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Reactive oxygen species, lipid peroxidation, protein oxidation and antioxidative enzymes in dehydrating Karanj (*Pongamia pinnata*) seeds during storage

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1. Introduction

Seed longevity, an important trait from ecological and agricultural perspectives, has been studied in considerable detail (Rajjou et al., 2008; Nonogaki et al., 2010). Seeds after harvest, undoubtedly deteriorate gradually and lose quality during extended storage (Rajjou et al., 2008). The seed storage behaviour has been categorized as orthodox and recalcitrant on the basis of desiccation tolerance and sensitivity, respectively. Orthodox seeds can be stored for longer periods if their moisture contents are reduced to 1–5% (Ellis et al., 1991a), whereas the recalcitrant seeds are killed when dehydrated below relatively high moisture content (30–50%) (Varghese and Naithani, 2008). Several seeds like *Carica papaya*, *Coffea arabica*, *Elaeis guineensis* (Ellis et al., 1991a,b) once categorized as recalcitrant have now been reclassified as intermediate in storage behaviour.

During ageing, loss of seed vigour and viability precedes the loss in germinability (Eksi and Demir, 2011). Membrane deterioration associated enhanced solute leakage, a measure of seed vigour (Eksi and Demir, 2011), has been reported in several recalcitrant seeds during ageing (Pukacka and Ratajczak, 2007; Varghese and Naithani, 2008). Similarly, the ageing related loss of viability was reported in seeds using TTC (triphenyl tetrazolium chloride), a quick and precise test (ISTA, 2003). In dry and viable seeds, leakage of reactive oxygen species (ROS) from electron transport chain of mitochondria during seed desiccation is inevitably enhanced that in turn promotes oxidative damage of nucleic acids, proteins and lipids. Active metabolism in the hydrated pockets, reported in restricted cellular areas of dry seeds, is one of the potential sources of ROS formation (Leubner-Metzger, 2005). Additionally, non-enzymatic ROS production in anhydrate sites of dry seeds also contributes to ageing associated cellular damage (Job et al., 2005). Excessive accumulation of ROS (superoxide radical and H_2O_2) in orthodox, recalcitrant and intermediate seeds (Bailly, 2004; Pukacka and Ratajczak, 2007; Varghese and Naithani, 2008; Sahu et al., 2017) has been discussed as a potential cause of viability loss. ROS induced oxidative damage of proteins and lipids (Balesevic-Tubic et al., 2007; Oracz et al., 2007; Varghese and Naithani, 2008; Parkhey et al., 2012) leads to severe cellular damage that eventually results in loss of viability (Halliwell and Gutteridge, 2007). Membrane damage and generation of toxic by-products are common features of lipid peroxidation (Parkhey et al., 2012). Many proteins are specific as they are regulatory and associated with particular stages of seed development (Tunnacliffe et al., 2010; Sahu et al., 2017), dormancy (Oracz et al., 2007), germination (Nonogaki et al., 2010; Tunnacliffe et al., 2010) and longevity/ageing (Rajjou et al., 2008). Carbonyls are produced by direct oxidative attack on Lys, Arg, Pro or Thr residues of proteins (Job et al., 2005). Accumulation of carbonyl derivatives, a diagnostic marker of oxidative stress, occurs due to ROS induced protein oxidation (Job et al., 2005).

Protection against ROS is offered by a battery of antioxidases, such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) (Pukacka and Ratajczak, 2007; Sahu et al., 2017). SOD, present in all aerobic organisms, catalyses the formation of H_2O_2 from superoxide radical. The CAT and APX protect the cell from potentially toxic H_2O_2 (Sahu et al., 2017). CAT is regulatory in the removal of the H_2O_2 produced under various stress conditions and also during β-oxidation of the fatty acids in germinating oily seeds (Bailly, 2004). APX regulates the intracellular H_2O_2 present in all cell compartments and unlike CAT, participates in cell detoxification due to its high affinity for H_2O_2 (Kranner and Birtic, 2005). The role of APX becomes decisive in the context of metabolizing excess amount of H_2O_2 in the desiccating seeds (Bailly, 2004; Sahu et al., 2017). Impairment of ROS scavenging enzymes is the key determinants in the loss of viability and vigour in several

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seeds (Kibinza et al., 2006; Varghese and Naithani, 2008; Sahu et al., 2017). Desiccation of non-orthodox seeds below critical moisture content leads to excessive promotion in the levels of ROS due to impairment of antioxidative enzymes (Sahu et al., 2017).

Karanj (*Pongamia pinnata* L.) an arboreal legume commonly known as Indian Beech tree, a member of subfamily Papilionaceae (Scott et al., 2008), is indigenous to the Indian subcontinent and South-East Asia (Sangwan et al., 2010). It has also been introduced to several countries as Australia, New Zealand and the United States of America (Scott et al., 2008) for controlling soil erosion, binding sand dunes because of its dense network of lateral roots (Sangwan et al., 2010). It is an excellent multipurpose tree and immensely used for its medicinal properties. Seeds yield a non-edible 40% Pongam or Karanga oil which is a potential source for biodiesel (Sangwan et al., 2010). The present work describes the seed storage behaviour and seed longevity of karanj during storage at natural ambience to offer standardized protocol for *ex-situ* storage in the germplasm banks. Attempt was also made to unravel the potential causes of seed ageing by monitoring the levels of ROS and its detoxifying enzymes to explain the mechanism of seed ageing as well as intermediate storage behaviour.

2. Material and methods

2.1. Seed collection, storage and determination of physical attributes

Freshly mature pods of karanj (*Pongamia pinnata*) were collected manually from the avenue trees in and around Raipur, Chhattisgarh, India in the month of April. The pods were brought to the laboratory immediately (within 2 h) and seeds were extracted by slight hammering of the pods or by pressing the knife along the suture. Freshly harvested, healthy and uniform sized seeds were stored (for natural ageing) in well aerated baskets at ambient temperature (27–30 °C and RH 45%) and were taken out from storage at different days of storage (*i.e.* 0, 90, 135, 225, 315 and 405 days) for various analyses. Average seed weight was determined by weighing hundred seeds individually (DFSC/IPGRI, 1999). The initial moisture content (IMC) of seeds was determined by Varghese and Naithani (2008). The seed size, length, breadth and other physical attributes were measured for hundred individual seeds and repeated five times independently. All the analysis were performed on the seeds sample harvested from the storage on 0 (same day), 90, 135, 225, 315 and 405 days.

2.2. Water content

Five independent replicates of ten seeds each were weighed before and after oven drying at 96 °C for 60 h for estimation of water content (WC) (ISTA, 1993). WC was calculated on dry mass (DM) basis and expressed as $gH_2O\ g^{-1}$ DM.

2.3. Germination and germination index

Seeds were surface sterilized (in 0.1% mercuric chloride) for 15 min, thoroughly washed four times with distilled water and placed on filter paper towels saturated with distilled water in a Petri dish. Petri dishes were kept in dark (karanj seeds are negative photoblastic, unpublished) at 28–30 °C and germination was scored every 24 h as the radicle emerges to 5 mm in length. Germination was expressed in percentage. To evaluate the speed of germination, the germination index (GI) of the seeds was estimated as described elsewhere (Sahu et al., 2017).

2.4. Electrolytic leakage

The electrolytic leakage of seeds was measured by determining the leachate conductivity (Sahu et al., 2017). Ten seeds were soaked in 30 ml of distilled water and leachates were collected after 24 h of imbibition. The conductivity of leachates was determined using Digital Direct Reading Conductivity Meter (Elico) and results were expressed as milli-Siemen (mS) $seed^{-1}$.

2.5. Viability

Seed viability was estimated by immersing the seeds in distilled water at 27–30 °C in dark for 18 h, as per details described in the germination test. After imbibition, the papery brown seed cover was removed carefully using forceps. The de-coated seeds were immersed in 1% solution of 2, 3, 5-triphenyl tetrazolium chloride (Sigma, USA) and incubated in dark at 27–30 °C for 12 h. The red-colored formazan formed after incubation was extracted from weighed amount of seeds with 5 ml of ethanol. The absorbance of the ethanolic solution obtained after centrifugation at 5000 rpm for 10 min was read at 520 nm (ISTA, 1996). Seed viability was expressed as $A_{520}\ g^{-1}$ FW. The experiment was performed with ten seeds in five independent replicates.

2.6. ROS determination

Embryonic axes were homogenized into powder using liquid nitrogen and used for ROS determination. The superoxide radical was estimated by the method of Sangeetha et al. (1990). Weighed amount (100 mg) of liquid nitrogen powder of axes was thoroughly mixed with 2 ml sodium phosphate buffer (0.2 M, pH 7.2) containing diethyle dithiocarbamate (10^{-3} M). The mixture was centrifuged for 15 min at 10,000 rpm and the superoxide radical in the supernatant was measured at 540 nm by its capacity to reduce nitro blue tetrazolium (2.5×10^{-4} M). The amounts of superoxide radical were expressed as $\bullet O_2^-\ min^{-1}\ g^{-1}$ FW.

The H_2O_2 was estimated following Schopfer et al. (2001). Liquid nitrogen powder of axes (100 mg) was dissolved in 3 ml of $KHPO_4$ (20 mM, pH 6.0) containing 5 μM Scopoletin (Sigma, USA) and 3 μg ml^{-1} horseradish peroxidase (Sigma, USA) in dark at 25 °C on a shaker for 1, 2, 3 and 4 h. The reaction mixture was centrifuged for 10 min at 10,000 rpm and the decline in fluorescence (Excitation: 346 nm, Emission: 455 nm) of scopoletin in supernatant was detected using spectrofluorometer (Shimadzu, Japan). H_2O_2 was expressed as nM $H_2O_2\ h^{-1}\ mg^{-1}$ FW.

2.7. Lipid peroxidation

The level of lipid peroxidation was measured by following Heath and Packer (1968). Liquid nitrogen powder of axes weighing 50 mg was homogenized with 0.5% (w/v) 2-thiobarbituric acid prepared in 20% (w/v) trichloroacetic acid, incubated for 30 min in a pre-heated water bath (100 °C) and transferred at 0 °C for 30 min. Thereafter, the homogenate was centrifuged at 10,000 rpm for 15 min and the volume of clear supernatant made up to 3 ml with distilled water. The amount of malondialdehyde-thiobarbituric acid complex was measured at 540 nm and corrected for the non-specific absorbance by subtracting the value obtained at 600 nm. Interfering absorbance was removed by recording absorbance at 440 nm to eliminate the interference by sucrose. The amount of MDA was calculated from the extinction coefficient of 150 $mM^{-1}\ cm^{-1}$ (Einset and Clark, 1958) and expressed as nM MDA g^{-1} FW.

2.8. Carbonylated protein

Carbonyl protein was estimated following Levine et al. (1994). Axes powder (50 mg) was homogenized in sodium phosphate buffer (10 mM, pH 7.2) containing 1 mM EDTA, 2 mM DTT, 0.2% (v/v) Triton X-100, 1 mM PMSF. Homogenate was centrifuged at 10,000 rpm for 20 min and protein concentration was determined in the supernatant following Bradford (1976). Protein extract, containing 50 mg protein, was incubated with 0.03% (v/v) Triton X-100 and 1% (w/v) streptomycin sulphate for 20 min to remove the nucleic acids and centrifuged at 10,000 rpm for 10 min. The supernatant was mixed with 300 μl of