academicJournals

Vol. 9(20), pp.1550-1559, 15 May, 2014
DOI:10.5897/AJAR2013.7923
Article Number: 3981F0B44665
ISSN 1991-637X

http://www.academicjournals.org/AJAR

African Journal of Agricultural Research

Full Length Research Paper

Physiological and biochemical performance of *Pouteria ramiflora (*Mart.) Radlk. seeds harvested at different maturation stages and subjected to drying

Lílian Abadia da Silva[1]*, Juliana de Fátima Sales[1], João Almir Oliveira[2], Heloisa Oliveira dos Santos[2], Túlio Silva Lara[2] and Marcos Antônio Soares[3]

[1]Federal Institute of Education, Science and Technology of, Cx. Postal 66, CEP: 75.901-970, Rio Verde, GO – Brasil.
[2]Department of Agriculture - Seed Section, Federal University of Lavras, Cx. Postal: 3037, CEP: 37.200-000 – Lavras, MG – Brasil.
[3]Department of Botany and Ecology, Federal University of Mato Grosso, Av. Fernando Corrêa da Costa, nº 2367 - 78060-900, Cuiabá, MT – Brasil.

Received 9 September, 2013; Accepted 7 May, 2014

This study aimed to evaluate alterations in the physiological and biochemical performance of *Pouteria ramiflora* (Mart.) Radlk. seeds at different maturation stages when subjected to drying. The seeds were harvested at two maturation stages (unripe and ripe). A sample of seeds at each maturation stage was not subjected to drying (control), and another sample was subjected to drying in a convection oven at 35°C until the seeds reached water content levels of 20 and 12% wet basis (w.b.). The drying of seeds up to 12% bu. negatively affected the physiological quality and vigor of the seeds, particularly in the unripe seeds. There was no detectable activity of catalase and peroxidase isoenzymes, and heat-resistant proteins were not observed on denaturing polyacrylamide gels. The α-amylase enzyme was most active in mature and moist seeds and showed the negative effect of drying, particularly with respect to unripe seeds. A greater intensity of esterase isoenzyme bands was detected in unripe seeds (regardless of water content) with little reduction of activity as the water content of mature seeds decreased. It can be concluded that the *P. ramiflora* (Mart.) Radlk. seeds show physiological and biochemical alterations when subjected to drying, they are intolerant to desiccation.

Key words: Forest species, physiological maturity, desiccation tolerance, isoenzymes.

INTRODUCTION

Pouteria ramiflora (Mart.) Radlk., locally known as ‘curriola’, ‘abiú-do-Cerrado’, ‘abiu-piloso’, ‘bacupari liso’, ‘fruta-de-veado’, ‘grão-de-galo’, ‘massaranduba’, ‘gunjara’, ‘mandapuca’ and ‘pitomba-de-leite’, is a woody fruit species that is widely distributed in the areas of Cerrado (Tropical Savanna); it is found in the Cerradão (Savanna Forest), Cerrado sensu stricto, Cerrado ralo (Open Cerrado), Borda de vereda (Palm-swamp border area) and mesophytic forest phytophysiognomies (Almeida et al., 1998). It has small hermaphrodite or

*Corresponding author. E-mail: lilianabadia5@gmail.com, Tel: 0(64) 8448-6707.

pistillate flowers arranged in racemose inflorescences with short axes that are densely arranged in leaf axils along the branches. Flowering occurs from May to September, with open flowers peaking in August (during the dry season); the fruit ripens during the rainy season, which promotes seed dispersal (Gama et al., 2011). The fruit has a strong smell and pleasant taste; it is greenish in color with white pulp and is appreciated by local people as juice or *in natura* (Dalponte and Lima, 1999). Permanent preservation areas may be sought because it is a species suited for open environments (Lorenzi, 1992). There were no reports found in the literature about the physiological performance of its seeds in response to water loss. Thus, the physiological characteristics of seeds must be assessed because of their ecological relevance and economic potential with the aim of establishing conservation strategies and contributing to create protocols for the production of seedlings of this species. The physiological condition of the seed was initially studied by Roberts (1973), who classified the seeds as orthodox or recalcitrant according to their performance in storage. Following this, Ellis et al. (1990) introduced the concept of intermediate seeds. Because this physiological aspect is related to the degree of seed tolerance to dehydration, these should be classified as desiccation-tolerant or orthodox seeds, desiccation-intolerant or recalcitrant seeds, and intermediate seeds (whose performance during drying and storage is sometimes similar to orthodox seeds and sometimes similar to recalcitrant seeds).

The standard orthodox seed development is characterized by the following three main phases: embryogenesis (histodifferentiation), in which the fertilized zygote undergoes multiple cell division; maturation or seed filling, in which synthesis of storage reserves, rapid cell expansion and dry matter accumulation occur; and, finally, desiccation and quiescence, in which dehydration occurs until reaching equilibrium with the relative humidity of the air and showing relatively low moisture content by harvest (Jiang and Kermode, 1994; Angelovici et al., 2010). Typically, orthodox seeds tolerate desiccation and (most likely) depend on it to redirect the metabolic processes of development towards germination. With respect to drying, there are two distinct periods during seed development: desiccation-intolerant (first phase and part of the second) and desiccation-tolerant (part of the second phase and the entire third phase). Moreover, recalcitrant seeds lose little water during maturation (moving directly from development metabolism to germination), are susceptible to desiccation, and normally maintain moisture contents between 30 and 70% (Barbedo and Marcos-Filho, 1998; Marcos-Filho, 2005).

Plant cells require physiological, biochemical and morphological adaptations that are temporally coordinated by differential expressions of genes capable of preventing cell damage or death as water content is removed during seed development. The mechanisms of desiccation tolerance in angiosperms appear to have originated ancestrally from algae and endosymbiotic progenitors of mitochondria and chloroplasts in plants (Gaff and Oliver, 2013). The acquisition of desiccation tolerance is regulated and the possible mechanisms of tolerance are activated only at precise moments following fertilization; vegetative tissues must respond to environmental signals to activate protection mechanisms for the entire plant. Environmental signals of extreme water loss may conceivably activate an existing repertoire of genes that specifically protects seeds and vegetative tissues (Illing et al., 2005).

Estimates suggest that approximately 50% of tropical species produce seeds sensitive to desiccation (Tweddle et al., 2003). Desiccation tolerance in species able to withstand that process is acquired in the phase of reserve accumulation and may be induced artificially by slow drying at certain developmental stages. Abscisic acid (ABA) has a key role in desiccation tolerance in orthodox seeds and also controls the accumulation of reserve substances (Nambara et al., 2010). The concentration of ABA during development decreases near the end of this phase while desiccation tolerance increases (Pammenter et al., 1994). Seeds undergo changes at different stages of development and become more tolerant to higher temperatures of drying with gradual water loss after physiological maturity, which indicates that events occur concomitantly with the reduction of water content (Rosa et al., 2004).

Several biochemical changes occur in seed cells in response to desiccation. Stresses associated with extreme water loss include mechanical stress associated with the loss of turgor, free radical oxidative stress, and destabilization or loss of macromolecular integrity (Walters et al., 2002). In *Arabidopsis*, the transition between reserve accumulation in seeds and desiccation is associated with a significant change in their metabolism that results from the accumulation of a variety of sugars, organic acids, nitrogen-rich amino acids and shikimate-derived metabolites (Fait et al., 2006).

There is a change in seed gene expression with the onset of drying, in which genes encoding protective molecules play a key role in desiccation-tolerant tissues (Ramanjulu and Bartels, 2002). The acquisition of desiccation tolerance, the ability to tolerate very low water potentials is correlated with the accumulation of various protective compounds, including sugars and proteins. These compounds include heat shock proteins (HSP), free radical-scavenging enzymes and non-reduced sugars or low molecular-weight solutes, which typically accumulate during the late stages of embryogenesis and coincide with the acquisition of desiccation tolerance. A special group of proteins known as late embryogenesis accumulated (LEA) proteins become abundant in the final phase of seed maturation with the genetic control exerted by the phytohormone

ABA; these proteins most likely act as chaperones, protecting macromolecular structures from damage caused by desiccation (Hoekstra et al., 2001; Manfre et al., 2009). Formation of reactive oxygen species (ROS), occurs during seed desiccation, which can lead to oxidative stress and cell damage and may result in deterioration. However, cells express detoxifying enzymes and produce antioxidant compounds that remove the ROS. Thus, the detoxification mechanisms play a key role in the acquisition of desiccation tolerance in developing seeds (Bailly, 2004). Therefore, this study was performed to evaluate desiccation tolerance enabled through changes in the physiological and biochemical performance of *Pouteria ramiflora* (Mart.) Radlk. seeds at different maturation stages after being subjected to drying.

MATERIALS AND METHODS

Fruits were collected at Fazenda Gameleira [Gameleira Farm] (16° 06' 20'' S – 51° 17' 11'' W, 592-m altitude), in the municipality of Montes Claros de Goiás, in December 2011. The research was performed in the Laboratory of Seed Analysis, Department of Agriculture, Federal University of Lavras, Minas Gerais (MG). The fruits were manually pulped and the seeds were washed in 2% sodium hypochlorite solution for one minute and then rinsed in distilled water. After washing, the seeds were placed in plastic trays covered with paper towels to remove excess water. The seeds were then divided into two groups according to the stage of their fruit ripening; the seeds from fruits picked from the ground under trees were considered mature, whereas seeds from fruits picked directly from the plant were considered unripe. The water content of seeds, unripe and ripe, was assessed using the air-oven method at 105±3°C for 24 h (Brasil, 2009) with four subsamples of 10 seeds. The calculation was performed on a wet basis (w.b.), and the result was expressed as a percentage. After assessing the initial water contents, some of the seeds were packed in paper bags and kept in a convection-drying oven at a temperature of 35±2°C. The sample mass was assessed using a precision scale every hour until values of water content of 20 and 12% w.b. were reached. Seed water loss was assessed using the formula recommended by the following rules for seed analysis (Brasil, 2009).

$$Wf = Wi \cdot \left(\frac{100 - WCi}{100 - WCf}\right)$$

where Wf: final sample weight (g); Wi: initial sample weight (g); WCi: initial seed water content (% w.b.); and WCf: desired seed water content (% w.b.).

Seeds at both maturation stages and with different water contents were evaluated with respect to the following parameters: germination (%); germination rate index; emergence (%); emergence rate index; X-ray test; electrical conductivity; respiration rate; quantification of reducing, non-reducing and total soluble sugars; the electrophoretic profile of enzymes (catalase, esterase and peroxidase) on a non-denaturing gel and of heat shock proteins by electrophoresis on a denaturing polyacrylamide gel; and the enzymatic activities of α-amylase and endo-β-mannanase. Seeds were subjected to manual scarification as a pre-germinative treatment, using sandpaper No. 50 (against the micropyle) to accelerate the process of soaking and, consequently, of germination.

Germination test

Sowing was performed on germitest paper rolls with two sheets as the base and one for the cover; for the germination test, these sheets were moistened in distilled water at a ratio of 2.5 times the dry substrate weight (Brasil, 2009) with four replicates of 25 seeds. The rolls containing the seeds were kept in a germinator set at 30°C. Daily assessments were performed until complete stabilization to calculate the germination rate index (GRI), according to Maguire (1962), and the seeds with 1.0 cm radicle protrusion were considered to be germinated. Only normal seedlings in which all essential structures were developed (well-developed primary root, and developed hypocotyl and epicotyl) 45 days after sowing were analyzed to assess the germination percentage.

Emergence test

A total of 100 seeds divided into four replicates of 25 each were used. Sowing was performed using a 40x30x8 cm plastic tray, containing a mixture of subsoil and sand at a ratio of 3:1 as a substrate. Sowing was performed at a depth of 3 cm; the substrate was moistened to 70% of its field capacity and subsequently irrigated when necessary. The trays were kept in a growth chamber (at 25°C) with an alternating light-dark cycle (12 h) for 30 days. Daily counts were conducted to assess the seedling emergence rate index (ERI), according to Maguire (1962), using as a criterion the emergence at a height of 1.5 cm (from the soil) for counting. After complete stabilization, the emergence percentage (E%) was also evaluated by analyzing only normal seedlings (well-developed primary and secondary roots, and expanded hypocotyl, epicotyl and foliage leaves).

X-ray test

Images of the internal structures of seeds were recorded using an X-ray device to evaluate the structural changes during desiccation and for showing possible damage resulting from the drying process. The seeds from each treatment were arranged in clear acrylic plates on double-sided adhesive tape and subjected to radiation using a Faxitron HP X-ray device, Model 43855A X, at 45 kV for 25 s to record digital images of the internal structures of seeds.

Electrical conductivity test

This test was performed with four subsamples of 10 seeds that were previously weighed on an electronic scale accurate to 0.001 g and then placed in plastic cups containing 75 ml of deionized water and kept in a biochemical oxygen demand (BOD)-type germination chamber set at 25°C for 24 h. After this period, the electrical conductivity of the soaking water was assessed using a benchtop digital conductivity meter, manufactured by Digimed, model CD 21A; the results were expressed as $\mu S.cm^{-1}.g^{-1}$.

Respiration rate

The respiration rate was assessed using the titration method, according to the methodology reported by Crispim et al. (1994). A glass Erlenmeyer flask with a fine screen attached to the mouth as support for the seeds was used for each replicate. Two sheets of blotting paper were wrapped in the screen and moistened with distilled water at a ratio equivalent to 2.5 times the dry weight; the seeds were placed over the wrapped screen. Forty-milliliter of 0.1 N KOH were added to the bottom of each Erlenmeyer flask. A control without seeds was prepared for each sample. Subsequently, the

Erlenmeyer flasks containing the samples were sealed with plastic wrap to avoid gas exchange, and kept in a BOD-type growth chamber for 24 h at a constant temperature of 25°C. After this period, 25 ml of 0.1 N KOH solution were taken from each sample to which three drops of phenolphthalein were added for titration with 0.1 N HCl. The volume of HCl spent to neutralize the KOH solution in each of the test samples was recorded at the turning point. Four subsamples of 10 seeds were used for each treatment, which were previously weighed to assess their dry weight. After titration, the respiration rate was calculated using the following equation:

$$RR = \frac{(B - L) \cdot C}{DW}$$

where B and L are the 0.1 N HCl volumes used in the blank and sample titrations, respectively; C is the constant termed correction factor with a value of 3.52; and DW is the dry mass of the analyzed seeds. The results were expressed as mg CO_2 per gram of dry seed.

Determination of the content of reducing, non-reducing and total soluble sugars

Four hundred milligram of seeds were macerated in a crucible with liquid nitrogen for quantification of reducing (RS), non-reducing (sucrose) and total soluble sugars (TSS). Thereafter, 10 ml of potassium phosphate buffer (100 mM pH 7.0) were added and the mixture was kept at 40°C for 30 min. The mash was then centrifuged at 10,000 rpm for 10 min, and the supernatant stored in the freezer at -20°C until analysis. The levels of reducing sugars (RS) were quantified adding the reagent 3,5-dinitrosalicylic acid (DNS) to 400 µl seed extract and the glucose standard curve was used (Miller, 1959). The readings were performed on a spectrophotometer at 540 nm. The levels of TSS and sucrose were quantified according to Willis and Yemm (1954) and using the anthrone reagent. The seed extract was diluted in potassium phosphate buffer (100 mM pH 7.0) at a 1:2 ratio for the TSS using a 30 µl aliquot of that solution for quantification. For the quantification of sucrose, 800 µl of the extract prepared were added to 800 µl of 30% KOH. The solution was incubated in a water bath at 37°C for 15 min, and 10 µl were subsequently taken from the solution for sucrose quantification. The TSS and sucrose standard curves were plotted based on known glucose concentrations and the reading was performed on a spectrophotometer at 620 nm.

Electrophoretic profile of enzymes

The enzymatic activities of α-amylase, catalase, esterase and peroxidase were assessed using non-denaturing polyacrylamide gel electrophoresis. Ten seeds from each treatment were ground in the presence of liquid nitrogen and PVP 40 (polyvinylpyrrolidone) antioxidant, and the extracts were immediately stored at -80±2°C until the analyses were performed. For the analysis of each enzyme, 100 mg aliquots were removed, Tris buffer (0.2 M Tris-HCl, pH 8 + 0.1% β-mercaptoethanol) was added at a ratio of 2.5 times its weight for catalase and esterase. A phosphate buffer (0.034 M disodium hydrogen phosphate, 0.2 M sucrose, 2.56% PVP; 0.003 M Dithiothreitol (DTT); 0.0057 M ascorbic acid; 0.0025 M sodium borate; 1% Polyethylene glycol (PEG) 6000; 0.2% β-mercaptoethanol) was used for the peroxidase extraction. Samples from the materials of each treatment were kept overnight in a refrigerator at 4°C and then centrifuged at 16,000 x g for 30 min at 4°C; 60 µl of supernatant from each sample were subsequently added to the discontinuous polyacrylamide gel system at 4.5% (stacking gel) and 7.5% (separating gel). The gel/electrode system was Tris-glycine pH 8.9 and the electrophoretic run performed at 4°C under constant 150-V voltage. The gels were stained after the electrophoretic run according to Alfenas (1998) for the esterase and catalase enzyme system and according to Tanksley and Orton (1983) for peroxidase.

The seeds were subjected to germination tests for 48 h for α-amylase analysis. Seedlings were removed after this period, and the reserve tissue was ground in a crucible (over ice) in the presence of liquid nitrogen and PVP; the samples were stored in an ultra-freezer at - 80±2°C until the time of analysis. Enzyme extraction was performed by adding 200 µl of 0.2 M Tris-HCl, pH 8.0 extraction buffer to 100 mg samples from each treatment. The homogenate was kept for 12 h in a refrigerator at a temperature of 5°C and centrifuged at 16,000 x g at 4°C for 30 min; 40 µl volumes of extract were then applied to a polyacrylamide gel at 4.5% (stacking gel) and 7.5% (separating gel – containing 5% soluble starch). The bands of α-amylase enzyme activity were stained according to Alfenas (1998), and the presence or absence of clear bands on a bluish background (negative staining) (resulting from the reaction with amylose) and the relative intensity of the isoforms were assessed following the treatment with iodine. The evaluation of gels was performed on transilluminator after staining, and the presence or absence of bands and relative intensity of the different isoforms were analyzed for each sample.

Assessment of the electrophoretic profile of heat shock proteins

The proteins of interest were separated and visualized by electrophoresis on a denaturing polyacrylamide gel (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis, SDS-PAGE). The seeds (wet and dry) were ground using liquid nitrogen and the extraction buffer was subsequently added buffer, consistent with Alfenas (1998), at a ratio of 10 parts buffer to 1 part sample. The samples were centrifuged at 16,000 x g for 30 min at 4°C. The supernatant was removed and incubated in a water bath at 85°C for 10 min to assess the heat shock proteins. The samples were again centrifuged as above. Prior to application on a denaturing polyacrylamide gel, 40 µl sample buffer (2.5 ml glycerol; 0.46 g SDS; 20 mg bromophenol blue; Tris-HCl pH 7.5) were added to 70 µl of each extract and boiled for five minutes. Next, 50 µl of each sample were applied to an SDS-PAGE of polyacrylamide gel at 12.5% (separating gel) and 6% (stacking gel); the running buffer used was Tris-glycine + SDS pH 8.9, and the electrophoretic run was performed on a vertical system at room temperature and constant voltage of 150 V for four hours. A molecular weight marker ranging from 10 to 100 KDa (Invitrogen) was used to assess the molecular mass of the polypeptide chains. Gels were stained after the run in 0.05% Coomassie Brilliant Blue solution for 24 h and destained in a solution of ethanol, acetic acid, and water at the ratios of 0.5:1:8.5 (v:v), respectively, consistent with Alfenas (1991). The evaluation of gels was performed on a transilluminator, which analyzed the presence and absence of bands.

Endo-β-mannanase enzymatic activity

The seeds from each treatment, pre-soaked in distilled water for 24 h and without the seed coat, were macerated in liquid nitrogen and PVP 40 for the extraction of endo-β-mannanase. Six hundred microlitre of extraction buffer [0.1 M Hepes; 0.5 M NaCl and 0.5% ascorbic acid, pH 8.0] were added to 200 mg of seed macerate from each treatment. Then, the microcentrifuge tubes containing the samples were stirred using a vortex-type stirrer for 1 min and centrifuged for 30 min at 10,000 x g at 4°C. Twenty microlitre of supernatant were run on a gel containing 6 ml of locust bean gum-Sigma nr 0753 (LBG), 0.24 g of agarose (Qbiogene) and 24 ml of buffer pH 5.0 (11 ml of 1 M citric acid, 50 ml of Na_2HPO_4 and 149

Table 1. Germination rate index (GRI); germination percentage (G (%)); emergence rate index (ERI) and seedling emergence percentage (E (%)) of *P. ramiflora* (Mart.) Radlk. seeds at two maturation stages and with different water contents.

Variables	Maturation	Water content (% w.b.)			CV (%)
		38	20	12	
GRI	Ripe	2.70 ^{bA*}	0.87 ^{aB}	0.00 ^{aC}	13.50
	Unripe	3.20 ^{aA}	0.00 ^{bB}	0.00 ^{aB}	
G (%)	Ripe	74.20 ^{aA}	36.81^{aB}	0.00 ^{aC}	19.62
	Unripe	80.27 ^{aA}	0.00 ^{bB}	0.00 ^{aB}	
ERI	Ripe	0.50 ^{aA}	0.06 ^{aB}	0.00 ^{aB}	16.34
	Unripe	0.17 ^{bA}	0.00 ^{aB}	0.00 ^{aB}	
E (%)	Ripe	54.98 ^{aA}	15.67 ^{aB}	0.00 ^{aC}	22.32
	Unripe	33.91 ^{bA}	0.00 ^{bB}	0.00 ^{aB}	

CV: coefficient of variation; *Means followed by the same letter (lowercase in columns and uppercase in rows) do not differ at a 5% significance level under Tukey's test.

ml of distilled water). The aliquots were applied on 2 mm holes made in the gel using a gel comb, and the gel containing the samples was then transferred to a germinator at 25°C for 21 h in a moist chamber in the dark. Thereafter, the gel was covered with the dye Congo red at 0.5% for 30 min and destained in ethanol for 10 min. The ethanol was removed with distilled water and a solution of 1 M NaCl was added until visual observation of the formation of white halos in the holes that will contain the samples. The halo diameters were then measured in two directions with a caliper to obtain an average value. A comparison with the standard curve generated by the commercial endo-β-mannanase of *Aspergillus niger* (Megazyme) was performed for the calculation of enzyme activity. The calculation of the endo-β-mannanase enzymatic activity was performed according to Downie et al. (1994). The assay was conducted with three replicates for each treatment.

Statistical design

The experimental design was entirely randomized in a 2x3 factorial scheme, with two maturation stages (ripe seeds and unripe seeds) and three water contents (38% - initial water content, 20% and 12% w.b.). The data were statistically interpreted by an analysis of variance and the means were compared using Tukey's test at 5% probability using the software Sisvar (Analysis of Variance System) for Windows (Ferreira, 2000). Four replicates were used to quantify the reducing sugars, total soluble sugars and sucrose, and the means were compared using the Scott-Knott test at 5% probability. The data expressed as percentages were transformed into arcsine $\sqrt{x}/100$, where x refers to the percentage and the numerical data were transformed into $\sqrt{x}+0.5$.

RESULTS AND DISCUSSION

The physiological quality (Table 1) of seeds harvested at the unripe stage and subjected to drying was below those harvested at the ripe stage and not dried (initial water content). Seed drying up to 12% w.b. negatively affected the physiological quality, regardless of the stage of seed maturation, and reached null values for germination and seedling emergence. Lower values were found when comparing the physiological performance between wet and dry seeds in each maturation stage for all variables when the seeds were dried to 12% water content, particularly for unripe seeds. Thus, seed drying caused changes in physiological performance, and such effects were more pronounced in seeds that had not reached full physiological maturity. These results corroborate those found by Nakada et al. (2011), who also found lower physiological quality and vigor in dry seeds at early stages of development when studying cucumber seeds at different stages of maturation subjected to shade drying. Coffee (*C. arabica* L.) seeds harvested at the green-cane and cherry stages had better physiological condition than those harvested unripe; thus, seeds harvested at the unripe stage were considered intolerant to desiccation (Brandao Junior et al., 2002).

The evaluation of integrity and internal morphology of seeds using X-ray testing (Figure 1) showed morphological changes in the endosperm that resulted from the drying process. The X-ray images showed an increase in the free space between the embryo and the seed coat (embryo/embryonic coelom) and a reduction in embryonic area with the decrease in the water content of seeds, particularly for unripe seeds. These results directly affect the physiological performance of seeds, which shows that the damage resulting from the drying process caused losses in physiological quality (as assessed by the germination and seedling emergence tests) and that these losses were more pronounced when seeds were dried to 12% w.b., particularly for immature seeds.

Similar result was found in the study conducted by Goodman et al. (2005), who also noted the recalcitrant nature of northern red oak (*Quercus rubra* L.) seeds, assessing the separation between cotyledon-cotyledon and cotyledon-pericarp with advanced drying and

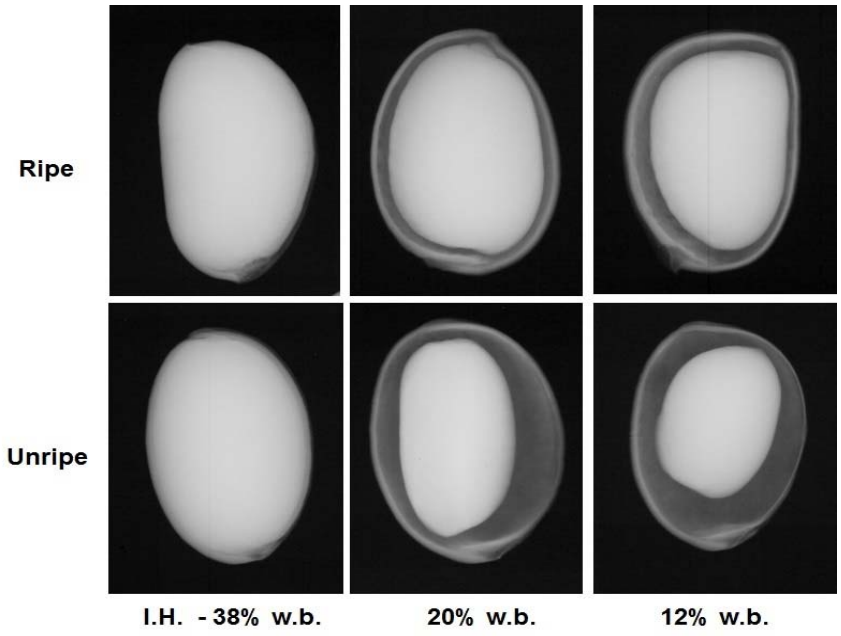

Figure 1. Radiographic images of *P. ramiflora* (Mart.) Radlk. seeds at two maturation stages and with different water contents. (I.H. – initial humidity - 37% w.b.; 20% w.b.; 12% w.b.). Federal University of Lavras (Universidade Federal de Lavras – UFLA), Lavras-MG, 2012.

establishing an important relationship between the damage assessed in X-ray images and the viability of seeds and seedlings. These authors emphasize that the morphological condition of seeds most likely reflects a cumulative result of all factors affecting viability loss; thus, the conditions observed in X-ray images can provide a more comprehensive representation of damages accumulated during desiccation, which confirms the potential of the analysis of X-ray images as a fast and non-destructive evaluation method of seed viability. According to Dell'Aquila (2007), the area of free space may be considered an indicator of germination potential and is closely related to the increase in abnormal seedlings because a gradual reduction in the formation of normal seedlings from radiographed pepper seeds (with a free area between the embryo and the endosperm exceeding 2.7%) was noted.

Regarding seed vigor, there was an effect of the maturation stage and water contents on electrical conductivity and respiration rate (Table 2).

Analysis of Table 2 shows that ripe seeds not subjected to drying had higher leachate electrical conductivity values than dried seeds. There was no difference in electrical conductivity between wet and dry seeds with respect to unripe seeds. Seeds that have not yet reached physiological maturity are more sensitive to desiccation and have lower vigor than ripe seeds because cell membrane repair may be ongoing in these seeds in response to damage after drying. Coffee (*Coffea arabica* L.) seeds harvested at the unripe stage are also more susceptible to damage from the drying process than seeds harvested at the green-cane and cherry stages, which indicates that the mechanisms of membrane protection occur between the unripe and cherry stages (Guimarães et al., 2002). The electrical conductivity of pepper (*Capsicum annuum* L.) seeds harvested from fruits picked 40 days after anthesis (immature) was high when compared to seeds derived from fruits picked at later periods (70 days after anthesis), which demonstrates that immature seeds have poor membrane organization and therefore greater solute leaching (Vidigal et al., 2009).

The respiration rate of ripe seeds was higher than that found in unripe seeds for all water contents, which indicates that ripe seeds were more efficient in reactivating their metabolism and respiration processes. Seeds with low water content had higher values of respiration rate in both stages of maturation. According to Wang et al. (2012), the survival of pea seeds after drying and soaking increases linearly with increased capacity to recover mitochondrial integrity, which indicates that the structural and functional recovery of mitochondria during germination has a key role in seed tolerance to desiccation.

The mean values regarding the concentrations of sugars are shown in Table 3. There was no difference between maturation stages and water contents with respect to the levels of reducing sugars. There were differences between water contents in both stages of maturation regarding total soluble sugars, with higher

Table 2. The electrical conductivity (EC) and respiration rate (RR) of *P. ramiflora* (Mart.) Radlk. seeds at two maturation stages and with different water contents.

Variables	Maturation	Water contents (% w.b.)			CV (%)
		38	20	12	
EC ($\mu S.cm^{-1}.g^{-1}$)	Ripe	5.12 aA*	3.39 bB	3.43 bB	11.35
	Unripe	3.61 bA	4.36 aA	4.27 aA	
RR (mg CO_2/ g D.M.)	Ripe	5.63 aB	7.19 aA	7.64 aA	13.13
	Unripe	4.36 bB	5.92 bA	5.78 bAB	

CV: coefficient of variation; *Means followed by the same letter (lowercase in columns and uppercase in rows) do not differ at a 5% significance level under Tukey's test.

Table 3. Concentrations of reducing sugars (RS), total soluble sugars (TSS), and sucrose (S) in *P. ramiflora* (Mart.) Radlk. seeds at two maturation stages and with different water contents.

Variables	Maturation	Water contents (% w.b.)			CV (%)
		38	20	12	
RS (mg/g^{-1})	Ripe	0.15 aA*	0.19 aA	0.21 aA	9.13
	Unripe	0.16 aA	0.20 aA	0.23 aA	
TSS (mg/g^{-1})	Ripe	2.81 aB	3.81 bB	7.67 bA	16.58
	Unripe	2.97 aB	8.20 aA	9.19 aA	
S (mg/g^{-1})	Ripe	18.18 aC	48.00 bB	66.56 aA	17.91
	Unripe	21.22 aB	77.90 aA	71.74 aA	

CV: coefficient of variation; *Means followed by the same letter (lowercase in columns and uppercase in rows) do not differ at a 5% significance level under the Scott-Knott test.

concentrations in seeds with lower water contents, both for unripe and ripe seeds. There was an increase in the concentrations of sucrose with the decrease in the water content of seeds in both maturation stages, which demonstrates the effect of drying on the accumulation of these sugars in seeds. The levels of total soluble sugars in Brazilwood (*Caesalpinia echinata* Lam.) seeds corresponded to approximately 10% dry matter of ripe seeds; sucrose was detected at high ratios throughout the entire process of development, and the accumulation of soluble carbon reserves is thus related to the degree of seed maturation and the final quality of the seeds (Borges et al., 2006). In *Acer platanoides* L. seeds, the concentration of sucrose in wet seeds harvested at early stages of development was higher than in seeds harvested upon reaching maturity. Rapid drying of immature seeds promoted the increase of sucrose concentration, whereas drying had no effect on the concentration of sucrose in ripe seeds (Hong et al., 2000). Starch and sucrose stand out among the reserve carbohydrates of seeds and may affect membrane stability during desiccation and the resumption of embryonic growth in addition to their function as reserves (Hellmann et al., 2008).

No catalase or peroxidase enzymatic activity was found in the electrophoretic profile of isoenzymes, regardless of maturation stage or water content. The activity and structure of certain seed enzymes or proteins sensitive to desiccation may be permanently altered by drying, which results in loss of activity (Nkang et al., 2000). Another possibility is that the extraction buffer used in this study may not be adequate for *P. ramiflora* seeds because the protocol was designed for different seeds than the studied species.

The electrophoretic pattern of the α-amylase and esterase enzymes in *P. ramiflora* (Mart.) Radlk. seeds at two stages of maturation and with different water contents is shown in Figure 2. According to the methodology used, the results showed the activity of only one isoform of α-amylase and esterase in this species under the conditions evaluated. The analyzed variables (maturation and water content) did not induce the expression of new isoforms, although reducing the water content to 12% w.b. in unripe fruits resulted in the enzymatic inhibition of α-amylase, which is shown by the disappearance of its isoform (Figure 2A).

The esterase and α-amylase of *P. ramiflora* (Mart.) Radlk. seeds showed enzymatic activity with reverse behavior, particularly with respect to the degree of maturation of their seeds (Figure 2). Higher activities in

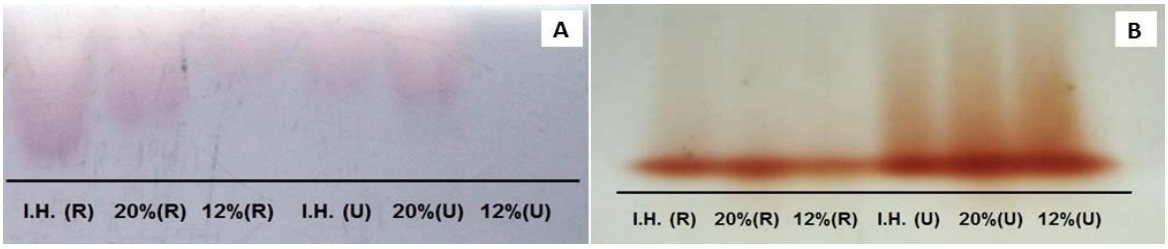

Figure 2. Electrophoretic pattern of α-amylase (A) and esterase (B) enzymes in *P. ramiflora* (Mart.) Radlk. seeds, at two maturation stages and with different water contents. (I.H. – Initial Humidity – 38%; 20% w.b.; 12% w.b.; R – ripe; U – unripe). Federal University of Lavras (Universidade Federal de Lavras – UFLA), Lavras-MG, 2012.

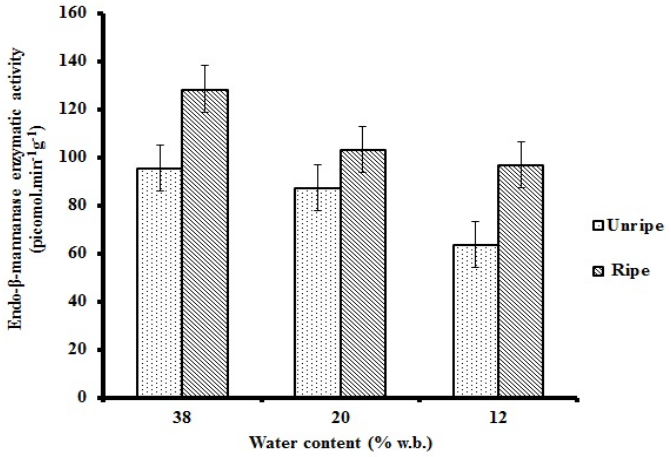

Figure 3. Endo-β-mannanase enzymatic activity in *Pouteria ramiflora* (Mart.) Radlk. seeds at two maturation stages and with different water contents. Federal University of Lavras (Universidade Federal de Lavras – UFLA), Lavras-MG, 2012.

α-amylase are found in ripe seeds than unripe seeds when fixing the water content in the seeds and varying their degree of maturation (Figure 2A); the opposite effect was observed in the activity in the esterase isoform, in which unripe seeds showed higher activity (Figure 2B). The reduction in seed moisture caused a reduction in α-amylase activity, and the effect was more drastic in unripe seeds with 12% w.b.; a slight decrease in esterase activity occurs as the water content is reduced in ripe seeds but the reducing water content apparently does not affect its activity in unripe seeds.

The α-amylase enzyme is essential for starch hydrolysis and its synthesis also occurs in response to the desiccation of cereal seeds (Rosa et al., 2004). Drying in the field and the artificial drying of corn seeds harvested at different stages of maturation led to increased α-amylase enzymatic activity, which favored seed germination (Faria et al., 2004). Esterase is a key enzyme that characterizes seed deterioration (Nakada et al., 2010) and presents increased activity during that physiological process (Walters, 1998; McDonald, 1999). The pattern observed for this enzyme corroborates the physiological and vigor results because its intense activity in unripe seeds may indicate cell membrane damage that culminates in reduced germination capacity and advanced deterioration. The esterase enzyme affects ester hydrolysis reactions and is directly linked to lipid metabolism, including total membrane phospholipids (Santos et al., 2005).

According to Tunes et al. (2011), esterase enzyme expression in barley seeds was barely affected by the harvest season. Nakada et al. (2011), when studying wet and dry Omega cucumber seeds (‘caipira’ type), found increased esterase activity in the first two and in the final stage of development (30, 35 and 55 days after anthesis, respectively), and that result correlated with the intensification of the deterioration process.

The endo-β-mannanase enzymatic activity in unripe and ripe seeds with different water contents is shown in Figure 3.

The endo-β-mannanase enzymatic activity was higher in ripe than unripe seeds, with a decrease in its activity in

response to the decrease in seed water content at both stages of maturation. Thus, the increased endo-β-mannanase activity can be correlated to the results found in germination and seedling emergence tests, in which the best values were found in ripe seeds with high water contents. The increase in the endo-β-mannanase enzymatic activity of tomato seeds coincided with an increase in seedling ERI, which explains why the increase in its activity may facilitate radicle protrusion because this is a main degradative enzyme of reserves in seeds (Albuquerque et al., 2010). That enzymatic activity, which is key to the germination of pepper seeds from the Habanero Yellow variety (*Capsicum chinense* Jacquin), was higher in more advanced stages of development when seeds had reached physiological maturity with full development of the enzymatic mechanisms that are involved in germination (Queiroz et al., 2011). Furthermore, Nascimento and Cantliffe (2001) found that the endo-β-mannanase enzymatic activity of lettuce seeds was higher one hour before radicle protrusion and in seeds of thermotolerant-genotypes when compared to thermosensitive seeds.

There was no detection of heat shock proteins by electrophoretic analysis of extracts prepared for that purpose. The absence of bands is presumably not an artifact of the technique because this is well-established methodology in other biological systems. Thus, drying did not induce changes in the pattern of heat-shock polypeptides in the species analyzed. Several recalcitrant seeds do not produce LEA proteins and, in this case, the presence of soluble sugars may minimize the effects of drying. The lack of LEA protein synthesis is one of the main differences between desiccation-tolerant and non-tolerant seeds (Marcos-Filho, 2005). LEA proteins are usually able to protect other proteins or membranes, similar to sugars, by acting as water replacement molecules (Goyal et al., 2005). Another possibility is that these proteins have different functions under different moisture contents and may yet have antioxidant properties that minimize the damaging effects of ROS (Mowla et al., 2006). The absence of such proteins is presumably a sign of intolerance to desiccation in *P. ramiflora* (Mart.) Radlk. seeds considering the relationship between the functions of these proteins and the acquisition of desiccation tolerance.

Conclusions

The physiological quality and vigor of *P. ramiflora* (Mart.) Radlk. seeds are affected by their maturation stage and drying activities. Ripe seeds performed better and were more tolerant to desiccation than unripe seeds. *P. ramiflora* (Mart.) Radlk. seeds showed only α-amylase and esterase isoforms; the first enzyme showed greater activity in ripe seeds, was sensitive to desiccation, and was not detected in seeds with 12% w.b.; conversely, the esterase isoform was much more active in unripe seeds, with little or no reduction in activity resulting from the reduction in water content. Catalase and peroxidase isoforms and heat shock proteins were not detected under the conditions evaluated. *P. ramiflora* (Mart.) Radlk. seeds are intolerant to desiccation, and this intolerance is maximal before seeds reach physiological maturity.

Conflict of Interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGMENTS

The authors acknowledge the financial support of CNPq-CAPES and FAPEG for granting the scholarship of the first author.

REFERENCES

Albuquerque KAD, Oliveira JA; Silva PA, Veiga AD, Carvalho BO, Alvim PO (2010). Armazenamento e qualidade de sementes de tomate enriquecidas com micronutrientes e reguladores de crescimento. Cienc. Agrotec. 34(1):20-28.http://dx.doi.org/10.1590/S1413-70542010000100002

Alfenas AC (1998). Eletroforese de izoenzimas e proteínas afins: fundamentos e aplicações em plantas e microorganismos. Viçosa: UFV, p. 574.

Alfenas AC, Petres I, Bruce W, Passados GC (1991). Eletroforese de proteínas e isoenzimas de fungos e essências florestais. Viçosa: UFV, p. 242.

Almeida SP, Proença CEB, Sano SM, Ribeiro JF (1998). Cerrado: espécies vegetais úteis. EMBRAPA-CPAC, Planaltina.

Angelovici R, Galili G, Fernie AR, Fait A (2010). Seed desiccation: A bridge between maturation and germination. Trends Plant Sci. 15:211-218. http://dx.doi.org/10.1016/j.tplants.2010.01.003

Bailly C (2004). Active oxygen species and antioxidants in seed biology. Seed Sci. Res. 14:93-107. http://dx.doi.org/10.1079/SSR2004159

Barbedo CJ, Marcos-Filho J (1998). Tolerância à dessecação de sementes. Acta Bot. Bras.12:145-164.

Borges IF, Barbedo CJ, Richter AA, Figueiredo-Ribeiro RCL (2006). Variations in sugars and cyclitols during development and maturation of seeds of Brazilwood (*Caesalpinia echinata* Lam., Leguminosae). Braz. J. Plant Physiol. 18:475-482. http://dx.doi.org/10.1590/S1677-04202006000400005

Brandao Junior DS, Vieira MGGC, Guimaraes RM, Hilhorst HWM (2002). Tolerância à dessecação de sementes de cafeeiro (*Coffea arabica* L.). Rev. Bras. sementes. 24(2):17-23.http://dx.doi.org/10.1590/S0101-31222002000100004

Brasil, Ministério da Agricultura, Pecuária e Abastecimento (2009). Regras para análise de sementes. Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. Brasília, DF: Mapa/ACS. p. 399.

Crispim JE, Martins JC, Pires JC, Rosolem CA, Cavariani C (1994). Determinação da taxa de respiração em sementes de soja pelo método da titulação. Pesq. Agropec. Bras. 29(10):1517-1521.

Dalponte JC, Lima ES (1999). Disponibilidade de frutos e a dieta de *Lycalopex vetulus* (Carnivora - Canidae) em um cerrado de Mato Grosso, Brasil. Rev. Bras. Bot. 22(2):325-332.http://dx.doi.org/10.1590/S0100-84041999000500015

Dell'aquila A (2007). Pepper seed germination assessed by combined X-radiography and computer-aided imaging analysis. Biol. Plant.

51(4):777-781.http://dx.doi.org/10.1007/s10535-007-0159-9
Downie B, Hillhorst HWM, Bewley JD (1994). A new assay for quantifying endo-β-mananase activity using Congo Red dye. Phytochemistry 36:829-835.http://dx.doi.org/10.1016/S0031-9422(00)90446-1
Ellis RH, Hong TD, Roberts EH (1990). An intermediate category of seed storage behaviour. J. Exp. Bot. 41(230):1167-1174. http://dx.doi.org/10.1093/jxb/41.9.1167
Fait A, Angelovici R, Less H, Ohad I, Urbanczyk-Wochniak, E, Fernie AR, Galili G (2006). Arabidopsis seed development and germination is associated with temporally distinct metabolic switches. Plant Physiol. 142(3):839-854. http://dx.doi.org/10.1104/pp.106.086694 PMid:16963520 PMCid:PMC1630763
Faria MAVR, Pinho RGV, Pinho EVRV, Guimarães RM, Freitas FEO (2004). Germinabilidade e tolerância à dessecação em sementes de milho colhidas em diferentes estádios de maturação. RBMS. 3(2):276-289.
Gaff DF, Oliver M (2013). The evolution of desiccation tolerance in angiosperm plants: a rare yet common phenomenon. Funct. Plant Biol. 40:315–328. http://dx.doi.org/10.1071/FP12321
Gama LU, Barbosa AAA, Oliveira PEAM (2011). Sistema sexual e biologia floral de Pouteria ramiflora e P. torta (Sapotaceae). Rev. Bras. Bot. 34(3):375-387.http://dx.doi.org/10.1590/S0100-84042011000300011
Goodman RC, Jacobs DF, Karrfalt RP (2005). Evaluating desiccation sensitivity of Quercus rubra acorns using X-ray image analysis. Can J. Forest. Res. 35:2823-2831. http://dx.doi.org/10.1139/x05-209
Goyal K, Walton LJ, Tunnacliffe A (2005). LEA proteins prevent protein aggregation due to water stress. Biochem. J. 388:151-157. http://dx.doi.org/10.1042/BJ20041931
Guimarães RM, Vieira MGGC, Fraga AC, Pinho EVRV, Ferraz VP (2002). Tolerância à dessecação em sementes de cafeeiro (*Coffea arabica* L.). Cienc. Agrotec. 26(1):128-139.
Hellmann ME, Mello JIO, Barbedo CJ, Figueiredo-Ribeiro RCL (2008). Variações dos carboidratos de reserva de sementes de Caesalpinia echinata (pau-brasil) armazenadas sob diferentes temperaturas. Hoehnea 35(2):255-264. http://dx.doi.org/10.1590/S2236-89062008000200007
Hoekstra FA, Golovina EA, Buitink, J (2001). Mechanisms of plant desiccation tolerance. Trends Plant. Sci. 6(9):431-438.http://dx.doi.org/10.1016/S1360-1385(01)02052-0
Hong TD, Gedebo A, Ellis RH (2000). Accumulation of sugars during the onset and development of desiccation tolerance in immature seeds of Norway maple (*Acer platanoides* L.) stored moist. Seed Sci. Res. 10:147-152.
Illing N, Denby JK, Collett H, Shen A, Farrant JM (2005). The Signature of Seeds in Resurrection Plants: A Molecular and Physiological Comparison of Desiccation Tolerance in Seeds and Vegetative Tissues. Integr. Comp. Biol. 45:771-787.http://dx.doi.org/10.1093/icb/45.5.771
Jiang L, Kermode A.R (1994). Role of desiccation in the termination of expression of genes for storage proteins. Seed Sci. Res. 4(2):149-173. http://dx.doi.org/10.1017/S0960258500002154
Lorenzi H (1992). Árvores brasileiras: Manual de identificação e cultivo de plantas arbóreas do Brasil. Editora Plantarum, Nova Odessa. PMCid:PMC225662
Maguire JD (1962). Speed of germination aid in selection and evaluation for seedling emergence and vigor. Crop Sci. 2:176-177.http://dx.doi.org/10.2135/cropsci1962.0011183X000200020033x
Manfre AJ, Lahatte GA, Climer CR, Marcotte Jr WR (2009). Seed Dehydration and the Establishment of Desiccation Tolerance During Seed Maturation is Altered in the Arabidopsis thaliana Mutant atem6-1. Plant Cell. Physiol. 50(2):243-253. http://dx.doi.org/10.1093/pcp/pcn185 PMid:19073649
Marcos-Filho J (2005). Fisiologia de sementes de plantas cultivadas.Piracicaba: Fealq. p. 495.
McDonald MB (1999). Seed deterioration: Physiology, repair and assessment. Seed Sci. Technol. 27(1):177-237.
Miller GL (1959). Use dinitrosalicylis acid reagent for determination of reducing sugars. Anal. Chem. 31(2):426-428. http://dx.doi.org/10.1021/ac60147a030
Mowla SB, Cuypers A, Driscoll SP, Kiddle G, Thomson J, Foyer CH, Theodoulou FL (2006). Yeast complementation reveals a role for an Arabidopsis thaliana late embryogenesis abundant (LEA)-like protein in oxidative stress tolerance. Plant J. 48:743-756. http://dx.doi.org/10.1111/j.1365-313X.2006.02911.xPMid:17092320
Nakada PG, Oliveira JA, Melo LC, Gomes LAA, Pinho EVRV (2011). Desempenho fisiológico e bioquímico de sementes de pepino nos diferentes estádios de maturação. Rev. Bras. sementes. 33(1):113-122.http://dx.doi.org/10.1590/S0101-31222011000100013
Nakada PG, Oliveira JA, Melo LC, Silva AA, Silva PA, Perina FJ (2010). Desempenho durante o armazenamento de sementes de pepino submetidas a diferentes métodos de secagem. Rev. Bras. sementes. 32(3):042-051.
Nambara E, Okamoto M, Tatematsu K, Yano R, Seo M, Kamiya Y (2010). Abscisic acid and the control of seed dormancy and germination. Seed Sci. Res. 20(2):55-67.http://dx.doi.org/10.1017/S0960258510000012
Nascimento WM, Cantliffe EDJ (2001). Composição química do endosperma, atividade enzimática e sua associação com a germinação das sementes de alface em altas temperaturas. Rev. Bras. sementes. 23(2):121-126.
Nkang A, Omokaro D, Egbe A (2000). Effects of desiccation on the lipid peroxidation and activities of peroxidase and polyphenoloxidase in seeds of *Telfairia occidentalis*. Seed Sci. Technol. 28:1-9.
Pammenter NW, Berjak P, Farrant JM, Smith MT, Ross G (1994). Why do stored, hydrated recalcitrant seeds die. Seed Sci. Res. 4(2):187-191. http://dx.doi.org/10.1017/S0960258500002178
Queiroz LAF, Pinho EVRV, Oliveira JA, Ferreira VF, Carvalho BO, Bueno ACR (2011). Época de colheita e secagem na qualidade de sementes de pimenta Habanero Yellow. Rev. Bras. sementes. 33(3):472-481.http://dx.doi.org/10.1590/S0101-31222011000300010
Ramanjulu S, Bartels D (2002). Drought- and desiccation-induced modulation of gene expression in plants. Plant Cell Environ. 25(2):141-151. http://dx.doi.org/10.1046/j.0016-8025.2001.00764.x PMid:11841659
Roberts EH (1973). Predicting the storage life of seeds. Seed Sci. Technol. 1(3):499-514.
Rosa SDVF, Pinho EVRV, Vieira MGGC, Veiga RD (2004). Indução de tolerância à alta temperatura de secagem em sementes de milho por meio de pré-condicionamento a baixa temperatura. RBMS. 3(2):290-310.
Santos CMR, Menezes NL, Villela FA (2005). Modificações fisiológicas e bioquímicas em sementes de feijão no armazenamento. Rev. Bras. sementes 27:104-114.http://dx.doi.org/10.1590/S0101-31222005000100013
Tanksley SD, Orton TJ (1983). Isozymes: Developments in plant genetic and breeding. Parte A (IA). New Yoek: Elsevier, p. 516.
Tunes LM, Badinelli PG, Barros ACSA, Meneghello GE, Amarante L (2011). Influência dos diferentes períodos de colheita na expressão de isoenzimas em sementes de cevada. Rev. Ceres. 58(2):178-184.
Tweddle JC, Dickie JB, Baskin CC, Baskin JM (2003). Ecological aspects of seed desiccation sensitivity. J. Ecol. 91(2):294-304.http://dx.doi.org/10.1046/j.1365-2745.2003.00760.x
Vidigal DS, Dias DCFS, Pinho EVRV, Dias LAS (2009). Alterações fisiológicas e enzimáticas durante a maturação de sementes de pimenta (*Capsicum annuum* L.). Rev. Bras. sementes. 31(2):129-136.http://dx.doi.org/10.1590/S0101-31222009000200015
Walters C9 (1998). Understanding the mechanisms and kinetics of seed aging. Seed Sci. Res. 8(2):223-244. http://dx.doi.org/10.1017/S096025850000413X
Walters C, Farrant JM, Pammenter NW, Berjak P (2002). Desiccation and Damage. In: Black M, Pritchard HW. (eds.) Desiccation and survival in plants. Drying without dying. pp. 263-291. http://dx.doi.org/10.1079/9780851995342.0263
Wang WQ, Cheng HY, Moller IM, Song SQ (2012). The role of recovery of mitochondrial structure and function in desiccation tolerance of pea seeds. Physiol. Plant. 144:20-34. http://dx.doi.org/10.1111/j.1399-3054.2011.01518.x
Yemm EW, Willis AJ (1954). The estimation of carbohydrates in plants extracts by anthrone. Biochem. J. 57(4):508-514.