Physiological and Biochemical Investigations into the Reinvigoration of Deteriorated *Brassica oleracea* L. (Cabbage) and *Lactuca sativa* L. (Lettuce) Seeds with Antioxidants and Inorganic Salt Solutions

by

Ademola Emmanuel Adetunji

216073827

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School of Life Sciences

College of Agriculture, Engineering and Science

University of KwaZulu-Natal, Durban

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ABSTRACT

This study focused on reinvigorating deteriorated *Brassica oleracea* L. (cabbage) and *Lactuca sativa* L. (lettuce) seeds with antioxidants and inorganic (electrolysed and non-electrolysed) salt solutions. All pre-treatment solutions were applied to fresh (control) and controlled deteriorated (CDd) seeds at 75% viability (P75), 50% viability (P50) and 25% viability (P25). The pre-hydration treatments were compared in terms of their effects on seed germination, seedling vigour, electrolyte conductivity (EC), accumulation of lipid peroxidation products, protein carbonylation (PC), antioxidant enzymes and germination enzymes. The study also investigated the effects of invigorating CDd seeds with selected antioxidants on subsequent seedling growth, gas exchange and photochemistry under greenhouse conditions. Controlled deterioration, in general, resulted in the loss of seed vigour and viability but at higher rates in lettuce than cabbage, and increased EC and PC, and lowered antioxidant and germination enzymes activities in both species. However, significant lipid peroxidation was only recorded in lettuce seeds. Antioxidant pre-treatments enhanced viability of CDd seeds of cabbage at P25 and lettuce at P50 and P25, and improved seedling vigour of fresh, P75 and P50 cabbage, and P50 and P25 lettuce seeds. Antioxidant pre-treatments reduced EC and lipid peroxidation in lettuce seeds, while PC was reduced in both species. The treatments also elevated antioxidant and germination enzymes activities in P25 cabbage seeds and P50 and P25 lettuce seeds. The inorganic salt pre-treatments did not enhance percentage seedling production in CDd cabbage seeds. However, in CDd lettuce seeds, Ca-containing solutions and electolysed (cathodic water) treatments promoted percentage normal seedling production and enhanced seedling vigour irrespective of pre-treatment solution pH or seed deterioration level. In the greenhouse studies, certain antioxidants promoted seedling vigour and leaf area in both species and enhanced shoot dry weight and gas exchange in lettuce. In summary, CDd lettuce seeds responded better to both types of invigoration (i.e., antioxidants and inorganic salt solutions) than cabbage seeds, while antioxidants appeared to be relatively more beneficial than inorganic salts in both species. The results suggest that the benefits of certain pre-treatment solutions were based on the enhancement of the activities of key antioxidant and germination enzymes, and the efficiency of photosynthesis at the early stages of growth. The results argue for the use of these seed pre-hydration treatments for mitigating poor stand establishment brought about by seed ageing, and as a useful approach to reinvigorating seeds in long-term storage collections for ensuring global food security.

PREFACE

The study described in this thesis was completed by the candidate while based in the Discipline of Biology, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville Campus, South Africa, under the supervision of Dr Boby Varghese, Professor Sershen Naidoo and Professor Norman W. Pammenter. The research was financially supported by a research grant by the National Research Foundation of South Africa to N.W. Pammenter (grant number CPRR13092145823).

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. The results reported are due to investigations by the candidate, and where use has been made of the work of others, it is duly acknowledged in the text.

As the candidate's supervisors we have approved this thesis for submission.

Name: Dr Boby Varghese **Date:** 12 March 2021

Name: Professor Sershen Naidoo

Name: Professor Norman W. Pammenter

DECLARATION 1: PLAGIARISM

I, Ademola Emmanuel Adetunji, declare that:

(i) the research reported in this thesis, except where otherwise indicated or acknowledged, is my original work;

(ii) this thesis has not been submitted in full or in part for any degree or examination to any other university;

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(v) where I have used material for which publications followed, I have indicated in detail my role in the work;

(vi) this dissertation is primarily a collection of material, prepared by myself, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;

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Ademola Emmanuel Adetunji

DECLARATION 2: PUBLICATIONS

Publication 1: **Adetunji, A.E.**, Sershen, Varghese, B., Pammenter, N.W., 2021. Effects of exogenous application of five antioxidants on vigour, viability, oxidative metabolism and germination enzymes in aged cabbage and lettuce seeds. South African Journal of Botany 137, 85–97. https://doi.org/10.1016/j.sajb.2020.10.001

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Ademola Emmanuel Adetunji

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

$-^{\bullet}CH-$	Lipid radical
$^{1}O_2$	Singlet oxygen
4-HHE	4-hydroxyhexenal
4-HNE	4-hydroxy-2-nonenal
8-oxoG	7-hydro-8-oxoguanine
AA	Ascorbic acid
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
Arg	Arginine
CaMg	Calcium magnesium
CAT	Catalase
CD	Controlled deterioration
CDd	Controlled deteriorated
$-CH_2-$	Methylene
CJD	Conjugated diene
CW	Cathodic water
Cys	Cysteine
DAS	Days after sowing
DM	Dry mass
DNA	Deoxyribonucleic acid
DNPH	2,4-Dinitrophenylhydrazine
DNS	Dinitrosalicylate

DW	Deionised water
EC	Electrolyte conductivity
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron transport chain
FapyGua	2,6-diamino-4-hydroxy-5-formamidopyrimidine
FM	Fresh mass
GA	Gallic acid
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
H_2O_2	Hydrogen peroxide
$HO^{\bullet}$	Hydroxyl
$HO_2^{\bullet}$	Hydroperoxyl
HOCl	Hypochlorous acid
ISTA	International Seed Testing Association
LN	Liquid nitrogen
$LOO^{\bullet}$	Lipid peroxyl radical
LOOH	Lipid hydroperoxide
LOX	Lipoxygenases
Lys	Lysine
MC	Moisture content
MDA	Malondialdehyde
Met	Methionine
NBT	Nitroblue tetrazolium

$O_2^{\bullet-}$	Superoxide
O_3	Ozone
$ONOO^-$	Peroxynitrite
PC	Protein carbonylation
PEG	Polyethylene glycol
Pro	Proline
PUFA	Polyunsaturated fatty acids
PVP	Polyvinylpyrrolidone
RH	Relative humidity
RNA	Ribonucleic acid
$RO^{\bullet}$	Alkoxyl
$RO_2^{\bullet}$	Peroxyl
$ROO^{\bullet}$	Peroxyl radicals
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SVI	Seedling vigour index
TCA	Trichloroacetic acid
Thr	Threonine

CHAPTER 1: INTRODUCTION

1.1 Research background

The demand for food security for a continually increasing global human population (with an estimated rise of about two to three billion by the year 2050 [Bongaarts, 1994; Foley, 2011]) remains high, especially in the face of the disproportionate production of crops (Fess et al., 2011). With the dwindling availability of resources like oil, phosphorous and water, intensified efforts such as high-input farming often geared towards ensuring high production of crops to cater for the growing need become less attainable and sustainable (Fess et al., 2011; Farooq et al., 2013). Several global economic and social challenges, including illiteracy, poverty, disease, amongst others, are recognised to be instrumental in poor crop production management, post-harvest and storage approach and the fall in regional agricultural productivity, which must be overcome to reduce the burden of catering for the rapidly rising population (Fess et al., 2011). Moreover, the effects of global warming as a symptom of climate change are further menace. It is anticipated to cause about 5% decline in world food production and even much more drop in developing regions by 2060, thus exacerbating the projected food security issues (Bongaarts, 1994). Even if some of these issues (for example, poverty) are resolved, the demand for efficient food production will still be doubled for sufficient global supply by mid-twenty-first-century (Foley, 2011; Ray et al., 2013), with an estimated population of nine billion people (Godfray et al., 2010).

It has been said that the way forward to meeting the future global food needs goes beyond increasing crop production (Huang et al., 2002; Ray et al., 2013). Despite the 18th- and 19th-century Agricultural and Industrial Revolutions and the 20th century Green Revolution successes previously recorded in improving crop production through technology, the world remains susceptible to food shortages (Huang et al., 2002; Godfray et al., 2010). However, food production can be improved upon provided that the necessary attention and support are given in the areas of management of resources, innovative technologies focusing on genetic

engineering, molecular biology (Huang et al., 2002), research and development based on post-harvest handling, and genetic resource conservation especially of quality seeds (Rao et al., 2017).

Most of the agricultural plants cultivated in the world begin with seed planting for the establishment of a new crop field (Finch-Savage and Bassel, 2016). Seeds are, therefore, primal to crop production, food security and human livelihood (Bewley, 1997; Wimalasekera, 2015; Finch-Savage and Bassel, 2016; FAO, 2017). Regardless of the plant species, planting season and agricultural region, using high-quality seeds will ensure synchronous germination (Ellis, 1992; Elias and Copeland, 1997; Ilyas, 2006), tolerance to abiotic and biotic stresses (Farooq et al., 2007; Finch-Savage and Bassel, 2016), vigorous growth (Elias and Copeland, 1997), and high yield (Wimalasekera, 2015; Ali, 2016) desirable in agriculture.

A major factor of seed performance is the complex attribute of seed vigour (Ellis, 1992; Finch-Savage and Bassel, 2016), a concept that goes beyond just a measurable variable such as germination (Hampton, 1999) as it is driven by a complex interplay between environmental and genetic components (Hodgkin and Hegarty, 1978; Holdsworth et al., 2001; Finch-Savage and Bassel, 2016). Good plant yield and field establishment across various environmental conditions rely heavily on the quality of seed defined by seed vigour (Hampton et al., 2013; Finch-Savage and Bassel, 2016). High seed vigour in terms of rate and uniformity of seedling emergence is crucial for seed performance as a low rate of germination often predisposes seedlings to harsh environmental conditions and diseases (Paparella et al., 2015). Quality seeds possess superior traits such as high final germination and rapid and uniform rates of germination to reduce the risk of seed-borne disorders and diseases. Other features include uniformity in colour, mass, shape, size, texture, and analytical purity (Wimalasekera, 2015). Improvement of seed vigour to enhance the crucial and yield determining stage of plant establishment is a principal objective of seed companies and agricultural industries (Finch-Savage and Bassel, 2016).

1.2 Statement of the research problem

Seed quality and vigour during germination have been studied intently by farmers, scientists and agronomists for many centuries (Gelmond et al., 1978; Sharma et al., 2015; Marcos-Filho, 2015). There has been further advancement in understanding the processes of

germination recently, especially with the use of various cutting edge scientific techniques, including studies on hormonal control of seed germination (Nonogaki, 2017), molecular mechanisms in germination and the use of mutants to test genes involved in dormancy and germination (Finch-Savage and Bassel, 2016; Finch-Savage and Footitt, 2017; Nishimura et al., 2018). However, our understanding of the basis of differences in seed vigour and, in consequence of that, seed performance during plant establishment is inadequate (Finch-Savage and Bassel, 2016). Increasing demand for quality seeds for crop production has hence necessitated prioritising seed quality improvement research. It is known that at physiological maturity, characterised by low moisture content in orthodox seeds such as cabbage and lettuce (Ibrahim and Roberts, 1983; Walters and Towill, 2004), germination and vigour are usually at the maximum level (TeKrony et al., 1979; TeKrony and Egli, 1997; Dayal et al., 2014). However, maintaining high seed vigour afterwards has become a pressing need to meet the demand for vigorous seeds. The challenge is that seeds gradually and continuously suffer post-harvest deterioration leading to quality loss during prolonged storage (Harrington, 1972; Vertucci and Roos, 1990; Walters et al., 2005; Sahu et al., 2017). Even desiccation-tolerant orthodox seeds are unable to hold on to their initial quality over an extended (years) storage during which they begin to deteriorate, proceeding inevitably towards death (Matthews, 1985; Basra et al., 2003; Poonguzhali, 2016).

This ageing-induced physiological deterioration of orthodox seeds in storage, commonly referred to as ageing, is exacerbated by increased seed moisture content, temperature, storage period and relative humidity (RH) during storage (Roberts, 1960; Ellis et al., 1982; Vertucci, 1993; Sivritepe and Eris, 2000; Kibinza et al., 2006; Poonguzhali, 2016) and is also influenced by the genetic make-up (Nagel et al., 2015) and initial quality of the seeds (Walters, 1998; Desheva, 2016). Susceptibility to ageing, and hence, the rate of deterioration in storage differs widely across species and among even varieties of related species (Tang et al., 1999; Jatoi et al., 2001). Even those stored in gene banks, a biorepository of vital plant genetic materials for decades or centuries, still suffer post-harvest deterioration and eventual mortality during the long-term storage (Walters et al., 2005; Lee et al., 2013). In several vegetable species, including *Brassica* spp, *Cucumis melo* (Saxena et al., 1987; Lee et al., 2013), *Capsicum annuum* and *Lactuca sativa*

(Walters et al., 2005; Hill et al., 2007), differences in the rates of seed deterioration during storage have been reported in relation to their storage conditions. Increasing the RH or reducing the temperature of the storage environment, for example, can result in an increased seed moisture level (Merritt et al., 2003), which in turn influences deterioration rate under air-dry storage condition (Ellis et al., 1990; Copeland and McDonald, 1999). In addition, the storage life of *Anigozanthos manglesii*, *Banksia ashbyi*, and *Mesomelaena tetragona* seeds kept at 50 °C was shown to be dependent on the RH of storage environment (Merritt et al., 2003).

Given the global need to ensure sustainable food security for a rapidly growing world population in a changing climate through *ex situ* seed banking, limiting seed deterioration in storage and/or reinvigorating seeds that have deteriorated to some extent in storage has been an increasingly important research focus (Bailly et al., 1998; Demir and Mavi, 2008; Wang et al., 2018; Singh et al., 2020). This process of ageing causes delayed germination, reduced vigour and eventual total viability loss (Copeland and McDonald, 1999; Boniecka et al., 2019). The higher the seed moisture level and temperature at which seeds are stored, the faster they lose viability (Simon, 1974; Ellis et al., 1990; Ellis et al., 1991; Ellis et al., 1995). Other factors, such as the physical state and physiological condition of seeds (Copeland and McDonald, 1999; TeKrony, 2003) also influence seed deterioration. Significant physiological and biochemical symptoms of ageing reported in various species include increased susceptibility to disease, reduced respiration (Ferguson et al., 1990), enzyme degradation and inactivation (Dell'Aquila, 1994), protein and genetic degradation (Sen and Osborne, 1977; Basra et al., 2003), loss of membrane integrity (Pesis and Ng, 1983; Mira et al., 2011) and increased incidence of morphologically abnormal seedlings (Simon, 1974; Roberts, 1986; Torres et al., 1997; Matthews et al., 2010).

The decline in seed quality during storage in most orthodox species investigated to date has been attributed to the generation of reactive oxygen species (ROS) (Smith, 1986; Bailly et al., 1998; Kim et al., 2010; Groot et al., 2015; Xia et al., 2020). The electron transport chain of mitochondria (Hawkins et al., 2009), and metabolically active hydrated pockets within restricted cellular regions of dry seeds (Sahu et al., 2017) are some of the possible sources of ROS production. An imbalance between the generation of ROS and antioxidant protection against them induces oxidative stress (Berjak and Pammenter, 2013; Choudhury et al., 2017; Chandra et

al., 2019), which brings about tissue damage by releasing prooxidants capable of driving the Fenton reaction and lipid peroxidation, and by degrading or deactivation of defence antioxidants (Gutteridge, 1995). Reactive oxygen species have also been implicated in protein oxidation (Mittler, 2002; Job et al., 2005; Ahmad et al., 2015), damage of DNA (Oracz et al., 2007; El-Maarouf-Bouteau et al., 2011; Zhou et al., 2020) and RNA (Fujikura and Karssen, 1992; Finch-Savage and Footitt, 2017; Kurek et al., 2019), and alteration of carbohydrate contents (Bernal-Lugo and Leopold, 1992; Piotrowicz-Cieślak, 2005; Lehner et al., 2008). The damages caused by ROS have been observed in a wide range of plant tissues such as embryonic axis, whole embryos, cotyledons, seedlings roots and shoots (Lehner et al., 2008; Yao et al., 2012), including those within seeds (Ferguson et al., 1990; Bailly et al., 2008).

Studies on ageing in orthodox seeds have also indicated that the mechanism(s) of seed deterioration may differ across species. For instance, while seed viability loss has been associated with accumulation of lipid peroxidation products in seeds (Al-maskri et al., 2002 on *Cucumis sativus*; Sahu et al., 2017 on *Pongamia pinnata* and Wiebach et al., 2020 on *Triticum aestivum* and *Hordeum vulgare*), van Staden et al. (1976) showed that this was unlikely the case in stored *Protea compacta* seeds. Priestley and Leopold (1979) and Chappell Jr. (2008) suggested that lipid peroxidation might be unconnected with ageing in *Glycine max* and *Spartina alterniflora* seeds, respectively; while deterioration was accompanied by lipid peroxidation in lettuce (Smith, 1986; Xue et al., 2001) but not cabbage seeds (Mira et al., 2011). A study on *Arachis hypogea* seeds (Pearce and Samad, 1980) may offer some explanation for these inconsistencies in terms of the involvement of lipid peroxidation in ageing. Those authors attributed ageing in *A. hypogea* seeds to the inability to regulate intracellular concentrations or subcellular segmentation of metabolites due to loss of membrane lipids and not peroxidation. Nonetheless, the above studies show that the reported biochemical mechanisms of seed deterioration in various orthodox seeds are different.

Given the above, the identification of biochemical markers of oxidative stress has become a popular area of investigation regarding seed deterioration (Murthy et al., 2003; Kim et al., 2010; Boniecka et al., 2019) irrespective of the cause of deterioration: be it ageing (Edje and Burris, 1970; Dell'Aquila, 1994; Mira et al., 2011; Boniecka et al., 2019), desiccation (Chaitanya and

Naithani, 1994; Sershen et al., 2016; Chandra et al., 2019) or any other biotic (Bolwell et al., 2002; Irfan et al., 2010; Delian et al., 2017) or abiotic (Karpinski et al., 1997; Kim et al., 2005; Anjum et al., 2015) factors. These markers range from the ROS (free radicals or compounds) and antioxidants (enzymic and non-enzymic) to estimating the products of oxidative processes and damage. For example, peroxidative alterations in the fatty acid composition of membrane lipids bring about changes in the structural and functional properties of the membrane (Pammenter et al., 1974; Smith, 1986), resulting in enhanced leakage of solute (Pukacka, 1991; Chaitanya and Naithani, 1994) as well as the formation of harmful by-products which can be assayed (Ponquett et al., 1992; Górecki et al., 1996; Parkhey et al., 2012). With regards to proteins, oxidative alterations of the side chains (arginine, lysine, proline or threonine residues) form carbonyls, impeding or modifying their activities and amplifying their predisposition to proteolytic attack (Job et al., 2005). Due to their ease of derivatisation and detection when chemically reacted with 2,4-dinitrophenylhydrazine (DNPH) to form 2,4-dinitrophenyl (DNP) hydrazine adduct, protein carbonyl groups are a common oxidative stress marker (Dalle-Donne et al., 2003; Shulaev and Oliver, 2006; Anjum et al., 2015). Also worthy of mentioning is the role of carbohydrates in seed ageing. Alterations to the contents of sucrose and non-reducing oligosaccharides such as stachyose and raffinose, which are known to preserve viability (Horbowicz, 1997; Pukacka et al., 2009) due to their function in the stabilisation of proteins and biological membranes (Crowe et al., 1987; Leprince et al., 1993), have been linked with seed deterioration in storage (Horbowicz, 1997; Zalewski and Lahuta, 1998; Piotrowicz-Cieślak, 2005; Nigam et al., 2019).

Assessing levels of antioxidant protection are just as important as measuring oxidative injury when investigating oxidative stress in biological systems (Moran et al., 1994; Roach et al., 2018). In this regard, antioxidants including detoxifying enzymes such as superoxide dismutase (SOD), peroxidase (POX), glutathione reductase (GR), catalase (CAT) and ascorbate peroxidase (APX) are often used as indicators of seed deterioration (Kim et al., 2010; Yan et al., 2016; Nagel et al., 2019) given their role in defending plant tissues against oxidative stress (Sahu et al., 2017; Wang et al., 2018).

Seed ageing has also been shown to be accompanied by a decline in the activity of enzymes associated with germination like α-amylase (Livesley and Bray, 1991; Ganguli and Sen-

Mandi, 1993) and β-1,3-glucanase during events leading to seed germination and radicle protrusion (Leubner-metzger and Meins, 1999; Koornneef et al., 2002; Leubner-Metzger, 2003). While α-amylase performs a major function in breaking down of stored starch present in the endosperm into sugars which supply the energy needed for root and shoot growth, β-1,3-glucanase has been suggested to play a role in the hydrolysing components of the cell wall leading to the weakening of endosperm at the region of protrusion of radicle (Farashah et al., 2011).

In the event of seed deterioration due to ageing in seed banks or controlled deterioration (CD) under laboratory conditions, as a consequence of high relative humidity and temperature, the impact on seed viability and vigour, and seedling establishment, recruitment and growth is of paramount importance. However, standard germination assessments in the laboratory, which is used to predict the actual field performance of seeds (Johnson and Wax, 1978) are not entirely reliable (Heydecker, 1972). Good field performance, especially in agricultural seeds, requires adequate emergence and establishment of seedlings for maximal yield. Studies have indicated differences in seed performance under optimal laboratory (optimal) conditions compared with field conditions, which are rarely optimal. Emergence success in the field is often substantially lower than the germination success measured in the laboratory (Hall and Wiesner, 1990; Mehrabadi and Bandani, 2009), especially when soil conditions become less favourable (Matthews and Collins, 1973; Perry and Harrison, 1977; Stormonth and Doling, 1979). Seedling emergence percentage, emergence rate, and shoot fresh and dry biomass was reported to be higher in pre-hydrated *Carum carvi* seeds sown in the soil as opposed to Petri dishes (Mirmazloum et al., 2020). Similarly, a massive difference between viability estimated by tetrazolium staining and laboratory germination techniques and soil establishment was discernible in 39 strains and cultivars of common bean seeds (Kolasinska et al., 2000). In the same vein, Lubbe et al. (2016) reported that the low germination capacity of *Amaranthus dubius* seeds treated with greywater in Petri dishes was rather enhanced than when seeds treated in the same way were sown in the soil. Such disparity in germination and seedling establishment is critical as it has an impact on crop productivity.

The situation may become more precarious when seeds that have been kept for long in seed banks are sown. More particularly, poor field performance has been reported in aged seeds

of several species. In a seed ageing test, using an International Seed Testing Association (ISTA, 1985) validated and frequently employed method of assessing vigour and quality of seed, the performance of soybean seeds subjected to accelerated ageing varied markedly from field responses (Johnson and Wax, 1978).

1.3 This Study

1.3.1 Rationale and motivation for this study

There are reports that some of the effects of seed deterioration (induced by ageing) may be reversed to an extent by hydration treatments which can restore lost viability as well as vigour (Bedi et al., 2006; Kibinza et al., 2011). Priming, a controlled pre-sowing seed hydration to a point close to, but before radicle protrusion (which allows for pre-germinative metabolism without actual germination [Poonguzhali, 2016]), is one of the most used techniques for enhancing seed performance. After priming, seeds may be dried down to the original water content before been sown or stored for future use (McDonald, 1998; Rakshit and Singh, 2018). Classical seed priming protocols originally developed decades ago (Khan et al., 1980; Rowse, 1992; Taylor et al., 1992; Taylor et al., 1998), involve but are not limited to bio priming (Callan et al., 1991; Carrozzi et al., 2012; Mahmood et al., 2016), hydro priming (Welbaum et al., 1998b; Waqas et al., 2019) and the use of non-permeating organic osmotica, termed osmoconditioning (Khan et al., 1980; Liu et al., 1996). These protocols have been shown to enhance germination rate (Bradford et al., 1990), germination/emergence capacity (Khan, 1992; Jett et al., 1996), seedling vigour and tolerance to stresses (Delian et al., 2017), speed of emergence, vigour, biomass accumulation and leaf photosynthetic efficiency (Draganić and Lekić, 2012; Malik and Ashraf, 2012; Shah et al., 2019). Other beneficial effects such as improved membrane integrity due to repair of membranes (Bray, 1995) and DNA (Osborne, 1983; Bray et al., 1989), antiperoxidative effects, mending of cellular injuries (Thornton and Powell, 1992) and metabolic elimination of harmful substances (Basu et al., 1973; Bose et al., 2018) induced by oxidants have also been reported (Mondal and Bose, 2014).

Seed pre-hydration treatments using synthetic and natural compounds have been reported to be quite effective in alleviating and repairing stress-induced cellular impairments in

several agriculturally important species, including the species of this study, *Brassica oleracea* (Abdolahi et al., 2012; Jisha et al., 2013) and *Lactuca sativa* (Varier et al., 2010; Carrozzi et al., 2012). Seeds treatments involving soaking in aqueous antioxidant solutions such as ascorbic acid (Burguieres et al., 2007; Yan et al., 2015), glutathione (Draganić and Lekić, 2012; Xia et al., 2020), trolox as tocopherol (Afzal et al., 2006; Draganić and Lekić, 2012) and gallic acid (Singh et al., 2017; Zeid et al., 2019) have been reported to neutralise the harmful oxidants capable of causing seed deterioration, and enhance seed germination, vigour and subsequent seedling growth in several species. Likewise, glycerol, a known radioprotectant (Chakrabarti et al., 1996; Yatim et al., 2016), has been suggested to scavenge and/or reduce the formation of harmful oxidants in seed embryonic axes exposed to desiccation and ultra-low temperature stress (Sershen et al., 2012). It is also known to maintain cellular stability under abiotic stress (Roopa et al., 2009; Sershen et al., 2012), increase cellular viscosity in dehydrating seed tissues (Morris et al., 2006) and enhance plant growth in several species (Tisserat and Stuff, 2011).

Furthermore, seed pre-germination treatments with inorganic salt solutions like NaCl (Khan et al., 2009), $CaCl_2$ (Abdolahi et al., 2012), $MgCl_2$ (Batool et al., 2015), and $MgSO_4$ (Carrozzi et al., 2012) have been shown to have restorative effects on vigour and enhance germination of debilitated seeds. The exact mechanisms via which these inorganic salts protect cells and tissues against oxidative stress and, in turn, have restorative effects in aged seeds are not well characterised. However, Ashraf and Rauf (2001) suggested that seeds take up ions from the respective saline solutions in which they are treated, leading to increased accumulation of ions in varying proportions in the different parts of seeds. Uptake of ions and existing ionic competition within cells can be affected by pH level (Pasqua et al., 2002; Jisha et al., 2013). The competition between protons, cations and anions is of key importance for plant mineral nutrition (Pasqua et al., 2002) as several findings have indicated that low pH levels are associated with cation uptake inhibition, while on the other hand, there may be slight or no influence on the uptake of anions (Jisha et al., 2013). This was part of the reasoning employed by Berjak et al. (2011) in their development of an invigoration approach referred to as cathodic protection, which involves treating zygotic embryos (Berjak et al., 2011), apical meristems (Gebashe, 2015) and seeds (Gondwe et al., 2016) with the cathodic fraction of an electrolysed solution of calcium and

magnesium chloride (CaMg). The reduced cathodic fraction of an electrolysed dilute ionic solution, henceforth referred to as cathodic water (CW), has a high pH and has been reported to possess strong reducing antioxidative power (Hanaoka, 2001; Hanaoka et al., 2004; Berjak et al., 2011). Priming with cathodic water has been shown to enhance or maintain high germination in stored seeds of *Cucurbita maxima*, *Lycopersicon esculentum* and *Pisum sativum* (Gondwe et al., 2016). However, unlike other restorative seed pre-hydration treatments that involve inorganic ions, cathodic protection has not yet gained popularity in germplasm banks since the mechanisms via which these solutions improve germination and vigour in stored seeds and other germplasms have not been established. Importantly, reports of studies that have looked at the restorative effects of inorganic ions suggest that their effects also appear to be species-specific (Nawaz et al., 2013; Gondwe et al., 2016). Since differences in seed vigour are mainly accredited to ageing (Powell and Matthews, 2005), an improvement in the germination of deteriorated seeds after application of soaking (or priming) treatments can be indicative of the amelioration of oxidative stress and could prove to be of great importance for restoration and recovery of debilitated and endangered germplasm.

While previous studies have only looked at CW generated from CaMg (Berjak et al., 2011; Naidoo et al., 2016) and NaCl (Hanaoka, 2001; Hanaoka et al., 2004), the present study compared the effects of cathodic fractions of $CaCl_2$ and $MgCl_2$ solutions as well. Where these treatments (antioxidants and inorganic salt solutions) alleviated the effects of ageing on vigour and viability, a range of established physiological (seedling growth and vigour measurements) and biochemical markers (electrolyte leakage, lipid peroxidation products [conjugated dienes and 4-hydroxy-2-nonenal], protein oxidation, defence enzymes and germination associated enzymes) of oxidative stress and germinability were assayed. Studying the physiological and biochemical lesions induced by ageing can be useful in identifying the factors that enhance and alleviate ageing in seeds. Also, the study investigated the effects of soaking controlled deteriorated (CDd; aged) seeds of cabbage and lettuce in selected exogenously applied antioxidants that resulted in the highest production of normal seedlings relative to seeds soaked in DW at P25 on the seedling emergence and performance, light-harvesting capacity and total chlorophyll, CO_2 assimilation rate, leaf gas exchange and chlorophyll fluorescence of seedlings produced from the soaked

seeds. These variables can be useful in identifying factors that promote recovery from ageing-induced oxidative stress and enhance the performance of seedling raised from aged seeds of both species in field circumstances. In this study, a greenhouse pot trial was used to mimic field conditions. The study adopted controlled deterioration (CD), an artificial seed ageing procedure involving seed exposure to unfavourable storage conditions (elevated moisture content and temperature) for a particular time (Coolbear et al., 1984), to simulate natural ageing which is common in orthodox seeds when stored for long periods.

1.3.2 Aim

The present study aimed to investigate the effects of pre-sowing hydration of aged *Brassica oleracea* L (cabbage) and *Lactuca sativa* L (lettuce) seeds with a range of exogenously applied antioxidants (ascorbic acid, gallic acid, glycerol, reduced glutathione, trolox) and inorganic salt solutions ($CaCl_2$ [non-electrolysed], $CaCl_2$ CW, CaMg [non-electrolysed], CaMg CW, CaMg generated CW adjusted to pH 6.5, $MgCl_2$ solution [non-electrolysed], $MgCl_2$ generated CW, NaCl solution [non-electrolysed], NaCl generated CW and NaCl generated CW adjusted to pH 6.5) on germination and seedling vigour.

1.3.3 Objectives

The specific objectives of the study included:

- ✓ identifying the time taken by *B. oleracea* (cabbage) and *L. sativa* (lettuce) seeds to reach 75%, 50% and 25% viability using a controlled deterioration curve;
- ✓ identifying the imbibition time associated with phase 2 of germination in both cabbage and lettuce seeds using a hydration curve;
- ✓ identifying and comparing the mechanism(s) of seed deterioration in these species by measuring the physiological and biochemical markers of oxidative stress in fresh and aged but unsoaked seeds;
- ✓ identifying the mechanism(s) of action of exogenously applied antioxidants on the invigoration of CDd seeds of these species by measuring germination, vigour, and

oxidative stress using physiological and biochemical markers;

- ✓ identifying the mechanism(s) through which inorganic salt solutions influence germinability, vigour and germination capacity of CDd seeds of these species by measuring oxidative stress using physiological and biochemical markers;
- ✓ investigating the possible influence of pH of randomly selected electrolysed inorganic salt solutions on germination vigour and capacity of CDd seeds of these species by measuring oxidative stress using physiological and biochemical markers; and
- ✓ evaluating the physiological responses of greenhouse-grown seedlings produced by CDd seeds of cabbage and lettuce to exogenously applied antioxidants that resulted in the highest production of normal seedlings relative to seeds soaked in DW at P25 by measuring seedling emergence, vigour and biomass accumulation, light-harvesting capacity (i.e., leaf area), total chlorophyll, CO_2 assimilation rate, leaf gas exchange and chlorophyll fluorescence using a greenhouse pot trial to mimic field conditions.

1.3.4 The research questions

The study attempted to answer the following questions:

- Is the mechanism of seed deterioration in cabbage and lettuce the same?
- What is the mechanism of action of the exogenously applied antioxidants in invigorating the artificially aged (CDd) cabbage and lettuce seeds?
- What is the mechanism of action of the exogenously applied inorganic salt solutions in invigorating the artificially aged (CDd) cabbage and lettuce seeds?
- Where the pre-treatment solutions (antioxidants and/or inorganic salt solutions) do invigorate both aged cabbage and lettuce seeds in terms of improved germination and vigour, do these benefits translate into improved plant growth and yield?

1.4 Thesis structure

Chapter 1 introduces the subject matter, causes, and factors implicated in seed deterioration and

presents plausible ameliorative treatments (exogenously applied antioxidants and inorganic salt solutions) and context for the rationale, aim, objectives and research questions of this study.

Chapter 2 presents an in-depth review of the literature on the physiological and biochemical concept and theory of seed deterioration. The principle and application of seed pre-hydration techniques as invigorative measures are outlined. The two species investigated are also described.

Chapter 3 describes the materials and methods used in this study. This section includes a description of the procedural steps of controlled (artificial) deterioration of seeds of both species investigated, techniques employed to alleviate the CD-induced oxidative stress, and the physiological measurements and biochemical assays used to evaluate the various established stress indicators.

Chapter 4 reports results on the CD trials, comparing rates and patterns of seed deterioration between the two species. The physiological and biochemical responses of fresh seeds of both species to invigorative pre-treatment solutions, CD and post-CD invigorative pre-treatment solutions are reported. Additionally, the observed responses in terms of selected photosynthetic attributes of both species in a greenhouse pot trial to the beneficial pre-treatment solutions common to both species after CD are reported.

Chapter 5 provides a discussion on the results of ageing trials, comparing rates and patterns of seed deterioration between the two species. The mechanism(s) of seed deterioration of both species using the biochemical indicators of oxidative stress measured are compared. The mechanisms of action of beneficial post-CD pre-treatment solutions in each species are discussed in relation to the physiological and biochemical indicators of oxidative stress measured. Also, the responses of photosynthetic attributes (under greenhouse conditions) of CDd seeds exposed to the beneficial pre-treatments solutions common to both species are discussed. The results are interpreted by drawing on broader literature on seed biology, plant biochemistry and stress physiology.

Chapter 6 shows the conclusions drawn from the study in relation to ageing-induced oxidative stress during storage of the two species and the ameliorative potentialities of the exogenously

applied antioxidants and inorganic salt solutions. Also, recommendations for future work on invigoration of debilitated orthodox germplasm, particularly cabbage and lettuce seeds, are suggested.

CHAPTER 2: LITERATURE REVIEW

2.1 Conservation of plant genetic resources

Given that global food demand is rising, it is needful to ensure the conservation of genetic resources to preserve ecosystem resilience and protect plant biodiversity for future agricultural food production (Hoban et al., 2013; Jacobsen et al., 2013). Over a billion people are estimated to be added to the already large and rapidly rising population of the developing world by this year 2020 (Conway and Toenniessen, 1999). If no pragmatic approach is deployed, the challenge of food security will worsen with the increasing impact of hunger and poverty, particularly in developing countries.

The worrisome widespread drop in crop yield due to a combination of factors, including but not limited to soil degradation and drastic changes in the climate (Challinor et al., 2014; Khan et al., 2020), and the negative crop production projections across the globe (Zinyengere et al., 2013; Challinor et al., 2014; Fahad et al., 2017) all point to a need for another Green Revolution with much more yield and better conservation of resources than the first (Conway and Toenniessen, 1999; Mann, 1999). For instance, by mid-twenty-first-century, up to 60% increase in food production is estimably needed to feed the growing population (Lipper et al., 2014; Thornton et al., 2018). This underscores the need to prioritise various approaches and research interventions towards increased crop production.

The need for the global food system to give attention to the production of vegetable crops, like cabbage and lettuce, thereby boosting dietary quality rather than focusing only on food quantity, is gaining increased recognition (Fischer and Tara, 2016; Willett et al., 2019; Ebert, 2020). Among economically useful crops that should be increased in production to solve the global food crises are vegetable crops which had bolstered food security historically when the main crops failed (Doughty, 1979). Cabbage and lettuce are in the list of major vegetable crops of global production based on farm gate value (Schreinemachers et al., 2018). However, there are clear indications of a low level of attention given to research efforts on vegetable crops relative to stable cereal crops, as shown in data reported by Schreinemachers et al. (2018) for 70

nations. While about five cereal researchers were reported per a million people in all national groups, low- and lower-middle-income countries, on average, had only a researcher working on vegetables (Schreinemachers et al., 2018). Agricultural investments and policies by both private and public sectors are still focused on the production of staple crops like tubers as well as cereals, while nutrient-rich vegetables are receiving inadequate attention (Haddad et al., 2016; Ebert, 2020). Therefore, it is needful that the production of vegetables is targeted for adequate investments, particularly in the section of Asia, the Pacific and sub-Saharan Africa with projected insufficient supply (Ebert, 2020).

Attempts being made to address the identified need includes the development of approaches like conservation agriculture, sustainable intensification (Garnett et al., 2013; Pretty and Bharucha, 2014), climate-smart agriculture (Lipper et al., 2014; Thornton et al., 2018) aimed to raise productivity, decrease emission and reduce susceptibility to environmental stresses (improved resilience). Thus, the application of cutting-edge techniques in the various aspects of agricultural science, including agroecology, ecophysiology, soil science and plant physiology (Cassman, 1999) for their benefits through investigative research efforts is a recognised approach. Furthermore, this can be by using dynamic approaches involving the use of modern biotechnological and physiological research techniques among others geared towards addressing low crop yield-related challenges, largely attributed to the low quality of genetic resources such as seeds – the principal yield determining factor (FAO, 2020), which forms the subject of this study.

Seed, as a genetic resource, may be regarded as the insurance system for the world food schemes. The depletion of this resource exposes the schemes to higher risks, which could ultimately lead to catastrophic failure. Without a systematic approach for seed genetic and physiological quality conservation, achieving the much-desired increased productivity and more resilience in the face of the rising world population and changing climate is a mirage. Moreover, seeds are considered the main basis for the sustenance of human as plants form over 80% of the human diet; promoting high-quality seed delivery is thus essential for enhancing crop production and plant tolerance to environmental challenges (FAO, 2020). Achieving food security, therefore, largely depends on seed security of seed-producing communities in all cropping seasons (FAO,

2020). The application of advances in plant physiology, particularly the various techniques of pre-hydration treatment (which uses priming technology to invigorate debilitated germplasms), is needed to improve seed performance, crop yields, maximum yield and allow for planting on less favourable lands by making seeds better able to withstand sub-optimal conditions and thereby reducing crop losses. Accordingly, agriculture in this century and afterwards can be more productive and provide for the improved conservation of plant genetic resources than in the previous times. Heightened efforts in that regards, therefore, will ensure that the idea of reaching several millions of the poor with crop production research benefits is achieved (Conway and Toenniessen, 1999).

2.1.1 Storage of orthodox seeds in gene banks

In terms of conserving plant genetic resources, seed capacity for prolonged storage is particularly essential for gene banks. As far back as 1908, Ewart had grouped seed longevity into short-, medium- and long-term, providing insight on the duration of seed storage before the setting in of severe viability loss (Solberg et al., 2020). Later, several experiments testing seed longevity were conducted under artificial and natural sowing conditions. The Beal (Telewski and Zeevaart, 2002) and the Vienna (Steiner and Ruckenbauer, 1995) germination studies have demonstrated the oldest (over 100 years) of seed longevity studies under natural conditions. Other pioneering seed longevity studies (Roberts and Ellis, 1989; Vertucci and Roos, 1990; Ellis et al., 1991) have shown that moisture content, temperature, relative humidity and oxygen are the essential factors influencing seed viability and vigour during storage; however, genetic factor and pre-storage condition are also important (Justice and Bass, 1978; Solberg et al., 2020).

At moisture levels as low as 5% (fresh mass basis) or less, and at sub-zero temperatures (usually -18 °C) in dry conditions, mature seeds of some species classified as orthodox such as cabbage and lettuce can be stored for long periods (Ellis and Roberts, 1980; Ibrahim and Roberts, 1983; Still, 1999 Walters and Towill, 2004). This is also the easiest method of conserving most spermatophytes genetic resources in conventional gene banks (Berjak and Pammenter, 2004), but seeds do not retain their initial quality with extended storage (years), gradually deteriorating, and inevitably proceeding towards death (Basra et al., 2003; Poonguzhali, 2016). For instance,

seeds that were initially stored in a gene bank at 5 °C but were later moved to -18 °C and stored between 15–19 years suffered a decline in germination capacity from 91% to 11% in cabbage and 97% to 2% in lettuce (Walters et al., 2005). The postharvest loss of physiological quality of seeds, even when seeds are stored in gene banks, has thus remained a major issue demanding attention for long-term storage (Chmielarz, 2009). As seeds deteriorate, vigour is first lost, after which comes the loss of viability (Trawatha et al., 1995; Shaban, 2013).

Moreover, some species classified as recalcitrant (not a purview of the present study) have desiccation-sensitive seeds and are not amenable to short or long-term storage under the conditions mentioned above (Berjak and Pammenter, 2004). With the development of cryostorage techniques, involving germplasm storage at ultra-low temperatures (-120 to -196 degrees, Chmielarz [2010]), the life span of seeds (including both orthodox and recalcitrant species) can be further extended, but not indefinitely (Pritchard, 1995; Walters et al., 2004; Walters et al., 2005; Chmielarz, 2009). This implies that though the degree and rate of deterioration of seeds stored at the enhanced conditions of the conventional seed gene banks can be reduced to an appreciable level (Poonguzhali, 2016), seed deterioration cannot be completely halted. Walters et al. (2004) documented in an experiment measuring changes in viability of seeds of cabbage, lettuce and several other plant species within 20 years of cryostorage that cryogenic temperatures could not sufficiently stop seed deterioration. They further mentioned that there could be as much as 300% variation in longevity among species and within accessions stored in these conditions as the degree of longevity in cryostorage depends on seed inherent properties and seed handling such as pre-storage temperature and harvest year. This implies that cryostorage temperatures do not completely halt all biological activities; molecules are still quite mobile enough at these low temperatures to permit the advancement of ageing reactions (Walters et al., 2004).

2.2 Germination related physiology

Under favourable conditions of moist, warmth and oxygen, quiescent but viable seed is vivified, forming an actively metabolising structure in a process described as germination (Brown, 1965). The progress of the germination can be roughly assessed by measuring respiration or

water uptake (Bewley and Black, 1994), while the completion of germination can be taken as when the system no longer depends on its stored food (Brown, 1965) or visibly marked by the protrusion of radicle (Bewley and Black, 1994). In instances where the radicle may grow before getting through the surrounding tissues, germination can be taken to have been completed from the time a sustained increase in seed fresh weight is recognised (Bewley and Black, 1994). So, the initiation of germinative activities gradually and eventually lead to the formation of normal, growing seedlings (Brown, 1965).

In cases where a viable seed fails to germinate under favourable germination conditions, dormancy is said to have set in as such seeds require additional condition(s) such as specific light, or temperature regime or exposure to some chemical or physical treatments (Bewley and Black, 1994). Dormant seeds that have been hydrated undergo almost all the metabolic processes that take place during the germination of nondormant seeds; yet, the protrusion of radicle does not occur (Bewley, 1997). Their germination later takes place when the additional conditions required for the release from dormancy are met. Three identified stages of seed germination include water imbibition, cell elongation and cell multiplication (Welbaum et al., 1998a). The events following germination, such as mobilisation of food reserve from the endosperm, supply the much-needed energy for seedling growth until the seedlings become photoautotrophic (Pritchard et al., 2002). Seed germination pattern usually follows sigmoid curves whereby a few seeds germinate earlier than the others in a population, followed by a rapid rise in percentage germination, and then relatively late germination of a few seeds is recorded. The curves are generally right-skewed as the occurrence of more germinations is recorded in the first half of the germination period than the second. Whilst the shape of the curves are generally similar, notable differences in germination patterns are noticeable among populations (Bewley and Black, 1994).

2.3 Oxidative stress in plants

Oxidative stress is widely described as the physiological state (response) in cells, tissues and organs, as a consequence of increased pro-oxidative activities (through the generation of reactive oxygen species [ROS]) compared with antioxidative (enzymic and non-enzymic) activities (Bartosz, 1997; Demidchik, 2017). This is as a consequence of aerobic metabolism during which

aerobic organisms produce ROS - incompletely paired oxygen-containing radicals that are formed by the unavoidable leakage of electrons on to molecular oxygen during electron transport in the mitochondria, chloroplast, and cell membranes (Sharma and Dubey, 2005). The generation of ROS may be triggered by severe abiotic and biotic stress conditions (Demidchik, 2015). The physiological responses are often characterised by a gradual accretion of various oxidised biomolecules such as nucleic acids, proteins, lipids, polysaccharides and metabolites, causing deleterious changes in normal biochemical, mechanical, and physical functions of cell components (Demidchik, 2017).

Oxidative stress, thus, functions as an injurious factor, and the main mechanisms involve altering the balance between the levels of generated and quenched ROS owing to upset of regular cellular metabolism, and ROS biosynthesis as a component of developmental processes like signalling response needed for adaptation and defence or programmed cell death (Khan and Wilson, 1995; Larson, 1995; Bolwell and Wojtaszek, 1997; Mittler, 2002; Baxter et al., 2014; Demidchik, 2017). Demidchik and Maathuis (2010) stated that plants could employ ROS accumulation for encoding and recognising various stress factors, including xenobiotic stressors like nanoparticles and herbicides that were not recognised before. Stress factors often engender secondary metabolic effects to be overcome by plant tissues for survival and restoration of growth and development (Zhu et al., 2002). For instance, salinity (Hasegawa et al., 2000), drought (Sharma and Dubey, 2005), desiccation (Varghese et al., 2011), light (Karpinski et al., 1997), temperature (Ali et al., 2005) and pathogens (Mittler, 2002) can induce oxidative stress by increasing the production of free radicals and reactive oxygen species (ROS). Uncontrolled production of ROS can offset the balance of ROS generated during aerobic events and the antioxidative defence system (Møller, 2001), leading to oxidative stress (Kranner, 1993).

2.4 Biochemical effects of ageing and oxidative stress in seeds

Oxidative stress has been implicated in the loss of vigour in plant tissues (Ramarathnam et al., 1987; Hendry, 1993). Loss of vigour in plant tissues is a fundamental physiological phenomenon observed when plant tissues are exposed to environmental stress of any type (abiotic and biotic) under suboptimal external (both agricultural and natural) conditions (Taiz and

Zeiger, 2010). It is a pressing global challenge for modern agriculture. Both abiotic and biotic stress types can cause oxidative stress (Saha et al., 2014), which is widely described as the physiological state (response) brought about by increased pro-oxidative activities (through a gradual generation and accumulation of reactive oxygen species [ROS]) over antioxidative (enzymic and non-enzymic) activities (Bartosz, 1997; Demidchik, 2017). In cabbage, for instance, seed deterioration has been related to changes in the levels of electrolyte leakage (Mirdad et al., 2006), proline, proteins, soluble sugars and phenolic compounds (Boniecka et al., 2019). However, Golovina et al. (1997a,b) reported no change in protein secondary structure in some 20–30 years stored seeds of orthodox species, including *Allium cepa*, *Raphanus sativus*, *Cucumis melo*, *Capsicum annuum* and a *Brassica* species (*B*. *napus*), despite the loss of membrane integrity. In lettuce, seed deterioration has been attributed to changes in the levels of lipid hydroperoxides (Smith, 1986) and volatile products such as aldehydes and alcohols (Mira et al., 2010). Of these environmental conditions, abiotic stress is recognised to constitute a major drawback to crop farming worldwide (Mittler, 2006; Jisha et al., 2013; Jenks and Hasegawa, 2014), accounting for about 51-82% loss of potential crop yield worldwide (Boyer, 1982; Jenks and Hasegawa, 2005; Taiz and Zeiger, 2010; Ghosh and Xu, 2014). In many cases, they engender secondary metabolic effects that need to be overcome by plant tissues for survival and restoration of growth and development (Zhu et al., 2002). For instance, salinity (Hasegawa et al., 2000), drought (Sharma and Dubey, 2005), light (Karpinski et al., 1997) and temperature (Ali et al., 2005) induce oxidative stress by increasing the production of free radicals and ROS thereby offsetting the balance of ROS generated during metabolic events and the defence system (Møller, 2001). The impacts [of oxidative stress] thereof are usually expressed in relation to the overall growth of plant, including vigour, yield, biomass accumulation or primary assimilation events (Taiz and Zeiger, 2010) as well as the quality of seed (Gouveia et al., 2017).

Seeds, due to their rich genetic diversity, are considered the most efficient natural means of protecting the variability of genetic material, as against somatic tissues. The challenge of loss of vigour, therefore, introduces a severe menace (Kurek et al., 2019) plaguing the conservation of millions of genetic materials kept in several world gene banks (Zhao et al., 2020) thereby making the understanding of vigour loss, and consequently seed ageing in storage, vital for plant

physiologists. Seed ageing has been intimately linked to oxidative stress involving ROS which are highly reactive, toxic and capable of causing degradative reactions on several biomolecules over an extended storage period (Bartosz, 1997; Leprince et al., 2000; Varghese et al., 2011; Saed-Moucheshi et al., 2014; Kurek et al., 2019). Biological molecules, including carbohydrate, lipids, proteins, and polynucleic acids such as DNA and RNA, are believed to be the main ROS targets during oxidative stress (Sies and Cadenas, 1985; Inzé and Van Montagu, 1995; Demidchik, 2017; Mittler, 2017), and the physiological lesions that result include loss of membrane integrity (through lipid peroxidation), reduced respiration, enzyme inactivation and degradation, and genetic degradation (Basra et al., 2003; Kibinza et al., 2006; Mira et al., 2011) leading to severely damaging effects on seed vigour, viability and germinability especially.

2.5 Oxidation of major biological molecules

2.5.1 Lipids

Among other biomolecules, ROS-mediated oxidation of polyunsaturated fatty acids (lipid peroxidation) is notably the most harmful as it allows for the chain reactions involving the formation and spread of ROS (Hailstones and Smith, 1988; Anjum et al., 2015). Lipid peroxidation is largely thought to be a significant bioindicator of oxidative stress (Hailstones and Smith, 1988; Gutteridge, 1995; Farmer and Mueller, 2013). The damaging effect is irreversible, causing severe degradation of the membrane, inactivation of enzymes, total loss of membrane-bound protein activities and resulting in cell death (Girotti, 1985; Demidchik, 2017). Lipid peroxidation has been implicated in the loss of viability during storage of seeds of many crop species (Feng et al., 2017; Sahu et al., 2017) and has been shown to lead to swelling of mitochondria, increased membrane viscosity and heightened bilayer permeability (measured as increased solute leakage) (Al-maskri et al., 2002; Basra *et al*., 2003). Products of lipid oxidation can also cause DNA damage and interrupt the normal functioning of several cellular systems (Al-maskri et al., 2002; Alexeyev, 2009).

Considering the mechanisms, lipid peroxidation can occur via non-enzymic and enzymic processes (Anjum et al., 2015; Oenel et al., 2017). The non-enzymic process of lipid peroxidation that is ROS-mediated entails an activation (initiation) stage involving ROS generation, distribution

(propagation) stage involving the ROS chain reactions, and a termination stage where non-radical products are formed (Gutteridge, 1995; Anjum et al., 2015). The peroxidation initiation stage is activated by the removal of a hydrogen atom from a methylene (−CH_2−) group (leaving behind −$^{\bullet}$CH− [lipid radical]) by sufficiently reactive species such as alkoxyl ($RO^{\bullet}$), hydroxyl radicals ($HO^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$), hydroperoxyl ($HO_2^{\bullet}$) and peroxynitrite but not superoxide ($O_2^{\bullet-}$) or hydrogen peroxide (H_2O_2). Saturated and monounsaturated fatty acids, for instance, oleic acid with one double bond and 18 carbon atoms, can be subjected to oxidation reaction but not the chain reaction of lipid peroxidation as they are less vulnerable (Catalá, 2006; Anjum et al., 2015). However, the polyunsaturated fatty acid of cellular membrane phospholipids contains double bonds, which makes it susceptible to peroxidation by facilitating hydrogen atom removal (Porter, 1984; Gutteridge, 1995; Demidchik, 2017). The lipid radical formed then triggers O_2-mediated chain reaction involving the formation of lipid peroxyl radical ($LOO^{\bullet}$) which in turn abstracts a hydrogen atom from nearby fatty acid forming a stable intermediate lipid hydroperoxide (LOOH) and another lipid radical of the propagation phase (Catalá, 2006; Demidchik, 2017). The process is limited by the termination reaction phase producing non-radical products. Also, non-radical peroxidation of lipids can occur by polyunsaturated fatty acids reacting with singlet oxygen ($^1O_2$) forming LOOH and no production of intermediate radicals (Halliwell and Chirico, 1993; Krieger-Liszkay et al., 2008; Przybyla et al., 2008; Nowicka et al., 2013). Though reasonably stable, lipid peroxides may be decomposed by metal complexes in a reaction catalysed by transition metals producing radicals that can reinitiate peroxidation via redox cycling of the metal ions forming products like 4-hydroxy-2-nonenal (4HNE), 4-hydroxyhexenal (4-HHE), and malonaldehyde (MDA) which are useful and most extensively studied biomarkers of lipid peroxidation (Esterbauer et al., 1991; Gutteridge, 1995; Catalá, 2006; Anjum et al., 2015). These aldehydes, in turn, bind with DNA or protein, causing more severe damage (Bentinger et al., 2007). Loss of membrane integrity, breakdown of organelles, oxidation and impairment of DNA, RNA and proteins result where there is severe lipid peroxidation reaction (Farmer and Mueller, 2013; Nowicka et al., 2013). Of these aldehydes, 4-HNE is considered the key product of the peroxidation of omega-6 fatty acid like linoleic acid (C18:2, n-6) and arachidonic acid (C20:4, n-6). The production of 4-HHE, the aldehyde thought to induce the permeability of mitochondrial

inner membrane (Kristal et al., 1996) and upset metabolic events (Yin et al., 2010), has been reported from peroxidation of omega-3 fatty acids like α-linolenic acid (C18:3, n-3) and docosahexaenoic acid (C22:6, n-3) (Catalá, 2006).

In enzymic peroxidation, dioxygenases including lipoxygenases (LOX enzymes) are considered the key oxidising enzymes of polyunsaturated fatty acids, having linoleic acids (C18:2 and C18:3) as the major substrates (Feussner et al., 2001; Feussner and Wasternack, 2002; Oenel et al., 2017). In plants, LOX enzymes can add oxygen molecule at carbon 9 or 13 of C18-fatty acids (Oenel et al., 2017), forming 9- and 13-hydroperoxyl derivatives of linoleic acid, respectively (Andreou and Feussner, 2009). The involvement of LOX enzymes in ageing-induced lipid peroxidation of seeds has been investigated in a few species where it was demonstrated that absence or lowering of LOX enzymes activities decreased the levels of MDA (*Zea mays* [Li et al., 2007]), MDA and LOOH (*Oryza sativa* seeds [Gayen et al., 2014]), promoted storability and germination (*Oryza sativa* seeds [Song et al., 2007]), and improved vigour and viability (*Nicotiana tabacum* [Li et al., 2018]).

2.5.2 Proteins

Since reactive oxidants can be indiscriminately generated in cells, especially at a heightened rate during abiotic or biotic stress, proteins are also a major target biomolecule (Davies, 2003; Møller et al., 2007) as they are abundant and readily reactive with several oxidants (Hawkins et al., 2009). Proteins constitute about 68% of oxidised biomolecules (Davies, 2003; Rinalducci et al., 2008); thus, protein oxidation is a useful bioindicator of oxidative stress (Møller et al., 2007). Specified as a covalent alteration of proteins by reactive oxidants or oxidative stress spinoffs (Møller et al., 2007), the ROS-mediated oxidation of proteins has been described extensively (Starke-Reed and Oliver, 1989; Stadtman, 1992; Oracz et al., 2007; Kumar et al., 2019). Protein oxidation often occurs even under normal physiological circumstances indicating that it is not always an injurious plant process (Johansson et al., 2004; Anjum et al., 2015). Avery (2011) stated that some proteins are considered more vulnerable to oxidation than others due to factors such as the easily oxidised amino acid residue content, metal-binding sites, localisation of protein in cells, molecular conformation and degradation rate. It is becoming increasingly clear

that newly synthesised proteins are highly susceptible to post-synthesis oxidative degradation, suggesting that attaining and conforming to a stable multimeric protein complex may be protective against oxidative injury (Medicherla and Goldberg, 2008; Avery, 2011). The oxidation of protein motivates the build-up of a toxic non-native protein capable of inducing programmed cell death in severe cases (Anjum et al., 2015; Demidchik, 2017). The production of unstable intermediates and the formation of stable products are useful for the estimation of protein damage (Hawkins et al., 2009). ROS-induced protein injury can vary since their properties are not all the same. The extremely reactive ROS, $HO^{\bullet}$, usually generated from H_2O_2 via the Fenton reaction, often lead to non-specific oxidation, unlike the specific type caused by the other ROS (Anjum et al., 2015). Other ROS causing oxidation of proteins include the radicals of alkoxyl ($RO^{\bullet}$), hydroperoxyl ($HO_2^{\bullet}$), peroxyl ($RO_2^{\bullet}$), superoxide ($O_2^{\bullet-}$) and non-radical species like hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2), ozone (O_3), peroxynitrite ($ONOO^-$), and singlet oxygen ($^1O_2$) (Gutteridge, 1995; Berlett and Stadtman, 1997; Dalle-Donne et al., 2003; Anjum et al., 2015). While the oxidation of certain amino acids (sulphur-containing) are reversible, most ROS-mediated modifications are characterised with irreversible loss or inactivation of parent amino acid residue, catalytic, metabolic, regulatory, structural or other activities and functions leading to protein damage or elimination (Ghezzi and Bonetto, 2003; Hawkins et al., 2009; Demidchik, 2017). Irreversible amino acid modifications like arginine and lysine, tryptophan and tyrosine, production of dityrosine, and protein to protein cross-linking are in most cases accountable for permanent shutdown of function in the affected proteins which are later on degraded (Berlett and Stadtman, 1997; Dean et al., 1997; Ghezzi and Bonetto, 2003). The reversible types of amino acid modifications like S-nitrosylation and glutathionylation may be playing redox regulatory role protecting from irreversible oxidation of cysteine as well as modulating protein function (Ghezzi and Bonetto, 2003; Møller et al., 2007). The main oxidative modifications of proteins are outlined in Table 1.

The most common mechanisms of ROS-mediated protein damage involve the direct metal-catalysed oxidation (primary carbonylation) of S-containing amino acid residues like

a. cysteine (Cys) to produce disulfide (cysteine), which is further oxidised through cysteine sulfenic acid to form cysteine sulfinic acid; these initial stages are reversible until the

highest oxidation and damaging level where cysteic acid is irreversibly formed (Ghezzi and Bonetto, 2003; Møller et al., 2007; Demidchik, 2017);

b. methionine (Met) to produce methionine sulfoxide. This stage is also reversible, but the final stage of Met oxidation to sulfone seems to be damaging and irreversible (Møller et al., 2007); and

c. most of the other amino acids, especially arginine (Arg), lysine (Lys), proline (Pro), and threonine (Thr) form stable aldehydes or ketones (carbonyls) in an irreversible reaction (Shacter, 2000; Møller et al., 2007; Anjum et al., 2015) that is not particular to any oxidants (Headlam and Davies, 2004; Hawkins and Davies, 2019). Thus, the extent of reactive oxidant-induced modification of proteins is generically measured as protein carbonyl (Moran et al., 1994; Morscher et al., 2015; Hawkins and Davies, 2019).

Table 1 Commonly reported ROS-induced modifications of polyunsaturated fatty acids (PUFA), proteins, carbohydrates and DNA

Examples of commonly reported ROS-induced modifications of PUFA (derived from Møller et al., 2007)	
PUFA	Oxidised product
Linoleic acid (18:2) Linolenic acid (18:3)	4-HNE Cyclic oxylipin, hydroxyoctadecatrieonic acid, MDA
Examples of commonly reported ROS-induced modifications of proteins (derived from Shacter, 2000; Møller et al., 2007; Demidchik, 2017; Hawkins and Davies, 2019)	
Amino acid	Oxidised product
Cysteine	Cysteic acid (cysteine sulfonic acid)
Methionine	Methionine sulfone
Arginine, Lysine, Proline, Threonine	Carbonyls (ketones, aldehydes): aminoadipic semialdehyde, pyrrolidone, acrolein, 4-HNE, MDA, glu γ-semialdehyde, 2-amino-3-ketobutyric acid
Glutamyl (glutathione, glutamine, glutamate)	Pyruvic acid, oxalic acid
Histidine	2-Oxohistidine, 4-HNE, aspartate, asparagine
Phenylalanine	Hydroxyphenylalanines
Tryptophan	Kynurenine
Tyrosine	3-Nitrotyrosine
Examples of commonly reported ROS-induced modifications of carbohydrates (derived from (Isbell et al., 1973; Møller et al., 2007)	
Sugar	Oxidised product
Aldohexose, polyol	Aldopentose, formic acid
Examples of commonly reported ROS-induced modifications of DNA (derived from Møller et al., 2007)	
DNA	Oxidised product
Purines (e.g., guanine)	8-Hydroxyguanine, FapyGua

Carbonyl formation [protein carbonylation, (PC)] demands higher energy inputs than the other AA residues oxidation and lead to deleterious alterations of protein structure and function (Demidchik, 2017). Secondary carbonylation reactions may occur by the reaction of protein with aggressive lipid peroxidation products like 4HNE, MDA (Wong et al., 2010; Wong et al., 2012; Anjum et al., 2015; Demidchik, 2017). Also, carbonyl formation can stem from protein glycation or glycoxidation (Berlett and Stadtman, 1997; Milkovska-Stamenova et al., 2015; Shumilina et al., 2019), and this may be a confounding factor in using carbonylation as an exclusive oxidation biomarker (Hawkins and Davies, 2019), or by direct protein backbone oxidation forming protein fragments with an N-terminal α-ketoacyl amino acid residue (Dean et al., 1997; Ghezzi and Bonetto, 2003). All these severely alter or inhibit the physiological and enzymatic activities of protein (Demidchik, 2017). Heightened PC has been reported for several plant oxidative stresses (Lounifi et al., 2013) induced by salinity (Tanou et al., 2009; Roychoudhury et al., 2011; Tanou et al., 2012), dehydration (Moran et al., 1994; Pyngrope et al., 2013), heavy metals (Pena et al., 2006; Rellán-Álvarez et al., 2006; Song et al., 2011), pathogen attack (Xu et al., 2008; Sundaram and Rathinasabapathi, 2010) and ROS-induced seed ageing (Rajjou et al., 2008; Cabiscol et al., 2014; Yin et al., 2017).

2.5.3 Carbohydrates

Studies on the oxidative modification of carbohydrates have received less attention even though carbohydrates are considered more abundant than the other plant biomolecules (Demidchik, 2017). Just as in other biomolecules, the oxidative modification of carbohydrates is conceivably injurious to living systems (Demidchik, 2017). Free polyols, like mannitol, pinitol and sorbitol (Smirnoff and Cumbes, 1989), and sugars are oxidised by $HO^{\bullet}$, mainly forming formic acid (Isbell et al., 1973; Møller et al., 2007). Miller (1986) stated that arabinogalactan, cellulose, pectin and such polysaccharides in the cell wall could be broken down by $HO^{\bullet}$**.** Auxin-mediated extension of cell induces the generation of ROS, which is used by cell wall-bound peroxidases to produce $HO^{\bullet}$ near scission site (Schopfer et al., 2002**;** Møller et al., 2007)**.** Moreover, cell wall Cu^{2+} reduced to Cu^{+} by $O_2^{\bullet-}$ and ascorbate can produce $HO^{\bullet}$ by reacting with apoplastic H_2O_2 (Fry, 1998; Fry et al., 2002). The $HO^{\bullet}$ formed cause non-enzymatic separation of pectins and

xyloglucans, leading to loosening of the cell wall (Fry *et al*., 2002; Demidchik, 2017). Similar Fenton reactions of H_2O_2 with Cu or Fe might substantially increase under stress conditions, leading to deleterious effects (Becana et al., 1998; Deák et al., 1999; Connolly and Guerinot, 2002; Demidchik, 2017). On the other hand, simple sugars, disaccharides (Couée et al., 2006; Demidchik, 2017) and some osmoprotectants (e.g., mannitol, sorbitol, proline and myo-inositol) are perhaps capable of scavenging ROS like $HO^{\bullet}$ (Smirnoff and Cumbes, 1989)**.** Increased levels of carbohydrate like mannitol, sucrose and glucose have also been correlated with oxidative stress resistance in several species of plant (Tschaplinski and Tuskan, 1994; Jouve et al., 2004, Patel and Williamson, 2016); however, there is a dearth of information on the direct connection between the physiology of plant and ROS -induced oxidation of carbohydrates (Demidchik, 2017).

2.5.4 Polynucleotides

Oxidative modification of DNA is often implicated in the ageing of seeds (Anderson and Baker, 1983; El-Maarouf-Bouteau et al., 2011; Kurek et al., 2019) and, in some cases, perennial plants (Britt, 1996; Demidchik, 2017). Essentially, ROS attack on DNA cause chemical modification of bases, fragmentation of deoxysugar and breaking of strands (Aruoma, 1999; Roldán-Arjona and Ariza, 2009). Again, $HO^{\bullet}$ being the most reactive, are particularly harmful to polynucleic acids (DNA and RNA) (Demidchik, 2017). $HO^{\bullet}$ attaches to double bonds of nucleotide bases and abstract H^+ from 2'-deoxyribose (resulting in sugar damage) (Roldán-Arjona and Ariza, 2009) and $-CH_3-$ of thymine (Demidchik, 2017). $HO^{\bullet}$ can also oxidise purines forming products such as 7-hydro-8-oxoguanine (8-oxoG), and the formation of 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) has also been reported as a product of polynucleic acids oxidation (Roldán-Arjona and Ariza, 2009; Demidchik, 2017). Guanine is often attacked by $^1O_2$, but not $O_2^{\bullet-}$ and H_2O_2, to form 8-Hydroxyguanine (Møller et al., 2007). ROS-modification of DNA can either be both direct and indirect. Often, MDA (breakdown product of PUFA) conjugation with guanine leads to the formation of an additional ring (Jeong, 2005; Møller et al., 2007). DNA impairment has both cytotoxic and genotoxic effects (Britt, 1996). Besides mutations, DNA oxidation can cause alterations of cytosines methylation needed for regulation of gene expression (Møller et al., 2007). Repair mechanisms of the oxidative damage of plant DNA include directly reversing the impairment caused as well as replacing the base or even the entire

nucleotide (Larsen et al., 2005; Møller et al., 2007; Yoshiyama et al., 2013). Defence system, both in cytosol and organelles, may also be implemented as a form of protection (Demidchik, 2017). Under oxidative stress, however, nuclear ROS-scavengers (glutathione and peroxiredoxin) inadequately protect the DNA (Tuteja et al., 2001; Larsen et al., 2005; Møller et al., 2007). Enzymes such as catalase and ascorbate peroxidase in the cytosol are required to protect nuclear DNA in such conditions (Vanderauwera et al., 2011; Demidchik, 2017).

2.6 Cellular generation of reactive oxygen species (ROS)

Reactive oxygen species are produced at several locations in the cells like chloroplast, mitochondria, plasma membrane, peroxisomes, apoplast, endoplasmic reticulum, and the cell wall. Conventionally, it is thought that ROS are unavoidably produced during metabolic processes of aerobic systems (Frei, 1994; Bartosz, 1997; Luikenhuis et al., 1998; Mullarky and Cantley, 2015). Several possible sources of ROS have been identified in plants, including reactions involving normal plant metabolisms like photosynthesis (Mittler, 2002) and mitochondrial respiration (Mittler, 2017). There are other ROS sources as well, which are produced from abiotic stress-induced pathways. For example, during photorespiration, the oxidation of glycolate by glycolate oxidase in peroxisomes accounts for the majority of ROS like hydrogen peroxide (Mittler, 2002). Recently, more plant ROS sources have been recognised, such as plasma membrane-bound peroxidases, amine oxidases and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases involved in events like apoptosis and defence against pathogens (Hammond-Kosack and Jones, 1996; Dat et al., 2000; Grant and Loake, 2000). While a low level of cellular ROS is formed in a standard condition of growth, ROS formation is heightened under stress conditions (Polle, 2001; Mittler, 2002).

Various enzymes (e.g., oxygenases) and non-enzymic processes "fix" oxygen atoms into various biological molecules (Elstner, 1987; Perl-Treves and Perl, 2002). Partly reduced forms of molecular oxygen (O_2) resulting from O_2 excitation to produce singlet oxygen ($^1O_2$), or from the transfer of one electron to O_2 forming superoxide radical ($O_2^{\bullet -}$), two electrons to O_2 forming hydrogen peroxide (H_2O_2), or three electrons to O_2 forming hydroxyl radical ($HO^{\bullet}$) (Mittler, 2002) are readily reactive than atmospheric oxygen. They hence are termed reactive oxygen species

(Bartosz, 1997). These ROS can cause unrestrained oxidation of various biomolecules leading to oxidative cellular damage (Inzé and Van Montagu, 1995; Mittler, 2002). Metabolically active organelles like the mitochondria, peroxisomes and chloroplasts, processing extremely oxidising reactions or that have high electron flow rates are the primary ROS sources within cells (Gill and Tuteja, 2010). Ubiquinone-cytochrome complexes I and III of the electron transport chain (ETC) are the main sites of $O_2^{\bullet-}$ production in mitochondria, while photosystem I and II are the main sites of $^1O_2$ and $O_2^{\bullet-}$ production in chloroplasts (Gill and Tuteja, 2010).

2.7 The dual capacity of ROS

Though ROS are harmful when in excess, they are still beneficial in cellular processes such as signalling cellular differentiation and proliferation (Mittler, 2017), ion transport and gene expression (Govindaraj et al., 2017) when produced moderately. Whilst plants can employ the ROS steady-state concentration for monitoring stress level within cells, this must be tightly controlled to avoid over-accretion of ROS that can cause cell death (Hammond-Kosack and Jones, 1996; Asada, 1999; Dat et al., 2000). ROS-induced death of cell can set in as a consequence of oxidative modifications of biomolecules like enzyme, DNA, RNA, protein and membrane lipid (the classical concept). On the other hand, heightened ROS levels can trigger programmed cell death, which has been shown by anti-apoptotic genes suppression of paraquat-induced oxidative stress cell death in *Nicotiana tabacum* (Mitsuhara et al., 1999; Mittler, 2002). Further, some cell death earlier believed to be directly caused by oxidative stress are now regarded as programmed cell death in favour of the view that ROS have beneficial effects on plants, promoting physiological function, cellular proliferation and viability (Mittler, 2017). In essence, plants require a regulatory system to ensure low ROS concentration, and another to allow for the quenching of surplus ROS production (Mittler, 2002). Balancing the different steady-state ROS level and generated ROS types, as driven by the interaction of different ROS-generating and ROS-quenching systems, is also important. The balance may be altered remarkably depending on the physiological state of the plant and the combination of various biochemical, developmental and environmental stimuli (Mittler, 2002). Apart from aggravating cellular impairment, ROS can stimulate the expression of defence gene. ROS like $O_2^{\bullet-}$ or H_2O_2 can separately or jointly induce various genes, thereby allowing for more ROS signalling flexibility. Besides, reports on plant responses to abiotic stress

shows that ROS may be more involved in regular signalling for adaptation to stress (Dat et al., 2000).

2.8 ROS scavenging in plant cells

The main plant defence system against ROS involves the activities of antioxidants – compounds that are capable of protecting cells from oxidative injury even when available in low quantity (Schuler, 1990; Govindaraj et al., 2017). These antioxidants can either be enzymatic or non-enzymatic. Major enzymic antioxidants include

i. Superoxide dismutases (SODs): These are ubiquitous metalloenzymes involved with essential defence against superoxide (Tanaka and Sugahara, 1980; Govindaraj et al., 2017) via a redox cycle where the active site metal gets deoxidised by one $O_2^{\bullet-}$ radical and re-oxidised by some other (Fridovich, 1981). The three (3) forms of identified SOD determined by the active site metals are iron-SOD, copper and zinc-SOD, and manganese-SOD (Fridovich, 1981; Tsang et al., 1991; del Río et al., 2018). SOD catalyses the dismutation of $O_2^{\bullet-}$ to O_2 and H_2O_2 (McCord and Fridovich, 1969; Perl-Treves and Perl, 2002), which can then be broken down by another essential enzyme – the catalases.

ii. Catalases (CATs): These are peroxisomes localised heme groups-containing enzymes (Anjum et al., 2016), but their presence has also been reported in mitochondria (Scandalios, 1990; Shugaev et al., 2011). They are involved in the breakdown of H_2O_2 to H_2O and O_2 (Perl-Treves and Perl, 2002). They are recognised as essential defence enzymes against ROS-induced oxidative stress (Bailly et al., 1996; Gallego et al., 1996; Kibinza et al., 2011). Also, they are involved in plant defence and metabolism as well as the perception of cellular signals (Redinbaugh et al., 1990; Mhamdi et al., 2010; Liu et al., 2015).

iii. Glutathione reductases (GRs): These flavoproteins are mostly in the chloroplasts but have also been reported in the cytosol, mitochondria and peroxisomes (Edwards et al., 1990; Jiménez et al., 1998; Yoshimura et al., 2004). They are extremely specific and are involved in the reduction of oxidised glutathione (GSSG) back to the reduced

form (GSH) using NADPH as reductant (Foyer and Halliwell, 1976; Inzé and Van Montagu, 1995; Perl-Treves and Perl, 2002), thereby sustaining a high GSH to GSSG ratio (Schaedle and Bassham, 1977; Carlberg and Mannervik, 1985; Yousuf et al., 2012). They sustain the reduced state of GSH through the ascorbate–glutathione cycle and are involved in maintaining (–SH) group and acts as a substrate for glutathione-S-transferases. In conjunction with superoxide dismutase and ascorbate–glutathione pathway enzymes, GRs constitute an important ROS scavenger (Yousuf et al., 2012). They have been demonstrated to enhance oxidative stress tolerance in transgenic *Nicotiana tabacum* (Yoshimura et al., 2004).

iv. Ascorbate peroxidases (APXs): These heme-containing enzymes are also involved in the decomposition of H_2O_2 using ascorbate as a reductant (Ozyigit et al., 2016). Different isoforms have been reported in the cytosol, chloroplast, mitochondria, thylakoid, stroma and peroxisome (Miyake and Asada, 1996; Chew et al., 2003; Anjum et al., 2016; Ozyigit et al., 2016). Increased APX activity has been reported under abiotic stress such as light (Yang et al., 2008), drought and heat (Koussevitzky et al., 2008) and heavy metal (Anjum et al., 2014).

v. Glutathione peroxidases (GPXs): These are non-heme containing antioxidant enzymes (Ozyigit et al., 2016) using glutathione as a reductant (Bela et al., 2015). They are ubiquitous and predicted to be localised in cytosol, chloroplast, endoplasmic reticulum and mitochondria and plastids (Rodriguez Milla et al., 2003; Rouhier and Jacquot, 2005). They have been demonstrated to play a role in lipid hydroperoxide detoxification, plant defence and response to biotic (Navrot et al., 2006) and abiotic stresses (Roxas et al., 1997; Bela et al., 2015).

A balance between the activities of antioxidant enzymes like APX, CAT and SOD is necessary to determine the steady-state ROS (e.g., $O_2^{\bullet -}$ and H_2O_2) level (Bowler et al., 1991; Mittler, 2002). In addition to metal ions sequestration, this balanced activity is considered crucial to forestalling the production of the extremely toxic $HO^{\bullet}$ through the metal-dependent Fenton or Haber–Weiss reactions (Mittler, 2002). APX and CAT are thought to be of different groups of H_2O_2 scavengers

due to their different affinity for H_2O_2 (µM and mM range, respectively). APX can reduce H_2O_2 to very low concentrations and is conceivably involved in ROS modulation for signalling, while the main role of CAT is to scavenging excess ROS under stress (Mittler, 2002). Since CAT is not reductant-dependent to play its role, it might not be sensitive to cell redox status, contrary to the other systems (Mittler, 2002). Interestingly, some intricate interactions between the mechanisms generating ROS and those scavenging ROS have been reported in transgenics having repressed ROS-quenching systems. Plants having repressed APX formation have their CAT, GR and SOD induced to make up for the absence of APX, while plants having inhibited CAT make up for it by inducing other antioxidant enzymes like GPX and APX thereby suggesting some level of redundancy (Willekens et al., 1997; Rizhsky et al., 2002).

The non-enzymic antioxidants also play vital roles in the antioxidant defence system, which forms a strong basis for their use as indicators of stress (Gill and Tuteja, 2010). Major non-enzymic antioxidants include

i. Ascorbic acid (AA): It is known to be abundant and one of the most potent antioxidants involved in ROS (e.g., $O_2^{\bullet -}$ [Foyer et al., 1991]) detoxification and prevention (Smirnoff, 2005; Athar et al., 2008; Gill and Tuteja, 2010; Govindaraj et al., 2017). This water-soluble antioxidant is found in all cellular compartments and at higher concentrations in photosynthetic cells (Mittler, 2002). AA is mostly present in its reduced form (Khan et al., 2011). It is crucial for the maintenance of membrane structure and capable of completely preventing lipid peroxidation initiation, scavenging ROS like singlet oxygen, hydroperoxyl radicals, superoxide and peroxynitrite, and protects other substrates from oxidative impairment (Gill and Tuteja, 2010; Govindaraj et al., 2017). Also, it has been documented to be involved in ROS scavenging by controlling redox balance in cells (Tommasi et al., 2001). AA has been reported to enhance abiotic stress tolerance (Azzedine et al., 2011; Khan et al., 2011; Mazid et al., 2011; Alamri et al., 2018). Ascorbic acid is involved in the modulation of the synthesis of tocopherol (Ahmad et al., 2014) and the regulation of plant defence responses over and above developmental processes (Conklin and Barth, 2004).

ii. Glutathione (GSH): In addition to AA, GSH is another non-enzymic antioxidant involved in the detoxification of ROS (Foyer et al., 1991). Both GSH and AA are involved in the ascorbate–glutathione cycle, where ascorbate peroxidase plays a role in the direct removal of H_2O_2 (Noctor and Foyer, 1998), singlet oxygen (Gill and Tuteja, 2010) and hydroxyl radical (Larson, 1988). AA is most abundant in its reduced and active form and found in various cellular compartments, including cytosol, mitochondria, endoplasmic reticulum, vacuole, peroxisomes, apoplast and chloroplasts (Mittler and Zilinskas, 1992; Jiménez et al., 1998). GSH provides a substrate for several reactions forming oxidised glutathione (GSSG). Balanced GSH to GSSG levels is key to maintaining a redox state in cells (Foyer and Noctor, 2005). A decline in GSH levels during stress often leads to an imbalanced redox state, thereby causing system deterioration (Tausz et al., 2004). Heightened biosynthesis of GSH in chloroplasts, instead of protecting cells, may cause oxidative impairment, perhaps by adjusting the general redox state of chloroplasts (Creissen et al., 1999). It has been reported that the ratio of reduced to oxidised antioxidants can signal the modulation of ROS-scavenging mechanisms (Karpinski et al., 1997; Mittler, 2002). GSH plays a major role in protecting oxidative attack on biological membranes (Moron et al., 1979; Govindaraj et al., 2017) and participates in various physiological events, including sulphate transport regulation, xenobiotics detoxification and signal transduction (Smirnoff, 2000; Gill and Tuteja, 2010). Heightened GSH level has been linked with plants' ability to withstand oxidative stress (De Paula et al., 1996; Hsu and Sung, 1997; Pietrini et al., 2003).

iii. Tocopherol (vitamin E): This lipophilic phenolic compound exists in eight similarly potent forms as alpha (α)-, beta (β)-, gamma (γ)- and delta (δ) -tocotrienols and -tocopherol (Zingg and Azzi, 2004). It forms part of the biological membrane, playing both non-antioxidant (Zingg and Azzi, 2004) and radical chain breaker functions (Marcus et al., 1998). It is regarded as potential ROS and lipid radical scavenger (Holländer-Czytko et al., 2005). Reduction in tocopherol levels following seed ageing suggests that it is involved in protection against oxidative stress-induced impairments

(Senaratna et al., 1988) thus making it a useful indicator of seed deterioration (Govindaraj et al., 2017). Its synthetic analogue, trolox, has also been reported to be similarly capable of preventing oxidative impairment (Hamad et al., 2010). Trolox has some advantages in being moderately soluble in water (Lúcio et al., 2009). Unlike α-tocopherol, trolox may be integrated directly into both lipid and water parts of cells (Hamad et al., 2010), thus making it suitable for conducting studies involving both living systems and model systems (Ross et al., 1995; Lúcio et al., 2009). The antioxidant power of trolox has been reported to be much more than that of α-tocopherol (Satoh et al., 1997; Hamad et al., 2010). Other synthesised analogues include Vitamin E acetate, α-tocopherylphosphate and α-tocopherylsuccinate (Birringer et al., 2003; Zingg and Azzi, 2004).

iv. β-carotene: Besides tocopherols, carotenoids play an important role in photoprotection of phototrophs by eliminating surplus energy as heat, directly scavenging reactive oxidants (Gill and Tuteja, 2010), including $^1O_2$, free radicals and protecting cells from oxidative impairment by suppressing lipid peroxidation (Govindaraj et al., 2017). Their antioxidant property is attributed to their extended conjugated double bonds system (Krinsky, 1992; Sies and Stahl, 1995; Collins, 2001). Low β-carotene levels have been shown to protect membrane lipids from peroxidative reactions (Krinsky, 1992; Govindaraj et al., 2017).

v. Gallic acid (GA): In plants, GA is a relatively ubiquitous (Haddock et al., 1982; Haslam and Cai, 1994) endogenous polyphenolic compound with several biological activities (Bate-Smith, 1962; Dewick and Haslam, 1969; Urquiaga and Leighton, 2000 Handique and Baruah, 2002), including reacting with active oxidants preventing their formation and accumulation (Handique and Baruah, 2002). GA occurs in the free or conjugate (as esterified hydrolysable tannins [Taiz and Zeiger, 2010]) form in several plants (Haddock et al., 1982; Ow and Stupans, 2005). Though polyphenols like quercetin (Metodiewa et al., 1999), as well as GA (Inoue et al., 1995; Sakagami and Satoh, 1997), may act as prooxidants depending on concentration and condition (Tückmantel et al., 1999; Verma et al., 2013), GA is primarily used as an antioxidant (van der Heijden et

al., 1986; Nakatani, 1992) due to their capacity to scavenge ROS like H_2O_2 (Yen et al., 2002).

2.9 Seed invigoration treatments

2.9.1 A short history of seed pre-hydration treatment

Seeds are continually faced with multiple challenges relating to production, post-harvest storage and subsequent quality. Moreover, in view of the effects of global warming as a symptom of climate change, different stress factors may cause poor seed performance in terms of reduced germination, uneven seedling emergence, poor seedling establishment, destructive alteration of root cell architecture, and thereby leading to a substantial yield loss (Rakshit and Singh, 2018). Hence, concerted efforts towards the improvement of seed performance have led to the development of different pre-sowing treatment techniques that can augment germination and synchronise seedling emergence under different suboptimal growth conditions dating back to the ancient Greeks (Evenari, 1984; Paparella et al., 2015). Seed pre-hydration was discovered by "Theophrastus, Democritus (5th century B.C.)" and "Mago (4th-3rd century B.C)" (Evenari, 1984). It was suggested that seed pre-hydration treatments in water or milk enhanced the germination of cucumber seeds (Theophrastus, D.H.P. Book VII, 1: 6). Democritus suggested steeping all seeds in some "roof tiles" plant extract before sowing (Plinius, N.H. Book XVIII, XLV: 159). Some other mentions include pre-hydrating almond seeds in a solution of honey or manure according to Carthaginian Mago (N.H. Book XVII, XI: 63), pre-hydrating pulses in "nitre" (Theophrastus, D.H.P. Book II, IV: 2), seed ripening of mistletoe in bird droppings (Plinius, N.H. Book XVI, XCII: 247) and pre-soaking cabbage seeds in houseleek extract to provide the cabbage with resistance to various insects (Plinius, N.H. Book XIX, LVIII: 180). The need to dry seeds artificially "to make them fertile" was also mentioned by Plinius (Plinius, N.H. Book XIX, XXXVI: 120) (Evenari, 1984).

In the 16th century, Olivier de Serres described the steeping of grains (*Hordeum*, *Secale* and *Triticum* spp.) in manure solution for 24 hours followed by drying back as a pre-sowing technique for enhanced seedling performance (Paparella et al., 2015; Waqas et al., 2019). In the 19th century (1855), Charles Darwin experimented with a seawater pre-hydration treatment and reported enhanced germination in treated cress and lettuce seeds. May et al. (1962)

demonstrated that drying of seeds for some time after hydration bestowed beneficial effects leading to increased germination rate under normal and adverse conditions. In 1963, Ells James presented the modern seed priming concept, pointing out the vital parameters of seed pre-hydration treatment and reporting that an increased rate of seedling emergence was observed in tomato seed exposed to the nutrient solution (Paparella et al., 2015; Waqas et al., 2019). Heydecker (1974) recognised the term seed "priming" as used by Malnassy, (1971), describing it as a seed pre-sowing treatment that can improve performance under suboptimal conditions (Parera and Cantliffe, 1994; Sivasubramaniam et al., 2011). Further, Heydecker (1974) described seed priming as a pre-hydration treatment in an osmotic solution that permits imbibition to the first germination phase before radical protrusion. Such seeds are sometimes dried back ('hardening' [Rowse, 1992]) to their initial moisture level and sown or stored (Bradford, 1986). Also, the use of specific terms like halopriming (imbibing in salt solutions) and osmotic priming (imbibing in other osmotic solutions) was proposed (Heydecker, 1974) to specify the priming agent. The technique thus far is recognised and widely used to improve seed performance in the field of agriculture (Parera and Cantliffe, 1994). During the pre-hydration treatment, the absorption of water is controlled to allow for the activation of pre-germinative metabolism without permitting radicle emergence by limiting the seed moisture content (Tarquis and Bradford, 1992; Welbaum et al., 1998b; Sivasubramaniam et al., 2011; Singh et al., 2020). The resultant seedlings assume a physiological (primed) state which enables faster growth and/or better activation of plant defence responses (Beckers and Conrath, 2007; Jisha et al., 2013).

2.9.2 Seed pre-hydration and pre-germinative metabolism

In the 'primed state', the hydration-induced specific metabolic changes are responsible for the ensuing beneficial effects of seed pre-hydration treatments (Osborne, 1983; Bray et al., 1989; Bray, 1995; Paparella et al., 2015). Upon seed imbibition, major cell functions and processes are activated, like the de novo proteins and nucleic acids biosynthesis, ATP formation, phospholipids and sterols accumulation, DNA repair and antioxidant system activation – the 'pre-germinative metabolism' (Paparella et al., 2015). Severe oxidative impairment of biomolecules like lipids, nucleic acids and proteins may occur in the early germination stages, during maturation on the mother plant as well as in post-harvest storage and under various stress

conditions (Cakmak et al., 1993; Kranner et al., 2010; Sahu et al., 2017). For seed vigour to be preserved and germination to be successful, embryonic DNA repair mechanisms must be well preserved. A good repair of impaired DNA allows for the resumption of cell cycle progression and DNA replication, while a defective repair system causes oxidative cell death (Osborne et al., 1980; Kranner et al., 2010; Waterworth et al., 2015). DNA impairments in seed embryo are repaired during early imbibition and are essential for performance in terms of germination and storability (Waterworth et al., 2010). Thus, DNA repair is a vital part of 'pre-germinative metabolism' triggered during imbibition and accompanied by unrestrained ROS activities (Paparella et al., 2015) capable of causing mutation in the meristematic tissues of the embryo (Vonarx et al., 1998). All major DNA repair pathways, such as the base- and nucleotide-excision repair, are triggered at the early imbibition phase for the maintenance of genome integrity (Vonarx et al., 1998; Macovei et al., 2010; Balestrazzi et al., 2011; Waterworth et al., 2015). Efficient ligase-dependent re-joining of strand breaks is key to most DNA repairs, and DNA ligase VI found only in plants has been described as a major deciding factor of seed quality and storability in *Arabidopsis thaliana* (Waterworth et al., 2010).

With regards to the regulatory roles of reactive oxidants in the germination of seeds, Møller et al. (2007) opined that comparatively long-lived oxidants like H_2O_2 takes the signal to a distant target, whereas the short-lived oxidants such as $HO^{\bullet}$ likely act near its production site and the product of oxidation (acting as a secondary messenger) then takes the signal to the target transcription factors. Besides signalling mediated by ROS, severe lesions to biomolecules can result from ROS activities. Though DNA impairments can be 'addressed' by certain repair functions, RNA is extremely sensitive to ROS-induced oxidative impairment owing to lack of specified mechanism of repair (El-Maarouf-Bouteau et al., 2013), while protein damage can be reversible (as in the oxidation of cysteine and methionine) and/or irreversible (as in carbonylation) (Shacter, 2000; Ghezzi and Bonetto, 2003; Møller et al., 2007; Anjum et al., 2015).

Nevertheless, enhanced activities of antioxidant (defence) enzymes such as APX, CAT, SOD, and GR allows for the control of ROS levels during imbibition (Bailly et al., 2000; Hsu et al., 2003). The ROS scavenging antioxidant potential of the seed is critical for the enhancement of germination and stress tolerance (Gidrol et al., 1994; Bailly et al., 2008; Dolatabadian et al., 2009;

Sahu et al., 2017; Xia et al., 2020). Also, gene expression profiling encoding antioxidant enzymes is a useful index of seed antioxidant response during germination. These safeguarding functions are triggered during pre-hydration treatments, thereby allowing seeds to undergo major metabolic and physiological pre-germinative phase changes up to the first cellular division, leading to improved germination and increased seedling vigour upon sowing (Paparella et al., 2015).

2.9.3 The seed priming technology overview

The priming concept usually refers to several approaches towards seed invigoration, all involving controlled hydration of seeds (Farooq et al., 2006). The seed priming technique is used to improve the overall post-harvest performance of seed (Khan et al., 1980; Taylor et al., 1992; Ghassemi-Golezani and Esmaeilpour, 2008; Mirmazloum et al., 2020), including longevity (storability) (Khan, 1992; Bruggink et al., 1999; Rajjou and Debeaujon, 2008; Chandra et al., 2019) and ability to withstand unfriendly environmental condition (Hegarty, 1977; Sivasubramaniam et al., 2011; Jisha et al., 2013; Ashraf et al., 2018). Priming enhances seed germination in three phases (Bewley, 1997): imbibition, germination, and growth (Waqas et al., 2019). During the first phase (imbibition), characterised by rapid water uptake owing to low seed water potential, respiratory activities and protein synthesis through existing DNA and mRNA are promoted. Phase II (germination) is a lag phase involving the initiation of various physiological functions relating to germination, including protein and mitochondria synthesis, degradation of stored food and reorganisation of cellular membrane, to support radicle protrusion and growth of seedling, which commences in Phase III (growth phase) (Varier et al., 2010; Ruttanaruangboworn et al., 2017; Waqas et al., 2019). The key determinant of seed priming is the controlled uptake of water up to Phase II, prior to radicle emergence (Varier et al., 2010; Waqas et al., 2019), which allows for vital physiological events like damaged DNA and mitochondria repair (Bewley, 1997). Priming duration can vary from less than 24 h (Cantliffe, 1981) to days (Bradford et al., 1990) or weeks (Khan et al., 1980), depending on cultivars, species and seed lot (Taylor et al., 1988). Phase II is more sensitive to environmental factors than Phase III. Hence, primed seeds that have undergone Phase II may be able to germinate better than unprimed seeds under suboptimal conditions (Waqas et al., 2019).

In many cases, primed seeds are dried back to a particular moisture level and stored (Mondal et al., 2011) or sown by the conventional method (Taylor et al., 1988; Parera and Cantliffe, 1994; Matsushima and Sakagami, 2013; Forti et al., 2020). Seed drying back is thought to confer a 'hardening' effect (Heydecker et al., 1973; Karivaratharaju and Ramakrishnan, 1985; Rowse, 1992; Basra et al., 2005). In the hardening technique, multiple (two to three) soakings with drying back cycles are suggested to yield a better result, although one cycle is enough for most species (Lee et al., 1998; Lee and Kim, 1999; Farooq et al., 2004; Solaimalai and Subburamu, 2004; Mondal et al., 2011). Seed hardening induced by pre-sowing treatments is attributed to some cytoplasmic physico-chemical changes such as decreased lipophilic and increased hydrophilic colloids, greater protoplasmic elasticity and viscosity, increased hydration of colloids, increased bond water level and increased protein coagulation temperature (Karivaratharaju and Ramakrishnan, 1985; Solaimalai and Subburamu, 2004). However, there have been reports of delayed germination and/or emergence in primed seeds that are dried back, relative to primed but not dried back seeds, owing to the extra time needed for rehydration though other beneficial effects of priming are conserved (Brocklehurst and Dearman, 1983; Brocklehurst et al., 1984; Akbar, 2008; Sivasubramaniam et al., 2011). Additionally, deterioration of seeds in storage has been reported when primed seeds were dried back in different species like *Lycopersicon esculentum* (Alvarado and Bradford, 1988), *Cichorium endivia* (Bekendam et al., 1987) and *Lactuca sativa* (Weges, 1987). Tarquis and Bradford (1992) stated that though pre-hydration treatments caused an increased germination rate, drying back predisposed lettuce seeds to loss of storability. This effect varies depending on initial seed quality (Sivasubramaniam et al., 2011). Thus, it has been suggested that the storage of primed seeds cannot extend beyond only a few weeks as mechanisms for repair of impaired DNA become reduced (van Pijlen et al., 1996). In a study on *Mimosa bimucronata*, Brancalion et al. (2008) added that priming benefits were partly lost in dried back seeds, recording lower performance in terms of percentage germination, seedling vigour, uniformity and germination speed index, and higher electrical conductivity relative to primed but not dried back seeds.

2.9.4 Seed priming methods

Seed priming methods are generally divided into classical (hydropriming, osmopriming,

redox priming, hormonal priming, cellular chemical priming, nutrient priming, priming with plant extracts, priming with plant growth regulators and biopriming [Jisha et al., 2013; Waqas et al., 2019; Singh et al., 2020]) and advanced (nanopriming [Mahakham et al., 2017], magnetopriming, irradiation with microwaves or ionising radiations and some other physical priming agents [Araújo et al., 2016]) techniques, some of which are described below.

2.9.4.1 Classical seed priming techniques

2.9.4.1.1 Hydropriming

Hydropriming is an age-old seed invigoration method popular with farmers as it is simple and economical. Hydropriming is of two types: drum-priming and on-farm priming (Singh et al., 2020). Drum-priming involves seed hydration by water vapour generated from a gentle rotation of a drum at a particular temperature (Rowse, 1992). In on-farm priming, seeds are pre-soaked in water for a period before sowing (Harris et al., 2001; Singh et al., 2020). Hydropriming technique is particularly useful under stressful conditions such as high heat and salinity and water deficit stress as seed hydration and water uptake efficiency in these conditions are enhanced (Waqas et al., 2019). However, maintaining optimum humidity and temperature is critical to preventing radicle protrusion, as hydropriming can allow for uncontrolled water uptake (Taylor et al., 1998). In contrast to unprimed (direct) sowing, the benefits of hydropriming have been demonstrated in several studies including, 3–4 times increase in biomass allocation and seedling length of *Cicer arietinum* under drought stress conditions (Kaur et al., 2002), rapid emergence and increased seedling vigour in rice seeds subjected to water-stress (Matsushima and Sakagami, 2013), and increased germination of three years stored seeds of napa cabbage (*Brassica rapa*) which correlated with decreased electrical leakage as well as enhanced antioxidant enzymes (superoxide dismutase and peroxidase) activities and soluble sugar level (Yan, 2015).

2.9.4.1.2 Osmopriming

In this pre-sowing treatment method, seeds are subjected to controlled hydration in an osmotic solution of low water potential generated from the addition of osmotica such as polyethylene glycol, sorbitol, glycerol and mannitol to priming water (Heydecker et al., 1973;

Ashraf and Foolad, 2005; Jisha et al., 2013; Singh et al., 2020). The low water potential of the osmotic solution is a crucial factor enabling seeds to be partially hydrated for pre-germinative metabolism but inhibited protrusion of the radicle (Taylor et al., 1998; Ashraf and Foolad, 2005; Bennett et al., 2018). Also, the use of various salt solutions (halopriming) has been widely reported, and their beneficial effects elucidated. For instance, Singh et al. (2014) osmoprimed *Vigna unguiculata* seeds with KNO_3 solution and reported improved germination, plant height, and biomass accumulation compared with unprimed and hydroprimed seeds. Fatokun et al. (2020) reported enhanced seedling emergence, photosynthetic and growth parameters of *Pisum sativum* and *Cucurbita pepo* seeds aged to 50% viability after priming with a mixture of $CaCl_2$ and $MgCl_2$ solutions relative to the unprimed seeds. Priming of cabbage seeds using varying levels (1%, 2% and 3%) of inorganic salts like KCl, KH_2PO_4, KNO_3, $MgCl_2$, $MgSO_4$, and NaCl significantly increased germination, seedling vigour, biomass accumulation and reduced mean germination time (Batool et al., 2015). Priming of artificially deteriorated *Brassica napus* seeds with $CaCl_2$ promoted seedling vigour (Abdolahi et al., 2012). Carrozzi et al. (2012) reported that priming with $MgSO_4$ increased germination of *Lactuca sativa* seeds stored for a year. Osmopriming is a low-cost priming option and allows for better water conservation (Moradi and Younesi, 2009).

2.9.4.1.3 Redoxpriming

This seed invigoration method refers to priming with antioxidative compounds (Jisha et al., 2013). Plant cell redox state is key to the regulation of growth, development and stress tolerance (Foyer and Noctor, 2005; Potters et al., 2010; Gupta et al., 2016; Kumar et al., 2016). Plant redox status is disturbed in response to external stimuli, and the severity of disturbance is determined by the kind of stimulus, the amount and the duration of tissue exposure (Jisha et al., 2013). Maintaining an appropriate redox environment (Schafer and Buettner, 2006) is thought to help in minimising the severity of stress-induced damage (Jisha et al., 2013). During oxidative stress, antioxidants are well-known redox buffers capable of reacting with ROS and functioning as a metabolic interface that moderate the proper induction of acclimation responses or programmed cell (Halliwell and Foyer, 1976; Takahashi and Asada, 1988; Miller et al., 2010). Among the compounds of major importance in the antioxidant pathway of plants, glutathione plays a significant role in the cellular redox signalling networks influencing growth, development

and defence (Foyer and Noctor, 2005; Jisha et al., 2013). Glutathione and tocopherol used as seed pre-hydration treatments resulted in increased seedling length of *Helianthus annuus* (Draganić and Lekić, 2012). In addition, pre-hydration treatment of seeds with other antioxidant solutions has been reported to improve seed performance in several species. For instance, pre-hydration with ascorbic acid (AA) solution improved agronomic and biochemical vigour of *Pisum sativum* seeds (Burguieres et al., 2007) and improved germinability and tolerance to deterioration of *Elymus sibiricus* artificially aged for 48 h (Yan et al., 2015). As mentioned by Afzal et al. (2006), seed pre-hydration treatment with AA and tocopherol enhanced vigour and storability of *Helianthus annuus* (Bhattacharjee and Gupta, 1985), maize, mustard (Dey and Mukherjee, 1988) and *Oryza sativa* (Bhattacharjee and Bhattacharyya, 1989).

2.9.4.2 Advanced seed priming techniques

2.9.4.2.1 Nanopriming

The use of nanomaterials in agriculture is somewhat recent relative to their application in biomedical and industrial sectors (Mahakham et al., 2017), and it is considered a promising approach that can transform food production and agriculture (Parisi et al., 2015; Servin et al., 2015) to meet the demand for food security in view of the envisaged rise in world population (Fraceto et al., 2016; Sundaria et al., 2019). Nanotechnology employs not more than 100 nm size of biocompatible nanoparticles (Waqas et al., 2019), often synthesised with plant extracts of desirable phytochemical properties as the nanopriming agents (phytosynthesised nanoparticles) (Mahakham et al., 2017). For example, Mahakham et al. (2017) primed *Oryza sativa* seeds stored for three years using phytosynthesised silver particles obtained from silver nitrate ($AgNO_3$) solution mixed with *Citrus hystrix* leaves extract (as reducing and stabilising agents). They reported enhanced performance in terms of germination and seedling vigour. Further, they proposed the mechanisms of action of nanopriming-induced invigoration of seed to include nanopores formation for the enhancement of water uptake, optimising ROS/antioxidant systems in seeds, production of $HO^{\bullet}$ for loosening of the cell wall and weakening of endosperm to enhance seed germination as well as nanocatalyst-enhanced hydrolysis of starch. In another study, Sundaria et al. (2019) demonstrated increased germination and shoot length in IITR26 and

WL711 wheat (*Triticum aestivum*) genotypes, respectively, using iron oxide synthesised nanoparticles as a priming agent. Further, they demonstrated and proposed nanopriming for wheat grain biofortification with iron which is a potential strategy for overcoming iron deficiency in humans.

2.9.4.2.2 Seed priming with physical agents

Thus far, various studies have shown that plant metabolic and developmental processes are sensitive to magnetic fields (Hirota et al., 1999; Răcuciu et al., 2008; Aladjadjiyan, 2010; Teixeira da Silva and Dobránszki, 2016). Magnetic fields are now being used for the invigoration of seeds and enhancement of agricultural productivity (Bilalis et al., 2012; Singh et al., 2020). Several beneficial effects of magnetopriming (priming with the magnetic field) have been documented in various studies for different plant species. For instance, Baby et al. (2011) reported improved germination, vigour, seedling biomass, the performance index of Chlorophyll a fluorescence and reduced level of $O_2^{\bullet-}$ in leaves of *Glycine max* seeds primed with a static magnetic field. Besides increased germination and germination speed, field emergence, vigour and seedling biomass, other beneficial effects such as improved membrane integrity and reduced electrolyte leakage were reported in *Helianthus annuus* seeds subjected to magnetopriming (Vashisth and Nagarajan, 2010). Further, they ascribed high germination rate and vigour to magnetopriming-induced rise in α-amylase, protease and dehydrogenase activities.

Gamma radiation (Hegazi and Hamideldin, 2010; Marcu et al., 2013; Araújo et al., 2016), UV radiation (Ouhibi et al., 2014; Thomas and Puthur, 2017; Thomas and Puthur, 2019), X-rays (Al-Enezi et al., 2012; De Micco et al., 2014), and microwaves (Randhir and Shetty, 2004; Han, 2010) are some other commonly used physical priming agents (Dutta, 2018; Waqas et al., 2019).

2.10 Study species description

The selected species (cabbage and lettuce) for this study are primarily propagated by seeds which have been considered to be orthodox and are usually stored below 10% water content and 10 °C, or as cool as possible (Ibrahim and Roberts, 1983; Still, 1999; Walters and Towill, 2004). The two species are outlined below in terms of their habit, origin, distribution and economic importance.

2.10.1 Cabbage

Brassica oleracea var. *capitata* f. *alba* L. (cabbage, 'Glory of Enkhuizen') is one of the 37 species in the *Brassica* genus (Gomez-Campo, 1980; Rakow, 2004) comprising about 338 genera belonging to Brassicaceae (or Cruciferae) family (Nosek et al., 2011; Šamec et al., 2017). *B*. *oleracea* is mostly herbaceous (Msikita et al., 1997), suffrutescent perennial species having a short thick vegetative stem (Rakow, 2004). The wild *Brassica oleracea,* which are considered the progenitor species of cabbage cultivars are restricted to the Coasts of North Spain, Western and Northern France, isle of Helgoland and the British Isles, (Snogerup et al., 1990; Panda et al., 2003). The domesticated *Brassica oleracea* cultivars, which are now of cosmopolitan distribution, are commonly called 'cole crops', including cauliflower, broccoli, Brussels sprouts, kohlrabi, kale and cabbage (Haynes et al., 2009), and are likely of the ancient Mediterranean origin (Maggioni et al., 2010). The cultivated *B. oleracea* are polymorphic, varying in leaf colour, size and shape, and grouped into savoy, red and white forms (Singh et al., 2006) and mostly seasonal (annuals or biennials) plants (Msikita et al., 1997). They are economically important dietary vegetables and one of the most grown cole crops across the world (Maggioni, 2015). The var. *capitata* f. *alba*, commonly called white cabbage (Jafary-Jahed and Razmjou, 2020), are recognised for their large leaves, which form a characteristic head having a substantial amount of vitamins, minerals and bioactive compounds essential to the human diet (Ryder, 1979; Nosek et al., 2011; Erdem et al., 2015; Šamec et al., 2017).

2.10.2 Lettuce

Lactuca sativa L. (lettuce, 'Great Lakes') belongs to the Asteraceae (Compositae) family, having approximately 100 species in *Lactuca* genus (de Vries, 1997). It is an annual herbaceous vegetable having spirally arranged leaves that form a dense rosette (Křístková *et al*., 2008). The origin of the cultivated *L*. *sativa* is regarded as polyphyletic (Křístková et al., 2008). The closest wild species and perhaps the progenitors are *L*. *serriola* (de Vries, 1997; Mou, 2009). The comestible genotypes are placed in seven cultivar groups (morphotypes), viz. Stalk, Latin, Cutting, Cos, Butterhead, Crisphead and Oilseed (de Vries, 1997; Křístková et al., 2008). They are regarded as the most important leafy vegetable and grown on a commercial scale across the world (Křístková

et al., 2008). The Crisphead group, including great lakes cultivar (Gray, 1975), provide a substantial amount of vitamins, minerals and phytochemicals essential to the human diet (Ryder, 1979; Mou, 2009; Midan and Sorial, 2011).

CHAPTER 3: MATERIALS AND METHODS

The present study was carried out in four major parts, viz. characterisation of seed ageing rates and patterns via controlled deterioration, antioxidant pre-hydration treatment experiments, inorganic salt pre-hydration treatment experiments and a greenhouse pot trial. All chemicals used were of analytical grade (Sigma-Aldrich, St. Louis, USA) unless otherwise stated.

3.1 Characterization of seed ageing rates and patterns via controlled deterioration

3.1.1 Seed material

Commercial seeds of *Brassica oleracea* L. (cabbage, 'Glory of Enkhuizen') and *Lactuca sativa* L. (lettuce, 'Great Lakes'), supplied in hermetically sealed plastic bags, were obtained from McDonalds Seeds (Pietermaritzburg, South Africa) and stored at 4 °C before being used in the experiments described below. Once the bags were opened, seeds were transferred into air-tight metal containers and used within 3 months of storage after purchase.

3.1.2 Seed vigour assessment

Seeds were removed from storage at 4 °C and maintained at room temperature overnight before use. Each seed lot was subjected to an initial germination and vigour test ($3 \times n = 25$) before being subjected to controlled deterioration (CD) and the application of pre-treatment solutions as described below. Only high vigour seed lots (germination >85% in cabbage and >95% in lettuce within 48 h of sowing) were used for experiments that follow.

3.1.3 Controlled deterioration

Seed moisture content (MC, %) was determined on a fresh mass (FM) basis using the adjusted low constant temperature oven method recommended by International Seed Testing Association (ISTA) for crop seeds (Komba et al., 2006). Controlled deterioration, a vigour declining approach that indicates storage longevity of seeds (Powell and Matthews, 2005; Mavi and Demir, 2007; Demir and Mavi, 2008) was used to simulate seed ageing. Controlled deterioration experiments performed using the methods of Mavi and Demir (2007) did not yield a reproducible

result (Fig. A1; Appendix A); hence, ageing methods described by TeKrony (2005) were used with slight modifications. Seed MC (%) was raised to 11% in 500 ml (134 x 102 x 70 mm) 4-side locked airtight plastic vessels (Addis®, Cape Town, South Africa) containing saturated potassium chloride (KCl) salt solution (Fig. 1). The saturated solution, made up of a slurry of KCl (50 g) and 20 ml of deionised water, produced a relative humidity of 85% at 25 °C within the vessels (Winston and Bates, 1960) as measured by a digital temperature and humidity meter (Sinotimer®, Wenzhou, China). Seeds were spread in a monolayer in aluminium weighing boats (100 seeds of each species [cabbage, 0.08 g; lettuce, 0.02 g] per boast and six boats per vessel) which were arranged on a mesh platform 5 cm above the saturated KCl solution. The inner surface of the plastic lid that sealed each vessel was lined with three layers of paper towel to prevent condensed vapour from dripping onto the seeds. The seed-containing vessels were maintained at 25 °C for 24 h to reach the target MC: MC was raised from 5.5% to 11% in cabbage and 5.7% to 11% in lettuce. Thereafter, the vessels were placed in an incubator (Heraeus FB 420, Hanau, Germany) set at 35 °C where the RH seed MC were kept at 83% and 11%, respectively for the duration of the experiment.

Figure 1 Setup of vessels in which seed moisture level was raised using saturated KCl solution: A) vessel containing saturated KCl solution, B) mesh platform with seeds spread in a monolayer in aluminium weighing boats, and C) seed-containing vessel with mesh platform raised 5 cm above saturated KCl solution and covered with lid having inner surface lined with a paper towel.

3.1.4 Germination test

A germination test was done daily until no germination was recorded. On each sampling day, four trials of 25 seeds each were removed from the incubator and sown between two layers of germination paper (Anchor Paper Co., Saint Paul, USA) moistened with 3 ml deionised water (DW), within Petri dishes (90 × 15 mm) and incubated in a growth room at 20 ± 2 °C and a 16:8 h photoperiod. Watering was done from time to time as required. Seedling production (%), discriminating between normal (including seedlings that were considered abnormal on germination but later became normal) and abnormal (with shoot growth but root length < 2 mm) growth, was assessed 14 days after sowing (DAS). Seeds that developed chlorophyllous cotyledons but failed to germinate and seedlings that died within 14 DAS were scored as dead. The experiment was repeated twice, and a CD curve was constructed for each species using the normal seedling (%) data (Fig. 2A, B). From the CD curve, times (days) taken to 75% viability (P75), 50% viability (P50) and 25% viability (P25), hereafter referred to as P75, P50 and P25 seeds, respectively were identified. Fresh seeds (viability: cabbage, >85%; lettuce, >95%) served as the control.

To identify seed ageing rates and patterns, CD curves from the % normal seedling data were converted to probit values and fitted using the Ellis and Roberts (1980) survival curve equation (Bam et al., 2008; Crawford et al., 2011):

$$v = Ki - \frac{p}{\sigma}$$

Where v = probit of % final viability after p days storage,

p = storage duration (days),

Ki = intercept, probit of % initial viability, and

1/σ = slope, which shows the rate of seed deterioration

The regression analyses of the probit values provided intercepts (*Ki*) and slopes (1/σ) for each CD curve.

3.2 The antioxidant pre-hydration treatment experiments

3.2.1 Application of exogenous antioxidant solutions

When seeds are pre-hydrated with exogenous solutions for invigoration, it is important to first determine the treatment duration as seeds should not germinate in the treatment solutions. This is because ROS are also involved in germination (Job et al., 2005; Verma et al., 2015) and exposure to antioxidants during germination can compromise radicle emergence. In the present study, an imbibition curve (Fig. A2) was firstly generated for each species by hydrating (using 3 mL DW) three replicates of 25 seeds placed between two discs of germination paper (Anchor Paper Co., Saint Paul, MN, USA), one below and the other above, in Petri dishes (90 × 15 mm) at laboratory temperature of 23 ± 2 ◦C. The Petri dishes were covered and sealed with Para film to prevent drying out. Then they were left for imbibition on a benchtop shaker (Labcon SPO 15-MP orbital, Maraisburg, South Africa) set at 100 rpm. At 2-h intervals, the seeds were blotted with a paper towel, weighed and returned to the moistened Petri dishes. The process was repeated until the first physical signs of germination (2 mm long radicle protrusion). The data was used to identify an imbibition time required to reach phase 2 of germination (period of early germination processes before radicle protrusion; Varier et al., 2010) (Fig. A2; Appendix A). Based on these data, imbibition times of 8 h for cabbage and 6 h for lettuce were used for all subsequent pre-hydration treatments.

For treatments application, four trials of 25 seeds each of fresh and controlled deteriorated (CDd) (P75, P50 and P25) seeds of both species were soaked in aqueous solutions of ascorbic acid (AA), gallic acid (GA), reduced glutathione (GSH), trolox (an analogue of α-tocopherol) and glycerol. The exogenous antioxidants were applied at three concentrations, 0.2 mM, 0.4 mM and 0.6 mM, and deionised water (DW) served as a control. This is based on the fact that a wide range of antioxidants applied exogenously at concentrations in this range has been shown to be beneficial in alleviating oxidative stress-induced lesions, enhancing germination, vigour and seed storability of several plant species (Amjad et al., 2007; Ozfidan-Konakci et al., 2015; Kuchlan et al., 2017). Applying the different antioxidants at three similar concentrations allowed for the comparison of effects within and across antioxidants. For each

treatment combination (CD level × antioxidant × concentration), four replicates of 25 seeds were soaked in 3 ml of antioxidant solution/DW for 8 h in cabbage and 6 h in lettuce. Seeds were then removed from soaking, blotted dry with a paper towel and assessed for germination as described for the CD experiments. Percentage seedling production (normal and abnormal) was assessed 14 DAS. Root and shoot length measurements were also taken 14 DAS and used to calculate the seedling vigour index (Abdul-Baki and Anderson, 1973).

3.2.2 Evaluation of biochemical markers of oxidative stress and germinability

Where specific CD level × antioxidant × antioxidant concentration combinations resulted in enhanced production of normal seedlings relative to seeds soaked in DW, seeds were exposed to these selected treatment combinations and assessed immediately after pre-hydration treatment for electrolyte leakage and a range of biochemical markers of oxidative stress and germinability (germination enzymes). For comparative purposes, two controls were used for the electrolyte leakage and biochemical marker assays: seeds aged to the appropriate CD level and (1) soaked in DW and (2) unsoaked. Seeds were blotted dry before processing them for all assays.

3.2.2.1 Electrolyte conductivity

Electrolyte leakage was measured in seeds using a CM100-2 multi-cell conductivity meter (Reid & Associates, Durban, South Africa) following Sershen et al. (2016) with slight modifications. Individual seeds (n = 5) were soaked in 2 ml of antioxidant solution/ DW for 8 h. The conductivity of the pre-hydration treatment (1.5 ml) was measured. Seeds were dried in an oven (Gallenkamp IH-150, London, England) at 80 °C for 48 h and weighed on a six-place balance (Mettler-Toledo MT5, Zürich, Switzerland) to determine the dry mass (DM). Leakage was represented as average conductivity after 8 h less the conductivity of DW and expressed as mS^{-1} cm^{-1} g^{-1} DM. The respective antioxidant solutions were used as blanks.

3.2.2.2 Conjugated dienes

Conjugated dienes were estimated as described by Parkhey et al. (2012). Seeds (three replicates of 0.25 g each) were homogenised in 4 ml methanol containing 0.02% (w/v) ethylenediaminetetraacetic acid (EDTA), 1% (w/v) sodium chloride (NaCl) and 2 ml of chloroform.

The homogenate was centrifuged (Model J-E, Beckman Coulter Avanti®, La Brea, CA, USA) at 11,000 *x g* for 20 min at 4 °C. An aliquot (100 µl) of the chloroform phase was taken and dried under a stream of nitrogen gas. This was dissolved in 2 ml of ethanol, and the absorbance was read using a UV-Vis spectrophotometer (Shimadzu UV-2600, Kyoto, Japan) at 234 nm using ethanol as a blank. Conjugated diene levels were calculated using an extinction coefficient of 25 mM^{-1} cm^{-1} and expressed as μmol^{-1} g FM.

3.2.2.3 4-Hydroxy-2-nonenal (4-HNE)

The estimation of 4-HNE followed the processes described by Parkhey et al. (2012). Seeds (three replicates of 0.25 g of seed each) were homogenized in 2 ml of 0.2 M borate buffer (pH 7.4) and 750 µl of 10% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 11,000 *x g* for 20 min at 4 °C. The supernatant (1 ml) was mixed with 1 ml of 1% (w/v) 2,4-dinitrophenylhydrazine (DNPH) dissolved in 0.5 M hydrochloric acid (HCl) and kept at room temperature for 2 h. This was precipitated with 2 ml of hexane, and the precipitate was dried under a stream of nitrogen gas. The precipitate was reconstituted in 2 ml of methanol, and the absorbance was read at 350 nm using methanol as the blank. The 4-HNE content was calculated using an extinction coefficient of 13,750 $M^{-1}cm^{-1}$ and expressed as $mmol^{-1}$ g FM.

3.2.2.4 Protein carbonylation

Total protein (three replicates of 2 g each) was extracted according to Juszczuk et al. (2008). Total protein content was determined according to (Bradford, 1976), and then the extracts were diluted with deionised water to a protein concentration of 10 mg/ml. Protein carbonyl content was estimated following the spectrophotometric method described by Augustyniak et al. (2015) with slight modifications. A solution (100 µl) of 10 mM DNPH dissolved in 2.5 M hydrochloric acid was added to 100 µl of each protein sample. This mixture was vortexed and incubated in the dark for 10 min at room temperature. Thereafter, 30 µl of 100% TCA solution was added. The solution was vortexed, incubated on ice for 5 min and centrifuged (Model J-E, Beckman Coulter Avanti®, La Brea, CA, USA) at 13,000 *x g* for 10 min. The supernatant was removed, and the pellet reconstituted in 500 µl of ice-cold acetone via sonication for 30 sec. This solution was incubated at −20 °C for 5 min and centrifuged (Model J-E, Beckman Coulter Avanti®,

La Brea, CA, USA) at 13,000 *x g* for 2 min at 4 °C. The remaining pellet was washed two times with acetone to remove excess DNPH and then sonicated briefly in 200 µl of 6 M guanidine hydrochloride. Absorbance was measured at 370 nm against guanidine hydrochloride (6 M) blank and protein carbonyl content was calculated using a molar absorption coefficient of 22,000 M^{-1} cm^{-1} and expressed as nM carbonyl mg^{-1} protein.

3.2.2.5 Enzymic antioxidant activity

The enzyme extraction procedure followed Farrant et al. (2004). Seeds (three replicates of 0.25 g each) were homogenized in 4 ml of extraction buffer (0.1 M sodium phosphate buffer [pH7.8], 0.1 mM EDTA, 2 mM dithiothreitol, 1.25 mM polyethylene glycol [PEG] 4000 and 0.1 g polyvinylpyrrolidone [PVP]). The extract was centrifuged (Model J-E, Beckman Coulter Avanti®, La Brea, CA, USA) at 16,000 *x g* for 30 min at 4 °C. The supernatant was collected and used for catalase, glutathione reductase and superoxide dismutase estimations as described below.

3.2.2.5.1 Catalase activity

Catalase (CAT) was assayed as described by (Claiborne, 1985). The assay mixture containing 37.5 mM of potassium phosphate buffer (pH 7.0), 10 mM of H_2O_2 and 100 µl of enzyme extract was prepared in the dark. A UV-Vis spectrophotometer (Shimadzu UV-2600, Kyoto, Japan) was used to measure the breakdown of H_2O_2 as a decline in absorbance at 240 nm. CAT activity was calculated using an extinction coefficient of 0.0436 mM^{-1} cm^{-1} and expressed as µmol H_2O_2 decomposed min^{-1} g^{-1} FM.

3.2.2.5.2 Glutathione reductase

The method described by Farrant et al. (2004) was used to estimate the activity of glutathione reductase (GR). The assay mixture comprised 50 mM potassium phosphate buffer (pH 7.8), 3mM $MgCl_2$, 10 mM oxidized glutathione, 0.5 mM reduced nicotinamide adenine dinucleotide phosphate and 50 µl enzyme extract. GR activity was based on the rate of NADPH oxidation in 5 min at 25 °C. The decline in absorbance was read at 340 nm using 50 mM potassium phosphate buffer (pH 7.8) as a blank. GR activity was calculated using the extinction coefficient 6.22 mM^{-1} cm^{-1} and expressed as µmol NADPH oxidized min^{-1} g^{-1} FM.

3.2.2.5.3 Superoxide dismutase

Superoxide dismutase (SOD) was estimated following the method of Beauchamp and Fridovich (1971) as modified by Varghese et al. (2011). The total assay mixture contained 50 mM sodium phosphate buffer, 1.17 µM riboflavin, 0.01 M methionine, 0.056 mM nitroblue tetrazolium (NBT) and 100 µl of enzyme extract. To initiate the reaction, a cuvette containing the assay mixture was placed in a vessel lined with aluminium foil and illuminated with a 55-W cool white fluorescent light (Philips, Johannesburg, South Africa) for 10 min. The enzyme activity was calculated using the enzymic inhibition of NBT photoreduction. A unit SOD corresponded to 50% inhibition of NBT photoreduction to blue formazan by the enzyme. SOD activity was expressed as units of SOD g^{-1} FM.

3.2.2.6 Germination enzymes activities

3.2.2.6.1 α-amylase activity

The enzyme was extracted following the method of Biswas et al. (1978) with slight modifications by Farashah et al. (2011). Seeds (three replicates of 0.25 g each) were homogenized in 2.5 ml of cold 0.1 M phosphate buffer (pH 7.2). The extract was centrifuged (Model J-E, Beckman Coulter Avanti®, La Brea, CA, USA) at 10,000 rpm for 25 min at 4 °C. The supernatant was used to measure α-amylase following the methods of Bernfeld (1955) and Baker (1991) with some modifications by Farashah et al. (2011). Briefly, 200 µl of 1% (w/v) starch solution prepared in phosphate buffer was added to 50 µl of enzyme extract. The reaction mixture was incubated for 30 min at 37 °C. To terminate the reaction, 100 µl dinitrosalicylate (DNS) reagent was added to the reaction mixture and placed in boiling water for 10 min. Thereafter, 350 µl of DW was added to the mixture, and the absorbance was read at 540 nm. The reducing sugar formed was estimated using a maltose standard curve and activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar (maltose) min^{-1} ml^{-1} g^{-1} FM.

3.2.2.6.2 β-1,3-glucanase activity

The enzyme extraction method followed Farashah et al. (2011). Seeds (three replicates of 0.25 g each) were homogenized in 2.5 ml of 15 mM sodium acetate buffer (pH 5.5). The extract

was centrifuged at 10,000 rpm for 5 min at 4 °C. β-1,3-glucanase activity was assayed following the description of Celestino et al. (2006) with slight modifications. The assay mixture contained 1% (w/v) substrate laminarin dissolved in 100 mM sodium acetate buffer (pH 5.0) and 50 µl of enzyme extract. The mixture was incubated at 50 °C for 30 min. The reaction was terminated by adding 300 µl DNS reagent and placing it in boiling water for 5 min, and the absorbance was read at 550 nm. The reducing sugar formed was estimated using a glucose standard curve, and activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar (glucose) min^{-1} ml^{-1} g^{-1} FM.

3.3 The inorganic salt pre-hydration treatment experiments

For these studies, the seed material and its vigour assessment and controlled deterioration followed the same methods (including levels of replication) applied for the antioxidant pre-hydration treatment experiments (sections 3.1.1, 3.1.2 and 3.1.3, respectively).

3.3.1 Preparation of inorganic salt solutions

In this study, seeds were treated with non-electrolysed and the electrolysed CW (after Berjak et al. (2011) of four inorganic salt solutions, viz. $CaCl_2$, CaMg (Mycock, 1999), $MgCl_2$ and NaCl (Table 2).

Table 2 Seed pre-hydration treatment solutions used in this study to hydrate fresh and controlled deteriorated seeds of cabbage and lettuce.

S/*n*	Pre-hydration treatment solution	Concentration of constituent(s)	pH
1	Control	Deionised water	5.6
2	$CaCl_2$ (non-electrolysed)	1 mM $CaCl_2$	6.0
3	$CaCl_2$ generated CW	1 mM $CaCl_2$	10.8
4	CaMg (non-electrolysed)	1 µm $CaCl_2$; 1 mM $MgCl_2$	5.9
5	CaMg generated CW	1 µm $CaCl_2$; 1 mM $MgCl_2$	10.7
6	CaMg generated CW adjusted to pH 6.5	1 µm $CaCl_2$; 1 mM $MgCl_2$	6.5
7	$MgCl_2$ solution (non-electrolysed)	1 mM $MgCl_2$	5.9
8	$MgCl_2$ generated CW	1 mM $MgCl_2$	10.6
9	NaCl solution (non-electrolysed)	1 mM NaCl	5.6
10	NaCl generated CW	1 mM NaCl	11.2
11	NaCl generated CW adjusted to pH 6.5	1 mM NaCl	6.5

To generate CW from any of the inorganic salt solutions used here, the solution was electrolysed using a BioRad™ Powerpac (BioRad, Hercules, California, USA) equipped with two platinum electrodes (Fig. A3; Appendix A). Each electrode was immersed in a 250 ml glass beaker containing 200 ml of the inorganic salt solution. Charge balance was maintained within the internal circuit by an agar-based salt bridge (30% KCl and 3% agar bacteriological), after which the solution was electrolysed at 60 V potential difference and 400 mA for 60 min. As mentioned above, only the cathodic fraction was used for seed treatments within 24 h of preparation (Gebashe, 2015).

To evaluate the possible influence, if any, of pH given that electrolysis of ionic solutions can raise the pH of the cathodic fraction significantly (in the range of 9 and above [Hanaoka et al., 2004] after 60 minutes – the duration used in the present study), the pH of CaMg and NaCl solutions (randomly selected) was adjusted to 6.5 with 5 M HCl solution.

3.3.2 Application of inorganic salt hydration treatment

The methods employed for this aspect of the study followed those described in section 3.2.1 of this Chapter, except that the inorganic salt solutions (Table 2) were the pre-hydration

treatment applied here.

3.3.3 Evaluation of biochemical markers of oxidative stress and germinability

Where inorganic salt hydration treatment of seeds enhanced normal seedling production relative to the control significantly ($p < 0.05$, ANOVA), estimation of electrical conductivity (EC) and other biomarkers of oxidative stress and germinability was performed. For comparison, unsoaked and DW-soaked seeds were used as controls. All soaked seeds were blotted before use in any assay.

The methods employed for the measurement of electrolyte conductivity, conjugated dienes, 4-Hydroxy-2-nonenal, Protein carbonylation; catalase activity, glutathione reductase activity, superoxide dismutase activity; α-amylase activity and β-1,3glucanase activity were the same methods (including levels of replication) applied for the antioxidant pre-hydration treatment experiments (sections 3.2.2.1, 3.2.2.2, 3.2.2.3, 3.2.2.4; 3.2.2.5.1, 3.2.2.5.2, 3.2.2.5.3; 3.2.2.6.1 and 3.2.2.6.2, respectively).

3.4 Greenhouse pot trial

For these studies, the seed material and its vigour assessment and controlled deterioration followed the same methods (including levels of replication) applied for the antioxidant pre-hydration treatment experiments (sections 3.1.1, 3.1.2 and 3.1.3, respectively).

3.4.1 Application of selected exogenous antioxidant solutions

The treatments (antioxidant × antioxidant concentration combinations) selected for this part of the study were those that resulted in the highest production of normal seedlings relative to seeds soaked in DW at P25 common to both cabbage and lettuce in the previous exogenous antioxidant experiment described in section 3.1.4 of this Chapter. The selected treatments were 0.4 mM glycerol, 0.6 mM GSH and 0.2 mM trolox for cabbage; and 0.6 mM glycerol, GSH and trolox for lettuce. Sixty P25 CD seeds of each species were soaked in 3 ml of antioxidant solution or DW (control) for 8 h in cabbage and 6 h in lettuce. The seeds were removed from the pre-hydration treatment, blotted dry with a paper towel and assessed for germination in a greenhouse as described below.

3.4.2 Seed sowing conditions

The pot trials for germination, seedling establishment and subsequent growth parameters were conducted during the spring season of 2019 (25 ± 2 °C and 70% relative humidity) within a polycarbonate greenhouse (29°49'04.0"S, 30°56'23.5"E), and the experiment was repeated three times. For each of the three experiments, control (DW) and antioxidant-treated seeds (*n* = 60) were sown individually 4-5 mm deep in 2 L potting bags filled with equal volumes of potting mix procured from Grovida Horticultural Products (Durban, South Africa). The potting mix was watered to field capacity before sowing and subsequently watered every second day. At 14 DAS, 60 ml of Fisher's Multifeed® fertiliser (1 gL^{-1}; Plaaskem, Boksburg, South Africa; 190 g nitrogen, 82 g phosphorous and 158 g potassium kg^{-1}) was applied to each pot.

3.4.3 Seedling performance assessment

3.4.3.1 Seedling emergence parameters

Seedling emergence was assessed daily for 14 days, and these data were used to calculate percentage emergence (% E) according to Patil et al. (2017), mean emergence time (MET) (Mahajan et al., 2011), mean daily emergence (MDE) (Patil et al., 2011) and time taken to 25% emergence (T_{25}) (Soltani et al., 2015) according to Coolbear et al. (1984) as modified by Hussain et al. (2013) as follows:

$$\%\,\mathrm{E} = \frac{\text{number of emergences}}{\text{number of seeds sown}} * 100$$

$$\mathrm{MET} = \frac{\sum \mathrm{Dn}}{\sum \mathrm{n}},$$

where, n is the number of seedlings that emerged on day D, and D is the number of DAS.

$$\mathrm{MDE} = \frac{\text{total emergence (\%)}}{\text{number of days to final emergence}}$$

$$\mathrm{T25} = \mathrm{ti} + \frac{(\mathrm{N}/2 - \mathrm{ni})(\mathrm{tj} - \mathrm{ti})}{\mathrm{nj} - \mathrm{ni}},$$

where, N is the final number of seedlings emerged, n_i and n_j is the total seedling emergence by adjacent counts at times t_i and t_j respectively, and $n_i < N/2 < n_j$.

3.4.3.2 Seedling vigour and biomass accumulation

Harvesting and all post-harvest measurements were carried out six weeks after sowing when plants had attained a steady-state (Jamil et al., 2007). From each experiment, seedlings (*n* = 10) were uprooted, separated into roots and shoots and the roots were rinsed under running tap water and thereafter blotted dry using blotting paper. Root and shoot lengths (mm) were measured for the calculation of the seedling vigour index (SVI) developed by Abdul-Baki and Anderson (1973):

$$\mathrm{SVI} = \text{seedling length (mm)} * \% \text{ seedling emergence}$$

The roots and shoots were dried in an oven (Gallenkamp Incubator, Model IH–150, London, England) at 80 °C for three days, and data were used to calculate the root:shoot ratio.

Leaf area (cm^2) of the ten seedlings from each experiment was measure using one young, fully expanded leaf (of the same age) from each seedling. Each leaf was scanned with an HP scanner (HP Scanjet G4050 L1957A, Shanghai, China), and the leaf area was estimated with a scientific image analysis program, ImageJ Ver. 1.52a (Rasband W.S, Maryland, USA). Leaf area ratio was calculated as the ratio of the leaf area to the unit total dry weight of leaf.

3.4.3.3 Leaf chlorophyll content, gas exchange and chlorophyll fluorescence

From each experiment, total chlorophyll was measured in five seedlings using a hand-held Soil Plant Analysis Development (SPAD) chlorophyll meter (Konica Minolta SPAD-502, Tokyo, Japan). The measurements were taken at the tip and on either side of the midrib of one of the second youngest, fully expanded leaves of each seedling, and the average reading was recorded for each leaf in SPAD units.

Photosynthetic rate (Pn), stomatal conductance (Gs) and transpiration rate (E) were measured on five seedlings using a portable photosynthesis and chlorophyll fluorescence system (Li-6400, LI-COR, Lincoln, NE, USA) between 11:00 and 13:00 when seedlings were six weeks old. Measurements were taken on one of the second youngest, fully expanded leaves per seedling for each experiment. Using the same instrument, chlorophyll fluorescence taken as the maximum quantum yield of PSII photochemistry (F_v/F_m) was measured in five (5) seedlings. Seedlings were subjected to dark adaptation for at least 1 h prior to measurements for electrons to drain off the photosystems (Kitajima and Butler, 1975; Sayed, 2003; Moradi

and Ismail, 2007). One measurement was taken on one of the second youngest leaves per seedling. Leaves exposed to chlorophyll fluorescence measurements were not reused for other readings.

3.5 Data processing and analysis

All data analyses were performed using IBM SPSS Statistics (Ver. 26.0. Armonk, NY, USA) and values were expressed as mean ± SD (standard deviation). All data were tested for normality using a Shapiro-Wilk test. All percentage data were arcsine transformed before analysis, but their original values were kept in figures and tables. The rates of seed deterioration in both species were compared by independent-samples t-test. To test for significant differences across treatments and controls, data for normal seedling (%), vigour index and all biochemical parameters were subjected to analysis of variance (ANOVA) where data were parametric. A Tukey post-hoc test was used for separation of means. Where data did not satisfy ANOVA assumptions, even after transformation, a Kruskal-Wallis test was used. All differences were considered significant at 0.05 significance level. In treatments where values obtained were not significantly different from those of control, these treatments were not considered in statistical comparisons.

CHAPTER 4: RESULTS

4.1 Characterisation of seed ageing rates and patterns via controlled deterioration

Unaged seeds of both cabbage and lettuce showed a high germinability (> 95%) within 48 h. When subjected to controlled deterioration (CD), cabbage seeds initially (day 0 to 2) exhibited no loss of vigour (asymptomatic phase) and maintained high initial normal seedling production (Fig. 2A). The asymptomatic phase was followed by a progressive decline in normal seedling production, with seeds reaching P75, P50 and P25 more or less on days 6, 13 and 17, respectively. This yielded deterioration rates of 0.13 ± 0.01, 0.15 ± 0.01 and 0.15 ± 0.01 probit/day, respectively (Table 3). All viability was lost by day 28.

Compared with cabbage, lettuce seeds exhibited a much longer asymptomatic phase when subjected to CD, with no loss of vigour recorded for 9 days (Fig. 2B). The asymptomatic phase was followed by a rather steep decline in viability, with P75, P50 and P25 being reached more or less on days 10, 11 and 13, respectively. This resulted in higher rates of deterioration (0.47 ± 0.02 probit/day for P75, 0.47 ± 0.02 probit/day for P50 and 0.48 ± 0.02 probit/day for P25) (Table 3) than cabbage under the same CD conditions. This was also evidenced by the fact that total viability loss in lettuce was recorded 9 days (day 19) earlier than in cabbage.

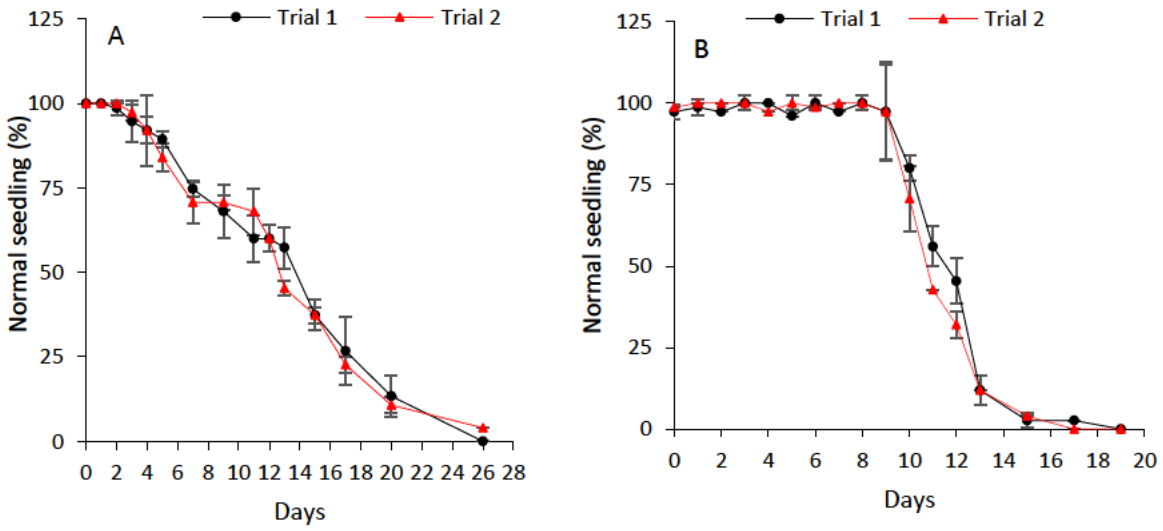

Figure 2 Controlled deterioration curves for cabbage (A) and lettuce (B) seeds subjected to controlled deterioration (using the methods of TeKrony [2005] with slight modifications). Normal seedling production (%) was assessed for 14 days. Data points represent mean ± SD ($4 \times n = 25$). The experiment was repeated twice for both species.

Table 3 Rate of deterioration in cabbage and lettuce seeds subjected to controlled deterioration (CD)

CD level	Cabbage seed deterioration rate (probit/day)	Lettuce seed deterioration rate (probit/day)
P75	0.13 ± 0.01 [b]	0.47 ± 0.02 [a]
P50	0.15 ± 0.01 [b]	0.47 ± 0.02 [a]
P25	0.15 ± 0.01 [b]	0.48 ± 0.02 [a]

Values represent mean ± SD (2 trials) of the rate of viability loss in *B*. *oleracea* (cabbage) and *L*. *sativa* (lettuce) seeds subjected to controlled deterioration. Values labelled with different letters are significantly different ($P < 0.05$, t-test) when compared across species within each CD level.

4.2 The antioxidant pre-hydration treatment experiments

4.2.1 Effect of controlled deterioration (CD) and the exogenous application of antioxidants on seedling growth and vigour of cabbage and lettuce seeds

Seeds subjected to CD produced normal and abnormal seedlings in both species (Fig. 3). The occurrence of abnormal seedlings was limited (ranging between 0.5% and 1.5%) in fresh cabbage seeds and observed in only three pre-hydration treatments but increased substantially in controlled deteriorated (CDd) seeds across all pre-hydration treatments (Table 3). Similarly, in lettuce, the occurrence of abnormal seedlings was minimal (ranging between 0.5% and 1.0%) in fresh seeds and limited to four pre-hydration treatments (Table 3). With CD, the abnormal seedlings were observed across relatively more pre-hydration treatments, but the percentage occurrence was only slightly higher (ranging between 0.5% and 4%) than those observed in fresh seeds. In L50 lettuce seeds, 0.6 mM GA, 0.2 mM glycerol and 0.2 mM GSH significantly increased abnormal seedlings produced relative to DW.

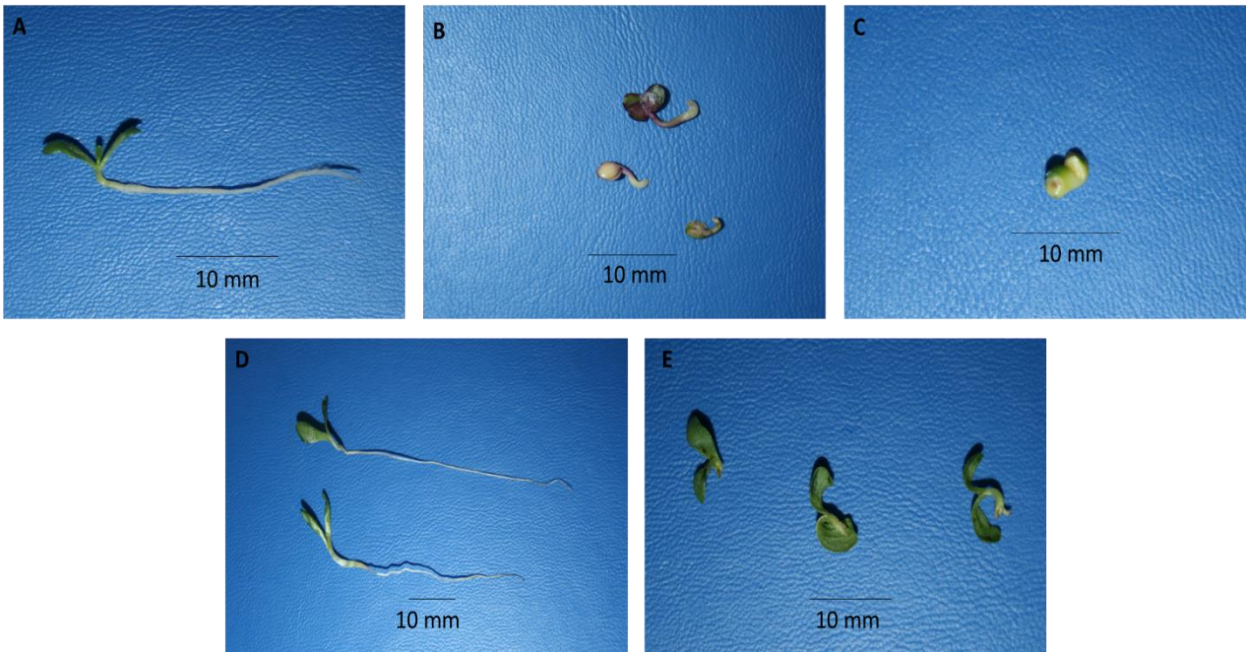

Figure 3 Normal and abnormal seedlings produced from controlled deteriorated *B. oleracea* (cabbage) and *L. sativa* (lettuce) seeds: A) normal cabbage seedling, B) abnormal cabbage seedlings, C) cabbage seed that developed chlorophyllous cotyledon only, D) normal lettuce seedlings, and E) abnormal lettuce seedlings.

Table 4 Effect of exogenous application of antioxidants on abnormal seedling production (%) in fresh and controlled deteriorated cabbage and lettuce seeds

Treatments	AS (%) for fresh cabbage seeds	AS (%) for P75 cabbage seeds	AS (%) for P50 cabbage seeds	AS (%) for P25 cabbage seeds	AS (%) for fresh lettuce seeds	AS (%) for P75 lettuce seeds	AS (%) for P50 lettuce seeds	AS (%) for P25 lettuce seeds
DW	0.00 ^{NS}	10.50 ± 5.63 ^{NS}	17.00 ± 11.66 ^{NS}	14.00 ± 9.32 ^{NS}	0.00 ^{NS}	0.00 ^{NS}	0.00 ^{b}	0.50 ± 1.41 ^{NS}
AA (0.2 mM)	0.00 ^{NS}	12.00 ± 4.78 ^{NS}	17.00 ± 10.20 ^{NS}	20.00 ± 11.31 ^{NS}	0.00 ^{NS}	1.00 ± 1.85 ^{NS}	0.00 ^{NS}	0.00 ^{NS}
AA (0.4 mM)	0.00 ^{NS}	10.50 ± 6.74 ^{NS}	14.50 ± 6.74 ^{NS}	28.50 ± 11.40 ^{NS}	0.00 ^{NS}	0.50 ± 1.41 ^{NS}	0.00 ^{NS}	0.50 ± 1.41 ^{NS}
AA (0.6 mM)	1.50 ± 1.41 ^{NS}	9.00 ± 7.69 ^{NS}	20.50 ± 6.41 ^{NS}	22.00 ± 10.47 ^{NS}	0.50 ± 0.00 ^{NS}	1.50 ± 0.00 ^{NS}	0.50 ± 1.41 ^{NS}	0.00 ^{NS}
GA (0.2 mM)	0.50 ± 0.00 ^{NS}	10.50 ± 4.28 ^{NS}	16.00 ± 7.09 ^{NS}	14.00 ± 11.06 ^{NS}	0.00 ^{NS}	0.00 ^{NS}	0.50 ± 1.41 ^{NS}	0.00 ^{NS}
GA (0.4 mM)	0.00 ^{NS}	10.50 ± 8.54 ^{NS}	11.50 ± 6.21 ^{NS}	11.00 ± 5.95 ^{NS}	0.00 ^{NS}	0.00 ^{NS}	0.00 ^{NS}	0.00 ^{NS}
GA (0.6 mM)	0.00 ^{NS}	10.50 ± 3.66 ^{NS}	16.00 ± 4.78 ^{NS}	10.50 ± 6.02 ^{NS}	0.50 ± 1.41 ^{NS}	1.50 ± 4.24 ^{NS}	3.50 ± 3.96 ^{a}	0.50 ± 1.41 ^{NS}
Glycerol (0.2 mM)	0.00 ^{NS}	6.50 ± 2.98 ^{NS}	13.00 ± 4.14 ^{NS}	12.50 ± 3.34 ^{NS}	0.00 ^{NS}	0.00 ^{NS}	4.00 ± 4.78 ^{a}	0.50 ± 1.41 ^{NS}
Glycerol (0.4 mM)	0.00 ^{NS}	7.50 ± 4.99 ^{NS}	15.50 ± 10.35 ^{NS}	9.00 ± 6.68 ^{NS}	0.00 ^{NS}	0.50 ± 1.42 ^{NS}	1.50 ± 2.07 ^{NS}	0.50 ± 1.41 ^{NS}
Glycerol (0.6 mM)	0.50 ± 1.41 ^{NS}	5.00 ± 4.14 ^{NS}	15.50 ± 5.42 ^{NS}	9.00 ± 4.66 ^{NS}	0.50 ± 1.41 ^{NS}	1.50 ± 2.98 ^{NS}	2.00 ± 3.70 ^{NS}	0.00 ^{NS}
GSH (0.2 mM)	0.00 ^{NS}	8.00 ± 4.78 ^{NS}	11.50 ± 5.83 ^{NS}	18.00 ± 5.66 ^{NS}	0.00 ^{NS}	0.50 ± 1.41 ^{NS}	3.00 ± 3.55 ^{a}	0.00 ^{NS}
GSH (0.4 mM)	0.00 ^{NS}	6.00 ± 4.78 ^{NS}	9.00 ± 7.01 ^{NS}	12.00 ± 8.00 ^{NS}	1.00 ± 2.83 ^{NS}	0.00 ^{NS}	0.00 ^{NS}	0.00 ^{NS}
GSH (0.6 mM)	0.00 ^{NS}	11.00 ± 4.14 ^{NS}	17.00 ± 5.95 ^{NS}	11.50 ± 4.99 ^{NS}	0.00 ^{NS}	0.00 ^{NS}	1.50 ± 2.98 ^{NS}	1.50 ± 2.98 ^{NS}
Trolox (0.2 mM)	0.00 ^{NS}	13.00 ± 5.13 ^{NS}	18.50 ± 9.30 ^{NS}	16.50 ± 12.73 ^{NS}	0.00 ^{NS}	0.00 ^{NS}	0.00 ^{NS}	0.00 ^{NS}
Trolox (0.4 mM)	0.00 ^{NS}	9.00 ± 6.32 ^{NS}	15.00 ± 5.95 ^{NS}	14.50 ± 5.63 ^{NS}	0.00 ^{NS}	0.50 ± 1.41 ^{NS}	0.00 ^{NS}	0.00 ^{NS}
Trolox (0.6 mM)	0.00 ^{NS}	4.00 ± 4.28 ^{NS}	16.50 ± 7.23 ^{NS}	13.50 ± 6.74 ^{NS}	0.00 ^{NS}	0.00 ^{NS}	0.50 ± 1.41 ^{NS}	1.50 ± 2.98 ^{NS}

Values represent mean ± SD (4 × *n* = 25) of % abnormal seedling production in fresh (control) and controlled deteriorated (P75, P50, P25) *B*. *oleracea* (cabbage) and *L*. *sativa* (lettuce) seeds soaked in deionized water (DW) and all the exogenously applied antioxidants. Values labelled with different letters are significantly different ($P < 0.05$, ANOVA) when compared across pre-hydration treatments within each CD level. Ascorbic acid, AA; gallic acid, GA; reduced glutathione; GSH; NS: not significantly different from value obtained with DW and therefore not considered in statistical comparisons.

Percentage normal seedling production in fresh, P75 and P50 cabbage seeds was not influenced significantly by the application of exogenous antioxidants when compared with seeds soaked in deionised water (DW) (Table 5). At P25, however, normal seedling production was increased significantly relative to P25-DW-treated seeds in the following pre-hydration treatments: 0.2 mM GA, 0.2 and 0.4 mM glycerol, 0.6 mM GSH and 0.2 mM trolox.

In lettuce, normal seedling production in fresh and P75 seeds was not influenced significantly by the application of exogenous antioxidants when compared with seeds soaked in DW (Table 6). At P50, however, normal seedling production was significantly increased relative to P50-DW-treated seeds in the following pre-hydration treatments: 0.6 mM of AA, GA, glycerol; 0.4 mM and 0.6 mM GSH. Additionally, at P25, normal seedling production was significantly increased relative to P25-DW-treated seeds in the following pre-hydration treatments: 0.2, 0.4 and 0.6 mM of AA, GSH; 0.6 mM glycerol; 0.2, 0.4 and 0.6 mM of trolox.

Table 5 Effect of exogenous application of antioxidants on normal seedling production (%) in fresh and controlled deteriorated cabbage seeds

Pre-hydration treatments	% Normal seedlings for fresh seeds	% Normal seedlings for P75 seeds	% Normal seedlings for P50 seeds	% Normal seedlings for P25 seeds
DW	90.50 ± 5.21 ^{NS}	68.50 ± 6.91 ^{NS}	43.50 ± 9.43 ^{NS}	23.50 ± 8.40 ^{b}
AA (0.2 mM)	85.50 ± 7.39 ^{NS}	76.00 ± 7.71 ^{NS}	49.50 ± 6.39 ^{NS}	34.50 ± 5.21 ^{NS}
AA (0.4 mM)	94.00 ± 2.14 ^{NS}	73.50 ± 8.54 ^{NS}	42.00 ± 7.41 ^{NS}	34.50 ± 2.98 ^{NS}
AA (0.6 mM)	87.50 ± 5.13 ^{NS}	74.50 ± 14.30 ^{NS}	47.00 ± 10.25 ^{NS}	31.00 ± 6.32 ^{NS}
GA (0.2 mM)	85.00 ± 7.41 ^{NS}	69.00 ± 7.09 ^{NS}	50.00 ± 10.13 ^{NS}	39.00 ± 9.01 ^{a}
GA (0.4 mM)	91.50 ± 7.23 ^{NS}	70.50 ± 14.80 ^{NS}	52.50 ± 10.57 ^{NS}	28.50 ± 4.50 ^{NS}
GA (0.6 mM)	88.50 ± 6.91 ^{NS}	71.00 ± 11.46 ^{NS}	47.50 ± 3.96 ^{NS}	27.00 ± 9.26 ^{NS}
Glycerol (0.2 mM)	90.50 ± 7.07 ^{NS}	69.00 ± 9.50 ^{NS}	49.50 ± 6.74 ^{NS}	42. 00 ± 8.28 ^{a}
Glycerol (0.4 mM)	90.50 ± 3.66 ^{NS}	75.00 ± 12.42 ^{NS}	47.00 ± 6.32 ^{NS}	43.50 ± 8.40 ^{a}
Glycerol (0.6 mM)	89.00 ± 4.66 ^{NS}	79.50 ± 9.43 ^{NS}	53.00 ± 8.75 ^{NS}	22.00 ± 3.02 ^{NS}
GSH (0.2 mM)	81.00 ± 13.65 ^{NS}	75.50 ± 10.99 ^{NS}	52.00 ± 3.70 ^{NS}	32.50 ± 9.18 ^{NS}
GSH (0.4 mM)	87.50 ± 7.54 ^{NS}	73.50 ± 15.41 ^{NS}	49.50 ± 5.21 ^{NS}	34.50 ± 6.74 ^{NS}
GSH (0.6 mM)	87.50 ± 4.99 ^{NS}	71.50 ± 8.67 ^{NS}	52.00 ± 6.41 ^{NS}	38.00 ± 7.71 ^{a}
Trolox (0.2 mM)	85.50 ± 7.39 ^{NS}	74.50 ± 9.78 ^{NS}	46.00 ± 11.31 ^{NS}	36.00 ± 8.00 ^{a}
Trolox (0.4 mM)	90.50 ± 4.24 ^{NS}	78.00 ± 5.66 ^{NS}	49.00 ± 5.55 ^{NS}	30.00 ± 5.24 ^{NS}
Trolox (0.6 mM)	87.50 ± 7.54 ^{NS}	73.00 ± 9.26 ^{NS}	43.00 ± 1.85 ^{NS}	32.50 ± 4.50 ^{NS}

Values represent mean ± SD ($4 \times n = 25$) of % normal seedling production in fresh (control) and controlled deteriorated (P75, P50, P25) *B*. *oleracea* (cabbage) seeds soaked in deionized water (DW) and all the exogenously applied antioxidants. Values labelled with different letters are significantly different ($P < 0.05$, ANOVA) when compared across pre-hydration treatments within each CD level. Ascorbic acid, AA; gallic acid, GA; reduced glutathione; GSH; NS: not significantly different from value obtained with DW and therefore not considered in statistical comparisons.

Table 6 Effect of exogenous application of antioxidants on normal seedling production (%) in fresh and controlled deteriorated lettuce seeds

Pre-hydration treatments	% Normal seedlings for fresh seeds	% Normal seedlings for P75 seeds	% Normal seedlings for P50 seeds	% Normal seedlings for P25 seeds
DW	98.00 ± 3.02 ^{NS}	75.00 ± 7.33 ^{NS}	62.00 ± 10.03 ^{b}	20.50 ± 5.42 ^{c}
AA (0.2 mM)	93.00 ± 4.14 ^{NS}	77.50 ± 4.24 ^{NS}	69.00 ± 4.14 ^{NS}	45.00 ± 8.21 ^{a}
AA (0.4 mM)	95.00 ± 6.32 ^{NS}	81.00 ± 11.06 ^{NS}	70.50 ± 10.89 ^{NS}	30.50 ± 4.24 ^{b}
AA (0.6 mM)	95.50 ± 4.78 ^{NS}	81.50 ± 4.14 ^{NS}	76.00 ± 7.41 ^{a}	30.50 ± 4.28 ^{b}
GA (0.2 mM)	94.00 ± 4.50 ^{NS}	71.00 ± 6.41 ^{NS}	72.00 ± 2.83 ^{NS}	20.00 ± 4.28 ^{NS}
GA (0.4 mM)	96.00 ± 5.24 ^{NS}	65.50 ± 10.46 ^{NS}	71.00 ± 6.68 ^{NS}	20.00 ± 6.76 ^{NS}
GA (0.6 mM)	95.50 ± 5.83 ^{NS}	73.00 ± 11.06 ^{NS}	76.00 ± 3.70 ^{a}	24.50 ± 4.99 ^{NS}
Glycerol (0.2 mM)	95.00 ± 7.33 ^{NS}	88.50 ± 5.42 ^{NS}	63.00 ± 6.68 ^{NS}	23.00 ± 5.13 ^{NS}
Glycerol (0.4 mM)	97.00 ± 4.66 ^{NS}	75.50 ± 8.67 ^{NS}	74.50 ± 4.24 ^{NS}	28.00 ± 9.56 ^{NS}
Glycerol (0.6 mM)	98.50 ± 2.98 ^{NS}	77.00 ± 5.95 ^{NS}	75.00 ± 5.95 ^{a}	30.50 ± 7.98 ^{b}
GSH (0.2 mM)	97.50 ± 5.63 ^{NS}	81.50 ± 6.74 ^{NS}	60.50 ± 7.23 ^{NS}	35.00 ± 6.68 ^{ab}
GSH (0.4 mM)	94.00 ± 4.78 ^{NS}	85.50 ± 6.74 ^{NS}	76.00 ± 8.82 ^{a}	30.00 ± 3.02 ^{b}
GSH (0.6 mM)	97.50 ± 2.98 ^{NS}	80.00 ± 5.66 ^{NS}	76.00 ± 2.14 ^{a}	39.50 ± 13.26 ^{ab}
Trolox (0.2 mM)	98.00 ± 2.14 ^{NS}	80.00 ± 10.47 ^{NS}	71.50 ± 7.23 ^{NS}	33.00 ± 5.95 ^{b}
Trolox (0.4 mM)	97.50 ± 3.66 ^{NS}	80.00 ± 6.05 ^{NS}	68.00 ± 12.83 ^{NS}	44.00 ± 10.47 ^{ab}
Trolox (0.6 mM)	95.00 ± 4.14 ^{NS}	81.00 ± 8.49 ^{NS}	69.00 ± 13.14 ^{NS}	44.50 ± 7.84 ^{a}

Values represent mean ± SD ($4 \times n = 25$) of % normal seedling production in fresh (control) and controlled deteriorated (P75, P50, P25) *L*. *sativa* (lettuce) seeds soaked in deionized water (DW) and all the exogenously applied antioxidants. Values labelled with different letters are significantly different ($P < 0.05$, ANOVA) when compared across pre-hydration treatments within each CD level. Ascorbic acid, AA; gallic acid, GA; reduced glutathione; GSH; NS: not significantly different from value obtained with DW and therefore not considered in statistical comparisons.

Seedling vigour index (SVI) in fresh cabbage seeds was significantly increased when treated with 0.2, 0.4 and 0.6 mM GA and glycerol, and 0.2 mM trolox when compared with fresh seeds soaked in DW (Table 7). However, SVI was only significantly increased in P75 cabbage seeds when they were treated with 0.4 mM trolox when compared with P75 seeds soaked in DW. Seedling vigour index in P50 cabbage seeds was not influenced significantly by antioxidant application compared with P50-DW-treated seeds, while SVI in P25 seeds was significantly increased relative to P25-DW-treated seeds when treated with 0.2 and 0.4 mM glycerol (Table 7).

In fresh and P75 lettuce seeds, SVI was not influenced significantly by antioxidant application compared with their DW-treated seeds (Table 8). However, SVI in P50 lettuce seeds was significantly increased relative to P50-DW-treated seeds when treated with GA (0.6 mM) and trolox (0.2, 0.4 and 0.6 mM), while SVI in P25 seeds was significantly increased when treated with 0.2 mM AA, 0.6 mM GSH and trolox (0.2, 0.4 and 0.6 mM) compared with P25-DW- treated seeds (Table 8).

Table 7 Effect of exogenous application of antioxidants on seedling vigour index in fresh and controlled deteriorated cabbage seeds

Treatments	SVI for fresh seeds	SVI for P75 seeds	SVI for P50 seeds	SVI for P25 seeds
DW	4923.20 ± 728.50 [b]	5414.50 ± 1099.41 [b]	4998.00 ± 813.96 [NS]	2305.20 ± 1366.14 [b]
AA (0.2 mM)	4126.50 ± 648.52 [NS]	4998.00 ± 1328.57 [NS]	4257.00 ± 498.45 [NS]	3265.50 ± 966.80 [NS]
AA (0.4 mM)	3312.00 ± 361.43 [NS]	4885.50 ± 1171.15 [NS]	2320.50 ± 579.52 [NS]	3624.00 ± 1069.05 [NS]
AA (0.6 mM)	4549.50 ± 1351.16 [NS]	5278.50 ± 1137.44 [NS]	2103.00 ± 639.37 [NS]	3084.00 ± 1569.18 [NS]
GA (0.2 mM)	7793.10 ± 890.57 [a]	5741.40 ± 1318.79 [NS]	6621.30 ± 756.85 [NS]	4613.70 ± 1001.24 [NS]
GA (0.4 mM)	7879.20 ± 638.02 [a]	6475.35 ± 1402.34 [NS]	6241.20 ± 974.23 [NS]	3815.70 ± 1555.03 [NS]
GA (0.6 mM)	7942.20 ± 965.41 [a]	6934.20 ± 1235.94 [NS]	7121.10 ± 1879.14 [NS]	2879.10 ± 2453.05 [NS]
Glycerol (0.2 mM)	7679.70 ± 1024.95 [a]	8353.80 ± 1418.06 [NS]	7110.60 ± 1237.70 [NS]	5798.10 ± 1185.47 [a]
Glycerol (0.4 mM)	7572.60 ± 1238.74 [a]	8225.70 ± 570.63 [NS]	5577.60 ± 1339.85 [NS]	5088.30 ± 2050.79 [a]
Glycerol (0.6 mM)	8162.70 ± 1161.01 [a]	6770.40 ± 1299.03 [NS]	7039.20 ± 1054.41 [NS]	3192.00 ± 2313.28 [NS]
GSH (0.2 mM)	3837.60 ± 669.83 [NS]	5058.30 ± 969.49 [NS]	5122.00 ± 896.90 [NS]	2328.30 ± 1038.52 [NS]
GSH (0.4 mM)	3809.00 ± 513.62 [NS]	3998.80 ± 562.45 [NS]	3060.20 ± 505.44 [NS]	3069.30 ± 543.66 [NS]
GSH (0.6 mM)	3641.30 ± 1013.48 [NS]	3692.00 ± 593.13 [NS]	4148.30 ± 823.31 [NS]	1592.50 ± 312.22 [NS]
Trolox (0.2 mM)	7871.60 ± 1112.36 [a]	6591.20 ± 1476.21 [NS]	6991.60 ± 1675.89 [NS]	4743.20 ± 1450.51 [NS]
Trolox (0.4 mM)	6551.60 ± 1341.89 [NS]	9319.20 ± 2533.07 [a]	5775.00 ± 1250.87 [NS]	3062.40 ± 1655.90 [NS]
Trolox (0.6 mM)	7027.70 ± 1559.13 [NS]	7268.80 ± 1901.68 [NS]	7097.20 ± 2193.01 [NS]	3711.40 ± 1465.64 [NS]

Values represent mean ± SD (4 × *n* = 25) of seedling vigour index (SVI) in fresh (control) and controlled deteriorated (P75, P50, P25) *B. oleracea* (cabbage) seeds soaked in deionized water (DW) and all the exogenously applied antioxidants. Values labelled with different letters are significantly different ($P < 0.05$, ANOVA) when compared across pre-hydration treatments within each CD level. Ascorbic acid, AA; gallic acid, GA; reduced glutathione; GSH; NS: not significantly different from value obtained with DW and therefore not considered in statistical comparisons.

Table 8 Effect of exogenous application of antioxidants on seedling vigour index in fresh and controlled deteriorated lettuce seeds

Treatments	SVI for fresh seeds	SVI for P75 seeds	SVI for P50 seeds	SVI for P25 seeds
DW	5077.60 ± 328.17 ^{NS}	4046.10 ± 532.21 ^{NS}	2799.00 ± 815.44 ^{b}	333.30 ± 175.13 ^{b}
AA (0.2 mM)	6218.60 ± 650.83 ^{NS}	5166.60 ± 647.61 ^{NS}	2929.10 ± 487.93 ^{NS}	1736.30 ± 507.99 ^{a}
AA (0.4 mM)	6635.20 ± 1116.61 ^{NS}	5504.60 ± 1093.68 ^{NS}	3747.40 ± 762.00 ^{NS}	1110.10 ± 287.88 ^{NS}
AA (0.6 mM)	4595.60 ± 989.48 ^{NS}	5461.40 ± 442.07 ^{NS}	4153.40 ± 551.82 ^{NS}	809.30 ± 645.70 ^{NS}
GA (0.2 mM)	5160.90 ± 898.73 ^{NS}	3982.00 ± 893.50 ^{NS}	3412.10 ± 569.21 ^{NS}	500.00 ± 847.56 ^{NS}
GA (0.4 mM)	6044.90 ± 490.37 ^{NS}	3959.00 ± 947.74 ^{NS}	4053.50 ± 517.51 ^{NS}	400.50 ± 127.43 ^{NS}
GA (0.6 mM)	5751.40 ± 443.09 ^{NS}	4162.90 ± 547.72 ^{NS}	4548.20 ± 680.37 ^{a}	860.20 ± 238.97 ^{NS}
Glycerol (0.2 mM)	5347.60 ± 911.97 ^{NS}	5441.30 ± 437.95 ^{NS}	3011.50 ± 621.05 ^{NS}	1432.40 ± 563.21 ^{NS}
Glycerol (0.4 mM)	6087.00 ± 713.95 ^{NS}	4428.20 ± 746.47 ^{NS}	4209.10 ± 419.54 ^{NS}	1010.10 ± 838.02 ^{NS}
Glycerol (0.6 mM)	5760.00 ± 506.87 ^{NS}	4414.70 ± 513.57 ^{NS}	4209.60 ± 455.99 ^{NS}	915.20 ± 359.82 ^{NS}
GSH (0.2 mM)	5285.00 ± 941.18 ^{NS}	5094.70 ± 674.65 ^{NS}	2353.80 ± 493.35 ^{NS}	1195.10 ± 375.55 ^{NS}
GSH (0.4 mM)	5299.50 ± 483.50 ^{NS}	5038.10 ± 389.80 ^{NS}	3571.60 ± 311.82 ^{NS}	691.00 ± 260.76 ^{NS}
GSH (0.6 mM)	4923.00 ± 538.53 ^{NS}	4887.40 ± 713.46 ^{NS}	5049.10 ± 480.58 ^{NS}	1746.40 ± 847.04 ^{a}
Trolox (0.2 mM)	5820.10 ± 625.38 ^{NS}	5708.50 ± 902.83 ^{NS}	3998.60 ± 610.33 ^{a}	1874.80 ± 352.21 ^{a}
Trolox (0.4 mM)	5677.70 ± 1024.38 ^{NS}	5004.70 ± 318.59 ^{NS}	4666.60 ± 942.22 ^{a}	2334.80 ± 550.57 ^{a}
Trolox (0.6 mM)	5214.70 ± 3179.31 ^{NS}	5392.50 ± 732.57 ^{NS}	4934.30 ± 1072.12 ^{a}	2344.70 ± 609.26 ^{a}

Values represent mean ± SD (4 × *n* = 25) of seedling vigour index (SVI) in fresh (control) and controlled deteriorated (P75, P50, P25) *L. sativa* (lettuce) seeds soaked in deionized water (DW) and all the exogenously applied antioxidants. Values labelled with different letters are significantly different ($P < 0.05$, ANOVA) when compared across pre-hydration treatments within each CD level. Ascorbic acid, AA; gallic acid, GA; reduced glutathione; GSH; NS: not significantly different from value obtained with DW and therefore not considered in statistical comparisons.

4.2.2 Effects of CD and the exogenous application of antioxidants on biomarkers of oxidative stress in cabbage and lettuce seeds

The oxidative stress biomarkers were measured in fresh and CDd seeds without soaking (unsoaked), and after soaking in DW, and all CD × antioxidant × concentration treatment combinations (termed 'beneficial pre-hydration treatments' henceforth) that changed normal seedling production (%) significantly relative to DW-treated seeds at a specific level of CD.

Controlled deterioration led to heightened solute leakage, measured as electrolyte conductivity (EC), in unsoaked cabbage and lettuce seeds relative to their unsoaked fresh seeds, but these differences were only significant at P50 and P25 (Table 9). The beneficial pre-treatment solutions did not extenuate leakage in P25 cabbage seeds relative to unsoaked P25 seeds; rather, DW, GA (0.2 mM) and trolox (0.2 mM) caused a further increase in EC levels in P25 cabbage seeds (Fig. 4A). However, in P50 lettuce seeds, soaking in 0.6 mM of AA, GA, glycerol and GSH significantly decreased EC levels relative to unsoaked and DW-treated P50 seeds; glycerol (0.6 mM) resulted in particularly low EC levels relative to the other beneficial pre-hydration treatments (Fig. 5A). Similarly, EC levels in P25 lettuce seeds soaked in 0.2 mM of AA, 0.6 mM glycerol, GSH and trolox were significantly reduced relative to unsoaked and DW-treated P25 seeds (Fig. 6A). Glycerol (0.6 mM) and GSH (0.6 mM) led to particularly low EC levels relative to the other beneficial pre-hydration treatments.

Controlled deterioration of cabbage seeds did not lead to a significant change in conjugated diene (CJD) levels relative to fresh cabbage seeds but significantly increased CJD levels in P75, P50 and P25 lettuce seeds relative to fresh lettuce seeds (Table 9). The beneficial pre-hydration treatments did not change CJD levels in P25 cabbage seeds relative to unsoaked P25 seeds (Fig. 4B). However, in P50 lettuce seeds, all pre-hydration treatments significantly decreased CJD levels relative to unsoaked P50 seeds; and 0.6 mM of GA, glycerol and GSH resulted in a greater reduction in CJD levels than DW (Fig. 5B). Similarly, all pre-hydration treatments significantly reduced CJD levels in P25 lettuce seeds relative to unsoaked P25 seeds; the antioxidants resulted in a greater reduction in CJD levels than DW (Fig. 6B).

In cabbage seeds, CD did not result in a significant change in 4-HNE levels relative to fresh seeds, but in lettuce, CD significantly increased 4-HNE levels in P50 and P25 seeds relative to

fresh seeds (Table 9). The levels of 4-HNE were significantly increased in P25 cabbage seeds pre-hydrated in 0.4 mM glycerol (Fig. 4C) and P50 lettuce seeds soaked in 0.6 mM glycerol (Fig. 5C), while the other pre-hydration treatments had no significant effect relative to unsoaked seeds; 0.6 mM of AA and GSH resulted in a greater reduction in 4-HNE levels of P50 lettuce seeds than DW. However, in P25 lettuce seeds, all exogenous antioxidants reduced 4-HNE levels significantly relative to unsoaked and DW-treated P25 seeds (Fig. 6C).

Controlled deterioration led to a significant rise in protein carbonylation (PC) levels in cabbage and lettuce seeds, but these differences were only significant at P50 and P25 in cabbage and P25 in lettuce seeds (Table 9). The PC levels were reduced significantly in P25 cabbage seeds soaked in all pre-hydration treatments relative to unsoaked seeds, and 0.2 mM GA, 0.4 mM glycerol and 0.6 mM GSH resulted in a greater reduction in PC levels than DW (Fig. 4D). In P50 lettuce seeds, pre-hydration in GA (0.6 mM) and GSH (0.6 mM) significantly reduced PC levels while other exogenous antioxidants had no significant effect relative to unsoaked and DW-treated seeds (Fig. 5D). The PC levels were reduced significantly in P25 lettuce seeds soaked in AA (0.2 mM), glycerol (0.6 mM) and GSH (0.6 mM), while trolox (0.6 mM) and DW had no significant effect relative to unsoaked seeds (Fig. 6D).

Table 9 Effect of CD on the biomarkers of oxidative stress and enzymes associated with germination in cabbage and lettuce seeds

Species		EC (mS/cm/g DW)	CJD (µmol/g FM)	4-HNE (µmol/g FM)	PC (nmol carbonyl/mg protein)	CAT (µmol/min/g FM)	GR (µMol NADPH oxidized min/g FM)	SOD (units/g FM)	α-amylase (µmol/min/ ml/g FM)	β-1,3-glucanase (µmol/min/ ml/g FM)
Cabbage	Fresh	0.49 ± 0.14 ^{b}	0.05 ± 0.01 ^{Ns}	0.80 ± 0.01 ^{Ns}	369.70 ± 35.46 ^{b}	42.95 ± 5.20 ^{a}	1.05 ± 0.21 ^{a}	230.86 ± 39.25 ^{Ns}	6.20 ± 1.74 ^{a}	1.94 ± 0.21 ^{a}
	P75	0.65 ± 0.15 ^{ab}	0.04 ± 0.00 ^{Ns}	0.13 ± 0.02 ^{Ns}	374.55 ± 46.60 ^{b}	37.25 ± 0.23 ^{a}	0.69 ± 0.18 ^{ab}	226.61 ± 7.86 ^{Ns}	4.24 ± 0.23 ^{ab}	1.66 ± 0.03 ^{ab}
	P50	0.76 ± 0.13 ^{a}	0.04 ± 0.00 ^{Ns}	0.12 ± 0.03 ^{Ns}	479.18 ± 42.99 ^{a}	25.41 ± 3.74 ^{b}	0.51 ± 0.16 ^{b}	213.88 ± 19.10 ^{Ns}	3.87 ± 0.033 ^{ab}	1.60 ± 0.13 ^{b}
	P25	0.84 ± 0.02 ^{a}	0.05 ± 0.00 ^{Ns}	0.09 ± 0.01 ^{Ns}	570.91 ± 22.34 ^{a}	6.33 ± 0.99 ^{c}	0.30 ± 0.05 ^{b}	203.43 ± 14.71 ^{Ns}	2.02 ± 0.27 ^{b}	0.77 ± 0.04 ^{c}
Lettuce	Fresh	0.45 ± 0.29 ^{c}	0.16 ± 0.03 ^{d}	0.18 ±0.00 ^{c}	483.03 ± 25.54 ^{b}	50.26 ± 6.58 ^{a}	0.55 ± 0.07 ^{a}	209.96 ± 10.57 ^{a}	1.95 ± 0.02 ^{a}	1.56 ± 0.09 ^{a}
	P75	0.63 ± 0.11 ^{c}	0.22 ± 0.01 ^{c}	0.21 ± 0.01 ^{c}	538.18 ± 33.18 ^{ab}	38.81 ± 7.36 ^{a}	0.31 ± 0.01 ^{b}	197.88 ± 8.48 ^{a}	1.71 ± 0.04 ^{ab}	1.47 ± 0.27 ^{ab}
	P50	1.38 ± 0.20 ^{b}	0.30 ± 0.02 ^{b}	0.44 ± 0.02 ^{b}	544.24 ± 33.79 ^{ab}	11.17 ± 0.20 ^{b}	0.27 ± 0.03 ^{b}	188.74 ± 18.75 ^{a}	1.60 ± 0.43 ^{ab}	1.08 ± 0.0.28 ^{ab}
	P25	1.81 ± 0.15 ^{a}	0.47 ± 0.00 ^{a}	0.53 ± 0.03 ^{a}	623.03 ± 44.62 ^{a}	10.29 ± 1.60 ^{b}	0.24 ± 0.08 ^{b}	101.88 ± 15.67 ^{b}	1.27 ± 0.20 ^{b}	0.95 ± 0.24 ^{b}

Values represent mean ± SD (n = 5 for EC and n = 3 for all other parameters). Values labelled with different letters indicate significant differences at $P < 0.05$ (ANOVA) in the biomarkers (electrical conductivity, EC; conjugated dienes, CJD; 4-hydroxy-2-nonenal, 4-HNE; protein carbonylation, PC; catalase, CAT; glutathione reductase, GR; superoxide dismutase, SOD) when compared within species, across the CD levels (Fresh, P75, P50, P25). NS: not significantly different.

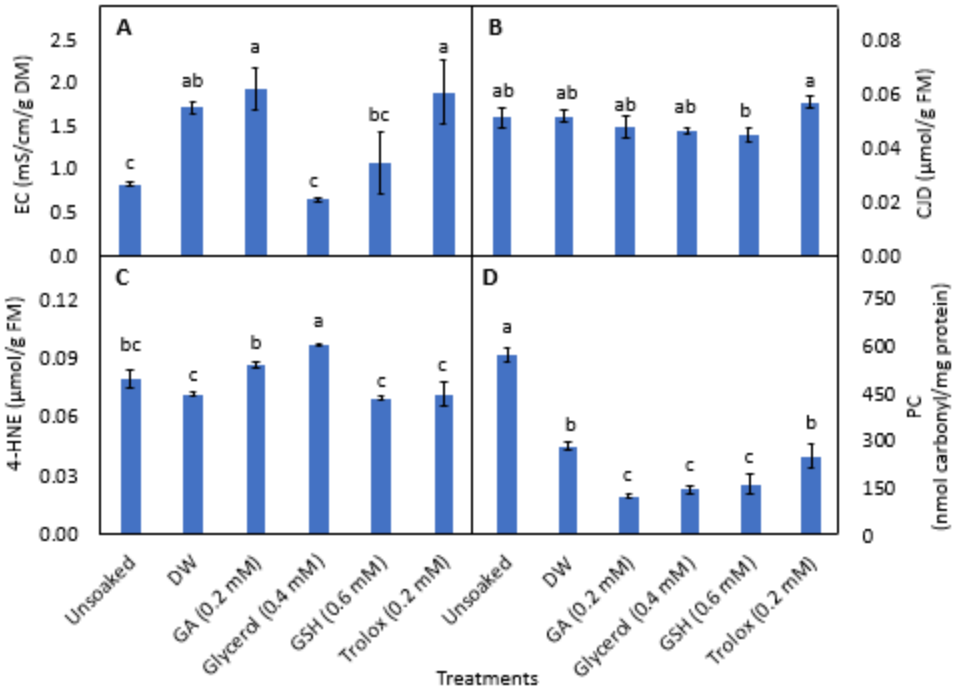

Figure 4 Effect of exogenous antioxidant application on biomarkers of oxidative stress in P25 *B. oleracea* (cabbage) seeds subjected to no soaking (unsoaked) or soaking in deionised water (DW), gallic acid (GA), glycerol, reduced glutathione (GSH) or trolox. A) electrical conductivity (EC), B) conjugated dienes (CJD), C) 4-hydroxy-2-nonenal (4-HNE) and D) protein carbonylation (PC) adduct. Values represent mean ± SD (n = 5 for EC and n = 3 for all other parameters). Bars labelled with different letters indicate significant differences at $P < 0.05$ (ANOVA).

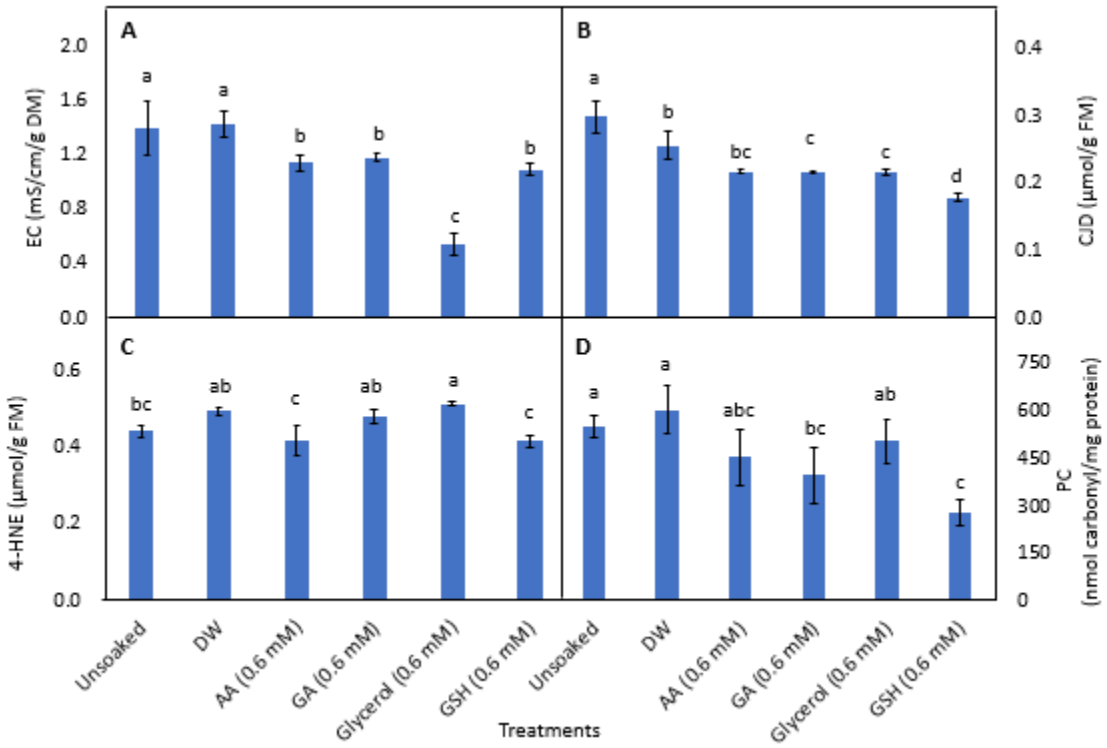

Figure 5 Effect of exogenous antioxidant application on biomarkers of oxidative stress in P50 *L. sativa* (lettuce) seeds subjected to no soaking (unsoaked) or soaking in deionised water (DW), ascorbic acid (AA), gallic acid (GA), glycerol or reduced glutathione (GSH). A) electrical conductivity (EC), B) conjugated dienes (CJD), C) 4-hydroxy-2-nonenal (4-HNE) and D) protein carbonylation (PC) adduct. Values represent mean ± SD (n = 5 for EC and n = 3 for all other parameters). Bars labelled with different letters indicate significant differences at $P < 0.05$ (ANOVA).

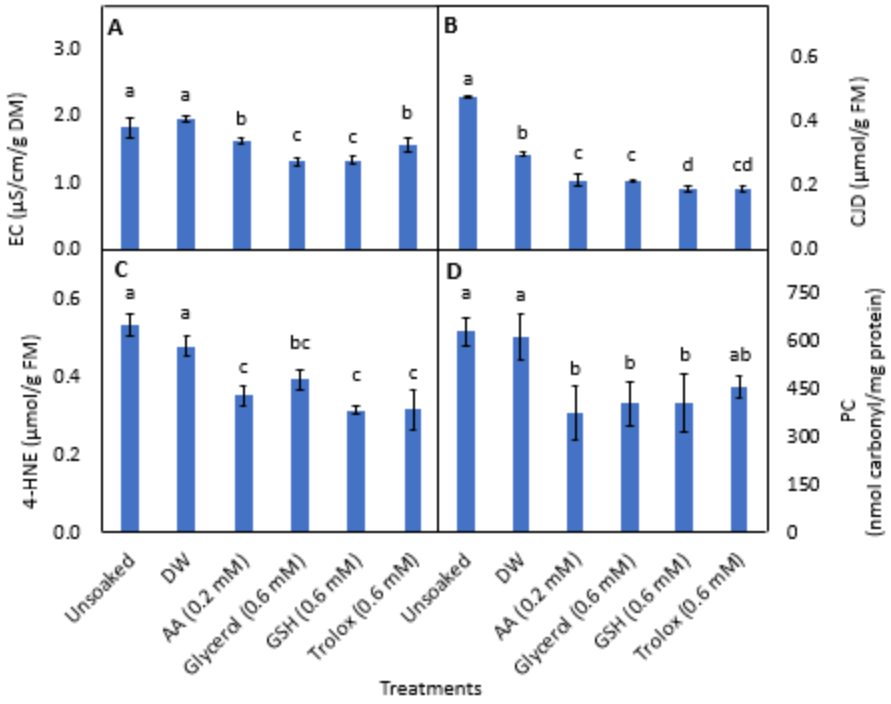

Figure 6 Effect of exogenous antioxidant application on biomarkers of oxidative stress in P25 *L. sativa* (lettuce) seeds subjected to no soaking (unsoaked) or soaking in deionised water (DW), ascorbic acid (AA), glycerol, reduced glutathione (GSH) or trolox. A) electrical conductivity (EC), B) conjugated dienes (CJD), C) 4-hydroxy-2-nonenal (4-HNE) and D) protein carbonylation (PC) adduct. Values represent mean ± SD (n = 5 for EC and n = 3 for all other parameters). Bars labelled with different letters indicate significant differences at $P < 0.05$ (ANOVA).

4.2.3 Effects of CD and the exogenous application of antioxidants on enzymic antioxidants activities in cabbage and lettuce seeds

In both species, CD to P50 and P25 led to a significant reduction in catalase (CAT) activity relative to fresh seeds (Table 9). CAT activity was significantly increased in P25 cabbage seed tissues pre-hydrated in GA (0.2 mM) and GSH (0.6 mM) relative to unsoaked and DW-treated P25 seeds (Fig. 7A). CAT activity was also significantly increased in 0.6 mM glycerol-treated P50 lettuce seeds relative to unsoaked seeds but decreased significantly in DW-treated seeds (Fig. 8A). In P25 lettuce seeds, CAT activity was significantly increased when pre-hydrated in GSH (0.6 mM) relative to unsoaked seeds but reduced in DW-treated seeds (Fig. 9A).

Controlled deterioration led to a significant reduction in glutathione reductase (GR) activity at P50 and P25 in cabbage seeds and at P75, P50 and P25 in lettuce seeds, relative to fresh seeds (Table 9). The activity of GR was only significantly increased in P25 cabbage seeds pre-hydrated in GSH (0.6 mM), relative to unsoaked P25 seeds (Fig. 7B). However, GR activity was significantly increased in P50 (Fig. 8B) and P25 (Fig. 9B) lettuce seeds treated with all pre-hydration treatments relative to unsoaked seeds; the exogenous antioxidants resulted in a higher GR activity than DW at both CD levels.

In cabbage, CD did not result in a significant change in superoxide dismutase (SOD) activity relative to fresh seeds, but it significantly increased SOD activity in P25 lettuce seeds relative to fresh seeds (Table 9). SOD activity was not significantly influenced by the pre-hydration treatments in P25 cabbage seeds; rather, it was significantly reduced in DW-treated seeds relative to unsoaked seeds (Fig. 7C). In P50 lettuce, SOD was significantly increased in seeds pre-hydrated in GSH (0.6 mM), relative to unsoaked and DW-treated seeds; 0.6 mM of GA and glycerol resulted in a higher SOD activity than DW (Fig. 8C). SOD activity was significantly increased in P25 lettuce seeds treated with all pre-hydration treatments, relative to unsoaked seeds (Fig. 9C).

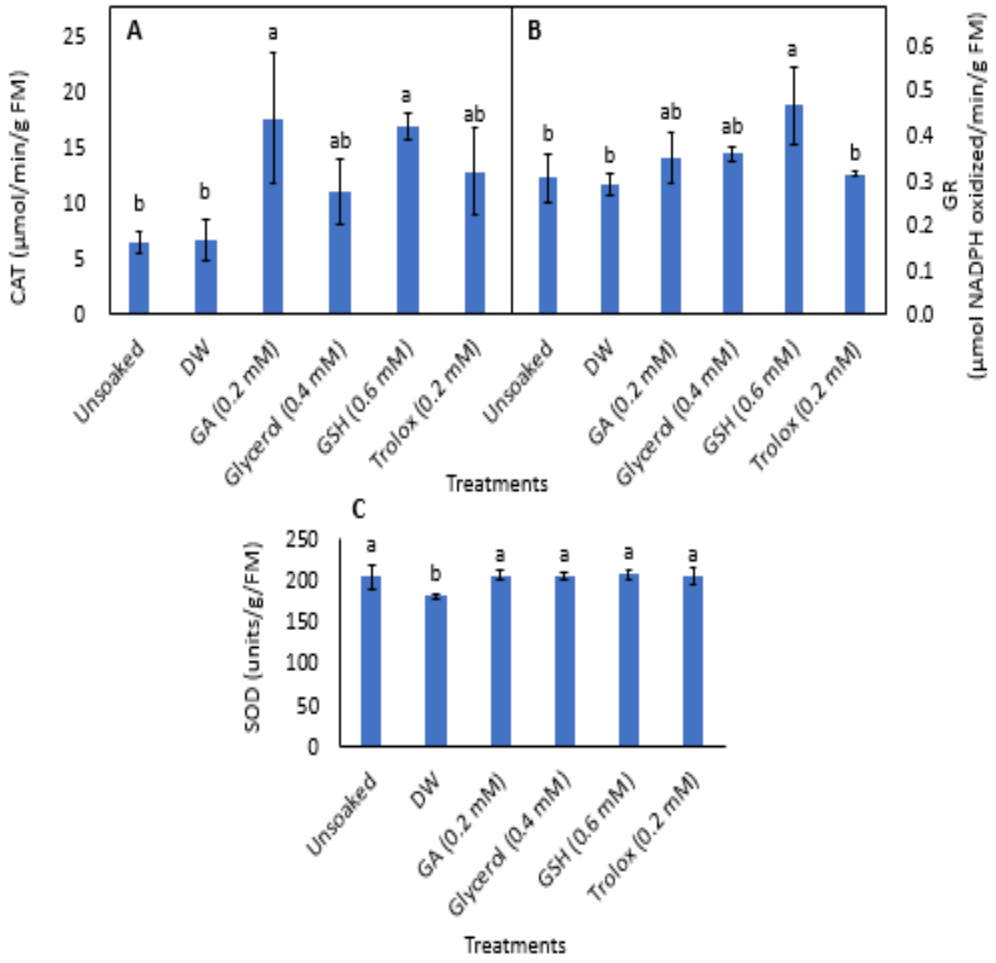

Figure 7 Effect of exogenous antioxidant application on antioxidant enzymes activities in P25 *B. oleracea* (cabbage) seeds subjected to no soaking (unsoaked) or soaked in deionised water (DW), gallic acid (GA), glycerol, reduced glutathione (GSH) or trolox. A) catalase (CAT), B) glutathione reductase (GR) and C) superoxide dismutase (SOD). Values represent mean ± SD (*n* = 5 for EC and *n* = 3 for all other parameters). Bars labelled with different letters indicate significant differences at $P < 0.05$ (ANOVA).

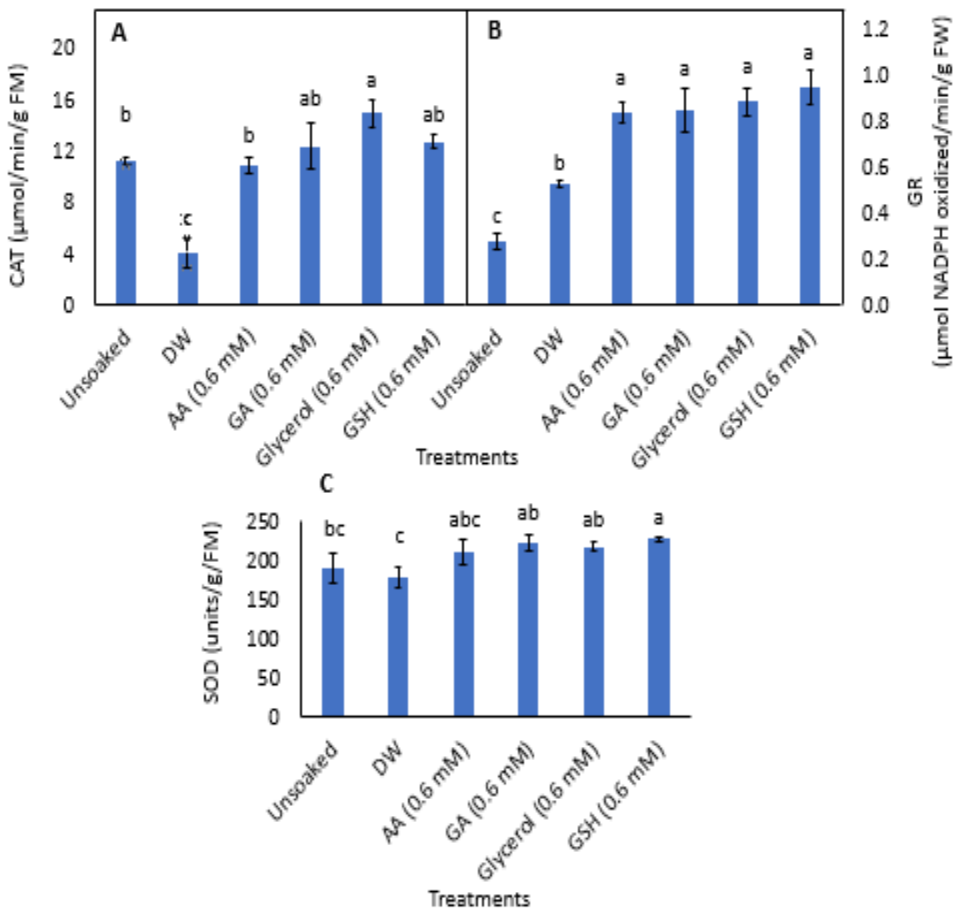

Figure 8 Effect of exogenous antioxidant application on antioxidant enzymes activities in P50 *L. sativa* (lettuce) seeds subjected to no soaking (unsoaked) or soaked in deionised water (DW), ascorbic acid (AA), gallic acid (GA), glycerol or reduced glutathione (GSH). A) catalase (CAT), B) glutathione reductase (GR) and C) superoxide dismutase (SOD). Values represent mean ± SD (n = 5 for EC and n = 3 for all other parameters). Bars labelled with different letters indicate significant differences at $P < 0.05$ (ANOVA).

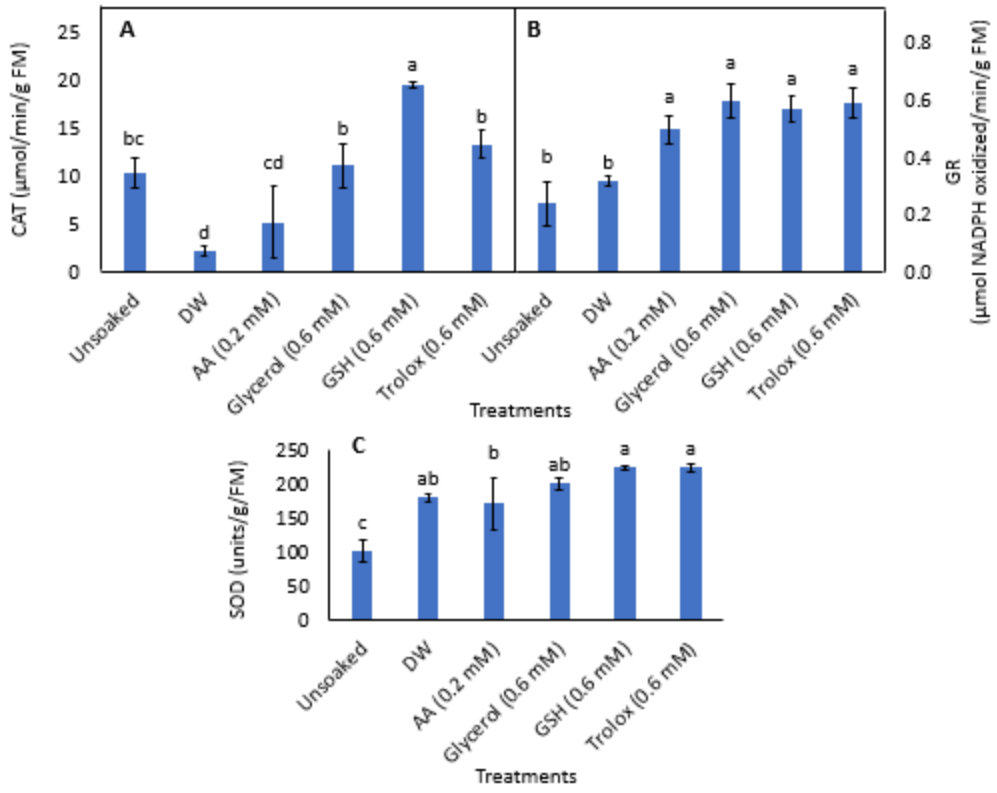

Figure 9 Effect of exogenous antioxidant application on antioxidant enzymes activities in P25 *L. sativa* (lettuce) seeds subjected to no soaking (unsoaked) or soaked in deionised water (DW), ascorbic acid (AA), glycerol, reduced glutathione (GSH) or trolox. A) catalase (CAT), B) glutathione reductase (GR) and C) superoxide dismutase (SOD). Values represent mean ± SD (*n* = 5 for EC and *n* = 3 for all other parameters). Bars labelled with different letters indicate significant differences at $P < 0.05$ (ANOVA).

4.2.4 Effects of CD and the exogenous application of antioxidants on germination-related enzymes in cabbage and lettuce seeds

In both species, CD led to a significant reduction in α-amylase activity relative to fresh seeds, but these differences were only significant at P25 (Table 9). However, α-amylase activity increased significantly in P25 cabbage seeds treated with glycerol (0.4 mM) relative to unsoaked seeds; trolox (0.2 mM) resulted in a higher α-amylase activity than DW (Fig. 10A). In P50 lettuce seeds, α-amylase activity was not significantly influenced by the pre-hydration treatments (Fig. 11A), but the enzyme's activity was significantly increased in P25 lettuce seeds soaked in GSH (0.6 mM) and trolox (0.6 mM), relative to unsoaked and DW-treated seeds; 0.6 mM glycerol

resulted in a greater increase in α-amylase activity than DW (Fig. 12A).

Controlled deterioration led to a significant reduction in β-1,3-glucanase activity in cabbage seeds at P50 and P25 and lettuce seeds at P25, compared with fresh seeds (Table 9). β-1,3-glucanase activity increased significantly in P25 cabbage seeds exposed to exogenous antioxidants, but those treated with DW had a significantly reduced activity relative to unsoaked seeds (Fig. 10B). However, β-1,3-glucanase activity was significantly increased in P50 lettuce seeds exposed to all pre-hydration treatments relative to unsoaked seeds (Fig. 11B). Similarly, β-1,3-glucanase activity increased significantly in P25 lettuce seeds exposed to all pre-hydration treatments relative to unsoaked seeds (Fig. 12B). The exogenous antioxidants resulted in higher β-1,3-glucanase activity than DW in both P50 and P25 lettuce seeds.

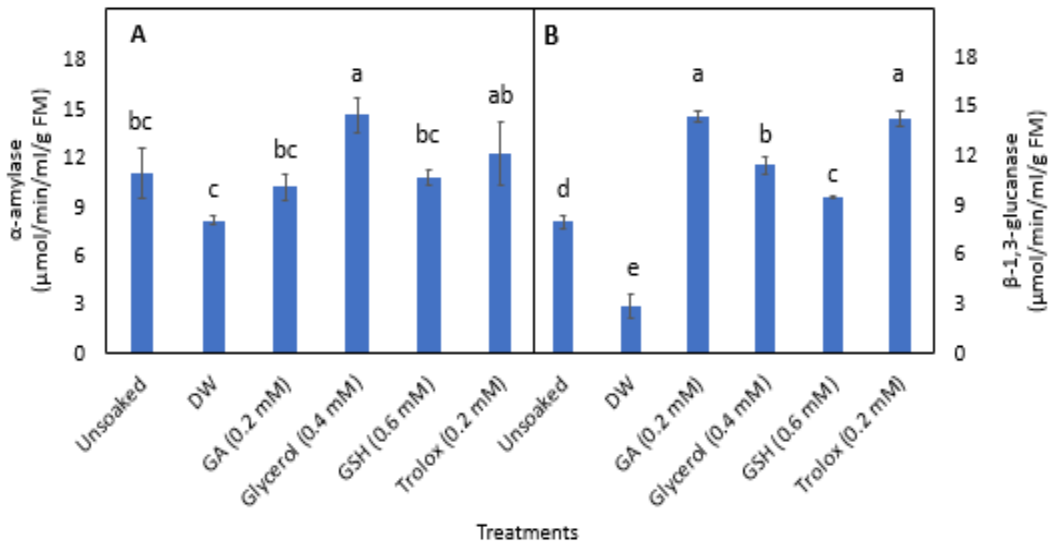

Figure 10 Effect of exogenous antioxidants application on germination enzymes activities: A) α-amylase and B) β-1,3-glucanase, in P25 *B. oleracea* (cabbage) seeds subjected to no soaking (unsoaked) or soaked in deionised water (DW), gallic acid (GA), glycerol, reduced glutathione (GSH) or trolox. Values represent mean ± SD (n = 5 for EC and n = 3 for all other parameters). Bars labelled with different letters indicate significant differences at $P < 0.05$ (ANOVA).

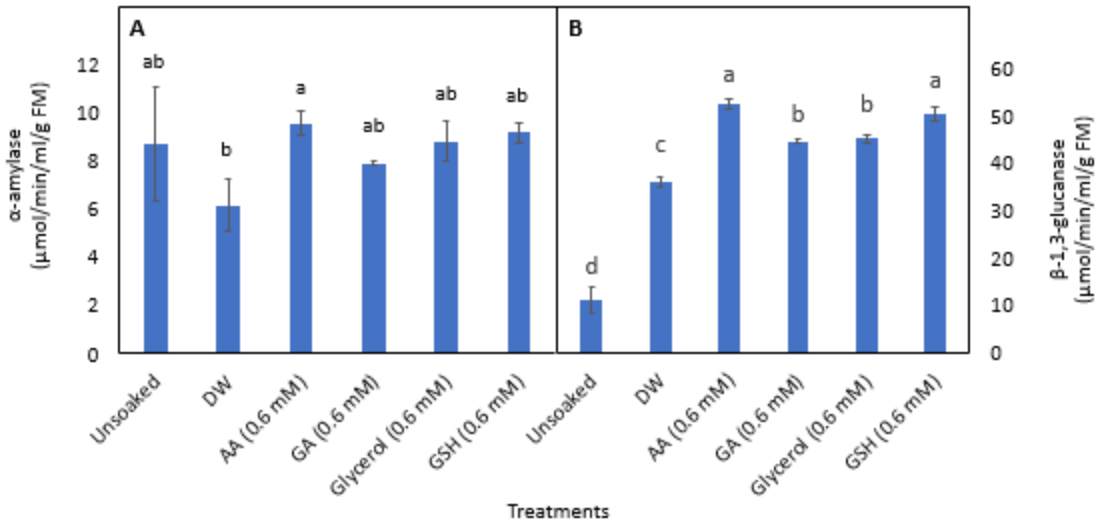

Figure 11 Effect of exogenous antioxidants application on germination enzymes activities: A) α-amylase and B) β-1,3-glucanase, in P50 *L. sativa* (lettuce) seeds subjected to no soaking (unsoaked) or soaked in deionised water (DW), ascorbic acid (AA), gallic acid (GA), glycerol or reduced glutathione (GSH). Values represent mean ± SD (n = 5 for EC and n = 3 for all other parameters). Bars labelled with different letters indicate significant differences at $P < 0.05$ (ANOVA).

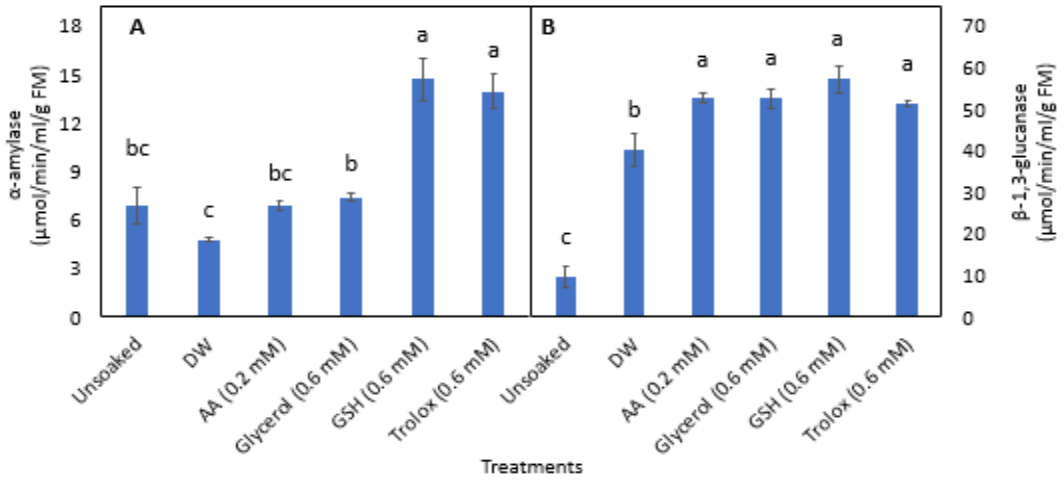

Figure 12 Effect of exogenous antioxidants application on germination enzymes activities: A) α-amylase and B) β-1,3-glucanase, in P25 *L. sativa* (lettuce) seed subjected to no soaking (unsoaked) or soaked in deionised water (DW), ascorbic acid (AA), glycerol, reduced glutathione (GSH) or trolox. Values represent mean ± SD (n = 5 for EC and n = 3 for all other parameters). Bars labelled with different letters indicate significant differences at $P < 0.05$ (ANOVA).

4.3 The inorganic salt pre-hydration treatment experiments

4.3.1 Effect of the application of inorganic salt solutions on % seedling production and vigour of cabbage and lettuce seeds

Abnormal seedling (AS) production, one of the known symptoms of ageing-induced damage in germinating seeds, was observed in the present study. The occurrence of AS ranging between 1.5% and 23.5% in cabbage and 0.5% and 19.0% in lettuce was observed only in the controlled deteriorated seeds across all inorganic salt pre-hydration treatments (Table 10). The proportion of AS produced was reduced significantly in P50 cabbage seeds treated with NaCl CW (6.5), and P25 cabbage seeds treated with $CaCl_2$ CW, CaMg, CaMg CW, CaMg CW (6.5), $MgCl_2$, NaCl, NaCl CW and NaCl CW (6.5) relative to DW-treated seeds. However, the proportion of AS produced was increased significantly in P25 lettuce seeds exposed to NaCl solution relative to DW-treated seeds.

Table 10 Effect of the application of inorganic salt solutions on abnormal seedling production (%) in controlled deteriorated cabbage and lettuce seeds

Pre-hydration Treatments	AS (%) for P75	AS (%) for P50	AS (%) for P25	AS (%) for P75	AS (%) for P50	AS (%) for P25
	Cabbage Seeds	Cabbage Seeds	Cabbage Seeds	Lettuce Seeds	Lettuce Seeds	Lettuce Seeds
DW	11.00 ± 8.75 ^{NS}	18.50 ± 10.89 ^{a}	20.00 ± 5.24 ^{a}	6.50 ± 6.74 ^{NS}	11.50 ± 9.18 ^{NS}	9.00 ± 4.14 ^{b}
$CaCl_2$	5.50 ± 5.21 ^{NS}	9.00 ± 8.49 ^{NS}	12.50 ± 8.67 ^{NS}	7.00 ± 5.13 ^{NS}	9.00 ± 7.33 ^{NS}	13.00 ± 5.95 ^{NS}
$CaCl_2$ CW	1.50 ± 2.98 ^{NS}	7.50 ± 6.95 ^{NS}	7.50 ±9.18 ^{b}	3.00 ± 4.66 ^{NS}	4.00 ± 5.66 ^{NS}	7.00 ± 7.63 ^{NS}
CaMg	6.50 ± 6.39 ^{NS}	12.50 ± 3.34 ^{NS}	8.00 ± 5.24 ^{b}	3.00 ± 04.66 ^{NS}	10.50 ± 5.21 ^{NS}	8.50 ± 6.21 ^{NS}
CaMg CW	5.00 ± 5.95 ^{NS}	17.50 ± 7.39 ^{NS}	6.00 ± 5.24 ^{b}	3.50 ± 4.50 ^{NS}	5.50 ± 7.07 ^{NS}	11.00 ± 4.66 ^{NS}
CaMg CW (6.5)	7.00 ± 5.55 ^{NS}	14.00 ± 4.78 ^{NS}	10.00 ± 4.78 ^{b}	3.00 ± 3.55 ^{NS}	4.00 ± 3.02 ^{NS}	8.00 ± 6.76 ^{NS}
$MgCl_2$	6.50 ± 6.39 ^{NS}	10.50 ± 4.24 ^{NS}	8.00 ± 4.78 ^{b}	4.00 ± 6.05 ^{NS}	2.50 ± 5.63 ^{NS}	12.00 ± 5.66 ^{NS}
$MgCl_2$ CW	7.00 ± 6.32 ^{NS}	13.50 ± 5.21 ^{NS}	14.00 ± 5.66 ^{NS}	1.50 ± 2.98 ^{NS}	6.50 ± 6.39 ^{NS}	11.00 ± 5.13 ^{NS}
NaCl	16.00 ± 8.00 ^{NS}	23.50 ± 6.57 ^{NS}	4.75 ± 1.58 ^{b}	2.75 ± 1.83 ^{NS}	18.00 ± 3.70 ^{NS}	19.00 ± 4.14 ^{a}
NaCl CW	9.50 ± 5.63 ^{NS}	7.50 ± 4.14 ^{NS}	9.00 ± 8.21 ^{b}	0.50 ± 1.41 ^{NS}	8.50 ± 5.83 ^{NS}	10.50 ± 6.02 ^{NS}
NaCl CW (6.5)	8.00 ± 4.78 ^{NS}	6.50 ± 5.95 ^{b}	9.00 ± 5.95 ^{b}	5.00 ± 4.14 ^{NS}	6.00 ± 5.66 ^{NS}	5.00 ± 6.32 ^{NS}

Values represent mean ± SD (4 × n = 25) of % abnormal seedling production in *B*. *oleracea* (cabbage) and *L*. *sativa* (lettuce) seeds exposed to deionised water (DW) and inorganic salt pre-hydration treatments after CD. Values labelled with different letters are significantly different ($p < 0.05$, ANOVA) when compared across pre-hydration treatments within each CD level. Cathodic water, CW; cathodic water adjusted to pH 6.5, CW (6.5); controlled deterioration, CD; abnormal seedling, AS; NS: not significantly different from value obtained with DW and therefore not considered in statistical comparisons.

Normal seedling production (%) in cabbage seeds was not enhanced significantly by the application of inorganic salt solutions; rather, this was reduced in $CaCl_2$- and $CaCl_2$ CW-treated P50 cabbage seeds relative to seeds soaked in deionised water (DW) (Table 11). In lettuce, however, normal seedling production was increased significantly in P50 seeds treated with $CaCl_2$ CW, CaMg and CaMg CW (6.5), and in P25 seeds treated with $CaCl_2$ CW, CaMg, $MgCl_2$ CW, NaCl CW and NaCl CW (6.5) relative to seeds soaked in DW (Table 12).

Table 11 Effect of the application of inorganic salt solutions on normal seedling production (%) in fresh and controlled deteriorated (CDd) cabbage seeds

Pre-hydration Treatments	% Normal Seedlings for Fresh Seeds	% Normal Seedlings for CDd (P75) Seeds	% Normal Seedlings for CDd (P50) Seeds	% Normal Seedlings for CDd (P25) Seeds
DW	89.00 ± 9.26 ^{NS}	72.00 ± 14.81 ^{NS}	43.00 ± 10.64 ^{a}	20.00 ± 3.02 ^{NS}
$CaCl_2$	94.00 ± 6.76 ^{NS}	71.00 ± 9.74 ^{NS}	26.00 ± 7.09 ^{b}	17.00 ± 7.33 ^{NS}
$CaCl_2$ CW	94.50 ± 5.21 ^{NS}	78.50 ± 10.01 ^{NS}	21.50 ± 10.24 ^{b}	19.00 ± 6.32 ^{NS}
CaMg	92.50 ± 7.54 ^{NS}	63.50 ± 12.91 ^{NS}	36.00 ± 6.05 ^{NS}	23.50 ± 10.13 ^{NS}
CaMg CW	91.50 ± 9.43 ^{NS}	72.5 ± 10.99 ^{NS}	49.50 ± 9.30 ^{NS}	22.00 ± 3.70 ^{NS}
CaMg CW (6.5)	96.50 ± 5.83 ^{NS}	69.50 ± 12.46 ^{NS}	48.00 ± 7.41 ^{NS}	26.50 ± 3.66 ^{NS}
$MgCl_2$	95.00 ± 7.01 ^{NS}	78.00 ± 8.28 ^{NS}	35.00 ± 9.50 ^{NS}	14.50 ± 8.26 ^{NS}
$MgCl_2$ CW	92.50 ± 9.90 ^{NS}	75.50+11.60 ^{NS}	40.00 ± 7.71 ^{NS}	21.50 ± 10.68 ^{NS}
NaCl	93.50 ± 5.47 ^{NS}	59.00 ± 4.14 ^{NS}	48.00 ± 9.80 ^{NS}	24.50 ± 7.84 ^{NS}
NaCl CW	95.50 ± 4.99 ^{NS}	75.00 ± 5.13 ^{NS}	34.00 ± 10.25 ^{NS}	25.50 ± 8.26 ^{NS}
NaCl CW (6.5)	94.00 ± 8.00 ^{NS}	69.50 ± 11.70 ^{NS}	48.50 ± 6.91 ^{NS}	32.00 ± 9.32 ^{NS}

Values represent mean ± SD ($4 \times n = 25$) % normal seedling production of the control (fresh seeds soaked in deionised water [DW] and all the inorganic salt solutions), and CDd (P75, P50 and P25) *B. oleracea* (cabbage) seeds exposed to DW and inorganic salt solutions. Values labelled with different letters are significantly different ($p < 0.05$, ANOVA) when compared across hydration treatments at P50. Cathodic water, CW; cathodic water adjusted to pH 6.5, CW (6.5); NS: not significantly different from value obtained with DW and therefore not considered in statistical comparisons.

Table 12 Effect of the application of inorganic salt solutions on normal seedling production (%) in fresh and controlled deteriorated (CDd) lettuce seeds

Pre-hydration Treatments	% Normal Seedling for Fresh Seeds	% Normal Seedling for CDd (P75) Seeds	% Normal Seedling for CDd (P50) Seeds	% Normal Seedling for CDd (P25) Seeds
DW	99.00 ± 1.85 ^{NS}	73.50 ± 9.78 ^{NS}	50.00 ± 10.03 ^{b}	21.00 ± 6.32 ^{b}
$CaCl_2$	100.00 ± 0.00 ^{NS}	70.00 ± 8.28 ^{NS}	58.00 ± 11.51 ^{NS}	32.00 ± 11.31 ^{NS}
$CaCl_2$ CW	100.00 ± 0.00 ^{NS}	74.00 ± 10.03 ^{NS}	67.00 ± 12.42 ^{a}	39.00 ± 11.06 ^{a}
CaMg	98.50 ± 4.24 ^{NS}	76.50 ± 10.57 ^{NS}	76.50 ± 10.57 ^{a}	40.00 ± 9.56 ^{a}
CaMg CW	100.00 ± 0.00 ^{NS}	72.50 ± 4.50 ^{NS}	63.00 ± 5.95 ^{NS}	23.00 ± 6.32 ^{NS}
CaMg CW (6.5)	99.00 ± 1.85 ^{NS}	76.50 ± 5.83 ^{NS}	68.00 ± 10.90 ^{a}	29.00 ± 5.13 ^{NS}
$MgCl_2$	99.00 ± 1.85 ^{NS}	83.00 ± 9.50 ^{NS}	68.00 ± 10.90 ^{NS}	28.00 ± 11.11 ^{NS}
$MgCl_2$ CW	99.50 ± 1.41 ^{NS}	71.00 ± 11.06 ^{NS}	57.00 ± 12.96 ^{NS}	39.00 ± 10.20 ^{a}
NaCl	98.00 ± 2.20 ^{NS}	76.25 ± 5.06 ^{NS}	50.00 ± 9.07 ^{NS}	24.50 ± 6.57 ^{NS}
NaCl CW	100.00 ± 0.00 ^{NS}	78.50 ± 6.02 ^{NS}	51.50 ± 11.99 ^{NS}	40.00 ± 14.18 ^{a}
NaCl CW (6.5)	99.50 ± 1.41 ^{NS}	74.00 ± 6.76 ^{NS}	68.00 ± 10.90 ^{NS}	48.00 ± 15.57 ^{a}

Values represent mean ± SD (4 × n = 25) % normal seedling production of the control (fresh seeds soaked in deionised water [DW] and all the inorganic salt solutions), and CDd (P75, P50 and P25) *L*. *sativa* (lettuce) seeds exposed to DW and inorganic salt solutions. Values labelled with different letters are significantly different (p < 0.05, ANOVA) when compared across hydration treatments within each controlled deterioration level. Cathodic water, CW; cathodic water adjusted to pH 6.5, CW (6.5); NS: not significantly different from value obtained with DW and therefore not considered in statistical comparisons.

In both fresh and controlled deteriorated (P75) cabbage seeds, SVI was not influenced significantly by soaking in inorganic salt solutions (Table 13). Seedling vigour index in P50 cabbage seeds, however, was significantly increased when soaked in CaMg CW (6.5) and NaCl CW (6.5) relative to P50 DW-treated seeds. In P25 cabbage seeds, SVI increased significantly relative to P25 DW-treated seeds when pre-hydrated in CaMg, NaCl CW and NaCl CW (6.5) (Table 13).

In both fresh and P75 lettuce seeds, SVI was not influenced significantly by inorganic salt solution application, compared with DW-treated seeds (Table 13). However, SVI in P50 lettuce seeds increased significantly relative to P50-DW-treated seeds when pre-hydrated in $CaCl_2$, $CaCl_2$ CW, CaMg, CaMg CW (6.5), $MgCl_2$ CW, NaCl CW and NaCl CW (6.5) while SVI in P25 lettuce seeds increased significantly when treated with $CaCl_2$ CW, NaCl CW and NaCl CW (6.5) compared with P25-DW-treated seeds (Table 13).

Table 13 Effect of the application of inorganic salt solutions on seedling vigour index in fresh and controlled deteriorated cabbage and lettuce seeds

Controlled deterioration Level	Treatments	Seedling Vigour Index	
		Cabbage	Lettuce
Fresh	DW	6721.20 ± 946.967 ^{NS}	3446.30 ± 530.44 ^{NS}
	$CaCl_2$	6820.20 ± 912.16 ^{NS}	3655.00 ± 457.42 ^{NS}
	$CaCl_2$ CW	6542.10 ± 1369.35 ^{NS}	3525.00 ± 244.66 ^{NS}
	CaMg	6284.90 ± 1346.14 ^{NS}	3749.80 ± 474.16 ^{NS}
	CaMg CW	5917.50 ± 1075.33 ^{NS}	3462.50 ± 289.32 ^{NS}
	CaMg CW (6.5)	7173.20 ± 1231.64 ^{NS}	3440.50 ± 333.14 ^{NS}
	$MgCl_2$	7004.70 ± 888.17 ^{NS}	3647.00 ± 414.42 ^{NS}
	$MgCl_2$ CW	6752.80 ± 765.97 ^{NS}	3404.50 ± 340.25 ^{NS}
	NaCl	4692.50 ± 1904.47 ^{NS}	3431.95 ± 231.65 ^{NS}
	NaCl CW	6906.30 ± 841.71 ^{NS}	3400.00 ± 153.44 ^{NS}
	NaCl CW (6.5)	6060.50 ± 1158.96 ^{NS}	3463.90 ± 482.66 ^{NS}
P75	DW	7554.50 ± 2623.67 ^{NS}	4191.90 ± 842.42 ^{NS}
	$CaCl_2$	5559.45 ± 1833.26 ^{NS}	3560.70 ± 7181.02 ^{NS}
	$CaCl_2$ CW	7126.30 ± 2643.67 ^{NS}	4387.50 ± 1315.91 ^{NS}
	CaMg	4357.00 ± 1044.53 ^{NS}	4047.90 ± 1256.79 ^{NS}
	CaMg CW	6512.00 ± 1503.23 ^{NS}	4157.10 ± 328.56 ^{NS}
	CaMg CW (6.5)	6627.20 ± 2031.09 ^{NS}	4512.70 ± 673.33 ^{NS}
	$MgCl_2$	8012.50 ± 2757.45 ^{NS}	4918.10 ± 831.24 ^{NS}
	$MgCl_2$ CW	6612.50 ± 1427.34 ^{NS}	3552.20 ± 880.11 ^{NS}
	NaCl	4672.20 ± 2007.78 ^{NS}	4491.45 ± 782.45 ^{NS}
	NaCl CW	6359.80 ± 1727.57 ^{NS}	4592.30 ± 1117.40 ^{NS}
	NaCl CW (6.5)	6435.00 ± 2780.75 ^{NS}	4390.60 ± 882.63 ^{NS}

Table 13 (Continued). Effect of the application of inorganic salt solutions on seedling vigour index in fresh and controlled deteriorated cabbage and lettuce seeds

Controlled Deterioration Level	Treatments	Seedling Vigour Index	
		Cabbage	Lettuce
P50	DW	1621.40 ± 911.97 [b]	1056.90 ± 323.25 [b]
	$CaCl_2$	1564.90 ± 522.56 [NS]	2705.20 ± 629.67 [a]
	$CaCl_2$ CW	1352.90 ± 797.91 [NS]	2819.10 ± 580.95 [a]
	CaMg	2855.00 ± 688.79 [NS]	2823.50 ± 585.35 [a]
	CaMg CW	2284.20 ± 781.18 [NS]	1772.80 ± 362.52 [NS]
	CaMg CW (6.5)	3395.10 ± 1073.31 [a]	3493.20 ± 1077.06 [a]
	$MgCl_2$	3042.80 ± 1570.89 [NS]	2525.20 ± 607.15 [NS]
	$MgCl_2$ CW	2938.70 ± 776.86 [NS]	2755.80 ± 1094.63 [a]
	NaCl	2644.10 ± 1514.80 [NS]	2000.80 ± 510.49 [NS]
	NaCl CW	2019.30 ± 1042.40 [NS]	2717.40 ± 906.07 [a]
	NaCl CW (6.5)	3520.30 ± 910.90 [a]	2981.20 ± 795.30 [a]
P25	DW	535.10 ± 222.14 [b]	403.30 ± 199.74 [b]
	$CaCl_2$	903.00 ± 597.52 [NS]	1090.93 ± 404.74 [NS]
	$CaCl_2$ CW	1198.40 ± 534.58 [NS]	1341.80 ± 362.03 [a]
	CaMg	1679.00 ± 796.61 [a]	1144.80 ± 405.07 [NS]
	CaMg CW	531.50 ± 294.91 [NS]	490.40 ± 296.44 [NS]
	CaMg CW (6.5)	1084.00 ± 321.11 [NS]	794.40 ± 395.29 [NS]
	$MgCl_2$	785.50 ± 627.33 [NS]	747.80 ± 511.32 [NS]
	$MgCl_2$ CW	1084.00 ± 648.73 [NS]	1205.80 ± 282.89 [NS]
	NaCl	1438.70 ± 426.89 [NS]	404.20 ± 147.05 [NS]
	NaCl CW	1633.10 ± 801.91 [a]	1493.80 ± 668.57 [a]
	NaCl CW (6.5)	2205.18 ± 1003.93 [a]	1562.30 ± 1038.11 [a]

Values represent mean ± SD (4 × *n* = 25) of seedling vigour index (SVI) in fresh and controlled deteriorated (P75, P50 and P25) *B*. *oleracea* (cabbage) and *L*. *sativa* (lettuce) seeds exposed to DW and inorganic salt solutions. Values labelled with different letters are significantly different ($p < 0.05$, ANOVA) when compared across hydration treatments within each CD level. Cathodic water, CW; cathodic water adjusted to pH 6.5, CW (6.5); controlled deterioration, CD; NS: not significantly different from value obtained with DW and therefore not considered in statistical comparisons.

4.3.2 Effect of the application of inorganic salt solutions on biomarkers of oxidative stress in controlled deteriorated lettuce seeds

The oxidative stress biomarkers were measured in control (fresh) and CDd lettuce seeds without soaking (unsoaked), and after soaking in DW; and in all those controlled deterioration (CD) × inorganic salt treatment combinations that enhanced normal seedling production (%) significantly relative to DW-treated lettuce seeds at a specific level of CD. Since the treatments did not promote % normal seedling production in cabbage seeds, these assays were not performed for this species.

Electrolyte leakage levels were significantly reduced in P50 lettuce seeds treated with CaMg CW (6.5) relative to DW-treated seeds (Fig. 13A), but no pre-hydration treatments (DW and inorganic salt) led to a significant reduction of electrolyte leakage in P25 lettuce seeds relative to unsoaked seeds (Fig. 14A).

Conjugated dienes (CJD) levels generally reduced after all pre-hydration treatments application, but this was only significant in P50 seeds soaked in $CaCl_2$ CW and CaMg CW (6.5), relative to unsoaked P50 seeds (Fig. 13B). In P25 seeds, DW and all inorganic salt pre-hydration treatments significantly reduced CJD levels relative to unsoaked P25 seeds; the inorganic salt treatments resulted in a greater reduction in CJD levels than DW (Fig. 14B).

The levels of 4-HNE were significantly increased in DW-treated P50 seeds but significantly reduced when treated with CaMg CW (6.5), relative to unsoaked P50 seeds (Fig. 13C). In P25 seeds, significantly reduced levels of 4-HNE relative to unsoaked P25 seeds were estimated for all inorganic salt treatments (Fig. 14C).

The inorganic salt treatments did not lead to a significant reduction in protein carbonylation (PC) levels of P50 seeds (Fig. 13D), while CaMg and NaCl CW (6.5) significantly reduced PC levels in P25 seeds relative to unsoaked and DW-treated seeds (Fig. 14D).

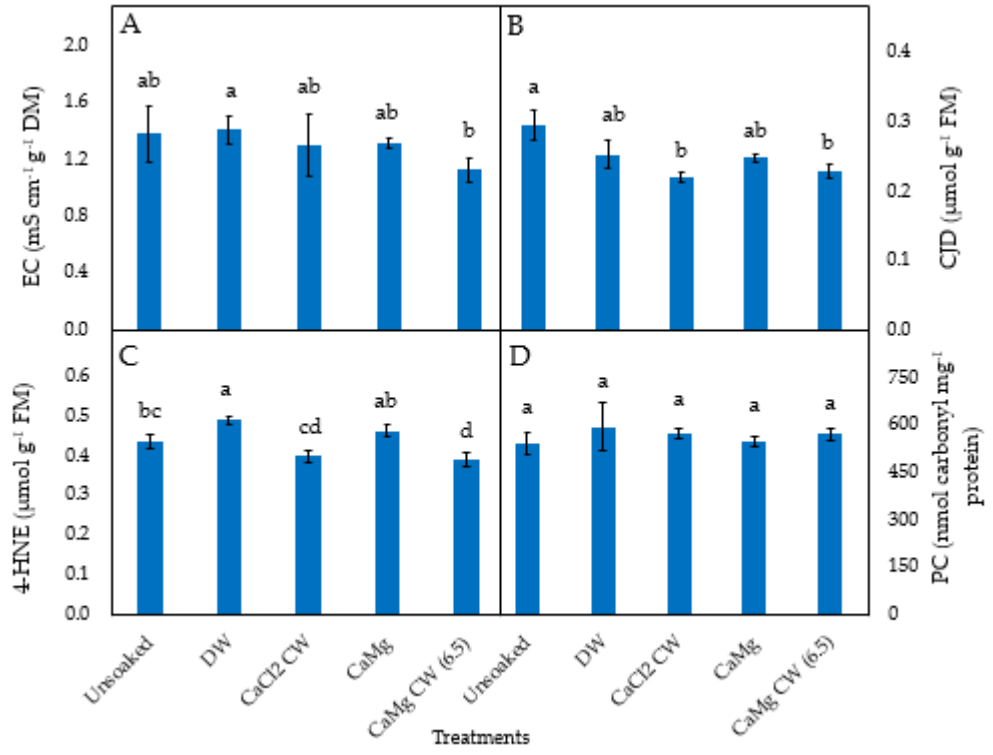

Figure 13 Effect of inorganic salt solution application on biomarkers of oxidative stress in P50 *L. sativa* (lettuce) seeds subjected to no soaking or soaking in deionised water (DW), $CaCl_2$ generated cathodic water ($CaCl_2$ CW), CaMg, or CaMg generated cathodic water adjusted to pH 6.5 (CaMg CW [6.5]). A) electrical conductivity (EC), B) conjugated dienes (CJD), C) 4-hydroxy-2-nonenal (4-HNE) and D) protein carbonylation (PC) adduct. Values represent mean ± SD (n = 5 for EC and n = 3 for all other parameters). Bars labelled with different letters indicate significant differences at $p < 0.05$ (ANOVA).

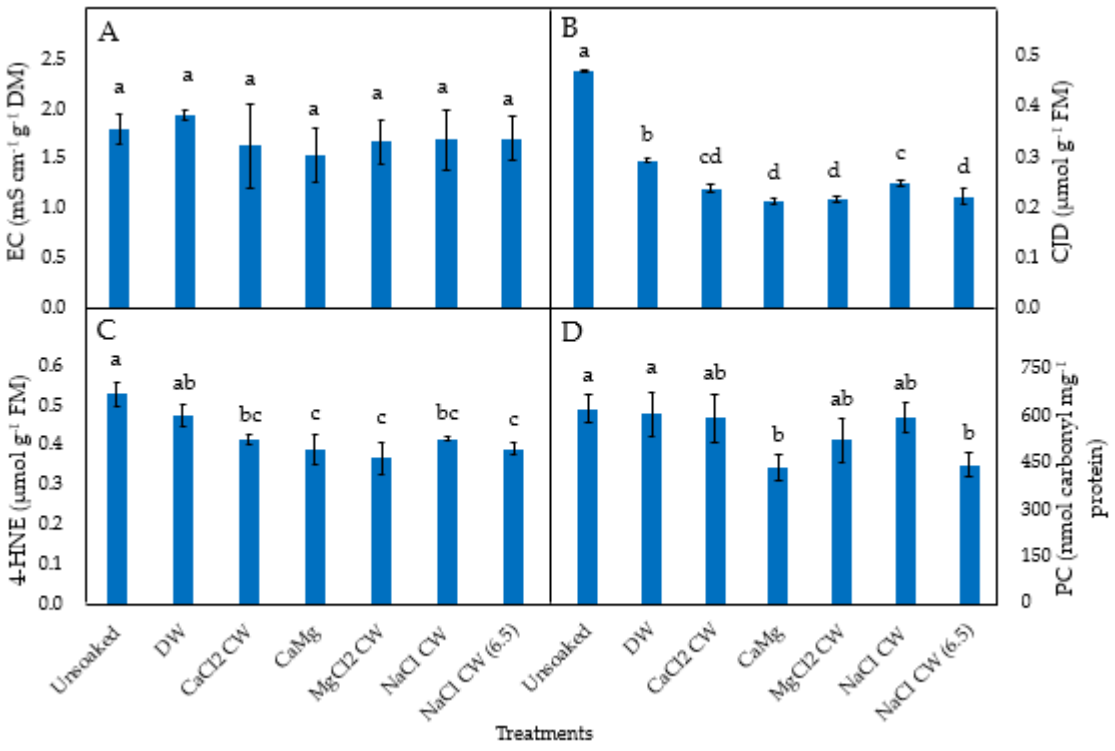

Figure 14 Effect of inorganic salt solution application on biomarkers of oxidative stress in P25 *L. sativa* (lettuce) seeds subjected to no soaking or soaking in deionised water (DW), $CaCl_2$ generated cathodic water ($CaCl_2$ CW), CaMg, $MgCl_2$ generated cathodic water ($MgCl_2$ CW), NaCl generated cathodic water (NaCl CW), or NaCl generated cathodic water adjusted to pH 6.5 (NaCl CW [6.5]). A) electrical conductivity (EC), B) conjugated dienes (CJD), C) 4-hydroxy-2-nonenal (4-HNE) and D) protein carbonylation (PC) adduct. Values represent mean ± SD (n = 5 for EC and n = 3 for all other parameters). Bars labelled with different letters indicate significant differences at $p < 0.05$ (ANOVA).

4.3.3 Effect of the application of inorganic salt solutions on enzymatic antioxidant activities of controlled deteriorated lettuce seeds

In CDd P50 lettuce seeds, catalase (CAT) activity was not influenced significantly by the inorganic salt treatments relative to unsoaked seed but decreased significantly in seeds soaked in DW (Fig. 15A). In P25 seeds, CAT activity was not influenced by NaCl CW and NaCl CW (6.5) soaking of seeds after deterioration, relative to the unsoaked seeds, but the enzyme activity decreased significantly in seeds soaked in DW, $CaCl_2$ CW, CaMg and $MgCl_2$ CW (Fig. 16A).

Glutathione reductase (GR) activity was increased significantly in P50 seeds in all pre-

hydration treatments relative to the unsoaked seeds (Fig. 15B); CaMg and CaMg CW (6.5) resulted in higher GR activity than DW. In CDd P25 seeds, GR activity was increased significantly by CaMg, NaCl CW and NaCl CW (6.5), relative to unsoaked seeds; NaCl CW resulted in higher GR activity than DW (Fig. 16B).

Superoxide dismutase (SOD) activity was not influenced significantly by any pre-hydration treatment in P50 seeds relative to unsoaked seeds (Fig. 15C). However, SOD activity in P25 seeds was increased significantly by all pre-hydration treatments relative to unsoaked seeds; the inorganic salt treatments enhanced enzyme activity more than DW (Fig. 16C).

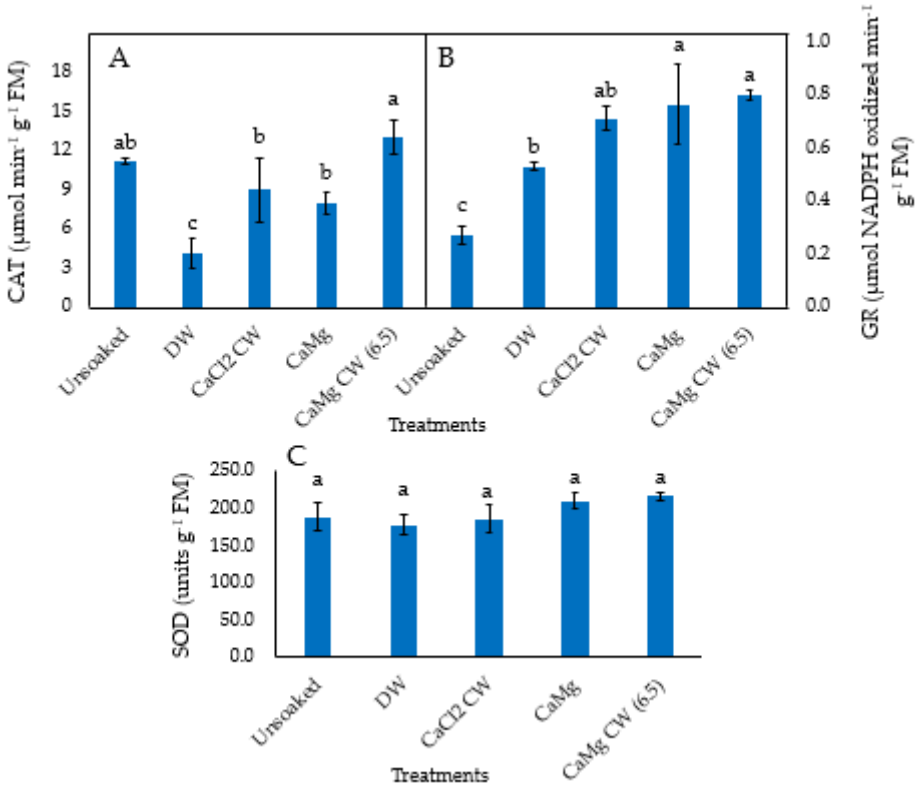

Figure 15 Effect of inorganic salt solution application on antioxidant enzymes activities in P50 *L. sativa* (lettuce) seeds subjected to no soaking or soaked in deionised water (DW), $CaCl_2$ generated cathodic water ($CaCl_2$ CW), CaMg, or CaMg generated cathodic water adjusted to pH 6.5 (CaMg CW [6.5]). A) catalase (CAT), B) glutathione reductase (GR) and C) superoxide dismutase (SOD). Values represent mean ± SD (n = 3). Bars labelled with different letters indicate significant differences at $p < 0.05$ (ANOVA).

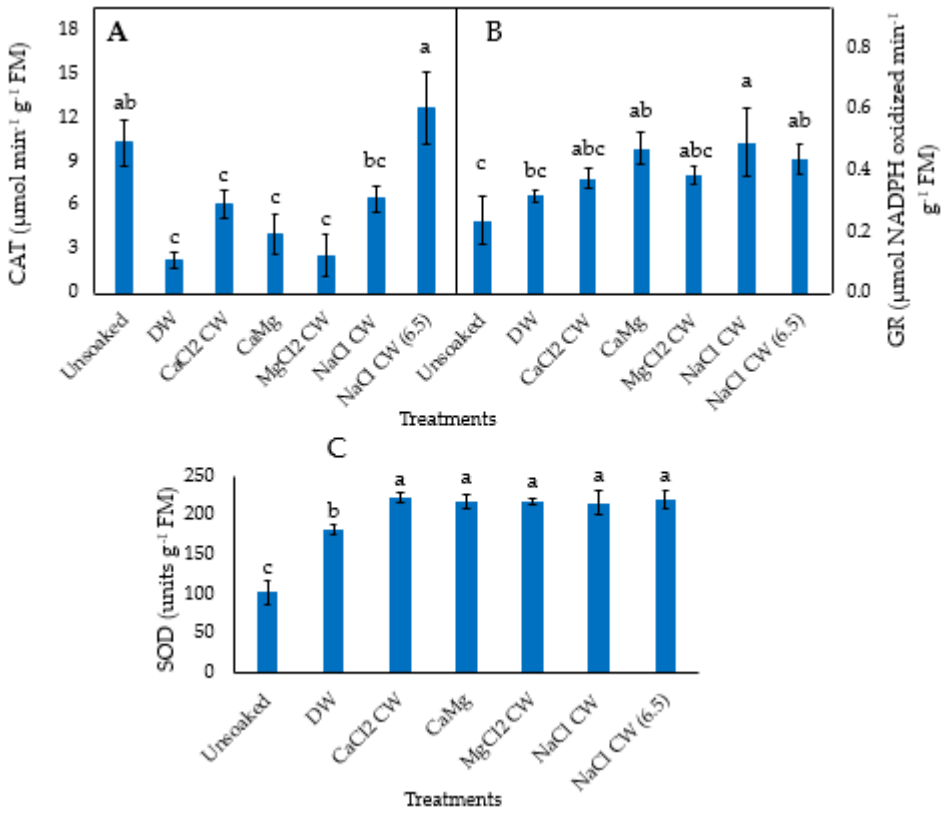

Figure 16 Effect of inorganic salt solution application on antioxidant enzymes activities in P25 *L. sativa* (lettuce) seeds subjected to no soaking or soaked in deionised water (DW), $CaCl_2$ generated cathodic water ($CaCl_2$ CW), CaMg, $MgCl_2$ generated cathodic water ($MgCl_2$ CW), NaCl generated cathodic water (NaCl CW), or NaCl generated cathodic water adjusted to pH 6.5 (NaCl CW [6.5]). A) catalase (CAT), B) glutathione reductase (GR) and C) superoxide dismutase (SOD). Values represent mean ± SD (n = 3). Bars labelled with different letters indicate significant differences at $p < 0.05$ (ANOVA).

4.3.4. Effect of the application of inorganic salt solutions on germination-related enzymes in controlled deteriorated lettuce seeds

The inorganic salt solutions had a promotive effect on the activities of germination enzymes in the CDd seeds. Though the pre-hydration treatments did not significantly enhance α-amylase activity in both P50 (Fig. 17A) and P25 (Fig. 18A) seeds relative to their unsoaked seeds, all pre-hydration treatments significantly increased β-1,3-glucanase activity in P50 seeds relative to unsoaked seeds; $CaCl_2$ CW and CaMg CW (6.5) soaked seeds exhibited higher β-1,3-glucanase activity than DW (Fig. 17B).

In P25 seeds, β-1,3-glucanase activity was increased significantly by all pre-hydration treatments relative to unsoaked seeds; $CaCl_2$ CW, NaCl CW and NaCl CW (6.5) resulted in a

greater rise in the enzyme activity than DW (Fig. 18B).

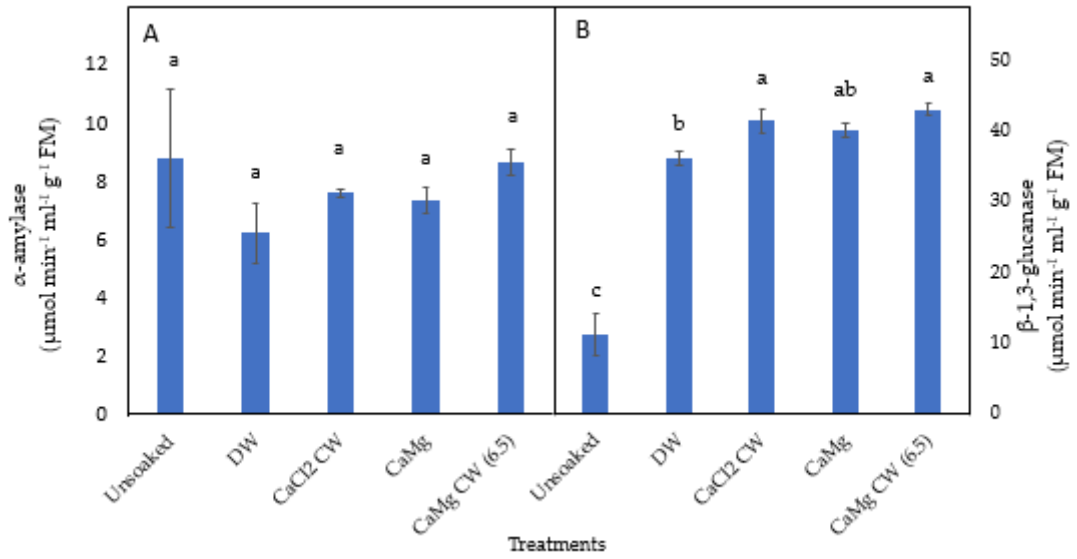

Figure 17 Effect of inorganic salt solution application on germination enzymes activities: (A) α-amylase and (B) β-1,3-glucanase, in P50 *L. sativa* (lettuce) seeds subjected to no soaking or soaked in deionised water (DW), $CaCl_2$ generated cathodic water ($CaCl_2$ CW), CaMg, or CaMg generated cathodic water adjusted to pH 6.5 (CaMg CW [6.5]). Values represent mean ± SD (n = 3). Bars labelled with different letters indicate significant differences at $p < 0.05$ (ANOVA).

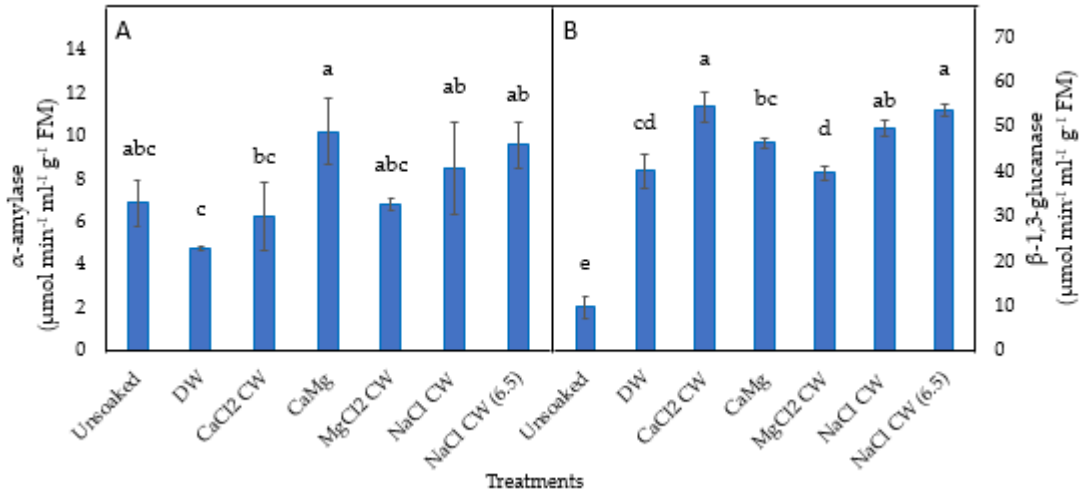

Figure 18 Effect of inorganic salt solution application on germination enzymes activities: (A) α-amylase and (B) β-1,3-glucanase, in P25 *L. sativa* (lettuce) seeds subjected to no soaking or soaked in deionised water (DW), $CaCl_2$ generated cathodic water ($CaCl_2$ CW), CaMg, $MgCl_2$ generated cathodic water ($MgCl_2$ CW), NaCl generated cathodic water (NaCl CW), or NaCl generated cathodic water adjusted to pH 6.5 (NaCl CW [6.5]). Values represent mean ± SD (n = 3). Bars labelled with different letters indicate significant differences at $p < 0.05$ (ANOVA).

4.4 The greenhouse pot trial

4.4.1 Effect of exogenous application of antioxidants on seedling emergence parameters of controlled deteriorated (P25) cabbage and lettuce seeds

The study showed that seedling emergence (%), mean emergence time (MET), mean daily emergence (MDE) and time to 25% emergence (T_{25}) were not influenced significantly by the exogenously applied antioxidants in both CDd cabbage (Fig. 19A–D) and lettuce (Fig. 20A–D) seeds relative to deionised water (DW).

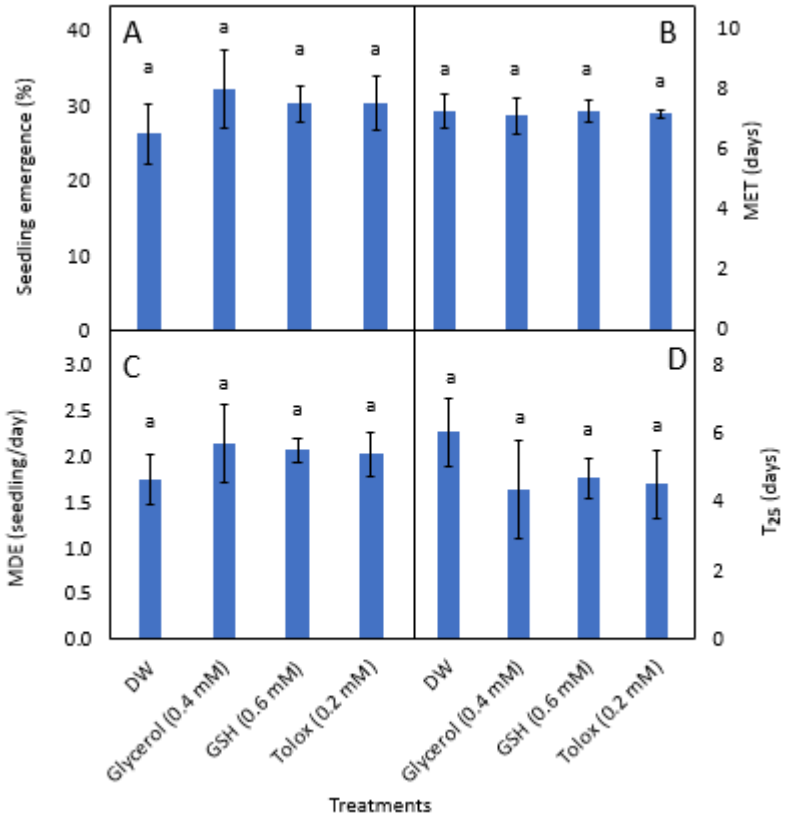

Figure 19 Effect of exogenous application of antioxidants on seedling emergence parameters in P25 *B. oleracea* (cabbage) seeds subjected to soaking in deionised water (DW), glycerol, reduced glutathione (GSH) or trolox. A) % seedling emergence, B) mean emergence time (MET), C) mean daily emergence (MDE) and D) time taken to 25% emergence (T_{25}). Values represent mean ± SD (3 × *n* = 60). There were no significant differences across the control (DW) and antioxidant treatments for all four parameters $P < 0.05$ (ANOVA).

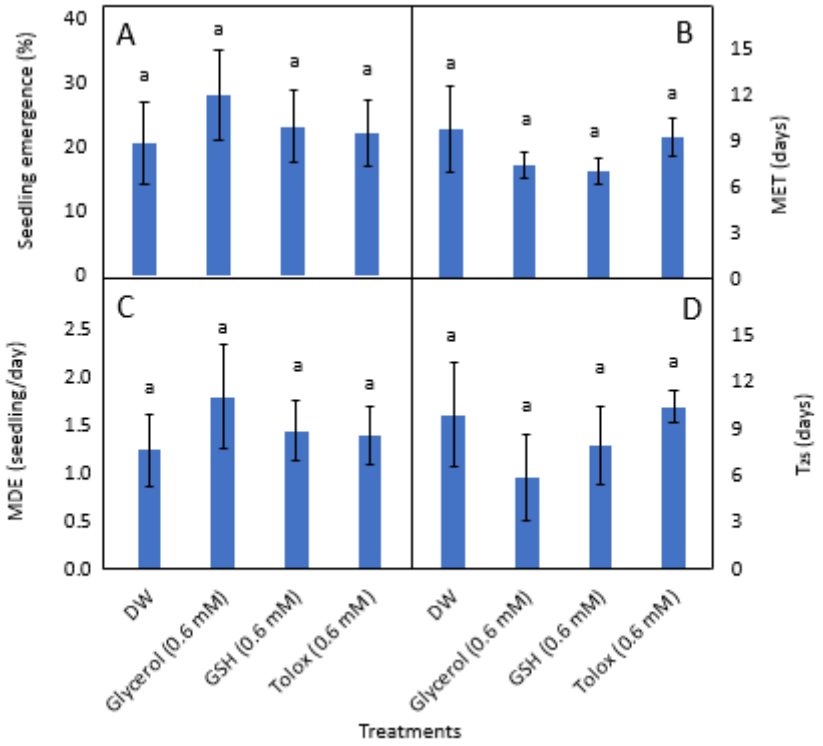

Figure 20 Effect of exogenous application of antioxidants on seedling emergence parameters in P25 *L. sativa* (lettuce) seeds subjected to soaking in deionised water (DW), glycerol, reduced glutathione (GSH) or trolox. A) % seedling emergence, B) mean emergence time (MET), C) mean daily emergence (MDE) and D) time taken to 25% emergence (T_{25}). Values represent mean ± SD (3 × *n* = 60). There were no significant differences across the control (DW) and antioxidant treatments for all four parameters $P < 0.05$ (ANOVA).

4.4.2 Effect of exogenous application of antioxidants on seedling vigour and biomass accumulation of seedlings produced from controlled deteriorated (P25) cabbage and lettuce seeds

Seedling vigour index (SVI) increased significantly in P25 cabbage seeds soaked in glycerol (0.4 mM) and GSH (0.6 mM), and in P25 lettuce seeds soaked in glycerol (0.6 mM), relative to DW-soaked seeds (Figs 21A and 22A, respectively). Root dry weight, however, was not influenced significantly by the application of the exogenous antioxidants in both cabbage and lettuce seeds relative to DW-soaked seeds (Figs 21B and 22B, respectively). Though shoot dry weight was not influenced significantly by the application of the exogenous antioxidants in CDd (P25) cabbage seeds, it increased significantly in seedlings produced from P25 lettuce seeds treated with glycerol relative to those produced from DW-soaked seeds (Figs 21C and

22C, respectively). The root:shoot ratio was not significantly influenced by the application of the exogenous antioxidant solutions in P25 seeds of both species relative to DW-treated seeds (Figs 21D and 22D, respectively).

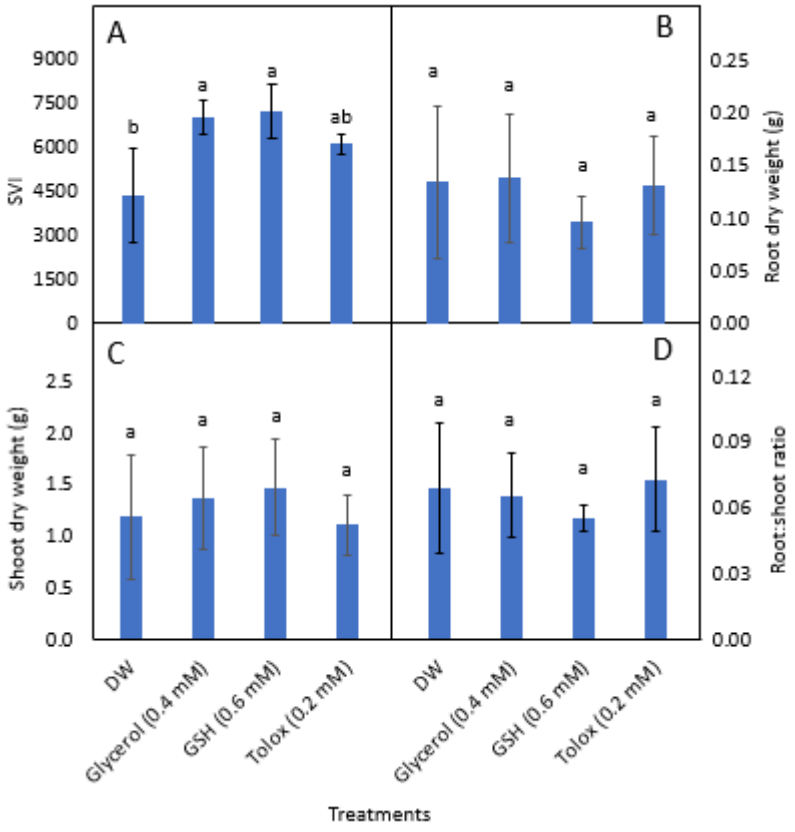

Figure 21 Effect of exogenous application of antioxidants on A) seedling vigour index (SVI), B) root dry weight, C) shoot dry weight and D) root:shoot ratio, in P25 *B. oleracea* (cabbage) seeds subjected to soaking in deionised water (DW), glycerol, reduced glutathione (GSH) or trolox. Values represent mean ± SD (3 × *n* = 10). Bars labelled with different letters indicate significant differences $P < 0.05$ (ANOVA).

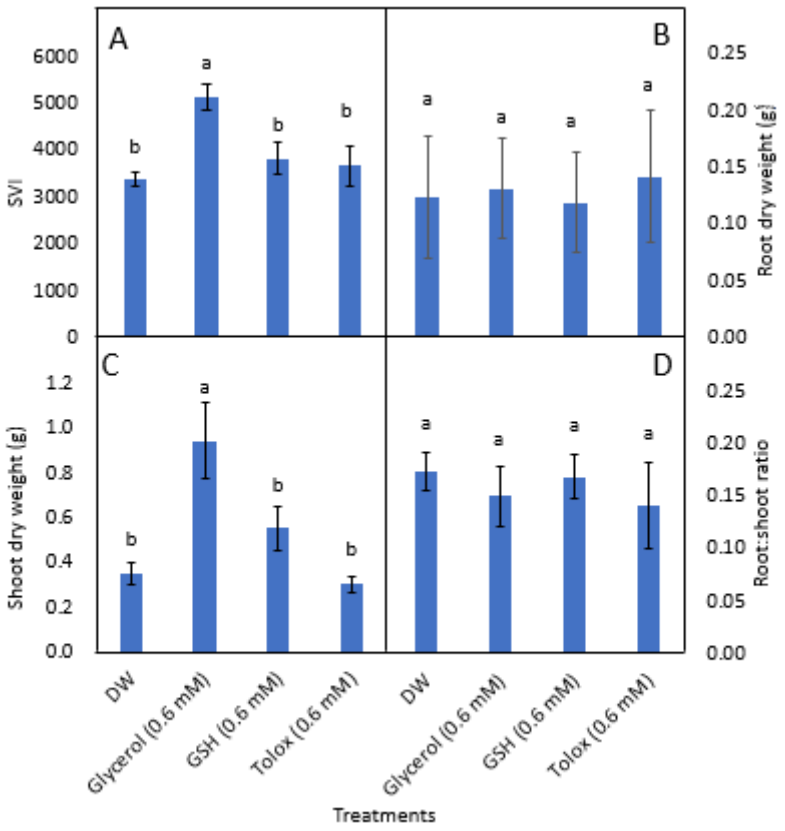

Figure 22 Effect of exogenous application of antioxidants on A) seedling vigour index (SVI), B) root dry weight, C) shoot dry weight and D) root:shoot ratio, in P25 *L. sativa* (lettuce) seeds subjected to soaking in deionised water (DW), glycerol, reduced glutathione (GSH) or trolox. Values represent mean ± SD (3 × n = 10). Bars labelled with different letters indicate significant differences at $P < 0.05$ (ANOVA).

4.4.3 Effect of exogenous application of antioxidants on leaf area, leaf area ratio and total chlorophyll content of leaves from seedlings produced from controlled deteriorated (P25) cabbage and lettuce seeds

The study showed that leaf area increased significantly in the seedlings produced from both P25 cabbage and lettuce seeds treated with glycerol (0.4 mM and 0.6 mM, respectively) relative to DW-soaked seeds (Figs 23A and 24A, respectively). However, the leaf area ratio (Figs 23B and 24B) and total chlorophyll content (Figs 23C and 24C) were not significantly influenced by the exogenously applied antioxidants in both cabbage and lettuce, respectively, relative to the DW-soaked seeds.

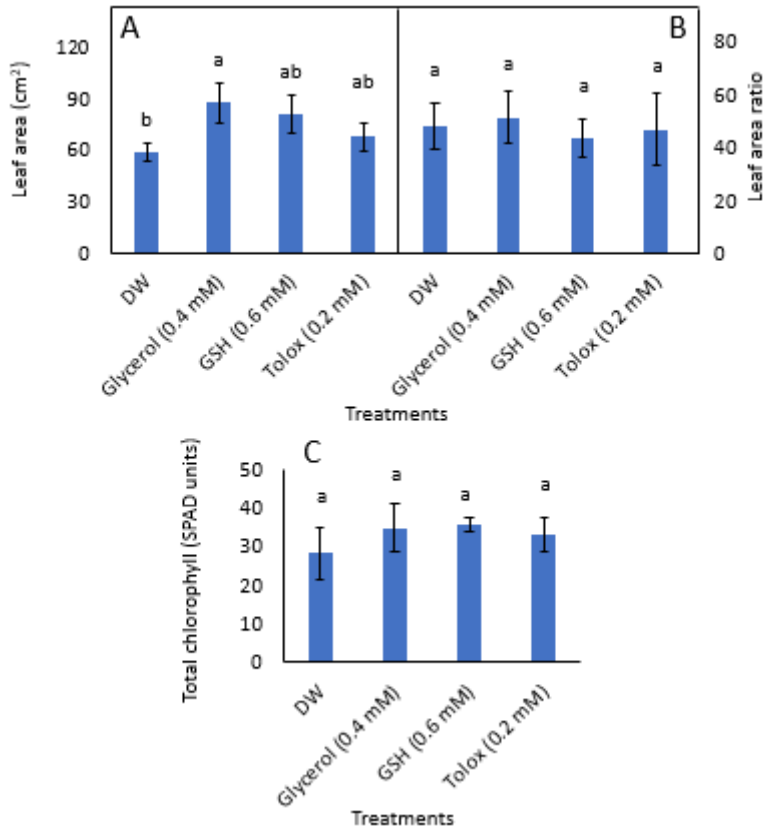

Figure 23 Effect of exogenous application of antioxidants on A) leaf area, B) leaf area ratio and C) total chlorophyll, in P25 *B*. *oleracea* (cabbage) seeds subjected to soaking in deionised water (DW), glycerol, reduced glutathione (GSH) or trolox. Values represent mean ± SD (3 × *n* = 10 for leaf area; *n* = 5 for total chlorophyll). Bars labelled with different letters indicate significant differences at $P < 0.05$ (ANOVA).

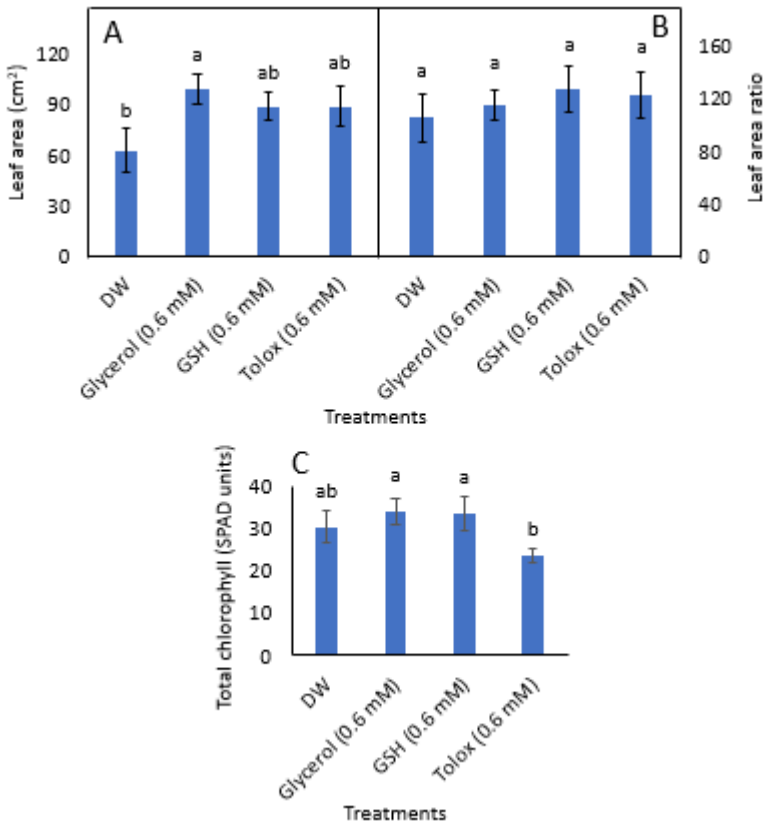

Figure 24 Effect of exogenous application of antioxidants on A) leaf area, B) leaf area ratio and C) total chlorophyll, in P25 *L. sativa* (lettuce) seeds subjected to soaking in deionised water (DW), glycerol, reduced glutathione (GSH) or trolox. Values represent mean ± SD (3 × *n* = 10 for leaf area; *n* = 5 for total chlorophyll). Bars labelled with different letters indicate significant differences at $P < 0.05$ (ANOVA).

4.4.4 Effect of exogenous application of antioxidants on photosynthetic efficiency and chlorophyll fluorescence of seedlings produced from controlled deteriorated (P25) cabbage and lettuce seeds

All parameters investigated, viz. seedling photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (E) and maximum quantum yield of PSII photochemistry (F_v/F_m) were not significantly influenced by the exogenous application of the antioxidants on P25 cabbage seeds (Fig. 25 A–D, respectively). In seedlings produced from P25 lettuce seeds, however, the photosynthetic rate increased significantly when these seeds were soaked in 0.6 mM of glycerol, GSH and trolox relative to DW-treated seeds (Fig. 26A). Stomatal conductance significantly increased when seeds were soaked in 0.6 mM of glycerol and GSH (Fig. 26B). Similarly, transpiration rate increased significantly in P25 lettuce when seeds were

treated with 0.6 mM of glycerol, GSH and trolox, relative to DW-treated seeds (Fig. 26C). The maximum quantum yield of PSII photochemistry was not significantly influenced by the exogenous application of the antioxidants relative to DW application (Fig. 26D).

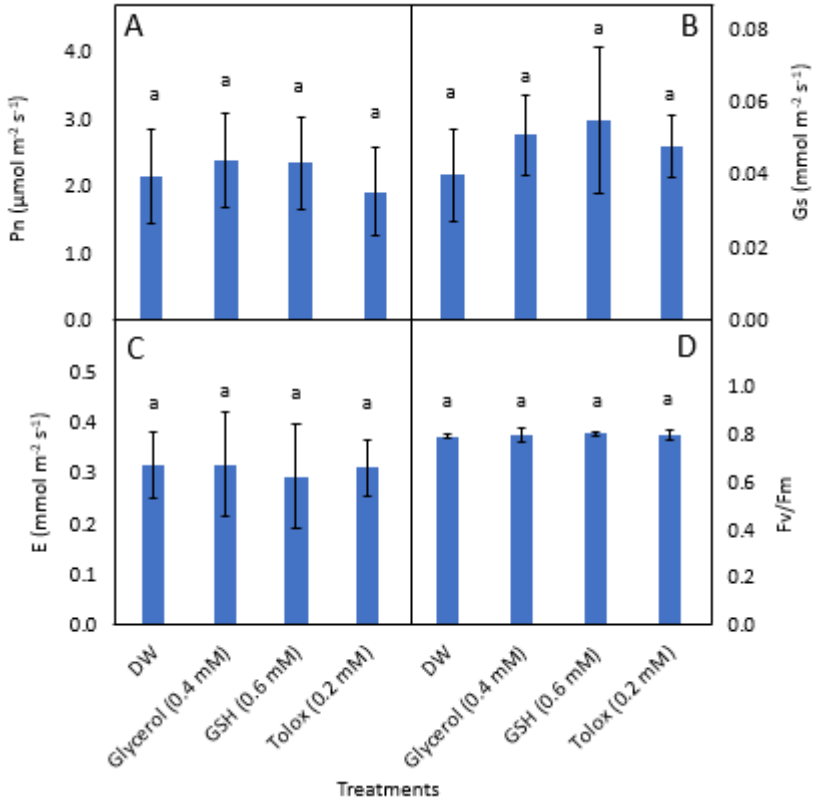

Figure 25 Effect of exogenous application of antioxidants on A) photosynthetic rate (Pn), B) stomatal conductance (Gs), C) transpiration rate (E) and D) chlorophyll fluorescence (F_v/F_m), in seedlings produced from P25 *B*. *oleracea* (cabbage) seeds subjected to soaking in deionised water (DW), glycerol, reduced glutathione (GSH) or trolox. Values represent mean ± SD (3 × *n* = 5).

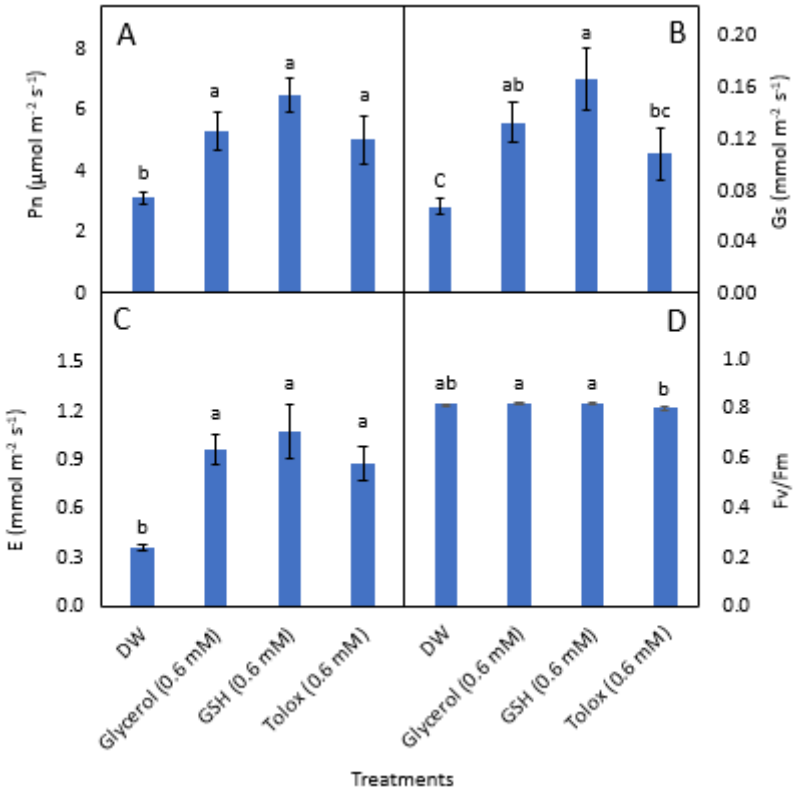

Figure 26 Effect of exogenous application of antioxidants on A) photosynthetic rate (Pn), B) stomatal conductance (Gs), C) transpiration rate (E) and D) chlorophyll fluorescence (F_v/F_m), in seedlings produced from P25 *L. sativa* (lettuce) seeds subjected to soaking in deionised water (DW), glycerol, reduced glutathione (GSH) or trolox. Values represent mean ± SD (3 × *n* = 5). Bars labelled with different letters indicate significant differences at $P < 0.05$ (ANOVA).

In summary, the results of this study reveal that with regards to the pre-treatments effects (in terms of the physiological, biochemical and photochemical markers evaluated), CDd lettuce seeds responded better to both antioxidant and inorganic salt pre-hydration treatments relative to cabbage seeds (Tables 14 and 15). However, the antioxidant pre-hydration treatments were better for both species than pre-treatment with inorganic salt solutions.

Table 14 Summary of the treatments with significant effects of exogenous application of antioxidant solutions on fresh (control) and controlled deteriorated (CDd) cabbage and lettuce seeds

Species		Normal seedling production	Seedling vigour index	Electrolyte conductivity	Lipid peroxidation products	Protein carbonylation	Antioxidant enzymes activities	Germination enzymes activities	Seedling growth	Gas exchange	Photochemistry
Brassica oleracea (Cabbage)	Control	NS	Enhanced by GA, glycerol and trolox	-	-	-	-	-	-	-	-
	P75	NS	Enhanced by trolox	-	-	-	-	-	-	-	-
	P25	Enhanced by GA, glycerol, GSH and trolox	Enhanced by glycerol	Enhanced by DW, GA and trolox	Glycerol enhanced 4HNE	Reduced by all pre-treatment solutions	GA and GSH enhanced CAT; GSH enhanced GR while DW reduced SOD	Glycerol enhanced α-amylase; β-1,3-glucanase enhanced by all antioxidants but reduced by DW	Glycerol enhanced SVI and leaf area; GSH enhanced SVI	NS	NS
Lactuca sativa (Lettuce)	P50	Enhanced by AA, GA, glycerol and GSH	Enhanced by GA and trolox	Reduced by AA, GA, glycerol and GSH	All pre-treatment solutions reduced CJD; Glycerol enhanced 4HNE; AA and GSH reduced 4HNE	Reduced by GA and GSH	CAT enhanced by glycerol, but reduced by DW; all pre-treatment solutions enhanced GR; GSH enhanced SOD	β-1,3-glucanase enhanced by all pre-treatment solutions	-	-	-
	P25	Enhanced by AA, glycerol, GSH and trolox	Enhanced by GSH and trolox	Reduced by AA, glycerol, GSH and trolox	All pre-treatment solutions reduced CJD; Glycerol enhanced 4HNE; all antioxidants reduced 4HNE	Reduced by AA, glycerol and GSH	CAT enhanced by GSH, but reduced by DW; all pre-treatment solutions enhanced GR and SOD	GSH and trolox enhanced α-amylase; β-1,3-glucanase enhanced by all pre-treatment solutions	Glycerol enhanced SVI, shoot dry weight and leaf area	Glycerol, GSH and trolox enhanced photosynthetic rate and transpiration rate; glycerol and GSH enhanced Stomatal conductance	NS

Summarised significant effects of exogenously applied antioxidants (ascorbic acid, AA; gallic acid, GA; glycerol, reduced glutathione, GSH; trolox) and deionised water, DW (control) on CDd seeds of *B. oleracea* (cabbage) and *L. sativa* (lettuce) in terms of the physiological, biochemical and

photochemical markers. Seedling vigour index, SVI; Conjugated dienes, CJD; 4-hydroxy-2-nonenal, 4-HNE; catalase, CAT; glutathione reductase, GR; superoxide dismutase, SOD. NS: not significantly different; -: not measured.

Table 15 Summary of the treatments with significant effects of the application of inorganic salt solutions on fresh (control) and controlled deteriorated cabbage and lettuce seeds

Species		Normal seedling production	Seedling vigour index	Electrolyte leakage	Lipid peroxidation products	Protein carbonylation	Antioxidant enzymes activities	Germination enzymes activities
Brassica oleracea (Cabbage)	P50	Reduced by $CaCl_2$ and $CaCl_2$ CW	Enhanced by CaMg CW (6.5) and NaCl CW (6.5)	-	-	-	-	-
	P25	NS	Enhanced by CaMg, NaCl CW and NaCl CW (6.5)	-	-	-	-	-
Lactuca sativa (Lettuce)	P50	Enhanced by $CaCl_2$ CW, CaMg and CaMg CW (6.5)	Enhanced by $CaCl_2$, $CaCl_2$ CW, CaMg, CaMg CW (6.5), $MgCl_2$ CW, NaCl CW and NaCl CW (6.5)	Enhanced by CaMg CW (6.5)	$CaCl_2$ CW and CaMg CW (6.5) reduced CJD; 4-HNE was reduced by CaMg CW (6.5) but enhanced by DW	NS	DW reduced CAT; all pre-treatment solutions enhanced GR	All pre-treatment solutions enhanced β-1,3-glucanase
	P25	Enhanced by $CaCl_2$ CW, CaMg, $MgCl_2$ CW, NaCl CW and NaCl CW (6.5)	Enhanced by $CaCl_2$ CW, NaCl CW and NaCl CW (6.5)	NS	All pre-treatment solutions reduced CJD; all inorganic salt solutions reduced 4-HNE	Reduced by CaMg and NaCl CW (6.5)	DW, $CaCl_2$ CW, CaMg and $MgCl_2$ CW Reduced CAT; CaMg, NaCl CW and NaCl CW (6.5) enhanced GR; all pre-treatment solutions enhanced SOD	All pre-treatment solutions enhanced β-1,3-glucanase

Summarised significant effects of inorganic salt solutions on CDd seeds of *B. oleracea* (cabbage) and *L. sativa* (lettuce) in terms of the physiological and biochemical markers of oxidative stress and germinability. Deionised water, DW (control); cathodic water, CW; cathodic water adjusted to pH 6.5, CW (6.5); Seedling vigour index, SVI; Conjugated dienes, CJD; 4-hydroxy-2-nonenal, 4-HNE; catalase, CAT; glutathione reductase, GR; superoxide dismutase, SOD. NS: not significantly different; -: not measured.

CHAPTER 5: DISCUSSION

Over the last few decades, many studies have attempted to understand the physiological, biochemical and molecular mechanisms underlying seed ageing in several crop species (Golovina et al., 1997a; Murthy et al., 2003; Boniecka et al., 2019). These attempts have become necessary considering the ongoing need to improve seed storage systems and methods to extend storage longevity and improve the field performance of stored seed. These efforts and an increased worldwide focus on *ex situ* conservation of plant germplasm (Pence et al., 2020; Hay and Sershen, 2021) have become even more important in the face of the growing global food demand and threats to food security posed by climate change-induced loss of crop biodiversity (Dash et al., 2020; Fatima et al., 2020; Tariq and Rashid, 2020).

Given that seed ageing occurs over extremely protracted periods of time in many domesticated species, a number of studies have employed controlled deterioration (CD) to investigate the phenomenon of ageing. These studies collectively showed that even when moisture level and temperature are controlled for, the rate of vigour and viability loss varies across species (Berjak and Villiers, 1972; Merritt et al., 2003; Shaban, 2013). It is clear that this variation is a function of the ability of seeds of different species to resist degradative changes through protective mechanisms to varying degrees (Shaban, 2013). However, there is a need for a more fundamental understanding of these mechanisms.

5.1 Differences in rates and patterns of ageing in cabbage and lettuce seeds subjected to controlled deterioration

As seeds deteriorate over time, two distinct phases are observable: the ageing resistance phase, during which high and reasonably steady germination is recorded, and the ageing susceptibility phase marked by rapid loss of ageing resistance and viability (Xu et al., 2020). In the present study, cabbage seeds subjected to CD had a short (2 days) ageing resistance (asymptotic) phase and extended period (28 days) of deterioration ending in total viability loss (Fig. 2A,B). However, though this rate of deterioration (in terms of probit/day for P75, P50 and P25) was significantly slower than that observed for controlled deteriorated lettuce seeds (Table 3), lettuce seeds had a more prolonged (9 days) ageing resistance phase and exhibited a much shorter time (19 days) to reach total viability loss (Fig. 2A,B). These results indicate differences in the rate of seed deterioration between both species when

subjected to CD; perhaps, due to differences in the type and degree of damage incurred.

The ageing pattern observed here is typical of high vigour seeds with initial high ageing resistance during cold storage (Ellis et al., 2018), high temperature (Walters et al., 2020) or accelerated ageing experimental conditions (Butler et al., 2009) in which a plateau at the early phase of a survival curve is recognisable. Whilst high vigour seeds can maintain high germination percentage for decades or even more under low-temperature storage in gene banks, making their deterioration (in terms of loss vigour/ageing resistance) hard to assess, seed deterioration progresses at varying rates towards eventual loss of viability. Once seeds with high viability become susceptible to ageing, they tend to suffer a loss of viability quickly afterwards, owing to the accumulation of ROS (Xu et al., 2020). The reports of previous studies have suggested differences in the rate of seed deterioration in storage across species and among even cultivars of related species (Tang et al., 1999; Jatoi et al., 2001) and seed lots within a species (Ellis and Roberts, 1980) in relation to their condition of storage. For instance, Walters et al. (2005) reported variations in the rate of seed deterioration in terms of viability loss in about 276 species and 207 cultivars from 42 species, including lettuce under cold storage in gene banks. Saxena et al. (1987) reported differences in the rate of vegetable seed deterioration among species in the Brassicaceae family, including cabbage stored under room temperature. Lee et al. (2013) reported variations in the rate of viability loss in 42 plant species stored for 10 years in “midterm storage (4°C, 30-40% RH)”. They imputed the variations in seed deterioration rate to the chemical and genetic make-up which outfitted some species for prolonged storability than the others under the same storage conditions.

Seed ability to resist deterioration is thought to be controlled in some ways by several genes distributed all through the genome and is greatly affected by environmental factors (Kochanek et al., 2010). These environmental factors include but are not limited to post-harvest processing (Hay et al., 2006) and storage environment, particularly, temperature and relative humidity (Roberts and Ellis, 1989; Walters, 1998), and seed moisture content (Merritt et al., 2003), which influences deterioration rate under air-dry storage condition (Ellis et al., 1990; Copeland and McDonald, 1999). Seeds suffer a rapid loss of ageing resistance with increasing seed moisture level and storage temperature (Simon, 1974; Ellis et al., 1990; Ellis et al., 1991; Ellis et al., 1995). Other factors, such as parental environment (Daws et al., 2004), harvest timing (Wang et al., 2008), the physical state and physiological condition of seeds

(Copeland and McDonald, 1999; TeKrony, 2003) have also been reported to influence seed deterioration. The variation in seed deterioration rate may or may not be due to differences in ageing mechanisms; where the former is the case, the effects of invigorative interventions could also differ.

5.2 Effects of exogenous application of antioxidants on vigour, viability, oxidative metabolism and germination enzymes in aged cabbage and lettuce seeds

In the present study, CD led to the loss of vigour and viability in both cabbage and lettuce seeds; however, this decline occurred at different rates in both species. This was accompanied by inter-species differences in physiological and biochemical markers of oxidative stress and germinability. Below P75, viability declined slower in cabbage seeds than in lettuce seeds. With CD, SVI in cabbage seeds was less severely compromised than in lettuce seeds (Tables 7 and 8, respectively). Moreover, there was no significant change in CJD and 4-HNE levels and SOD activity in cabbage seeds but in lettuce seeds (Table 9). However, even though cabbage seeds exhibited lower levels of viability loss and oxidative stress when deteriorated, they produced much more abnormal seedlings than lettuce seeds (Table 10). These differences suggest that the mechanisms of seed deterioration in both species may not be the same. While CD led to oxidative stress in lettuce seeds, it appears to affect developmental processes in cabbage seeds. Nevertheless, CD increased the frequency of abnormal seedling (AS) production in both species, and this occurred in the soaking treatments as well (Table 10). A rising proportion of AS production in seed lots that are still viable and capable of producing a high germination capacity is a symptom of deterioration (Matthews, 1985). A previous study on the effect of exogenous application of antioxidants prior to the ageing of sunflower seed showed an increased number of AS (Draganić and Lekić, 2012). A gradual loss of seed vigour resulting in the eventual loss of viability has been reported to characterise seed deterioration (Argerich and Bradford, 1989; Lehner et al., 2008). This may be attributed to various factors, including deterioration-induced cellular, chemical and metabolic modifications (Bailly et al., 2008; Boniecka et al., 2019). Uncontrolled reactive oxygen species (ROS) accumulation during hydration of aged seeds inhibits radicle protrusion (germination) or causes seeds to develop into AS (Bailly et al., 2008). El-Maarouf-Bouteau et al. (2011) proposed the involvement of programmed cell death, mitochondria dysfunction, DNA modification and oxidative burst in seed deterioration.

Importantly, in the present study, selected (beneficial) soaking treatments improved normal seedling production of cabbage and lettuce seeds but not necessarily at the same concentrations and CD levels. Glycerol, GSH and trolox, for instance, increased normal seedling production in both species at P25 while AA, glycerol and GSH increased normal seedling production at P50 and P25 in lettuce seeds (Tables 5 and 6). In lettuce, 0.6 mM of all exogenous antioxidants significantly increased normal seedling production but not necessarily at the same CD levels. Additionally, GA was effective in both species but at different concentrations and CD levels. In previous studies, exogenous application of antioxidants such as AA enhanced viability in aged seeds of *Elymus sibiricus* (Yan et al., 2016) and maximum growth and yield of salt-stressed *Ablemoschus esculentus* seeds (Raza et al., 2013). Increased level of endogenous glycerol through seed soaking treatments is speculated to have a protective effect on plants in extreme environmental conditions (Ali et al., 2008; Tiryaki and Buyukcingil, 2009). The improvement of normal seedling production induced by the beneficial soaking treatments identified here may be related to the suggested role of exogenous antioxidants in stimulating and activating plant endogenous antioxidant contents, which are conjectured to be contingent upon NADPH (reductant) given off as carbon flux product via the pentose phosphate pathway (Burguieres et al., 2007). Under stress conditions, the exogenously supplied antioxidant possibly function in the indirect stimulation of the biosynthesis of growth-promoting substances such as amino acids (e.g., proline) associated with pentose phosphate pathway activity, which is a source of precursors of sugar phosphate needed for the synthesis of phenolics (Burguieres et al., 2007). Phenolics are known for their inherent potent antioxidant properties (Ghasemzadeh and Ghasemzadeh, 2011).

The beneficial soaking treatments also improved SVI of cabbage and lettuce seeds but not necessarily at the same concentrations and CD levels (Table 7 and 8, respectively). The application of GA and trolox, for instance, improved SVI in both species but at varying concentrations and different CD levels. Glycerol improved SVI in Fresh and P25 cabbage seeds, while AA and GSH improved SVI in P25 lettuce seeds only. The effect of the beneficial soaking treatments on SVI was, however, not observed in P50 cabbage seeds and fresh and P75 lettuce seeds. The effects of antioxidants on plant growth extend to several physiological processes, which include the regulation of plant growth, cell differentiation and metabolism (Shao et al., 2008). Havas (1935) reported accelerated seedling growth and improved shoot

and root length of wheat as a result of AA application. Draganić and Lekić (2012) reported that application of exogenous antioxidants such as AA, glutathione, trolox and their combination favoured shoot and root growth of normal sunflower seedlings, while antioxidants, especially AA, have also been reported to stimulate shoot development in *in vitro* systems (Gupta and Datta, 2004). Ascorbic acid-linked improvement of growth was correlated with meristematic activity in shoots of *Pisum sativum* (Noctor, 2006). Exogenous antioxidants-linked improvement of seedling growth response may be related to the role of antioxidants in the modification of processes, including cell division and elongation (Shao et al., 2008). They may be stimulating the synthesis of endogenous antioxidants (Burguieres et al., 2007), which in influencing plant growth regulators, modulate growth (Pastori et al., 2003).

The present study demonstrates that CD led to the loss of membrane integrity, as measured by EC, in cabbage and lettuce seeds at P50 and P25 (Table 9). Although the application of exogenous antioxidants has been reported to extenuate the injurious effect of stress in several species (Ahmad et al., 2012; Ejaz et al., 2012; Raza et al., 2013), the beneficial soaking treatments (as applied in this study) did not reduce EC in P25 cabbage seeds (Fig. 4A). In lettuce seeds, however, the beneficial soaking treatments (0.6 mM of AA, GA, glycerol and GSH in P50; 0.2 mM of AA, 0.6 mM of glycerol, GSH and trolox in P25) resulted in a marked reduction in EC (Figs 5A and 6A, respectively). Seed deterioration has been previously reported to be characterised by increased leakage of electrolyte (Mira et al., 2011). Stress-induced modifications such as lipid peroxidation and the disappearance of membrane phospholipids from plant tissues to a level that they are no longer adequate for the fabrication of entire cell membranes make plant tissues leaky (Simon, 1974). The free movement of solutes and water, an indicator of cell membrane permeability, resulting from such modification has been attributed to seed deterioration (Simon, 1974; Mira et al., 2011) in terms of vigour and viability (Bedi et al., 2006; Sahu et al., 2017). Reduction of EC levels by the beneficial soaking treatments is indicative of their potentiality for promoting the retention of membrane integrity during CD in lettuce seeds. This again points to the potential disparity in the mechanisms of ageing in both species. For leakage of solutes to be suppressed, membranes need to regain their integrity of bilayer conformation (Simon, 1974). Chain-breaking antioxidants, particularly AA, tocopherols, and GSH can directly “repair” ROS attack

on lipid structures, thereby defending against membrane injury (Buettner, 1993); this may have been the case in deteriorated lettuce seeds here.

Seed deterioration has been previously reported to be characterised by the accumulation of lipid peroxidation products in various species (*Acer Platanoides*, Pukacka, 1991; sunflower seeds, Kibinza et al., 2006; *Zygophyllum xanthoxylon*, Li et al., 2008). On the contrary, other studies have shown that lipid peroxidation, as measured by its products, was not related to seed deterioration in *Zea mays* (Lin and Pearce, 1990), wheat grains (Lehner et al., 2008), and Brassicaceae species (Mira et al., 2011). As alluded to earlier, CD led to little or no accumulation of lipid peroxidation products (CJD and 4-HNE) in cabbage seeds, while controlled deteriorated (CDd) lettuce seeds exhibited heightened levels of CJD and 4-HNE (Table 9). In P50 and P25 lettuce seeds, CJD levels were reduced by all beneficial pre-hydration treatments (Figs 5B and 6B, respectively), but this was not the case for 4-HNE (Fig. 5C) as it was only in P25 lettuce seeds that 4-HNE levels were reduced by the exogenous antioxidants (Fig. 6C). Mira et al. (2011) showed that though electrolyte conductivity increased with seed deterioration, no accumulation of malondialdehyde (MDA) was recorded in ageing processes in four wild Brassicaceae species. The present results indicate that there are uncertainties about what mechanism(s) of membrane damage occur in deteriorated cabbage seeds as ageing was not accompanied by the accumulation of lipid peroxides despite the increase in electrolyte leakage; other researchers have made similar observations (Golovina, 2019, pers. comm.[1]). Additionally, Lehner et al. (2008) reported that there was no change in MDA during ageing in wheat grains and thus opined that lipid peroxidation was not necessary for ageing to occur. The beneficial soaking treatments most likely reduced lipid peroxidation events in P50 and P25 lettuce seeds by scavenging ROS and/or enhancing the antioxidant defence system, leading to improved normal seedling production and SVI. Antioxidants such as tocopherol, a primary lipophilic antioxidant, and AA, a hydrophilic antioxidant, work together to defend lipids from peroxidative reactions (Buettner, 1993). These antioxidants were also beneficial in lettuce seeds.

Increased PC is an indication of oxidative damage to protein and did characterise CD in P50 and P25 cabbage seeds and P25 lettuce seeds (Table 9). All beneficial soaking treatments were effective in reducing PC levels in P25 cabbage seeds (Fig. 4D). In both cabbage and lettuce seeds, GSH (0.6 mM) reduced PC formation, while DW was effective at

[1] Dr E.A. Golovina, Laboratory of Biophysics, Wageningen University, Droevendaalsesteeg 1, 6708 PB, Wageningen, The Netherlands

doing this in P25 cabbage seeds only. In P50 lettuce seeds, 0.6 mM GA was also effective at reducing PC level, while 0.2 mM AA and 0.6 mM of glycerol also brought about a marked reduction in the carbonyl derivatives in P25 lettuce seeds (Figs 5D and 6D, respectively). In a study on *Arabidopsis thaliana* seeds, CD substantially raised PC levels (Rajjou et al., 2008), and those authors opined that this might cause loss of functional properties of proteins and enzymes or heightened seed susceptibility to proteolysis. They suggested further that loss of germinative vigour may be attributed to changes in seed protein and the inability of deteriorated seeds to display a normal proteome while germinating. Kalemba and Pukacka (2014), Sahu et al. (2017) and Yin et al. (2017) similarly implicated carbonylation of proteins in oxidative stress damaged seeds of *Fagus sylvatica*, *Pongamia pinnata* and *Oryza sativa,* respectively. On the contrary, Golovina et al. (1997a,b) reported the conservation of proteins during long term seed storage of several other orthodo*x* species, including *Brassica napus*. From the results presented here, loss of seed viability and vigour with CD in both species was related to increased protein oxidation, and the benefits of soaking treatments appear to have been partly based on the mitigation thereof. Proteins may be a major ROS target due to their rapid reactivity with several reactive oxidants, in addition to their presence in large quantity in cells, plasma, and nearly all tissues (Hawkins et al., 2009). With protein being the main seed reserve used after the protrusion of radicle in cabbage (Still, 1999), its oxidative modification can severely impact developmental and post germinative events.

An efficient defence system capable of quelling high levels of reactive oxidants is essential for seed survival and recovery from deterioration. The results of the present work show that CD was accompanied by a progressive decline in the antioxidant capacity (specifically catalase [CAT] activity at P50 and P25) in both cabbage and lettuce seeds (Table 9). The activity of CAT was, however, increased by GSH (0.6 mM) in both species at P25 (Figs 7A and 9A), while GA (0.2 mM) and glycerol (0.6 mM) induced a substantial rise in CAT activity in P25 cabbage (Fig. 7A) and P50 lettuce (Fig. 8A) seeds, respectively. A decline in CAT activity was reported for *Sesamum indicum* seeds (Tabatabaei, 2013) and was cited as one of the reasons for the loss of viability in aged *Triticum aestivum* grains (Lehner et al., 2008) and *Helianthus annuus* seeds (Kibinza et al., 2006). Possible production of cyanide, an effective inhibitor of the synthesis of mitochondrial ascorbate in plants, during seed deterioration can compromise the activities of hemeproteins like catalases and peroxidases, thereby

constraining defence against ROS attack (Rajjou et al., 2008). Moreover, CAT has been described as a major enzyme involved in the recovery of seeds from deterioration (Kibinza et al., 2011). Like peroxidase (POX), CAT is mainly involved in the hydrolysis of hydrogen peroxide (H_2O_2), a critically toxic by-product of oxidative metabolism, to water and oxygen (Kibinza et al., 2011; Sahu et al., 2017).

Controlled deterioration also led to a significant reduction in GR activity in P50 and P25 cabbage seeds and in P75, P50 and P25 lettuce seeds (Table 9). In P25 cabbage seeds, only GSH (0.6 mM) caused a marked rise in GR activity (Fig. 7B), while all beneficial pre-hydration treatments enhanced GR activity in P50 and P25 lettuce seeds (Figs 8B and 9B, respectively). Previous studies have documented that seed deterioration was characterised by reduced GR activity in *Citrullus vulgaris* (Hsu and Sung, 1997), *Gossypium hirsutum* (Goel and Sheoran, 2003) and *Helianthus annuus* (Kibinza et al., 2006) seeds. The role of GR includes regeneration of GSH, a substance that itself is involved in removing ROS, from oxidized glutathione (GSSG) to maintain the redox status of glutathione (Kibinza et al., 2011). The GSH protects -SH groups in enzymes and structural proteins against oxidation by either scavenging oxidants or mending -SH groups through GSH-disulfide exchange reaction. The GSSG produced is then rapidly reduced by GR (Esterbauer and Grill, 1978). Heightening endogenous antioxidants levels and enhancing the activity of antioxidative enzymes, such as GR, may be plants strategy of limiting harmful peroxidation (Schmidt and Kunert, 1986).

Controlled deterioration did not influence SOD activity of cabbage seeds but caused a significant reduction in the enzyme's activity in P25 lettuce seeds (Table 9). In P25 cabbage seeds, SOD activity was not enhanced by the soaking treatments (Fig. 7C) but was enhanced by GSH (0.6 mM) in P50 lettuce seeds (Fig. 8C) and by all beneficial soaking treatments in P25 lettuce seeds (Fig. 9C). Ageing-induced loss of seed viability has been demonstrated to involve reduced SOD activity in several plant species, including *Gossypium hirsutum* (Goel and Sheoran, 2003), *H. annuus* (Kibinza et al., 2006) and *T. aestivum* (Lehner et al., 2008). SOD is known to be directly responsible for the dismutation of superoxide anion radical ($\cdot O_2^-$) by removing oxygen radicals and forming H_2O_2 (Saed-Moucheshi et al., 2014). It is able to act as a preventive and chain-breaking antioxidant (Buettner, 1993). Furthermore, the longevity of seeds in storage is dependent on their ability to produce and employ antioxidative enzymes for the scavenging of surplus reactive oxidants (Sahu et al., 2017). The reduced enzymes

activities in seeds decrease the respiratory capacity, which in turn decreases the assimilates and supply of energy (ATP). Therefore, various alterations in the macromolecular structure of enzymes may add up to their lowered viability (Shaban, 2013). Since enzymes are involved in the advancement of seed deterioration, shifts in their activity can be indicative of loss of quality (Shaban, 2013). Enhanced activity of CAT in *Ablemoschus esculentus* (Raza et al., 2013), GR and SOD in *H. annuus* (Bailly et al., 1998; Bailly et al., 2000; Kibinza et al., 2011), CAT and SOD in *P. sativum* (Burguieres et al., 2007) were linked with the recovery of seed vigour. The defensive capacity of antioxidative enzymes including CAT, GR and SOD against cell membrane injury (Tyiso, 2003; Bailly, 2004) and biomolecules oxidation (Job et al., 2005; Varghese and Naithani, 2008; Sahu et al., 2017) are widely reported. Collectively, the present results show that certain exogenously applied antioxidants can enhance the activities of endogenous enzymic antioxidants and subsequent seedling production and SVI, but the effects appear to be enzyme and treatment specific.

Controlled deterioration led to a reduction in the α-amylase activity of cabbage and lettuce seeds at P25 (Table 9). Glycerol (0.4 mM) induced hydrolysis of starch by increasing α-amylase activity in P25 cabbage (Fig. 10A). The activity of α-amylase was not influenced by the soaking treatments in P50 lettuce seeds (Fig. 11A) but was heightened by GSH (0.6 mM) and trolox (0.6 mM) in P25 lettuce seeds (Fig. 12A). Reduced amylase activity was reported in aged wheat grains (Livesley and Bray, 1991; Das and Sen-Mandi, 1992) and *Brassica campestris* (Bedi et al., 2006). Loss of viability may be linked to their inability to adequately produce α-amylase, which in turn is presumed to be due to ageing-induced inadequacy of plant growth regulators such as gibberellins (Petruzzelli and Taranto, 1990) and other ageing-related impairments (Ganguli and Sen-Mandi, 1993). Additionally, CD resulted in a significant decline in β-1,3-glucanase activity of P50 and P25 cabbage and P25 lettuce (Table 9). The exogenous antioxidants enhanced β-1,3-glucannase activity in P25 cabbage seeds (Fig. 10B) while all beneficial soaking treatments enhanced its activity in both P50 and P25 lettuce seeds (Figs 11B and 12B, respectively). Increased β-1,3-glucanase activity related to increased seed germination of *Origanum vulgare* (Farashah et al., 2011). The present results (summarised in Table 14) suggest that treatment of aged cabbage and lettuce seeds with antioxidants can enhance seedling production and SVI by promoting the activity of key germination enzymes.

5.3 Effects of inorganic salt solutions on vigour, viability, oxidative metabolism and germination enzymes in aged cabbage and lettuce seeds

Apart from antioxidant treatments, studies on the invigoration of debilitated orthodox seeds have employed various seed preconditioning treatments, including organic (Brocklehurst et al., 1987; Sivritepe and Sivritepe, 2008) and inorganic (Taylor et al., 1988; Poonguzhali, 2016) hydration agents and exposure to a magnetic field (Abdul-Baki and Anderson, 1973) and ionising radiations (Macovei et al., 2014). Where preconditioning treatments allow deteriorated seeds partially to regain vigour, the proportion of AS produced consequently reduces (Rao et al., 1987). In the present study, the significant reduction in the proportion of AS produced in cabbage seeds treated with inorganic salt (electrolysed and non-electrolysed) solutions relative to DW-treated CD seeds (Table 10) was mainly accompanied by a significant increase in mortality (Table A1; Appendix A) and no significant increase in normal seedling production relative to DW-treated seeds (Table 11). Abnormal seedling production and delayed germination resulting from loss of vigour are expressions of advanced seed deterioration. Carrozzi et al. (2012) reported that AS production was not changed compared with control in one year aged lettuce (*L*. *sativa*) seeds hydrated with an inorganic salt ($MgSO_4$) solution. Tarquis and Bradford (1992) also reported increased AS production in *L*. *sativa* seeds pre-hydrated beyond 1 hour after CD. In the present study, the treatment of CDd lettuce seeds with the solutions of inorganic ions ($CaCl_2$ CW, CaMg, CaMg CW [6.5], $MgCl_2$ CW, NaCl CW and NaCl CW [6.5]) improved normal seedling production significantly (Table 12). Comparison with the results obtained for aged cabbage seeds with the same treatments suggests a species-specific effect of these inorganic ions on post CD invigoration. These species-specific effects of specific inorganic salt solutions might be based on differences in the nature of the main oxidants and oxidant targets at the cellular level. According to Hawkins et al. (2009), the nature of the predominant reactive oxidant has a substantial role to play in determining the level of impairment inflicted on their targets. Additionally, the potency of seed preconditioning agents on germination and seedling growth has been said to vary with species and the type of stress imposed (Kaczmarek et al., 2017).

However, it is worth noting that in CDd lettuce seeds, there were also differences in the effects of inorganic salt treatments between CD levels: CaMg CW (6.5) treatment improved normal seedling production at P50 and not in P25; $MgCl_2$ CW, NaCl CW and NaCl

CW (6.5) were significantly effective at P25 only (Table 12). On the other hand, $CaCl_2$ CW and CaMg application improved normal seedling production relative to DW soaking at both P50 and P25 in lettuce. In a previous study, Gondwe et al. (2016) reported that preconditioning of seeds with electrolysed and non-electrolysed solutions of CaMg improved germination in seeds of *Lycopersicon esculentum, Pisum sativum* and *Cucurbita maxima* after storage for four months at 5 °C. They attributed the beneficial effects to the strong reductive property of the electrolysed salt solution on ROS. In the present study, the enhancement of normal seedling production of lettuce seeds by the Ca containing solutions might be related to the supplementation of this divalent cation, a key second messenger that has been implicated in several oxidative stress alleviation responses (Gong et al., 1998; Larkindale and Knight, 2002; Zhao and Tan, 2005), and/or the antioxidative potentials of CW (Hanaoka, 2001; Gondwe et al., 2016) given that all solutions that showed a significant effect were CW solutions except where Ca was a component of the non-electrolysed inorganic salt solution. Whilst the ameliorative antioxidant effects of CW (generated using 2 mM NaCl solution) have been suggested to be related to the increased ionic product of the solvent water (Hanaoka et al., 2004), the present study also underscores the relevance of the nature of the ionised solutes used to prepare the CW. Moreover, the mechanisms of action of the hydration treatments, perhaps, involves the fixing of ageing-induced oxidative stress alterations of ion channel activity (Demidchik, 2010), allowing for improved cellular functioning. The ion channel activity mentioned in the homeostatic modulation of the cellular ion channel is necessary to regulate cellular ion metabolic equilibrium that impacts plant responses (adaptation to and dealing with) to various stress (abiotic and biotic) factors at the cellular level (Demidchik, 2010). Extensive oxidative damage to receptors, transport proteins and ion channels can cause impaired cellular function (Bailly, 2004; Gill and Tuteja, 2010). It is becoming evident that reactive oxidants can trigger Ca^{2+}- and K^+-permeable channels in cell membranes leading to the rise in cytosolic Ca^{2+} (Foreman et al., 2003) and leakage of K^+ (Demidchik et al., 2003) from the cell, respectively. As a second messenger known to be involved in several signalling responses (Sanders et al., 1999; Larkindale and Knight, 2002; Demidchik and Maathuis, 2010), the productivity and stress tolerance (abiotic and biotic) capacity of plants are influenced by their Ca^{2+} status (Kaczmarek et al., 2017), while oxidative stress-induced leakage of K^+ can stimulate programmed cell death (Remillard and Yuan, 2004; Demidchik et al., 2014). How

the hydration treatment employed in the present study affects Ca^{2+} and K^{+} status in cells post CD should form part of future studies.

Unlike the normal seedling production, which was only improved in CDd lettuce seeds, the inorganic salt hydration treatment had a positive effect on SVI in both cabbage and lettuce seeds at P50 and P25 CD levels (Table 13). Post CD treatment with NaCl CW (6.5) showed a significant promotive effect on SVI in both cabbage and lettuce seeds at both P50 and P25. Hydration with CaMg CW (6.5) also significantly improved the SVI in both species after CD to P50, whereas five other inorganic salt solutions ($CaCl_2$, $CaCl_2$ CW, CaMg, $MgCl_2$ CW and NaCl CW) were beneficial in improving SVI relative to the DW-treated lettuce seeds only. At P25, NaCl CW was beneficial in both species, while CaMg and $CaCl_2$ CW were beneficial in exhibiting higher SVI in cabbage and lettuce seeds, respectively. Treatment of aged seeds with $MgSO_4$ solution has been reported to improve vigour in *L. sativa* (Carrozzi et al., 2012), but at the time of this study, there were no previous reports of any of the inorganic ion solutions applied here shown to improve vigour in this species. Again, the beneficial effect recorded here is attributed to the ionic effect of the divalent cations. Gondwe et al. (2016) reported that seed preconditioning with electrolysed and non-electrolysed solutions of CaMg improved SVI in seeds of *L. esculentum, P. sativum* and *C. maxima*. Similarly, Iqbal and Ashraf (2007a) reported that seed preconditioning with $CaCl_2$ solution promoted seedling vigour of *Triticum aestivum* seeds under non-saline and even saline conditions. Other studies have shown the promotive effect of seed pre-hydration on seedling vigour using low water potential osmotica solutions, including NaCl (1 mM) in *Capsicum annuum* seeds subjected to salt stress (Khan et al., 2009), $CaCl_2$ in *Brassica napus* seeds subjected to accelerated ageing (Abdolahi et al., 2012) and $MgCl_2$ (Batool et al., 2015) in *B. oleracea* seeds under standard germination conditions. However, almost all these studies have not gone to the extent of trying to understand the underlying biochemical changes responsible for improved normal seedling production and SVI due to the inorganic salt treatment.

On this note, the present study demonstrated that treatment with inorganic salt solutions significantly influenced certain markers of stress in CDd lettuce seeds. In effect, CaMg CW (6.5) significantly reduced EC levels in P50 lettuce seeds (Fig. 13A), indicating that the inorganic salt hydration treatment had a restorative effect on lettuce seed membranes and reduced the heightened leakage of ions traditionally associated with ageing in seeds.

Inorganic ions have been suggested to be involved in protecting cytoskeleton or cell membrane from injury (Ree and Guerra, 2020) due to their promotive effect on the structure and stability of membrane lipid bilayer (Binder and Zschörnig, 2002; Redondo-Morata et al., 2012; Ree and Guerra, 2020). In addition, cation-enhanced interactions between lipids allow for closeness between lipid molecules, thereby increasing membrane density (Kagawa et al., 2013; Ree and Guerra, 2020). In previous studies, Abdolahi et al. (2012) reported that KH_2PO_4 solution lessened EC in three cultivars (RGS, 'Hyola 401' and 'Pacific') of *Brassica napus* seeds subjected to accelerated ageing for different durations (48 h and 96 h). However, they also showed that $CaCl_2$ solution performed otherwise in the same study, while Sathish and Sundareswaran (2010) found $CaCl_2$, KH_2PO_4 and KNO_3 inorganic salt hydration treatments to have no significant effect on the EC of three genotypes (UMI 61, UMI 285 and COH[M] 5) of aged *Zea mays* seeds. Overall, it seems that seed hydration treatments may have a promotive effect or be ineffective or even detrimental in some species (Tarquis and Bradford, 1992).

The basis of exposing aged seeds to different soaking treatments is generally to enhance recovery from and/or reduce ageing-induced peroxidative changes. Lipid peroxidation products (CJD and 4-HNE) levels were reduced significantly by CaMg CW (6.5) in P50 lettuce seeds, and $CaCl_2$ CW reduced CJD levels in both P50 (Fig. 13B) and P25 (Fig. 14B) in this species. In P25 seeds of the same species, five inorganic salt treatment solutions (viz. $CaCl_2$ CW, CaMg, $MgCl_2$ CW, NaCl CW and NaCl CW [6.5]) significantly reduced CJD and 4-HNE, while DW was only effective in CJD reduction (Fig. 14B,C). Previous studies have reported that seed preconditioning led to the reduction of lipid peroxidation products in different plant organs and under various stress conditions. Chowdhury and Choudhuri (1989), for instance, reported $CaCl_2$ seed pre-hydration to reduce lipid peroxidation (MDA) in water-stressed seeds of *Corchorus capsularis* and *C. olitorius*. Additionally, Khorshidi and Nojavan (2006) reported reduced levels of MDA in the roots and shoots of cold stressed *Zea mays* seedlings produced from seeds pre-treated with $CaCl_2$ solution. Those authors suggested that the inorganic salt hydration treatment enhanced antioxidative enzymes activity, thereby leading to reduced cold stress injury. Seed pre-soaking in water also reduced total peroxide and MDA levels in *Momordica charantia* seeds (Wang et al., 2003). The present study shows that in addition to the strong reductive properties of CW, the inorganic ions in the CW might be improving hydrolytic and antiperoxidative enzyme activities to offset lipid peroxidation effects (Farooq

et al., 2009). Seed quality enhancement by seed hydration treatments has been ascribed to lessened ROS-mediated lipid peroxidation (de Oliveira et al., 2012).

As products of stress-induced lipid peroxidation, certain reactive carbonyl species (α,β-unsaturated ketones and aldehydes) have been implicated in the mediation of ROS signals leading to the modification of proteins (Mano et al., 2019). The resultant oxidation of proteins leads to alterations in protein structural and functional properties (Juszczuk et al., 2008). Specific germination enzymes can also be carbonylated (Bailly et al., 2008; Kalemba and Pukacka, 2014), resulting in loss of seed vigour. In the present study, the levels of the product of oxidative modification of proteins, measured as PC, were lowered significantly by CaMg and NaCl CW (6.5) in CD lettuce P25 seeds only (Fig. 14D). This effect may be ascribed to the direct scavenging of ROS by CW or enhanced activities of endogenous antioxidative enzymes. Though not assessed in the present study, perhaps, the action of the inorganic salt hydration treatment may involve enhancement of injurious oxidant and carbonyl scavengers that constitute the non-enzymatic antioxidant defence system. As reported by Dell'Aquila (1994), ageing stress led to the oxidative degradation of proteins in *Triticum durum* seeds. Oxidised (carbonylated) proteins are targeted for proteolysis (Pyngrope et al., 2013), and if not degraded, can constitute a large molecular weight assemblage, which accrues with age (Kalemba and Pukacka, 2014). The intracellular proteolysis resulting from the oxidation of specific amino acids may be involved in the stress-induced restructuring of plant metabolic process, as some reports have indicated that some species are more prone to proteolytic reactions than others when exposed to stress (Pyngrope et al., 2013).

High antioxidant activity is known to defend plants from oxidative damage accumulated due to oxidative stress, thereby increasing plant survival under stress conditions (Larson, 1995; Kim et al., 2005; Siadat et al., 2012). This is achieved through the activities of free radicals and ROS detoxifying antioxidative enzymes such as CAT, GR, SOD, amongst others, which scavenge oxidants capable of attacking amino acids, proteins and lipids that are essential for cell functioning and integrity (Larson, 1995). Reduced activities of antioxidant enzymes such as CAT, GR, SOD and POX in aged seeds cause lowered seed respiratory competency and energy supply resulting in loss of viability (Demirkaya et al., 2010). The results of the present study show that the post CD hydration treatments did not have any significant effect on CAT activity but enhanced GR and SOD activities in lettuce seeds (Figs

15A–C and 16A–C). More specifically, post CD soaking of seeds with DW and all tested inorganic salt solutions enhanced GR and SOD activities in P50 and P25 lettuce seeds, respectively, while CaMg, NaCl CW and NaCl CW (6.5) improved GR activity in P25 lettuce seeds only. The direct antioxidative properties of CW on ROS and the stimulation of endogenous antioxidant enzymes activities by the inorganic salt hydration treatments may, therefore, have contributed to the improvement in normal seedling production and seedling vigour observed in lettuce. Khorshidi and Nojavan (2006) stated that cations, particularly Ca^{2+}, can enhance the activities of most enzymic antioxidants. At the same time, it is worth mentioning that soaking in water can also result in a rise in antioxidant enzymes, including GR and SOD activities (*Momordica charantia* [Wang et al., 2003]).

Seed germination enzymes are critical in the early growth stages of a germinating seed; most importantly, some of them are responsible for solubilising excess food stored as protein, lipid and starch to release energy for embryo development (Nawaz et al., 2013; Joshi, 2018). During germination, the process of mobilisation of stored food to the embryo is ubiquitous (Nawaz et al., 2013) but can be disturbed when already damaged seeds are exposed to stress conditions during germination, thereby exacerbating the damage. The degree of such disturbance depends on the levels and efficacy of germination associated enzymes involved in chemical reserve hydrolysis. It is envisaged that the hydration of CD seeds with inorganic salt solutions would have enhanced the germination enzymes activities, thereby contributing to organic substances mobilisation to various embryonic regions resulting in better germination and normal seedling establishment (Nawaz et al., 2013). Hence, a rise in germination enzyme activity can result in improved vigour and viability. The present study revealed that the inorganic salt hydration treatments (electrolysed or non-electrolysed) did not influence α-amylase activity (Figs 17A and 18A) significantly; however, all inorganic salt solutions examined as well as DW enhanced β-1,3-glucanase activity in both P50 and P25 lettuce seeds (Figs 17B and 18B, respectively). β-glucanases function in the hydrolysis of β-linked glucans, and their activities can be regulated by abiotic and biotic stresses (Simmons, 1994). As various forms of β-glucans contribute to cell wall composition, the involvement of β-glucanases in processes that might alter cell walls structure and function is reasonable. There are suggestions that they influence cell wall matrix composition and viscoelastic properties which contribute to cell expansion (Simmons, 1994; Brummell et al.,

1997; Kotake et al., 2000). The results of the present study (summarised in Table 15), therefore, suggest that the enhancement of β-1,3-glucanase activity by the hydration treatments may have contributed to the increased production of normal seedlings and vigour of debilitated lettuce seeds.

5.4 Influence of exogenous antioxidant invigoration of aged cabbage and lettuce seeds on subsequent seedling emergence, growth, gas exchange and photochemistry

This section discusses the influence of invigorating aged cabbage and lettuce seeds using exogenous antioxidants on the subsequent seedling emergence, growth and biomass accumulation, gas exchange and photochemistry (Table 14). Previous studies on the effects of exogenous application of antioxidants on seed performance had mostly shown promotive effects such as an improved speed of emergence, seedling growth and/or yield (Farooq et al., 2006b; Ahmad et al., 2012; Shah et al., 2019). For instance, soaking of unaged *Oryza sativa* seeds in antioxidants including trolox (as tocopherol) promoted germination (Yousof et al., 2010), while the use of glycerol as an osmotic treatment enhanced germination rate in unaged *Apium graveolens*, *Allium ampeloprasum* and *Allium cepa*, % seedling emergence in *A. graveolens* seeds, and mean emergence time in all the species (Brocklehurst and Dearman, 1984). It should be noted that in the same study, however, glycerol did lead to a reduction in *Daucus carota* % seedling emergence. The use of exogenously applied antioxidants in the present study not only differs from the above studies in that the antioxidant solutions were exogenously applied to aged cabbage and lettuce seeds, but also the effects on seedling emergence parameters appeared to be neither promotive nor detrimental relative to DW-treated seeds (Figs 19A–D and 20A–D, respectively). While Yousof et al. (2010) showed that seed hydration in tocopherol before sowing did not change mean germination time and time taken to 50% germination in unaged *Oryza sativa* seeds, Draganić and Lekić (2012) reported reduced germination in *Helianthus annuus* seeds subjected to accelerated ageing after hydration treatment with ascorbic acid (AA), reduced glutathione (GSH) and tocopherol.

Despite the lack of effect on seedling emergence parameters in this study, the exogenously applied antioxidants had a significant influence on seedling growth in both species. More specifically, glycerol enhanced SVI in P25 cabbage and lettuce seeds (Figs 21A and 22A, respectively) and enhanced shoot biomass in P25 lettuce seeds (Fig. 22C), while GSH enhanced SVI in P25 cabbage seeds only (Fig. 21A). The promotive effects on SVI and shoot

biomass may be attributed to the improvement of physiological functions during the early stages of development, possibly owing to enhanced antioxidant protection. Previous studies have shown similar promotive effects of several exogenously applied antioxidants on SVI and biomass accumulation. In the study by Roopa et al. (2009), for example, the exogenous application of glycerol to *Pennisetum glaucum* seeds promoted SVI, seedling height and shoot dry weight.

Other examples of the stimulatory effect of exogenously applied antioxidants on seedling growth after seed hydration exist in the literature: *Helianthus annus* shoot length increased when seeds were treated with AA, GSH and tocopherol (Draganić and Lekić, 2012). In another study, *Brassica napus* and *Helianthus annus* root and shoot length and dry weight, and seedling dry weight were increased when seeds were pre-hydrated with AA (Dolatabadian et al., 2008). The authors opined that the promotive effects might be a consequence of the enhancement of cell division and differentiation of meristematic cells. In fact, it has been said that the exogenous application of antioxidants may have diverse effects on several plant metabolic and physiological processes, including germination and uptake and transport of ion (Dolatabadian et al., 2008). Exogenous application of antioxidants has also been shown to promote plant growth in terms of vigour and yield by enhancing nutrient uptake and increasing stress tolerance (Shah et al., 2019), possibly through enhanced antioxidant defence (Ahmad et al., 2012) during the early stages of seedling development when seedlings are quite vulnerable to stress. This not only helps in effective seedling recruitment but also increased yield, which is highly beneficial.

Given that plant leaves are the main organs associated with photosynthesis in a plant, leaf area has a direct effect on plant photosynthetic performance and growth (Weraduwage et al., 2015). Leaf area defines the light-harvesting capacity and is, therefore, a useful index of evaluating plant growth (Gifford et al., 1984; Koester et al., 2014; Weraduwage et al., 2015). In the present study, seed treatment of P25 cabbage and lettuce seeds with certain antioxidants had a significant effect on leaf area in the seedlings subsequently produced by both species: glycerol enhanced leaf area in seedlings produced by both P25 cabbage and lettuce seeds (Figs 23A and 24A, respectively) relative to DW-treated seeds. However, it should be noted that the leaf area ratio and total chlorophyll content were not significantly affected in both species (Figs 23B,C and 24B,D, respectively). Similarly, in a greenhouse and

field study on *Pennisetum glaucum* seeds, the application of glycerol as a pre-hydration treatment led to increased leaf area in the seedlings produced (Roopa et al., 2009). The promotive effect of glycerol, as also observed in the present study, may be linked to the reported stimulatory effects on the activities of certain enzymes involved in photosynthesis, such as phosphoenolpyruvate carboxylase (Vu et al., 1993). Seed pre-hydration treatment with other exogenous antioxidants such as AA has also been suggested to enhance the retention of stay-green traits, eventually leading to more leaf area to capture radiant energy in *Triticum aestivum* (winter wheat) (Shah et al., 2019).

High growth rates are generally accompanied by high photosynthetic efficiency (Ashraf and Harris, 2013; Lubbe et al., 2016). In the present study, the effects of seed pre-hydration treatment with antioxidants did affect gas exchange, but these effects were limited to lettuce. In fact, gas exchange and chlorophyll fluorescence in seedlings produced from P25 cabbage seeds were statistically comparable to seedlings produced from DW-treated seeds (Fig. 25A–D). In contrast, seed pre-hydration treatment of P25 lettuce seeds with glycerol and GSH enhanced Pn, Gs and E in seedlings compared with those produced from seeds treated with DW (Fig. 26A–C). However, Fv/Fm in seedlings was not significantly affected by any antioxidant treatment (Fig. 26D). Pre-hydration treatment of P25 lettuce seeds with trolox also enhanced Pn and E in the seedlings produced. Increased Gs often leads to increased Pn and E (Xu et al., 1997; Jarvis and Davies, 1998; Miyashita et al., 2005; Hayat et al., 2011; Kusumi et al., 2012), but Pn can be enhanced through non-stomatal effects as well (Gong and Chen, 2012), which appears to have been the case in seedlings produced from trolox-treated seeds. In a study on the oxidative stress effects of drought on seeds of two *Triticum aestivum* genotypes, seed pre-hydration with the antioxidant AA, enhanced photosynthetic parameters, including Pn (Malik and Ashraf, 2012). As alluded to earlier, the early seedling development stage is particularly susceptible to oxidative stress (Kumutha et al., 2009), which may affect genome integrity, seed quality, and viability. Though naturally occurring antioxidant molecules should operate as ROS scavengers, in debilitated seeds, these may be irreversibly affected. The provision of antioxidants through seed pre-hydration treatments may bolster antioxidant protection during this stage, thereby mitigating the injurious effects of oxidative stress such as the impairment of chloroplast thylakoid membranes and reaction centres (Miyake and Asada, 1992; Zhang et al., 2003; Dolatabadian et al., 2009). It is known

that photosynthetic processes, even under favourable conditions, is intrinsically accompanied by the generation of high levels of cellular oxidants such as hydrogen peroxides, superoxide radical and singlet oxygen through the functioning of the electron transport chain in the chloroplasts (Zhao and Zou, 2002; Foyer and Noctor, 2003; Foyer and Shigeoka, 2011). An efficient antioxidant defence system, comprising of antioxidative enzymes as well as antioxidants such as GSH, α-tocopherol, etc., is required to prevent toxic concentrations of reactive oxygen species in the chloroplasts (Zhao and Zou, 2002), which could lead to photo-oxidative injury to photosynthetic apparatus and consequently reduced plant growth. The results of the present study, therefore, suggest that the enhancement of photosynthetic rate and gas exchange by the exogenous antioxidant treatments, particularly glycerol, in seedlings from P25 lettuce seeds may have contributed to the improved performance, in terms of shoot growth relative to those of cabbage.

CHAPTER 6: CONCLUDING REMARKS AND RECOMMENDATIONS

6.1 Introduction

There is little doubt that ageing-induced loss of crop seed vigour and viability is a serious threat to food security, particularly in countries where farmers are dependent on seed storage. Seed deterioration during long-term storage also poses a significant threat to germplasm conservation**.** Seed ageing, therefore, represents a challenge for the agri-food sector and seed industry, threatening the world's ability to meet the global food demand. With increasing populations, especially in developing nations, crop production losses owing to poor seed vigour have already resulted in market instability and enormous pressure on the governments. This is projected to worsen when combined with the effects of climate change and human mass migrations (Perch-Nielsen et al., 2008). As a result, the United Nations (UN) in the 2030 Agenda for Sustainable Development places great emphasis on food security, improved nutrition and sustainable agriculture (UN General Assembly, 2015). A lot of resources have also been invested in research on seed storage, priming, and invigoration to increase crop production (Copeland and McDonald, 2001; Fuglie et al., 2013).

In that regard, slowing down the deterioration of seeds and enhancing seed viability and vigour have become crucial for seed preservation (Xu et al., 2020)**,** given the inevitability of seed viability loss even under enhanced storage conditions in gene banks. Seed treatments before storage for enhancing ageing resistance are useful and urgently needed, mainly where long-term storage facilities are not available or seeds are stored using poor and/or ageing infrastructure (Xu et al., 2020). Focused research involving the use of state-of-the-art techniques on seed invigoration, as in the present study, and at both academic and industrial levels can provide an improved understanding of the mechanisms of ageing-induced loss of seed vigour and promising invigorative methods. On this note, the key conclusions of the present research are discussed below in relation to their significance to our current understanding of alleviating the effects of seed ageing and the knowledge gaps that still exist. Furthermore, the shortcomings and salient findings of the study are used to generate a set of recommendations for future studies on seed ageing and seed soaking/priming treatments for reinvigoration of aged seed.

6.2 Concluding remarks

The two species, *Brassica oleracea* L. (cabbage) and *Lactuca sativa* L. (lettuce), investigated in this study, are widely planted around the world due to their economic importance. Their selection was also based on the fact that they are representative of the many orthodox seed crop species stored in formal and informal gene banks for long-term conservation or use in the next planting season.

In reflecting on the results of the various experiments conducted in this study, it was clear that seed ageing resulted in the loss of vigour and viability in both species but at different rates. The differences in seed deterioration rates emerged despite subjecting both species (with similarly high initial viability of > 95%) to the same CD conditions. This suggests inter-species differences in mechanisms of ageing, which might be the case for other orthodox crop species. Understanding the differences in seed deterioration rates and storability is vital for effective management of seed collections and conservation as it is useful in the selection of intervals for viability retesting and thus recollection or regeneration strategies (Probert et al., 2009; Lee et al., 2013).

This study revealed that CD increased the frequency of abnormal seedlings in both cabbage and lettuce seeds. Oxidative stress appears to be a significant contributor to seed viability loss during ageing in both cabbage and lettuce seeds. However, as alluded to above, the data associated with the oxidative stress indicators suggest that the mechanisms of seed deterioration differed between the two species. For example, CD was accompanied by a rise in electrolyte conductivity (EC) and protein carbonylation (PC), and a decline in catalase (CAT), glutathione reductase (GR), superoxide dismutase (SOD), α-amylase and β-1,3-glucanase activities in both species, while a rise in lipid peroxidation products investigated (conjugated dienes [CJD] and 4-hydroxy-2-nonenal [4-HNE]) was observed in lettuce seeds only. These results are in line with the views of Golovina (2019, pers. comm.[1]) as well as the reports by Mira et al. (2010) and Mira et al. (2011), which indicate that seed deterioration is accompanied by lipid peroxidation in lettuce seeds, but not in *Brassica* seeds.

Invigoration attempts for the controlled deteriorated (CDd) seeds indicate that the use of exogenous antioxidative compounds, as applied in the present study, could be an effective ameliorative approach for debilitated cabbage and lettuce seeds and perhaps orthodox crop seeds in general. Notably, out of the five antioxidants (ascorbic acid [AA], gallic

acid [GA], glycerol, reduced glutathione [GSH] and trolox) applied to seeds, four (GA, glycerol, GSH and trolox) were beneficial in cabbage, and all five in lettuce, in terms of improving viability in CDd seeds. Depending on plant species, the mechanisms behind the beneficial effects of these antioxidants appear to be based on triggering pre-germinative metabolic events during early seed hydration phase and inducing tissue repair responses (e.g., activation of antioxidant mechanisms), necessary to maintain genome integrity and ensure proper germination, seedling growth and development. In general, the results suggest that treatment with exogenously applied antioxidants can have wide applicability by seed scientists on commercial seed lots for the enhancement of seed vigour and germination capacity in aged seeds and possibly oxidative stress tolerance in seeds subjected to harsh environmental factors. Additionally, it will be useful to seed bank technologists in need of improved protocols for the recovery of stored germplasm. It is envisaged that the promising results of this study will motivate other scientists to try and implement these pre-hydration treatments for other debilitated orthodox seeds.

The promotive effects of the experimental approach involving the application of electrolysed (cathodic water; CW) and non-electrolysed inorganic salt pre-hydration treatments on normal seedling production appear to be based on the stimulation of antioxidative and germination-related enzyme activities in lettuce, which in turn enhances energy metabolisms, early mobilisation of stored food and endosperm weakening. At the same time, the promotive effect on seedling vigour could be a functional nutrient (Subbarao et al., 2003; Kronzucker et al., 2013) effect for growth-related events. The results also suggest that the essentiality of calcium ions (Ca^{2+}) in conjunction with the plausible restorative strength of CW contributes to a substantial increase in normal seedling production in lettuce, irrespective of the pH of the treatment solutions or extent of deterioration (for P50 and P25, at least).

Notably, the results suggest that the mechanisms behind the inorganic salt hydration treatment effects are based on the direct reductive power of CW and the stimulation of endogenous antioxidants (particularly, GR and SOD), which in turn mitigated the effects of stress-induced oxidative injury (e.g., reduced EC, lipid peroxidation and PC) in lettuce seeds. Additionally, pre-hydration treatment with specific inorganic ion solutions (and even DW) enhance germination enzyme activities in lettuce seeds.

There could be various other reasons for the influence of the inorganic salt pre-hydration treatments on germination, normal seedling establishment and SVI, especially those with calcium ions. For instance, calcium is well documented as an essential element having several underlying physiological functions in the signalling and structure of the plant. Calcium ion (Ca^{2+}) is regarded as a pervasive second messenger (McAinsh and Pittman, 2009). It has been reported to be involved in the regulation of the flow of water, signalling the regulation of cell physiology and several ensuing reactions to numerous stimuli of plants (McAinsh and Pittman 2009; Dodd et al., 2010; Gilliham et al., 2011). An unequivocal basic function of Ca^{2+} in graviperception has been indicated through the metabolic processes of starch, and in statolith morphology determination, which might be related to maintenance of the actin constituent of statocytes cytoskeleton located in root-cap (Belyavskaya, 1996; Berjak and Mycock, 2004). A rise in the exogenous supply of Ca^{2+} could function in the maintenance of an optimal K^+/Na^+ ratio in the cytosol by regulating the transport of K^+ across the cytoplasmic membrane (Iqbal and Ashraf, 2007b). Other divalent cations, such as Mg^{2+}, have been reported to show similar effects (Shabala et al., 2005; Kaczmarek et al., 2017).

The study revealed the prospect of the various pre-hydration treatments (with antioxidant and inorganic salt solutions) employed in the present study in promoting recovery from ageing-induced oxidative stress and enhancing the performance of seedlings raised from aged seeds of both species. Additionally, it was shown that the approach, particularly those involving exogenous antioxidants application, can be used to alleviate poor stand establishment as demonstrated under greenhouse conditions. In the greenhouse pot trial, seed pre-hydration treatments with antioxidants did not affect seedling emergence capacity or rate significantly; however, these treatments had a positive influence on growth in both species, especially a significantly improved shoot dry weight, gas exchange and CO_2 assimilation rate in lettuce. Importantly, glycerol enhanced seedling vigour and light-harvesting capacity (i.e., leaf area) in each species at their respective concentrations and shoot dry weight in lettuce. Additionally, glycerol and GSH stimulated Pn, Gs and E while trolox promoted Pn and E in lettuce. Reference to the literature suggests that this stimulation of growth and photosynthesis may be related to the protection of photosystems from oxidative stress and/or stimulation of enzymes involved in photosynthesis, possibly by enhancing the antioxidant defence system during the early stages of seedling development

when seedlings are particularly vulnerable to stress. The results suggest that seed pre-hydration treatments may mitigate poor stand establishment brought about by seed ageing.

6.3 Recommendations

Whilst this study tried to overcome the shortcomings of studies of this nature, some limitations could not be overcome. For instance, in the asymptotic phase, the loss of resistance to ageing was hard to recognise owing to lack of a visible change in normal seedling production, even though the reduction in normal seedling production was of significant interest and not difficult to detect. Whether the loss of ageing resistance occurs before recognisable changes in normal seedling production, particularly in lettuce seeds, is worth investigating in greater detail. Monitoring seed ageing resistance, as well as seed vigour and viability, may help to identify early deterioration signs and proper timing for earlier invigorative intervention to improve ageing resistance and prolong storability. Other researchers also recommend that characterising non-lethal injury within seeds may allow for the detection of early deterioration signs and accurate prediction of storability (Walters et al., 2020).

Given that the CD-induced rise in EC in cabbage seeds was not accompanied by lipid peroxidation, the exact causes of membrane impairment leading to solute leakage in cabbage seeds during ageing could not be identified here. Hence, research towards understanding what leads to membrane damage in cabbage during ageing is worth pursuing. Furthermore, investigations on the lesions imposed by ageing at the molecular level (e.g., via protein profiling and assessment of DNA integrity by oxidative DNA damage profiling) and the possible influence of exogenous antioxidants on obviating and/or repairing these injuries are recommended.

Though the present study could not identify the main and all oxidants in cabbage and lettuce seeds that lead to ageing, the study noted that the oxidant(s) and oxidant target(s) in cabbage appear to be different from those in lettuce. By implication, the promotive effects of specific pre-hydration treatments differ between species. Hence, investigations towards understanding the nature of the primary oxidants and oxidant targets in specific species will be useful in determining the most suitable pre-hydration treatment application(s). Such investigations should extend to microscopical analyses, including cytochemical and histochemical localisation of reactive oxidants, crosstalk between plant growth regulators,

reactive oxidants and germination (to prevent the occurrence of temporal conflict between antioxidative inhibition of damaging oxidants and those involved in germinative processes).

Up until now, the benefits of seed pre-hydration treatments as a way to extend seed storability are not largely accepted due to cost or doubt of its validity, perhaps. For example, during *ex situ* seed conservation in gene banks, regeneration becomes necessary in all probability when percentage normal seedling production declines to about 85% (Yin et al., 2017). Between the period of early viability loss and regeneration, hardly is there any approach in place to slow down seed deterioration during storage (Xu et al., 2020). Treatments involving the exogenous application of antioxidants could eliminate ROS accumulated during storage, reinvigorate seed, extend seed storability, thereby holding off seed regeneration. Seed bank practitioners should, however, note that the effects of the antioxidant pre-hydration treatments might be dependent on the nature of the main oxidants and oxidant targets in specific species.

Additionally, the benefits of seed pre-hydration treatment suggest that the approach could be especially useful for the regeneration of valuable, endangered or rare germplasm (Venudevan and Srimathi, 2013), with which even little improvement in germination, vigour and establishment can be highly advantageous since such species already have small population size and perhaps limited genetic integrity. As such, the beneficial seed pre-hydration treatments, as applied in this study, could salvage individuals as genetic resources for plant propagation and breeding.

The present results further suggest that specific inorganic salt hydration treatments may improve cellular function in deteriorated seeds by influencing intracellular Ca^{2+} and K^{+} levels, and this should form part of future studies. The lack of information on the influence of pH on the effects of antioxidant-based soaking solutions on aged seeds suggests that this also may represent a potential future area of research.

The positive effects of seed pre-hydration treatment with antioxidants on plant growth demand further investigation since the application of antioxidant seed pre-hydration treatments could be used to alleviate poor stand establishment, which is one of the major consequences of seed ageing during germination and early seedling growth. The pre-hydration treatments, as demonstrated in this study, have the potential to be extended to other varieties and species in various stress conditions to improve the recovery of stored

germplasm.

Considering the beneficial effects of the pre-hydration treatments in terms of improved germination and seeding vigour, CDd lettuce seeds responded better to both antioxidant and inorganic salt solution pre-treatments compared with cabbage seeds. However, antioxidant pre-treatments were better for both species than pre-treatment with inorganic salt solutions. These pre-hydration treatments may be useful particularly for seed bank practitioners trying to reinvigorate valuable, endangered or rare seed collections. Studies to assess the effects of the beneficial pre-hydration treatments on seeds that have lost viability due to poor handling, storage under unconventional storage or even those stored for long-term in gene bank could form part of future studies.

Despite the attempts reported so far on improving seed enhancement techniques, innovative ideas and state-of-the-art investigations should be regularly introduced into this applied science sector of the seed industry. Interdisciplinary translational investigation combining bioinformatics and molecular tools will allow for the expansion of the range of seed pre-hydration treatment applications to other relevant commercial sectors, such as the native seed market.

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APPENDIX A

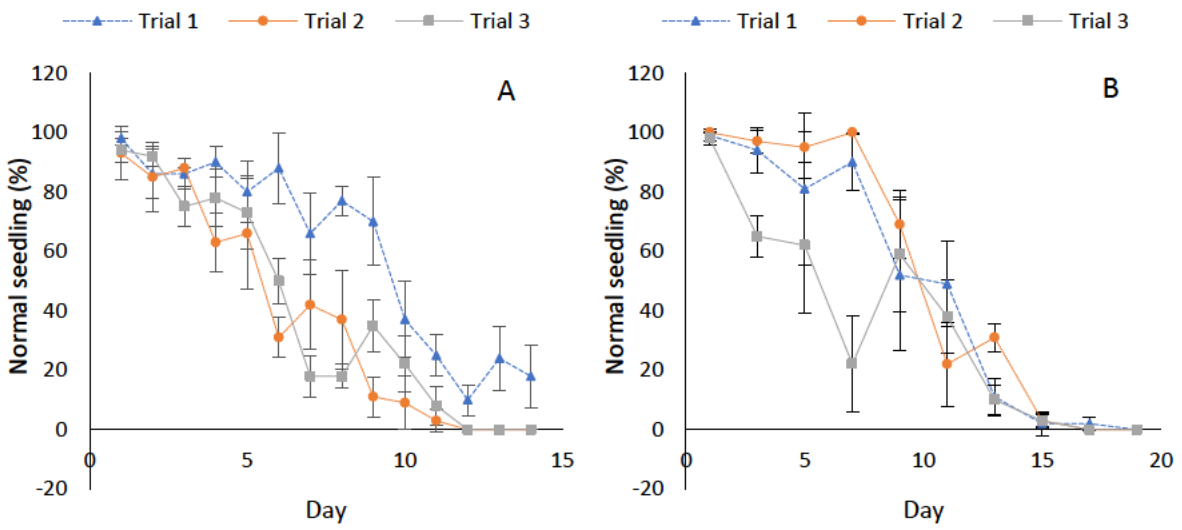

Figure A27 Normal seedling production (%) 14 days after germination of cabbage (A) and lettuce (B) seeds subjected to controlled deterioration using the methods of Mavi and Demir (2007). Data points represent mean ± SD (4 × *n* = 25).

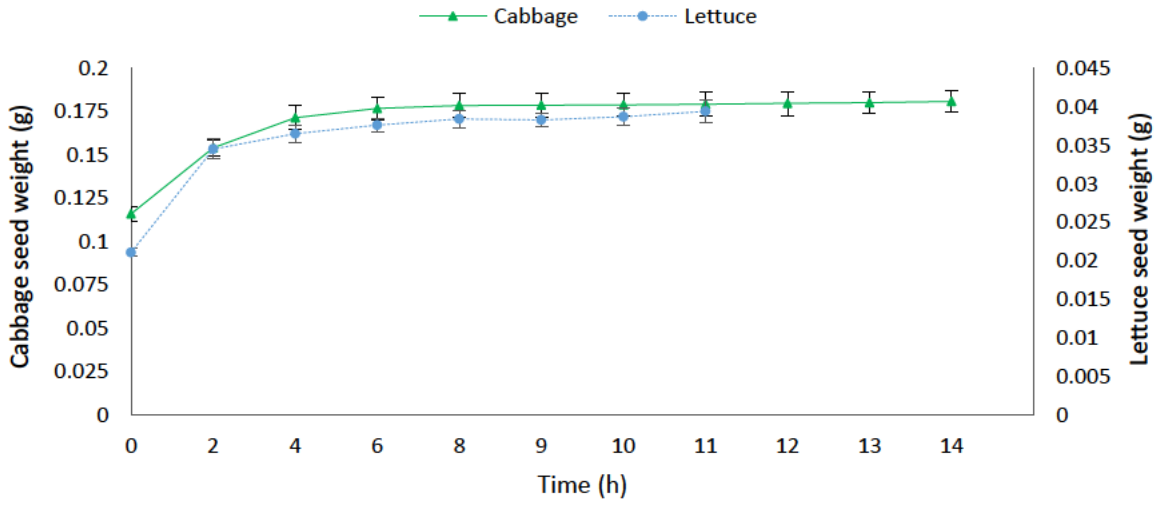

Figure A28 Water uptake in cabbage and lettuce seeds. Data points represent mean ± SD (3 × *n* = 25).

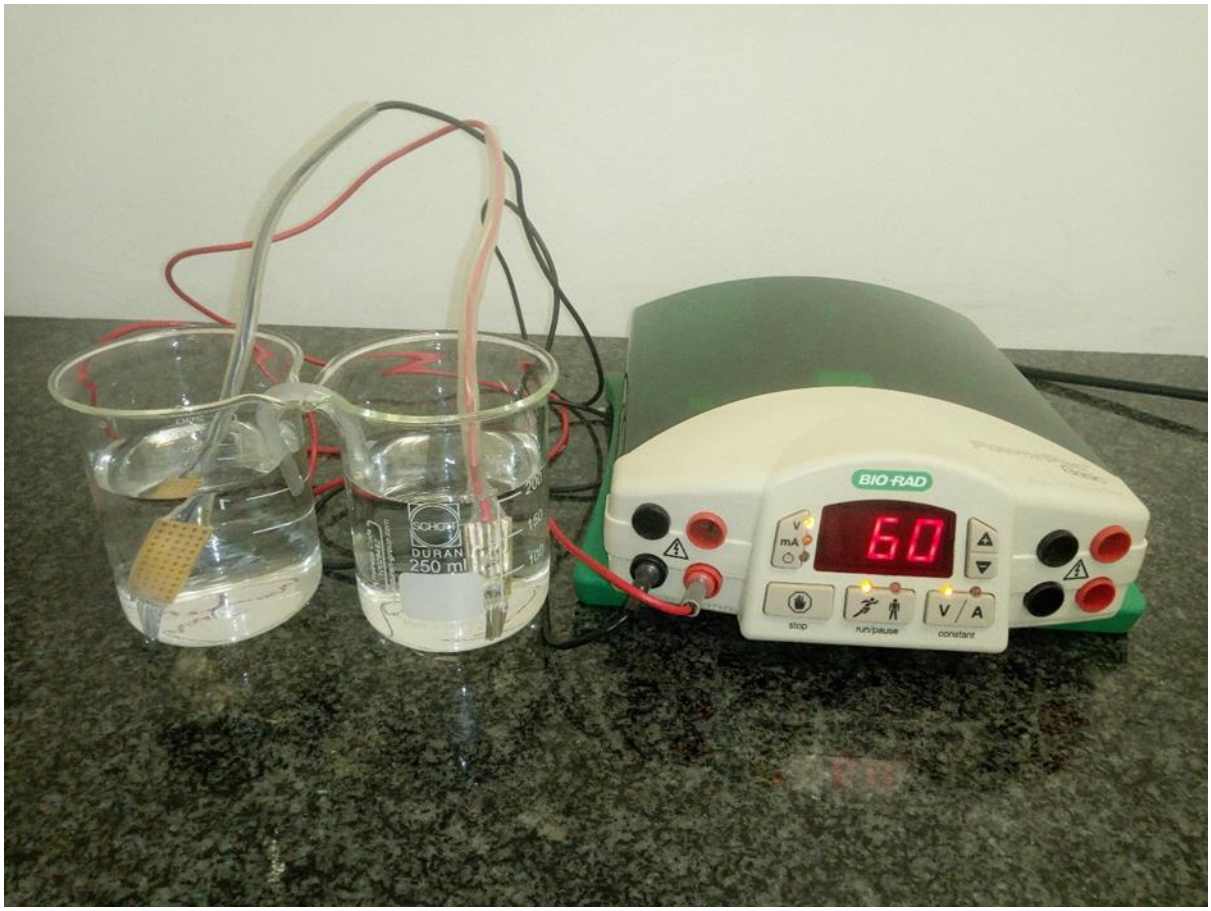

Figure 29 Apparatus used to generate cathodic water of inorganic salt solutions.

Table A16 Effect of the application of inorganic salt solutions on mortality (%) in fresh and controlled deteriorated cabbage and lettuce seeds

	Cabbage				Lettuce			
Hydration treatments	% mortality for fresh seeds	% mortality for CDd (P75) seeds	% mortality for CDd (P50) seeds	% mortality for CDd (P25) seeds	% mortality for fresh seeds	% mortality for CDd (P75) seeds	% mortality for CDd (P50) seeds	% mortality for CDd (P25) seeds
DW	11.00 ± 9.26 ^{NS}	17.00 ± 8.21 ^{NS}	38.50 ± 5.63 ^{b}	60.00 ± 3.02 ^{b}	1.00 ± 1.85 ^{NS}	20.00 ± 7.09 ^{NS}	38.50 ± 11.70 ^{a}	70.00 ± 5.24 ^{a}
$CaCl_2$	6.00 ± 6.76 ^{NS}	23.50 ± 6.57 ^{NS}	65.00 ± 7.33 ^{a}	70.50 ± 11.10 ^{a}	0.00 ± 0.00 ^{NS}	23.00 ± 5.95 ^{NS}	33.00 ± 8.75 ^{NS}	55.00 ± 11.66 ^{NS}
$CaCl_2$ CW	5.50 ± 5.21 ^{NS}	20.00 ± 7.09 ^{NS}	71.00 ± 12.78 ^{a}	73.50 ± 5.21 ^{a}	0.00 ± 0.00 ^{NS}	23.00 ± 7.63 ^{NS}	29.00 ± 7.93 ^{NS}	54.00 ± 11.51 ^{NS}
CaMg	7.50 ± 7.54 ^{NS}	30.00 ± 4.28 ^{NS}	51.50 ± 6.91 ^{NS}	68.50 ± 4.50 ^{a}	1.50 ± 4.24 ^{NS}	20.50 ± 6.57 ^{NS}	13.00 ± 5.13 ^{b}	51.50 ± 13.60 ^{b}
CaMg CW	8.50 ± 9.43 ^{NS}	22.50 ± 7.98 ^{NS}	33.00 ± 11.46 ^{NS}	72.00 ± 3.70 ^{a}	0.00 ± 0.00 ^{NS}	24.00 ± 4.28 ^{NS}	31.50 ± 10.13 ^{NS}	66.00 ± 10.03 ^{NS}
CaMg CW (6.5)	3.50 ± 5.83 ^{NS}	23.50 ± 7.23 ^{NS}	38.00 ± 7.09 ^{NS}	63.50 ± 2.56 ^{NS}	1.00 ± 1.85 ^{NS}	20.50 ± 3.96 ^{NS}	28.00 ± 11.90 ^{NS}	63.00 ± 9.74 ^{NS}
$MgCl_2$	5.00 ± 7.01 ^{NS}	15.50 ± 10.13 ^{NS}	54.50 ± 8.80 ^{NS}	77.50 ± 6.39 ^{a}	1.00 ± 1.85 ^{NS}	13.00 ± 7.33 ^{NS}	36.50 ± 7.84 ^{NS}	60.00 ± 6.41 ^{NS}
$MgCl_2$ CW	7.50 ± 9.90 ^{NS}	17.50 ± 6.74 ^{NS}	46.50 ± 6.02 ^{NS}	64.50 ± 9.67 ^{NS}	0.50 ± 1.41 ^{NS}	27.50 ± 11.80 ^{NS}	36.50 ± 5.42 ^{NS}	50.00 ± 11.11 ^{b}
NaCl	6.50 ± 5.47 ^{NS}	25.00 ± 12.42 ^{NS}	28.50 ± 11.40 ^{NS}	70.75 ± 3.01 ^{a}	1.50 ± 1.85 ^{NS}	21.00 ± 5.55 ^{NS}	32.00 ± 12.28 ^{NS}	56.50 ± 13.43 ^{NS}
NaCl CW	4.50 ± 4.99 ^{NS}	15.50 ± 6.21 ^{NS}	57.00 ± 13.31 ^{a}	65.50 ± 4.75 ^{NS}	0.00 ± 0.00 ^{NS}	21.00 ± 5.95 ^{NS}	40.00 ± 10.03 ^{NS}	49.50 ± 10.03 ^{b}
NaCl CW (6.5)	6.00 ± 8.00 ^{NS}	22.50 ± 7.69 ^{NS}	46.50 ± 4.24 ^{NS}	59.00 ± 7.33 ^{NS}	0.50 ± 1.41 ^{NS}	21.00 ± 5.55 ^{NS}	36.00 ± 5.66 ^{NS}	47.00 ± 8.75 ^{b}

Values represent mean ± SD (4 × *n* = 25) of % mortality of the control (fresh seeds soaked in deionised water [DW] and all inorganic salt solutions), and CDd (P75, P50 and P25) cabbage and lettuce seeds exposed to inorganic salt solutions. Values labelled with different letters are significantly different at $P < 0.05$ (ANOVA) when compared across hydration treatments within each controlled deterioration level. Cathodic water, CW; cathodic water adjusted to pH 6.5, CW (6.5); controlled deteriorated, CDd; not significant, NS.