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Biochemical basis of seed deterioration —an overview

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ABSTRACT Seeds die inevitably but unexpectedly during storage. Seed deterioration is loss of seed quality, viability and vigor due to effect of adverse environmental factors. It is a natural process and once seed deterioration has started, this catabolic process cannot be reversed. Biochemical processes of lipid peroxidation and associated free radical oxidative stress are one of the major causes of seed deterioration. Reactive oxygen species (ROS) are highly reactive, unstable and may react and damage nearby molecules. They may modify and inactivate proteins, lipids, DNA, RNA leading to inactivation of enzymes or decrease in proteins, disintegration of cell membranes, genetic damage and also induce cellular dysfunctions. In addition, loss of the glassy state and cellular repair mechanisms is also considered as driving force for seed deterioration. Thus, knowing and understanding the complexities of factors governing seed deterioration are therefore of major agronomical and economical importance.

Keywords: Lipid peroxidation, protein metabolism, enzyme activity, antioxidant molecules, genetic functions, hydrolysis, glassy state, seed performance, longevity

Seed deterioration is a complex and an inevitable physiological process leading to loss of viability and / or seed vigour during various post harvest treatments including storage, underadverse conditions. It does not differ substantially from degeneration or the senescence and include all the progressive detrimental changes that occur in seeds as they die. The rate of seed deterioration is influenced by environmental as well as biological factors and varies within species, varieties and seed lots. Environmentally, high temperatures during storage enhance seed deterioration, as does high moisture content. The interaction of the two, however, is greater than their sum and hence they may interact and cause seed deterioration synergistically. Biologically, storage fungi increase as seed deteriorate and reduce seed germination independent of other physiological cause(s) of seed deterioration. The process of seed deterioration could be associated with following physiological changes:

- Progressive decrease in germinability.
- Increased mean time of germination.
- Increase in the number of abnormal seedlings.
- Lower tolerance to adverse storage conditions.

All of these physiological changes during seed deterioration have been attributed to various biophysical and biochemical changes in seed components, such as the loss of enzymatic activities, the loss of membrane integrity, accumulation of toxic substances and genetic alterations [1-2]. However, the exact cause of loss of seed viability is still not well defined. Lipid peroxidation and associated free radical oxidative stresses are considered to be major contributors to seed deterioration [3]. However, different mechanisms of seed deterioration may exist under different storage conditions. While at comparatively low temperature free radical damage may be the primary event of seed deterioration, the loss of seed viability at higher temperatures is closely related to thermal inactivation of proteins [4]. Water content is another important factor affecting the rate of seed deterioration. In dry seeds, enzymatic reactions may play little role in seed deterioration, because dry seeds lack active enzymatic metabolism. Under these conditions, certain non-enzymatic reactions such as Amadori and Maillard reactions could play an important role [5]. In this article various mechanisms that have been proposed to explain the process of seed deterioration have been discussed critically.

MODERN THEORIES ON SEED DETERIORATION

Major constituents of the seeds are the lipids, carbohydrates, proteins and, of course, the nucleic acids. However, the proportion of each component varies in seeds of various species. Some seeds are rich

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in lipids or proteins or both while the others are rich in carbohydrates. Any of these components may be damaged during the process of seed deterioration. However, the various reactions leading to this damage are not clearly understood. Several models have been proposed to explain the process of seed deterioration. However, there is no evidence for a single determinant of any one of the symptoms of deterioration. Probably, the exact mechanism is the combination of all these models. Nevertheless, lipid peroxidation model has stimulated the greatest interest of the scientists all over the world and seems to be the major cause of seed deterioration especially in oilseeds. The various models proposed are explained below:

1. Lipid peroxidation

The phenomenon of incipient death during germination of aged seeds suggests the presence of a destructive element which becomes active only after imbibition. The lipid peroxidation model proposes that this destructive element is oxygenated fatty acids. They can be produced through autoxidation or enzymatically [3]. During autoxidation, fatty acid hydrocarbon chains spontaneously oxidize in the presence of oxygen, thereby producing reactive free radical intermediates, known as hydroperoxides. The polyunsaturated fatty acids are more susceptible to this reaction, as they contain methylene group between the two double bonds which is highly reactive and can very easily lose hydrogen free radical ($H^{\cdot}$). The rate of this reaction is greatly enhanced by the class of enzymes called lipoxygenases (LOXs), which are found in many seeds. The reaction sequence for the non-enzymatic autoxidation is given in figure 1. Once a free radical is produced, a chain reaction is initiated which creates additional reaction cycles and free radicals. The reaction is terminated by combination of two free radicals producing stable end products.

Further, the mechanism of lipid peroxidation may differ in seeds depending upon their moisture content. It has been found that autoxidation may be the primary cause of seed deterioration at low seed moisture content (<6%) while action of LOX is more when seed moisture content exceeds 14% [6]. Between 6 % and 14 % moisture content, lipid peroxidation is likely to be its minimum because sufficient water is available to serve as a buffer against autoxidative free radical attack but not enough water is present to activate lipoxygenase-mediated free radical production. Thus, the mechanism of lipid peroxidation may be different under accelerated (high relative humidity) and natural (low relative humidity) aging.

Initiation

$RH + O_2$ ——— Free radicals ($R^{\bullet}$)

ROOH ——— Free radicals ($ROO^{\bullet}$)

Propagation

$R^{\cdot} + O_2$ ——— $RO_2^{\cdot}$

$RO_2^{\cdot} + RH$ ——— $R^{\cdot} + ROOH$

Termination

$R^{\cdot} + R^{\cdot}$

Stable products

$R^{\cdot} + RO^{\cdot}$

Fig. 1. Free radical chain reaction resulting in autoxidation.

The free radicals possess a potential for short distance damage due to their short life time. The long distance detrimental effects of lipid peroxidation are due to their conversion into more stable chemical species [3]. It has actually been found that the fatty acid hydroperoxides may further be reduced or degraded resulting in the formation of wide array of α,β-unsaturated aldehydes of which 4-hydroxynon -2-enal (4-HNE) is the most abundant. At physiological concentrations, 4-HNE is a signaling molecule that modulates a variety of fundamental biological processes. In addition, 4-HNE is covalently bound to proteins and no enzymatic mechanisms are known for its removal. Thus, proteins that carry 4-HNE adducts are resistant to degradation by proteasomes. Modification by 4-HNE makes it therefore, an attractive candidate for damage that contributes to aging (Figure 2) [7]. The factors triggering hydroperoxide breakdown include heat, enzymes, cytochromes and transition metal ions.

Lipid peroxidation is potentially damaging to seeds in three ways:

By destruction of membrane lipids- Biomembranes represent a key site of direct injury from lipid peroxidation as they possess an inherently large surface area and usually have more unsaturated fatty acids than storage lipids. Lipid peroxidation of membranes may lead to decline in membrane integrity and to an increase in membrane permeability [8]. A loss in the integrity of the plasma membrane has been demonstrated in aged seeds by the extent of leakage

of cytoplasmic components to the external medium [9]. Increased membrane permeability is more common in mitochondrial membranes as they are rich in unsaturated acyl chains [10]. Here, it may have fatal effects as it can lead to a breakdown of the proton gradient necessary to maintain respiratory coupling.

By co-oxidation of associated cellular components by free radical transfer from oxidized lipid- Normally stable molecules are incorporated into the autoxidative chain reaction by abstraction of hydrogen atoms. In addition lipid peroxide and their secondary products can react with terminal groups of amino acids in proteins and enzymes. The lipid peroxidation has been reported to stimulate the formation of schiffbases between peroxidized phospholipids and membrane proteins [11]. This non-enzymatic reaction may lead to polymerization of proteins.

Formation of cytotoxic aldehydes- It has been reported that deteriorated seeds produce 20 times more volatile aldehydes during imbibition than fresh seeds. The aldehydes formed by hydroperoxide break down produce a variety of cytotoxic effects. These aldehydes react with sulfhydryl groups leading to an inactivation of proteins. For example, they have been shown to inhibit tubulin, the main protein of microtubules which is necessary for mitotic spindle formation [12]. In addition aldehydes strongly inhibit protein and DNA synthesis [13].

II. Alterations of protein metabolism

The mechanisms by which proteins become damaged and subsequently targeted for degradation during deterioration are unknown. The processes that may lead to alteration of protein metabolism during aging include (a) decreased protein synthesis, (b) increased protein inactivation, and (c) increased protein hydrolysis.

Protein synthesis-The studies with radioactive amino acids in many seed species have shown that seed deterioration is associated with reduced capacity for protein synthesis [14]. The inability to synthesize proteins is accompanied by an excessive loss of capacity to synthesize RNA [15], which could be a consequence of damage to nuclear DNA. However, protein synthesis is also impaired at the level of translation. It has been found that ribosomes from non-viable seeds have reduced activity. This could result from adverse structural modifications of the ribosomes, or from loss of one or more rRNAspecies or ribosomal proteins [16]. The retardation of protein synthesis could also be due to failure of the polyribosomes to dissociate [17-18]. However, the loss of protein synthesis is not associated with changes in tRNA or aminoacyl-tRNA synthetase activity. In addition, reduction of ATP (and possibly GTP) synthesis in non-viable seeds can affect protein and RNA synthesis and it is likely that there is an important link between nucleoside triphosphate levels in seeds and their capacity to carry out essential metabolic functions.

Protein inactivation- The inactivation of proteins during seed storage may lead to deterioration as it depresses the metabolic capacity and reduces the ability of metabolic system to repair the damages incurred during storage. The proteins may be inactivated by losing or gaining certain functional groups, by oxidation of sulfhydryl groups or by conversion of amino acids within the protein structure. The spontaneous deamidation, isomerization and racemization of L-asparaginyl and L-aspartyl residues of proteins have been observed during cellular aging [19]. This leads to the accumulation of detrimental residues like L-isoaspartyl in the proteins which have been shown to alter the structure and function of proteins. Changes in the protein structure could also be attributed to attack of free radicals. The soluble proteins may be attacked by different classes of oxidants than membrane proteins. The most reactive amino acids susceptible to oxidative damage appear to be cysteine, histidine, tryptophan, methionine, and phenylalanine, usually in that order [20]. Further, proteins may be modified by non-enzymatic glycation through Amadori and Maillard reaction [5]. The Amadori reaction involves attack on amino groups of protein by reducing sugars to form glycated proteins. The Maillard reaction represents subsequent complex interactions between the glycated Amadori products to form polymeric, brown coloured products, hence the term "browning reaction". The cytotoxic volatile aldehydes are also produced following Strecker degradation of Maillard products [21]. These non-enzymatic reactions seem to be the most probable cause for inactivation of proteins during seed deterioration, because dry seeds lack active enzymatic metabolism. As an example, Amadori and Maillard products were found in soybean seeds subjected to accelerated ageing, and formed most rapidly in seeds at 40% to 80% relative humidity [5]. A marked increase in reducing sugars such as glucose and galactose has been observed in deteriorating maize seeds [22] that may enhance Amadori and Maillard reactions because of the presence of reactive semi-aldehyde group in these

sugars. The structure and function of proteins and DNA is known to be altered by Amadori-Maillard reaction. The products of these reactions, termed also as advanced glycosylation end (AGE) products, are able to disrupt the membranes. Therefore, early aging-dependent disorders in membrane permeability arise in the protein phase as a result of nonenzymatic glycosylation [23].

Protein hydrolysis-The researchers have reported a lower level of protein in non-viable seeds than in viable seeds [24]. This could be due to the hydrolysis of proteins as is indicated by the fact that there is increase in total free amino acids after aging treatment. Further, leaching of the amino acids into the imbibing medium has also been reported. This assumption is further supported by the high protease activity in the seeds exposed to accelerated aging conditions [25]. The oxidative attack on Lys, Arg, Pro, or Thr residues of proteins result in protein carbonylation which has been widely used as marker for protein oxidation [26-27]. This in turn inhibits or alters their activities and increases their susceptibility toward sproteolytic attack [28].

III. Changes in the activity of enzymes

Metabolism of the seeds is greatly affected during their storage. This is mainly due to modulation of various enzyme activities present in the seeds. The alterations in the activity of enzymes could be brought about by changes in composition or configuration of their structure. The major configuration changes include: (1) partial folding or unfolding (2) degradation to subunits and (3) condensation to form polymers. There is activation of some enzymes especially the hydrolytic enzymes, during storage. But, this is highly dependent upon the moisture level of the seeds. If moisture content reaches higher level, normal germination may occur. However, if moisture levels for germination are not attained, the seed deteriorates because of energy expenditure or accumulation of breakdown products. The various hydrolytic enzymes activated by high moisture levels are: lipase, phospholipase, protease, DNase, phosphatase and amylase [25]. Further, a depression in the activity of free radical and peroxide scavenging enzymes such as catalase, peroxidase, superoxide dismutase, ascorbate peroxidase and glutathione reductase, has been observed during accelerated aging [29]. This may make the seed more sensitive to the effects of oxygen and free radicals on the unsaturated fatty acids present in the membranes. Furthermore, reduction in the activity of respiratory enzymes particularly dehydrogenases, involved in generation of ATP, has been observed during aging of the seeds.

IV. Impairment of genetic functions

Studies by a number of workers on a variety of seeds and grains have shown that almost any combination of time, temperature and moisture content that leads to a loss of viability during storage also leads to genetic damage in the survivors. This damage could be in the form of fragmentation of DNA into low molecular weight components, perhaps due to activation of DNase (s). Also, damage to nucleic acids could be a consequence of a build-up of chemical mutagens during storage. The following observations have been made in support of above theory: (1) extracts from aged seeds retard the germination of fresh seeds, and (2) mutations in seeds are highly correlated with seed age and decline in seed viability. Free radicals are also suspected of assault on chromosomal DNA. Potential targets for oxidative damage in the DNA chain include the purines and pyrimidine bases as well as the deoxyribose sugar moities [20]. Specific damage to the bases may leave the strand intact but modification of sugar residues can also lead to strand breakage. The hydroxyl radical mediates the removal of hydrogen from the 2-deoxyribose moiety at C3, C4 and C5 of the DNA molecule, causing the formation of SSBs. Moreover, a large number of single oxidized bases is produced following the reaction with thymine, cytosine, 5- methylcytosine, adenine and guanine. Among them is 7, 8-dihydro- 8-oxoguanine (8-oxoG), a ubiquitous product of DNA oxidation generated by the reaction of hydroxyl radical (Figure 2) [30]. This could be one of the explanations for the increased propensity of genetic mutations as seeds age.

Further it has been reported that there is an increase in chromosome damage with increase in the period of storage. Extensive chromosomal damage could result in impaired template activity of DNA, and reduced RNA and protein synthesis. However, damage to segments of the DNA containing the genes for repair enzymes could be more deleterious than damage to less essential DNA fragments.

It is well established that DNA degradation occurs during seed deterioration. However, several factors argue against it [6]. First, nuclear DNA is highly conserved against free radical attack. The molecule is enclosed by a protective nuclear membrane and is surrounded by histone proteins. Therefore, it is more

likely that free radicals must first penetrate the nuclear membrane and destroy protective histones before causing any damage to nuclear DNA. However, the mitochondrial DNA seems to be more susceptible to free radical attack as it lacks any protective membrane and no histone proteins are associated with it. Secondly, the integrity of DNA has little effect on transcription of the earliest events of germination though they are the first markers of seed deterioration.

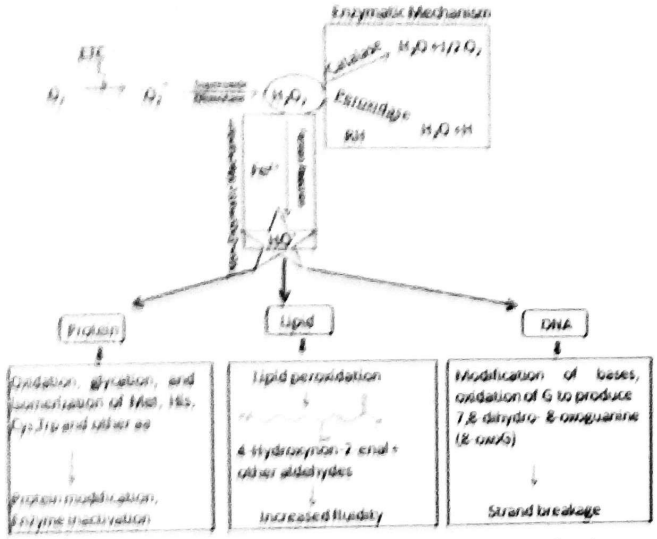

Fig. 2. Effect on different macromolecules during seed deterioration

V. Hydrolysis of sugars

The occurrence of D-galactose-containing oligosaccharides in plant seeds is almost ubiquitous. The most widespread are raffinose family oligosaccharides (RFOs), which consist of linear chains of galactosyl residues, attached to the glucose moiety of sucrose *via* an α-(1→6) glycosidic linkage [31]. The first member of this series, raffinose is the main RFO in most monocotyledon seeds, while its higher homologues, the tetrasaccharide stachyose and the pentasaccharide verbascose, accumulate predominantly in seeds of dicotyledons. Usually, higher homologues, such as ajugose, are only found in trace quantities in seeds. Isomers of RFOs containing α-galactosidic linkages at other carbons of the glucose or at the fructose moiety (planteose and the sesamose series) are of restrictive occurrence in higher plants [32].

During accelerated ageing the decrease in oligosaccharides like stachyose has been observed in soybean seeds [33]. This decline could be due to their non enzymatic degradation or due to hydrolysis by α galactosidase. Sugar hydrolysis has also been observed in seeds stored under natural ageing conditions [22, 34]. The hydrolysis of oligosaccharides would significantly increase the formation of reducing sugars like fructose, glucose and galactose. The presence of reducing sugars is a driving force of Maillard reactions, which initiate non-enzymatic protein degradation during seed aging.

VI. Loss of glassy state

A glass can be defined as an amorphous, non-equilibrium condition in which a liquid achieves such a high viscosity that it resembles a solid. The occurrence of glassy state in the cytoplasm of dry seeds has been reported in numerous instances [35-41]. This physical state of the seeds may be one of the most important factors in determining the rate of physical and chemical changes associated with the seed deterioration. When seeds are in glassy state, the cytoplasm is so viscous that diffusional movements and many deterioration reactions are arrested [35, 42]. In dehydrated vegetables and model systems, the glassy state is reported to prevent lipid peroxidation [43] and Amadori and Maillard reactions [44]. Therefore, it is reasonable to assume that the decline of seed germinability and vigour during seed aging might be associated with a loss of the glassy state. The glassy state may be lost either by increasing the water content, or by increasing the temperature [35, 45]. This in turn may soften the solid-like glassy state into the rubbery state or even 'melt' into the liquid state since the glass transition temperature (Tg) will fall below the storage temperature. The low viscosity and enhanced molecular mobility in the rubbery or liquid state would permit certain deteriorative reactions to proceed rapidly, which are otherwise retarded in the glassy state [46].

The formation of glassy state in the cytoplasm of seeds is facilitated by raffinose family oligosaccharides such as sucrose, raffinose, stachyose, and verbascose that accumulate during seed maturation [47]. These sugars are somewhat more favorable to glass formation in seeds than the reducing sugars, in part because of higher molecular weight, and hence they have a higher glass transition temperature (Tg), which is the temperature at which the glassy state is lost. Furthermore, these sugars have lesser tendency for crystallization, which suppresses or even obliterates the formation of glass. The sugars in the glassy state can maintain the structural integrity of membranes and proteins under dry conditions [48]. Sugars also prevent damage by the increased level of chaotropic ions such

as Na^+, K^+ or Cl^- within the cytoplasm during water withdrawal [49]. However, it is possible that in addition to the soluble sugars, some contribution to the glassy state may be made from other seed constituents including glycosides, larger carbohydrates and even proteins.

VII. Loss of antioxidant molecules

Since free radicals are naturally produced during plant metabolism, particularly in chloroplasts and mitochondria [50], plants are well endowed with antioxidant molecules and scavenging systems. Antioxidant molecules include lipid-soluble antioxidants (Tocopherols: vitamin E and β-carotene) or water soluble ones (vitamin C and glutathione). Tocopherols are chain-blocking antioxidants, therefore blocking lipid peroxidation. Reduced supply of these antioxidant molecules during oxidative stress could be one of the causes of seed deterioration. Thiols are among the cellular compounds that are firstly affected upon oxidative stress in general, due to the susceptibility of their sulfhydryl group(s) to oxidation. The major non-protein thiol in most plant species is the water soluble antioxidant glutathione (GSH), a tripeptide that has an important function in maintaining the cellular redox status and in the storage and transport of sulfur necessary for protein synthesis [51]. The primary oxidation product of GSH is its disulfide GSSG. The oxidised form of GSH can be reduced back to GSH by the action of glutathione reductase at the expense of NADPH. With regard to glutathione in seeds, it has been reported that GSSG inhibits protein synthesis, while GSH is involved in the degradation of the harmful oxidants like hydrogen peroxide. In addition, as shown for animal systems, changes in the glutathione redox status may affect cellular processes *via* thiol-disulfide exchange with proteins [52]. Glutathion Reductase plays an important role in maintaining a high ratio of GSH/GSSG in cells and its activity decreased during ageing. A rise in GSSG has been suggested to precede the loss of mitochondrial integrity, cytochrome c release and caspase-3 activation. Glutathione redox state has also been reported to influence the activity of the ubiquitin/26S protein degradation pathway in ageing of pea seeds [53]. There are several reports indicating that seed aging may involve oxidation of thiols like glutathione. It is harmful to the cellular metabolism and therefore, may contribute to aging-induced seed deterioration.

CONCLUSION

Seed deterioration appears to be caused by lethal damage to different cellular components including membranes, proteins, nucleic acids and cytoplasm. At least three main mechanisms have been suggested for seed deterioration: (1) Free radical attack and lipid peroxidation; (2) Amadori and Maillard reactions; and (3) finally, loss of glassy state of the cytoplasm. However, none of those mechanisms fully or exclusively account for seed deterioration. We believe that seed deterioration is not likely to be ascribed to a single mechanism. Instead, the process of seed deterioration seems to be a multi factorial event where each component is equally critical and act in synergism. In this respect, studies investigating the relationship between different mechanisms must be encouraged. The different mechanisms of seed deterioration are not induced as a universal sequence of events, but are most probably independent processes, which vary among seed species, and with aging conditions. However, common denominator to seed deterioration seems to be lipid peroxidation and any practical approach to reduce this process might improve seed vigour and longevity.

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