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CROP PRODUCTION

Lipid peroxidation and antioxidant enzymes of *Jatropha curcas* L. seeds stored at different maturity stages

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ABSTRACT. The objective of the present work was to investigate the antioxidant enzyme activities and the occurrence of lipid peroxidation during storage of *J. curcas* seeds obtained from fruits at different maturity stages. Seeds extracted from fruits collected at three stages of maturity were used, i.e., yellow, brownish-yellow, and brown (dry fruits). The seeds were subsequently stored for 18 months in Kraft paper bags in a laboratory environment. Initially as well as every three months, the seeds were evaluated for germination and electrical conductivity, and the seed embryos were evaluated for lipid peroxidation; protein content; and superoxide dismutase, peroxidase, and ascorbate peroxidase enzyme activity at the same time points. In general, the seeds from the three stages of fruit maturity did not differ regarding germination throughout the entire period of storage, although a reduction in viability was observed after nine months of storage. The electrical conductivity increased during storage and was greater for the seeds obtained from brown fruits. There was a reduction in protein content and in enzyme activities in the seed embryo, except for superoxide dismutase. No relationship was observed between reduction in seed viability and lipid peroxidation. Thus, *J. curcas* seeds with high initial quality can be stored under environmental conditions for up to 9, 15 and 15 months for brown (dry), yellow and brownish-yellow fruits, respectively, without a significant reduction in germination but with a reduction in seed vigour.

Keywords: physic nut, seed maturation and deterioration, enzyme activity, malondialdehyde content.

Peroxidação de lipídios e enzimas antioxidativas em sementes de *Jatropha curcas* L. armazenadas em diferentes estágios de maturação

RESUMO. O objetivo do presente trabalho foi avaliar a atividade de enzimas antioxidativas e a peroxidação de lipídios durante o armazenamento de sementes de *J. curcas* em diferentes estádios de maturação. Foram utilizadas sementes extraídas de frutos colhidos com coloração externa da casca amarela, amarela-marrom e marrom (frutos secos). As sementes foram armazenadas por 18 meses, em embalagem de papel Kraft em ambiente de laboratório. Inicialmente e a cada três meses, as sementes foram avaliadas quanto à germinação e condutividade elétrica, e os embriões das sementes foram avaliados quanto a peroxidação de lipídios, conteúdo de proteínas e atividade das enzimas superóxido dismutase, peroxidases e peroxidase do ascorbato. Em geral, as sementes dos três estádios de maturação dos frutos não diferiram quanto à germinação durante todo o período de armazenamento, embora tenha sido observada uma redução da germinação a partir de nove meses de armazenamento. Houve redução no conteúdo de proteína e na atividade das enzimas antioxidantes no embrião das sementes dos três estádios de maturação, com exceção da superóxido dismutase. Não foi observada relação entre a redução da viabilidade das sementes e a peroxidação de lipídios. Sementes de *J. curcas* com elevada qualidade inicial podem ser armazenadas sob condições ambientais por até 9, 15 e 15 meses, para os estádios de coloração dos frutos marrom (frutos secos), amarelo-marrom e amarelo, respectivamente, sem redução significativa da germinação, mas com redução no vigor das sementes.

Palavras-chave: pinhão manso, maturação e deterioração de sementes, atividade enzimática, conteúdo de malonaldeído.

Introduction

Jatropha curcas L. Seeds contain between 30 - 40% oil (Parawira, 2010). This species is considered a promising source of raw material for biodiesel and bio kerosene production due to the excellent quality of the oil obtained from its seeds (Dias, 2011; Koh & Ghazi, 2011).

The propagation of this species may be performed vegetatively; however, currently, propagation by seeds is more common. Thus, obtaining high-quality seeds has great importance, and this factor is affected by suitable harvest time, which for *J. curcas* is based on the outside colour of the fruits, with yellow and brownish-yellow fruits being the most recommended (Dranski

et al., 2010; Silva, Dias, Milagres, & Dias, 2012; Sowmya, Gowda, Balakrishna, & Rao, 2012). At these stages, the seeds are near the physiological maturity point (Silva et al., 2012).

In addition to the ideal harvest time, the storage condition and storage behaviour of the seeds have great effects on the maintenance of viability after harvest. In this sense, although *J. curcas* seeds are considered orthodox (Duong, Shen, Luangviriyasaeng, Ha, & Pinyopusarerk, 2013), i.e., they can be dried to low moisture content and can tolerate low temperatures during storage, the seeds are reported to have a short viability period, between six and 12 months (Guzman & Aquino, 2009; Moncaleano-Escando et al., 2013; Dias, Oliveira, Vallory, Silva, & Soares, 2016).

There are various mechanisms involved in the loss of seed viability during storage; among these mechanisms are lipid peroxidation, enzyme inactivation or protein degradation, cell membrane rupture, and the loss of DNA integrity (McDonald, 1999; Mittler, 2002; Bailly, 2004; Hu et al., 2012; Kumar, Prasad, Banerjee, & Thammineni, 2015). The generation of reactive oxygen species (ROS) and resulting lipid peroxidation, together with the ability of the seeds to remove these radicals, are considered the main causes of seed deterioration (McDonlad, 1999; Bailly, 2004), especially for oilseeds such as *J. curcas* seeds.

ROS are molecules resulting from the reduction of molecular oxygen that consequently form toxic products (Bailly, El-Maarouf-Bouteau, & Corbineau, 2008). Therefore, to maintain seed viability, the regulation of ROS levels by enzymatic and non-enzymatic (antioxidant) activities, in conjunction with DNA repair mechanisms, should be at appropriate levels in the embryo (Kibinza, Bazin, Bailly, Farrant, & Corbineau, 2011; Donà et al., 2013). In seeds, the main enzymes present that scavenge these radicals are superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), and peroxiredoxin (PrxR) (Mittler, 2002; Rajjou & Debeaujon, 2008). It was previously reported that increased activity of SOD during *J. curcas* seed germination is related to protection against the ROS during this process (Cai et al., 2011).

Lipid peroxidation is one of the harmful effects caused by ROS (McDonald, 1999). Malondialdehyde (MDA) content is considered a biomarker of oxidative damage (Bailly et al., 1996) and is a commonly used method for the determination of lipid peroxidation (Cai et al., 2011); the accumulation of this compound is observed in the deterioration of lipid-rich seeds of some species, such as cotton (Goel, Goel, & Sheoran, 2003), sunflower (Kibinza, Vinel, Côme, Bailly, & Corbineau, 2006), and soybean (Sharma, Kaur, Bansal, & Gill, 2013).

The seed deterioration process is poorly understood; however, it is an important area of research (Hu et al., 2012). Moreover, *J. curcas* is progressively gaining importance in several countries due to its high oil content and suitability for biodiesel production (Kumar & Sharma, 2008; Parawira, 2010). Therefore, information regarding the storage ability of its seeds is important and can help define strategies for conservation, which is particularly important for the establishment of new production fields by low-technology farmers, which are predominant in Brazil (Arruda et al., 2004).

Thus, the objectives of this work were to assess the viability and vigour of *J. curcas* seeds at different maturity stages during short-term storage in ambient conditions and to investigate the deterioration process of this species in relation to the activation of the enzymes of the antioxidant defence system and the lipid peroxidation.

Material and methods

Jatropha curcas fruits were harvested from a commercial production area planted in 2006, located in the county of Viçosa, Minas Gerais State, Brazil (latitude 20°48'24.008" S and longitude 42°52'53.306" W).

At least 50 similar plants were randomly selected, and their fruits were harvested at distinct stages of maturity (at least 2,000 fruits per stage) based on the outside colouring of the skin, i.e., yellow, brownish-yellow, and completely brown (dry fruit), which corresponded to approximately 60, 70, and 80 days after anthesis, respectively. The seeds from each stage of maturity, with initial moisture contents of 48%, 48%, and 18% for yellow, brownish-yellow and brown fruits, respectively, were manually extracted and set to dry naturally until equilibrium moisture content was reached (approximately 8%). Afterwards, the seeds were placed in Kraft paper bags and remained in storage in a laboratory environment for 18 months, after which they were evaluated every three months. Ambient temperature and relative humidity were monitored daily with the aid of a thermo-hygrograph during the storage period.

For germination tests, eight repetitions of 25 seeds distributed in a paper towel roll wetted with a quantity of water equivalent to 2.7 times the dry weight of the substrate were used. The rolls were

kept in a growth chamber at 25°C, and the evaluations of the percentage of normal seedlings were made at seven (first count of germination) and 12 days (final germination) after sowing (Oliveira, Dias, Hilst, Silva, & Dias, 2014).

Bulk electrical conductivity was measured (ISTA, 1995). For this purpose, four repetitions of 25 seeds were weighed and placed into plastic cups containing 75 mL of distilled water (methodology determined by preliminary tests). The set was then kept in a BOD (Biochemical Oxygen Demand) chamber at 25°C for 24 hours. Afterward, electrical conductivity was determined using a Digmed model DM-31 electrical conductivity meter. The results were expressed in micro Siemens per centimetre per gram of seeds.

The malondialdehyde (MDA) content was determined by the thiobarbituric acid (TBA) reaction following the methodology described by Peixoto, Cambraia, Sant'Anna, Mosquim, and Moreira (1999). Twenty-five embryos for each replicate were ground with a mortar and pestle, and then samples of 200 mg of embryo tissue were homogenized with 2 mL of 0.1% trichloroacetic acid (TCA)(w/v). The homogenized product was centrifuged at 12,000 xg for 15 min. An aliquot of 0.5 mL of the supernatant was added to 1.5 mL of 0.5% TBA (w/v) and 20% TCA. The mixture was incubated in boiling water for 30 minutes and then immediately cooled in an ice bath. Afterwards, the samples were centrifuged at 10,000 xg for 10 min. The absorbance of the sample was read at the wavelengths of 532 nm and 600 nm (Cakmak & Horst, 1991) using a spectrophotometer (Thermo Scientific™ model UV-Vis Genesys 105). Lipid peroxidation was expressed as the MDA content in nanomoles per gram of fresh matter based on the difference between absorbance at 535 and 600 nm, using a molar extinction coefficient of 155 mM^{-1} cm^{-1}.

For the determination of enzymatic activity and soluble protein content, four repetitions of 50 seeds each were imbibed for 36 hours, after which the embryos were extracted and frozen in liquid nitrogen. The embryos (embryonic axes and cotyledons) were ground with a mortar and pestle, and then the crude enzymatic extracts were obtained by homogenization of 300 mg of embryo tissue in liquid nitrogen followed by the addition of 2 mL of extraction medium, which consisted of 0.1 M potassium phosphate buffer (pH 6.8), 0.1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM phenylmethanesulfonyl fluoride (PMSF), and 1% polyvinyl polypyrrolidone (PVPP) (w/v) (Peixoto et al., 1999). The homogenized product was centrifuged at 12,000 xg for 15 min. at 4°C. Subsequent analyses were performed immediately after removing the embryo for each storage period.

The soluble protein content of the enzymatic extracts was determined in accordance with the method of Bradford (1976), using BSA as a standard. 100 microlitres of the enzyme extract was added to 1 mL of Bradford reagent, followed by shaking. After 20 minutes, the absorbance was read with a spectrophotometer at a wavelength of 595 nm.

Superoxide dismutase (SOD) activity was determined by the addition of 30 μL of the crude enzymatic extract to 2.97 mL of reaction medium, which consisted of 50 mM sodium phosphate buffer (pH 7.8),13 mM methionine, 75 μM p-nitroblue tetrazolium (NBT), 0.1 mM EDTA, and 2 μM riboflavin (Del Longo, González, Pastori, & Trippi, 1993). The reaction was conducted at 25°C in a reaction chamber under lighting from a 15-W fluorescent bulb, and the reaction vessel was kept inside a box covered with aluminium foil. After 5 minutes of exposure to light, the lighting was interrupted; formazan blue, produced by the photoreduction of the NBT, was then measured according to its absorbance at 560 nm. The absorbance value of a reaction medium equal to the previous medium but which was kept in the dark for an equal period served as a control and was subtracted from the absorbance reading of the sample that was exposed to light (Giannopolitis & Ries, 1977). One SOD unit was defined as the quantity of enzyme necessary to inhibit the photoreduction of NBT by 50% (Beauchamp & Fridovich, 1971).

Peroxidase (POX) activity was determined by the addition of 100 μL of the crude enzymatic extract to 2.9 mL of reaction medium, which consisted of 25 mM potassium phosphate buffer (pH 6.8), 20 mM guaiacol, and 20 mM H_2O_2 (Kar & Mishra, 1976). Purpurogallin production was determined by the increase in absorbance at 420 nm at 25°C for the first minute of the reaction. Enzymatic activity was calculated using a molar extinction coefficient of 2.47 mM^{-1} cm^{-1} (Chance & Maehley, 1955) and was expressed as micromoles per minute per milligram of protein.

Ascorbate peroxidase (APX) activity was determined by adopting the methodology described by Peixoto et al. (1999). One hundred microlitres of the crude enzymatic extract was added to 2.9 mL of reaction medium, which consisted of 50 mM potassium phosphate buffer (pH 7.8), 0.25 mM ascorbic acid, 0.1 mM EDTA, and 0.3 mM H_2O_2. The decrease in absorbance at 210 nm at 25°C was determined for the first minute of the reaction. Enzymatic activity was calculated using a molar extinction coefficient of 2.8 mM^{-1} cm^{-1} (Nakano & Asada, 1981), and the result was expressed as micromoles per minute per milligram of protein.

The experiment was carried out in a completely randomized design in a split-plot arrangement with

four replications. The stages of fruit maturity were allocated to the plots and the periods of evaluation to the split-plots. The assumptions of normality and homogeneity of variance for the data obtained were tested using Shapiro-Wilk's test and Bartlett's test ($p < 0.05$), respectively. The data were subjected to the ANOVA, and the mean values obtained for the stages of fruit maturity for each analysis and in each period were compared using Tukey's test ($p < 0.05$). The germination data did not follow a normal distribution, and the mean values obtained for the stages of fruit maturity were compared by the Wilcox on rank test ($p < 0.05$).

Results

The temperature and relativity humidity during the eighteen months of storage ranged from 19.7 to 28.2°C and from 49.0 to 77.2%, respectively (Figure 1). In general, there was greater fluctuation in the relative humidity (64 ± 11%, standard deviation - S.D.) than in the temperature (23.2 ± 2.7°C, S.D.). Although the storage condition has a direct effect on the seed moisture content, which is reflected in the metabolism, the average water content of the seeds of the three maturity stages remained relatively stable throughout the storage period (8.7% ± 0.6%, S.D.), which is important for seed conservation.

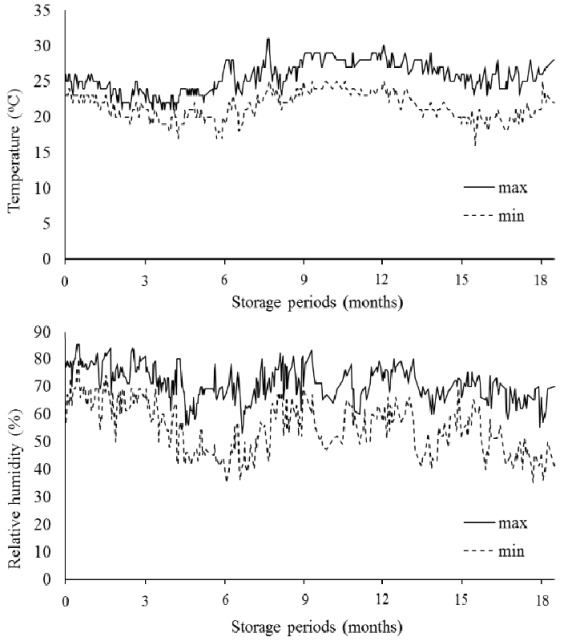

Figure 1. Ambient temperature (a) and relative humidity (b) during the storage of *Jatropha curcas* L. seeds.

At the beginning of storage, the seeds from the three stages of maturity had germination values greater than 87% (Figure 2a), which means that the seeds were harvested with high physiological quality. Additionally, there was no difference in germination between the seeds of the three stages of fruit maturity throughout the entire period of storage, except at nine months of storage, at which the seeds obtained from yellow fruits showed lower germination compared to the seeds obtained from brown fruits. When comparing the periods of storage for each stage of maturity, a decrease in viability was observed, especially after nine months of storage for seeds extracted from brown fruits and after 15 months for seeds extracted from brownish-yellow and yellow fruits.

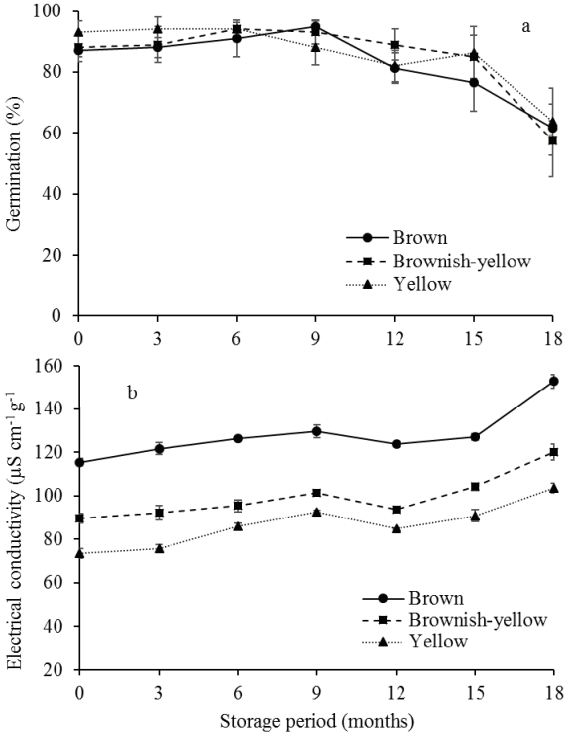

Figure 2. Germination (a) and electrical conductivity (b) of *Jatropha curcas* L. seeds extracted from fruits at different stages of maturity as a function of the storage period. The minimum significant difference (Tukey's test, $p < 0.05$) between the stages of maturity for electrical conductivity was 4.05. Bars represent the average of four repetitions ± standard error.

Throughout the entire storage period, lower values of electrical conductivity were obtained for seeds extracted from yellow fruit, whereas the greatest values were observed in seeds obtained from brown fruit (Figure 2b). In addition, there was an increase in electrical conductivity values with increasing seed storage period for the three stages of fruit maturity (Figure 2b). This result indicates reduced seed vigour throughout the storage period, which was previously observed compared with decreased viability; this reduced seed vigour started after nine months of storage for seeds extracted from

the brown fruits and after 15 months for seeds extracted from brownish-yellow and yellow fruits (Figure 2a). The increase in electrical conductivity was more evident after 18 months of storage at all stages of maturity. This period coincided with a sharper reduction in seed germination, i.e., after 18 months of storage, when there was a decrease of 15, 27, and 22% in seed germination of the fruits at the brown, brownish-yellow, and yellow stages, respectively, in relation to the previous period (Figure 2a).

A reduction in the initial values of MDA content was observed in the embryos of the *J. curcas* seeds during storage, regardless of the stage of fruit maturity; this effect was observed for seeds stored for up to the nine months (Figure 3a). After 12 months, there was an increase in the MDA content; however, after this period, there was a reduction in the values. There was no difference in the MDA content of the seeds across the three stages of maturity, either at the beginning or throughout the entire period of storage.

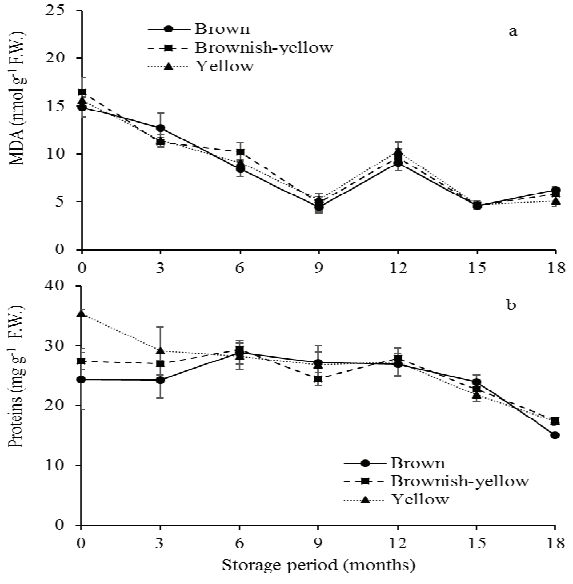

Figure 3. Malondialdehyde (MDA) content (a) and protein content (b) in the embryos of *Jatropha. curcas* L. seeds obtained from fruit at different stages of maturity as a function of storage period. The minimum significant difference (Tukey's test, $p < 0.05$) between the stages of maturity for MDA content was 1.51; for protein content, 4.56. Bars represent the average of four repetitions ± standard error.

At the beginning of storage, lower contents of soluble proteins were observed in the embryonic axis of the seeds obtained from brown fruits followed by those of brownish-yellow fruits, compared to the seeds from yellow fruits (Figure 3b). Afterward, there was a slight increase in the protein content after six months of storage for the first two stages, followed by a reduction in protein content. For the seeds extracted from yellow fruits, the protein content decreased at the beginning of storage, and for all stages of maturity, a larger reduction was observed after 12 months of storage. This period coincided with a sharper reduction in seed germination (Figure 2a), when lower values were observed for the seeds extracted from brown fruits.

SOD enzyme activity was initially 1.26, 1.05, and 0.78 U min.$^{-1}$ mg protein^{-1} in the seeds of the fruits at the brown, brownish-yellow, and yellow stages, respectively (Figure 4a). During seed storage, there was an increase in the activity for the three stages of maturity (Figure 4a). The increase in the activity of this enzyme was more accentuated through nine months of storage, especially for the seeds extracted from brown fruits, followed by a slight decrease until 15 months of storage and another increase thereafter.

There was a reduction in peroxidase (POX) enzyme activity during the storage of *J. curcas* seeds at the three stages of fruit maturity. After nine months of storage, the activity of this enzyme was not detected (Figure 4b). When comparing the fruit maturity stages, greater POX activity at the beginning of storage was observed in seeds extracted from brown fruit, as was observed for the SOD enzyme. However, after three months of storage, there was a sharp decrease in the activity of this enzyme in seeds extracted from brown and brownish-yellow fruits but greater activity in the seeds obtained from yellow fruits. After three months of storage, there was also reduction in POX activity at all stages of maturity, and after nine months of storage, which was the last period in which it was possible to detect the activity of this enzyme, there was no difference between the stages of fruit maturity.

For APX, there was a reduction in enzyme activity during the storage of *J. curcas* seeds (Figure 4c). When comparing the stages of fruit maturity, greater APX activity was observed in seeds obtained from brown fruits, followed by those obtained from brownish-yellow fruits, until three months and after nine months of storage (Figure 4c). At six months, there was a sharp decrease in APX activity for the seeds obtained from brown and brownish-yellow fruits, while the activity in the seeds extracted from yellow fruits showed a steady decrease until 12 months of storage. After 12 months of storage, APX activity reached its lowest values (< 42 nmol min.$^{-1}$ mg^{-1} of protein) for all three stages of maturity (Figure 4c).

In seeds from all three stages of maturity, APX activity was not detected after 18 months of storage, due to reduced activity of this enzyme to undetectable levels or to total loss of activity.

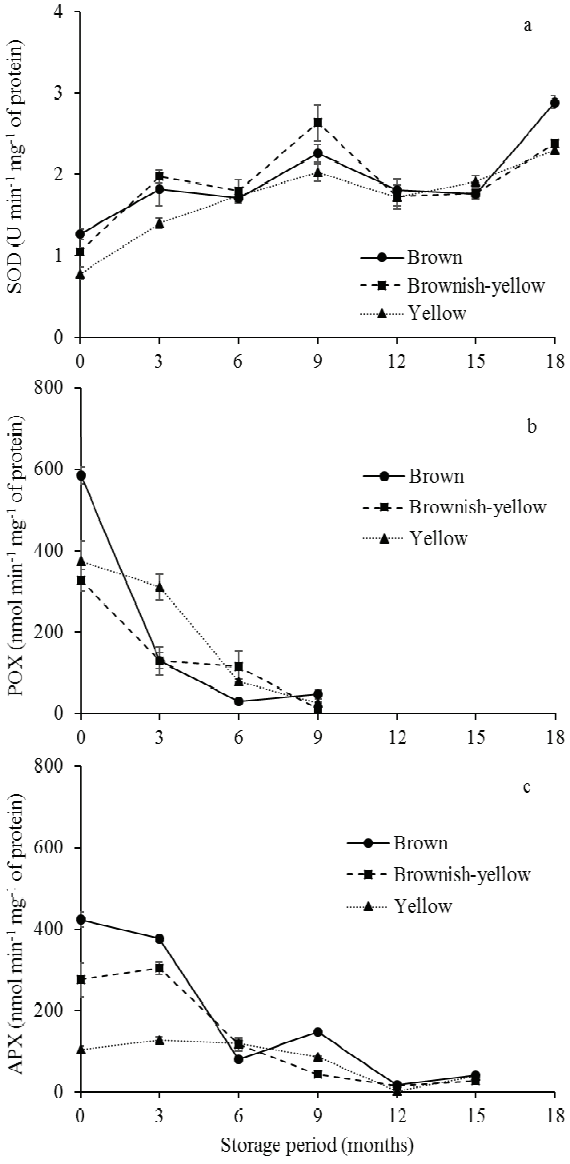

Figure 4 Activity of the enzymes superoxide dismutase (SOD) (a), peroxidase (POX) (b), and ascorbate peroxidase (APX) (c) in the embryos of *Jatropha curcas* seeds obtained from fruit at different stages of maturity as a function of storage period. The minimum significant difference (Tukey's test, $p < 0.05$) between the stages of maturity for SOD was 0.2, for POX was 55.7, and for APX was 24.0. Bars represent the average of four repetitions ± standard error.

Discussion

The initial quality of seeds to be stored is important to their survival during storage (Bewley et al., 2013). The seed germination percentages obtained in this study were greater than those obtained in other studies for shorter periods of storage. This result is attributed to the high initial quality of the seeds, which were stored just after being harvested and had initial germination greater than 87% (Figure 2a). Previously, for *J. curcas* seeds that initially had 86% germination, Pereira et al. (2013) obtained 76% germination after 6 months of storage, while Guzman and Aquino (2009) observed total loss of viability of two lots with initial germination percentages of 94% and 80% after 12 months of storage. Additionally, Moncaleano-Escando et al. (2013) reported that these seeds have a short viability period (less than 6 months), although for their experiments they used seeds with low viability (65%). In the present study, the viability of *J. Curcas* seeds had begun to decrease after nine months of storage for seeds obtained from brown fruits and after 15 months of storage for seeds obtained from brownish-yellow and yellow fruits. These results suggest that the longevity of these seeds is longer than that previously reported and that high-quality seeds can be stored for extended periods.

There was an increase in the values of electrical conductivity in the *J. curcas* seeds during the storage period (Figure 2b). These results are in accordance with the hypothesis proposed by Delouche and Baskin (1973) that reduction in seed viability is associated with loss of integrity of the cell membrane system. Thus, an increase in the values of electrical conductivity, although in low levels compared to the initial values for each stage of seed maturity stage, shows loss of integrity of the cell membrane system, which can be associated with the accumulation of harmful alterations in the seeds. According to McDonald (1999), these alterations can be associated with lipid peroxidation, membrane disruption, DNA damage, impairment of RNA and protein synthesis as a result of ROS generation. The results for soluble protein content (Figure 3b) and enzyme activity (Figure 4) support this statement, although it is necessary to correlate these data with the ROS analysis to assure that the deterioration process observed is due to the oxidation of cellular components, as the MDA content did not increase during storage (Figure 3a).

Lower values of electrical conductivity were obtained for seeds extracted from yellow fruits (Figure 2b). These data suggest that in the seeds obtained from brown fruits (dry fruits), which presented high germination (Figure 2a), greater values of EC are associated with seed maturation drying phenomena. During the natural desiccation of the seeds attached to the plants, harmful alterations can occur, leading to greater values of

EC. These phenomena are related to the production of ROS and to greater antioxidant enzyme activity (Kranner & Birtic, 2005). This can be confirmed by our results regarding the differences between the enzymatic activity before storage across the three stages of seed maturity (Figure 4), which were highest in the embryos of the seeds extracted from brown fruits. Additionally, seeds extracted from the yellow and brownish-yellow fruits presented greater water contents just after harvest and more rapid drying (outside the fruit tissue) than did seeds from brown fruits, which dried relatively slowly inside the fruits on the plants. This could lead to less harmful alteration to the seeds of fruits of the yellow and brownish-yellow stages and could consequently lower EC values. It was previously reported that rapid drying is better for *J. curcas* seed quality (Zonta, Araújo, Araújo, & Dias, 2011).

In the present study, a relationship between the loss of seed viability and the accumulation of MDA was not observed (Figure 3a). The accumulation of MDA is associated with lipid peroxidation as a result of the attack of ROS on polyunsaturated fatty acids, leading to seed deterioration and reduced viability (El-Maarouf-Bouteau, Mazuy, Corbineau, & Bailly, 2011; Kumar et al., 2015). The accumulation of MDA during ageing is reported in several species, including sunflower (Kibinza et al., 2006), wheat (Lehner et al., 2008), cotton (Goel et al., 2003), and soybean (Sharma et al., 2013), during artificial and natural ageing. Despite these observations, Kibinza et al. (2006) also observed a reduction in MDA content in sunflower seeds stored at low moisture content and concluded that seed deterioration was not associated with lipid peroxidation under those conditions. Additionally, Mira, Estrelles, González-Benito, and Corbineau (2011) showed that conductivity tests effectively detected seed deterioration in four Brassicaceae species, which was similar to the results obtained in the present work, but the loss of seed viability was not associated with malondialdehyde accumulation, suggesting that lipid peroxidation did not cause seed deterioration.

During dry storage, there is no free water in the dry seeds, and non-enzymatic mechanisms such as lipid peroxidation are likely to be involved in ROS accumulation (El-Maarouf-Bouteau et al., 2011). In the present study, seeds were stored dry, at a moisture content of approximately 8.0%. During storage, the moisture content of the seeds ranged from 7.5% to 9.6% (data not shown). Lipid peroxidation is a process that depends on seed moisture content, i.e., it occurs through autoxidation at moisture levels less than 6%, while production of ROS by oxidative enzymes is responsible for lipid peroxidation at moisture contents greater than 14% (McDonald, 1999). Between these moisture levels, it is probable that lipid peroxidation is minimal because there is sufficient moisture content to serve as protection against the autoxidation due to the ROS attack but not sufficient to activate oxidative enzymes.

Thus, even in the event of increased electrical conductivity of seeds throughout the period of storage (Figure 2b) and reduced germination (Figure 2a), the data obtained for MDA content in the embryos suggest that in the conditions in which the seeds were stored, i.e., under low moisture content (approximately 8%) and ambient temperature and relative humidity (Figure 1), lipid peroxidation did not occur.

A greater initial value of soluble protein content was observed for seeds of fruits at the yellow stage of maturity, followed by the brownish-yellow and brown stages (Figure 3b). This reflects metabolic activity and is related to the maturation process (Rajjou & Debeaujon, 2008), as the yellow stage is near physiological maturity (Silva et al., 2011); these seeds still have a high moisture content and are metabolically active. During storage, a reduction in soluble protein content in the embryos of *J. curcas* seeds was observed (Figure 3b). This reduction can reflect the respiratory activity, which increases throughout storage, leading to protein degradation. Protein degradation is one of the causal mechanisms indicated in the loss of seed viability (McDonald, 1999).

The colour of the *J. curcas* fruit changes according to seed maturation – from green to yellow to brown (Silva, Dias, Dias, & Hilst, 2011). The point of physiological maturity of the seeds corresponds to the brownish-yellow stage, and these seeds have a high moisture content. Then, changing fruit colour accompanies the natural maturation drying phenomena in this species. In the present work, seeds extracted from fruits at the brown stage (dry fruits) had a moisture content of approximately 18% just after harvest, while for yellow and brownish-yellow stages these values were greater than 48%.

A greater activity of SOD enzyme, and also of the POX and APX enzymes, was observed at the most advanced stage of fruit maturity (Figure 4a), i.e., brown fruits (dry fruits), and a lower degree of activity was observed for fruits at the yellow stage, i.e., near the point of physiological maturity of the seeds (Silva et al., 2011), before storage and after drying. According to Martins, Vilela, Guimarães, Gomes, and Silva (2012), higher SOD activity is required at the beginning and at the end of the seed maturation process. This is an indication that its

action in the defence against the formation of ROS is mainly required during the stages in which the seeds are not completely formed, exhibiting intense metabolic and respiratory activity, and during the final stage of maturation, as a protection against the ROS generation during desiccation.

According to Rajjou and Debeaujon (2008), seed tolerance to desiccation may be related to seed capacity for eliminating ROS to avoid harmful events, such as lipid peroxidation induced by those compounds. Thus, greater activity of the enzymes related to the antioxidant defence system in the seeds obtained from brown fruits, i.e., dry fruit, may be related to the production of ROS during the process of natural desiccation of these seeds. In addition, the greater activity may also be related to the protection acquired by these seeds at the end of the maturation phase, ensuring reduced effects caused by the attack of ROS on compounds essential for metabolism during germination. This was shown by the viability (Figure 2a) and MDA content in the embryos (Figure 3a) of the seeds extracted from brown fruits, which did not differ from those of the other stages.

In the present study, there was an increase in SOD activity throughout seed storage (Figure 4a). Cai et al. (2011) observed an increase in SOD enzyme activity during the germination of *J. curcas* seeds. According to these authors, the increase in activity of this enzyme may be triggered by the increase in production of ROS or may be a protective mechanism adopted by the species against oxidative damage. This enzyme seemed effective in the protection of cells against the anion superoxide, as the MDA content (Figure 3a) suggests that lipid peroxidation is not responsible for the reduction in seed viability. In addition, high values of seed germination were observed up to 15 months of storage for fruits of the yellow and brownish-yellow stages (Figure 2a), i.e., the seeds remained viable for an extended period, which is associated with increased SOD activity.

Higher activity of SOD increases the H_2O_2 content in cells, leading to an increase in the activity of antioxidant enzymes, whose action eliminates this ROS. As such, it was expected that the activity of POX and APX would increase, which is associated with increased SOD activity (Figure 4a), but this was not observed (Figure 4b and c). According to Mittler (2002), there are different affinities of APX (micromolar range) and CAT (millimolar range) for H_2O_2, which suggests that they belong to two different classes of H_2O_2-scavenging enzymes. This claim can be extended to POX, as was observed in the present work (Figure 4b). Therefore, APX could be responsible for the fine modulation of ROS for signalling, whereas CAT could be responsible for the removal of excess ROS during stress (Mittler, 2002), as occurs during seed deterioration.

We therefore hypothesize that the H_2O_2 generated by SOD was scavenged mainly by CAT activity (not assessed in this work), as there was an increase in the activity of SOD (Figure 4a), and the viability of the seeds, mainly of the fruits of the yellow and brownish-yellow stages, remained high during storage, although this hypothesis needs to be confirmed.

A reduction in POX (Figure 4b) and APX (Figure 4c) enzyme activities during storage of *J. curcas* seeds at the three stages of fruit maturity was observed. According to Mittler (2002), the reduction or loss of enzyme activity is associated with the deterioration process, which includes reductions in both the synthesis and activity of enzymes; loss of DNA integrity; and, mainly, oxidative stress.

According McDonald (1999), reduced enzyme activity during seed ageing is one of the causes and indications of deterioration. This process leads to harmful alterations and to reduced seed vigour, as was observed for EC (Figure 2b), and this process ultimately leads to reduced seed viability (Figure 2a). The decrease in antioxidant potential of the cells associated with the generation of ROS is the prerequisite event for seed deterioration due to ageing (Kibinza et al., 2011). However, these enzymes exhibit different sensitivities to seed ageing depending on the species and the storage conditions (Lehner et al., 2008), as was observed for the SOD enzyme in this study, for which increased enzyme activity was observed.

Conclusion

Jatropha curcas seeds with high initial quality obtained from fruits harvested at the yellow, brownish-yellow and brown (dry) stages can be stored under environmental conditions for up to 15, 15, and 9 months, respectively. Seed vigour decreases during storage, although it does not compromise the seed viability during these periods.

Seed deterioration during storage under environmental conditions and low moisture content (8%) is not related to lipid peroxidation in this species.

The deterioration of seeds is associated with changes in enzyme activities of the antioxidant defence system in the embryos during storage, with an increase in SOD activity and a reduction in POX and APX activities.

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