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Reserve metabolism of stored and germinated *Araucaria angustifolia* seeds

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ABSTRACT. Germination metabolism of recalcitrant seeds of *Araucaria angustifolia* is activated in storage, which complicates the seeds conservation and utilization. This study aimed to identify the changes in the reserve metabolites of *A. angustifolia* seeds throughout storage in order to understand the processes of hydrolysis caused by germination metabolism. Mature seeds were harvested in southern Brazil and stored in an ambient environment and cold chamber conditions. Biochemical analyses were performed for embryos and megagametophytes from seeds stored for 0, 15, 30, 45, and 90 days. Due to seeds being in advanced germination in storage, they were evaluated at 90 days in different early developmental categories: I – seeds with mature embryos, II – seeds with embryos showing apparent elongation along the embryonic axis, and III – seeds with root protrusion. Higher contents of carbohydrate, protein, and amino acids were observed in embryos compared to megagametophytes, and these metabolites were decreased after onset of germination, especially in the embryo tissue. Mobilization of metabolites in megagametophytes would probably increase in later stages of germination. It is suggested that such alterations are not due to deterioration of reserve components, but instead are based on seed metabolism, which remains active after harvest with hydrolysis of metabolites providing energy for germination.

Keywords: Brazilian pine; conifer; protein profile; reserve mobilization.

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Introduction

Araucaria angustifolia is an endangered (Brasil, 2008; Thomas, 2013) native Brazilian conifer tree. However, seeds from local varieties of *A. angustifolia* showed great diversity of physical and physiological parameters, which may reflect that a relevant genetic diversity is still present (Shibata, Coelho, Araldi, Adan, & Peroni, 2016; Araldi, Coelho, & Shibata, 2018). Its mature seeds are formed by a bulky megagametophyte, the major storage tissue, and an embryo, which represents only 1.3% of the total weight of the seed (Araldi & Coelho, 2015). Starch and protein have been considered the most important reserve compounds in mature seeds of *A. angustifolia* (Panza, Láinez, Maroder, Prego, & Maldonado, 2002; Balbuena et al., 2011; Araldi, Coelho, & Maraschin, 2016). Starch corresponds to 73% of the megagametophyte tissue, with soluble protein representing 1 to 2.5% of the embryo and 0.3 to 2% of the megagametophyte, and insoluble protein representing 0.8% of the embryo and 0.5% of the megagametophyte (Piriz Carrillo, Chaves, Fassola, & Mugridge, 2003; Astarita, Floh, & Handro, 2004, Silveira et al., 2008, Balbuena et al., 2009; Garcia, Shibata, Coelho, Soares, & Guerra, 2012; Araldi et al., 2016b).

Germination of *A. angustifolia* seeds is cryptogeal, a pattern typical of large-seeded species in which the root-hypocotyl axis emerges on the soil surface and penetrates into the soil before shoot elongation (Dillenburg, Rosa, & Mósena, 2010; Capocchi et al., 2011). Complete germination, when all of the seedling structures are formed (root, hypocotyl and shoot), takes approximately 60 to 70 days (Caçola, Amarante, Fleig, & Mota, 2006; Garcia et al., 2012; Garcia, Coelho, Maraschin, & Oliveira, 2014; Shibata, Coelho, & Steiner, 2013).

Storage proteins and starch in mature embryos of *A. angustifolia* are accumulated in cells of a variety of tissues but especially in radicle cells (Panza et al., 2002), the first seedling structures to emerge during germination. The hypocotyl represents a major sink for the seed reserves in the initial growth of *A.*

angustifolia, acting as an underground storage structure for root growth and shoot emergence, but it may also play a role in storage of photosynthates after shoot emergence (Dillenburg et al., 2010). The shoot emerges before seed reserves are fully exhausted (Ferreira & Handro, 1979), which apparently occurs 100 days after planting when 50% of cotyledons remain green; cotyledons dry and break their connections with the growing plant at approximately 160 days after planting (Dillenburg et al., 2010).

Several biochemical alterations have been reported in *A. angustifolia* seeds during development (Astarita et al., 2004; Silveira et al., 2008; Balbuena et al., 2009; Garcia et al., 2012; Navarro et al., 2017; Oliveira et al., 2017), storage (Ramos & Souza, 1991; Piriz Carrillo et al., 2003; Araldi, Coelho, Gaziola, & Azevedo, 2016), and germination (Rosado, Ferreira, Mariath, & Cocucci, 1994; Pieruzzi et al., 2011). However, to determine the exact moment when biochemical changes related to the germination process start is difficult because seeds of *A. angustifolia* are considered recalcitrant, with a few months of longevity under natural conditions (Piriz Carrillo et al., 2003; Caçola et al., 2006; Amarante, Mota, Megguer, & Ide, 2007; Garcia et al., 2014).

However, there is no standard characterization of recalcitrant seeds, and recent studies indicate a need to better describe the metabolism of seeds characterized as such since each species has its own peculiarities and cannot be combined into a single group. This is because many recalcitrant seeds lack a clear distinction between maturation and germination (Berjak & Pammenter, 2008; Walters, 2015), and differences between recalcitrant and orthodox seeds could arise from the stage of maturity in which they detach from the mother plant, with recalcitrant seeds being dispersed at a very immature stage (Barbedo, Centeno, & Figueiredo-Ribeiro, 2013). Thus, biochemical alterations observed after the harvest of recalcitrant seeds may not be due to the process of deterioration, and it is possible to observe subcellular germination events of recalcitrant seeds quickly after shedding (Berjak & Pammenter, 2008; Obroucheva, Lityagina, Novikova, & Sin'Kevich, 2012; Berjak & Pammenter, 2013; Walters, 2015).

In fact, cell features and type of reserves in mature seeds of *A. angustifolia* indicate continuous development without the interposition of a dry state (Panza et al., 2002). Germination process events, including an increase in the levels of protein synthesis and meristem cell metabolism, initiate shortly after shedding of *A. angustifolia* seeds and continue during storage (Farrant, Pammenter & Berjak, 1989). Additionally, there is visual evidence of the onset of germination in seeds stored for 30 days inside transparent plastic containers in the natural environment without the supply of additional water (Araldi & Coelho, 2015). These authors suggested that heterogeneity in the degree of seed maturity is an important aspect in determining the storability of *A. angustifolia* seeds.

As demonstrated in the literature, germination of recalcitrant *A. angustifolia* seeds will inevitably occur during storage, hampering the actions for natural population recovery. Therefore, the aim of this work was to identify the changes in reserve metabolites in mature seeds of *Araucaria angustifolia* throughout storage in order to understand the processes of hydrolysis and mobilization based on germination metabolism imposed after harvest.

Material and methods

Seed harvest, storage, and characterization

Mature seeds of *A. angustifolia* were harvested from a natural population located in the region of São José do Cerrito (27° 36' S, 50°39' W; average elevation of 918 meters) in southern Brazil. Seeds were collected at the beginning of dispersal from 63 cones of 15 mother trees, totaling approximately 7,100 seeds. The seeds were randomly distributed among three replicates and subjected to storage by being placed in sealed, semipermeable (porosity of 0.015 μm) and transparent plastic containers, which permitted gaseous exchange and limited water loss. The containers were then placed in two different storage conditions: the ambient laboratory conditions and a cold chamber (temperature of 10 ± 3°C, and relative humidity of 45 ± 5%), where they were kept for a period of 90 days (each storage condition containing three replicates). Reference values for storage temperature and relative humidity for the ambient laboratory conditions according to Epagri/Ciram (2014) were -0.5 to 24.8°C and 35 to 98%, with averages of 12.5°C and 84%, respectively.

At 90 days of storage at least 40% of the ambient and 10% of the cold chamber seeds exhibited root protrusion due to advanced germination under the storage conditions and inside the containers

(spontaneous seed sprouting). Germination during storage is characteristic of recalcitrant seeds (Pammenter & Berjak, 2013). These seeds were classified into early developmental categories (EDC) as described by Araldi and Coelho (2015): I – seeds with mature (but not germinated) embryos; II – seeds with embryos showing apparent elongation along the embryonic axis, indicating the beginning of germination; and III – seeds with embryos that started root protrusion with coat rupture. To better characterize the metabolic events associated with seed storage, the following analyses were performed on the embryos and megagametophytes, separately, from newly harvested seeds and those stored for 15, 30, 45, and 90 days, with the seeds from categories I, II, and III being evaluated only at 90 days of storage.

Carbohydrate and starch extraction and quantification

Soluble carbohydrates and starch were extracted using a pool containing embryos and megagametophytes from 10 seeds per replicate. Then, 200 mg of dry biomass was macerated with mortar and pestle in liquid nitrogen, followed by triple extraction with ethyl alcohol 80% (McCready, Guggolz, Silviera, & Owens, 1950). Supernatants were collected for quantification of soluble carbohydrates and the residue was used for starch extraction by adding perchloric acid (52%). After filtering aliquots of soluble carbohydrates and starch using a fiberglass filter, quantification was proceeded by colorimetric analysis using a spectrophotometer at 490 nm by the phenol-sulfuric method (Dubois, Giles, & Hamilton, 1956), with D-glucose as a standard.

Protein extraction and quantification

Soluble protein content was determined using a pool of ten embryos and megagametophytes per replicate, separately homogenized (3:1 buffer volume/macerated samples in liquid nitrogen) with a pestle with 100 mM of potassium phosphate buffer (pH 7.5) containing 1 mM of ethylene diaminetetraacetic acid (EDTA), 3 mM of dithiothreitol, and 4% (w/v) insoluble polyvinylpolypyrrolidone (PVPP) (Azevedo, Alas, Smith, & Lea, 1998). The homogenate was centrifuged at 10,000 *g* for 30 min at 4°C. Protein content was determined spectrophotometrically at 595 nm according to Bradford (1976) using bovine serum albumin (BSA) as a standard.

Total protein content of embryos and megagametophytes was determined from 200 mg of dry matter through sulfuric acid digestion by adding 1 mL of hydrogen peroxide (H_2O_2) and 2 mL of sulfuric acid (H_2SO_4) in a digester block at 360°C. After digestion, 5 mL of 10 M sodium hydroxide (NaOH) was added to 10 mL of digestion extract. Samples were titrated with 0.25 M of H_2SO_4 and converted to crude protein by a factor of 6.25 (AOAC, 1995).

Amino acid extraction and quantification

Amino acid extraction was performed with 2 mL of MCW (methanol:chloroform:water in the ratio of 12:5:3) added to 200 mg of dry matter and kept overnight at 4°C. The homogenate was centrifuged at 10,000 *g* for 20 min., the supernatant was removed, and 500 µL of chloroform and 750 µL of water was added (Bieleski & Turner, 1966). After further centrifugation, the aqueous phase was removed and placed at 38°C for 1 hour. Sample quantification was performed by adding 500 µL of 0.2 M sodium citrate buffer (pH 5.0), 200 µL of reactive ninhydrin (5% in methylglycol), and 1 mL of potassium cyanide (2% of 0.01 M solution in methylglycol) (Yemm & Cocking, 1955). Samples were kept in a water bath at 100°C for 20 min. Absorbance readings were performed using a spectrophotometer at 570 nm using Leucine as the standard.

One dimensional gel electrophoresis (SDS-PAGE)

Aliquots (38 µg) of soluble proteins were mixed with an equal volume of loading buffer containing 0.5 M of tris (pH 6.7), 20% glycerol, 2% SDS, 5% 2-mercaptoethanol and traces of bromophenol blue. Protein separation was performed in a polyacrylamide SDS-PAGE gel of 12% separating gel and 3% concentrating gel (Laemmli, 1970) at a constant voltage of 15 mA per gel for 150 min. with three replications. The gels were stained with 0.1% Coomassie Brilliant Blue (Alfenas, 1998).

Reserve mobilization

The mobilization of embryo reserves for seedling formation, expressed as a percentage, was calculated based on the initial and final content of the following reserve metabolites: soluble carbohydrate, starch, soluble protein, total protein, and amino acids.

Experimental design and statistical analysis

The experiment was conducted using a completely randomized split-plot design, with two storage conditions (ambient environment and cold chamber) and seven storage periods (0, 15, 30, 45, 90_{I}, 90_{II}, and 90_{III} days). Analysis of variance and Tukey's test of means at 5% probability were performed using the statistical software SAS, 2009). The results were expressed as the mean and standard error of mean (±S.E.M.) of three independent replicates. Assessment of the presence and intensity of bands in the gel analysis was done qualitatively.

Results

Soluble carbohydrate content was higher in embryos (113.3 mg g^{-1} dw) compared to megagametophytes (32.5 mg g^{-1} dw) of newly harvested seeds (Figure 1A-B), and the starch content already showed slight differences between the tissues (588.6 mg g^{-1} dw in embryos, and 648.4 mg g^{-1} dw in megagametophytes) (Figure 1C-D). The availability of soluble carbohydrates increased in embryos prior to the beginning of germination (45 and 90 days after harvest – DAH) for samples stored in the cold chamber, and there was a 60% reduction after the onset of germination (EDC 90 II). For the ambient samples, however, there was an increase in the availability of soluble carbohydrates in megagametophytes in the first stages of germination (EDC 90 II), followed by an increase in embryos (162.3 mg g^{-1} dw at EDC 90 III). Starch content (insoluble carbohydrates) decreased during storage and germination, in both embryos and megagametophytes beginning at 30 days of storage.

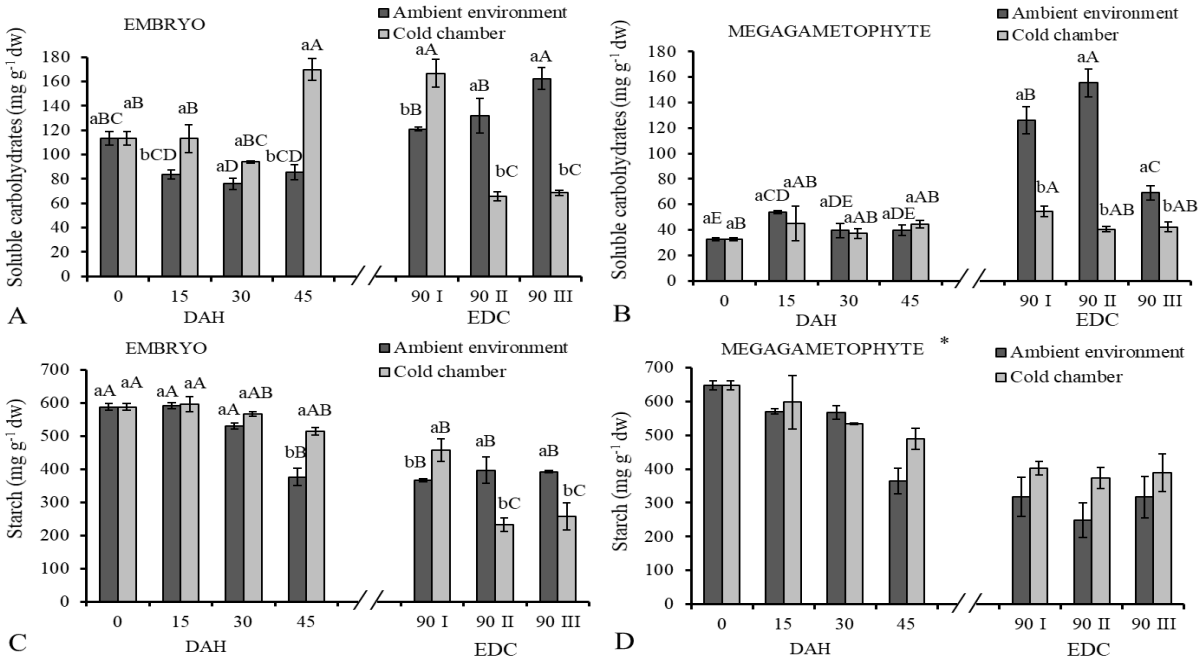

Figure 1. Soluble carbohydrate content in embryos (A) and megagametophytes (B), and starch content in embryos (C) and megagametophytes (D) during storage of *Araucaria angustifolia* seeds in the ambient environment and cold chamber conditions. Vertical bars are the pooled standard errors of the mean (ANOVA). Lowercase letters compare storage conditions, and uppercase letters compare the days after harvest (DAH) and early development categories (EDC) by Tukey's test ($p \leq 0.05$). *Indicates the absence of a significant interaction between storage condition x DAH or EDC.

Soluble protein content in newly harvested seeds was 25.7 mg g^{-1} dw in embryos and 0.8 mg g^{-1} dw in megagametophytes and reduced in embryos after harvest (7.4 mg g^{-1} dw in ambient and 13.4 mg g^{-1} dw in cold chamber at EDC 90 I) and during germination (0.2 mg g^{-1} dw in ambient and 0.3 mg g^{-1} dw in cold chamber at EDC 90 III) for both storage conditions (Figure 2A-B). In megagametophytes, there was a reduction in soluble proteins only for seeds stored in the ambient condition during germination, with an increasing trend in cold chamber samples. Total protein content in newly harvested seeds was also higher in embryos (46.7 mg g^{-1} dw) compared to megagametophytes (20.4 mg g^{-1} dw) (Figure 2C-D). ANOVA demonstrated a reduction in total protein content of embryos after harvest and during germination, but there was no significant interaction between these treatments.

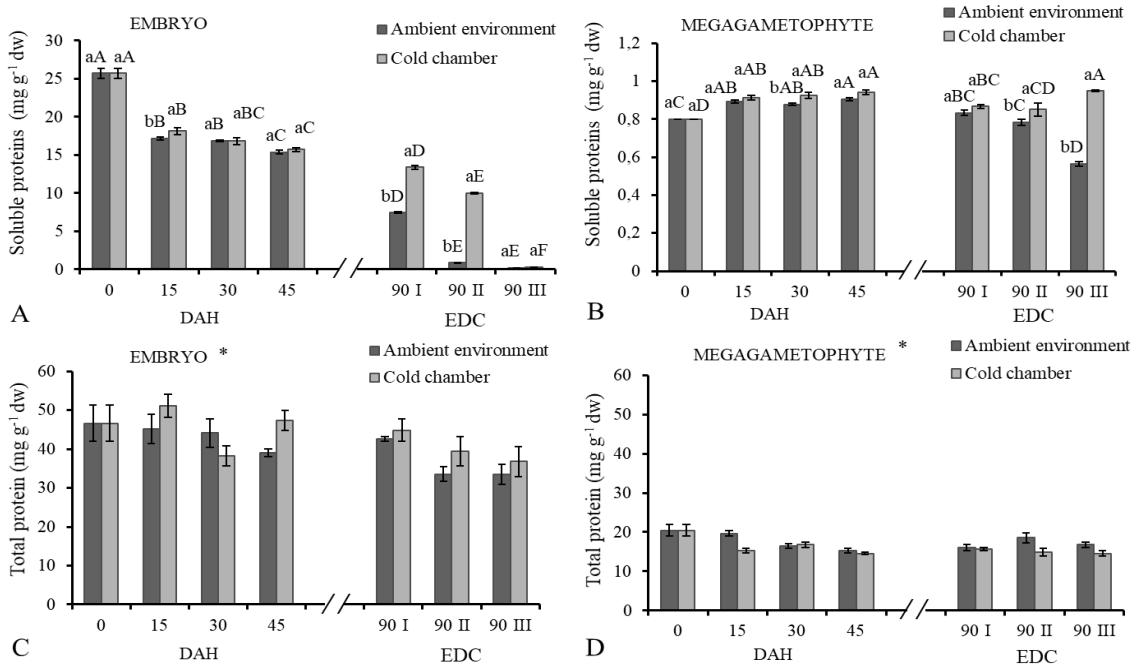

Figure 2. Soluble protein content in embryos (A) and megagametophytes (B), and total protein content in embryos (C) and megagametophytes (D) during storage of *Araucaria angustifolia* seeds in the ambient environment and cold chamber conditions. Vertical bars are the pooled standard errors of the mean (ANOVA). Lowercase letters compare storage conditions, and uppercase letters compare the days after harvest (DAH) and early development categories (EDC) by Tukey's test ($p \leq 0.05$). *Indicates the absence of a significant interaction between storage condition x DAH or EDC.

Soluble amino acids in the embryos of newly harvested seeds were 10.1 mg g^{-1} dw and 3.6 mg g^{-1} dw in megagametophytes (Figure 3A-B). In embryos, the availability of soluble amino acids increased until 90 days of storage and was then followed by a reduction during germination. In megagametophytes, amino acid content increased at 90 days of storage only for ambient samples, whereas it remained stable for samples stored in the cold chamber.

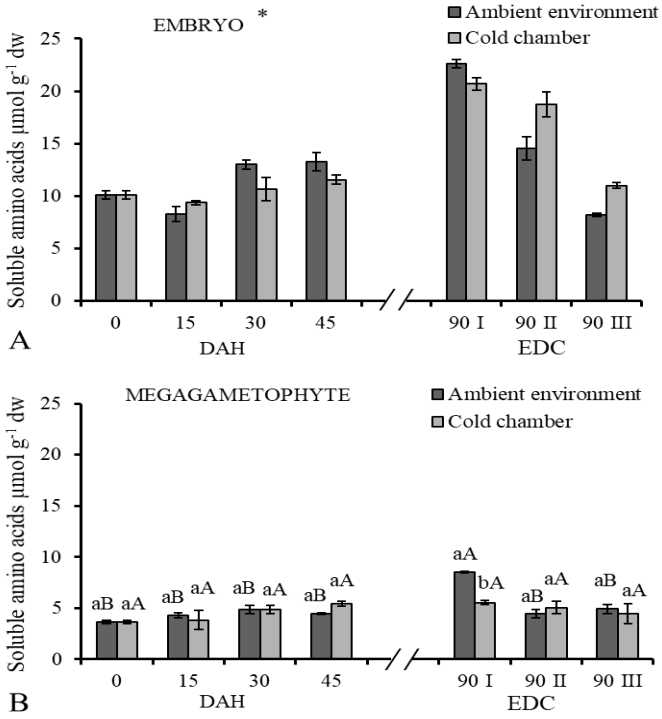

Figure 3. Soluble amino acid content in embryos (A) and megagametophytes (B) during storage of *Araucaria angustifolia* seeds in the ambient environment and cold chamber conditions. Vertical bars are the pooled standard errors of the mean (ANOVA). Lowercase letters compare storage conditions, and uppercase letters compare the days after harvested (DAH) and early development categories (EDC) by Tukey's test ($p \leq 0.05$). *Indicates the absence of a significant interaction between storage condition x DAH or EDC.

The SDS-PAGE pattern of the soluble protein fraction from embryos and megagametophytes was characterized by the presence of six major protein bands with molecular weights approximately between 43 and 6 kDa (Figure 4A-B). At 90 DAH, a significant decrease in the intensity of these bands was observed, especially for bands of approximately 30 and 26 kDa for both embryos and megagametophytes. During germination (EDC), the reduction in the intensity of protein bands was even more pronounced; at EDC 90 III, no bands were clearly detected in embryos (in both ambient and cold chamber conditions), and only bands at 10 kDa remained in megagametophytes (in ambient and cold chamber conditions).

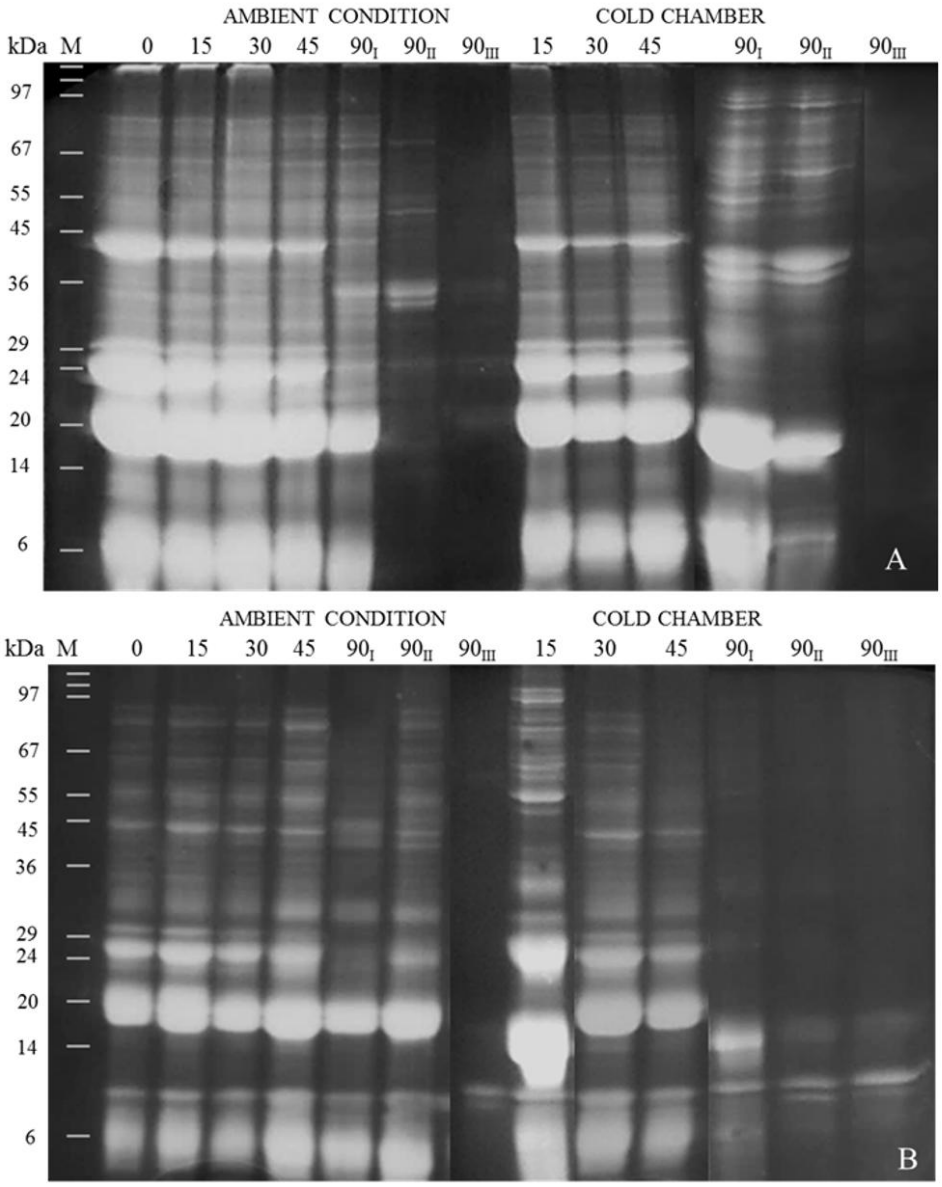

Figure 4. SDS-PAGE profiles of soluble proteins of *Araucaria angustifolia* embryos (A) and megagametophytes (B) during storage in the ambient laboratory and cold chamber conditions. Lane M – molecular weight markers.

Analysis of reserve mobilization demonstrated that reserve hydrolysis started in embryos due to the higher initial content of all metabolites except starch, with soluble protein being the first metabolite compound to be mobilized from these structures to seedling formation (Figure 5A-B). Metabolite mobilization in embryos did not increase after EDC II, with the exception of soluble protein and amino acid content, for both storage conditions. Soluble protein mobilization in embryos was almost complete at EDC III, with 97% and 98% of proteins mobilized in ambient and cold chamber conditions, respectively. The reserve mobilization of the megagametophyte tissue was lower, with carbohydrates and amino acids showing the highest mobilization but only for samples stored at ambient condition, with approximately 45% and 42% of mobilization at EDC III, respectively. The mobilization of starch increased between stages I and

II of germination and was followed by a decrease, except for ambient embryo samples, for which there was no mobilization. In megagametophytes, an increase was observed in the mobilization of carbohydrates and soluble protein between EDC II and III in samples stored in ambient conditions, and a subtle increase was observed in total protein and amino acids content for samples stored in the cold chamber.

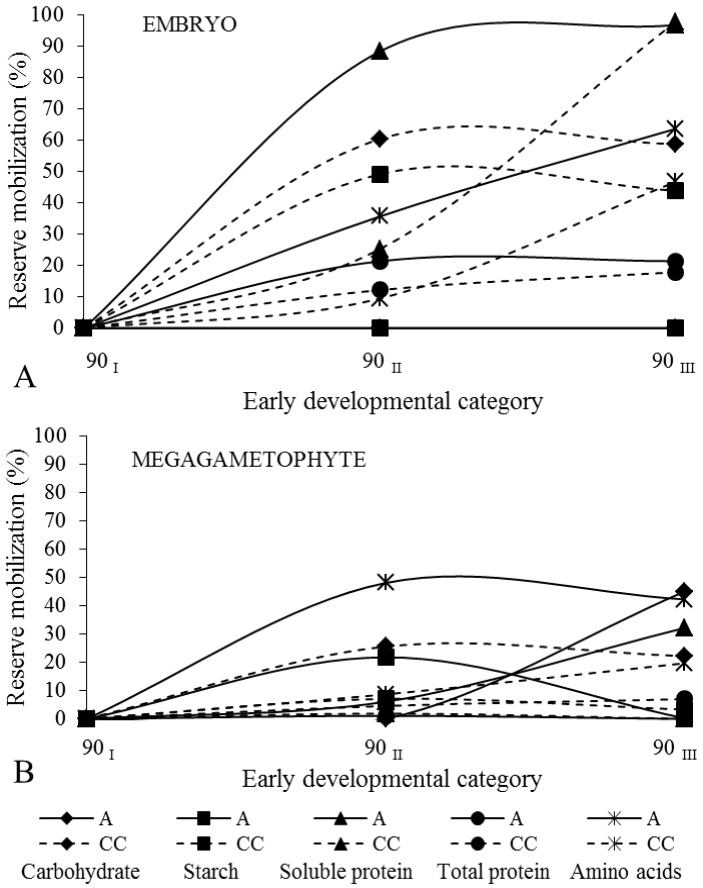

Figure 5. Reserve mobilization in embryos (A) and megagametophytes (B) during storage of *Araucaria angustifolia* seeds in the ambient laboratory and cold chamber conditions. A – ambient condition; CC – cold chamber condition.

Discussion

The key part of biochemical evaluation for understanding the metabolism of recalcitrant seeds after harvest is to characterize the stage of seed development or "maturation degree" (Barbedo et al., 2013; Silva, Centeno, Ribeiro, & Barbedo, 2015; Lamarca et al., 2016). This characterization is very difficult since there is not a clear division between the processes of maturation and germination (Berjak & Pammenter, 2008; Walters, 2015). In this study, only seeds with no visual evidence of germination (without root protrusion) were assessed at 0, 15, 30, and 45 DAH, and thus, some alterations in metabolite content were observed as a function of storage. Higher contents of soluble carbohydrate, soluble protein, total protein, and amino acids were observed in embryos compared to megagametophytes in newly harvested seeds, which is associated with the increased availability of these metabolites for embryo growth. In megagametophytes, there was no reduction in the levels of the evaluated reserve metabolites until 90 days of storage.

Even without visual evidence of germination, there are indications that biochemical alterations related to germination metabolism start soon after seed harvest (Panza et al., 2002) and may even start before that. Under optimal conditions, at 27°C, physiological germination of *A. angustifolia* ended 8 days after sowing, when at least 50% of the seeds exhibited root protrusion, indicating the beginning of the seedling growth phase, but moisture content showed no significant changes during the germination process (Balbuena et al., 2011). Thus, even without an additional water supply, visual evidence of germination was detected after 30 days while stored under ambient laboratory or cold chamber conditions (Araldi & Coelho, 2015).

After the onset of germination (EDC 90_I, 90_{II}, and 90_{III}), the contents of starch, soluble protein, total protein, and amino acids reduced, especially in embryo tissue. In general, there was a higher mobilization of metabolites in embryos compared to megagametophytes, in which mobilization would probably increase in later stages. In fact, some authors agree that reserve mobilization begins in the embryo during the first phase of germination (imbibition), and most mobilization in reserve tissues occurs only in phase three of germination (after root protrusion, also designated 'post-germination') because the products of hydrolysis in reserve tissues might be important in supporting germination, but especially because they support early seedling growth and establishment until it becomes autotrophic (Bewley, Bradford, Hilhorst, & Nonogaki, 2013).

The availability of reserve metabolites ensures early development of seedlings and subsequent field establishment. During germination, starch is converted to sugars within the starch granules, and proteins are converted to amino acids within the protein storage vacuoles, before these catabolites are moved into the cytosol (Bewley et al., 2013). Starch and proteins, as well as hemicelluloses and triacylglycerols, are mobilized by distinct suites of enzymes, many of which are transcribed and synthesized *de novo* (Bewley et al., 2013). Quantitatively, starch was the major reserve compound in embryos and megagametophytes of *A. angustifolia* seeds, providing sugars (as oligosaccharides) for the embryo to utilize during germination. In newly harvested seeds, Piriz Carrillo et al. (2003) found starch content to be 10% higher on average, and there are reports that starch content reduces in *A. angustifolia* seeds at 180 days of storage at 5°C (Ramos & Souza 1991; Piriz Carrillo et al., 2003).

At 15 days of storage, soluble proteins were the only metabolites to decrease in embryos, which occurred for both storage conditions, and the first metabolite to be mobilized in embryos during germination. Despite the reduction in soluble protein content, amino acid content does not decrease or even increase until 90 days of storage (EDC 90_I). The amount of amino acids was similar to that observed by others authors, which reported the presence of approximately 20, 12, and 8 μmol g^{-1} fw of free amino acids in the embryonic axis, cotyledon and megagametophyte of mature seeds of *A. angustifolia*, respectively (Astarita et al., 2004). Among the total amino acids observed, the most important were aspartic acid (2.7 μmol g^{-1} fw), glutamic acid (2.4 μmol g^{-1} fw), and arginine (1.7 μmol g^{-1} fw) in embryos and glutamic acid (2.7 μmol g^{-1} fw) followed by aspartic acid (2.3 μmol g^{-1} fw) in megagametophytes, where arginine correspond only to 0.2 μmol g^{-1} fw (Astarita et al., 2004). The balance between proteins and amino acids depends on the initial content of these metabolites and on the rate of hydrolysis, in addition to protein synthesis in the cytoplasm. During germination, storage proteins in the protein storage vacuoles (PSVs) of both the embryonic axis and the cotyledons are subjected to proteolysis to provide nutrients and amino acids; at the same time, amino acids are reutilized for the *de novo* synthesis of more proteins in the same regions (Rajjou et al., 2004, Bewley et al., 2013).

Regarding the protein profile, other authors have observed that the most important bands in the embryos and megagametophytes of mature seeds of *A. angustifolia* have approximately 48, 30, and 25 kDa (Garcia et al., 2012), and the megagametophytes of *A. bidwillii* have approximately 21, 20, 7, and 4 kDa (Capocchi et al., 2011). Additionally, most proteins in the embryogenic cultures of *A. angustifolia* have molecular weights between 20 and 60 kDa (Jo, Santos, Bueno, Barbosa, & Floh, 2014).

The most intense bands detected on the gel may correspond to a vicilin-like protein group (reserve globulins 7S), since these are the most abundant storage proteins in *A. angustifolia* seeds, and whose molecular weights have been reported to be approximately 12 to 15 kDa, 22 kDa, 24 kDa, 27 to 30 kDa, and 41 kDa (Silveira et al., 2008; Balbuena et al., 2009; Balbuena et al., 2011). The protein profile using two-dimensional electrophoresis allowed for the identification of 32 spots, of which 14 corresponded to a vicilin-like protein (43% of identified spots) in *A. angustifolia* seeds, with some exclusive vicilin-like protein spots detected in mature and germinated embryos, with a great abundance of proteins related to the processes of hydrolysis in germinated embryos (Balbuena et al., 2011). The two most abundant amino acids in the vicilin-like proteins are glutamic acid and arginine, comprising 10.7 and 10.5% of their primary sequences, respectively (Balbuena et al., 2011). The amino acid arginine fluxes to protein biosynthesis in mature embryos but does not persist until the end of germination (Balbuena et al., 2011), which may be associated with decreased intensity of protein bands observed in the gels of germinated seeds.

Conclusion

Notable changes in reserve metabolites of *A. angustifolia* seeds occur within the first three months of storage, such as decreases in the starch content (embryo and megagametophyte) and soluble protein (embryo), a decrease in the intensity of bands in the protein profile, and an increase in amino acid content (embryo). Notable changes also occur during germination imposed by storage, such as reductions in the starch content (embryo and megagametophyte), soluble protein (embryo) and amino acids (embryo) and a decrease in the intensity and number of bands in the protein profile (embryo and megagametophyte). Reserve mobilization started in embryos, and soluble protein was the first metabolite compound to mobilize for seedling formation in these structures. It is suggested that such alterations are not due to the deterioration of reserve components that typically occurs in the natural aging of seeds but are based on seed metabolism, which remains active after harvest with the hydrolysis of metabolites providing energy for germination. These findings bring new insights to the scientific basis of studies on recalcitrant seeds.

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