PHYSIOLOGICAL CHARACTERIZATION ON SEED AGING OF SIX NATIVE SHRUB SPECIES

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ABSTRACT

Vegetation reclamation in oil-sands requires a consistent and adequate supply of seeds of native shrubs. However, annual seed production is erratic and seeds are usually short lived and insufficient for the reclamation projects. Seeds of six native shrub species including: *Prunus virginiana, Prunus pensylvanica, Arctostaphylos uva-ursi, Shepherdia canadensis, Cornus sericea,* and *Viburnum edule* were used to analyze physiological changes during storage and artificial aging processes. The shrub seeds were studied for one year during storage under eight different combinations of temperature (-20, 4, 22.5 °C), atmosphere (Air / N_2) and relative humidity (RH; 7-8 % / 3-4 %). No significant differences were detected among the storage parameters after one year; however, sub-zero and N_2 environments showed a potential in maintaining a higher seed vigour during storage. In the artificial aging experiment, seeds were subjected to 45 ^{o}C, 60 % RH for 5-25 d. For most shrub species, the seed viability decreased significantly after 10-15 d artificial aging and was down to 0 % after 20 d. The germination percentage declined already after 5 d; therefore, there was a delay in detecting viability loss using the tetrazolium test. Non-aged seeds and aged seeds of most collections showed significantly different seedling lengths, which indicated a negative effect of accelerated aging process on the seedling growth. The electrolyte conductivity, as well as seed dehydrin protein expression, is strongly correlated with the seed vigour, which can be used as seed quality assessment methods in seed longevity predicting. A loss of membrane integrity occurred during the accelerated seed aging processes, as indicated by an increased electrolyte conductivity that was negatively correlated with the seed viability and germination. During artificial aging process, heat stress of *Prunus virginiana* induced expression of dehydrins with a molecular mass of 27 kDa, which reached a detectable level after 5 d.

The storage protocol developed in this study would benefit the adequate supply of viable shrub seeds for reclamation. With species-specific parameters taken into consideration, the artificial aging technique to predict seed longevity can be further expanded to other non-crop species used in reclamation of lands after oil extraction.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
RH	Relative humidity
CONRAD	Canadian Oil-sands Network for Research and Development
EC	Electrolyte conductivity
GLM	General Linear Model
GRI	Seed germination rate index
HSP	Heat shock protein
LEA	Late embryogenesis abundant
LSD	Least significant difference
MC	Seed moisture content
MGT	Mean seed germination time
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SVI	Seed vigour index
Tris	2-Amino-2- (hydroxymethyl)-1,3-propanediol
TZ	Tetrazolium
UVS	Percentage of viable but un-germinated seed
VAG	Seed viability after germination (including germinated seeds)
VBG	Seed viability before germination test

1 INTRODUCTION

The Alberta oil-sands contain an estimated 1.7 to 2.5 trillion barrels of oil (Alberta, 1999). Utilization of this vast natural reserve is contingent upon oil extraction, during which all vegetation and soil cover is removed. Therefore, responsible utilization of this resource requires adequate strategies to reclaim the oil-sands to their original boreal forest ecosystem. The boreal forest ecosystem is complex and primarily made up of grasses, forbs, shrubs, trees and other life forms including animals. Over the last 30 years, oil-sands reclamation in Alberta had been focusing more on grasses and forbs but less on shrubs and trees (Rowland et al., 2009). Shrubs are key to the successful re-establishment of the boreal forest ecosystem, but the natural regeneration of shrubs is low at oil-sands sites (Mackenzie et al., 2010). Successful establishment of native shrubs by planting accelerates soil development, plants and animals diversity and forage production and provides faunal cover and food for wildlife (Booth, 1985; Mech, 2011). A major limitation preventing the use of shrubs for oil-sands reclamation project is the unavailability of a consistent supply of high quality native shrub propagules. Native shrubs in this region are erratic in their seed production; the seed collection is costly, and the seeds have a relatively short longevity (Way, 2003; Price et al., 2010). Therefore, an understanding of the seed aging mechanism and proper storage protocols is ecologically and economically critical for efficient reclamation of oil-sands sites.

Seed aging during storage is a complex process (Walters et al., 2010) that is affected by seed quality (Probert et al., 2009b), pre-storage treatments (drying process and desiccation tolerance of seeds), seed moisture content, storage temperature (Walters et al., 1998; Ellis et al., 2006), and the make-up of storage containers (Gómez-Campo, 2006). Generally, seeds from cool and wet environments are more short-lived than those

from hot and dry environments (Probert et al., 2009b). Seeds showing higher longevity at reduced moisture content and temperature are called orthodox seeds (Ellis et al., 1981; Mohammadi et al., 2011), which can be stored over a wide range of environments. However, the longevity of orthodox seeds differs quantitatively among diverse species and among seed lots within a species (Dickie et al., 1990; Bewley et al., 2013).

The knowledge of physiological and biochemical changes during seed storage for non-agricultural crops, such as native shrubs in the boreal forest. In addition, there are no guidelines showing how to evaluate the variation in seed longevity within species. Therefore, understanding the process and mechanisms underlying seed aging will not only help to predict longevity of seeds and optimize seed storage protocols, but also to help the conservation of plant diversity.

Subjecting seeds to elevated temperature and/or moisture (referred to as accelerated or artificial aging conditions) is often used to evaluate the seed responses to more conventional storage conditions (Powell et al., 1977; Hay et al., 2003; Rao et al., 2006). The procedure of accelerated seed aging has been standardized for several crops (Priestley, 1986; Rodo et al., 2003), but not for non-crop species.

Objectives

Six native shrub species from the boreal forest were selected for this study and included *Prunus virginiana*, *Prunus pensylvanica*, *Arctostaphylos uva-ursi*, *Shepherdia canadensis, Cornus sericea,* and *Viburnum edule*. For these native shrub species, the objectives of this study were to:

1) evaluate the effects of different storage conditions on seed viability, germination, and seedling vigour;

2) identify biophysical parameters for the prediction of seed viability loss by analysing physiological and biochemical changes in seeds exposed to accelerated aging; and
3) determine the optimal temperature, seed moisture content, and optimal atmosphere for seed storage with focus on identifying physiological and biochemical changes affecting seed longevity.

Hypothesis

It was hypothesized that:

1) low temperature and low oxygen and low seed moisture contents prolong seed viability during storage;
2) decreased viability in accelerated seed aging process is associated with the loss of membrane integrity; and
3) accumulation of LEA protein (dehydrins) is associated with seed viability loss.

2 LITERATURE REVIEW

2.1 Importance of shrubs in oil sand reclamation

2.1.1 Alberta oil-sands mining and the disturbance of boreal forest ecosystem

Oil-sands contain an estimated 1.7 to 2.5 trillion barrels of oil in the north-eastern Alberta (Kelly et al., 2009). For oil extraction, all vegetation and soil cover is removed to create open pits several kilometers wide and up to 100 m deep (Rowland et al., 2009). Without a reclamation programme, the development scale of Alberta oil sand industry would lead to the loss of biodiversity and irreversible ecological damage of boreal forest ecosystem. However, the oil industry is obliged to reclaim land to its original state prior to oil extraction according to Land Surface Conservation and Reclamation Act 1973 and the Environmental Protection and Enhancement Act 1992 (Goverment-Alberta, 1999). Currently, only 1.04 km^2 out of 686 km^2 of land disturbed by oil-sands mining is certified as reclaimed by the Alberta government (Mech, 2011).

2.1.2 Conservation and reclamation of boreal forest

Barley (*Hordeum vulgare* L.) is always planted to provide a quick vegetation cover and soil erosion control during the first growing season after capping (Goverment-Alberta, 1999). However, barley is a poor competitor and could be invaded readily by domestic flora within the first year. Although reclamation in these areas has been underway since 1970s, reclamation treatments have left more bare ground, some grasses and forbs, but less shrubs, trees, woody debris, moss, and lichen than natural forests (Rowland et al., 2009).

2.1.3 Shrubs as a key component of boreal forest

Succession is a slow, natural, orderly change in plant and animal communities that occurs over time (Pérez et al., 2013). The earliest succession stage is dominated by herbs and

small shrubs, followed by an intermediate stage dominated by tall shrubs and young trees, and the final stage resulting in a mature forest. Fire, resource mining, flood, or extreme weather can disturb the existing environment. As a result, the ecosystem reverts to its earlier succession stage. The establishment of native shrubs accelerate soil development, enhance plant and animal diversity, and provide faunal cover and wildlife food (Booth, 1985; McKell, 2012). In a reclaimed 'Mixed Wood Region' of Alberta, shrubs enhance the development of soil organic matter (Rowland et al., 2009), which is the key component for a successful reestablishment of the natural ecosystem.

2.1.4 Challenges in shrub reclamation

There is a considerable variation in annual seed production in shrubs, thus often creating uncertain supply of seeds for a particular year when needed for reclamation. Seed collection of native shrub species is a resource demanding work, and seed longevity of native shrub species from this area is usually limited to 2-3 years after harvesting (Stevens et al., 1996; Shaw et al., 2012).

2.1.5 Ecology of native shrub species

2.1.5.1 Bearberry (*Arctostaphylos uva-ursi*)

Bearberry (*Arctostaphylos uva-ursi*) is a prostrate evergreen shrub with a number of ecotypes (Stebel et al., 2013). Bearberry can stay out of the wind chill and is widely distributed in Canada and the northern United States (Kaplan, 2012). Shade intolerant bearberry grows best in open areas and tolerant a wide range of soil type and pH. The species is frequently found on nutrition-poor dry soil with limited clay and silt (Kaplan, 2012) such as beaches, dunes and mountains where it may play an important role in preventing soil erosion (Stebel et al., 2013). Bearberry propagates vegetatively and regenerates rapidly from dormant buds especially after forest fires. Flowering occurs

between March and June (the precise period depends on location and altitude), and fruits ripen in autumn and stay on the plant throughout the winter (Kaplan, 2012; Stebel et al., 2013).

2.1.5.2 Buffalo berry (*Shepherdia canadensis* Nutt.)

Buffalo berry (*Shepherdia canadensis*) is a native deciduous shrub present across the entire forest region in Canada and northern United States (Hamer, 1996). Buffalo berry's height ranges from 1-9 m and is ideal for reclamation of disturbed areas as it provides food and cover for wildlife (Riedl et al., 2013). Buffalo berry can grow well in nutrient poor soils because of its nitrogen fixing ability, which also enhances the growth of associated species by enriching soil with nitrogen around its perimeter (Bourassa et al., 2011). Buffalo berry can regenerate by both sexual and vegetative means. Seed production begins at four to six years of age; the small seed size is always associated with delayed germination that requires 40 to 60 d cold stratification for embryo development (Rosner et al., 2003; Morales et al., 2012b). This species can also regenerate by producing sprouts from surviving root crowns and dormant buds. Buffalo berry plays a dominant role in all successional stages and it is also a dominant species in climax vegetation of ponderosa pine forest (Morales et al., 2012a; Morales Rivera, 2012).

2.1.5.3 Chokecherry (*Prunus virginiana* L.)

Chokecherry is a medium to large multi-stemmed fruiting shrub (1-7 m) or a small tree (9-13 m) (St-Pierre, 1993). It is native to Canada with distribution from British Columbia to Newfoundland (Bainard et al., 2011). Chokecherry can out-compete shade-loving plants in well-lighted openings and clearings of forests (Wang et al., 2012). Chokecherry is usually dominant during secondary succession, since it is well adapted to disturbance

by fire and can recover rapidly (Webb et al., 1985; Wang et al., 2012). When light increases after opening of the forest canopy, sprouts can develop from the root crown of chokecherry stumps and dying bushes. Chokecherry can dominate a site for a prolonged period of time by continuously producing new sprouts (Johnson et al., 1995; Suarez-Gonzalez et al., 2013). When a large group of chokecherries grows together, they can provide habitat for larger mammals (Rurek, 2010). The fruit of chokecherry is a single-seed of 4-9 mm in diameter with color varying from yellow, orange, red to purple-black (St-Pierre et al., 2005). Preliminary experiments in our group showed that the thousand seed weight of chokecherry seed is usually between 60 g to 65 g. Optimal conditions for the germination of chokecherry seed require nine weeks of warm stratification at 30/20 °C day/night followed by 10 weeks of cold stratification at 4 °C (Lockley, 1980; Rowley et al., 2007). Chokecherry seeds can be subsequently germinated at 30-20 °C with 14 h photoperiod (Lockley, 1980; Wang et al., 2012). A previous study showed that subsequent growth conditions of 7/-4 °C day/night temperatures in vermiculite promoted maximal root growth and minimal root-tip browning (St-Pierre, 1993).

2.1.5.4 Dogwood (*Cornus sericea* L. ssp. sericea)

Dogwood (*Cornus sericea*) is a medium to large multi-stem deciduous shrub with average height from 1.6 - 4 m (Shannon et al., 2012). This shrub is not only a beautiful ornamental for its red branches, but it also provides food and protection for wildlife (Charles-Dominique et al., 2014). North American aboriginal people use dogwood branches to make baskets, and fruit as food and in traditional medicine. The plant has also been combined with grasses to make a ceremonial tobacco (Fuchigami et al., 1971). Dogwood is a native species that is most common in boreal, temperate, and cool meso-

thermal climates (Davis et al., 2014). Dogwood can tolerate a wide range of soil conditions, ranging from moderately acidic to alkaline with moderate to high nutrient levels (Woźnicka et al., 2014). Dogwood is shade tolerant, however, generally grows better in intermediate to high light conditions. It occurs around communities such as grassland, forest, shrub land, and woodland (Shannon et al., 2012). Dogwood starts to produce seed at the age of three to four years and seeds require 60-90 d cold stratification before germination. Birds and mammal disperse dogwood seeds that naturally establish within one year (Woźnicka et al., 2014). Even though dogwood is typically present throughout all stages of succession, it is more abundant in the earlier stages (Shannon et al., 2012).

2.1.5.5 Low bush cranberry (*Viburnum edule* (Michx.) Raf.)

Low bush cranberry (*Viburnum edule*) is an erect, deciduous shrub with height from 0.6 to 2 m (Chai et al., 2013). It is widely distributed throughout Alaska and across Canada. Low bush cranberry is an important component of the forest edge and hedgerow habitats that provides cover for small mammals and birds (Wiese et al., 2012). The late fall ripened fruits are consumed by small mammals and foliage serves as habitat for beavers and rabbits (Solarik et al., 2010). Low bush cranberry can reproduce vegetatively by sprouting from damaged rootstock and stumps (Daust, 2013). Seeds are produced at the age of five years (Solarik et al., 2010) and require a two-stage stratification (a warm period followed by cold stratification) to break dormancy (Chai et al., 2013). Low bush cranberry is a seed banking species, as the cleaned and air-dried seeds can be stored up to 10 years without losing viability (Wiese et al., 2012). Low bush cranberry is moderately shade tolerant and important during all stages of forest succession (Clement et al., 2011).

2.1.5.6 Pin cherry (*Prunus pensylvanica*)

Pin cherry is a short-lived native shrub or small tree, which has a narrow, round-topped crown (Allison et al., 2003). Pin cherry regenerates both by seeds and sprouts (Ghayyad et al., 2010). Seedlings of pin cherry have been found one year after clear-cut in a 25-year-old Maine hardwood forest (White, 1991; Ghayyad et al., 2010). Sexual maturity can be reached as early as two years growth resulting in abundant seed production at early ages (Wendel, 1990).

Pin cherry seeds are 4-6 mm in diameter with a thick seed coat (Halls, 1977; Belcher, 1985; Ghayyad et al., 2010). Dormancy of pin cherry seeds is usually caused by several years' initial physiological inhibition and followed by a secondary dormancy that remains until germination is stimulated by disturbance (Marks, 1974; Ghayyad et al., 2010). Thus, there is often a long delay between pin cherry seed dispersal and germination. Germinate is stimulated by changes in light and soil temperature (Marks, 1974; Willis et al., 2012), but the specific aspect of the changed environment that triggers germination is not known (Canham et al., 1985). A break down of germination inhibitor or increase permeability is proposed mechanisms for germination initiation (Marks, 1974; Wendel, 1990; Callahan et al., 2009). A study in Nova Scotia on pin cheery stand development found higher frequency of regeneration on soil disturbed by logging machine than on undisturbed soil (Allison et al., 2003). Laboratory experiments revealed that germination in open areas might be related to more extreme temperature fluctuations (Marks, 1974; Callahan et al., 2009). According to Peterson et al., (1995), germination of pin cherry may also be promoted by nitrogen (especially nitrate) fertilization.

2.2 Storage conditions affecting seed longevity

Seed storage is a major contributing factor to the quality of planting materials for propagation in subsequent seasons, and food storage and conservation of genetic recourses. The aging process of seeds during storage is complex (Walters, 2007) and it is affected by seed quality at harvest (Probert et al., 2009b), pre-storage treatments (drying process and desiccation tolerance of seeds) (Hong et al., 1998), seed moisture content, temperature (Walters et al., 1998; Willis et al., 2012), and type of seed containers used to maintain optimal moisture levels (Gómez-Campo, 2006; Walters, 2007). Probert et al., (2009a) concluded the three important environmental factors affecting seed storage: moisture content (RH), temperature, and gaseous environment.

2.2.1 Effects of temperature on seed longevity

The longevity of orthodox seeds decreases as the temperature increases when relative humidity is constant during storage (Dickie et al., 1990; Balešević-Tubić et al., 2010). This is primarily due to a higher respiration rate at higher temperature causing increased utilization of storage proteins and carbohydrates (Kochanek et al., 2009). High temperature also promotes protein denaturation and lipid peroxidation in seeds (Probert et al., 2009a) and increases infections by fungi, bacteria, and pests with negative effects on seed longevity. As orthodox seeds are well tolerant to low temperature, the use of subfreezing temperature may prolong seed life by up to 20 years (Nagel et al., 2010).

2.2.2 Effects of seed moisture levels and humidity on seed longevity

The relative humidity and seed moisture content are other important factors that affect the maintenance of seed viability. In a closed environment, seed moisture content changes according to the relative humidity in the storage container (Mayer et al., 2014). Therefore, in a high humidity environment, seed moisture content will increase due to

water absorption (Probert et al., 2009b). As seed moisture levels increase, the respiration rate increases and this activates various catabolic enzymes, which accelerate the consumption of storage proteins and carbohydrates (Bass, 1980; Nagel et al., 2010). A high relative humidity level in the storage environment also increases the activity of microorganisms, which severely affect seed storage life (Mayer et al., 2014). At ambient temperature, a reduction of seed moisture content by 4-7% essentially assures retention of high germination percentage for one or more years (Roberts, 1972).

2.2.3 Effects of storage atmosphere on seed longevity

Besides temperature and relative humidity, the gaseous composition of the storage environment has also been implicated in seed longevity. Under constant temperature and moisture conditions in a closed environment, the gaseous environment changes due to respiration by the seeds and associated micro-flora (Rao et al., 2006). For many species, a high oxygen concentration reduces seed viability (Bewley et al., 1982; Schwember et al., 2010). Thus, many seeds retain their viability better in sealed containers than in open storage conditions (Schwember et al., 2011). Roberts (1961) found that rice seeds were better preserved in N_2 atmosphere with 12% relative humidity than stored with oxygen or air. In case of lettuce, seed of 5-6% moisture content deteriorates slower in sealed containers with CO_2 atmosphere than in air (Pérez-García et al., 2009). However, Schwember et al. (2011), argued that there was limited advantage in using a controlled atmosphere for seeds stored at low temperature and low moisture conditions.

2.3 Changes of seed viability and vigour during the aging process

The mechanisms by which seeds lose their germination potential are not well understood (Walters et al., 2010; Walters et al., 2011). Therefore, the knowledge of physiological and structural changes at the biochemical and molecular level during seed aging process

can help understand the reasons of seed viability loss and can be used to optimize seed storage protocols.

Seed vigour is the sum of all seed properties and it can be used to determine the potential activity and performance of seeds (Baalbaki et al., 2009). To get accurate information on seed performance in storage or field, germination test, viability test, as well as other seed characteristics are needed to be assayed (Thuzar et al., 2010). The standard germination test is designed to provide a first and final count of germinated seeds, which are then interpreted as the germination percentage; it is the sum of both strong and weak seedlings (Pérez-García et al., 2009). Seed germinability represents the capacity of seed to retain enzymes activities needed to catalyze metabolic reactions, which determine the germination and seed growth (Basra, 1995).

Seed viability test is a measure of viable and dead seeds. A viable seed is one that germinates under favorable conditions. Seeds that cannot germinate due to their age and / or dormancy, but have viable tissues and cells capable of metabolism are also considered viable (Priestley, 1986). There is no doubt that reduced seed vigour decreases the field emergence. A Strong but non-linear correlation is observed between percentage seed viability and various attributes of seed vigour (McDonald Jr et al., 1986; Pérez-García et al., 2009). Aged seeds that retain their capacity to germinate generally show a postponed and slower germination with an enhanced sensitivity to external stress. Aging is just one determinant of vigour, which is also influenced by seed genotype, degree of maturation, and physical integrity (Roberts, 1972; Mayer et al., 2014). Seed deterioration is used in this thesis in a general sense to indicate declining vigour or viability (Baalbaki et al., 2009). The relationship between the seed viability and the vigour of a hypothetical seed

lot with increasing seed deterioration is illustrated in Figure 2-1 (Ritenour et al., 2001). Since the standard germination test is conducted under optimal conditions, even weak seeds have the ability to germinate. In a vigour test, low quality seeds with reduced vigour will deteriorate more rapidly over time.

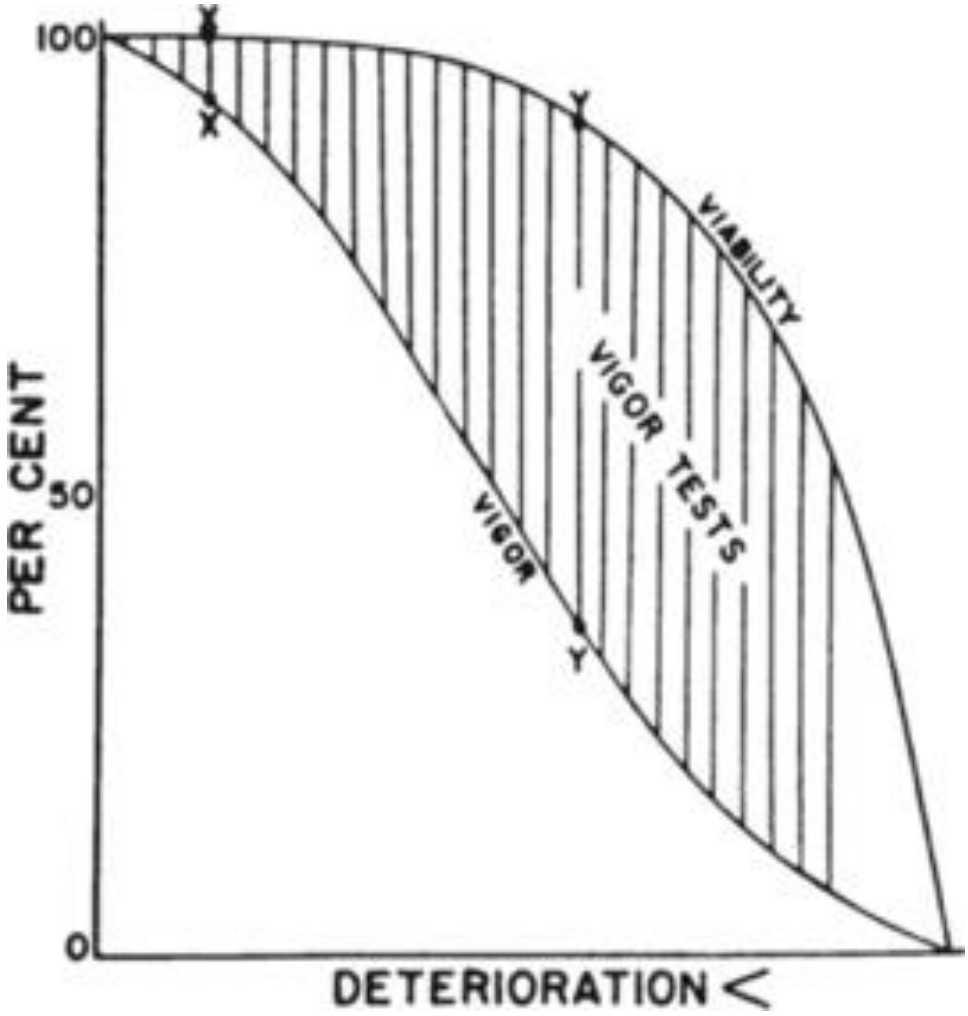

Figure 2-1. The relationship among seed vigour, viability, deterioration and the area application of seed vigour test (Delouche, 1960)

2.3.1 Seed dormancy

The term seed dormancy that may last for several hours to several years has been used to describe two inactive conditions, one resulting from unfavourable environmental conditions and the other due to cellular metabolism imposed germination blocks (Baskin et al., 1998; Nambara et al., 2010). In non-growing conditions, seed moisture content and metabolism rate are low, thus, seeds can survive on their nutrient reserves (Dickie et al., 1990; Desai, 2004). This process is a remarkably complex, effective, and the protective mechanism would ensure the long survival of seeds until when favourable conditions of germination and growth are available (Baskin et al., 1998; Desai, 2004). Such prolonged survival of the embryo within the seed ensures dispersal and distribution to long distance destinations. Many seeds have the ability to adapt to seasonal changes. For example, they have a delayed action mechanism that ensures that the seeds will remain dormant until another growing season comes. The length of dormancy differs greatly among various species and cultivars. For example, lotus seed can remain viable for hundreds of year (Desai, 2004). According to Black et al. (2000) the following factors may prevent the embryo germinate: interference with water uptake and gaseous exchange, presence of chemical inhibitors, seed coats functions as a barrier for release of inhibitors from the embryo, modification of light reaching the embryo, and seed coats exerting a mechanical restraint (Bewley et al., 1982; Dickie et al., 1990; Nambara et al., 2010).

The dormancy process is reversible; sometimes, the seed may turn in to secondary dormancy by exposure to oxygen deficiency, an excess of water, or a temperature too high for germination (Desai, 2004). Dormancy can be terminated by some factors that are not required for germination, but are needed to prime the seed so that it can respond to conditions favourable for germination. Some seeds may need several weeks chilling at

4°C before germination; however, a higher temperature is generally needed to support germination (Baskin et al., 1998; Desai, 2004). Also dormancy can be broken by light, however, most seed germinate under a dark condition (Dickie et al., 1990). Previous studies suggest that chilling and light are factors not strictly required throughout germination but only to trigger or potentiate the process (Bewley et al., 1982; Walck et al., 2011).

The mechanisms of dormancy and its release involve the interaction of inhibitors and growth-promoting hormones, such as gibberellins (GA), cytokines, ethylene, and abscisic acid (ABA) (Desai, 2004). According to this model illustrated in Figure 2-2, ambient environmental factors affect the balance of ABA and GA. The dormant state is induced by ABA synthesis and GA catabolism, whereas germination is promoted by ABA catabolism and GA synthesis. Hence the dormancy cycling is caused by a complex interplay among hormone degradation and synthesis pathways in accordance with ambient environmental changes (Finch‐Savage et al., 2006).

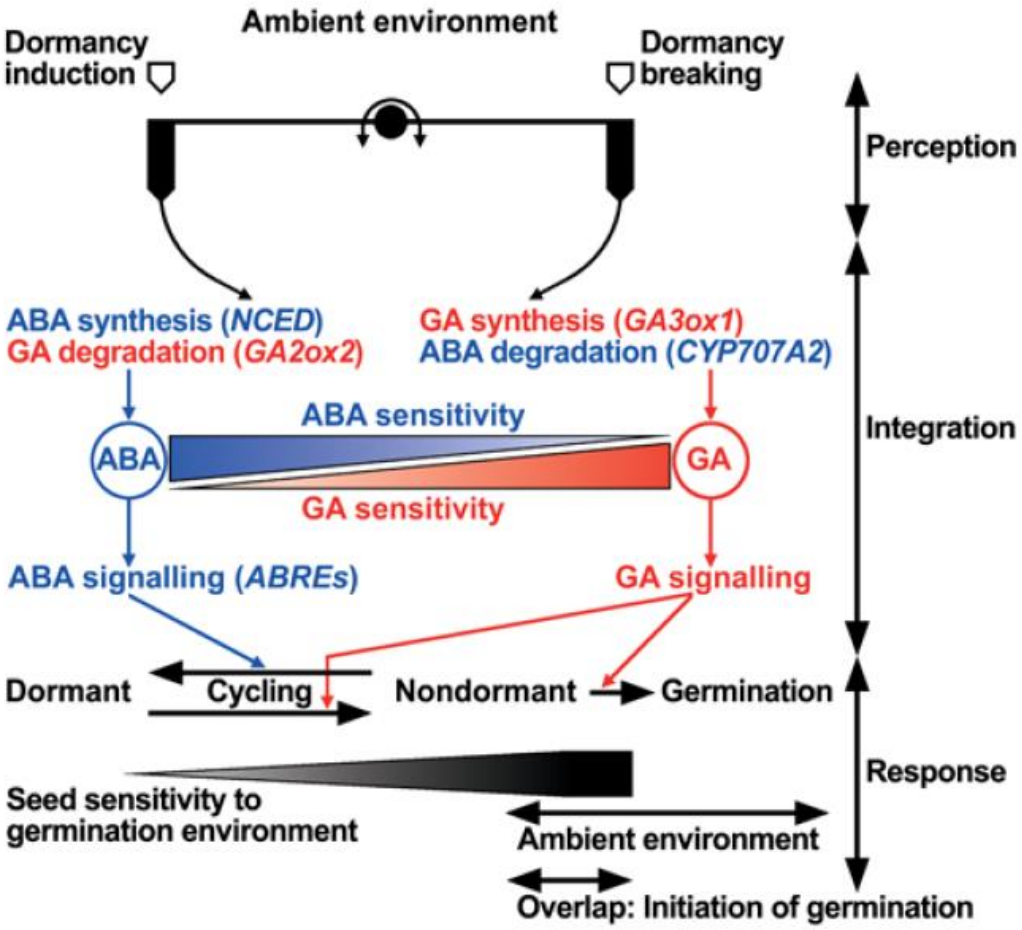

Figure 2-2 Model for the regulation of dormancy and germination by abscisic acid (ABA) and gibberellin acid (GA) in response to the environment (Finch-Savage et al., 2006).

2.3.2 Seed membrane integrity

Cellular membranes are proposed as the primary sites of injury during storage of seeds (Bailly, 2004). The accelerated seed aging treatment reduces the cell membrane phospholipids, which leads to a reduction in seed viability. However, such marked changes do not occur during normal long-term aging. Seed aging at high relative humidity may cause hydrolysis of important cellular component including membranes, which cannot be replaced or repaired due to insufficient activity of appropriate synthetic processes (Powell, 1986; Morales et al., 2012b). Also, along the aging process, free radicles are formed and may cause damages to the membrane lipids. Therefore, the slow germination and growth, abnormal growth, or even no growth could have a strong link to fundamental changes in membranes and macromolecules (Bewley et al., 1982).

2.3.3 Protein expression

Changes characterized during the aging process in seeds include alterations in membrane protein composition, protein degradation, decreases in lipid content and decreases in mRNA translation (Noodén, 2012; Delahaie et al., 2013). Increased accessibility of proteins to proteinases due to decompartmentation, enhanced proteolysis by molecular modifications to polypeptides, and the increased activity of proteinases are considered to be three main leading reasons for protein degradation (Kumar et al., 1999; Shaban, 2013). Proteins in dry seeds are considered to be resistant to degradation in adverse environment conditions (Golovina et al., 1997) although current hypotheses on seed aging have implicated per-oxidative damage to enzymes as a major cause of seed aging (Walters, 2007). In orthodox seeds, the presence of specific proteins such as the late embryogenesis abundant (LEA) proteins, heat shock proteins (HSPs), and some seed storage proteins are associated with desiccation tolerance and maintenance of a quiescent

state. A close relationship seems to exist between the abundance of some of these stress proteins and seed longevity (Rajjou, 2008a). One of the most striking characteristics of mature orthodox seeds is their ability to withstand severe desiccation, as water content decreases during the late maturation stage of seed development. The 'late embryogenesis abundant' (LEA) proteins have been found associated with several kinds of desiccation tolerance, and they are especially more abundant during late embryogenesis than during mid-embryogenesis as their name indicates (Shih et al., 2008). Under stress conditions in seeds, specific stress proteins may be formed and be involved in avoiding stress, repairing damage, or protecting cellular machinery from the effects of stress (Rajjou, 2008b).

2.4 Detecting seed aging

Because a standard germination test is a less sensitive index of seed quality than the vigour test, many studies suggest that the loss of germination could serve as a basis for vigour test. According to ISTA (International Seed Testing Association), vigour test can be classified into various categories based on the method (Baalbaki et al., 2009). For example, vigour test can be classified into physiological and biochemical test based on which component is measured. Biochemical tests look at protein analysis, respiration analysis, or reactions related to the gene expression, while physiological tests measure seedling vigour and viability (Moore, 1973).

2.4.1 Seed vigour measurements

The test relies on activity of dehydrogenase enzymes in the embryo tissue (Moore, 1973; Baalbaki et al., 2009) and provides a quick estimate of seed viability (Neljubow, 1925). In 2000, both AOSA (Association of Official Seed Analysts) and ISTA recommended the

Tetrazolium test (Baalbaki et al., 2009) for seed viability determination. All living tissues, display dehydrogenase activity, which in highly reduced state produces hydrogen ions that reduce the colorless tetrazolium salt 2,3,5-triphenyl-tetrazolium-chloride (TTC) to generate the stable, red-coloured product formazan (Figure 2-3). Five classes of staining patterns can be distinguished according to the intensity of red color by formazan in the seed tissues (Steiner, 2003).

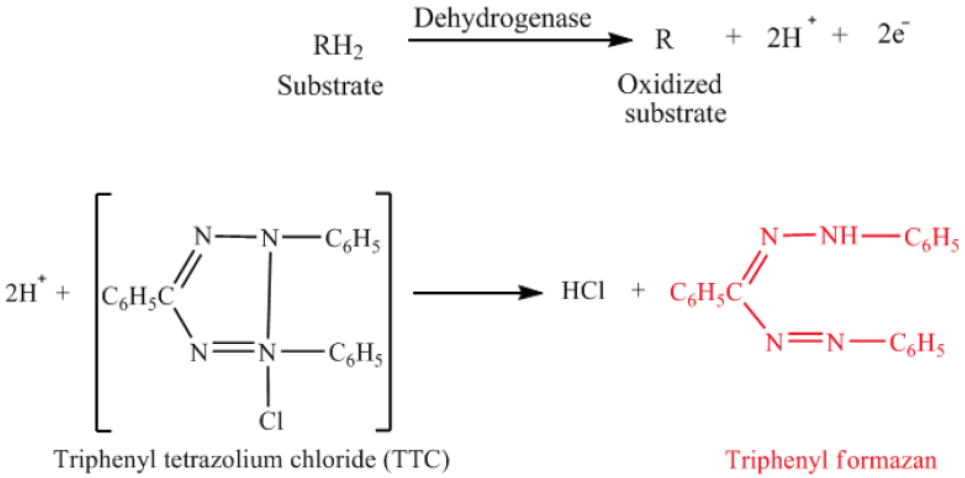

Figure 2-3 Mechanism showing the role of dehydrogenase in the reduction of 2,3,5-triphenyl-tetrazolium-chloride (TTC) to triphenyl formazan (TF) (Brooks, 2011)

When formazan is formed in the seed tissue, the resulting red colour is a positive indication of viability by indirectly detecting cellular respiratory activity occurring in the mitochondria. The non-viable seed and low-viable seed do not react with TTC and consequently do not show the red colour. Respiring tissue in seed can be found within the embryo of a seed, in cotyledons and radicle, in some nutritive endosperm tissues, and also in the female gametophyte tissue of gymnosperms.

The Tetrazolium test generally shows a significant correlation between reduction of the dye, respiration and seedling vigour. However, under aging treatments and particularly during accelerated seed aging, a loss of seed viability occurs without concomitant loss of dehydrogenase enzymes leading to an overestimating of seed viability (Bewley et al., 1982).

2.4.2 Electrolyte conductivity test

The electrolyte conductivity (EC) test is a simple valuable quantitative test to assess seed vigour for many plant species. The test measures seed electrolyte leakage caused by inorganic ions passing through cell membranes (Baalbaki et al., 2009). During seed soaking, ions diffuse out through the cell membrane that can be detected by monitoring electrolytes. Low quality seeds with membrane damage caused by stress or aging leak high amounts of solutes (Powell, 1986). In contrast, seeds with high vigour and germination ability show low electrolyte conductivity.

In living cells, membrane integrity is not only important for keeping cellular compartmentalization but is also the site for biochemical reactions (Abdul-Baki, 1980). Leakage of soluble compounds also occurs during the rehydration of dry seeds. Seed membrane reorganize during this process, which means the sooner the seed membranes are reestablish, the lower is the electrolyte conductivity (Black et al., 2000).

There are many factors that affect seed electrolyte conductivity test results. Since seed membrane integrity changes during the seed development as well as during storage, the seed mortality and quality are strongly associated with seed solute leakage (Powell, 1986). Loss of protection by seed coat or the presences of mechanically damaged seed significantly increase seed conductivity. To keep the accuracy of the electrolyte conductivity test, damaged seeds should be removed before the test (Desai, 2004). Large

seeds leak more electrolyte than the small seeds of equivalent quality. To eliminate the difference in seed size, the electrolyte conductivity tests are usually expressed per gram seeds. If the seeds have been fungicide treated before the test, the treated seeds need to be washed prior to the test (Baalbaki et al., 2009).

2.4.3 Methods of seed protein analysis

2.4.3.1 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a widely used method to separate proteins according to their molecular mass (Schägger et al., 1987). It is reliable for determination of molecular weights for most polypeptides, but can also be used for assay of protein purity (Cohen et al., 1987). Protein samples are mixed with Tris buffer containing SDS and the reducing agent β-mercaptoethanol followed by boiling to denature the proteins and reduce their disulphide bonds. The anionic detergent SDS aids in the denaturation of secondary and non-disulphide- linked tertiary structures and adds negative charges to each polypeptide in proportion to their mass (Pitt-Rivers et al., 1968) (Bergfors, 2009).

Electrophoresis using a gel composed of a lower resolving gel and an upper stacking gel with pH 8.8 and 6.8, respectively, separates individual polypeptides. The gel sections are made up by an acrylamide: bisacrylamide mixture that is polymerized to a network by the action of APS (Amonium persulphate) and TEMED (Tetramethylethylenediamine) in the presence of 10 % SDS and appropriate Tris-HCl buffer. The concentration of acrylamide in resolving gel may vary from 5% to 25 % (w/v) to generate different resolution power during electrophoresis (Raymond et al., 1959). A low percentage polyacrylamide gel generates larger pore sizes, which is preferred for separation of high molecular weight proteins. Bromophenol blue is commonly used as tracking dye for the electrophoresis as

the small negatively charged dye molecules migrate faster than the SDS-coated polypeptides (Flores, 1978). The separated polypeptides can subsequently be visualized as distinct bands in the gel with low background using the Coomassive brilliant blue staining technique (Steinberg, 2009). For low-abundant polypeptides, silver staining is preferred as it is a more sensitive protein staining method than Coomassive staining (Bergfors, 2009).

2.4.3.2 Two-dimensional polyacrylamide gel electrophoresis (2D–PAGE)

Two dimensional gel electrophoresis (2-DE) is a well-established method for high resolution profiling of low abundance proteins (Issaq et al., 2008). 2-DE starts with one-dimensional gel electrophoresis, and followed by separation of molecules are isoelectric point (isoelectric focusing, IEF) in a direction 90 degree from the first separation (Janson, 2012). Thereby, a gradient of pH is applied to the gel besides the applied electrical potential. In the pH gradient gel, proteins will be charged if the pH value different than their isoelectric points (Janson, 2012). The positive charged proteins will migrates towards the negative side of the gel according to their mass to charge ratio. Protein can be detected after the separation by silver or Commasive brilliant blue staining (Issaq et al., 2008). Even though the 2-DE technique is relative sensitive for protein analysis, however, it is still considered to be a time consuming and labour intensive technique. Also the potential of 2-DE for proteome analysis is limited to proteins of medium to low abundance (Janson, 2012). The difficulty in reproducibility between gels leads to increased system variations, which made it more difficult to tell whether the difference was caused by system or the biological change (Rabilloud et al., 2010).

2.4.3.3 Zymography

Zymography is a widely used functional assay for analysing proteolytic activity; in particular for the matrix metalloproteinases (MMPs) (Kupai et al., 2010). This simple, sensitive quantitative standard method was first introduced by Granelli-Piperno and Reich, and modified by Heussen, which then became a routine technique in cancer research around the world (Granelli-Piperno et al., 1978; Heussen et al., 1980; Leber et al., 1997). The zymography provides reliable identification of proteolytic active polypeptide based on the molecular mass of inactive and active forms after SDS-PAGE. Proteases analyzed by zymography cleave protein by peptide bond hydrolysis, but can also after gel separation re-nature as well as exert proteolytic activity on a copolymerized substrate upon removal of the SDS (Hu et al., 2010). The size of proteolysis product can be revealed by Commasive blue staining of SDS-PAGE gel. The amount of proteases correlates linear with the band intensity within a certain size range (Leber et al., 1997). The zymography techniques offers several benefits features when compare to other methods, for example: no requirement of expensive materials such as antibodies, as well as the flexibility in changing of substrate types, and reaction buffers, etc. However, there still some concern about the accuracy of proteolytic activity determination (Hu et al., 2010). Besides, the two-steps standing/ distaining is critical and complicated for the accurate measurement of proteolytic activity (Hughes et al., 2010).

2.4.3.4 Western blot assay

The Western blot (also called protein immunoblot) is a widely used technique to detect specific proteins in given samples, which was developed by Harry Towbin (Towbin et al., 1979). Proteins are separated by SDS-PAGE, and then transferred from gel to membrane

by electrophoretic transfer. Generally, two type of membranes are used in the western blot, the nitrocellulose and polyvinylidene fluoride (PVDF) membranes. The nitrocellulose membrane has high affinity for protein and preserves protein integrity and gives low background signals (Fernandez et al., 1992). However, nitrocellulose is fragile and does not allow for re-probing of membrane. Compared with the nitrocellulose membrane, PVDF membrane has a superior on protein retention capacity, and has high mechanical strength, but gives higher background signals (Fernandez et al., 1992).

The membrane containing bound proteins is incubated with primary antibody specific for the target protein (Towbin et al., 1979). Subsequently, un-bound proteins are washed off before membrane is incubated with the secondary antibody specific for target primary antibody. The identification of the target is ensured by the specificity of the antibody-antigen interaction, and the thickness of the band can be a good indicator of protein concentration.

2.4.3.5 MudPIT (Multidimensional Protein Identification Technology)

Multidimensional protein identification technology is a widely used non-gel technique for separating and identifying individual components of the mixture of protein and peptide (Kislinger et al., 2005). It has been proven to be a qualitative and quantitative tool for proteomic analyses (Chen et al., 2006). In MudPIT, a complex mixture of protein and peptide is digested and loaded directly to a triphasic micro capillary column packed with reversed phase, strong cation exchange, and high-pressure liquid chromatography (HPLC) grade materials (Delahunty et al., 2007). The column is placed directly in-line with a tandem mass spectrometer after the load of complex peptide mixture onto the triphasic micro capillary column. The tandem mass spectrometry peptide data generated

from a MudPIT run is then analyzed to determine the protein composition of the original sample (Delahunty et al., 2007).

2.4.4 Artificial aging tests

Seeds can be subjected to rapid aging processes (artificial aging) by accelerated seed aging, saturated salt seed aging or controlled seed deterioration procedures. In most artificial aging tests, seeds are exposed to high temperature and high humidity conditions, followed by physiological and biochemical tests. High quality seed lots can withstand those stress conditions and show slower deterioration rate and germinate faster than seeds with poor vigour (Ching, 1972; Rice et al., 2001; Rodo et al., 2003). Thus, artificial aging test is an indicator of seed quality and vigour. Compared with the natural aging test, artificial aging tests are rapid and correlate with field performance of seeds. Therefore, artificial aging can provide valuable information of both storage potential of seeds and their field emergence (Baalbaki et al., 2009).

2.4.4.1 Accelerated aging test

Accelerated seed aging test was initially developed to estimate the longevity of seed in commercial storage (Delouche et al., 1973), but has also been used to predict the life span of a number of other seeds. In the accelerated aging test, seeds are exposed to high temperature (45°C) and high humidity (95% RH), which are the two main factors causing seed aging (Walters, 1998). When conducting accelerated seed aging test, seeds are place inside a two layer sealed plastic box (one layer is for water) or a desiccator. The two layer plastic box/desiccator is placed in a water jacketed accelerated aging chamber. The temperature is controlled at 41-45 °C and relative humidity is maintained at 95%. Seeds

are sampled during the selected aging period and tested by germination and seedling number as well as by biochemical tests (Baalbaki et al., 2009).

2.4.4.2 Saturated salt accelerated seed aging test

Saturated salt accelerated seed aging test is a modification of accelerated seed aging, where saturated salt solutions such as LiCl, NaCl or KCl are used instead of water to create a suitable aging environment for both large and small seeds. The saturated salt solution reduces the relative humidity, allowing soaking, and thus slows down the seed deterioration rate. The relative humidity below 80% prevents the storage fungal growth, which can influence the accelerated seed aging process (Zhang et al., 1997).

2.4.4.3 Controlled deterioration test

The controlled deterioration test was initially developed to estimates the difference in seed vigour among small seeded species (Hampton et al., 1995). It is also a modification of accelerated seed aging test where seeds are placed in high temperature and high humidity stress for a short period of time. The main difference between controlled deterioration and accelerated seed aging tests is that in the controlled aging test, seeds are pre-moist at a certain level before being subjected to high temperature stress conditions. The equilibrium process minimizes the seed moisture change that would cause the difference in seed deterioration but not the real difference in seed quality (Baalbaki et al., 2009). Besides, the equilibrated seeds eliminate the difference in water absorbance, electrolyte leakage and conductivity test, thus enhancing the accuracy of the test. One limitation of controlled deterioration test is that it may need a long time to reach the equilibrium moisture stage (Rodo et al., 2003).

3 MATERIALS AND METHODS

3.1 Plant materials

Six shrub species identified by the vegetation cooperative of CONRAD (Canadian Oil-sands Network for Research and Development) were used in this study. The species included *Prunus virginiana*, *Prunus pensylvanica*, *Arctostaphylos uva-ursi*, *Shepherdia canadensis, Cornus sericea,* and *Viburnum edule*. Seeds were collected from the species grown in the central mix-wood region of north-eastern Alberta during 2009 to 2012 (Table 3-1) and seed lots were cleaned following the procedures described in the fact sheets from Wild Rose Consulting. Cleaned seeds were air dried at room temperature to constant weight (approximately 7-8% dry weight water content) and then transported to Department of Plant Sciences, University of Saskatchewan for initial seed quality assessment and storage.

Table 3-1 Shrub seed lots collected from central mixed-wood region of north-eastern Alberta from 2009 to 2012 used in the study.

Species	Seed lot and collection year			
	1984	2009	2010	2012
Bearberry (*Arctostaphylos uva-ursi*)	BUF 8484		BEAR 1117 BEAR 1618	
Buffalo berry (*Shepherdia canadensis*)		BUF 14093 BUF 4906		BUF 9134 BUF 1424
Chokecherry (*Prunus virginiana*)			CHK 1410 CHK 6259	CHK 1114
Dogwood (*Cornus sericea*)		DOG 3914 DOG 6930	DOG 3119 DOG 3259	
Low bush cranberry (*Viburnum edule*)			LBC 3119 LBC 7278	
Pin cherry (*Prunus pensylvanica*)	PIN 8484		PIN 1298	

Table 3-2. Seed germination conditions.

Species	Stratification (weeks)		Light	Germination temperature day/night (°C)
	Warm	Cold		
Bearberry (*Arctostaphylos uva-ursi*)	9	7	NO	15/5
Buffalo berry (*Shepherdia canadensis*)	0	3	YES	15/5
Chokecherry (*Prunus virginiana*)	9	8	NO	30/20
Dogwood (*Cornus sericea*)	3	0	NO	30/20
Low bush cranberry (*Viburnum edule*)	16	2	NO	15/5
Pin cherry (*Prunus pensylvanica*)	9	10	NO	15/5

Warm stratification was conducted at 30/20 °C and cold stratification was conducted at 4°C.

3.2 Methods

3.2.1 Initial seed quality assessments

3.2.1.1 Determination of seed moisture content

Determination of the initial seed moisture content (MC) by the oven-drying method (Baalbaki et al., 2009) was done before storage and within one month of seeds being received. The analysis included three samples of 10-30 seeds per collection depending on seed size and seed availability. Moisture content was calculated using the formula:

$$MC\ \% = \frac{Fresh\ Weight\ -\ Dry\ Weight}{Fresh\ Weight} \times 100 \qquad \text{Equation 3-1}$$

3.2.1.2 Determination of thousand seed weight

According AOSA (Baalbaki et al., 2009), the determination of thousand seed weight was completed by weight 100 seeds for eight replicate samples, separately; then calculate the average weight of 100 seeds, and multiply by 10.

3.2.1.3 Analysis of seed viability by TZ test

Tetrazolium (TZ) tests were conducted to assess seed viability following procedures established by the International Seed Testing Association (1985). Three replicates of 10 seeds were placed in Petri dishes containing two layers of filter papers soaked with 5 mL distilled water. Upon 24 h incubation at room temperature, the seeds were cut and submerged in a 1% (w/v) solution of 2,3,5 triphrnyl tetrazolium chloride (TTC) for 24 h at room temperature. The production of the red substance formazan from TTC in living tissues by the action of dehydrogenase enzymes was examined on the radicle, plumule and cotyledons.

3.2.1.4 Cold and warm stratification of seeds

Seeds of all shrub species were in different degrees of dormancy. Thus, treatments such as cold stratification (4 °C) and/or warm stratification (30/20 °C, 12/12 h) were required to break dormancy before subjecting the seeds to germination tests (Table 3-1) (Baskin et al., 1998).

3.2.1.5 Seed germination

Seeds were germinated at different sets of alternating temperatures (15/5, 20/10, 25/15, 30/20, and 35/25 °C) with or without light (12 h) depending on the dormancy-breaking requirements of each species. The seeds were placed on top of two layers of filter papers moistened with 5 mL distilled water and kept in 9 cm Petri dishes. The Petri dishes were enclosed in clear plastic bags to reduce the evaporation. During the course of the experiments, the seeds with any sign of fungal infection were sprayed with 0.05% Benomyl 50 Systemic Fungicide solution. The germinated seeds were counted and removed every 24 h. The seeds were considered germinated when the radicle was no less than 2 mm in length. These Un-germinated seeds at the end of the germination test were used by TZ test for viability. The seed viability of each collection was then corrected using germination data. The germinated seeds were incubated at 30/20 °C with light. Seedling length, as an indicator of seed vigour, was measured after 7 d (Vertucci et al., 1990).

3.2.2 Seed storage conditions

3.2.2.1 Effects of moisture and temperature on seed longevity during storage

Two chokecherry and two pin cherry seed collections: CHK 1410, CHK 6259, PIN 1298, PIN 1618 were used for this experiment (Table 3-1). The seeds obtained from 2010 were

stored at various storage conditions with target seed moisture contents (MC) of 1-3% and 7-8%, respectively, within one month after receiving. The target seed moisture content was achieved by placing seeds in sealed glass desiccators with re-generable humidity sponges (VWR international, Mississauga, Ontario, Canada). They were then stored in separate vacuum desiccators. Six spot humidity indicator cards (6HIC200-SCC, Static Control Components, USA) and Incand multi-channel in-out thermo-hydrometers (Traceable® 15551-258, Control Company, USA) were placed in desiccators to monitor relative humidity (RH) and temperature, respectively. The RH inside desiccators was maintained at 20 % and 30 % for target seed moisture contents 1-3 % and 7-8 %, respectively, by changing humidity sponges when needed. Three replicates were used for each storage treatment.

3.2.2.2 Gas storage experiment

In the N_2 treatment, air in desiccators was drawn out and N_2 was injected into desiccators, which was repeated three times. A small balloon was placed inside the desiccator as an indicator for air pressure. N_2 was replaced monthly. The seeds with moisture content of 7-8% and stored at room temperature in air are set to be the control group.

3.2.3 Artificial aging

Seventeen collections of six native shrub species from boreal forest region, BEAR 1117, BEAR 1618, BUF 14903, BUF1424, BUF 4906, BUF 9134, BUF8484, CHK 1410, CHK 1114, PIN 1298, PIN 8484, DOG 3119, DOG 3259, DOG 6930, DOG 9314, LBC 7278 and LBC 3119, were used for this experiment. The seeds were first rehydrated at 22.5 °C with 45% RH, and then aged at 45 °C with 60% RH by using lithium chloride to provide the desired RH environments (Zhang et al., 1997; Rodo et al., 2003).

3.2.3.1 Rehydration

Seeds were placed in paper envelopes over chemical-porcelain desiccator plates placed in sealed KIMAX desiccators with detachable stopcock valve (VWR international, Mississauga, Ontario, Canada) for four weeks, following a procedure developed by Butler (2009). The 45% RH inside the desiccator was generated by placing 1L 40.5 % w/v lithium chloride solution at the bottom of the desiccator. Seed moisture content was monitored every two days.

3.2.3.2 Accelerated seed aging

Equilibrated seeds were subject to accelerated aging treatments in growth chamber (Sanyo Versatile Environment Chamber MLR-350H, Sanyo Scientific, USA) set at 60 % RH, 45°C on chemical-porcelain desiccator plates (VWR desiccator plates, VWR International, Mississauga, Ontario, Canada) in sealed glass desiccators (KIMAX Desiccators with detachable stopcock valve, Kimble Chase) following a method described by Kochanek (2009). The 60 % RH was generated by 1L, 30 % w/v LiCl solution placed at the bottom of the desiccator. The relative humidity and temperature inside the desiccator monitored by using radio-signal remote hygrometer/thermometer (Traceable® 15551-258, VWR International, Mississauga, Ontario, Canada). Three samples of 25-30 seeds for each collection were removed after each of 0, 5, 10, 15, and 20 d, respectively, for seed viability, germination, electrolyte conductivity and protein analysis. This experiment was repeated once. 0 d aged seeds are considered to be controlled group.

3.2.4 Biochemical and molecular changes during seed storage

3.2.4.1 Analysis of electrolyte conductivity

Three replicates of 15 seeds were weighted and soaked in 10 mL distilled water for 48 h at 22.5 °C (Baalbaki et al., 2009). The conductivity of seed soaked water was measured using an Oakton portable waterproof pH/CON 300 Meter (Oakton Instruments, Vernon Hills, IL, USA) after 0, 2, 5, 12, 24 and 48 h soaking, respectively. Total ion leakage was expressed as per g of seeds ($\mu S\ cm^{-1}g^{-1}$). Average electrolyte conductivity was calculated using mean and SE of 0, 2, 5, 12, 24 and 48 h ion electrolyte conductivity measurements.

3.2.4.2 Phenol extraction of seed proteins

Total proteins from seeds were extracted using a modified phenol extraction procedure described by Van Etten et al (1979). Seed samples (0.5 g) were pulverized by mortar and pestle grinding, and then transferred to test tubes containing 50 mg polyvinylpolypyrrolidone (PVPP). In rapid succession, 2.5 mL of cold (4 °C) extraction buffer (250 mM Tris-HCl, pH 7.5, 700 mM sucrose, 100 mM KCl, 50 mM EDTA) and 25 µl β-mercaptoethanol were added. Samples were then homogenized for 30 sec using a Polytron homogenizer (Polytron Corp, Elkhart, Ind. USA) at high-speed setting. Thereafter, 5 mL of Tris-HCl (Molarity mass: 121.4 g mol^{-1}) buffered phenol (pH 7.9) was added and homogenates were vortexed for 2 min. After centrifugation at 5,000 ×g for 30 min, the upper phenol layer was recovered. To further purify the phenol-soluble proteins, the phenol solution was re-extracted twice adding an equal volume extraction buffer, vortexing for 1 min followed by 20 min centrifugation at 5,000 ×g. A 12.5 mL cold methanol solution containing 100 mM ammonium acetate and 10 mM β-

mercaptoethanol was added to the phenol solution followed by overnight precipitation of proteins at 4 °C.

Precipitated proteins were recovered by centrifugation at 12,000 ×g for 10 min and washed twice with 1 mL methanol, and twice with 1 mL acetone containing 10 mM β-mercaptoethanol. Sample protein (dissolved in sample buffer) concentration was determined in triplicates by a dye-binding assay (Bradford, Bio-Rad. Hercules, CA, USA) using Coomassie Blue reagent (Bradford, 1976).

3.2.4.3 Tris-HCl buffer extraction of seed proteins

Seed samples (1 g) were crushed in liquid nitrogen into fine powder using a mortar. Proteins were extracted from 500 mg powder by re-suspension in 2 mL extraction buffer (0.5 M Tris-HCl pH 6.8, 10 % (w/v) sodium-dodecyl-sulphate, 10 % (v/v) glycerol, 25 µL β-mercaptoethanol and 0.05 % bromophenol blue) followed by boiling for 5 min, cooling on ice for 5 min and centrifugation at 12,500 ×g, 12 min, room temperature. The protein concentration in soluble extract was determined in triplicates using a Coomassie brilliant blue dye-binding assay (Bradford, 1976).

3.2.4.4 SDS-PAGE

Protein samples (35 µg) were analyzed by one-dimensional sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE), as described (Demeke et al., 1997).

For SDS-PAGE, the seed protein pellet was air-dried, re-suspended in SDS buffer (25 mM Tris, pH 7.5, 1% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, and 0.002% (w/v) bromphenol blue), and boiled for 2 min. The 12% resolving gel contained acrylamide/ bis-acrylamide (37.5:1), 1.5 M Tris-HCl pH 8.8, 10% (w/v) sodium-dodecyl-sulphate, 10% ammonium persulphate, and 0.05% N, N, N', N'-

tetramethylethylenediamine (TEMED). The 5% stacking gel contained 30% acrylamide/ bisacrylamide, 1 M Tris-HCl pH 6.8, 10% (w/v) sodium-dodecyl-sulphate, 10% ammonium persulphate and 0.05% TEMED. Running buffer contained 25 mM TRIS-base, 192 mM glycine and 0.1% sodium-dodecyl-sulphate. A 35 µL of the extracted protein was loaded and proteins were separated using a Bio-Rad electrophoresis unit (Protean II, Bio-Rad. Hercules, CA, USA) for 15 h at 10 mA.

Following electrophoresis, the stacking gel was removed and resolving gel was soaked in fixative (40% methanol, 10% glacial acetic acid) for 1/2 h. Separated polypeptides were then visualized by Coomassie blue staining as described by Fenner (1975). The gel was destained by agitation in 40% methanol, 10% glacial acetic acid) solution for 4 to 8 h as described by Demeke (1997).

3.2.4.5 Western blotting of dehydrins

The stacking gel was removed from the SDS-PAGE gel with separated polypeptides and a notch was made at the bottom left corner to mark gel orientation. The gel was equilibrated in transfer buffer (40 mM TRIS/ 20 mM NaAc3H_2O pH 7.4, 2 mM EDTA, 20% methanol, 0.05% (w/v) sodium-dodecyl-sulphate) for 30 min. Four, 1.5 mm blotting papers and 1 nitrocellulose (NC) membrane (0.45 m, GE Healthcare, Montreal, Quebec, Canada) pre-soaked in transfer buffer were assembled into a sandwich in tray with transfer buffer to minimize the risk of trapping bubbles during assembly. The transfer sandwich was assembled as follows: support pad, two sheets blotting paper, nitrocellulose membrane, polyacrylamide gel, two sheets blotting papers and support pad. The complete sandwich was placed in the transfer tank (Protean II, Bio-Rad. Hercules, CA, USA) with the membrane closest to the positive electrode, and the tank was filled

with cooled transfer buffer (4 °C). Electro-blot was done at 10 volts with 1.4-1.7 v/cm for at least 4 h. Membrane was dried at room temperature and stored in a dry place (Ganeshan et al., 2008).

The membrane was placed in 200 mL blocking buffer [5% (w/v) low fat Carnation Milk, 137 mM NaCl, 2.68 mM KCl, 10 mM Na_2HPO_4/1.76 mM KH_2PO_4 pH 7.4, 0.1% Tween 20], and incubated with slow agitation for at least 2 h at room temperature Then the nitrocellulose membrane was incubated with 50 mL 1:5,000 diluted anti-dehydrin antibodies (StressGen, Ann Arbor, Michigan, USA) in blocking buffer for at least 2 h (Ganeshan et al., 2008).

To remove excess primary antibodies, the membrane was washed four times with blocking buffer. The membrane was then incubated for 2 h with 50 mL 1:5000 diluted secondary antibodies (alkaline phosphatase labelled goat anti-rabbit IgG (KPL, Inc., Maryland, USA) in blocking buffer with slow agitation. The membrane was washed four times with blocking buffer, and then washed three times with New Buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl].

The BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) -NBT (nitro-blue tetrazolium chloride) color substrate solution was prepared just prior to use and protected from light. NBT stock (75 mg/mL Nitroblue tetrazolium in 70% N, N-dimethylformamide) was diluted in color development solution to a final concentration of 0.3 mg/mL BCIP (50 mg/mL) was added to a final concentration of 0.15 mg/mL. To remove excess buffer, the membrane was blotted with Whatman 3 mm paper, and then immersed in 25 mL color development solution for color band developement in darkness. When desired development was reached, usually within 20-30 min, membrane was rinsed

twice with New buffer before immersing the membrane in (20 mM Tris-HCl (pH 2.9), 1 mM EDTA) to stop color development (Ganeshan et al., 2008).

3.2.5 Data analysis

The speed of germination was calculated by using germination rate index (G.R.I.) (Caldwell, 1960; Brar et al., 1991), which is computed by using the following formula:

$$G.R.I=\sum_{i=1}^{c}\frac{N_i}{N_t*T_i}*100\% \qquad \text{Equation 3-2}$$

Where, N_i is the number of seeds germinated in day *i*, N_t is the total number of seed germination, T_i is the *i*th day since germination started and c is the number of days counted in the experiment (Baalbaki et al., 2009).

Seed vigour index (S.V.I) was calculated using the following formula (Baalbaki et al., 2009):

$$S.V.I= \text{Final germination percentage}* \text{Seedling length} \qquad \text{Equation 3-3}$$

Mean seeds germination time (MGT) was proposed by Ellis and Roberts (1980) as an indicator of vigour, calculated as following:

$$MGT = \frac{\sum N_i T_i}{\sum N_i} \qquad \text{Equation 3-4}$$

where N_i is the number of normal seedlings present on day *i*, T_i is the *i*th day since germination begins and ΣN_i is final germination.

The Viability after germination (including germinated seeds) after germination test (VAG) was calculated as following:

$$\text{VAG} = \frac{N_G + N_V}{N_A} * 100\ \% \qquad \text{Equation 3-5}$$

where N_G is the number of total germinated seeds; N_V is the number of viable un-germinated seeds, N_A is the total number of seeds.

Analysis of Variance was conducted using general linear model (GLM) to determine the difference in one-year storage experiment and artificial aging treatments on seed viability, final germination percentage, mean germination time, electrolyte conductivity, Germination rate index and vigour index for shrub species. Seed species, storage temperature, germination temperature, gas, and moisture content were considered as fixed factors in the storage experiment; while duration of aging and species were considered as fixed factors in artificial aging test. Final germination percentage, viability, electrolyte conductivity value, mean germination time, un-germinated viable seeds, germination rate index, vigour index and radicle / hypocotyl were treated as response variables. Pearson's bivariate correlations were performed for all the measurement in accelerated seed aging test. All analysis for this study was done using in R 3.0.2 (R Development Core Team 2013).

4 RESULTS

4.1 Effect of seed storage conditions on seed physiological properties

4.1.1 Storage of chokecherry seeds

Storage of two chokecherry collections under different conditions revealed no significant difference (P>0.05) on most vigour and viability attributes of seeds after one-year (Figure 4-1, Table 4-1, Table 4-2). Furthermore, total germination percentage was not significantly (P>0.05) altered upon one-year storage. For the CHK 1410 seed lot, the viability after germination percentage (including germinated seeds) was 30-58% at 5/15 °C, and 7-61% at 15/25 °C, respectively. For CHK 6259, seed viability after germination percentage (including germinated seeds) was 40-67% at 5/15 °C, and 45-71% at 15/25 °C, respectably.

For CHK 1410, seeds stored in air at 22.5 °C with 7-8% as well as seeds stored in N_2 at 4 °C showed significant higher values in percentage of un-germinated but viable seeds than seeds stored in air at -20 °C with 1-3% moisture content when germinated at 15/5 °C. For CHK 6259, seeds stored in air at 4 °C with 7-8% moisture content showed significantly higher percentage of un-germinated but viable seeds than seeds stored in air at -20 °C with 1-3% moisture content, 25 °C with two moisture contents and in N_2 at 4 °C when germinated at 25/15 °C.

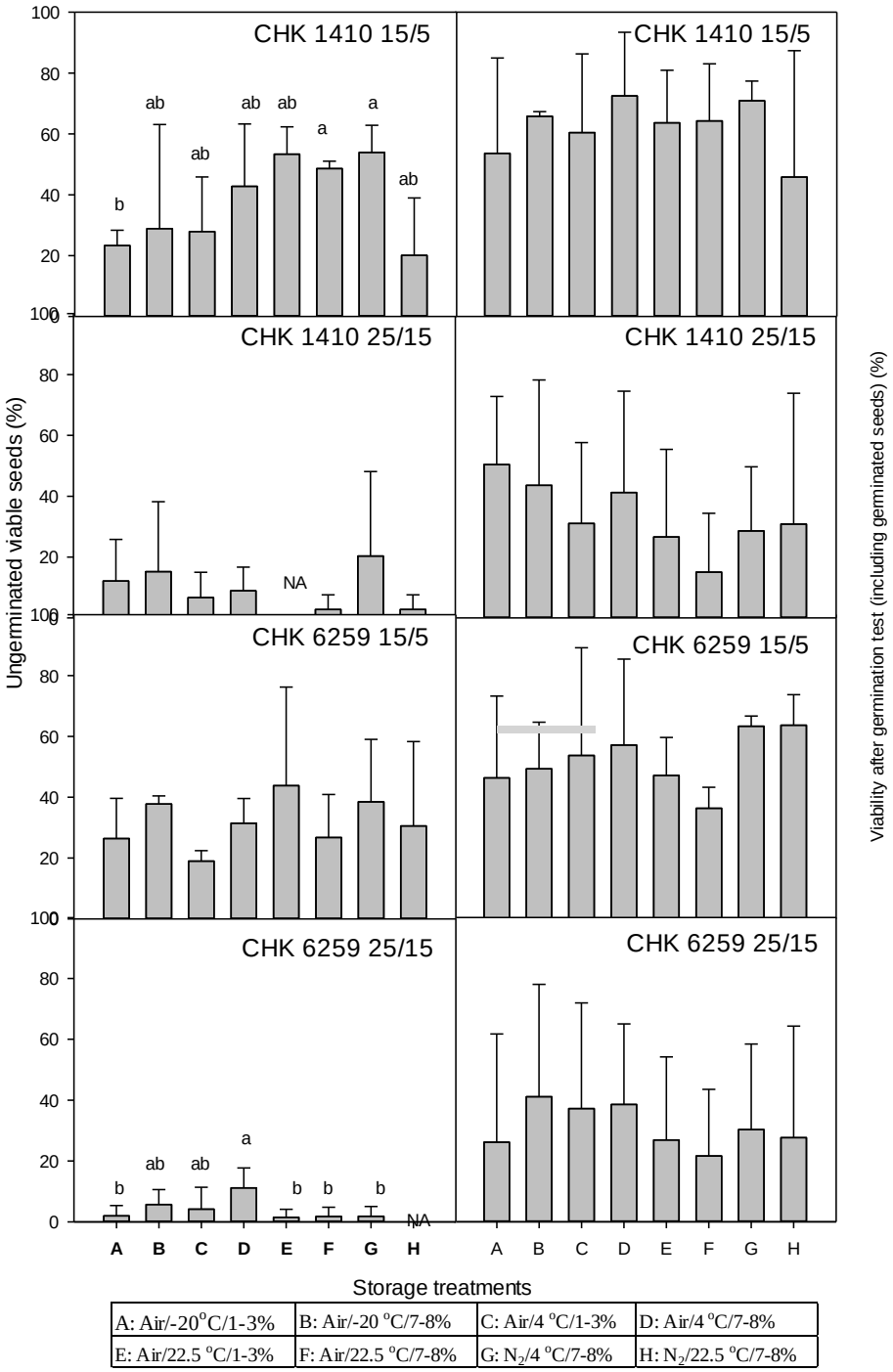

Figure 4-1 Effects of one-year storage treatments on seed viability after germination percentage (including germinated seeds) (right) and percent of un-germinated but viable seeds (left) in two collections of chokecherry at two germination temperatures. Means with same letters within a collection, germination temperature and parameter are not significantly different at $p \leq 0.05$

Table 4-1 Effects of one-year storage treatments on seed vigour and viability in CHK 1410. Data are Means ± SE. Means with the same letters within a collection and parameter are not significantly different at *P≤0.05*.

Gas	T(°C)	MC (%)	EC ($\mu S*cm^{-1}g^{-1}$)	V (%)	GT (15/5°C)				GT (15/25°C)			
					G (%)	UVS (%)	GRI ($\%*d^{-1}$)	VI (mm*%)	G (%)	UVS (%)	GRI ($\%*d^{-1}$)	VI (mm*%)
Initial			-	93±3a	10±2a	-	-	-	33±17a	-	-	-
Air	-20	7/8	109±14a	92±5a	37±34a	29±34ab	1.3±1.1a	831±774a	28±36a	15±23a	3.7±3.4a	801±959a
		1/3	104±14a	96±4a	30±27a	23±5b	1.5±1.4a	731±635a	38±33a	12±14a	2.3±2.1a	1067±929a
	22.5	7/8	106±3a	92±2a	16±17a	49±2ab	0.6±0.6a	393±352a	12±21a	3±5a	1.2±2.1a	474±822a
		1/3	112±25a	93±5a	10±9a	53±9a	1.1±1.5a	206±189a	27±29a	0±0a	2.1±2.6a	662±657a
	4	7/8	87±5a	83±5a	30±7a	43±21ab	1.5±0.2a	710±570a	32±41a	9±8a	2.3±3.1a	833±976a
		1/3	106±9a	96±2a	32±30a	28±18ab	1.0±1.0a	621±928a	24±29a	7±8a	1.9±1.9a	745±757a
N_2	4	7/8	91±23a	93±7a	17±15a	54±9a	1.5±1.9a	373±578a	8±7a	20±28a	0.8±0.7a	255±274a
	22.5	7/8	105±12a	93±5a	26±37a	20±19b	1.6±2.7a	805±961a	28±45a	3±5a	3.0±4.9a	1043±1794a

T= storage temperature, MC=Seed moisture content, G=Final germination percentage, EC=Electrolyte conductivity, UVS= Un-germinated viable seeds, GT= Germination temperature, V= Viability, GRI= Germination rate index, VI= Vigour index

Table 4-2 Effects of one-year storage treatments on seed vigour and viability in CHK 6259. Data are Means ± SE. Means with the same letters within a collection and parameter are not significantly different at *P≤0.05*.

Gas	T (°C)	MC (%)	EC (µS*cm^{-1}g^{-1})	V (%)	GT (15/5°C)				GT (15/25°C)			
					G (%)	UVS (%)	GRI (%*d^{-1})	VI (mm*%)	G (%)	UVS (%)	GRI (%*d^{-1})	VI (mm*%)
Initial			-	98±1a	16±5a	-	-	-	53±4a	-	-	-
Air	-20	7/8	96±13a	95±3a	21±20a	38±3a	1.6±1.5a	478±500a	35±42a	6±5ab	2.3±2.0a	793±807a
		1/3	93±17a	95±4a	29±26a	26±13ab	2.4±2.6a	1040±979a	24±37a	2±3b	1.5±2.3a	936±1502a
	22.5	7/8	107±23a	97±1a	17±22a	27±14ab	1.1±1.0a	233±222a	20±22a	2±3b	1.6±1.7a	958±1223a
		1/3	115±16a	100±0a	13±22a	44±32a	0.7±1.3a	388±672a	25±25a	2±3b	2.2±2.0a	688±678a
	4	7/8	98±14a	97±1a	37±36a	31±8ab	1.5±1.6a	699±623a	21±28a	17±22a	1.8±2.6a	320±437a
		1/3	93±17a	99±1a	45±40a	19±3b	3.8±3.3a	940±851a	33±29a	4±7ab	1.9±1.9a	877±774a
N_2	4	7/8	115±11a	95±3a	31±28a	38±20a	2.4±2.5a	774±677a	28±26a	2±3b	2.2±1.9a	806±750a
	22.5	7/8	126±5a	98±2a	46±40a	31±28ab	2.0±1.8a	1364±1186a	28±37a	0±0b	2.1±2.5a	300±325a

T= storage temperature, MC=Seed moisture content, G=Final germination percentage, EC=Electrolyte conductivity, UVS= Un-germinated viable seeds, GT= Germination temperature, V= Viability, GRI= Germination rate index, VI= Vigour index

4.1.2 Storage of pin cherry seeds

The germination and viability of PIN 1298 did not show any significant difference (P>0.05) between fresh and one-year-stored seeds (Table 4-3, Table 4-4). The highest vigour and highest total germination percentage was found in PIN 1298 stored at -20 °C with 1-3% moisture content comparing to other storage treatments (Figure 4-2). The seed viability after germination (including germinated seeds) was significantly lower than the viability before germination. For PIN 1298, seed viability after germination percentage (including germinated seeds) was 9-60% at 5/15 °C, and 17-34% at 15/25 °C, respectively. For PIN 1298, seeds stored in air with 1-3% at – 20 °C and 4 °C showed higher percentage in un-germinated but viable seed than seeds stored in N_2 at 22.5 °C. For PIN 1618, seeds stored in air with 1-3% moisture content at – 20 °C had higher value in un-germinated but viable seeds than seeds stored at 22.5 °C. There was no germination at 25/15 °C for PIN 1298 stored at 4 °C with 1-3% moisture content, and PIN 1618 stored at 4 °C with 7-8% moisture content at both 15/5 °C and 25/15 °C, or stored at 22.5 °C with 1-3% moisture content, stored in nitrogen with germination temperature of 15/5 °C.

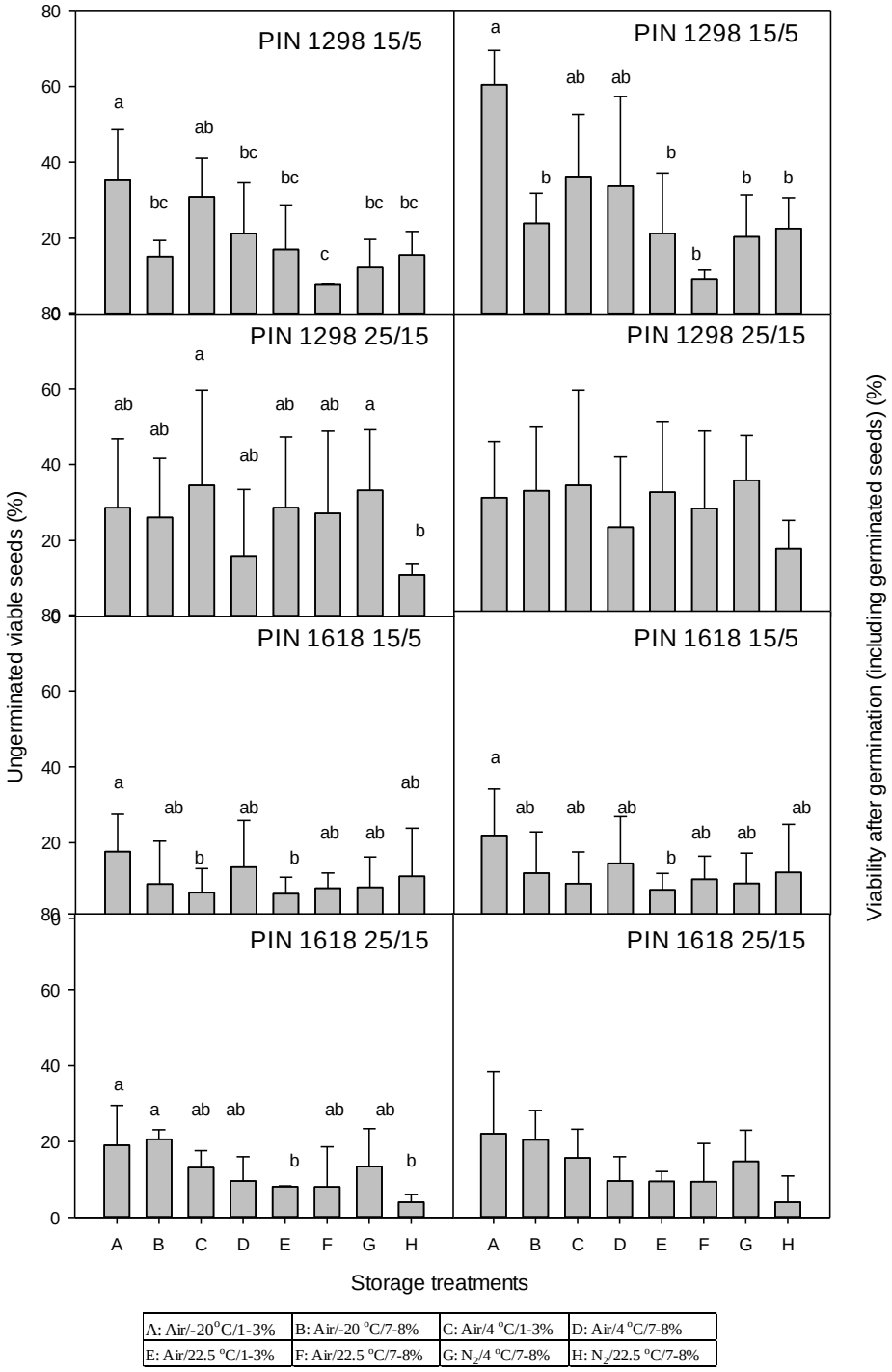

Figure 4-2 Effects of one-year storage treatments on seed viability after germination percentage (including germinated seeds) (right) and percent of un-germinated but viable seeds (left) in two collections of pin cherry at two germination temperatures. Means with same letters within a collection, germination temperature and parameter are not significantly different at $p \leq 0.05$.

Table 4-3 Effects of one-year storage treatments on seed vigour and viability in PIN 1298. Data are Means ± SE. Means with the same letters within a collection and parameter are not significantly different at P≤0.05.

Gas	T (°C)	MC (%)	EC (µS*cm^{-1}g^{-1})	V (%)	GT (15/5°C)				GT (15/25°C)			
					G (%)	UVS (%)	GRI (%*d^{-1})	VI (mm*%)	G (%)	UVS (%)	GRI (%*d^{-1})	VI (mm*%)
Initial			-	95±2a	8±2b		-	-	5±3a		-	-
Air	-20	7/8	190±28a	88±4ab	9±8b	15±4bc	0.5±0.5a	476±541b	7±3a	26±16ab	0.5±0.3a	62±17a
		1/3	204±14a	96±4a	24±4a	35±13a	1.9±1.3b	1354±56a	3±5a	29±18ab	1.2±2.1a	96±166a
	22.5	7/8	206±39a	93±4ab	1±8b	8±0c	0.1±0.2a	27±46b	1±2a	27±22ab	0.2±0.3a	129±223a
		1/3	189±37a	92±4ab	4±9b	17±12bc	0.2±0.2a	246±262b	4±0a	29±19ab	0.4±0.1a	74±27a
	4	7/8	185±16a	87±5ab	5±5b	21±13bc	1.8±2.8a	485±767b	3±0a	16±18ab	1.0±1.1a	258±357a
		1/3	219±52a	92±0ab	8±4b	31±10ab	0.6±1.1a	116±200b	0±0a	35±25a	-	-
N_2	4	7/8	182±4a	83±4b	7±2b	12±7c	0.3±0.3a	114±130b	8±5a	33±16a	0.2±0.4a	59±102a
	22.5	7/8	192±24a	97±1a	13±2b	16±6bc	0.3±0.2a	143±58b	7±2a	11±3b	0.7±0.5a	305±384a

T= storage temperature, MC=Seed moisture content, G=Final germination percentage, EC= Electrolyte conductivity, UVS= Un-germinated viable seeds, GT= Germination temperature, V= Viability, GRI= Germination rate index, VI= Vigour index

Table 4-4 Effects of one-year storage treatments on seed vigour and viability in PIN 1618. Data are Means ± SE. Means with the same letters within a collection and parameter are not significantly different at P≤0.05.

Gas	T (°C)	MC (%)	EC ($\mu S*cm^{-1}g^{-1}$)	V (%)	GT (15/5°C)				GT (15/25°C)			
					G (%)	UVS (%)	GRI ($\%*d^{-1}$)	VI (mm*%)	G (%)	UVS (%)	GRI ($\%*d^{-1}$)	VI (mm*%)
	Initial		-	98±1a	25±3a		-	-	8±1a		-	-
Air	-20	7/8	165±30a	97±1a	3±5b	9±11ab	0.1±0.1a	19±32a	1±2b	19±10a	0.2±0.3a	70±121a
		1/3	158±11a	95±3a	2±3b	18±14a	0.3±0.5a	116±202a	3±5b	19±18a	0.5±0.8a	61±105a
	22.5	7/8	145±10a	99±1a	0±2b	8±4ab	0.1±0.1a	5±9a	1±0b	8±11b	0.1±0.2a	7±12a
		1/3	168±24a	97±3a	-	7±4b	-	-	1±4b	8±0b	0.2±0.4a	21±36a
	4	7/8	166±19a	97±3a	-	14±12ab	-	-	-	10±6ab	-	-
		1/3	156±13a	94±6a	1±0b	7±6b	0.1±0.1a	35±61a	3±2b	13±4ab	0.1±0.2a	59±102a
N2	4	7/8	190±12a	94±6a	-	8±8ab	-	-	1±6b	13±10ab	0.4±0.6a	28±48a
	22.5	7/8	175±4a	97±1a	-	15±9ab	-	-	4±2ab	3±5b	0.5±0.7a	110±156a

T= storage temperature, MC=Seed moisture content, G=Final germination percentage, EC=Electrolyte electrolyte conductivity, UVS= Un-germinated viable seeds, GT= Germination temperature, V= Viability, GRI= Germination rate index, VI= Vigour index

4.1.3 Analysis of seed protein profiles after one-year storage treatments

4.1.3.1 Comparison of two protein extraction methods

Two methods were tested and compared for protein extraction from chokecherry and pin cherry seeds. Analysis of proteins extracted by phenol and Tris-HCl extraction methods by SDS-PAGE revealed both methods extracted polypeptides with a wide range of molecular weights from 10 to 120 kDa (Figure 4-3). However, the phenol extraction method showed slightly more polypeptide bands in the 15 and 80 kDa size range, and was therefore used in subsequent experiments.

4.1.3.2 Analysis of seed protein profiles after one year storage treatments

Total phenol-soluble proteins were extracted from chokecherry seeds stored for one year under different combinations of temperature, humidity and gaseous environments. Western blot analyses using dehydrin specific antibodies revealed detecteble amounts of dehydrin proteins in seeds stored under different storage conditions (Figure 4-4). The highest abundance of the 25.9 kDa polypeptide band was observed in two N_2 treatments stored at different temperatures with 7-8% moisture content, and air treatment stored at -20 °C with 7-8% moisture content; no band was detected above the 25.9 kDa for these three treatments. Seeds stored at room temperature (22.5°C) in the air showed lower accumulation of 25.9 kDa dehydrin protein and seeds stored with 1-3% moisture content tended to have less dehydrin polypeptides with molecular masses lower than 25.9 kDa. Therefore, the different storage conditions had effects on the production of dehydrins.

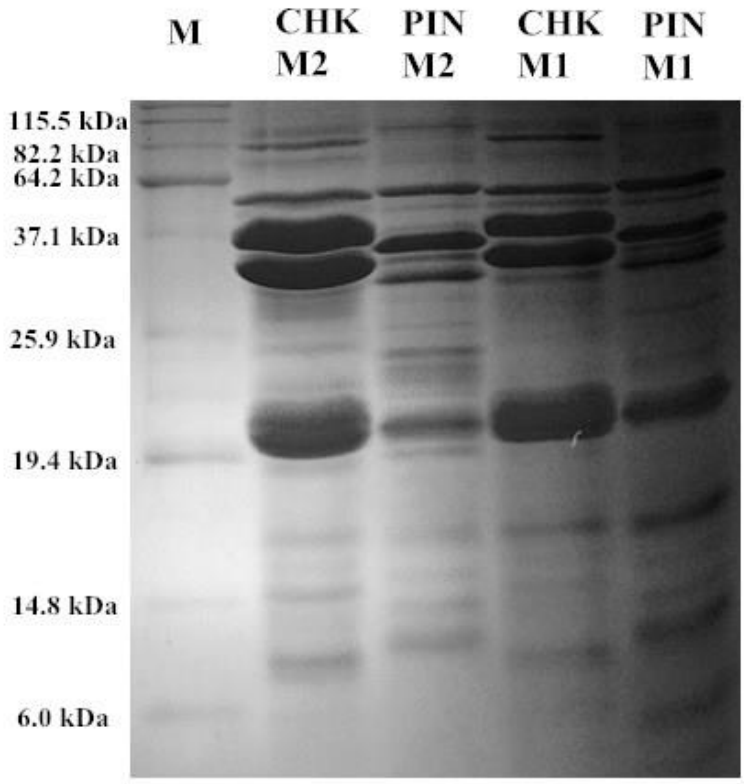

Figure 4-3 SDS-PAGE analysis of chokecherry and pin cherry seed proteins. The proteins were extracted using Tris-HCl buffer (M2) or phenol (M1) extraction method. The left lane indicates unigram of standard polypeptides. CHK: chokecherry, PIN: pin cherry.

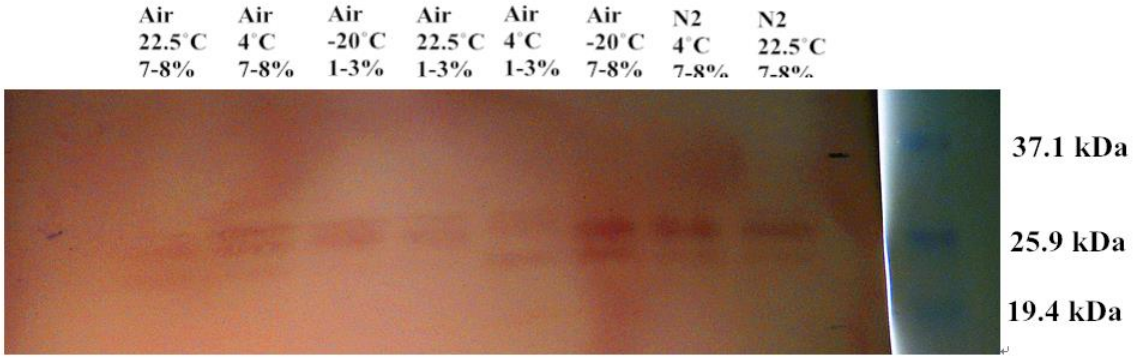

Figure 4-4 Immunoblot analysis of dehydrins in chokecherry seeds upon different one-year storage treatments. The western blot of total protein (35 μL/lane) was probed with antibodies against dehydrin proteins. The right lane indicates unigram of standard polypeptides

4.2 Accelerated seed aging

4.2.1 Seed viability and germination changes during accelerated seed aging

Seed viability before germination test decreased with increasing aging duration in both pin cherry and chokecherry collections (Figure 4-5). A significant decrease in viability and germination showed after 10 d aging PIN 1298 and CHK 1410. No seeds were viable in chokecherry and pin cherry after 25 d aging treatment. Both germination and viability (before and after germination test) declined during accelerated seed aging duration with high R^2 (0.59-0.86) (Table 3-1). Seed viability after germination test, which included germinated seeds and un-germinated but viable seed, was lower than the viability before germination test. CHK 1410 collection showed a significant ($P \leq 0.05$) decline in seed viability after 5 d accelerated aging. The percentage of viable un-germinated seed was also significantly reduced after 10 d aging treatment in two chokecherry collections, and after 15 d in pin cherry (Table 4-5)

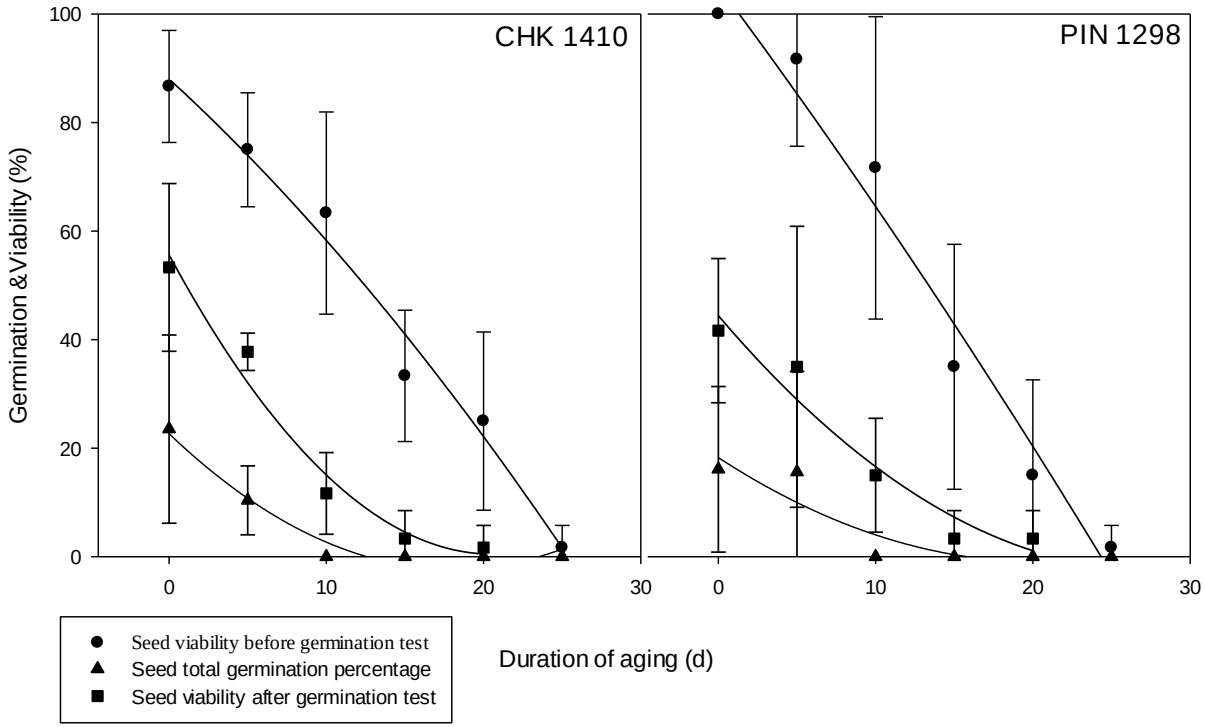

Figure 4-5 Observed (symbol) and simulated (solid line) seed viability and germination in chokecherry and pin cherry collections during artificial aging process at 45°C with 60 % RH. Values are mean ± SE (n=6).

No germination was found in CHK 1410 and PIN 1298 collection after 10 d aging treatments (Figure 4-5). For CHK 1410, the total germination percentage was significantly reduced from 25% to 10% ($P \leq 0.05$); the germination rate index declined from 1.0 to 0.4% / d, and the vigour index decreased from 279 to 26 mm×% after 5 d aging treatment (Table 4-5). The length of radicle and hypocotyl also decreased with accelerated aging from 7 mm to 1 mm, and 9 mm to 1 mm after 5 d aging, respectively. No significant increase was found in the mean germination time between non-aged and 5 d aged seeds.

For PIN 1298, the total germination percentages were 15% for 0 d and 5 d aged seeds (Table 4-5, Figure 4-5). No germination was observed after 10 d aging duration. No significant ($P \leq 0.05$) difference was found between non-aged and 5 d aged seeds in germination rate index, seedling length, vigour index, and mean germination time. Regression equations represent total germination percentage and viability [before and after (including germinated seeds) germination test] list in the Table 4-6.

Table 4-5 Seed germination rate index, seedling vigour and seed viability after various duration of accelerated seed aging treatment for chokecherry and pin cherry collections.

Collections	Duration (d)	GRI (% *d^{-1})	R (mm)	H (mm)	TT (mm)	VI (mm*%)	UVS (n)	MGT (d)
CHK 1410	0	1.0±0.6a	7±6a	9±7a	16±12a	279±209a	3±1a	27±7a
	5	0.4±0.3b	1±0b	1±0b	2±0b	26±15b	3±1a	19±10a
	10	-	-	-	-	-	1±1b	-
	15	-	-	-	-	-	0±1b	-
	20	-	-	-	-	-	-	-
PIN 1298	0	0.7±0.7a	6±4a	9±5a	15±6a	262±264a	3±1a	30±8a
	5	0.6±0.7a	4±4a	7±4a	11±8a	183±292a	2±1ab	25±6a
	10	-	-	-	-	-	2±1bc	-
	15	-	-	-	-	-	0±1c	-
	20	-	-	-	-	-	-	-

R=Radicle length, H=Hypocotyl length, TT =Total seedling length, GRI = Germination rate index, VI = Vigour index, UVS = Un-germinated viable seed, MGT=Mean germination time; "-" indicates data are not available.

Values are mean ± SE; values with the same letter within a collection and parameter are not significantly different at $p \leq 0.05$.

TZ tests showed evenly stained entire embryo in non-aged chokecherry seeds (Figure 4-6.). After 10 d aging treatment, part of the embryo and cotyledon could not be stained. Embryo was completely unstained after 20-25 d aging treatment, which indicated the total loss of seed viability.

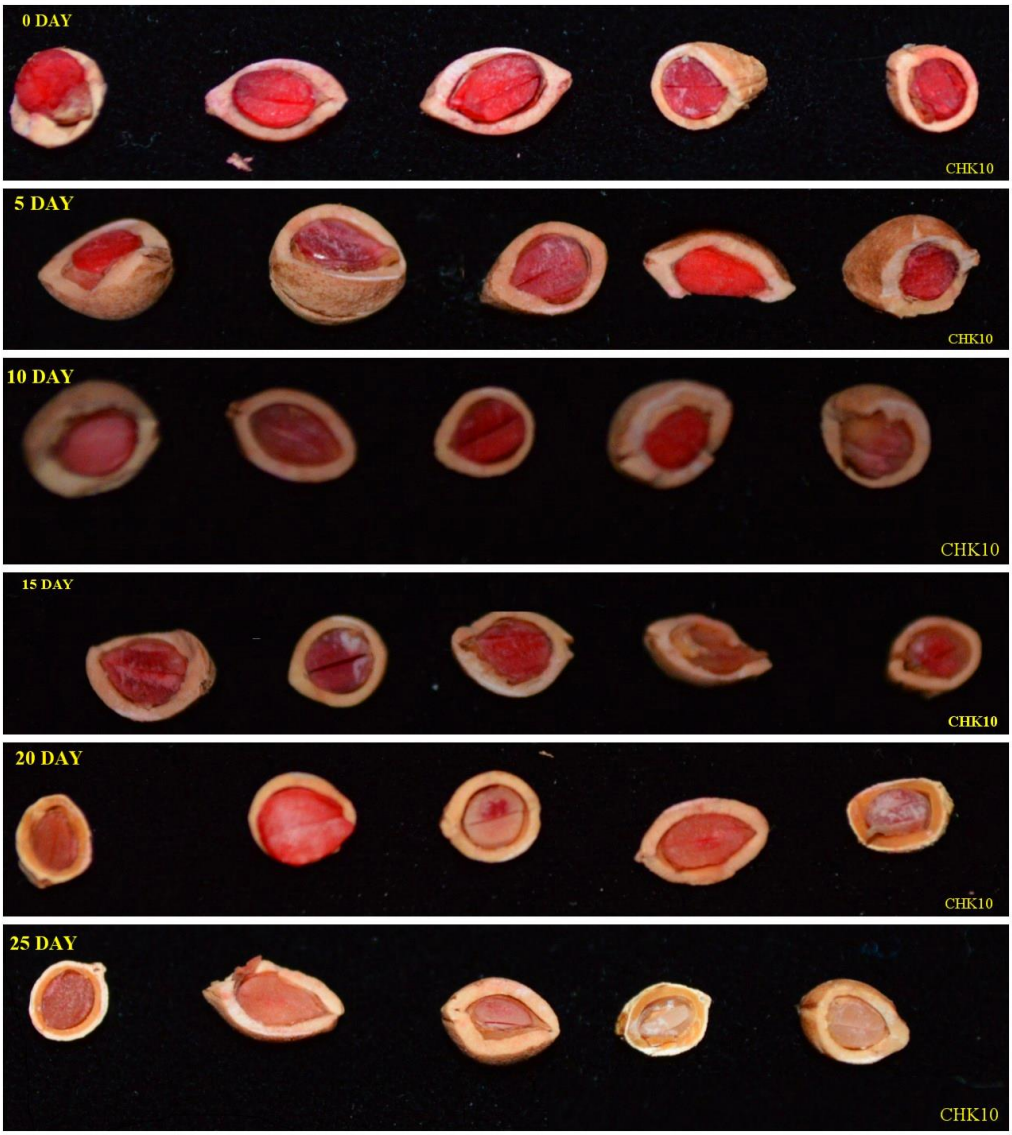

Figure 4-6 Seed viability evaluation of chokecherry. Half seeds of CHK 1410 accession stained with TZ at various days of aging.

Table 4-6 Regression equations for seed viability and total germination percentage as a function of artificial aging time.

Species	Equation	R^2	P-value
CHK 1410	VBG=88.04-2.65t-0.03t^2	0.84	<0.0001
	VAG=55.62-5.36t+0.13 t^2	0.84	<0.0001
	G=22.56-2.76*t*-0.08 t^2	0.60	<0.0001
PIN 1298	VBG=100-3.89t-0.02 t^2	0.81	<0.0001
	VAG=44.43-3.41t+0.06 t^2	0.76	<0.0001
	G=18.25-1.89*t*-0.05 t^2	0.59	<0.0001
BUF 1424	VBG=91.67-2.15t-0.06 t^2	0.93	<0.0001
	VAG=68.80-5.15t+0.09 t^2	0.84	<0.0001
	G=57.23-4.50*t*+0.08 t^2	0.75	<0.0001
BUF 14903	VBG=92.86-2.21t+0.06 t^2	0.92	<0.0001
	VAG=67.57-5.01t+0.08 t^2	0.75	<0.0001
	G=51.69-4.43*t*+0.08 t^2	0.69	<0.0001
BUF 9134	VBG=97.20-1.59t+0.09 t^2	0.97	<0.0001
	VAG=47.39-3.91t+0.09 t^2	0.73	<0.0001
	G=29.81-3.27*t*+0.09 t^2	0.79	<0.0001
BUF 4096	VBG=90.89-2.16t-0.07 t^2	0.91	<0.0001
	VAG=67.34-5.71t+0.12 t^2	0.76	<0.0001
	G=52.82-4.62*t*+0.09 t^2	0.68	<0.0001
DOG 3259	VBG=61.24-2.47t+0.26 t^2	0.82	<0.0001
	VAG=59.64-5.03t+0.11 t^2	0.75	<0.0001
	G=36.98-3.43t+0.13 t^2	0.69	<0.0001
DOG 3119	VBG=76.00-1.21t+0.13 t^2	0.78	<0.0001
	VAG=72.64-6.71t+0.16 t^2	0.87	<0.0001
	G=33.02-4.11*t*+0.12 t^2	0.82	<0.0001
DOG 6930	VBG=64.38-0.52t+0.12 t^2	0.87	<0.0001
	VAG=59.01-4.44t+0.08 t^2	0.76	<0.0001
	G=55.67-6.65*t*+0.19 t^2	0.89	<0.0001
DOG 3914	VBG=71.62-0.15t+0.18 t^2	0.94	<0.0001
	VAG=51.18-5.15t+0.10 t^2	0.78	<0.0001
	G=41.81-5.01*t*+0.14 t^2	0.74	<0.0001

VAG: Viability after germination (including germinated seeds), VBG: Viability before germination, G: total germination percentage

All buffalo berry collections showed high viability before aging treatment, especially BUF 14903 with 100% viability before germination test (Figure 4-7). Seed viability of BUF 1424 and BUF 4096 showed significant ($P \leq 0.05$) decline after 10 d aging while that of BUF 14093 and BUF 9134 decreased after 5 d aging treatment. All buffalo berry collections lost their viability after 25 d accelerated aging. Seed viability after germination (including germinated seeds) was lower than the viability before germination test in all buffalo berry collections. In BUF 4096, BUF 1424, and BUF 14093 collections, viability after germination (including germinated seeds) decreased significantly after 10 d aging; seed viability after germination (including germinated seeds) declined after 5 d aging in BUF 9134 (Table 4-7). The percentage of viable but un-germinated seeds was also significantly reduced after 5 d for BUF 9134, 15 d for BUF 14093, BUF 1424, and BUF 4906, respectively. Regression equations represent total germination percentage and viability [before and after (including germinated seeds) germination test] list in the Table 4-6.

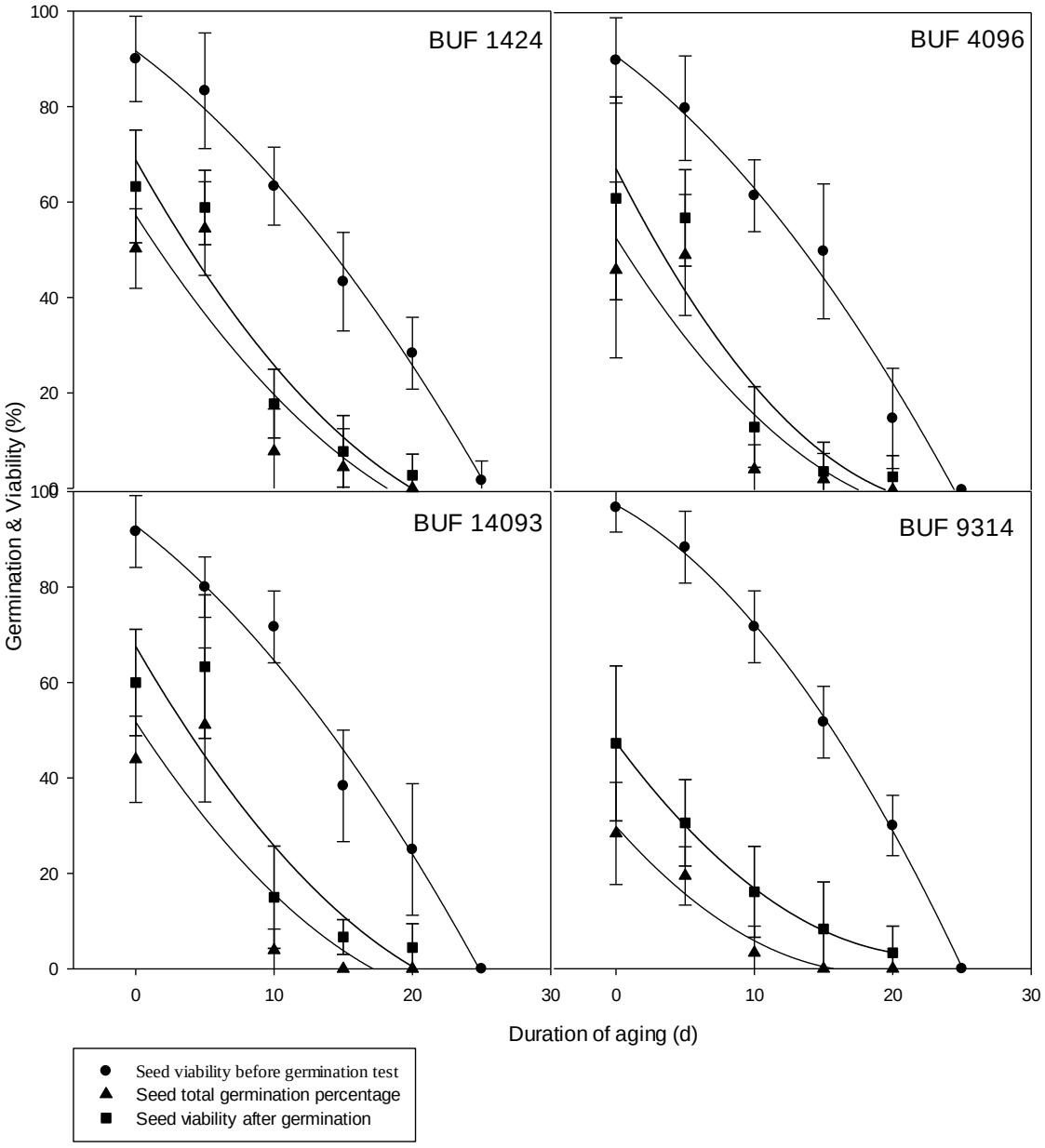

Figure 4-7 Observed (symbol) and simulated (solid line) seed viability and germination in four buffalo berry collections and during artificial aging process at 45 °C with 60% RH. Values are mean ± SE (n=6).

Seeds failed to germinate in BUF 1424 and BUF 4906 after 20 d aging treatments, and BUF 14903 and BUF 9134 after 15 d aging treatments (Figure 4-7). The total germination percentage of non-aged seeds was high, 50%, 42%, 45%, for BUF 1424, BUF 14093, and BUF 4906 collection, respectively. In BUF 1424, BUF 14903, and BUF 4906, no significant decrease was found between 0 d and 5 d aged seeds in total germination percentage ($P \leq 0.05$). After 10 d of aging treatment, however, the total germination percentage was reduced significantly ($P \leq 0.05$). In BUF 9134 collection, total germination percentage was significantly reduced from 30% to 19% after 5 d of aging treatment ($P \leq 0.05$).

The germination rate index was significantly ($P \leq 0.05$) decreased from 2.7-5.2 to 0.2-0.4 %/d after 10 d accelerated seed aging treatments (Table 4-7). In BUF 9134 collection, the vigour index was significantly decreased from 249 to 103 mm*% after 5 d aging treatments. In BUF 14093, BUF 4906, and BUF 1424 collections, the average vigour index was significantly decreased from 348-532 to 18-20 mm*% after 10 d aging treatment ($P < 0.05$). Hypocotyl and seedling length was significantly reduced after 15 d aging treatment in BUF 1424 and BUF 4096 ($P < 0.05$). However, no significant difference was found in radicle length in the accelerated aging duration in all buffalo berry collections ($P > 0.05$). No significant difference was found in the mean germination for BUF 1424 collection. After 10 d aging treatments, the mean germination time increased significantly from 11 to 26 d in BUF 14903 and from 10 to 21 d in BUF 9134, respectively. In BUF 4906 collection, significant difference was found after 15 d aging treatment with an increase in the meantime from 12 d (non-aged) to 21 d (15 d aged).

Table 4-7 Seed germination rate index, seedling vigour and seed viability after various durations of accelerated seed aging treatment for four buffalo berry collections

Collections	Duration (d)	GRI (% *d^{-1})	VI (mm*%)	UVS (%)	R (mm)	H (mm)	TT (mm)	MGT
BUF 1424	0	4.8±1.3a	348±96a	13±6a	3±3a	5±4a	8±6a	13±4a
	5	4.8±1.1a	284±93a	4±3ab	2±1a	3±3ab	6±4ab	14±2a
	10	0.4±0.5b	32±50b	10±13ab	2±1a	2±2ab	4±2ab	18±5a
	15	0.2±0.4b	28±45b	3±5b	1±1a	1±1b	2±1b	18±0a
	20	-	-	3±4b	-	-	-	-
BUF 14093	0	5.0±1.2a	403±195a	16±4a	4±4a	6±5a	10±8a	11±2a
	5	4.7±2.1a	447±272a	12±8ab	3±2a	6±4a	9±6a	13±2a
	10	0.2±0.3b	18±21b	11±12ab	3±2a	4±2a	7±3a	26±12b
	15	-	-	7±4b	-	-	-	-
	20	-	-	4±5b	-	-	-	-
BUF 4096	0	5.2±2.4a	532±409a	15±13a	4±3a	7±5a	11±7a	12±3a
	5	3.9±1.1a	399±195a	8±12ab	3±2a	5±4ab	8±5ab	15±3a
	10	0.3±0.3b	20±30b	9±10ab	2±2a	3±1ab	5±3ab	15±5a
	15	0.1±0.2b	13±33b	2±4b	1±1a	1±1b	2±0b	31±9b
	20	-	-	3±4b	-	-	-	
BUF 9134	0	2.7±1.5a	249±144a	22±9a	3±2a	6±3a	8±5a	10±2a
	5	2.0±0.8a	103±88b	11±5b	2±1a	3±3a	5±3a	11±3a
	10	0.2±0.5b	13±22b	13±9b	2±1a	2±2a	4±2a	21±14b
	15	-	-	8±10b	-	-	-	-
	20	-	-	3±6b	-	-	-	-

R=Radicle length, H=Hypocotyl length, TT =Total seedling length, GRI = Germination rate index, VI = Vigour index, UVS = Un-germinated viable seed, MGT=Mean germination time; "-" indicates data are not available.

Values are mean ± SE; values with the same letter within a collection and parameter are not significantly different at $p \leq 0.05$.

The initial seed viability (before germination test) was around 80% before the aging test, and then decreased in all collections of dogwood with increased aging duration (Figure 4-8). After 10 d aging treatment, DOG 3914, DOG 6930 and DOG 3119 showed significant decline in seed viability (before germination test) ($P<0.05$); and DOG 3259 decreased after 15 d accelerated aging. After 25 d aging, all dogwood collections lost their viability. In non-aged seeds, the viability after germination (including germinated seeds) was similar to before germination viability in non-aged DOG 3119, DOG 3259 and DOG 6930 collections, while the viability after germination (including germinated seeds) of DOG 3914 was significant lower than the original one. Seed viability after germination (including germinated seeds) decreased after 5 d aging for DOG 3119 and DOG 3419, and 10 d aging for DOG 3259 and DOG 6930, respectively. The percentage of un-germinated but viable seeds declined after accelerated aging of 10 d for DOG 3259, and 20 d for DOG 6930, DOG 3914, DOG 3119, respectively. Regression equations represent total germination percentage and viability [before and after (including germinated seeds) germination test] list in the Table 4-6.

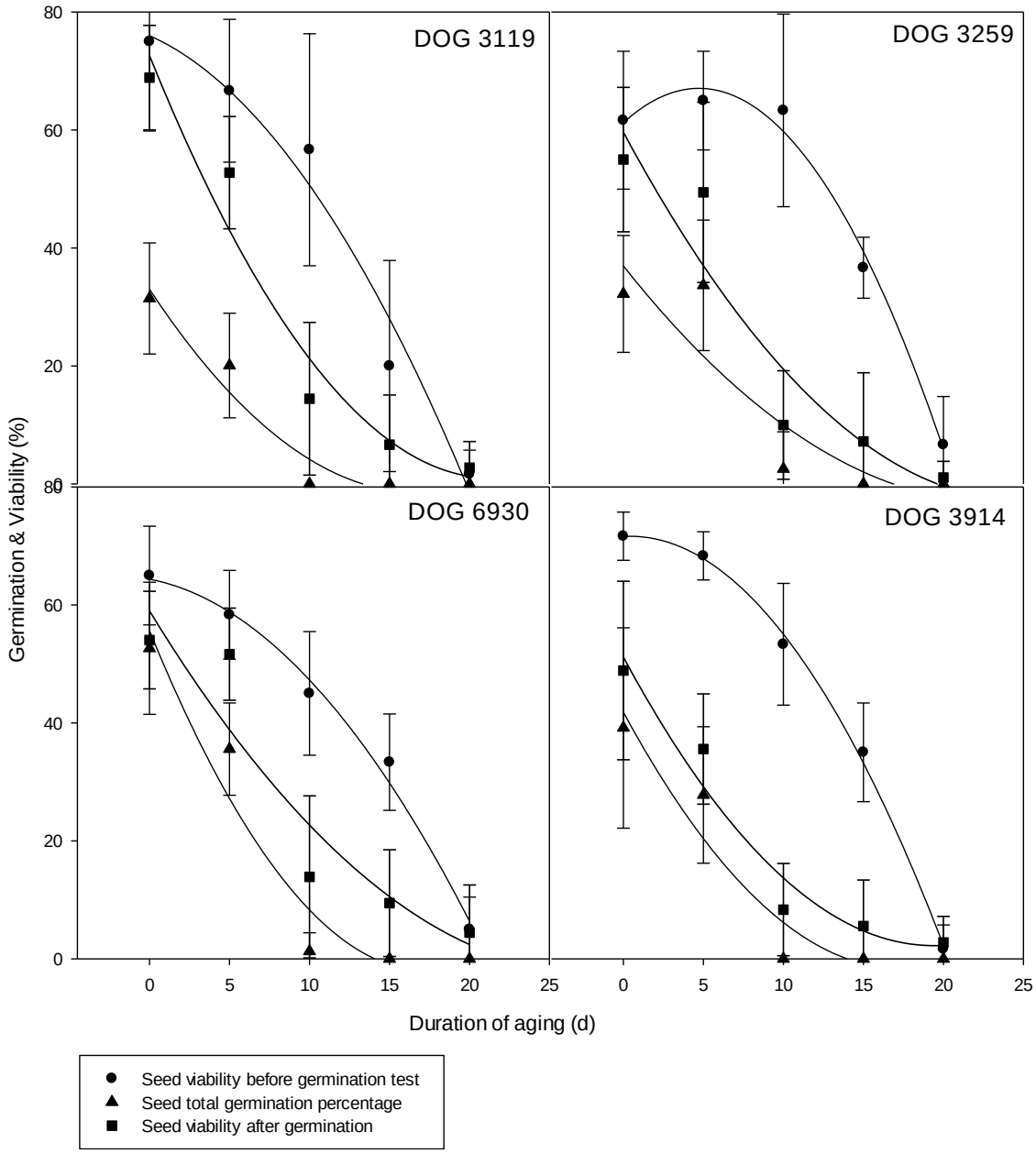

Figure 4-8 Observed (symbol) and simulated (solid line) seed viability and germination in four dogwood collections during artificial aging process at 45 °C with 60 % RH. Values are mean ± SE (n=6).

No seeds germinated for DOG 6930 and DOG 3259 collections after 15 d aging treatment and for DOG 3119 and DOG 3914 after 10 d aging treatments (Figure 4-8). The total germination percentage of non-aged seeds were 53% for DOG 6930, 42% for DOG 3914, 32% for DOG 3119, and 36% for DOG 3259, respectively. Total germination percentage were significantly reduced to 36% for DOG 6930 ($P<0.05$), 28% for DOG 3914, and 20 for DOG 3119 after 5 d aging treatments, respectively. In DOG 3259 collection, no significant difference was observed between 0 d and 5 d aged seed in total germination percentage ($P>0.05$), and significant difference was found after 10 d aging treatments ($P<0.05$).

There was significant ($P<0.05$) difference in germination rate index of all dogwood collections after 10 d accelerated aging treatments (Table 4-8). The four collections gradually reached their germination peak around 40-45 d after three weeks warm stratification with the average germination index of 0.1-10.8 %/d. Seed vigour index was observed significantly decreased in DOG 6930, DOG 3914, and DOG 3119 after 5 d aging treatments ($P<0.05$), and 10 d for DOG 3259. Seed radicle length, hypocotyl length, seedling length, and radicle -hypocotyl length also showed significant decrease after the 10 d aging treatments ($P<0.05$). No significant increase was observed in mean germination time for all dogwood collections in non-aged and 5 d aged seeds. The mean germination time increased significantly from 10 to 23 d for DOG 6930 and DOG 3259 after 10 d accelerated aging ($P<0.05$), respectively.

Table 4-8 Seed germination rate index, seedling vigour and seed viability after various duration of accelerated seed aging treatment for four dogwood collections.

Collections	Duration (d)	GRI (%*d^{-1})	R (mm)	H (mm)	TT (mm)	VI (mm* %)	UVS (n)	MGT (d)
DOG 6930	0	10.8±6.7a	7±3a	13±6a	19±8a	1003±225a	3±1a	11±5a
	5	4.4±0.8b	4±2a	9±5a	13±6a	457±79b	1±1b	12±5a
	10	0.1±0.2b	0±1b	2±4a	2±5a	14±35c	2±1b	24±2b
	15	-	-	-	-	-	1±1b	-
	20	-	-	-	-	-	1±1b	-
DOG 3259	0	4.7±3.1a	4±2a	15±12a	20±13a	577±247a	2±1a	14±2a
	5	3.4±0.4a	3±2a	9±5ab	12±7ab	450±208a	1±1b	14±8a
	10	0.1±0.3b	2±1a	2±1b	4±2b	9±22b	1±1b	24±1b
	15	-	-	-	-	-	1±1b	-
	20	-	-	-	-	-	-	-
DOG 3914	0	9.9±3.6a	5±3a	12±6a	17±8a	625±182a	2±1a	11±9a
	5	7.1±6.2a	4±2a	10±5a	14±6a	393±114b	2±1ab	10±4a
	10	-	-	-	-	-	2±2ab	-
	15	-	-	-	-	-	1±1ab	-
	20	-	-	-	-	-	-	
DOG 3119	0	7.4±4.6a	5±3a	13±10a	18±12a	570±259a	2±1a	10±2a
	5	3.4±1.2a	3±2a	9±5a	12±7a	252±199b	2±1a	10±3a
	10	-	-	-	-	-	1±1a	-
	15	-	-	-	-	-	1±1a	-
	20	-	-	-	-	-	-	-

R=Radicle length, H=Hypocotyl length, TT =Total seedling length, GRI = Germination rate index, VI = Vigour index, UVS = Un-germinated viable seed, MGT=Mean germination time; "-" indicates data are not available.

Values are mean ± SE; values with the same letter within a collection and parameter are not significantly different at $p \leq 0.05$.

4.2.2 Electrolyte conductivity changes during accelerated seed aging

The electrolyte conductivity of buffalo berry seeds soaked in water increased with increasing aging and soaking time in all collections (Figure 4-9). Significant difference was found in electrolyte conductivity after accelerated aging of 20 d for BUF 4093, 15 d for BUF 4096, 5 d for BUF 1424, 10 d for BUF 9134 comparing to the control (*P<0.05*), respectively. After 20 d aging treatment, average electrolyte conductivity was elevated to 632, 276, 312, and 539 $\mu S \cdot cm^{-1}g^{-1}$ in BUF 1424, BUF 14093, BUF 4906, and BUF 9134, respectively. For BUF 9134 and BUF 1424, seed electrolyte conductivity was significantly higher in 20 d aged seeds than the 0 d aged seeds when soaked for 48 h with distilled, deionized water *(P<0.05)*. For BUF 9134, significant difference was found after 10 d ageing treatments after soaked for 2-48 h *(P<0.05).* For BUF 4906, however, only the electrolyte conductivity of 0 h and 12 h soaking didn't show significant difference among different aging duration. In BUF 4096, no significant difference was found in electrolyte conductivity among 0-10 d aged seeds after soaked for 0-12 h. However, significant difference was found between 0 d and aged seeds after soaked for 48 h *(P<0.05)*. In BUF 14903, no significant difference was found in electrolyte conductivity of 0-15 d aged seed's when soaked for 0-24 h. After soaked for 48 h, significant difference was found between non-aged and aged seeds *(P<0.05)*.

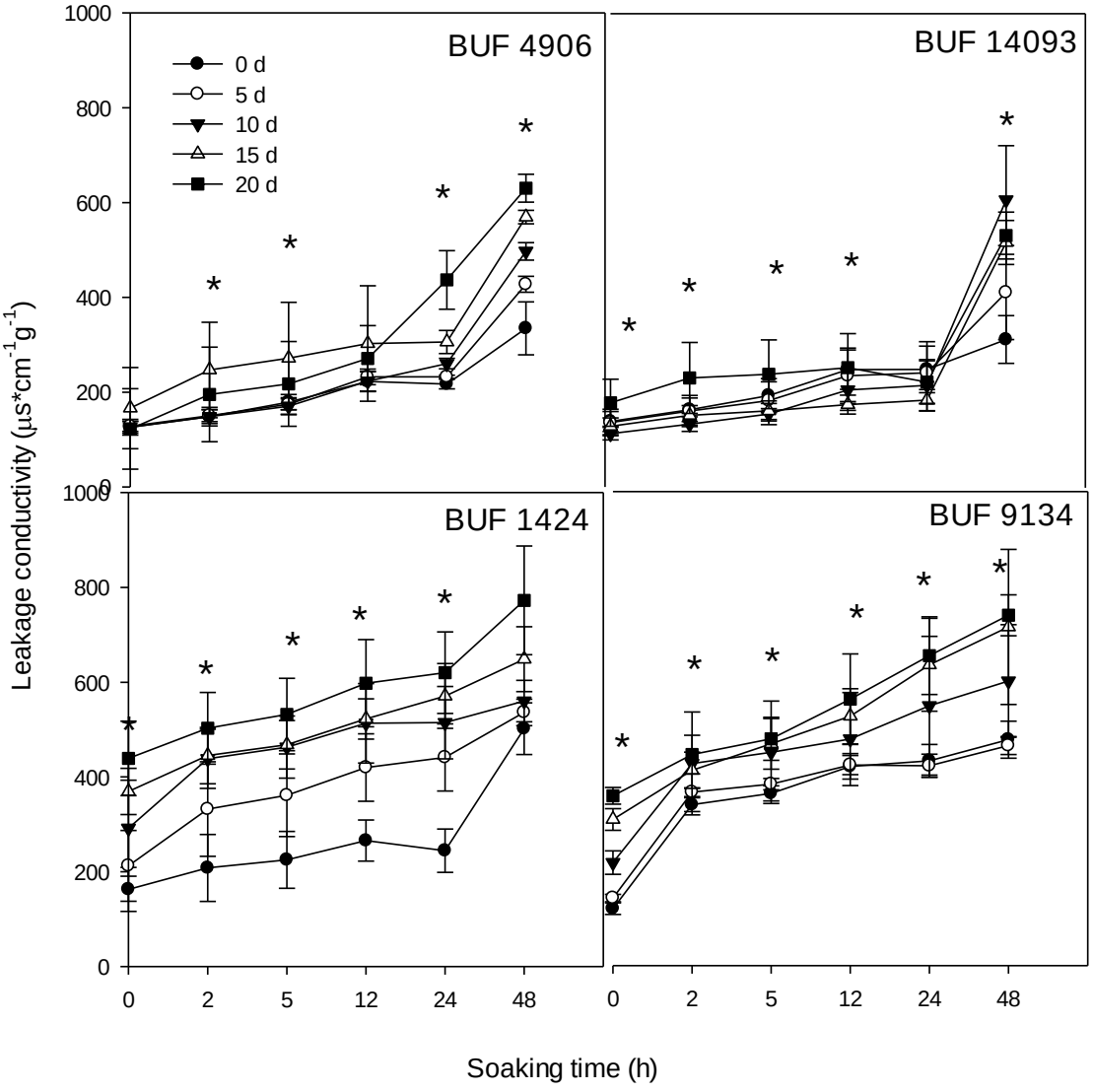

Figure 4-9 Electrolyte conductivity of four buffalo berry collections during artificial aging at 45°C with 60% RH. Values are mean ± SE (n=6). '*' indicates significant difference among aging time within the same soaking time ($P \leq 0.05$).

The electrolyte conductivity of dogwood seed increased with soaking time in all collections (Figure 4-10). For DOG 3914, the highest electrolyte conductivity appeared to 20 d aged seeds, however, 5 d and 10 d aged seeds showed significantly higher than other aged seeds after soaking for 24 h *(P<0.05)*. In DOG 3119, significant difference was found after 10 d aging treatment in 0 h soaking, after 20 d aging during 2-5 h soaking, and after 15 d aging treatment during 12-48 h soaking *(P<0.05)*. For DOG 3259, significant difference was observed after 5 d aging treatment during 0 and 12-48 h's soaking; significant difference was also found after 10 d aging during 2-5 h soaking *(P<0.05)*. For DOG 6930, no significant difference was found among electrolyte conductivity of different aging duration when soaking for same time until 24 h's soaking. After 20 d aging treatments, the average electrolyte conductivity was elevated to 201, 168, 296, and 118 $\mu S \cdot cm^{-1}g^{-1}$ in DOG 6930, DOG 3259, DOG 3914 and DOG 3119 respectively .The leakage was significantly elevated after 15 d aging treatment for DOG 3119, 5 d for DOG 3259, and 10 d for DOG 3914, respectively *(P≤0.05)*.

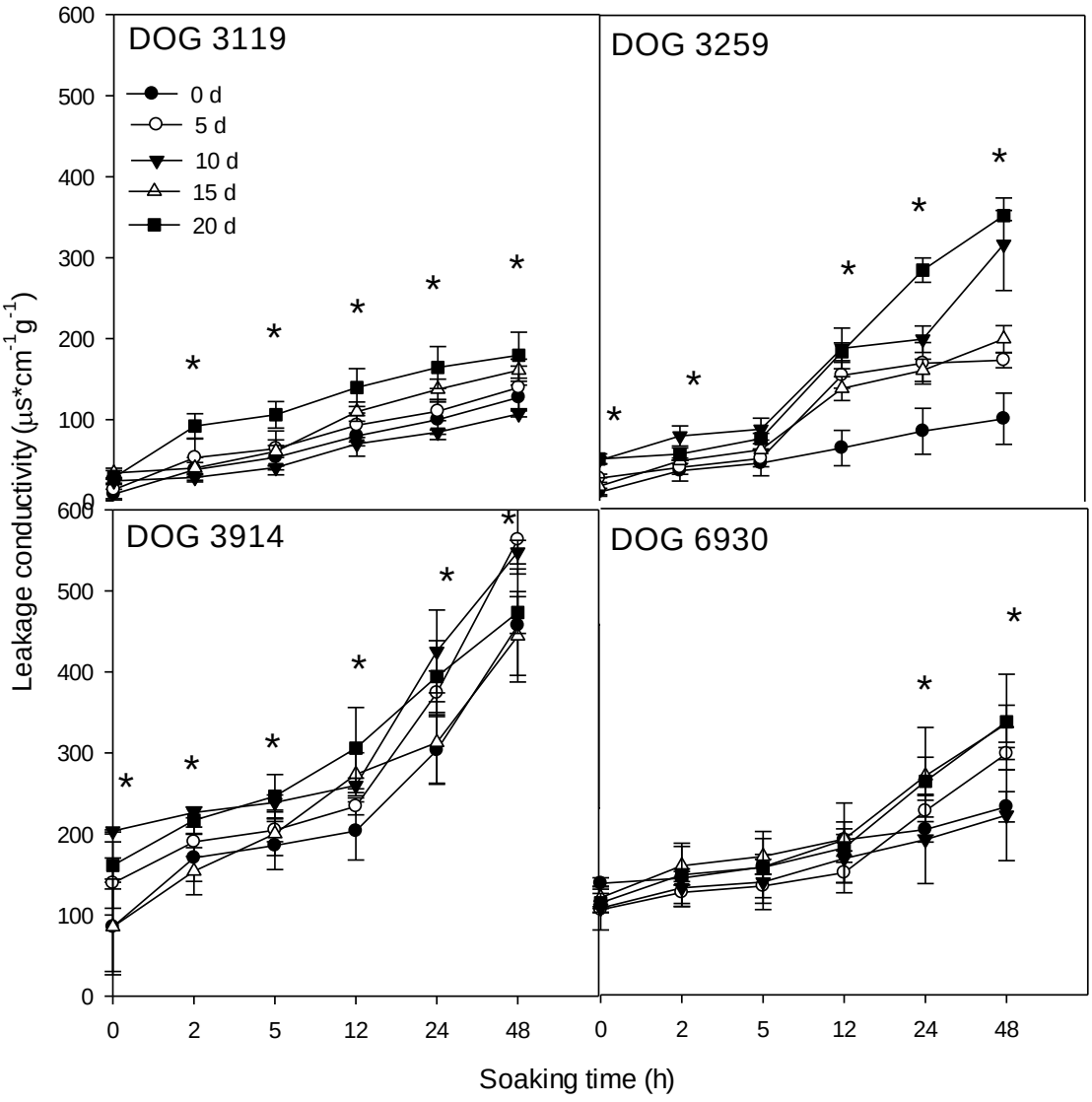

Figure 4-10 Electrolyte conductivity of four dogwood collections during artificial aging at 45°C with 60% RH. Values are mean ± SE (n=6). '*' indicates significant difference among aging time within the same soaking time ($P \leq 0.05$).

The electrolyte conductivity in all chokecherry and pin cherry collections increased with soaking time (Figure 4-11). Electrolyte conductivity significantly increased after 5 d aging for CHK 1410, and 10 d for PIN 1298. For CHK 1410 and PIN 1298, the electrolyte conductivity significantly increased with the increment of the aging duration after the same soaking time ($P \leq 0.05$). After 15 d aging treatment, significant differences were detected in seed electrolyte conductivity for CHK 1410, and PIN 1298($P \leq 0.05$). In CHK 1410, electrolyte conductivity of 5 d and 10 d aged seeds showed significantly higher values than seeds of other durations. For PIN 1298, no significant difference was detected between 0 d and 5 d, 10d and 15 d aged seeds at any soaking time. Due to the increased leakage, aged pin cherry seeds (5 d &10 d) became more vulnerable to fungi infection during the germination period than non-aged seeds (0 d). (Figure 4-12, A)

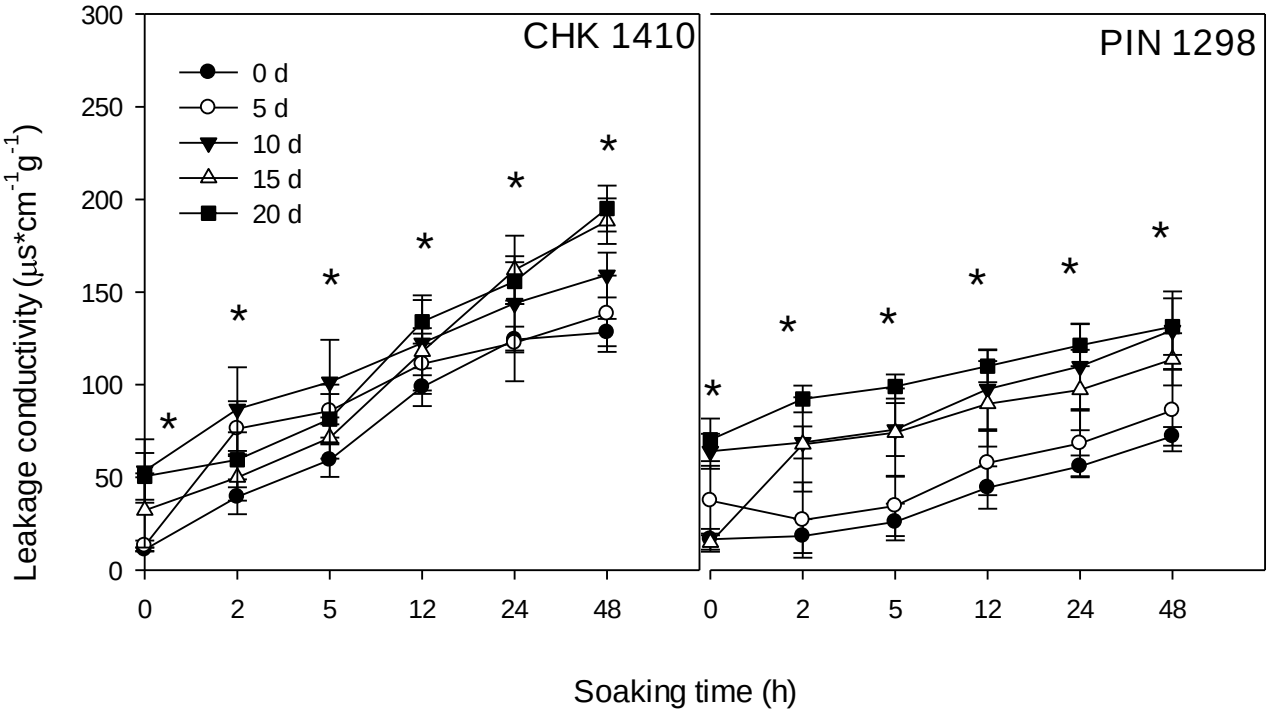

Figure 4-11 Electrolyte conductivity of a chokecherry and a pin cherry collection during artificial aging at 45°C with 60%RH. Values are mean ± SE (n=6). ‘ * ’ indicates significant difference among aging time within the same soaking time ($P \leq 0.05$)

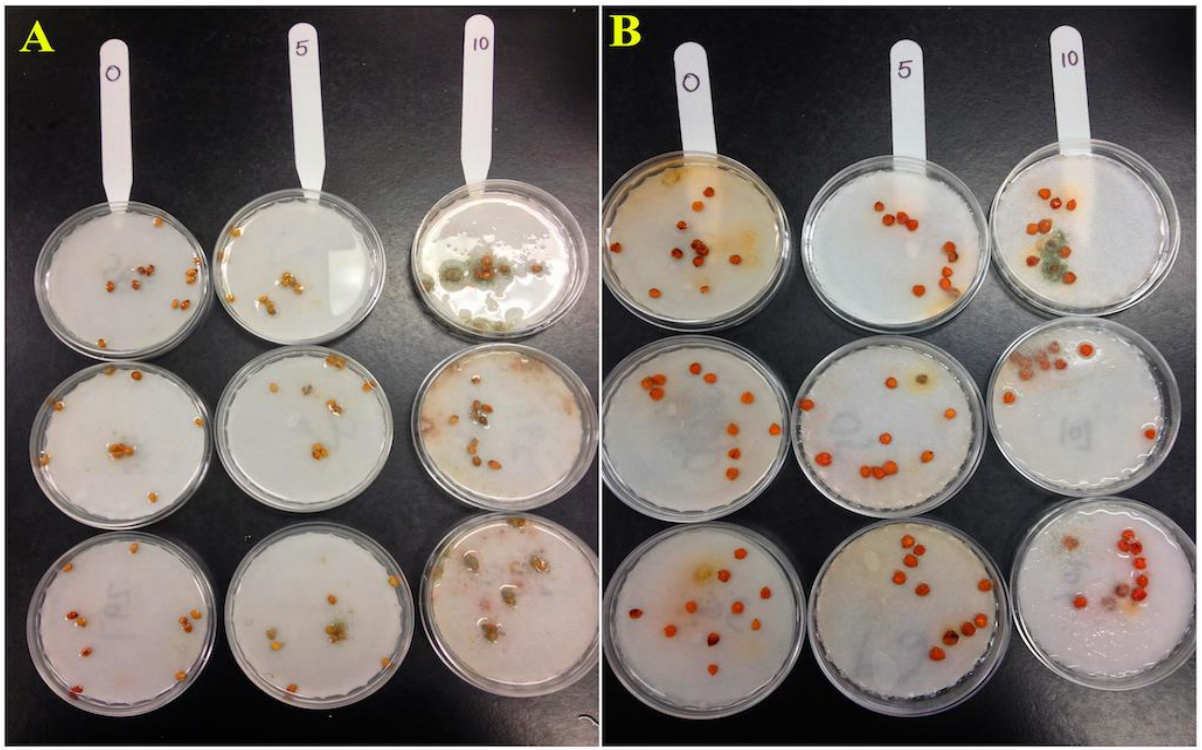

Figure 4-12 Aged seeds were more vulnerable to fungal infection than non-aged seeds during germination. A: PIN 1298; B: LBC 7278, 0: 0 d, 5: 5 d, and 10: 10 d. Petri-dishes in the same column are aged for same duration.

4.2.3 Effects of accelerated seed aging on seed properties

For BUF 14903, significant correlations were observed between aging durations (Table 4-9). Electrolyte conductivity showed a strong, negative correlation with seed viability, and positive correlations with aging duration and mean germination time. Viability before and after (including germinated seeds) germination test showed strong correlations with most parameters, except mean germination time.

For BUF 4096, negative correlations were found between aging duration and most measurements; strong, positive correlations were observed between aging duration and mean germination time and electrolyte conductivity (Table 4-10). Positive correlation was also found between electrolyte conductivity and mean germination time. The viability after germination (including germinated seeds) showed significant correlations with germination rate index, viability before germination test, and aging duration.

Table 4-9 Simple correlation coefficients between various measures of seed quality for BUF 14903 during accelerated seed aging.

Correlations									
BUF 14903	UVS	VAG	GRI	G	V	EC	TT	VI	MGT
AD	-.82**	-.86**	-.81**	-.81**	-.89**	.49**	-.59*	-.55*	.67**
UVS	1	.86*	.89**	.85**	.71**	-0.2	0.39	.52*	-.58*
VAG		1	.90**	.84**	.73**	-0.3	0.43	.52*	-.62*
GRI			1	.82**	.66**	-0.29	0.18	0.34	-.66**
G				1	.71**	-0.36	.63**	.88**	-.63*
V					1	-.54**	.63**	.48*	-0.21
EC						1	-0.17	-0.29	.51*
TT							1	.87**	-0.42
VI								1	-0.48
MGT									1

** Significant at $P \leq 0.01$ (2-tailed).

*. Significant at $P \leq 0.05$ (2-tailed).

UVS= Un-germinated but viable seeds, VAG= Viability after germination (including germinated seeds), GRI=Germination rate index, G= Total germination percentage, V=Viability before germination, EC=Electrolyte conductivity, TT= Seedling length, VI=Vigour index, MGT=Mean germination time.

Table 4-10 Simple correlation coefficients between various measures of seed quality for BUF 4096 during accelerated seed aging.

Correlations									
BUF 4096	UVS	VAG	GRI	G	V	EC	TT	VI	MGT
AD	-.83**	-.85**	-.81**	-.80**	-.81**	.78**	-.66**	-.58*	.82**
UVS	1	.96**	.86**	.78**	.61**	-.68**	0.42	0.39	-.54*
VAG		1	.81**	.85**	.60**	-.69**	.58*	.62**	-.56*
GRI			1	.73**	.54**	-.63**	0.15	0.12	-.54*
G				1	.55**	-.65**	.62**	.84**	-0.37
V					1	-.76**	0.29	0.12	-.78**
EC						1	-0.46	-0.36	.80**
TT							1	.86**	-0.42
VI								1	-0.28
MGT									1

** Significant at $P \leq 0.01$ (2-tailed).

*. Significant at $P \leq 0.05$ (2-tailed).

UVS= Un-germinated but viable seeds, VAG= Viability after germination (including germinated seeds), GRI=Germination rate index, G= Total germination percentage, V=Viability before germination, EC=Electrolyte conductivity, TT= Seedling length, VI=Vigour index, MGT=Mean germination time.

For BUF 9134, negative correlations were found between aging duration and most measurements (UVS, VAG, G, V), and a strong, positive correlation was observed between aging duration and electrolyte conductivity; however no significant correlations were found with mean germination time (Table 4-11). Mean germination time did not show any significant correlation with any measurements. Electrolyte conductivity showed significant negative correlations with percentage of un-germinated but viable seeds, germination rate index, total germination percentage and viability after germination.

For BUF 1424, a strong, positive correlation was observed between aging duration with electrolyte conductivity, and negative correlations were found between aging duration and most measurements (Table 4-12). However, no significant correlation was found between aging duration with seedling length and mean germination time. Electrolyte conductivity showed significant negative correlations with viability after germination, percentage of un-germinated but viable seeds, and final germination percentage. No significant correlation was found between seedling lengths and all other measurements.

Table 4-11 Simple correlation coefficients between various measures of seed quality for BUF 9134 during accelerated seed aging.

Correlations									
BUF 9134	UVS	VAG	GRI	G	V	EC	TT	VI	MGT
AD	-.81**	-.83**	-.77**	-.82**	-.85**	.92**	-.58*	-.61*	0.52
UVS	1	.95**	.71**	.74**	.69**	-.82**	0.26	0.22	-0.06
VAG		1	.61**	.78**	.69**	-.82**	.63*	.56*	-0.21
GRI			1	.78**	.64**	-.79**	-0.19	0.01	-0.48
G				1	.68**	-.82**	.53*	.85**	-0.45
V					1	-.83**	0.47	0.21	-0.16
EC						1	-0.13	-0.26	0.42
TT							1	.87**	-0.26
VI								1	-0.34
MGT									1

** Significant at $P \leq 0.01$ (2-tailed).

*. Significant at $P \leq 0.05$ (2-tailed).

UVS= Un-germinated but viable seeds, VAG= Viability after germination (including germinated seeds), GRI=Germination rate index, G= Total germination percentage, V=Viability before germination, EC=Electrolyte conductivity, TT= Seedling length, VI=Vigour index, MGT=Mean germination time.

Table 4-12 Simple correlation coefficients between various measures of seed quality for BUF 1424 during accelerated seed aging.

Correlations									
BUF 1424	UVS	VAG	GRI	G	V	EC	TT	VI	MGT
AD	-0.91**	-0.90**	-.84**	-.84**	-.92**	.93**	-0.32	-.80**	0.48
UVS	1	.92**	.94**	.94**	.81**	-.85**	0.28	.85**	-.59*
VAG		1	.92**	.92**	.81**	-.82**	0.28	.84**	-.66**
GRI			1	.90**	.70**	-.81**	0.13	.62**	-.72**
G				1	.75**	-.82**	0.18	.83**	-0.44
V					1	-.88**	0.26	.77**	-0.39
EC						1	-0.2	-.69**	0.33
TT							1	.58*	-0.14
VI								1	-0.42
MGT									1

** Significant at $P \leq 0.01$ (2-tailed).

*. Significant at $P \leq 0.05$ (2-tailed).

UVS= Un-germinated but viable seeds, VAG= Viability after germination (including germinated seeds), GRI=Germination rate index, G= Total germination percentage, V=Viability before germination, EC=Electrolyte conductivity, TT= Seedling length, VI=Vigour index, MGT=Mean germination time.

For CHK 1410, negative correlations were found between aging duration and most measurements (Table 4-13). However, no significant positive correlation was found between aging duration and electrolyte conductivity and mean germination time Electrolyte conductivity showed strong, negative correlations with germination rate index, final germination percentage, and viability before germination. The viability after germination (including germinated seeds) showed positive correlations with germination rate index, viability before germination, and seedling length. The total germination percentage showed strong negative correlation with electrolyte conductivity and aging duration; and strong positive correlations with the percentage of un-germinated but viable seeds, and viability before germination.

For PIN 1298, strong negative correlations were found between aging duration with the percentage of un-germinated but viable seeds, viability before / after germination, final germination percentage, and positive correlations between aging duration and electrolyte conductivity (Table 4-14). Electrolyte conductivity tests showed strong negative correlations with the percentage of un-germinated but viable seeds, viability before/ after germination, and final germination percentage.

Table 4-13 Simple correlation coefficients between various measures of seed quality for CHK 1410 during accelerated seed aging.

Correlations									
CHK 1410	UVS	VAG	GRI	G	V	EC	TT	VI	MGT
AD	-.88**	-.89**	-.74**	-.67**	-.86**	0.36	-0.58	-.66*	-0.34
UVS	1	.90**	.76**	.64**	.77**	-0.29	.64*	0.37	0.42
VAG		1	.78**	.67**	.78**	-0.32	.61*	0.37	0.47
GRI			1	.85**	.65**	-.49**	0.2	0.35	0.02
G				1	.63**	-.53**	0.22	0.53	0.49
V					1	-.56**	.68*	.76**	0.47
EC						1	0.14	-0.06	-0.05
TT							1	.88**	.65*
VI								1	.61*
MGT									1

** Significant at $P \leq 0.01$ (2-tailed).

*. Significant at $P \leq 0.05$ (2-tailed).

UVS= Un-germinated but viable seeds, VAG= Viability after germination (including germinated seeds), GRI=Germination rate index, G= Total germination percentage, V=Viability before germination, EC=Electrolyte conductivity, TT= Seedling length, VI=Vigour index, MGT=Mean germination time.

Table 4-14 Simple correlation coefficients between various measures of seed quality for PIN 1298 during accelerated seed aging.

Correlations									
PIN 1298	UVS	VAG	GRI	G	V	EC	TT	VI	MGT
AD	-.75**	-.74**	-0.45	-.54**	-.87**	.77**	-0.04	0.02	-0.39
UVS	1	.94**	0.42	.63**	.70**	-.55**	0.23	0.56	.70*
VAG		1	.57*	.84**	.68**	-.63**	0.54	.85**	0.4
GRI			1	.62**	0.42	-.51*	0.32	0.48	-0.03
G				1	.47**	-.62**	.78**	.96**	-0.17
V					1	-.75**	0.06	0.21	0.44
EC						1	-.68*	-0.26	0.67
TT							1	.81**	-0.38
VI								1	-0.03
MGT									1

** Significant at $P \leq 0.01$ (2-tailed).

*. Significant at $P \leq 0.05$ (2-tailed).

UVS= Un-germinated but viable seeds, VAG= Viability after germination (including germinated seeds), GRI=Germination rate index, G= Total germination percentage, V=Viability before germination, EC=Electrolyte conductivity, TT= Seedling length, VI=Vigour index, MGT=Mean germination time.

For DOG 3119, strong negative correlations were observed between aging duration and most measurements (Table 4-15). The electrolyte conductivity showed a strong positive correlation with aging duration, and negative correlation with viability before germination. No significant correlations were found between mean germination times and other measurements.

For DOG 6930, negative correlations were found between aging duration and most measurements; however, no significant positive correlation was found between aging duration and electrolyte conductivity, and mean germination time (Table 4-16). Negative correlation was observed between electrolyte conductivity and viability before germination.

For DOG 3914, negative correlations were found between aging duration and most measurements (Table 4-17); however, no significant strong positive correlations were found between aging duration with before germination viability, electrolyte conductivity, and mean germination time. Strong negative correlations were also observed between electrolyte conductivity and total germination percentage and viability before germination. The viability after germination (including germinated seeds) was positively correlated with germination rate index, seedling length, and vigour index.

For DOG 3259, negative correlations were found between aging duration and most measurements (Table 4-18). However, a strong positive correlation was observed between aging duration and electrolyte conductivity, and no significant correlations were found between aging duration and vigour index and mean germination time. Strong negative correlations were observed between electrolyte conductivity with the percentage of un-germinated but viable seeds, viability before/ after germination, final germination percentage, and germination rate index.

Table 4-15 Simple correlation coefficients between various measures of seed quality for DOG 3119 during accelerated seed aging.

Correlations									
DOG 3119	UVS	VAG	GRI	G	V	EC	TT	VI	MGT
AD	-.87**	-.84**	-.72**	-.83**	-.90**	.47**	-0.51	-.60*	-0.11
UVS	1	.96**	.79**	.89**	.72**	-0.24	0.31	0.48	0.09
VAG		1	.69**	.83**	.69**	-0.34	-0.25	-0.06	0.41
GRI			1	.90**	.62**	-0.18	0.47	.77**	-0.49
G				1	.71**	-0.22	0.52	.84**	-0.04
V					1	-.63**	0.54	.59*	0.25
EC						1	.66*	0.36	-0.29
TT							1	.87**	-0.21
VI								1	-0.22
MGT									1

** Significant at $P \leq 0.01$ (2-tailed).

*. Significant at $P \leq 0.05$ (2-tailed).

UVS= Un-germinated but viable seeds, VAG= Viability after germination (including germinated seeds), GRI=Germination rate index, G= Total germination percentage, V=Viability before germination, EC=Electrolyte conductivity, TT= Seedling length, VI=Vigour index, MGT=Mean germination time.

Table 4-16 Simple correlation coefficients between various measures of seed quality for DOG 6930 during accelerated seed aging.

Correlations									
DOG 6930	UVS	VAG	GRI	G	V	EC	TT	VI	MGT
AD	-.89**	-.88**	-.72**	-.87**	-.66**	0.25	-.91**	-.89**	0.42
UVS	1	.97**	.83**	.92**	.52**	-0.19	.81**	.84**	-0.36
VAG		1	.79**	.92**	.57**	-0.27	.63*	.78**	-0.27
GRI			1	.82**	.41*	-0.07	.68*	.71**	-0.42
G				1	.51**	-0.16	.68*	.93**	-0.37
V					1	-.62**	-0.05	0.08	0.37
EC						1	0.41	0.31	-0.53
TT							1	.88**	-0.48
VI								1	-0.41
MGT									1

** Significant at $P \leq 0.01$ (2-tailed).

*. Significant at $P \leq 0.05$ (2-tailed).

UVS= Un-germinated but viable seeds, VAG= Viability after germination (including germinated seeds), GRI=Germination rate index, G= Total germination percentage, V=Viability before germination, EC=Electrolyte conductivity, TT= Seedling length, VI=Vigour index, MGT=Mean germination time.

Table 4-17 Simple correlation coefficients between various measures of seed quality for DOG 3914 during accelerated seed aging.

Correlations									
DOG 3914	UVS	VAG	GRI	G	V	EC	TT	VI	MGT
AD	-.88**	-.89**	-.74**	-.79**	-0.34	0.14	-.76**	-.83**	-0.09
UVS	1	.95**	.74**	.75**	0.2	0.17	.89**	.61*	0.17
VAG		1	.75**	.85**	0.3	-0.14	.69**	.75**	0.3
GRI			1	.85**	.48**	-0.21	0.42	.74**	-0.31
G				1	.53**	-.36*	0.31	.94**	0.04
V					1	-.52**	-0.34	0.32	0.03
EC						1	0.33	-0.37	0.05
TT							1	.54*	0.04
VI								1	0.02
MGT									1

** Significant at $P \leq 0.01$ (2-tailed).

*. Significant at $P \leq 0.05$ (2-tailed).

UVS= Un-germinated but viable seeds, VAG= Viability after germination (including germinated seeds), GRI=Germination rate index, G= Total germination percentage, V=Viability before germination, EC=Electrolyte conductivity, TT= Seedling length, VI=Vigour index, MGT=Mean germination time.

Table 4-18 Simple correlation coefficients between various measures of seed quality for DOG 3259 during accelerated seed aging.

Correlations									
DOG 3259	UVS	VAG	GRI	G	V	EC	TT	VI	MGT
AD	-.86**	-.85**	-.76**	-.81**	-.93**	.49**	-.62*	-0.51	0.25
UVS	1	.96**	.83**	.85**	.74**	-.42*	0.55	0.51	-0.11
VAG		1	.83**	.82**	.74**	-.49**	0.09	0.04	0.31
GRI			1	.87**	.63**	-.47**	-0.01	0.32	-0.13
G				1	.72**	-.48**	0.14	.69**	-0.55
V					1	-.54**	0.43	0.25	-0.4
EC						1	0.08	0.02	-0.23
TT							1	.79**	-0.43
VI								1	-.61*
MGT									1

** Significant at $P \leq 0.01$ (2-tailed).

*. Significant at $P \leq 0.05$ (2-tailed).

UVS= Un-germinated but viable seeds, VAG= Viability after germination (including germinated seeds), GRI=Germination rate index, G= Total germination percentage, V=Viability before germination, EC=Electrolyte conductivity, TT= Seedling length, VI=Vigour index, MGT=Mean germination time.

4.2.4 Seed protein changes during accelerated seed aging

Proteins extracted from all treatments in chokecherry and pin cherry showed a 25.9 kDa polypeptide that reacted with dehydrin antibodies (Figure 4-13). In chokecherry species, the dehydrin antibodies recognized two clear bands around 25.9 kDa non-aged seeds. One clear band appeared above the two already exsisting bands after 5 d aging treatment. For the pin cherry collection, the protein patterns of seed changed after artificial aging. The concentration of 25.9 kDa protein increased steadily with aging period, and a new protein band appeared after 10 d aging treatments at 19.4 kDa.

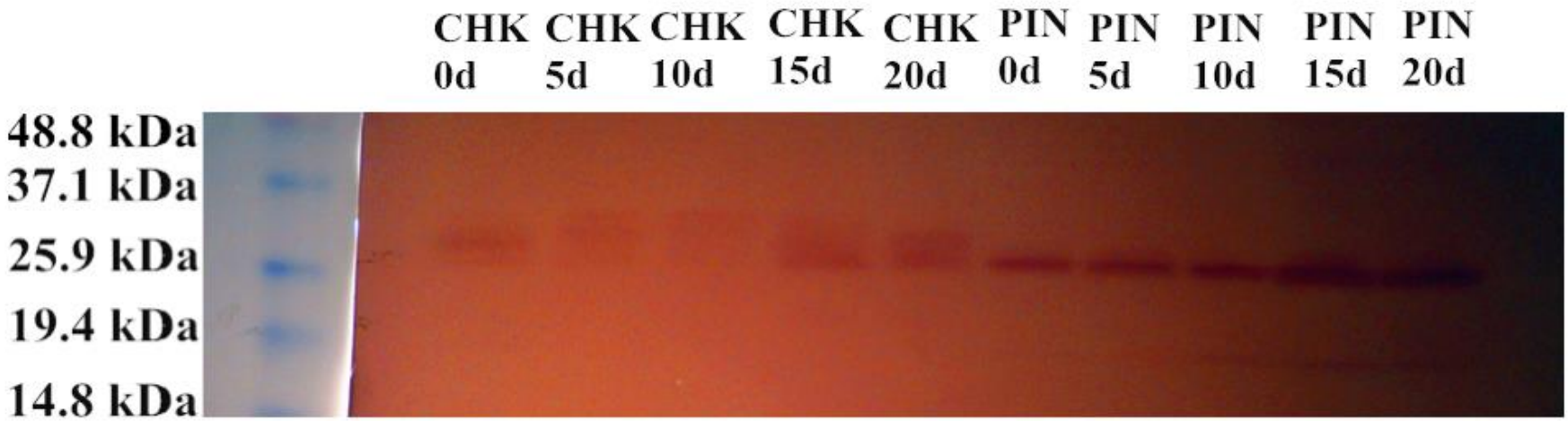

Figure 4-13. Accumulation of dehydrins during seed aging. The western blot of total protein (35 µL/ lane) was probed with antibodies against dehydrin protein. Numbers on the left indicate the approximate molecular weights. CHK = chokecherry; PIN = pin cherry.

5 DISCUSSION

5.1 Seed properties as affected by one year storage under various temperature/ RH/ gas conditions

One-year storage of two chokecherry collections and two pin cherry collections did not reveal any significant differences in most seed biochemical and physiological properties with the exception of total seed viability. Seeds stored at -20 °C had higher final germination and vigour index, and lower percentage of un-germinated but viable seeds than seeds stored at room temperature (22.5 °C). Seeds lose vigour and become more sensitive to stresses during storage (Butler et al., 2009; Ballesteros et al., 2011). Storage temperature, relative humidity, and atmosphere are considered to be the main factors affecting seed aging (Ballesteros et al., 2011). Elevated temperatures can increase seed deterioration rate (Priestley, 1986; Ballesteros et al., 2011) while sub-zero temperatures may prolong seed survive for several decades (Roberts, 1972; Vertucci et al., 1990; Walters, 2007). According to Stevens et al. (1981), shrub seeds can maintain germination ability up to 10 years when stored in an open, unheated and uncooled warehouse.

The two seed moisture contents tested in these experiments, 3~4% (ultra-dry) and 7~8% (normal), did not have significant effects on seed properties after one-year storage. Seeds with high moisture content may be damaged during freezing due to the presence of free water (Roberts, 1972; Vertucci et al., 1990; Coolbear, 1995; Schwember et al., 2011). On the other hand, desiccation damage may occur when seeds are too dry (Hong et al., 1996; Bewley et al., 2013).

One year after storage, N_2 treatment showed a trend of higher total germination percentage and seed vigour index. The presence of O_2 during storage not only accelerates seed respiration rate, but also stimulates the growth of aerobic microorganisms (Roberts,

1961; Osonubi et al., 1980; Schwember et al., 2011). CO_2 can also promote the growth of obligate anaerobes (Negm et al., 1972; Reilly, 1980; Mohammadi et al., 2011). Hence, the replacement of O_2 and CO_2 with N_2 in storage can extend seed viability (Bass et al., 1978; Bass, 1980; Schwember et al., 2011)

Even though one-year storage is not long enough to observe significant changes in seed quality of native shrubs, the trends observed in sub-zero temperature N_2 treatments showed potential to slow down seed deterioration rate and prolong seed longevity.

5.2 Seed viability and germination as affected by artificial aging process

Seed vigour can be predicted by subjecting seeds to a precise degree of aging in controlled deterioration tests (Ching, 1972; Gidrol et al., 1989; Rao et al., 2006). In the current study, native shrub seeds showed a decline in seed vigour after artificial aging treatment based on germination and tetrazolium tests. Most seeds failed to germinate after 10 to 15 d aging treatments at 45 ^{o}C and 60% RH. Seeds deteriorate rapidly after exposure to both high temperature and high humidity under laboratory conditions (Gidrol et al., 1989; Hyatt et al., 2008). Vegetative seeds such as carrot (*Daucus carota*), onion (*Allium cepa*), or lettuce (*Lactuca sativa*) lose vigour rapidly after being exposed to high temperature (45 ^{o}C) and high humidity (100%) conditions for 72 h (Delouche et al., 1973; Bailly et al., 1996; Rodo et al., 2003). Shrub seeds are more resistant than crop seeds to accelerated aging since most of them have hard seed coats. Generally seeds with hard seed coats are long lived (Bass, 1980; Priestley, 1986; Rao et al., 2006) because hard seed coats prevent seeds not only from mechanical injury, but also temperature and humidity stress as well as the invasion of microorganisms (Mohamed-Yasseen et al., 1994).

Aged seeds exhibited delayed and reduced germination especially after 10 d aging treatments. Slower germination is considered an indicator of vigour loss (Ching, 1972;

Carjuzaa et al., 2008; Hyatt et al., 2008). The prolonged germination period /slower growth caused by artificial aging in soybean (*Glycine max* II Mef. var. Wayne) and onion seeds is contributed by reduced vigour (Berjak et al., 1972; Parrish et al., 1978; Priestley, 1986; Rice et al., 2001). Fresh seeds usually germinate faster and more uniformly than stored / aged seeds (Demir et al., 2008). This kind of delay is considered to be associated with the time required for internal repair of damaged membranes (Rice et al., 2001). In most shrub species in this study, no germination was observed after 15 d aging, possibly due to degradation of nucleic acids, proteins and/or membranes (Priestley, 1986; Mtwisha et al., 1998; Bewley et al., 2013), accumulation of toxic compounds (Butler et al., 2009), and loss of vitamins or hormones (Walters, 2007). There was a delayed response to aging treatments in seed viability compared to germination as reported by several researchers (Delouche et al., 1973; Rodo et al., 2003; Hyatt et al., 2008).

Accelerated aging reduced seedling length of shrubs, likely due to degradation of mitochondria DNA in membrane (Gidrol et al., 1989; Bailly, 2004; Bewley et al., 2013). DNA degradation leads to impaired transcription causing incomplete or faulty enzyme synthesis essential for earlier stages of germination (Walters et al., 2010; Schwember et al., 2011).

5.3 Seed electrolyte conductivity as affected by aging process

During accelerated seed aging process, the electrolyte conductivity was negatively correlated with seed viability and germination. Aging can cause inability of seeds to maintain membrane integrity and cause reduction in germination ability (Fessel et al., 2006). Seeds lose vigour and become more vulnerable to stresses due to leaky membranes, accumulated chromosome mutations, and enzymes losing catalytic activity (Walters, 1998). Biological membranes support the transportation of chemicals and ions

thus play a key role in maintaining seed vigour (Rahoui et al., 2010). The damage on cell membrane determines the rate of solute leakage in response to seed aging, which can be an indication of seed deterioration (Priestley et al., 1979; Thuzar et al., 2010). The increased leakage associated with aging may be the result of a more permeable membrane or a larger pool of electrolytes (Powell, 1986; Takos et al., 2012). Ching (1972) considered this possibility in amino acid leakage studies of vigorous and aged, non-germinable seeds.

The increase of membrane permeability caused by artificial aging was also indicated by the stimulated fungal infection in our study. Leakage substances include ionic solutes, small organic molecules such as amino acids and sugar (Lee et al., 1995), which are favourable for fungi growth (Overath et al., 1973; Rahoui et al., 2010).

5.4 Rate of aging between species

Buffalo berry and dogwood have thin seed coats and lower electrolyte leakage rates than other shrub species studied. The hard, thick seed coat serves as primary defense against adverse environmental conditions and leakage, but also inhibits germination (Mohamed-Yasseen et al., 1994; Butler et al., 2009). Seeds of dogwood and buffalo berry are 'shallow' dormant than other shrub species studied and only required 2-3 weeks of stratification to break the dormancy. These two species also showed a more uniform and rapid seedling growth.

5.5 Seed protein changes as affected by aging process

Dehydrins are proteins that are induced and accumulate during seed maturation, which protect tissues from abiotic stresses (Delahaie et al., 2013). According to Yamane et al. (2006), dehydrins play an important role in *Prunus* species during the dormancy period. Cold stress can induce dehydrins production, which can enhance the performance of

future seedlings (Delseny et al., 2001; Brooks, 2011). After one-year storage treatments, the accumulation of dehydrins was greater in chokecherry seeds that were stored with N_2 at room temperature, as well as seeds stored at sub-zero temperature.

In response to stress, plants may produce specific proteins to protect the cellular machinery and damage repair (Rajjou, 2008b). Immunological detection by western blot revealed the expression of dehydrins with a molecular mass of ~27 kDa under heat stress. In both species chokecherry and pin cherry, the concentration of dehydrins increased after aging treatments, indicating a protective role of these proteins just like other heat stress proteins (Wang et al., 2003; Rurek, 2010). Dehydrins share many physiological properties such as function and structure with small heat shock proteins (Mtwisha et al., 1998; Delahaie et al., 2013). This kind of water-soluble and heat stable proteins showed maintenance of structural stability under a variety of stresses (Delseny et al., 2001). According to Wahid et al. (2007), an increase in leaf water pressure potential is correlated with the expression of dehydrins and heat shock proteins under heat stress. It has been suggested that dehydrins can bind to macro molecular structures to prevent cellular damage during stresses conditions, serving as molecular chaperones and being associated to the cytoplasmic membranes (Sales et al., 2000; Wahid et al., 2007; Carjuzaa et al., 2008; Delahaie et al., 2013).

5.6 Discussion on methods

The extraction and preparation of protein samples are two critical steps in the proteomic study. Different protein extraction methods promote certain groups of proteins, which may not be identified by all extraction methods. There is no universe protein extraction method that can capture all the proteomes due to the diversity of cellular proteins (Saravanan et al., 2004). Degradation minimization, reproduction comprehensiveness and

contaminant removal are considered to be the main characteristics of ideal extraction methods. In this study, two widely used protein methods were assessed by examining characterises of extracts quantitatively and qualitatively. Different solubilisation of protein in different extraction buffers are the foundation of protein extraction protocols (Isaacson et al., 2006). Unlike Tris-HCl buffer extraction method, in which proteins are obtained from cellular extracts used directly for further analysis, the phenol extraction method result relies on precipitating proteins from the phenol extract. According to Saravanan (2004), the phenol extraction method is ideal to extract proteins from resistant tissues such as wood, olive leaves and seeds (Ritenour et al., 2001; Saravanan et al., 2004). Phenol extraction method is more time consuming and laborious than the Tris-HCl extraction method. However, the phenol extraction is more efficient than Tris-HCl in generating high purity protein as determined by SDS-PAGE analysis, especially in the region 14.5 kDa to 82.2 kDa. The high protein purity obtained by phenol extraction is mainly because the water-soluble contaminants are eliminated from the non-water based protein-enriched phenol layer (Benndorf et al., 2009).

Seeds viability from tetrazolium test after artificial aging test showed a delay and overestimation in viability as validated by germination. The level of overestimation in triphenyl-tetrazolium chloride assay was greater for heat tolerance estimates than for salt and freezing tolerance estimates (Ishikawa et al., 1995). According to (Bewley et al., 1982), the overestimating of seed viability using TZ test was caused by the delay in concomitant loss of dehydrogenase enzymes. Similar observation was also found in accelerated aging experiments in pea and onion seeds (Bass et al., 1978; Rodo et al., 2003; Hyatt et al., 2008).

5.7 Conclusions and practical implications

Six shrub species identified by the vegetation cooperative of CONRAD, *Prunus virginiana*, *Prunus pensylvanica*, *Arctostaphylos uva-ursi*, *Shepherdia canadensis*, *Cornus sericea*, and *Viburnum edule* were used in this study. Two *Prunus virginiana* and two *Prunus pensylvanica* collections were stored under different gas-temperature-RH conditions for one year. The mechanism of seed aging was studied by subjecting seventeen collections of all the shrubs mentioned above to the accelerated aging test.

In general, the duration seeds can be stored without serious loss of viability is determined largely by storage environment. The effects of storage conditions including the combination of gas, relative humidity, as well as temperature on seed physiological properties did not show significantly different changes after one-year storage. However, seed storage at room temperature with N_2, as well as sub-zero temperature showed a trend of reduced deterioration. One year aged pin cherry showed significant decrease in total germination percentage after one year storage while seed viability remains very high, which indicate the low vigour seeds might experience deep dormancy.

Aged seeds consistently exhibited lower performance when controlled and aged seeds were compared on the basis of several criteria of seed vigour. Changes in seeds of native shrub species that occur during accelerated aging (45^{o}C, 60% relative humidity) showed subsequent loss of vigour, a decline in seedling length, germination percentage, viability, and overexpression of protein band at 27 kDa that was recognised by dehydrin antibody.

The current study also suggests that electrolyte conductivity values were strongly correlated with seed vigour. Increased leaching of electrolytes was observed in the artificial aged seeds. High electrolyte conductivity in solution of aged seeds indicates faster deterioration. The weakening of cell membrane might have caused increased

leaching of metabolites and electrolyte through the semi permeable membrane into the inhibiting medium.

In conclusion, this study found the trend of optimal storage protocol for seed storage and improved the basic understanding of physiological and biochemical for native shrub species. The storage protocol developed by this study can ensure an adequate supply of viable shrub seeds for reclamation, which can be immediately transferred for the use by industrial partner. Artificial aging technique for predict seed longevity can also be expended for other non-crop species that can be used in reclamation of lands related to oil extraction; however, species specific parameters must be taken into consideration.

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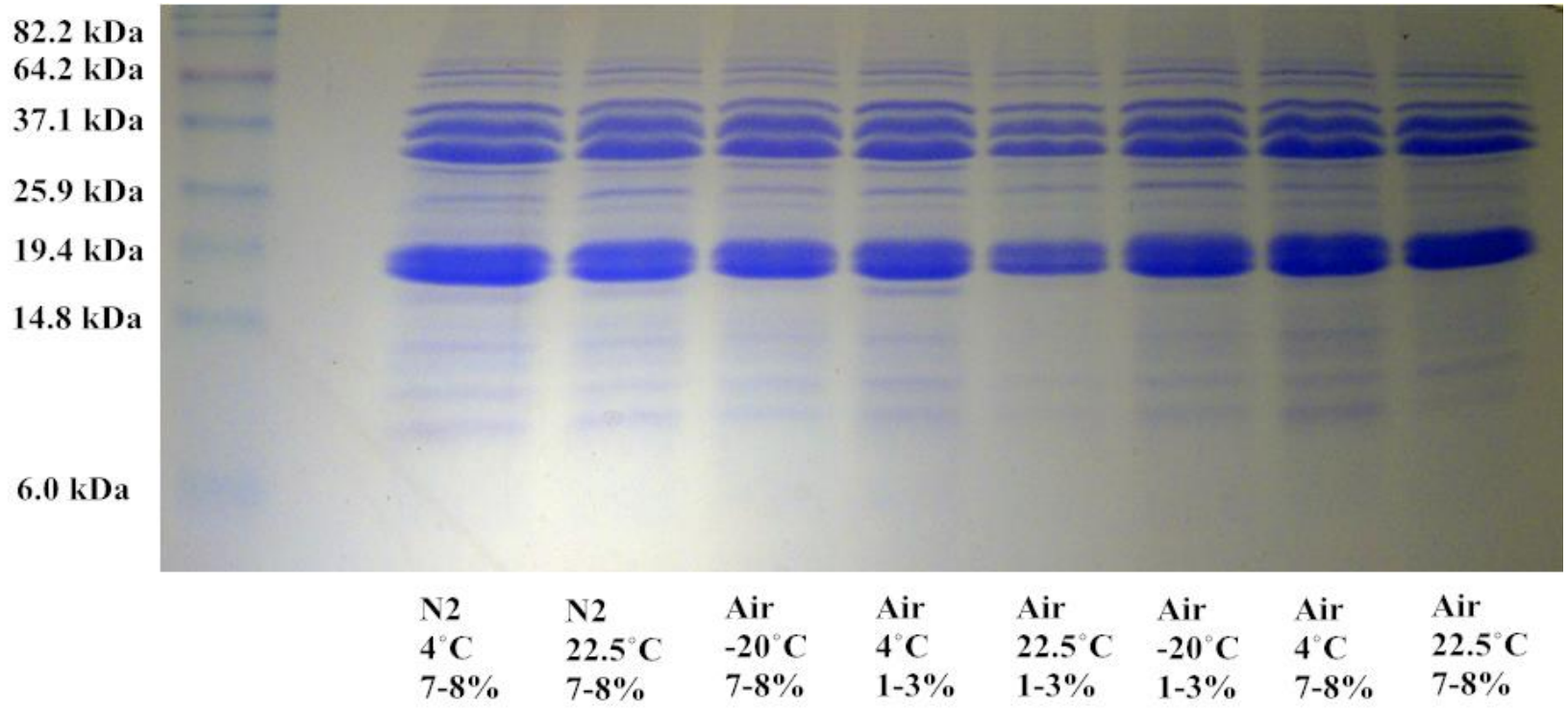
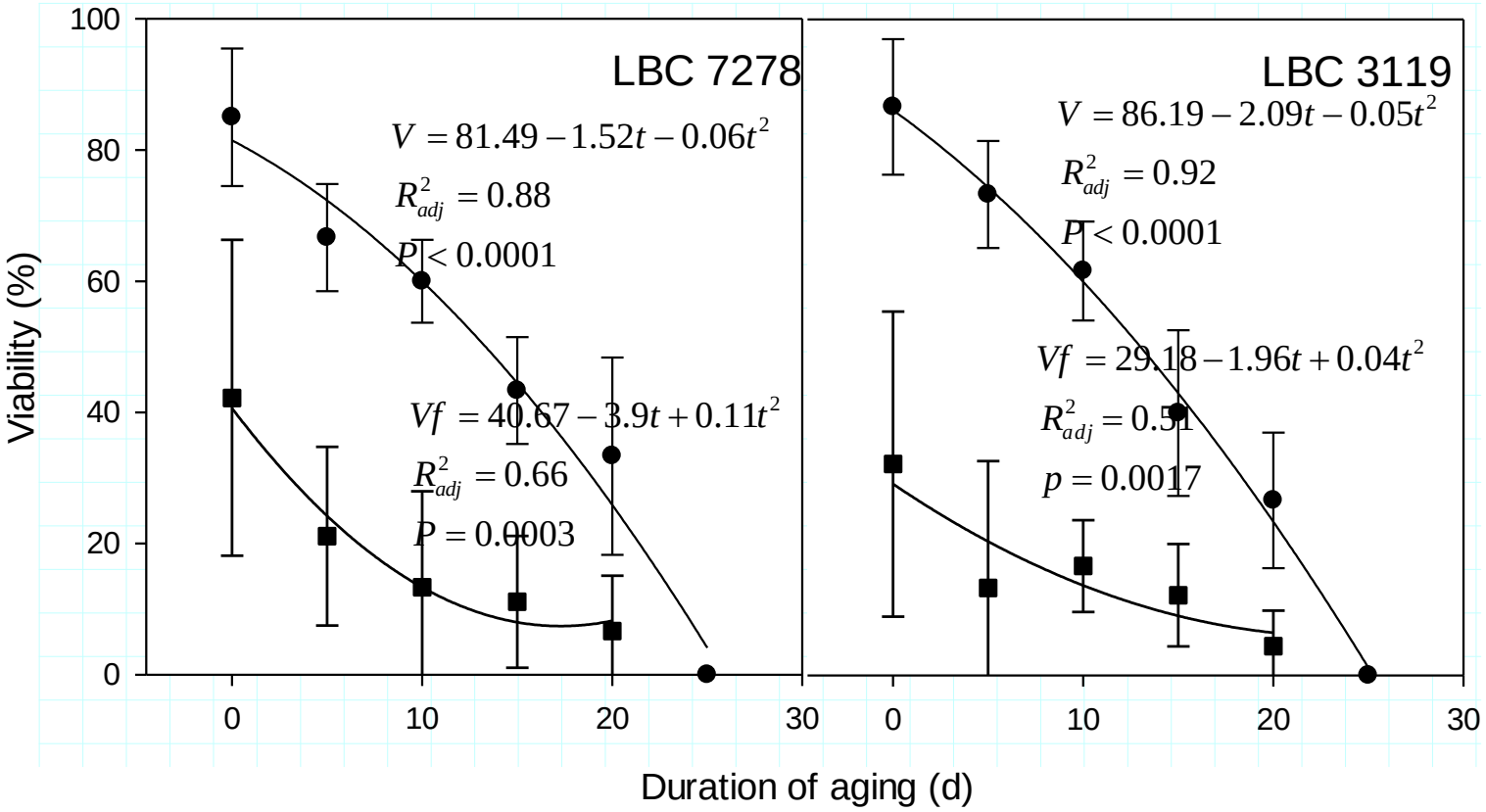
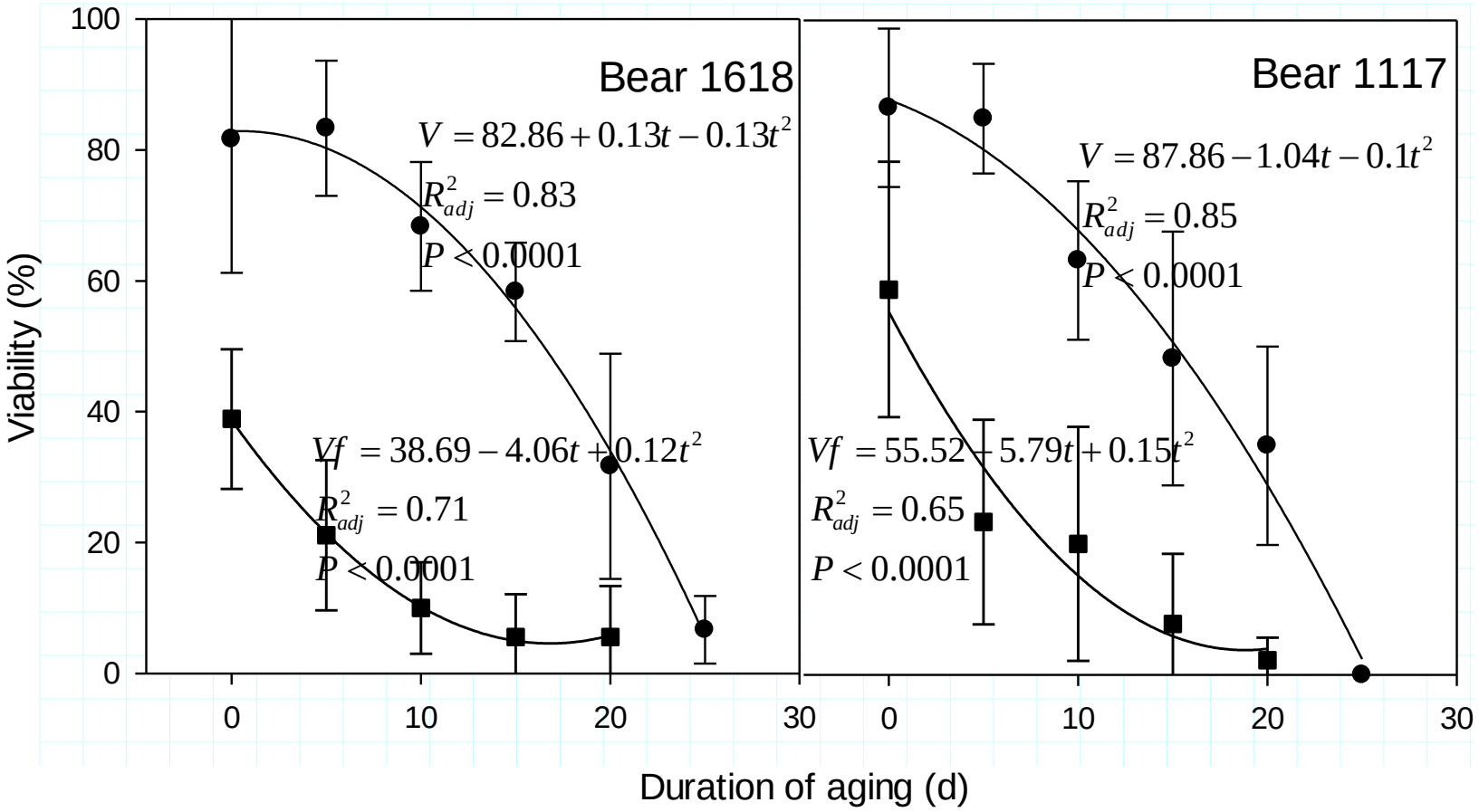
7 APPENDIX



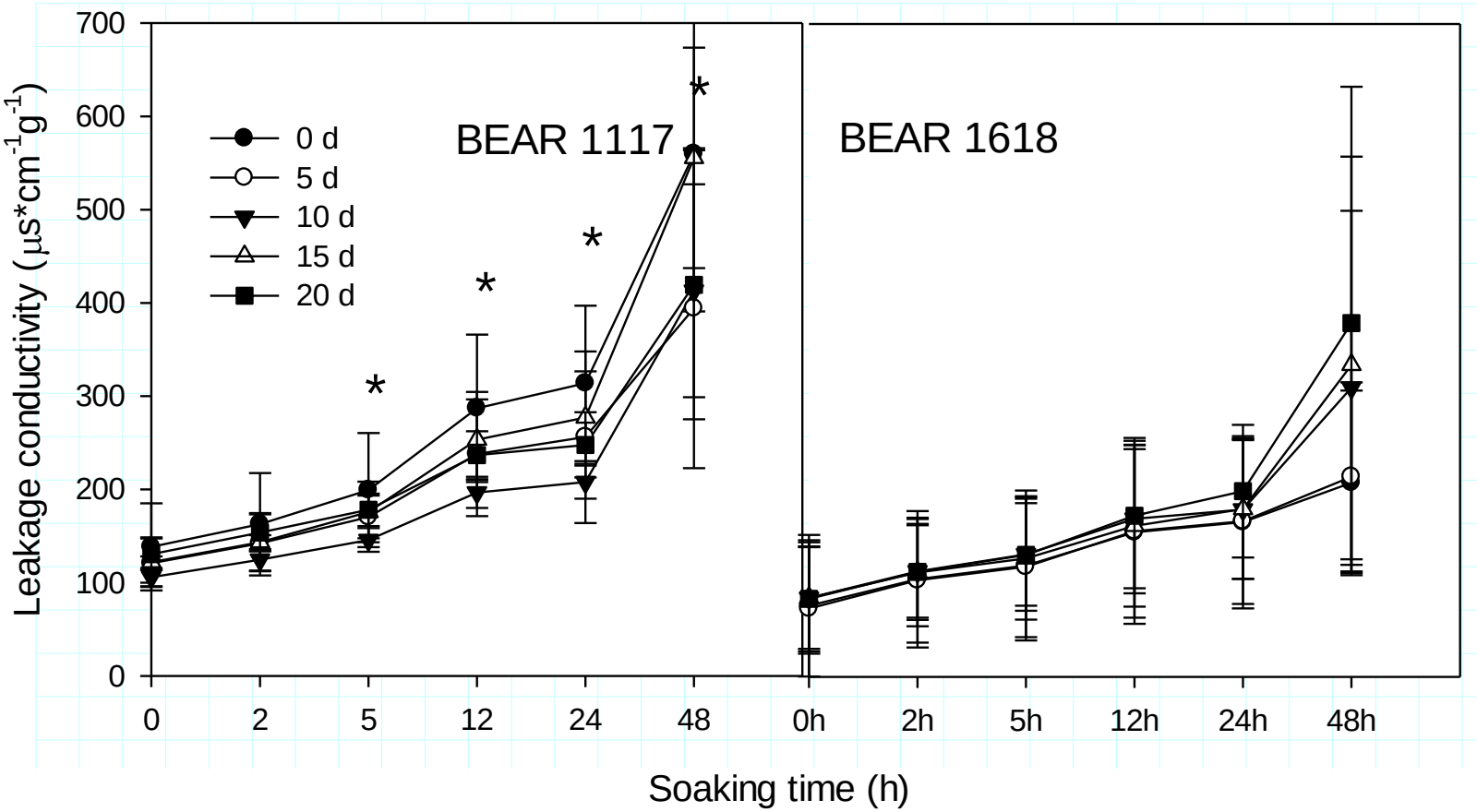
Appendix 1: Influence of one-year storage treatments on protein patterns of chokecherry collected in 2010. An equal amount (35 µL) of total soluble protein extracts was loaded on each lane. Numbers on the left lane indicates the approximate molecular weights.



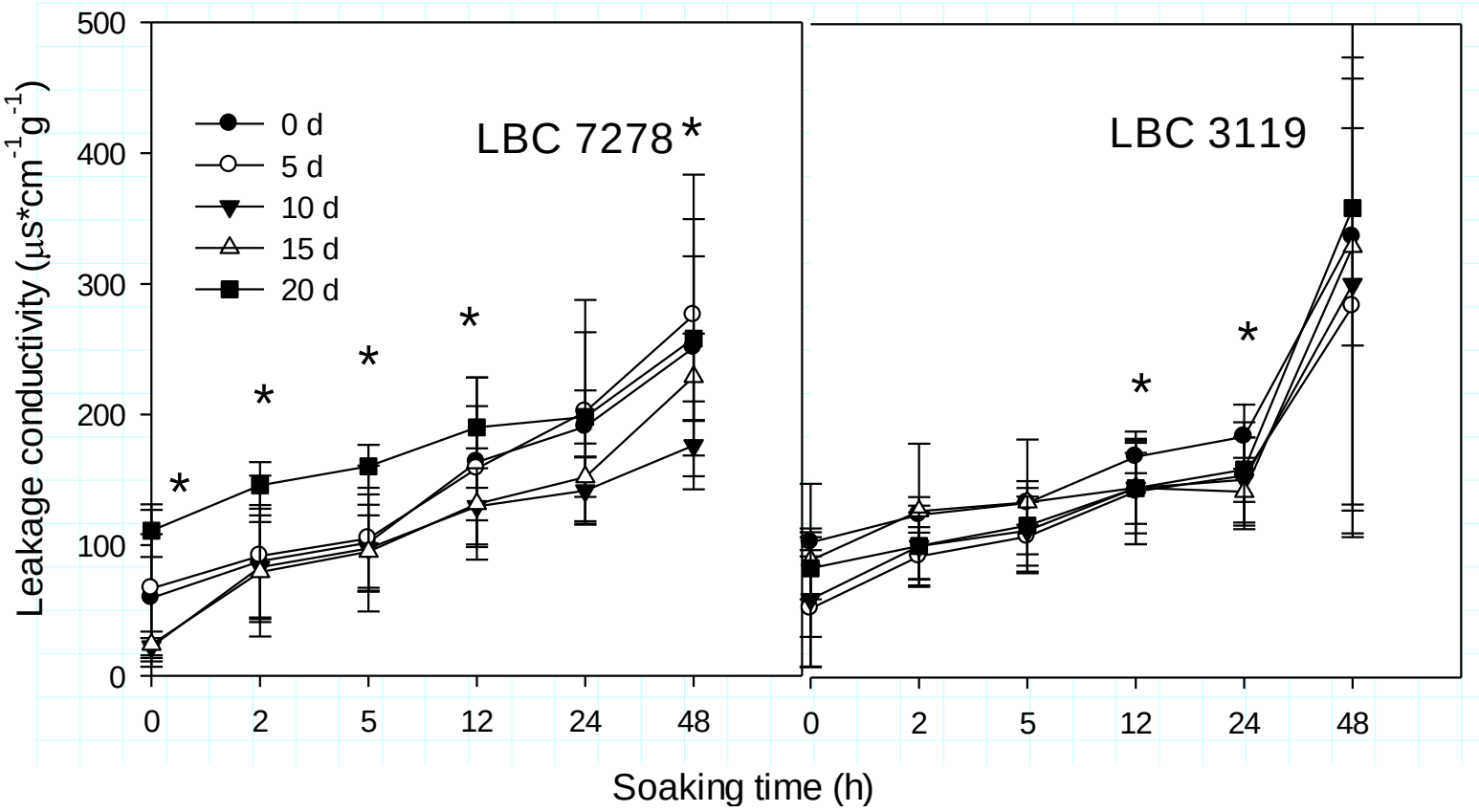
Appendix 4: Observed (symbol) and simulated (solid line) viability before germination and viability after germination (including germinated seeds) test of two low bush cranberry collections during artificial aging process at 45 °C with 60 % RH. Data are Means ± SE. Bars are standard errors (n=6).



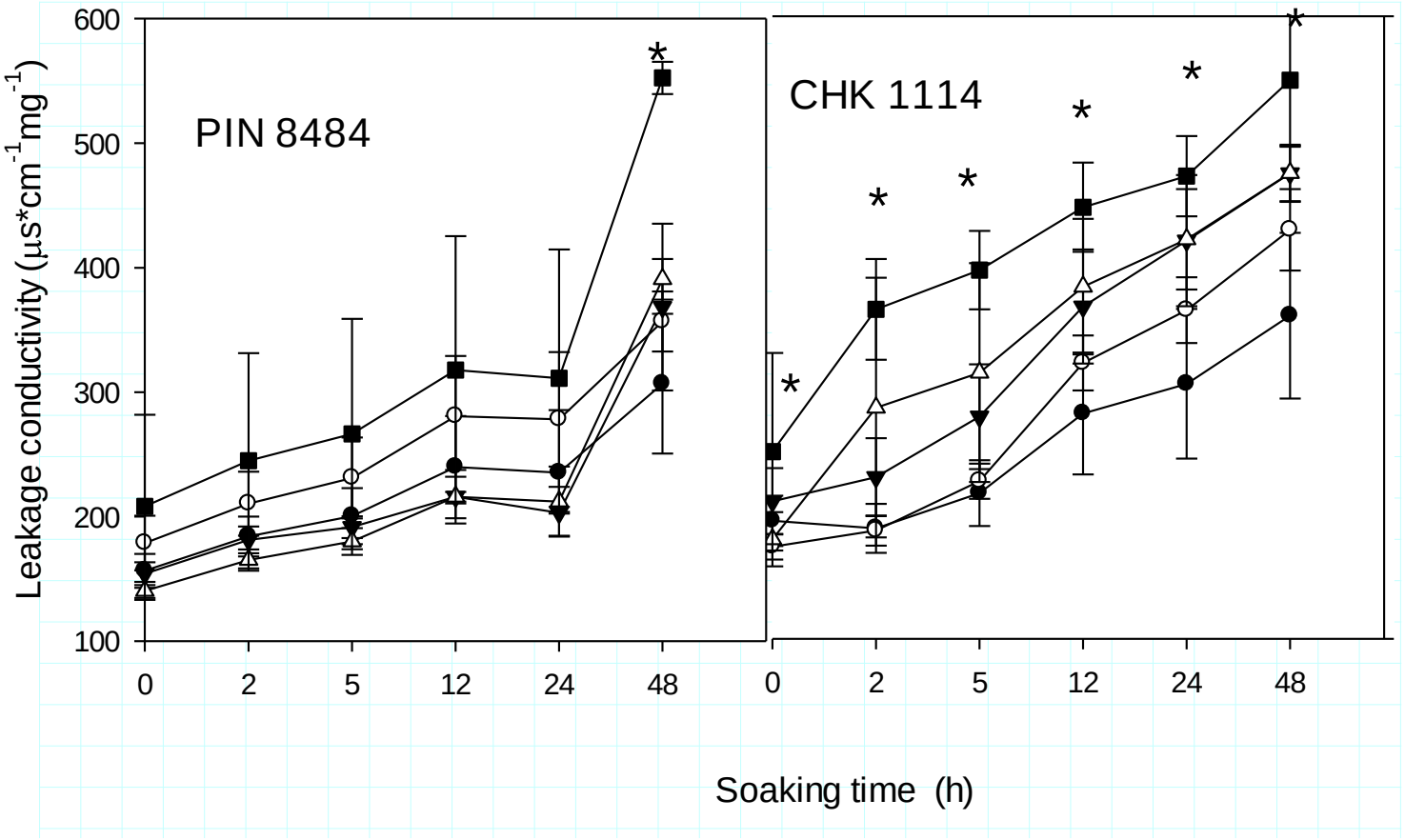
Appendix 5: Observed (symbol) and simulated (solid line) viability before germination test and viability after germination (including germinated seeds) test of two bearberry collections during artificial aging process at 45°C with 60%RH. Data are Means ± SE (n=6).



Appendix 6: Electrolyte conductivity of two bearberry collections after different artificial aging duration at 45°C with 60% RH. Bars are ±SE (n=6). ' * ' indicates statistically significant difference in same soaking time *(P < 0.05)*.



Appendix 7: Electrolyte conductivity of two low bush cranberry collections after different artificial aging duration at 45°C with 60% RH. Bars are ±SE (n=6). ‘ * ’ indicates statistically significant difference in same soaking time *(P < 0.05)*.



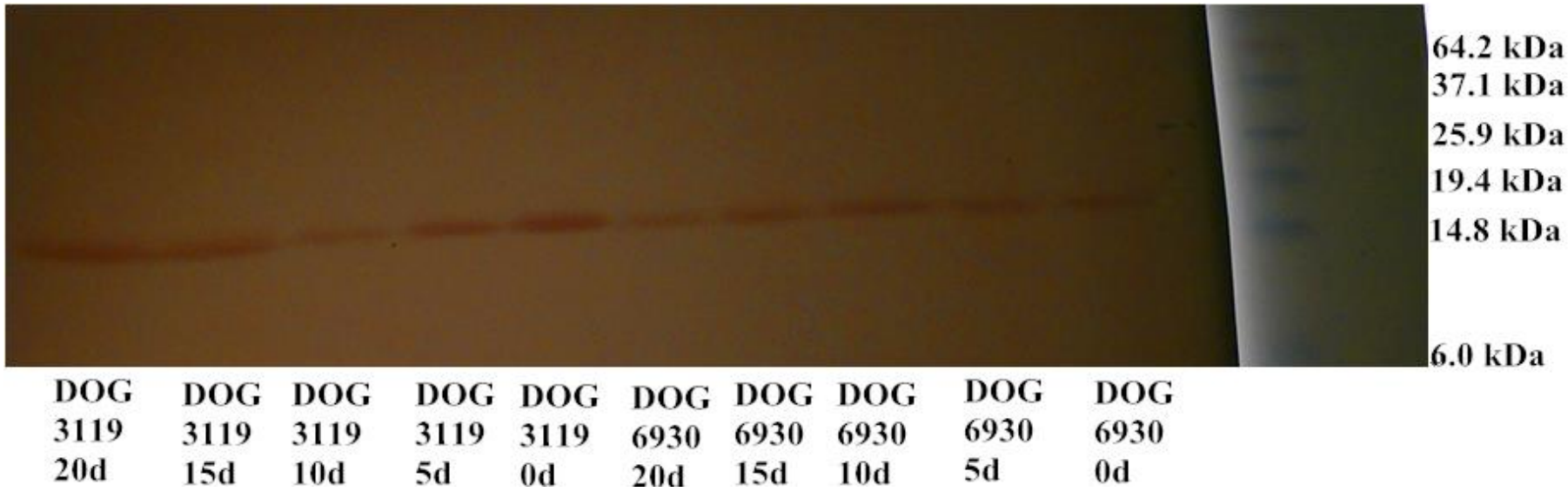
Appendix 8: Electrolyte leakage conductivities of low bush cranberry collections after different artificial aging duration at 45°C with 60% RH. Bars are ±SE (n=6). ' * ' indicates statistically significant difference in same soaking time *(P < 0.05)*.

Appendix 9: Simple correlation coefficients between various measures of seed quality for several low bush cranberry, bear berry and chokecherry collections during accelerated seed aging. VAG= Viability after germination, V=Viability before germination, EC=Electrolyte conductivity

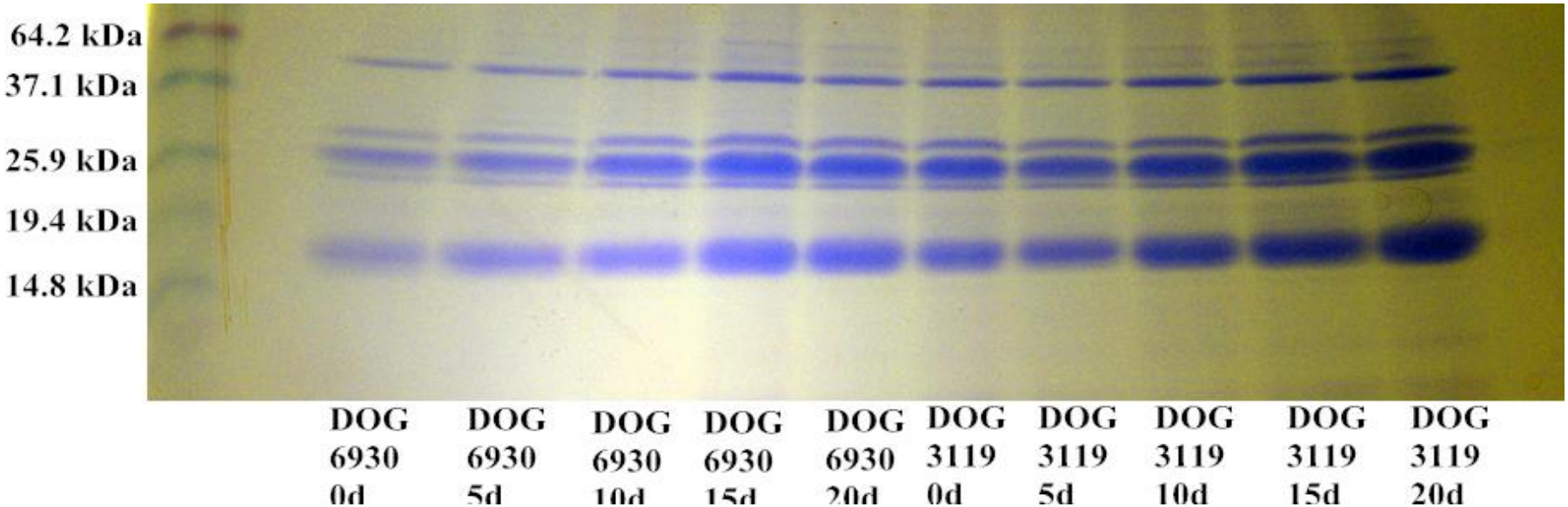
Correlation									
Species		VAG	V	EC	Species		VAG	V	EC
LBC 3119	AD	-.50**	-.85**	-0.02	LBC 7278	AD	-.61**	-.82**	.12
	VAG	1.00	.34	.24		VAG	1.00	.43*	-.06
	V		1.00	-.38*		V		1.00	-.53**
	EC			1.00		EC			1.00
BEAR 1117	AD	-.76**	-.94**	-.11	BEAR 1618	AD	-.77**	-.74**	.32
	VAG	1.00	.61**	.22		VAG	1.00	.51**	-.39*
	V		1.00	-.17		V		1.00	-.60**
	EC			1.00		EC			1.00
CHK 1114	AD	-.62**	-.88**	.67**					
	VAG	1.00	.55**	-.44*					
	V		1.00	-.87**					
	EC			1.00					

**. Correlation is significant at the 0.01 level (2-tailed).

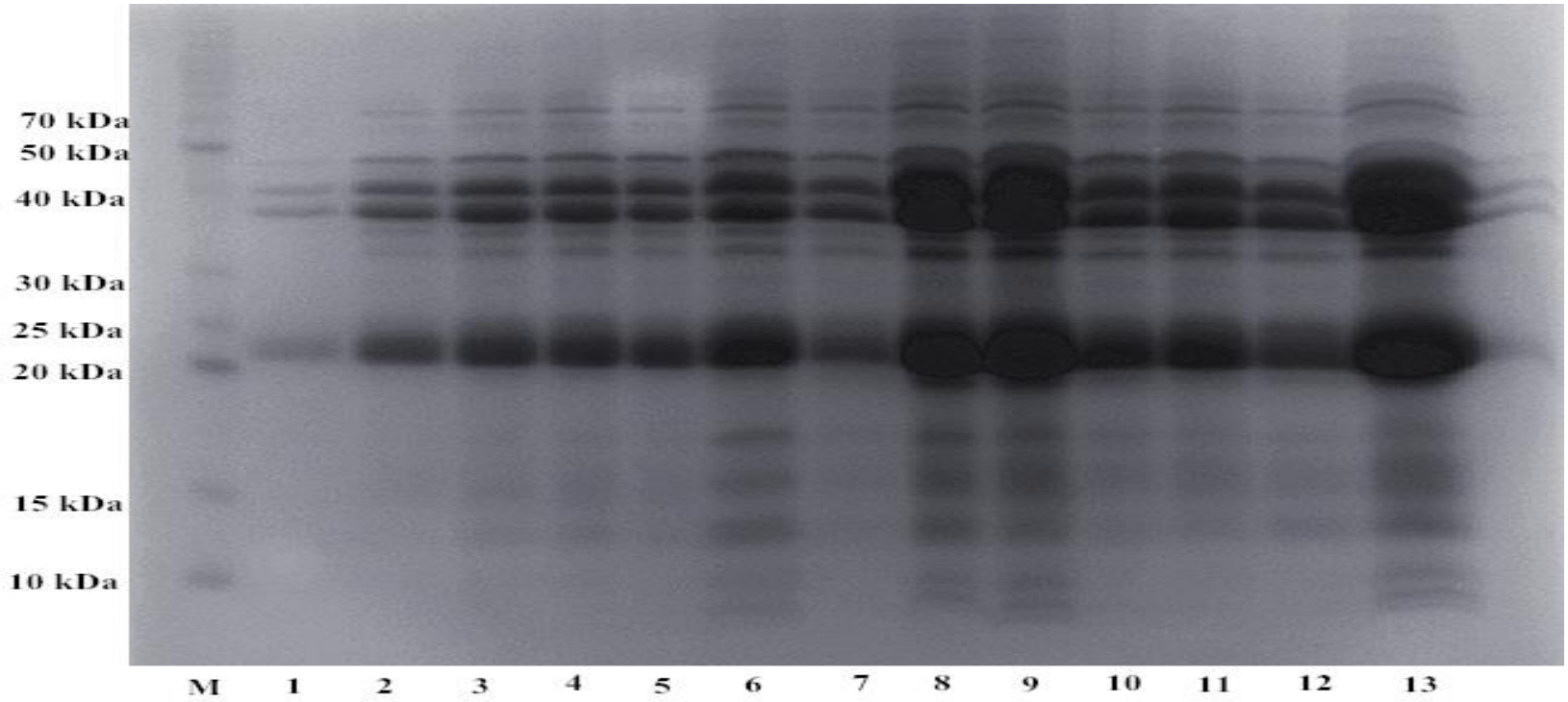
*. Correlation is significant at the 0.05 level (2-tailed).



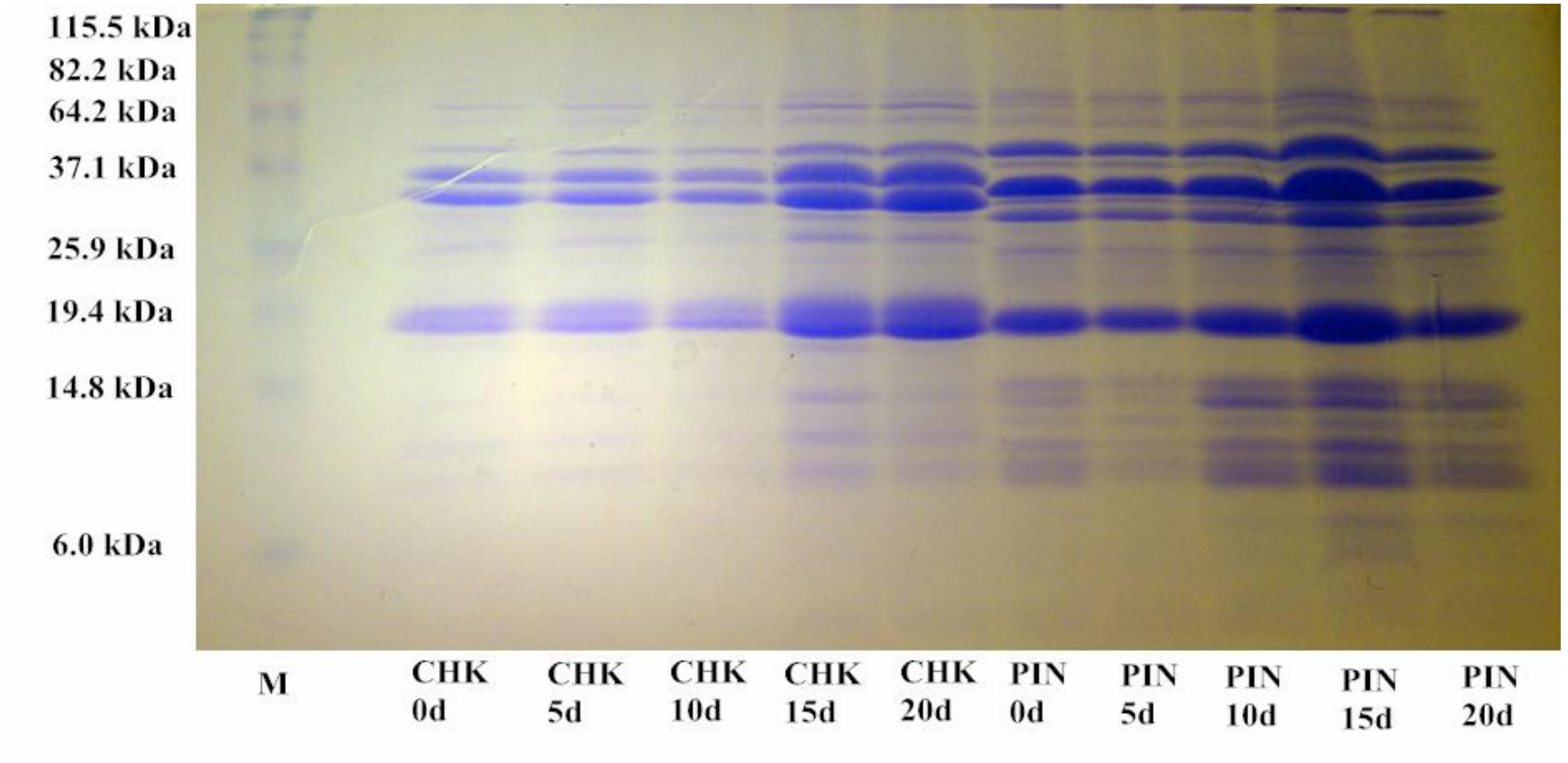
Appendix 10: Accumulation of dehydrins in dogwood seeds during seed aging process. The western bolt of total protein (35 µL/lane) was probed with antibodies against dehydrin protein. Numbers on the right lane indicate approximate molecular weights.



Appendix 11: Protein profiles of two dogwood collections during seed aging process. An equal amount (35 µL) of total soluble protein extracts was loaded on each lane. Numbers on the left indicate the approximate molecular weights.



Appendix 12: Changes of protein patterns of CHK 1410 and CHK 1114 after different aging duration. Numbers on the left indicate the approximate molecular weights. M: Marker, lane 1-5： 0 day aged CHK 1410 with different loading concentration; lane 1: 10 μL, lane 2: 15 μL, lane 3: 25 μL, lane 4: 30 μL, lane 5: 20 μL. Lane 6-7：5 d aged CHK 1410 with different loading concentration， lane 6: 30 μL, lane 7: 20 μL. Lane 8-13: CHK 1114 with different aging duration, an equal amount (35 μL) of total soluble protein extracts was loaded on each lane; lane 8: 0 d aged, lane 9: 5 d aged, lane 10: 10 d aged, lane 11: 15 d aged, lane 12: 20 d aged, lane 13: 25 d aged.



Appendix 13 Protein profiles of chokecherry and pin cherry seeds samples collected at different accelerate aging duration. An equal amount (35 µL) of total soluble protein extracts was loaded on each lane. Numbers on the left indicate the approximate molecular weights.