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Germination, protein contents and soluble carbohydrates during storage of sugar apple seeds (*Annona squamosa* L.)

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Summary

The aim of this study was to evaluate changes in the germination potential, protein contents and soluble carbohydrates in sugar apple (*Annona squamosa* L.) seeds from Castilla-Tolima and Apulo-Cundinamarca, Colombia during storage for 30, 60 and 120 days in a cool room, refrigeration and ambient conditions. In each period, the seed moisture contents, germination percentage (GP), mean germination time (MGT), mean germination rate (MGR), soluble protein content, and contents of sucrose, glucose and fructose were evaluated. The highest GP (> 80 %) and MGR (0.15 germinated seeds / day) and the lowest MGT (< 7 days) were reached after 60 and 120 days of storage under ambient conditions. In the three storage conditions, the protein contents declined at 60 days and 120 days in seeds from Castilla and Apulo, respectively. Under the ambient conditions (AC), there was an increase of 72.3 % in the sucrose content, 61 % increase in the fructose content and 70 % decrease in the glucose content. The results show that this environment temperature for 60 and 120 days was best suited for maintaining short-term germination. The high GP and MGT values and low MGR value indicated a possible dormancy release that occurred naturally.

Introduction

The sugar apple belongs to the *Annona* genus and is widely distributed in the wild worldwide and in Colombia (Martínez et al., 2013). It grows in the dry areas of the valleys in the provinces of Valle, Caldas, Tolima, Cundinamarca and Santander (Colombia) (Guerrero and Fischer, 2007), between 450 and 1,500 meters above sea level (Hoyos, 1989) and requires no cold periods; meaning it develops and grows well in relatively stable temperature conditions (George and Nissen, 1993). The minimum temperature is in a range of 10 to 20 °C and the maximum is 22 to 28 °C (Guerrero and Fischer, 2007). An important process of seed production is storage; this procedure allows growers to save seeds from one season to another. Storage is also important for germplasm conservation, where one of the objectives is to maintain the viability of seeds of a wide range of species for an indefinite period (a long-term period is often considered 10 to 100 years or more) (Hong and Ellis, 1996). However, during storage, chemical and physiological alterations result in deterioration and loss or reduction in germination (Kapoor et al., 2011). This is related to the depletion of reserves in seeds designed to provide energy for the growing embryo during storage (Villela and Peres, 2004; Veiga et al., 1997). It has been demonstrated before that soluble carbohydrates play a key role in desiccation tolerance and seed storage. Increases in the levels of sucrose, particularly in the contents of sugars of the oligosaccharide raffinose family, have been correlated with desiccation tolerance and longevity in orthodox seeds (Obendorf, 1997; Horbowicz and Obendorf, 1994)

In *Anonaceae*, it was reported that seeds have an orthodox behavior. Lobo et al. (2007) showed that cherimoya (*Annona cherimola* Mill.) and soursop (*Annona muricata* L.) seeds exhibit orthodox storage conditions. Dornelles et al. (2002) reported that a decrease in moisture contents in sugar apple (*Annona squamosa* L.) seeds promotes seed vigor, indicating that they are tolerant to desiccation and are orthodox. According to Villela and Peres (2004), orthodox seeds can be stored at low temperatures and can withstand adverse conditions during the dormancy period when exposed to moisture and temperature conditions that are suitable for resuming their development and complete the germination process.

The longevity of seeds varies greatly between species and is conditioned by the prevailing environment during the formation of fruits, seed maturation, processing, and handling of seeds after they are collected (Catunda et al., 2003; Santana and De Cavalho, 2006). In each species and, in some cases, in different varieties, specific conservation needs may be required for maintaining their longevity, so it is important to determine the optimum conditions for seed storage in each particular case. Therefore, knowledge of the effect of storage on seed physiology is important for maintaining gene banks as well as processes of propagation and conservation of species (Pereira et al., 2008). The present study aimed to determine the effect of short-term storage on germination, protein and soluble carbohydrates in sugar apple seeds (*Annona squamosa* L.) collected from two geographical areas of Colombia.

Materials and methods

The plant material was obtained from the accessions T7AS180, T7AS181, C2AS224, and C2AS225, collected in Apulo (Cundinamarca province) and Castilla (Tolima province), Colombia. The seeds were obtained from ripe fruits, soft to the touch, that were disinfected with immersion for 9 minutes in 1 % sodium hypochlorite, washed with 96 % ethanol:distilled water (1:1) three times, and, finally, rinsed three times with distilled water to remove any ethanol residue that might have remained on the seeds (Hou et al., 2009).

Storage conditions

The seeds collected in Apulo (Cundinamarca) were stored with moisture contents between 10 and 12 %, while the moisture contents of the seeds from Castilla (Tolima) during the storage varied between 12 and 14 %. The seeds were stored for 0, 30, 60, and 120 d in craft paper bags under three conditions: cool room (CR) at 4 °C and RH = 79 %, refrigeration (R) at 10 °C and RH = 79 %, and ambient storage (storage at room temperature) (AC) at 19 °C and RH = 60 %; the latter corresponded to the conditions in Laboratory of Crop Physiology, Faculty of Agrarian Sciences, National University of Colombia, Bogotá, Colombia. The period of storage, storage condition, and locations were arranged in a 3×3×2 factorial arrangement.

Seven replicates per 4 treatments were employed. For each replicate, 120 seeds were used. In total, 840 seeds per treatment were employed.

Germination

After each storage period, the seeds were removed to assess the germination. The seeds were sown in disinfected peat substrate Klasmann® (Klasmann-Deilmann GmbH, Germany) without nutrients at a depth equal to twice the seed length and subsequently placed in a controlled environment cabinet (SGC066, Sanyo Gallenkamp, Leicester, UK) for 30 days at a constant temperature of 35 °C in absence of light because the germination of *Annona squamosa* L. seeds is indifferent to light conditions (Ferreira et al., 2002). Observations were made every 5 days for 30 days because germinating seeds after this period could be considered dormant (Baskin and Baskin, 2001). Germination was recorded in those seeds that had radicle protrusion.

With sampling data, the following parameters were evaluated: *germination percentage* (GP) = $\left(\frac{N}{Ns}\right) * 100$, where N = number of germinated seeds and N_s = total number of seeds; *mean germination time* (MGT) = $\sum_{i=1}^{k} ni/ti$ and *mean germination rate* (MGR) = $\sum_{i=1}^{k} ni * ti \ / \ \sum_{i=1}^{k} ni$ where n_i = number of germinated seeds in the i^{th} data collection; t_i = time (in days) of the i^{th} data collection and K = time (in days) duration of the germination test.

Protein and soluble carbohydrate contents

The protein determination was made based on the methodologies described by Bradford (1976) and Zor and Selinger (1996), and the contents of carbohydrates sucrose, glucose, fructose were analyzed with high-performance liquid chromatography (Chinnici et al., 2005; Solarte et al., 2014). 1.00 g of seeds per replicate were employed. Due to similarities in the germination response and protein content of plant material from two locations, the soluble carbohydrates were determined only in seeds from Castilla (Tolima) after two periods of storage with responses in terms of germination (0 to 120 days). To determine the content of sucrose, glucose and fructose, extraction was performed from 100 mg of macerated seeds using 1 mL of 80% ethanol at 90 °C, which were then centrifuged 10 minutes at 6000 rpm at 4 °C and the supernatant was removed. The pellet was resuspended two more times with 1 mL of 80% ethanol at 90 °C following the same process to obtain 3 mL of supernatant. The supernatant was dried in a Speed Vac and resuspended in 1 mL of distilled water (Hou et al., 2009). The concentrated extract was centrifuged again at 6000 rpm for 15 minutes at 4 °C and filtered with 0.22 µm before the HPLC analysis. The contents of individual sugars were measured by liquid chromatography (Waters-Milford, MA USA) using a 300 × 7.8 mm Rezex RCM-monosaccharide column Ca+ (8%), 8 µm particle sizes and a refractive index detector 2414 (Waters). The conditions of analysis were as follows: 86 °C oven temperature, 44 °C detector temperature, and a 1 ml·min^{-1} flow of deionized water was employed as the mobile phase. Sugars were distinguished by comparing retention time and calibration curves of external standards by means of the software Empower 2 2005-2008.

We assessed the assumptions of normality and homogeneity of variances, and the data that showed no normality in the residuals were transformed with the arcosen function $\sqrt{}$ (pg/100) usually used in germination studies (Wagner et al., 2006). ANOVA was performed and the differences between the treatments were determined with a Tukey test ($P < 0.05$). We used the statistical package SAS (Statistical Analysis System), version 9.1.

Results and discussion

Seed storage of *Annona squamosa* L. at ambient temperature and humidity (AC) for 60 and 120 d generated the best responses in the variables of seed germination from two locations (Fig. 1). In both periods, the GP (Fig. 1A) had similar values for both Castilla-Tolima and Apulo-Cundinamarca (80 - 85% for 60 and 120 d). The maximum MGR (Fig. 1B) obtained with AC storage for 60 days was for Castilla-Tolima (0.17 germinated seeds / d) and for 120 d for Apulo-Cundinamarca (0.18 germinated seeds / d), which was an MGR that was more than 3-fold higher than that found in the seeds without storage (0.05 and 0.06 germinated seeds / d, respectively, for Castilla and Apulo). The MGT values (Fig. 1C) showed variation ($P < 0.0001$) over time with a tendency to decrease as storage progressed, resulting in lower values of MGT storage in AC for 60 and 120 days for the seeds of Castilla-Tolima (5.9 and 6.3 d, respectively) and Apulo-Cundinamarca (6.8 and 5.7 d, respectively). But, although these values did not differ from those found in CR and R at 60 days of storage, they were significantly lower than those found for seeds that were not stored (19.4 and 17 d for Castilla-Tolima and Apulo-Cundinamarca, respectively) (Fig. 1C).

In *A. squamosa* L. seeds, other authors have reported the same behavior in seed storage conditions. Dornelles et al. (2002) verified a gradual increase in the MGR and PG for up to three months of storage. Magalhães et al. (2009) found germination percentages of 88% when sugar apple seeds were stored at room temperature (maximum: 25.3 °C, minimum 16.1 °C) in paper for 6 months. However, Sousa (2008) reported no influence from storage for 0, 30, and 60 d on the germination of sugar apple seeds when they received no treatment to break the dormancy. In a similar study by Magalhães et al. (2014), the speed and percentage of germination and seedling dry weight in response to 5 storage periods (0, 3, 6, 9 and 12 months), two storage conditions (environment and cool conditions at 6 - 8 °C), and two packaging types (paper bag and plastic bag) were assessed. The authors found that, with a moisture content of 8.49%, the seed vigor was maintained during the storage and the seeds stored under ambient conditions in paper bags remained viable for six months.

These results indicated that the dormancy induced by embryo immaturity that is typical for Annonaceae was possibly overcome with storage. In particular, the period (60 and 120 days) and the conditions (temperature and humidity) of storage directly influenced the natural overcoming of dormancy seen in *A. squamosa* L. seeds, which was also indicated by Dornelles et al. (2002). It has been noted that Annonaceae seeds being dispersed have a small embryo, considered underdeveloped and immature (Noorman et al., 1992). In the case of *Annona squamosa* L., the embryo is rudimentary and undeveloped but differentiated with a slow growth rate, which later causes the seed to germinate after 1 to 3 months (Hayat, 1963). In cherimoya and soursop seeds, it was observed that morphophysiological dormancy could also be easily overcome with storage (Lobo et al., 2007). In *A. crassiflora* seeds, Silva et al. (2007) observed that the seeds had underdeveloped embryos at the time of fruit maturity and required a period subsequent to the dispersion to terminate their development. Likewise, for Pinto and Genu (1984), in soursop seeds, the endogenous dormancy could be completely overcome with storage. Hopkinson and English (2005) found that dormancy persisted longer under cool storage than under ambient storage conditions.

Changes in protein content during seed storage

The protein contents in seeds without storage (0 d) from both locations had similar values (9.61 and 9.92 mg / g for Castilla-Tolima and Apulo-Cundinamarca, respectively). In seeds of Castilla-Tolima, the protein content declined at 60 and 120 days in all of the storage conditions, presenting the lowest values at 60 days (5.21 mg / g (AC), 5.62 mg / g (CR) and 5.79 mg / g (R)). In the seeds of Apulo-Cundinamarca, the content significantly decreased at day 120 in all of the storage conditions (5.36 mg / g (AC), 5.90 mg / g (CR) and 7.52 mg / g (R)), as compared to the seeds without storage (9.92 mg / g) (Fig. 2).

The effect of time and storage conditions on the germination and

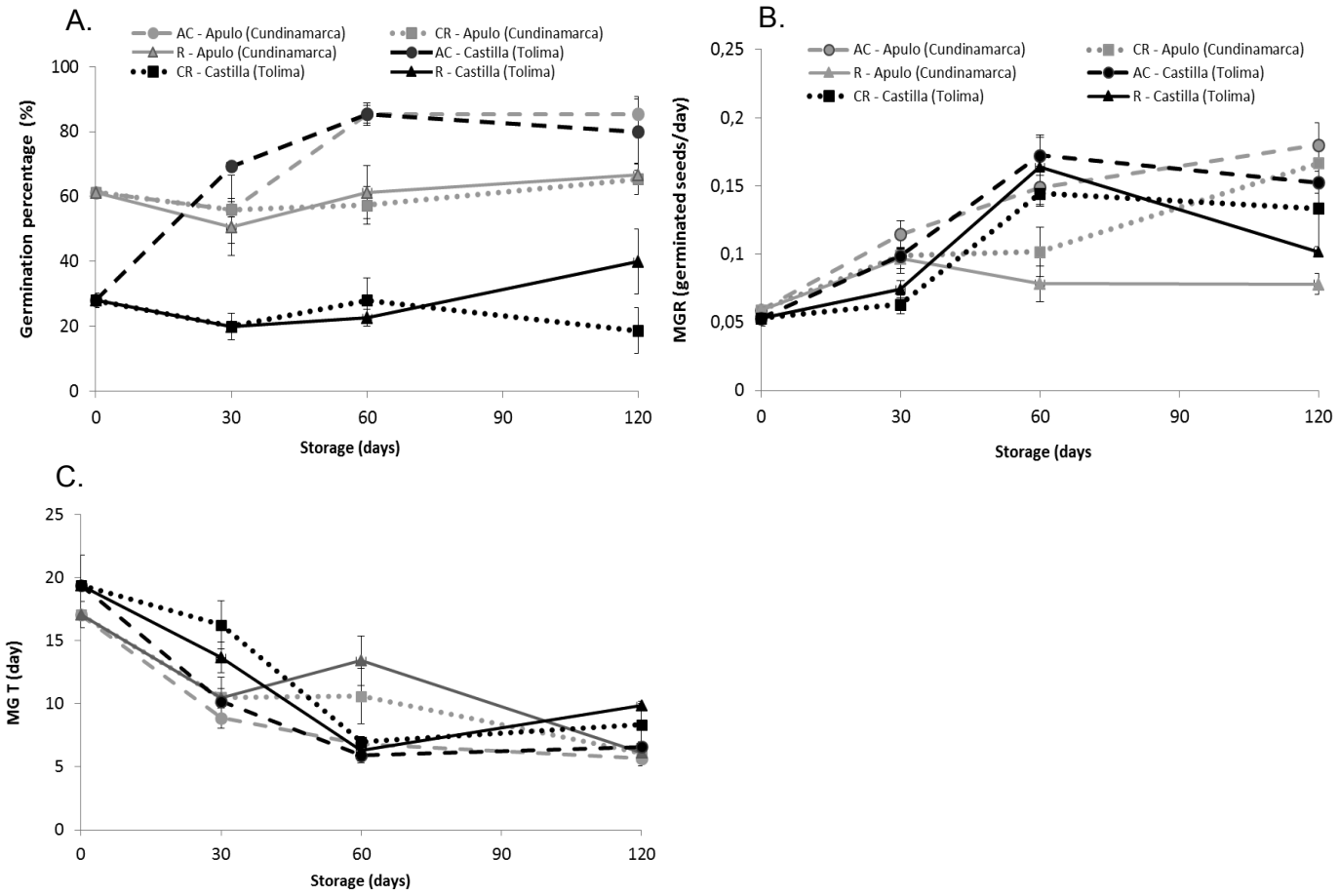

Fig. 1: Germination percentage GP (A), mean germination rate MGR (B), and mean germination time MGT (C) of *A. squamosa* L. seeds from Castilla-Tolima (black lines) and Apulo-Cundinamarca (gray lines) under ambient conditions AC (dashed lines), cool room CR (continuous points) and refrigeration R (solid lines). Germination incubation was at 35 °C and 60% RH in wet peat. Vertical bars indicate standard error (±), n = 4.

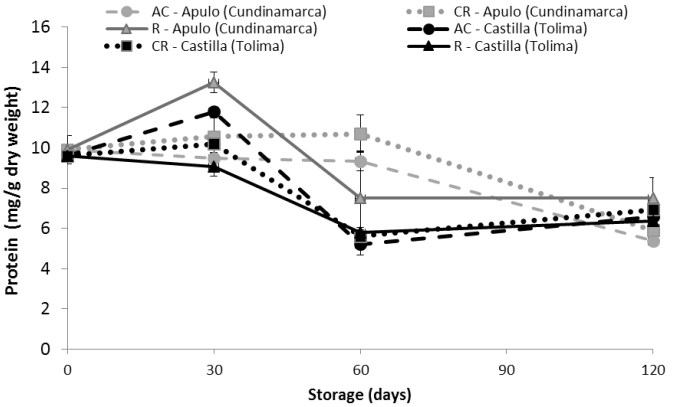

Fig. 2: Soluble protein content of *A. squamosa* L. seeds from Castilla-Tolima (black lines) and Apulo-Cundinamarca (gray lines) for the four periods of storage under ambient conditions, AC (dashed lines), cool room CR (continuous points) and refrigeration R (solid lines). Germination incubation was at 35 °C and 60% RH in wet peat. Vertical bars indicate standard error (±), n = 4.

conservation of the physiological quality of seeds is associated with the metabolism of sugars and proteins. The protein content decreased with storage time, presenting the lowest values at 120 d, for the Apulo-Cundinamarca seeds and, for seeds from Castilla-Tolima, the lowest values between 60 and 120 days (Fig. 2). This reduction in the protein content may be due to several factors that are within the increased rate of protein loss in cotyledons and embryonic axes, the damage to the protein synthesizing system, and the synthesis or activation of large quantities of proteolytic enzymes during the deterioration of seeds (BEWLEY and BLACK, 1994). It has been shown that the seeds of *Vigna radiata* and *Cicer arietinum,* under natural or artificial aging, increased protease activity (AGARWAL and KHARLUKHI, 1987); likewise, in *Shorea robusta* seeds, increases in protease activity were recorded in this embryonic axis and cotyledons (KRISHNA CHAITANYA et al., 2000). The massive loss of cell protein was also observed during storage in seeds of rice (PRABHAKAR and MUKHERJEE, 1980), *Shorea robusta* (NAUTIYAL and PUROHIT, 1985), *Pisum sativum*, *Phaseolus aureus*, and *Citrus reticulata* (SAMSHERY and BANERJEE, 1979).

Changes in sugar content during seed storage

In the seeds without storage (Fig. 3), a higher sucrose content (7.82 mg / g) than those of glucose and fructose was observed, which had similar levels (4.11 and 4.37 mg / g, respectively). With the CR storage for 120 days, no significant variation was detected in the levels of sucrose, although the content of glucose and fructose showed a slight decrease (3.13 and 2.69 mg / g). When the seeds were stored in R conditions, significant changes in the content of sugars were proven ($P < 0{,}0001$), a 22% increase in sucrose levels (9.62 mg / g) with respect to the seeds without storage and a decrease in the content of the reducing sugars glucose (1.24 mg / g) and fructose (0.94 mg / g)

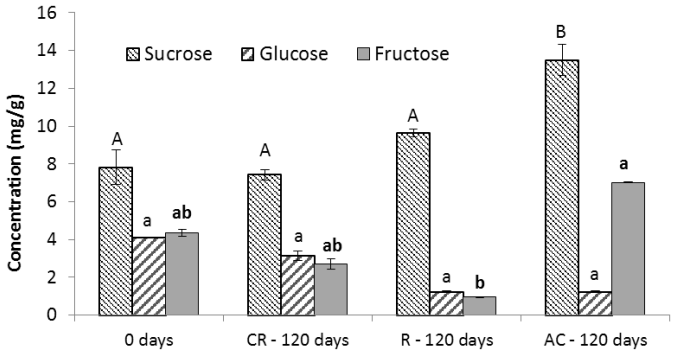

Fig. 3: Changes in the contents (mg / g dry weight) of sucrose, glucose and fructose in seeds of *A. squamosa* L. from Castilla-Tolima. without storage (0 days) and stored for 120 days under ambient conditions, AC (19 °C and RH = 60 %), cool room CR (4 °C and RH = 79 %) and refrigeration R (10 °C and RH = 79 %). n = 3. The ANOVA showed a significant effect of storage on the glucose ($P \leq 0.05$), fructose and sucrose ($P \leq 0.01$) concentrations. Means with different letters indicate significant differences according to the Tukey test ($P \leq 0.05$).

were shown. The seeds stored under ambient temperature conditions (AC) showed an increase of 72.3 % in the content of sucrose (13.49 mg / g), as compared to the sugar content of the seeds without storage. The glucose content at this time in AC (1.24 mg / g) decreased by 70 % and the fructose levels (7 mg / g) increased by 61 %, as compared to the seeds without storage.

The storage conditions for 120 d highly affected the sugar content in the *Annona squamosa* L. seeds. At ambient temperature and relative humidity (AC), the sucrose levels were much higher than those observed in the seeds stored in cool room (CR) and refrigeration (R). This could be possible due to the fact that the activities of enzymes controlling the level of sucrose, such as sucrose phosphate synthase (sucrose synthesizing enzyme from fructose and glucose) and invertase (sucrose-degrading enzyme producing fructose and glucose) were decreased at low temperatures and moisture (IYARE and EKWUKANA, 1992), which would explain the differences in the levels of sucrose. In CR at 4 °C, there was no change in the levels of reducing sugars; however, in refrigeration at 10 °C and storage at ambient condition, reductions in the glucose levels accompanied by increases in the level of sucrose were much higher than those in the seeds stored at CR. This might be because the reducing sugars were used as a substrate for the formation of sucrose (HUBBARD et al., 1991) for respiration and metabolism for other processes that consume energy (KOCH, 2004); however, when the seeds were stored at low temperatures, the levels of reducing sugars were constant (MEDLICOTT et al., 1986), as was observed in the seeds stored in cool room (CR).

In this study, the glucose and fructose contents in the seeds decreased during the refrigerated storage (R), but there was a considerable increase in the levels of sucrose (23 %), as compared to the non-stored seeds. In contrast to the AC storage, the glucose levels decreased and there was a large increase in sucrose, suggesting that, in this condition, the metabolism of both the respiration and action of sucrose phosphate synthetase functioned normally (TURHAN and ERGIN, 2012). These variations in the content of sugars, such as sucrose, might also be associated with increases in solubilization reserves, as reported during the physiological maturity in other seeds with an orthodox behavior (NKANG, 2002).

The influence of storage temperature on the metabolism of sugars in seeds has also been recorded for other species. In *Caesalpinia echinata,* GARCIA et al. (2004) found that glucose and fructose values were constant in seeds stored in paper bags for 18 months in a cool room (6 ± 1 °C and RH = 85 % ± 5 %), with a considerably decreasing temperature (25 ± 10 °C and RH = 80 % ± 15 %). At low temperatures, the sucrose content remained stable, which was also recorded for corn seeds (BERNAL-LUGO and LEOPOLD, 1998).

The results of this study contribute to the understanding of germination in *Annona squamosa* L. seeds through the carbohydrate metabolism in seeds during storage. The best responses in the PG germination variables MGR and MGT for both locations were seen under the ambient conditions, AC, at 60 and 120 days; a condition in which the levels of sucrose and fructose increased (at 120 days). In contrast, the lowest PG and MGR and the highest MGT were detected in the non-stored seeds (0 days) and seeds stored in CR and R, where the possible limitations for metabolism by the responsible enzymes were associated with lower sucrose contents (TURHAN and ERGIN, 2012). The differences in the soluble sugars and germination capacity of *A. squamosa* L. seeds suggest that seed longevity is related to seed respiration rates, which may be different depending on storage conditions as indicated by GARCIA et al. (2004) for *Caesalpinia echinata* LAM seeds. The data indicate that the storage in cool room (4 °C) and in refrigeration (10 °C) reduced the seed quality during storage, expressed as a decrease in germination rate and, subsequently, a resulting loss of germinability; this could be due to changes in the content of soluble carbohydrates. At 4 ºC, the sucrose levels decreased or remained stable with respect to the seeds without storage ($P < 0.05$), resulting in limited availability of respiratory substrates for germination. Another possibility is that, with the limited contents of disaccharides, the protective effect of sugars on the structural integrity of membranes and the ability of seed cells to maintain a vitrified state in the cytosol were decreased (BERNAL-LUGO and LEOPOLD, 1998). This indicates that adequate levels of sucrose are required to maintain the viability of seeds and ensure germination during storage of *A. squamosa* L. seeds. Sucrose and other forms of non-reducing sugars contribute to the structural stability of organelles, membranes, enzymes, and other macromolecules (OBENDORF, 1997; PETERBAUER and RICHTER, 2001). In particular, sucrose is exceptionally effective at protecting cell membranes in systems exposed to desiccation; it is one of the best sugars for vitrification process in plant cells (BERNAL-LUGO and LEOPOLD, 1998) because it protects the structure and function of phospholipids during cell drying (LEPRINCE and BUITINK, 2010).

Conclusion

The period and conditions (temperature and RH) of storage directly influenced overcoming the dormancy that was naturally present in the *A. squamosa* L seeds and, therefore, the physiological quality of the seeds. The best germination responses of the variables PG, VMG and TMG were present in the ambient storage conditions at 60 and 120 d, when the sucrose and fructose levels increased up to 120 d. In contrast, the PG and VMG were lower and the TMG was higher in the seeds without storage (0 d) and stored in CF and R, where the lower levels of sucrose were detected due to possible limitations for the metabolism of corresponding enzymes. Seed longevity could be related to seed respiration rates that might be different at different storage conditions. Adequate levels of sucrose were required for maintaining the seed viability and germination of *A. squamosa* during storage.

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