EFFECT OF METHYL JASMONATE AND SALICYLIC ACID ON CHILLING INJURY OF 'EUREKA' LEMONS

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DECLARATION

I hereby declare that the research work reported in this thesis is the result of my own investigation, except where acknowledged.

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We certify that the above statement is correct.

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DEDICATION

This thesis is dedicated to my late beloved grandmother Mrs Lubisi Masevase (1919-2009) for her endless support all the way from the beginning of my studies. May her soul rest in peace.

I also dedicate this work to my wife to be.

ABSTRACT

South Africa is the second largest exporter of citrus fruit in the world. There has recently emerged a strong demand for lemons in the world market due to their nutritional value, culinary and non-culinary uses. During exportation, fruit are subjected to low temperature (-0.5°C) for varying periods of time as an obligatory quarantine treatment. However, lemons are sensitive to low temperatures and easily develop chilling injury during this obligatory quarantine treatment. This has become a major limitation to the expansion of South Africa's lemon industry. Postharvest treatments with methyl jasmonate (MJ) and / or salicylic acid (SA) have been successfully used in horticultural crops to reduce chilling injury. A similar treatment was applied to 'Eureka' lemons. During the 2008 harvest season, postharvest fruit were either dipped in 10 or 50 µM MJ or 2 or 2.5 mM SA solutions. A control or no dip treatment was also applied. Three replicates of 15 fruits per treatment were used. During the 2009 harvest season the following postharvest treatments were applied as dips: 10 µM MJ, 2 mM SA, 10 µM MJ & 2 mM SA, 1 µM MJ & 0.2 mM SA, or 0.1 µM MJ & 0.02 mM SA solutions. A control or no dip treatment was also applied. Three replicates of 15 fruits per treatment were used.

Subsequently fruit were stored at -0.5ºC for 0, 7, 14, 21, 28, 35, and 42 days, before being transferred to room temperature (25°C) for 7 days where after chilling injury was rated. Treatments with 10 µM MJ and / or 2 mM SA reduced chilling injury symptoms in lemons harvested during the 2009 season. Although no visual symptoms of chilling injury were observed during the 2008 harvest season, treatments with 10 µM MJ and / or 2 mM SA reduced fruit mass loss, delayed the occurrence of stress symptoms such as lipid peroxidation and suppressed accumulation of ROS in the rind. Treatments with 10 µM MJ and / or 2 mM SA were more effective in inducing antioxidant capacity and other defence compounds such as phenolics, ascorbic acid, carbohydrates and chilling injury responses such as accumulation of proline in the rind. This may have increased the chilling tolerance of fruit during the cold storage. Therefore, this study revealed that MJ and SA have the potential to reduce and delay symptoms of chilling injury in lemons. This lead to the suggestion that both, MJ and SA dips should be further tested as treatments to mitigate chilling injury in lemons. Future studies

should focus more on preventing the injury itself or preventing the primary event of chilling injury. This could probably reduce the chances of secondary events to take place.

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GENERAL INTRODUCTION

1.1 Introduction

Lemon (*Citrus limon* L.) belongs to the genus Citrus of the Rutaceae family (Ladaniya, 2008). Citrus is the most important fruit consumed in the world (Li *et al*., 2009). It was recently reported that lemons constitute the main source of vitamins such as ascorbic acid (C), thiamin (B1), niacin (B3), pyroxidal phosphate (B6) and folic acid (B9) (Iglesias *et al*., 2007). In general, consumers prefer fresh rather than processed or canned fruit. Ladaniya (2008) suggests that consumption of fresh fruit is vital to improving blood circulation as well as health-promotion as antioxidants and this plays a crucial role in human health. According to Iglesias *et al*. (2007) there is increasing demand for lemon fruit driven by World Health Organization recommendations.

1.2 The South African Citrus Industry

South Africa is one of the top countries that produce and export lemons to Europe, Japan, USA and other countries around the world. According to Ladaniya (2008), South Africa recently reached a milestone of 100 years of citrus exports to the U.K. However, the industry loses fruit during postharvest storage due to physiological disorders (Mukhopadhyay, 2004). Despite the beneficial nutritive value of lemons, there is still a need to improve fruit quality in order to meet current consumer demands (Iglesias *et al.*, 2007). It remains a major challenge to the industry to reduce postharvest losses while maintaining fruit quality until consumption (Tasneem, 2004). The export of fruit to distant markets requires special postharvest practices to ensure that consumers receive a high quality product and value for their money (Tasneem, 2004). Cold storage of citrus fruit is one of the most widely used technologies for maintaining fruit quality as it is used as a quarantine treatment in exporting countries. It slows down respiration and other metabolic processes (Ghasemnezhad *et al*., 2007; Biolatto *et al*., 2005).

South Africa, as a citrus exporter, requires its fruit to be quarantined against Mediterranean fruit fly (Medfly, *Ceratitis capitata*); fruit must be certified free of

Mediterranean fruit fly before exportation (Schirra *et al*., 2004). Quarantine treatment is an obligatory practice which involves exposing fruit to sub-zero temperature (-0.5°C) for a specific number of days depending on a country's protocol. The objective is to control infestations of Medfly in citrus fruit as per the regulations of most importing countries (Schirra *et al*., 2004). When exporting lemons from South Africa to the United States of America, lemons undergo cold sterilization at a pulp temperature of -0.6ºC for 25-32 days (Van Wyk *et al*., 2009). However, lemons often do not tolerate such temperatures and develop chilling injury symptoms, especially when returned to warm temperatures; hence, this practice reduces fruit marketability (Mukhopadhyay, 2004; Schirra *et al*., 2004; Biolatto *et al*., 2005). For the citrus industry to remain globally competitive, it must minimize physiological disorders such as chilling injury. However, chilling injury remains one of the major hurdles in the industry (Mukhopadhyay, 2004).

1.1 Chilling Injury

Chilling injury is a physiological disorder caused by exposure of fruit to low temperatures above the freezing point (Soto-Zamora *et al*., 2005; Jing *et al*., 2009). The development of symptoms on the fruit varies with species, with most symptoms remaining invisible until after fruit has been transferred to room temperature (25°C); this usually only happens when the fruit is displayed at the market (Sala, 1998; Porat *et al*., 2004). Symptoms manifest in various forms including: mass loss, staining or browning of the flavedo and albedo, necrosis of the flavedo, water soaking, pitting and pit-like depressions in the flavedo that extend over time (Sala, 1998; Porat *et al*., 2004; Lafuente *et al*., 2005). Eventually postharvest losses during storage and at the market are realised (Lafuente *et al*., 2005). It is therefore, important to establish techniques that can reduce chilling injury (Tasneem, 2004).

1.2 Postharvest Treatment for Reducing Chilling Injury Symptoms

Salicylic acid and methyl jasmonate are hormones that are naturally occurring in the plant kingdom and are able to induce physiological processes in the fruit that offer protection against chilling injury (González-Aguilar *et al*., 2001; Tasneem, 2004). Salicylic acid has been shown to reduce chilling injury in peaches (Wang *et al*., 2006) and pomegranates (Sayyari *et al*., 2009). Treatment with methyl jasmonate significantly reduced the incidence of chilling injury in mangoes (González-Aguilar *et al*., 2001) and guavas (González-Aguilar *et al*., 2004).

However, the mode of action of methyl jasmonate in reducing chilling injury has not yet been fully elucidated (Cao *et al*., 2007). Similarly, no mechanism by which salicylic acid might control the occurrence of quality-related symptoms has been reported yet.

1.3 Problem Statement

The occurrence of chilling injury in lemons during postharvest storage still remains an unsolved problem and is reducing the expansion of South African citrus exports. Cold storage is important and cannot be avoided since it is used for maintaining fruit quality and as a quarantine treatment.

1.4 Hypothesis

Treating lemon fruit with an effective concentration of methyl jasmonate or salicylic acid or a combination of the two prior to cold storage at -0.5°C for 42 days, can reduce chilling injury during an extended cold storage time.

1.5 Objectives

1.5.1. To evaluate the potential of methyl jasmonate and salicylic acid in reducing chilling injury symptoms in lemons during quarantine treatment.

1.5.2. To determine the role of methyl jasmonate and salicylic acid in suppressing the production of reactive oxygen species and stimulating defence mechanisms against chilling injury in lemons during extended cold storage time.

1.5.3. To investigate the effect of methyl jasmonate and salicylic acid on fruit mass loss, respiration rate and ethylene evolution as non-destructive indicators of chilling injury.

1.5.4. To investigate physiological responses of lemons to chilling stress.

1.5.5. To determine the effect of methyl jasmonate and salicylic acid in inducing antioxidants defence mechanisms or systems associated with chilling injury in cold stored lemons.

1.6 Significance of the Study

It is hoped that the results of this study will make a significant contribution to the South African citrus industry in helping reduce chilling injury symptoms in lemons. Moreover, the

results of this study will probably aid future research to the citrus industry to meet the demand for fresh fruit in the world citrus market and to reduce postharvest losses of citrus fruit during exportation.

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CHAPTER 2

LITERATURE REVIEW

The ‘eureka’ lemon is commercially the most important lemon cultivar in the world (Ladaniya, 2008). It is grown in large parts of the world including South Africa, Australia, and the United States of America (Ladaniya, 2008). Lemon fruit usually range in size, from small to medium (50-70 mm diameter), with an oval shape (de Villiars and Joubert, 2006). They range from seedless to seedy with high juice and acid content (Ladaniya, 2008). Botanically, lemon fruit are classified as hesperidium, a specialised berry with a leathery rind and the mesocarp is divided internally into segments (Wardowski *et al*., 1986; Iglesias *et al*., 2007). In addition, lemon fruit are also covered by a medium to thick rind or peel which is ridged longitudinally (Wardowski *et al*., 1986). This rind is composed of two major morphologically distinct regions, which are known as the pericarp and endocarp (Iglesias *et al*., 2007). The pericarp covers and protects the fruit from damage and is divided into two layers, the flavedo and the albedo (de Villiars and Joubert, 2006, Iglesias *et al*., 2007).

The flavedo is the thin, rough, external, coloured portion of the peel which consists of the hypodermis, outer mesocarp and oil glands (Wardowski *et al*., 1986; de Villiars and Joubert, 2006, Iglesias *et al*., 2007). The albedo is the second outermost white, thick and spongy layer of the peel, which consists of a loose anastomosed network of parenchymatous cells with large air spaces as part of the inner mesocarp (Wardowski *et al*., 1986; Iglesias *et al*., 2007). The endocarp is the internal part and forms the pulp which is rich in soluble sugars, vitamin C, pectin, fibers, organic acids and potassium salt (de Villiars and Joubert, 2006). According to Iglesias *et al.* (2007), the pulp contains a high percentage of water (85-90%), carbohydrates, minerals, vitamin C and small quantities of lipids and proteins.

2.1 THE IMPORTANCE OF LEMON FRUIT

Lemon fruit are an economically important and are grown in developing and developed countries and are valued as part of a nutritious diet (Economos and Clay, 1999; Iglesias *et al*., 2007). Fruit are a rich source of essential nutrients such as minerals, vitamins, glycaemic and non-glycaemic carbohydrates (sugars and fibers), potassium, folate, calcium, thianin, niacin, phosphorus, magnesium, riboflavin, pantothenic acid and phytochemicals (Economos and Clay, 1990). This array of chemicals in lemon fruit may help reduce the risk

or retard the progression of many diseases and disorders, including cardiovascular diseases, stroke, cancer, anaemia, heart diseases and hypertension (Economos and Clay, 1990). In addition to the nutritional value of lemons the fruit also supply anticancer agents and other compounds with antioxidant activity (Iglesias *et al.*, 2007).

2.2 SCOPE OF THE CITRUS INDUSTRY IN SOUTH AFRICA

The Republic of South Africa is the largest citrus producer and exporter within the Southern African region. The industry is, and always has been, export oriented (Wardowski *et al*., 1986). During the 2008 season alone, the industry exported 61% of the fruit and distributed only 30% to local markets (Citrus Growers' Association, 2008). Today, the South African citrus industry is the second largest exporter of citrus fruit in the world (Figure 2.1). The industry produces and supplies citrus fruit to many parts of the world, including Japan, Canada, Europe, the Middle East, South East Asia, the USA and the UK (Figure 2.2). Fruit exported by the industry include grapefruit, lemons, soft citrus, valencia and navels (Citrus Growers' Association, 2008).

The South African citrus industry is now ranked as the fifth largest exporter of lemon fruit in the world (Figure 2.3). However, the industry still can increase lemon exports, especially in accessing a market like Japan. During the 2008 season, only 4% of lemon fruit were approved for the Japanese market compared with 81% and 15% for grape fruit and oranges, respectively (Citrus Growers' Association, 2008). Iglesias *et al.* (2007) advise that there is still a major need to improve fruit quality and solve issues affecting global trade of lemons in order to meet current consumer demands. One of the major factors hindering trade in lemons is their susceptibility to chilling injury during postharvest storage.

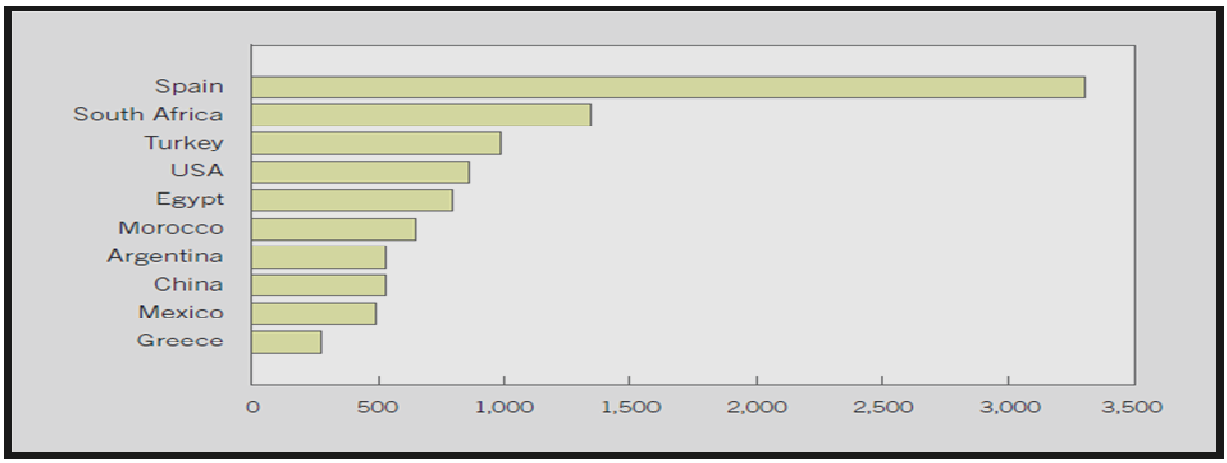

Figure 2.1: Total world exportation of fresh citrus fruit in thousand tons produced in main citrus producing countries during the 2008/2009 harvest season (Citrus Growers' Association, 2010).

Figure 2.2: Distribution (local, processed and export) of lemon fruit in tons produced in South Africa from 2000-2009 harvest seasons (Citrus Growers' Association, 2010).

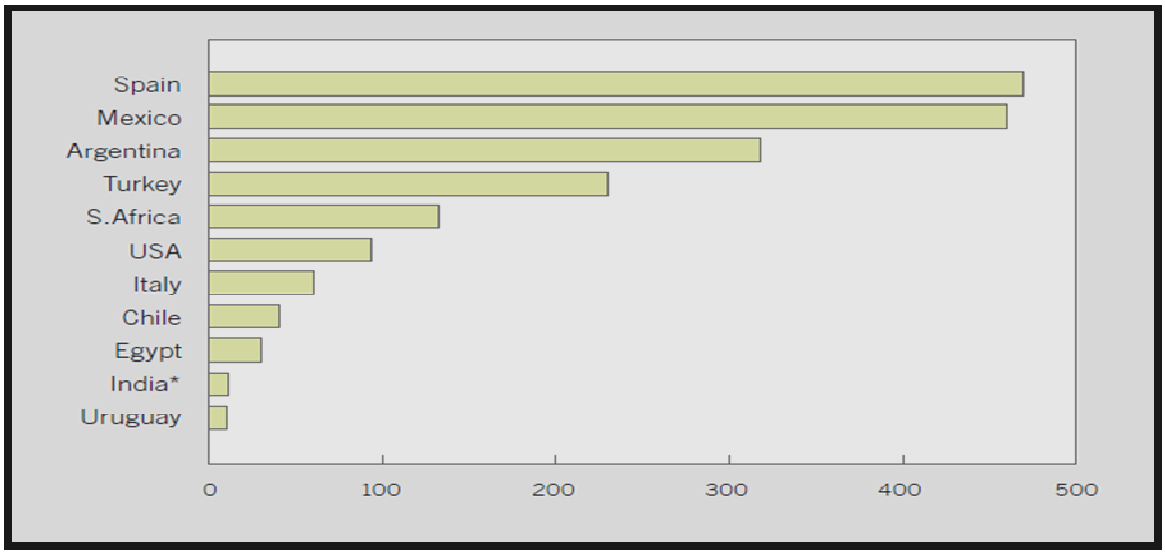

Figure 2.3: Total world exportation of lemon fruit (in thousand tons) produced in the lemon fruit producing countries during the 2008/2009 season (Citrus Growers' Association, 2010).

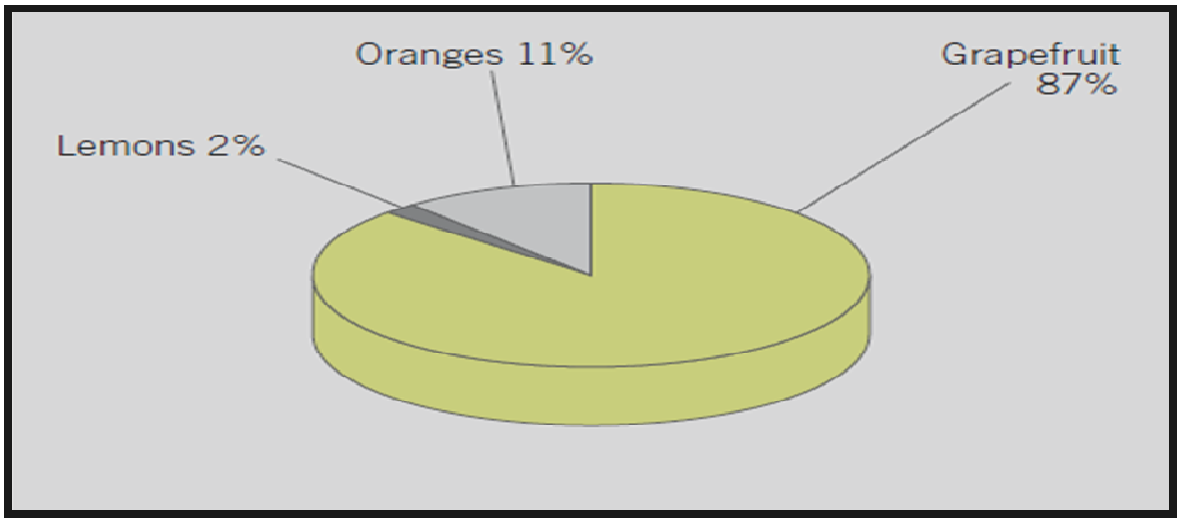

Figure 2.4: Total percentage of South African citrus fruit (lemons, oranges and grapefruit) approved for Japan exportation in 2009.

2.3 CHILLING INJURY

Chilling injury is a physiological disorder that occurs in most horticultural crops of tropical and subtropical origin and reduces fruit marketability. It is a major obstacle to the

expansion of the lemon trade on the world market (Siriphanich, 2002; Lurie and Crisosto, 2005). Chilling injury can occur during storage, transportation, market distribution and even holding in household refrigerators (Raison and Orr, 1990). Lafuente *et al.* (2005) report that citrus fruit, such as lemons are sensitive to low temperatures and develop chilling injury symptoms when exposed to temperatures below 9°C. Lafuente *et al.* (2005) report that sensitivity of lemons to chilling injury may be due to a low ability to rearrange membrane lipids in response to chilling stress.

According to Raison and Orr (1990), responses of plant tissues to chilling injury may be separated into 'primary' and 'secondary events' (Figure 2.5). The primary events depend on the duration of exposure. Exposing fruit to chilling temperature (-0.5°C) for a short period (about 16 days) can affect metabolism and cause changes in the proteins and lipids of the fruit as a primary event (Raison and Orr, 1990). During the primary events, chilling temperature affects fruit metabolism and results in metabolic dysfunction as a primary response to chilling injury. Metabolic dysfunction in horticultural crops involves high or abnormal respiration and production of ethylene in the fruit, coupled with increased electrolyte leakage as well as high amino acid incorporation into proteins (Raison and Orr, 1990).

Lafuente *et al*. (2005) suggested that changes in membrane permeability were associated with membrane-lipids' physical phase transition, from a 'flexible liquid-crystalline' to a 'solid gel' structure. However, symptoms resulting from the primary events of chilling injury are reversible; hence, fruit can recover from metabolic dysfunction. Wang (1990) advised that if the chilling storage was terminated and the temperature rose above the chilling temperature (-0.5°C), the tissue of the fruit could either recover from the metabolic imbalances or the higher temperature can accelerate the development of visible chilling injury symptoms. On the other hand, if chilling storage time was extended, then the primary event would lead to a cascade of secondary events (Raison and Orr, 1990; Lafuente *et al*., 2005).

During the secondary events, extended chilling temperature can cause hydrolysis of membrane lipids resulting in loss of regulatory control, an imbalance in metabolism and cell death leading to the development of the visual symptoms of chilling injury (Lyons, 1973; Raison and Orr, 1990; Lafuente *et al*., 2005). Unlike the primary event, damages resulting from the secondary event are irreversible, even if chilling storage is discontinued. However,

transferring fruit from chilling temperature (-0.5°C) to room temperature (25°C) after a long cold exposure, 28 days or more, could increase the development or visibility of chilling injury symptoms. El-hilali (2003) argued that chilling injury symptoms may already be present during low temperature storage but become more visible when fruit are transferred to room temperature.

Symptoms of Chilling Injury

Lafuente *et al*. (2005) remarked that chilling injury symptoms varied among crops due to different mechanisms involved. Some of the chilling injury symptoms pointed out by Raison and Orr (1990) and Lafuente *et al*. (2005) include increased electrolyte leakage, abnormal respiration, increased ethylene evolution in the fruit, surface lesions (brown pit like depressions in the flavedo, pitting, large sunken areas, discolouration of the surface), water-soaking of the tissue and increased decay.

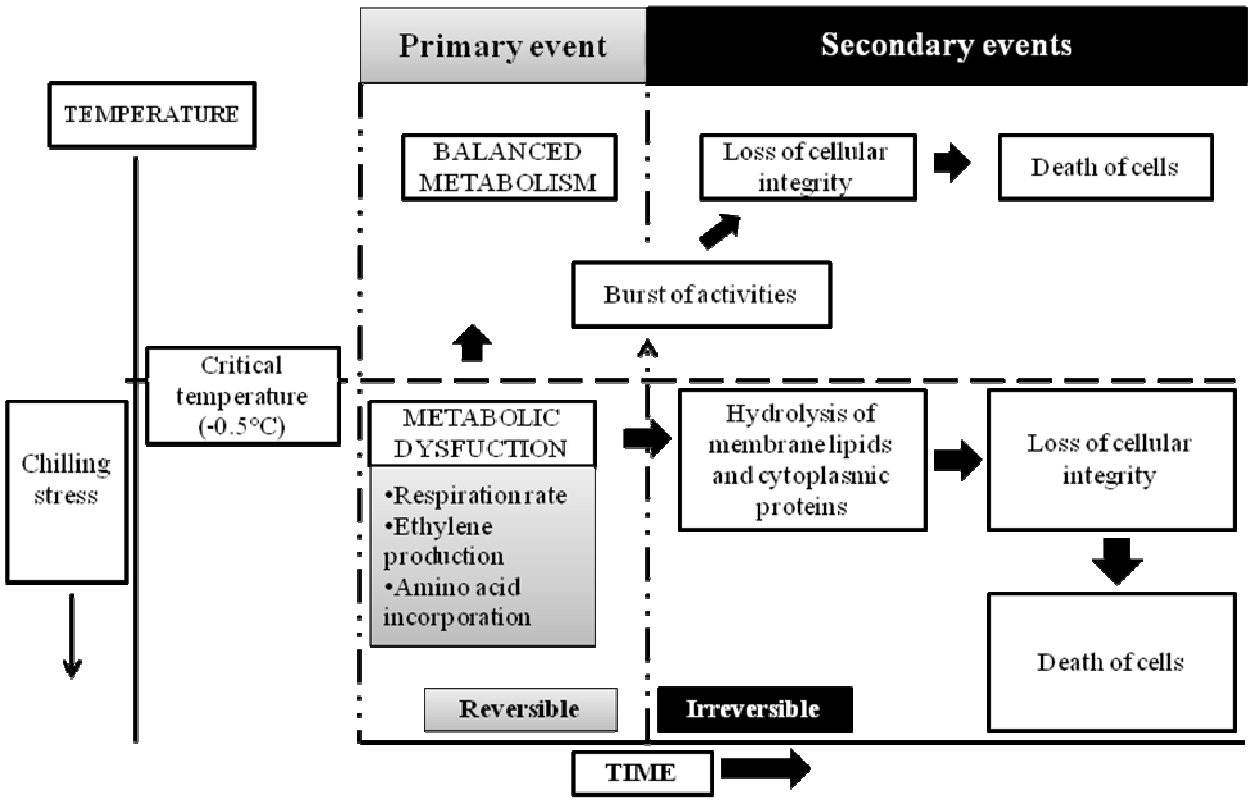

Figure 2.5: Modified Schematic representation of the relationship between the 'primary event' and 'secondary events' of chilling injury (Raison and Orr (1990).

2.4 PHYSIOLOGICAL ASPECTS OF CHILLING INJURY

2.4.1. Reactive Oxygen Species

Reactive oxygen species (ROS), like superoxide radicals ($O_2^{\cdot -}$), hydroxyl radicals ($^{\cdot}OH^-$) and hydrogen peroxide (H_2O_2) are the products of dysfunctional oxidative biochemical reactions within cells (Paliyath *et al*., 2008). During the course of normal metabolic processes ROS are continuously produced by plant tissue as unwanted by-products of various metabolic pathways (Maxwell *et al.*, 1999; Pitzschke *et al.,* 2006). Studies have revealed that most cellular compartments such as chloroplasts, mitochondria, peroxisomes and nitrogen-fixing nodules have the potential to become sources of ROS (Davey *et al.,* 2000; Møller, 2001; Bhattacharjee, 2005). Furthermore, ROS are formed via many processes such as dismutation of superoxide in chloroplasts and oxidation of gylcolate in the C2 pathway of peroxisomes (Figure 2.5). Moreover, ROS can also be formed via the mitochondrial electron transport system and the detoxification reactions catalysed by cytochrome P450 in the cytoplasm and endoplasmic reticulum (Asada, 1994; Bhattacharjee, 2005).

The production of ROS in plant tissues has been suggested to be enhanced by plant stresses such as chilling temperatures (Davey *et al.,* 2000; Mittler *et al*., 2004). Møller (2001) warned that if the production of ROS was not controlled or prohibited in plant tissue, cell damage will result. During chilling stress, ROS can attack cell membranes and react with proteins, DNA and lipids causing extensive damage such as decreased enzyme activity, increased membrane permeability and lipid peroxidation resulting to chilling injury (Møller, 2001; Sarkar *et al*., 2009). Thus, ROS must be effectively inhibited or, at least, either removed or avoided to reduce chilling injury symptoms in the fruit (Møller, 2001). Therefore, ROS can be effectively scavenged by a variety of antioxidative defence components that are found in plant tissue (Alscher *et al*., 1997; Pitzschke *et al*., 2006).

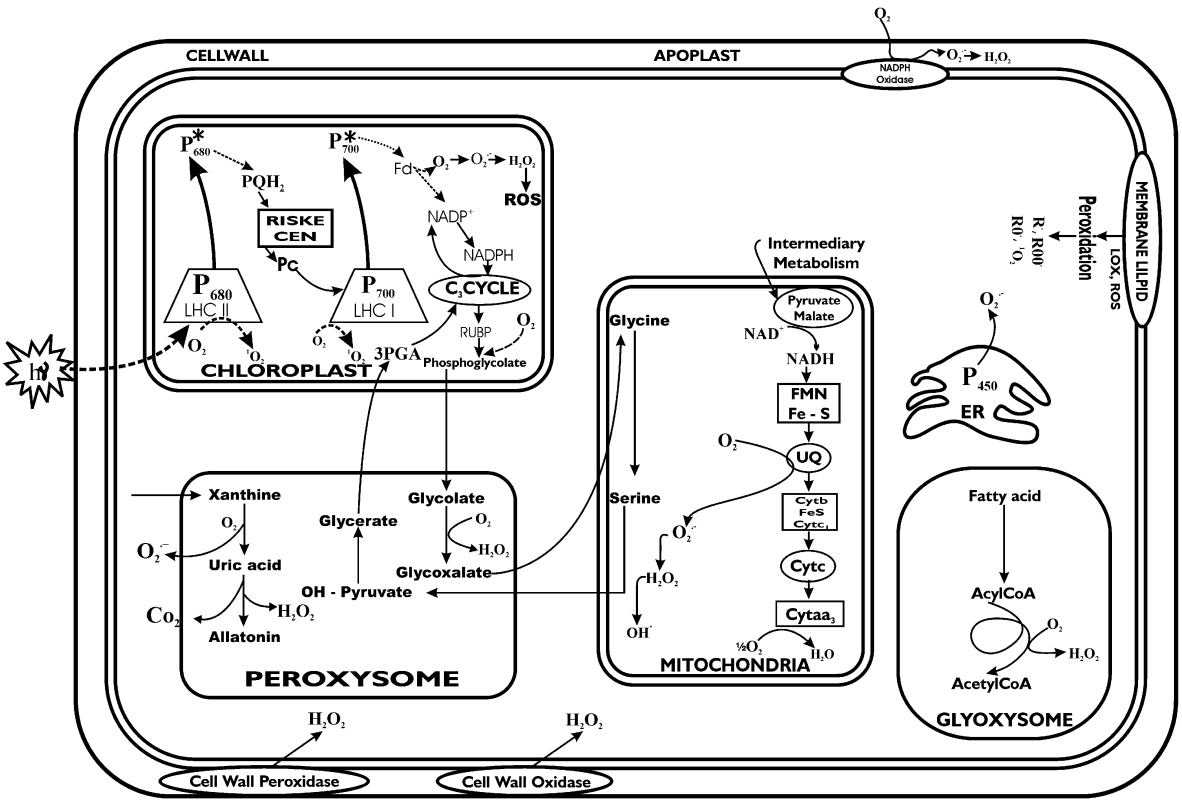

Figure 2.6: Source of reactive oxygen species in a plant cells (Bhattacharjee, 2005). (PGA = polygalacturonic acid, LOX = lipoxygenase, ROS = reactive oxygen species, FMN = flavin mononucleotide, UQ = ubiquinone, Fe-S = Iron-Sulfur cluster, NAD = nicotinamide adenine dinucleotide, P_{680} LHC II = low reaction chlorophyll center in photosystem II, P_{700} LHC I =the low reaction chlorophyll center in photosystem I, PC = plastocyanin, PQH_2 = plastoquinone, P = primary electron donor, Fd = ferredoxin, RuBP = ribulose 1.5-bisphosphate, ER = endoplasmic reticulum, Cyt = cytochrome, Cyt b = cytochrome b, Cyt c = cytochrome c).

2.4.2. Ethylene Production

Ethylene is a gaseous plant hormone that plays a major role in plant defence mechanisms (Davies, 2004). It is also known as a stress hormone, with its levels increasing in response to stress (Ladaniya, 2008). However, lemons produce relatively low levels of ethylene (<0.1 µl/kg/h) after harvest (Ladaniya, 2008). This could be because lemons lack an autocatalytic rise in ethylene production (Fujii *et al*., 2007). However, studies have revealed that the same small amount of ethylene produced by the fruit may play a role in protecting the fruit against plant stresses including chilling (Cajuste and Lafuente, 2007). In addition, it has

been suggested that the presence of ethylene in lemon fruit stored at chilling temperatures may be beneficial in promoting chilling tolerance in some fruit (Martinez-Téllez *et al*., 1995; Lurie and Crisosto, 2005).

In contrast, certain studies have reported that a burst in ethylene in fruit is often a sign of damage to cellular membranes, which is usually followed by the development of chilling injury symptoms (Zhou *et al*., 2001). Ladaniya (2008) concurred that high production of ethylene in citrus fruit may be associated with cell damage due to chilling injury. Murata (1990), however, argued there was no direct association between chilling injury and ethylene production. The author also reported that the role of ethylene in preventing chilling injury in fruit depends on the origin of the commodity. It is thus important to establish whether ethylene production in fruit promotes chilling injury or if it is involved in defence mechanisms, or if it is of any significance whatsoever (Raison and Orr, 1990).

2.4.3. Respiration Rate

Respiration (aerobic) is the oxidative breakdown of carbohydrates, lipids, proteins and organic acids into simple products such as carbon dioxide and water with the production of energy and other molecules (Kader, 2002; Arafat, 2005). Fruit require energy from aerobic respiration to break down organic compounds (carbohydrates) for carrying out metabolic reactions and to maintain membrane permeability (Ladaniya, 2008). Lemons are non-climacteric fruit characterized by a relatively low respiration rate (Porat, 2004; Fujii, 2007). The respiration rate of lemons can be affected by changes in storage temperature (Ladaniya, 2008). High temperatures accelerated respiration rate while lower temperatures reduced the respiration rate of lemon fruit (Ladaniya, 2008). Therefore, storing lemons at lower temperatures is desirable for extending normal metabolism of the fruit for a given period. Chilling temperature (-0.5 °C) may damage membranes leading to exposure of mitochondria to harmful substances such as ROS (Huang and Romani, 1991). Similarly, Murata (1990) found that a high respiration rate can be associated with increased membrane permeability due to tissue damage.

2.4.4. Lipid Peroxidation

Lipids are biochemical compounds which contain one or more long chains of fatty acids and are soluble in organic solvents. Lipids include diacyl-and triacylglycerols, phospholipids, sterols and waxes that provide an external barrier to the fruit (Paliyath *et al*., 2008). Lemons contain lipids, however, they are not a rich source of fats and only low amounts can be found either in seeds or the rind (Ladaniya, 2008). Exposing lemons to extended cold storage may result in the oxidation of lipids, also known as lipid peroxidation (Blokhina, 2000).

Lipid peroxidation is thought to be initiated by ROS in plant cells and is considered to be a major cause of cell damage when fruit are exposed to chilling temperatures (Aruoma *et al.*, 1989; Bhattacharjee, 2005). According to Arafat (2005) as well as Blokhina (2000), lipid peroxidation can be divided into three distinct stages: initiation, propagation and termination (Figure 2.7). In initiation it has been suggested that lipid peroxidation can be triggered, either by hydroxyl radicals that are responsible for abstracting hydrogen atoms from fatty-acid side chains or by the enzyme lipoxygenase which is capable of breaking down the lipid component of membranes (Bhattacharjee, 2005). The initiation stage includes activation of oxygen and involves transition metal complexes, such as iron and copper to form an activated oxygen complex that can abstract allylic hydrogens or act as catalysts in the decomposition of existing lipid hydro-peroxides, resulting in the formation of alkoxy and peroxy radicals (Bhattacharjee, 2005). According to Bhattacharjee (2005) alkoxy and peroxy radicals are toxic and could pose a threat to bio-molecules.

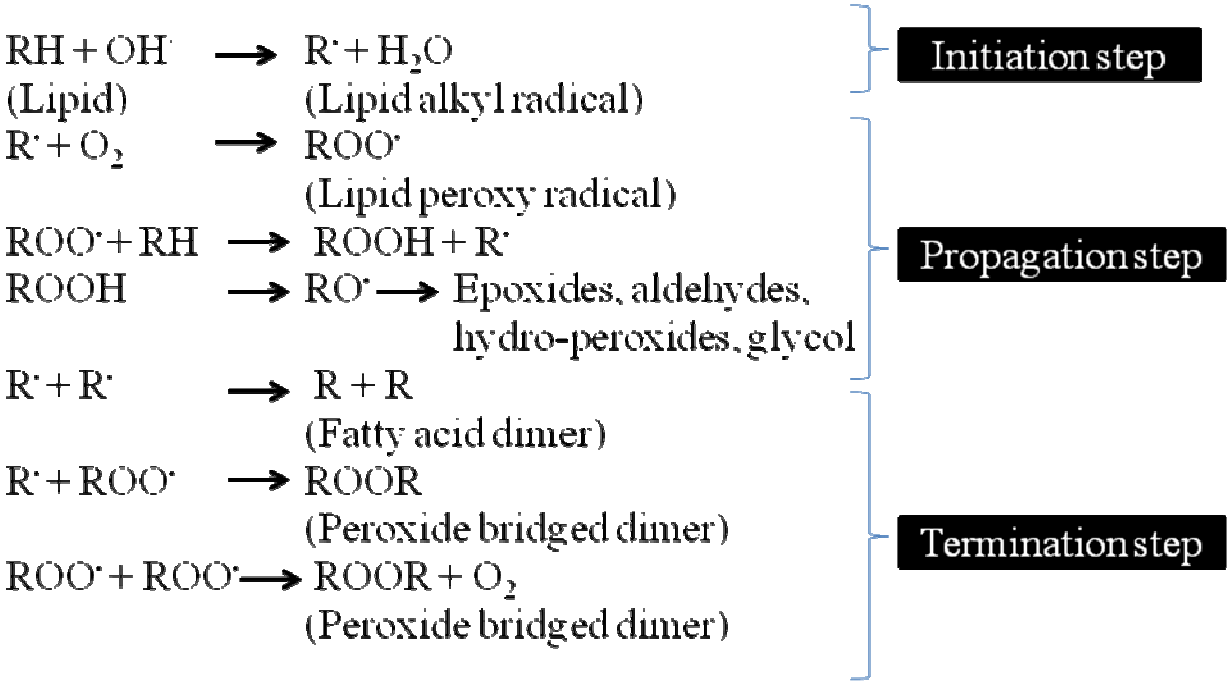

Figure 2.7: Three stages (initiation, propagation and termination) involved in lipid peroxidation of plant cells (modified from Bhattacharjee, 2005).

2.4.5. Antioxidants

Antioxidants are compounds capable of quenching ROS without undergoing conversion, themselves, to destructive radicals (Hodges, 2003). Antioxidants are also defined as compounds capable of delaying or inhibiting oxidation of molecules, such as lipids, by inhibiting the initiation or propagation stage of oxidizing chain reactions (Velioglu *et al*., 1998; Pennycooke *et al.*, 2005). Fruit contain important antioxidants such as phenolics, ascorbic acid, and carbohydrates (Chanjirakul *et al*., 2006). These antioxidants play a vital role in protecting fruit against the effects of ROS which are produced in response to stresses such as chilling (Sala, 1998). Antioxidants are thought to protect fruit against chilling stress by acting as free radical scavengers, peroxide decomposers, singlet and triplet oxygen quenchers, enzyme inhibitors and synergists in fruit (Chanjirakul *et al*., 2006). Therefore, efficient antioxidant activity in fruit stored at chilling temperatures is essential in order to maintain the concentrations of ROS at relatively low levels (Hodges and Forney, 2000; Cao *et al.*, 2008).

Ascorbic Acid

Ascorbic acid is one of the potent antioxidants found abundantly in lemon fruit (Koca *et al.,* 2003; Ladaniya, 2008). The antioxidant is known for removing ROS in plant tissue and for protecting plant tissue from oxidative damage (Mittler, 2002; Om-arun and Siriphanich, 2005). In addition, ascorbic acid has the ability to act as an enzyme co-factor by interacting enzymatically and non-enzymatically with ROS or as a radical scavenger in fruit tissue stored at chilling temperatures (Davey *et al.,* 2000). However, ascorbic acid has also been reported to decrease with cold storage time in fruit (Tatsumi *et al*., 2006). The decrease in ascorbic acid content during cold storage of horticultural crops is mainly due to a reduction in the protective mechanism of the fruit from temperature damage (Om-arun and Siriphanich, 2005). Therefore, extended cold storage time could result in a decline in ascorbic acid in the rind, causing occurrence of rind storage disorders.

Carbohydrates

Simple carbohydrates are mainly mono- and disaccharides and play a significant role in plant cells by acting as building blocks in plant biosynthesis reactions and are also building blocks for compounds providing protection as antioxidants (Couée *et al.,* 2006). Chilling stress, which causes direct or indirect accumulation of ROS, is associated with accumulation of soluble sugars; this is considered an adaptive response to stress (Roitsch, 1999; Couée *et al*., 2006). The composition and concentration of soluble sugars in fruit may influence the sensitivity of tissue to chilling temperatures and protect cellular membranes from chilling injury (Leprince *et al*., 1992; Ingram and Bartels, 1996; Holland *et al.,* 2002). Soluble sugars can either have a direct or indirect role in protecting or influencing chilling tolerance mechanisms in fruit at chilling temperatures (Raison and Orr, 1990). This role includes the protection of fruit tissues against oxidative stress, the ability to scavenge ROS, induction of antioxidant defences, reduction of water loss from tissue and stabilization of cell membranes (Raison and Orr, 1990; Couée *et al*., 2006).

Phenolic compounds

A phenolic compound is characterized by at least one aromatic ring (C6) bearing one or more hydroxyl groups (Paliyath *et al*., 2008). Phenolic compounds are derivatives of the pentose phosphate, shikimate and phenylpropanoid pathway in plants (Balasundram *et al*., 2006). They display a variety of physiological functions including antioxidant activity (Othman *et al.,* 2007). Phenolic compounds are often classified as secondary metabolites widely distributed in plants and known for their role in defence against plant stress (Ćetković *et al*., 2007). In general, the role of phenolics in plants is to adapt the plant to changing environmental conditions and to act as a signalling molecule (Paliyath *et al*., 2008). In lemon fruit, phenolic compounds are abundant in the rind where they play a role as antioxidants (Balasundram *et al*., 2006). Beneficial effects derived from phenolic compounds have been attributed to their antioxidant activity in plants (Heim *et al*., 2002; Balasundram *et al*., 2006). Phenolic compounds, like any other antioxidant, protect cells from ROS, either by scavenging or by neutralizing free radicals (Pennycooke *et al.*, 2005).

Proline

Proline is one of the most abundant amino acids found in citrus fruit (Ladaniya, 2008). However, proline levels in fruit can be affected by plant stresses (Yelenosky, 1979). Plant stress, such as chilling stress, can cause an increase in proline synthesis and proline accumulation in horticultural crops; it is this increase in proline that is considered to be an indicator of plant stress (Yelenosky, 1979, Paliyath *et al*., 2008). Plant stress can cause the concentration of proline in fruit to increase, probably by up to 100 times the normal level, which makes up to 80% of the total amino acid pool (Matysik *et al*., 2002). The role of proline in plants varies from an adaptive response to salinity and drought to helping fruit withstand the effect of stress during cold storage (Chen and Li, 2002; Matysik *et al*., 2002).

The involvement of proline in plant stress, such as chilling stress, includes roles where proline acts as stabilizer of proteins and membranes (Rudolph *et al*., 1986; Chen and Li, 2002), as scavenger of ROS (Saradhi *et al.,* 1995; Chen and Li, 2002) and as a source of reduction equivalents during recovery from stress (Hare and Cress, 1997; Chen and Li, 2002). Proline protects plant tissues from free radical induced damage by the quenching of singlet oxygen (Matysik *et al.,* 2002; Misra and Saxena, 2009). Therefore, proline may not be just a

by-product of stress defence, but a chemically active compound, involved in plant stress protection (Matysik *et al.*,2002). In addition, proline synthesis has been considered key in regulating inducible antioxidant responses in fruit subjected to stress (Sarkar *et al*., 2009).

Proline-linked pentose phosphate pathway and phenolic synthesis in fruit at chilling temperatures

The role of proline in regulating antioxidants in fruit stored at chilling temperature (-0.5°C) is linked to the pentose-phosphate pathway together with phenolic photosynthesis (Paliyath *et al*., 2008). Recently, a proline synthesis model involving pentose-phosphate pathway and phenolic synthesis has been proposed (Figure 2.8). In the model, proline's response to chilling stress is to induce antioxidants and other defence mechanisms or molecules (Shetty and Wahlqvist, 2004; Paliyath *et al.*, 2008; Sarkar *et al*., 2009). Shetty (2004) points out that the model is based on the role of proline and pyrroline-5-carboxylate (P5C) in regulating redox and hydride ion-mediated stimulation of the pentose phosphate pathway.

Together, P5C and proline function as a redox couple and are known to be metabolic regulators (Shetty, 2004). Proline is synthesized via the reduction of glutamate to P5C (Paliyath *et al.*, 2008). During fruit respiration, oxidation reactions produce hydride ions, to accelerate the reduction of P5C to proline in the cytosol (Sarkar *et al*., 2009). Paliyath *et al.* (2008) remarked that since the process of P5C reduction required NADPH as a reductant, then an increase in proline synthesis would result in a reduced NADPH/$NADP^+$ ratio. Moreover, Shetty (2004) advised that the reduction of P5C will then provide $NADP^+$, which is the co-factor for glucose-6-phosphate dehydrogenase (G6PDH). The enzyme plays a crucial role catalysing the first rate limiting steps of the pentose phosphate pathway (Sarkar *et al*., 2009).

It has been suggested that during cold storage of horticultural crops, different stress factors may induce proline synthesis, which in turn stimulates the pentose phosphate pathway (Shetty, 2004; Shetty and Wahlqvist, 2004; Paliyath *et al*., 2008). The stimulation of the pentose phosphate pathway is essential for inducing an efficient antioxidant response and for acting along with NDAPH to support pathways for the synthesis of antioxidants, phenolics and other protective compounds (Shetty, 2004; Shetty and Wahlqvist, 2004; Paliyath *et al*.,

2008). Sarkar *et al.* (2009) recommended that the model could further stimulate both, the shikimate and phenylpropanoid pathways.

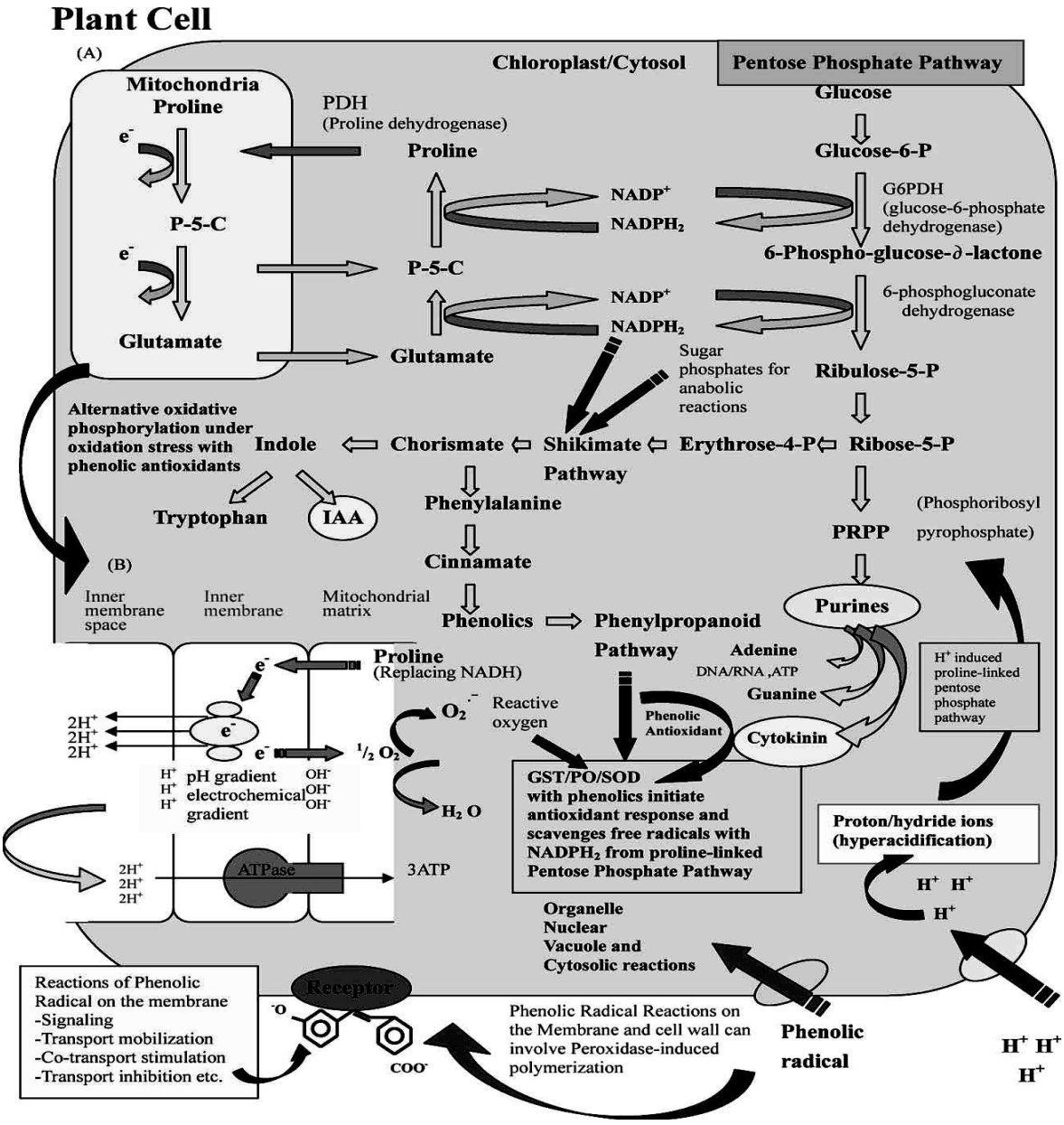

Figure 2.8: Role of proline-linked pentose phosphate pathway for effective antioxidant activity and for regulating phenolic synthesis in fruit tissues at cold storage (adapted from Shetty, 2004; Sarkar *et al.*, 2009). (GST = glutathione S-transferase, SOD = superoxide dismutase, PO = phenolic oxidation, DNA = deoxyribonucleic acid, RNA = ribonucleic acid, ATP = adenosine triphosphate, $NADP^+$ = nicotinamide adenine dinucleotide phosphate, IAA = indole-3-acetic acid).

2.5 ALLEVIATION OF CHILLING INJURY SYMPTOMS IN FRUIT

Raison and Orr (1990) advised that, if chilling of fruit cannot be avoided, then treatments should be developed to either increase the tolerance of tissue before chilling or to reduce the development of chilling injury symptoms. Different techniques such as pre-storage hardening and non-lethal high temperature conditioning treatments which include benzimidazole have been used to increase chilling tolerance in citrus fruit (Lafuente *et al.,* 2005). However, the efficacy of these treatments in reducing chilling injury may be largely influenced by pre-harvest factors and also by the type of heat treatment selected, which may also affect fruit quality (Schirra and Mulas, 1995).

2.5.1. Jasmonates

Jasmonic acid and methyl jasmonate are grouped together as jasmonates and are widely distributed in plants as stress hormones (Creelman and Mullet, 1997). Jasmonates are fatty acids that play a role in plant development and also act as signalling molecules increasing plant defence mechanisms during environmental stress (Creelman and Mullet, 1997; Nilprapruck *et al*., 2008). The hormones are known for their strong activity when exogenously applied postharvest particularly methyl jasmonate (Cleland, 1999). Methyl jasmonate has been studied for its effectiveness in maintaining fruit quality in horticultural crops during the postharvest period (Ghasemnezhad and Javaherdashti, 2008). The compound has the ability to increase the resistance decay by enhancing fruit antioxidant systems (Ghasemnezhad and Javaherdashti, 2008). Recently, methyl jasmonate has been used to reduce chilling injury in a variety of horticultural crops such as guava (González-Aguilar, 2004), pineapple (Nilprapruck *et al*., 2008) and peach (Jin *et al*., 2009). Application of methyl jasmonate reduced chilling injury in zucchini squash (Wang and Buta, 1994), grapefruit (Meir *et al*., 1996), mango (González-Aguilar *et al*., 2000) tomato (Ding *et al*., 2002), guava (González-Aguilar, 2004) and peppers (Wang *et al*., 2005). However, methyl jasmonate's mode of action in reducing chilling injury has not yet been clearly elucidated (Cao *et al*., 2007*).*

2.5.2. Salicylic acid

Salicylic acid belongs to a group of plant phenolics and is widespread in plants and now considered as a naturally occurring plant hormone (Raskin, 1992; Kang *et al*., 2003). It

can act as a potential non-enzymatic antioxidant and plays an important role in regulating several plant physiological processes such as stomatal closure (Rai *et al*., 1986), increasing plant tolerance to abiotic stress (Li *et al*., 1998), regulating activities of antioxidant enzymes (Arfan *et al.*, 2007; Noreen *et al*., 2009). Salicylic acid plays a role in plant development, growth and pathogen tolerance by inducing the production of 'pathogenesis-related proteins' (Li *et al.*, 1998; Zheng and Zhang, 2004). Salicylic acid has received attention for its action in signalling pathways that are induced by cold stress (Yordanova and Popova, 2007). Recently, salicylic acid has been used to reduce chilling injury in horticultural crops. Treatment with salicylic acid has been repeated to increase chilling tolerance in maize (Janda *et al*., 2000) and peaches (Wang *et al.*, 2006). Moreover, postharvest treatment with salicylic acid reduced chilling injury in tomato fruit (Ding *et al.*, 2002) and pomegranates (Sayyari *et al*., 2009). The mode of action of salicylic acid in increasing chilling tolerance in crops is related to its influence on antioxidative enzyme activities, oxidative stress responses and hydrogen peroxide metabolism (Wang *et al.*, 2006; Yordanova and Popova, 2007).

2.6 CONCLUSION

The South African citrus industry still loses large quantities of lemons during postharvest storage, transit and at the market due to chilling injury. Chilling injury, a physiological disorder that occurs in fruit exposed to low but not freezing temperatures. Storing lemons at low temperature (-0.5°C) cannot be avoided, since it is an obligatory quarantine treatment for the control of Mediterranean fruit fly. The physiological disorder is an unsolved problem in the citrus industry, which reduces fruit quality and marketability of the fruit. Postharvest treatment with either methyl jasmonate or salicylic acid has been successfully used to reduce chilling injury in several other horticultural crops. In this study, it was hypothesised that postharvest treatment with a correct concentration of methyl jasmonate and/or salicylic acid could reduce chilling injury symptoms in lemons during extended cold storage.

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CHAPTER 3

THE ROLE OF METHYL JASMONATE AND SALICYLIC ACID IN SUPPRESSING REACTIVE OXYGEN SPECIES AND STIMULATING DEFENSE MECHANISMS TOWARDS CHILLING INJURY OF EUREKA LEMONS

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SUMMARY

Certain markets have phytosanitary requirements which involve exposing lemons to chilling temperature (-0.5°C) during exportation. This exposure can result in the development of chilling injury (CI) symptoms. Accumulation of reactive oxygen species (ROS) is thought to be a primary cause of CI. Therefore, the roles of methyl jasmonate (MJ) and salicylic acid (SA) in delaying accumulation of ROS and oxidative damage during cold storage were investigated. During 2008, fruit were sterilized, air-dried and dipped in 10 or 50 µM MJ or 2 or 2.5 mM SA solution for 30 seconds. Fruit were also dipped into 10 µM MJ, 2 mM SA, 10 µM MJ & 2 mM SA, 1 µM MJ & 0.2 mM SA or 0.1 µM MJ &0.02 mM SA for 30 seconds (during 2009 season). Fruit were thereafter, waxed with Avoshine® and stored at -0.5ºC for 0, 7, 14, 21, 28, 35, or 42 days, before being transferred to room temperature for 7 days. Measurements of ROS, membrane lipid peroxidation and CI in the fruit rind were taken. During 2008, no fruit developed CI. Treatment with 10 µM MJ, 2 mM SA, or 10 µM MJ & 2 mM SA significantly ($P \leq 0.05$) reduced the production of ROS and suppressed membrane lipid peroxidation and thus reduced the CI index during extended cold storage in 2009. Treatment with MJ or SA probably triggered defence mechanisms such as antioxidants in order to enhance cell membrane stability and protect cells from possible ROS damage. Therefore, MJ and / or SA could be used to reduce CI in lemons.

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Cold storage of lemons has been widely practised for slowing down respiration, delaying senescence, reducing water loss and maintaining fruit quality (Meng *et al*., 2009). It is considered an obligatory quarantine treatment for shipping South African fruit to distant markets such as Europe, Japan and the USA. However, the practice results in postharvest losses due to chilling injury of susceptible fruit. Sanchez-Ballesta *et al.* (2006) defined chilling injury as a physiological disorder induced by low, but not freezing, temperatures, which affect fruit quality due to loss of cellular integrity caused by membrane lipid degradation. Chilling injury is still one of the major obstacles to the expansion of the world lemon trade (Siriphanich, 2002). In lemons, chilling injury is visibly manifested by pitting of the peel (Sala, 1998). The condition is characterised by dark brown varying to black blemishes with increasing damage, thereby affecting fruit quality and reducing fruit shelf life and marketability (Sala, 1998; Tasneem, 2004; Lurie and Crisosto, 2005).

Shewfelt and del Rosario (2000) developed a hypothesis describing the mechanism of chilling injury (Figure 3.1). They suggested that exposing lemons to chilling temperature (-0.5°) may cause oxygen to act primarily as an electron acceptor leading to the formation of ROS (Asada, 1993; Sarkar *et al*., 2009). These ROS, if not rapidly removed from the cells or controlled, can attack cell membranes resulting in extensive damage to membrane lipids and other cellular structures (Maxwell *et al*., 1999; Møller, 2001; Sarkar *et al*., 2009). In addition, if cold storage time is extended, membrane breakdown may cause further cell damage leading to visible symptoms of chilling injury (Siriphanich, 2002). Nonetheless, fruit have defensive systems to mitigate chilling injury either by controlling or detoxifying ROS and delaying oxidative damage (membrane lipid peroxidation) when exposed to chilling stress (Shewfeft and del Rosario, 2000; Siriphanich, 2002).

Salicylic acid is a hormonal substance belonging to a group of phenolic compounds known to increase tolerance to chilling injury in tomatoes (Ding *et al*., 2002), sweet peppers (Fung *et al*., 2004), peach fruit (Wang *et al*., 2006) and pomegranates (Sayyari *et al*., 2009). According to Wang *et al*. (2006), salicylic acid is linked to oxidative stress responses. Similarly, methyl jasmonate is known for its ability to increase tolerance to chilling injury by enhancing both the antioxidant system, and scavenging capacity of fruit (Ghasemnezhad and Javaherdashti, 2008). Methyl jasmonate significantly reduces chilling injury symptoms in

mango (González-Aguilar *et al*., 2000) and zucchini (Wang and Buta, 1994). In this study, the roles of methyl jasmonate and salicylic acid in suppressing the production of ROS and stimulating defence mechanisms to chilling injury in lemons during extended cold storage were investigated.

MATERIALS AND METHODS

Plant material and treatments

During the 2008 harvest season, 'Eureka' lemons, with no visible damage, were harvested from the Ukulinga Research Farm (29° 40' S 30° 24' E, 806 m elevation) in Pietermaritzburg, KwaZulu-Natal, South Africa. The location is characterized by a mean annual rainfall and temperature of 738 mm and 18°C respectively, with a light to moderate frost in winter. Fruit were surface sterilized with Sporekill® (Hygrotech Pty Ltd.), air-dried for 5 minutes and randomly divided into treatments. For each postharvest treatment, fruit were either dipped into 10 or 50 µM methyl jasmonate (MJ) (Droby *et al*., 1999) for 30 sec or into 2 or 2.5 mM salicylic acid (SA) solutions for the same time period (Xu and Tian, 2008). A control or no dip treatment was also adopted. Three replicates of 15 fruits per treatment were used. After dipping, fruit were waxed with Citrashine wax® (Citrashine Pty Ltd, Johannesburg, South Africa) and stored at -0.5ºC for 0, 7, 14, 21, 28, 35, or 42 days before being transferred to room temperature (25°C) for a 7 day shelf life period.

During the 2009 harvest season, 'Eureka' lemons were obtained from a local commercial farm called Ithala (29° 52' S 30° 16' E, approximately 700 m above sea level) in Pietermaritzburg, KwaZulu-Natal, South Africa. Fruit of uniform colour and size were transported to the laboratory and randomly divided into five treatments. For each treatment, fruit were either dipped into the following hormone solutions for 30 sec: 10 µM MJ; 2 mM SA; 10 µM MJ & 2 mM SA; 1 µM MJ & 0.2 mM SA; or 0.1 µM MJ & 0.02 mM SA. A control or no dip treatment was also applied. Three replicates of 15 fruits per treatment were used. After dipping, fruit were waxed with Citrashine wax and stored at -0.5ºC for 0, 7, 14, 21, 28, 35, or 42 days before being transferred to room temperature (25°C) for a 7 day shelf life period.

Detection of reactive oxygen species (ROS)

The formation of ROS in the rind of lemons was determined according to the method of Maxwell *et al*. (1999) using 2', 7'-dichlorofluorescein diacetate (H_2DCF-DA) which is converted to the membrane-impermeable polar derivative 2', 7'-dichlorofluorescein (H_2DCF) when taken up by the cells; within the cell H_2DCF is rapidly oxidized to the highly fluorescent DCF (dichlorofluorescein) by intracellular hydrogen peroxide (H_2O_2) and other peroxides. A 5 mM H_2DCF-DA solution was prepared in ethanol and stored in the dark at -80°C under argon pending use. Freeze-dried flavedo tissue (0.1 g) was mixed with 5 ml of H_2DCF-DA solution and incubated for 30 min at room temperature before being centrifuged at 3000 x g for 10 min. The supernatant was removed and diluted 50-fold. Fluorescence was measured using fully automated micro-plate based multi detection reader (FLUOstar OPTIMA, BMG LABTECH, Germany) with excitation and emission wavelengths set at 485 nm and 520 nm, respectively.

Determination of lipid peroxidation

The level of lipid peroxidation was measured using a modified method of Gülen *et al*. (2008), as the amount of malondialdehyde (MDA), reacted with thiobarbituric acid (TBA) (Sigma Chemical Co., St. Louis, MO 63178 USA) to form a TBA–MDA complex. Lemon flavedo tissue (0.1 g DW) was homogenized in 4 ml 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 3000 x g for 10 min. A 1 ml supernatant from the crude extract was added to a test tube containing 1 ml 20% (w/v) TCA, 0.01% (w/v) butylated hydroxytoluene and 0.65% (w/v) TBA solution. Samples were then mixed vigorously, heated to 95^{o}C and kept at that temperature for 30 min. Subsequently samples were cooled in ice, before centrifuged at 3000 × *g* for 10 min. Absorbance was read at 532 nm and 600 nm using a UV–Visible Spectrophotometer (DU® 800, Beckman Coulter, CA, USA). Total MDA equivalents were calculated according to Heath and Packer (1968) as following:

$$\text{Total MDA (nmol g}^{-1}\text{ DW)} = (\text{Amount of extraction buffer (ml)} \times \text{amount of supernatant (ml)} \times [(\text{Abs } 532_{nm} - \text{Abs } 600_{nm}) / 155] \times 10^{3}) \times \text{Amount of sample (g)}^{-1}$$

Assessment of chilling injury

Chilling injury symptoms were evaluated according to the method previously described by Sala (1998). The method involves a rating scale based on surface necrosis and

intensity of browning (0 = no; 1 = slight; 2 = medium; 3 = severe). Chilling injury index (CI) was calculated according to El-hilali *et al*. (2003):

$$CI = \sum (\text{number of fruit with chilling injury} \times \text{score of severity}) \div \text{total number of fruit}$$

Statistical analysis

A randomized complete block design (RCBD) was followed. Data were subjected to analysis of variance (ANOVA) using GenStat® Version 12 (VSN International, Hemel Hempstead, UK). Means were separated using least significant differences (LSD) at the 5% level of significance.

RESULTS

Effect of salicylic acid and /or methyl jasmonate treatment on reactive oxygen species (ROS) in lemon fruit during cold storage

During the 2008 harvest season, rind ROS were significantly ($P \leq 0.05$) affected by cold storage time, treatment and the interaction between the two factors (Figure 3.2). Production of ROS increased 14 days after treatment (DAT) and decreased slowly at 28 DAT for both treated and untreated fruit (control). Rind of untreated fruit had lower levels of ROS compared with treated fruit. The production of ROS was higher in fruit treated with SA as compared with fruit treated with MJ (Figure 3.2). In addition, fruit treated with the higher MJ concentration (50 µM) had higher levels of ROS 14 DAT as compared with fruit treated with the lower concentration (10 µM). The production of ROS increased rapidly from 28 DAT for untreated fruit and for fruit treated with the lower MJ concentration (10 µM). However, treatments with higher concentrations of either MJ (50 µM) or SA (2.5 mM) significantly suppressed the production of ROS even after the extended cold storage time (Appendix 1).

During the 2009 harvest season, rind ROS were similarly significantly ($P \leq 0.05$) affected by cold storage time, treatment and the interactions between these two factors (Figure 3.3). Production of ROS increased with an extension of the cold storage time reaching a peak at 42 DAT. Postharvest treatments with MJ and SA significantly ($P \leq 0.05$) reduced the production of ROS (Figure 3.3). Fruit treated with 10 µM MJ had lower levels of ROS as compared with other treatments (Figure 3.3). Treatment with SA (2 mM) was the second most

effective in reducing ROS in the rind (Figure 3.3). Extending the cold storage time from 28 to 42 days resulted in an increase in rind ROS production. However, MJ and SA treatment were effective in reducing and / or delaying the production of ROS in fruit during extended cold storage time (Appendix 2). Total production of ROS in the rind of fruit treated with 0.1 µM MJ & 0.02 mM SA was lower than total production of ROS in the rind of fruit treated with 1 µM MJ & 0.2 mM SA. The rind of fruit treated with 10 µM MJ & 2 mM SA had a lower total production of ROS than the rind of untreated fruit (Figure 3.3). The rind of untreated fruit had higher total production of ROS compared with the rind of treated fruit (Figure 3.3).

Effect of salicylic acid and /or methyl jasmonate treatment on lipid peroxidation in lemon fruit during cold storage

Lipid peroxidation was significantly ($P \leq 0.05$) affected by both, treatment and cold storage time; the interaction between the two factors during the 2008 season was also significant ($P \leq 0.05$) (Figure 3.4). During 2008, lipid peroxidation increased in the rind of fruit treated with higher concentrations of MJ (50 µM) or SA (2.5 mM). Treatment with 10 µM MJ or 2 mM SA significantly ($P \leq 0.05$) reduced lipid peroxidation during cold storage (Figure 3.4). During the 2009 season, lipid peroxidation was significantly ($P \leq 0.05$) affected by treatment, cold storage time and the interaction between treatment and cold storage time (Figure 3.5). Lipid peroxidation increased with cold storage time, reaching a peak at 14, 35 and 42 DAT. Untreated fruit had high levels of rind lipid peroxidation during cold storage. Postharvest treatment with MJ and SA significantly ($P \leq 0.05$) suppressed lipid peroxidation during cold storage.

Treatment with 10 µM MJ & 2 mM SA was more effective in reducing the levels of rind lipid peroxidation as compared with other treatments (Figure 3.5). Fruit treated with 10 µM MJ & 2 mM SA had lower levels of lipid peroxidation than other treatments. Treatment with SA at 2 mM was the second most effective treatment in reducing rind lipid peroxidation which 10 µM MJ was the third most effective treatment (Figure 3.5). The treatment combinations of MJ with SA were effective in suppressing rind lipid peroxidation. The effectiveness of treatments in suppressing lipid peroxidation in the rind of the fruit was decreasing with the level of treatment concentrations. Treatment combination of 1 µM MJ &

0.2 mM SA was more effective than 0.1 µM MJ & 0.02 mM SA in suppressing lipid peroxidation, while the treatment combination with 0.1 µM MJ & 0.02 mM SA was less effective (Figure 3.5).

Effect of salicylic acid and /or methyl jasmonate treatment on chilling injury in lemon fruit during cold storage

For the 2008 harvest season, fruit did not develop visual symptoms of chilling injury and there were no significant differences ($P>0.05$) observed among treatments and cold storage period. There was no significant interaction ($P>0.05$) between the two factors, with respect to the chilling injury (CI) index (see appendix 1). During the 2009 harvest season, however, CI symptoms were significantly ($P\leq0.05$) affected by treatment, cold storage period and the interaction of the two factors (Figure 3.6). The CI symptoms were first visible as pitting and water-soaking after 14 days of cold storage, developing rapidly thereafter in untreated fruit (see appendix 3). However, postharvest treatment with MJ and / or SA significantly ($P\leq0.05$) delayed and reduced CI symptoms. Treatment with 10 µM MJ & 2 mM SA was more effective in protecting fruit from chilling damage by delaying and reducing CI symptoms than other treatments with a total chilling percentage of only 0.076% (Figure 3.6).

The 2 mM SA treatment was the second most effective treatment in delaying and reducing CI with a total of 0.133% of CI symptoms, followed by 10 µM MJ with a total of 0.243% of CI symptoms. Overall, the ability of SA and MJ to reduce CI was dependent on the levels of the treatment concentration. Fruit treated with high concentrations were more effective in reducing CI than the low concentrations. Fruit treated with 1 µM MJ & 0.2 mM SA had low CI compared with fruit treated with 0.1 µM MJ & 0.02 mM SA. Fruit treated with 1 µM MJ & 0.2 mM SA had a total of 0.36% CI compared with fruit treated with 0.1 µM MJ & 0.02 mM SA which had 0.91% CI. Nonetheless, treatment with 0.1 µM MJ & 0.02 mM SA reduced CI than the untreated fruit, which had a high total of 0.93% CI symptoms.

DISCUSSION

Effect of salicylic acid and / or methyl jasmonate on the accumulation of reactive oxygen species during cold storage of lemons

Exposing lemons to chilling temperature (-0.5°C) for disinfestation of Mediterranean fruit fly can cause accumulation of ROS resulting in oxidative damage (membrane lipid peroxidation) (Figure 3.4). Cell death will result followed by the development of CI in response to extended exposure to cold temperatures. Treatment with either MJ (10 µM) and / or SA (2 mM) was effective in suppressing the production of ROS in lemon rind during extended cold storage (Figure 3.5). Untreated fruit significantly showed reduced ROS production up to the end of 28 day storage period. However, the compensatory abilities of the fruit to withstand cold storage were exceeded and the production of ROS increased rapidly after 28 days. It has been reported that fruit can withstand chilling stress for a short period through biochemical changes that involve production of antioxidants and alterations in lipid composition (Gülen *et al*., 2008). However, if the cold exposure period is extended, the compensatory abilities of the fruit will be exceeded and damages due to ROS accumulation and oxidation will occur (Lichtenthaler, 1996; Lurie, 2003).

Treating fruit with low concentrations of MJ (10 µM) or SA (2 mM) or a combination of the two treatments (10 µM MJ & 2 mM SA) was effective in slowing down the production of ROS during the extended cold storage. Treatment with higher MJ (50 µM) or SA (2.5 mM) concentration suppressed ROS production. However, these treatments had a negative effect on fruit quality, with respect to appearance. Fruit treated with moderate concentrations (10 µM MJ or 2 mM SA) or a combination (10 µM MJ & 2 mM SA) had a better appearance than fruit treated with higher concentrations during the cold and shelf life storage. This is similar to results reported in a previous study on grapefruit (Droby *et al.*, 1999), where treatment with MJ at moderate concentration (10 µM) was found to be a potential postharvest treatment to enhance natural resistance and to reduce chilling injury in citrus fruit. Moreover, treatment with SA at moderate concentration (2 mM), significantly reduced decay in harvested sweet cheery fruit (Xu and Tian, 2008). Furthermore, in a similar manner, Ding *et al*. (2002) and Cao *et al*. (2009) reported that when MJ or SA are applied at moderate concentrations, they

can provide protection against ROS rind and cell damage in fruit; however, when the compounds are applied at higher concentration, a negative effect on fruit quality can result.

Effect of salicylic acid and / or methyl jasmonate on membrane lipid peroxidation during cold storage

Lipid peroxidation is one of the damages caused by cold stress on cell membranes of lemons and can be used as a direct indicator of membrane injury (Hodges *et al*., 1999; Cao *et al*., 2009). In the present study, treatment with 10 µM MJ or 2 mM SA or a combination of these treatments (10 µM MJ & 2 mM SA) significantly decreased or delayed the incidence of lipid peroxidation in the rind of lemons cold-stored. Results agree with those of Wang *et al*. (2006) where SA treatment significantly delayed membrane lipid peroxidation in peach fruit during cold storage. Similarly, MJ treatment significantly decreased membrane lipid peroxidation in strawberries by maintaining antioxidant activity of the fruit (Kondo *et al*., 2005). However, treatment with 50 µM MJ or 2.5 mM SA was ineffective in reducing lipid peroxidation. In addition, lipid peroxidation levels were higher in fruit treated with higher concentrations of both chemicals as compared with medium concentration of either SA or MJ.

Effect of salicylic acid and / or methyl jasmonate treatment on chilling injury in lemon fruit during cold storage

Despite the extended cold storage time, lemon fruit harvested from Ukulinga Research Farm in 2008 season did not develop chilling injury. However, fruit were stressed during cold storage as indicated by the production of high levels of ROS and increased lipid peroxidation in the rind. Several reasons could have caused fruit not develop CI: CI was at the primary event stage, where accumulation of ROS and oxidative damage such as lipid peroxidation were observed as a metabolic dysfunction. In addition, it may also be that the damage caused by cold stress was probably reversed due to the presence of antioxidants which were able to stimulate recovery from CI. Christie *et al*. (1994) and Sarkar *et al*. (2009) proposed that recovery from CI is possibly due to antioxidant protection. A further reason for the recovery from CI is that symptom development is not only dependent on the conditions during cold storage but also on other factors such as temperature and water status of the fruit prior to chilling (Smeets and Wehner, 1997; Szalai *et al*., 1996; Bafeel and Ibrahim, 2008). Van

Rooyen and Bower (2003) reported that preharvest conditions, such as crop management also play a role in the final fruit quality. It could be that fruit did not develop CI symptoms probably because fruit were already stressed in the field and were resistant to stress due to stress levels that fruit were exposed to preharvest.

During the 2009 season, postharvest treatment with MJ and / or SA significantly ($P \leq 0.05$) delayed and reduced CI symptoms in lemon fruit. Treatment with (10 µM MJ & 2 mM SA) was more effective in protecting fruit from chilling damage by delaying and reducing CI symptoms as compared with other treatments. The results showed that a treatment combination of MJ and SA at low to medium concentrations could be used to reduce and delay CI symptoms. However, lower concentrations of treatment combinations were least effective in alleviating CI in lemon fruit as compared with medium concentrations. Fruit treated with (1 µM MJ & 0.2 mM SA) had low CI symptoms compared with when treated with (0.1 µM MJ & 0.02 mM SA). Treatment with (0.1 µM MJ & 0.02 mM SA) was less effective in reducing CI symptoms in lemons during cold storage.

However, fruit treated with (0.1 µM MJ & 0.02 mM SA) had low CI symptoms compared with untreated fruit. High CI symptoms were observed in untreated fruit. CI symptoms were initially observed after 14 days in both untreated (control) and (0.1 µM MJ & 0.02 mM SA) treated fruit followed by fruit treated with (1 µM MJ & 2 mM SA) after 21 days in cold storage. Fruit treated with a combination of (10 µM MJ & 2 mM SA) showed delayed CI symptoms at 42 days and therefore showed good quality, in terms of colour and shape, when compared with fruit in other treatments. Therefore, treatment with a combination of 10 µM MJ & 2 mM SA was more effective in alleviating CI in lemon fruit (Figure 3.8) as compared with other treatments. It was also reported that when a combination of 1 mM of MJ and SA was applied on sweet peppers, it induced the expression of a set of defence genes, thus enhancing tolerance to CI (Fung *et al*., 2004; Cao *et al*., 2009).

This study agrees with previous findings reported by Ding *et al.* (2002), Zolfagharinasab and Hadian, (2007) and Cao *et al*. (2009). They found that treatment with MJ or SA or the combination of the two at moderate concentrations could induce some defence mechanisms that indirectly provided protection against chilling damage. They further reported that treatment with higher concentrations caused negative effects on fruit quality.

CONCLUSION

Treatment with MJ or SA at medium concentrations (10 µM MJ or 2 mM SA) or combined (10 µM MJ & 2 mM SA) suppressed the accumulation of ROS, delayed membrane lipid peroxidation and reduced the chilling injury index in lemon fruit subjected to extended cold storage. Treatment with MJ or SA probably could have triggered defence mechanisms such as antioxidants in order to enhance cell membrane stability and protect cells from damage caused by ROS. The role of antioxidants in fruit during cold storage should be further evaluated. Preharvest conditions such as crop management, temperature and water status of the fruit prior to chilling stress for the disinfestation of Mediterranean fruit fly should also be considered.

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FIGURES

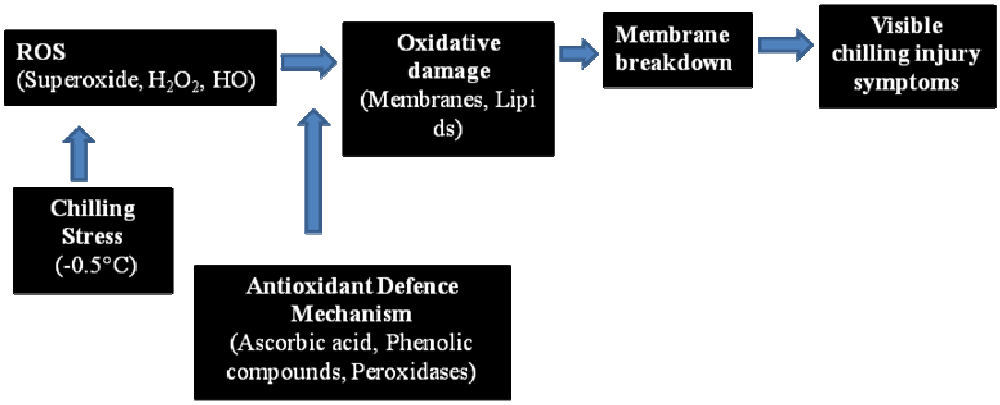

Figure 3.1: Mechanism of chilling injury (Shewfeft and del Rosario, 2000).

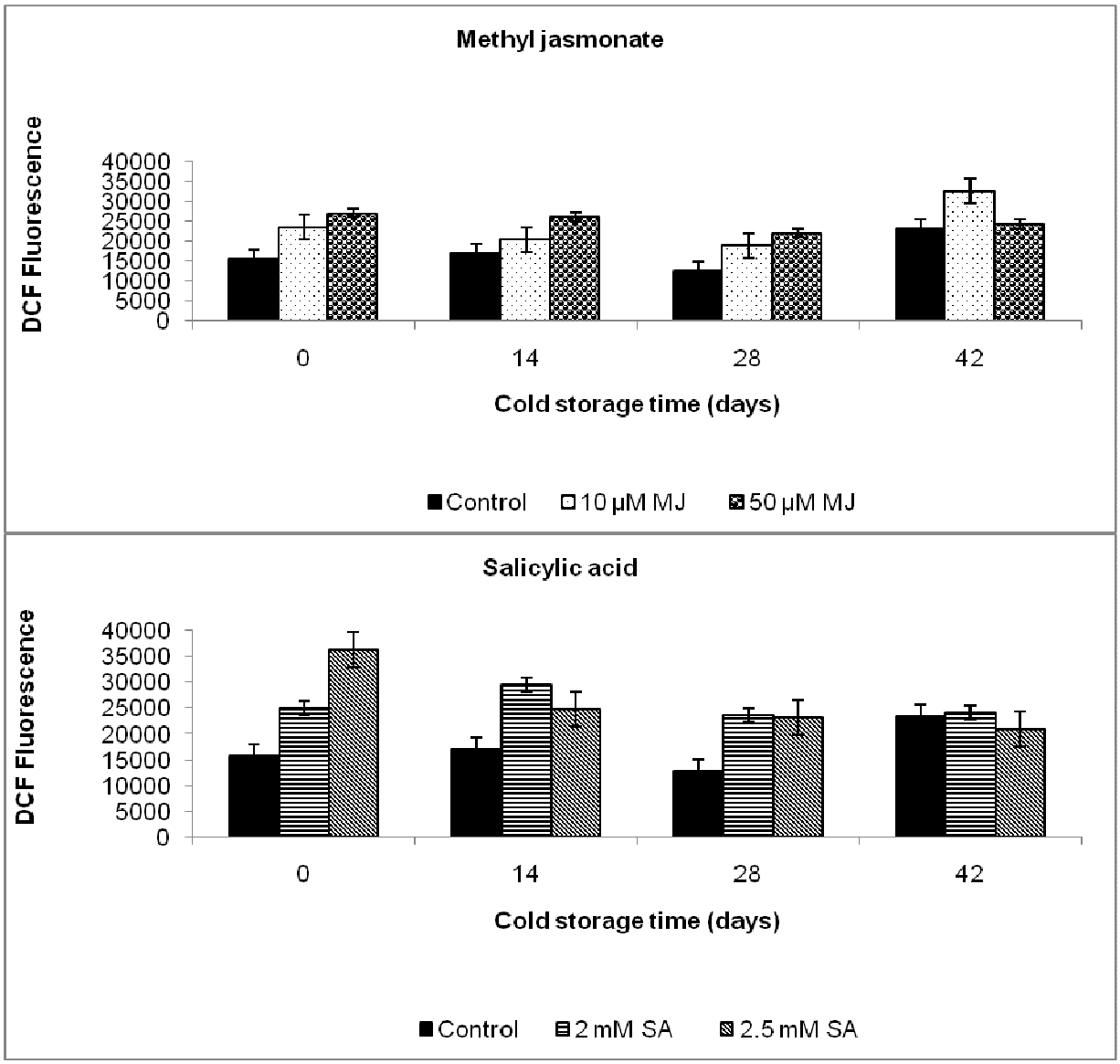

Figure 3.2: Formation of reactive oxygen species on the rind of lemons treated with MJ (10 µM; 50 µM) or SA (2 mM; 2.5 mM) and stored at -0.5℃ for 42 days before being transferred to room temperature for 7 days (2008 season). LSD $_{(0.05)}$ = 4155.1.

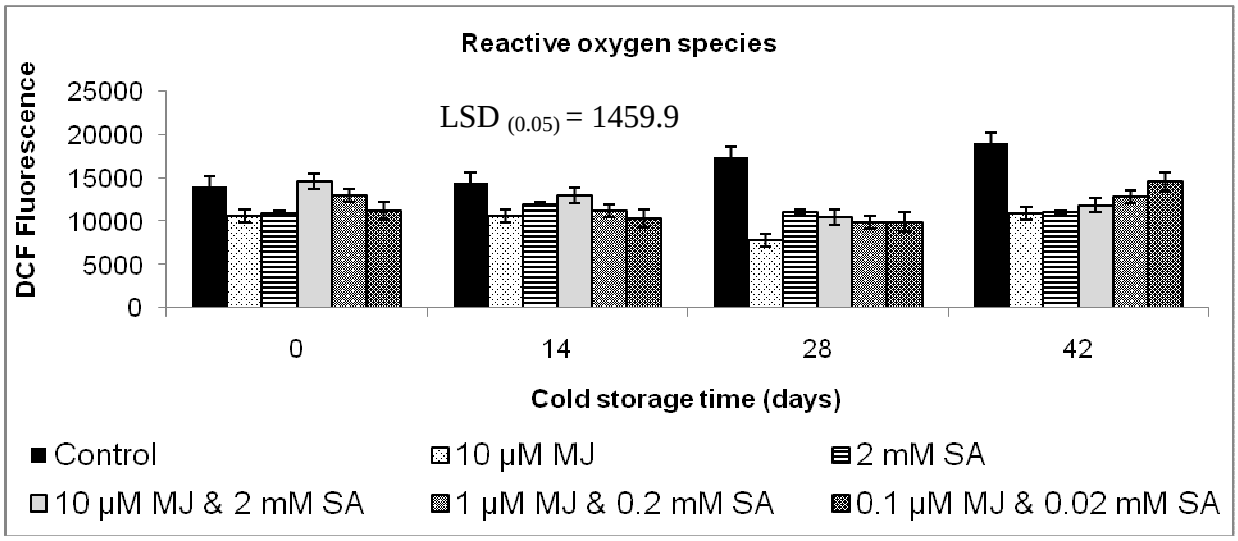

Figure 3.3: Reactive oxygen species formation in the rind of lemons treated with either 10 µM MJ and / or 2 mM SA; 1 µM MJ & 0.2 mM SA; or 0.1 µM MJ & 0.02 mM SA before stored at -0.5°C for 42 days and transferred to room temperature for 7 days (2009 season).

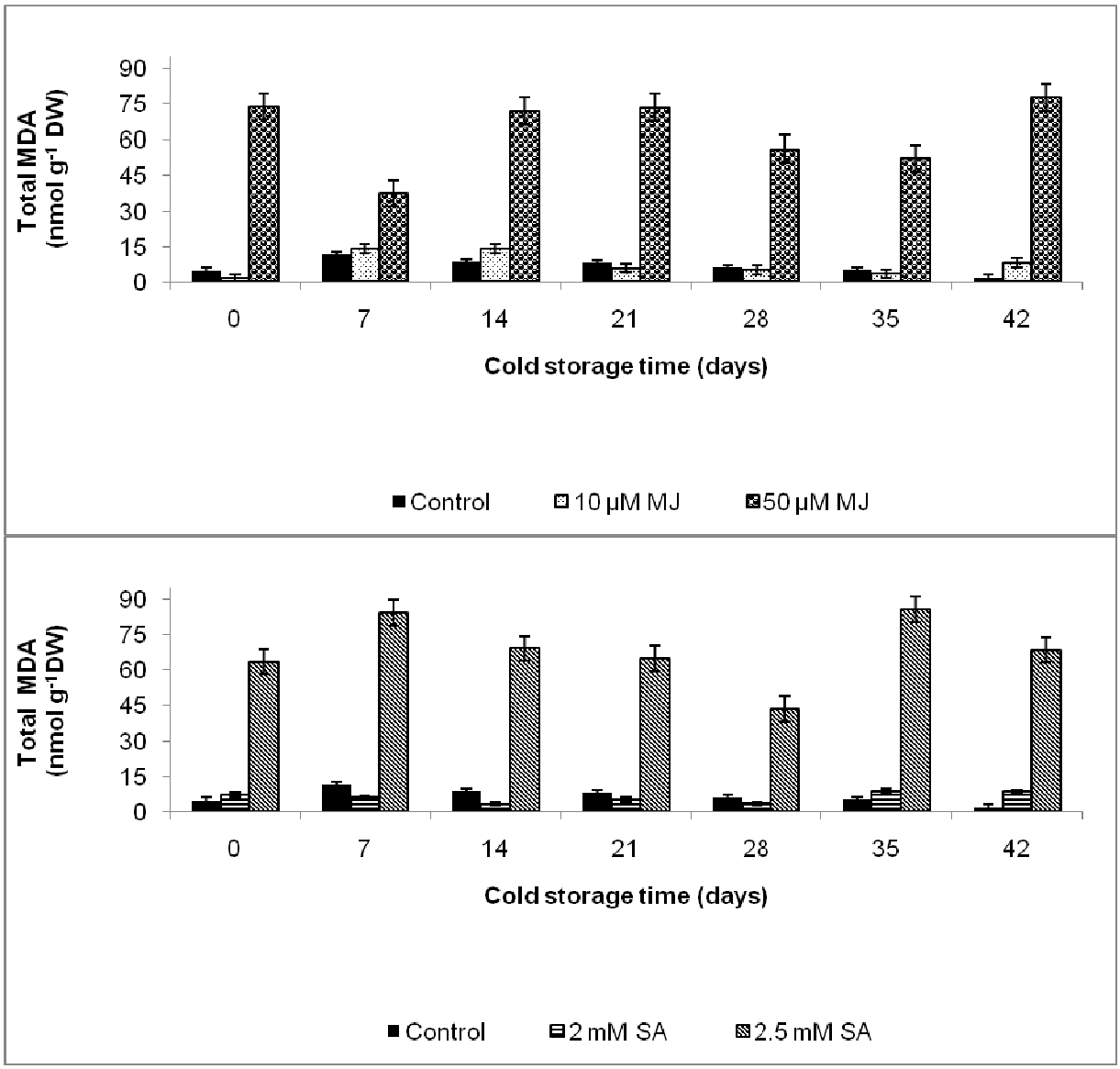

Figure 3.4: Effect of different concentrations of MJ (10 or 50 µM) or SA (2 or 2.5 mM) on membrane lipid peroxidation of fruit during cold storage (-0.5˚C) for 42 days and at the end of 7 days shelf life period (2008 season). LSD $_{(0.05)}$ = 1.841.

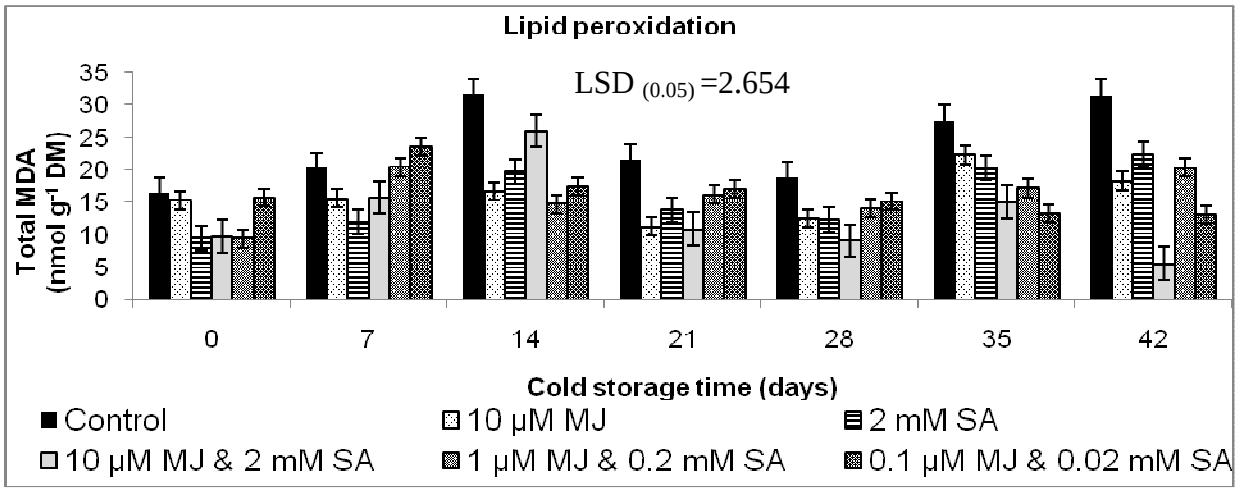

Figure 3.5: Effect of 10 µM MJ, 2 mM SA, 10 µM MJ &2 mM SA, 1 µM MJ & 0.2 mM SA, or 0.1 µM MJ & 0.02 mM SA on lipid peroxidation of fruit stored at -0.5˚C for 42 days before transfer to room temperature for 7 days (2009 season).

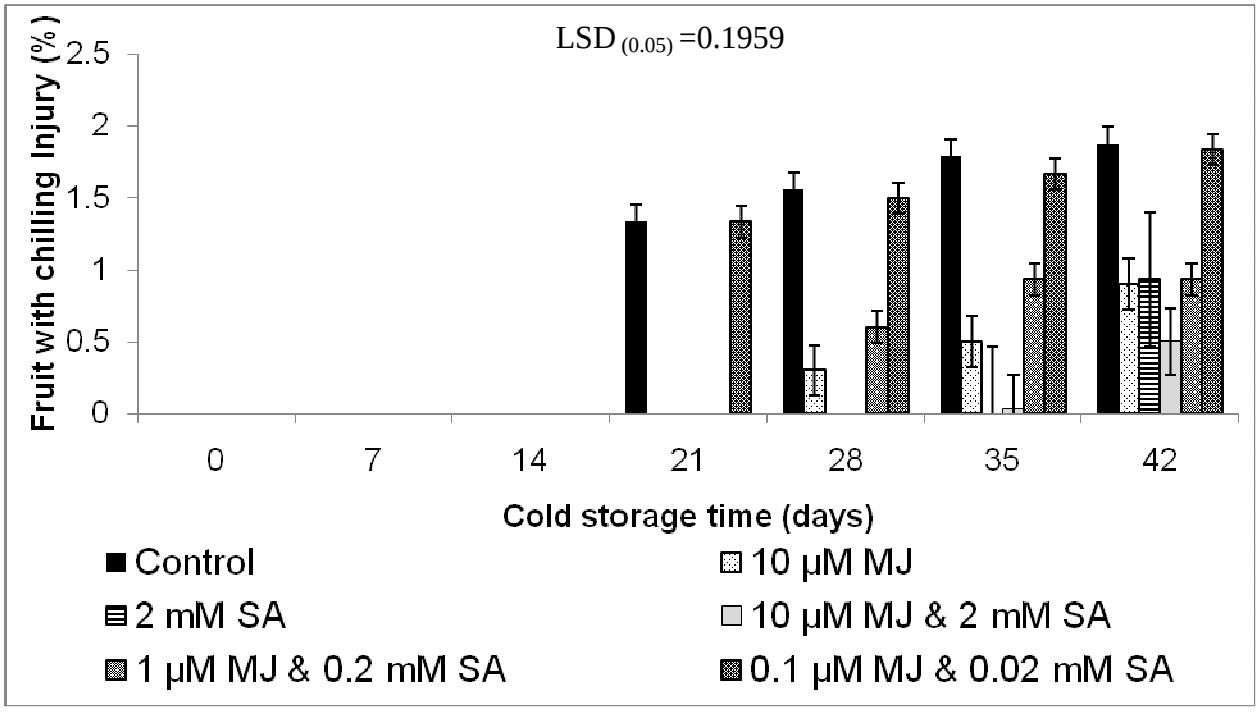

Figure 3.6: Effect of postharvest treatments on chilling injury (%) in lemons using 10 µM MJ, 2 mM SA, 10 µM MJ & 2 mM SA, 1 µM MJ & 0.2 mM SA, or 0.1 µM MJ & 0.02 mM SA and stored at -0.5˚C for 42 days before transfer to room temperature for 7 days (2009 season).

CHAPTER 4

EFFECT OF METHYL JASMONATE AND SALICYLIC ACID ON PROLINE AND OTHER PHYSIOLOGICAL ASPECTS (ETHYLENE, RESPIRATION RATE, AND FRUIT MASS LOSS) IN *EUREKA* LEMONS

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(Chapter formatted to be submitted to the Postharvest Biology and Technology Journal)

ABSTRACT

Exposing lemons to chilling temperature disrupts several metabolic processes such as respiration rate, amino acid incorporation and ethylene production resulting in physiological dysfunction- a primary event of chilling injury. The effect of methyl jasmonate (MJ) and salicylic acid (SA) on physiological factors, such as fruit mass loss, respiration rate and ethylene evolution, during cold storage of lemons was evaluated as well as proline accumulation, a common physiological response to cold stress. During the 2008 harvest season, 'Eureka' lemons were dipped in 10 or 50 µM MJ or 2 or 2.5 mM SA solutions for 30 s. A control or no dip treatment was also applied. Three replicates of 15 fruits per treatment were used. During the 2009 harvest season, 'Eureka' lemons were sterilized, air-dried and dipped in (10 µM MJ), (2 mM SA), (10 µM MJ & 2 mM SA), (1 µM MJ & 0.2 mM SA), or (0.1 µM MJ & 0.02 mM SA) for 30 s. A control or no dip treatment was also applied. Three replicates of 15 fruits per treatment were used. Therefore fruit were waxed with Avoshine® and stored at -0.5ºC for 0, 7, 14, 21, 28, 35, or 42 days, before being transferred to room temperature (25°C) for 7 days. Fruit mass, ethylene evolution, respiration rate electrolyte leakage as well as proline metabolism in the rind were determined. Reduction in fruit mass was probably due to stomatal closure induced by the treatments in the fruit. Excessive fruit mass loss during the 2009 harvest season was associated with fruit susceptibility to chilling injury. Membrane electrolyte leakage was not associated with chilling injury in fruit harvested during the 2008 harvest season. However, treatment with MJ and SA significantly ($P \leq 0.05$)

reduced membrane electrolyte leakage of the fruit during cold storage during the 2009 harvest season. Ethylene production was not associated with chilling injury. Nonetheless, high respiration rate, excess mass loss and accumulation of proline confirmed that fruit were stressed by the cold treatment.

Keywords: methyl jasmonate, salicylic acid, respiration rate, proline, electrolyte leakage, ethylene evolution.

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1. Introduction

The demand for fresh fruit has increased over the last two decades due to their high nutritional value (Pérez-Tello *et al*., 2009). Lemons (*Citrus limon* L.), belonging to the citrus family, are also in demand in many countries. As lemon fruit are non-climacteric and can normally be stored for long periods of time (6-8 weeks) (Kader, 2002; Porat *et al*., 2004). However, the postharvest storage life of lemon fruit is limited by ultra-low temperature exposure which results in chilling injury (Pérez-Tello *et al*., 2009), an economically important postharvest disorder (Arafat, 2005). This disorder has been found to be the most important obstacle to the expansion of the world lemon trade (Siriphanich, 2002; Arafat, 2005). Some of the symptoms of chilling injury in lemons include excessive mass loss, dehydrated-looking depressions, increased decay and enhanced ethylene and respiration rates (Leguizamon *et al*., 2001; El-hilali *et al*., 2003; Porat *et al*., 2004; Lafuente *et al*., 2005).

Kluge *et al.* (2003) established that the primary cause of chilling injury in lemons is cell membrane damage. This damage initiates a cascade of secondary reactions, which include accelerated or high respiration and ethylene production as physiological dysfunctions (Biolatto *et al*., 2005; Murata, 1990). This excessive production of ethylene in lemons during cold storage is often a sign of damage to cellular membranes, which is usually followed by the development of chilling injury symptoms (Zhou *et al*., 2001). Changes in respiratory responses of fruit have been related to chilling temperatures (Pérez-Tello *et al*., 2009).

Chilling stress also results in an accumulation proline in fruit (Chen and Li, 2002); proline accumulation is often considered to be a stress tolerance mechanism (Misra and

Saxena, 2009) and varies in its role occurring as an adaptive response, helping fruit to withstand the effect of cold stress (Matysik *et al*., 2002), acting as a membrane stabilizer (Rudolph *et al*., 1986), a scavenger of reactive oxygen species (Saradhi *et al*., 1995) and / or a source of reduction equivalents during recovery from stress (Hare and Cress, 1997). Several studies have demonstrated that fruit which are not able to produce proline when exposed to low temperatures have a significantly lower cold stress tolerance (Xin and Browse, 1998; Matysik *et al*., 2002). Matysik *et al*. (2002) concluded that proline may not be just a by-product of cold stress defence, but a chemically active compound involved in the physiology of cold stress protection in fruit. High proline levels in the rind of grapefruit during cold storage were associated with increased chilling resistance (Purvis, 1981). However, information on the effect of MJ and SA on proline is in scarce. The objectives of this study were to investigate the effect of MJ and SA on fruit mass loss, respiration rate and ethylene evolution as non destructive indicators of chilling injury and to investigate physiological responses (proline) of lemons to chilling stress.

2. Materials and methods

2.1 Chemicals

All chemicals were obtained either from Fluka®, Glycoteam GmbH, Sigma-Aldrich®, or Saarchem®,

2.2 Plant material

During the 2008 harvest season, ‘Eureka’ lemons were harvested from the Ukulinga Research Farm (29° 40' S 30° 24' E, 806 m elevation) in Pietermaritzburg, KwaZulu-Natal, South Africa. The location has a mean annual rainfall and temperature of 738 mm and 18°C respectively, with a light to moderate frost in winter. During the 2009 harvest season, the same cultivars were obtained from a local commercial farm called Ithala (29° 52' S 30° 16' E, approximately 700 m above sea level) near Pietermaritzburg, KwaZulu-Natal, South Africa. Fruit of uniform colour and size were selected and transported to the laboratory for analysis.

2.3 Treatments and storage conditions

During the 2008 harvest season, fruit were treated with Sporekill® (Hygrotech Pty Ltd.), air-dried for 5 min and randomly divided into treatments. As postharvest treatments, fruit were dipped into 10 or 50 µM methyl jasmonate (MJ) (Droby *et al*., 1999) or 2 or 2.5

mM salicylic acid (SA) solutions for 30 sec (Xu and Tian, 2008). A control or no dip treatment was also included. Three replicates of 15 fruits per treatment were used. During the 2009 harvest season, fruit were randomly divided into treatments and dipped into 10 µM MJ or 2 mM SA or 10 µM MJ & 2 mM SA or 1 µM MJ & 0.2 mM SA; or 0.1 µM MJ & 0.02 mM SA solutions for 30 sec. A control or no dip treatment was also applied. Three replicates of 15 fruits per treatment were used. After dipping, fruit were waxed with Citrashine (Citrashine Pty Ltd, Johannesburg, South Africa) and stored at -0.5ºC for 0, 7, 14, 21, 28, 35 or 42 days before being transferred to room temperature (25°C) for a 7 day shelf life period.

2.4 Fruit mass loss

In order to calculate percentage fresh mass loss of all individual fruit, fruit were weighed before and after the storage intervals. The percentage mass loss was then calculated according to Gómez, *et al*. (2005) as follows:

Fruit mass loss (%) = [{initial fruit mass (g)-final fruit mass (g)} / initial fruit mass (g)] ×100%

2.5 Respiration rate measurement

Fruit respiration rate was measured before and after the storage intervals using an infrared gas analyser (EGM-1, PP Systems, Hitchin, Hertfordshire, UK). Respiration rate of the fruit was measured by incubating fruit in 1 ℓ jars for 15 min as previously described by Van Rooyen and Bower (2003). The net respiration rate of the atmosphere in the jars per gram fruit was calculated by adjusting ambient carbon dioxide in the jar, fruit volume, head space and fruit mass.

2.6 Determination of cell membrane electrolyte leakage

Electrolyte leakage was used to evaluate membrane damage following cold storage using a multi-range conductivity meter (HI 9033, Hanna Instruments, Johannesburg, R.S.A.). The procedure used was based on the method of Zhu *et al*. (2004). Three discs (1.8 mm in diameter) were excised from the flavedo using a cork borer and rinsed in distilled water. Following this, the discs were placed in test tubes containing 10 ml distilled water. Test tubes were incubated at 25ºC in a shaker for 3 h. Electrical conductivity of the water bath (Initial EC) was measured after incubation. Samples were then placed in (thermostatic) water bath at

100ºC for 1 h and the second reading (Final EC) was determined after cooling the solution to room temperature. The percentage electrolyte leakage was calculated as follows:

$$EC\% = [(\text{Initial EC}) / (\text{Final EC})] \times 100\%$$

where (EC%) the percentage electrolyte leakage, (Initial EC) the initial reading of electrolyte leakage of the disc solution and (Final EC) the final reading of the electrolyte leakage of the disc solution.

2.7 Proline Determination

Proline was determined according to Claussen (2005) using acidic ninhydrin reagent (2.5 g ninhydrin / 100 ml of a solution containing glacial acetic acid, distilled water and 85% ortho-phosphoric acid at a ratio of 6:3:1). Flavedo tissue (0.1 g DW) was extracted in 10 ml 3% (w/v) aqueous sulfosalicylic acid solution. The homogenate was filtered through two layers of glass-fiber filter and the filtrate was then used in the assay. Glacial acetic acid and ninhydrin reagent (1 ml each) were added to 1 ml filtrate. The closed test tubes, with the reaction mixture, were kept in a boiling water bath for 1 h, and the reaction was terminated by holding samples in a water bath at room temperature (21°C) for 5 min. Sample absorbance was immediately determined at a wavelength of 546 nm. The proline concentration was determined by comparison with a standard curve. The flavedo proline concentration was calculated on a dry weight basis as mmol proline $(g\ DW)^{-1}$.

2.8 Ethylene measurement

Ethylene production of the fruit was measured according to Blakey *et al*. (2009) using a gas chromatograph (DANI 1000, DANI Instruments S.p.A., Monzese, Italy) equipped with a flame ionization detector (FID), and a stainless steel packed column with an alumina-F1 stationary phase. The injector, column and detector temperatures were set at 160ºC, 80ºC and 180°C, respectively. Measurements were taken before and after the storage intervals by incubating the fruit in a sealed 1 ℓ jar containing a 20 ml glass vial for 30 min (Van Rooyen and Bower, 2003). Thereafter, the glass vial was sealed and transferred to the gas chromatograph autosampler (HT250D, HTA S.r.L., Brescia, Italy).

2.9 Statistical analysis

Experiments were performed using a complete randomized block design. Data was subjected to analysis of variance (ANOVA) using GenStat® Version 11 (VSN International, Hemel Hempstead, UK). Least significant differences (LSD) at the 5% level were used to separate treatment means

3. Results

3.1 Effect of MJ and / or SA on fruit mass loss during cold storage

During the 2008 harvest season, fruit mass loss was significantly ($P \leq 0.05$) affected by treatment, cold storage time and the interaction between treatment and cold storage time (Figure 4.1). Fruit lost mass during and after cold storage. Treatment with 10 µM MJ was more effective in reducing fruit mass loss than treatment with 50 µM MJ. Treatment with 2 mM SA was the second most effective in reducing fruit mass loss. Greatest fruit mass losses were observed in fruit treated with 2.5 mM SA. All MJ and SA treatment suppressed fruit mass loss compared with control fruit. Excessive fruit mass loss was observed after fruit were transferred to room temperature (Figure 4.1). Untreated fruit lost 4.7% of initial mass, this increased to 8.6 after 42 days of cold storage when fruit were transferred to room temperature.

On the other hand, fruit treated with 10 µM MJ had an initial mass loss of 3.2%, and after 42 days of cold storage this increased to 6.7% at room temperature (Figure 4.1). Initial fruit mass loss for fruit treated with 50 µM MJ was 4.2% during cold storage increasing to 8.6% when fruits were transferred to room temperature (Figure 4.1). At 42 days, fruit treated with 2 mM SA lost 3.8% of initial mass during cold storage and a further 7.7% at room temperature (Figure 4.1). Fruit treated with 2.5 mM SA lost 4.4% of mass during cold storage and 9.2% after being transferred to room temperature (Figure 4.1). During the 2009 harvest season, fruit mass loss was significantly ($P \leq 0.05$) affected by cold storage time and treatment (Figure 4.2a). Fruit mass loss increased with cold storage time (Figure 4.2 a). However, MJ and SA treatments were effective in reducing fruit mass loss during cold storage. Treatment with 10 µM MJ & 2 mM SA was more effective in reducing initial fruit mass loss than other treatments (Figure 4.2). Greatest fruit mass losses were observed in untreated fruit (Figure 4.2 a). Higher mass loss was observed when fruit were transferred to room temperature for 7 days

(shelf life). Fruit mass loss was significantly (P≤0.05) reduced by 10 µM MJ & 2 mM SA treatment (Figure 4.2 b). Excessive fruit mass loss was observed at 42 days in untreated fruit (Figure 4.2 b). No significant significance differences (P.>0.05) were found between the other treatment combinations with respect to fruit mass loss after shelf life (Figure 4.2 b).

3.2 Effect of MJ and /or SA on respiration rate during cold storage

During the 2008 harvest season, respiration rate of the lemon fruit was significantly (P≤0.05) affected by cold storage time (Figure 4.3). Respiration rate of the fruit was high when fruit were exposed to chilling temperatures. However, fruit initially showed a decrease in respiration rate with increasing cold storage time until 28 days; after this, a trend increased respiration rate occurred with increasing cold storage time (Figure 4.3). Untreated fruit showed a higher respiration rate at 35 days compared with treated fruit (Figure 4.3). Treatment with MJ and SA effectively slowed down the respiration rate at 35 days (Figure 4.3 a & c), as well as after the 7 days shelf life period (Figure 4.3 b & c). These figures suggest a trend in the ability of the treatments to slow down the respiration rate although no significant significance differences (P>0.05) were found between treatments or between treatments and cold storage time with respect to fruit respiration rate.

During the 2009 harvest season, respiration rate of the fruit during cold storage was significantly (P≤0.05) affected by treatment, cold storage time and the interaction of the two factors (Figure 4.4 a & b). Increasing cold storage time reduced the respiration rate of the fruit (Figure 4.4 b). Respiration rate of the fruit was highest at 0 days before exposure to cold storage (Figure 4.4 a). Exposing fruit to cold storage (-0.5°C) slowed down the respiration rate from 0 to 14 days. An increase of respiration rate observed 21 days into the cold storage followed by a slight decrease one further week into the cold storage. The results showed a gradual increase in respiration rate during cold storage at 35 days (Figure 4.4). However MJ and SA treatments significantly (P≤0.05) slowed down fruit respiration rate after shelf life period (Figure 4.4).

An increase in fruit respiration rate was observed in control fruit after these were transferred to room temperature. Treatment with 2 mM SA was the most effective in slowing down the respiration rate (Figure 4.4). Treatment with 10 µM MJ was the second best in slowing down the respiration rate. The respiration rate of fruit treated with 0.1 µM MJ & 0.02

mM SA was higher than when treated with other concentrations of MJ and / or SA. However, higher fruit respiration rates were observed in untreated fruit with (Figure 4.4).

3.3 Effect of MJ and / or SA on cell membrane electrolyte leakage

During the 2008 harvest season, the average cell membrane electrolyte leakage for all treatments was 29.3% (Figure 4.5). A noticeable increase in electrolyte leakage in the rind was observed after 7 days of cold storage. There were no clear statistical differences between electrolyte leakage percentages during the 2008 season. However, obvious differences were observed on fruit treated with MJ at 14, 28 or 42 days and on fruit treated with SA at 7, 14, 21 or 28 days. Percentage electrolyte leakage of fruit treated with 10 µM MJ was lower than for fruit treated with 50 µM MJ. During the 2009 harvest season, cell membrane electrolyte leakage was significant ($P \leq 0.05$) affected by treatments, cold storage time and the interaction between the two factors (Figure 4.6). Membrane electrolyte leakage was increasing with cold storage time.

A noticeable increase in percentage cell membrane electrolyte leakage was observed at 14 days. Higher percentage cell membrane electrolyte leakage was observed in untreated fruit with 88% at 42 days (Figure 4.6). However, treatment with MJ and SA significantly ($P \leq 0.05$) reduced membrane electrolyte leakage of the fruit during cold storage (Figure 4.6). Fruit treated with (10 µM MJ & 2 mM SA) had lower cell membrane electrolyte leakage at 42 days than fruit treated with 10 µM MJ. Treatment with 2 mM SA was the third best treatment in reducing cell membrane electrolyte leakage whereas 0.1 µM MJ & 0.02 mM SA was the fourth best treatment. Treatment with 1 µM MJ & 0.2 mM SA was less effective in reducing cell membrane electrolyte leakage during cold storage probably due to its low concentration level.

3.4 Effect of MJ and /or SA on proline metabolism during cold storage

Proline was significantly ($P \leq 0.05$) affected by treatments, cold storage time and the interaction between the two factors during the 2008 season (Figure 4.7). The highest proline accumulation was observed in fruit treated with higher concentration of SA (2.5 mM) followed by fruit treated with 10 µM MJ at 7 and 28 days of cold storage (Figure 4.7). Fruit treated with 2.5 mM SA showed a highest total proline accumulation followed by MJ (10 µM) treated fruit. Treatment with 2 mM SA was the third best treatment in inducing proline.

Treatment with MJ at 50 µM was less effective in inducing proline in the rind compared with the control with a slight different.

Proline was significantly ($P \leq 0.05$) affected by treatments, cold storage time and the interaction between the two factors during the 2009 season (Figure 4.8). Untreated fruit had low proline levels in the rind. However, treatment with MJ and SA significantly ($P \leq 0.05$) induced proline levels in the rind during cold storage (Figure 4.8). The results showed that treatment with 10 µM MJ gave the highest significant increase in rind proline levels followed by the 2 mM SA treatment (Figure 4.8). The level of proline accumulation in the rind of fruit treated with MJ and SA decreased with the level of concentration. Fruit treated with a combination of 0.1 µM MJ & 0.02 mM SA had the lowest level of proline as compared with other treatments. Proline was also significantly ($P \leq 0.05$) affected by cold storage time, increasing with cold storage time till reaching a peak at 28 days. A slight decrease in proline accumulation was observed in fruit from 35 to 42 days during cold storage.

3.5 Effect of MJ and SA on ethylene evolution of lemons during cold storage

No ethylene production was detected in the fruit during the 2008 and 2009 harvest seasons. Therefore, no significance differences ($P > 0.05$) were observed between treatments, cold storage time and the interaction between treatments and cold storage time.

4. Discussion

Results obtained in the present study are consistent with information in the literature stating that storing lemons near freezing temperature (-0.5°C) for extended periods can result in primary events of chilling injury which involve physiological dysfunction (Raison and Orr, 1990). The study showed that fruit lost mass during the 42 days of cold storage. Mass loss increased after fruit were transferred to room temperature for a week (shelf life). In this study, fruit mass loss was associated with water loss (Bower and Jackson, 2003), which means that fruit were losing more water when transferred to room temperature than when stored at lower temperatures. According to (Pérez-Tello et al., 2009), it is expected of fruit stored at higher temperatures to have higher weight loss than when stored at lower temperatures. This may explain why fruit are stored at low temperatures, postharvest, in order to slow down water loss. Results of this study agree with previous studies (Zolfagharinasab and Hadian, 2007;

González-Aguilar *et al.,* 2001) where fruit mass loss increased when fruit were transferred to room temperatures.

During the 2008 harvest season, the reduction in fruit mass loss when fruit were treated with 10 µM MJ or 2 mM SA led to the suggestion that it may be as a result of stomatal closure being induced by the treatments thus reducing transpiration of the fruit. According to González-Aguilar *et al.* (2001), the reduction of mass loss in mangoes treated with MJ was associated with stomatal closure induced by MJ, which reduced transpiration of fruit. Fruit mass loss was generally higher during the 2009 harvest season for most treatments compared with 2008 figures. Excessive fruit mass loss during the 2009 harvest season compared with 2008 harvest season was probably related to fruit source and location.

The study revealed that storing lemons at low temperatures resulted in an increased respiration rate probably as a chilling stress symptom. During the 2008 harvest season, fruit had high respiration rate during cold storage (0-21 days) as compared with when transferred to room temperature. Conversely, during the 2009 harvest season, fruit showed a normal respiration rate at 0-14 days of cold storage and thereafter the respiration rate increase significantly. Highest fruit respiration rates were observed in untreated fruit. However, postharvest treatment with MJ and SA significantly ($P \leq 0.05$) reduced fruit respiration rate during cold storage. Treatment with 2 mM SA was the most effective in slowing down respiration rate of the fruit. Fruit had a lower respiration rate during cold storage compared with when transferred to room temperature. This study agrees with the study by Bower and Jackson (2003), which reported that fruit had a high respiration rate when transferred to room temperatures. However in this study, the extension of cold storage time to 21 days, led to abrupt changes in fruit respiration rate. The respiration rate of the fruit when transferred to room temperature was now lower than when stored at cold storage, indicating cold stress on the membrane at 21 and 42 days.

The high respiration rate was probably an indication that fruit were more stressed during the four weeks of cold storage. Murata (1990) suggested that abnormal respiration was a typical indicator of chilling injury in horticultural crops during cold storage. Again, it was discovered that extended cold storage could cause high membrane permeability, eventually resulting in the exposure of mitochondria to harmful substances; such a process may cause mitochondria to respond by increasing ATP synthesis as a way to retain respiratory control

(Huang and Romani, 1991; Van Rooyen, 2005). The abnormal respiration rate during cold storage led to the suggestion that fruit were highly stressed during cold storage. However, termination of cold storage may have allowed for fruit to recover. Thus, respiration rate was a good indicator of chilling injury in lemons during cold storage.

The fluctuations in cell membrane electrolyte leakage observed during the 2008 and 2009 harvest season showed a loss of membrane integrity; this may have recovered upon termination of cold storage. Arafat (2005) found that high percentage electrolyte leakage in fruit showed membrane damage due to chilling injury. In this study, membrane damage increased with increasing cold storage duration. However, there were no significant differences ($P \leq 0.05$) between treatments and interaction, of treatment and cold storage time during the 2008 harvest season. This led to the suggestion that perhaps electrolyte leakage was not be a good indicator of chilling injury in lemons harvested during the 2008 season. Results agree with those of Pérez-Tello *et al*. (2009) where electrolyte leakage was not a good indicator of chilling injury in fruit.

Conversely, for the 2009 harvest season, cell membrane electrolyte leakage was significantly ($P \leq 0.05$) affected by treatments, cold storage time and the interaction between the two factors. Cell membrane electrolyte leakage of the fruit was increasing with cold storage time. This was probably due to membrane malfunction which is usually caused by the leakage of water and electrolytes from the cells during chilling stress (Nilprapruck *et al*., 2008). However, treatment with MJ and SA significantly ($P \leq 0.05$) reduced membrane electrolyte leakage of the fruit during cold storage. Treatment with 10 µM MJ & 2 mM SA was most effective in reducing cell membrane electrolyte leakage followed by treatment with MJ at 10 µM. Results agree with those of González-Aguilar *et al.*(2000) where electrolyte leakage was consistently lower in MJ treated mango fruit than in untreated fruit during cold storage.

The efficiency of 10 µM MJ & 2 mM SA treatment in reducing cell membrane electrolyte leakage was associated with its ability to inhibit the malfunction of the membrane permeability compared with other treatments which were less effective probably due to low concentration levels (González-Aguilar *et al.*, 2000). Therefore, electrolyte leakage was perhaps a good indicator of chilling injury in lemons harvested during the 2009 season. The

differences between the 2008 and 2009 harvest seasons, with respect to electrolyte leakage were associated with chilling injury sensitivity. The results suggest that fruit harvested during the 2009 season were more sensitive to chilling injury than fruit harvested during the 2008 season, due to high electrolyte leakage which increased with cold storage time. Furthermore, the differences between the 2008 and 2009 harvest seasons also associated with cultivation practices, other physiological and biochemical factors which may have contributed to the chilling sensitivity of the fruit which subsequently resulted in difference electrolyte leakage during the two seasons.

The study showed that during the 2008 and 2009 harvest seasons, fruit were able to accumulated proline as a response to cold stress. The ability of fruit to accumulate proline during cold storage may be a sign of tolerance to cold stress. According to Matysik *et al*. (2002), fruit that are able to accumulate proline have a significantly higher stress tolerance than fruit which are unable to accumulate proline. During the 2008 harvest season, high proline levels were observed in fruit treated with 2.5 mM SA followed by fruit treated with 10 µM MJ. This led to observation that fruit treated with 2.5 mM SA or 10 µM MJ were better able to withstand the stress of chilling compared with other treatments. The results agree with those of Purvis (1981) where proline levels in the rind of grapefruit were found to be higher in fruit which were more resistance to chilling injury during cold storage.

During the 2009 harvest season, high proline levels were observed in fruit treated with 10 µM of MJ, followed by fruited treated with 2 mM SA. The level of proline accumulation in the rind of fruit treated with MJ and SA decreased with the level of concentration. Fruit treated with 10 µM MJ & 2 mM SA had higher levels of proline in the rind than when treated with 1 µM MJ & 0.2 mM SA. Moreover, fruit treated with 0.1 µM MJ & 0.02 mM SA had high levels of proline in the rind compared with untreated fruit. Proline levels increased with cold storage time in treated fruit, suggesting that intensity of cold stress increased with cold storage time. In addition, extending cold storage time to 28 days resulted in increased proline levels in fruit; this was an indication that fruit were experiencing more stress during that time Purvis (1981). After 28 days of cold storage, proline levels in fruit were slowly decreasing, indicating the compensatory ability of fruit to withstand cold stress was now over-exceeded. Probably, this is when symptoms of chilling injury may be expected to develop due to a drop in cold

stress defense mechanisms such as proline metabolism. This could also have resulted in the loss of membrane integrity, instability of proteins and the accumulation of ROS (Hare and Cress, 1997; Matysik *et al*., 2002; Misra and Saxena, 2009).

Lemons are non-climacteric fruit characterized by low ethylene production and lacking an auto-catalytic rise in ethylene production (Fujii *et al*., 2007). Fruit ethylene production on untreated fruit was below the limits of detection of the gas chromatagraph. Treatments did not appear to affect the production of ethylene during and after cold storage. However, this does not mean that fruit did not produce ethylene during cold storage. It is possible that a more sensitive sampling method and analysis would reveal differences in ethylene production. Some studies have reported that even small amounts of ethylene produced by fruit could play a role in protecting fruit against tissue damage caused by stress (Cajuste and Lafuente, 2007). While other studies found that the burst in ethylene production in citrus during postharvest storage was often a sign of cellular membrane damage, which was usually followed by the development of chilling injury symptoms (Zhou *et al*., 2001).

Field (1990) reported that there was no common association between chilling injury and ethylene production during postharvest of fruit. It is possible that the role of ethylene in chilling injury might be commodity dependent. According to Paull and Armstrong (1994), and Pérez-Tello *et al*. (2009), for some commodities, ethylene production is stimulated during chilling injury; on the other hand, for other commodities ethylene production does not occur until chilled fruit are transferred to room temperature. It is therefore important to establish whether ethylene production in fruit promotes physiological changes associated with chilling injury, or if it is involved in mitigating cell membrane damage, or if it is of no significance whatsoever (Field, 1990). This study revealed that ethylene production may not be a good indicator of chilling injury in lemons, despite extended cold storage time, due to the limited production in lemons and the difficulties in measurement. This is similar to earlier reports on chilling injury in mamey sapote fruit stored at 2 or 10°C (Pérez-Tello *et al.*, 2009) whereby ethylene production was also not a good indicator of chilling injury.

In conclusion, the study confirmed that exposing lemons to cold storage could lead to physiological dysfunction as a primary event of chilling injury. Some of the symptoms developed by the fruit during the extended cold storage were excess mass loss and chilling

enhanced respiration rate. However, ethylene production was not associated with chilling injury symptoms. Excessive fruit mass loss during the 2009 harvest season when compared with the 2008 harvest season was associated with low chilling injury tolerance. Fruit harvested during the 2008 season, were tolerance to chilling injury while fruit harvested during the 2009 harvest season were susceptible to chilling injury. This could be associated with cultivation practises and other physiological and biochemical factors involved during chilling stress of the fruit.

The cell membrane electrolyte leakage of the fruit harvested during the 2009 harvest season increased with cold storage time in untreated fruit. This was an indication that fruit harvested during the 2009 harvest season were stressed during cold storage. However, postharvest treatment with 10 µM MJ & 2 mM SA significantly ($P \leq 0.05$) reduced cell membrane electrolyte leakage of the fruit during cold storage as an indicator of chilling stress. Electrolyte leakage was probably not associated with chilling symptoms in fruit harvested during the 2008 season. The effective reduction of fruit mass loss using MJ and / or SA was probably associated with stomatal closure induced by the treatments (González-Aguilar *et al.* 2001).

Postharvest treatment with (10 µM MJ & 2 mM SA) significantly ($P \leq 0.05$) induced proline accumulation in the rind during cold stress. Fruit with high levels of proline were able to withstand chilling stress. Proline accumulation in the rind of fruit may have helped fruit to withstand cold stress by controlling the production of ROS (Saradhi *et al*., 1995), acting as stabiliser of protein in membranes (Rudolph *et al*., 1986), and a source of reduction equivalents (Hare and Cress, 1997) during recovery after chilling. MJ and SA appeared to enhance the effect, hence decreasing chilling damage. Further studies should be done to evaluate the effect of SA or MJ on the production of ROS during cold storage of lemons.

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Figures

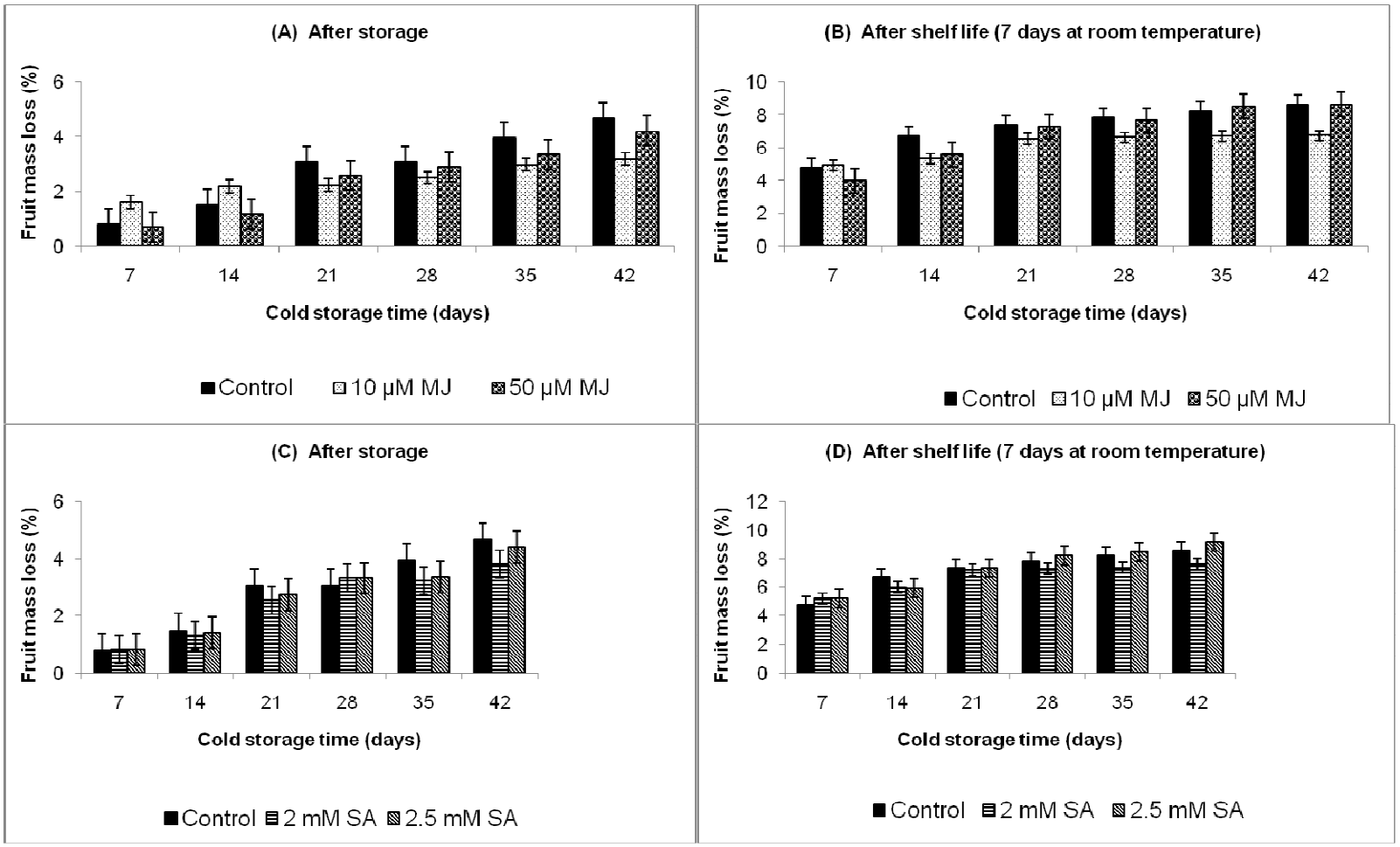

Figure 4.1: Mass loss (%) of fruit treated with 10 or 50 µM methyl jasmonate (MJ) or 2 or 2.5 mM salicylic acid (SA) and stored at -0.5°C. Fruit were removed after 7, 14, 21, 28 or 42 days of storage and transferred to room temperature for 7 days (2008 season). LSD $_{(0.05)}$ = 0.453.

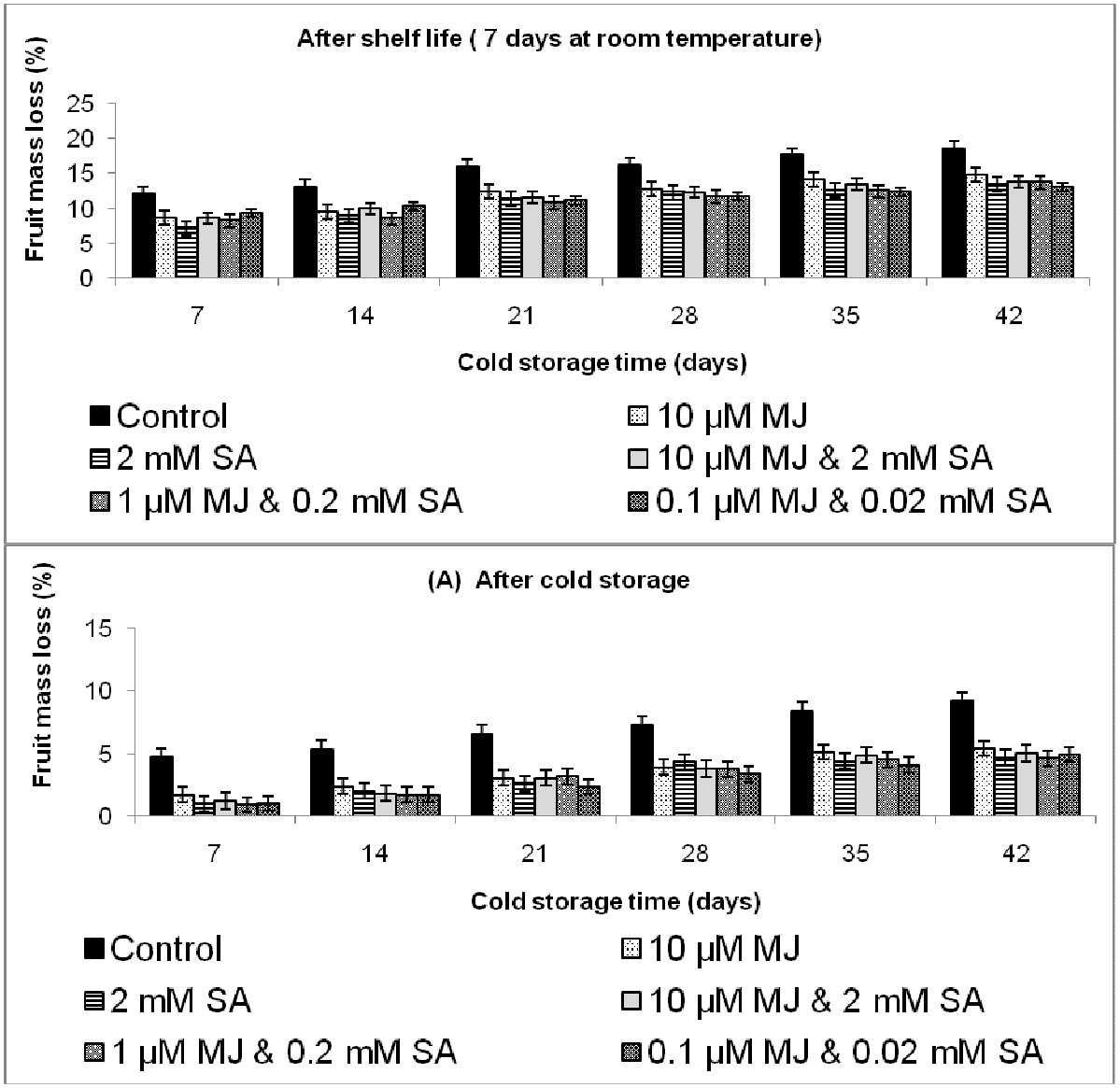

Figure 4.2: Mass loss (%) of fruit treated with 10 µM MJ, 2 mM SA, 10 µM MJ & 2 mM SA, 1 µM MJ and 0.2 mM SA, or 0.1 µM MJ & 0.02 mM SA before storage at -0.5°C for 7, 14, 21, 28 or 42 days at cold storage and transferring to the room temperature for 7 days (2009 season). $LSD_{(0.05)} = 0.4933$.

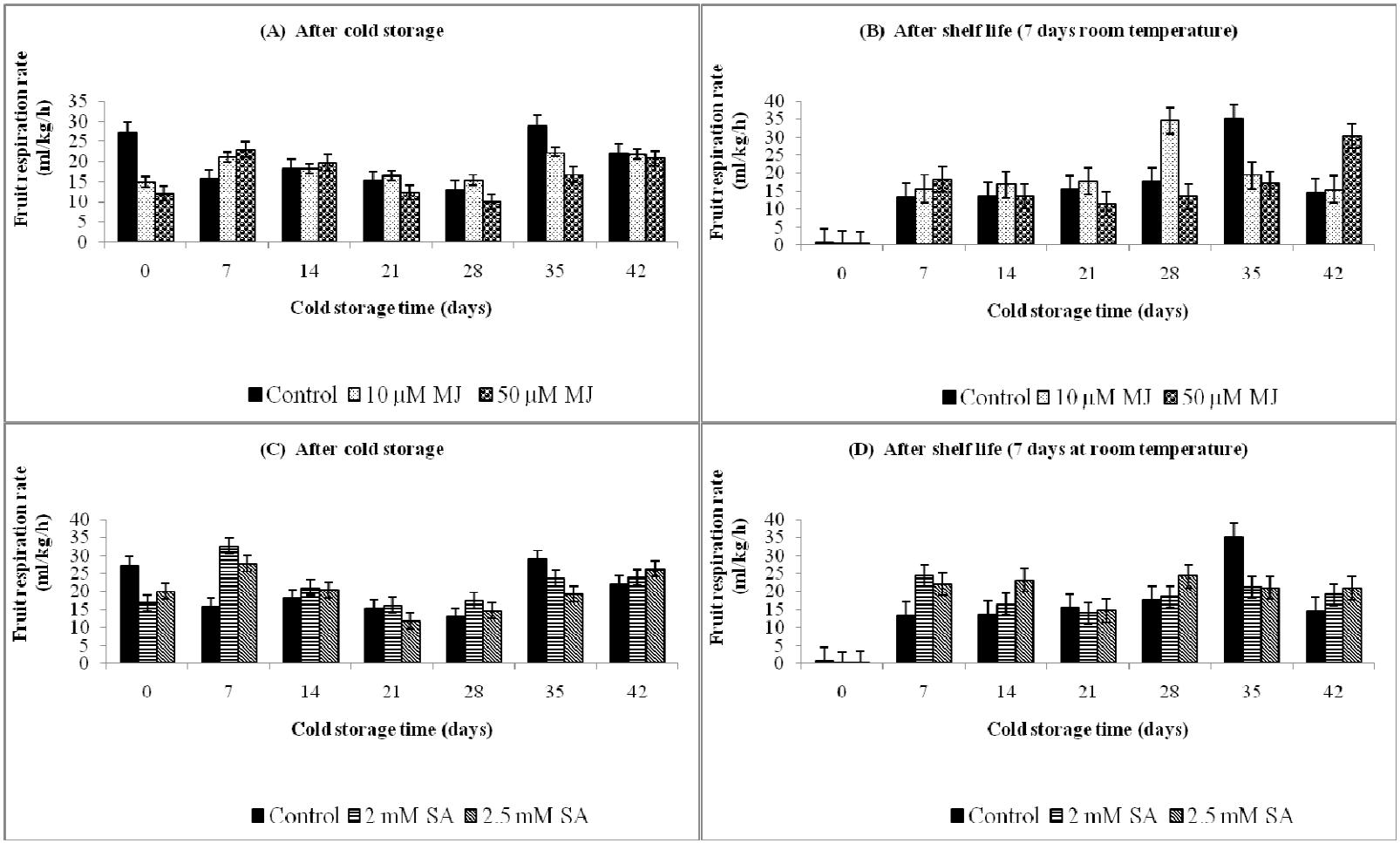

Figure 4.3: Respiration rate (ml kg^{-1} h^{-1}) of fruit treated with 10 or 50 µM MJ or 2 or 2.5 mM SA and stored at -0.5˚C. Fruit were measured directly after removal from 7, 14, 21, 28, or 42 days of cold storage and after storage at room temperature for 7 days (2008 season). $LSD_{(0.05)} = 4.919$.

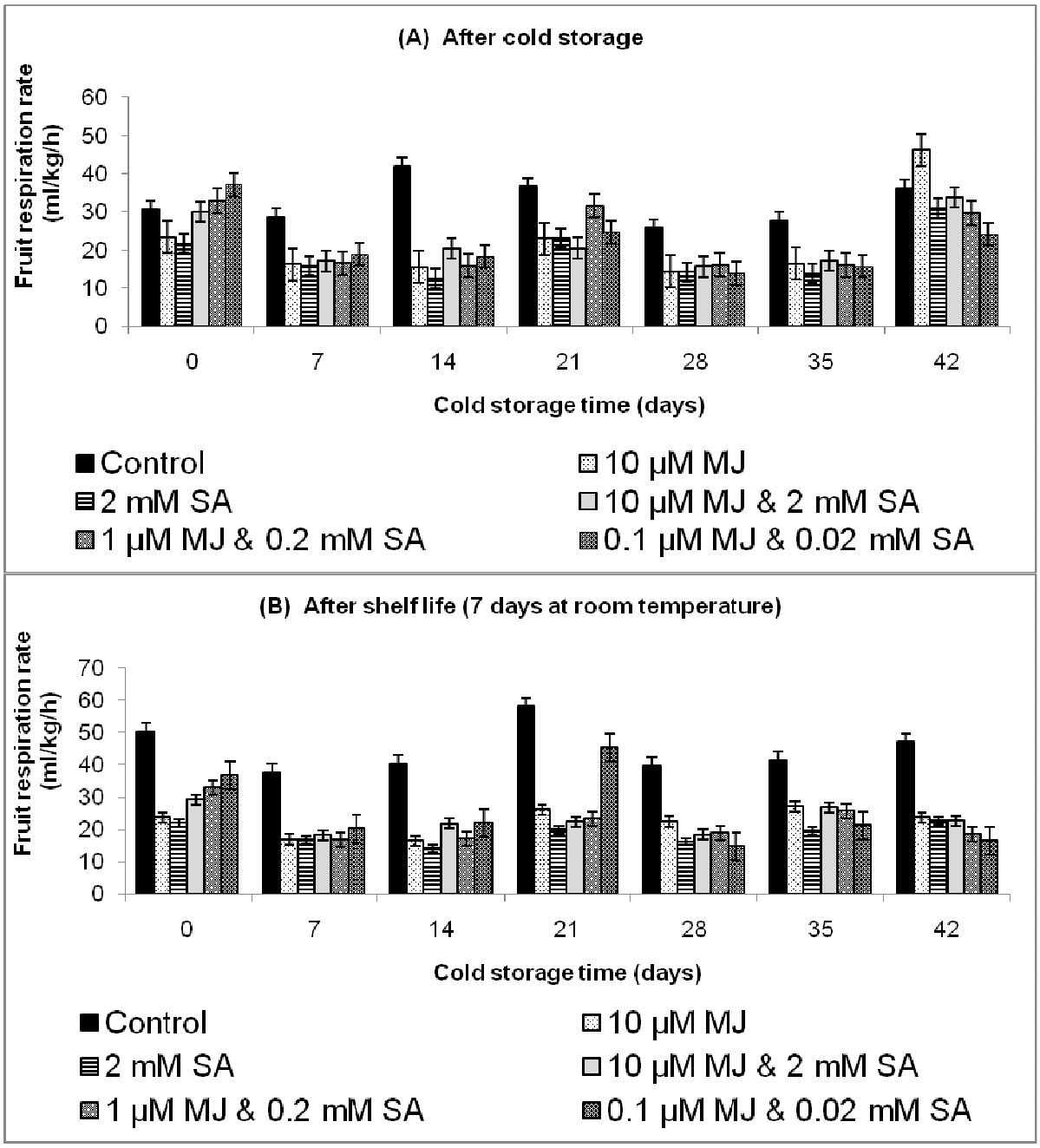

Figure 4.4: Respiration rate (ml kg^{-1} h^{-1}) of lemon fruit treated with 10 µM MJ, 2 mM SA, 10 µM MJ & 2 mM SA, 1 µM MJ & 0.2 mM SA, or 0.1 µM MJ& 0.02 mM SA. Fruit were measured directly after removal from 7, 14, 21, 28, or 42 days of cold storage or before transfer to room temperature for 7 days (2009 season). $LSD_{(0.05)}$ =3.259.

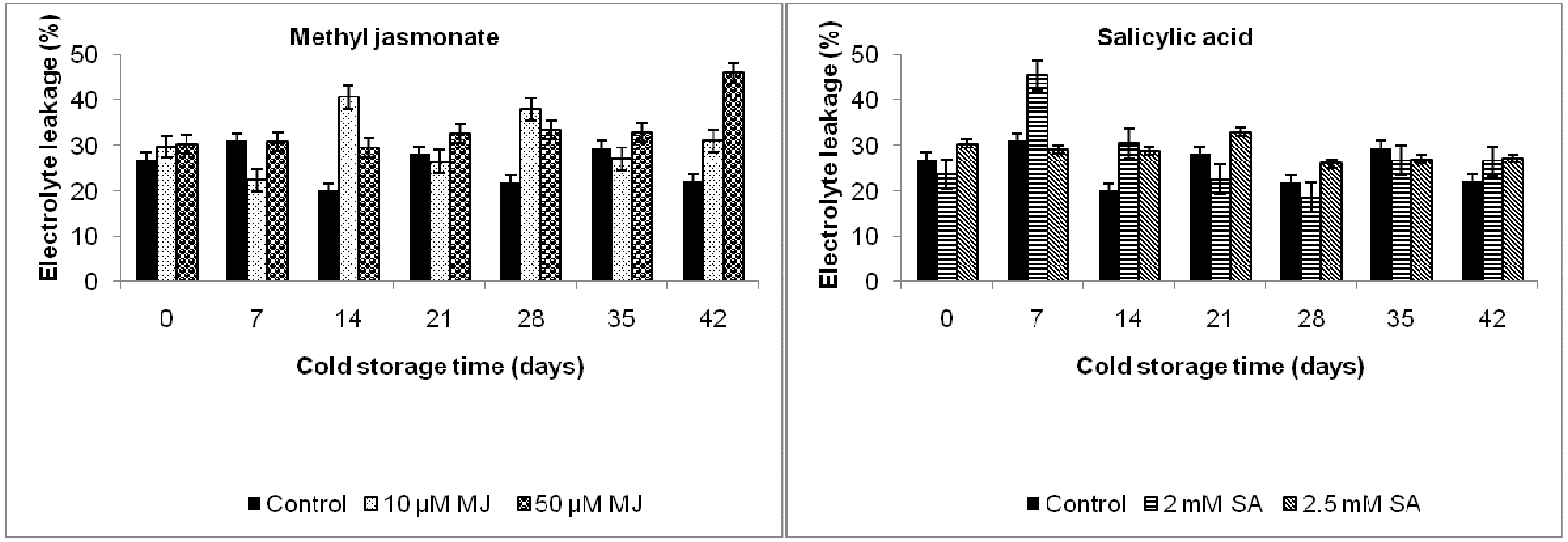

Figure 4.5: Electrolyte leakage % of rind of lemon fruit treated with 10, 50 µM MJ or 2, 2.5 mM SA and stored at -0.5°C for 0, 7, 14, 21, 28, 35 or 42 days before transfer to room temperature for 7 days (2008 season). LSD (0.05) = 6.214.

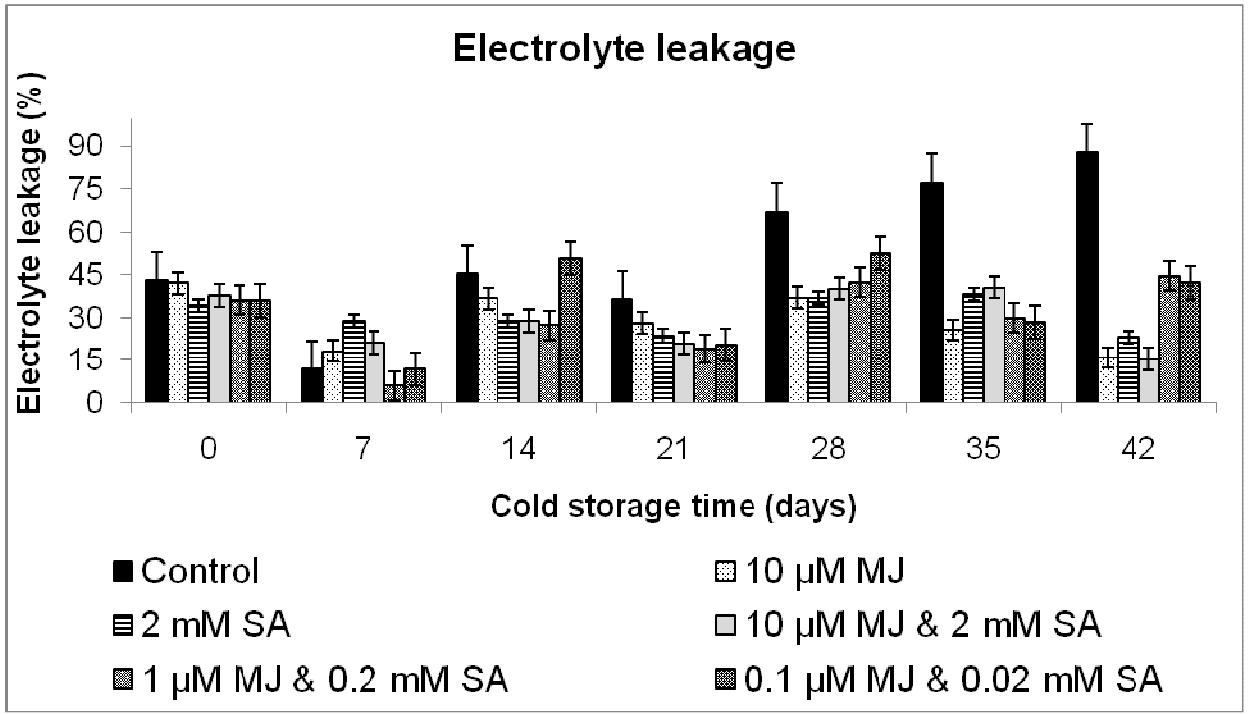

Figure 4.6:Percentage electrolyte leakage for fruit treated with 10 µM MJ, 2 mM SA, 10 µM MJ & 2 mM SA, 1 µM MJ & 0.2 mM SA, or 0.1 µM MJ & 0.02 mM SA before stored at -0.5°C for 0, 7, 14, 21, 28, 35 or 42 days. Fruit were subsequently transferred to room temperature for 7 days (2009 season). $LSD_{(0.05)} = 8.47$.

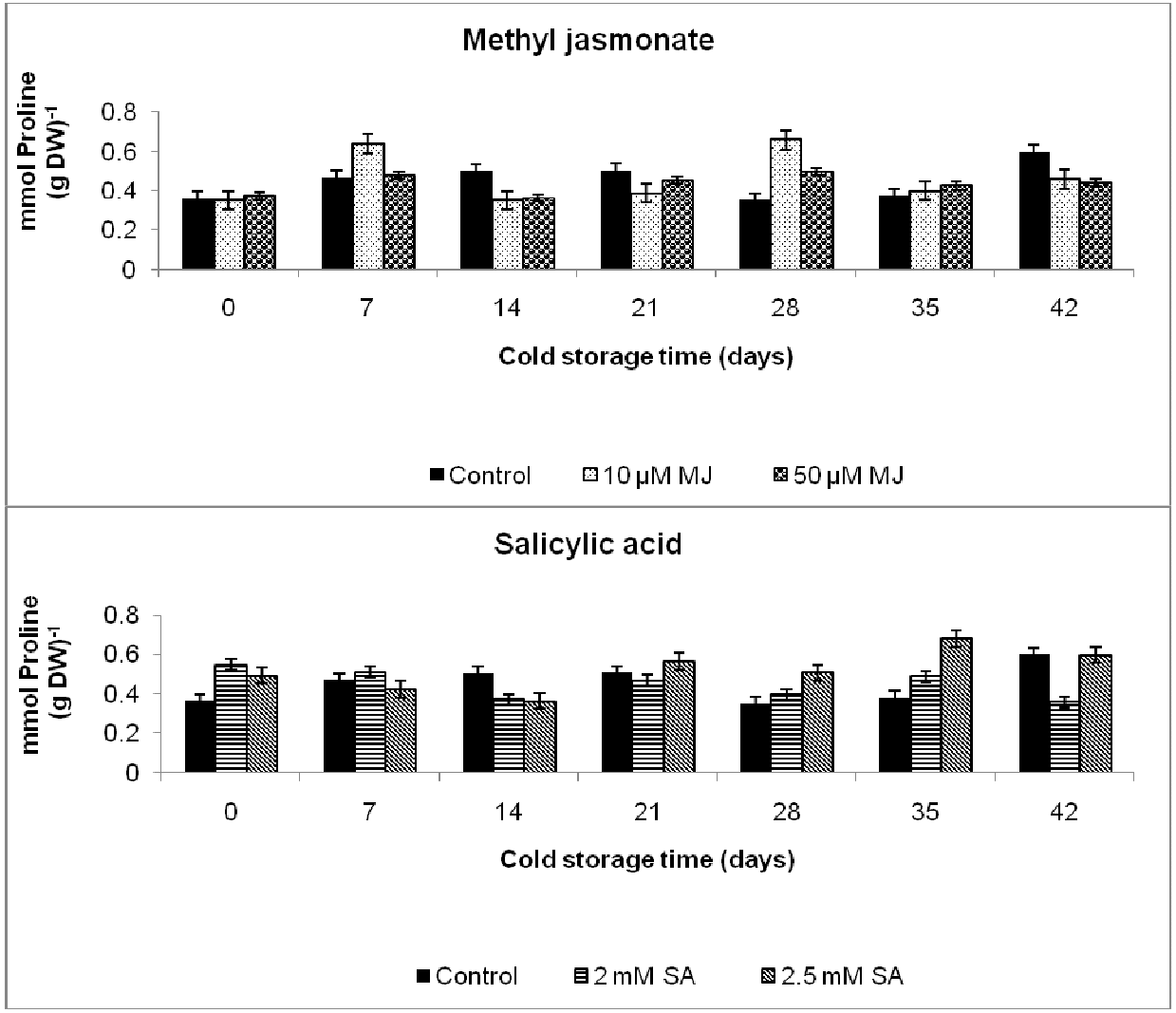

Figure 4.7: Effect of different concentrations of MJ (10 and 50 µM) or SA (2 and 2.5 mM) on proline accumulation in fruit during cold storage (-0.5˚C) for 0, 7, 14, 21, 28, 35, or 42 days and at the end of 7 days shelf life period (2008 season). LSD (0.05) = 0.01195.

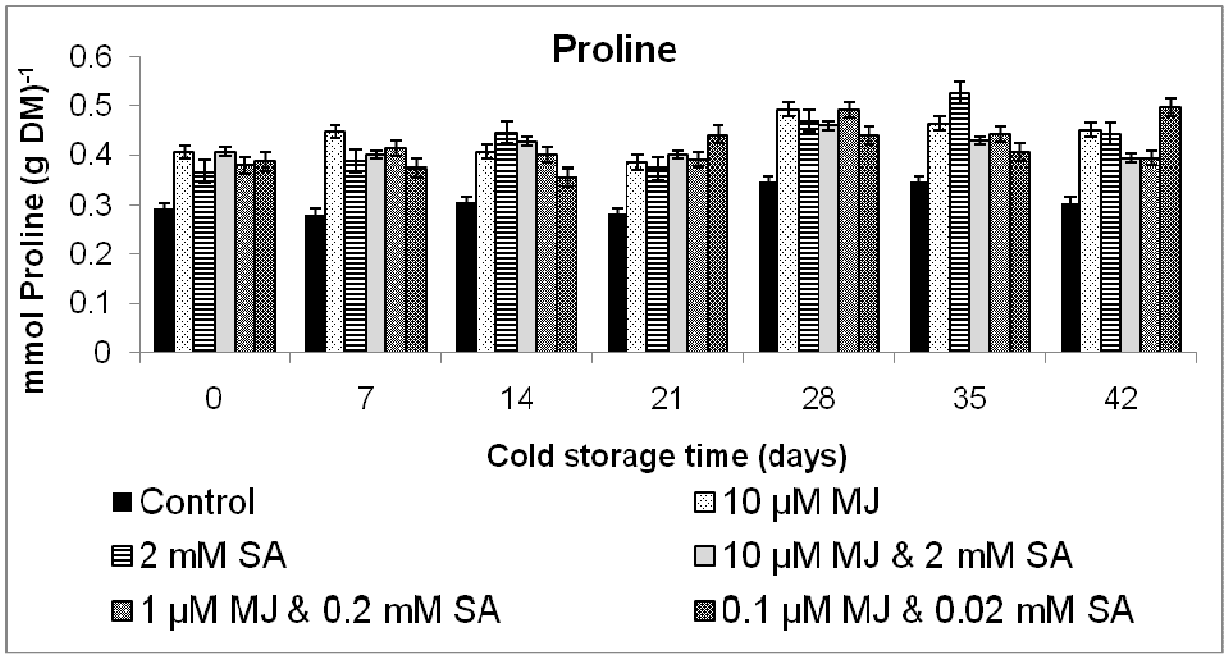

Figure 4.8: Proline accumulation in fruit treated with 10 µM MJ, 2 mM SA, 10 µM MJ & 2 mM SA, 1 µM MJ& 0.2 mM SA, or 0.1 µM MJ & 0.02 mM SA before being stored at -0.5℃ for 0, 7, 14, 21, 28, 35, or 42 days. Fruit were subsequently transferred to room temperature for 7 days (2009 season). $LSD_{(0.05)} = 0.02179$.

CHAPTER 5

EFFECT OF METHYL JASMONATE AND SALICYLIC ACID POSTHARVEST TREATMENT ON MECHANISMS AND ACTIVITIES OF ANTIOXIDANTS IN LEMON FRUIT RIND DURING COLD STORAGE

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SUMARY

Lemon fruit are chilling sensitive and develop chilling injury during cold quarantine treatment. The fruit may contain antioxidants which may either enhance chilling tolerance or induce defensive responses to protect fruit from damages caused by chilling injury. Carbohydrate levels in the rind may increase chilling tolerance in the fruit during quarantine treatment. With the aim of inducing antioxidant activities and carbohydrate levels, fruit were treated with different concentrations of methyl jasmonate (10, 50 µM) or salicylic acid (2 or 2.5 mM) during the 2008 harvest season. Fruit were also treated with 10 µM methyl jasmonate (MJ), 2 mM salicylic acid (SA), 10 µM MJ & 2 mM SA, 1 µM MJ & 0.2 mM SA, or 0.1 µM MJ & 0.02 mM SA during the 2009 harvest season. Following treatment, fruit were waxed and stored at -0.5°C for up to 42 days plus 7 days at room temperature (25°C). Measurements of total antioxidant scavenging capacity, ascorbic acid, total phenolics and carbohydrates (sucrose, glucose and fructose) in the rind were taken at seven-day intervals. The total antioxidant scavenging capacity in the rind was higher during the 2008 harvest season than during the 2009 harvest season. This led to the suggestion that fruit harvested during the 2008 harvest season were probably chilling tolerant due to high antioxidant activities. High levels of antioxidants may have induced defence mechanisms to protect fruit against chilling stress. High levels of total antioxidant scavenging capacity in the rind of fruit were observed at 14 days. This showed that fruit were probably stressed at 14 days. Treatment with MJ at 10 µM or SA at 2 mM or 10

µM MJ & 2 mM SA was effective in inducing antioxidant such as ascorbic acid and total phenolic compounds which could have enhanced chilling tolerance or at least protected fruit from chilling damage. Furthermore, treatment with MJ at 10 µM or SA at 2 mM or 10 µM MJ & 2 mM SA was also effective in increasing rind sucrose, fructose and glucose concentrations. This could have enhanced chilling tolerance by increasing the pool of sugar that can act as osmoprotectants or protecting fruit from chilling damage. Therefore, this study suggests that 10 µM MJ or 2 mM SA or 10 µM MJ & 2 mM SA may be used to increase chilling tolerance of lemons during phytosanitory treatment. It is suggested that 10 µM MJ or 2 mM SA or a combination of 10 µM MJ & 2 mM SA may be used to increase chilling tolerance of lemons during quarantine cold treatment.

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Lemons are subjected to an obligatory quarantine treatment in order to comply with export regulations for certain countries (Aung *et al*., 1998). Lemons need to be exposed to temperatures not higher than -0.6°C for 25-32 continuous days when being exported from South Africa to countries like the United States of America (Van Wyk *et al*., 2009). However, this treatment results in chilling injury leading to a down-grading of fruit quality (Cao *et al*., 2009). Chilling injury can be a consequence of oxidative stress (an early response to chilling injury) owing to over-production of ROS (Imahori *et al*., 2008; Cao *et al.* 2009). According to Scandalios (1993) and Cao *et al.* (2009), ROS are highly active and can damage membrane lipids, proteins and nucleic acids, leading to disruption of cellular homeostasis. Nevertheless, plants have evolved efficient antioxidant defence mechanisms that prevent the accumulation of ROS and repair oxidative damage (Imahori *et al.*, 2008; Wang *et al*., 2009). According to Shetty (2004) and Sarkar *et al*. (2009), antioxidants could either directly enhance low temperature tolerance or induce defence systems that prevent chilling injury. In addition, chilling tolerant crops have higher antioxidant activities than susceptible crops (Sala, 1998; Cao *et al*., 2009).

Phenolic compounds are naturally occurring antioxidants in plants. They are thought to play physiological roles in maintaining cellular viability and scavenging of radicals (González-Aguilar *et al.*, 2004; Ali *et al.*, 2007). The antioxidant compounds include flavonoids, hydroxycinnamate esters and tannins. They are considered as powerful antioxidants (Grace and Logan, 2000; Blokhina *et al*., 2003) due to their ability to decrease fluidity of membranes, resulting in changes that hinder the

diffusion of free radicals thus restricting peroxidative reactions (Arora *et al*., 2000; Blokhina *et al*., 2003). In lemons, phenolics are primarily found in the flavedo and are thought to play a significant role in conferring chilling tolerance to fruit (Su *et al*., 2008). However, despite the possible role of phenolic compounds in plant cold stress tolerance, they have received scant attention (Pennycooke *et al*., 2005).

Ascorbic acid is an effective antioxidant found in high concentrations in lemon tissue, where it plays a defence role in response to cold stress or by removing ROS (Om-arun and Siriphanich, 2005; Cao *et al*., 2009; Proietti *et al*., 2009). It provides membrane protection by detoxifying ROS (Blokhina *et al*., 2003; Rapisarda *et al.*, 2008). Cold storage of fruit may affect ascorbic acid levels in some horticultural crops. Tatsumi *et al.* (2006) found that, for certain commodities, ascorbic acid level decreased with cold storage time. Decreased levels of ascorbic acid due to cold storage time were observed in cucumbers (Tatsumi *et al*., 2006). This was associated with chilling injury (Yamauchi, 1975; Tatsumi *et al*., 2006). It has been suggested that recovery from the chilling injury indicates antioxidant protection from phenolic compounds and ascorbic acid (Christie *et al*., 1994; Sarkar *et al*., 2009).

Previous studies indicate that an increase in carbohydrate concentration in cold stored fruit could be associated with tolerance to chilling injury in such treated fruit (Levitt, 1980; Lafuente *et al*., 2005; Pérez-Tello *et al*., 2009). Furthermore, carbohydrates are involved in chilling stress tolerance through cellular membrane protection from chilling stress (Leprince *et al*., 1992; Ingram and Bartels, 1996; Aung *et al*., 1998). In lemons, carbohydrates are abundant in the rind (Sinclair and Crandell, 1949; Bean, 1960) and have been suggested to be involved in cold stress responses (Ingram and Bartels, 1996; Aung *et al.*, 1998). The accumulation of carbohydrates in lemons during cold storage could induce antioxidant defence mechanisms or modify the expression of genes involved in resisting chilling injury (Levitt, 1980; Purvis and Grierson, 1982; Holland *et al.*, 2002; Couée *et al.*, 2006).

Many studies have revealed that some postharvest treatments, such as MJ and SA, which induce chilling tolerance, had an ability to enhance antioxidant activities (Sala, 1998; Zheng *et al.,* 2008; Cao *et al* 2009). Methyl jasmonate (MJ) significantly reduced chilling injury in a variety of fruit types, such as sweet pepper (Fung *et al*., 2004) and guava (González-Aguilar *et al.,* 2004). Salicylic acid (SA) has similarly been applied to significantly reduce chilling injury in pomegranates (Sayyari *et al*., 2009). Therefore, the aim of this study was to determine the effect of MJ and / or SA on inducing antioxidant defence mechanisms or systems associated with chilling injury in cold stored lemons. Also, to investigate the effect of postharvest treatment with MJ and / or SA on inducing carbohydrate content in lemon fruit in order to increase chilling tolerance at cold storage.

MATERIALS AND METHODS

Plant materials and postharvest treatments

Eureka lemons were obtained during the 2008 and 2009 harvest seasons from two different farms near Pietermaritzburg, South Africa. During the 2008 harvest season, fruit were harvested from the Ukulinga Research Farm (29° 40' S 30° 24' E, 806 m elevation). The location is characterized by a mean annual rainfall and temperature of 738 mm and 18°C respectively, with a light to moderate frost in winter. Fruit were washed with Sporekill® (Hygrotech Pty Ltd.), air-dried for 5 minutes and randomly divided into treatments. For each postharvest treatment, fruit were either dipped into 10 or 50 µM MJ (Droby *et al*., 1999) or 2 or 2.5 mM SA solutions for 30 sec (Xu and Tian, 2008). A control or no dip treatment was also applied. Three replicates of 15 fruits per treatment were used with 5 fruit being sampled for evaluation. During the 2009 harvest season, fruit were obtained from Ithala commercial farm (29° 52' S 30° 16' E, approximately 700 m above sea level). Fruit were randomly divided into treatments and were either dipped into 10 µM MJ; 2 mM SA; 10 µM MJ & 2 mM SA; 1 µM MJ & 0.2 mM SA; or 0.1 µM MJ & 0.02 mM SA solutions for 30 sec. A control or no dip treatment was also adopted. Three replicates of 15 fruits per treatment were used. After dipping, fruit were waxed with Citrashine (Citrashine Pty Ltd, Johannesburg, South Africa) and stored at -0.5ºC for 0, 7, 14, 21, 28, 35 and 42 days before being transferred to room temperature (25°C) for a 7 day storage period.

Measurement of total antioxidant capacity

Total antioxidant capacity in the rind of fruit was determined using ABTS (2,2´-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) assay according to Tesfay *et al*. (2010). Flavedo freeze-dried tissue (0.2 g) was mixed with 5 ml of 1N perchloric acid and centrifuged (Sorvall RC-5C Plus, Newtown, CT, USA) at 12, 402 x g for 10 min at 4^{o}C. ABTS assay was prepared by reacting ABTS stock solution (7 mM concentration) with 2.45 mM potassium persulfate as proposed by Re *et al*. (1999) The mixed solution was stored in the dark at room temperature for 12 h; the radical cation solution was diluted with ethanol to an absorbance of 0.7 at 734 nm. After dilution, 1.0 ml of fresh prepared ABTS radical cation was placed into a cuvette and the absorbance at 734 nm read after 1 min, after which 10 µL of sample extraction was added to 1.0 ml of ABTS radical cation. The mixed solution was incubated for 6 min in a Spectrophotometer at 743 nm. Antioxidant activity measured as the percentage inhibition of absorbance at 734 nm was calculated and plotted as a function of concentration of antioxidants.

Determination of total phenolics content

Total phenolics in the rind of lemon were determined using the method of Pérez-Conesa *et al*. (2009). A 0.1 g sample of freeze-dried flavedo tissue was mixed with 1 ml of 1 M HCL, vortexed for 1 min and incubated at 37°C for 30 min. After incubation, 1 ml NaOH (2 M in 75 % methanol) solution was used for alkaline hydrolysis and the resulting mixture vortexed for 1 min and incubated at 37°C for 30 min. The samples were vortexed before mixing with 1.0 ml of 0.75 M metaphosphoric acid and centrifuged at 5, 000 rpm (2,510) for 10 min. The supernatants were removed, transferred into a 10 ml volumetric flask and the pellets re-suspended in 1.0 ml of acetone: water (1:1, v/v), vortexed for 1 min and centrifuged at 5, 000 rpm (2,510) for 10 min. Both extracts were combined and made up to 10 ml with acetone: water (1:1, v/v). Total phenolics were measured using the Folin-Ciocalteu reagent. Briefly, 5 ml of nanopure water, 1 ml of sample and 1 ml of Folin-Ciocalteu reagent were added to a 25 ml volumeric flask and allowed to stand for 5-8 min at room temperature. Therefore, 10 ml of a 7% sodium carbonate solution was added, followed by the addition of 8 ml of nanopure water placed to volume. The solution was vortexed thoroughly and allowed to stand at room temperature for 2 h before filtered through a Whatman® 0.45 µm poly filter prior to the determination of total

phenolics at 750 nm absorbance using a Beckman DU 800 spectrophotometer. Gallic acid monohydrate was used as the standard to prepare the calibration curve. The results were expressed as milligrams of gallic acid equivalents (GAE) per g DM of lemon peel.

Determination of Ascorbic acid

Ascorbic acid concentrations in the rind were determined using the method previously described by Böhm *et al*. (2006). One gram (DW) of the rind samples was mixed with 5 ml 0.56 M metaphosphoric acid, vigorously shaken and centrifuged at 2988 x g and the supernatant transferred into a volumetric flask. This procedure was repeated twice and thereafter the combined extracts made up to 20 ml using 0.56 M meta-phosphoric acid. Subsequently, 200 µL of the extract was mixed with 300 µL 0.3 M trichloroacetic acid and centrifuged at 17212 x g for 10 min. Subsamples of the supernatant (300 µL aliquots) were mixed with 100 µL 2,4-dinitrophenylhydrazine reagent (0.013 M in 30 % perchloric acid), and heated to 60°C for 1 h and subsequently cooled in an ice bath for 5 min. Thereafter, 400 µL 15.75 M sulphuric acid was added to the sample and the absorbance read at 520 nm after 20 min. The ascorbic acid concentration was calculated by comparison to the values obtained with an L-ascorbic acid standard curve. Results were expressed as mg/g DW.

Determination of sugar concentrations

Soluble sugars were extracted from freeze-dried flavedo tissue according to Tesfay *et al*. (2010). Freeze-dried flavedo samples (0.1 g each) were mixed with 10 ml of 80 % (v/v) ethanol and homogenized for 60 seconds. Following this, the mixture was incubated for an hour in an 80°C water bath. Thereafter, the mixture was kept at 4°C overnight. The following day the mixture was centrifuged at 12,000 x g for 15 min at 4°C. The supernatant was filtered through glass wool before being allowed to dry in a Savant Vacuum Concentrator (Speed Vac; Savant, NY, USA). Ultrapure water (2 ml) was used to re-suspend the dried samples. Thereafter, the re-suspended samples were re-centrifuged before being filtered through a 0.45 µm nylon filter and analysed using an isocratic High-Performance Liquid Chromatography system (LC-20AT, Shimadzu Corporation, Kyoto, Japan) equipped with a

refractive index detector (RID-10A, Shimadzu Corporation, Kyoto, Japan) and a Rezex RCM-Monosaccharide column (300 mm x 7.8 mm) (8 micron pore size; Phenomenex®, Torrance, CA, USA).

Statistical analysis

The experimental design was a completely randomised block design. All data were subjected to analysis of variance (ANOVA) using GenStat® Version 11 statistical package (VSN International Ltd, Hemel Hempstead, UK). Least significant difference (LSD) was used to evaluate the differences among treatment means where $P \leq 0.05$ were considered as significant.

RESULTS

Changes in total antioxidant capacity induced by methyl jasmonate and / or salicylic acid in cold stored lemons

During the 2008 harvest season, total antioxidant scavenging capacity of the fruit was significantly ($P \leq 0.05$) affected by cold storage time (Figure 5.1). There was a significant ($P \leq 0.05$) interaction between cold storage time and treatments. Results showed that fruit were highly stressed at 0 days at-0.5°C with an average total antioxidant scavenging capacity of 71.28%. Total antioxidant scavenging capacity of the fruit declined to 57.09% (average) at 7 days. Even so, extending cold storage time caused a slow decline of total antioxidants scavenging capacity. The total antioxidant capacity of untreated fruit declined to 32.07% (average) at 42 days. Treatment with MJ was effective in reducing total antioxidants scavenging capacity at 7-14 days. Similarly, treatment with SA was effective in reducing total antioxidants scavenging capacity at 14 days. However, there were no clear statistical differences between treatments with respect to total antioxidants scavenging capacity.

During the 2009 harvest season, total antioxidant capacity of the fruit was significantly ($P \leq 0.05$) affected by treatment and cold storage time (Figure 5.2). Exposing lemons to low temperature (-0.5°C) caused an increase in total antioxidant capacity of the fruit. After 7 days total antioxidant capacity increased from 0.45% to 0.55% (average). This remained constant for 21 days. Thereafter a rapid decline was noted. The total antioxidant capacity was 0.33% (average) after 28 days and decreased to 0.30% (average) after 42 days at cold storage. However, postharvest treatment with MJ and SA significantly ($P \leq 0.05$) induced and delayed

the degradation of total antioxidant capacity in lemons during cold storage (Figure 5.2). Results were however, inconsistence. Untreated fruit (control) had low levels of rind total antioxidant capacity, which appeared to increase until 21 days of storage, thereafter declining rapidly. The treatments, however, appeared to remain enhanced levels for longer.

Effect of methyl jasmonate and / or salicylic acid on phenolic metabolism of lemons at cold storage

During the 2008 harvest season, total phenolics of untreated fruit varied over time with increased decreases. However, treatment with MJ significantly ($P \leq 0.05$) enhanced the total phenolics of the fruit. This was noted at 21 days storage. Treatment with SA appeared more effective in increasing total phenolics than MJ, as the increasing effect were more sustained. Fruit treated with a moderate concentration of SA (2 mM) had higher total phenolics than fruit treated with higher concentrations (2.5 mM). In addition, total phenolics of fruit treated with 2 mM SA increased and reached a peak at 7 days. During the 2009 harvest season total phenolic content in the rind was significantly ($P \leq 0.05$) affected by treatments, cold storage time and the interaction between the two factors (Figure 5.4). Rind total phenolics content decreased with cold storage time in untreated fruit. Untreated fruit also had lower levels of rind total phenolics compared with MJ and SA treated fruit, where phenolics compounds appeared to have been induced significantly ($P \leq 0.05$). At 42 days of storage little different between the MJ and SA treatment was evident.

Effect of methyl jasmonate and / or salicylic acid on ascorbic acid content in lemons stored at cold storage

In the 2008 season, ascorbic acid tended to decline during storage with low levels rated after 28 days. Overall, both MJ and SA significantly enhanced and maintained higher levels in the rind, although there was some variation with concentration (Figure 5.5). In the 2009 season, control fruit showed lower levels of ascorbic acid which declined further with storage. Both MJ and SA enhanced and maintained higher levels. Again, there was none consistent variation with compound and concentration (Figure 5.6). A sharp decrease in ascorbic acid content was also observed at 14 days of storage but increase thereafter until 42 days. Treatment with the combination of 0.1 µM of MJ and 0.02 mM of SA had an effect in delaying the decline of ascorbic acid in the rind, with a total production of ascorbic acid of 0.20 mg/g DM after postharvest storage. Similarly, fruit treated with (1µM of MJ and 0.2 mM of SA) had a total production of ascorbic acid of 0.13 mg/g DM after postharvest storage. Treatment with 10 µM of MJ increased

ascorbic acid with a total production of 0.10 mg/g DM after postharvest storage whereas treatment with 2 mM of SA induced ascorbic acid with a total ascorbic acid production of 0.06 mg/g DM after postharvest. Treatment with 10 µM of MJ and 2 mM of SA significantly induced ascorbic acid in the rind of the fruit during cold storage with a total ascorbic of 0.06 mg/g DM after postharvest. Results showed that untreated fruit had lower levels of ascorbic in the rind of the fruit during cold storage compared to methyl jasmonate and salicylic acid treated fruit. Untreated fruit had a total ascorbic acid of 0.01 mg/g DM after postharvest.

Changes in carbohydrates induced by methyl jasmonate and / or salicylic acid in cold stored lemons during the 2008 harvest season

For the 2008 harvest season, major carbohydrates detected in the rind of cold stored lemons were sucrose, glucose and fructose. The amount of sucrose detected in the rind of fruit was lower than the glucose and fructose content. Sucrose levels were significantly ($P \leq 0.05$) affected by treatment, cold storage time and the interaction of these two factors (Figure 5.7). Sucrose level increased and reached a peak at 14 days (Figure 5.7). This was probably in response to cold stress. On the other hand, the sucrose level of untreated fruit continued to increase and reached a peak at 28 days. A gradual decline in sucrose level in treated fruit was observed between 14 to 28 days. Extending cold storage time caused sucrose level to decline in untreated fruit. However, treating fruit with MJ (10 or 50 µM) or SA (2.5 mM) gradually increased the sucrose level during extended cold storage while treatment with 2 mM SA decreased the rate of depletion of sucrose level in the rind. Therefore, treatment with MJ at 10 µM significantly increased and prolonged sucrose level during cold storage of lemons.

Glucose levels were higher than fructose and sucrose. The rind glucose concentration was significantly ($P \leq 0.05$) affected by treatments (Figure 5.7). Salicylic acid was effective in increasing the glucose level compared with untreated (control) fruit. Rind glucose levels were not significantly affected ($P > 0.05$) by cold storage time and the interaction between treatments and cold storage time. Fructose levels were significantly ($P \leq 0.05$) affected by cold storage time (Figure 5.7). Exposing lemons to –0.5°C for 14 days caused a slight decrease in fructose in the rind of fruit. Treatment with SA caused fruit to lose fructose at 14 days compared with treatment with MJ. Extending cold storage time resulted in an increase in fructose in the rind. Fructose level increased and reached

a peak at 28 days. This was followed by a depletion of fructose that was observed in control fruit from 28-42 days. Fruit treated with either MJ or SA, were able to delay this effect. Even so, there was no significant difference ($P>0.05$) between treatment and the interaction between cold storage time and treatment.

Changes in carbohydrates induced by methyl jasmonate and / or salicylic acid in cold stored lemons during the 2009 harvest season

During the 2009 harvest season, the main carbohydrates detected in the rind were sucrose, glucose and fructose (Figure 5.8). Carbohydrate content (sucrose, glucose and fructose) was significantly ($P\leq0.05$) affected by treatment, cold storage time and the interaction between these two factors. Fructose level detected in the rind was generally higher than that of glucose and sucrose. The increase in fructose level in the rind of fruit may be in response to cold stress. Cold storage (-0.5°C) time significantly affected ($P\leq0.05$) carbohydrate content in the rind. Depletion in carbohydrate content was observed when cold storage time was extended from 14 to 42 days (Figure 5.8). Generally, the amount carbohydrate content in the rind was significantly ($P\leq0.05$) induced by postharvest treatment with either MJ and / or SA.

High levels of rind carbohydrate content were observed in fruit treated with 10 µM MJ followed by fruit treated with 1 µM MJ & 0.2 mM SA (Figure 5.8). Treatment with 2 mM SA was the third most effective treatment in inducing carbohydrate content in the rind followed by .0.1 µM MJ & 0.02 mM SA treatment. Fruit treated with 10 µM MJ & 2 mM SA had higher levels of carbohydrate content in the rind compared with untreated fruit (Figure 5.7). Therefore, carbohydrate content in the rind of fruit treated with either MJ and / or SA was higher than that of untreated fruit (control). For fruit treated with 10 µM MJ & 2 mM SA, sucrose content gradually increased in response to extended cold storage time. However, a decrease in sucrose level was observed at 42 days (Figure 5.8). The glucose level in fruit treated with 10 µM MJ & 2 mM SA gradually increased with extended cold storage time, reaching a peak at 28 days (Figure 5.8). A slow decrease in glucose level was observed from 35 to 42 days. Fructose was very high in the rind of fruit treated with 2 mM SA or 10 µM MJ (Figure 5.8). However, treatments were ineffective in sustaining the initial fructose level resulting in fructose level decreasing dramatically after 28 days (Figure 5.8).

DISCUSSION

Exposing lemons to chilling temperature (-0.5°C) may result in chilling stress, thus inducing ROS (Siriphanich, 2002). These ROS can attack cell membranes, resulting in lipid peroxidation, degradation of proteins and other molecules in the cells (Pennycooke *et al*., 2005). Such damage could lead to the breakdown of membranes, finally leading to the appearance of chilling injury symptoms (Shewfelt and del Rosario, 2000; Siriphanich, 2002). Nevertheless, fruit have antioxidants which accumulate to mediate chilling stress by controlling the production of ROS or by acting as defence mechanisms (Shewfelt and del Rosario, 2000; Siriphanich, 2002; Pennycooke *et al*., 2005). If the ability of the fruit to respond can be enhanced or the response maintained for longer, the potential for chilling injury will be decreased. Therefore, in this study the effect of MJ or SA in inducing antioxidant defence systems during the cold storage of lemons were investigated.

The total antioxidant scavenging capacity in the rind was significantly different during the 2008 and 2009 harvest seasons. The reasons for differences are probably complex fruit and environmental interaction (Shivashankara *et al*., 2004). Fruit harvested during the 2008 harvest season had higher total antioxidant scavenging capacity than the 2009 fruit. The higher total antioxidant scavenging capacity of the fruit during the 2008 harvest season was probably associated with chilling tolerance. Sala (1998) and Cao *et al*. (2009) reported similar findings in that chilling tolerant fruit had higher antioxidant activities as well as higher total antioxidant scavenging capacity compared with susceptible fruit. The cause of high antioxidant activities of the fruit during the 2008 harvest season could be a response to environmental stress due to soil conditions at Ukulinga. Fruit from Ukulinga probably have developed a stress resistance mechanism which may involve an increase in antioxidant activities which may allow the fruit to withstand and recover from harsh conditions such as biotic and abiotic stress. Moreover, the difference in antioxidant capacity between the two farms could also involve the selection of planting material.

During the 2008 harvest season, the total antioxidant scavenging capacity in the rind of fruit was significantly ($P \leq 0.05$) affected by cold storage time. The highest levels of total antioxidant scavenging capacity were observed at 0 days. However, storing lemons at -0.5°C reduced the total antioxidant scavenging capacity in the fruit at 7 days (Figure 5.1). Furthermore, an increase in

total antioxidant scavenging capacity in the rind was observed at 14 days of cold storage in untreated fruit. This suggested that this increase in total antioxidant scavenging capacity was associated with a response to chilling stress. This could indicate that fruit were significantly stressed at 14 days and antioxidant compounds were probably responding by either scavenging harmful radicals or repairing the damage caused by chilling stress. The decline in total antioxidant scavenging capacity observed after 14 days may be associated with depletion of antioxidants. This further suggested that the decline in total antioxidant scavenging capacity may have been due to a strong relationship with ascorbic acid; ascorbic acid decreased rapidly during cold storage of fruit compared with other antioxidants such as total phenols. This is similar to results reported in a previous study on mango fruit, where a reduction in total antioxidant capacity after 20 days was associated with a greater contribution of ascorbic acid than other antioxidants such as total phenolics (Shivashankara *et al*., 2004). However, there were no significant differences between treatments with respect to total antioxidant scavenging capacity in the rind.

During the 2009 harvest season, exposing lemons to chilling temperature (-0.5°C) resulted in accelerated total antioxidant scavenging capacity from 0-14 days presumable in response to cold stress. However, the percentage of total antioxidant scavenging capacity decreased with extended cold storage time from 21-42 days. Postharvest treatment with MJ and SA significantly ($P \leq 0.05$) induced and delayed the degradation of total antioxidant capacity in lemons during cold storage. Nevertheless, there were no clear differences between treatments and concentration levels used. Previous studies (Srilaong and Tatsumi, 2003; Kondo *et al*., 2005) suggest that as senescence occurs with extended storage, antioxidant capacity will decline. A similar tendency was found in this study where capacity was particularly low (2009 season). The addition of MJ and SA appeared to have a positive effect, in that both compounds initially enhanced and later more successfully maintained antioxidant levels than was in untreated fruit. This may have enhanced the ability of fruit to endure long periods of storage. From the increase in antioxidant capacity (untreated fruit) up to 14 days of storage, it is suggested that this is the stage at which the ability to reduce the chilling stress becomes critical. If fruit cannot maintain the capacity, a decline in antioxidant capacity will be evident with a subsequently higher potential for damage. Both MJ and SA may be useful in preventing this decline.

Ascorbic acid is a potent antioxidant found in high concentrations in lemons and is known for conferring protection against oxidative stress (Om-arun and Siriphanich, 2005). During the 2008 harvest season, ascorbic acid in the rind decreased with cold storage time from 0-28 days. The antioxidant ascorbic acid is known for decreasing with extended cold storage time in other horticultural crops such as cucumber (Tatsumi *et al*., 2006). The decrease in ascorbic acid levels in fruit during cold storage is associated with chilling injury (Yamauchi, 1975; Tatsumi *et al*., 2006). Again, postharvest treatment with MJ and SA significantly ($P \leq 0.05$) induced ascorbic acid in the rind of fruit, resulting in increased ascorbic acid in the rind. Ascorbic acid levels were higher in fruit treated with higher concentrations of either MJ (50 µM) or SA (2.5 mM) than in fruit treated with medium concentrations 10 µM or 2 mM.

Higher concentrations of MJ or SA were more effective than moderate concentrations of the two chemicals in inducing ascorbic acid production. Despite an earlier decline in ascorbic acid, a regeneration phase was observed after 28 days in fruit treated with 10 µM MJ. Treatment with 10 µM MJ prolonged the levels of ascorbic acid in the rind; this perhaps played a role against oxidative stress by removing ROS from tissue during extended cold storage. Fruit are important sources of antioxidants (Chanjirakul *et al*., 2006). These antioxidants include phenolic compounds, which may include beneficial effects by scavenging free radicals and protect cells against oxidative damage (Chun *et al*., 2003; Chanjirakul *et al*., 2006). Several studies have reported that phenols may play a role in maintaining cellular viability and are involved in chilling tolerance of citrus fruit (Tamagnone *et al.*, 1998; Sala and Lafuente, 1999; González-Aguilar *et al*., 2004).

It has been suggested by Levitt (1980); Purvis and Grierson (1982) and Holland *et al*. (2002) that carbohydrate levels in the rind of fruit may influence the sensitivity of fruit tissues to low temperature. Therefore, in this study the effect of MJ or SA in inducing carbohydrate content during the cold storage of lemons were investigated. There appears to be a relationship between carbohydrate content and chilling tolerance. Treatment with either MJ (10 µM MJ) and / or SA (2 mM SA) increased rind carbohydrates and this probably caused fruit to withstand chilling injury during cold storage (Table 1). Holland *et al.* (1999) confirmed that carbohydrates are involved in protecting citrus fruit such as ‘Fortune’ mandarins against chilling injury, with sucrose

being the predominant and probably the most important carbohydrate. During the 2008 season, rind glucose and fructose content were found to be higher than sucrose level during cold storage. High levels of glucose and fructose in the rind were seemingly associated with chilling tolerance. This increase in glucose and fructose levels may be a defence mechanism to resist chilling stress as in grapefruit (Purvis *et al*., 1979) and other citrus fruit (Lafuente *et al*., 2005). The rind sucrose concentrations were generally lower than those of glucose and fructose. This is in agreement with previous reports that sucrose is lower than glucose and fructose in the rind of other citrus cultivars during chilling stress (Purvis and Grierson, 1982; Holland *et al*., 1999).

The low content of sucrose in the rind may have been due to the fact that most fruit store energy in the form of sucrose and this sucrose is transformed to glucose and fructose at postharvest (Nilpraprucк *et al*., 2008). The sucrose content increased and reached a peak at day 14 of cold storage. The increase in sucrose could be associated with physiological responses to chilling injury (Pérez-Tello *et al*., 2009). Moreover, sucrose was found to be more effective than glucose and fructose in reducing chilling injury in tomato seedlings (Holland *et al.*, 1999). Extending cold storage time from 14 to 28 days resulted in a decline in sucrose content in untreated fruit. Treatment with MJ (10 or 50 µM) or SA (2.5 mM) however showed an increased sucrose concentration in the rind of fruit during cold storage, while treatment with 2 mM SA only delayed the decline in sucrose during cold storage.

During the 2009 harvest season, rind carbohydrate content was significantly affected by treatment, cold storage time and the interaction of these two factors. Treatment with MJ (10 µM) increased carbohydrate contents to 88.5 mg/g, the highest value of all treatments. Treatment with 2 mM SA was the second best treatment in inducing carbohydrate contents in the rind. This study showed that the rind of fruit treated with 10 µM MJ or 2 mM SA was stressed during cold storage and the carbohydrates content was probably responding to chilling injury. The carbohydrate content in the rind was decreasing with cold storage time in untreated fruit. Untreated fruit had the lowest level of carbohydrate content in the rind during cold storage than treated fruit. However, postharvest treatment with MJ and SA delayed the degradation of carbohydrate content in the rind during cold storage. The low level of carbohydrate content was observed in the rind of fruit treated with a combination of MJ at 10 µM and SA at 2 mM with

56.4 mg/g. This led to the suggestion that applying MJ and SA on fruit at moderate concentrations (10 µM MJ & 2 mM SA) may induce defence mechanisms that indirectly provide protection against chilling injury.

The fructose concentration of the rind was generally higher than that of glucose and sucrose. High levels of fructose were observed in the rind of fruit treated with either 10 µM MJ, 1 µM MJ & 0.2 mM SA, 2 mM SA or 0.1 µM MJ& 0.02 mM SA. A similar increase in fructose was reported by Purvis *et al*. (1979) in grapefruit under chilling stress. The increase in rind fructose could be a defence mechanism to resist chilling stress. All treatments in our study were, however, ineffective in maintaining fructose levels after 28 days. Cold storage indicates that fruit were unable to withstand cold storage exceeding 28 days.

This study suggests that storing lemons at -0.5°C could result in chilling injury which was shown to increase with storage time (chapter 3). This was confirmed by the amount of sucrose which increased with cold storage time in response to chilling injury in the rind of fruit treated with 10 µM MJ & 2 mM SA. In addition, the glucose level in the rind of fruit treated with 10 µM MJ & 2 mM SA gradually increased with cold storage time until it reached a peak at 28 days. However, a decrease in sucrose and glucose levels observed at 42 days showed that the ability of the fruit to withstand chilling stress had been exceeded. Thus, the study agreed with other previous studies reporting that increased tolerance to chilling injury was strongly associated with increased carbohydrate content (Pérez-Tello *et al*., 2009; Lafuente *et al*., 2005).

In conclusion, citrus flavedo contains various compounds, amongst which are phenolics and ascorbic acid which act as antioxidants or free radical scavengers. These are likely to be important in protecting cells against the stress of prolonged low temperature storage. The levels may be affected by the length of the low temperature storage time as well as pre-harvest factors, many which are unknown. The postharvest treatments of MJ and SA show promise in that there is evidence of enhanced and prolonged concentrations of important antioxidants especially where antioxidant levels are low, such as phenolics and ascorbic acid. The low levels of carbohydrates in the rind of fruit harvested during the 2009 harvest season (Ithala farm) indicate that fruit were

probably susceptible to chilling injury than fruit harvested during the 2008 harvest season (Ukulinga farm), which had generally higher rind carbohydrate levels.

Postharvest treatment with MJ or SA induced carbohydrate levels in the rind. Carbohydrate content increased and was shown to be linked to chilling tolerance (Table 1). Carbohydrates, collectively, may comprise fruit defence mechanisms against chilling injury during extended cold storage. This study suggested that applying MJ and /or SA on fruit at moderate concentration (10 µM MJ, 2 mM SA or 10 µM MJ & 2 mM SA) may induce defence mechanisms such as carbohydrates in the rind that indirectly provide protection against chilling injury. Therefore, applying postharvest treatments of 10 µM MJ or 2 mM SA or 10 µM MJ & 2 mM SA may be used to mitigate chilling injury in lemons during quarantine treatment. However, the mode of action and optional concentrations are not as yet clear and it is suggested that further work be conducted.

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FIGURES

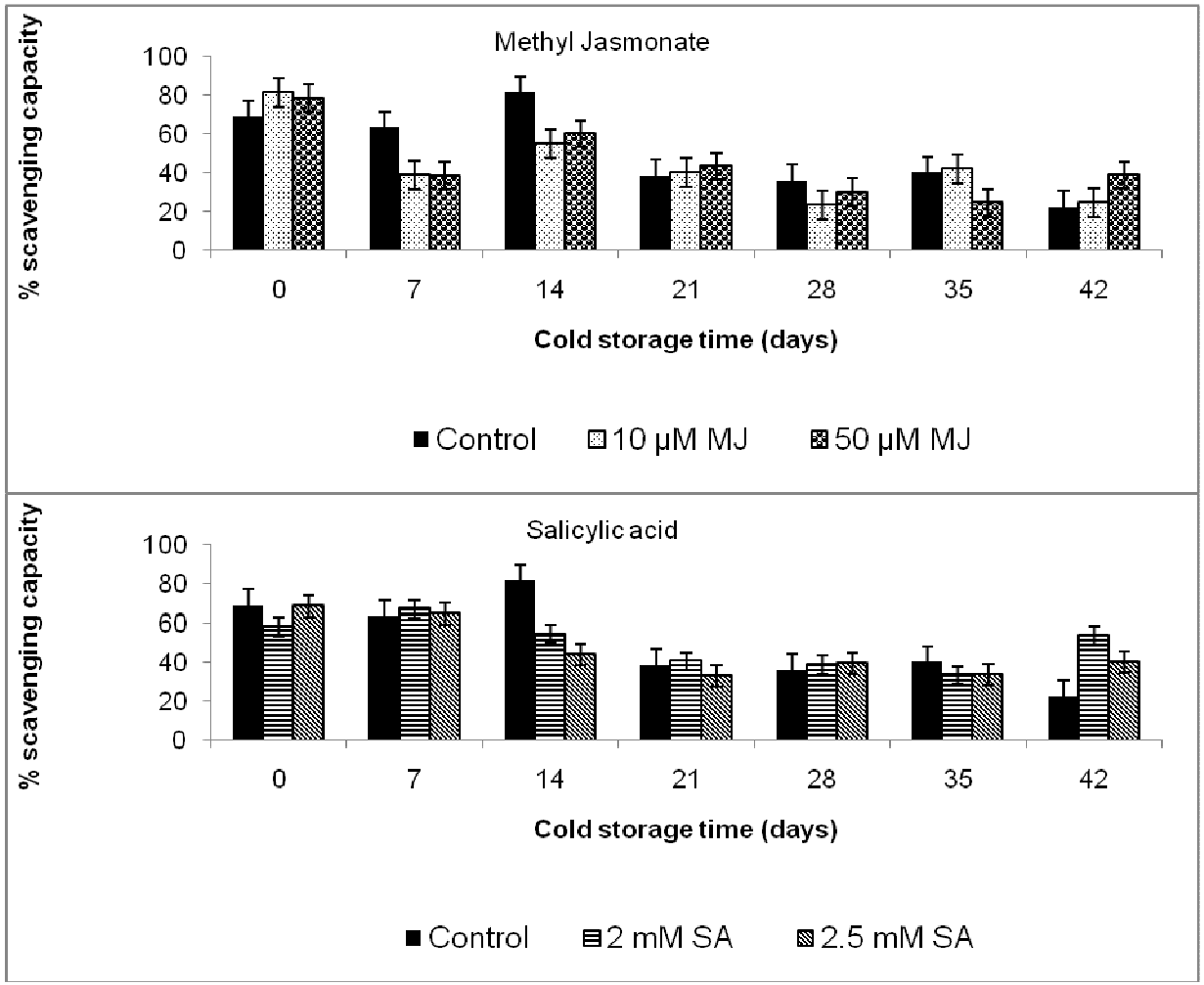

Figure 5.1:Total antioxidant capacity of fruit treated with (10 or 50 µM MJ) or (2 or 2.5 mM SA) and stored at -0.5°C for 42 days before being transferred to room temperature for 7 days (2008 season). $LSD_{(0.05)} = 6.372$.

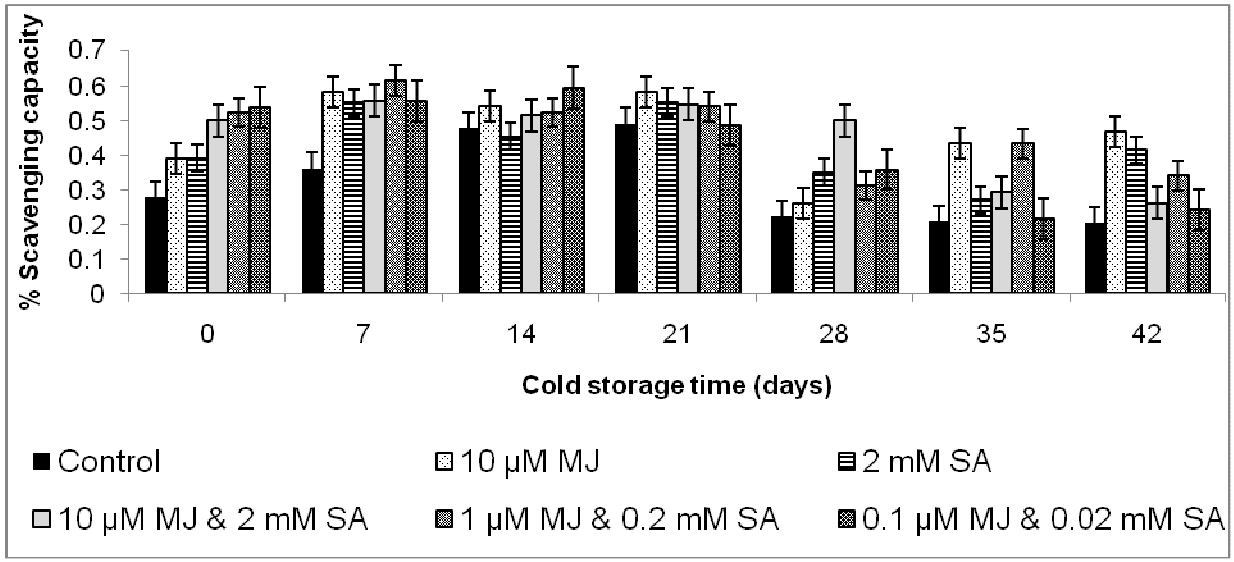

Figure 5.2: Total antioxidant capacity of fruit rind treated with (10 µM MJ), (2 mM SA), (10 µM MJ & 2 mM SA), (1 µM MJ & 0.2 mM SA), or (0.1 µM MJ & 0.02 mM SA) and stored at -0.5°C for 42 days before being transferred to room temperature for 7 days (2009 season). $LSD_{(0.05)} = 0.0715$.

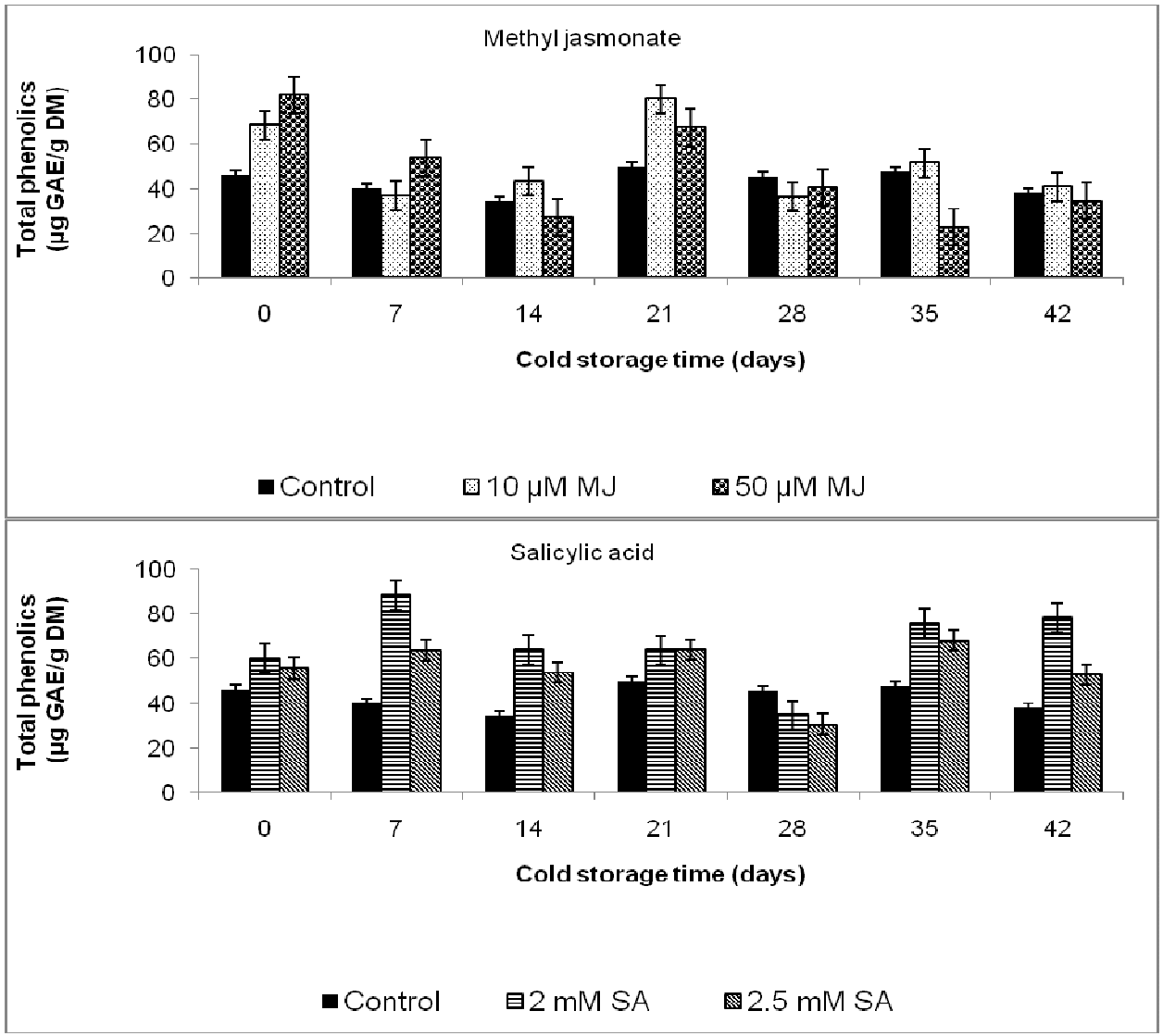

Figure 5.3: Total phenolics in fruit rind treated with (10 or 50 µM MJ) or (2 or 2.5 mM SA) and stored at -0.5°C for 42 days before being transferred to room temperature for 7 days (2008 season). $LSD_{(0.05)} = 7.026$.

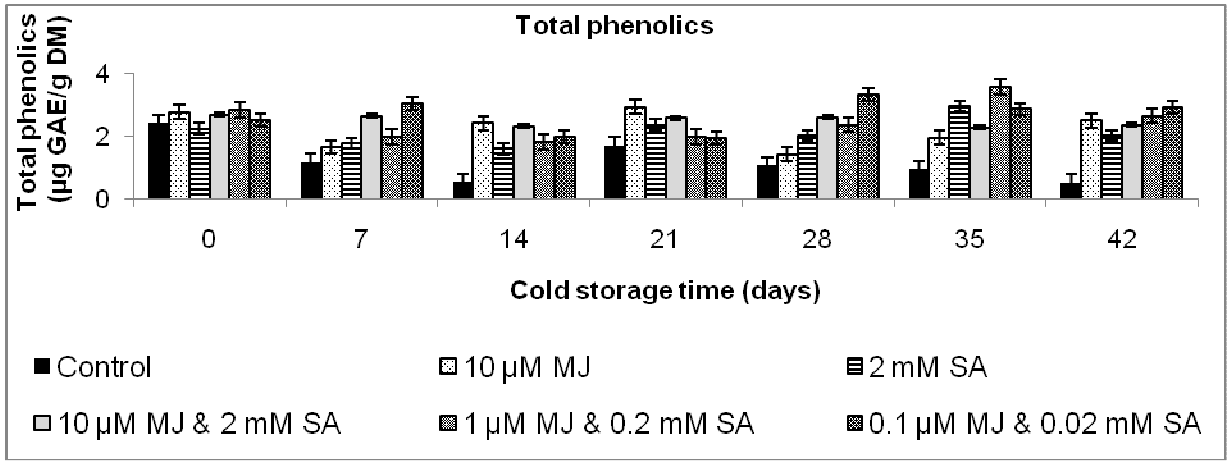

Figure 5.4: Total phenolics in rind of fruit treated with 10 µM MJ, 2 mM SA, 10 µM MJ & 2 mM SA, 1 µMMJ & 0.2 mM SA, or 0.1 µM MJ & 0.02 mM SA and stored at -0.5°C for 42 days before being transferred to room temperature for 7 days (2009 season). $LSD_{(0.05)}$ =0.3118.

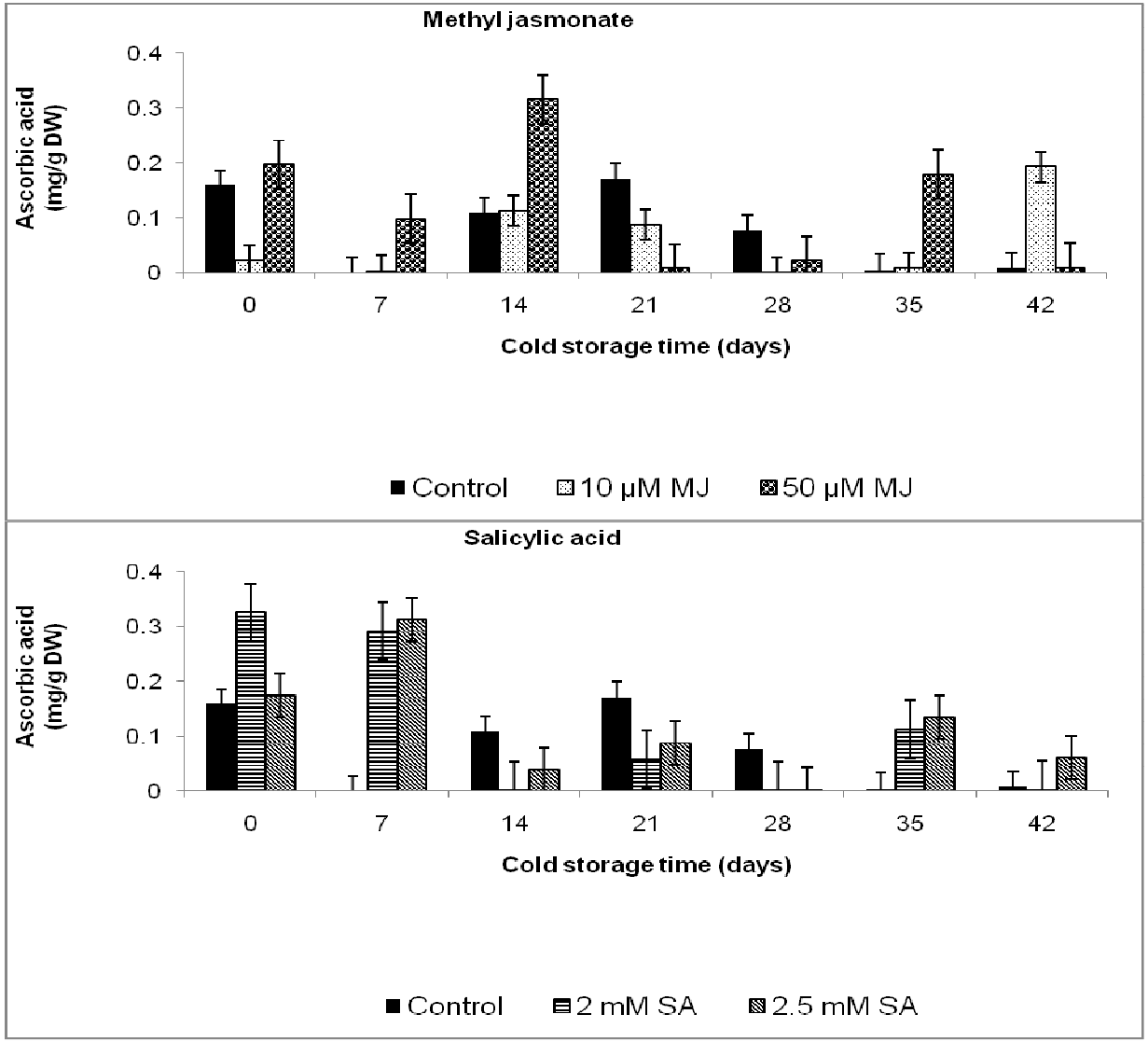

Figure 5.5: Effects of MJ at 10 or 50 µM or SA at 2 or 2 mM on ascorbic acid of fruit during cold storage (-0.5°C) for 42 days and at the end of 7 days shelf life (2008 season). $LSD_{(0.05)} = 0.001606$.

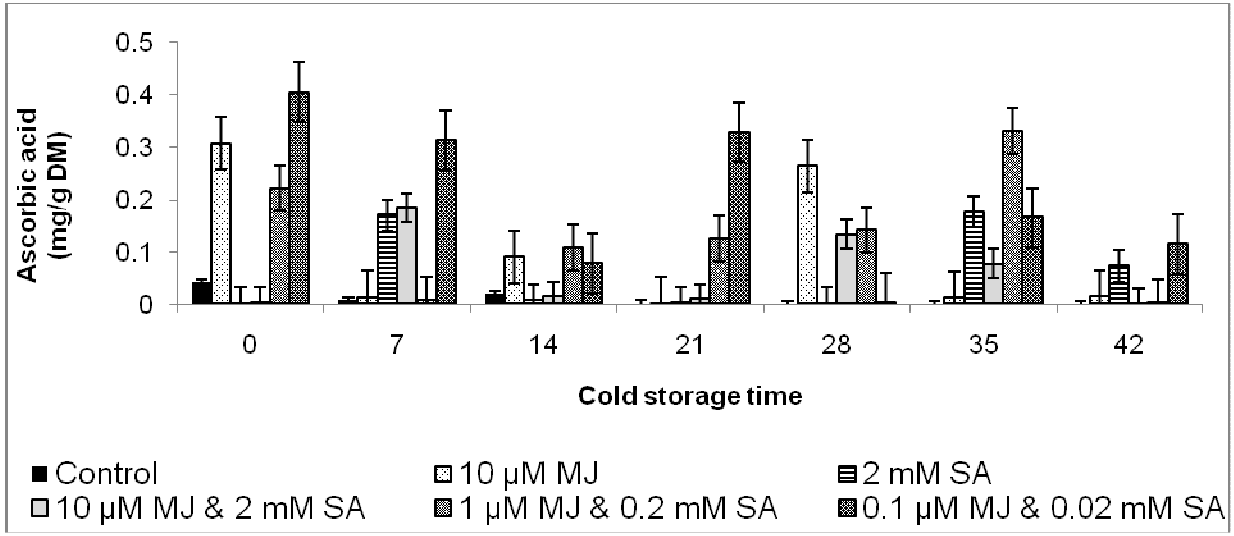

Figure 5.6: Effect of 10 µM MJ, 2 mM SA, 10 µM MJ & 2 mM SA, 1 µM MJ & 0.2 mM SA, or 0.1 µM MJ & 0.02 mM SA on ascorbic acid in lemons when stored at -0.5°C for 42 days before being transferred to room temperature for 7 days (2009 season). $LSD_{(0.05)} = 0.001324$.

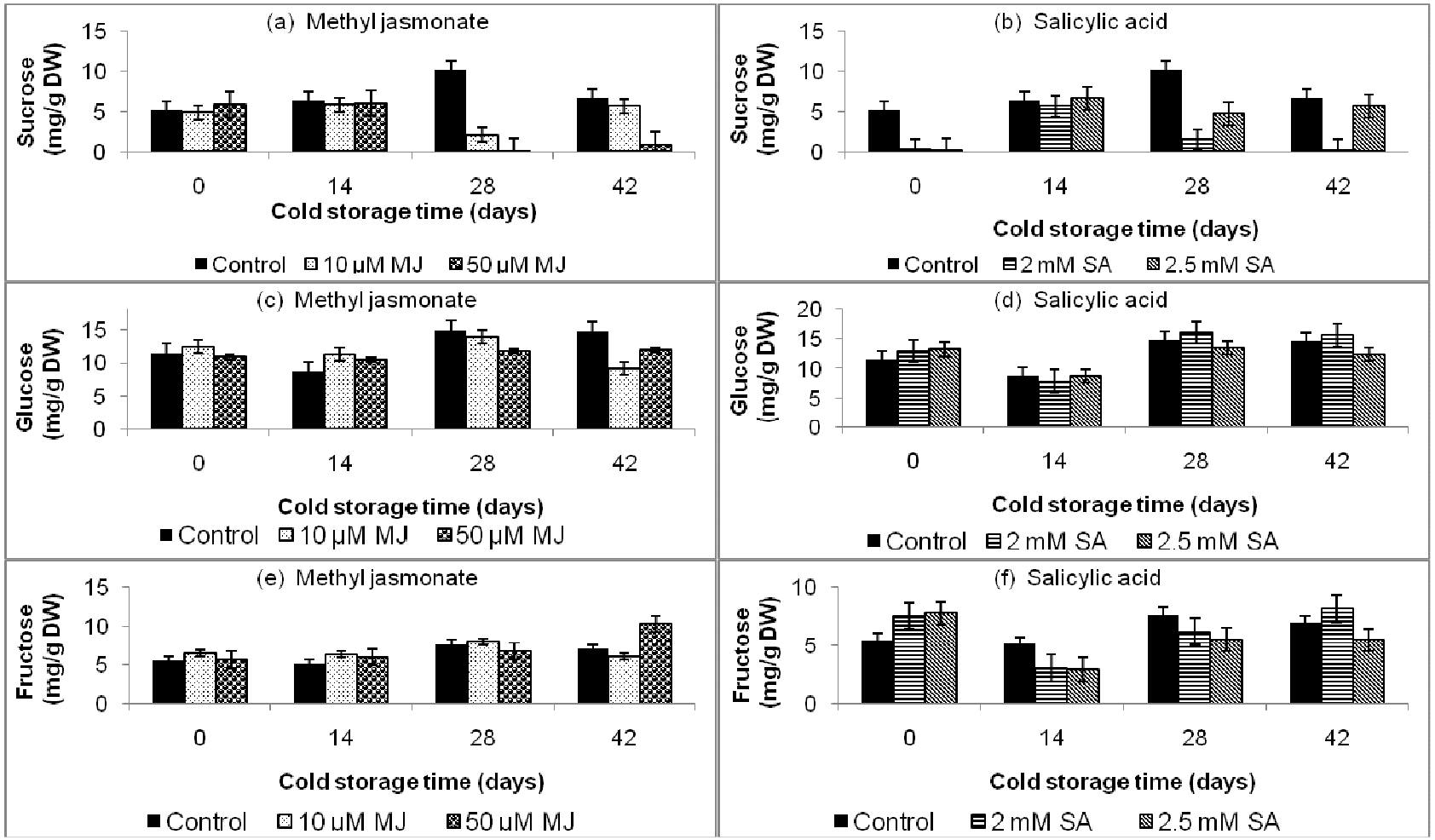

Figure 5.7: Changes in carbohydrates (mg/g DW) in the rind of fruit treated with different concentrations of MJ (10 or 50 µM) or SA (2 or 2.5 mM) and stored at -0.5°C for 42 days before being transferred to room temperature for 7 days (2008 season). LSD $_{(0.05)}$ = 1.861.

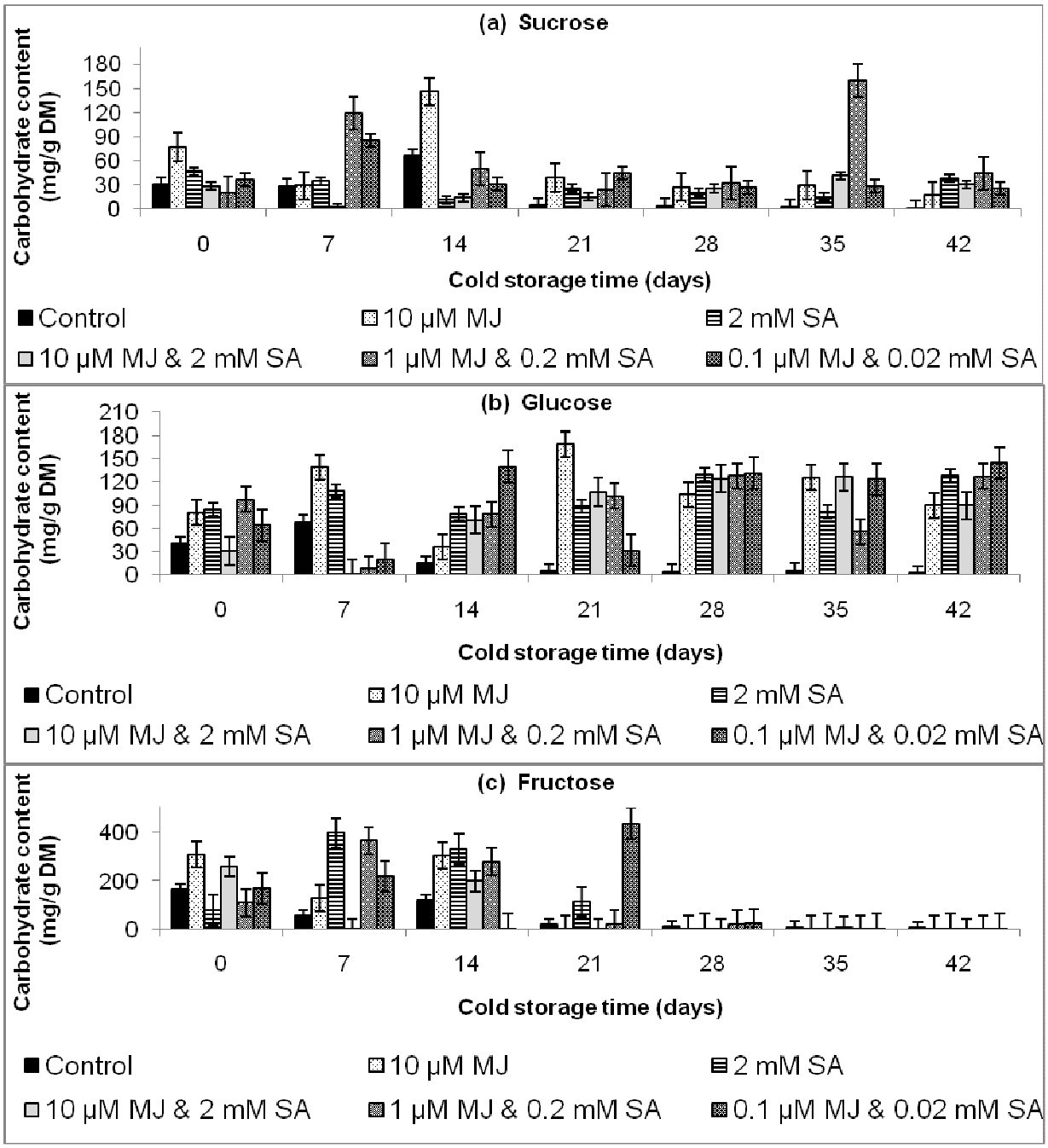

Figure 5.8: Changes in carbohydrates (mg/g DM) in the rind of fruit treated with 10 µM MJ, 2 mM SA, 10 µM MJ & 2 mM SA, 1 µM MJ & 0.2 mM SA, or 0.1 µM MJ & 0.02 mM SA. Fruit were stored at -0.5ºC for 0, 7, 14, 21, 28, 35 and 42 days before being transferred to room temperature for a 7 day storage period (2009 season). $LSD_{(0.05)}$ = 129.55.

TABLE 1: Fruit with CI (%) after were treated with MJ and / or SA, and stored at -0.5°C for 0, 7, 14, 21, 28, 35, or 42 days, before being transferred to room temperature for 7 days.

	Fruit with Chilling Injury (%)	
Treatment	Ukulinga farm (2008 season)	Ithala farm (2009 season)
10 µM MJ & 2 mM SA	*	0.076
2 mM SA	No visible symptoms	0.133
10 µM MJ	No visible symptoms	0.243
1 µM MJ & 0.2 mM SA	*	0.629
0.1 µM MJ & 0.02 mM SA	*	0.905
Control	No visible symptoms	0.936
50 µM MJ	No visible symptoms	*
2.5 mM SA	No visible symptoms	*
LSD (P= 0.05)	**0.00**	**0.1959**

Values are means.

*Treatment was not applied.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

The South African citrus industry is the world second largest exporter of citrus fruit exporting fruit to countries in America, Europe and Asia. However, there are still challenges occurring during fruit exportation that reduce fruit exportation and cause fruit loss at postharvest. This includes physiological disorders, such as chilling injury. Siriphanich (2002) defined chilling injury as the greatest obstacle to the expansion of trade in horticultural crops in the world market. Chilling injury affects fruit quality and reduces fruit marketability due to its symptoms. The physiological disorder occurs during quarantine treatment which involves exposing fruit to chilling temperature (-0.5°C) for a certain period depending on the targeted market (Lafuente *et al*., 2005), thereby complying with quarantine export regulations and controlling pests (Aung *et al*., 1998).

However, chilling-sensitive citrus cultivars, such as lemons, do not tolerate this obligatory treatment and often develop chilling injury (Lafuente *et al*., 2005). Therefore, the aim of this study was to reduce chilling injury in lemons during the quarantine treatment. It was hypothesized that treating lemons with either methyl jasmonate (MJ) and / or salicylic acid (SA) could reduce the development of chilling injury symptoms during prolonged quarantine treatment (42 days), as these compounds have been described to strengthen the resistance of chilling injury. Exposing lemons to chilling temperature (-0.5°C) to fulfil quarantine requirement affected fruit metabolism and caused metabolic dysfunction such as fruit mass loss, abnormal respiration rate, typical indicators of primary events of chilling injury (Figure 7.1). Metabolic dysfunction of the fruit involved an abnormal respiration rate and an increased accumulation of proline in the rind (Chapter 4). These parameters indicate that fruit were probably stressed during cold storage.

Biolatto *et al*. (2005) argue that the primary cause of chilling injury is plant cell membrane damage manifested as increased ethylene production, respiration as well as accumulation of toxic compounds, such as reactive oxygen species (ROS). Chilling stress was evidenced by the high amount of ROS produced in the rind of cold exposed fruit which was evidenced in the intensive damage to cell membrane resulting in lipid peroxidation (Chapter

3). However, postharvest treatment with MJ (10 µM) and / or SA (2 mM) effectively induced antioxidant defence responses in the rind, measured as total antioxidant scavenging capacity, as well as ascorbic acid and total phenolics concentration (Chapter 5). These induced antioxidants defence responses by the postharvest treatment with MJ (10 µM) and / or SA (2 mM) probably caused MJ and SA to effectively slow the respiration rate of the fruit, reduce fruit mass loss, and suppress the accumulation of ROS. Suppressing ROS production possibly resulted in the reduction of membrane lipid peroxidation of the rind.

Treatment with MJ (10 µM) and / or SA (2 mM) effectively increased proline level in the rind during cold storage (Chapter 4). Proline was probably accumulated in the rind as a cold stress response mechanism, since proline has the ability to help fruit withstand of cold stress by scavenging ROS (Saradhi *et al*., 1995; Matysik *et al*., 2002). Moreover, Matysik *et al*. (2002) reported that fruit which are not able to produce proline when exposed to chilling stress, have a significantly lower chilling stress tolerance than those that produce proline. In this study, lemons harvested during the 2008 season did not develop visual symptoms of chilling injury despite the extended cold storage time (42 days). However, the results of this study reveal that fruit were stressed as established in an abnormal respiration rate of the fruit, high rind proline accumulation and rind ROS concentrations (Chapter 3 and 4).

In the fruit used during the 2008 season chilling stress was presumable not effective to lead the damages to the secondary event of chilling injury. The chilling stress was probably in the primary event stage and damages were reversible due to high rind antioxidant capacity of the fruit. During the 2008 season, rind glucose and fructose content were found to be higher than sucrose level during cold storage (Chapter 5). This increase in glucose and fructose levels may be a defence mechanism to resist chilling stress as in grapefruit (Purvis *et al*., 1979). For the fruit not to develop visual symptoms of chilling injury could be some form of harvesting (physiological, biochemical or genetical factors) which must have been present, possibly cultivation practices. It was suggested by Van Rooyen and Bower (2003) that preharvest conditions, such as crop management, also play a role in final fruit quality. On the other hand, during the 2009 harvest season, fruit location was changed and fruit developed chilling injury symptoms, hence environmental conditions made fruit more prone to the development of

chilling injury (Appendix 2). However, treatment with MJ and SA significantly reduced visual symptoms, such as pitting, surface lesion and water-soaking in the flavedo.

This study revealed that the effectiveness of MJ and / or SA as chilling injury reducing compounds depends on their concentration, which may induce defence mechanisms such as antioxidants, proline and as well as carbohydrates in the rind that indirectly provide protection against chilling injury. Treatment with 0.1 µM MJ & 0.02 mM SA delayed chilling injury symptoms by 14 days, on the other hand 1 µM MJ & 0.2 mM SA delayed chilling injury symptoms by 21 days. Treatment with 2 mM SA was more effective than 10 µM MJ in reducing or delaying chilling injury. Treatment with 2 mM SA delayed the symptoms by 35 days; conversely 10 µM MJ delayed the symptoms by only 21 days. Therefore this study reveal that treatment with 10 µM MJ & 2 mM SA is most effective in reducing and delaying chilling injury symptoms by 35 days (Chapter 3).

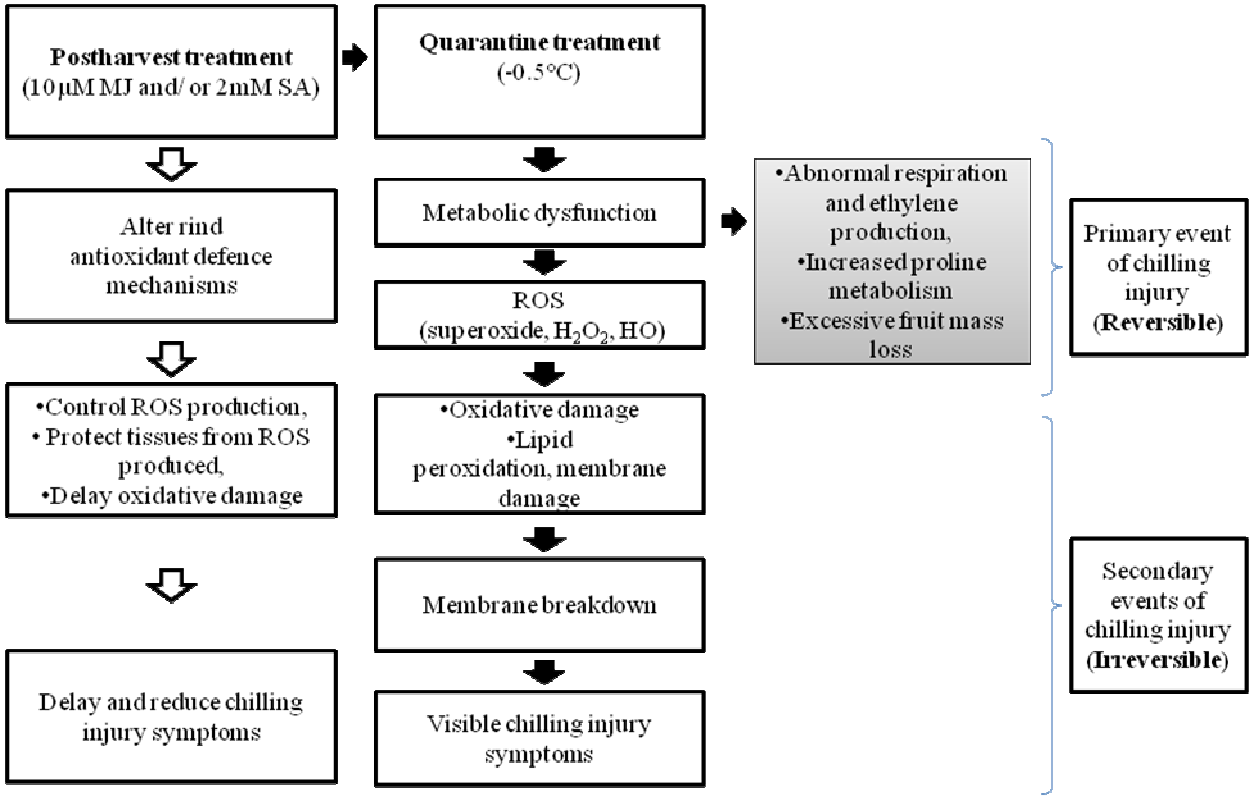

Figure 6.1: Possible mode of action of methyl jasmonate and salicylic acid in decreasing chilling injury in lemons during the present study (Shewfelt and del Rosario, 2000).

CONCLUSIONS

The exposure of lemon fruit to chilling temperature (-0.5°C) for 42 days caused metabolic dysfunction in the fruit indicating the primary event of chilling injury. High accumulation of proline levels in the fruit's rind was probably an indication of chilling stress. In addition, extending the cold storage time caused fruit to produce more ROS which probably resulted in lipid peroxidation followed by membrane damage. These damages caused by ROS possibly could have lead to the development of visual chilling injury symptoms. However, no visual chilling injury symptoms were detected in fruit harvested from Ukulinga farm during the 2008 season. Treatments with MJ (10, 50 µM) or SA (2, 2.5 mM) were found to alter rind antioxidant compounds and this triggered defence mechanisms possibly to increase chilling tolerance of the fruit. However, medium concentrations of either MJ (10 µM) or SA (2 mM) were more effective in inducing defence compounds such as ascorbic acid and phenolics as well as the carbohydrate content and chilling injury response products such as proline, unlike higher concentrations (50 µM or 2.5 mM). It was therefore concluded that the antioxidant compounds induced by MJ (10 µM) or SA (2 mM) were possible compounds used by the fruit as a defence mechanism to prevent chilling injury during an extended cold storage period. On the other hand, during the 2009 harvest season, fruit did developed visual chilling injury symptoms. The symptoms were increasing with cold storage time in untreated fruit. However, this study revealed that MJ (10 µM) or SA (2 mM) can be used to reduce chilling injury symptoms in lemon fruit. Moreover, treatment with 10 µM MJ & 2 mM SA was more effective in reducing and delaying chilling injury in lemons probably by maintaining defensive mechanisms or antioxidants compounds such as phenolics, ascorbic acid, carbohydrate metabolism and proline.

FINAL RECOMMENDATIONS

This study suggests that dipping lemons in either MJ and / or SA could become an alternative treatment to reduce chilling injury symptoms during cold storage quarantine treatment of lemons. In addition, treatment with MJ (10 µM) and /or SA (2 mM) effectively reduced and delayed chilling injury symptoms in the fruit. Moreover, MJ (10 µM) and / or SA (2 mM) treatment was found to alter rind antioxidant compounds such as phenolics, ascorbic acid as well as carbohydrate concentration and the previously reported chilling response, proline, which perhaps could have increased chilling tolerance in the fruit. This treatment was also effective in suppressing ROS and delaying lipid peroxidation.

FUTURE DIRECTIONS

This study revealed that MJ and SA have the potential to reduce and delay symptoms of chilling injury in lemons. This lead to the suggestion that both, MJ and SA dips should be further tested as treatments to enhance cold storage tolerance in lemons. Currently, many studies focus on the alleviation of chilling injury by reducing or delaying symptoms of chilling injury. Therefore, future studies should focus more on preventing the injury itself or preventing the primary event of chilling injury. This could probably reduce the chances of secondary events to take place. Perhaps the occurrence of secondary events could be reduced by using postharvest treatments that trigger the expression of genes resulting in an increase in protective compounds such as the rind antioxidant, phenolics, ascorbic acid and other precursors should follow. Once these cascades of events have been elucidated, strategies to increase the production of these protective compounds can be devised to maintain the quality of cold stored lemon fruit.

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APPENDICES

Appendix 1: Fruit harvested during the 2008 harvest season at Ukulinga farm.

Figure 1: Lemon fruit treated with either MJ (10, 50 µM) or SA (2, 2.5 mM), stored for 35 days at -0.5°C and transferred to room temperature for 7 days

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Appendix 2: Fruit harvested during the 2009 harvest season at Ithala farm.

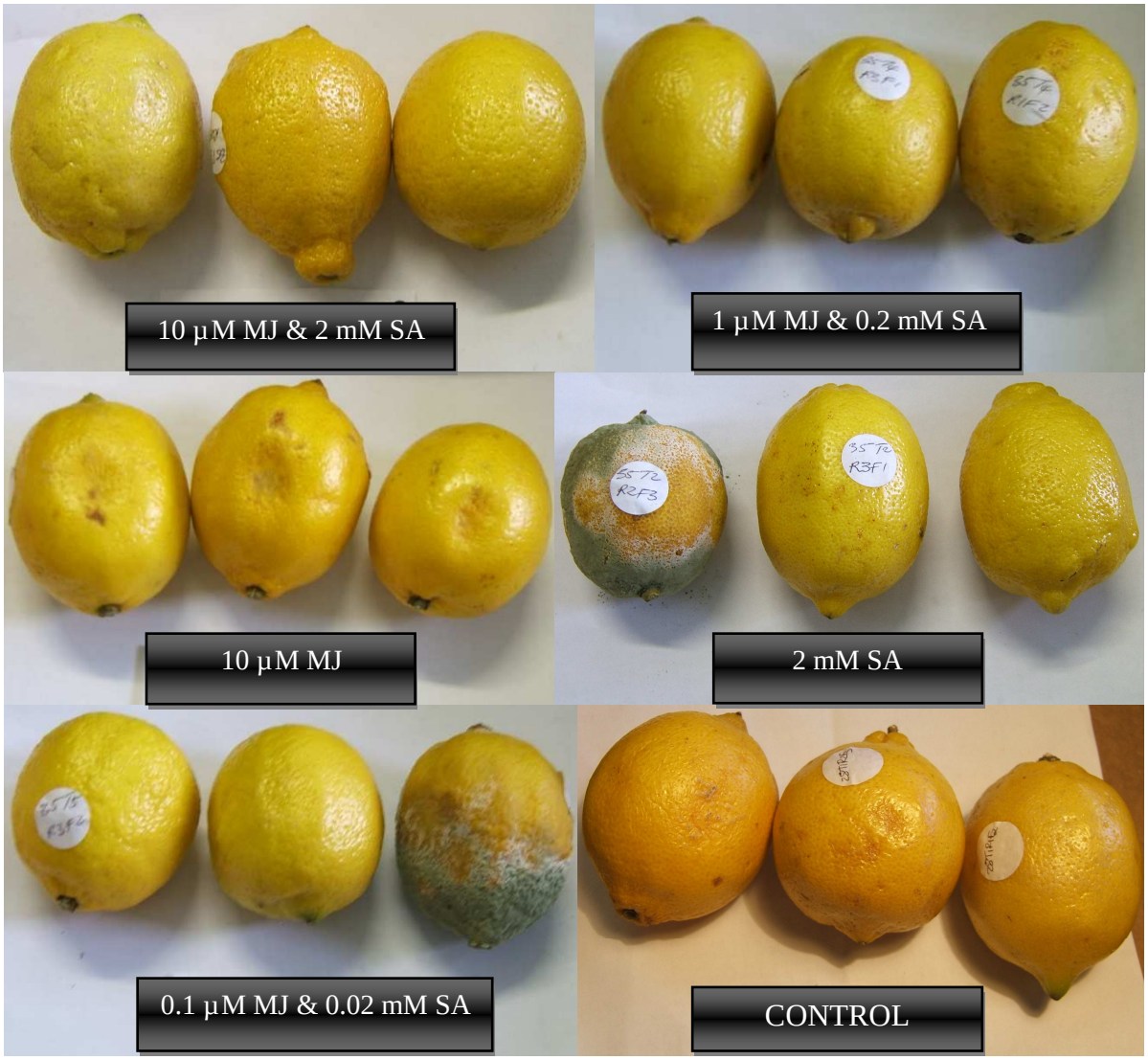

Figure 2: Lemon fruit treated with either methyl jasmonate (MJ) and /or salicylic acid (SA), stored for 35 days at -0.5°C and transferred to room temperature for 7 days.

Appendix 3:Chilling injury symptoms during the 2009 harvest season.

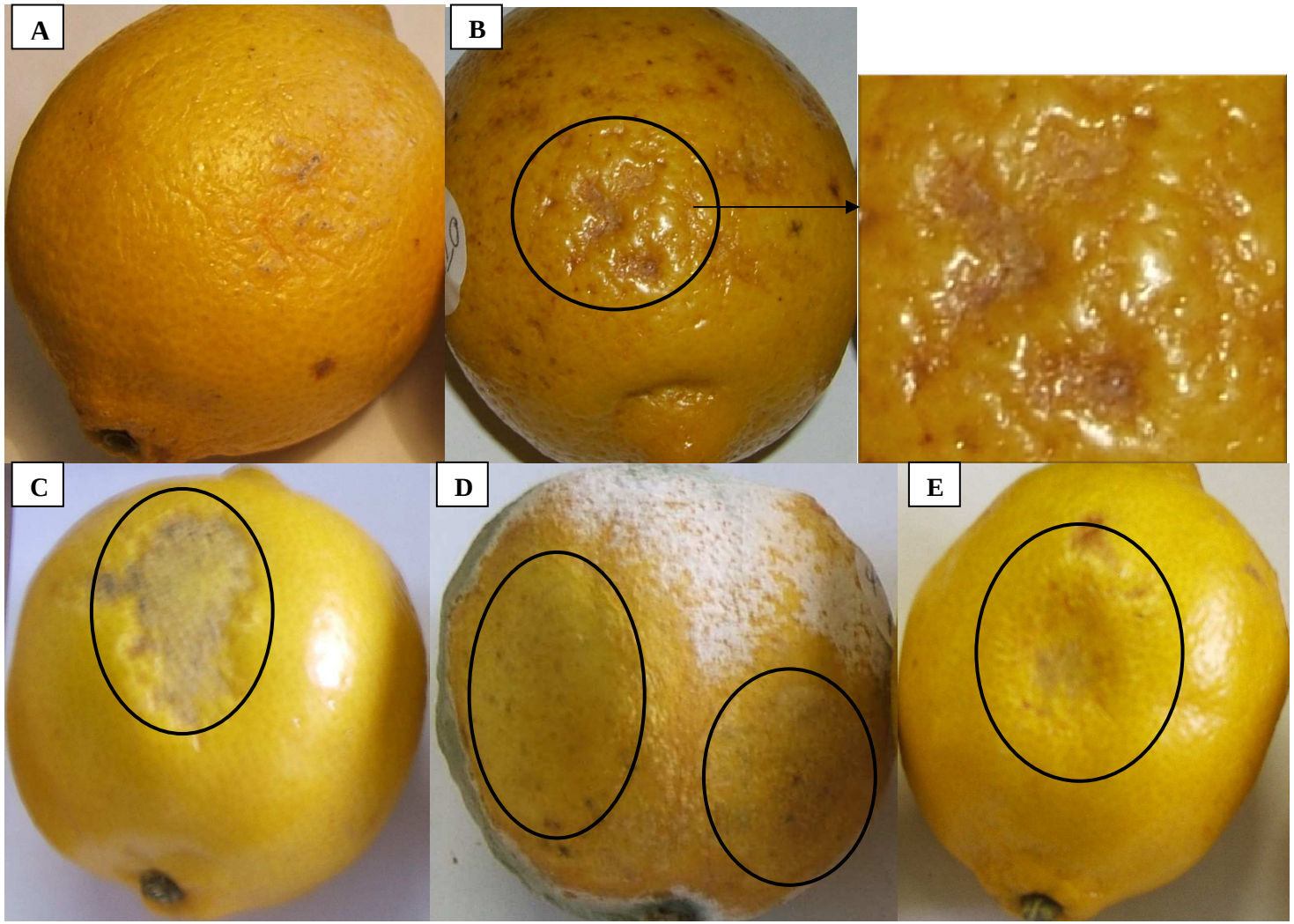

Figure 3.5: Chilling symptoms were first visible as excess mass loss (A), surface pitting (B or C), or water-soaking (D), which developed rapidly thereafter in untreated fruit leading to penicillium development, and collapsed cells (E).

Appendix 4: Pending Acta Horticulturae publications

Siboza, X.I., Bower, J.P. and Bertling, I. 2009. Effect of Methyl Jasmonate and Salicylic Acid on Chilling Injury of 'Eureka' Lemons. Acta Horticulturae. All Africa Horticulture Congress. Nairobi, Kenya.

Siboza, X.I., Bower, J.P. and Bertling, I. 2010. Effect of Methyl Jasmonate and Salicylic Acid on Chilling Injury of 'Eureka' Lemons. Acta Horticulturae. 28th International Horticultural Congress. Lisboa, Portugal.