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Cryopreservation of achenes of caju-de-árvore-do-cerrado (*Anacardium othonianum* Rizz)

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The aim of the present study was to determine the water content limit for the cryopreservation of caju-de-árvore-do-Cerrado (*Anacardium othonianum* Rizz) achenes and assess the effect of the thawing method on their performance. Achenes were dried in silica gel until water contents of 14, 12, 8 and 4% w.b. (wet basis) were reached and then were stored in cryogenic cylinders in direct contact with liquid nitrogen (-196°C) for 20 days; then, the achenes were subjected to slow, fast and microwave thawing. After thawing, the achenes underwent physiological evaluation and vigor tests. The water content limit for the cryopreservation of *A. othonianum* Rizz achenes was determined to be between 14 and 12% w.b. Water contents of less than 12% reduced the physiological quality and vigor of the achenes. The thawing method used did not affect the viability of the achenes for most features assessed; however, when thawed in a microwave, the achenes exhibited poorer performance, especially those that had 8% water content.

Key words: Preservation, desiccation, cryogeny, liquid nitrogen.

INTRODUCTION

The Cerrado (savannah) region is the second largest Brazilian biome, originally covering 21% of the country's territory. Intense pressure from livestock and extractive activities in the Cerrado has endangered many ecologically, socially and economically important species. Approximately, half of the original 2 million km^2 of the Cerrado has been transformed into cultivated pastures and annual crops and for other uses (Costa, 2009; Klink and Machado, 2005). The species locally known as cajú-de-árvore-do-cerrado (*Anacardium othonianum* Rizz.) is a typical species that is well known and much appreciated in the Cerrado region of the Brazilian Central Plateau. This tree species occurs throughout the Federal district and in the state of Goiás (GO) and is 3-4 m in height with a crown diameter of 3 to 4 m; propagation occurs predominantly through seeds. The pseudofruits of this tree are 2 to 4 cm in length and 2 to 3 cm in diameter and weigh between 5 to 12 g. The color of the ripe pseudofruit peel varies from yellowish to reddish. The plant is consumed in the form of *in natura* pulp or juice, liquor or sweets. The nut also constitutes an alternative food source and can be consumed when toasted (Vieira et al., 2006).

Brazilian biomes are under severe environmental and anthropic pressure; the *in situ* conservation of native species in their natural habitat through the creation of protected areas is the predominant approach used for biodiversity protection and germplasm conservation (Pilatti et al., 2011). Conversely, the storage of seeds represents the simplest and most feasible and cost-

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Abbreviations: GRI, Germination rate index; **ERI,** emergence rate index; **SBD,** stem base diameter.

effective way for preserving *ex situ* plant genetic variability; this is being achieved through the establishment of germplasm banks (Van Slageren, 2003). *Ex situ* seed conservation provides an additional protection against the risks inherent in field conservation, for example, losses due to climatic changes or diseases and pest attacks (Pilatti et al., 2011). Nevertheless, the deterioration and loss of physiological quality of seeds in germplasm banks may compromise long-term seed storage (Walters et al., 2004). Success in the preservation of the seed physiological quality is affected by factors including water content, relative humidity and air temperature (Carvalho and Nakagawa, 2000). Environments with lower relative humidity and temperature have been shown to be more suitable for preserving orthodox seeds, preserving their germinative capacity and vigor (Marcos Filho, 2005).

Strategies of *ex situ* conservation involving biotechnological approaches for the conservation of seeds, tissues, pollen and DNA in germplasm banks should be fully explored in view of global climate change (Ahuja, 2011). A biotechnological approach, cryopreservation consists of the storage of biological material at ultra-low temperatures, usually at liquid-nitrogen temperature (-196°C). This technique is an efficient method for the long-term storage of genetic resources because most biological activity ceases at extremely low temperatures (below -150°C), consequently preventing deterioration. Cryopreservation can provide optimum conditions for germplasm banks (Engelmann, 2004; Hirano et al., 2006; Walters et al., 2004). Therefore, cryopreservation is an increasingly important technique for the conservation of endangered species, especially for the germplasm of tropical forest plants, for which cryopreservation protocols must be rapidly established. The acquisition and conservation of genetic resources from tropical forests is difficult and requires strategic planning (Muthusamy et al., 2005). Although the use of cryopreservation remains limited, the number of examples of large-scale cryopreservation of various types of material is increasing, including seeds with orthodox behavior and intermediate storage requirements, dormant buds, pollen and biotechnology products (Engelmann, 2004).

The success of cryopreserving desiccation-tolerant seeds depends on two major steps: (1) dehydration of the specimen in an environment that ensures that the water content is below the high-moisture freezing limit; and (2) transfer to and maintenance at a temperature below zero, which can be optimized with respect to the humidity of the seed lot and the species (Pritchard, 2007). Several protocols have been developed for the cryopreservation of many plant species, including crop plants, grasses, ornamental plants, tropical and temperate fruits, legumes and oilseeds (Santos, 2001). In this regard, Walters et al. (2004) predicted that the viability of lettuce seeds (*Lactuca sativa* L.), when stored in direct contact with liquid nitrogen can be maintained for about 3400 years. However, the development of cryopreservation protocols for genetic material obtained from native Brazilian plants remains limited, and most of the efforts are directed at preserving economically important species (Pilatti et al., 2011).

Water content, chemical composition and freezing and thawing rates are limiting factors for the maintenance of physiological quality and metabolic integrity of seeds after the cryopreservation process (Cavalcante-Mata, 2004). However, among these factors, the water content of seeds at the time of immersion in liquid nitrogen should be considered the most critical factor in cryopreservation. The water content should be reduced to a level that prevents the formation of intra-cellular ice crystals during the ultra-fast freezing process (promoted by the immersion in liquid nitrogen), thereby preserving the integrity of the seed tissues (Lambardi et al., 2004). If the water content is too low, the seeds might lose plasticity, causing fissures in their physical structure and the rupture of cell structures (Cavalcanti-Mata, 2004).

Therefore, the aims of the present study were to determine the water content limit required for the cryopreservation of caju-de-árvore-do-Cerrado (*Anacardium othonianum* Rizz) achenes and to assess the effect of the thawing method on their subsequent performance.

MATERIALS AND METHODS

Fruits were collected at Fazenda Gameleira (Gameleira Farm) located in the municipality of Montes Claros, state of Goiás (GO) (16° 06' 20'' S - 51° 17' 11'' W, 592-m altitude), during September 2011. Fruits were collected from 10 mother trees; the experiment was conducted at the seed laboratory of the Federal Institute of Goiás (Instituto Federal Goiano - IF Goiano) - Rio Verde Campus, GO. The achenes were extracted manually from the fruits, subjected to beneficiation and then treated with Vitavax-Thiram® fungicide [active ingredient (carboxin + thiram): 200 + 200 g/L] at a concentration of 300 mL of fungicide diluted in 500 mL of water to 100 kg of achenes.

The initial water content of the achenes was determined in three sub-samples using the oven-drying method at 105±3°C for 24 h according to the Guidelines for Seed Analysis [Regras para Análise de Sementes] (Brasil, 2009), and the results were expressed as the percentage on a wet basis. The achenes were then dried until they reached the water contents established for determining the moisture content limit for cryopreservation (14, 12, 8 and 4% w.b.). The samples were dried by arranging them evenly in trays covered with blue silica gel and were weighed every hour until the weights corresponding to the desired water contents were achieved. The samples were weighed using a precision electronic scale (0.001 g). The water loss of the achenes was determined according to the following equation:

$$wf = wi.\left(\frac{100 - WCi}{100 - WCf}\right)$$

Where, Wf, final weight of the sample (g); Wi, initial weight of the sample (g); WCi, initial water content of the achenes (% w.b.); and WCf, desired water content of the achenes (% w.b.).

The achenes were then wrapped in aluminum foil, placed inside

aluminum canisters, sorted by water content and stored in cryogenic cylinders that were placed in direct contact with liquid nitrogen at -196°C for 20 days. After the cryostorage period, the achenes were thawed using the following methods: slow thawing at room temperature of 25°C for 2 h, fast thawing in a water-bath at 37°C for 5 min and microwave thawing at 150 W power for 2 min. After thawing, the achenes were rinsed with distilled water and placed on paper towels to eliminate excess water. After thawing, the physiological quality and vigor of the achenes was assessed based on the following parameters: percentage of germination, germination rate index (GRI), percentage of emergence, emergence rate index (ERI), accelerated aging (%) and electrical conductivity. The ability of the plant structures to regenerate after cryopreservation and thawing was assessed based on the following parameters: stem base diameter (SBD), length of the aerial parts and root and dry mass of the leaf, stem and root.

Germination tests

In the germination test, three replicates of 25 achenes were used; these were arranged on germitest paper, which was rolled and moistened with distilled water in an amount equivalent to 2.5 times the weight of the dry substrate. The rolls were maintained in Mangelsdorf-type germination chambers at 25°C. Assessments were performed daily for 30 days to determine the rate of germination speed (GSI) according to the equation proposed by Maguire (1962), considering that the seeds had germinated protrusion radiular 0.5 cm. In determining the germination percentage, only normal seedlings (in which all essential structures were developed, without any damage) after 30 days of evaluations were considered.

Accelerated aging test

In the accelerated aging test, three replicates of 25 achenes were used; the method adopted was that proposed by Valentini and Piña-Rodrigues (1995). The test was conducted in germination boxes (gerbox) with aluminum mesh inside, in which the achenes were evenly arranged. To each box, 40 mL of distilled water was added, and 25 achenes were arranged on the mesh. The capped gerboxes were maintained in a temperature-regulated germination chamber at 40°C for 72 h. After aging, the achenes were subjected to the germination test as previously described, and the results were expressed as a percentage of normal seedlings.

Electrical conductivity test

The electrical conductivity test was conducted on three sub-samples of 10 achenes. The samples were weighed using a precision electronic scale (0.001 g), placed in plastic cups with 75 mL of deionized water and maintained in a germination chamber at 25°C for 24 h. After this time, the electric conductivity of the solution was measured using a Technal digital conductivity meter (model TEC-4 MP). The results were expressed as $\mu S.cm^{-1}.g^{-1}$.

Emergence tests

The emergence test was conducted in a greenhouse at a mean temperature of 24.9±4°C and a relative humidity of 76±14.5% during the evaluation period. Three replicates of 25 achenes were used, which were sown in sand at a 3-cm depth. The substrate was moistened up to 70% of its field capacity and irrigated by spraying three times daily. Daily counts were performed, recording the emergence of seedlings above a 1 cm height; from this, the ERI of the seedlings was determined according to Maguire (1962). Thirty (30) days after sowing, the percentage of emergence corresponded to the percentage of normal seedlings (with all essential structures formed and showing no damage).

For the assessment of the stem base diameter (SBD) and the length and dry mass of seedlings, the normal seedlings from the emergence test were measured 30 days after sowing. The SBD was measured using a digital caliper. A ruler was used to measure the length of the aerial parts and root of normal seedlings. To determine dry mass, the leaves, stem and roots of the seedlingsin each replicate were sorted, placed in paper bags and dried in an air-circulating oven-dryer at 65°C for 48 h. After drying, the samples were weighed using a precision electronic scale (0.001 g).

Statistical design

The experiment consisted of a completely randomized design in a 4x3 factorial arrangement with four water contents (4, 8, 12 and 14% w.b.) and three thawing methods (slow, fast and microwaved) with three replicates. The analysis of variance (ANOVA) and comparison of means using the Tukey test at 5% of significance were performed using Sisvar software (Ferreira, 2003). Simple linear regressions and polynomial regressions were used to compare the water content of the achenes. Data expressed as percentages were transformed into arcsin $\sqrt{x}/100$, in which x refers to percentage, and numerical data were transformed into $\sqrt{x}+0.5$.

RESULTS AND DISCUSSION

Water content limit for cryopreservation

The highest values for germination (Figure 1A) and GRI (Figure 1B) were obtained for achenes with water contents of 12 and 14%, respectively; these values decreased with a decrease in water content. These results demonstrate that reducing the water content to below 12% w.b. negatively affects the vigor and seedling development during cryopreservation of achenes. A similar result was found for seeds of cinchona (*Strychnos pseudoquina* A. St. Hil) in the cryopreservation of seeds with moisture content below 7%, caused a reduction in the percentage of germination, suggesting a negative response to freezing for seeds with this water content (Silva et al., 2012). Conversely, the freezing of Brazilwood (*Caesalpinia echinata* Lam.) seeds with high water content (21.9%) resulted in a significant reduction in germination capacity, indicating that such seeds are freeze tolerant as long as their water content is not high (Hellmann et al., 2006). Seeds of purple tabebuia [*Tabebuia impetiginosa* (Mart. ex DC.) Standl.] cryopreserved with moisture levels of 12.5, 8.4 and 4.2% maintained their physiological performance in liquid nitrogen. However, the higher water content (18.3%) caused decreased germination, indicating a detrimental effect on seed preservation (Martins et al., 2009). The germination of common ash (*Fraxinus excelsior* L.) seeds when stored in liquid nitrogen within a water content range of 5.66 to 19.35% w.b. varied between 6% and 74%, depending on the water content (Chmielarz, 2009). Thus, the water content suitable for cryopreservation and

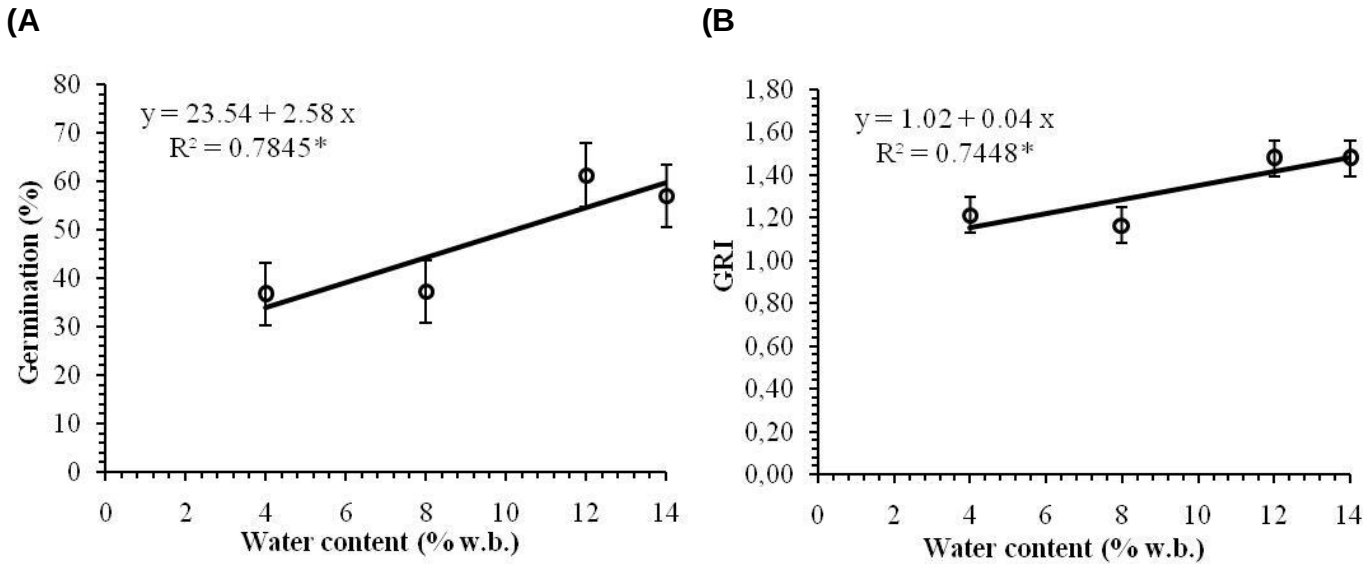

Figure 1. Percentage of germination (A) and germination rate index - GRI (B) for achenes of *A. othonianum* Rizz with different water contents and subjected to cryopreservation in liquid nitrogen at -196°C for 20 days. *Significant at 5% probability according to the F test. Bars: standard error of the mean.

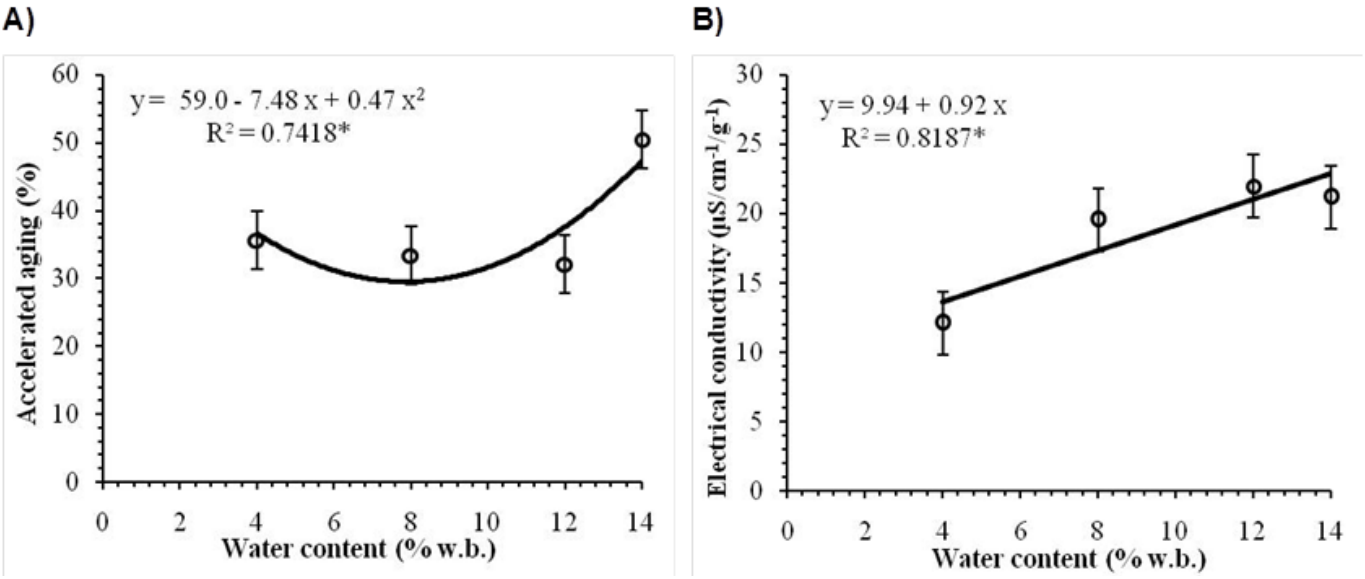

Figure 2. Accelerated aging (A) and electrical conductivity (B) for *A. othonianum* Rizz achenes with various water contents subjected to cryopreservation in liquid nitrogen at -196°C for 20 days. *Significant at 5% probability according to the F test. Bars: standard error of the mean.

maintaining the quality of seeds and propagules of dispersion varies according to the species tested and the freezing method, which requires investigation of moisture content limit for each species.

With regard to accelerated aging, achenes with a water content of 14% exhibited the highest germination value (Figure 2A); however, as the water content of *A. othonianum* Rizz achenes decreased, a decrease in the percentage of germination after aging was observed. These data confirm the negative effect of reducing the water content in the vigor and force of achenes demonstrated by germination test. While studying the cryopreservation of *Citrus* seeds (*C. sinensis, C. paradisi*and *C. reticulata*), Graiver et al. (2011) observed

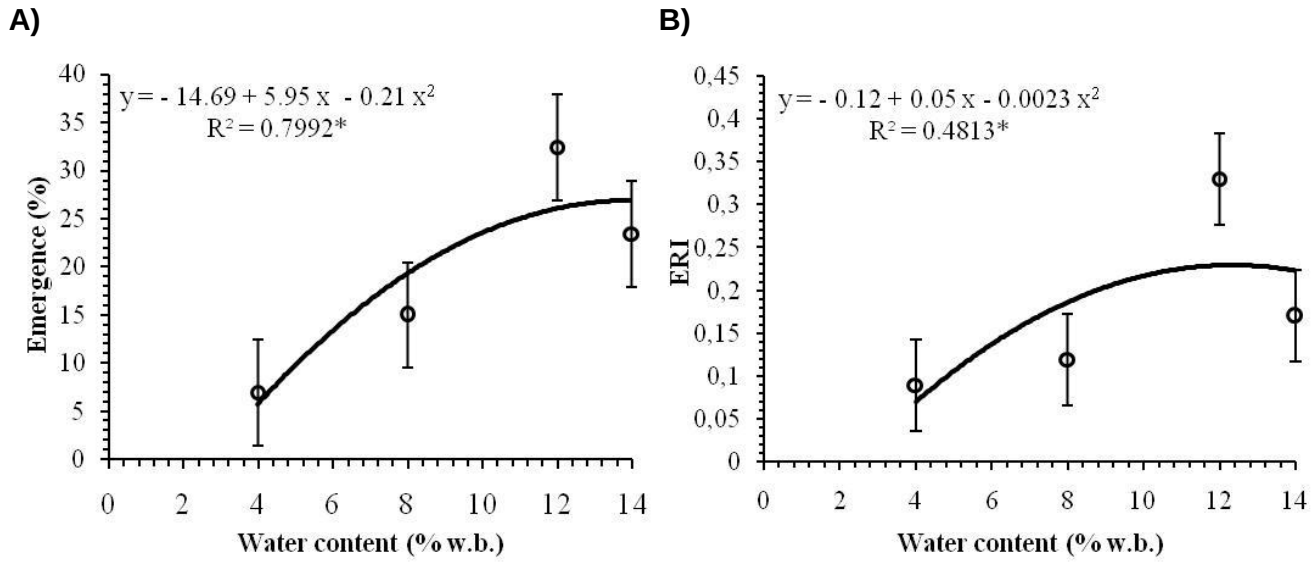

Figure 3. Emergence (A) and emergence rate index - ERI (B) for *A. othonianum* Rizz achenes with various water contents subjected to cryopreservation in liquid nitrogen at -196°C for 20 days. *Significant at 5% probability according to the F test. Bars: standard error of the mean.

a decrease in the percentage of germination in all tested seeds when desiccation was performed to an equilibrium relative humidity of less than 75%, with the lowest values observed at 11%.

The highest values for electrical conductivity (Figure 2B) were found for achenes with 12% w.b., and this decreased as their water content decreased. Therefore, the lowest values of ion leaching in the electrical conductivity test were obtained for achenes with low water contents, demonstrating that achenes with high water content exhibit disorganized membrane systems when subjected to cryopreservation and thawing by various methods.

The pattern found for the electrical conductivity contrasts with that observed for the physiological quality, since in this case showed higher membrane damage in seeds with high water contents. However, the existence of membrane damage did not cause significant effects on seedling development, and thereby, it is believed that repair mechanisms may act during the reactivation of metabolism, protecting the membrane system and preventing loss of viability achenes. For sunflower seeds stored at sub-zero temperatures, a loss of membrane structure was observed for seeds dried in silica until lower water contents were reached, causing a greater leaching of seed exudates (Jose et al., 2010). The highest values for emergence (Figure 3A) and ERI (Figure 3B) were obtained for achenes cryopreserved with 12% water content, and a decrease in the values of these parameters occurred in response to decreased water content. Silva et al. (2011) observed a similar result for Barbados nut (*Jatropha curcas* L.) seeds, in which desiccation at a 6.4% water content associated with longer periods of liquid nitrogen exposure caused abnormalities in seedlings, damage to leaf cells and tissues and a negative effect on germination.

For common ash (*F. excelsior* L.) seeds with water contents of 7.40 to 19.35% w.b., the emergence of seedlings after freezing in liquid nitrogen ranged from 49 to 65%, and this result did not differ from thatfor non-cryostored seeds (Chmielarz, 2009). According to Hor et al. (2005), seeds of four different species of the genus *Citrus* (*Citrus aurantifolia, Citrus grandis, Citrus madurensis* and *Citrus reticulata*) after exposure to liquid nitrogen exhibited no survival at high water contents, and a dramatic increase in the percentage of emergence was found by decreasing the water content up to 75 to 80%; subsequently, a low rate of decrease was observed as the desiccation was decreased to 75 to 80%.

For the remaining parameters (Figure 4A, B, C, D and E), except for root dry mass (Figure 4F), the best results were achieved with achenes cryopreserved with a 12% water content, and decreases occurred in response to decreases in their water content; the lowest values were achieved at a 4% water content. The highest values for root dry mass were obtained for achenes with a 14% water content, and the lowest values were achieved for achenes with an 8% water content.

In this sense, achenes cryopreserved with higher water contents tested produced more vigorous seedlings, confirming the negative effect of cryopreservation of achenes with low water content in the development and seedling vigor, so it is recommended the adoption of the levels 14 and 12% wb for the cryopreservation of

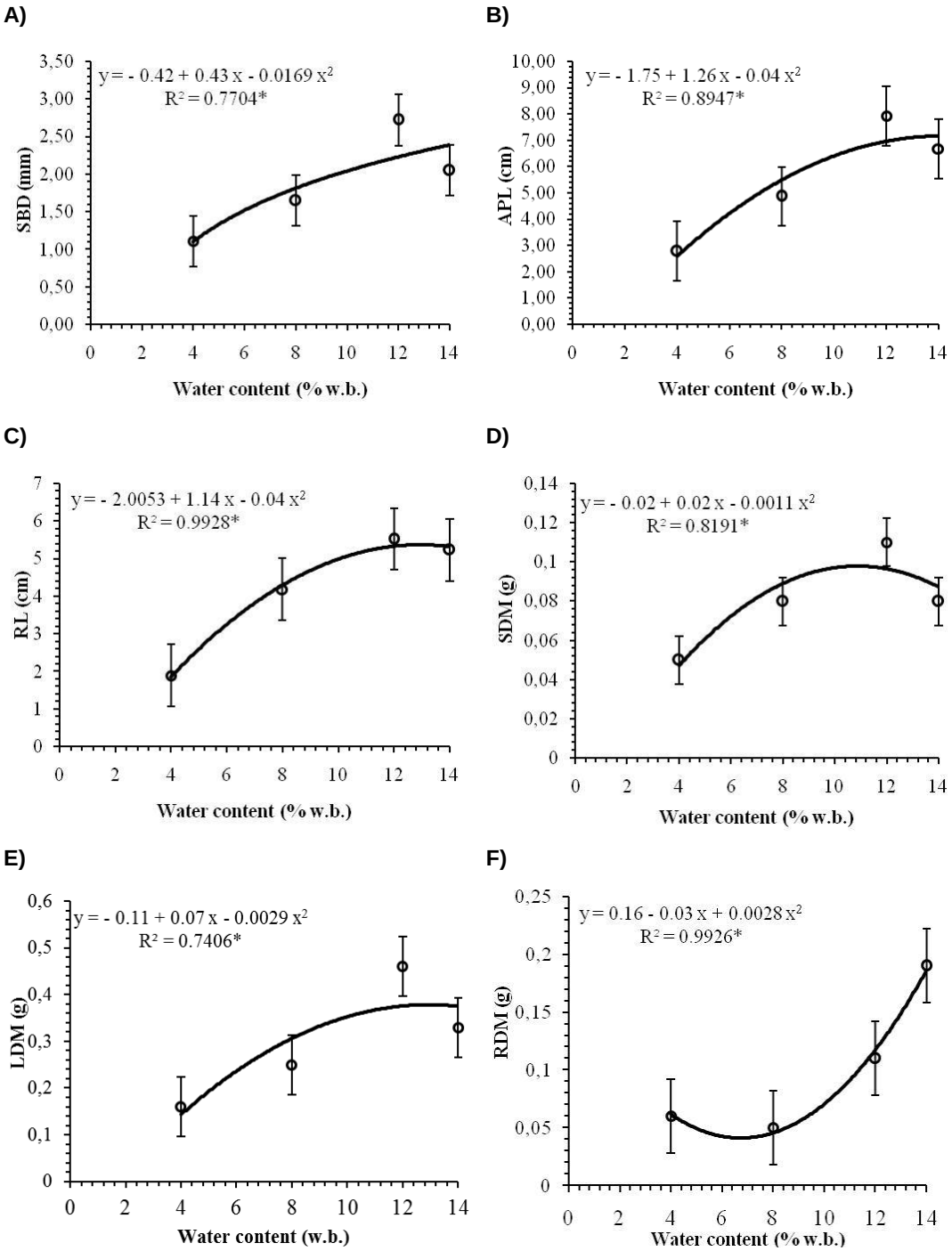

Figure 4. Stem base diameter - SBD (A); aerial parts' length - APL (B); root length - RL (C); stem dry mass - SDM (D); leaf dry mass - LDM (E); root dry mass - RDM (F) for *A. othonianum* Rizz seedlings obtained from achenes with various water contents subjected to cryopreservation in liquid nitrogen at -196°C for 20 days. *Significant at 5% probability according to the F test. Bars: standard error of the mean.

achenes of the species in question. On the other hand, for seeds of Jatropha (*Jatropha curcas* L.) a cryogenic storage, within the range of water content of 4 to 8% but does not affect seed germination, providing higher values of dry mass and length seedlings (Goldfarb et al. 2008). The vigor of Pink Trumpet tree [*Tabebuia heptaphylla* (Vell.) Toledo] seedlings from cryopreserved seeds was not affected by storage in liquid nitrogen Higa et al.

Table 1. Percentage of germination, germination rate index (GRI) and accelerated aging for *A. othonianum* Rizz achenes cryopreserved with various water contents and subjected to different thawing methods.

Thawing method	Water content (% w.b.)					
	14	12	8	4	Linear	Square
Germination (%)						
Slow	46.05[az]	65.51a	50.84a	32.09a	ns	ns
Fast	54.82a	59.74a	55.00a	27.09a	*1	ns
Microwave	70.31a	58.77a	6.14b	50.79a	ns	*2
GRI						
Slow	1.37a	1.51a	1.30a	1.13a	ns	ns
Fast	1.45a	1.50a	1.42a	1.03a	*3	ns
Microwave	1.60a	1.41a	0.77b	1.46a	ns	*4
Accelerated aging (%)						
Slow	45.00a	46.92a	49.03a[z]	36.63a	ns	ns
Fast	59.24a	23.55a	50.84a	32.35a	ns	ns
Microwave	46.92a	25.50a	0.00b	35.59a	ns	*5

[z] Means followed by the same letter in the columns did not differ significantly between each other at 5% probability according to Tukey's test. In the rows, * represents significance at 5% probability. ns = Non-significant. [1]$Y = 22.66 - 2.78\ X$. $R^2 = 0.6900$; [2]$Y = 137.08 - 29.61\ X + 1.81\ X^2$. $R^2 = 0.8214$; [3]$Y = 2.21 - -0.31\ X$. $R^2 = 0.6900$; [4]$Y = 4.62 - 2.86\ X + 0.52\ X^2$. $R^2 = 0.6574$; [5]$Y = 114.64 - 79.11\ X + 14.75\ X^2$. $R^2 = 0.8179$.

2011).

According to Lima et al. (2012), *A. othonianum* Rizz seeds with water contents of 20 and 16.8%, which were stored in germination chamber (BOD) bottles at 18°C, maintained their viability for up to 12 months. However, seeds stored at 29.5% humidity (humidity at harvest) lost their viability during all of the storage periods assessed.

Thawing methods

Interactions were observed between the percentage of germination, GRI and accelerated aging. The achenes cryopreserved at 8% water content and thawed in a microwave exhibited the lowest values of these variables (Table 1).

In onion seeds, thawing at room temperature led to the greatest reduction in physiological quality; the microwave thawing method was the most suitable because it maintained the physiological quality and the starch, protein and soluble sugar contents of the seeds (Molina et al., 2006). *S. pseudoquina* A. St. Hil. seeds cryopreserved with a 6% water content showed no difference between the slow and fast thawing methods (Silva et al., 2012). Pink Trumpet tree seeds [*T. heptaphylla* (Vell.) Toledo] with 7.5 and 8.4% water contents maintained their germination capacity after storage in liquid nitrogen. The storage period (15 min to 26 weeks) and the type of thawing (slow and fast) did not affect germination, which varied between 54 and 67% (Higa et al., 2011).

According to Taiz and Zeiger (2009), fully hydrated plant cells can retain viability if quickly cooled to prevent the formation and growth of ice crystals, which could otherwise destroy intracellular structures. Rapid heating of frozen tissues is also necessary to prevent the transformation of small ice crystals into larger crystals that can cause damage or prevent the loss of water vapor by sublimation because both process occur at intermediate temperatures (-100 to -10°C).

Conclusions

The water content limit for the cryopreservation of *Anacardium othonianum* Rizz achenes is between 14 and 12% w.b.; water contents of less than 12% reduced the physiological quality and vigor of achenes. The thawing method did not affect the viability of achenes for most of the evaluated parameters; however, achenes with 8% water content and thawed in a microwave exhibited poorer results.

REFERENCES

Ahuja MR (2011). Strategies for conservation of germplasm in endemic redwoods in the face of climate change: a review. Plant Genet. Resour. 9(3): 411-422.

Brasil (2009). Ministério da Agricultura, Pecuária e Abastecimento. Regras para análise de sementes. Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. Brasília.

Carvalho NM and Nakagawa J (2000). Sementes: Ciência, tecnologia e produção. Jaboticabal: FUNEP.

Cavalcanti-Mata MERM, Rocha MS, Duarte MEM (2004). Teor de água

limite para crioconservação de sementes de algodão arbóreo variedade 6M. Rev. Bras. Prod. Agroind. 6(2): 179-189.
Chmielarz P (2009). Cryopreservation of dormant European ash (*Fraxinus excelsior*) orthodox seeds.Tree Physiol. 29:1279-1285.
Costa CJ (2009). Armazenamento e conservação de sementes de espécies do Cerrado. Embrapa Cerrados, Planaltina - DF.
Engelmann F (2004). Plant cryopreservation: progress and prospects. In Vitro Cell. Dev. Biol.Plant. 40: 427-433.
Ferreira DF (2003). Programa de análises estatísticas (Statistical Analysis Software) e planejamento de experimentos - SISVAR 5.0 (Build 67). Lavras: UFLA.
Goldfarb M, Queiroga VP, Martins MED, Severino LS (2008). Teor de água limite para crioconservação das sementes de pinhão manso (*Jatropha curcas* L.). Rev. Bras. Prod. Agroind. 10(2): 121-129.
Graiver N, Califano A, Zaritzky N (2011). Partial dehydration and cryopreservation of Citrus seeds. J. Sci. Food Agric. 91: 2544-2550.
Hellmann ME, Mello JIO, Figueiredo-Ribeiro RCL, Barbedo, CJ (2006). Tolerância ao congelamento de sementes de pau-brasil (*Caesalpinia echinata* Lam.) influenciada pelo teor de água inicial. Rev. Bras. Bot. 29(1): 93-101.
Higa TC, Paulilo MTS, Benson EE, Pedrotti E, Viana AM (2011). Developing seed cryobank strategies for *Tabebuia heptaphylla* (Bignoniaceae), a hardwood tree of the Brazilian South Atlantic Forest. Cryo Lett. 32(4): 329-38.
Hirano T, Ishikawa K, Mii M (2006). Advances in orchid cryopreservation. In: Teixeira da Silva JA (ed) Floriculture ornamental and plant biotechnology: advances and topical issues, vol 2, 1st edn. Global Science Book, London.
Hor YL, Kim YJ, Ugap A, Chabrillange N, Sinniah UR, Engelmann F, Dussert S (2005). Optimal Hydration Status for Cryopreservation of Intermediate Oily Seeds: Citrus as a Case Study. Ann. Bot. 95: 1153-1161.
Jose SCBR, Salomão AN, Costa TSA, Silva JTTT, Curi CCS (2010). Armazenamento de sementes de girassol em temperaturas subzero: aspectos fisiológicos e bioquímicos. Rev. Bra. Sem. 32(4): 29 - 38.
Klink CA and Machado RB (2005). Conservation of the Brazilian Cerrado. Conserv. Biol. 19(3): 707-713.
Lambardi M, De Carlo A, Biricolti S, Puglia AM, Lombardo G, Siragusa M, De Pasquale F (2004). Zigotic and nucellar embryo survival following dehydration/cryopreservation of Citrus intact seeds. Cryo Lett. 25(2): 81-90.
Lima RE, Rubio Neto A, Silva FG, Sales JF, Santana JG, Correa RM (2012). Effect of water contents and storage on Caju-de-árvore do cerrado seed germination. Global Sci. Tech. 5: 78-82.
Maguire JD (1962). Speed of germination aid in selection and evaluation for seedling emergence and vigor. Crop Science. 2: 176-177.
Marcos Filho J (2005). Deterioração de sementes. In: MARCOS FILHO, J. (Ed.). Fisiologia de sementes de plantas cultivadas. Piracicaba: FEALQ.
Martins L, Lago AA, Andrade ACS, Sales WRM (2009). Conservação de sementes de ipê-roxo [*Tabebuia impetiginosa* (Mart. ex DC.) Standl.] em nitrogênio líquido. Rev. Bras. sem. 31(2): 71-76.
Molina TF, Tillmann MAA, Dode LB, Viegas J (2006). Crioconservação em sementes de cebola. Rev. Bra. Sem. 28(3): 72-81.
Muthusamy J, Staines HJ, Benson EE, Mansor M, Krishnapillay B (2005). Investigating the use of fractional replication and Taguchi techniques in cryopreservation: a case study using orthodox seeds of a tropical rainforest tree species. Biodivers. Conserv. 14: 3169-3185.
Pilatti FK, Aguiar T, Simões T, Benson EE, Viana AM (2011). In vitro and cryogenic preservation of plant biodiversity in Brazil. In Vitro Cell. Dev. Biol. Plant. 47: 82-98.
Pritchard HW (2007). Cryopreservation of desiccation-tolerant seeds. Methods Mol. Biol. 368:185-201.
Santos IRI (2001). Criopreservação de germoplasma vegetal. Biotecnologia cienc. desenvolv. 20: 60-65.
Silva RC, Camillo J, Luis ZG, Scherwinski-Pereira JE (2011). Potencial germinativo e morfoanatomia foliar de plântulas de pinhão-manso originadas de germoplasma criopreservado. Pesq. Agrop. Bras. 46(8): 836-845.
Silva VF, Sales JF, Silva FG, Campos RC, Branquinho AC, Silva VA (2012). Cryopreservation of quina seeds (*Strychnos pseudoquina* A. St. Hil). Int. Res. J. Biotechnol. 3(4): 55-60.
Taiz L and Zeiger E (2009). Fisiologia vegetal**.** 4ª ed. Porto Alegre: Artmed.
Valentini SRT, Piña-Rodrigues FCM (1995). Aplicações do teste de vigor em sementes. In: Silva, A.; Piña-Rodrigues, F.C.M.; Figliolia, M.B. Manual técnico de sementes florestais.
Van Slageren MW (2003). The millennium seed bank: building partnerchips in arid regions for the conservacion of wild species. J. Arid Environ. 54(1): 195-201.
Vieira RF, Costa TSA, Silva DB, Ferreira FR, Sano SM (2006). Frutas nativas da região Centro-Oeste. Brasília: Embrapa Recursos Genéticos e Biotecnologia.
Walters C, Wheeler L, Stanwood PC (2004). Longevity of cryogenically stored seeds. Cryobiology. 48: 229-244.