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Enzyme activity and reserve mobilization during Macaw palm (*Acrocomia aculeata*) seed germination

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ABSTRACT.

Reserve mobilization in seeds occurs after visible germination, which is marked by the protrusion of the radicle or cotyledonary petiole, as in species of Arecaceae. *Acrocomia aculeata* (macaw palm), usually produces hard seeds whose endosperm has mannan-rich cell walls. We investigated the composition of storage compounds in macaw palm seed and the roles of two enzymes (endo-β-mannanase, α-galactosidase) during and after germination. The seeds were firstly submitted to pre-established protocol to overcome dormancy and promote germination. Enzyme activity in both embryo and endosperm were assayed from the initiation of germinative activities until leaf sheath appearance, and the status of seed structures and reserve compounds were evaluated. Protein content of the embryo decreased with the initiation of imbibition while the lipid content began decreasing six days after removal of the operculum. Increases in enzyme activity and starch content were both observed after visible germination. We suggest that endo-β-mannanase and α-galactosidase become active immediately at germination, facilitating haustorium expansion and providing carbohydrates for initial seedling development. Protein is the first storage compound mobilized during early imbibition, and the observed increase in the starch content of the haustorium was related to lipid degradation in that organ and mannan degradation in the adjacent endosperm.

Keywords: α-galactosidase, Arecaceae, endo-β-mannanase, galactomannan, lipid, palm seed, protein, starch

Introduction

Seeds must first imbibe water for germination to occur – which allows metabolism to restart with an overall increase in respiratory activity (Bewley *et al*. 2013). Energy is needed for post-germination events, fueling early developmental stages of the seedling before it becomes autotrophic (Mayer & Shain 1974). Major reserve mobilization therefore occurs after germination *sensu strictu*, when compounds, including hemicellulose, are degraded to provide carbohydrates to the developing tissues (Bewley *et al*. 2013).

Seeds of Arecaceae species can store fatty acids, proteins and, less commonly, starch in granum or in specific organelles (DeMason *et al*. 1983; Alang *et al*. 1988; Buckeridge *et al*. 2000; Panza *et al*. 2004). Some palm species produce seeds with endosperm tissues rich in hemicelluloses (DeMason *et al*. 1983; DeMason 1986; Alang *et al*. 1988), placing them in the hard-seed category (Buckeridge 2010). To access the principal cellular reserves and promote seedling development the cell walls of the surrounding tissues of the embryo must be weakened (DeMason *et al*. 1985). Galactomannan is degraded by enzymes during storage mobilization (Gong *et*

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al. 2005) or during germination, with consequent weakening of the micropilar and adjacent endosperm, allowing embryo protrusion (Reid & Meier 1973). β-mannanase hydrolyses the β(1→4) links between mannose residues of the backbone chain, while α-galactosidase removes galactose side chains by severing α(1→6) links (Nonogaki *et al*. 2007). In palm species, seedling development shows some particularities. From visible germination, cotiledonary petiole grows outside the seed while haustorium increases inside and actively participates on mobilization of storage compounds from endosperm (Alang *et al*. 1988; DeMason *et al*. 1985).

Acrocomia aculeata (macaw palm) is a species that grows in the tropical Americas, producing fruits and seeds with high lipid contents (Hiane *et al*. 2005) that can be used for biofuel production (Aguieiras *et al*. 2014). In addition to storage lipids, macaw palm seeds (endosperm and embryo) contain other storage compounds such as protein (Moura *et al*. 2010); the endosperm has cells with thickened non-cellulosic and non-lignified walls (Moura *et al*. 2010) composed of non-soluble carbohydrates characteristic of mannan – although how macaw palm seeds use these embryo and endosperm storage compounds and how they are mobilized during germination is still unclear. Macaw palms only multiply through seeds (Ellis *et al*. 1985), but their dormancy (Ribeiro *et al*. 2011; Oliveira *et al*. 2013b) will hinder the establishment of future commercial plantations. Thus, a better understanding of seed physiology and initial seedling development will be important to assure successful germination rates, and while the hormonal dynamics involved in overcoming dormancy and allowing the early germination of macaw palm seeds have recently been determined (Bicalho *et al*. 2015; Ribeiro *et al*. 2015), the physiology of reserve mobilization during germination and post-germination events is still poorly understood.

We therefore investigated storage mobilization processes and the roles of the enzymes β-mannanase and α-galactosidase in the germinating embryos and endosperm of macaw palm seeds starting at early imbibition until initial seedling development (emerging of primary root and leaf sheath); their storage compounds were also quantified over time to better understand how embryos mobilize reserves during those events.

Materials and methods

Plant material, germination procedures, sampling, and seed water content

Mature fruits of the macaw palm (*Acrocomia aculeata* (Jacq.) Lodd. ex Mart.) were collected in December of 2010 from 50 plants of a native population located in Montes Claros, Minas Gerais State, Brazil (16º 44' 06" S x 43º 51' 42" W).

For the germination protocol (Tab. 1), the fruits were sorted, the endocarp shells removed and the seeds extracted (using a vise) – and then treated to overcome dormancy and promote germination (Motoike *et al*. 2007. Patent register PI0703180-7 A2) in a protocol consisting of a number of different steps distributed over 7 days that includes sterilization, imbibition, mechanical scarification (operculum removal), and exposure to growth regulators and saline and oxidizing solutions. The seeds were then held in transparent polystyrene boxes with two layers of germination paper humidified with water, covered with lids and kept at 27 °C to germinate. The seeds were maintained under these conditions until the seedlings completed their initial developmental steps - primary root and leaf sheath appearance (Fig.1), for a total of 29 days (Tab. 1).

Sampling of the embryo, haustorium, and adjacent endosperm (surrounding embryo or haustorium) tissues were performed before, during, and after the period comprising the treatments to overcome dormancy as described above: i) Embryos – were sampled during the days before primary root appearance (0, 1, 3, 5, 7, 10 and 13 days); ii) Haustorium – sampled during the days after primary root appearance (22 and 29 days) ; iii) Endosperm – during the entire period, although on specific days: 0 (dry seeds, before treatment), 1, 3, 5 and 7, 10, 13, 22 and 29. All of the samples (n = 4) were immediately frozen in liquid nitrogen and kept at -80 °C until analyzed.

The seeds (five replicates of 15 seeds) were weighted daily during the first 15 days of the germination protocol to monitor their water contents (%), following Brasil (2009).

Biochemical analyses
Storage compound quantifications

Lipids, starch, and proteins were quantified in the embryos (days 0 to 13) and haustorium (days 22 to 29), using 4 replicates. For lipid quantification, 1g of sample (n=4) were dried, powdered in a blender and the lipids subsequently cold-extracted with hexane by the gravimetric method using Soxhlet equipment as described by Ataíde *et al*. (2013). For starch quantification, 0.2g of the dried samples (n=4) were digested in 35% perchloric acid, followed by phenol-sulfuric method (Dubois *et al*. 1956), and measured spectrophotometrically at 490 nm; the results were adjusted using the glycosidic linkage correction factor (0.93) (FAO 2003). Protein extraction was performed on 0.1g of fresh samples (n=4) in 100 mM sodium acetate buffer (pH 7); quantification followed the Bradford (1976) method, with spectrophotometric measurements at 595 nm.

Mannan concentration was determined in endosperm samples adjacent to the haustorium on days 10, 13, 22 and 29 of the germination protocol, which corresponded to events from cotyledonary petiole protrusion (visible germination) to the appearance of the primary root and leaf sheath. After drying, mannan was extracted from 0.2g of the samples (n=4) using deionized water in a thermostatic

Table 1. Phases, days, steps and events of sampling during germination procedure of macaw palm seeds (*Acrocomia aculeata*).

	Phase	Days	Step	Event	Sampling material		
					Endosperm	Embryo	Haustorium[3]
Germination procedure	Pre-treatment[1]	0	Endocarp rupture	Seed extraction (dry seed)	✓	✓	
	Treatment to overcome dormancy	1	Immersion in NaClO solution (5%)	Seed disinfestation	✓	✓	
		2	Immersion in H_2O_2 solution (0.03%)	Seed imbibition			
		3			✓	✓	
		4					
		5			✓	✓	
		6					
		7	Scarification and Immersion in gibberellin solution (0.5%)	Operculum removal	✓	✓	
	Sowing	8					
	After Sowing	10		Cotiledonary petiole appearance (visible germination)	✓	✓	
		13		Growth of cotiledonary petiole	✓	✓	
		22		Primary root appearance[2]	✓		✓
		29		Leaf sheath appearance[2]	✓		✓

[1] Pre-treatment phase comprises the period before seeds were submitted to the dormancy-breaking protocol, from fruit harvest up to seed acquirement.

[2] Events marking the initial seedling development (according to Ribeiro et al. 2012)

[3] In dry macaw palm seeds (after dispersion) embryo is a single structure in which cotiledonary petiole and haustorium are fused. During germination, both cotiledonary petiole and haustorium develop different tissues with different functions (see Ribeiro *et al*. 2012).

bath at 90 °C during 2 hours. The filtrate was then diluted in 90% ethanol, lyophilized, and the mannan digested with 2 M trifluoroacetic acid at 120 °C at 1.5 atm, neutralized with 2.5 M ammonium hydroxide, and solubilized in deionized water. Mannan derivatization followed the alditol-acetate method (Englyst & Cummings 1984). Samples were injected into a Shimadzu 14AS GC, using mannose and galactose (Sigma) as standards. The monosaccharide content was adjusted using the glycosidic linkage correction factor (0.93) (FAO 2003).

All storage compound contents are expressed as milligrams per gram of dry weight (mg g^{-1} DW) of embryo / haustorium or endosperm.

Enzymatic assays

Fresh samples of embryo, haustorium, or adjacent endosperm (0.1g/each) were previously frozen in liquid nitrogen. Then were homogenized with 1.5 mL of 100 mM sodium acetate buffer (pH 5) and centrifuged at 9000 g during 20 min at 4 °C. The supernatant was used as the enzymatic extract of β-mannanase and α-galactosidase.

The β-mannanase assay followed Gusakov *et al*. (2011), with adaptations, in which 700 µL of 0.5% guar gum substrate and 300 µL of the enzyme extract were incubated in a thermostatic bath at 37 °C. The reactions were halted at one minute intervals during 30 minutes by adding 1 mL of 1% dinitrosalicylic acid (DNS) (Miller 1959); the mixture was then brought to a boil, cooled, and measured spectrophotometrically at 550 nm. One unit of the enzyme was defined as the equivalent quantity of reducing sugars formed per minute of reaction per gram of fresh matter.

The α-galactosidase assay followed Guimarães *et al*. (2001), in which a mixture of 250 µL of 2mM ρNPGal (*para*-nitrophenyl α-d-galactopyranoside) substrate, 730 µL of extraction buffer (100 mM sodium acetate, pH 5), and 20 µL of the enzyme extract were incubated in thermostatic bath at 37 °C during 15 minutes. The reactions were halted at one-minute intervals with 1 mL of 500 mM sodium carbonate (Na_2CO_3) and measured spectrophotometrically

at 410 nm. One unit of the enzyme was defined as the equivalent necessary to produce 1 μmol of ρ-nitrophenyl per minute per gram of fresh matter.

Data analysis

The significance level was set at $P < 0.05$ in one-way ANOVA, taking the "days of exposure to the germination protocol" as the only factor in all biochemical analysis. When significant differences were found, the Tukey post-hoc comparison was applied to determine individual differences between the means.

Results

Imbibition and germination of macaw palm seeds

The initial water content of the seeds was 6.3% (dry seeds – day 0), increasing until the end of the imbibition period and reaching 31% of the seed fresh weight at day 9. The dormancy-breaking protocol promoted 80% (± 5%) germination (cotyledonary petiole appearance – visible germination) three days after operculum removal.

Main morphological changes during macaw palm seed germination and early seedling development

Macaw palm dried seeds – before imbibition, day 0 is shown in Fig. 1A. After whole imbibition, day 6 (Fig. 1B), seeds presented tumid endosperm and embryo. The visible germination, on day 10, was characterized by cotiledonary petiole appearance (Fig. 1C). From this event onward, we verified seedling development. By day 13, the cotyledonary petiole had elongated to approximately 3 mm beyond the seed (Fig. 1D); the primary root appeared at day 22 (Fig. 1E). Day 29 (21 days after operculum removal) was characterized by the appearance of the leaf sheath (Fig. 1F). The haustorium expanded inside the endosperm from day 10 onward, and notably during the events of initial seedling development (Fig. 1C-F).

Lipid, protein and starch dynamics during germination and post germination events

Embryos from dry seeds showed 470 mg g^{-1} DW (47%) of lipids, which remained unchanged until the day 13, when the growth of the cotyledonary petiole was evident. From that day onward, lipid concentration decreased by 26% in relation to day 0, reaching 350 mg g^{-1} DW ($P < 0.01$; Fig. 2). Proteins composed initially 18 mg g^{-1} DW of embryo dry weight on day 0. The content of protein gradually diminished during imbibition period, and more pronouncedly from day 13 onward (cotyledonary petiole appearance and early seedling development), attaining an average of 6 mg g^{-1} DW (33% of the initial levels) by day 29 ($P < 0.01$; Fig. 2). The dynamics of the starch content were different from those of lipids and proteins – with starch accumulation rather than consumption. The starch content remained at ~40 mg g^{-1} DW from day 0 to 10 (imbibition and visible germination), although from day 13 onward (growth of cotyledonary petiole until leaf sheath appearance) the starch content gradually increased to a final average of 120 mg g^{-1} DW ($P < 0.01$; Fig. 2).

Mannan degradation during germination and early seedling development

β-mannanase activity was detected in both the embryo (days 0 to 13) or haustorium (days 22 and 29) and adjacent endosperm during all of the days of the germination process (Fig. 3). In the embryo/haustorium, β-mannanase activity remained at low levels during seed imbibition, only increasing significantly at day 29 ($P < 0.01$). In the adjacent endosperm, this increase of β-mannanase occurred earlier, from day 13 onward (i.e., from visible germination until initial seedling development), with enzyme activity being 3.5-fold greater than that during imbibition (0 to 10 days; $P < 0.01$; Fig. 3). α-galactosidase activity was likewise observed in both structures analyzed, and demonstrated the same dynamics as β-mannanase, although at lower levels ($P < 0.01$) in both the embryo, haustorium and endosperm (Fig. 3).

Both mannose and galactose were detected in the endosperm from days 10 to 29, although with notable differences in their proportions and dynamics (Tab. 2). Mannose contents were approx.31.3 mg g^{-1} DW on day 10, significantly decreasing to 0.89 mg g^{-1} DW on day 29 ($P = 0.01$). Galactose content, on the other hand, was initially 6.2 mg g^{-1} DW and remained without significant variation during all of the sampling days (Tab. 2).

Discussion

We have reported here the dynamics of macaw palm embryo reserve mobilization as well as the relationships between cell wall degrading enzymes and early seedling development. Initially, macaw palm embryos showed lipids and proteins as the main storage compounds, as has been observed in other Arecaceae species (Alang *et al*. 1988; DeMason 1988; Panza *et al*. 2009). Protein content decreased in the macaw palm embryos during late imbibition events and, more pronouncedly, during the emergence of seedling structures from the seed and their growth, as also reported by Oliveira *et al*. (2013a) for seeds of *Butia capitata*. According to Murray *et al*. (1979), protein degradation in embryos precedes protein mobilization

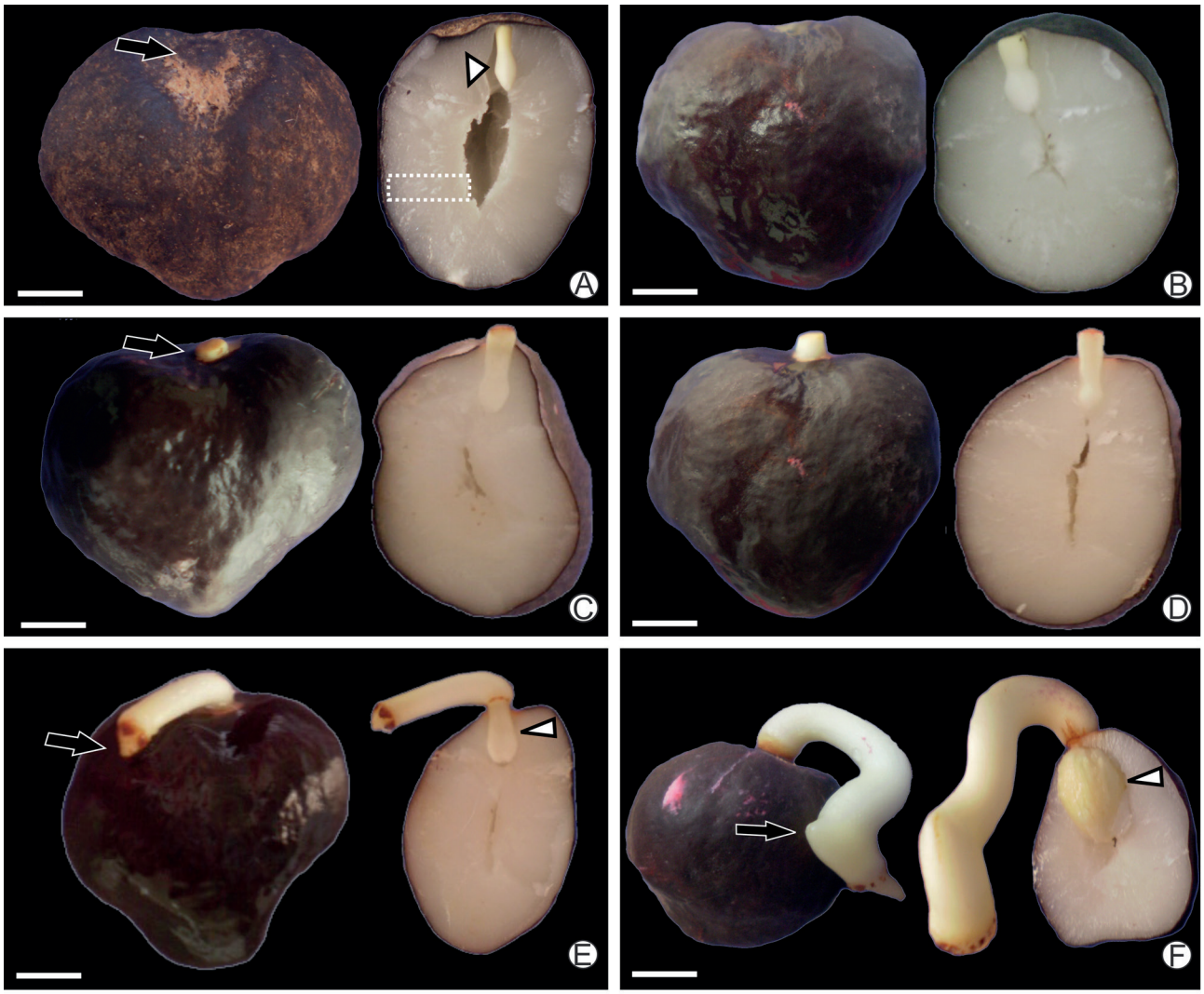

Figure 1. Stages of macaw palm seed germination process and early seedling development. A – dry seeds (day 0), black arrow, operculum; white arrow, embryo; trace, endosperm; B – imbibed seed and operculum removal (day 7); C –visible germination, 3 days after operculum removal (day 10), black arrow, cotyledonary petiole; D – initial growth of cotiledonary petiole, 6 days after operculum removal (day 13); E – Elongation of cotiledonary petiole and primary root appearance (black arrow) 15 days after operculum removal (day 22), white arrow, haustorium; F –Leaf sheath appearance (black arrow), 21 days after operculum removal (day 29), note the haustorium (white arrow) enlargement the initial seedling development. Bar: 0.5cm.

in storage tissues (cotyledons), as proteins are necessary for the first events during imbibition – providing carbon skeletons for carbohydrates, amino acids, and enzymes (Bewley *et al*. 2013).

Lipids accounted for almost 50% of dry weight of macaw palm embryos, making this structure as rich in lipids as the endosperm (Hiane *et al*. 2005). Although lipids were the major reserve compound in embryos, lipid content there only diminished during the events of initial seedling development (day 22 and 29), overlapping the period of haustorium expansion inside the seed. Similarly, lipid stores in *Elaeis guineensis* embryos were catabolized during later seedling development, when haustorium had enlarged inside the seed (Alang *et al*. 1988). Therefore, lipid mobilization occurs during seedling establishment but is not essential for seed germination itself (Graham 2008).

The low starch content observed in the embryo from day 0 to 10 indicated that this compound is not a significant reserve compound in macaw palm seeds, as has been reported for other Arecaceae species (DeMason *et al*. 1983; Alang *et al*. 1988; Buckeridge *et al*. 2000; Panza *et al*. 2004). Starch likewise was not detected by histochemical tests in the embryos, nor in the in endosperm tissues of macaw palm seeds (Moura *et al*. 2010). During early seedling development events (day 22 to 29) there was a considerable increase in starch content as opposed to lipid content, indicating the gluconeogenic origin of the polysaccharides. Gluconeogenesis is common in oilseeds, and is induced after visible germination (Graham 2008). Starch formation and accumulation during seedling development has likewise

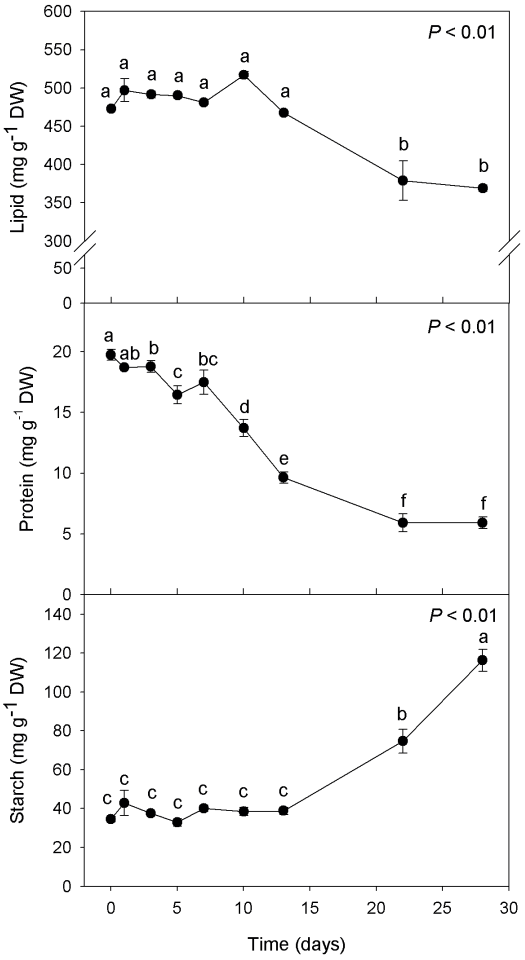

Figure 2. Lipid, protein and starch concentration (mg g^{-1} DW) in macaw palm seed embryos and haustorium during germination process. Circles are mean ± SE. Means followed by the same letter in each graphic do not differ each other by Tukey post-hoc comparisons ($P < 0.05$).

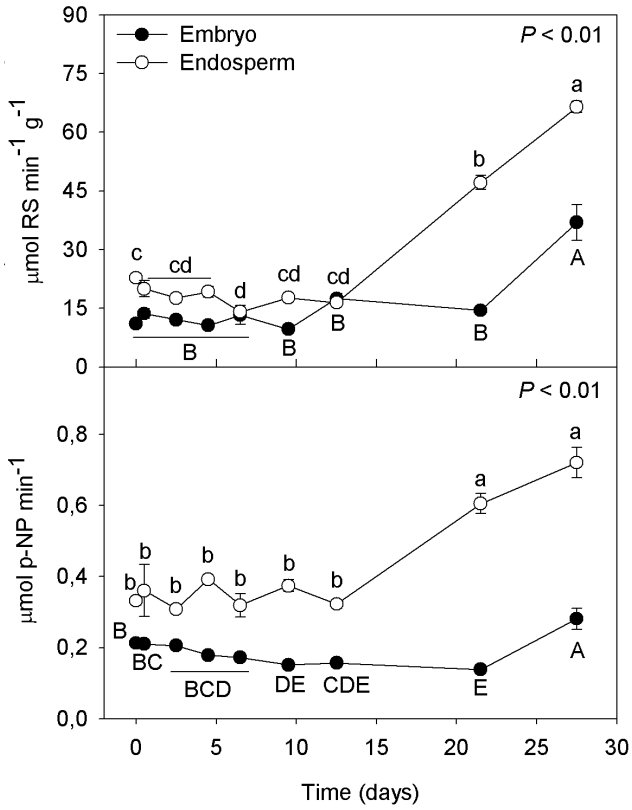

Figure 3. Endo-β-mannanase and α-galactosidase activities on embryo, haustorium and adjacent endosperm during macaw palm seed germination and early seedling development. Circles are mean ± SE. Means followed by the same lowercase do not differ each other by Tukey post-hoc comparison for endosperm analysis. Means followed by the same uppercase do not differ each other by Tukey post-hoc comparison for embryo analysis ($P < 0.05$). RS: reducing sugar; ρNP: *para*-nitrophenyl α-d-galactopyranoside.

Table 2. Monosaccharides concentration (mannose and galactose) found in adjacent endosperm of macaw palm seeds in the days of the germination procedure after operculum removal. Values are means ± SE. Means followed by identical letters in the same column do not differ each other by Tukey post-hoc comparison (P < 0.05).

Days of germination procedure (Sampling time, days)	Mannose (mg.g^{-1} DW)	Galactose (mg.g^{-1} DW)
10	31.3±3.2 a	6.2±1.3
13	18.3±4.2 a	5.1±1.1
22	1.96±0.5 b	3.8±2.3
29	0.89±0.6 b	2.2±1.1

been reported in other oily palms, *viz.*, *E. guineensis* (Oo & Stumpf 1983), *Cocos nucifera* (Sugimuma & Murakami 1990), and *B. capitata* (Oliveira *et al*. 2013a).

Because of the abundant mannose and galactose contents in the endosperm tissue, we assume that galactomannan is a storage compound for macaw palm seeds, and Moura *et al*. (2010) suggested that the cell walls of macaw palm endosperm were composed of a type of mannan. Seed mannans and galactomannans are, in fact, important reserve compounds in the endosperm cell walls of many Arecaceae species because of this conversion into living sugars and possibly involvement in gluconeogenesis (Alang *et al*. 1988; Buckeridge *et al*. 2000; Buckeridge 2010). The decrease in mannose content in the endosperm noted here during initial seedling development coincided with increasing β-mannanase activity and haustorium expansion. In some hard seeds, galactomannans present in the endosperm cell wall are preferentially degraded before other major reserves (such as lipids; Oo & Stumpf 1983) during germination (Buckeridge 2010) or haustorium expansion (Alang *et al*. 1988; Balasubramaniam *et al*. 1973). In some Arecaceae species, the haustorium expands inside the seed while secreting enzymes that degrade endosperm storage compounds to furnish energy for germination and early seedling growth (Oo & Stumpf 1983). Although β-mannanase activity has been related to overcoming

dormancy in the seeds of some species (Bewley 1997; Gong *et al*. 2005; Queiroz *et al*. 2012), our results indicated the important role of this enzyme in storage mobilization and endosperm weakening – allowing haustorium expansion during seedling development (a post-germination event).

Despite the fact that no alterations in galactose contents were observed during the first 29 days of germination, increases in α-galactosidase activity accompanied that of β-mannanase, with both enzymes acting concomitantly on galactomannan degradation during endosperm weakening (Bewley *et al*. 2013). The activities of both enzymes increased by day 29 (leaf sheath appearance) in macaw palm embryos, and were probably more related to seedling growth and development than reserve mobilization.

Macaw palm embryos were shown here to have distinct storage compounds that are mobilized at different times. Lipids are the major reserve and are mobilized during early seedling growth and development. Storage proteins are also present, and their mobilization starts earlier, at imbibition, and continues up until shoot emergence. The expansion of both the haustorium and cotyledonary petiole (day 10 onward) appeared to have induced storage reserve mobilization and increased β-mannanase and α-galactosidase activities. Storage mobilization in macaw palm seeds therefore appears to involve both the embryo and endosperm to provide enough energy for seedling development.

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