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Storage of *Alibertia edulis* seeds: Influence of water content and storage conditions

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Knowledge of the desiccation sensitivity of *Alibertia edulis* seeds is essential to provide adequate conditions for maintaining viability during storage. Thus, the objective of this study was to evaluate the effect of variations in the water content, environmental conditions, and storage periods on the conservation of *A. edulis* seeds. After processing, the seeds were dried under ambient conditions to water contents of 20, 15, 10 and 5±2%, and then subjected to storage under laboratory (25°C), cold chamber (16°C), refrigerator (8°C), and freezer (-18°C) conditions for zero, 30, 60, 90, 120, 150 and 180 days. To assess the physiological potential of the seeds, protrusion of the primary root, percentage of normal seedlings, germination speed index, total seedling length and total dry mass of seedlings were performed. A completely randomized factorial split-plot design (4 water contents × 4 temperature × 7 storage periods) was used for the experiment. *A. edulis* seeds tolerated water content reduction to 5% and storage under room temperature and cold chamber storage conditions for 150 days. Seeds with water content between 10 and 5% did not tolerate more than 60 days of freezing conditions, confirming their physiological behavior as intermediate.

Key words: Brazilian savanna, conservation, drying, Rubiaceae.

INTRODUCTION

The Brazilian Savanna holds 5% of the planet's biodiversity and is considered the richest savanna in the world, but one of the most threatened biomes (MMA, 2010). Unfortunately, many of the natural resources present in this biome, such as medicinal plants and fruit species, some endemic (Klink and Machado, 2005), are disappearing because of uncontrolled deforestation.

Many of these native species could be used to produce seedlings, among other objectives, to recover or enhance degraded areas and maintain germplasm banks to guarantee the conservation of biodiversity (Martins et al., 2009). Seed storage is of fundamental importance in the preservation of the physical, physiological, and health qualities of seeds, ensuring the conservation of the genetic diversity of many species for scientific research and agriculture (Medeiros and Eira, 2006). However, the

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success of storing seeds depends on understanding their behavior during the process, which enables the use of appropriate conditions for maintaining their viability (Hong and Ellis, 1996).

Regarding their behavior in storage, seeds are usually classified into two distinct groups: orthodox and recalcitrant. Orthodox seeds remain viable after drying to a moisture content of down to 5% and remain viable for long periods at low temperatures. Recalcitrant seeds are sensitive to desiccation and do not survive under low levels of moisture, which prevents their long-term storage (Roberts, 1973). Besides these groups, there is a third group of seeds with intermediate storage behavior (Ellis et al., 1990). Intermediate seeds tolerate dehydration of 7.0 to 10% moisture and do not tolerate low temperatures for extended periods of time (Hong and Ellis, 1996).

Tolerance to desiccation is one of the most important properties of seeds, and is defined as the ability to survive without irreversible damage after the complete or almost complete removal of intracellular water (Leprince and Buitink, 2010). Several mechanisms have been associated with desiccation tolerance in seeds, such as: reduction in the degree of vacuolation; the accumulation of insoluble reserves; reaction of the cytoskeleton; nuclear DNA conformation; intracellular differentiation; metabolic shutdown; presence and efficiency of antioxidant systems; and accumulation of protective molecules (Berjak and Pammenter, 2001). However, further studies are needed to determine the behavior of seeds of native tropical species in terms of drying, duration of drying, and necessary storage conditions for maintaining the physiological potential of seeds, in order to conserve the germplasm of species occurring naturally in the Savanna.

Alibertia edulis (Rich.) A. Rich. ex DC. (Rubiaceae), popularly known as Apuruí, Marmelada-Nativa, Marmelão, and Marmelo do Cerrado, has nutritional and medicinal importance. The fruits are globular shape with size of about 2 to 4 cm long and 2 to 4 cm diameter black color when ripe and greatly appreciated by the public, can be consumed fresh or as jelly, sweets and soft drinks (Silva et al., 2001; Chiquieri et al., 2004). The roasted seed is used to replace coffee, and the fruit can also be fed to livestock (Felfili et al., 2000).

There is some information in the literature regarding the occurrence of desiccation sensitivity in the seeds of other species of Rubiaceae, as reported for *Coffea canephora* Pierre ex A. Froehner; its seeds showed tolerance to desiccation down to 15% moisture and could be stored for four months at 10°C (Rosa et al., 2005); and for *Coffea arabica* and *Coffea congensis* A. Froehner, where desiccation up to levels less than 9% can damage the seeds, thus demonstrating the sensitivity of *Coffea* species to desiccation (Eira et al., 2006). *Genipa americana* L. seeds dried to 10 and 5% moisture had reduced viability after 30 days of storage, but there was no total loss of germination after this period (Salomão, 2004; Magistrali et al., 2013).

Thus, the lack of knowledge regarding the longevity of *A. edulis* seeds hinders the exploitation of the properties of fruits and optimization of the cultivation of this species for commercial purposes, in reforestation, and maintenance in germplasm bank programs. The hypothesis of this study is that seeds of *A. edulis* can be stored for a period exceeding 30 days at low temperatures provided their water content is maintained around 5%.

To test this hypothesis and to determine the physiological behavior of the seeds of this species, this study evaluated the effect of different water contents, environmental conditions, and storage periods on the physiological potential of *A. edulis* seeds.

MATERIALS AND METHODS

Vegetal material

A. edulis fruits were collected at the end of July, 2013 from 15 arrays located in an region of the Savanna (*sensu stricto*) in Dourados - MS. After collection, the fruits were brought to the Laboratory of Nutrition and Metabolism of Plants, Universidade Federal da Grande Dourados (UFGD) in Dourados - MS, where they were washed in running water and any damaged fruits were discarded. Subsequently, the fruits were processed manually and on sieves to separate the seeds. The seeds were then washed with tap water and placed on Germitest® paper for 40 min at room temperature (25°C±2°C, 32% relative humidity) to remove excess moisture.

After water was removed from the seed surfaces, the seeds were dried under laboratory (25°C) conditions on plastic trays and weighed until they reached the pre-established water content (20, 15, 10 and 5%, respectively), as calculated according to the formula of Sacandé et al. (2004). To obtain 20, 15, 10, and 5% moisture content water, 7, 25, 28, and 30 h of drying, respectively, were required at room temperature.

When the water content levels of the seeds were found to be near those desired, samples were taken, homogenized, and divided into fractions packaged aluminum foil bags. These samples were subjected to the following storage conditions: 25±2°C, 35% relative humidity (laboratory); 16±2°C, 40% relative humidity (cold chamber); 8±2°C, 35% relative humidity (refrigeration); and -18±2°C, 42% relative humidity (freezing). After 0 (newly processed), 30, 60, 90, 120, 150, and 180 days of storage, the seeds were pre-humidified to 100% relative humidity and 25°C under continuous white light for 24 h, thereby preventing damage from soaking.

Evaluation of seed physiological quality

The following physiological characteristics were then determined. The water content was determined at 105°C±3°C for 24 h using the oven-drying (Brasil, 2009) with three replicates of 5 g of seeds each, expressed as percentage on a fresh weight basis.

Protrusion of the primary root was measured on Germitest® paper rolls with four replications of 25 seeds each, which were kept in 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 (%).

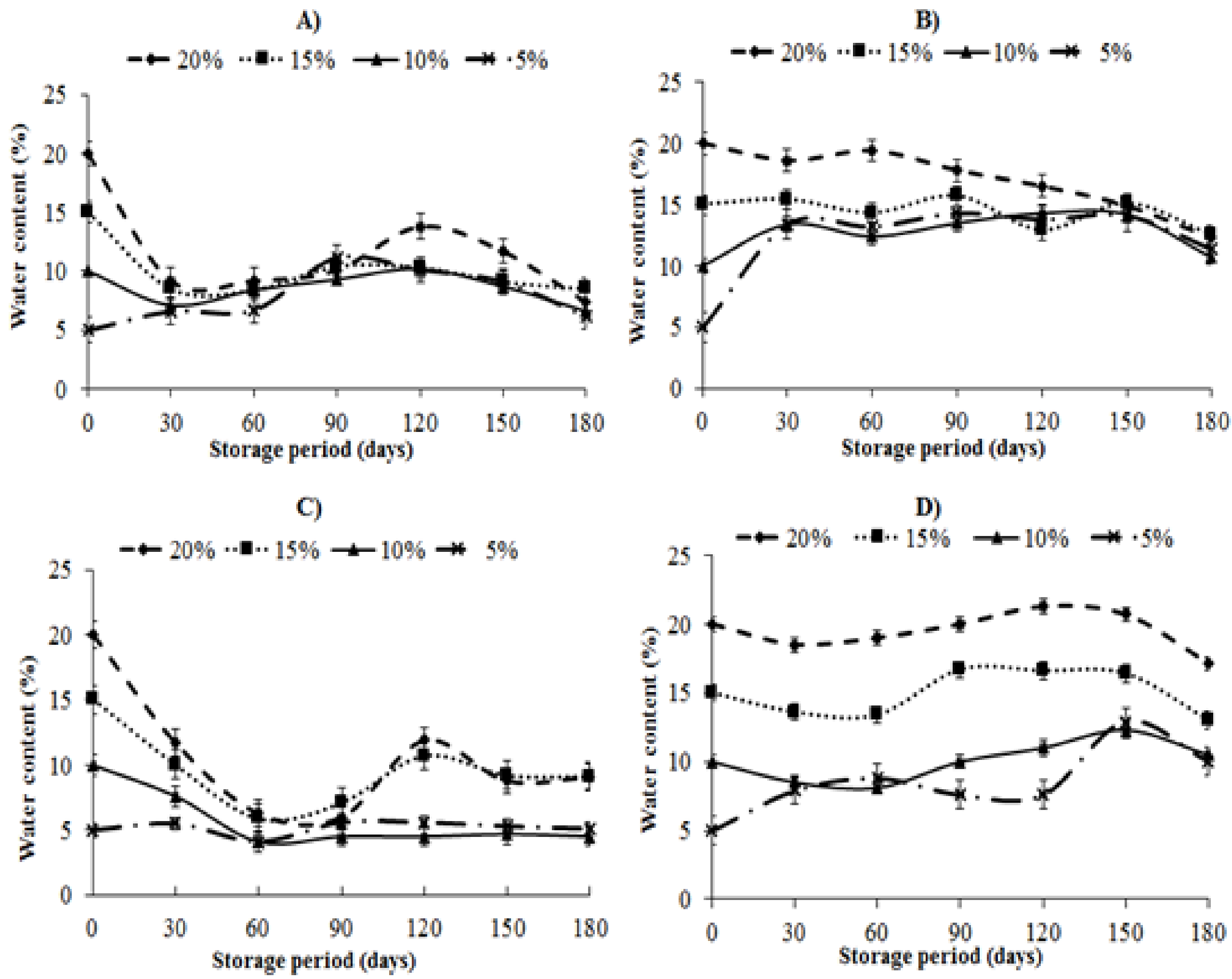

Figure 1. Water content (%) of *A. edulis* seeds packed with different water contents (%), environmental temperature (A - Laboratory, B - cold chamber, C- refrigerator and D - freezer) and stored for different periods.

The percentage of normal seedlings was determined in Germitest® paper rolls with four replications of 25 seeds each, which were put in BOD at 25°C under continuous white light. Evaluations were performed forty-five days after sowing by computing the percentages of normal seedlings, using the issuance of shoots and root system development. The results were expressed in percentages (%).

Germination speed index (GSI) was calculated using the numbers of seedlings that germinated each day divided by the numbers of days between sowing and germination, according to the formula proposed by Maguire (1962). Seedling length was obtained by measuring the lengths of the primary root, shoot and total plant using a millimeter ruler. The results were expressed in centimeters (cm seedlings^{-1}).

The total dry mass was obtained from seedlings that had been dried in an oven at 60°C for 48 h using an analytical balance (0.0001 g), with the results expressed in grams (g seedlings^{-1}).

Experimental design/ statistical analysis

A completely randomized factorial split-plot design (4 temperature/environment conditions × 4 water contents × 7 storage periods) was used for the experiment. For data showing significance in the analysis of variance, the temperature data were compared by a Tukey's test and the data on water content and storage period were adjusted by regression equations at 5% probability using the SISVAR software (Ferreira, 2011).

RESULTS AND DISCUSSION

Seeds stored under laboratory conditions (25°C), a cold chamber (16°C), a refrigerator (8°C) and a freezer (-18°C) showed variations in the water content during storage (Figure 1). The variations observed in the water content of seeds stored under different conditions evaluated over 180 days demonstrated it can be a semi-permeable packing, there was an exchange of water vapor between the seed and the external environment, thus changing the level of hydration of the seeds during storage. However, this type of semi-permeable packaging

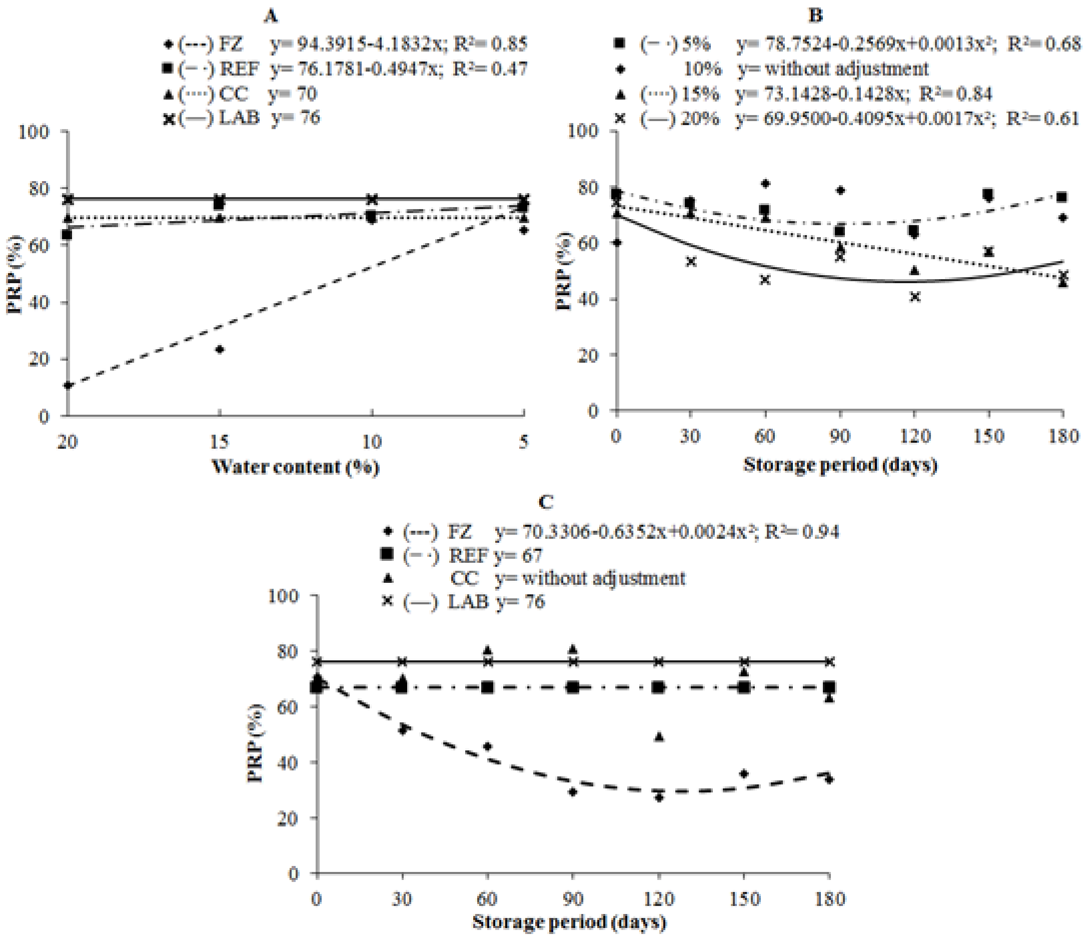

Figure 2. Primary root protrusion (PRP) (%) of *A. edulis* seeds according interactions of water content x storage conditions (A), storage periods x water content (B) and storage periods x storage conditions (C) LAB (Laboratory), CC (cold chamber), refrigerator (REF) and freezer (FZ).

is often used for the storage of seeds in banks. It is noteworthy that the quality of packaging varies, but it also highlights the tremendous opportunities to customize packaging properties by combining thermal plastics and water vapor barriers, and by manipulating layer thicknesses (Gómez-Campos, 2006; Walters, 2007).

In laboratory, cold chamber and refrigerator conditions, significant reductions were observed in the water content after 30 days of storage, mainly for the 20 and 15% water content. The seeds stored under freezer conditions showed little variation in the levels of 20, 15, 10, and 5% water, presenting at the end of the 180 days storage mean results of 17, 13, 11, and 9%, respectively.

For the protrusion of the primary, there were significant interactions for water contents × storage conditions, storage period × storage conditions, and storage periods × water contents (Figure 2). With the water content used, the storage conditions of higher temperatures such as laboratory temperature (25°C) and cold chamber (16°C) gave average values of 76 and 70% germination data respectively. Under the refrigerator conditions (8°C), the primary root protrusion increased as the water content was reduced, and this behavior was most significant in freezing conditions (-18°C) (Figure 2A).

In the interaction between storage period and water content, the quadratic equation the water content of 5% showed a minimum value for primary root protrusion of 67% at 92 days of storage, although no differences were observed during the storage time for the seeds with a water content of 10% (Figure 2B). The different storage conditions did not affect primary root protrusion over the 180 days of storage, except under freezing conditions showing minimum values of 30% at 128 days (Figure 2C).

The triple interaction (water content × storage conditions × storage period) was significant for the percentage of normal seedlings and germination speed index (GSI), this being represented by the split depending on the storage conditions. Under laboratory storage conditions (25°C), the maximum percentage of normal

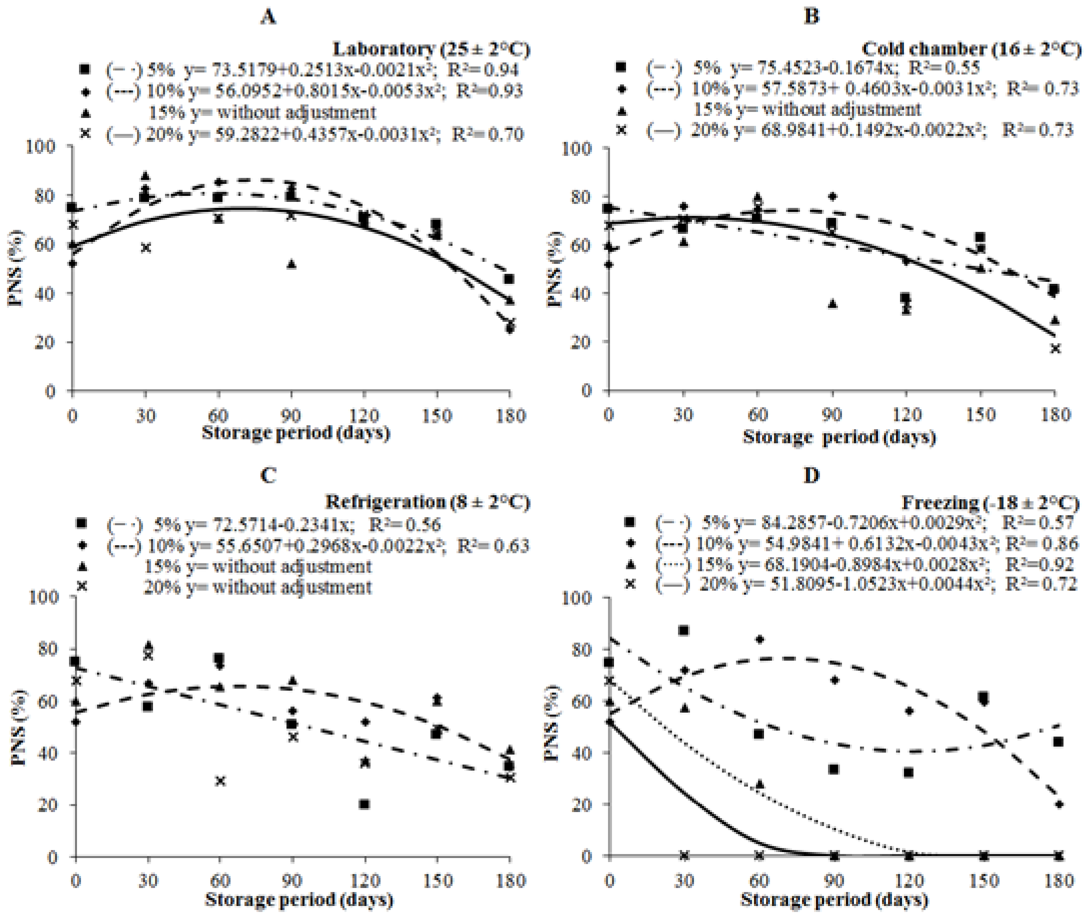

Figure 3. Percentage of normal seedlings (PNS) (%) of *A. edulis* according interaction water content × storage period in the storage conditions of laboratory (A), cold chamber (B), refrigerator (C) and freezer (D).

seedlings was observed for seeds with a water content of 10% (86%) after 75 days of storage, followed by seeds stored with a water content of 5% (82%) after 54 days of storage (Figure 3A).

For seeds stored under cold chamber storage conditions (16°C), the maximum percentage of normal seedlings was observed for seeds with a water content of 10% (74%) after 73 days of storage (Figure 3B). Seeds stored with initial water content of 5 and 20% showed a reduction in the percentage of normal seedlings over the 180 days of storage. Seeds stored under refrigerator conditions (8°C) showed a maximum percentage of normal seedlings after 67 days of storage (66%) at 10% (Figure 3C). For freezing conditions (-18°C), the highest percentage of normal seedlings (76%) was observed in seeds stored with a water content of 10% after 70 days of storage (Figure 3D). However, seeds with 5% water content had higher than 50% normal seedlings for up to 60 days of storage and the seeds with water content of 15 and 20% had values below 50% at 30 days of storage.

The classification criterion which distinguishes intermediate and orthodox seeds is seed storage after drying to 5% moisture content at -20°C for a period of 90 days in order to maintain viability (Hong and Ellis 1996). However, it has been reported that an important feature related to intermediate seeds of tropical origin is the fact that their longevity is reduced when stored at temperatures below 10°C (Ellis et al., 1991; Hong and Ellis, 1992). Thus, it was demonstrated that *A. edulis* seeds tolerated a reduction in water content between 5 and 10%, and remain viable during 150 days of storage; however, they lost viability when exposed to freezing temperatures (-18°C) during storage, indicating that they can be classified as intermediate as far as drying and storage is concerned.

The sensitivity to freezing was observed in seeds of other species of Rubiaceae; Coffee seeds are now considered to have storage behavior defined as intermediate (Ellis et al., 1990, 1991; Hong and Ellis, 1996). Seeds of *Genipa americana* L. (Rubiaceae) dried to water contents between 10 and 5% and stored at -20°C showed high percentages of germination and normal seedlings for up to 30 days of storage, and after this period showed complete loss of viability, indicating

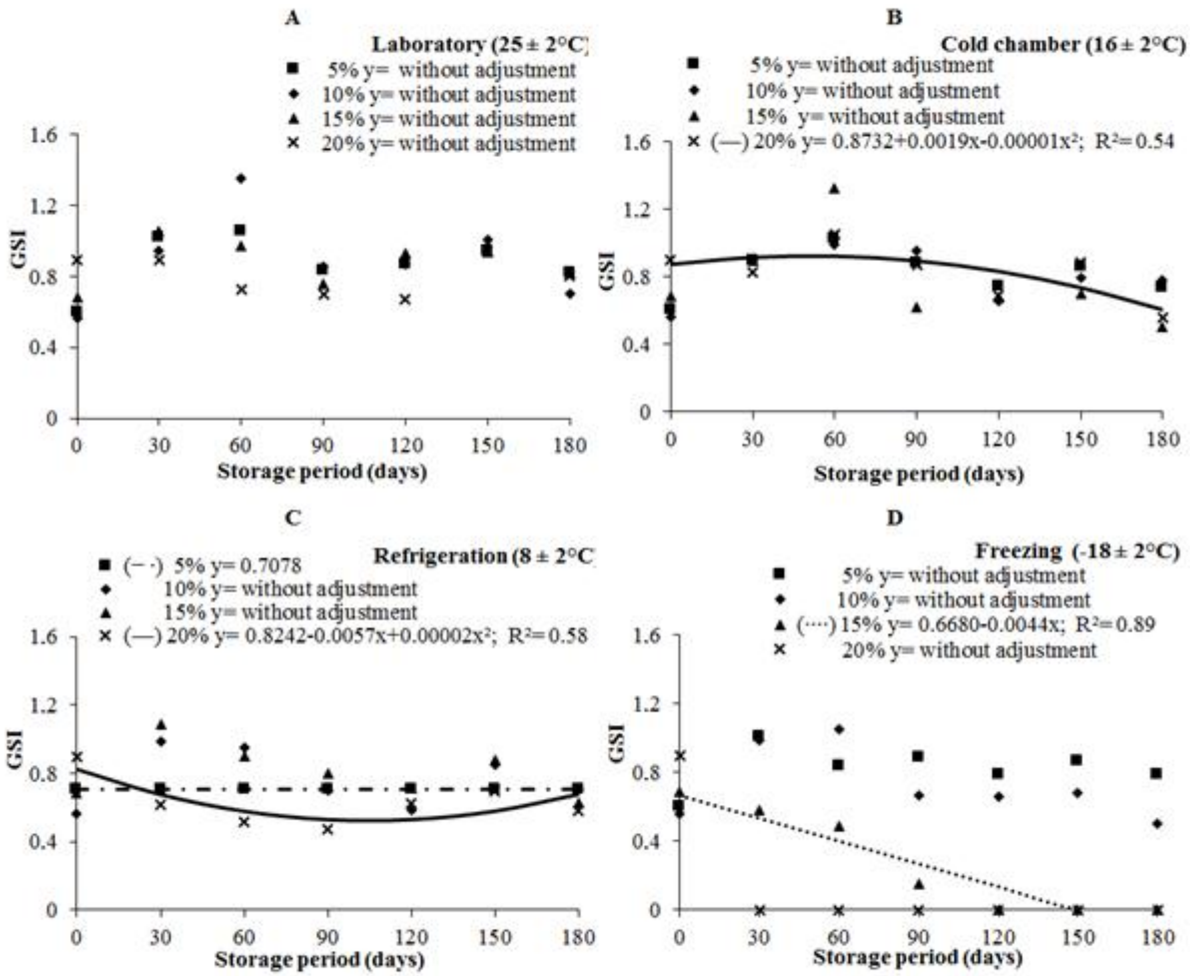

Figure 4. Germination speed index (GSI) of *A. edulis* seeds according interaction water content × storage period in the storage conditions of laboratory (A), cold chamber (B), refrigerator (C) and freezer (D).

an intermediate behavior in relation to drying and storage (Magistrali et al., 2013). *Alibertia patinoi* (Cuatrec.) Delprete & C.H. Perss. (Rubiaceae) seeds were classified as recalcitrant, and have high water content (44%) and exhibit continuous metabolism even after dispersion (Escobar and Torres, 2013). Drying of seeds can cause extensive ultrastructural damage to cells, including protein denaturation, crystallization of solutes, and damage to the membranes (Black et al., 2002). Thus, the ability to maintain cellular integrity and repair damage caused by drying is of fundamental importance for the seeds to tolerate desiccation (Pammenter and Berjak, 1999).

The sensitivity of the *A. edulis* seeds at low temperatures (-18°C, freezer) was more pronounced in seeds stored with higher water content (15 and 20%), with the formation of normal seedlings for only up to 30 days of storage. Possibly, the intracellular water formed ice crystals during storage, causing damage to the cellular compartment; thus, the seeds lost viability when rehydrated. Damage caused by freezing may be due to membrane lipid peroxidation promoted by both dehydration and freezing (Wen et al., 2012); however, intracellular ice formation per se is not lethal, at least for the size and density of crystals (Wesley-Smith et al., 2014).

Regarding the germination speed, seeds stored under laboratory conditions (25°C) did not show significantly adjusted equations (Figure 4A). Under cold room conditions (16°C), the maximum germination speed was recorded after 51 days of storage in seeds stored with an initial moisture content of 20% (0.92) (Figure 4B). Seeds stored in a refrigerator (8°C) with an initial moisture content of 5% showed a medium germination speed of 0.70 over the 180 days of storage (Figure 4C), although for the water content of 20%, a minimum GSI value (0.52) was observed for 106 days of storage (Figure 4C). Freezer conditions (-18°C) negatively affected the germination rate of seeds stored with an initial moisture content of 15%, being terminated at 150 days (Figure 4D).

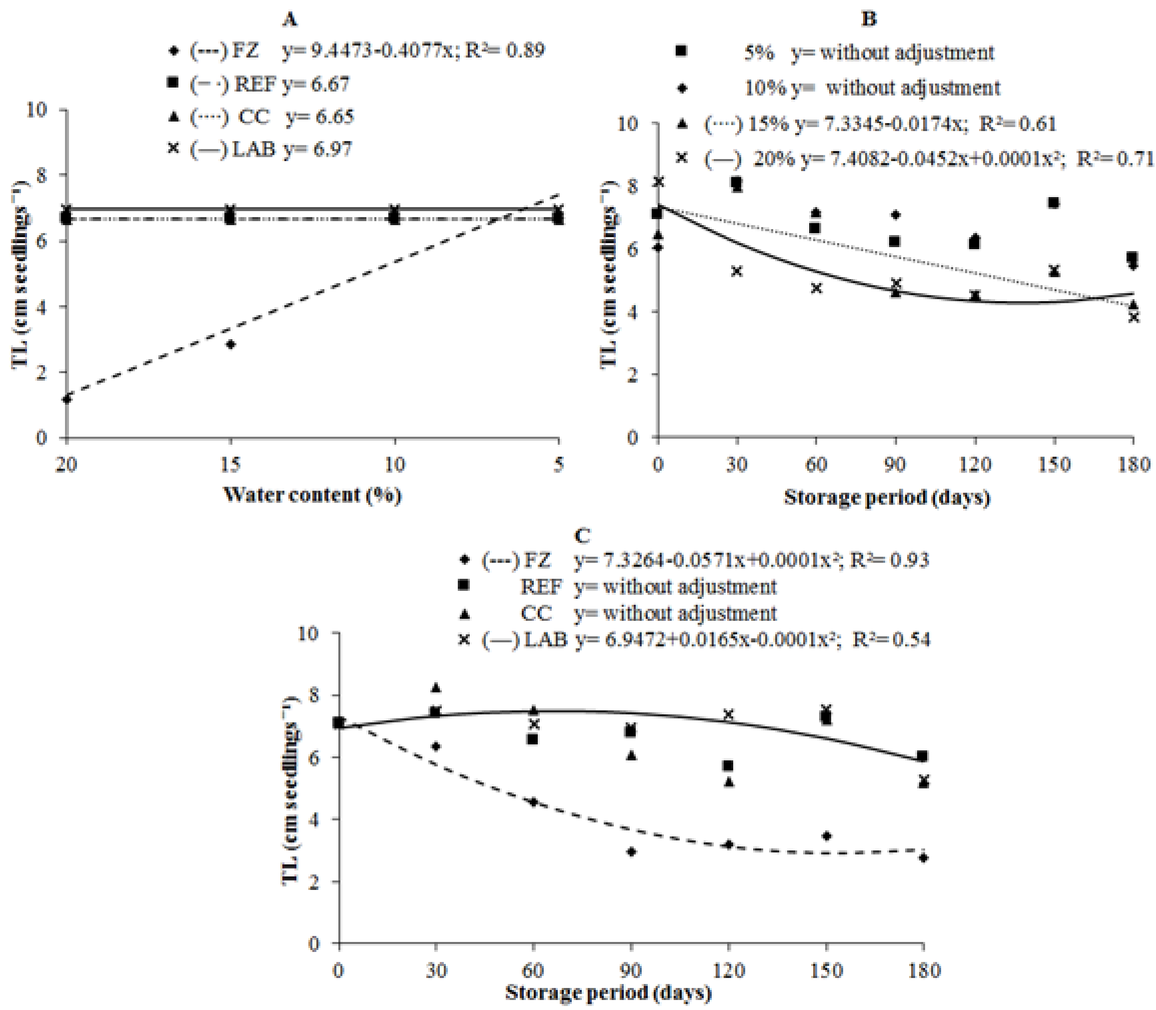

Figure 5. Total length (TL) (cm seedlings^{-1}) of *A. edulis* seedling according interactions of water content x storage conditions (A), storage periods x storage conditions (B) and storage periods x water content (C). LAB (Laboratory), CC (cold chamber), refrigerator (REF) and freezer (FZ).

The interaction of water content × storage conditions, storage period × storage conditions, and storage periods × water content were significant for the total seedling length (Figure 5). Seeds stored under freezing conditions (-18°C) with an initial moisture content of 5% had higher seedling growth throughout the storage period (7.40 cm seedlings^{-1}) (Figure 5A).

For interaction of water with storage periods, there were no differences in total seedling growth over the 180 days of storage for seeds stored with a water content of 5 and 10% (Figure 5B); however, for seeds stored with a moisture content of 20%, the minimum value observed for total seedling growth (4.28 cm seedlings^{-1}) was at 138 days of storage (Figure 5B).

Seeds stored under laboratory conditions (25°C) showed maximum total growth (7.49 cm seedlings^{-1}) after 66 days of storage (Figure 5C). Under cold chamber (16°C) and refrigerator conditions (8°C), there were no differences in total seedling growth over the 180 days of storage; however, under freezing conditions (-18°C), a significant reduction in seedling growth was verified, while a minimum length (2.91 cm seedlings^{-1}) was observed at 154 days of storage (Figure 5).

The triple interaction (water content × storage conditions × storage period) was significant for the accumulation of total dry matter (TDM), this being represented by the split depending on the storage condition (Figure 6). With respect to the accumulation of total dry mass, seeds stored under laboratory (25°C) (Figure 6A) and cold chamber (16°C) (Figure 6B) conditions with an initial moisture content of 5% had a significant accumulation of total dry mass of 0.0054 g seedlings^{-1} over the 180 days, evidencing the translocation of reservations for the formation of seedlings. On the other hand, seeds stored with a water content of 10 and 20% under laboratory conditions

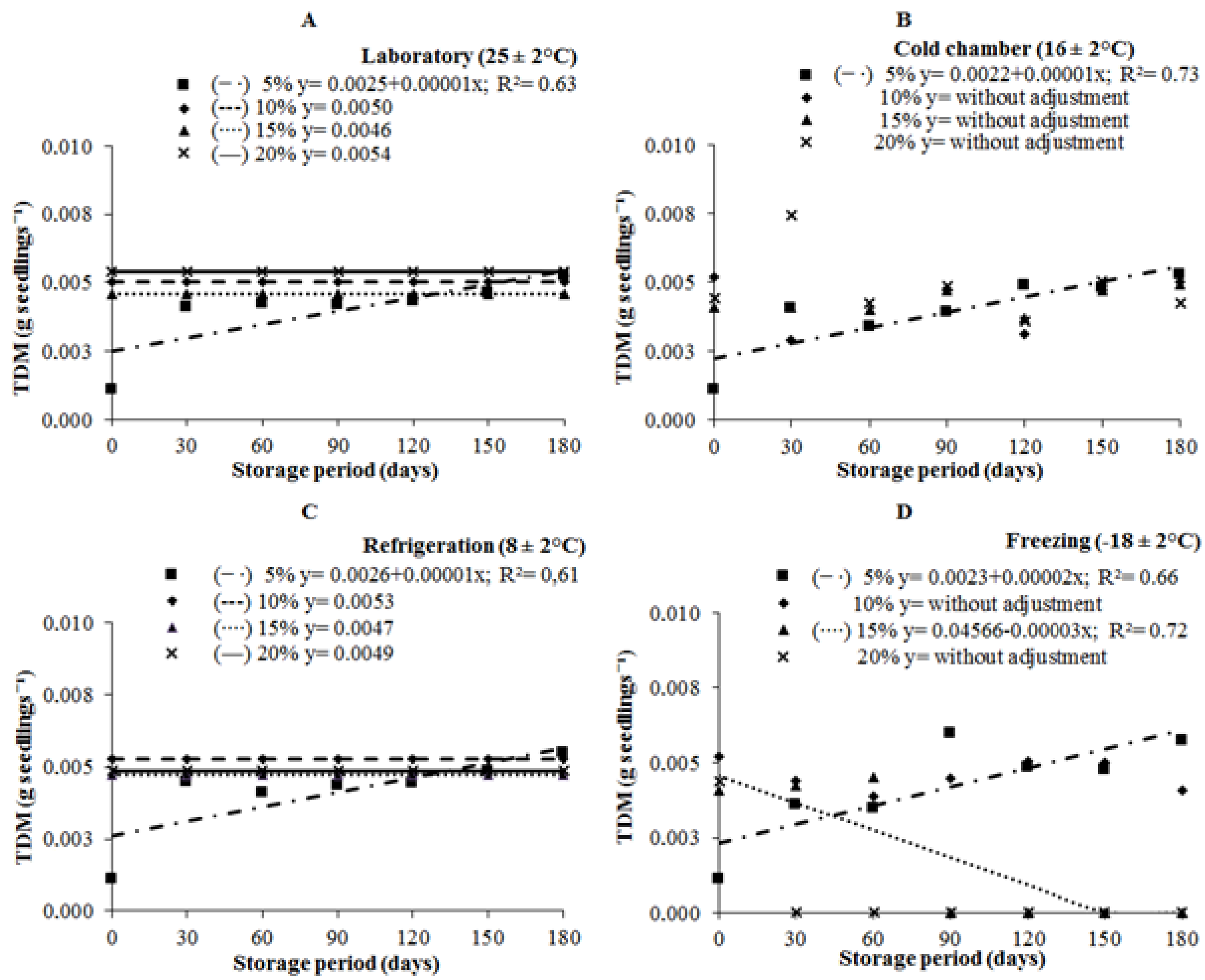

Figure 6. Total dry mass (TDM) (g) of *Alibertia edulis* seedlings according interaction water content × storage period in the storage conditions of laboratory (A), cold chamber (B), refrigerator (C) and freezer (D).

(25°C) yielded an average accumulation of dry matter of 0.0050 g seedlings^{-1} over the 180 days of storage (Figure 6A).

Seeds stored under refrigerator conditions (8°C) with an initial moisture content of 5% had a significant increase in the average accumulation of total dry mass (0.0050 g seedlings^{-1}) over 180 days of storage; however, the seeds with 10% water content had an average accumulation of 0.0053 g seedlings^{-1} throughout the storage period (Figure 6C). Freezing conditions (-18°C) negatively influenced the accumulation of seed mass stored with 15% water content, totally ceasing at 150 days (Figure 6D). On the other hand, seeds stored with 5% water content had an average accumulation of total dry mass of 0.0050 g seedlings^{-1} at 180 days of storage.

The negative effect of seed storage with high water content (15% and 20%) under low temperature conditions (-18°C freezer) was also observed in other parameters such as germination speed index, which stopped at 150 days (content of 15% water) and 30 days (content 20% water) of storage (Figure 4D), causing reduction in seedling growth, and thus interfering with the accumulation of total dry mass (Figure 5 and 6D). These results, together with the percentages of radicle protrusion and normal seedlings, suggest that the nature of the damage due to freezing is different from the mechanisms of damage caused by the drying of the seeds in water contents between 10 and 5%. Although the seeds of *A. edulis* demonstrated some tolerance to desiccation, storage at temperatures below 10°C adversely affected the maintenance of the longevity of these seeds, confirming the intermediate physiological behavior of the seeds.

Therefore, it is evident that seeds with intermediate behavior cannot be stored in accordance with the protocols usually recommended for the storage of seeds, because although they apparently survive conditions of low water content, they do not survive the additional stress of exposure to -18°C (Black et al., 2002). The major impediment to storing seeds with intermediate physiologies (that is, Coffee) is an understanding of the limit to which seeds can be dried and the interaction of temperature and water content on seed survival. For non-orthodox seed species, cryopreservation is the only technique available for long-term germplasm conservation. In the case of intermediate seed-propagated species, seeds are partially desiccation tolerant and, therefore, the option which has to be always tested first is whole-seed cryopreservation (Eira et al., 2006).

Thus, it is worth mentioning the importance of prior knowledge of the physiological behavior of the seeds to determine the proper technique for safe storage. This aspect becomes more relevant for native species from threatened biomes that require specific criteria for their long-term storage, such as the species investigated in the present work. Accordingly, the reduction of water content associated with favorable storage conditions is crucial for maintaining the physiological quality of seeds of *A. edulis*. For seeds that are sensitive to drying and storing, maintenance of viability is still one of the biggest challenges faced by researchers for conservation in gene banks.

One of the considerations for the physiological behavior of *A. edulis* seeds is the ecological relationship with the habitat (Daws et al., 2004; Kochanek et al., 2010). From an ecological perspective it is worth noting that seeds classified as intermediate are essentially desiccation tolerant (Tweddle et al., 2003). In terms of habitat, most sensitive seeds (89%) occupy wet forest, riverine, flooded, or coastal environments. Most species (79%) are native to the tropics and many of the desiccation-intolerant species produce seeds that mature during tropical monsoons and rainy seasons, and are unlikely to experience dry conditions (Farnsworth, 2000).

The effects of water content and temperature are interdependent and therefore, current results of *A. edulis* show that critical water content always increases with decreasing temperature. So, there is a narrow window of seed moisture status at which the storage of *A. edulis* seeds is feasible. Just as it was reported the occurrence of intermediate behavior in seeds of other species of the family Rubiaceae, further research on the different levels of desiccation sensitivity among others *Alibertia* species should result in a better understanding of seed longevity and appropriate commercial seed and germplasm storage protocols.

Conclusions

A. edulis seeds tolerate a reduction in their water content to 5% and room temperature (25°C), and cold chamber (16°C) storage conditions for 150 days. Seeds with water content of between 10 and 5% did not tolerate more than 60 days under freezing (-18°C) storage conditions, confirming their physiological behavior as intermediate. The storage of seeds with high water content (15 and 20%) in conditions of low temperature (-18°C freezing) reduced seedling growth and accumulation of total dry mass.

Conflict of Interests

The authors have not declared any conflict of interests.

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