UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
Departamento de Botânica
Programa de Pós-Graduação em Biologia Vegetal

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LEILANE CARVALHO BARRETO

ASPECTOS FISIOLÓGICOS RELACIONADOS AO ARMAZENAMENTO DE FRUTOS E ENVELHECIMENTO DE SEMENTES DE MACAÚBA [*Acrocomia aculeata* (JACQ.) LODD. EX MART.]

Tese apresentada ao Programa de Pós-Graduação em Biologia Vegetal do Departamento de Botânica do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de Doutor em Biologia Vegetal.

Área de Concentração: Fisiologia Vegetal

BELO HORIZONTE – MG

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Área de Concentração: Fisiologia Vegetal

Orientador: Profª. Drª. Queila de Souza Garcia
Universidade Federal de Minas Gerais

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RESUMO GERAL

Acrocomia aculeata (Jacq.) Lood. ex Mart., popularmente conhecida como macaúba, possui ampla ocorrência nas Américas, sendo considerada a palmeira de maior dispersão no território brasileiro e com ampla distribuição nas áreas de Cerrado. Devido ao grande potencial oleaginoso dos frutos, a espécie possui reconhecida importância econômica, podendo ser utilizada nas indústrias farmacêutica, alimentícia, cosmética e de biodiesel. Neste estudo objetivou-se analisar as alterações fisiológicas e bioquímicas que ocorrem em sementes durante o envelhecimento sob condições naturais e artificais. Foram testados os efeitos do armazenamento do fruto em diferentes condições (laboratório, viveiro, câmara fria e freezer) sobre o crescimento de embriões em cultura *in vitro* e avaliado o possível papel da vitamina E e de fitormônios relacionados à defesa como marcadores do crescimento do embrião. O perfil de ácidos graxos e as alterações dos principais compostos de reserva do mesocarpo e das sementes durante o armazenamento foram determinados. Foram avaliados também os efeitos do envelhecimento acelerado na viabilidade dos embriões, na peroxidação de lipídeos, na perda de eletrólitos, na formação de peróxido de hidrogênio (H_2O_2) e na atividade de enzimas antioxidantes no embrião após a embebição de sementes artificialmente envelhecidas. Os resultados mostraram que a condição de armazenamento em laboratório foi a mais eficiente para a manutenção da viabilidade dos embriões. A vitamina E, juntamente com o ácido abscísico, foram eficientes em proteger o endosperma e o embrião contra o estresse oxidativo durante o armazenamento, além de terem sido considerados marcadores do crescimento do embrião para a obtenção de plântulas *in vitro*. O perfil de ácidos graxos das diferentes estruturas dos frutos de macaúba não foi alterado durante o armazenamento, independentemente da condição avaliada. A composição de ácidos graxos do embrião é semelhante à do mesocarpo, com predominância de ácidos graxos monoinsaturados, enquanto no endosperma predominam os ácidos graxos saturados. O processo de envelhecimento acelerado revelou que existe uma relação entre a perda de viabilidade das sementes, a peroxidação lipídica e os danos de membrana durante o envelhecimento. A produção de H_2O_2 observada em embriões embebidos após o envelhecimento acelerado mostrou que o sistema antioxidante enzimático não foi completamente eficiente na remoção das espécies reativas de oxigênio durante a embebição. Essas informações contribuem para que frutos e sementes de macaúba sejam armazenados em condições que minimizam os

danos causados pelo envelhecimento, mantendo a viabilidade do embrião por mais tempo.

Palavras-chave: *Acrocomia aculeata*, antioxidantes, armazenamento, envelhecimento

ABSTRACT

Acrocomia aculeata (Jacq.) Lood. ex Mart., popularly known as macaúba, is widely spread in the Americas and considered the palm tree most widely distributed in Brazil, existing largely in Cerrado's areas. Due to the large oleaginous potential of its fruits, the economic importance of the species is recognized and it may be used in the pharmaceutical, cosmetics, food and biodiesel industries. This study aimed to analyze the physiological and biochemical changes occurring in macaw palm seeds during ageing under natural and artificial conditions. The effects of fruit storage under different conditions (laboratory, nursery, cold chamber and freezer) were tested on the embryo growth cultured *in vitro,* and it was evaluated the possible role of vitamin E and defense related phytohormones as markers of embryo growth. Fatty acids profile and changes in the main seed reserve compounds were determined. We also evaluated the effects of accelerated ageing on macaw palm embryos viability, on lipid peroxidation, on electrolyte leakage, on hydrogen peroxide (H_2O_2) production and on antioxidant enzymes activity in the embryo after imbibition of artificially aged seeds. Results showed that storing fruits in laboratory was the most efficient method for maintaining embryo viability. The antioxidants mechanisms in the seeds (vitamin E), along with abscisic acid were effective in protecting the endosperm and the embryo against oxidative stress during storage, and were considered good indicators of embryo growth to obtain plantlets *in vitro*. Fatty acids profile of the different fruit structures did not change during storage, regardless the condition evaluated. The fatty acids composition of the embryo was very similar to that of the mesocarp, predominating monounsaturated fatty acids, while in the endosperm, saturated fatty acids are predominant. Accelerated ageing showed that loss of seed viability, lipid peroxidation and membrane damage during ageing are related. The production of H_2O_2 in imbibed embryos after ageing revealed that the enzymatic antioxidant system was not completely efficient in removing reactive oxygen species during imbibition. Such information contributes to storage macaw palm fruits in conditions that minimize the damages caused by ageing, maintaining embryo viability longer.

Key words: *Acrocomia aculeata,* ageing, antioxidants, storage

INTRODUÇÃO GERAL

Acrocomia aculeata (Jacq.) Lood. ex Mart. é uma palmeira com ampla distribuição geográfica, podendo ser encontrada desde o sul do México ao sul do Brasil, Paraguai e Argentina (Azevedo Filho et al. 2012), em regiões com precipitação entre 1500 e 2000 mm, temperaturas entre 15 e 35 ºC e altitudes entre 150 e 1000 m (Motta et al. 2002). É considerada a palmeira de maior dispersão no território brasileiro, encontrando-se amplamente distribuída pelas áreas de Cerrado, com as maiores concentrações localizadas em Minas Gerais, Goiás, Mato Grosso e Mato Grosso do Sul (Henderson et al. 1997; Azevedo Filho et al. 2012; Borcioni 2012). No Brasil é popularmente conhecida como macaúba, bocaiuva, coquinho de catarro, coco de espinho, macacauba, macaíba, macaibeira, macajuba, macaúva, mucaia, mucajá e mucajaba ou outros nomes, dependendo da região de distribuição (Fig. 1a) (Azevedo Filho et al. 2012; Borcioni 2012).

Os frutos, cuja produção ocorre entre os meses de setembro e janeiro, dependendo das condições climáticas, são esféricos ou ligeiramente achatados, em forma de drupa globosa com diâmetro de 2 a 5 cm (Fig. 1b). O mesocarpo é fibroso e mucilaginoso e possui coloração amarela (Henderson et al. 1997). A espécie tem potencial de produzir até 30 toneladas de frutos por hectare por ano (Lopes et al. 2013; Lescano et al. 2015), os quais constituem o produto de maior importância econômica, devido principalmente ao seu potencial oleaginoso, cuja produtividade pode chegar a quatro toneladas de óleo por hectare (César et al. 2015). O óleo derivado do mesocarpo é destinado primeiramente à indústria de cosméticos e tem sido estudado para suprir a cadeia de produção de biodiesel no Brasil. Por outro lado, este óleo é altamente perecível, estando sujeito ao crescimento de microrganismos e à deterioração devido a reações físico-químicas (Ciconini 2013). Da semente é extraído um óleo de alta qualidade, que representa cerca de 10% do total de óleo da planta e tem potencial para utilização na indústria alimentícia, farmacêutica, cosmética e de biodiesel (Bora and Rocha 2004; Hiane et al. 2006; Ramos et al. 2008; César et al. 2015).

Apesar de possuir diversos usos práticos, o estabelecimento de plantios comerciais da macaúba ainda é muito limitado devido às dificuldades de propagação da espécie, uma vez que as sementes apresentam dormência e a germinação ocorre lentamente e em baixas porcentagens (Bandeira 2008; Ribeiro et al. 2011). A macaúba produz sementes

ortodoxas e pode formar bancos de sementes do tipo persistente (Fig. 1c e 1d); o embrião possui grandes quantidades de lipídeos e proteínas de reserva, e a viabilidade é mantida quando as sementes são desidratadas a um conteúdo de água de 5% ou quando armazenadas a -20 ºC por 90 dias (Ribeiro et al 2012).

Sementes ortodoxas, no entanto, envelhecem e eventualmente perdem sua capacidade de germinar durante períodos prolongados de armazenamento (Murthy et al. 2003). Os dois fatores ambientais mais importantes que influenciam a velocidade dos processos de deterioração durante o envelhecimento de sementes são a umidade relativa do ar, que controla o conteúdo de água da semente, e a temperatura (Galleschi et al. 2002). Dentre as modificações observadas durante o envelhecimento de sementes, as mais comuns são a peroxidação lipídica, o rompimento de membranas celulares, as alterações nos ácidos nucleicos, a inativação de enzimas e a degradação de proteínas (Murthy et al. 2003; Arc et al. 2011). Muitos desses danos têm sido associados aos efeitos deletérios das espécies reativas de oxigênio (reactive oxygen species, ROS), devido à sua alta reatividade com biomoléculas (McDonald 1999; Arc et al. 2011).

As deteriorações bioquímicas que ocorrem durante o envelhecimento têm sido estudadas, principalmente, sob condições de envelhecimento acelerado. Este processo, inicialmente proposto como um método para avaliar a capacidade de armazenamento das sementes, é rápido, barato, simples e útil para a maioria das espécies (McDonald 1999). O envelhecimento acelerado é conduzido usando alta temperatura combinada com alta umidade e tem grande utilidade para o entendimento dos mecanismos do envelhecimento e dos processos de deterioração associados a ele. Sob tais condições, as sementes geralmente perdem a viabilidade em poucos dias ou semanas, e acredita-se que a peroxidação lipídica e a perda da integridade da membrana mediada por ROS sejam também as principais causas do envelhecimento de sementes sob condições artificiais (Murthy et al. 2003).

Espécies reativas de oxigênio são naturalmente produzidas em plantas durante os processos metabólicos normais e atuam como moléculas sinalizadoras nos processos de crescimento e desenvolvimento (Bhattachrjee 2005; Foyer and Noctor 2005). No entanto, o equilíbrio entre a produção e a remoção de ROS pode ser perturbado por vários fatores abióticos adversos, como alta intensidade de luz, seca, baixas ou altas temperaturas, estresses mecânicos, etc (Apel and Hirt 2004). Essas situações de produção excessiva de ROS são caracterizadas como estresse oxidativo, e também são

componentes essenciais do repertório de sinalizações que as plantas usam para fazer os ajustes apropriados de expressão gênica e estrutura celular em resposta aos fatores adversos (Foyer and Noctor 2005).

As plantas possuem diferentes mecanismos de controle e remoção de ROS, os quais as protegem das reações oxidativas destrutivas sob condições de estresse. Esses mecanismos podem ser tanto não enzimáticos como enzimáticos (Arora et al. 2002; Blokhina 2003). Os sistemas de defesa não enzimáticos consistem de compostos de baixo peso molecular, como o ácido ascórbico e a glutationa, os quais são solúveis em água, enquanto os antioxidantes lipofílicos, como tocoferois, tocotrienois e carotenoides são ativos na membrana (Waskiewicz et al. 2014). Com relação aos sistemas antioxidantes enzimáticos, as principais enzimas envolvidas nos processos de remoção de espécies reativas de oxigênio são a superóxido dismutase (SOD), catalase (CAT), ascorbato peroxidase (APX) e glutationa redutase (GR) (Blokhina 2003).

Além disso, é possível que alguns fitormônios também estejam envolvidos na defesa contra o estresse oxidativo em sementes. O ácido salicílico, ácido jasmônico e ácido abscísico, em particular, têm sido associados a respostas de defesa contra os processos de estresse oxidativo, apesar dos mecanismos de ação desses hormônios ainda não estarem completamente elucidados (Anderson et al. 2004; Bari and Jones 2009; Barba-Espin et al. 2010).

O objetivo principal neste estudo foi analisar as alterações fisiológicas e bioquímicas que ocorrem em sementes de macaúba durante o envelhecimento sob condições naturais e artificais (Fig. 2). Os resultados das investigações foram organizados em três capítulos. No primeiro, intitulado "Vitamin E and defense-related phytohormones are reliable markers of embryo growth in macaw palm fruits exposed to various storage conditions" foram testados os efeitos do armazenamento durante um ano em quatro diferentes condições sobre o crescimento de embriões em cultura *in vitro* e avaliados os possíveis usos da vitamina E e de fitormônios relacionados à defesa como marcadores do crescimento do embrião de macaúba. No segundo capítulo, "Dynamic of reserve compounds of mesocarp and seeds of macaw palm (*Acrocomia aculeata*) submitted to different storage conditions" foram analisadas as alterações dos principais compostos de reserva de frutos e sementes de macaúba armazenados durante um ano em diferentes condições, além de ter sido determinado o perfil de ácidos graxos do mesocarpo, endosperma e embrião durante o armazenamento. No terceiro capítulo, "Accelerated

ageing and subsequent imbibition affect seed viability and the efficiency of antioxidant system in macaw palm seeds" foram avaliados os efeitos do envelhecimento acelerado em embriões de macaúba e a sua influência na viabilidade e vigor das sementes (peroxidação de lipídeos e perda de eletrólitos), na formação de peróxido de hidrogênio e na atividade das enzimas antioxidantes durante a embebição de sementes artificialmente envelhecidas.

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Figura 1. **A.** Vista geral da macaúba no *campus* da UFV em Florestal, MG (Foto: Leilane Barreto). **B.** Frutos maduros de macaúba (Foto: Biodieselbr). **C.** Vista longitudinal de fruto aberto de macaúba com detalhe das sementes (Foto: Rubio Neto, 2012). **D.** Vista longitudinal do fruto e da semente abertos (Foto: Paulo Hilst).

Figura 2 A. Vista do armazenamento em viveiro. **B.** Vista do armazenamento em laboratório. **C.** Vista do armazenamento em câmara fria. (Fotos: Elisa Bicalho)

CAPÍTULO 1

Vitamin E and defense-related phytohormones are reliable markers of embryo growth in Macaw Palm fruits exposed to various storage conditions

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ABSTRACT

The fruit of the macaw palm (*Acrocomia aculeata*) may be used for biofuel production, but its exploitation as a crop is currently limited by its low germinability. Therefore, obtaining plantlets *in vitro* is an excellent way to solve this problem. Here we aimed to identify the optimal conditions for storing the fruit before obtaining plantlets and testing to what extent vitamin E and defense-related phytohormones are good indicators of embryo growth *in vitro*. We tested the effects of four storage conditions (nursery, laboratory, cold chamber and freezer) on seed germinability and embryo growth, and evaluated endogenous levels of vitamin E and defense-related phytohormones (abscisic acid, salicylic acid and jasmonic acid) in the endosperm and embryos. Low temperatures [both cold chamber (5 °C) and freezer (−18 °C) methods] killed the embryos, while storing the fruit in the laboratory was the most efficient method of obtaining plantlets, even after a year. Vitamin E and abscisic acid turned out to be good indicators of embryo growth. Enhanced vitamin E and abscisic acid levels had a strong positive correlation with successful embryo growth, thus indicating that these compounds are needed to protect the embryo during fruit storage. Furthermore, abscisic acid levels had a negative correlation with the percentage of contaminated embryos, thus suggesting that the endogenous physiological stage of the seeds affects subsequent contamination in *in vitro* cultures. We concluded that (1) storing fruit under laboratory conditions is the most efficient means of obtaining plantlets successfully, and (2) vitamin E and abscisic acid can be used as reliable indicators of embryo growth during *in vitro* culture.

Keywords: Abscisic acid; *Acrocomia aculeata*; Biofuel; Fruit storage; Seed embryo; Vitamin E

INTRODUCTION

The use of vegetable oils for the production of alternative fuels has been motivated by growing concerns about environmental issues, the rising price of fossil fuels and the rapid depletion of its reserves in the world. Biodiesel is an interesting alternative since its performance is similar to that of conventional fuel, it reduces greenhouse gas emissions due to being non-toxic, biodegradable and recyclable, and promotes job creation and social inclusion by empowering family agriculture (Leite and Leal 2007). The Macaw Palm (*Acrocomia aculeata* (Jacq.) Lodd. ex Martius) is a palm tree that is widely distributed in the Americas and is distinguished from other palm species due to its high productivity: its fruits generate more than five tons of oil per hectare per year (Ferrari and Azevedo Filho 2012). Aside from its high productivity and the quality of the oil extracted from the mesocarp and seed, the macaw palm has adapted to a wide range of environmental conditions, which makes it a potentially important agro-industrial species as a source of raw materials for biofuel production (Moura et al. 2008; Pires et al. 2013). However, macaw palm propagation is very difficult due to its high level of seed dormancy, which limits the creation of commercial plantations and exploitation of natural populations (Bandeira 2008). Experiments with *in vitro* culture of macaw palm embryos can be used to improve species propagation on a commercial scale for biofuel production (Moura et al. 2008; Ribeiro et al. 2012a). However, there is still some uncertainty about the effects of fruit storage conditions on the subsequent *in vitro* growth culture of macaw palm embryos.

High temperatures and humidity during storage generally favor fruit and seed deterioration (Rao et al. 2006). Long storage periods induce seed deterioration by ageing processes associated with biochemical changes that result in the weakening or loss of membrane integrity, caused mainly by reactive oxygen species accumulation and increased lipid peroxidation (Bailly 2004; Parkhey et al. 2012). Seeds have antioxidant mechanisms that ensure cells are protected against reactive oxygen species (Goel et al. 2003). Antioxidants can be enzymatic or non-enzymatic, and some of the most effective inhibitors of lipid peroxidation propagation in biological membranes are tocopherols and tocotrienols, which are lipid-soluble molecules belonging to the group of vitamin E compounds (Falk and Munné-Bosch 2010). Both tocopherols and tocotrienols may be present in four forms (α, β, γ and δ) in seeds, and tocotrienols are the major compounds in seeds of palm trees (Siles et al. 2013). Some phytohormones are also likely to be

involved in defense against oxidative stress in seeds. Salicylic acid, jasmonic acid and abscisic acid in particular have been associated with defense responses against oxidative stress processes in seeds (Bailly et al. 2008; Barba-Espín et al. 2010).

In previous studies, Ribeiro et al. (2011, 2012a, b) examined the effects of hormonal treatments for overcoming seed dormancy in the macaw palm, changes that show embryos at ultrastructural level during germination and treatments for seed storage in this species. In the latter study, fruits were also kept in the open for one year under natural conditions, or were buried, left in the shade or stored in a cold chamber (10 ºC) for 12 months. Storing the fruits at 10 ºC maintains 85 % embryo viability, has the advantage of not requiring prior processing and can thus be used for ex situ conservation (Ribeiro et al. 2012b). Here we tested additional fruit storage conditions that may affect the growth of embryos in in vitro culture and aimed to evaluate the possible use of vitamin E and defense-related phytohormones as markers of successful embryo growth. This information will help provide the optimal conditions for storing macaw palm fruit before obtaining plantlets, and contribute to the generation of technologies that support the sustainable use of this species for biofuel production.

MATERIALS AND METHODS

Plant material, treatments and sampling

Mature fruits of *A. aculeata* were collected immediately after their natural abscission from a natural population in Montes Claros, Minas Gerais, Brazil (16°42'34"S; 43°52'48"W) between six and eight February 2012. The fruits, which were obtained from at least 100 individuals, were pooled and immediately used for experiments. The fruits were randomly divided into four groups and stored at the Universidade Federal de Viçosa, in Florestal, Minas Gerais, Brazil (19º52'59"S; 44º25'16"W) under the following conditions: (1) nursery, exposed to rainfall and natural climatic conditions; (2) laboratory, at room temperature (25 ºC); (3) cold chamber at 5 °C and (4) freezer at -18 ºC. For the nursery, laboratory and cold chamber conditions, samplings were performed at 0, 45, 90, 180, 270 and 360 days of storage. For the freezer method, samples were collected at 180 and 360 days of storage. Time 0 corresponds to the freshly harvested, immediately processed fruits. For nursery and laboratory treatments, fruits were kept in polyethylene bags; for cold chamber and freezer treatments, fruits

were kept in polypropylene bags. For each time and storage condition four bags, containing approximately 180 fruits each, were used.

At the end of each storage time, the fruits were removed from each condition. The exocarp was then broken with the aid of a hammer, and the seeds were extracted from the fruits using a manual bench-top vise. Some of the seeds were immediately used for germination tests and *in vitro* embryo culture. The endosperm and embryo were obtained from the rest of the seeds and kept in the freezer (-18°C) until biochemical analyses.

Seed germinability

Seeds were disinfected with a 6% solution of sodium hipochlorite for 15 min, followed by three rinses in running water. The germination test was conducted with four replicates of 25 seeds for each treatment and time, by maintaining the seeds in plastic boxes with autoclaved vermiculite with 90% field capacity for 15 days in a growth chamber at 30ºC. After that, the seeds were transferred to vermiculite with 50% of field capacity, and the germination was evaluated for 120 days.

***In vitro* embryo culture**

The *in vitro* embryo culture was performed with five replicates of ten embryos for each time and storage condition. Seeds were disinfected with a 6% solution of chlorine for 15 min, followed by three rinses in running water. In a laminar air flow chamber, embryos were extracted from the seeds using a scalpel and then disinfected with a 0.5% solution of sodium hypochlorite for 10 min, followed by three rinses in distilled and autoclaved water. The embryos were inoculated in test tubes of 12 x 1 cm, containing 2 mL of MS (Murashige and Skoog 1962) medium at 75% original strength (previously autoclaved at 121°C for 20 min). The test tubes containing the embryos were kept in the dark at a constant temperature of 24°C. After 30 days, germination, contamination and development stage of the embryos were evaluated.

Vitamin E and lipid peroxidation

The vitamin E composition of seeds, including both tocopherols and tocotrienols, was measured in methanol extracts by HPLC as described by Cela et al. (2011). In short, the seed samples were ground in liquid nitrogen and extracted with ice-cold methanol using a Branson 2510 Ultrasonic Cleaner (Bransonic) for 45 min. The samples were then

centrifuged for 15 min at 4ºC and transferred to vials for analysis. The HPLC equipment consisted of an integrated system with a Waters 600 controller pump, a Waters 714 plus auto-sampler and an FP-1520 fluorescence detector (Jasco). Tocopherols and tocotrienols were separated on an Inertsil 100A (5 mm, 30 250 mm, GL Sciences Inc.) normal-phase column operating at room temperature. The mobile phase used was a mixture of n-hexane and 1,4-dioxane (95,5 : 4.5, v/v) at a flow rate of 0,7 $ml \cdot min^{-1}$, and the injection volume was 10 µl. Detection was carried out for excitation at 295 nm and emission at 330 nm. Quantification was based on the fluorescence signal response compared with authentic standards of each compound (Sigma-Aldrich).

Levels of lipid peroxidation were estimated from the amount of malondialdehyde (MDA) equivalents in the seeds, following the method described by Hodges et al. (1999), that takes into account the possible effects of interfering compounds in the thiobarbituric acid-reactive substances (TBARS) assay.

Phytohormone analyses

The extraction and UPLC-MS/MS analyses of the endogenous concentrations of abscisic acid, salicylic acid and jasmonic acid were performed as described by Müller and Munné-Bosch (2001). Deuterium labelled phytohormones (d_6-abscisic acid, d_4-salicylic acid and d_5-jasmonic acid) were used as internal standards.

Statistical analyses

Differences in the time-course evolution of vitamin E, malondialdehyde and hormone levels in the endosperm and embryo of fruits stored under different conditions were tested by analysis of variance (ANOVA), using storage time as a factor. In addition, Spearman's rank correlation analyses (Bonferroni corrected) were used to correlate the parameters of embryo development (elongated, contaminated, without growth) with vitamin E, lipid peroxidation and hormone levels in the endosperm and embryo. All statistical tests were performed using SPSS package v.12 (Chicago, IL) and differences or correlations were considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

Storage of macaw palm fruits under laboratory conditions maintains the capacity for embryo growth

The germinability of macaw palm seeds is very low, and the germination capacity, which was around 7%, was not altered significantly during storage in the nursery, laboratory or cold chamber in the 360 evaluation days (data not shown). The seeds of the fruits kept in the freezer, however, did not germinate after storage, and therefore showed a significant reduction in germinability over time (ANOVA, P=0.002). The low germination levels of macaw palm seeds are related to physiological dormancy, as previously shown for this species (Ribeiro et al. 2011). The *in vitro* embryo culture test revealed that the percentage of developed, non-developed and contaminated embryos changed significantly over time in each of the storage conditions tested (ANOVA, P<0.001). Successful embryo development was reduced during storage in the nursery, particularly after 45 days and between 270 and 360 days (Fig. 1). In the laboratory, the percentage of elongated embryos remained high (>80%) after 360 days of storage. In both conditions, the percentage of contaminated embryos was low, except at 45 days. Cold chamber storage resulted in a reduction in the number of elongated embryos due to an increase in both contaminated and non-developed embryos. Embryos did not develop at all after storage in the freezer (Fig. 1). Laboratory storage conditions were therefore the most effective for obtaining plantlets in *in vitro* culture, followed by storage in the nursery, while storage at temperatures of 5 ºC or below (cold chamber and freezer) dramatically reduced embryo growth. Ribeiro et al. (2012b) showed that storing the fruits at 10 ºC for a year maintained 85 % embryo viability. Taken together, these findings demonstrate that embryo viability during fruit storage is maintained at temperatures ranging from 10 to 25 ºC. Therefore, storing fruits at these temperatures has the advantage of not requiring prior processing and can thus be used for *ex situ* conservation.

Vitamin E levels and extent of lipid peroxidation as markers of embryo growth

α-Tocopherol and all four forms of tocotrienols (α-, β-, γ- and δ-tocotrienol) were present in the embryo and endosperm. α-Tocopherol, followed by α -tocotrienol, was the most abundant vitamin E form in the embryo, while α- and γ-tocotrienols were the most abundant forms in the endosperm (Fig. 2). Levels of both α-tocopherol and α-tocotrienol in the embryo tended to decrease with time under all storage conditions, but

changes were only significant in the nursery and freezer (Table 1A). No significant changes in α- and γ-tocotrienol levels in the endosperm over time were observed, except in the cold chamber, where the levels of these compounds peaked at 180 days and decreased afterwards (Fig. 2, Table 1A). Tocopherols have been show to protect lipids against oxidation during storage in *Arabidopsis thaliana* (Sattler et al. 2004), and several palm trees accumulate very high levels of tocotrienols in seeds (in some is the only vitamin E form), which would suggest that tocotrienols may substitute tocopherols in seeds, at least in some cases (Siles et al. 2013). Here we show that vitamin E levels decrease with storage time in the embryo, but not in the endosperm, thus suggesting that the embryo suffers more oxidative stress than the endosperm during storage. Furthermore, α-tocopherol and α-tocotrienol levels in the embryo decreased in the nursery and cold chamber, but not in the laboratory, which would indicate that increased oxidative stress is responsible for reduced embryo growth in fruits stored in the nursery and cold chamber. In contrast, the reduced viability of embryos in the freezer appears to be unrelated to oxidative stress; vitamin E compounds were therefore not reliable indicators of embryo growth in the freezer. Since freezing reduces cellular metabolic activity, it is reasonable to suggest that oxidation processes such as vitamin E loss cannot be indicative of embryo growth reduction under this condition. A correlative analysis of all data (Table 2) revealed that all vitamin E forms present in the embryo, but not in the endosperm, have a strong positive correlation with successful *in vitro* growth (% elongated embryos), thus indicating that vitamin E levels can be used as a reliable marker of potential embryo growth.

The extent of lipid peroxidation, estimated as MDA concentrations, was generally low in both the endosperm and embryo, particularly in the latter (Fig. 3). MDA levels in the embryo tended to decrease with time, reaching non-detectable levels at 360 days in all storage conditions. MDA levels in the endosperm tended to be higher and more stable than those of the embryo. Results suggest that neither tissue suffered lipid peroxidation and that the embryo was protected from oxidative stress during storage. As embryos are more susceptible to oxidative damage and are also need to ensure the survival and germination of new plants, it is crucial that the antioxidant mechanisms provide this tissue with greater protection, since the endosperm is used by the embryo during germination (Bailly 2004; Falk et al. 2004). Thus, the strong positive correlation observed between vitamin E levels in the embryo and embryo growth indicates that high

vitamin E levels are needed to protect the embryo during storage of macaw palm fruits. Unexpectedly, MDA levels also positively correlated with embryo growth (Table 2). In this case, however, significant correlations were observed with the MDA levels of both the embryo and endosperm. Since lipid peroxidation did not increase during storage, this correlation may simply reflect the correlation between the amounts of fatty acids (MDA precursors) and embryo growth.

Defense-related phytohormones as markers of embryo growth

Among the hormones evaluated, abscisic acid was the only one with much (up to ninefold) higher levels in the embryo compared to the endosperm (Fig. 4). Abscisic acid levels remained constant over time in all storage conditions in the endosperm, while they decreased over time in all storage conditions, except in the laboratory (Table 1C). Correlation tests revealed a positive correlation between abscisic acid levels and embryo growth, and a negative correlation with the percentage of contaminated embryos (Table 2), thus suggesting that abscisic acid, along with vitamin E plays a role in protecting embryos during storage. The action of abscisic acid in plant-defense responses is not yet fully understood, and it can either be a positive or negative defense regulator against pathogenic fungi and bacteria, depending on the individual plant-pathogen interactions (Anderson et al. 2004; Bari and Jones 2009). The relation between abscisic acid and contamination suggests that the endogenous physiological status of the embryo affects subsequent contamination in *in vitro* culture, exerting in this case a protective role against biotic stress. It is interesting to note that contaminated embryos were not alive, but were all non-developing embryos, mostly because of fungal attack. Variability in the levels of contamination may respond to occasional surface sterilization problems or other manipulation effects.

Salicylic acid and jasmonic acid concentrations varied over time in all storage conditions, in both the embryo and the endosperm. Salicylic acid in the embryo decreased under all storage conditions, but also under laboratory conditions (Fig. 5). Therefore, salicylic acid levels in the embryo did not correlate with embryo growth (Table 2). Jasmonic acid levels in the embryo peaked after 180 days in all storage conditions, and decreased afterwards (Fig. 6). However, no correlation was found between jasmonic acid concentrations in the embryo and embryo growth (Table 2). Salicylic acid and jasmonic acid levels in the endosperm were, however, negatively correlated with embryo growth. Since both compounds play a defensive role against

bacteria and fungi, respectively (Davies 2010), it is tempting to speculate that they may serve as a first defense barrier against biotic stress in macaw palm seeds.

CONCLUSION

We concluded that the laboratory conditions were the most efficient for storing the fruits and successfully obtaining plantlets. Seeds did not suffer lipid peroxidation, and the embryo was protected during the storage, mainly by α- tocopherol and α-tocotrienol. Vitamin E and abscisic acid levels in the embryo may be used as reliable markers of embryo growth to obtain plantlets *in vitro*, an essential factor in the sustainable exploitation of the macaw palm for biofuel production.

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Table 1 *P* values of the analysis of variance (ANOVA) of the time course evolution of vitamin E (A), malondialdehyde (B) and hormone (C) levels in the endosperm and embryo of fruits stored under different conditions.

(A)		Nursery	Laboratory	Cold Chamber	Freezer
Endosperm	**αT**	-	-	-	-
	αTT	NS	NS	0,030	NS
	βTT	NS	NS	NS	NS
	γTT	NS	NS	0,032	NS
	δTT	NS	NS	NS	NS
Embryo	**αT**	0,004	NS	NS	0,030
	αTT	0,029	NS	NS	0,021
	βTT	< 0,001	< 0,001	0,003	< 0,001
	γTT	< 0,001	< 0,001	0,014	0,001
	δTT	-	-	-	-

(B)	Nursery	Laboratory	Cold Chamber	Freezer
Endosperm	NS	< 0,001	< 0,001	0,003
Embryo	NS	0,022	0,008	0,002

(C)		Nursery	Laboratory	Cold Chamber	Freezer
Endosperm	**ABA**	NS	NS	NS	NS
	SA	0,012	0,002	0,005	< 0,001
	JA	0,005	NS	NS	NS
Embryo	**ABA**	0,001	NS	< 0,001	< 0,001
	SA	0,010	<0,001	<0,001	0,035
	JA	<0,001	<0,001	<0,001	0,004

T, tocopherol; TT, tocotrienol; ABA, abscisic acid; SA, salicylic acid; JA, jasmonic acid. NS, not significant

Table 2 Correlation coefficient (r^2) and P-values (shown in parentheses) of Spearman rank correlation analysis (Bonferroni corrected) to correlate the parameters of embryo development (elongated, contaminated, without growth) with tocopherol (αT), tocotrienol (αTT, βTT, γTT, δTT), lipid peroxidation (MDA) and hormone (ABA, SA and JA) levels in the endosperm and embryo.

	Endosperm								Embryo							
	αTT	βTT	γTT	Δtt	MDA	ABA	SA	JA	αT	αTT	βTT	γTT	MDA	ABA	SA	JA
Elongated	-0,135 (0,889)	-0,079 (1,000)	-0,094 (1,000)	-0,119 (1,000)	0,375 **(0,002)**	-0,034 (1,000)	-0,378 **(0,002)**	-0,344 **(0,005)**	0,290 **(0,030)**	0,324 **(0,010)**	0,393 **(<0,001)**	0,433 **(<0,001)**	0,278 **(0,042)**	0,693 **(<0,001)**	0,175 (0,454)	-0,217 (0,197)
Contaminated	0,026 (1,000)	-0,006 (1,000)	0,049 (1,000)	0,132 (0,924)	-0,170 (0,492)	0,082 (1,000)	0,011 (1,000)	0,246 (0,096)	-0,118 (0,143)	-0,171 (0,480)	0,040 (1,000)	-0,006 (1,000)	0,061 (1,000)	-0,371 **(0,002)**	0,006 (1,000)	-0,220 (0,182)
Without growth	0,209 (0,228)	0,150 (0,697)	0,185 (0,371)	0,036 (1,000)	-0,418 **(<0,001)**	-0,039 (1,000)	0,540 **(<0,001)**	0,228 (0,150)	-0,403 **(<0,001)**	-0,352 **(0,004)**	-0,631 **(<0,001)**	-0,631 **(<0,001**)	-0,429 **(<0,001)**	-0,618 **(<0,001)**	-0,265 (0,062)	0,079 (1,000)

T, tocopherol; TT, tocotrienol; MDA, malondialdehyde; ABA, abscisic acid; SA, salicylic acid; JA, jasmonic acid. P <0,05 shown in bold

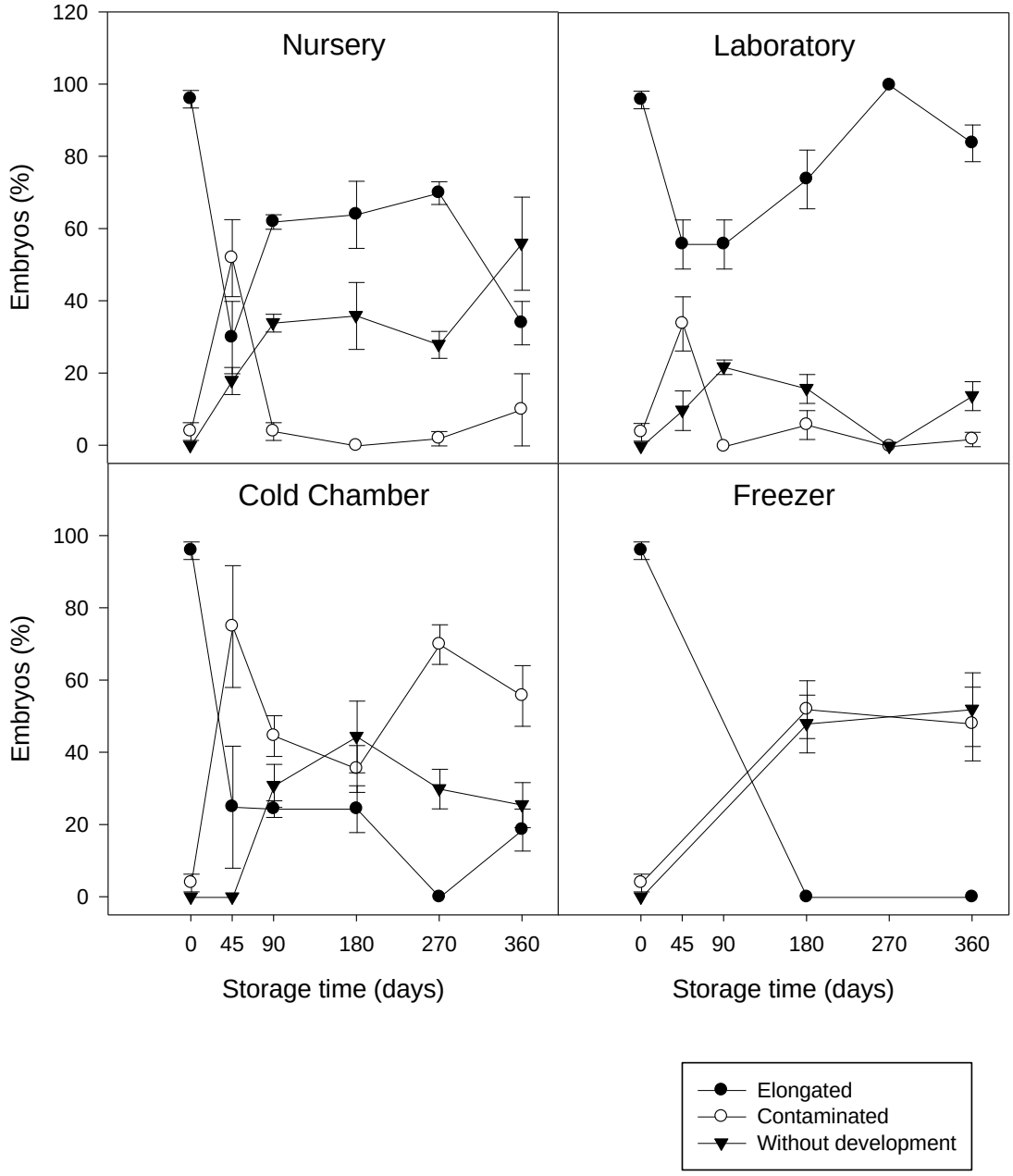

Fig. 1 Time-course evolution of embryo growth in *in vitro* culture (including elongated, contaminated and non-developing embryos) of seeds from fruits stored at different conditions for 360 days. Data correspond to the mean ± SE of n=4. Elongated, contaminated and non-developing embryos showed significant differences with time of storage in all treatments as indicated by one-way analysis of variance (ANOVA, $P<0.001$ in all cases)

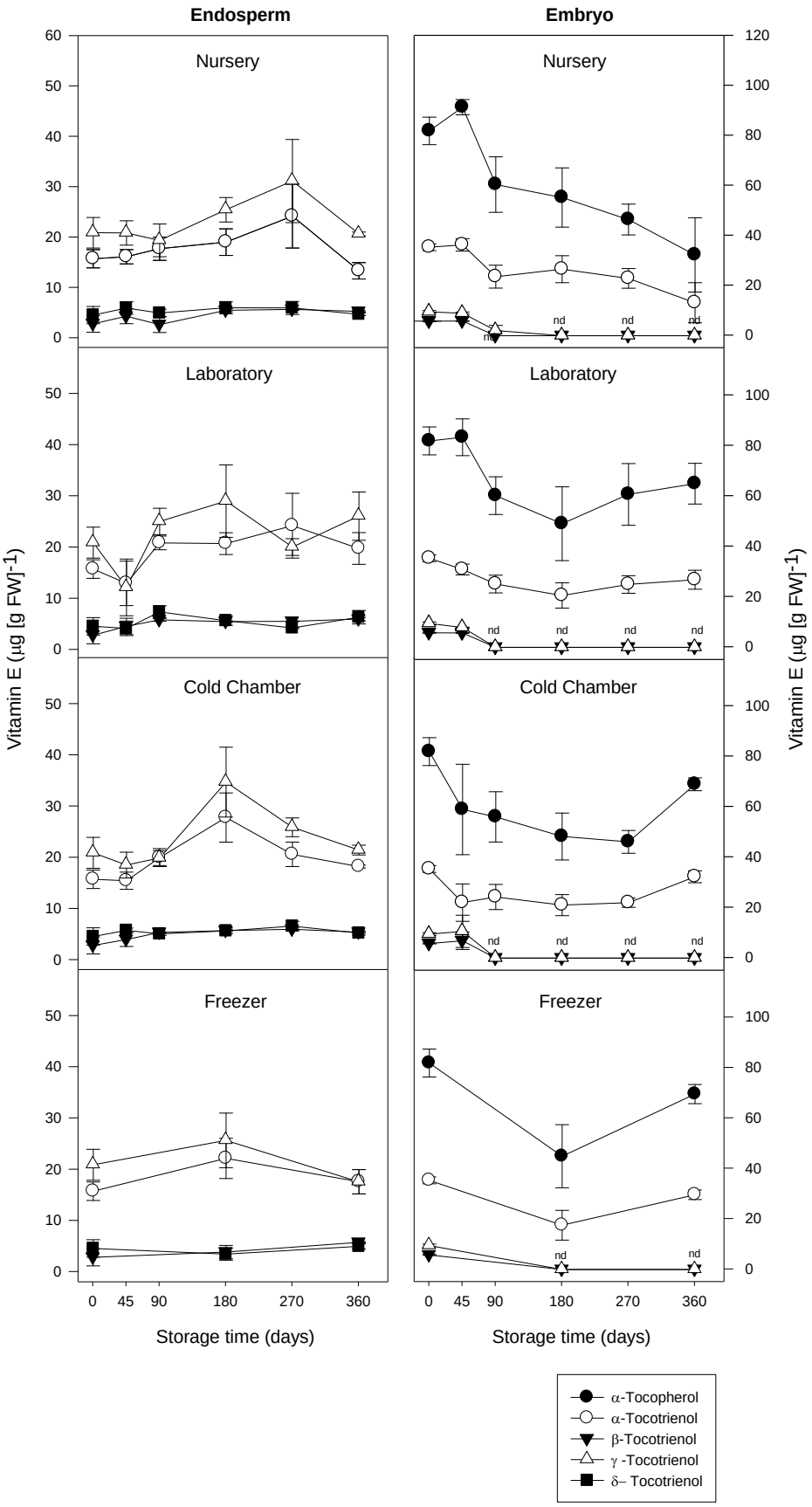

Fig. 2 Time-course evolution of vitamin E levels (including tocopherols and tocotrienols) in the seed endosperm and embryo of fruits stored at different conditions for 360 days. Data correspond to the mean ± SE of n=4. Results of one-way analysis of variance (ANOVA) are shown in Table 1A. nd, not detected

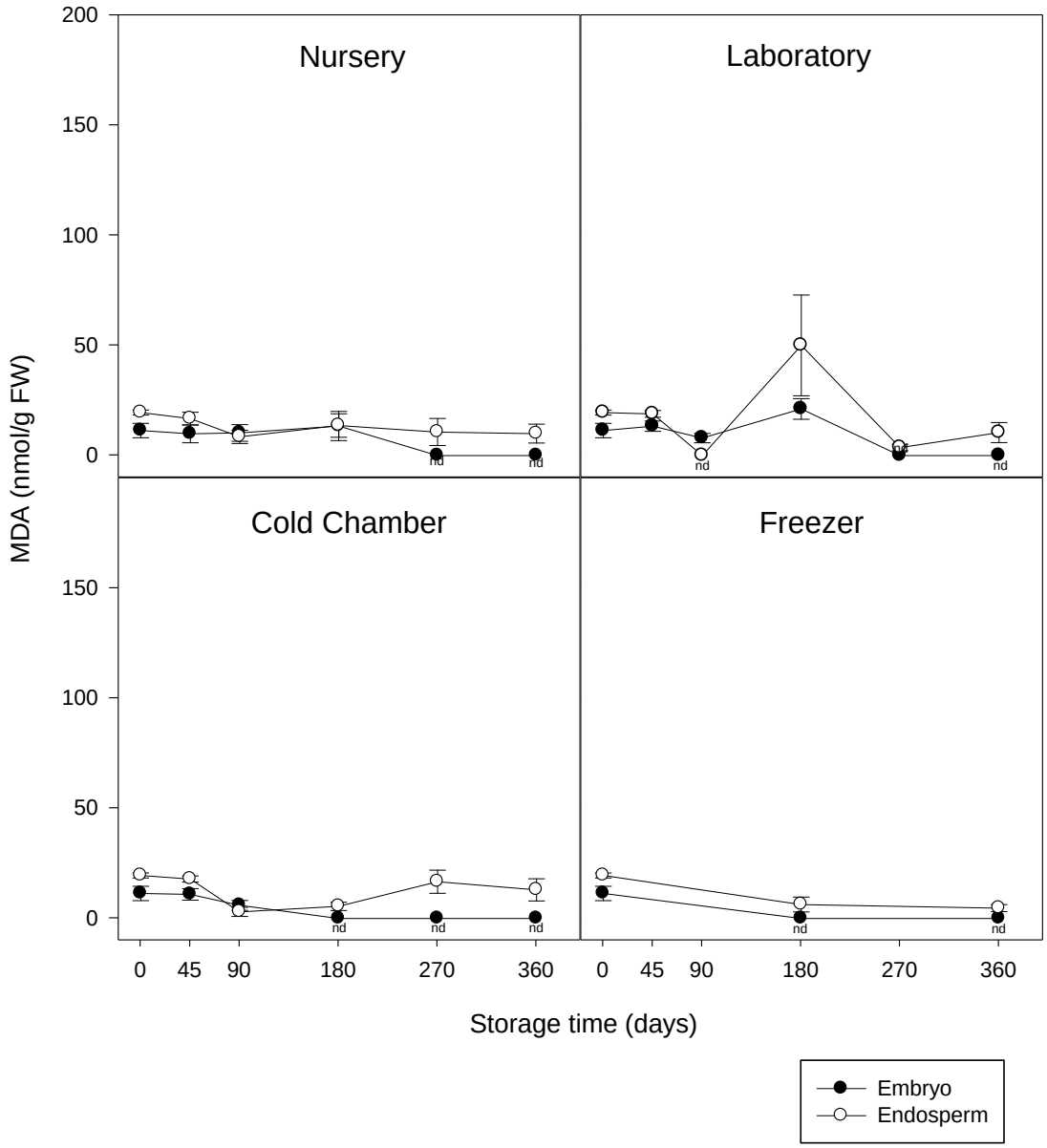

Fig. 3 Time-course evolution of melondialdehyde levels (an indicator of lipid peroxidation) in the seed endosperm and embryo of fruits stored at different conditions for 360 days. Data correspond to the mean ± SE of n=4. Results of one-way analysis of variance (ANOVA) are shown in Table 1B. nd, not detected

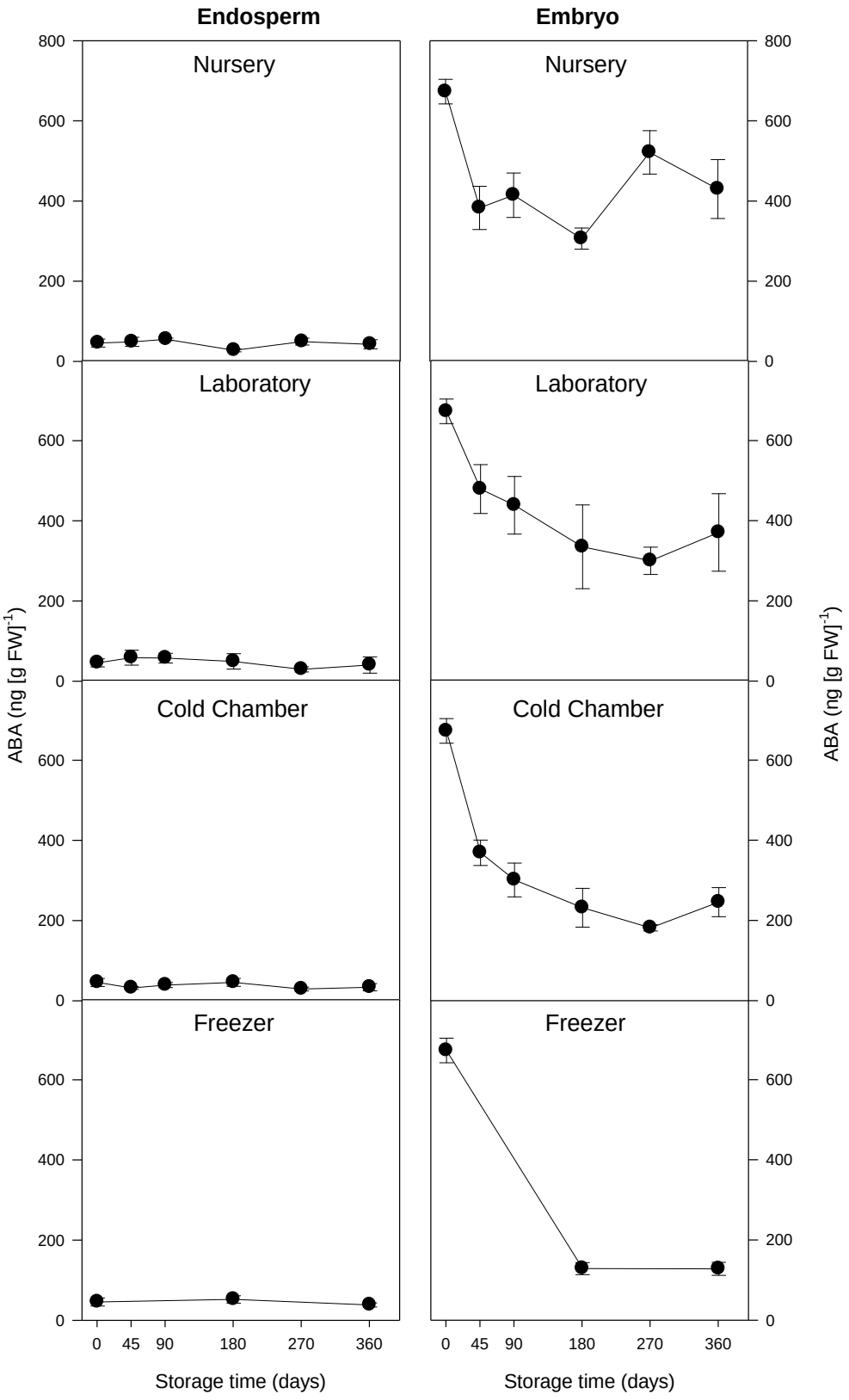

Fig. 4 Time-course evolution of abscisic acid (ABA) levels in the seed endosperm and embryo of fruits stored at different conditions for 360 days. Data correspond to the mean ± SE of n=4. Results of one-way analysis of variance (ANOVA) are shown in Table 1C

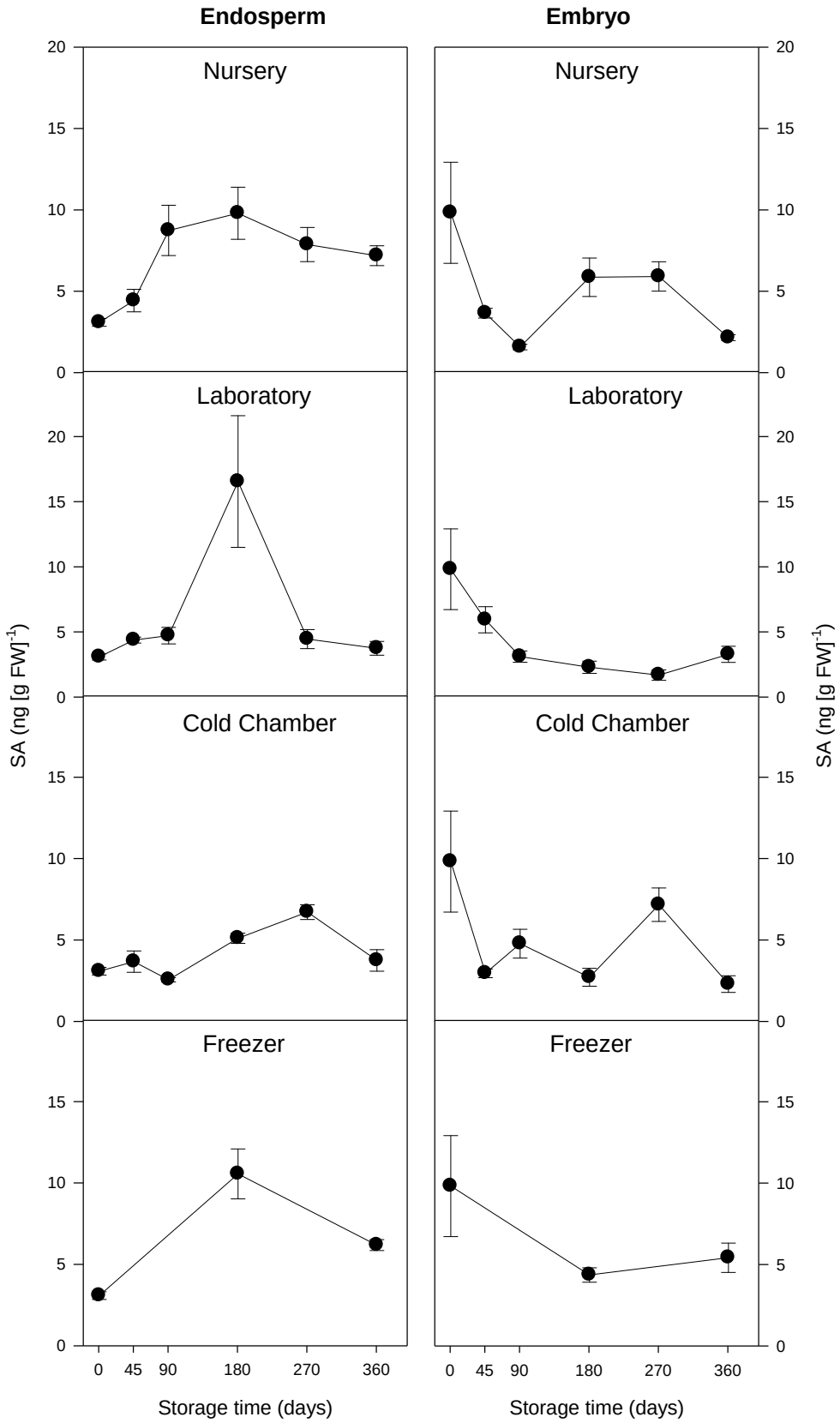

Fig. 5 Time-course evolution of salicylic acid (SA) levels in the seed endosperm and embryo of fruits stored at different conditions for 360 days. Data correspond to the mean ± SE of n=4. Results of one-way analysis of variance (ANOVA) are shown in Table 1C

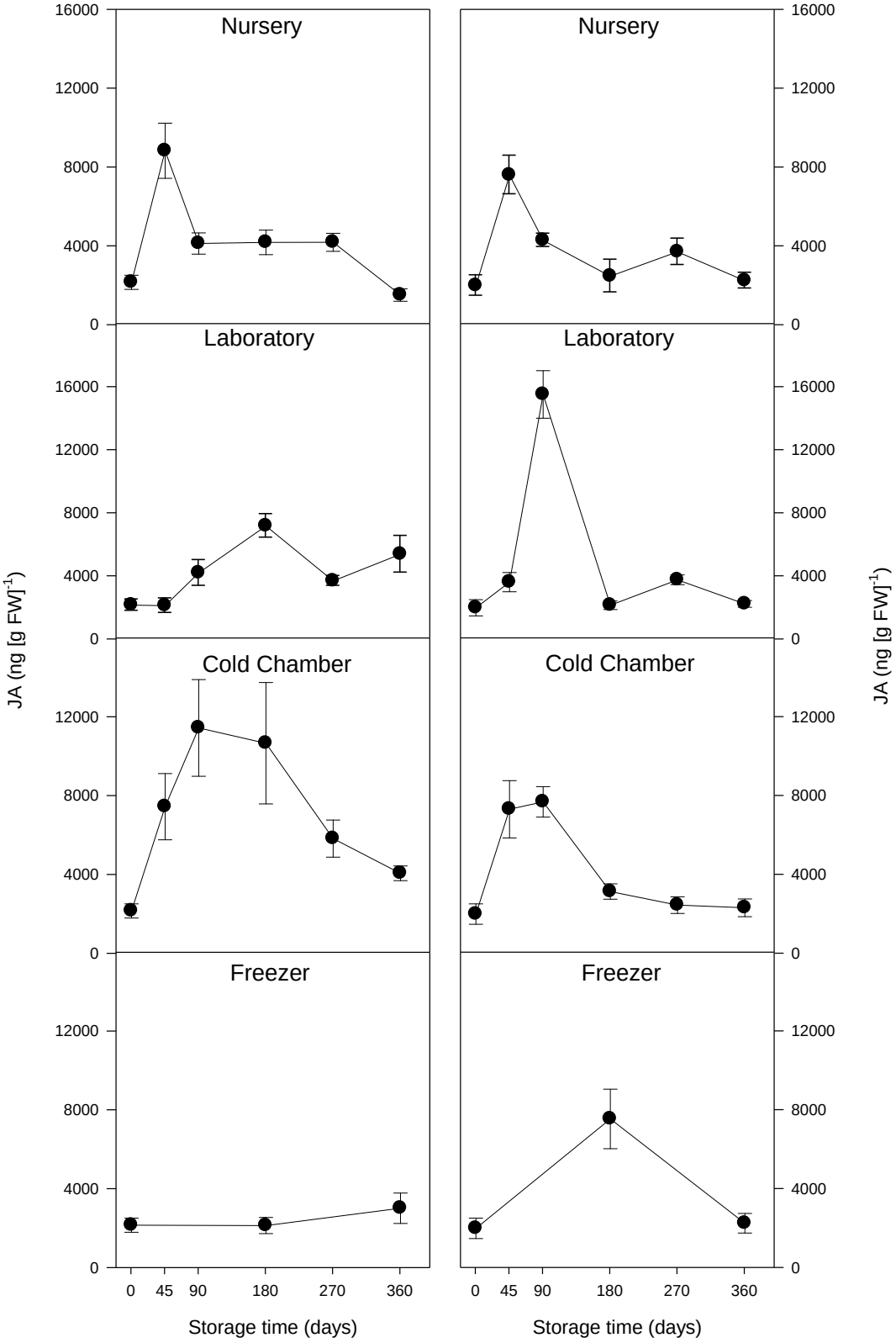

Fig. 6 Time-course evolution of jasmonic acid (JA) levels in the seed endosperm and embryo of fruits stored at different conditions for 360 days. Data correspond to the mean ± SE of n=4. Results of one-way analysis of variance (ANOVA) are shown in Table 1C

CAPÍTULO 2

Dynamic of reserve compounds of mesocarp and seeds of macaw palm (*Acrocomia aculeata)* submitted to different storage conditions

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ABSTRACT

Macaw palm fruits have a diverse biochemical constitution, and there is significant commercial interest in this species among food, pharmaceutical, cosmetics, and bioenergy industries. We evaluated changes in the reserve compounds of macaw palm fruits and seeds stored for one year under three different conditions. Protein and carbohydrates levels were highest in the embryo than in the endosperm. Fatty acid profiles were very similar over time under all storage conditions and in each structure evaluated, with the embryo composition being very similar to the mesocarp. Macaw palm oil remained well-preserved under all storage conditions tested, but seed reserves and seed viability are best maintained at room temperatures. The endosperm contained higher levels of saturated fatty acids than either the embryo or mesocarp, making seeds more resistant to oxidative deterioration than the mesocarp. The results showed that the composition of the mesocarp oil promises the production of high-quality biodiesel from this structure, and changes in carbohydrates and protein levels show that laboratory conditions are the most efficient for maintaining seed quality during storage.

Key words: Arecaceae; biofuel; carbohydrates; fatty acids; fruit storage; proteins

INTRODUCTION

The macaw palm (*Acrocomia aculeata* (Jacq.) Lodd Martius ex) is an oleaginous palm tree of Arecaceae family widely distributed in the Americas and adapted to a wide range of environmental conditions (Ferrari and Azevedo Filho 2012). Its fruits and seeds have high oil contents, and plantations are able to generate more than 5000 kg of oil per hectare (Moura et al. 2010; Ferrari and Azevedo Filho 2012). The biochemical constituents and fatty acid compositions of the fruits are remarkably diverse, and the economic value of macaw palms reflects their high oil productivity and the variety of uses of the products harvested from the different parts of its fruits. The pulp and kernel can be consumed *in natura,* or in ice creams, pastries, cakes and biscuits, and the biomass derived from the endocarp and epicarp can be used directly for different purposes, such as fuel or converted into charcoal briquettes (Hiane et al. 2006; Poetsch et al. 2010; Lescano et al. 2015), resulting in a huge commercial interest in this species, especially by the food, pharmaceutical, cosmetic, and bioenergy industries (Moura et al. 2008; Pires et al. 2013).

The food reserves of palm seeds (proteins, lipids and carbohydrates) are stored in the endosperm and embryo. Storage polysaccharides are found in the thickened cell walls of the endosperm, while lipids, proteins, and mineral nutrients are present in the cytoplasm; embryo reserves are mainly composed of lipids and protein bodies (DeMason et al. 1985; Alang et al. 1988; DeMason 1988; Sekhar and DeMason 1988; Moura et al. 2010). Reserves stored in the seeds are mobilized during germination when the water contents of the endosperm and embryo increase, with resulting cell expansion. Seed reserves have critical roles in initial seedling growth and establishment, being mobilized and consumed during embryo development after germination (Bewley and Black 1982; Baud et al. 2002). Carbohydrates and lipids are mainly used as carbon and energy sources, while proteins are the main source of nitrogen and sulfur for the synthesis of new proteins, nucleic acids, and secondary metabolic compounds during seedling development (Gallardo et al. 2008).

Orthodox palm seeds, like those of the macaw palm, acquire desiccation tolerance during maturation and are dispersed with low water contents; they can remain in a quiescent state for many years until favorable germination conditions appear (Ellis and Hong 1991; Arc et al. 2011; Ribeiro et al. 2012). These seeds therefore age before germination, a slow process that progressively alters their cell constituents (lipids, sugars, proteins, nucleic acids), with resulting losses of vigor and, eventually, viability. Much of the damage that occurs during seed aging is related to the deleterious effects of free radicals, reactive oxygen species (ROS) accumulation, and lipid peroxidation (Corbineau et al. 2000;

Merritt et al. 2003; Farrant et al. 2004; Walters et al. 2005; Mira et al. 2011). Deterioration is also related to decreases in carbohydrate reserves (Obendorf 1997; Pichardo-González et al. 2014), and their sugar contents can be an indicator of seed storability or aging (Bernal-Lugo and Leopold 1992; Górecki et al. 1997; Piotrowicz-Cieślak 2005; Garcia et al. 2006; Lehner et al. 2008).

Seed storage, for both economic and reproduction purposes, must be conducted under conditions that maintain seed quality and minimize any damage caused by ageing (Costa et al. 2013). In light of the economic interest in macaw palms and the fact that its reproduction is seasonal, the present study analyzed the dynamics of macaw palm fruits and seeds reserve compounds under different storage conditions.

MATERIALS AND METHODS

Plant material and Storage

Mature fruits of *Acrocomia aculeata* were collected following natural abscission from palms growing in Montes Claros, Minas Gerais State, Brazil, and were stored under the following conditions: in a plant nursery (exposed to natural climatic conditions); under laboratory conditions (room temperature, ~25 °C); in cold chambers (5 °C). The fruits were kept in polyethylene bags during the nursery and laboratory treatments, and in polypropylene bags during the cold chamber treatments. Testing was performed after 0, 45, 90, 180, 270 and 360 days of storage (time 0 corresponds to the freshly harvested fruits), using 180 fruits (four bags) at each storage period under each storage condition.

At the end of each storage period, the fruits were removed from the different storage conditions, the exocarps broken (with the aid of a hammer), and the seeds extracted from the fruits (using a manual bench-top vice). Parts of mesocarp, endosperm, and embryo were then removed and kept at -18 °C for biochemical analysis.

Fatty acid profiles

Fatty acid extraction

80 mg samples each of the embryo, endosperm, and mesocarp were ground in 80 mL of hexane, followed by homogenization in vortex mixer for 15 s. The resulting mixture was vacuum filtered through filter paper in a Büchner funnel. The residue was re-extracted twice, and the filtrates from the three extractions were combined and then held in an exhaust chamber for 24 h to remove the remaining solvent.

Sample preparation: hydrolysis and methylation

Approximately 10 mg of each oil sample was dissolved in 100 µL of a 1 mol/L ethanol and potassium hydroxide solution (95%:5%) in a 2 mL capacity cryogenic tube. After vortex agitation for 10 s, the oil was hydrolyzed in a microwave oven at 80 W for 5 min. After cooling, 400 µL of 20% chloridric acid, 20 mg of NaCl, and 600 µL of ethyl acetate were added and the mixture agitated in a vortex for 10 s. After five minutes rest, 300 µL of the organic layer was removed, placed in a microcentrifuge tube, and subsequently dried by evaporation – yielding the free fatty acid extract (adapted from Christie 1989). The free fatty acids were then methylated with 100 µL of BF_3 / methanol (14%), heated for 10 minutes in a water bath at 60 °C, and subsequently diluted with 400 µL of methanol for analysis by gas chromatography.

Gas Chromatography

The analyses were performed using a HP7820 Gas Chromatograph equipped with a flame ionization detector. An INNOWAX (HP) 15 m x 0.25 mm x 0.20 µ column was used in a temperature gradient, starting at 100 ºC until 220 ºC (raising 7 ºC/min); injector (split of 1/30) at 250 ºC, detector at 260 ºC, hydrogen as the carrier gas (2 mL/min), and an injection volume of 1 µL.

Oxidative Stability

To evaluate the oxidative stability of the analyzed oils, their oxidation index (OX) were calculated considering the positions and numbers of unsaturations in the carbon chains (as unsaturated compounds are significantly more susceptible to oxidation than saturated compounds) (Waynick 2005). These calculations were performed using the formula: OX= [0.02 (%O) + (%L) +2(%Ln)]/100, were O refers to oleic acid (18:1), L refers to linoleic acid (18:2), and Ln refers to linolenic acid (18:3).

Carbohydrate extraction and quantification

Endosperm and embryo samples were ground with hexane to remove any oil, and then dried at 45 °C for 30 minutes. For extraction, 1 mL of 80% ethanol was added to 80 mg of the dry material (endosperm or embryo), followed by agitation in a vortex for 10 s. The material was held in a water bath at 75 °C for 30 minutes and then centrifuged for 5 min (9000 g at 4 °C). The supernatants were removed and the pellets re-extracted twice again. The total sugar contents of the supernatants were quantified using the phenol-sulfuric method, with glucose as the standard (DuBois et al. 1956).

Soluble protein extraction and quantification

Samples containing 30 mg of embryo or endosperm were ground in 400 µL of sodium phosphate buffer (pH 6.8) and centrifuged for 15 min (9000 g and 20 °C). After centrifuging, 10 µL of the supernatant was transferred to an ELISA plate (in triplicate) for protein determination. Quantifications were performed following Bradford (1976) using a "Bio Rad Protein Assay" kit. For each sample, 200 µL of the diluted reagent (4:1) was added.

Statistical analyses

Differences in the fatty acid, carbohydrate, and protein levels of the embryo, endosperm, and mesocarp under different storage conditions were tested by analysis of variance (ANOVA), using storage time as the variable. Differences were considered significant when $P < 0.05$; in these cases, the Tukey test at a 5% level of probability was used for comparing the means. All statistical tests were performed using SPSS package v.12 software (Chicago, IL).

RESULTS

Analyses of the fatty acid profiles detected the presence of the following fatty acids in the three structures analyzed (embryo, endosperm, and mesocarp): caprylic (C8:0), capric (C10:0), lauric (C12:0), myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), margaric (C17:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), α-linolenic (C18:3) and arachidic (C20:0) (Table 1). Figure 1 demonstrates the chromatograms of the fatty acids found in the mesocarp, endosperm and embryo of freshly harvested fruits, showing differences in the fatty acids profiles and the concentrations of each fatty acid in the structures evaluated.

Six saturated, two monounsaturated, and two polyunsaturated fatty acids were observed in the mesocarp, corresponding to 21.5%, 59% and 18.4% of its total fatty acid content, respectively. Eight saturated, one monounsaturated, and one polyunsaturated fatty acid were detected in the endosperm, corresponding to 76.1%, 21.5% and 1.4% of its total fatty acids, respectively. Eight saturated, two monounsaturated and two polyunsaturated fatty acids were detected in the embryo, corresponding to 32.6%, 54.2% and 9.02% of its total fatty acid content, respectively (Figure 2).

The fatty acid profiles of the embryo and mesocarp were very similar, with a predominance of oleic acid (C18:1) (53.8% and 52.8% respectively), palmitic acid (C16:0) (13.3% and 19.4%), and linoleic acid (C18:2) (8.8% and 17.2%) (Figure 3). The

highest fatty acid concentrations in the endosperm were of lauric acid (C12:0) (41.2%), oleic acid (C18:1) (21.5%), and myristic acid (C14:0) (10.9%) (Figure 3). The three predominant fatty acids constituted 75.3% of the mesocarp, 89.4% of the embryo, and 73.6% of the endosperm. Additionally, lauric acid (C12:0) was detected in the embryo (8.7%), palmitoleic acid (C16:1) in the mesocarp (6.31%), and caprylic (C8:0) and palmitic (C16:0) acids in the endosperm (6.64% and 7.4% respectively). The other fatty acids were present in concentrations <5% (Table 1). The oxidation index of the mesocarps, endosperms, and embryos of freshly harvested fruits were 0.208, 0.018 and 0.101 respectively.

The profiles of the three main fatty acids in each structure were very similar under the three storage conditions tested (Figure 3), although some alterations were observed (Table 2): the levels of palmitic acid (C16:0) in the embryo (nursery), oleic acid (C18:1) in the endosperm (nursery and cold chamber), linoleic acid (C18:2) in the mesocarp (laboratory and nursery), and lauric acid (C12:0) in the endosperm (nursery) were significantly different after 360 days of storage as compared to freshly harvested fruits.

The levels of soluble proteins in all of the samples varied from 0.7 to 1.6 $mg.g^{-1}$ fm. Significant increases in the proteins levels of the endosperm were observed during storage, except among fruits stored in the cold chamber. Soluble protein levels in the embryo increased with storage, especially after 45 days, achieving levels of 3.38, 2.7 and 2.4 $mg.g^{-1}$ fm under laboratory, nursery and cold chamber conditions respectively (Figure 4).

Carbohydrates concentrations were higher in the embryo (6.23 $mg.g^{-1}$dm) than in the endosperm (between 0.88 and 1.4 $mg.g^{-1}$dm) in freshly harvested seeds, although decreasing with storage under all of the conditions tested. The embryos of seeds stored under laboratory and nursery conditions contained approximately 4.7 $mg.g^{-1}$ dm of carbohydrates at the end of storage (360 days), with decreases being more pronounced under cold chamber conditions (1.47 $mg.g^{-1}$ dm). Carbohydrate levels in the endosperm varied between 0.88 and 1.4 $mg.g^{-1}$ dm, and although demonstrated significant changes during storage in the laboratory and in the cold chamber, after 360 days of storage their carbohydrates levels did not differ from levels observed in the freshly harvested seeds (Figure 5).

DISCUSSION

Although the macaw palm embryo is contained within the endosperm (forming the seed structure), we have shown here for the first time that its fatty acid composition is most similar to that of the mesocarp. The predominance of medium chain fatty acids (such as oleic, lauric, myristic and palmitic acids) in the endosperm of macaw palms has been reported by other authors (Hiane and Ramos 1992; Belén-Camacho et al. 2005; Hiane et al. 2005; Amaral et al. 2011; Coimbra and Jorge 2011a; Coimbra and Jorge 2011b), but these studies did not evaluate the fatty acid profiles of the isolated tissues (endosperm and embryo). Some of these studies described fatty acid profiles for the seeds that were similar to our results for the endosperm (with lauric, oleic, and myristic acids as the major constituents), while oleic, palmitic or linoleic acids appeared as the major seed constituents in other studies – although they are, in fact, present in high quantities in the embryo but not in the endosperm (Belén-Camacho et al. 2005; Hiane et al. 2005; Arveláez et al. 2008; Amaral et al. 2011; Coimbra and Jorge 2011a; Coimbra and Jorge 2011b; Lescano et al. 2015).

Except for variations in the lauric acid (C12:0) content in the endosperm of fruits stored in the nursery and in the linoleic acid (18:2) content in the mesocarp in all storage conditions, no significant changes were observed in any of the fatty acid concentrations at the end of storage period (360 days) in relation to initial concentrations in any of the structures evaluated – indicating that these fatty acids remain intact during storage. These results corroborated those of Barreto et al. (2014), who observed no lipid peroxidation in macaw palm seeds during storage (low levels of MDA), and concluded that the high levels of vitamin E detected in the endosperm and embryo served to protect those seeds.

The low concentrations of unsaturated fatty acids in the endosperm resulted in a low oxidation index (OX) in that tissue as compared to the embryo and mesocarp (which have higher levels of unsaturated fatty acids, especially monounsaturated oleic acid). Freedman and Bagby (1989) noted that the oxidation of unsaturated fatty substances occur at different velocities depending on the number and positions of their double bonds. The high saturated fatty acid contents observed in *A. aculeata*, especially in the endosperm, lend the seed high stability against diverse oxidative mechanisms that could culminate in its deterioration (Hiane et al. 2005; Arveláez et al. 2008).

Resistance to oxidation is considered a very important chemical property in the development of industrial products, and this quality is not only related to the degree of unsaturation of any given oil, but also to the presence of antioxidant compounds. The susceptibility of some oils to oxidation can limit their usefulness to food, cosmetics, and

pharmaceutical industries and decreased their commercial values (Belén-Camacho et al. 2005; Coimbra and Jorge 2011b). Coimbra and Jorge (2011b) reported that macaw palm mesocarp demonstrated high potential for use in the food industry due to their high percentages of unsaturated fatty acids and low concentrations of saturated fatty acids – considered ideal profiles for edible oil production. As the oil extracted from the mesocarp is rich in oleic acid, it can also be used to generate high quality biodiesel with a high monounsaturated fatty acids content (Moura et al. 2008; Pires et al. 2013; Aguieiras et al. 2014). Additionally, high proportions of saturated and monounsaturated fatty acids are considered optimal for fuel quality as they largely avoid undesirable polymerization that can occur during the combustion of fuels derived from polyunsaturated fatty acids (Demirbas 2009).

Our results demonstrated that the embryos of macaw palm fruits stored under laboratory and nursery conditions maintained their initial carbohydrate levels until the end of the experimental period (360 days of storage), although these final levels were lower in the embryos of fruits stored in cold chamber. Barreto et al. (2014) likewise demonstrated that macaw palm fruits stored in the laboratory and nursery gave the best results in terms of embryo viability, while viability was significantly reduced after storage under cold chamber conditions. The results of the present study suggest that the maintenance of high levels of carbohydrates under laboratory and nursery storage conditions contributed to the physiological quality of those seeds.

The concentrations of soluble protein have also been associated with seed viability during storage (Shibata et al. 2012). Loss of viability accompanied by reductions in the protein contents was observed in *Lolium perene* L. and *Tabebuia roseoalba* (Ridl.) Sandwith (Bignoniaceae) seeds (Ching and Schoolcraft 1968; Abbade and Takaki 2014). We observed significant increases in protein levels in macaw palm seeds after 90 days of storage, especially in embryos. The higher levels of soluble proteins observed in embryos stored under laboratory and nursery conditions coincided with the higher levels of embryo viability under those same storage conditions reported by Barreto et al. (2014) Taken together, these results indicate that seed viability during storage is associated with high soluble protein levels.

Storing macaw palm fruits for 360 days did not alter the fatty acid profiles of their different structures. The endosperm contained higher levels of saturated fatty acids than either the embryo or mesocarp, making seeds more resistant to oxidative deterioration than the mesocarp. The composition of the mesocarp oil promises the production of high-quality biodiesel from this structure. Fruit storage is associated with changes in

carbohydrate and protein contents, and the decreased sugar levels of embryos stored in the cold chamber, along with the increased proteins levels of embryos stored under laboratory and nursery conditions can be related to their viability, confirming that laboratory conditions are the most efficient for maintaining seed quality during storage.

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Table 1 Fatty acids composition of mesocarp, endosperm and embryo of *Acrocomia aculeata* in time 0 of storage.

Fatty acids	**% of total fatty acids**		
	Mesocarp	**Endosperm**	**Embryo**
Saturated			
C8:0 (caprylic acid)	Tr	6.64	1.62
C10:0 (capric acid)	Tr	4.89	1
C12:0 (lauric acid)	0.32	41.27	8.72
C14:0 (myristic acid)	0.15	10.9	2.95
C16:0 (palmitic acid)	19.49	7.4	13.31
C17:0 (margaric acid)	0.15	0.16	0.63
C18:0 (stearic acid)	1.2	4.44	3.69
C20:0 (arachidic acid)	0.22	0.4	0.57
Monounsaturated			
C16:1 (palmitoleic acid)	6.31	Tr	0.44
C18:1 (oleic acid)	52.77	21.5	53.78
Polyunsaturated			
C18:2 (linoleic acid)	17.24	1.4	8.88
C18:3 (α-linolenic acid)	1.24	Tr	0.14

Tr = traces (<0.06% of total fatty acids).

Table 2 P values corresponding to analysis of variance (ANOVA) of main fatty acids performed with mesocarp, endosperm and embryo of *Acrocomia aculeata* stored in laboratory, nursery and cold chamber.

		Laboratory	Nursery	Cold Chamber
Mesocarp	C18:1	NS	<0.001	<0.001
	C16:0	NS	0.046	0.012
	C18:2	<0.001	<0.001	0.021
Endosperm	C12:0	0.01	<0.001	0.002
	C18:1	NS	<0.001	<0.001
	C14:0	0.021	NS	0.025
Embryo	C18:1	NS	NS	NS
	C16:0	NS	<0.001	NS
	C18:2	NS	NS	NS

Differences were considered significant when $p<0.05$. NS = not significant.

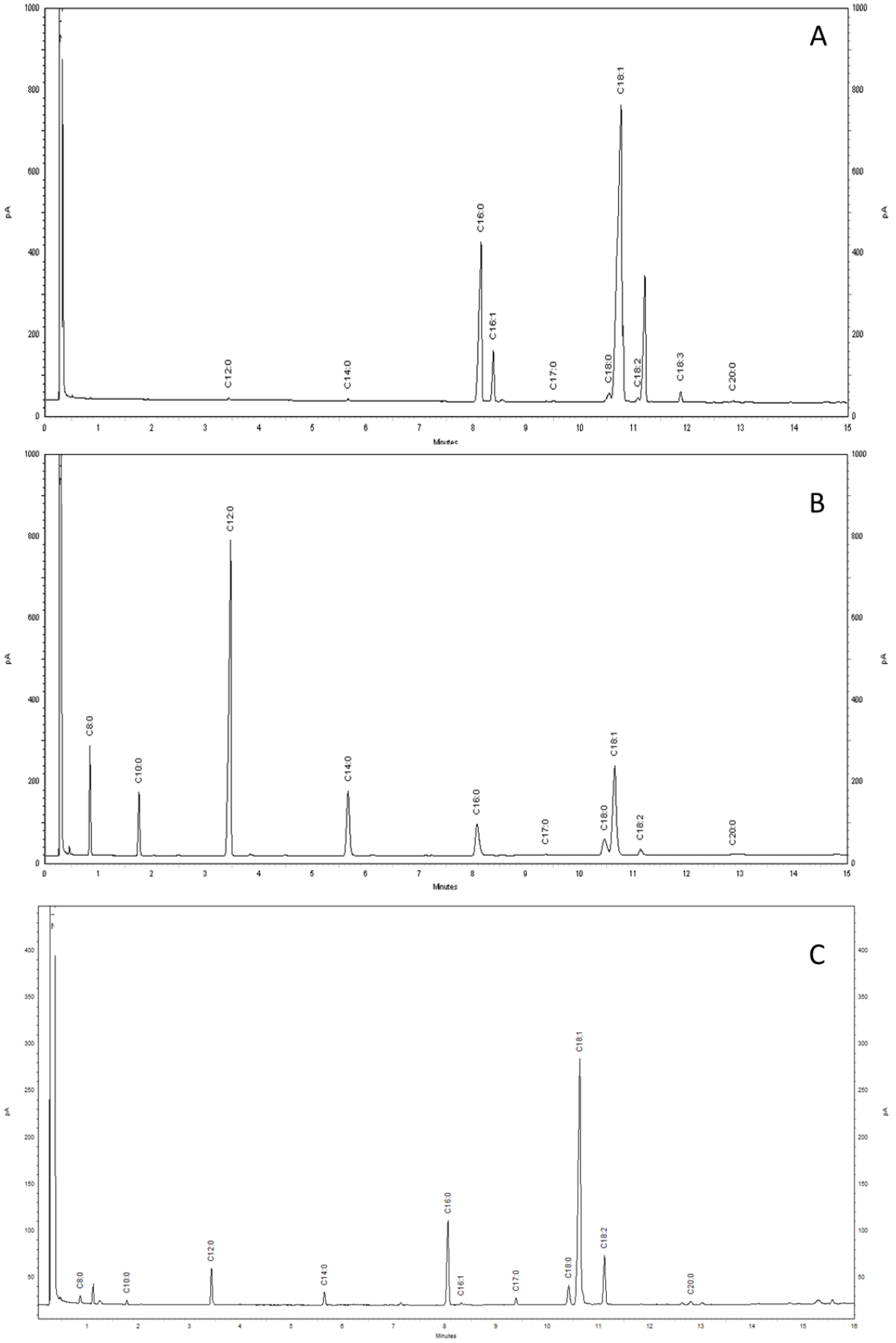

Figure 1. Chromatographic profiles of fatty acid methyl esters from mesocarp (A), endosperm (B) and embryo (C) of *Acrocomia aculeata* before storage (time 0).

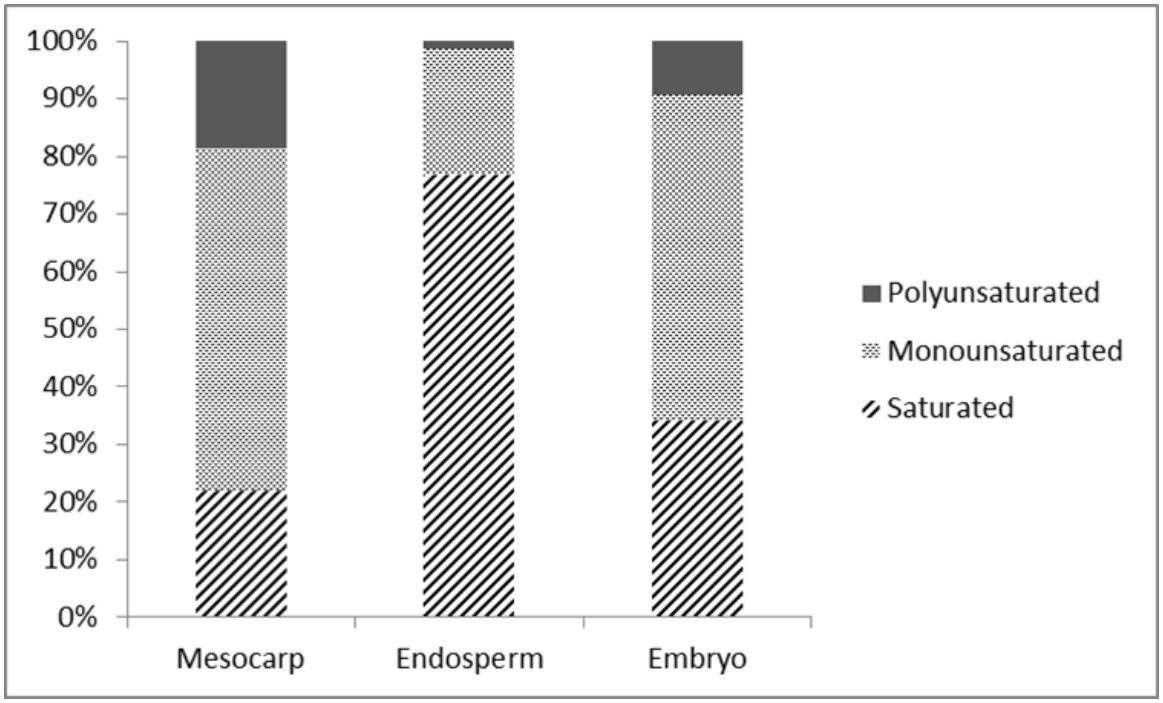

Figure 2. Average proportions of fatty acid methyl esters distributions classified into saturated, monounsaturated and polyunsaturated extracted from mesocarp, endosperm and embryo of *A. aculeata* before storage (time 0).

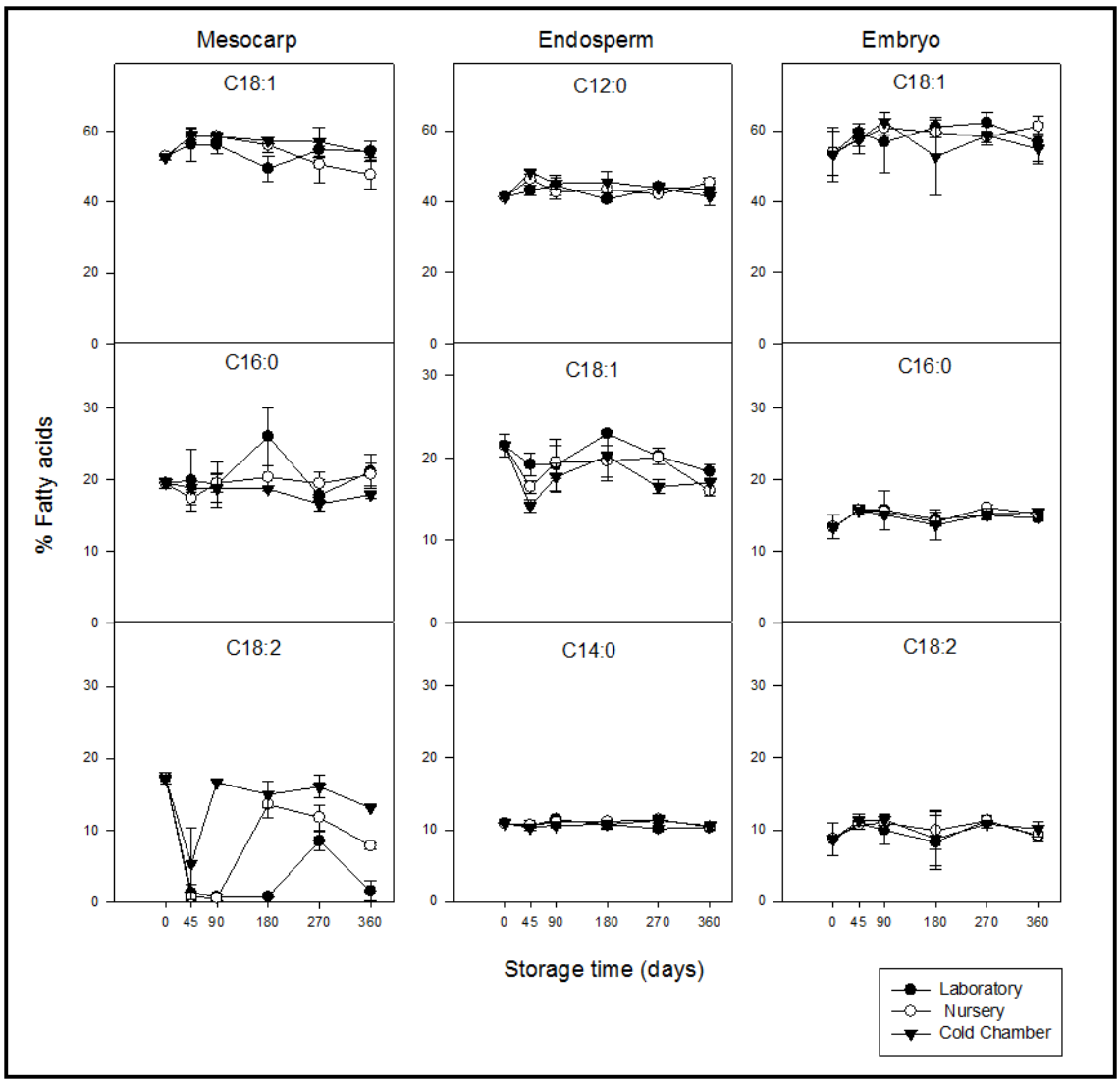

Figure 3. Variation of content of main fatty acids (%) found in mesocarp, endosperm and embryo of *A. aculeata* stored in laboratory, nursery and cold chamber.

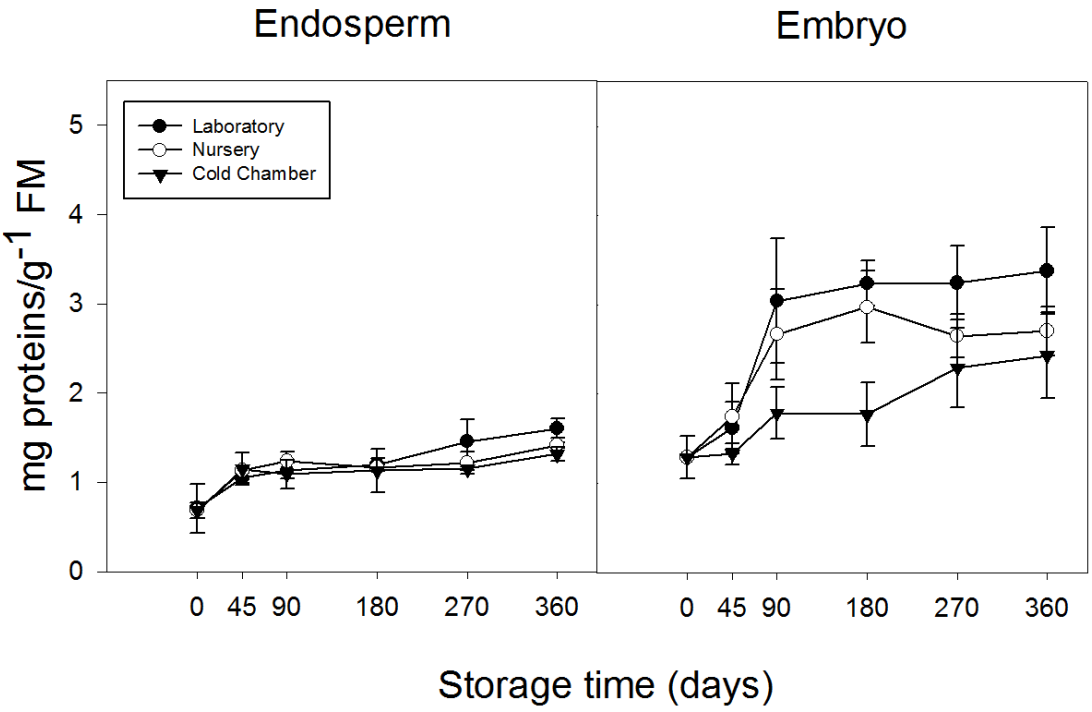

Figure 4. Variation of total proteins content (mg/g^{-1} FM) found in endosperm and embryo of *A. aculeata* stored in laboratory, nursery and cold chamber.

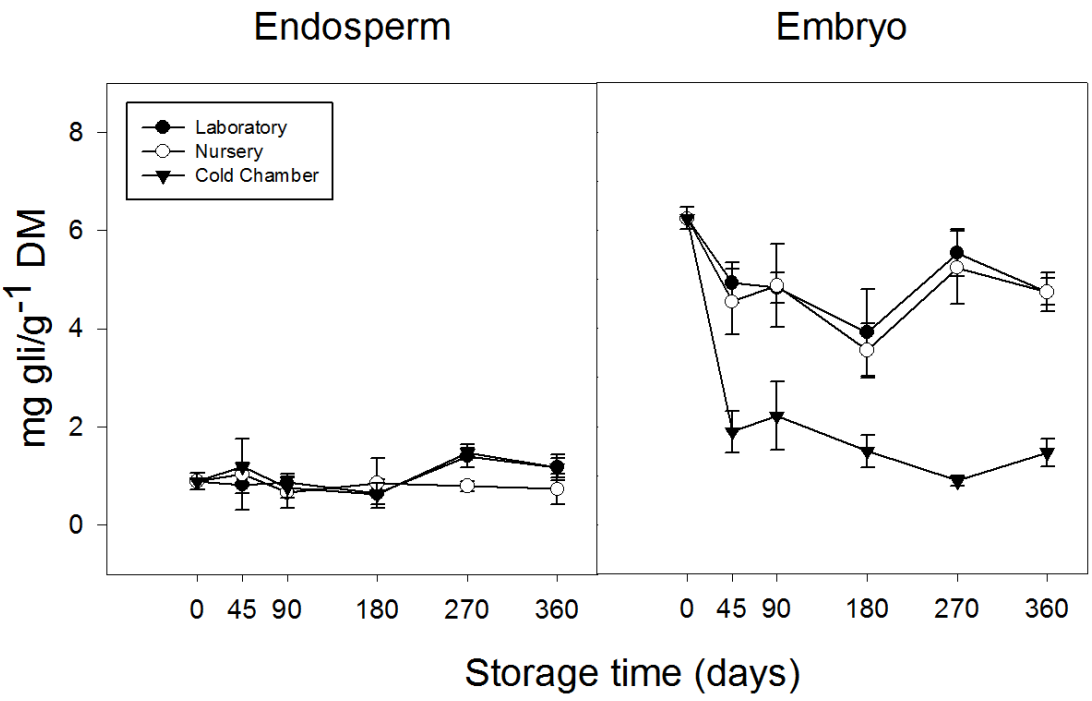

Figure 5. Variation of soluble carbohydrates content (mg/g^{-1} DM) found in endosperm and embryo of *A. aculeata* stored in laboratory, nursery and cold chamber.

CAPÍTULO 3

Accelerated ageing and subsequent imbibition affect seed viability and the efficiency of antioxidant system in macaw palm seeds

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ABSTRACT

Accelerated ageing is an accurate indicator of seed vigor and storability, helping the understanding of cellular and biochemical deterioration that occur during seed ageing. The present study was carried out to elucidate the mechanisms of ageing in macaw palm embryos. Seeds were artificially aged during 4, 8 and 12 days at 45 ºC and 100% relative humidity.After ageing, seeds were tested for viability (tetrazolium), electrical conductivity, lipid peroxidation (MDA) and hydrogen peroxide (H_2O_2) content. Part of the aged seeds was imbibed for 8 days to determine the hydrogen peroxide content and the activity of antioxidant system enzymes (superoxide dismutase, catalase and glutathione reductase). Ageing reduced the embryo viability from 8 days of treatment and increased malondialdehyde content (MDA) and solute leakage. Hence, membrane permeability correlated with both loss of viability and lipid peroxidation. After imbibition, H_2O_2 content significantly increased along with superoxide dismutase activity. Catalase activity was significantly higher than control in embryos aged from 8 days, and glutathione reductase activity did not change. Our results suggest that macaw palm seed deterioration during accelerated ageing is closely related to lipid peroxidation, and that enzymatic antioxidant system is not completely efficient in reducing reactive oxygen species after imbibition, a critical phase to germination. Moreover, accelerated ageing test can be used as a reliable model to understand the mechanisms involved in palm seeds deterioration.

Key words: Antioxidant enzymes, electrolyte leakage, hydrogen peroxide, lipid peroxidation, reactive oxygen species, seed water content

INTRODUCTION

During storage, seeds gradually deteriorate, as they undergo ageing processes that lead to a progressive decrease in their vigor, and then to a loss of their viability (Stewart and Bewley, 1980; Lehner et al., 2008; Arc et al., 2011). This loss of viability varies among species, and it is mainly dependent of temperature and seed water content (Robert and Ellis, 1989). It has already been shown that high seed moisture and high temperature further accelerate seed deterioration (Ellis and Hong, 1991; Goel et al. 2003).

Many of the processes associated to seed ageing during storage include genetic damage, protein degradation, enzyme inactivation and loss of membrane integrity (Bailly et al., 1996, 2002; Merritt et al., 2003; Bailly, 2004; Arc et al., 2011; Wang et al., 2011). Although the mechanisms responsible for the loss of seed viability are still not completely elucidated (Walters et al. 2005; Mira et al., 2011), lipid peroxidation and reactive oxygen species (ROS) accumulation along with the resulting oxidative events induced by them are considered the major cause of seed deterioration (Corbineau et al., 2000; Hay et al., 2010; Mira et al., 2011).

ROS are continuously produced in plants as co-products of various metabolic pathways, and in seeds they also play important roles in signaling associated with germination and dormancy alleviation (Bailly et al., 2008). However, when plants are subjected to abiotic stresses, there are significantly increases of ROS levels that cause a redox imbalance and consequently, oxidative stress (Hossain et al., 2015). ROS are originated from the incomplete or partial reduction of oxygen, which leads to the formation of superoxide (O_2•−), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH) and also singlet oxygen ($^1O_2$), that is produced by direct energy transfer from triplet chlorophyll to oxygen (Blokhina, 2003; Bhattachrjee, 2005; Hendry, 2008).

Seeds are known to contain numerous antioxidant compounds, which can be enzymatic or non-enzymatic, and they act to prevent oxidative damage by scavenging ROS before they attack membranes or other seed components (Leprince et al. 1993). The main enzymes involved in cell detoxification are superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), peroxidase (POD) and ascorbate peroxidase (APX) (Bailly et al., 1996, 2002; Goel et al., 2003). SOD is generally considered a key enzyme in the regulation of intracellular concentrations of superoxide radical and peroxides, catalyzing the disproportionation of two molecules of superoxide into molecular oxygen and H_2O_2 (Beyer and Fridovich, 1987). CAT and POD are implicated in the removal of

hydrogen peroxide, and similarly, GR can also play a part in the control of endogenous hydrogen peroxide through an oxide-reduction cycle involving glutathione and ascorbate. APX reduces H_2O_2 to water, using ascorbate as an electron donor (Nakano and Asada, 1981; Cakmak and Horst, 1991; Bailly et al., 2002).

Most of the studies on the cellular and biochemical deterioration during seed ageing have been performed under accelerated ageing conditions (i.e., at high temperature and high humidity), which is recognized as an accurate indicator of seed vigor and storability. Seeds that deteriorate rapidly under accelerated ageing conditions generally show a marked depression in their ability to germinate (McDonald, 1999; Lehner et al., 2008). On the other hand, the mechanisms involved in seed deterioration under such extreme conditions might be different from those occurring on natural ageing, and this is still a question for discussion (Lehner et al., 2008). Some authors consider that accelerated ageing is the same as natural ageing, with the only difference being the rate at which they occur (McDonald, 1999; Galleschi et al., 2002). Considering that only few comparative studies of natural and accelerated ageing of seeds are reported in the literature, the answer to this question is further complicated. Moreover, studies on accelerated ageing do not include an evaluation of aged seeds during the imbibition phase. To establish a clear relationship between the production/removal of ROS and accelerated ageing, it is necessary to consider the effects of storage conditions on the antioxidant system efficiency during the imbibition, which is an important and critical step for germination occurs (Lehner et al., 2008).

Information about accelerated ageing in palm seeds are still scarce (Negreiros and Perez, 2004; Murugesan et al., 2005). In macaw palm [*Acrocomia aculeata* (Jacq.) Lodd. ex. Mart.], some studies regarding its germination and storage have been conducted (Azevedo Filho et al., 2012; Barreto et al., 2014; Neto et al., 2015, 2012; Ribeiro et al., 2011; Silva et al., 2013), but none comprises accelerated ageing. This species is adapted to the dry tropical climate of the "Cerrado" and known for the large quantities of high quality oil concentrated in the mesocarp and seed, what turns it a significant economic potential, especially in terms of biofuel production (Hiane et al., 2005; Moura et al., 2010; chapter 2). Due to its pronounced seed dormancy, germination speed is very slow and also the germination rates (taking up to four years), what makes the establishment of commercial cultivations of macaw palm very limited (Ribeiro et al., 2011). Therefore, additional information concerning fruit storage and viability could

aid in developing methods for *ex situ* conservation of macaw palm, especially when we consider the economic importance of the species.

In a previous study performed by our research group (Barreto et al., 2014), the effects of different storage conditions on vitamin E and defense related phytohormones in macaw palm seeds were investigated. In the present work, other parameters were measured, but they can act as a basis to compare to those results previously obtained to evaluate if physiological and biochemical processes involved in accelerated ageing and in other storage conditions are related. Here we aimed characterize the sensitivity of macaw palm seeds to accelerated ageing conditions; investigate whether seed viability is related to lipid peroxidation, membrane damage and hydrogen peroxide accumulation during accelerated ageing; and determine whether the imbibition of aged seeds changes the antioxidant enzymatic system of the embryos.

MATERIALS AND METHODS

Plant material

Experiments were performed on fresh seeds of *Acrocomia aculeata* collected in 2015 from a wild population in Mirabela (16°200 45″S 44°130 17″W), Minas Gerais State, Brazil and obtained from COOPERIACHAO (Cooperativa do Riachão no Norte de Minas Gerais, Brazil).

Accelerated ageing treatment

Accelerated ageing was performed by exposing the seeds to a temperature of 45 ºC for 4, 8 and 12 days in tightly closed plastic boxes with 100% of relative humidity (RH). These temperature conditions were chosen based on previous work by Rubio Neto et al. (2015) that showed that drying macaw palm fruits at 57 ºC was detrimental to embryo viability and germination after 8 days, while embryos whose fruits were dried at 37 ºC were still able to germinate. Besides, numerous studies concerning accelerated ageing are conducted at 45 ºC (Stewart and Bewley 1980; Sung and Jeng 1994; Bailly et al 1996, 1998; Al-Maskiri et al 2003; Lehner et al 2008; Mira et al 2011; Gordin et al 2015; Krainart et al 2015), and considering the scarce information about palm seeds, this temperature was chose as an initial step. After the ageing treatment, seeds were dried for four days at room temperature until they reach the original moisture content

(MC) (~ 0.23 g H2O g-¹ dm controlled by weighting). After drying, part of the seeds of each period of ageing (4, 8 and 12 days) was imbibed for 8 days (time in which occurs a stabilization of the water content absorbed) at 35 ºC and 12h photoperiod for H_2O_2 content determination and enzymatic assays. Seeds which were not submitted to any treatment were considered as control. Biochemical measurements were carried out on isolated embryos that were frozen in liquid nitrogen until analysis take place

Water content determination

Four replicates of 10 embryos were weighed immediately after each period of ageing. Then, they were dried at 105 ºC ± 3 for 24 h for dry mass (DM) determination. Results were expressed as g H_2O g^{-1} DM.

Viability

Embryo viability was measured by the tetrazolium test, which was performed according to Ribeiro et al. (2010). Tests were conducted with four replicates of 10 embryos and results were expressed as percentage of viable embryos.

Electrical conductivity

Solute leakage of control and aged embryos was estimated by placing four replicates of 10 embryos each in 10 ml of ultrapure water at 25 ºC for 6 h. The electrical conductivity of the medium was measured using a conductivity meter (Gehaka CG 1800). The conductivity was expressed as $\mu S.cm^{-1}.embryo^{-1}$.

Malondialdehyde measurements

Lipid peroxidation were evaluated by measuring malondialdehyde (MDA) content of embryos, according to Hodges et al. (1999), that consider the possible effects of interfering compounds in the thiobarbituric acid-reactive substances (TBARS) assay. There were used four replicates of 50 mg (fresh weight) of embryos and results were expressed as nmol g^{-1} DM.

Evaluation of hydrogen peroxide content

H_2O_2 in control, aged embryos and in embryos during subsequent imbibition was measured according to the method described by Velikova et al. (2000). Fifteen embryos were ground in a mortar using liquid nitrogen and homogenized with 800 µl of thiobarbituric acid (TCA) and then centrifuged for 15 min at 15 000 g at 4 °C. The

resulting supernatant was collected and putted on tubes with 10 mM potassium phosphate buffer, and then it was added 1 M potassium iodide (KI). The absorbance was read at 390 nm. The content of H_2O_2 was given on a standard curve and results were expressed as mg H_2O_2 g^{-1} DM.

Enzymes extraction

Four replications of 100 mg of embryos from each treatment were used for the preparation of enzyme extract. Embryos were ground in liquid nitrogen in a mortar and homogenized with 1 mL of 100 mM potassium phosphate buffer (pH 7.8) containing 100 µM ethylenediaminetetraacetic acid (EDTA) and 5% PVP (m/v). The homogenate was centrifuged at 15 000 g for 15 min at 4 ºC. The resultant supernatant was separated and used for enzyme assays. 500 µl of the supernatant were used for protein determination. The enzyme assays refer only to seeds after imbibition.

Enzyme assays

Catalase activity was estimated based on the method of Aebi (1984). The reaction mixture contained 150 µL of enzyme extract, 15 µL of 100 µM H_2O_2 and 150 µL of 100 mM potassium phosphate buffer (pH 7.0). The absorbance was read at 240nm immediately after addition of the enzyme extract at an interval of 15 sec for 2 min (ε, 0,036 mM^{-1} cm^{-1}). The blank was without enzyme extract. The enzyme activity was expressed as mM H_2O_2 decomposed $(mg\ protein)^{-1}min^{-1}$.

GR activity was assayed based on the method of Foyer and Halliwell (1976). The reaction mixture contained 50 µL of enzyme extract, 150 µL of 100 mM phosphate buffer (pH 7.8) and 50 µL of 10 mM oxidised glutathione. After 3 min, 10 µL of 5 mM NADPH was added and mixed thoroughly. The rate of NADPH oxidation was monitored at 340 nm for 3 min every 15 sec (ε, 6.2 mM^{-1} cm^{-1}). The enzyme activity was expressed as µM NADPH oxidised $(mg\ protein)^{-1}min^{-1}$.

Superoxide dismutase (SOD) activity was measured according to Giannopolitis and Ries (1977), with modifications. The reaction mixture contained 2 µM riboflavine, 13 mM methionine, 75µM nitroblue tetrazolium (NBT) and 100 µM EDTA in 50 mM potassium phosphate buffer (pH 7.8), and 20 µL of enzyme extract. The reaction systems were maintained for 10 min at room temperature in a chamber equipped with a 15 W fluorescent light. Control reactions were performed in the dark. SOD activity was assayed by measuring the ability of the enzyme extract to inhibit the photochemical

reduction of NBT, quantified at 560 nm. One unit of SOD was defined as the enzyme activity which inhibited the photoreduction of NBT to blue formazan by 50%, and SOD activity of the extracts was expressed as units SOD (mg protein)$^{-1}$.

Protein determination

Protein content of the extracts was determined according to the method of Bradford (1976) using the BioRad protein assay kit with bovine serum albumin as calibration standard.

Statistical Analyses

The results were expressed as the averages of four replicates ± SD, and the data statistically evaluated using one-way analysis of variance, employing JMP software (*SAS Institute Ins*.). The means were compared using the Tukey test, at a 5% level of probability.

RESULTS

Effect of ageing on embryo water content and viability

Figure 1 shows the changes in embryo water content and viability during accelerated ageing performed at 45 ºC and 100% RH. Before ageing treatment, the embryo MC was around 0.23 g H_2O g^{-1} DM and 90% of the embryos were viable, according to the tetrazolium test. Ageing was associated with a progressive increase in embryo water content, which reached about 0.34, 0.55 and 0.64 g H_2O g^{-1} DM after 4, 8 and 12 days of treatment, respectively. It also resulted in a decrease in embryo viability, which was markedly reduced from 8 days of treatment (Fig. 1, P<0.05).

Lipid peroxidation and hydrogen peroxide content

The MDA content in control embryos was 30.67 nmol g^{-1} DM, and it significantly increased from 8 days, reaching 43.46 and 44.32 nmol g^{-1} DW after 8 and 12 days of ageing, respectively (Table 1). After imbibition, MDA content did not significantly change, and the values remained between 26.85 and 32.55 nmol g^{-1} DM, close to its initial value before ageing (26.22 nmol g^{-1} DM) (data not shown).

Hydrogen peroxide levels did not change during ageing process (Table 2); after 12 days of ageing, H_2O_2 values were close to its initial value (120.98 and 112.2 µg H_2O_2 g^{-1} DM,

respectively). In contrast, imbibition of control and aged seeds leaded to significant changes in hydrogen peroxide content (Table 2). H_2O_2 content in the embryo slightly increased after imbibition, and embryos aged for 8 and 12 days had H_2O_2 levels significantly higher than control embryos (125.07, 105.84 and 97.35 µg H_2O_2 g^{-1} DM, respectively).

Electrolyte leakage

Before ageing, the initial value of embryo electrolyte leakage was 2.30 µS cm^{-1} $embryo^{-1}$. Seed ageing resulted in a significant increase in electrolyte leakage from 8 days of treatment, whose values reached 3.313 and 3.245 µS cm^{-1} $embryo^{-1}$ after 8 and 12 days, respectively (Table 3). Figure 2 shows the relationship between electrolyte leakage and viability and between electrolyte leakage and MDA content. These date show there was a negative linear relationship between electrolyte leakage and viability and a positive relationship between electrolyte leakage and MDA levels.

Antioxidant enzyme activities

Catalase activity significantly increased (by 200%) from 8 days of ageing in relation to the imbibed control. In dry control, CAT activity was not detected (Fig. 3a). GR activity was higher in dry control embryos (0.57 µM NADPH min^{-1} mg^{-1} protein) in relation to the imbibed control (0.21 µM NADPH min^{-1} mg^{-1} protein) and did not change after imbibition, regardless the time of ageing (Fig. 3b). SOD activity was not affected in embryos aged for 4 days ($P > 0.05$), but it increased by almost 50% in embryos aged for 8 and 12 days, in comparison to the imbibed control. In dry control embryos, SOD activity was the same as in imbibed embryos (0.92 and 0.885 U min^{-1} mg^{-1} protein, respectively) (Fig. 3c).

DISCUSSION

Macaw palm seeds exposed to 45 °C in an atmosphere saturated with water vapor (100% RH) had their viability decreased from 8 days of ageing. Besides temperature, MC is a key factor of seed deterioration during storage, and both factors have been integrated in viability equations to predict seed lifespan (Walters, 1998). It is assumed that low temperature and low moisture content are favorable conditions to improve orthodox seed quality during storage (Belletti et al., 1991; Ellis and Hong, 1991;

Walters, 1998). In macaw palm embryos, the increase in MC during ageing at high temperature had a negative effect on seed survival (viability). Until 4 days of ageing, embryo MC was 0.34 g H_2O g^{-1} DM and the viability remained high (97%), but after 8 days, with an embryo MC of 0.55 g H_2O g^{-1} DM, viability started to decrease (67%). As observed by Kibinza et al. (2006), the higher water content along with high temperature could lead to a faster deterioration during ageing, explaining the rapid loss of viability.

Macaw palm embryos accumulated significant amounts of MDA from 8 days of ageing. Peroxidation of membrane lipids causes increased permeability of lipid bilayers and a decrease in membrane fluidity (Priestley and Leopold, 1979). The determination of MDA is a convenient method to quantify the extent of lipid peroxidation, especially in oil rich seeds with high linoleic acid content (Priestley and Leopold, 1979; Goel et al., 2003). In macaw palm embryo, linoleic acid is the third most occurring (chapter 2), which demonstrates the embryo susceptibility to damage induced by free radical. Besides, loss of seed viability observed during accelerated ageing has been associated with an accumulation of MDA in several species (Bailly et al., 1996; Zacheo et al., 1998; Al-Maskri et al., 2003; Kibinza et al., 2006; Xia et al., 2015).

Alterations in membranes of aged seeds are well reported in literature and can lead to a greatly enhanced leakage of solutes during seed imbibition, which can be indicative of an inability to reform coherent membranes during seed rehydration, resulting in loss of vigor and viability (Priestley and Leopold, 1979; Bailly et al., 1996; Goel et al., 2003; Mira et al., 2011; Liu et al., 2013). In this study, we observed a significant increase in electrical conductivity after 8 days of ageing; and membrane integrity loss, evaluated by electrolyte leakage, was linearly related to both embryo viability and MDA content. As viability decreased with ageing, electrolyte leakage linearly increased, along with MDA levels. The results obtained demonstrate that there is a direct relationship between lipid peroxidation and electric conductivity, with both factors significantly responding to environmental stress (high temperature and high humidity). Similarly, Al-Maskri et al. (2003) and Goel et al. (2003) found a relationship between lipid peroxidation products and seed ageing and suggested that ageing had damaging effect on seed membrane and on the resulting lipid peroxidation products, increasing electrical conductivity.

The production of H_2O_2 during imbibition is a common feature, as the resumption of respiration in imbibed seeds can lead to electron leakage and increased production of ROS (Bailly et al., 1998; Goel, Goel and Sheoran, 2003; Bailly et al., 2008; Lehner et

al., 2008; Gomes and Garcia, 2013). Hydrogen peroxide (like other ROS) can be either beneficial of detrimental, depending on its accumulation level within the embryonic cells (Bailly et al., 2008; Jeevan Kumar et al., 2015). Changes in hydrogen peroxide levels were only observed after imbibition in aged embryos, but not during ageing, indicating that membrane damage during accelerated ageing were caused by other ROS and that H_2O_2 levels observed in aged embryos may be the result of normal metabolism. However, the higher levels after imbibition in embryos that were already damaged by ageing show that the excess of H_2O_2 was not completely scavenged. Increases in the concentration of ROS could be considered as an aspect of oxidative stress, but it has been also suggested that production of ROS and their release in the surrounding medium during seed imbibition play a part in protecting the embryo against pathogens (Wojtyla et al., 2006).

The imbibition resulted in an increase in SOD activity in embryos from 8 days of ageing. SOD is the first defense enzyme against oxidative stress and is considered a key enzyme in the regulation of intracellular concentrations of superoxide radical and peroxides. This converts $O_2\bullet^-$ into H_2O_2, decreasing the risk of hydroxyl radical formation (Goel et al., 2003; Ahmad et al., 2008). The increase in SOD activity is in agreement with the increase in H_2O_2 levels that we observed in imbibed embryos, and although CAT activity was also higher in embryos from 8 days of ageing, we can assume that its activity was not marked enough to prevent hydrogen peroxide accumulation from the eighth day of treatment. Catalase is the principal scavenging enzyme which can directly dismutate H_2O_2 and is indispensable for ROS detoxification during stress (Ahmad et al., 2008).

Glutathione reductase activity significantly decreased in imbibed control embryos in relation to dry control embryos, which suggests that in the latter, reduced glutathione levels (GSH) are higher than oxidized glutathione (GSSG), as it occurs in healthy cells, in which glutathione occurs in the reduced state (GSH) in a higher concentration than the oxidized state (GSSG), and the GSH/GSSG ratio is about 20:1 or even 100:1 (Meyer, 2008; Gill and Tuteja, 2010). In the presence of ROS, GSH easily react with them, generating GSSG through an enzymatic reversible process catalyzed by glutathione peroxidase (GPx). The opposite way (GSSG reduction) is catalyzed by GR and NADPH. Therefore, GR prevents an excess of GSSG, ensuring accumulation of higher levels of GSH and conferring tolerance to plants (Apel and Hirt, 2004; Meyer,

2008; Gill and Tuteja, 2010; Foyer and Noctor, 2011; Noctor et al., 2012), what did not occur in this study after imbibition of macaw palm aged seeds.

The percentage of viable embryos after 8 and 12 days of accelerated ageing was similar to that reported by Barreto et al. (2014) with the storage of macaw palm fruits in nursery condition during 270 days. In that study, authors showed that embryo is more susceptible to suffer oxidative stress than the endosperm during storage and that vitamin E and abscisic acid are responsible for protecting embryos. Here we also observed that antioxidant mechanisms have an important role in protecting macaw palm embryos during ageing, however, we cannot exclude the possibility that the antioxidant machinery became unable to remove the ROS produced in the reactivated metabolism during imbibition. Rehydration of a seed from the dry state through imbibition is essential to set in motion the metabolic events essential for germination to start, however, this process impose considerable stresses upon the component cells, then efficient mechanisms are required to minimize these damages (Nonogaki et al., 2010).

In conclusion, our results establish a clear relationship among loss of viability, lipid peroxidation and membrane damage in macaw palm embryos during accelerated ageing, highlighting the key role of the embryo water content at high temperature on the mechanisms involved in seed deterioration. Moreover, the higher production of H_2O_2 in embryos that suffered damages during ageing shows that the enzymatic antioxidant system was efficient to some extent in reducing ROS levels during the critical step of imbibition of aged seeds.

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Table 1 Effect of the duration of accelerated ageing (45 °C, 100% RH) on malondialdehyde (MDA) contents in aged macaw palm embryos. Means of 4 replicates ± SD. Values followed by the same letter are not significantly different (P<0.05) by Tukey test.

Duration of ageing (days)	MDA (nmol g^{-1} DM)
0	30.67 ± 2.81 a
4	29.56 ± 1.52 a
8	43.46 ± 0.59 b
12	44.32 ± 2.97 b

Table 2 Content of hydrogen peroxide (µg H_2O_2 g^{-1} DM) in macaw palm embryos imbibed for 8 days after accelerated ageing (45 °C, 100% RH). Means of 4 replicates ± SD. Values followed by the same letter are not significantly different (P<0.05) by Tukey test.

Duration of ageing (days)	Aged embryos	Aged and imbibed embryos
0	112.2 ± 2.11	97.35 ± 5.49 b
4	104.97 ± 5.56	101.88 ± 2.31b
8	124.47 ± 5.36	105.84 ± 3.70 ab
12	120.98 ± 3.37	125.07 ± 4.94 a

Table 3 Values of electrolyte leakage for macaw palm embryos during accelerated ageing at 45 ºC and 100% RH. Means of 4 replicates ± SD. Values followed by the same letter are not significantly different (P<0.05) by Tukey test.

Duration of ageing (days)	Electrolyte leakage (µS cm^{-1} $embryo^{-1}$)
0	2.30 ± 0.03 a
4	2.11 ± 017 a
8	3.31 ± 0.20 b
12	3.56 ± 0.26 b

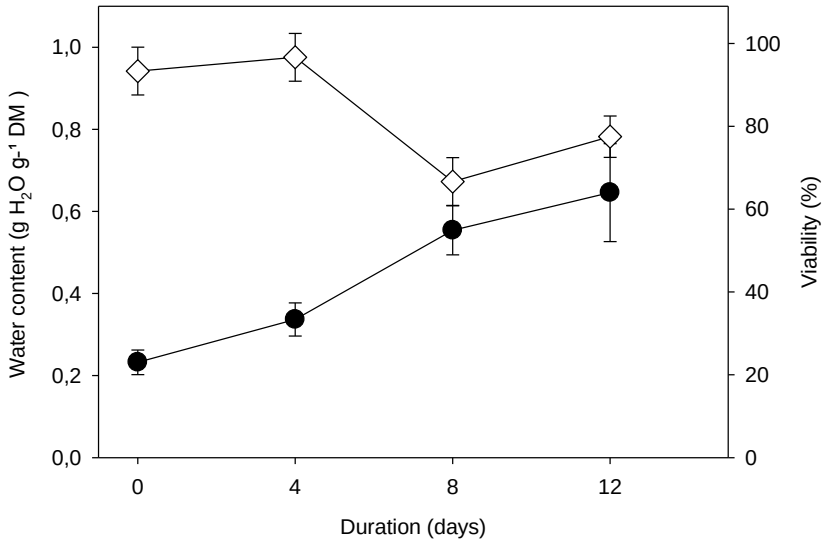

Figure 1 Changes during accelerated ageing at 45 ºC and 100% RH in embryo viability (◊) and embryo water content (●). Means of 4 replicates ± SD

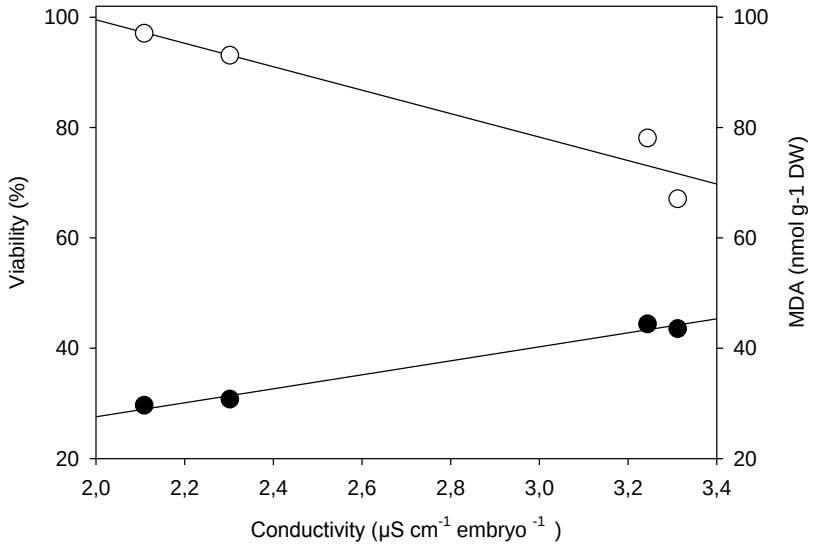

Figure 2 Relationships between electric conductivity (µS cm^{-1} $embryo^{-1}$) and viability (○) (r^2 = 0.9204) and between electrolyte leakage (µS cm^{-1} $embryo^{-1}$) and MDA nmol g^{-1} DM (●) (r^2 = 0.9874) during accelerated ageing (45 °C, 100% RH) of macaw palm seeds.

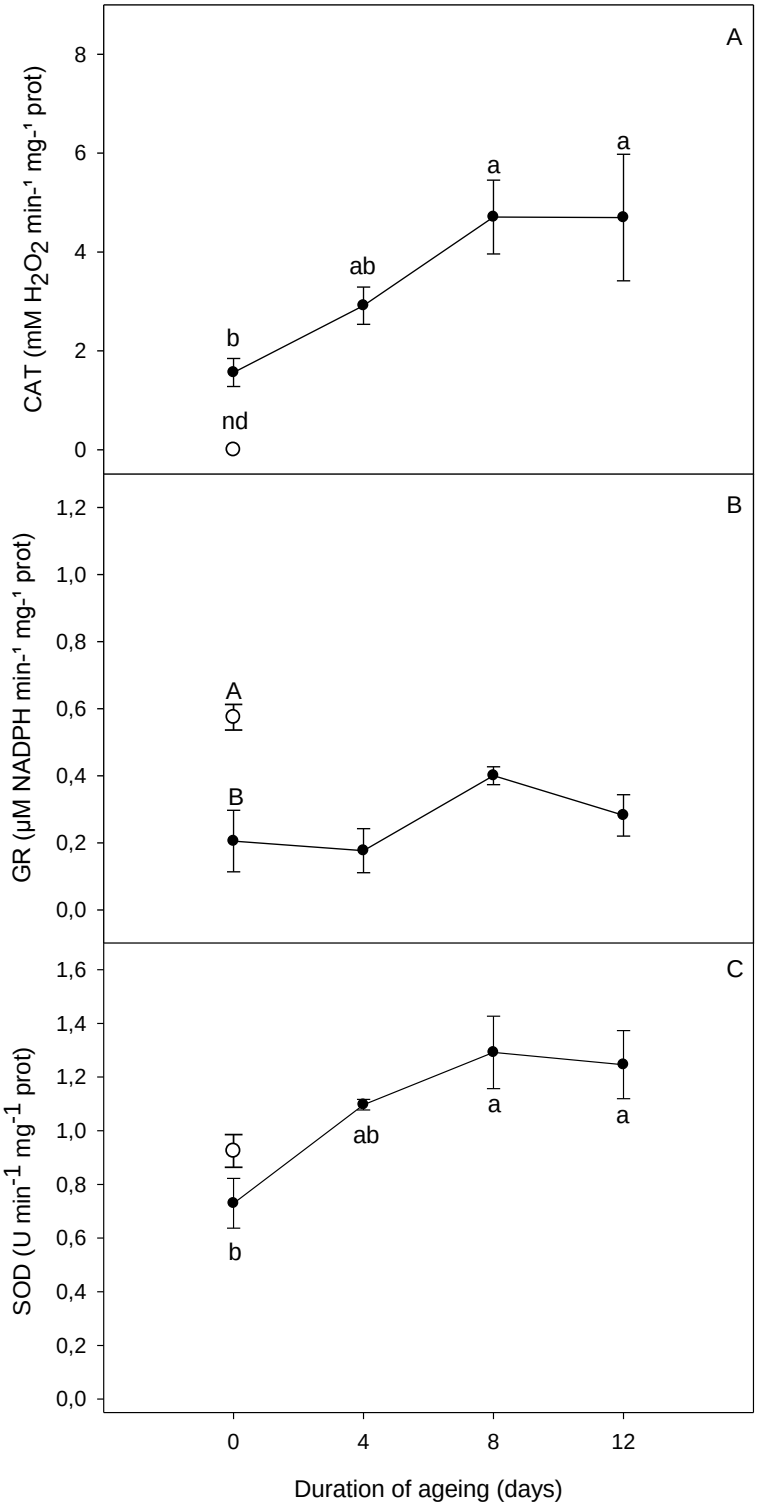

Figure 3. Changes in CAT (A), GR (B) and SOD (C) activities in macaw palm embryos accelerated aged for 4, 8 and 12 days and submitted to 8 days of imbibition (●). Data correspond to the mean of 4 replications ± SD. Different letters indicate significant differences ($P<0.05$) by Tukey test. Capital letters refer to differences between dry control (○) and imbibed control (●). Small letters refer to differences in imbibed embryos. nd = not detected.

CONSIDERAÇÕES FINAIS

A macaúba possui importância econômica reconhecida e, devido às potencialidades da espécie, estudos sobre armazenamento, germinação e manutenção da viabilidade do embrião são importantes para o estabelecimento de plantios comerciais, contribuindo para disseminar uma alternativa para a produção de alimentos, cosméticos, fármacos e biocombustível. O presente trabalho gerou informações relevantes para que o armazenamento dos frutos seja realizado de forma eficiente, além de revelar as principais alterações bioquímicas e fisiológicas que ocorrem nas sementes durante o processo de envelhecimento.

Os resultados do cultivo *in vitro* de embriões, juntamente com as alterações observadas nos níveis de proteínas e carboidratos, durante o armazenamento de frutos de macaúba por até 360 dias mostraram que, dentre as quatro condições testadas, o armazenamento em laboratório foi o método mais eficiente para a manutenção da viabilidade dos embriões e a posterior obtenção de plântulas. Os níveis de vitamina E e ABA no embrião foram considerados importantes marcadores do crescimento do embrião para a obtenção de plântulas *in vitro*. Este estudo também ressaltou o fato de que condições adequadas de temperatura e umidade são essenciais durante o armazenamento, uma vez que apenas temperaturas baixas (câmara fria e freezer) foram prejudiciais para a manutenção da viabilidade dos embriões.

Tanto o armazenamento por 360 dias como o envelhecimento acelerado mostraram que os mecanismos antioxidantes (enzimáticos e não enzimáticos) estão envolvidos na proteção do endosperma e principalmente do embrião contra o estresse oxidativo, o que é de grande importância para o estabelecimento de plantios de macaúba. Considerando o valor econômico e ambiental que a espécie apresenta, os resultados obtidos com esse estudo mostraram novas questões que devem ser respondidas a partir de estudos futuros, principalmente no que diz respeito a investigações mais detalhadas dos níveis de carboidratos e proteínas, os quais parecem estar relacionados com a manutenção da qualidade das sementes durante o armazenamento.