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Campomanesia adamantium (Cambess.) O. Berg seed desiccation: influence on vigor and nucleic acids

DAIANE M. DRESCH, TATHIANA E. MASETTO and SILVANA P.Q. SCALON

Faculdade de Ciências Agrárias, FCA, Universidade Federal da Grande Dourados, UFGD, Rodovia Dourados-Itahum, Km 12, Caixa Postal 533, Cidade Universitária, 79804-970 Dourados, MS, Brasil

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ABSTRACT

The aim of this study was to evaluate the sensitivity of *Campomanesia adamantium* seeds to desiccation by drying in activated silica gel (fast) and under laboratory conditions (slow). To assess the sensitivity of the seeds to desiccation, we used drying with silica gel and drying under laboratory conditions (25 °C), in order to obtain seeds with moisture content of 45, 35, 30, 25, 20, 15, 10 and 5%. The physiological potential of the seeds after desiccation was evaluated by measuring primary root protrusion, percentage of normal seedlings, germination seed index, seedling length, total seedling dry mass, electrical conductivity and DNA and RNA integrities. The *C. adamantium* seeds were sensitive to desiccation and to a reduction in moisture content to 21.1% or less by desiccation using silica gel, and to 17.2% or less by desiccation under laboratory conditions; impairment of the physiological potential of the seeds was observed at these low moisture content levels. The integrity of the seed genomic DNA was not affected after drying seeds in the two methods. However, drying in silica gel to 4.5% moisture content and drying under laboratory conditions to 5.4% moisture content resulted in the loss of seed RNA integrity.

Key words: Cerrado, DNA, drying, RNA, viability.

INTRODUCTION

Occupying 21% of Brazilian territory, the landscape known as the Brazilian Savanna (Cerrado) is the second largest biome in Brazil, after the Amazon, with considerable fauna and flora diversity. However, it has suffered rapid devastation over the last 35 years, and several plant species are now endangered (Klink and Machado 2005). In the state of Mato Grosso do Sul, the Brazilian Savanna is home to numerous groups of plant species, including members of the Myrtaceae group, which comprise ornamental species and species that are used commercially in local agriculture owing to the quality of their fruits (Donadio and Moro 2004, Scalon et al. 2012).

Among the fruit plants, *Campomanesia adamantium* (Cambess.) O. Berg, commonly known as "guabiroba" or "guavira" grows as shrubs measuring 0.5 to 1.5 m in height. It blooms between September and November and fruits from November to December. The fruits have a round shape and a color ranging from dark to light green and yellow, with a sweet and pleasant aroma. They

Correspondence to: Daiane Mugnol Dresch
E-mail: daiamugnol@gmail.com

can be consumed *in natura* or as juices, sweets, and jam, and are used as main ingredients in liqueurs, popsicles, ice cream, and spirits. The propagation of *C. adamantium* occurs via seeds (Vallilo et al. 2004, 2006, Lorenzi, 2008) that are sensitive to desiccation (Zaidan and Carreira 2008, Scalon et al. 2013).

The development of seeds, which are tolerant to water loss, ends with a predetermined drying stage, with a considerable reduction in the moisture content (between 90% and 95% of the initial value), leading to a reduction in the metabolism of the seed and the embryo, characterizing the onset of a quiescent state (Black and Pritchard 2002). The recalcitrant seeds, however, remain sensitive to desiccation throughout their development until their release from the mother plant (Berjak and Pammenter 2008). These seeds have limited longevity, even when kept in an environment with high moisture content (Pammenter and Berjak 2000).

The main challenge for the *ex situ* conservation of seeds from species sensitive to desiccation is to determinate their behavior towards drying, and, if they are sensitive, to store them only for a short period (usually of weeks to months) (Daws et al. 2006). Therefore, one of the factors affecting sensitivity to desiccation is the drying rate (Wesley-Smith et al. 2001, Kermode and Finch-Savage 2002, Berjak and Pammenter 2008), which depends on inherent seed properties such as the nature of the seed coat, size, and developmental stage (Berjak and Pammenter 2008). The processes and mechanisms involved in the protection of these seeds from desiccation damages are being identified through the study of various aspects. Together, these methods promote seed tolerance to drying; however, the way they work and interact has not yet been fully understood (Berjak et al. 2007). The stability of DNA during drying and its ability to undergo repair during rehydration are essential components of a complete tolerance mechanism (Boubriak et al. 1997).

DNA and RNA are involved in the control of vital cellular processes. Recently, the integrity of nucleic acids has been the subject of investigation of studies related to the dynamics of seed viability loss (Faria et al. 2005, Kranner et al. 2006, Masetto et al. 2008, Kranner et al. 2011). The loss of DNA integrity in seeds sensitive to desiccation, is characterized by programmed cell death, which is a complex process that eliminates redundant or damaged cells (Viannelo et al. 2007). This is clearly exemplified in seeds of *Medicago truncatula* Gaertn. (alfalfa) (Buitink et al. 2003, Faria et al. 2005) and *Pisum sativum* L. (Kranner et al. 2011) and by the passive death of cells, a non-physiological process dissociated from morphogenetic events and without any DNA changes (Xu et al. 2004), as observed in *Eugenia pleurantha* O. Berg (popularly known as "pitanga-do-mato") (Masetto et al. 2008).

To elucidate the effect of desiccation on *C. adamantium* seeds in the context of long-term storage, we evaluated the effects of slow drying at room temperature and fast drying using silica gel on the physiological potential of seeds and on the integrity of their DNA and RNA.

MATERIALS AND METHODS

Campomanesia adamantium fruits were harvested at the end of December 2011 from 30 regions located in areas of the Cerrado (*sensu stricto*), in the city of Ponta Porã-MS. After harvesting, the fruits were brought to the Laboratory of Plant Nutrition and Metabolism at the Federal University of Grande Dourados (UFGD), in Dourados-MS, where they were washed with tap water and, where the damaged fruits were discarded. The fruits were then manually processed using sieves to isolate seeds from the fruit waste. Next, the seeds were washed and placed on Germitest® paper for 40 min at room temperature (25 °C ± 2 °C, 35% RH). The seeds were later subjected to fast drying methods usually applied for Chmielarz (2009) using

activated silica gel (25 °C ± 2 °C, 8% RH) or slow drying under laboratory conditions (25 °C ± 2 °C, 35% RH).

For fast drying, the seeds were placed on a steel screen inside closed "gerbox" plastic boxes with silica gel at the bottom. The silica gel was replaced as soon as it lost its indicative blue coloration. For slow drying, the seeds were placed inside open plastic containers. The seeds were then weighed every h until they achieved predetermined weights according to Sacandé et al. (2004). After the desired humidity levels were reached using both drying methods, the seeds were pre-humidified in a humidity chamber (25 ºC, 100% RH under constant white light) for 24 h, in order to prevent damages by imbibition. The following aspects were studied in order to evaluate the physiological potential:

Moisture content: was determined at 105 °C ± 3 °C for 24 h using the greenhouse method (Brasil 2009) with three replicates of 5 g of seeds each, and the results were expressed on a wet basis.

Primary root protrusion: was measured on Germitest® paper rolls with four replications of 25 seeds each, germinated with B.O.D. (Biochemical Oxygen Demand) at 25 °C under continuous white light. Assessments were conducted daily, and the root was considered protruded when it reached a length of 5 mm. The results were expressed in percentages (%).

Percentage of normal seedlings: was determined in Germitest® paper rolls with four replications of 25 seeds each, which were germinated with BOD at 25 °C under continuous white light. Evaluations were performed 42 d after sowing by computing the percentages of normal seedlings, using the issuance of shoots and root system development as the criteria. The results were expressed in percentages (%).

Germination speed index (GSI): was calculated according to Maguire (1962).

Seedling length: was obtained by measuring the lengths of the primary root, shoot and total plant, with four replications of 10 seedlings each using a millimeter ruler. The results were expressed in centimeters (cm).

Total dry mass: was obtained from seedlings that had been dried in an oven at 60 ºC for 48 h using an analytical balance (0.0001 g), with the results expressed in grams (g).

Electrical conductivity: was assessed using repeated four times by using 25 seeds in each test. Seeds were weighed and placed into 75 mL of distilled water in 200 mL plastic cups at 25 °C for 2 h (Okuhisa et al. 2009). The readings were obtained using a bench-top conductivity meter**,** where each container was gently stirred in order to homogenize the electrolytes lixiviated in the solution. The results are expressed in $\mu S.cm^{-1}g^{-1}$.

DNA and RNA extraction and analysis: The samples were stored in a freezer (-18 °C, 42% RH) until the moment of extraction. Samples were then macerated in liquid nitrogen until a fine powder was formed. This was transferred to a 1.5 mL Eppendorf tube and 600 µL of 1 × extraction buffer (2.5 mL 2 × buffer [0.6M NaCl; 0.1M TRIS-HCl pH 8.0; 0,04M EDTA pH 8.0; 4% Sarcosyl (p/v), and 1% SDS]; 2 mL urea 12M; 0.25 mL phenol; 0.25 mL distilled water was added. Next, the microtubes were vortexed to homogenize the samples before adding 600 µL of chloroform-isoamyl alcohol (24:1). The microtubes were centrifuged at 14.000 rpm for 15 min at 20 °C, and the supernatant was transferred to a new microtube in which 80% of the total sample volume of isopropanol was added. The microtubes were then inverted for 3 min to permit pellet formation, after which the tubes were incubated for 10 min at room temperature and centrifuged at 3.500 rpm for 10 min at room temperature. The supernatants were discarded and 500 µL of 70% ethanol was added to the microtubes, which were then centrifuged at 2.000 rpm for 5 min at room temperature. The microtubes were then inverted on a clean tissue paper in order to dry the pellet, which

was later dissolved in 200 µL TRIS-EDTA buffer (10 mM TRIS HCl; 1 mM EDTA; pH 8.0).

Samples were analyzed by electrophoresis by loading 3 µL DNA onto a 1% agarose gel. DNA bands were stained with ethidium bromide, visualized using ultraviolet radiation, and documented using Sony equipment.

A completely randomized experimental design in a factorial scheme (2 drying methods × 8 moisture content values) was used. The data were submitted to variance and regression analysis at a 5% probability level by using the SISVAR software (Ferreira 2011). The results obtained from electrophoresis were qualitatively analyzed via visual evaluation of the integrity and quality of the bands.

RESULTS

Slow drying of *C. adamantium* seeds under laboratory conditions took more than 19 h to achieve a moisture content of 7.5%. However, this time was reduced by fast drying (silica gel), which took 17 h to achieve a moisture content of 7.9% (Fig. 1).

For primary root protrusion, the maximum value of 72.3% was observed in the seeds with a moisture content of 46.5% (Fig. 2a). During slow drying, there was a linear reduction in the primary root protrusion as the moisture content decreased, and the percentage of embryos that resumed growth reduced from 74% (45.3% moisture content) to 47% (7.9% moisture content).

The percentage of normal seedlings was influenced by the seed drying method and decreased linearly with the moisture content. There was an initial survival rate of 70%, which decreased to 26% and 44% with fast and slow drying, respectively (Fig. 2b). The employed drying methods exposed different levels of sensitivity of seeds to desiccation at the observed moisture content values, with more than 50% normal seedlings at 21.1% moisture content after fast drying (56% normal seedlings), and 17.2% moisture content after slow drying (56% normal seedlings) (Fig. 2b). The germination speed index (GSI) decreased linearly with desiccation by both drying methods (Fig. 2c). Seeds subjected to fast drying had lower GSI values (0.32) than those of seeds subjected to slow drying (0.83), indicating the damage caused by fast drying on the seed germination rate.

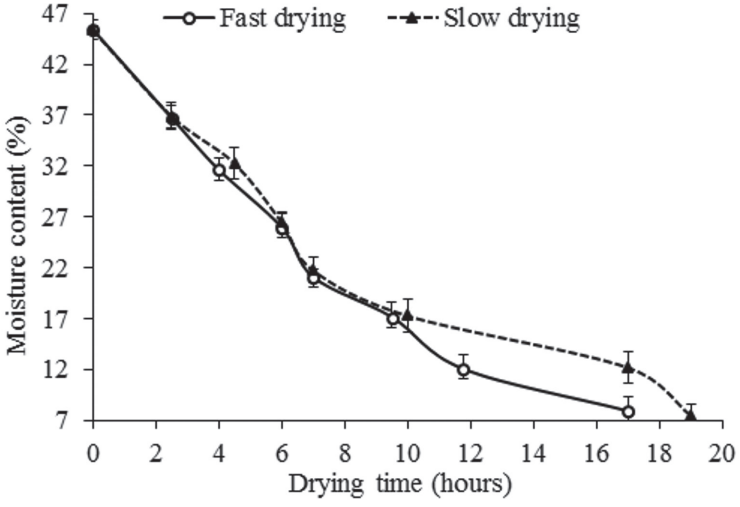

Figure 1 - Drying rate of *Campomanesia adamantium* seeds: fast drying (silica gel) and slow drying (room temperature).

The shoot length was negatively affected by desiccation (Fig. 3a). After fast drying, the smallest shoot length observed was 3.03 cm at 12.9% moisture content. After slow drying, the increasing drying intensified the damage caused to the seeds, which was reflected by the smaller shoot length of 3.12 cm at 7.5% moisture content.

The primary root length was affected by desiccation after both drying methods (Fig. 3b). With fast drying, the smallest value of 3.98 cm was observed at 6.2% moisture content, while after slow drying, the highest value of 8.47 cm was observed at 37.8% moisture content. For total length, seeds subjected to fast drying showed a minimum value of 7.08 cm at 9.25% moisture content, whereas after slow drying, the highest value of 13.65 cm was observed at 42.1% moisture content (Fig. 4a).

For total dry mass, the highest values were obtained at 33.5% (0.0330 g) and 40.1% (0.0285 g) moisture content for fast and slow drying, respectively (Fig. 4b). However, after fast drying, a remarkable reduction in the accumulation of seedling dry mass was observed at the lowest levels of seed, that is, moisture content at the 11.7% (0.0102 g) and 7.9% (0.0023 g).

For electrical conductivity, the lowest values were observed at 27.7% (6.22 $\mu S\ cm^{-1}g^{-1}$) and 35.8% (5.92 $\mu S\ cm^{-1}g^{-1}$) moisture content, after fast and slow drying, respectively (Fig. 5). On the other hand, the highest electrical conductivity values were obtained with a reduction in the seed

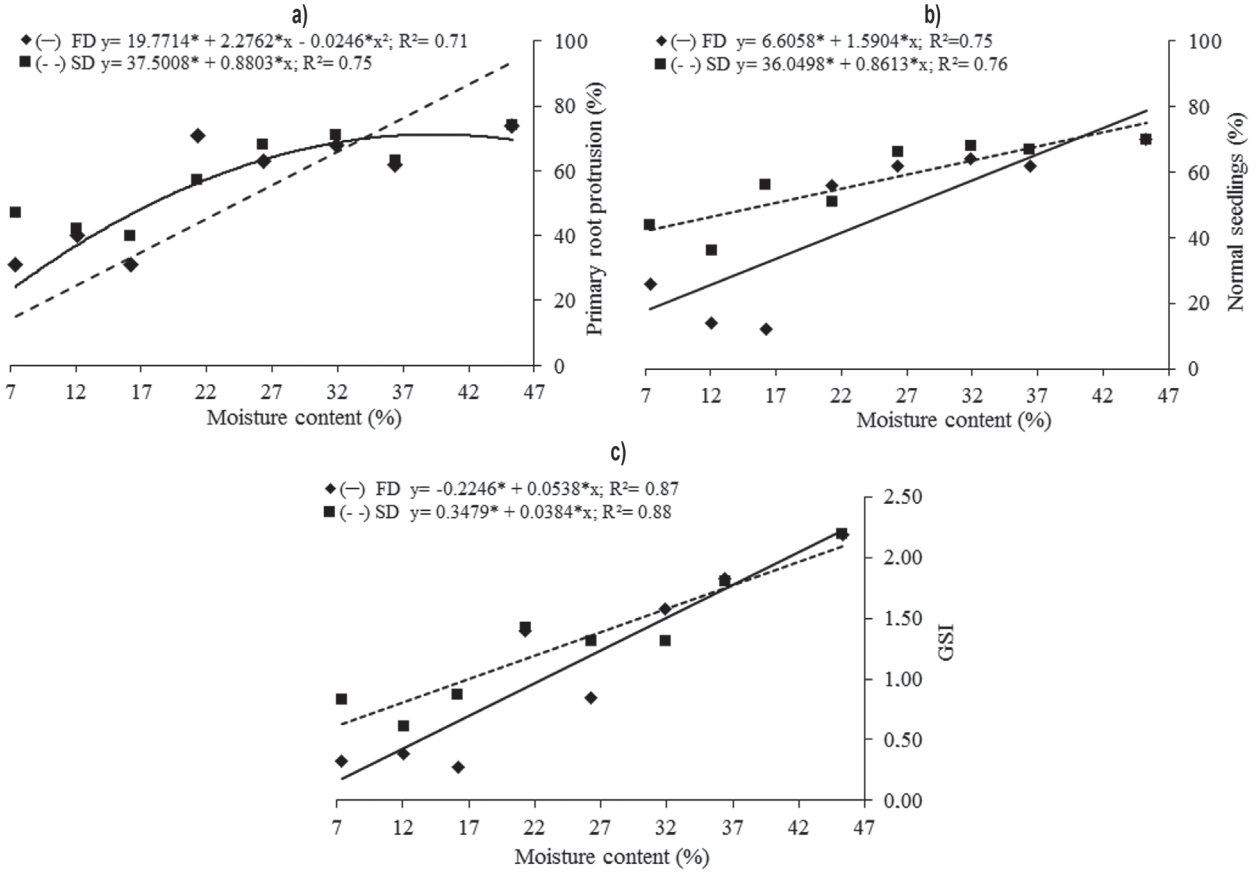

Figure 2 - Effect of drying rate on primary root protrusion **(a)**, percentage of normal seedlings (%) **(b)** and germination speed index (GSI) **(c)** of *Campomanesia adamantium* seeds subjected to fast drying (FD) (silica) and slow drying (SD) (room temperature) at different water contents.

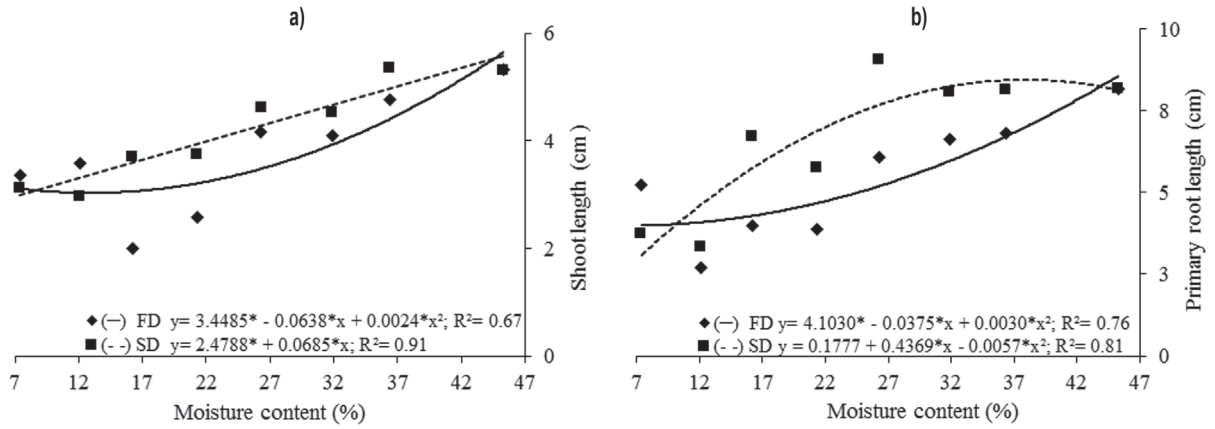

Figure 3 - Effect of drying rate on shoot length (cm) **(a)** and primary root length (cm) **(b)** of *Campomanesia adamantium* seedlings subjected to fast drying (FD) (silica) and slow drying (SD) (room temperature) at different water contents.

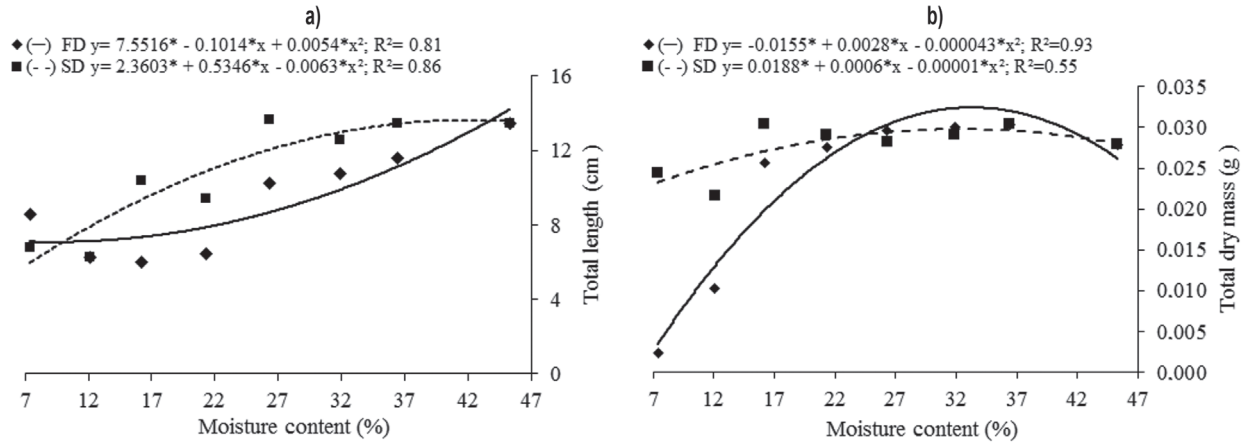

Figure 4 - Effect of drying rate on total length (cm) **(a)** and total dry mass seedlings (g) **(b)** of *Campomanesia adamantium* seedlings subjected to fast drying (FD) (silica) and slow drying (SD) (room temperature) at different water contents.

moisture content to 11.7% (12.92 μS $cm^{-1}g^{-1}$) and to 7.5% (12.02 μS $cm^{-1}g^{-1}$) for fast and slow drying, respectively. These results indicate that compared to the physiological potential of seeds subjected to fast drying, the physiological potential of seeds subjected to slow drying is less damaged at lower moisture content levels.

The electrophoretic profile of genomic DNA extracted from *C. adamantium* seeds indicated that the DNA integrity was preserved after both drying methods (Fig. 6). However, analysis revealed that the seed RNA was degraded following fast and slow drying at 4.5% and 5.4% moisture content, respectively, demonstrated by the degradation of the 28S and 18S bands, as observed by electrophoresis (Fig. 7).

DISCUSSION

Seed desiccation by different drying methods influenced the seed germination process, suggesting that seeds have different levels of sensitivity to desiccation, depending on their moisture content. In this study, we found that the percentage of normal plants was higher than

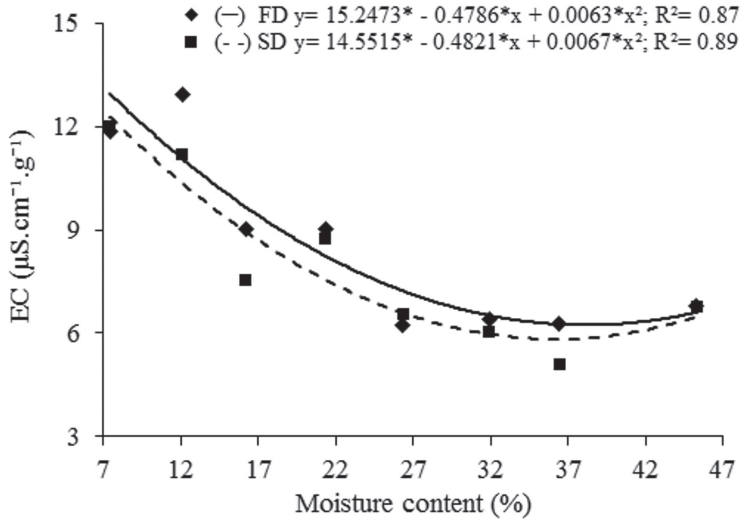

Figure 5 - Effect of drying rate on electrical conductivity ($\mu S.cm^{-1}g^{-1}$) of *Campomanesia adamantium* seeds subjected to fast drying (FD) (silica) and slow drying (SD) (room temperature) at different water contents.

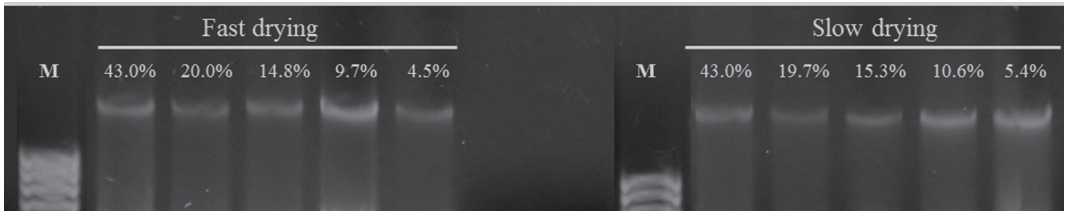

Figure 6 - Agarose gel (1%) of genomic DNA extracted of *Campomanesia adamantium* seeds subjected to fast drying (silica) and slow drying (room temperature) at different water contents. M: marker 100 bp (molecular weight).

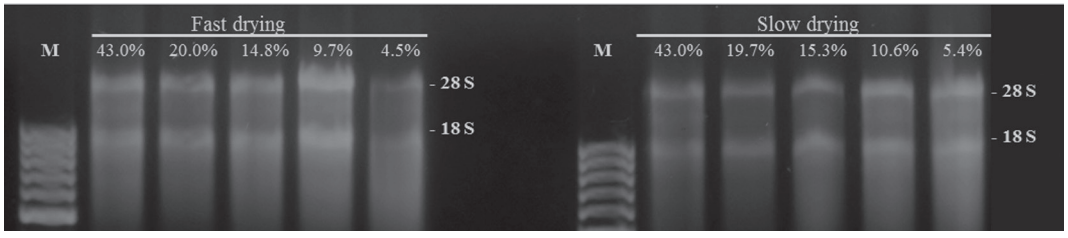

Figure 7 - Agarose gel (1%) of genomic RNA extracted of *Campomanesia adamantium* seeds subjected to fast drying (silica) and slow drying (room temperature) at different water contents. M: marker 100 bp (molecular weight).

50% at moisture contents of 21.1% and 17.2% for fast and slow drying, respectively. Thus, it is clear that fast drying was more severe than slow drying, considering that the damage caused by the reduction of moisture content to 17.3% resulted in shortened primary root protrusion and a decrease in the percentage of normal seedlings to values lower than 50%. Furthermore, following slow drying, these values were lower only when the seeds had a moisture content of 12.2%. The mechanisms by which rapid drying allows lower moisture contents to be tolerated in recalcitrant material have not yet

been fully elucidated, but evidence indicates that the duration of the benefit, is short (Wesley-Smith et al. 2001).

Therefore, these results contradict the idea that fast drying, leading to lower moisture content, results in more viable seeds, suggesting that the limitation for desiccation tolerance, and that exposure to low moisture content may result in desiccation damage. The germination potential of *C. adamantium* seeds is directly correlated with the tolerable moisture content associated with the drying method used; hence, the desiccation at 17.2% and 12.2% moisture content levels with fast and slow drying, respectively, may be considered critical for this species.

Sensitivity to desiccation was reported for the seeds of several species of the genus *Campomanesia*. In *C. pubescens* (DC.) O. Berg ("gabiroba") seeds, reduction in the moisture content from 35% to 4% by desiccation reduced the germination potential and vigor, suggesting that the seeds are intolerant to desiccation and can be classified as recalcitrant (Dousseau et al. 2011). In *C. lineatifolia* Ruiz and Pav. ("guabiraba") seeds, a reduction to 16% moisture content resulted in decreased seed germination rate, rendering these seeds as recalcitrant as well (Carvalho et al. 1997). In *C. adamantium* (Cambess.) O. Berg seeds, reduction in the moisture content from 35.7% to 9.8% after drying under laboratory conditions (slow) negatively affected the germination potential of isolated seeds, suggesting that this species can be classified as recalcitrant for not being able to survive storage at low temperatures and for being intolerant to desiccation (Melchior et al. 2006). However, the critical desiccation level was not specified.

The primary root length was negatively affected by seed desiccation in both drying methods, indicating that the primary root is the first structure to show sensitivity to desiccation (Buitink et al. 2003). These results suggest that seed desiccation impairs primary root growth and may consequently affect fixation in soil and the absorption of water and organic nutrients. A reduction in the root length was also observed in seeds of *Euterpe oleracea* (Mart.) (Martins et al. 1999) ("açaí"), *Hancornia speciosa* Gomes ("mangaba") (Santos et al. 2010), and *Eugenia pyriformis* Camb. ("uvaia") (Scalon et al. 2012).

Seed desiccation compromised the total seedling growth and the accumulation of total dry mass, where, after fast drying, a drastic reduction in seedling dry mass accumulation was detected at the lowest moisture content level. The reduction in seed moisture content possibly affected the ability of seedlings to translocate nutrients. Similar results were observed for seeds of *Euterpe oleracea* Mart. ("açaí") (Nascimento et al. 2007) and *Cinnamomum zeylanicum* Blume (cinnamon) (Silva et al. 2012).

The negative effects of seed desiccation were evidenced by the results of electrical conductivity measurements, where the highest values were obtained when moisture content was reduced from 11.7% to 7.5% after fast and slow drying, respectively. These results reveal that the drying methods may have damaged the integrity of cellular membrane systems owing to an elevation in the concentration of exudates lixiviated by the seeds. The reduction in the seed physiological potential is generally accompanied by an increase in the levels of electrolytes and sugars released by the seeds imbedded in water. This is due to loss of the selective permeability of cellular membranes since desiccation harms the cell structure in several ways, including solute crystallization, protein denaturation, and damage to membranes (Vieira 1994, Black and Pritchard 2002). Therefore, the integrity of membranes is critically important for seed viability, since any undue rupture caused by desiccation may have immediate consequences to the seeds during rehydration (Kermode and Finch-Savage 2002).

The results of total-body electrical conductivity measurements indicate that this assay can be

potentially used to evaluate seed quality in *C. adamantium*, as demonstrated for seeds of Brazilian fruit species such as *Inga uruguensis* Hook. and Arn. ("ingá") (Barbedo and Cicero 1998) and *Eugenia brasiliensis* Lam. ("grumixameira") (Kohama et al. 2006).

The electrophoretic profile of genomic DNA extracted from *C. adamantium* seeds showed that DNA integrity was preserved after seed desiccation with both drying methods. This may also be reflected by the percentage of normal plants observed, despite a decrease in the number of normal plants after gradual seed desiccation, the reduction of moisture content to 4.5% with fast drying and 5.4% with slow drying, did not result in complete seed mortality, characterized by the respective occurrence of 20% and 28% of primary root protrusion. Thus, preserved DNA integrity is an essential component of seed desiccation tolerance. However, changes of DNA that cause death (i.e. loss of telomere repeats or unrepaired breaks) are minor, compared to the fragmentation that occurs after death or even during simple extraction procedures. Even after death, DNA is extremely stable in seeds, as is demonstrated by the retrieval of genetic information from 1000-year-old specimens found in archaeological sites (Walters et al. 2006).

Nevertheless, analysis after fast and slow drying showed that RNA was degraded in seeds with 4.5% and 5.4% moisture content, characterized by the loss of integrity in the 28S and 18S bands. Therefore, the rRNA degradation occurred without DNA fragmentation (Samali et al. 1997). It is important to mention that the results shown in Figures 6 and 7 correspond to populations of seeds and may not reflect the state of nucleic acids in a single seed. Thus, in a population with 20% mortality and 80% viability, the partial rRNA degradation after desiccation might be attributed to dead seeds that completely lost rRNA integrity. However, as previously by Kranner et al. (2011), these changes can be used as markers of early degradation in batches of seeds subjected to desiccation.

Even though a pattern of DNA degradation that is characteristic of programmed and passive cell death was not observed, desiccation resulted in the loss of RNA integrity (Fig. 6 and 7). There is evidence that RNA degradation is caused by RNases, and increased enzymatic activity has been associated with programmed cell death (Panavas et al. 1998, Xu and Hanson 2000). Only a partial rRNA degradation, possibly meant that the remaining RNA copies were sufficient for the transcriptional activity required for seed germination. In this context, the reduction in the RNA content during tissue desiccation may be the result of either decreased transcript synthesis or degradation (Dinakar and Bartels 2012). As it is, future investigation on gene transcripts associated with seed desiccation tolerance may help to determine mRNA stability during seed desiccation in *C. adamantium*.

Based on these results, it can be concluded that *C. adamantium* seeds show a recalcitrant behavior due to their sensitivity to desiccation after fast and slow drying, which was reflected by the reduced physiological potential of the seed. The damages caused by desiccation are observed by the decreased percentage of normal seedlings originating from seeds subjected to fast drying in the moisture content of 21.1% and slow drying in the moisture content of 17.2%. The genomic DNA remained intact during drying. However, the absence of protection mechanisms, which resulted in seed viability loss, could be attributed to a loss of RNA integrity during desiccation. These results indicate that the loss of nucleic acid stability during drying is one of the components underlying sensitivity to desiccation.

C. adamantium seeds are sensitive to desiccation and a reduction in the moisture content beyond 21.1% following drying using silica

gel (fast) and beyond 17.2% following drying under laboratory conditions (slow), damages the physiological potential of the seeds.

DNA integrity was not affected during seed desiccation using either methods. However, drying using silica gel (fast) in the moisture content of 4.5% and drying under laboratory conditions (slow) in the moisture content of 5.4% resulted in damage to seed RNA.

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RESUMO

O objetivo desse trabalho foi avaliar a sensibilidade à dessecação em sementes de *Campomanesia adamantium*, por meio de secagem em sílica gel ativada (rápida) e em condições de laboratório (lenta). Para avaliar a sensibilidade à dessecação das sementes, realizou-se a secagem com sílica gel e secagem em condições de laboratório (25 °C) visando à obtenção de sementes com os teores de água de 45, 35, 30, 25, 20, 15, 10 e 5%. O potencial fisiológico das sementes após a secagem foi avaliado por meio da medição da protrusão da raiz primária, porcentagem de plântulas normais, índice de germinação da semente, comprimento de plântulas, massa seca total de plântulas, condutividade elétrica e integridade do DNA e RNA. As sementes de *C. adamantium* são sensíveis à dessecação e à redução do teor de água a partir de 21,1% na secagem em sílica gel e teor de água de 17,2% para secagem em condições de laboratório, sendo verificados prejuízos ao potencial fisiológico das sementes. A integridade do DNA genômico não foi afetada após a secagem das sementes nos dois métodos. Porém, a secagem em sílica gel no teor de água de 4,5% e a secagem em condições de laboratório com o teor de água de 5,4% resultaram na perda da integridade do RNA da semente.

Palavras-chave: Cerrado, DNA, secagem, RNA, viabilidade.

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