

# Establishment of post-harvest early-developmental categories for viability maintenance of *Araucaria angustifolia* seeds

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## ABSTRACT

*Araucaria angustifolia* seeds are recalcitrant, and their metabolism remains high during storage. This research aimed to describe the initiation of germination in *A. angustifolia* seeds during storage in order to standardize the assessment of physiological quality and to promote seed conservation. Seeds were collected from two populations and stored for 270 days in the natural laboratory environment and cold chamber. Seeds were classified according to four early developmental stages: I – mature seeds; II – seeds with elongation along the embryonic axis; III – beginning of root protrusion; IV – advanced germination stage, with seedling shoots. After categorization, physical and physiological quality was assessed. In freshly collected seeds, only category I was observed. At 270 days, approximately 40% of seeds were in category III in laboratory conditions, while the maintenance in a cold chamber delayed germinative metabolism. Viability tests showed that seeds in categories III and IV were more susceptible to damage caused by storage. In conclusion, the percentage of viable *A. angustifolia* seeds depends on the development stage after collection. Seeds that have reached early developmental category III should be prioritized for propagation, while those remaining in categories I and II should be longer stored with periodic assessment for reduction in physiological quality.

**Keywords:** Brazilian pine, longevity, recalcitrant seeds, seed conservation, seed development

## Introduction

*Araucaria angustifolia* (Brazilian pine) is a key species of the Brazilian Atlantic Rain Forest (Veloso *et al.* 1991; Coutinho & Dillenburg 2010), being its only native gymnosperm of economic importance (Silveira *et al.* 2008; Elbl *et al.* 2014). Its seeds are consumed by humans and are the most important food source for several wild mammals and birds during the winter (Stefenon *et al.* 2009; Reis *et al.* 2014). The seeds are recalcitrant, with a short conservation period under natural conditions, with at least a 60% viability reduction at 4 months post-harvest (Fowler *et al.* 1998; Amarante *et al.* 2007; Garcia *et al.* 2014). Given the need for the conservation of genetic resources, it is important to note that *A. angustifolia* has been classified as critically endangered by IUCN (2013).

Studies on the metabolism of recalcitrant seeds have been performed by several authors (Barbedo & Bilia 1998; Song *et al.* 2003; Berjak & Pammenter 2008; Caccere *et al.* 2013; Pammenter & Berjak 2014; Walters 2015), including studies on *ex situ* conservation by storage (Pammenter *et al.* 1994; Drew *et al.* 2000; Li & Pritchard 2009; Pasquini *et al.* 2012; Charloq *et al.* 2013; Walters *et al.* 2013; Bonjovani & Barbedo 2014; Liu *et al.* 2014). Storage of recalcitrant seeds

under desiccating conditions resulted in the initiation of subcellular damage, which may be repaired when seeds are set-out to germinate (Farrant *et al.* 1989; Tarquis & Bradford 1992). However, when a critical proportion of cells are damaged, there will be total viability loss (Farrant *et al.* 1989). Storage is possible if the conditions preclude water loss, but such hydrated storage is strictly a short-term option because it will also promote germination metabolism with an accompanying increase in respiratory metabolism, favor a microbial contamination, and cause damage if water is not supplied in appropriate amounts (Farrant *et al.* 1989; Pammenter *et al.* 1994; Barbedo & Marcos Filho 1998; Drew *et al.* 2000; Berjak & Pammenter 2013). Therefore, high humidity also promotes the deterioration process (Barbedo & Cicero 2000).

Some researchers have reported the early germination of *A. angustifolia* seeds during storage (Farrant *et al.* 1989; Garcia *et al.* 2014). Subcellular germination events, including an increase in the levels of protein synthesis and meristem cell metabolism, initiate shortly after the seeds of *A. angustifolia* are shed, and continue on during storage (Farrant *et al.* 1989).

Recalcitrant seed quality is influenced by drying after harvest, genetic potential, environmental conditions, harvest date, mechanical damage, and storage conditions

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(Demir *et al.* 2008; Ligterink *et al.* 2012), and the determination of seed quality is a critical step for conservation, cultivation, breeding and research activities (Corbineau 2012). However, studies evaluating the quality of recalcitrant seeds, in general, do not consider the fact that germination metabolism may be active, and so assess seed quality in a manner similar to that done for more orthodox seeds.

Some authors have evaluated the decline in physiological quality of *A. angustifolia* seeds during storage (Fowler *et al.* 1998; Fontes *et al.* 2001; Piriz Carrillo *et al.* 2003; Caçola *et al.* 2006; Amarante *et al.* 2007; Garcia *et al.* 2014). However, there have been no published reports on the characterization and standardization of germination metabolism after harvest, which begins immediately after physiological maturity and thus prior to quality evaluation. In view of the intraspecific variation typical of recalcitrant seeds (Li & Pritchard 2009), a standardized assessment of physiological quality is necessary in order to determine a seeds developmental stage (Shibata *et al.* 2013). Thus, the aim of this work was to standardize the assessment of physiological quality of *Araucaria angustifolia* by identifying the initiation of the germination process during storage and to categorize seeds according to early developmental stage. More specifically the present study aimed to identify what stage of early development allows for the longest storage period, thus promoting seed conservation and providing a basis for further research on physiological quality of recalcitrant species.

## Material and methods

### Plant material

Mature cones of *Araucaria angustifolia* (Bert.) O. Ktze were collected in May 2012 from two natural populations: Lot 1 from the region of São José do Cerrito (27°36' S, 50°39' W, average elevation of 918 meters); and Lot 2 from Painel (27°55' S, 50°04' W, average elevation of 1171 meters), both in the state of Santa Catarina in southern Brazil. Both populations are located in areas of secondary forest (Mixed Rain Forest), with temperate Cfb climate according to Köppen classification, and relief from flat to slightly rolling. Cones

were collected from  $15 \pm 2$  matrices/population, for a total of  $65 \pm 2$  cones/population. The collection of samples from two different populations was intended to better represent the species given the typical intraspecific diversity of recalcitrant seeds, and thus provide a stronger hypothesis test.

### Seed storage and determination of physiological quality

Seed samples were homogenized and distributed among four replicates per lot, from which fractions were withdrawn and placed in sealed, semipermeable (porosity of 0.015  $\mu\text{m}$ ), transparent plastic containers which permitted gaseous exchange yet limited water loss. The containers were then placed in two different storage conditions: the natural laboratory environment, and a cold chamber (temperature of  $10 \pm 3^\circ\text{C}$ , and relative humidity of  $45 \pm 5\%$ ), where they were kept for a period of 270 days (each storage condition containing the four replicates per lot). Reference values for storage temperature and relative humidity for the natural laboratory environment are listed in Tab. 1.

Prior to assessment of quality, seeds were categorized according to early developmental stage. Determination of early developmental stage, and tests of moisture content, viability and vigor were performed at zero, 15, 30, 45, 90, 135, 180, 225 and 270 days, for both lots and both storage conditions.

### Categorization of early developmental stages

In order to standardize the assessment of viability and vigor, 35 seeds/replicate were assessed from both lots and both storage conditions. Seeds were separated into early developmental stages by visual characterization of seeds and/or embryos, which were manually extracted using a stylus and scalpel. This analysis allowed grouping seeds into four distinct categories (Fig. 1):

- Category I: seeds with mature (but not germinated) embryos, with whitish, pinkish or greenish cotyledons (Fig. 1A-B);
- Category II: seeds with embryos showing apparent elongation along the embryonic axis, indicating the beginning of germination (Fig. 1C-D);

**Table 1.** Reference temperature and relative humidity data for the city of Lages, SC, during storage of *Araucaria angustifolia* seed lots, according to Epagri/Ciram (2014).

Storage Period (days)	Temperature ( $^\circ\text{C}$ )			Relative humidity (%)		
	Mean	Maximum	Minimum	Mean	Maximum	Minimum
15 – 30	13.3	24.8	0.9	83	98	35
45	12.4	24.3	0.4	85	98	41
90	11.7	24.0	-0.5	85	98	43
135	12.4	24.4	1.1	85	98	43
180	13.4	26.0	1.6	83	98	42
225	14.1	26.0	2.4	82	98	43
270	15.5	27.0	4.3	81	98	42

- Category III: seeds with embryos that started root protrusion, with seed coat rupture, and hypocotyl thickening; in general the cotyledons are greenish (Fig. 1E-F);
- Category IV: seeds with embryos in advanced stages of germination, with seedling shoots; cotyledons present, and the primary root being brownish (Fig. 1G-H).

In addition to visual analysis, embryo mass was determined using 10 seeds/replicate for each condition and storage period. Embryos were then extracted and categorized according to early developmental stage. Mean embryo mass was determined for each category, as was the percentage of seed mass represented by the embryo, regardless of the condition and storage period, in order to better characterize early developmental stages.

#### Seed quality analysis during storage

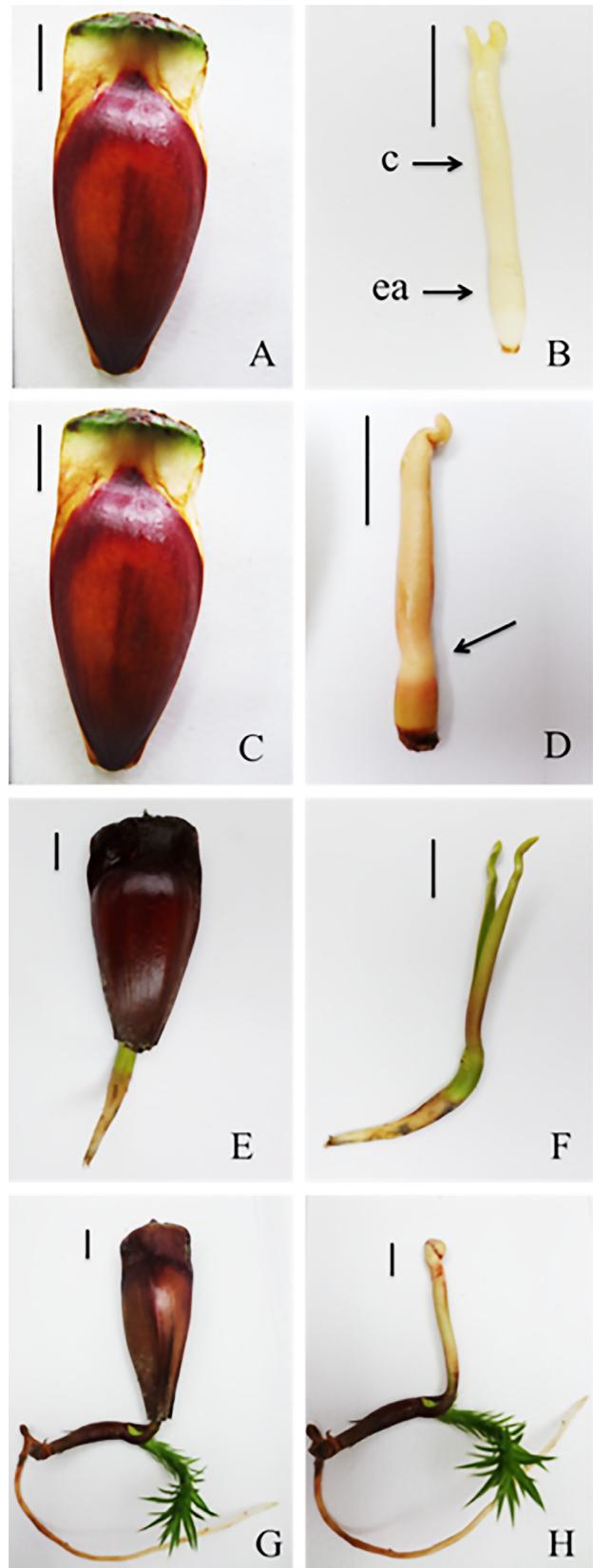
Moisture content was assessed through weight loss after oven drying at  $105^{\circ}\text{C} \pm 3^{\circ}\text{C}$  for 24 hours, using three transversely cut seeds/replicate, (Brazil 2009). For analysis of seed viability, the Rules for Seed Analysis - RAS were used (Brazil 2009), which recommend the use of the tetrazolium test instead of the germination test, because of the extensive time-period before the formation of normal seedlings.

Therefore, viability was assessed using the tetrazolium test on 25 embryos/replicate, (according to methodology of Brazil 2009, with adaptations by Oliveira *et al.* 2014), and exudate pH, according to methodology of Araldi *et al.* (2015). Both viability tests were based on the identification of viable structures associated with tissues appearance. An electrical conductivity test was performed with 10 embryos/replicate immersed in 75 ml of ultrapure water where they were kept for 12 hours at  $25 \pm 1^{\circ}\text{C}$ , and the results reported in  $\mu\text{S cm}^{-1} \text{g}^{-1}$  (Medeiros & Abreu 2007).

Seeds from all categories were used for analysis of seed quality, however, only categories I and II will be presented in the results of viability analysis (tetrazolium and exudate pH exudate), since these categories better exhibited the differences between storage conditions and had a less substantial decline in viability.

#### Experimental design and statistical analysis

The experiment was conducted using a completely randomized design in split plot, with two storage conditions (natural environment and cold chamber) and nine storage periods (0, 15, 30, 45, 90, 135, 180, 225 and 270 days). Percentage data were transformed into  $\text{arc sin } \sqrt{\%}$ . Analysis of variance, Tukey test of means at 5% probability, and regression analysis were performed using the statistical program SAS (2009). Since there were no significant differences between seed lots, viability analyses (tetrazolium and exudate pH), moisture content and electrical conductivity were presented as a function of lot average.



**Figure 1.** Appearance of *Araucaria angustifolia* seeds and embryos at early developmental stages I (A, B), II (C, D), III (E, F) and IV (G, H), observed during the storage period, showing cotyledons (c) and embryonic axes (ea). Bars indicate 1 cm.

## Results

### Early development of seeds during storage

The four early-developmental categories showed pronounced differences in embryo mass. Embryos belonging to category I averaged 0.12 g (Lot 1) and 0.14 g (Lot 2), representing 1.35% of the total seed mass (Tab. 2). There was an increase in embryo mass in category II (0.25 and 0.22 g, for Lots 1 and 2, respectively) and category III (0.87 and 0.82 g, for Lots 1 and 2, respectively). However, after root protrusion, embryo mass-gain was much higher, with it reaching close to 50% of total seed mass (Tab. 2).

**Table 2.** Embryo mass and relative percentage of total seed mass according to early developmental stage of *Araucaria angustifolia*.

	Early developmental category	Mass/embryo (g)	% relative to the seed total weight
Lot 1	I	0.12	1.35
	II	0.25	3.06
	III	0.87	10.39
	IV	2.98	48.64
CV (%)		26	30
Lot 2	I	0.14	1.35
	II	0.22	2.31
	III	0.82	11.11
	IV	3.25	47.12
CV (%)		31	29

Mass per embryo refers to the overall mean of embryos of each category, regardless of the condition and period of storage.

In freshly collected seeds, only embryos in category I were observed (Tab. 3). After 30 days of storage, embryos were found to be in categories II and III for both lots, and a decrease in the percentage of embryos in category I. After 45 days the percentage of embryos in category III increased substantially, with values close to 40% at 270 days for samples stored in the natural laboratory environment. The same trend was observed for the cold chamber samples, but with slightly lower percentages, approximately 33% of the embryos being in category III for both lots at 270 days, but embryos in category IV were not observed in the cold chamber.

The results show morphological changes typical of each early-developmental category, resulting in significant alterations in embryo mass, for all lots, conditions, and storage periods.

### Seed quality after storage

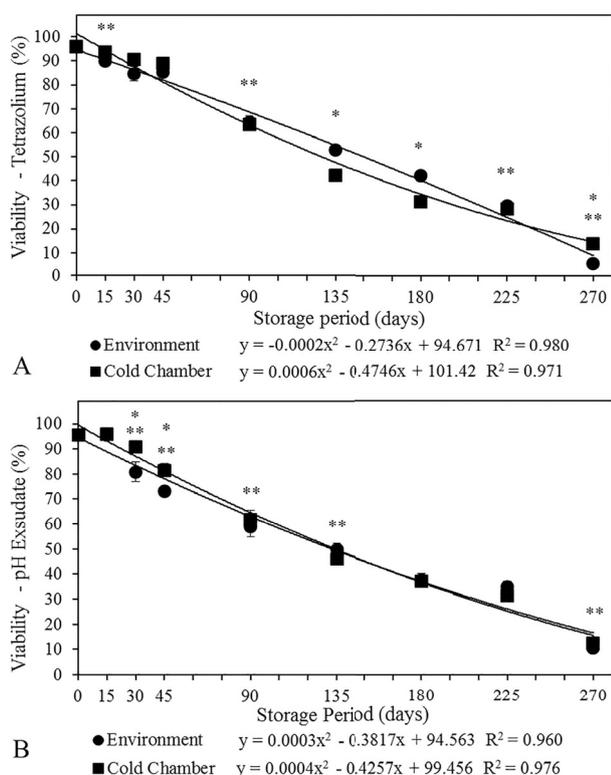
Viability of embryos from freshly collected seeds, as determined by tetrazolium and exudate pH tests, was 96% and 95%, respectively (Fig. 2A-B). Viability reduced sharply after 45 days of storage, and at the end of experimental period (270 days) viability was 4% (tetrazolium), and 5% (exudate pH) for embryos stored at natural laboratory environment, and 9% (tetrazolium), and 13% (exudate pH) for embryos stored in the cold chamber.

Viability should be related to moisture content, especially in recalcitrant seeds. Freshly collected seeds showed 49.5% moisture, and these values decreased during storage (Fig. 3). The sharpest reductions in moisture content occurred after 180 days of storage (moisture below 38%), coinciding with the period in which embryo viability decreased to lower than 42% (tetrazolium) or 38% (exudate pH) for both storage conditions (Fig. 2A-B). At the end of the storage

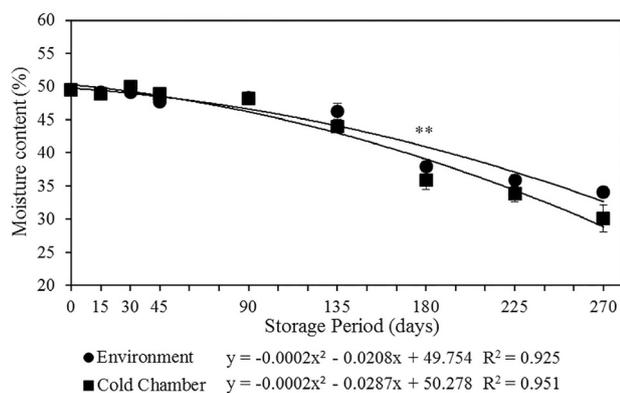
**Table 3.** Percentage of *Araucaria angustifolia* seeds in early developmental categories I, II, III and IV, observed during storage in the natural laboratory environment and cold chamber.

S.C.	E.D.	Lot 1										Lot 2									
		Storage Period (days)																			
		0	15	30	45	90	135	180	225	270	0	15	30	45	90	135	180	225	270		
Natural Environment	I	100	100	89	84	42	31	22	49	38	100	98	90	72	49	20	40	46	28		
	II	-	-	9	8	15	17	9	5	7	-	1	6	14	15	11	8	8	11		
	III	-	-	2	8	43	48	55	40	39	-	1	4	14	36	48	39	41	41		
	IV	-	-	-	-	-	4	14	6	16	-	-	-	-	-	21	13	5	20		
CV (%)		0	0	18	17	18	24	26	28	27	0	1	3	12	12	26	22	20	13		
Cold Chamber	I	100	100	98	98	68	27	47	49	49	100	100	100	96	46	26	40	44	45		
	II	-	-	2	1	19	20	22	13	15	-	-	-	3	20	36	17	18	26		
	III	-	-	-	1	13	53	31	38	36	-	-	-	1	34	38	43	37	29		
	IV	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-		
CV (%)		0	0	2	1	10	17	27	26	21	0	0	0	7	13	28	22	25	27		

Being: S.C. – storage condition; E.D. – early developmental category



**Figure 2.** Viability of *Araucaria angustifolia* embryos assessed by tetrazolium (A) and pH exudate tests (B) of freshly collected seeds, and seeds in storage in the natural environment and dry chamber. Values represent the mean of seed lots from 4 replications ( $n=25$ ) for each treatment (from early developmental categories I and II), and vertical bars are the pooled standard errors of the mean (ANOVA). \* indicates the presence of significant differences between the mean of at least one storage condition treatment ( $P \leq 0.05$ ) in each storage period. \*\* indicates the presence of significant differences between the mean of the storage period treatment ( $P \leq 0.05$ ) in relation to the previous period, for at least one storage condition.



**Figure 3.** Moisture content of seeds of *Araucaria angustifolia* freshly collected and in storage in the natural environment and cold chamber. Values represent the mean of seed lots from 4 replicates ( $n=3$ ) for each treatment (from early developmental categories I, II, III and IV), and vertical bars are the pooled standard errors of the mean (ANOVA). \* indicates the presence of significant differences between the mean of at least one storage condition treatment ( $P \leq 0.05$ ) in each storage period. \*\* indicates the presence of significant differences between the mean of the storage period treatment ( $P \leq 0.05$ ) in relation to the previous period, for at least one storage condition.

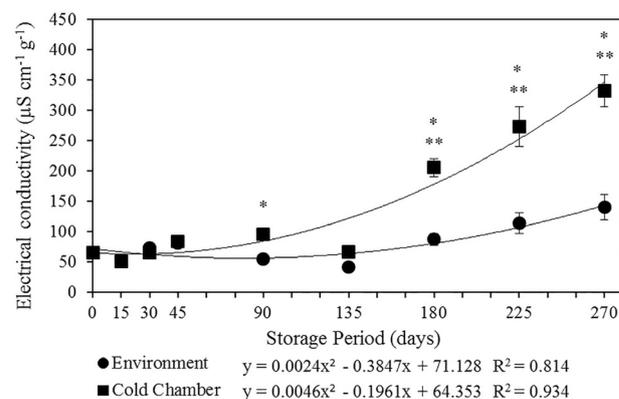
period (270 days), moisture content of seeds reached 34% (natural environment), and 30% (cold chamber).

Differences in embryo vigor, as assessed by electrical conductivity, between storage conditions were pronounced. In freshly collected samples, electrical conductivity was  $64.3 \mu\text{S cm}^{-1} \text{g}^{-1}$ , and the most significant differences between storage conditions occurred after 180 days (Fig. 4). At 270 days, electrical conductivity was  $140.3 \mu\text{S cm}^{-1} \text{g}^{-1}$  (natural environment), and  $332.4 \mu\text{S cm}^{-1} \text{g}^{-1}$  (cold chamber).

## Discussion

Subcellular germination events of recalcitrant seeds initiate early after shedding (Drew *et al.* 2000; Obroucheva *et al.* 2012; Berjak & Pammenter 2013). In fact, a decrease in the percentage of embryos in category I was observed after only 30 days of storage of *A. angustifolia* seeds, which suggests that germination metabolism certainly starts prior to this period. Recalcitrant seeds, including *A. angustifolia*, stored for 28 days at high relative humidity show protein digestion resulting in the formation of several vacuoles, plastids devoid of starch, and an increase in the number of mitochondria, all indicative of increased respiratory activity (Farrant *et al.* 1989). Therefore, *A. angustifolia* seeds remain metabolically active, and show changes associated with the process of germination while stored, a characteristic that forms the basis of recalcitrant behavior (Pammenter & Berjak 2013).

In general, the percentage of embryos in category II was low in all treatments. This is because category II is a transition between the end of embryonic development (mature but not germinated embryos, typical category I), and the early formation of seedlings (physiologically germinated



**Figure 4.** Electrical conductivity of *Araucaria angustifolia* embryos from freshly collected seeds, and from seeds in storage in the natural environment and dry chamber. Values represent the mean of seed lots from 4 replicates ( $n=10$ ) for each treatment (from early developmental categories I, II, III and IV), and vertical bars are the pooled standard errors of the mean (ANOVA). \* indicates the presence of significant differences between the mean of at least one storage condition treatment ( $P \leq 0.05$ ) in each storage period. \*\* indicates the presence of significant differences between the mean of the storage period treatment ( $P \leq 0.05$ ) in relation to the previous period, for at least one storage condition.

embryos, typical category III). At the end of the experiment, most of the embryos that remained in categories I and II had deteriorated, being discolored and having visually softened tissue and/or damage from microorganisms. This is confirmed by results of the tests of physiological quality, which found a low percentage of viable seeds at 270 days of storage.

Viability decreased over the experimental period for both storage conditions, however, considering all early-developmental categories (I to IV), viability values were, on average, 10% lower than considering only categories I and II for all storage periods (data not shown). This indicates that embryos in categories III and IV were more susceptible to deterioration. Furthermore, a more significant reduction in viability occurred with about 10% water loss, which favors deterioration. Once germination starts during storage, when there is a gradual increase in metabolic activity and water is necessary to complete the process, recalcitrant seeds including those of *A. angustifolia*, become increasingly sensitive to drought stress, and the damage caused by the lack of water triggers deterioration (Farrant *et al.* 1989; Fowler *et al.* 1998; Pammenter & Berjak 2013; Walters 2015). Therefore, at the time when the water demand of seeds increased, it was also period of the lowest moisture levels, thus contributing to a sharp decline in viability. This makes it clear that the establishment of quality standards prior to physiological analysis is important for accurate assessment, and the segregation into early developmental categories should be considered. For seedling formation, seeds remaining in early developmental categories I and II can be kept in storage for future use, provided their physiological changes are periodically assessed during the storage period. Moreover, it is not possible to distinguish seeds in category I from those in category II without opening them and observing their embryos. Seeds with root protrusion (early developmental category III) should be used for propagation as quickly as possible. Following these measures would allow the most optimal storage and use of seeds of *A. angustifolia*.

The results presented herein suggest that heterogeneity in the degree of maturity of seeds is one of the most important aspects of determining the storability of *A. angustifolia* seed lots. Some authors have proposed that the period of viability of recalcitrant seeds during storage is dependent upon how developed the seeds are, considering that recalcitrant seeds differ from orthodox seeds only in the stage of maturity at which they were disconnected from mother plant (i.e., recalcitrant are immature dispersed seeds, Barbedo *et al.* 2013). Besides, there is natural variation in seed longevity, and the assignment of seeds to particular categories based on seed responses at full maturity is a difficult task because many recalcitrant seeds lack a clear punctuation between maturation and germination (Berjak & Pammenter 2008; Walters 2015).

Decrease in embryo viability does not appear to have been strongly influenced by storage condition, possibly due to the use of sealed containers with small poros-

ity that limited water loss. Furthermore, although there was a great temperature range in the natural laboratory environment, the average temperature in this condition (15°C) was only slightly higher than that of the cold chamber (10°C). More pronounced differences between storage conditions were observed in early developmental categories, wherein the samples in the natural laboratory environment reached categories II, III, and IV earlier than those in the cold chamber, since seeds of *A. angustifolia* germinated easily at temperatures ranging from 10°C to 30°C (Espindola *et al.* 1994).

Electrical conductivity increased over the storage period and may be indicative of the onset of deterioration, given a lowered integrity of the cell membrane system of the seeds, which represents the initiation of the deterioration process (Matthews *et al.* 2012; Silva *et al.* 2014). The assessment of vigor using electrical conductivity showed a difference between storage conditions because the conductivity was higher in the cold chamber samples (lower vigor), compared to that of the samples in the natural laboratory environment (higher vigor), especially beginning at 135 days. Due to the sensitivity of the test, and since the natural laboratory environment condition had a higher average temperature and a higher temperature range than cold chamber, the laboratory environment samples were expected to have a higher rate of solute leaching. However, in the early developmental categories observed, the samples kept in the cold chamber had higher conductivity. This result can be explained by the correlation between physical characteristics and electrolyte leakage by seeds (Miceli & Miceli 2012).

The process of the digestion of reserves during the development of seedlings of *A. angustifolia* originates in the embryo (Rosado *et al.* 1994), which leads to several morphological changes after they reach early-developmental categories III and IV, such as the thickening of the hypocotyl and primary root. At the seedling stage, the primary root is well-developed, cylindrical, woody, rusty in color, and with longitudinal ridges (Kuniyoshi 1983), and the hypocotyl is distinguished not only by the lack of lateral roots, but also by its extension, and its slightly greenish color (Dillenburg *et al.* 2010). Therefore, considering that seeds stored in the natural environment reached early developmental categories III and IV first, the morphological characteristics of developing seedlings provided a physical barrier preventing the leaching of solutes, and thus reducing electrical conductivity. Meanwhile, about 77% of seeds kept in the cold chamber were in categories I and II at 270 days (both lots), and thereby were more susceptible to solute leaching due to the disruption of the membrane system during the storage period. Thus, the electrical conductivity test was not sensitive enough to differentiate the physiological quality of seeds, unless their early developmental stages were previously established.

In summary, the results of this study demonstrate that the initiation of germination in stored seeds of *A. angustifolia*

can be verified by visual analysis at about 30 days after collection. Seed storage in cold-chamber conditions delays germination, but does not prevent its occurrence. After reaching category III, embryos are more likely to deteriorate. Heterogeneity in the degree of maturation is one of the major causes of seed deterioration in *A. angustifolia*. Viability of *A. angustifolia* embryos can be kept around 12% after 270 days of storage in cold-chamber conditions. The lots of assessed seeds showed the same behavior with regard to physiological quality analysis, and were able to be separated into early developmental categories. Propagation purposes should prioritize the use of *A. angustifolia* seeds that have reached early developmental category III (with root protrusion), while those that remain in category I and II could be stored for 270 days in a cold chamber while maintaining at least 12% viability, provided there is periodic assessment for reduction in physiological quality.

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