The impact of different storage temperatures and storage period on the antioxidant activity of pomegranate

Aphiwe Angela Gule

A thesis submitted in fulfilment of the requirements for the degree of Magister Scientiae in the Department of Biotechnology, University of the Western Cape

Supervisor: Prof. N. Ludidi

January 2021

KEYWORDS

Pomegranate

Oxidative stress

Antioxidant activity

Chilling injury

The impact of different storage temperatures and storage period on the antioxidant activity of pomegranate

ABSTRACT

Fruit storage at cold temperatures is normally used to delay decay while maintaining the fruit's quality. However, storage at low temperatures for a prolonged period causes some important metabolic functions within the fruit to stop functioning properly. This phenomenon is even more critical and complex in chilling-sensitive fruits. Fruits that originated in the tropical and sub-tropical regions are known to be chilling sensitive; their storage at low but non-freezing temperatures causes them to experience chilling injury (CI). Chilling injury causes irreversible damage to the external and internal attributes of the fruits. Pomegranate, which is investigated in this study, is consumed as fresh arils in many regions of the world. Its external appearance is a shiny-red, with an attractive waxy peel. Pomegranate is classified as a non-climacteric fruit, which means that after harvest it does not ripen any further, thus it should be harvested when it is fully ripe. Moreover, it is highly admired for its medicinal benefits owing to its high antioxidant activity. However, it is susceptible to chilling injury when stored below 5^{o}C. The symptoms of chilling injury are expressed as skin browning, surface pitting, fungal growth and higher vulnerability to decay, this thus results in quality loss, especially when these symptoms reach the arils, which are the most consumed. These lead to less consumer acceptability, which highly determines pomegranate market growth. South Africa is one of the new yet rapidly growing countries when it comes to pomegranate market. However, it has been negatively affected by post-harvest fruit quality losses caused by chilling injury. Managing temperature is important for maintaining the quality of the fruit, including its nutritional value. Hence, in this study, temperature was used to study the impact of temperature on chilling injury on the quality losses of the fruit. Numerous studies have been done to study the changes that occur biochemically and physiologically on the antioxidant defence system and they have shown that the antioxidant defence system is involved in the chilling acclimation process. Despite this important knowledge, few studies have focused on the responses of antioxidant enzymes against chilling injury, which enhances the overproduction of reactive oxygen species. In order to understand the protective mechanism of antioxidant enzymes against chilling stress, visual quality changes, changes in hydrogen

peroxide (H_2O_2) content, malondialdehyde (MDA) content as well as the changes in the activity of antioxidant enzymes were studied in the seeds and arils of pomegranate. This study therefore, aimed to research the effect of two different temperatures (4°C and 6.5°C) on the antioxidants and metabolomics products of pomegranate seeds and arils over a period of 5 months taken at a monthly interval for the first two months, thereafter taken during the 5th month. Low temperature (4°C) triggered CI in pomegranate that was stored for 2 months and also triggered external decay; both the CI index and external decay were lower in the pomegranate stored at 6.5°C for a period of 5 months. Chilling injury increased H_2O_2 levels, thus causing an increase in MDA. However, the levels of MDA confirm that the H_2O_2 increase did not cause any oxidative stress damage in the first 2 months of storage and led to cell damage at 5 months of storage. Moreover, enzymatic activity of ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD) and dehydroascorbate reductase (DHAR) showed upregulation of activity in response to chilling stress in the seeds at both temperatures. However, there was downregulation in the arils for APX, CAT and SOD. A decrease in H_2O_2 at 6.5°C was observed, whereas there was no DHAR activity observed in the arils at both temperatures where high levels of H_2O_2 were found. The activities of antioxidant enzymes were more up-regulated at 6.5°C than at 4°C. Here, it is illustrated that 6.5°C causes less chilling injury in pomegranate cv. 'Wonderful' for a period of 5 months. Analysis of antioxidant enzymes and oxidative stress caused by H_2O_2 during chilling injury when coupled with physiological and biochemical analysis provided new insights into the chilling tolerance process.

DECLARATION

I declare that, “The impact of different storage temperatures and storage period on the antioxidant activity of pomegranate” is my own work, it has not been submitted before for any degree or examination in any other university and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Aphiwe Angela Gule

Signature

January 2021

ACKNOWLEDGEMENTS

Firstly, I would like to thank the Almighty for protection and strength.

Secondly, I would also like to thank the Bovenvlei orchard staff and owners for making this project possible by generously donating the fruits and helping us pick them.

Thirdly, I would like to thank my supervisor Prof. Ludidi and Dr. Oluwafemi Caleb from Stellenbosch University for their support. I would also like to thank Dr. Kyle Phillips for helping me with the transportation of the fruits from ARC to UWC. I would like to also acknowledge MaRhadebe for taking my son (Royale) in so that I can finish my studies. I would also like to acknowledge my mother, my nephew and my sisters for cheering me at all times and my late grandmother for teaching me that education is important.

LIST OF ABBREVIATIONS

$^1O_2$	Singlet oxygen
AOX	Alternative oxidase
APX	Ascorbate peroxidase
AsA	Ascorbate
BSA	Bovine serum albumin
CAT	Catalase
CI	Chilling injury
CU/ZN-SOD	Copper/Zinc superoxide dismutase
DHA	Dehydro-L-ascorbate
DHAR	Dehydroascorbate reductase
EDTA	Ethylenediaminetetraacetic acid
Fe-SOD	Iron superoxide dismutase
GR	Glutathione reductase
GSH	Glutathione
H_2O	Water
H_2O_2	Hydrogen peroxide
KCN	Potassium cyanide
LOOH	Lipid hydroperoxides
LP	Lipid peroxidation
MDA	Malondialdehyde
Mn-SOD	Manganese Superoxide dismutase
NAMC	National agricultural marketing council
NBT	Nitrotetrazolium Blue chloride

NFPMs	National fresh produce markets
PAGE	Polyacrylamide gel electrophoresis
POMASA	Pomegranate producers association of South Africa
PUFAs	Polyunsaturated fatty acids
PVPP	Polyvinylpolypyrrolidone
SOD	Superoxide dismutase
TA	Titratable acidity
TBA	Thiobarbituric Acid
TCA	Trichloroacetic acid
TEMED	N, N, N', N'-Tetramethylethylenediamine
TSS	Total soluble solids
USA	United States of America

LIST OF FIGURES

LIST OF TABLES

TABLE OF CONTENTS

AIM AND OBJECTIVES

The aim of this study was to examine the impact of chilling in terms of storage temperature and storage duration on oxidative stress and the activity of antioxidant enzymes in pomegranate seeds and arils.

The aim of this thesis was achieved by completing the following objectives:

1. To elucidate the impact of temperature and duration of storage on visual quality of pomegranate cv. 'Wonderful' that was stored at 4°C and 6.5°C for an overall period of 5 months and sampled at 1, 2 and 5 months of storage by studying
   - Chilling injury score and
   - External decay.
2. To elucidate the impact of temperature and duration of storage on the antioxidant enzymes of pomegranate seeds and arils that were stored at 4°C and 6.5°C for 1, 2 and 5 months by measuring:
   - Oxidative stress parameters such as H_2O_2 and lipid peroxidation
   - Antioxidant enzymes such as SOD, CAT, APX and DHAR.

HYPOTHESIS

1. Prolonged storage of pomegranate cv. 'Wonderful' at lower temperatures (4°C) leads to chilling injury that is characterised by poor visual quality (skin browning) and an increase in ROS levels and a decrease in antioxidant activity.

CHAPTER 1: LITERATURE REVIEW

1. Introduction

Pomegranate (*Punica granatum* L.) is consumed as fresh arils which are juicy and sweet, the arils have seeds within them, depending on the cultivar (Tayyari *et al*., 2017). It is usually grown in subtropical and tropical countries and in almost all Mediterranean countries. The 'Wonderful' cultivar was discovered in Florida and brought to California in 1896. This is the primary cultivar of commerce in the USA. The 'Wonderful' cultivar is widely-cultivated in California, while in the last few years, it has replaced the native Greek cultivars. Now, 'Wonderful' is the main pomegranate cultivar in Greece. It is also grown in Western Europe, Israel and Chile (Thomidis, 2014).

Cultivar 'Wonderful' is described as one of the most deeply coloured pomegranates in both husk and juice, with a rich flavour, good juice yield and both sprightly acidity and slight thirst-quenching astringency similar to that of a grapefruit juice. Many pomegranate lovers consider it to be among the best-tasting cultivars (Pareek *et al*., 2015). Moreover, cv. 'Wonderful' is nearly ideal for juicing, with excellent juice yield as well as quality. Its's health benefits are not limited to but include helping with heart disease, cancer and problems associated with aging. It has plenty of antioxidants, vitamins, potassium, folic acid and iron (Thomidis, 2014).

Due to diet trends and nutritional benefits, its production and consumption has increased worldwide in the recent years (Mphahlele *et al*., 2014). These trends have led to the concept of a diet in terms of the amounts of nutrients covering the daily requirements of an organism but also to its perception as a factor improving human health, thus including also components preventing or reducing the risk of chronic diseases. In view of the new perception of food products by consumers, producers have felt the pressure to develop novel different kinds of foods meeting the requirements of the customers (Mphahlele *et al*., 2014; Gumienna *et al*., 2016). Increasing consumer awareness of the effect of diet on the incidence of risk factors leading to chronic diseases promotes the development of sectors of the food industry involved in the production of functional foods (Mphahlele *et al*., 2014; Gumienna *et al*., 2016).

With these trends, there is a growing demand for high quality pomegranate fruit both for fresh use and for processing into juice, syrup, squash, wine and anardana (dried pomegranate arils). As a result, the extent of pomegranate cultivation has increased significantly in many regions throughout the world and industries that make pomegranate products have also developed (Mphahlele *et al.,* 2014). Besides, during the production of agricultural food by-products disposal waste is created during handling and processing of fruits and vegetables these include peels, seeds, leaves, bracts, stems, roots and bark (Durante *et al*., 2017). Besides, these by-products have large amounts of storage and cell wall structural carbohydrates, proteins and lipids, potentially useful for animal and human food supplementation and/or bioenergy production, high-value natural compounds such as carotenoids, phenols, tocochromanols, vitamins, and phytosterols, many of them have health-promoting properties (Durante *et al*., 2017). Increased awareness of the potential commercial value of most agricultural food by-products has stimulated the exploitation of efficient extraction techniques of their bioactive compounds with undeniable environmental sustainability benefits and a more effective use of the harvested plant material (Durante *et al*., 2017).

With that being said, pomegranate seed is one of the agri-food by-products (Durante *et al*., 2017). The oil of the seed is a major source of punicic acid, a distinctive ω-5 trienoic fatty acid with emerging evidence for important therapeutic uses in human health, including inhibition of cancer cell proliferation. Seeds represent a quantitatively abundant by-product of fruit industrial processing. Seeds are often discarded within the so-called pomace, together with skins and vascular tissues of the fruits, but can be easily recovered by separation and sifting technologies. Seeds account for approximately 22% of the waste (rind and the seeds) of the pomegranate juice industry. Seeds represent the portion of the fruit with the highest concentration of antioxidants, hence their waste represents a double loss for the agri-food industry that has to face the cost of disposal and the loss of profits for their re-use and valorisation (Durante *et al*., 2017).

Moreover, pomegranate is grown globally in many different geographical regions, satisfying the nutritional and medicinal needs of populations of various countries (Gumienna *et al*., 2016). The Western Cape province of South Africa is responsible for 70% of the country's pomegranate production. In recent years, there has been an expansion of pomegranate

production in the arid Northern Cape. The South African pomegranate season has a promising market with the harvest of the 'Wonderful' variety, which is providing bigger fruit than the earlier varieties such as 'Akko' and 'Hershkovitz'. However, drought remained a challenge in the year 2016, which led to these fruit varieties being substantially smaller than usual, thus putting pressure on the prices attained on export markets (Fresh Plaza, 2019).

In order to meet the demand for pomegranates and its products, the marketing period of pomegranates should be extended (Matityahu *et al*., 2014). This extension requires a better understanding of the processes that take place during the post-harvest storage (Matityahu *et al.,* 2014). Among the important factors that limit the long-term storage of pomegranates are disorders occurring on the fruit husk, namely fungal decay, chilling injury, shrinkage due to weight loss and husk scald, all leading to loss of visual quality (Matityahu *et al*., 2014). Pomegranate is a non-climacteric fruit that is often exposed to continuous physiological and biochemical changes post-harvest (Jaakola, 2013). Post-harvest quality changes are almost impossible to improve. However, the quality of the fruit post-harvest can be maintained by slowing down the deteriorative changes through the application of post-harvest treatments and cutting-edge modern technology (Jaakola, 2013, Kader *et al*., 1989).

Many factors play a role in changes and the rate at which they take place during post-harvest, these include like harvest maturity, temperature, relative humidity and atmospheric composition during storage and transportation (Kader *et al*., 1989). The application of post-harvest treatments such as heat, maintaining optimum storage temperature, modified atmosphere packaging, controlled atmosphere storage, shrink wrapping, coating and drying have been reported to nutritional quality, as well as antioxidants in pomegranate (Jaakola, 2013).

The pomegranate post-harvest handling system must aim to ensure that the pomegranates' nutritious benefits are not compromised through detoriation during storage in the market or during transportation. The study of chilling injury (CI) not only answers questions about how CI leads to fruit quality loss and how it affects antioxidant capacity of the fruit, but also further shows how this phenomenon can negatively affect the market of pomegranate cv. 'Wonderful' in South Africa.

1.2 Analysis of the pomegranate market in South Africa

South Africa is one of the new countries in the pomegranate market in the Southern Hemisphere, competing with countries like Chile, Australia, Peru and Argentina (Gumienna *et al*., 2016). Pomegranate production in South Africa was at about 1000 hectares, with an estimated 2250 tons of fruit exports in 2012 (Gumienna *et al*., 2016). Subsequently, due to the counter-season market window in the Northern Hemisphere during summer and spring, production and the export of pomegranate in South Africa is estimated to have a good chance of increasing its market (Gumienna *et al*., 2016). Hence, extensive studies on any factors that may hinder the market should be investigated. This justifies studies seeking understanding of chilling injury during post-harvest storage of pomegranate (Matityahu *et al.,* 2014).

Chilling injury is one of the major causes of pomegranate quality deterioration while they are in the market or transportation, following exposure to temperatures below 5^{o}C for longer than 4 weeks (Isolcell, 2012). In India, even though the information about pomegranates' nutritious benefits is recognised and the global demand for their pomegranates is high, the pomegranate industry is not well developed. This is due to poorly developed technological commercialisation, lack of resource personnel and lack of scientific research database. Thus, the post-harvest losses are very high, accounting for 20-40% (Dhinesh and Ramasamy, 2016).

Damage caused by CI is one of the main reasons for the declining market value and quality of agricultural products between the point of harvesting and consumption (Shaffie *et al*., 2014). Pomegranate requires extreme care and proper storage in order to maintain its quality both in terms of nutrition value and visual quality to prevent losses due to chilling injury (Shaffie *et al*., 2014). When the pomegranates lose the red colour of their rind due to improper post-harvest storage, they are estimated to cause about 10-15% of their market value losses since the consumers link the pomegranates and its products to its intense red colour (Fawole and Opara, 2013; Dhinesh and Ramasamy, 2016).

Reducing losses due to CI can increase the fruit's supply without farmers having to cultivate extra land (Dhinesh and Ramasamy, 2016). It is suggested that in order to minimise losses, a number of processed products can be manufactured and preserved for future use, satisfying consumer perception of a high nutritional quality and convenience produce. These can also help with increasing the market value and make the fruit available throughout the year, thus

increasing the shelf life of the pomegranate fruits (Dhinesh and Ramasamy, 2016). Figure 1.1 shows the quantity of pomegranates sold on the national fresh produce markets (NFPMs), including the associated price trends for 2016 and 2017 (Ntshangase *et al*., 2017).

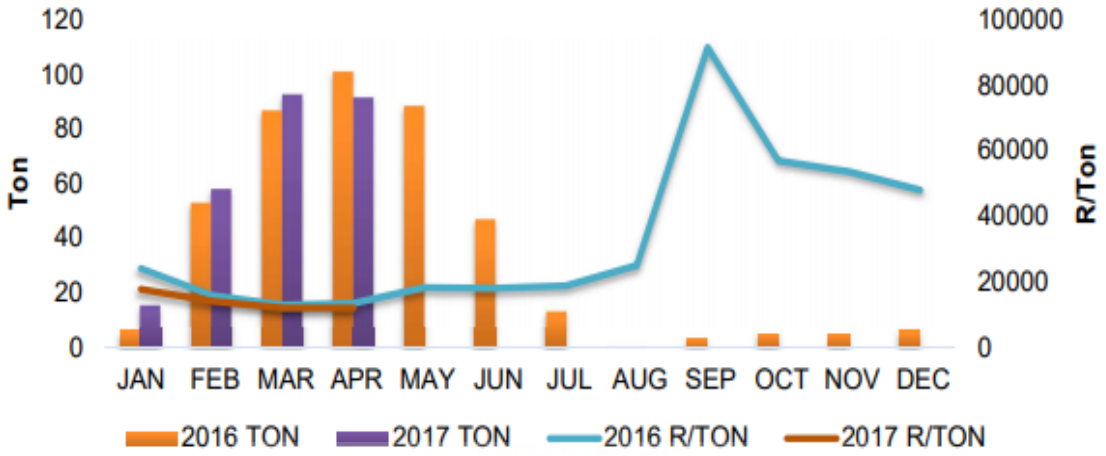


Figure1. 1: Local market sale of pomegranates.
Source: Ntshangase *et al.,* (2017).

Ntshangase *et al.,* (2017) reported that approximately 413 tons of pomegranate were sold on the local market in 2016 with the month of April (2016) reaching the highest record with 101 tons of fruits as indicated in Figure 1.1. The 2017 season recorded a volume of 257 tons between January and April, which topped the volume sold during the same period in 2016. The volume of pomegranates sold on the local market between January and March of 2017 also topped the volume of pomegranates sold during the same period in 2016. The average price reached a peak in September 2016 at R91 644 per ton (Ntshangase *et al*., 2017).

South Africa's production of pomegranates amounted to approximately 7 337 tons in 2016, with 69% destined for exports and the remainder for the local market (5.4%) and processing (25.5%) (Ntshangase *et al*., 2017). The export of pomegranates increased during in 2017 while local consumption remained stable. In 2016, South Africa exported 5072 tons of pomegranates and consumed 398 tons on the local market (Ntshangase *et al*., 2017). Table 1.1 shows the export of pomegranates per variety grown in South Africa over the past five years. It is important to note that the largest volume of pomegranates exported was of the variety 'Wonderful', with a share of 70%, which was followed by 'Hershkovitz' and 'Acco' with shares of 19% and 10% respectively (Ntshangase *et al*., 2017).

Table 1.1: South Africa's exports of pomegranate per cultivar (Ntshangase *et al*., 2017)

Cultivar	2012	2013	2014	2015	2016
Wonderful	248 400	393 177	520 332	611 288	823 782
Hershkovitz	82 326	59 523	134 441	205 241	228 115
Acco	35 000	30 927	73 660	134 345	113 999
Kessari	15 523	13 100	2 086	520	4 346
Shir	2 093	3 140	3 663	0	2 337
Rosy	0	0	3 684	6 105	1 865
Other	44 033	26 637	2 030	12 739	5 010
Total	427 375	526 233	739 895	970 237	1 173 455

Figure 1.2 shows the export destinations for South Africa's pomegranates during the 2016 season (Ntshangase *et al*., 2017). Europe was the leading market for South Africa's pomegranates, with 59% of total exports, followed by the Middle East with 15%, the Far East and Asia with 8% and the UK with 8%. The Indian Ocean Islands accounted for the least export destination for pomegranates from South Africa, while Africa and Russia constituted an equal export share of 4% (Ntshangase *et al*., 2017). In Europe there is a great demand for South African pomegranates. However, the best-selling pomegranate cultivar 'Wonderful' does not get as much rind colour depth as pomegranates grown in other countries. According to Fresh Plaza (2019), the prices at which South Africa sells its pomegranate to the European countries is much higher than in the Middle East. The exports of pomegranate from South Africa started in Europe and as the number of pomegranate producers increased South Africa had to find new markets in new countries (Fresh Plaza, 2019).

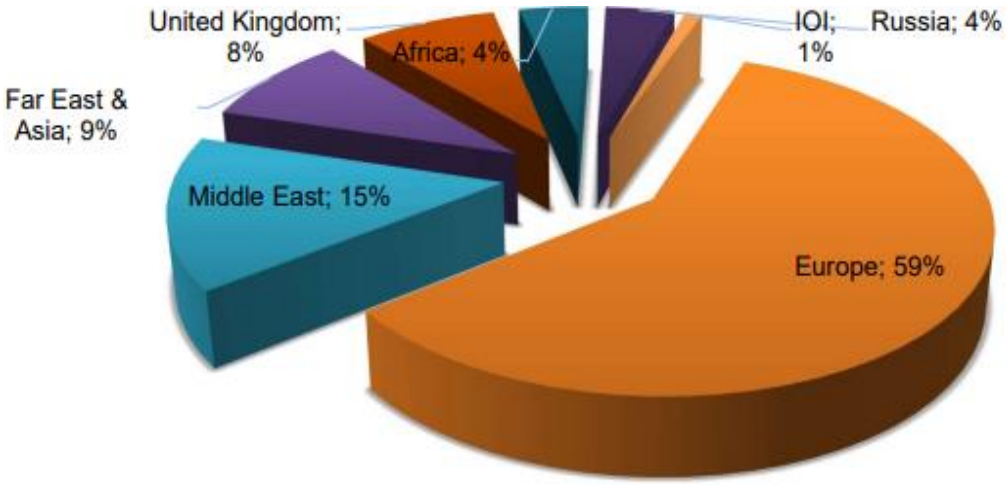


Figure 1.2: Main export destinations for South Africa's pomegranates. Europe was South Africa's main pomegranate export destination, followed by Middle East.

Source: Ntshangase *et al*., (2017).

1.3 Storage conditions affect pomegranate quality

Various studies have shown that storage conditions have a notable influence on phytochemicals in pomegranates (Gumienna *et al.,* 2016). Temperature management procedures are important for maintenance of quality attributes, including the nutritional components. Pomegranate is known to be highly susceptible to weight loss and decay during post-harvest handling and storage. For instance, one study the storage time for cv. 'Wonderful' pomegranate kept at 20°C or 30°C was about 1–4 weeks, with high incidence of quality losses which affected the transportation time (Gumienna *et al*., 2016).

Apart from the common visible post-harvest quality defects such as decay and water loss, browning symptoms in the peel and arils, invisible quality losses also occur (Gumienna *et al*., 2016). Other researchers have reported colour loss as a result of degradation of anthocyanins, as well as a decrease in total soluble solids (TSS) and titratable acidity (TA), which was accompanied by a reduction in consumer acceptability in terms of freshness, taste and loss of potential medicinal properties. Incidence and severity of sensory quality losses and physiological disorders in pomegranate have been linked to pre-harvest temperatures, fruit maturity and cultural practices (Gumienna *et al*., 2016).

The incidence and severity of chilling injury depend upon storage temperature and duration (Isolcell, 2012). The optimum storage temperature recommended for pomegranates varies from 0°C to 10°C with storage life ranging from 2 weeks to 5 months, depending on the cultivar (Gumienna *et al*., 2016). Kader (2006) recommended 2 months storage duration for 'Wonderful' pomegranate stored at 5°C and 90–95% relative humidity.

Unfortunately, the production of pomegranate in South Africa is currently plagued by a high incidence of post-harvest losses, especially during handling, due to limited knowledge on storage requirements of several commercial cultivars such as 'Bhagwa' and 'Ruby' (Gumienna *et al*., 2016). Higher storage temperature (compared to the optimum storage temperature) has significant influence on pomegranate aril physiology and quality attributes (Mphahlele *et al*., 2014). Furthermore, several researchers have shown that fruit quality of pomegranates differs significantly among growing regions and cultivars. However, there is limited information regarding the effect that storage temperatures have on the nutritional and bioactive properties of pomegranate arils (Mphahlele *et al*., 2014).

1.4 Post-harvest physiological disorder: the phenomenon of chilling injury

Physiological disorders are known as a breakdown of tissue that is not caused by disease causing organisms (Wills *et al*., 1998). Post-harvest physiological disorders develop as a result of a response to external stimuli such as temperature in the case of chilling injury. Fruits are preferably stored at low temperatures in order to decrease the rates at which respiration and metabolism occur. Nevertheless, these processes are not inhibited to the same point at low temperatures and some of the reactions that occur within these processes are sensitive at low temperatures and, as a result, they stop functioning (Wills *et al*., 1998). Moreover, low temperatures do not reduce the activity of other systems the way they affect respiration. This causes differences in the way the processes respond to low temperatures. This imbalance in metabolism, as time progresses, causes the cells to stop functioning in a normal manner. The cells ultimately lose their integrity and structure and collapse because they are unable to carry out normal metabolic processes. These collapsed cells are visible as brown patches on the rind of the fruit and this phenomenon is known as chilling injury (Figure 1.3.1) (Wills *et al*., 1998).

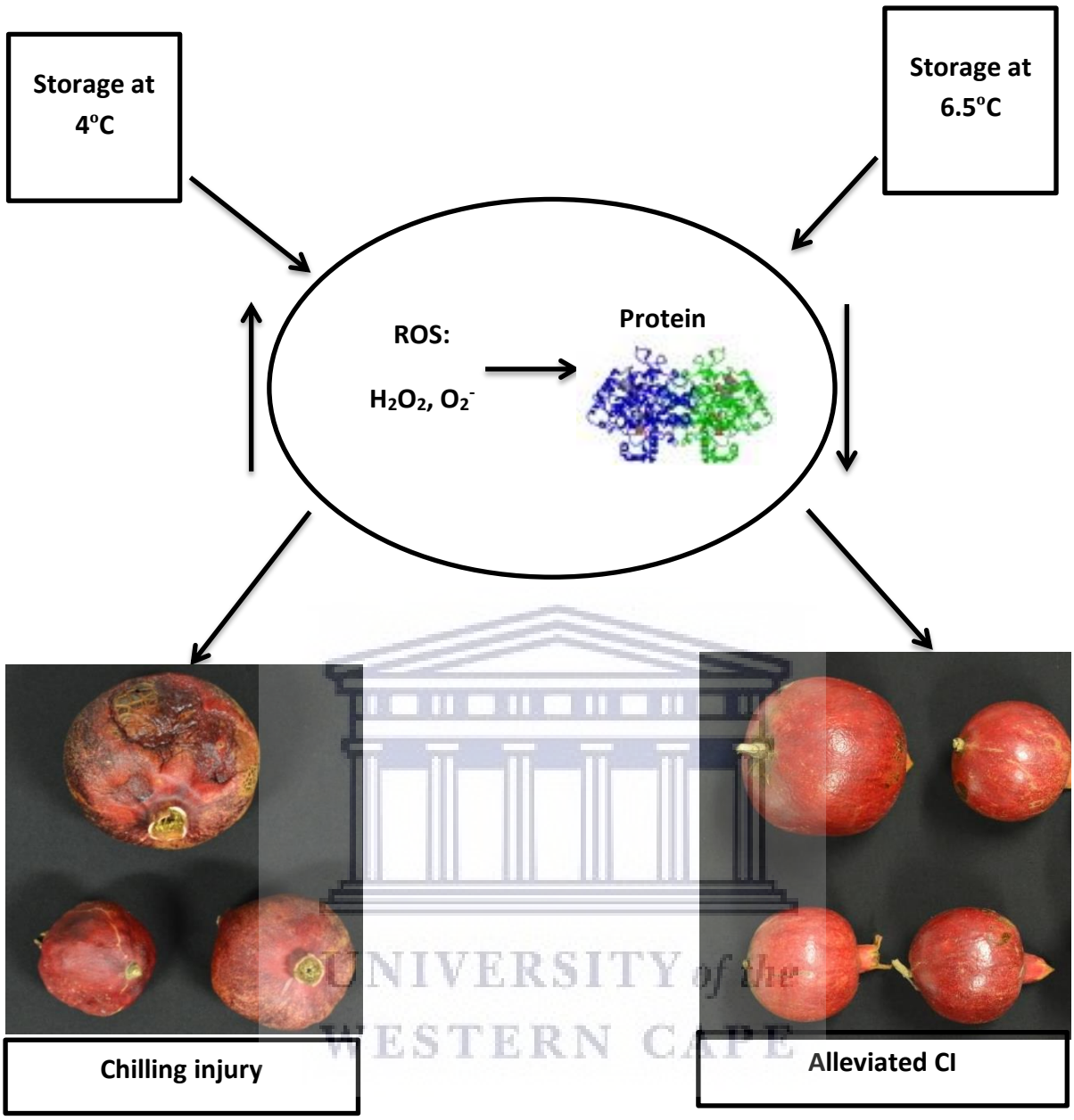


Figure 1.3.1: Visual demonstration and physiological appearance of ROS mediated chilling in pomegranate cv. 'Wonderful'.

ROS mediated CI led to changes in proteins of pomegranates. Storage at 4°C causes ROS to be upregulated thus causing changes in the protein structure and lead to chilling injury, whereas storage at 6.5°C maintains ROS levels at minimum thus causing the fruit to tolerate CI. The protein structure is adapted from Houston *et al.*, (2017).

1.4.1 In-depth analysis of chilling injury

Temperature is an important factor when it comes to storage of fruit (Mphahlele *et al*., 2014). Low temperature storage is a post-harvest technology that is used generally to prolong the post-harvest life of fruits and vegetables (Mphahlele *et al*., 2014, Li *et al*., 2016). Crops that are chilling sensitive have a short half-life since the low temperature storage cannot delay deterioration and pathogen growth (Li *et al*., 2016). Scientists have been studying chilling injury for more than 200 years; however, the understanding of its early phases of development and its fundamental causes at a molecular level are not fully known, causing failure to finding a long-term solution to chilling injury (Albornoz *et al*., 2019). Fruits that are originally from the tropical or subtropical regions are sensitive to chilling injury that manifests after their storage at low temperatures (i.e. below 10^{o}C) but above their freezing point (Gross *et al*., 2016).

During chilling injury, most physiological and biochemical functions are changed thus causing cellular dysfunction (Wang, 2004). These changes include enhancement of ethylene production, respiration rate, changes in energy production, and membrane dysfunction among others. In the case of pomegranate, if the duration of chilling injury is prolonged, these changes may lead to loss of internal and external qualities like whitening of the arils, development of fungi, surface lesions, discolouration of the albedo, water soaking of tissue, off-flavour and post-harvest decay. Pre-harvest chilling injury may lead to failure to ripen normally or to fully ripen (Wang, 2004). However, these processes can be reversed if the fruit is retained at a higher temperature before damage occurs (Li *et al*., 2016).

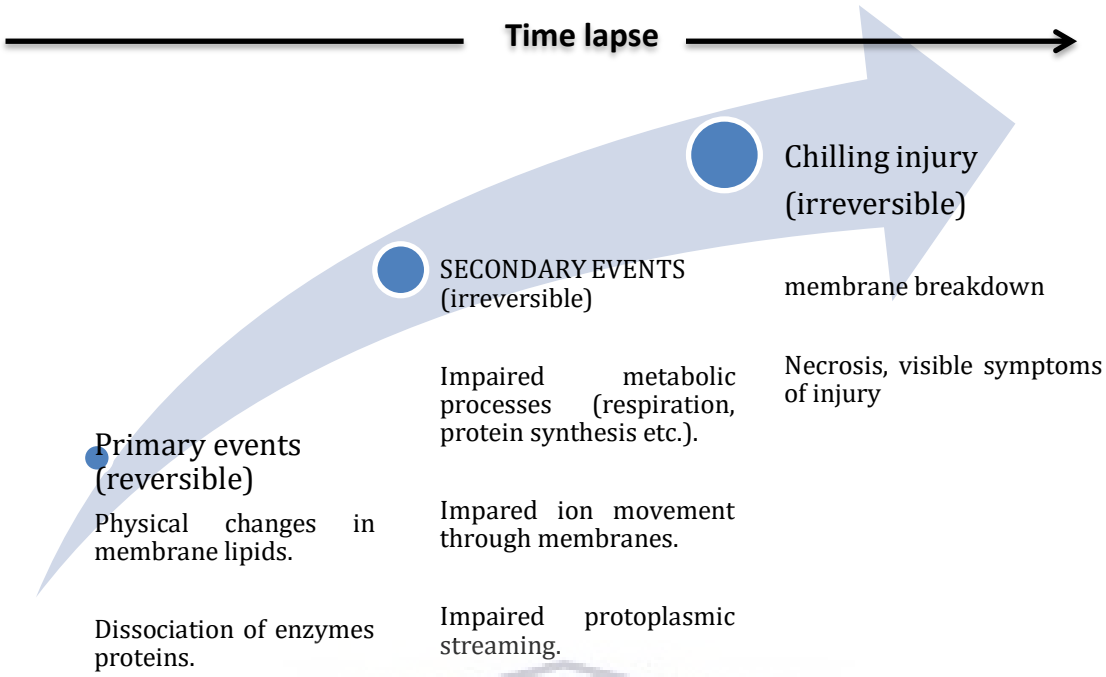


Figure 1.3.2: Depiction of the 3 main stages of CI.

In the primary event, low temperatures cause physiological dysfunction that can be reversed since low temperatures cause disturbances in the physiological functions. These disturbances do not cause physical changes of injury hence they are said to be reversible. The secondary events, low temperature cause the physical properties of cell membranes to be disrupted due to impaired metabolic processes. CI increases as the storage time progresses. Source: Wills *et al*., 1998; Biswas, (2012).

Wang (2004) suggested that there are 2 hypotheses that may help in understanding how temperature leads to chilling injury and how its detrimental effects come about (Wang, 2004). The first hypothesis suggested that there is one unifying response to temperatures that cause chilling injury for all chilling sensitive species and this serves as the primary response which would lead to secondary events which consequently turn to chilling injury symptoms (Figure 1.3.2). Wang could have suggested that the primary response entails that there is a unifying response because according to Li *et al*., (2016), although there are many diverse factors affecting CI, the signs at a microscopic level are the same in all plants. Moreover, the change in membrane lipids, changes in kinetics or substrate specificity of a regulatory enzyme, the modifications of the cytoskeletal structure, or a rise in cytosolic calcium, were proposed as prospective events that occur in the primary response (Wang, 2004).

When sensitive fruits have been stored for a long period of time low temperatures, the primary response trigger secondary events that consist of a loss of membrane integrity, leakage of solutes, loss of compartmentalization, decrease in the rate of mitochondrial oxidative activity, rise in the activation energy of membrane-associated enzymes, termination of protoplasmic streaming, decline in energy supply and utilization, disorganisation of cellular and subcellular structures, dysfunction and imbalance of metabolism, accumulation of toxic substances, stimulation of ethylene production, increase in respiration rate and manifestation of a variety of chilling injury symptoms (Wang, 2004).

The second hypothesis suggests that chilling injury initiates from a wide range of responses to low temperature. This hypothesis rejects the primary response hypothesis on the grounds that tropical and subtropical fruits are diverse both in structure and expression of chilling symptoms so it would not be reasonable to believe that one unified response can lead a chain of events and cascade into a number of chilling symptoms. As a result, the second hypothesis advocates that there are numerous responses over numerous conditions (Wang, 2004). These responses include swelling and disorganisation of the mitochondria and the chloroplasts where thylakoid dilation and unstacking of grana occur, reduction in the size and number of starch granules, accumulation of lipid droplets inside the chloroplasts and condensation of nuclear chromatin (Li et al., 2016).

The macroscopic symptoms of CI in fruits and vegetables are diverse and two categories can be distinguished, which often develop simultaneously (Li *et al*., 2016). However, some symptoms, e.g. internal bleaching and browning or development of an abnormal texture in the pulp, are not externally visible. These different symptoms show that the problem of CI is not simple, because the mechanisms involved differ as a function of the crop and become apparent in many different ways. Some studies suggest that the textural changes of certain CI-sensitive fruits during low temperature storage are due mainly to disorders of cell wall metabolism, including a decrease in the solubilisation and depolymerisation of pectin (Sevillano *et al*., 2009).

Fast pre-refrigeration using forced air is one of the simplest ways to extend pomegranate commercial life (Mphahlele *et al*., 2014). The temperature has to be around 5^{o}C to prevent the development of physiological disorders during 2–3 months of storage. Not all cultivars

prefer the same storage conditions, for example Spanish sweet pomegranates will suffer from chilling injury if they are stored for a period of more than 2 months below 5°C while cv. 'Wonderful' can be stored for 2 months at 5°C without showing any signs of chilling injury (Mphahlele *et al.*, 2014). On the other hand, studies have shown that the conventional storage of cv. 'Mollar' at 5°C and 90–96% relative humidity for up to 8 weeks resulted in a decrease in fungal decay losses, even though the fruit was not completely prevented from developing CI symptoms (Mphahlele *et al.*, 2014).

1.4.2 Factors affecting chilling injury susceptibility

Factors affecting the susceptibility of pomegranates to chilling injury involve the origin of the crop, genetic makeup of the commodity, stage of development, metabolic status of the tissue and a number of external stimuli such as temperature, light, relative humidity and atmospheric composition (Wang, 2004). These factors determine if the plant would be susceptible or tolerant to chilling injury. In chilling-susceptible species, the critical threshold temperature may vary with the stage of development or maturity. For example, avocados, mangos and tomatoes are more sensitive to chilling when they are immature. The metabolic status and the chemical composition of the tissue during the period of chilling can disturb the tolerance of tissue to chilling (Wang, 2004).

A study by Wang (2004) revealed that chilling-resistant tissues have a higher level of unsaturated fatty acids in the lipid membrane than in the fruits that tend to be chilling tolerant. High amounts of reducing sugars and proline are positively linked to chilling injury. Light is another fact that induces chilling injury under certain conditions. Structural changes in proteins that are chilling-induced may be primarily triggered for light-induced damage if they are linked to photosystem II. On the other hand, relative humidity is also associated with chilling injury since studies have proven that chilling injury symptoms are more severe under high relative humidity (Wang, 2004). 'Wonderful' pomegranates develop CI symptoms, namely brown discoloration or scald of the skin and surface pitting, if stored in conventional cold storage at 5°C or lower temperatures (Wang, 2004)

1.4.3 Post-harvest quality losses due to chilling injury

Pomegranates show quality losses during post-harvest storage as a result of chilling injury disorders (for instance physiological and metabolic disorders) which lead to internal and external quality losses (Arendse *et al*., 2015). These may be mainly caused by the loss of water in the process of chilling injury. As stated above, these symptoms worsen at temperatures below 5^{o}C. Nonetheless, temperature can still be manipulated in order to avoid pomegranate decay by storing the fruits at low temperatures (Arendse *et al*., 2015). It is believed that the first qualitative symptom of metabolic disorders is incomplete ripening (at the pre-harvest stage) and rancidity (at the both the pre-harvest and post-harvest stage) (Li *et al*., 2016).

The second loss of qualitative attributes include visual qualities, for example skin shrinkage, abnormal skin yellowing, tissue decomposition, internal or surface browning, water infiltration in intercellular spaces, development of a woolly or dry texture in the pulp, browning of membranes and lesser resistance to mechanical injury and fungal and microbial infestation (Li *et al*., 2016). Chilling injury, weight loss and decay are the most important apparent limitations of storability of pomegranate. As temperature is reduced, a specific temperature determined by the ratio of saturated to unsaturated fatty acids accelerates the conversion of lipids from a liquid-crystalline condition into that of a solid condition in plant cell membranes. The conversion of fatty acids may give rise to chilling resistance at lower temperatures in the plant cells (Lee and Lee, 2000).

1.4.3.1 Chilling induces weight loss

One of the main problems linked to chilling injury in pomegranates is weight loss (Arendse *et al.,* 2015). Weight loss can be caused by water loss during cold storage and it can consequently cause rancidity and undesirable texture of the rind thus causing poor visual quality. This water loss is happens because of the natural porosity of the skin. Moreover, shrivelling symptoms on fruit occur when the weight loss exceeds 5% or more of the initial weight. In order to avoid the weight loss, Yahia (2011) suggested that the fruits should not be stored at high temperatures that are accompanied by low relative humidity as these two factors are likely to cause high incidence of water loss. Meanwhile Kahramanoğlu *et al*., (2019) suggested that

the pomegranate must be stored at temperatures between 5-10°C accompanied by a relative humidity of ~90%.

1.4.3.2 Mould

High relative humidity breeds the growth of mould, which consequently leads to fruit decay (Kahramanoğlu *et al*., 2019). *Botrytis cinerea* Pers. is the major cause of grey mould in pomegranate. This type of infection usually starts in orchards when the fruits have been infected and extends to storage, after which it is easy to infect uninfected fruits nearby. *Botrytis cinerea* is known to infect the flowers of the pomegranate during their development, and crowns of the fruit. The infection starts in the crown and extends to the rest of the fruit. As a result, 30% of pomegranates are lost due to visible mould and due to a lack of fungal control measures (Kahramanoğlu *et al*., 2019).

1.4.3.3 Sensory quality

Sensory quality attributes and nutritional value of fruit play an important role in consumer satisfaction and repeated purchase (Fawole & Opara, 2013). However, like other fruits, pomegranate also experiences post-harvest quality losses during handling and storage. Quality assessment of pomegranate at harvest is based on a wide range of physico-chemical characteristics, including fruit colour, TSS (total soluble solids), TA (titratable acidity), TSS/TA and texture (Fawole & Opara, 2013). The flavour, sensation and aroma produced from non-volatile compounds generate a characteristic sweetness, saltiness, bitterness, sourness and pungent or astringent feeling in the mouth. Pomegranate flavour has been attributed to a combination of sweetness and sourness. This combination is often derived from the ratio between TSS and TA (TSS: TA). The overall sensory sweetness of pomegranate juice depends on sugar types, namely fructose, glucose and sucrose, whereas its acidic tastes is as a result of its organic acids, mainly malic, tartaric and citric acids (Arendse *et al*., 2015).

1.5 Reactive oxygen species (ROS) responses to chilling

The storage of fruit at low temperature is essential in order to reduce fruit respiration, thus avoiding post-harvest losses (Valenzuela *et al*., 2017). Fruits are structures with high respiratory activity during respiration so they use oxygen to generate energy. However, cold storage generates chilling injury in many fruits, in part by inducing uncoupling in the respiratory chain thus giving rise to ROS production (Valenzuela *et al*., 2017). The term ROS defines all the molecules that are derived from the molecular oxygen (O_2) that are more reactive than O_2 itself (Mhamdi *et al*., 2010). ROS refers to free radicals such as superoxide (O_2^-) and the hydroxyl radical (OH), but also to non-radicals like singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) (Huang *et al*., 2012; Valenzuela *et al*., 2017).

Chloroplasts, mitochondria and peroxisomes are a major source of ROS in plant cells. The role ROS plays in the physiology of a plant can be compared to a double-edged sword since they are important secondary signalling molecules at low levels, but at the same time, they are toxic products of aerobic metabolism when they accumulate to high levels within cells during stress (Huang *et al*., 2012). As a result of the toxicity of ROS, many fruits have evolved efficient enzymatic antioxidant defence mechanisms (Gill and Tuteja, 2010). Nonetheless, the equilibrium between the production and scavenging of ROS either by the enzymatic or non–enzymatic means may be disturbed by chilling injury and other environmental stresses (Caverzan *et al*., 2016). During chilling injury to be specific, the disturbances in equilibrium cause an abrupt increase cellular oxidative potential, which has a potential to damage many cell components including proteins, lipids and DNA (Caverzan *et al*., 2016). According to Lee and Lee (2000) major targets of chilling injury are cell membranes.

Sometimes during stress, the ability of the plant to detoxify ROS may become weak thus leading to ROS levels increasing beyond the capacity of the plant to scavenge them. This causes lipid peroxidation (LP) in biological membranes to increase, in that way affecting the physiological processes of the cell (Sharma *et. al*., 2012). During the modification of lipids, malondialdehyde (MDA) forms. It is an end product of the oxidative modification of lipids, and it is responsible for cell membrane damage including changes to the intrinsic properties

of the membrane, such as fluidity, ion transport, loss of membrane-bound enzyme activity and protein cross–linking. These changes eventually result in cell death (Sharma *et. al*., 2012).

1.5.1 Production of hydrogen peroxide (H_2O_2)

Hydrogen peroxide is one of the key signalling compounds involved in responses to chilling and it is the most stable ROS (Lin *et al*., 2011; Ślesak *et al*., 2007). H_2O_2 has two effects such as leading to cell death when it is at high concentrations or used as a signalling molecule at low concentrations (Lin *et al*., 2011). However, according to Ślesak *et al*. (2007) plants are tolerant to high concentrations of H_2O_2 since their antioxidant response system is made to scavenge H_2O_2 at high levels thus maintaining the cellular redox state. Since plants tolerate high levels of H_2O_2 it can be assumed that H_2O_2 cannot only act as a signalling molecule (in physiological processes such as photosynthesis, respiration, translocation and transpiration) since signalling molecules are mostly present in low concentrations (Ślesak *et al*., 2007; Siti *et al*., 2015). This brings about an assumption that H_2O_2 also plays a key role in primary plant metabolism as well as controlling the expression of various genes (Ślesak *et al*., 2007).

Hydrogen peroxidase produces NADPH oxidase as a major source during its systemic signalling responses (Si *et al*., 2018). Respiratory burst oxidase homolog (RBOH)-mediated H_2O_2 production is suggested to be the first response to environmental stresses and it is essential for systemic translocation of the signal, in this manner triggering responses of plants to deal with future stress events such as cold, heat, high light intensity and salinity (Si *et al*., 2018). H_2O_2 is normally produced when plants are responding to external stimuli such as an environmental stress, which can be biotic or abiotic stresses. During external stimuli, H_2O_2 is used by the plant to trigger the antioxidant systems and this could lead to tolerance to the consequent chilling stress (Krasensky-Wrzaczek and Kangasjärvi, 2018). Not only does H_2O_2 play a role in controlling many stress responses but it also plays a role in physiological adjustment in response to the stress. According to Ślesak *et al*., (2007) the rate at which H_2O_2 is produced depends on how long the stress takes and how severe it is. For example, chilling stress as a result of accumulation of high levels of H_2O_2 over a long period of time can cause oxidative stress (Lin *et al*., 2011).

H_2O_2 can be formed by a two-electron reduction process of O_2, catalysed by certain enzymes known as oxidases or indirectly via reduction or dismutation of O_2^- that is formed by oxidases, peroxidases, or by photosynthetic and respiratory electron transport chains (Mhamdi *et al*., 2010). These reactions lead to the formation of H_2O_2 in numerous subcellular compartments of the cell; thus, the impact of H_2O_2 will be influenced by the degree to which the antioxidative system scavenges this ROS (Mhamdi *et al*., 2010). In peroxisomes, H_2O_2 can be formed directly from O_2 by photorespiratory glycolate oxidase or by other enzyme systems such as xanthine oxidase and superoxide dismutase (Mhamdi *et al*., 2010).

Moreover, H_2O_2 is not a free radical as it does not have any unpaired electrons. However, it is a precursor to certain radical species including the peroxyl radical, hydroxyl radical, and superoxide (Kurutas, 2015). One of its most important characteristics is the ability to be mobile thus crossing cell membranes freely contrary to the superoxide radical. The effect of H_2O_2 mobility is defined as the "greatest toxic effect" since it can be formed in one location of the plant and move to distant parts of the plant before decomposing to yield the highly reactive hydroxyl radical. In this way, it acts as a channel to transmit free radical-induced damage to cells (Kurutas, 2015). Early studies have shown that during abiotic stresses, high H_2O_2 levels have adverse impact in plants including membrane lipid peroxidation, toxicity and cell death (Si *et al*., 2018).

1.5.2 Lipid peroxidation as a result of ROS accumulation

Lipid peroxidation is a process by which oxidants such as free radical and non-radical species form an attack against lipids that have carbon-carbon bonds (Ayala *et al*., 2014). LP produces numerous oxidation products. Among the LP products, lipid hydroperoxides (LOOH) are the main primary products and malondialdehyde (MDA) is the most mutagenic product (Ayala *et al*., 2014). Malondialdehyde known as a product of lipid peroxidation and is used as an indicator of stress in some tissues (Valenzuela *et al*., 2017). Malondialdehyde is generated by an end-product of decomposition of arachidonic acid and larger polyunsaturated fatty acids (PUFAs), through enzymatic or non-enzymatic processes. Malondialdehyde may also have activities in redox signalling (Ayala *et al*., 2014). Cells have a potential to promote cell survival

or initiate cell death according to the how they respond to membrane LP and according to specific cellular metabolic circumstances and repairing capacities death (Ayala *et al*., 2014).

At low lipid peroxidation rates, the cells are able to maintain survival via antioxidant defence systems or via activation of signalling pathways that enhance antioxidant proteins, resulting in tolerance to the stress (Sharma *et al*., 2012). However, during high levels of lipid peroxidation, the degree of oxidative damage overcomes the capacity and they initiate either apoptosis or necrosis (Sharma *et al*., 2012). A decline in lipid stability may lead to loss of membrane structural integrity and membrane fluidity that consequently affects proper functions of proteins by either direct attack on the proteins by ROS or loss of activity due to an unfavourable lipid membrane environment (Shewfelt and Del Rosario, 2000; Sharma *et al*., 2012).

Failure of a critical membrane-associated enzyme would lead to metabolic imbalances within the cell manifested at the tissue level as visible symptoms of a disorder (Shewfelt and Del Rosario, 2000). The symptoms would be dependent on the membrane affected, its role in cell physiology, and the type of tissue affected (Shewfelt and Del Rosario, 2000). The general process of lipid peroxidation occurs in three phases; namely the initiation phase, propagation phase and termination phase (Ayala *et al*., 2014). During the initiation step, the pro-oxidants like the hydroxyl radical abstract the allylic hydrogen to form the carbon-centred lipid radicals. During the propagation phase, lipid radicals rapidly react with oxygen to produce a lipid peroxy-radical, which extracts a hydrogen molecule from another lipid molecule, thus generating a new lipid radical that will continue the chain reaction and LOOH. During the termination phase, antioxidants donate a hydrogen atom to the lipid-peroxy radical species and form a corresponding vitamin E radical that reacts with another lipid peroxy-radical forming a non-radical product (Alaya *et al*., 2014).

When the susceptibility of fatty acids increases as a result of oxidation by ROS, increases the rate of desaturation increases, thus the membranes rich in PUFAs are susceptible to oxidative damage (Schmid-Siegert *et al*., 2016). The active sites of the formation of ROS, which are the mitochondria and chloroplasts, have a high oxidative metabolism and contain high percentages of PUFAs (Ayala *et al*., 2014). For instance, thylakoid membranes in chloroplasts

are susceptible to photo-oxidative damage by singlet oxygen ($^1O_2$) that is generated through the interaction of excited chlorophyll with oxygen (O_2). Moreover, the major site of $^1O_2$ generation in chloroplasts, photosystem II, is surrounded by PUFA-rich lipids and, extraordinarily, two thirds of the fatty acids in thylakoids are tri-unsaturated fatty acids, primarily α-linolenic acid. A further layer of protection that uses PUFA-rich membranes as structural antioxidants exists (Ayala *et al*., 2014).

Moreover, it is assumed that membrane-lipid peroxidation is a point of commonality of many storage disorders and it could be caused by a number of different stresses and manifest itself in a number of different external symptoms (Shewfelt and Del Rosario, 2000). The simplicity of a common point in the mechanism is covered by the complexity of membrane response as affected by composition of lipids and antioxidants, function of key enzymes and their sensitivity to the physical environment of the membrane. On the other hand, elicitation of antioxidants and defence or repair systems in response to an initial stress could limit the peroxidation normally elicited in unconditioned tissue or in response to a subsequent stress (Shewfelt and Del Rosario, 2000). Enzymes important in protecting membranes from lipid peroxidation include catalase, peroxidase, and superoxide dismutase. Increased lipid peroxidation has been observed in the development of chilling injury in many crops, such as cucumber (*Cucumis sativus* L.) and zucchini squash (*Curcurbita sp.*) (Shewfelt and Del Rosario, 2000).

1.6 Antioxidant enzymes' response to chilling

Pomegranates have tremendous health benefits that are mainly due to their high antioxidant capacity (Fawole and Opara, 2013). The health benefits are influenced by the pomegranates' high content and unique composition of soluble polyphenolic compounds (Fawole and Opara, 2013). An antioxidant component is not necessarily a medicinal component, however, when it is consumed even at small amounts it has beneficial effects on overall human health due to its nutritional value (Gumienna *et al*., 2016). It has been proven that addition of antioxidants in a daily diet can lead to reduction of chronic diseases (Gumienna *et al*., 2016). In food, antioxidants maintain stability and thus protect polyunsaturated fatty acids; they have

reactions with free radicals, chelation of metals and disturbing or interrupting the propagation phase of lipid oxidation. Antioxidants also influence sensory qualities of fruit-derived products. This is confirmed by the tart taste caused by their high contents of polyphenols in pomegranates (Gumienna *et al*., 2016).

Moreover, antioxidants exhibit qualities that render them advantageous to human health, for example inhibition of oxidation reactions, binding pro-oxidative substances, inhibition of enzymes involved in oxidation reactions or scavenging free radicals (Gumienna *et al*., 2016). Antioxidants that are found in plants have many functions and are known as secondary metabolites. They are substances that attract insects and act as components to protect against UV radiation, or factors regulating osmotic pressure, as well as exhibiting an astringent effect (Gumienna *et al*., 2016).

Despite the importance of antioxidants, they can be affected by storage of fruits at low temperatures since low temperatures are known to cause chilling injury after a prolonged storage (Valenzuela *et al*., 2017). Storage at low temperature causes a decrease in antioxidant enzymatic activity by decreasing the fruits' ability to tolerate increased ROS production which leads to oxidative stress. Oxidative stress is initiated through high production of ROS due to a number of processes such as photosynthesis, respiration, photorespiration and oxidative burst, which occur at different cellular locations (Valenzuela *et al*., 2017). If the production of ROS increases intensely, as occurs under environmental stress, hydroxyl radical reacts with membrane lipids, inducing peroxidation, which leads to membrane degradation and thus leads to visible signs of chilling injury (Figure 1.4) (Jan *et al*., 2019).

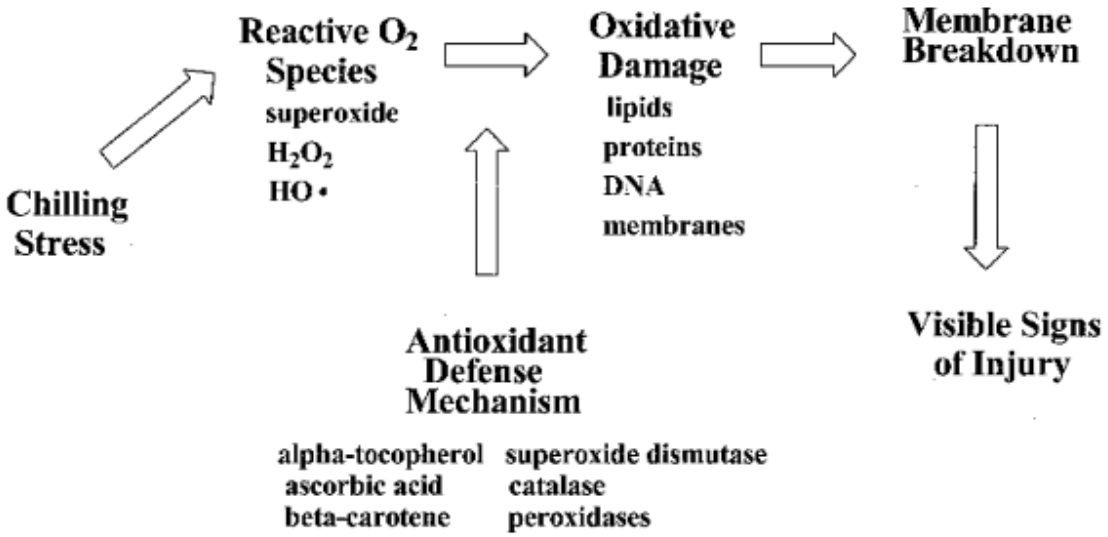


Figure 1.4: Demonstration of how chilling induces ROS and how it leads to visible signs of chilling injury.

When Chilling stress occurs as a result of ROS, the antioxidant defence mechanism is switched on to balance. However, if the ROS level becomes higher damage to the lipids is caused which lead to membrane breakdown that causes visible signs of injury. Source: Jan *et al.* (2019).

In addition, treatments for decreasing damage in fruits caused by storage at low temperatures in fruits that are sensitive to low temperatures have relied on reducing oxidative stress by increasing antioxidant enzyme defence mechanisms (Valenzuela *et al*., 2017). The environmental and physical changes that the fruit undergoes pre- and post-harvest can significantly increase the production of ROS, which causes oxidative damage thus inducing decay of the product and loss of quality post-harvest. During post-harvest, especially of immature fruits, oxidative stress avoidance is substantial for the maintenance of fruit quality. To avoid or tolerate stress Valenzuela *et al*., (2017) advised 2 ways in which these can be achieved, generating a response that includes metabolic changes at biochemical and at molecular levels (Valenzuela *et al*., 2017). These changes can be further divided into 3 categories namely: (i) the induction of the enzymatic, (ii) non-enzymatic antioxidant defence mechanisms and (iii) enzymes that belong to the Halliwell-Asada cycle (Valenzuela *et al*., 2017).

During the Halliwell-Asada cycle, ascorbate peroxidase (APX) involves the use of ascorbate to reduce H_2O_2 (Valenzuela *et al*., 2017). The oxidized ascorbate is recycled by

monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) yielding oxidized glutathione. At this point, oxidized glutathione is reduced by glutathione reductase (GR). This cycle occurs in the chloroplast and cytosol and in the mitochondria (Valenzuela *et al*., 2017; Lee and Lee, 2000). Furthermore, the detoxification of cellular H_2O_2 in this cycle acts as a crucial defensive mechanism against harmful reactive oxygen species. Non-enzymatic antioxidants, involve numerous metabolites in plants that can be used as ROS defence mechanisms, these include carotenoids, phenolic compounds, ascorbate and glutathione (Valenzuela *et al*., 2017).

During the antioxidant defence mechanism H_2O_2 is produced from the detoxification of superoxide anions by superoxide dismutase (SOD) in the chloroplast and cytosol (Valenzuela *et al*., 2017, Lee and Lee, 2000). The resultant product, H_2O_2, can be scavenged by CAT and several peroxidases (POD) such as thioredoxin peroxidase and glutathione peroxidase (Valenzuela *et al*., 2017). Antioxidants can be grouped into three categories namely: (i) lipid soluble and membrane-associated tocophenols, (ii) water soluble reductants such as ascorbic acid and glutathione, and (iii) antioxidant enzymes such as SOD, catalase, peroxidase, APX and GR (Lee and Lee, 2000).

In addition, fruits that experience high rates of respiration have an additional system known as alternative oxidase (AOX) that can reduce the production of ROS in mitochondria (Valenzuela *et al*., 2017). Alternative oxidase provides an alternative electron flow route for electrons passing through electron transport chain to reduce oxygen when the chain is saturated or suffers some damage. However, if all methods mentioned above fail to maintain ROS, the production of ROS may exceed the capacity of the plant tissue to maintain cellular redox homeostasis and cause oxidative stress. The oxidative stress in fruit can be detected either directly as accumulation of ROS, MDA, and the appearance of brown pigments associated with electrolyte leakage, or indirectly as changes in enzymatic and non-enzymatic antioxidant systems (Valenzuela *et al*., 2017).

1.6.1 Catalase (CAT)

Catalase was the first antioxidant enzyme to be found and identified (Sharma *et al*., 2011). It is a ubiquitous, tetrameric, heme-containing enzyme that catalyses the dismutation of 2 molecules of H_2O_2 into H_2O and O_2. Sharma *et al*., (2012) suggested that CAT has higher affinity for H_2O_2 compared to APX, however, this is contradictory to a study by Lee and Lee, (2000) who showed that CAT has a low affinity for H_2O_2 when compared to APX. Despite the controversy, unlike other antioxidant enzymes, CAT does not need a reducing equivalent to scavenge H_2O_2. According to Sharma *et al*., (2012), H_2O_2 is mainly produced in the peroxisomes where CAT is normally found. However, Lee and Lee, (2000) suggested that H_2O_2 diffuses into the cytosol and only then it diffuses to the peroxisomes. There are reports that CAT is also present in the cytosol, chloroplast and mitochondria, although there is poor understanding and little information about the CAT activity in these parts (Sharma *et al*., 2011).

Catalase is classified into three categories, the first one being Class I CATs which are expressed in photosynthetic tissues and are regulated by light, Class II CATs are expressed at high levels in vascular tissues while Class III CATs are highly abundant in seeds and young seedlings (Sharma *et al*., 2011). Hydrogen peroxide has been shown to be involved in many stress conditions. Under stressful conditions the cells may produce toxic levels of H_2O_2, which will be scavenged by CAT in an energy efficient manner. CAT is very important for maintaining the redox balance during the oxidative stress. Abiotic stresses can either increase or deplete CAT activity, depending on how intense the stress is, how long it lasts and the type of stress the cells are going through. Salt stress decreases the level of CAT activity, whereas chilling stress does not (Sharma *et al*., 2011).

1.6.2 Ascorbate peroxidase (APX)

Ascorbate peroxidase, which is a part of ascorbate-glutathione (AsA-GSH) is one of the most vital ROS scavenging antioxidant enzymes (Sharma *et al.,* 2011). In order to reduce H_2O_2 to H_2O and 2 molecules of monodehydroascorbate (MDHA), APX uses 2 molecules of ascorbate (Sharma *et al.,* 2011). It is found in many cells of the plant as compared to other antioxidant enzymes (Sharma *et al.,* 2011). APX is said to be an efficient H_2O_2 scavenger during stress due to its ability to scavenge H_2O_2 efficiently as compared to CAT (Sharma *et al.,* 2011). In other words APX has a higher affinity for H_2O_2 and it belongs to heme peroxidases in the Class I super-family, as well as, being regulated by redox signals and H_2O_2 (Sharma *et al.,* 2011).

According to amino acid sequences, there are only 5 distinct isoenzymes in terms of chemical and enzymatic characteristics that belong to APX (Sharma *et al*., 2011). These were found at different subcellular localisations in higher plants. These are known as cytosolic, stromal, thylakoidal, mitochondrial and peroxisomal isoforms. APX scavenges H_2O_2 in the specific regions/organelles where they are found. However, this is with the exception of the cytosolic APX, which scavenges H_2O_2 found in the cytosol, apoplast or H_2O_2 that is diffused from organelles (Sharma *et al.,* 2011). Sharma *et al*. (2011) reported that the overexpression of a cytosolic APX-gene derived from pea (*Pisum sativum* L.) in transgenic tomato plants (*Solanum lycopersicum* L.) had a capability to increase tolerance to oxidative injury that is caused by chilling and salt stress. Furthermore, the overexpression of the thylakoidal APX gene in either tobacco or in *Arabidopsis* had a capability to increase tolerance to oxidative stress (Sharma *et al.,* 2011).

1.6.3 Dehydroascorbate reductase (DHAR)

Dehydroascorbate reductase (DHAR) is the main enzyme that recycles ascorbate, which catalyses the glutathione (GSH)-dependent reduction of oxidized ascorbate (dehydroascorbate, DHA) (Asada, 1999; Mittler, 2002). This enables DHAR to regenerate ascorbate and detoxify ROS. Ascorbate is a well-known cofactor in enzymatic reactions and it is an important antioxidant enzyme for defensive mechanisms against oxidative stress (Do *et*

al., 2016).. However, ascorbate (AsA) is always oxidized through the scavenging of ROS generated by environmental stressors such as drought, salinity, ultraviolet (UV) light and extreme temperatures (Do *et al*., 2016).

Ascorbate is a major antioxidant that plays a role in several functions of plants (Do *et al*., 2016). Ascorbate indirectly detoxifies ROS, e.g. superoxide, $^1O_2$, ozone, and H_2O_2, which are produced during aerobic metabolic processes such as photosynthesis or respiration (Do *et al*., 2016). Not only does AsA play a role in the is regulation of cell elongation and progression but it also plays a part in the regeneration of α-tocopherol also known as vitamin E from the tocopheroxyl radical (Do *et al*., 2016). In addition, AsA is a cofactor for enzymes such as prolyl and lysyl hydrolases and ethylene-forming enzyme, and for 2-oxoacid-dependent dioxygenases essential for the production of abscisic acid and gibberellic acid (Do *et al*., 2016).

Once used, AsA is oxidized to MDHA radical that can be reduced to AsA in the chloroplast or cytosol by MDHAR in an NAD(P)H-dependent reaction (Figure 1.5.1) (Do *et al*., 2016). In the chloroplast, MDHA radical can also be reduced to AsA by thylakoid-associated ferredoxin that is more effective than reduction by MDHAR (Do *et al*., 2016). Dehydroascorbate (DHA) is then reduced to AsA by DHAR in a reaction requiring glutathione. Because the apoplast contains low levels of glutathione or DHAR, DHA, which predominates in the apoplast, must re-enter the cell for reduction to AsA (Do *et al*., 2016). In the absence of sufficient DHAR, however, DHA undergoes irreversible hydrolysis to 2 AsA (Do *et al*., 2016). It has been reported that the overexpression of DHAR increased grain yield and biomass in transgenic sweet potato, rice, tomato, acerola and tobacco plants (Do *et al*., 2016).

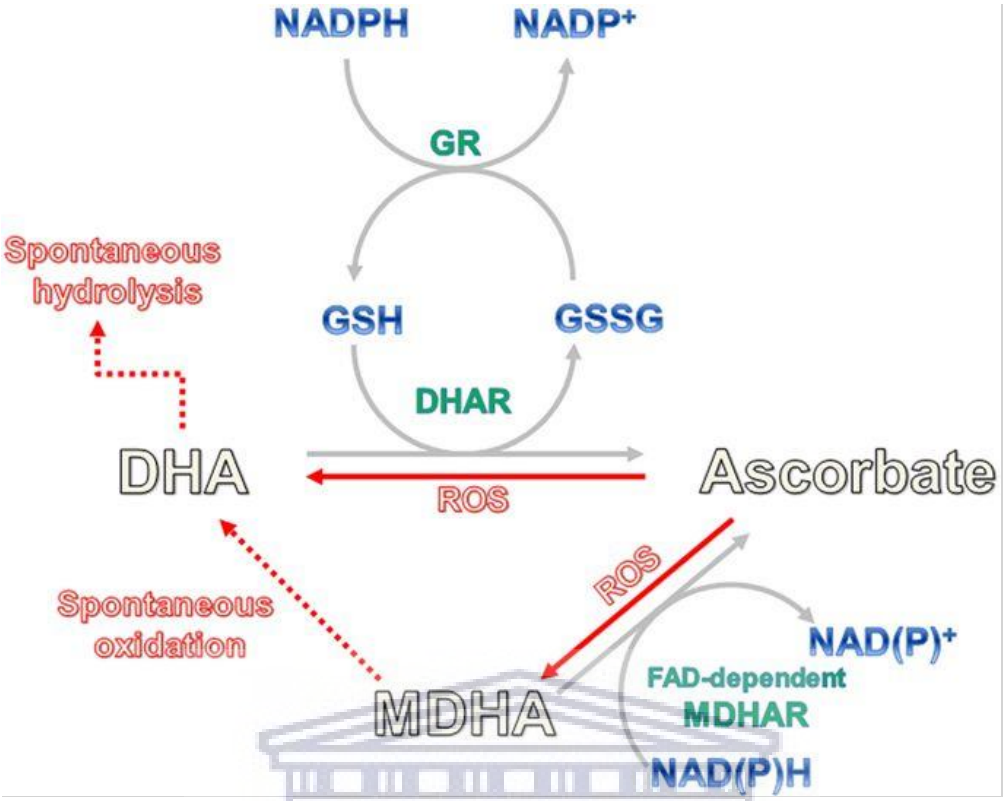


Figure 1.5.1: Halliwell-Asada Pathway depicting enzymatic and non-enzymatic scavenging of ROS.

Ascorbate is oxidized by ROS and converted into two different oxidized forms. The 2 forms consist of dehydroascorbate (DHA) and monodehydroascorbate (MDHA): The ascorbate (AsA) recycling is catalysed by three enzymes namely glutathione (GSH)-dependent dehydroascorbate reductase (DHAR), FAD-dependent monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR). Source: Do *et al.,* (2016).

1.6.4 Superoxide dismutase (SOD)

Superoxide dismutase (SOD) plays central role in defence against oxidative stress in all aerobic organisms (Yousuf *et al.,* 2011). The enzyme SOD belongs to the group of metalloenzymes and catalyses the dismutation of O_2^- to O_2 and H_2O_2 (Figure 1.5.2 a) (Yousuf *et al.,* 2011). Superoxide dismutase is present in most of the subcellular compartments that generate activated oxygen (Yousuf *et al.,* 2011). Superoxide dismutase is classified into three isozymes, namely copper/zinc SOD (Cu/Zn-SOD), manganese SOD (Mn-SOD) and iron SOD (Fe-SOD) (Yousuf *et al.,* 2011). All forms of SOD are nuclear encoded and targeted to their respective subcellular compartments by an amino terminal targeting sequence (Yousuf *et al.,* 2011).

Manganese superoxide dismutase is localized in mitochondria, whereas Fe/SOD is localized in chloroplasts. Copper/zinc superoxide dismutase (Figure 1.6.2 b) is found in the cytosol, chloroplast, peroxisome and mitochondria (Yousuf *et al.,* 2011). Eukaryotic Cu/Zn-SOD is cyanide sensitive and present as a dimer, whereas Mn-SOD and Fe-SOD are cyanide insensitive and may be dimers or tetramers (Yousuf *et al.,* 2011). SOD activity increases in plants exposed to various environmental stresses, including drought and metal toxicity (Sharma *et al*., 2011; Yousuf *et al.,* 2011).

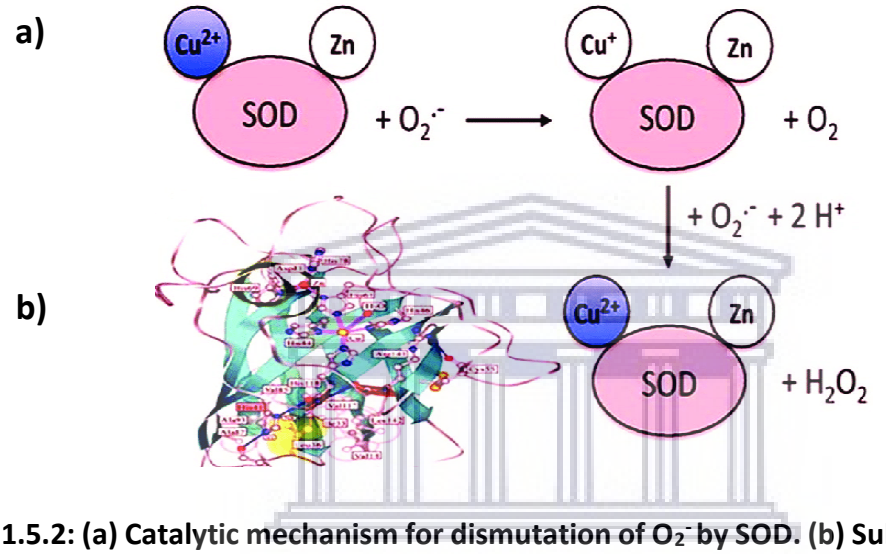


Figure 1.5.2: (a) Catalytic mechanism for dismutation of O_2^- by SOD. (b) Subunit structure of Cu/Zn-SOD structure.

Source: Younus (2018).

Increased activity of SOD in plants is often associated with an increase in tolerance against environmental stresses (Sharma *et al.,* 2011). Sharma *et al.,* (2011) suggested that SOD can be used as an indirect selection criterion for screening chilling-sensitive plant materials. Superoxide dismutase plays a major role in protecting the cells against oxidative stress by conversion of O_2^- to O_2 and H_2O_2. However, the idea that the superoxide radical is a potent cell-damaging agent has remained controversial for a long time because O_2^- is not very reactive (Benov, 2001; Sharma *et al.,* 2011). It has been proposed that the superoxide radical causes cell damage by indirectly participating in the iron-mediated Haber-Weiss reaction or superoxide-driven Fenton chemistry to generate the highly reactive hydroxyl radical (HO') (Sharma *et al.,* 2011).

1.7 Adjustment of temperature as a possible solution for CI in pomegranate

A useful way to extend the shelf life of pomegranates is to adjust the environmental conditions that will maintain the quality of the fruits within economic limitations. Previous studies have shown that temperature is the most significant factor in controlling the respiratory activity, transpiration and the growth of microbial pathogens (Serry, 2019). This temperature has to be around 5^{o}C to prevent the physiological disorders from occurring during 2-3 months storage. Pomegranates are susceptible to chilling injury when stored for more than 2 months at a temperature below 5^{o}C (Serry, 2019).

However, since in technological approaches the biochemical control of senescence and chilling injury is poorly understood, some studies have a tendency to manipulate temperature (Shewfelt and Del Rosario, 2000). Response to one type of stress may change how other forms of stresses respond. Despite the knowledge gap, experimental studies that try to optimise storage conditions have been remarkably effective at extending shelf life of fresh fruits while minimising storage disorders (Shewfelt and Del Rosario, 2000).

This thesis reports on a study which investigated the influence of temperature on ROS-induced oxidative stress in pomegranate cv. 'Wonderful' and examining activities of ROS-scavenging antioxidant enzymes (catalase, ascorbate peroxidase, dehydroascorbate reductase and superoxide dismutase) in relation to chilling injury. To date, there have been no studies about antioxidant enzyme isoform-specific activity assays (mentioned above) to understand how different isoforms of these antioxidant enzymes are associated with chilling injury in pomegranate cv. 'Wonderful'. This study will provide new insights into the protective mechanisms against chilling injury in pomegranate cv. 'Wonderful'.

CHAPTER 2: MATERIALS AND METHODS

The chemicals used in this research were bought from Sigma-Aldrich unless stated otherwise.

2.1 Fruit sampling and storage conditions

Fully ripened pomegranate fruit cv. 'Wonderful' were handpicked during the commercial harvest period in March 2018 at Bovenvlei farm in the Western Cape, South Africa. A total of 200 fruits were selected and randomly divided into four groups. Each group had 40 fruits which were packed inside standard open top cartons with dimensions of 0.4 m long, 0.3 m wide and 0.133 m high and each carton had a total of 22 perforations. Each group, comprising of 2 cartons (twenty fruits per carton) were stored at the following conditions: 4°C and 6.5°C for a total period of 5 months. The seeds and arils were harvested from the fruits that were stored at 4°C and 6.5°C during the 1st month, 2nd month and 5th month of storage. Each time when the seeds and arils were harvested they were frozen in liquid nitrogen and ground using a mortar and pestle, and stored at -80°C for further experiments.

The control/fresh seeds and arils in this experiment were harvested from the fruits that were straight from the farm (apart from the ones mentioned above). The seeds and arils were frozen in liquid nitrogen, ground and stored at -80°C for future experiments.

2.2 PHYSIOLOGICAL DISORDER MEASUREMENTS: Visual quality post-harvest

Secondary disease index (SDI) measurements which included external decay were performed according to Wang and Zhu (2017) with minor modifications. Secondary disease index was evaluated using a hedonic scale based on percentage of fruit surface with decay where 0= (no signs of surface decay), 1= (<25%), 2= (25%), 3= (50%), 4= (75%) and 5= (>75%). Intermediate values were assigned when appropriate. The external decay was calculated using the equation below:

$$\text{External decay} = \frac{number\ of\ fruits\ with\ CI}{total\ number\ of\ fruits\ recorded}\ \text{X}\ 100\%$$

Chilling injury (CI) of intact pomegranate fruit during storage at 4°C and 6.5°C for an overall period of 5 months was estimated using the score of CI symptoms (browning fleck) on the

fruit peel according to a revised method from Supapvanich *et al*., (2012). The CI score was rated using a hedonic scale of 1 to 5; where: 1= no CI symptom, 2= slight, 3= moderate, 4= moderately severe and 5 = severe. Intermediate values were assigned when appropriate.

2.3 OXIDATIVE STRESS STUDY

2.3.3 Trichloroacetic acid (TCA) extraction

Hydrogen peroxide content was measured according to a method modified from Junglee *et al.,* (2014) and MDA concentration was measured according to a modified method based on Tatar and Gevrek (2008). The material was frozen and ground in liquid nitrogen. Approximately 100 mg of ground seeds and arils material (that were stored at 4^{o}C and 6.5^{o}C for a period of 1, 2 and 5 months of storage) were weighed and homogenized in 6% TCA. The samples were centrifuged at 12 000 rpm for 30 minutes at 4°C. Independent triplicates were conducted for each treatment.

2.3.3.1 Hydrogen Peroxide (H_2O_2) content

The H_2O_2 content was determined using a method by Velikova *et al.* (2000). Hydrogen peroxide content was assayed by homogenising 6% of TCA extract with 150 µl of reaction buffer containing 5 mM K_2HPO_4, pH 5.0 and 0.5 M KI. Reactions were incubated at 25^{o}C for 20 minutes, followed by recording of absorbance in triplicates at 390 nm. Hydrogen peroxide content was calculated using a standard curve constructed from the absorbance of H_2O_2 standards read at 390 nm. Independent triplicates were used for each treatment. The results were expressed as µmolg^{-1}FW.

2.3.3.2 Malondialdehyde (MDA) content

The thiobarbituric acid reactive substances (TBARS) assay is a method used for monitoring lipid peroxidation. To assess the MDA content a modified method by Terzi and Kadioglu, (2006) was used where 100 mg of each frozen sample was homogenized with 20% TCA, followed by addition of TBA to a final concentration of 5%. The mixture was vortexed and boiled at 90^{o}C for 20 minutes. The extracts were chilled on ice for 10 minutes and centrifuged at 13 000 rpm for 5 minutes. The thiobarbituric acid reactive substances (TBRAS), reflective of MDA, were detected by reading their absorbance at 532 nm and subtracting non-specific

absorbance at 600 nm. The amount of MDA was calculated using a molar extinction coefficient of 155$mM^{-1}cm^{-1}$. Independent triplicates were conducted for each treatment, with results expressed as $\mu molg^{-1}FW$.

2.4 ANTIOXIDANT ASSAYS

2.4.1 Protein extraction and protein quantification

Ground fruit tissue (seeds and arils) for each sample was homogenized in 5% polyvinylpolypyrrolidone (PVPP) (w/v) in extraction buffer [(40mM Potassium phosphate (KPO_4) (pH 7.4), 1 µM ethylenediaminetetraacetic acid (EDTA)] in a 3:1 ratio (buffer: tissue). The samples were centrifuged at 13 000 rpm at 4°C for 15 minutes. The supernatant was transferred to a new tube and quantified for protein concentration using the Pierce™ Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific). The protein standard curve was constructed using BSA (1mg/ml). The experiment was conducted in independent triplicates for each treatment / storage condition and the absorbances were read at 590 nm. The protein concentration was calculated using an equation derived from the standard curve.

2.4.1.1 Superoxide Dismutase (SOD) activity assay

The determination of SOD isoenzyme activity was performed according to a modified method of Beauchamp and Fridovich (1971). Three native PAGE gels were prepared with 12% separating gel [constituted of 40% acrylamide, 1.5M Tris-HCl pH 8.8, 10% ammonium persulfate (APS) and N, N, N', N'-tetramethylethylenediaminese (TEMED)] and 5% stacking gel (constituted of 40% acrylamide, 1 M Tris-HCl pH 6.8, 10% APS and TEMED) were prepared. The gels were solidified at room temperature. Protein extracts (250 µg) from each treatment were loaded into each well of the non-denaturing gel. The gels were electrophoresed on a running buffer that contained 25 mM Tris, pH 8.3, and 192 mM glycine. The electrophoretic separation was performed at 4°C for 4 hours at a constant voltage of 80 V. After the electrophoresis was completed, the gels were incubated in the dark in 50 mM potassium phosphate (pH 7.8) buffer, and the two other gels were incubated in 50 mM potassium phosphate (pH 7.8) containing either 5 mM KCN or 6 mM H_2O_2 as inhibitors to aid in the identification of SOD isoforms.

To observe SOD activity, the gels were incubated in 5 mM phosphate buffer and 2.5 mM nitrotrezolium blue chloride (NBT) for 20 minutes in the dark with constant shaking. This was followed by incubation in 50 mM KPO_4 buffer (pH 7.8) containing 28 mM N, N, N, N-tetramethylethylenediamine (TEMED) and 28 mM riboflavin for 15 minutes on a light box. When achromatic bands were observed, the reaction was stopped by rinsing the gels with dH_2O.

2.4.1.2 SOD spectrophotometric assay

A method modified from Singh *et al.,* (2007) was used to determine superoxide dismutase activity. An appropriate (to give 100 μg of protein) volume of the protein samples were mixed with 20 mM phosphate buffer (pH 7.8) containing 0.1 mM nitrotrezolium blue chloride (NBT), 0.005 mM riboflavin, 10 mM L-methionine and 0.1 mM (EDTA) to a total volume of 200 μl. The mixture was incubated for 20 minutes at room temperature on a light box to initiate the reaction. Following the incubation, the samples were spectrophotometrically read at 560 nm. One unit of SOD is defined as the amount required to inhibit NBT photo-reduction by 50%.

2.4.1.3 Catalase (CAT) activity assay

The determination of CAT activity was performed according to a method modified from Yamashita *et al.,* (2007). A native PAGE gel was prepared with 7% separating gel (constituted of 40% acrylamide, 1.5M Tris-HCL pH 8.8, 10% APS and TEMED) and 5% stacking gel (constituted of 40% acrylamide, 1M Tris-HCL pH 6.8, 10% APS and TEMED). The gels were solidified at room temperature. A total of 250 μg protein extract for each sample was loaded into the native PAGE gel. The gel was equilibrated in running buffer containing 25 mM Tris and 192 mM glycine. Electrophoresis separation was performed at 4^{o}C for 6 hours at a constant voltage of 80 V. In order to identify CAT isoforms, the gel was washed three times with dH_2O under constant shaking for 10 minutes, followed by incubation in 0.006% H_2O_2 (v/v) for 10 minutes. The gel was stained with 2% ferric chloride (w/v) and 2% potassium hexoferricyanide (w/v) simultaneously. When the achromatic bands were observed, the stain was discarded and the gel was rinsed with distilled water.

2.4.1.4 CAT spectrophotometric assay

Catalase activity was determined using a method described by Aebi (1974). The protein sample of an appropriate volume to give 100 μg of protein was mixed with a reaction mixture of 50 mM potassium phosphate buffer (pH 7.0) that contained 1.5 mM EDTA. The reaction was initiated by adding 10 mM H_2O_2. CAT activity was measured at 240 nm. The CAT activity was calculated from the decomposition of H_2O_2 (extinction coefficient 39.4 $mM^{-1}cm^{-1}$). One unit of catalase was defined as the amount of enzyme which liberated half the peroxide oxygen from 10 mM H_2O_2 solution in 100 seconds at 25°C. The results were expressed as nmol H_2O_2 $seconds^{-1}$ mg^{-1}.

2.4.1.5 Ascorbate Peroxidase (APX) activity assay

The determination of APX activity was performed according to Mittler and Zilinskas (1993). The native PAGE gel was prepared with a 12% separating gel (constituted of 40% acrylamide, 1.5 M Tris-HCl pH 8.8, 10% APS and TEMED) and a 5% stacking gel (constituted of 40% acrylamide, 1 M Tris-HCl pH 6.8, 10% APS and TEMED). Upon solidification of the gels at room temperature, 250 μg of protein from each sample were loaded into the gel and equilibrated in running buffer containing 4 mM ascorbic acid, 25 mM Tris and 192 mM glycine. Electrophoresis separation was performed at 4°C for 4 hours at a constant voltage of 80 V.

Upon electrophoresis completion, the APX isoforms were identified following equilibration of the gel for 10 minutes in 50 mM KPO_4 (pH 7.0) containing 2 mM ascorbic acid. The stain was discarded and the gel was incubated for 10 minutes in the dark with constant shaking in 50 mM KPO_4 (pH 7.8) containing 4 mM ascorbic acid and 2 mM H_2O_2. The gel was incubated for 1 minute in 50mM KPO_4 (pH 7.8) and lastly, the gel was incubated for 10 minutes on a light box with gentle shaking in 50 mM KPO_4 buffer (pH 7.8) containing 28 mM TEMED and 0.5 mM NBT. The APX isoforms were visible as white bands on a dark blue background.

2.4.1.6 APX spectrophotometric assay

A method described by Singh *et al*. (2007) was used to determine the total ascorbate peroxidase activity. An appropriate volume of protein (to yield 100 μg for each sample) was mixed with 50 mM K_2HPO_4 containing 2 mM ascorbate, 10 mM H_2O_2 was added last to initiate the reaction. The spectrophotometer readings were taken immediately at 290nm for 1

minute. The total APX activity was calculated using the extinction coefficient of 2.8 mM^{-1} cm^{-1}.

2.4.1.7 Dehydroascorbate Reductase (DHAR) activity asssay

The determination of DHAR activity was performed according to a method described by De Tullio *et al*. (1998). A native PAGE gel was prepared with 7.5% separating gel [constituted of 40% acrylamide, 1.5 M Tris-HCl pH 8.8, 10% APS and TEMED) and 5% stacking gel (constituted of 40% acrylamide, 1 M Tris-HCl pH 6.8, 10% APS and TEMED). The gels were allowed to solidify at room temperature. A total of 250 µg protein extract for each sample was loaded into each well of the native PAGE gel. The gel was equilibrated in running buffer containing 25 mM Tris and 192 mM glycine. The electrophoresis separation was performed at 4^{o}C for 4 hours at a constant voltage of 80 V, until the loading dye had run off the gel, followed by continuation of the electrophoretic runs for a further 45 minutes. The gel was incubated for 20 minutes with shaking in 0.1 M KPO_4, pH 6.2, containing 4 mM glutathione (GSH) and 2 mM dehydro-L-ascorbate (DHA). The gel was washed with distilled water (dH_2O) for 30 seconds. Thereafter, the gel was stained in a light box with gentle shaking in a 0.125 M HCl (hydrochloric acid) solution containing 0.1% W/V ferric chloride and 0.1% W/V potassium hexoferricyanide until the formation of dark blue bands against a light blue background. The gel was rinsed with distilled water to stop the reaction.

2.5 Statistical analysis

The statistical validity of all the data was tested by means of a One-way Analysis of Variance (ANOVA) and the Tukey's multiple comparisons test at 5% level of significance was completed to compare the means, using GraphPad Prism 6.01 software.

CHAPTER 3: RESULTS

3.1 VISUAL QUALITY SCORE: Chilling affected CI score, caused external decay and led to mould growth

Pomegranate fruit is usually susceptible to chilling injury, which is visible as skin browning, skin pitting, husk scald, high sensitivity to fungal growth and susceptibility to decay (Martínez-Romero *et al*., 2012). The CI symptoms were visible on the rind of pomegranate (Figure 3.1 D-G) from the 1st month of storage until the end of storage at 5 months when the fruits were stored at 4°C. In the storage at 6.5°C, the CI symptoms were not visible in the 1st month and 2nd month of storage (Figure 3.1 D-F) but manifested during the 5th month of storage with fungal growth appearing on the crown of the fruit (Figure 3.1 G).

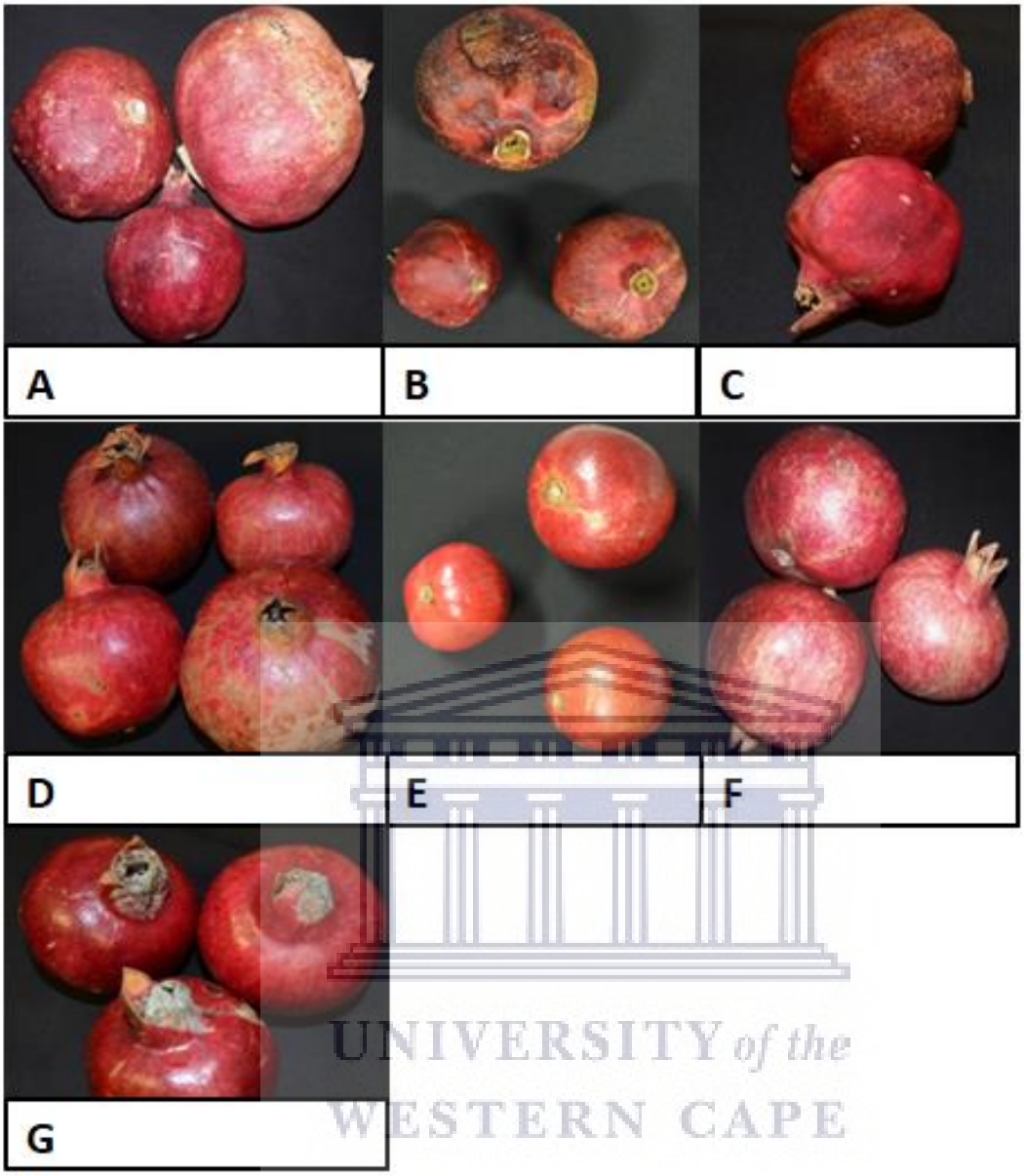


Figure 3.1: Appearance of chilling injury symptoms on cv. 'Wonderful' pomegranate.
Fruit exposed to chilling injury at 4°C for a period of 1 month (a), 2 months (b) and 5 months (c). Fruit stored at 6.5°C for a period of 1 month (d), 2 months (e)and 5 months (f) and mould growth on pomegranate crown stored at 6.5°C (g).

In order to quantify the chilling damage in pomegranate stored at 4°C and at 6.5°C over a period of 5 months, the external decay % (Figure 3.2 A and B) and chilling injury score (Figure 3.2 c and d) were conducted using a hedonic scale. The chilling index and external decay increased gradually during storage at both 4°C and 6.5°C with time, thus showing a directly proportional relationship. The external decay at 4°C increased by 27.5%, 31% and 47.3% compared to control during storage during the 1st month, 2nd month and 5th month of storage

respectively. At 6.5°C the external decay increased by 12.5%, 24.2% and 28% during 1st , 2nd and 5th month of storage respectively. It is worth noting that the external decay was higher in pomegranate that was stored at 4°C than the pomegranate that was stored at 6.5°C.

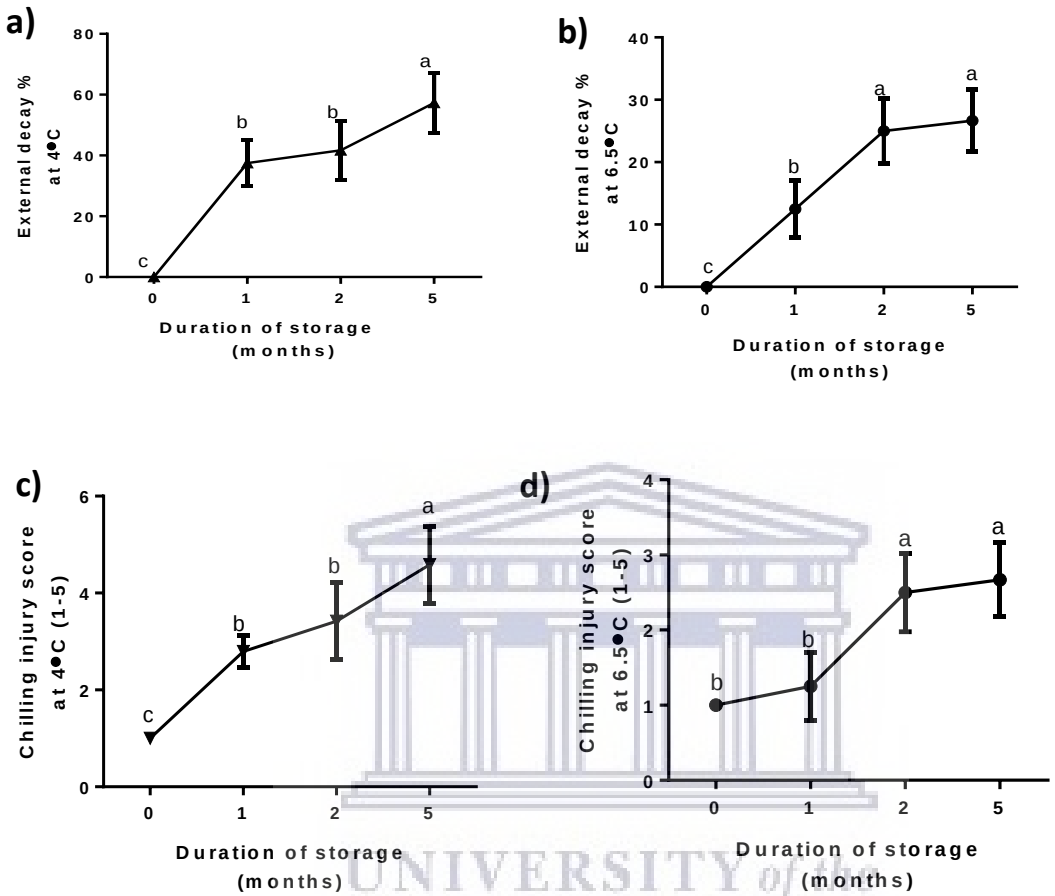


Figure 3.2: Changes in visual quality of pomegranate during a period of 5 months of storage. A) external decay in pomegranate at 4°C (▲) and b) external decay in pomegranate at 6.5°C (●) and chilling injury score of pomegranate at c) 4°C (▲) and d) chilling injury score in pomegranate at 6.5°C (●). A total number of 12 panels were asked to visually evaluate pomegranates for chilling injury score and external decay stored at 4°C and 6.5°C for 1 month, 2 months and 5 months, the control was a fresh fruit from the farm. The scores represent the standard deviation of the mean (*n* = 12). Scores: external decay, where 0= no signs of external decay, 1= 1-25%, score 2= 25%, 3= 50%, 4= 75% and 5= 75-100%. CI score: 1 = no CI symptom, 2= slight, 3= moderate, 4=slightly moderate and 5 =severe. Values different letters within the same figure are significantly different at P<0.05.

The same trend that was observed in the chilling injury was also observed in the CI score. Figure 3.2 c and d showed that chilling injury score at both temperatures increased gradually. Chilling injury score at 4°C increased by 3-fold, 3.3-fold and 4.5-fold during the 1st month, 2nd and 5th months of storage respectively compared to the control while at 6.5°C the CI score

increased by 1.3-fold, 2.4-fold and 2.8-fold at 1 month, 2 and 5 months of storage respectively compared to the control. The CI score was higher in the pomegranate that was stored at 4°C than in the pomegranate that was stored at 6.5°C.

3.2 OXIDATIVE STRESS STUDY: Oxidative stress affected pomegranate seeds and arils that were stored at 4°C more than those that were stored at 6.5°C

Figure 3.3 (a -d) represents H_2O_2 content and MDA content (to measure lipid peroxidation) in the seeds and arils of pomegranate stored at 4°C and at 6.5°C for 1, 2 and 5 months. Chilling injury triggered changes in H_2O_2 content both in the seeds and arils (Figure 3.3 A and B). After 1 month of storage the chilling injury was 1.6-fold higher as compared to the control at 4°C, while there was an increase at 6.5°C of 1.5-fold during the same period of storage, after 2 months of storage there was an observed increase of 2.1-fold while there was an increase at 6.5°C of 1.6-fold and lastly, at 5 months of storage the H_2O_2 content at 4°C was similar to the H_2O_2 content observed at the 2nd month of storage while the H_2O_2 content at 6.5°C increased by 1.9-fold. Figure 3.3 B shows H_2O_2 content in arils of pomegranate stored at 4°C and 6.5°C. After 5 months of storage, the H_2O_2 content increased by 1.15-fold at 4°C and increased by 1.05-fold at 6.5°C.

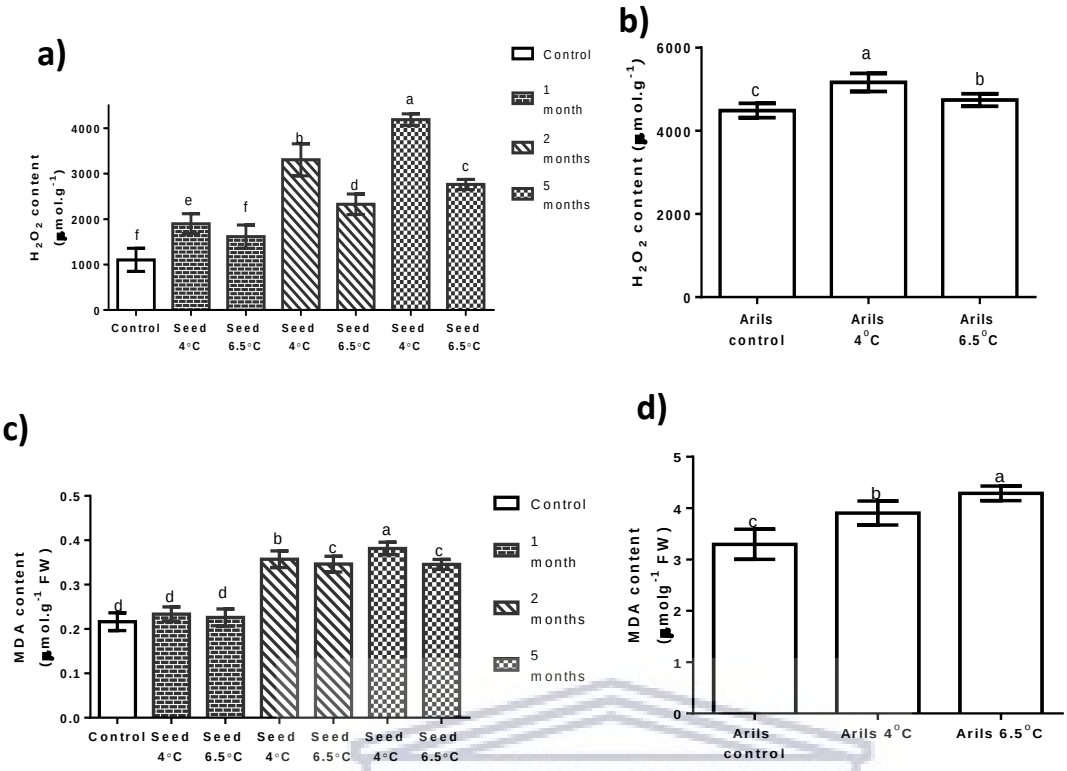


Figure 3.3: The effects of temperature and storage duration on H_2O_2 content.

H_2O_2 content was measured in seeds (a) and arils (b). Hydrogen peroxide was measured to estimate ROS accumulation. The effect of temperature and storage duration on lipid peroxidation (MDA) content seeds (c) and arils (d). Lipid peroxidation was measured as the indication of oxidative stress. Error bars represent the means ± SE; n= 3. Different letters indicate the significant difference between means at $p<0.05$ (Tukey's multiple comparisons test).

Chilling also triggered changes in lipid peroxidation (Figure 3.3 C and D) of both the seeds and the arils. The MDA content of the seeds that were stored at 4°C and 6.5°C during the 1st month of storage was not significantly different from the control. However, after 2 months of storage there was an increase of 1.7-fold at 4°C in MDA content while there was an increase of 1.6-fold at 6.5°C compared to the control. During the 5th month of storage, there was an increase of 1.8-fold in the pomegranate that was stored at 4°C in MDA content whereas there was a decrease of 1.7-fold in the pomegranate that was stored at 6.5°C compared to the control. Figure 3.3 d shows lipid peroxidation in arils of pomegranate stored at 4°C and 6.5°C. After 5 months of storage, the MDA content increased by 1.2-fold at 4°C and increased by 1.3-fold 6.5°C.

3.3 ANTIOXIDANT ENZYME ANALYSIS: Storage of pomegranate at 6.5°C reduces chilling

3.3.1 Superoxide dismutase activity was up-regulated by chilling stress

Superoxide dismutase activity of pomegranate fruit exposed to chilling injury was studied in the seeds and arils using native PAGE. Pomegranate fruits were stored at 4°C and 6.5°C, with the seeds and the arils of the pomegranate sampled at 1, 2 and 5 months of storage (Figure 3.4). A total of 7 (seven) SOD isoforms were identified, with Cu/Zn-SOD being the most abundant. The SOD isoforms were classified according to their response (being sensitive or resistant) to SOD inhibitors (5 mM KCN and 6 mM H_2O_2). The Cu/Zn-SOD-2 had the same activity in all storage conditions except at 6.5°C during the 2nd month of storage, where the activity was down-regulated and at 4°C during the 5 months storage where there was no activity observed.

Superoxide dismutase activity during the 1st month of storage remained the same for both 4°C and 6.5°C. However, at 2 months of storage, the SOD activity at 6.5°C was downregulated and it was upregulated at 5 months of storage during the same storage temperature. At 4°C during the 2nd month of storage, the SOD activity was upregulated and then it was downregulated at 5 months of storage with only Mn-SOD isoform visible. During the 5th month of storage Cu/Zn-SOD-5 was only observed in the arils that were stored at 6.5°C. Densitometry analysis was also performed using the Spot Denso tool of AlphaEaseFC in order to determine the pixel intensities of SOD isoforms (Figure 3.5 A- E) from the SOD native PAGE. The same trends observed in the in-gel assay were also observed in the densitometry analysis.

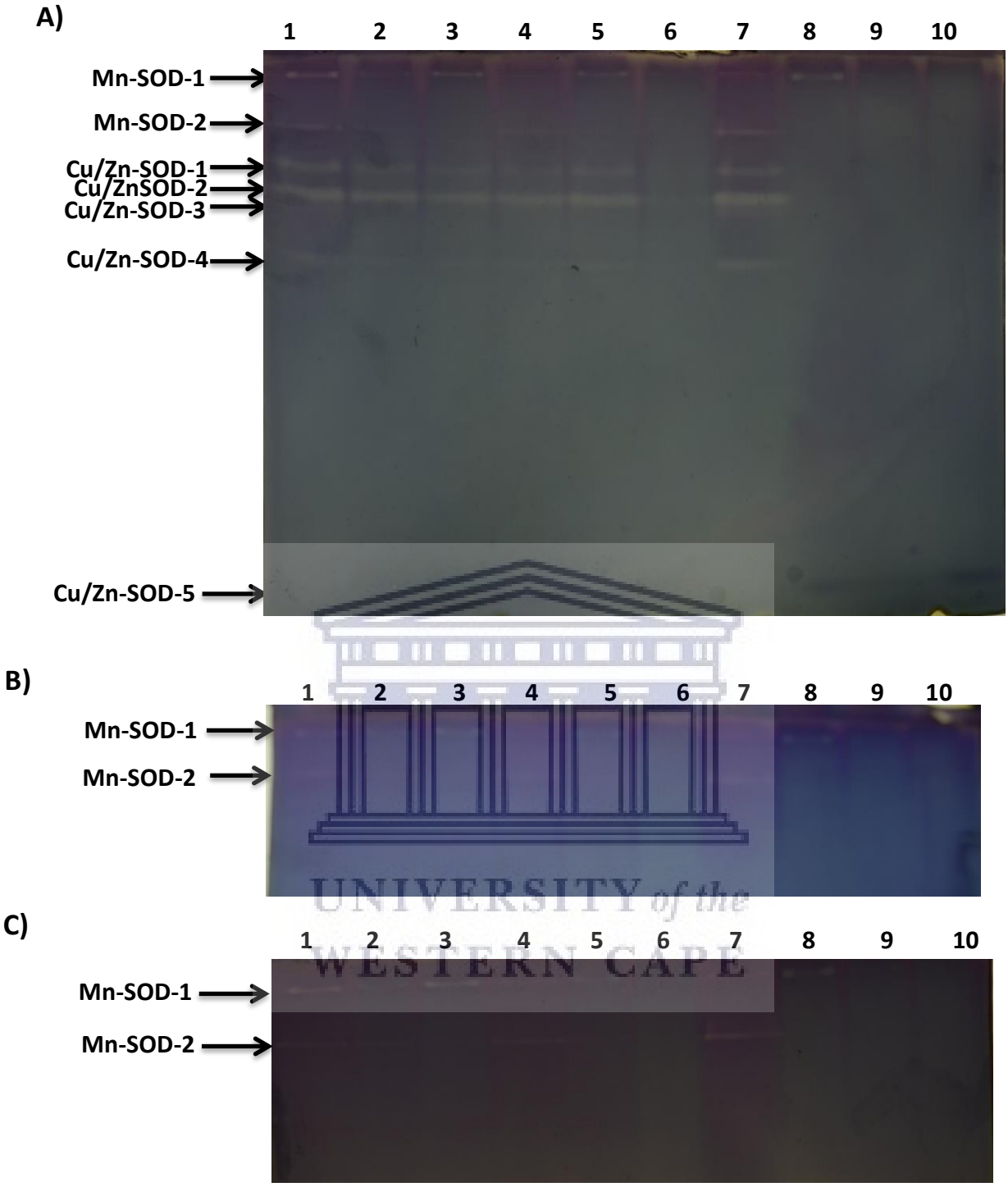


Figure 3.4: Determination of differences in superoxide dismutase activity in seeds and arils that were exposed to chilling stress.

In-gel assays showing (a) gel without isozyme inhibitors, (b) Gel with 5 mM KCN as SOD isoform Cu/Zn-SOD inhibitor and, (c) Gel with 6 mM H_2O_2 to inhibit both Cu/Zn-SOD and Fe-SOD. Lane 1: Control seeds, lane 2: Seeds stored for 1 month at 4^{o}C, lane 3: Seeds stored for 1 month at 6.5^{o}C, lane 4: Seeds stored for 2 months at 4^{o}C, lane 5: Seeds stored for 2 months at 6.5^{o}C, lane 6: Seeds stored for 5 months at 4^{o}C, lane 7: Seeds stored for 5 months at 6.5^{o}C, lane 8: Arils control, lane 9: Arils stored for 5 months at 4^{o}C and lane 10: Arils stored for 5 months at 6.5^{o}C.

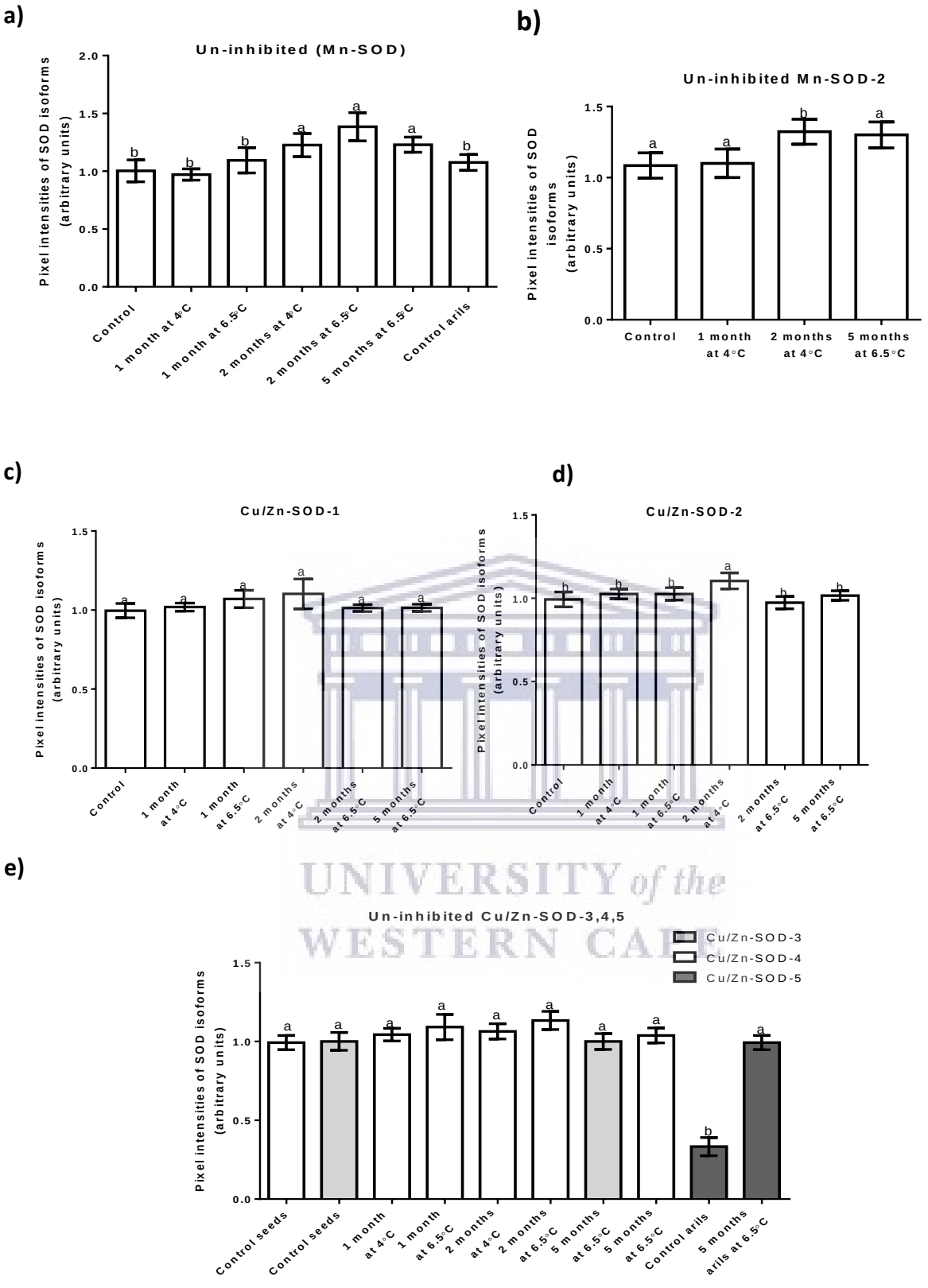


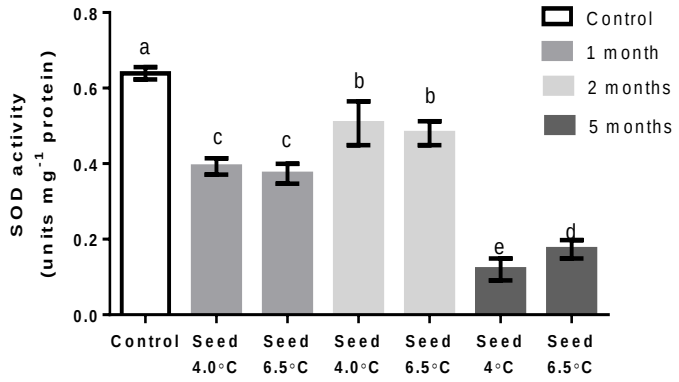
Figure 3.5: Pixel intensities of SOD isoforms in response to chilling.

Pixel intensities determined from SOD in-gel assay: Mn-SOD-1 (a), Mn-SOD-2 (b), Cu/Zn-SOD-1 (c), Cu/Zn-SOD-2 (d) and, Cu/Zn-SOD-3, 4 and 5 (e). The pixel intensities were measured from SOD native PAGE using the Spot Denso tool of AlphaEaseFC and the data was expressed

in arbitrary units. Error bars represent the means ± SE; n= 3. Different letters indicate the significant difference between means at $p<0.05$ (Tukey's multiple comparisons test).

The total SOD activity (Figure 3.6) was measured by a spectrophotometric assay, where the total SOD activity represented a combined action of Cu/Zn-SOD, Mn-SOD and Fe-SOD. In comparison to the control, chilling injury decreased total SOD activity at 4^{o}C during the first month of storage by 0,39-fold compared to the control, while during the 2nd month of storage there was a decrease of 0.21-fold. During the 5th month of storage, total SOD activity decreased by 0.73-fold. During the storage at 6.5^{o}C, the total SOD activity decreased by 0.42-fold at 1 month of storage, whereas it decreased by 0.25-fold and 0.81-fold at 2 months and 5 months of storage, respectively. In the arils at 5 months of storage, the total SOD activity of the arils at both temperatures was statistically similar to the total SOD activity of the control.

a)



b)

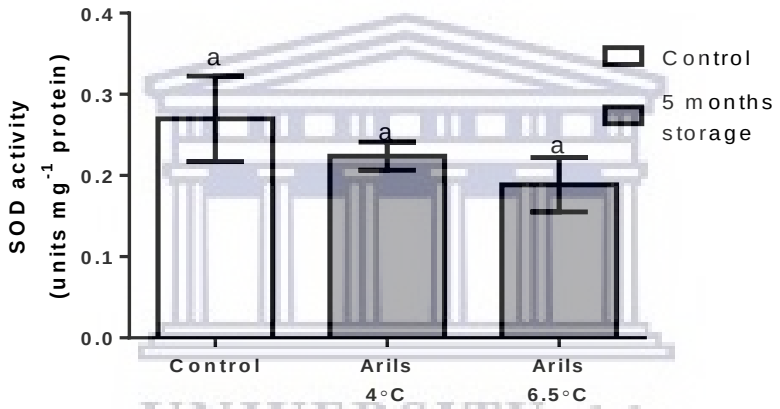


Figure 3.6: Total SOD activity of pomegranate in response to chilling.

Total SOD activity was measured in seeds (a) stored at 4°C and 6.5°C sampled at 1, 2 and 5 months of storage and arils (b) stored at 4°C and 6.5°C for 5 months. One unit of SOD is defined as the amount of SOD enzyme that is required to cause a decrease of 50% of SOD-inhibitable NBT reduction. Error bars represent the means ± SE; n= 3. Different letters indicate the difference between means at $p<0.05$ (Dunnett's multiple comparisons test).

3.3.2 Catalase activity was affected by chilling stress

Catalase activity in pomegranate seeds and in the arils that were stored at 4°C and 6.5°C for either 2 months or 5 months was investigated using native PAGE (Figure 3.7 a and b). Catalase activity was down-regulated during the 2 months storage at both 4°C and 6.5°C. However, at 5 months of storage, the CAT activity was upregulated at both temperatures. Densitometry

analysis was also performed using the Spot Denso tool of AlphaEaseFC in order to determine the changes in pixel intensities of CAT isoforms in seeds and arils (Figure 3.7 c and d) from the native PAGE. Differences in pixel intensities were observed in seeds and arils stored at 4^{o}C and 6.5^{o}C for different storage periods. The results corresponded with those observed in the in-gel assay, where at 2 months of storage the CAT activity was downregulated by approximately 0.9-fold at 4^{o}C, whereas at 5 months the CAT activity was upregulated by 0.15-fold at 4^{o}C and 0.19-fold at 6.5^{o}C. In the arils, the CAT activity was downregulated by 0.09-fold.

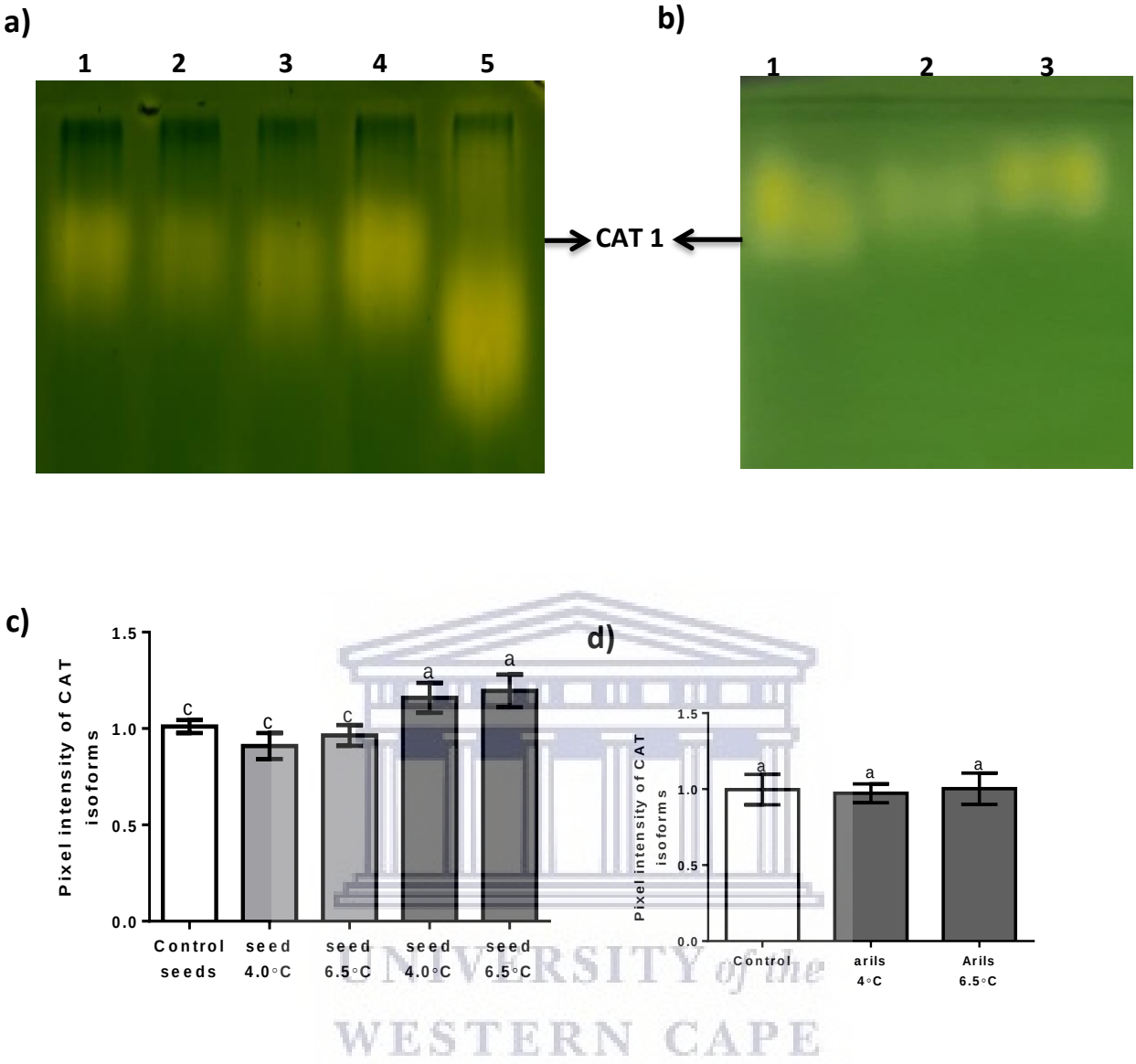


Figure 3.7: Response of catalase to chilling.

Catalase in-gel assay of pomegranate seeds (a) and arils (b) stored at 4°C and 6.5°C for either 2 or 5 months. In-gel assay: lane 1: Control, lane 2: Seeds stored for 2 months at 4°C, lane 3: Seeds stored for 2 months at 6.5°C, lane 4: Seeds stored for 5 months at 4°C, lane 5: Seeds stored for 5 months at 6.5°C, lane 6: Arils control, lane 7: Arils stored for 5 months at 4°C, and lane 8: Arils stored for 5 months at 6.5°C. Pixel intensities of CAT in-gel isoforms are represented in seeds (c) and arils (d). The white bars represent the control, the light grey bars represent storage of 2 months and the dark grey bars represent storage of 5 months. The pixel intensities were measured from the CAT non-denaturing gel using Spot Denso tool of AlphaEaseFC and the data was expressed in arbitrary units. Error bars represent the means ± SE; n= 3. Different letters indicate the difference between means at $p<0.05$ (Tukey's multiple comparisons test).

Total CAT activity (Figure 3.8 a and b) was also studied by spectrophotometry to further understand the involvement of CAT in the scavenging of H_2O_2. There were no differences observed in the CAT activity of the pomegranate seeds that were stored for 2 months at 4°C and 6.5°C when they were compared to the control. However, an increase of CAT activity in the seeds that were stored at 6.5°C for 5 months was observed. In the arils stored for 5 months at 4°C, the CAT activity was down-regulated and the contrary was observed during the 5 months of storage at 6.5°C where the CAT activity was similar to that of the control.

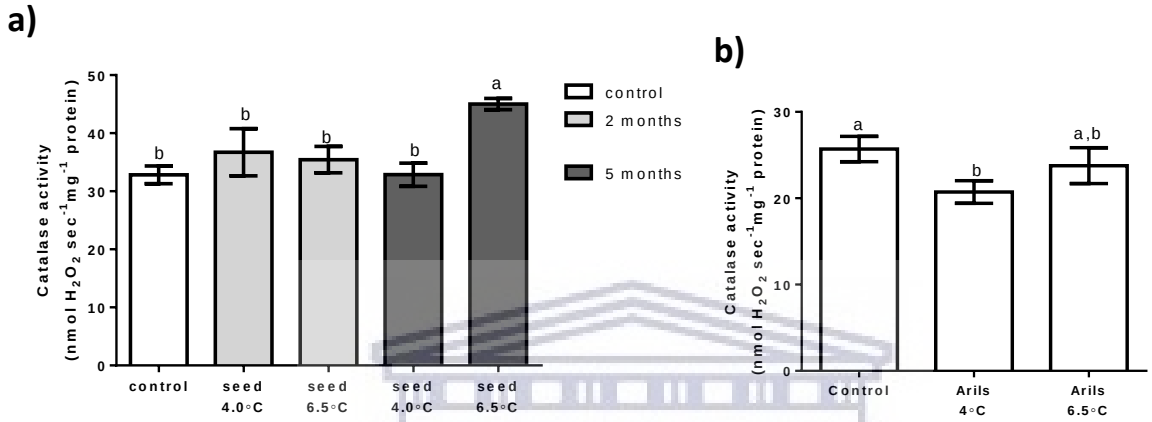


Figure 3.8: Determination of total catalase activity in pomegranate seeds and arils in response to chilling stress.

Total CAT activity was measured in pomegranate seeds were stored 4°C and 6.5°C and sampled at 2 and 5 months of storage (A), and the arils were stored at 4°C and 6.5°C for 5 months (B). One unit of CAT is defined as the amount of enzyme that liberates half of the peroxide oxygen from 10 mM H_2O_2 solution in 100 s at 25°C. Error bars represent the means ± SE; n= 3. Different letters indicate the difference between means at $p<0.05$ (Tukey's multiple comparisons test).

3.3.3 APX activity was upregulated by chilling stress

To determine whether there were differences in individual APX isoforms that were induced by chilling stress, the changes in APX activity in the pomegranate seeds and arils that were subjected to chilling stress were examined. In the in-gel assay (Figure 3.9) whereby the individual APX isoforms were studied, five (5) isoforms were identified. Chilling stress did not cause any detectable changes in APX-3 when it was compared to the control in all the storage conditions except during the 5 months of storage at 4°C whereby the activity was down-regulated. APX-1 was only activated during the 1st month of storage at 6.5°C with APX-2 being visible during both 1st and 5 months of storage at 6.5°C. However, both isoforms were not

visible in the control. In the arils during the 5 months of storage, APX-3 was down-regulated at 4°C and was not detected at 6.5°C for the same storage duration. APX-5 was down-regulated in arils that were stored at 4°C and 6.5°C for 5 months.

Figure 3.9: Determination of differences in APX activity in response to chilling.
APX activity was determined in pomegranate seeds and arils that were stored at 4°C and 6.5°C for a total period of 5 months sampled at different time intervals. Lane 1: Control, lane 2: Seeds stored for 1 months at 4°C, lane 3: Seeds stored for 1 months at 6.5°C, lane 4: Seeds stored for 2 months at 4°C, lane 5: Seeds stored for 2 months at 6.5°C, lane 6: Seeds stored for 5 months at 4°C, lane 7: Seeds stored for 5 months at 6.5°C, lane 8: Arils control, lane 9: Arils stored for 5 months at 4°C, and lane 10: Arils stored for 5 months at 6.5°C.

Densitometry analysis of APX isoforms in terms of pixel intensities was performed to determine changes caused by chilling injury in the pomegranate seeds and arils stored at 4°C or 6.5°C for a total period of 5 months. The pixel intensities were measured from APX native PAGE using AlphaEaseFC and the data was expressed in arbitrary units. The trends of the data were similar to those of the native PAGE results.

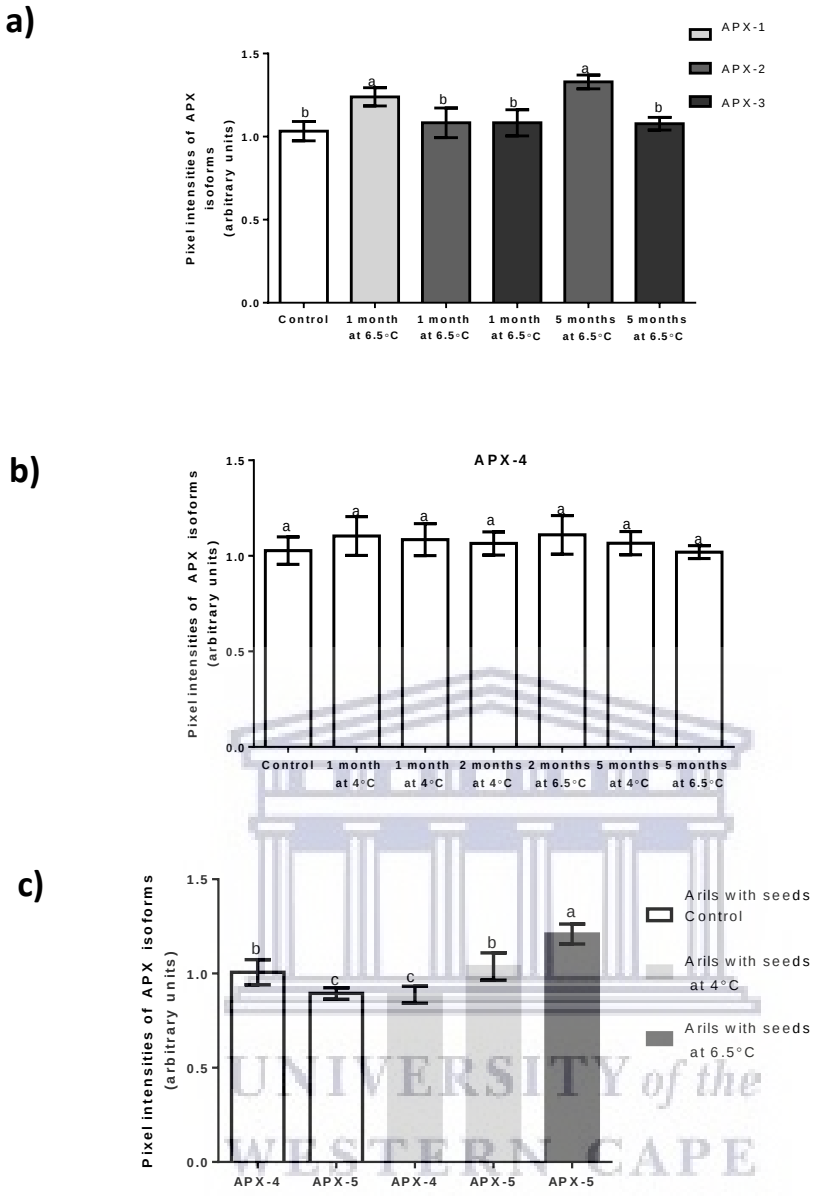


Figure 3.10: Pixel intensities for APX activity in response to chilling.

APX in-gel pixel intensities for seeds stored at 6.5^{o}C for 1, 2 and 5 months for isoform APX-1, 2, 3 (a), APX in-gel pixel intensities for seeds stored 4^{o}C for 1, 2 and 5 months for isozymes APX-4 (b) and APX in-gel pixel intensities for arils with seeds stored at 4^{o}C and 6.5^{o}C for 5 months of isozymes APX-4 and APX-5 (c). The pixel intensities were measured from APX native PAGE gel using the Spot Denso tool of AlphaEaseFC and the data was expressed in arbitrary units. Error bars represent the means ± SE; n= 3. Different letters indicate the difference between means at $p<0.05$ (Dunnett's multiple comparisons test and Sidak's multiple comparisons test).

Spectrophotometric analysis was conducted in order to study the effect of chilling injury on total APX activity in pomegranate that was exposed to chilling at different temperatures. After

1 month of storage total APX activity (Figure 3.11 a) decreased in both the seeds that were stored at 4°C and 6.5°C when they were compared to the control. In the seeds that were stored for 2 months at 6.5°C showed a significant increase compared to the control.

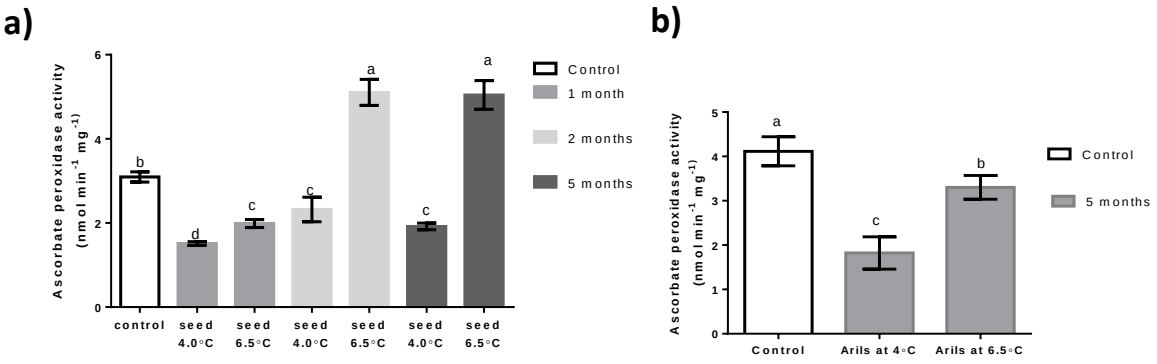


Figure 3.11: Determination of total APX activity in response to chilling.

Total ascorbate peroxidase activity was measured in the seeds of pomegranate (a) and, arils stored at 4°C or 6.5°C for an overall period of 5 months (b) sampled during the 1st, 2nd and 5th month of storage. Error bars represent the means ± SE; n= 3. Different letters indicate the difference between means at $p<0.05$ (Tukey's multiple comparisons test).

The APX activity in the seeds that were stored for 2 months increased as compared to that of the seeds that were stored for a month at the same temperature. Total APX activity was highly up-regulated in the seeds that were stored at 6.5°C for 5 months. On the contrary, the activity was down-regulated in the seeds that were stored at 4°C for the same period of time. In the arils, the total APX activity was more down-regulated in the arils that were stored at 4°C for 5 months than in the arils that were stored at 6.5°C for the same period of storage (Figure 3.11 b).

3.2.4 Chilling upregulated DHAR activity in the seeds that were stored at 6.5°C

Since APX uses AsA to reduce H_2O_2 to H_2O, which requires regeneration of AsA from dehydroascorbate. This regeneration is catalysed by dehydroascorbate reductase. As a result, changes in DHAR activity in pomegranate seeds and arils that were subjected to chilling were studied. Native PAGE (Figure 3.12 a) showed that at 1 month and 2 months of storage at both 4°C and 6.5°C, there were no notable changes observed in DHAR activity. However, at 5 months of storage at 4°C, there was no DHAR activity observed. Seeds that were stored at 6.5°C showed upregulation of DHAR activity. In the arils and seeds no activity was detected (results not shown).

The pixel intensities (Figure 3.12 b) were measured from the DHAR in-gel assay where there was an increase of 1.09-fold and 1.23-fold during the 1st month and 2nd months of storage, respectively, at 4°C. During the 6.5°C storage, an increase in DHAR activity was observed, with an increase of 1.23-fold during the first month, 1.26-fold and 1.29-fold increase as compared to the control during the 2 month and 5 months of storage, respectively.

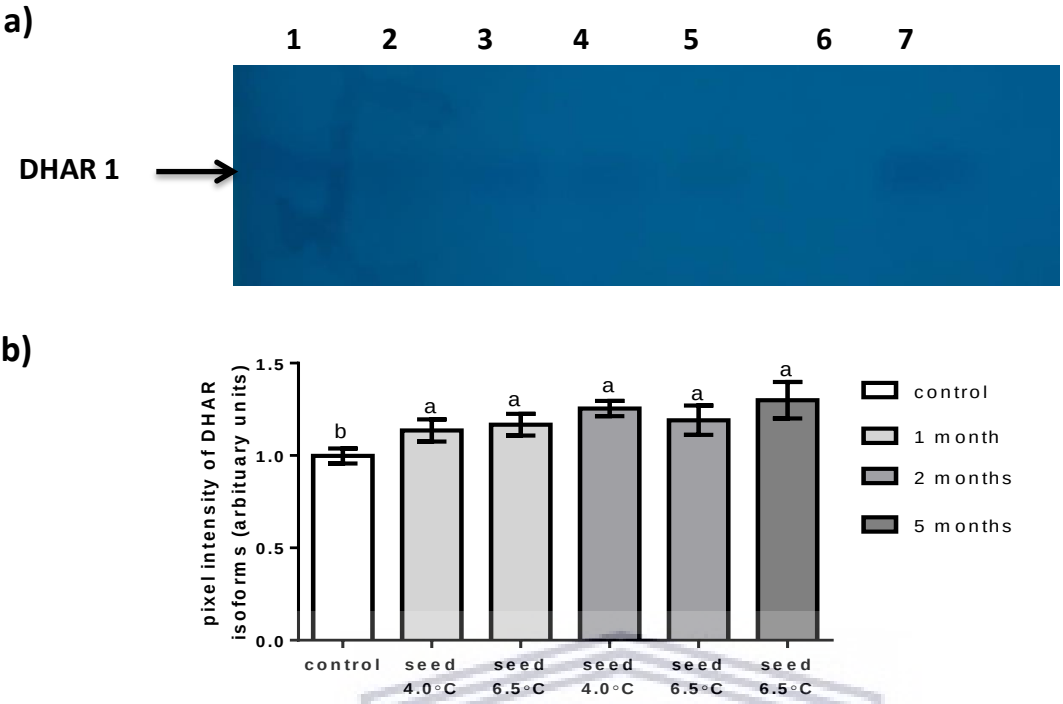


Figure 3.12: Determination of dehydroascorbate reductase activity in the seeds of pomegranate stored at 4°C or 6.5°C for 1, 2 or 5 months.

DHAR isoforms in response to chilling are shown in an in-gel assay (a). Lane 1: Control, lane 2: Seeds stored for 1 months at 4°C, lane 3: Seeds stored for 1 months at 6.5°C, lane 4: Seeds stored for 2 months at 4°C, lane 5: Seeds stored for 2 months at 6.5°C, lane 6: Seeds stored for 5 months at 4°C, lane 7: Seeds stored for 5 months at 6.5°C. Pixel intensities of DHAR isoforms (b). The pixel intensities were measured from DHAR native PAGE using the Spot Denso of AlphaEaseFC and the data was expressed in arbitrary units. Error bars represent the means ± SE; n= 3. Different letters indicate the difference between means at $p<0.05$ (Tukey's multiple comparisons test).

CHAPTER 4: DISCUSSION

Fruit storage in cold non-freezing temperatures mainly at 4-5^{o}C is a commonly used type of storage to delay chilling injury in fruits and to avoid fruit decay, thus, maintaining the fruit quality by slowing down some metabolic processes (Valenzuela *et al*., 2017). Damages caused by chilling injury are firstly noticed on the external appearance of the fruit (Valenzuela *et al*., 2017). This study therefore aimed to investigate the effect of CI damage in pomegranate fruits that were stored at 4^{o}C and at 6.5^{o}C.

In order to assess the damage caused by CI in pomegranate cv. 'Wonderful', this study was conducted over a period of 5 months in pomegranate that was stored at 4^{o}C and at 6.5^{o}C. The samples were taken during the 1st month, 2nd month and the 5th month of storage, meanwhile the control was a fruit tissue extracted from a fruit coming straight from the farm. This study included assessment of visual quality, which included chilling injury score and external decay percentage. Both studies were based on the rind (outer part) of the fruit.

In figure 3.1 mould growth was observed on the crown of the fruit that was stored at 6.5^{o}C during the 5th month of storage but no mould was found in the fruits that were stored at 4^{o}C at 5 months of storage (Figure 3.1). These results correlated with a study by O'Grady *et al.,* (2014) where no visual mould growth was detected in arils that were kept at low temperatures but mould was observed when the arils were stored at higher temperature. These studies were also similar in that neither of the fruits that were used in the studies were disinfected prior to storage. The infection could not have originated at the orchard since at 4°C no infection was observed while at 6.5°C it was observed. These results suggest that in order to avoid mould growth a temperature of 4^{o}C must be used, these findings correlates with a study by Valenzuela *et al*., (2017) where they proposed that a temperature of 0–5°C must be used to store fruits in order to prevent mould growth.

Moreover, necrotic regions were also observed in the rind of the fruit's tissues during chilling injury (Figure 3.1). According to Albornoz *et al*., (2019), the reason for limited necrotic regions while the neighbouring cells remained healthy in the fruit is because of a secondary messenger (possibly H_2O_2) that could have limited cell death within the region of injury. The H_2O_2 signalling mechanism could have triggered the antioxidant enzymes that respond to

chilling injury to stop the peroxidation, thus limiting it to the already affected areas. One of the first events to be triggered under CI is the accumulation of ROS (Albornoz *et al*., 2019).

In addition to visual quality, the external decay was affected by storage temperature and the storage duration (Figure 3.2 a and b), where the fruit quality deteriorated with prolonged storage and duration at both 4°C and 6.5°C. It was observed that, at 4°C after 1st month of storage, the fruits experienced low external decay which quantitatively accounted for an increase of 3-fold when compared to the control. During the 2nd month of storage, the fruits had moderate chilling injury and at 5 months of storage the fruits experienced severe chilling injury which accounted for 3.3-fold and 4.6-fold increase respectively. Moreover, the results of the chilling injury score (Figure 3.2 c and d) at 4°C differed with those of the fruit that was stored at 6.5°C. As hypothesised, there were no chilling injury symptoms observed during the 1st month of storage, there were slight chilling symptoms observed during the 2nd month of storage, which accounted for an increase of 2.4-fold when compared to the control. There were moderate chilling symptoms after the 5th month of storage, which accounted for an increase of 2.8-fold when compared to the control.

Visual quality detoriated as the storage duration increased. Also, it is worth noting that CI symptoms were more visible at 4°C than at 6.5°C during all the storage times. This could be because of the poor response of the antioxidative system, whereby there was an imbalance between antioxidant enzyme (e.g. SOD, CAT, APX and DHAR) activities and their ability to scavenge reactive oxygen species (e.g. H_2O_2). The low temperature (4°C in this case) and prolonged exposure (especially at 5 months of storage) to chilling are known to cause lipid peroxidation, thus leading to the visible signs of CI on the external and internal attributes of the fruit (Valenzuela *et al*., 2017). The results of this study agree with results obtained by Serry (2019), whereby no chilling injury symptoms appeared on the fruits within the first six weeks of cold storage.

The same trend that was observed in the CI index (Figure 3.2 C and D) was also observed for external decay (Figure 3.2 A and B). External decay was affected by storage temperature and the duration of storage. The external decay results displayed a directly proportional relationship where the external decay increased with increasing storage duration at both storage temperatures. The changes in the visual quality (in both CI index and external decay)

of pomegranate observed at 2 months as compared to 1 month of storage at 4°C could be due to the fact that low temperatures trigger the disturbances of physiological functions but those disturbances did not lead to visible manifestations of injury. However, for prolonged storage, even the low temperatures can lead to visible manifestations of injury, hence there were huge visible changes that occurred during the 5th month of storage at 4°C. According to Lukatkin *et al*., (2012) the disturbances of the physiological processes are reversible if the fruit is taken back to a normal temperature before the CI symptoms become visible (i.e. before the CI becomes stable).

Moreover, irreversible damage, like the one observed at 5 months of storage, might have been caused by the prolonged chilling which in turn may be caused by the accumulation of toxic metabolites (Lukatkin *et al*., 2012). In a study by Taghipour *et al.* (2015), cold storage up to 1 month had no significant effect on CI index, but afterwards, an increasing trend was observed in which all differences were statistically significant. Also, Tahipour *et al.* (2015) showed that fruits stored up to 1 month had no significant incidence of CI symptoms. This led to a conclusion that the undesirable changes that occur in a short period of time are reversible and can help to keep the quality of pomegranate fruits. This was further confirmed by Serry (2019), whereby the external decay increased with the duration of storage of pomegranate.

It is therefore worthy to note that the external decay was higher at 4°C than 6.5°C (Figure 3.2 a and b). The high external decay observed at 4°C than at 6.5°C could also be due to the humidity loss from the fruits at 4°C. The water vapour transmission rate would have been higher at 6.5°C, thus preventing external visible signs of CI. However, fungal infection was visible at the 6.5°C storage temperature during the 5th month of storage. It has been documented that pomegranates are profoundly susceptible to chilling injury because of high porosity of the fruit's rind, which allows free water vapour development and movement (Serry, 2019). The differences in external decay could also be caused by the dermal tissue system of the fruit, which is responsible for regulation of water loss (Valenzuela *et al*., 2017). As the outermost plant barrier, the cuticle plays several protective functions (Lara *et al*., 2019). Its hydrophobic nature allows the cuticle to protect the plant against water loss and regulate the exchange of water, carbon dioxide and other solutes with the environment, thus protecting the fruit from chilling injury (Lara *et al*., 2019).

To further understand whether chilling injury induced levels of H_2O_2 that were within limits to be used for signalling purposes or to cause damage, H_2O_2 content in the fruits was studied. In this study, the chilling injury triggered the accumulation of H_2O_2 in both seeds and arils (Figure3.3 a and b). The H_2O_2 content in seeds that were stored at 4°C was higher than in seeds that were stored at 6.5°C, for both the 1st month and the 5th month storage period. Since the H_2O_2 content was higher at 4°C as compared to the H_2O_2 content at 6.5°C, it can be deduced that the chilling injury could have caused oxidative stress more in the seeds that were stored at 4°C than at 6.5 °C.

In the arils (Figure 3.3 b), the H_2O_2 content was measured at 5 months of storage and an increase of 1.2–fold was observed at 4°C, which was significantly higher than the 1.1–fold increase that was observed at 6.5°C. There was a higher H_2O_2 content in the arils compared to the seeds at 5 months of storage in both storage temperatures. This could be attributed to the fact that chilling stress affects various tissues differently (Valenzuela *et al*., 2017). According to Valenzuela *et al*. (2017), high H_2O_2 content leads to membrane degradation, hence it would be reasonable to suggest that the high H_2O_2 content caused more lipid peroxidation in the arils as compared to the seeds. The results observed in this study are in agreement with the study by Kader *et al*., (2011) whose results revealed that chilling stress induces a significant increase in H_2O_2 level of wheat seedlings and that chilling could cause H_2O_2 accumulation in *Zea mays* and mung bean plants. A study by Lee and Lee (2000) also revealed that chilling stress enhances H_2O_2 production in cucumber leaves.

Changes in plant cellular redox status as a result of accumulation of H_2O_2 are co-ordinated in order to control cellular changes that occur as a result of an environmental stress for example chilling injury. However, the rate at which ROS is produced may not be equal to the rate at which it is scavenged by antioxidant enzymes, which can lead to a phenomenon known as oxidative stress (Kader *et al*., 2011). Oxidative stress could lead to oxidative damage in lipids which can impair their functions. These changes are usually shown by elevated MDA content. Lipid peroxidation (which is measured by MDA content) is one of the causes of quality losses in food (Shiban *et al*., 2012). The oxidative deterioration of food components in agricultural by-products is responsible for rancidity and off flavours and thus leads to a decrease in nutritional quality and safety due to the formation of secondary compounds that may potentially be toxic (Shiban *et al*., 2012).

To evaluate whether H_2O_2 led to a lipid peroxidation and whether MDA had an effect on the visual quality of the fruit, MDA was measured. Chilling injury triggered changes in MDA of both the seeds and the arils (Figure 3.3 c and d). The MDA content during the 1st month of storage at 4°C was low compared to the MDA content of the fruits stored for 2 and 5 months in the same temperature. This was due to the fact that shorter duration of storage of the fruits at a chilling inducing temperature (4°C) does not cause a lot of increase in the downregulation of metabolism, thus leading to lower MDA accumulation. At 6.5°C, the MDA content was lower than the MDA content observed at 4°C, as hypothesised. Since high H_2O_2 content and MDA content were observed at 5 months of storage at 4°C in the seeds, it can be concluded that the fruit's external decay occurred due to the prevalence of oxidative stress. According to Wang and Zhu (2017), the metabolic changes in chilling-sensitive fruits occur only after prolonged exposure to chilling temperatures, and the main cause of visible chilling symptoms is metabolic disorder, hence there was a high CI score and external decay % at 5 months of storage.

According to Aghdam *et al*. 2014, MDA content is generally an indirect measurement of loss of membrane integrity and thus it reflects the manifestation of CI. This was shown to be true in this study because a directly proportional relationship between the MDA content and the visual quality (external decay and CI score) was observed, meaning that where MDA was high (Figure 3.3 c and d), the external decay of the fruit and chilling injury score were also high (Figure 3.2 c and d). On the other hand, there was a great variation between how the arils and seeds responded to chilling injury. In the arils when the MDA content was evaluated at 5 months of storage, the results showed that the MDA content was higher at 4°C than at 6.5°C.

Also, there seems to be no correlation between MDA content and H_2O_2 content in the arils, since the H_2O_2 content at 4°C was higher compared to the H_2O_2 content in arils that were stored at 6.5°C. However, higher MDA was observed in the arils that were stored at 6.5°C for 5 months. This could be because there is little or no correlation or that the H_2O_2 observed at 4°C was there for signalling purposes. It is also possible that, at 4°C, the rate of disappearance of MDA from the decaying tissues is equivalent to the rate of its formation. This data shows that even though there can be variations in the degree to which cell components show net oxidation, there may be no correlation in the oxidation process of the seeds and arils.

Moreover, it is worth noting that there were higher levels of MDA in pomegranate arils than in the seeds only at 5 months, suggesting that there is membrane deterioration and the loss of membrane integrity and tissue structure. These differences could be owed to the fact that CI affects different tissues differently; also CI targets processes occurring across different biological levels and different time frames. Moreover, due to the fact that different tissues carry out different functions and the number of processes that lead to CI and the rate at which the CI affects different tissues would also be expected to be different across tissues (Albornoz *et al*., 2019).

These results were in agreement with results of a study by Wang and Zhu (2017), where pre-storage cold acclimation (PsCA) reduced MDA compared to the control in cucumber stored at 5^{o}C, which again suggested that PsCA maintained membrane integrity. Pre-storage cold acclimation at 10°C for 72 hours reduced levels of H_2O_2 during cold storage. These suggest PsCA maintained quality of cucumber by reducing ROS levels to protect cell membrane integrity by (Wang and Zhu 2017). Maintenance of membrane integrity at low temperature is important in resistance to chilling temperature (Wongsheree *et al.*, 2009). Since the results between external decay, CI injury score, H_2O_2 and MDA correlate except in the arils, it is clear that MDA can be used to measure lipid peroxidation in pomegranate exposed to chilling injury as previously suggested by Valenzuela *et al*., (2017).

Lipid peroxidation is directly linked to oxidation and displays a better correlation with antioxidant levels. It is hypothesised that there is a directly proportional relationship between chilling injury and antioxidant enzymes, meaning that when there is an increase in CI score, external decay, H_2O_2 and MDA, there should also be an increase in antioxidant activity. Therefore, this study also focused on the antioxidant enzyme capacity to combat oxidative damage caused by CI.

To determine the role of ROS scavenging systems in combating the oxidative stress in this study, ROS scavenging enzymes were characterised in pomegranate seeds and arils stored at 4^{o}C and 6.5^{o}C for an overall period of 5 months, sampled during the 1st, 2nd and 5th month of storage. Before going into the analysis of the results of this study, it is worth keeping in mind that that the chain of events related to the induction of specific isoforms is not known. Nonetheless, Edwards *et al*. (1994) proposed that plants synthesise new isoforms of

antioxidant enzymes in response to environmental stress, with changes in their kinetic properties. Hence, responses of the antioxidative system against CI were investigated using in-gel assays.

Superoxide dismutase acts as the first line of defence against ROS and it catalyses the dismutation of superoxide radicals to hydrogen peroxide and oxygen (Yousuf *et al.,* 2011). SOD activity of pomegranate fruit exposed to chilling injury was studied in the seeds and arils using a non-denaturing gel (Figure 3.4). In this study only Cu/Zn-SOD and Mn-SOD isoforms were observed; according to Lee and Lee, (2000) both Cu/Zn-SOD and Mn-SOD are preferentially induced by O_3^- and UV-B. Chilling injury inhibited SOD activity in seeds and the activity of Cu/Zn-SOD-5 was only observed in the in the arils that were stored at 6.5^{o}C for 5 months while there was an inhibition of SOD in the arils that were stored for the same duration at 4^{o}C. The inhibition of SOD could be due to the increase of O_2^-. This study is in accordance with a study by Lee and Lee, (2000) whereby the SOD activity of cucumber leaves exposed to CI increased with increasing storage.

The total SOD activity was measured by a spectrophotometric assay (Figure 3.6), where the total SOD activity represented a combined action of Cu/Zn-SOD, Mn-SOD and Fe-SOD. The results had a similar trend to the in-gel assay. In comparison to the control chilling injury decreased total SOD activity in the seeds, on the contrary, total SOD activity remained the same in the arils stored at 4^{o}C and in the control. In the arils at 5 months of storage the total SOD activity of the arils at both temperatures was similar to the total SOD activity of the control. The total SOD activity decreased more in the seeds that were stored at 6.5^{o}C than in the storage at 4^{o}C, this could mean that fruits stored at 4^{o}C have more capacity to dismutate superoxide. Moreover, the decrease in SOD activity could mean that the overproduction of H_2O_2 was mainly due to SOD dismutating the O_2^-, or because of self-dismutation of superoxide or reduction of superoxide by ascorbate. The results of SOD do correlate with those of H_2O_2, since superoxide dismutation by SOD releases H_2O_2.

Moreover, the low content of H_2O_2 during the 5th month of storage at 6.5^{o}C compared to the H_2O_2 content at 4^{o}C could mean that Cu/Zn-SOD in a specific cell-compartment would correlate to H_2O_2 formation, since Cu/Zn-SOD was highly upregulated (Figure 3.4). The accumulation of MDA appears to be largely dependent on SOD activity in the seeds and arils,

based on the notion that SOD was releasing H_2O_2, which consequently led to high MDA levels. This would imply that changes in SOD activity did not play a critical role in the decrease of lipid peroxidation. However, this does not imply that SOD is not important since it is required for aerobic life. From experiments involving temperature stress Raychaudhuri and Deng, (2000) suggested that cytosolic Cu/Zn-SOD appeared to be the most responsive of the SOD mRNAs, it is the only one that was strongly induced by heat shock and during the recovery period after chilling stress. This information could show for the first time that, under chilling stress in pomegranate, Cu/Zn-SOD is more expressed than Mn-SOD and Fe-SOD.

Another antioxidant enzyme that is known to defend a fruit against environmental stress is CAT. CAT is known to catalyse reduction of H_2O_2 into H_2O and O_2 using energy in the cells exposed to chilling injury. Catalase is present in cell compartments that are known to be the main producers of H_2O_2 (Sharma and Ahmad, 2014; Liu and Kokare, 2017). Differences in how chilling injury affected catalase activity in the seed and arils that were stored at either 6.5^{o}C and 4^{o}C were observed. Catalase activity was downregulated during the 2nd month of storage at 4^{o}C, which was in contrast to the seeds that were stored at 6.5^{o}C at 2 months, where CAT activity was upregulated. Catalase activity was highly upregulated at 5 months of storage at 6.5^{o}C compared to the control. In the arils that were stored for 5 months at 4^{o}C, the CAT activity (Figure 3.7 B) was downregulated and the contrary was observed for arils stored at 6.5^{o}C for the same duration, whereby the CAT activity remained similar to that of the control. There was upregulation in CAT activity of the pomegranate seeds stored at 6.5^{o}C compared to the control at all storage periods. However, the CAT activity of the seeds stored at 4^{o}C decreased.

The results of the total CAT activity (Figure 3.8) correlated with the results found in the in-gel activity and pixel intensities (Figure 3.6 c and d). The decrease of CAT activity in the seeds and arils stored at 4^{o}C for 5 months could be manifest as an increase in H_2O_2 content, since Kader *et al*., (2011) showed that an increase in H_2O_2 content could be attributed to decreased activity of key antioxidant enzymes. The increase of CAT activity at 5 months of storage at 6.5^{o}C could be due to the high demands of high levels of H_2O_2 to be removed by CAT. In a different study, the overexpression of a CAT gene from *Brassica juncea* introduced into tobacco enhanced tolerance of the transgenic tobacco to Cadmium-induced oxidative stress (Sharma *et al*., 2011). Moreover, CAT is known to scavenge H_2O_2 hand in hand with APX

(Mizuno *et al*., 1998; Lee and Lee, 2000); as a result, the effect of CI on APX activity was also investigated.

APX is also an important antioxidant enzyme in scavenging H_2O_2 using ascorbate as a reducing agent (Shigeoka *et al*., 2002). During this study, chilling stress induced APX activity. At 1 month of storage, the total APX activity (Figure 3.10 B) decreased in the pomegranate seeds that were stored at 4°C and 6.5°C compared to the control. At 2 months of storage at 4°C, the APX activity decreased. On the contrary, at 6.5°C during the 1st month and 5th month of storage, the APX activity was upregulated due to preferential induction of APX-2 (Figure 3.9). At 5 months of storage, the total APX activity was downregulated at 4°C, this decrease is likely due to the significant increase in H_2O_2 under these conditions. APX, as a defence mechanism, is upregulated during stressful conditions in order to combat the stress as it intensifies (Lee and Lee, 2000). During the 5 months of storage, APX activity increased as H_2O_2 was increased by CI. The trend of total APX activity in the arils followed that of the total CAT activity, whereby at 4°C the CAT and APX activity were highly reduced as compared to the arils that were stored at 6.5°C. High levels of APX were observed in seeds than in the arils at 5 months of storage at 6.5°C.

An increase in SOD activity at low temperatures leads to high levels of H_2O_2 that could move freely among organelles and cytosol (Mizuno *et al*., 1998). Consequently, the newly accumulated H_2O_2 in turn could elicit the increase of APX, or H_2O_2 may function as a secondary messenger during the low temperature stress since the induction of APX activity occurred at a low temperature and during a short period of storage. APX activity in the seeds that were stored for 2 months and 5 months at 4°C and 6.5°C increased in a manner similar to the increase seen in SOD. This could be because the SOD-mediated dismutation of the superoxide radical to H_2O_2 and O^{-}_2 resulted in increased APX and CAT activity to reduce H_2O_2 to H_2O in order to protect the fruit.

Even though the CAT activity was higher than the APX activity, CAT activity decreased in both storage conditions at 2 months, whereas APX activity increased. These results were similar to those obtained by Mizuno *et al*. (1998), where CAT activity decreased first until APX activity was downregulated and then started to increase slowly to basal levels during storage at 1°C and 5°C. This might have been because CAT did not scavenge H_2O_2 until the H_2O_2

concentration increased to a critical level, since CAT has a very low affinity for scavenging H_2O_2 compared to APX. According to Mizuno *et al*. (1998), APX has a higher affinity for H_2O_2 than CAT. Hydrogen peroxide is produced in the intracellular parts of the fruit and moves to the cytosol first where cytosolic APX is found and thereafter moves to peroxisomes where CAT is usually localised (Mizuno *et al*., 1998). It is possible that the increase in APX activity when the CAT activity decreased was to compensate for the CAT activity that was lost. Mizuno *et al*., (1998) suggested that the collaboration between APX and CAT is important for the protection of fruits against the CI-mediated H_2O_2 produced when the fruits are kept at low temperature storages.

Since APX uses ascorbate which is generated by DHAR, the role of DHAR in combating ROS had to be investigated. DHAR uses glutathione (GSH) as the reducing agent to regenerate DHA back to ascorbate (Asada 1999; Mittler 2002; Do *et al*., 2016). The level of DHAR expression is important for appropriate ROS scavenging because it establishes the efficiency of H_2O_2 scavenging. Increasing DHAR expression also provides enhanced tolerance to environmental stress (Chen and Gallie, 2006). In this study it was demonstrated that the activity of DHAR was most sensitive to low temperatures (Figure 3.12). DHAR activity was more upregulated at 6,5^{o}C during the 2nd month of storage.

During the 5th month of storage at 4^{o}C, DHAR activity was not detected in the in-gel assay, these results were in contradiction to the DHAR activity of the seeds that were stored at 6.5^{o}C whereby the DHAR activity was upregulated during the same storage period. The upregulation of DHAR activity at 6.5^{o}C during the 5th month of storage could have been due to the system producing more H_2O_2, thus leading to DHAR producing more AsA for use by APX in order to scavenge H_2O_2, hence the levels of APX during the 5 months of storage at 6.5^{o}C were also high. The undetected activity of DHAR during the 5th month of storage at 4^{o}C correlates with the results of APX activity, where there was no activity of APX detected in the in-gel activity during the 5th month of storage at 4^{o}C and there was little activity detected in the total APX activity.

Moreover, the inactivation of APX and DHAR at 4^{o}C during the 5th month of storage was coupled with high increase in H_2O_2 content (Figure 3.3 A and B), this could have happened because DHAR is not regenerating ascorbate to scavenge H_2O_2. This would also suggest that

the oxidative stress caused by H_2O_2 led to a decrease in APX, which consequently resulted in the decrease in the observed external quality of the fruit that was stored at 4°C for 5 months. At this stage the CI score indicated that the fruit had severe chilling symptoms. This also indicates that the fruits had low to no DHAR activity and thus showed less tolerance to chilling injury.

According to literature, increased levels of H_2O_2 result to the downregulation of DHAR activity (Chen and Gallie, 2006). In this study, the arils and seeds that were stored at 4°C and at 6.5°C for 5 months, the H_2O_2 levels were elevated and no DHAR activity was observed. In a study by Chen and Gallie (2006), guard cells of plants expressing DHAR showed a higher AsA redox state, reduced levels of H_2O_2 and suppression of DHAR expression accompanied by higher levels of H_2O_2. In a study by Mizuno *et al*., (1998), the concentration of DHAR decreased more rapidly during storage at 1°C than at higher a temperature. These results contradict the results of this study, where the higher DHAR activity was observed at 6.5°C than at 4°C. Therefore, the decrease in APX activity during the 5th month of storage at 4°C might be caused by the depletion of AsA as an electron donor.

Moreover, the results of this study are further supported by a series of studies that proved that a decrease in DHAR leads to intolerance to chilling injury. In a different study, *Arabidopsis* with increased DHAR expression maintained higher levels of AsA with reduced levels of membrane damage than control plants did following exposure to high temperature (Wang *et al*., 2010). Ushimaru *et al*., (2006) suggested that even small changes in DHAR activity improved tolerance to some environmental stresses because the researchers noticed that, regardless of even a slight increase in AsA content, *Arabidopsis* expressing rice DHAR was more tolerant to salt stress. In a study by Yin *et al*., (2010) expressing a cytosolic *Arabidopsis* DHAR in tobacco resulted in better tolerance to aluminium stress and retained a higher level of AsA in roots, which also had lower levels of H_2O_2, lipid peroxidation and DNA damage, and had improved root growth. In this study, the seeds that were stored at 6.5°C showed higher CI tolerance since they showed high DHAR activity. Also, the seeds showed greater CI tolerance as compared to the arils when they were stored at 6.5°C for 5 months.

CHAPTER 5: GENERAL CONCLUSION AND FUTURE WORK

A major breakthrough in storage temperature to achieve the expectations of maintaining and improving fruit quality, and extend its shelf life, is hard to imagine. Nonetheless, studies on adjusting storage conditions have been remarkably effective at extending the shelf life of fresh fruits while minimising storage disorders. According to our knowledge, this study is the first to explore antioxidant activities of pomegranate fruit exposed to chilling injury using in-gel assays. Generally, this work potentially provides new insights into how pomegranate reacts to oxidative stress caused by storage at low but non-freezing temperatures.

This study showed that low temperature and extended storage duration lead to chilling injury in pomegranate. Since the external decay and CI index was low even at 5 months of storage at 6.5^{o}C, this study has showed that pomegranate can be stored for at least 5 months to successfully control the respiratory activity, metabolism activity and the antioxidant activity of pomegranate. The increases in enzyme activity occurring at 4°C at 2 months of storage implied that even though this is a chilling sensitive fruit it is still capable of protein biosynthesis, which implies a high level of metabolic control and co-ordination. CI increased ROS accumulation and lipid peroxidation, especially at 4^{o}C during the 5 months storage, which in turn inactivated the APX, DHAR and SOD activity. This means that protein synthesis and protective metabolites may not sustain cellular integrity for long (5 months) periods of storage at lower (4^{o}C) temperatures; whereas the contrary was observed at 6.5^{o}C for the same storage duration. It has been shown in this study that the preferential induction of APX-2 at 6.5^{o}C could have caused the high levels of H_2O_2 scavenging.

In all experiments of this study, there were differences in how the seeds and arils responded to chilling injury. The differences could have been due to degradation reaching arils first and the seeds last. This could further explain why there was more damage in the arils than in the seeds. The arils could have shielded the seeds from the chilling injury. The data presented here shows that temperature at 6.5^{o}C should be used as the storage temperature for longer storage duration.

Moreover, since this study focused only on two parameters, namley temperature and storage duration, future work could include studies of relative humidity since it is also an important aspect that influences the quality of pomegranate during storage and would influence the organoleptic characteristics of pomegranate. Gel-free and label-free proteomics approaches could be used to identify proteins that are expressed when the pomegranate is exposed to chilling injury in order to identify genes that determine chilling tolerance.

CHAPTER 6: REFERENCE LIST

1. Abdel Kader, D.Z., Saleh, A.A., Elmeleigy, S.A. and Dosoky, N.S., 2011. Chilling-induced oxidative stress and polyamines regulatory role in two wheat varieties. *Journal of Taibah University for Science*, *5*(1), pp.14-24.
2. Aebi, H., 1974. Catalase. *In Methods of enzymatic analysis* (pp. 673-684). Academic press.
3. Aghdam, M.S. and Mohammadkhani, N., 2014. Enhancement of chilling stress tolerance of tomato fruit by post-harvest brassinolide treatment. *Food and bioprocess technology,* 7(3), pp.909-914.
4. Albornoz, K., Cantwell, M.I., Zhang, L. and Beckles, D.M., 2019. Integrative analysis of post-harvest chilling injury in cherry tomato fruit reveals contrapuntal spatio-temporal responses to ripening and cold stress. *Scientific reports*, *9*(1), pp.1-14.
5. Arendse, E., Fawole, O.A. and Opara, U.L., 2015. Effects of postharvest handling and storage on the physiological attributes and qualityof pomegranate fruit (Punica granatum L.); a review. *International Journal of Postharvest Technology and innovation*, 5(1), pp. 13-31.
6. Asada K (1999). The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. *Annu Rev Plant Biol* 50: 601–639
7. Ayala, A., Muñoz, M.F. and Argüelles, S., 2014. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxidative medicine and cellular longevity*, *2014*.
8. Beauchamp, C. and Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Analytical biochemistry*, 44(1), pp.276-287.
9. Benov, L., 2001. How superoxide radical damages the cell. *Protoplasma*, *217*(1-3), pp.33-36.
10. Biswas, P., 2012. *Role of intermittent warming in reducing chilling injury in tomato: a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Food Technology at Massey University, Palmerston North, New Zealand* (Doctoral dissertation, Massey University).

11. Caverzan, A., Casassola, A. and Brammer, S.P., 2016. Reactive oxygen species and antioxidant enzymes involved in plant tolerance stress. SHANKER AK & SHANKER C. *Abiotic and biotic stress in plants- Recent advances and future perspectives. Publisher InTech*, pp. 463-480.
12. Chen, Z. and Gallie, D.R., 2006. Dehydroascorbate reductase affects leaf growth, development, and function. *Plant Physiology*, *142*(2), pp.775-787.
13. De Tullio, M.C., De Gara, L., Paciolla, C., and Arrigoni, O., 1998. Dehydroascorbate reducing proteins in maize are induced by the ascorbate biosynthesis inhibitor lycorine. *Plant Physiology and Biochemistry*, 36(6), pp. 433-440.
14. Dhinesh, K.V. and Ramasamy, D., 2016. Pomegranate processing and value addition. *Journal of Food Processing and Technology*, *7*(3).
15. Do, H., Kim, I.S., Jeon, B.W., Lee, C.W., Park, A.K., Wi, A.R., Shin, S.C., Park, H., Kim, Y.S., Yoon, H.S. and Kim, H.W., 2016. Structural understanding of the recycling of oxidized ascorbate by dehydroascorbate reductase (OsDHAR) from Oryza sativa L. japonica. *Scientific reports*, *6*, pp.19498.
16. Durante, M., Montefusco, A., Marrese, P.P., Soccio, M., Pastore, D., Piro, G., Mita, G. and Lenucci, M.S., 2017. Seeds of pomegranate, tomato and grapes: An underestimated source of natural biotactive molecules and antioxidants from agri-food by-products. *Journal of Food Composition and Analysis*, 63, pp.65-72.
17. Fawole, O.A. and Opara, U.L., 2013. Effects of storage temperature and duration on physiological responses of pomegranate fruit. *Industrial crops and products*, *47*, pp.300-309.
18. Fresh Plaza. 2019. South Africa: Start of pomegranate season hampered by logistical challenges. Available on: https://www.farmingportal.co.za/index.php/agri-index/68-crops/1778-south-africa-start-of-pomegranate-season-hampered-by-logistical-challenges accessed on: 27 November 2019.
19. Gill, S.S. and Tuteja, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant physiology and biochemistry,* 48(12), pp.909-930.

20. Gross, K.C., Wang, C.Y. and Saltveit, M.E. eds., 2016. *The commercial storage of fruits, vegetables, and florist and nursery stocks*. United States Department of Agriculture, Agricultural Research Service.
21. Gumienna, M., Szwengiel, A., and Górna, B., 2016. Bioactive components of pomegranate fruit and their transformation by fermentation processes. *European Food Research and Technology*, *242*(5), pp.631-640.
22. Houston, D.M., Bugert, J., Denyer, S.P. and Heard, C.M., 2017. Anti-inflammatory activity of Punica granatum L. (Pomegranate) rind extracts applied topically to ex vivo skin. *European Journal of Pharmaceutics and Biopharmaceutics*, *112*, pp.30-37.
23. Huang, T.L., Nguyen, Q.T.T., Fu, S.F., Lin, C.Y., Chen, Y.C. and Huang, H.J., 2012. Transcriptomic changes and signalling pathways induced by arsenic stress in rice roots. *Plant molecular biology*, *80*(6), pp.587-608.
24. Isolcell. 2012. Pomegranate Chilling Injury occurrence. Available from: http://www.post-harvest.biz/en/news/pomegranate-chilling-injury-occurrence/ id:79153/
25. Jaakola, L., 2013. New insights into the regulation of anthocyanin biosynthesis in fruits *Trends In Plant Science*, Vol. 18, No. 9
26. Jan, N., Qazi, H.A., Raja, V. and John, R., 2019. Proteomics: A tool to decipher cold tolerance. *Theoretical and Experimental Plant Physiology*, *31*(1), pp.183-213.
27. Junglee, S., Urban, L., Sallanon, H. and Lopez-Lauri, F., 2014. Optimized assay for hydrogen peroxide determination in plant tissue using potassium iodide. *American Journal of Analytical Chemistry*, *5*(11), p.730.
28. Kader, A.A., 2006. Postharvest biology and technology of pomegranates. *In Pomegranates* (pp. 229-238). CRC press.
29. Kader, A.A., Zagory, D., Kerbel, E.L. and Wang, C.Y., 1989. Modified atmosphere packaging of fruits and vegetables. *Critical Reviews in Food Science & Nutrition*, 28(1), pp. 1-30.
30. Kader, D.Z., Saleh, A.A., Elmeleigy, S.A. and Dosoky, N.S., 2011. Chilling-induced oxidative stress and polyamines regulatory role in two wheat varieties. *Journal of Taibah University for Science*, *5*(1), pp.14-24.

31. Kahramanoğlu, İ., Usanmaz, S. and Alas, T., 2019. Degradation of post-harvest applied fludioxonil in pomegranate fruit cv. 'Wonderful' and efficacy on the gray mould.

32. Krasensky-Wrzaczek, J. and Kangasjärvi, J., 2018. The role of reactive oxygen species in the integration of temperature and light signals. *Journal of experimental botany*, *69*(14), pp.3347-3358.

33. Kurutas, E.B., 2015. The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: current state. *Nutrition journal*, *15*(1), p.71.

34. Lara, I., Heredia, A. and Domínguez, E., 2019. Shelf life potential and the fruit cuticle: the unexpected player. *Frontiers in plant science*, *10*, p.770.

35. Lee, D.H. and Lee, C.B., 2000. Chilling stress-induced changes of antioxidant enzymes in the leaves of cucumber: in gel enzyme activity assays. *Plant science*, *159*(1), pp.75-85.

36. Li, L., Lichter, A., Chalupowicz, D., Gamrasni, D., Goldberg, T., Nerya, O., Ben-Arie, R. and Porat, R., 2016. Effects of the ethylene-action inhibitor 1-methylcyclopropene on post-harvest quality of non-climacteric fruit crops. *Post-harvest Biology and Technology*, *111*, pp.322-329.

37. Lin, W.C., Block, G.S., and Ehret, D.L., 2011. The effect of H_2O_2 application during production on greenhouse pepper shelf life. *Am. J. Plant Sci. Biotechnol*, *5*, pp.101-104.

38. Liu, X., and Kokare, C., 2017. Microbial enzymes of use in industry. In *Biotechnology of Microbial Enzymes* (pp. 267-298). Academic Press.

39. Lukatkin A.S, Brazaitytė A, Bobinas Č ,Duchovskis P. 2012. Chilling injury in chilling-sensitive plants: a review. *Žemdirbystė=agriculture,* 99 (22), pp.111–124.

40. Martínez-Romero, D., Mirdehghan, S.M., Valverde, J.M., Serrano, M. and Guillén, F., Reduction of chilling injury of pomegranate by heat treatment before cold storage.

41. Matityahu, I., Glazer, I., Holland, D., Bar-Ya'akov, I., Ben-Arie, R. and Amir, R., 2014. Total antioxidative capacity and total phenolic levels in pomegranate husks correlate

to several post-harvest fruit quality parameters. *Food and Bioprocess Technology*, *7*(7), pp.1938-1949.

42. Mhamdi, A., Queval, G., Chaouch, S., Vanderauwera, S., Van Breusegem, F. and Noctor, G., 2010. Catalase function in plants: a focus on Arabidopsis mutants as stress-mimic models. *Journal of experimental botany*, *61*(15), pp.4197-4220.
43. Mittler, R. and Zilinskas, B.A., 1993. Detection of ascorbate peroxidase activity in native gels by inhibition of the ascorbate-dependent reduction of nitroblue tetrazolium. *Analytical biochemistry,* 212(2), pp.540-546.
44. Mittler, R., 2002.Oxidative stress, antioxidants and stress tolerance. *Trends in plant science*, 7(9), pp.405-410.
45. Mizuno, M., Kamei, M. and Tsuchida, H., 1998. Ascorbate peroxidase and catalase cooperate for protection against hydrogen peroxide generated in potato tubers during low-temperature storage. *IUBMB Life, 44*(4), pp.717-726.
46. Mphahlele, R.R., Fawole, O.A., Stander, M.A. and Opara, U.L., 2014. Preharvest and post-harvest factors influencing bioactive compounds in pomegranate (*Punica granatum L.*)—A review. *Scientia Horticulturae*, *178*, pp.114-123.
47. Ntshangase, T., Phaleng, L., and Potelwa, Y., 2017. SOUTH AFRICAN FRUIT TRADE FLOW Issue No. 26 available from: http://www.namc.co.za/research-portal/wp-content/uploads/2017/07/South-African-Fruit-flow-report-June-2017-Issue-26-1-final_EDITED.pdf
48. O'Grady, L., Sigge, G., Caleb, O.J. and Opara, U.L., 2014. Effects of storage temperature and duration on chemical properties, proximate composition and selected bioactive components of pomegranate (Punica granatum L.) arils. *LWT-Food Science and Technology*, *57*(2), pp.508-515.
49. Pareek, S., Valero, D. and Serrano, M., 2015. Post-harvest biology and technology of pomegranate. *Journal of the Science of Food and Agriculture*, *95*(12), pp.2360-2379.
50. Raychaudhuri, S.S. and Deng, X.W., 2000. The role of superoxide dismutase in combating oxidative stress in higher plants. *The Botanical Review*, *66*(1), pp.89-98.
51. Schmid-Siegert, E., Stepushenko, O., Glauser, G. and Farmer, E.E., 2016. Membranes as structural antioxidants: recycling of malondialdehyde to its source in oxidation-

sensitive chloroplast fatty acids. *Journal of Biological Chemistry*, *291*(25), pp.13005-13013.

52. Serry, K.H. 2019. Post-harvest quality and chilling injury of "succary" pomegranates as affected by different packaging types. *The future Journal of Biology. Available from: https://www.researchgate.net/publication/334448490 accessed on: 19/08/19*

53. Sevillano, L., Sanchez-Ballesta, M.T., Romojaro, F. and Flores, F.B., 2009. Physiological, hormonal and molecular mechanisms regulating chilling injury in horticultural species. Post-harvest technologies applied to reduce its impact. *Journal of the Science of Food and Agriculture*, *89*(4), pp.555-573.

54. Shafie, M., Rajabipour, A., Castro-García, S., Jiménez-Jiménez, F. and Mobli, H., 2015. Effect of fruit properties on pomegranate bruising. International Journal of Food Properties, 18(8), pp.1837-1846.

55. Sharma, I. and Ahmad, P., 2014. Catalase: a versatile antioxidant in plants. In *Oxidative Damage to Plants* (pp. 131-148). Academic Press.

56. Sharma, P., Jha, A.B., Dubey, R.S. and Pessarakli, M., 2012. Reactive oxygen species, oxidative damage, and antioxidative defence mechanism in plants under stressful conditions. *Journal of botany*, *2012*.

57. Shewfelt, R.L., and Del Rosario, B.A., 2000. The role of lipid peroxidation in storage disorders of fresh fruits and vegetables. *HortScience*, *35*(4), pp.575-579.

58. Shiban, M.S., Al-Otaibi, M.M. and Al-Zoreky, N.S., 2012. Antioxidant activity of pomegranate (*Punica granatum L.*) fruit peels. *Food and Nutrition Sciences*, *3*(07), p.991.

59. Shigeoka, S., Ishikawa, T., Tamoi, M., Miyagawa, Y., Takeda, T., Yabuta, Y. and Yoshimura, K., 2002. Regulation and function of ascorbate peroxidase isoenzymes. *Journal of experimental botany*, *53*(372), pp.1305-1319.

60. Si, T., Wang, X., Zhao, C., Huang, M., Cai, J., Zhou, Q., Dai, T. and Jiang, D., 2018. The Role of Hydrogen Peroxide in Mediating the Mechanical Wounding-Induced Freezing Tolerance in Wheat. *Frontiers in plant science*, *9*, p.327.

61. Singh, H.P., Batish, D.R., Kohli, R.K. and Arora, K., 2007. Arsenic-induced root growth inhibition in mung bean (Phaseolus aureus Roxb.) is due to oxidative stress resulting from enhanced lipid peroxidation. *Plant Growth Regulation*, *53*(1), pp.65-73.
62. Siti, Z.I., Mohammad, M.K., Nashriyah, M. and Amru, N.B., 2015. Effects of hydrogen peroxide on growth, development and quality of fruits: a review. *Journal of Agronomy*, *14*(4), pp.331-336.
63. Ślesak, I., Libik, M., Karpinska, B., Karpinski, S. and Miszalski, Z., 2007. The role of hydrogen peroxide in regulation of plant metabolism and cellular signalling in response to environmental stresses. *Acta Biochimica Polonica*, *54*(1), pp.39-50.
64. Supapvanich, S., Arkajak, R. and Yalai, K., 2012. Maintenance of post-harvest quality and bioactive compounds of fresh-cut sweet leaf bush (*S auropus androgynus L. M err*.) through hot $CaCl_2$ dips. *International Journal of Food Science & Technology*, *47*(12), pp.2662-2670.
65. Taghipour, L., Rahemi, M. and Assar, P., 2015. Determining the physiochemical changes and time of chilling injury incidence during cold storage of pomegranate fruit. *Journal of Agricultural Sciences*, *60*(4), pp.465-476.
66. Tatar, O. and Gevrek, I., 2008. Lipid peroxidation and water content of wheat. *Asian J Plant Sci*, *7*, pp.409-412.
67. Tayyari, F., Khazaei, J., Rajaei, P. and Jouki, M., 2017. Effects of modified atmosphere packaging systems, low temperature and storage time on the quality of fresh minimally processed pomegranate arils. *Carpathian Journal of Food Science & Technology*, *9*(1).
68. Terzi, R. and Kadioglu, A., 2006. Drought stress tolerance and the antioxidant enzyme system. *Acta Biologica Cracoviensia Series Botanica*, 48, pp.89-96
69. Thomidis, T., 2014. Fruit rots of pomegranate (cv. Wonderful) in Greece. *Australasian Plant Pathology*, *43*(5), pp.583-588.
70. Ushimaru, T., Nakagawa, T., Fujioka, Y., Daicho, K., Naito, M., Yamauchi, Y., Nonaka, H., Amako, K., Yamawaki, K., Murata, N., 2006. Transgenic Arabidopsis plants expressing the rice dehydroascorbate reductase gene are resistant to salt stress. *Journal of Plant Physiology*, 163, pp.1179–1184.

71. Valenzuela, J.L., Manzano, S., Palma, F., Carvajal, F., Garrido, D. and Jamilena, M., 2017. Oxidative stress associated with chilling injury in immature fruit: post-harvest technological and biotechnological solutions. *International journal of molecular sciences*, *18*(7), p.1467.
72. Velikova, V., Yordanov, I. and Edreva, A., 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. *Plant Science*, 151(1), pp.59-66.
73. Wang, C.Y., 2004, September. Alleviation of chilling injury in tropical and subtropical fruits. In *International Symposium on Tropical and Subtropical Fruits 864,* pp.267-273.
74. Wang, Z., Xiao, Y., Chen, W., Tang, K., Zhang, L., 2010. Increased vitamin C content accompanied by an enhanced recycling pathway confers oxidative stress tolerance in Arabidopsis. *Journal of Integrative Plant Biology,* 52, pp.400–409.
75. Wills, R.B.H., McGlasson, B., Graham, D. and Joyce, D., 1998. Post-harvest: An introduction to the physiology and handling of fruits, vegetables and ornamentals. Univ. *New South Wales Press*, *262*.
76. Wongsheree, T., Ketsa, S. and van Doorn, W.G., 2009. The relationship between chilling injury and membrane damage in lemon basil (Ocimum× citriodourum) leaves. *Post-harvest Biology and Technology*, *51*(1), pp.91-96.
77. Yahia, E.M. ed., 2011. *Post-harvest biology and technology of tropical and subtropical fruits: Mangosteen to white sapote*. Elsevier.
78. Yamashita, K., Shiozawa, A., Banno, S., Fukumori, F., Ichiishi, A., Kimura, M. and Fujimura, M., 2007. Involvement of OS-2 MAP kinase in regulation of the large-subunit catalases CAT-1 and CAT-3 in Neurospora crassa. *Genes & genetic systems*, 82(4), pp.301-310
79. Yin. L., Wang. S., Eltayeb, A.E., Uddin, M.I., Yamamoto, Y., Tsuji, W., Takeuchi. Y., Tanaka, K., 2010. Overexpression of dehydroascorbate reductase, but not monodehydroascorbate reductase, confers tolerance to aluminum stress in transgenic tobacco. *Planta* 231, pp.609–621.
80. Younus, H., 2018. Therapeutic potentials of superoxide dismutase. *International journal of health sciences*, *12*(3), p.88.

81. Yusuf, M., Fariduddin, Q., Hayat, S., Hasan, S.A. and Ahmad, A., 2011. Protective response of 28-homobrassinolide in cultivars of Triticum aestivum with different levels of nickel. *Archives of environmental contamination and toxicology*, *60*(1), pp.68-76.