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An integrative analysis of physiological and biochemical changes during pod and seed development in the tree legume *Acacia mangium*

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Abstract

Seed development has been studied in many woody species due to its role in seed production for afforestation and reforestation programs. Still, which biochemical changes co-occur in fruits and seeds during development are poorly known. Thus, this work is an integrative analysis of physiological and biochemical alterations during pod and seed development in *Acacia mangium*, a fast-growing tree legume cultivated worldwide. Pods were harvested between 30 and 165 days after anthesis, but seeds could be detached from 105 days after anthesis. The content of water, dry weight, non-structural carbohydrates, and soluble proteins was evaluated in both pods and seeds, and the activity of hydrolases was assessed only in pods. Early pod growth was governed by water uptake and was associated with low levels of soluble sugars and soluble proteins and high activity of acid proteases. Late pod growth was marked by reserve deposition, considering the accumulation of non-structural carbohydrates and soluble proteins. The remobilization of these reserves in the pods was preceded by high activity of amylases and acid proteases, and this process may have contributed to reserve deposition in the seeds during late filling, regarding an increase in the levels of starch and soluble proteins. Seed physiological maturity was achieved before harvesting time, as minimum water content and maximum germinability and vigour were attained after the end of dry weight accumulation. Seeds should not be harvested after the end of maturation drying to avoid losses in quality.

Keywords Harvesting time · Physiological maturity · Pod development · Reserve deposition · Seed quality

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Introduction

The degradation of forest ecosystems by anthropic activities, especially in tropical regions, has been related to global warming and climate change (Li et al. 2022). These processes may be mitigated by afforestation and reforestation programs, which can benefit carbon sequestration and water and thermal balance (Rohatyn et al. 2021). Since tree species are usually propagated from seeds, producing high-quality seed lots plays a vital role in afforestation and reforestation programs (Syamsuwida et al. 2020).

Seed quality is a complex feature encompassing different components, including germinability, desiccation tolerance, vigour, and storage life, which are expected to be sequentially acquired during seed development (Bewley et al. 2013). Thus, comprehending this process is fundamental to revealing how seeds attain quality and when they should be harvested. Seed development is usually triggered by egg cell fertilization followed by histodifferentiation, in which the zygote undergoes sequential cell divisions, and the embryo develops its growth patterns. After that, reserve deposition results in seed filling, and water loss marks maturation drying in orthodox seeds (Bareke 2018).

In angiosperms, the seeds typically develop inside the fruit. Besides its roles in seed protection and dispersion, the fruit is also responsible for the transference of nutrients and assimilates from the mother plant to the seeds (Goméz-Martín et al. 2020). Since the fruits and seeds develop in parallel, the fruits are expected to exert physiological and biochemical influences on the acquisition of quality components by the seeds. In dry fruits like pods, the photosynthetic activity of green tissues plays a part in fruit growth and seed filling, whereas fruit maturation culminates in dehiscence and enables seed dispersion (Bennet et al. 2011).

Despite transcriptomic approaches have been recently used to identify genes involved in pod development in herbaceous plants such as *Arabidopsis thaliana* (Gómez et al. 2014), *Brassica napus* (Liu et al. 2015), and *Phaseolus vulgaris* (Gómez-Martín et al. 2020), only a few efforts have been made to characterize which biochemical changes coincide during pod and seed development in woody species. Several studies only describe how growth and colour alterations in pods are related to the acquisition of germinability and vigour by seeds in legume trees like *Erythrina variegata* (Matheus et al. 2011), *Amburana cearensis* (Lopes et al. 2014), *Poincianella pluviosa* (Silva et al. 2015), *Albizia hasslerii* (Ristau et al. 2020), and *Anadenanthera colubrina* (Cruz et al. 2021).

According to this rationale, this work aims to carry out an integrative analysis of physiological and biochemical changes during pod and seed development in *Acacia mangium* Willd. This species is a fast-growing tree legume native to regions of Indonesia, Papua New Guinea, and Australia. It has been introduced to different parts of the world due to its uses in the production of timber, paper, and charcoal (Hegde et al. 2013). *A. mangium* has been utilized in commercial plantations and restoration of degraded areas considering its capacity to sequester carbon, improve soil fertility, and stimulate forest productivity. In addition, *A. mangium* exhibits massive production of long-lived orthodox seeds, characterized by coat-imposed dormancy and heat tolerance (Koutika and Richardson 2019).

Although many features underlying *A. mangium* cultivation have been studied, the literature still lacks pod and seed development information. The experiments presented here assessed the levels of non-structural carbohydrates (NSC, i.e., soluble sugars and starch) and soluble proteins in the pods and seeds as well as the activity of hydrolytic enzymes in the pods to verify possible connections with seed physiological maturity, harvesting time,

and quality. The clarification of biochemical changes during fruit and seed development may not only contribute to the understanding of biological mechanisms but may also assist in the management of seed production.

Materials and methods

Study area and pod harvest

This study was carried out from August 2014 to February 2015 in the forest experimental field (5°54'S and 35°2'W) at the Escola Agrícola de Jundiaí in Macaíba, State of Rio Grande do Norte, North-eastern Brazil. The study area presents a tropical climate with an average annual rainfall of 1,000 mm, mean annual temperature of 27.1 °C, and average relative humidity of 76%; the rainy season extends from March to July (Instituto de Defesa e Meio Ambiente do Rio Grande do Norte - IDEMA 2013). *A. mangium* fruits were harvested from 30 mother trees at 30, 60, 90, 105, 120, 135, 150, and 165 days after anthesis (DAA). It was possible to separate fruits and seeds only from 105 DAA. The developmental stages of fruits and seeds were characterized according to the Munsell (1976) colour chart. Each colour was identified by a code like 5.0 GY 8/8, in which 5.0 GY is the colour, 8 is the lightness/darkness, and 8 is the chroma.

Water content and dry weight

The water content (WC) and dry weight (DW) of fruits and seeds were determined with the low constant temperature oven method as recommended by the International Rules for Seed Testing (International Seed Testing Association – ISTA 2008). After measuring the fresh weight (FW), samples were maintained at 101–105 °C for 17 h and weighed to assess the DW. The WC was calculated on FW basis and expressed in percentage.

Germination tests

The germination tests were performed following the International Rules for Seed Testing (ISTA 2006). Seeds were mechanically scarified on the opposite side of the hilum, surface-sterilized with 2.5% (w/v) sodium hypochlorite for 5 min and washed with sterile distilled water. Afterwards, seeds were placed between towel paper sheets moistened with sterile distilled water (2.5 mL per gram of dry paper) and incubated at 27 ± 2 °C under 12-h photoperiod for 21 days. Germination was evaluated daily; a seed was considered to have germinated if it could produce a normal seedling, i.e., if it had developed roots, hypocotyl, cotyledons, epicotyl, and apical bud. Germination percentage was taken as a marker of germinability (Black et al. 2006), whereas germination speed index (Maguire 1962) and seedling length (Black et al. 2006) were considered markers of seed vigour. Seedling length was measured at the end of the germination tests, when the epicotyl had emerged in normal seedlings.

Soluble metabolites, starch, and soluble proteins

To extract soluble metabolites, samples of fruits and seeds were triturated and exposed to 80% (v/v) ethanol at 60 °C for 30 min in hermetically closed tubes. Supernatants were collected and residues were re-extracted. Total soluble sugars (TSS) were measured with the phenol-sulfuric method (Dubois et al. 1956), employing D-glucose as a standard. Non-reducing sugars (NRS) were determined by the anthrone assay (Van Handel 1968), using a sucrose calibration curve. Total free amino acids (TFAA) were quantified by the ninhydrin method (Yemm and Cooking 1955), utilizing L-glutamine as a standard. The content of these metabolites was expressed as μmol g^{-1} DW.

Starch was extracted from the residues obtained after the removal of soluble metabolites. These residues were macerated with chilled 30% (v/v) perchloric acid and samples were centrifuged at 10,000 x g for 10 min at 4 °C. Supernatants were collected and residues were re-extracted two times. Starch was measured by the anthrone assay (Morris 1948), utilizing a D-glucose calibration curve. The values were multiplied by 0.9 for conversion to starch (McCready et al. 1950) and the content of this reserve was expressed as mg g^{-1} DW.

Soluble proteins were extracted from fruits by maceration with ice-cold 100 mM Tris-HCl buffer pH 7.0. Samples were centrifuged at 10,000 x g for 10 min at 4 °C, supernatants were harvested, and residues were extracted again. The extraction buffer was supplemented with 500 mM NaCl and 200 mM β-mercaptoethanol to obtain an extract enriched in storage proteins from seeds (Barros-Galvão et al. 2017). Soluble proteins were determined with the Coomassie Brilliant Blue reagent (Bradford 1976), using bovine serum albumin as a standard and expressed as mg g^{-1} DW.

Enzyme assays

Amylases were extracted from fruits by maceration with chilled 100 mM potassium acetate buffer pH 6.0 containing 500 mM $CaCl_2$. Extracts were centrifuged at 10,000 x g for 15 min at 4 °C and supernatants were employed as a source of enzymes. In enzyme assays, soluble starch was used as substrate (Elarbi et al. 2009), and free reducing sugars in the reaction medium were quantified with the 3,5-dinitrosalicylate method (Miller 1959), utilizing a D-glucose calibration curve. The activity of amylases was expressed as μg g^{-1} DW min^{-1}.

Acid proteases were extracted from fruits by maceration with ice-cold 50 mM Tris-HCl buffer pH 7.2 supplemented with 200 mM β-mercaptoethanol. Samples were centrifuged at 10,000 x g for 30 min at 4 °C and supernatants were used as enzyme extracts. Enzyme assays were performed with casein as substrate (Beevers 1968) and free amino acids in the reaction medium were determined with the ninhydrin method (Yemm and Cooking 1955). The activity of acid proteases was expressed as μmol g^{-1} DW min^{-1}.

Experimental design and statistical analysis

Composite samples of pods and seeds were randomly obtained from different mother plants during harvests. For determination of the physiological markers and biochemical measurements were used four and five replicates, respectively. The results of each marker (dependent variable) were analysed in function of DAA (independent variable) through linear regression adjusting polynomial models. To choose the best model, the following criteria

(Littell et al. 1991) were respected: type I sum of squares and the probability value for the F test associated with this sum of squares; the coefficient of multiple determination (R^2 and adjusted R^2 for degrees of freedom) and the coefficient of variation (CV). The statistical analyses were performed using R version 3.6.1 software (R development core team 2011).

Results

The pods and seeds of *A. mangium* exhibited simultaneous colour changes during development. The pods were classified as green-yellow 5.0 GY 8/8 (Fig. 1a, b, c, and d) at the early stages (from 30 to 105 DAA), exhibited zones similar to green-yellow 5.0 GY 8/6 and red yellow-red 10. R 7/4 to 7/6 (Fig. 1e) at 120 DAA and acquired a yellowish yellow-red colour 7.5 YR 6/4 (Fig. 1f, g, and h) at the late stages (from 135 to 165 DAA). In turn, the seeds displayed a greenish yellow-green colour 7.5 GY 8/8 (Fig. 1i) at 105 DAA, changed to a greenish yellow colour 7.5 Y 4/4 (Fig. 1j) at 120 DAA, and were classified as red-purple 5.0 RP 3/10 (Fig. 1k, l, and m) at the late stages.

The colour changes observed during the experiment were associated with alterations in the DW and WC of pods and seeds of *A. mangium* (Fig. 2). Pod development presented a typical sigmoidal pattern in terms of DW accumulation. In fact, the pod DW was approximately 1.97 mg at the first harvest (30 DAA), remained almost unchanged until 60 DAA, and increased 200 times from 30 to 105 DAA, when maximum DW was reached (Fig. 2a). In parallel, the pod WC was 28% at 30 DAA, increased to 78% at 60 DAA, and slowly decreased to 61% until 120 DAA, overlapping with DW accumulation; from 120 to 135 DAA, however, the pod WC rapidly decreased to 11% (Fig. 2a), coinciding with colour

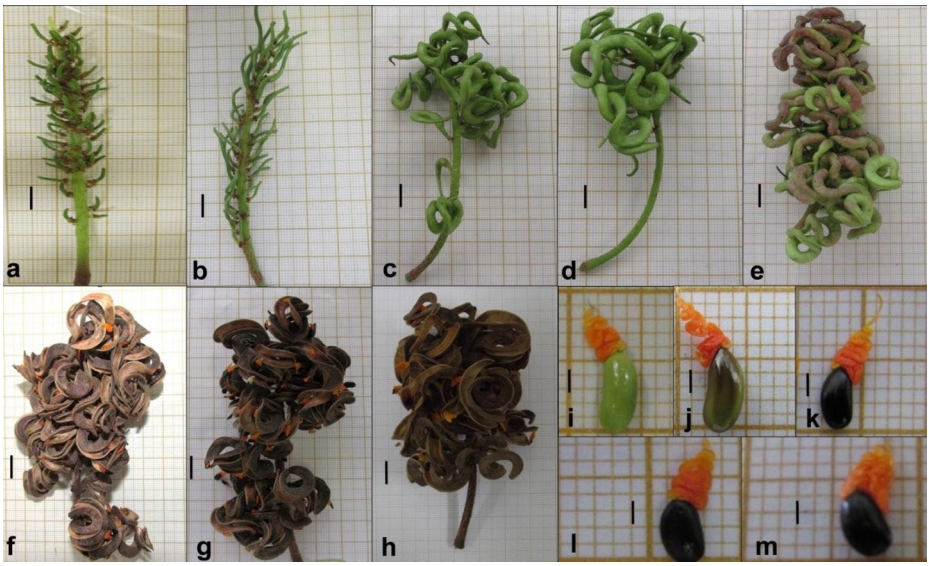

Fig. 1 Morphology of *Acacia mangium* pods and seeds during development. Pods were harvested at 30 (**a**), 60 (**b**), 90 (**c**), 105 (**d**), 120 (**e**), 135 (**f**), 150 (**g**), and 165 (**h**) days after anthesis, and seeds could be separated from pods at 105 (**i**), 120 (**j**), 135 (**k**), 150 (**l**), and 165 (**m**) days after anthesis. Vertical bars correspond to 10 mm

change and dehiscence (Fig. 1f). According to these results, it is suggested that *A. mangium* pods exhibited typical developmental phases, since growth ended by 105 DAA and then maturation became evident, followed by senescence.

Regarding the development of *A. mangium* seeds, DW and WC corresponded to about 6.3 mg and 62%, respectively, at 105 DAA, when seeds could be separated from pods (Fig. 2b). The seed DW increased 57% from 105 to 135 DAA and was not significantly changed until the last harvest (165 DAA), while the seed WC gradually decreased from 105 to 150 DAA, remaining only 3.4% (Fig. 2b). It is noteworthy that maximum seed DW was reached during pod maturation and dehiscence (Fig. 1f) and seed colour change (Fig. 1k). Based on these results, it was possible to assess late seed filling from 105 to 135 DAA and seed maturation drying from then on.

Germinability and vigour were simultaneously acquired by *A. mangium* seeds at the end of seed filling (Fig. 3). Indeed, the seeds harvested at 105 DAA were still green (Fig. 1i) and were unable to germinate (Fig. 3a), whereas those harvested at 120 DAA exhibited chlorophyll loss (Fig. 1j) and 45% germination (Fig. 3a). Maximum germinability (93%) was acquired at 150 DAA (Fig. 3a), coinciding with chlorophyll loss (Fig. 1l) and minimal WC (Fig. 2b). Concerning seed vigour, both germination speed index (Fig. 3b) and seedling length (Fig. 3c) progressively increased from 105 to 150 DAA, when maximum values were registered. Nevertheless, a decrease in germinability (Fig. 3a) and germination speed index (Fig. 3b) was verified at the last harvest (165 DAA), indicating loss of seed quality.

It is notable that the phases of pod development in *A. mangium* exhibited alterations in the content of NSC and soluble proteins. As expected, the growth phase involved reserve deposition; for instance, the content of TSS (Fig. 4a), NRS (Fig. 4a), starch (Fig. 4b), and soluble proteins (Fig. 4c) increased 384, 341, 42, and 419%, respectively, in *A. mangium* pods from

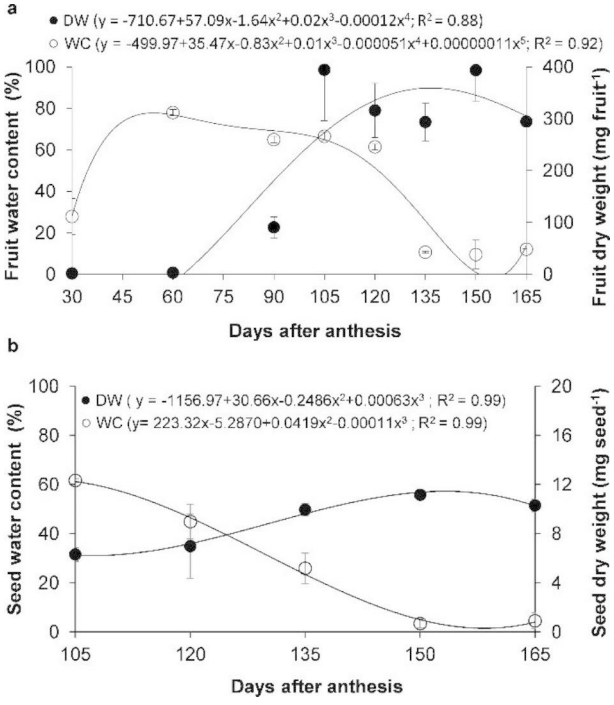

Fig. 2 Water content and dry weight of pods (**a**) and seeds (**b**) of *Acacia mangium* during development. Dots represent means and vertical bars represent standard deviation of four replicates

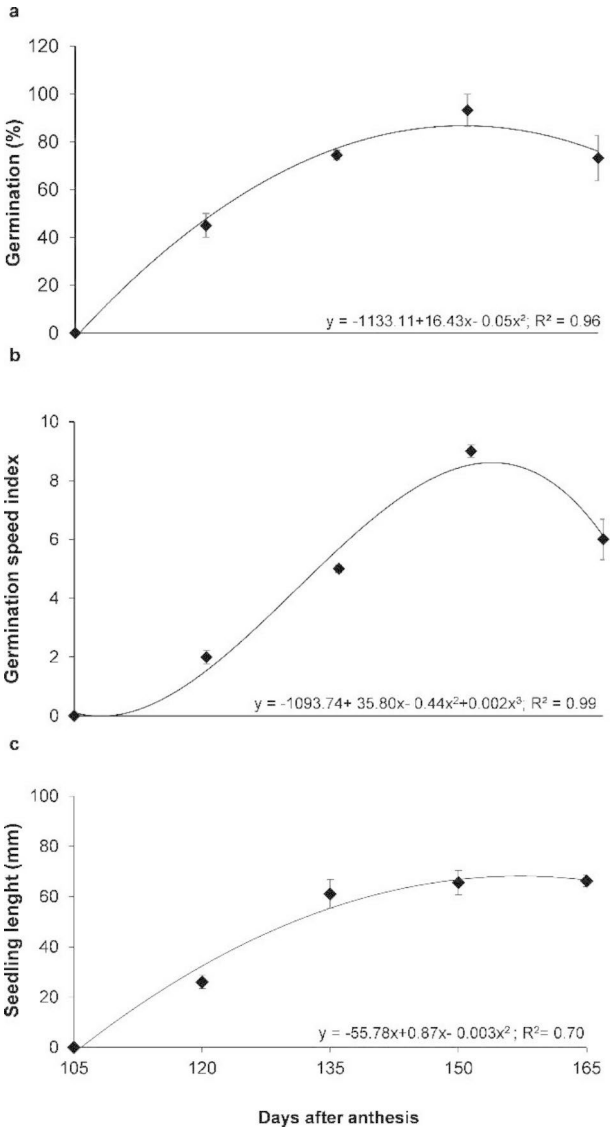

Fig. 3 Germination percentage (**a**), germination speed index (**b**), and seedling length (**c**) obtained for *Acacia mangium* seeds during development. Dots represent means and vertical bars represent standard deviation of four replicates

30 to 105 DAA. By contrast, reserve remobilization occurred in the course of maturation and senescence, since the content of TSS (Fig. 4a), NRS (Fig. 4a), starch (Fig. 4b), and soluble proteins (Fig. 4c) decreased 55, 27, 25, and 45%, in that order, in *A. mangium* pods from 105 to 165 DAA. Curiously, the activity of amylases in the pods revealed two peaks; the first one was at 60 DAA during starch deposition, and the second one was at 120 DAA preceding starch degradation (Fig. 4b). In addition, the activity of acid proteases in the pods decreased 60% from 30 to 60 DAA and peaked at 105 DAA (Fig. 4c).

The content of NSC and soluble proteins also changed from mid to late development of *A. mangium* seeds (Fig. 5a and b). The starch content increased 35% from 105 to 120 DAA and then reverted to the value of the first harvest at 165 DAA (Fig. 5a). By contrast,

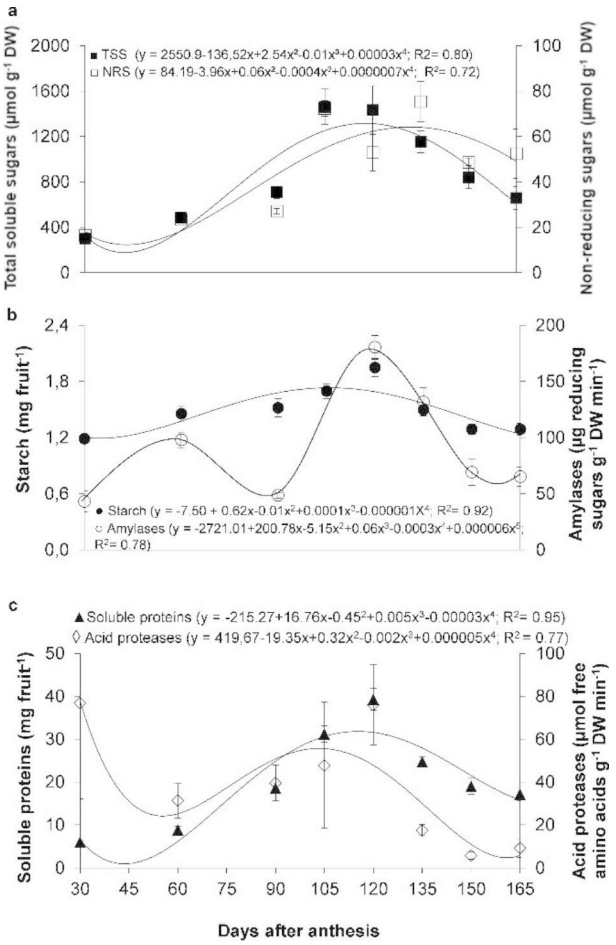

Fig. 4 Biochemical alterations in *Acacia mangium* pods during development, including the content of total soluble sugars and non-reducing sugars (**a**), the starch content and the amylase activity (**b**), and the soluble protein content and the acid protease activity (**c**). Dots represent means and vertical bars represent standard deviation of five replicates

the content of TSS and NRS reduced 34 and 18%, respectively, from105 to 120 DAA and unexpectedly increased about 15% from 150 to 165 DAA (Fig. 5a). Different from starch, soluble proteins were accumulated until the end of seed filling and were retained during seed maturation. In fact, the content of soluble proteins increased 83% from the first harvest to 135 DAA and remained almost unaltered until the last harvest (Fig. 5b). In parallel, the TFAA content dropped 40% from 105 to 120 DAA, peaked at 135 DAA and then decreased 30% until 165 DAA (Fig. 5b).

Discussion

The development of pods and seeds is remarkably coordinated in *A. mangium*. In the pods, changes in colour (Fig. 1a, b, c, d, e, and f), WC (Fig. 2a), and DW (Fig. 2a) evidence that the growth phase extends until 105 DAA; as dehiscence is already observed at 135 DAA, the maturation phase is possibly ended between 120 and 135 DAA and senescence may take place from then on. In the seeds, in turn, changes in colour (Fig. 1i, j, k, and l), WC (Fig. 2b),

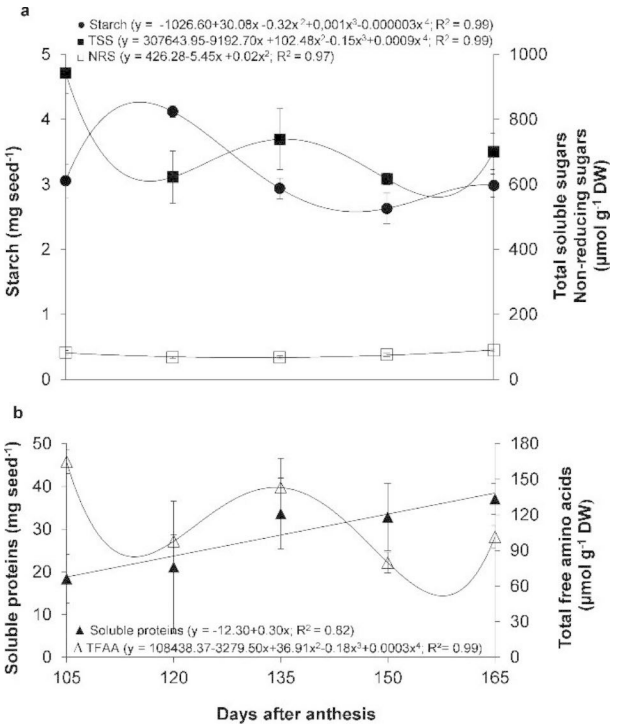

Fig. 5 Biochemical alterations in *Acacia mangium* seeds during development, including the content of starch, total soluble sugars, and non-reducing sugars (**a**), and the soluble protein content and the acid protease activity (**b**). Dots represent means and vertical bars represent standard deviation of five replicates

DW (Fig. 2b), and quality (Fig. 3) indicate that late filling is verified from 105 to 135 DAA and maturation drying is completed between 135 and 150 DAA. In this way, the end of pod growth precedes the end of reserve deposition in the seeds, as well as dehiscence occurs before the seeds complete water loss and acquire maximum germinability and vigour.

Although seed development has been widely studied in crops and native species, physiological maturity and harvesting time are controversial issues. In general, physiological maturity results from successive developmental processes and is achieved when seeds exhibit maximum DW (Bewley et al. 2013), while harvesting time is based on technological markers and defined according to maximum seed quality (Bareke 2018). In this study, *A. mangium* seeds are physiologically mature at 135 DAA (Fig. 2b), but they may be harvested at 150 DAA, since the DW is maintained (Fig. 2b) and the WC decreases to 3.4% (Fig. 2b), whereas viability (Fig. 3a) and vigour (Fig. 3b and c) reach maximum values.

Changes in fruit colour have been used as markers to predict seed harvesting time, since the loss of chlorophylls and the accumulation of carotenoids and anthocyanins commonly occur during fruit maturation (Gómez-Martín et al. 2020). In several tree legumes, like *E. variegata* (Matheus et al. 2011), *A. cearensis* (Lopes et al. 2014), *P. pluviosa* (Silva et al. 2015), *A. hasslerii* (Ristau et al. 2020), and *A. colubrina* (Cruz et al. 2021), chlorophyll loss happens before fruit dehiscence and when seeds reach high germinability and vigour. Herein, however, chlorophyll loss starts between 105 and 120 DAA and it ends until 135 DAA in both pods (Fig. 1d, e and f) and seeds (Fig. 1i, j and k) of *A. mangium*, coinciding with dehiscence but preceding maximum seed quality (Figs. 2b and 3). Thus, the harvest of *A. mangium* seeds may not be exclusively managed according to changes in fruit colour.

In order to obtain high-quality seed lots, harvests may be carried out when seeds exhibit minimum WC and maximum DW, germinability, and vigour. Although it is generally expected that germinability is acquired before vigour during seed maturation (Bewley et al. 2013), both qualities are maximally attained by *A. mangium* seeds at 150 DAA (Fig. 3) in this work, when maturation drying is completed (Fig. 2b). In other woody species, including *A. cearensis* (Lopes et al. 2014), *Vernonanthura discolor* (Grzybowski et al. 2016) *P. pluviosa* (Silva et al. 2015), *Lophantera lactescens* (Silva et al. 2019), *Tabebuia aurea* (Santos et al. 2019),and *A. hasslerii* (Ristau et al. 2020), germinability and vigour are also simultaneously acquired during late maturation.

It is noteworthy that the growth phase of pod development in *A. mangium* is initially directed by water uptake, considering an increase in the pod WC from 30 to 60 DAA (Fig. 2a). After that, this phase depends on reserve deposition according to DW accumulation in the pods from 60 to 105 DAA (Fig. 2a). Similar changes in WC and DW are verified during fruit growth in other woody species, as *E. variegata* (Matheus et al. 2011), *P. pluviosa* (Silva et al. 2015), and *L. lactescens* (Silva et al. 2019). In *A. mangium*, early pod growth may overlap with seed histodifferentiation, while late pod growth is likely to parallel seed filling. Considering that seed histodifferentiation encompasses extensive cell divisions and the establishment of the embryo developmental planes (Bareke 2018), low levels of soluble sugars (Fig. 4a) and soluble proteins (Fig. 4c) associated with high activity of acid proteases (Fig. 4c) in *A. mangium* pods until 60 DAA may be related to intense metabolic activity and protein turnover. Moreover, the accumulation of soluble sugars (Fig. 4a), starch (Fig. 4b) and soluble proteins (Fig. 4c) in *A. mangium* pods from 60 to 105 DAA is an evidence of reserve deposition. As the pods are still green until 105 DAA (Fig. 1e), this process is possibly supported by the uptake of assimilates from the mother plant combined with the photosynthetic activity of the pod wall (Bennet et al. 2011).

Regarding our results, there is a time lapse between the end of pod growth (105 DAA) and the end of seed filling (135 DAA), in which pod maturation takes place in *A. mangium*. During this process, the vascular connection between pods and seeds may be severed because chlorophyll loss is evident in both at 120 DAA (Fig. 1e and j) whereas pod desiccation and dehiscence is completed at 135 DAA (Fig. 1f). Thus, reserve remobilization in the pods during maturation may have contributed to reserve deposition in the seeds at late filling. Accordingly, high activity of amylases (Fig. 4b) and acid proteases (Fig. 4c) is consistent with a decrease in the levels of TSS (Fig. 4a), starch (Fig. 4b), and soluble proteins (Fig. 4c) in the pods after 105 DAA. From 105 to 120 DAA, the levels of TSS (Fig. 5a) and TFAA (Fig. 5b) reduce as starch (Fig. 5a) and soluble proteins (Fig. 5b) accumulate in the seeds, suggesting reserve deposition. Starch and lipid deposition in the seeds also occur at late fruit maturation in *T. aurea* (Santos et al. 2019) and a decrease in the levels of water-soluble polysaccharides in the pods is associated with the accumulation of these compounds in the seeds during their final developmental stages in *Senna macranthera* (Áquila et al. 2012).

In practice, seed harvest should not be made long after pod shattering taking into account possible losses in seed quality (Bewley et al. 2013). Herein, no significant changes in WC (Fig. 2b), DW (Fig. 2b), NSC content (Fig. 5a), and soluble protein content (Fig. 5b) are verified in *A. mangium* seeds from 150 to 165 DAA. However, it is notable that the germination percentage (Fig. 3a) and the germination speed index (Fig. 3b) reduce during this period, revealing a decrease in both viability and vigour, respectively. Losses in seed quality are also verified after pod shattering in other legume trees like *E. variegata* (Matheus

et al. 2011), *A. cearensis* (Lopes et al. 2014), and *P. pluviosa* (Silva et al. 2015). Hence, *A. mangium* seeds should be harvested when maturation drying is completed to avoid losses in viability and vigour.

Given that forest reproductive material plays a central role in afforestation and reforestation strategies (Hazarika et al. 2021), our findings link basic aspects of pod and seed development to practical features of seed production in *A. mangium*. Briefly, pod color should be used with caution to define seed harvest, physiological maturity may not coincide with harvesting time, and viability and vigor could be acquired simultaneously at late seed maturation. In addition, since seed production is affected by inter-annual (Pearse et al. 2017) and provenance (Mohammed et al. 2022) variation due to weather and resources, further studies are needed to reveal how the developmental patterns described in this work may be influenced by these variations.

Conclusion

It is possible to highlight that the early stages of pod growth in *A. mangium* are governed by water uptake while the late ones are marked by reserve deposition. The remobilization of NSC and soluble proteins in the pods during maturation may have contributed to reserve deposition in the seeds during late filling. *A. mangium* seeds achieve physiological maturity before harvesting time, as maturation drying is completed and maximum germinability and vigour are attained after the end of DW accumulation. Seed harvest should be made when minimum WC is achieved to avoid losses in quality long after pod shattering.

Abbreviations

DW	Dry weight
FW	Fresh weight
ISTA	International Seed Testing Association
NRS	Non-reducing sugars
NSC	Non-structural carbohydrates
TFAA	Total free amino acids
TSS	Total soluble sugars

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Authors' contributions MVP and CSF conceived and designed the experiment; MDS, FCF, and CSF harvested the plant material and carried out the physiological measurements under the supervision of MVP; MDS, FCF, and DFAO performed the biochemical determinations under the supervision of ELV; MDS and EEC carried out the statistical analysis and adjusted polynomial models; MVP, CSF, and ELV wrote the manuscript.

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Declarations

Competing interests The authors declare no competing interests.

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