

**UNIVERSITY OF KWAZULU-NATAL**

**METHYL JASMONATE AND SALICYLIC ACID ENHANCE  
CHILLING TOLERANCE IN LEMON (*Citrus limon*) FRUIT**

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## Abstract

South African 'Eureka' lemon fruit must be exposed to chilling temperatures ( $\pm 0.6^{\circ}\text{C}$ ) as a mandatory quarantine treatment against insect pests for all its overseas markets. Chilling lemon fruit at such temperatures may develop chilling injury (CI) symptoms on the flavedo. This negative effect on fruit quality reduces fruit marketability. This study evaluated postharvest factors influencing physiological, biochemical and ultra-structural mechanisms involved in alleviating CI in lemon fruit. It was hypothesised that treatment with methyl jasmonate (MJ) and salicylic acid (SA) may enhance chilling tolerance in lemon fruit by maintaining cellular integrity and inducing synthesis of enzymatic and non-enzymatic antioxidants. Furthermore, fruit susceptibility to CI was associated with the source of fruit. Lemon fruit were harvested from three locations representative of moderate subtropical, warm temperate and cool subtropical environments. Harvested fruit were treated either with 10  $\mu\text{M}$  MJ, 2 mM SA or 10  $\mu\text{M}$  MJ plus 2 mM SA, stored either at  $-0.5$ , 2 or  $4.5^{\circ}\text{C}$  for 0, 7, 14, 21, or 28 days and afterwards transferred to  $23^{\circ}\text{C}$  for a week as shelf-life simulation. Thereafter, fruit were evaluated for alterations in physiological, biochemical and ultra-structural features involved in the manifestation of CI symptoms.

Chilling damage was more severe in untreated lemon fruit than in treated lemon fruit. Storing lemon fruit at  $4.5^{\circ}\text{C}$  accelerated the manifestation of CI symptoms more so than at  $2^{\circ}\text{C}$  while storage at  $-0.5^{\circ}\text{C}$  delayed the manifestation of CI symptoms. Lemon fruit of moderate subtropical origin were more chilling-tolerant than lemon fruit of warm temperate and cool subtropical origin. Treatment with 10  $\mu\text{M}$  MJ plus 2 mM SA significantly ( $P < 0.05$ ) improved chilling tolerance in lemon fruit. This treatment effectively maintained membrane integrity, thereby retarding electrolyte leakage and membrane lipid peroxidation as well as mass loss and respiration rate. Treatment with 10  $\mu\text{M}$  MJ plus 2 mM SA was also effective in enhancing the antioxidant concentrations of

vitamin E and carotenoids. The production of these antioxidants could have been part of a defence system against chilling damage, reducing CI and maintaining fruit quality.

Treatment with 10  $\mu$ M MJ plus 2 mM SA enhanced the concentration of compounds involved in chilling resistance, such as proline, soluble sugars, ascorbic acid and total phenolics as well as the enzyme phenylalanine ammonia-lyase (PAL). The enhancement of the defence mechanisms may have played a role in enhancing chilling tolerance in lemon fruit. The treatment also inhibited certain enzymes involved in tissue browning, such as peroxidase (POD) which might have contributed to delaying manifestation of symptoms. Polyphenol oxidase (PPO) was found to not be a good biochemical marker of the occurrence of CI. Treatment with 10  $\mu$ M MJ plus 2 mM SA appeared to be able to enhance chilling tolerance in lemon fruit by maintaining the ultra-structure of the cuticle, cell wall integrity, cell membrane of parenchyma cells of the flavedo. This treatment also preserved the mineral nutrients of the flavedo (carbon, oxygen, phosphorus, potassium, calcium, magnesium, sulphur, sodium, silicon and aluminium) during cold storage. This could have played a role in protecting the fruit against chilling stress and maintaining fruit quality.

Treatment with 10  $\mu$ M MJ plus 2 mM SA reduced ROS production, while the activity of enzymatic antioxidants such as catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR), and accumulation of essential proteins was enhanced. This increase in activity of enzymatic antioxidants and the presence of stress-responsive proteins in the lemon flavedo could have been directly involved in enhancing chilling tolerance. The CI symptoms were accompanied by an increase in membrane permeability, membrane lipid peroxidation as well as phospholipase D (PLD) and lipoxygenase (LOX) activity; however, treatment with 10  $\mu$ M MJ plus 2 mM SA effectively reduced the membrane permeability, membrane lipid peroxidation, and PLD and LOX activity induced by the cold treatment. This could have contributed to the efficacy of 10  $\mu$ M MJ plus 2 mM SA in inhibiting the manifestation of CI symptoms.

Treatment with 10  $\mu$ M MJ plus 2 mM SA enhanced flavedo total antioxidant capacity measured by ferric reducing ability of plasma; 2,2-diphenyl-1-picrylhydrazyl; 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) and the oxygen radical absorption capacity assays. The enhancement of antioxidant capacity in lemon flavedo could have contributed to the fruit's chilling tolerance. Therefore, the effect of 10  $\mu$ M MJ plus 2 mM SA treatment, enhancing chilling tolerance, may be attributed to its ability to enhance enzymatic and non-enzymatic antioxidants; activate essential proteins and mitigate the effect of ROS accumulation. With the use of 10  $\mu$ M MJ plus 2 mM SA treatments, the South African citrus industry will be able to meet the quarantine temperature requirements for exportation of lemon fruit whilst reducing economic losses, depending on the preharvest conditions experienced by the fruit in each shipment.

## Declaration

I, Xolani Irvin Sibozza, declare that:

1. The work reported in this thesis, except where otherwise indicated, is my original work.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This thesis does not contain other persons' writing, unless specifically acknowledge as being sourced from other persons. Where other written sources have been quoted then:
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Above all, I praise and worship God the Almighty, for his love, grace, and for granting me this wonderful opportunity.

## **Dedication**

This thesis is dedicated to the loving memory of my father Envoy, my mother Margaret, my grandfather Luckson and my grandmother Masevase, who regretfully did not live to see this work.

This thesis is also dedicated to my aunt, Leayah P. Lubisi, for being there for me through thick and thin. Her emphasis on education and encouragement significantly contributed to the fulfilment of my dreams. She dedicated her life to nurturing a no-body like me, so that one day I could become somebody. Surely, without her unconditional support, my life goals could not have been successfully achieved.

## A Lesson from a Mad Hatter

*“Alice: Where I come from, people study what they are not good at in order to be able to do what they are good at.*

*Mad Hatter: We only go around in circles in Wonderland, but we always end up where we started. Would you mind explaining yourself?*

*Alice: Well, grown-ups tell us to find out what we did wrong, and never do it again.*

*Mad Hatter: That's odd! It seems to me that in order to find out about something, you have to study it. And when you study it, you should become better at it. Why should you want to become better at something and then never do it again? But please continue.*

*Alice: Nobody ever tells us to study the right things we do. We're only supposed to learn from the wrong things. But we are permitted to study the right things other people do. And sometimes we're even told to copy them.*

*Mad Hatter: That's cheating!*

*Alice: You're quite right, Mr. Hatter. I do live in a topsy-turvy world. It seems like I have to do something wrong first, in order to learn from what not to do. And then, by not doing what I'm not supposed to do, perhaps I'll be right. But I'd rather be right the first time, wouldn't you?”*

*Meir Liraz*

*Alice and the Mad Hatter in Wonderland*

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## Prologue

Temperature is one of the major factors pivotal in postharvest storage of fruit (Ma et al., 1990). In lemon fruit (*Citrus limon Burm. f.*), cold storage is commonly used to prolong the postharvest life by reducing fruit respiration rate, mass loss and general decay (Maul et al., 2011). Citrus fruit, like other crops originating from the tropical and subtropical regions are chilling sensitive to low temperature during storage (Wang and Wallace, 2004). Storage temperatures can be regarded either as chilling, ranging from below 0 to 15°C (Lyons, 1973), or as freezing, ranging between -1.1 to -0.6°C (Wang and Wallace, 2004). Recently, chilling temperatures above freezing have been used for the disinfestations of fruit possibly carrying eggs or larvae of the Mediterranean fruit fly (Medfly, *Ceratitis capitata*), a pest resident in South Africa and hindering fruit exportation.

Lemon fruit from South Africa are shipped to distant markets such as Korea, Thailand, China, Russia, Europe, the United Kingdom and United States of America under cold temperature  $\pm 0.6^{\circ}\text{C}$  as a cold sterilisation and obligatory quarantine treatment imposed for all citrus fruit exporting countries. Fruit must be cold sterilised at  $\pm 0.6^{\circ}\text{C}$  for 24 successive days when shipped to China, Korea, Thailand and the United States of America. The consignment must be cold sterilised for 12 days prior to arrival in Japan; however, lemon fruit like other citrus fruit are chilling-sensitive and prone to the physiological disorder chilling injury (CI). Chilling injury symptoms mainly manifest when fruit are returned from cold storage to warm temperatures (Lafuente et al., 2005). According to Lafuente et al. (2005), CI is caused by exposure of fruit to low, non-freezing temperatures causing red blotch, pitting, surface lesion, staining, water-soaking, superficial scald and necrosis on the fruit's exocarp, the coloured flavedo, and eventually results in cell death (Wang et al., 2001; Sanchez-Ballesta et al., 2003) and finally necrotic spots.

The disorder not only affects fruit quality but also reduces marketability and limits long-term storage (Porat et al., 2004). Currently, there are no reliable commercial methods to alleviate CI of lemon fruit (Porat et al., 2004). During exposure to chilling conditions, an array of mechanisms enables the fruit to minimise the negative effects of chilling stress (Ruelland et al., 2009). These stress coping mechanisms involve the production of antioxidants to scavenge reactive oxygen species (ROS), and control or inhibit their production, which may be triggered by the stress, as well as the activation of cold responsive genes (Knight and Knight, 2001; Sanchez-Ballesta et al., 2006).

In citrus, the physiological and molecular mechanisms that enable fruit to tolerate CI have not been fully elucidated, possibly as these may vary with season (Lafuente et al., 1997; Holland et al., 1999; Sanchez-Ballesta et al., 2000). Fruit contain an abundance of antioxidants which are capable of acting as free radical scavengers, ROS quenchers as well as enzyme synergists and inhibitors (Chanjirakul et al., 2006). Activation of enzymatic antioxidant systems, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR); and other enzymes such as phenylalanine ammonia-lyase (PAL) and heat shock proteins (HSP), may increase chilling tolerance in fruit during cold storage (Sanchez-Ballesta et al., 2000; Lafuente et al., 2004). Antioxidant defence systems protect fruit against CI by scavenging harmful radicals and controlling ROS production in fruit (Sala and Lafuente, 1999; Lafuente et al., 2004).

The plant hormones methyl jasmonate (MJ) and salicylic acid (SA) play major roles in maintaining fruit postharvest. Both MJ and SA are signalling molecules involved in the signal transduction systems of plants. These hormonal substances induce the production of defences compounds, such as enzymatic and non-enzymatic antioxidants mainly under stress conditions (Ali et al., 2007). Methyl jasmonate application was found to significantly reduce CI symptoms in zucchini (Wang and Buta, 1994), mango (González-Aguilar et al., 2000) and suppressed fungal growth in grapefruit (Droby et al., 1999). Furthermore, MJ

inhibited microbial contamination of peppers during postharvest (Buta and Moline, 1998) and reduced postharvest decay while maintaining the internal and external quality of papayas (González-Aguilar et al., 2003) and raspberries (Chanjirakul et al., 2006). Previous studies have reported that postharvest treatments with SA have the potential to reduce CI and maintain fruit quality in peaches (Wang et al., 2006; Cao et al., 2010; Yang et al., 2012), pomegranate (Sayyari et al., 2009), plum (Luo et al., 2011) and lemon (Siboza et al., 2011) fruit.

The reduction of CI by SA was associated with the enhanced concentration of enzymatic and non-enzymatic antioxidants in the peach fruit (Yang et al., 2012). However, the mode of action of combined physiological effect of MJ and SA to reduce CI in lemon fruit remains unknown. Furthermore, the genomics, proteomics, and metabolomics responsible for moderating cold acclimation remain to be identified (Ruelland et al., 2009).

### **Hypothesis**

This study focuses on the evaluation of pre- and postharvest factors which may be involved in influencing the physiological, biochemical and ultra-structural mechanisms that alleviate CI in lemon fruit. It is hypothesized that postharvest treatments with certain MJ or SA concentrations or a combination of the two compounds prior to cold storage can enhance chilling tolerance and thereby maintaining fruit quality of 'Eureka' lemon fruit. Furthermore, it is hypothesized that the sensitivity of lemon fruit to chilling injury is affected by farm location (environmental conditions), storage temperature, and storage duration.

## **Research objectives**

The specific objectives of this study were:

1. To investigate the influence of lemon preharvest and postharvest factors on susceptibility to CI during cold storage.
2. To determine the effect of MJ and SA treatment on fruit quality aspects, such as membrane integrity, fruit mass and respiration rate and metabolic status (vitamin E and carotenoids).
3. To investigate the involvement of soluble sugars, proline and ascorbic acid in chilling tolerance of lemon fruit.
4. To determine the impact of MJ and SA application on membrane phenolics and PAL activity as well as on the activity of enzymes oxidising phenolic compounds, such as polyphenol oxidase (PPO) and peroxidase (POD). In addition, to investigate the efficacy of MJ and SA treatment in: (1) Inducing total phenolic compounds and PAL activity in lemon fruit, as defence mechanisms against CI; (2) Inhibiting PPO and POD activity in lemon fruit, as these enzymes are involved in phenolic oxidation, enzymatic browning and fruit deterioration.
5. To investigate ultrastructural alterations in responses of MJ- and SA-treated lemon fruit under chilling conditions.
6. To investigate if the activity of enzymatic antioxidants and accumulation of heat-shock proteins (HSP) as potential molecular markers involved in chilling tolerance can either be influenced by farm locations or by subjecting fruit to certain storage temperatures.
7. To investigate if phospholipase D (PLD) and lipoxygenase (LOX) activities are affected by chilling; and if so, how these activities are altered by MJ and SA treatment.

8. To investigate the efficacy of MJ and SA treatments in enhancing chilling tolerance by triggering antioxidant activities in lemon fruit measured by ferric reducing ability of plasma (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and the oxygen radical absorption capacity (ORAC) assays.
9. To examine the effects of MJ and SA as postharvest treatments to enhance proteins as a mode of action to increase chilling tolerance in lemon fruit during cold storage.

### **Practical implication of the study**

This study evaluates the use of MJ and SA as postharvest treatments to enhance chilling tolerance of lemon fruit. If chilling tolerance can be enhanced, prolonging storage of lemon fruit to allow shipment to distant markets is made possible. Furthermore, the results will be beneficial to citrus growers, researchers, shippers, distributors and the citrus industry in meeting consumer's demands for high fruit quality.

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# Chapter 1

## Review: chilling injury in lemon fruit

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## 1. INTRODUCTION

Citrus fruit belong to the *Citrus* L. genus of the *Rutaceae* family and are botanically defined as *hesperidium*, a particular kind of berry with a leathery rind. The fruit is internally divided into segments (Soule and Grierson, 1986). Citrus ranks first among the commonly produced fruit crops, such as banana, grapes, and apples with respect to global production (Murata, 1997; Ladaniya, 2008). The fruit is known for its health benefits and its consumption is beneficial to human health by providing nutrients, reducing incidence of cardiovascular diseases and improving blood circulation (Ladaniya, 2008). They are a major source of vitamin C, carotenoids, flavanones, limonoids, thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxine, folic acid, biotin, and inositol pectine in the human diet (Ladaniya, 2008). Different citrus fruit types commercially produced and exported to many countries around the world include grapefruit (*Citrus paradise*), orange (*Citrus sinensis*), pomelo (*Citrus maxima*), mandarin (*Citrus reticulata*), tangerine (*Citrus tangerina*), satsuma (*Citrus unshiu*), lemon (*Citrus limon*) and lime (*Citrus aurantifolia*) (Murata, 1997).

### 1.1. The characteristics of 'Eureka' lemons

Lemons (*Citrus limon* (L.) Burm.f.) are economically important citrus fruit grown in many parts of the world, including South Africa, Australia, Israel and other Mediterranean countries (Ladaniya, 2008). The characteristics of 'Eureka' include a yellow peel at maturity (Plate 1) and a fruit with varying peel thickness, surface texture and oil glands (Young, 1986). The cultivar is ovate to round with rounded apical, and is well-known for its high juice, citric and ascorbic acid content, and is deemed either commercially seedless or with 4-10 seeds per fruit (Ladaniya, 2008).

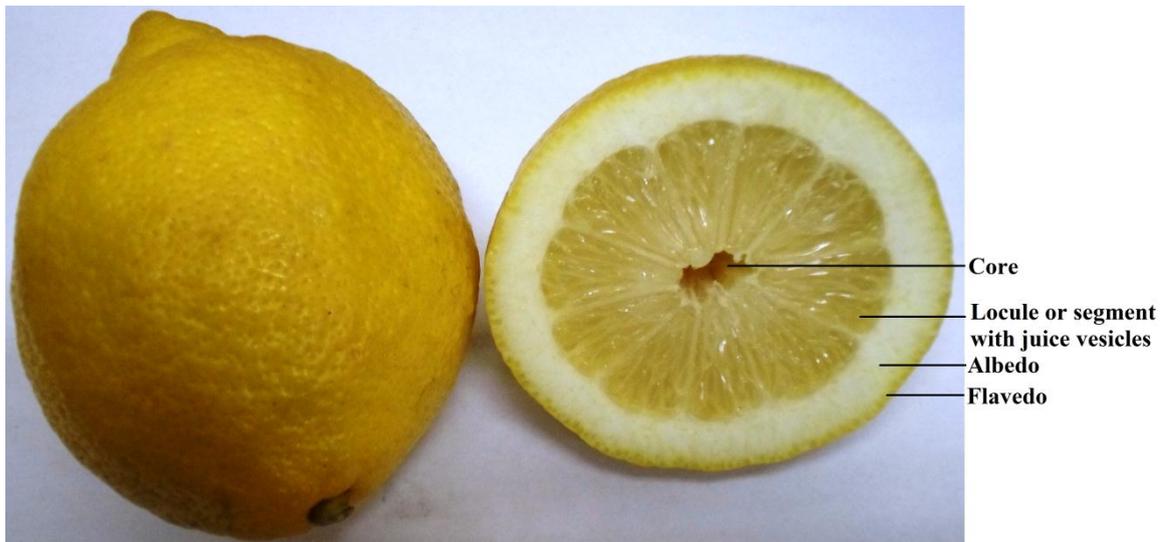


Plate 1: Lemon fruit with transverse section showing the core, locule or segment, albedo and flavedo.

### **1.2. Background to citrus industry: production and exportation**

The South African citrus industry is a well-recognised citrus producer and ranks number 13 in terms of global fresh citrus production, marketing 70% of its produce on the world market. South Africa is the third largest exporter of citrus fruit after Spain and Turkey. Fruit are exported to different destinations in the world (CGA, 2012). The major export destinations include Northern Europe (25%), Middle East (19%), Far East (13%), Russia (13%), United Kingdom (10%), the Mediterranean (6%), Asia (3%), United States (3%), Canada (3%) and other (5%) (CGA, 2012).

The industry has gained market access to export lemons to high paying countries in the world. However, lemon exports are only 12% compared with 'Valencia' orange which accounts for 44% of the total exports. Currently, the industry faces many postharvest challenges such as rind damage, physiological disorders and pathogen attack. These challenges result in postharvest economic losses. The South African citrus industry was once ranked second world largest exporter from 2000 to 2011, until a drop by one level in 2012, which affected the industry's world position in exports. Therefore, understanding the

possible factors that led to this decline in market share may generate knowledge that may contribute to the development of new postharvest management practises which may reduce losses, improve the standard of the South African citrus industry and increase exports.

### **1.3. Postharvest handling of lemons**

#### *1.3.1 Cold storage*

Generally, fruit quality (appearance, mineral nutrients, taste and shelf-life) of fresh lemons gradually decreases during postharvest handling and storage. Postharvest management practices should therefore aim at retaining and maintaining fruit quality during storage. Postharvest losses of citrus fruit have been reported to range between 5 to 10% for most developed countries and 25 to 30% for developing countries (Ladaniya, 2008). This is possibly due to limited postharvest facilities such as cold-chain systems (Ladaniya, 2008).

#### *1.3.2 Problems of cold storage*

Cold storage is widely used to maintain and prolong fruit quality, delay senescence and extend shelf-life of lemons (Meng et al., 2009; Zhu et al., 2011). Cold storage as a postharvest technology is beneficial for the preservation of fruit quality after harvest and for slowing the rate of cell metabolism (Sevillano et al., 2009). Cold storage is vital for preserving mineral nutrients in the storage of fruit such as lemons (Lafuente et al., 2005). However, cold storage also has negative effects on fruit quality during extend storage periods. Extended cold storage may result in chilling induced physiological disorders such as chilling injury (CI) especially to tropical and subtropical crops. Chilling causes severe damage and induces rigidification of membranes, leading to loss of membrane integrity and membrane-bound enzyme activities (Kosova et al., 2007; Lattanzio et al., 2012).

### 1.3.3 Chilling injury

Chilling injury (CI) is a physiological disorder that manifests when subtropical and tropical fruit are stored at temperatures ranging from 0-8°C and above 12°C for vegetables (Sevillano et al., 2009). Lemons are very sensitive to CI, and the recommended storage temperature is 9°C and above (Lafuente et al., 2005). Susceptibility to CI may be influenced by cultivar, origin, environmental conditions during growth, seasonal changes and fruit maturity (Lafuente et al., 1997; González-Aguilar et al., 2000b). Despite documented evidence of lemon susceptibility to CI, it is a requirement that lemon fruit must be exposed to sub-zero temperatures (-0.5°C) during exportation as an obligatory quarantine treatment for the disinfection of Mediterranean fruit fly (*Ceratitidis capitata*). This requirement poses a major challenge to postharvest handling, storage and shelf-life of lemon fruit which are inclined to develop CI (Lafuente et al., 2005). Therefore, physiological and molecular mechanisms, implicated in the activation and manifestation of CI in lemon fruit are reviewed below.

## 2. THE PHYSIOLOGICAL BASIS OF CHILLING INJURY (CI)

### 2.1. Primary response to CI: The first physiological response to cold storage

A range of hypotheses formulated by various authors have been proposed to explain the physiological bases of plant/fruit response to chilling and the development of CI symptoms. Cell membranes are thought to play an important role in response to chilling. Lyons (1973) hypothesised that lipid-phase transition of the cell membrane occurs, suggesting that lipids are majorly involved in the development of CI. According to this hypothesis, chilling affects cell membranes by decreasing membrane fluidity governed by the degree of fatty acid unsaturation in membrane lipids.

Enhancing the Lyons' model, Raison and Orr (1990) suggested that CI can be divided into primary (reversible damage) and secondary (irreversible damage) events. In this hypothesis, primary events of chilling may include changes in membrane permeability, alterations of membrane lipids and in the kinetics of regulatory enzymes, changes in the cytoskeletal structure and an increase in respiration rate (Raison and Orr, 1990; Sevillano et al., 2009). Shewfelt and del Rosario (2000) later offered a different perspective from that of Raison and Orr (1990) which suggested that chilling induces reactive oxygen species (ROS) accumulation (Mittler, 2002; Sharma et al., 2012); these species attack and further damage membranes finally causing CI (Siriphanich, 2002).

Subtropical crops, such as lemons, continuously produce ROS either as by-products of aerobic metabolism or when exposed to chilling (Li et al., 2008b). Under normal conditions, there is a balance between ROS production and antioxidants able to scavenge ROS (Pitzschke et al., 2006). This balance ensures that ROS are either removed or detoxified from the cell membrane and that there is no damage to membranes and organelles. Therefore, an imbalance between ROS production and antioxidant concentration in a fruit may cause damage to membranes leading to the development of CI symptoms. However, ROS are not only produced as a toxic by-product but also play a role in plant response to stress such as chilling (Karuppanapandian et al., 2011). These responses to stress include gene activation, hormone signalling and stomatal regulation (Bolouri-Moghaddam et al., 2010; Sharma et al., 2012).

Studies have shown that at low moderate concentrations, ROS can act as secondary messengers influencing expression of various genes as well as signal transduction pathways including those leading to chilling tolerance (Pitzschke et al., 2006; Karuppanapandian et al., 2011; Sharma et al., 2012). This suggests that cells have mechanisms to employ ROS as stress signals (Pitzschke et al., 2006); possibly functioning as signalling molecules in stress responses (Rivera et al., 2007).

Enhanced ROS production can either act positively to signal or induce protective mechanisms, or negatively, to accelerate cellular damage (Dat et al., 2000; Kim et al., 2009). Sharma et al. (2012) argued that whether ROS will act as damaging or signalling molecules depends on the equilibrium between ROS production and scavenging antioxidants. Spatial and temporal fluctuations of ROS in cells can act as signals required for acquiring tolerance to chilling stress (Apel and Hirt, 2004). Accumulation of ROS in fruit during chilling exposure appears to have a strong influence on the cold regulation of gene expression by cold and, thereby, increases chilling tolerance of fruit (Yadav, 2010). Therefore, a need arises to search for postharvest treatments that enhance the antioxidant scavenging capacity of fruit and stabilise their enzymatic systems during cold storage.

## **2.2.Secondary Response to CI: Oxidative damage**

Exposure of fruit to chilling temperature results in excessive generation of ROS in the cell compartments (Maruthasalam et al., 2010). The enhanced ROS concentration poses a threat to cells by causing lipid peroxidation, protein oxidation, enzyme inhibition and damage to nucleic acids resulting in cell death (apoptosis) (Mittler, 2002; Sharma and Dubey, 2005; Yang et al., 2010; Sharma et al., 2012). This process is known as oxidative damage. Studies by Mittler et al. (2004) and Karuppanapandian et al. (2011) emphasise that ROS can cause irreversible membrane damage leading to cell death and CI symptoms. However, the extent to which chilling conditions may lead to oxidative damage will depend on the degree of severity and the duration of exposure (Maruthasalam et al., 2010). Secondary responses could be caused by several factors, resulting in alteration of fruit physiological processes.

### *a) Fruit respiration rate*

The main purpose of storing fruit at low temperature is to slow down respiration rates (Huang et al., 2008). In subtropical crops, chilling temperatures can alter fruit respiration, leading to a respiration burst (González-Aguilar et al., 2000a).

#### *b) Membrane lipid peroxidation*

During chilling, ROS levels exceed what can be counteracted by antioxidants, accelerating lipid peroxidation (Sharma et al., 2012) in the cell membranes (Yang et al., 2010; Toivonen and Hodges, 2011). An increase in lipid peroxidation is concomitant to increased production of ROS (Sharma et al., 2012), making ROS accumulation the most damaging process to cell membranes. Lipid peroxidation occurs in both, enzymatic and non-enzymatic processes; enzymatic, lipid peroxidation occurs through the activity of lipoxygenases (Skórzyńsk-Polit, 2007).

Lipoxygenases catalyse the dioxygenation of fatty acid chains to form lipid hydroperoxides, which are converted to secondary compounds (Feussner and Wasternack, 2002; Skórzyńsk-Polit, 2007); while in non-enzymatic processes, ROS are engaged in the initiation of lipid peroxidation (Skórzyńsk-Polit, 2007). This process has been well studied and discussed by Bhattacharjee (2005). The impact of lipid peroxidation in cell membranes results in decreased membrane fluidity, increased membrane permeability and damage of membrane proteins (Gill and Tuteja, 2010). Therefore, lipid peroxidation has been widely used as an indicator of ROS-mediated damage of cell membranes (Sharma et al., 2012).

#### *c) Membrane permeability*

Oxidative damage leads to increased membrane permeability. Chilling induces rigidification of membranes in fruit cells, leading to a disturbance of membrane processes, such as electron transfer reactions (Ruelland et al., 2009). Cell membranes are the primary site of CI; when damaged, leakage of soluble cytosolic compounds occurs, leading to cell death (Kosova et al., 2007).

#### *d) Membrane protein oxidation*

Protein oxidation is one of the irreversible reactions caused by ROS attack (Gill and Tuteja, 2010), resulting in protein modification (Sharma et al., 2012). According to

Ruelland et al. (2009), increased susceptibility of protein to proteolysis, disturbance of protein stability, complexes and metabolic regulations are results of protein oxidation. Previous studies suggest that injured tissues contain increased concentrations of carboxylated proteins – a widely used marker of protein oxidation (Møller and Kristensen, 2004; Sharma et al., 2012).

*e) Development of CI symptoms*

During postharvest storage, lemons are subjected to chilling temperatures leading to chilling stress (Lafuente et al., 2004). Extended exposure to chilling can lead to the development of CI symptoms, especially after chilled fruit have been removed from chilling temperatures to room temperature (Schirra and Cohen, 1999). In lemons, CI symptoms may manifest as pitting, red blotch and necrosis of the flavedo. This is usually followed by the collapse of affected areas resulting in depressions on the fruit surface (Cohen et al., 1994). The manifestation of CI symptoms on the flavedo reduces consumer acceptance of fruit and causes economic losses to the citrus industry (Dong et al., 2012).

*f) Other symptoms including ultra-structure damage*

Several studies have shown that ultra-structural changes (swelling and disintegration of mitochondria and chloroplasts, thylakoid dilation) are more extensive in crops that are highly sensitive to chilling (Wise and Naylor, 1987; Kratsch and Wise, 2000). Furthermore, the longer a fruit is exposed to chilling conditions, the more extensive and irreversible the damage to the ultra-structure of the fruit (Ma et al., 1990; Kratsch and Wise, 2000).

### **2.3. Control measures to mitigating CI**

To withstand chilling postharvest, lemons, like any other crop, have developed defence mechanisms (Guy, 1990; Dong et al., 2012); albeit, to a certain extent (Janská et al., 2010). The mode of activation by which fruit tolerate chilling is of scientific interest (Lattanzio et

al., 2012). Exposure of fruit to chilling temperatures triggers a variety of physiological, biochemical and molecular responses that allow fruit to adjust to chilling conditions and resist injury (Janská et al., 2010; Lattanzio et al., 2012). These include the activation of signal transduction pathways (ROS, Ca<sup>2+</sup>), the alteration in unsaturated fatty acid to saturated fatty acid ratio, proteins and carbohydrate composition (Heidarvand and Amir, 2010).

In citrus fruit, many responses during cold storage have been associated with cold-induced tolerance to CI without a complete and clear understanding of the mechanisms involved (Schirra and Cohen, 1999). Fruit under chilling stress may exhibit chilling tolerance by modifying their metabolism either by adjusting the cellular metabolism altered during chilling exposure or by enhancing tolerance mechanisms (Yordanova and Popova, 2007; Yuanyuan et al., 2009). During chilling, cellular metabolism, structure, catalytic properties and enzyme function are regulated to restore normal metabolism levels in response to stress (Fernie et al., 2005; Yordanova and Popova, 2007). Additionally, fruit produce stress-responsive metabolites to enhance chilling tolerance (Thomashow, 1999; Yordanova and Popova, 2007).

This enhancement of tolerance involves genomics, proteomics, metabolomics and other cellular components (Guy, 1990; Yordanova and Popova, 2007; Yuanyuan et al., 2009). The stress responsive metabolites in conjunction with cold-responsive proteins (Gálvez et al., 2010) and heat-shock proteins (HSPs) can stabilise both membrane phospholipids and cytoplasmic proteins, scavenge ROS, and maintain hydrophobic interactions and ion homeostasis (Janská et al., 2010). Therefore, mechanisms to maintain homeostasis result in chilling tolerance through alterations in gene expression and metabolic adjustment (Yordanova and Popova, 2007). The increased presence or activity of antioxidants (enzymatic and non-enzymatic) in the fruit is also important (Janská et al., 2010).

### 2.3.1 *Cell membrane modification*

Membranes are dynamic structures that support physiological and biochemical reactions; however, they are also sites where damage due to chilling occurs (Campos et al., 2003). When exposed to chilling temperatures, fruit cell membranes undergo changes in lipid and, particularly, fatty acid composition in order to maintain metabolic and normal cell functionality (Routaboul et al., 2000; Campos et al., 2003). Membrane lipids are primarily composed of saturated and unsaturated fatty acids (Heidarvand and Amir, 2010), with a high saturated to unsaturated fatty acid ratio providing greater membrane rigidity, while a lower saturated to unsaturated fatty acid ratio results in greater membrane fluidity. Therefore, a higher ratio of unsaturated to saturated fatty acids may be responsible for increasing chilling tolerance (Cao et al., 2009a).

### 2.3.2 *Mineral nutrients*

Mineral nutrients are essential for the completion of a plant's life cycle (Waraich et al., 2011b) and are also involved in stress defence. Information on the role of mineral-mineral nutrients with regard to fruit susceptibility to postharvest CI is very limited (Toivonen and Hodges, 2011). Certain mineral nutrients that have been reported to play a significant role in increasing chilling tolerance in crops including calcium, phosphorus, potassium, zinc, and silicon. The role of calcium in regulating responses to biotic and abiotic stress, as well as calcium as a putative signalling molecule, have been well-documented by Hu and Schmidhalter (2005) and Toivonen and Hodges (2011).

Potassium plays a significant role in glycolytic enzymes, protein synthesis and in mediating cell expansion (Hu and Schmidhalter, 2005). Improvement in the potassium nutritional status in plants can greatly lower ROS production by reducing activity of NAD(P)H oxidases (Cakmak, 2005). Low supply of potassium in plants is associated with chilling sensitivity, while chilling tolerance can be enhanced by increasing the potassium supply in crops such as tomato and pepper (Cakmak, 2005).

Other minerals that have been reported to play a crucial role in increasing chilling tolerance include zinc and silicon. Zinc possibly acts through its involvement in stabilising cellular structures involved in protein metabolism and auxin metabolism (particularly IAA) (Waraich et al., 2011a). Silicon on the other hand alleviates CI by stimulating antioxidant systems in plants (Liang et al., 2007).

### 2.3.3 *Antioxidant defence mechanisms*

Antioxidants are defined as compounds that can either delay or inhibit the oxidation of lipids, proteins and other molecules such as deoxyribonucleic acid (DNA), by inhibiting the initiation of oxidising chain reactions (Velioglu et al., 1998; Wang, 2010c). In fruit, antioxidant systems are a defence mechanism to regulate ROS levels (Hossain and Teixeira da Silva, 2011). Antioxidant defence systems also protect fruit from oxidative damage during stress such as chilling (Rivera et al., 2007). An induction of antioxidant defence mechanisms by one type of stress has been reported to produce tolerance to another, possibly more severe stress, such as chilling (Sala and Lafuente, 1999; Rivera et al., 2007). In citrus fruit, chilling-tolerant cultivars were found to have more efficient antioxidant defence systems than chilling-sensitive cultivars (Sala, 1998; Rivera et al., 2007).

Activation and regulation of antioxidants is important for the protection of fruit against chilling (Zhu et al., 2011). However, the level and activity of antioxidants in fruit can be affected by both pre-and postharvest factors such as genotype, climate and maturity (Wang, 2010c). During chilling, the enzymatic activities that detoxify ROS production may be less efficient due to chilling stress effect (Ruelland et al., 2009). Therefore, an efficient antioxidant system comprising of enzymatic and non-enzymatic antioxidants is necessary to scavenge or detoxify excess ROS, thereby increasing the chilling tolerance of fruit (Noctor and Foyer, 1998; Baek and Skinner, 2003; Sharma et al., 2012).

### A. *Enzymatic defence mechanisms*

#### i. *Superoxide dismutase (EC1.15.1.1)*

Superoxide dismutase (SOD) is the first line of defence from potential damages that may be caused by ROS. It is present in all aerobic organisms and in cellular compartments that generate ROS (Mittler, 2002; Sala et al., 2005; Rivera et al., 2007). During chilling, SOD plays a significant role preventing superoxide radicals from producing hydroxyl radicals (Rivera et al., 2007). Increased activity of SOD is often correlated with increased tolerance of plants against environmental stresses and enhanced oxidative stress tolerance in crops (Sharma et al., 2012). Sala (1998) observed that SOD activity was high in chilling tolerant mandarin fruit during cold storage at 2.5°C for 8 weeks.

#### ii. *Guaiacol peroxidase (EC 1.11.1.9)*

Guaiacol peroxidase (GPX) is widely known as a stress enzyme associated with many important biosynthesis processes, including lignification of cell walls and defence against environmental stresses (Kobayashi et al., 1996; Sharma et al., 2012). The enzyme protects cell membranes from oxidative damage (Gasper et al., 1991; Sarkar et al., 2009) by taking part in redox reactions in plasma membranes and in cell wall modifications such as lignification and suberisation (Sarkar et al., 2009). Furthermore, GPX is responsible for the cross-linking of phenolic moieties during the biosynthesis of lignins and existing lignans in plant cell walls (Morales and Barcelo, 1997; Sarkar et al., 2009). In citrus fruit, GPX may protect fruit against CI by scavenging ROS, its accumulation could be related to a defence mechanism of fruit to cope with chilling stress (Lafuente et al., 2004).

#### iii. *Catalase (EC 1.11.1.6)*

Catalase (CAT) was the first antioxidant enzyme to be discovered (Hossain and Teixeira da Silva, 2011). Studies indicate that an increase in CAT activity may be an adaptation to oxidative stress (Sala and Lafuente, 1999). The enzyme was found to be present in higher

concentrations in chilling-tolerant than chilling-susceptible citrus cultivars (Sala, 1998; Maul et al., 2011). This suggests that CAT is a major enzyme involved in the defence against CI in citrus fruit (Sala and Lafuente, 2000; Sala et al., 2005). The effectiveness of CAT in increasing chilling tolerance involves its efficiency in removing excess ROS (Lafuente et al., 2004).

*iv. Glutathione Reductase (EC 1.6.4.2)*

Glutathione reductase (GR) is an NAD(P)-H-dependent enzyme that catalyses the reduction of glutathione disulphide (GSSG) to glutathione (GSH) to maintain a high cellular GSH:GSSG ratio (Hossain and Teixeira da Silva, 2011; Sharma et al., 2012). An increased GR activity has been associated with improved tolerance to oxidative stress (Sharma and Dubey, 2005; Sharma et al., 2012). According to Sala (1998), GR activity in mandarin fruit was higher in chilling tolerant fruit than in chilling susceptible fruit cold stored at 2.5°C for 8 weeks.

*v. Ascorbate peroxidase (EC 1.11.1.11)*

Ascorbate peroxidase (APX) is regarded as one of the most widely distributed antioxidant enzymes, playing an essential role in controlling ROS levels (Sharma et al., 2012). In citrus fruit, APX is one of the most important antioxidant enzymes potentially deployed in the defence against CI (Lafuente et al., 2004). The enzyme plays a role in eliminating H<sub>2</sub>O<sub>2</sub> by utilising ascorbate as its specific electron donor to reduce H<sub>2</sub>O<sub>2</sub> to water with the concomitant generation of monodehydroascorbate (Song et al., 2005). Studies by Sharma and Dubey (2005) and Sharma et al. (2012) reported enhanced APX activity in response to environmental stress.

## *B. Non- enzymatic defence mechanisms*

### *i. Ascorbic acid*

Ascorbic acid (AA) is one of the most abundant and potent antioxidants in plants (Davey et al., 2000; Ioannidi et al., 2009). As a major antioxidant protecting plant tissues against oxidative damage caused by ROS, AA also serves as an enzyme co-factor (Smirnoff, 1996; Badejo et al., 2009). It is also involved in plant stress resistance by acting as a signalling compound and scavenging ROS via the APX reaction (Smirnoff, 2000; Pastori et al., 2003; Ioannidi et al., 2009). Understanding the role of AA in fruit physiology can provide opportunities to alter its concentration in fruit and thereby potentially minimise postharvest losses (Ioannidi et al., 2009).

### *ii. Tocopherols*

Tocopherols ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ), also known as vitamin E, are recognised as lipid bound antioxidants with the ability to protect polyunsaturated fatty acid chains from lipid peroxidation (Li et al., 2008b). The role of tocopherols in enhancing chilling tolerance in horticultural crops has been associated with the ability of these compounds to efficiently scavenge ROS, quench singlet oxygen ( $O^{\cdot}$ ), and terminate lipid peroxidation chain reactions (Schneider, 2005; Maeda et al., 2006).

### *iii. Carotenoids*

Carotenoids are antioxidants (Sharma et al., 2012) known for playing a crucial role in acquisition of oxidative stress tolerance (Karuppanapandian et al., 2011). As antioxidants, carotenoids have been reported to detoxify ROS (Young, 1991) and serve as precursors to signalling molecules that influence environmental stress responses (Li et al., 2008a).

#### *iv. Soluble sugars*

Chilling can cause cellular energy starvation, triggering metabolic reprogramming in crops (Bolouri-Moghaddam et al., 2010). Soluble sugars have been reported to play important roles as key molecules and regulatory molecules in crops (Bolouri-Moghaddam et al., 2010). The involvement of soluble sugars allows crops to adapt to constantly changing environments (Bolouri-Moghaddam et al., 2010) and influence the sensitivity of plant tissues to chilling (Purvis and Grierson, 1982). During chilling, soluble sugars form a glass-like layer preventing the intracellular compartment from mechanical collapse and enables the cell to avoid the formation of intracellular ice crystals (Ingram and Bartels, 1996; Kosova et al., 2007). These compounds are recognized as regulatory molecules, controlling gene expression related to plant metabolism and chilling tolerance (Bolouri-Moghaddam et al., 2010).

The roles of soluble sugars include influencing the sensitivity of tissues to chilling temperatures (Holland et al., 2002; Zhu et al., 2011) and protecting fruit against CI (Ingram and Bartels, 1996; Holland et al., 2002). Soluble sugars play crucial roles as signaling molecules in response to chilling stress; this results in increased chilling tolerance (Couee et al., 2006; Zhu et al., 2011). Soluble sugars have been reported to protect crops from CI by changing the osmotic potential of the cell and decreasing cellular dehydration by 50% (Ruelland et al., 2009).

#### *v. Proline*

Proline is one of the most abundant amino acids in citrus fruit reported to be involved in plant stress (Yelenosky, 1979). The amino acid accumulates in response to an array of plant stresses, including chilling (Ruelland et al., 2009). Proline plays a protective role by conferring osmotic adjustment together with increased ROS detoxification to fruit tissues and by interacting with the hydrophobic residue of proteins during chilling (Valliyodan and

Nguyen, 2006). Other protective roles of proline include enzyme protection from denaturation, water-binding capacity intensification, regulation of cytosolic acid, and protein synthesis stabilisation as well as inducing gene expression against chilling (Ruelland et al., 2009). Other roles of proline, such as the proline-linked pentose phosphate pathway for phenolic synthesis and efficient antioxidant response have been previously discussed in detail by Shetty and Wahlqvist (2004).

#### *vi. Phenolic compounds*

Phenolics are secondary metabolites (hydroxycinnamate esters, flavonoids, lignin and tannins) (Sharma et al., 2012), which are synthesised through the shikimic acid pathway (Dixon and Paiva, 1995; Tomás-Barberán and Espín, 2001). Phenolics are one of the most important metabolites found in plants. They are synthesised from cinnamic acid formed by PAL, the key enzyme in regulating the shikimic acid biosynthetic pathway (Javanmardi et al., 2003; Michalak, 2006). The role of phenolics in plants includes absorbing and neutralising free radicals (Michalak, 2006), quenching singlet and triplet oxygen or decomposing peroxides (Javanmardi et al., 2003).

The role of phenolics in chilling has received much less attention (Pennycooke et al., 2005). Phenolics play a key role as defence compounds against ROS during chilling (Lattanzio et al., 2012). As secondary metabolites, phenolics stabilise membranes by decreasing membrane fluidity, restricting peroxidative reactions and delaying the diffusion of free radicals (Blokhina et al., 2003; Michalak, 2006). It has been suggested that chilling enhances phenolic metabolism; therefore, the threshold temperature for increasing phenolic metabolism is related to the threshold temperature at which CI is induced (Lattanzio et al., 2012).

#### *2.3.4 Phenylalanine ammonia-lyase (EC 4.3.1.5)*

Phenylalanine ammonia-lyase (PAL) is a principal enzyme at the entry-point of the phenylpropanoid pathway that catalyses the deamination of L-phenylalanine to form *trans*-cinnamic acid, which is the primary intermediate in the biosynthesis of phenolics (Levine et al., 1994; Sanchez-Ballesta et al., 2000). The increase in PAL activity in plants under stress has been associated with a defence mechanism operating in stress afflicted cells (Dixon and Paiva, 1995; Sanchez-Ballesta et al., 2000). The enzyme is encoded by a small multigene family of 2-6 members which are differentially expressed in plant tissues in response to different stress conditions, including chilling (Zhu et al., 1995; Sanchez-Ballesta et al., 2000). Therefore, chilling stress often affects PAL activity levels in plants (Graham and Patterson, 1982; Sanchez-Ballesta et al., 2000).

Exposure to chilling increases PAL synthesis in plants (Pereyra et al., 2005; Gálvez et al., 2010). Previous studies suggest that PAL is one of the key enzymes in the phenolic metabolism that protects fruit against CI (Sanchez-Ballesta et al., 2000; Gálvez et al., 2010). Very little is known about the regulation of PAL expression in many fruit (Sanchez-Ballesta et al., 2000); however, in citrus, the induction of PAL activity has been associated with chilling tolerance (Martínez-Téllez and Lafuente, 1993; Lafuente et al., 2001).

### 2.3.5 *Chilling responsive proteins (Heat shock proteins)*

Heat shock proteins (HSPs) are usually associated with responses to high temperatures; however, studies indicate that these proteins also occur in response to chilling (Heidarvand and Amir, 2010). Accumulation of HSPs has been reported to enhance the chilling tolerance level of affected tissues of crops (Mittler, 2002; Toivonen and Hodges, 2011). Studies by different authors (Nover and Sharf, 1997; Yan et al., 2006; Heidarvand and Amir, 2010) reported that HSPs act as molecular chaperones, preventing aggregation of denatured proteins and supporting protein translocation into organelles. Furthermore, HSPs are thought to have a pivotal function in protecting plant tissues against chilling stress by

stabilising proteins and membranes, enabling protein refolding and maintaining cellular homeostasis (Toivonen and Hodges, 2011).

### **3. STRATEGIES TO ALLEVIATE CI**

Fruit antioxidant capacity may not be sufficient to mitigate oxidative damage caused by ROS under chilling conditions (Huang et al., 2008). If the chilling duration is extended, the defence system may be easily overwhelmed by excess ROS, which may result in severe damage to the fruit (Zhang et al., 2008; Sharma et al., 2012) leading to CI. Postharvest treatment can directly or indirectly alter antioxidant systems, maintain fruit quality during storage and activate other metabolic processes enabling fruit to withstand chilling conditions (Huang et al., 2008).

Methods for alleviating CI in horticultural crops have been studied intensely (Pantastico et al., 1975; Jackman et al., 1988; Lafuente et al., 2005; Wang, 2010b) to mitigate CI. Several postharvest treatments, such as intermittent warming, heat treatments, high- and low-temperature conditioning have been developed to reduce CI in citrus fruit (Porat et al., 2002; Porat et al., 2004). Wang (2010a) stated that some techniques are more effective in alleviating CI in certain cultivars than others; however, the optimum treatment conditions vary with different fruit. Despite the importance of CI in the citrus industry, progress has been slow and no confirmed reliable postharvest treatments are yet available to reduce the development of CI in citrus fruit (Siriphanich, 2002; Porat et al., 2004). Wang (2010a) advised that the more the mechanisms of CI are understood, the greater the likelihood of finding more effective methods to alleviate CI. Some of the naturally occurring substances that have shown potential to reduce CI in horticultural crops include the following plant growth regulators.

*i. Jasmonic acid and methyl jasmonate*

Jasmonic acid (JA) and its methyl ester, methyl jasmonate (MJ), are natural plant growth regulators reported to be biologically active when applied to plants (Parthier, 1990). Research indicated that MJ is much more volatile than JA, and can act as a hormonal communication compound between plants as well as within the plant (Farmer and Ryan, 1990). Various experiments indicate that MJ application activates the synthesis of proteins identical to those activated by wounding, and osmotic, water and salt stresses (Anderson et al., 1989; Farmer et al., 1991; Meir et al., 1996) and produced in responses to various environmental stresses (Creelman and Mullet, 1997).

Depending on the concentration applied, MJ could enhance resistance to pathogen attacks as well as to CI (Sembdner and Parthier, 1993; Meir et al., 1996; González-Aguilar et al., 2003). When applied at postharvest, MJ significantly reduced CI in tomato (Ding et al., 2002), peach (Feng et al., 2003), and guava (González-Aguilar et al., 2004). Similarly results were reported by Wang and Buta (1994) on zucchini squash fruit treated with MJ prior to cold storage at 5°C. Dipping avocado cultivars on MJ for 30 seconds ('Fuerte and 'Hass' at 2.5 µM, avocado 'Etinger' or grapefruit at 10 µM, and red bell pepper fruit at 25 µM) was reported by Meir et al. (1996), to reduce CI symptoms when such fruit were subsequently stored at 2°C.

Recent findings by Sibozza and Bertling (2013) reported that treatment with 10 µMJ reduced CI symptoms in 'Eureka' lemon fruit stored at -0.5°C for up to 42 days plus 7 days at 23°C. Seemingly, the effectiveness of MJ in reducing CI depends on the type of commodity and the concentration applied. According to Meir et al. (1996), MJ is receiving renewed biological interest as a potential important signalling molecule in plants. The role of MJ in enhancing chilling tolerance in fruit is usually associated with its effect in increasing HSPs and activating the expression of antioxidant defence genes (Meir et al.,

1996). However, little is known about the effect and the mode of action of MJ in CI in lemon fruit applied postharvest.

*i. Salicylic acid*

Salicylic acid (SA) is a natural phenolic compound, a hormone-like signalling molecule (Kang et al., 2003; Huang et al., 2008), which is derived from the shikimate-phenylpropanoid pathway (Ghasemzadeh and Jaafar, 2013). This hormone acts as a potential non-enzymatic antioxidant and plays an important role in the regulation of plant growth and development (Raskin, 1992; Kang et al., 2003; Noreen et al., 2009). Recently, SA has been identified as essential for the expression of plant stress resistance (Clarke et al., 2004; Wen et al., 2008). Application of SA influences physiological process, including the induction of several antioxidant system (Yordanova and Popova, 2007; Ghasemzadeh and Jaafar, 2013).

Salicylic acid is also involved in mediating plant defence against pathogens (Raskin, 1992; Yordanova and Popova, 2007), high temperature stress (Dat et al., 1998) and drought stress in particular (Singh and Usha, 2003). As a hormone which plays a role in plants stress, SA has been associated with chilling tolerance in horticultural crops. Treatment with SA reduced CI in tomato (Ding et al., 2001, 2002), pomegranate (Sayyari et al., 2009), cucumber (Cao et al., 2009b), pineapple (Lu et al., 2010) and lemon (Siboza and Bertling, 2013). According to Yordanova and Popova (2007), the role of SA in chilling tolerance has been associated with its influence on the activity of antioxidative enzymes.

The hormone has also been reported to be involved in the activation of stress-induced antioxidant systems (Huanga et al., 2008 ). The effectiveness of SA in inducing chilling tolerance in crops has been suggested to depend either on the type of species or concentration of SA applied (Noreen et al., 2009). The reduction of CI in SA treated crops

(cucumber and maize) was associated with an increase in the activity of peroxidase (POX) and glutathione reductase (GR) (Yordanova and Popova, 2007).

#### **4. SUMMARY AND CONCLUSION**

The development of CI in horticultural crops has been under investigation for many decades, with the main aim of finding method of alleviating this physiological disorder at postharvest stage. It is clear from literature that lemon fruit are chilling sensitive and develop a lot of CI symptoms postharvest, negatively affecting the fruit marketability and causing huge economic losses to the South African citrus industry. Currently, no reliable commercial method to lessen the development of CI in citrus fruit exists (Porat et al., 2004), let alone on lemon fruit. Therefore, a basic understanding of the physiological, biochemical and molecular mechanisms involved in chilling tolerance of lemon fruit postharvest would allow for the design of appropriate strategies to mitigate CI (Sevillano et al., 2009). Beneficial effects of MJ and SA in alleviating CI have been demonstrated in several horticultural crops; however, little is known about the potential beneficial effects of MJ and SA treatments combination in alleviating CI in lemons during cold storage. These naturally occurring products which are beneficial in reducing CI are also effective in enhancing gene expression and antioxidant activity in fruit tissues (Wang, 2006). Therefore, an understanding of the mechanisms by which fruit perceive chilling stress and transmit signals to activate adaptive responses is of fundamental importance to improving chilling tolerance during cold storage (Xiong et al., 2002).

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## Chapter 2

# Influence of preharvest farm location and postharvest treatment on chilling injury of lemons (*Citrus limon*)

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## **Abstract**

Treatments with methyl jasmonate (MJ) and salicylic acid (SA) have shown promising results in reducing chilling injury (CI) of many horticultural crops, including lemons. This study investigated whether the production environment could influence the development of chilling injury in lemon fruit treated with MJ and SA, and stored at different temperatures (-0.5, 2 and 4.5°C) for up to 28 days. Lemons were sourced from three production sites (New Venture Farm: 31° 02' S 29° 25' E, 68-483 meter above sea level), (Tala Valley Citrus Estate: 29° 52' S 30° 30' E, 416-922 meter above sea level) and (Sun Valley Estate: 28° 83' S 30° 06' E, 820-1003 meter above sea level) characterised by moderate subtropical (mean annual temperature: 14.5-26.5°C; mean annual rainfall: 712-717 mm), warm temperate (mean annual temperature: 12.6-23.7°C; mean annual rainfall: 612-771 mm) and cool subtropical conditions (mean annual temperature: 10.7-26.5°C; mean annual rainfall: 669-675 mm), respectively. Fruit were treated with MJ and SA solutions, waxed and afterwards stored either at -0.5, 2 or 4.5°C for 0, 7, 14, 21 or 28 days. After cold storage, fruit were transferred to 23°C for 7 days as shelf-life simulation. Treatment with 10 µM MJ plus 2 mM SA was more effective in alleviating CI compared with either 10 µM MJ or 2 mM SA, regardless of farm climatic conditions, storage temperatures and storage duration. Furthermore, storing lemons at -0.5°C increased chilling tolerance, while at 2°C delayed the manifestation of CI symptoms. Moreover, storing lemons at 4.5°C stimulated the CI symptoms as compared with ultra-low storage temperatures. The inhibition of CI symptoms by storing lemons at -0.5°C suggested a non-damaging physiological response triggered by such a storage temperature. Lemons from the moderate subtropical location were chilling tolerant compared with those from the warm temperate location. Symptoms were more severe in lemons from the cool subtropical location.

*Keywords:* Chilling injury; Climatic conditions; Storage duration; Storage temperature; Methyl jasmonate; Salicylic acid.

## 1. Introduction

Cold storage is a widely used method to maintain fruit quality and extend shelf-life of many horticultural crops (Meng et al., 2009; Zhu et al., 2011). However, the challenge with cold storage include fruit susceptibility to chilling injury (CI) (González-Aguilar et al., 2004). In citrus, pitting, red blotch, and membrane staining followed by collapse of cell membrane are the main CI symptoms (Cohen et al., 1994). Fruit susceptibility to CI has been suggested to depend on fruit cultivar, origin and environmental conditions of the fruit during growth (Lafuente et al., 1997; Wang, 2010). Ladaniya (2008) suggested that postharvest losses resulting from CI are influenced by pre- and postharvest factors such as climatic conditions (relative humidity, rain, temperature). Symptoms are not always visible during cold storage, but mainly develop after cold storage (shelf-life), when fruit reach consumers (Bruhn et al., 1991; Crisosto et al., 1999; Schirra and Cohen, 1999). This does not only limit fruit storage life but also reduces marketability and consumer acceptability, thereby resulting in postharvest losses (Crisosto et al., 1997; González-Aguilar et al., 2000).

There is a growing concern by consumers about the use of potentially hazardous chemicals in food production. Such chemicals raise health concerns, and therefore, interest in the use of more naturally occurring compounds in mitigating CI in fruit is increasing (Wang, 2006). Methyl jasmonate (MJ) and salicylic acid (SA) are not only naturally occurring compounds, but also plant growth regulators found in plants. Previous research have shown that MJ and SA have potential to reduce CI in zucchini squash (*Cucurbita pepo*) (Wang and Buta, 1994), red bell pepper (*Capsicum annum* cv. Maor), avocado (*Persea Americana* Mill., cvs. Hass, Etinger, and Fuerte), grapefruit (*Citrus paradise* cv. Marsh seedless) (Meir et al., 1996), mango (*Mangifera indica* cv 'Kent') (González-Aguilar et al., 2001), peach (*Prunus persica* Batsch cv Baifeng) (Feng et al., 2003; Yang et al., 2012) and lemon (*Citrus limon* cv Eureka) (Siboza et al., 2012). However, the beneficial use of MJ

and SA in reducing CI in 'Eureka' lemon grown under different climatic conditions is not well understood. Therefore, the objective of this study was to determine whether differences in the development of CI symptoms in lemon stored at different temperatures can be associated with environmental conditions during fruit development at the production site. Furthermore, such variation in the development of CI in lemon can be mitigated by postharvest MJ and SA dips or applications.

## **2. Materials and Methods**

### *2.1. Plant material*

At physiological maturity, 'Eureka' lemons [*Citrus limon* (L.) Burm.] were obtained from three commercial farms located in different climatic zones of KwaZulu-Natal, South Africa. Lemon fruit were sourced from New Venture Farm (31° 02' S 29° 25' E, 68-483 meter above sea level), Tala Valley Citrus Estate (29° 52' S 30° 30' E, 416-922 meter above sea level) and Sun Valley Estates (28° 83' S 30° 06' E, 820-1003 meter above sea level). The New Venture Farm (Table 3), Tala Valley Citrus Estate (Table 1) and Sun Valley Estates (Table 2) farms are located in the moderate subtropical zone, the warm temperate zone and the cool subtropical zone; respectively. Lemon fruit were harvested on the 6<sup>th</sup> of June 2011 and 2012 seasons, and the trends were similar. Fruit were immediately transported on paved roads from the collection site to the laboratory in a ventilated vehicle. Fruit transportation distances from farm locations to the laboratory site were 39.2 km (Tala Valley Citrus Estates), 130 km (Sun Valley Estates) and 228 km (New Venture Farm). Lemon fruit were selected for uniformity of parameters such as size, shape and colour.

### *2.2. Experimental procedure*

Lemons were washed with Sporekill® (Hygrotech Pty Ltd), allowed to air-dry, and randomly allocated to postharvest chemical treatment of MJ and SA. Fruit were either

soaked in 10  $\mu$ M MJ, 2 mM SA or 10  $\mu$ M MJ plus 2 mM SA solutions for 30 s (Siboza and Bertling, 2013). Furthermore, a control / no dip were included. Afterwards, fruit were waxed with Citrashine® (Citrashine Pty Ltd, Johannesburg, South Africa) according to Sibozza and Bertling (2013). Fruit were cold stored either at -0.5; 2 or 4.5°C (air delivery temperature). The relative humidity was maintained at 85-90%. Five replicates of 20 fruit per treatment were sampled at 0, 7, 14, 21 or 28 days into the cold storage. After removal from cold storage, lemons were transferred to room temperature (23°C) for 7 days and monitored for the development of CI symptoms.

### *2.3. Experimental design*

The experiment was laid out as a factorial treatment structure in split-split-split plot arrangement with the following factors: main plot - production environment (3 levels: Sun Valley Estate, Tala Valley Citrus Estate and New Venture Farm); sub-plot – post harvest treatments (4 levels: 10  $\mu$ M MJ, 2 mM SA or 10  $\mu$ M MJ plus 2 mM SA solutions and a control – untreated fruit); and sub-sub plot – sampling interval (5 levels: 0, 7, 14, 21 or 28 days). Storage conditions were used as a blocking factor (3 levels: -0.5, 2 or 4.5°C).

### *2.4. Evaluation of chilling injury on the fruit*

Lemons were evaluated for the CI symptom (pitting) after 0, 7, 14, 21 or 28 days of cold storage either at -0.5, 2 or 4.5°C and a week at shelf-life based on the following scale (Lafuente et al., 1997; Sala, 1998): 0 = normal ( no pitting), 1 = slight pitting (a few scattered pits), 2 = moderate pitting (pitting covering up to 30% of the fruit surface), 3 = severe pitting (extensive pitting covering > 30% of the fruit surface) and expressed as CI index (Fig. 1).

### *2.5. Statistical analysis*

Five replicates of 20 fruit per treatment were used in this experiment and the statistical analyses were performed using GenStat® 14<sup>th</sup> Edition (VSN International, Hemel

Hempstead, UK). The data were subjected to analysis of variance (ANOVA) to answer the following question: (a) whether there was an interaction between the production environment and postharvest treatments that influenced the development of CI under different storage temperature conditions.

### **3. Results**

#### *3.1. Effects of postharvest treatments with MJ and SA in enhancing chilling tolerance in lemon fruit*

Cold storage of lemons at postharvest induced physiological responses, which mostly appeared as pitting in the flavedo (Fig. 1). The results show that the manifestation of CI was significantly ( $P < 0.05$ ) influenced by farm location, postharvest treatments, storage temperature, cold storage duration and the interaction of these factors (Table 1). The susceptibility of untreated lemons to CI was higher compared with MJ and SA treated lemons from all farm locations. Interestingly, postharvest treatments with MJ and SA significantly ( $P < 0.05$ ) reduced CI in lemons (Fig. 2) from all farm locations. Treatment with 10  $\mu$ M MJ plus 2 mM SA was more effective in reducing CI in lemons for all locations, followed by the treatment with 10  $\mu$ M MJ (Fig. 2).

#### *3.2. Effects of farm location on the effectiveness of postharvest treatments with MJ and SA at enhancing chilling tolerance in lemon fruit*

Severe CI symptoms were observed in lemons from Sun Valley Estates compared with lemons from Tala Valley Estates (Fig. 2). Lemons from the New Venture Farm were chilling-tolerant. The untreated lemons from Sun Valley Estates had more severe CI symptoms compared with untreated lemons from Tala Valley Citrus Estate, while untreated lemons from New Venture Farm had the lowest (Fig. 2). Treatment with 10  $\mu$ M MJ plus 2 mM SA effectively enhanced chilling tolerance in lemon fruit from both New Venture Farm and Tala Valley Citrus Estate.

### *3.3. Effects of storage temperature on postharvest treatments with MJ and SA in enhancing chilling tolerance in lemon fruit*

Unexpectedly, symptoms of CI were more severe on lemons stored at 4.5°C than at 2°C; and, storing lemons at -0.5°C delayed the manifestation of CI symptoms in lemons (Fig. 2). Lemons stored at 4.5°C sustained a higher incidence of CI than those stored either at 2 or -0.5°C. Treatment with 10 µM MJ plus 2 mM SA reduced symptoms in lemons at all storage temperatures. However, there was no consistent effect on symptoms reduction on lemons by treatment with 10 µM MJ. This treatment was more effective in reducing symptoms on lemons stored at 4.5°C than on lemons stored at -0.5 or 2°C. Treatments with either 2 mM SA or 10 µM MJ plus 2 mM SA showed a similar trend in reducing CI symptoms in lemons stored at -0.5°C (Fig. 2). However, treatment with 10 µM MJ plus 2 mM SA was the most effective in reducing CI symptoms on lemons stored either at -0.5, 2 or 4.5°C.

## **4. Discussion**

Postharvest cold storage is used to prolong shelf-life and is an obligatory quarantine treatment when exporting fruit to countries without insect pests such as fruit fly (Meng et al., 2009; Zhu et al., 2011). Previous studies indicate that storing lemons at temperatures below 13°C causes CI (Wild and Rippon, 1973; Cohen et al., 1983). Recently, temperatures above 9°C were recommended for long term storage of lemons (Lafuente et al., 2005). However, such temperatures cannot be used for exporting purposes due to an obligatory quarantine treatment which involves exposing fruit to -0.5°C. At this temperature, the CI symptoms in lemons were manifested as pitting, red blotch and necrosis on the flavedo. The effect of SA in reducing CI may be associated either with its ability to influence enzyme activities (Yordanova and Popova, 2007) or its role in plants as

a stress signal molecule (Raskin, 1992; Cai et al., 2005; Yang et al., 2012). Therefore, SA could have directly or indirectly enhanced chilling tolerance in lemon fruit during chilling.

The mechanisms by which MJ induces tolerance to CI is still unclear (González-Aguilar et al., 2001). However, previous researchers suggested that MJ mediates the natural response of fruit to cold stress (Meir et al., 1996; González-Aguilar et al., 2001). Furthermore, it was suggested that MJ can activate defence mechanisms such as antioxidants and cold responsive genes which can enhance chilling tolerance in fruit (Pauwels et al., 2008; Jin et al., 2009). In peach fruit, the effect of MJ in alleviating CI was associated with enhanced antioxidant activity in the fruit (Jin et al., 2009). Therefore, the efficacy of 10 µM MJ in reducing CI in lemons may be attributed to its ability in activating expression of antioxidant defence genes (Meir et al., 1996).

In this study, the postharvest treatment combination of 10 µM MJ plus 2 mM SA was more effective in inducing chilling tolerance and maintaining fruit quality of lemons than each separate compound (10 µM MJ or 2 mM SA). This suggests that the combination of these two treatments activate different defence mechanisms (antioxidants and cold responsive genes) in increasing chilling tolerance in lemons during cold storage (Sapitnitskaya et al., 2006). Similar results were found in lemons that were stored at -0.5°C for 42 days (Siboza and Bertling, 2013). Therefore, the efficacy of 10 µM MJ plus 2 mM SA in enhancing chilling tolerance in lemons may be attributed to its double activation of defence mechanisms involved in chilling tolerance.

Preharvest environmental conditions under which crops are grown have great influence on CI susceptibility (Wang, 2010). In this study, the susceptibility of the lemons to CI was significantly ( $P < 0.05$ ) influenced by the farm locations. Lemons from cooler locations (Sun Valley Estates and Tala Valley Estate) showed significantly higher CI symptoms when compared with lemons from the warmer location (New Venture Farm). Lemons from

the coolest location (Sun Valley Estates) were highly susceptible to CI, probably as a consequence of the chilling stress in the field prior to postharvest treatment (Lafuente et al., 1997). Wang and Wallace (2004) advised that preharvest chilling temperatures in the field before harvest may add to chilling susceptibility during cold storage. This trend is similar to that observed in mandarin fruit (*Citrus reticulata*) stored for 28 days either at 2.5 or 12°C by Lafuente et al. (1997), who reported that mandarin fruit harvested during the coolest months showed the maximum susceptibility to CI.

Storage temperature played a role in chilling susceptibility of lemons to CI and symptom development varied with differences in storage temperatures. The results of this study suggest that chilling tolerance of lemon fruit is triggered by storing fruit at -0.5°C but not at 4.5°C. A similar trend was also observed in peach fruit stored for 30 days, where fruit stored at 0°C showed less CI symptoms compared to those stored at 5°C (Zhang and Tian, 2009). The benefit of reducing CI in peach fruit stored at 0°C was associated with higher level of polyunsaturated fatty acids (Zhang and Tian, 2009). Therefore, in this study, the mode of action with regards to the physiological responses of lemons stored at -0.5°C in inhibiting CI symptoms still needs further investigation.

In conclusion, differences in the development of CI in lemons stored at different temperatures can be associated with environmental conditions during fruit development at the farm location. Lemons originating from different farm locations did not develop CI symptoms at the same cold storage temperature. Environmental factors during fruit development at the farm location such as temperature and relative humidity may play a crucial role in chilling susceptibility of lemon during postharvest storage. Lemons from the moderate subtropical farm location were found to be more chilling tolerant than lemons from the warm temperate location. Symptoms were more severe on lemons from the cool subtropical location. Lemons were more chilling tolerant when stored at -0.5°C than at 2°C or 4.5 °C. Postharvest treatment with 10 µM MJ plus 2 mM SA was more effective than

treatment with either 10  $\mu$ M MJ or 2 mM SA alone. Treatment with 10  $\mu$ M MJ plus 2 mM SA has significant potential to enhance chilling tolerance in lemons regardless of preharvest farm locations, storage temperature and storage duration. Therefore, more extensive research was undertaken to understand the mode of action of the postharvest treatment with 10  $\mu$ M MJ plus 2 mM SA in inducing chilling resistance in lemons.

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### **Table legend**

Table 1. Climatic conditions prevailing at the Tala Valley Citrus Estate (BRU, 2007).

Table 2. Climatic conditions at the Sun Valley Estates (BRU, 2007).

Table 3. Climatic conditions prevailing at the New Venture Farm (BRU, 2007).

Table 4. The mean squares for the influence of farm locations, treatments, storage temperatures, cold storage duration and the interactions of these factors in the development of chilling injury (CI) in lemons at postharvest

### **Figure legend**

Fig. 1. Lemon fruit were evaluated for chilling injury symptom based on the following scale: A (no pitting), B (slight pitting), C (moderate pitting), 3 (severe pitting).

Fig. 2. Postharvest treatments with 10  $\mu$ M methyl jasmonate (MJ), 2 mM salicylic acid (SA) or 10  $\mu$ M MJ plus 2 mM SA on chilling injury (CI) of 'Eureka' lemons during cold storage at -0.5, 2 or 4.5°C for 28 days plus 7 days at 24°C shelf-life. Lemons were evaluated for CI based on the following scale: 0 (no pitting), 1 (slight pitting), 2 (moderate pitting), 3 (severe pitting). Values are means of five replicate with  $\pm$  standard errors.

Table 1

	<b>Annual</b>	<b>Jan</b>	<b>Feb</b>	<b>Mar</b>	<b>Apr</b>	<b>May</b>	<b>Jun</b>	<b>Jul</b>	<b>Aug</b>	<b>Sep</b>	<b>Oct</b>	<b>Nov</b>	<b>Dec</b>
<b>Rainfall</b>													
Median rainfall (mm)	612	93	82	80	37	17	7	8	17	35	67	82	87
Mean rainfall (mm)	771	107	97	98	50	33	18	14	29	47	77	101	100
<b>Mean daily temperature</b>													
Average (°C)	18.1	21.3	21.9	20.8	18.8	16.6	14.0	14.1	15.5	16.9	17.7	19.0	20.4
Minimum (°C)	12.6	16.7	17.1	15.9	13.4	10.4	7.4	7.3	9.0	11.2	12.4	14.1	15.7
Maximum (°C)	23.7	25.8	26.6	25.7	24.4	22.9	20.7	20.9	22.1	22.7	23.0	24.0	25.2
<b>Relative humidity</b>													
Mean daily (%)	70	78	77	74	71	65	62	63	63	67	72	74	76
<b>Positive chilling units</b>													
Mean		0	0	0	0	0	71	62	0	0	0	0	0
Maximum		0	0	0	0	0	111	113	0	0	0	0	0
Minimum		0	0	0	0	0	0	0	0	0	0	0	0
Std deviation		0	0	0	0	0	31	45	0	0	0	0	0
<b>Frost severity</b>	Rare												

Table 2

	<b>Annual</b>	<b>Jan</b>	<b>Feb</b>	<b>Mar</b>	<b>Apr</b>	<b>May</b>	<b>Jun</b>	<b>Jul</b>	<b>Aug</b>	<b>Sep</b>	<b>Oct</b>	<b>Nov</b>	<b>Dec</b>
<b>Rainfall</b>													
Median rainfall (mm)	669	132	94	92	34	8	3	3	9	29	63	84	118
Mean rainfall (mm)	675	111	98	89	36	18	9	12	19	34	58	86	105
<b>Mean daily temperature</b>													
Average (°C)	18.6	23.4	23.6	22.0	19.1	14.9	11.6	11.7	14.5	17.9	19.9	21.6	23.0
Minimum (°C)	10.7	16.8	17.0	15.4	11.3	5.9	1.7	1.9	5.3	9.4	12.5	14.6	16.2
Maximum (°C)	26.5	30.0	30.2	28.8	26.8	23.8	21.4	21.5	23.7	26.3	27.3	28.6	29.9
<b>Relative humidity</b>													
Mean daily (%)	60	66	65	67	63	62	51	54	53	54	60	60	63
<b>Positive chilling units</b>													
Mean		0	0	0	0	0	102	110	2	0	0	0	0
Maximum		0	0	0	0	0	127	132	14	0	0	0	0
Minimum		0	0	0	0	0	84	91	0	0	0	0	0
Std deviation		0	0	0	0	0	13	12	4	0	0	0	0
<b>Frost severity</b>	Severe frosts are expected in 96% of the years												

Table 3

	<b>Annual</b>	<b>Jan</b>	<b>Feb</b>	<b>Mar</b>	<b>Apr</b>	<b>May</b>	<b>Jun</b>	<b>Jul</b>	<b>Aug</b>	<b>Sep</b>	<b>Oct</b>	<b>Nov</b>	<b>Dec</b>
<b>Rainfall</b>													
Median rainfall (mm)	712	106	100	82	43	24	13	11	19	39	77	94	104
Mean rainfall (mm)	717	103	94	79	48	26	16	14	23	58	78	81	97
<b>Mean daily temperature</b>													
Average (°C)	20.5	24.1	24.2	23.4	21.1	18.6	16.0	15.9	17.4	19.3	20.5	21.8	23.4
Minimum (°C)	14.5	19.0	19.1	18.1	15.2	12.0	8.7	8.6	10.4	13.2	15.1	16.8	18.2
Maximum (°C)	26.5	29.2	29.2	28.8	27.1	25.3	23.3	23.3	24.4	25.4	25.9	27.0	28.5
<b>Relative humidity</b>													
Mean daily (%)	65	72	71	69	66	62	57	59	59	63	66	68	70
<b>Positive chilling units</b>													
Mean		0	0	0	0	0	0	0	0	0	0	0	0
Maximum		0	0	0	0	0	0	0	0	0	0	0	0
Minimum		0	0	0	0	0	0	0	0	0	0	0	0
Std deviation		0	0	0	0	0	0	0	0	0	0	0	0
<b>Frost severity</b>	None												

Table 4.

Source	d.f	m.s	F pr
Replicates	4	0,08	
Treatments (T)	3	16,24	< 0.001
Storage temperatures (ST)	2	5,71	< 0.001
Farm locations (FL)	2	30,49	< 0.001
Storage Duration (SD)	4	59,33	< 0.001
T × ST	6	1,20	< 0.001
T × FL	6	0,80	< 0.001
ST × FL	4	3,47	< 0.001
T × SD	12	3,81	< 0.001
ST × SD	8	0,99	< 0.001
FL × SD	8	2,57	< 0.001
T × ST × FL	12	1,03	< 0.001
T × ST × SD	24	0,22	< 0.001
T × FL × SD	24	0,36	< 0.001
ST × FL × SD	16	0,70	< 0.001
T × ST × FL × SD	48	0,25	< 0.001
Residual	716	0,03	
Total	899		

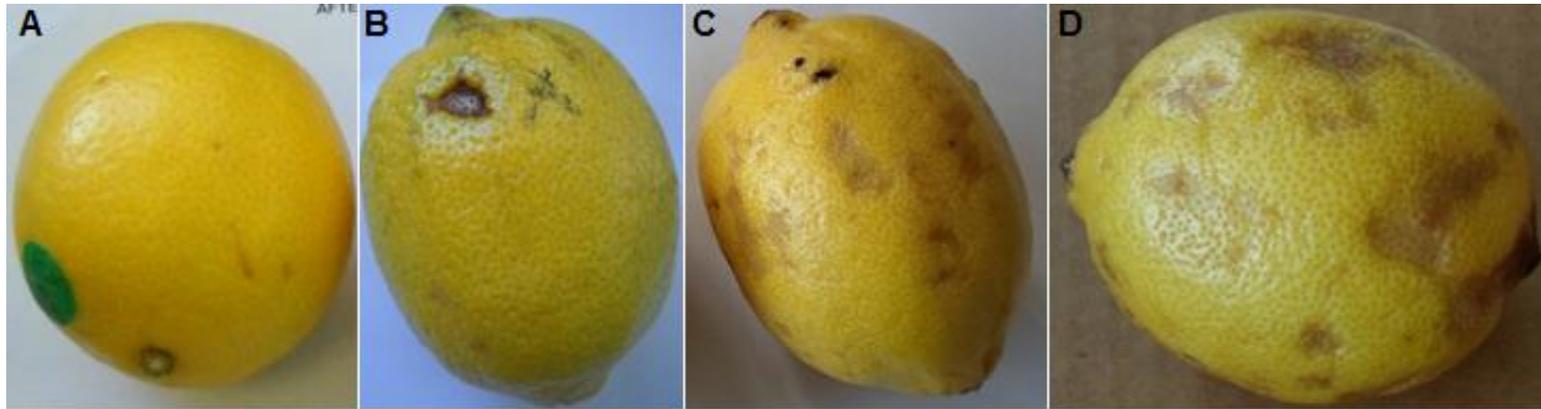


Fig. 1.

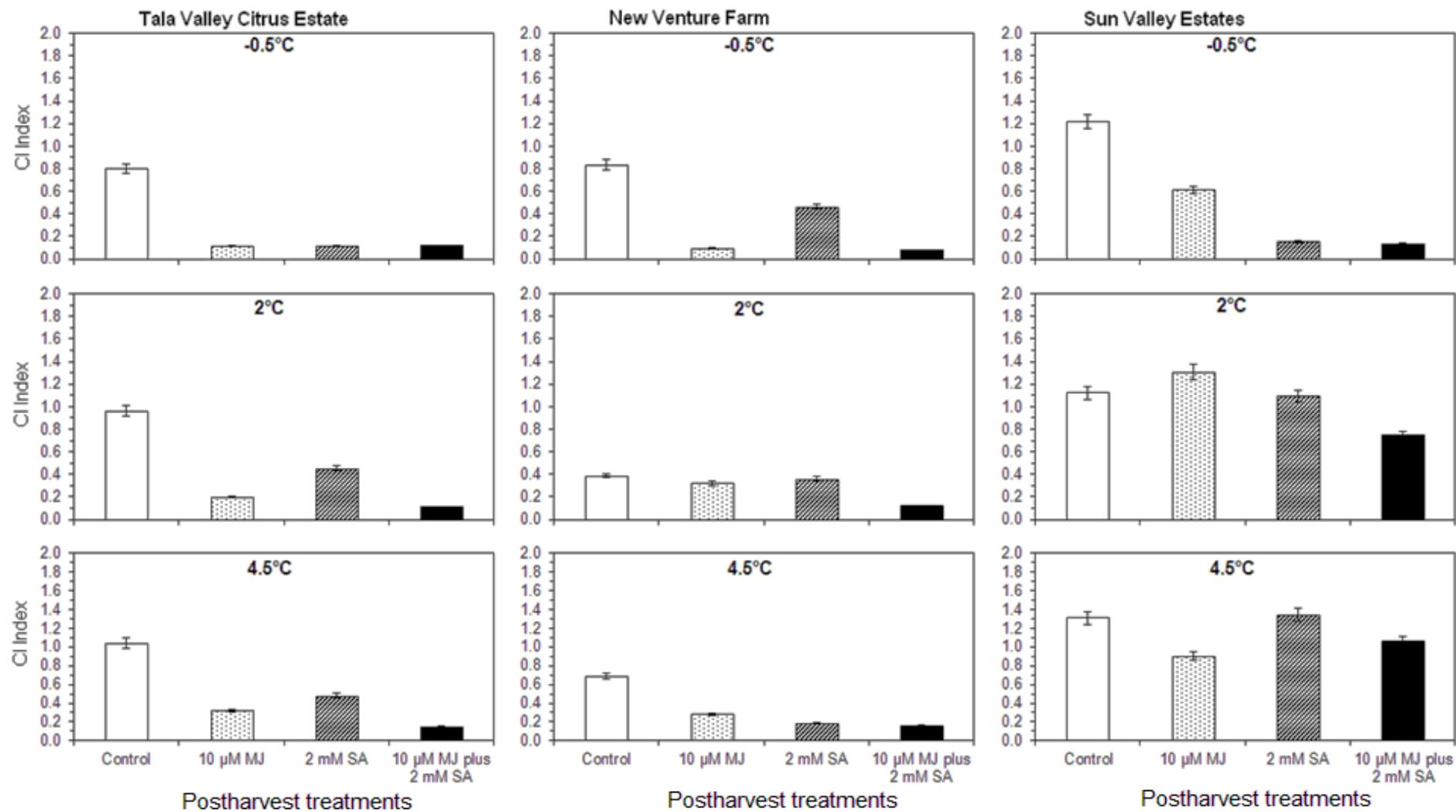


Fig. 2.

## Chapter 3

# The role of methyl jasmonate and salicylic acid in enhancing chilling tolerance in lemon [*Citrus limon* (L.) Burm. F.]

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## **Abstract**

Lipid peroxidation results in membrane damage through the action of reactive oxygen species (ROS); this causes rapid alterations in membrane permeability, respiration rate and increase in mass loss which leads to chilling injury (CI) in subtropical fruit kept under cold storage. Postharvest treatments such as methyl jasmonate (MJ) together with salicylic acid (SA) have shown potential to mitigate CI in lemons; however, the mechanisms by which these compounds are able to enhance tolerance are poorly understood. The objective of this study was to determine the possible mechanisms by which MJ and SA are able to enhance chilling tolerance in lemon fruit and maintain fruit quality. Lemon fruit were treated with 10  $\mu$ M MJ, 2 mM or 10  $\mu$ M MJ plus 2 mM SA and were stored either at -0.5, 2 or 4.5°C for up to 28 days and at 23°C for 7 days. This treatment was best able to maintain membrane integrity, thereby retarding electrolyte leakage, membrane lipid peroxidation as well as mass loss and respiration rate also inducing rind antioxidants, such as vitamin E and carotenoids, probably enhancing chilling tolerance. Treatment with 10  $\mu$ M MJ plus 2 mM SA also had an effect on reducing mass loss and respiration rate and in inducing antioxidants such as vitamin E and carotenoids. The production of antioxidants could have probably been part of a defence system against chilling damage, and maintaining fruit quality. Antioxidants such as Vitamin E and carotenoids could probably be implicated in the action of postharvest chemical treatments with 10  $\mu$ M MJ plus 2 mM SA to enhance chilling tolerance in lemons.

*Keywords:* Carotenoids; Chilling injury; Lipid peroxidation; Mass loss; Respiration rate; Vitamin E

## 1. Introduction

Postharvest losses in citrus fruit are mainly caused by physical (mass loss) and biological (decay), nutritional and physiological disorders (Ladaniya, 2008). Chilling injury (CI) is a physiological disorder that commonly occurs in tropical and subtropical crops during cold storage (Holland et al., 2002; Kluge et al., 2003; Sanchez-Ballesta et al., 2004). Cold storage below 10°C compromises marketability of lemon fruit, which are very prone to CI (Lafuente et al., 2005). The symptoms of which are pitting and necrosis manifest on the flavedo (Lafuente et al., 2004).

Chilling affects membrane integrity and increases electrolyte leakage (Campos et al., 2003) and lipid peroxidation (Siboza and Bertling, 2013). Cell membrane damage has been suggested to be the cause of CI, setting off a cascade of secondary reactions, such as an increase in respiration rate and production of reactive oxygen species (ROS) (Kluge et al., 2003). Basically, electrolyte leakage contributes to the secondary chain reactions. However, fruit contain lipid-soluble antioxidants such as vitamin E and carotenoids, which act as free radical scavengers (Chanjirakul et al., 2006) protecting cell membranes from lipid peroxidation, and maintaining cellular viability. Within the citrus family, the increase in ion leakage has been reported to be a sensitive indicator of CI in grapefruit flavedo (Forney and Peterson, 1990; Cohen et al., 1994).

Lemon fruit are non-climacteric and exhibit a low respiration rate (Kader and Arpaia, 2002; Porat et al., 2004). Cold storage plays a pivotal role by lowering fruit respiration rate further (Vines et al., 1968). The ability of citrus fruit to continue losing water during postharvest storage depends on the amount of water present in the fruit at harvest (Hung et al., 2011). Postharvest fruit water loss causes fruit shrinkage and mass loss (Hung et al., 2011),

negatively affecting fruit appearance as consumers dislike shrivelled fruit (Ladaniya, 2008). An eco-friendly postharvest treatment to maintain fruit quality whilst reducing CI is needed.

Methyl jasmonate (MJ), a plant hormone that plays a role in responses to various stresses (Creelman and Mullet, 1997), has shown potential to reduce CI in tomato (*Lycopersicon esculentum* L. cv. Beefstake) stored at 5°C for 4 weeks (Ding et al., 2001), and guava (*Psidium guajava*) stored at 5°C for up to 15 days and 2 days at 20°C (González-Aguilar et al., 2004). Salicylic acid (SA), also a plant hormone, that can act as an antioxidant which plays a role in regulating plant growth, and mediating defence against high temperature stress and pathogens (Yordanova and Popova, 2007). Recently, SA has been associated with increasing CI tolerance in certain fruit, such as peach (*Prunus persica* (L.) Batch.cv. Beijing 24) stored at 0°C for 28 days, then at 20°C for 3 days (Wang et al., 2006) and pomegranate (*Punica granatum* cv. Malas Saveh) stored at 2°C for 3 months (Sayyari et al., 2009).

The susceptibility of horticultural crops to CI depends on cultivar, genetic make-up and origin of the fruit, environmental conditions during growth and metabolic status of the flavedo (Wang, 2010). Although MJ and SA may be able to reduce CI in lemon fruit during cold storage at -0.5°C for up to 42 days plus 7 days at 23°C (Siboza and Bertling, 2013), the mechanism afforded by these compounds in maintaining fruit quality while reducing CI is not well known. Therefore, in this study, physiological aspects, such as membrane integrity and respiration rate were examined. In addition fruit quality aspects such as mass loss, vitamin E and total carotenoids of fruit from different origin were evaluated.

## 2. Materials and Methods

### 2.1 Plant material

'Eureka' lemon fruit [*Citrus limon* (L.) Burm. F.] were harvested from two commercial farms in KwaZulu-Natal, South Africa. New Venture Farm [moderate sub-tropical (mean annual temperature: 14.5-26.5°C; mean annual rainfall: 712-717 mm; 31° 02' S 29° 25' E, 68-483 m asl)]. Tala Valley Citrus Estate [warm temperate (mean annual temperature: 12.6-23.7°C; mean annual rainfall: 612-771 mm; 29° 52' S 30° 30' E, 416-922 m asl)]. Lemon fruit were harvested on the 6<sup>th</sup> of June 2011 and 2012 seasons, and the trends were similar. Fruit were immediately transported on paved roads from the collection site to the laboratory in a ventilated vehicle. Fruit transportation distances from farm locations to the laboratory site were 39.2 km (Tala Valley Citrus Estates), 130 km (Sun Valley Estates) and 228 km (New Venture Farm). Upon arrival, fruit were selected for uniformity of parameters such as size, shape and colour.

### 2.2 Postharvest treatments and storage

Lemon fruit were treated according to Sibozza and Bertling (2013). Fruit were dipped either into 10 µM MJ, 2 mM SA or 10 µM MJ plus 2 mM SA for 30 s. Another group of lemon fruit, not dipped in any solution, was used as the control. Following treatment the fruit were allowed to air-dry at 23°C for 2 h. Subsequently, fruit were waxed, weighed and cold-stored at -0.5, 2, or 4.5°C (air delivery temperature). The relative humidity was maintained at 85-90% in the shipping container. Five replicates of 20 fruit per treatment were sampled after 0, 7, 14, 21 or 28 days into cold storage. After removal from the cold storage, fruit were transferred to 23°C for a week (shelf-life) to determine CI on the fruit. The flavedo was carefully removed from the fruit and immediately frozen in liquid nitrogen, ground in a mortar to a fine powder and stored at -70°C until further analysis.

### *2.3 Estimation of CI index*

Fruit were evaluated for CI prior to storage, as well as after 7, 14, 21, and 28 days into cold storage as well as after shelf-life. The CI severity on the lemon fruit was visually rated according to (Lafuente et al., 2004): 0 = normal (pitting), 1 = slight pitting (a few scattered pits), 2 = moderate pitting (pitting covering up to 30% of the fruit surface), 3 = severe pitting (extensive pitting covering > 30% of the fruit surface) and expressed as CI index.

### *2.4 Determination of fruit mass loss*

The assumption that fruit mass loss is fruit water loss is common in postharvest research (Gómez et al., 2005). Therefore, fruit mass for each lemon fruit was determined before and after cold storage, as well as after shelf life. The percentage fruit mass loss was calculated as follows:  $\text{fruit mass loss (\%)} = [(\text{initial fruit mass (g)} - \text{final fruit mass (g)}) / \text{initial fruit mass (g)}] \times 100\%$  (Gómez et al., 2005).

### *2.5 Measurement of fruit respiration rate*

The fruit respiration rate was measured as CO<sub>2</sub> production at each sampling date by sealing each fruit in a 1 L jar for 15 min. The CO<sub>2</sub> of the atmosphere in the jars was measured using an environmental gas analyser (EGM-4; PP Systems, Hitchin, Hertfordshire, UK) and the respiration rate was expressed in ml kg<sup>-1</sup> FW h<sup>-1</sup> (Blakey et al., 2009).

### *2.6 Determination of membrane electrolyte leakage*

Membrane permeability was expressed as tissue electrolyte leakage (EC) and determined according to Cohen et al. (1994), with slight modification. Five fruit disks of lemon flavedo were immersed in 15 ml distilled water and incubated at 23°C with constant shaking for 3 hrs. The initial EC (EC<sub>1</sub>) of the solution containing the fruit disks was measured using a conductivity meter (HIM 9033; Hanna Instruments, Johannesburg, RSA). The solution

containing the fruit disks was then incubated with constant shaking at 100°C for 1 hr, before the final EC (EC 2) was measured. The EC % was calculated as the ratio of the initial reading to the final reading.

### *2.7 Flavedo sample preparation*

After the evaluation of CI, flavedo tissue of each fruit was carefully removed and immediately dipped in liquid nitrogen, and thereafter stored at -20°C. The flavedo tissue was later freeze dried and returned to -20 °C storage. Subsequently, the freeze dried flavedo tissue was pulverised in a mortar and pestle under liquid nitrogen and stored at -20°C for further evaluation of chilling damage in ultra-structural condition and for mineral-nutrient analysis.

### *2.8 Determination of lipid peroxidation*

The level of lipid peroxidation in lemon flavedo was measured according Chong et al. (2005). Lemon flavedo tissue (0.2 g DW) was homogenised in 4 ml of 0.1% (w/v) trichloroacetic acid (TCA) in a pre-chilled mortar and pestle at 4°C. The homogenate was centrifuged at 3000 × g for 5 min. A mixture of 10 ml supernatant was added to a test tube containing 1 ml solution comprising 20% (w/v) TCA, 0.01% (w/v) butylated hydroxytoluene, and 0.65% (w/v) thiobarbituric acid (TBA). Samples were then mixed vigorously, heated at 95°C for 30 min and cooled to 4°C, before centrifugation at 3,000 × g for 10 min. Absorbance was then read at 532 nm and 600 nm. Total malondialdehyde (MDA) equivalents were calculated and expressed as Total MDA (nmol g<sup>-1</sup> DW) according to Sibozza and Bertling (2013).

### *2.9 Extraction and determination of vitamin E*

Vitamin E was extracted from lyophilized lemon flavedo powder according to Chong et al. (2005) using 0.2 g DW flavedo. The sample was homogenised in 1 ml acetone at 4°C, 0.5 ml

hexane was added, the homogenate vortexed for 30 s and then centrifuged at  $1000 \times g$  for 10 min. The upper hexane layer was discharged and the extraction process was repeated twice by adding 0.5 ml hexane to the remaining pellets. Following this, an amount of 0.4 ml 0.1% (w/v) 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine (PDT) was added to 0.2 ml of pooled supernatant and the volume was made up to 3 ml with absolute ethanol. After adding 0.4 ml 0.1% (w/v) ferric chloride ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ), the sample was gently mixed under dim light in a dark room to avoid photochemical reduction. After 4 min reaction at room temperature, 0.2 ml of 0.2 M ortho-phosphoric acid was added and the mixture was allowed to stand for 30 min. Absorbance was read at 554 nm, the maximum absorbance of the PDT reaction mixture, ferric chloride and  $\alpha$ -tocopherol (vitamin E). A blank was prepared in the same manner except that absolute ethanol was used instead of the sample. Vitamin E was used as a standard and expressed as vitamin E ( $\mu\text{g g}^{-1}$  DW).

#### 2.10 Determination of total carotenoids

Total carotenoids were determined according to Lichtenthaler (1987). Flavedo tissue (0.2 g DW) was ground in 2 ml 80% (v/v) acetone and centrifuged at  $3000 \times g$  for 10 min at  $4^\circ\text{C}$ . For maximum detection of carotenoids, as well as chlorophyll *a* and *b*, the absorbance of the supernatant was read at 470, 646.8 and 663.2 nm to allow calculation of the concentration of total carotenoids, chlorophyll *a* and *b* as follows:

$$C_a = 12.25 A_{663.2} - 2.79 A_{646.8}$$

$$C_b = 21.50 A_{646.8} - 5.10 A_{663.2}$$

$$C_{x+c} = (1000 A_{470} - 1.82 C_a - 85.02 C_b) / 198,$$

where  $C_a$  represents chlorophyll *a*,  $C_b$  chlorophyll *b* and  $C_{x+c}$  total carotenoids.

### 2.11 Data analysis

Data obtained from the analytical determinations were subjected to analysis of variance (ANOVA) using GenStat® 14<sup>th</sup> Edition statistical package (VSN International Ltd., Hemel Hempstead, UK). A least significant difference test (t-test) was used to compare the two farms. Differences at  $P < 0.05$  were considered to be significant.

## 3. Results

### *3.1. The manifestation of CI symptoms in lemon fruit from different farm locations in response to MJ and SA postharvest treatment, storage temperature and cold storage duration.*

Lemon fruit were chilling sensitive and noticeable CI symptoms developed during shelf-life (Fig. 1). The manifestation of CI significantly ( $P < 0.05$ ) increased with shelf-life storage. The symptoms were significantly ( $P < 0.05$ ) influenced by postharvest treatments (MJ and SA), storage temperatures (-0.5, 2 or 4.5°C), farm locations (New Venture Farm and Tala Valley Citrus Estate) and, cold storage duration (0, 7, 14, 21, or 28 plus week shelf-life). In untreated lemon fruit, CI symptoms were observed after 7 days of storage and were most severe when lemon fruit were transferred from cold storage to room temperature for one week (Fig. 1). While the severity of CI significantly increased with cold storage time in untreated lemon fruit (Fig. 1), postharvest treatment with 10 µM MJ plus 2 mM SA significantly ( $P < 0.05$ ) reduced and delayed the occurrence of CI symptoms. Treatment with 10 µM MJ plus 2 mM SA effectively reduced CI (Fig. 1). Storing lemon fruit at -0.5°C resulted in reduced CI compared with 2°C storage, while at 4.5°C the development of CI symptoms was accelerated (Fig. 1).

*3.1. The effect of MJ and SA postharvest treatment, storage temperature, farm location and cold storage duration on fruit mass loss.*

Fruit mass loss was significantly ( $P < 0.05$ ) affected by postharvest treatment with MJ and SA, storage temperature, farm location, storage duration and the interactions of these factors (Fig. 2). Higher mass loss was detected in susceptible lemon fruit (untreated) than in chilling tolerant lemon fruit (MJ and SA treated). Storing lemon fruit at room temperature for an additional 7 days (shelf-life) after cold storage led to a significant increase ( $P < 0.05$ ) in fruit mass loss. Postharvest treatment with 10  $\mu\text{M}$  MJ plus 2 mM SA effectively reduced fruit mass loss, allowing lemon fruit treated with 10  $\mu\text{M}$  MJ plus 2 mM SA to remain turgid after shelf-life. When treated with either 10  $\mu\text{M}$  MJ or 2 mM SA the observed mass loss was less than in untreated in lemon fruit, but these single treatments were not as effective as the combination of the two. Lemon fruit held at 4.5°C, which developed severe chilling injury, had the highest mass loss followed by those held at 2°C. Significant ( $P < 0.05$ ) low mass loss was observed in lemon fruit held at -0.5°C which also displayed no signs of chilling injury.

*3.2. Changes in respiration rates of lemon fruit from different farm locations in response to MJ and SA postharvest treatment, storage temperature, farm location and cold storage duration.*

Significant ( $P < 0.05$ ) differences with respect to fruit respiration rate were observed in lemon fruit from different farm locations in response to postharvest treatment with MJ and SA, storage temperature and duration (Fig. 3). Untreated, chilling susceptible, lemon fruit displayed high mass loss and were found to have high respiration rates, whereas MJ and SA treated (chilling tolerant lemon fruit) were found to have low mass loss and a low respiration rate, with 10  $\mu\text{M}$  MJ plus 2 mM SA being most effective in retarding respiration, followed by the individual treatments of either 10  $\mu\text{M}$  MJ or 2 mM SA (Fig. 3). The lowest respiration rate

was observed in lemon fruit treated with 10  $\mu$ M MJ plus 2 mM SA, which remained turgid and displayed minimal CI; however, there were no significant differences in respiration rates of lemon fruit treated with either 10  $\mu$ M MJ or 2 mM SA, similar to the trend observed in mass loss. Storing lemon fruit at 4.5°C resulted in severe CI and was aligned with a high respiration rate that gradually increased throughout cold storage and shelf-life.

*3.3.Changes in electrolyte leakage in lemon flavedo of fruit from different farm locations in response to MJ and SA postharvest treatment, storage temperature and cold storage duration.*

The electrolyte leakage of lemon flavedo was significantly ( $P < 0.05$ ) influenced by treatments, storage temperature, farm location, storage duration and the interactions of these factors. In control fruit, electrolyte leakage increased with cold storage time and reached a peak by 14 days into cold storage (Fig. 4). The increase in electrolyte leakage at 14 days paralleled with the development of CI. However, the differences in electrolyte leakage were much smaller than those found in CI. The electrolyte leakage in MJ and SA treated lemon fruits was consistently lower than in untreated ones (Fig. 4). Treatment with MJ and SA significantly ( $P < 0.05$ ) inhibited electrolyte leakage with 10  $\mu$ M MJ concentration being the most effective concentration followed by 2 mM SA. Treatment with 10  $\mu$ M MJ plus 2 mM SA was the third most effective concentration in inhibiting this electrolyte leakage during cold storage (Fig. 2).

*3.4.The effect of MJ and SA postharvest treatment, storage temperature and cold storage duration on MDA content in lemon flavedo of fruit from different farm locations.*

Total MDA concentration was significantly ( $P < 0.05$ ) affected by treatment, storage temperature, farm location, storage duration and their interactions; cold storage at -0.5, 2, or 4.5°C significantly increased total MDA concentration to a peak 14 days into cold storage

(Fig. 5), while postharvest treatment with MJ and SA inhibited total MDA concentration significantly ( $P < 0.05$ ). Treatments with 10  $\mu$ M MJ plus 2 mM SA consistently inhibited Total MDA concentration (Fig. 5). Total MDA concentration significantly increased with storage temperature, particularly in untreated lemon fruit stored at 4.5°C. However, postharvest treatment with 10  $\mu$ M MJ plus 2 mM SA significantly ( $P < 0.05$ ) inhibited total MDA concentration, even at this higher temperature.

*3.5.Changes in vitamin E ( $\alpha$ -tocopherol) concentration in lemon flavedo of fruit from different farm locations in response to MJ and SA postharvest treatment, storage temperature and cold storage duration.*

Tocopherol concentrations in lemon flavedo were significantly ( $P < 0.05$ ) affected by treatment, storage temperature, farm location, storage duration and the interaction of these factors (Fig. 6). Tocopherol levels increased with cold storage time up to 14 days of storage and then gradually declined (Fig. 6). The decrease in tocopherols after 14 days of storage was associated with the manifestation of CI symptoms in untreated lemon fruit (Fig. 1); however, particularly, postharvest treatment with 2 mM SA and the 10  $\mu$ M MJ plus 2 mM SA combination resulted in higher tocopherol levels and significantly ( $P < 0.05$ ) delayed the reduction of these antioxidants after 14 days of cold storage. Low tocopherol levels were observed in untreated lemon fruit with severe CI symptoms, while tocopherol levels were high in chilling tolerant lemon fruit stored at -0.5°C, as well as those stored at 2°C.

*3.6.The effect of MJ and SA postharvest treatment, storage temperature, farm location and cold storage duration on total carotenoids in lemon rinds*

The total carotenoid concentration in lemon flavedo was significantly ( $P < 0.05$ ) affected by treatment, storage temperature, and duration as well as by the interaction between treatment and storage temperature, treatment and farm location and storage temperature and storage

duration (Fig. 7) A decline in total carotenoids was observed in untreated lemon fruit that showed severe CI. Treatment with MJ and SA prevented the depletion of total carotenoids in lemon flavedo during cold storage, with 10  $\mu$ M MJ plus 2 mM SA being most effective (Fig. 7).

#### **4. Discussion**

Consumers' awareness of quality has led to demand for high quality fresh fruit. To meet these needs, farmers, exporters and distributors have to produce fruit of high quality and maintain quality at postharvest. Therefore, sound postharvest management practices aimed at minimising losses whilst preserving the nutritional quality and freshness of fruit are crucial for ensuring better economic returns to growers and availability of high quality fruit to consumers (Ladaniya, 2008). To minimise the high dependence on potentially hazardous chemicals used to reduce CI and maintain fruit quality, the use of naturally occurring substances and sustainable non-chemical techniques such as MJ and SA have been suggested (Wang, 2006).

Our results are in agreement with previous research, which show that MJ and SA reduced CI in 'Eureka' lemon fruit during cold storage at  $-0.5^{\circ}\text{C}$  for 42 days plus 7 days at  $23^{\circ}\text{C}$  (Siboza and Bertling, 2013). In this study, untreated 'Eureka' lemon fruit showed CI symptoms, which mainly manifested as pitting, red blotch and necrosis. It has been reported that CI increases with cold storage duration and symptoms are often visible only after fruit have been transferred to non-chilling temperatures (Posmyk et al., 2005), making this physiological disorder a challenge to control.

The effective protection against CI with 10  $\mu$ M MJ plus 2 mM SA treatment may trigger flavedo defence mechanisms (Siboza and Bertling, 2013). Both hormones, MJ as well as SA,

are small signalling molecules involved in environmental stress responses in plants (Jin et al., 2009; Yang et al., 2012), possibly triggering defence mechanisms against CI in lemon fruit. Transcript levels of heat shock proteins, pathogenesis, and alternative oxidase protein genes have been reported to increase after MJ treatments in horticultural crops (Ding et al., 2001; Fung et al., 2004; Jin et al., 2009). Similarly, SA has been reported to reduce CI in horticultural crops thereby, enhancing enzymatic antioxidants (Yordanova and Popova, 2007).

At  $-0.5^{\circ}\text{C}$  storage, less CI was observed than at  $2^{\circ}\text{C}$ , while  $4.5^{\circ}\text{C}$  storage accelerated the CI symptom development (Fig. 1). A similar trend was observed in peaches and nectarines, where CI developed faster and more intense on fruit stored between  $2$  and  $5^{\circ}\text{C}$  than on those stored at  $0^{\circ}\text{C}$  (Lurie and Crisosto, 2005). Using  $10\ \mu\text{M}$  MJ plus  $2\ \text{mM}$  SA and a storage temperature of  $-0.5^{\circ}\text{C}$  can, however, effectively reduced CI symptoms (Fig. 1).

Ladaniya (2008) advised that high water loss has greater consequences, as it affects fruit appearance and mass, thereby reducing fruit marketability. In this study, the increase of mass loss at room temperature could be due to high transpiration rates when fruit were transferred to room temperature (Hung et al., 2011). The high mass loss was associated with CI and respiration rate. It has been reported that mass loss in fruit and vegetables is usually due to loss of water through transpiration, leading to shrivelling which could reduce consumer acceptability (Žnidarčič et al., 2010).

The results of this study suggest that fruit mass loss could be related to CI. The high fruit mass loss in untreated lemon fruit was possibly due to the high respiration rate leading to membrane damage. It has been reported that respiration of citrus fruit is affected by temperature, humidity and handling practises (Ladaniya, 2008). Lemon fruit are non-climacteric fruit with relatively low rates of respiration. In this study, lemon fruit exhibited respiration levels which

were associated to membrane damage or CI. Postharvest treatment with 10  $\mu$ M MJ plus 2 mM SA proved to be a potential tool for reducing respiration, retarding respiration rate and alleviated CI in lemon fruit. Previous studies show that as much as citrus fruit do not exhibit remarkably high respiration rate; increasing storage temperature increases respiration rate, while lowering the storage temperature restores the respiration rate (Vines et al., 1968). In this study, the same trend was observed and the high respiration rate in lemon fruit stored at 4.5°C was associated with irreversible membrane damage indicated by lipid peroxidation which was later followed by severe CI symptoms.

Low respiration rate was observed in lemon fruit stored at -0.5°C and this is suggestive of low CI. The decreased respiration rate in lemon fruit stored at -0.5°C paralleled lower fruit mass loss at that temperature. This suggested that using -0.5°C as a postharvest storage temperature may be good for maintaining fruit quality thereby, reducing respiration rate leading to low fruit mass loss, and CI symptoms. Our results agree with Baladrán-Quintana et al. (2003) that respiration rate of plants decreases with decreasing temperature, which could be a result of an increased Arrhenius activation energy in enzyme system. High respiration rate was observed when fruit were transferred from cold storage to shelf-life (23°C) and was followed by high mass loss and severe CI symptoms. An increase in respiration rate occurred at post-chilling as in chilling-injured tissue (Lyons and Breidenbach, 1990; Baladrán-Quintana et al., 2003).

An increase in respiration rates in zucchini after cold storage at 2.5°C was suggested to be due to the oxidation of compounds that accumulated during chilling stress (Baladrán-Quintana et al., 2003). It has been reported that high respiration rate in fruit and vegetables indicates permanent and irreversible tissue damage (Baladrán-Quintana et al., 2003). This was consistent with our results; the high respiration rate in lemon fruit was accompanied by an

increased fruit mass loss and CI symptoms. However, postharvest treatment with 10  $\mu$ M MJ plus 2 mM SA reduced respiration rate, fruit mass loss, and CI.

Electrolyte leakage (Zhao et al., 2009) increased with cold storage time. This may be indicative of membrane damage which could be associated with the occurrence of CI symptoms. In the flavedo of 'Marsh' grapefruit and 'Villa franca' lemon fruit stored at 2°C for 12 weeks and 4 days at 20°C, an increase in electrolyte leakage was reported to be a sensitive indicator of CI (Forney and Peterson, 1990; Cohen et al., 1994). In this study, electrolyte leakage was a sensitive indicator of CI as high electrolyte leakage was observed in untreated fruit (with severe CI). This was consistent with the suggestion that electrolyte leakage mainly occurs in a variety of chilled tissues (Campos et al., 2003). Postharvest treatment with 10  $\mu$ M MJ plus 2 mM SA could therefore, have reduced CI symptoms by maintaining membrane integrity and preventing solute leakage.

Posmyk et al. (2005) and Imahori et al. (2008) stated that membrane lipid peroxidation may be one of the first events in the development of CI, making membrane lipid peroxidation a good indicator of membrane disintegration during cold storage. The increased total MDA concentration (Fig. 5) which is indicative of membrane lipid peroxidation (Ke, 2007) was accompanied by a high respiration rate (Fig. 3) and mass loss (Fig. 2), was associated with the loss of membrane integrity (Fig. 4) and development of CI (Fig. 1). This study suggest that 10  $\mu$ M MJ plus 2 mM SA treatments has the potential to maintain external lemon fruit quality. It has been reported that the extent by which lipid peroxidation is activated depends on the severity of cold stress and is usually correlated with intensity of CI development (Imahori et al., 2008). The inhibition of total MDA concentration by 10  $\mu$ M MJ plus 2 mM SA treatment, suggests that MJ and SA have the potential to maintain membrane integrity during chilling.

It has been reported that plants synthesise compounds such as antioxidants which reduce the damaging effects of oxidative and other stresses (Posmyk et al., 2005). Therefore, plants have evolved an efficient antioxidant defence system to repair oxidative damage (Imahori et al., 2008). Antioxidants play a vital role in protecting cellular membranes, thus improving chilling tolerance in plants (Yang et al., 2011). Previous studies suggest that treatments with MJ and SA in lemon fruit may increase chilling tolerance by enhancing antioxidants, thereby reducing CI (Siboza and Bertling, 2013).

This study suggests that tocopherols were involved in chilling tolerance of MJ and SA-treated lemon fruit. The enhanced tocopherol levels in chilling tolerant lemon fruit (MJ and SA treated) may have maintained membrane integrity by inhibiting lipid peroxidation. The degradation of tocopherols in lemon fruit at cold storage temperatures paralleled with the severance of CI, mass loss, respiration rate and lipid peroxidation. The results of this study suggest that -0.5 and 2°C were good storage temperatures for maintaining antioxidants such as tocopherols which may be involved in chilling tolerance in lemon fruit. In citrus, flavedo colour is likely the most important external quality parameter used in determining consumer acceptance (Oberholster and Cowan, 2001). In this study, the decrease in carotenoids contents in control fruit may have increased ROS content (Ke, 2007). The accumulation of total carotenoids in flavedo of fruit treated with 10 µM MJ plus 2 mM SA could have protected lemon fruit from chilling thereby acting as antioxidant and preventing membrane lipid peroxidation. The results of this study suggest that total carotenoids may be involved in inhibiting lipid peroxidation in the flavedo of lemon fruit, thereby enhancing chilling tolerance.

In conclusion, cold storage temperature, farm location and storage duration are the most important factors affecting fruit quality of 'Eureka' lemon fruit during postharvest. From the

results of this study it can be concluded that cold storage of 'Eureka' lemon can trigger mass loss and increased respiration rate as indications of stress. The manifestation of CI symptoms in 'Eureka' lemon fruit was effectively reduced by treatment with 10  $\mu$ M MJ plus 2 mM SA. Treatment with 10  $\mu$ M MJ plus 2 mM SA maintained membrane integrity thereby retarding mass loss, respiration rate, electrolyte leakage and membrane lipid peroxidation. This treatment also enhanced lipid-soluble antioxidants such as tocopherols (vitamin E) and carotenoids which could have contributed to defence mechanisms, thereby reducing CI. This study provides evidence that fruit mass loss, respiration and membrane lipid peroxidation may be good indicators of CI. The enhancement of lipid-soluble antioxidants (total carotenoids and tocopherols) in lemon flavedo may have played a role in chilling tolerance. Therefore, the mode of action of 10  $\mu$ M MJ plus 2 mM SA in enhancing chilling tolerance in 'Eureka' lemon may be attributed to its ability to reduce membrane electrolyte leakage and lipid peroxidation, and maintain lipid-soluble-antioxidant concentrations and by retarding mass loss, and respiration rate.

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Fig. 7. The effects of 10  $\mu\text{M}$  methyl jasmonate (MJ), 2 mM salicylic acid (SA) or 10  $\mu\text{M}$  MJ plus 2 mM SA treatments on the accumulation of total carotenoids ( $\mu\text{g g}^{-1}$  DW) in the flavedo of lemon fruit during cold storage. Values are the mean of five replicate samples  $\pm$  S.E.  $\text{LSD}_{0.05} = 0.47$ .

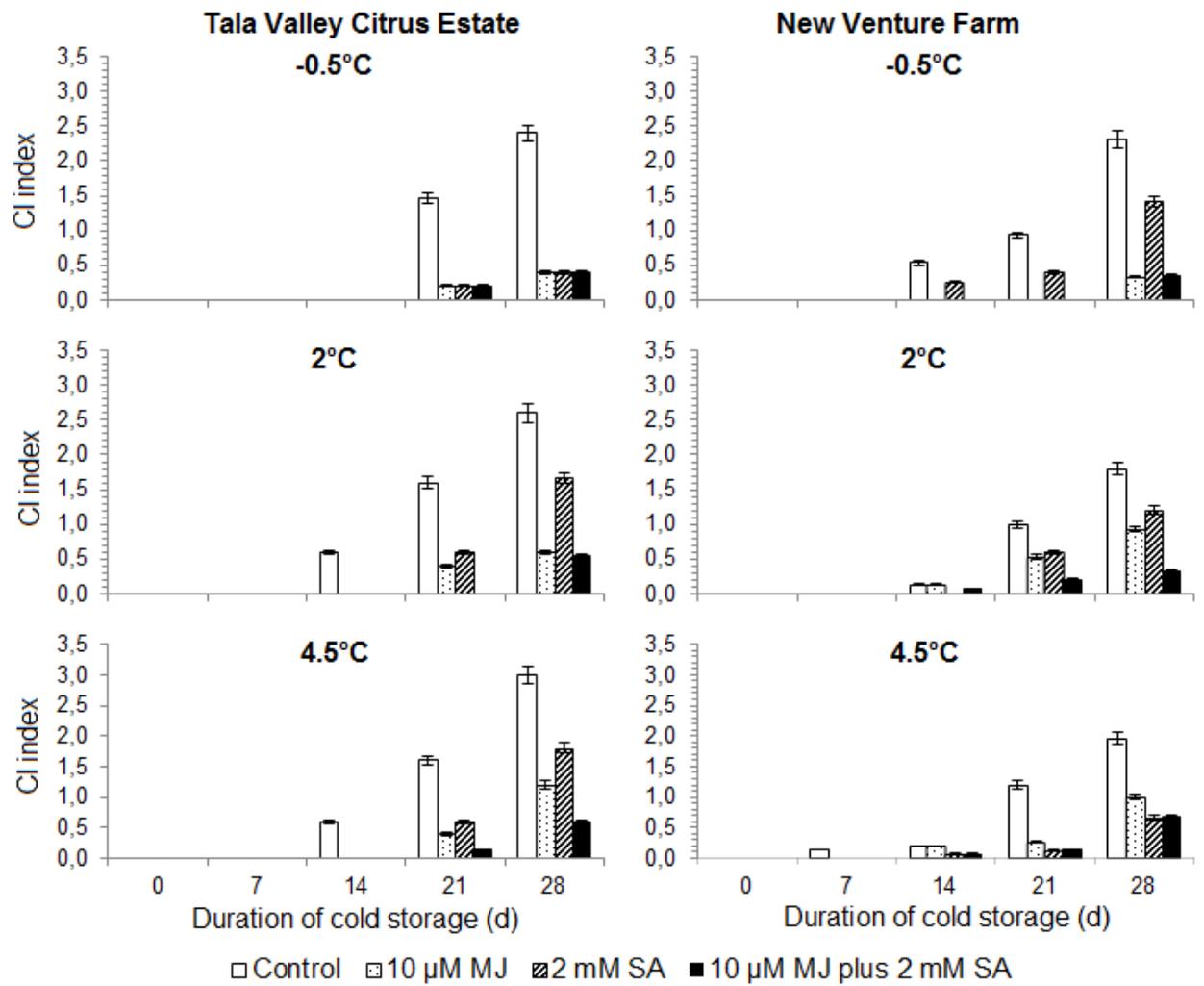


Fig. 1.

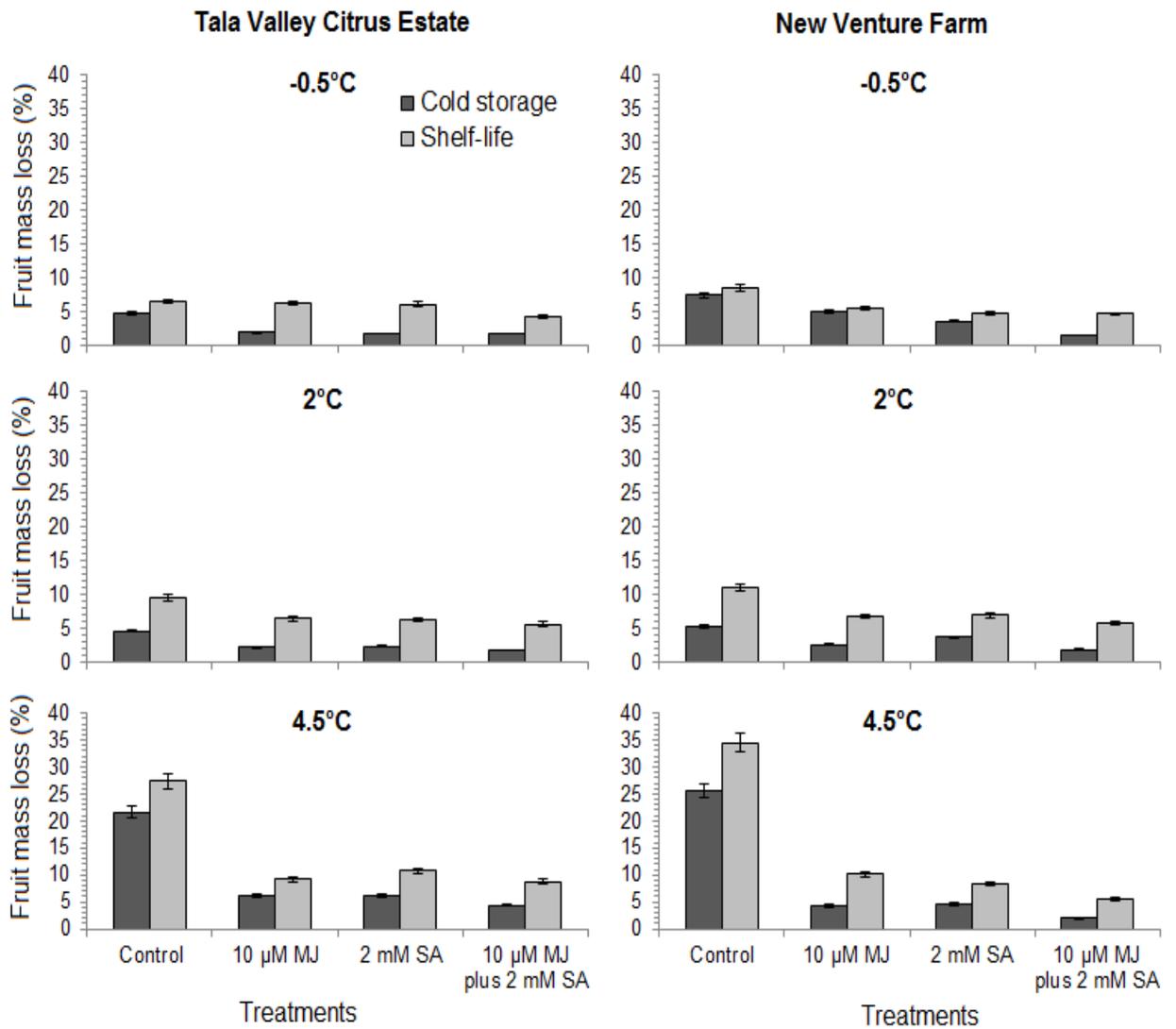


Fig. 2.

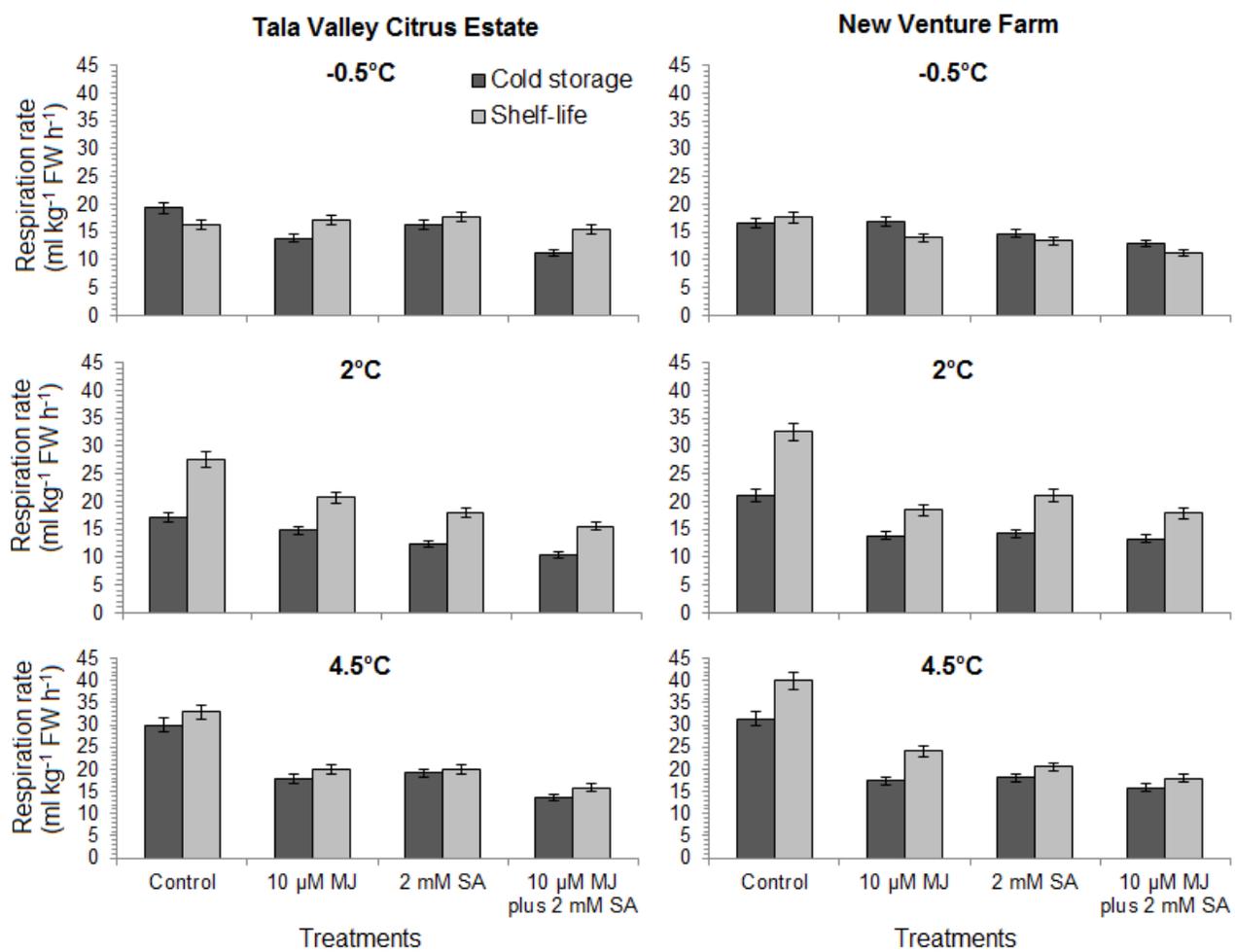


Fig. 3.

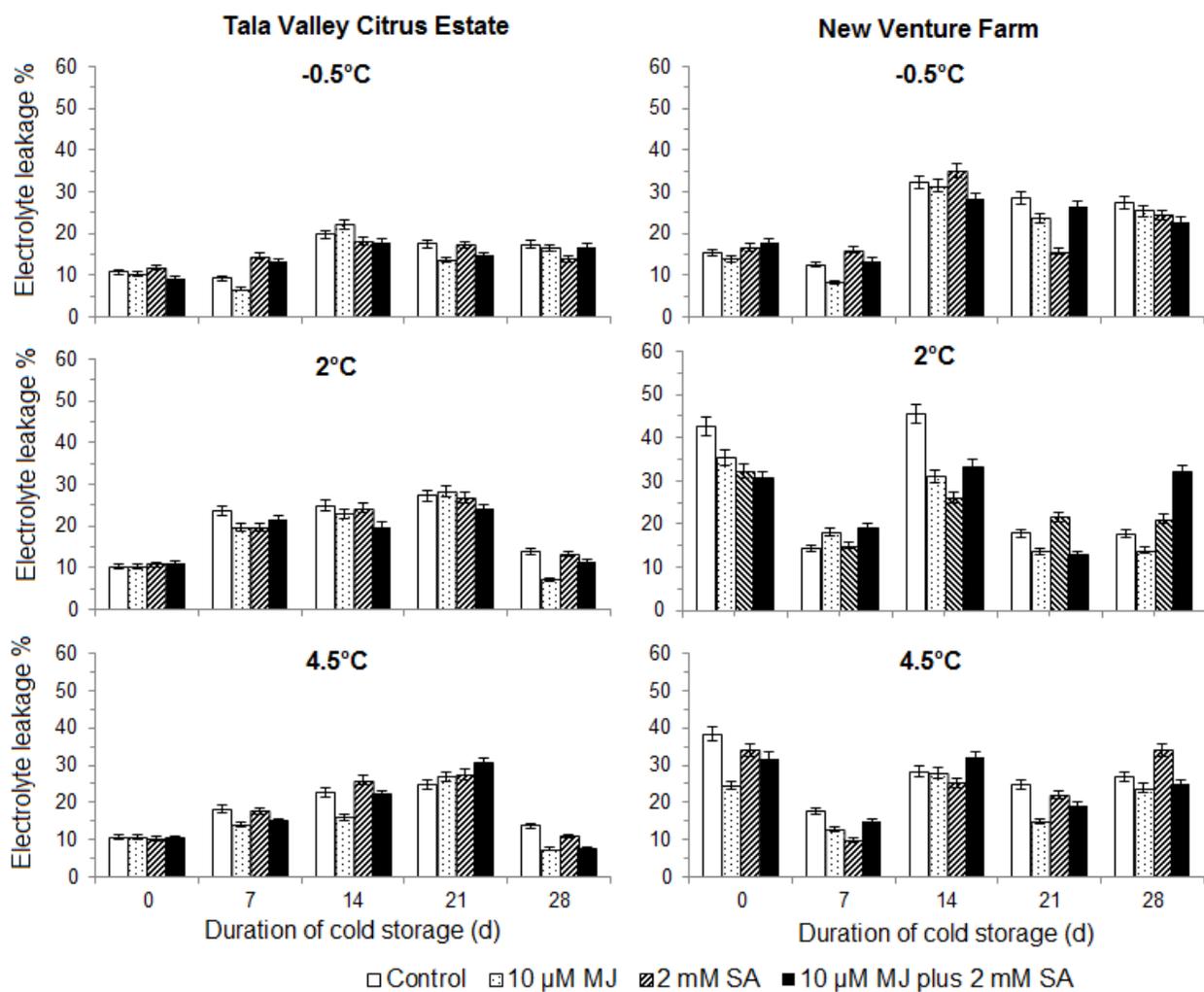


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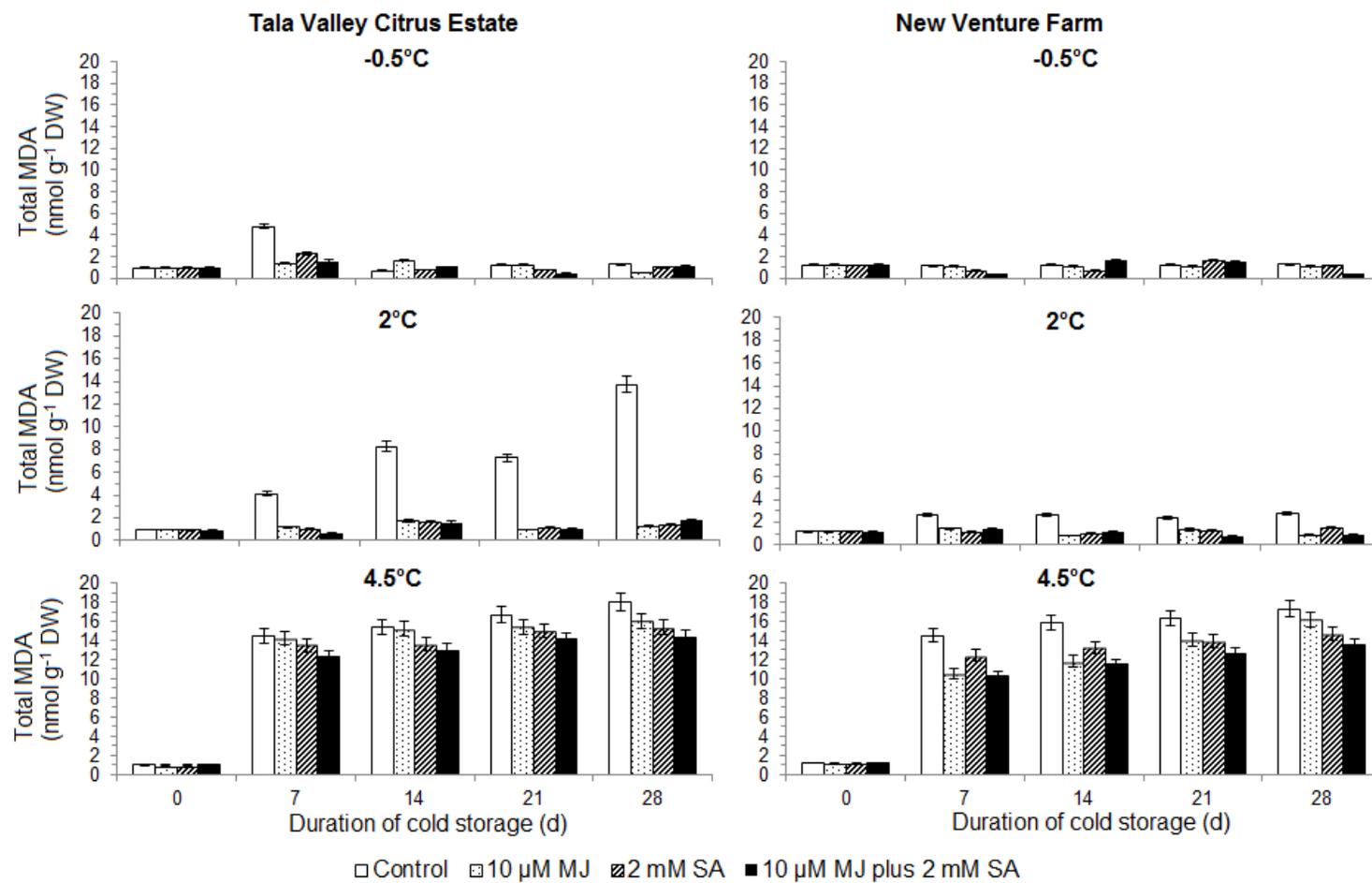


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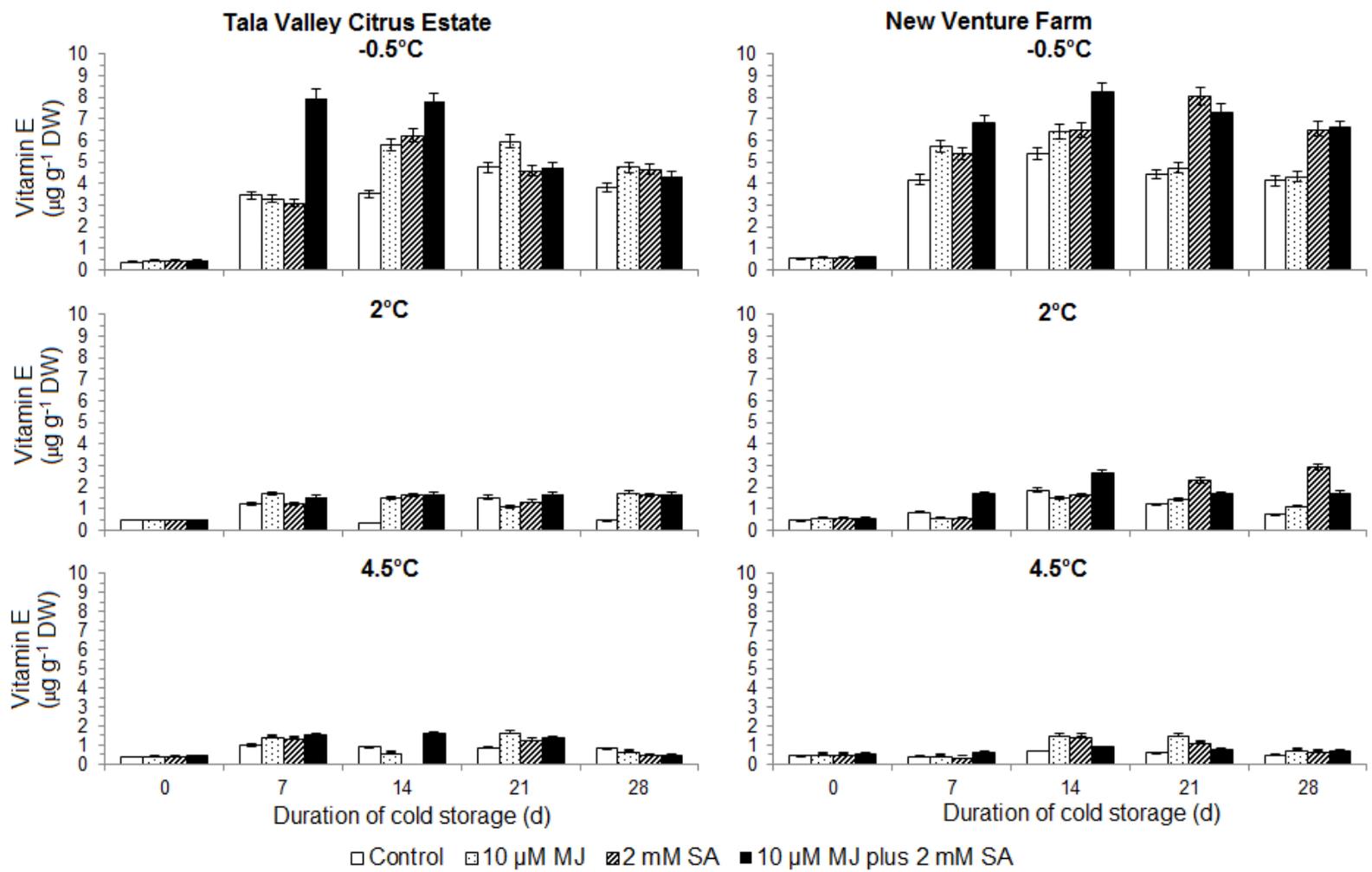


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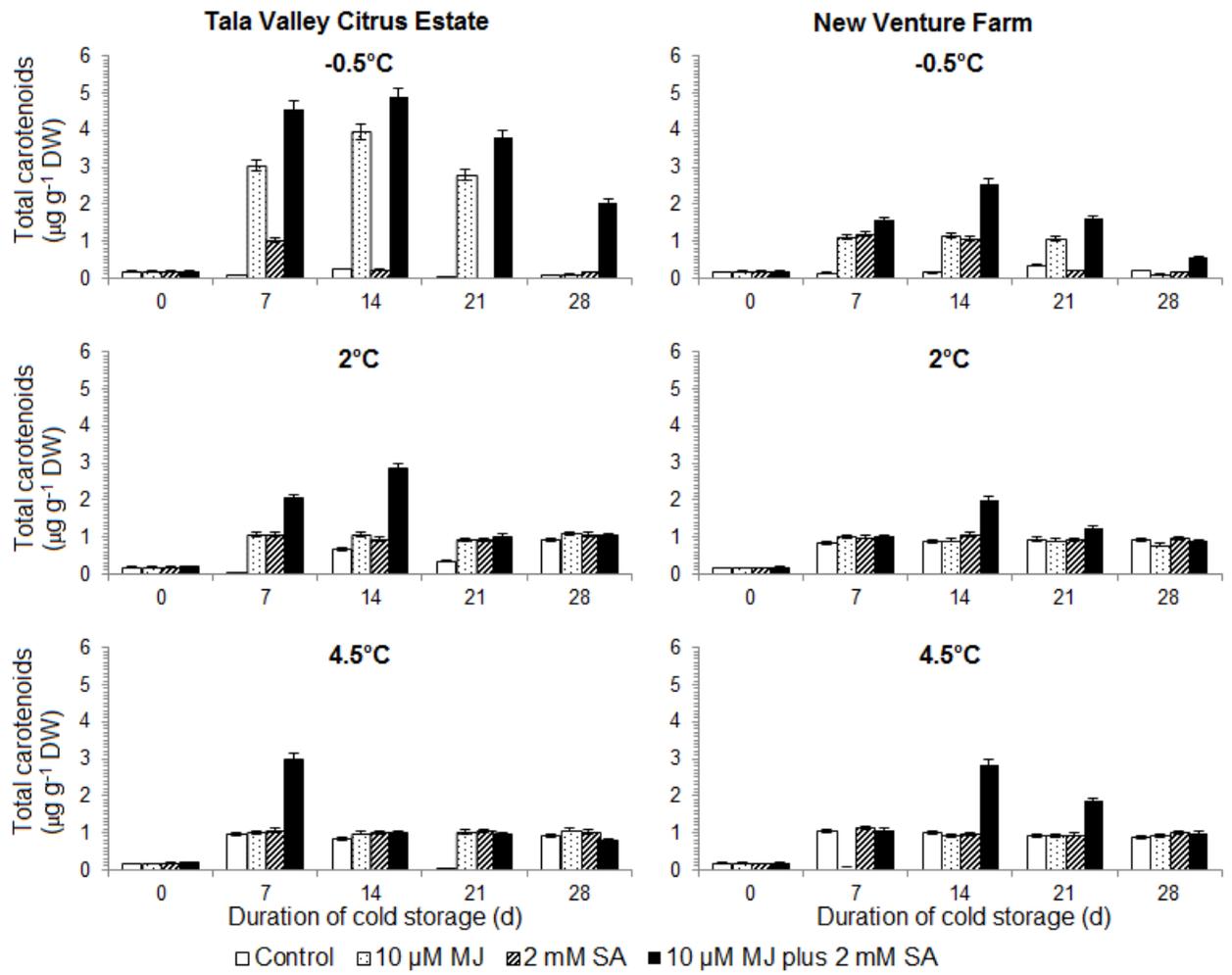


Fig. 7.

## Chapter 4

Soluble sugars, proline and ascorbic acid; are they involved in the response of lemons (*Citrus limon*) to chilling stress?

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## **Abstract**

Cold storage of lemons often leads to chilling injury (CI), a physiological disorder that causes pitting, red blotch, surface lesion and staining in the lemon rind, limiting fruit marketability. It was hypothesised that treatment with methyl jasmonate (MJ) and salicylic acid (SA) may enhance chilling tolerance in lemon fruit by enhancing the synthesis of biochemical compounds such as proline, ascorbic acid and soluble sugars in lemon flavedo. These biochemical compounds are thought to be involved in the response of lemon fruit to chilling temperatures and the action of MJ and SA mediated reduction of CI in lemon fruit. Lemon fruit were collected from moderate subtropical (mean annual temperature: 14.5-26.5°C; mean annual rainfall: 712-717 mm), warm temperate (mean annual temperature: 12.6-23.7°C; mean annual rainfall: 612-771 mm) and cool subtropical (mean annual temperature: 10.7-26.5°C; mean annual rainfall: 669-675 mm) locations. Lemon fruit were treated with 10 µM MJ, 2 mM SA, or 10 µM MJ plus 2 mM SA before cold storage at -0.5°C for up to 28 days plus 7 days at 23°C. The concentration of soluble sugars, proline, and ascorbic acid increased in chilling tolerant fruit treated with MJ and SA. Postharvest treatment with MJ and SA significantly ( $P < 0.05$ ) enhanced the accumulation of proline, ascorbic acid and soluble sugars thought to be involved in chilling resistance. The treatment combination of 10 µM MJ plus 2 mM SA was more effective in alleviating CI and the synthesis of soluble sugars, proline and ascorbic acid than individual applications of either 10 µM MJ or 2 mM SA. This study suggests that biochemical compounds such as proline, ascorbic acid and soluble sugars in lemon flavedo during cold storage may be involved in chilling tolerance mediated by MJ and SA treatment.

*Keywords:* Ascorbic acid; Methyl jasmonate; Proline; Salicylic acid; Sugars

## 1. Introduction

The appearance of chilling injury (CI) symptoms, as pitting, red blotch and necrosis, on lemon fruit during postharvest storage and on the market affects consumer acceptability and may result in fruit rejection. The occurrence of this disorder is therefore a major threat to the citrus industry, limiting fruit marketability and causing economic losses (Maul et al., 2011; Zhang et al., 2011). Soluble sugars may influence the sensitivity of plant tissues to chilling stress (Purvis and Grierson, 1982; Holland et al., 2002) and increase cold acclimation (Holland et al., 2005), thereby limiting CI. The role of soluble sugars in chilling tolerance of citrus fruit has been investigated and conflicting results have been reported (Holland et al., 1999). In lemon fruit (*Citrus limon*), the accumulation of soluble sugars has been associated with chilling tolerance (Siboza et al., 2011). However, little is known about the impact of weather conditions at specific farm locations on the accumulation of soluble sugars in lemon fruit.

Proline is a protein involved in stress protection by scavenging reactive oxygen species (ROS) in plant tissues (Matysik et al., 2002; Claussen, 2005). Ascorbic acid, known to protect plant tissues against ROS, is also involved in chilling resistance of plant (Om-Arun and Siriphanich, 2005; Badejo et al., 2009). High accumulation of ascorbic acid has been associated with chilling resistance in pineapples (*Ananas comosus*. L) stored at 10°C for 3 weeks (Om-Arun and Siriphanich, 2005). When used as postharvest treatments, methyl jasmonate (MJ) and salicylic acid (SA) modulate stress defence responses in crops (Ding et al., 2001; Zhang et al., 2011). In citrus, MJ reduced CI in grapefruit (*Citrus paradisi*) stored at 2°C for 4-10 weeks (Meir et al., 1996; Droby et al., 1999) and lemon fruit (*Citrus limon*) stored at -0.5°C for up to 42 days plus 7 days at 23°C (Siboza and Bertling, 2013).

Postharvest application of MJ to citrus fruit might provide a means to reduce CI in susceptible cultivars while maintaining fruit quality (Meir et al., 1996). Treatment with SA increased CI

resistance in tomato (*Solanum lycopersicon* L.) stored at 5°C for 4 weeks (Ding et al., 2001), pomegranate (*Punica granatum* L.) stored at 2°C for 3 months (Sayyari et al., 2009), and peach (*Prunus persica*) stored at 1°C for up to 5°C weeks (Yang et al., 2012). Little is known about the mode of action of MJ and SA in reducing CI (Siboza et al., 2011, 2012). Previous studies suggest that proline (Shang et al., 2011), soluble sugars (Holland et al., 2005) and ascorbic acid (Smirnoff and Wheeler, 2000) serve as defence mechanisms against various plant stresses by mainly by scavenging ROS and stabilising cell membrane homeostasis. Therefore, in this study the apparent changes in some biochemical compounds (proline, ascorbic acid and soluble sugars) were investigated as possible mechanisms triggered by MJ and SA treatment in alleviating CI in lemon fruit.

## **2. Materials and Methods**

### *2.1. Plant material*

Mature 'Eureka' lemon fruit [*Citrus limon* (L.) Burm.] were obtained from three commercial farms in KwaZulu-Natal, South Africa. The farms were classified according to climatic conditions (BRU, 2007). Fruit were obtained from New Venture Farm (moderate subtropical zone; mean annual temperature: 14.5-26.5°C; mean annual rainfall: 712-717 mm; 31° 02' S 29° 25' E, 68-483 m.asl), Tala Valley Citrus Estate (warm temperate zone; mean annual temperature: 12.6-23.7°C; mean annual rainfall: 612-771 mm; 29° 52' S 30° 30' E, 416-922 m asl) and Sun Valley Estates (cool subtropical zone: mean annual temperature: 10.7-26.5°C; mean annual rainfall: 669-675 mm; 28° 83' S 30° 06' E, 820-1003 m asl). Lemon fruit were harvested on the 6<sup>th</sup> of June 2011 and 2012, and the trends were similar. Fruit were immediately transported on paved roads from the collection site to the laboratory in a ventilated vehicle. Fruit transportation distances from farm locations to the laboratory site

were 39.2 km (Tala Valley Citrus Estates), 130 km (Sun Valley Estates) and 228 km (New Venture Farm).

Lemon fruit were carefully selected for uniformity in colour, size, and absence of defects.

### *2.2. Experimental design*

The experiment used a randomised complete block design with the following factors: farm location (3 levels; Tala Valley Citrus Estate, New Venture Farm and Sun Valley Estates); postharvest treatments (4 levels; Control, 10  $\mu$ M MJ, 2 mM SA, or 10  $\mu$ M MJ plus 2 mM SA); storage duration (5 levels; 0, 7, 14, 21 or 28 days) and storage temperature (1 level;  $-0.5^{\circ}\text{C}$ ). Farm location was not part of the treatment structure. Each treatment was replicated three times.

### *2.3. Postharvest treatment with MJ and SA and storage*

Lemon fruit were surface-sterilised, air dried and randomly allotted to the MJ and SA treatment concentrations according to Sibozza and Bertling (2013). Fruit were soaked into one of the following solutions: 10  $\mu$ M MJ, 2 mM SA, or 10  $\mu$ M MJ plus 2 mM SA (Sibozza and Bertling, 2013) for 30 s (Droby et al., 1999). A control (untreated lemon fruit) was also included. Fruit were waxed and air dried before cold storage at  $-0.5^{\circ}\text{C}$  (air delivery temperature) for 0, 7, 14, 21 or 28 days. Relative humidity was maintained at  $\approx 90\%$ . Five replicates of 20 lemon fruit per treatment were sampled at 0, 7, 14, 21 or 28 days into the cold storage. After removal from cold storage, fruit were transferred to room temperature ( $23^{\circ}\text{C}$ ) for 7 days to simulate shelf-life.

### *2.4. Estimation of CI Index*

After shelf-life simulation, all lemon fruit were visually inspected for CI symptoms on the flavedo. CI was scored using a scale of 0 (no pitting), 1 (slight pitting), 2 (medium pitting) and

3 (severe pitting). The CI index calculated as reported by Lafuente et al. (2004). Results are expressed as means of five replicate samples containing 20 fruit  $\pm$  SE.

### *2.5. Flavedo tissue sample preparation*

After the evaluation of CI symptoms, flavedo tissue was removed from lemon fruit and was immediately frozen in liquid nitrogen and lyophilized. For biochemical analysis the freeze-dried flavedo tissue was pulverized in a cold mortar using a pestle and liquid nitrogen.

### *2.6. Biochemical analyses*

All chemicals were obtained from Sigma-Aldrich Chemical Co. (St Louis, Missouri, United State of America).

*Determination of soluble sugars:* Soluble sugars were extracted from the previously prepared flavedo tissue by the method previously described by Tesfay et al. (2010). Flavedo tissue (0.5 g) was homogenised in 10 ml 80 % cold ethanol (4°C) using an UltraTurrax (Model T250, IKA-Germany) for 2 min. The homogenate was incubated at 80°C for one hour and cooled to 4°C; centrifuged at 12 000  $\times$  g for 15 min at 4°C, and filtered through glass wool. The supernatant was dried in a Savant SpeedVac® Concentrator (New York, United States of America) for 24 hrs. Dried samples were re-suspended in 2 ml ultrapure water, vortexed for 1 min and re-centrifuged. Samples were filtered through nylon syringe filters (0.45  $\mu$ m pore size) prior to High-Performance Liquid Chromatography (HPLC) (LC-20AT, Shimadzu Corporation, Kyoto, Japan) analysis. The HPLC was 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, California, United States of America) using distilled water as the mobile phase. The soluble sugar concentration of the

lemon flavedo was determined by comparison with that of sucrose, glucose and fructose standards.

*Determination of proline:* The proline concentration of lemon flavedo was determined according to Claussen (2005). Flavedo tissue (0.5 g) was extracted in 10 ml of a 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 incubated for 1 h in a boiling water bath. After incubation, samples were cooled to 21°C and kept at that temperature for 5 min. Sample absorbance was then spectrophotometrically determined at 546 nm. The proline concentration was determined by comparison with a standard curve and the flavedo proline concentration calculated on a dry weight basis as mmol proline (g DM)<sup>-1</sup>.

*Determination of ascorbic acid concentration:* The ascorbic acid concentration of flavedo tissue was determined according to Abeysinghe et al. (2007) with slight modifications. Previously prepared flavedo tissue (1 g DM) was extracted at 4°C with 10 ml 1% oxalic acid and homogenised for 10 min using an UltraTurrax (Model T250, IKA-Germany). The mixture was centrifuged at 20 000 × g for 10 min at 4°C and the supernatant was separated and subjected to the HPLC system. The HPLC was equipped with a UV detector (Photodiode Array detector PDA-100, Colombia, United States of America) set at 251 nm; ODS C18 column (4.6 x 250 mm). The flow rate was one ml per min with an injection volume of 20 µl. The mobile phase contained a mixture of 0.02 M ammonium acetate buffer (pH 5.4) and 1 mM octylamine in methanol. The ascorbic acid concentration of the lemon flavedo was expressed as mg / 100 g DM.

### *2.7. Data analysis*

Data were subjected to the statistical package, GenStat® 14<sup>th</sup> Edition (VSN International, Hemel Hempstead, UK). Means were separated using least significant difference at the 5% level of significance.

## **3. Results**

### *3.1. Postharvest treatment with MJ and SA in reducing CI on lemon fruit from Tala Valley, New Venture Farm and Sun Valley Estates during cold storage at -0.5°C*

Cold storage of lemon fruit at -0.5°C for 0, 7, 14, 21 or 28 days and 7 days at 23°C to simulate shelf-life resulted in the appearance of CI symptoms, mainly as pitting, necrosis and red blotch on the flavedo. Untreated lemon fruit were more chilling susceptible than MJ- and SA-treated lemon fruit; moreover, the severity of CI symptoms on lemon fruit varied with farm location (Fig. 1). Chilling injury symptoms were observed later in untreated lemon fruit at Tala Valley Citrus Estate (21 days) compared to New Venture Farm and Sun Valley Estates which showed CI symptoms earlier in the untreated fruit after 14 days. Postharvest treatment with MJ as well as SA significantly ( $P < 0.05$ ) reduced and retarded occurrence of CI symptoms; this varied with treatment (Fig. 1). Treatment with the 10 µM MJ plus 2 mM SA mixture was the most effective in reducing CI.

The 2 mM SA treatment was the second most efficient, followed by 10 µM MJ. The MJ as a treatment was more effective than SA at reducing CI on fruit from Sun Valley Estates, and the opposite was true for fruit gathered from New Venture Farm. The effects of all three

treatments (10  $\mu$ M MJ, 2 mM SA, and 10  $\mu$ M MJ plus 2 mM SA) with respect to CI index did not differ significantly between 14 and 28 days of storage fruit from Tala Valley Citrus Estate (Fig. 1).

### *3.2. Effect of MJ and SA treatments on proline in the flavedo of 'Eureka' lemon fruit*

Proline concentration in the flavedo of the lemon fruit was generally low, however, significant ( $P < 0.05$ ) differences were observed between treatment, cold storage time, location and interaction between these factors (Fig. 2). Postharvest treatment with MJ and SA significantly ( $P < 0.05$ ) enhanced proline concentrations during cold storage. Fruit treated with MJ and SA had higher proline concentration in the flavedo than in untreated ones. Treatment with 10  $\mu$ M MJ plus 2 mM SA was more effective in enhancing proline concentrations than 2 mM SA (Fig. 2). Treatment with 10  $\mu$ M MJ was the second most effective concentration in enhancing proline concentrations in lemon flavedo.

### *3.3. Changes in soluble sugars in the lemon flavedo of fruit treated with MJ and SA*

Soluble sugars (sucrose, glucose and fructose) in the flavedo of lemon fruit were significantly ( $P < 0.05$ ) affected by treatment, cold storage time and fruit location. Untreated lemon fruit had low concentrations of soluble sugars. Treatment with MJ and SA at various concentrations significantly ( $P < 0.05$ ) enhanced soluble sugars in lemon flavedo. The accumulation of soluble sugars in the flavedo of lemon fruit varied, with glucose being the most abundant, followed by sucrose and fructose. Fructose was the least abundant soluble sugar in the flavedo of the lemon fruit. Cold storage significantly ( $P < 0.05$ ) affected sucrose concentrations in the flavedo (Fig. 3). Sucrose concentration was significantly ( $P < 0.05$ ) higher in MJ and SA treated lemon fruit than in untreated lemon fruit (Fig. 3).

Treatment with 10  $\mu$ M MJ plus 2 mM SA was the most effective concentration in enhancing glucose and fructose (Fig. 3) in lemon flavedo, followed by the treatment with 10  $\mu$ M MJ. Soluble sugars were significantly ( $P < 0.05$ ) affected by the interactions between postharvest treatments and the farm locations. Treatment with 10  $\mu$ M MJ was the most effective concentration followed by 2 mM SA in enhancing sucrose concentration in the lemon flavedo from Tala Valley Citrus Estate. Low sucrose concentrations were observed in the flavedo of untreated lemon fruit from New Venture Farm and Sun Valley Estate; whereas, untreated lemon fruit from Tala Valley Citrus Estate tended to have high levels of sucrose in the flavedo, levels even comparable to those enhanced by the 10  $\mu$ M MJ treatment in lemon fruit from Sun Valley Estates.

Treatment with various concentrations of MJ and SA had an effect in enhancing glucose concentration in lemon fruit (Fig. 3). There were other similarities of treatment concentrations effectiveness in enhancing glucose concentrations in lemon fruit. There were no clear differences between glucose concentrations of lemon fruit from Sun Valley Estates treated with the combination of 10  $\mu$ M MJ plus 2 mM SA and those from Tala Valley Citrus Estate treated with 2 mM SA. The differences in glucose accumulation between untreated and 10  $\mu$ M MJ plus 2 mM SA treated lemon fruit from Tala Valley Citrus Estate were not significant. Similar trend was observed when there was no significant differences in glucose concentration of lemon fruit from New Venture Farm treated with 10  $\mu$ M MJ plus 2 mM SA or 10  $\mu$ M MJ.

The differences in glucose concentrations between untreated lemon from Sun Valley Estates and New Venture Farm lemon fruit treated with 2 mM SA or untreated lemon fruit from New Venture Farm were not significant. Treatment of lemon fruit from New Venture Farm with 10  $\mu$ M MJ enhanced more fructose concentration than the other treatments. No clear differences in fructose concentration were found between Sun Valley Estate lemon fruit treated with 2

mM SA and those from New Venture Farm treated with 10  $\mu$ M MJ plus 2 mM SA. The concentrations of fructose in New Venture Farm lemon fruit treated with 2 mM SA was the same as that of those left untreated. These concentrations were also the same in Tala Valley Citrus Estate lemon fruit treated with 10  $\mu$ M MJ. There were no significant differences in fructose concentration between Tala Valley Citrus Estate lemon fruit treated with 2 mM SA and Sun Valley Estate lemon fruit treated with 10  $\mu$ M MJ. Low concentrations of fructose were observed in untreated lemon fruit either from Tala Valley Citrus Estate or Sun Valley Estate. Lemon fruit from the Sun Valley Estate had the lowest concentrations of fructose concentration compared to lemon fruit from other locations.

#### *3.4.Changes in ascorbic acid concentration in lemon flavedo of fruit treated with MJ and SA*

Ascorbic acid in the flavedo of lemon fruit during cold storage was significantly ( $P < 0.05$ ) affected by treatments, location, and the interaction of these two factors. The concentration of ascorbic acid was low and decreasing in untreated lemon fruit (Fig. 4). However, concentrations of ascorbic acid in the flavedo of MJ and SA treated lemon fruit were significantly higher ( $P < 0.05$ ) than in untreated lemon fruit. The effect of MJ and SA in enhancing ascorbic acid in the flavedo of lemon fruit depended on the treatment concentration. Postharvest treatment with 10  $\mu$ M MJ plus 2 mM SA was more effective in enhancing ascorbic acid (Fig. 4). No significant differences ( $P > 0.05$ ) in ascorbic acid concentration were found in the flavedo of lemon fruit treated with either 10  $\mu$ M MJ or 2 mM SA. However, treatment with 10  $\mu$ M MJ plus 2 mM SA was the most effective in enhancing ascorbic acid in lemon fruit from Sun Valley Estates. Ascorbic acid in the lemon flavedo was significantly ( $P < 0.05$ ) affected by the interaction between farm location and treatments.

No significant difference ( $P > 0.05$ ) in ascorbic acid concentration was observed between lemon fruit from Tala Valley Citrus Estate treated with 10  $\mu\text{M}$  MJ plus 2 mM SA and those treated with 10  $\mu\text{M}$  MJ. The concentrations of ascorbic were similar in lemon fruit from Sun Valley Estates treated with 10  $\mu\text{M}$  MJ or untreated, to those from Tala Valley Citrus Estate treated with 2 mM SA. When treated with 2 mM SA, no significant difference was found in the level ascorbic acid between lemon fruit from Sun Valley Estates and New Venture Farm. Untreated lemon fruit from both Tala Valley Citrus Estate and New Venture Farm accumulated almost the same amount of ascorbic acid. The lowest concentrations of ascorbic acid was observed in lemon fruit from New Venture Farm treated with 10  $\mu\text{M}$  MJ. Ascorbic acid level of lemon fruit was significantly affected by the farm locations. Lemon fruit from New Venture Farm accumulated high concentrations of ascorbic acid, followed by the lemon fruit from the Sun Valley Estate. Low concentrations of ascorbic acid were observed in lemon fruit from Tala Valley Citrus Estate.

#### **4. Discussion**

In the present work, cold storage of lemon fruit caused the manifestation of CI symptoms which were firstly observed on untreated lemon fruit. The CI symptoms were manifested mainly as pitting, red blotch and necrosis on the lemon flavedo of untreated fruit. The results of this study indicate that MJ and SA treatments are effective in inhibiting CI in lemon fruit during cold storage.

It has been reported that the plant hormones, MJ and SA are signal molecules that directly or indirectly increase chilling tolerance in horticultural crops (Droby et al., 1999; Yang et al., 2012). This was an indication that treatment combination of MJ and SA effectively triggered

the production of biochemical molecules that may be involved in the defence mechanisms against CI. These compounds may have an effect in increase chilling tolerance in the fruit (Sapitnitskaya et al., 2006). This study suggests that lemon susceptibility to CI depends on the origin of the fruit and preharvest factors (such as rainfall and temperature) during fruit growth (Wang, 2010).

To protect from oxidative damage, fruit have evolved efficient protective mechanisms or antioxidant systems to scavenge ROS (Beak and Skinner, 2003; Yang et al., 2012). Antioxidants as protective mechanisms work in a complex co-operative network to detoxify ROS (Bolouri-Moghaddam et al., 2010; Yang et al., 2012). In this study, the activation of other protective mechanisms was investigated to understand the mode of action of MJ and SA in reducing CI in lemon fruit. One of the biochemical compounds that have been associated with defence mechanisms against CI is proline. Proline accumulation in crops under chilling stress serves as an adaptive mechanism, preventing protein degradation (Shang et al., 2011). It was also suggested that chilling tolerant crops generally have a large capacity to enhance proline biosynthesis in response to chilling (Shang et al., 2011). The accumulation of proline concentration in tomato stored at  $2 \pm 1^\circ\text{C}$  for up to 3 weeks (Zhao et al., 2009) and peach stored at  $1^\circ\text{C}$  for up to 3 weeks (Shang et al., 2011) under chilling stress was associated with chilling tolerance. In this study, cold storage of lemon fruit caused an increase in proline concentration in the flavedo of MJ and SA treated lemon fruit.

Similarly, in cold stored grapefruit, proline concentrations in flavedo were found to be higher in fruit that were more resistant to CI than in susceptible ones (Purvis, 1981; Nordby et al., 1987). In this study, the increase of proline concentration in lemon fruit during cold storage was a response of lemon fruit to chilling stress. It was suggested that crops accumulate proline in response to chilling stress (Shang et al. (2011). Our results agree with the previous authors.

The increased accumulation of proline concentration in MJ and SA treated fruit in parallel with increased chilling tolerance of lemon fruit suggested that MJ and SA are important for triggering proline accumulation and support its role as a protective mechanism in response to chilling stress.

Soluble sugars operate in a complex network with plant hormone signalling and stress-related pathways to help plant withstand chilling stress (Bolouri-Moghaddam et al., 2010). In this study, the abundance of soluble sugars in chilling tolerant lemon fruit (MJ and SA treated) could have played a role in retarding or delaying the onset of CI symptoms. In citrus, soluble sugars have been reported to increase chilling tolerance (Holland et al., 2005). The role of soluble sugars in increasing chilling tolerance in citrus has been associated with its ability to protect fruit against oxidative damage (Holland et al., 2005; Bolouri-Moghaddam et al., 2010). Our results agree with the previous authors, that soluble sugars are involved in increasing chilling tolerance in fruit (Holland et al., 2005; Bolouri-Moghaddam et al., 2010). Furthermore, postharvest application of MJ and SA significantly enhanced and activated the accumulation of soluble sugars to resist CI. However, there is no clear physiological process evidence on how the soluble sugars were enhanced by MJ and SA treatment.

Glucose acts as regulatory metabolite, modulating gene expression in plants (Bolouri-Moghaddam et al., 2010). In this study, the glucose concentrations were most abundant, followed by the sucrose in the flavedo. A similar trend was observed in the flavedo of lemon fruit (Aung et al., 1998). Citrus fruit stabilise proteins by increasing glucose and sucrose concentration in the flavedo under chilling stress (Aung et al., 1998). It was also reported that glucose and sucrose are important for maintaining membrane integrity during chilling stress by preserving the physical characteristics of the membrane (Crowe et al., 1988; Patton et al., 2007).

Glucose and sucrose are recognised as regulatory molecules controlling gene expression related to plant metabolism and chilling stress resistance (Rolland et al., 2006; Bolouri-Moghaddam et al., 2010). In this study, the high accumulation of glucose in the flavedo of MJ and SA treated lemon fruit was associated with enhanced chilling tolerance in the lemon fruit. High accumulation of soluble sugars, especially glucose, in MJ and SA treated lemon fruit was associated with the cold acclimation. The results of this study suggest that the efficacy of MJ and SA in enhancing proline and soluble sugars such as glucose and fructose is influenced by farm location. This could be due to the factors prevailing on the farm locations such temperature, rainfall, relative humidity and mineral nutrients in the soil.

Generally, lemon fruit are considered a good source of ascorbic acid. Ascorbic acid is an ubiquitous molecule in plants that functions as an enzyme co-factor and as a major cellular antioxidant protecting tissues against damage caused by ROS (Smirnoff and Wheeler, 2000). In this study, the enhancement of ascorbic acid by 10  $\mu$ M MJ plus 2 mM SA treatment could have contributed to the chilling tolerance of the lemon fruit. Ascorbic acid can scavenge ROS that may cause oxidative damage, hence it can play a role as part of the defence mechanisms against oxidative stress (Smirnoff and Wheeler, 2000). Both, MJ and SA are involved in various signal transduction systems, which induce particular enzymes to form defence compounds such as antioxidants (Ali et al., 2007). In this study MJ and SA significantly triggered plant defence compounds in lemon flavedo at cold storage, which could have played a role as chilling mechanisms to increase chilling tolerance in lemon fruit. This showed that treatment with either MJ and SA may mediate the natural response of lemon fruit to chilling stress and increase chilling tolerance in the lemon fruit (Meir et al., 1996).

In conclusion, this study provides evidence that MJ and SA reduce CI in Eureka' lemon fruit at postharvest storage. The treatment with 10  $\mu$ M MJ plus 2 mM SA was more effective in

alleviating CI than the individual application (10  $\mu$ M MJ or 2 mM SA) of the two treatments MJ or SA. The effect of 10  $\mu$ M MJ plus 2 mM SA treatment on chilling tolerance of lemon was associated with the treatment's ability to induce the accumulation of biochemical compounds such as proline, ascorbic acid and soluble sugars that are believed to be involved in the defence mechanisms against CI in the lemon fruit. These results indicate that MJ and SA treatment in lemon fruit make tissue more resistant to CI by increasing biochemical compounds such as soluble sugars, ascorbic acid, proline and other compounds that may be linked, play a role or function in the defence mechanisms.

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## Figure legends

Fig. 1. Chilling injury (CI) index of lemon fruit from Tala Valley Citrus Estate, New Venture Farm and Sun Valley Estates treated with 10  $\mu$ M methyl jasmonate (MJ), 2 mM salicylic acid (SA) or 10  $\mu$ M MJ plus 2 mM SA solutions. Lemon fruit were stored at -0.5°C for up to 28 days plus 7 days at 23°C. Values are means of five replicate samples containing 20 fruit  $\pm$  SE. Least significant differences of means (5% level) = 0.15.

Fig. 2. Proline concentration in the lemon flavedo treated with methyl jasmonate (MJ) and salicylic acid (SA) solutions prior to cold storage at -0.5°C for 0, 7, 14, 21 or 28 days and 7 days at 23°C to simulate shelf-life. Values on the figures are means of three replicate samples containing 20 fruit  $\pm$  SE. Least significant differences of means (5% level) = 0.01.

Fig. 3. Changes of sucrose (LSD<sub>0.05</sub> = 0.60), glucose (LSD<sub>0.05</sub> = 2.70) and fructose (LSD<sub>0.05</sub> = 0.84) concentrations in lemon flavedo either from Tala Valley Citrus Estate, New Venture Farm or Sun Valley Estates treated with 10  $\mu$ M methyl jasmonate (MJ), 2 mM salicylic acid (SA) or 10  $\mu$ M MJ plus 2 mM SA. Lemon fruit were stored at -0.5°C for 0, 7, 14, 21 or 28 days and 7 days at 23°C to simulate shelf-life. Values on the figures are means of three replicate samples containing 20 fruit  $\pm$  SE.

Fig. 4. The effects of postharvest treatment with 10  $\mu$ M methyl jasmonate (MJ), 2 mM salicylic acid (SA) or 10  $\mu$ M MJ plus 2 mM SA on ascorbic acid concentration of lemon fruit from Tala Valley Citrus Estate, New Venture Farm and Sun Valley Estates. Lemon fruit were stored at -0.5°C for up to 28 days plus 7 days at 23°C to simulate shelf-life. Values on the figures are means of three replicate samples containing 20 fruit  $\pm$  SE. Least significant differences of means (5% level) = 2.14.

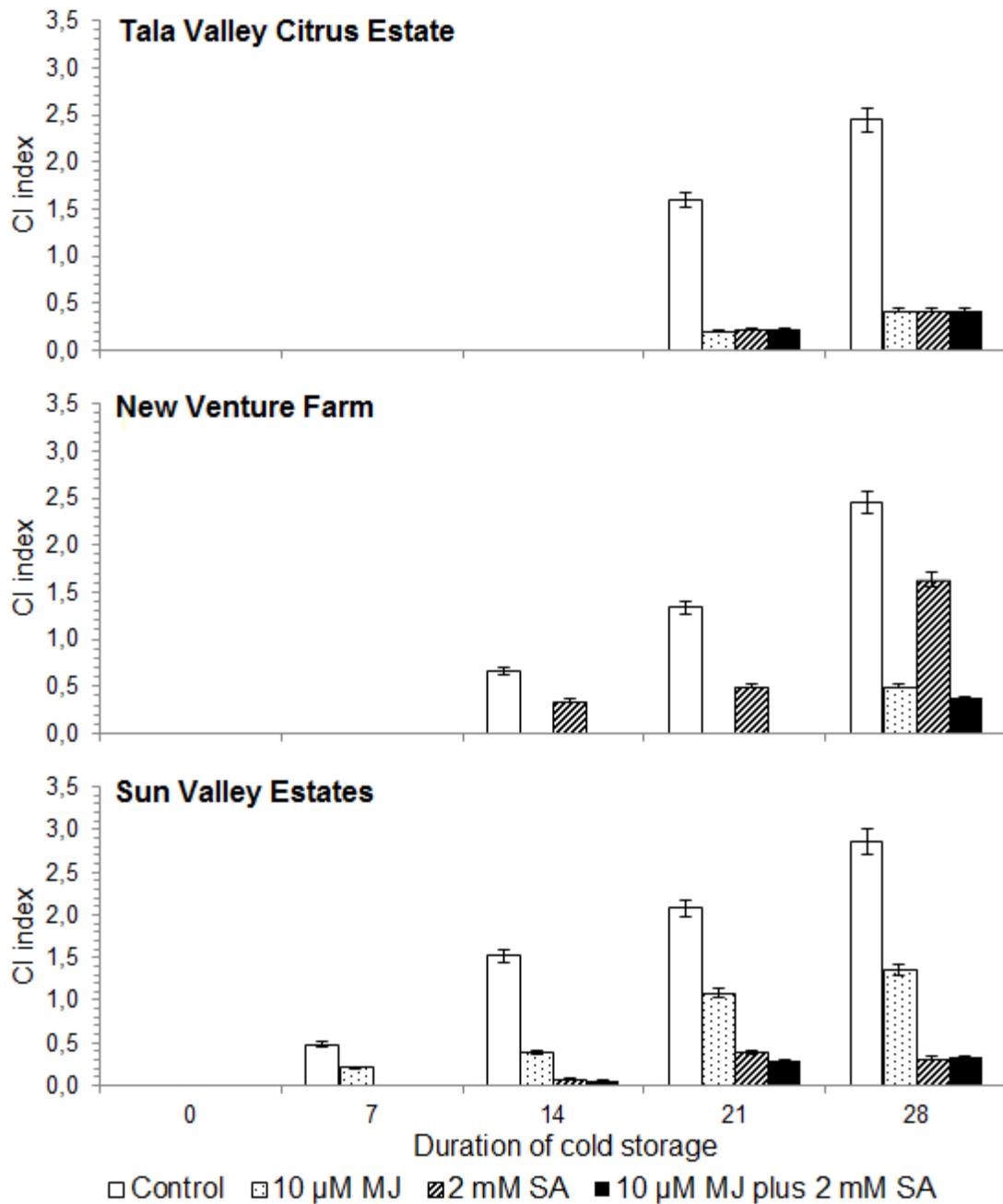


Fig. 1.

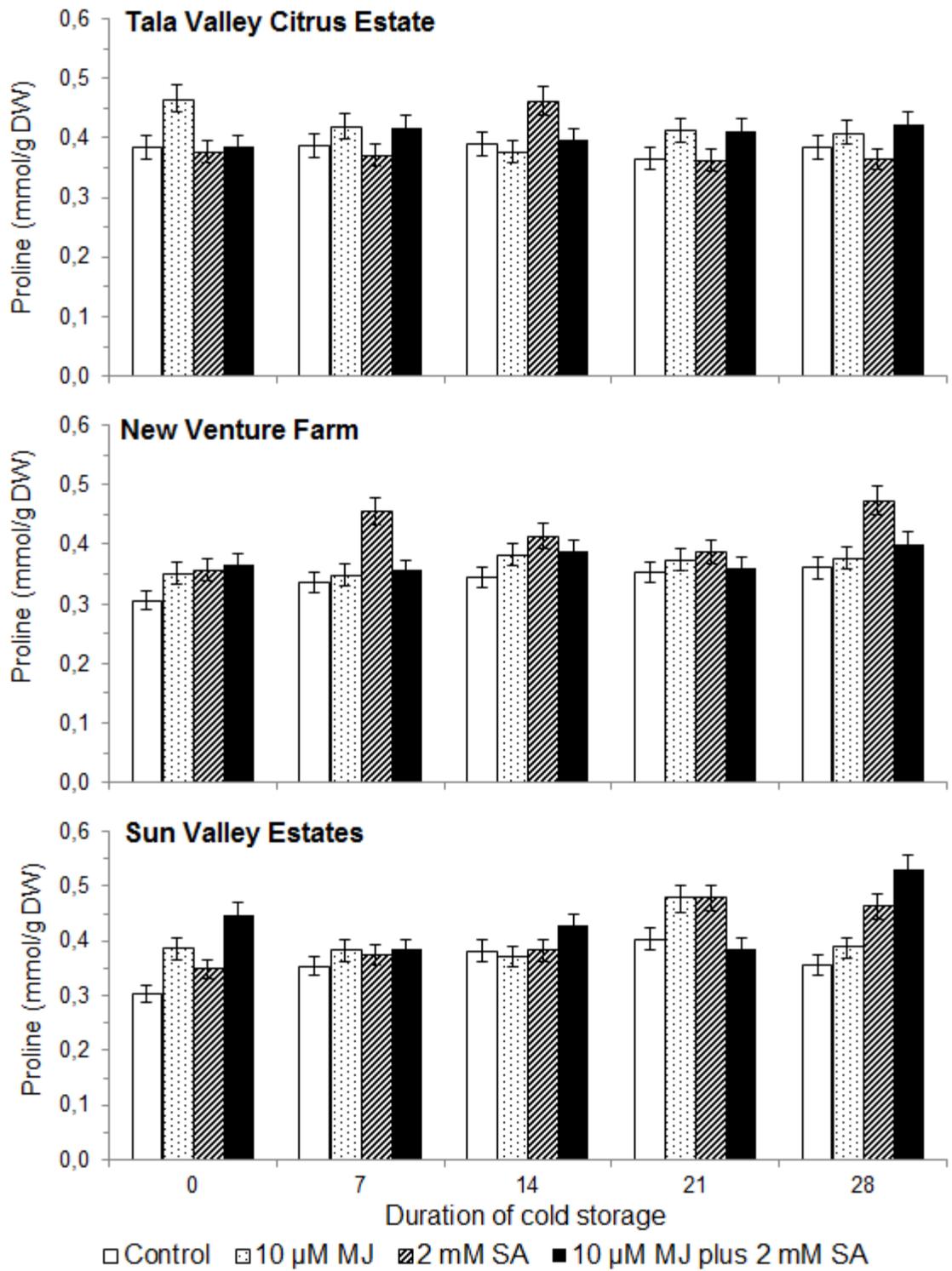


Fig. 2.

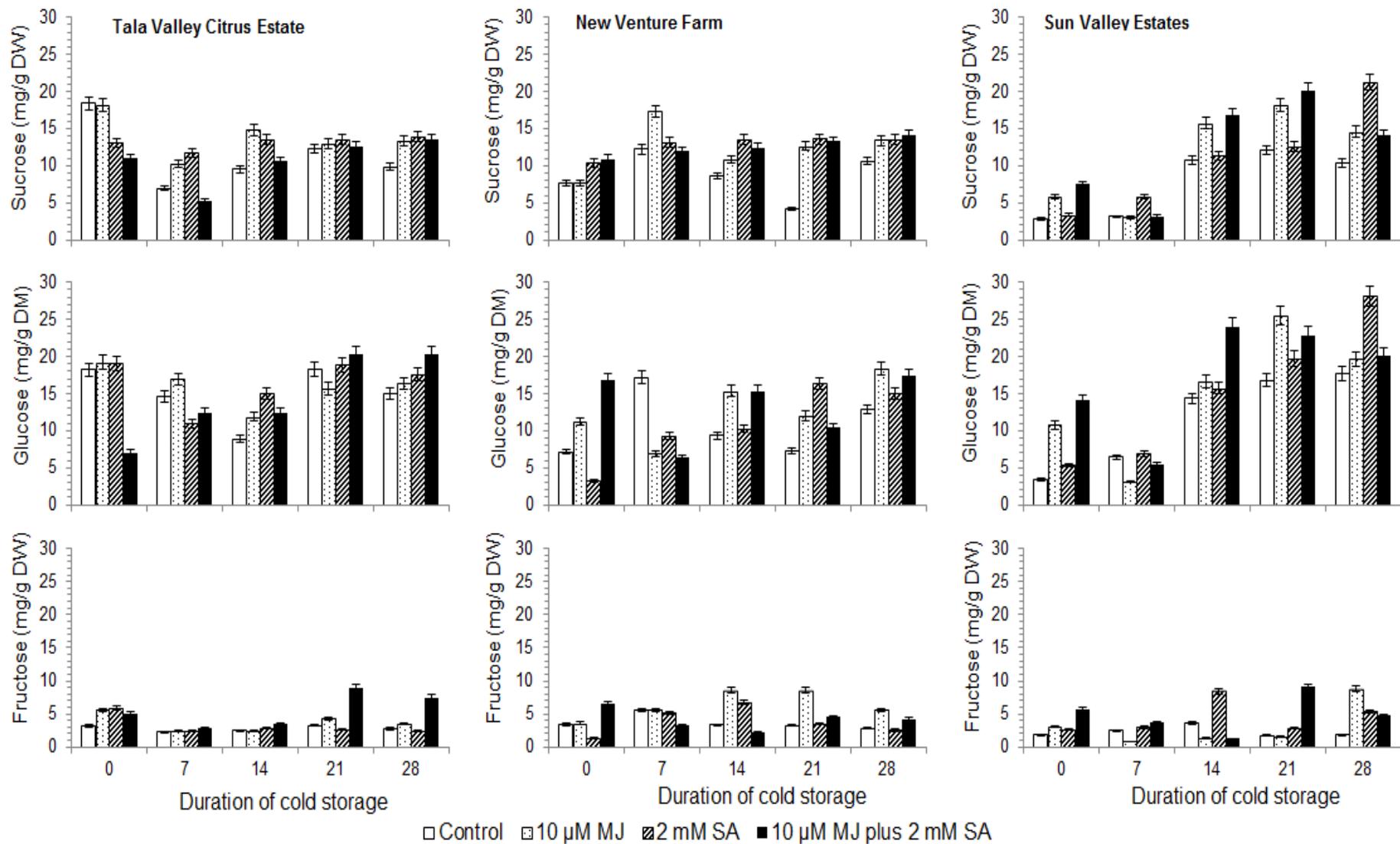


Fig. 3.

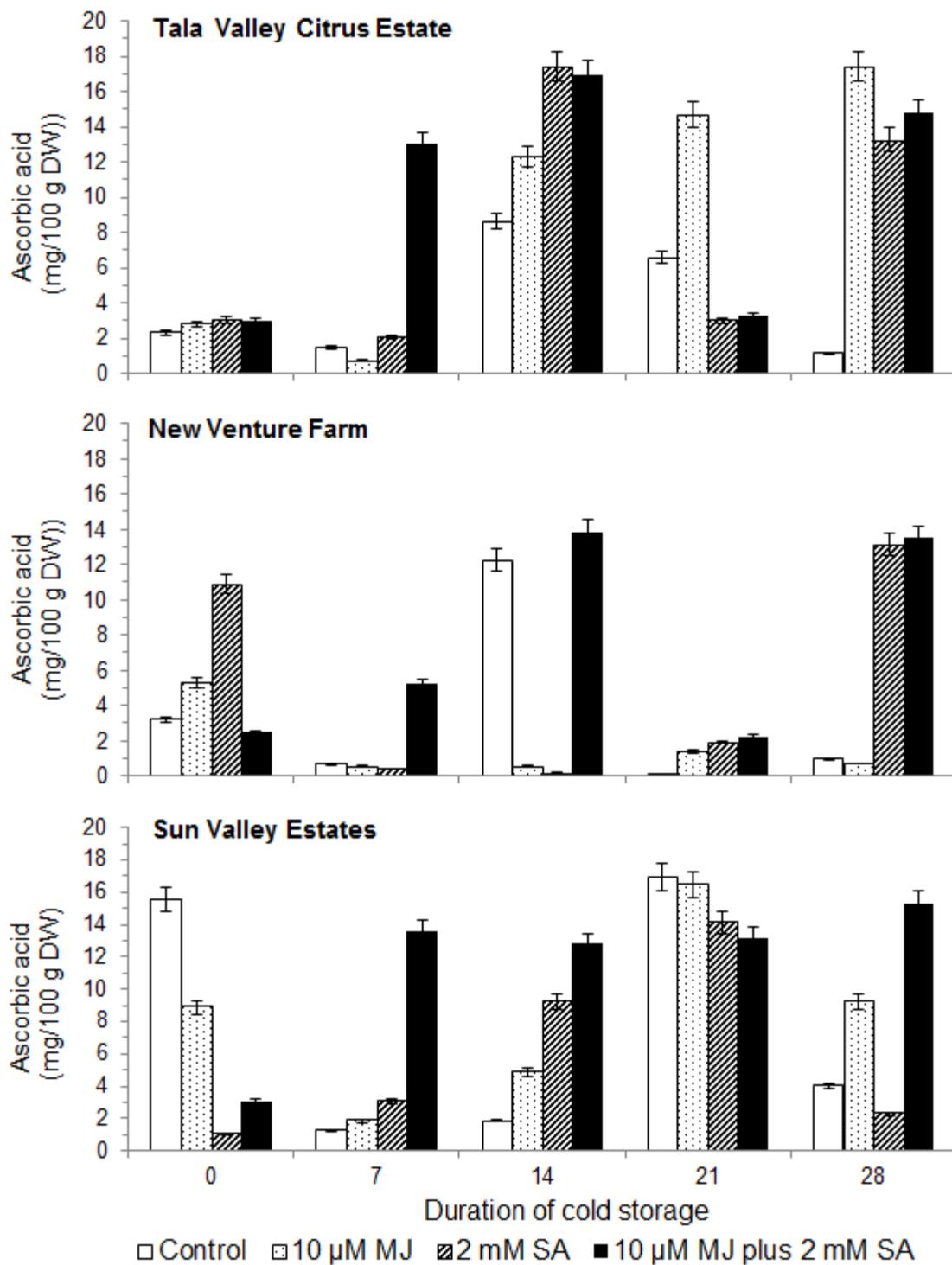


Fig. 4.

## Chapter 5

Salicylic acid and methyl jasmonate increase phenolic compounds and phenylalanine ammonia-lyase to improve chilling tolerance in cold-stored lemon fruit (*Citrus limon*)

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## **Abstract**

Chilling injury (CI) may result from the degradation of membrane integrity which can be aligned to phenolic oxidation activated by polyphenol oxidase (PPO; EC 1.14.18.1) and peroxidase (POD; EC1.11.1.7), enzymes responsible for tissue browning. Phenolics phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) are involved in acclimation against chilling stress in crops. It was hypothesised that treatment with methyl jasmonate (MJ) and salicylic acid (SA) may enhance chilling tolerance in lemon fruit by increasing the synthesis of total phenolics and PAL by activating the key enzyme regulating the shikimic acid pathway whilst inhibiting the activity of POD and PPO. Lemon fruit were treated with 10  $\mu$ M MJ, 2 mM SA or 10  $\mu$ M MJ plus 2 mM SA, waxed, stored at -0.5, 2 or 4.5°C for up to 28 days plus 7 days at 23°C. Treatment with 10  $\mu$ M MJ plus 2 mM SA enhanced chilling tolerance in lemon fruit, and increased total phenolics and PAL activity in lemon flavedo. Treatment with 10  $\mu$ M MJ plus 2 mM SA inhibited POD activity in lemon flavedo, which might have contributed in delaying the manifestation of CI symptoms. PPO activity was found to be a poor biochemical marker of CI. Treatment with 10  $\mu$ M MJ plus 2 mM SA was effective in enhancing chilling tolerance, possibly as a result of increased production of total phenolics through the activation of PAL and inhibition of POD.

*Keywords:* Methyl jasmonate; Peroxidase; Phenylalanine ammonia-lyase; Polyphenol oxidase; Salicylic acid; Total phenolic content

## **1. Introduction**

Chilling injury (CI), a physiological disorder disrupting normal cell metabolism and is one of the most significant reasons for postharvest losses in citrus fruit (Sala et al., 2005). In citrus fruit, CI is manifested as pitting, rind staining, red blotch and necrosis on the flavedo (Sala and Lafuente, 1999). To tolerate CI, fruit contain antioxidants, such as phenolic compounds, that can mitigate cellular damage under chilling stress (Grace and Logan, 2000). The concentration of phenolic antioxidants in fruit can be negatively affected by cold storage (Tomás-Barberán and Espín, 2001), resulting in lower antioxidant concentrations and poor resistance to stress, ultimately leading to membrane damage and CI.

Phenolic compounds are plant secondary metabolites involved in fruit quality (flavour, appearance and health-promoting properties) (Tomás-Barberán and Espín, 2001; Su et al., 2008). These compounds are powerful antioxidants (Grace and Logan, 2000) capable of scavenging reactive oxygen species (González-Aguilar et al., 2004), it is through this action they seem to be involved in chilling tolerance of lemon fruit (Siboza and Bertling, 2013). Conversely, phenolic compounds can be associated with injury resulting from plant stress (Dixon and Paiva, 1995; Grace and Logan, 2000). Decreased membrane integrity following CI can result in leakage of phenolic compounds and denaturation of proteins. The role of phenolics and their metabolism in enhancing chilling tolerance in lemon fruit still needs further investigations.

Phenylalanine ammonia-lyase (PAL) is the key enzyme at the entry-point of the phenylpropanoid pathway (Rivero et al., 2001); involved in defence mechanism and acclimation against chilling (Dixon and Paiva, 1995; Rivero et al., 2001). The induction of PAL activity in response to stress has been considered as a defence mechanism of fruit to withstand stress (Martínez-Téllez and Lafuente, 1997; Lafuente et al., 2001), therefore,

chilling can affect PAL activity (Sanchez-Ballesta et al., 2000). Chilling induce the production of phenolic compounds and PAL activity (Dixon and Paiva, 1995; Rivero et al., 2001); not surprisingly, PAL activity has been shown to directly increase in response to chilling in a variety of species (Tan, 1980; Graham and Patterson, 1982; Leyva et al., 1995). In citrus, the induction of PAL activity by chilling has been associated with chilling tolerance and is considered a good marker of CI (Martínez-Téllez and Lafuente, 1993; Sanchez-Ballesta et al., 2000). As higher PAL activity is aligned with reduced CI during cold storage (Lafuente et al., 2004), the induction of PAL activity in mandarin has been considered a good marker of this fruit's susceptibility to CI during cold storage (Sanchez-Ballesta et al., 2000). However, the role of PAL activity during cold storage and how this activity is possibly aligned with increased chilling tolerance has not been investigated.

Phenolic compounds are oxidised by peroxidase and polyphenol oxidase (Rivero et al., 2001), resulting in tissue browning in stressed horticultural crops as a common symptom associated with increased POD and PPO activity (Raimbault et al., 2011). Martínez-Téllez and Lafuente (1997) advised that PPO and POD are involved in enzymatic browning and fruit deterioration. The resultant tissue browning in fruit injured by cold temperature exposure is one of the most notable CI symptoms that affects fruit marketability causing economic losses (Vela et al., 2003). The activity of POD and PPO has been reported to increase in response to chilling stress, causing CI symptoms, such as necrosis, pitting and browning (Rivero et al., 2001). Tomás-Barberán and Espín (2001) also reported that POD and PPO are involved in the oxidation of antioxidants, thereby decreasing the antioxidant status of the fruit which ultimately leads to fruit being susceptible to CI during cold storage. On the other hand, POD is an important enzyme in the antioxidant enzymatic system. The enzymes POD and PPO have

been associated with tissue browning during plant stress; however, their physiological functions are not totally elucidated (Raimbault et al., 2011).

The beneficial effect of plant hormones such as methyl jasmonate (MJ) and salicylic acid (SA) in alleviating CI has been demonstrated in zucchini squash (*Cucurbita pepo* L.) (Wang and Buta, 1994), avocado (*Persea Americana* Mill), grapefruit (*Citrus paradise*), bell pepper (*Capsicum annum*) (Meir et al., 1996), tomato (*Solanum lycopersicon* L.) (Ding et al., 2002) and lemon fruit (*Citrus limon*) (Siboza and Bertling, 2013). However, the mode of action of MJ and SA in reducing CI is unknown. Preharvest environmental conditions and postharvest storage conditions are factors that can impact antioxidant activity of fruit during cold storage (Connor et al., 2002). Generally, PAL, POD and PPO all play crucial roles in the phenolic metabolism and may be involved in the manifestation of CI symptoms (Martínez-Téllez and Lafuente, 1993). Therefore, in this study, the impact of postharvest treatment with MJ and SA in inducing PAL activity and phenolic accumulation while inhibiting enzymes oxidising phenolic compounds, such as PPO and POD, in lemon fruit grown under different environmental conditions were investigated.

## **2. Materials and Methods**

### *2.1. Plant material*

'Eureka' lemon fruit [*Citrus limon* (L.) Burm.], were obtained from commercial farms located in KwaZulu-Natal, South Africa. New Venture Farm :moderate subtropical (mean annual temperature: 14.5-26.5°C; mean annual rainfall: 712-717 mm; 31° 02' S 29° 25' E, 68-483 m asl), Tala Valley Citrus Estate :warm temperate (mean annual temperature: 12.6-23.7°C; mean annual rainfall: 612-771 mm; 29° 52' S 30° 30' E, 416-922 m asl) and Sun Valley Estates :cool

subtropical zone (mean annual temperature: 10.7-26.5°C; mean annual rainfall: 669-675 mm); 28° 83' S 30° 06' E, 820-1003 m asl). Lemon fruit were harvested on the 6<sup>th</sup> of June 2011 and 2012 seasons, and the trends were similar. Fruit were immediately transported on paved roads from the collection site to the laboratory in a ventilated vehicle. Fruit transportation distances from farm locations to the laboratory site were 39.2 km (Tala Valley Citrus Estates), 130 km (Sun Valley Estates) and 228 km (New Venture Farm). Lemon fruit were selected for uniformity in colour and size.

### *2.2. Treatment and storage*

Lemon fruit were treated as described by Sibozza and Bertling (2013). Following treatment with a disinfectant (Sporekill®) and air-drying at room temperature (23°C), fruit were randomly divided into four of treatments. Fruit were soaked in one of the following solutions: 10 µM MJ, 2 mM SA, 10 µM MJ plus 2 mM SA for 30 s; one lot was the no soaking (control). Fruit were waxed with Citrashine® and allowed to dry at room temperature for one hour, before cold storage either at -0.5; 2 or 4.5°C (air delivery temperature) for 28 days with sampling at 0, 7, 14, 21, or 28 days. The relative humidity was maintained at 85-90%. After cold storage, lemon fruit were transferred to room temperature (23°C) for one week (simulation of shelf life), to allow the manifestations of CI symptoms.

### *2.3. Experimental design*

The experiment was design using a randomised complete block design with the following factors: preharvest farm locations (3 levels; Tala Valley Citrus Estate, New Venture Farm and Sun Valley Estates); postharvest treatments (4 levels; Control, 10 µM MJ, 2 mM SA, or 10 µM MJ plus 2 mM SA); storage temperature (3 level; -0.5, 2 or 4.5°C) and storage duration (5 levels; 0, 7, 14, 21 or 28 days).

### *2.4. Determination of CI on lemon fruit*

Following the one week simulated shelf life period, the lemon fruit were evaluated for CI symptoms according to Lafuente et al. (1997). The evaluation was based on the following scale: 0 (no pitting), 1 (slight pitting), 2 (moderate pitting), 3 (severe pitting). The results are presented as mean of five replicates of 20 fruit  $\pm$  S.E.M.

### *2.5. Sample preparation and storage*

Lemon samples were prepared according to Lafuente et al. (2004), with slight modifications. Flavedo tissue was collected from the entire surface of the lemon fruit, pulverised using mortar and pestle with liquid nitrogen to a fine powder and stored at  $-70^{\circ}\text{C}$  prior to total phenolic and enzyme assays.

### *2.6. Extraction and assay of total phenolics*

Total phenolics of the flavedo were extracted and determined using the method of Pérez-Tello et al. (2009) with some modifications. Flavedo tissue powder (0.1 g DW) was extracted at  $4^{\circ}\text{C}$  with 1 ml of 1 M HCl, vortexed for 1 min and incubated at  $37^{\circ}\text{C}$  for 30 min. After incubation, 1 ml NaOH (2 M in 75 % methanol) solution was added before vortexing the solution for 1 min. The solution was incubated for a second time at  $37^{\circ}\text{C}$  for 30 min. After incubation, 1 ml of 0.75 M metaphosphoric acid was added to the solution and vortexed for 1 min. This solution was then centrifuged at  $2510 \times g$  for 10 min to separate the supernatant from the tissue pellets. To completely extract phenolics, the remaining pellets were re-suspended in a 1 ml acetone: water (1:1, v/v) before centrifugation. Both extracts were combined and made up to 10 ml with acetone: water (1:1, v/v). Total phenolics were determined by adding 5 ml nanopure water and 1 ml Folin-Ciocalteu reagent to 1 ml sample in a 25 ml test tubes, samples were mixed and allowed to stand for 8 min at  $24^{\circ}\text{C}$ . Thereafter, 10 ml 7% sodium carbonate and 8 ml nanopure water were added to the sample. This solution was vortexed thoroughly for 1 min and allowed to stand at  $24^{\circ}\text{C}$  for 2 h. The solution was filtered through a Whatman®

0.45  $\mu\text{m}$  poly filter prior to the determination of total phenolics at 750 nm. Gallic acid monohydrate was used as standard to prepare a calibration curve; results were expressed as milligrams gallic acid equivalents (GAE)  $\text{g}^{-1}$  DW lemon flavedo. Results are presented as mean of three replicate samples of 20 fruit  $\pm$  S.E.M.

#### *2.7.Determination of PAL activity*

The enzyme PAL (EC 4.3.1.5) was determined as previously described by Martínez-Téllez and Lafuente (1997). The enzyme PAL was extracted from 0.4 g DW flavedo acetone powder (prepared as previously described by Sala et al. (2005) with 15 ml 0.1 M sodium borate buffer pH 8.8, containing 0.02 M  $\beta$ -mercaptoethanol at 4°C). The assay medium contained 0.1 ml PAL enzyme extract, 1.9 ml 0.05 M sodium borate buffer (pH 8.8) and 1 ml 20 mM L-phenylalanine in a final volume of 3 ml. The PAL activity was measured by determining the absorbance of cinnamic acid at 290 nm over a period of 2 h at 40 °C. The PAL activity was expressed on a dry-matter basis as nmoles of cinnamic acid per gram of flavedo acetone powder per hour ( $\text{nmol h}^{-1} \text{g}^{-1}$  DW). Results are the mean of three replicate samples of 20 fruit  $\pm$  S.E.M.

#### *2.8.Determination of POD activity*

The enzyme, POD (EC1.11.1.7) activity was determined following the method described by Martínez-Téllez and Lafuente (1993) with slight modifications. Flavedo acetone powder (0.2 g DW) was ground for 10 min in 6 ml 100 mM Tris-HCL buffer, pH 8, containing 5 mM  $\beta$ -mercaptoethanol and centrifuged for 30 min at  $15\,000 \times g$  at 4°C. Following this, POD activity was measured at 470 nm in reaction mixtures of 2.15 ml 10 mM sodium acetate, pH 5.3, containing guaiacol, 0.25 ml 0.1% hydrogen peroxide and 0.1 ml supernatant. The mixture was incubated at 30°C for 2 min. One unit of POD activity was defined as an increase of

absorbance at 470 nm per min. The POD activity was expressed as units of POD  $\text{min}^{-1} \text{g}^{-1}$  of flavedo acetone powder. Results are mean of three replicate samples of 20 fruit  $\pm$  S.E.M.

### *2.9. Determination of PPO activity*

The enzyme PPO (EC 1.14.18.1) was extracted as previously described by Martínez-Téllez and Lafuente (1993) with slight modifications. Flavedo acetone powder (0.2 g DW) was mixed with 6 ml 0.05 M potassium phosphate buffer pH 7.2 containing 1 M KCl and 5% (w/w) polyvinyl-polyrrolidone at 4°C. The homogenate was centrifuged at  $27\,000 \times g$  for 10 min at 4°C and the enzyme extract used to assay total PPO activity. The assay medium contained 25  $\mu\text{l}$  enzyme extract and 1.25 ml 0.02 M caffeic acid. Activity of PPO was determined by measuring the rate of increase in absorbance at 410 nm at 30°C. One unit of PPO activity was defined as an increase of 0.01 absorbance unit per min at 410 nm. The PPO activity of lemon flavedo was expressed as unit of PPO  $\text{min}^{-1} \text{g}^{-1}$  DW of flavedo tissue.

### *2.10. Statistical analysis*

All statistical analyses were performed using GenStat® 14<sup>th</sup> Edition (VSN International, Hemel Hempstead, UK). Data were subjected to analysis of variance with treatments, farm location, storage temperature and cold storage time as factors. Means were compared by Duncan's Multiple Range Tests at a significance level of  $P < 0.05$ .

## **3. Results**

### *3.1. Effect of MJ and SA on CI of lemon fruit during cold storage*

Cold storage of lemon fruit either at -0.5, 2 or 4.5°C, significantly ( $P < 0.05$ ) affected the occurrence of CI, with symptoms developing as pitting, necrosis and red blotch. Untreated fruit developed severe CI symptoms. The highest CI was detected in Sun Valley Estates fruit

stored at 4.5°C, followed by the Tala Valley Citrus Estate fruit when stored at 4.5°C. Treatment with MJ and SA significantly ( $P < 0.05$ ) reduced CI symptoms. Generally from all locations, when lemon fruit were stored at 2°C, treatment with 10 µM MJ plus 2 mM SA was most effective in reducing CI, followed by the 10 µM MJ or 2 mM SA treatment; a similar trend was observed when lemon fruit were stored at 4.5°C (Fig. 1). At -0.5°C storage, treatment with 10 µM MJ plus 2 mM SA was the most effective treatment, followed by the 2 mM SA application.

### *3.2. Postharvest phenolics concentration in lemon flavedo of MJ and SA treated lemon fruit*

The concentration of total phenolics in lemon flavedo was significantly ( $P < 0.05$ ) affected by farm location, treatment, cold storage temperature as well cold storage time, with storage at -0.5°C resulting in the highest concentration of total phenolics (Fig. 2). Untreated lemon fruit (the most chilling-susceptible fruit; Fig. 1) stored at 4.5°C had the lowest phenolic levels, independent of location (Fig. 2). All fruit had low levels of phenolics in the flavedo at the end of the storage period (Fig. 2). This was accompanied by CI development, resulting in the highest CI index in control lemon fruit (Fig. 1). However, postharvest treatment with either 10 µM MJ, 2 mM SA or 10 µM MJ plus 2 mM SA significantly ( $P < 0.05$ ) enhanced the concentration of total phenolics in the flavedo during cold storage (Fig. 2).

Total phenolics in lemon flavedo varied significantly ( $P < 0.05$ ) following 10 µM MJ, 2 mM SA or 10 µM MJ plus 2 mM SA treatment and depended on cold storage temperature, location and storage duration. The highest phenolic concentration during cold storage was observed in lemon fruit treated with 10 µM MJ plus 2 mM SA (Fig. 2). The increase in total phenolics following 10 µM MJ, 2 mM SA as well as 10 µM MJ plus 2 mM SA treatment was significantly ( $P < 0.05$ ) affected by farm location and storage temperature. The increase in total phenolics varied with farm location and chilling susceptibility. Total phenolics were

higher in the flavedo of fruit harvested from New Venture Farm (highly chilling tolerant) than in those from Tala Valley Citrus Estate (chilling tolerant).

### *3.3. Postharvest metabolism of PAL in lemon flavedo of MJ and SA treated lemon fruit*

The exposure of lemon fruit either to -0.5, 2 or 4.5°C significantly ( $P < 0.05$ ) affected PAL activity (Fig. 3). Low levels of PAL activity were observed in untreated lemon fruit. However, PAL activity was significantly ( $P < 0.05$ ) enhanced by postharvest treatment with either 10 µM MJ, 2 mM SA or 10 µM MJ plus 2 mM SA (Fig. 3). The effectiveness of MJ and SA in enhancing PAL activity of lemon flavedo depended on the concentration applied, farm location and storage temperature. Postharvest treatment with 10 µM MJ plus 2 mM SA was most effective in enhancing PAL activity, followed by treatment with 10 µM MJ (Fig. 3). Treatment with 2 mM SA was less effective in inducing PAL activity of lemon flavedo.

The postharvest PAL activity of the lemon flavedo was significantly ( $P < 0.05$ ) influenced by cold storage temperature (Fig. 3). Significant high PAL activity was observed in the flavedo of fruit stored at -0.5°C, followed by those stored at 2°C (Fig. 3). The PAL activity in the flavedo of all lemon fruit stored at 4.5°C was lower than in those stored at other temperatures (-0.5 or 2°C). This pattern was same for all farms. Farm location was a further factor affecting PAL activity. The decrease in PAL activity during cold storage was particularly evident in the flavedo of lemon fruit from Sun Valley Estate characterised by the highest CI followed by the flavedo of lemon fruit from Tala Valley Citrus Estate. High PAL activity (Fig. 3) and low CI (Fig. 1) were observed in lemon fruit from New Venture Farm.

### *3.4. Postharvest POD activity in lemon flavedo of MJ and SA treated lemon fruit*

During cold storage, POD activity in lemon flavedo was significantly ( $P < 0.05$ ) affected by MJ and SA treatment, cold storage temperature and farm location. Storing lemon fruit at -0.5,

2 or 4.5°C significantly ( $P < 0.05$ ) triggered the accumulation of POD activity in lemon flavedo. This activity increased with cold storage time (Fig. 4). The highest POD activity was observed in the flavedo of untreated fruit (Fig. 4). After 21 days at cold storage, the POD activity in the flavedo of 2 mM SA treated fruit was found to be high in lemon fruit from New Venture Farm. However, postharvest treatment with 10  $\mu$ M MJ plus 2 mM SA significantly ( $P < 0.05$ ) reduced POD activity (Fig. 4). In general, POD activity of MJ and SA treated lemon fruit was significantly less than that of untreated lemon fruit (Fig. 4).

As with PAL activity, the efficacy of postharvest treatment with MJ and SA on POD activity significantly ( $P < 0.05$ ) depended on the treatment concentrations, storage temperature, and farm location (Fig. 4). The treatment combination of 10  $\mu$ M MJ plus 2 mM SA was significantly ( $P < 0.05$ ) effective in inhibiting POD activity in lemon flavedo, followed by treatment with 2 mM SA. Treatment with 10  $\mu$ M MJ was less effective in inhibiting POD activity with the POD activity significantly ( $P < 0.05$ ) varying with farm location and storage temperature (Fig. 4). The significant highest POD activity was observed in chilling-susceptible lemon fruit from Sun Valley Estate, followed by lemon fruit from Tala Valley Citrus Estate. Furthermore, POD did not increase at -0.5°C in fruit from Tala Valley Citrus Estate and Sun Valley Estate. On the other hand, little POD activity was observed on chilling-tolerant lemon fruit (10  $\mu$ M MJ plus 2 mM SA) from New Venture Farm.

### *3.5. Postharvest PPO activity in lemon flavedo of MJ and SA treated fruit*

The amount of PPO activity in the flavedo of all lemon fruit was miniscule with the control fruit showing an increase of the activity (Fig. 5). However, postharvest treatment with either 10  $\mu$ M MJ, 2 mM SA or 10  $\mu$ M MJ plus 2 mM SA significantly ( $P < 0.05$ ) inhibited the PPO activity in lemon flavedo during cold storage (Fig. 5). Combination treatment with 10  $\mu$ M MJ

plus 2 mM SA showed a relatively huge increase of PPO activity compared to the individual treatments (10  $\mu$ M MJ or 2 mM SA).

#### **4. Discussion**

As plant hormones widely distributed in plants, MJ and SA play an important role in regulating physiological mechanisms and mediating plant defence mechanisms against chilling stress (Yordanova and Popova, 2007; Yang et al., 2012). In this study, both MJ and SA were used as postharvest treatments on lemon fruit to investigate whether they play a role in maintaining fruit quality during cold storage by triggering the activity of PAL (Fig. 3) and increasing phenolic concentrations in the flavedo (Fig. 2). Phenolics and PAL are thought to be involved in alleviation of CI (Fig. 1) during cold storage. However, PPO and POD are believed to be antagonistic to the action of PAL with regards to CI. The results indicate that storage temperature had a significant influence on CI susceptibility of lemon fruit; however, consistent reduction of CI in lemon fruit from all farm locations was achieved by treatment with 10  $\mu$ M MJ plus 2 mM SA, regardless of storage temperature with 28 days of treatment.

The role of MJ in alleviating CI has been associated with its potential ability to activate the production of defence mechanisms, such as phenolic compounds and heat shock proteins (Meir et al., 1996; Meng et al., 2009). There is evidence that the antioxidant pool in the lemon flavedo plays a pivotal role in the resistance to CI (Siboza and Bertling, 2013). Similarly, the reduction of CI in SA-treated horticultural crops is associated with increased levels of heat-shock proteins, and activation of stress-induced antioxidant systems (Yordanova and Popova, 2007; Huang et al., 2008 ). In this study, the combination of 10  $\mu$ M MJ plus 2 mM SA was the most effective in reducing CI. This suggested that this combination (10  $\mu$ M MJ plus 2 mM

SA) doubles the efficacy of each compound (10  $\mu$ M MJ or 2 mM SA) in triggering defence reactions against CI. However, this tendency was not effectively achieved in inhibiting PPO activity which was extremely low in the lemon flavedo.

Chilling tolerance in horticultural crops is improved by activation of antioxidants (Cao et al., 2009). The high concentration of total phenolic content in chilling tolerant fruit (10  $\mu$ M MJ plus 2 mM SA treated; Fig. 2) was associated with the resistance to CI (Fig. 1). The increase in phenolics compounds could have played a role in stabilising cell membranes by decreasing membrane fluidity, absorbing and neutralising free radicals (Osawa, 1994; Blokhina et al., 2003). Our study is in agreement with the results by Galli et al. (2009), who found an increase in total phenolics content in chilling tolerant pawpaw fruit stored at 4°C for 8 weeks.

The high accumulation of total phenolics in the flavedo of lemon fruit from New Venture and Tala Valley Citrus Estate was associated with chilling tolerance, while the low phenolic concentration observed in the flavedo of lemon fruit from Sun Valley Estates was associated with chilling susceptibility. This is in agreement with Wang (2010), that preharvest environmental conditions prevailing at a farm location can affect the fruit antioxidant concentration. In this study, environmental conditions at the farm location may have contributed to the production of total phenolics before harvesting and this may have resulted in increased chilling tolerance in lemon fruit during postharvest storage. The results in this study also showed that storage temperature had an effect on total phenolics content of the flavedo. The results of this study, suggest that -0.5°C is a better storage temperature for lemon fruit, than the warmer 2 and 4.5°C, as the colder condition seems to allow for the maintenance of a certain size antioxidant pool, thereby prolonging lemon shelf life through the inhibition of CI occurrence.

The enzyme PAL is the key enzyme between primary (shikimate pathway) and secondary (phenylpropanoid pathway) metabolism; induced by stresses including chilling (Dixon et al., 1992; González-Aguilar et al., 2004; Cao et al., 2009). Both, PAL activity and phenylpropanoid pathway have been related to plant defence mechanisms (Dixon and Paiva, 1995; Cao et al., 2009). The results suggest that postharvest treatment with MJ and SA can increase PAL activity which is aligned with increased chilling tolerance in lemon fruit. Generally, PAL activity is regulated through gene expression (Tomás-Barberán and Espín, 2001), with the PAL genes likely to be expressed in response to cold stress (Lois et al., 1989; Sanchez-Ballesta et al., 2000). This study suggests that storage temperature is important for enhancing PAL activity. Storage at -0.5 or 2°C effectively triggered PAL activity in chilling tolerant lemon flavedo. The enhancement of PAL activity in lemon flavedo could have affected PAL genes. The up regulation of PAL genes is associated with cold acclimation (Martínez-Téllez and Lafuente, 1997). Therefore, the increase in PAL activity in chilling tolerant lemon fruit might be a defence mechanism to withstand chilling.

Treatment with 10 µM MJ plus 2 mM SA resulted in highest PAL activity in lemon flavedo, the likely cause why these fruit were able to withstand chilling stress and have reduce CI symptoms. The mode of action of MJ ( $10^{-4}$  or  $10^{-5}$  M) in reducing CI in guava fruit at 5°C for up to 15 days plus two days at 20°C, has been associated with increased PAL activity (González-Aguilar et al., 2004). Similarly, the role of SA (0.5 mM) in reducing CI in cucumber fruit stored at 1°C for up to 18 days, was associated with its ability to enhance PAL activity in fruit (Cao et al., 2009). Our study is in agreement with other studies that the induction of PAL activity could serve as a good biochemical marker for chilling tolerance in horticultural crops during cold storage (Lafuente et al., 2001; Lafuente et al., 2004; Sala et al., 2005; Cao et al., 2009).

When changes in oxidative phenolic enzymes (POD and PPO) in the flavedo of lemon fruit during cold storage were examined, it was found that POD activity was inhibited by 10  $\mu$ M MJ plus 2 mM SA treatment. Choosing an effective treatment concentration is very important in inhibiting this enzyme responsible for tissue browning. Wang (1995) reported an increase in POD activity in zucchini squash preconditioned at 15°C for 2 days before cold storage at 5°C for 12 days, a temperature that induced cold damage. In our study, an increase of POD activity was found in chilling susceptible lemon fruit (control) that were stored at 2 and 4.5°C (Fig. 4), indicating that currently used storage regime will aggravate the occurrence of CI. Furthermore, the relatively higher POD activity observed during storage at 4.5°C may be associated with an increase in CI symptoms. Low POD activity in the flavedo fruit stored at -0.5°C could probably be explained by such ultra-low temperature in inhibiting POD activity in fruit, as Tomás-Barberán and Espín (2001) reported an inhibition of POD activity in crops stored at low temperatures (0-4.5°C). This was attributed to the fact that low temperatures were far from optimal for the enzyme POD (Tomás-Barberán and Espín, 2001). This could explain why POD activity was generally low at -0.5°C storage but increased with higher storage temperatures, where it might be involved in the appearance of CI in lemon fruit.

In many horticultural crops, PPO activity has been reported to be involved in tissue browning (Tomás-Barberán and Espín, 2001), a CI symptom that reduces fruit marketability. The negligible PPO activity in lemon flavedo (Fig. 5), may be caused by low levels of ethylene and high levels of citric acid, typical for lemon fruit as non-climacteric fruit with a relatively low level of ethylene production and high levels of citric acid (Kader, 1992; Fujii et al., 2007). However, these exact conditions reduce PPO activity (Vela et al., 2003). The development of CI in lemon fruit, could, therefore, not be attributed to PPO activity. This together with the very low PPO activity nullifies PPO as a possible biochemical marker of CI in lemon fruit.

Despite the association of PPO activity with tissue browning, the activity of this enzyme in response to chilling stress could vary between fruit species, cultivar and storage conditions (Pérez-Tello et al., 2009). The presented results are in agreement with previous studies by Martínez-Téllez and Lafuente (1993) and Pérez-Tello et al. (2009) who found no relationship between PPO activity and CI of cold stored citrus and sapote fruit, respectively. The substantial difference in PPO and POD activity indicates that the mechanisms triggering POD and PPO activity during cold storage are likely to be different (Raimbault et al., 2011).

In summary, moderate subtropical climatic conditions prevailing at farm locations are conveying a certain chilling tolerance to lemon fruit, followed by warm temperate climatic conditions do so to a lesser extent; cool subtropical climate conditions may pre-dispose lemon fruit to CI development. Postharvest storage temperatures may also play a role in chilling susceptibility of lemon fruit. Lemon fruit were more chilling tolerant when stored at  $-0.5^{\circ}\text{C}$  than when stored at  $2^{\circ}\text{C}$ . Storing lemon fruit at  $-0.5^{\circ}\text{C}$  may have led to suitable conditions for phenolic production which could have been enhanced by  $10\ \mu\text{M}$  MJ plus  $2\ \text{mM}$  SA treatment. The combined effect of temperature and postharvest chemical treatment with  $10\ \mu\text{M}$  MJ plus  $2\ \text{mM}$  SA may have played a role in cold acclimation against CI. Storing lemon fruit at  $4.5^{\circ}\text{C}$  seemed to favour the manifestation of CI symptoms. Moreover, postharvest treatment with  $10\ \mu\text{M}$  MJ plus  $2\ \text{mM}$  SA resulted in a decrease in CI index, probably through inducing defence mechanisms involved in cold acclimation (phenolics and PAL activity) and inhibiting the accumulation of POD activity. As postharvest treatment with  $10\ \mu\text{M}$  MJ plus  $2\ \text{mM}$  SA reduces CI by inducing cold acclimation increases the potential benefit of these hormones as commercial tools to manage CI on lemon fruit. The hypothesis that treatment with MJ and SA may enhance chilling tolerance in lemon fruit by inducing the synthesis of total phenolics content and PAL activity while inhibiting the production of POD and PPO activity is accepted.

However, the effectiveness of 10  $\mu$ M MJ plus 2 mM SA may depend on storage temperature. The PPO activity was negligible and this compound may not be a good biochemical marker for CI in lemon fruit. The potential benefit of using the combination of these hormones (MJ and SA) as commercial postharvest chemical treatments to manage to CI in lemon fruit should be explored further.

### **Acknowledgements**

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## Figure Legends

Fig. 1. The manifestation of chilling injury (CI) symptoms in lemon fruit from New Venture Farm, Tala Valley Citrus Estate and Sun Valley Estates treated with 10  $\mu$ M MJ, 2 mM SA or 10  $\mu$ M MJ plus 2 mM SA before cold storage either at -0.5, 2 or 4.5°C for up to 28 days plus 7 days at 23°C. Lemons were evaluated for CI symptom based on the following scale: 0 (no pitting), 1 (slight pitting), 2 (moderate pitting), 3 (severe pitting). Data are means of five replicates of 20 fruit  $\pm$  S.E. Least significant differences of means (5% level) = 0.26.

Fig. 2. Changes in the total phenolics (mg GAE  $g^{-1}$  DW) in the lemon flavedo of fruit from New Venture Farm, Tala Valley Citrus Estate and Sun Valley Estates treated either with various concentrations of MJ and SA before cold storage either at -0.5, 2 or 4.5°C for up to 28 days plus 7 days at 23°C shelf-life. Data are means of three replicates of 20 fruit  $\pm$  S.E. Least significant differences of means (5% level) = 0.20.

Fig. 3. Changes in the PAL activity (nmol $^{-1}$   $g^{-1}$  h) in the lemon flavedo of fruit from New Venture Farm, Tala Valley Citrus Estate and Sun Valley Estates when treated either with 10  $\mu$ M methyl jasmonate (MJ), 2 mM salicylic acid (SA) or 10  $\mu$ M MJ plus 2 mM SA before cold storage either at -0.5, 2 or 4.5°C for up to 28 days plus 7 days at 23°C. Data are means of three replicates of 20 fruit  $\pm$  S.E. Least significant differences of means (5% level) = 0.09.

Fig. 4. Changes in POD activity (units min $^{-1}$   $g^{-1}$ ) in the lemon flavedo of fruit obtained from New Venture Farm, Tala Valley Citrus Estate and Sun Valley Estates. Lemon fruit were treated either with 10  $\mu$ M methyl jasmonate (MJ), 2 mM salicylic acid (SA) or 10  $\mu$ M MJ plus 2 mM SA before cold storage either at -0.5, 2 or 4.5°C for 0, 7, 14, 21 or

28 days and 7 days at shelf-life. Least significant differences of means (5% level) = 0.14.

Fig. 5. Changes in the PPO activity ( $\text{units min}^{-1} \text{g}^{-1}$ ) in the lemon flavedo, after postharvest treatment with 10  $\mu\text{M}$  methyl jasmonate (MJ), 2 mM salicylic acid (SA) or 10  $\mu\text{M}$  MJ plus 2 mM SA. Least significant differences of means (5% level) = 0.01.

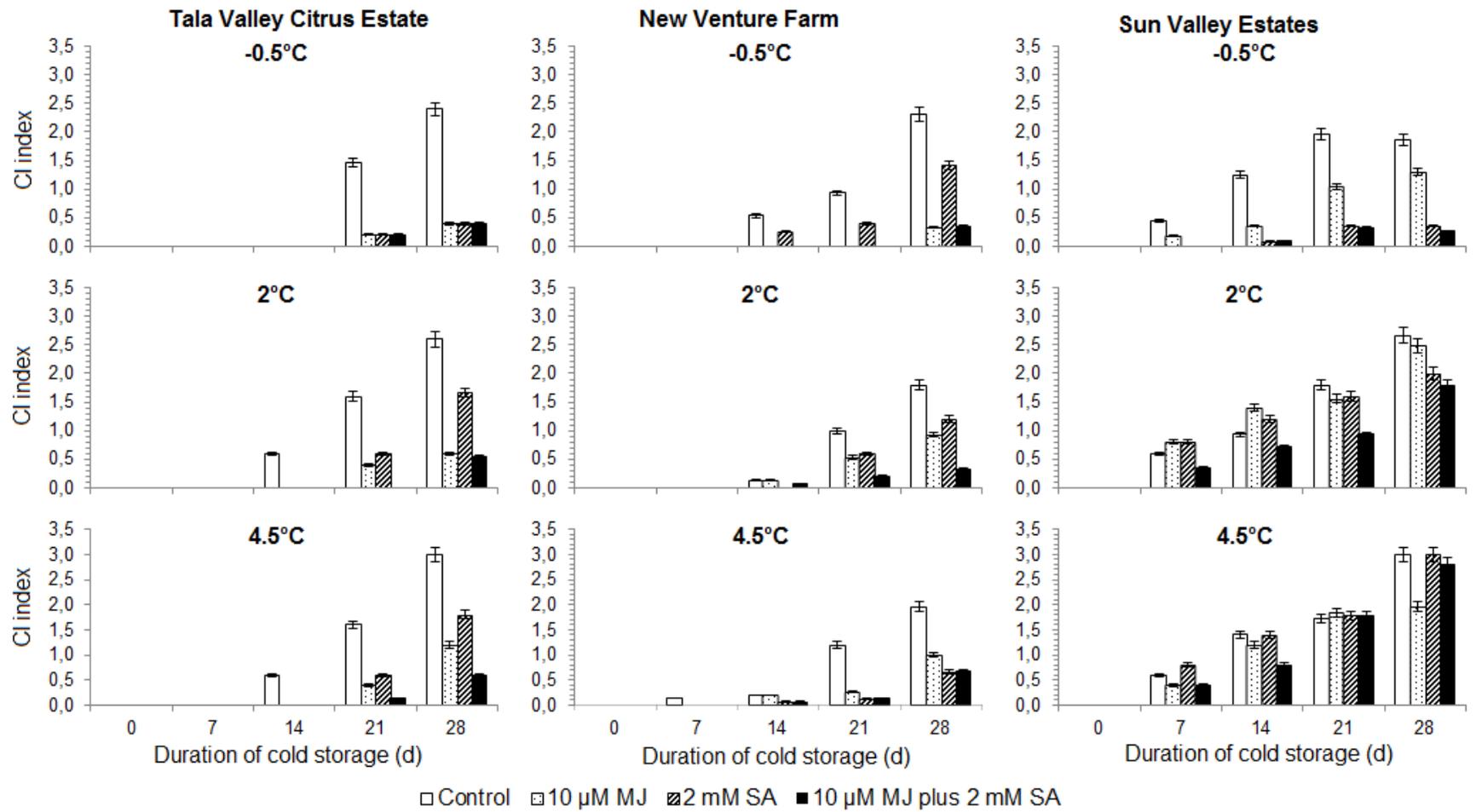


Fig. 1.

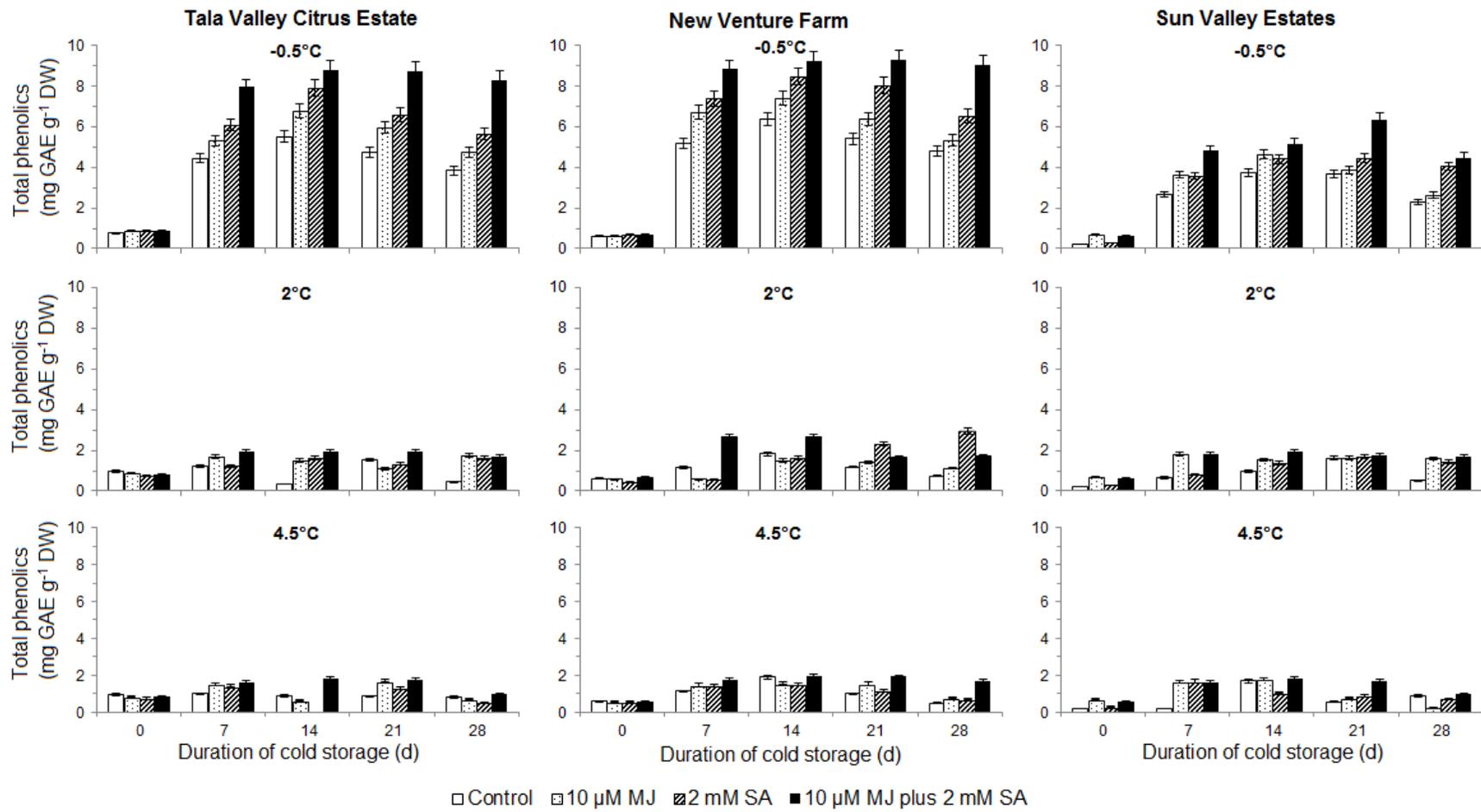


Fig. 2.

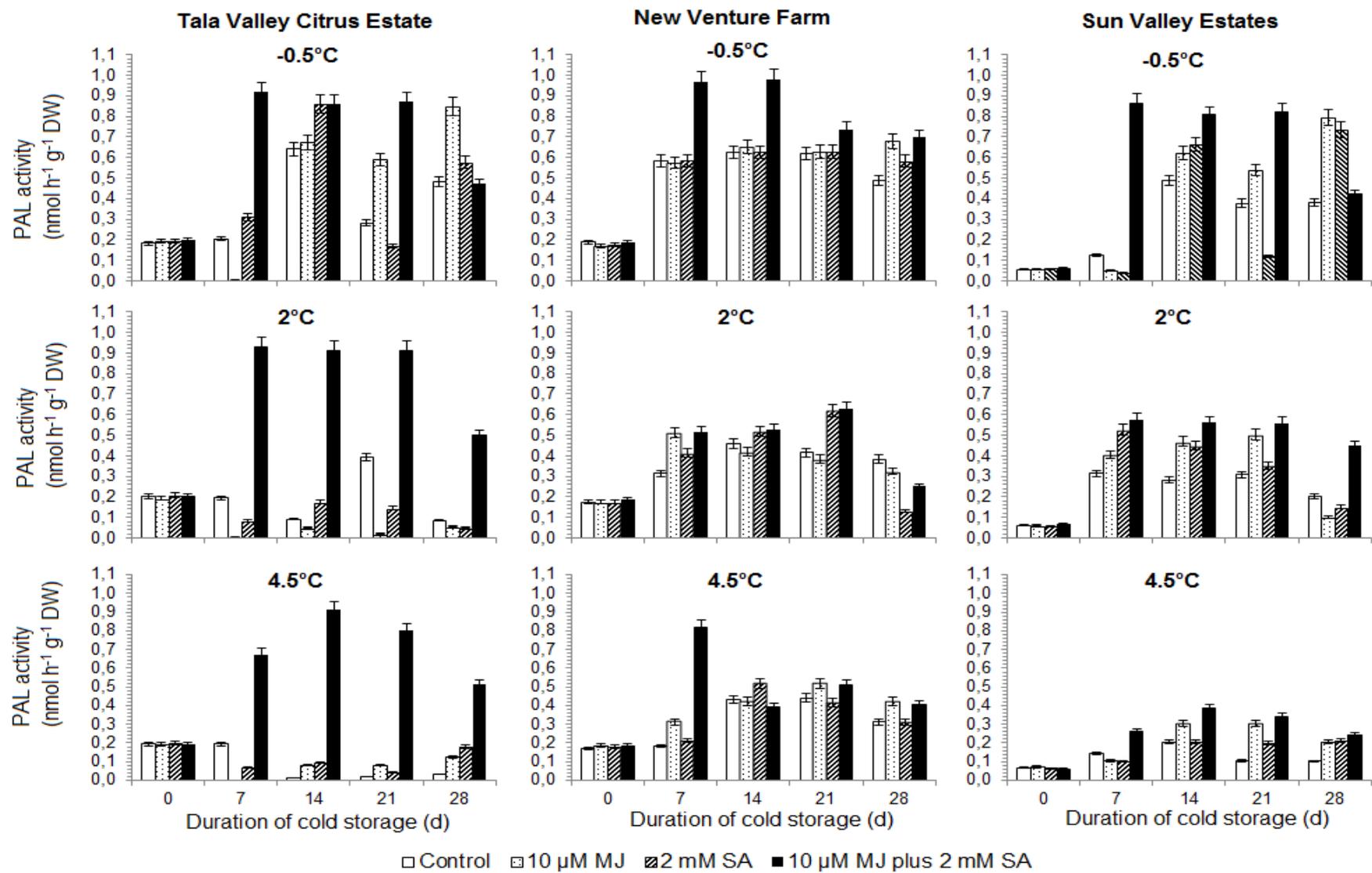


Fig. 3.

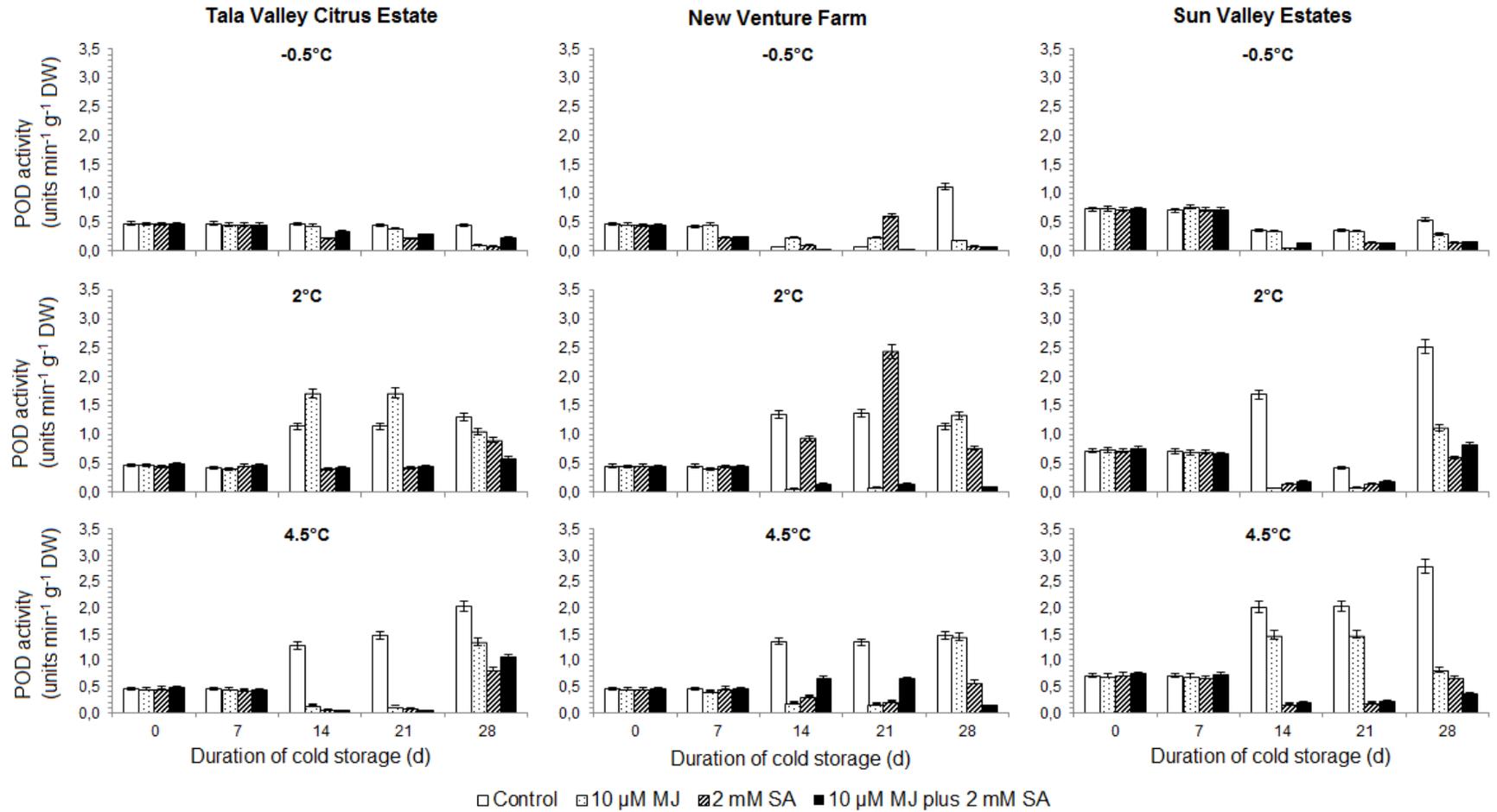


Fig. 4.

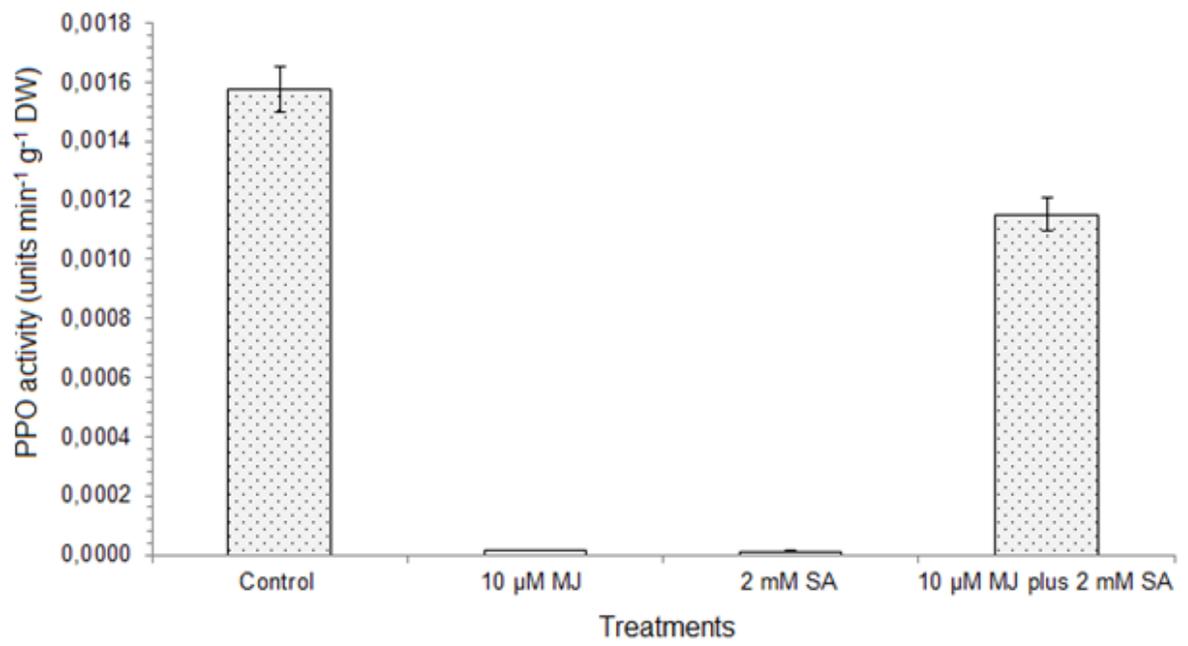


Fig. 5.

## **Chapter 6**

# Ultra-structural alterations and nutrient responses of methyl jasmonate and salicylic acid treated lemons (cv. Eureka) stored under chilling conditions

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## **Abstract**

Lemon fruit often sustain chilling injury (CI) during postharvest. With the aim of enhancing chilling tolerance and preserving tissue ultra-structure to maintain mineral nutrient concentrations of the flavedo, lemon fruit were treated with methyl jasmonate (MJ) and salicylic acid (SA), known chilling resistance inducers. Lemon fruit were obtained from the moderate subtropical (mean annual temperature: 14.5-26.5°C; mean annual rainfall: 712-717 mm), the warm temperate (mean annual temperature: 12.6-23.7°C; mean annual rainfall: 612-771 mm) and cool subtropical zone (mean annual temperature: 10.7-26.5°C; mean annual rainfall: 669-675 mm). Fruit were treated with MJ and SA solutions before storage at -0.5°C for 28 days plus 7 days at 23°C. The chilling damage in the flavedo of control fruit was marked by swollen, cracked, collapsed cuticle and disintegrated cell wall as well as flattened parenchyma cells. These damages could have impaired the physiological function of the cuticle and cell membrane. Treatment with 10 µM MJ plus 2 mM SA appeared to have a potential in enhancing chilling tolerance in lemon fruit by maintaining the integrity of the cuticle, cell wall and parenchyma cells in the lemon flavedo. This treatment also significantly ( $P < 0.05$ ) preserved the mineral nutrients of the flavedo during cold storage. The accumulation of mineral nutrients in the flavedo could have played a role in protecting the fruit against chilling stress and maintaining fruit quality. Therefore, treatment with 10 µM MJ plus 2 mM SA could be a useful tool to enhance chilling tolerance and preserving ultra-structural condition and mineral nutrients in lemon fruit at postharvest. Further studies, are required to understand ultra-structural changes in MJ and SA-treated lemon fruit.

*Keywords:* Chilling injury; Methyl jasmonate; Mineral nutrients; Salicylic acid; Ultra-structure

## 1. Introduction

Chilling stress has been recognised as a unique environmental factor influencing crop plant physiology for several decades (Kratsch and Wise, 2000). Most tropical and subtropical crops including citrus are susceptible to chilling injury (CI) (Wang, 2010) at postharvest. The symptoms of CI affect fruit quality and cause economic losses in the citrus industry. The reduction of CI symptoms and maintenance of postharvest quality becomes an important issue (Gómez et al., 2009). Considerable effort has been exerted to understand the basis of CI in citrus fruit; lemon fruit are regarded as very sensitive to CI (Lafuente et al., 2005).

Many studies suggest that the more sensitive a crop is to chilling, the sooner and more extensive are the ultra-structural changes (Wise and Naylor, 1987; Ma et al., 1990; Kratsch and Wise, 2000). To our knowledge, the ultra-structural changes of 'Eureka' lemon fruit during cold storage have not yet been investigated. Storage conditions have been suggested to be the major external factor influencing CI; however, preharvest factors seem to be the major precursors involved, such as mineral nutrients (Ezz and Awad, 2009) and environmental factors associated with farm locations. Mineral-nutrient status of horticultural crops plays a critical role in increasing resistance against biotic and abiotic stress (Waraich et al., 2011).

Exogenous postharvest treatments with plant growth regulators such as methyl jasmonate (MJ) and salicylic acid (SA) have been found to effectively reduce CI in grapefruit (*Citrus paradise* cv. Marsh seedless) stored at 2°C for 4-10 weeks (Meir et al., 1996), tomato (*Lycopersicon esculentum* L. cv. Beefstake) stored at 5°C for up to 4 weeks (Ding et al., 2001, 2002), loquat (*Eriobotrya japonica* Lindl.) stored at 1°C for 35 days (Cao et al., 2009a), and cucumber (*Cucumis sativus* L.) stored for 18 days at 1°C (Cao et al., 2009b). Recently, Sibozza et al. (2012) found that combining the two compounds (MJ and SA) enhanced chilling tolerance in lemon fruit (*Citrus limon* cv Eureka) during cold storage at -

0.5°C for up to 42 days plus 7 days at 23°C. However, the mode of action of treatment combination of MJ and SA in enhancing chilling tolerance still needs further understanding. Ladaniya (2008) emphasised that every postharvest treatment should aim to reduce losses and preserve nutritional quality of fruit. To our knowledge the influence of low temperature on mineral nutrients of lemon flavedo during cold storage is unknown. Therefore, the role of MJ and SA in reducing CI, whilst preserving nutritional quality and maintaining ultra-structural of lemon fruit during cold stress, were investigated.

## **2. Materials and Methods**

### *2.1 Plant material and farm locations*

Lemon fruit (c.v. Eureka) were obtained from three commercial farms located in KwaZulu-Natal, South Africa. New Venture Farm is located in the moderate subtropical zone (mean annual temperature: 14.5-26.5°C; mean annual rainfall: 712-717 mm; 31° 02' S 29° 25' E, 68-483 m asl). Tala Valley Citrus Estate is located in the warm temperate zone (mean annual temperature: 12.6-23.7°C; mean annual rainfall: 612-771 mm; 29° 52' S 30° 30' E, 416-922 m asl). Sun Valley Estates is located in the cool subtropical zone (mean annual temperature: 10.7-26.5°C; mean annual rainfall: 669-675 mm; 28° 83' S 30° 06' E, 820-1003 m asl). Lemon fruit were harvested on the 6<sup>th</sup> of June 2011 and 2012 seasons, and the trends were similar. Fruit were immediately transported on paved roads from the collection site to the laboratory in a ventilated vehicle. Fruit transportation distances from farm locations to the laboratory site were 39.2 km (Tala Valley Citrus Estates), 130 km (Sun Valley Estates) and 228 km (New Venture Farm). Lemon fruit were carefully selected for uniformity in colour, size, and absence of defects.

## *2.2 Postharvest treatments and storage conditions*

Lemon fruit were immediately transported by a ventilated vehicle to the laboratory and selected for uniformity in colour, size and freedom from defects. Subsequently, lemon fruit were washed with 0.1% Sporekill® (v/v) (Hygrotech Pty Ltd., Pretoria, South Africa) for 3 min and allowed to dry at 23°C for 1 hr. Lemon fruit were randomly divided into four lots of treatments, each containing 105 fruit, and treated according to Sibozza and Bertling (2013). Fruit were soaked into the following solutions: ultra-pure water (control), 10 µM MJ, 2 mM SA, or 10 µM MJ plus 2 mM SA for 30 s. After postharvest treatments, Citrashine® (Citrashine Pty Ltd, Johannesburg, South Africa) was used to wax all the lemon fruit. Lemon fruit were stored at -0.5 °C (air delivery temperature) for 0, 7, 14, 21 or 28 days. The relative humidity was maintained at ≈ 90%. After storage, lemon fruit were transferred to 23°C for 7 days (shelf-life), to allow the manifestations of CI symptoms.

## *2.3 Evaluation of chilling injury*

After 28 days at cold storage, with sampling intervals (0, 7, 14, 21, or 28 days) and an additional 7 days at shelf life, lemon fruit were evaluated for CI severity in the flavedo (the outer coloured part of the peel). The CI index was determined according to Lafuente et al. (2004), based on the following scale: 0 = normal ( no pitting), 1 = slight pitting (a few scattered pits), 2 = moderate pitting (pitting covering up to 30% of the fruit surface), 3 = severe pitting (extensive pitting covering > 30% of the fruit surface) and expressed as CI index.

## *2.4 Flavedo sample preparation*

After the evaluation of CI, flavedo tissue of each lemon was carefully removed using a potato peeler and immediately dipped in liquid nitrogen, and thereafter stored at -20°C. The flavedo

tissue was later freeze dried and returned to -20 °C storage. The samples were prepared according to Petracek et al. (1995) with slight modifications. Subsequently, the freeze dried flavedo tissue was pulverised in a mortar and pestle under liquid nitrogen. Samples were pulverised to be able to amount these on aluminium stubs.

### *2.5 Chilling damage in ultra-structural condition and mineral nutrients analysis in the lemon flavedo using Scanning Electron Microscope (SEM) in combination with Energy Dispersive X-ray spectroscopy (EDX)*

Chilling damage in the ultra-structure and mineral nutrients analysis in the flavedo were detected and accomplished according to Cohen et al. (1994) with slight modifications. Flavedo samples were attached to the microscope aluminium stub and observed at 10 to 20 kV; 0° tilt angle, 0.85 tor gas pressure, H<sub>2</sub>O gas type, 8.5 mm working distance. This was done using SEM in combination with EDX equipped with EDX detector (Zeiss Evo LS 15, Oxford Xmax detector, and INCA Energy EDX software). The SEM/EDX is an advanced tool that is used to identify and quantify the elemental composition of a sample. The nutrient content and ultra-structure condition of the lemon flavedo were studied in a wide range of magnification: x50 to x15,000.

### *2.6 Statistical evaluation*

Data were subjected to analysis of variance (ANOVA) using the GenStat<sup>®</sup> 14<sup>th</sup> Edition statistical package (VSN International, Hemel Hempstead, UK) with the treatments, farm locations and cold storage durations as factors. Least significant difference (LSD) at the 5% level was considered to be significant. Five replicates per four treatments (Control, 10 µM MJ, 2 mM SA or 10 µM MJ plus 2 mM SA) from all three locations (New Venture Farm, Tala Valley Citrus Estate or Sun Valley Estates) in 28 days at cold storage, with sampling intervals (0, 7, 14, 21, or 28 days) and an additional 7 days at shelf life were used in this experiment.

### 3. Results

#### 3.1. *The manifestation of CI symptoms in MJ and SA treated lemon fruit*

Cold storage of lemon fruit resulted in the manifestation of CI symptoms in the flavedo. The most visual symptoms were necrosis, pitting and red blotch. The symptoms were more severe in control fruit from Sun Valley Estates followed by New Venture Farm and Tala Valley Citrus Estate (Table 1). The development of CI appeared mainly after 14 days and gradually increased with prolongation of storage duration. Lemon fruit treated with 10  $\mu$ M MJ plus 2 mM SA did not suffer from CI until at the end of cold storage (at 28 days) (Table 1). Treatment with 10  $\mu$ M MJ plus 2 mM SA significantly ( $P < 0.05$ ) delayed the manifestation of CI and enhanced chilling tolerance compared to the fruit treated with the individual concentrations (10  $\mu$ M MJ or 2 mM SA).

#### 3.2. *Ultra-structural changes in lemon flavedo of fruit treated with MJ and SA*

The lemon flavedo observed under SEM contained various size and shape cells. As CI progressed, SEM observations showed swollen (Fig. 1), cracked (Fig. 2) and collapsed cuticles (wax) (Fig. 3). As cold storage prolonged (14-28 days), the parenchyma cells exhibited mild (Fig. 4) to severe (Fig. 5) chilling damage in control fruit. The parenchyma cells of the chilling susceptible lemon fruit (control) were flattened and elongated (Fig. 4 and 5) while the parenchyma cells of chilling tolerant lemon (MJ and SA treated) remained turgid and round (Fig. 7). Furthermore, the cuticle of chilling susceptible lemon fruit (control) appeared cracked and collapsing while the cuticle of chilling susceptible lemon fruit (MJ and SA treated) lemon fruit consistently appeared smooth and intact. Postharvest treatment with 10  $\mu$ M MJ plus 2 mM SA appeared to enhance chilling tolerance in lemon fruit by maintaining ultra-structural integrity in the flavedo (Fig. 6 and 7).

#### 2.1. *Response of mineral nutrients in flavedo of lemon fruit treated with MJ and SA*

During cold storage, mineral nutrients were significantly ( $P < 0.05$ ) affected by treatments, farm locations, cold storage durations and the interactions of this factors. Postharvest treatments with either 10  $\mu\text{M}$  MJ, 2 mM SA or 10  $\mu\text{M}$  MJ plus 2 mM SA significantly ( $P < 0.05$ ) preserved and enhanced the mineral nutrients in the lemon flavedo (Fig. 8, 9 and 10). The level of effectiveness of MJ and SA concentrations in maintaining nutrients were not consistent with all the mineral nutrients. The most abundant mineral nutrients detected were sodium, phosphorus, calcium, and potassium (Fig. 8). The levels of magnesium, silicon, sulphur and aluminium (Fig. 9) were also significantly ( $P < 0.05$ ) increased by MJ and SA treatments in the lemon flavedo.

#### **4. Discussion**

Every sound postharvest management program aims to reduce losses and preserve nutritional quality (Ladaniya, 2008). Han et al. (2006) proposed that CI limits the distribution and storage of horticultural crops, resulting in extensive postharvest losses. In this study, postharvest treatment with 10  $\mu\text{M}$  MJ plus 2 mM SA appeared to have a potential in enhancing chilling tolerance in lemon fruit (Table 1). The role of MJ and SA treatment combination in reducing CI in 'Eureka' lemon fruit during cold storage at  $-0.5^{\circ}\text{C}$  for up to 42 days and 7 days at  $23^{\circ}\text{C}$ , has been previously discussed by Sibozza and Bertling (2013). In this study, combination treatment with 10  $\mu\text{M}$  MJ plus 2 mM SA enhanced chilling tolerance in parallel with maintaining the ultra-structural changes in lemon flavedo.

The ultra-structural damages (swollen cuticle, cracked cuticle, chilling damaged parenchyma cells and cell wall disintegration) in the flavedo of control fruit were associated with chilling damage, suggesting that fruit were chilling stressed. This affected the ultra-structural conditions of the fruit. The swelling of the cuticle in control lemon flavedo corresponded with

the development of CI. Ma et al. (1990) advised that swelling is a common feature of CI in chilling-sensitive crops. In this study, the cracks and disintegration of the cuticle and disruption of cell wall (Fig. 1, 2, 3, 4, and 5) in control fruit were associated with the chilling damage of the fruit.

Previous studies (Medeira et al., 1999; Maia et al., 2004) suggested that collapse and flattening of the parenchyma cell layers of flavedo is the most evident alteration due to chilling damage in citrus. In this study, the parenchyma cells in control fruit (chilling susceptible) were flattened, disinterested and elongated (Fig. 4) while the parenchyma cells in chilling tolerant lemon (MJ and SA treated) were turgid and rounded (Fig. 7). Similarly, cold storage of Kiwifruit at  $-0.5^{\circ}\text{C}$  for 24 weeks resulted in cell wall disintegration due to chilling damage (Sfakiotakis et al., 2005). Ma et al. (1990) reported that the swelling of the chloroplast in mungbean leaves as induced by the chilling stress, may be due to the enzyme responsible for the degradation of starch in the stroma of chloroplasts remaining active.

Our results are consistent with previous studies suggesting an important contribution of micro-cracking to the extent of external CI symptoms (Rhee and Iwata, 1982; Fernández-Trujillo and Martínez, 2006; Martínez and Fernández-Trujillo, 2007). Cuticle plays an essential role in maintaining high water content within tissues necessary for normal metabolism (Ladaniya, 2008). In this study, the observed cracks on the cuticle could have impaired the physiological function of the cuticle and increased water loss (Medeira et al., 1999; Jaitrong et al., 2005). Jaitrong et al. (2005) suggested that chilling injured cell membrane would accelerate the oxidation of phenolics resulting to tissue browning (CI symptoms). The chilling damage in ultra-structure in control fruit, were not observed in chilling tolerant lemon (MJ and SA treated) flavedo tissue.

The maintenance of normal ultra-structural in lemon fruit treated with 10  $\mu$ M MJ plus 2 mM SA could explain the role of MJ and SA in enhancing chilling tolerance in 'Eureka' lemon fruit. Han et al. (2006) suggested that ultra-structural changes in the cell wall in response to chilling stress still need to be further explored. The results of this study suggest that postharvest treatment with 10  $\mu$ M MJ plus 2 mM SA enhances chilling tolerance in lemon fruit by maintaining the cell wall integrity and parenchyma cells and preventing cuticle cracking and disintegration. Further studies, are required to understand the role of MJ and SA in maintaining the ultra-structure of the peel of lemon fruit during cold storage.

Mineral-nutrient status of crops has been suggested to play a crucial role in increasing crop resistance to environmental stresses including chilling (Marschner, 1995; Waraich et al., 2011). This study suggests that the levels of mineral nutrients in lemon flavedo of susceptible fruit (control) were lower than in chilling tolerant fruit (MJ and SA treated). Postharvest treatments with 10  $\mu$ M MJ, 2 mM SA or 10  $\mu$ M MJ plus 2 mM SA could preserve and enhance the levels of mineral nutrients of lemon fruit during cold storage. The enhanced levels of mineral nutrients (carbon, oxygen, phosphorus, potassium, calcium, magnesium, sulphur, sodium, silicon and aluminium) in lemon flavedo could have played a role in protecting the fruit against chilling stress and maintained fruit quality. The levels of these nutrients stay high because the cuticle cell walls of the peel did not disintegrate.

The enhanced levels of calcium in lemon flavedo of MJ and SA treated fruit could have been involved in increasing chilling tolerance. Calcium has been suggested to increase abiotic stress tolerance in fruit at postharvest (Bowler and Fluhr, 2000; Toivonen and Hodges, 2011). Wang (2010) advised that calcium may be involved in chilling tolerance in crop tissues, possibly by improving fruit quality (Ezz and Awad, 2009). The results of our study suggest that MJ and SA treatment enhance chilling tolerance in lemon fruit by inducing higher calcium levels. The

involvement of calcium in mediating chilling stress response is suggested to be by activating plasma membrane enzyme ATPase required to pump back lost nutrients during cell membrane damage (Palta, 2000; Waraich et al., 2012). In this study, chilling tolerant lemon fruit were found to have higher calcium content than susceptible fruit. Therefore, our results are consistent with findings by Slutzky et al. (1981) and Wang (2010) that calcium may be involved in chilling tolerance in horticultural crops.

Phosphorus is a mineral-nutrient known to be involved in enzyme regulation, transportation of carbohydrates, energy reservation and transfer (Hu and Schmidhalter, 2001; Waraich et al., 2011). In this study, the enhanced content of phosphorus in the flavedo of MJ and SA treated fruit, could have played a crucial role by regulating enzymes, and maintaining cell turgidity by increasing stomatal conductance (Waraich et al., 2011). The enhanced levels of potassium in chilling tolerant lemon fruit treated with 10  $\mu$ M MJ plus 2 mM SA treatments was associated with chilling tolerance. Potassium is reported to be important for survival of crops under environmental stress by activating enzymatic antioxidants (Marschner, 1995; Hu and Schmidhalter, 2005). In our study, potassium could have contributed in the enhancement of chilling tolerance by activating enzymatic antioxidant and maintaining ultra-structure of lemon flavedo.

Magnesium is another mineral-nutrient reported to enhance resistance against physiological disorders in horticultural crops (Slutzky et al., 1981; Ezz and Awad, 2009) by protecting thylakoid membrane (necessary for the reduction of oxidative damage) and increasing antioxidant enzymes (Candan and Tarhan, 2003; Tewari et al., 2004; Waraich et al., 2012). In this study, chilling tolerant lemon fruit (MJ and SA treated) were found to have high magnesium content while chilling susceptible lemon fruit (control) had low magnesium content. In addition, chilling susceptible lemon fruit were found to have higher amount of

reactive oxygen species and lipid peroxidation than chilling tolerant lemon fruit (data not shown). Studies suggest that low magnesium content in crops shown to accumulate significantly higher amount of lipid peroxidation (Candan and Tarhan, 2003; Tewari et al., 2004; Waraich et al., 2012) as a result to chilling stress. Therefore, our results indicate that the high content of magnesium in chilling tolerant lemon was associated with chilling tolerance in fruit. This suggested that postharvest treatments with MJ and SA enhance chilling tolerance in lemon fruit by preserving and inducing mineral nutrients involved in defence mechanisms such as magnesium.

Other mineral nutrients detected were micro-nutrients such as copper, zinc and molybdenum. However, these micro-nutrients did not show a significant and consistent response except for zinc and silicon. Ezz and Awad (2009) advised that the role of zinc is to provide resistance against chilling stress. In this study, the enhancement of zinc content by MJ and SA treatments could have contributed to chilling tolerance. This study provides evidence that treatment with 10  $\mu$ M MJ plus 2 mM SA enhances chilling tolerance by inducing defensive compounds such as mineral nutrients involved in plant stress responses.

Silicon is one of the most studied mineral-nutrient in plants. Studies report that silicon is beneficial for the healthy growth and development of plants, and is involved in plant environmental stress (Epstein, 1999; Ma, 2004; Liang et al., 2005). One of the major roles of silicon in alleviating abiotic stresses in plants has been associated with simulation of antioxidant system (Liang et al., 2005). In this study, the role of silicon in simulating antioxidant was supported by chilling tolerance in lemon fruit with high silicon content. This suggested that silicon was involved in chilling tolerance of lemon fruit. Therefore, treatment with 10  $\mu$ M MJ plus 2 mM SA enhanced silicon content in lemon flavedo during chilling stress which could have contributed to chilling tolerance.

In conclusion, postharvest treatment with 10  $\mu\text{M}$  MJ plus 2 mM SA significantly ( $P < 0.05$ ) reduced CI index and enhanced chilling tolerance, maintained the cell and cuticle ultra-structural of the peel and preserved nutritional quality of 'Eureka' lemon fruit during cold storage. The effect of 10  $\mu\text{M}$  MJ plus 2 mM SA at enhancing chilling tolerance in lemon fruit may be attributed to its ability to maintain the integrity of parenchyma cells, by maintaining cuticle and cell wall, and at the same time preserving mineral-nutrient quality of the fruit.

### **Acknowledgements**

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## **Table legends**

Table 1. The expression of chilling injury (CI) index in lemon fruit from Tala Valley Citrus Estate, New Venture Farm and Sun Valley Estates treated with methyl jasmonate (MJ) and salicylic acid (SA) and stored at  $-0.5^{\circ}\text{C}$  for 28 days plus 7 days at  $23^{\circ}\text{C}$  for simulation shelf-life. The evaluation was based on the following scale: 0 (no pitting), 1 (slight pitting), 2 (moderate pitting), 3 (severe pitting).

## **Figure legends**

Fig. 1. Swollen cuticle (wax) in lemon flavedo of chilling susceptible fruit during cold storage at  $-0.5^{\circ}\text{C}$  for 28 days plus 7 days at  $23^{\circ}\text{C}$  for simulation shelf-life.

Fig. 2. Cracked wax in lemon flavedo of untreated lemon fruit during cold storage at  $-0.5^{\circ}\text{C}$  for 28 days plus 7 days at  $23^{\circ}\text{C}$  for simulation shelf-life.

Fig. 3. Collapsed cuticle in lemon flavedo of the untreated lemon after cold storage at  $-0.5^{\circ}\text{C}$  for 28 days and 7 days at  $23^{\circ}\text{C}$  for simulation shelf-life.

Fig. 4. Chilling damaged parenchyma cells in lemon flavedo of the untreated lemon fruit during postharvest storage at  $-0.5^{\circ}\text{C}$  for 28 days plus 7 days at  $23^{\circ}\text{C}$  for simulation shelf-life.

Fig. 5. Severe chilling damage in lemon flavedo of the parenchyma cells of susceptible fruit after cold storage at  $-0.5^{\circ}\text{C}$  for 28 days and 7 days at  $23^{\circ}\text{C}$  for simulation shelf-life.

Fig. 6. Cuticle in lemon flavedo of the chilling tolerant lemon fruit (methyl jasmonate: MJ and salicylic acid: SA treated).

Fig. 7. Parenchyma cells in lemon flavedo of the chilling tolerant lemon fruit (methyl jasmonate: MJ and salicylic acid: SA treated) after cold storage at  $-0.5^{\circ}\text{C}$  for 28 days plus 7 days at  $23^{\circ}\text{C}$  for simulation shelf-life.

Fig. 8. Postharvest treatments with methyl jasmonate (MJ) and salicylic acid (SA) in maintaining phosphorus ( $LSD_{0.05} = 0.05$ ), sodium ( $LSD_{0.05} = 0.06$ ), potassium ( $LSD_{0.05} = 0.68$ ), and calcium content ( $LSD_{0.05} = 0.32$ ) in the lemon flavedo of fruit during cold storage at  $-0.5^{\circ}\text{C}$  for 28 days plus 7 days at  $23^{\circ}\text{C}$  for simulation shelf-life.

Fig. 9. Influence of postharvest treatments with methyl jasmonate (MJ) and salicylic acid (SA) in silicon ( $LSD_{0.05} = 0.07$ ), magnesium ( $LSD_{0.05} = 0.05$ ), aluminium ( $LSD_{0.05} = 0.22$ ), and sulphur content ( $LSD_{0.05} = 0.04$ ) in lemon flavedo during cold storage at  $-0.5^{\circ}\text{C}$  for 28 days plus 7 days at  $23^{\circ}\text{C}$  for simulation shelf-life.

Table 1

Treatments	CI index			
	Cold storage time (days)	Tala Valley Citrus Estate	New Venture Farm	Sun Valley Estates
Control	0	0.00	0.00	0.00
	7	0.00	0.00	0.48
	14	0.00	0.66	1.52
	21	1.60	1.33	2.08
	28	2.44	2.45	2.86
	<b>Mean</b>	<b>0.81<sup>c</sup></b>	<b>0.89<sup>b</sup></b>	<b>1.39<sup>a</sup></b>
10 $\mu$ M MJ	0	0.00	0.00	0.00
	7	0.00	0.00	0.21
	14	0.00	0.00	0.39
	21	0.20	0.00	1.08
	28	0.42	0.51	1.35
	<b>Mean</b>	<b>0.13<sup>fg</sup></b>	<b>0.10<sup>fg</sup></b>	<b>0.61<sup>d</sup></b>
2 mM SA	0	0.00	0.00	0.00
	7	0.00	0.00	0.00
	14	0.00	0.35	0.08
	21	0.22	0.50	0.39
	28	0.42	1.63	0.32
	<b>Mean</b>	<b>0.13<sup>fg</sup></b>	<b>0.50<sup>e</sup></b>	<b>0.16<sup>f</sup></b>
10 $\mu$ M MJ plus 2 mM SA	0	0.00	0.00	0.00
	7	0.00	0.00	0.00
	14	0.00	0.00	0.07
	21	0.22	0.00	0.29
	28	0.42	0.38	0.33
	<b>Mean</b>	<b>0.13<sup>fg</sup></b>	<b>0.08<sup>g</sup></b>	<b>0.14<sup>fg</sup></b>

Means followed by different lower-case letters are significantly different at ( $P < 0.05$ ) by the Duncan's Multiple Range Test.

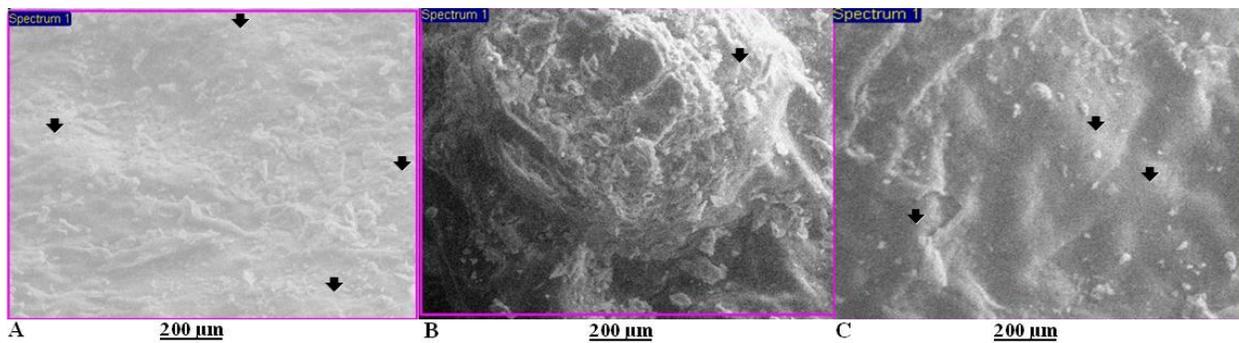


Fig. 1.

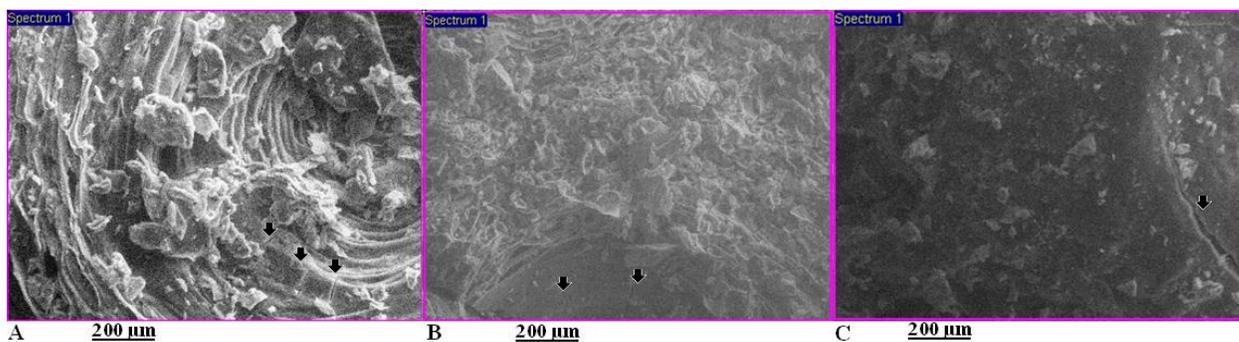


Fig. 2.

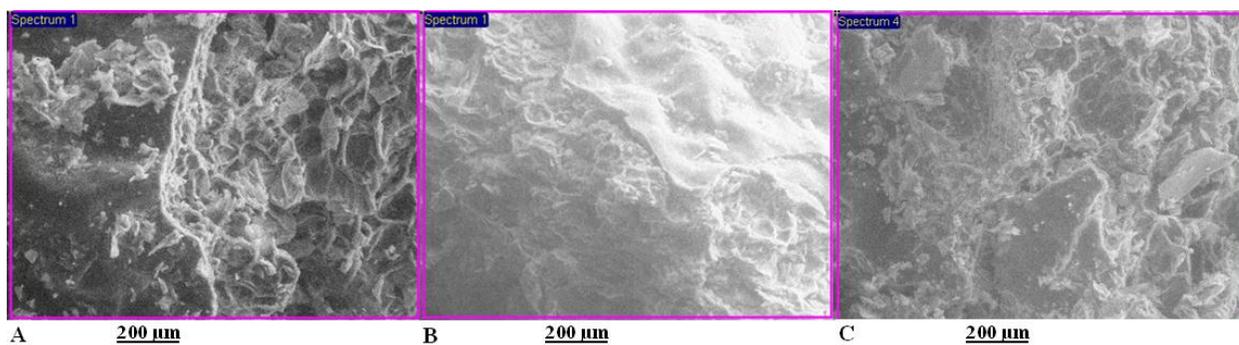


Fig. 3.

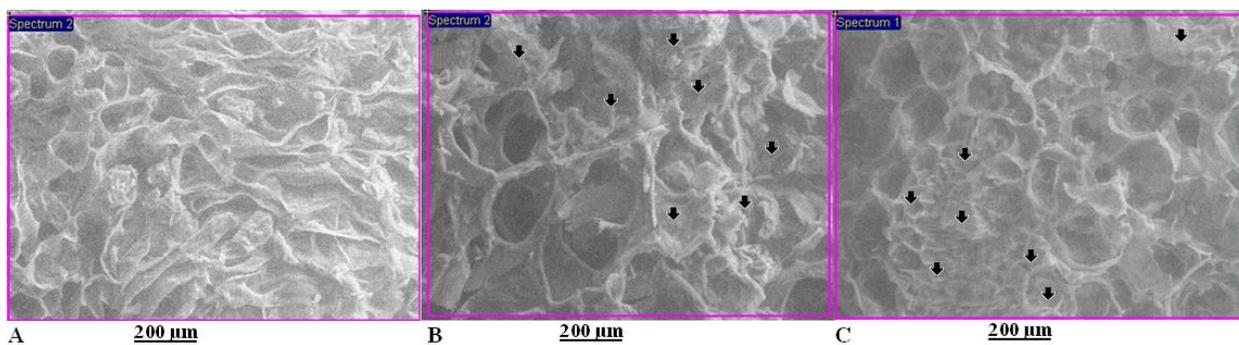


Fig. 4.

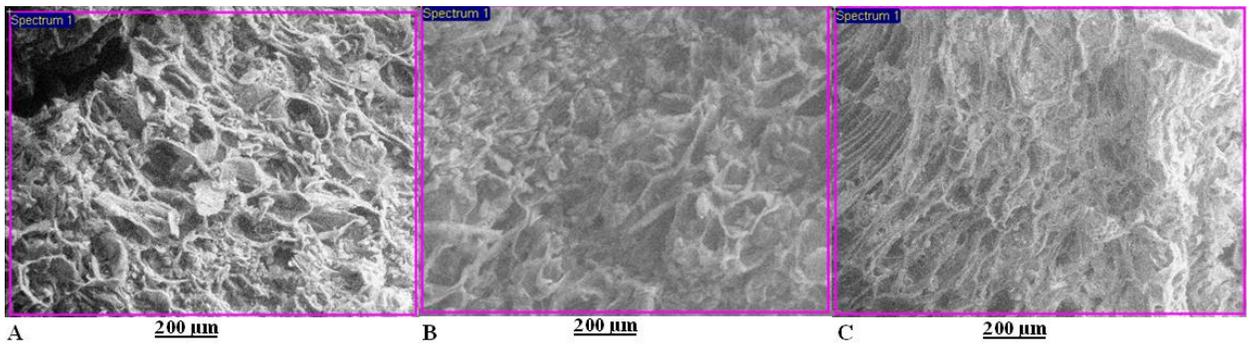


Fig. 5.

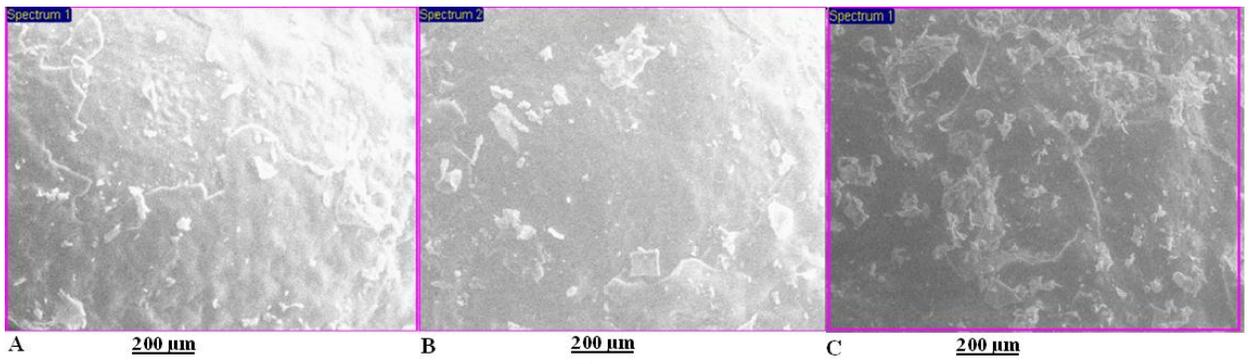


Fig. 6.

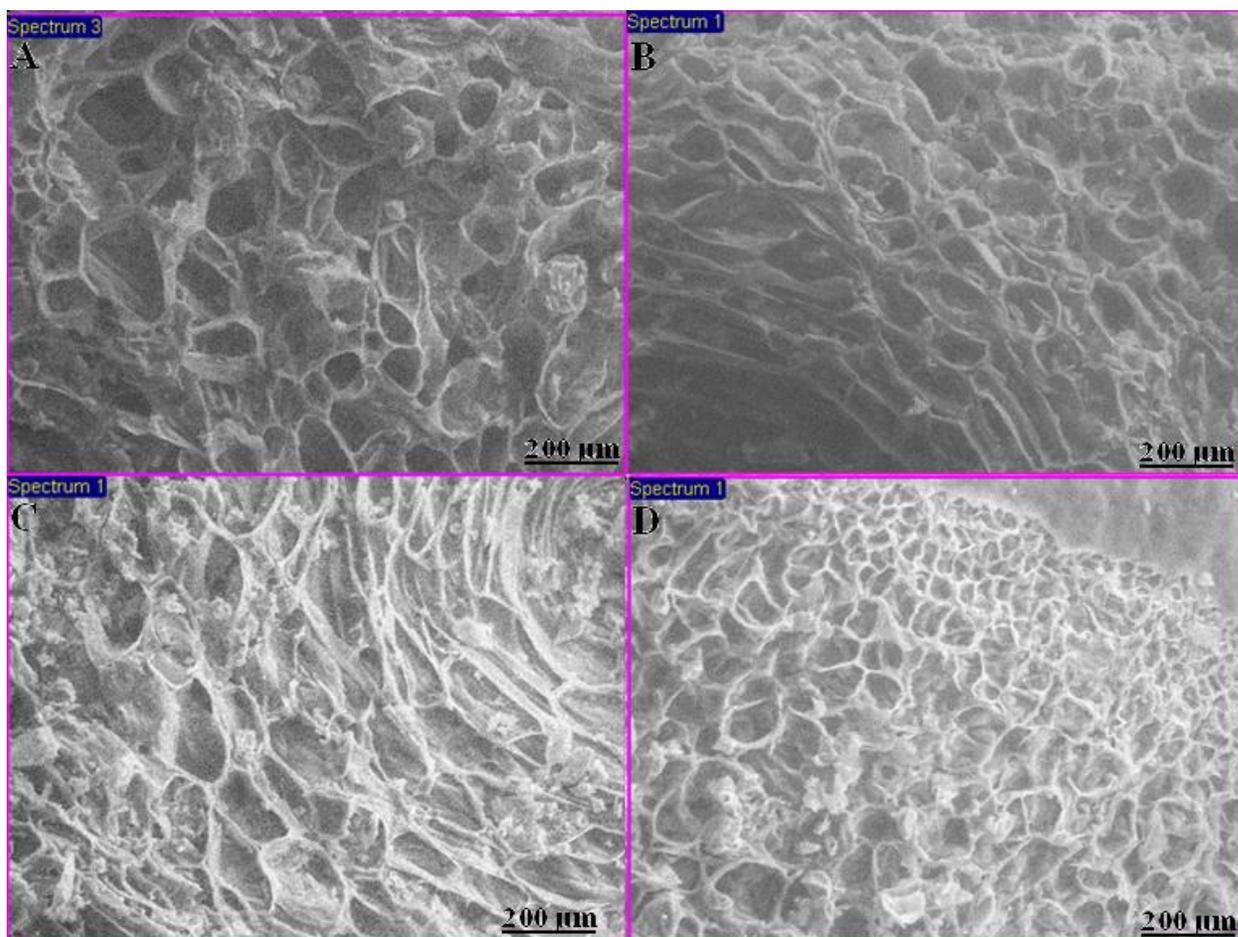


Fig. 7.

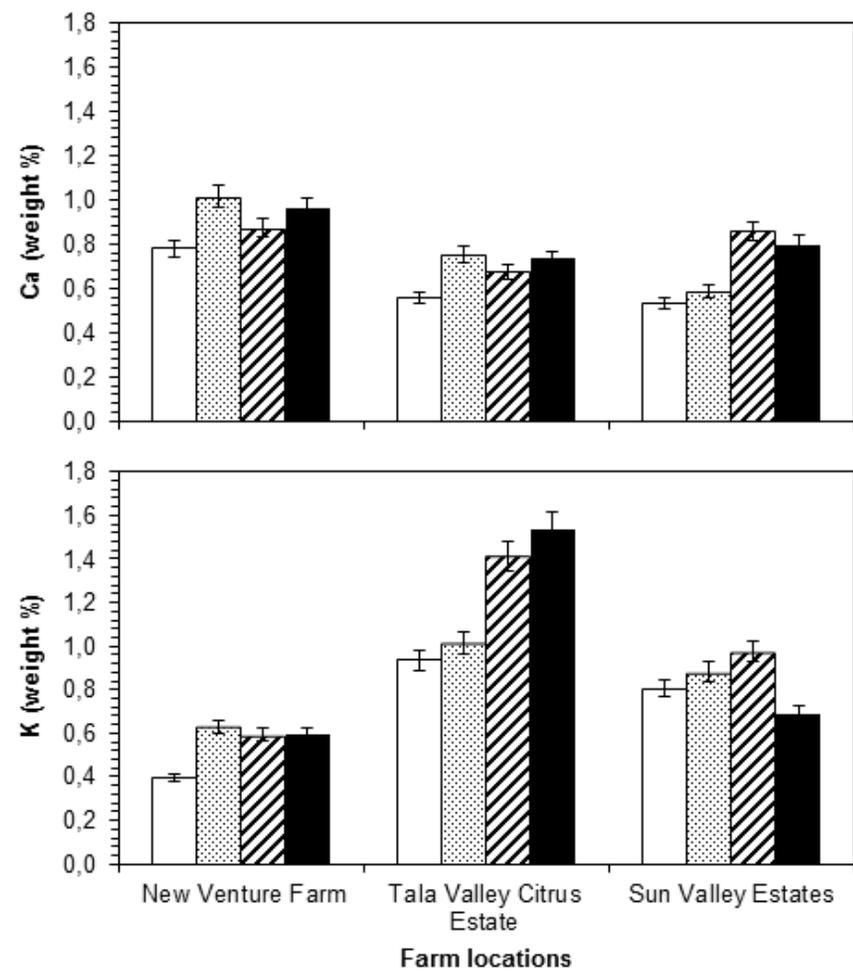
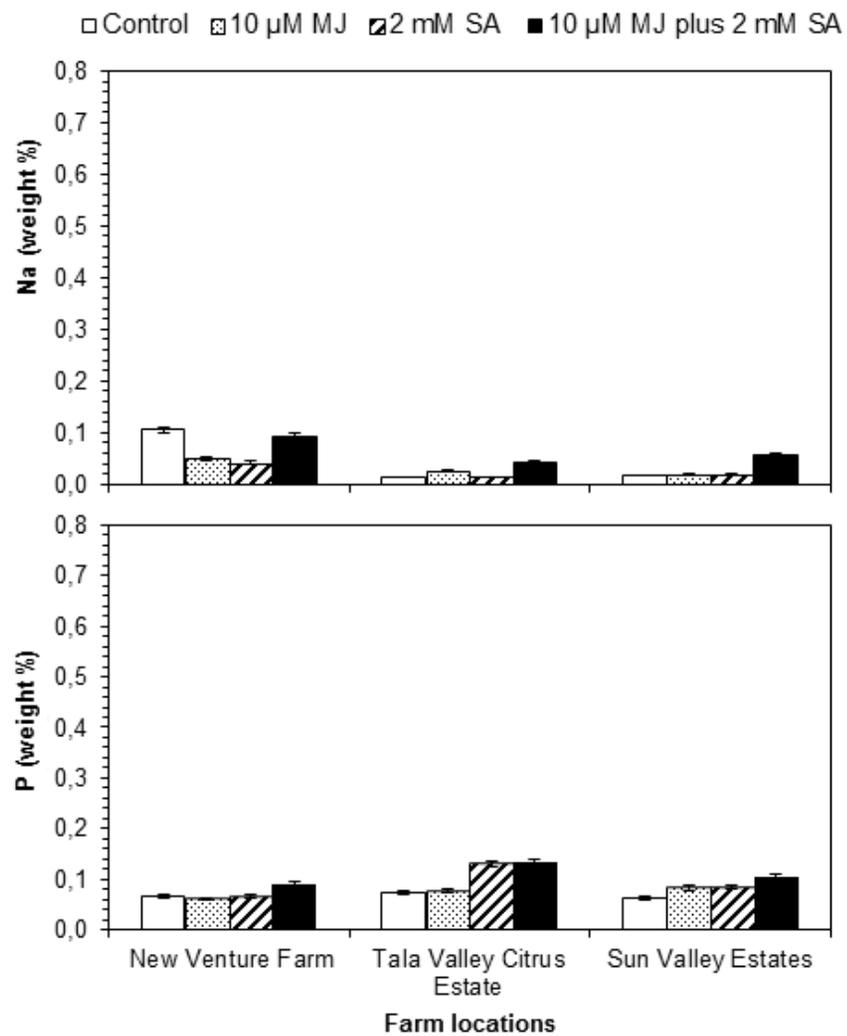


Fig. 8.

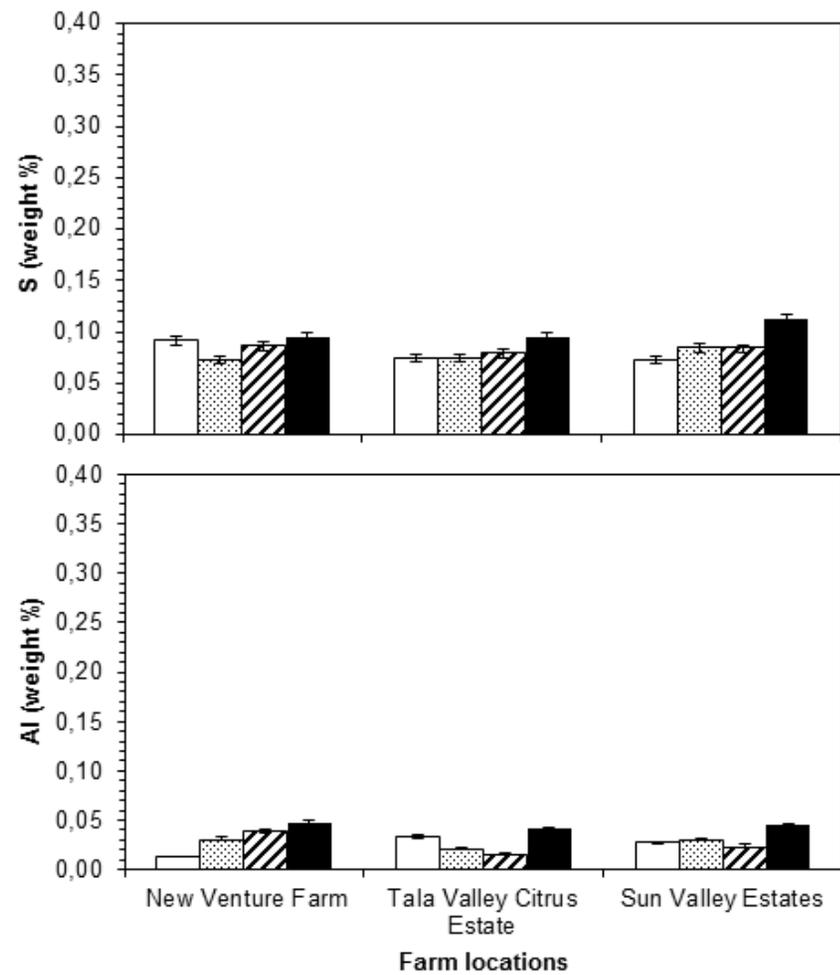
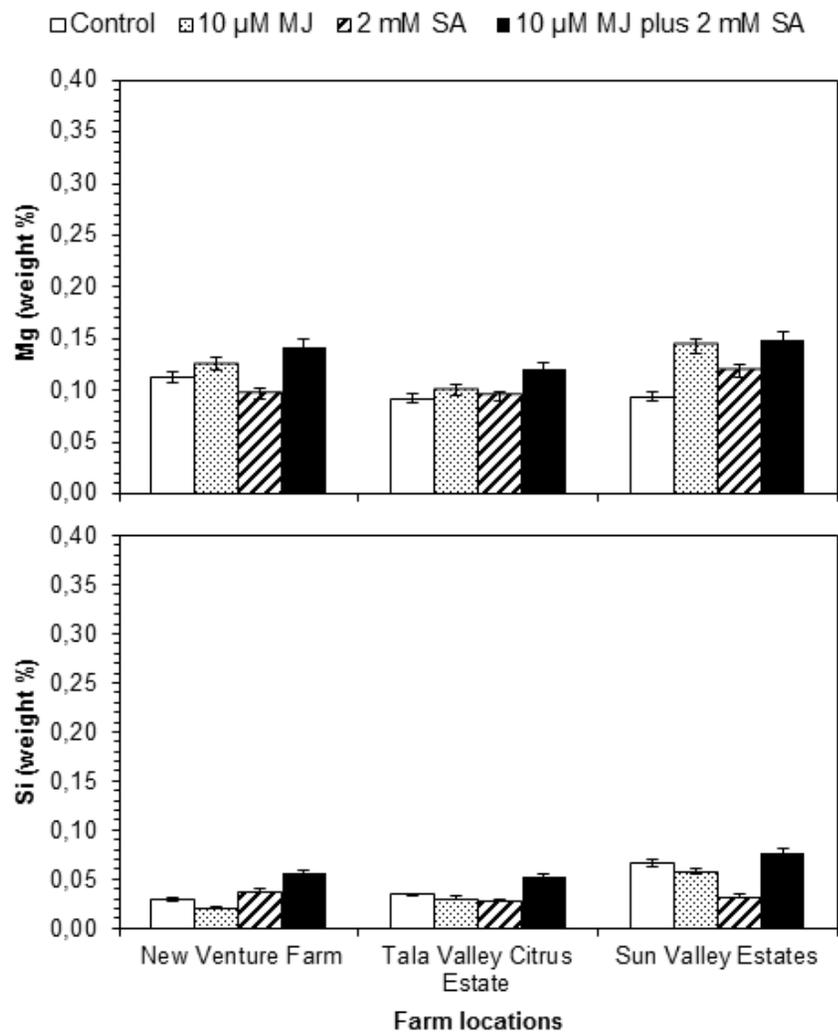


Fig. 9.

## Chapter 7

Alteration of enzymatic antioxidants and inhibition of oxidative stress by methyl jasmonate and salicylic acid to increase chilling tolerance in lemon fruit [*Citrus limon* (L.) Burm. F.]

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## **Abstract**

To prevent excessive accumulation of reactive oxygen species (ROS), as typically occurs during cold storage, fruit have evolved antioxidant defence mechanisms and heat shock proteins (HSPs) that reduce chilling injury (CI). It was hypothesised that treatments with methyl jasmonate (MJ) and salicylic acid (SA) may enhance chilling tolerance in lemon fruit by inducing the production of enzymatic antioxidant systems. Lemon fruit from different climatic zones were treated with MJ and SA concentrations and stored at -0.5, 2, or 4.5°C for 28 days plus 7 days at 23°C. The manifestation of CI and changes in ROS as well as in enzymatic antioxidant systems, such as catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11) and glutathione reductase (GR; EC 1.6.4.2); and HSPs were investigated in lemon flavedo. Symptoms of CI were more severe in control fruit stored at 4.5°C than at 2 or -0.5°C. Fruit grown in the moderate subtropical zone (mean annual temperature: 14.5-26.5°C; mean annual rainfall: 712-717 mm) were more chilling tolerant than fruit from the warm temperate zone (mean annual temperature: 12.6-23.7°C; mean annual rainfall: 612-771 mm). Fruit from the cool subtropical zone (mean annual temperature: 10.7-26.5°C; mean annual rainfall: 669-675 mm) were chilling susceptible and developed severe CI symptoms. Treatment with 10 µM MJ plus 2 mM SA reduced CI, suppressed ROS production, increased CAT, APX and GR activity, and enhanced the accumulation of HSPs; suggesting that enzymatic antioxidants and HSPs are involved in conveying chilling tolerance in MJ- and SA-treated lemon fruit. The increase in activity of these antioxidant enzymes together with HSPs could have contributed to the mode of action by which MJ and SA convey chilling tolerance to lemon fruit.

*Keywords:* Ascorbate peroxidase; Catalase; Chilling injury; Glutathione reductase; Heat shock proteins; Methyl jasmonate; Reactive oxygen species; Salicylic acid.

## 1. Introduction

The high sensitivity of lemon fruit to chilling imposes a major limitation to the postharvest handling of this fruit (Porat et al., 2004). Chilling injury (CI) visible in the flavedo arises from oxidative damage triggered by the uncontrolled production of reactive oxygen species (ROS) (Siboza and Bertling, 2013). Such an accumulation of ROS is harmful to cellular components and causes oxidative damage, resulting in lipid peroxidation, protein oxidation and polysaccharide degradation (García-Limones et al., 2002; Baek and Skinner, 2003). Cold storage of fruit and vegetables accelerates ROS production and causes oxidative stress (Baek and Skinner, 2003), thereby contributing to CI. Lipid peroxidation and the reduction of antioxidant mechanisms are signs of ROS over-production and thereby of oxidative stress (Ádám et al., 1988; Baek and Skinner, 2003). Therefore, to be protected against oxidative damage, fruit have evolved efficient systems (enzymatic antioxidants, liposoluble or membrane-associated antioxidants and water soluble antioxidants) to scavenge ROS (Baek and Skinner, 2003). Amongst the first group, the enzymatic antioxidant systems, catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), glutathione reductase (GR; EC 1.6.4.2) and superoxide dismutase (SOD; EC 1.15.1.1) are most prominent in horticultural crops (Halliwell and Gutteridge, 1999; Baek and Skinner, 2003).

Previous studies have demonstrated that treatments with the combination of plant the growth regulators methyl jasmonate (MJ) and salicylic acid (SA) can enhance lemon chilling tolerance by enhancing the flavedo antioxidant concentration (Siboza and Bertling, 2013). To gain deeper insight into the mode of action of MJ and SA treatments in lemon fruit isolating and identifying proteins that are involved in chilling stress resistance may be beneficial. Heat shock proteins (HSPs) are additional proteins known to confer chilling tolerance (Waters et al., 1996; Schoffl et al., 1998; Rozentzvieg et al., 2004). The accumulation of HSPs has been

reported to enhance chilling resistance in affected tissues (Mittler, 2002; Toivonen and Hodges, 2011). Studies indicate that HSPs act as molecular chaperones preventing the aggregation of denatured proteins and supporting their translocation to organelles (Parsell and Lindquist, 1993; Yan et al., 2006; Heidarvand and Amir, 2010). Furthermore, HSPs are thought to have a pivotal function in protecting tissues against CI by stabilising proteins and membranes, enabling protein refolding and the maintenance of cellular homeostasis (Toivonen and Hodges, 2011). There is only limited information on the influence of low temperature in triggering HSP expression (Ding et al., 2001). Despite the potential role of MJ and SA in reducing CI (Siboza and Bertling, 2013), little attention has been directed to the enhancement of HSPs triggered by MJ and SA in association with chilling tolerance in horticultural crops (Ding et al., 2001). The purpose of this study was, therefore, to quantify the activity of enzymatic antioxidant systems and the accumulation of HSPs as potential molecular markers of chilling tolerance during low temperature storage of chilling sensitive lemon fruit.

## **2. Materials and Methods**

### *2.1 Plant materials*

Export grade 'Eureka' lemon fruit [*Citrus limon* (L.) Burm. F.] were obtained from three farms in KwaZulu-Natal, South Africa, located in different climatic regions: New Venture Farm [moderate subtropical zone (mean annual temperature: 14.5-26.5°C; mean annual rainfall: 712-717 mm); 31° 02' S 29° 25' E, 68-483 m asl], Tala Valley Citrus Estate [warm temperate zone (mean annual temperature: 12.6-23.7°C; mean annual rainfall: 612-771 mm); 29° 52' S 30° 30' E, 416-922 m asl] and Sun Valley Estates [cool subtropical zone (mean annual temperature: 10.7-26.5°C; mean annual rainfall: 669-675 mm); 28° 83' S 30° 06' E, 820-1003 m asl]. Lemon fruit were harvested on the 6<sup>th</sup> of June 2011 and 2012 seasons, and

the trends were similar. Fruit were immediately transported on paved roads from the collection site to the laboratory in a ventilated vehicle. Fruit transportation distances from farm locations to the laboratory site were 39.2 km (Tala Valley Citrus Estates), 130 km (Sun Valley Estates) and 228 km (New Venture Farm). Fruit were selected for uniformity of size and colour, surface-sterilised and allowed to dry at 23°C for one hour before being randomly apportioned to treatments.

### *2.2 Postharvest treatments and storage*

Fruit were either dipped into 10 µM MJ, 2 mM SA or 10 µM MJ plus 2 mM SA for 30 s (Siboza and Bertling, 2013); control fruit were left untreated. Following treatments, lemon fruit were allowed to dry at 23°C for 2 h, thereafter waxed with Citrashine® and again allowed to dry at 23°C for 2 h. Fruit were stored either at -0.5, 2 or 4.5°C (air delivery temperature) in the refrigerated container with a relative humidity of 85-90%. Fruit were sampled at 0, 7, 14, 21 and 28 days into the cold storage. Following removal from cold storage, lemon fruit were transferred to 23°C for 7 days to observe development of CI symptoms (shelf-life). Five replicates of 20 fruit per treatment were used.

### *2.3 Determination of CI*

Lemon fruit were evaluated for CI by the method previously described by Sala and Lafuente (2000). 0 = normal (no pitting), 1 = slight pitting (a few scattered pits), 2 = moderate pitting (pitting covering up to 30% of the fruit surface), 3 = severe pitting (extensive pitting covering > 30% of the fruit surface) and expressed as CI index.

### *2.3.Flavedo tissue preparation*

Following the evaluation of CI, flavedo tissue was collected from the fruit surface, flash-frozen in liquid nitrogen and stored at -70°C. The flavedo tissue was then lyophilised, ground

to a powder with mortar and pestle in liquid nitrogen, and retained at  $-70^{\circ}\text{C}$  for ROS and enzyme analysis.

#### *2.4 Determination of reactive oxygen species (ROS)*

The formation of ROS in the flavedo was determined according to Maxwell et al. (1999) with improvements by Sibozza and Bertling (2013). Flavedo tissue (0.1 g DW) was mixed with 5 ml 5 mM 2',7'-dichlorofluorescence in diacetate solution. The sample was incubated for 30 min at room temperature and centrifuged at  $3000 \times g$  for 10 min. The supernatant was retained and diluted 50 times. Fluorescence was measured using a fully automated micro-plate-based multi-detection reader with excitation and emission wavelengths set at 485 nm and 520 nm, respectively. Results were validated according to Sibozza and Bertling (2013). Each value represents the mean of three replicates of 20 fruit per treatment  $\pm$  S.E.M.

#### *2.5 Flavedo preparation for protein extraction*

Samples were prepared according to the method of Sala et al. (2005). Flavedo tissue (3 g DW) was homogenised in 10 ml of acetone, previously chilled to  $-20^{\circ}\text{C}$ . The homogenate was extracted under vacuum using cold acetone until a colourless solution was obtained. The supernatant was discarded and the flavedo acetone powder was freeze-dried. The flavedo acetone powder was stored at  $-70^{\circ}\text{C}$  for enzyme (CAT, APX and GR) activity analysis. Each value represents the mean of three replicates of 20 fruit per treatment  $\pm$  S.E.M.

#### *2.6 Determination of catalase (CAT) activity*

The activity of CAT was determined according to Bergmeyer (1970). Flavedo acetone powder (0.5 g) was pulverised using mortar and pestle with 1.5 ml 50 mM Tris-hydrochloride (Tris-HCl), pH 7.8 containing 0.1 mM ethylenediamine tetraacetic acid (EDTA), 0.2% TritonX-100, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 2 mM dithiothreitol (DTT). The extract

was centrifuged at  $27\,000 \times g$  for 30 min at  $4^{\circ}\text{C}$ . The supernatant was used to determine CAT activity by recording the decrease in  $\text{H}_2\text{O}_2$  concentration as the change in absorbance at 240 nm in 10 seconds intervals for 180 seconds. One unit of CAT was defined as the amount of enzyme, which decomposes  $1\ \mu\text{mol}$   $\text{H}_2\text{O}_2$  per minute. The protein concentration was determined according to Bradford (1976), using bovine serum albumin (BSA) as a standard.

### *2.7 Determination of ascorbate peroxidase (APX) activity*

The activity of APX was measured according to Nakano and Asada (1981). Flavedo acetone powder (0.5 g DW) was pulverised using mortar and pestle with 1.5 ml cold 50 mM potassium phosphate buffer (pH 7.0) containing 2 % polyvinylpyrrolidone, 0.1 mM EDTA, and 2 mM ascorbic acid. The homogenate was centrifuged at  $27\,000 \times g$  for 30 min at  $4^{\circ}\text{C}$  and the supernatant was used to assay APX activity. The absorbance was read at 290 nm in 10 seconds intervals for 180 seconds. The amount of enzyme that oxidized  $1\ \mu\text{mol}$  ascorbic acid per minute at  $25^{\circ}\text{C}$  was defined as 1 unit of APX. The flavedo protein concentration was determined by the method of Bradford (1976), using BSA as a standard.

### *2.8 Determination of glutathione reductase (GR) activity*

Extraction of GR was achieved by following the method of Sala and Lafuente (2000). Flavedo acetone powder (1 g DW) was mixed with 10 ml 100 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM EDTA at  $4^{\circ}\text{C}$ . The solution was centrifuged at  $27\,000 \times g$  for 15 min at  $4^{\circ}\text{C}$ . The supernatant was used to assay GR activity according to Smith et al. (1988). One unit of GR was defined as the amount of enzyme that catalysed the oxidation of  $1\ \mu\text{mol}$  NADPH per minute. The protein concentration was determined by the method of Bradford (1976), using BSA as a standard.

### 2.9 Total protein extraction

Proteins were extracted as described previously by Isaacson et al. (2006) with slight modifications. The colourless flavedo powder (0.2 g DM) was suspended in 10 ml extraction buffer (0.5 M Tris-HCl pH 7.5; 50 mM EDTA; 0.1 M KCl; 0.7 M sucrose, 2%  $\beta$ -mercaptoethanol (v/v) and 1 mM PMSF). The mixture was vortexed for 30 s and centrifuged at  $5050 \times g$  for 10 min at 4°C. The homogenate was filtered through three layers of extraction buffer-wetted Miracloth. The supernatant was stored at -20°C for further protein analysis.

### 2.10 Protein separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Protein samples were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described by Laemmli (1970). PageRuler™ Prestained Protein Ladder Plus with a broad range of 10-250 kDa was used to monitor protein separation of the SDS-PAGE. Visualization of protein bands was enhanced by silver-staining the gels as previously described by Rabilloud et al. (1988). The experiment was repeated three times.

### 2.11 Data analysis

The experimental setup was as follows: three farm locations (Tala Valley Citrus Estate, New Venture Farm or Sun Valley Estates), four treatments (control, 10  $\mu$ M MJ, 2 mM SA or 10  $\mu$ M MJ plus 2 mM SA), three storage temperatures (-0.5, 2, or 4.5°C) and five storage durations (0, 7, 14, 21 or 28 days) and five replicates per treatment were used. Data were subjected to analysis of variance (ANOVA) using the GenStat® 14<sup>th</sup> Edition statistical package (VSN, International Ltd., Hemel Hempstead, UK). Differences at  $P < 0.05$  were considered to be significant.

### 3. Results

#### 3.1. *Chilling tolerance in MJ- and SA-treated lemon fruit during cold storage*

Cold storage of lemon fruit induced CI; this injury was significantly ( $P < 0.05$ ) affected by postharvest treatment, storage temperature, farm location and the interaction of these factors. The CI index increased with increasing cold storage temperature (-0.5, 2 to 4.5°C) and severity differed between farm locations (Table 1). Postharvest treatments with 10 µM MJ, 2 mM SA or 10 µM MJ plus 2 mM SA significantly ( $P < 0.05$ ) reduced CI with 10 µM MJ plus 2 mM SA being the most effective concentration (Table 1).

#### 3.2. *Alteration in ROS concentration in lemon flavedo of MJ- and SA-treated lemon fruit*

The lemon flavedo of 10 µM MJ plus 2 mM SA treated fruit was found to have both lower ROS levels (Fig. 1) and less CI symptoms (Table 1) than the flavedo of fruit treated either with 10 µM MJ or 2 mM SA . Furthermore, storing lemon fruit at -0.5°C retarded the accumulation of ROS in lemon fruit significantly ( $P < 0.05$ ) more than storage at 2°C (Fig. 1). The low accumulation of ROS in lemon fruit stored at -0.5°C was accompanied by minor CI symptoms. Contrary, storing lemon fruit at 4.5°C resulted in high ROS accumulation accompanied by severe CI symptoms.

#### 3.3. *Changes in CAT activity in lemon flavedo of MJ- and SA-treated lemon fruit*

During cold storage of lemon fruit, CAT activity of lemon flavedo declined in all treatments. Moreover, this activity was significantly ( $P < 0.05$ ) affected by postharvest treatments, storage temperature, farm location, cold storage time and the interactions of these factors. Flavedo CAT activity declined with an increase in storage temperature (Fig. 2), so that the CAT activity of lemon fruit stored at -0.5°C was higher than that of lemon fruit stored at 2°C. Lemon fruit stored at 4.5°C showed a gradual decline in CAT activity (Fig. 2). The decrease in

CAT activity corresponded with the development of CI. The decline in CAT activity in lemon flavedo of control fruit was accompanied by CI symptoms. Postharvest treatment with 10  $\mu$ M MJ, 2 mM SA, or 10  $\mu$ M MJ plus 2 mM SA significantly enhanced CAT activity in lemon flavedo (Fig. 2).

#### *3.4. Changes in APX activity in lemon flavedo of MJ- and SA-treated lemon fruit*

Lemon flavedo APX activity was significantly ( $P < 0.05$ ) affected by postharvest treatment, storage temperature, farm location and the interactions of these factors (Fig. 3). Treatment with 10  $\mu$ M MJ plus 2 mM SA was found to be the most effective treatment increasing APX activity (Fig. 3). In all treated lemon fruit, the APX activity increased more rapidly than in control fruit (Fig. 3). High APX activity was observed in lemon fruit stored at  $-0.5^{\circ}\text{C}$  followed by lemon fruit that were stored at  $2^{\circ}\text{C}$ . Low APX activity was observed in chilling susceptible lemon fruit that were stored at  $4.5^{\circ}\text{C}$  (Fig. 3).

#### *3.5. Glutathione reductase activity in lemon flavedo of MJ- and SA-treated lemon fruit*

The GR enzyme activity was significantly ( $P < 0.05$ ) affected by treatment, storage temperature, farm location and the interactions of these factors. Postharvest treatment with 10  $\mu$ M MJ plus 2 mM SA increased GR activity during cold storage (Fig. 4). An increased GR activity of the lemon flavedo was achieved by treatments with 10  $\mu$ M MJ, 2 mM SA or 10  $\mu$ M MJ plus 2 mM SA (Fig. 4). During cold storage, GR activity was higher in lemon flavedo of chilling tolerant lemon fruit stored at  $-0.5^{\circ}\text{C}$  than either at  $2^{\circ}\text{C}$  or  $4.5^{\circ}\text{C}$  (Fig. 4).

#### *3.6. Accumulation of a 70 kDa HSP in lemon flavedo of fruit treated with MJ and SA*

The accumulation of a 70 kDa protein in lemon flavedo was detected in fruit from certain farm locations and in those subjected to certain storage temperatures (Fig. 5). The 70 kDa protein was observed in both, treated and untreated fruit (Fig. 5 A and B). Storing lemon fruit at -

0.5°C resulted in stronger presence of this protein in the flavedo of Tala Valley Citrus Estate and New Venture Farm fruit than in the 2 or 4.5°C stored fruit (Fig. 5 A). On the other hand, fruit from Sun Valley lacked the accumulation of 70 kDa protein in (Fig. 5 D and E).

#### **4. Discussion**

Cold storage of lemon fruit resulted in CI symptoms. The injury was manifested mainly as pitting, red blotch and necrosis, symptoms previously noted by Sala and Lafuente (2000) in citrus fruit. The results of this study suggest that the treatment combination of SA and MJ greatly enhances the effects of reducing CI compared with using SA or MJ separately. Such positive effect of MJ and SA on reducing CI in lemon fruit has been previously reported to stem from antioxidant defence mechanisms (Siboza and Bertling, 2013). Similarly, in this study results suggest that postharvest treatment with 10 µM MJ plus 2 mM SA is involved in the induction of chilling tolerance. Furthermore, results suggest that storing lemon fruit at -0.5°C may be able to delay the manifestation of CI symptoms in lemon fruit.

The phenomenon that storing lemon fruit at -0.5°C results in less CI than storing fruit at 2 or 4.5°C could be explained by the enhanced enzymatic antioxidant levels, HSP 70 kDa and low ROS levels in the flavedo of such fruit which could have prevented oxidative damage to membranes. Similarly, Zhang and Tian (2009) observed that peach fruit stored at 0°C did not suffer from CI, but CI symptoms developed under 5°C storage. The authors concluded that the higher levels of linolenic acid and higher membrane lipid unsaturation in peach fruit stored at 0°C, compared with that of fruit stored at 5°C, was beneficial to maintaining membrane integrity and enhancing chilling tolerance in peach fruit

Previous studies suggest that oxidative stress is involved in the development of CI in a variety of fruit crops (Shewfelt and Purvis, 1995; Mittler, 2002; Rivera et al., 2004; Sibozza and Bertling, 2013). In 'Eureka' lemon flavedo, the accumulation of ROS (Fig. 1) was found to be aligned with the manifestation of CI symptoms which increased with cold storage time in control fruit as well as in treated fruit (Table 1). Several authors reported that ROS in cell membranes alter the function of a variety of biomolecules, (lipids, proteins and nucleic acids) with the peroxidation of lipids in particular eventually leading to the loss of membrane integrity (Shewfelt and Purvis, 1995; Mittler, 2002; Rivera et al., 2004). In this study, the low levels of ROS in MJ-and SA-treated lemon fruit could have prevented oxidative damage in membranes.

The results of this study suggest that storing lemon fruit at  $-0.5^{\circ}\text{C}$  delay oxidative damage by limiting ROS generation and protecting lemon fruit against CI, whereas storage at warmer temperatures ( $4.5^{\circ}\text{C}$ ) favoured oxidative damage due to higher accumulation of ROS resulting in CI. Therefore, treatment with  $10\ \mu\text{M}$  MJ plus  $2\ \text{mM}$  SA protects lemon fruit from oxidative damage and enhances their chilling tolerance. Such treatment may detoxify ROS which otherwise would have led to severe CI. Sala (1998) advises that plants are protected against oxidative stress by a complex antioxidant system. The effect of MJ and SA treatment preventing oxidative damage and increasing chilling tolerance in lemon fruit could be associated with such a complex antioxidant defence system.

Of the various enzymatic antioxidants, CAT is a major player involved in citrus fruit defence mechanisms against CI (Sala et al., 2005). Therefore, the enhanced CAT activity in the flavedo of  $10\ \mu\text{M}$  MJ plus  $2\ \text{mM}$  SA treated lemon fruit could have contributed to the detoxification of ROS and prevented oxidative damage, thereby increasing chilling tolerance and reducing occurrence of CI symptoms. Enzymatic antioxidants play an important role in

inhibiting or controlling ROS production during chilling (Foyer et al., 1997; Rivera et al., 2004). Protection of cells against oxidative damage can be afforded by CAT scavenging ROS (Imahori et al., 2008); in lemon flavedo such an increased CAT activity through 10  $\mu$ M MJ plus 2 mM SA treatment could, through scavenging free radicals during cold storage, have increased the chilling tolerance of the fruit.

Previous studies suggest that an incremental increase in cell ROS detoxification mechanisms can be an important factor preventing oxidative stress (Foyer et al., 1997; Rivera et al., 2004). In MJ and SA treated lemon fruit, APX activity increased more rapidly than in control fruit (Fig. 3). Similarly, in mandarin, chilling-tolerant fruit were found to have more APX activity than chilling-sensitive fruit (Sala, 1998). The present study provides evidence that storage temperature plays a crucial role in regulating enzymatic antioxidant in lemon flavedo. Storing lemon fruit at 4.5°C resulted in a reduced APX activity (Fig. 3), bringing about high oxidative damage (ROS) (Fig. 1) and severe CI symptoms, independent of farm location (Table 1). However, the increase in APX activity in lemon flavedo of fruit treated with 10  $\mu$ M MJ plus 2 mM SA may explain the observed chilling tolerance. The increase in APX activity could have enhanced ROS detoxification which may have contributed to the fruit's chilling tolerance.

Previous studies report that GR plays a crucial role in scavenging ROS and activation of protective mechanisms against chilling stress (Foyer et al., 1997; Kocsy et al., 2001). In this study, GR activity was higher in the lemon flavedo of chilling tolerant lemon fruit (-0.5°C) from Tala Valley Citrus Estate treated with 10  $\mu$ M MJ plus 2 mM SA than in chilling susceptible lemon fruit (control; Fig. 4). The higher GR activity in 10  $\mu$ M MJ plus 2 mM SA treated lemon fruit (chilling tolerant) could have been one of the reasons for the chilling tolerance of these fruit, particularly as such behaviour was not observed in chilling susceptible lemon fruit from Sun Valley Estates after 28 days of storage. The induction of GR activity by

treatment with 10  $\mu$ M MJ plus 2 mM SA could have increased chilling tolerance by controlling ROS production, as previous studies on chilling tolerance in maize, soybean (Pinhero et al., 1997; Kocsy et al., 2001) and mandarin fruit (Sala, 1998) suggested that an increase in GR activity may contribute to an enhanced chilling tolerance. Therefore, the induction of GR activity, and that of other enzymatic antioxidants, by MJ and SA treatments could be the mode of action by which these compounds increase chilling tolerance during cold storage.

One of the main postharvest challenges affecting citrus fruit exportation is their sensitivity to low temperature resulting to CI (Ding et al., 2001). This study suggests that the efficacy of 10  $\mu$ M MJ plus 2 mM SA in inhibiting CI may be enhanced by the right choice of storage temperature. The up-regulation of 70 kDa reportedly a group of HSPs (Ding et al., 2001), following storage of lemon fruit from Tala Valley Citrus Estate and New Venture Farm at -0.5°C (Fig. 5), was aligned with an enhanced chilling tolerance of such fruit. It has been reported that exposure of horticultural crops to chilling may enhance the presence of HSPs (Ding et al., 2001). In this study, the transcripts of the 70 kDa HSP were detected in all chilling tolerant lemon fruit. This suggested that 70 kDa HSPs are involved in chilling tolerance of lemon fruit.

This accumulation of 70 kDa HSPs during cold storage confirms previous findings by Ding et al. (2001) that HSPs contribute to the acquisition of chilling tolerance. Similarly, the accumulation of HSPs was associated with the increased chilling tolerance of tomato fruit (Whitaker, 1994; Sabehat et al., 1996; Ding et al., 2001). The up-regulation of 70 kDa HSPs could have played a crucial role in protecting lemon fruit against CI. Increasing storage temperature from -0.5°C to 2°C affected the appearance of protein bands. The HSP 70 kDa

protein bands appeared to be faint in fruit stored at 2°C in both, Tala Valley Citrus Estate and New Venture Farm. Storage temperature, therefore, plays a significant role in enhancing the presence of HSPs 70, possible inducers of chilling tolerance in lemon fruit. These proteins, however, accumulated in both, untreated and treated lemon fruit from Tala Valley Citrus Estate and New Venture Farm, possibly suggesting that farm location is further factor contributing to the production of HSP 70. These proteins appeared in the flavedo of lemon fruit from Tala Valley Citrus Estate and New Venture. Such protein presence was accompanied by chilling tolerance. On the other hand, fruit from Sun Valley Estates, unlike fruit from other locations, were chilling susceptible and did not accumulate HSP 70, regardless of the length of the cold storage period.

In conclusion, cold storage of lemon fruit results in increased ROS accumulation, which if not controlled, causes oxidative damage leading to CI. Postharvest treatment with 10 µM MJ plus 2 mM SA is effective in inducing mechanisms reducing CI, such as increasing the pool of enzymatic antioxidants (CAT, APX, GR), which will be able to scavenge the ROS produced during cold storage. This increase in enzymatic antioxidants varies with treatment concentration, farm location, storage temperature and cold storage duration. Postharvest treatment with 10 µM MJ plus 2 mM SA was effective in enhancing chilling tolerance in lemon fruit, probably by enhancing enzymatic antioxidants during cold storage. Storing lemon fruit at -0.5°C stimulated the accumulation of enzymatic antioxidants and HSPs, thereby probably delaying the manifestation of CI. The ability of -0.5°C storage to suppress CI was associated with the high stimulation of enzymatic antioxidant HSP 70 kDa. Furthermore, the ability of -0.5°C storage in enhancing chilling tolerance in lemon fruit could be associated with low generation of ROS. The induction of enzymatic antioxidant and HSP 70 kDa in the

lemon flavedo by the postharvest treatments with 10  $\mu$ M MJ plus 2 mM SA could be the means by which MJ and SA enhance chilling tolerance of lemon fruit during cold storage.

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## Table Legends

Table 1. The influence of postharvest treatments with 10  $\mu$ M MJ, 2 mM SA or 10  $\mu$ M MJ plus 2 mM SA on chilling injury (CI) index of lemon fruit from the New Venture Farm, Tala Valley Citrus Estate and Sun Valley Estates subjected to cold storage at at -0.5, 2 or 4.5  $^{\circ}$ C for up to 28 days plus 7 days at 23 $^{\circ}$  C.

## Figure Legends

Fig. 1. Levels of reactive oxygen species (ROS) in lemon flavedo of fruit treated with different concentrations of methyl jasmonate (MJ) and salicylic acid (SA) during cold storage at -0.5, 2 or 4.5  $^{\circ}$ C for up to 28 days plus 7 days at 23 $^{\circ}$  C. Least significant differences of means (5% level) = 1992.1.

Fig. 2. Changes in catalase (CAT) activity in flavedo of lemon fruit treated with 10  $\mu$ M methyl jasmonate (MJ), 2 mM salicylic acid (SA) or 10  $\mu$ M MJ plus 2 mM SA prior to cold storage at -0.5, 2 or 4.5  $^{\circ}$ C for up to 28 days plus 7 days at 23 $^{\circ}$  C. Least significant differences of means (5% level) = 0.44.

Fig. 3. Changes in ascorbate peroxidase (APX) activity in lemon flavedo of fruit treated with 10  $\mu$ M methyl jasmonate (MJ), 2 mM salicylic acid (SA) or 10  $\mu$ M MJ plus 2 mM SA and stored at -0.5, 2 or 4.5  $^{\circ}$ C for either at 0, 7, 14, 21 or 28 days plus 7 days at 23 $^{\circ}$  C. Least significant differences of means (5% level) = 0.27.

Fig. 4. Changes in glutathione reductase (GR) activity in lemon flavedo of fruit treated with 10  $\mu$ M methyl jasmonate MJ, 2 mM salicylic acid (SA) or 10  $\mu$ M MJ plus 2 mM SA and stored at -0.5, 2 or 4.5  $^{\circ}$ C for up to 28 days and 7 days at shelf life. Least significant differences of means (5% level) = 0.51.

Fig. 5. Effects of storage temperature of lemon fruit either at  $-0.5^{\circ}\text{C}$  (A),  $2^{\circ}\text{C}$  (B) or  $4.5^{\circ}\text{C}$  (C) on the accumulation of 70 kDa heat shock proteins (HSP) in the flavedo. Fruit from Sun Valley Estates lacked the accumulation of HSP 70 kDa (D) and (E). Where M = molecular marker; T0 = Control; T1 =  $10\ \mu\text{M}$  methyl jasmonate (MJ); T2 =  $2\ \text{mM}$  salicylic acid (SA); T3 =  $10\ \mu\text{M}$  MJ plus  $2\ \text{mM}$  SA; P = pawpaw.

Table 1

Treatments	Duration of cold storage (days)	CI index								
		Tala Valley Citrus Estate			New Venture Farm			Sun Valley Estates		
		-0.5°C	2°C	4.5°C	-0.5°C	2°C	4.5°C	-0.5°C	2°C	4.5°C
Control	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	14	0.00	0.60	0.60	0.64	0.13	0.20	1.31	0.88	1.03
	28	2.42	2.60	3.00	2.43	0.80	1.91	2.24	2.80	3.00
10 $\mu$ M MJ	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	14	0.00	0.00	0.00	0.00	0.13	0.20	0.43	1.40	1.04
	28	0.40	0.60	1.20	0.48	0.93	0.96	1.35	2.61	2.05
2 mM SA	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	14	0.00	0.00	0.00	0.35	0.00	0.07	0.08	1.20	1.40
	28	0.40	1.67	1.80	1.49	1.20	0.72	0.32	2.08	2.80
10 $\mu$ M MJ plus 2 mM SA	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	14	0.00	0.00	0.00	0.00	0.07	0.07	0.08	0.64	0.96
	28	0.40	0.53	0.60	0.37	0.33	0.60	0.32	1.80	2.05

LSD of means (5% level) = 0.24  
CV % = 31.7

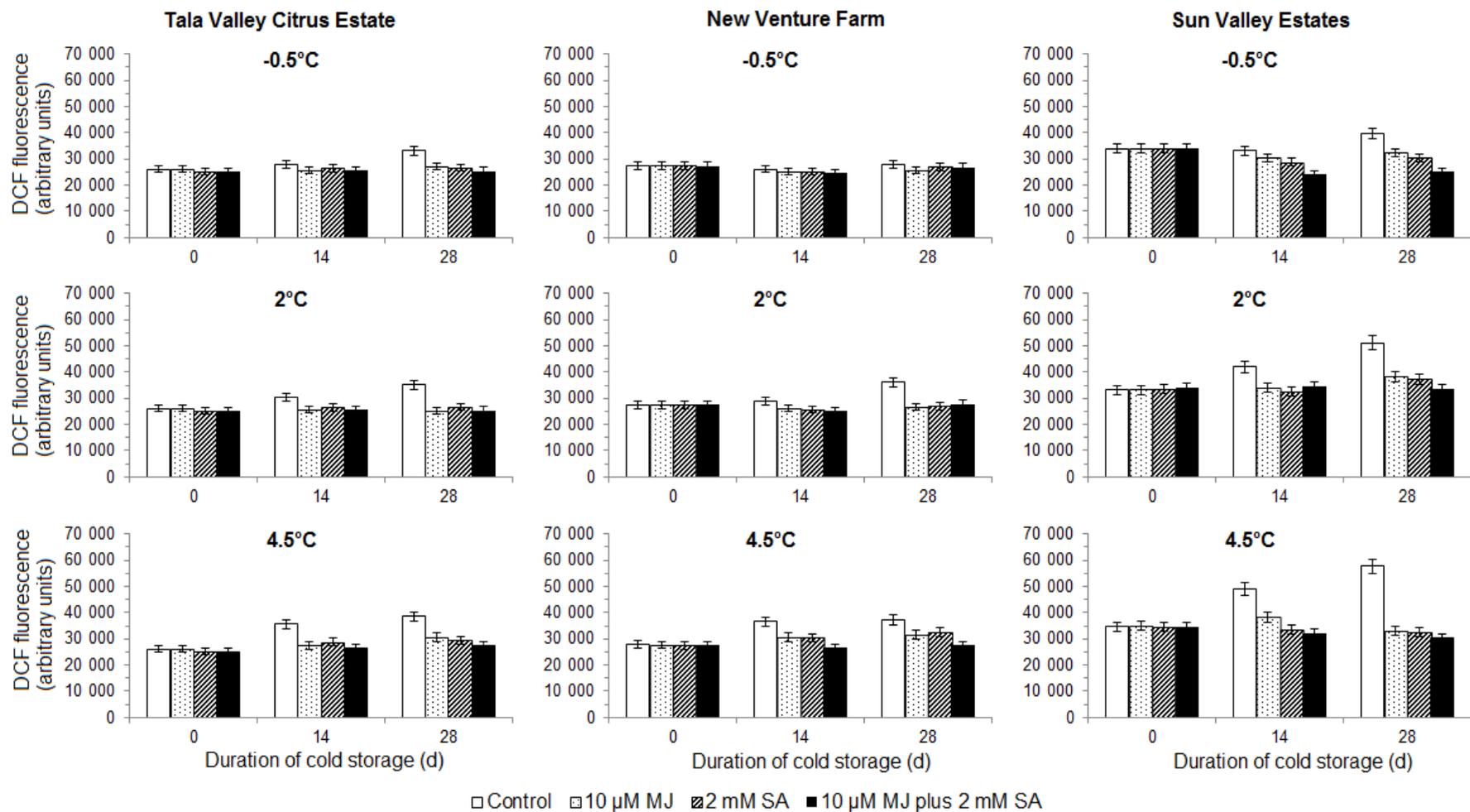


Fig. 1.

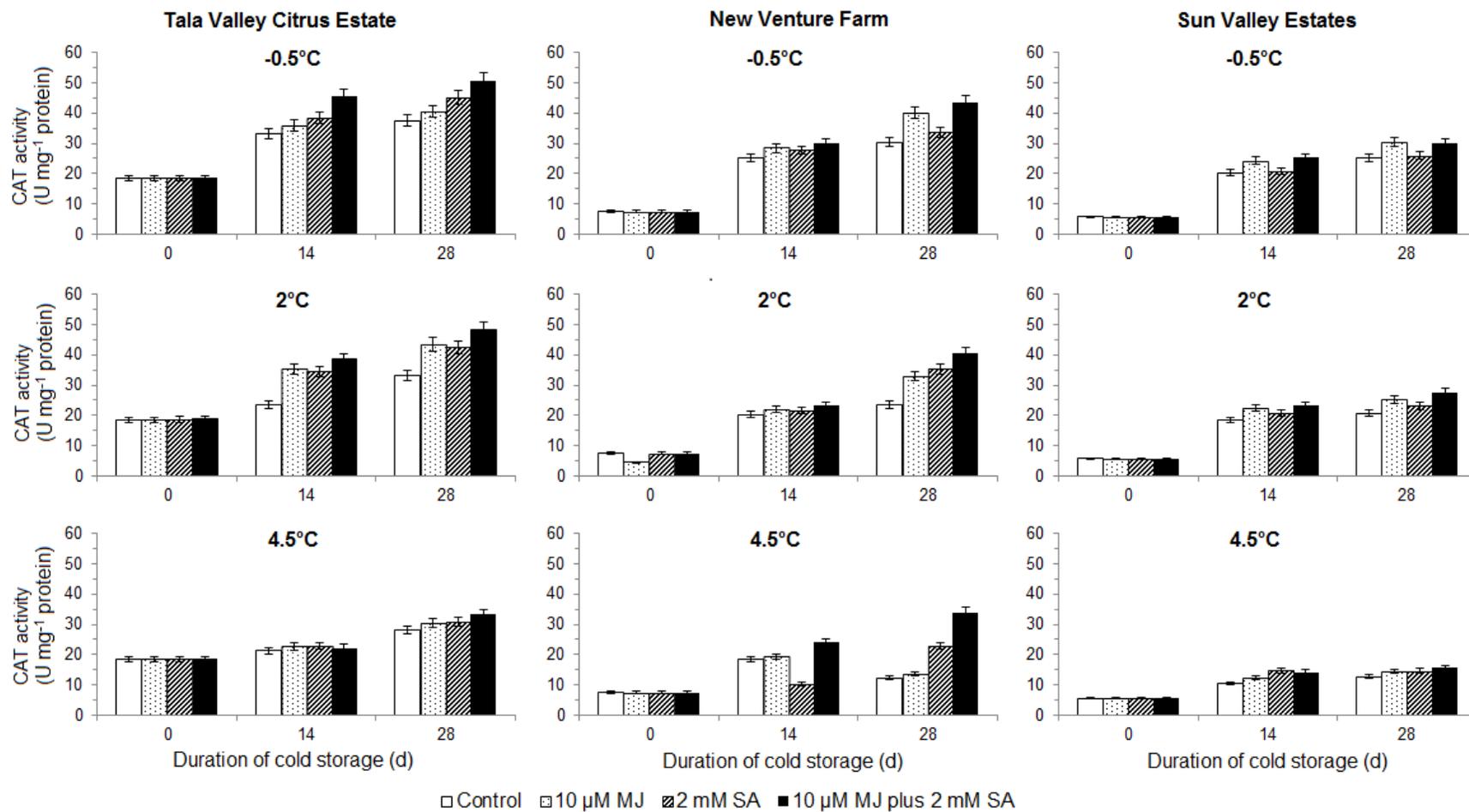


Fig. 2.

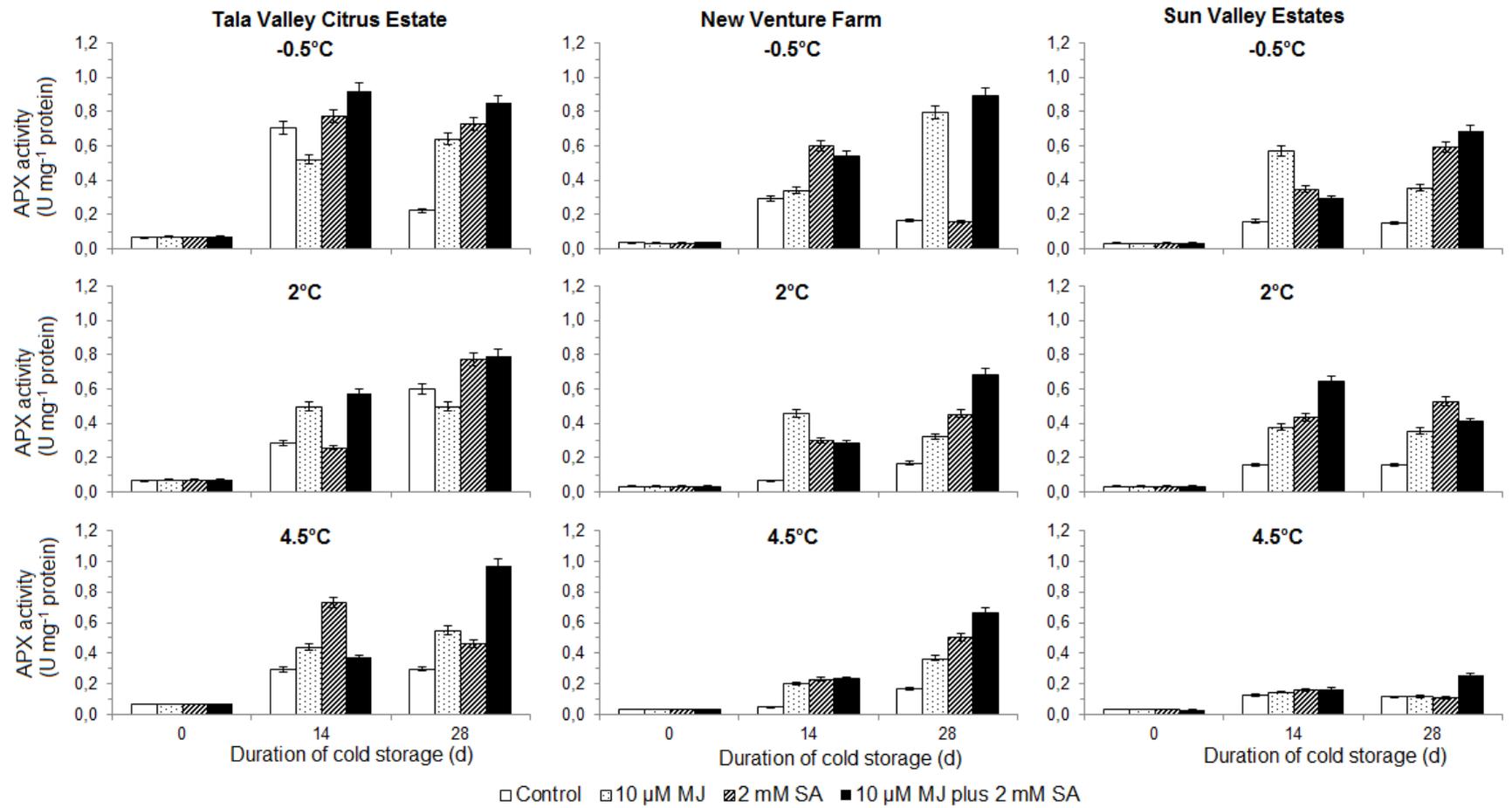


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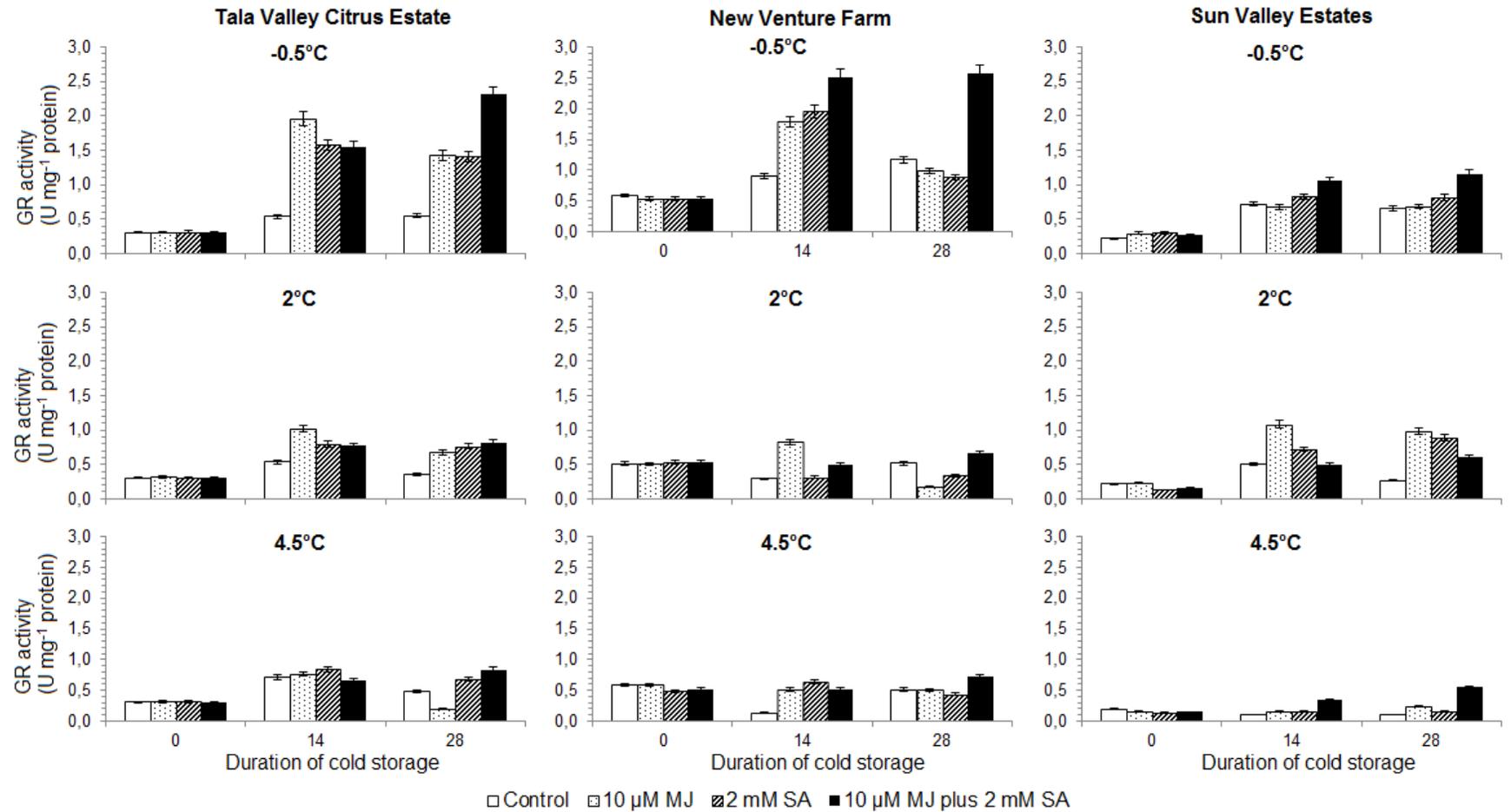


Fig. 4.

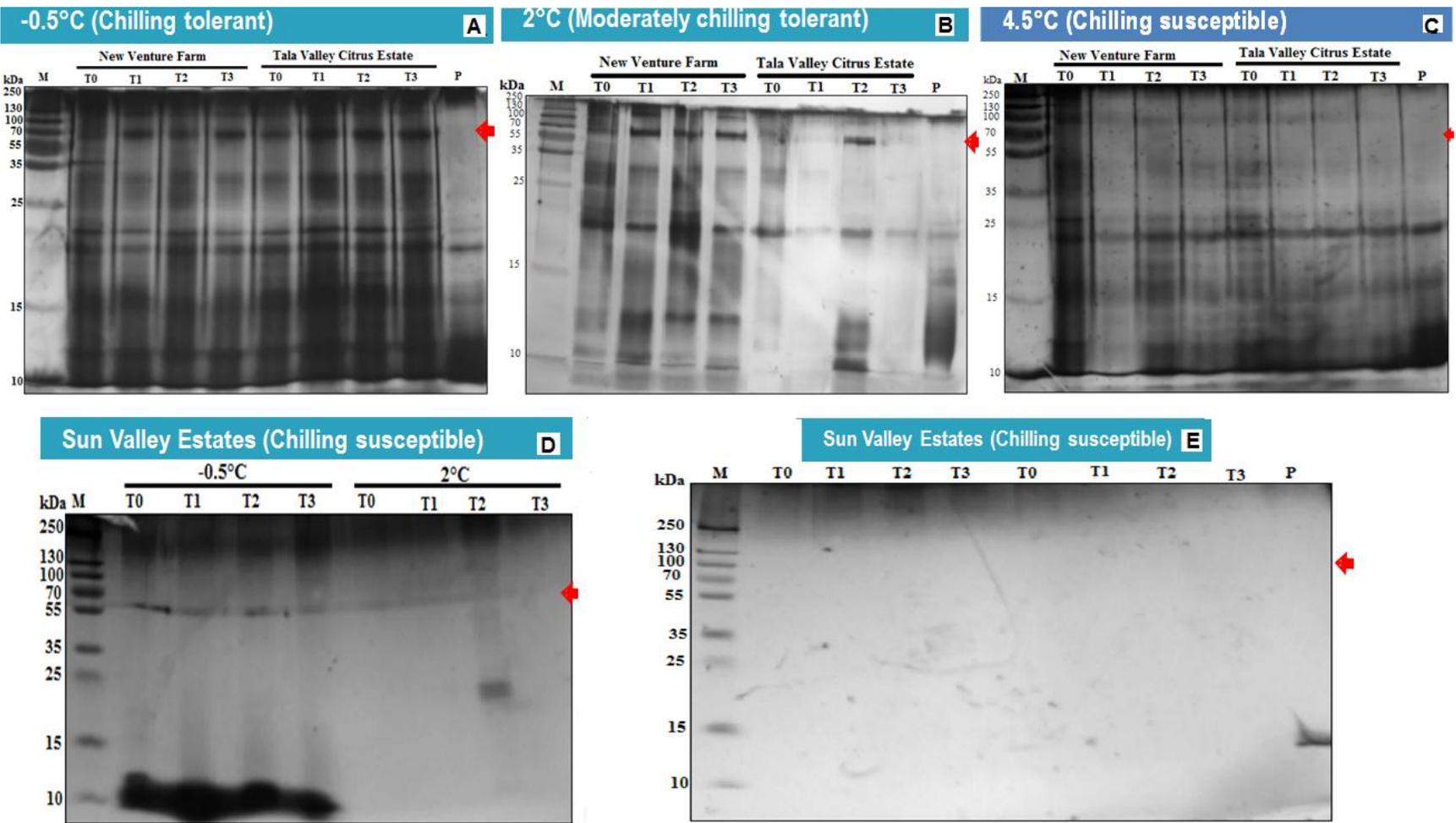


Fig. 5.

## Chapter 8

The influence of methyl jasmonate and salicylic acid on maintaining cell membrane integrity of lemons [*Citrus limon* (L.) Burm. F.]

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## **Abstract**

Cold storage is a widely used technology to maintain fruit quality and extend shelf-life. However, such storage causes chilling stress which is manifested as an increase in membrane permeability and peroxidation of polyunsaturated fatty acids. The resultant leakiness of membranes leads to the degradation of membrane lipids by activating the enzymes phospholipase D (PLD) and lipoxygenase (LOX), resulting in the loss of membrane integrity and ultimately cell death. Membrane integrity was studied by investigating membrane permeability, the degree of membrane lipid peroxidation as well as PLD and LOX activity in lemon flavedo following cold storage at -0.5, 2 or 4.5°C for up to 28 days. Enzyme activities of untreated fruit were compared with those of fruit treated with methyl jasmonate (MJ) and salicylic acid (SA). Chilling injury (CI) symptoms were accompanied by an increase in membrane permeability, membrane lipid peroxidation as well as an increase PLD and LOX activities. The 10 µM MJ plus 2 mM SA postharvest treatment was the most effective treatment in reducing the chilling induced membrane permeability, membrane lipid peroxidation, and PLD and LOX activity of the lemon rind and CI symptoms.

*Keywords:* Lipid peroxidation; Lipoxygenase; Membrane permeability; Methyl jasmonate; Phospholipase D; Salicylic acid

## **1. Introduction**

Cold storage is widely used to maintain fruit quality, prolong shelf-life and to comply with quarantine treatment to cold-sterilize fruit. However, such exposure to low temperature commonly results in chilling stress, leading, upon exposure of fruit to room temperature, to chilling injury (CI); a physiological disorder which leads to the activation of degradation enzymes, such as phospholipases caused by the rigidification of membranes (Pinhero et al., 1998; Mao et al., 2007). Exposure of fruit to chilling temperatures in general leads to membrane degradation and lipid peroxidation, particularly of the polyunsaturated fatty acids of the bipolar membrane; their disintegration gives rise to the production of reactive oxygen species (ROS) (Todd et al., 1992; Palma et al., 1995; Antunes and Sfakiotakis, 2008).

Once CI occurs, membrane lipids are degraded by phospholipase D (PLD), an enzyme responsible for the release of free fatty acids (Bhattacharjee, 2005). The enzyme PLD plays a major role in breaking down membrane lipids and thereby dismantling membranes (Bargmann and Munnik, 2006). If not controlled, PLD activity can cause loss of cell membrane integrity and destroy cell viability. Lipid peroxidation is catalysed by lipoxygenase (LOX), while non-enzymatically ROS are engaged in the initiation of lipid peroxidation (Skórzyńsk-Polit, 2007). During cold storage of corn leaves and the kernel, an increase in PLD and LOX activities was associated with the response to an exposure to chilling stress (Pinhero et al., 1998; Paliyath et al., 1999; Mao et al., 2007).

The enzyme LOX plays a primary role in oxidizing membrane lipids through catalysing peroxidation reactions of plasma membrane lipids, resulting in a decrease in lipid unsaturation and hereby a decreased membrane fluidity (Lee et al., 2005; Mao et al., 2007). This may result in increased membrane permeability, which is attributed to chilling of

sensitive tissue or senescence (Kappus, 1985; Skórzyńsk-Polit, 2007). Studies indicate that changes in membrane structure and composition are considered primary events of CI leading to membrane permeability and thereby metabolic dysfunction of fruit (Marangoni et al., 1996; Valdenegro et al., 2005; Antunes and Sfakiotakis, 2008). The manifestation of CI as pitting, necrosis and red blotch on lemon fruit remains one of the major reasons for postharvest and economic losses in the citrus industry.

Plant growth regulators, particularly methyl jasmonate (MJ) and salicylic acid (SA), have been found to reduce CI in zucchini (*Cucurbita pepo*) stored at 5°C for up to 14 days (Wang and Buta, 1994), avocado (*Persea Americana* Mill) stored at 2°C for 4-10 weeks (Meir et al., 1996). This also include guava stored at 5°C for up to 15 days (González-Aguilar et al., 2004), tomato (*Lycopersicon esculentum* L) stored at 5°C for 4 weeks (Ding et al., 2001), peach (*Prunus persica*) stored at 0°C for 28 days (Wang et al., 2006), pomegranate (*Punica granatum* L.) stored at 2°C for 3 months (Sayyari et al., 2009); and lemon (*Citrus limon*) stored at -0.5°C for up to 42 days (Siboza et al., 2012; Sibozza and Bertling, 2013). The mode of action of these compounds in reducing CI has however, not been clearly elucidated. Fundamental understanding of physiological and biochemical responses of lemon to chilling during quarantine treatment and shipment could allow for the development of new methodologies enhancing the chilling tolerance of lemon fruit. Therefore, the goal of this study was to determine the changes in PLD and LOX activity responses to chilling stress in MJ-and SA-treated lemon during cold storage.

### 3. Materials and Methods

#### 2.1. Plant material

'Eureka' lemon fruit [*Citrus limon* (L.) Burm. F.] were obtained from commercial growers in three different climatic zones of KwaZulu-Natal, South Africa. New Venture Farm: moderate subtropical zone (mean annual temperature: 14.5-26.5°C; mean annual rainfall 712-717 mm; 31° 02' S 29° 25' E, 68-483 m asl), Tala Valley Citrus Estate: warm temperate zone (mean annual temperature: 12.6-23.7°C; mean annual rainfall: 612-771 mm; 29° 52' S 30° 30' E, 416-922 m asl) and Sun Valley Estates: cool subtropical zone (mean annual temperature: 10.7-26.5°C; mean annual rainfall: 669-675 mm; 28° 83' S 30° 06' E, 820-1003 m asl). Lemon fruit were harvested on the 6<sup>th</sup> of June 2011 and 2012 seasons, and the trends were similar. Fruit were immediately transported on paved roads from the collection site to the laboratory in a ventilated vehicle. Fruit transportation distances from farm locations to the laboratory site were 39.2 km (Tala Valley Citrus Estates), 130 km (Sun Valley Estates) and 228 km (New Venture Farm). Lemon fruit were carefully selected for uniformity (size and colour) and absence of defects. Fruit were surface sterilised (Siboza and Bertling, 2013) and air-dried for an hour at 23°C.

#### 2.2. Treatments and storage conditions

Fruit were randomly divided into four lots containing five replicates of 20 fruit per treatment. Lemon fruit were soaked in different MJ and SA solutions: 10 µM MJ (Droby et al., 1999); 2 mM SA (Xu and Tian, 2008), 10 µM MJ plus 2 mM SA (Siboza and Bertling, 2013) for 30 s. Lemon fruit were subsequently waxed according to Sibozza and Bertling (2013) and stored either at -0.5, 2 or 4.5°C (air delivery temperature) for 0, 7, 14, 21, or 28 days. The relative humidity during cold storage was maintained at 85-90%. After cold storage, fruit were transferred to room temperature for a 7 day shelf-life simulation period.

### *2.3. Experimental design*

The experiment was design using a randomised complete block design with the following factors: postharvest treatments (4 levels; Control, 10  $\mu$ M MJ, 2 mM SA, or 10  $\mu$ M MJ plus 2 mM SA); storage temperature (3 level; -0.5, 2 or 4.5°C); farm location (3 levels; Tala Valley Citrus Estate, New Venture Farm and Sun Valley Estates) and storage duration (5 levels; 0, 7, 14, 21 or 28 days).

### *2.4. Determination of chilling injury index*

After the 7 days of shelf-life simulation, lemon fruit were evaluated for CI symptoms using a rating scale based on surface necrosis, pitting, where 0 = normal (pitting), 1 = slight pitting (a few scattered pits), 2 = moderate pitting (pitting covering up to 30% of the fruit surface), 3 = severe pitting (extensive pitting covering > 30% of the fruit surface). The CI index was calculated using the method described by Sala and Lafuente (2000). Five replicate samples of 20 fruit per treatment were used in this experiment.

### *2.5. Determination of membrane permeability*

Membrane permeability was expressed by tissue electrolyte leakage (EC) and was determined according to Cohen et al. (1994). Five fruit disks of the lemon rind were immersed in 15 ml distilled water and incubated at 23°C with constant shaking for 3 hrs. The initial EC (EC1) of the flavedo tissue solution was measured using a conductivity meter (HI 9033, Hanna Instruments, Johannesburg, South Africa). The flavedo tissue solution was then incubated with constant shaking at 100°C for 1 hr, before the final EC (EC 2) was measured. The increase in EC was calculated as the ratio of the initial reading to the final reading. Three replicate samples of 20 fruit per treatment were used in this experiment.

### *2.6. Lemon flavedo sample preparation*

Lemon flavedo samples were prepared according to Lafuente et al. (2004), with slight modifications. Flavedo tissues were collected from the total surface of the lemon fruit using a potato peeler, freeze-dried and pulverised in a mortar using a pestle with liquid nitrogen. The resultant fine powder was stored at  $-70^{\circ}\text{C}$  for analysis of lipid peroxidation and ROS concentration.

### *2.7. Determination of membrane lipid peroxidation*

Lemon flavedo tissue (0.1 g DW) was homogenised in 4 ml 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at  $3000 \times g$  for 10 min according to Dhindsa *et al.* (1981). A mixture of 10 ml supernatant, 1 ml 20% (w/v) TCA, 0.01% (w/v) butylated hydroxytoluene, and 0.65% (w/v) thiobarbituric acid was heated to  $95^{\circ}\text{C}$ , and kept at that temperature for 30 min before being cooled at  $4^{\circ}\text{C}$  and then centrifuged at  $3000 \times g$  for 10 min. The absorbance of the sample was read at 532 nm and 600 nm, using a UV–Visible Spectrophotometer (DU® 800; Beckman Coulter, Fullerton, CA, USA). Total malondialdehyde (MDA) equivalents were calculated according to Sibozza and Bertling (2013). Three replicate samples of 20 fruit per treatment were used in this experiment.

### *2.8. Sample preparation for enzyme activity (flavedo acetone powder)*

Samples were prepared according to the method of Sala et al. (2005). Flavedo tissue (6 g DW) was homogenised in 10 ml 100% acetone previously chilled to  $-20^{\circ}\text{C}$ . The homogenate was filtered under vacuum using chilled 100% acetone until a colourless powder was obtained. The supernatant was discarded and the acetone powder freeze-dried and stored at  $-20^{\circ}\text{C}$  for determination of PLD and LOX activity.

### *2.10. Extraction of PLD and LOX from flavedo acetone powder*

Proteins were extracted at 4°C to avoid any enzyme degradation. Acetone powder (5 g DW) was mixed with 5 ml 50 mM Tris-HCl (pH 8), containing 10 mM KCl, 500 mM sucrose and 0.5 mM phenylmethylsulfonylfluoride prepared at 4°C according to Rui et al. (2010). The extracts were homogenized and centrifuged at 12 000 × g for 10 min at 4°C. Subsequently, the supernatants were used for the assay of PLD and LOX activity.

#### *2.11. Determination of PLD activity*

Activity of PLD (EC3.1.4.4) was determined according to Rui et al. (2010). The reaction mixture contained 1.8 ml 50 mM calcium acetate (pH 5.6) mixed with 27.4 mM nitrophenylphosphorylcholine, 0.2 ml acid phosphatase dissolved in 50 mM calcium acetate (pH 5.6) and 0.6 ml enzyme extract. After incubating the reaction mixture at 37°C for 60 min, 0.1 ml 50 mM NaOH was added. The D-nitrophenol content was determined by reading the absorbance of the sample at 400 nm. One unit of PLD was defined as the amount of enzyme that catalysed the formation of 1 nmol D -nitrophenol per hour. The PLD activity was expressed as units mg<sup>-1</sup> protein. Three replicate samples of 20 fruit per treatment were used in this experiment.

#### *2.12. Determination of LOX activity*

Using the method of Rui et al. (2010), LOX (EC1.13.11.12) activity was determined by mixing 200 µl Tween 20 and 40 µl linoleic acid in 40 ml 0.1 mM phosphate, pH 7.0 as a standard assay mixture. The reaction mixture contained 0.2 ml enzyme extract and 1 ml of the standard assay mixture. The absorbance read at 234 nm at room temperature (approximately 25°C). One unit of LOX was defined as the amount of enzyme which causes an increase in A<sub>234</sub> of 0.01 min<sup>-1</sup> at 234 nm. The protein concentration of the enzyme extract was estimated according to Bradford (1976). The LOX activity was expressed as

units  $\text{mg}^{-1}$  protein. Three replicate samples of 20 fruit per treatment were used in this experiment.

### *2.13. Statistical Analysis*

Data were analysed using GenStat 14.1 (2011, VSN International Ltd). Mean separations were performed by Duncan's Multiple Range Tests. Differences at  $P < 0.05$  were considered significant.

## **4. Results**

### *3.1. The influence of postharvest treatments with MJ and SA in enhancing chilling tolerance in lemon fruit.*

Cold storage of 'Eureka' lemon fruit significantly ( $P < 0.05$ ) induced CI in the lemon flavedo (Fig. 1). The manifestation of CI in 'Eureka' lemon fruit was significantly ( $P < 0.05$ ) dependent on postharvest treatments, farm location, storage temperature and duration, and the interaction of these factors (Fig. 1). Symptoms started to appear on lemon fruit after 7 days of cold storage and increased with cold storage duration in untreated lemon fruit at all sites (Fig. 1). Postharvest treatment with MJ and SA significantly ( $P < 0.05$ ) reduced CI symptoms. The effectiveness of MJ and SA in reducing CI symptoms was concentration dependent, with 10  $\mu\text{M}$  MJ plus 2 mM SA being the most effective, followed by 10  $\mu\text{M}$  MJ. Treatment with 2 mM SA was less effective. Cold storage of lemon fruit at  $-0.5^\circ\text{C}$  produced low CI symptoms than when stored at  $2^\circ\text{C}$ . Increasing the storage temperature to  $4.5^\circ\text{C}$  resulted in high CI symptoms. Severe CI symptoms were observed in lemon stored at  $4.5^\circ\text{C}$ .

Farm location was another factor affecting the development of CI. Lemon fruit from New Venture Farm were chilling tolerant while lemon fruit from Tala Valley Citrus Estate were moderately chilling tolerant. Lemon fruit harvested from Sun Valley Estates were chilling susceptible with severe symptoms. The development of CI was affected by the interaction of treatments, storage temperature, farm location and storage duration. There were no significant differences in CI for lemon fruit treated with 10  $\mu$ M MJ plus 2 mM SA from New Venture Farm and those from Tala Valley Citrus Estate.

### *3.2. Changes in electrolyte leakage in lemon flavedo of fruit in response to MJ and SA treatment and storage temperature.*

Electrolyte leakage of lemon flavedo was significantly ( $P < 0.05$ ) affected by treatments, cold storage time, storage temperature, storage duration and the interaction of these factors as well as CI (Fig. 2 and 1). During cold storage of lemon fruit, electrolyte leakage increased with cold storage duration in chilling susceptible lemon fruit (Fig. 2). The electrolyte leakage of lemon fruit was delayed by the postharvest treatment with MJ and SA when compared to the untreated lemon fruit.

### *3.3. The influence of postharvest treatments with MJ and SA in inhibiting MDA concentration in lemon flavedo*

During cold storage of lemon fruit, total MDA concentration was significantly ( $P < 0.05$ ) affected by treatment, storage temperature, farm location, cold storage duration and the interaction of these factors (Fig. 3). During cold storage, total MDA concentration was increasing with cold duration in untreated lemon fruit (Fig. 3). The effectiveness of MJ and SA in reducing total MDA concentration constantly depended on concentration of the treatment. Lemon fruit treated with 10  $\mu$ M MJ plus 2 mM SA showed maximum reduction in lipid peroxidation followed by lemon fruit treated with 10  $\mu$ M MJ or 2 mM SA. High

concentration of total MDA was observed in untreated lemon fruit. Total MDA concentration in lemon flavedo was significantly affected by storage temperature, it was minimal in lemon stored at  $-0.5^{\circ}\text{C}$  (chilling tolerant) followed by the lemon fruit stored at  $2^{\circ}\text{C}$  (medium chilling tolerant). Observations also revealed that total MDA concentration was high on lemon fruit stored at  $4.5^{\circ}\text{C}$  (chilling susceptible).

#### *3.4. The influence of MJ and SA treatments in inhibiting PLD activity in lemon flavedo.*

In this study, PLD enzyme in lemon flavedo was significantly ( $P < 0.05$ ) affected by postharvest treatments, cold storage temperatures, farm locations, cold storage duration and interactions of these factors (Fig. 4). Cold storage of lemon fruit triggered the accumulation of PLD enzyme which increased with cold storage duration in untreated lemon fruit (Fig. 4). The efficacy of MJ and SA in inhibiting PLD activity in lemon was concentration dependent. Treatment with  $10\ \mu\text{M}$  MJ plus  $2\ \text{mM}$  SA was consistently effective followed by  $10\ \mu\text{M}$  MJ or  $2\ \text{mM}$  SA in inhibiting PLD activity in lemon.

#### *3.5. The effect of treatments with MJ and SA in inhibiting LOX activity in lemon flavedo*

During cold storage, the LOX activity in lemon flavedo was significantly ( $P < 0.05$ ) affected by postharvest treatments, cold storage temperatures, farm locations, cold storage duration and the interactions of these factors (Fig. 5). As in PLD activity, exposure of lemon fruit to chilling stress triggered the accumulation of LOX enzyme in untreated lemon fruit. The LOX activity increased with cold storage duration. However, postharvest treatment with  $10\ \mu\text{M}$  MJ plus  $2\ \text{mM}$  SA significantly ( $P < 0.05$ ) inhibited LOX activity in lemon flavedo (Fig. 5).

## **5. Discussion**

Several naturally occurring growth regulators (MJ and SA) have been successfully used to reduce CI in horticultural crops. However, the mode of action of these chemicals by which

CI is reduced is not yet fully understood. Therefore, evaluating biochemical and physiological responses of lemon fruit to MJ and SA treatment could improve understanding of the mode of action of these chemicals in reducing CI. Storage temperature is one element of cold storage that affects the development of CI in lemon fruit (Fig. 1). This was in agreement with previous study by Zhang and Tian (2009) who reported that CI symptoms in peach fruit developed more intensely when store at temperatures between 5°C than those stored at 0°C.

During chilling, cell membrane permeability increases leading to the leakage of electrolytes (Mao et al., 2007). The development of CI is related to tissue deterioration which leads to changes in membrane permeability (Imahori et al., 2008). Therefore, the loss of membrane integrity due to chilling was investigated by measuring electrolyte leakage and lipid peroxidation in the lemon flavedo. The electrolyte leakage was consistently lower in chilling tolerant lemon fruit (MJ and SA treated) than in highly chilling susceptible ones (control). The results of this study confirm that storage temperature plays a significant role in membrane permeability. The electrolyte leakage levels were lower in chilling tolerant lemon fruit than in susceptible lemon fruit.

It has been reported that cold exposure can change the structure of membranes by initiating the degradation of the polyunsaturated fatty acids due to lipid peroxidation while inducing membrane rigidification and cell death (Posmyk et al., 2005; Imahori et al., 2008). Lipid peroxidation, one of the main causes of membrane deterioration is suggested to be the first events in the manifestation of CI (Imahori et al., 2008). Therefore, in this study, the loss of membrane integrity in lemon flavedo was determined by evaluating the levels of MDA as one product of lipid peroxidation (Ke, 2007). The progression of lipid peroxidation in lemon flavedo (Fig. 3) could have contributed to the development of CI (Fig. 1), as it has

been found that chilling induce lipid degradation (Wang et al., 1992; Imahori et al., 2008). Lipid peroxidation was significantly lower in chilling tolerant lemon fruit (10  $\mu$ M MJ plus 2 mM SA) while greater lipid peroxidation was observed in chilling susceptible lemon fruit (untreated). This was associated with loss of membrane integrity which was accompanied by CI symptoms.

The occurrence of lipid peroxidation in lemon flavedo indicated that lemon fruit were stressed during cold storage and that the effect of the stress increased with cold storage temperature. This membrane damage increased with cold storage temperature. This was in line with the observation of Sairam et al. (2002) and Zhao et al. (2011) who reported that lipid peroxidation levels are related to the extent of damage to the ultrastructure and are an important marker for evaluating stress tolerance. Lipid peroxidation was therefore, a good marker for CI injury in 'Eureka' lemon fruit. A positive correlation between lipid peroxidation and PLD activity ( $R = 0.733$ ) was also observed. This suggests that lipid peroxidation and PLD activity were both involved in phospholipid catabolism and in the initiation of a lipolytic cascade in membrane deterioration during chilling (Paliyath and Droillard, 1992; Pinhero et al., 1998; Mao et al., 2007).

Chilling may induce phospholipid catabolism by activating PLD enzyme, leading to cellular membrane breakdown (Bargmann and Munnik, 2006). The enzyme PLD is known to increase in crops subjected to stress (Bargmann and Munnik, 2006). In this study, the intense increase of PLD enzyme in untreated lemon fruit was associated chilling sensitivity. The high PLD activity in untreated lemon fruit likely caused a drastic degradation of phospholipids leading to the initiation and progression of CI (Mao et al., 2007). Postharvest treatment with 10  $\mu$ M MJ plus 2 mM SA inhibited the activation of PLD activity and resulted in low CI symptoms.

This study reveals that storage temperatures play a significant role in triggering PLD activity. The PLD activity was lower in chilling tolerant lemon stored at  $-0.5^{\circ}\text{C}$  than in moderately chilling tolerant lemon fruit stored at  $2^{\circ}\text{C}$ . Chilling susceptible lemon fruit stored at  $4.5^{\circ}\text{C}$  had high accumulation of PLD activity. This confirmed that chilling tolerant lemon fruit had lower PLD activity levels than chilling susceptible lemon. Therefore, PLD activity was a good biochemical marker for CI and this was in agreement with Mao et al. (2007).

It was reported that LOX plays a primary role in generating peroxidative damage in membrane lipids through peroxidation reactions on plasma membrane lipids, resulting in low level of lipid unsaturation and membrane fluidity (Lee et al., 2005; Mao et al., 2007). In this study, a similar trend of PLD activity was observed as in LOX activity (Fig. 5) with the positive correlation ( $R = 0.960$ ). The consistent trend of PLD and LOX activity could be that LOX depends on PLD activity in deteriorating membranes, since it cannot directly act on phospholipids (Mao et al., 2007).

The development of CI was accompanied with increase of PLD and LOX activity. This was in agreement with previous study by Mao et al. (2007) that the development of CI is associated with activation of PLD and LOX activities. Furthermore, this study reveals that electrolyte leakage and lipid peroxidation are also involved in initiating CI in lemon, and that alleviation of CI can be achieved by maintaining membrane integrity. The membrane integrity of lemon fruit was maintained by  $10\ \mu\text{M}$  MJ plus  $2\ \text{mM}$  SA postharvest treatment, thereby inhibiting electrolyte leakage, lipid peroxidation, PLD and LOX activities thus resulted in chilling tolerance. The differences in MDA, PLD and LOX were much lower (or no different in some cases) than those found in CI in samples exposed to the treatments.

In conclusion, cold storage of lemon results in chilling stress which leads to lipid peroxidation and it triggers enzymes involved in membrane degradation such as PLD and LOX which subsequently cause a loss in membrane integrity and CI symptoms. The development of CI was accompanied by an increase of membrane electrolyte leakage, lipid peroxidation, and PLD and LOX activity. The increased membrane lipid peroxidation was related to the increased levels of PLD and LOX activity and electrolyte leakage suggesting that the production of reactive oxygen species was probably higher than antioxidant levels in control fruit. Postharvest treatment with the combination of MJ and SA (10  $\mu$ M MJ plus 2 mM SA) was an effective concentration to alleviate CI in lemon during cold storage. This study reveals that the mode of action of treatment with 10  $\mu$ M MJ plus 2 mM SA involves maintaining membrane integrity by preventing lipid peroxidation, and reducing PLD and LOX enzyme activities, thereby delaying the development of CI.

### **Acknowledgement**

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## Figure legends

Fig. 1. The manifestations of chilling injury (CI) on methyl jasmonate (MJ) and salicylic acid (SA) treated lemon fruit during cold storage either at -0.5, 2 or 4.5°C for up to 28 days and 7 days at 23°C. Five replicate samples of 20 fruit per treatment were used in this experiment. Least significant differences of means (5% level) = 0.26.

Fig. 2. Levels of electrolyte leakage in lemon flavedo treated with different concentrations of methyl jasmonate (MJ) and salicylic acid (SA) before cold storage either at -0.5, 2 or 4.5°C for up to 28 days and 7 days at 23°C. Three replicate samples of 20 fruit per treatment were used in this experiment. Least significant differences of means (5% level) = 5.27.

Fig. 3. Changes in levels of lipid peroxidation in lemon flavedo treated with postharvest treatments of methyl jasmonate (MJ) and salicylic acid (SA) prior to cold storage either at -0.5, 2 or 4.5°C for up to 28 days and 7 days at 23°C. Three replicate samples of 20 fruit per treatment were used in this experiment. Least significant differences of means (5% level) = 0.68.

Fig. 4. Changes in phospholipase D (PLD) activity in MJ and SA treated lemon fruit from Tala Valley Citrus Estate, New Venture Farm and Sun Valley Estates stored either at -0.5, 2 or 4.5°C for up to 28 days and 7 days at 23°C. Three replicate samples of 20 fruit per treatment were used in this experiment. Least significant differences of means (5% level) = 0.17.

Fig. 5. Changes in the activity of lipoxygenase (LOX) in the flavedo of lemon fruit treated with methyl jasmonate (MJ) and salicylic acid (SA) during cold storage either at -

0.5, 2 or 4.5°C either for up to 28 days and 7 days at 23°C. Five replicate samples of 20 fruit per treatment were used in this experiment. 0.17.

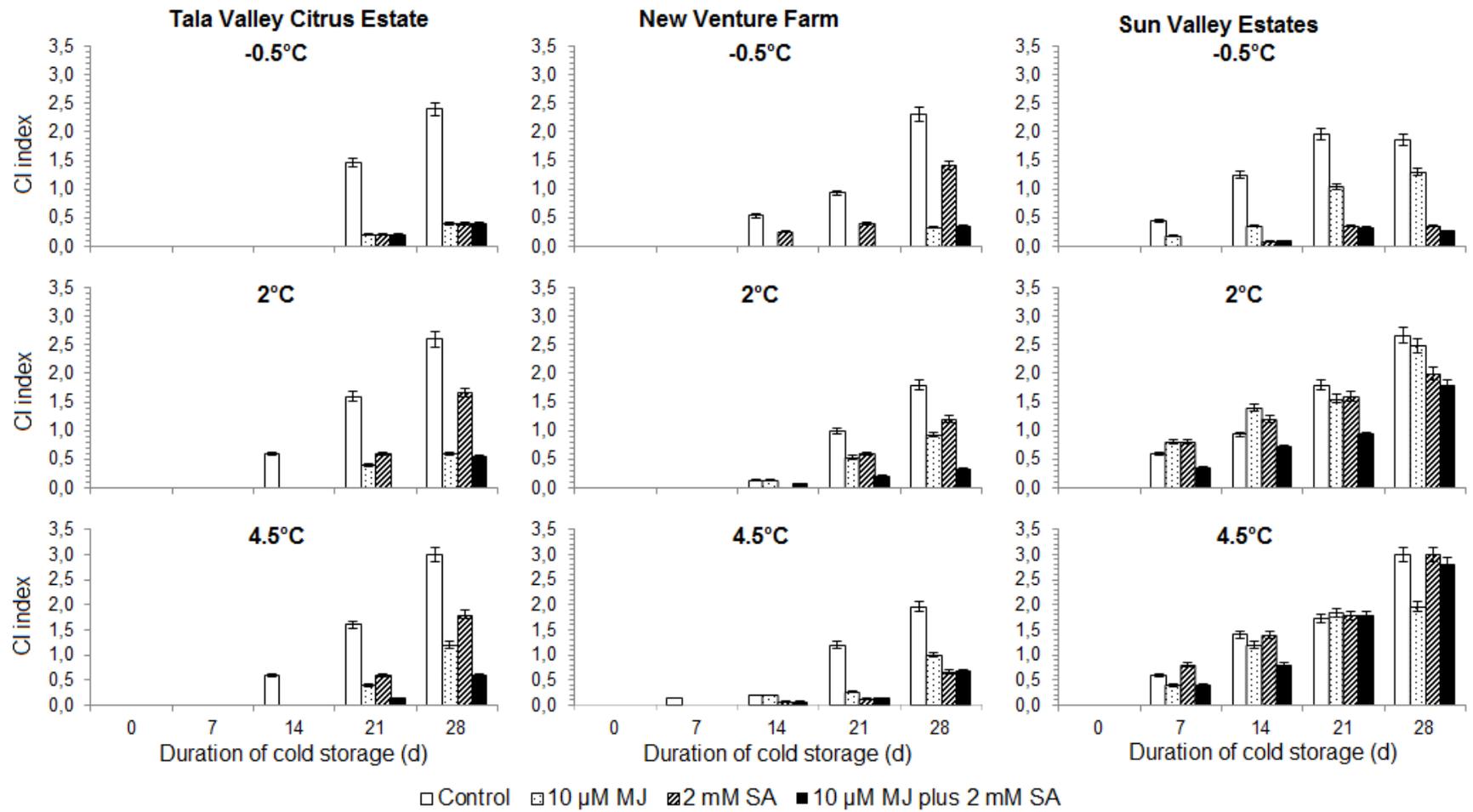


Fig. 1.

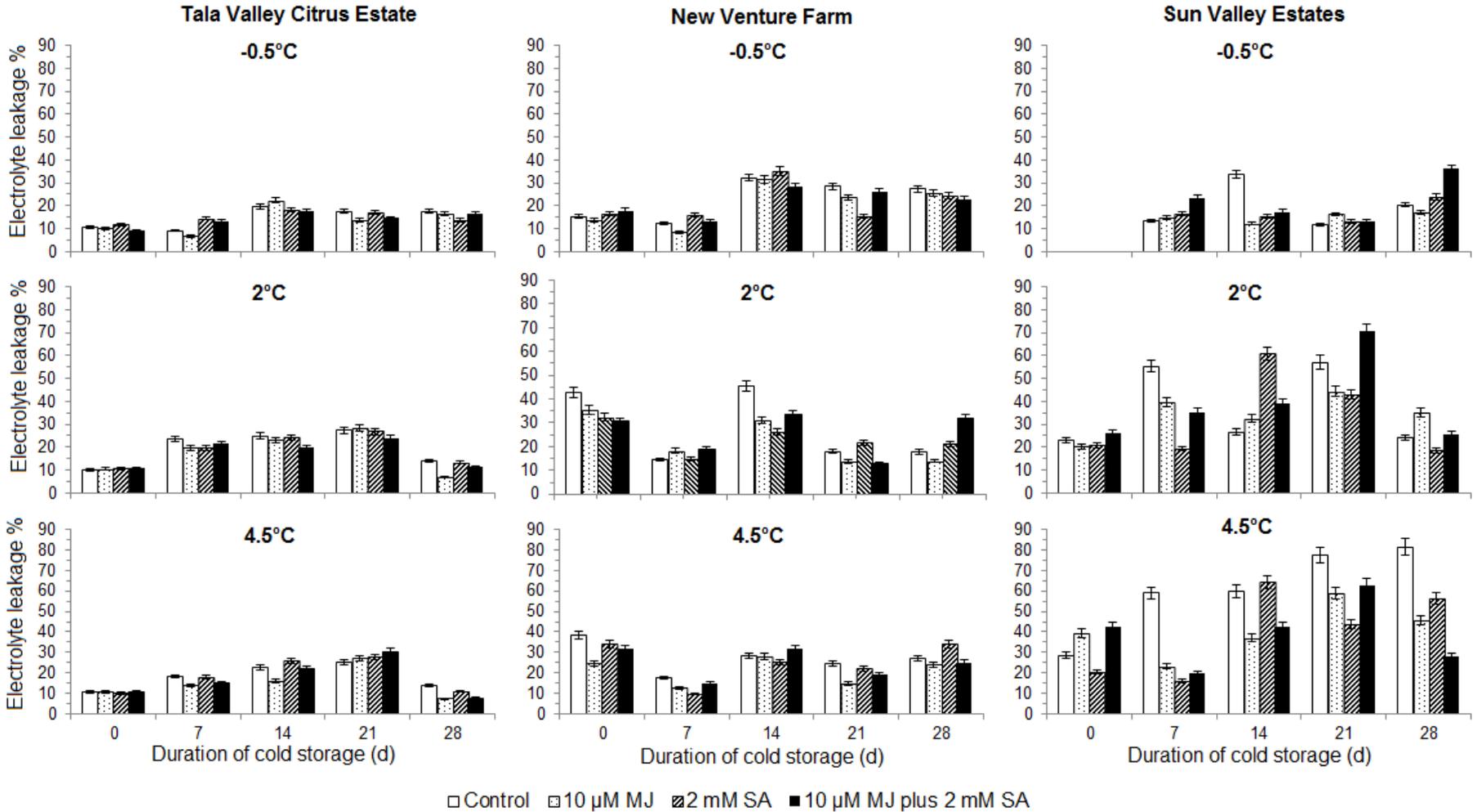


Fig. 2.

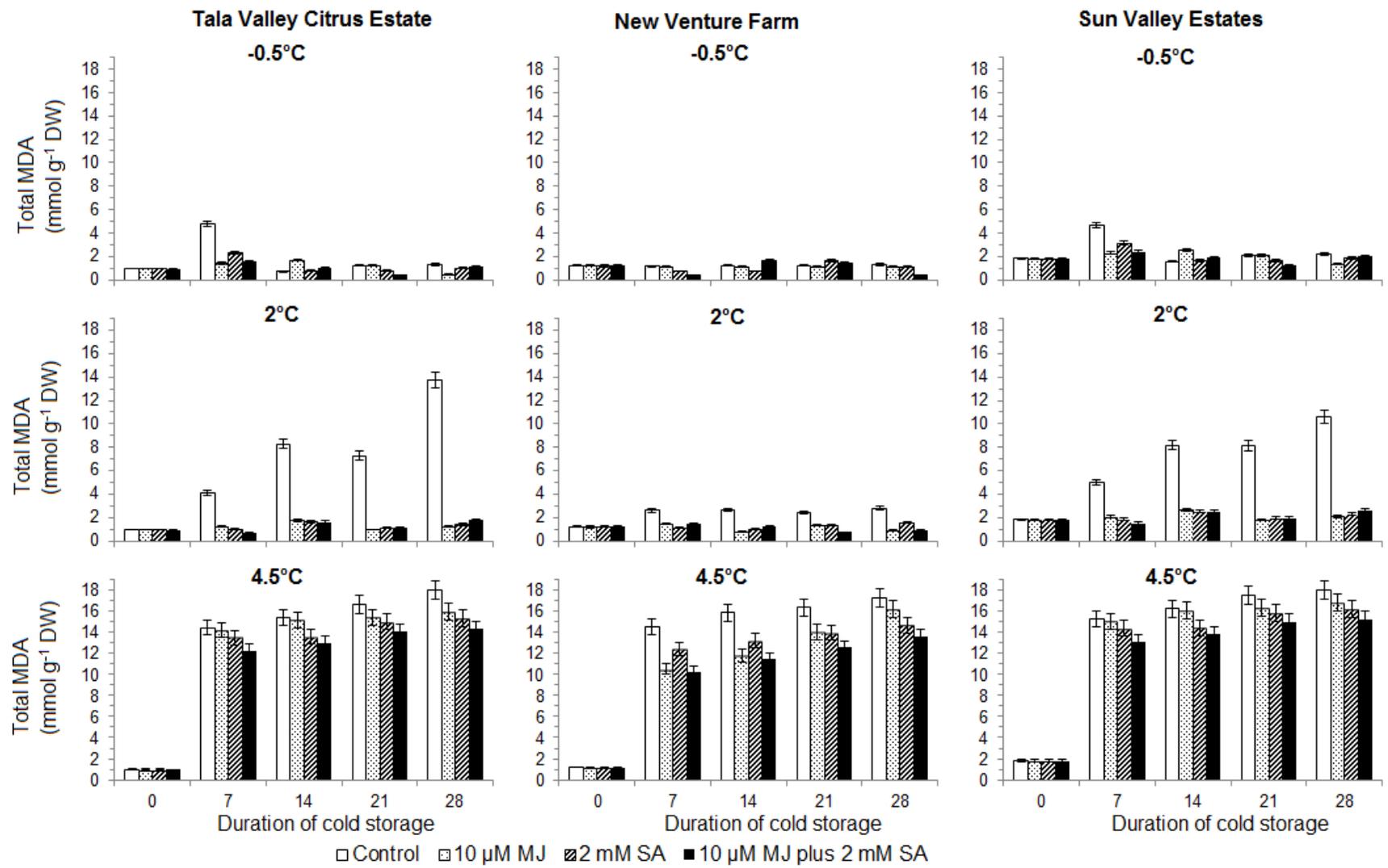


Fig. 3.

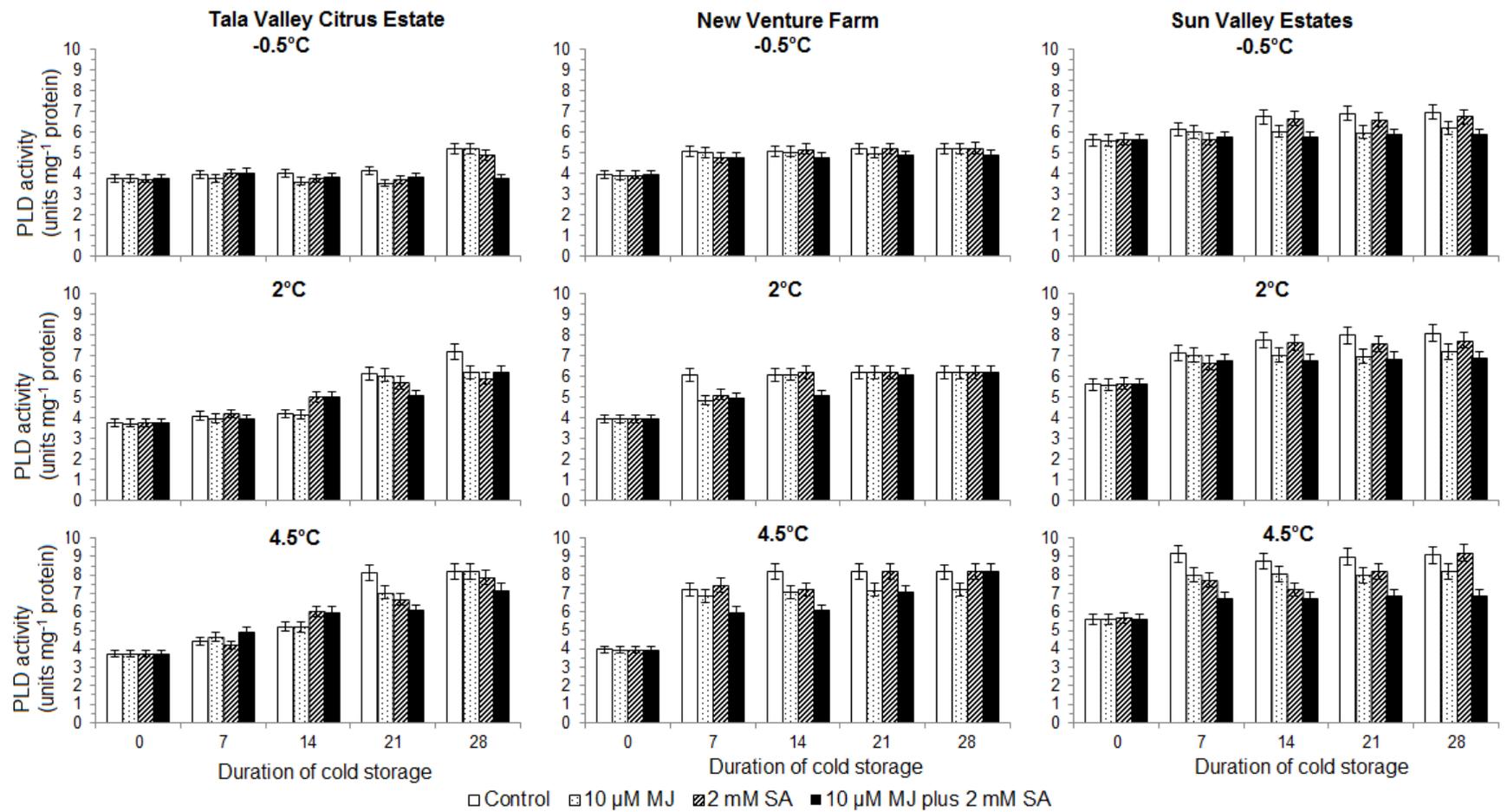


Fig. 4.

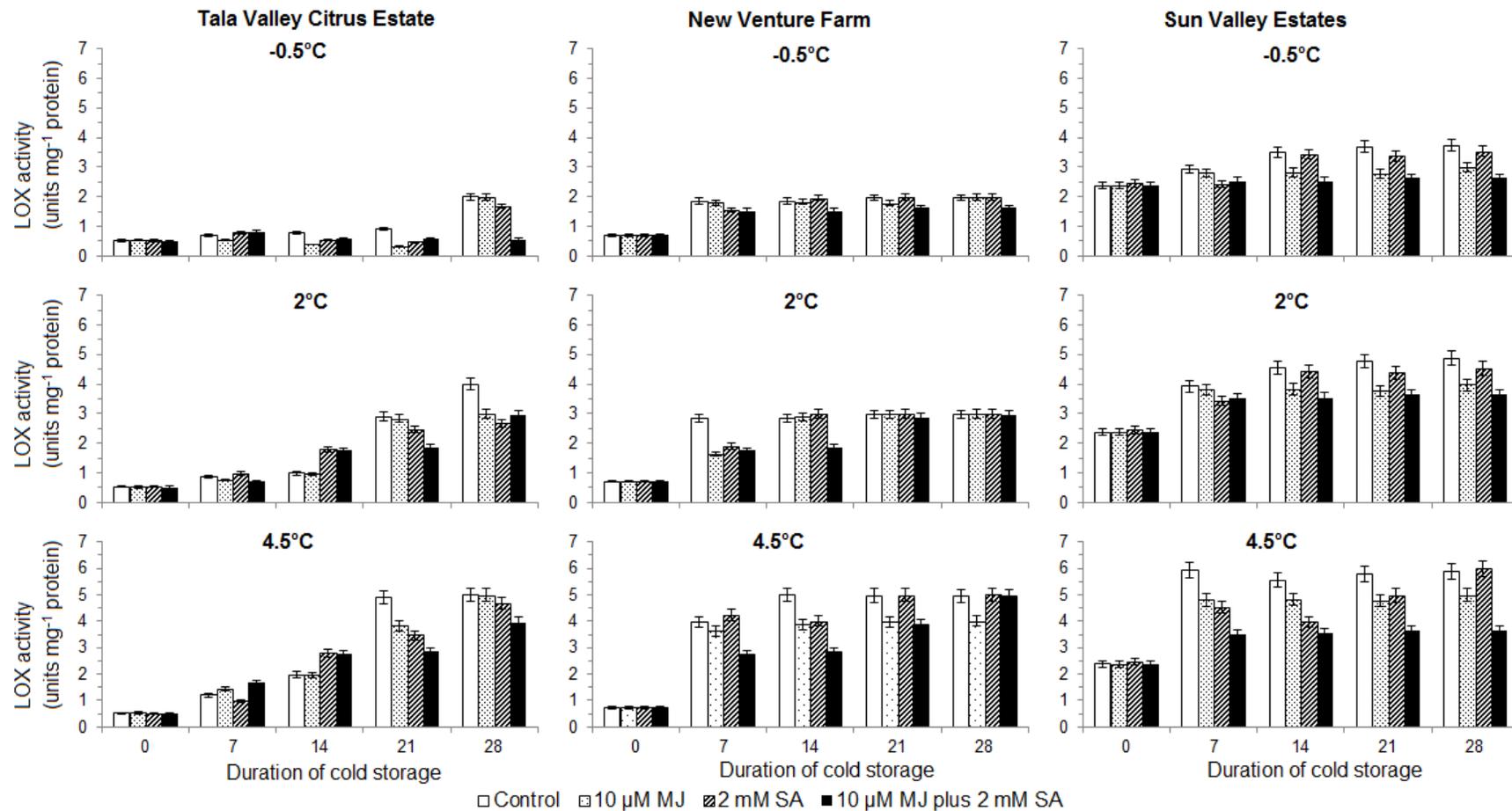


Fig. 5.

## Chapter 9

The induction of antioxidant activity in cold stored 'Eureka' lemon by postharvest treatments with methyl jasmonate and salicylic acid

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## **Abstract**

The efficacy of methyl jasmonate (MJ) and salicylic acid (SA) in enhancing chilling tolerance by inducing antioxidant capacity in 'Eureka' lemon fruit, measured by ferric reducing ability of plasma (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and the oxygen radical absorption capacity (ORAC) assays, were investigated. Lemon fruit were obtained from moderate sub-tropical (mean annual temperature: 14.5-26.5°C; mean annual rainfall: 712-717 mm), warm temperate (mean annual temperature: 12.6-23.7°C; mean annual rainfall: 612-771 mm) and cool sub-tropical zone (mean annual temperature: 10.7-26.5°C; mean annual rainfall: 669-675 mm). Fruit were treated with 10 µM MJ, 2 mM SA or 10 µM MJ plus 2 mM SA and stored either at 2 or 4.5 °C for up to 28 days and a week at 23°C. The ability of 10 µM MJ plus 2 mM SA treatment in enhancing chilling tolerance was greatly regulated by storage temperature. It seems that storing lemon fruit at 2°C plays a profound role in maintaining antioxidant capacity in lemon fruit. This could have contributed to chilling tolerance and treatment with 10 µM MJ plus 2 mM SA enhanced this effect significantly. Lemon fruit from the warm temperate zone were found to have high antioxidant capacity followed by lemon fruit from the moderate sub-tropical zone. This was consistent with results for CI; lemon fruit from the warm temperate zone were more chilling tolerant than lemon fruit from the moderate sub-tropical zone. The low antioxidant capacity in lemon fruit from the cool sub-tropical zone paralleled with the high CI symptoms. This study provides evidence that lemon fruit with abundant amount of antioxidant activity withstand chilling stress better than lemon fruit with low antioxidant capacity. This effect was significantly enhanced by treatment with 10 µM MJ plus 2 mM SA on lemon fruit stored at 2 °C.

*Keywords:* Antioxidants; Ascorbic acid; Methyl jasmonate; Salicylic acid

## **1. Introduction**

The defence effects of natural antioxidants consumed in fruit and vegetables in lowering the risk of cancer and cardiovascular diseases, have gained increasing interest by both consumers and scientists (Temple, 2000; Thaipong et al., 2006). Fruit and vegetables at postharvest use antioxidant system as a defence mechanism to regulate reactive oxygen species (ROS) (Hossain and Teixeira da Silva, 2011) and prevent against oxidative damage. Therefore, the enhancement of antioxidant defence mechanisms in fruit and vegetables has been reported to produce tolerance to chilling stress (Sala and Lafuente, 1999; Rivera et al., 2007). Chilling causes chilling injury (CI) in lemon fruit (Siboza et al., 2012). This is an economically important postharvest problem that reduces the overall quality and marketability of horticultural crops (Dong et al., 2012).

Chilling tolerant citrus cultivars have been found to have more efficient antioxidant defence system than chilling-sensitive cultivars (Sala, 1998; Rivera et al., 2007). However, Wang (2010) emphasised that the level and activity of antioxidants in horticultural crops can be affected by preharvest factors (environmental conditions) and postharvest factors (storage conditions). Several antioxidant assays have been successfully used to determine antioxidant capacity of horticultural crops. These include, ferric reducing ability of plasma (FRAP) (Benzie and Strain, 1996; Thaipong et al., 2006) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Mokbel and Hashinaga, 2006; Thaipong et al., 2006). Some of the assays include 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) (Leong and Shui, 2002; Thaipong et al., 2006) and the oxygen radical absorption capacity (ORAC) (Cao et al., 1993; Thaipong et al., 2006).

These assays have produced different results among plants tested and across laboratories; this is caused by substantial differences in sample preparation, extraction procedures, and

expression of results for the same assay (Pérez-Jiménez et al., 2008; Dudonné et al., 2009). Again, measuring total antioxidant activity on the basis of individual active components in crops is very difficult (Patthamakanokporn et al., 2008). Furthermore, main drawbacks associated with common antioxidant assays have been previously discussed by Pérez-Jiménez et al. (2008) and summarised in Table 1. Therefore, Pérez-Jiménez et al. (2008) advised that if possible, more than one method for determining antioxidant capacity (FRAP, ABTS, DPPH and ORAC) needed to be combined to provide comprehensive information on the total antioxidant capacity of a crop.

In lemon fruit that were stored at  $-0.5^{\circ}\text{C}$  for up to 42 days and 7 days at  $23^{\circ}\text{C}$ , antioxidant capacity measured by the ABTS assay was enhanced by postharvest treatments with methyl jasmonate (MJ) and salicylic acid (SA) (Siboza et al., 2011, 2012). However, the mode of action of MJ and SA in enhancing CI in lemon fruit (*Citrus limon* cv Eureka) is unclear and requires further investigation. Meir et al. (1996) suggested that MJ might mediate the natural responses of fruit to chilling. Studies by Sibozza et al. (2012) and Sibozza and Bertling (2013) suggested that combining both MJ and SA at an effective concentration may reduce CI in lemon fruit by enhancing defence mechanisms. However, the mechanisms by which the MJ and SA combination triggers defence chilling tolerance remain unknown. Furthermore, previous studies suggest that different postharvest aspects such storage temperature and duration affect antioxidant capacity in crops (Srivastava et al., 2007; Pérez-Jiménez et al., 2008). Therefore, the aim of this research was to investigate the effect of preharvest farm location (the moderate sub-tropical, warm temperate or cool sub-tropical zone), storage temperature (2 or  $4.5^{\circ}\text{C}$ ) on antioxidant activities measured by FRAP, DPPH, ABTS and ORAC in MJ-and SA-treated lemon fruit.

## **2. Materials and methods**

### *2.1 Chemical reagents*

All chemicals reagents were of analytical grade and purchased either from Aldrich Chemical Co. (Steineheim, Germany), Merk (Darmstadt, Germany) and Sigma Chemical Co. (St Louis, MO, USA).

### *2.2 Fruit samples*

Lemon fruit (c.v. Eureka) were obtained from three commercial farms located in different climatic zones in KwaZulu-Natal, South Africa. New Venture Farm: moderate sub-tropical zone (mean annual temperature: 14.5-26.5°C; mean annual rainfall: 712-717 mm; 31° 02' S 29° 25' E, 68-483 m asl). Tala Valley Citrus Estate: warm temperate zone (mean annual temperature: 12.6-23.7°C; mean annual rainfall: 612-771 mm; 29° 52' S 30° 30' E, 416-922 m asl). Sun Valley Estates: cool sub-tropical zone (mean annual temperature: 10.7-26.5°C; mean annual rainfall: 669-675 mm; 28° 83' S 30° 06' E, 820-1003 m asl). Lemon fruit were harvested on the 6<sup>th</sup> of June 2011 and 2012, and the trends were similar. Fruit were immediately transported on paved roads from the collection site to the laboratory in a ventilated vehicle. Fruit transportation distances from farm locations to the laboratory site were 39.2 km (Tala Valley Citrus Estates), 130 km (Sun Valley Estates) and 228 km (New Venture Farm).

Lemon fruit were carefully selected for uniformity (size and colour) and absence of defects.

### *2.3 Postharvest treatments and storage conditions*

Lemon fruit were washed with 0.1% Sporekill® (v/v) (Hygrotech Pty Ltd., Pretoria, South Africa) for 3 min and air dried for 1 hr (Siboza and Bertling, 2013). Fruit were soaked either in 10 µM MJ, 2 mM SA, or 10 µM MJ plus 2 mM SA for 30 s. A control (no soaking) was included (Siboza and Bertling, 2013). All fruit were waxed, air dried for 1 hr,

and cold stored either at 2 or 4.5°C (air delivery temperature) with a relative humidity of about 90% in the refrigerated container. Fruit were stored for up to 28 days plus a week at 23°C (shelf-life), to allow the manifestations of CI symptoms.

#### *2.4 Evaluation of CI*

After 0, 7, 14, 21, or 28 days at cold storage and an additional week at shelf-life, lemon fruit were evaluated for CI severity in the flavedo (the outer coloured part of the peel) and the CI index was determined according to Sala and Lafuente (2000). The CI severity on the lemon fruit was visually rated according to the following scale: 0 = normal (pitting), 1 = slight pitting (a few scattered pits), 2 = moderate pitting (pitting covering up to 30% of the fruit surface), 3 = severe pitting (extensive pitting covering > 30% of the fruit surface) and expressed as CI index. The CI symptoms were evaluated at every sampling interval (0, 7, 14, 21, or 28 days).

#### *2.5 Flavedo sample preparation*

After the determination of CI, the flavedo tissue of each lemon was carefully removed. The flavedo tissue was immediately pulverised in a mortar and pestle with liquid nitrogen and stored at -20°C for antioxidant analysis.

#### *2.6 Antioxidant extraction*

Antioxidant in flavedo was extracted according to Thaipong et al. (2006). Frozen flavedo tissue (3 g DM) was mixed with 25 ml 100% methanol (v/v) and homogenised for 2 min with the Ultra-Turrax homogenizer at 4°C. The homogenates were kept at 4°C for 12 hours before being centrifuged at 20 000 × g for 20 min at 4°C. The supernatant was recovered and used for antioxidant activity determination. All antioxidant assays were done at 4°C.

### *2.7 Determination of antioxidant activity estimated by the FRAP assay*

The FRAP assay was done according to Benzie and Strain (1996) with some modifications by Thaipong et al. (2006). The stock solutions included 300 mM acetate buffer (3.1 g  $C_2H_3NaO_2 \cdot 3H_2O$  and 16 ml  $C_2H_4O_2$ ), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-*s*-triazine) solution in 40 mM HCl and 20 mM  $FeCl_3 \cdot 6H_2O$  solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml  $FeCl_3 \cdot 6H_2O$  solution. Antioxidant extract (150  $\mu$ l) was allowed to react with FRAP solution (2850  $\mu$ l) for 30 min in the dark. The absorbance was taken at 593 nm. The standard curve was linear between 25 and 800  $\mu$ M Trolox. Results were expressed in  $\mu$ M Trolox equivalents  $g^{-1}$  DW ( $\mu$ M TE  $g^{-1}$  DM).

### *2.8 Determination of antioxidant activity estimated by the DPPH assay*

The DPPH assay was done according to the method of Thaipong et al. (2006). The stock solution was prepared by dissolving 0.024 g DPPH with 100 ml 100% methanol (v/v). The working solution was obtained by mixing 10 ml stock solution with 45 ml 100% methanol (v/v) to obtain an absorbance of  $1.1 \pm 0.02$  units at 515 nm. Antioxidant extract (150  $\mu$ l) was allowed to react with DPPH solution (2850  $\mu$ l) for 24h in the dark. The absorbance was taken at 515 nm. The standard curve was linear between 25 and 800  $\mu$ M Trolox. Results were expressed in  $\mu$ M TE  $g^{-1}$  DM.

### *2.9 Determination of antioxidant activity estimated by the ABTS assay*

The ABTS assay was performed according to Thaipong et al. (2006). The stock solutions included 7.4 mM ABTS<sup>•+</sup> solution and 2.6 mM potassium persulfate solution. The working solution was prepared according (Re et al., 1999) and allowed to react for 12 h at 25°C in the dark. The solution was diluted by mixing 1 ml ABTS<sup>•+</sup> solution with 60 ml 100% methanol (v/v) to obtain an absorbance of  $1.1 \pm 0.02$  units at 734 nm. Antioxidant extract

(150  $\mu\text{l}$ ) was allowed to react with ABTS<sup>•+</sup> solution (2850  $\mu\text{l}$ ) for 2h in the dark. The absorbance was taken at 734 nm. The standard curve was linear between 25 and 600  $\mu\text{M}$  Trolox. Results were expressed in  $\mu\text{M TE g}^{-1}\text{ DM}$ .

### *2.11 Determination of antioxidant activity estimated by the ORAC assay*

The ORAC assay was performed according to Wang (2003) with some modifications. Frozen flavedo tissue (5 g DM) was extracted in 45 ml phosphate buffer (75 mM, pH 7.0) using the Ultra-Turrax homogenizer at 4°C for 2 min. The mixture was then centrifuged at 20,000 rpm for 20 min at 4°C. The supernatant was used for the ORAC assay. The ORAC assay measures the effect of antioxidant components in fruit extracts on the decline in R-phycoerythrin (R-PE) fluorescence. The R-PE fluorescence is induced by a peroxy radical generator, which was prepared fresh all the time. The reaction mixture contained of 3.4 mg/l of R-PE (100  $\mu\text{l}$ ), 75 mM phosphate buffer (pH 7.0) (1.7 ml) and sample (100  $\mu\text{l}$ ). The reaction mixture was incubated at 37°C for 15 min and the reaction was started by the addition of 320 mM 2,2'-azobis (2-amidinopropane) dihydrochloride (100  $\mu\text{l}$ ). Using a fully automated microplate-based multi-detection reader (FLUOstar OPTIMA; BMG Labtech, Offenburg, Germany), fluorescence was measured and recorded every 5 min using excitation at 485 nm and emission at 520 nm. The phosphate buffer was used as a blank and 1  $\mu\text{M}$  Trolox was used as a standard during each run. The results are expressed as  $\mu\text{M TE g}^{-1}\text{ DM}$ .

### *2.12 Statistical analysis*

Experiments were performed using a randomised complete block design. For CI determination, 600 fruit were used: 4 treatments x 2 storage temperatures x 3 farm locations x 5 storage duration x 5 replicates were used in this experiment. For the determination of

antioxidant activities, three replicates of 5 fruit per 4 treatments of 2 storage temperatures, 3 farm locations and 5 storage durations were used. Data for the analytical determinations were subjected to analysis of variance (ANOVA) using GenStat® 14<sup>th</sup> Edition. Differences at  $P < 0.05$  were considered to be significant.

### **3. Results**

#### *3.1. The effect of MJ and SA on the development of CI in lemon fruit*

Cold storage of lemon fruit either at 2 or 4.5 °C for up to 28 days plus a week at shelf life resulted in the manifestation of CI symptoms. The symptoms were manifested in the flavedo mainly as pitting, red blotch and necrosis. Postharvest treatments, storage temperature, farm location, cold storage duration and the interactions of these factors had a profound effect on the development of CI. Severe CI symptoms were observed in control fruit than in MJ and SA treated fruit (Table 2). Postharvest treatment with either 10 µM MJ, 2 mM SA or 10 µM MJ plus 2 mM SA significantly ( $P < 0.05$ ) reduced the CI incidence. Treatment with 10 µM MJ plus 2 mM SA was the most effect treatment in reducing CI (Table 2). The results of this study indicate that the CI index in lemon fruit from New Venture Farm (moderate sub-tropical zone) was lower than that of lemon fruit from Tala Valley Citrus Estate (warm temperate zone) (Table 2). High CI index was observed in lemon fruit from Sun Valley Estates (cool sub-tropical zone).

#### *3.2. Changes in antioxidant activity in lemon flavedo estimated by the FRAP assay in response to MJ and SA postharvest treatment and storage temperature.*

The antioxidant capacity estimated by FRAP assay was significantly ( $P < 0.05$ ) affected by the postharvest treatments, storage temperature, storage duration and farm location. Cold storage of lemon fruit triggered an increase in the antioxidant capacity measured by the FRAP assay in control fruit. Postharvest treatments with MJ and SA significantly ( $P <$

0.05) enhanced the antioxidant activity measured by FRAP assay (Fig. 1). Treatments with 10  $\mu$ M MJ plus 2 mM SA was the most effective concentration in enhancing FRAP compared to the other concentrations (Fig. 1). Therefore, the lemon fruit treated with 10  $\mu$ M MJ plus 2 mM SA were found to have high levels of antioxidant measured by FRAP assay and this was followed by low CI index than the other concentrations (Fig. 1). High FRAP values were observed in lemon fruit harvested from the warm temperate zone followed by lemon fruit from moderate sub-tropical zone. Low FRAP values were observed in lemon fruit harvested from cool sub-tropical zone (Fig. 1).

*3.3. The effect of MJ and SA postharvest treatment, and storage temperature in antioxidant activity in lemon flavedo estimated by the DPPH assay.*

The antioxidant capacity estimated by DPPH assay was significantly ( $P < 0.05$ ) affected by treatments, storage temperature, farm location and the interactions of these factors (Fig. 2). The DPPH levels in lemon flavedo was increasing with cold storage time and reached the highest peak after 14 days at postharvest storage (Fig. 2). Thereafter, the DPPH levels were gradually decreasing with cold storage time from 21 to 28 days. The low levels of DPPH were observed after 28 days of postharvest storage. This was followed by the manifestation of CI symptoms in control fruit. Postharvest treatments with 10  $\mu$ M MJ plus 2 mM SA significantly ( $P < 0.05$ ) enhanced DPPH levels in lemon flavedo (Fig. 2). This was followed by low levels of CI symptoms (Table 1).

High DPPH levels were found in lemon fruit stored at 2 °C followed by the lemon fruit stored at 4.5 °C. The high DPPH levels in lemon fruit stored at 2 °C was followed by chilling tolerance. Low DPPH levels in lemon fruit stored at 4.5 °C was followed by severe CI. After postharvest storage at 2 or 4.5 °C for 28 days and a week at shelf-life, the changes in DPPH levels were significant in farm locations. Lemon fruit harvested from warm

temperate zone were found to have the higher DPPH levels in the flavedo than in lemon fruit harvested from moderate sub-tropical zone. Lemon fruit harvested from cool sub-tropical zone were found to have lower DPPH levels than the other climatic zone.

*3.4. Enhancement of antioxidant activity in lemon flavedo estimated by the ABTS assay in fruit from different farm locations.*

Cold storage of lemon fruit significantly ( $P < 0.05$ ) affected the antioxidant capacity determined by ABTS assay (Fig. 3). The trend of ABTS activity was constant with that of CI in respect to postharvest treatments. Postharvest treatments with 10  $\mu$ M MJ plus 2 mM SA significantly ( $P < 0.05$ ) enhanced ABTS levels in lemon flavedo more than the other concentrations (10  $\mu$ M MJ or 2 mM SA) (Fig. 3). Treatment with 2 mM SA was the second effective concentration in inducing ABTS followed by 10  $\mu$ M MJ (Fig. 3). The results of this study provide evidence that storage temperature plays a profound role in maintaining antioxidant capacity in the lemon flavedo. Storing lemon fruit at 2 °C maintained antioxidant capacity measured by ABTS assay. In contrast, increasing storage temperature to 4.5 °C resulted in the reduction of antioxidant capacity measured by ABTS assay (Fig. 3). The ABTS levels were high in lemon fruit from warm temperate zone (Tala Valley Citrus Estate) followed by the lemon fruit from the moderate sub-tropical zone (New Venture Farm).

*3.5. Changes in antioxidant activity in lemon flavedo estimated by the ORAC assay*

The ORAC assay was significantly ( $P < 0.05$ ) affected by treatments, storage temperature, farm location and the interactions of these factors (Fig. 5). The ORAC-antioxidant levels were low in lemon flavedo. However, in response to chilling stress, the ORAC-antioxidant levels gradually increased during cold storage reaching a peak after 21 days. The levels of ORAC-antioxidant in lemon flavedo gradually decline in cold storage after 28 days (Fig. 5)

and this was followed by severe CI symptoms (Table 1). The levels of ORAC-antioxidant were low in lemon flavedo compared to levels measured by other assays in control fruit. However, treatment with 10  $\mu$ M MJ plus 2 mM SA significantly ( $P < 0.05$ ) induced the ORAC-antioxidant levels more than the separate concentrations (10  $\mu$ M MJ or 2 mM SA). The ORAC-antioxidant levels in lemon fruit from warm temperate zone were more effectively maintained than in lemon fruit from the moderate sub-tropical zone (Fig. 5). However, the ORAC-antioxidant levels in lemon fruit from the cool sub-tropical zone, which had severe CI symptoms, were found to be the lowest as per location.

#### **4. Discussion**

Horticultural crops contain vast amounts of antioxidants which may contribute to protection against oxidative damage (Blokhina et al., 2003; Duan et al., 2007). Several assays such as FRAP (Benzie and Strain, 1996), DPPH (Mokbel and Hashinaga, 2006), ABTS (Leong and Shui, 2002), and ORAC (Cao et al., 1993) have been frequently used to estimate antioxidant capacity in crops (Thaipong et al., 2006). In this study, the chilling tolerance of MJ-and SA-treated lemon fruit from three climatic zones was associated with the induction of antioxidant capacity as estimated by FRAP, DPPH, ABTS and ORAC assays. The results of this study suggest that 10  $\mu$ M MJ plus 2 mM SA treatment reduces CI in lemon fruit by enhancing antioxidant activity in the flavedo during cold storage. The enhanced antioxidant activities in lemon flavedo could have contributed to chilling tolerance. The effect of MJ and SA in reducing and retarding CI effectively in lemon fruit stored at  $-0.5^{\circ}\text{C}$  for up to 42 days plus 7 days at  $23^{\circ}\text{C}$  was explained by Sibozza et al. (2012).

This study suggests that environmental conditions such as temperature and rainfall from the warm temperate zone were more favourable for CI resistance than the environmental conditions from moderate sub-tropical zone. Environmental conditions from cool sub-tropical zones are favourable for the development of CI in lemon fruit. Therefore, farm locations play a significant role in influencing the development of CI in lemon fruit.

Storage temperature plays a profound role at postharvest of lemon fruit. This study indicates that storage temperature influences CI development. The effect of temperature on influencing FRAP antioxidant capacity was profound. Low temperature (2 °C) maintained and enhanced the FRAP activity in lemon flavedo than the 4.5 °C temperature regime. This suggested that low temperature, slow fruit metabolism, biochemical and physiological activities lead to chilling tolerance and high antioxidant capacity. In contrast, high temperature (4.5 °C) accelerates biochemical, physiological and metabolic activities in the fruit, resulting in high depletion of defence systems such as antioxidants. This was supported by the low manifestation of CI in lemon fruit stored at 2 °C compared to CI on fruit stored at 4.5 °C.

The results of this study suggest that environmental conditions from the warm temperate zone were favourable for maintaining antioxidant capacity measured by FRAP, followed by the environmental conditions from moderate sub-tropical zones. The high antioxidant capacity in lemon fruit was accompanied by minimal manifestation of CI symptoms. This result also suggests that environmental conditions such as temperature and rainfall from cool sub-tropical zones are not favourable for maintaining antioxidant capacity in lemon flavedo. Therefore, future research must aim at comparing cultural practises and orchard management to see the differences among locations. The low antioxidant capacity in lemon fruit was accompanied by severe manifestation of CI symptoms.

The levels of antioxidant capacity measured by DPPH were increasing with cold storage time (0-14 days) probably as a response to chilling stress. However, extending cold storage from 14-21 days may cause a depletion of antioxidant capacity in lemon flavedo. This was followed by the development of CI in control fruit. The results suggested that the treatment combination (10  $\mu$ M MJ plus 2 mM SA) can be used to enhance the DPPH levels. Lemon fruit with low DPPH levels (control) were followed by severe CI development compared to lemon fruit with high accumulation of DPPH levels (MJ and SA treated). Again lemon fruit treated with 10  $\mu$ M MJ plus 2 mM SA was found to have high DPPH levels, and to be high chilling tolerant. This suggested that the antioxidant capacity estimated by DPPH levels could have been involved in enhancing chilling tolerance in MJ and SA treated lemon fruit.

Storage temperature plays an important role at postharvest storage of lemon fruit. As previously observed in antioxidant capacity estimated by FRAP, the DPPH levels were affected by storage temperature. The high accumulation of DPPH levels in lemon fruit stored 2 °C, could have contributed in enhancing chilling tolerance in lemon fruit. The low levels of DPPH in lemon fruit stored at 4.5 °C could not have been enough in withstanding chilling stress and enhancing chilling tolerance.

The results of this study suggest that lemon fruit from different farm locations may have different biochemical, physiological and metabolic changes even when stored at the same conditions at postharvest. Chilling susceptible lemon fruit were found to have low antioxidant capacity measured by DPPH levels. This was more evident in the lemon fruit harvested from the cool sub-tropical zone. The high induction of DPPH levels in 10  $\mu$ M MJ plus 2 mM SA treated lemon fruit from warm temperate zone and moderate sub-tropical zone could have been used by the fruit as a defence mechanism against chilling. In contrast, the low DPPH levels in lemon fruit harvested from the cool sub-tropical zone may not have

been enough for the fruit to withstand chilling stress and enhance chilling tolerance compared to fruit from the other locations. However, postharvest treatment with the combination of 10  $\mu$ M MJ plus 2 mM SA was always consistent in enhancing the levels of DPPH in lemon flavedo which was associated with the defence mechanisms against CI (Fig.3).

The induction of ABTS levels in lemon fruit flavedo was associated with the contribution to chilling tolerance in the fruit. Chilling tolerant lemon fruit (10  $\mu$ M MJ plus 2 mM SA treated) were found to have high ABTS levels than chilling susceptible lemon fruit (control) (Fig. 1). This was in agreement with Sala (1998) that chilling tolerant fruit have high antioxidant capacity than chilling susceptible fruit. Therefore, the results provide evidence that postharvest treatment with 10  $\mu$ M MJ plus 2 mM SA can increase chilling tolerance in lemon fruit by inducing antioxidant capacity in the flavedo. The adequate amount of antioxidant capacity measure by ABTS in lemon stored at 2 °C was supported by the chilling resistance in the fruit while the depletion of antioxidant capacity measured by ABTS in lemon flavedo was followed by the severe CI symptoms.

Farm location, is another factor that plays a significant role in changes of antioxidant capacity measured by ABTS assay. Environmental conditions prevailing at farm locations may have an influence on fruit quality at postharvest. Conditions from the warm temperate zone were found to be consistent with high antioxidant capacity in lemon flavedo. Lemon fruit harvested from these climatic zones were also found to be chilling resistant than the lemon fruit that were harvested from the cool sub-tropical zone. The lemon fruit from the cool sub-tropical zone were found to have low ABTS levels and suffered severe chilling damage. The low ABTS levels in lemon fruit from the cool sub-tropical zone was associated with poor defence mechanisms against CI flavedo whereas the high ABTS levels

in lemon fruit from the warm temperate and moderate sub-tropical zones were associated with defence mechanism against CI.

This study suggests that the combination of MJ and SA at the right concentration (10  $\mu$ M MJ plus 2 mM SA) can be used to enhance antioxidant capacity measured by ORAC assay. The low levels of ORAC assay in lemon flavedo were associated with the low contribution of antioxidants measured by ORAC in defence against CI. The results of this study also provide evidence that postharvest storage temperatures may influence the antioxidant capacity in lemon flavedo measured by ORAC assay. Lemon fruit that were stored at 2 °C were chilling tolerant with high ORAC levels than lemon fruit that were stored at 4.5 °C which were chilling susceptible (Fig. 5). This suggested that storage of lemon fruit at 2 °C reduces CI by enhancing antioxidant capacity in the fruit. The induced antioxidant capacity measured by ORAC assay could have been involved in defence mechanisms against the manifestations of CI in lemon fruit.

The accumulation antioxidant capacity measured by ORAC assay in lemon flavedo depended on the farm location. The difference of this accumulation was associated with the environmental factors in the farm locations. This study suggest that environmental conditions from the warm temperate zone were favourable for triggering defence mechanisms in the fruit followed by the moderate sub-tropical zone conditions. Environmental conditions from the cool sub-tropical zone were found to be unfavourable for antioxidant capacity measured by ORAC assay. Similarly, this was also observed in antioxidant capacities measured by the other assays and by the development of CI symptoms.

Both, MJ and SA are considered to be plant signal molecules involved in defence responses and signal transduction pathways by inducing secondary metabolic pathway enzymes to form defence compounds such as antioxidants (Ding et al., 2002; Ali et al., 2007). It has been reported that MJ and SA can act as secondary messengers and be involved by triggering defence genes in response to plant stress such as chilling (Ali et al., 2007). In this study, the high induction of antioxidant capacities in MJ-and SA-treated lemon fruit was associated with the defence response against chilling stress triggered by MJ and SA treatment.

In conclusion, postharvest application of 10  $\mu$ M MJ plus 2 mM SA significantly ( $P < 0.05$ ) enhanced chilling tolerance in lemon, thereby maintaining higher levels of antioxidant activities as measured by FRAP, DPPH, ABTS and ORAC assays. Storage temperature played a profound role in the development of CI. Storing lemon fruit at 2 °C delayed the onset of CI symptoms, thereby maintaining the antioxidant capacity, whilst 4.5 °C storage accelerated the onset of CI symptoms, thereby limiting antioxidant capacity. Higher antioxidant capacities were observed in lemon flavedo of fruit from the warm temperate zone followed by the fruit from the moderate sub-tropical zone than the cool sub-tropical zone. Lemon fruit with high antioxidant capacity (MJ and SA treated) were found to be chilling tolerant, whereas lemon fruit with low antioxidant capacity (control) were found to be chilling susceptible. The findings of this study, suggest that lemon fruit treated with 10  $\mu$ M MJ plus 2 mM SA should be stored at 2°C in order to trigger maximum antioxidant concentration in the flavedo needed to increase chilling tolerance. Therefore, MJ and SA when combined at an effective concentration (10  $\mu$ M MJ plus 2 mM SA) has a potential in enhancing antioxidant capacity, thereby enhancing chilling tolerance in ‘Eureka’ lemon fruit.

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Table 1. Main drawbacks associated with antioxidant assays such as ferric reducing ability of plasma (FRAP); 2,2-diphenyl-1-picrylhydrazyl (DPPH); 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS); and the oxygen radical absorption capacity (ORAC), by Pérez-Jiménez et al. (2008).

Table 2. Changes in chilling injury (CI) index of 'Eureka' lemon fruit from the New Venture Farm, Tala Valley Citrus Estate and Sun Valley Estates when treated with either 10  $\mu$ M methyl jasmonate (MJ), 2 mM salicylic acid (SA) or 10  $\mu$ M MJ plus 2 mM SA prior to cold storage either at 2 or 4.5 °C for up to 28 days plus 7 days shelf-life. Least significant differences of means (5% level) = 0.23.

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Fig. 1. Effects of postharvest treatments either with 10  $\mu$ M methyl jasmonate (MJ), 2 mM salicylic acid (SA) or 10  $\mu$ M MJ plus 2 mM SA in antioxidant capacity of lemon flavedo measured by ferric reducing ability of plasma (FRAP) assay. Values are means of three replicate with  $\pm$  standard errors. Least significant differences of means (5% level) = 3.06.

Fig. 2. The influence of 10  $\mu$ M methyl jasmonate (MJ), 2 mM salicylic acid (SA) or 10  $\mu$ M MJ plus 2 mM SA treatments in enhancing antioxidant capacity of lemon flavedo measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Values are means of three replicate with  $\pm$  standard errors. Least significant differences of means (5% level) = 0.66.

Fig. 3. Changes in antioxidant capacity in lemon flavedo measured by 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) assay in fruit treated with 10  $\mu$ M methyl jasmonate (MJ), 2 mM salicylic acid (SA) or 10  $\mu$ M MJ plus 2 mM SA. Values are means of three replicate with  $\pm$  standard errors. Least significant differences of means (5% level) = 0.82.

Fig. 4. The effect of 10  $\mu$ M methyl jasmonate (MJ), 2 mM salicylic acid (SA) or 10  $\mu$ M MJ plus 2 mM SA on lemon flavedo antioxidant capacity measured by oxygen radical absorption capacity (ORAC) assay. Values are means of three replicate with  $\pm$  standard errors. Least significant differences of means (5% level) = 0.43.

Table 1.

Antioxidant assays	Main drawbacks
FRAP	<p>Other compounds may absorb at 595 nm,  Any compound with a redox potential lower than 0.77 v, although it does not behave <i>in vivo</i> as an antioxidant, may reduce ion,  It is performed at non-physiological pH.</p>
DPPH	<p>Other compounds may absorb at 515 nm,  There may be steric hindrance for molecules with higher molecular weight,  The free radical used is not present <i>in vivo</i> and is quite stable, unlike radicals present in living organisms.</p>
ABTS	<p>Antioxidants, besides reacting with the radical to yield the original molecule, generate other compounds,  Reaction is not over at the usual 6 min taken,  The free radical used is not present <i>in vivo</i>.</p>
ORAC	<p>The kinetics of reaction may vary on the concentration of the antioxidant; what enables this method to be used for kinetics study,  It measures the ability of antioxidants to scavenge peroxy radical, present <i>in vivo</i>; however, the procedure to generate these peroxy radicals is not physiological,  Protein may have an interfering effect.</p>

Table 2.

Chilling injury index <sup>a</sup>							
Treatments	Duration of cold storage (d)	New Venture Farm		Tala Valley Citrus Estate		Sun Valley Estates	
		2°C	4.5°C	2°C	4.5°C	2°C	4.5°C
Control	0	0.00	0.00	0.00	0.00	0.00	0.00
	7	0.00	0.13	0.00	0.00	0.60	0.60
	14	0.13	0.20	0.60	0.60	0.88	1.03
	21	1.00	1.20	1.60	1.60	1.32	1.91
	28	0.80	1.91	2.60	3.00	2.80	3.00
Mean		0.39 fgh	0.69 e	0.96 cd	1.04 bc	1.12 b	1.31 a
10 µM MJ	0	0.00	0.00	0.00	0.00	0.00	0.00
	7	0.00	0.00	0.00	0.00	0.80	0.40
	14	0.13	0.20	0.00	0.00	1.40	1.04
	21	0.53	0.27	0.40	0.40	1.73	1.04
	28	0.93	0.96	0.60	1.20	2.61	2.05
Mean		0.32 h	0.28 hi	0.20 ij	0.32 h	1.31 a	0.91 d
2 mM SA	0	0.00	0.00	0.00	0.00	0.00	0.00
	7	0.00	0.00	0.00	0.00	0.80	0.80
	14	0.00	0.07	0.00	0.00	1.20	1.40
	21	0.60	0.13	0.60	0.60	1.39	1.73
	28	1.20	0.72	1.67	1.80	2.08	2.80
Mean		0.36 gh	0.18 ij	0.45 fg	0.48 f	1.09 b	1.35 a
10 µM MJ plus 2 mM SA	0	0.00	0.00	0.00	0.00	0.00	0.00
	7	0.00	0.00	0.00	0.00	0.32	0.40
	14	0.07	0.07	0.00	0.00	0.64	0.96
	21	0.20	0.13	0.00	0.13	0.96	1.87
	28	0.33	0.60	0.53	0.60	1.80	2.05
Mean		0.12 j	0.16 j	0.11 j	0.15 j	0.74 e	1.06 bc

<sup>a</sup>Mean values followed by different letters are significantly different at  $P < 0.05$  according to Duncan's Multiple Range Test.

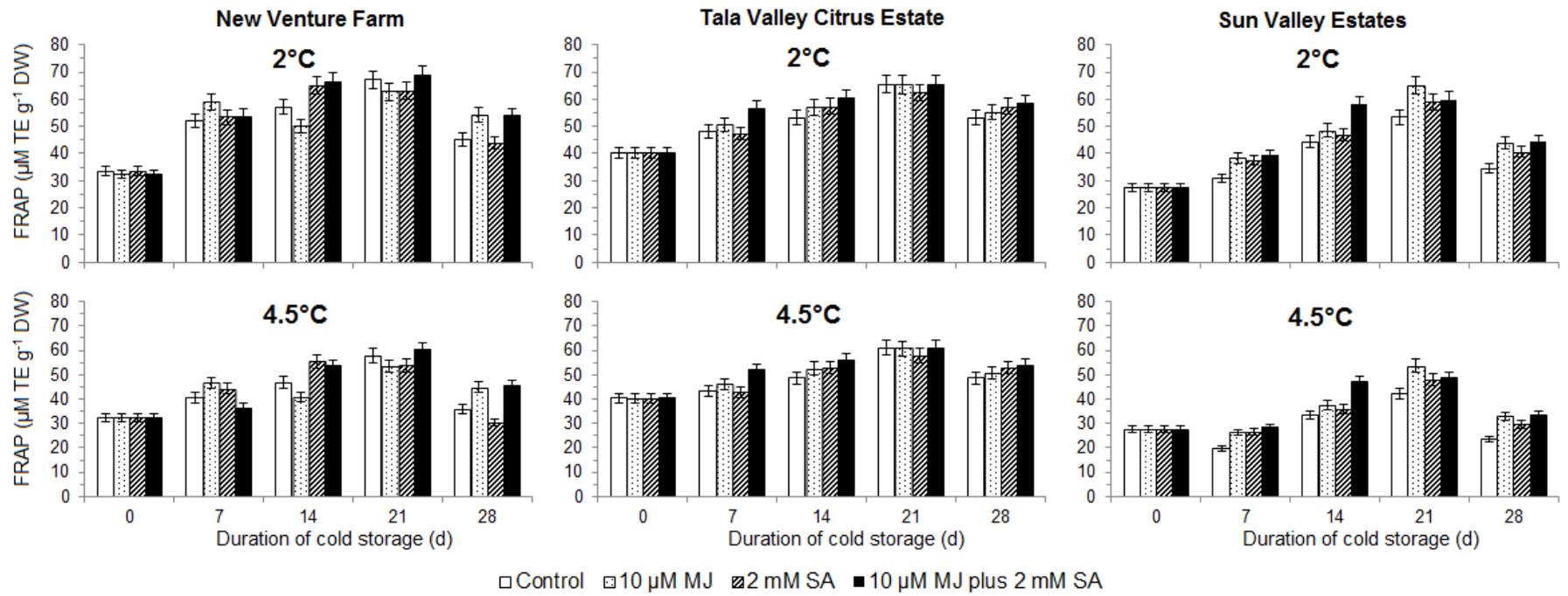


Fig. 1.

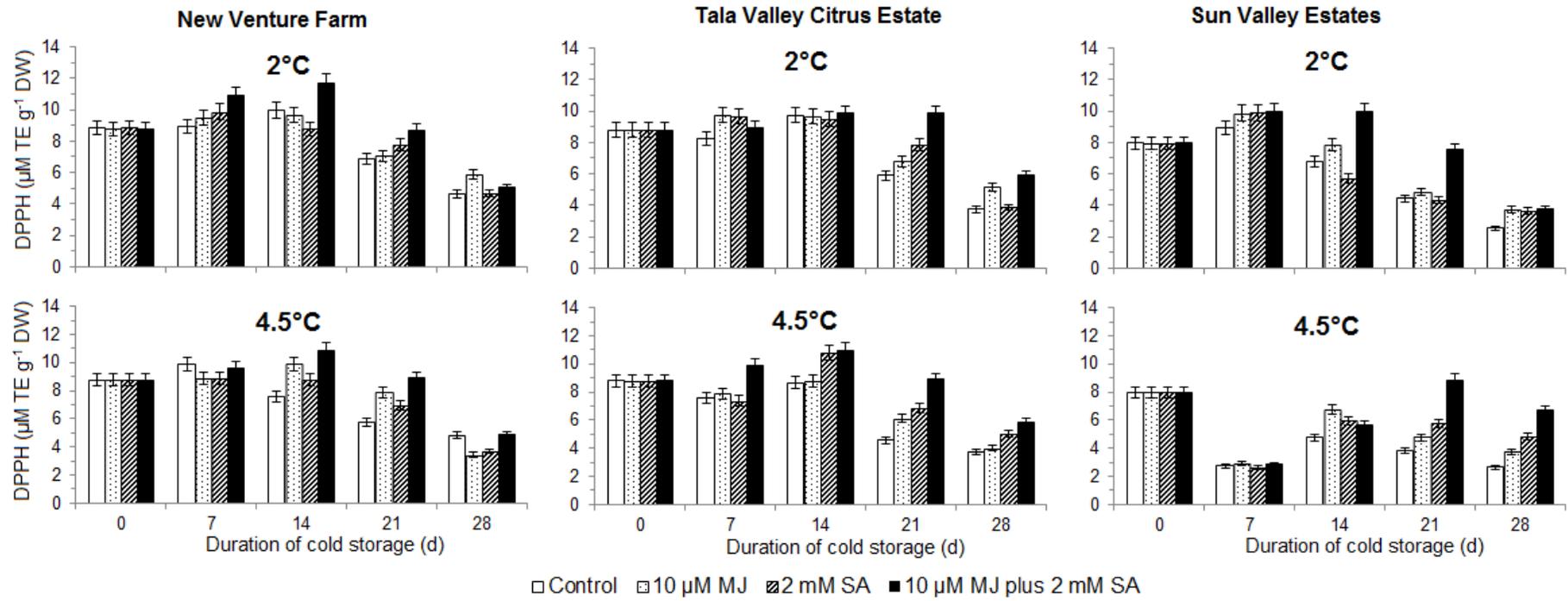


Fig. 2.

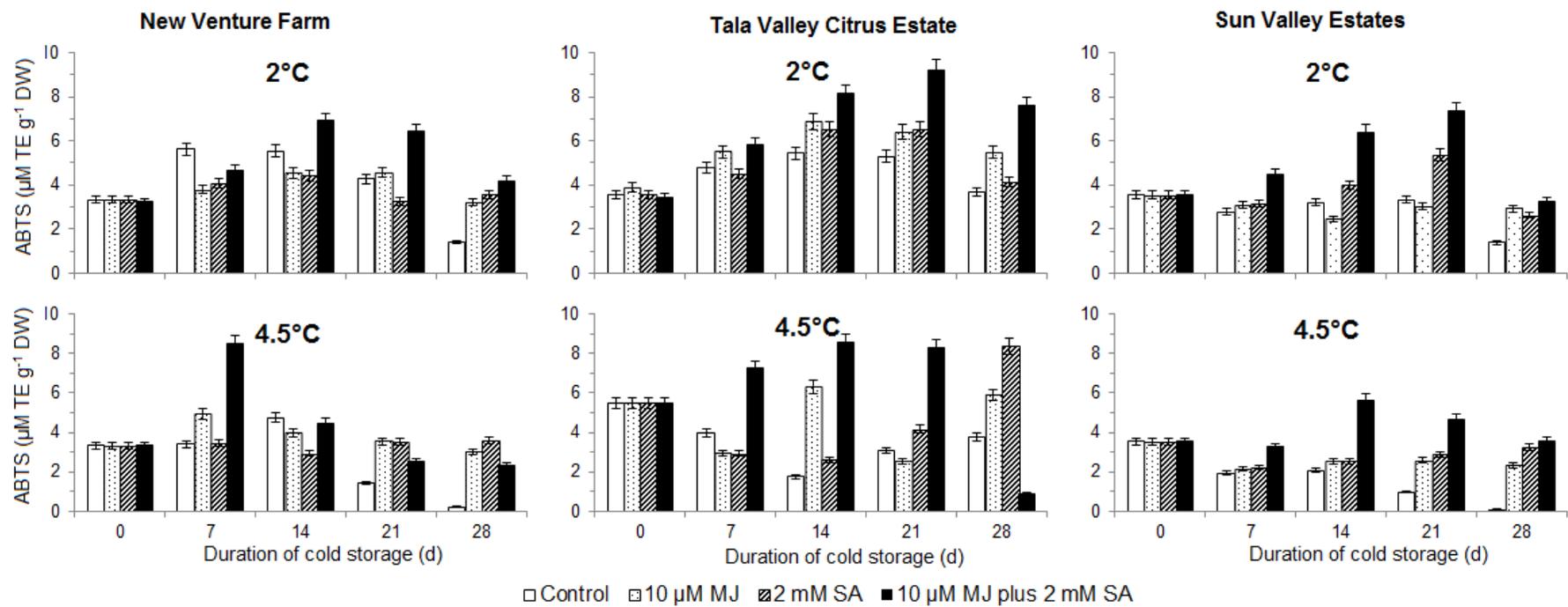


Fig.3.

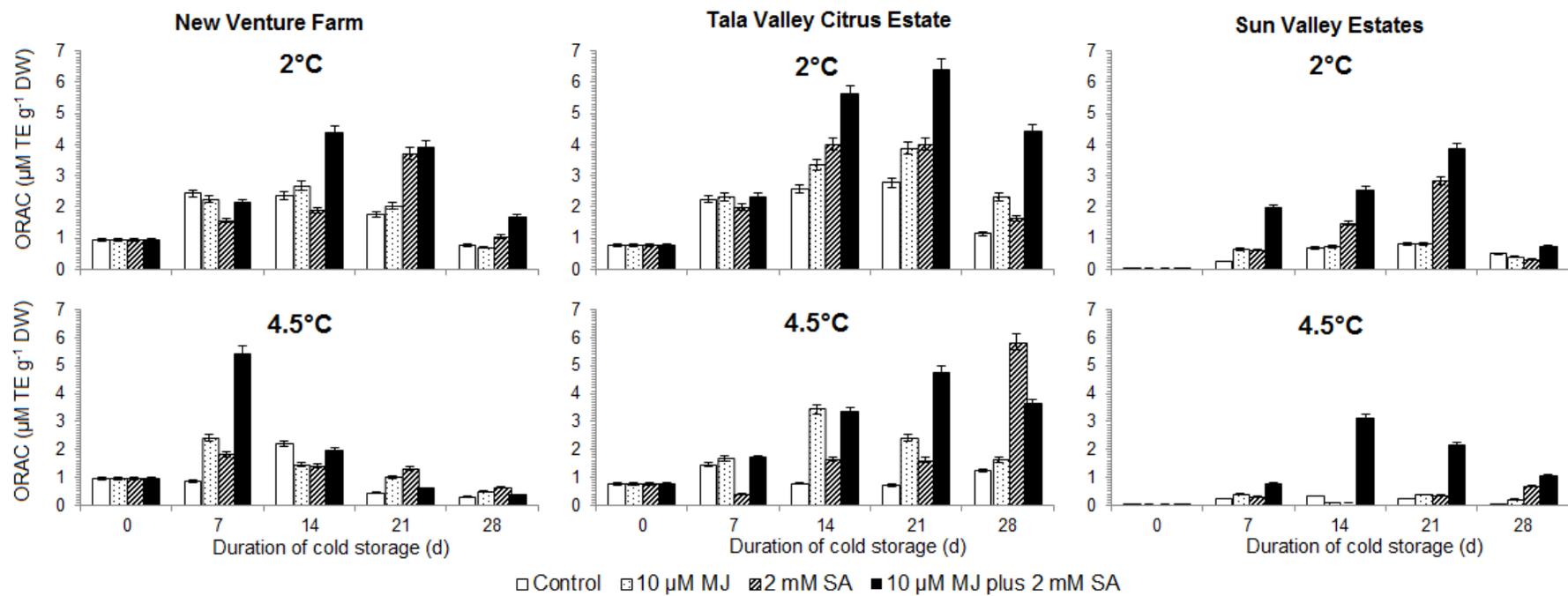


Fig. 4.

## Chapter 10

### General Discussion and Future Directions

The manifestation of CI (chilling injury) in cold-stored lemon fruit remains a challenge to the South African citrus industry. Symptoms of CI developing in the flavedo affect consumer acceptability of fruit, resulting in lower returns to the grower. An understanding of the physiological responses to chilling temperatures and the manifestation of CI in lemon fruit is, thus, of great importance to the citrus industry. In this study, the response of lemon fruit to cold storage temperatures and the manifestation of CI, as well as the mechanisms involved in chilling tolerance were studied. This could be useful in developing recommendations for alleviating this physiological disorder. Commercially mature fruit were obtained from three locations across KwaZulu-Natal, South Africa, representative of moderate subtropical (Chapter 2: Table 3), warm temperate (Chapter 2: Table 1) and cool subtropical (Chapter 2: Table 2) climatic zones. The fruits were treated with either 10  $\mu$ M methyl jasmonate (MJ), 2 mM salicylic acid (SA) or 10  $\mu$ M MJ plus 2 mM SA and stored at -0.5, 2 or 4.5°C for up to 28 days.

The findings of this study revealed that chilling sensitivity of lemon fruit was strongly influenced by preharvest climatic conditions and postharvest treatments, as well as storage temperature and duration (Chapter 2). Untreated lemon fruit (control) exhibited severe CI symptoms; these symptoms increased with increasing cold storage duration. Postharvest application of MJ and SA showed potential in alleviating CI; however, the efficacy of MJ and SA treatment in alleviating CI was associated with additive benefit of the two compounds or a synergy between them. Treatment with 10  $\mu$ M MJ plus 2 mM SA was more effective in enhancing chilling tolerance, than treatment with either 10  $\mu$ M MJ or 2 mM SA. The enhanced chilling tolerance following 10  $\mu$ M MJ plus 2 mM SA treatment implies that these two compounds when applied in combination are involved in

mechanisms that trigger physiological processes conferring protection against damage caused by chilling. These effects were not evident when the treatments (MJ or SA) were applied separately.

The compounds MJ and SA are plant growth regulators that act as signalling molecules during plant stress. We found that postharvest treatment with these compounds significantly ( $P < 0.05$ ) conferred protection against CI and enhanced chilling tolerance in lemon fruit by inducing the synthesis of heat shock proteins translation, and enzymatic and non-enzymatic antioxidants (Chapter 7); and maintaining the flavedo mineral composition and ultra-structure of the flavedo (Chapter 6). These results further suggest that the efficacy of MJ and SA treatment in enhancing chilling tolerance in lemon fruit is significantly ( $P < 0.05$ ) influenced by farm location, storage temperature and cold storage duration. Storage temperature was one of the major factors involved in the chilling susceptibility of lemon fruit. Storing lemon fruit at  $-0.5^{\circ}\text{C}$  and at  $2^{\circ}\text{C}$ , effectively suppressed the development of CI symptoms. This could probably be attributed to a slowing of metabolic processes at these low temperatures. However, storing lemon fruit at  $4.5^{\circ}\text{C}$  favoured the development of CI symptoms probably due to higher metabolic rates at the relatively higher temperature.

Preharvest climatic conditions influenced chilling sensitivity of lemon fruit significantly. Lemon fruit from New Venture Farm (moderate subtropical region) were found to be chilling tolerant while fruit from Tala Valley Citrus Estate (warm temperate region) were found to be only moderately chilling tolerant. Lemon fruit from Sun Valley Estates (cool subtropical region) showed the maximum susceptibility to CI, with severe CI symptoms (Chapter 2). This was possibly a consequence of the accumulation of chilling in the field prior to harvest (Lafuente et al., 1997). Lafuente et al. (1997) did not state temperatures at harvest. In our study, the mean daily temperatures were about  $8.7\text{-}23.3^{\circ}\text{C}$  (New Venture Farm),  $7.4\text{-}20.7^{\circ}\text{C}$  (Tala Valley Citrus Estates), and  $1.7\text{-}21.4^{\circ}\text{C}$  (Sun Valley Estates) in the

field prior to harvest. The presented findings suggest that the combination treatment of MJ and SA can be used to enhance chilling tolerance in lemon fruit, particularly in fruit from the cool subtropical region.

The observed effect of the treatment with 10  $\mu\text{M}$  MJ plus 2 mM SA on enhancing chilling tolerance in lemon fruit during cold storage suggests that the two compounds when applied together may be involved in several physiological processes that could trigger more than one protective mechanisms against chilling stress. To test this hypothesis, the action of reactive oxygen species (ROS) and the measurements on increased total antioxidant capacity in fruit treated with both MJ and SA were done (Chapter 7). The individual treatments did not seem to have an effect on ROS produced in response to chilling. However, the combination of the two treatments (10  $\mu\text{M}$  MJ plus 2 mM SA) significantly ( $P < 0.05$ ) enhanced the activity and concentration of the defence mechanism by inducing the production of antioxidants thus controlling the production of ROS, thereby enhancing chilling tolerance and extending the shelf-life of lemon fruit.

Postharvest application of 10  $\mu\text{M}$  MJ plus 2 mM SA was also effective in maintaining fruit quality (Chapter 3). This was achieved through reduced lipid peroxidation which prevented excessive membrane leakage. Furthermore, the postharvest treatments played a role in retarding fruit respiration rates aligned with mass loss. Furthermore, application of 10  $\mu\text{M}$  MJ plus 2 mM SA effectively maintained vitamin E and total carotenoid levels. This could have played a role in maintaining fruit quality and contributed to the enhancement of chilling tolerance in lemon fruit. Chilling susceptible lemon fruit were found to have a high respiration rate, fruit mass loss, lipid peroxidation, membrane leakage and lower levels of vitamin E and total carotenoids (Chapter 3). These factors may serve as markers of CI in lemon fruit. Storing lemon fruit at 4.5°C resulted in high respiration rates coupled with severe mass loss compared with storage at 2 °C and -0.5°C, respectively. This was inevitably followed by a more severe manifestation of CI symptoms in fruit stored at 4.5°C

than at 2 or -0.5°C, respectively. This suggested that -0.5°C was an effective storage temperature with regard to reducing respiration rate and mass loss without severe CI.

The responses of phospholipase D (PLD) and lipoxygenase (LOX) activity to chilling in MJ and SA treated lemon, including the effect of MJ and SA treatments in maintaining membrane integrity of lemon flavedo during cold storage were investigated (Chapter 8). Cold storage of lemon fruit resulted in an increased lipid peroxidation, membrane permeability, and activation of phospholipase D (PLD) and lipoxygenase (LOX) in chilling susceptible lemon fruit. This was associated with loss of membrane integrity which was accompanied by the onset of CI symptoms. However, postharvest treatment with 10 µM MJ plus 2 mM SA significantly maintained membrane integrity, thereby reducing electrolyte leakage, lipid peroxidation, and PLD and LOX activity. This was associated with chilling tolerance and enhanced by storage at -0.5°C. The ability of the combination treatment, MJ and SA, to maintain membrane integrity renders this treatment a crucial in the management of chilling stress.

Fruits have developed efficient mechanisms to scavenge ROS as a protection against oxidative stress (Beak and Skinner, 2003; Yang et al., 2012). In this study, treatment with 10 µM MJ plus 2 mM SA significantly ( $P < 0.05$ ) enhanced the concentration of flavedo proline, soluble sugars and ascorbic acid content (Chapter 4). Proline accumulation in lemon flavedo during cold storage could serve as a defensive mechanism against chilling. Soluble sugars have been reported to increase chilling tolerance (Holland et al., 2005). Soluble sugars and proline are compatible solutes that function as osmotic to maintain cell water potential to prevent loss of water during low temperature stress and thus, stabilise proteins and membranes. Soluble sugars are known to protect membranes against chilling (Valluru and Van Den Ende, 2008). Therefore, in this study, the enhanced levels of soluble sugars could have improved chilling tolerance by protecting fruit membranes against chilling. Ascorbic acid is an abundant compound known as a major cellular antioxidant

protecting cellular tissue against oxidative stress (Smirnoff and Wheeler, 2000). Our study showed that ascorbic acid increased in response to treatment with 10  $\mu$ M MJ plus 2 mM SA. This suggests that this treatment was able to confer/enhance chilling tolerance in lemon fruit. In that respect, the findings of this study indicate that MJ and SA treatment enhances chilling tolerance in lemon fruit by enhancing the levels of metabolites associated with chilling tolerance.

Chapter 5 investigated the efficacy of MJ and SA treatment in enhancing total phenolic compounds and PAL activity in lemon fruit, as defence mechanisms against CI, and inhibiting PPO and POD activity in lemon fruit, as they are involved in phenolic oxidation, enzymatic browning and fruit deterioration. The results showed that the effect of treatment with 10  $\mu$ M MJ plus 2 mM SA significantly ( $P < 0.05$ ) induced defence mechanisms involved in cold acclimation such as enhancing flavonoid phenolics and PAL activity while inhibiting accumulation of POD. This increase in phenolic compounds could have played a role in stabilising membranes by decreasing membrane fluidity, and by absorbing and neutralising free radicals (Osawa, 1994; Blokhina et al., 2003; Javanmardi et al., 2003; Michalak, 2006). In citrus fruit, high PAL activity has been previously associated with regulating the rate of substrate supply for polyphenol synthesis in response to chilling tolerance (Lafuente et al., 2004). An induction of PAL activity by chilling has been associated with chilling tolerance and considered as a good marker of CI in citrus fruit (Martínez-Téllez and Lafuente, 1993; Sanchez-Ballesta et al., 2000). The inhibition of POD activity could explain the potential of MJ and SA in enhancing chilling tolerance. The results of this study suggest that PPO was not involved in CI and may have limited use as a biochemical marker of CI.

Chilling damage was visible in the ultra-structure of chilling susceptible lemon fruit and was marked by swollen, cracked, and collapsed cuticles, and disintegrated parenchyma cells and cell walls (Chapter 6). These damages could have affected the performance of the

fruit; however, application of 10  $\mu\text{M}$  MJ plus 2 mM SA protected the flavedo ultra-structure of lemon fruit peel from chilling damage and preserved nutrient concentration. The ability of 10  $\mu\text{M}$  MJ plus 2 mM SA in protecting the flavedo ultra-structure from chilling damage and preserving nutrients present in the flavedo could explain the potential of this treatment in enhancing chilling tolerance. The most prevalent elements detected in the flavedo were carbon, oxygen, phosphorus, potassium, calcium, magnesium, sulphur, sodium, silicon and aluminium. The higher nutrient concentration in the flavedo of fruit treated with MJ and SA could have played a role in protecting fruit against chilling stress, thereby maintaining fruit quality. Calcium has been suggested to increase abiotic stress tolerance (Bowler and Fluhr, 2000; Toivonen and Hodges, 2011). The enhanced calcium level by MJ and SA treatment may account for the increase in chilling tolerance.

Chilling susceptible lemon fruit were found to have high ROS accumulation in the flavedo. This would have, in turn, caused oxidative damage which resulted in high membrane permeability, high PLD and LOX activity as well as lipid peroxidation. Treatment with 10  $\mu\text{M}$  MJ plus 2 mM SA retarded the accumulation of ROS by increasing catalase, ascorbate peroxidase, glutathione reductase activities (Chapter 7). The induced enzymatic antioxidant systems could have detoxified ROS, prevented oxidative damage, and increased chilling tolerance. Storing lemon fruit at  $-0.5^{\circ}\text{C}$  enhanced the beneficial effects of MJ and SA. Furthermore, the effects of MJ and SA as postharvest treatments in enhancing proteins as a mode of action in increasing chilling tolerance in lemon fruit at cold storage were investigated. Heat shock proteins were triggered during cold storage of lemon fruit. The enhancement of heat shock proteins was probably a defence mechanism against chilling. The proteins were triggered according to the severity of stress. Fruit stored at  $-0.5^{\circ}\text{C}$  had high expression of proteins than fruit stored at 2 or  $4.5^{\circ}\text{C}$ .

Improvement of antioxidant defence mechanisms in crops produces tolerance to chilling (Sala and Lafuente, 1999; Rivera et al., 2007). Chilling susceptible lemon fruit were found to have low antioxidant defence capacity (Chapter 9). Treatment with 10  $\mu$ M MJ plus 2 mM SA significantly ( $P < 0.05$ ) enhanced total antioxidant capacity measured by ferric reducing ability of plasma (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and the oxygen radical absorption capacity (ORAC) assays. This was greatly regulated by storing lemon fruit at 2°C than 4.5 °C. Chilling tolerant citrus cultivars usually have more efficient antioxidant defence system than chilling-sensitive cultivars (Sala, 1998; Rivera et al., 2007). In this study, the application of 10  $\mu$ M MJ plus 2 mM SA significantly ( $P < 0.05$ ) enhanced chilling tolerance in lemon, thereby maintaining higher levels of antioxidant activities as measured by FRAP, DPPH, ABTS and ORAC assays. The enhancement of antioxidant capacity in lemon fruit triggered by MJ and SA treatment was associated with reduced CI.

### **General conclusions**

The chilling sensitivity of lemon fruit results in the development of CI symptoms, leading to economic losses in the South African citrus industry. The development of CI symptoms in lemon fruit was influenced by preharvest climatic conditions (farm locations), postharvest treatments, storage temperatures and storage duration. Treatment with 10  $\mu$ M MJ plus 2 mM SA effectively enhanced chilling tolerance in lemon fruit by maintaining fruit quality and membrane integrity as well as enhancing phenolic compounds and mechanisms involved in chilling tolerance. This treatment effectively enhanced chilling tolerance by enhancing total antioxidant capacity as measured by ABTS, DPPH, FRAP assays, enzymatic and non-enzymatic antioxidants, maintaining the ultrastructure condition and preserving mineral-nutrients in the flavedo. The induction of these antioxidant defence systems likely prevented oxidative damage by scavenging ROS and strengthening defence

mechanisms against chilling stress. The efficacy of 10  $\mu$ M MJ plus 2 mM SA treatment was more enhanced when lemon fruit were stored at  $-0.5^{\circ}\text{C}$  than at  $2^{\circ}\text{C}$ . Increasing cold storage temperature to  $4.5^{\circ}\text{C}$  favoured the onset of CI and accelerated depletion of resources involved in defence processes. Therefore, postharvest treatment with 10  $\mu$ M MJ plus 2 mM SA may be commercially used to enhance chilling tolerance in lemon fruit during quarantine treatments.

### **Recommendations to the citrus industry**

Postharvest treatments using MJ and SA can be effective in improving the storage of lemon fruit, preserve quality and extend the cold storage period. Treating lemon fruit with these two compounds may enhance chilling tolerance in lemon fruit during quarantine treatment at low temperatures. It is recommended that the use of  $-0.5^{\circ}\text{C}$  as a quarantine treatment temperature is beneficial for slowing metabolism of lemon fruit, thereby delaying the onset of CI symptoms. Appendix 2 gives an indication of recommended postharvest treatments, safe storage temperatures and durations for lemon fruit grown in SA.

### **Future research**

An understanding on the relationships that may exist if any on the role of compounds such as proteins, proline, soluble sugars and antioxidants that may be triggered in response to the application of MJ and SA postharvest treatment and whether these compounds are implicated in the enhancement of chilling tolerance in lemon fruit deserves further detailed investigation. The effect of orchard management practices and other parameters such as soil test analysis should also be further considered when studying the manifestation of CI. Future studies should focus on investigating fruit internal quality of MJ and SA treated lemon fruit at postharvest. Future research should involve non-destructive measurement by

the use of near infrared spectroscopy to predict and evaluate internal and external fruit quality.

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## **List of publications included as part of the thesis**

- Siboza, X.I., Bower, J.P. and Bertling, I., 2013. Effect of methyl jasmonate and salicylic acid postharvest treatment on mechanisms and activities of antioxidants in lemon fruit rind during cold storage. *Acta Hort.* 1007, 127–133.
- Siboza, X.I., Bertling, I., 2013. The effects of methyl jasmonate and salicylic acid on suppressing the production of reactive oxygen species and increasing chilling tolerance in 'Eureka' lemon [*Citrus limon* (L.) Burm.F.]. *J. Hortic. Sci. Biotech.* 88, 269–276.
- Siboza, X.I., Bower, J.P., Bertling, I., 2011. Effect of methyl jasmonate and salicylic acid on chilling injury of 'Eureka' lemons. *Acta Hort.* 911, 409–414.
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## Appendices

### Appendix 1: Farm description

#### *Tala Valley Citrus Estate*

Tala Valley Citrus Estate (29° 52' S 30° 30' E, 416-922 m asl) is located in the warm temperate zone (Figure 2). Climatically, this zone is known to have ground frost (1%).

#### *Sun Valley Estates*

Sun Valley Estates (28° 83' S 30° 06' E, 820-1003 m asl) is located in the cool subtropical climatic zone (Figure 3). The area is known to have ground frost (5%). Severe frosts are expected in 96% of the year.

#### *New Venture Farm*

New Venture Farm (31° 02' S 29° 25' E, 68-483 m asl) is located in the moderate subtropical zone (Figure 4). The area has no ground frost.

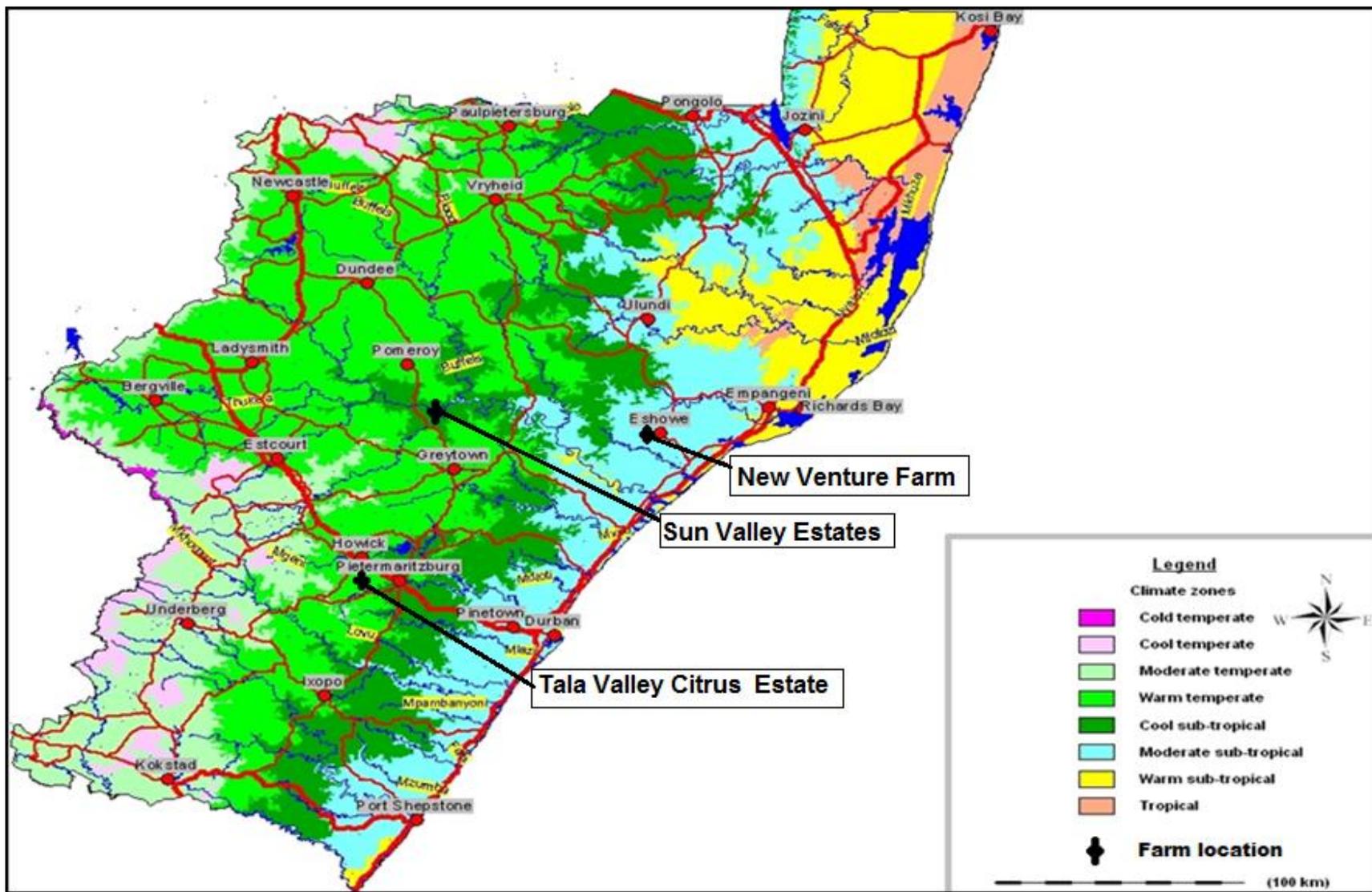


Figure 1. Map of the KwaZulu-Natal province indicating major climatic zones (BRU, 2007).

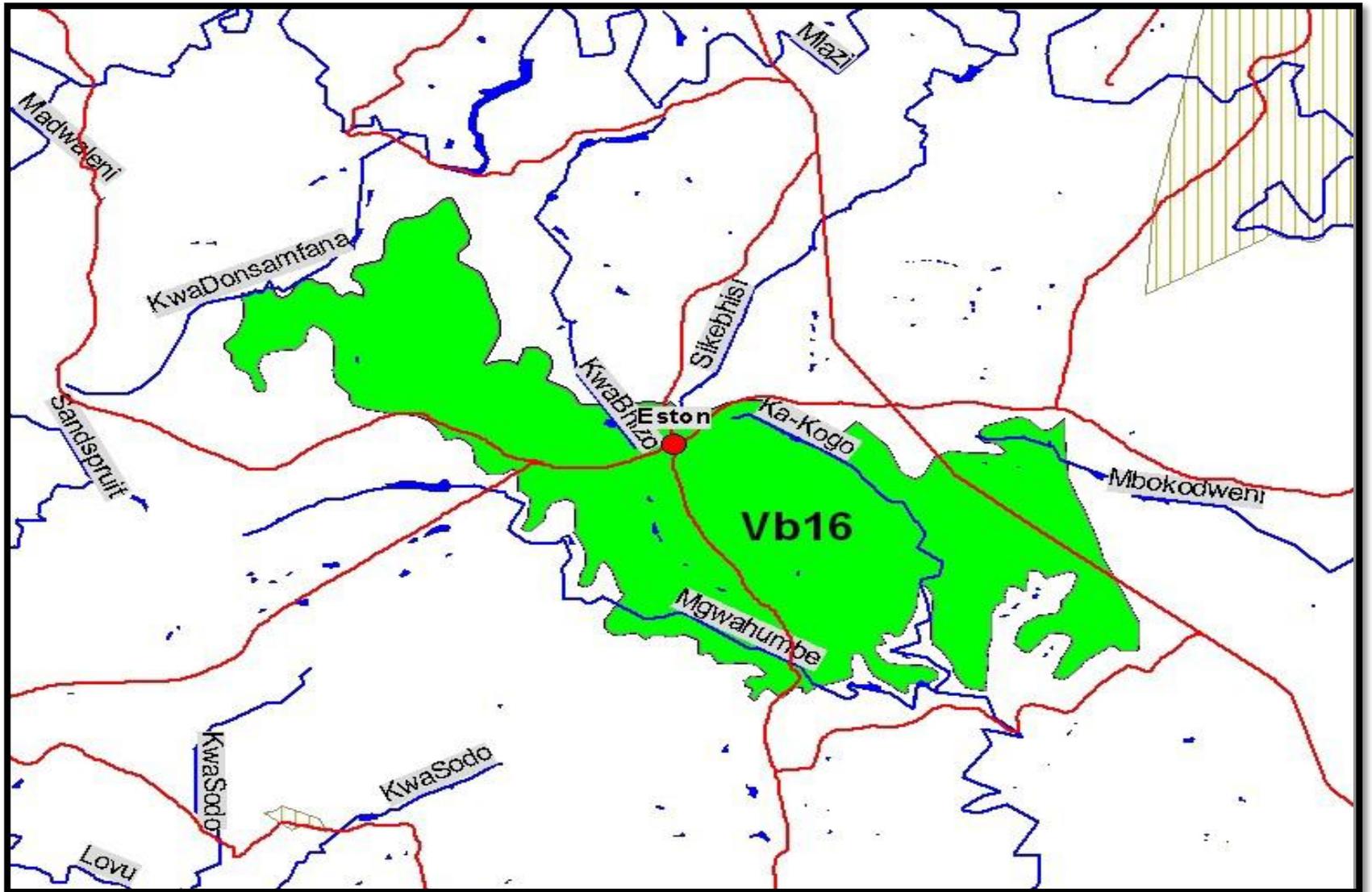


Figure 2. Eston map indicating the location of Tala Valley Citrus Estate (Vb 16) (BRU, 2007).

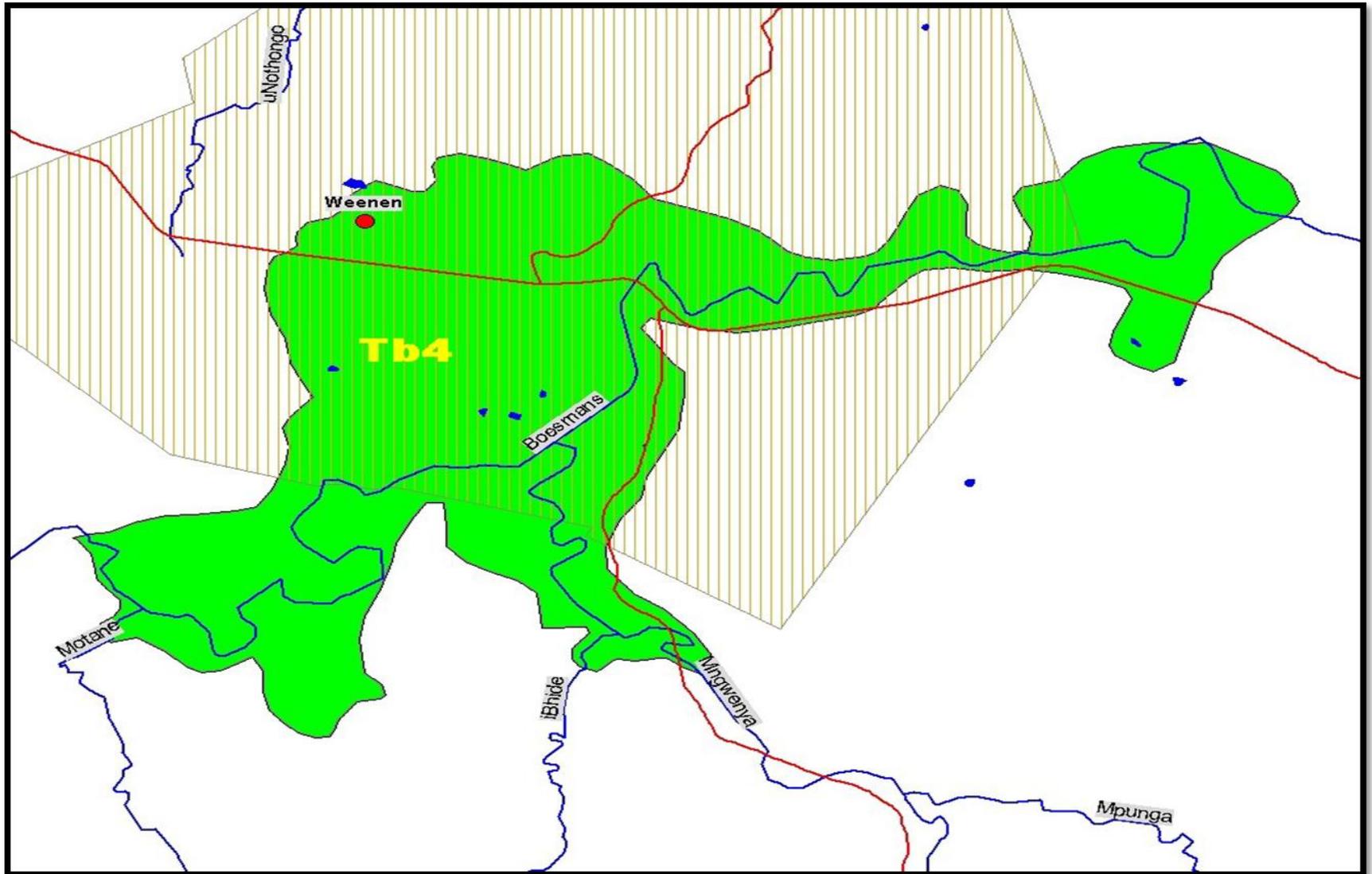


Figure 3. Weenen map indicating the location of Sun Valley Estates (Tb4) (BRU, 2007).

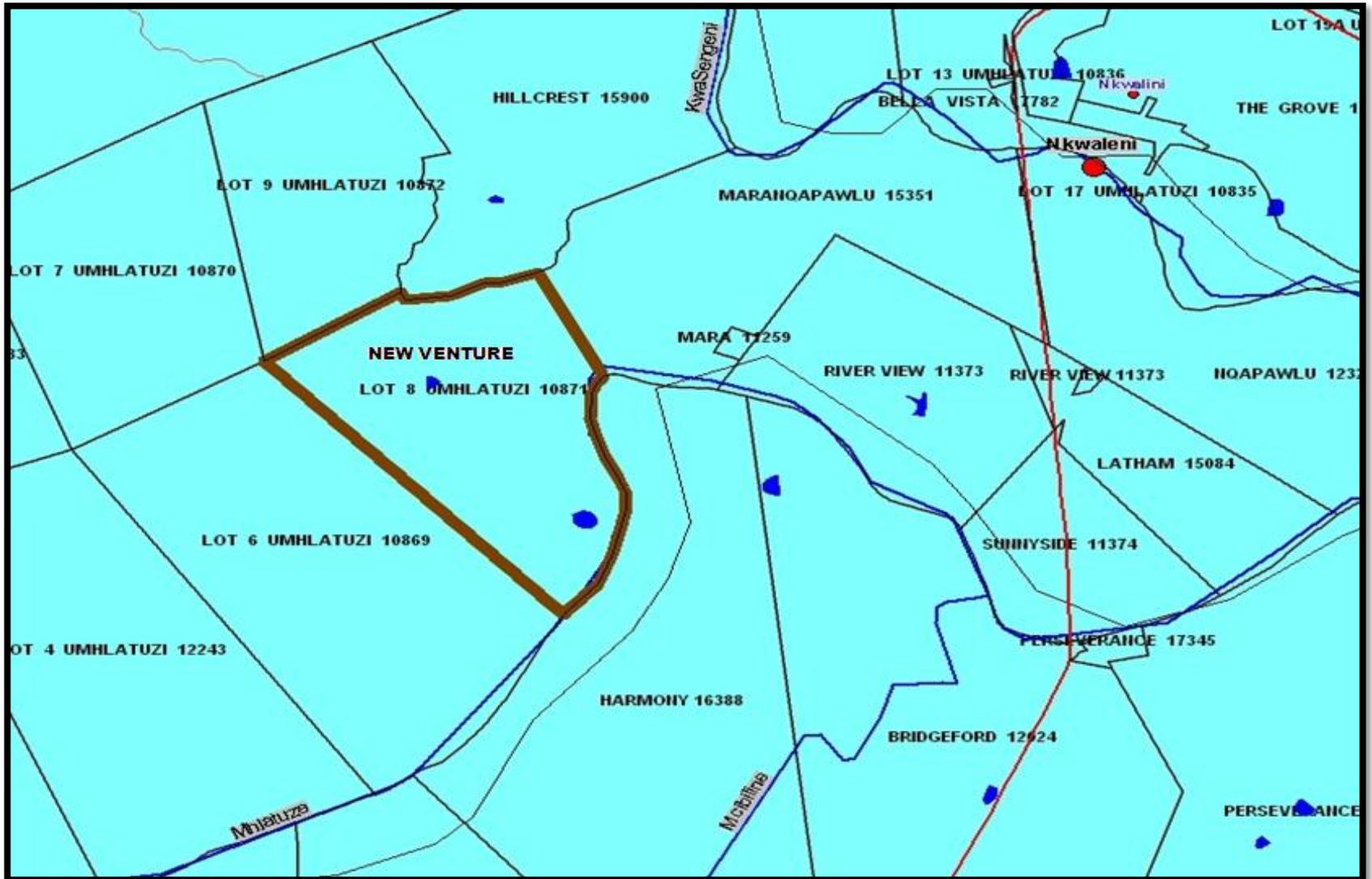


Figure 4. Nkwaleni map indicating the location of New Venture Farm (BRU, 2007).

## **References**

BRU, 2007. Bioresource Unit climate information (other than for mean rainfall) estimated from the South African Atlas of climatology and hydrology. Province of KwaZulu-Natal, Department of Agriculture, Environmental Affairs & Rural Development, Pietermaritzburg.

**Appendix 2: Recommended postharvest treatment with methyl jasmonate (MJ) and salicylic acid (SA), safe storage temperatures and durations in lemon fruit**

Treatments	Storage	Storage duration (days)			
		7	14	21	28
Control	-0.5°C	Safe storage	Medium risk storage	High risk storage	
	2°C	Safe storage	Medium risk storage	High risk storage	
	4.5°C	Safe storage	High risk storage		
10 µM MJ	-0.5°C	Safe storage		Medium risk storage	
	2°C	Safe storage	Medium risk storage		
	4.5°C	Safe storage	Medium risk storage		
2 mM SA	-0.5°C	Safe storage		Medium risk storage	
	2°C	Safe storage	Optional storage	High risk storage	
	4.5°C	Safe storage	Medium risk storage	High risk storage	
10 µM MJ plus 2 mM SA	-0.5°C	Safe storage			
	2°C	Safe storage		Medium risk storage	
	4.5°C	Safe storage	Medium risk storage		