CHAPTER 3

ACTIVE OXYGEN SPECIES

Mature Fruits of *Syzygium cuminii*
**Introduction**

**Active** oxygen species (AOS) are produced as an important facet of cellular metabolism of all aerobic systems. AOS homeostasis is maintained by AOS producing and scavenging enzymes and the activities of these enzymes in all the cellular compartments of the seed is under tight control of cellular environment in close association with various developmental phases of cell metabolism (Greggains *et al.*, 2001; Dussert *et al.*, 2006; Kibinza *et al.*, 2006; Pukacka and Ratajczak, 2007; Roach *et al.*, 2007). Excess formation of AOS as a net result of unregulated metabolism is considered responsible for oxidative stress induced loss of seed viability. Nearly 2-3% of the oxygen consumed by the mitochondrial ETC is converted into superoxide that subsequently generates H$_2$O$_2$ in hydrated seeds (Bailly *et al.*, 2008). The superoxide and later other forms of AOS are also formed in the series of reactions occurring extracellularly primarily by the catalytic action of NADPH oxidase (Lamb and Dixon, 1997) and peroxidases (Bolwell, 1995). In contrast, the source of AOS formation in relatively dry seeds is non-enzymic like lipid peroxidation or Amadori and Maillard reaction (Priestley, 1986; Sun and Leopold, 1995). Damaging levels of AOS accumulated due to failure of antioxidant enzymes has been reported in low viable seeds of wide range of species (Priestley, 1986; Chaitanya and Naithani, 1994, 1998; McDonald, 1999; Varghese and Naithani, 2002; Bailly, 2004; Pukacka and Ratajczak, 2007; Varghese and Naithani, 2008). High reactivity of AOS ($\bullet$O$_2^\cdot$, H$_2$O$_2$, OH•) towards proteins, sugars, lipids and nucleic acids plays key role during oxidative stress mediated cellular injury (Benson and Bremner, 2004; Kranner and Britic, 2005; Roach, 2009; Roach *et al.*, 2010; Varghese *et al.*, 2011; Parkhey *et al.*, 2012). In addition, H$_2$O$_2$ inactivates
SH-groups of several structural and functional proteins involved in transport, receptors and ion channels etc. leading to extensive cellular damage (Halliwell and Gutteridge, 1999) (Refer Chapter V for detail). According to Bailly (2004), the detoxifying potential of scavenging enzymes may be reduced due to H$_2$O$_2$ mediated inactivation of their functional integrity.

During seed storage, endogenous AOS are directly involved in viability loss (Priestley, 1986; McDonald, 1999; Bailly, 2004; Pukacka and Ratajczak, 2007; Cai et al., 2011). Reduced viability in sunflower seeds dried below CWC ($\approx 0.21$ g H$_2$O g$^{-1}$ DM) was accompanied with rapid accumulation of H$_2$O$_2$ (El-Maarouf-Bouteau et al., 2011). Similarly, the endogenous levels of AOS was elevated in ageing recalcitrant seeds like Shorea robusta (Chaitanya and Naithani, 1998), Azadirachta indica (Varghese and Naithani, 2002), Castanea sativa (Roach et al., 2007), Antiaris toxicaria (Xin et al., 2010) etc. Accelerated decline in seed viability and vigour during early stages of imbibition in sunflower is associated with altered ability of H$_2$O$_2$ detoxification due to impaired catalase activity (Bailly et al., 1996, 1998, 2002). Impaired activities of SOD, CAT and GR in the embryonic axis of ageing sunflower seed is responsible for accumulation of H$_2$O$_2$ and primary/secondary products of lipid peroxidation (Kibinza et al., 2006). Several lines of evidence (Leprince et al., 1993; Chaitanya and Naithani, 1994, 1998; Chaitanya et al., 2000b; Varghese and Naithani, 2002, 2008; Ahmad et al., 2010) suggest that AOS produced during ageing causes severe membrane perturbations. It triggers phospholipid degradation, leading to irreversible formation of gel phase domains and loss of membrane function (Senaratna et al., 1988; McDonald, 1999). AOS, together with lipid-peroxidized products, are widely considered to be major contributors to
seed deterioration (Hendry, 1993; Chaitanya and Naithani, 1994, 1998; Chaitanya et al., 2000b; Varghese and Naithani, 2002, 2008). They generate changes in unsaturated fatty acids that affect the structural and functional properties of cell membranes such as the inactivation of membrane bound proteins and an increase in membrane permeability (Hendry, 1993; McDonald, 1999). In recalcitrant seeds, the desiccation induced loss of seed viability and vigour is closely related to elevated levels of AOS (Bailly et al., 2008). Drying of excised axis of Castanea sativa exhibited a classical Gaussian pattern in •O₂⁻ production in response to drying (Roach et al., 2007). Analysis of pattern and rate of •O₂⁻ generation, H₂O₂ and TBARS content studied in the axis of Antiaris toxicaria during slow drying revealed correlation between loss of desiccation with the increase in rate of •O₂⁻ accumulation, content of H₂O₂ and TBARS with concomitant decline of antioxidant enzyme activities (Cheng and Song, 2008). Desiccation induced water loss is linked with an upsurge of free radicals that mediates substantial damage in the axis and cotyledon of desiccated seeds (Côme and Corbineau, 1996; Kermode and Finch-Savage, 2002; Corbineau et al., 2004). In fact, decline in viability and vigour in response to dehydration is explained by the incompetence of seed ability to protect the seed from AOS injury (Côme and Corbineau, 1996; Varghese and Naithani, 2002; Corbineau et al., 2004; Pukacka and Ratajczak, 2007; Varghese and Naithani, 2008). Competence to survive the oxidative assault imposed by AOS depends on the scavenging capacity of the cell. In fact, dehydration induced oxidative stress is promoted when AOS production exceeds the capacity of antioxidant system (Bailly et al., 1998, 2002; Corbineau et al., 2004; Pukacka and Ratajczak, 2007). Loss of viability in most of the dehydration sensitive seeds is accompanied by reduced antioxidant capacity; antioxidant molecules (Kranner et al., 2006) and enzymes
(Varghese and Naithani, 2008; Pukacka et al., 2011). Leprince et al.
(1999) concluded that desiccation intolerance in the recalcitrant seeds
may be linked to their inability to down regulate their metabolism during
dehydration, thereby enhancing drying induced oxidative cellular
damage. Dehydration of recalcitrant seeds to intermediate water content
perturbs the metabolic balance towards overproduction of AOS finally
leading to alteration at physiological, biochemical and molecular level
ultimately converging into reduced seed viability and vigour (Berjak et
al., 2000, 2007; Berjak and Pammenter, 2008; Kranner et al., 2010).

The aforementioned literature clearly indicated the active and
direct involvement of AOS in seeds, 1-during ageing (Slow drying) and,
2-dehydration induced loss of viability. The present investigation was
designed to probe the role of AOS during loss of viability in dehydrating
jamun seeds. Superoxide and hydrogen peroxide, important AOS, were
estimated in the axis and cotyledon of jamun seeds to confirm their
relative role during desiccation induced loss of germination. The
differential response of drying rate on germinability in desiccating jamun
seeds was evaluated by measuring AOS levels in the slow and rapid dried
seeds.

**Materials and Methods**

**Superoxide Radical**

Superoxide radical in slow and rapidly dried jamun seeds at
various stages was estimated by the method of Sangeetha et al. (1990)
utilizing nitroblue tetrazolium (NBT) as a detection system. The
reduction of NBT by superoxide radical was detected by recording blue
formazon (reduced NBT) at 540 nm. The calibration curve was obtained
by determining the rate of NBT reduction using pyrogallol as a superoxide radical source at 540 nm. To 2.8 ml of sodium phosphate buffer (0.2 M, pH 7.2) containing 100 µl of NBT (2.5 x 10⁻⁴ M) was added in a 3 ml quartz cuvette. The absorbance was zeroed. The reaction was triggered by adding 100 µl of 0.2 mM pyrogallol. The change in absorbance was recorded at 540 nm for 15 minutes. The superoxide formation in the extract was estimated by recording the kinetics of the reaction mixture containing 2.8 ml of sodium phosphate buffer, 200 µl extract and 100 µl NBT solution at 540 nm. The superoxide radical was expressed as •O₂⁻ min⁻¹ g⁻¹ FW

**Hydrogen Peroxide**

Batches of slow and rapidly dried jamun seeds of various stages were preincubated for 30 minutes in 3 ml of potassium phosphate buffer (20 mM, pH 6.0) to remove preformed H₂O₂ and then incubated in the incubation medium containing 5 µM Scopoletin (Sigma, USA) and 30 µg ml⁻¹ horse radish peroxidase (Sigma, USA) in dark at 25ºC on a shaker. H₂O₂ was estimated following Schopfer et al. (2001). Principally, the decline in fluorescence was monitored using spectroflurometer (Shimadzu, Japan) at excitation: 346 nm and emission: 455 nm. The hydrogen peroxide radical was expressed as µM H₂O₂
### Results

#### Superoxide Radical – Slow Drying

**Figure 3.1** Accumulation of superoxide radical in the axis and cotyledon of *Syzygium cuminii* seeds with decline in water content during slow drying. Correlation between superoxide radical and water content of dehydrating seed was $r = -0.89$ (axis) and $r = -0.94$ (cotyledon), whereas correlation between superoxide radical and days of slow drying was $r = 0.95$ (axis) and $r = 0.92$ (cotyledon). Data are mean of 4 replicates $\pm$ SD, where no bars are shown, the spread of $\pm$ SD is less than the size of the symbol.

Throughout the analysis, the accumulation of superoxide radical ($\bullet$O$_2^-$) was comparatively higher in the axis than in the cotyledon. Significantly low levels of superoxide radical ($\bullet$O$_2^-$) were observed in the axis (210.40 $\bullet$O$_2^-$ min$^{-1}$ g$^{-1}$ FW) and cotyledon (101.11 $\bullet$O$_2^-$ min$^{-1}$ g$^{-1}$ FW) of fresh undesiccated seeds that increased sharply in the axis whereas gradually in the cotyledon with the advance in dehydration of seeds during slow drying. Dehydration of fresh seeds from 0.93 to 0.23 g H$_2$O g$^{-1}$ DM resulted in almost 4.5-fold enhancement in superoxide radical ($\bullet$O$_2^-$) levels i.e. from 210.40 to 890.97 $\bullet$O$_2^-$ min$^{-1}$ g$^{-1}$ FW in axis, whereas 2.5-fold increase i.e. from 101.11 to 275.40 $\bullet$O$_2^-$ min$^{-1}$ g$^{-1}$ FW in the cotyledons. A strong negative correlation was established between superoxide radical and the decrease in water content in axis ($r = -0.89$) and cotyledon ($r = -0.94$), while a positive correlation was established...
between superoxide radical in the axis \((r = 0.95)\) and cotyledon \((r = 0.92)\) with the days of slow drying.
Superoxide Radical – Rapid Drying

Figure 3.2 Accumulation of superoxide radical in the embryonic axis and cotyledon of Syzygium cuminii seeds during the rapid drying (by silica gel) of water content. Correlation between superoxide radical and water content of dehydrating seed was \( r = -0.98 \) (axis) and \( r = -0.96 \) (cotyledon), while the correlation established between superoxide radical and days of rapid drying was \( r = 0.99 \) (axis) and \( r = 0.97 \) (cotyledon). Data are mean of 4 replicates \( \pm \) SD, where no bars are shown, the spread of \( \pm \) SD is less than the size of the symbol.

Significantly low levels of superoxide radical (\( \cdot \text{O}_2^- \)) was estimated in the axis and cotyledon of fresh jamun seeds but it was increased sharply in the axis and gradually in the cotyledon with rapid drying. The amount of superoxide radical (\( \cdot \text{O}_2^- \)) was comparatively higher in the axis than the cotyledon. For example, the levels of superoxide estimated in the axis were 210.40 \( \cdot \text{O}_2^- \) min\(^{-1}\) g\(^{-1}\) FW whereas it was 101.11 \( \cdot \text{O}_2^- \) min\(^{-1}\) g\(^{-1}\) FW in cotyledon. The levels of superoxide radical were promoted nearly 2-folds in axis and cotyledons when the seeds were rapidly-dried from 0.93 to 0.48 g H\(_2\)O g\(^{-1}\) DM during first 4 days. Later the accumulation of superoxide radical was gradual in cotyledon from 182.22, 221.05, 244.50 and 257.40 \( \cdot \text{O}_2^- \) min\(^{-1}\) g\(^{-1}\) FW in seeds dried to 0.48, 0.39, 0.26 and 0.12 g H\(_2\)O g\(^{-1}\) DM. The levels of superoxide radical in the axis increased from 416.83 to 489.43 \( \cdot \text{O}_2^- \) min\(^{-1}\) g\(^{-1}\) FW in seeds rapid-dried from 0.48 to 0.39 g H\(_2\)O g\(^{-1}\) DM but further rapid drying to 0.26 and 0.12 resulted in in accumulation of superoxide radical to 554.39 and 650.38 \( \cdot \text{O}_2^- \) min\(^{-1}\) g\(^{-1}\)}
FW, in the axis respectively. A negative correlation was established between superoxide radical in the axis \( r = -0.98 \) and cotyledon \( r = -0.96 \) with decline in water content, while a positive correlation was established between superoxide radical in axis \( r = 0.99 \) and cotyledon \( r = 0.97 \) with the days of rapid drying.
Like superoxide, the levels of hydrogen peroxide were promoted in the embryonic axis and cotyledon during slow drying of fresh jamun seeds. In the embryonic axis of fresh undesiccated seeds (0.93 g H$_2$O g$^{-1}$ DM) the level of hydrogen peroxide was 37.11 µM H$_2$O$_2$. The level of hydrogen peroxide in the axis was promoted to 153.80 µM H$_2$O$_2$, nearly 3.5-fold, as the seeds were dehydrated to 0.23 g H$_2$O g$^{-1}$ DM during slow drying. Unlike the axis, the level of hydrogen peroxide in the undesiccated cotyledon (0.93 g H$_2$O g$^{-1}$ DM) was 53.80 µM H$_2$O$_2$. It was not much altered as the seeds were dehydrated to 0.81 g H$_2$O g$^{-1}$ DM. But further drying of seeds elevated gradually the accumulation of hydrogen peroxide and showed highest i.e. 92.90 µM H$_2$O$_2$ in the cotyledon of most desiccated seeds (0.23 g H$_2$O g$^{-1}$ DM). Axis recorded relatively higher levels of hydrogen peroxide than the cotyledon. A negative correlation was established between hydrogen peroxide and the decrease in water content in axis ($r = -0.94$) and cotyledon ($r = -0.97$), while a positive
correlation was established between hydrogen peroxide in the axis ($r = 0.99$) and cotyledon ($r = 0.99$) with the days of slow drying.
Hydrogen Peroxide – Rapid Drying

Figure 3.4 Changes in hydrogen peroxide level in the embryonic axis and cotyledon of Syzygium cuminiit seeds in response to decline in water content during the rapid drying (by silica gel). Correlation between hydrogen peroxide and water content of dehydrating seed was r = - 0.94 (axis) and r = - 0.95 (cotyledon) while the correlation between hydrogen peroxide and days of rapid drying was r = 0.98 (axis) and r = 0.97 (cotyledon). Data are mean of 4 replicates ± SD, where no bars are shown, the spread of ± SD is less than the size of the symbol.

Rapid drying of jamun induced promotion in the level of hydrogen peroxide accumulation both in axis and cotyledon as well. The cotyledon of the fresh seed registered higher amounts hydrogen peroxide (53.80 µM H₂O₂) then the axis (37.11 µM H₂O₂). Later with the rapid drying of seeds the accumulation of hydrogen peroxide in the cotyledon remained comparatively low than the axis. For example, the axis observed increase in the level of hydrogen peroxide from 42.69 to 130.64 µM H₂O₂ whereas it was 54.85 to 72.70 µM H₂O₂ in the cotyledon of seeds dried from 0.63 to 0.12 g H₂O g⁻¹ DM. Significantly sharp promotion in the level of hydrogen peroxide was discernible in axis (70.55 to 130.64 µM H₂O₂) during rapid-drying from 0.48 to 0.12 g H₂O g⁻¹ DM. A strong negative correlation established with the accumulation of hydrogen peroxide in the axis (r = - 0.94) and cotyledon (r = - 0.95) and the decline in water content, while a positive correlation was established between accumulation of hydrogen peroxide in the axis (r = 0.98) and cotyledon (r
= 0.97) with the days of rapid drying.

**Discussion**

The formation and accumulation of active oxygen species (AOS) have been reported in ageing seeds (Buchvarov and Gantcheff, 1984; Hendry, 1993; McDonald, 1999; Pukacka and Ratajczak, 2005; Oracz et al., 2007). During mitochondrial respiration, nearly 2-3% of the oxygen consumed escaped ETC to produce superoxide and hydrogen peroxide (Nelson et al., 1992). Loss of seed germinability and GI was inversely proportional to the accumulation of active oxygen species, namely, superoxide and hydrogen peroxide during dehydration; slow and rapid in the axis and cotyledon of jamun seeds. Strong negative correlation obtained between accumulation of AOS and rate of drying in jamun seeds whereas positive correlation was noticed between levels of AOS and total period of drying.

Seed deterioration during ageing and dehydration especially in recalcitrant seeds has been widely quoted with AOS metabolism. AOS has been extensively discussed as a foremost cause of loss of viability in recalcitrant - *Acer platanoides* (Pukacka, 1991), *Quercus robur* (Hendry et al., 1992), *Shorea robusta* (Chaitanya and Naithani, 1994, 1998) and intermediate - *Azadirachta indica* (Varghese and Naithani, 2002, 2008) seeds. AOS is central in all oxidative damage during seed ageing (Chaitanya and Naithani, 1994; Varghese and Naithani, 2002) and the magnitude of accumulation has been shown to be positively correlated with degree of viability loss in various recalcitrant seeds (Chaitanya and Naithani, 1994, 1998; Varghese and Naithani, 2002; Pukacka and Ratajczak, 2005; Dussert et al., 2006; Cai et al., 2011).
AOS are potentially highly toxic and are, therefore, tightly controlled by antioxidants (Foyer and Noctor, 2005). Fresh jamun seeds recorded lowest levels of AOS in the axis and cotyledon that were enhanced with the advance in drying. Presumably, low levels of AOS in the freshly harvested high viable and vigour jamun seeds may be due to presence of higher amounts of antioxidant enzyme system (Refer Chapter IV). Vigorous seeds showing high germination capacity are invariably supported by higher antioxidant capacity (as also reported for jamun seeds and discussed in next chapter) that controls the levels of AOS by enzymatic detoxification thus preventing the excess accumulation that may directly or indirectly mediate cell damage through lipid peroxidation.

The levels of superoxide and its rate of accumulation were exceptionally high in the axis than the cotyledon of jamun seeds. Nearly 4-fold rise in the levels of superoxide was noticed in axis compared to 2.5-fold in cotyledon. During slow-dehydration the net rise of superoxide was relatively higher \(135 \cdot \text{O}_2^- \text{min}^{-1} \text{g}^{-1} \text{FW}\) in the cotyledon of the seeds initially dried from 0.93 to 0.42 than during the drying at later stages from 0.42 to 0.23 \(39 \cdot \text{O}_2^- \text{min}^{-1} \text{g}^{-1} \text{FW}\). In contrast, the axis showed gradual promotion of superoxide through slow-dehydration. On the other hand, accumulation pattern of superoxide in the axis and cotyledon in response to rapid drying was slightly sluggish than the slow drying. In axis, the rapid drying of seeds initially (0.93 to 0.48 g H\(_2\)O g\(^{-1}\) DM) induced relatively 2-fold amounts of superoxide whereas 1.5-fold in the later phase (0.48 to 0.12 g H\(_2\)O g\(^{-1}\) DM) of drying. Like axis, the accumulation pattern of superoxide observed was similar showing 1.8-fold promotion in the initial period (from 0.93 to 0.48 g H\(_2\)O g\(^{-1}\) DM) of drying whereas it was 1.4-fold during later phase (from 0.48 to 0.12 g H\(_2\)O g\(^{-1}\) DM) of drying. Activities of scavenging enzymes, responsible for
regulating AOS concentrations, are decreased in response to desiccation of recalcitrant seeds (Kranner, 2002; Kranner et al., 2002). Consequently, enhanced levels of AOS mediate deleterious reactions leading to seed ageing (Harman, 1987; Beckmann and Ames, 1998). In germinating maize, loss of desiccation tolerance is associated with increased levels of AOS (Leprince et al., 1990). Desiccation induced AOS burst was also reported in various recalcitrant seeds/axis, like Shorea robusta (Chaitanya and Naithani, 1994), Theobroma cacao (Li and Sun, 1999), Azadirachta indica (Varghese and Naithani, 2002), Araucaria bidwillii (Francinic et al., 2006), Acer pseudoplatanus (Pukacka and Ratajczak, 2007) etc.

$\text{H}_2\text{O}_2$ levels estimated in the axis and cotyledon of jamun seeds desiccated from 0.93 to 0.48 g H$_2$O g$^{-1}$ DM promoted comparatively higher amounts of H$_2$O$_2$ in slow dried than in the rapid dried seeds. The amounts of H$_2$O$_2$ increased from 53.8 to 92.9 and 37.11 to 153.8 μM H$_2$O$_2$ respectively in cotyledon and axis of seeds dried from 0.93 to 0.48 g H$_2$O g$^{-1}$ DM. Rapid drying induced net increase in the amounts of H$_2$O$_2$ was relatively less than the slow drying as the cotyledon and axis of seeds dried from 0.93 to 0.12 g H$_2$O g$^{-1}$ DM observed accumulation of H$_2$O$_2$ respectively from 53.8 to 72.7 and 37.11 to 130.64 μM H$_2$O$_2$. Loss of desiccation tolerance in seeds of Antiaris toxicaria was correlated with the increase in •O$_2$ rate of accumulation, content of H$_2$O$_2$ and TBARS, and concomitant decline of antioxidant enzyme activities (Cheng and Song, 2008).

Surprisingly, initially seeds exhibiting 100% germination showed comparatively lower amounts of H$_2$O$_2$ in the axis than the cotyledon of jamun seeds, both during slow and rapid drying, but dehydration of seed below CWC (0.86 g H$_2$O g$^{-1}$ DM in slow dried seeds whereas 0.63 in
rapid dried seeds) leads to higher levels of $\text{H}_2\text{O}_2$ in the axis than in the cotyledon, in the later period of drying. Different deleterious mechanisms have been shown to operate under different drying rates in the axis of *Ekebergia capensis* (Pammenter *et al.*, 1998) and *Artocarpus heterophyllus* (Wesley-Smith *et al.*, 2001). Three categories of cellular damage namely mechanical damage, metabolism-induced damage and macromolecular denaturation have been proposed during seed ageing.

Our data clearly indicated the active role of AOS in desiccation induced loss of germinability in jamun seeds. Substantially low or signaling levels of AOS in the undried seeds allow smooth activation of germination related events as the seeds imbibe water. But desiccation of hydrated seeds induced loss of water (perhaps structured water) in the axis and cotyledon of jamun seeds and initiated a cascade of enzymatic and non-enzymatic reactions as a result of unregulated metabolism that promoted over accumulation of AOS. Higher amounts of AOS are perhaps regulatory in promoting the loss of seed viability in jamun seeds. Ameliorative effect of rapid drying under intermediate MC level was ascribed to minimize unbalance of AOS metabolism as a consequence, restricting oxidative damage, and dehydration induced damage (Pammenter and Berjak, 1999; Wesley-Smith *et al.*, 2001). Desiccation sensitivity developed in recalcitrant seeds/axis especially below CWC is generally due to increased synthesis of AOS along with reduced capacity of antioxidants (Chaitanya and Naithani, 1994, 1998; Li and Sun, 1999; Pammenter and Berjak, 1999; Varghese and Naithani, 2008; Varghese *et al.*, 2011). The significant differential response of slow and rapid rate of dehydration on viability and vigour of recalcitrant seed underline the influence of AOS metabolism on desiccation-induced seed deterioration. During dehydration, respiration was unregulated leading to enhanced
production of AOS due to partial reductions of oxygen (Hendry, 1993; Finch-Savage et al., 1994; Côme and Corbineau, 1996; Kranner et al., 2006). Drying induced loss of viability observed in *Quercus robur* (Finch-Savage et al., 1994), *Shorea robusta* (Chaitanya and Naithani, 1994) and *Theobroma cacao* (Li and Sun, 1999) was accompanied by a loss of the cellular antioxidant potential and an accumulation of free radicals. Desiccation and rehydration of the resurrection plant *Xerophyta viscosa* (Sherwin and Farrant, 1998), and germinated maize (Leprince et al., 1990) or wheat seeds (Farrant et al., 2004), are also associated with imbalance in the AOS content and detoxifying enzyme activities.