Exploring Mechanisms of Seed Aging in Oat and Barley under Artificial Aging

A Thesis submitted to the

College of Graduate and Postdoctoral Studies

In partial fulfillment of the Requirements

For the Degree of Master of Science

In the Department of Plant Sciences

University of Saskatchewan

Saskatoon

By

Solomon Sakyi-Quartey

Permission to use

In presenting this thesis in partial fulfillment of the requirements of a postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised this thesis work, or in their absence, by the Head of the Department or the Dean of the College in which this thesis work was done. It is understood that any copying or publication or use of this thesis or part thereof for financial gain shall not be allowed without my written permission.

It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this dissertation in whole or part should be addressed to:

Head of the Department of Plant Sciences University of Saskatchewan

51 Campus Drive

Saskatoon, SK S7N 5A8

OR

Dean

College of Graduate and Postdoctoral Studies University of Saskatchewan

116 – 110 Science Place

Saskatoon SK S7N 5C

Abstract

Seeds are the major propagules of plants, for which long-term storage requires maintenance of genetic integrity and physiological quality to assure seed viability. Generally, the seed quality declines during storage, but the physiological and biochemical mechanisms associated with reduced seeds quality and vigour are not well understood. Exposure of seeds to artificial aging was used in this study to understand natural seed deterioration for two oat (*Avena sativa* L.) cultivars (CDC Dancer and CDC Minstrel) and two barley (*Hordeum vulgare* L.) cultivars (CDC Copeland and CDC Kindersley). Accelerated aging was accomplished by exposing seeds to high temperature and high moisture conditions until seeds lost their viability. A significant ($p \leq 0.05$) decrease in germination frequencies, viability, and vigour was noted for all genotypes during a five-day aging process. A faster and similar decline in seed quality was initially noted for the oat seeds as compared to the barley seeds. For barley, the initial seed deterioration was higher for CDC Kindersley as compared to CDC Copeland. A catalytic seed treatment (CAT™) on eight days aged seeds could partially reverse the effects of aging for all seed sources, except for CDC Kindersley. The SDS-PAGE gel profiles of major seed storage proteins did not reveal any noticeable changes during aging, whereas biochemical analyses showed increased DNA damage and various changes to seed carbohydrate composition during aging for all genotypes studied. A high raffinose to sucrose ratio in CDC Copeland barley seeds correlated with a significantly ($p \leq 0.05$) lower decline in germination frequencies during the five day long aging process, but when aging was extended for another 10 days, a more rapid deterioration of the seeds occurred. Seeds of the oat cultivar CDC Minstrel, showing the smallest changes in carbohydrate metabolism during aging, exhibited the highest endurance to seed degeneration when aging was done for 15 days. Thus, seed

deterioration progressed differently in the oat and barley genotypes and could not conclusively be correlated with differences in carbohydrate metabolism.

Acknowledgements

I would like to express my very great appreciation to my supervisors Dr. Yuguang Bai and Ravi Chibbar for valuable and constructive suggestions during the planning and development of this research thesis. Their dynamism, vision, sincerity, friendly guidance, and expert advice have been invaluable throughout all stages of this research. I would also like to acknowledge members of my Advisory Committee; Dr. K. Tanino, Dr. Monica Båga and Dr. Curtis Pozniak who offered me extensive personal and professional support and taught me a great deal about scientific research.

I would like to give special thanks to the Dr. Aaron Beattie and the Oat and Barley group for providing plant materials for this research. My appreciation also goes to Dr. Tanino and Rensong Liu of the Abiotic Stress Lab for providing seed treatment solutions. I am also grateful for the help provided Manu Gangola, Mike Hepp and Bharathi Raja of Cereal Quality Lab with my molecular experiments. I would like to thank members of my lab colleagues, Lei Ren, Hu Wang and Rashim Bibi for support.

I would like to dedicate the thesis in memory of my late Joseph Sakyi-Quartey who had been my number one supporter. My deepest gratitude goes to my mum Lina and my siblings Danny, Sally and Wendy for their continuous support and motivation through good and bad times. I am also grateful to my friends Gideon, Leon, George, Kobby, Yusuf, Paapa and Kevin for their support.

Table of Contents

List of Tables

List of Figures

Chapter 1. Introduction

Seeds carry the plant's genetic blueprint and have the ability to produce the next generation when conditions for germination become favorable (West and Heywood 1993). Thus, preservation of seeds is vital for the maintenance of most agricultural and horticultural plants (Benz 2012). For plant breeders, a reliable seed conservation system assures development of new industrial crops, alternative food crops, and new medicinal plants will be possible in the future (Li and Pritchard 2009). Plants can be preserved by using *in situ* or *ex situ* methods, whereby populations, species and/or germplasms are maintained within or outside their site of origin. Cryopreservation and cultivation of plants *in vitro* or *in vivo* are methods to preserve entire plant parts. However, the maintenance of most plant resources is done by storing seeds in seed banks (FAO 1997; Linington and Pritchard, 2001). Having seeds stored away serves as an insurance against threats such as genetic erosion and loss of biodiversity (CBD 2002; Nascimento and Meiado 2017).

Upon seed dispersal from the mother plant, many seeds will under natural conditions deteriorate and die due to exposure to the natural elements. The surviving seeds become part of the soil seed bank where they may lay dormant for long periods before germinating (Baskin and Baskin 1998; Fenner and Thompson 2005). The environmental conditions in the soil influences the length of seed dormancy and longevity and this can have severe implications on local plant populations. For example, extended seed longevity for invasive plant species may help their distribution in the environment and contribute to weed infestation in crop fields. In contrast, environmental conditions having a negative effect on particular seeds in the soil may lead to extinction of sensitive species.

Storing seeds as *ex situ* germplasm is very important for plant genetic resources conservation (Heywood et al. 2007). However, to completely utilize the potential of *ex situ* conservation efforts, long-term seed storage needs to be monitored for seed viability by periodical germination assays to determine regeneration frequencies with minimal seed consumption (Li and Pritchard 2009).

Different techniques have been used to maintain seed viability during storage with varying degrees of success. The longevity of stored seeds depends on storage duration, environmental conditions, seed morphology, physiological stage and genetic makeup of seeds (Bewley and Black 1994). In general, seeds stored under dry and cool conditions maintain a higher viability and vigour than seeds stored under humid and warm conditions (Walters et al. 2013). Alterations in cell membrane integrity, protein synthesis, and various enzyme activities are some of the changes noted during seed storage (Bailly 2004; Kibinza et al. 2006). When seeds are stored under high temperature and high humidity, there is an increase in the metabolic state of cells and seeds become more exposed to oxidizing agents that alter biomolecules and promote chemical degradation of cellular components (Roberts and Ellis 1989; McDonald 1999). However, the biochemical changes in seeds during storage and aging are not well understood (Treuren et al. 2013) and can differ between species and even subspecies of the same genus (Arc et al. 2013). Thus, seed viability and vigour in the same seed lot often varies depending on the seed storage condition. In agriculture, unforeseen damage in seed viability can adversely affect stored seeds, seed sales and production programs, and be very costly for the industry (McDonald 1999).

The study of seed deterioration and aging during storage can take a very long time especially for desiccation tolerant seeds, which are also called orthodox seeds (Walters 1998). Thus, artificial aging methods such as exposure to high humidity and high temperature are generally used to study the effects of storage conditions on seed deterioration (Black et al., 2006). Elevated partial pressure

of oxygen may be used to study seed deterioration or ageing under dry conditions (Groot et al. 2012). Through the use of artificial aging techniques, the physiological and biochemical changes during seed storage can been studied. The results of these aging studies are being used to develop predictive models and devise strategies to enhance seed viability during storage (Long et al. 2008; Long et al., 2015).

The objective of this study was to investigate the physiological and biochemical changes during seed storage and artificial aging in cereal species. The major focus was to elucidate changes in seed storage compounds and selected biomolecular changes which affect the seed physiology during aging. The knowledge gained can be used to systematically develop strategies to improve seed vitality and vigour during long term storage.

Hypotheses:

1. Major biomolecular changes during artificial seed aging affect seed viability and germination.

2. Changes in oligosaccharides influence seed germination.

3. A catalytic seed treatment can reverse the effect of rapid seed aging.

Chapter 2. Literature Review

The following sections summarise the importance of seed storage and germplasm preservation, factors affecting seed storage, changes in seed properties during storage and the utilization of artificial aging methods to study the seed aging process to develop strategies for long-term seed storage.

2.1 Seed development and germination

Mature seeds are the terminal physiological status of plant development, and in higher plant development, seed production ensures the maintenance of the plant species (Bewley and Black, 1994). The formation of seeds starts inside the botanical fruit, which consists of a mature ovary containing one or more ovules. Fertilization of the female gametophyte (ovule) by male gametophyte (pollen) to form a zygotic diploid embryo is the start of seed development. A second fertilization event gives rise to the triploid endosperm, with two doses of female and one dose of male genetic components. The seed coat (or testa) surrounding the embryo and endosperm is maternal as it is solely derived from the ovule tissue. The main role of the seed coat is to protect the seed from biotic and abiotic stresses (Haughn and Chaudhury 2005).

During seed development, there is a gradual increase in seed weight due to extensive cell growth and elongation. Initially, the seed moisture content is high, but as the seed grows, the dry weight increases and becomes stable at maturity. Carbohydrates, proteins, and oils in varying proportions depending on the seed species are the main seed storage components. In monocotyledonous seeds, carbohydrates (mainly starch) and proteins are the most abundant reserves stored in the endosperm and embryo tissues, which provide most of the nourishment needed during germination (Bewley et al. 2013; Bradford and Nonogaki 2008).

One of the most significant stages of the plant life cycle is seed germination. Germination involves a switch from quiescent dry state to a metabolically active stage. In many cases, fresh seeds germinate readily, whereas others require after-ripening to eliminate dormancy acquired during the last stage of seed development (Bewley and Black 1994). The germination process is complex and starts with water uptake by the embryo (Bewley et al. 2013). Upon imbibition, seeds swell, cells become more turgid and gas exchanges within the seeds is increased. This causes a revival of metabolism, cellular respiration, the biogenesis of mitochondria, DNA repair, the transcription and translation of new mRNAs, and the beginning of reserve utilization (Koornneef et al. 2008; Nonogaki et al. 2010). Certain enzyme systems that metabolize stored carbohydrates, proteins and lipids are activated followed by transport of released nutrients to the growing point of the seed. Normally, the radicle emerges first by rupturing the seed coat; later, both root and shoot systems develop, and the young seedling becomes independent and synthesizes its own cell material needed for growth and development. The appearance of the embryonic axis through the embryo is considered the last phase of germination (Weitbrecht et al. 2011).

2.2 Importance of seed storage and germplasm preservation

Reliable seed storage conditions are crucial to preserve seed quality, vigour, and viability for future seed use by farmers or breeders (Rajjou and Debeaujou 2008). Storage of seeds as off-site germplasm of the plant is also essential for the 00 of plant genetic resources and the survival of most the plant species (FAO 1997; Linington and Pritchard, 2001). Therefore, storage of seeds which acts as the germplasm of a higher plant is also essential for biodiversity conservation especially for plants producing orthodox seeds, as they offer desiccation tolerance and allow propagation after long-term dry storage. The ability of seeds to endure desiccation or be stored for

extra periods is a vital functional characteristic for species continuity. Good seed storage practices also help to prevent seed losses (Tweddle et al. 2003).

The main objective of seed storage is to preserve seed vigour and prolong viability. Seed vigour represents the physiological potential of a seed (Marcos-Filho 2015) and it plays an essential role during storage of seeds. Seeds with high vigour have greater storage potential, preserve well for longer periods, and produce healthy plants. Under optimal storage conditions, the activity of metabolic processes in the seeds are kept very low, which slows down the ageing process and increases the seed's life span for future use.

2.3 Factors affecting seed storage

Seed storage is influenced by several factors including nature and type of seeds, temperature, seed moisture and ambient relative humidity (Abdul-Baki 1980; Kijak and Ratajczak, 2020) which are also the major factors affecting seed viability (Roberts and Ellis 1989). In general, a seed deteriorates progressively after its separation from the mother plant; however, proper control of these variables can sustain seed viability over a considerable storage period (Li and Pritchard 2009).

2.3.1 Seed type

The physical and physiological stage influences if the seed can be stored by conventional means (Roberts and Ellis 1989). As the rate of seed deterioration during storage is genus and species specific (Priestley 1986; Roberts and Ellis 1989), seed types are generally classified as orthodox, intermediate, or recalcitrant. Studies during the late 1950's revealed that for successful storage, the seed must have great tolerance to loss of most of its moisture content, also known as desiccation tolerant or 'orthodox' seeds. Harrington (1960) coined a 'rule of thumb' that for each one percent

decrease in moisture content or 5.6°C decrease in temperature, the storage life of a seed was doubled. Therefore, orthodox seeds have good tolerance to dry conditions, as evident by no loss in viability even at five percent moisture content or below. Later, Roberts (1973) proposed that seeds that did not meet the criterion for orthodox seeds should be called 'recalcitrant seeds'. These seeds loose viability when desiccated to 15-20% of moisture and cannot be stored for long periods. During the late 1990s, it was realised that some plant species such as legumes did no fall into any of the two extremes, but shared characteristics similar to recalcitrant seeds but tolerated desiccation to about 10–12% moisture . These types were then classified as 'intermediate seeds.'

Seeds may also be classified as mesobiotic (medium life span) or macrobiotic (long-lived). Seed type may also be associated with the biotic factors of the seed that include genetic makeup, initial seed quality, provenance and seed moisture content. Generally, a high initial seed quality assures an extended viability in storage due to higher resistance to unfavourable storage conditions. Seed morphology may also play a role for storability, as small spherical seeds are less likely to be externally damaged.

Provenance refers to the source or area of the origin of the seed. It reflects the conditions that the plant and seed endured prior to harvest and storage (Moshki et al. 2012). It includes the climatic state, soil quality and cultivation type. Fluctuations in weather conditions during seed formation also affect seed quality and thereby affect its storage (Schmidt 2007).

2.3.2 Relative humidity

The residual moisture of seeds varies among species and even between plant to plant (Finch-Savage and Bassel 2016) and is the most important factor that influences seed viability during storage. Generally, the higher moisture content in seeds, the shorter life-time to expect in storage

(Tangney *et al*. 2019). Often, the reduced storage potential by high seed moisture content is a result of increased mold growth that irreversibly damages the seeds (Saxena et al. 2015) .

Humidity levels in storage have an effect on germination times, as exemplified by *Brassica spp.* and *Eruca sativa* seeds showing longer germination time when stored at high relative humidity as compared to seeds kept at low humidity (Suma et al. 2013). The same study also showed the seedling length, which is a predictor of seed vigour, declines when seeds are stored at high relative humidity. Artificial aging tests confirm higher losses in seed quality for seeds stored at high humidity compared to those kept at low relative humidity (Farhadi et al. 2012). Generally, storage environments with humidity levels exceeding 18 to 20% are to be avoided, as these conditions are known to promote growth of microorganism (fungi) that causes rapid deterioration of the seed (Rao et al. 2014).

2.3.3 Temperature

Low temperature during storage maintains seed viability, although there are some exceptions (Roberts 1972). Temperature affects the rate of seed vigour loss during storage, and for each 5ºC reduction in temperature, the storage life of seeds is expected to double (Harrington 1972). Storage time is another factor for viability loss, as demonstrated by 73% of *Swertia chirayita* seeds showing 90% germination frequency upon six months storage at 4°C, but only 64% of the seeds showed 80% germination after 12 months (Pradhan and Badola 2012). The same study also demonstrated that seeds stored at 4°C have higher germination rates than those stored at 15°C followed by those kept at room temperature. However, germination was significantly reduced with increasing storage duration with higher reduction in germination percentage when the seeds were stored at higher temperature (Pradhan and Badola 2012). Even slight increases in temperature and moisture content may promote fungal growth and insect development in seeds contributing to loss in seed viability

(Christensen 1972). Therefore, if the seeds are not properly dried before storage, the high moisture content stimulating fungal growth may reduce seed viability.

2.4 Changes in seed properties during storage

Seed physical properties and composition start changing as soon as it is harvested from the mother plant, and it continues during storage. The change in seed properties and composition is accelerated if seeds are stored improperly, such as at high temperatures and/or high relative humidity (Farhadi et al. 2012). Temperature and moisture content during storage are environmental factors determining the rate of seed aging (Elllis and Roberts 1980; Priestley 1986). As seeds age, membranes become leaky, enzymes lose catalytic activity and chromosomes accumulate mutations (Arc et al. 2011).

During storage, changes occur in the physiology of seeds and/or alteration of its biomolecules (Walters et al. 2010). Singular factors or combinations of time, temperature, and moisture content during seed storage have been investigated in relation to their effect on the growth and yield of surviving seeds (Abdalla and Roberts 1969). Seed deterioration is defined as the loss of quality, viability and vigour either due to ageing or adverse environmental factors (Kapoor et al. 2011). Ageing is the progressive decline in biological functions, accompanied by an increased risk of degenerative changes and cell death over time. The chemical degradation of seed components during storage occurs through damage caused by oxidizing agents (Fu et al. 2015), but the rate of such reactions is also influenced by seed properties and composition, which are affected by temperature and moisture.

2.4.1 Physiological factors

A number of physiological changes in cells and tissues are associated with aging in seeds. During ageing, seeds gradually lose their vigour and viability for germination (Maity et al. 2000) determined as decrease in seed moisture content, loss in seedling vigour and vigour index, and finally reduction in germination.

The concomitant decrease in germination percentage that accompany seeds during storage determines seed viability (Gidrol et al. 1988). The reduction in seed viability may be attributed to degradation of mitochondrial membranes leading to suboptimal energy generation for germination. Seed ageing can cause a rapid decrease in germination percentage; a reduction from 85% to 10% germination frequency within seven days has been reported for carrots (Al-Maskri et al. 2003). However, proper control of the seed storage conditions can effectively sustain fairly good seed viability over a considerable storage period (Pradhan and Badola 2008).

A gradual decrease in seed moisture content accompanies seed deterioration during storage. The moisture threshold for seed desiccation is genotype and species specific and also varies considerably among orthodox and recalcitrant seeds (Roberts and Ellis 1989).

Seedling vigour can be defined as a function of root and shoot length combined with germination speed. Seed vigour measurements such as germination speed have been known to be reduced during aging (Farhadi et al. 2012). The seedling vigour tends to be reduced during seed storage, where, aged seeds often produce reduced root and shoot lengths of germinated seeds. In rice, the germination speed is faster in recently harvested grain than in aged or stored rice (Kapoor et al. 2011), concurring with the results of another similar study in tomato (Jain and Van Staden 2006).

2.4.2 Molecular and biochemical aspects of seed ageing and longevity

At the biochemical and molecular level, seed ageing is associated with various alterations in transcription, translation, and cellular composition of macro and micro-molecules. These activities include DNA degradation, impairment of RNA and protein synthesis, reduced energy metabolism, loss of membrane integrity and solute leakage (Sano et al. 2016).

Membrane disruption is one of the main reasons attributed to seed deterioration (Jyoti and Malik 2013). Solute leakage may follow membrane disruption or damage during ageing. Cell membranes are important regulators for cell-to-cell interactions and their disruption leads to loss of function in certain parts of the seed. The major source of membrane disruption as seeds deteriorate is an increased free fatty acid (FFA) level and release of free radicals by lipid peroxidation (Goel et al. 2003). Progressive increase in the accumulation of lipid peroxidation as the seeds age was shown in an artificial/accelerated aging study of carrot seeds (Farhadi et al. 2012). Sveinsdóttir et al. (2009) showed that the addition of FFAs increases fusion of plant vesicles, which leads to an increase in membrane leakage. There is a positive correlation between the storage period and content of FFAs in soybean (Saxena et al. 2015), and caraway seeds (Regina and Raman 1992). The accumulation of FFAs results in a concomitant decline in the seeds cellular pH, which has been proven to be harmful to seed health. Gradual increase in the quantity of FFAs in stored seeds is a measure of deterioration; therefore, it defines seed aging and consequently its viability. Increase in saponification value, which is the amount of alkali (in milligrams) required to saponify one gram of fat, was observed due to increase in formation of short chain fatty acid glycerides (Saxena et al. 2015).

The respiration during seed storage is accompanied by the loss of food reserves, decrease in some proteins and non-reducing sugars accompanied by increase in reducing sugars and FFAs.

(Moncaleano-Escandon et al. 2013). Gradual loss of enzyme activities, such as alpha–amylase in rice (Marques et al. 2014) or endo-β-mannanase in lettuce seeds, increases when storage temperature increases from 15°C to 25°C to 35°C (Wang et al. 2010). Conversion of aminocyclopropane-1-carboxylic acid (ACC) to ethylene decreases rapidly during aging. Therefore, strategies to decrease ACC conversion to ethylene during storage have been used to sustain long term seed viability (Khan 1994).

A reduction in protein concentration has been observed in seeds during storage (Saxena et al. 2015; Siadat et al., 2012; Rao et al. 2014). In aged seeds under controlled conditions, an 80 kilodaltons (kDa) polypeptide was present in seeds stored for about one year while polypeptides of predominantly 60 kDa were detected in seeds stored for 14 years (Khan et al. 2003). In oil seeds such as sunflower, a decrease in the seed oil content was also reported during storage (Singh and Prasad 1977).

Seed ageing has been shown to cause chromosomal aberrations and DNA degradation (Parrish and Leopold 1978). Chromosomal aberrations such as fragmentation, fusion, and ring formations have been reported during seed storage (Mahjabin and Abidi 2015). Alterations to DNA in the cell nucleus, causes genetic mutations as well as physiological damage (Dourado and Roberts 1984).

2.3.2.1 Reactive Oxygen Species (ROS)

Reactive Oxygen Species (ROS), the most common oxidants in cells, are produced during seed development, from embryogenesis to germination, but also during seed storage (Bailly et al. 2008). ROS are formed by the partial reduction of molecular oxygen to superoxide (O_2-), hydrogen peroxide (H_2O_2), lipid peroxides (ROOH), or the corresponding hydroxyl (HO•) and peroxyl radicals (ROO•) (Gaschler and Stockwell 2017). ROS can be produced by means of enzyme

activity or by non-enzymatic chemical reduction of molecular oxygen (O_2). ROS are highly reactive and attack in their vicinity various classes of biomolecules including proteins, DNA and lipids such as polyunsaturated fatty acids (PUFAs) (Tsikas 2017). Peroxidation of cellular molecules, such as lipids (cell membranes) (Bailly 2004; Rajjou et al. 2008; Rajjou and Debeaujon 2008), is facilitated by ROS and can cause alterations in the cellular membranes (Groot et al. 2012). The PUFA arachidonic acid is peroxidized to finally form malondialdehyde (MDA), 4-hydroxy-2- nonenal (HNE) and other reaction products such as F2-isoprostanes. The loss of grain viability at 45ºC and 100% RH is associated with a build-up of H_2O_2, a progressive reduction in catalase (CAT) and superoxide dismutase (SOD) activities, and with an upsurge in glutathione reductase (GR) activity (Lehner et al. 2008). In addition to SOD, GR, and CAT, the major enzymes that contribute to cell detoxification are peroxidases such as ascorbate peroxidase (APX), glutathione peroxidase (GPX) and thioredoxin peroxidase (TPX) (Scandalios 1997; Bailly 2004; Milesi 2006). Also, ageing has been shown to be associated with a depletion of antioxidant enzyme activities in cotton (Goel et al. 2003), soybean (Murthy et al. 2002), beech (Pukacka and Ratajczak 2005) and sunflower seeds (Bailly et al. 1996; Torres et al. 1997; Kibinza et al. 2006). The ability of seeds to scavenge ROS is thought to be associated with seed storage potential (Priestley 1986; Bailly 2004).

2.3.2.2 Lipid peroxidation

Disruptions in redox homeostasis are caused by an accumulation of oxidizing molecules either by overproduction or loss of cellular reducing ability. In either case, the accumulated oxidizing agents are able to oxidize DNA, proteins, and lipids thereby altering their structure, activity, and physical properties. Excessive oxidation of lipids alters the physical properties of cellular membranes and can cause covalent modification of proteins and nucleic acids (Gaschler and Stockwell 2017). The specific reaction of ROS with lipids is generally known as “lipid peroxidation”. The peroxidation

of lipids may be the most frequent cause of deterioration and loss of viability of seeds, since it leads to the reduction of lipids. Thus, the amount of oil in seeds may differ according to storage conditions (Koutroubas et al. 2000), particularly temperature and relative humidity as such conditions directly impact oil degradation during seed storage.

Lipid peroxides cause toxic effects through two general mechanisms. Lipids are responsible for preserving the integrity of cellular membranes. Widespread peroxidation of lipids alters the composition, assembly, structure, and dynamics of lipid membranes. As highly reactive compounds, lipid peroxides are also able to propagate further generation of ROS, or transform into reactive compounds capable of crosslinking DNA and proteins. MDA, HNE and F2 isoprostanes are accepted biomarkers of oxidative stress, namely of lipid peroxidation. Over the last two decades the number of scientific publications reporting on oxidative stress in general and lipid peroxidation have steadily increased, with the proportion of lipid peroxidation in the form MDA, seeming to increase in favor of lipid peroxidation.

2.5 Using artificial aging to study seed aging

Artificial aging also known as accelerated aging refers to the exposure of seeds to two environmental variables; high temperature and relative humidity to cause rapid or accelerated deterioration of seeds during storage (Hampton and TeKrony 1995). It is used to assess seed vigour and storability (Barreto and Garcia 2017). The principle of this aging method is that high vigour seeds will have higher germination rates after exposure to these two unfavourable conditions than low vigour seeds (Moncaleano-Escandon et al. 2013). It also helps to elucidate the seed deterioration process by assessing changes occurring during seed aging (Barreto and Garcia 2017). The method was initially developed by Crocker and Groves (1915) by suggesting that higher temperatures accelerated protein coagulation. Delouche and Baskin (1973) and McDonald and

Phaneendranath (1978) made further modifications to the method, which shows great accuracy in estimating the aging of soybean and corn seeds. In the optimized artificial aging method, the seeds are subjected to rapid deterioration by placing them in artificial aging boxes with high temperatures (41 ± 0.3°C) and humidity for about 48 to 96 hours depending upon the crop. After the requisite time period, the seeds are removed and subjected to a quick germination test. A good artificial aging result is more than or equal to 80% germination (Rodo and Marcos Filho 2003).

Accelerated ageing significantly decreases both seed germination capacity and relative growth rate (Farhadi et al. 2012). Exposure of seeds to artificial aging leads to increased ROS production, cell damages such as lipid peroxidation that reduces the seed vigour (Qin et al. 2011). The peroxidation of membranes enhances cell permeability, mitochondrial enlargement and later cellular breakdown, resulting in increased seed conductivity due to electrolyte leakage (Priestley 1986; Al-Maskri et al. 2003). The peroxides also undergo chemical splitting to produce ketones, aldehydes and acids responsible for physical signs of seed deterioration such as bad odour (Basra et al. 2003).

Most artificial aging studies support the concept that seeds aged rapidly or artificially will show profound reduction in seed viability and vigour mainly due to increased seed conductivity, build-up of lipid peroxidation and accumulation of unsaturated fatty acids (Al-Maskri et al. 2003; Pukacka and Ratajczak 2005). However, no significant changes in MDA content were found in *Brassicaceae* wild species (Mira et al. 2010) or wheat (Lehner et al. 2008) during aging. This difference concerning the role of MDA or lipid peroxidation in loss of seed viability may be explained by the reaction of ROS with other molecules such as proteins and nucleic acids which could also destroy membranes (Helbock et al. 1998).

In summary, artificial aging can be used to assess seed behaviour during seed storage. Seeds that perform well after artificial aging stress conditions have a greater likelihood to survive under a wide range of field and storage conditions (Marcos-Filho 2015). In addition, artificial aging can be used to develop strategies for long term seed conservation.

2.6 Plant material to study seed aging

In this research, monocotyledonous cereals oat (*Avena sativa* L.) and barley *Hordeum vulgare* L) were the plant material used to understand seed aging. Both species are mainly grown purposefully for their grain. Oat thrives well in moist climatic areas on the European and North American continents (Varma *et al.*, 2016). Oat and other small grain cereals were introduced to the Canadian Prairies in the mid-1700s and became an important feed crop for early settlers in Western Canada. Oat continues to be a major cereal crop in Canada, with an average annual production in recent years in excess of four million tonnes on approximately 1.2 million acres (AAFC, 2020). Canada is a major supplier of oat, making up the majority of world oat trade. Recently, demand for oat has been strong from the United States food market, as well as the livestock market. The majority of oat trade is with the United States, with Canada being their major supplier. The main uses for oat grains are general livestock feed, human consumption, and recreation horse feed (pony oat).

The human food market for oat is growing as consumers recognize the dietary benefits of whole grains. Oat offers a plentiful source of bran, fibre and beta-glucan. Oat is recognised world-wide as a healthy and highly nutritious cereal that contains high amounts of soluble fibre, lipid, protein and natural compounds beneficial to man and livestock health (Izydorczyk 2014). It contains proteins such as albumin, globulin and glutelin as the major prolamin. The oat kernel is covered by 23-32 straw-like materials known as the hull and is composed of bracts of florals (Salo and

Kotilainen, 1969). It is a delicacy to the human community and feed to livestock. The processing and storage of oat strives to keep its vigor and viability. However, ageing is a natural phenomenon and irrespective of storage under ideal and controlled conditions, the seeds lose their viability and vigour at rates that varies with cultivars (Shaban and Motlagh, 2014).

Barley is like oat one of the ancient grains and in the modern agriculture a leading cereal crop. It is a self-pollinated diploid species belonging to the Triticeae tribe. Like oat, it is also a temperate cereal crop with Western European countries being the largest producers (Friedt et al, 2011). The hardy nature of barley allows it to be cultivated in adverse environments such as on dry and saline soil (Schmidt et al 2019). Its production has increased throughout decades mainly due to the development of disease and insect resistant cultivars (Friedt et al, 2011). Barley grain is highly nutritious, and its nutritive value is almost equal to that of corn. It contains a high concentration of soluble and insoluble dietary fibre, B and E vitamins, selenium, flavonoids and anthocyanins (Behall et al, 2004) with proven health effects such as reducing blood pressure, blood cholesterol, blood glucose levels (type 2 diabetes) and colon cancer incidence.

Barley (*Hordeum vulgare* L.) is a versatile crop grown for malting, food, and general purposes (feed and forage) across the Canadian Prairies. In the recent three crop years (2019 – 2021) in Canada 2.6 to 3 million hectares of barley was seeded with a total production around 8 to 10 million tonnes (AAFC, 2020). In Western Canada, malting barley accounted for 60.8% of the barley seeded area in 2017 compared to 56.2% in 2016; general purpose barley accounted for 31.8% of seeded area in 2017 compared to 37.3% in 2016.

Chapter 3. Materials and methods

3.1 Plant material

Oat and barley seeds used in the study were developed by the Oat and Barley Breeding Program, Crop Development Centre, Department of Plant Sciences, University of Saskatchewan. The seeds were from the 2015 bulk collection being stored at –20 °C. Four cultivars, two each for oat (CDC Dancer and CDC Minstrel) and barley (CDC Copeland and CDC Kindersley) were used in the experiments. Both CDC Dancer (registered 2002) and CDC Minstrel (registered 2009) are spring type producing white milling quality oat grain. CDC Copeland (registered 1999) and CDC Kindersley (registered 2011) are two-row malting barley varieties.

3.2 Experimental and treatment design

A randomized complete design with replications and repeats was used in all experiments. Days of aging (d), species/cultivar and concentrations of (CAT™) were considered as independent factors and viability and germination percentages were results. Seeds were selected randomly in each replication by using a seed counter. Physically broken seeds were discarded from the selection.

A completely randomized design with replications and repeats was used to determine the effects of aging treatments in all experiments. Days of aging and cultivar were considered as factors. Soluble sugar content, germination percentages, SDS gel electrophoresis, and DNA comet assay were then observed as results.

3.3 Artificial aging and seed assessment

3.3.1 Artificial aging

Artificial aging of seeds was done by using a KIMAX desiccator with detachable stopcock valve (VWR international, Mississauga, Ontario, Canada) as described by (Baalbaki 2009). Seeds were

placed in paper envelopes and rehydrated in the desiccator for 25 to 30 days at 45% RH and 22.5ºC temperature. Rehydration leads to equilibration to ensure that all moisture changes that would lead to seed deterioration by artificial aging is uniform for all seeds. Thereafter, the seeds were aged by equilibration at 60% RH, 45ºC for 14 days. The 45% RH inside the desiccator was achieved by placing 1L, 40.5 % w/v lithium chloride (LiCl) solution and 60 % RH was achieved by 1L, 30 % w/v LiCl solution. Equilibrated seeds were then subjected to accelerated aging treatments by placing them in a growth chamber (Sanyo Versatile Environment Chamber MLR-350H, Sanyo Scientific, USA) set at 45 °C. TZ (1% (w/v) 2,3,5 triphenyl tetrazolium chloride) viability evaluations were done every five days until the TZ tests showed total loss of viability. Viability and germination scores were all recorded as described below.

3.3.2 TZ Viability Test

Three replicates of 30 seeds were placed in Petri dishes containing two layers of filter papers soaked with 5 mL distilled water. After 24-hour incubation at room temperature, seeds were cut longitudinally in half to expose the embryo tissue and then submerged in a 1% (w/v) 2,3,5 triphenyl tetrazolium chloride (TZ) solution for 18 hours at room temperature. The production of the red, non-diffusible substance formazan from the colorless TZ compound in the embryo tissue showed presence of active dehydrogenase enzymes, and thus was an indicator for viable seeds. The absence of color change was considered a loss of seed viability (Souza et al. 2010). Each TZ test was repeated four times.

3.3.3 Standard Germination Test

Optimum germination temperatures for oat (20º C) and barley (24º C) according to (ISTA 2019) were used. Five replicates of 30 seeds were placed in 9 cm Petri dishes containing two layers of filter papers moistened with 5 mL distilled water. The Petri dishes were placed in clear plastic bags

to minimise evaporation and incubated at room temperature (22°C) in dark. Germinated seeds were counted and removed every 24 hours for seven days. Seeds were considered germinated when the radicle protruded 2 mm. The experiment was repeated four times.

3.3.4 Seed Vigour Measurement

Germination Rate Index (GRI) as a form of seed vigour was determined by using the following formula (Melville et al. 1980).

$$GRI = {}^{\sum k}_{i=1} N_i / {}^{k}_{\sum i=1} NiTi$$

Where, $\underline{T_{iv}}$is the time from the start of the experiment to the *i*th day of observation, N_i is the number of seeds germinated in the *i*th day and *k* is the last day of germination.

Data were analyzed using the R Statistics software package (version 3.3.2, R Core Team 2017). An analysis of variance (ANOVA) was performed using the mixed model procedure to compare each evaluation day and physiological parameters (viability, germination, and germination rate). Cultivar, days of aging and their interactions were considered as fixed effects, while replications and repeats were taken as random effects. For each trait, if the ANOVA indicated significant differences at the $P<0.05$ level, means were separated using the least significant difference.

3.4 Seed priming with CAT™

To prevent fungal growth during the germination tests, the seeds were sterilized with 3% formaldehyde for 10 minutes and then washed three times with distilled water (Asgharipour and Rafiei 2011). Fifty surface-sterilized seeds were placed in each Petri dish containing two filter papers wetted with 75 mL CAT™ (Olkowski et al 2014) solution provided by the Abiotic Stress Lab, Department of Plant Sciences, and University of Saskatchewan. The seed priming was done using undiluted (100%), 50%, 25%, 12.5%, 6%, 3% dilutions of CAT™, and no CAT™ (0%).

Petri dishes were placed randomly in sealable plastic bags and covered in aluminium foil to ensure darkness during the germination process performed as described above. Three germination experiments were made, and each experiment contained four replicate samples. Germination data for each cultivar were analyzed separately due to significant days of aging × cultivar interactions for observed germination and viability percentages.

3.5 Biochemical analysis of seed components

3.5.1 Extraction and purification of soluble sugars

Extraction of soluble sugars in seeds was done essentially as previously described (Gangola et al. 2014). Samples of 200 seeds per cultivar were ground in a Udy Cyclone Mill (Udy Corporation, Fort Collins, CO, USA) mill to produce seed meal. Approximately 500 mg seed meal was added to a 15 mL falcon tube containing 10 mL 80% (v/v) ethanol and mixed using a vortex mixer. Extractions of soluble sugars was achieved by incubation in a 60 °C shaking water bath for 45 minutes with regular mixing at 15 min intervals. The resulting slurry was centrifuged at 12,100 x *g* for 10 min to collect the supernatant. Supernatants pooled from three extractions per ground seed meal sample were used for purification of soluble sugars.

Removal of proteins and lipids present in the soluble sugar extract was done by filtration through a C18 cartridge (Honeywell Burdick & Jackson, Muskegon, MI, USA) using a vacuum manifold. The cartridge was first prewashed by filtration of 5 mL of 99% (v/v) methanol followed by 5 mL distilled water, before 3 mL sample extract was applied. A 1.6 mL aliquot of filtered extract was vacuum dried to dryness using a Speed vac® concentrator and universal vacuum system (Thermo Savant, Holbrook, NY, USA)

3.5.2 Instrumentation and chromatographic conditions

An Ion Chromatography System 5000 [ICS 5000 comprising of an autosampler, a single gradient pump (Model SP-5) and an electrochemical detection cell with disposable working gold electrode and Ag/AgCl reference electrode (Thermo Fisher Scientific, Stevens Point, WI, USA] was used to perform anion exchange chromatography of soluble sugar extracts as described (Gangola et al. 2014). During sample integration and reactivation of the detector gold standard, PAD waveform at four different potentials was used for a combined time of 500 milliseconds (msec). The four working potentials (E) were: +0.1 V for 400 msec (E1), -2.0 V for 20 msec (E2), +0.6 V for 10 msec (E3) and -0.1 V for 70 msec (E4). A Chromeleon 7.0 software (Dionex Canada Ltd., Oakville, ON, Canada) installed on a Dell Optiplex 780 desktop was used to control the whole assembly. The soluble sugars were separated using a CarboPac PA200 (3 × 50 mm) column preceded by a CarboPac PA100 (4 × 50 mm) guard column. Both columns were maintained at 30°C and a mobile phase of 50 % (w/w) sodium hydroxide was used.

3.5.3 Estimation of total starch content of seeds

The amount of total starch concentration was determined using a method approved by the American Association of Cereal Chemists International (AACCI approved method 76-13.01, AACCI 2000). In this method, the D-glucose concentration in hydrolysed starch was determined. In summary, duplicate samples of approximately 100 mg ground seed material obtained from 50 seeds per genotype were prepared and suspended in 0.2 mL 80% (v/v) ethanol. Three mL of thermostable α-amylase (100 U/mL, Megazyme International Ltd., Wicklow, Ireland) in 100 mM sodium acetate buffer pH 5.0 was added to ground seed material suspension and it was incubated at 100°C for 6 min. The suspension was intermittently mixed every two mins to hydrolyze starch into maltodextrins. The samples were then incubated with 0.1 mL 330 U amyloglucosidase (3,300

U/mL, Megazyme International Ltd., Wicklow, Ireland) in 200 mM sodium acetate buffer pH 4.5 at 50°C for 30 min, to further hydrolyze maltodextrins into glucose. On completion of the reaction, sample volume was adjusted to 10 mL using nano-pure water. Three aliquots (10 µL each) were each added to three mL glucose determination reagent, GOPOD and incubated at 50°C for 20 min. Samples containing a glucose standard were also made.

Total starch concentration was measured as free glucose by recording the absorbance at 510 nm (Hucl and Chibbar 1996) using a spectrophotometer (DU 800, Beckman Coulter Inc., Mississauga ON, Canada). Total starch concentration was calculated on a percentage dry weight basis (Mccleary et al. 1997).

Starch (%) = ΔA x F x FV/0.01 x 1/1000 x 100/W x 162/180 where,

ΔA = Absorbance (reaction) read against the reagent blank

$$F = \frac{100\ (\mu g\ of\ D\text{-}glucose)}{Absorbance\ for\ 100\ \mu g\ of\ glucose}$$ (conversion from absorbance to µg)

FV = Final volume i.e. 10 mL.

0.01 = volume of sample analyzed.

1/1000 = Conversion from µg to mg.

100/W = Factor to express starch as a percentage of dry flour weight.

W = weight (in mg) of flour analyzed 162/180 = Adjustment from free D-glucose to anhydro D-glucose (as occurs in starch).

3.5.4 Protein extraction

Seed samples (10 g) were frozen in liquid nitrogen and crushed into a fine powder using mortar and pestle. Proteins in 500 mg ground seeds were extracted by re-suspension in 2 mL extraction

buffer (0.05 M Tris-HCl pH 6.8, 10 % (w/v) sodium-dodecyl-sulphate, 10 % (v/v) glycerol, 1.25 % (v/v) β-mercaptoethanol and 0.05 % bromophenol blue) followed by 5 min boiling, 5 min cooling on ice, and 12 min centrifugation at 12,500 × g at room temperature. The protein concentration in soluble extract was determined in triplicates using a Coomassie brilliant blue dye-binding assay (BioRad, Hercules, CA, USA).

3.5.4 Gel electrophoresis

Ground seed meal (100 mg) were re-suspended in 0.1 mL sample buffer containing 25 mM Tris-HCl pH 7.5, 1% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, and 0.002% (w/v) bromphenol blue), boiled for 2 min and centrifuged at 12,000 x g for 5 min. A 35 µl aliquot from the generated supernatant was analyzed by sodium-dodecyl-sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Laemmli 1970). The resolving gel portion of the 20 x 20 cm gel system contained 12 % acrylamide/ bis-acrylamide (37.5:1), 0.375 M Tris-HCl pH 8.8, 0.1% (w/v) SDS, 0.033 % ammonium persulphate, and 0.05 % tetramethylethylenediamine (TEMED). The stacking gel portion consisted of 5% acrylamide/bisacrylamide (37.5:1), 0.125 M Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.033 % ammonium persulphate, and 0.05% TEMED. The polypeptides were separated for 15 h at constant 10 mA using a Protean II electrophoresis unit (Bio-Rad. Hercules, CA, USA) and a running buffer containing 25 mM Tris-HCL, 192 mM glycine, and 0.1% SDS. Following electrophoresis, the stacking gel was removed and resolving gel was soaked in fixing solution (40% methanol, 10% glacial acetic acid) for 30 min. Separated polypeptides were then visualized by Coomassie blue staining for 1 hour using 3% perchloric acid, 0.06% Coomassie Brilliant Blue R (Sigma, Aldrich, St Louis, MO, USA). The gel was destained by agitation in 5% acetic acid) for 4 to 8 h.

3.5.5 Estimation of DNA damage using Comet assay

To perform Comet assays (Einset and Collins 2015), seed embryo tissues (20 mg fresh weight) in 0.2 mL cold phosphate-buffered saline (PBS), 50 mM EDTA were transferred to a plastic Petri dish placed on ice. The tissues placed under safe red light were then sliced within 25 s using a scalpel to release nuclei. The suspension containing released nuclei was mixed with 0.2 mL 1% melted (w/v) low melting point agarose (Sigma-Aldrich) in PBS and held at 37°C. A 70 µL aliquot of the nuclei/agarose mixture was transferred to a glass slide on ice and a cover slip was placed over the suspension. After 1 min gel formation, the cover slip was removed and the slide transferred to a 4°C, dark electrophoresis chamber containing cold 0.3 M NaOH, 1 mM Na_2-EDTA solution. The time from the beginning of slicing to transfer of slides to the electrophoresis chamber was 5 min. After at least 20 min cell lysis and DNA unwinding, the slides were subjected to electrophoresis in the dark at 25 V (300 mA) for 15 min at 4°C. Afterwards, the slide was soaked in cold PBS for 10 min to neutralise pH, then dipped in 95% ethanol and dried at room temperature. After staining with 1µg/mL 4′-6-diamidino-2-phenylindole, analysis of the comets was performed using a fluorescence microscope and using the Comet Assay IV image analysis program (Instem Solutions for Life, Stone, Staffordshire, U.K.).

Fifty comets were scored from analysis of five seeds and the degree of DNA damage was expressed as the percentage of DNA in the tail (% tail DNA) with error bars representing 95% confidence levels based on analysis of variance. Data with three replicates were analysed using general linear model in R statistical software (R-Project.org). Means were differentiated using Tukey HSD (Highest Significant Difference) for cultivar. As recommended, significance was also confirmed using the t-test and P values were calculated.

Chapter 4. Results

4.1 Effects of artificial aging on seed viability, germination, and germination rate index

Two cultivars each of oat (CDC Dancer and CDC Minstrel) and barley (CDC Copeland and CDC Kindersley) were artificially aged and their performance during the aging process was assessed by germination tests, vigour, and viability tests. The standard germination test provided germination counts for each day, from which total germination percentage and germination rate index (an indicator of seed vigour) were estimated. Seed viability was assessed by the triphenyl tetrazolium chloride (TZ) assay, which rapidly shows presence or absence of active dehydrogenase enzymes in tissues.

During artificial aging, germination frequencies and seed viability showed a gradual decline for all cultivars (Figs. 4.1, 4.2, 4.3, 4.4). Most of the viability and germination curves were negatively sloped (Figs. 4.1, 4.2, 4.4), with the exception of the seed viability curves for the two barley cultivars, which were slightly sigmoid (Fig. 4.3) and similar to those reported in previous studies on seed aging (Walters et al. 2010). Overall, the decreases in seed viability and germination frequencies varied between the cultivars. Loss of viability was fastest for CDC Dancer (50% viability loss after seven days) and slowest for CDC Copeland (50% viability loss after 13 days). CDC Copeland also showed the slowest decay for germination frequency (50% germination after six days; Fig. 4.4), whereas the three remaining cultivars reached 50% germination already after two-three days of accelerated aging (Figs.4.2, 4.4). A total loss of germination ability was noted within the 10-30 days period for the two oat cultivars, 15-30 days for CDC Kindersley and 25-30 days for CDC Copeland. For all cultivars, the loss of seed viability appeared to occur later than the total loss of germination ability.

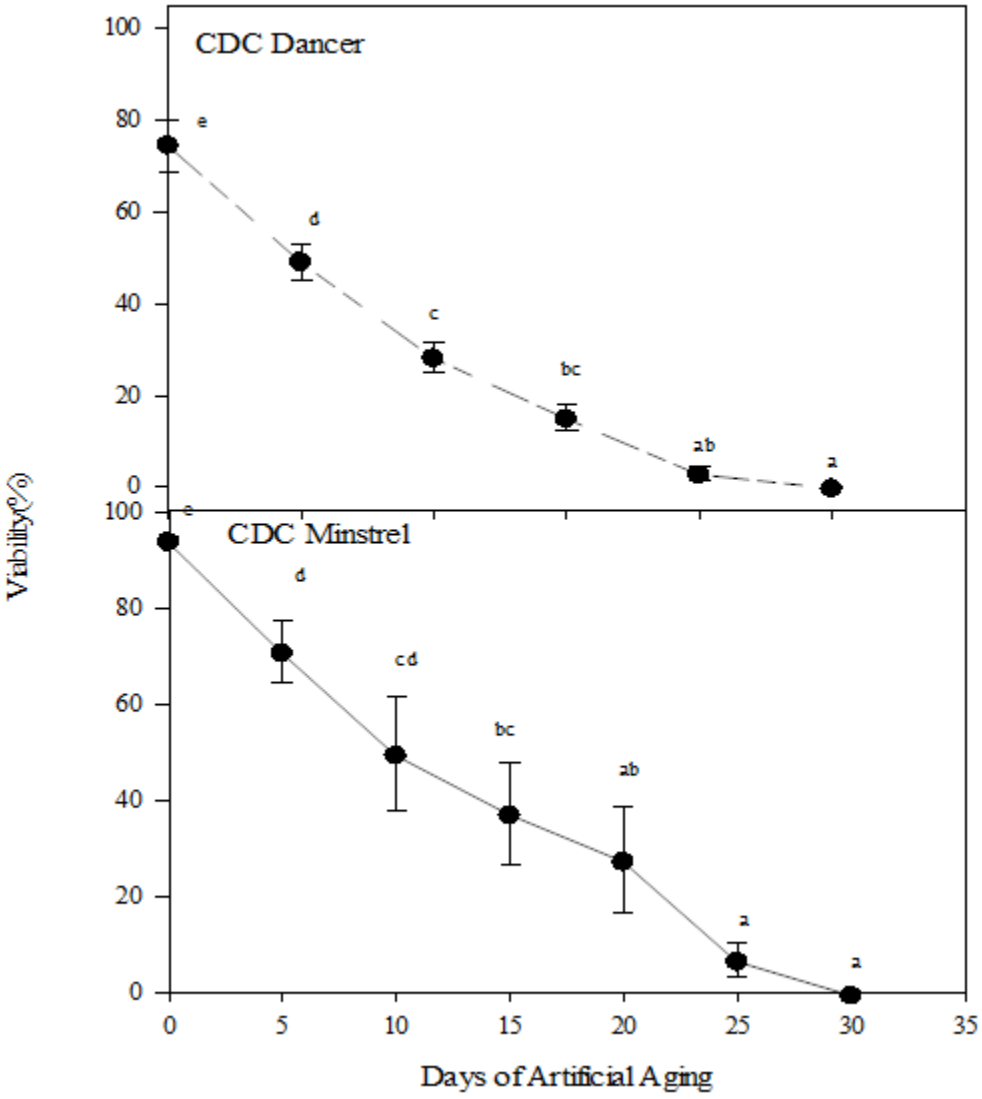

Figure 4.1 Seed viability in two oat cultivars during 30-d artificial aging. Percent viability are mean ± SE where means with the same letter within a graph are not significantly different at $p \leq 0.05$.

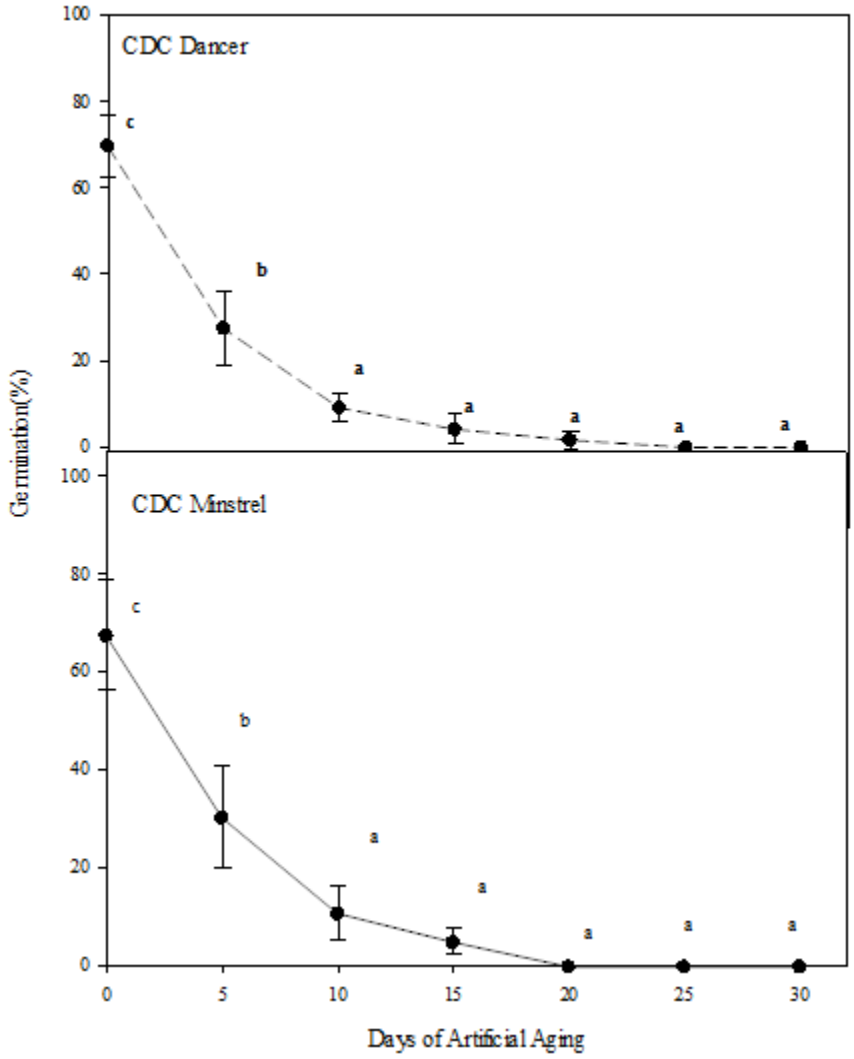

Figure 4.2 Germination frequencies for two oat cultivars during 30-d artificial aging. Germination frequencies are mean ± SE where means with the same letter within a graph are not significantly different at $p \leq 0.05$.

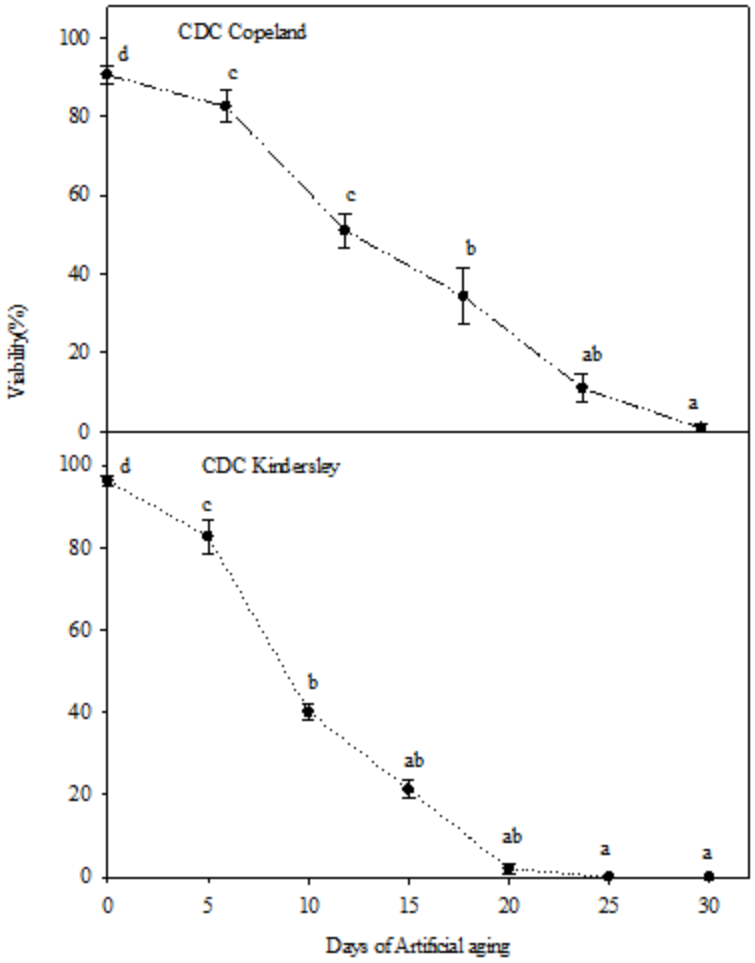

Figure 4. 3 Seed viability in two barley cultivars during 30-d artificial aging. Percent viability are mean ± SE where means with the same letter within a graph are not significantly different at $p \leq 0.05$.

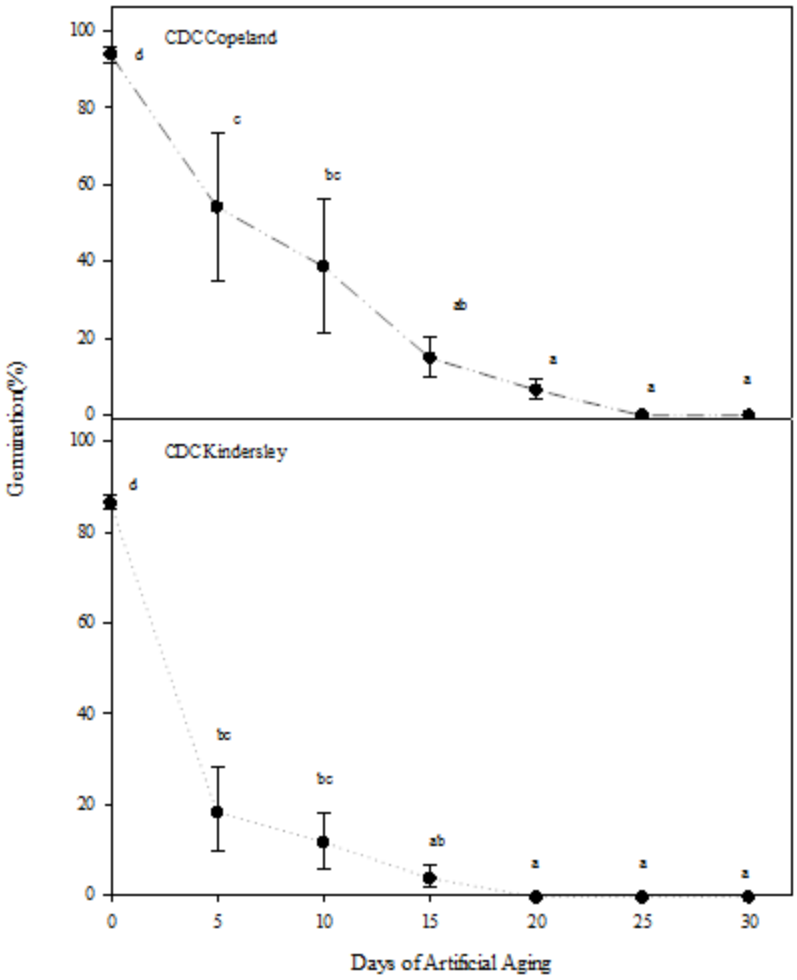

Figure 4. 4. Germination frequencies for two oat cultivars during 30-d artificial aging. Germination frequencies are mean ± SE where means with the same letter within a graph are not significantly different at $p \leq 0.05$.

Seed vigour measured by germination rate index (GRI) was low (about 0.4) for the two oat cultivars at the start of the accelerated aging incubation step at 45°C (day 0; Table 4.1), suggesting pre-treatment of these seeds at high temperature (45°C) and high moisture levels (60% RH) had negatively affected vigour for these seeds. For the barley cultivars, initial GRI indexes were

notably higher (0.8-0.9; day 0 in Table 4.1) supporting a higher resistance to the pre-treatment than oat seeds. Nevertheless, the GRI scores for the two barley lines started a drastic decline when the pre-treated seeds were exposed to the accelerated aging treatment at 45°C. Only after five days of 45°C exposure, the CDC Kindersley GRI score had declined from 0.82 to 0.26 (68% reduction; Table 4.1) and the GRI score for CDC Copeland had decreased from 0.92 to 0.58 (37% reduction; Table 4.1). Five days of accelerated aging at 45°C, a slower decline in GRI scores was noted for CDC Dancer (0.37 to 0.31; 16% reduction), whereas the GRI score declined from 0.42 to 0.25 (40% reduction) for CDC Minstrel, which was similar to that of CDC Kindersley (Table 4.1). Eventually, the aging process caused total loss of seed vigour for all cultivars within the 30-day time span of the experiment. The slowest loss of vigour was noted for CDC Copeland, which showed the slowest decline in germination frequency (Fig. 4.4) and viability (Fig. 4.3) and the highest vigour upon exposure to the pre-treatment conditions at 45°C, 60% RH (Table 4.1). Thus, the experiments suggest that the seed incubation conditions had very different effects on seed vigour for the various seed sources.

4.2 Reversing effects aging with CAT™ treatments on aged seeds

A positive concentration-specific effect of CAT™ on seed germination was found in seeds aged for eight days, but the enhancing effect was more prominent for the two oat cultivars than for the two barley cultivars (Table 4.2). Highest increase in germination frequency was noted for CDC Dancer, for which 50 to 100 % CAT™ enhanced germination by more than two-fold (Table 4.2). Germination for aged CDC Minstrel seeds also benefitted from the treatment, but at an optimal concentration of 6% CAT™. CDC Copeland germination was also slightly enhanced (about 1.4-fold) by 6% CAT™ treatment, but CDC Kindersley was not significantly affected by any of the

CAT™ concentrations. Thus, positive effects from treatment of aged seeds with CAT™ was not found to general but depended on the individual seed source.

Table 4.1 Effect of 30-day artificial aging treatments on seed germination rate in oat and barley cultivars.

Days of Aging	Germination Rate Index (Day^{-1})			
	Oat		Barley	
	CDC Dancer	CDC Minstrel	CDC Copeland	CDC Kindersley
0	0.37 ± .04a	0.42 ± .04a	0.92 ± .02a	0.82 ± .05a
5	0.31 ± .03a	0.25 ± .01ab	0.58 ± .08b	0.26 ± .08b
10	0.16 ± .05b	0.29 ± .06a	0.33 ± .06c	0.26 ± .08b
15	0.13 ± .04bc	0.08 ± .04c	0.41 ± .07bc	0.28 ± .12b
20	0c	0.09 ± .06bc	0.35 ± .06bc	0b
25	0c	0c	0.03 ± .03d	0b
30	0c	0c	0d	0b

Data are mean ± SE. Means with the same letters within a collection are not significantly different at $p \leq 0.05$.

Table 4.2 Effects of catalytic seed treatment on seed germination frequencies.

Concentration of CAT™ (%)	Germination frequency (%)			
	CDC Dancer (oat)	CDC Minstrel (oat)	CDC Copeland (barley)	CDC Kindersley (barley)
0	12 ± 1cd	4 ± 1b	59 ± 2bc	19 ± 2ab
3	16 ± 2cd	4 ± 1b	57 ± 1bc	18 ± 2ab
6	19 ± 1bc	14 ± 2a	78 ± 1a	21 ± 1a
12.5	15 ± 1cd	6 ± 1b	59 ± 2bc	14 ± 1b
25	12 ± 2d	5 ± 1b	52 ± 2c	20 ± 2ab
50	33 ± 2a	6 ± 1b	62 ± 1b	18 ± 1ab
100	24 ± 1b	5 ± 1b	55 ± 3bc	21 ± 1ab

Treatment was done on seeds aged for eight days. Data are mean ± SE. Means with the same letters within a collection are not significantly different at $p \leq 0.05$.

Although seed treatment with CAT™ caused a significant increase in germination frequencies for three of the four cultivars tested, its effect on seed vigour measured by the germination rate was mostly not significant (Table 4.3).

Table 4.3 Effect of CAT™ seed treatment on seed germination rate.

Concentration of CAT™ (%)	Germination Rate Index (Day^{-1})			
	CDC Dancer (oat)	CDC Minstrel (oat)	CDC Copeland (barley)	CDC Kindersley (barley)
0	0.24 ± .02bc	0.24 ±. 07a	0.59 ± .01a	0.55 ± .03a
3	0.27 ± .02ac	0.19 ± .04a	0.58 ± .02a	0.60 ± .06a
6	0.29 ± .01ac	0.28 ± .01a	0.60 ± .03a	0.51 ± .03a
12.5	0.26 ± .02ac	0.27 ± .05a	0.54 ± .02a	0.55 ± .04a
25	0.23 ± .04c	0.36 ± .09a	0.55 ± .02a	0.52 ± .04a
50	0.36 ± .04a	0.29 ± .03a	0.55 ± .03a	0.54 ± .05a
100	0.35 ± .03b	0.37 ± .06a	0.53 ± .03a	0.57 ± .03a

Data are mean ± SE. Means with the same letters within a collection are not significantly different at $p \leq 0.05$.

4.3 Effect of artificial aging on biomolecules

4.4.1 Changes in seed carbohydrates

4.4.1.1 Total starch

All living cells require energy in the form of ATP and/or GTP. The energy produced in seeds is primarily derived from stored carbohydrates such as starch in the endosperm, but also from breakdown of proteins and lipids available in the embryo and endosperm. The abundant starch component in oat and barley seeds is easily broken down by hydrolysis to glucose in the simplest form to provide energy through the Krebs cycle (Alberts et al. 2002). To determine if artificial aging of oat and barley seeds involves any significant amount of starch hydrolysis, the percentage of total available starch in 15 days aged seeds were compared to unaged seeds. The experiment showed a significant reduced total starch percentage in seeds aged for 15 days as compared to unaged seeds, with the exception of CDC Kindersley seeds. For this cultivar, the measured total starch content increased from 48% to 51% upon 15 days aging; however, the increase was not statistically significant. For the other barley cultivar, CDC Copeland, the measured starch concentration declined slightly from 51% to 49% upon aging.

The starch content in unaged oat seeds was lower than in the two malting barley seeds, but aging showed a higher impact on starch concentration in oat than in barley. For CDC Dancer, unaged seeds had a total starch percentage of 40%, that was reduced by five percentage points after 15

days of aging. In CDC Minstrel, the starting starch concentration was 38% and after 15 of aging, a loss of 10% was observed.

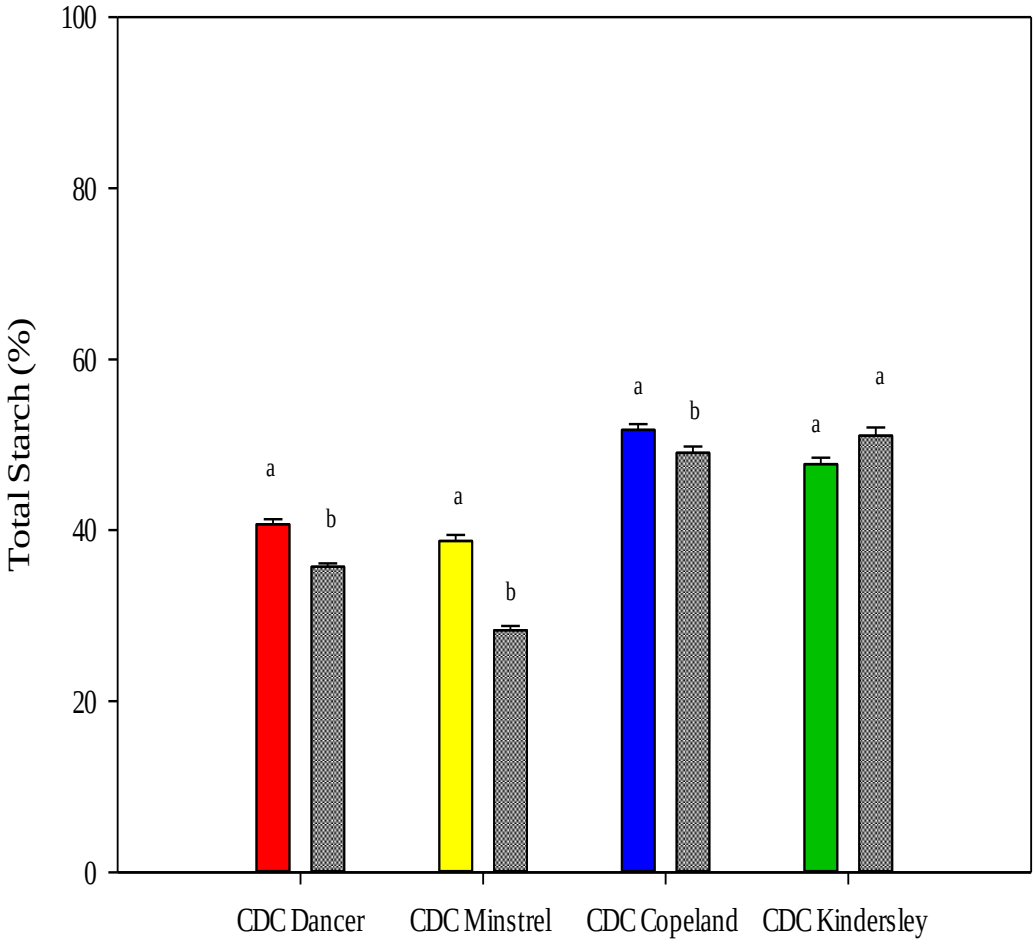

Figure 4.5 Effects of 15 days of aging on total starch concentration in barley and oat seeds. For each cultivar, the unaged seeds are represented by the left bar and aged seeds by the right bar. Data are Means ± SE. Means with the same letters within a collection and parameters are not significantly different at $p \leq 0.05$.

4.4.2 Soluble Sugars

4.4.2.1 Glucose

The two barley cultivars showed consistent higher glucose concentrations in seeds than the two oat cultivars during the aging process. A slight increase in seed glucose concentrations was noted for the two barley cultivars during the first three days of aging, but it was followed by a slight decline. Of the two oat cultivars, CDC Minstrel showed up to two-fold higher glucose concentration in seeds than CDC Dancer. Although slight, but significant, increase in glucose concentrations was observed for CDC Dancer at the end of the aging process. No such increase was noted for CDC Minstrel.

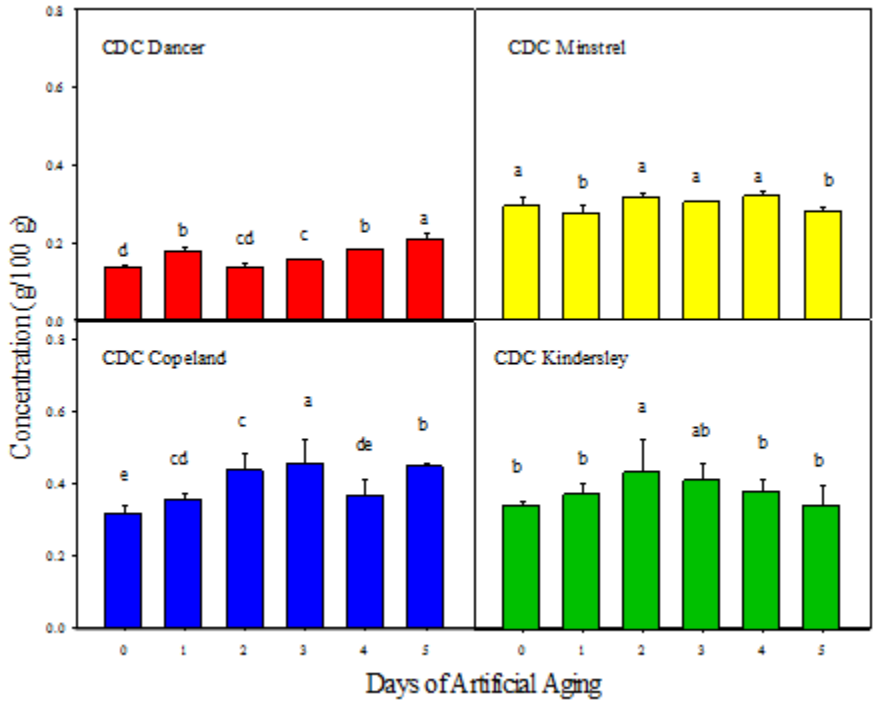

Figure 4.6 Glucose concentration in barley and oat seeds during five days of aging. Data are mean ± SE. Means with the same letters within a collection and parameters are not significantly different at $p \leq 0.05$.

4.4.2.2 Fructose

The fructose concentration in seeds from the two barley cultivars was about two-fold higher than that seen in the two oat seed sources during aging (Fig. 4.7). Aging caused the fructose concentration in the barley seeds to rise slightly higher. For CDC Copeland, the fructose increase appeared multi-phased with a first peak at three days, followed by a decline, and a second increase at the end of the aging process. A slight fructose increase was also noted for the seeds of CDC Dancer during aging, but no such increase was noted for CDC Minstrel, which overall showed higher fructose content than CDC Dancer.

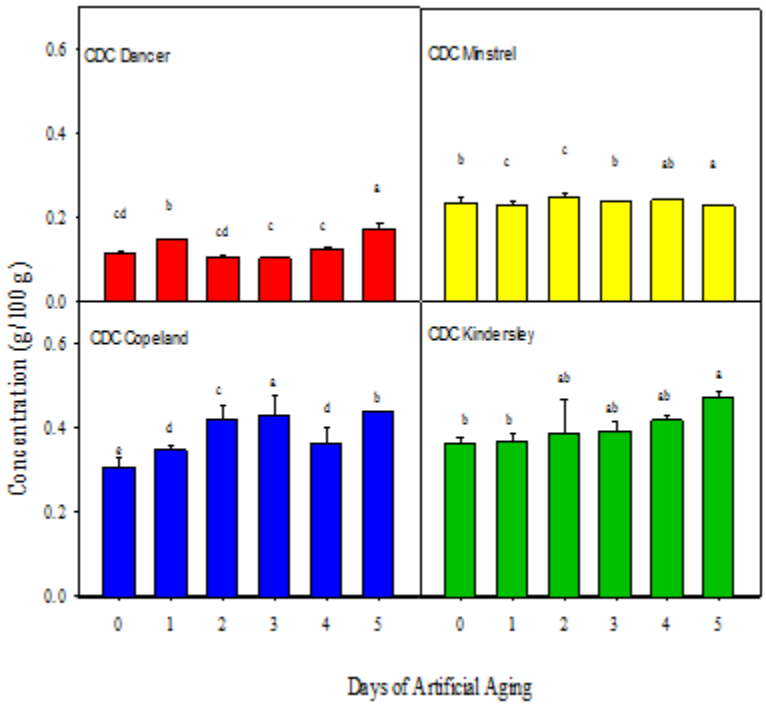

Figure 4.7 Fructose concentration during five days of ageing in oat and barley cultivars. Data are mean ± SE. Means with the same letters within a collection and parameters are not significantly different at $p \leq 0.05$

4.4.2.3 Sucrose

The sucrose concentrations measured for the oat and barley seeds were similar (about 8-10 g/100g) and only small changes were noted during aging (Fig. 4.8). For the oat cultivar CDC Dancer, the sucrose concentration in seeds decreased slightly during the five days of artificial ageing. In contrast, the sucrose concentration CDC Minstrel seeds remained, like the fructose concentration, relatively constant during aging. For the barley CDC Copeland seeds, the sucrose concentration displayed very similar pattern of increase as noted for the fructose concentration in the same seeds during aging. In CDC Kindersley seeds, the sucrose concentration decreased with aging.

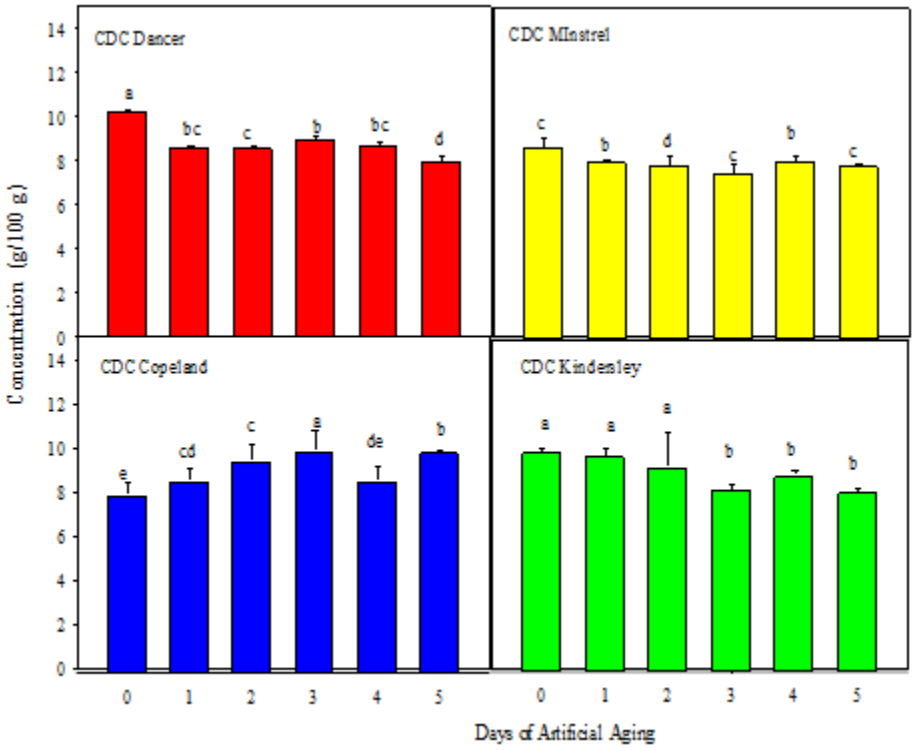

Figure 4.8 Sucrose concentration during five days of ageing in oat and barley cultivars. Data are mean ± SE. Means with the same letters within a collection and parameters are not significantly different at $p \leq 0.05$

4.4.2.4 Myo–Inositol

For the oat seeds, the initial myo-inositol concentrations were about three-fold higher than for the barley seeds (Fig. 4.9). Both oat cultivars showed decreased myo-inositol concentrations as the five days of aging progressed. In barley, the myo-inositol concentrations showed a different pattern, where seeds of both cultivars demonstrated increased myo-inositol concentrations during the first two (CDC Copeland) or three (CDC Kindersley) days, followed by decrease.

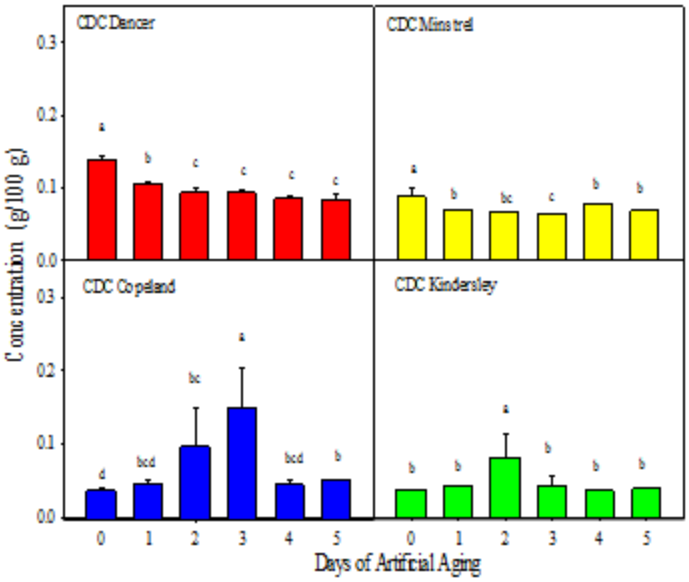

Figure 4.9 Myoinositol concentration during five days of ageing in oat and barley cultivars. Data are mean ± SE. Means with the same letters within a collection and parameters are not significantly different at $p \leq 0.05$

4.4.2.5 Galactinol

At the start of the five days aging, the galactinol concentration was highest in seeds of the two oat cultivars, but it declined with aging (Fig. 4.10). In contrast, the galactinol concentrations showed

a similar pattern to myo-inositol, where an increase was seen during the first two to three days, and thereafter a decline. CDC Copeland seeds showed a two-fold higher galactinol concentration throughout the aging process as compared to CDC Kindersley seeds.

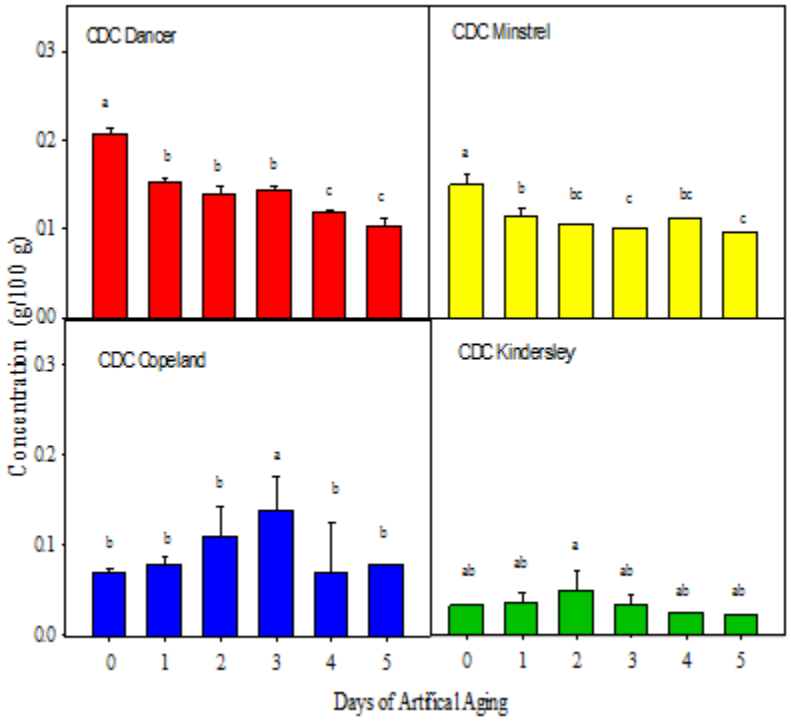

Figure 4.10 Galactinol concentration during five days of ageing in oat and barley cultivars. Data are mean ± SE. Means with the same letters within a collection and parameters are not significantly different at $p \leq 0.05$.

4.4.2.6 Raffinose

The raffinose concentration was found to be more than two-fold higher in CDC Copeland seeds throughout the aging process as compared to the other three seed sources (Fig. 4.11). Aging further increased the raffinose concentration for CDC Copeland. Oat CDC Minstrel also showed an increased raffinose content when aged, but the raffinose concentration remained relatively constant in the oat CDC Dancer and barley CDC Kindersley seeds.

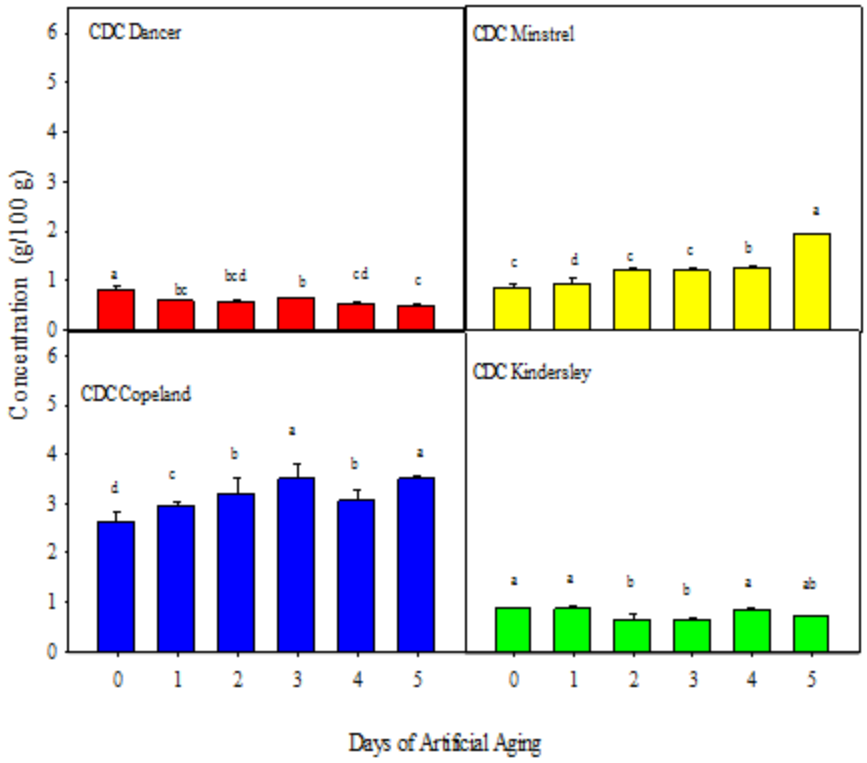

Figure 4.11 Raffinose concentration during five days of ageing in oat and barley cultivars. Data are mean ± SE. Means with the same letters within a collection and parameters are not significantly different at $p \leq 0.05$.

4.4.2.7 Stachyose

Similar to raffinose concentration, the stachyose concentration in aged seeds of CDC Copeland was more than two-fold higher than that observed in seeds of the three other seed sources (Fig. 4.12). Only the CDC Copeland seeds showed a clear increase in stachyose content during aging. For the remaining three cultivars, the raffinose concentration in seeds was relatively constant with only a slight decrease noted for CDC Dancer.

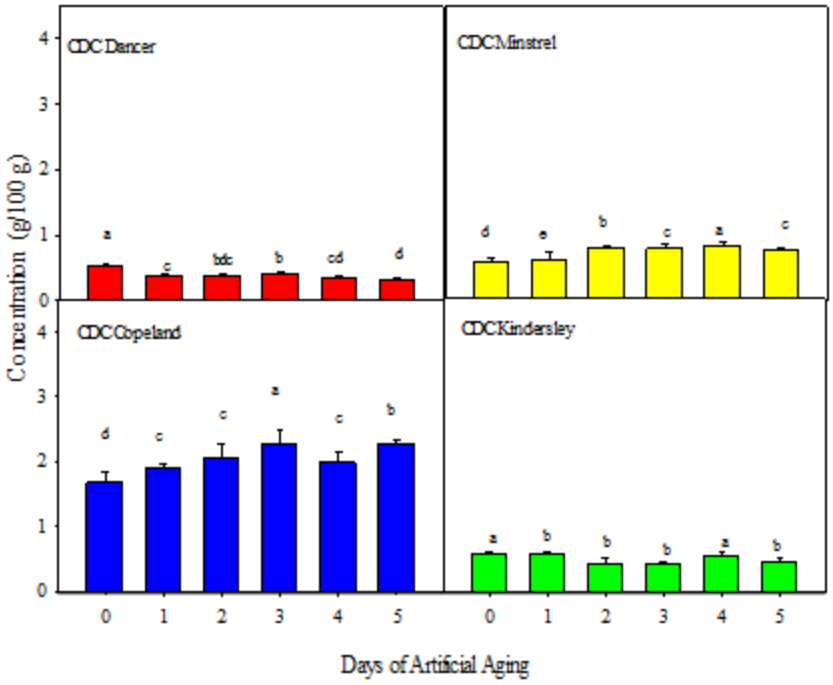

Figure 4.12 Stachyose concentration during five days of ageing in oat and barley cultivars. Data are mean ± SE. Means with the same letters within a collection and parameters are not significantly different at $p \leq 0.05$.

4.4.2.8 Verbascose

The verbascose concentration in barley seeds were below the limit of detection by HPLC analysis (Gangola et al. 2014), whereas oat seeds contained detectable amounts (Fig. 4.13). The CDC Dancer seeds showed a gradual decrease for verbascose with ageing, where the decrease was most notable early in the aging process. No major change in verbascose concentration was noted for CDC Mistrel seeds.

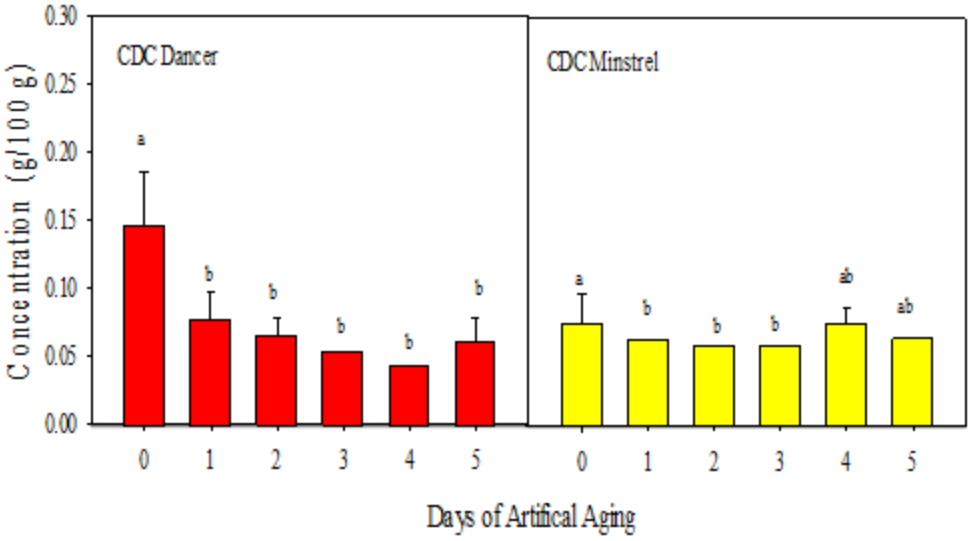

Figure 4.13 Verbascose concentration during five days of ageing in two oat cultivars. Data are mean ± SE. Means with the same letters within a collection and parameters are not significantly different at $p \leq 0.05$.

4.4.3 Ratio of Raffinose to Sucrose

The ratio between RFO and sucrose is suggested to affect the process of seed aging (Koster and Leopold, 1988; Blochl et al 2008) and was determined for the genotypes in this study. The barley CDC Copeland seeds showed the highest raffinose/sucrose ratios, which increased from 0.32 to 0.36 during the five days accelerated aging process. Ratios for the other three seeds sources were around 0.1, with a clear increase during aging noted for oat CDC Minstrel seeds, a slight decrease for CDC Dancer seeds and no significant change for CDC Kindersley seeds. Overall, the different changes in raffinose/sucrose ratios did not conclusive explain the consistent decline in germination frequencies for all cultivars as aging progressed. However, the high raffinose/sucrose ratio for CDC Copeland may have slowed down the aging process and contributed to a lower reduction in germination frequency seen for this cultivar during the five-day aging period.

Table 4.4 Effect of aging on germination and ratio of raffinose to sucrose in oat cultivars.

	CDC Dancer		CDC Minstrel	
Day	Ratio	Germination (%)	Ratio	Germination (%)
0	0.08a	74 ± 3a	0.11c	63 ± 2b
1	0.07b	42 ± 3bc	0.1d	72 ± 1b
2	0.07b	47 ± 4b	0.16a	91 ± 3a
3	0.07b	36 ± 3c	0.16a	79 ± 2b
4	0.06b	37 ± 2c	0.16a	51 ± 2c
5	0.06b	12 ± 1d	0.15b	26 ± 4d

Data are means ± SE. Means with the same letters within a collection are not significantly different at $p \leq 0.05$.

Table 4.5 Effect of aging on germination and ratio of raffinose to sucrose in barley cultivars.

	CDC Copeland		CDC Kindersley	
Day	Ratio	Germination (%)	Ratio	Germination (%)
0	0.32d	96 ± 1a	0.09b	70 ± 2a
1	0.35b	91 ± 2a	0.09b	21 ± 3b
2	0.33c	80 ± 4b	0.07d	13 ± 3c
3	0.36a	77 ± 2b	0.08c	8 ± 1d
4	0.36a	78 ± 1b	0.1a	3de
5	0.36a	58 ± 4.4c	0.09b	0e

Data are mean ± SE. Means with the same letters within a collection are not significantly different at $p \leq 0.05$.

To study if the amount of sucrose and raffinose affected seed germination and/or viability over a longer timespan than five days, the sucrose and raffinose concentrations were determined after 15 days of ageing. The relatively high raffinose concentration and high raffinose/sucrose ratio in CDC Copeland seeds observed during the five days long aging (Table 4.5) were maintained after 15 days aging (Table 4.6). However, the germination frequency for CDC Copeland seeds dropped from 58% at five days aging (Table 4.5) to only 4% in 15-day aged seeds (Table 4.6). Thus, high

raffinose/sucrose ratio may not have been sufficient to reduce seeds vitality losses when aging was is prolonged for a longer time. In contrast, CDC Minstrel seeds, , were more resistant to the longer aging process and showed 11% germination frequency after 15 days. In summary, CDC Copeland seeds managed aging better in the short term, but oat CDC Minstrel seemed to have more endurance to combat aging in the long term. These differences could not be explained solely by changes in the oligosaccharide reserves during the aging process.

Table 4.6 Effect of 15 d of aging on sucrose and raffinose concentration and their ratio on seed viability and germination in barley and oat cultivars.

Cultivar	Sucrose	Raffinose	Ratio Raffinose/Sucrose	Viability	Germination
CDC Dancer Unaged	4.2	0.4	0.10a	93a	44a
CDC Dancer Aged	3.1	0.2	0.06b	39b	0b
CDC Minstrel Unaged	4.0	0.6	0.14a	93a	56a
CDC Minstrel Aged	3.4	0.4	0.13b	73b	11b
CDC Copeland Unaged	3.9	1.2	0.3a	99a	96a
CDC Copeland Aged	3.2	1.0	0.3a	65b	4b
CDC Kindersley Unaged	4.9	0.4	0.08a	99a	87a
CDC Kindersley Aged	2.7	0.1	0.05b	27b	0b

Data are mean ± SE. Means with the same letters within a collection are not significantly different at $p \leq 0.05$.

4.5.1 Changes in storage proteins

To study any major changes in storage proteins, the seed polypeptides were extracted from unaged and 15-days aged seeds and analyzed by SDS-PAGE. Visualization of the extracted and gel separated polypeptides by Coomassie blue staining showed, as expected, different polypeptide profiles between oat and barley cultivars (Fig. 4.14). No notable differences in these patterns were

observed upon comparison of polypeptide from unaged and aged seeds of each cultivar. Thus, the accelerated aging for 15 days did not lead to any large breakdowns of storage proteins in seeds.

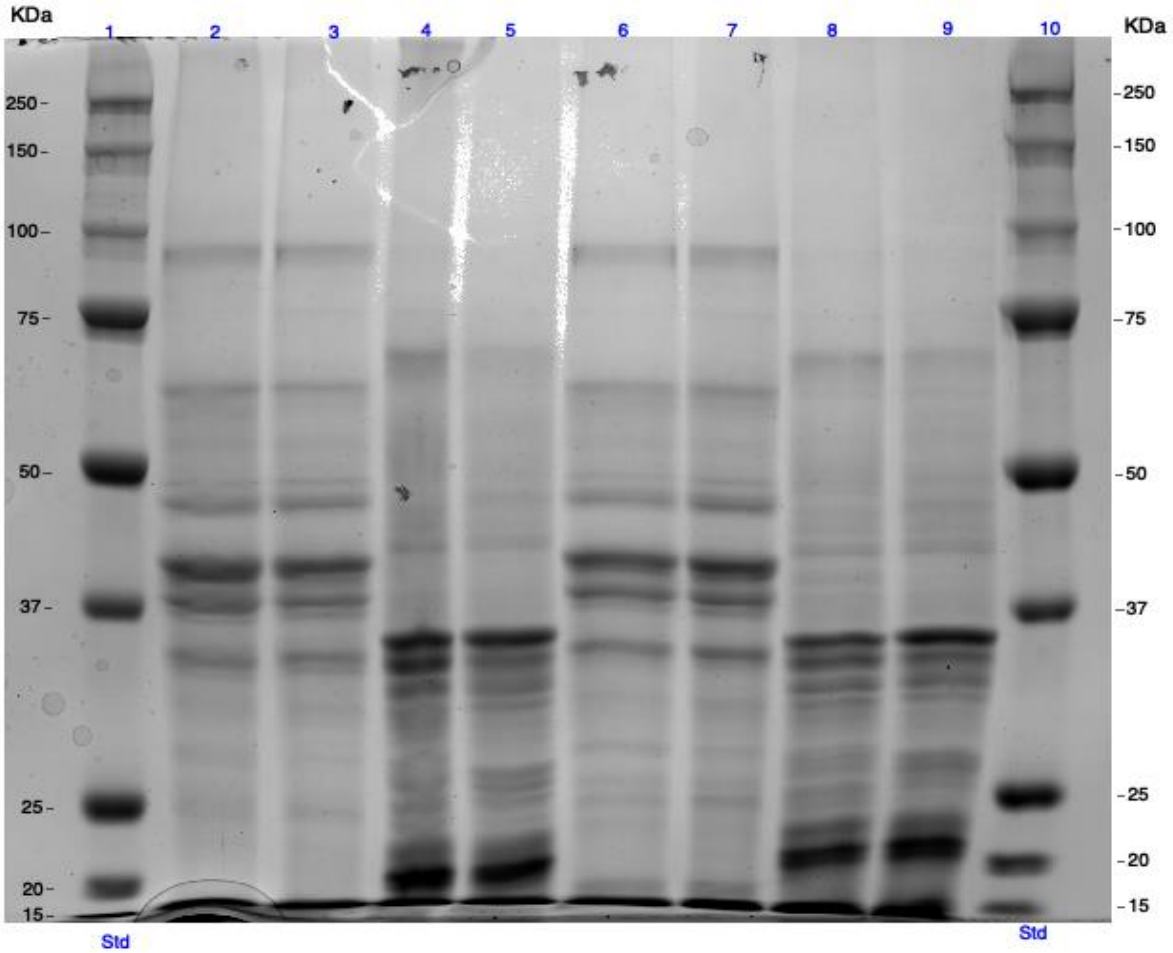

Figure 4.14 SDS-PAGE analysis of oat and barley seed proteins. Visualization of gel-separated seed storage proteins by Coomassie blue staining.

Analyzed samples were extracted from unaged and aged oat and barley seeds, respectively. Migration of standard proteins (St) is indicated to the left. Lane 1 & 10 are molecular weight standards, Lane 2, 3, 4 & 5 represent unaged seeds of CDC Dancer, CDC Minstrel, CDC Copeland and CDC Kindersley, respectively. Lanes 6, 7, 8 and 9 represent aged seeds of CDC Dancer, CDC Minstrel, CDC Copeland and CDC Kindersley.

4.6.1 Comet Assay for DNA status

To study if there are any major changes in the DNA integrity during seed ageing, crude nuclei extract from unaged and aged seeds were analyzed by the Comet assay (Einset and Collins 2015). In this analysis, the intactness of DNA is determined by the distance between the head (green line; undamaged DNA) and tail (blue line; damaged DNA) regions of the comet (Fig. 4.15). The analysis of both oat and barley seeds showed the tail region, indicated by area between the green line and the tail end of the comet, was significantly larger in eight days aged seeds as compared to non-aged seeds. To quantify the differences, the two areas of the comets were measured and showed the increase in the tail was several folds. (Table 4.7). The analysis showed major changes in the DNA structure and integrity occurred during seed ageing.

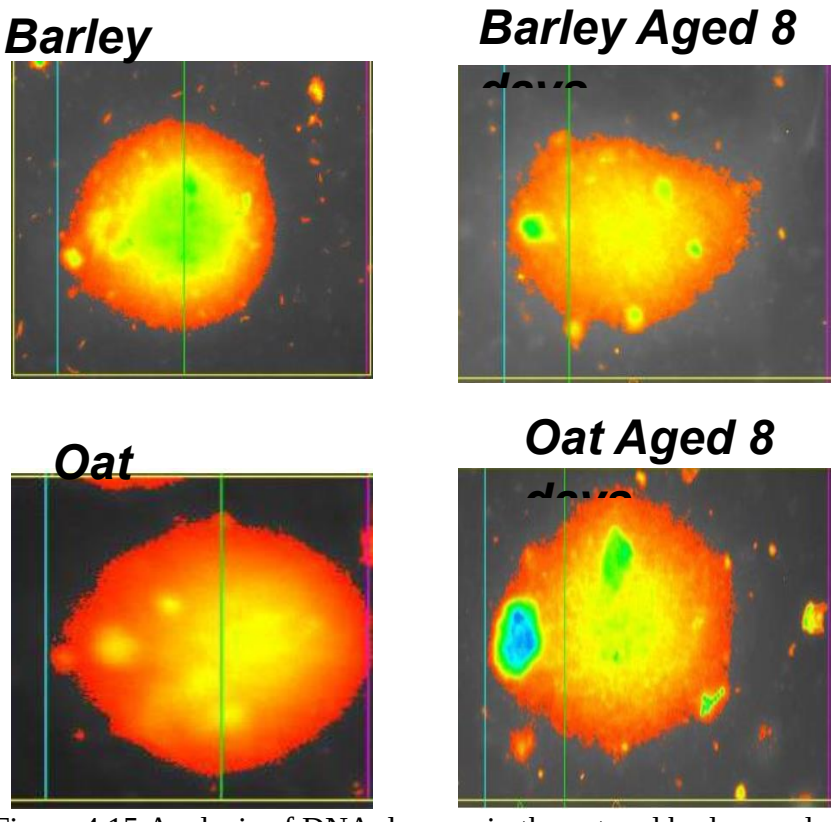

Figure 4.15 Analysis of DNA damage in the oat and barley seeds upon aging. Fluorescent image of comets produced from analysis of unaged and aged seeds.

Table 4.7 Comet Assay to show the increase in the tail region during seed ageing in oat and barley.

	Comet Assay Score	
	Score	Standard error
Barley unaged seeds	3.5% DNA tail	+/- 0.9
Barley aged seeds	62.0% DNA tail	+/- 3.4
Oat unaged seeds	2.4% DNA tail	+/- 0.7
Oat aged seeds	53% DNA tail	+/- 6.7

Chapter 5. Discussion, conclusions and future implications

5.1 Artificial aging and seed performance

Seed longevity is not assured under the most optimal conditions and seeds aging causes significant loss of seed viability and vigour (Walters et al. 2005; Kong et al. 2014). Seeds in storage are continuously subjected to oxidative stress (e.g. ROS) acting on cell constituents such as proteins, lipids, nucleic acids, sugars, etc. through processes such as lipid peroxidation (Wilson and McDonald 1986; Galletti et al. 1995; Harman and Mattick 1976), Amadori and Maillard reactions (Murthy and Sun 2000) and protein carbonylation (Arc et al. 2011). To combat the stresses, dry seeds are fortified with protection mechanisms such as glassy state transformation, RFOs, antioxidant enzymes, heat shock and LEA proteins (Koster and Leopold 1988; Nishizawa et al. 2008). Lipid peroxidation and accumulation of ROS are considered to be major contributors to seed deterioration (Mittler et al. 2004). The balance between destructive and protective mechanisms under a particular environmental condition will ultimately determine the seed's lifespan.

Rapid loss of seed viability and vigour during artificial aging has been reported for many species (Walters 1998, McDonald 1999; Arc et al. 2011; Groot et al. 2012) and is generally associated with depletion or chemical modification of storage reserves (Lehner et al. 2008). A gradual decline of both seed viability and germination followed by a phase of rapid deterioration is often seen during seed aging and defined by a sigmoid pattern (Walters et al. 2010). This type of seed deterioration was indicated for the barley seeds in this study, (Fig. 4.3), but not observed for the oat seeds (Fig. 4.1), suggesting seed decline progressed differently in the two species.

Seed treatments to enhance germination are widely used and the positive effects are associated with a wide range of metabolic and physiological improvements (Ashraf and Foolad 2005, Catusse et al. 2008). Activation of protective enzymes, such as are superoxide dismutase (SOD), glutathione reductase (GR), catalase (CAT) and peroxidases and accumulating osmoprotectants, such as proline, soluble sugar and soluble protein are the typical stress-avoidance responses (Masoudi-Sadaghiani et al. 2011). Activation of enzymatic antioxidants can reduce ROS-induced oxidative damages. However, treating aged seeds to improve germination of the portion of seeds that are viable but otherwise would not germinate has not been previously reported. The fact that seed viability percentage was consistently greater than germination percentage after aging (Figs. 4.1-4.4) indicates that there is a delaying effect of aging on viability loss, and treatments such as CAT™ may reverse such damaging effects. Positive effects of CAT application were achieved in our study, with the exception of CDC Kindersley seeds (Table 4.2). The reason for lack of response for CDC Kinderley seeds may be the timing of CAT™ treatment, which was done on seeds aged for eight days. At this stage, the CDC Kindersley seeds had reached the rapid deterioration stage for viability (Fig. 4.3) and thus, are on the road of no recovery as suggested by the extensive DNA damage at this stage (Fig. 4.10; Table 4.7). It would be interesting to test the effect of CAT application on seeds aged for different time periods.

5.2 Carbohydrates

This study showed seeds with higher oligosaccharide (raffinose) to sucrose contents most of the time had a significantly higher germination potential when compared to other cultivars in the same treatment or condition (Table 4.2, Table 4.3). This concurs with earlier work on maize, pea and rape seeds suggesting soluble sugars, mainly the raffinose family oligosaccharides (RFOs), are also involved in the fortification of seeds against the deleterious effects happening during

dehydration and/or ageing (Obendorf 2008). This protection includes scavenging of ROS, which is thought to be associated with seed storage potential (Priestley 1986; Bailly 2004). Thus, it can be suggested that CDC Copeland seeds have better coping mechanisms to deal with the physiological damage caused during artificial aging due to the higher RFO content and higher RFO/sucrose ratio in seeds (Table 4.5). However, this coping mechanism did not seem to be long-lasting for CDC Copeland, as the seeds rapidly deteriorated when the artificial aging was done for a longer time (15 days aging; Table 4.6).

Additionally, other authors (Bernal-Lugo and Leopold 1992; Horbowicz and Obendorf 1994; Steadman et al. 1996; Sinniah 1998; Piotrowicz-Cieślak 2005) have shown that sugar content, predominantly the ratio of oligosaccharide to sucrose contents, might be used as a gauge of seed storage potential or ageing. A study in maize showed that a RFO/sucrose ratio higher than 0.2 predicts a good storage potential (Bernal-Lugo and Leopold 1995). According to this prediction, only CDC Copeland seeds exceeded this threshold in our study (0.3; Table 4.5), suggesting it would have the highest storage potential; this was also shown in the short-term aging experiment (Table 4.5). The positive effects of oligosaccharides may involve stabilization of intracellular glass transition by increasing cytoplasm viscosity and the glass-to-liquid transition temperature, which normally slows down the ageing process (Leopold et al. 1994; Bernal-Lugo and Leopold 1995).

In our study, a decrease in total starch was observed in all aged cultivars except CDC Kindersley (Figure 4.1). Starch is a major energy source during germination for most seeds (Zhao et al. 2018), but the amount of starch utilization during germination varies with species and this could be due to soluble sugars being available in sufficient amounts. Results from (Zhao et al. 2018) showed that from the dry seed to highest germination, the starch content of the starchy seeds decreased by a small margin due to the relative abundance of starch, while that of the fatty seeds decreased more

significantly. Thus, it is likely the great amount of starch in the barley seeds (Fig. 4.5) made it difficult to measure with accuracy the small amount of starch that was actually hydrolyzed in our experiments.

5.3 Proteins

In this study, all the major seed storage polypeptides were present in both the aged and unaged cultivars, possibly due to the assertion that seed storage proteins do not carry out any enzymatic functions (Krishnan and Coe, 2001). In addition, the SDS-PAGE followed by Coomassie blue staining would only detect major changes in polypeptide profiles. There may have been minor changes which could not be detected using the one-dimensional SDS-PAGE analysis method. Soluble proteins are utilized for a large proportion from imbibition to the highest germination and even the early seedling stage irrespective of the relative percentage of starch, protein, and fat in dry seeds (Zhao et al. 2018). Some components of seed reserves may promote the transition from the quiescent to the active state during germination (Catusse et al., 2008), which is certainly important and not concentration dependent.

5.4 DNA

The comet assay in this study was done to study if seed aging was associated with increased DNA damage. Evidently, the comet assay showed aged seeds had a larger tail percentage as compared to unaged aged (Fig. 4.4). As the percent head DNA usually represent the amount of healthy DNA available, whereas percent tail correlates to amount damaged DNA, the comet assays clearly demonstrated that seed aging caused increased DNA damage. It was evident that eight days of artificial ageing had a very significant impact on the DNA status in seeds. The extent of the DNA damage at this stage may have been well beyond repair by any DNA repair systems in cells or rescue by a CAT^{TM} treatment.

5.5 Seed variability

Based on the evidence gathered in this study, a wide variation is seen among the species and also within the species (cultivars). This could be attributed to the fact that some cultivars e.g. CDC Copeland had better adaptive mechanisms to deal with the physiological damages induced by short-term artificial aging when compared to the other cultivars. The multiple factors involved in seed longevity (Clerkx et al. 2004) are evident in this study as observed results showed an array of differences between cultivars of the same species for each measured trait. The various physiological states are exhibited by the number of days of artificial aging and the obtained results. Generally, the longer seeds were aged, the more physiologically impaired they were. Longer aged seeds suffered more physiological damage as seen in the decrease in soluble sugars and total starch. Also, the comet assay showed a wide difference between aged seed and unaged seeds. All this evidence improves the general understanding of seed behaviour during aging. The physiology and internal adaption to stress by aging is unique. Further insights into these mechanisms will be key to improving our understanding of seed aging.

5.5 General discussion

From the present study, artificial aging was observed to cause a significant change in the viability and germination of seeds as time progressed in all the experiments. This finding was consistent with reports by (Pradhan and Badola 2012) in their study on the effect of storage period and storage conditions on seed germination in eleven populations of *Swertia chirayit*. In their study, a multivariate ANOVA showed a significant effect of storage condition and storage period on seed germination and mean germination time ($p < 0.0001$). Moreover, in a study on the effect of artificial aging on wheat quality deterioration during storage, (Tian et al. 2019) concurred with the findings of the present study and reported that seed germination in both wheat cultivars used in

their study almost completely ceased during ten months of storage whereas the contents of free fatty acids and MDA increased significantly. Comparatively, in the present study, ageing affected all physiological parameters examined in all cultivars though all cultivars had lost their germination and viability properties after 30 days. Results from a study by Abbas et al. (2004) on the effect of artificial aging on viability and leachate exudation in Fenugreek seeds agreed with the results in this thesis. In Fenugreek, the germination percentage was reduced up to 58% after seven days of artificial aging as compared to the non-aged control (100%). Similar results were also reported by Walters (1998) in which the cultivars lost more than 50% of total germination following five days of artificial aging (Walters 1998).

A study on the effect of artificial aging on germination components and seedling growth of Basil (*Ociumum basilicm*) seeds by (Farhadi et al. 2012) in Iran found that the percentage and speed of germination, shoot and root length decreased with artificial aging following five to ten days of aging. Meanwhile, in the case of seed viability, a significant decrease was observed after five days of artificial aging. These results were similar to observed results in this study. A report by Da Rosa et al. (2011) concurred with this observation in their study on the effect of storage conditions on coffee seed and seedling quality. In their study, only the germination of seeds harvested at the cherry stage, evaluated before and after the storage at 10°C, was not affected by moisture content, but the seeds lost viability and did not produce suitable seedlings for planting when stored for nine months. The differences among cultivars in terms observed results agreed with findings by Rodo and Marcos Filho (2003) in which seed viability and integrity was higher in certain species when compared to others during artificial treatment.

Notwithstanding all the protective mechanisms, seeds accumulate some form cellular damage during aging. Upon imbibition, the cytoplasm of seed cells is transformed from a glassy to a fluid

state and this activates cellular metabolism (Rajjou and Debeaujon 2008). In this state, seeds can initiate process that can repair the damage, ultimately improving seed vigour. As reported earlier by (Tilden and West 2008; Khan et al 2016), seed priming treatments can lead to a possible reversal of the detrimental effects of aging which can increase physiological performance (Panuccio et al. 2014). In this study, concentrations of CAT^{TM} produced significant higher germination percentages than in the control seeds for all observed cultivars except CDC Kindersley. The effects of CAT^{TM} was highly specific, various concentrations produced specific effects on germinations. Further investigation could lead to the exact mechanism of how this treatment "repairs" damage done by aging. This result supports the hypothesis that damage done by aging to seeds can be repaired or mitigated.

Soluble sugars such glucose, fructose and sucrose play important roles in the storage and germination potential of seeds (Bernal-Lugo and Leopold 1992). Sucrose and some oligosaccharides play an important role in seeds by stabilizing proteins during stress by helping seeds maintain their physiological functions (Hoekstra et al. 2001). Alterations in soluble carbohydrate contents may possibly contribute to both the reduction of seed vigour and germination potential (Bernal-Lugo and Leopold 1992). Studies by Petruzzelli and Taranto (1989) showed that soluble carbohydrates in general diminish as seed aging progresses. Aging has also been known to cause the depletion or chemical modification of storage reserves (Lehner et al. 2008), which affects seedling growth (vigour) upon germination.

Buitink et al. (2000) showed that increased cellular viscosity (glassy state) and molecular mobility within the cytoplasm were positively associated with seed longevity. Decreasing temperature and moisture content contributed to the intracellular glass. Seeds in the glass formation favored the replacement of water by oligosaccharides i.e. sucrose and raffinose family oligosaccharides

(RFOs), which distorted normal crystal matrices (Koster and Leopold 1988). RFOs and sucrose have been known to serve as energy sources during seed during germination. The oligosaccharides raffinose and stachyose have been distinguished as very capable inhibitors of sucrose crystallization (Blöchl et al. 2008). Oligosaccharides protect seed cell membrane from damage caused by dehydration and allow lipids to be in a fluid state during the dehydration or desiccation phase when the seed is in the glass state (Crowe et al. 1987). They also safeguard the structure and function of proteins (Imamura et al. 2003) which are susceptible to change. Embryonic proteins are often held stable and physiologically functional even in long term storage due to the action of these sugars. Seeds in the glassy state are protected to an extent from deleterious processes, e.g. Maillard reactions. The glassy formation of the cytoplasm is done by a mix of sugars and late embryogenesis abundant (LEA) proteins. This combination is very hydrophilic and protects seeds against desiccation stress. In this study, especially in CDC Copeland, the ratio of raffinose to sucrose was high and support previous reports that higher oligosaccharide to sucrose ratio in seeds will result in better germination (Koster and Leopold, 1988).

5.6 Conclusions

There have been significant efforts in previous studies to comprehend the mechanisms underlying seed longevity especially the molecular aspects of seed longevity. A general consensus from artificial ageing studies is that it causes physiological damage seeds as evidenced by a decrease in seed germination, viability, and germination rate over time. As seen in this study, the seed deterioration varies greatly between cultivars and species, but also as the aging process progresses. This suggests seed aging is complex and involves factors that are very dynamic. Seed treatments such as CAT™ enhanced germination for three out of four cultivars in this study, but the optimal CAT™ concentration varied between species and cultivars (Table 4.2). Nevertheless, the results

indicated that seed damage due to rapid aging treatments have the potential to be reversed, which may have practical implications in plant germplasms conservation. Selection of seed lots of a particular species for long term storage will be very important for conservation of delicate and rare seeds species in the future. In contrast, analysis of seed storage proteins was found to be of no value for explaining the physiological status of seeds during aging. The seed protein analysis needs to be more focused on seed proteins that are known to be associated with seed aging. Heat shock proteins and LEA proteins shown to have a protective role against aging are good candidates for such an analysis (Kalemba and Pukacka, 2007). The comet assay showed the extent of damage being done at the nuclei acid level. The comet assay promises to provide answers to important questions concerning, for example, background levels of DNA damage in normal cells, the variation in DNA repair capacity within populations, and the regulation of DNA repair at the molecular level within the nucleus. Thus, the comet assay could also be a useful tool for selection of seed lots for storage.

5.7 Future implications

Seed longevity and conservation is an important issue not only for plants but for all living organisms. The issue surrounding conservation and longevity is a complex trait of interlocking factors determined by multiple events. Starting from seed development, storage and environmental conditions, seeds have to use several adaptive mechanisms to be viable and germinate under optimal conditions. In this study, we evaluated some physiological differences in oat and barley seeds. We observed differential results when cultivars of the same species were given the same treatment. All the experimental seeds in this study, generally lost more than 30% viability and germination ability after five days of artificial aging. Upon further investigation, we found out that in certain cultivars, seeds with a higher raffinose to sucrose ratio performed better when they were

artificially aged for a shorter time. This is useful information for long term studies of seed conservation of cereals in particular. However, the scientific question of exact mechanisms still lingers on and the future challenge will be on how to understand multiple factors affecting the biochemical and molecular mechanisms underlying seed ageing. Advances in molecular-based studies will help in the identification of genes contributing to seed longevity. This will provide more insight to our current knowledge of the molecular mechanisms associated with seed aging and longevity. The future challenge, therefore, will be to understand how these multiple factors are integrated and can be used to better preserve germplasm for future use.

Chapter 6. References

AAFC (2020). Agriculture and Agri-Food Canada, Canada Outlook for principal food crops, May 22, 2020 pp 1-13.

American Association of Cereal Chemists. Approved Methods Committee. (2000). *Approved methods of the American Association of Cereal Chemists* (10th ed.). St. Paul, Minn., USA

Abbas, M., Mumtaz Khan, M., Iqbal, M.J. & Khan, R.W. (2004). Effects of artificial ageing on viability and leachate exudation in fenugreek (*Trigonella foenum-graecum L.*) seeds. *Journal of the Korean Society for Horticultural Science,* 45: 238-242.

Abdalla, F., & Roberts, E. (1969). The effect of seed storage conditions on the growth and yield of barley, broad beans, and peas. *Annals of Botany*, 33:169-184.

Abdul Baki A.A. (1980). Biochemical aspects of seed vigor. *HortScience*, 15: 765-771.

Al-Maskri, A. Y., Khan, M. M., & Al-Habsi, K. (2003). Effect of accelerated ageing on viability, vigor (rgr), lipid peroxidation and leakage in carrot (*Daucus carota L.*) seeds. *International Journal of Agriculture & Biology*, 5: 580–584.

Alberts, B., Bray, D., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P. (2002). How Cells Obtain Energy from Food: In *Molecular Biology of the Cell.* (4th edition). Garland Science New York, USA: 419–445.

Arc, E., Ogé, L., Grappin, P., & Rajjou, L. (2011). Plant seed: a relevant model to study aging processes: In *The Field of Biological Aging: Past, Present and Future*, Transworld Research Network, Kerala, India: 87-102.

Arc, E., Galland, M., Godin, B., Cueff, G., & Rajjou, L. (2013). Nitric oxide implication in the control of seed dormancy and germination. *Frontiers in Plant Science*, 4: 346.

Asgharipour, M. R., & Rafiei, M. (2011). Effect of salinity on germination and seedling growth of lentils. *Australian Journal of Basic and Applied Sciences*, 5:2002–2004.

Ashraf, N. & Foolad, M. (2005). Pre-sowing seed treatment – A shotgun approach to improve germination, plant growth and crop yield under saline ad non-saline conditions. *Advances in Agronomy* 88: 223-271.

Baalbaki, R. (2009). *Seed vigor testing handbook*. Association of Official Seed Analysis Ithaca, NY, USA: 32.

Bailly, C. (2004). Active oxygen species and antioxidants in seed biology. *Seed Science Research*, 14: 93-107.

Bailly, C., Benamar, A., Corbineau, F., & Come, D. (1996). Changes in malondialdehyde content and in superoxide dismutase, catalase and glutathione reductase activities in sunflower seeds as related to deterioration during accelerated aging. *Physiologia Plantarum,* 97: 104-110.

Bailly, C., El-Maarouf-Bouteau, H., & Corbineau, F. (2008). From intracellular signaling networks to cell death: The dual role of reactive oxygen species in seed physiology. *Comptes Rendus - Biologies, 331*: 806-814.

Barreto, L., & Garcia, C. (2017). Accelerated ageing and subsequent imbibition affect seed viability and the efficiency of antioxidant system in macaw palm seeds. *Acta Physiologiae Plantarum,* 39: 1-8.

Baskin CC & Baskin JM. 1998. *Seeds. Ecology, biogeography, and evolution of dormancy and germination*, Academic Press, San Diego, USA, pp 666.

Basra, S. M. A., Ahmad, N., Khan, M. M., Iqbal, N., & Cheema, M. A. (2003). Assessment of cottonseed deterioration during accelerated ageing. *Seed Science and Technology*, 31, 531–540.

Behall, K., Scholfield, D., & Hallfrisch, J. (2004). Diets containing barley significantly reduce lipids in mildly hypercholesterolemic men and women. *American Journal Of Clinical Nutrition*, 80: 1185-1193.

Bentsink, L., & Koornneef, M. (2008). Seed dormancy and germination. *The Arabidopsis Book/American Society of Plant Biologists*, 6: e0119

Benz, B. (2012). The Conservation of Cultivated Plants. *Nature Education Knowledge*, 3: 4.

Bernal-Lugo, I., & Leopold, a C. (1992). Changes in soluble carbohydrates during seed storage. *Plant Physiology*, 98: 1207–1210.

Bernal-Lugo, I., & Leopold, A. C. (1995). Seed stability during storage: Raffinose content and seed glassy state. *Seed Science Research*, 5: 75–80.

Bewley, J., & Black, M. (1994). *Seeds: Physiology of development and germination* (2nd ed., Plenum Press, New York, USA.

Bewley, J., Bradford, Kent J., Hilhorst, Henk W., Nonogaki, Hiro., (2013). *Seeds: Physiology of Development, Germination and Dormancy,* 3rd Edition, SpringerLink, New York, USA.

Black, M., Bewley, J.D. & Halmer, P. (Eds.) (2006). The encyclopedia of seeds: science, technology and uses. CABI publishing series, Wallingford, U.K. pp 828.

Blöchl, A., Peterbauer, T., Hofmann, J., & Richter, A. (2008). Enzymatic breakdown of raffinose oligosaccharides in pea seeds. *Planta*, 228: 99-110.

Bradford, K., & Nonogaki, H. (Eds.). (2008). *Annual plant reviews, seed development, dormancy, and germination*., Blackwell Publishing, Oxford, UK pp 27.

Buitink, J., Leprince, O., Hemminga, M. A., & Hoekstra, F. A. (2000). Molecular mobility in the cytoplasm: an approach to describe and predict lifespan of dry germplasm. *Proceedings of the National Academy of Sciences, USA*, 97: 2385-2390.

Catusse, J., Strub, J-M., Claudette, J., Van Dorssealer & Job, D. (2008) Proteome-wide characterization of sugarbeet seed vigor and its tissue specific expression. *Proc Natl Acad Sci (USA)*, 105: 10262-10267.

CBD (2002) Global strategy for plant conservation. The Secretariat of the Convention on Biological Diversity, Montreal, Canada.

Christensen C.M. (1972). Microflora and seed deterioration. In: Roberts E.H. (eds) *Viability of Seed*s. Springer, Dordrecht, Netherlands: 55-93.

Clerkx, E.J., Blankestjin-De Vries, H., Ruys, G.J., Groot, S.P. C. & Koormneef, M. (2004) Genetic differences in seed longevity of various *Arabidopsis* mutants. *Physiologia Plantarum* 121: 448 – 461.

Crocker, W., & Groves, J. F. (1915). A method of prophesying the life duration of seeds. *Proceedings of the National Academy of Sciences* USA, 1: 152–155

Crowe, J. H., Crowe, L. M., Carpenter, J. F., & Aurell Wistrom, C. (1987). Stabilization of dry phospholipid bilayers and proteins by sugars. *Biochemical Journal*, 242: 1-10.

Da Rosa, S. D. V. F., Carvalho, A. M., McDonald, M. B., Von Pinho, E. R. V., Silva, A. P., & Veiga, A. D. (2011). The effect of storage conditions on coffee seed and seedling quality. *Seed Science and Technology*, 39: 151-164.

Delouche, J.C., & Baskin, C.C. (1973). Accelerated aging techniques for predicting the relative storability of seed lots. *Seed Science and Technology*, 2: 427-452.

Dourado, A. M., & Roberts, E. H. (1984). Chromosome aberrations induced during storage in barley and pea seeds. *Annals of Botany*, *54*(6), 767-779.

Einset, J., & Collins, A. R. (2015). DNA repair after X-irradiation: Lessons from plants. *Mutagenesis*, 30, 45–50.

Ellis, R., & Roberts, E. (1980). Improved Equations for the Prediction of Seed Longevity. *Annals of Botany,* 45: 13-30

FAO (1997) The State of the World's Plant Genetic Resources for Food and Agriocultire, FAO, Rome.

Farhadi, R., Rahmani, M. R., Salehi, B. M., & Sadeghi, M. (2012). The effect of artificial ageing on germination components and seedling growth of basil (*Ociumum basilicm L*.) seeds. *Journal of Agriculture and Food Technology*, 2: 69-72.

Fenner, M., & Thompson, Ken. (2005). *The ecology of seeds*. Cambridge University Press, UK.

Finch-Savage, W.E. & Bassel, G.W. (2016) Seed vigour and crop establishment: extending performance beyond adaptation. *J. Experimental Botany*, 67: 567 – 591.

Friedt, W. D., Horsley, R. L., Harvey, B. M., Poulsen, D. C., Lance, R., Ceccarelli, S., Capettini, F. (2011). Barley Breeding History, Progress, Objectives, and Technology. In *Barley: Production, Improvement, and Uses,* Wiley-Blackwell, Ames, Iowa, USA: 160-220.

Fu, Y., Ahmed, Z., & Diederichsen, A. (2015). Towards a better monitoring of seed ageing under ex situ seed conservation. *Conservation Physiology,* 3: 1- 16.

Galletti, P., Ingrosso, D., Manna, C., Clemente, G., & Zappia, V. (1995). Protein damage and methylation-mediated repair in the erythrocyte. *Biochemical Journal,* 306: 313-325.

Gangola, M. P., Jaiswal, S., Khedikar, Y. P., & Chibbar, R. N. (2014). A reliable and rapid method for soluble sugars and RFO analysis in chickpea using HPAEC-PAD and its comparison with HPLC-RI. *Food Chemistry*, 154: 127–133.

Gaschler, M., & Stockwell, B. (2017). Lipid peroxidation in cell death. *Biochemical and Biophysical Research Communications,* 482: 419-425.

Gidrol X., Noubhani A., Mocquot B., Fournier A., & Pradet A. (1988). Effect of accelerated aging on protein synthesis in two legume seeds. *Plant Physiology and Biochemistry*, 26: 281-288.

Goel, A., Goel, A. K., & Sheoran, I. S. (2003). Changes in oxidative stress enzymes during artificial ageing in cotton (*Gossypium hirsutum L.*) seeds. In *Journal of Plant Physiology* 160: 1093-1100.

Groot, S. P. C., Surki, A. A., De Vos, R. C. H., & Kodde, J. (2012). Seed storage at elevated partial pressure of oxygen, a fast method for analysing seed ageing under dry conditions. *Annals of Botany*, 110: 1149-1159.

Hampton, J. G., & TeKrony, D. M. (1995)., *Handbook of vigour test methods.* The International Seed Testing Association, Zurich, Switzerland

Harman, G.E. & Mattick, L.R. (1976) Association of lipid oxidation with seed ageing and death. *Nature* 260: 323–324.

Harrington, J. F. (1960). Drying, storing and packaging seed to maintain germination. *Seed Man's Digest*, 1: 16-8.

Harrington, J. F. (1972). Seed storage and longevity. In *Seed biology*, 3, Academic Press, New York, USA: 145-245.

Haughn, G. & Chaudhary, A. (2005). Genetic analysis of seed coat development in Arabidopsis. Trtends in Plant Science 10:472 - 479.

Helbock, H.J., Beckman, K. B., Shigenaga, M K., Walter, P. B., Woodall, A. A., Yeo, H. C., & Ames, B. N. (1998). DNA oxidation matters: The HPLC-electrochemical detection assay of 8-oxo-deoxyguanosine and 8-oxo-guanine. *Proc. Natl Acad. Sciences USA*, 95: 288-293.

Heywood, V., Casas, A., Ford-Lloyd, B., Kell, S., & Maxted, N. (2007). Conservation and sustainable use of crop wild relatives. *Agriculture, Ecosystems and Environment*, 121: 245–255.

Hoekstra, F. A., Golovina, E. A., & Buitink, J. (2001). Mechinisms of Plant desiccation tolerence. *Trends in Plant Science*, 6: 431–438.

Horbowicz, M., & Obendorf, R. L. (1994). Seed desiccation tolerance and storability: Dependence on flatulence-producing oligosaccharides and cyclitols—review and survey. In *Seed Science Research* 4: 385-405.

Hucl, P., & Chibbar, R. N. (1996). Variation for starch concentration in spring wheat and its repeatability relative to protein concentration. *Cereal Chemistry*. 73: 756-758.

Imamura, K., Ogawa, T., Sakiyama, T., & Nakanishi, K. (2003). Effects of types of sugar on the stabilization of protein in the dried state. *Journal of Pharmaceutical Sciences*, 92: 266-274.

ISTA. (2019). International Rules for Seed Testing, *International Seed Testing Association*, Zurich, Switzerland.

Izydorczyk, M. S., McMillan, T., Bazin, S., Kletke, J., Dushnicky, L., Dexter, J., & Rossnagel, B. (2014). Milling of Canadian oat and barley for functional food ingredients: Oat bran and barley fibre-rich fractions. *Canadian Journal of Plant Science*, 94: 573-586.

Jain, N., & Van Staden, J. (2006). A smoke-derived butenolide improves early growth of tomato seedlings. *Plant Growth Regulation*, 50: 139–148.

Jyoti & Malik, C. P. (2013). Seed deterioration: A review. *International Journal of Life Sciences Biotechnology and Pharma Research*, 2: 374-385.

Kapoor, N., Arya, A., Siddiqui, M. A., Kumar, H., & Amir, A. (2011). Physiological and biochemical changes during seed deterioration in aged seeds of rice (Oryza sativa L.). *American Journal of Plant Physiology*, 6: 28–35.

Khan, A. A. (1994). ACC-derived ethylene production, a sensitive test for seed vigor. *Journal of the American Society for Horticultural Science*, 119: 1083–1090.

Khan, M.M., Iqbal, M.J., Abbas, M. & Usman, M. (2003). Effect of accelerated aging on viability, vigor and chromosomal damage in pea (*Pisum sativum* L.) seeds. *Pakistan J Agric Sci.*, 40: 50-54.

Khan, F. A., Maqbool, R., Narayan, S., Bhat, S. A., Narayan, R., & Khan, F. U. (2016). Reversal of age-induced seed deterioration through priming in vegetable crops–A review. *Advances in Plants & Agriculture Research*, 4: 159.

Kibinza, S., Vinel, D., Côme, D., Bailly, C., & Corbineau, F. (2006). Sunflower seed deterioration as related to moisture content during ageing, energy metabolism and active oxygen species scavenging. *Physiologia Plantarum*, 128: 496–506.

Kijak, H. & Ratajczak, E. (2020) What do we know about the genetic basis of seed desiccation tolerance and longevity? *Int J Mol Sci.* 21: 3612.

Kong, Q., Mao, P. S., Yu, X. D., & Xia, F. S. (2014). Physiological changes in oat seeds aged at different moisture contents. *Seed Science and Technology*, 42: 190-201.

Koster, K. L., & Leopold, A. C. (1988). Sugars and desiccation tolerance in seeds. *Plant Physiology*, 88: 829–832.

Koutroubas, S. D., Papakosta, D. K., & Doitsinis, A. (2000). Water requirements for castor oil crop (*Ricinus communis L.*) in a mediterranean climate. *Journal of Agronomy and Crop Science*, 18 4: 33–41.

Krishnan, H.B. & Coe, E.H. (2001) Seed storage proteins. In Encyclopedia of Genetics (eds. S. Brenner & J.H. Miller) Academic Press, San Diego, CA, USA, pp 1782 – 1787.

Lehner, A., Mamadou, N., Poels, P., Côme, D., Bailly, C., & Corbineau, F. (2008). Changes in soluble carbohydrates, lipid peroxidation and antioxidant enzyme activities in the embryo during ageing in wheat grains. *Journal of Cereal Science*, 47: 555–565.

Leopold, A., Sun, W., & Bernal-Lugo, I. (1994). The glassy state in seeds: Analysis and function. *Seed Science Research*, 4: 267-274.

Li, D., & Pritchard, H. (2009). The science and economics of ex situ plant conservation. *Trends in Plant Science*, 14: 614-621.

Linington, S.E. & Pritchard, H.W. (2001). Genebanks. In Encyclopedia of Biodiversity, ed. S.A. Levin, volume 3, Academic Press, San Francisco, USA, pp 165-181.

Long, R.L., Panetta, F.D., Steadman, K.J., Probert, R.J., Bakker, R.M., Brooks, S., & Adkins, S.W. (2008) Seed persistence in the field may be predicted by laboratory-controlled aging. *Weed Science* 56: 523 – 528.

Long, R.L., Gorecki, M.J., Renton, M., Carles, A., Li, Y., Colville, L., Goggin, D.E., Commander, L.E., Westcott, Chery, H., & Finch-Savaga, W.E. (2015) The ecophysiology of seed persistance: a mechanistic view of the journey to germination or demise. *Biological Reviews*, 90: 31-59.

Mahjabin, S. B., & Abidi, A. B. (2015). Physiological and biochemical changes during seed deterioration: a review. *International Journal of Recent Scientific Research*, 6: 3416-3422.

Maity, S., Banerjee, G., Roy, M., Pal, C., Pal, B., Chakrabarti, D., & Bhattacharjee, A. (2000). Chemical induced prolongation of seed viability and stress tolerance capacity of mung bean seedlings. *Seed Science and Technology*, 28: 155–162.

Malik, C. P. (2013). Seed deterioration: a review. *International Journal of Life Sciences Biotechnology and Pharma Research*, 2: 374-385.

Marcos-Filho, J. (2015). Seed vigor testing: An overview of the past, present and future perspective. *Scientia Agricola*, 72: 363–374.

Marques, E. R., Araújo, R. F., Araújo, E. F., Martins Filho, S., Soares, P. C., & Mendonça, E. G. (2014). Dormancy and enzymatic activity of rice cultivars seeds stored in different environments. *Journal of Seed Science*, *36*: 435–442.

Masoudi, S., F., Babak, A. M., Zardoshti, M. R., Hassan, R. S. M., & Tavakoli, A. (2011). Response of proline, soluble sugars, photosynthetic pigments and antioxidant enzymes in potato (*Solanum tuberosum L*.) to different irrigation regimes in greenhouse condition. *Australian Journal of Crop Science*, 5: 55-60.

McCleary, B. V., Gibson, T. S., & Mugford, D. C. (1997). Measurement of total starch in cereal products by amyloglucosidase-α-amylase method: Collaborative study. *Journal of AOAC International, 80*: 571-579.

Mcdonald, M. (1999). Seed deterioration: Physiology, repair and assessment. *Seed Science And Technology*, 27: 177-237.

McDonald Jr, M. B., & Phaneendranath, B. R. (1978). A modified accelerated aging seed vigor test for soybeans. *Journal of Seed Technology*, 3: 27-37.

Melville A.H., Galletta G.J., Draper A.D., & Ng T.J. (1980). Seed germination and early seedling vigor in progenies of inbred strawberry selections. *HortScience,* 15: 749-750.

Milesi, M. (2006). Oxidative stress, diseases and antioxidants. *Agro Food Industry Hi-Tech*, 17: 6-9.

Mira, S., González-Benito, M. E., Hill, L. M., & Walters, C. (2010). Characterization of volatile production during storage of lettuce (*Lactuca sativa*) seed. *Journal of Experimental Botany*, 61: 3915–3924.

Mittler, R., Vanderauwera, S., Gollery, M., & Van Breusegem, F. (2004). Reactive oxygen gene network of plants. *Trends in plant science*, 9: 490-498.

Moncaleano-Escandon, J., Silva, B. C. F., Silva, S. R. S., Granja, J. A. A., Alves, M. C. J. L., & Pompelli, M. F. (2013). Germination responses of *Jatropha curcas L*. seeds to storage and aging. *Industrial Crops and Products*, 44: 684–690.

Moshki, A., Hodjati, S. M., Bakhshande, N., & Lamersdorf, N. P. (2012). The role of seed provenance in the growth and nutrient status of black locust (*Robinia pseudoacacia L*.). *American Journal of Plant Nutrition and Fertilization Technology*, 2: 27–31.

Murthy, U., Liang, Y., Kumar, P., & Sun, W. (2002). Non-enzymatic protein modification by the Maillard reaction reduces the activities of scavenging enzymes in *Vigna radiata*. *Physiologia Plantarum*, 115: 213-220.

Nascimento, J. P. B., & Meiado, M. V. (2017). In situ or ex situ seed conservation: which is the more effective way to maintain seed longevity of an endangered cactus? *Plant Species Biology*, 32: 115–120

Nishizawa, A., Yabuta, Y., & Shigeoka, S. (2008). Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. *Plant Physiology*, 147: 1251–1263.

Nonogaki, H., Bassel, G. W., & Bewley, J. D. (2010). Germination-still a mystery. *Plant Science*, 179: 574–581.

Obendorf, R. L. (2008). Oligosaccharides and galactosyl cyclitols in seed desiccation tolerance. In *Seed Science Research* 7: 63-74.

Olkowski, A.A., Laarveld, B., & Tanino, K.K. (2014) Enhancement and control of seed germination. Filed Canadian patent (CA2949750A1), Canada assignee, University of Saskatchewan - inventors.

Panuccio, M., Jacobsen, S., Akhtar, S., & Muscolo, A. (2014). Effect of saline water on seed germination and early seedling growth of the halophyte quinoa. *AoB PLANTS*, 6: 47.

Parrish, D. J., & Leopold, A. C. (1978). On the mechanism of aging in soybean seeds. *Plant Physiology*, 61: 365–368.

Pérez-García, F., Gómez-Campo, C., & Ellis, R. H. (2009). Successful long-term ultra dry storage of seed of 15 species of *Brassicaceae* in a genebank: variation in ability to germinate over 40 years and dormancy. *Seed Science and Technology*, 37: 640-649.

Petruzzelli, L., & Taranto, G. (1989). Wheat aging: the contribution of embryonic and non-embryonic lesions to loss of seed viability. *Physiologia Plantarum*, 76: 289-294.

Piotrowicz-Cieslak, A.I. (2005). Changes in soluble carbohydrates in yellow lupin seed under prolonged storage. *Seed Science and Technology,* 33: 141-145.

Pradhan, B., & Badola, H. (2008). Seed germination response of populations of *Swertia chirayita* [(Roxb. ex Fleming) H. Karst] following periodical storage. *Seed Technology,* 30: 63-69.

Pradhan, B., & Badola, H. (2012). Effect of storage conditions and storage periods on seed germination in eleven populations of *Swertia chirayita*: A critically endangered medicinal herb in Himalaya. *The Scientific World Journal*, 2012: 128105.

Priestley, D. (1986). *Seed aging : Implications for seed storage and persistence in the soil*. Ithaca, New York, USA

Pukacka, S., & Ratajczak, E. (2005). Production and scavenging of reactive oxygen species in Fagus sylvatica seeds during storage at varied temperature and humidity. *Journal of Plant Physiology,* 162: 873-885.

Qin, P., Kong, Z., Liao, X., & Liu, Y. (2011). Effects of accelerated aging on physiological and biochemical characteristics of waxy and non-waxy wheat seeds. *Journal of Northeast Agricultural University (English Edition),* 18: 7-12.

Rajjou, L., Lovigny, Y., Groot, S. P. C., Belghazi, M., Job, C., & Job, D. (2008). Proteome-Wide characterization of seed aging in arabidopsis: A comparison between artificial and natural aging protocols. *Plant Physiology,* 148: 620–641.

Rajjou, L., & Debeaujon, I. (2008). Seed longevity: Survival and maintenance of high germination ability of dry seeds. *Comptes Rendus - Biologies*, 331: 796–805.

Rao, G. S., Narayana, S. L., Bhadraiah, B., & Manoharachary, C. (2014). Biochemical changes due to fungal infestation in stored seeds of some vegetable crops. *Indian Phytopathology*, 67: 159-163.

Regina, M., & Raman, T. (1992). Biochemical changes in stored caraway seeds due to fungi. *Indian Phytopathology*, 45: 384.

Roberts E.H. (1972) Storage Environment and the Control of Viability. In: Roberts E.H. (eds) *Viability of Seeds*. Springer, Dordrecht, Netherlands, pp 14-58.

Roberts, E. H. (1973). Predicting the storage life of seeds. In *Seed Science and Technology* 1: 499-514.

Roberts, E. H., & Ellis, R. H. (1989). Water and seed survival. *Annals of botany*, 63: 39-39.

Rodo, A. B., & Marcos Filho, J. (2003). Accelerated aging and controlled deterioration for the determination of the physiological potential of onion seeds. *Scientia Agricola*, 60: 465–469.

Salo, M. L., & Kotilainen, K. (1970). On the carbohydrate composition and nutritive value of some cereals. *Agricultural and Food Science*, 42: 21-29.

Sano, N., Rajjou, L., North, H.M., Debeaujon, I., Marion-Poll, & Seo, M. (2016) Staying Alive: Molecular sapects of seed longevity. *Plant and Cell Physiol.* 57: 660 – 674.

Saxena, N., Rani, S. S., & Deepika, M. (2015). Biodeterioration of Soybean (*Glycine max L.*) seeds during storage by Fungi. *International Journal of Current Microbiology and Applied Sciences*, 4: 1118-1126.

Scandalios, J. G. (1997). *Oxidative stress and the molecular biology of antioxidant defenses*. Cold Spring Harbor Laboratory Press, New York, USA: 343-406.

Schmidt, L. (2007). *Tropical Forest Seed*. Springer Science & Business Media, Berlin, Germany, pp 12-14.

Schmidt, S. B., George, T. S., Brown, L. K., Booth, A., Wishart, J., Hedley, P. E., ... & Husted, S. (2019). Ancient barley landraces adapted to marginal soils demonstrate exceptional tolerance to manganese limitation. *Annals of Botany*, 123: 831-843.

Shaban, M., & Rahmati Motlagh, Z. (2014). Physiology of plants affected by ageing. *International Journal of Advanced Biological and Biomedical Research (IJABBR)*, 2: 2301-2305.

Siadat, S. A., Moosavi, A., & Sharafizadeh, M. (2012). Effects of seed priming on antioxidant activity and germination characteristics of maize seeds under different ageing reatment. *Research Journal of Seed Science*, 5: 51-62.

Singh, B., & Prasad, T. (1977). Effect of seed-borne fungi on the physico-chemical properties of sunflower oil. *Journal of Phytopathology*, 90: 337-341.

Sinniah, U., Ellis, R., & John, P. (1998). Irrigation and seed quality development in rapid-cycling brassica: soluble carbohydrates and heat-stable proteins. *Annals of Botany*, 82: 647-655.

Souza, C., Ohlson, O., Gavazza, M., & Panobianco, M. (2010). Tetrazolium test for evaluating triticale seed viability. *Revista Brasileira De Sementes*, 32: 163-169.

Steadman, K., Pritchard, H., & Dey, P. (1996). Tissue-specific Soluble Sugars in Seeds as Indicators of Storage Category. *Annals of Botany*, 77: 667-674.

Suma, A., Sreenivasan, K., Singh, A. K., & Radhamani, J. (2013). Role of relative humidity in processing and storage of seeds and assessment of variability in storage behaviour in brassica spp. and Eruca sativa. *The Scientific World Journal*, 2013: 9.

Sveinsdóttir, H., Yan, F., Zhu, Y., Peiter-Volk, T., & Schubert, S. (2009). Seed ageing-induced inhibition of germination and post-germination root growth is related to lower activity of plasma membrane H -ATPase in maize roots. *Journal of Plant Physiology*, 166: 128-135.

Tangney, R. J., Merritt, D. P., Miller, B. B., & Fontaine, J. (2019). Seed moisture content as a primary trait regulating the lethal temperature thresholds of seeds. *Journal of Ecology*, 107: 1093-1105.

Tian, P., Lv, Y., Yuan, W., Zhang, S., & Hu, Y. (2019). Effect of artificial aging on wheat quality deterioration during storage. *Journal of Stored Products Research*, 80: 50-56.

Tilden, R. L., & West, S. H. (1985). Reversal of the effects of aging in soybean seeds. *Plant Physiology*, 77 : 584-586.

Torres, M., De Paula, M., Pérez-Otaola, M., Darder, M., Frutos, G., & Martínez-Honduvilla, C. J. (1997). Ageing-induced changes in glutathione system of sunflower seeds. *Physiologia Plantarum* 101: 807-814.

Treuren, R., Groot, E., & Hintum, C. (2013). Preservation of seed viability during 25 years of storage under standard genebank conditions. *Genetic Resources and Crop Evolution*, 60: 1407-1421.

Tsikas, D. (2017). Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: Analytical and biological challenges. *Analytical Biochemistry*, 524: 13–30.

Tweddle, J. C., Dickie, J. B., Baskin, C. C., & Baskin, J. M. (2003). Ecological aspects of seed desiccation sensitivity. *Journal of Ecology*, 91: 294–304.

Varma, P., Bhankharia, H., & Bhatia, S. (2016). Oat: A multi-functional grain. *Journal of Clinical and Preventive Cardiology*, 5: 9-17.

Walters, C. (1998). Understanding the mechanisms and kinetics of seed aging. *Seed Science Research*, 8: 223-244.

Walters, C., Ballesteros, D., & Vertucci, V. A. (2010). Structural mechanics of seed deterioration: Standing the test of time. *Plant Science*, 179: 565–573.

Walters, C., Berjak, P., Pammenter, N., Kennedy, K., & Raven, P. (2013). Preservation of recalcitrant seeds. *Science*, 339: 915-916.

Walters, C., Wheeler, L. M., & Grotenhuis, J. M. (2005). Longevity of seeds stored in a genebank: species characteristics. *Seed Science Research*, 15: 1-20.

Wang, J. H., Baskin, C. C., Chen, W., & Du, G. Z. (2010). Variation in seed germination between populations of five sub-alpine woody species from eastern Qinghai-Tibet Plateau following dry storage at low temperatures. *Ecological Research*, 25: 195–203.

Weitbrecht, K., Müller, K., & Leubner-Metzger, G. (2011). First off the mark: early seed germination. *Journal of experimental botany*, 62: 3289-3309.

West, M., & Heywood, J. J. (1993). Embryogenesis in higher plants: an overview. *The Plant Cell*, 5: 1361.

Wilson, D.O. & McDonald, M.B. (1986) The lipid peroxidation model of sed ageing. *Seed Sci. Technl.* 14: 269 – 300.

Zhao, M., Zhang, H., Yan, H., Qiu, L., & Baskin, C. C. (2018). Mobilization and Role of Starch, Protein, and Fat Reserves during Seed Germination of Six Wild Grassland Species. *Frontiers in Plant Science, 9*: 234.