are live plants in germplasm collections in the field. This type of conservation is not considered ideal because it requires large land areas, maintenance costs are high, and the plants conserved in the field are vulnerable to disease, pathogens, and climate and environmental disasters, which can destroy the entire collection (EIRA; REIS; RIBEIRO, 2005; DUSSERT et al., 2012)

For orthodox seeds, a viable alternative for conserving plant germplasm is in seed banks at temperatures around -18°C (WALTERS, 2015). However, recalcitrant seeds are sensitive to desiccation and storage at negative temperatures, requiring alternative methods for long-term conservation (BARBEDO; CENTENO; RIBEIRO, 2013).

One possibility for the safe storage of coffee genetic resources for an extended period is cryopreservation at -196°C (DUSSERT; ENGELMANN, 2006; TRIGIANO; GRAY, 2011), but the technique still needs adjustments. So far, there are certain questions regarding the behavior of coffee seeds about tolerance to desiccation, cooling in liquid nitrogen (LN) and warming after cryopreservation, which are pertinent and should be investigated.

## 2.3 Drying

Prior to immersion and contact with liquid nitrogen, the seeds must undergo drying, and this process must be carried out in such a way as to avoid the formation of ice crystals in the seeds, which can lead to cell death (PAMMENTER; BERKAK, 2014).

Seed longevity is closely related to their tolerance to desiccation (DEKKERS et al., 2015). Drying reduces cellular metabolism, which increases seed longevity during storage. However,

some seed species are intolerant to desiccation, and thus specific technologies for long-term conservation of plant germplasm are necessary (KOHOMA et al., 2006).

Tolerance to desiccation is acquired at the end of the maturation phase and can vary among species and even among seeds from the same lot (PAMMENTER; BERKAK, 2014). Drying removes the remaining water present in the seeds (MARCOS FILHO, 2015), and the speed of water removal can affect the type of damage caused, the degree of desiccation tolerance, and viability, especially for seeds with recalcitrant characteristics (ROSA et al., 2005; JOSÉ et al., 2011; COELHO et al., 2015). Generally, desiccation-sensitive seeds such as coffee seeds can survive lower water contents if subjected to faster drying, avoiding the accumulation of damage during the process (ROSA et al., 2005; COELHO et al., 2015). On the other hand, other authors claim that slow drying increases the tolerance of coffee seeds to desiccation due to the longer time for induction of their protective mechanisms (VEIGA et al., 2007; VIEIRA et al., 2007; SANTOS; VON PINHO;ROSA, 2013; ABREU et al., 2014).

## 2.4 Cryopreservation

*In vitro* conservation through cryopreservation is the most suitable technique for preserving plant genetic resources for species that produce recalcitrant or intermediate seeds, such as those in the *Coffea* genus (TRIGIANO; GRAY, 2011; BERJAK; PAMMENTER, 2014). Its main advantages are safety, stability, do not require a lot of space and maintenance requirements, possibility of long-term storage, and lower cost per adoption (ENGELMANN, 2004; DULLOO et al., 2009; CHEN et al., 2011), and furthermore, there is no need for any electrical system (RAO, 2004).Besides it is safe method for conserving plant species that are not physiologically capable of remaining viable in conventional germplasm banks at -18°C or -20°C (JENDEREK; REED, 2017).

It involves the conservation of biological material in liquid nitrogen at -196ºC, or in its vapor phase at -150ºC, under which metabolic reactions do not occur, ensuring the viability of the stored material without any modifications or genetic alterations for an indefinite period (KARTHA, 1985; SANTOS, 2004).

Studies conducted in recent decades have resulted in significant advances in the cryopreservation of plant cells and systems.

Cryopreservation varies depending on the biological material to be used, the species, and even among cultivars of the same species (PANIS; SWENNEN; ENGELMAN, 2001; SANT et al., 2008). The need for adjusting the technique for each material is crucial for success, in addition to the tissue's ability to survive dehydration and exposure to ultra-low temperatures (BERJAK; PAMMENTER, 2007). That is the reason it is difficult to generalize and develop a universal protocol (REED, 2017; VOLLMER et al., 2017).

In the case of coffee, seeds have been the primary choice as explants for cryopreservation (DUSSERT, et al., 2002). However, within the *Coffea* genus, species with varying degrees of tolerance to desiccation and cryopreservation are found, and due to the rapid loss of physiological viability of the seeds, it is necessary that the batch used has high initial quality (DUSSERT et al., 1997; DUSSERT; ENGELMANN, 2006).

Drying, cooling, and warming are crucial steps that influence the success of cryopreservation and are related to the formation of intracellular ice crystals (SANTOS, 2000).

Therefore, the major challenge of the technique is to avoid the formation of intracellular ice crystals, which can cause the destruction of cell membranes (PANIS; PIETTE; SWENNEN, 2005). This is because water, when frozen, forms irregular and pointed structures that, depending on the extent, puncture the membrane and cell wall, causing cell death (WESLEY-SMITH et al., 2015).

Ideally, inducing vitrification during cryopreservation would be preferable because, in the vitreous state, the viscosity is extremely high, and molecular mobility is extremely low (ENGELMANN, 2004). The vitreous state will prevent the growth of ice crystals (WESLEY-SMITH et al., 2014). The low molecular capacity of the vitreous matrix will also impose a stasis in the generation of free radicals (BERJAK; PAMMENTER, 2007).

Nucleation is the first step in ice formation, and this process will define the characteristics of ice crystals and can be divided into two forms: homogeneous nucleation and heterogeneous nucleation (GONZALEZ-ARNAO et al., 2008). Homogeneous nucleation starts from the clustering of individual water molecules, and thus, the process will occur at -40 °C in pure water. For this reason, biological solutions will never undergo the process of homogeneous nucleation (WILSON; HENEGHAN; HAYMET, 2003). As pure water is cooled, the mobility of individual water molecules is reduced, decreasing the probability of an ice crystal growing. Meanwhile, other substances or particles (COGER; TONER, 2000) and irregularities in the water (as

predicted by intracellular surfaces) provide nucleation sites where ice crystals can form and grow, leading to heterogeneous nucleation, a process that occurs at temperatures above -40 °C (MAZUR, 2004). Thus, the cooling temperature will always vary depending on the volume of the solution and the osmotic concentration (WILSON; HENEGHAN; HAYMET, 2003).

It is likely that the formation of ice crystals in biological tissues occurs through heterogeneous nucleation, and the cooling of hydrated cells will begin at around -0°C (MAZUR, 2004; WESLEY-SMITH et al., 2014). Although homogeneous nucleation occurs at -40 °C, at temperatures below -80 °C, molecular mobility is further reduced, and the ice crystal does not form (GONZALEZ-ARNAO et al., 2008). Thus, in the temperature range between 0 to -80 °C, ice crystal formation and growth are possible.

The longer the tissue resides in this temperature range, the more likely damage due to ice crystals will occur. This is why in most cryopreservation protocols for complex multicellular tissues, such as seeds, cooling needs to be very fast, in the range of -200°C/minute (BERJAK; PAMMENTER, 2007). Similarly, in the same temperature range of 0 to -80 °C, during warming, the vitreous state of water can convert to the crystalline state, generating physical damage associated with ice crystals (BERJAK; PAMMENTER, 2007). As with cooling, the longer the tissue is exposed during the warming process, the higher the probability of ice crystal formation, and rapid warming is as important as rapid cooling (BERJAK; PAMMENTER, 2007; WESLEY-SMITH et al., 2014).

In complex structures, such as seeds, drying is one of the most important and critical processes in cryopreservation. Water present in the cells during cooling needs to be adjusted with the minimum amount of freezable water possible. Additionally, the speed at which water is removed influences the final quality of the seeds since recalcitrant species are sensitive to desiccation (COELHO, 2018).

Performing some evaluations can help understand the damages caused by different stages of cryopreservation. Physiological evaluation of seeds allows for the assessment of the extent of quality-related damages, with germination and tetrazolium tests being the most commonly used in coffee seed research (ABREU et al., 2014; CLEMENTE et al., 2011; COELHO et al., 2015). The electrical conductivity test in seeds indicates possible damage to the membrane systems through the analysis of solute leaching and is also widely used in coffee seeds (PRETE, 1992; MALTA; PEREIRA; CHAGAS, 2005; REINATO et al., 2007).

Biochemical, ultrastructural, and gene expression evaluations are also important to clarify the effects of cellular damage caused by different stresses. Therefore, research with coffee seeds usually involves biochemical (ABREU et al., 2014; SANTOS; VON PINHO; ROSA, 2013; SAATH et al., 2014) and ultrastructural (BORÉM; MARQUES; ALVES, 2008; BORÉM et al., 2013; SAATH et al., 2010) studies of the effects of drying, with cryopreservation effects being uncommon. Ultrastructural studies of recalcitrant and orthodox seeds have contributed to understanding different responses to dehydration and cooling, which can lead to quality loss by damaging organelle membrane systems, cytoskeleton, and skeletal nucleus (BERJAK; PAMMANTER, 2000, COELHO et al., 2015, FIGUEIREDO et al., 2021). There are few studies relating gene expression of coffee seeds and cryopreservation (VELOSO, 2017).

In the results of research conducted with coffee seeds subjected to cryopreservation (DUSSERT et al., 1997; 2001; 2012; DUSSERT; ENGELMANN, 2006, COEHO et al., 2015, FIGUEIREDO et al., 2017; 2021), low percentages of germination or formation of normal seedlings are observed after exposure to liquid nitrogen. Therefore, for cryopreservation to be successful, it is necessary to consider and study the effects that the cryopreservation process steps, i.e., drying, cooling, and warming can cause to the seeds.

According to Figueiredo et al. (2021), for the cryopreservation of *Coffea arabica* L, the ideal moisture content is 17% (wb), as with 17% moisture content, the seeds show better physiological quality and more preserved cellular structures. The authors tested different warming times at 40°C and (2, 4, and 6 minutes) and after warming used cathodic water (as an antioxidant treatment) and concluded that the tested warming times and the use of cathodic water do not alter the physiological, biochemical, and structural quality of cryopreserved *Coffea arabica* L seeds.

For the *Coffea canephora* species, Coelho et al. (2018) concluded that the seeds show better results when subjected to rapid cooling, i.e., when they are placed directly in liquid nitrogen. Additionally, the authors note that drying up to 20% (wb) does not harm seed viability and in their research, they also observed that although still with low germination percentages, this was the moisture level that provided the best physiological performance.

## 2.5 Seed warming

In order to achieve better results in seed cryopreservation, it is necessary to avoid the formation of ice crystals during the process, which can occur mainly during the cooling and warming stages (MAZUR, 2004).

Therefore, the warming stage is also essential to ensure the success of cryopreservation, as intracellular ice recrystallization can occur during this process and if conducted improperly, may hinder the successful regeneration of seedlingsderived from cryopreserved seeds (BENSON, 2008). The process of ice recrystallization in residual freezable water in tissue will occur between -80 and -40°C if the warming is slow (BERJAK; PAMMENTER, 2014; PAMMENTER; BERJAK, 2014). One way to avoid this recrystallization is to subject the seeds to rapid warming, in a water bath at temperatures between 38°C and 45°C, for one or two minutes for stress-sensitive species (BENSON, 2008; BERJAK; PAMMENTER, 2014).

However, for coffee seeds, the temperature and warming method of cryopreserved materials still need to be systematically investigated (DUSSERT; ENGELMANN, 2006). An important factor to be observed in cooling and warming is that these processes can lead to enzyme denaturation and the generation of reactive oxygen species (ROS) (BENSON; BREMNER, 2004; BERJAK; SERSHEN; PAMMENTER, 2011; ROACH et al., 2008; WHITAKER et al., 2010).

ROS are reduced, transient molecules with high reactivity (MEHDY et al., 1996). They are produced in chloroplasts, mitochondria, and peroxisomes as secondary products of photosynthesis and respiration (APEL; HIRT, 2004). Their occurrence can generate necrotic damage to the apical meristem, decrease production, and impair root development, especially in tropical or subtropical species that produce recalcitrant seeds (BERJAK et al., 2011).

Coelho et al. (2019), working with *Coffea canephora*, followed a warming protocol in a water bath for 2 minutes at 40°C (DUSSERT et al., 1998) and achieved 43% survival of cryopreserved seeds.

## 2.6 Gene expression

Through the rapid advancement in biotechnology, expectations increase regarding work that expands the knowledge of regulatory pathways and genes that command the tolerance of seeds and explants to drying and low temperatures (VELOSO, 2017).

Several studies that depict the impact of abiotic stresses on plants use quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) as a tool to understand the mechanism of plant stress tolerance. This is because, in addition to being a predominant method for gene expression assays, RT-qPCR has high specificity, precision, efficiency, and sensitivity (JIANG et al., 2012).

Real-time PCR monitoring (RT-qPCR) revolutionized the quantification of DNA and RNA fragments, as it allows for the precise quantification of these nucleic acids in real-time with greater reproducibility by quantifying values during the exponential phase of the reaction. The accumulation of PCR products is detected and monitored directly by the fluorescence increase of the used fluorophore, unlike traditional PCR, where products must be analyzed by electrophoresis afterwards (VARGAS, 2014).

The main principle in the use of this method is the so-called cycle threshold (Ct). The Ct can be defined as the PCR cycle in which the fluorescent signal of the reporter dye passes through an arbitrary threshold line. The presentation of data in the form of Ct should ensure that the threshold is delimited in the exponential phase of amplification, that is, above the background noise and below the plateau phase of the PCR reaction. The Ct value is inversely proportional to the initial amount of the transcript of interest in the reaction, which indicates that the lower the Ct value, the greater the initial amount of the transcript in the sample (SCHMITTGEN; LIVAK, 2008).

RT-qPCR requires essential components for proper functioning, including a thermocycler with an optical system for fluorescence excitation and a computer with software for data acquisition and final analysis of the reaction (AMBION, 2019).

Absolute and relative quantification are the two existing ways to analyze results obtained in studies that use RT-qPCR. Absolute quantification is represented by the quantity of copies of the transcript of interest, used when there is a need to know the quantity of amplicons, such as in viral load calculations, while in relative quantification, there is a change in the expression of the target gene relative to some reference group such as a control that did not receive treatment or a sample at time zero, for example (SUGDEN; WINTER, 2008).

Statistical analysis methods have been developed to classify the best reference genes for a given organism or experimental condition. This can be done by comparing the expression of the gene of interest under different conditions with reference genes whose expressions are stable under various experimental conditions. These include *geNorm* (VANDESOMPELE et al., 2002), *NormFinder* (ANDERSEN; JENSEN; ORNTOFT, 2004), *BestKeeper* (PFAFFL et al., 2004), comparative ΔCt method (SILVER et al., 2006), and the *Refinder* software (XIE et al., 2011).

Performing tests to choose the appropriate reference gene for normalizing RT-qPCR data is extremely important for relative analysis of gene expression, as the use of reference genes without prior analysis of their expression and stability can lead to incorrect data interpretation and may influence results (SILVEIRA et al., 2009).

There are not many studies in the literature aimed at understanding how genes that are responsible for molecular changes in plants exposed to adverse conditions of low temperatures and desiccation act, with a view to cryopreservation of the material. In this regard, Harding (2004) stated that this area of study requires investment in basic and applied research. The few genetic studies related to cryopreservation commonly investigate the expression of genes related to protection against oxidative damage from lipid peroxidation that occur during desiccation and rehydration processes (DUSSERT et al., 2006; VOLK, 2010).

Performing molecular evaluations of coffee (*C. arabica*) for cryopreservation, Veloso (2017) concluded that the expression of the studied genes varies according to the applied methodologies (drying, cooling, and warming time), and that the telomerase, apoptosis (APOP), DNA-methylase (DNA-met), ascorbate peroxidase (APX), superoxide dismutase (SOD), and peroxidase (POX) genes showed similar expression patterns, suggesting joint action in DNA repair and removal of ROS.

Therefore, due to the scarcity of studies elucidating what happens during the stages of cryopreservation and seed warming, it is important to continue studying genes associated with damage at each stage of the process for greater understanding.

### 2.7 Genes related to oxidative stress

For seed germination to occur, efficient sources of energy are needed, which are provided by lipids, which also have a reserve and structural function. Lipases hydrolyze triglycerides, generating fatty acids and glycerol, some of which are converted into sugar, thus releasing energy

for germination to occur. Lipids are primary constituents of cell membranes, and their arrangement directly affects the physiological processes of seeds (MARCOS-FILHO, 2015).

One of the main causes of seed deterioration is lipid peroxidation, where free radicals, hydroperoxides, and secondary products are produced through the action of oxidative enzymes such as lipoxygenases. This process affects membrane phospholipids and stored lipids and damages cell membranes and other cellular compounds. Lipid autoxidation is common in all cells but is predominant in seeds with low water content, around 6%. The mechanism of lipid peroxidation is also dependent on the water content of the seeds. In seeds with water content between 6 to 14%, lipid peroxidation is very low, while in seeds with water content close to or above 14%, lipid peroxidation is much more significant and is stimulated by hydrolytic enzymes such as lipoxygenases (McDONALD, 1999; MARCOS-FILHO, 2015).

Lipid peroxidation is harmful to seeds because it leads to the formation of free radicals, hydroperoxides, and various secondary products. Conversely, seeds can be protected by enzymatic mechanisms, as well as by the action of antioxidants. Thus, enzymatic mechanisms are capable of reducing or even neutralizing the harmful effects.

Various environmental conditions cause stress in seeds and negatively affect metabolism, growth and development, as well as causing their deterioration and death when exposed for long periods to such conditions. These stresses can be of biotic nature, such as when exposed to pathogens, or abiotic, such as water restriction, salinity, high or low temperatures, flooding, and drying (WANG et al., 2012).

In addition to biotic and abiotic stresses, physiological stress conditions as well as photosynthesis and respiration lead to the production of reactive oxygen species (ROS). The production of ROS can occur during seed desiccation, germination, and aging, leading to oxidative stress and cellular damage (WANG et al., 2012).

ROS cause molecular damage, especially to nucleic acids, proteins, and lipids, compromising metabolism and cell membrane integrity (SMITH et al., 2009). They are by-products of redox reactions (KOVALCHUK, 2010), appearing as either free radicals or molecules. The most well-known free radicals are superoxide anion ($O_{2}^{-}$), hydroxyl radical (OH), and hydroperoxyl radical ($HO_2$), while singlet oxygen ($O_2$) and hydrogen peroxide ($H_2O_2$) are the main examples of molecules that act as ROS (D'AUTRÉAUX; TOLEDANO, 2007; BHATTACHARJEE, 2010).

Oxidative stress occurs when there is an imbalance between ROS production and elimination (KIM; KWAK, 2010). In general, there is a balance between the generation and elimination of ROS, but when subjected to some stressful situation, an imbalance occurs, resulting in their accumulation. In response to excessive ROS production, plants have developed defense mechanisms to remove or minimize their harmful effects (CHOUDHARY; KUMAR; KAUR, 2019).

These mechanisms have enzymatic and non-enzymatic nature responsible for the elimination of ROS, both produced as a result of normal metabolism and resulting from any stress situation, and are found in various cellular compartments in plants (APEL; HIRT, 2004).

Among the antioxidant enzymes, superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GSH), peroxidases (POX), catalase (CAT), and polyphenol oxidase (PPO) stand out. The main antioxidant metabolites include ascorbic acid (AsA), glutathione (GSH), α-tocopherol, and carotenoids. All of these occur in chloroplasts, mitochondria, and peroxisomes (MITTLER, 2002; KIM & KWAK, 2010; DINAKAR et al., 2012).

# 3 MATERIALS AND METHODS

## 3.1 Harvesting and processing of fruits

Fruits from *Coffea canephora* Pierre, Apoatã cultivar, were harvested in the crops of the Varginha Experimental Farm - Procafé Foundation (Integrated Program for Coffee Technology Support) during the 2020/2021 harvest season. The fruits were selectively harvested at the cherry ripening stage from the middle branches of the plants and from the middle parts of these branches.

After harvesting, the fruits were selected to standardize the ripening stage and mechanically hulled. The seeds were then demucilaged by fermentation in water for 48 hours at room temperature and subsequently pre-dried in the shade to remove surface moisture.Before drying, the seeds were classified in sieves to standardize the size used.

The seeds were then subjected to initial moisture content determination by the laboratory oven method at 105°C for 24 hours(BRASIL, 2009), initial physiological quality evaluation through the germination test (BRASIL, 2009), and seed viability assessment in the tetrazolium test (CLEMENTE et al., 2011).

## 3.2 Drying and cryopreservation of seeds

The seeds were dried to a moisture content of 20% wb (wet basis), according to Coelho et al. (2019). The author observed that a moisture content of 20% (wb) (which corresponds to 0.25 g.g-1 db) allowed for greater survival of *Coffea canephora* seeds, as well as zygotic embryos after cryopreservation.

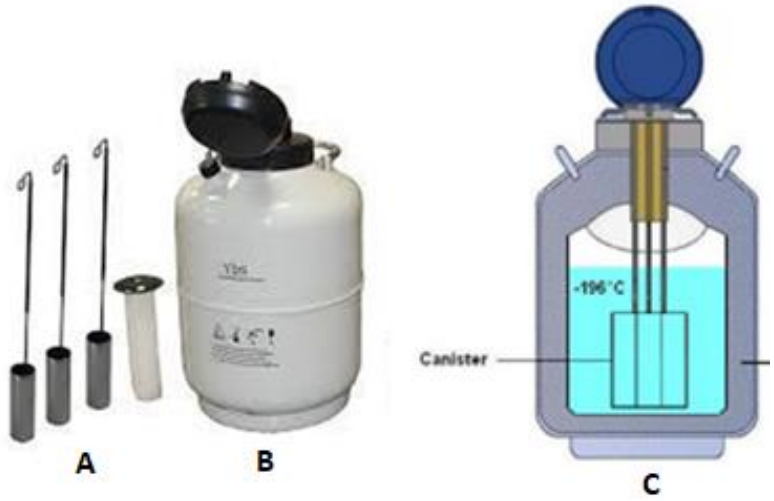
Two drying methods were tested, using activated silica gel and a stationary dryer. For the silica gel drying, the seeds were placed in a single layer on the metal screens of acrylic gerbox type boxes containing 80 grams of activated silica gel inside and below the screens. During the drying process, the silica gel was changed daily at the same time to regenerate its drying power.

The stationary dryer consists of blowing heated air through a static volume of seeds. Operational care was taken to prevent excessive drying of the seed layer near the hot air inlet and delay drying of the more distant layers.

After reaching the ideal moisture content of 20% (wb), and before immersion in liquid nitrogen, the seeds were subjected to physiological tests to evaluate seed quality before cryopreservation.

After drying, five hundreds of seeds with parchmentwere packaged in mesh bags and directly immersed in a tank containing liquid nitrogen, which provides ultra-rapid cooling at a speed of approximately -200°C/minute (DUSSERT et al., 2001) (FIGURE 1).

Figure 1 - Illustration of the cryopreservation system: stainless steel canister (A); cryogenic tank with liquid nitrogen at -196°C (B) and cut of the cryogenic tank with liquid nitrogen at -196°C (C).




Source: By the author (2023).

For warming, the seeds were quickly removed from their respective packages and immersed directly in a water bath at the temperatures and times presented in Table 1.

Subsequently, the seeds were dried on paper towels to remove surface water and had their parchment removed manually to be subjected to physiological, biochemical, ultrastructural, and molecular evaluation.

Table 1 - Temperatures and times tested in the warming of *Coffea canephora* Pierre seeds.

| Warming | Temperatures (ºC) | Time (min) |
|---|---|---|
| Water Bath | 40 | 1, 2, 3 |
| | 45 | 1, 2, 3 |
| | 50 | 1, 2, 3 |
| | 55 | 1, 2, 3 |
| | 60 | 1, 2, 3 |
| | 70 | 1, 2, 3 |

Source: By the author (2023).

### 3.3 Seed moisture content determination

The seed moisture content was determined by the laboratory oven method at 105°C for 24 hours (BRASIL, 2009), with two replicates of 10 seeds. Results were expressed as a percentage based on the wet weight of the seeds.

### 3.4 Physiological evaluations

The physiological evaluation of control seeds, i.e., moist seeds without undergoing any treatment, as well as seeds dried to a water content of 20% (wb) and cryopreserved seeds were carried out.

The germination test was performed with four replicates of 25 seeds for each sample, sowed on germination paper moistened with distilled water equivalent to 2.5 times the weight of the dry paper. The seeds were placed in a seed germinator at a constant temperature of 30°C under light(BRASIL, 2009).Were determined the percentage of radicle emergence at 15 days and the percentage of normal seedlings (presence of the primary root and at least two lateral roots) at 30 days after sowing. The percentage of strong normal seedlings were determined at 30 days (seedlings that exhibited hypocotyls of three centimeters or more) and the percentage of seedlings with expanded cotyledonary leaves at 45 days after sowing were also determined (FRANÇA NETO; KRZYZANOWSKI; COSTA, 1999).

For the tetrazolium test, four replicates of 10 seeds were used, which were soaked in distilled water for 48 hours at 30ºC (CLEMENTE et al., 2011). After soaking, the embryos were removed with a scalpel, avoiding damage to them. After extraction, the embryos were maintained

in a polyvinylpyrrolidone (PVP) antioxidant solution up to the time of immersion in 0.5% tetrazolium solution. The samples were maintained in dark containers at a temperature of 30 °C for 3 hours (Brasil, 2009). Analysis of the viability of the embryos was performed with the aid of a stereoscopic magnifier according to the location and extent of the damage after a longitudinal cut in the embryonic axis (BRASIL, 2009).

The mass electrical conductivity test was conducted using the bulk system, with four replicates of 25 seeds, according to the methodology of Krzyzanowsky; França Neto; Henning (1991) with modifications. The seeds were weighed and and were then immersed in 37.5 mL of distilled water. After 24 hours of soaking at a temperature of 25ºC, the electrical conductivity was determined using a conductivity meter and the results were expressed in $\mu S\ cm^{-1}\ g^{-1}$.

### 3.5 Molecular and isoenzyme evaluation

#### 3.5.1 Selection of treatments and evaluated genes

For the molecular analyses, the following treatments were selected: seeds dried in silica gel and in a dryer up to 20% (wb) before cryopreservation, and samples that were warmed for 2 minutes, as detailed in Table 2.

Table 2 - Selection of cryopreserved seeds for molecular analysis, based on drying methods and warming temperatures and times.

| | **Drying Method** | **Warming Conditions** | |
|---|---|---|---|
| | | **Temperature (**ºC) | **Time (min)** |
| **Waterbath** | Silica/Dryer | 40 | 2 |
| | Silica/Dryer | 50 | |
| | Silica/Dryer | 60 | |

Source: By the author (2023).

The treatments were chosen based on the results of physiological tests and since the warming treatment at 50°C for 2 min showed superior performance compared to others, it was selected as the calibrator and the other treatments were selected for a better understanding of the effects of each drying and warming method.

Regarding the genes to be studied, two genes related to oxidative stress, lipoxygenase and superoxide dismutase (SOD), were selected. The superoxide dismutase (SOD) gene was chosen

because it acts in the elimination of ROS during oxidative stress caused by desiccation (FRANCA; PANEK; ELEUTHERIO, 2007) and low temperatures (BUDIARTO, 2009), both factors studied in this work. Lipoxygenase has been chosen because in many times it is present after lipid peroxidation, which occurs following damage to the seeds.

Table 3 - Identification and sequence of amplification primers used in molecular analyses.

| **Identification** | **Sequence** |
|---|---|
| Genes related to oxidative stress | |
| Lipoxygenase | F- TTCCGAATAGAACCGTTTGG<br>R- GGATGCGGAGTACCTGAAAG |
| Superoxide Dismutase (SOD) | F- AAGGGTTCTTTAGGCTGGGC<br>R- AAGCCACACCCAACCAGATC |

Source: By the author (2023).

The Primer 3 Plus software (UNTERGASSER et al., 2007) was used for designing the primers, to verify the annealing temperature (58-60°C), the amplified fragment size (80-150 bp), the value of variation of Gibbs free energy (positive value), and the harpin formation temperature (preferably negative), using the OligoAnalyser 3.1 software (INTEGRATED DNA TECHNOLOGIES, 2014).

### 3.5.2 RNA extraction

RNA extraction was performed using the protocol suggested by Onate-Sánchez and Vicente Carbajosa (2003) with lithium chloride. Samples of 10 seeds that underwent different methodologies for cryopreservation and were stored in a deep freezer at -86°C until they were lyophilized and sent to Iowa State University, where they were ground in an electric grinder with liquid nitrogen until a fine powder was obtained.

To evaluate the integrity of the extracted material, samples were quantified in a spectrophotometer (Thermo Scientific 2000 1-position Spectrophotometer), and the 260 nm/280 nm reading ratio was between 1.8 and 2.1.After extraction, the samples were ready for use in reactions since the Go-Taq 1-Step RTqPCR System Master Mix (Promega) was used.

### 3.5.3 Standardization of RT-qPCR

In order to determine primer efficiency, PCR were performed using the primer at a concentration of 10 pM to amplify different concentrations of RNA samples. The objective was to obtain the standard curve estimate based on the Ct values and, from this curve, determine the slope and $R^2$ values. Primer pair efficiency was calculated using the following expression:

$$\text{Efficiency} = [10(\frac{-1}{\text{Slope}})]^{-1}$$

The RNA concentration used was 40 ng/µl in the reactions and the normalizing gene used was Actin, which had already been used in studies with the same objective. Table 4 lists the identification and sequence of the primer used as a reference or normalizing gene.

Table 4 - Identification and sequence of the primer used as a reference gene.

| Identification | Sequence |
|---|---|
| Actin | F- TCAGCACATTCCAGCAGATG<br>R- TAAAAGCTCACCACCCCAAG |

Source: By the author (2023).

### 3.5.4 RT-qPCR

The RT-qPCR were performed using the GoTaq® Probe 1-Step RT-qPCR System (Promega) Master Mix Kit in a final volume of 15 µl.

The reaction conditions were: 1 cycle at 37 °C for 15 minutes and 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 10 seconds, 56 ºC for 30 seconds, and 72 ºC for 30 seconds. Fluorescence readings were taken using the Agilent Stratagene Mx3000P system thermocycler at each amplification cycle and subsequently analyzed with the software of the same equipment.

The result was expressed as Ct value. All samples were analyzed in triplicate, and a control without RNA was included for each primer pair to check for contamination.

### 3.5.5 Isoenzymes

For biochemical analysis, through the expression of isoenzymes by the electrophoresis technique, the seeds were macerated in liquid nitrogen, in the presence of polyvinylpyrrolidone (PVP) and the samples were stored at -86°C (deep freezer) until the moment of analysis. The methodology used was proposed by Alfenas et al. (2006) for extraction, electrophoretic run, and revelation of peroxidase (PO), glutamate oxaloacetate transaminase (GOT), esterase (EST), and catalase (CAT) enzymes involved in oxidation, respiration, and lipid peroxidation pathways.

## 3.6 Ultrastructural evaluation by scanning electron microscopy

The analysis of scanning electron microscopy (SEM) was carried out by sectioning coffee seeds stored in modified Karnovsky fixative solution (25% glutaraldehyde, 10% formaldehyde in 0.2 M sodium cacodylate buffer, pH 7.2) and stored in a refrigerator at 4ºC until preparation for SEM. For preparation, the materials (sections of endosperms) were washed in 0.05 M cacodylate buffer (three times for 10 min), post-fixed in 1% osmium tetroxide for one hour, and subsequently dehydrated in an ascending series of acetone concentrations (30, 50, 70, 90, and 100% three times). The final dehydration was carried out in a critical point dryer (BAL-TEC CPD 030).

The endosperm sections were mounted on aluminum supports, covered with gold in a Sputter device (BAL-TEC SCD 050), and observed in a LEO EVO 40XVP scanning electron microscope.

The images were generated and digitally recorded, using the same magnification for all samples.

## 3.7 Experimental design and data analysis

The experimental design was completely randomized, in a 2x6x3 factorial scheme, with two drying methods (silica and dryer), six warming temperatures in a water bath (40, 45, 50, 55, 60, and 70ºC), and three time periods (1, 2, and 3 minutes).

The results of the physiological tests were subjected to analysis of variance using the SISVAR statistical program (FERREIRA, 2014), and the means were compared by the Scott-

Knott test. Comparisons were also made between each treatment resulting from the 2x3x6 factorial and the control treatment, using the *Student*'s t-test at a 5% probability level.

For the biochemical analyses, the interpretation of the results obtained for each isoenzyme was performed by considering the presence/absence and intensity of bands in gels. The images of ultrastructural analyses, through electron microscopy, were also visually analyzed and interpreted.

# 4 RESULTS AND DISCUSSION

## 4.1 Physiological analyses of cryopreserved seeds

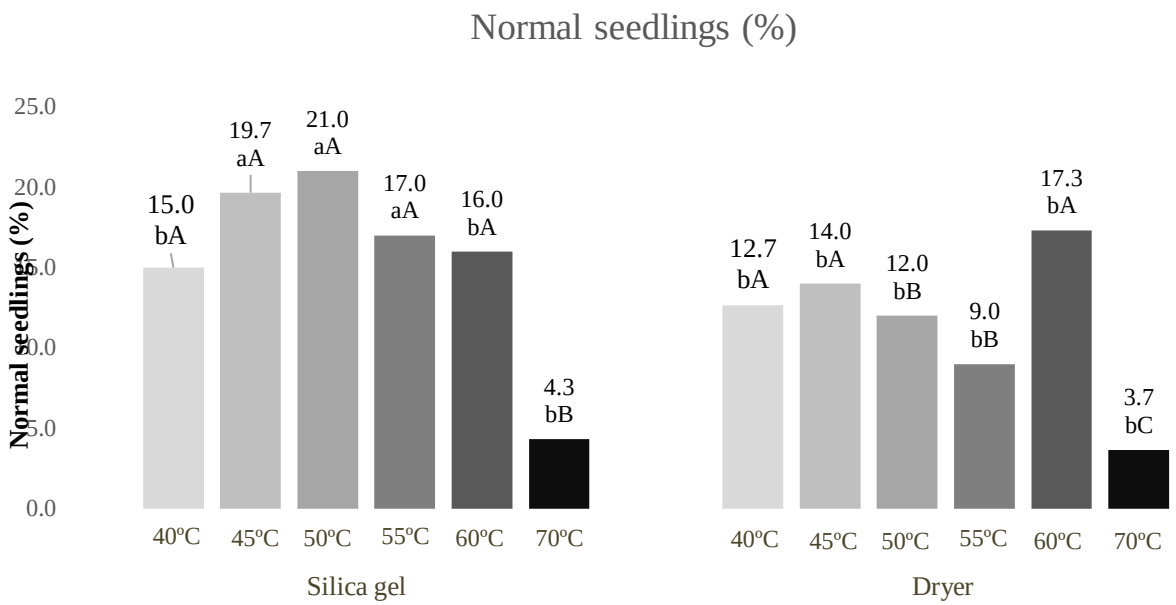
The freshly harvested seeds with 31% moisture content were evaluated for their initial quality and showed average values of 83% germination and 90% viability in the tetrazolium test (data not shown).

Prior to exposure to cryopreservation and warming, the seeds exhibited high quality, which was also observed by Coelho et al. (2019) in their study of different drying methods and moisture contents in *Coffea canephora* seeds after immersion in liquid nitrogen.

Following the warming step, a variation of 29% to 41% in seed moisture content was observed, depending on the different warming treatments. According to the analysis of variance of the physiological evaluation results (Appendix A), there was a triple interaction of factors for the electrical conductivity variable; a double interaction of temperature and warming time for seed viability and strong normal seedlings; and a double interactions between drying method and warming temperature for germination, as well as a double interaction between temperature and warming time. For the root emergence variable, there were no significant effects of factors or interactions.

The results of the percentage of normal seedlings in the germination test as affected by drying and warming temperature are presented in Figure 2. At temperatures of 40, 60, and 70°C, there were no significant differences between drying methods. However, at temperatures of 45, 50, and 55°C, drying with silica gel was more efficient than drying in a dryer (FIGURE 2).

Figure 2 - Normal seedlings from*Coffea canephora* Pierre seeds following cryopreservation, after drying with silica gel orin a dryer, and then warming at different temperatures.




Means followed by the same letter do not differ from each other by the Scott-Knott test at a 5% probability level. Lowercase letters compare the drying methods within the same temperature, and uppercase letters compare the temperatures within the same drying method.

Source: By the author (2023).

In silica gel drying, the warming temperature of 70ºC resulted in the lowest germination rate, while other temperatures did not show significant differences. In dryer drying, the temperature of 70ºC was also the most harmful, while the best drying temperatures were 40ºC, 45ºC, and 60ºC. Generally, silica gel drying provides a higher percentage of normal seedlings after cryopreservation compared to dryer drying, making the latter less suitable for *Coffea canephora* seed cryopreservation (FIGURE 2).

These results corroborate those of Coelho et al. (2019), who observed superior seed quality after cryopreservation with silica gel drying compared to drying with saturated NaCl solution in *Coffea canephora* seeds. Figueiredo et al. (2021) also found better physiological quality in cryopreserved *Coffea arabica* seeds after silica gel drying compared to drying with saturated $(NH_4)_2SO_4$ solution and saturated NaCl solution. Therefore, the manner of drying has an impact on the quality and vigor of cryopreserved seeds.

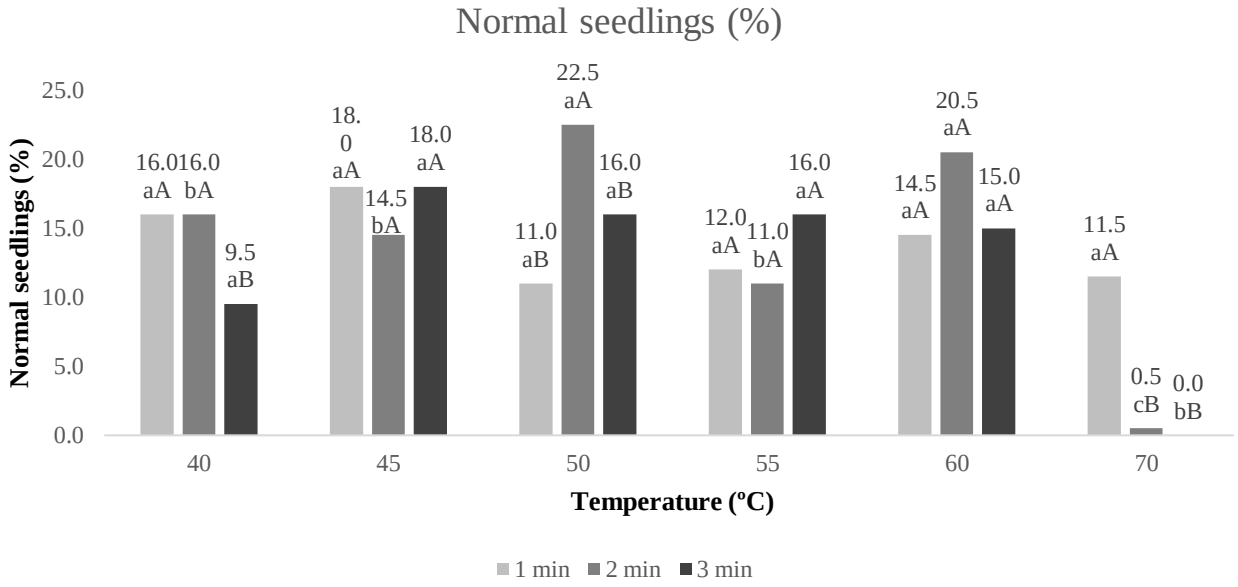
In contrast to other coffee seed cryopreservation studies (FIGUEIREDO et al., 2021; COELHO et al., 2017), the present study found that seeds dried more quickly in a dryer exhibited inferior physiological performance compared to those dried with silica gel, which may be

attributed to other factors associated with the drying method.

Seed quality may be affected by differences in theimbibition rate, as the drying process causes disorganization of the cell membrane structure, resulting in water removal. Therefore, during rehydration, careful attention is required to prevent damage caused by rapid water uptake. This damage may occur because membranes may not have enough time to rearrange and convert from a crystalline to a liquid state (FERREIRA; BORGHETTI, 2004), resulting in the release of exudates caused by their rupture.

In dryer drying, the best seed quality was achieved at drying temperatures of 40, 45 and 60ºC, while the temperature of 70ºC was harmful to the seeds, regardless of the drying method used (FIGURE 2). At 1 minute of warming, there was no significant differencefor percentage of normal seedlingsamongwarming temperatures. At 2 minutes, temperatures of 50 and 60ºC had superior results, while the temperature of 70ºC resulted in the worst performance at 2 and 3 minutes (FIGURE 3).

Figure 3 - Normal seedlings from*Coffea canephora* Pierre seeds following warming at different temperatures and for different times after cryopreservation.




Means followed by the same letter do not differ from each other by the Scott-Knott test at a 5% probability level. Lowercase letters compare warming temperatures within the same time period, while uppercase letters compare times within the same warming temperature.

Source: By the author (2023).

Regarding temperature, there was no difference among the warming times in the germination test results at 45, 55, and 60°C (FIGURE 3). At 40°C, 1 and 2 minutes were better,

and at 70°C, 2 and 3 minutes were very harmful. The best combination of temperature and warming time of the seeds after removal from liquid nitrogen was the temperature of 50°C for 2 minutes. It was the most favorable for the formation of normal seedlings, with an average of 22.5% (FIGURE 3).

One of the main problems related to the low survival rate of seeds/explants after cryopreservation is the formation of intracellular ice. Ice crystals can cause damage to cell membranes (STEPONKUS et al., 1993) or even problems associated with compression of cellular constituents (SARAGUSTY et al., 2009), and are thus potentially lethal. Factors such as the cooling/warming rate, the addition of cryoprotectants, and water content in the cell have been studied to minimize this problem (BENSON, 2008; LIU; PHY; YEOMANS, 2012). Results like these may indicate compromised cell integrity, since the occurrence of ice crystals in the apoplast and in the cellular simplast can cause membrane damage (TAIZ et al., 2017).

Dussert et al. (1988) studied the cryopreservation of seeds of some *Coffea* species (*Coffea arabica, C. costatifructa, C. racemosa*, and *C. sessiliflora*) and observed that the best warming temperature for *C. arabica* seeds is 40°C for 2 minutes. Figueiredo et al. (2021) also investigated the physiological, biochemical, and ultrastructural aspects of *Coffea arabica* L. seeds subjected to different cryopreservation protocols and observed a higher percentage of normal seedlings under warming at 40°C for 2 minutes.

After the cryopreservation step, Coelho (2018) warmed *Coffea canephora* seeds for 2 minutes at 40°C and observed 43% seedling survival in the germination test, a survival rate that was not achieved in the presentstudy when the same conditions were applied to the seeds. Therefore, as *C. canephora* is a more recalcitrant species than *C. arabica*, there is still no consensus on the best warming temperature.

The results of strong normal seedlings, a characteristic of seed vigor, showed that drying in a dryer provided a higher percentage of strong normal seedlings, although the values were very low (TABLE 5).

Table 5 –Percentage of strong normal seedlings from*Coffea canephora* Pierre seeds that were cryopreserved after drying with silica gel or in a dryer.

| Drying Methods | Strong normal seedlings (%) |
|---|---|
| **Silica** | 2.78 b |
| **Dryer** | 4.33 a |
| CV (%) 54.46 | |

Means followed by the same lowercase letter in the row do not differ from each other according to the Scott-Knott test at a 5% probability level.

Source: By the author (2023).

Comparing the effects of different temperatures and warming times on the vigor of *Coffea canephora* seeds showed that at the warming time of 1 minute, temperature had no effect on the percentage of strong normal seedlings. At 2 minutes, the temperature of 50ºC provided the best growth of strong normal seedlings, corroborating the result of the germination test. At the 3-minute warming time, the median temperatures of 45, 50, 55, and 60ºC provided for greater growth of strong normal seedlings, while the temperature of 70ºC is not recommended for post-cryopreservation warming of *Coffea canephora* Pierre as it causes seed death (TABLE 6).

Table 6 - Percentage of strong normal seedlings from*Coffea canephora* Pierre seeds cryopreserved and subsequently warmed at different temperatures and for different times.

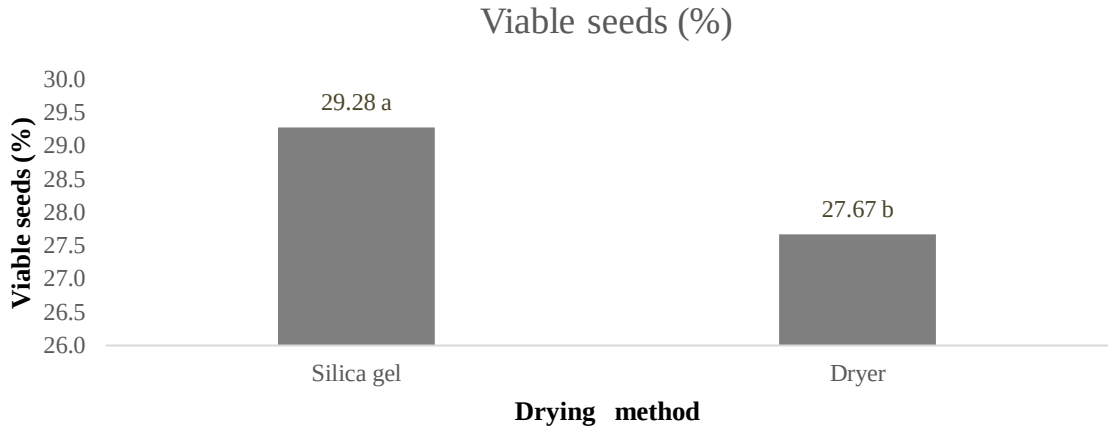
| | **Temperature (ºC)** | | | | | |
|---|---|---|---|---|---|---|
| **Time (min)** | 40 | 45 | 50 | 55 | 60 | 70 |
| **1** | 2.5 aA | 3.0 aA | 3.0 aB | 1.5 aB | 2.5 aA | 3.0 aA |
| **2** | 2.5 bA | 4.0 bA | 8.5 aA | 3.0 bB | 4.0 bA | 0 bA |
| **3** | 1.0 bA | 4.5 aA | 3.5 aB | 7.5 aA | 5.5 aA | 0 bA |
| CV (%) | 54.46 | | | | | |

Means followed by the same lowercase letter in the row and uppercase letter in the column do not differ from each other by the Scott-Knott test at a 5% level of probability.

Source: By the author (2023).

Warming at a temperature of 50°C for 2 minutes and 55°C for 3 minutes were the combinations that provided the best conditions for the development of strong normal coffee seedlings, obtaining higher percentages (TABLE 6). The results of the tetrazolium test show that the percentage of viable cryopreserved seeds is higher when the seeds were dried with silica gel, which corroborates the germination results (FIGURE 4).

Figure 4 - Viability in the tetrazolium test of *Coffea canephora* Pierre seeds cryopreserved after drying with silica gel or in a dryer.

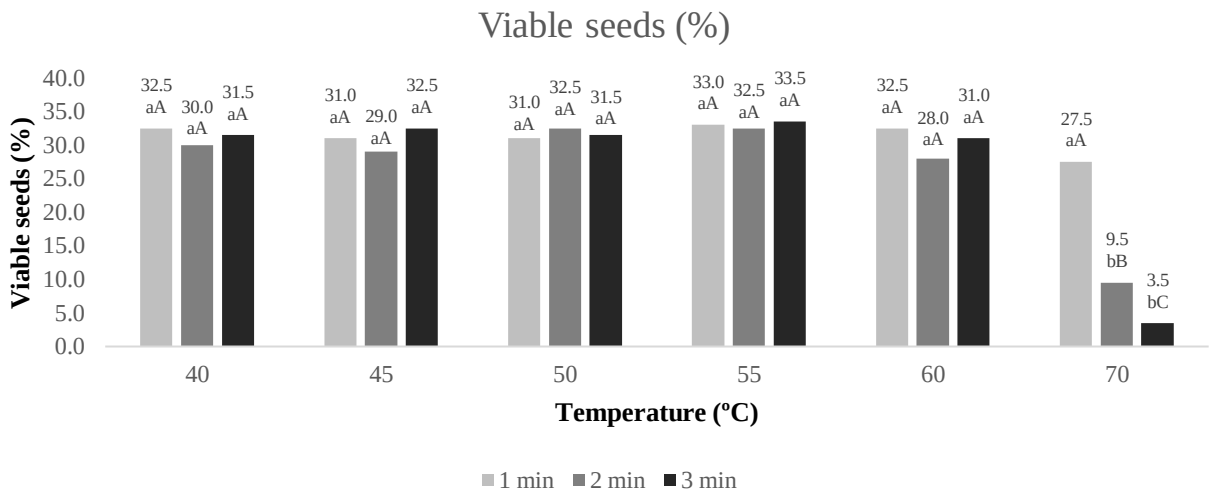



Means followed by the same letter do not differ from each other according to the Scott-Knott test at a 5% probability level.

Source: By the author (2023).

The different temperatures and warming times did not affect seed viability in the tetrazolium test as the means were very close to each other, except at the temperature of 70ºC, where low physiological quality was observed, especially for the warming times of 2 and 3 minutes (FIGURE 5).

Figure 5 - Viability in the tetrazolium test of *Coffea canephora* Pierre seeds cryopreserved and warmed at different temperatures and for different times.




Means followed by the same letter do not differ from each other by the Scott-Knott test at 5% probability. Lowercase letters compare temperatures within the same time, and uppercase letters compare times within the same temperature.

Source: By the author (2023).

Dussert et al. (2001), studying the tolerance of *Coffea* species to cryopreservation techniques, reported that regardless of the seed water content or cooling rate, *Coffea canephora* seeds are not able to germinate after being immersed in liquid nitrogen. However, the authors concluded that the damage occurs in the endosperms, since the embryos survive when extracted from these seeds.

Comparing the results of the germination test (FIGURE 3) with those of the tetrazolium test (FIGURE 5), a discrepancy in the results can be observed, with the percentage of viable seeds being higher than the percentage of normal seedlings. In the tetrazolium test, there was no significant effect of seed exposure to the different cryopreservation protocols, unlike what was observed in the germination results.

Figueiredo et al. (2016) also observed greater sensitivity of the endosperms compared to the embryos extracted from these seeds in relation to stresses caused in the different stages of the *Coffea arabica* seed cryopreservation process. Dussert; Engelmann (2006) and Coelho et al. (2015) also found high viability in *Coffea arabica* L. seeds that had extremely low germination percentages.

A triple interaction of the drying, warming temperature, and warming time factors was observed in the electrical conductivity data of the *Coffea canephora* Pierre cryopreserved seeds (TABLES 7 and 8).

Table 7 - Electrical conductivity (µS $cm^{-1}$ $g^{-1}$) of *Coffea canephora* Pierre seeds cryopreserved after drying with silica gel or in a dryer and warmed at different temperaturesand for different times.

| **Warming temperature (ºC)** | **Water bath warming time (min)** | | | | | |
|---|---|---|---|---|---|---|
| | **1** | | **2** | | **3** | |
| | **Silica gel** | **Dryer** | **Silica gel** | **Dryer** | **Silica gel** | **Dryer** |
| **40** | 35.01 a | 28.19 a | 27.15 a | 32.23 a | 33.33 a | 40.51 a |
| **45** | 45.90 a | 36.75 a | 37.42 a | 33.71 a | 31.90 a | 50.39 a |
| **50** | 45.47 a | 35.50 a | 37.41 a | 64.92 b | 36.67 a | 31.51 a |
| **55** | 30.14 a | 40.81 a | 32.46 a | 52.80 a | 34.66 a | 34.66 a |
| **60** | 36.87 a | 53.00 a | 34.23 a | 49.26 a | 49.21 a | 73.86 b |
| **70** | 40.59 a | 124.20 b | 191.33 b | 332.06 b | 294.08b | 483.53 b |
| **CV (%)** | 25.81 | | | | | |

Means followed by the same lowercase letter in the row comparing drying methods for each warming time do not differ from each other by the Scott-Knott test at a 5% probability level.

Source: By the author (2023).

Table 8 - Electrical conductivity (µS $cm^{-1}$ $g^{-1}$) of *Coffea canephora* Pierre seeds cryopreserved after drying with silica gel or in a dryer and warmed at different temperaturesand for different times.

| Drying method | Warming time (min) | Warming temperature (ºC) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 40 | 45 | 50 | 55 | 60 | 70 |
| Silica gel | 1 | 35.01 aA | 45.90 aA | 45.47 aA | 30.14 aA | 36.87 aA | 40.59 aA |
| | 2 | 27.15 aA | 37.42 aA | 37.41 aA | 32.46 aA | 34.23 aA | 191.33 bB |
| | 3 | 33.33 aA | 31.90 aA | 36.67 aA | 34.66 aA | 49.20 aA | 294.08 bC |
| Dryer | 1 | 28.19 aA | 36.75 aA | 35.50 aA | 40.81 aA | 53.00 aA | 124.20 bA |
| | 2 | 32.23 aA | 33.71 aA | 64.92 aB | 52.79 aA | 49.26 aA | 332.06 bB |
| | 3 | 40.51 aA | 50.39 aA | 31.51 aA | 34.66 aA | 73.86bA | 483.53 cC |
| CV (%) | | 25.81 | | | | | |

Means followed by the same lowercase letter in the rows and uppercase letter in the columns in the same drying method do not differ from each other by the Scott-Knott test at a 5% probability level.

Source: By the author (2023).

The results of the electrical conductivity test show that both drying methods, silica gel and dryer, at a warming temperature of 70ºC, lead to a greater release of exudates compared to other temperatures, with conductivity values increasing aswarming time increases, indicating greater damage to cell membranes (except for 1 minute of warming with drying with silica gel) (TABLE 7). A temperature of 60ºC for 3 minutes and a temperature of 50ºC for 2 minutes for seeds dried in a dryeralso led to statistically significant differences, with higher electrical conductivity values.

However, at the other temperatures of 40, 45, and 55ºC, there were no significant differences amongwarming time, drying method, and warming temperature treatments for electrical conductivity (TABLES 7 and 8).

The longer the warming time at a temperature of 70ºC, the higher the electrical conductivity values in the seeds, indicating a loss of quality and corroborating the germination data.

These results are consistent with the findings of Coelho et al. (2018), who emphasized that factors such as the initial quality of the seeds, drying conditions, cooling rate, and warming procedures are critical for the survival of species after cryopreservation. In the present study, the coffee seeds had high initial quality, and even after drying with silica gel, it can be inferred that the cooling and warming steps are the most sensitive steps in achieving success in cryopreservation of *Coffea canephora* seeds.

Although seeds dried in a dryer also had quality, this method proved not to be the most suitable when the final goal is cryopreservation of *Coffea canephora* seeds.

The response of seeds to cryopreservation may depend on the species being analyzed, since some species undergo the process without major problems, while others do not survive (NIKISHIMA et al.2007). Thammasiri and Soamkul (2007) developed cryopreservation tests for *Vanda coerulea* seeds using only the PVS2 solution and achieved survival rates of up to 67% in seeds that initially contained 33% moisture. PVS2 was used to dehydrate the seeds and protect the plant material from the stresses of freezing. When these same seeds were exposed only to NL, there was no survival, due to the high moisture content of the plant tissues. Seeds of angico dried to a water content of 7.5% can be stored at a temperature of -196ºC without change in their physiological quality (SANTOS et al., 2012). Similarly, pomegranate seeds can be dried to 10% (wb) and cryopreserved without complications (SILVA et al., 2015);paineira rosa seeds can be desiccated to 6.8% moisture content, and storage in liquid nitrogen does not affect their germination(PRUDENTE et al., 2016).

Thus, it is clear that moisture content is one of the obstacles to achieving greater seed survival after cryopreservation.

Ideally, before exposure to liquid nitrogen, the seeds should have only non-freezable water. However, in practice, this is impossible to achieve. The amount of freezable water is affected by the difference in moistureamong the seeds in the drying step, and this water remains in the cells during the cryopreservation period (BERJAK; PAMMENTER, 2014). This may be the reason why germination was lower after the cryopreservation process in the present study, regardless of the warming time and temperature.

## 4.2 Comparison of physiological analyses between cryopreserved and non-cryopreserved (control) seeds

Statistical analysis was not performed on the results of physiological evaluation of the control seeds, i.e., those dried with silica gel or in a stationary dryer but not cryopreserved, as they were not part of the factorial arrangement of the study. These results were then compared to the temperature and warming time treatments for each drying method separately.

Tables 9 and 10 present the results of these comparisons performed by Student's t-test for root emergence, germination, strong normal seedlings, viability, and electrical conductivity of

cryopreserved and non-cryopreserved (control) *Coffea canephora* Pierre seeds, previously driedwith silica gel (TABLE 9) orin a dryer (TABLE 10).

Overall, all the results of the evaluations of cryopreserved seeds, both those dried with silica gel and in a dryer, were poor compared to those of the control seeds, as expected. However, this analysis was carried out considering the great difficulties in obtaining an efficient protocol for the cryopreservation of canephora seeds. These analyses may, however, provide indications of possible paths to be followed in future research.

Table 9 - Root emergence values, germination, strong normal seedlings, percentage of viable seeds by the tetrazolium test, and electrical conductivity of *Coffea canephora* Pierre seeds cryopreserved and non-cryopreserved (control) after drying with silica gel.

| Temperature (ºC) | a) Radicle emergence(%) | | | b) Germination (%) | | |
|---|---|---|---|---|---|---|
| | Time (min) | | | | | |
| | 1 | 2 | 3 | 1 | 2 | 3 |
| 40 | 35 b | 32 b | 23 b | 14 b | 17 b | 14 b |
| 45 | 31 b | 30 b | 24 b | 21 b | 17 b | 21 b |
| 50 | 20 b | 37 b | 28 b | 13 b | 27 b | 23 b |
| 55 | 34 b | 29 b | 34 b | 19 b | 15 b | 17 b |
| 60 | 36 b | 40 b | 29 b | 15 b | 19 b | 14 b |
| 70 | 31 b | 2 b | 0 b | 12 b | 1 b | 0 b |
| Control | | 92 a | | | 82 a | |
| | c) Strong normal seedlings(%) | | | d) Viability (%) | | |
| Temperature (ºC) | 1 | 2 | 3 | 1 | 2 | 3 |
| 40 | 3 b | 3 b | 2 b | 87 a | 75 a | 77 a |
| 45 | 3 b | 6 b | 7 b | 77 a | 75 a | 80 a |
| 50 | 4 b | 13 a | 6 b | 85 a | 77 a | 80 a |
| 55 | 3 b | 4 b | 8 b | 82 a | 87 a | 87 a |
| 60 | 4 b | 4 b | 4 b | 85 a | 75 a | 80 a |
| 70 | 4 b | 0 b | 0 b | 65 b | 22 b | 17 b |
| Control | | 34 a | | | 90 a | |
| | e) Electrical conductivity ($\mu S\ cm^{-1}\ g^{-1}$) | | | | | |
| Temperature (ºC) | 1 | 2 | 3 | | | |
| 40 | 35.01 a | 27.15 a | 33.33 a | | | |
| 45 | 45.90 a | 37.42 a | 31.90 a | | | |
| 50 | 45.47 a | 37.41 a | 36.67 a | | | |
| 55 | 30.14 a | 32.46 a | 34.66 a | | | |
| 60 | 36.87 a | 34.23 a | 49.20 a | | | |
| 70 | 40.59 a | 191.33 b | 294.08 b | | | |
| Control | | 30.53 a | | | | |

Means followed by the same lowercase letter as the control do not differ from each other by Student's t-test at a 5% probability level. Source: By the author (2023).

Table 10 - Root emergence values, germination, strong normal seedlings, percentage of viable seeds by the tetrazolium test, and electrical conductivity of *Coffea canephora* Pierre seeds cryopreserved and non-cryopreserved (control) after drying in a dryer.

| Temperature (ºC) | f) Radicle emergence (%) | | | g) Germination (%) | | |
|---|---|---|---|---|---|---|
| | Time (min) | | | | | |
| | 1 | 2 | 3 | 1 | 2 | 3 |
| 40 | 42 b | 37 b | 8 b | 18 b | 15 b | 5 b |
| 45 | 22 b | 26 b | 20 b | 15 b | 12 b | 15 b |
| 50 | 17 b | 35 b | 21 b | 9 b | 18 b | 9 b |
| 55 | 14 b | 20 b | 30 b | 5 b | 7 b | 15 b |
| 60 | 32 b | 26 b | 21 b | 14 b | 22 b | 16 b |
| 70 | 20 b | 0 b | 0 b | 11 b | 0 b | 0 b |
| Control | | 73a | | | 64a | |

| Temperature (ºC) | h) Strong normal seedlings (%) | | | i) Viability (%) | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 |
| 40 | 2b | 2b | 0 b | 75 b | 75 b | 80 b |
| 45 | 3b | 2b | 2b | 77 b | 80 b | 82 a |
| 50 | 2b | 4a | 1b | 70 b | 85 a | 77 b |
| 55 | 0b | 2b | 7a | 82 b | 75 b | 80 a |
| 60 | 1b | 4a | 7a | 77 b | 65 b | 75 b |
| 70 | 2b | 0b | 0b | 72 b | 25 b | 0 b |
| Control | | 17a | | | 97a | |

| Temperature (ºC) | j) Electrical conductivity ($\mu S\ cm^{-1}\ g^{-1}$) | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| 40 | 28.19 a | 32.23 a | 40.51 a |
| 45 | 36.78 a | 33.71 a | 50.39 a |
| 50 | 35.50 a | 64.92 b | 31.51 a |
| 55 | 40.81 a | 52.79 b | 34.66 a |
| 60 | 53.00 b | 49.26 a | 73.86 b |
| 70 | 124.20 b | 332.06 b | 483.53 b |
| Control | | 29.99 a | |

Means followed by the same lowercase letter as the control do not differ from each other by Student's t-test at a 5% probability level

Source: By the author (2023).

Comparing the results of the evaluations of root emergence (a) and germination (b) of cryopreserved seeds with the results of the control seeds, there were no significant differences among the warming temperatures at all drying times for silica gel (TABLE 9) or the dryer (TABLE 10). However, some treatments showed percentages of strong normal seeds similar to the control, such as the seeds warmed at 50°C/2 minutes for drying with silica gel (TABLE 9)

and warmed at 50°C/2min, 55°C/3min, and 60°C/2 and 3 minutes for drying in the dryer (TABLE 10).

Regarding the results of the tetrazolium test (d) of seeds dried with silica gel (TABLE 9), there were no significant differences in the viability of cryopreserved seeds and control seeds at all temperatures, regardless of the warming time, except for 70°C. However, for most treatments of seeds dried in the dryer (TABLE 10), the viability of cryopreserved seeds exhibited lower percentages than control seeds, except for treatments warmed at 50°C/2 minutes and 45°C/3 minutes, and 55°C/3 minutes.

The worst warming temperature for seeds was 70°C, as can also be observed by the electrical conductivity results (e), since these treatments had a largerquantity of leachates, as shown by the high values in the test (TABLE 9 and 10). This indicates that the temperature of 70°C is too aggressive for warming *Coffea canephora* seeds, regardless of the time, since cell tissues and membrane systems are not preserved, compromising the physiological performance of the seeds.

For other temperatures and drying times of seeds dried with silica gel, however, there were no significant differences in electrical conductivity between cryopreserved seeds and control seeds.

One of the main causes of loss of physiological quality in coffee seeds has been observed in processing and drying, where the seeds undergo physical changes that can reduce the percentage and speed of germination, and contribute to an increase in abnormal seedlings (ZONTA, 2008).

There are reports that seeds with degrees of recalcitrance may suffer mechanical damage if dried at low water contents (UMARANI; AADHAVAN; FAISAL, 2015). According to Pritchard (2007), the water content present in the seeds is the factor that most affects their cryopreservation. Coelho (2018) observed that *Coffea canephora* seeds dried to 17% (wb) with silica gel show a drastic reduction in physiological quality compared to seeds with 20% (wb), reinforcing the importance of adjusting the appropriate water content for each species to withstand the stresses of the cryopreservation stages, especially cooling and warming.

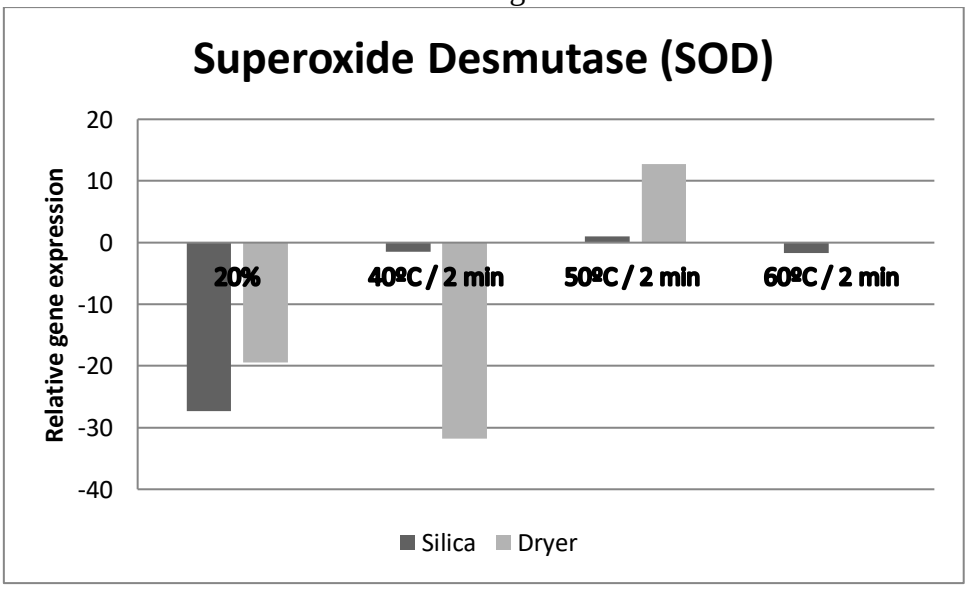
Figueiredo (2016) tested different water contents in *Coffea arabica* seeds in her pre-cooling study and found that a water content of 17% (wb) allows for greater survival of the seeds

in cryopreservation when cooled at a rate of -1°C $min.^{-1}$ to a temperature of -40°C before immersion in liquid nitrogen. The author found that at this moisture level, *Coffea arabica* seeds show integrity of cellular membranes, without the occurrence of cytoplasmic leakage, as occurs in drying to 20% (wb). These studies tested drying with silica gel, drying with saturated $(NH_4)_2 SO_4$ solution, and drying with a saturated NaCl solution, with silica gel being the most suitable.

### 4.3 Biochemical and molecular evaluations

SOD is the first enzyme of defense against damage caused by ROS, responsible for the dismutation of the superoxide anion ($O_{2-}$), produced in different locations in the cell, into molecular oxygen ($O_2$) and hydrogen peroxide ($H_2O_2$). Since they dismutate $O_{2-}$, they indirectly reduce the risk of OH- formation from $O_{2-}$ (PAUL; ROYCHOUDHURY, 2017; CHOUDHARY; KUMAR; KAUR, 2019; LIU; SOUNDARARAJAN; MANIVANNAN, 2019). The expression of the superoxide dismutase (SOD) gene, observed in Figure 6, showed that seeds not subjected to cryopreservation had down-regulated gene expression, especially when drying occurred with silica gel (FIGURE 6).

FIGURE 6 - Relative gene expression of superoxide dismutase (SOD) in *Coffea canephora* Pierre seeds before and after cryopreservation. The following treatments were used: seeds dried with silica gel and in a dryer to 20% (wb), and cryopreserved seeds warmed at 40, 50, and 60°C for 2 minutes. Data show the average of three replicates using the 50°C/2 min. dried with the silica gel treatment as a calibrator.




Source: By the author (2023).

After the cryopreservation and warming step, up-regulated expression of the SOD gene was observed in seeds dried with silica gel, with little difference among the warming temperatures. When warming was carried out at 50°C for 2 minutes and drying was performed with silica gel, the expression of SOD was down-regulated compared to seeds dried in a dryer, which is consistent with the results of physiological tests, as a higher germination percentage was observed in this treatment (drying with silica gel and warming at 50°C/2 minutes) (FIGURE 3).

From the results in Figure 6, it can be inferred that drying in a dryer and warming at 50°C caused more stress in the seeds and, consequently, higher production of ROS (reactive oxygen species), as the SOD gene had greater expression under these conditions.

Veloso (2017), performing molecular evaluation of *Coffea arabica* seeds under cryopreservation, observed higher expression of the SOD gene in treatments that received slow drying with a saturated solution of ammonium sulfate and sodium chloride salt, suggesting a possible relationship between drying rate and gene expression. In this study, the highest gene expression was observed at the fastest drying rate, in a dryer. As the SOD gene catalyzes the formation of hydrogen peroxide from superoxide radicals ($O_{2}$-), it is possible that these radicals were in greater quantity when drying occurred in the dryer.

In the same study, Veloso (2017) also related gene expression to the warming time and seed moisture content, observing that seeds dried with silica gel to 20% moisture content tended to have reduced SOD expression as warming time increased. In the present study, we could not compare these results, as the treatments analyzed for gene expression had the same warming time.

Increasing activity of antioxidant enzymes is associated with the function of neutralizing the deleterious effect of ROS, as they act as signaling molecules of plant responses, from growth to stress responses (SHARMA et al., 2012; ALVES et al., 2012; EL-ENANY et al., 2013; VERMA et al., 2014).

Barros et al. (2021) found higher SOD activity in low physiological vigor seeds under the thermal stress of 35°C. The technique of analyzing the activity of superoxide dismutase (SOD) has been widely used as an auxiliary tool to evaluate the quality of many seeds, such as sesame, soybean, corn, coffee, pepper, and eggplant (SANTOS et al., 2016; ALVES et al., 2017; PIRES et al., 2016, VELOSO, 2017).

After the action of SOD, ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT) enzymes begin their action by removing hydrogen peroxide (APEL; HIRT, 2004).

In the expression of CAT in electrophoresis gel (FIGURE 7), a lower expression was observed in seeds warmed at temperatures of 60 and 70°C, in both types of drying. There was also a decrease in CAT activity aswarming temperature increased, especially in silica gel drying. This may have occurred due to the denaturation of these enzymes, as temperature is one of the factors that most affect the functionality of these proteins (BORZANI et al., 2001).

Greater expression of the enzyme was observed in treatments that received silica gel drying, and these were superior in physiological tests when warmed at temperatures of 45, 50, and 55°C. It is important to highlight that in dryer drying and warming at 70°C/3min, in which there was high seed mortality, CAT expression was not found. In silica gel drying under these same conditions, a decrease in CAT expression and in seed physiological potential was also observed (FIGURE 7).

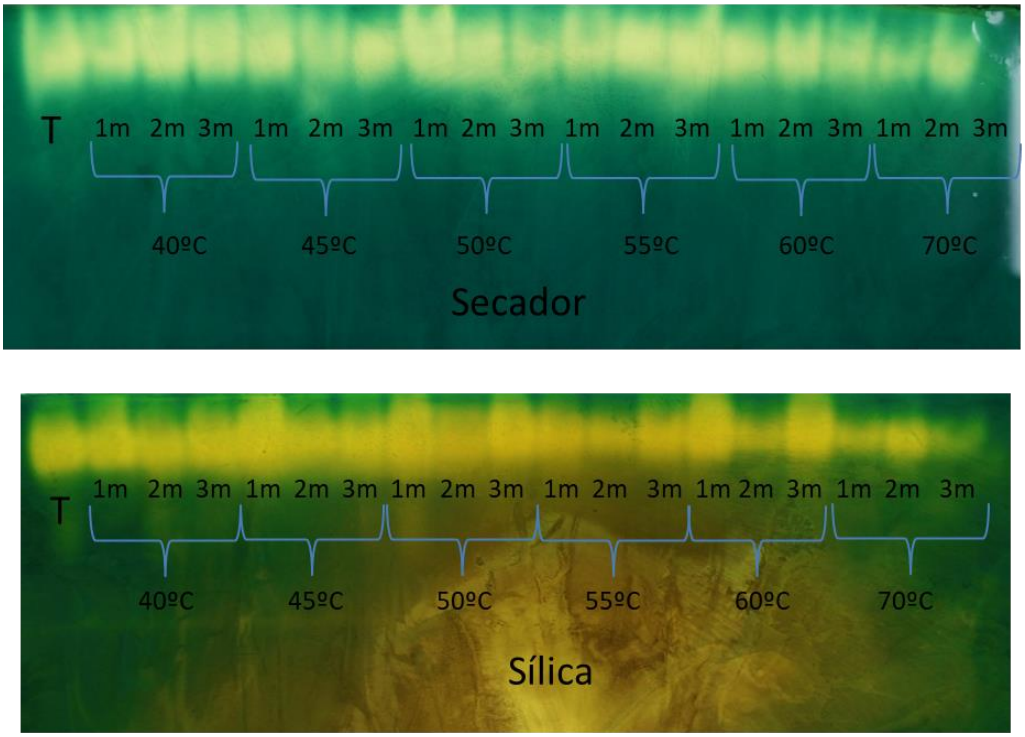
These results corroborate those of Brandão Júnior et al. (2002), who found a decrease in CAT activity in coffee seeds with lower physiological performance. Coelho et al. (2018) also associated low enzyme expression with lower physiological quality in seeds dried with a saturated NaCl solution with 22% (wb) moisture content. Nkang et al. (2000) observed a decrease in CAT activity with an increase in hydroperoxide levels during the drying process of *Telfairia occidentalis* seeds.

According to Barbosa et al. (2010), the CAT enzyme acts by preventing the accumulation of hydrogen peroxide, which is of great importance, since this reactive species, through the Fenton and Haber-Weiss reactions and the participation of iron and copper metals, results in generation of the OH radical, against which there is no enzymatic defense system.

The OH radical has been identified as having the highest reactive potential and extreme instability. According to the same authors, these characteristics make the OH radicalthe free radical most likely to produce oxidative damage. In addition to being the main initiator of lipid peroxidation, resulting in the alteration of the biological function of cell membranes, this radical can act on proteins, altering them in relation to their structure and/or biological function. Its attack on DNA enables the occurrence of mutations (BARBOSA et al., 2010).

Li and Sun (1999) observed increases in lipid peroxidation in embryonic axes of *Theobroma cacao* during desiccation, related to decreases in the activity of protective enzymatic systems. Such results may indicate an increase in the content of oxidative free radicals which, although not quantified in the present study, can be confirmed by the increase in electrolyte leaching in the electrical conductivity test and the loss of seed viability in the germination test.

Figure 7 - Expression of the catalase (CAT) enzyme in cryopreserved *Coffea canephora* Pierre seeds previously dried with silica gel orin a dryer.




Source: By the author (2023).

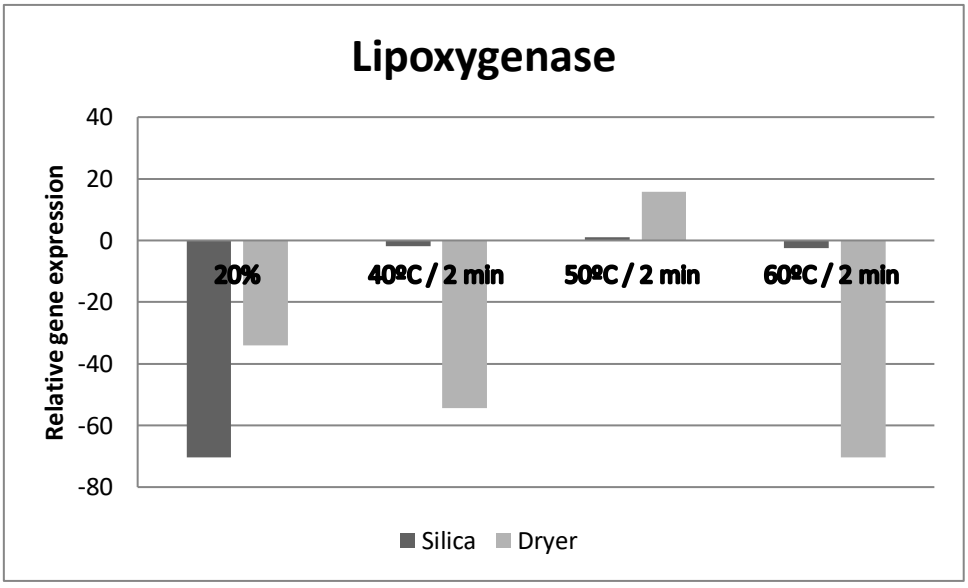
Enzymatic oxidation by the lipoxygenase enzyme, belonging to the oxidoreductase group, is one of the routes that directly form hydroperoxides. Lipoxygenases are present in almost all living cells and are capable of catalyzing reactions between oxygen and cis-cis unsaturated fatty acids to form peroxides. The main substrates of these enzymes are free fatty acids, although some also use triacylglycerols as a substrate, but with less specificity (MATTHÄUS, 2010).

The function of the lipoxygenase enzyme is lipid peroxidation, since lipids present in the membrane have a greater disposition due to their larger surface area, and most unsaturated lipids are quite sensitive to degradation, which can lead to imbalances in the viscosity and permeability

of the membranes. After the action of this enzyme, free radicals are formed, which, in turn, are related to seed deterioration (NAKADA et al., 2010).

This was observed in seeds after drying in a dryer, as the down-regulated gene expression of the lipoxygenasewas observed in the treatment with warming at 60°C for 2 minutes, followed by the 40°C/2 minutes treatment. These same treatments obtained an up-regulated percentage of germination after the seeds were dried in a dryer (FIGURES 8 and 2). The highest gene expression of lipoxygenase was found after warming cryopreserved seeds at 50°C for 2 minutes (dried in a dryer) (FIGURE 8), with this treatment being inferior in the germination test to the treatment warmed at 60°C for 2 minutes (dried in a dryer). In agreement with Nakada et al. (2010), it is possible that after the action of the enzyme, free radicals may have formed, and they are related to the seed deterioration process.

Figure 8 - Relative gene expression of lipoxygenase in *Coffea canephora* Pierre seeds before and after cryopreservation. The following treatments were used: seeds dried with silica gel and in a dryer to 20% moisture content (wb), and cryopreserved seeds warmed at 40, 50, and 60°C for 2 minutes. Data show the average of three replicates using the 50°C/2 min treatment dried with silica gel as a calibrator.




Source: By the author (2023).

In silica gel dried seeds that were not cryopreserved, down-regulated expression of the gene was observed, and this treatment showed better physiological results compared to cryopreserved treatments (TABLE 9). Generally, in silica gel drying, the gene activity of the gene was up-regulated after cryopreservation, but without much difference between the

treatmentsstudied; this result was expected since these seeds did not undergo the action of low temperature and subsequent warming.

Lima et al. (2007) studied delayed harvest as a method of differentiating soybean genotypes and concluded that genotypes without the lipoxygenase enzyme in their constitution were able to produce seeds of better physiological quality, as found by germination, first count, accelerated aging, emergence, and emergence speed index in sand tests.

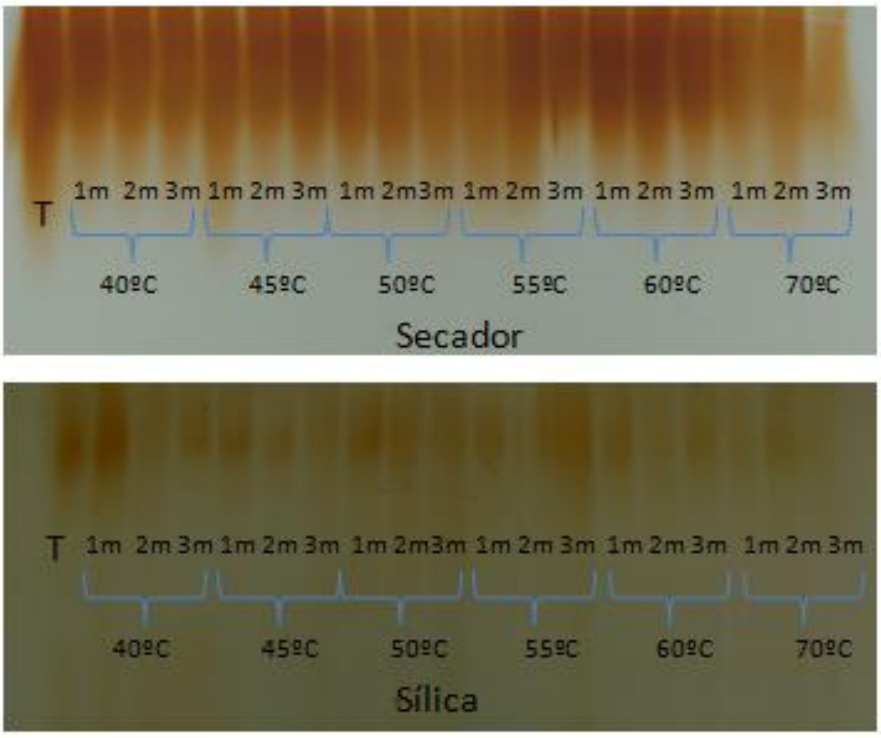
However, in a study by Oliveira et al. (2006), the authors found that the germination speed of soybean seeds was higher when the lipoxygenase enzyme was present, and it helped in lipid mobilization. In their study on cotton seeds, Freitas et al. (2006) observed a reduction in lipoxygenase activity with an increase in the accelerated aging period in the seeds.

Evaluation of isoenzyme expression of PO in cryopreserved *C. canephora* seeds (FIGURE 9) showed higher expression after dryer drying compared to silica gel drying in all the treatments evaluated. As seen in the results of physiological tests, dryer drying is more harmful to seeds, confirmed by the high expression of this enzyme, which is responsible for activating defense mechanisms. PO plays an important role in seed metabolism by using peroxides as hydrogen acceptors, contributing to an increase in defense mechanisms and preventing quality loss (USHIMARU et al., 2001).

In both drying methods, a reduction in PO expression is observed aswarming time increases, especially at lower temperatures. This reduction can be understood through the effect of seed water content, which also increased over time in the water bath. The effect of water content in seeds on enzyme expression in electrophoresis gels was well documented by Fávaris et al. (2022). The authors analyzed the expression of several enzymes in seeds with different moisture contents, showing that the presence of water in seeds affects their expression in electrophoresis gels.

Among the warming treatments, with different temperatures and times, higher PO expression was observed at 60°C after dryer drying. The control treatment, in which the seeds were dried in a dryer, also showed high activity of this enzyme, indicating that damage to enzymatic systems can occur after drying, even if they do not immediately affect physiological quality (FIGURE 9). At 70°C, the lower PO expression may have occurred due to severe damage to the seeds, which also compromises enzymatic systems.

Figure 9 - Expression of peroxidase enzyme (PO) in *Coffea canephora* Pierre seeds dried with silica gel and in a dryer and then cryopreserved.




Source: By the author (2023).

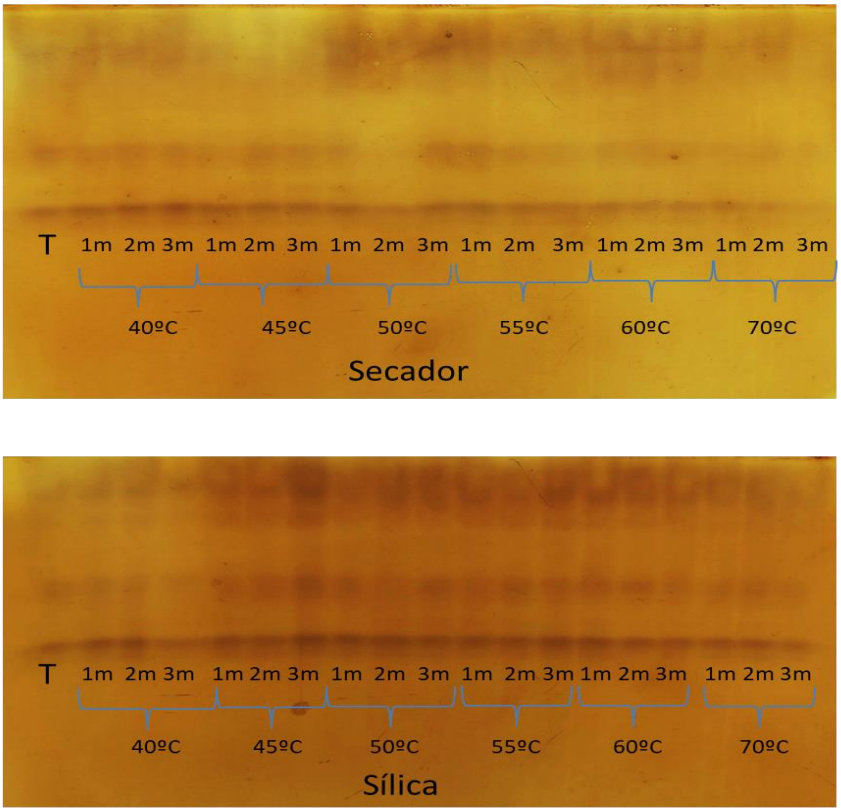
The activity of the GOT enzyme (FIGURE 10) was higher at lower warming temperatures, i.e., at 40 and 45°C, when dried in a dryer. A decrease in the expression of this enzyme was observed as the seed warming temperature increased, especially at 60°C and 70°C. Furthermore, in analysis of drying in a dryer, there was almost no expression of this enzyme after warming the cryopreserved seeds at 70°C for 3 minutes, corroborating the germination data; high temperature along with longer seed contact time likely caused biochemical damage, destabilizing proteins.

In seeds dried with silica gel, there is greater uniformity in expression of this enzyme, as well as slightly higher expression compared to dryer drying treatments. Lower activity was observed at more extreme warming temperatures, such as 40°C and 70°C (FIGURE 10). Coelho et al. (2018) studied different drying methods and different water contents in cryopreserved *Coffea canephora* Pierre seeds and observed that seeds dried more slowly (saturated NaCl solution) showed higher expression of GOT activity than seeds dried more quickly, regardless of the water content of the seeds. This supports the results found in the present study, where drying

with silica gel, slower drying, resulted in higher expression compared to drying in the dryer (FIGURE 10).

Stresses applied to the seeds can directly or indirectly affect GOT, since it acts in the oxidation of amino acids, which provides energy to the Krebs cycle or reduces α-ketoglutarate, leading to biosynthesis of new amino acids for the growth of the zygotic embryo (MALONE et al., 2007). In both types of drying studied, the highestwarming temperature of 70°C caused an extreme decline in the physiological quality of *C. canephora* seeds, which may have affected the activity of GOT, decreasing its expression in gel electrophoresis (FIGURE 10).

Figure 10 - Expression of the enzyme glutamate-oxaloacetate transaminase (GOT) in cryopreserved *Coffea canephora* Pierre seeds that had been dried with silica gel or in a dryer.




Source: By the author (2023).

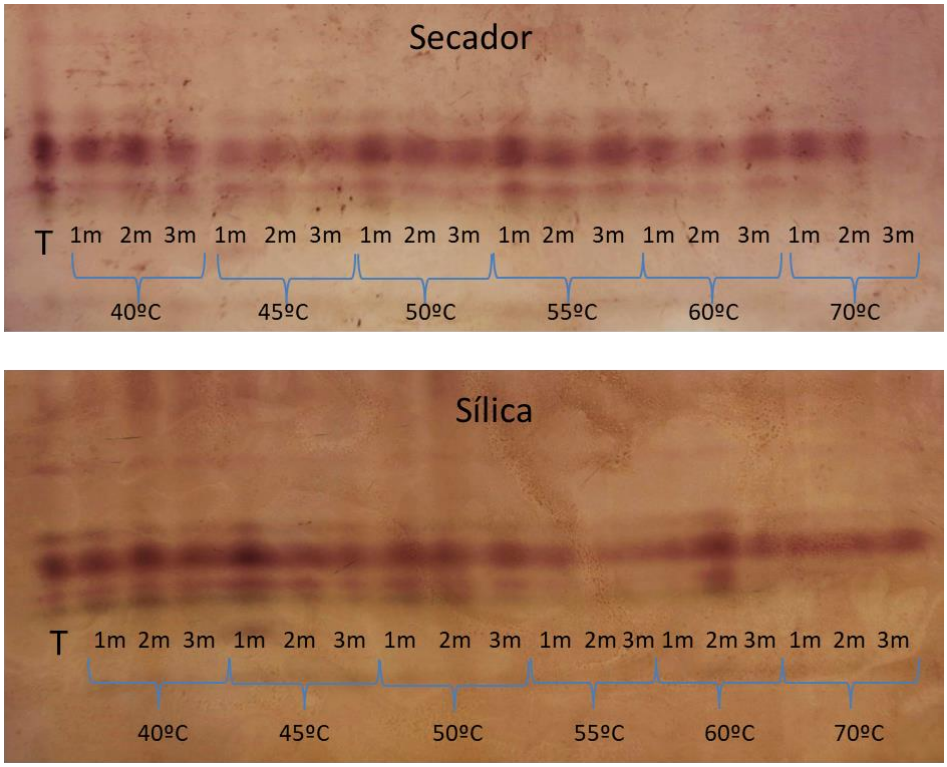
In the expression of EST, after cryopreservation and warming of seeds dried in a dryer (FIGURE 11), lower enzyme activity was observed at the temperature of 45°C and at 70°C; there was no enzymatic activity after 3 minutes of seed exposure to the warming. The control treatment

showed higher expression than the other treatments. In general, expression of EST activity within each warming temperaturewas higher at the shortest warming time of 1 minute (FIGURE 11).

In seeds dried with silica gel, there was higher expression in seeds warmed at lower temperatures, up to 50°C, and a decrease in the intensity of this enzyme was observed above that temperature, indicating a decrease in seed quality, as lower physiological performance was observed in these treatments (FIGURE 11).

EST participates in ester hydrolysis reactions and can also act on membrane phospholipids (TAIZ; ZEIGER, 2004). According to Nakada et al. (2010), an increase in the activity of this enzyme is indicative of seed deterioration. Brandão Júnior et al. (2002) observed an increase in EST activity and number of bands in aged or damaged seeds. However, in this study, increased expression of this enzyme did not coincide with lower physiological quality, as seeds warmed at 70°C and dried with silica gel had lower germination and enzyme activity, while higher expression was observed in seeds with better germination (warmed at 50°C) and in the control.

Figure 11 - Expression of esterase (EST) enzyme in cryopreserved *Coffea canephora* Pierre seeds that had been driedwith silica gel or in a dryer.




Source: By the author (2023).

### 4.4 Ultrastructural evaluations

The accumulation of ROS (reactive oxygen species) resulting from oxidative stress causes several metabolic alterations, such as disruption of the cell membrane, inactivation of enzymes, degradation of lipids and proteins, and loss of integrity of DNA molecules, resulting in cell death, since cell biological functions are compromised. Although all cellular components are susceptible to the action of ROS, it is noteworthy that cell membranes, which are mainly formed of phospholipids, are the main site of peroxidation and ROS formation. Thus, changes in the membrane system are described as one of the first manifestations of the deterioration process (KUMAR et al., 2015; EBONE et al., 2019).

The results of structural alterations in cell membranes of *Coffea canephora* P. endosperms from before to after the cryopreservation process are shown in Figures 12, 13, 14, and 15 in analysis by scanning electron microscopy (SEM).

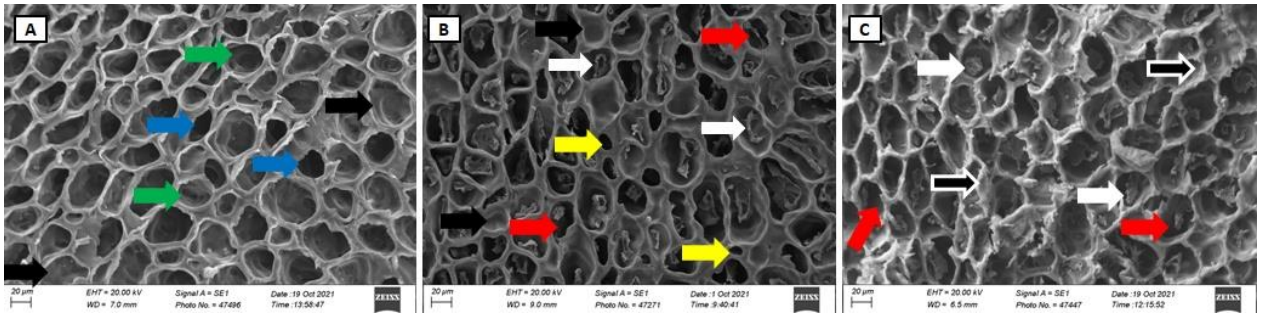
The following treatments were selected for the SEM analyses: seeds after harvesting and before drying, seeds with 31% (wb) moisture content, seeds dried with silica gel or in a dryer to 20% moisture content (wb) before cryopreservation, and the cryopreserved seeds described in Table 11.

Table 11 – Warming temperatures of the samples selected for scanning electron microscopy analysis warmed in a water bath for 2 minutes.

| **Drying method** | **Warming temperature (**ºC) |
|---|---|
| Silica gel and dryer | 40 |
| Silica gel and dryer | 50 |
| Silica gel and dryer | 60 |
| Silica gel | 70 |

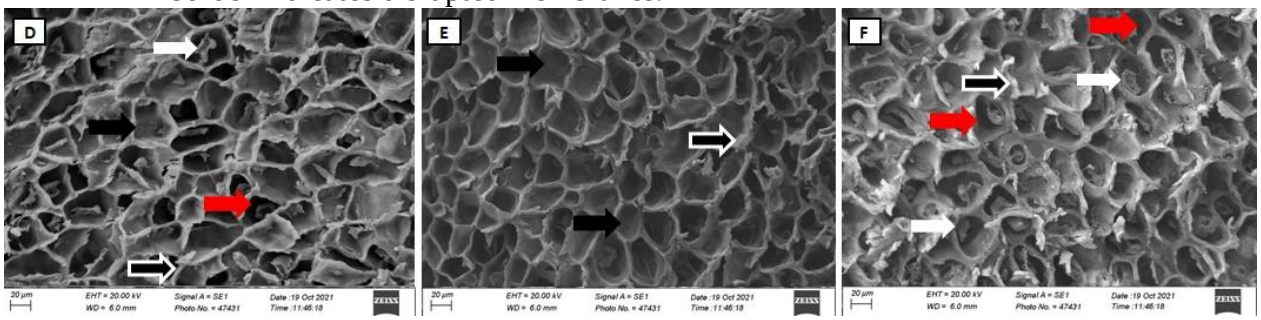
Source: By the author (2023).

Figure 12 - Scanning electron microscopy of *Coffea canephora* P. seeds freshly harvested (A); under silica gel drying (B); or under dryer drying (C), to 20% moisture content. Arrows of black color indicate intact cells; green color indicates partially filled cells; blue color indicates empty cells; white color indicates shrunken cells; red color indicates the cell lumen; yellow color indicates empty intracellular spaces; and black color with a white border indicates disrupted membranes.



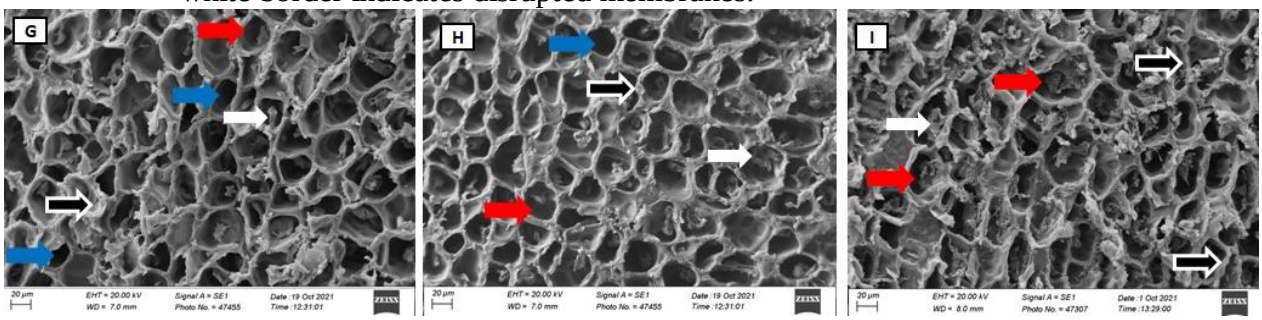

Source: By the author (2023).

Figure 13 - Scanning electron microscopy of *Coffea canephora* P. seeds dried with silica gel, cryopreserved, and then warmed for 2 minutes at different temperatures: 40°C (D); 50°C (E); 60°C (F). Arrows of black color indicate intact cells; white color indicates shrunken cells; red color indicates the cell lumen; and black color with a white border indicates disrupted membranes.



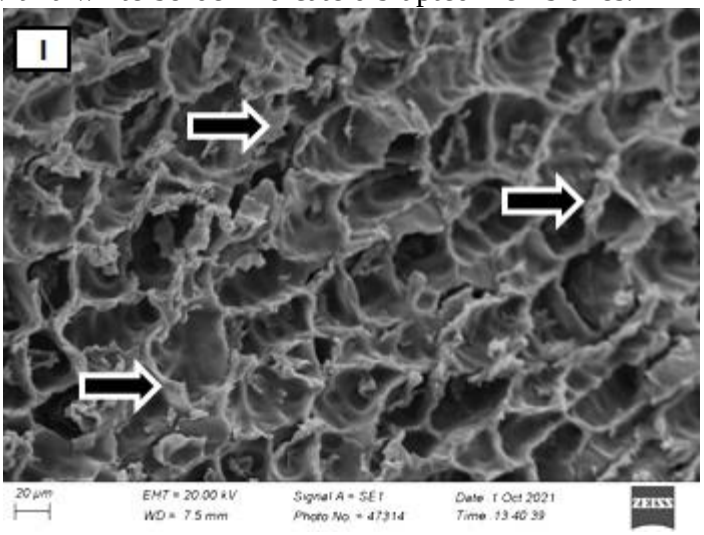

Source: By the author (2023).

Figure 14 - Scanning electron microscopy of *Coffea canephora* P. seeds dried in a dryer, cryopreserved, and then warmed for 2 minutes at different temperatures: 40°C (G); 50°C (H); 60°C (I). Arrows of black color indicate intact cells; white color indicates shrunken cells; red color indicates the cell lumen; and black color with a white border indicates disrupted membranes.




Source: By the author (2023).

Figure 15 - Scanning electron microscopy of *Coffea canephora* P. seeds dried with silica gel, cryopreserved, and then warmed at a temperature of 70°C for 2 minutes (J). Black arrows with a white border indicate disrupted membranes.




Source: By the author (2023).

In the seeds that were not cryopreserved (FIGURE 12), turgid or partially turgid cells were observed before drying, as well as empty cells (FIGURE 12A). In seeds dried to 20% moisture content (wb) with silica gel, some turgid cells, many contracted cells, and empty intracellular spaces were observed, but there were no clear signs of cell leakage and rupture (FIGURE 12B). The cell walls of these two treatments are highly structured (FIGURE 12A and 12B).

In seeds dried to 20% (wb) in a dryer, contracted cells and destruction of cell walls were observed, causing a decrease in cell content (FIGURE 12C). According to Saath et al. (2010), water removal during drying induces cell wall contraction, leading to cell wall destruction.Cell volume is also reduced, causing an agglomeration of cytoplasmic components, making cell content considerably viscous.

The images show that some damage had already occurred in the stage preceding seed immersion in liquid nitrogen, but after cryopreservation, the damage is more pronounced, especially when warming is performed at higher temperatures (FIGURE 12-15).

Figueiredo et al. (2016) also observed damage in the drying stage preceding immersion in liquid nitrogen through SEM; this damage can compromise seed viability after cryopreservation.

The compromised state of cell structures in endosperm cells in coffee beans dried to moisture levels between 30-20% (wb) was also observed in the studies of Saath et al. (2010).

Seeds dried with silica gel and then cryopreserved exhibited less disruption of cell membranes compared to drying in a dryer. However, even in seeds dried with silica gel, i.e., less damaged seeds, the membranes are more disorganized compared to control treatment seeds, indicating a significant loss of structure and cell volume (FIGURE 13).

When warming occurred at 40°C, intact cells, many contracted cells, and the cell lumen were observed (FIGURE 13D). These same characteristics were observed when the seeds were warmed at 60°C, but to a greater extent (FIGURE 13F).

In Figure 13E, a larger number of intact cells were observed among all the cryopreserved treatments, corroborating the results of the physiological tests performed in this study, where germination showed the best results at 50°C for 2 minutes in seeds dried with silica gel.

For seeds dried in a dryer and then cryopreserved, there were no intact cells, and a large number of empty and contracted cells were observed; cell membranes were severely compromised, with increased disorganization according to the warming temperature (FIGURE 14). Borém; Marques; Alves (2008) also observed greater ultrastructural damage in coffee beans that underwent faster drying.

Seeds dried with silica gel, cryopreserved, and then warmed at 70°C showed the greatest damage to cell walls, resulting in ruptures and greater cell disorganization (FIGURE 15). This helpsexplain the lower survival rate in the germination test for seeds in this treatment compared to others. Warming at a temperature of 70°C is very aggressive for *Coffea canephora* seeds.

Figueiredo et al. (2016) also observed the relationship between greater damage to cell membranes and inferior physiological results in their studyon cryopreservation of *Coffea arabica* seeds when the seeds were not subjected to the electrolyzed solution. In the same study, the authors noted that embryo cells in the seeds had less visible damage and had a better appearance compared to endosperm cells. This is an issue that has been raised by other researchers, such as Dussert; Engelmann (2006) and Coelho et al. (2015), indicating greater sensitivity of endosperms to ultra-low temperatures.

The images also corroborate the results of the electrical conductivity and germination tests, where warming at 70°C was found to be quite detrimental to *Coffea canephora* seeds. Thus,

greater cell damage can be correlated with greater exudate release and a lower percentage of survival in the germination test.

Thus, it is possible to observe the importance of seed moisture for the success of cryopreservation, as well as the drying speed, in this work the faster drying (dryer) was not more efficient for C. canephora seeds, since it was lower physiological, molecular and ultrastructural potential was observed in seeds subjected to drying in a dryer and then cryopreserved.

# 5 CONCLUSIONS

Warming at a temperature of 70ºC is detrimental to the seeds and is not recommended for *Coffea canephora* Pierre seed cryopreservation protocols, regardless of the exposure time.

Drying seeds with silica gel for subsequent cryopreservation increases the success rate of the cryopreservation technique compared to drying in a dryer for *Coffea canephora* Pierre seeds.

Drying with silica gel to a water content of 20% (wb), followed by direct immersion in liquid nitrogen and warming at a temperature of 50ºC for 2 minutes in a water bath led to greater survival of *Coffea canephora* Pierre seeds. However, further studies on this topic are necessary, as the survival rate of this species is still low.

# REFERENCES


ABDELNOUR-ESQUIVEL, A. et al. Cryopreservation of zygotic embryos of *Coffea spp*. **CryoLetters**, Cambridge, v. 13, n. 5, p. 297-302, 1992.

ABREU, L.A.S. et al. Behavior of coffee seeds to desiccation tolerance and storage. **Journal of Seed Science**, Londrina, v. 36, n. 4, p. 399-406, Oct./Dec., 2014.

ALFENAS, A.C. et al. **Eletroforese e marcadores bioquímicos em plantas e microorganismos**. 2. ed. Viçosa: Ed. UFV, 627 p. 2006.

ALVES M.V.P. et al. Physiological and biochemical characterization of jiló seeds (*Solanum gilo*) in different harvest times. **American Journal of Plant Sciences**, [s.l.], v. 8, n. 10, p. 2569-2595, 2017.

ALVES, J.D. et al. Antioxidative responses and morpho-anatomical adaptations to aterlogging in *Sesbania virgata***. Trees**, Berlin, v. 27, n. 3, p. 717-728, 2012.

AMBION. Real-Time PCR (qPCR) guide. [2019]. Disponível em: http://www.ambion.com/techlib/tn/81/813.html. Acesso em: 18 set.2023.

ANDERSEN, C.L.; JENSEN, J.L.; ORNTOFT, T.F. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. **Cancer Research**, v.64, p.5245–5250, 2004.

APEL, K.;HIRT, H. Reactive Oxygen Species: Metabolism, Oxidative Stress, and Signal Transduction. **Annual Review of Plant Biology**, v.55, p. 373-379, 2004.

ARAUJO, R.F. et al. Conservação de sementes de café (*Coffea arabica* L .) despolpado e não despolpado. **Revista Brasileira de Sementes, Londrina**, v. 30, n. 3, p. 71-78, maio, 2008.

BARBEDO, C.J.; CENTENO, D.C.; RIBEIRO, R.C.L.F. Do recalcitrant seeds really exist?. **Hoehnea**, São Paulo, v. 40, n. 4, p. 583-593, 2013.

BARBOSA, K.B.F. et al. Estresse oxidativo: conceito, implicações e fatores modulatórios. **Revista de Nutrição**, Campinas, v. 23, n. 4, p. 629-643, jul./ago., 2010.

BARROS, T.T.V. et al. **Semina:** Ciências Agrária, Londrina, v. 42, n. 3, suplemento 1, p. 1435-1452, 2021.

BENSON, E.E. Cryopreservation of phytodiversity: a critical appraisal of theory and practice. Critical Reviews in **Plant Sciences**, v. 27, n. 3, p. 141-219, 2008.

BENSON, E.E.; BREMNER, D. Oxidative stress in the frozen plant: a free radical point of view. In: FULLER, B.J.; LANE, N.; BENSON, E.E. (Eds.). **Life in the frozen state**. 1st ed. Boca Raton: CRC Press, cap. 6, p. 205-241,2004.

BERJAK, P. Catodic amelioration of the adverse effects of oxidative stress accompanying procedures necessary for cryopreservation of embryonic axes of recalcitrant seeded species**. Seed Science Research**, Wallingford, v. 21, n. 3, p.187-203. Set,. 2011.

BERJAK, P.; PAMMENTER, N.W. Cryostorage of germplasm of tropical recalcitrant-seeded species: Approaches and problems. **International Journal Plant Science**, Chicago, v. 175, n. 1, p. 29-39, Jan., 2014.

BERJAK, P.; PAMMENTER, N.W. From Avicennia to Zizania: seed recalcitrance in perspective. **Annals of Botany**, London, v. 101, n. 2, p. 213-228, 2007.

BERJAK, P.; PAMMENTER, N.W. What ultrastructure has told us about recalcitrant seeds? **Revista Brasileira de Fisiologia Vegetal**, Edição Especial. Londrina, v. 12, p. 22-55, 2000.

BERJAK, P.; SERSHEN, B.V.; PAMMENTER, N.W. Cathodic amelioration of the adverse effects of oxidative stress accompanying procedures necessary for cryopreservation of embryonic axes of recalcitrant-seeded species. **Seed Science Research**, Wageningen, v. 21, n. 3, p. 187-203, Sept., 2011.

BHATTACHARJEE, S. Sites of generation and physicochemical basis of formation of reactive oxygen species in plant cell. In: GUPTA, S.D. **Reactive oxygen species and antioxidants in higher plants.**Enfield: Science Publishers, p.1-30. 2010.

BORÉM, F.M. et al. Microscopia eletrônica de varredura de grãos de café submetidos a diferentes formas de processamento e secagem. **Coffee Science**, Lavras, v. 8, n. 2, p. 227-237, abr./jun., 2013.

BORÉM, F.M.; MARQUES, E.R.; ALVES, E. Ultrastructural analysis of drying damage in parchment Arabica coffee endosperm cells. **Biosystems engineering**, Harpenden, v. 99, n. 1, p. 62-66, Jan., 2008.

BORZANI, W. et al . **Biotecnologia Industrial**. São Paulo: Editora Edgard Blücher Ltda, v. 1, 2001.

BRANDÃO JÚNIOR, D.S.; VIEIRA, M. das G.G.C.; HILHOST, H.W.M. Aquisição da tolerância à dessecação nos diferentes estádios de desenvolvimento de sementes de cafeeiro (*Coffea arabica* L.). **Ciência e Agrotecnologia**, v.26, p.673-681, 2002.

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

BUDIARTO, K. The occurrence of hyperydricity on several carnations (*Dianthus carriophyllus* L.) cultivars during low temperature storage. **Biodiversitas**, Belo Horizonte, v. 10, n. 3, p. 104-107, Jul., 2009.

CARVALHO, C.H.S. **Cultivares de café: origem, características e recomendações**. Brasília: Embrapa café, 2008.

CHEN, X.L. et al. Cryopreservation of in vitro-grow apical meristems of Lilium by droplet-vitrification. **South African Journal of Botany**, Amsterdam, v. 77, n. 2, p. 397-403, Apr., 2011.

CHOUDHARY, A.; KUMAR, A.; KAUR, N. ROS and oxidative burst: Roots in plant development. **Plant Diversity**, Beijing, v. 28, n. 42, p. 33-43, 2019.

CLEMENTE, A.C.S. et al. Preparo das sementes de café para a avaliação da viabilidade pelo teste de tetrazólio. **Revista Brasileira de Sementes**, Londrina, v. 33, n. 1, p. 38-44, 2011.

COELHO, S.V.B. **Criopreservação de sementes do gênero Coffea: Melhoria de protocolo para *Coffea arabica* L. e estudos preliminares para *Coffea canephora* Pierre.** 2018. 85 p. Tese (Doutorado em Agronomia/Fitotecnia) -Universidade Federal de Lavras, Lavras, 2018.

COELHO, S.V.B. et al. Alterações fisiológicas e bioquímicas em sementes de café secas em sílica gel e soluções salinas saturadas. **Pesquisa Agropecuária Brasileira**, Brasília, v. 50, n. 6, p. 483-491, 2015.

COELHO, S.V.B; et al. Cryopreservation of *Coffea canephora* Pierre ex A. Froehner seeds: importance of drying rate and moisture content. **Australian Journal of Crop Science**, v. 13, p. 1335-1342, 2019.

COGER R, TONER M. Preservation Techniques for Biomaterials. In: **The Biomedical Engineering Handbook 2º edição**. Boca Raton: CRC Press LLC. 2000.

CONAB - COMPANHIA NACIONAL DE ABASTECIMENTO. Acompanhamento da safra brasileira: café, **Primeiro levantamento**, janeiro, 2023. Brasília: Conab, v. 10, n. 1, p. 1-22, 2023.

D'AUTRÉAUX, B.; TOLEDANO, M.B. ROS as signaling molecules: mechanisms that generate specificity in ROS homeostasis. **Nature Reviews Molecular Cell Biology**, [s.l.], v. 8, p. 813-824, 2007.

DAVIS, A.P. et al. An annotated taxonomic conspectus of the genus *Coffea* (Rubiaceae). **Botanical Journal of the Linnean Society**, London, v. 152, n. 4, p. 465–512, 2006.

DEKKERS, B.J.W. et al. Acquisition and loss of desiccation tolerance in seeds: from experimental model to biological relevance. **Planta**, Berlin, v. 241, n. 3, p. 563-577, 2015.

DINAKAR, C.; DJILIANOV, D.; BARTELS, D. Photosynthesis in desiccation tolerant plants: Energy metabolism and antioxidative stress defense. **Plant Science**, 2012.

DULLOO, M.E. et al. Cost efficiency of cryopreservation as a long-term conservation method for coffee genetic resources. **Crop Science**, v. 49, n. 6, p. 2123-2138, 2009.

DUSSERT, S. et al. Biologie de la conservation des semences de caféiers: aspects fondamentaux et conséquences pratiques: une revue. **Cahiers Agricultures**, Les Ulis, v. 21, n. 2-3, p. 106-114, 2012.

DUSSERT, S. et al. Cryopreservation of *Coffea* (Coffee) In: TOWILL, L.E.T.; Bajaj, Y.PS. (Eds). **Biotechnology in Agriculture and Forestry**: Cryopreservation of Plant Germplasm II. v. 50, Berlin, Heidelberg: Springer Berlin Heidelberg. p. 224-233, 2002.

DUSSERT, S. et al. Cryopreservation of seed of four coffee species (*Coffea arabica, C. costatifructa, C. racemosa* and *C. sessiliflora*): importance of water content and cooling rate. **Seed Science Research**, Wageningen, v. 8, n. 1, p. 9-15, 1998.

DUSSERT, S. et al. Oxidative stress phospholipid loss and lipid hydrolysis during drying and storage of intermediate seeds. **Physiologia Plantarum**, Lund, v. 127, n. 2, p. 192-204, Apr., 2006.

DUSSERT, S. et al. Tolerance of coffee (*Coffea* spp.) seeds to ultra-low temperature exposure in relation to calorimetric properties of tissue water, lipid composition, and cooling procedure. **Physiologia Plantarum**, Malden, v. 112, n. 4, p. 495- 504, 2001.

DUSSERT, S. et al. Variability in storage response within a coffee (*Coffea* spp.) core collection under slow growth conditions. **Plant Cell Reports**, Heidelberg, v. 16, n. 5, p. 344-348, Feb., 1997.

DUSSERT, S.; ENGELMANN, F. New determinants for tolerance of coffee (*Coffea arabica* L.) seeds to liquid nitrogen exposure. **CryoLetters**, Cambridge v. 27, n. 3, p. 169-178, 2006.

EIRA, M.T.S.; REIS, R.B.; RIBEIRO, F.N.S. Banco de sementes de café em criopreservação: experiência inédita do Brasil, In: SIMPÓSIO DE PESQUISA DOS CAFÉS DO BRASIL, 4., 2005, Londrina. **Anais eletrônicos**... Brasília: Embrapa Café, 2005. 1 CDROM

EBONE, L.A.; CAVERZAN, A.; CHAVARRIA, G. Physiological alterations in orthodox seeds due to deterioration processes. **Plant Physiology and Biochemestry**, v. 145, p. 34-42, 2019.

EL-ENANY, A.E. et al. Role of antioxidant enzymes in amelioration of water deficit and waterlogging stresses on Vigna sinensis plants. **Journal of Biology and Earth Sicences**, Suchy Las, v. 3, n. 1, p. B144-B153, 2013.

ELLIS, R.H.; HONG, T.D.; ROBERTS, E.H. An intermediate category of seed storage behaviour?: I. Coffee. **Journal of Experimental Botany**, Oxford, v. 41, n. 9, p. 1167-1174, Sept., 1990.

ENGELMANN, F. Plant cryopreservation: progress and prospects. In Vitro Cellular and Developmental Biology - **Plant,** New York, v. 40, n. 5, p. 427433, Sept., 2004.

FAGAN, E.B. et al. Efeito do tempo de formação do grão de café (*Coffea* sp) na qualidade da bebida. **Bioscience Journal**, Uberlândia, v. 27, n. 5, p. 729-738, set./out., 2011.

FÁVARIS, N.A.B. et al. Protein profile in arabica coffee seeds in electrophoresis gel: importance of freeze-drying. **Journal of Seed Science**, Londrina, v.44 e202244017, 2022.

FERREIRA, A.G.; BORGHETTI, F. (Orgs.). **Germinação: do básico ao aplicado**. Porto Alegre: Artmed, 2004, 324 p.

FERREIRA D.F. Sisvar: A guide for its bootstrap procedures in multiple comparisons. **Ciência e Agrotecnologia**, Lavras, v. 38, n. 2, p. 109-112, 2014.

FIGUEIREDO, M.A. et al. Exploratory studies for cryopreservation of *Coffea arabica* L. seeds. **Journal of Seed Science**, Londrina, v.39, n.2, p.150-158, 2017.

FIGUEIREDO, M.A. et al. Physiological, biochemical, and ultrastructural aspects of *Coffea arabica* L. seeds under different cryopreservation protocols. **Ciência e Agrotecnologia**, Lavras, v. 45:e027020, 2021.

FIGUEIREDO, M.A.**Secagem e resfriamento de sementes de *Coffea arabica* L. visando à criopreservação.**2016. 199 p. Tese (Doutorado em Agronomia/Fitotecnia) -Universidade Federal de Lavras, Lavras, 2016.

FRANCA, M.B.; PANEK, A.D.; ELEUTHERIO, E.C. Oxidative stress and its effects during dehydratation. **Comparative Biochemistry and PhysiologyPart A: Molecular & Integrative Physiology**, v. 146, n. 4, p. 621-631, Apr., 2007.

FRANÇA NETO, J. B.; KRZYZANOWSKI, F. C.; COSTA, N. P. Metodologia do teste de tetrazólio em sementes de soja. In: KRZYZANOWSKI, F. C.; VIEIRA, R. D.; FRANÇA NETO, J. B. **Vigor de sementes**: Conceitos e testes. Londrina: ABRATES. Cap 8.5, p.1-27, 1999.

FREITAS, F.A. et al. Absorção de P, Mg, Ca e K e tolerância de genótipos de arroz submetidos a estresse por alumínio em sistemas hidropônicos. **Ciência Rural**, v. 36, n. 1, p. 72-79, 2006.

GONZALEZ-ARNAO, M.T. et al. Development and large scale application of cryopreservation techniques for shoot and somatic embryo cultures of tropical crops. **Plant Cell** Tissue and Organ Culture, Dordrecht, v. 92, n. 1, p. 1-13, 2008.

HARDING, K. Genetic integrity of cryopreserved plant cells: a review. **Cryo Letters**, Cambridge, v. 25, n. 1, p. 3-21, Jan/Fev., 2004.

INTEGRATED DNA TECHNOLOGIES. **OLIGOANALYZER 3.1 SOFTWARE**. Coralville, 2014. Disponível em: <https://www.idtdna.com/calc/analyzer>. Acesso em: 18 Set. 2022.

JENDEREK, M.; REED, B.M. Cryopreserved storage of clonal germplasm in the USDA National Plant Germplasm System. In vitro Cell and Developmental Biology – **Plant,** v. 53, p. 299-308, 2017.

JIANG, H. et al.. Global Analysis of Gene Expression Profiles in Developing Phisic Nut (*Jatropha curcas* L.) Seeds. **Plos One**, vol. 7, p. 1-12, 2012.

JOSÉ, A.C. et al. Effects of drying rate and storage time on *Magnolia ovata* Spreng. seed viability. **Seed Science and Technology**, Zurich, v. 39, n. 2, p. 425-434, 2011.

KARTHA, K.K. Meristem culture and germplasm preservation. In: _____ (Ed.). **Cryopreservation of plant cells and organs**. Boca Raton: CRC Press. p. 115-134. 1985.

KIM, Y.H.; KWAK, S.S. The role of antioxidant enzymes during leaf development. In: GUPTA, S.D. Reactive oxygen species and antioxidants in higher plants. Enfield: **Science Publishers**. p., 129-150. 2010.

KOHOMA, S. et al. Secagem e armazenamento de sementes de *Eugenia brasiliensis* LAM. (Grumixameira). **Revista Brasileira de Sementes**, Londrina, v. 28, n. 1, p. 72-78, 2006.

KOVALCHUK, I. Multiple roles of radicals in plants. In: GUPTA, S.D. Reactive oxygen species and antioxidants in higher plants. Enfield: **Science Publishers**. p. 31-44, 2010.

KRZYZANOWSKY, F.C.; FRANÇA NETO, J.B.; HENNING, A.A. Relatos dos testes de vigor disponíveis para as grandes culturas. **Informativo ABRATES**, Brasília, v. 1, n. 2, p. 15-50, mar., 1991.

KUMAR, J.S.P.; et al. Seed birth to death: dual functions of reactive oxygen species in seed physiology. **Annals of Botany**, v. 116, n. 4, p. 663- 668, 2015.

LI C; SUN W. Desiccation sensitivity and activities of free radical-scavenging enzymes in recalcitrant *Theobroma cacao* seeds. **Seed Sci Res** 9:207-217, 1999.

LIMA, W.A.A.De. . et al. Retardamento de colheita como método de diferenciação de genótipos de soja para qualidade de sementes. **Revista Brasileira de Sementes**, v. 29, n. 1, p. 186–192, 2007.

LIU, B.; SOUNDARARAJAN, P.; MANIVANNAN, A. Mechanisms of Silicon-Mediated Amelioration of Salt Stress in Plants. **Plants**, Basel, v. 8, p. 307, 2019.

LIU, J.; PHY, J.; YEOMANS, E. Theoretic considerations regarding slow cooling and vitrification during cryopreservation. **Theriogenology**, v. 78, n.8, p. 1641-1652, 2012.

MALONE, G. et al. Expressão diferencial de isoenzimas durante o processo de germinação de sementes de arroz em grandes profundidades de semeadura. **Revista Brasileira de Sementes**, Londrina, v. 29, n. 1, p. 61-67, 2007.

MALTA, M.R.; PEREIRA, R.G.F.A.; CHAGAS, S.J. de R. Condutividade elétrica e lixiviação de potássio do exsudato de grãos de café: alguns fatores que podem influenciar essas avaliações. **Ciência e Agrotecnologia**, Lavras, v. 29, n. 5, p. 1015-1020, set./out., 2005.

MARCOS-FILHO, J. **Fisiologia de sementes de plantas cultivadas**. 2°. ed., Londrina: ABRATES. 660p. 2015.

MATTHÄUS, B. Oxidation of edible oils. In: **Oxidation in foods and beverages and antioxidant applications.** Woodhead Publishing. p. 183–238,2010.

MAZUR, P. Principles of cryobiology. In: FULLER, B. J.; LANE, N.; BENSON, E. E. (Eds.). **Life in the frozen state**. 1st ed. Boca Raton: CRC Press. cap. 1, p. 3-65, 2004.

McDONALD, M.B. Seed deterioration: physiology, repair and assessment. **Seed Science and Technology**, Wageningen, v. 27, n. 1, p. 177-237, 1999.

MEHDY, M.C.; et al. The role of activated oxygen species in plant disease resistance. **Physiologia Plantarum** 98:365-74. 1996.

MITTLER, R. Oxidative stress, antioxidants and stress tolerance. **Trends in Plant in Science**, Cambridge, v. 7, n. 9, p. 405-410, 2002.

NAKADA, P.G. et al. Desempenho durante o armazenamento de sementes de pepino submetidas a diferentes métodos de secagem. **Revista Brasileira de Sementes**, Londrina, v. 32, n. 3, p. 42-51, 2010.

NASS, L.L. et al. Pré-melhoramento de germoplasma vegetal. In: NASS, L. L. (Org). **Recursos genéticos vegetais**. Brasília: Embrapa Recursos genéticos e Biotecnologia. p. 683-716. 2008.

NIKISHINA, T.V. et al. Cryopreservation of seeds and protocorms of rare temperate orchids. **Russian Journal of Plant Physiology**, New York, v. 54, n. 1, p. 121–127, 2007.

NKANG, A.; OMOKARO, D.; EGBE, A. Effects of desiccation on the lipid peroxidation and activities of peroxidase and polyphenoloxidase in seeds of *Telfairia occidentalis*. **Seed Science and Technology**, v.28, p.1-9, 2000.

OLIVEIRA, D.A. et al. Lipoxigenases e teor de ácido linolênico relacionados à qualidade de sementes de soja. **Revista Brasileira de Sementes**, v. 28, n.1, p. 30-35, 2006.

PAMMENTER, N.W.; BERJAK, P. Physiology of desiccation-sensitive (recalcitrant) seeds and the implications for cryopreservation. **International Journal Plant Science**, Chicago, v. 175, n. 1, p. 21-28, Jan., 2014.

PANIS, B.; PIETTE, B.; SWENNEN, R. Droplet vitrification of apical meristems: A cryopreservation protocol applicable to all Musaceae. **Plant Science**, Limerick, v. 168, n. 1, p. 45–55, 2005.

PANIS, B.; SWENNEN, R.; ENGELMAN, F. Cryopreservation of plant germplasm. In: International Symposium on In Vitro Culture and Horticultural Breeding, 4., 2001, Tampere. **Proceedings**... Tampere: ISHS Acta Horticulturae, v. 650, p. 79-86, 2001.

PAUL, S.; ROYCHOUDHURY, A. Seed priming with spermine and spermidine regulates the expression of diverse groups of abiotic stress-responsive genes during salinity stress in the seedlings of indica rice varieties. **Plant Gene**, Kidlington v. 11, p. 124-132, 2017.

PFAFFL, M.W. et al. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper - Excel-based tool using pair-wise correlations. **Biotechnology Letters**, v.26, p. 509–515, 2004.

PIRES, R. et al. Action of nitric oxide in sesame seeds (*Sesamum indicum* L.) submitted to stress by cadmium. **Journal of Seed Science**, Londrina, v. 38, n.1, p. 022-029, 2016.

PRETE, C.E.C. **Condutividade elétrica do exsudato de grãos de café (*Coffea arabica* L.) e sua relação com a qualidade da bebida**. 1992. 125 f. Tese (Doutorado em Fitotecnia) - Escola Superior de Agricultura "Luiz de Queiroz", Piracicaba, 1992.

PRITCHARD, H.W. Cryopreservation of desiccation-tolerant seeds. In: DAY, J. G.; STACEY, G. N. (Eds.). Cryopreservation and freeze-drying protocols. New Jersey: **Human Press Inc Totowa**. p. 185-201, 2007.

PRUDENTE, D.O. et al. Germinação in vitro e criopreservação de sementes de paineira-rosa. **Scientia Agraria Paranaensis**. v.15, n.3, p.272- 276, 2016.

RAO, N.K. Plant genetic resources: Advancing conservation and use through biotechnology. **African Journal of Biotechnology**, v. 3, n. 2, p.136-145, Feb., 2004.

REED, B.M. Plant cryopreservation: a continuing requirement for foos and ecosystem security. In vitro Cell and Developmental Biology – **Plant,** v. 53, p. 285-288, 2017.

REINATO, C.H.R. et al. Influência da secagem, em diferentes tipos de terreiro, sobre a qualidade do café ao longo do armazenamento. **Coffee Science**, Lavras, v. 2, n. 1, p. 48-60, jan./jun., 2007.

ROACH, T. et al. An oxidative burst of superoxide in embryonic axes of recalcitrant sweet chestnut seeds as induced by excision and desiccation. **Physiologia Plantarum**, Malden, v. 133, n. 2, p. 131-139, June, 2008.

ROSA, S.D.V.F. et al. Effects of different drying rates on the physiological quality of *Coffea canephora* Pierre seeds. **Brazilian Journal of Plant Physiology**, Piracicaba, v. 17, n. 2, p. 199-205, 2005.

SAATH, R. et al. Activity of some isoenzymatic systems in stored coffee grains. **Ciência e Agrotecnologia**, Lavras, v. 38, n. 1, p. 15-24, Jan./Feb., 2014.

SAATH, R. et al. Microscopia eletrônica de varredura do endosperma de café (*Coffea arabica* L.) durante o processo de secagem. **Ciência e Agrotecnologia**, Lavras, v. 34, n. 1, p. 196-203, jan./fev., 2010.

SANT, R. et al. Cryopreservation of shoot-tips by droplet vitrification applicable to all taro (*Colocasia esculenta* var. esculenta) accessions. **Plant Cell**, Tissue and Organ Culture, Dordrecht, v. 92, n. 1, p. 107-111, 2008.

SANTOS, C.A. et al. Germinação de duas espécies da caatinga sob déficit hídrico e salinidade**.** **Pesquisa Florestal Brasileira**, Colombo, v. 36, v. 87, p. 219-224, 2016

SANTOS, G.C.; VON PINHO, E.V.R.; ROSA, S.D.V.F. Gene expression of coffee seed oxidation and germination processes during drying. **Genetics and Molecular Research**, Ribeirão Preto, v. 12, n. 4, p. 6968-6982, Dec., 2013.

SANTOS, I.R.I. Criopreservação de eixos embrionários de espécies de Citrus usando encapsulamento e desidratação. Brasília: **Embrapa Recursos Genéticos e Biotecnologia**, (EMBRAPA-CERNAGEN. Documentos, 115). 23p. 2004.

SANTOS, I.R.I. Criopreservação: potencial e perspectivas para a conservação de germoplasma vegetal. **Revista Brasileira de Fisiologia Vegetal**, Londrina, v.12,p. 70-94, 2000.

SANTOS, R.S. et al. Efeito do congelamento na qualidade fisiológica de semente de angico (*Anadenanthera colubrina*) (Vell.) Brenan var. Cebil (Griseb) Altshul – Fabaceae – Mimisoideae) com diferentes teores de água. **Heringeriana**. v.6. n.1, p.80-81, 2012.

SCHMITTGEN, T.D.; LIVAK, K. J. Analyzing real-time PCR data by the comparative CT method. **Nature Protocols**, London, v. 3, n. 6, p. 1101-1108, June, 2008.

SHARMA, P. et al Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. **Journal of Botany**, [s.l.], p. 1-26, 2012.

SILVA, L.M.M.; MATA, M.E.R.M.C.; DUARTE, M.E.M. Teor de água limite para crioconservação de sementes de romã (*Punica granatum*L.). **Engenharia Agrícola**. V.35, n.2, p.313-321, 2015.

SILVEIRA, É.D. et al. Selection of reference genes for quantitative real-time PCR expression studies in the apomictic and sexual grass Brachiaria brizantha. **BMC Plant Biology**, v. 9, n. 1, p. 84, 2009.

SILVER, N. et al. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. **BMC Molecular Biology**, v.7, p.1–9, 2006.

SMITH, A.M. et al. **Plant Biology**. Garland Science. p. 679. 2009.

STEPONKUS, P.L.; UEMURA, M.; WEBB, M.S. The contrast of the cryostability of the plasma membrane of winter rye and spring oat – two species that widely differ in their freezing tolerance and plasma membrane lipid composition. In: STEPONKUS, P.L. (ed.). **Advances in low temperature biology**, vol. 2, London: JAI Press., p. 211–312. 1993.

SUGDEN, D.; WINTER, P. **Molecular biomethods handbook**. Berlin: Springer, 2008.

TAIZ, L. et al. **Fisiologia e desenvolvimento vegetal**. 6. ed. Porto Alegre: Artmed. 858 p. 2017.

TAIZ, L.; ZEIGER, E. **Fisiologia vegetal** 3.ed. Porto Alegre: Artmed, 719p. 2004.

THAMMASIRI, K.; SOAMKUL, L. Cryopreservation of *Vanda coerulea* Griff. ex Lindl. seeds by vitrification. **Science Asia**, Bangkok, v. 33, n. 2, p. 223–227, 2007.

TRIGIANO, R. N.; GRAY, D. J. **Plant tissue culture, development and biotechnology**. London: CRC Press. 583 p. 2011.

UMARANI, R.; AADHAVAN, E.K.; FAISAL, M.M. Understanding poor storage potential of recalcitrant seeds. **Current Science**, v. 108, n. 11, p. 2023- 2034, June, 2015.

UNTERGASSER, A. et al. **PRIMER3 PLUS SOFTWARE**. Boston, 2007. Disponível em: <https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>. Acesso em: 18 Set. 2022.

USHIMARU, T. et al. Antioxicidative enzymes in seedlling of Nelumbo nucifera germinated under water. **Physiologia Plantarum**, v.112, n.1, p.39- 46, may., 2001.

VAN DER VOSSEN, H.; BERTRAND, B.; CHARRIER, A. Next generation variety development for sustainable production of arabica coffee (*Coffea arabica* L.): a review. **Euphytica**, Wageningen, v. 204, n. 2, p. 243-256, 2015.

VANDESOMPELE, J. et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. **Genome Biology**, v.3, p.1–12, 2002.

VARGAS, L.H.G. **Prospecção de Gens de Tecido Específico e Metabólitos em Elaeis spp.** 2014. 139 p. Dissertação (Mestrado em Biotecnologia Vegetal) - Universidade Federal de Lavras, Lavras, 2014.

VEIGA, A.D. et al. Armazenabilidade de sementes de cafeeiro colhidas em diferentes estádios de maturação e submetidas a diferentes métodos de secagem. **Revista Brasileira de Sementes**, Londrina, v. 29, n. 1, p. 83-91, abr. 2007.

VELOSO, J.S.**Avaliacão molecular de sementes de café submetidas à criopreservação**. 2017. 56 p. Dissertação (Mestrado em Agronomia/Fitotecnia)- -Universidade Federal de Lavras, Lavras, 2017.

VERMA, K.K. et al. Photosynthetic gas exchange, chlorophyll fluorescence, antioxidant enzymes, and growth responses of Jatropha curcas during soil flooding. **Turkish Journal of Botany, Ankara**, v. 38, p. 130-140, 2014.

VIEIRA, A.R. et al. Armazenamento de sementes de cafeeiro: ambientes e métodos de secagem. **Revista Brasileira de Sementes**, Londrina, v. 29, n. 1, p. 76 82, abr., 2007.

VOLK, G.M. Application of Functional genomics and Proteomics to Plant Cryopreservation. **Current Genomics**, Hilversum, v. 11, n. 1, p. 24-29, Mar. 2010.

VOLLMER, R. et al. A large-scale viability assessment of the potato cryobank at the International Potato Center (CIP). In Vitro Cell and Developmental Biology **– Plant**, v. 53, p. 309-317, 2017.

WALTERS, C. Orthodoxy, recalcitrance and in-between: describing variation in seed storage characteristics using threshold responses to water loss. **Planta**, Berlin, v. 242, n. 2, p. 397-406, 2015.

WANG, P. et al. Delayed senescence of apple leaves by exogenous melatonin treatment: toward regulating the ascorbate–glutathione cycle. **Journal of Pineal Research**, v. 53, n. 1, p. 11-20, 2012.

WESLEY-SMITH, J.et al. 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. **Annals of Botany,** London, v. 113, n. 4, p. 695-709, 2014.

WESLEY-SMITH, J. et al. Why is intracellular ice lethal? A microscopical study showing evidence of programmed cell death in cryo-exposed embryonic axes of recalcitrant seeds of Acer saccharinum. **Annals of Botany**, London, v. 115, n. 6, p. 991-1000, 2015.

WHITAKER, C. et al. Production of reactive oxygen species in excised and cryopreserved explants of *Trichilia dregeana* Sond. **South African Journal of Botany**, Matieland, v. 76, n. 1, p. 112-118, Jan., 2010.

WILSON, P.W.; HENEGHAN, A.F.; HAYMET, A.D.J. Ice nucleation in nature: supercooling point (SCP) measurements and the role of heterogeneous nucleation. **Cryobiology**, San Diego, v. 46, n. 1, p. 88-98, Feb. 2003.

XIE, F. et al. . miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. **Plant Molecular Biology**, v.80, p.75–84, 2012.

# APPENDIX A

## Analysis of variance table

Table 1. Summary of the analysis of variance of the physiological evaluations of cryopreserved *Coffea canephora* seeds.

| Response variables | Drying (D) | Temperature (TºC) | Time (T) | D * TºC | D * T | TºC * T | D * TºC * T | CV (%) |
|---|---|---|---|---|---|---|---|---|
| Radicle protusion | | | | | | | | 32.72 |
| Germination | | | | * | | * | | 40,06 |
| Strong normal seedlings | * | | | | | * | | 54,46 |
| Viability | * | | | | | * | | 15,04 |
| Electrical conductivity | | | | | | | * | 25,81 |

*Significant at the 5% level of probability, by the Scott-Knott test.