Desiccation-induced ROS accumulation and lipid catabolism in recalcitrant *Madhuca latifolia* seeds

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Abstract Loss of viability in desiccation-sensitive seeds of *Madhuca latifolia* (Roxb.) J. F. Macbr., an important multipurpose tropical tree, was correlated with seed water content (WC). WC declined from 0.59 to 0.19 g g⁻¹ fresh mass, 35 days after harvest from mother plant, at ambient conditions (temperature 25 ± 2 °C, relative humidity 50 ± 2%). The desiccation-induced reduction in viability was related with an accumulation of reactive oxygen species (ROS) that promoted lipid peroxidation associated loss of membrane integrity. Conducted study revealed 1.6–19 folds rise in lipid peroxidized products in desiccated *M. latifolia* seeds, and was found to be linked inversely with WC and germination percentage. Additionally, increased activities (7 and 13 folds) of lipid hydrolyzing enzymes; lipase (EC 3.1.1.3) and lipoxxygenase (EC 1.13.11.12) respectively, were discernible in desiccating *M. latifolia* seeds. In summary, increased ROS, lipid oxidation, lipase and lipoxxygenase were strongly correlated with viability loss in desiccating *M. latifolia* seeds.

Keywords Desiccation · *Madhuca latifolia* · Lipase · Lipid peroxidation · Lipoxxygenase · Reactive oxygen species · Water content

Introduction

Plants have been an indispensable part of human life for ages. Ever since ancient times, their fruits, seeds even roots and branches have been used to meet personal and social needs such as food, curing diseases and beautifying the planet (Canan et al. 2016; Sorkheh and Khaleghi 2016; Yazici and Sahin 2016). *Madhuca latifolia* (Roxb.) J. F. Macbr., a commercially important tropical tree, is mostly propagated through seeds (Orwa et al. 2009; Royal Botanic Gardens Kew 2016). Recalcitrant, desiccation-sensitive seeds are widespread in nature and their loss of viability has been ascribed to a variety of factors, including oxidative stress via reactive oxygen species (ROS) and resulting physical damage to cell membranes and other organelles (Berjak and Pammenter 2008). However, the desiccation sensitivity and associated mechanisms of seed death in *M. latifolia* have not been reported.

Recalcitrant seeds possess relatively high [0.4–4.4 g g⁻¹ dry mass (DM)] water content (WC) at the time of shedding from their mother plant, hence are metabolically highly active, and quite sensitive towards desiccation and low temperature (Berjak and Pammenter 2004). Such seeds deliberately lose water even under ambient temperature and optimum relative humidity (Umarani et al. 2015). Desiccation leads to imbalanced metabolism, intracellular damage and death of embryos even at higher WCs within weeks or months time, depending on the species (Pammenter and Berjak 1999). This metabolism related seed deteriorations are intimately related with the over production of ROS due to inefficient functioning or failure of ROS scavenging system (Anjum et al. 2015). Accelerated production of ROS causes oxidation of membrane lipids, proteins and nucleic acids (Berjak and Pammenter 2008). These types of desiccation-

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induced ROS mediated damage in membranes and accumulation of toxic by-products of oxidative metabolism may be the basic cause for viability loss in recalcitrant seeds under ambient storage (Umarani et al. 2015).

A number of ROS are known, among which superoxide anion (O_2^-), hydroxyl radical ($\cdot OH$) and hydrogen peroxide (H_2O_2) are most potent in causing damage to macromolecules (Gill and Tuteja 2010). Among the macromolecules, cellular lipids, particularly poly unsaturated fatty acids (PUFAs), are quite sensitive to ROS, and its peroxidation is the major cause of quality, vigour and viability loss of seeds, under ambient storage (Shaban et al. 2013). In addition to ROS, lipids are also catabolised by lipoxygenase (LOX), particularly by hydroperoxidation of the *cis-cis*-1,4-pentadiene structures of fatty acids, and releases lipid hydroperoxide (LOOH), a more reactive form of fatty acid. The LOOH thus formed is further degraded into several other cytotoxic reactive products viz.; malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE) and conjugated dienes (CD), which can reduce seed viability (Anjum et al. 2015). Among these products, the 4-HNE is highly toxic, and even trace of it is sufficient to attack protein, nucleic acids and mitochondrial respiration leading to loss of viability. Additionally, due to high instability/reactivity, it readily makes conjugates with proteins and nucleic acids, which are again toxic to the cell (Parkhey et al. 2012).

Further, ROS are also shown to cause de-esterification of seed phospholipids, therefore, leading to accumulation of free fatty acids (FFAs) in desiccating recalcitrant seeds (Ratajczak and Pukacka 2005). However, in addition to ROS, activity of lipase (EC 3.1.1.3) also adds FFA by hydrolysing the ester-carboxylate bonds of lipids, particularly at the organic-aqueous interface, thus playing critical role in loss of viability (Anjum et al. 2015). Free fatty acid also serves as membrane destabilizing agent, and substrate for the LOX, hence may be the initiator of degenerative reactions. Moreover, due to the detergent like chemistry, FFAs are found to damage mitochondrial membranes resulting in reduced energy production (Mosavi Nik et al. 2011). Accumulation of FFA also cause reduced cellular pH which is highly detrimental to normal metabolism and functioning of enzymes leading to loss of viability (Xia et al. 2015). Considering the above discussed facts, the present study was aimed to monitor changes in the membrane integrity (by measuring electrolyte leakage), tissue viability status, ROS (O_2^- , H_2O_2 and $\cdot OH$), total lipid content and its oxidized products (FFA, CD, LOOH, MDA and 4-HNE), and activities of lipase and LOX enzymes in tissues of desiccating *M. latifolia* seeds.

Materials and methods

Seed collection

Madhuca latifolia (Roxb.) J. F. Macbr. seeds were harvested by manual plucking off the ripened greenish-yellow drupes (66 days after flowering), which differentiate them from unripe green drupes during their development/maturation (Chandra and Keshavkant 2016), from the randomly selected healthy trees growing at Village Attari, 8 km to North-West of Pt. Ravishankar Shukla University Campus, Raipur (22°33'N to 21°14'N, 82°6' to 81°38'E, 305 MSL), India. Within an hour of collection, drupes were brought to the laboratory and immediately processed to separate mature seeds. Healthy, uniform sized seeds were sorted out and kept in perforated plastic trays at ambient conditions ($25 \pm 2^\circ C$ temperature, $50 \pm 2\%$ relative humidity). These seeds and their parts were assayed for physiological estimations at every five-day intervals until germination capacity was lost. For biochemical estimations, embryonic axes (EA) and cotyledon tissues were separately frozen in liquid nitrogen, on each of the harvest dates, and were then stored in sterile vials at $-80^\circ C$ (U410, Eppendorf, Germany). All the experiments were performed twice and in five replications, and within 2 months of post harvest storage of liquid nitrogen frozen tissues at $-80^\circ C$.

Germination assessment

To assess germination percentage, 20 seeds each in five replicates, at five-day intervals, were surface sterilized (5 min) with 1% (v/v) sodium hypochlorite solution (50 ml), followed by thorough washing (5 times) with MilliQ water (MW) (Millipore Gradient A-10, USA). These seeds were allowed to germinate inside the two layers of MW saturated filter paper (Whatman No. 1) towels, rolled inside the plastic sheet, in the dark at $25-27^\circ C$, and till appearance of the radicle (at least 5 mm) in each of the seeds (Varghese et al. 2002). Germination test was assessed for 15 days because after this duration the seeds became black in colour and/or undergo fungal manifestations.

Determination of WC

Water content of the whole seed was determined following the procedure of Parkhey et al. (2014). Five independent sets containing 10 seeds each were weighed using an electronic balance (BSA 224S-CW, Sartorius, Sweden), before and after oven drying at $102^\circ C$ for 48 h. Water content was calculated gravimetrically on the fresh mass (FM) basis and expressed as $g\ g^{-1}$ FM.

Monitoring of electrolyte leakage

Leakage of electrolyte was measured in five replicates, following the protocol of Blum and Ebercon (1981). For this, two seeds in each replicate were dipped in 20 ml of MW and kept on shaker for 24 h at 50 rpm and at ambient temperature. Thereafter, electrical conductivity of the solution was measured (C_0) using EC-TDS analyzer (CM-183, Elico, India). Then, samples were boiled (100 °C) for 20 min and electrolyte content was determined (C_1). Results were expressed as percentage of electrolyte leakage $\text{Seed}^{-1} = C_0/C_1 \times 100$.

Assessment of tissue viability

The tetrazolium test (TZ test) was performed following Chandra et al. (2015), and in five replicates. Ten de-coated *M. latifolia* seeds were immersed in 1% (w/v) solution of 2,3,5-triphenyl tetrazolium chloride and incubated in the dark at ambient condition for 12 h. Red coloured formazon thus formed were screened visually and then extracted separately for EA and cotyledons, with 100% ethanol. Absorbance of extracted formazon was read at 520 nm using a UV–Vis spectrophotometer (Lambda 25, Perkin Elmer, USA) and expressed as $A_{520} \text{ g}^{-1} \text{ FM}$.

Release of ROS

Weighed amounts (0.2 g) of EA and cotyledons, in five replicates, were extracted in cold (4 °C) sodium phosphate buffer (0.2 M, pH 7.2) comprising diethyl dithiocarbamate (DDC, 10^{-3} M) to inhibit SOD activity (Sangeetha et al. 1990). The homogenate was immediately centrifuged for 5 min at 10,000 g. In the supernatant, O_2^- was measured by its capacity to reduce nitro blue tetrazolium ($2.5 \times 10^{-4} \text{ M}$). The absorbance of the end product was measured at 540 nm. Amount of the O_2^- was calculated following the extinction coefficient of $2.16 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ and values were expressed as $\mu\text{mol g}^{-1} \text{ FM}$.

Amount of H_2O_2 was assessed in five replicates following the method of Velikova et al. (2000). Seed tissues (0.2 g) were homogenized with 2 ml of 0.1% (w/v) trichloroacetic acid (TCA) followed by centrifugation at 12,000g for 15 min at 25 °C. Absorbance was noted at 390 nm by adding 1 ml of supernatant into 1 ml of 10 mM sodium phosphate buffer (pH 7) and 2 ml of 1 M potassium iodide. Content of H_2O_2 was calculated following an extinction coefficient of $0.28 \mu\text{M}^{-1} \text{ cm}^{-1}$ and expressed as $\mu\text{mol g}^{-1} \text{ FM}$.

Hydroxyl radicals were estimated following the protocol of Halliwell et al. (1987). For this, seed tissues (0.2 g) were incubated in sodium phosphate buffer (10 mM, pH 7.4) comprising 15 mM 2-deoxyribose, at 37 °C for 2 h. The 0.7 ml of this solution was further incubated with 3 ml of 0.5% (w/v) thiobarbituric acid (TBA) prepared in 5 mM NaOH and 1 ml glacial acetic acid, at 100 °C for 30 min and then cooled at 4 °C for 10 min. After centrifugation, absorbance of the supernatant was recorded at 600 and 532 nm. Its amount was estimated using the extinction coefficient $155 \text{ mM}^{-1} \text{ cm}^{-1}$ and mean of five replicates was expressed as $\text{nmol g}^{-1} \text{ FM}$.

Total lipid: extraction and estimation

Five replicates having 4 g each of liquid nitrogen frozen EA or cotyledons were ground into fine powder and extracted in petroleum ether (40–60 °C) using soxhlet apparatus for 8 h (Raheja et al. 1973). Content of total lipid was determined gravimetrically and expressed as $\text{g g}^{-1} \text{ FM}$.

Assessment of lipase activity

The enzyme source was extracted in five replicates. Weighed amounts (0.2 g) of liquid nitrogen frozen fine tissue powders were homogenized with 2 ml of borate buffer (0.2 M, pH 7.4) consisting 20% polyvinyl pyrrolidone, and centrifuged at 12,000g for 15 min (Keshavkant and Naithani 2001). An aliquot (100 μl) of extracted enzyme was mixed properly with 1 ml of substrate (0.98% (w/v) sodium chloride, 5 g gum acacia and 5 ml olive oil), and allowed to stand at 37 °C for 1 h. The tube was then kept in a water bath (90 °C) for 2 min to stop the reaction. Thereafter, 6 ml chloroform and 2 ml sodium phosphate buffer (0.66 mM, pH 6.2) were added to it and kept for 30 min at ambient condition. Then, in the bottom layer, 3 ml of copper triethanolamine reagent (1 M triethanolamine, 1 N acetic acid, 6.45% (w/v) copper nitrate) was added and once again incubated for 30 min. Now, in the bottom layer, 11 mM DDC (100 μl) was mixed and absorbance was read at 440 nm (Itaya and Ui 1965). A standard curve of stearic acid was prepared and lipase (EC 3.1.1.3) activity was expressed in terms of $\mu\text{mol min}^{-1} \text{ g}^{-1} \text{ FM}$.

Determination of FFA

Estimation of FFA was performed in five replicates following the method of Itaya and Ui (1965). The 4 ml of chloroform and 1 ml of 0.66 mM sodium phosphate buffer (pH 6.2) were added to 0.1 ml of extracted lipid (Raheja et al. 1973), and were allowed to stand for 30 min at

ambient condition. The 3 ml of copper triethanolamine reagent (as prepared previously) was added to the chloroform layer of it and then once again incubated for 30 min. Now, 20 μl of 11 mM DDC was added and absorbance of the sample was recorded at 440 nm. Content of FFA was expressed as mg g^{-1} Lipid.

Assay of LOX

Activity of LOX (EC 1.13.11.12) was assayed following the method of Grossman and Zakut (1979). A mixture of 0.25 ml of Tween-20 and 7 ml of linoleic acid, dissolved in ethanol, was used as substrate. For LOX assay, borate buffer (0.2 M, pH 7.4) extracted enzyme (10 μl) was added to 1990 μl of substrate, in five separate replicates. Initial and final absorbance of the complex was recorded after 0 and 5 min respectively, at 234 nm. Activity of LOX was calculated using extinction coefficient $25 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed as $\mu\text{mol min}^{-1} \text{ g}^{-1}$ FM.

Estimation of CD

Weighed amounts (0.2 g) of liquid nitrogen crushed samples, in each five replicates, were homogenized with methanol containing 0.02% (w/v) ethylenediaminetetraacetic acid, 1% (w/v) sodium chloride and 2 ml of chloroform. Homogenate was centrifuged at 12,000 g for 15 min at 4 °C. An aliquot of supernatant was taken out and dried under liquid nitrogen. The residue was dissolved in ethanol and absorbance was recorded at 234 nm (Gidrol et al. 1989). An extinction coefficient of $25 \text{ mM}^{-1} \text{ cm}^{-1}$ was used to deduce the amount of CD and values were expressed as $\mu\text{mol g}^{-1}$ FM.

Monitoring of LOOH

To measure LOOH, procedure of Delong et al. (2002) was followed, and assessed in five replicates. Liquid nitrogen crushed (0.2 g) EA and cotyledon tissues were homogenized in 2 ml of ice-cold ethanol: MW (80:20, v/v) mixture and centrifuged at 4 °C for 10 min at 11,000g. Supernatant (500 μl) thus obtained was mixed with 500 μl of 10 mM triphenyl phosphine, prepared in methanol, and allowed to stand at 25 °C for 30 min. Thereafter, 2 ml of the ferrous xynol orange reagent (4 mM butylated hydroxytoluene, 250 μM ferrous ammonium sulphate hexahydrate, 100 μM xynol orange, 250 mM H_2SO_4 and 90% methanol) was added in it, incubated at room temperature for 60 min, and absorbance was measured at 560 nm. Content of LOOH was calculated using extinction coefficient $60,000 \text{ M}^{-1} \text{ cm}^{-1}$ and were expressed as mmol g^{-1} FM.

Monitoring of lipid peroxidation

To measure lipid peroxidized products, weighed amounts (0.2 g) of liquid nitrogen crushed samples, in five replicates, were homogenized with 0.5% (w/v) TBA dissolved in 20% (w/v) TCA (Hodges et al. 1999). The homogenate was incubated for 30 min in a water bath at 100 °C and then shifted to 0 °C for 15 min, so as to stop the reaction. Thereafter, centrifuged the sample at 11,000g for 10 min, and absorbance of the supernatant was recorded at 532 and 600 nm. The quantity of MDA present was calculated from the extinction coefficient of $157 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed as mmol g^{-1} FM.

Assessment of 4-HNE

The 4-HNE contents were estimated in five replicates following the method of Ray et al. (2007). Weighed amounts (0.2 g) of liquid nitrogen grinded seed tissues were homogenized with 0.2 M chilled borate buffer (pH 7.4) and 1.5 ml of 10% (w/v) TCA, centrifuged at 11,000g for 15 min. Collected supernatant was incubated for 2 h at ambient condition after addition of 1 ml 2,4-dinitrophenylhydrazine (1%, w/v), prepared in 0.5 M HCl. Samples were now extracted with hexane and then evaporated under liquid nitrogen. Absorbance of the product was measured at 350 nm by addition of 2 ml of methanol to it. Amount of the 4-HNE was calculated using an extinction coefficient of $13,750 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed in terms of mmol g^{-1} FM.

Statistical analyses

One-way ANOVA was employed to assess the influence of desiccation on different parameters. Moreover, associations between various parameters were tested using Pearson's correlation coefficient test. All the statistical analyses were performed using SPSS software (Version 16.0). Values presented are mean $\pm$ standard deviation of five separate replicates, and all the experiments were performed twice.

Results

Freshly harvested 0 day seeds of *M. latifolia* with 0.59 g g^{-1} FM WC showed 100% germination, which was maintained up to 10 days after harvest (DAH, 0.48 g g^{-1} FM WC). Later, germination percentage declined gradually, and was lost completely at 35 DAH (0.19 g g^{-1} FM WC) (Table 1). The decline in seed WC was strongly correlated with loss of germination percentage (Table 2). The critical water content (CWC) for *M. latifolia* seed was 0.48 g g^{-1} FM.

Table 1 Changes in water content, germination percentage, electrolyte leakage, viability and reactive oxygen species (superoxide, hydrogen peroxide and hydroxyl radical) levels in desiccating (temperature 25 ± 2 °C, relative humidity $50 \pm 2\%$) *Madhuca latifolia* seeds

	Days after harvest							
	0	5	10	15	20	25	30	35
WC (g g ⁻¹ FM)	0.59 ± 0.010 ^A	0.59 ± 0.010 ^A	0.48 ± 0.027 ^B	0.43 ± 0.029 ^C	0.40 ± 0.012 ^D	0.28 ± 0.019 ^E	0.25 ± 0.020 ^F	0.19 ± 0.015 ^G
GP (%)	100 ± 0 ^A	100 ± 0 ^A	100 ± 0 ^A	95 ± 4 ^B	70 ± 6 ^C	55 ± 8 ^D	20 ± 4 ^E	0 ± 0 ^F
EL (%)	0.130 ± 0.005 ^F	0.147 ± 0.006 ^E	0.146 ± 0.005 ^E	0.158 ± 0.007 ^D	0.168 ± 0.006 ^{DC}	0.176 ± 0.007 ^C	0.217 ± 0.010 ^B	0.285 ± 0.010 ^A
TZ staining

Viability (A ₅₂₀ g ⁻¹ FM)								
Cotyledons	14.51 ± 0.69 ^A	13.62 ± 1.01 ^B	10.97 ± 0.65 ^C	6.12 ± 0.45 ^D	2.56 ± 0.27 ^E	1.84 ± 0.012 ^F	0.58 ± 0.04 ^G	0.21 ± 0.02 ^G
Embryonic axes	41.82 ± 0.95 ^a	40.39 ± 0.64 ^b	39.63 ± 0.70 ^b	27.64 ± 0.90 ^c	13.81 ± 0.89 ^d	11.58 ± 0.90 ^e	5.23 ± 0.28 ^f	3.11 ± 0.20 ^g
O ₂ ⁻ (μmol g ⁻¹ FM)								
Cotyledons	1.06 ± 0.19 ^H	1.54 ± 0.03 ^G	1.81 ± 0.05 ^F	2.02 ± 0.04 ^E	2.39 ± 0.02 ^D	2.6 ± 0.05 ^C	3.08 ± 0.08 ^B	4.66 ± 0.08 ^A
Embryonic axes	1.13 ± 0.01 ^h	2.32 ± 0.01 ^g	2.88 ± 0.01 ^f	4.57 ± 0.07 ^e	5.06 ± 0.05 ^d	5.36 ± 0.05 ^c	6.02 ± 0.07 ^b	7.58 ± 0.4 ^a
H ₂ O ₂ (μmol g ⁻¹ FM)								
Cotyledons	0.86 ± 0.02 ^G	0.99 ± 0.06 ^G	1.27 ± 0.06 ^F	1.7 ± 0.04 ^E	2.21 ± 0.17 ^D	2.93 ± 0.06 ^C	3.94 ± 0.28 ^B	6.39 ± 0.46 ^A
Embryonic axes	7.82 ± 0.40 ^h	9.41 ± 0.15 ^g	11.32 ± 0.33 ^f	13.3 ± 0.17 ^e	15.25 ± 0.36 ^d	28.52 ± 1.12 ^c	32.73 ± 0.62 ^b	37.8 ± 1.37 ^a
·OH (nmol g ⁻¹ FM)								
Cotyledons	0.72 ± 0.03 ^G	0.76 ± 0.01 ^G	0.91 ± 0.02 ^F	1.01 ± 0.01 ^E	1.16 ± 0.02 ^D	1.27 ± 0.01 ^C	1.4 ± 0.04 ^B	1.56 ± 0.03 ^A
Embryonic axes	3.25 ± 0.14 ^h	4.24 ± 0.11 ^g	5.14 ± 0.12 ^f	5.84 ± 0.09 ^e	6.15 ± 0.06 ^d	6.71 ± 0.17 ^c	7.42 ± 0.30 ^b	9.28 ± 0.32 ^a

Values are means of five replicates ± SD. Data point showing the same letter are insignificant at $p < 0.05$ level

WC water content, GP germination percentage, EL electrolyte leakage, TZ tetrazolium, O₂⁻ superoxide anion, H₂O₂ hydrogen peroxide, ·OH hydroxyl radical

Table 2 Pearson's correlation coefficients of the studied parameters in desiccated (25 ± 2 °C temperature, $50 \pm 2\%$ relative humidity) *Madhuca latifolia* seeds

	DAH	WC	GP	EL	TZ		O_2^-		H_2O_2		OH		TL	
					Cot	EA	Cot	EA	Cot	EA	Cot	EA	Cot	EA
WC	-0.98													
GP	-0.93	0.92												
EL	0.88	-0.86	-0.95											
TZ														
Cot	-0.96	0.95	0.84	-0.76										
EA	-0.96	0.95	0.91	-0.82	0.97									
O_2^-														
Cot	0.93	-0.91	-0.94	0.98	-0.83	-0.87								
EA	0.98	-0.96	-0.88	0.88	-0.96	-0.95	0.93							
H_2O_2														
Cot	0.91	-0.91	-0.96	0.99	-0.80	-0.86	0.98	0.90						
EA	0.94	-0.96	-0.97	0.91	-0.86	-0.91	0.92	0.90	0.94					
OH														
Cot	0.99	-0.99	-0.95	0.90	-0.95	-0.97	0.94	0.97	0.93	0.95				
EA	0.97	-0.96	-0.91	0.93	-0.91	-0.91	0.97	0.98	0.94	0.92	0.97			
TL														
Cot	-0.89	0.92	0.90	-0.76	0.89	0.93	-0.79	-0.83	-0.83	-0.92	-0.91	-0.82		
EA	-0.96	0.98	0.94	-0.84	0.94	0.97	-0.87	-0.92	-0.89	-0.96	-0.97	-0.91	0.97	
Lipase														
Cot	0.95	-0.94	-0.95	0.97	-0.87	-0.90	0.99	0.95	0.98	0.94	0.96	0.98	-0.83	-0.91
EA	0.93	-0.93	-0.97	0.98	-0.83	-0.88	0.98	0.92	0.99	0.95	0.95	0.96	-0.84	-0.91
FFA														
Cot	0.99	-0.98	-0.95	0.92	-0.94	-0.95	0.95	0.97	0.94	0.95	0.99	0.98	-0.88	-0.96
EA	0.97	-0.96	-0.98	0.95	-0.89	-0.94	0.97	0.94	0.97	0.97	0.98	0.96	-0.90	-0.95
LOX														
Cot	0.90	-0.89	-0.97	0.98	-0.78	-0.85	0.97	0.87	0.99	0.95	0.92	0.92	-0.84	-0.89
EA	0.90	-0.89	-0.95	0.99	-0.77	-0.83	0.98	0.89	0.99	0.93	0.92	0.94	-0.79	-0.86
CD														
Cot	0.99	-0.97	-0.91	0.89	-0.94	-0.94	0.94	0.99	0.91	0.93	0.98	0.98	-0.84	-0.93
EA	0.96	-0.95	-0.98	0.96	-0.87	-0.93	0.97	0.93	0.98	0.97	0.97	0.96	-0.89	-0.94
LOOH														
Cot	0.99	-0.97	-0.92	0.90	-0.94	-0.95	0.95	0.99	0.93	0.93	0.99	0.98	-0.87	-0.94
EA	0.98	-0.95	-0.85	0.83	-0.95	-0.93	0.90	0.98	0.85	0.88	0.96	0.97	-0.80	-0.90
MDA														
Cot	0.99	-0.97	-0.95	0.91	-0.94	-0.96	0.95	0.97	0.93	0.96	0.99	0.97	-0.89	-0.96
EA	0.99	-0.98	-0.93	0.90	-0.95	-0.96	0.95	0.98	0.93	0.95	0.99	0.98	-0.89	-0.96
4-HNE														
Cot	-0.16	0.18	0.49	-0.42	0.02	0.21	-0.31	-0.07	-0.43	-0.41	-0.22	-0.15	-0.09	-0.11
EA	0.06	-0.06	0.29	-0.27	-0.21	-0.01	-0.13	0.13	-0.24	-0.18	0.00	0.05	0.14	0.12

Table 2 continued

	Lipase		FFA		LOX		CD		LOOH		MDA	
	<i>Cot</i>	<i>EA</i>	<i>Cot</i>	<i>EA</i>	<i>Cot</i>	<i>EA</i>	<i>Cot</i>	<i>EA</i>	<i>Cot</i>	<i>EA</i>	<i>Cot</i>	<i>EA</i>
WC												
GP												
EL												
TZ												
<i>Cot</i>												
<i>EA</i>												
O ₂ ⁻												
<i>Cot</i>												
<i>EA</i>												
H ₂ O ₂												
<i>Cot</i>												
<i>EA</i>												
·OH												
<i>Cot</i>												
<i>EA</i>												
TL												
<i>Cot</i>												
<i>EA</i>												
Lipase												
<i>Cot</i>	0.99											
<i>EA</i>												
FFA												
<i>Cot</i>	0.97	0.96										
<i>EA</i>	0.98	0.98	0.97									
LOX												
<i>Cot</i>	0.97	0.99	0.92	0.97								
<i>EA</i>	0.98	0.99	0.93	0.96	0.99							
CD												
<i>Cot</i>	0.96	0.94	0.98	0.96	0.89	0.90						
<i>EA</i>	0.98	0.99	0.97	0.99	0.98	0.97	0.95					
LOOH												
<i>Cot</i>	0.97	0.95	0.98	0.97	0.91	0.91	0.98	0.96				
<i>EA</i>	0.92	0.88	0.96	0.92	0.83	0.84	0.98	0.90	0.98			
MDA												
<i>Cot</i>	0.96	0.95	0.98	0.98	0.92	0.92	0.98	0.97	0.99	0.96		
<i>EA</i>	0.97	0.95	0.98	0.97	0.91	0.91	0.99	0.96	0.96	0.97	0.99	
4-HNE												

Table 2 continued

	Lipase		FFA		LOX		CD		LOOH		MDA	
	Cot	EA	Cot	EA	Cot	EA	Cot	EA	Cot	EA	Cot	EA
<i>Cot</i>	– 0.29	– 0.39	– 0.20	0.36	– 0.50	– 0.42	– 0.12	0.39	– 0.17	0.00	– 0.24	– 0.18
<i>EA</i>	– 0.10	– 0.20	– 0.01	– 0.14	– 0.31	– 0.24	– 0.08	– 0.18	– 0.04	0.20	– 0.02	0.04

GP germination percentage, *EA* embryonic axes, *EL* electrolyte leakage, *TL* total lipid, *Cot* cotyledons

Values are significantly different at $p < 0.05$ level. Negative symbol (–) shows negative correlation between two variables, while no symbol meant positive correlation between two variables. Bold values denotes no significant relationship between two variables at $p < 0.05$ level

Fig. 1 Variation in total lipid content in embryonic axes and cotyledons of desiccating (temperature 25 ± 2 °C, relative humidity $50 \pm 2\%$) *Madhuca latifolia* seeds. Values are means of five replicates $\pm$ SD. Data point showing the same letter are not significantly different at $p < 0.05$ level

Leakage of electrolyte increased as seeds desiccated. With increased desiccation, a significant rise in leachate conductivity was observed (Table 1). Rate of electrolyte leakage was negatively correlated with seed WC and germination percentage, but positively with levels of MDA and ROS (Table 2).

The results of the TZ staining test revealed gradual loss of viability of *M. latifolia* seeds with desiccation and DAH (Table 1). With increased desiccation, a precipitous fall in TZ colouration ability of *M. latifolia* seed was discernible (Table 1). Loss of viability was negatively correlated with DAH and level of ROS while positively with seed WC (Table 2).

All the three ROS (Table 1) gradually increased in *M. latifolia* seeds during desiccation. Least levels of all the three ROS were observed in 0 day seeds, which shoots up to 2.1–7.4 folds in 35 DAH seeds. A close dependency of ROS generation was discernible over seed WC and DAH (Table 2).

In response to desiccation, a gradual decline in total lipid content was discernible in both EA and cotyledons of *M. latifolia* seeds (Fig. 1). In this study, cotyledons contained more total lipids than the EA. Declining lipid content was positively associated with tissue WC and germination percentage, but negatively with ROS, lipase and LOX (Table 2).

Consistent with this, the lipase activity (Fig. 2) and FFA content (Fig. 3) increased with DAH and desiccation. Higher lipase activity and FFA content were measured in the EA compared to the cotyledons. Content of FFA and activity of lipase were related positively with DAH and ROS, while negatively with seed WC and germination percentage (Table 2).

Fig. 2 Alteration in lipase activity in embryonic axes and cotyledons of desiccating (temperature $25 \pm 2^\circ\text{C}$, relative humidity $50 \pm 2\%$) *Madhuca latifolia* seeds. Values are means of five replicates $\pm$ SD. Data point showing the same letter are not significantly different at $p < 0.05$ level

Fig. 4 Changes in lipoygenase activity in *Madhuca latifolia* seed tissues with increased rate of desiccation (temperature $25 \pm 2^\circ\text{C}$, relative humidity $50 \pm 2\%$). Values are means of five replicates $\pm$ SD. Data point showing the same letter are not significantly different at $p < 0.05$ level

Fig. 3 Deviation in free fatty acids in embryonic axes and cotyledons of *Madhuca latifolia* seeds during desiccation (temperature $25 \pm 2^\circ\text{C}$, relative humidity $50 \pm 2\%$). Values are means of five replicates $\pm$ SD. Data point showing the same letter are not significantly different at $p < 0.05$ level

Fig. 5 Contents of conjugated dienes in embryonic axes and cotyledons of desiccating (temperature $25 \pm 2^\circ\text{C}$, relative humidity $50 \pm 2\%$) *Madhuca latifolia* seeds. Values are means of five replicates $\pm$ SD. Data point showing the same letter are not significantly different at $p < 0.05$ level

Activity of LOX increased in response to desiccation and along with DAH in *M. latifolia* seeds (Fig. 4) with higher activity in the EA particularly in low viability seeds. Accumulated data exhibited its positive relationship with DAH and ROS, but negative with seed WC, germination percentage and total lipid content (Table 2).

Like LOX, CD too exhibited increasing trend with DAH and loss of tissue WC (Fig. 5). The level of CD was higher in the EA compared to the cotyledons throughout. Content of CD revealed negative association with seed WC, germination percentage and total lipid content, but positive with DAH and ROS (Table 2).

Accumulation of LOOH increased along with desiccation and DAH in *M. latifolia* seeds (Fig. 6). Its levels measured in the EA were higher than in the cotyledons, all

through the investigation. LOOH exhibited positive association with DAH and ROS, but negative with seed WC, and germination percentage (Table 2).

Alike LOOH, MDA too was increased in *M. latifolia* seeds with extent of desiccation (Fig. 7), approving positive relationship with DAH, leakage of electrolyte, and ROS accumulation, but negative with seed WC and tissue viability (Table 2). Its levels were higher in the EA than the cotyledons, in general.

In desiccating *M. latifolia* seeds, an initial gradual rise in 4-HNE was discernible until 15 DAH ($0.43 \text{ g g}^{-1} \text{ FM WC}$) (Fig. 8), thereafter it declined gradually. Higher amount of 4-HNE was measured in the EA, than the cotyledons, throughout.

Fig. 6 Changes in lipid hydroperoxide in embryonic axes and cotyledons of desiccating (temperature $25 \pm 2^\circ\text{C}$, relative humidity $50 \pm 2\%$) *Madhuca latifolia* seeds. Values are means of five replicates $\pm$ SD. Data point showing the same letter are not significantly different at $p < 0.05$ level

Fig. 7 Deviation in lipid peroxidized product malondialdehyde in embryonic axes and cotyledons of dehydrating *Madhuca latifolia* seeds at temperature $25 \pm 2^\circ\text{C}$ and relative humidity $50 \pm 2\%$. Values are means of five replicates $\pm$ SD. Data point showing the same letter are not significantly different at $p < 0.05$ level

Discussion

Water content is an important factor influencing vigour and viability of any seed under long term storage (Pammenter and Berjak 1999; Xia et al. 2015). Our results have confirmed recalcitrant nature of *M. latifolia* seeds that lost their viability rapidly when desiccated below the CWC (Table 1). Our findings are in congruence with the reports of Varghese et al. (2002) who showed a dependence of viability over the WC of *Madhuca indica* seeds. *M. latifolia* seed became completely non-viable as the WC reached to $0.19 \text{ g g}^{-1} \text{ FM}$ on 35 DAH (Table 1). Similar observations of desiccation promoted viability loss was also reported for several recalcitrant seeds like *M. indica*,

Fig. 8 Pattern of 4-HNE content in embryonic axes and cotyledons of *Madhuca latifolia* seeds with extended dehydration at temperature $25 \pm 2^\circ\text{C}$ and relative humidity $50 \pm 2\%$. Values are means of five replicates $\pm$ SD. Data point showing the same letter are not significantly different at $p < 0.05$ level

Knema attenuata, *Shorea robusta*, etc. (Varghese et al. 2002; Vinayachandra and Chandrashekar 2012; Parkhey et al. 2014). In addition, results of TZ staining test also exhibited a steep decline in the tissue coloration ability suggesting rapid loss of viability in *M. latifolia* seeds with DAH and reduced seed WC (Table 1), as was also published for desiccating *S. robusta* seeds (Parkhey 2013).

Increased rate of electrolyte leakage, an indicator of enhanced permeability of cell membranes (Shaban 2013), was discernible during loss of viability in *M. latifolia* seeds, which showed close relationship with seed WC, MDA and ROS levels (Table 2). Involvement of the lipid peroxidation reaction in membrane deterioration and associated loss of viability has already been documented for recalcitrant seeds by Berjak et al. (1992) and Parkhey et al. (2012), among many others. In general, over production of ROS due to desiccation-induced disruption in the metabolism, are responsible for peroxidation of lipid moieties, particularly PUFAs (Berjak and Pammenter 2008; Roach et al. 2010). Similar was the situation in *M. latifolia* seeds, in which all the three ROS significantly increased in desiccated seeds during 35 DAH, revealing negative correlation with loss of viability (Table 2). Just like *Quercus robur* (Pukacka et al. 2011) and *S. robusta* (Parkhey et al. 2012), in *M. latifolia* ROS seem to have imposed damage to membrane lipids (PUFA fraction) associated with the loss of viability during desiccation of seeds. Peroxidation of lipids also results in the production of free radical intermediates such as LOOH and an array of secondary products, which have potential to cause seed deterioration and reduced germination (Gill and Tuteja 2010; Umarani et al. 2015). In the EA and cotyledons of desiccating *M. latifolia* seeds, a gradual loss in total lipid content (3.8 fold) was discernible with simultaneous rise in

LOOH (3.6 fold) and MDA (2.5 fold) contents (Figs. 6 and 7). A good correlation of DAH was calculated for both LOOH and MDA, which were related negatively with seed WC, germination percentage and total lipid content (Table 2). Our findings are in congruence with the observations reported for desiccating *Quercus robur* and *Knema attenuata* seeds by Pukacka et al. (2011) and Vinayachandra and Chandrashekar (2012) respectively.

In the present study, increased lipase was found to be associated with loss of total lipid by enhanced production of FFA and LOOH in the EA and cotyledons of desiccating *M. latifolia* seeds (Figs. 1, 2, 3, 6). The FFA was a key source of LOOH and free radicals in the presence of LOX (Zacheo et al. 2000). Being a membrane destabilizing agent, accumulation of FFA has largely been shown to be related with the loss of viability in desiccating seeds (Xia et al. 2015). Correlation of FFA in deterioration/loss of viability in *M. latifolia* seeds have been established with DAH and germination percentage (Table 2). Similar to our data, lipase promoted a rise in FFA accumulation and loss of viability in oil rich seeds of *S. robusta* (Parkhey et al. 2012). A positive correlation between lipase and FFA accumulation (Table 2), thereby suggesting the involvement of lipase in de-esterification of lipid moieties, was found in desiccating *M. latifolia* seeds.

LOX also exhibited up-regulation (13–69 folds) in the activity, in both EA and cotyledons of *M. latifolia* seeds, in response to desiccation and with extended DAH (Fig. 4). Our findings suggest that LOX enhanced enzymatic oxidation of lipid fractions by degrading FFA and releasing toxic aldehydic products such as LOOH, CD and MDA (Table 2), in the EA and cotyledons respectively. In the species like *Prunus dulcis* and *S. robusta*, existence of active LOX has been detected even in the low water containing (less than $0.4 \text{ g}^{-1} \text{ WC}$) seed tissues (Zacheo et al. 2000; Parkhey et al. 2012). In the EA and cotyledons of *S. robusta* seeds, Parkhey et al. (2012) estimated LOX regulated enhancement in the CD levels by 5.2 and 2.7 folds, respectively. In addition to CD content, accumulation of MDA was also related with loss of viability in *Avicennia marina* and *Antiaris toxicaria* seeds during desiccation (Greggains et al. 2001; Xin et al. 2010). The data obtained here suggested that desiccation promoted LOX-regulated lipid peroxidation, resulting in the accumulation of LOOH, CD and MDA, is involved in the loss of viability in *M. latifolia* seeds. Zacheo et al. (2000) stated that both CD and LOOH may be later broken down or enter into the phenomenon of chemical re-arrangement, thereby forming secondary cytotoxic products. Similar change in the magnitude of MDA and many other lipid oxidized products were also recorded for desiccating *Theobroma cacao* and *S. robusta* seeds by Li and Sun (1999) and Parkhey et al. (2012).

Under oxidative conditions, the lipid peroxidation reaction also produces highly toxic 4-HNE from its chief precursor LOOH inside the mitochondria with subsequent apoptotic cell death (Yin et al. 2015). A significant upsurge in the 4-HNE content occurred in pathogen-infected *Phaseolus vulgaris* and desiccating *S. robusta* seeds, which was found to be linked closely with their physiological quality or viability status (Muckenschnabel et al. 2001; Parkhey et al. 2012). In contrast to the other products of lipid peroxidation reaction, an initial, gradual rise in 4-HNE content was noticed until 15 DAH, which thereafter declined abruptly to their initial levels in both EA and cotyledons of *M. latifolia* seeds on 35 DAH (Fig. 8). In this regard, it could be proposed that most of the 4-HNE formed during the later stages of desiccation of *M. latifolia* seed could not be measured following the protocol employed, due to their conjugation with proteins formed during oxidative stress. Further, our spectrophotometric procedure was able to detect only free forms of 4-HNE; therefore, conjugated forms remained undetermined in this study. Additionally, it could also be argued that the high reactivity and unstable nature of 4-HNE are also possible reasons for its decline after 15 DAH in desiccating *M. latifolia* seeds. A similar decline of 4-HNE was recently reported in the EA and cotyledons of desiccating *S. robusta* seeds after 5 DAH by Parkhey et al. (2012).

Conclusions

Desiccation promoted loss of viability in *M. latifolia* seeds was correlated with accumulation of ROS and its lipid peroxidation products resulting in loss of membrane integrity. Extended desiccation contributed peroxidation of PUFAs, correlating with the loss of viability in *M. latifolia* seeds. In addition, activity of both lipase and LOX also linked with the loss of viability through production of FFA, CD, LOOH, MDA and 4-HNE, in desiccating *M. latifolia* seeds. In summary, our findings suggested that several mechanisms (ROS, lipid oxidation, and lipase and lipoxygenase) were simultaneously operative in desiccating *M. latifolia* seeds finally contributing to loss of viability.

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Authors' contributions JC performed the experiments, generated and analyzed the data, and drafted the manuscript. SK conceptualized the project, designed the experimental protocols, and finalized the manuscript draft. Both the authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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