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LIPID, PROTEIN AND CARBOHYDRATE DURING SEED DEVELOPMENT IN *Araucaria angustifolia*

HIGHLIGHTS

A. angustifolia embryo continued to accumulate dry matter until the seed dispersion.

Dry matter of *A. angustifolia* seeds is formed by a large amount of starch.

The highest content of soluble carbohydrate at Stage IV collaborated to faster germination.

A transition from developmental to germination in seed metabolism were observed. .

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ABSTRACT

This study aimed to evaluate changes in germination and in the amount of carbohydrates, starch, proteins, and lipids of *A. angustifolia* seeds at different stages of development. Seeds were collected in the Cotyledonary Stage and Stages I, II, III and IV. Germination, moisture content, dry matter amount, and levels of carbohydrates, starch, proteins, lipids and fatty acids were determined. Both embryo and megagametophyte contained the starch as the large reserve content, while proteins and mainly lipids represented only a small proportion. During seed development, the highest values for germination, starch and soluble carbohydrate content were observed at stage IV. Although, soluble protein content had decreased from Cotyledonary Stage to Stage III, an increase at Stage IV was observed in the embryo. No difference was observed in lipid content. Linoleic acid was more abundant in fatty acids. Thus, *A. angustifolia* embryo showed an increase soluble protein content, an accumulation of dry matter and soluble carbohydrate content at Stage IV. It seems that these compounds collaborate to faster and higher germination.

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INTRODUCTION

Araucaria angustifolia (Bertol.) Kuntze is a critically endangered dioecious tree species native to southern Brazil (IUCN, 2018). The reproductive cycle of *A. angustifolia* takes 20-24 months (ANSELMINI et al., 2008; MANTOVANI et al., 2004; ROGGE-RENNER et al., 2017) from the appearance of reproductive structures and pollen dispersion; however, the exact moment of fertilization has not yet to be determined easily (GUERRA et al., 2008; MANTOVANI et al., 2004). In the initial stages of seed development, cell division and elongation takes place with formation of more than one embryo (polyembryony). Subsequently, reserve compounds are accumulated and the development of the dominant embryo occurs (AGAPITO-TENFEN et al., 2012). At the end of development, seeds do not undergo maturation drying nor do they acquire desiccation tolerance; such seeds have been described as recalcitrant (GASPARIN et al., 2017).

Zygotic embryo development of *A. angustifolia* has been extensively studied (ASTARITA et al., 2003; BALBUENA et al., 2009, 2011; ELBL et al., 2015, 2015; FARIAS-SOARES et al., 2013; GARCIA et al., 2015; NAVARRO et al., 2017; OLIVEIRA et al., 2017; ROGGE-RENNER et al., 2013; SILVEIRA et al., 2008). These studies focused on the morphological characteristics of the embryo, which mature in April in southern Brazil. As a complement, other studies have investigated the physiological changes of seeds until the moment of their dispersion (SHIBATA et al., 2013). However, nothing is known about the accumulation of reserve compounds by the seeds of *A. angustifolia* during the months of collecting seeds – from April to July.

Seed reserves deposited during maturation stage have an essential metabolic function, i.e. they are mobilized to fuel seedling growth (BAUD et al., 2008). In general, seeds accumulate large amounts of reserve compounds such as carbohydrates, proteins and oils in the embryo and/or in the extra-embryonic tissues (BEWLEY et al., 2013).

In *A. angustifolia* seeds, starch is the carbohydrate most commonly found (ARALDI et al., 2016; SHIBATA et al., 2016), although soluble carbohydrates may be present. This compound act as a substrate for respiration during germination and may provide protection against the effects of desiccation (BERJAK et al., 2008; BONOME et al., 2011). Lipids and fatty acids are considered to be important for protection against injury during seed dehydration (MELLO et al., 2010), whereas proteins can act in many different ways. Some protein groups form a protective layer of moisture around intracellular structures and macromolecules, while others are able to sequester ions during dehydration and in a dry state (BERJAK et al., 2008). Accumulate of these reserves will reflect in seed germination and conservation. Thus, knowledge of the metabolism is essential for understanding of seed characteristics and, consequently, obtain better seed quality and developing sound conservation practices for recalcitrant species, considering it is of major scientific and practical importance (BERJAK et al., 2008; DEVIC et al., 2016) .

This study aimed to evaluate changes in germination and in the amount of carbohydrates, starch, proteins, and lipids during seed development of *A. angustifolia*, in order to verify if their seeds show a fast germination, commonly observed in recalcitrant seed, considering that stored reserve at different development stage can affect germination capacity.

MATERIAL AND METHODS

Seed sampling

Cones (megastrobili) of *A. angustifolia* were collected from a natural population located in the town of Curitibanos, Santa Catarina, Brazil (altitude: 960 m; 27° 18' 11" S and 50° 38' 12" W). Collecting began in April, when the cotyledons were elongate and embryos were able to germinate, at Cotyledonary Stage (GUERRA et al., 2008), and continued on a monthly basis until July. Stages were classified according to development (Figure 1) (SHIBATA et al., 2013).

Embryo and megagametophyte were separated and lyophilized (Liotop L101 - Liobras) for 9 hours for carbohydrate and protein analysis.

Measurement of dry mass and moisture content

For each development stage two replicates of three seeds each were cut transversally, weighed (wet weight), dried at 105°C ±3°C for 24 hours and reweighed to determine dry weight and moisture content (BRASIL., 2009).

Germination test

After each collect, four replicates of 25 seeds were surface-decontaminated with hypochlorite solution (2%, v/v) for three minutes and was subsequently cut 3 mm of the tip of each seed (MOREIRA-SOUZA et al., 2003). Germination test were performed on the vermiculite substrate with controlled conditions in a germination chamber at 25 °C and 12 h of photoperiod.
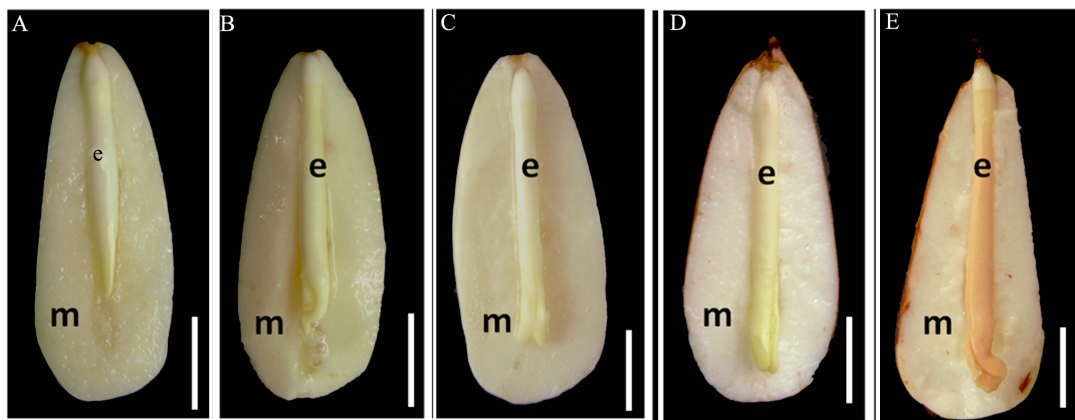
FIGURE 1 Longitudinal section of *Araucaria angustifolia* seeds at development stages: Cotyledonary (A); I (B); II (C); III (D) and IV (E). Bars: 1 cm; e: embryo; m: megagametophyte.

Seeds were considered as germinated when emerging radicles were at least 3 mm in length. Final germination percentage was recorded after 70 days and time to 50% of germination (T_{50}) was calculated according to the formula (COOLBEAR et al., 1984): where N is the final number of seeds germinating and n_i, n_j, total number of seeds germinated by adjacent counts at time t_i, t_j, where 2.

$$T_{50} = t_i + [(N + 1)/2 - n_i / n_j - n_i] . (t_j - t_i) \qquad [1]$$

$$n_i < (N + 1)/2 < n_j \qquad [2]$$

Soluble carbohydrate content

A sample of 0.5 g of dry material was macerated in a mortar with liquid nitrogen and transferred to tubes. The tubes were then placed in a water bath at 100°C for 5 min and 5 mL of 80% ethanol was added to each tube. After boiling, the extracts were centrifuged at 1500 g for 10 min, filtered through a glass microfiber filter and dispensed into test tubes with the volume being adjusted to 10 mL with 80% ethanol. This process was repeated three times.

Total soluble carbohydrate was estimated through colorimetric analysis using the phenol-sulfuric method (DUBOIS et al., 1956), and measuring the absorbance at 490 nm using a spectrophotometer. Total sugar content was calculated based on a standard curve of D-glucose.

Starch content

To the precipitate resulting from the extraction of total soluble carbohydrate, 10 mL of cold distilled water and 13 mL of 52% perchloric acid were added. The mixtures were incubated for 15 minutes with occasional stirring. Then, 20 mL of distilled water was added, followed by centrifugation at 1500 g for 15 minutes. The supernatant was discarded, and 5 mL of cold distilled water and 6.5 mL of 52% perchloric acid were added to the residue and stirred for 15 minutes, followed by

another round of centrifugation (1500 g, 15 min). The supernatant was decanted, and the combined fraction of starch was extracted in a 100-mL beaker. The solution was homogenized and filtered (MCCREADY et al., 1950), and the starch dosage determined following the steps described for soluble carbohydrate content using the phenol-sulfuric method (DUBOIS et al., 1956).

Soluble protein content

Protein was extracted following Garcia et al. (2015). Extraction buffer composed of 20 mM dibasic sodium phosphate (pH 7.5), 1 mM EDTA, 50 mM NaCl, 10% glycerol (v/v), 1 mM PMSF and 1.5% β-mercaptoethanol (v/v) was added to 100 mg samples of dry matter. After centrifugation at 10000 g at 4 °C for 25 minutes, the supernatant was collected. Total protein was precipitated in the presence of absolute ethanol. The supernatant was discarded and the total protein resuspended in a 20 mM solution of dibasic sodium phosphate (pH 7.5). Total protein levels, which served as a reference for the remaining analyses, were quantified spectrophotometrically by the method of Bradford (1976) using bovine serum albumin as a standard (BSA $0 - 800 \, \mu g/mL$, $R^2 = 0.9845$, $y = 2078.7 \, x - 29.82$).

Lipid content

Lipid extraction followed Bligh and Dyer (1959) with modifications by Brum et al. (2009). A 1.5 g fresh sample was extracted with 10 mL of chloroform, 20 ml methanol and 8 ml water with occasional vortex mixing for 30 min, followed by the addition of chloroform and water (1:1). Subsequently, samples were stored at 4 ° C for 1 hour. The upper aqueous layer containing methanol, water and non-lipid compounds was discarded and the lower chloroform layer was filtered through filter paper (80 g / m², porosity 8μm) with 14mM sodium sulfate.

The solvent was rotatory-evaporated at 30 ° C for 5 minutes. Lipid content was calculated based in the initial and final weight of the samples.

Fatty acids

Fatty acid was extracted from 40 uL of total lipid by the addition of 0.7 mL of 10 M KOH in water and 5.3 mL of MeOH. Samples were incubated in a 55 °C water bath for 1.5 h with vigorous hand shaking for 5 s every 20 min to properly permeate, dissolve, and hydrolyze the sample. After the tubes were cooled in a cold tap-water bath for 15 min, 0.58 mL of 12M H_2SO_4 was added. The samples were incubated again in a 55 °C water bath for 1.5 h with hand shaking for 5 s every 20 min. After the tubes were cooled in a cold tap-water bath again, 3 mL of hexane was added and the samples were centrifuged for 5 min at 20 °C. Analysis of fatty acids was performed using gas chromatography (GC 2010 –Shimadzu).

Statistical analysis

The experimental design was completely randomized, with five treatments (development stages) and three replicates for biochemistry variables and 25 seeds of four replicates for the germination test. First, the data were tested for normality (Lilliefors test) and germination results were arcsine transformed. Variance analysis was performed and means compared by SNK's test at 5% significance.

The exploratory techniques of multivariate statistics were applied through the Principal Component Analysis. Variables were standardized, and analysis was performed in R software (R DEVELOPMENT CORE TEAM, 2010).

RESULTS AND DISCUSSION

Recalcitrant seeds have some specific features in contrast to orthodox seeds, as lack of maturation drying (desiccation), with little or no loss of water at the end of seed development (BERJAK et al., 2008; NEWTON et al., 2013; OBROUCHEVA et al., 2016). High values of moisture content were observed in the seed tissues (embryo and megagametophyte) of *A. angustifolia* during all development, however, both tissues showed a slight decrease from earlier stages (Cotyledonary Stage or Stage I) to others stages. The megagametophyte had the highest moisture content (66.3%) at the Cotyledonary Stage and then remained stable from Stage II onwards (48.2%). Similarly, the moisture content of the embryo was close to 63% both Cotyledonary Stage and Stage

I, and decreased at Stage II reaching 53.4% and no difference was observed between others stages (Figure I A). Other studies were also found variation in moisture content both tissue of *A. angustifolia* seed and a decrease with the progress of the seed development (ASTARITA et al., 2003; GARCIA et al., 2012; PANZA et al., 2002).

High values of moisture content maintain active metabolism of seeds and they may still be accumulating dry matter up to the time of their dispersal (NEWTON et al., 2013; OBROUCHEVA et al., 2016). Our results showed that *A. angustifolia* embryo exhibit a continuing dry matter accumulation up to the time of seed shedding, with 134 mg.embryo^{-1} at Stage IV. However, megagametophyte reached maximum to the dry matter at Stage II and it remained stable until Stage IV (around 4.8 g.seed^{-1}). Seeds of *A. angustifolia* have a reserve tissue that alone represents a large proportion of water and dry matter content (GARCIA et al., 2012) and thus, maximum dry matter accumulation at Stage II, indicate that the seeds have reached maturity, however, the embryo continued to store reserves. It is probable that these reserves came from the megagametophyte.

For many species, maximum accumulation of dry matter indicates interruption of assimilating translocation from mother plant to seed and, in this point, seeds acquire the maximum germination (DE SOUZA et al., 2018; SILVA et al., 2017). During development, seed germination at Cotyledonary Stage was lower (46%) than Stage I, II, III (around 88%). At Stage IV an increase to 99% was observed (Figure 2C). Germination test also revealed differences in germination speed by T_{50} (Figure 2D). Late stages showed rapid germination and low T_{50}, with 5 and 6 days at Stage III and IV, respectively. Thus, the continuum accumulation of dry matter up to Stage IV of embryo seem to collaborate with a rapid and high seed's germination. However, these effect in germination depends on amount and types of seed reserves (SORIANO et al., 2014; ZHAO et al., 2018).

The main reserves which make up the dry matter of seeds include carbohydrates, proteins and oils present within both embryo and extra-embryonic tissues (BEWLEY et al., 2013). In *A. angustifolia* tissues were observed starch as the large reserve content, while proteins and mainly lipids represents only a small proportion. In others studies with microscopy analysis of *A. angustifolia* revealed the presence of starch, protein and lipid in embryo cells, however, in megagametophyte, the starch is the most abundance reserve, although proteins and lipid bodies are also present (PANZA et al., 2002; ROGGE-RENNER et al., 2013). These reserves showed different accumulate during seed development of *A. angustifolia* and their metabolism seem to be linked with seeds physiology.
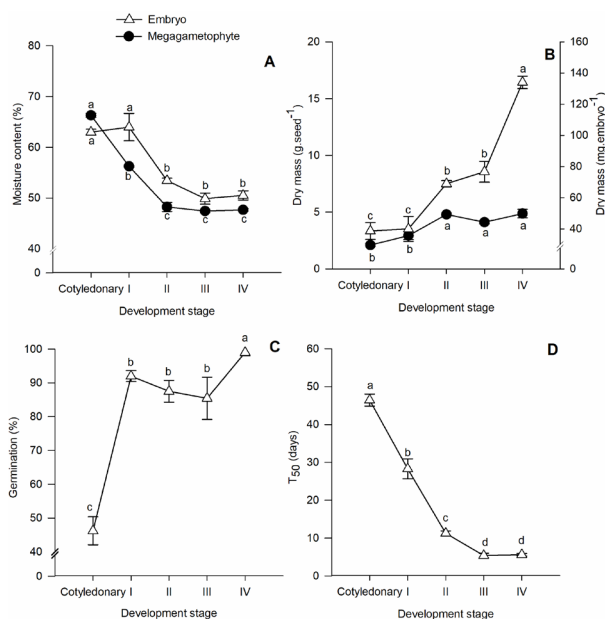
FIGURE 2 (A) Moisture content (mean ± SE) and (B) dry matter (mean ± SE) of embryo (Δ) and megagametophyte (●), (C) germination (mean ± SE) and (D) time to 50% germination (T50) (mean ± SE) of *Araucaria angustifolia* at Cotyledonary Stage, Stage I, II, III and IV. Means followed by same letter do not differ by SNK's test (P < 0.05). Note differences of dry matter and length of the embryo at different development stage (B) and the highest values of germination and lowest T50 at later stages.FIGURE 3(A) starch, (B) Soluble carbohydrate and (C) protein of embryo (Δ) and megagametophyte (●) of *Araucaria angustifolia* at different development stages. Means followed by same letter do not differ by SNK's test (P < 0.05).

The starch content remained stable from Cotyledonary Stage to Stage III, around 350.4 and 367.1 mg·g^{-1} in embryo and megagametophyte, respectively (Figure 3A). At Stage IV, an increase to 465.2 (embryo) and 519.7 mg·g^{-1} (megagametophyte) was found, despite dry matter in nutritive tissue remained stable from Stage II onwards. Higher starch content was also found at Mature and Cotyledonary Stage (also referred to cotyledonal) in *A. angustifolia* embryo, however this compound was prevalent in the megagametophytes at late embryogenesis (NAVARRO et al., 2017). For other recalcitrant seeds, as *Inga vera*, Caccere et al. (2013) observed a continuous accumulation of starch in the embryonic axis with the greatest accumulation occurring in stage IV (350 mg·g^{-1}). In *Hevea brasiliensis* seeds, an increase in starch in the embryo from 2 mg·g^{-1} to 5.5 mg·g^{-1} was also reported (BONOME et al., 2011). The embryo utilizes sugars released by starch degradation for its growth (YAN et al., 2014), that it will act as the energy source mainly imbibition phase (ZHAO et al., 2018).

Others reserves, such as soluble carbohydrate can also collaborate with germination (ZHAO et al., 2018) and play a fundamental role in seed development (AGUIRRE
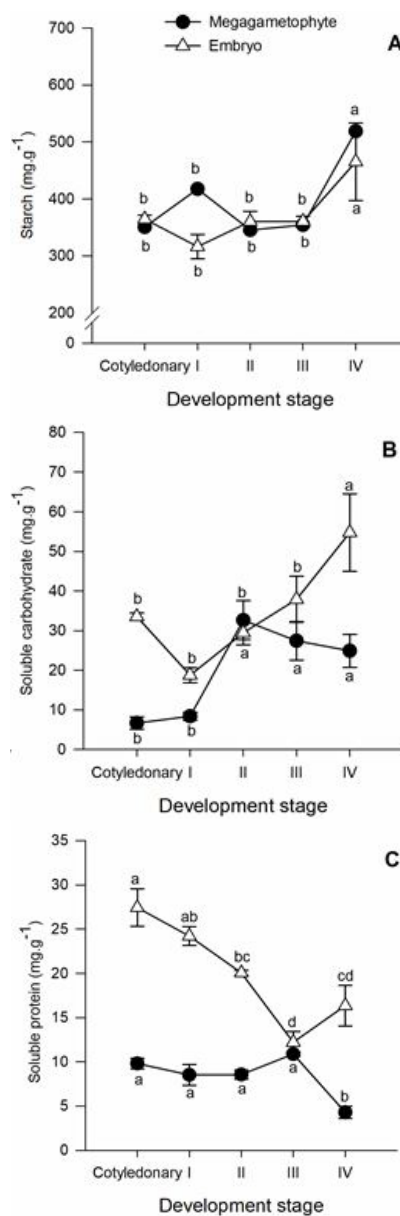

FIGURE 3 (A) starch, (B) Soluble carbohydrate and (C) protein of embryo (Δ) and megagametophyte (●) of *Araucaria angustifolia* at different development stages. Means followed by same letter do not differ by SNK's test (P < 0.05).

et al., 2018; KESARI et al., 2011). *A. angustifolia* embryo had the highest soluble carbohydrate content at Stage IV (54.7 mg·g^{-1}), while the megagametophyte showed an increased from the Cotyledonary Stage (6.7 mg·g^{-1}) to stage II (32.6 mg·g^{-1}) (Figure 3B). It is possible that mobilization of reserves from the megagametophyte to the embryo was occurring at Stage IV. Such a mobilization may be associated with the preparation of the embryo for the beginning of germination because, usually, soluble sugars are used as a source of energy at the beginning of

this process (NKANG, 2002). Thus, the highest content of soluble carbohydrate at Stage IV in embryo collaborated to faster germination in these stages, because they ensure an adequate supply of energy and necessary reserve for the developing seedling. An increasing of soluble carbohydrate was also observed in progress of seed development of *Hevea brasiliensis* (BONOME et al., 2011; DE SOUZA et al., 2018). Similarly, Navarro et al. (2017) found higher soluble carbohydrates (sucrose and raffinose) content in *A. angustifolia* mature zygotic embryos and lower content in megagametophyte. Additionally, another soluble reserve that also enhanced at Stage IV, were the soluble proteins.

The proteins have been considered characteristic markers of the maturity of the zygotic embryo in angiosperms and gymnosperms (DUNSTAN et al., 1998) and they also serve as a substrate for the germination process. During seed development of *A. angustifolia*, protein decreased from 9.5 to 4.3 mg.g^{-1} in the megagametophyte from the cotyledonary stage to stage IV. The embryo exhibited a significant decrease in protein, reaching 12.2 mg.g^{-1} at stage III (Figure 3 C), these changes suggest that proteins are being broken-down into amino acids and peptides so that these components can be used to build new tissue or to participate in the respiratory chain of reactions. Balbuena et al. (2009) reported a decrease in protein from late cotyledonary stage to mature stage in *A. angustifolia* zygotic embryos, with 19 μg.mg^{-1} and 12 μg.mg^{-1}, respectively. This decrease is similar to what has been reported for other recalcitrant species, such as *Durio zibethinus*, where the protein content declined markedly before of the fruit abscission (BROWN et al., 2001). Despite these decrease for some recalcitrant seeds, *A. angustifolia* showed a soluble protein content transitions, with an increase in the embryo and a decrease in the megagametophyte at Stage IV. This event coincides with the highest soluble carbohydrates content and dry matter in the embryo. Possibly, preparatory events to fast germination can occur prior seed dispersion and these enabled fast germination of *A. angustifolia* in the last stage.

In addition to carbohydrates and protein, *A. angustifolia* seeds have lipids content, but in low amount, around 1% (ABE et al., 2010; GONÇALVES et al., 2014; SILVA et al., 2016). This compound is most abundant in the megagametophyte in conifer seeds (GRIGOVÁ et al., 2007; KRUPEK et al., 2010). Thus, only *A. angustifolia* megagametophyte was analyzed at different stages of seed development.

The lipid content in *A. angustifolia* was low, around 2%, and no differences were found during seed development. Large quantities of unsaturated fatty acids (mainly oleic and linoleic acid) was present at all development stages, representing more 75% of components lipids (Table 1). During seed development, linoleic and oleic acids increased from Cotyledonary Stage to others stages, reaching the highest values at Stage III with 68.75 and 7.84%, respectively. Among the saturated fatty acids were observed major proportions of palmitic acids, with 15.89% at Cotyledonary Stage and a gradual decrease thereafter to 13.07% (Stage IV). Similarly, Silva et al. (2016) were found that the major fatty acids of *A. angustifolia* seeds were linoleic, oleic and palmitic acids.

TABLE 1 Fatty acids (%) composition of *Araucaria angustifolia* megagametophyte at different development stages (n = 3, means ± SE).

Component	Development stage				
	Cotyledonary	I	II	III	IV
Total lipids	2.3 ± 1.15	2.20 ± 2.77	2.17 ± 2.29	2.09 ± 0.75	1.94 ± 3.44
Fatty acids					
Palmitic acid	15.89 ± 0.01	11.85 ± 2.33	15.60 ± 0.10	13.14 ± 0.45	13.07 ± 0.65
Stearic acid	1.58 ± 0.03	1.35 ± 0.34	1.87 ± -.17	1.55 ± 0.05	1.27 ± 0.11
Total saturated	17.47	13.2	17.47	14.69	14.34
Oleic acid	12.54 ± 0.06	11.27 ± 0.34	8.69 ± 0.27	7.84 ± 0.03	8.54 ± 0.21
Linoleic acid	63.13 ± 0.26	66.93 ± 1.96	63.61 ± 0.10	68.75 ± 3.46	66.90 ± 0.73
Linolenic acid	1.30 ± 0.06	1.73 ± 0.06	2.40± 0.28	2.92 ± 0.21	2.36 ± 0.19
Eicosenoic acid	0.52 ± 0.01	0.57 ± 0.20	0.93 ± 0.17	0.85 ± 0.06	0.66 ± 0.09
Total insatured	77.49	80.50	75.61	80.36	78.46
Others	5.04± 0.11	6.29 ± 0.65	6.90 ± 0.21	4.95 ± 4.04	7.21 ± 0.14

Many studies have reported unsaturated fatty acids to maintain membrane lipid fluidity during low temperatures (CHEN et al., 2014). Furthermore, seeds with higher unsaturated fatty acids content may germinate more rapidly and earlier at low temperatures, due to unsaturated fatty acids hold much lower melting points (LINDER, 2000).

Thus, the higher proportion of unsaturated fatty acids can avoid possible damages after seed dispersal of *A. angustifolia*, since that their shedding occurs between the end of autumn and winter (MATTOS, 2011) and to contribute to faster germination during this period. Other species that have recalcitrant behavior and usually is shedding in autumn is *Quercus robur*, their seeds were also showed higher levels of oleic and linoleic acids (COLVILLE et al., 2012). Thereby, unsaturated fatty acids might act as protection against the low-temperature during seeds dispersion, in the autumn or winter seasons, but for this confirmation, ecological studies should be performed.

To visualize the information obtained by biochemical and physiological variables in each

development stage, the principal component analysis (PCA) were performed. This analysis showed a separation of samples and explained of 63.9% of the total variance, with 51.2% and 12.7% for component 1 and component 2, respectively (Figure 4). Component 1 was influenced by T_{50} and moisture content of megagametophyte, and for component 2, in turn, the starch and soluble protein of megagametophyte were the most important variables. Early stages have higher values for moisture content of megagametophyte with 66.31% (Cotyledonary Stage) and 56.26 % (Stage I) and these stages also showed longer time to germination (T_{50}). Distinct groups according to their similarities can be observed, where Cotyledonary Stage and Stage I formed two groups to the left of the axis and three groups formed by Stages II, III and IV to the right of component 1.
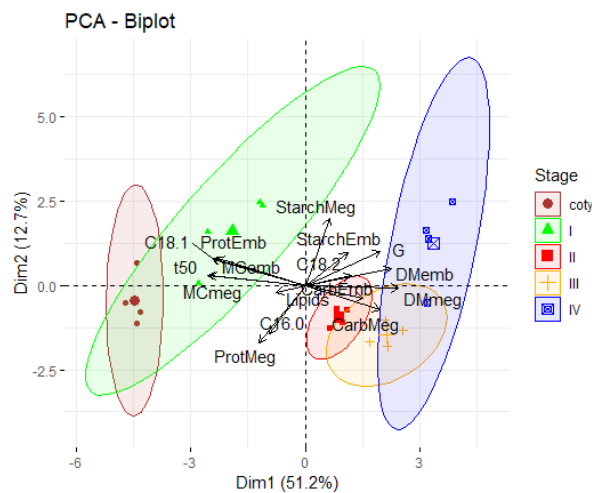

FIGURE 4 Ordination diagram on axes 1 and 2 achieved by Principal Component Analysis, representing 66.5% of the original variability. Biplot graph showing the physiological and biochemistry performance at Cotyledonary Stage (coty - •), Stage I (▲), II (■), III (+) and IV(▣). Abbreviations: soluble carbohydrate of embryo (CarbEmb) and megagametophyte (CarbMeg); dry mass of embryo (DMemb) and megagametophyte (DMmeg); germination (G); linoleic acid (C18:2); lipids content (Lipids); moisture content of embryo (MCemb) and megagametophyte (MCmeg); oleic acid (C18:1); protein content of embryo (ProtEmb) and megagametophyte (ProtMeg); starch content of embryo (StarchEmb) and megagametophyte (StarchMeg); time to 50% germination (t50).

In summary, the water content of the both tissues decrease from Cotyledonary Stage to Stage II and the maximum dry matter content in the megagametophyte occurred at Stage II. However, the embryo continued to accumulate dry matter until the seed dispersion (Stage IV), indicating that reserves were being mobilized from the megagametophyte to the embryo. Dry matter of *A.*

angustifolia seeds is formed a large quantifies by starch that increased at Stage IV, collaborating for increase dry matter of embryo at Stage IV. In this stage were also observed an increase in soluble carbohydrate and soluble protein in the embryo. When all variables were summarized by PCA, were possible noted a separation between of early stages (Cotyledonary and Stage I) and others stages. The results obtained in this study seem to indicate a metabolic change between early and late stages, with a transition from developmental metabolism to germination metabolism, and consequently, such events contributed to faster and higher germination at Stage III and IV and it highlights how maturation stage is the crucial phase for recalcitrant seed. In this moment, the seeds maint high metabolic activity, unlike orthodox seeds, which to contribute to a short lifespan. A comprehension over control in this stage is essential to better seed conservation, mainly for endangered species and considerable ecological and economic importance, as *A. angustifolia*.

CONCLUSIONS

In *A. angustifolia* seeds had starch as the large reserve content, while proteins and mainly lipids represent only a small proportion. Dry matter in megagametophyte was stable from Stage II onwards, however, the embryo showed the highest dry matter and soluble carbohydrate content and an increase soluble protein content at Stage IV and these compounds seem to collaborate to faster and higher germination in this stage.

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