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PHYSIOLOGICAL AND BIOCHEMICAL CHANGES DURING SEED DEVELOPMENT AND MATURATION IN *MADHUCA LATIFOLIA* ROXB.

JIPSI CHANDRA AND S KESHAVKANT*

School of Studies in Biotechnology, Pt. Ravishankar Shukla University, Raipur 492 010, India

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Abstract

Seed development comprises a series of events involving cell division/histo-differentiation, reserves deposition and desiccation. Development of Madhuca (*Madhuca latifolia* Roxb.) seed has been divided into eight consecutive stages; 10, 18, 26, 34, 42, 50, 58, 66 days after fertilization (DAF). Seed colour was finally found to turn dark-brown from initial creamish-white. Length, circumference, fresh and dry mass increased gradually throughout. In contrast, water content (WC) and electrical conductance were declined (1.4- and 94-folds, respectively) suggesting deposition of reserves and improvement in membrane integrity in parallel to development. During seed development, sugar was declined (3-folds), while starch and protein contents were increased (4- and 13-folds, respectively) suggesting their supportive role in germination and early seedling growth. Activities of antioxidants exhibited rising trend (1.6 - 21-folds) between initial and last stages of development advocating their defensive function. Data suggested that ideal time for Madhuca drupes/seeds harvesting was 66 DAF.

Introduction

Seed development in the life cycle of any higher plant is a crucial process providing the link between two different sporophytic generations (Baud *et al*. 2002). Generally, seed development involves an orchestrate programme of pattern, which includes formation, reserves deposition and desiccation. Formation of a seed is initiated after double fertilization that takes place in an embryo sac, and then repeated cell division occurs in order to establish the seed structures (Baud *et al*. 2002). Then, seed filling phase starts during which the water content (WC) declines and storage reserves accumulate, resulting into cell expansion. Carbohydrates, lipids and storage proteins are three chief storage reserves generally accumulated in most of the seeds. Seed filling is highly complex process, as many genes and a number of enzymes are involved to regulate storage of each component (Weber *et al*. 2005). For the process of seed filling, the monomers of various macromolecules *viz*., amino acids, fatty acids and monosaccharides are translocated through sieve elements of phloem to synthesize their long chain polymers (Weber *et al*. 2005). Next, the seed enters into maturation drying phase where changes in the seed size and huge fall in WC occurs (Berjak and Pammenter 2008). Maturation drying phase is absolutely absent in recalcitrants therefore, these seeds possess high amount of water (30 - 70%) at the time of shedding from mother plant (Berjak and Pammenter 2008).

Process of seed development is genetically programmed, and developing seed converts the precursors of carbon and nitrogen, into stable reserves required later for various processes (Weber *et al*. 2005). During development, as a by-product of metabolic reactions reactive oxygen species (ROS) are generated, which are shown to play dual functions either cytotoxic (when exists in high amount) or playing a role in development, dormancy breakage and defence against biotic and abiotic stresses (Berjak and Pammenter 2008). These ROS are popularly known to react with all

*Author for correspondence: <skeshavkant@gmail.com>.

sorts of macromolecules, pigments and other cellular components, leading their degradation (Parkhey *et al*. 2014). Plant cells are having tremendous potency to prevent themselves from such oxidative injury by keeping the ROS under control via an efficient and highly redundant network of antioxidants (Chandra *et al*. 2015). Superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POX) and ascorbate peroxidase (APX) constitute the major enzymatic systems by which cells catabolize free radicals, thus minimizes the severity of oxidative damage (Chandra *et al*. 2015). SOD plays key role in conversion of superoxide into oxygen and hydrogen peroxide. While CAT, POX and APX catalyzes the conversion of hydrogen peroxide into water and molecular oxygen and therefore prevent further generation of free radicals (Chandra *et al*. 2015). Very limited reports are available concerning to the function of antioxidants during seed development however; no one has yet attempted to explore them in developing Madhuca seeds.

The physiological and biochemical investigations conducted by He and Gao (2008) and Kok *et al*. (2013) on developing seeds of *Chimonanthus praecox* and *Elaeis guineensis*, respectively, provided comprehensive knowledge regarding seed metabolism. Certainly, information regarding developmental biochemical changes is an indispensable base in understanding the regulatory set-up coordinating seed development and metabolism. Additionally, in-depth knowledge regarding pattern and mechanisms of reserves accumulation in seed is pre-requisite in any plant-breeding programme (Kok *et al*. 2013). The metabolic changes taking place during seed development have been studied for several species (Baud *et al*. 2002, Silveira *et al*. 2004, Saldivar *et al*. 2011, Pavithra *et al*. 2014), however there is an absolute lack of knowledge for Madhuca.

Madhuca (*Madhuca latifolia* Roxb., family Sapotaceae) seeds are quite sensitive towards desiccation and low temperature hence got damaged even at 15°C (DFSC/IPGRI 2000). No information is available regarding biochemical changes taking place during developmental stages of Madhuca seeds. In this context, experiments were designed to profile the morphological (colour, length, circumference), physiological (Fresh mass (FM), Dry mass (DM), WC and electrolyte leakage) and biochemical (contents of sugar, starch and protein, and activities of SOD, POX and APX) changes that occur during the development of Madhuca seeds.

Materials and Methods

Madhuca (*Madhuca latifolia* Roxb.) drupe bunches were obtained from 10 - 15 identified trees growing in the school campus of Attari village, located 8 km away from the Pt. Ravishankar Shukla University, Raipur, India. The flowers appear in March, which takes about 32 - 35 days for complete development. During April, the inflorescences were constantly and keenly monitored based on the appropriate physiological state at anthesis. Flowers having only pistil without stamen and corolla, indicating completion of fertilization, were tagged. The drupe bunches were harvested manually at defined developmental stages i.e. 10, 18, 26, 34, 42, 50, 58, 66 days after fertilization (DAF). Five bunches of drupes from each developmental stage were plucked from different Madhuca trees. Each bunch represents a single replicate for each analysis. Due to the small amount of endosperm available at stages 10 - 34 DAF, two bunches of Madhuca drupes were collected from each tree and pooled as a single replicate. The drupes were plucked from the bunch. The kernels in the form of liquid/gel-like (up to 18 DAF) were collected from the drupes using an aspirator, whereas for the solid kernels, the fruits were immersed into liquid nitrogen (LN_2) and then the seed was cracked to free the kernel. Thereafter, samples were grinded with LN_2 and were stored at –80°C (U410, Eppendorf, Germany) for further use.

Seeds of different developmental stages were scored for their size and colour. Length and circumference data of seeds were determined using scale and vernire-callipers, respectively and expressed in terms of cm.

To monitor change in FM and DM data, five independent sets of 5 seeds each, were weighed using a four digit electronic balance (Sartorius, Sweden), before and after oven drying at 103 ºC for 72 hrs (Parkhey *et al*. 2014), and expressed in g.

Seed WC was determined gravimetrically (Parkhey *et al*. 2014) and expressed as g H_2O/g FM.

Ten seeds in five replications were soaked in 30 ml MilliQ water (MW) (Millipore, Gradient A-10, USA) and allowed to stand at room temperature (26 - 28ºC) for 24 hrs. The electrical conductivity was measured using a digital conductivity meter (CM 183, Elico, India) and expressed as mS/g FM.

Amounts of total sugar and starch were determined following the method of Siloto *et al*. (2006) and Hodge and Hofreiter (1962), respectively and were expressed in mg/g FM.

Protein was extracted following the method of Wang *et al*. (2006), and was assayed by Bradford (1976). Data was expressed as g protein/g FM, and BSA was used as standard.

Weighed (0.2 g) amount of LN_2 crushed seed powder was homogenized with 2 ml of borate buffer (0.2 M, pH 7.4) consisting 20% (w/v) polyvinyl polypyrolidone. The slury was centrifuged at 14,000 rpm for 15 mins at 4°C. The supernatant thus obtained was used as a source of antioxidant enzymes.

Activity of SOD (EC 1.15.1.1) was estimated following Marklund and Marklund (1974) by estimating per cent inhibition of pyrogallol auto-oxidation at 490 nm. One unit of SOD was defined as the quantity of enzyme required to inhibit the nitro blue tetrazolium photoreduction by 50%. Activity was expressed as SOD units /min /g FM.

POX (EC 1.11.1.7) was measured following Chance and Maehly (1955). The tetraguaiacol formed was measured at 470 nm, and its activity was expressed as µM/min/g FM.

Activity of APX (EC 1.11.1.1) was assayed following Nakano and Asada (1981) by monitoring the rate of ascorbate oxidation at 290 nm. Its activity was expressed as mM/min/g FM.

All the experiments were performed in five individual biological replicates. The data obtained were subjected to one-way ANOVA, and the mean differences were compared by the DMRT at $p < 0.05$ level, using the SPSS software (Ver. 16.0). The correlation analyses between studied parameters were also performed.

Results and Discussion

Madhuca trees are potentially important due to various applications of its different parts. Madhuca trees started flowering during March and from April onwards drupe setting was initiated which ripens up to July. In practice, a seedling of it is propagated from small embryo enclosed within the hardy seed. Theoretically, the strength of any developing plant depends on the vigour of seed; therefore proper development of a seed is a crucial phase of plants life cycle. Madhuca seed takes 66 days to attain physiological maturity. Therefore, eight developmental stages were identified to study various changes taking place, at the interval of 8 - 10 days. The embryo started developing and become visible after around 10 days of fall off corolla, which was used for first sampling. The early drupes are gray in colour having small furs over its surface, and when attains maturity, furs disappear and colour of drupes turn from gray to green and then greenish-yellow, finally.

In this study, eight distinct stages (10, 18, 26, 34, 42, 50, 58, 66 DAF) of seed development are identified based on the morphological characteristics (Table 1). The colour of developing seed was initially creamish-white (10 - 34 DAF) that passes from creamish-yellow (42 DAF) to light brown (58 DAF) and finally become dark brown (66 DAF). Similar change has also been observed in developing *Pongamia pinnata* seed (Kesari and Rangan 2011). Length and

circumference of Madhuca seeds increased gradually across the stages and around 3.7- and 3.96-folds increments respectively, in above parameters were observed at the end of development, which were found to be linked intimately ($r = 0.98$, $p < 0.05$) with development phenomenon (Table 1). Matching observations were documented by Kesari and Rangan (2011).

In this investigation, both FM and DM data gradually increased throughout the analysis period. The FM increase was gradual from 10 - 34 DAF and was pronounced ($p < 0.05$) from 34-50 DAF (Table 1). A slight fall (0.52 g) in FM was discernible between 58 and 66 DAF (Table 1). Matching trend was observed for the DM, which increased gradually up to 34 DAF and thereafter increasingly ($p < 0.05$) up to 58 DAF. Highest value (1.6 g) of DM was recorded for 66 DAF seeds (Table 1). A positive correlation ($r = 0.98$, $p < 0.05$) between DM accumulation and seed development was established. Similar trend was noticed during *Glycine max* (Sitthiwong *et al*. 2005) and *Vigna unguiculata* (Deshmukh *et al*. 2011) seed development. The increase in seed weight along with developmental stages was perhaps due to rapid cell division and expansion, accompanied by a progressive gathering of storage reserves. Reverse to FM and DM data, a high WC (0.87 g H_2O /g FM) was recorded in 10 DAF seeds, which was tended to decline gradually with developmental stages and reached least (0.61 g H_2O /g FM) in 66 DAF seed (Table 1). Data reflected a negative ($r = –0.99$, $p < 0.05$) relationship among WC and developmental stages. Availability of a high WC during initial stages of seed development is crucial for maintenance of metabolic activity and adequate seed growth (Westgate and Grant 1989). And, at the later stages, loss of WC is most probably due to the accumulation of reserves which displaces water from the storage cells (Kermode 1990). Carbohydrate and lipid accumulation in the developing *Elaeis guineensis* and *Pongamia pinnata* seeds closely paralleled the increase of DM and reduction of WC (Kok *et al*. 2013, Pavithra *et al*. 2014).

High (2.35 mS/g FM) rate of electrolyte leakage was measured from 10 DAF seeds, which decreased gradually and finally reached 0.025 mS/g FM at the end of development (66 DAF), which is 94-folds less than the earlier (Table 1). Being a measure of membrane integrity, high rate of leakage at initial stages (10 - 26 DAF) of seed development suggested the poor cellular membrane integrity (Table 1). Thereafter, a low amount of leakage from 34 DAF onwards seeds, indicated substantial improvement in membrane integrity and rise in reserves accumulation. Data witnessed a negative association of it with reserve deposition ($r = –0.74$, $p < 0.05$) and seed developmental stages ($r = –0.71$, $p < 0.05$). The results of current investigation are in accordance with that observed in *Pongamia pinnata* (Pavithra *et al*. 2014).

Further, a remarkable (3-folds, $p < 0.05$) fall in total sugar content was discernible from 10 to 66 DAF, while significant (4-folds, $p < 0.05$) upsurge in starch accumulation was observed (Table 2). Data showed a positive ($r = 0.98$, $p < 0.05$) link between developmental stages and starch deposition, while it was negative ($r = –0.97$, $p < 0.05$) for total sugar. Matching observations has also been observed in developing seeds of *Zea mays* (Cao *et al*. 2008) and *Pongamia pinnata* (Pavithra *et al*. 2014). Weber *et al*. (2005) suggested that seed coat plays crucial role in the metabolic control of developing seed. In *Pongamia pinnata*, activity of wall bound invertase is negligible in the cotyledons and high in the seed coat thus resulting significant assimilation of sugars in cotyledons during initial stages of development (Pavithra *et al*. 2014). The simultaneous decline in sugar content and increase in starch accumulation indicated the role of starch as carbon source for lipid synthesis (Pavithra *et al*. 2014). Generally, during early stages of development starch synthesis was accompanied by deposition of photosynthetic enzymes which help in recycling of CO_2 released during fatty acid synthesis as a source of carbon which once again executes the lipid biosynthesis during later phases of seed development (King *et al*. 1997). It was also suggested that seed development associated decline in sugar was possibly due to the inter

Table 1. Physiological changes in *Madhuca latifolia* Roxb. seeds during development.

Parameters	Days after fertilization (DAF)							
	10	18	26	34	42	50	58	**66**
								
Colour	Creamish-white	Creamish-white	Creamish-white	Creamish-white	Creamish-yellow	Yellowish	Light brown	Brown
Length (cm)	$1.08^{g} \pm 0.13$	$1.82^{f} \pm 0.2$	$2.16^{e} \pm 0.08$	$2.60^{d} \pm 0.14$	$3.00^{c} \pm 0.18$	$3.10^{c} \pm 0.1$	$3.38^{b} \pm 0.08$	$4.00^{a} \pm 0.12$
Circumference (cm)	$1.84^{h} \pm 0.11$	$2.60^{g} \pm 0.1$	$3.62^{f} \pm 0.17$	$4.78^{e} \pm 0.16$	$5.06^{d} \pm 0.19$	$5.54^{c} \pm 0.11$	$7.30^{a} \pm 0.21$	$6.62^{b} \pm 0.27$
Fresh mass (g)	$0.23^{g} \pm 0.06$	$0.83^{f} \pm 0.01$	$1.46^{e} \pm 0.08$	$2.13^{d} \pm 0.02$	$3.19^{c} \pm 0.08$	$4.07^{b} \pm 0.07$	$4.67^{a} \pm 0.2$	$4.15^{a} \pm 0.48$
Dry mass (g)	$0.03^{f} \pm 0.04$	$0.12^{f} \pm 0.02$	$0.28^{e} \pm 0.03$	$0.45^{d} \pm 0.07$	$0.75^{c} \pm 0.12$	$1.19^{b} \pm 0.08$	$1.48^{a} \pm 0.04$	$1.60^{a} \pm 0.16$
Water content (g /g FM)	$0.87^{a} \pm 0.005$	$0.85^{b} \pm 0.01$	$0.81^{c} \pm 0.01$	$0.77^{d} \pm 0.014$	$0.75^{e} \pm 0.01$	$0.69^{f} \pm 0.01$	$0.66^{g} \pm 0.006$	$0.61^{h} \pm 0.018$
Electrolyte leakage (mS /g FM)	$2.35^{a} \pm 0.27$	$0.59^{b} \pm 0.001$	$0.21^{c} \pm 0.018$	$0.10^{d} \pm 0.003$	$0.06^{e} \pm 0.005$	$0.037^{f} \pm 0.003$	$0.027^{g} \pm 0.003$	$0.025^{g} \pm 0.002$

Each value is mean of five replicates ±Sd. Data were significantly different at the level of ($p < 0.05$). Values with the matching letter are not significantly different.

Table 2. Developmental changes in total sugar, starch, protein and antioxidants (SOD, POX & APX) in developing seeds of *Madhuca latifolia* Roxb.

Parameters	Days after fertilization (DAF)							
	10	18	26	34	42	50	58	66
Total sugar (mg/g FM)	$59.06^{a} \pm 4.2$	$51.39^{b} \pm 1.79$	$47.31^{c} \pm 0.69$	$38.98^{d} \pm 1.52$	$28.36^{e} \pm 1.81$	$20.26^{f} \pm 2.7$	$19.17^{f} \pm 0.71$	$19.14^{f} \pm 1.35$
Starch (mg/g FM)	$6.89^{g} \pm 0.26$	$8.60^{g} \pm 0.62$	$11.23^{f} \pm 0.29$	$13.80^{e} \pm 0.75$	$16.01^{d} \pm 0.41$	$18.36^{c} \pm 0.66$	$23.54^{b} \pm 0.94$	$27.50^{a} \pm 3.1$
Protein (g/g FM)	$0.03^{h} \pm 0.002$	$0.05^{g} \pm 0.006$	$0.17^{f} \pm 0.01$	$0.22^{e} \pm 0.01$	$0.26^{d} \pm 0.01$	$0.30^{c} \pm 0.008$	$0.34^{b} \pm 0.02$	$0.39^{a} \pm 0.009$
SOD (units/min /g FM)	$1.46^{c} \pm 0.067$	$1.62^{c} \pm 0.11$	$1.63^{c} \pm 0.06$	$1.98^{b} \pm 0.10$	$2.08^{ab} \pm 0.13$	$2.19^{ab} \pm 0.12$	$2.29^{a} \pm 0.09$	$2.36^{a} \pm 0.07$
POX (µM /min /g FM)	$0.18^{e} \pm 0.03$	$0.51^{d} \pm 0.034$	$0.57^{d} \pm 0.14$	$0.87^{c} \pm 0.13$	$0.94^{bc} \pm 0.11$	$1.04^{bc} \pm 0.18$	$1.19^{b} \pm 0.09$	$3.76^{a} \pm 0.44$
APX (mM/min/g FM)	$0.02^{f} \pm 0.0007$	$0.04^{e} \pm 0.003$	$0.06^{d} \pm 0.003$	$0.08^{c} \pm 0.016$	$0.14^{b} \pm 0.008$	$0.15^{b} \pm 0.014$	$0.17^{a} \pm 0.012$	$0.19^{a} \pm 0.01$

Each value is mean of five replicates ±Sd SD. Data were significantly different at the level of ($p < 0.05$). Values with the matching letter are not significantly different.

conversion of different classes of sugars (Pavithra *et al*. 2014). Generally, monosaccharides are in abundance during initial stages of development, while oligosaccharides are high at later phases, impairing membrane fusion and increasing seed longevity (Blackman *et al*. 1992).

Similar to starch, significant (13-folds, $p < 0.05$) accumulation of protein in Madhuca seed was observed in between 10 and 66 DAF (Table 2), exhibiting a proximity ($r = 0.98$, $p < 0.05$) between protein content and development. Similar trend of protein deposition has earlier been observed by Silveira *et al*. (2004) and Saldivar *et al*. (2011) in developing seeds of *Pinus taeda* and *Glycine max*, respectively. During seed maturation most of the storage proteins are generally accumulated in the vacuole or as membrane-bound protein bodies within the cell (Hoekstra *et al*. 2001). Further, storage proteins like albumins and globulins are expressed abundantly during the deposition phase through the mid of the maturation phase (He and Gao 2008). Gallardo *et al*. (2008) suggested that total protein content estimated in legume seeds is compilation of storage proteins, house-keeping proteins, protease inhibitors, biologically active enzyme, lectins and allergens. Authors has also proposed that the high content of protein stored in mature seeds became dissipated during germination and early seedling growth that is why most of them are undetectable during later phases of seedling growth (Kesari and Rangan 2011). During seed maturation, protein synthesis is a complex phenomenon and requires further investigation.

The tolerance of plants or its organs to damage-inducing conditions depends on efficiency of ROS detoxifying enzymes (Chandra *et al*. 2015). In this investigation, activities of SOD, POX and APX were observed to be increased gradually across the seed developmental stages and reached their highest (SOD: 2.36 units/min/g FM, POX: 3.76 µM/min/g FM, APX: 0.19 mM/min/g FM) in 66 DAF seeds, which were 1.61-, 21- and 9.5-folds higher than their initials (10 DAF) (Table 2). Accumulated data exhibited a strong correlation ($r = 0.98$, $p < 0.05$) between antioxidants level and seeds development. Among the antioxidants, SOD was found to perform best althrough. The increases in the activities of antioxidants parallel to seed development indicated that they play an important role in combating oxidative condition, and are well connected with the increased deposition of protein and reduced WC in maturing seed (Bailly *et al*. 2004). Similar changes in the antioxidants were observed during seed or fruit development in several other species (Bailly *et al*. 2004, Montavon and Bortlik 2004).

In summary, the process of seed development is a complex phenomenon and comprises a series of physiological and metabolic changes. The results obtained in this study give snapshots of several morphological (colour, length and circumference), physiological (FM, DM, WC and electrolyte leakage) and biochemical (sugar, starch, protein and antioxidant enzymes *viz*., SOD, POX and APX) changes hardwired in seed development programme of Madhuca. Further, the ideal time for harvesting Madhuca drupes/seeds was recommended to be at/after 66 DAF when drupes become completely mature with maximum reserves deposition. Information gathered on Madhuca seed development could be exploited for profiling of enzymes implicated in the biosynthetic pathway of oil. The current findings will also provide a foundation for identification and characterization of specific fatty acid, protein species and gene expression analysis, which are essentially required for implementing a genetic engineering approach for crop improvement in Madhuca.

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