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Original Paper

Reserve mobilisation exhibits a biphasic pattern during seedling establishment in the Caatinga pioneer species *Pityrocarpa moniliformis*

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Abstract

Reserve mobilisation and metabolite utilisation were characterised during seed germination and seedling establishment in *Pityrocarpa moniliformis*, an endospermic legume from Caatinga. Seeds were germinated under controlled conditions for four days and seedlings were transferred to hydroponics and maintained at a greenhouse during four days. Samples were collected at distinct physiological stages and reserves, metabolites, and enzymatic activities were determined in different seedling parts. Galactomannans stored in the endosperm and non-reducing sugars accumulated in the cotyledons were mobilised from seed germination to hypocotyl emergence. During these processes, the accumulation of reducing sugars in the endosperm coincided with an increase in the starch content in the cotyledons and seedling axis, indicating that sugars released from galactomannans may have been utilised in starch biosynthesis. Starch and storage protein mobilisation in the cotyledons occurred later, from hypocotyl elongation to cotyledon expansion. Starch degradation possibly supported the accumulation of starch and soluble sugars in the root, while storage protein hydrolysis was associated with cotyledon greening and first leaf emergence. Accordingly, reserve mobilisation exhibits a biphasic pattern, enabling fast seedling establishment as a physiological strategy to ensure environmental colonisation.

Key words: hydrolytic enzymes, seed germination, soluble metabolites, stored reserves, tree legume

Resumo

A mobilização de reservas e a utilização de metabólitos foram caracterizadas durante a germinação da semente e o estabelecimento da plântula em *Pityrocarpa moniliformis*, uma leguminosa endospérmica da Caatinga. As sementes foram germinadas sob condições controladas por quatro dias e as plântulas foram transferidas para hidroponia e mantidas em casa de vegetação durante quatro dias. As coletas foram realizadas em estágios fisiológicos distintos e reservas, metabólitos e atividades enzimáticas foram determinadas nas diferentes partes da plântula. Galactomananas armazenadas no endosperma e açúcares não redutores acumulados nos cotilédones foram mobilizados da germinação da semente à emergência do hipocótilo. Durante estes processos, a acumulação de açúcares redutores no endosperma coincidiu com o aumento do conteúdo de amido nos cotilédones e no eixo da plântula, indicando que os açúcares liberados das galactomananas podem ter sido utilizados na biossíntese do amido. A mobilização do amido e das proteínas de reserva nos cotilédones ocorreu mais tarde, do alongamento do hipocótilo à expansão dos cotilédones. A degradação do amido possivelmente sustentou a acumulação de amido e açúcares solúveis na raiz, enquanto a hidrólise das proteínas de reserva foi associada à acumulação de clorofilas nos cotilédones e à emergência da primeira folha. Assim, a mobilização das reservas exibe um padrão bifásico, permitindo o rápido estabelecimento da plântula como uma estratégia fisiológica para garantir a colonização do ambiente.

Palavras-chave: enzimas hidrolíticas, germinação da semente, metabólitos solúveis, reservas armazenadas, leguminosa arbórea.

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Introduction

During seed germination and seedling establishment, reserves are mobilised in the storage tissues, producing metabolites that are transported to the embryo or seedling axis. In the growing tissues, these metabolites are consumed as cellular fuels to maintain the ATP supply and are also utilised as biosynthetic precursors to enable cell repair, proliferation, and growth (Bewley *et al*. 2013). Thus, reserve mobilisation supports seedling growth until the root system and the photosynthetic apparatus are sufficiently developed for autotrophic conditions (Kitajima & Myers 2008).

In forest ecosystems, reserve mobilisation plays a central role in gap colonisation by woody species (Kidson & Westoby 2000; El-Keblawy *et al*. 2018). Pioneer species generally produce large numbers of small seeds with low reserve content, whereas many shade-tolerant species are characterised by producing a small number of large seeds that store a high content of reserves (Melo *et al*. 2004). Therefore, species that produce small seeds tend to rapidly establish in forest gaps, supported by photosynthetic cotyledons, while species having large seeds resist the carbon deficit imposed by shading through reserve cotyledons (Kidson & Westoby 2000; Baraloto *et al*. 2005; El-Keblawy *et al*. 2018).

Among the woody species commonly found in tropical forests, tree legumes (Fabaceae) are evident not only for their abundance, but also for the diversity of reserves stored in their seeds (Buckeridge *et al*. 2000; Gulewicz *et al*. 2014). Legume seeds are generally rich in storage proteins, as *Andira parviflora* Ducke and *Hymenaea parviflora* Huber (Gonçalves *et al*. 2002) from the Amazonic Forest and *Acacia farnesiana* (L.) Willd, *Mimosa arenosa* (Willd.) Poir, *Senna spectabilis* (DC.) H.S.Irwin & Barneby (Mayworm *et al*. 1998), *Albizia lebbeck* (L.) Benth, and *Enterolobium contortisiliquum* (Vell.) Morong (Carvalho *et al*. 2011) found in the Caatinga. Some tree legumes produce oilseeds, including *Caesalpinia echinata* Lam. (Mello *et al*. 2010) and *Caesalpinia peltophoroides* Benth. (Corte *et al*. 2006) from the Atlantic Forest, and the Caatinga native species *Caesalpinia pyramidalis* Tul., *Poecilanthe ulei* (Harms) Arroyo & Rudd (Mayworm *et al*. 1998), and *Lonchocarpus sericeus* (Poir.) Kunth *ex* DC. (Carvalho *et al*. 2011).

Polysaccharides stored in the seeds of tree legumes are diversified in terms of structure and localisation. In non-endospermic legumes, cotyledons accumulate starch granules in amyloplasts or xyloglucans in thickened cell walls (Bewley *et al*. 2013). *A. parviflora* (Gonçalves *et al*. 2002), *C. echinata*, and *Inga vera* Willd. produce starchy seeds (Mello *et al*. 2010), whereas the Atlantic Forest species *Copaifera langsdorffii* Desf. and *Hymenaea courbaril* L. produce seeds with a high content of xyloglucans (Franco *et al*. 1996). In endospermic legumes, galactomannans deposited in thickened cell walls are the major reserve in the endosperm (Buckeridge *et al*. 2000; Sharma *et al*. 2021). Galactomannans have been characterised in many exotic species, such as *Prosopis juliflora* (Sw.) DC. (Vieira *et al*. 2007), *Cassia pleurocarpa* F. Muell. (Singh *et al*. 2009), *Sesbania* spp. (Pollard *et al*. 2011), *Delonix regia* (Bojer *ex* Hook.) Raf. (Bento *et al*. 2013), and *Senna tora* L. (Harshal & Lalitha 2014), as well as in native species, including *Cassia grandis* L.f. and *Senna reticulata* (Willd.) H.S.Irwin & Barneby from the Amazonic Forest (Buckeridge *et al*. 1995b), *Schizolobium paraybae* (Vell.) Blake. (Ganter *et al*. 1993) from the Atlantic Forest, *Stryphnodendron barbatiman* (Vell.) Mart. (Ganter *et al*. 1993), and *Bowdichia virgilioides* Kunth (Buckeridge *et al*. 1995b) from the Cerrado.

Some efforts have been made to elucidate the process of reserve mobilisation in native tree legumes. Indeed, galactomannan mobilisation is well understood in economically used species, like *Ceratonia siliqua* L. (Spyropoulos & Lambiris 1980), *Cyamopsis tetragonolobus* (L.) Taub. (McCleary 1983; Singh *et al*. 1987), *Trigonella foenum-graecum* L. (Dirk *et al*. 1999), and *Trigonella persica* Boiss. (Bakhshy *et al*. 2019). This process has been also characterised in *Dimorphandra mollis* Benth. from the Cerrado (Buckeridge *et al*. 1995a), *Apuleia leiocarpa* (Vogel) J.F. Macbr. (Pontes *et al*. 2002), *S. paraybae* (Petkowicz *et al*. 2007) from the Atlantic Forest, and *Erytrina velutina* Willd. from the Caatinga (Reis *et al*. 2012).

Considering the representativity of tree legumes in the Caatinga, as well as the ecological and economic importance of this biome (Santos *et al*. 2008; Apgaua *et al*. 2014; Silva *et al*. 2014), initiatives to elucidate how the reserves are utilised during seed germination and seedling establishment are essential to uncover the physiological strategies employed by these species to colonise the environment (Kidson & Westoby 2000; Baraloto *et al*. 2005). These strategies explain, at least in part, the mechanisms of ecological succession and

allow the development of techniques applied to the restoration of degraded areas (Borges *et al*. 2002).

Pityrocarpa moniliformis (Benth) Luckow & R.W. Jobson (Jobson & Luckow 2007), previously denominated *Piptadenia moniliformis* (Benth) is an endospermic legume native to the Caatinga (Lorenzi 2002). As a pioneer species, *P. moniliformis* is a rustic and fast-growing tree that is recommended for heterogeneous reforestation with preservationist purposes (Azerêdo *et al*. 2010; Benedito *et al*. 2011). Beyond its ecological importance, *P. moniliformis* is economically used, providing wood, firewood, coal, and feed; it also presents medicinal properties and honey potential (Azerêdo *et al*. 2010, 2011).

Taking into account that studies on reserve mobilisation in legume species emphasise the degradation of storage carbohydrates, especially galactomannans in exotic species with economic importance (Spyropoulos & Lambiris 1980; McCleary 1983; Singh *et al*. 1987; Dirk *et al*. 1999; Bakhshy *et al*. 2019), the literature lacks efforts to elucidate not only the degradation of different reserves but also the utilisation of produced metabolites in native species. Therefore, this work aims to characterise the mobilisation of different reserves and how the resulting metabolites are used during seed germination and seedling establishment in *P. moniliforms*. In this way, we intend to contribute to the comprehension of physiological strategies that allow this pioneer species to colonise stressful environments.

Materials and Methods

Plant material

Mature pods of *P. moniliformis* were collected from different mother trees located in Nisía Floresta, Rio Grande do Norte, Brazil (06°5012.4”S, 35°1104”W). These pods were opened manually for the removal of seeds, which were stored in plastic bags and kept under refrigeration (Benedito *et al*. 2011). To overcome seed coat-imposed dormancy, seeds were mechanically scarified in the region opposite to the hilum (Benedito *et al*. 2008). As determined by previous tests, seeds were washed in a diluted solution of commercial detergent (1:500), rinsed in running tap water, and surface-sterilized with 70% (v/v) ethanol for 30 s followed by 0.25% (w/v) NaClO for 3 min. After that, seeds were washed three times and imbibed for 1 h in sterile distilled water.

After imbibition, seeds were sown on towel paper humidified with sterile distilled water in the proportion of 2.5 mL per gram of dry paper, arranged in rolls (Vieira & Carvalho 1994), and incubated in a controlled environment (80 µmol $m^{-2}s^{-1}$ of photosynthetically active radiation, 12 h photoperiod, and 28 ± 2 °C) for 4 d. Then, four-day-old seedlings were transferred to plastic pots containing distilled water and cultivated in a greenhouse for 4 d to assess reserve mobilisation in the absence of external nutrient inputs. At this physiological stage, the seedlings exhibited a size that allowed them to be transferred to the hydroponic system; as the cotyledons were still covered by the testa, the seedlings were also protected against desiccation.

Samples were collected at different physiological stages: freshly-imbibed seeds (stage I), 1 to 2 d imbibed seeds (stage II), germinated seeds - radicle protrusion (stage III), hypocotyl emergence - 5 to 10 mm seedling axis (stage IV), hypocotyl elongation - 25 to 30 mm seedling axis (stage V), cotyledon emergence - testa falling (stage VI), expanded cotyledons (stage VII), and first leaf emergence (stage VIII), as shown in Fig. 1. The cotyledons were collected at all stages. As the testa and endosperm could not be separated, the testa/endosperm were collected until stage VI. The axis was collected from stages III to V, while the root and hypocotyl were separated from stages VI to VIII. Samples containing approximately 200 mg of cotyledons and 100 mg of the other seedling parts were frozen and maintained at -20 °C until biochemical quantifications.

Neutral lipids

Neutral Lipids (NL) were measured by the gravimetric method (Soxhlet 1879). Samples of dry cotyledons were extracted with 2 mL of n-hexane at 60 °C for 5 h with occasional stirring. After evaporation of n-hexane at 80 °C, the NL content was expressed as mg $cotyledon^{-1}$.

Soluble proteins

Soluble proteins were extracted from cotyledons by maceration with 100 mM Tris-HCl buffer pH 7.0 supplemented with 500 mM NaCl and 2 mM 2-mercaptoethanol. After centrifugation at 10.000 xg for 10 min, the supernatants were collected, and the precipitates were re-extracted twice with the extraction buffer. These steps

were carried out at 4 °C. Soluble proteins were extracted from the other seedling parts with 100 mM Tris-HCl buffer pH 7.0 following the same procedures (Barros-Galvão *et al*. 2017). The soluble protein content was determined according to Bradford (1976) using bovine serum albumin as a standard and expressed as mg part^{-1}.

Soluble metabolites

Soluble metabolites, including total soluble sugars (TSS), non-reducing sugars (NRS), and total free amino acids (TFAA) were extracted from frozen samples with 80% (v/v) ethanol at 60 °C in sealed tubes. The supernatants were collected, and the residues were re-extracted under the same conditions (McCready *et al*. 1950). The final residues were reserved and used for the extraction and determination of starch.

TSS were determined according to Morris (1948), using the anthrone reagent (Yemm & Willis 1954) and D-glucose as a standard. NRS were quantified by the anthrone method with modifications as described by Van Handel (1968), utilising a sucrose standard curve. TFAA were estimated according to Yemm & Cocking (1955), using the ninhydrin reagent, based on a standard curve of L-glutamine. The content of all soluble metabolites was calculated and expressed as µmol g^{-1} dry weight (DW).

Starch

Starch was determined using the residues from the extraction of soluble metabolites. These residues were macerated with chilled 30% (v/v) percloric acid and centrifuged at 10.000 xg for 10 min. The supernatants were reserved, and the precipitates were re-extracted twice. Starch was quantified with the anthrone reagent, utilising a D-glucose standard curve. Values were multiplied by 0.9 to convert glucose into starch (McCready *et al*. 1950) and the starch content was expressed in mg part^{-1}.

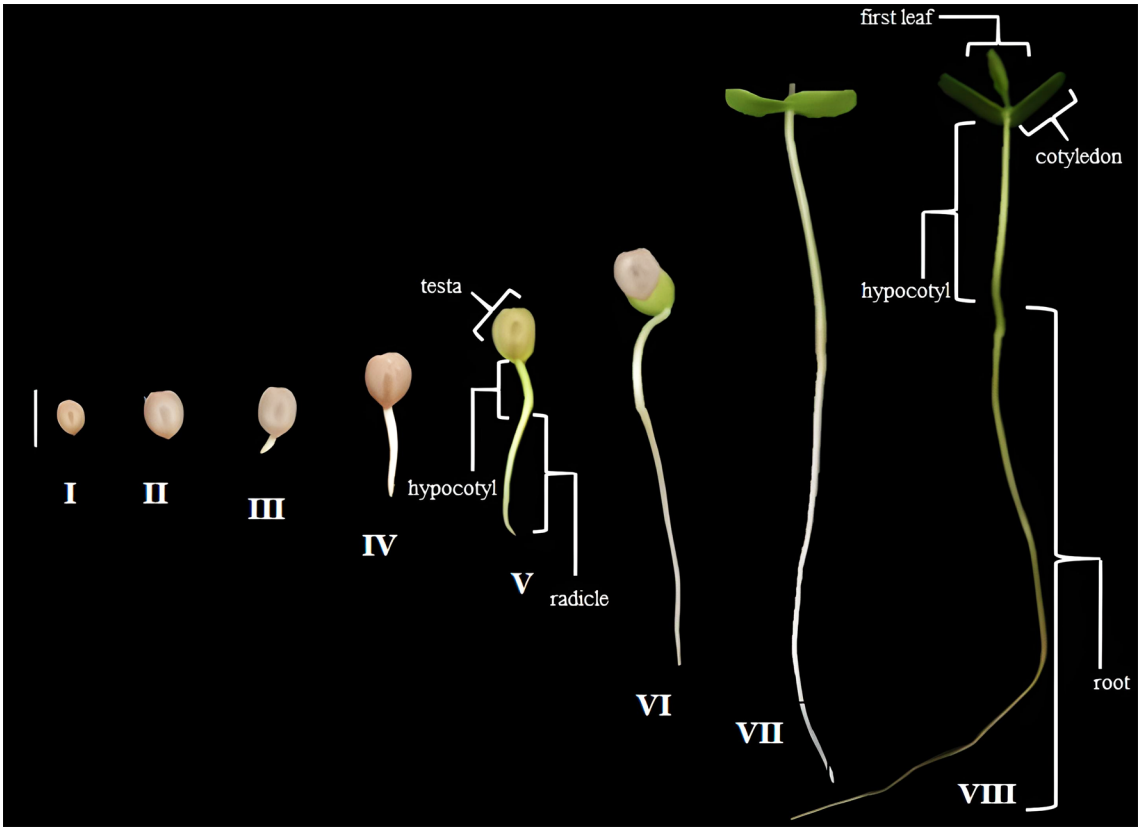

Figure 1 – Morphology of *Pityrocarpa moniliformis* during seed germination (stages I to III) and seedling establishment (stages IV to VIII). The vertical bar represents 10 mm.

Cell wall storage polysaccharides

Water-soluble cell wall storage polysaccharides (CWSP) were extracted from seed coat/endosperm samples by maceration with distilled water at room temperature followed by incubation at 80 °C for 2 h. After centrifugation at 10.000 xg for 20 min, the supernatants were reserved, and the pellets were re-extracted twice (Buckeridge *et al*. 1995b). The CWSP were determined with the anthrone assay (Yemm & Willis 1954), using D-glucose as a standard, and the results were expressed in mg $part^{-1}$.

Enzyme activities

The activity of amylases was estimated by the release of reducing sugars (RS) using soluble starch as a substrate (Elarbi *et al*. 2009). Amylases were extracted from 100 mg of frozen cotyledons, which were macerated with 100 mM potassium acetate buffer pH 6.0 containing 5 mM $CaCl_2$. After centrifugation at 10.000 xg for 20 min, the supernatants were collected and used as enzymatic extracts. These procedures were performed at 4 °C. In each quantification, 200 µL of extract, 400 µL of 100 mM potassium acetate buffer pH 6.0 containing 5 mM $CaCl_2$ and 0,5% (m/v) soluble starch were mixed and incubated at 55 °C for 10 min. The reaction was stopped on ice. The concentration of RS was determined according to Miller (1959), using the 3,5-dinitro-salicylate reagent and the activity of amylases was expressed as µmol g^{-1} DW min^{-1}.

The activity of acid proteases was estimated by the production of free amino acids from casein as a substrate (Beevers 1968, modified). Samples of 100 mg of frozen cotyledons were macerated with 50 mM tris-HCl buffer pH 7,2 containing 2 mM 2-mercaptoethanol. Samples were centrifuged at 10.000 xg for 20 min and the supernatants were utilised as a source of enzymes. All procedures were carried out at 4 °C. In each determination, 250 µL of extract, 250 µL of 100 mM potassium acetate buffer pH 5.5, and 250 µL of 1% (m/v) casein were mixed and incubated at 60 °C for 60 min. The reaction was stopped by adding 250 µL of 20% (w/v) trichloroacetic acid. The samples were kept on ice for 15 min and then centrifuged at 10.000 xg for 20 min at 4 °C. The concentration of free amino acids in the supernatants was measured as described by Yemm & Cocking (1955), utilising the ninhydrin reagent. The activity of acid proteases was expressed as µmol g^{-1} DW min^{-1}.

Experimental design and statistical analysis

During the experiment, the collections were performed randomly, obtaining five replications for each physiological stage. Considering the physiological stages as categories, the results were submitted to ANOVA followed by the Tukey test at a significance level of 5%, using R version 3.6.1. software (R development core team 2011).

Results

The endosperm and the cotyledons supported the growth of the hypocotyl-radicle axis at two distinct moments during the establishment of *P. moniliformis* seedlings. In the seed coat/endosperm, the DW loss started from radicle protrusion, as a 24% decrease in the DW content was observed

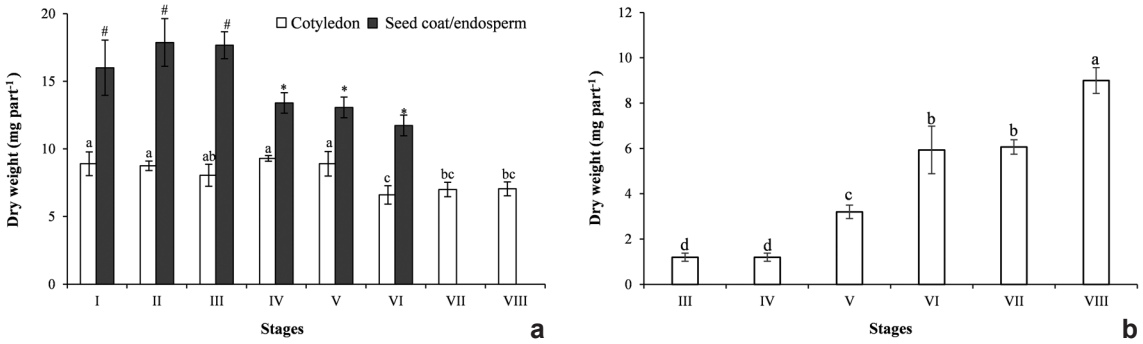

Figure 2 – a-b. Dry weight content in – a. seed coat/endosperm, cotyledons; b. seedling axis of *Pityrocarpa moniliformis* during seed germination (stages I to III) and seedling establishment (stages IV to VIII). The bars represent the mean, and the error bars are the standard deviation of five replicates. The values marked with the same character do not differ significantly according to the Tukey test ($P < 0.05$).

from stage III to IV (Fig. 2a). In the cotyledons, however, the DW loss was evident from hypocotyl elongation, when a 26% decrease in the DW content was verified between stages V and VI (Fig. 2a). The DW accumulation in the hypocotyl-radicle axis showed a biphasic pattern, succeeding the DW loss in the different storage tissues. In fact, the DW content of the axis increased 166% from stage IV to V and 48% from stage VII to VIII (Fig. 2b), evidencing the transference of DW from the endosperm and the cotyledons, respectively.

The mobilisation of water-soluble CWSP in the endosperm occurred from seed germination to early seedling establishment in *P. moniliformis*, as the content of these compounds in the seed coat/endosperm decreased 49% from freshly-imbibed seeds (stage I) to hypocotyl emergence (stage IV) (Fig. 3a). By classic paper chromatography, it was possible to confirm that these water-soluble CWSP were mainly galactomannans (data not shown). In parallel, the content of TSS and NRS increased 7 times and 53%, respectively, from stage I to IV (Fig. 3a), decreasing from then on. It is noteworthy that the galactomannans were intensively mobilised during the stages III and IV, accompanying a peak of the TSS content. Significant contents of soluble proteins and TFAA in the seed coat/endosperm were not observed (data not shown).

The content of starch in the cotyledons increased 60% from stage I to V (Fig. 3b), coinciding with the peak of TSS in the seed coat/endosperm (Fig. 3a) and a 50 and 73% decrease in the content of TSS and NRS in the cotyledons, respectively, from freshly-imbibed seeds (stage I) to hypocotyl elongation (stage V) (Fig. 3c). These results evidenced a probable interchange and interconversion of carbohydrates between these storage tissues. In addition, the activity of amylases doubled from hypocotyl elongation (stage V) to cotyledon emergence (stage VI), when a 30% decrease in the starch content in the cotyledons was verified (Fig. 3b). Thus, the mobilisation of the storage polysaccharides in the endosperm and cotyledons occurred at different moments of seedling establishment. Indeed, the galactomannans were mainly hydrolysed after seed germination

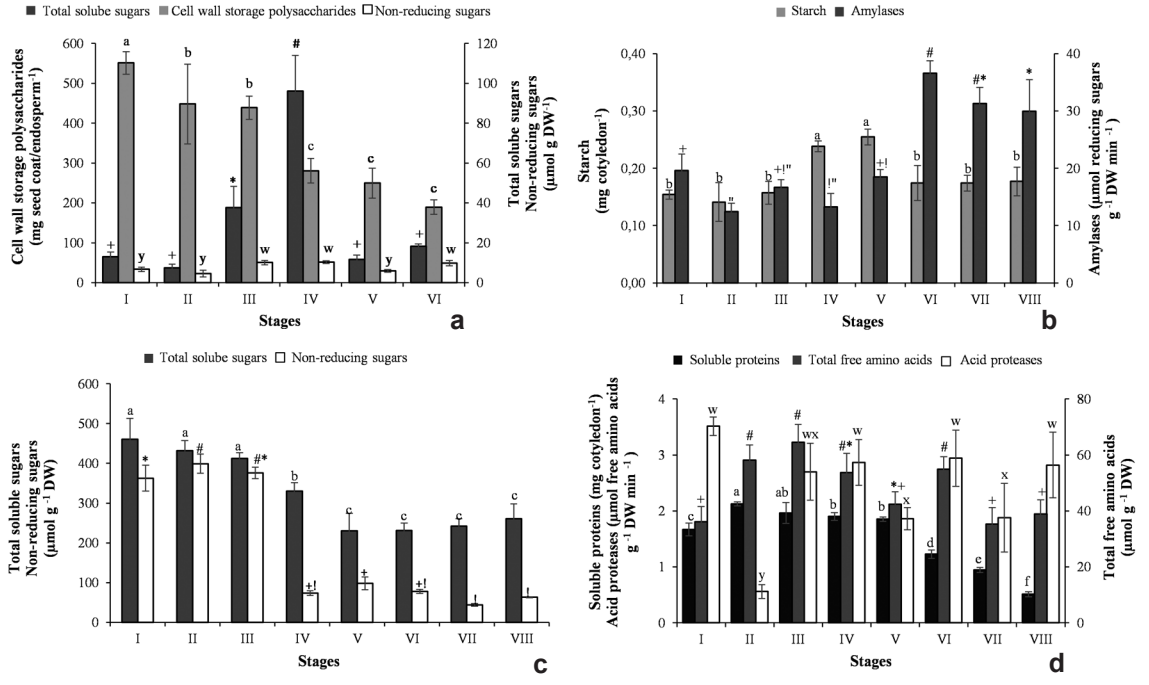

Figure 3 – Content of – a. water-soluble cell wall storage polysaccharides, total soluble sugars, and non-reducing sugars in the seed coat/endosperm; b. starch and activity of amylases; c. total soluble sugars and non-reducing sugars; d. soluble proteins, activity of acid proteases, and content of total free amino acids in the cotyledons of *Pityrocarpa moniliformis* during seed germination (stages I to III) and seedling establishment (stages IV to VIII). The bars represent the mean, and the error bars are the standard deviation of five replicates. The values marked with the same character do not differ significantly according to the Tukey test ($P < 0.05$).

(stages III to IV) (Fig. 3a), whereas starch was predominantly degraded after hypocotyl elongation (stage V) (Fig. 3b). Significant contents of NL were not detected in the cotyledons throughout the experiment (data not shown).

The soluble protein content in the cotyledons decreased 72% from hypocotyl elongation (stage V) to first leaf emergence (stage VIII) (Fig. 3d). Furthermore, the content of TFAA and the activity of acid proteases in the cotyledons showed a peak at radicle protrusion (stage III) and another one at cotyledon emergence (stage VI) (Fig. 3d). It is remarkable that the second peak of TFAA and acid protease activity was associated with storage protein mobilisation.

Changes in the content of starch, soluble sugars, proteins, and free amino acids in the seedling axis showed a biphasic pattern during the establishment of *P. moniliformis* seedlings, in agreement with the mobilisation of galactomannans in the endosperm after seed germination and the mobilisation of starch and storage proteins in the cotyledons during late seedling growth. In the seedling axis, the starch content increased 166% from radicle protrusion (stage III) to hypocotyl elongation (stage V) (Fig. 4a), while the TSS content decreased 60% from stage IV to V (Fig. 4b) and the NRS content decreased 57% between stages III and V (Fig. 4c); these alterations accompanied the axis growth during early seedling establishment (Figs. 1; 2b). In the root, the content of starch (Fig. 4a), TSS (Fig. 4b), and NRS (Fig. 4c) increased 125, 90, and 137%, in that order, from cotyledon emergence (stage VI) to first leaf emergence (stage VIII). During this period, the starch content remained unchanged (Fig. 4a), the TSS content decreased 29% (Fig. 4b), and the NRS content increased 2.6-fold (Fig. 4c) in the hypocotyl. It is noteworthy that variations in carbohydrate accumulation accompanied cotyledon greening and the emergence of the first leaf at late seedling establishment (Fig. 1).

In the seedling axis, the soluble protein content increased 62% from radicle protrusion (stage III) to hypocotyl emergence (stage IV) (Fig. 4d) and the TFAA content increased 2.2-fold between radicle protrusion and hypocotyl elongation (stage V) (Fig. 4e), coinciding with a 31% decrease in the TFAA content in the cotyledons from stage III to V (Fig. 3d). In the hypocotyl, a 57% reduction in the content of soluble proteins took place from cotyledon emergence (stage VI) to first leaf emergence (stage VIII) (Fig. 4d) and

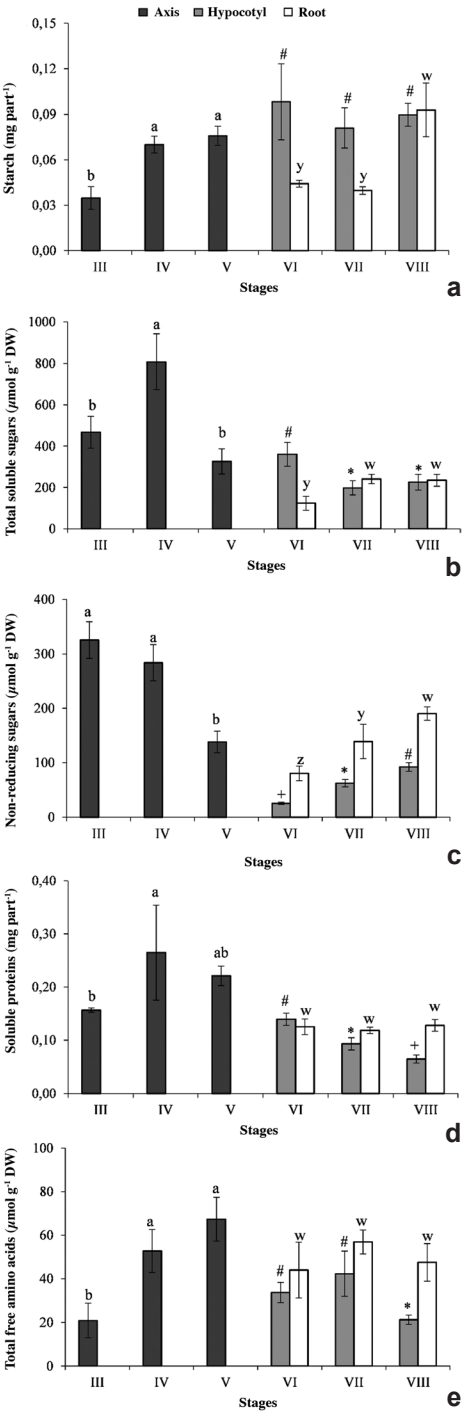

Figure 4 – Content of – a. starch; b. total soluble sugars; c. non-reducing sugars; d. soluble proteins; e. total free amino acids in the seedling axis of *Pityrocarpa moniliformis* during seed germination (stages I to III) and seedling establishment (stages IV to VIII). The bars represent the mean, and the error bars are the standard deviation of five replicates. The values marked with the same character do not differ significantly according to the Tukey test ($P < 0.05$).

a 50% decrease in the TFAA content occurred between cotyledon expansion (stage VII) and stage VIII (Fig. 4e). These changes were associated with storage protein mobilisation in the cotyledons (Fig. 3d) and axis growth (Figs. 1; 2b). No significant alterations were detected in the content of soluble proteins and TFAA in the root during late seedling establishment (from stage VI to VIII) (Fig. 4d-e).

Discussion

Based on the content of storage polysaccharides, soluble sugars, and storage proteins found in freshly-imbibed seeds (stage I), it is possible to verify that *P. moniliformis* seeds store low content of reserves. Indeed, freshly-imbibed seeds contain approximately 2% galactomannans, 1% starch, 10% soluble proteins, and 5% NRS, corresponding to only 18% in a DW basis (data not shown). Among the endospermic legumes, Caesalpinioideae species tend to exhibit seeds containing a high content of galactomannans, whereas Mimosoideae and Faboideae species usually produce seeds poor in these reserves (Buckeridge *et al*. 2000; Bento *et al*. 2013). As a Mimosoideae member, *P. moniliformis* also produces seeds with low content of galactomannans. Although legume seeds are normally rich in storage proteins (Bewley *et al*. 2013), *P. moniliforms* seeds contain only 10% of an enriched fraction of storage proteins in a DW basis. This soluble protein content is notably lower than that found in other Mimosoideae species from the Caatinga, including *A. bahiensis*, *A. farnesiana*, *M. arenosa* (Mayworm *et al*. 1998), *A. lebbeck* and *E. contortisiliquum* (Carvalho *et al*. 2011), which accumulate approximately 28, 55, 44, 42, and 50% total proteins, respectively, as determined by the Kjeldahl method. Since *P. moniliforms* produces a large amount of small and viable seeds annually (Lorenzi 2002), low reserve content may be related to low seed mass, as it has been proved that smaller seeds contain less storage tissues and more protective tissues than larger seeds (Wu *et al*. 2019).

The mobilisation of the different reserves stored in *P. moniliformis* seeds occur at distinct moments during seed germination and seedling establishment. In fact, galactomannan mobilisation starts in the endosperm (Fig. 3a) before radicle protrusion (stage III), while NRS are intensively mobilised in the cotyledons (Fig. 3c) immediately after seed germination. Accordingly, NRS have been recognised as accessible reserves at the first stages of seedling growth due to their small and soluble nature (Rosental *et al*. 2014). In addition, the concomitant mobilisation of galactomannans in the endosperm and raffinose family oligosaccharides in the cotyledons also occurs in other endospermic legumes, such as guar (McCleary 1983; Singh *et al*. 1987), fenugreek (Dirk *et al*. 1999), *D. mollis* (Buckeridge *et al*. 1995a), *S. virgata* (Buckeridge & Dietrich 1996), *Platymiscium pubecens* (Borges *et al*. 2002), and *Peltophorum dubium* (Veronesi *et al*. 2014).

It is noteworthy that galactomannan mobilisation (Fig. 3a) is followed by a peak of TSS (Fig. 3a) in the endosperm of *P. moniliformis* between stages III and V. Taking into account that the NRS content corresponds to only 3.6% of the TSS pool at stage IV, RS are remarkably accumulated in the endosperm. During the hydrolysis of galactomannans in guar (Singh *et al*. 1987), *S. virgata* (Buckeridge & Dietrich 1996), and *P. dubium* (Veronesi *et al*. 2014) the RS content also increases in the endosperm. Additionally, mannose and galactose are accumulated while the content of galactomannans decreases in the endosperm of fenugreek (Dirk *et al*. 1999) and *A. leiocarpa* (Pontes *et al*. 2002). According to this evidence, it is likely that the RS accumulated in the endosperm of *P. moniliformis* are products of galactomannan hydrolysis, which are available for transport to the cotyledons during early seedling establishment, as previously proposed (McCleary 1983; Singh *et al*. 1987; Bakhshy *et al*. 2019).

In parallel with galactomannan mobilisation in the endosperm (Fig. 3a), starch is accumulated while NRS are consumed in the cotyledons (Fig. 3b-c) and axis (Fig. 4a-c) of *P. moniliforms* seedlings from stage III to V. According to these results, it is possible to suggest that RS released from galactomannans are, at least in part, used to produce starch, since starch is accumulated in the cotyledons of guar (Singh *et al*. 1987), in the cotyledons and axis of fenugreek (Dirk *et al*. 1999), and in the embryo of *P. dubium* (Veronesi *et al*. 2014) as a transitory reserve derived from galactomannan mobilisation. As the NRS content markedly decreases in the cotyledons (Fig. 3c) and TSS accumulate in the seedling axis (Fig. 4b) from radicle protrusion to hypocotyl emergence, it is reasonable to assume that part of NRS have been utilised as transport sugars. Moreover, the consumption of TSS (Fig. 4b) and NRS (Fig. 4c) in the seedling axis might have supported its growth from stage IV to V (Figs. 1; 2b).

In contrast to galactomannan degradation, the mobilisation of starch (Fig. 3b) and storage proteins (Fig. 3d) in the cotyledons of *P. moniliformis* seedlings initiates later, from cotyledon emergence

(stage VI). Starch degradation also takes place during late seedling establishment in the cotyledons of guar (Singh *et al*. 1987) and fenugreek (Dirk *et al*. 1999) seedlings. In *P. moniliformis* seedlings, starch mobilisation precedes cotyledon greening (stage VII) (Fig. 1), starch (Fig. 4a) and TSS (Fig. 4b) accumulation in the root, and the increase of NRS content (Fig. 4c) in the root and hypocotyl. Therefore, the accumulation of these carbohydrates in the seedling axis could be attributed to the transference of NRS from the cotyledons, which is possibly derived from both starch mobilisation (Fig. 3b) and photosynthetic activity (Fig. 1).

Although the soluble protein content has not decreased in the cotyledons of *P. moniliformis* seedlings from seed imbibition (stage II) to radicle protrusion (stage III), the activity of acids proteases and the TFAA content increase in these organs during this period (Fig. 3d). These results may be related to protein turnover during seed germination, since it is a developmental transition in which proteins produced at the end of seed maturation are replaced by *de novo* synthetized proteins (Rajjou *et al*. 2012). Moreover, a decrease in the TFAA content in the cotyledons after radicle protrusion (Fig. 3d) is accompanied by the accumulation of soluble proteins (Fig. 4d) and TFAA (Fig. 4e) in the seedling axis until hypocotyl elongation (stage V), evidencing the transference of free amino acids from the source to the sink tissues at early seedling development.

A second peak of TFAA content and acid protease activity in the cotyledons of *P. moniliformis* seedlings is evident between hypocotyl elongation (stage V) and cotyledon expansion (stage VII), which is accompanied by storage protein mobilisation (Fig. 3d). It is known that acid proteases play a central role in the hydrolysis of storage proteins during the transition from storage to lytic vacuoles (Müntz 2007). In the cotyledons of the tree legume *E. velutina*, amino acid accumulation also occurs in parallel with storage protein mobilisation at later stages of seedling establishment (Reis *et al*. 2012). Curiously, the content of soluble proteins (Fig. 4d) and TFAA decreases in the hypocotyl (Fig. 4e) and remains unchanged in the root (Fig. 4e), while storage proteins are hydrolysed in the cotyledons of *P. moniliformis* seedlings at late establishment (Fig. 3d). Soluble proteins and TFAA may have supported the biogenesis of the photosynthetic apparatus in the cotyledons or the growth of the first leaf (Fig. 1).

Our results confirm that seed germination and seedling establishment in *P. moniliformis*,

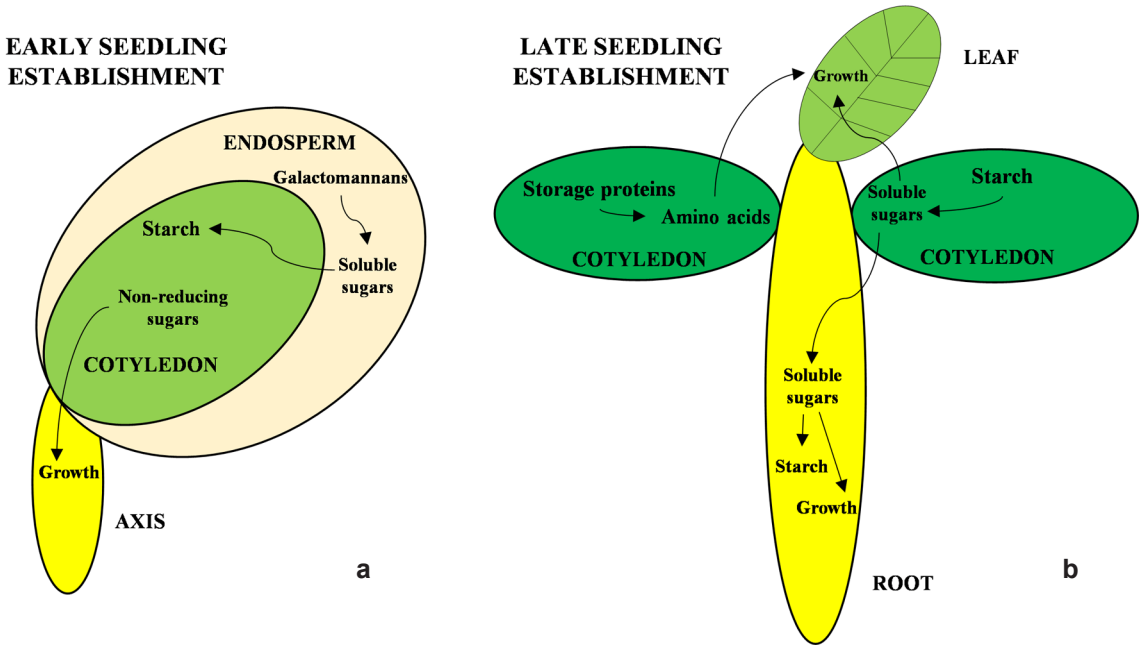

Figure 5 – a-b. Proposed model to summarize the biphasic pattern of reserve mobilisation in *Pityrocarpa moniliformis* – a. early seedling establishment may depend on the degradation of galactomannans in the endosperm and the consumption of non-reducing sugars in the cotyledons; b. late seedling establishment may be supported by the degradation of starch and storage proteins in the cotyledons.

as a typical pioneer species, occur in a few days and a biphasic pattern of reserve mobilisation is observed in the course of these processes (Fig. 5). Galactomannans stored in the endosperm (Fig. 3a) and NRS accumulated in the cotyledons (Fig. 3c) sustain the growth of the embryo axis until approximately 3 d after imbibition, when the hypocotyl emerges (stage VI) (Fig. 5a). From then on, starch (Fig. 3b) and storage protein (Fig. 3d) mobilisation probably supports the transition to autotrophy, as the cotyledons are plenty expanded nearly 8 d after imbibition, when the first leaf emerges (stage VIII) (Fig. 5b). As *P. monilifomis* seeds exhibit small size and limited reserves, the biphasic pattern of reserve mobilisation underlies fast seedling establishment as a physiological strategy to ensure gap colonisation.

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