Chapter: II

Active Oxygen Species
2.1 Introduction

Oxidative stress and AOS:
The plant experiences oxidative stress only when the production of AOS [Active Oxygen Species] surpasses the antioxidant capability of the cell to detoxify AOS [45]. AOS (Active oxygen species), till recent past, have traditionally been considered to be toxic products of aerobic metabolism. In plants, oxidative stress is produced by drought, low and high temperatures, high light intensities, ozone, heavy metals, salinity mechanical wounding, and so on [455, 456, 92, 457, 458] and also during pathogen infection [459]. However, in recent years, it has become apparent that plant cells generate low levels of AOS in response to a number of biotic and abiotic stresses, such as pathogenic elicitors and even ozone [460, 461] and recognized as potent signalling molecule. AOS production is enhanced by many abiotic stresses, such as drought stress, salt stress, heat shock, low temperature, nutrient deprivation, and high light [104, 92]. The paradox of self-imposed stress points to a role of AOS in signalling, where increased accumulation of \( \text{H}_2\text{O}_2 \) and changes in redox status alter in response to environmental change [462, 463]. Several AOS-induced signalling pathways play a significant role in plant growth and development, and interaction with biotic and abiotic environments [464, 465].

Unfavorable environmental conditions during seed storage is widely implicated in causing oxidative stress and development of various types of AOS; superoxide radical, hydrogen peroxide and hydroxyl radical, which are most active and toxic products of oxidative stress [466]. AOS contribute to the accumulation of oxidative damage to cellular constituents. Thus, a more modern version of this tenet is the ‘oxidative stress theory’ of aging, which holds that increases in AOS accompany aging, leading to functional alterations pathological conditions, and even death in seeds [467].

Oxygen during mitochondrial electron transport processes give rise to superoxide, a potentially harmful active oxygen species (AOS) which in turn may reduce to hydrogen peroxide (\( \text{H}_2\text{O}_2 \)), singlet oxygen (\( ^1\text{O}_2 \)) and the hydroxyl radical (OH\(^-\)) [107]. Sequential reduction of molecular oxygen produces various AOS. Superoxide is formed by one-electron reduction of molecular O\(_2\).
[85] whereas reduction of second electron forms hydrogen peroxide \((H_2O_2)\), and a third one-electron reduction produces the hydroxyl radical \((OH^-)\).

Comparatively, \(H_2O_2\) is a more stable AOS than superoxide radical \((O_2^-)\) and can diffuse across membranes including mitochondrial membrane through water channels [468] and can cause oxidative modifications of biomolecules at distant sites from its production [469]. The reactivity of the hydroxyl radical \((OH^-)\) is very high [85, 88] and known to oxidize proteins, lipids, and other biomolecules in close proximity to site of \(OH^-\) production.

**Control of AOS levels: detoxifying mechanisms**

Plants are well adapted for the efficient elimination (in the case of oxidative stresses) or homeostasis of AOS (for cellular signaling) through activation of multiple pathways. Various enzymatic and non-enzymatic mechanisms are known for the removal or degradation of AOS in seeds. Superoxide dismutase, which can be mitochondrial (MnSOD), cytosolic (Cu/ZnSOD) or chloroplastic (CuZnSOD, FeSOD) dismutates \(O_2^-\) into \(H_2O_2\) and \(O_2\) [470]. \(H_2O_2\) is eliminated by the action of catalase (CAT), which is located in glyoxysomes and peroxisomes [471], except the isoform Cat-3 of maize, which is located in mitochondria [472]. The ascorbate–glutathione cycle (also called the Halliwell–Asada cycle) may also take part in \(H_2O_2\) scavenging; it involves ascorbate peroxidase (APX), monodehydroascorbatereductase (MDAR), dehydroascorbatereductase (DHAR) and glutathione reductase (GR). The enzymes of this cycle, which are present in chloroplasts, cytoplasm, mitochondria, peroxisomes and the apoplast [92], involved in the regeneration of the powerful antioxidants ascorbic acid (vitamin C), reduced glutathione and \(\alpha\)-tocopherol (vitamin E). The role of ascorbate system in seeds has been reviewed recently [473]. Glutathione peroxidases (GPX) also catalyse the reduction of \(H_2O_2\) [474]. Non-enzymatic antioxidants such as tocopherols, ascorbic acid (AsA), and glutathione (GSH) detoxify AOS [462, 476, 477]. Various compounds, such as polyphenols, flavonoids and peroxiredoxins [475] also have a strong antioxidant function.
**Functions of AOS:**

AOS are produced as by-products of oxidation–reduction reactions. In the last decades, there has been a substantial increase in our understanding of the dual role of AOS in plant tissues, as signal molecules and as damaging species when they are overproduced. At low levels, they are known to act as important signalling molecules. For instance, in plants, AOS are used to facilitate an array of essential biological processes [85, 98, 478], including immune defense [85], growth and development [479,478], seed germination [480], seed dormancy [230], programmed cell death [481, 482, 483], stress acclimation [484, 478] and in biotic and abiotic stresses [485].

As plants are sessile, they have developed a broad range of strategies, collectively known as 'defence' or 'stress' responses, to protect themselves against biotic and abiotic stresses [488]. The oxidative burst, a rapid and transient production of huge amounts of AOS, is one of the earliest aspects of a plant's defense strategy. Increased extracellular AOS production in response to wounding has also been reported in plant roots [489, 490], lichens [491] and marine macro alga [492]. Such extracellularly produced AOS can be implicated in cell wall strengthening [493, 494], whereas a putative role for AOS in cell wall loosening has been suggested in germinating orthodox seeds [495].

H$_2$O$_2$ has multiple effects on almost all organisms and can influence the life of every single cell [496]. On one hand, H$_2$O$_2$ is highly reactive and toxic and can lead to oxidative destruction of cells; on the other hand, it acts as a signalling molecule in regulating cell growth and development, cell proliferation, cell stress response, and signal transduction [94]. Many processes involving H$_2$O$_2$ have been identified in plants, including programmed cell death (PCD) [497, 498, 499, 500, 501], somatic embryogenesis [502], wounding [503], root gravitropism [504] and ABA-mediated stomatal movement [97, 505]. At the cellular level, events regulated by H$_2$O$_2$ are beginning to be identified. They include protein phosphorylation through mitogen-activated protein kinase (MAP kinase) cascades [506, 507, 508], calcium mobilization [509, 510] and regulation of gene expression [511, 512]. The roles of superoxide and other AOS in
signalling pathways are less well described so far; however indications are that $\text{O}_2^-$ plays role in cell death and plant defense [513, 514, 515].

At high levels these highly reactive molecules are involved in oxidative stress on the cell and invoke profound changes in gene expression [85]. Uncontrolled accumulation of AOS during oxidative stress will result in cumulative oxidative damage to DNA, RNA, lipids and proteins in the cell [516, 517, 518]. High amounts of $\text{H}_2\text{O}_2$ can directly or indirectly oxidize large variety of biomolecules such as lipids, proteins, and nucleic acids [92] and at the same time alter the overall redox state of the cells by degrading cellular ascorbic acid and glutathione pool. $\text{H}_2\text{O}_2$ is also known to react with thiol groups and can lead directly to inactivation of some enzymes, e.g. those of the Calvin cycle [520].

The uncontrolled accumulation of AOS, particularly of $\text{OH}^-$, which cannot be eliminated enzymatically, is highly toxic for the cell. Hydroxyl radical can react with the majority of biomolecules thus resulting in irreversible oxidative stress mediated cellular damage. Many harmful effects of hydroxyl radical on cellular macromolecules such as nucleic acids and proteins have been identified [122, 52]. Hydroxyl radical and not $\text{O}_2^-$ nor $\text{H}_2\text{O}_2$ can directly damage both nuclear and organelle DNA as it attacks deoxyribose, purines and pyrimidines [522]. The OH- radical is most reactive species regarding protein sensitivity to oxidative stress, since it can damage a great range of amino acids and proteins involved in transport, receptors and ion channels leading to extensive cellular dysfunction [83, 122].

**AOS and Seed Ageing:**

In ageing seed AOS are usually considered as toxic molecules, the accumulation of which leads to cell injury and disturbances in during seed storage and germination. The deleterious role of these compounds in seed ageing is now quite well established and documented [172, 126, 302]. The probable involvement of AOS in seed ageing has been reviewed by many authors [172, 126, 302]. AOS may also play a role in desiccation-related damage particularly in dehydration-
intolerant recalcitrant seeds [70]. On the other hand, alteration in respiratory pathway and energy metabolism in aged seeds have been demonstrated by various authors [523, 524, 302]. Oxidation processes that occur in ageing seeds during storage [184, 525, 466] is one of the important factors in the lowering of seed germination ability. Seed deterioration is partially due to AOS induced membrane lipid peroxidation that causes leaky membrane. The eventual ageing related loss of seed germination, viability and vigour in seeds during storage is most often attributed to accumulated AOS and its induced oxidative degradation of cellular constituents [302, 172, 122, 284].

According to Smith and Berjak [526] the possible involvement of oxidative processes in seed ageing must consider seed storage behaviour. In orthodox seeds the low moisture content during storage supports auto-oxidation reactions leading to AOS production [302]. In such conditions, *in vivo* detoxifying enzyme activities are almost impaired and therefore unable to remove AOS, which may either have a direct deleterious effect on cellular components (lipids, enzymatic and structure proteins, nucleic acids) or be trapped in the intracellular structures [466]. Plethora of literature demonstrated that seed ageing is associated with a loss of antioxidant enzyme activity [527, 528, 529, 530, 531, 532]. Prolonged dry storage or inappropriate conditions of storage (high temperature and relative humidity) magnify the magnitude of the processes. Secondly, imbibition and germination of previously stored seeds should be considered as the critical steps of the oxidative phenomena related to ageing, since it is at these times that the cellular dysfunctions resulting from AOS accumulation are expressed. Imbibition results in the release of trapped AOS during storage and in the production of new AOS by reactivation of metabolism. The delay in germination of aged seeds might correspond to the time necessary for the cells to re-initiate the antioxidant machinery and then escape from an oxidative stress [533, 148].

Dehydration mediated production of AOS seed has been widely quoted as being the main factor causing seed death in desiccation sensitive recalcitrant seeds [172, 126, 56, 121, 149]. An array of AOS molecules, explicitly; $O_2^-$, $H_2O_2$, $OH^-$ are reported to be produced during varied stress situations including desiccation and ageing in seeds [466, 122, 534, 535, 316, 8, 172, 536, 302, 121, 537, 315, 538]. Desiccation of recalcitrant seeds below CWC, perturbs the metabolic
balance towards accumulation of AOS which finally leads to alteration at morphological, physiological, biochemical and molecular level that ultimately results in to reduced seed viability, vigor and cell death [539, 466, 69, 540, 8, 89].

Significant stimulation of extracellular production of superoxide radical during slight dehydration or drying of seeds to lower water contents in *Azadirachta indica* [121, 22], *Castanea sativa* [541], *Antiaris toxicaria* [542], *Pongamia pinnata* [367], *Syzygium cumini* [16], *Trichilia emetic* [543], *Trichilia dregeana* [544], *Shorearo busta* [17, 149]. Elevated level of AOS accumulation leads to loss of seed viability and vigor in recalcitrant seeds like *Aesculus hippocastaneum* [8], *Quercus* sp. [539], *Castanea sativa* [123] and *Trichilia dregeana* [58], *Antiaris toxicaria* [542], *Acer platanoides* [528]. The present investigation reports, in detail, the levels of AOS namely superoxide, H$_2$O$_2$ and OH-radical in the desiccation sensitive neem seeds exposed to slow and rapid drying during storage.

### 2.2 Materials and Methods

#### 2.2.1 Superoxide radical

Superoxide radicals were estimated following the method of Sutherland and Learmonth [552]; Berridge and Tan [553]. Batches of five seeds were incubated in potassium phosphate buffer (20 mM, pH 6.0) containing 500 μM 2,3- Bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT Sodium Salt, Sigma) in dark, at 25°C on a shaker for 6 h. The change in absorbance of the incubation media was measured at 470 nm, using a spectrophotometer (ATI-Unicam, UK) and expressed as A$_{470}$ relative unit.

#### 2.2.2 Hydrogen peroxide

To estimate hydrogen peroxide, five seeds in five replicates were pre-incubated for 30 min in potassium phosphate buffer (20 mM, pH 6.0) to remove pre-formed H$_2$O$_2$. Seeds were then again incubated for 5 h in the same buffer containing 5 μM scopoletin (Sigma) and 3 μg mL$^{-1}$ horseradish peroxidase (HRP) (Sigma) in dark, at 25°C on a shaker [554]. The change in fluorescence of the incubation medium was measured using spectrofluorometer (Shimadzu,
Japan) at excitation $\lambda$: 346 nm, and emission $\lambda$: 455 nm taking reagent blank as reference. The estimated hydrogen peroxide was expressed as nM H$_2$O$_2$ seed$^{-1}$.

2.2.3 Hydroxyl radical

The OH radicals were estimated following the method of Gutteridge [555]. Batches of five seeds were incubated in an incubation medium (20 mM potassium phosphate buffer, pH 6.0) having 2.5 mM sodium benzoate (HiMedia) in dark at 25$^\circ$C for 6 h on a shaker. Incubation mediums were then centrifuged at 8000xg for 15 min at room temperature. Supernatants were collected and change in fluorescence (excitation $\lambda$/ emission $\lambda$: 305 nm / 407 nm) was measured and expressed as relative fluorescence unit. Without sodium benzoate run in parallel for blanks that were used for correction of unspecific fluorescence originating from substances eluted in plant material.

2.3 Results

2.3.1 Superoxide radical (O$_2^-$)

The release of superoxide radical revealed a low profile in the initial stages, but increased rapidly with storage during natural drying (Figure 2.1a). The minimum level of superoxide radical recorded at 0 days of storage (3.45 A$^4$70 relative unit) and thereafter a gradual increase up to 20 days (nearly 1.6 fold), after which the levels raised sharply to the maximum at 130 days of storage (14.15 A$^4$70 relative unit). About 4.1 fold increment in O$_2^-$ level were estimated in nonviable seed (130 days of storage) as compared to fresh seed (0 days of storage). Negatively correlated with WC ($r = -0.64$), %G ($r = -0.99$) and positively correlated with SP ($r = 0.99$). In rapid drying desiccation of seeds from 1.45 to 0.18 g H$_2$O g$^{-1}$ DM was accompanied by increased O$_2^-$ (3.45-8.3 A$^4$70 relative unit) (Figure 2.1b). A steady increase in level of O$_2^-$ with in a period of 3 h (3.85 A$^4$70 relative unit) of drying. Maximum value achieved in a period of 8 h drying (seeds with 0.18 g H$_2$O g$^{-1}$ DM and 60% germination). Approximately 2.4 fold increment in the value of seeds of WC 0.18 g H$_2$O g$^{-1}$ DM as that of WC 1.454 g H$_2$O g$^{-1}$ DM. Correlated negatively with WC ($r = -0.93$), %G ($r = -0.996$) and positively with drying ($r = 0.93$).
Figure 2.1a

Figure 2.1. Superoxide radical in naturally dried (a) and rapidly dried (b) neem seeds. For each data point ± SD, five replicates of five seeds each were used, tested in a significance level of $p \leq 0.05$.

2.3.2 Hydrogen peroxide (H$_2$O$_2$)

The pattern of H$_2$O$_2$ accumulation in naturally dried seeds was similar to that of O$_2^-$ (Figure 2.2a). The value of H$_2$O$_2$ accumulation gradually increased from 64.08 nM H$_2$O$_2$ seed$^{-1}$ (0 days of storage) to 78.84 nM H$_2$O$_2$ seed$^{-1}$ (20 days of storage). But sharply increase to the value of 120 nM H$_2$O$_2$ seed$^{-1}$ in seeds of 60 days of storage. A final value of 168 nM H$_2$O$_2$ seed$^{-1}$ was achieved by the seeds of 130 days of storage. An almost 2.6 fold increment in level was enumerated in naturally dried neem seeds. It had a strong positive correlation with SP ($r = 0.996$) and a strong negative correlation with %G ($r = -0.999$). With WC correlation value was $r = -0.61$.

But in case of rapid drying, at 8 h of drying period the value amplified from 64.08 nM H$_2$O$_2$ seed$^{-1}$(0 h) to 85.44 nM H$_2$O$_2$ seed$^{-1}$(Figure 2.2b). 69.8 nM H$_2$O$_2$ seed$^{-1}$ and 78.52 nM H$_2$O$_2$ seed$^{-1}$ value was observed in seeds of WC 0.906 g H$_2$O g$^{-1}$ DM and 0.483 g H$_2$O g$^{-1}$ DM. Seeds having WC 0.18 g H$_2$O g$^{-1}$ DM showed a 1.3 fold increment as that of the seeds of WC 1.454 g H$_2$O g$^{-1}$ DM. Correlation values were: with drying ($r = 0.98$), with WC ($r = -0.98$) and with %G ($r = -0.96$).
2.3.3 Hydroxyl radical (OH⁻)

Like the other two (O₂⁻ and H₂O₂) hydroxyl radical (OH⁻) also followed the same trend during natural drying (Figure 2.3a) i.e. low profile at the initial stage and sharp increment after 20 days (9.1 relative fluorescence unit) of storage. Nonviable seeds (130 days of storage) achieved a highest value of 17.65 relative fluorescence unit whereas in fresh viable seeds value was 6.84 relative fluorescence units. Around 2.6 times increment in the value of OH⁻ in nonviable seeds (130 days of storage) as of fresh seeds (0 days of storage). A strong positive and a strong negative correlation with SP (r = 0.99) and with %G (r = -0.97) were expressed respectively. With WC it was correlated (r = -0.61) negatively.

OH⁻ in seeds accumulated slightly during rapid drying (Figure 2.3b). After a drying period of 8 h value achieved was 9.22 relative fluorescence units. The values obtained at WC 0.906 g H₂O g⁻¹ DM (3 h) and 0.483 g H₂O g⁻¹ DM (7 h) were 7.91 and 8.92 relative fluorescence units respectively. Strong positive correlation with drying (r = 0.997) but negative correlation with %G (r = -0.87) and with WC (r = -0.99) were revealed.
Figure 2.3. Accumulation of hydroxyl radical in naturally dried (a) and rapidly dried (b) neem seeds. Values are mean ± SD of five replicates of five seeds each. Data are significantly different ($p \leq 0.05$).

2.4 Discussion

The loss of seed viability is often associated with the overproduction of AOS. Seeds accumulate high amounts of AOS in response to drying during last phase of development i.e. maturation phase. The AOS production in dry seeds would probably result mainly from non-enzymatic mechanisms, such as those of Amadori and Maillard [172, 223] and lipid peroxidation [556] that is favored during drying [302]. Accumulation of AOS is favoured in drying seeds especially at low water content when the AOS-detoxifying enzymes are not active [332, 454].

Active oxygen species (AOS) are capable of oxidizing all potential biomolecules [122, 91] such as protein [83], lipid [83] and DNA [521] thus widely known for disturbing various metabolic pathways. The accumulation of intermediate metabolites of proteins and lipids are equally highly cytotoxic and are involved in cellular damage [Please refer details in chapter 3 and 4]. Drying, slow or rapid, of neem seeds promoted the formation of AOS, although the content was different. Comparatively the levels of all the AOS estimated were higher in the slow dried seeds than the
rapid dried seeds (Figs. 2.1a, 2.1b, 2.2a, 2.2b, 2.3a, 2.3b). Initially, the levels of AOS were very low in the fresh and hydrated seeds. For example, slow drying of neem seeds from 1.45 to 0.167 g H$_2$O g$^{-1}$ DM promoted the levels of AOS slightly from 3.45 to 5.35 A$_{470}$ relative units. But, prolonged slow drying of seeds from 0.167 to 0.123 and 0.116 g H$_2$O g$^{-1}$ DM increased the levels of O$_2^-$ enormously from 5.35 to respectively 9.15 and 12 A$_{470}$ relative units (Figs. 2.1a, 2.1b). The substantial increment in the superoxide levels in slow dried seeds was closely related with relatively very low germinability [50 and 30% germination], GI [187.5 and 68.75] and viability [38.65 and 35.07 A$_{520}$ g$^{-1}$ DM in axes and 19.31 and 17.42 A$_{520}$ g$^{-1}$ DM in cotyledons]. The close correlation of superoxide levels with the germinability ($r = -0.99$) (Fig. 2.1a) and viability clearly reveals its important role in promoting the loss of germinability in neem seeds. High levels of superoxide (O$_2^-$) was positively related with the loss of seed viability during prolonged storage in sun flower (Kibinza et al., 2006), Antiaris toxicaria [557], Fagus sylvatica [316]. In several recalcitrant and intermediate seeds the loss of seed germination is associated with the AOS induced unregulated metabolism [558, 121, 22]. The drying induced accumulation of AOS and its intermediates in the desiccation sensitive seeds are potential causative factor in promoting loss of seed germinability, viability and vigour [558, 466, 56]. In the low viable seeds it is the substantial levels of superoxide that accounts for the loss of germinability and definitely not the close relationship between germinability and levels of superoxide which is almost similar in the slow [r=0.99] and rapid [r= 0.99] dried seeds. Comparatively, the amounts of superoxide accumulated in the rapid dried seeds were significantly low than the slow dried seeds (Figs. 2.1a, 2.1b). The desiccation tolerance was enhanced in the rapid dried neem seeds perhaps due to accumulation of low amounts of superoxide in the rapid dried seeds than the slow-dried seeds. Our conclusion is substantiated by recording the levels of all AOS in rapid dried neem seeds. The rapid drying of neem seeds for 3, 7 and 8hrs exhibited steady promotion in the level of superoxide with close negative correlation with germination [r = -0.99] but comparatively low levels of all AOS at all stages of drying. For example, the levels of O$_2^-$ increased from 3.45 in fresh seeds with 100% germination to 3.85, 6.15 and 8.3 A$_{470}$ relative unit observing respectively 100, 80 and 60% germination. It is suggested that the levels of superoxide produced in response to drying; slow or rapid is more important than the correlation of superoxide levels with germination per se. We also conclude that rapid drying induced enhanced desiccation tolerance was mainly due to lowering of the AOS levels and its mediated oxidative catabolism [70, 68].
The pattern of \( \text{H}_2\text{O}_2 \) and OH radical measured in the slow drying seeds was similar to superoxide. Significantly high amounts of \( \text{H}_2\text{O}_2 \) were accumulated in the slow dried seeds exhibiting 50\%, 30\% and 0\% viability than the rapid drying neem seeds. For example, nearly 78 nM\( \text{H}_2\text{O}_2 \) seed\(^{-1}\) of \( \text{H}_2\text{O}_2 \) was registered in slow dried seeds of 90\% germinability whereas in rapid dried it was registered in 80\% germination seeds. Similarly, the slow dried neem seeds with 50\% germination noticed very high amounts of \( \text{H}_2\text{O}_2 \) formation [120 nM\( \text{H}_2\text{O}_2 \) seed\(^{-1}\)] compared to rapid dried seeds of 60\% germination [85.44 nM\( \text{H}_2\text{O}_2 \) seed\(^{-1}\)] (Fig. 2.2a). Even the levels of OH-radical detected by fluorescence technique in slow and rapid drying seeds of various germinability showed comparatively higher levels during slow drying than the rapid drying. For example, the amount of OH-radical formed is almost same in the seeds showing germination 60\% [9.22 relative fluorescence unit] and 90\% [9.1 relative fluorescence unit] respectively in rapid and slow dried neem seeds (Figs 2.3a, 2.3b).

A linear relationship recorded between amounts of AOS estimated and the percent germination both under slow and rapid drying approves the key role of AOS in loss of germination. In slow drying neem seeds, the AOS induced massive cellular damage (especially membranes, DNA, proteins and lipids that results in severe cellular disorder) that finally leads to loss of viability, vigour and germinability is due to overproduction of AOS that exceeds the antioxidant capacity of the seeds finally enhancing cascade of pathways related to oxidative stress [126, 122]. The loss of seed viability is often associated with severe dysfunction of cellular, metabolic activities including loss of membrane integrity, reduced energy metabolism, impairment of scavenging enzymes and protein synthesis, and DNA degradation [172, 559, 560, 302]. The dehydration induced deterioration in desiccation seeds is associated with ultrastructural changes in the axis of \textit{Artocarpus heterophyllus} [68] and \textit{Ekebergia capensis} [54].

Our data clearly revealed that levels of all AOS are enhanced in neem seeds after exposure to drying condition, however, the levels of AOS are distinctly different in slow and rapid dried neem seeds. The levels of all the AOS estimated in our study are comparatively higher in the slow dried seeds than the rapid dried seeds. It is suggested that the dehydration induced cell injury in the slow dried seeds is perhaps due to exposure of neem seeds for longer duration at intermediate water content that invokes and permits the excessive accumulation of AOS [17,
by simultaneous impairment of antioxidant enzymes resulting in metabolic disorder and oxidative induced cellular damage ultimately leading to loss of germinability [332, 563, 567]. Similar results that were reported [330] explain the differences between slow and rapid drying induced cellular damage. It was suggested that the cellular damage during rapid drying is a consequence of “physical stress” whereas in slow drying it is due to “unregulated metabolism”. In contrast, accumulation of insignificant amounts of all AOS in the rapid dried seeds to very low water contents may account to rapid removal of water without concomitant metabolic perturbations finally enhancing desiccation tolerance. Insignificant alteration at the metabolic levels permits higher viability at very low water content during rapid drying.

It is suggested that the desiccation induced cellular injury that leads to loss of germination in neem seeds can be attributed to overproduction of AOS mainly due to 1-impairment of antioxidant mechanism and 2-formation of AOS from different sources [mitochondrial ETC, extracellular NAPH oxidase etc.] thus enhancing the unregulated oxidative stress induced cellular damage [56]. Over accumulation of AOS mainly due to impairment of antioxidant system and enhanced leakage of AOS from mitochondrial ETC is a characteristics feature of a poor viable or non-viable desiccation sensitive seeds [17, 118, 314, 70, 121]. The superoxide generated from the ETC is subsequently converted into H$_2$O$_2$ in the presence of mitochondrial SOD [565] and then diffused across the membranes including mitochondrial membrane through water channels [468] and finally causes oxidative modifications of biomolecules at distant sites from its production [469].