CATHODIC WATER INVIGORATION OF DETERIORATED ORTHODOX SEEDS – IMPLICATIONS ON SUBSEQUENT PLANT GROWTH

By

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As the candidate's supervisor, I have approved this thesis for submission

Name: Professor RP Beckett

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ABSTRACT

Orthodox seeds deteriorate even when kept under the best of conditions leading to loss of germination, poor seedling growth. In this study, a novel approach via the use of cathodic water, an electrolysed form of calcium magnesium solution, was used to invigorate fresh and controlled deteriorated seeds of wild (*Bolusanthus speciosus* (Bolus) Harms*, Combretum erythrophyllum* (Burch.) Sond*., Erythrina caffra* Thumb.) and agricultural (*Pisum sativum* L.*, Cucurbita pepo* L.) species. Other treatment solutions investigated alongside cathodic water were un-electrolysed calcium-magnesium solution and deionized water. Fresh seeds of the test species were controlled deteriorated to 50% germination (P_{50}) at 40°C and 100% relative humidity. Thereafter, the seeds were invigorated with the treatment solutions. Fresh and un-primed controlled deteriorated seeds served as the control. In addition, to study the mechanism of invigoration, the effects of priming on the membrane stability index (MSI), amylase activities, lipid peroxidation products malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) were investigated. The effects of priming on the reactive oxygen species (ROS) scavenging enzymes superoxide dismutase, and catalase and DNA (concentration and purity). All priming treatments improved germination, emergence, growth parameters and subsequent seedling photosynthesis relative to the unprimed seeds. In general, cathodic water was most effective at invigorating seeds in the all test species. Also, controlled deteriorated seeds benefitted more than the fresh seeds treatments. Analyses of the lipid peroxidation products and antioxidant enzyme activities in invigorated seeds provided support for the hypothesis that the effectiveness of cathodic water in the invigoration of debilitated orthodox seeds derive from its ability to act as a potent antioxidant. This study, which is a novel approach at bringing the concept of electrochemistry into germplasm conservation via the use of cathodic water, has also confirmed the efficacy of cathodic water in invigorating debilitated seeds. This is especially critical for seeds containing traits that may be under the threat of being lost due to various reasons. The current study reinforces the strong potential of cathodic water in the recovery of aged germplasm and improved yield of orthodox seeded species.

Keywords: cathodic water, controlled deterioration, germination, growth, invigoration, orthodox seeds.

PREFACE

The experimental work described in this thesis was carried out in the laboratories of Plant Germplasm Conservation Research Unit and one of the green houses of the School of Life Sciences, University of KwaZulu-Natal, WestvilleCampus, from January 2016 to November 2018, under the supervision of Professor Norman Pammenter, and from May 2019 to May 2020 by Professor RP Beckett. Dr Boby Varghese co-supervised the project from January 2016 – May 2020.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others, it is duly acknowledged in the text.

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Kayode FATOKUN

DECLARATION 1 - PLAGIARISM

I, Kayode FATOKUN declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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DECLARATION 2 - PUBLICATIONS

Fatokun, K.; Beckett, R.P.; Varghese, B. & Pammenter, N.W. (2020). Influence of Cathodic Water Invigoration on the Emergence and Subsequent Growth of Controlled Deteriorated Pea and Pumpkin Seeds. *Plants*, *9*(8), 955; doi:10.3390/plants9080955

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The research was conceptualized by late Professor Patricia Berjak (1939-2015) and Professor Norman Pammenter. All authors were involved in the research design. The research was carried out by Kayode Fatokun for his Doctoral study and was responsible for data collection, data analyses and writing of all the articles. Supervision, corrections and guidance were provided by Professor RP Beckett, Dr Boby Varghese, and Emeritus Professor N.W. Pammenter.

Signed: 

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DEDICATION

This thesis is dedicated to my sons Joshua and Paul Fatokun, who in their formative lives had to contend with the challenges of an absentee student-daddy

'At the time I have decided, my words will come true. You can trust what I say about the future. It may take a long time, but keep on waiting--it will happen' (Habakkuk 2:3)

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CHAPTER 1

INTRODUCTION

This chapter contains literature that gives a proper background to the study; it includes topics such as; desiccation tolerance in orthodox seeds, seed deterioration/controlled deterioration of seed, effects of seed ageing on germination and plant growth, seed invigoration, seed priming techniques, seed germination and seedling vigour, effects of seed priming on germination and subsequent seedling growth, imbibition damage in seeds/ seeds electrical conductivity, antioxidants and reactive oxygen species (ROS) in the context of normal germination, desiccation sensitivity and tolerance. This chapter also provide literature in respect to the main subject of this research - cathodic water. The problem statement and research justification for this research were also covered. The aim of this study and the intended contribution of the study to the body of knowledge/ novelty was also included in this introductory chapter.

1.1 Background

Seeds are the genetic resource by which most higher-plants are propagated (Sacandé et al., 2004). Seeds are classified based on their desiccation tolerance; recalcitrant and orthodox seeds. While recalcitrant seeds are desiccation sensitive and cannot be stored for a long time, orthodox seeds are tolerant of desiccation (Dickie and Pritchard, 2002; Engelmann, 2011) and can be stored for a long time (Engelmann, 2011), especially at low water contents and temperatures (McDonald, 2004). It has been reported that about 90% of the species being studied are orthodox seeds producing (Dickie and Pritchard, 2002; Sacandé et al., 2004). Orthodox seeds are either stored in the short or the long-term. Short-term orthodox seed storage provides high-quality planting material for reintroduction and rehabilitation (Felix et al., 2020; Tweddle et al., 2003). Also, seeds are stored in the long time as active collection samples that are distributed. Perhaps, the most important reason for long term seed storage is to serve as long-term base collections which are ultimately used to conserve genetic resources and diversity of plants (Ferrando-Pardo et al., 2016; Vagera, 2007). For example, a major function of *ex-situ* seed conservation is preserving plant germplasm, which is suitable for restoring wild populations. Conservation of plant genetic resources is critical especially in the face of deforestation, land-use changes, and climate change. It is therefore very important to maintain seed quality for a long period (Ferrando-Pardo et al., 2016; Vagera, 2007).

Gene banking of seeds is the ideal means of *ex-situ* conservation of plant genetic resources of orthodox seeds (Berjak and Villiers, 1972; Cuena-Lombraña et al., 2020; Walters et al., 2005). However, during long-term storage, even under very good conditions, orthodox seeds deteriorate. The best that can be done is to lower the rate of deterioration during storage of such seeds in the dry state (Chhabra and Singh 2019; Walters et al., 2005). The deterioration of seeds during long-term storage is known as ageing, and in the process, both vigour and viability are gradually lost (Chhabra and Singh 2019; Garza-Caligaris et al., 2012). Seed deterioration and the consequent reduction in seed 'performance' are of substantial financial worry to the seed industry. At a global level, it poses considerable threat to long-term conservation of wild species' genetic diversity and that of agricultural and horticultural species (Ferrando-Pardo et al., 2016; Robin et al., 2009). For a long time, the primary focus of long-term seed conservation has been on the critically endangered species (Raimondo, 2015). However, there is an urgent need to include genetic resources of species of "actual or potential economic concern" especially with aggressive desert encroachment on farmlands, the ever-increasing world population and shortage of food especially in Sub-Saharan Africa (Jorgenson and Burns, 2007).

In many seed species, the loss of seed vigour often precedes the gradual or abrupt decline in viability during storage (Cuena-Lombraña et al., 2020; Finch-Savage and Bassel, 2016). In fact, the loss in seed vigour is often the first indication of seed deterioration in the gene banks. A principal indicator of seed vigour is the rate of germination occurring after imbibition (Finch-Savage and Bassel, 2016). Consequences of reduced seed vigour include abnormal growth, lowered seedling growth, lack of seedling uniformity (Chhabra and Singh 2019; McDonald, 2004; Qu et al., 2008; Ramamurthy et al., 2015). The physiological parameters of seed deterioration, which include decreased germination percentage and lower seedling growth, are related to the low rate of metabolism in the embryo of aged seeds (Khanum et al., 2019). Loss in seed vigour is also associated with loss of cell membrane integrity, which occurs as a result of metabolic and biochemical alterations in the seed. It must be noted that even when ageing effects such as reduced germination are absent, seedling development may be abnormal showing stunted roots/shoot growth (Cuena-Lombraña et al., 2020; Tarquis and Bradford, 1992). The occurrence and severity of the observed consequence of ageing on germination and subsequent seedling growth increase with ageing time and the level of applied stress/ seed storage conditions (Walters et al., 2005). Other factors that contribute to seed deterioration are relative humidity, temperature of the storage environment, genetics, mechanical damage, seed water content, presence of microflora and seed maturity (McDonald, 2004; Yari et al., 2011). Similarly, when seeds are artificially

subjected to stress such as heat and humidity (controlled deterioration), the deterioration of the seeds is inexorable (Leão-Araújo et al., 2017). The consequence of seed deterioration due to various stresses is the reduction in seed vigour, and the eventual decrease in productivity (López-Fernández et al., 2018; Yari et al., 2011).

The old consensus of seed deterioration was that DNA degradation in deteriorated seeds leads to weakened transcription leading to faulty translation of enzymes and possible degradation of “long-lived” mRNAs scheduled for enzymes liable for the first stages of germination (Tarquis and Bradford, 1992). However, it has also been reported that many other systems within seed tissues become deteriorated during the process of ageing (Clerkx et al., 2003; Khanum et al., 2019; Tarquis and Bradford, 1992). Deterioration of seeds during storage has been linked to the production of reactive oxygen species (ROS) (Kranner et al., 2010). Examples of major ROS implicated in damage to seeds are the hydroxyl radical (•OH), hydrogen peroxide (H_2O_2) and superoxide ($•O_2^-$). Reactive oxygen species cause cell death, which can be necrotic or programmed cell death (Kranner et al., 2010). McDonald (2004) attributed changes in protein structure and nucleic acid damage to ROS production in stored seeds. These ultimately lead to mutation in chromosomes and delay in cell division and germination (Berjak, 2006; McDonald, 2004). Reactive oxygen species-mediated lipid peroxidation has now been accepted as a central theme responsible for cellular degradation of important cellular molecules and structures of seeds (Galleschi et al., 2002; Walters et al., 2005). Other factors linked with ageing/deterioration in seeds are degeneration and inactivation of enzymes on account of alterations in their macromolecular structures (Lehner et al., 2008; Mc-Donald, 2004). Several reports have associated seed deterioration with the reduction in enzyme activities. Such enzymes include peroxidases, catalase, glutathione reductase and superoxide dismutase (Mc-Donald, 2004). The reduction in enzymatic activities has been reported to diminish respiratory activities, consequently reducing the energy [(Adenosine triphosphate (ATP)] and assimilates supply of the germinating seeds (Lehner et al., 2008; Mc-Donald, 2004). Hence, negative changes in molecular structures of enzymes can decrease the germination efficiency of seeds (Lehner et al., 2008).

As stated earlier, a major cause of damage to stressed seeds is the generation of reactive oxygen species, which leads to severe oxidative damage and tissue necrosis (Berjak et al., 2011). Plants are imbued with internal processes to counteract the damaging consequences of ROS generated as a result of various abiotic stresses which include desiccation. Such systems include antioxidative systems, which are composed of metabolites, such as glutathione, tocopherol, ascorbate, and enzymatic scavengers, such as superoxide dismutase, peroxidases, catalases and enzymes in the ascorbate-glutathione pathways

(Berjak et al., 2011). Normally, ROS would be quenched by the endogenous antioxidant system in seeds by converting toxic free radicals and molecules to oxygen and water. At high level of stress, the strength of the plant's internal protective mechanisms is not sufficient to neutralize the damage caused by ROS bursts within the plant/seed. An approach to solving this problem is to supply exogenous antioxidants such as ascorbic acid; however, success is variable, and some anti-oxidants are cytotoxic at high concentrations (Berjak et al., 2011; Lehner et al., 2008). This study suggests that cathodic water (the cathodic fraction of an electrolysed, dilute ionic solution of calcium and magnesium), may be used to treat control deteriorated (stressed) seeds to counteract the potential damage caused by ROS. While the ameliorative effects of cathodic water have been reported on the germination of zygotic embryos (Berjak et al., 2011), embryonic axes (Naidoo et al., 2010), shoot tips (Gebashe, 2015) and some orthodox seeds (Gondwe et al., 2016), its long-term effect on seedling establishment, plant growth and productivity are not well documented.

1.2 Desiccation Tolerance in Orthodox Seeds

Desiccation tolerance is the ability of seeds to withstand stress imposed by an almost complete loss of cellular water during a state of extreme dryness and to resume normal metabolic activities upon imbibition (Pammenter and Berjak, 2014). Recalcitrant seeds are desiccation sensitive due to non-existence or incomplete expression of a set of mechanisms and processes that together bestow desiccation tolerance on orthodox seeds, which is the outstanding feature of orthodox seeds and the characteristic that distinguishes orthodox seeds from recalcitrant seeds (Pammenter and Berjak, 2014). Because of this adaptive strategy, orthodox seeds can be stored for many years at low moisture contents and sub-zero temperatures without significant loss in viability. They can also tolerate harsher environmental conditions that may not be ideal for germination and seedling establishment (Engelmann, 2011). However, from a state of extreme dryness, these seeds can resume normal metabolic activities upon imbibition (Engelmann, 2011; Pammenter and Berjak, 2014). Recalcitrant seeds undergo neither intracellular differentiation nor any significant metabolic shutdown. Embryos of recalcitrant seeds remain metabolically active, with little or no reduction in the extent of the extensive intracellular membranes (Farrant and Walters, 1998; Naidoo et al., 2010; Pammenter and Berjak, 2014). Most angiosperms (75-80%) such as those used in the present study - *Pisum sativum, Cucurbita pepo, Erythrina caffra, Bolusanthus speciosus, Combretum erythrophyllum* and *Oryza sativa* produce orthodox seeds (Tweddle et al., 2003).

Orthodox seeds acquire desiccation tolerance during the early maturation phase. This phase is prior to the onset of desiccation; it is as the embryo matures that desiccation tolerance

increases (Farrant and Walters, 1998). The changes in levels of desiccation tolerance during seed development are related to changes in ultra-structural, biochemical and biophysical characteristics of embryonic axes of the seed (Farrant and Walters, 1998). During the period of acquisition of desiccation tolerance, accumulation of storage compounds, such as proteins, oils, and carbohydrates occurs (Farrant and Walters, 1998). Proteins are stored in protein storage vacuoles, while lipids are stored in oil bodies. These offer physical resistance during drying, which occurs at late maturation phase. The rate and degree of drying vary among species, but over 90% of the original water in the seed/embryo may be lost during post maturation drying (Rodrigues-Junior et al., 2015). For example, Farrant and Walters (1998) reported that the dry mass of embryonic axes of *Aesculus hippocastanum* increased from about 0.5 to 4 mg, and the water content decreased from 10.2 to 2.0 g H_2O g^{-1} dry mass (g g^{-1}). Accumulation of dry matter reserves, reduced levels of monosaccharides, the presence of dehydrin-like proteins and an ability to form glasses appear to be associated with an increase in desiccation tolerance. These changes protect cellular structures, membranes and other proteins by acting as a hydration buffer, sequestering ions and renaturing unfolded proteins (Farrant and Walters, 1998). Non-reducing sugars fill the free volume between large molecules, created during dehydration, allowing less molecular mobility in the matrix (Rodrigues-Junior et al., 2015). The role of late embryogenesis abundant (LEA) proteins in the reduction of desiccation-induced cellular damage has been proven by the consistent correlation between desiccation tolerance in orthodox seed tissue and accumulation of certain LEA proteins (Blackman et al., 1995). Other structural adaptations that occur during this stage are chromatin compaction and nuclear size reduction (Bailly et al., 2001; Wang et al., 2012). Adaptation at the late maturation phase leads to a gradual increase in seed longevity (Rodrigues-Junior et al., 2015). After the acquisition of desiccation tolerance, the seed embryo enters a state of quiescence when metabolism activities in the seed are almost non-existent (Pammenter and Berjak, 2014).

Desiccation tolerance in orthodox seeds is lost once germination commences though they remain desiccation-tolerant before the third phase of germination just before the emergence of the radicle. As germination advances, orthodox seeds lose desiccation tolerance progressively. However, desiccation tolerance is lost in different embryonic tissues at different times (Koster et al., 2003; Reisdorph and Koster., 1999). Desiccation tolerance is lost when the radicle emerges and at this state, desiccation becomes damaging and ultimately can lead to the death of the seed (Blackman et al., 1995; Castro et al., 2017; Maia et al., 2014). As germination of orthodox seeds advances, desiccation tolerance is lost completely, which coincides with the loss of heat-stable proteins (Castro et al., 2017).

However, the timing of desiccation loss varies among different species of orthodox seeds (Rodrigues-Junior et al., 2015). The hydration methods of the seeds have also been reported as influencing desiccation tolerance.

1.3 Orthodox Seed Storage

In seeds that mature on the mother plant, the slow process of seed deterioration commences following seed maturity on the mother plant. When such seeds are harvested and kept at ambient temperatures and relative humidity, deterioration of the seeds continues and unless actions are taken to slow down the rate of deterioration the seeds lose viability quite fast (Andreev et al., 2004; Kranner et al., 2007; López-Fernández et al., 2018; McDonald and Wilson, 1980). Hence, seeds need to be appropriately stored to prolong the life span or “viability” of the seed. The act of preserving the seeds under controlled environment with the aim of prolonging the viability of the seeds is known as seed storage (Ghahfarokhi et al., 2014).

It is important that harvested seeds are cleaned, dried, packaged shortly after harvesting and processed for storage (IBPGR, 1985). Seeds for short term storage are mainly used for propagation while long term seed storage is mainly for conservation of genetic resources. Long term storage is normally done in gene banks. Accessions held in a gene bank are valuable and represent plants which may no longer be available or which are endangered in their natural environment. Seeds held in gene banks are used for future plant breeding, research, characterization, and evaluation or the regeneration of fresh seeds of each accession. The optimum storage conditions such as seed moisture content, temperatures and relative humidity vary depending on the species (Khanum et al., 2019; Kranner et al., 2007). The conditions that prolong viability during storage have been well defined for orthodox seeds. The appropriate storage conditions as recommended by the International Board for Plant Genetic Resources (IBPGR) advisory committee on seed storage are as follows. For base collections, seeds must be stored in sealed containers, the seeds moisture content must be 3-7%, and preferably the temperature must be -18°C or less (IBPGR, 1985). It is recommended that seeds for active collections be stored in sealed containers, and the seed moisture content must be 7% or less, and stored at temperatures of less than 15°C (IBPGR, 1985). A base collection is defined as, ‘’a set of accessions, each of which should be distinct and in terms of genetic purity as close as possible to the sample provided originally, which is preserved for the long term future, while active collections are the accessions which are immediately available for use’’ (Sackville and Chorlton, 1997). Accessions in the active collections play an important role in breeding either by gene bank staff or other researchers.

Either seeds are being stored as base or active collections, the viability of stored seeds must be determined before the seeds are stored. For seeds meant for commercial purposes, the initial viability must be higher than 94% (Hay and Whitehouse, 2017; Marcondes et al, 2011). It has been recommended that seeds' viability be monitored at least once in every ten years (for base collection seeds that are stored under the preferred standard storage conditions) or five years (for active collection seed and base collection with poor initial viability (IBGR, 1985). The essence of monitoring the viability of stored seeds is to predict the right time for regeneration of the accession. It is crucial that the viability of stored seeds is monitored at regular intervals. The intervals for monitoring seeds viability are determined by the curators subject to the initial germination of the species at the start of storage, type of species, initial moisture content of the seed and other seed storage conditions such as temperature, relative humidity (Hay and Whitehouse 2017; Marcondes et al., 2011). The longevity of seeds in storage is prolonged by reduced seed moisture content and reduced storage temperature (Kranner et al., 2007; Marcondes et al., 2011). Seed germination is the most accurate test used to determine or monitor seed viability, and it has been used to determine the viability of *Abelmoschus esculentus* (Daniel et al., 2013), *Lactuca sativa* (Fatokun et al., 2015), *Pisum sativum* (Gondwe et al., 2016).

1.4 Seed Germination and Seedling Vigour

Germination is one of the most vulnerable stages in the life cycle of plants (Liu et al., 2016; Vange et al., 2004). It is the process of reactivation of metabolic activities of the seed leading to the emergence of a radicle and plumule. Under suitable conditions, it is the resumption in the growth of a previously inactive embryonic plant contained in a seed. The suitable conditions which may be internal or external to the seed include water, suitable temperature, oxygen, the absence of inhibitory substances and sometimes light or darkness (McDonald, 1999**;** McDonald, 2004). The conditions for successful seed germination vary between species, and it is sometimes closely associated with the ecological conditions of a plant's natural habitat. In plants propagated vegetatively, germination could be conceived of including the re-growth of the vegetative part of a plant into a new plant (Fatokun et al., 2015). Germination eventually leads to the formation of a seedling.

Generally, the starting process of germination is considered to be imbibition. Under optimal conditions, water uptake by a dry seed is divided into three phases (Phases I, II and III). Phase I is characterised by rapid water intake due largely to the matric forces exerted by the seed. During phase II, repairs of DNA and mitochondria take place; using existing

messenger ribonucleic acid (mRNA) proteins are synthesized (Balestrazzi et al., 2011). Immediately after phase I, the seeds move to phase II, where there is very little net gain of water. This phase is called the lag phase or the activation phase. In the lag phase, considerable metabolic activity that prepares viable non-dormant seeds for radicle emergence occurs. The synthesis of mitochondria and proteins by new mRNA occurs in this phase. In the final phase, otherwise called phase III, water uptake increases, coupled with radicle elongation (Balestrazzi et al., 2011; McDonald, 1999). According to ISTA (1985), a seed is considered germinated when there is 1 mm radicle protrusion. In some seeds, future germination response is affected by environmental conditions the mother plant is exposed to during seed formation; most often, these responses are types of seed dormancy.

1.4.1 Germination rate, germination capacity, and germination index

The level and rapidity of germination are of utmost concern to seed companies, farmers, and seed scientists (Das et al., 2020; Kader, 2005). Germination rate (GR) is the number of seeds of a particular plant species, variety or seed lot that is likely to germinate over a given period. It is a measure of germination time course; GR is usually expressed as a percentage. For example, a 76% germination rate indicates that in the presence of optimum germination conditions, about 76 out of 100 seeds will germinate over a period. Germination rate can also be defined as the reciprocal of time taken for the process of germination to be completed in a given seed lot (Roberts, 1988). The germination period given is usually 14 days for most plants (ISTA, 1995). However, this period of 14 days for the completion of germination may not be true for many wild species, including *Combretum erythrophyllum*. This may be due to many factors including physical and chemical characteristics of seeds and availability of conditions necessary for successful and uniform germination. The seed requirements and desired number of plant stand in a given area are calculated using the germination rate. In other words, it is the time taken from the actual commencement of seed germination to the actual completion of germination.

Other parameters used to monitor seed germination include Germination Capacity (GC), Mean Germination Time (MGT), Germination Rate Index (GRI) and Germination Index (GI) (Kader, 2005). Germination capacity is the number of seeds able to complete germination in a population (i.e., seed lot). The MGT is an accurate measure of the time taken for a seed lot to germinate. The focus of MGT is on the day that most germination took place. Mean Germination Time is not a reflection of time spread or uniformity of germination, unlike GRI, which shows the percentage of germination per day. In other words, the higher the germination percentage and the shorter the duration for germination to be completed, the higher the GRI. Germination Rate Index lacks any correlation with the ‘high’ and ‘low’

germination days because it spreads the percentage germination evenly across the time spread. Finally, Germination Index focuses on the germination percentage/speed relationship (Kader, 2005). Germination Index is the most comprehensive measurement parameter because it combines both speed of germination (spread, duration and 'high/low' germination events) and germination percentage (Kader, 2005). With GI, variation among seed lots is magnified. Hence, in this regard, an easily compared numerical measurement is obtained (Kader, 2005). Germination events and parameters are important indicators of seed vigour (Fatokun et al., 2020).

1.4.2 Seed vigour

The field emergence (the visible penetration of the shoot above the soil surface) of high germinating seed lots of the same species with similar germination values may differ significantly even when exposed to similar conditions such as sowing date and field characteristics. The seeds may also differ in performance after storage when planted in the same environment or when transported and seeded in the same destination. Hence, it is clear that the germination test is not reliable enough to indicate all significant quality differences among high germinating seed lots. These significant differences are attributable to a component of seed quality called seed vigour (Farooq et al., 2006; Patel et al., 2017). Seed vigour has therefore been defined by two scientific bodies as: "the sum total of those properties of the seed which determine the level of activity and performance of the seed or seed lot during germination and seedling emergence" (ISTA, 1995). Seed vigour encompasses all seed properties which determine the potential for rapid, uniform emergence and development of normal seedlings under a wide range of prevailing field conditions at the time of sowing (ISTA, 1995).

As seeds deteriorate, the ability of the seeds or seed lot to carry out all the physiological functions that allow them to bring about good performance on the field is reduced; seed vigour is lost. This loss in performance is called physiological ageing. Seed vigour differs in seed lots of agricultural, horticultural and wild/ silvicultural species. Physiological ageing of seeds begins before seeds are harvested (immediately after seed physiological maturity), and it progresses as seeds are harvested, processed, and stored (Rodrigues-Junior et al., 2015). Biochemical changes such as enzymatic activities, cell membrane integrity, and accumulation of toxic metabolites**,** impaired protein synthesis and RNA synthesis during periods of low-temperature stress have been implicated in seed physiological deterioration (Amanpour-Balaneji and Sedghi, 2012; López-Fernández et al., 2018). Several authors have reported that the rate of seed deterioration is dependent on several factors, among which are genetic, seed production conditions, and environmental factors that are not yet fully

understood (Daniel et al., 2013). In recalcitrant seeds, deterioration occurs very quickly, and seed death (end point of deterioration) may be reached in a few days. However, in orthodox seeds deterioration occurs very slowly and may take some years before complete seed death.

Germination test results, in combination with seed vigour tests, provide a complete performance profile for a wide range of field conditions and can help guide important seeding decisions (Rodrigues-Junior et al., 2015). Such decisions include the appropriate time of the year to sow seeds, soil conditions, the temperature of the seed-bed, and soil moisture. The value of seed germination is usually higher than seed vigour, but in optimal field conditions, the two values may be very close. The seed vigour value represents the lowest performance level that is expected from the seed lot under stressful field conditions. The results of seed vigour tests factor in field mortality; hence, it helps the farmer in seed drill calculations. It must be noted that using the appropriate vigour test method for a particular species of the crop is very important in getting the actual vigour of the seed/seed lot under consideration.

1.4.3 Seed dormancy

Seeds sometimes fail to germinate over an expected period even though the prevailing conditions are suitable for germination; such seeds are considered to be dormant (Bernareggi et al., 2016; Koutouan-Kontchoi et al., 2020). In other words, the embryos of dormant seeds are viable but inactive. While dormancy may be a problem in an agricultural context, it plays a significant role in ecological context in that it helps in optimizing the distribution of germination in time or space. For example, germination is prevented during an unsuitable ecological condition, especially the period when the probability of seed survival is low (Bernareggi et al., 2016). The staggering of germination is advantageous as it protects some seeds and seedlings from being exposed to damage or death during short periods of unfavourable environmental conditions. Such conditions include weather, transient herbivores and undesirable competition from other plants for light and water. That occurs when a plant disperses seeds with different levels of dormancy, and the plant can spread the germination of its offspring in time; this reduces the exposure and risk of losing a whole generation due to transient unsuitable condition or an environmental disaster (Patykowski et al., 2016; Ungar, 2017). Hence, dormancy is especially important in wild plants.

Based on the part of the seed that is responsible for the expressed dormancy, seed dormancy has been classified into (i) endogenous dormancy, which can further be classified into mechanical dormancy, physical dormancy, and chemical dormancy, and (ii) exogenous dormancy (Baskin and Baskin, 2014; Fenner and Thompson, 2005; Koutouan-Kontchoi et

al., 2020). Endogenous dormancy is classified into morphological dormancy, physiological dormancy and combined dormancy. While endogenous dormancy is caused by conditions inherent in the embryo, exogenous dormancy is caused by conditions external to the embryo. Seed dormancy can also be classified into physiological, morphological, and physical dormancy based on their mode of actions (Fenner and Thompson, 2005; Ibarra et al., 2016). For this review, only physical dormancy, physiological and secondary dormancy are considered here.

Physical dormancy in seeds is caused by a seed coat or the presence of some structures such as samara in *Combretum erythrophyllum* that act as a barrier to water intake by seeds or prevent the entrance of gases into the seeds. Generally, such seed coats contain one or more palisade layers that is/are lignified with malphigian cells, which are tightly packed together. Often the malphigian layers contain phenolics which are water-repellent (Baskin, 2003; Yan et al., 2014). The impermeable layer(s) or seed coats evolve during maturation and drying phase of the seed or fruit on the mother plant (Bernareggi et al., 2016). Hence, the seed is prevented from germinating (even though other conditions that favour seed germination are present) until dormancy is broken. Physical dormancy has been reported in angiosperms such as cucurbita and legumes. In the wild, factors such as high and or fluctuating temperatures, freezing and drying are responsible for breaking physical dormancy. Repeated cycles of hot and cold temperatures over time, which may be months or years result in the weakening and eventual break the seed coats of seeds present in soil seed banks (Baskin, 2003; Baskin and Baskin, 2014; Silveira et al., 2013). Other factors include passage through the digestive tracts of animals and fire (Baskin, 2003; Rhie et al., 2015). In the laboratory, physical dormancy is broken with several methods which include nicking, scrubbing with sandpaper and acid treatments. The most common type of seed dormancy is physiological, and it is endogenous to the seed. It occurs due to the presence of a physiological inhibiting mechanism. Physiological dormancy is overcome by periods of moist chilling stratification. In the winter, seeds get their necessary moist chilling in the ground, which synchronizes germination in the spring (Baskin, 2003; Baskin and Baskin, 2014). Secondary dormancy occurs when otherwise non-dormant (at the time of seed harvesting or dispersal in the wild) and post dormant seeds (after dormancy of the seeds have been broken) are exposed to unfavourable conditions such as high temperatures (Soltani et al., 2017), water stress, and light/darkness (Ibarra et al., 2016). Genetic predisposition is sometimes necessary for the induction of secondary dormancy in seeds (Soltani et al, 2017). Although the mechanisms of secondary seed dormancy remain complex and are not yet fully understood, some researchers have suggested that it might be linked to changes in hormones and seed metabolism and loss of sensitivity in receptors

present in the seeds' plasma membrane (Bewley and Black, 1994; Houman et al, 2009; Kildisheva et al., 2020).

1.5 Seedling Emergence and Plant Growth

Seedling emergence and its establishment are of primary importance for optimising field productivity of any seed producing plant. The consequences of unfavourable or sub optimal environmental conditions include poor seed germination, insufficient seedling emergence, and subsequently poor field establishment and productivity (Ashraf and Foolad, 2005; Finch-Savage and Bassel, 2016), which is capable of causing a significant reduction in productivity (Vange et al., 2004). It has been reported that one of the major stumbling blocks to high yield in crop plants is the absence of synchronised crop establishment due to unfavourable conditions such as poor weather and poor soil conditions (Mwale et al., 2003). On the other hand, good establishment increases productivity, improves drought tolerance, and enhances competitiveness against weeds, thereby reducing or eliminating the time-consuming need for re-sowing, an additional strain on the cost of farming (Bhattacharya et al., 2015). It is a well-accepted fact that seed priming improves on the speed and uniformity of germination, reduces seedling emergence time, and improves on total stand establishment and its uniformity (Bhattacharya et al., 2015; Guha et al., 2012).

The appearance of the radicle marks the end of seed germination and the beginning of stand "establishment" (Ortega-Baes and Rojas-Aréchiga, 2007). At this stage of plant growth, food reserves stored in the seed are utilized (Ortega-Baes and Rojas-Aréchiga, 2007). It is one of the critical phases in plant growth, as plants are very vulnerable to injury, disease, and water stress (Vange et al., 2004). Even mortality is sometimes high at this stage. Hence, to counteract this loss, some species have adapted by producing many seeds (Liu et al., 2016).

In plants propagated by seeds, the formation of the embryo, otherwise called embryogenesis occurs before the maturation of the seeds. The embryo contains one (monocotyledon) or two (dicotyledon) seed leaves. The seed embryo contains all the parts necessary to begin its life by the end of embryogenesis (Liu et al., 2016). After seed germination, cells in the embryo multiply and grow. This leads to a process called organogenesis, which is the production of plant organs such as leaves, stem and roots. While the roots of the plant grow from root meristems, the new shoot (stem and leaves) grows from shoot meristems (Mohammadi et al., 2011). Branches of plants are formed when small clumps of meristematic cells grow to form new root or shoot. The cells involved are those that are yet to differentiate to form a specialized tissue (Mohammadi et al., 2011). This is called primary growth and it gives rise to an increase in length of that particular root or shoots. Secondary growth occurs when cells

in the cambium of a plant divide, leading to the widening of the root or shoot (Barlow, 2005). A cell may also grow longer, otherwise known as cell elongation (Ross et al., 1983).

1.6 Seed Deterioration/Controlled Deterioration of Orthodox Seeds

Seed deterioration can be defined as the deteriorative changes in seeds occurring with time that increase the seeds' vulnerability to external challenges such as high temperature and decrease the capacity of the seed to survive (Andreev et al., 2004; Felix et al., 2020; Kranner et al., 2007). Seed deterioration can be controlled or uncontrolled. While in uncontrolled seed deterioration, seeds are left at the mercy of the prevailing environmental conditions, in controlled deterioration, seeds are subjected to predetermined storage conditions such as temperature and humidity (Ghahfarokhi et al., 2014). Seed deterioration may be slow under natural conditions. Hence, for experimental purposes, there is the need to shorten the time required for seed to deteriorate. Thus, seeds are subjected to predetermined and aggravated conditions of heat and humidity to accelerate the rate of deterioration (Ghahfarokhi et al., 2014). Controlled deterioration of seeds have been used in the study of *Triticum durum*, (Galleschi et al., 2002), *Capsicum annumm* Linn (Kaewnaree et al., 2011), *Phaseolus vulgaris* (Amanpour-Balaneji and Sedghi, 2012) and *Pisum sativum* (Gondwe et al., 2016; Veselova et al., 2015).

Seed deterioration is cumulative; as seed ageing increases, seed vigour and germination progressively decrease. Seed deterioration is inevitable; the best that can be done is to reduce its rate (Berjak and Villiers, 1972; Felix et al., 2020; Garza-Caligaris et al., 2012). Factors that predispose seeds to deterioration include genetics, seed structures which include seed coat permeability, seed size and surface area and seed chemical composition (lipid, protein, starch and mucilage). Other factors making seed to be vulnerable to deterioration are physical/ physiological seed quality (maturity, physical damage and seed dormancy), temperature and relative humidity of the storage environment (Andreev et al., 2004). The temperature of the storage environment affects the ability of air to support water vapour, and it enhances the physiological speed of deteriorative reactions in seeds. The other important environmental and biological factors that contribute to seed deterioration include fungal infections which aggravate as seeds deteriorate and have other separate effects on deterioration (Andreev et al., 2004). The moisture content for most orthodox seeds at maturity is 5-14%. The rule of thumb of the relationship between seed moisture content/seed storage temperature and seed storage life is that every 1% decrease in seed moisture content and every 5 ˚C decrease in storage temperature doubles seed storage life (Kim, 2018; Walters et al., 2005). As a rule of thumb, to best store seeds % RH + ˚C should be < 45.5.

Seed deterioration does not occur uniformly in seeds (Robin et al., 2009). In monocotyledonous plants, seed deterioration starts in the root tip, causing radicle extension to be decreased more than the coleoptile extension (Jeng and Sung, 1994). The deterioration of seeds starts in the growing points (shoot and root) of the embryonic axis in dicotyledonous plants (Jeng and Sung, 1994). The mechanism of deterioration in the field, which is considered short term, differs from long-term deterioration, which occurs during storage. Mechanisms of seed deterioration can be explained from a decline in free radical scavenging enzymes such as SOD and catalase (Jeng and Sung, 1994), a decline in protein or increased amino acid content (Kalpana and Madhava-Rao, 1997), to decreased DNA synthesis. It is reported that increased degradation of nucleic acids leads to faulty transcription and translation of enzymes essential for germination and increased membrane permeability (Perez and Arguello, 1995).

Earlier reports of seed deterioration were that DNA degradation results in weakened transcription leading to an incorrect translation of enzymes and possible degradation of “long-lived” mRNA scheduled for enzymes that are responsible for the first stages of germination (Lagouge and Larsson, 2013). However, the old consensus has given way to the new consensus, which is that free radicals cause profound cellular damage. Free radical production, primarily initiated by oxygen, has been related to the peroxidation of lipids and other essential compounds found in cells causing a host of negative events which include decreased lipid content, reduced respiratory competence, and increased evolution of volatiles compounds ranging from hexanal to aldehydes (Esashi et al., 1997; Smith and Berjak, 2017). The greatest free radical “sink” is the mitochondrion, most of which are found in meristematic cells. As a consequence of seed deterioration, mtDNA replication is reduced. Consequently, fewer mitochondria lead to reduced energy [adenosine triphosphate (ATP)] and slower seedling growth (Lagouge and Larsson, 2013). Also, free radicals initiate a chain of reactions which results in considerable rearrangement of molecules altering their structures and functions (McDonald, 2004). If these are proteins (enzymes), lipids (membranes), or nucleic acids (DNA), normal biological function is compromised, and deterioration is increased (Kalpana and Madhava-Rao, 1997; Smith and Berjak, 2017).

The harmful effects of free radicals in seeds tissues are influenced by seed moisture content. When seed moisture is below 6% fresh weight, seed deterioration is non-enzymatic. However, at moisture content greater than 14% deterioration of seeds is caused by enzymes such as lipoxygenase (Kranner et al., 2010). Free radicals cause changes in protein structure, altering their functions, for example, in enzymes cleavage of protein produces

lower-molecular-weight secondary products, whereas cross-linkage of protein yields higher-molecular-weight product. Also, catalytic and structural functions are changed due to distortion in their secondary and tertiary structures (Kranner et al., 2010). It has also been reported that free radicals attack DNA resulting in the breakage of its strands and that of deoxyribose sugar. Damage caused by free radicals to DNA increases genetic mutations as seeds become aged. Such mutation may lead to delay in mitosis necessary for cell division and germination. Free radicals have also been reported to damage mitochondria resulting in leakages from its membranes during respiration. Mitochondria are essential for normal cell function. Usually reduced seedling growth from plants generated from poor quality seed may be a consequence of reduced mitochondrial function (Dao-Fu et al., 2014; Zorov et al., 2014). Some of the effects of free radicals on mtDNA include active cell division, production of new mitochondria and enzymes. The enzymes are absolutely essential for oxidative phosphorylation. Mitochondrial DNA is more susceptible to free radical attack than nuclear DNA because it has no protective histones; hence, it is considered naked. More free radicals are produced in the mitochondria making it more difficult for repairs to take place, unlike nuclear DNA (McDonald, 2004).

An array of protective enzymes such as superoxide dismutase, catalase, and various enzymes of the ascorbate glutathione pathway including ascorbate peroxidase and glutathione reductase protect seeds from free radicals (Ardebili et al., 2019; Chin et al., 1995; McDonald and Wilson, 1980). Besides, non-enzymatic compounds that react with free radicals also protect seeds from free radicals (McDonald, 2004). Examples of such compounds include glutathione, vitamin C (ascorbic acid) and Vitamin E (tocopherol). Some enzymes protect seeds from free radicals; such enzymes include the ones that are involved in base and nucleotide excisions and DNA mismatch (McDonald, 1999). Figure 1.1 shows a model of seed deterioration, according to McDonald (1999).

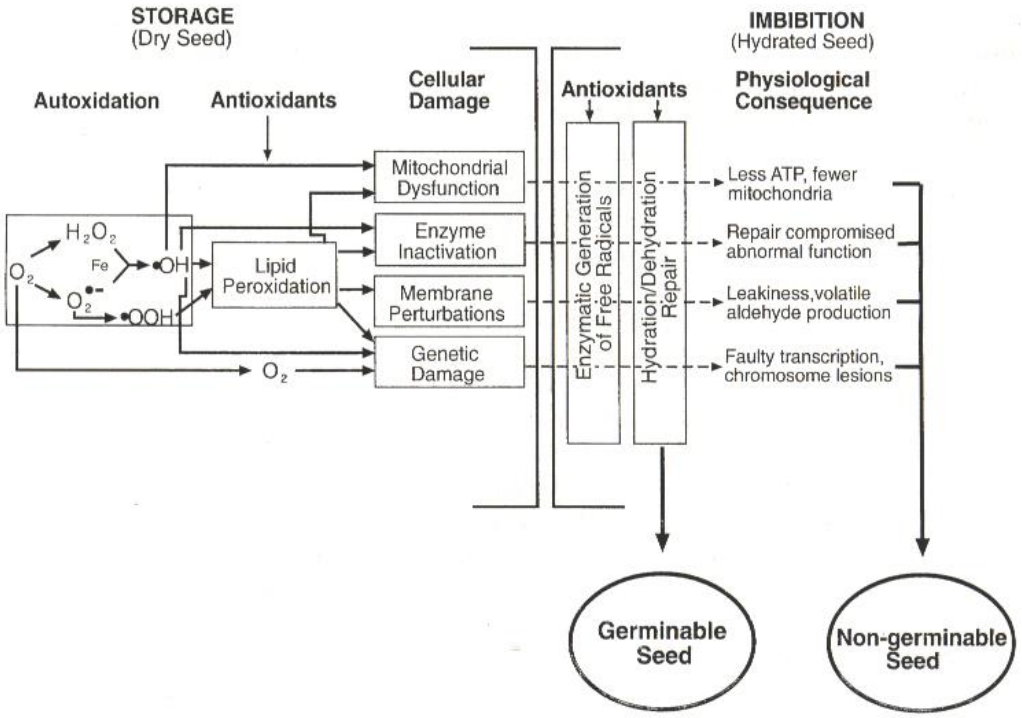

Figure 1.1 A model of seed germination and its physiological consequences during seed storage and imbibition (McDonald, 1999). Lipid peroxidation is the major cause of deterioration in orthodox seeds. In the course of seed storage, seed deterioration occurs majorly due to activities of free radicals. The activities of free radicals can be counteracted by the presence of antioxidants, especially during storage, and hydration, either during water imbibition by seeds or seed priming.

As stated earlier, in gene banks, seeds are stored under optimal storage conditions (low seed moisture content, low relative humidity, and low temperature). Seeds deteriorate even under these optimal conditions leading to a loss in viability and germination vigour. As discussed above, the rate of seed deterioration varies significantly between species, among varieties of same species and seed lots of same varieties (Jatoi et al., 2001; McDonald and Wilson, 1980). In a study of five varieties of *Pisum sativum*, storage temperature of 25°C, 35°C and 45°C (100% relative humidity) and storage durations of 48, 72 and 96 hours led to significant variety differences in growth rate (Khan et al., 2017). In the study, seed deterioration was assessed by the rate of germination, shoot and root length, shoot, and root dry weight. The result indicated that seed deterioration increased with an increase in storage temperature and storage period (Khan et al., 2017).

1.7 Effects of Controlled Deterioration of Seeds on Germination and Plant Growth

Seed deterioration, also known as natural ageing, is a very slow process especially under good storage conditions. It is therefore very important to speed up the rate of deterioration

for experimental purpose via controlled deterioration of seeds. According to Rajjou et al. (2008), molecular events accompanying both natural and controlled deterioration (CD) or artificial ageing are similar. Like natural seed ageing, CD of seeds has been reported to have deleterious effects on seed germination indices and subsequent plant growth (Amanpour-Balaneji and Sedghi, 2012; Mohammadi et al., 2011; Ouzouline et al., 2009; Rajjou et al., 2008). The authors of those and many other reports have advanced several reasons for the deleterious effects of seed ageing on plants. Some of those reports are discussed in the paragraphs. In a study of the effect of seed controlled deterioration on *Glycine max*, seed deterioration resulted in decreased percentage germination and rate of seed germination (Mohammadi et al., 2011). The decrease in germination is associated with decreased excision of chromatin loop domains (Mohammadi et al., 2011). In addition, production of abnormal seedlings increased in plants derived from deteriorated seed in the study (Mohammadi et al., 2011).

The more seeds are deteriorated, the more seedling growth declined (Mohammadi et al., 2011). In a study of *Secale cereale*, Andreev et al. (2004) reported that loss in germination is correlated with reductions in soluble nuclear protein and DNA topoisomerase II. DNA topoisomerase II increases or decreases the linking number of a DNA loop by 2 units, and it enhances chromosome separation. Andreev et al. (2004) concluded that the changes in DNA fragmentation patterns in ageing seeds were primarily caused by a decreased activity of the enzymes accounting for the excision of chromatin loop domains (Andreev et al., 2004). Similarly, Chin et al. (1995) reported significant differences in both the germination percentage and germination rate between aged and unaged seeds of watermelon. In the study, ageing increased lipid peroxidation and lowered the activities of peroxide-scavenging enzymes. Further reports in the study indicated that despite differences in the germination performance, seed leakage, and extent of lipid peroxidation and activities of peroxide-scavenging enzymes in the seeds with different ploidy, the changes in germination and related physiological responses in relation to ageing and hydration were similar (Chin et al., 1995).

Using *Arabidopsis thaliana* as a model, Rajjou et al. (2008) reported that subjecting seeds to CD lead to significant loss of germination vigour. This loss in vigour was directly related to the level and duration of deterioration of CD seeds. Proteomic analyses revealed that this loss in seed vigour could be accounted for by substantial protein changes in the aged seeds and by an inability of the low-vigour seeds to display a normal proteome during germination. The same study further revealed that CD sharply increased the extent of protein oxidation

(carbonylation), which induced a loss of functional properties of seed proteins and enzymes and/or enhance their susceptibility toward proteolysis (Rajjou et al., 2008).

The effects of accelerated ageing on the seeds lipid composition of two varieties of *Triticum aestivum* was studied by Ouzouline et al. (2009). The result of the study indicated that eight days of accelerated ageing resulted in total inhibition/complete loss of seed viability/germinability as well as a decrease in their total and especially unsaturated fatty acid contents. In the phosphatidylcholine of the seeds, oleic and linoleic acid contents decreased. Polar lipids declined more than the neutral lipids. After ageing, the composition of lipids classes as well as the compositions of total fatty acids present in the seeds. The conclusion drawn from the study was that loss of seed viability in the study is directly related to a decline in the lipid content of the deteriorated seeds (Ouzouline et al., 2009).

Smith (1982) studied the relationship between loss of seed germination, storage conditions, total lipid hydroperoxides and the fatty acid levels of seeds of *Lactuca sativa* L. The result indicated that the loss in germination of *Lactuca sativa* L. following seed deterioration was caused by the deterioration of membranes and damage to nucleic acids. The report linked increased leakage of solutes observed in the study during imbibition to peroxidative damage to membrane lipids. The fatty acid level did not change on imbibition, but lipid hydroperoxides underwent considerable change. Hence, although peroxidation of lipids may occur under certain conditions of storage, this may not lead to loss of saturation of fatty acids (Smith, 1982).

1.8 Seed Invigoration

Seed invigoration, although sometimes used interchangeably with seed priming, is an encompassing term of treatments used to enhance the field performance of a given seed lot. Basically, it is a pre-sowing technique or treatments used to improve germination, emergence and seedling growth of a seed lot or to facilitate the delivery of seeds and other materials needed at the time of sowing (Sharma et al., 2015; Taylor et al., 1998; Taylor et al., 2008). Seed invigoration includes pre-sowing hydration treatments (Basra et al., 2006; Farooq et al., 2007; Sharma et al., 2015), seed treatment with low molecular weight osmoprotectant (Taylor et al., 1998; Taylor et al., 2008), seed coating technologies/ techniques (Song et al., 2005) and more recently, a pre-sowing dry heat treatment (Farooq et al., 2006). The focus of seed invigoration is to enhance germination and emergence, reduce seedling emergence time, and increase the uniformity of emergence and protection of seeds from biotic and abiotic factors during early phases of seedling establishment, which is a very critical stage.

A detailed schematic flowchart on seed invigoration is given in Figure 1.2 (below). Seed hydration treatments will be discussed in detail below, in section 1.8. In seed coating, seeds are treated by dusting the seeds with chemicals such as fungicides. Earlier in seed coating techniques, the seeds are placed on a solid surface and dusted with fungicide (Gowda et al., 2020; Mancini and Romanazzi, 2014; Rocha et al., 2019; Taylor et al., 2008). The seeds are then hand-mixed using a spade/shovel until each seed is evenly coated with fungicide. Later advances in seed coating involved the use of a rotating drum to mix the seed with the desired fungicide/dust formulation such as copper carbonate. The barrels are mounted at angles, loaded with seeds, and desired formulation (powder) is then sprinkled on the seeds. The barrel is turned by a hand crank with the seeds tumbling back and forth, becoming coated with the fungicide (Gowda et al., 2020; Taylor et al., 2008).

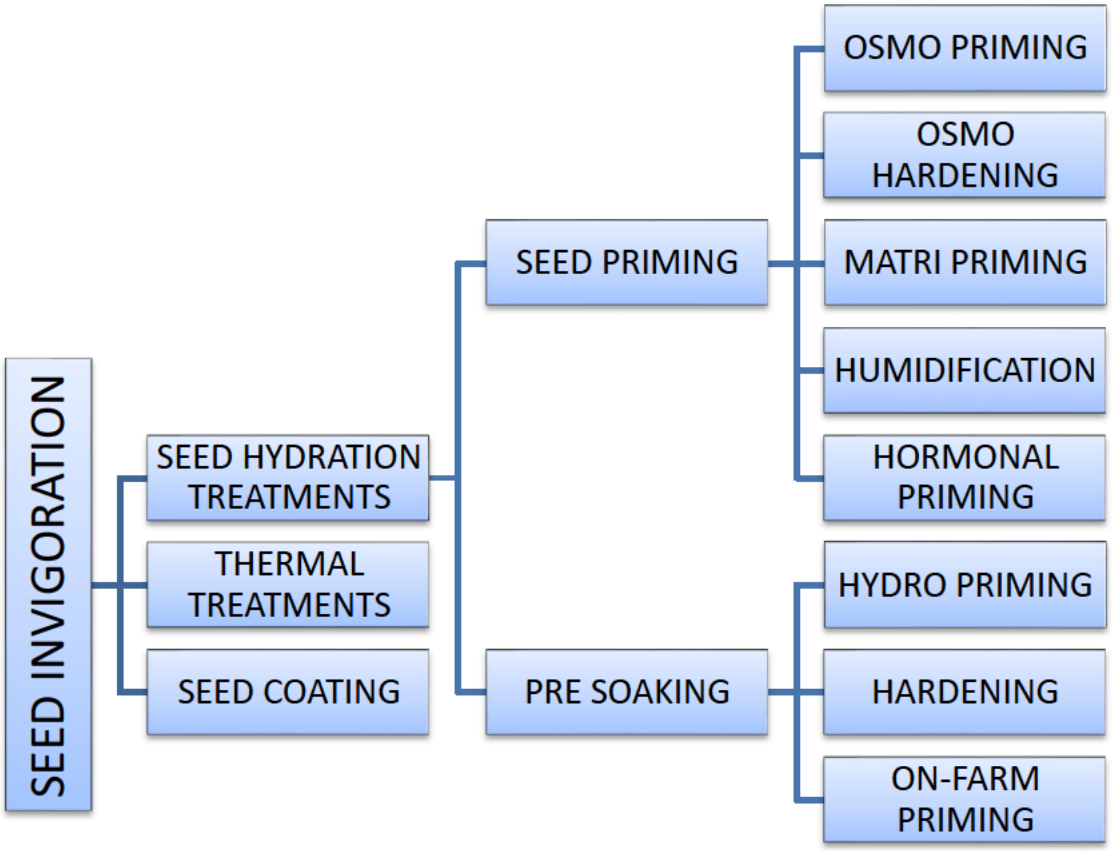

Figure 1.2 Classification of seed invigoration techniques (Farooq et al., 2008)

Seed invigoration technologies are broadly divided into seed hydration treatments, thermal treatments and seed coating. The seed hydration method may be uncontrolled, otherwise known as pre-soaking, or controlled. Controlled seed hydration treatments are called seed priming. Seed priming may be called hormonal priming (hormones), osmopriming, humidification, osmohardening, matripriming, nutrient seed priming, hydro priming (water) and antioxidants seed priming depending on the nature of the priming agent (osmoticum) used (Farooq et al., 2008).

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The most common method of seed treatment is seed dressing. In seed dressing, seeds are dressed with modern pesticides. The chemicals are applied as dry powder or in the form of a slurry (Gowda et al., 2020; Mancini and Romanazzi, 2014; Upadhyaya et al., 2013). Seed dressing is applied at both farm and industries. In industries such as seed companies, motor or hand-driven seed treatment drums are used, the right quantity of the required chemicals is calculated and sprinkled on the seed (Sharma et al., 2015). Mixing is subsequently done mechanically. Among the small scale farmers, generally, shovels are used for mixing the chemicals with the seeds. Low-cost earthen pots are also used. However, these local methods often lead to uneven mixing and are not considered as standard methods. Effective extension services are required to educate farmers on the need for seed treatments, especially among low-income small scale farmers. For the purpose of this study, I shall focus more on seed priming in the next subsection (subsection 1.9).

1.9 Seed Priming

In order to address the problem associated with poor germination rate/ seed vigour, rapid and uniform seedling emergence, poor seedling vigour, poor seedling establishment and ultimately better crop yields, seed priming has become popular among scientists and farmers (Anwar et al., 2020; Ardebili et al., 2019; Das et al., 2020; Farooq et al., 2008; Yari et al., 2011). Seed priming, also called pre-sowing physiological treatment for seed enhancement, plays a pivotal role in seed treatment technology. First proposed by Heydecker (1973), seed priming is a controlled hydration technique in which seeds are partially hydrated to a point that allows pre-germinative metabolism to commence, but prevents actual germination/ radicle emergence (to avoid seed embryos becoming desiccation-sensitive) and then re-dried until close to the original mass (Ashraf and Foolad, 2005; Bradford, 1986; Das et al., 2020; Heydecker and Coolbear, 1977; Singh et al., 2015).

In some cases of priming, the hydrated seeds are not re-dried but sown immediately after hydration. However, for orthodox seeds that are not re-dried after priming treatment, reduction in the lag time of imbibition occurs (Heydecker and Coolbear, 1977; McDonald, 2000). Usually, for seeds that are not re-dried, the seeds are imbibed in solutions with high osmotic potential to prevent the seeds from taking in enough water and move to Phase III of seed hydration. The notion is to hold the seeds in phase II and therefore essentially restricting the seed within the lag phase (Singh et al., 2015; Taylor et al., 1998). Seeds are metabolically active during this period. The stored reserves are used during germination. After imbibition, the seeds are removed from the priming solution, rinsed in water, either dried and stored or not dried and sown in the field (Anwar et al., 2020; McDonald, 2004).

Seed hydration causes activation or repair of enzymes (McDonald, 2004). In the drying phase to original water content after priming, damaged seeds are repaired and stabilized, and this has been reported as being responsible for the improved performance of primed seeds (Anwar et al., 2020; McDonald, 2004). Repair of the seed occurs in the embryonic axes (containing the most mitochondria).

Many techniques have been employed to prime seeds by hydrating them in solutions to allow sufficient imbibition to enable the early events in the germination process to occur, but not enough to permit radicle emergence. These techniques include: (a) hydropriming - soaking seeds in water, (b) halopriming - hydration of seeds in inorganic salt solutions, (c) osmopriming - soaking seeds in osmotic solutions, for example, polyethylene glycol (PEG), (d) thermopriming - treatment of seeds with low or high temperatures (Ashraf and Foolad, 2005), (e) matri-priming or treatment of seeds with solid matrices or placing seeds between saturated jute mat layers (examples include hydrated sand) (Hu et al., 2005), and (f) bio-priming - coating seeds with bacteria, e.g., *Trichoderma* spp. and *Pseudomonas aureofaciens* (Prabha et al., 2019; Rocha et al., 2019). Another seed priming technique involves alternate soaking of seeds in tap water and drying before sowing (hardening) (Ghassemi-Golezani et al., 2008), or solutions of plant nutrients (nutrient seed priming) and plant growth regulators (Subedi and Ma, 2005).

Numerous advantages of priming orthodox seeds have been reported for seed species around the world (Eskandari and Kazemi, 2011; Farooq et al., 2008; Khan et al., 2016). Primed seeds germinate rapidly when re-imbibed under normal or stress conditions. Taken together, these studies confirm the importance of priming to achieve good germination, seedling establishment, and higher productivity in many crops of the tropical region such as *Oryza sativa, Zea mays, Sorghum bicolor* and *Cajanus cajan*. A more detailed discussion of the effects of seed priming is done in section 1.10

1.9.1 Seed priming with antioxidants

Antioxidant priming involves hydration of seeds in antioxidants such as ascorbic acid, tocopherol, and glutathione (Ardebili et al., 2019; Guha et al., 2012). During some germination processes such as imbibition and radicle elongation, ROS are produced. The production of ROS has been implicated in making germination processes vulnerable to environmental stress with the consequence of germination failure. Antioxidants are required to counteract the activities of ROS (Guha et al., 2012). However, the strength of the endogenous antioxidants in the seed may not be sufficient in this regard. Priming with antioxidants has been reported to augment the activities of the endogenous antioxidant

during seed germination and early seedling growth (Guha et al., 2012). In the study pre- and mid-storage seed priming treatments with ascorbic acid of high-medium vigour (2-month-old) and medium vigour (5-month-old) okra seeds at 500 mg kg^{-1} seed significantly reduced loss of vigour and viability of okra seeds during subsequent storage under various ageing conditions (Guha et al., 2012).

1.9.2 Hydro priming

Hydro priming is the hydration of seeds in water to a point where germination processes are activated before sowing. This may or may not be followed by air-drying of the seeds. Seeds that were hydro-primed have been reported to exhibit rapid germination and seedling emergence in saline (Pedrero-López et al., 2016) and non-saline conditions (Maiti et al., 2013). Bourioug et al. (2020) reported that hydro priming on sunflower seeds was used to break dormancy and improve crop performance under water stress.

Besides an increase in germination, emergence and plant growth, hydro-priming using hot water has also been used to control many seed-borne diseases. This is done by treating seeds with water that is hot enough to kill the pathogenic organisms but not hot enough to kill the seeds' embryo (Floyd and Melvin-Carter, 2005; Rahman et al., 2008). The process involves heating water to the desired temperature in a water bath. The seeds are subsequently placed in the water for the prescribed duration. The length of treatment and temperature of the water must be strictly adhered to avoid killing the seeds or inefficiency in the control of the disease/pathogen. The seeds are thereafter transferred to cold water at room temperature, which may be followed by drying back or not, before sowing. This method controls pathogen in addition to other benefits associated with hydro-priming as stated in the paragraph above. Hydro-priming at a temperature of 55-60°C for 15 min was used to control blight in *Pisum sativum* caused by *Pseudomonas syringae pv pisi* (Rahman et al., 2008).

1.9.3 Nutrient seed priming

Nutrient seed priming is the hydration of seeds in nutrient solution before sowing (Kundrát et al., 2017). It is an approach that combines the benefit of priming with the provision of nutrients to the germinating seeds leading to improved seed quality and enhanced germination, seedling emergence, and establishment. During extended periods of low temperature, nutrient acquisition by plant roots is confronted with a major problem due to limited solubility and availability of certain nutrients (Yan et al., 2012). The solubility and speed of diffusion of nutrients in the soil are affected negatively. The situation is usually worse for sparingly soluble nutrients such as phosphorus, calcium, iron, zinc, manganese, and copper, mainly delivered by diffusion (Jungk, 2002). A negative correlation exists

between the viscosity of water and soil temperature. As the viscosity of water increases under low temperature, it leads to a reduction in the speed of nutrients that are transported to plant roots via diffusion or mass flow (Jungk, 1991, 2002; Wan et al., 2001). In this condition of low soil temperature, priming with the required nutrients will assist the plant in overcoming or reducing the problem of nutrient deficiency as a result of poor transport of nutrients to plant roots.

The second problem associated with low root zone temperatures is limited root activity (Pregitzer and King, 2005; Sowinski and Maleszewski, 1989). Root growth for spatial nutrient acquisition is inhibited by low temperatures (Imram et al. 2013) and adaptive root exudation for nutrient mobilization in soils, for example, the release of phytosiderophores for iron and zinc acquisition is also inhibited (Marschner et al., 1987). Under low root zone temperature, nutrient uptake, and root to shoot translocation of nutrients are also inhibited. Reports have indicated adverse effects of low root zone temperature on the uptake and translocation of nutrients such as phosphorus, potassium, zinc, manganese, iron, copper, calcium and nitrogen (Ehdaie et al., 2010).

Nutrient seed priming is done to invigorate seeds because of problems associated with nutrients acquisition by plants, especially at the early stages of seedling growth. Although there is a limitation as to the concentration of fertilizer that can be used for seed priming to avoid killing the seeds, priming seeds with macronutrients has been reported to improve early plant growth (Farooq et al., 2012; Miraj et al., 2013). Coating seeds with peroxide compounds such as calcium dioxide (CaO_2) which supplies oxygen to seeds has been reported to be beneficial under anoxic or near anoxic soil conditions. For example, improvements have been observed in rice grown in flooded field conditions (Leaver and Roberts, 1984).

1.10 Effect of Priming on Germination and Seedling Growth

Priming is a simple and low-cost technique that is commercially used for seed enhancement. Primed seeds perform better in normal, saline or diseased soil, and other suboptimal seed growth conditions (Ghassemi-Golezani et al., 2011; Elouaer and Hannachi, 2012). Seed priming has been reported to improve on the germination, emergence and growth of some vegetables, floriculture and some field crops (Farooq et al., 2008; Yari et al., 2011). Priming increases seed vigour via the rate and homogeny of germination, improved uniformity of emergence and higher stand establishment and better crop yields (Ghassemi-Golezani et al., 2011; Yari et al., 2011). However, the improvement brought about by priming or success

of seed priming is influenced by many factors such as the plant species, type and concentration of priming media, water potential of the priming agent, duration of exposure to priming agent, seed size, seed vigour, temperature and storage conditions of the primed seeds (Elouaer and Hannachi, 2012; Saha and Mandal, 2016).

The effects of halopriming, hydropriming and osmopriming on the seedling growth and yield of *Solanum lycopersicum* and *Capsicum annuum L.* were compared; the reports indicated that halopriming performed better than hydropriming leading to significant increase in emergence rate, seedling vigour index in *Solanum lycopersicum* and *Capsicum annuum* (Maiti et al., 2013). This study also showed that under field conditions, halopriming showed better performance than the control and hydropriming in terms of plant height, root length, shoot length, flowering time and yield (Maiti et al., 2013). Toklu et al. (2015) reported that priming seeds with distilled water, 100 ppm indole-3-acetic acid (IAA) and 10% polyethylene glycol (PEG-6000) increased the germination, emergence percentage, and growth rate of seedlings of *Triticum aestivum* under field conditions (Toklu et al., 2015). Under post-rainy conditions, hydropriming, and osmopriming improved germination, seedling growth and yield of *Brassica oleracea, Capsicum annuum L., Solanum lycopersicum* and *Cucumis sativus* when compared with the control (Maiti et al., 2009).

Bhattacharya et al. (2015) examined the influence of some pre-storage seed priming treatments in fresh seeds of *Glycine max*. In the study, various priming agents such as powdered crude plant materials (*Capsicum annuum* powder at 1 g kg^{-1} of seed; *Azadirachta indica* (neem) leaf powder at 2 g kg^{-1} of seed); chemicals (common bleaching powder and iodinated calcium carbonate at 2 g kg^{-1} of seed; para-amino benzoic acid and ferulic acid at 500mg kg^{-1} of seed) and pharmaceuticals (aspirin at 50mg kg^{-1} of seed) were used to invigorate seeds. The result of the study indicated that all treatments significantly ($p<0.05$) increased germination, field establishment and yield over control. The improvement caused by ferulic acid, aspirin and para-amino benzoic acid treatments was greater than those caused by other treatments in terms of improving storability and field performance. With respect to the physiological and biochemical mode of actions of the treatments, pre-storage dry treated reduced electrolyte leakage, sugars, and amino acid; the formation of lipid peroxide was reduced more than control (Bhattacharya et al., 2015). Based on the results of the study, pre-storage dry treatments with ferulic acid, aspirin, and para-amino benzoic acid were suggested for improved storability and field performance of stored *Glycine max* seed. Saha and Mandal (2016) investigated the effect of treating seed with *Capsicum annuum* (red chilli) and bleaching powders invigoration in different seed sizes of *Helianthus annuus*. The invigoration was done pre-storage. The seed sizes were categorised into three (viz.

composite, large, medium and small). The seeds were invigorated with 1 g kg^{-1} of seeds of red chilli powder and 2 g kg^{-1} of seed of bleaching pre-storage. Both treatments significantly reduced the rate of natural seed ageing; field performance and yield were increased, electrolyte leakage and volatile aldehyde production were lowered with higher dehydrogenase enzyme activities in comparison with the controls. In comparison with both small and medium-sized seeds, post storage germination and yields were higher in large seeds. It was therefore concluded that pre-storage dry treatments of large seeds with red chilli powder and bleaching powder might be suggested for improved germination and yield of high-vigour *Helianthus annuus* seeds in storage (Saha and Mandal, 2016).

Gondwe et al. (2016) primed the seeds of *Pisum sativum*, *Cucurbita maxima* and *Lycopersicon esculentum* with a tap water solution containing 2 mM ascorbic acid, 1 µM calcium chloride, 1 mM magnesium chloride solution and cathodic water. Half of the seeds were dried under ambient condition while the other half was rapidly dried under laminar flow. The seeds were subsequently stored at 5°C for four months after which the effect of seed priming and subsequent storage on the germinability and seed vigour were examined. The result indicated that rapid drying significantly reduced germination in *Pisum sativum* and *Cucurbita maxima.* The result further showed that seed priming enhanced seed vigour in all the test species. Germination was enhanced in *Pisum sativum* and *Cucurbita maxima.* Priming with cathodic water showed more improvement both in germination and seed vigour in all the species (Gondwe et al., 2016). In another study Abdolahi et al. (2012) investigated the effects of seed priming with KH_2PO_4 (−0.625 MPa) and $CaCl_2$ (−1.25 MPa) solutions on the germination of three cultivars of *Brassica napus L*. In the study seeds of the three cultivars were controlled deteriorated at 40ºC and 100% relative humidity for 48 and 96 h. In comparison with control, both treatment solutions enhanced seed germination and seed vigour. However, KH_2PO_4 performed better than $CaCl_2$. The results further indicated that KH_2PO_4 was more effective than $CaCl_2$ in minimizing leakage of electrolytes. Mean germination time and germination percentage were enhanced as were shoot length, root length, shoot dry mass, root dry mass and seedling vigour and germination indices (Abdolahi et al., 2012).

Anwar et al. (2012) reported the beneficial effect of seed priming in integrated weed management of *Oryza sativa* under aerobic soil conditions (Anwar et al., 2012). In this study, four treatments (viz, hydro priming, hardening of seeds, priming with Zappa® and control) were investigated. Two weeding treatments (weed-free and weedy) served as weed control treatments. Seed priming significantly improved germination indices such as mean germination time, germination percentage, germination index and seedling vigour index; the

ability to suppress weeds and yield were also improved. The plants generated from the control exhibited inconsistent germination, poor seedling establishment and was less efficient in competing with weeds resulting in poor yield. Seed priming increased yields by an average of 0.4 t ha^{-1} over control. Weed-inflicted relative yield loss of rice was reduced by 10% in response to seed priming. Zappa® solution was the best priming treatment in the study (Anwar et al., 2012).

Elouaer et al. (2012) investigated the effects of seed priming with sodium chloride (NaCl) and potassium chloride (KCl) on the germinability and seedling growth of *Carthamus tinctorius* exposed to salt stress, a condition which is sub-optimal. In the study, at 20°C *Carthamus tinctorius* seeds were primed with (5 g l^{-1} NaCl for 12 hours and 5 g l^{-1} KCl for 24 hours. The seeds were subsequently exposed to five concentrations level of NaCl saline solutions (0, 5, 10, 15 and 20 g l^{-1}) in Petri dishes. Both treatments improved germination indices such as germination percentage, mean germination time, germination index and germination speed. Growth parameters such as radicle and seedling length, seedling fresh and dry weight and vigour index of *Carthamus tinctorius* were also investigated. This study indicated that NaCl and KCl priming could be beneficial on *Carthamus tinctorius* germination and growth under saline condition (Elouaer et al., 2012).

1.11 Imbibition Damage in Seeds/ Seeds Electrical Conductivity

Water uptake into the outer cells of seed cotyledons increases as a consequence of damaged seed testa. The loss in seed quality in response to testa damage is called imbibition damage and it has been reported as a leading cause of loss in seed quality (Powell, 1998). The increased water uptake into the outer layer of the cotyledons as a result of damaged testa has been reported to damage cell membrane leading to increased electrolyte leakage from seed embryo and consequently leading to cell death (Powell and Matthews, 1980, 1981). Hence, electrolyte conductivity test, an index of membrane stability, has been recommended to evaluate seed vigour for several plants, for example, *Cucurbita pepo* (Vieira et al., 2006) and *Pisum sativum* (Panobianco et al., 2007). However, at low temperatures, electrolyte conductivity test was not a good indicator of deterioration in orthodox seeds. For example, at 10ºC, seed deterioration was not directly correlated to a loss in membrane integrity, probably due to repair of the cell membrane during storage at that temperature (Panobianco et al., 2007).

Other complications in seed quality caused by imbibition damage include reduced respiration, germination, and seedling emergence. Seedling growth is affected due to disruption in the transfer of assimilates from the cotyledons to the growing seed embryo due

to imbibition damage. This has been reported in grain legumes and some other species such as *Pisum sativum* (Duke and Kakefuda, 1981; Wang et al., 2012), *Vigna unguiculata* (Legesse and Powell, 1992), *Glycine max* (Blackman et al., 1995), *Arabidopsis thaliana* (Maia et al., 2014), and *Senna multijuga* (Rodrigues-Junior et al., 2015).

Differences in susceptibility of seeds to imbibition damage have been linked to genotypic variations between and within plant species. For example, Powell et al. (1986) reported a variation in field emergence of some cultivars of *Phaseolus vulgaris*. In the study, ten cultivars with unpigmented testae emerged much more poorly in contrast to twenty-one cultivars that were pigmented, which emerged well (an indication of good seed vigour). The poor emergence of the unpigmented cultivars was linked to rapid water imbibition resulting in high imbibition damage (Powell et al., 1986). In another study, Powell and Matthews (1979; 1981) demonstrated the role of testa damage in seed quality by scarifying some pea seeds and using unscarified seeds as a control. The scarified seeds imbibed water more rapidly, showed increased dead tissue and higher electrolyte leakage when compared with the control. Field emergence of the scarified seeds was poor. Generally, priming leads to higher cell division and DNA synthesis when such seeds are re-imbibed in water (McDonald, 2004). The reduction in the conductivity readings is associated with repairs that take place during seed priming. Other associated changes during seed priming include an increase in enzymatic activities and reduction in lipid peroxidation. Generally, the purpose of seed priming is the reparation of deteriorated seeds so that the negative effects of deterioration/ageing could be reversed. Reports have indicated that aged/deteriorated seed exhibit increased antioxidant enzyme activities to combat the deleterious ROS effects (McDonald, 2004).

The physiological death of seeds due to imbibition damage or increased pre-disposition of seeds to infection by soil-borne fungi may be responsible for reduced emergence associated with imbibition damage of some seeds. For instance, infection by *Pythium,* a soil-borne fungus, has been reported as a major cause of emergence failure in *Pisum sativum* (You et al., 2017). Dead cotyledons of seeds act as a food base for the growth of the fungi a situation that thereby predisposes the seeds to infection (Matthews, 1971). Leakage of solutes from damaged seeds into the soil results in increased inoculum potential, thus predisposing the seeds to further fungal attacks (Matthews, 1971; Rahoui et al., 2010). Greater infection by *Pythium* has also been reported in *Glycine max* seeds having cracked testae when compared with control with undamaged testa (You et al., 2017).

1.12 Anti-Oxidants and ROS in the Context of Normal Germination, Desiccation Tolerant and Sensitive Seeds

Although some of the contents of this paragraph have been mentioned earlier in different sections, it is important to discuss them here for proper flow and understanding of the other paragraphs in this subsection. The main content of a cell is water as it constitutes up to 80% of the entire cellular content. It is therefore expected that cellular disruption of normal cellular events/ functions may occur when water is removed from the cells through the process of drying. However, in orthodox seeds, due to the development of a suite of protective mechanisms during seed development and maturation on mother plant (Berjak and Pammenter, 2008; Pammenter and Berjak, 1999), orthodox seeds are able to tolerate drying to very low water contents (Pammenter and Berjak, 1999). Hence, they are regarded as being desiccation-tolerant (Berjak and Pammenter, 2008). Such suites of protective mechanisms are either completely absent or not expressed in the recalcitrant seeds, and hence, unlike the orthodox seeds, they cannot be stored for a long time (Pammenter and Berjak 2014). In orthodox seeds, a change in the pattern of enzymatic and non-enzymatic antioxidants activities occur as desiccation tolerance is acquired, sustained and lost (Bailly, 2004) during different stages of seed maturation and germination. It must be emphasized again that although an orthodox seed can be stored for a long time, its storage is not indefinite even under the best of conditions. This is true even under conditions used in gene banks which are primarily focused on long-term conservation of orthodox seeds, either 'conventionally' in freezers at -18°C, or in liquid nitrogen (Walters et al., 2005). Orthodox seeds lose vigour and ultimately viability in storage, the rate of deterioration being inversely related to the storage temperature (Kranner, 2010).

Free radicals and ROS are molecular species containing one or more unpaired electrons, which are produced as a result of dehydration, deterioration and germination of orthodox seeds (Bailly, 2019; Kranner et al., 2010). It has been reported that uncontrolled production and accumulation of ROS may result in cellular damage and/or death of seeds; such seeds fail to germinate even when water and other optimum germination conditions are provided (Berjak, 2006; Smith and Berjak, 2017). Studies in the past two decades have confirmed the role of free radical activities in seed deterioration in germinating orthodox (Liszkay et al., 2004; Schopfer et al., 2002; Whitaker et al., 2010) and recalcitrant seeds (Varghese and Naithani, 2002; Varghese et al., 2011; Ntuli et al., 2013). Berjak and Pammenter (2008) suggested that the interest may be a result of the implication of ROS in an intracellular signalling network. Consequently, ROS needs to be stringently controlled by the antioxidants (Kurek et al., 2019). Active oxygen species include the radical derivatives of oxygen ($\bullet O_2^-$,

OH, alkoxyl and hydroperoxyl radicals) and/or non-radical derivatives of oxygen such as hydrogen peroxide (H_2O_2), ozone and singlet oxygen (Kurek et al., 2019). Although oxygen is only a slightly reactive molecule, it may give rise to strongly reactive and/or potentially harmful reactive oxygen species during electron transport processes of photosynthesis and/or respiration (Bailly, 2004). The reduction of oxygen gives rise to superoxide radical ($\bullet O^{2-}$). Superoxide radical ($\bullet O^{2-}$) has an uncoupled electron and it can react with other molecules to stabilize its energy. Although superoxide is not highly reactive and short-lived, it can form hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH) which are very reactive.

Except in the early developmental phase, orthodox seeds lack the major source of ROS in green plants, which is from leakage in the photosynthetic electron transport chains in chloroplasts. From the beginning of development to the end of germination, a drastic change is evident in the water content and metabolic activities of the seeds. Hence, the sources of ROS in seeds also vary considerably. One of the primary sources of ROS is the mitochondrial respiratory chain as electron leakage from the transport chain generates superoxide and subsequently H_2O_2 by dismutation of the former (Möller, 2001). In normoxic conditions, approximately 2 - 3 % of the oxygen used by the mitochondria can be converted into superoxide and H_2O_2 (Puntarulo et al., 1988). Thus, the amount of H_2O_2 produced is directly proportional to respiratory activities (Staniek and Nohl, 2000; Zorov et al., 2014).

In orthodox seeds, respiration is significantly reduced during the desiccation phase on the mother plant. Respiratory activities are shut down as the seed become quiescent (Bewley and Black, 1994). When the seed water content falls below 0.25 g g^{-1} dry matter, mitochondrial respiration stops (Vertucci and Farrant, 1995; Zorov et al., 2014). However, respiration resumes during seed germination. As respiratory activities increase, the production of ROS is also enhanced. This may be responsible for the high sensitivity of germinating seed to adverse environmental conditions such as temperature, salinity and environmental pollutants (Fatokun et al., 2015; Vange et al., 2004). Although the potentially toxic effects of ROS are the central focus during seed development, they may play some beneficial roles such as cell growth and development in embryo growth (Puntarulo et al., 1988; Staniek and Nohl, 2000), endosperm breaking (Müller et al., 2007; Müller et al., 2009) and cell signalling (Kranner et al., 2010; Müller et al., 2009). The variations in ROS contents observed during seed maturation could, therefore, be involved in the shift of gene function from a developmental to a germinative mode, which is supposed to be initiated by seed dehydration (Kermode, 1995). Reactive oxygen species are known to regulate the expression of many genes, for example, in *Arabidopsis*, H_2O_2 induces 113 genes and represses 62 others (Desikan et al., 2001). However, the mechanisms allowing control of gene expression by ROS in plants are still largely unknown. One of the most cited

possibilities concerns the activation of transcription factors by redox status changes (Foyer and Noctor, 2003; Vranová et al., 2002).

1.13 Cathodic Water

Cathodic water is the cathodic fraction of an electrolysed, dilute ionic solution of calcium and magnesium solution (CaMg). To overcome the negative impact of ROS as a consequence of various abiotic stress such as excision of explants, desiccation and exposure to liquid nitrogen temperatures (Berjak et al., 2011; Gebashe, 2015; Naidoo et al., 2016), and storage (Bam et al., 2017; Gondwe et al., 2016), various authors have recently reported the successful use of cathodic water as an antioxidant pre-treatment. Cathodic water has strong reducing power and its use obviates the need for exogenously-supplied potentially toxic chemical antioxidants (Berjak et al., 2011; Pammenter et al., 1974). Cathodic protection as commonly understood, is a process by which electrons generated at a cathode counteract oxidative corrosion. Harnessing the reducing power of cathodic water in plant germplasm conservation was based on intuition by late Professor Patricia Berjak (Berjak et al., 2011). The intuition emanated from an earlier study, in which the deterioration of maize seed was counteracted by cathodic protection. In that study, maize seeds were placed on an aluminium foil disc with the disc attached to the cathode of a power pack (Berjak, 1978; Pammenter et al., 1974). The intuition has been substantiated with remarkable successes. For example, cathodic protection was used as a medium during explant excision, as the solvent for cryoprotectant solutions, and as the medium for post-cryo thawing and rehydration in the cryopreservation of *Strychnos gerrardii* (Berjak et al., 2011). Prior to that study, despite many attempts, no one had been able to produce a viable plant from cryopreserved axes of *Strychnos gerrardii* (Berjak et al., 2011). In another study, Gondwe et al. (2016) working on orthodox seed storage reported successful invigoration of gene bank stored seeds of *Pisum sativum* and *Cucurbita pepo* with cathodic water. In this study, 22 year-old gene bank stored seeds of pumpkin with 0% initial germination were invigorated with cathodic water resulting in 57% germination (Gondwe et al., 2016). In this study, cathodic water was used to invigorate controlled deteriorated orthodox seeds to counteract the potential damage caused by ROS, and ultimately invigorate seeds that have lost vigour (become debilitated) due to controlled deterioration.

The successes recorded with cathodic water treatments of recalcitrant and orthodox seeds present the possibility that the capability of endogenous anti-oxidants may be amplified/ augmented to a level where they are effective in coping with stress-induced ROS generation (Gondwe et al., 2016). Hence, in this study, cathodic treatment was used in addition to other exogenous application of selected treatments to reinvigorate deteriorated seeds of *P.*

sativum, Cu. pepo, E. caffra, B. speciosus and *Co. erythrophyllum*. They were evaluated and compared to determine their ameliorative effects on CD of the five orthodox species. The evaluation was done in terms of germination and seedling growth (rate of germination, seedling emergence, plant growth and plant tissue mineral composition), plant physiology (chlorophyll fluorescence, photosynthesis and transpiration); plant biochemical activities (α amylase, MDA and 4-HNE) and DNA (concentration and purity).

1.14 Problem Statement and Research Justification

The world is facing unpredictable and radical unfavourable climate change coupled with human population explosion. To meet the food requirement of the ever-increasing world population, emphasis on present day agriculture is to produce more food with lesser input such as land, water, and manpower. A basic and very important input in agricultural production is the seed; about 90% of food crops are grown from seeds (Etebu and Nwauzoma, 2017). When produced, seeds needed for agricultural production are stored in the short term to provide high-quality planting materials from one season to the other. Seeds are also stored to conserve genetic resources and diversity plants, especially that of wild species that is to maintain germplasm suitable for restoring wild populations, especially in the face of climate change. Such seed storage is considered long-term base collections. Also of great importance is the maintenance and conservation of the genetic resources of agricultural species of economic importance. Seed quality, therefore, must be maintained over extended periods. However, no matter how good the storage conditions, all seeds deteriorate during long-term storage, which leads to a decline in seed germination, seedling vigour, seedling emergence, plant growth and productivity. This reduction in seed 'performance' is of considerable financial concern to any country in general and seed/agricultural industry in particular. It is also of global concern with respect to the long-term conservation of genetic diversity, both of wild species and in the agricultural and horticultural sectors.

It has been reported that physiological parameters of seed deterioration such as decreased germination percentage and seedling growth are linked to low rate of metabolism in embryo of aged seeds but the changes in metabolic activities associated with seed deterioration due to ageing remains complex and not yet fully understood (Leão-Araújo et al., 2017; Smith and Berjak, 2017). Loss in seed vigour of aged seeds is associated with loss of membrane integrity as a result of metabolic and biochemical alterations in the seed, uncontrolled production and release of free radicals, decrease in proteins, inactivation of enzymes, genetic damage and lipid peroxidation (Arun et al., 2017; Berjak and Pammenter, 2008; Murthy et al., 2003; Smith and Berjak, 1995). To address the problem associated with loss in

seed vigour, various priming techniques such as hydropriming, halopriming, nutrient priming, and matripriming have been used for reinvigorating debilitated seeds with varying level of successes (Farooq et al., 2008; Gondwe et al., 2016; Maiti et al., 2013). In this study, cathodic water, an electrolysed form of calcium magnesium solution (Berjak et al., 2011) was used, thereby bringing the concept of electrochemistry into plant germplasm conservation and crop establishment and production. It is important to say that the use of cathodic water is a novel approach to address the problem of reinvigorating debilitated orthodox seeds. Although the ability of cathodic water to reinvigorate deteriorated seeds may be complex and is not yet understood, the ameliorative effects of cathodic water on the germination of *P. sativum* and *Cu. pepo* has been reported in our laboratory (Gondwe et al., 2016); however, its effects on the growth of the various species were not explored in the study. Also, the ameliorative effects of cathodic water on the germination, seedling emergence and growth of wild species (*E. caffra, B. speciosus*, and *Co. erythrophyllum*) have not been well documented (if they do exist at all). Hence, this study was carried out to bridge the gaps in knowledge enumerated above.

1.15 Aim of the Study

This study aimed to test the possibility of reinvigorating orthodox seeds from selected wild and agricultural species that were subjected to controlled deterioration using cathodic water. It was also hypothesised that the possible beneficial effects of cathodic water on these seeds would be carried forward in the seedling establishment and growth. In the study, priming of these seeds was carried out by exogenous application of selected anti-oxidants/treatments: ascorbic acid (used only in the preliminary study), CaMg solution and deionized water. They were evaluated and compared to determine their comparative effects on some growth parameters of the test species. The assessments were done in terms of germination, seedling emergence, and growth parameters of the test plants. However, as strictly controlled ROS activity is crucial for certain metabolic events during germination (Bailly, 2004), the timing of cathodic exposure was determined with imbibition curves so as not to interfere with these processes.

This study was designed to examine the impact of the ameliorative effects of cathodic water treatment on germination, seedling establishment and growth of plants derived from controlled deteriorated seeds of *P. sativum*, *Cu. pepo*, *B. specious*, *Co. erythrophyllum* and *E. caffra*. The biochemical basis of seed deterioration and CD repairs via priming were examined using, membrane stability index, α amylase, 4-HNE, MDA and crude protein. The project also explained the mode of action of the ameliorative effects of cathodic water in plants (using some physiological parameters such as photosynthesis, chlorophyll

fluorescence and transpiration rate) on the growth of plants derived from invigoration of debilitated seeds of the test plants with cathodic water. In terms of outcomes, the successful reinvigoration of CD seeds will have profound implications on the productivity of plants derived from CD orthodox seeds. The need to use potentially toxic exogenously supplied chemical anti-oxidants in the course of reinvigorating debilitated seeds of the test plants was also obviated.

The aims of this study were:

1. To assess the effects of invigoration with cathodic water on the germination and seed vigour of deteriorated seeds of *P. sativum, Cu. pepo, B. specious, Co. erythrophyllum* and *E. caffra*.
2. To assess the effects of cathodic water invigoration on seedling emergence and the growth of plants derived from controlled deteriorated seeds of the test species.
3. To assess the physiological modes of actions of cathodic water on the growth of the test plant species.

1.16 Contribution to the Body of Knowledge/ Novelty

The aim of this was to expand our knowledge of the use of electrochemistry (cathodic water) in plant germplasm conservation. It assessed differences between the effects of cathodic water and other seed treatments on some highly bred agricultural and wild species in terms of germination, seedling emergence and growth. In terms of outcomes, successful cathodic quenching of ROS, as observed in this study, will have profound implications for the invigoration of debilitated orthodox seeds. It has also provided a good direction on the use of cathodic water in invigorating other species that were not investigated in this study. In terms of practical applicability, this study has no doubt advanced the frontiers of knowledge in confirming the use of cathodic water in reinvigorating debilitated orthodox seeds and by extension, plant germplasm conservation of especially the genetic resources of critically endangered species. Some gene banked materials which may be difficult or impossible to collect again will also benefit from the outcome of this research. Although gene banked material was not used in this study, successful reinvigoration of controlled deteriorated seed by means of cathodic treatment would not only be practically important but would undoubtedly advance the knowledge base in terms of ROS implication in seed damage and its amelioration. The inclusion of genetic resources of species of “actual economic concern” (pea and pumpkin) in this study will also contribute to their germplasm conservation and improvement in their productivities considering the need to increase food production to meet the need of ever-increasing world population, especially in the sub-Saharan Africa.

1.17 Brief Overview of Study

The project started with a preliminary study that was carried out to investigate the effects of cathodic water on the germination and growth of *P. sativum* and *Cu. pepo* seeds subjected to controlled deterioration. The cathodic treatment was used in addition to other exogenous applications of selected antioxidants and other treatments. The six other treatments investigated alongside that of cathodic water included cathodic water (usually with a pH of ~11) adjusted to pH 7.0, calcium magnesium solution (usually with a pH of ~7), calcium magnesium solution (with pH adjusted to 11, which is the pH of cathodic water), ascorbic acid and distilled water. Fresh seeds (not primed and not subjected to controlled deterioration) served as the control. The treatments were evaluated and compared to determine their comparative effects on germination and some growth parameters of *P. sativum* and *Cu. pepo*. The assessments were done in terms of germination, seedling emergence, plant mortality, and plant growth. Electrolyte conductivities on both the fresh and deteriorated seeds were also measured. The study was conducted by subjecting the seeds of *P. sativum* and *Cu. pepo* to controlled deterioration in an oven (40°C) until almost complete loss of germination. The deteriorated seeds were thereafter invigorated with cathodic water and other treatments. Fresh, unprimed seeds of both species served as the control.

Results showed that invigorating seeds with cathodic water led to significantly higher germination in both *P. sativum* and *Cu. pepo*. Even though all the invigoration treatments lead to an improvement in both germination and growth in both species, cathodic water generally performed better when compared with any of the other five treatments. It was also observed that adjusting the pH of both cathodic water and calcium magnesium did not result in any significant difference in germination or growth when compared to the original solutions (cathodic water and calcium magnesium solution). In terms of comparison between the two species investigated, *Cu. pepo* responded better than *P. sativum* both in terms of improvement in germination and growth. Hence, the preliminary study was a good indication of the efficacy of cathodic water in invigorating debilitated seeds obtained as a consequence of CD. The preliminary study is presented as Chapter 3 of this thesis.

The success of the preliminary study gave the needed encouragement for the main study. However, in the main study, some adjustments were made in the treatments investigated. Some of the changes made included dropping the adjustment in the pH of cathodic water and calcium magnesium solution because adjusting the pH of the two solutions did not result in any significant effect on the test plants as stated earlier. Ascorbic acid treatment was also

dropped from the main study for its poor performance when compared to other treatments. A significant change that was also made in the main study was the reduction in the extent of deteriorating the seeds of the test species. Contrary to deteriorating to the point of almost complete loss of germination/viability in the preliminary study, deterioration to 50% loss in germination (P_{50}) was adopted in the main study. Three wild species (*E. caffra*, *Co. erythrophyllum*, and *B. speciosus*) were also added as test plants in the main study. In the preliminary study, the following data were collected, germination percentage, electrolyte conductivity of aged and controlled deteriorated seeds, chlorophyll content, chlorophyll fluorescence and the biomass of plant parts. In addition to the parameters investigated in the preliminary study, the following were also investigated in the main study; germination (rate of germination, root and shoot biomass), growth (steady-state gas exchange, transpiration) and cell membrane stability index of the seeds. Also investigated were lipid peroxidation products MDA and 4-HNE and membrane stability index, antioxidant enzymes SOD and catalase, MDA (purity and concentration.

The methodology used in this study was thus: water content of the seeds of test species was raised to 14% (fresh weight basis). The seeds were then subjected to controlled deterioration at 40°C and 100% relative humidity. The seeds were thereafter hydrated with cathodic water and dried back to close to the original water content (priming). The appropriate time of exposure to cathodic exposure was determined from the imbibition curve of the species investigated. There were six priming treatments in the study viz; fresh seeds primed with cathodic water (FSP.CW), fresh seeds primed with calcium-magnesium solution (FSP.CM), fresh seeds primed with deionized water (FSP.DW), CD primed with cathodic water (ASP.CW), CD primed with calcium-magnesium solution (ASP.CW), CD primed with deionized water (ASP.DW). Unprimed controlled deteriorated seeds (ASC) and unprimed fresh seeds (FSC) served as the two controls (Table 1.1). Germination study was conducted in a growth room and the seedling vigour assessed using the dry mass of the seedlings. A greenhouse pot experiment was used for the growth study. The seedling growth was assessed to determine the influence of invigorating treatments on the seedling emergence, and subsequent growth of the plants. The influence of cathodic water and other treatments on the growth of the test plants were examined using morphological and physiological parameters.

Table 1.1 Seed treatments and their abbreviations

S/N	TREATMENTS	ABBREVIATIONS
1	Fresh seeds not primed - Control	FSC
2	Fresh seeds primed with cathodic water	FSP.CW
3	Fresh seeds primed with calcium-magnesium solution	FSP.CM
4	Fresh seeds primed with deionized water	FSP.DW
5	CD seeds that were not primed - Control	ASC
6	CD seeds primed with cathodic water	ASP.CW
7	CD seeds primed with calcium-magnesium solution	ASP.CW
8	CD seeds primed with deionized water	ASP.DW

1.18 Outline of Thesis/ Linkages of Scientific Manuscripts

The thesis format adopted in this study is 'thesis by manuscripts' also known as 'thesis by publications'. The thesis contains a total of five manuscripts out of which three (Chapters 4, 5 and 6) have been published. The other two manuscripts (chapters 2 and 3) are considered as supporting the 3 chapters earlier mentioned.

Chapter 1: This chapter contains the introduction and literature review.

Chapter 2: A COMPARISON OF WATER IMBIBITION AND CONTROLLED DETERIORATION IN FIVE ORTHODOX SPECIES. To deteriorate the seeds, the seeds were subjected to an aggravated temperature of 40°C, 14% moisture content at 100% relative humidity. This chapter compared water imbibition and controlled deterioration in the five test species. The comparison was made in terms of the progressive loss of germination as the age of controlled deterioration increased. These changes were compared using electrolyte leakage and Fourier Transform Infrared-Spectroscopy (FTIR). An unintended finding in the course of the comparison was that FTIR could be used as a measure of monitoring the deterioration of stored seeds. The report is attached as a separate manuscript (Appendix 1). In this chapter, a comparison of water imbibition in the five test species was also made. While the report of the progressive loss in germination was used to determine the 50% viable seeds used in the main study; the water imbibition was used to determine the imbibition period for the see priming treatment in the germination and growth study.

Chapter 3: CATHODIC WATER SEED PRIMING – A NEW APPROACH TO INVIGORATE DETERIORATED ORTHODOX SEEDS. This chapter, as mentioned in subsection 1.17

above, contains the report of the preliminary study. The chapter is included in this study to provide the necessary information, which leads to the 'main' studies, which are reported in chapters 4, 5, and 6.

Chapter 4: GERMINATION INDICES OF ORTHODOX SEEDS AS INFLUENCED BY CONTROLLED DETERIORATION AND CATHODIC WATER INVIGORATION. In this chapter, the influence of cathodic water, calcium magnesium solution and deionised water on the germination of *B. speciosus*, *E. caffra*, *Co. erythrophyllum*, *P. sativum* and *Cu. pepo* was compared using germination indices such as the first day of germination, germination percentage (%), mean germination time, germination index and uniformity of germination. Concentration and purity of deoxyribonucleic acid (DNA) as affected by seed deterioration and seed priming were investigated in the test species. Also examined was the response of the germination enzyme, amylase, to both seed deterioration and priming. The chapter has been published by the Journal of Environmental Biology (Appendix 2-Acceptance letter). Although the germination study was a success, there was the need to investigate if the performance of cathodic water which resulted in significant improvement in germination could be carried through to seedling emergence and later growth of the test species.

Chapter 5: CATHODIC WATER ENHANCES SEEDLING EMERGENCE AND GROWTH OF CONTROLLED DETERIORATED ORTHODOX SEEDS. In this chapter *B. speciosus*, *Co. erythrophyllum* and *E.caffra* were used to investigate the influence of the priming solution on the emergence indices of the three species. Among the emergence indices considered were the first day of emergence, mean emergence time, and uniformity of emergence. The possible carryover of the observed effects of seed priming to later stages of growth was also examined. Among the growth parameters investigated were total biomass, leaf area, root length, shoot length and aspects of plant physiology (chlorophyll fluorescence and photosynthesis). Besides the growth and plant physiology data, investigation, lipid peroxidation products (MDA and 4-HNE) and the membrane stability index (MSI) were investigated in all the treatments. This chapter is currently being reviewed by an accredited journal, Seed Science Research. Both lipid peroxidation products and MSI investigated in this manuscript are indicators of seed deterioration. There was the need to investigate the ameliorative action of cathodic water with the focus on its antioxidant properties. Two of the test species (*P. sativum* and *Cu. pepo*) were used for the investigations. This chapter has been published by Plants-MDPI (Appendix 4 – acceptance letter)

Chapter 6: INFLUENCE OF CATHODIC WATER INVIGORATION ON THE EMERGENCE AND SUBSEQUENT GROWTH OF CONTROLLED DETERIORATED PEA AND PUMPKIN SEEDS. A further investigation into the possible carryover of the effect of seed priming with cathodic water and other solution was investigated using *P. sativum* and *Cu. pepo.* Besides the emergence, growth, plant physiology data further investigations were made into the mechanisms of actions of cathodic water seed invigoration using antioxidative enzymes, catalase and superoxide dismutase. As stated earlier, cathodic water is an electrolysed form of calcium magnesium solution, hence, to provide some explanation as regards the differences in the observed effects of cathodic water and calcium magnesium solution, plant tissue analyses were made. This chapter has been published by Plants-MDPI (Appendix 3 – acceptance letter)

Chapter 7: GENERAL OVERVIEW, SUMMARY, RECOMMENDATIONS AND PRACTICAL RELEVANCE OF STUDY. In this chapter, a brief summary of the whole study was presented. Also presented were possible practical applications of cathodic water seed invigoration. Several recommendations were also made for people who may be interested in future researches that deal with cathodic water seed invigoration.

Chapter 8: All references were consolidated and presented in chapter 8.

CHAPTER 2

A COMPARISON OF WATER IMBIBITION AND CONTROLLED DETERIORATION IN FIVE ORTHODOX SPECIES

This is a supplementary chapter that provides the necessary background for other manuscripts contained in this thesis: seed water imbibition, controlled deterioration.

Abstract

Orthodox seeds deteriorate even when stored in the best of conditions. Hence, it is very important to monitor germination in stored seeds. To assess orthodox seed deterioration, germination test is usually employed. This study assessed and compared seed deterioration in five orthodox species using the electrolyte leakage and Fourier transform infrared spectroscopy (FTIR). The study also compared water imbibition by the test orthodox seeds. To achieve this, a set of wild (*Bolusanthus speciosus, Combretum erythrophyllum*, *Erythrina caffra*) and agricultural (*Pisum sativum*, and *Cucurbita pepo*) seeds were imbibed in between 20 layers of single-ply paper towel. The other set was subjected to controlled deterioration at 40°C and 100% relative humidity for 32 days with samples taken for germination and electrolyte leakage measurement at 4 days intervals. FTIR measurements were done at 0, 20 and 32 days of controlled deterioration. The results indicated that there are some significant interspecies differences in the imbibition time and seed water content, but these are not large. In all species, uptake of water was complete between about 15 and 25 h. The wild species showed higher sensitivity to controlled deterioration. Complete loss in germinability occurred much earlier in the wild species (20 d in *B. speciosus* and *E. caffra* and 16 d in *Co. erythrophyllum*) compared with 36 d for agricultural species, *P. sativum* and *Cu. pepo*. There was a negative correlation between electrolyte leakage and seed germination in all wild and agricultural species. A strong positive correlation occurred between the age of controlled deterioration, electrolyte leakage and FTIR transmission in all the species. While controlled deterioration may help in decisions relating to storage of orthodox seeds, the water imbibition results from this study will no doubt help set the priming time of the species. The study reaffirms electrolyte leakage as an indicator of seed viability in *P. sativum* and *Cu. pepo*; it also recommends the use of electrolyte leakage as an indicator of seed deterioration in *B. speciosus*, *Co. erythrophyllum* and *E. caffra*. The study also recommends FTIR as a tool for monitoring germination of stored seeds in all the test species.

Keywords: Controlled deterioration, electrolyte leakage, FTIR, germination, imbibition, orthodox seeds

2.1 INTRODUCTION

Seeds are the genetic resource by which most higher-plants are propagated (Sacandé et al., 2004). Based on their desiccation tolerance, seeds are classified into recalcitrant and orthodox seeds. While recalcitrant seeds are desiccation sensitive and cannot be stored for a long time, orthodox seeds are tolerant of desiccation (Dickie and Pritchard, 2002; Engelmann, 2011) and can be stored for a long time (Engelmann, 2011), especially at low water contents and temperatures (McDonald, 2004). Of all the plants that have been studied, about 90% produce orthodox seeds (Sacandé et al., 2004). Orthodox seeds are either stored in the short or the long-term. Short-term orthodox seed storage provides high-quality planting material for planting, re-introduction, and rehabilitation (Tweddle et al., 2003). Seeds are also stored in the longer term in genebanks for distribution (active collections) and to conserve genetic resources and diversity of orthodox seeded species (base collections) (Vagera, 2007).

Natural ageing is a key factor affecting the germination of stored orthodox seeds (Amanpour-Balaneji and Sedghi, 2012). As the ageing time increases, organic compounds present in seeds become deteriorated, germination and seed vigour are reduced, and ultimately complete death of the seed occurs (Amanpour-Balaneji and Sedghi, 2012; Mohammadi et al., 2012; Rajjou et al., 2008). Seed ageing and the eventual death is a complex biological trait. It involves a consortium of metabolic, biochemical, physiological and molecular processes. Although significant efforts are being made to understand the causes and mechanisms involved in seed deterioration, the causes of seed deterioration and death remain largely not well known. Seed deterioration may be slow under normal, ambient conditions. However, for experimental purposes, there is the need to shorten the time required for seed to deteriorate. Hence, seed deterioration is controlled by subjecting the seeds to predetermined and aggravated conditions of heat and humidity to accelerate the rate of deterioration (Ghahfarokhi et al., 2014; Sharma et al., 2018). Controlled deterioration of seeds has been used in the study of *Capsicum annumm* (Kaewnaree et al., 2011), *Phaseolus vulgaris* (Amanpour-Balaneji and Sedghi, 2012); *Hordeum vulgare* (Nagel et al., 2016) and *Vigna radiata* (Sharma et al., 2018).

Gene banking of seeds is the ideal means of *ex situ* conservation of plant genetic resources of orthodox seeds (Berjak and Villiers, 1972; Chacko, 2019; Walters et al., 2005). However, during long-term storage, even under very good conditions, orthodox seeds deteriorate. The best that can be done is to reduce the rate of deterioration during storage of such seeds in the dry state (Walters et al., 2005). The deterioration of seeds during long-term storage is known as seed ageing, and in the process, both vigour and viability are gradually

compromised (Garza-Caligaris et al., 2012). Seed deterioration and its consequence of reduction in seed 'performance' is of significant financial concerns to the seed industry. On the global scale, such concerns relate to the long-term conservation of genetic diversity of wild, agricultural, and horticultural plants (Tweddle et al., 2003). Seed deterioration has been implicated in the loss of diversity in natural ecosystems (Harlan and Martini, 1936). It is a threat to global efforts at conserving genetic resources of plants because it causes loss of genetic diversity in plants (Frankel and Bennett, 1970).

Seed deterioration is directly related to seed longevity. However, the rate of seed deterioration differs between and within species (Santos et al., 2016; Sharma et al., 2018). As seed deterioration progresses, seed longevity, and by extension, seed viability is compromised (Sharma et al., 2018). As the ageing time increases, a point is reached where regeneration of stored seed becomes inevitable (Chacko, 2019; van Treuren et al*.,* 2013; Walters et al*.,* 2005). Seed regeneration is costly and may affect the genetic integrity of an accession. It is therefore crucial that *ex-situ* conserved seeds must be managed in a way that ensures maximum longevity. Assessment of deterioration in stored seeds is the basic tool for managing the germplasm of *ex-situ* conserved seeds (Engels and Visser, 2003).

Germination test is currently the standard method of assessing the viability of *ex-situ* conserved seeds (Engels and Visser, 2003). However, conventional germination method is destructive, labour intensive and time-consuming, especially when a large amount of plant germplasms present in gene banks are considered. Also, traditional germination method does not elucidate the mechanisms involved in seed deterioration. Therefore, it has become very imperative to develop new and equally reliable tools which are time efficient, low cost and non-destructive methods to assess the deterioration of stored seeds to supplement the traditional germination tests for more effective *ex situ* conservation of seed germplasm (Colville et al*.,* 2012; Donà et al*.,* 2013; Engels and Visser, 2003). Among the recently developed tools used for seed deterioration assessments are electrolyte leakage, genomic and some biochemical markers. The recent advances have provided some light in the understanding of the complexities involved in seed ageing. It must be noted that most of the tools are species-dependent; hence, the need to test the tools on a particular species to know the applicability before its adoption. This study used both electrolyte leakage and Fourier Transmission Infra Red (FTIR) spectroscopy to assess and compare controlled deterioration in five orthodox seeded species.

Generally, the starting process of germination is considered to be water imbibition. Under optimal conditions, water uptake by a dry seed is divided into three phases. The first phase, known as phase I, is mainly characterised by rapid water imbibition. The rapid water intake

which occurs in phase I is largely due to the matric forces exerted by the seed. In phase I, biochemical, genetic, and physiological activities such as DNA, mitochondria repairs take place; protein synthesis also occurs using existing messenger ribonucleic acid (mRNA) (Balestrazzi, 2011; Ventura et al., 2012). Immediately after phase I, the seeds move to phase II, also known as lag phase or activation phase. In phase II only little net gain of water takes place. However, considerable metabolic activities occur, which prepares viable non-dormant seeds for radicle emergence. A major occurrence in phase II is the synthesis of mitochondria and proteins by new mRNA (Sano et al., 2015). In the final phase, otherwise called phase III, water uptake increases coupled with radicle elongation (da Silva Moura et al., 2016; Lev and Blahovec, 2018; Varier et al., 2010).

Water uptake into the outer part of seed cotyledons occurs as a consequence of damaged or broken testae, causing some damage to the cell membrane. Cell membrane damage results in increased electrolyte leakage from the seed embryo and ultimately leading to increased imbibitional stress and cell death (Lev and Blahovec, 2018; Powell et al., 1986). The loss in seed quality due to water uptake is called imbibition damage and it has been described as a leading cause of loss in seed quality (Lev and Blahovec, 2018; Powel et al., 1986). Imbibition damage reduces seed respiratory activities and germination, lower seedling emergence and growth. The negative influence imbibitional damage on seedling growth is due to disruption in the transfer of food from the cotyledon(s) to the growing embryonic of seed. Electrolyte leakage and seed germination are positively correlated, hence, electrolyte conductivity test, which as an index of membrane stability, has been recommended to evaluate seed vigour for a number of species, for example, *Pisum sativum* (Panobianco et al., 2007) and *Cucurbita pepo* (Vieira and Dutra, 2006).

The concepts of water imbibition in orthodox seeds, artificial ageing otherwise known as controlled deterioration, and electrolyte leakages are very important in seed storage, seed priming and seed germination studies. Although, some studies have reported a positive correlation between seed deterioration and electrolyte leakage and has therefore recommended electrolyte leakage to measure seed deterioration in *P. sativum* and *Cu. pepo*; similar recommendations have not been reported for *B. speciosus*, *Co. erythrophyllum* and *E. caffra.* The use of FTIR as a tool for monitoring seed deterioration is also novel. This study is aimed at assessing and comparing seed deterioration in five orthodox species using the electrolyte leakage and Fourier transform infrared spectroscopy (FTIR). The study also compared water imbibition by the test orthodox seeds.

2.2 MATERIALS AND METHODS

2.2.1 Plant Material

Five orthodox seed species which include *Bolusanthus speciosus* (Bolus) Harms, *Combretum erythrophyllum* (Burch.) Sond, *Erythrina caffra* Thumb., *Pisum sativum* L. (pea), and *Cucurbita pepo* L. (pumpkin) were used in this study. In terms of economic usage, *P.* and *Cu. pepo* are agricultural and they were purchased from a local seed company, Grovida Seeds, Durban South Africa. The other three species can be regarded as wild/horticultural species. They were purchased from Silverhill Seeds, Cape Town, South Africa. The seeds were collected in paper bags from around the city of Cape Town, kept in airtight containers and stored at 4°C until use.

Initial testing indicated that all the wild species had physical dormancy due to their seed coats. While the dormancy in *B. speciosus* and *E. caffra* was broken by mechanical scarification that of *Co. erythrophyllum* was broken by removing the samara covering the seeds with the aid of a scalpel. Seeds sizes were examined and only seeds of similar sizes were used in the study (Al-Karaki, 1998; Saha and Mandal, 2016). It must be noted that the use of agricultural and wild species in this study was only meant to say that test species were selected from both categories of seeds. It is not meant to generalise the result on the basis of wild/agricultural species.

2.2.2 Determination of Seeds Water Imbibition

The initial water contents of the seeds were determined gravimetrically. Thereafter, ten seeds of each test species were hydrated in between 20 ply of single-layer paper towel. The seeds were initially weighed at intervals of 2 h as they imbibed water. The interval used was then increased to 4 h for *P. sativum* and *Cu. pepo*, *E. caffra* and *B. speciosus*, and 12 h for *Co. erythrophylum* until germination started. Imbibition curves were fitted to determine the triphasic pattern of seed water imbibition.

2.2.3 Controlled Deterioration of Seeds

The water content for all the species was raised to 14% using a vapour chamber. The seeds were then sealed in airtight glass jars and kept in a digital oven (Series 2000, Scientific, USA) at 40°C and 100% relative humidity. Samples (100 seeds per species) were retrieved at 4 d interval until 36 d and germination and electrolyte leakages measured.

2.2.4 Seed Germination

Germination was done in 90 mm Petri dishes with five layers of germination paper placed inside. Another layer of germination paper was placed on top of the seeds. The germination papers were kept moist with distilled water. As indicated above, at each sampling occasion,

100 seeds were retrieved, and then divided into four replicates of 25 seeds (ISTA, 1995). The Petri dishes were placed on shelves in the germination room. Each replicate of 25 seeds was separated into five Petri dishes with five seeds each to minimize competition between the seedlings. The photoperiod of the germination room was 16 h lights (52 µmol $m^{-2}s^{-1}$) / 8 h dark and the temperature was 25 ± 2°C. A seed was considered germinated when 1 mm radicle protrusion was observed.

2.2.5 Determination of Seed Electrolyte Leakage

Electrolyte leakage (S m^{-1} g^{-1}) was measured using the method of Hampton and Tekrony (1995). Three seeds (1 g) were immersed in 50 ml of deionized water in glass tubes. The glass tubes were placed in a water bath for a period of 24 h at 25°C. Thereafter, electrolyte conductivity of the leachate was measured using a conductivity meter: CM 100-2 multi-cell conductivity meter (Reid and Associates, Durban, South Africa).

2.2.6 Fourier-Transform Infrared Spectroscopy (FTIR) - Spectra Acquisition

Forty seeds of each test species were ground to prepare seed meals. The spectra of the seed meal were acquired using a Fourier-Transform Infrared Spectroscopy (FTIR) spectrometer (Burker Tensor 27 FTIR Spectrometer). The FTIR had a spectral range of 4000–10,000 cm^{-1} (1000–2500 nm) and a resolution of 4 cm^{-1}. The reflectance spectra of the seeds were acquired by placing about 0.01g of seed meal at the centre of the FTIR scanning glass window and covering with the instrument lid. The instrument lid had a black background. Each treatment was replicated three times. Each sample was scanned two times. The mean of the two scans was reported as the spectra of the sample. The FTIR provided some semi-quantitative data that were used to characterise the compounds present in the seeds at the various ageing times investigated in this study.

2.2.7 Statistical Analyses

Data were subjected to analyses of variance. Means of replicates were separated using 5% LSD. Post hoc was done using the Tukey test. FTIR wave numbers and the corresponding transmittances were extrapolated with the aid of Sigma plot.

2.3 RESULTS AND DISCUSSIONS

2.3.1 Seed Water Imbibition

When expressed on a percentage water content basis, there were some interspecies differences in the imbibition time and seed water content. Although the seeds used for this study had initial water contents of 11.8% (*B. speciosus*), 7.5% (*Co. erythrophyllum*), 11.2% (*E. caffra*), 11.7% (*P. sativum*) and 6.7% (*Cu. pepo*); the actual critical water contents of the

various species did not vary widely; 63% (*B. speciosus*), 57% (*Co. erythrophyllum*), 60% (*E. caffra*), 62% (*P. sativum*) and 56% (*Cu. Pepo*) (Figure 2.1). In all species, uptake of water was complete between about 15 and 25 h. However, it must be noted that these rates of water uptake were achieved only when the seed coats of *B. speciosus* and *E. caffra* were nicked and the samara covering the seeds of *Co. erythrophyllum* were removed. As a result, water uptake was almost similar for all species (Figure 2.1).

The seed water content and the time of imbibition were highly positively correlated in all the species (Figure 2.1). As proposed by Bewley and Black (1994), seeds water intake is triphasic in nature, that is, it is characterised into phases I, II and III. The triphasic pattern of seed water uptake as observed in this study has been reported by many authors, for example, Manz et al., 2005 (*Nicotiana tabacum*), Mei and Song, 2008 (*Zea mays*) and Gimenez et al., 2014 (*Annona emarginata*)**.** In priming, determination of seed imbibition curves helped in setting the time of invigoration treatments which were 18 h (*B. speciosus*), 20 h (*Co erythrophyllum*), 20 h (*E. caffra*), 24 h (*P. sativum*) and 24 h (*Cu. pepo*) (Figure 3.2). Basically, duration of seed invigoration treatments is defined according to the duration of phase I (Varier et al., 2010).

The time required for the seeds to reach the critical water level (point beyond which the seeds transit from phase I to phase II or minimum seed water content, below which germination is blocked) varied among the species ranging from 18 h in *B. speciosus* and *Co. erythrophyllum* to 24 h in *E. caffra* and *Cu. pepo* (Figure 2.1). *P. sativum* reached the critical water level at 32 h of imbibition. The variation in the time required for the seeds to reach the critical water level among the species may be due to variations in the seed tissue water potential of the test species and or resistance of the seed coat. It has been reported that water intake occurs in seeds as a result of the huge difference between seed tissue water potential and the ambient water potential of pure water (Tonini et al., 2010). These huge differences between the dry seed tissue water potential and the ambient water potential (in the case of pure water) result in rapid influx of water during phase I of water imbibition. The rapid water inflow is responsible for the steep slope in phase I of water imbibition (Figure 2.2).

Phase II is the lag period. During this phase, there is little or no uptake of water, resulting in only a small change in fresh seed mass and a slight decline in mass in some instances (Figure 2.1). The slight loss in seed mass may be due to loss of mucilage surrounding the seeds. Water always flows from an area of higher to lower water potential and the net flux of the flow stops when the difference in water potential becomes zero, an indication of the beginning of phase II of the water imbibition process. While dry seed tissues have been

reported to have a water potential (Ψ) of between -350 and -50 Mega Pascal (MPa), pure water has a water potential of zero MPa. Uptake of water by seeds is a physical process, and it results in seed mass increase (Figure 2.2). Although not investigated in this study, as the water imbibition increases, a series of physiological, metabolic and biochemical processes are triggered (Tonini et al., 2010). The activation of different metabolic processes with water uptake by the seeds may lead to an increase in respiration and the beginning of sugar consumption (Tonini et al., 2010). The sugar serves as a substrate for seed embryo respiration. Sugars such as sucrose and glucose are carbon sources for metabolites production including amino acids, lipids, proteins and complex carbohydrates such as starch and cellulose (da Silva et al., 2016; Min et al., 2017). All these lead to initial radicle extension due to reversible ("elastic") growth driven by osmotic water uptake (Varier et al., 2010). The small amount of water uptake by seeds in phase II may also be due to changes in osmotic potential, due to by seed reserves degradation (da Silva et al., 2016; Varier et al., 2010).

There were wide variations in the duration of phase II among the species with *Co. erythrophyllum* having the longest duration of 230 h (data not shown). The duration of phase II for other species was 22 h (*B. speciosus*), 8 h (*E. caffra*), 12 h (*P. sativum*) and 8 h (*Cu. pepo*) (Figure 2.1). The wide variations in the duration of phase II among the species may be due to differences in the activation and duration of metabolic activities required for seed germination. Although there is little or no increase in the water uptake in phase II, this phase is associated with considerable levels of metabolic activities (da Silva et al., 2016; Reis et al., 2011). Stored reserves in seeds endosperms such as protein, fats and lipids are converted into compounds such as sugar needed for germination (Rajjou et al., 2012; Reis et al., 2011). The tissues surrounding the embryos are weakened by enzymes which are activated by gibberellin (Ogawa et al., 2003). Similarly, smaller compounds needed to supply the required energy for embryo growth are released as a result of seed reserve degradation in the endosperms (Rajjou et al., 2012; Reis et al., 2011). All these biochemical and physiological changes facilitate the protrusion of radicles (Ogawa et al., 2003), an indication of the start of phase III of the imbibition process.

Phase III, where radicle protrusion takes place, is characterised with resumed water uptake by the germinating seed (Figure 2.1). The higher water intake in this phase may be associated with activities such as respiration and mitochondrial functions of the embryo (da Silva et al., 2016; Tonini et al., 2010). Further growth of the embryo after the completion of seed germination requires cell wall loosening to allow phase III water uptake. Suitable water potentials and temperatures are of utmost importance at this stage of the germination

process. The growth potential of the embryo and the constraining force of the endosperm and testa layers determine the completion of germination.

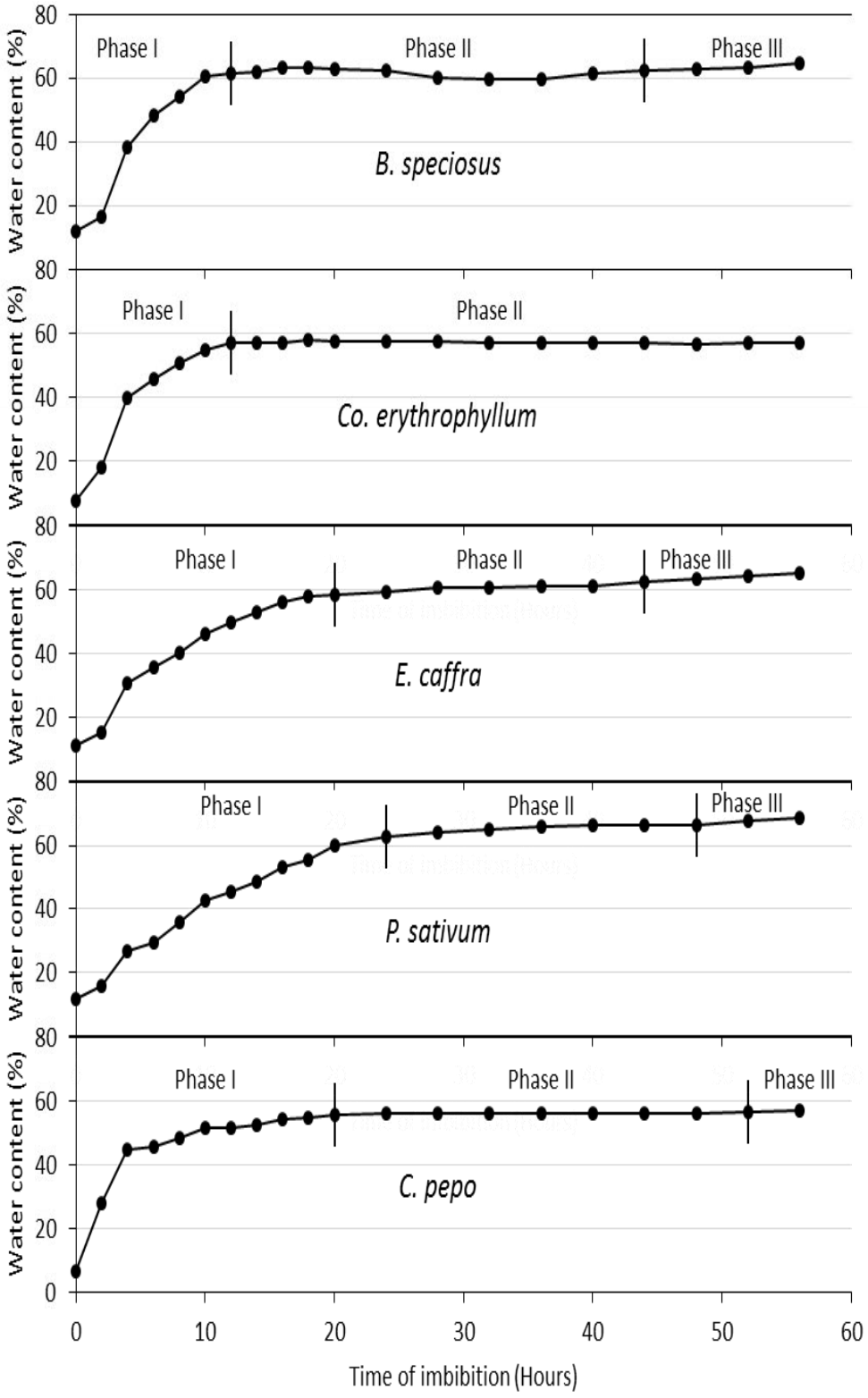

Figure 2.1 Water imbibition curves of *B. speciosus*, *P. sativum*, and *Cu. pepo*, *E. caffra* and *Co. erythrophyllum* for 56 h. *Co. erythrophyllum* transited to Phase III at 248 h (data not shown).

2.3.2 Controlled Deterioration of Seeds and Electrolyte Leakage

There were marked differences in the response of the test agricultural species and the wild species to controlled deterioration (CD). In the agricultural species, a gradual decline in germination started at 8 d for *P. sativum* with a 5.0% loss in germination (Figure 2.3). In *Cu. pepo* germination loss began later with a 6.7% loss in germination at 12 d of controlled deterioration (Figure 2.3). The relationships of the ageing time in all the species and seed germination were represented by polynomial equations (Figure 2.3). The wild species showed greater sensitivity to controlled deterioration. Germination loss began much earlier than the agricultural species with germination loss beginning at 4 d for *E. caffra, B. speciosus* and *Co. erythrophyllum.* As the ageing time increased, gradual but continuous decline in germination continued in all the species. The critical periods for rapid decrease in percentage of germination (in d) were determined to be 0.5 for *B. speciosus*, 3 for *Co. erythrophyllum*, 1 for *E. caffra,* 10 for *P. sativum* and 14 d for *Cu. pepo* (Figure 2.3).

Consistent with their greater sensitivity to controlled deterioration, total inhibition of seed germinability occurred much earlier in the wild species, at 20 d in *B. speciosus* and *E. caffra* and 16 d in *Co. erythrophyllum*. In the agricultural species, complete loss of germination occurred at 36 d for both *P. sativum* and *Cu. pepo*. Unlike the agricultural species which had their seed coats unbroken, the greater sensitivity of the wild species may be due to the process of breaking their dormancy, which involved breaking the seed coats (nicking) in *E. caffra, B. speciosus* and removal of the samara in *Co. erythrophyllum.* Factors such as seed coat permeability, physical damage and seed dormancy have been reported to predispose seeds to deterioration (Baskin and Baskin, 2014). For example, Powell and Matthews (1980) demonstrated the role of testa damage in seed quality by scarifying some pea seeds and using unscarified seeds as a control. The scarified seeds imbibed water rapidly, showed increased dead tissue and higher electrolyte leakage (which are indications of imbibition damage) and emerged poorly in the field. Other crops where similar reports exist include grain legumes and some other species such as *Phaseolus vulgaris* (Powell et al., 1986), *Glycine max* (Blackman et al., 1995), *Pisum sativum* (Wang et al., 2012), *Arabidopsis thaliana* (Maia et al., 2014), *Senna multijuga* (Rodrigues-Junior et al., 2015) and *Triticum aestivum* (Lev and Blahovec, 2018). Other factors that predispose seeds to deterioration are genetic factors, seed size, physical/physiological and seed maturity (Baskin and Baskin, 2014; Boniecka et al., 2019; Rodrigues-Junior et al., 2015). Hence, the differences observed germination/tolerance to CD among species in this study might be attributable to some of these factors.

In all the species, germination was negatively correlated with age of controlled deterioration (Figure 2.3). Seed deterioration occurs as a result of free radical attacks. Reactive oxygen species, in particular the hydroxyl radical, is a major cause of peroxidation of the unsaturated fatty acids of cell membranes (Ratajczak et al., 2015), leading to membrane damage and electrolyte leakage (Lazar et al., 2014; Ratajczak et al., 2015). Other biochemical changes during seed deterioration leading to a loss in germination include chromosome aberrations and damage to the DNA, changes in the synthesis of RNA and protein, changes in enzymes, differences in respiratory activity caused by ATP production and membrane alteration (Boniecka et al., 2019; Nagel et al., 2016; Sharma et al., 2018). Although, seeds are protected against free radicals by an array of protective enzymes such as superoxide dismutase, catalase, glutathione peroxidase and non-enzymatic compounds (glutathione, ascorbic acid, tocopherol) and other antioxidants that react with free radicals (Boniecka et al., 2019; Santos et al., 2016). Seed deterioration is cumulative; as seed CD increases, seed performance is compromised (Lazar et al., 2014). In each of the species investigated, the point at which decline in germination started may be the point at which the seeds' endogenous antioxidant became insufficient to protect the seeds. It has been reported that, when the strength of antioxidant protection becomes insufficient, oxidation occurs, resulting in free radical accumulation and subsequent deterioration of cells and seeds (Colville et al., 2012).

In general, there were steep increases in electrolyte leakage at the early stages of controlled deterioration in the wild species when compared with the agricultural species (Figure 2.4). This was followed by smaller increases as the ageing time increased. In the agricultural species, increases in electrolyte leakages at the early stages of CD were small when compared with the wild species. Electrolyte leakage was highly positively correlated (r^2= 0.956) with days of deterioration (Figure 2.4). However, a negative correlation occurred between electrolyte leakage and seed germination in all wild and agricultural species (Figure 2.4). The relatively higher leakages at the early stages of CD in the wild species may explain the earlier loss of germination in the wild species. In the agricultural species, increases in electrolyte leakages at the early stages of CD were small when compared with the wild species. Many authors have implicated cell membrane damage as being responsible for the leakage of electrolytes from seeds because the cell membrane is the first part of the cells to interact with the environments (Colville et al., 2012; Lazar et al., 2014). In the case of the wild species, physical damage was done to the seed coat in the course of breaking dormancy. Lipid peroxidation of cell membranes which occur in the course of CD of seeds is species-dependent; the differences in the rate of lipid peroxidation in the species may have

been responsible for the differences in electrolyte leakage among species and consequently differences in the rate and age of germination loss (Colville et al., 2012; Lazar et al., 2014).

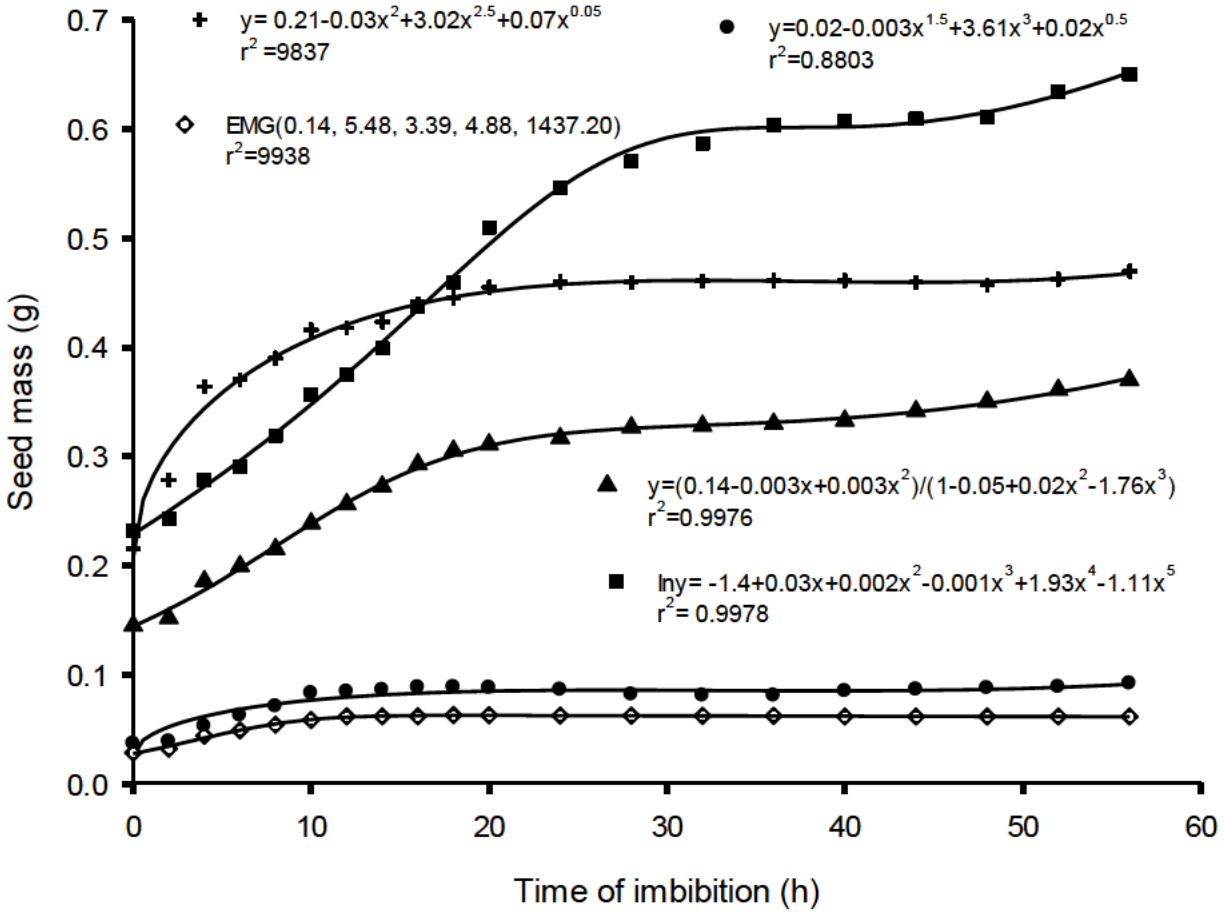

Figure 2.2 Change in seed mass of *Bolusanthus speciosus* (•), *Combretum erythrophyllum* (◊), *Erythrina caffra* (▲), *Pisum sativum* (■) and *Cucurbita pepo* (+) as the species imbibed water

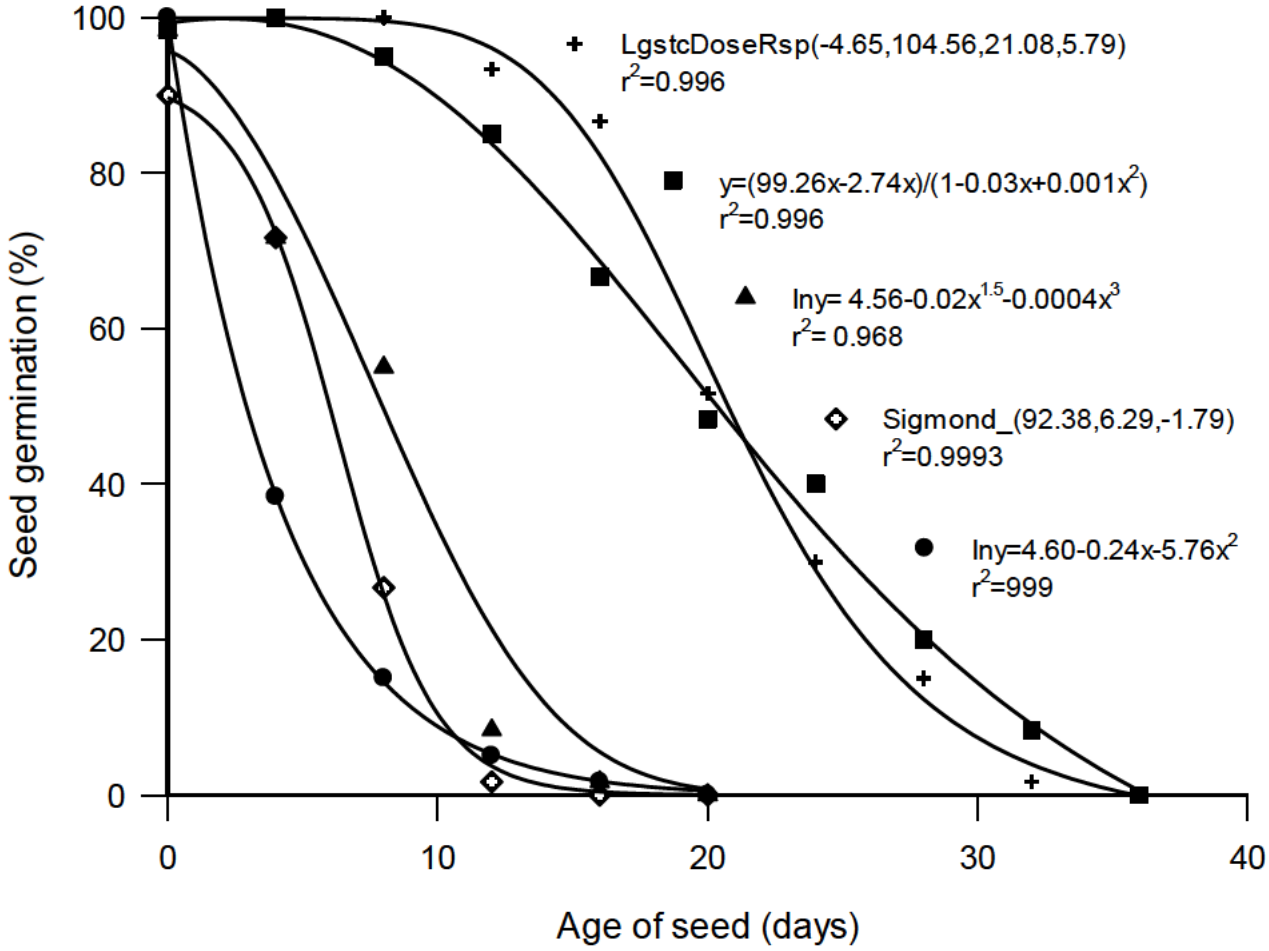

Figure 2.3 Loss of germination of *Bolusanthus speciosus* (•), *Combretum erythrophyllum* (◊), *Erythrina caffra* (▲), *Pisum sativum* (■) and *Cucurbita pepo* (+) subjected to controlled deterioration at 40°C and 100% relative humidity

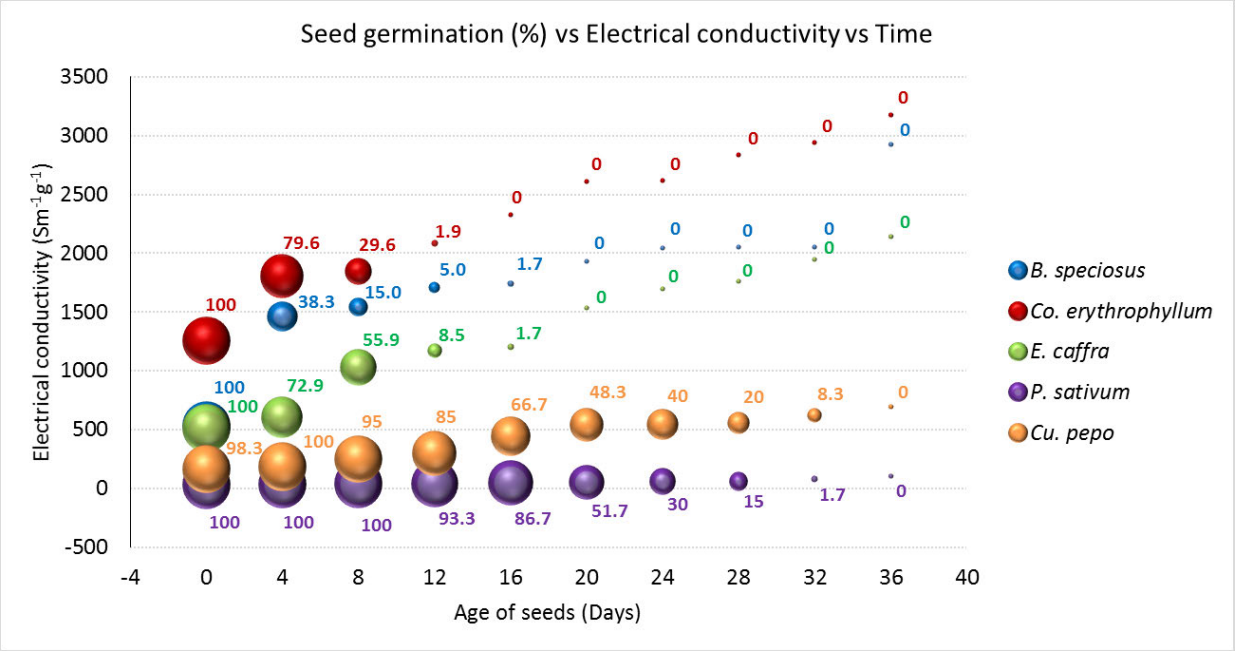

Figure 2.4 Germination and electrolyte leakages in *Pisum sativum, Cucurbita pepo, Erythrina caffra, Bolusanthus speciosus* and *Combretum erythrophyllum* subjected to controlled deterioration at 40 °C and 100% relative humidity for 36 d. Samples were taken at an interval of 4 d. The samples were subjected to both germination and electrolyte conductivity measurements.

2.3.3 Fourier-Transform Infrared Spectroscopy (FTIR)

There was a positive correlation between the transmittance (%) and the age of seeds in both the agricultural (Figure 2.5) and the wild species (Figures 2.6). When compared to the controls, there were significant reductions in the value of the transmittance at 20 d of controlled deterioration in all the test species. This is an indication of loss of seed integrity in all the species. As the ageing time increased from 20 to 32 days, the transmittance increased significantly in both *P. sativum* and *Cu. pepo*, however, in *B. speciosus* and *E. caffra* the changes were benign. This is an indication that further exposure to CD beyond 20 d was no longer significantly affecting the organic compound present in the seeds of *B. speciosus* and *E. caffra*. This observation was in agreement with the results of the germination and electrolyte leakages tests conducted viz. at 20 d of ageing there was complete loss of germination in the wild species (Figures 2.3 and 2.4).

The numbers of peaks present in the spectra vary according to species, ranging from 6 in *E. caffra* and 11 in *Cu. pepo*. The other species had *P. sativum* (8), *B. speciosus* (9), and *Co. erythrophyllum* (10). The class of compounds identified from each species are as contained in Tables 2.1 and 2.2. Generally, the amines, alkanes and the halo compound were identified as compounds that got degraded as the ageing time increased in all the test species (Tables 2.1 and 2.2). Other identified compound classes which were present in one or more of the test species include anhydrides, sulphates, isothiocyanates, carboxylic acids, alcohols, alkynes, phenols, α, β unsaturated esters, nitro and aromatic compounds (Tables 2.1 and 2.2). In most of these compounds, the transmittance is negatively correlated with the age of seeds. The progressive decline in the transmittance of the compounds present in the seeds is an indication that the concentration of the organic compounds present in the seeds reduced as a result controlled deterioration the seeds were subjected to. It has been reported that when seeds are subjected to controlled deterioration the production of ROS such as superoxide anion, hydrogen peroxide, hydroxyl radicals and singlet oxygen increases, ROS attack the organic molecules in seeds, particularly the polyunsaturated fatty acids causing some damages (McDonald, 2004). Seed deterioration involves structural, cytological, physiological, biochemical and physical changes such as lipid peroxidation, membrane disruption, and DNA damage, impairment of RNA and protein synthesis. The ROS attacks on the polyunsaturated fatty acids in cell membrane weaken the membranes making it easy for solutes leakage. Also, ageing induced ROS attacks on the organic compounds such as enzymes negatively alter their macromolecular structures and activities (Lehner et al., 2008; Mc-Donald, 2004). The lowering of enzymatic activities as a result of enzyme degradation by ROS reduces respiration in seeds which results in lower energy

(ATP) production. All these results in a reduction in assimilate supply to the germinating seeds, leading to decreased germination and weaker seedling growth (Lehner et al., 2008; Mc-Donald, 2004).

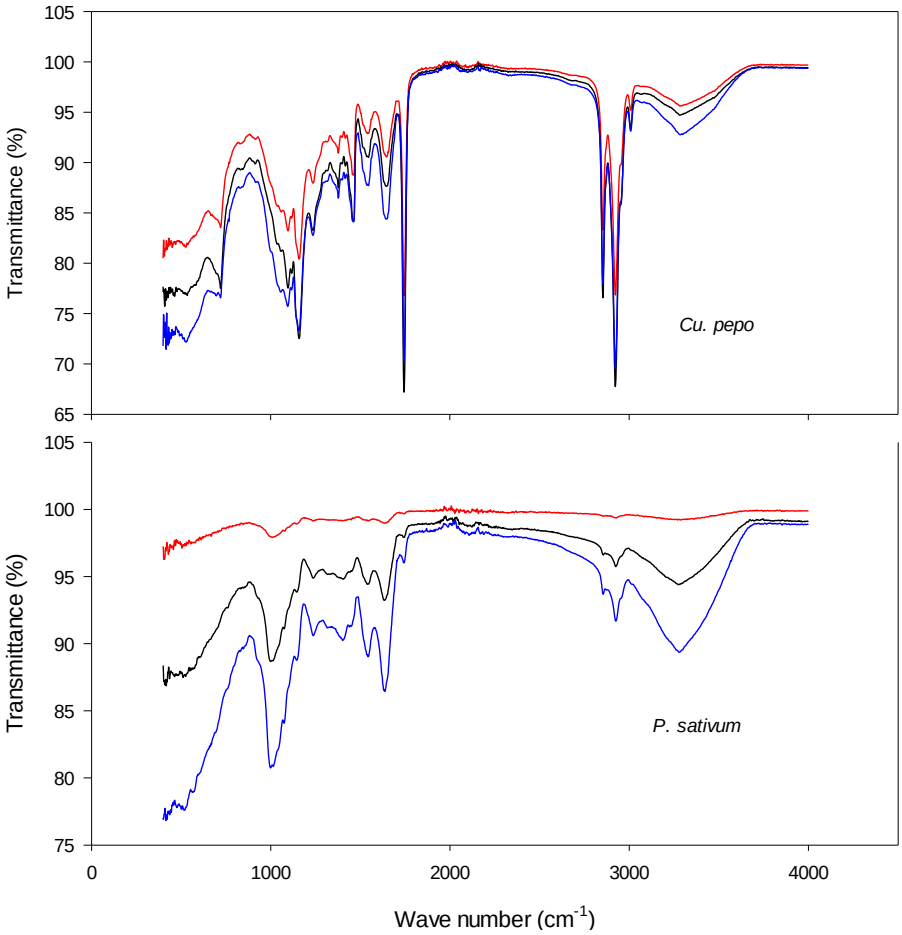

Figure 2.5 FTIR of *Cucurbita pepo* and *Pisum sativum* at 0 (--), 20 (--) and 32 (--) d of controlled deterioration. Seeds were subjected to controlled deterioration at 40 °C and 100% relative humidity. Samples were taken at an interval of 4 d intervals and the samples were subjected to both germination and electrolyte conductivity measurements.

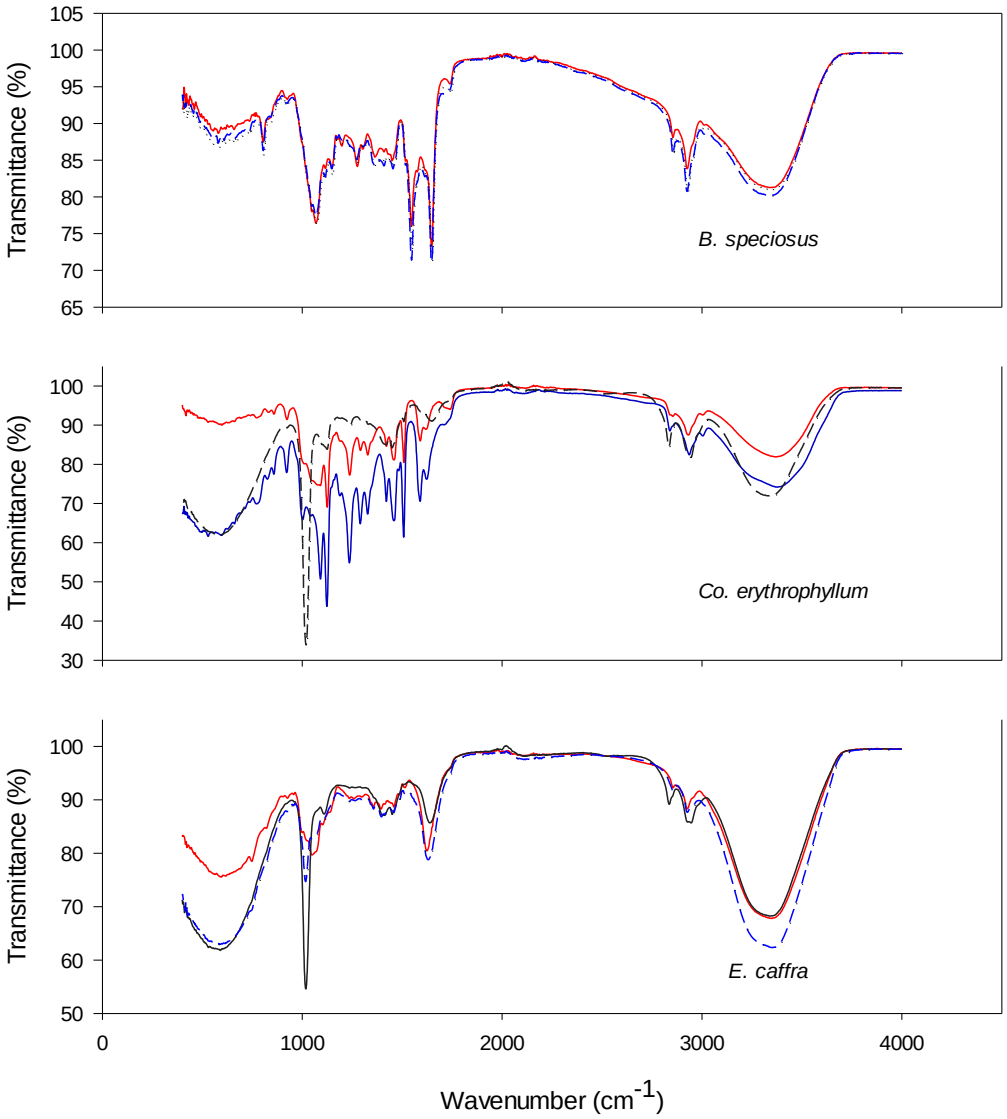

Figure 2.6 FTIR of *Bolusanthus speciosus*, *Combretum erythrophyllum* and *Erythrina caffra* at 0 (--), 20 (--) and 32 (--) d of controlled deterioration. Seeds were subjected to controlled deterioration at 40 °C and 100% relative humidity. Samples were taken at an interval of 4 d intervals and the samples were subjected to both germination and electrolyte conductivity measurements.

Table 2.1 FTIR Table and the compounds present in *Pisum sativum* and *Cucurbita pepo* at 0 (control), 20 and 32 days of controlled deterioration

WAVE NUMBER (cm^{-1})	TRANSMITTANCE (%)			GROUP	COMPOUND CLASS
	0d	20d	32d		
			P. sativum		
527	97	88	77	C-I stretching	Halo compound
1020	98	89	81	C-N stretching	Amine
1247	99	95	90	C-N stretching	Amine
1398	99	95	90	O-H bending	Carboxylic acid
1543	99	94	89	N-O stretching	Nitro compound
1628	99	93	86	N-H bending	Amine
2919	99	96	91	C-H stretching	Alkane
3300	99	94	89	C-H stretching	Alkyne
			Cu. pepo		
527	81	77	72	C-I stretching	Halo compound
718	83	77	71	C=C bending	Alkene
1099	83	77	71	C-O stretching	Secondary alcohol
1162	80	72	73	C-O stretching	Tertiary alcohol
1226	87	85	82	C-N stretching	Amine
1374	90	88	86	O-H bending	Phenol
1465	89	86	86	C-H bending	Alkane
1650	90	87	84	C=C stretching	Cyclic alkene
1734	92	67	70	C=O stretching	α,β, unsaturated ester
2919	79	68	68	C-H stretching	Alkane
3300	96	95	92	C-H stretching	Alkyne

Class and the group of compounds were identified using the IR Spectrum Table of Sigma Aldrich available at https://www.sigmaaldrich.com/technical-documents/articles/biology/ir-spectrum-table.html#ir-table-by-compound

Table 2.2 FTIR Table and the compounds present in *Bolusanthus speciosus*, *Combretum erythrophyllum* and *Erythrina caffra* at 0 (control), 20 and 32 days of controlled deterioration

WAVE NUMBER (cm^{-1})	TRANSMITTANCE (%)			GROUP	COMPOUND CLASS
	0d	20d	32d		
			B. speciosus		
591	89	88	86	C-I stretching	Halo compound
781	91	88	85	C-H bending	1,2,3 tri substituted
1056	76	76	76	CO-O-CO	anhydride
1268	84	84	84	C-N stretching	Aromatic amine
1415	86	85	84	S-O stretching	sulphate
1550	80	79	71	N-O stretching	Nitro compound
1650	83	83	71	C-H bending	Aromatic compound
2940	85	85	80	C-H stretching	Alkane
3342	81	81	80	N-H stretching	Secondary amine
			Co. erythrophyllum		
591	90	62	62	C-I stretching	Halo compound
1020	79	33	17	S=O stretching	Sulfoxide
1120	83	68	44	C-O stretching	Secondary alcohol
1242	89	77	54	C-N stretching	Amine
1501	84	81	61	N-O stretching	Nitro compound
1607	92	86	70	N-H bending	Amine
2094	99	98	97	N=C=S stretching	Isothiocyanate
2834	92	87	84	N-H stretching	Amine salt
2940	88	82	81	C-H stretching	Alkane
3321	81	80	75	N-H stretching	Secondary amine
			E. caffra		
591	75	62	62	C-I stretching	Halo compound
1035	79	61	62	C-N stretching	Amine
1416	89	88	87	O-H bending	Alcohol
1630	90	79	78	N-H bending	Amine
2940	88	86	87	C-H stretching	Alkane
3350	68	68	62	N-H stretching	Secondary amine

Class and the group of compounds were identified using the IR Spectrum Table of Sigma Aldrich, available at https://www.sigmaaldrich.com/technical-documents/articles/biology/ir-spectrum-table.html#ir-table-by-compound

It can be observed that more transmittance peaks emerged in the 21 and 32 d of controlled deterioration when compared to the control (Figures 2.5 and 2.6). This may have occurred due to the breaking of some biomolecules or compounds to smaller end products. Peroxidation of biochemical molecules as a result of ROS attacks in the course of seed deterioration leads to the formation of reactive smaller molecules such as carbonyl or nitrogen groups that easily diffuse through cell membranes, thereby increasing the concentration of the electrolyte leakages. The smaller biomolecules may also adduct between carbohydrates, proteins and nucleic acids causing intermolecular cross-linking, which may further degrade into advanced glycation end-products (Walters et al., 2010). It has also been reported that as the ageing time increase, some compounds are biochemically converted to new compounds. The consequence of all these increases in end products of lipid peroxidation is a reduction in seed quality, and hence, germination is compromised. Some volatile compounds present in seed have been implicated in seed deterioration. The concentrations of some volatile compounds are related to seed ageing; hence, they serve as biochemical markers of seed ageing for example aldehyde have been reported to non-enzymatically attack protein and DNA (Taylor et al., 1999). The peaks of the spectra were examined, while there were significant differences in the peaks of some spectra, others either overlapped or have very little differences and were not very distinctive for individual component (Tables 2.1 and 2.2). Weaker bands, which were not very distinctive for individual components, were common with the control treatments. It must be noted that while some of the compounds exhibit a change in concentration as indicated by the transmittance obtained from the FTIR spectroscopy, others did not. While some compounds are present in the control and seeds aged to 20 and 32 days, some other compounds were only identified in the aged seeds. Hence this study supports the findings of some earlier studies that differences in the viable and non-viable seeds were due to differences in the chemical composition of the seeds (Mukasa et al., 2019). However, this study further indicated that the observed differences at different ageing times could be attributed to the changes in the concentrations of the chemical compounds present in the seeds (Tables 2.1 and 2.2).

In conclusion, this study was able to provide critical relationships between the concepts of water imbibition in orthodox seeds, controlled deterioration and electrolyte leakages between and within the agricultural and wild species studied. This study has no doubt reaffirm electrolyte leakage as an indicator of seed viability and consequently seed vigour in *P. sativum* (Panobianco et al., 2007) and *Cu. pepo* (Vieira and Dutra, 2006). Also, this study recommends electrolyte leakage as an indicator of seed quality in *B. speciosus*, *Co. erythrophyllum* and *E. caffra,* which to the best of our knowledge, do not exist in literature.

The imbibition curves will no doubt assist interested farmers, seed scientists and gene banks in the course of setting imbibition time in the course of invigorating debilitated seeds of the test species. The study also recommends FTIR as a tool for monitoring deterioration of *ex-situ* stored seeds in all the test species. This study has no doubt contributes to better monitoring of seed deterioration under *ex-situ* seed conservation.

CHAPTER 3

CATHODIC WATER SEED PRIMING – A NEW APPROACH TO INVIGORATE DETERIORATED ORTHODOX SEEDS

Kayode FATOKUN: Candidate
Investigation, methodology, writing - original draft, statistical analysis and preparation of Tables and Figures

Norman W. PAMMENTER: Supervisor
Supervision and funding acquisition

This chapter contains the report of 'the preliminary study, the success of which gave the necessary encouragement for the main study reported in chapters 4, 5 and 6.

Abstract

A greenhouse pot experiment was conducted to investigate the effects of cathodic water (an electrolysed form of calcium magnesium solution) on the emergence and growth of deteriorated seeds of pea and pumpkin. Treatments that were investigated alongside that of cathodic water were; cathodic water with pH adjusted to 7, calcium magnesium solution, calcium magnesium solution (with pH adjusted to 11), ascorbic acid and distilled water. Electrolyte leakages from both the fresh and deteriorated seeds were also investigated. The study was conducted by subjecting seeds of the test species to controlled deterioration in an oven at 40°C for 32 days. The deteriorated seeds were thereafter invigorated with cathodic water and other treatments. Fresh seeds of both species served as the control. The results of invigorating the seeds with cathodic water lead to significant ($p<0.05$) emergence in both pea and pumpkin. Even though all the invigoration treatments lead to an improvement in both emergence and growth in both species, seeds treated with cathodic water generally performed best when compared with other treatments. More improvement in emergence and growth was also observed with pumpkin when compared with pea. Electrolyte leakage from the deteriorated seeds was significantly higher than that of fresh seeds in both species. While the successful invigoration of the deteriorated seeds which lead to the emergence and subsequent growth may be linked to cell repairs and other biochemical changes in the invigorated seeds (not investigated in this study), the positive effects on the growth and biomass of the plant may be associated with improvement in chlorophyll content and chlorophyll fluorescence in pumpkin, and an increase in chlorophyll content in pea. This study provides good evidence that cathodic water can invigorate deteriorated orthodox seeds, and may therefore be useful in conserving Germplasm.

Keywords: cathodic water, controlled deterioration, emergence, growth, invigoration

3.1 INTRODUCTION

Seeds are the genetic resource by which most higher-plants are propagated. On the basis of their desiccation tolerance, seeds are classed as either recalcitrant or orthodox. While the recalcitrant seeds are desiccation sensitive and can not be stored for a long time; orthodox seeds are tolerant of desiccation and can be stored for a long time. Orthodox seeds genetic resources can be conserved via conventional gene banking. However, during long-term storage, orthodox seeds deteriorate even under very good conditions the best that can be done is to lower the rate of deterioration (Tweddle et al., 2003; Walters et al., 2005). Deterioration of seeds during storage has been linked to the production of reactive oxygen species (ROS). Other factors that contribute to seed deterioration, also known as “seed ageing”, are relative humidity and temperature of the storage environment, genetics, mechanical damage, seed water content, presence of microbiome and seed maturity (McDonald, 2004; Yari et al., 2011).

The occurrence and severity of the observed effects of ageing on germination and subsequent seedling growth increase with ageing time and the level of applied stress (Walters et al., 2005). Loss in seed vigour is associated with loss of membrane integrity (Murthy et al., 2003; Smith and Berjak, 2017). In particular, membrane damage is ROS-mediated which may lead to electrolyte leakage. Hence, electrolyte leakage has been recommended as an indicator of seed deterioration and physiological stress in some species (Bermudez and Pignata, 2011; Valavanidis et al., 2006). Other factors linked with ageing in seeds are degradation and inactivation of enzymes due to changes in their macromolecular structures (Lehner et al., 2008; Mc-Donald, 2004). The reduction in enzymatic activities as a result of enzyme degradation has been reported to lower respiratory activities which in turn lowers the energy (ATP) and assimilates supply of the germinating seeds, which consequently results in decreased germination and weaker seedling growth (Lehner et al., 2008; Mc-Donald, 2004).

It has been proposed that seed priming (a seed invigoration technique) can be used to improve seed vigour, and the rate and uniformity of seedling emergence, which will ultimately improve crop yields. Many techniques have been employed to prime seeds. The first step often involves hydrating seeds in solutions to allow sufficient imbibition to enable the early events in the germination process to occur, but not enough to permit radicle emergence. Solutions that are used for seed priming include water (hydropriming), osmotically active solutions such as polyethylene glycol (osmopriming) or salts solutions (halopriming). Other seed priming techniques include placing seeds between saturated jute

mat layers (matripriming) and alternate soaking of seeds in tap water and drying before sowing (hardening) (Ghassemi-Golezani et al., 2008; Maiti et al., 2013). Seed priming has been reported to have positive effects on the germination and growth of some vegetables, floriculture and some field crops (Farooq et al., 2008; Toklu et al., 2015; Yari et al., 2011).

Plants have been reported to have internal mechanisms to counteract the damaging effect of ROS bursts, such systems include antioxidative systems, which are composed of metabolites, such as ascorbate, glutathione, tocopherol, and enzymatic scavengers, such as superoxide dismutase (SOD), peroxidases, and catalases (Berjak et al., 2011). ROS would normally be quenched by the endogenous antioxidant system. However, at a high level of stress, the ability of protective mechanisms are insufficient to neutralize the damage caused by ROS production within the seed. An approach to solving this problem is to supply exogenous antioxidants such as ascorbic acid; however, success has been variable and some anti-oxidants are cytotoxic at high concentration (Berjak et al., 2011; Lehner et al., 2008). This study proposes the use of cathodic water in seed priming. Cathodic water, developed in our laboratory, is the cathodic fraction of an electrolysed, dilute ionic solution of calcium and magnesium chloride (Berjak, 1978; Pammenter et al., 1974). Cathodic water has strong reducing power and its use in seed priming obviates the need for exogenously-supplied potentially toxic chemical antioxidants. It was hypothesised that cathodic water might be able to counteract the potential damage caused by ROS and ultimately invigorate seeds that have lost vigour.

The intuition leading to the discovery of cathodic water has been substantiated with remarkable successes. For example, cathodic protection was used as a medium during explant excision, as the solvent for cryoprotectant solutions, and as the medium for post-cryo thawing and rehydration in the cryopreservation of *Strychnos gerrardii* (Berjak et al., 2011). Prior to that study, even though a lot of efforts have been made, no one has produced a viable plant from cryopreserved axes of *Strychnos gerrardii* (Berjak et al., 2011). In this study, twenty-two year old, gene banked seeds of pumpkin used in the previous experiment by a colleague was mimicked by subjecting seeds of the test species (pea and pumpkin) to controlled deterioration at 40°C and 100 % relative humidity to allow for gradual seed deterioration and to avoid accelerated death of seed embryos. While the ameliorative effects of cathodic water in the previous study focused on the germination of the test species (Gondwe et al., 2016), this is the first study that examined the effects of cathodic water seed priming on seedling emergence and plant growth of orthodox species.

Although the aim of this study is to bring the concept of electrochemistry into plant germplasm conservation via the use of cathodic water, this study also examined the influence of some other treatments which include cathodic water with pH adjusted to 7, calcium magnesium solution, calcium magnesium solution with pH adjusted to 11, ascorbic acid and distilled water and compared them with that of cathodic water. The comparisons were done in terms of seedling emergence, plant mortality, and plant growth.

3.2 METHODOLOGY

3.2.1 Study Area

Seed germination was done in a growth room set at 16 h dark / 8 h light (52 µmol m^{-2} s^{-1}) photoperiod. The pot experiment was done in a greenhouse, with an average temperature of 23.5°C and average relative humidity of 67%, at GPS *coordinates*: S 29°48'59.8'' E 30°56'37.4''. Seeds used were purchased a year before the trial from Grovida seeds, a local seed company based in Durban. The seeds were stored at -5°C.

3.2.2 Controlled deterioration of seeds

The seeds of each species were examined for size, and seeds of similar sizes were used for this study. After the examination of seed sizes, the initial water contents of the test species were determined gravimetrically. The water contents for both species were subsequently raised to 14% in a vapour chamber. The seeds were then sealed in airtight glass jars and kept in an oven at 40°C. Different glass jars were used for each of the species. Samples (25 seeds x 4 replicate) were taken at 4 d interval and sterilized for 10 min in 1% aqueous sodium hypochlorite. The seeds were germinated on water agar. Sampling was done until almost complete loss of germination vigour in both species (32 d).

3.2.3 Seed Electrolyte Leakage

Electrolyte leakage (S m^{-1} g^{-1}) was measured for both the deteriorated seeds and the control (fresh seeds). Three seeds (1 g) were immersed in 50 ml of distilled water in glass tubes placed in a water bath for 24 hours at 25°C. Thereafter, the leachate was measured for electrical conductivity using a CM 100-2 multi-cell conductivity meter (Reid and Associates, South Africa) (Hampton and Tekrony, 1995)

3.2.4 Preparation of Calcium Magnesium (CaMg) Solution and Cathodic Water

A solution containing 1 µM $CaCl_2$ and 1 µM $MgCl_2$ known as calcium magnesium (CaMg) solution was prepared, autoclaved and stored at -5 °C until needed. The pH of CaMg solution was ~ 7.

Cathodic water was prepared by electrolysing the CaMg solution (Mycock, 1999). Two 200 ml glass beakers were filled with water containing CaMg solution and platinum electrodes were immersed in the solution, the anode in one beaker and the cathode in the other. To form a complete circuit, an agar-based salt bridge was inserted to connect the two beakers. The CaMg solution was electrolysed by the provision of a 60 V potential difference using a Bio-Rad Powerpac (BioRad, Hercules, California, USA) at 400 mA for 1 h at room temperature. The electrolysis yielded anodic (oxidizing) water at with a pH of *c*. 2.4, and cathodic (reducing) water with a pH of *c*. 11.2. The anodic water was discarded while the cathodic water was used within one hour of preparation.

3.2.5 Seed Priming with Cathodic Water and Other Treatments

Hydration of the controlled deteriorated (CD) seeds was done in between 20 layers of single-ply paper towel with 50 ml of the priming solutions for 24 h. Domestic aluminium foil was folded around the paper towels and sealed with cellophane tape to allow hydration of the seeds to take place. After hydration, the seeds were dried back to the original mass in the open air (on tables in the seed laboratory) for 7 days. The seeds were subsequently kept in airtight glass jars and stored in a refrigerator at 4°C until use.

Controlled deteriorated seeds were hydrated in six priming solutions treatments; cathodic water (CW), cathodic water with pH adjusted to 7 (CW pH=7), calcium magnesium solution (CaMg), calcium magnesium solution with pH adjusted to 11 (CaMg pH=11), ascorbic acid (As) and distilled water (DW). Unprimed fresh (FSC) and aged seeds (ASC) served as the controls.

3.2.6 Plant Growth

The growth study was conducted as a greenhouse pot experiment. Eight hundred grams of Grovida potting mix was weighed into each pot of 2 litter size. In each pot, five seeds from each treatment were sown. Nutrients were supplied to the plants using multi-feed fertilizer (at 1 g multi-feed fertilizer 100 ml H_2O^{-1}). Watering was done based on observation. Plants were thinned to 2 and 1 plants/ pot at 4 and 6 weeks after planting respectively. The plants were grown for 14 weeks. Pots were arranged in the greenhouse in a completely randomised design (CRD)

3.2.7 Data Collection

Seedling emergence was observed daily and recorded. Time to the first seedling emergence in each pot was also observed. Dead plants were counted and recorded as mortalities

The chlorophyll ($C_{55}H_{72}O_5N_4Mg$) content was measured with SPAD chlorophyll content meter on three fully expanded, non-senescing leaves of similar physiological maturity (the

third, fourth and fifth leaf from the terminal bud). Three measurements were taken per leaf and values expressed as chlorophyll content index (CCI). Chlorophyll content index is a measure of the transmission (T) ratio of light at wavelengths of 653 nm to 931 nm light in the transmission spectrum of a green leaf (CCI = %T931nm/%T653nm).

Chlorophyll fluorescence (Fv/Fm), the ratio of the variable (Fv) to maximum fluorescence (Fm), which is a measure of potential photochemical efficiency of photosystem II (PSII) was done at eight weeks after planting. The measurement was done using a Li-Cor 6400XT portable photosynthesis measuring system (Li-Cor, Lincoln, NE). Three measurements were taken on the third leaf from the terminal bud across all treatments and replicates (n=12 per treatment). All leaves were fully expanded and non-senescing. Measurements were taken after the plants were dark-adapted for 40 min (Sershen et al., 2010). The stem girth was measured with vernier calipers a day preceding harvesting 5 cm above soil level.

At harvesting, the plants were carefully pulled out of the potting mix to avoid damage to the roots, washed and separated into root, stem, and leaves. The root and stem lengths were measured, and then plant parts dried at 70°C until constant mass (4 d) and then their mass determined. All the leaves were counted for all treatments and replicates and the control. Individual leaf area (cm^2) was measured using a leaf area meter (Licor, CI-202 Area Meter, Lincoln, Nebraska, USA). Measurement was done across all treatments and the control (Tiwari et al. 2006).

3.2.8 Data Analyses

The data collected were subjected to analysis of variance (ANOVA) procedure using GenStat Release 18.1 (PC/Windows Vista) (VSN International Ltd., 2009). The means of the treatments were separated for the least significant difference at 5% ($LSD_{0.05}$). The data for seed ageing were further subjected to correlation and regression analyses. Thus, mathematical functions expressing correlations and regression relationships between the number of days seeds were in the oven and the germination percentage of seeds were obtained using the curve fitting programme of TableCurve 2D v5.01.01 (Systat Software Inc., San Jose, CA, USA, 2002).

3.3 RESULTS

Controlled deterioration at 40°C and 100% relative humidity was done to allow for gradual seed deterioration and to avoid accelerated death of seed embryos. At 4 d of ageing both species maintained 100% germination which was similar to the initial germination obtained at the start of the experiment (Figure 3.1). As the ageing time increased, gradual but

continuous decline in germination continued in both species. Controlled deterioration was terminated at 32 d as deterioration beyond 32 d would have killed the seeds. The percentage loss in germination at 32 d of controlled deterioration was 92% for pea and 98% for pumpkin. A measure of the electrolyte leakages from the CD and fresh seed indicated that there was a significant leakage of electrolyte in the CD seeds when compared with the fresh seeds in both species (Figure 3.2). In both species, the fresh seed control had 100% emergence which is significantly ($P>0.05$) higher than any of the controlled deteriorated seed treatments (CW, CW (pH=7), CaMg, CaMg (pH=11), DW and As) (Table 3.1). Although the initial germination of pea and pumpkin were 8.34 and 1.67% respectively; mean emergence of the test species were 21.0% (pea) and 28.9% (pumpkin).

Of the seedlings derived from the controlled deteriorated seeds treatments, mortality occurred in all the treatments except CW treatments. All plants from the fresh seed control survived to maturity (Table 3.1). CW and CW (pH=7) treatments with 32.50 % emergence had the best performance among the controlled deteriorated seed treatments (Table 3.1). However, there was significant ($p<0.05$) delay in seedling emergence from all the deteriorated seed treatments when compared with the fresh seed control. While the first emergence in the fresh seed treatment occurred in both species at 5 d after planting, the first seedling emergence occurred at 7 d (pea) and 14 d (pumpkin) after planting for the CD seed treatments (Table 3.1). There was no emergence in the aged seeds control treatment; hence, the treatment was ignored in further study and analyses.

Cathodic water treated seeds had the best growth parameters such as total biomass, root length, and stem girth and shoot lengths among the CD seed treatments (Table 3.2). In pumpkin, cathodic water treatment actually performed better than fresh seed control treatment in terms of shoot weight, root weight, stem girth and leaf area (Table 3.3). Invigoration with CW increased leaf size, number, and chlorophyll content with some of the values being significant ($p<0.05$) (Table 3.3). In pea, the chlorophyll fluorescence for all treatments except the control was lower than $0.75 \leq Fv/Fm \leq 0.86$, which is the established range for stress-free plants (Table 3.4). Similarly, in pumpkin, all treatments, including the control, were below the range. CW with 0.749 may be considered as being the only treatment within the range (Table 3.4).

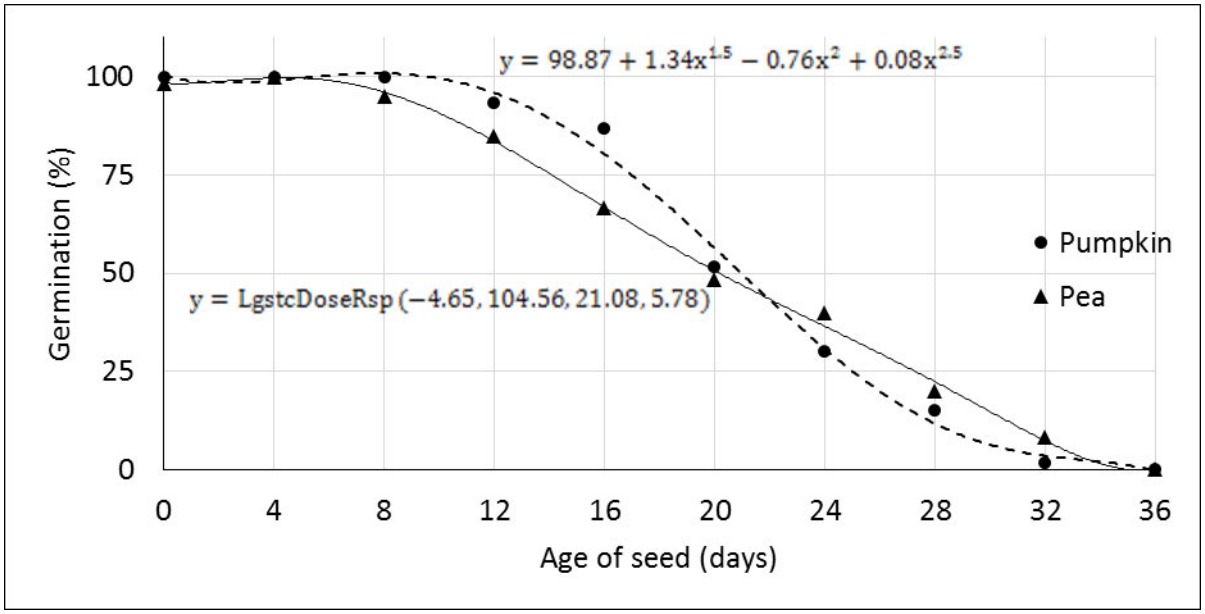

Figure 3.1 Ageing curve for pea and pumpkin at 40 °C and 100% relative humidity with sampling done at 4 d intervals for a period of 36 d

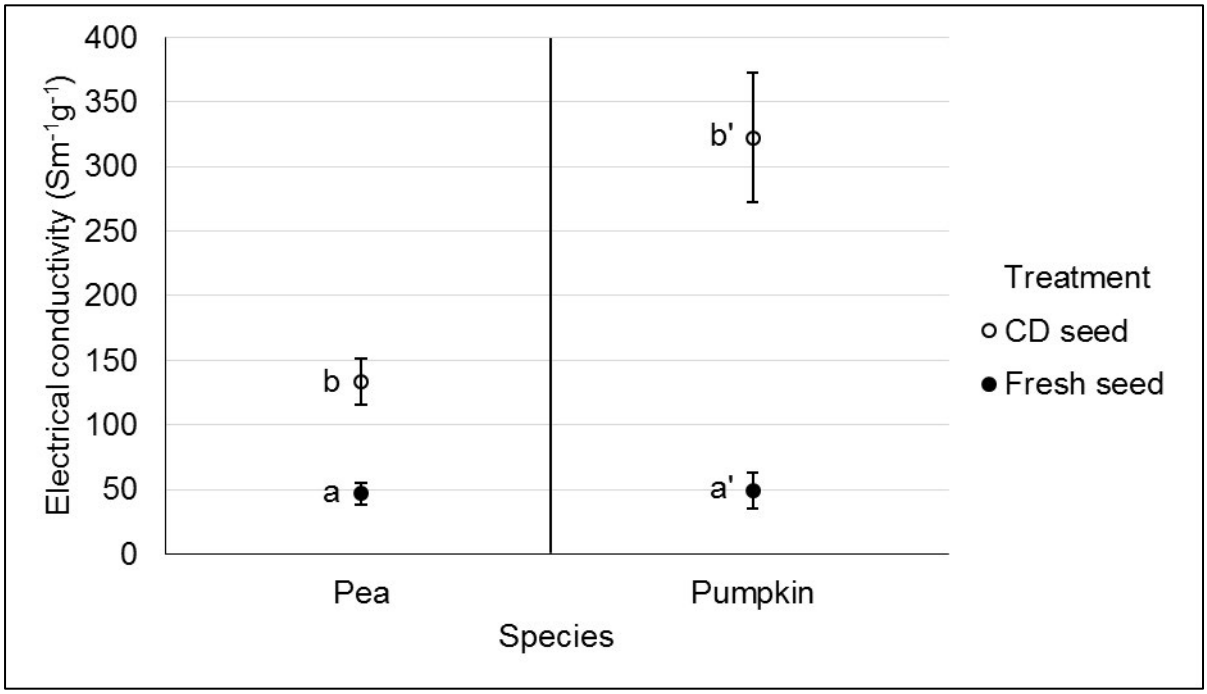

Figure 3.2 Electrolyte leakages of the fresh (•) and controlled deteriorated (o) seeds of pea and pumpkin measured after 32 d of controlled deterioration at 40°C and 100% relative humidity. Bars are mean ± 2se with letters that are not similar, indicating that the electrolyte leakage of the fresh and controlled deteriorated seeds are significantly ($p<0.05$) different

Table 3.1 Emergence and mortality of control deteriorated seeds of pea and pumpkin treated with cathodic water and other treatments after 18 days of seeding

	*FD Emergence		Emergence (%)		Mortality (%)	
Treatments	Pumpkin	Pea	Pumpkin	Pea	Pumpkin	Pea
Fresh seed control	5.0^{d}	5.0^{cd}	100.0^{e}	100.0^{e}	0.0^{a}	0.0^{a}
Cathodic water	14.0^{c}	7.0^{abc}	32.5^{d}	30.0^{d}	0.0^{a}	0.0^{a}
Cathodic water (pH=7)	15.0^{bc}	7.0^{abc}	30.0^{cd}	27.5^{cd}	6.3^{ab}	8.3^{a}
CaMg solution	18.0^{ab}	7.0^{abc}	20.0^{b}	22.5^{cd}	33.3^{c}	45.8^{c}
Camg (pH=11)	18.0^{ab}	8.0^{ab}	22.5^{bc}	20.0^{bc}	41.6^{c}	37.5^{bc}
Deionized water	17.0^{abc}	5.0^{cd}	22.5^{bc}	22.5^{cd}	29.1^{c}	19.2^{ab}
Ascorbic acid	19.0^{a}	8.0^{ab}	17.5^{b}	12.5^{b}	25.0^{bc}	37.5^{bc}
Mean	15.1	6.6	30.6	29.4	19.3	21.2
Lsd $_{0.5}$	-	-	8.94	7.88	13.56	13.09

*FD emergence (First Day of Emergence) is the number of days before the emergence of the first seedling in each treatment. Figures along the same column with different letters are significantly ($p<0.05$) different.

Table 3.2 Effect of cathodic water treatment on the root length, stem girth shoot length and shoot/root ratio of pea and pumpkin at 14 weeks after planting

Treatments	Pumpkin			Pea		
	Root length (cm)	Stem girth (mm)	Shoot length (cm)	Root length (cm)	Stem girth (mm)	Shoot length (cm)
Fresh seed control	28.0^{cd}	9.5^{ab}	22.5^{c}	31.7^{b}	2.4^{c}	35.7^{c}
Cathodic water	25.3^{bcd}	12.4^{b}	15.3^{abc}	26.5^{ab}	2.2^{bc}	35.0^{bc}
Cathodic water (pH=7)	30.0^{d}	9.7^{ab}	17.5^{bc}	24.7^{ab}	2.1^{bc}	33.3^{abc}
CaMg solution	17.0^{a}	7.9^{a}	7.0^{a}	24.3^{ab}	2.1^{bc}	34.3^{bc}
CaMg (pH=11)	20.0^{ab}	6.5^{a}	11.0^{ab}	22.0^{ab}	2.4^{c}	27.0^{ab}
Deionized water	21.3^{abc}	8.5^{a}	17.3^{bc}	19.7^{ab}	1.9^{b}	27.5^{abc}
Ascorbic acid	17.0^{a}	7.1^{a}	10^{ab}	18.0^{a}	1.37^{a}	25.5^{a}
Mean	22.7	8.8	14.38	23.8	2.1	31.2
$Lsd_{0.05}$	4.369	2.342	5.761	7.92	0.267	5.455

Figures along the same column with different letters are significantly ($p<0.05$) different. Cathodic water (pH=7) is cathodic water with its pH adjusted to 7, CaMg (pH=11) is CaMg solution with its pH adjusted to 11.

Table 3.3 Effect of cathodic water seed treatment on the root mass, stem mass, leaves mass, and total biomass of pea and pumpkin at 14 weeks after planting

Treatments	Root	Stem	Leaf	Shoot	Total biomass (g)	S/R ratio
	mass (g)					
	Pumpkin					
Fresh seed control	0.14^{a}	0.35^{ab}	1.27^{ab}	1.62^{ab}	1.76^{a}	12.06^{b}
Cathodic water	0.43^{b}	0.81^{c}	2.65^{d}	3.46^{c}	3.89^{c}	8.58^{ab}
Cathodic water (pH=7)	0.48^{b}	0.76^{c}	2.50^{cd}	3.26^{c}	3.74^{bc}	7.07^{a}
CaMg solution	0.29^{ab}	0.43^{ab}	1.76^{bcd}	2.20^{bc}	2.48^{abc}	7.65^{a}
CaMg (pH=11)	0.21^{a}	0.54^{c}	1.57^{abc}	2.11^{abc}	2.32^{ab}	10.05^{ab}
Deionized water	0.20^{a}	0.35^{ab}	0.94^{ab}	1.29^{ab}	1.48^{a}	6.98^{a}
Ascorbic acid	0.13^{a}	0.18^{a}	0.61^{a}	0.79^{a}	0.92^{a}	6.22^{a}
Mean	0.27	0.49	1.62	2.11	2.37	8.37
$Lsd_{0.05}$	0.19	0.31	0.99	1.26	1.44	2.50
	Pea					
Control	1.42^{c}	1.47^{c}	0.46^{b}	2.89^{c}	3.35^{c}	7.05^{abc}
Cathodic water	1.21^{c}	1.48^{c}	0.29^{ab}	2.68^{c}	2.98^{c}	9.67^{bc}
Cathodic water(pH=7)	1.35^{c}	1.46^{c}	0.28^{a}	2.82^{c}	3.10^{c}	9.96^{c}
CaMg solution	0.57^{ab}	0.73^{ab}	0.33^{ab}	1.29^{ab}	1.62^{b}	4.33^{a}
CaMg (pH=11)	0.84^{b}	0.91^{b}	0.30^{a}	1.75^{b}	2.05^{ab}	5.94^{abc}
Deionized water	0.55^{ab}	0.49^{a}	0.15^{a}	1.04^{ab}	1.19^{a}	7.29^{abc}
Ascorbic acid	0.43^{a}	0.52^{a}	0.21^{a}	0.95^{a}	1.16^{a}	4.49^{ab}
Mean	0.91	1.01	0.29	1.92	2.21	6.96
$Lsd_{0.05}$	0.35	0.37	0.16	0.69	0.79	3.38

Figures along the same column (each species) with different letters are significantly ($p<0.05$) different.

Table 3.4 Effect of cathodic water seed treatment on the number of leaves, leaves areas, chlorophyll content and chlorophyll fluorescence of pea and pumpkin at 14 weeks after planting

Treatments	Number of leaves	Leaves Area (cm^2)	Chlorophyll content (CCI)	Chlorophyll fluorescence (Fv/Fm)
			Pumpkin	
Fresh seed control	8.0^{ab}	1025.0^{c}	50.30^{b}	0.738^{bc}
Cathodic water	11.7^{d}	1295.0^{d}	50.63^{b}	0.749^{c}
Cathodic water (pH= 7)	10.0^{bcd}	1225.0^{d}	50.77^{b}	0.681^{abc}
CaMg solution	11.0^{cd}	336.0^{a}	44.10^{a}	0.740^{bc}
CaMg (pH= 11)	9.0^{bc}	292.0^{a}	44.36^{a}	0.612^{a}
Deionized water	6.7^{a}	710.0^{b}	41.13^{a}	0.647^{ab}
Ascorbic acid	8.0^{ab}	227.0^{a}	42.80^{a}	0.670^{abc}
Mean	9.2	730.0	46.30	0.691
Lsd (5%)	1.9	141.6	3.92	0.091
			Pea	
Fresh seed control	120.0^{b}	628.0^{b}	68.80^{c}	0.768^{d}
Cathodic water	105.3^{b}	573.0^{b}	68.80^{c}	0.702^{c}
Cathodic water (pH= 7)	105.3^{b}	573.0^{b}	68.80^{c}	0.702^{c}
CaMg solution	80.5^{a}	422.0^{a}	48.60^{a}	0.638^{a}
CaMg (pH= 11)	69.0^{a}	412.0^{a}	51.90^{ab}	0.686^{bc}
Deionized water	59.0^{a}	321.0^{a}	55.17^{b}	0.658^{ab}
Ascorbic acid	66.0^{a}	359.0^{a}	54.17^{b}	0.638^{a}
Mean	86.3	477.00	57.45	0.685
Lsd (5%)	21.95	111.90	4.73	0.032

3.4 DISCUSSION

The intuition which led to harnessing the reducing power of cathodic water in plant germplasm conservation has been substantiated with remarkable successes in some recalcitrant seeded species and the germination of some orthodox seeds (Berjak et al., 2011; Gondwe et al., 2016). Although seedling emergence was significantly ($p<0.05$) delayed in all the aged seeds treatments despite seed priming with any of the priming solution (Table 3.1). However, it is noteworthy that seeds that were subjected to controlled deterioration for 32 days which recorded germinations of 8.34 (pea), 1.67 % (pumpkin) and 0% emergence in both species without priming achieved 30.0 (pea) and 32.5% (pumpkin)

emergence after CW treatment. Other treatments also achieved some level of emergence, but none of them (with the exception of cathodic water (pH=7) was able to perform as much as cathodic water treatments (Table 3.1).

Although emergence was significantly delayed in the primed controlled deteriorated seed treatments when compared with the fresh seed control. It is clear from the results obtained in this study that CW enhanced earlier emergence when compared with CD seeds treated with other priming solution. With the exception of fresh seed treatment and CW primed treatments, seed deterioration caused post-emergence mortality in both species (Table 3.1). The occurrence of post-emergence mortality in this study may be due to hang over effects of controlled deterioration of seeds used (Sershen et al., 2010). It seems likely that the absence of mortality in seeds treated with CW was due to the strong reducing power of cathodic water on ROS. Physiological activities leading to seed repairs occur, particularly in phase 2 of seed priming (McDonald, 2004), and include DNA repairs, increase in enzymatic activities, cell membrane repairs and protein refolding. Generally, the most significant improvements in emergence both in total and early seedling emergence and the most significant reductions in mortality occurred in seeds primed with cathodic water treatment and CW (pH= 7). These results are, consistent with other studies on the reinvigoration orthodox seeds (Amanpour-Balaneji and Sedghi, 2012; Berjak et al., 2011; Ramamurthy et al., 2015).

The general delay in emergence observed in all the aged seeds treatments may be linked to loss of seed vigour and induction of secondary dormancy in the seeds (Baskin and Baskin, 2004; Maiti et al., 2013). As stated above, after deterioration for 32 d there was no seedling emergence in either test species even though some germination occurred (Table 3.1). The lack of seedling emergence in the aged seed control may be due to lack of sufficient vigour to cause seedling emergence in the treatment and probably complete death of some seeds embryos. Plants have been reported to have internal mechanisms to counteract the damaging effect of ROS bursts. These include antioxidant systems comprising metabolites, such as ascorbate, glutathione, tocopherol, and enzymatic scavengers, such as superoxide dismutase (SOD), peroxidases, and catalases (Berjak et al., 2011). However, at a high level of stress, the power of the internal protective mechanism of the seeds may not be able to neutralise the damaging effects of ROS activities. A delay in emergence as a result of the loss of seed vigour has been reported in maize (Ghassemi-Golezani et al., 2010) and wheat (Yari et al., 2011).

Other reports in this study established a strong correlation between seed germination and duration of controlled deterioration of pea and pumpkin (Figure 3.1). The decline in

germination as a result of controlled deterioration of seed may be due to the production of reactive oxygen species. The ROS may have resulted in seed damage and consequently, reduction in germination due to ROS attack. Reduction in germination may also be occasioned by the death of some seed embryo in the course of controlled deterioration of seed (Tian et al., 2006). It has been reported that many systems within seeds tissues become deteriorated in the process of ageing (Clerkx et al., 2003; Tarquis and Bradford, 1992). Electrolyte leakage in the CD seeds of both pea and pumpkin were significantly higher than those of fresh seeds (Figure 3.2). The significant leakage of electrolyte in the deteriorated seed and subsequent seed damage may have been influenced by reactive oxygen species (ROS) (Kaewnaree et al., 2011). Hence, loss or reduction in germination in the deteriorated seeds may be attributed to electrolyte leakage from the deteriorated seeds of the test species. Seed germination has been reported to have strong inverse correlation with electrolyte leakage. For example, strong inverse correlation has been reported between loss of seed germination and electrolyte leakage in *Diplotaxis tenuifolia* and *D. erucoides* (Lazar et al., 2014) and wheat (Goodarzian et al., 2014). Hence, electrolyte leakage has been recommended as a measure of seed vigour for pea and pumpkin (ISTA, 1995).

After growth for 14 w the total biomass, root and shoot length and stem girth of CW invigorated CD of pumpkin and pea were not significantly different from control (Tables 3.2). While other treatments permitted some successful germination, their growth was usually significantly poorer than that of the controls. Surprisingly, deteriorated seeds of pumpkin invigorated with cathodic water produced seedling that grew better than fresh seeds, possibly because of a poor initial vigour of the seeds used in this study. Although the seeds were stored at 5°C, the seeds may have lost some vigour due to natural ageing (Walters et al., 2005). Some measure of ageing in the fresh seeds was suggested by the photochemical efficiency of photosystem II (Fv/Fm) of the plants derived from the fresh seeds which was 0.75 rather than values over 8 that occur in healthy plant (Table 3.4) (Bjorkman and Demming, 1987). Cathodic water treatment may have also favoured the production of chlorophyll, in both test species, chlorophyll content of the plants derived from CW and CW (pH=7) were significantly higher than those of plants derived from other CD treatments. The improvement in chlorophyll content may have resulted in higher photosynthetic efficiency of the plants which is crucial to growth and dry matter accumulation in plants. Chlorophyll plays a role in light energy harvest by plants (Shu et al., 2013). Also, larger leaf areas as observed in CW treatments may have provided greater surface area for photosynthetic activities, and subsequent partitioning of photoassimilates in favour of vegetative growth (Fatokun and Zharare, 2015; Gerardeaux et al., 2009). Cathodic water may have reduced the oxidative stress damage in the leaves of plants derived from CW treatments (Demisrkaya et al., 2010;

Ramamurthy et al., 2015) and probably reduction in the controlled deterioration induced hang over effects on the plants derived from CW and CW (pH=7) treatments (Sershen et al., 2010).

Generally, the performance of the 6 CD treatments is in the order CW ~ CW (pH=7) > CaMg ~ CaMg (pH=11) > DW > As. While the outstanding performance of CW and CW (pH=7) treatments may be due to the reducing power of CW, that of CaMg may be linked to its nutritional role in plant growth. Calcium and magnesium are readily soluble and absorbable plant macronutrients; hence they may have served as an additional source of plant nutrients, especially at the early stages of seedling growth. Seed priming with plant nutrients, otherwise known as nutrients priming such as zinc, manganese, boron, and phosphate, has been reported to have a significant influence on the growth of maize (Muhammad et al., 2015). The successful use of ascorbic acid may be attributed to its antioxidant properties. However, from the result of this study, the antioxidant properties of ascorbic acid may not be as strong as that of cathodic water.

3.5 CONCLUSION AND RECOMMENDATIONS

The positive response of both species to cathodic water reinvigoration has indicated its potential relevance in the conservation of orthodox seeds. This will be particularly true if the seeds are of species or genotypes in danger of becoming extinct in nature. In general, seeds invigorated with CW performed better than those primed using existing techniques such as water (hydro priming), ascorbic acid (osmo priming), calcium magnesium solution (nutrient priming). Adjusting the pH of CW and CaMg solution did not result in striking differences in plant emergence and growth when compared with the original solutions. Hence, it may be unnecessary to consider such pH adjustments as separate treatments in future studies. The mechanism of action of the ameliorative effects of cathodic water should be further investigated using biochemical germination enzyme amylase, deoxyribonucleic acid (DNA) concentration and purity should be explored in future germination studies.

CHAPTER 4

GERMINATION INDICES OF ORTHODOX SEEDS AS INFLUENCED BY CONTROLLED DETERIORATION AND CATHODIC WATER INVIGORATION

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Abstract

The study aimed to test the effects of priming on the germination of deteriorated orthodox seeds of five plant species. The water contents of seeds were raised to 14%, and the seeds then deteriorated to 50% viability at 40°C and 100% relative humidity. Deteriorated seeds and fresh seeds of the test species were then primed with cathodic water, un-electrophorized calcium magnesium (CaMg) solution and deionised water. Unprimed fresh and unprimed controlled deteriorated seeds served as the controls. Germination indices were then determined. In general, all priming treatments had positive effects on the germination indices of deteriorated seeds of all species, and to a lesser extent fresh seeds. However, seeds treated with cathodic water performed better than seeds treated with either CaMg solution or deionised water. While controlled deterioration reduced DNA concentrations and the DNA purity of the seeds, priming, particularly with cathodic water had a protecting effect on DNA. Priming also boosted amylase activities in both fresh and deteriorated seeds. Results are consistent with cathodic water-reducing oxidative stress during imbibition. Cathodic water seed priming, therefore, has the potential to play a significant role in the conservation of orthodox seeds.

KeyWords: Cathodic water, Controlled deterioration, Germination indices, Orthodox seeds, Priming

4.1 INTRODUCTION

Plants produce seeds that are either “orthodox”, which tolerate dehydration and can be stored dry, or “recalcitrant”, which are damaged by the loss of only a small amount of water and cannot be stored for practical purposes (Berjak and Pammenter, 2008). Orthodox seeds, and the genetic resources they contain, can be effectively conserved in seeds banks (Walters et al., 2005). However, during long-term storage, even under optimal conditions, orthodox seeds deteriorate. The deterioration of seeds during long-term storage is known as ageing, and in the process, both germination and seedling vigour are gradually lost (Garza-Caligaris et al., 2012). Seed deterioration is of global concern with respect to the long-term conservation of genetic diversity of both wild species and agricultural plants (Zhang et al., 2017). The major focus of long-term seed conservation has been on the critically endangered species (Raimondo, 2017). However, there is also an urgent need to conserve the genetic resources of species of “actual or potential economic concern”. Preservation of genetic diversity of crop plants is essential for future breeding programs to produce varieties that perform well under future climate change scenarios, particularly in sub-Saharan Africa where the effects of climate change are likely to be severe (Jorgenson and Burns, 2007; FAO, 2018).

In the seeds of many plants, a loss of vigour, indicated by a reduction in the rate of germination, often precedes a gradual or abrupt decline in viability during storage (Finch-Savage and Bassel, 2016). The main factors contributing to the loss of vigour are storage time, relative humidity and temperature of the storage environment, mechanical damage, seed water content, presence of microflora and seed maturity (McDonald, 2004; Yari et al., 2011; Vijayakumar et al., 2019). The underlying cause of seed deterioration is unclear, but a major contributing factor is believed to be the production of reactive oxygen species (ROS), which can attack any biomolecule, and induce programmed cell death (Kranner et al., 2010). For example, ROS-mediated lipid peroxidation has now been accepted as a major cause of cellular damage in deteriorated seeds (Walters et al., 2005; Mahjabin and Abidi, 2015). Ultimately, DNA can be damaged, resulting in a delay in cell division and germination (Berjak, 2006).

While a major cause of the inability of recalcitrant seeds to tolerate drying appears to be uncontrolled ROS production (Whitaker et al., 2010), orthodox seeds have internal mechanisms that reduce the damaging effect of ROS. Such systems include both ROS scavenging enzymes such as superoxide dismutase (SOD), peroxidases (POX) and catalases (CAT) and also non-enzymatic antioxidants such as ascorbate, glutathione, and

tocopherol (Berjak et al., 2011). As seeds are stored for a progressively longer period, these mechanisms start breaking down, and seeds begin to deteriorate. It has been suggested that one way of slowing the rate of deterioration would be to exogenously supply antioxidants such as ascorbic acid; however, success has been variable and some antioxidants are cytotoxic at high concentration (Lehner et al., 2008; Berjak et al., 2011). Recently, a novel approach that allows desiccation of at least the embryonic axis of recalcitrant seeds has been described, involving treatment with “cathodic water” (Berjak et al., 2011; Naidoo et al., 2010). Cathodic water is a powerful antioxidant and is the cathodic fraction of an electrolysed, dilute ionic solution of calcium and magnesium solution. A preliminary report suggests that treatment with cathodic water may improve the subsequent storage of orthodox seeds (Gondwe et al., 2016). However, it is unclear whether cathodic water can invigorate deteriorated orthodox seeds. Therefore, the aim of the present study was to test whether cathodic water can improve the vigour of deteriorated orthodox seeds of agricultural (*Pisum sativum* and *Cucurbita pepo*) and wild (*Bolusanthus speciosus*, *Combretum erythrophyllum* and *Erythrina caffra*) plants.

4.2 METHODS AND MATERIALS

4.2.1 Plant Materials

Seeds of five orthodox species were acquired from local seed companies around South Africa. The agricultural plants selected for the study were *Pisum sativum* L. (pea) and *Cucurbita pepo* L. (pumpkin), while the wild species included *Bolusanthus speciosus* (Bolus) Harms (tree wisteria), *Combretum erythrophyllum* (Burch.) Sond (River bush willow) and *Erythrina caffra* Thumb. All seeds were stored in airtight containers at 4°C until further use. Wild species had seed coat-induced or “physical” dormancy, and therefore the seed coats of *B. speciosus* and *E. caffra* were mechanically scarified, and the samara covering the seeds of *Co. erythrophyllum* were excised. Seeds of similar size were selected for further study.

4.2.2 Controlled Deterioration of Seeds

The initial water contents of the test species were determined gravimetrically. Using a water chamber, the water contents of seeds were raised to 14%, after which the seeds were subjected to controlled deterioration at 40°C and 100% relative humidity (RH) in a digital oven (Series 2000, Scientific, USA). Seeds were sampled from the oven at 4 d intervals and germination was tested until complete loss of germination occurred. The time required for 50% inhibition of germination was estimated (P_{50}), and the process was repeated to confirm the repeatability of the estimation. Seeds aged to P_{50} were used for further study.

4.2.3 Preparation of Calcium-Magnesium Solution and Cathodic Water

A calcium-magnesium solution (CaMg) of 0.5 µM $CaCl_2$ and 0.5 mM $MgCl_2$ was prepared. The CaMg solution was autoclaved for sterilization and stored in a refrigerator. When required, 200 ml of CaMg solution was decanted into two glass beakers, and then platinum electrodes were immersed in the solutions, anode in one beaker and cathode in another. The circuit was completed with an agar-based salt bridge, and the solution was electrolysed at 60 V potential difference using a Bio-Rad PowerPac™ Basic (Bio-Rad, USA) power pack for 1 hr at room temperature yielding anodic (oxidizing) water at pH c. 2.4, and cathodic (reducing) water at pH c. 11.2 (Berjak et al., 2011). The anodic water was discarded, whereas cathodic water was used within 1 hr.

4.2.4 Seed Priming

The three priming solutions used were cathodic water, CaMg solution and deionized water. Seeds were hydrated by placing them between 20 layers of single-ply paper towel, which was placed on aluminium foil. To prime the seeds, either 50 ml (for *P. sativum*, *Cu. pepo* and *E. caffra*) or 30 ml (for *B. speciosus* and *Co. erythrophyllum*) of solutions were added. The seeds were hydrated in the priming solutions for 24 hr (*P. sativum* and *Cu. pepo*), 48 hr (*Co. erythrophyllum*) or 18 hr (*E. caffra* and *B. speciosus*), corresponding to a point close to, but just before the emergence of radicle. After hydration, the seeds were dried back to their original masses under ambient laboratory conditions for 7 (*P. sativum* and *Cu. pepo)*, 6 (*E. caffra*), 5 (*Co. erythrophyllum*) or 4 d (*B. speciosus*). The seeds were kept refrigerated in air-tight bottles until further use.

4.2.5 Determination of DNA Purity and Amylase Activity

DNA was extracted at the end of phase 2 from seeds hydrated in priming solutions (cathodic water, calcium magnesium solution and deionised water). Briefly, seeds were ground into a fine powder (“seed meal”) and approximately 70 mg of powdered seeds were transferred to a 1.5 ml Eppendorf tubes containing 700 µl of extraction buffer. The tubes were incubated for 10 min at 65°C, and then 200 µl of 5 M potassium acetate was added. Tubes were vortexed, stored in ice for 10 min, and then centrifuged at 12000 rpm for 10 min at 4°C. Supernatant (400 µl) was transferred into a new tube and 400 µl of iso-propyl alcohol was added. The supernatant was discarded and the pellets were washed twice with 500 µl ethanol, centrifuging at 8000 rpm for 3 min for each wash. The pellets were then dried at room temperature, re-suspended in 50 - 100 µl of deionized water and stored at -40°C. The concentration and purity of DNA was read with Multiskan Sky NanoDrop™ Spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware USA).

P. sativum and *Cu. pepo* were selected to investigate the effect of priming on amylase activity. The seeds were hydrated in the priming solution until the beginning of phase III. Thereafter, 1 g was homogenized with 10 ml of ice-cold 10 mM $CaCl_2$ solution and amylase activity was measured following the method of Devi et al. (2014).

4.2.6 Seed Germination

Germination was tested at 25°C using a 16 hr light / 8 hr dark cycle. Seeds were placed in 90 mm Petri dishes with five layers of moist germination paper underneath and another layer on top. Each treatment comprised 100 seeds divided in four replicates of 25 seeds arranged in a randomized design. Each replicate of 25 seeds was separated into five Petri dishes with five seeds per Petri dish to minimize competition between the seedlings. There were six seed priming treatments and two controls. The seed priming treatments were as follows: aged seeds primed with cathodic water (ASP.CW); aged seeds primed with CaMg solution (ASP.CM); aged seeds primed with distilled water (ASP.DW); fresh seed (unaged) primed with cathodic water (FSP.CW); fresh seeds primed with CaMg solution (FSP.CM); and fresh seeds primed with distilled water (FSP.DW) The two controls were seeds that were first control deteriorated and unprimed (ASC) and the second fresh seeds that were neither deteriorated nor primed (FSC). A seed was considered germinated when 1 mm radicle protrusion was observed.

Germination counts were taken once a day for 14 d for *B. speciosus, E. caffra P. sativum* and *Cu. Pepo* and 21 d for *Co. erythrophyllum*. First day germination (FDG), final germination percentage (FGP) and germination index (GI) were calculated according to the method of Czabator (1962).

At the end of germination study, five seedlings were randomly selected per replicate across all treatments and plant species, separated into root and shoot, oven-dried at 65°C to constant mass and then weighed. In addition, the seedlings' vigour index (SVI) was calculated as described by Abdul-Baki and Anderson (1973) using the formula SVI = DM (g) × FGP, where DM is dry mass of seedling (g) and FGP is final germination percentage.

4.2.7 Statistical Analyses

The data collected were subjected to analysis of variance (ANOVA) using Genstat Release 12.1 (VSN International Ltd., 2009). The means of the treatments were separated by the least significant difference at 5% ($LSD_{0.05}$). Post-hoc analysis was performed using the Tukey test. However, for the "first day of germination" data a non-parametric ANOVA (Kruskal-Wallis rank sum test for multiple independent samples) was used, followed by a Dunn's post-hoc test adjusted by the Benjamini-Hochberg FDR method.

4.3 RESULTS AND DISCUSSIONS

Seeds stored under optimum conditions deteriorate with time, and seed banks need to regularly evaluate the quality by using germination indices (Kader 2005; Abdolahi et al., 2012). The effects of seed deterioration, to some extent, can be reversed by using various priming methods, also known as seed invigoration (Amanpour-Balaneji et al., 2012; Sadeghi and Shekafandeh, 2015). The aim of the present study was to evaluate a novel method of invigoration using cathodic water; taken together, results strongly suggest that cathodic water has considerable potential for invigorating deteriorated seeds.

In the present study, rather than using naturally aged seeds, seeds were subjected to controlled deterioration until they had lost 50% germination. In all species, controlled deterioration of seeds led to significant ($p<0.05$) delay in germination and a reduction in the germination index, reflecting a reduction in germination rate (Table 4.1). Deterioration also reduced seedling vigour (Figure 4.1) and biomass (Figure 4.2), and the concentration and purity of DNA (Figure 4.4). It seems likely that these changes are at least in part, a result of acceleration in the accumulation of ROS that occurs during deterioration (Kranner et al., 2010). ROS can attack DNA, causing breakage of DNA strands and deoxyribose sugar and peroxidise membrane lipids (Suresh et al., 2019). Such oxidative damage inhibits mitosis (McDonald, 1999; Bailly, 2004), resulting in delayed germination and reduced seed vigour and seedling biomass (Suresh et al. 2019), as observed in this study.

Table 4.1 Effects of cathodic water, calcium magnesium solution and deionized water treatments on the germination parameters of *Bolusanthus speciosus*

GERMINATION PARAMETERS	FSC	FSP.CW	FSP.CM	FSP.DW	ASC	ASP.CW	ASP.CM	ASP.DW	$LSD_{0.05}$	SE(±)
FIRST DAY OF GERMINATION*	3.00^{a}	3.00^{a}	3.00^{a}	3.00^{a}	5.00^{bc}	4.00^{b}	4.00^{b}	4.00^{b}	-	-
LAST DAY OF GERMINATION	5.75^{c}	4.00^{a}	5.00^{b}	5.00^{b}	8.00^{e}	7.00^{d}	7.00^{d}	8.00^{e}	0.258	0.125
TOTAL GERMINATION	24.50^{d}	25.00^{d}	25.00^{d}	24.75^{d}	13.00^{a}	19.75^{c}	17.25^{b}	17.25^{b}	1.192	0.577
GERMINATION PERCENTAGE	98.00^{d}	100.00^{d}	100.00^{d}	99.00^{d}	52.00^{a}	79.00^{c}	69.00^{b}	69.00^{b}	4.766	2.309
MEAN GERMINATION TIME	3.32^{d}	4.21^{f}	3.83^{e}	3.70^{e}	1.12^{a}	2.58^{c}	2.00^{b}	1.79^{b}	0.155	0.075
GERMINATION INDEX	18.90^{d}	26.70^{f}	23.26^{e}	22.20^{e}	5.48^{a}	14.31^{c}	10.83^{b}	9.42^{b}	1.039	0.504
UNIFORMITY OF GERMINATION	0.10^{c}	0.21^{e}	0.15^{d}	0.13^{d}	0.03^{a}	0.06^{b}	0.05^{ab}	0.04^{a}	0.012	0.006

*ANOVA was performed across treatments with the means of replicate separated at $LSD_{0.05}$. Post hoc was done using Tukey test ($p<0.05$, n=32). Means along the same row with different letters were significantly different. *Due to the nature of the data, a non-parametric ANOVA was used. After performing the Kruskal-Wallis rank sum test for multiple independent samples, Dunn's post-hoc test adjusted by the Benjamini-Hochberg FDR method, was used.*

Table 4.2 Effects of cathodic water, calcium magnesium solution and deionized water treatments on the germination parameters of *Combretum erythrophyllum*

GERMINATION PARAMETERS	FSC	FSP.CW	FSP.CM	FSP.DW	ASC	ASP.CW	ASP.CM	ASP.DW	$LSD_{0.05}$	SE(±)
FIRST DAY OF GERMINATION	11.00^{a}	10.50^{a}	11.00^{a}	11.00^{a}	12.75^{c}	12.00^{b}	12.00^{b}	12.00^{b}	0.394	0.191
LAST DAY OF GERMINATION*	16.00^{bc}	14.00^{a}	15.00^{ab}	15.00^{ab}	17.00^{cd}	16.00^{bc}	16.00^{bc}	17.00^{cd}	-	-
TOTAL GERMINATION	20.75^{c}	22.75^{c}	21.50^{c}	20.50^{c}	11.75^{a}	16.50^{b}	13.75^{ab}	14.00^{ab}	2.346	1.137
GERMINATION PERCENTAGE	83.00^{c}	91.00^{c}	86.00^{c}	82.00^{c}	47.00^{a}	66.00^{b}	55.00^{ab}	56.00^{ab}	9.380	4.550
MEAN GERMINATION TIME	1.00^{c}	1.25^{d}	1.05^{c}	1.02^{c}	0.39^{a}	0.62^{b}	0.58^{b}	0.55^{ab}	0.107	0.052
GERMINATION INDEX	7.43^{c}	9.53^{d}	7.85^{c}	7.63^{c}	2.77^{a}	4.50^{b}	4.19^{b}	4.00^{ab}	0.827	0.401
UNIFORMITY OF GERMINATION	0.0050^{c}	0.0056^{d}	0.0053^{c}	0.0053^{c}	0.0044^{a}	0.0048^{b}	0.0047^{b}	0.0047^{b}	0.0002	0.0001

*ANOVA was performed across treatments with the means of replicate separated at $LSD_{0.05}$. Post hoc was done using Tukey test ($p<0.05$, n=32). Means along the same row with different letters were significantly different. *Due to the nature of the data, a non-parametric ANOVA was used. After performing the Kruskal-Wallis rank sum test for multiple independent samples, Dunn's post-hoc test adjusted by the Benjamini-Hochberg FDR method, was used.*

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Table 4.3 Effects of cathodic water, calcium magnesium solution and deionized water treatments on the germination parameters of *Erythrina caffra*

GERMINATION PARAMETERS	FSC	FSP.CW	FSP.CM	FSP.DW	ASC	ASP.CW	ASP.CM	ASP.DW	$LSD_{0.05}$	SE(±)
FIRST DAY OF GERMINATION	3.00^{a}	3.00^{a}	3.00^{a}	3.00^{a}	5.00^{bc}	4.00^{b}	4.00^{b}	4.00^{b}	-	-
LAST DAY OF GERMINATION	5.00^{b}	4.00^{a}	4.75^{ab}	5.00^{b}	7.50^{cd}	7.00^{c}	8.00^{d}	7.75^{cd}	0.471	0.228
TOTAL GERMINATION	24.75^{d}	25.00^{d}	24.75^{d}	25.00^{d}	12.75^{a}	19.25^{c}	17.25^{bc}	16.25^{b}	1.349	0.654
GERMINATION PERCENTAGE	99.00^{d}	100.00^{d}	99.00^{d}	100.00^{d}	51.00^{a}	77.00^{c}	69.00^{bc}	65.00^{b}	5.395	2.614
MEAN GERMINATION TIME	3.85^{d}	4.26^{e}	4.07^{e}	4.08^{e}	1.197^{a}	2.17^{c}	1.92^{b}	1.78^{b}	0.136	0.066
GERMINATION INDEX	23.22^{d}	27.36^{f}	25.39^{e}	25.32^{e}	5.98^{a}	11.69^{c}	10.44^{bc}	9.36^{b}	0.928	0.45
UNIFORMITY OF GERMINATION	0.154^{c}	0.216^{e}	0.187^{d}	0.186^{d}	0.031^{a}	0.050^{b}	0.046^{ab}	0.041^{ab}	0.012	0.006

*ANOVA was performed across treatments with the means of replicate separated at $LSD_{0.05}$. Post hoc was done using Tukey test ($p<0.05$, $n=32$). Means along the same row with different letters were significantly different. *Due to the nature of the data, a non-parametric ANOVA was used. After performing the Kruskal-Wallis rank sum test for multiple independent samples, Dunn's post-hoc test adjusted by the Benjamini-Hochberg FDR method, was used.*

Table 4.4 Effects of cathodic water, calcium magnesium solution and deionized water treatments on the germination parameters of *Pisum sativum*

GERMINATION PARAMETERS	FSC	FSP.CW	FSP.CM	FSP.DW	ASC	ASP.CW	ASP.CM	ASP.DW	$LSD_{0.05}$	SE(±)
FIRST DAY OF GERMINATION*	3.00^{a}	3.00^{a}	3.00^{a}	3.00^{a}	5.00^{bc}	4.00^{b}	4.00^{b}	4.00^{b}	-	-
LAST DAY OF GERMINATION	6.00^{c}	4.00^{a}	5.00^{b}	5.25^{b}	8.50^{e}	6.00^{c}	7.00^{d}	7.00^{d}	0.394	0.191
TOTAL GERMINATION	24.75^{d}	25.00^{d}	24.75^{d}	24.75^{d}	13.00^{a}	20.25^{c}	18.75^{bc}	17.75^{b}	1.210	0.586
GERMINATION PERCENTAGE	99.00^{d}	100.00^{d}	99.00^{d}	99.00^{d}	52.00^{a}	81.00^{c}	75.00^{bc}	71.00^{b}	4.840	2.345
MEAN GERMINATION TIME	3.24^{d}	3.89^{f}	3.60^{e}	3.48^{e}	1.23^{a}	2.50^{c}	2.01^{b}	1.89^{b}	0.128	0.062
GERMINATION INDEX	22.16^{d}	29.39^{f}	26.18^{e}	24.83^{e}	7.25^{a}	16.50^{c}	12.99^{b}	11.93^{b}	0.997	0.483
UNIFORMITY OF GERMINATION	0.07^{d}	0.11^{f}	0.09^{e}	0.08^{e}	0.025^{a}	0.05^{c}	0.04^{b}	0.03^{b}	0.005	0.002

*ANOVA was performed across treatments with the means of replicate separated at $LSD_{0.05}$. Post hoc was done using Tukey test ($p<0.05$, n=32). Means along the same row with different letters were significantly different. *Due to the nature of the data, a non-parametric ANOVA was used. After performing the Kruskal-Wallis rank sum test for multiple independent samples, Dunn's post-hoc test adjusted by the Benjamini-Hochberg FDR method, was used.*

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Table 4.5 Effects of cathodic water, calcium magnesium solution and deionized water treatments on the germination parameters of *Cucurbita pepo*

GERMINATION PARAMETERS	FSC	FSP.CW	FSP.CM	FSP.DW	ASC	ASP.CW	ASP.CM	ASP.DW	$LSD_{0.05}$	SE(±)
FIRST DAY OF GERMINATION*	4.00^{a}	4.00^{a}	4.00^{a}	4.00^{a}	7.00^{c}	5.00^{bc}	5.00^{bc}	5.00^{bc}	-	-
LAST DAY OF GERMINATION	7.00^{bc}	5.00^{a}	6.00^{ab}	6.50^{b}	10.00^{e}	8.00^{cd}	8.25^{d}	8.50^{d}	0.649	0.315
TOTAL GERMINATION	25.00^{d}	25.00^{d}	24.75^{d}	24.75^{d}	13.00^{a}	21.25^{c}	17.75^{b}	16.75^{b}	1.389	0.673
GERMINATION PERCENTAGE	100.00^{d}	100.00^{d}	99.00^{d}	99.00^{d}	52.00^{a}	85.00^{c}	71.00^{b}	67.00^{b}	5.557	2.693
MEAN GERMINATION TIME	2.597^{d}	3.383^{f}	3.173^{e}	3.02^{e}	1.02^{a}	2.23^{c}	1.73^{b}	1.56^{b}	0.114	0.055
GERMINATION INDEX	17.17^{d}	25.08^{g}	22.80^{f}	21.31^{e}	6.06^{a}	14.65^{c}	11.19^{b}	9.96^{b}	0.910	0.441
UNIFORMITY OF GERMINATION	0.033^{d}	0.055^{g}	0.048^{f}	0.044^{e}	0.018^{a}	0.029^{c}	0.024^{b}	0.023^{b}	0.002	0.001

*ANOVA was performed across treatments with the means of replicate separated at $LSD_{0.05}$. Post hoc was done using Tukey test ($p<0.05$, n=32). Means along the same row with different letters were significantly different. *Due to the nature of the data, a non-parametric ANOVA was used. After performing the Kruskal-Wallis rank sum test for multiple independent samples, Dunn's post-hoc test adjusted by the Benjamini-Hochberg FDR method, was used.*

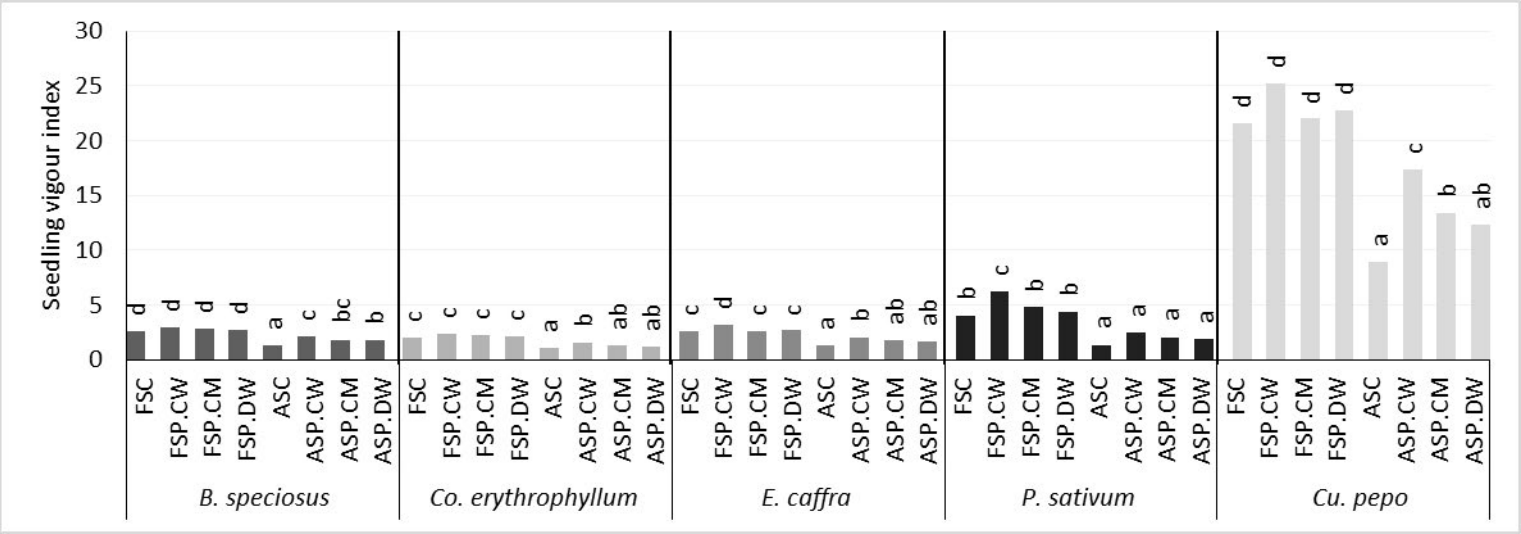

Figure 4.1 Effects of controlled deterioration of seeds and seed priming with cathodic water, calcium magnesium solution and deionized water treatments on the seedling vigour of five orthodox species
***Means with different letters were significantly different**

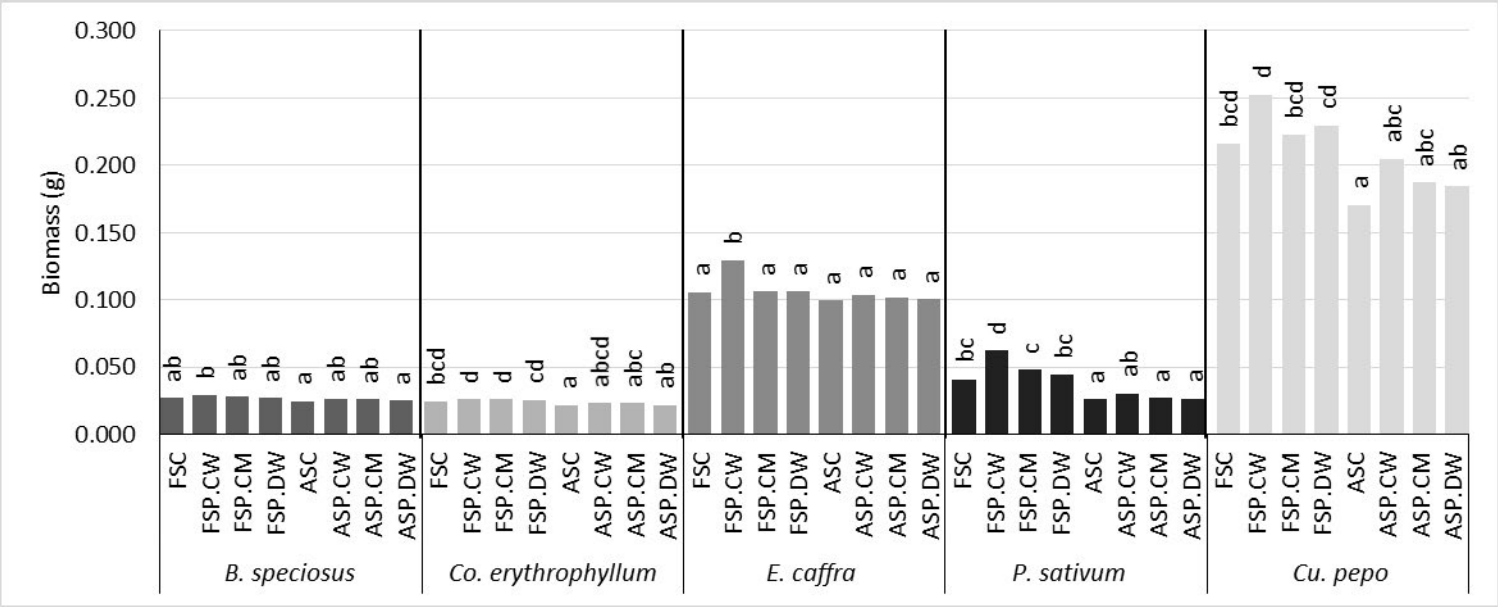

Figure 4.2 Effects of controlled deterioration of seeds and seed priming with cathodic water, calcium magnesium solution and deionized water on the seedling biomass of five orthodox species

*Means with different letters were significantly different

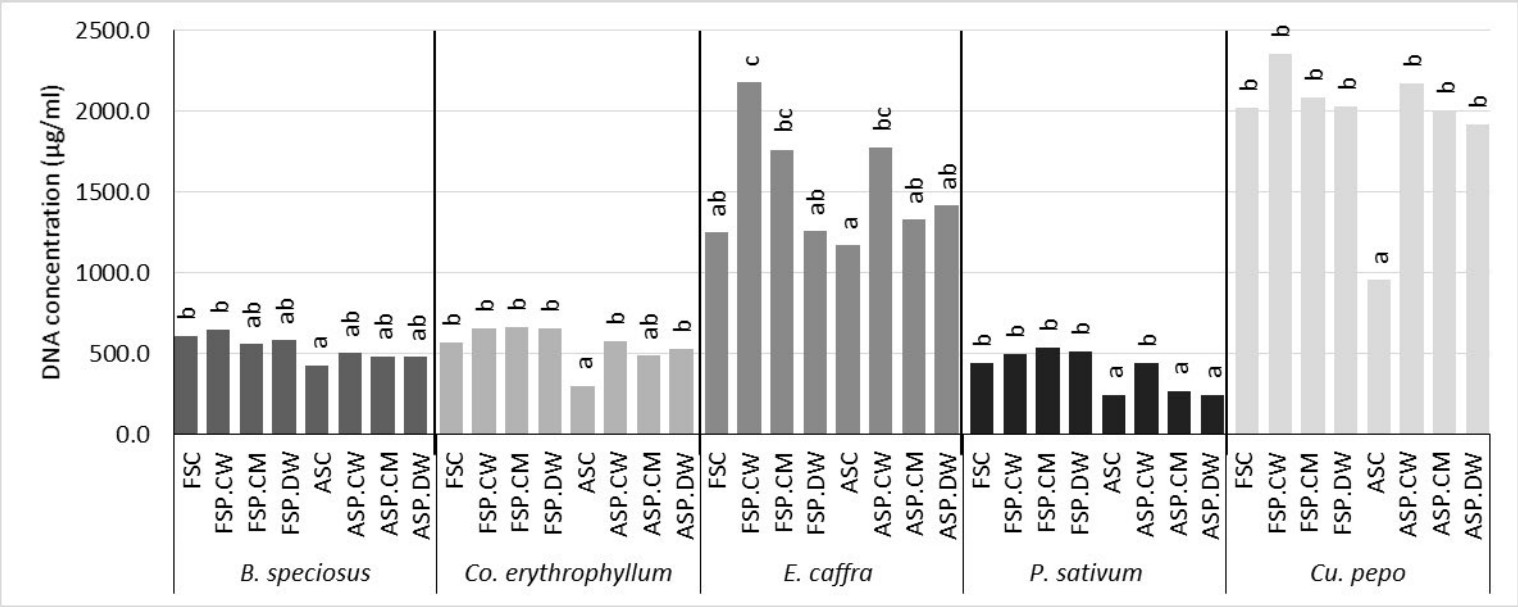

Figure 4.3 Effects of controlled deterioration of seeds and seed priming with cathodic water, calcium magnesium solution and deionized water on the DNA concentration of five orthodox species

*Means with different letters were significantly different

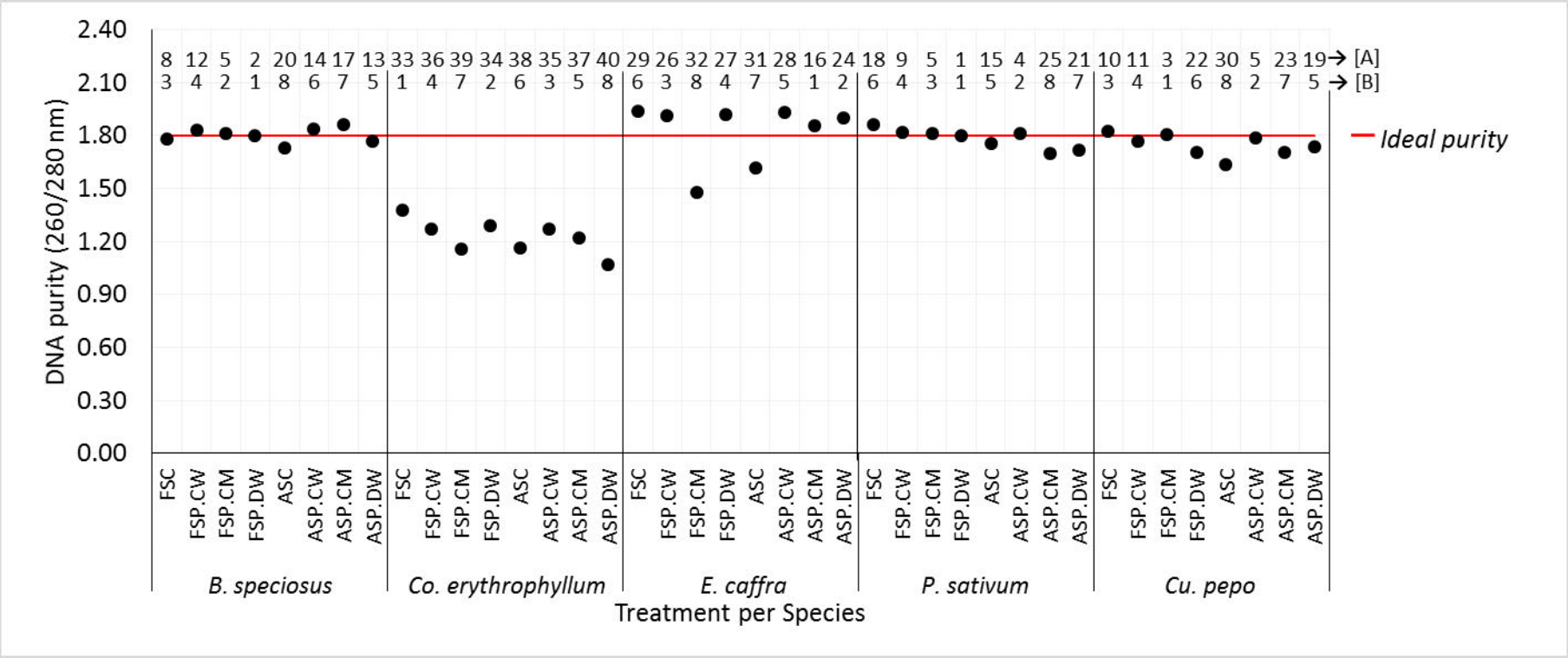

Figure 4.4 Effects of controlled deterioration of seeds and seed priming with cathodic water, calcium magnesium solution and deionized water on the DNA purity of five orthodox species

[A] – Purity of all the five species were ranked from the most pure (1) to the least pure (40)

[B] - Purity of each of the five species was ranked from the most pure (1) to the least pure (8)

Each point of the DNA purity on the graph represents mean of 3 replicates

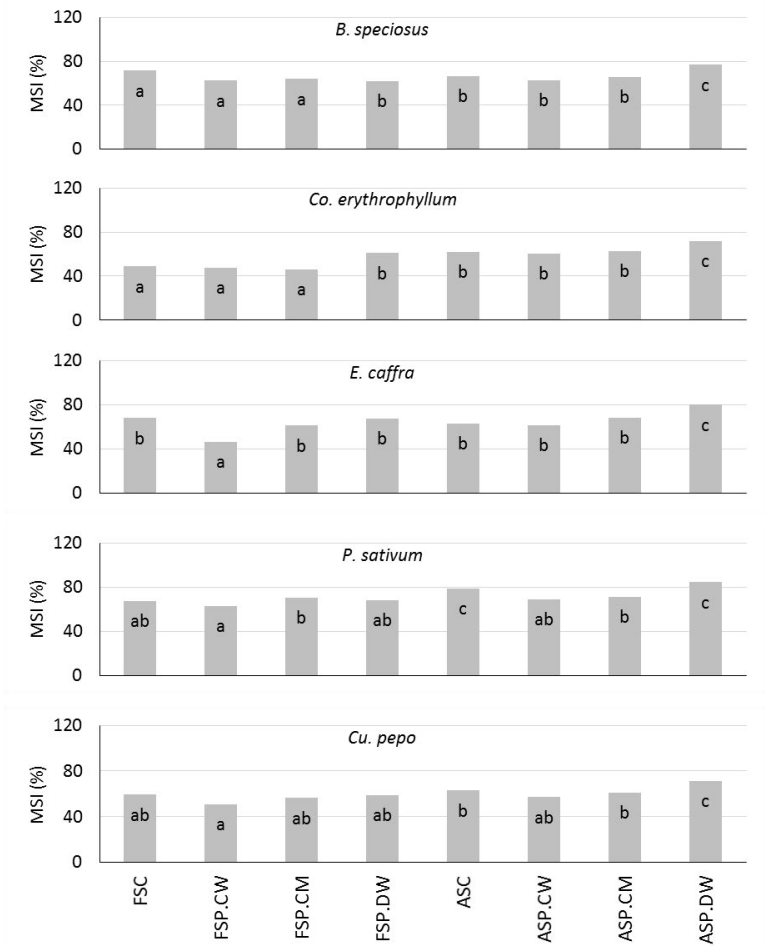

Figure 4.5 Effects of controlled deterioration of seeds and seed priming with cathodic water, calcium magnesium solution and deionized water on the MSI of five orthodox species

*Means with different letters were significantly different

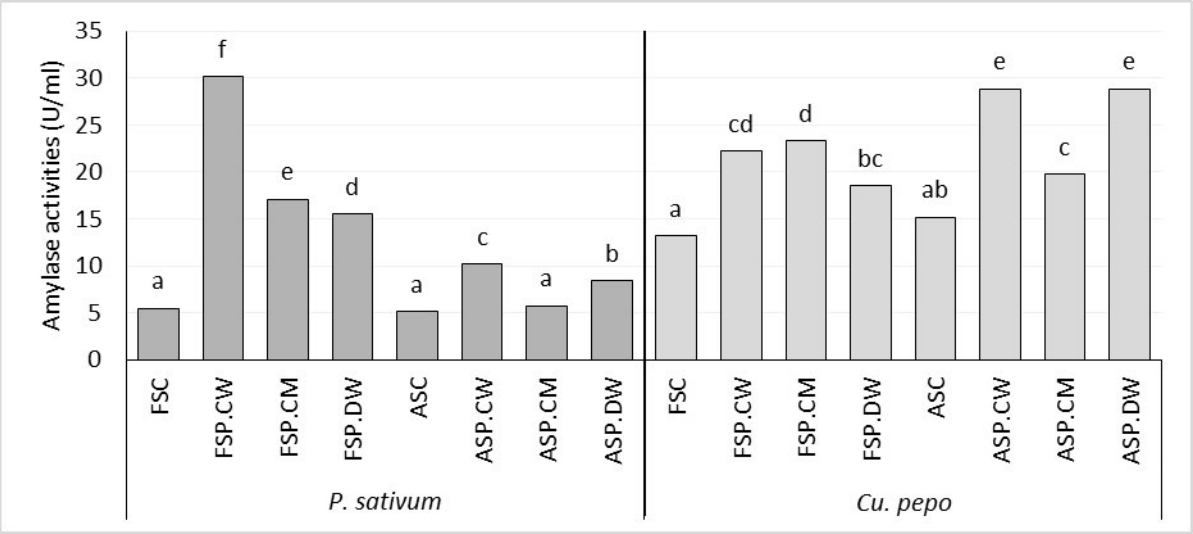

Figure 4.6 Effects of controlled deterioration of seeds and seed priming with cathodic water, calcium magnesium solution and deionized water on the amylase activities of *P. sativum and Cu. pepo*
*Means with different letters were significantly different

Priming the deteriorated seeds of all species with cathodic water, CaMg solution and deionized water significantly ($p<0.05$) improved both the rate of germination and the final germination percentage compared with un-primed seeds (Table 4.1). Furthermore, in all species, cathodic water was more effective than CaMg or deionized water, although differences between priming treatments were only significant for *B. speciosus, Cu. pepo* (rate of germination and final germination percentage), and *P. sativum* (rate of germination). Interestingly, cathodic water even increased the rate of germination in fresh seeds (Table 4.1), while other priming treatments had similar, but smaller effects.

In all species, deterioration significantly ($p<0.05$) reduced seedling vigour compared with fresh seed (Table 4.1). Cathodic water priming significantly improved seedling vigour in the deteriorated seeds of all species, except *P. sativum*, while priming with CaMg or distilled water was less effective (Figure 4.1). Even in fresh seeds, cathodic water significantly improved vigour in *E. caffra* and *P. sativum* (Figure 4.1). Effects of deterioration and priming on seedling dry mass were less pronounced (Figure 4.2).

In all species, controlled deterioration reduced the concentrations (Figure 4.3) and purity (Figure 4.4) of DNA in the seeds. A ratio of ~1.8 between A_{260} and A_{280} is generally accepted as normal for “pure” DNA. DNA extracted from all treatments of *Co. erythrophyllum* sharply deviated from normal values, suggesting that protein, phenol or other contaminants that absorb strongly at or near 280 nm were present (Khare et al*.,* 2014). However, the decline in DNA concentrations and purity caused by deterioration were probably a result of breakage or damage of DNA strands, which is likely to cause a delay in mitosis (McDonald, 1999; Bailly, 2004). Delay in mitosis delays cell division, and consequently retards germination and reduces seed vigour and seedling biomass (McDonald, 1999; Bailly, 2004; Balestrazzi et al., 2011). In deteriorated seeds, priming increased both the amount and purity of DNA, and in most species cathodic water was most effective, often significantly so. The most likely explanation is that cathodic water reduced oxidative stress, reducing DNA damage during imbibition, improving the performance of primed deteriorated seeds.

Imbibition is known to trigger the release of gibberellins, which in turn stimulates the synthesis of amylase (Damaris et al., 2019), a key enzyme in germination. Amylase provides the food materials for growth and development of germinating embryo by breaking down starch into low molecular mass carbohydrates that can be used for embryo growth. Deterioration had no significant effect on amylase activity in *P. sativum* and *Cu. pepo* (Figure

4.5). However, in both species, priming with any of the solutions significantly ($p<0.05$) increased amylase activities. In *P. sativum*, cathodic water increased amylase activity significantly more than the other two priming solutions, while in *Cu. pepo* the CaMg solution was best for fresh seeds, while cathodic water and distilled water were best for deteriorated seeds. Stimulation of amylase activity by priming may in part contribute to the improved performance of primed seeds, particularly seeds primed with cathodic water.

The study concludes that cathodic water seed priming has the potential to play a significant role in the conservation of orthodox seeds by way of reducing oxidative stress during imbibition.

CHAPTER 5

CATHODIC WATER ENHANCES SEEDLING EMERGENCE AND GROWTH OF CONTROLLED DETERIORATED ORTHODOX SEEDS

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Abstract

All orthodox seeds eventually deteriorate during storage, a well-known problem in seed banking. Here we used a greenhouse study to test if priming deteriorated seeds with cathodic water can improve the emergence and subsequent seedling growth of three South African tree species, *Bolusanthus speciosus*, *Combretum erythrophyllum* and *Erythrina caffra.* Other priming solution investigated were calcium magnesium (CaMg) solution and deionised water. In the present study, seeds were subjected to an artificial deterioration by increasing their water content to 14% and keeping them at 40°C and 100% RH until they had lost 50% of their germination under laboratory conditions. Fresh and deteriorated seeds were primed with cathodic water, CaMg solution and deionised water, with non-primed fresh and deteriorated seeds as controls. Controlled deterioration significantly reduced total emergence and the biomass and photosynthetic parameters of the resulting seedlings. In one species (*Bolusanthus speciosus*), priming the deteriorated seeds with cathodic water significantly improved emergence parameters. However, in all species cathodic water significantly improved the total biomasses and other growth parameters of the seedlings derived from deteriorated seeds. Priming with CaMg solution and deionised water had little effect on emergence, and while improving the growth of seedlings derived from deteriorated seeds, they were less effective than cathodic water. In fresh seeds, priming with all solutions resulted in a small improvement in some parameters. Controlled deterioration of fresh seeds reduced the membrane stability index (MSI) in two of the three species, and in all species increased the levels of the lipid oxidation products MDA and 4-HNE. Priming deteriorated seeds with cathodic water increased the MSI and reduced the MDA contents in all species, and the 4-HNE content in one species. Other priming solutions were generally less effective in ameliorating oxidative stress. Results suggest the strong antioxidative property of cathodic water is an important reason for its ability to ameliorate deterioration. In conclusion, the present study shows that priming with cathodic water is an effective way of invigorating deteriorated seeds of *Bolusanthus speciosus*, *Combretum erythrophyllum* and *Erythrina caffra* and may have considerable potential in orthodox seed conservation.

Keywords: Cathodic water, deterioration, membrane, orthodox seeds, priming

5.1 INTRODUCTION

Orthodox seeds need to be stored in the short-term so that they can produce high-quality plants in the next growing season. Seeds also must be stored in long-term base collections with the aim of conserving genetic resources so that germplasm can be maintained and used in future breeding programs and for restoring wild populations (Farooq et al., 2019). Therefore, seed quality must be sustained over extended periods, but even though the seeds are stored in very good conditions, seeds deteriorate (also known as ageing) during long-term storage. The consequences of seed deterioration include a decline in seed germination, reduction in seedling emergence and poor plant growth (Sreepriya and Girija, 2019). The reduction in seed 'performance' as a result of seed deterioration is of significant concern with respect to the long-term conservation of genetic diversity, both of wild species and in both agricultural and horticultural plants (Farooq et al., 2019).

A major cause of ageing is oxidative stress. During storage, seeds accumulate reactive oxygen species (ROS) such as superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\cdot OH$) (Bailly et al., 2008; Sahu et al., 2017). ROS attack many biomolecules, and in particular the lipids in the cell membrane. Seeds have internal protective mechanisms such as antioxidant enzymes, which include superoxide dismutase (SOD) and catalase (CAT), to counter the deleterious effects of ROS. However, as deterioration progresses the ability of these internal protective mechanisms becomes overwhelmed and damage occurs. Lipid peroxidation is believed to be a significant factor in seed deterioration, and principal among the final products of lipid peroxidation are 4-hydroxynonenal (4-HNE) and malondialdehyde **(**MDA) (Barrera et al., 2018). The presence of 4-HNE and MDA indicates that the cell membrane may have become permeable and that solutes may leak from the cell. Clear evidence now exists that losses in membrane integrity are a result of the uncontrolled production of ROS (Berjak and Pammenter, 2008; Mohaddes et al., 2019; Smith and Berjak, 1995). While seeds suffering oxidative stress will probably display reduced germination and emergence, in addition, “hangover effects” of deterioration may be carried through to later growth stages of the plant. Seedlings of deteriorated seeds may display reduced photosynthesis and transpiration, slower growth and ultimately lower yields (for review see Finch-Savage and Bassel (2016)). Thus, even if poor quality seeds germinate or emerge, the quality of plants generated from such seed is not guaranteed.

Seed invigoration, although sometimes used alternatively with seed priming, is an encompassing term of various treatments that are used to improve the germination; field emergence and growth of a given seed lot (Farooq et al., 2019). It is basically a pre-sowing

technique used to improve germination and seedling growth or to facilitate the performance of seeds at the time of sowing (Farooq et al., 2019; Taylor et al*.,* 1998). Seed invigoration includes pre-sowing hydration treatments (Basra et al*.*, 2006; Farooq et al., 2005; Farooq et al., 2007), osmoprotectant (low molecular weight) seed treatments (Taylor et al*.*, 1998), coating techniques (Song et al., 2005) and more recently, pre-sowing dry heat treatment (Farooq et al*.,* 2005). The focus of seed invigoration is to improve germination, increase field seedling emergence, reduce emergence time and enhance uniformity. Seed invigoration also protects seeds from unfavourable biotic and abiotic environmental factors during critical phases of seedling establishment.

In some cases of priming, the hydrated seeds are not re-dried but sown immediately after hydration (Ella et al., 2011), reducing the lag time of imbibition (Heydecker and Coolbear, 1977; McDonald, 2000). Usually for seeds that are not re-dried, the seeds are placed in solutions with a high osmotic potential which prevents the seeds from imbibing enough water to enter phase III of seed hydration. The notion is to hold the seeds in phase II and therefore essentially keeping the seed within the lag phase (Taylor et al*.,* 1998). During this period, the seeds are metabolically active; conversion of stored reserves that enhances germination also takes place. After imbibition, the seeds are removed from the priming treatments rinse with water, and sowing in the field is done (Ella et al., 2011; McDonald, 2004). However, it is also possible to dry seeds before they are sown. In both methods, seed hydration causes activation or repair of enzymes (McDonald, 2004). If seeds are dried after priming, it has been suggested that damaged seeds undergo repair during drying, possibly explaining the improved performance of primed seeds (McDonald, 2004). Numerous advantages of priming orthodox seeds have been reported for plants from around the world (Eskandari and Kazemi, 2011; Farooq et al., 2008). Primed seeds can exhibit faster and more uniform germination when sown under normal or stress conditions. Priming has been shown to benefit a range of crops from tropical regions, for example, *Oryza sativa, Zea mays, Sorghum bicolor* and *Cajanus cajan* (Arun et al., 2017; Ella et al., 2011).

In this study, we tested the ability of a novel seed priming agent, cathodic water, to ameliorate deterioration in three South African tree species, *Bolusanthus speciosus*, *Combretum erythrophyllum* and *Erythrina caffra*. Cathodic water is an electrolysed form of calcium magnesium (CaMg) solution (Berjak et al., 2011). Cathodic water has strong antioxidative properties, and in addition to the benefits conferred by conventional priming solutions, may counteract the damage caused by ROS (Berjak et al., 2011). Our study therefore brings the concept of electrochemistry into plant germplasm conservation and crop establishment and production. Preliminary reports from our group showed that under

laboratory conditions priming with cathodic water had beneficial effects on the germination of deteriorated seeds from a variety of species (Fatokun et al. 2020; Gondwe et al., 2016). However, its effects on seedling emergence and subsequent growth of the resulting seedlings have not been adequately documented. Rather than use naturally aged seeds, here we used controlled deterioration, involving exposing seeds to predetermined aggravated temperature and humidity (Bewley and Black, 1994; Bhattacharya et al., 2019). This enabled us to deteriorate the seeds of all species to the same extent.

5.2 MATERIALS AND METHODS

5.2.1 Study area

Seed ageing and germination were carried out in the laboratories of the Plant Germplasm Conservation Research Unit, University of KwaZulu-Natal, Durban, South Africa. Plants were grown in a greenhouse of the same School (Average temperature: 23.5°C, relative humidity: 67%.

5.2.2 Plant management and data collection

Potting mix and multifeed fertilizer used in this study were purchased from Grovida, a local seed company in Durban. The seeds of the test species were bought from Silverhill seeds, Cape Town, South Africa. Initial germination test carried out on the seeds indicated that both *B. speciosus* and *E. caffra* have seed coat imposed dormancy, while *C. erythrophyllum* also has a form of physical dormancy due to the samara covering the seed. Nicking was carried to break *E. caffra* and *B. speciosus* dormancy, while dormancy in *C. erythrophyllum* was broken by the removing the samara covering the seeds. (Preparation of calcium magnesium solution and cathodic water – subsection 3.2.4).

The water content for all the species was raised to 14% using a vapour chamber. The seeds were then sealed in airtight glass jars and kept in a digital oven (Series 2000, Scientific, USA) at 40°C and 100% relative humidity. Samples were taken every few days to assess the time needed to achieve a 50% reduction in germination. To prime the seeds (50 seeds per treatment) they were placed between 20 layers of single-ply paper towel, which was placed on aluminium foil. Priming solutions (50 ml) were poured onto these paper towels. The aluminium foil containing the seeds were placed in plastic pouches, and after 24 hr, just before radicle emergence, the seeds were dried back to their original masses under ambient laboratory conditions for 7 d and kept at 4°C in air-tight bottles until required. There were eight treatments: six seed priming treatments and two controls. The seed priming treatments were: aged seeds primed with cathodic water (ASP.CW); aged seeds primed with CaMg

solution (ASP.CM); aged seeds primed with distilled water (ASP.DW); fresh seed (unaged) primed with cathodic water (FSP.CW); fresh seeds primed with CaMg solution (FSP.CM); fresh seeds primed with distilled water (FSP.DW) and two controls; unprimed fresh seeds (FSC) and unprimed aged seeds (ASC). Each treatment was replicated four times.

To test emergence and subsequent seedling growth, a completely randomized experimental design was used. The plants were grown in 2 l pots containing 800 g of potting mix and watered as required. After CD and priming, five seeds were planted in each pot, with four pots per treatment arranged in a completely randomized design. Emerged seedlings were counted and recorded daily. Thinning was done at four weeks after planting to reduce the number of plants per pot from five to one. Plants were irrigated as and when required throughout the period of the experiment. Grovida multifeed water soluble fertilizer was used to supply nutrients to the growing plants at 1 g l^{-1}. The composition of the fertilizer was Nitrogen (N), phosphorus (P), potassium (K), sulphur (S), and magnesium (Mg) at 193, 83, 153, 6.1 and 4.6 g kg^{-1}respectively. Others minerals were zinc (Zn), boron (B), molybdenum (Mo), iron (Fe), manganese (Mn) and copper (Cu) at 700, 1054, 63, 751, 273 and 75 mg kg^{-1} Physiological data such as photosynthesis, transpiration and chlorophyll fluorescence were taken at 8 WAP. The study was terminated at 12 WAP. The plants were harvested and separated into root, stem and leaves. The plant parts were measured and the leaves were counted. All plant parts were oven-dried at 65°C to constant mass. The oven-dried plant parts were weighed and recorded.

5.2.3 Emergence

Seedling emergence counts were taken daily until constant counts were achieved. The value was expressed in percentage. The following were determined from the emergence data taken: First Day of Emergence (FDE), Final Emergence Percentage (FEP), Mean Emergence Time (MET) – MET was calculated according to the equation of Ellis and Roberts (1981): MET = $\Sigma Dn/n$ (Where n is the number of emerged seedlings on day D and D is the number of days counted from the beginning of seedling emergence.

Emergence Index (EI) – EI is also called speed of emergence and it was calculated using the method of Czabator (1962)

$$= \frac{\text{Number of emerged seedlings}}{\text{Days of first count}} + \cdots + \frac{\text{Number of emerged seedlings}}{\text{Days of final coun}}$$

Uniformity of Emergence (UE) – UE was calculated using the formula of Abdolahi et al. (2012), = $\Sigma n/ [(T-t)^2 n]$ (**n** is the number of emerged seedlings counted on a particular day, **t** is the time (number of days) beginning from day 0, T is the MET

5.2.4 Leaf chlorophyll content and chlorophyll fluorescence

Leaf Chlorophyll content was measured from the third, fourth and fifth leaves (counting from the terminal bud) across all treatments using a SPAD chlorophyll meter (model SPAD-502; Minolta Corp., Ramsey, N.J.). Three measurements were taken on each leaf at ten weeks of growth. The chlorophyll content was estimated as the mean of the nine readings and expressed as the chlorophyll content index (CCI).

Chlorophyll fluorescence was measured using Li-Cor 6400XT portable photosynthesis measuring system (Li-Cor, Lincoln, NE). Chlorophyll fluorescence transients were measured on the third leaf from the terminal bud across all treatments and replicates at eight weeks after planting. Measurements were taken after the plants were dark-adapted for 40 minutes. Fv/Fm, the ratio of variable (Fv) to maximum fluorescence (Fm), was used as a measure of potential photochemical efficiency of photosystem II (PSII).

5.2.5 Photosynthetic capacity – Steady state gas exchange

Gas exchange was measured with Li-Cor 6400 portable photosynthesis measuring system, fitted with a standard chamber and configured as an open system (Li-Cor, Lincoln, NE). Measurements were taken at 8 weeks after planting across all treatments and replicates.

Instantaneous measurement of leaf based CO_2 assimilation and transpiration rates were carried out at a CO_2 concentration of 400 µmol CO_2 mol^{-1}, a light intensity of 1000 µmol m^{-2} s^{-1} and a temperature of 25°C; measurements were done at about 11:00 am to 2:00 pm when conditions were most stable. The measurements were taken on fully expanded, non-senescing leaves. Three measurements were taken per plant on the third, fourth and fifth leaves from the terminal bud. The mean of these three measurements was used as the average rate of photosynthesis for each plant.

5.2.6 Harvesting and post-harvest data collection

Shoot heights were measured using a meter rule at twelve weeks of growth. At harvesting, the plants were carefully pulled out of the potting mix to avoid damage to the roots. Potting mix which adhered to the roots was removed using tap water. The lengths of the roots and stems were measured, the number of leaves was counted and the leaf area was measured with a leaf area meter (CI-202 Area Meter, CID, Inc., USA). The plants were subsequently separated into leaves, stems and roots. The plant parts (root, stem and leaves) were oven-dried at 65˚C until constant mass and then weighed.

5.2.7 Determination of MDA and 4HNE contents in seeds

At about the end of phase II of the seed hydration, 1 g of seeds were homogenised in 5 ml of 20% (w/v) trichloro acetic acid (TCA) consisting of 0.5% (w/v) TBA. The homogenate was then incubated for 30 min at 95°C (Hodges et al., 1999), after which it was placed in an ice bath for 10 min and thereafter centrifuged at 10000 *g* for 10 min. The absorbance of the supernatant was read at 600 nm using PowerWave™ microplate spectrophotometer (BioTek Instruments, Inc, USA). The content of MDA was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1}. Content of MDA was expressed as mmol g^{-1} fresh mass.

The content of 4-HNE was estimated at about the end of phase II of the seed hydration: 1 g of seeds were homogenised in 5 ml of cold borate buffer (0.2 M, pH 7.4) at 4°C. The homogenate was then mixed with 10% (w/v) TCA and centrifuged at 12000 *g* for 15 min. The supernatant obtained was thoroughly mixed with 2, 4-dinitrophenyl hydrazine (1 mg ml^{-1} in 0.5 M HCl). The complex obtained was kept at laboratory conditions for 2 h after which it was extracted in hexane and evaporated under liquid nitrogen. The residue was dissolved in methanol and absorbance was read at 350 nm against methanol as blank (Ray et al., 2007). Content of 4-HNE in the samples was expressed as mmol g^{-1} fresh mass.

5.2.8 Determination of seeds Membrane Stability Index

Membrane stability index of the seeds was assessed using the method of Yuan et al. (2014). Into two test tubes were added 20 ml of milliQ water (MW) (Millipore, Gradient A-10, USA). Seeds (1 g) were added to the tubes, and one tube was placed in a water bath at 40°C for 40 min (T1) and the other in a water bath at 100°C for 15 min (T2). The electrical conductance of the water in both test tubes after incubation was measured with a multi-cell electrical conductivity meter (Reid and Associates, Durban, South Africa). The measurement was repeated four times and the average of the four trials was reported as the electrical conductance and use in calculating the membrane stability index (MSI) of the seeds; MSI % = [1- (T1/T2)] X 100

5.2.9 Statistical analysis

The data collected were subjected to analysis of variance (ANOVA) using GenStat Release 12.1 (PC/Windows Vista) (VSN International Ltd., 2009). Means of the treatments were compared using the Tukey Test at 5% least significant difference ($LSD_{0.05}$).

5.3 RESULTS

5.3.1 Effect of cathodic water on seedling emergence

Seedling emergence was delayed as a result of controlled deterioration of seeds in all the test species. Emergence was delayed for about 2 d in *E. caffra*, and 4 d in *B. speciosus* and *C. erythrophyllum* (Tables 5.1). Total seedling emergence, mean emergence time, emergence index and uniformity of emergence were also adversely affected by controlled deterioration when compared with the fresh unprimed seeds. Priming deteriorated seeds promoted early and uniform emergence. While for *B. speciosus* and *E. caffra* the effects were mostly significant for cathodic water, the other priming solutions had smaller effects that were often not significant. In *C. erythrophyllum*, while priming tended to improve emergence, the effects were mostly not significant. In contrast, priming fresh seeds only resulted in small increases in total emergence in all species.

Table 5.1 Effects of cathodic water, calcium magnesium solution and deionized water treatments on the emergence of *Bolusanthus speciosus*, *Combretum erythrophyllum* and *Erythrina caffra*

EMERGENCE		FSC	FSP.CW	FSP.CM	FSP.DW	ASC	ASP.CW	ASP.CM	ASP.DW	LSD
First day of emergence	*B. speciosus*	6.0±0.8^{abc}	4.5±0.5^{a}	5.0±0.6^{ab}	7.0±0.6^{abcd}	9.5±1.0^{e}	7.0±1.0^{abcd}	8.0±0.8^{bcd}	8.5±0.5^{cd}	2.2±1.05
Emergence %		85.0±5.0^{cd}	100.0±0.0^{d}	90.0±5.8^{d}	90.0±5.8^{d}	30.0±5.8^{a}	60.0±8.2^{bc}	50.0±5.8^{ab}	50.0±5.8^{ab}	16.6±8.04
Mean emergence time		3.3±0.1^{c}	4.6±0.041^{d}	3.6±0.2^{c}	3.3±0.2^{c}	0.7±0.2^{a}	2.3±0.3^{b}	1.5±0.3^{ab}	1.6±0.2^{ab}	0.6±0.30
Emergence index		1.6±0.1^{d}	2.6±0.1^{e}	1.7±0.2^{d}	1.4±0.1^{cd}	0.3±0.1^{a}	1.0±0.1^{bc}	0.6±0.1^{ab}	0.6±0.1^{ab}	0.3±0.16
Uniformity of emergence		0.023±0.001^{d}	0.037±0.001^{e}	0.024±0.001^{d}	0.021±0.002^{cd}	0.010±0.001^{a}	0.016±0.001^{bc}	0.012±0.002^{ab}	0.012±0.001^{ab}	0.004±0.002
First day of emergence	*C. erythrophyllum*	12.5±0.5^{a}	11.5±0.3^{a}	12.8±0.6^{a}	11.8±0.5^{a}	15.8±1.1^{b}	13.0±0.6^{ab}	13.0±0.4^{ab}	12.8±0.6^{a}	1.8±0.88
Emergence %		60.0±8.2^{bc}	70.0±5.8^{c}	60.0±8.2^{bc}	45.0 ±9.6^{abc}	25.0±5.0^{a}	45.0±5.0^{abc}	35.0±5.0^{ab}	30.0±5.8^{ab}	19.6±9.57
Mean emergence time		2.3±0.3^{bc}	2.8±0.3^{c}	2.5±0.4^{bc}	1.9±0.5^{abc}	0.7±0.229^{a}	1.5±0.2^{abc}	1.3±0.2^{abc}	1.2±0.3^{ab}	0.9±0.43
Emergence index		1.2±0.2^{bc}	1.5±0.2^{c}	1.3±0.2^{bc}	1.1±0.3^{abc}	0.3±0.111^{a}	0.7±0.095^{abc}	0.7±0.128^{abc}	0.6±0.144^{ab}	0.5±0.25
Uniformity of emergence		0.005±0.0002^{b}	0.006±0.0003^{b}	0.005±0.0004^{b}	0.005±0.0005^{b}	0.004±0.0003^{a}	0.004±0.0002^{ab}	0.004±0.0002^{ab}	0.005±0.0002^{ab}	0.001±0.0004
First day of emergence	*E. caffra*	5.3±0.3^{ab}	4.5±0.3^{a}	4.8±0.3^{ab}	4.8±0.3^{ab}	6.8±0.3^{c}	5.8±0.3^{bc}	6.5±0.3^{c}	6.8±0.3^{c}	0.8±0.37
Emergence %		100.0±0.0^{b}	100.0±0.0^{b}	100.0±0.0^{b}	100.0±0.0^{b}	40.0±0.0^{a}	50.0±5.8^{a}	45.0 ±5.0^{a}	50.0±5.8^{a}	9.9±4.79
Mean emergence time		3.6±0.1^{b}	4.6±0.1^{c}	4.2±0.2^{bc}	4.1±0.1^{bc}	1.0±0.074^{a}	1.5±0.1^{a}	1.4±0.2^{a}	1.3±0.2^{a}	0.4±019
Emergence index		2.5±0.1^{b}	4.1±0.2^{d}	3.3±0.3^{c}	3.2±0.2^{bc}	0.6±0.060^{a}	1.0±0.092^{a}	0.9±0.139^{a}	0.8±0.119^{a}	0.4±0.22
Uniformity of emergence		0.059±0.004^{b}	0.136±0.012^{d}	0.095±0.013^{c}	0.086±0.007^{bc}	0.020±0.001^{a}	0.025±0.001^{a}	0.023±0.002^{a}	0.022±0.002^{a}	0.02±0.01

There were eight treatments: FSC = fresh seeds that were neither aged nor primed; ASC= seeds that were aged but not primed; FSP.CW = fresh seeds that were not aged but primed with cathodic water; FSP.CM = fresh seeds that were not aged but primed with calcium magnesium solution; FSP.DW = fresh seeds that were not aged but primed with deionized water; ASP.CW = aged seeds that were prime with cathodic water; ASP.CM = aged seeds that were prime with calcium magnesium solution; ASP.DW = aged seeds that were primed with deionized water. ANOVA was performed across treatments with the means of replicate separated at $LSD_{0.05}$. Post hoc was done using Tukey test. Means along the same row with different letters were significantly different ($p<0.05$, n=32).

Table 5.2 Effects of cathodic water, calcium magnesium solution and deionized water treatments on the root length, stem length, number of leaves and leaf area of *Bolusanthus speciosus*, *Combretum erythrophyllum* and *Erythrina caffra*

	Bolusanthus speciosus				***Combretum erythrophyllum***				***Erythrina caffra***			
TREATMENT	Root Length (cm)	Stem Length (cm)	Number of Leaves	Leaf Area (cm^2)	Root Length (cm)	Stem Length (cm)	Number of Leaves (cm)	Leaf Area (cm^2)	Root Length (cm)	Stem Length (cm)	Number of Leaves	Leaf Area (cm^2)
FSC	34.3 ± 0.6^{d}	13.0 ± 0.8^{c}	13.0 ± 0.7^{bc}	58.3 ± 4.2^{bc}	36.0 ± 1.1^{bcd}	16.5 ± 1.7^{c}	15.0 ± 1.1^{ab}	59.0 ± 1.1^{d}	25.8 ± 1.1^{c}	17.5 ± 1.0^{cde}	12.8 ± 0.5^{b}	716.2 ± 15.4^{d}
FSP.CW	33 ± 1.1^{cd}	12.8 ± 0.3^{c}	16.0 ± 0.7^{d}	112.4 ± 5.3^{e}	41.3 ± 0.3^{d}	21.5 ± 1.3^{d}	22.5 ± 0.6^{c}	78.8 ± 0.6^{f}	28.5 ± 0.6^{cd}	20.9 ± 1.4^{e}	16.0 ± 0.4^{c}	779.4 ± 17.2^{d}
FSP.CM	33 ± 0.7^{cd}	14.0 ± 0.7^{c}	12.0 ± 0.4^{bc}	72.6 ± 4.2^{cd}	32.8 ± 1.0^{bc}	16.6 ± 0.7^{c}	16.5 ± 0.6^{b}	77.9 ± 2.0^{f}	24.8 ± 0.5^{c}	17.0 ± 0.7^{cd}	13.8 ± 0.3^{bc}	739.8 ± 26.0^{d}
FSP.DW	36.5 ± 2^{d}	14.3 ± 0.2^{c}	13.8 ± 0.6^{cd}	74.1 ± 2^{d}	36.3 ± 1.4^{cd}	12.5 ± 0.6^{bc}	15.3 ± 0.3^{ab}	69.3 ± 1.4^{e}	30.5 ± 1.0^{d}	19.3 ± 0.6^{de}	15.0 ± 0.4^{bc}	736.2 ± 14.6^{d}
ASC	18.3 ± 1.1^{a}	7.5 ± 0.2^{a}	8.5 ± 0.6^{a}	21.2 ± 1.3^{a}	18.5 ± 0.6^{a}	7.2 ± 0.2^{a}	11.5 ± 0.6^{a}	20.5 ± 0.5^{a}	11.9 ± 0.9^{a}	9.9 ± 0.4^{a}	7.3 ± 0.5^{a}	86.3 ± 0.6^{a}
ASP.CW	31.3 ± 1.3^{bcd}	12.5 ± 1.2^{c}	11.5 ± 0.6^{bc}	58.0 ± 1.8^{bc}	30.8 ± 0.5^{b}	12.1 ± 1.1^{bc}	14.3 ± 0.8^{ab}	45.9 ± 1.7^{c}	26.3 ± 0.6^{c}	14.3 ± 0.5^{bc}	13.5 ± 0.6^{b}	642.7 ± 9.8^{c}
ASP.CM	28.5 ± 1.2^{bc}	9.0 ± 0.9^{ab}	10.5 ± 0.3^{ab}	31.0 ± 2.1^{a}	21.9 ± 2.2^{a}	11.4 ± 0.7^{ab}	14.2 ± 0.8^{ab}	27.0 ± 1.7^{b}	18.8 ± 1.0^{b}	12.6 ± 0.6^{ab}	8.5 ± 0.6^{a}	122.7 ± 1.2^{a}
ASP.DW	25.7 ± 1.2^{b}	12.0 ± 0.4^{bc}	8 ± 0.4^{a}	50.5 ± 2^{b}	23.9 ± 1.1^{a}	8.5 ± 0.6^{ab}	13.5 ± 1.4^{ab}	21.4 ± 1.3^{ab}	18.5 ± 1.0^{b}	12.0 ± 0.5^{ab}	9.5 ± 0.6^{a}	214.1 ± 17.1^{b}
$LSD_{0.05}$	3.6±1.7	2.0±1.0	1.7±0.8	9.3±4.5	3.4±1.6	2.9±1.4	2.4±1.2	4.0±2.0	2.5±1.2	2.3±1.1	1.5±0.7	43.9±21.3

There were eight treatments: FSC = fresh seeds that were neither aged nor primed; ASC= seeds that were aged but not primed; FSP.CW = fresh seeds that were not aged but primed with cathodic water; FSP.CM = fresh seeds that were not aged but primed with calcium magnesium solution; FSP.DW = fresh seeds that were not aged but primed with deionized water; ASP.CW = aged seeds that were prime with cathodic water; ASP.CM = aged seeds that were prime with calcium magnesium solution; ASP.DW = aged seeds that were primed with deionized water. ANOVA was performed across treatments with the means of replicate separated at $LSD_{0.05}$. Post hoc was done using Tukey test. Means along the same row with different letters were significantly different ($p<0.05$, n=32).

Table 5.3 Effect of cathodic water, calcium magnesium solution and deionized water treatment on the root mass, stem mass, leaves mass, shoot mass, total biomass, and shoot/root ratio of *Bolusanthus speciosus*, *Combretum erythrophyllum* and *Erythrina caffra*

DRY MASS (g plant^{-1})		**FSC**	**FSP.CW**	**FSP.CM**	**FSP.DW**	**ASC**	**ASP.CW**	**ASP.CM**	**ASP.DW**	**$LSD_{0.05}$**
Root mass	*B. speciosus*	0.12±0.004^{cd}	0.20±0.013^{e}	0.15±0.013^{de}	0.18±0.015^{e}	0.04±0.004^{a}	0.12±0.009^{bcd}	0.07±0.008^{ab}	0.10±0.011^{bc}	0.03±0.01
Stem mass		0.13±0.008^{b}	0.20±0.003^{c}	0.13±0.004^{b}	0.12±0.030^{b}	0.05±0.001^{a}	0.11±0.004^{b}	0.09±0.002^{ab}	0.10±0.002^{ab}	0.03±0.02
Leaf mass		0.19±0.006^{b}	0.28±0.016^{c}	0.31±0.007^{c}	0.30±0.036^{c}	0.10±0.004^{a}	0.19±0.007^{b}	0.11±0.003^{a}	0.16±0.006^{ab}	0.04±0.02
Shoot mass		0.32±0.012^{cd}	0.48±0.014^{e}	0.44±0.009^{e}	0.42±0.066^{de}	0.16±0.004^{a}	0.30±0.009^{bc}	0.20±0.003^{ab}	0.26±0.004^{abc}	0.07±0.03
Total biomass		0.45±0.014^{c}	0.68±0.023^{d}	0.59±0.019^{d}	0.59±0.068^{d}	0.20±0.007^{a}	0.41±0.011^{c}	0.27±0.009^{ab}	0.37±0.012^{bc}	0.08±0.04
Shoot/root ratio		2.70±0.091^{a}	2.44±0.158^{a}	2.93±0.198^{a}	2.38±0.371^{a}	3.60±0.318^{a}	2.60±0.260^{a}	2.91±0.357^{a}	2.69±0.298^{a}	0.80±0.39
Root mass	Co. erythrophyllum	0.34±0.035^{c}	0.68±0.008^{e}	0.53±0.018^{d}	0.33±0.031^{c}	0.08±0.009^{a}	0.25±0.012^{c}	0.16±0.009^{ab}	0.16±0.004^{b}	0.05±0.03
Stem mass		0.20±0.025^{b}	0.51±0.041^{d}	0.39±0.018^{c}	0.25±0.019^{b}	0.06±0.004^{a}	0.19±0.006^{b}	0.09±0.006^{a}	0.05±0.003^{a}	0.06±0.03
Leaf mass		0.35±0.018^{b}	0.65±0.02^{c}	0.65±0.016^{c}	0.37±0.016^{b}	0.067±0.004^{a}	0.29±0.032^{b}	0.13±0.011^{a}	0.12±0.009^{a}	0.05±0.02
Shoot mass		0.55±0.027^{bc}	1.16±0.036^{e}	1.04±0.022^{d}	0.62±0.015^{c}	0.13±0.007^{a}	0.48±0.034^{b}	0.23±0.016^{a}	0.18±0.008^{a}	0.07±0.03
Total biomass		0.90±0.061^{cd}	1.84±0.043^{f}	1.57±0.036^{e}	0.96±0.039^{d}	0.20±0.009^{a}	0.74±0.025^{c}	0.38±0.025^{b}	0.34±0.007^{ab}	0.10±0.05
Shoot/root ratio		1.66±0.096^{ab}	1.70±0.037^{ab}	1.95±0.048^{b}	1.91±0.177^{b}	1.74±0.328^{ab}	1.93±0.224^{b}	1.45±0.024^{ab}	1.07±0.070^{a}	0.47±0.23
Root mass	E. caffra	0.87±0.106^{cd}	0.82±0.029^{bcd}	0.85±0.072^{cd}	1.08±0.153^{d}	0.26±0.025^{a}	0.73±0.035^{bcd}	0.55±0.085^{abc}	0.46±0.058^{ab}	0.24±0.12
Stem mass		1.06±0.074^{bcd}	1.56±0.149^{d}	1.30±0.181^{cd}	1.37±0.144^{cd}	0.20±0.038^{a}	0.93±0.170^{bc}	0.27±0.038^{a}	0.56±0.069^{ab}	0.35±0.17
Leaf mass		2.02±0.175^{bc}	3.31±0.202^{d}	2.26±0.163^{bc}	2.70±0.302^{cd}	0.21±0.041^{a}	1.46±0.247^{a}	0.42±0.064^{b}	0.58±0.075^{a}	0.53±0.26
Shoot mass		3.08±0.208^{bc}	4.88±0.306^{d}	3.56±0.308^{bc}	4.07±0.434^{cd}	0.41±0.068^{a}	2.39±0.292^{b}	0.69±0.101^{a}	1.15±0.140^{a}	0.76±0.37
Total biomass		3.95±0.225^{bc}	5.70±0.278^{d}	4.41±0.355^{bcd}	5.15±0.579^{cd}	0.68±0.089^{a}	3.12±0.274^{b}	1.24±0.159^{a}	1.61±0.197^{a}	0.88±0.43
Shoot/root ratio		3.75±0.596^{c}	6.01±0.594^{d}	4.20±0.293^{cd}	3.83±0.245^{c}	1.57±0.176^{ab}	3.34±0.524^{bc}	1.32±0.190^{a}	2.50±0.086^{abc}	1.13±0.55

xThere were eight treatments: FSC = fresh seeds that were neither aged nor primed; ASC= seeds that were aged but not primed; FSP.CW = fresh seeds that were not aged but primed with cathodic water; FSP.CM = fresh seeds that were not aged but primed with calcium magnesium solution; FSP.DW = fresh seeds that were not aged but primed with deionized water; ASP.CW = aged seeds that were prime with cathodic water; ASP.CM = aged seeds that were prime with calcium magnesium solution; ASP.DW = aged seeds that were primed with deionized water. ANOVA was performed across treatments with the means of replicate separated at $LSD_{0.05}$. Post hoc was done using Tukey test. Means along the same row with different letters were significantly different ($p<0.05$, n=32).

5.3.2 Effect of priming on seedling growth

Priming fresh seeds with all solutions tended to increased growth parameters (Tables 5.2, 5.3). In general, cathodic water was most effective, although the differences between cathodic water and the other solutions were not always significant. The increases in total dry mass were significant for all priming solutions for *B. speciosus* and *C. erythrophyllum*, but only for cathodic water for *E. caffra*. Controlled deterioration of seeds significantly reduced the subsequent growth of plants derived from the deteriorated seeds. For example, root length was typically reduced by *c*. 50% (Table 5.2). The numbers of leaves were reduced to 23% in *Co. erythrophyllum*, 35% in *B speciosus* and 43% in *E. caffra* (Table 5.2). The biomasses of the individual plant parts and the total biomasses of all species were very significantly reduced in seedling derived from deteriorated seeds (Table 5.3). Invigorating deteriorated seeds with any of priming solutions greatly increased the growth parameters of all species. Most of the improvements were significant for the seeds primed with cathodic water, while the improvement in the seeds primed with CaMg solution and deionised water treatments were smaller, and not always significant (Table 5.3). In some cases, the parameters were more than double compared with plants derived from unprimed aged seeds

5.3.3 Effect of priming on photosynthetic parameters

For all species, seedlings derived from deteriorated rather than fresh seeds had significantly lower chlorophyll contents, photochemical efficiencies, and rates of photosynthesis and transpiration in all species (Figures 5.1, 5.2). While having little effect on fresh seeds, priming tended to increase these parameters in deteriorated seeds. In *B. speciosus*, all effects of cathodic water were significant, while the effects of the other priming solutions tended to be less and were not always significant. In the other two species, the effects of priming were smaller and usually not significant, although general, cathodic water gave the best results.

5.3.4 Effect of priming on membrane stability and the levels of oxidized lipids

In fresh seeds, priming tend to increase the MSI significantly for cathodic water and distilled water in *B. speciosus*, and cathodic water in *C. erytrophyllum*. Primed fresh seeds had significantly lower MDA levels than un-primed seeds in *B. speciosus* and *C. erytrophyllum*, with cathodic water-reducing MDA levels significantly more than the other priming solutions. *E. caffra* priming did not affect MDA levels in fresh seeds. Priming fresh seeds with cathodic water significantly reduced 4-HNE in all species, but the other priming solutions had no significant effect. Controlled deterioration of the seeds of *C. erythrophyllum* and *E. caffra* significantly reduced their MSI compared with fresh seeds, but the reduction was not smaller and not significant in *B. speciosus* (Figure 5.3). For all species, deterioration significantly increased the levels of the lipid peroxidation products MDA and 4-HNE (Figure 5.4). The MSI of deteriorated seeds primed with all solutions had significantly better MSI than un-primed seeds, except in *B. speciosus* where only cathodic water had a significant effect (Figure 5.3). All priming solutions significantly reduced the amounts of lipid peroxidation product MDA in the seeds, and cathodic water was significantly more effective than the other priming solutions for *C. erythrophyllum* and *B. speciosus* (Figure 5.4). However, priming only reduced 4-HNE levels in *E. caffra*, and all solutions were equally effective.

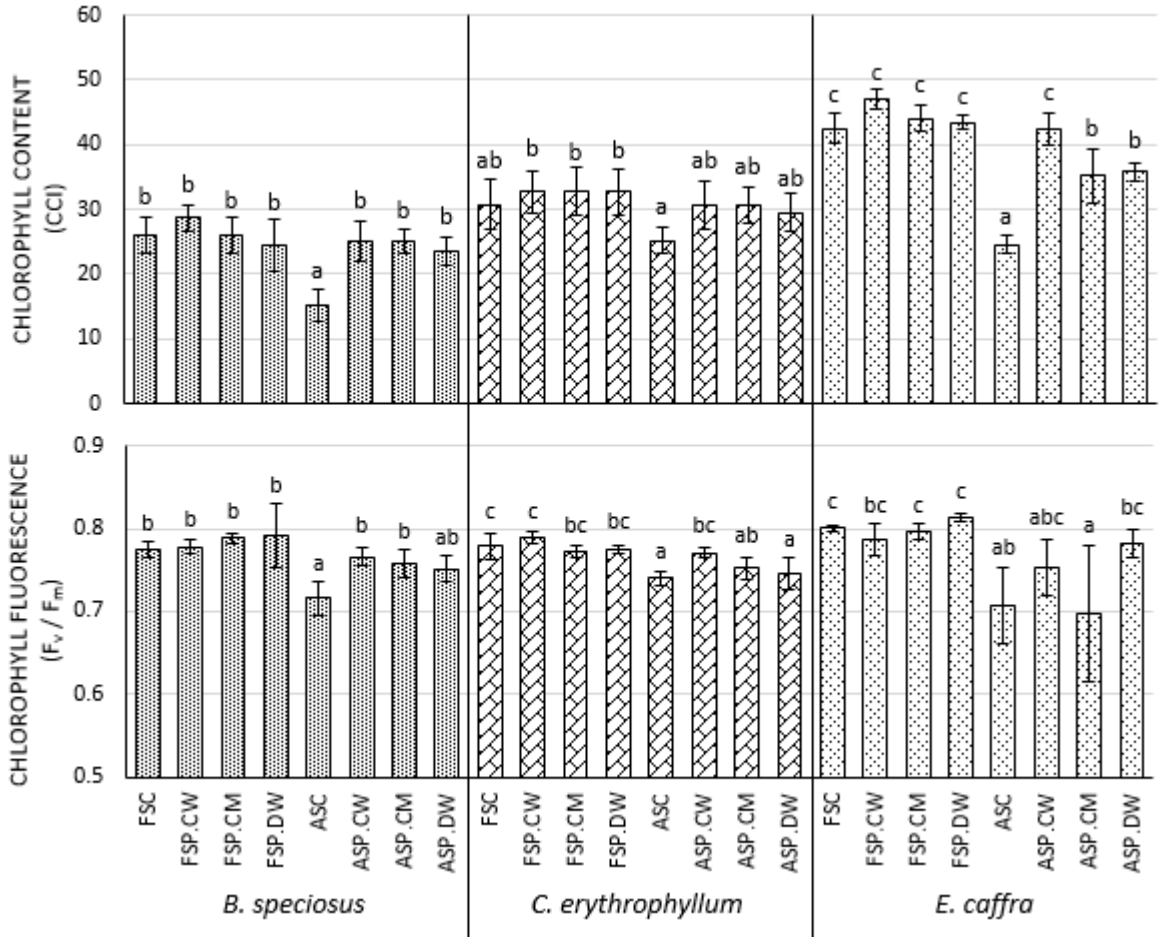

Figure 5.1 Effect of cathodic water, calcium magnesium solution and deionized water seed invigoration on the chlorophyll fluorescence and chlorophyll content in the leaves of *Bolusanthus speciosus*, *Combretum erythrophyllum* and *Erythrina caffra*.

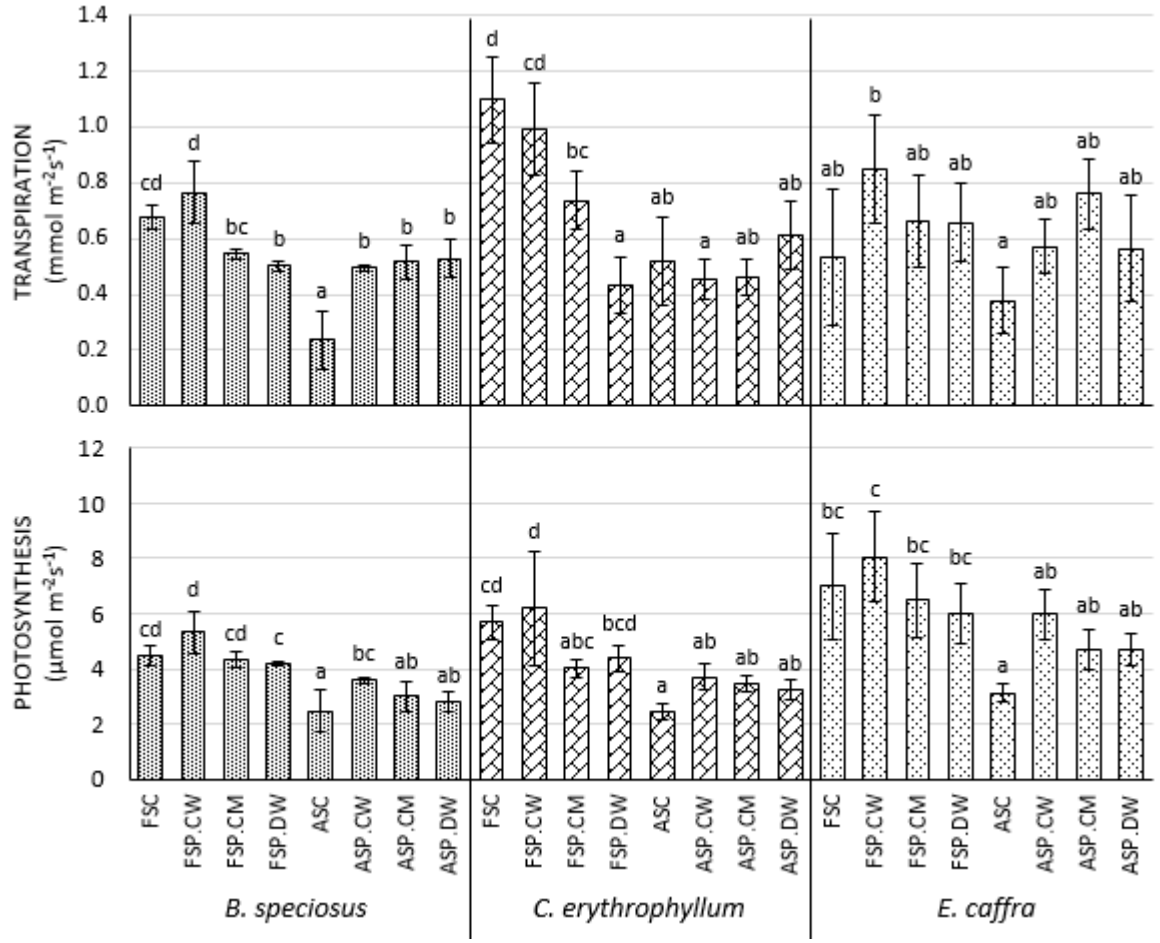

Figure 5.2 Effects of cathodic water seed invigoration on the photosynthesis of *Bolusanthus speciosus, Combretum erythrophyllum* and *Erythrina caffra*

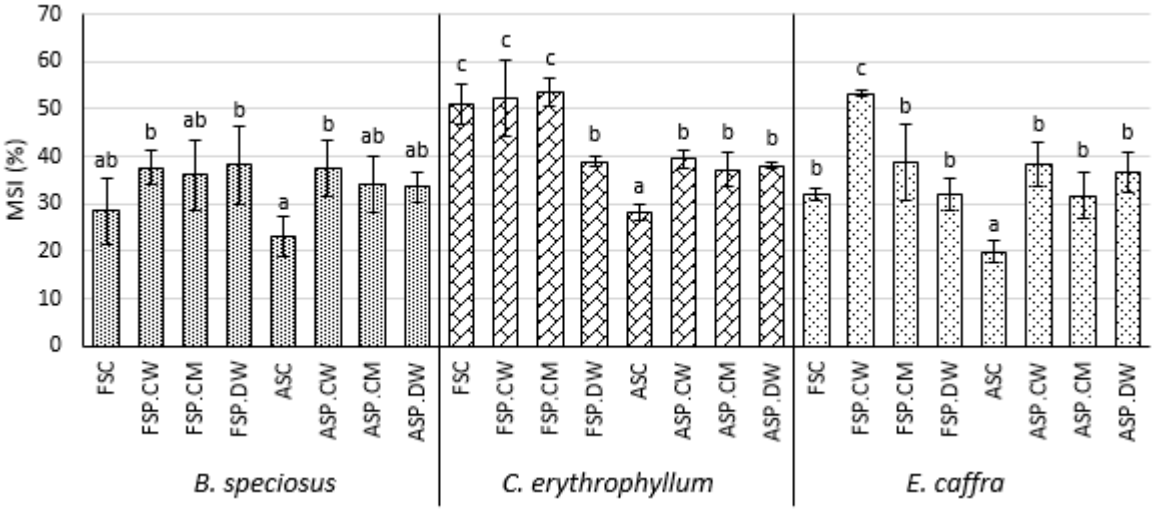

Figure 5.3 Effects of controlled deterioration and cathodic water seed invigoration on the membrane stability index of *Bolusanthus speciosus, Combretum erythrophyllum* and *Erythrina caffra*

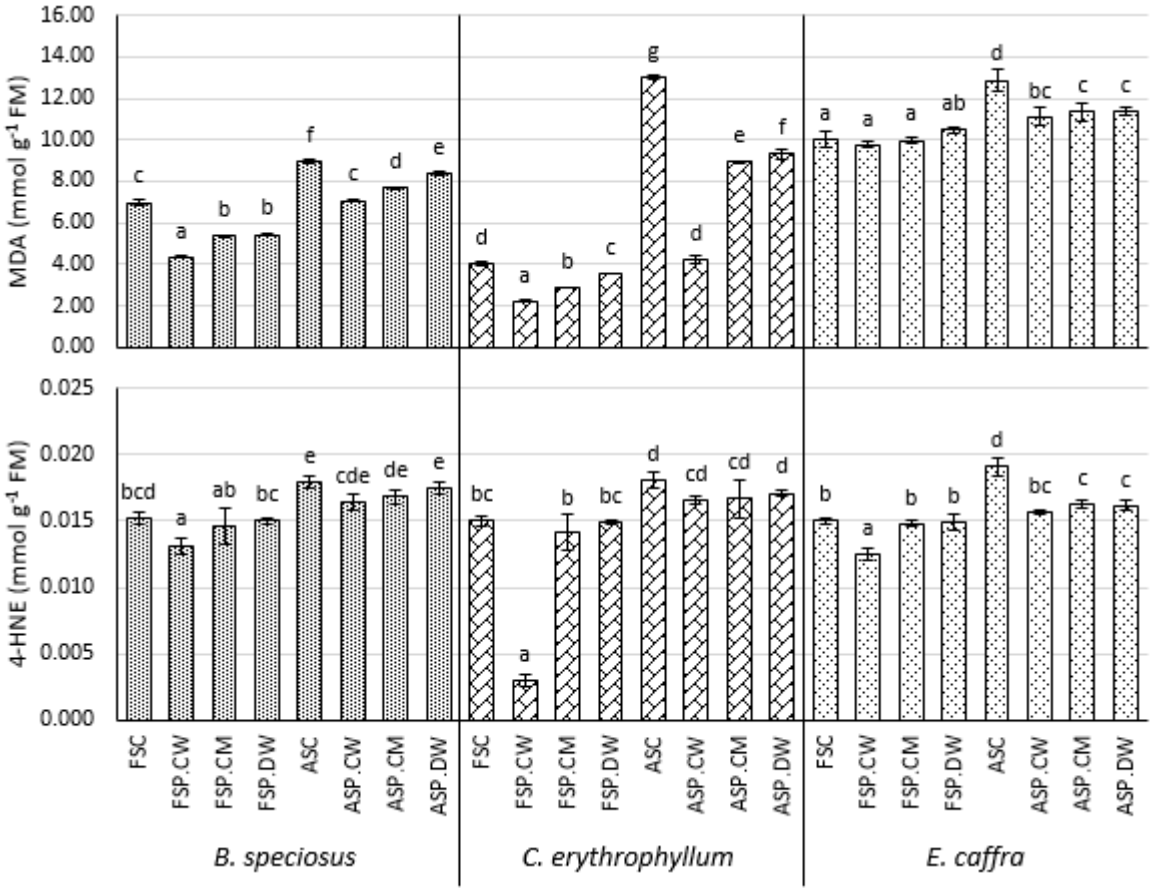

Figure 5.4 Effects of controlled deterioration of seeds and cathodic water seed invigoration on MDA and 4-HNE contents in the seeds of *Bolusanthus speciosus, Combretum erythrophyllum* and *Erythrina caffra*.

5.4 DISCUSSIONS

The main conclusions of the work presented here are that for all three of the species tested, priming with the strong reducing agent cathodic water significantly improves the growth of seedlings derived from deteriorated seeds. Furthermore, in one of the species, *B. speciosus*, the emergence of deteriorated orthodox seeds was also significantly increased by priming with cathodic water. While our earlier laboratory studies showed that cathodic water could improve the germination of deteriorated seeds (Fatokun et al., 2020; Gondwe et al., 2016), here we show that the benefits extend to emergence and the later stages of seedlings development. The effects of cathodic water on the levels of lipid

peroxidation products are consistent with the view that cathodic water acts by reducing the levels of ROS in aged seeds. We suggest that priming with cathodic water can be recommended as a useful tool to improve the conservation of orthodox seeds.

5.4.1 Effects of priming on emergence

In all species, controlled deterioration delayed seedling emergence and reduced the uniformity of emergence and the emergence index (Table 5.1). The first day of emergence was delayed by 3 d in *B. speciosus* and *C. erythrophyllum* and about 2 d in *E. caffra*. In *B. speciosus* priming deteriorated seeds promoted early and uniform emergence. The effects were most significant for cathodic water, while the other priming solutions had smaller effects that were often not significant. In the other two species, while priming tended to improve the emergence parameters, the effects were smaller and mostly not significant. Although the effects of cathodic water on emergence have not been tested before, priming with other solutions has been shown to improve emergence parameters. For example, priming was reported to promote the earlier emergence of seedlings in *Oryza sativa* (Wang et al., 2018) and *Zea mays* (Ghassemi-Golezani et al., 2011). A particularly important emergence parameter is uniformity, because uniformity can improve stand establishment and increase biomass, particularly when conditions are suboptimal, such as during drought, salinity and water stress (Ghassemi-Golezani et al., 2011; Wang et al., 2018). Uniformity of emergence can also reduce weed-inflicted yield loss, for example, by up to 10% in rice (Farooq et al., 2006). While the improvement in emergence in *C. erythrophyllum* and *E. caffra* were too small to be significant, priming with cathodic water significantly improves emergence in aged *B. speciosus*.

5.4.2 Effect of priming on seedling growth

Priming fresh seeds with cathodic water and other solutions resulted in small and in most cases not significant, increases in the growth of the resulting seedlings (Tables 5.2, 5.3). The small improvements that occurred may be due to the repair of the natural deterioration that occurred before harvesting and during seed storage (Andreev et al., 2004; Kranner et

al., 2007). Seedlings derived from seeds subjected to controlled deterioration displayed significantly reduced growth (Tables 5.2, 5.3). Invigoration of deteriorated seeds with all priming solutions resulted in better seedling growth for all species (Tables 5.2, 5.3). Most of the improvements were significant for the seeds primed with cathodic water, while the improvement in the seeds primed with the other priming solutions was smaller and often not significant. The improvement in seedling growth in plants derived from primed seeds may have occurred due to increase in the activities of enzymes such as *α-amylase*. Such increases in enzymatic activities have been reported to promote the hydrolysis of starch into soluble sugars for seed respiration and better growth (Fatokun et al., 2020). Interestingly, while priming only improved emergence in one of the species tested here (Table 5.1), cathodic water significantly improved the growth parameters of seedlings derived from deteriorated seeds in all species tested here.

5.4.3 Effects of priming on photosynthetic parameters

In all species, seed deterioration significantly reduced the chlorophyll contents, photochemical efficiencies, and rates of photosynthesis and transpiration in the resulting seedlings (Figures 5.1, 5.2). While having little effect on fresh seeds, priming tended to increase these parameters in deteriorated seeds. In *B. speciosus*, all effects of cathodic water were significant, while the effects of the other priming solutions tended to be less and were not always significant. In the other two species, the effects of priming were smaller and usually not significant, although in general, cathodic water gave the best results. Although not tested for cathodic water, priming with other solutions is well known to improve photosynthesis in the resulting seedlings, e.g., Anwar et al. (2020) (for review see Pawar and Laware (2018)). Theoretically, priming may have increased the capacity for photosynthetic electron transport, or possibly a greater investment in enzymes of the Calvin cycle (Osmond et al., 1997). While we did not study the mechanisms of the improvement in photosynthesis in detail, the net result of the general improvements in photosynthesis was an increase in plant growth (Tables 5.2, 5.3)

5.4.4 Effects on membrane leakage and lipid peroxidation products

Controlled deterioration significantly reduced the MSI of *C. erythrophyllum* and *E. caffra*, but not *B. speciosus* (Figure 5.3). For all species, deterioration significantly increased the levels of the lipid oxidation products MDA and 4-HNE (Figure 5.4). All priming solutions significantly increased the MSI, except in *B. speciosus* where only cathodic water had a significant effect (Figure 5.3). All priming solutions significantly reduced the amounts of lipid peroxidation product MDA in the seeds, and cathodic water was significantly more effective than the other priming solutions for *C. erythrophyllum* and *E. caffra* (Figure 5.4). However, priming only reduced levels of the other peroxidation product we tested, 4-HNE, in *E. caffra*, and all solutions were equally effective. Lipid peroxidation and MSI are intimately linked, as lipid peroxidation is well known to increase membrane permeability (Simon, 1974). There have been numerous studies that have shown that priming reduces oxidative stress in seeds (for review see Lutts et al., 2016). It is possible that priming up-regulates ROS scavenging enzymes in the seeds. For example, Kubala et al. (2013, 2015) reported that priming seeds of *Spinacia oleracea* up-regulates APX, SOD, CAT and GR. Kibinza et al. (2011) showed that during the ageing of *Helianthus annuus* seeds H_2O_2 accumulated and the expression of CAT was reduced; priming both invigorated the seeds increased the expression and activity of CAT. Alternatively, or perhaps additionally, the priming-induced reductions in lipid oxidation products and increases in MSI may have been because priming more generally reduced ROS formation (Fallah et al., 2018). For cathodic water, this may have resulted from direct reactions of the priming solutions with ROS, but as other priming solutions were also beneficial, reductions in ROS may have occurred by other ways as yet unknown. The precise mechanisms whereby priming increased MSI and reduced lipid peroxidation were not investigated in the present study. However, while priming with all solutions reduced oxidative stress, and improved the MSI cathodic water tended to have a slighter stronger ameliorative effect, particularly in its ability to reduce MDA formation (Figure 5.4). This may explain why the strongly reducing

cathodic water was better overall at improving the growth of seedlings derived from deteriorated seeds.

5.4.5 Conclusions

Our original motivation for testing the effectiveness of cathodic water in germplasm conservation came from an earlier study, in which the deterioration of maize seed was counteracted by cathodic protection (Pammenter et al., 1974). In that study, maize seeds were placed on an aluminium foil disc with the disc attached to the cathode of a power pack (Berjak, 1978; Pammenter et al., 1974). However, a solution of cathodic water is more practical to use than foil discs, and in later experiments, we showed that cathodic water can improve the cryopreservation of the embryonic axes and embryos of recalcitrant seeds (Berjak et al., 2011; Naidoo, 2010) and shoot tips (Gebashe et al., 2015), and could improve the germination of orthodox seeds (Fatokun et al., 2020; Gondwe et al., 2016). In the work presented here, we showed that cathodic water is a particularly effective priming agent for improving the emergence and subsequent seedling growth of deteriorated seeds. While all priming solutions were capable of some measure of invigoration, priming with cathodic water was most effective, and results suggested that the benefits probably derived from its strong antioxidant capacity. The deterioration of seeds in seed banks is of global concern, as it affects the long-term conservation of genetic diversity of both wild species and agricultural plants (Zhang et al., 2016), essential for future breeding programs. In the future, it will be necessary to produce varieties that perform well under future climate change scenarios, particularly in sub-Saharan Africa where the effects of climate change are likely to be severe (FAO, 2018; Jorgenson and Burns, 2007). Results presented here show that priming deteriorated seeds with cathodic can be an effective means of improving the conservation of *Bolusanthus speciosus, Combretum erythrophyllum* and *Erythrina caffra* and by extension some other orthodox seeded species that may be tested in future.

CHAPTER 6

INFLUENCE OF CATHODIC WATER INVIGORATION ON THE EMERGENCE AND SUBSEQUENT GROWTH OF CONTROLLED DETERIORATED PEA AND PUMPKIN SEEDS

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Abstract

The quality of seeds in gene banks gradually deteriorates during long term storage, which is probably at least in part a result of the progressive development of oxidative stress. Here, we report a greenhouse study that was carried out to test whether a novel approach of seed invigoration using priming with cathodic water (cathodic portion of an electrolysed calcium-magnesium solution) could improve seedling emergence and growth in two deteriorated crop seeds. Fresh seeds of *Pisum sativum* and *Cucurbita pepo* were subjected to controlled deterioration to 50% viability at 14% seed moisture content (fresh weight basis), 40°C and 100% relative humidity. The deteriorated seeds were thereafter primed with cathodic water, calcium magnesium solution and deionized water. In addition, to study the mechanism of the impacts of invigoration, the effects of such priming on the lipid peroxidation products malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) and the reactive oxygen species (ROS) scavenging enzymes superoxide dismutase, and catalase were also determined in the fresh and deteriorated seeds. All priming treatments improved seed emergence parameters, subsequent seedling photosynthesis and growth relative to the unprimed seeds. In general, cathodic water was most effective at invigorating deteriorated seeds. Analysis of the lipid peroxidation products and antioxidant enzyme activities in invigorated seeds provided support for the hypothesis that the effectiveness of cathodic water in the invigoration of debilitated orthodox seeds in general, and pea and pumpkin seeds in particular, derive from its ability to act as an antioxidant.

Keywords: Antioxidant enzymes, cathodic water, controlled deterioration, invigoration, seedling emergence, viability

6.1 INTRODUCTION

Orthodox seeds can be effectively conserved in seeds banks, but even during careful storage eventually deteriorate in vigour and viability (Desheva, 2016; Garza-Calgaris et al., 2012). The deterioration of seeds in gene banks is of global concern, as it affects the long-term conservation of genetic diversity of both wild and agricultural plants (Andjelkovic et al., 2018; Skorupińska 2019), essential for future breeding programs. In the future, it will be necessary to produce varieties that perform well under future climate change scenarios, particularly in sub-Saharan Africa where the effects of climate change are likely to be severe (FAO, 2018; Jorgenson and Burns, 2007). Deterioration of seeds can cause reduced or complete loss of seedling emergence on the field (Amanpour-Balaneji et al., 2012; Sreepriya and Girija, 2019; Ullah et al., 2019). Furthermore, "hangover effects" of deterioration may be carried through to later growth stages of the plant. Seedlings of deteriorated seeds may display reduced photosynthesis and transpiration (Sershen et al., 2010; Wang et al. 2019), slower growth and ultimately lower yields (Chhabra et al., 2019). Thus, even if poor quality seeds germinate or emerge, the quality of plants generated from such seeds is not guaranteed (Amanpour-Balaneji et al., 2012; Sershen et al., 2010; Wang et al., 2019).

The "oxidative stress" model for seed deterioration suggests that deterioration is a result of the continued production of reactive oxygen species (ROS) such as superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\cdot OH$) (Junio da Silva et al., 2018; Sahu et al., 2017). These ROS damage many biomolecules, and in particular cause cellular dysfunction through lipid peroxidation (Sahu et al., 2017; Junio da Silva et al., 2018). Furthermore, when the moisture content of seeds rises above c. 14%, for example during early stages of imbibition, lipid peroxidation also occurs through the activities of the enzyme lipoxygenase (Chandrakar et al., 2016). This enzyme causes the release of the cytotoxic lipid peroxidation products such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) (Sahu et al., 2017). Seeds possess housekeeping enzymes which ameliorate the cytotoxic effects of ROS and lipid peroxidation products on seeds, including superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX). In addition, they also contain non-enzymatic antioxidants such as ascorbic acid, glutathione, flavonoids and α-tocopherols (Karalija and Selović, 2018; Sahu et al., 2017). As seeds age/deteriorate, ROS production increases and anti-oxidative mechanisms are overwhelmed as biomolecules become oxidized, and the result is poor seedling emergence on the field and poor subsequent seedling growth and productivity (Bhattacharya et al., 2019; Thirusendura and Saraswathy, 2018).

While seed companies and seed banks try to reduce deterioration, for example by storing seeds at low temperatures and low humidity, ways of improving or “invigorating” seeds that have already become partly deteriorated are regularly investigated (Bhattacharya et al., 2019; Sreepriya and Girija, 2019). One approach is “seed priming”, first proposed by Heydecker (1973), involves hydrating seeds to a point that allows pre-germinative metabolism to start, but it is not high enough to allow actual radicle emergence (to avoid seed embryos becoming desiccation-sensitive). Seeds are then either re-dried until they return to their original dry weight for later planting (Ashraf and Foolad, 2005; Bradford, 1986; Fatokun et al., 2020; Heydecker and Coolbear, 1977) or are planted immediately (Ella et al., 2011). Priming agents used for seed invigoration include water (hydro priming), inorganic salt (halo priming), solid matrices (matrix priming) and plant nutrients (nutrient seed priming) (Fatokun et al., 2020; Umair et al., 2017). Where plant mineral nutrients are used for seed priming, besides ameliorating the impact of lipid peroxidation, the nutrients have been reported as providing a transient source of plant nutrition at the early stages of the plant growth (Muhammad et al., 2017; Umair et al., 2017).

While the mechanism of seed priming is uncertain, considering that the oxidative stress model for seed ageing associated with uncontrolled and unbalanced production of ROS, priming probably reduces the production of ROS or reduces their harmful effects. We therefore hypothesised that priming with solutions containing an external supply of antioxidants might be particularly effective in reinvigorating deteriorated seeds leading to earlier and higher field emergence and more vigorous seedling growth. In our previous studies on recalcitrant species (Berjak et al., 2011), cathodic water (CW) has shown itself to be remarkably efficacious in reducing ROS activity and permitting the production of 70% viable seedlings (root and shoot) from axes of cryopreserved *Strychnos gerrardii*, which had never before been achieved (Berjak et al., 2011). Also, exposure of explants to cathodic water has achieved what seemed to be impossible, viz. callus production (for indirect morphogenesis) of an endangered plant species, and has resulted in plantlet formation from 100% of nodal explants of a heavy-metal accumulator (Mycock et al., 2013). Our earlier work on orthodox species (Fatokun et al., 2020; Gondwe et al., 2016) studied the effects of priming seeds with cathodic water; results clearly showed that cathodic water could improve the germination of deteriorated seeds from a range of species.

In the work presented here, we used *Pisum sativum* and *Cucurbita pepo* to further investigate the benefits of priming or reinvigorating controlled deteriorated seeds. Specifically, we tested the ability of priming with cathodic water to improve seedling

emergence and subsequent seedling growth; unelectrolysed CaMg solution (nutrient priming) and deionised water (hydro priming) were investigated alongside cathodic water for comparison. In addition, to study the mechanism of invigoration, we tested the effects of priming on the lipid peroxidation and the activities of ROS scavenging enzymes in seeds at about the beginning of phase III of seed imbibition (da Silva Moura et al., 2016).

6.2 MATERIALS AND METHODS

The study was conducted in a greenhouse and the laboratories of the School of Life Sciences, University of KwaZulu-Natal, Westville Campus, Durban South Africa. The average temperature was 23.52°C and relative humidity: 67%.

6.2.1 Controlled deterioration of seeds

The initial water contents of the seeds used were determined gravimetrically on a fresh weight basis as 11.7% for *P. sativum* and 6.7% for pumpkin. Seed water contents were raised to 14% for both species in a vapour chamber, and the seeds were then subjected to controlled deterioration (CD; accelerated ageing) at 40°C and 100% relative humidity (RH) in a digital oven (Series 2000, Scientific, USA). Seeds were sampled from the oven at 4 d intervals and germination was tested. CD was continued until complete loss of germination and the time required for 50% inhibition of germination estimated.

6.2.2 Preparation of cathodic water and seed priming

A solution of 0.5 µM $CaCl_2.2H_2O$ and 0.5 mM $MgCl_2.6H_2O$ (Mycock, 1999) (CaMg solution) was autoclaved, 200 ml decanted into each of the two glass beakers, and platinum electrodes immersed in the solutions, the anode in one beaker and the cathode in another. The circuit was completed with an agar-based potassium chloride salt bridge (30% KCl), and the solution was electrolysed at 60 V potential difference using a Bio-Rad PowerPacTM Basic (Bio-Rad, USA) power pack for 1 hr at room temperature yielding anodic (oxidizing) water at pH 2.4, and cathodic (reducing) water at pH 11.2 (Berjak et al., 2011). The anodic water was discarded, and the cathodic water was used for invigoration of seeds within 1 hr.

The priming solutions used were: cathodic water; un-electrolysed CaMg solution and deionized water. Seeds (50 seeds per treatment) were hydrated by placing them between 20 layers of single-ply paper towel, which was placed on aluminium foil. To prime the seeds, 50 ml of the solutions were poured onto these paper towels. The aluminium foil containing the seeds were placed in plastic pouches, and after 24 hr, just before radicle

emergence, the seeds were dried back to their original masses under ambient laboratory conditions for 7 d and kept at 4°C in air-tight bottles until required.

6.2.3 Plant Management/ Experimental design

Potting mix and multi-feed fertilizer used in this study were purchased from Grovida, Durban, South Africa. In all, there were eight treatments (six seed priming treatments and two controls). The seed priming treatments were: aged seeds primed with cathodic water (ASP.CW); aged seeds primed with CaMg solution (ASP.CM); aged seeds primed with deionized water (ASP.DW); fresh seed (unaged) primed with cathodic water (FSP.CW); fresh seeds primed with CaMg solution (FSP.CM); fresh seeds primed with deionized water (FSP.DW) and two controls. The first control comprised seeds that were controlled deteriorated (CD) and unprimed (ASC) and the second control comprised fresh seeds that were neither subjected to control deterioration nor priming (FSC).

The plants were grown in 2 l pots containing 800 g of potting mix and watered as required. After CD and priming, five seeds were planted in each pot, with four pots per treatment arranged in a completely randomized design. Four weeks after planting plants were thinned to one plant per pot. Thinning was carried out by removing the weakest plants in the pots (Smith, 2008). Plants were supplied with nutrients once every two weeks with Grovida water-soluble “multi-feed fertilizer” (1 g l^{-1}) comprising N, P, K, S, and Mg at 193, 83, 153, 6.1 and 4.6 g kg^{-1} respectively. The fertilizer also contained the micronutrients Zn, B, Mo, Fe, Mn and Cu at 700, 1054, 63, 751, 273 and 75 mg kg^{-1}.

6.2.4 Seedling Emergence

Seedling emergence counts were taken daily until no further change was observed. The following parameters were measured: first day of emergence (FDE), final emergence percentage (FEP), mean emergence time (MET), emergence index (EI), and uniformity of emergence (UE) (Abdolahi et al., 2012; Czabator 1962; Ellis and Roberts, 1981).

6.2.5 Physiological measurements

Leaf Chlorophyll content was measured in the third, fourth and fifth leaves (counting from the terminal bud) across all treatments using a hand-held, self-calibrating, and non-destructive SPAD chlorophyll meter (model SPAD-502; Minolta Corp., Ramsey, NJ). Three measurements were taken on each of the three leaves at ten weeks of growth. Carbon fixation and the maximal efficiency of PSII (F_V/F_M) were measured with Li-Cor 6400 portable photosynthesis measuring system, fitted with a standard chamber and configured as an open system (Li-Cor, Lincoln, NE). Measurements were taken at eight weeks after

planting across all treatments and replicates. Instantaneous measurements of CO_2 assimilation rates were carried out at a CO_2 concentration of 400 ppm, a flow rate of 500 ml min^{-1}, a temperature of 25ºC, and a light intensity of 1000 µmol m^{-2} s^{-1} between 11:00 am and 2:00 pm. Three measurements were taken per plant on the third, fourth and fifth leaves from the terminal bud. F_V/F_M was determined on the third leaf from the terminal bud 8 weeks after planting following dark adaptation for 40 min.

6.2.6 Harvesting and Plant Tissue Analyses

Shoot heights were measured after twelve weeks of growth, after which plants were harvested. Harvested plants were separated into leaves, stems and roots. The lengths of the roots and stems were measured, the leaves counted and their area measured with a leaf area meter (CI-202 Area Meter, CID, Inc., USA). The plant parts (root, stem and leaves) were oven-dried at 65˚C until constant mass, after which their biomass were determined.

For plant tissue analyses, plants were grown in separate pots as contained in section 6.2.3, except that no fertilizer was applied. After 4 weeks of growth, the leaves were harvested, and oven-dried to constant mass. The leaves were then ground to pass 1 mm screen; 0.5 g wet digested, filtered and analysed using an Inductively Coupled Plasma Atomic Emission Spectrometer (ICPAES). Similarly, treated seeds and control were also analysed.

6.2.7 Determination of MDA and 4HNE

In an additional experiment, fresh and deteriorated seeds were hydrated in the priming solutions as described above. Seeds were not dried, but at about the beginning of phase III of the seed hydration, 1 g was homogenised in 5 ml of 20 % (w/v) trichloro acetic acid (TCA) consisting of 0.5 % (w/v) thiobarbituric acid. The homogenate was then incubated for 30 min at 95°C (Hodges et al., 1999), placed in an ice bath for 10 min and then centrifuged at 10 000*g* for 10 min. The absorbance of the supernatant was read at 600 nm using a PowerWave™ microplate spectrophotometer (BioTek Instruments, Inc, USA). The content of MDA was calculated spectrophotometrically using ε_{532} = 155 mM^{-1} cm^{-1} and expressed as mmol g^{-1} fresh mass (FM).

The content of 4-HNE was also estimated at about the beginning of phase III of seed hydration. Seeds (1 g) were homogenised in 5 ml of cold borate buffer (0.2 M, pH 7.4) at 4°C. The homogenate was then mixed with 10% (w/v) TCA and centrifuged at 12 000*g* for 15 min. The supernatant obtained was thoroughly mixed with 2, 4-dinitrophenyl hydrazine

(1 mg ml^{-1} in 0.5 M HCl). The complex obtained was kept at ambient laboratory conditions for 2 hr after and then extracted in hexane and evaporated under liquid nitrogen. The residue was dissolved in methanol the concentration of 4-HNE calculated spectrophotometrically using ε_{350} = 13.8 mM^{-1} cm^{-1} (Ray et al., 2007), and results expressed as mmol g^{-1} FM.

6.2.8 Quantification of Antioxidant Enzymes

The effects of priming solutions on the activities of SOD and CAT in seeds of fresh and deteriorated seeds of *C. pepo* were tested. Seeds were hydrated in the priming solution until about the beginning of phase III of seed imbibition. Extraction was done in phosphate buffer saline at pH 7.4 (1g seed/5 ml buffer). The mixture was centrifuged for 10 min at 5,000 rpm at 4ºC. The supernatant obtained was used to measure SOD and CAT activities. SOD activity was determined by measuring the per cent inhibition of pyrogallol auto-oxidation by the enzyme at 420 nm (Marklund and Marklund, 1974). Enzyme activity was expressed as units of SOD min^{-1} g^{-1} FM. CAT activity was measured using the method of Chance and Maehly (1955) based on the breakdown of H_2O_2 (ε_{240} = 39.4 mM^{-1} cm^{-1}) and activity was expressed as µmoles min^{-1} mg^{-1} protein. CAT activity was measured in all treated seeds and the two controls. Each of the eight treatments was replicated three times.

6.2.9 Statistical Analyses

The data collected were subjected to analysis of variance (ANOVA) using GenStat Release 12.1 (PC/Windows Vista) (VSN International Ltd., 2009). Means of the treatments were compared using the Tukey Test at 5% least significant difference ($LSD_{0.05}$).

6.3 RESULTS

6.3.1 Effect of priming on seedling emergence and growth

Seedling emergence was delayed as a result of controlled deterioration of seeds, for 2 d in *P. sativum* and 4 d in *C. pepo* (Table 6.1). Total seedling emergence, mean emergence time, emergence index and uniformity of emergence were also adversely affected by controlled deterioration when compared with the fresh unprimed seeds. In response to priming the controlled deteriorated seeds of *P. sativum* with cathodic water, a significant improvement of 33% occurred. The benefits of priming deteriorated seeds with CaMg solution and deionized water were smaller and did not differ significantly from un-primed seeds (Table 6.1).

With the exception of root length in *C. pepo*, controlled deterioration significantly ($p<0.05$) reduced all growth parameters of both species (Tables 6.2, 6.3). Priming significantly improved most of the growth parameters in both control and deteriorated seeds; these effects were however much pronounced in the deteriorated seeds. Almost invariably, cathodic water was more effective than CaMg or deionized water. For example, in *P. sativum* the total biomass of seedlings derived from aged seeds, cathodic water, CaMg water and deionized water increased yields from 0.8 g plant^{-1} to 2.9, 2.1 and 2.7 g plant^{-1} respectively. In the seedlings derived from fresh seeds only cathodic water treatment increased total biomass significantly from 4.1 to 6.9 g plant^{-1}(Table 6.3).

Table 6.1 Effects of cathodic water, calcium magnesium solution and deionized water treatments on the emergence of *Pisum sativum* and *Cucurbita pepo*

EMERGENCE	FSC	FSP.CW	FSP.CM	FSP.DW	ASC	ASP.CW	ASP.CM	ASP.DW	LSD
Pisum sativum									
First day of emergence	4.3±0.3^{ab}	4.0±0^{a}	4.3±0.2^{ab}	4.5±0.3^{abc}	6.3±0.2^{d}	4.5±0.3^{abc}	5.3±0.2^{bcd}	5.5±0.3^{abc}	0.7±0.4
Last day of emergence	6.3±0.3^{bc}	4.5±0.3^{a}	5.8±0.2^{b}	6.3±0.2^{bc}	7.5±0.3^{d}	6.3±0.2^{bc}	7.5±0.3^{d}	7.3±0.2^{cd}	0.8±0.4
Emergence %	100.0±0^{c}	100.0±0^{c}	100.0±0^{c}	100.0±0^{c}	60.0±0^{a}	80±8.2^{b}	75±5.0^{ab}	70±5.8^{ab}	11.5±5.6
Mean emergence time	4.1±0.2^{cd}	4.9±0.04^{d}	4.3±0.2^{cd}	3.9±0.2^{cd}	1.7±0.2^{a}	3.3±0.4^{bc}	2.6±0.2^{ab}	2.1±0.2^{a}	0.7±0.3
Emergence index	3.0±0.3^{d}	4.3±0.1^{e}	3.2±0.3^{de}	2.8±0.3^{cd}	1.0±0.2^{a}	2.4±0.3^{bcd}	1.7±0.2^{abc}	1.3±0.2^{ab}	0.7±0.3
Uniformity of emergence	0.14±0.03^{bc}	0.31±0.01^{d}	0.18±0.04^{c}	0.12±0.02^{abc}	0.03±0.01^{a}	0.09±0.02^{abc}	0.05±0.01^{ab}	0.04±0.01^{a}	0.06±0.03
Cucurbita pepo									
First day of emergence	6.5±0.5^{b}	4.0±0^{a}	6.0±0^{ab}	6.0±0^{ab}	10.0±0.8^{c}	6.0±0^{ab}	6.5±0.5^{b}	7.0±0.6^{b}	1.2±0.6
Last day of emergence	9.5±0.5^{b}	6.5±0.50^{a}	10.0±0^{b}	10.0±0^{b}	14.5±0.5^{d}	10.5±0.5^{bc}	11.0±0.6^{bc}	12.5±0.5^{cd}	1.3±0.6
Emergence %	100.0±0^{c}	100.0±0^{c}	100.0±0^{c}	100.0±0^{c}	60.0±0.0^{a}	80.0±8.2^{b}	70.0±5.8^{ab}	70.0±5.8^{ab}	11.9±5.8
Mean emergence time	4.3±0.10^{d}	4.8±0.03^{d}	4.3±0.04^{d}	4.12±0.04^{cd}	1.6±0.10^{a}	3.4±0.30^{bc}	2.8±0.21^{b}	2.6±0.21^{b}	0.5±0.20
Emergence index	2.4±0.10^{d}	3.6±0.09^{e}	2.4±0.06^{d}	2.2±0.08^{d}	0.6±0.10^{a}	1.9±0.20^{cd}	1.4±0.12^{bc}	1.3±0.16^{b}	0.35±0.17
Uniformity of emergence	0.015±0.001^{de}	0.021±0.0004^{f}	0.016±0.0004^{e}	0.014±0.001^{de}	0.006±0.001^{a}	0.013±0.001^{cd}	0.010±0.001^{bc}	0.009±0.001^{cd}	0.002±0.001

There were eight treatments: FSC = fresh seeds that were neither aged nor primed; ASC= seeds that were aged but not primed; FSP.CW = fresh seeds that were not aged but primed with cathodic water; FSP.CM = fresh seeds that were not aged but primed with calcium magnesium solution; FSP.DW = fresh seeds that were not aged but primed with deionised water; ASP.CW = aged seeds that were prime with cathodic water; ASP.CM = aged seeds that were prime with calcium magnesium solution; ASP.DW = aged seeds that were primed with deionised water. ANOVA was performed across treatments with the means of replicate separated at $LSD_{0.05}$. Post hoc was done using Tukey test. Means along the same row with different letters were significantly different ($p<0.05$, n=32).

Table 6.2 Effects of cathodic water, calcium magnesium solution and deionized water treatments on the root length, stem length, number of leaves, number of inflorescence and leaf area of *Pisum sativum* and *Cucurbita pepo*

	Pisum sativum					***Cucurbita pepo***				
TREATMENT	Root Length (cm)	Stem Length (cm)	Number of leaves	Number of Inflorescence	Leaves area (cm^2)	Root Length (cm)	Stem Length (cm)	Number of Leaves	Number of Inflorescence	Leaves Area (cm^2)
FSC	39.8 ± 0.7^{c}	36.2 ± 0.5^{e}	79.5 ± 1.0^{c}	5.5 ± 0.3^{cd}	266.8 ± 1.3^{d}	30.5 ± 1.7^{abc}	28.6 ± 3.0^{de}	9 ± 0.4^{bcd}	9.3 ± 0.6^{c}	681.2 ± 8.3^{c}
FSP.CW	45.5 ± 1.0^{d}	45.5 ± 0.6^{f}	97 ± 3.1^{d}	9.5 ± 0.6^{e}	309.4 ± 3.3^{d}	51.6 ± 4.9^{d}	42.8 ± 2.6^{f}	11.3 ± 0.7^{d}	10.8 ± 1.0^{c}	768.4 ± 12.2^{d}
FSP.CM	44.8 ± 0.9^{d}	37.0 ± 0.9^{e}	87.3 ± 1.3^{c}	6.8 ± 0.5^{d}	271.6 ± 12.8^{d}	38.5 ± 2.4^{bc}	21.4 ± 1.3^{bcd}	9.5 ± 1.2^{bcd}	9.5 ± 0.3^{c}	680.9 ± 10.1^{c}
FSP.DW	40.8 ± 1.6^{cd}	36.6 ± 1.0^{e}	85.3 ± 2.3^{c}	6.5 ± 0.6^{d}	269.4 ± 15.4^{d}	38.8 ± 1.1^{bc}	32.3 ± 1.1^{e}	10.5 ± 0.3^{cd}	6.5 ± 0.3^{b}	701.2 ± 4.9^{cd}
ASC	15.7 ± 0.8^{a}	18.8 ± 0.4^{a}	36.2 ± 1.2^{a}	2.3 ± 0.2^{a}	67.5 ± 1.1^{a}	26.0 ± 1.6^{a}	11.0 ± 0^{a}	5.5 ± 0.2^{a}	0.0 ± 0^{a}	350.1 ± 4.3^{a}
ASP.CW	37.3 ± 1.3^{c}	31.1 ± 0.4^{d}	52.5 ± 0.3^{b}	4.5 ± 0.3^{bc}	205.6 ± 3.8^{c}	40.8 ± 0.7^{cd}	27.1 ± 1.1^{cde}	8.5 ± 0.3^{bc}	6.5 ± 0.3^{b}	645.9 ± 9.5^{c}
ASP.CM	26.8 ± 0.7^{b}	27.7 ± 0.6^{c}	38.7 ± 1.6^{a}	3.3 ± 0.2^{ab}	143.3 ± 6.1^{b}	35.8 ± 1.6^{abc}	20.1 ± 2.2^{bc}	7.5 ± 0.3^{ab}	2.5 ± 0.3^{a}	635.6 ± 11.1^{c}
ASP.DW	23.0 ± 0.7^{b}	22.8 ± 0.7^{b}	51.4 ± 1.1^{b}	2.8 ± 0.2^{ab}	132.4 ± 1.7^{b}	27.8 ± 2.14^{ab}	13.4 ± 1.07^{ab}	7.0 ± 0.41^{ab}	2.5 ± 0.7^{a}	457.4 ± 9.5^{b}
$LSD_{0.05}$	3.1±1.5	2.0±1.0	4.9±2.3	1.2±0.6	37.6±2.2	6.7±3.3	5.3±2.5	1.7±0.8	1.6±0.8	48.2±23.4

Seeds were subjected to controlled deterioration (ageing) at 100% relative humidity and 40℃. There were eight treatments: FSC = fresh seeds that were neither aged nor primed; ASC= seeds that were aged but not primed; FSP.CW = fresh seeds that were not aged but primed with cathodic water; FSP.CM = fresh seeds that were not aged but primed with calcium magnesium solution; FSP.DW = fresh seeds that were not aged but primed with deionized water; ASP.CW = aged seeds that were prime with cathodic water; ASP.CM = aged seeds that were prime with calcium magnesium solution; ASP.DW = aged seeds that were primed with deionised water. ANOVA was performed across treatments with the means of replicate separated at $LSD_{0.05}$. Post hoc was done using Tukey test. Means along the same columns with different letters were significantly different ($p<0.05$, n=32). Means were sorted in ascending order.

Table 6.3 Effect of cathodic water, calcium magnesium solution and deionized water treatment on the root mass, stem mass, leaves mass, shoot mass, total biomass and shoot/root ratio of *Pisum sativum* and *Cucurbita pepo*

DRY MASS (g plant^{-1})	FSC	FSP.CW	FSP.CM	FSP.DW	ASC	ASP.CW	ASP.CM	ASP.DW	$LSD_{0.05}$
Pisum sativum									
Root Mass	1.2 ± 0.01^{bc}	1.6 ± 0.16^{c}	1.6 ± 0.2^{c}	1.2 ± 0.14^{bc}	0.1 ± 0.01^{a}	0.9 ± 0.07^{bc}	0.6 ± 0.27^{ab}	0.8 ± 0.19^{ab}	0.5 ± 0.24
Stem Mass	1.0 ± 0.08^{c}	1.5 ± 0.09^{d}	1.1 ± 0.1^{c}	1.1 ± 0.02^{c}	0.3 ± 0.03^{a}	0.7 ± 0.09^{b}	0.5 ± 0.02^{ab}	0.7 ± 0.03^{b}	2.0 ± 0.09
Leaf Mass	1.1 ± 0.05^{cd}	1.2 ± 0.09^{d}	0.9 ± 0.12^{c}	1.0 ± 0.04^{cd}	0.2 ± 0.02^{a}	0.6 ± 0.03^{b}	0.5 ± 0.03^{b}	0.5 ± 0.01^{b}	0.2 ± 0.08
Shoot Mass	2.9 ± 0.11^{d}	5.3 ± 0.08^{e}	2.8 ± 0.15^{d}	2.9 ± 0.04^{d}	0.7 ± 0.03^{a}	2.1 ± 0.12^{c}	1.4 ± 0.03^{b}	1.9 ± 0.03^{c}	0.3 ± 0.12
Flower Mass	0.8 ± 0.03^{b}	2.6 ± 0.11^{c}	0.9 ± 0.06^{b}	0.8 ± 0.01^{b}	0.2 ± 0.01^{a}	0.8 ± 0.05^{b}	0.4 ± 0.01^{a}	0.7 ± 0.01^{b}	0.1 ± 0.07
Total Biomass	4.1 ± 0.09^{c}	6.9 ± 0.16^{d}	4.4 ± 0.34^{c}	4.0 ± 0.16^{c}	0.8 ± 0.04^{a}	2.9 ± 0.10^{b}	2.1 ± 0.30^{b}	2.7 ± 0.23^{b}	0.6 ± 0.29
Shoot/Root Ratio	2.4 ± 0.10^{a}	3.4 ± 0.34^{a}	1.9 ± 0.37^{a}	2.6 ± 0.29^{a}	14.4 ± 0.66^{b}	2.4 ± 0.28^{a}	5.1 ± 2.39^{a}	2.9 ± 0.66^{a}	2.7 ± 1.32
Cucurbita pepo									
Root Mass	1.0 ± 0.1^{c}	1.8 ± 0.14^{d}	1.0 ± 0.09^{c}	1.1 ± 0.14^{c}	0.3 ± 0.002^{a}	0.8 ± 0.05^{bc}	0.5 ± 0.02^{ab}	0.4 ± 0.08^{a}	0.26 ± 0.13
Stem Mass	1.6 ± 0.1^{de}	2.2 ± 0.18^{f}	2.0 ± 0.17^{def}	2.1 ± 0.09^{ef}	0.3 ± 0.02^{a}	1.5 ± 0.03^{cd}	1.0 ± 0.11^{bc}	0.6 ± 0.09^{ab}	0.32 ± 0.16
Leaf Mass	1.8 ± 0.2^{bc}	2.1 ± 0.29^{c}	2.0 ± 0.15^{bc}	2.1 ± 0.15^{c}	0.9 ± 0.01^{a}	2.0 ± 0.17^{bc}	1.4 ± 0.1^{abc}	1.3 ± 0.16^{abc}	0.51 ± 0.24
Shoot Mass	3.7 ± 0.3^{cd}	4.8 ± 0.45^{d}	4.1 ± 0.23^{cd}	4.4 ± 0.10^{cd}	1.2 ± 0.01^{a}	3.6 ± 0.18^{c}	2.5 ± 0.19^{b}	2.0 ± 0.21^{ab}	0.70 ± 0.34
Flower Mass	0.3 ± 0.02^{de}	0.4 ± 0.03^{e}	0.2 ± 0.03^{c}	0.2 ± 0.01^{cd}	0.0 ± 0^{a}	0.2 ± 0.01^{bc}	0.1 ± 0.01^{ab}	0.08 ± 0.03^{a}	0.07 ± 0.03
Total Biomass	4.7 ± 0.2^{c}	6.5 ± 0.53^{d}	5.1 ± 0.17^{c}	5.5 ± 0.14^{cd}	1.5 ± 0.01^{a}	4.5 ± 0.22^{c}	3.0 ± 0.21^{b}	2.3 ± 0.28^{ab}	0.80 ± 0.38
Shoot/Root Ratio	4.0 ± 0.5^{ab}	2.70 ± 0.26^{a}	4.4 ± 0.66^{ab}	4.4 ± 0.6^{ab}	4.2 ± 0.07^{ab}	4.4 ± 0.17^{ab}	5.0 ± 0.27^{b}	5.7 ± 0.78^{b}	1.40 ± 0.68

There were eight treatments: FSC = fresh seeds that were neither aged nor primed; ASC= seeds that were aged but not primed; FSP.CW = fresh seeds that were not aged but primed with cathodic water; FSP.CM = fresh seeds that were not aged but primed with calcium magnesium solution; FSP.DW = fresh seeds that were not aged but primed with deionised water; ASP.CW = aged seeds that were prime with cathodic water; ASP.CM = aged seeds that were prime with calcium magnesium solution; ASP.DW = aged seeds that were primed with deionized water. ANOVA was performed across treatments with the means of replicate separated at $LSD_{0.05}$. Post hoc was done using Tukey test. Means along the same row with different letters were significantly different ($p<0.05$, n=32).

6.3.2 Effect of priming on physiological parameters

The seedlings produced from seeds derived from deteriorated seeds of both species contained significantly less chlorophyll than those derived from fresh seeds. The decline in chlorophyll contents were 33.4% and 22.1% for *P. sativum* and *C. pepo,* respectively (Figure 6.1). In *P. sativum*, all priming treatments increased the chlorophyll content of the leaves of plants derived from deteriorated seeds to a similar extent. In *C. pepo*, only cathodic water was effective in increasing the chlorophyll content of the leaves of seedling derived from deteriorated seeds. Priming had little effect on the chlorophyll content of seedlings derived from fresh seeds.

For both species, controlled deterioration significantly reduced the maximal efficiency of PSII (F_V/F_M) of the resulting seedlings (Figure 6.1). All priming treatments restored values to those of the controls. By contrast, priming had little effect on fresh seed treatments. There was significant reduction 50% (*P. sativum*) and 36.9% (*C. pepo*) in the rates of carbon fixation in the plants generated from fresh seeds were compared to plants produced from deteriorated seedlings - ASC (Figure 6.2), but priming with cathodic water increased rates of fixation to values similar of those in seedlings derived from control seeds (FSC) in *P. sativum* and *C. pepo*. Priming with CaMg solution and deionized water increased rates of fixation, but rates were not significantly higher than those in plants derived from un-primed seeds. In seedlings produced from fresh seeds, all priming treatments tended to slightly increase rates of fixation.

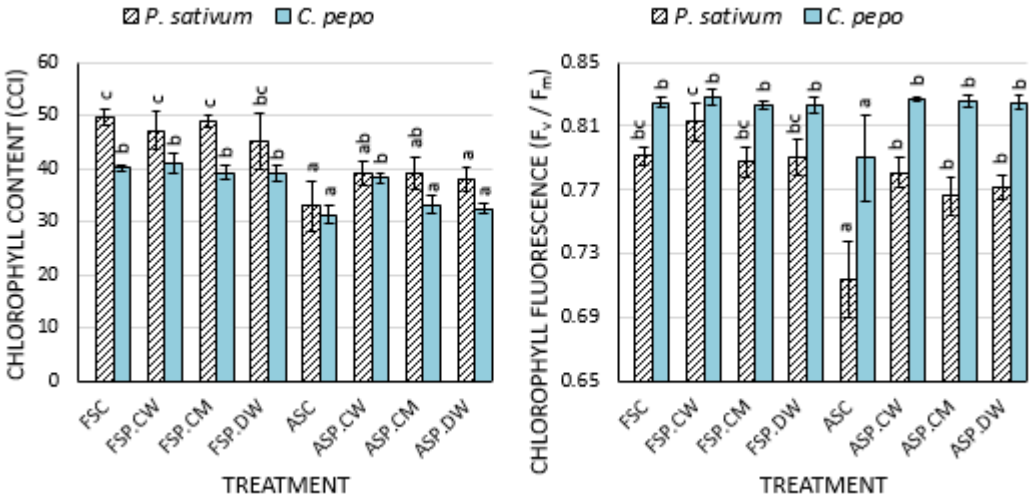

Figure 6.1 Effects of cathodic water, calcium magnesium solution and deionized water seed invigoration on the chlorophyll content and chlorophyll fluorescence of the leaves of *Pisum sativum* and *C. pepo* plants. Each treatment was replicated 4 times. Bars with different letters in each species are significantly (p<0.05) different. Chlorophyll fluorescence: Measurements were taken 1 time from 3 leaves per plant (n= 12/ treatment). Chlorophyll content: measurements were taken 3 times per each leaf per plant (= 36 measurements/ treatment

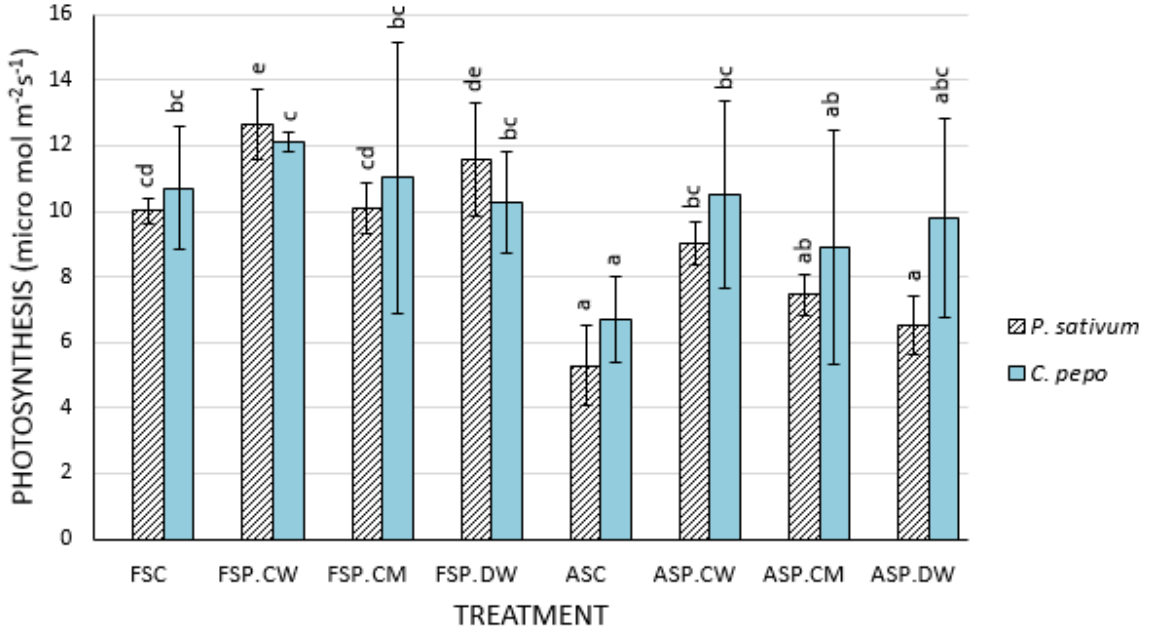

Figure 6.2 Effects of cathodic water seed invigoration on the photosynthesis of *Pisum sativum* and *Cucurbita pepo* plants. Bars with different letters in each species are significantly (p<0.05) different. Each bar represents the mean of 3 replicates; n=12/ treatment.

6.3.3 Effect of priming on lipid peroxidation and antioxidant enzymes

Controlled deterioration significantly ($p<0.05$) increased the content of both MDA and 4-HNE in *P. sativum* and *C. pepo* seeds (Figure 6.3). While the 4-HNE content increased by 1.74 in *P*. *sativum*, in *C*. *pepo* there was a lower increase of 1.22 folds. Similarly MDA content increased in the deteriorated seeds of *P. sativum*, in *C. pepo*, 1.69, 1.51 folds, respectively (Figure 6.3). Cathodic water significantly reduced MDA and 4-HNE contents in controlled deteriorated seeds treatments in both species. For MDA, the reductions were significant for cathodic and CaMg solution, but not deionized water. For 4-HNE the reductions were significant for all priming treatments in *P. sativum*, and cathodic water was significantly more effective than either CaMg solution or deionized water. *In C. pepo*, only cathodic water significantly reduced 4-HNE levels. Priming had little effect on the levels of lipid peroxidation products in fresh seeds.

Controlled deterioration significantly reduced the activities of CAT and SOD in the seeds of *C. pepo* (Figure 6.4). For SOD, all priming treatments significantly increased SOD activity, but cathodic water and CaMg solution were significantly more effective than deionized water. Generally controlled deteriorated seeds treatments benefited more than fresh seeds treatments. While increases of 18.23, 18.23, and 16.33% occurred in the fresh seeds treated with cathodic water, calcium magnesium solution and deionised water respectively; much greater increases of 80.0, 79.9 and 68.5% when similar treatments were applied to aged seeds (Figure 6.4). For CAT, only cathodic water and CaMg solution significantly increased enzyme activity, and cathodic water was significantly more effective than CaMg solution.

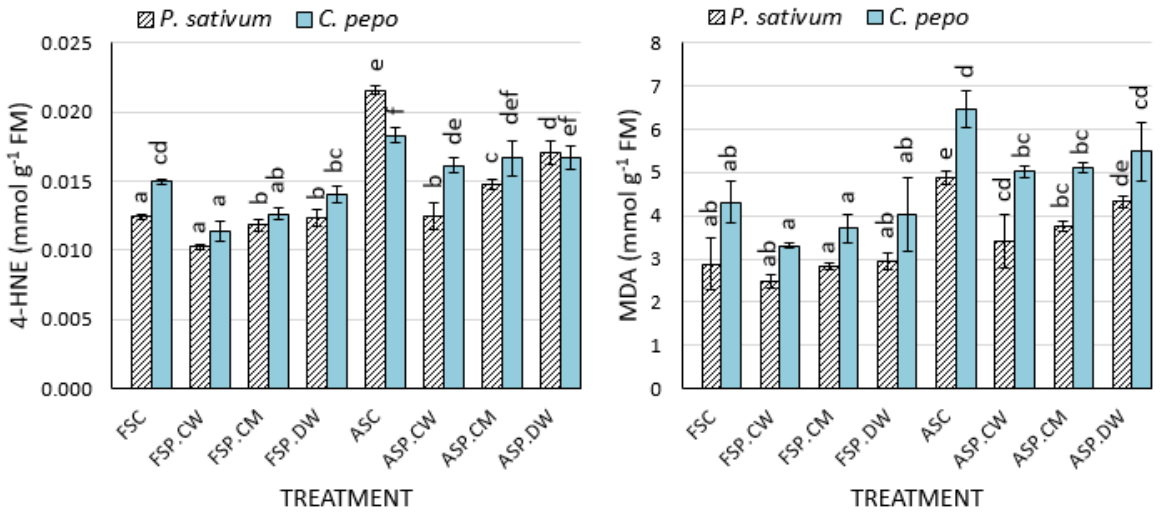

Figure 6.3 Effect of cathodic water, calcium magnesium solution and deionized water on the MDA and 4-HNE content in the aged and primed seeds of *P. sativum* and *C. pepo.* Bars with different letters in a species are significantly different (p<0.05). Each bar represents the mean of 4 replicates.

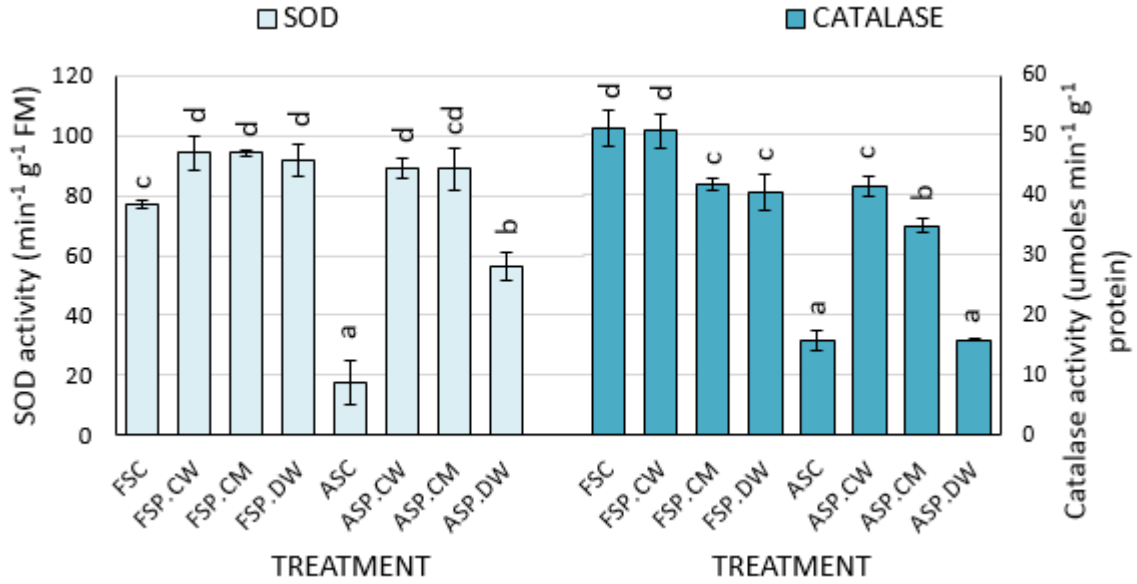

Figure 6.4 Effect of cathodic water, calcium magnesium solution and deionized water on the activities of SOD and catalase in *C. pepo.* Bars with different letters in a species are significantly different (p<0.05). Each bar represents the mean of 4 replicates.

6.3.4 Effect of Cathodic Water, Calcium Magnesium Solution and Deionized Water on Mineral Content of seeds and Shoot of *Pisum sativum* and *Cucurbita pepo*

Priming the controlled deteriorated seeds of *P. satsivum* with cathodic water and CaMg solution led to increase in the concentration of magnesium in the seeds and calcium in the shoot of *P. sativum* when compared with the unprimed controlled deteriorated (Figure 6.5). In *C. pepo,* there were no significant changes in any of the treatments, neither was there any significant elevation of Ca and Mg in any of the other *P. sativum* treatments (Figure 6.5).

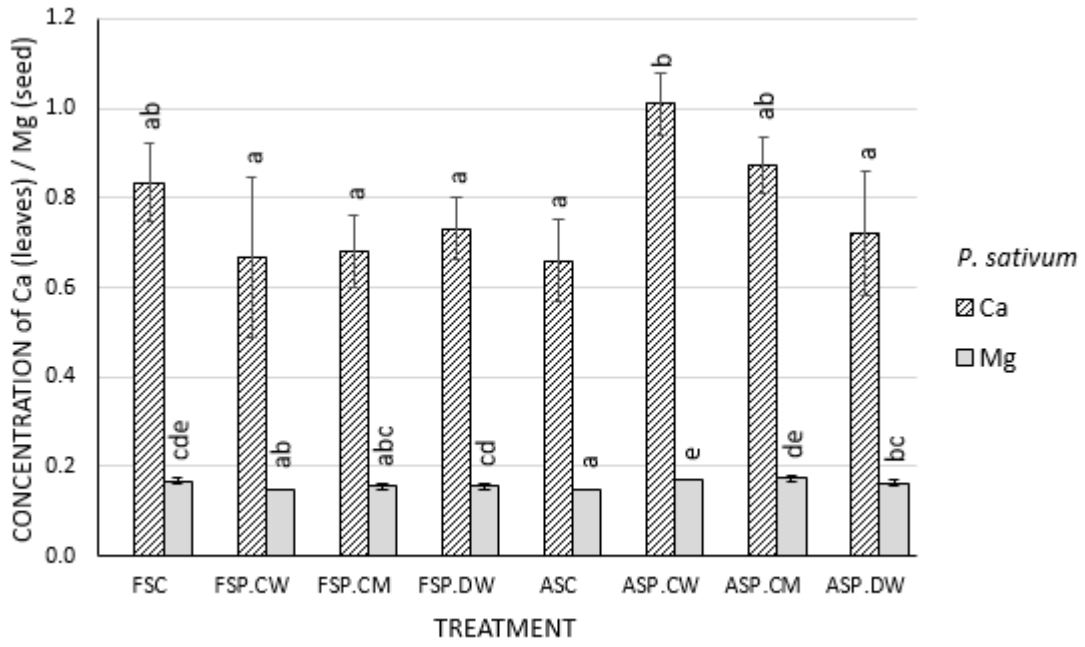

Figure 6.5 Concentration of magnesium in the seeds and calcium in the leaves of plants generated from primed CD seeds of *Pisum sativum*. In both cases, Ca and Mg concentrations are higher in controlled deteriorated seeds primed with calcium magnesium solution and cathodic water treatments. Bar with different letters are significantly ($p<0.05$) different.

6.4 DISCUSSION

Cathodic protection as commonly understood, is a process by which electrons generated at a cathode counteract oxidative corrosion. In the work presented here, a novel approach to cathodic protection was used, involving the reduced, cathodic fraction ("cathodic water") of an electrolysed dilute solution of calcium and magnesium chloride (Berjak et al., 2011) to invigorate controlled deteriorated seeds of *P. sativum* and *C. pepo*. Here, we compared the effects of priming with cathodic water with priming with un-electrolysed CaMg solution and deionized water.

6.4.1 Effects of priming on emergence and later seedling growth

Seedling emergence was delayed as a result of the controlled deterioration of seeds in both species (Table 6.1). The delay in emergence may be linked to a reduction in seed vigour and possibly also the induction of secondary dormancy (Bewley and Michael 1994; Ghassemi-Golezani et al., 2011). Delays in emergence as a result of the loss of seed vigour have been reported in many plants including *Phaseolus vulgaris* L. (Andjelkovic et al., 2018) *Glycine max* (Bhattacharya et al., 2019) and *Zea mays* (Ghassemi-Golezani et al., 2011). Generally, seed priming with cathodic water improved the emergence parameters in both species, with most of the improvements being statistically significant (Table 6.1). In particular, uniformity is

an important parameter, as high uniformity has been reported to improve stand establishment, particularly important under suboptimal conditions such as drought, salinity and water stress. Uniformity of emergence due to priming has also been reported to suppress weeds growth; for example, priming has been reported to reduce weed-induced yield loss in *Oryza sativa* by 10% (Anwar et al., 2012). While priming with CaMg solution or deionized water also generally improved the emergence parameters of deteriorated seeds, they were less effective than cathodic water and their effects were not always significant. In deteriorated seeds, cathodic water appears to have performed better than CaMg solution and deionized water although the values may not be statistically different in this study due to large variations. For instance, in *C. pepo,* emergence in ASP.CW was 12.5% greater than ASP.CM/ASP (Table 6.1). In terms of practical applications such increase is considered significant in terms of plant productivity (Anwar et al., 2012; Farooq et al., 2006) and may have huge impact both in terms of recovery of germplasm and profitability (economic value/farm income). It is well known that priming can improve emergence in a variety of species e.g *Cicer arietinum* (Ullah et al., 2019), *Zea mays* (Ghassemi-Golezani et al., 2011), *Oryza sativa* (Wang et al., 2018) and *Triticum aestivum* (Ahmed et al., 2019), cathodic water appears to be superior to other commonly used priming solutions.

Interestingly, in the present study seeds were deteriorated to 50% emergence in petri dishes in the laboratory, in pots in the greenhouse emergence was greater than 50% in both species. The greater emergence may mean that the growth medium used was more favourable to the seeds than the germinating papers used for the germination study. The implication is that that laboratory germination data may not reflect emergence under field conditions.

While good germination is important, the subsequent growth of seedlings was reduced if they were derived from deteriorated seeds (Tables 6.2 and 6.3). It is well known that “hangover effects” of deterioration may be carried through to later growth stages of the plant. Seedlings of deteriorated seeds may display reduced photosynthesis, slower growth and ultimately lower yields (Jorgenson and Burns, 2007). Thus, even though deteriorated seeds may germinate and emerge well, the resulting seedlings may have low “vigour”. Current models of seed deterioration suggest that as seeds age the accumulation of free radicals causes profound cellular damage, disrupting normal cell functions (Ahmed et al., 2019; Bhattacharya et al., 2019). Lipid peroxidation impairs critical cellular function such as oxidative phosphorylation, reducing energy (ATP) production, resulting in slower cell division (mitosis) (Bailly et al., 2004; Chhabra et al., 2019; McDonald, 1999), ultimately leading to a delay in emergence and plant growth (Balestrazzi et al., 2011; Chhabra et al., 2019).

In both species, controlled seed deterioration significantly reduced growth parameters such as root length and mass, stem length and mass, the number of leaves, leaf area and total biomass (Tables 6.2 and 6.3). Invigoration of seeds of the test species with any of the priming solutions had some measure of improvement on root and stem length, number of leaves and leaf area, and the masses of the root, stem, leaf, and shoot, as also the total biomass of the plants derived from deteriorated seeds but not significant (Tables 6.2, 6.3). However, cathodic water was almost invariably the best priming solution. For instance, seed priming with cathodic water did not just have significant impact on total biomass in both fresh and aged seeds treatments, its improvement on total biomass was also significantly better than what was obtainable from calcium magnesium solution and deionized water (with the exception of aged seeds in *P. sativum*). Also, priming was particularly effective in improving flower production. While deterioration completely suppressed flower production in the seedlings of *C. pepo* (Table 6.2), priming with cathodic water produced *c*. two thirds the number of flowers as those from fresh seeds. Seedlings derived from deteriorated seeds primed with CaMg solution or deionized water produced less than one-third of the flowers produced by seedlings derived from fresh seeds. Similar, but less marked effects were observed in *P. sativum*. Reasons for the particularly strong effect of cathodic water priming on flower production remain unclear. The reduction in biomass of plant/plant parts as observed in this study might be due to the reduced nutrient uptake by the plants as a result of reduced root length/mass (Tables 6.2, 6.3). Our results are in agreement with the observations of many other researchers on a variety of species, for example, Amanpour-Balaneji and Sedghi, 2012 (*Triticum aestivum*), Sreepriya and Girija, 2019 (*Sesamum indicum L*.), Wang et al., 2018 (*Oryza sativa*) and Umair et al., 2017 (*Zea mays*). Reductions in seedling growth a result of controlled deterioration of seeds were effectively alleviated by seed priming. It has been reported that seed priming enhances antioxidant activities, nutrient uptake, photosynthesis, rate of cell division, cell elongation (Bosco de Oliveira et al., 2012; Goltsev et al., 2016; He et al., 2018; Li et al., 2017; Sahu et al., 2017;).

6.4.2 Effects of priming on photosynthetic parameters

The effects of deterioration and priming on the photosynthetic parameters measured here were consistent with the growth data; deterioration strongly reduced the efficiency of PSII (F_V/F_M) (Figure 6.1). Generally, priming deteriorated seeds improved the photochemical efficiencies. Lower F_V/F_M values in the seedlings derived from un-primed deteriorated seeds undoubtedly contributed to their poor growth (Tables 6.2, 6.3). Results from other photosynthetic parameters also support the view that improved photosynthesis in seedlings

derived from primed seeds contributes to improved seedling growth. Deterioration reduced the chlorophyll content in all species (Figure 6.1). Cathodic water reversed the effects of deterioration, and although not significant in all instances, the improvements were generally better than those in plants primed with CaMg solution or deionized water. Seedlings of both *C. pepo* and *P. sativum* produced from deteriorated seeds possessed significantly reduced rates of photosynthesis (Figure 6.2) compared with seedlings derived from fresh seeds. Priming, particularly with cathodic water increased the photosynthetic rates of seedlings derived from deteriorated seeds and even to some extent in seedlings derived from fresh seeds (Figure 6.2). While we did not study in detail the mechanisms whereby priming improved photosynthesis, the, greater leaf area and leaf number in the primed seed treatments (Table 6.2) would have provided greater surface area for photosynthetic activities, and subsequent growth of the plants (Fatokun and Zharare, 2015; Li et al., 2017; Mouradi et al., 2016). The longer roots produced by cathodic water treated seeds may have also enhanced nutrient uptake which ultimately would have improved on photosynthesis (Bosco de Oliveira et al., 2012; Li et al., 2017).

6.4.3 Effects of priming on lipid peroxidation products, ROS scavenging enzymes and mineral composition

In both species, cathodic water significantly reduced both MDA and 4-HNE levels in deteriorated seeds. Although CaMg solution reduced MDA level in *P. sativum* and *C. pepo* it failed to significantly reduce 4-HNE level in *C. pepo.* All priming solutions significantly reduced 4-HNE in *P. sativum* cathodic water was significantly better at reducing the levels of 4-HNE than CaMg solution and deionized water (Figure 6.3). In this study, MDA contents increased in the deteriorated seeds of *P. sativum* and *C. pepo,* 1.69, 1.51 times respectively, while the 4-HNE content increased 1.74 times in *P. sativum* and 1.22 folds in *C. pepo* (Figure 6.3). The increases in both MDA and 4-HNE indicate that deterioration of seeds occur as a result of lipid peroxidation (Bhattacharya et al., 2019; Chandrakar et al., 2016). Although seeds have internal mechanisms to counteract the damaging effect of ROS, at high level of stress the internal protection of the seeds may not be enough (Chandrakar et al., 2016). Priming with cathodic water significantly reduced the content of MDA and 4-HNE in the seeds of the test species. All other treatments appear to have reduced MDA and 4-HNE content in seeds but some of the reductions were statistically insignificant. Reductions in lipid peroxidation products by cathodic water strongly support the view that priming with cathodic water boosts or augments the internal antioxidative system in the seeds (Bhattacharya et al., 2019; Sahu et al., 2017).

Oxidative stress occurs as a result of over production of ROS leading to lipid peroxidation (Bhattacharya et al., 2019; Sahu et al., 2017). The major ROS implicated in damage to seeds are superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\cdot OH$), all of which would normally be quenched by the endogenous antioxidant system (Varghese et al., 2011). Damage results when the seeds internal anti-oxidant systems are inadequate to cope with ROS produced under conditions promoting oxidative stress such as controlled deterioration (Sahu et al., 2017). Controlled deterioration significantly reduced the activities of the ROS scavenging enzymes SOD and CAT in the seeds of both species, presumably as a result of repeated reactions with ROS (Figure 4). Priming increased enzyme activity, most likely as a result of reductions in ROS formation prolonging enzyme life, but possibly in addition by up-regulating enzyme synthesis/activities.

Consistent with earlier submission that priming is more beneficial to deteriorated seeds when compared with fresh seed, priming-induced increases in SOD activities were higher in aged seeds compared with fresh seeds. For example, in *C. pepo* while 'increases' of 18.23, 18.23, and 16.33 % occurred in the fresh seeds treated with cathodic water, calcium magnesium solution and deionised water respectively, much greater increases of 80.03, 79.91 and 68.48% occurred when similar treatments were applied to aged seeds (Figure 6.4). The main motivation for the present study was that cathodic water may be a potent priming agent because of its powerful antioxidative capacity. By scavenging ROS, cathodic water should therefore ameliorate the impact of controlled deterioration on emergence and later growth of the plants. The results obtained provided some support for this hypothesis.

Calcium and Mg are macronutrients required for plant growth, their presence in cathodic water but at 1 mM concentration in CaMg solution is short of the optimum soil level of Ca, Mg required for plant growth (Hochmuth, 2012). However, the result of the plant tissue analyses indicated that the contributions of Ca and Mg as plant nutrients can not be completely ignored (He et al., 2018; Li et al., 2017) because priming led to increase in the concentration of magnesium in the seeds and calcium in the shoot of *P. sativum* when compared with the unprimed controlled deteriorated seeds (Figure 6.5). It can therefore be suggested that the better performance of CaMg solution when compared with deionized water or control was due to the little contributions of Ca and Mg present in the treatment (He et al., 2018; Li et al., 2017). Since it is clear that the effect of Ca and Mg as plant nutrients to the growth of the test species is benign in most cases, it may be suggested that the main strength of cathodic water lies in its strong antioxidative properties; which may have helped in the control of ROS activities associated with seed deterioration thereby alleviating oxidative stress damage in cathodic water treated seeds (Demirkaya et al., 2010; Min et al.,

2017; Ramamurthy et al., 2015) and probably reduction in the seed CD induced hang over effects (Sershen et al., 2010).

The strong antioxidant properties of cathodic water (Hanaoka et al., 2004) facilitated the repair of controlled deteriorated seeds of the test species. However, there are inter-species differences in terms of the intensity of the sensitivity of each species. The biochemical assays and physiological data all supported the growth data and further lend credence to the strong reducing power of cathodic water. It has no doubt contributed to a deeper and fundamental understanding of the processes involved in cathodic protection in plant material especially reinvigoration of *P. sativum* and *C. pepo* in particular and orthodox seeded species in general. Although priming is usually recommended for poor quality seeds, this study has shown that freshly harvested seeds may also benefit from priming.

6.4.4 Conclusions

The deterioration of seeds in seed banks is of global concern, as it affects the long-term conservation of genetic diversity of both wild species and agricultural species, essential for future breeding programs. In the future, it will be necessary to produce varieties that perform well under future climate change scenarios, particularly in sub-Saharan Africa, where the effects of climate change are likely to be severe. Here we show that priming can invigorate deteriorated seeds of two crop species. Priming improves seed emergence, and moreover, the effects of invigoration were carried forward into plant growth. While all priming solutions were capable of some measure of invigoration, priming with cathodic water was most effective. This may have been as a result of the strong antioxidant capacity of cathodic water or other mechanisms of cathodic water actions not yet known. Either cathodic water exerts its beneficial effect in ways other than ameliorating oxidative stress or not further work is needed to more accurately assess amelioration of oxidative stress by cathodic water, for example protein carbonyl levels, the glutathione redox couple, or direct measurements of ROS levels in plant tissues. In conclusion, priming deteriorated orthodox seeds with cathodic water can improve both the emergence and subsequent growth of *P. sativum* and *C. pepo*. While requiring extra time to prepare, the additional benefits of cathodic water suggest that it can be used as an effective tool in orthodox seed conservation.

CHAPTER 7

GENERAL OVERVIEW, SUMMARY, RECOMMENDATIONS AND PRACTICAL RELEVANCE OF STUDY

This chapter takes a general look at the whole study. A summary of the study is presented in subsection 7.1 (GENERAL OVERVIEW OF STUDY/SUMMARY). There is no doubt that cathodic water is a novel approach to seed invigoration. Hence, there are many opportunities for future researchers to explore in the use of cathodic water. Some of these were suggested in subsection 7.2 (RECOMMENDATIONS). The third subsection (Subsection 7.3 - PRACTICAL RELEVANCE OF STUDY) dealt with how cathodic water could be used to solve real-life problems in the field of crop production and crop protection, horticulture, and seed conservation.

7.1 GENERAL OVERVIEW / SUMMARY OF STUDY

This study was aimed at investigating a novel approach to seed invigoration via the use of cathodic water an electrolysed form of calcium magnesium solution. Germination and greenhouse studies were carried out to investigate the efficacy of cathodic water on the germination, emergence and growth of five orthodox species. Other existing treatments that were investigated alongside cathodic water included; deionized water (hydro priming) and calcium magnesium solution (nutrient priming). The test orthodox seeds were; *E. caffra*, *Co. erythrophyllum*, *B. speciosus* (wild species) and *P. sativum* and *Cu. pepo* (agricultural species). The investigation was done using seeds that were controlled deteriorated to 50% germination.

To achieve a 50% deterioration of seeds, the initial water contents of the seeds were raised to 14% and the seeds were thereafter subjected to controlled deterioration at 100% relative humidity and 40°C in a digital oven. Sample of the seeds were taken at regular intervals and germinated until complete loss of germination. Some of the samples taken were also used for electrolyte leakage test. This part of the study successfully compared the tolerance of the test species to controlled deterioration. Tolerance to controlled deterioration was species-dependent. A clear distinction was seen between the agricultural and the wild species, with the wild species being less tolerant of controlled deterioration (Figure 3.3). Although the activities of ROS is widely believed to be responsible for seed deterioration, in this study in

addition to ROS activities, the physical dormancy procedure may be responsible for the less tolerance of the wild species when compared with the agricultural species. In *B. speciosus* and *E. caffra*, nicking which involved abrading the seeds on sand paper was carried out to break the seed coat dormancy. In *Co. erythrophyllum* the samara covering the seeds were removed using a scalpel. All these physical dormancy breaking procedures may have further exposed the germinating seeds embryos of the wild species to further environmental stress besides those caused by ROS and electrolyte leakage hence the lack of tolerance to controlled deterioration when compared to the agricultural species that were not scarified.

In all the test species, age of deterioration and electrolyte leakage were negatively correlated with seed germination (Figure 3.4). An FTIR analysis conducted on the seeds also supported both the controlled deterioration and electrolyte leakage data. The transmittances (%) of the seeds were negatively correlated with age of seed deterioration and the electrolyte leakage. The FTIR further revealed the class and some changes that occurred in the compound involved in the deterioration of each species. The FTIR data is a novel approach to investigating orthodox seeds' deterioration. The FTIR approach is not only less destructive, but also very fast when compared to the standard germination procedures that are currently being used for testing seed viability. For instance, while the standard germination requires a minimum of 100 seeds and 14 days (agricultural) and 21 days (wild) species to be carried out. The FTIR requires just 5-10 seeds and can be completed in a couple of hours.

In a separate experiment, imbibition curves of the test species were determined by hydrating the seeds in between 20 layers of single-ply paper towel. The imbibition curves revealed the triphasic nature of seed water imbibition in all the test species. While the water content of the test species did not vary widely, the critical water contents of the various species were almost similar; 63% (*B. speciosus*), 57% (*Co. erythrophyllum*), 60% (*E. caffra*), 62% (*P. sativum*) and 56% (*Cu. Pepo*), however, the phase II period vary very widely ranging from 8 hours in *E. caffra* to 230 hours in *Co. erythrophyllum.* The duration of phase II for other species were; 22 hours (*B. speciosus*), 12 hours (*P. sativum*) and 8 hours (*Cu. pepo*) (Figure 3.1). The imbibition curve generated was used to determine the appropriate time of exposure of the seeds to invigoration treatments.

The assessment of the effect of the invigoration treatments was determined using seed germination, seedling emergence and subsequent plant growth. To achieve this, fresh and controlled deteriorated seeds were invigorated with the priming solutions (cathodic water, calcium magnesium solution and deionized water). Unprimed fresh and unprimed controlled deteriorated seeds served as the controls. In the germination study, germination indices

such as; first day of germination, final germination percentage, seed vigour, mean germination time, germination index, mean germination time, uniformity of germination and seedling mass were determined. Seedling emergence was also assessed using the following emergence indices; first day of emergence, final emergence percentage, mean emergence time, emergence index, mean emergence time and uniformity of emergence. The influence of cathodic water and other treatments on the growth of the test plants was examined using morphological/growth parameters (root length, shoot length, number of leaves, leaf area, root mass, shoot mass and total biomass).

The results of the germination and greenhouse studies indicated that to some extent, the negative effects of controlled deterioration on seed germination, emergence, and subsequent growth of the five test species were ameliorated by all priming solutions. The effects on germination were also observed in the physiological responses (photosynthesis, chlorophyll fluorescence and transpiration) of the plants derived from these seeds. Generally, cathodic water was the most effective priming solution. Deteriorated seeds benefitted more from cathodic water priming than fresh seeds in all the species. Results suggested that cathodic water may be highly useful in reinvigorating orthodox seeds that have become deteriorated e.g. by long term storage in a seed bank. Analyses of the whole study indicated that effects of cathodic water on deteriorated seed reported observed on seed germination were carried through to seedling growth in all the test species. It must be noted here that contrary to the popular use of priming to invigorate deteriorated seeds, fresh seeds can also benefit from priming.

Beside the physiological data biochemical data such as lipid peroxidation products (MDA and 4-HNE), DNA (concentration and purity), germination enzyme (amylase) and MSI all supported the germination and growth data taken in the study. Alterations in the activities of antioxidant enzymes (SOD and CAT), were observed in response to controlled deterioration of seeds, and the result indicated that CD reduced the activities of the enzymes which are in agreement with the findings of many other researchers (Asada 2006; Bhattacharya et al., 2019; Garcia et al., 2015; Sahu et al., 2017; Zoeller et al. 2012). In contrast, CW priming significantly enhanced the activities of SOD and CAT. Deionized water and CaMg solution also resulted in some marginal improvement, in the activities of the enzymes. An increase in the activities of antioxidant enzymes as a result of CW priming of orthodox seeds has not really been explored although it has been demonstrated in recalcitrant seeded species.

Plant tissue analyses led to significant elevation in Mg concentrations in the seeds and Ca in the shoot of *P. sativum* when compared with the unprimed controlled deteriorated (Figure

6.5). In *Cu. pepo,* there were no significant changes in any of the treatments, neither was there any significant elevation of Ca and Mg in any of the other *P. sativum* treatments (Figure 6.5). Hence, it can therefore be suggested that the better performance of CaMg solution when compared with deionized water or the control was due to the little contributions of Ca and Mg present in the treatment which probably was used up in the process of phosphorylation (Bailly et al., 2004; Chhabra et al 2019; He et al., 2018; Li et al., 2017; McDonald 1999). Since it is clear that the effects of Ca and Mg as plant nutrients in the growth of the test species is generally benign, it may be suggested that the main strength of cathodic water lies in its strong antioxidative properties. The antioxidant property of CW may have boosted the internal antioxidants present in the seeds thereby help in the control of ROS activities which are associated with seed deterioration resulting in the alleviation of oxidative stress damage in the CW treated seeds (Demirkaya et al., 2010; Min et al., 2017; Ramamurthy et al., 2015).

7.2 RECOMMENDATIONS

Cathodic water as stated earlier, is the cathodic fraction of an electrolysed, dilute ionic solution of calcium and magnesium solution (CaMg). Ca and Mg are macronutrients required for plant growth; their probable contributions to plant growth should not be ignored. Hence, future researches should not only be designed around the strong antioxidant properties of cathodic water; designing an experiment to further investigate cathodic water as an advanced form of nutrient priming should be explored; this is important considering the fact that CaMg solution performed better than deionized water. The investigation can be done using other species. If the nutrients (Ca and Mg) are actually made available to the seedling, the nutrient release and uptake kinetics and probably compartmentalization need to be investigated in future studies.

Questions such as the following need to be answered by future researchers;

1. If the nutrients calcium and magnesium are used up by plants, at what stage of growth are they used up?
2. What are the effects of cathodic water on plant mineral contents and phytonutrients?
3. How does cathodic water affect plant growth regulators such as gibberellins, abscisic acid, auxins and cytokinins?

The mechanism of action of the ameliorative effects of cathodic water should be further investigated using other biochemical and physiological markers not covered in this study. It will also not be out of place to investigate the cost benefits of cathodic water seed priming in future researches. Cathodic water as used in this study focused on sexual propagation

through the use of orthodox seeds, it will be good to investigate its usefulness in asexual propagation such as budding and grafting. A lot of failures in the course of grafting and budding are due to oxidative stress (Adams 2016; Bidabadi et al. 2018; Sharma 2019). Hence, designing an experiment along this line may be a good way to go in future researches.

This study showed cathodic water (as an electrolysed form of calcium magnesium solution) has a large potential for priming/ invigoration, then what to expect from the other 'cathodic water' using other salts; is it going to be better than CaMg solution? In other words; is there any room to improve 'cathodic water' even more? In a bid to explore further improvement of cathodic water, increasing the concentrations of calcium and magnesium in CaMg solution is suggested.

This study has shown clearly that the use of cathodic water increased biomass in the test species it will be good to investigate the effect on actual economic yield (such as fruits and seeds) especially in the agricultural species.

7.3 PRACTICAL RELEVANCE OF STUDY

Some of the practical relevance/ potential applications or the uses to which cathodic water can be put to solve real-life problems are:

Conservation of genetic traits of orthodox seeds

The main aim of this study was to test whether invigoration techniques, particularly the use of cathodic water, can help in the preservation of orthodox seeds, for example, those in seed banks. The results of this study indicate that orthodox seeds that have lost vigour due to deterioration can be reinvigorated with cathodic water. Hence, cathodic water is especially useful in reinvigorating deteriorated seeds in the seed banks, especially when those seeds are no longer available in the wild. In the agricultural species, seeds with certain desirable traits (which have become extinct) may benefit from cathodic water seed priming as the seeds may be needed for breeding purposes.

Ameliorating the impact of drought

The ability of cathodic water to ensure early germination, improvement in seedling vigour, plants with longer roots makes cathodic water a good candidate to combat drought. This is especially good for South Africa because it is one of the countries that are experiencing recurrent and severe droughts in many of its provinces. For example, droughts have been reported in the 1960s, 1980s, 1990s, 2002, 2003, 2005 and 2015 to date (Adger 2001; Kihupi et al., 2003; SAWS, 2016). In 2016, the government of South Africa declared five

(KwaZulu-Natal, Mpumalanga, North West, Limpopo and the Free State) of its nine provinces drought disaster areas for agriculture due to the severity of drought experienced (Kapambwe, 2016). Small scale farmers may be the hardest hit as they can not afford the capital intensive irrigation system being used by the commercial farmers (Kapambwe, 2016). Future projections have also indicated that drought should be expected, hence, a low cost method of combating drought through the use of cathodic water will have a great impact on agriculture, with its multiplier effects on job creation, improved economy and reduction in poverty.

Amelioration of the impact of adverse environmental, weather and climatic conditions

Improvement in seedling vigour, growth, plant physiology (leaf areas, number of leaves, chlorophyll content, chlorophyll fluorescence, and photosynthetic rate) as observed in this study has severally been reported as contributing to the ability of plant to cope with plants exposed to stress (Anwar et al., 2012). Hence, plants exposed stressors such as water stress (Ghassemi-Golezani et al., 2011); salinity (Basra et al., 2006) and elevated temperatures stand to benefit from cathodic water seed invigoration. This is very important considering changes being experienced all over the world due to global warming, desert encroachment and other natural disasters. There may be very little anyone or government can do to control these natural disaster but adoption of cathodic water may help in reducing the adverse effects of natural negative changes in weather and climatic conditions.

Restoration of forest loss due to acid rain

Cathodic water may also be relevant in the restoration of forest lost due to acid rain. Acid rain has been experienced in many countries including South Africa, leading to loss of forest resources. This may be done by amending soil with cathodic water. It has been reported that calcium level in the soil reduced the sensitivity of trees to acid rain. Hence, cathodic water soil amendment may reduce the degree of forest decline caused by long-term acid deposition in the soil (Liu et al., 2011).

CHAPTER 8

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Appendix 1 - Electrolyte Leakage and FTIR – Fast and Less Destructive Ways of Monitoring Orthodox Seeds Deterioration

Abstract

During the storage of orthodox seed progressive deterioration or loss of viability occur, which is of considerable importance for seed banks. The most commonly used method for determining seed viability is a simple germination test. However, this method is sample-destructive and labour-intensive. Non-destructive methods for determining seed viability are thus highly sought after by the seed industry. The present study tested the utility of non-destructive methods to assess deterioration. Seeds of *Bolusanthus speciosus*, *Combretum erythrophyllum*, *Erythrina caffra*, *Pisum sativum*, and *Cucurbita pepo* were subjected to controlled deterioration. The ability of seeds to germinate was progressively lost, with the wild species losing viability faster than the crop species. As possibly non-destructive indicators of viability loss, ion leakage and FTIR spectra from the seeds were measured during deterioration. Results indicated that the results from the non-destructive tests correlated well with germination tests, suggesting that they can be used as alternatives to assess seed deterioration.

Introduction

Seeds are classified based on their desiccation tolerance as being recalcitrant or orthodox. While recalcitrant seeds are desiccation sensitive and are intolerant of storage, orthodox seeds are desiccation tolerant and can be stored for long period (Dickie and Pritchard 2002; Engelmann, 2011). According to Dickie and Pritchard (2002), *c*. 90% of the plant species studied produced orthodox seeds (Sacandé et al., 2004). While freshly collected orthodox seeds typically germinate readily after any inherent dormancy mechanisms have been broken, sometimes it is desirable to store seeds, for example in seed banks to conserve genetic diversity (Vagera, 2007). Unfortunately, even when stored under optimal conditions, typically at low water contents and temperatures (McDonald, 2004), in all species total germination and seedling vigour are progressively reduced and ultimately the seed dies (Amanpour-Balaneji and Sedghi, 2012; Mohammadi et al., 2011; Rajjou et al., 2008). The causes of seed deterioration and death are not fully understood, although the rate varies between and within varieties of same species (dos Santos, 2016; Sharma et al., 2018). Once viability is lost, costly replacement of the accession will be needed. It is therefore very important that *ex situ* conserved seeds must be managed in a way that ensures maximum longevity. Assessment of deterioration in stored seeds is the basic tool for managing the germplasm of *ex situ* conserved seeds (Engels and Visser, 2003).

Many different methods are available for determining seed viability, such as the germination test (Engels and Visser, 2003), tetrazolium-based tests, biochemical test, and human inspection (McDonald, 1975). However, these methods have several disadvantages. They are not only sample-destructive and labour-intensive, but also require complicated and time-consuming procedures to be performed by personnel with specialized training. Non-destructive methods for determining seed viability are thus highly sought after by the seed industry (Lee et al., 2017). These methods have included electrolyte leakage, and the use of genomic and biochemical markers. It must be noted that most of the tools are species dependent, hence, the need to test the tools on a particular species to know the applicability before its adoption. The aim of the present study was to test electrolyte leakage and changes in Fourier Transmission Infra-Red (FTIR) spectra to assess deterioration during storage in five orthodox seeded species.

While seed deterioration is normally slow under ambient conditions, for practical purposes, there is the need to shorten the time required for seed to deteriorate. Artificial deterioration can be carried out by subjecting the seeds to predetermined and aggravated conditions of heat and humidity to accelerate the rate of deterioration (Ghahfarokhi et al., 2014; Sharma et al., 2018). Artificial deterioration was used in the present study to allow the assessment methods to be compared in a reasonable time.

Materials and Methods

Plant Material

The five species used *Bolusanthus speciosus* (Bolus) Harms, *Combretum erythrophyllum* (Burch.) Sond, *Erythrina caffra* Thumb., *Pisum sativum* L. (pea), and *Cucurbita pepo* L. (pumpkin). Were acquired and initial testing were done as described in subsection 2.2.1. Also, controlled deterioration, seed germination, and measurement of electrolyte leakage; and acquisition of FTIR speactra were done as described in subsections 2.2.3, 2.2.4, 2.2.5, and 2.2.6 respectively.

Statistical Analyses

Data were subjected to analyses of variance. Means of replicates were separated using 5% lsd. Post hoc comparisons were made using the Tukey test. FTIR wave numbers and the corresponding transmittances were extrapolated using Sigma plot.

Results

Figures (3.1, 3.2, 3.3 and 3.4) and Tables (3.1 and 3.2) are as presented in chapter 3 of this thesis

Figure 3.1 shows the effect of controlled deterioration on seed germination. The agricultural species were more resistant to deterioration than the wild seed, with seeds losing 50% viability after *c*. 20 d, and 100% after *c*. 35 d. By contrast, the wild species lost 50% of their viability after 10 d or less, and 100% after 20 d or less. Loss of ability to germinate was strongly correlated with electrolyte leakage (Figure 3.2); in the wild species electrolyte leakage increased more rapidly during the early stages of controlled deterioration compared with the agricultural species.

Analysing seeds with FTIR spectroscopy indicated that a positive correlation existed between the transmittance (%) and the age of seeds in both the agricultural (Figure 3.3) and the wild species (Figure 3.4). Compared to the controls, there were significant increases in transmittance at 20 d of controlled deterioration in all the test species, indicating a loss of seed integrity in all species. As the ageing time increased from 20 to 32 days the transmittance increased significantly in both *P. sativum* and *Cu. pepo*, however, in *B. speciosus* and *E. caffra* the changes were slight. The numbers of peaks present in the spectra vary according to species, ranging from 6 in *E. caffra* and 11 in *Cu. pepo*. As seeds aged, more transmittance peaks appeared, probably due to the breaking of some biomolecules or compounds to smaller end products. Tables 3.1 and 3.2 present the class of compounds identified from each. Generally, the amines, alkanes and the halo compounds were degraded as the ageing time increased in all the test species (Tables 3.1 and 3.2). Other compounds present in one or more of the test species include anhydrides, sulphates, isothiocyanates, carboxylic acids, alcohols, alkynes, phenols, unsaturated esters, nitro and some aromatic compounds.

Discussions

Results presented here showed that it is not necessary to measure germination of stored seeds by germination, but that non-destructive methods can usefully be used. The germination trials indicated that the agricultural species tested here were more resistant to deterioration than the wild species (Figure 3.1). However, rather than being genuine, this difference was more likely to because the seeds from the wild species were scarified. Increased seed coat permeability has been reported to predispose seeds to deterioration (Baskin and Baskin, 2014). Electrolyte leakage was closely related to loss in the ability of the seed to germinate (Figure 3.2), and therefore can be used to test seed viability. While this

has been reported for *P. sativum* (Panobianco et al., 2007) and *Cu. pepo* (Vieira and Dutra, 2006), work presented here indicates that this method can also be used for the wild species *B. speciosus*, *Co. erythrophyllum* and *E. caffra*. The “oxidative stress” model for seed deterioration suggests that deterioration is a result of the continued production of ROS such as superoxide ($\cdot O_2$-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\cdot OH$) (Sahu et al. 2017). These ROS damage many biomolecules, and in particular cause cellular dysfunction through lipid peroxidation. The membranes of seeds therefore become progressively more leaky. In theory, after measuring electrolyte leakage seeds could be dried and returned to storage, i.e. the method is non-destructive and will conserve stocks of seeds.

It has been reported that measuring FTIR spectra can reliably indicate deterioration and eventual loss of viability in seeds such as soybean and watermelon (e.g. Baek et al., 2019; Yasmin et al., 2019). In the present study, the FTIR spectra from the seeds changed during controlled ageing (Figures 3.3 and 3.4). These changes were probably as a result of the breaking of some biomolecules or compounds to smaller end products, possibly as a result of oxidative damage (Walters et al., 2010). However, while some compounds are present in the control and seeds aged to 20 and 32 days, some other compounds were only identified in the aged seeds. Probably a more sophisticated analysis of the spectra is needed before clear recommendations can be made, but clearly FTIR has great potential as a non-destructive method for assessing seed deterioration.

Conclusion

In conclusion, this study showed that both electrolyte leakage and FTIR can be used to assess deterioration in the species studied here.

References

As contained in chapter 8 (Consolidated list of references)

Appendix 2 – Acceptance Letter for the Paper: Germination Indices of Orthodox Seeds as Influenced by Controlled Deterioration and Cathodic Water Seed Invigoration

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Acceptance Letter

Date	August 13,2019
MS Registration NO.	**MRN/1175**
Title	**Germination indices of orthodox seeds as influenced by controlled deterioration and cathodic water seed invigoration**
Author(s)	*Kayode Fatokun, R.P. Beckett, B. Varghese, Sershen and N.W. Pammenter*

Dear Dr. R.P. Beckett,

We are pleased to inform you that as per recommendations of the review-panel and approval from R & D Division, your above-mentioned manuscript has been **accepted** for publication in the *Journal of Environmental Biology* and is expected to be published in Volume **41 (2020)**.

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We thank you for opting *Journal of Environmental Biology* for the publication of your research.

To,
Dr. R.P. Beckett,
School of Life Sciences,
University of KwaZulu-Natal Pietermaritzburg,
Private Bag X01, Scottsville 3209, South Africa.

Sincerely yours,

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Appendix 3 – Acceptance Letter for the Paper: Influence of Cathodic Water Invigoration on the Emergence and Subsequent Growth of Controlled Deteriorated Pea and Pumpkin Seeds

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