The Cascade of Physiological Events Leading to Chilling Injury: The Effect of Post-harvest Hot Water and Molybdenum Applications to Lemon (*Citrus limon*) Fruit

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DECLARATION

I, **Nhlanhla Mathaba**, declare that the research reported in this thesis, except where otherwise indicated, is an original work. This thesis has not been submitted for any degree or examination at any other university.

Nhlanhla Mathaba

December 2012

We certify that we supervised the student.

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‘A few scholars have put forward the neat and simple proposition that the Old Testament uses mercy while the New Testament speaks of grace

The truth is more elusive, like the words themselves. Think of it this way: Mercy is God withholding the punishment we rightfully deserve. Grace is God not only withholding that punishment but offering the most precious of gifts instead.

Mercy withholds the knife from the heart of Isaac.
Grace provides a ram in the thicket.

Mercy runs forgive the Prodical Son.
Grace throws a party with every extravagance.

Mercy bandages the wounds of the man beaten by the robbers.
Grace covers the cost of his full recovery.

Mercy hears the cry of the thief on the cross.
Grace promises paradise that very day.

Mercy pays the penalty for our sin at the cross.
Grace substitutes the righteousness of Christ for our wickedness.

Mercy converts Paul on the road to Damascus.
Grace calls him to be an apostle.

Mercy saves John Newton from life of rebellion and sin.
Grace makes him a pastor and author of a timeless hymn.

Mercy closes the door to hell.
Grace opens the door to heaven.

Mercy withholds what we have earned.
Grace provides blessing we have not earned.’

‘Jeremiah D., 2006, Captured by grace: No one is beyond the reach of a loving God, Integrity Publishers, INC, Brentwood, TN, USA’

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- To my mom, for unconditional support, the backbone, the strength of my life and wind beneath my wings.

DEDICATION

This thesis is dedicated to a lovely memory of three important people in my life who sadly did not live to see this work, my father Mr A Mathaba, Aunt Nelisiwe Mathaba and my spiritual father Ps J Mkhize for their impact and influence in my life within a short time.

ABSTRACT

New emerging markets such as Japan and the United States require cold sterilisation of South African citrus fruit as a phytosanitary standard against fruit fly. However, citrus fruit are chilling susceptible, with lemons being the second-most chilling susceptible after grapefruit. Chilling injury is a physiological rind disorder; the occurrence of which is despite its prevalence in horticultural commodities, not well understood. Therefore, the aim of this study was to investigate physiological compounds regulating chilling susceptibility or resistance in citrus fruit, with special emphasis on lemons. Furthermore, the potential of hot water dips or “molybdenum soaks” to maintain a certain level of physiological compounds which determine manifestation of chilling injury symptoms in citrus fruit was investigated. Moreover, it was attempted to create an understanding of the order in which physiological compounds mitigate chilling injury.

Lemon fruit from different farms known to be chilling susceptible or resistant were obtained during the 2007 and 2008 harvest season. Thereafter, fruit were treated by soaking for 30 min in 1μM $NaMoO_4.2H_2O$ solution followed by a 2 min HWD 47 or 53°C. Treated fruit were waxed, weighed and stored at -0.5°C for up to 28 days and sampled for chilling injury evaluation 7, 14, 21, or 28 days into cold storage. A second evaluation was carried out five days after withdrawal from cold storage to allow development of chilling injury symptoms as a shelf-life simulation. After the second evaluation fruit were peeled, peel freeze-dried, milled using mortar and pestle and stored at -21°C for further physiological analysis. Freeze-dried peel was analysed for soluble sugars (glucose, fructose, sucrose), vitamin C (ascorbic acid), vitamin E (α-tocopherol), β-carotene, polyamines (putrescine, spermine, spermidine), specific flavanones (naringin and hesperidin) using HPLC-UV-Vis detector and proline, total antioxidant assays (FRAP, ABTS, DPPH), total phenolics, total flavonoids, lipid peroxidation using spectrophotometry, as well as for the heat shock protein (HSP70) using electrophoresis and silver-staining.

Chilling susceptibility of lemon fruit varied with fruit source; those sourced from Ukulinga and Eston Estates were chilling resistant, while fruit from Sun Valley Estates showed chilling injury symptoms after 28 days of cold storage plus five days shelf-life. Furthermore, hot water dips (HW) 53°C, 1 μM Molybdenum (Mo) and 10 μM Mo plus HW 53°C significantly reduced chilling injury symptoms compared with the control and HW 47°C. In addition, Sun Valley Estates fruit also showed higher fruit weight loss compared with non-chilling resistant lemons. The alignment of higher fruit weight loss during storage with chilling susceptibility ascertains the use of weight loss as a non-destructive parameter for chilling susceptibility.

With respect to flavedo sugars, glucose was found to be the dominant soluble sugar with multi-functional roles during cold storage. This plays a significant role in mitigating cellular stress. Chilling susceptible lemons from Sun Valley Estates had low flavedo glucose concentrations and, therefore, little conversion of glucose to ascorbic acid was possible resulting in a low antioxidant capacity. However, treatments with HW 53°C and Mo soaks seemed to enhance the enzymatic conversion of glucose to ascorbic acid leading to a higher antioxidant capacity in the flavedo of such treated fruit. Furthermore, glucose also feeds into the pentose phosphate pathway which is coupled with the shikimate pathway synthesizing secondary metabolites, especially of the phenolics group. The decrease in glucose was aligned to the levels of total phenolics, but not to that of β-carotene, naringin and hesperidin through 28 days into cold storage period. Moreover, as glucose also feeds into shikimate pathway, simultaneously an increase in proline flavedo concentration was observed. Proline is an antioxidant synthesized from glutamate; as cellular glucose decreases so does the total antioxidant capacity during cold storage.

Ascorbic acid is a dominant and potent antioxidant in lemon flavedo as proven with the FRAP, ABTS and DPPH assays. Chilling resistant fruit have significantly higher ascorbic acid conversion. Furthermore, ascorbic acid also acts to generate the α-tocopheroxy radical to further important membrane-bound antioxidant, vitamin E (α-tocopherol equivalent). Furthermore, the DPPH assay was found to be effective in quantifying total antioxidants in lemon flavedo since it detects both lipophilic and hydrophilic antioxidants compared with the ABTS and FRAP assays which are bias to the estimation of liphophilic or hydrophilic antioxidants, respectively. The hot water and molybdenum treatments increased total antioxidants (DPPH assay) with reduced lipid peroxidation 7 days into cold storage and therefore, reduced chilling symptoms in fruit from Sun Valley Estates.

The capacity of antioxidant to scavenge reactive oxygen species (ROS) was increased during cold storage and membrane stability significantly improved. Furthermore, putrescine as low valency polyamine was reduced as such compound acted as precursor to the synthesis of the high valency polyamines, spermine and spermidine. Chilling susceptible lemons from Sun Valley Estates showed increased soluble-conjugated polyamines as a response to stress. Furthermore, HW 53°C, 1 μM Mo and 10 μM Mo plus HW 53°C significantly increased the protein concentration and, therefore, likely also the occurrence of proteins with 70kDa (as estimator of HSP70). Additionally, the concentration of conjugated high valency polyamines was also increased, resulting in reduced chilling injury symptoms.

The effect of ROS has only been viewed as damaging, while recently their role has also been viewed as stress acclamatory signalling compounds when produced concentrations below critical damaging threshold. Therefore, hot water dips seems to signals synthesis of total protein which include HSPs which then act throughout cold stress to protect other protein and channel other damaged proteins towards proteolysis. While molybdenum increased ROS production below damaging critical threshold, with ROS signalling stress acclimation by further signalling production of bioactive compound with antioxidant properties.

TABLE OF CONTENTS

DECLARATION i

ACKNOWLEDGEMENTS iii

DEDICATION iv

ABSTRACT v

TABLE OF CONTENTS viii

CHAPTER 1: Literature Review 1

1.1 GENERAL INTRODUCTION 1

1.2 Cold storage as the phytosanitary standard in fruit industry 5

1.3 Chilling injury as the main rind disorder in citrus fruit 5

1.3.1 Physiological and chemical events leading to the appearance of chilling symptoms 5

1.3.2 Reactive oxygen species as the primary cause of chilling injury 10

1.3.3 Lipid peroxidation 11

1.4. Role of carbohydrates and proline in chilling stress 12

1.4.1 Carbohydrates 12

1.4.2 Proline 13

1.5. Hot water treatments and polyamines: Background, past, present and future 15

1.5.1 Background and physiological effect of hot water treatments in plants 15

1.5.2 Physiological role of polyamines in mitigating plant stress 17

1.6. Involvement of molybdenum in plant metabolism 19

1.6.1 General function of molybdenum in plant metabolism 19

1.6.2 Xanthine dehydrogenase (XDH) as a Mo-cofactor enzyme and its involvement in oxidative stress/signalling 19

1.7. Quantification of antioxidants 21

1.7.1 Total antioxidant assay 21

1.7.2 Bioactive compound with antioxidants properties in citrus 23

1.8 Aim of research 25

1.9 References 27

CHAPTER 2: Research Note – Effect of Hot Water and Molybdenum Dips on Lemon Fruit Weight Loss: a Non-destructive Parameter for Chilling Susceptibility during Cold Storage 38

Abstract 39
1. Introduction 40
2. Materials and Methods 40
3. Results and Discussion 41
4. Conclusions 43
5. Reference: 44
List of Figures 46
List of Tables 46

CHAPTER 3: Effect of Postharvest Hot Water and Molybdenum Dips on Soluble Sugars, Glucose-Ascorbic Relationship and Proline Accumulation in Lemon Rind during Cold Storage 49

SUMMARY 50
Introduction 51
Materials and Methods 52
Chemicals 52
Plant materials 53
Estimation of chilling injury 53
Soluble sugar extraction and HPLC determination 53
Determination of ascorbic acid 54
Determination of proline 54
Statistical analysis 55
RESULTS 55
Effect of HW and Mo dips on chilling injury symptoms 55
Effect of fruit source on pre and post-harvest soluble sugars, ascorbic acid and proline on chilling susceptibility 56
Principal component analysis 56
Effect of cold storage time on reducing sugars, and the glucose-ascorbic acid and glucose-proline relationship during cold storage 57
Effect of HW and Mo dips on lemon flavedo glucose-ascorbic acid, glucose-proline, and reducing sugar relationships during cold storage 57
Discussion 58
Acknowledgements 61
REFERENCES 61

List of Tables 68
List of Figures 68

CHAPTER 4: Hot Water and Molybdenum Dips: The Case of Antioxidant Assays in Lemon Flavedo during Cold Storage 77
Abstract 78
1. Introduction 79
2. Materials and Methods 81
3. Results 85
4. Discussion 88
References 92
List of tables 98
List of figures 98

Chapter 5: Effect of Hot Water and Molybdenum Dips on Bioactive Compounds with Antioxidant Properties in Lemon Flavedo during Cold Storage 105
Abstract 106
1. Introduction 106
2. Materials and Methods 108
3. Results 111
4. Discussion 115
5. Conclusions 118
Acknowledgements 119
References 119
List of Tables 124
List of Figures 124

CHAPTER 6: Effect of Hot Water and Molybdenum Dips on Endogenous Polyamines and 70 kDa Protein (estimator of HSP70) in Lemon Flavedo and their Ability to Alleviate Chilling Injury during Cold Storage 132
Abstract 133
1. Introduction 134
2. Methods and Material 136
2.1 Plant material 136
3. Results 138

4. Discussion 141
5. Conclusion 143
List of Tables 148
List of Figures 148

CHAPTER 7: A Holistic View: General discussion, conclusion and outlook 158
Future research and commercial implications 160
References: 163

CHAPTER 1

Literature Review

1.1 GENERAL INTRODUCTION

The South African citrus industry is the largest fruit industry in the country in terms of volumes and the second agricultural industry in terms of value after the deciduous fruit industry (National Agricultural Directory 2009/2010) and currently ranks second (2009) amongst global citrus exporters (Table 1, CRI, 2009). In 2004/2005 it earned about two billion Rands, approximately 4.5% of the total Agricultural Gross Value Production (National Agricultural Directory 2009/2010). The South African citrus industry is a lucrative fruit industry, owing to be mainly export-orientated. In 2009, 64% of the total production went into export, 28% into local markets and 8% into the processing sector (Fig 1A, Key Industry Statistics, 2009). The leading export cultivars are 'Valencia' oranges – 44%, 'Navels' oranges – 22.2%, grapefruit – 16.0%, lemons – 9.9% and soft citrus – 7.8% (Fig 1B, CRI, 2009). The industry has its traditional major importing countries; however, Japan and the USA have been the new growing and high profitable markets (Fig 1C). In 2009, Japan and USA imported 6.5 and 2.8% of the total production, respectively (Fig 1C, CRI, 2009). The industry encounters some challenges in maintaining and continuing the pursuit of such high paying markets.

Competition from other exporters (Table 1) is a constant threat to the South African citrus industry. New emerging high paying markets (Japan and USA) further require subjecting high quality fruit to cold sterilization to comply with phytosanitary standards to avoid spreading of undesirable insect pests (National Directory 2004/5). Cold sterilization has been found to be an effective measure to avoid pests' movement from exporting to importing countries, as it eradicates eggs as well as larvae (Underhill et al., 1999). Furthermore, cold sterilization has the potential of replacing chemical fumigation, an added advantage as chemical residues are often regarded as dangerous to consumers and the environment (Jacobi et al., 2001). Even though cold sterilization is a chemical-free method to meet phytosanitary standards for these new emerging markets, it comes with some disadvantages that can reduce marketability and quality of fruit.

Table 1: World fresh citrus exports (thousand tons) from 2001-2009

Country	01/02	02/03	03/04	04/05	05/06	06/07	07/08	08/09	09/10
Spain	3,142	3,313	3,564	3,117	3,238	3,627	3,036	3,300	3,083
South Africa	**981**	**1,043**	**1,049**	**1,080**	**1,213**	**1,290**	**1,323**	**1,346**	**1,485**
Turkey	826	740	607	877	948	947	725	990	1,103
USA	1,018	1,117	1,136	919	930	1,068	1,072	863	1,065
Egypt	303	414	491	605	650	790	560	794	989
Morocco	430	484	438	490	543	583	582	652	924
Argentina	421	486	548	642	580	539	537	531	501
China	184	241	344	386	380	500	720	530	478
Mexico	225	347	399	413	399	414	433	491	461
Greece	317	398	303	235	337	289	259	280	365

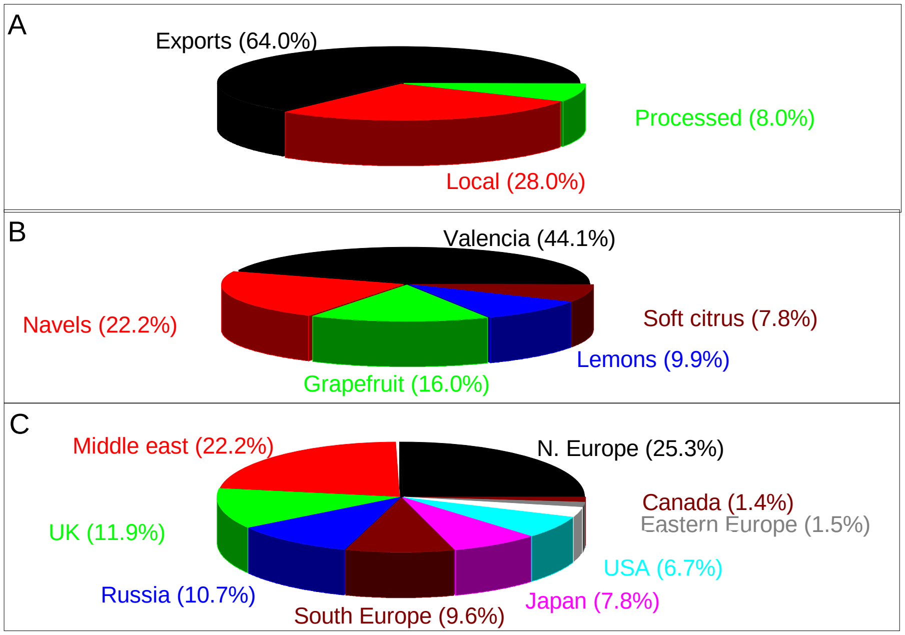


Figure 1: A. Percentage production sector volumes, B. Export products, C. Major export destinations during the 2009 harvest season (CRI, 2009)

Citrus fruit quality is based one internal parameters (unique flavour, nutritional value, juiciness, TSS:Acid ratio) as well as external parameters including, good rind colour and no manifestation of post-harvest diseases resulting in extended shelf-life (Ladaniya, 2008). While, internal quality parameters, such as nutritional value, juice percentage, TSS:Acid ratio can improve due to post-harvest low temperature storage because of reduced metabolic rate, the external quality declines as physiological disorders manifest mainly chilling injury (Ladaniya

2008). Chilling injury is a flavedo (Fig 2) disorder which manifests as sunken lesions and discolouration of the peel due to permanent irreversible damage to plant cells as a result of exposing plant parts to temperatures below critical threshold temperatures (Donkin, 1995). Appearance is a major marketing tool in citrus fruit compared with taste; appearance of chilling damage, therefore, severely reduces marketability of citrus fruit (McLaunchlan et al., 1997).

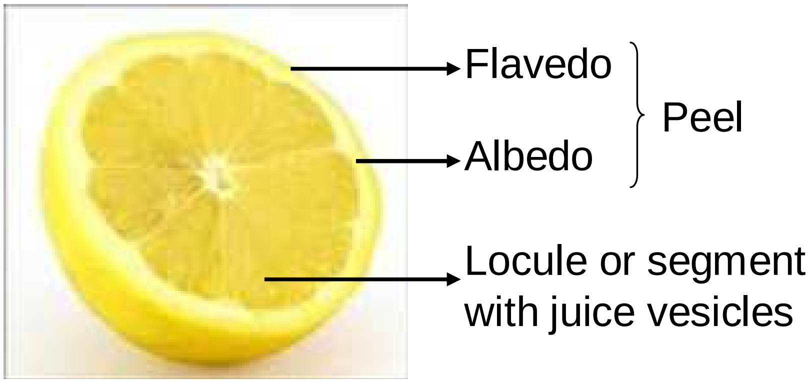


Figure 2: Transverse section of a lemon

Physiologically, the appearance of chilling symptoms is a multifactorial problem with several cascades of cellular events. Several authors have associated resistance of citrus fruit to chilling injury with higher soluble flavedo sugars, especially glucose, fructose and sucrose (Aung et al., 1998; 1999; 2001; Ryan and Aung, 1999). However, other researchers have argued that chilling injury is a result of the imbalance between antioxidants and reactive oxygen species (ROS), with plant cells producing higher amount of ROS during exposure to cold stress than can be dissipated by the antioxidants present (Huang et al., 2007; Xu et al., 2008). If ROS are in excess, oxidative stress is inevitable and susbsquently chilling symptoms develops. The antioxidant potential of a certain tissue can be estimated as the total antioxidant capacity, and the potential of enzymatic and non-enzymatic (bioactive compounds) antioxidant system to scavenge ROS (Re et al., 2008). Furthermore, the latter antioxidants can be divided into water-soluble (hydrophibic) and lipid-soluble (lipophilic) antioxidants (Huang et al., 2007).

The antioxidant capacity of non-enzymatic antioxidants has been shown to increase by polyamines (PA's) application (Kuznetsov and Sheyakova, 2007). Polyamines are positively charged compounds at normal physiological cell pH and, therefore, can react with any negatively charged cellular compound, such as proteins, membrane phospholipids, DNA and non-enzymatic antioxidants (Kuznetsov and Sheyakova, 2007; Königshofer and Lechner, 2002). Conjugation to these compounds improves the resistance of such cell component to ROS damage by enhancing the antioxidant scavenging capacity and, hence, reduces membrane damage (Kuznetsov and Sheyakova, 2007). This ultimately leads to improved chilling resistance. Methods to predict chilling injury and mitigate its appearance are essential for the South African citrus industry not to incur losses due to unmarketable fruit.

Hot water dips (HWD) or intermittent warming are established non-chemical methods to mitigate chilling stress in various fruit commodities. Wang (1995) defined intermittent warming as the prestorage warming of fruit and vegetables in order to increase chilling resistance. The effect of intermittent warming is based on the enhancement and activation of heat shock proteins (HSPs), compounds protecting other proteins during stress exposure (Vierling, 1999; Lindquist, 1986). Previous reports on cold-stored grapefruit (Rozenzvieg et al., 2004), and tomato fruit (Sabehat et al., 1996) have confirmed stimulation of HSPs following cold-exposure that resulted in increased stress resistance. It has been concluded that HSPs play a significant role in basic plant metabolism with short-time heat stress up-regulating most HSPs (Vierling, 1999; Lindquist, 1986).

Certain chemical formulations containing essential micro-elements or plant hormones have been reported to enhance the chilling resistance of fruit and vegetables when applied as post-harvest dips. Post-harvest exogenous application of methyl jasmonates (MJ) to peaches (Budde et al., 2005) and zucchini squash (Wang, 1994) enhance the concentration of antioxidant compounds during cold storage and therefore chilling resistance (Wang, 1994). Salicylic acid (SA) combined with calcium dips on peaches and strawberries mitigate the appearance of internal browning during cold storage (Wang et al., 2006; Shafiee et al., 2010). Furthermore, post-harvest exogenous application of polyamines, particularly putrescine has shown potential to alleviate the appearance of chilling symptoms on cold stored plums (Valero et al., 2002; and bell peppers (González-Aguilar, 2000). Recently, molybdenum has been found to also reduce the appearance of chilling symptoms in cold-stored lemon fruit (Mathaba et al., 2008).

There is a need to further investigate the ability of HW and molybdenum dips to mitigate chilling injury appearance and to identify the roles played by these post-harvest treatments. The possible function of lemon rind sugars, the increase of different antioxidants by HW and Mo to reduce membrane damage, and increase the concentration of other secondary metabolites, such as polyamines in the flavedo. Finally the interaction of these sugars and such metabolites within the pentose phosphate pathway to reduce oxidative stress requires investigation.

1.2 Cold storage as the phytosanitary standard in fruit industry

In 1951 the global fresh produce community, under supervision of Food and Agricultural Organization (FAO) established the International Plant Protection Convention (IPPC). This treaty was signed to a common understanding on the prevention and to continuously monitor the movement of pest/diseases during international trading of any fresh produce (Ladaniya, 2008). The agreement binds the importing/exporting countries to have professional inspectors at ports to approve and certify (phytosanitary certificate) exportable plant material as free from pest and diseases. For the citrus industry, important diseases and insect pests that need to be control are citrus canker (Ladaniya, 2008) and fruit fly (Ladaniya, 2008; Underhill et al., 1999; McLaunchlan et al., 1997). The South African citrus has to treat fruit for fruit fly to be able to export to new, emerging, high profitable markets such as Japan and the US. However, Japan banned chemical fumigation against fruit fly and requires exporting countries to cold-treat citrus fruit at -0.6°C for 12-20 days (Kitagawa et al., 1988), depending on the type of citrus fruit imported (Ladaniya, 2008 Schirra et al., 2004; Houck et al., 1990). These cold treatments have been proven to kill larvae or eggs of fruit fly when fruit are exposed to temperature close to 0°C (Ladaniya, 2008). The requirements are, however, potentially harmful to the fruit, because citrus, as a subtropical fruit is susceptible to chilling injury when exposed to cold storage (Erkan et al., 2005; Porat et al., 2002; Ezze et al., 2004). It is therefore important for the South African citrus industry to develop non-chemical methods to predict and mitigate the appearance of chilling symptoms in order to maintain supply to the Japanese and US markets.

1.3 Chilling injury as the main rind disorder in citrus fruit

1.3.1 Physiological and chemical events leading to the appearance of chilling symptoms

The susceptibility of citrus fruit to chilling conditions varies with cultivar (Table 2), climatic conditions, fruit morphology, flavedo chemical composition (Sinclair, 1984), fruit colour and maturity (Bower et al., 1997) as well as the position on the tree (inner or outer canopy) (Cronje et al., 2011). Furthermore, variation in chilling susceptibility has been correlated with cold storage temperature, duration of cold storage and relative humidity, during cold storage (Table 2).

Within the citrus family, lemons are the second most susceptible citrus type after grapefruit, while sweet oranges are the most chilling resistance citrus type (Table 2) (Ladayina, 2008; Chalutz et al., 1985). The South Africa citrus industry mainly produces the lemon cultivar 'Eureka' which is chilling-susceptible when exposed to cold storage temperatures of 14.5-15.5°C cold storage. It is common packhouse practice is to keep 'Eureka' lemons at 0-4.5°C for short-term storage and at 14.5°C for prolonged cold storage; because any temperature less than 14.5°C results in the development of chilling symptoms, such as surface pitting, membrane staining, and red blotch (Ladayina, 2008).

The physiological and chemical events to chilling injury of chilling-sensitive tissues have been modelled by Lyons (1976, Fig 3). However, Raison and Orr (1990) simplified the model and made clear divisions between primary events, which are reversible and irreversible secondary events, leading to cell death (Fig 4). According to Lyons (1976) plant tissue subjected to chilling conditions undergoes physical cell membrane phase transition from a normal flexible, liquid-crystalline to a solid gel structure (Fig 3). When membranes solidify, membrane fluidity changes and membrane lipids undergo phase transition which results in the configuration of certain essential membrane-bound proteins and enzymes. Finally, depletion of activation energy results in limiting the reaction time of key enzymes (Wolfe, 1978).

A continuous chilling temperature causes membrane lipids to solidify leading to a contraction of membrane which subsequently cracks, resulting in increased cell permeability (Fig 3) (Lyons, 1976). Change in the membrane bilayer due to extended chilling temperature is associated with increased ion movement and water molecules through the membrane. This results in increased membrane permeability which manifests itself as solute leakage (Fig 3). This leakage is aligned to the loss of cellular integrity leading to irreversible secondary chilling events (Raison and Orr, 1990) (Fig 4).

The solute leakage, determined as electrolyte leakage, has been associated with fruit weight loss and can be used a non-destructive indicator of potential chilling injury symptoms (Cohen et al., 1994). Studies investigating fruit weight loss as a chilling injury parameter were carried out on grapefruit, lemons (Cohen et al., 1994), in 'Washington Navel' oranges (Erkan and Pekmezci, 2000), in Satsuma fruit (Jemric et al., 2003). These investigations have shown that fruit weight loss during cold storage correlates positively with the appearance of chilling symptoms.

Table 2: Recommended cold storage temperature, relative humidity, and cold storage time for different citrus fruit (Ladaniya, 2008).

Citrus fruit	Temperature (°C)	Relative humidity (%)	Storage time (weeks)
(1) Sweet oranges			
Baladi orange (Egypt)	2-3	85-90	8
Blood Red	5-7	85-90	8-10
Valencia (California and Arizona)	3-9	85-90	4-8
Valencia (Florida and Texas)	1-2	85-90	8-12
Valencia, Navel (Spain)	2-3	85-90	4-8
Washington Navel (California)	5-7	85-90	6
Shamouti	5-6	85-90	8-12
Malta, Mosambi	5-7	90-95	12
Sathgudi	5-7	90-95	12
(2) Mandarins, Tangerines and hybrids			
Ponkan	4-6	85-90	4-5
Satsuma	2-3	80-85	12-18
Clementine	3-4	85-90	4-6
Clementine (Spain, Lee, Ellendale, Murcott)	4-5	85-90	4
‘Nagpur’	6-7	85-90	6-8
‘Coorg’ (main crop)	5-6	85-90	6-8
Temple, Orlando, Dancy	4-6	90-95	2-5
Kinnaw mandarin	3-4	85-90	8-12
(3) Lemons			
Dark green	13-14	85-90	16-24
Light green	13-14	85-90	8-16
Bright Yellow	10-12	85-90	3-4
Sicilian	7-8	85-90	8
Australian	10	85-90	24
California	12.5-15	85-90	8-12
(4) Limes			
Tahiti	9-10	90-95	6-8
‘Kagzi’, Mexican	9-10	90-92	10-12
Dark green	8-9	90-92	8-12
Yellow	8-9	90-92	8-12
(5) Grapefruit	10-14	90	6-8
Texas and Florida grapefruit	10	90-92	4
California and Arizona grapefruit	14-15	90	5-6
Foster, Ruby, Saharanpur Special	9-10	90	16-20
(6) Pummelo	8-9	85-90	10-12

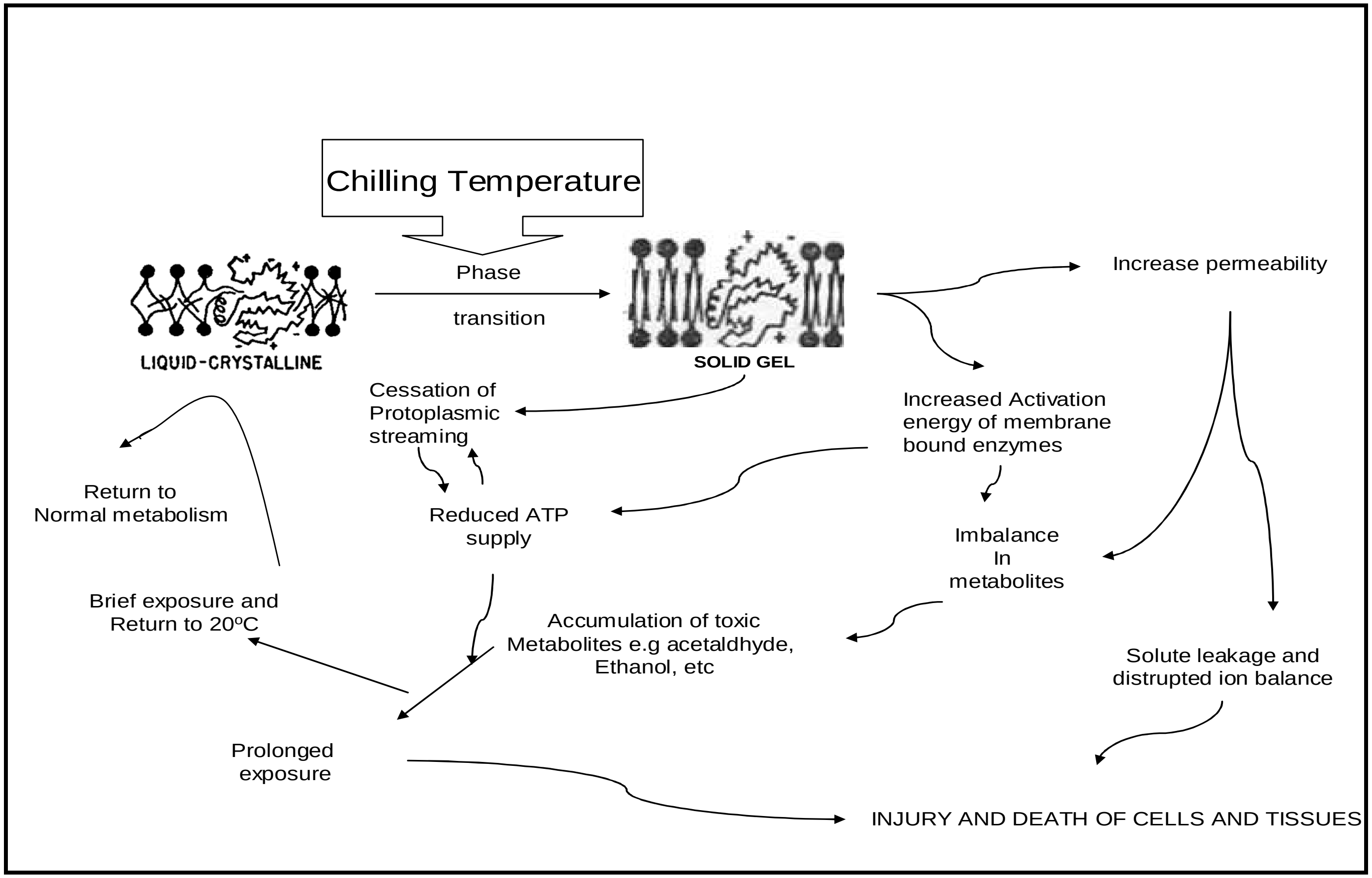

Chilling Temperature
Phase
transition
LIQUID-CRYSTALLINE
SOLID GEL
Increase permeability
Increased Activation energy of membrane bound enzymes
Cessation of Protoplasmic streaming
Return to Normal metabolism
Reduced ATP supply
Imbalance In metabolites
Brief exposure and Return to 20ºC
Accumulation of toxic Metabolites e.g acetaldhyde, Ethanol, etc
Solute leakage and distrupted ion balance
Prolonged exposure
INJURY AND DEATH OF CELLS AND TISSUES

Figure 3: Schematic representation of cellular events associated with chilling injury in plant cells sensitive to chilling temperatures (Lyons, 1976).

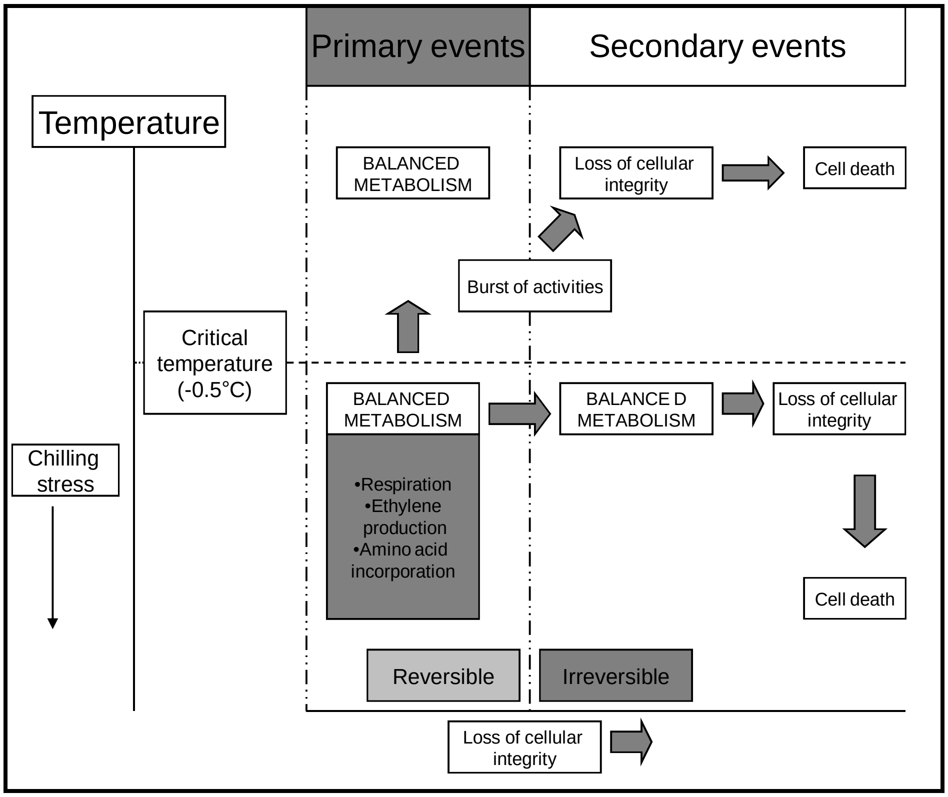

Primary events
Secondary events
Temperature
BALANCED METABOLISM
Loss of cellular integrity
Cell death
Burst of activities
Critical temperature (-0.5°C)
BALANCED METABOLISM
BALANCE D METABOLISM
Loss of cellular integrity
Chilling stress
•Respiration
•Ethylene production
•Amino acid incorporation
Cell death
Reversible
Irreversible
Loss of cellular integrity

Figure 4: Modified schematic presentation of cellular events during exposure to chilling stress (Raison and Orr, 1990)

Furthermore, increased cell membrane permeability also affects other reactions within the cell, such as the reduction in activation energy of membrane-bound enzymes. This may lead to a suppressed reaction rate and imbalances in non-membrane bound enzymes activity. These metabolic imbalances or dysfunctions result in the accumulation of pyruvate, acetaldehyde and ethanol (Lyons, 1976). Raison and Orr (1990) classified such metabolic dysfunction as primary events of chilling injury. Ultimately, the extended accumulation of dysfunction metabolites leads to secondary events and cell death, an indication that plant cells can not withstand the accumulation of such secondary metabolites above a critical threshold level. The critical threshold level is cultivar and species dependent. Cultivars such as ‘Fortune’ and ‘Nova’ mandarins and, in general, lemons and grapefruit, are very prone to chilling injury (Lafuente et al., 1997).

Cessation of protoplasmic streaming is the final process contributing to cell death under cold exposure; it refers to the reduced movement of fluid substances within the cell cytoplasm. Ceasing of cellular activities, like protoplasmic streaming, will negatively affect transport of nutrients and proteins within the cell. In addition, reduction in supplied energy (ATP) is a primary cause for the cessation of protoplasmic streaming because of the significantly reduced energy supply in chilling sensitive plant tissues (Lyons, 1976).

1.3.2 Reactive oxygen species as the primary cause of chilling injury

About 1% of oxygen consumed by plants is, normally, converted to ROS (Bhattacharjee, 2005). Under biotic or abiot stress, however, this conversion percentage is increased (Alscher et al., 1997). When the production of ROS exceeds a critical threshold level, oxidative damage to membrane lipids, DNA, proteins, enzymes and amino acids occurs (Huang et al., 2007). Chilling damage is as result of oxidative damage by various ROS, including the superoxide, perhydroxy, hydrogen peroxide, alkoxy radical, peroxy radical, organic hydroperoxide, singlet oxygen, excited carbonyl (Bhattacharjee, 2005).

Non-harmful oxygen is relatively stable at the triplet state with two unpaired electrons and, therefore, restricted of reacting with other molecules because of slow kinetics and therefore does not cause damage (Krieger-Liszkay, 2004). In the presence of an electron transfer reaction, oxygen can produce a reactive intermediate having the potential to damage cell walls, DNA, proteins and enzymes (Halliwell and Gutteridge, 1998). A highly reactive singlet oxygen species is a produce of energy addition because energy remove spin restriction and spontaneously increase the oxidizing and damaging potential of oxygen (Krieger-Liszkay, 2004).

However, plants have evolved antioxidant defence systems against ROS damage (Alscher et al., 1997; Yesbergenova et al., 2007) during normal and during stress metabolism. Antioxidants are molecular compounds which maintain ROS levels below a certain critical threshold level, thereby reducing oxidative stress, which can result in chilling injury.

1.3.3 Lipid peroxidation

Besides membrane deterioration, oxidation of amino acid side chains, oxidation of polypeptide backbones resulting from protein fragmentation and DNA strand break-down also lipid peroxidation is an important result of ROS damage to cellular micromolecules (Tomizawa et al., 2005). Lipid peroxidation refers to oxidative damage caused mainly by ROS to membrane lipids, resulting in a toxic product quantified as malondialdehyde (MDA), a secondary end-product of polyunsaturated fatty acid oxidation (Imahori et al., 2008; Eraslan et al., 2007). Malondialdehyde is often used to as an index of oxidative damage. Its accumulation results in multiple cellular dysfunctions such as intercrossed and conjugated protein structures, membrane permeability increase, electrolyte leakage and finally cell death (Leng and Qi, 2003). Furthermore, Bhattacharjee (2005) proposed lipid peroxidation to be mechanically involved in free radical production, initiated by ROS reacting with membrane lipids.

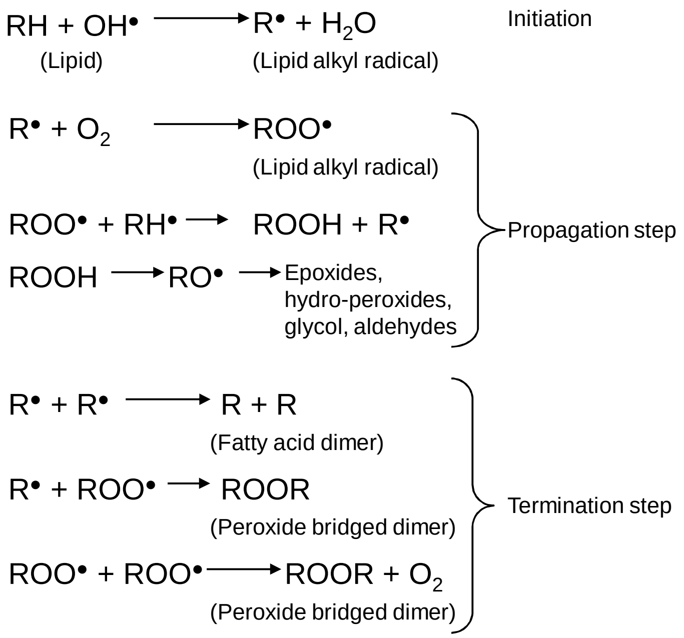

RH + OH• → R• + H2O
(Lipid)
(Lipid alkyl radical)
Initiation
R• + O2 → ROO•
(Lipid alkyl radical)
ROO• + RH• → ROOH + R•
ROOH → RO• → Epoxides, hydro-peroxides, glycol, aldehydes
Propagation step
R• + R• → R + R
(Fatty acid dimer)
R• + ROO• → ROOR
(Peroxide bridged dimer)
ROO• + ROO• → ROOR + O2
(Peroxide bridged dimer)
Termination step

Figure 5: Three step mechanism of membrane lipid peroxidation as a 3-step mechanism to generation of free radicals in plant cells (Bhattacharjee, 2005)

1.4. Role of carbohydrates and proline in chilling stress

1.4.1 Carbohydrates

Carbohydrates are molecular compounds made up of carbon, hydrogen and oxygen and following the formula $(CH_2O)n$, where n ≥ 3 (Duff and Duff, 1984), these compounds play a major role in citrus fruit development and post-harvest maintenance (Ladaniya, 2008). The appealing properties of citrus fruit, such as colour, texture, and flavour are accounted to a high concentration of free-state or derivatives of carbohydrate (Ladaniya, 2008). Carbohydrates can be classified as simple (monosaccharide sugars) or more complex carbohydrates (oligosaccharides sugars) and polysaccaride sugars (Duff and Duff, 1984; Ladaniya, 2008).

D-Glucose and *D*-fructose (Fig 6 a and b) are the dominant monosaccharide sugars in citrus (Ladaniya, 2008). The combination of these two monosaccharides form a reducing disaccharide sugar, which is the main translocatable sugar in plants and therefore, also in citrus (Fig 6 c and d) (Ladaniya, 2008). Carbohydrate metabolism has been linked with plant stress responses (Tognetti, et al., 1990; King, et al., 1988), specifically in citrus flavedo. Holland et al. (2002) found sucrose to be involved in chilling resistance of 'Fortune' mandarin fruit stored at 2°C for up to 28 days and in 'Fortune' mandarin harvested over different years and seasons (Holland et al., 1999). There are several proposed mechanisms which aim to explain the involvement of carbohydrates in cold stress resistance in plants.

Sugars can reduce the freezing point of cell sap and therefore the appearance of freezing injury in grape berries and lettuce leaves. This is achieved by changing the osmotic potential and thereby depressing the solute freezing point of the cytoplasm (Wills et al., 2008). Crowe et al. (1998) further clarify that an increased cellular osmotic potential allows water movement into the cell, hence reducing dehydration of cell membranes under chilling or freezing temperature. In addition, sugars have proven to maintain labile membrane protein and cell integrity.

Furthermore, as the cellular osmotic potential is increased by high sugars, cold stress is mitigated through the replacement of polar water residue in the membrane and proteins resulting in a stabilization of the membrane and protein and therefore reducing the temperature for protein denaturation (Back et al., 1979; Crowe et al., 1998). The formation of a metastable 'glassy' state by high cellular sugar was proposed by Roos, (1993). Sugars can enhance stress resistance, and when in a 'glass' state, armophous carbohydrates can act as thermoplastics and improve membrane, stabilizing protein and other compounds (Roos, 1993). Finally, sugars especially glucose, are involved in the production of proline and other secondary metabolites as mechanisms to mitigate stress (Shetty, 2004; Ashraf and Foolad, 2007).

1.4.2 Proline

Proline is an amino acid which is known to be associated with plant stress response. It typically accumulates in cells of stressed plants (Ashraf and Foolad, 2007). The multiple metabolic functions of proline are similar to sugars; however, this amino acid also possesses antioxidant properties (Shetty, 2004). A further major cellular function of proline is its osmolyte property (Ozden et al., 2009). Osmolytes regulate water channeling into plant cells under external stress to maintain normal cell volume (Wehner et al., 2003). During plant stress proline can enhance protein and membrane stability. As an osmolyte proline must be having properties similar to sugars replacing the polar water residue of the membrane phospholipids (Crowe et al., 1998). Proline can also interact with peptide bond of proteins to maintain their structure and stability during stress exposure (Harries and Rösgen, 2008).

During proline synthesis cellular cytoplasmic pH is significantly reduced leading to redox imbalances when plant cells are exposed to stress (Shetty, 2004). Proline is synthesized from glutamate, $NADPH_2$ is recycled (Fig 6) while any proton ions from phytochemicals, microbial elicitors or acid itself are generated (redox recycle). This stimulates the pentose phosphate pathway and normalizes the cytosol pH or buffering cellular redox potential (Shetty, 2004; Ashraf and Foolad, 2007).

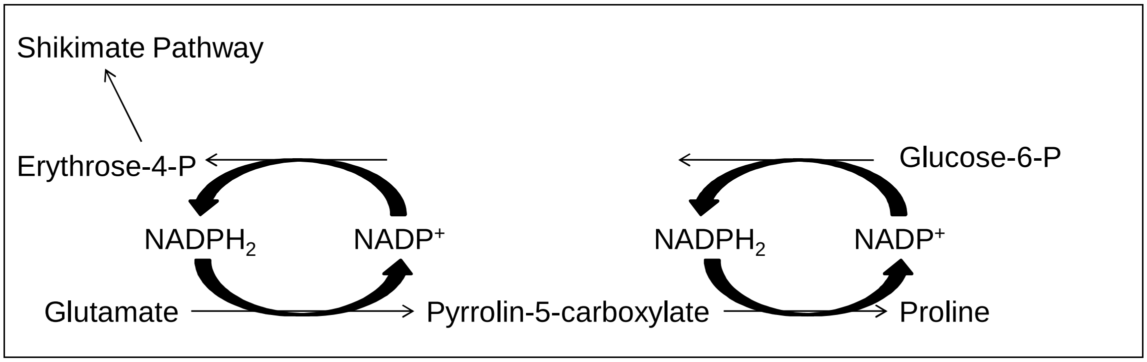
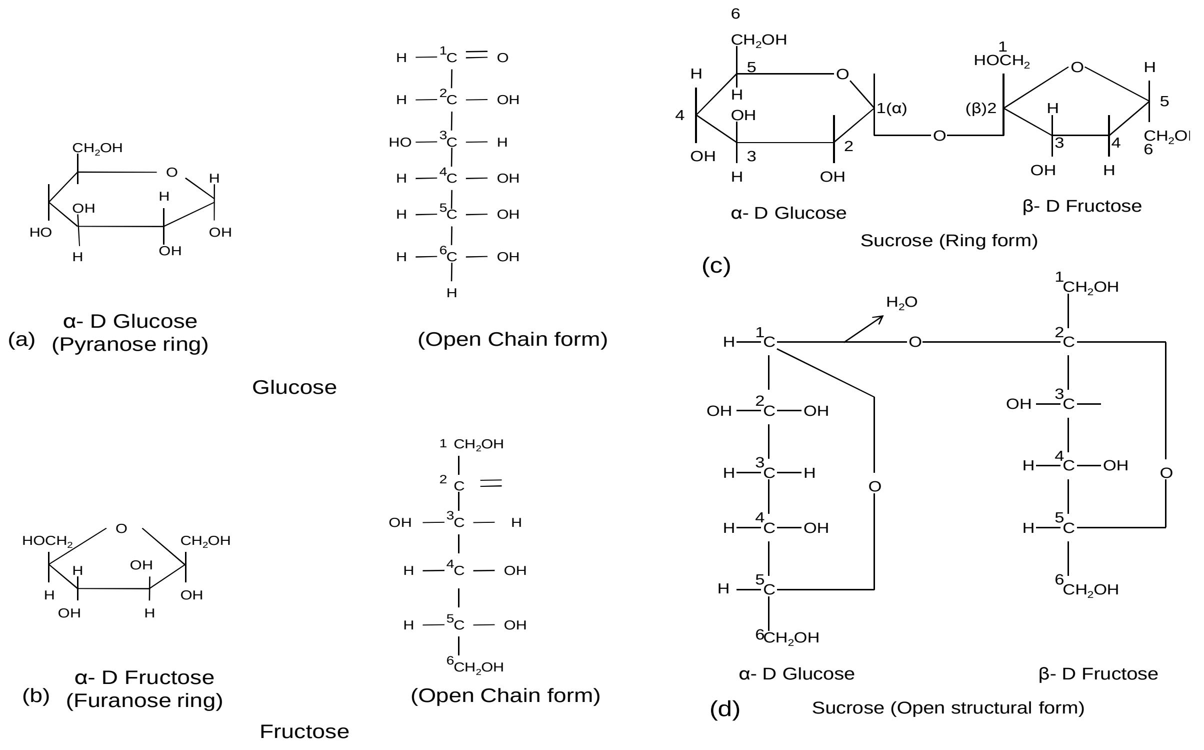


Figure 6: Biosynthesis of proline from glutamate in plant cells (Zheng et al., 2001)



α- D Glucose
(a)
(Pyranose ring)
(Open Chain form)
Glucose
α- D Fructose
(b)
(Furanose ring)
(Open Chain form)
Fructose
α- D Glucose
β- D Fructose
Sucrose (Ring form)
(c)
1(α)
(β)2
H_2O
α- D Glucose
β- D Fructose
(d)
Sucrose (Open structural form)

Figure 7: Chemical structure of sugars, a) close and open chain structure of glucose, b) close and open structure of fructose, c) close structure of sucrose and d) open chemical structure of sucrose

Moreover, production of erythrose in the pentose phosphate pathway links proline with the shikimate acid pathway. Proline stimulates the commitment of pantose phosphate pathway into phenolics synthesis. According to Shetty (2004), proline linked pentose phosphate pathway is involved in the synthesis of phenolics phytochemicals by stimulating the shikimate and phenylpropanoid pathway (Ashraf and Foolad, 2007; Zheng et al., 2001), thereby increasing the cellular antioxidant capacity during stress exposure. The study on such a multi-functional biological compound is essential in understand plant's response to cold stress.

1.5. Hot water treatments and polyamines: Background, past, present and future

1.5.1 Background and physiological effect of hot water treatments in plants

There is a growing concern about the negative effect of agro-chemicals on the environment and human health; therefore, consumers demand non-chemical methods to be used during production and post-harvest management of agricultural produce (Erkan et al., 2005; Klein and Lurie, 1992). Hot water treatments have shown potential to fulfil the requirements of a non-chemical method to mitigate post-harvest disorders of both pathological and physiological in nature for fruit and vegetables (Klein and Lurie, 1992). Different forms of heat treatments exist depending on the commodity and the aim of such treatment (either disease or physiological disorder) (Table 3). Originally, hot water was used to control insects and fungal disinfestations; vapour heat treatment was specifically used for insect control (Erkan et al., 2005). Furthermore, heat treatments have been used as post-harvest treatments to inducing chilling-resistance in tomato fruit stored at 2°C (Sabehat et al., 1996) and protection against pathogens in different horticultural produce (Klein and Lurie, 1992).

The efficacy of hot water treatments to mitigate chilling injury depends on the sensitivity of the species and the duration of heat application. Heat damage might occur due to duration-species incompatibility. It has been proven that heat treatment activates heat shock proteins (HSPs), molecular chaperones that assist in protein folding, assembly, and transportation; these proteins also re-direct damaged proteins towards proteolysis (Rozenzvieg et al., 2004). There is a diverse group of HSPs in plants ranging in molecular weight from 15 to 115 kDa. Previous research on 'Star Ruby' grapefruit found that hot water dips at 62°C for 2 min enhance the production of four speciefic HSPs. The production of these significantly contributes to reduced chilling symptoms compared with untreated fruit. The HSPs isolated and identified ranged in molecular weight from 18 to 70kDa (HSP18-I, HSP18-II, HSP22 and HSP70) (Rozenzvieg et al., 2004).

Table 3: Effect of different hot water treatments, optimal temperature and aim of heat treatment on different citrus cultivars (Fallik 2004).

Crop	Treatment	Optimal temperature °C (time)	Aim
Clementine	HWT	45 (2.5 min)	Decay control
Grapefruit	HWRB	59-62 (20 s)	Decay control, chilling injury and decay resistance, better quality
Kumquat	HWRB	58 (20 s)	Decay control, better quality
Lemon	HWT	52-53 (2 min)	Decay control, decay resistance
Lemon	HWRB	62.8 (15 s)	Decay control, quality maintenance
Mandarin (cv. Fortune)	HWT	50-54 (3 min)	Decay control
Orange (cv. Shamouti)	HWRB	56 (20 s)	Decay control, better quality
Orange (cv. Tarocco)	HWRB	62.8 (15 s)	Decay control
	HWT	53 (3 min)	Decay control, chilling injury
Tangerine (cv. Minneola)	HWRB	56 (20 s)	Decay control

HWT-Hot water treatment/dips

HWRB-Hot water rinsing plus brushing

Heat shock proteins, according to Vierling (1991), are classified according to their approximate molecular weight in kDa; HSP110, HSP90, HSP70, HSP60 are classified high molecular weight while HSPs less than 60 kDa are low molecular weight HSPs. Heat treatment of tomato fruit (cv. Daniella) was found to stimulate expression of several proteins with a molecular mass between 60 to 70 kDa; these proteins were not found in untreated tomato fruit (Sabehat et al., 1996). In addition, HSPs of a molecular weight between 60 to 70 kDa were dominant in grapefruit treated with hot water brushing (HWB) at 62°C for 20 s; such treatment enhances chilling resistance (Rozenzvieg et al., 2004) and disease resistance (Pavoncello et al., 2001). However, such a reduction in physiological disorders may not be solely dependent on the presence of HSPs and their action (Lurie, 1976), other compounds are likey to also contribute to chilling resistance.

1.5.2 Physiological role of polyamines in mitigating plant stress

Polyamines (PA) are positively charged low molecular weight plant compounds containing an amine group associated with counteracting any form of stress by binding to negatively charged plant molecules, such as proteins, membrane phospholipids and DNA (Valero et al., 2002; Kuznetsov and Shevyakova, 2007; Naka et al., 2010). Once conjugated to these negatively charged molecules, their function reactive oxygen species scavenging capacity is improved (Kuznetsov and Shevyalova, 2007), while cell wall strength is enhanced, maintaining cohesion of the cell wall during cell expansion. Such strengthening of the cell wall is particularly important during stress when conjugated with pectins (Lenecci et al., 2005; Messiaen et a., 1997). Pectins, cellulose-xyloglucans which make up about 30% of the plant cell wall form a covalent bond with PAs improving wall structure during stress exposure (Kuznetsov and Shevyalova, 2007).

In plants the dominant PAs are putrescine (*put*), spermidine (*spd*) and spermine (*spm*) (Fig 8); the affinity for conjugation with negatively charged molecules varies with the greater affinity by spm^{4+} followed by spd^{3+} and the lowest affinity by put^{2+} (Messiaen et al., 1997, Lenucci et al., 2005). Putrisce is synthesized from ornithine through an enzyme ornithine decarboxylase (ODC) in the nucleus of actively dividing cells and through arginine decarboxylase (ADC) in the mitochondria (Fig 9) as a plant response to stress (Kuznetsov and Shevyakova, 2007; Tiburcio et al., 1994b).

Polyamines are synthesized in different cell organelles, depending on the presence of key enzymes involved in their production (Fig 9); however, *put* is a precursor of cellular production of the other high affinity PAs, spd and spm (Kuznetsov et al., 2007).

A. $^{+}H_3N — (CH_2)_4 — NH_3^{+}$

Putrescine

B. $^{+}H_3N — (CH_2)_3 — {}^{+}NH_2 — (CH_2)_4 — {}^{+}H_3N$

Spermidine

C. $^{+}H_3N — (CH_2)_3 — {}^{+}NH_2 — (CH_2)_4 — {}^{+}NH_2 — (CH_2)_3 — {}^{+}H_3N$

Spermine

Figure 8: Chemical structure of plant dominant polyamines; A. Putrescine, B. Spermidine and C. Spermine

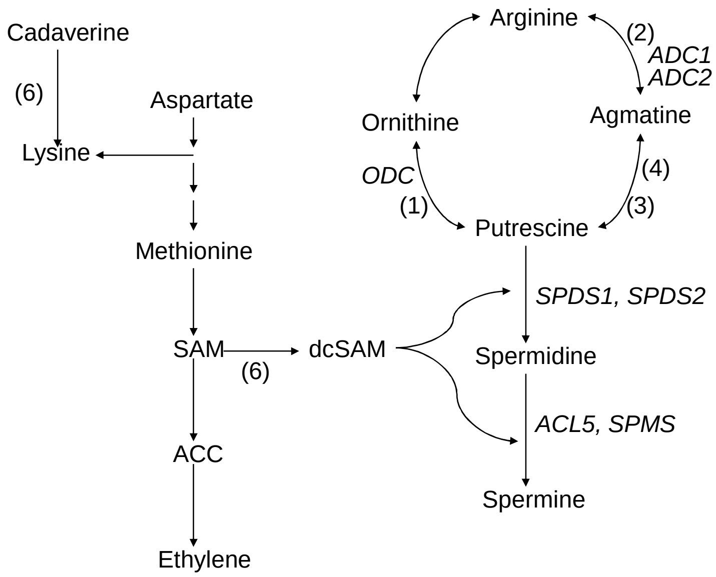

Cadaverine
(6)
Lysine
Aspartate
Methionine
SAM
(6)
dcSAM
ACC
Ethylene
Arginine
(2)
ADC1
ADC2
Ornithine
Agmatine
ODC
(1)
(4)
(3)
Putrescine
SPDS1, SPDS2
Spermidine
ACL5, SPMS
Spermine

Figure 9: Pathway of biosynthesis of main plant polyamines (Put, spd and spm) (Kuznetsov et al., 2007). Enzymes involved: (1) ODC, ornithine decarboxylase, (2) ADC, arginine decarbaxylase, (3) agmatine iminohydrolase, (4) N-carba-molyputrescine amidhydrolase, (5) SAMDC, SAM decarboxylase, (6) SPDS, spermidine synthase, (7) SPMS, spermine synthase, (8) LDC, Lysine decarboxylase; ACC, 1-aminocyclopropane-1-carboxylic acid; dcSAM, decarboxylates S-adenosyl-methionine. The genes involved are *ADC1, ADC2, ODC, SPDS1, SPDS2, ACL5,* and *SPMS*

The conversion of *put* to *spm* and *spd* is stress severity dependent, and furthermore, signalled by ethylene synthesis, decarboxylated S-adenosyl-methionine (dcSAM) (Fig 9) (Kuznetsov and Shevyakova, 2007). However, citrus have low climacteric proprieties and therefore, slow peak in ethylene production (Eaks, 1970). This low ethylene production could lead to inherently high put levels and a low conversion to other high affinity PA in citrus, *spm* and *spd*, during cold storage.

1.6. Involvement of molybdenum in plant metabolism

1.6.1 General function of molybdenum in plant metabolism

Molybdenum (Mo) and tungsten (W) are the only within group 6 transition metals that are essential for plant growth and development, particularly Mo which is important for most living organisms including plants, animals and microorganisms (Kisket et al., 1997). Molybdenum by itself seems to be biological inactive; however, it plays a pivotal role as a cofactor (Kisket et al., 1997; Mendel and Schwarz, 1999). According to Mendel and Schwarz (1999), Mo is incorporated into a apoprotein (as Mo-cofactor) and gains the ability to associate with a diverse range of redox active enzymes that catalyse basic reactions of the nitrogen and sulfur metabolism and the carbon cycle (Kisket et al., 1997; Mendel and Schwarz, 1999). In addition, nitrogen-fixing crops have been found to have higher Mo concentration than non-nitrogen fixing crops; such as lima beans, cowpeas, and navy beans (Triplett et al., 1982) which is further proof for involvement of Mo in plant metabolism.

1.6.2 Xanthine dehydrogenase (XDH) as a Mo-cofactor enzyme and its involvement in oxidative stress/signalling

There are many plant enzymes to which Mo serves as a cofactor, including nitrogen reductase (NR), sulfate oxidase (SO), aldehyde oxidase (AO), and xanthine dehydrogenase (XDH). Xanthine dehydrogenase and AO are similar in amino acid sequence and are classified as Mo-hydroxylases (Yesbergenova et al., 2005, Mendel and Schwarz, 1999). These hydroxylases are cytoplasmic homodimeric enzymes with a molecular mass of approximately 300 kDa (Yesbergenova et al., 2005; Hesberg et al., 2003; Datta et al., 1991). The affinity of hypoxanthine and xanthine as substrates to XDH is highest than that of possible substrates (Hesberg et al., 2003; Taylor, 2002).

There has been a suggestion that mammalian Mo-hydroxylases are involved in the production of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide (Fig. 10).

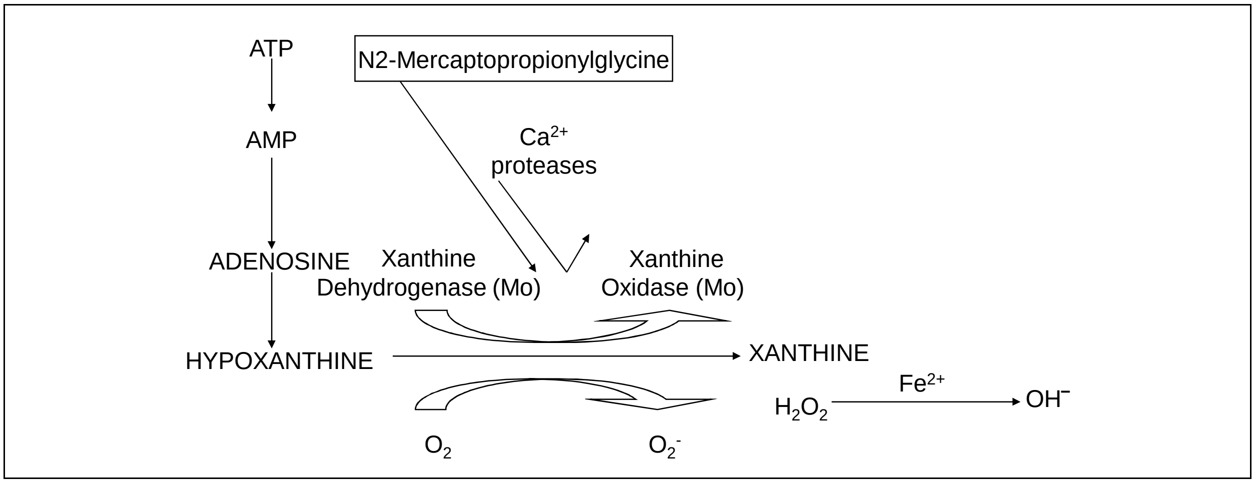


Figure 10: Possible involvement of the Mo-cofactor enzyme xanthine denydrogenase (XDH) in oxidative stress through production of reactive oxygen species (ROS). Model adopted from Adbo et al. (2003)

In plants XDH is primarily involved in the catabolism of ureide and purine during the fixition of nitrogen (Mendel and Schwarz, 1999). Furthermore, XDH activity can donate electrons to NAD+ with a reduction in molecular oxygen resulting in generation of ROS only if xanthine, hypoxanthine or NADH acts as electron (Yesbergenova et al., 2005). Moreover, XDH has been directly linked with plant stress response; the activity of XDH was found to increase in senescent leaves of *Arabidopsis thaliana* in response to salinity and cold stress (Hesberg et al., 2003); increased XDH activity was associated with increased ROS levels in water-stressed leaves and roots of A. *thaliana* (Yesbergenova et al., 2005). In addition, XDH activity has been reported to increase several-fold due to pathogen attack (Mendel and Schwarz, 1999; Kesker et al., 1997).

The Mo concentration of a certain tissue might play a key role in regulating the XDH activity during stress and normal metabolism; furthermore, Mo is involved in the production of ROS and, therefore, the occurrence of oxidative damage. Recently, ROS has been suggested to not only have a negative effect on cellular metabolism but also to play an essential role in signaling acclimatory plant response mechanisms against stress (Foyer and Noctor, 2005; Foyer and Shigeoka, 2010). The latter authors have revealed that the “concept of ROS functioning” is dual; these compounds can not only have “damage” functions but also act as “singaling” molecules. In the past, it has been suggested that ROS should be kept at a level as low as possible therefore, undermining its potential role within the context of cellular redox homeostasis and redox signaling (Foyer and Shigeoka, 2010). Therefore, addition of Mo could balance the plant cell homoeostasis by increasing XDH parallel activity by production of ROS which plays an oxidative stress signalling. However, ROS must be produced to allow signalling, but its concentration must be kept below a certain critical threshold level. An increase in antioxidants could be a ROS-signaled mechanism signaling stress response.

1.7. Quantification of antioxidants

1.7.1 Total antioxidant assay

Oxidative stress is a result of an array of ROS producing radicals including superoxide ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and the hydroxyl radicals ($OH^{\bullet}$) (Huang et al., 2007). Plants have developed a well-integrated defence system of antioxidants to scavenge and maintain the ROS formation below certain critical threshold levels (Yesbergenova et al., 2007). The antioxidant scavenging efficacy is increased by diverse, natural antioxidants, which can either be lipo-soluble or hydro-soluble (Sies and Stahl, 1995).

Recently, the possible manipulation of plant antioxidant production has been investigated in order to enhance this natural defence system against any form of stress. This research interest has led to the introduction of new assays to robustly quantify and classify total antioxidants. Newer total antioxidant assays include the ferric sulphate reducing ability of plasma (FRAP) assay (Halvorsen et al., 2002), the hydrogen atom-transfer reaction, measuring the oxygen radical absorbance capacity (ORAC) assay (Gorinstein et al., 2006); the 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) assay (Re et al., 1998); the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) (Xu et al., 2008) and the β-carotenebleaching assay (Gorinstein et al., 2006).

These antioxidant assays can be classified into two groups: the inhibition assays and assays that involve an antioxidant to generate a free radical. Inhibition assays include the ABTS and DPPH assays where the donation of hydrogen or an electron of a preformed free radical is measured (Re et al., 2008). The ABTS is based on the reaction of the very stable radical ($ABTS^{\bullet+}$) and antioxidant in the extract which is a blue/green product of ABTS-potassium persulfate reaction. This assay can estimate both hydrophilic and lipophilic antioxidants (Re et al., 2008). The DPPH assay involves a stable free radical with an unpaired electron that is oxidized when in contact with the antioxidant in the extract (Fig. 11) (Jin and Chen, 1998). The resultant colour change for the DPPH radical from purple to yellow can be a measure of photo-inhition of antioxidant within the extract.

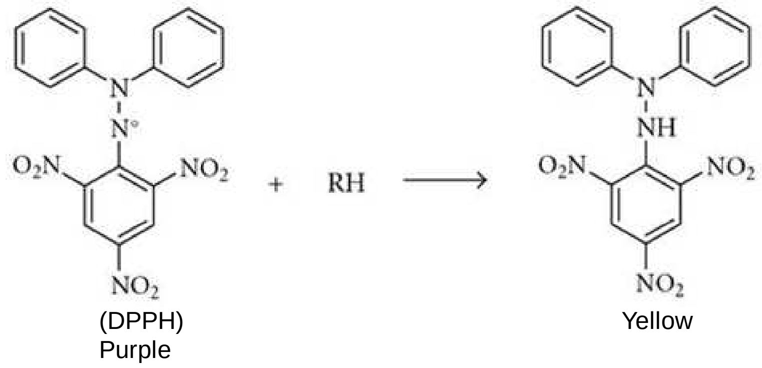


Figure 11: Chemical structure of DPPH plus an antioxidant reaction product (Rolman et al., 2010)

The ORAC and β-carotene bleaching assays are examples of antioxidant assays involving the production of a free radical. The FRAP assay is the mostly used assay and is based on the reduction of the ferric tripyridyltriazine Fe^{3+}-TPTZ complex to ferrous tripyridyltriazine Fe^{2+}-TPTZ by a certain reductant at low pH. The reaction produces a deep blue colour (Réka and Varga, 2002). Drawbacks of common total antioxidant assays have been summarized in Table 4.

Table 4: Main drawbacks associated with common antioxidant assays (Pérez-Jiménez et al., 2008)

Method	Main drawbacks
FRAP	Other compounds may absorb at 595 nm Any compound with a redox potential lower than 0.77 V, although it does not behave *in vivo* as an antioxidant, may reduce iron It is performed at non-physiological pH
ABTS	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 *in vivo*
DPPH	Other compounds may absorb at 515 nm There may be a steric hindrance for molecules with higher molecular weight The free radical used is not present *in vivo* and is quite stable, unlike radicals present in living organisms
ORAC	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 peroxyl radicals, present *in vivo* ; however, the procedure to generate these peroxyl radical is not physiological Protein may have an interfering effect

It is recommended to use a minimum of three assays to gain an understanding of a tissue's antioxidant capacity. The choice of antioxidant assay will depend on available resources, for an example most laboratories do not have a fluorimeter for the ORAC assay, however, FRAP, ABTS, DPPH and β-carotene bleaching assay are commonly used because of the availability of a spectrophotometer in most laboratories (Pérez-Jiménez et al., 2008).

1.7.2 Bioactive compound with antioxidants properties in citrus

Plants subjected to stress increase the synthesis of bioactive compounds as physiological means to tolerate stress (Cogo et al., 2011). Bioactive compounds are secondary metabolites and some have antioxidant properties (Plaza et al., 2011; Cogo et al., 2011). When plant cells are exposed to stress, ROS production increases and bioactive compounds with antioxidant properties protect against oxidative stress reducing the risk of stress related disorders (Al-Weshahy et al., 2011), including chilling injury. In citrus, common bioactive compounds with antioxidant properties include ascorbic acid, polyphenolic groups, and certain vitamins (Abeysinghe et al. 2007). Antioxidants can be divided into enzymatic, water, and lipo-soluble (Fig 12).

Within water-soluble bioactive compounds, ascorbic acid (vitamin C) is the dominant and a potent antioxidant, with polyphenols also contributing significantly to the antioxidant pool in citrus (Abeysinghe et al., 2007; Plaza et al., 2011). Polyphenols are abundant in citrus and are present in a wide range of different compounds, including flavonoids (namely flavonones, flavonols, flavones, isoflavonoids, flavanols and anthocyanidins), phenolic acids, stilbenes, coumarins and tannins (Abeysinghe et al., 2007; Khanizadeh et al., 2008; Xu et al., 2008). In citrus, heperidin and naringin are dominant flavonones, prviding a bitter taste in grapefruit (Li et al., 2006; Abeysinghe et al., 2007).

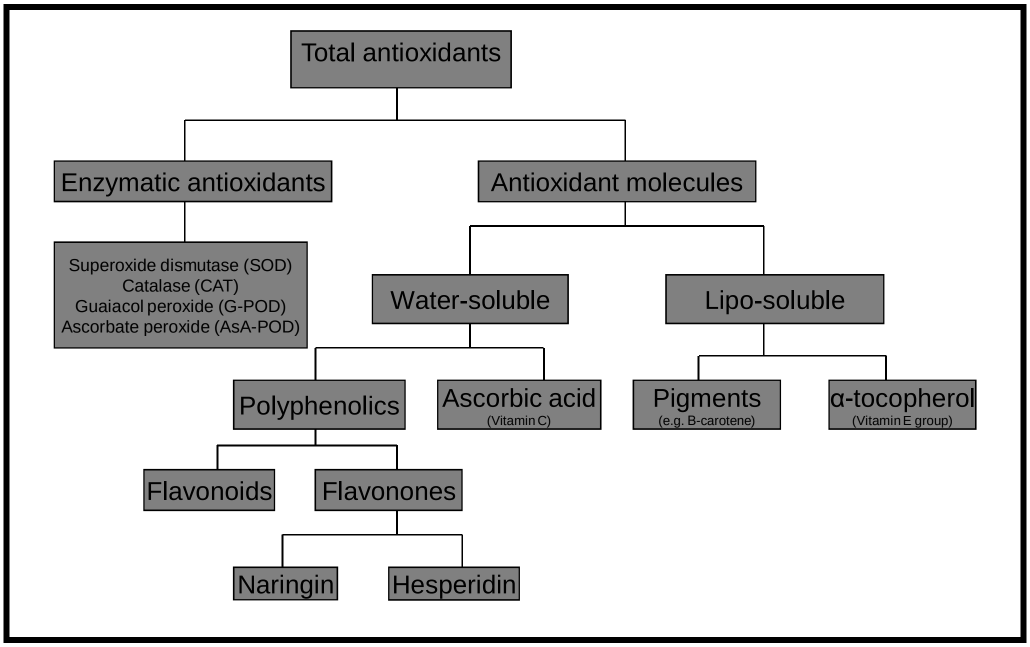

Total antioxidants
Enzymatic antioxidants
Antioxidant molecules
Superoxide dismutase (SOD)
Catalase (CAT)
Guaiacol peroxide (G-POD)
Ascorbate peroxide (AsA-POD)
Water-soluble
Lipo-soluble
Polyphenolics
Ascorbic acid
(Vitamin C)
Pigments
(e.g. B-carotene)
α-tocopherol
(Vitamin E group)
Flavonoids
Flavonones
Naringin
Hesperidin

Figure 12: Schematic representation of antioxidant systems in citrus species

Within the lipo-soluble bioactive compounds, α-tocopherol is a dominant compound of the vitamin E group (Fig. 12) followed by pigments, mainly β-carotene and other carotenoids with bioactive properties (Sies and Stahl, 1995). The molecular action of carotenoids is specifically for scavenging singlet oxygen and other electronically excited molecules produced by photo-excitation and chemi-excitation reactions (Sies and Stahl, 1995).

Therefore, there is need to unravel the holistic contribution of bioactive compounds and provide an understanding of their action sequence in lemon flavedo during cold storage. Furthermore, the study aims at providing an understanding of the action/role of these bioactive compounds and secondly, developing possible treatments to increase their concentration as a stress acclimatory means inducing oxidative stress resistance.

1.8 Aim of research

Mechanisms that lead to chilling injury in citrus fruit are complex and involve interactions of multiple physiological compounds and pathways. So far, a holistic understanding of cellular events leading to the appearance of chilling injury and post-harvest treatments that could mitigate such injury are lacking. Therefore, this research was initiated to unravel physiological mechanisms leading to chilling injury and to deeply investigate the potential of hot water dips to mitigate chilling injury. Furthermore, the physiological role of Mo and its potential to contribute to reducing appearance of chilling injury in citrus fruit, with special emphasis on lemon (cv. Eureka) was investigated. The following studies were therefore undertaken:

1.8.1 High-paying markets require cold-sterilization of lemon fruit during storage as a remedy against fruit fly. However, citrus fruit are susceptible to chilling; exposure at -0.5°C for more than 21 days is necessary for cold sterilization. It is therefore hypothesized that -0.5°C cold storage could meet the obligatory cold sterilization without appearance of chilling injury symptoms; certain treatments are applied preventing the occurrence of chilling injury.

1.8.2 Farmers require an easy, cheap, chemical-free method to non-destructively predict the potential appearance of chilling injury symptoms. Cold storage damages cellular membranes leading to increased electrolyte leakage and fruit weight loss. Therefore it was hypothesized that chilling-susceptible lemon fruit will show increased water loss compared with chilling resistant fruit.

1.8.3 Sugars are the main source of energy in plants. However, they have been proposed to also play other important roles in maintaining product quality, by driving the production of secondary metabolites. The synthesis of ascorbic acid (AA), a potent antioxidant, and the synthesis of proline, an antioxidant commonly appearing during stress is linked to the presence of symptoms. Therefore, it is hypothesized that sugars, especially glucose, play a major role in determining chilling-susceptibility by increasing the AA and proline concentration during cold stress. Furthermore, hot water and molybdenum treatments might enhance chilling stress resistance of lemon rind.

1.8.4 Citrus peel contains an array of antioxidants which scavenge ROS and, therefore, reduce potential ROS damage causing oxidative damage to membranes during cold storage.

However, antioxidants are diverse in chemical structure, functionality and location within the plant tissue and therefore, methods (assays) to estimate these antioxidants are specific to certain antioxidant properties. Therefore, it is hypothesized that the assays used to determine antioxidants differ in their efficacy to estimate both hydrophilic and lipophilic antioxidants. This necessitates determination of antioxidants using different antioxidant assays. Furthermore, it was intended to understand the role of HW and Mo post-harvest treatments to maintaining antioxidants quantified by different antioxidant assays (FRAP, ABTS and DPPH assays).

1.8.5 The occurrence or presence of antioxidant as plant defence against oxidative stress; has been reported; however, the order in the cascade of events is not well-understood. Furthermore, the interaction of specific bioactive compounds with antioxidant property has been proposed but is also not fully understood. Therefore, we hypothesize that antioxidants act in a certain order when scavenging ROS. Certain antioxidants function within the cytoplasm (hydrophilic antioxidants) while others are membrane bound (lipid-soluble antioxidants); interacting to holistically reduce oxidative stress. Hot water and Mo treatments could enhance the function and interaction of these antioxidants under stress conditions.

1.8.6 Polyamines are biological compounds with antioxidant properties reacting with negatively charged cellular compounds such as proteins (specifically HSP70), DNA and membranes; such reaction improves their resistance to damage during exposure to stress. However, PAs differ in affinity towards negatively charged compounds; while *put* is the dominant PA. Therefore, it is hypothesized that HW and Mo treatment enhance the conversion of the low affinity polyamine (*put*) to the higher affinity PAs (spm^{+4} and spd^{+3}) in order to increase the chilling-resistance of lemon flavedo during cold storage.

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CHAPTER 2

Research Note

Effect of Hot Water and Molybdenum Dips on Lemon Fruit Weight Loss: a Non-destructive Parameter for Chilling Susceptibility during Cold Storage

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Abstract

Lemons (c.v. Eureka) are among the most chilling susceptible citrus fruit. However, as cold sterilization is a quarantine requirement for countries exporting fruit to certain high paying markets, this necessary cold storage may result in rind disorders, especially chilling injury. This disorder appears as dark sunken lesions or pitting on the fruit surface, ultimately reducing fruit marketability. Physiologically, chilling injury is a result of alterations in cellular membranes due to cold temperatures which leads to increased membrane permeability, electrolyte leakage and, ultimately, high fruit weight loss. Preliminary research has shown that a combination of hot water (HW) and molybdenum (Mo) dips has the potential to mitigate chilling injury. The main objective of this work was to determine whether fruit weight loss can be used as a non-destructive parameter for the occurrence of chilling symptoms. Furthermore, it was investigated if HW and Mo dips can reduce fruit weight loss under cold storage thereby reducing chilling injury. Fruit from two growing regions differing in chilling occurrence were treated. Treatments included HW 53°C for 2 min and 1 µM or 10 µM Mo for 30 min. Fruit were then stored at -0.5°C for up to 28 days and evaluated weekly for chilling injury. Lemon fruit from Sun Valley showed chilling symptoms after 28 days of cold storage paralleled by high fruit weight loss. Furthermore, fruit from the chilling susceptible region treated with HW at 53°C plus 1 µM Mo showed reduced chilling injury and fruit weight loss, however, fruit weight loss can not be used as a reliable parameter for prediction of chilling injury.

Keywords: *Lemon, chilling injury, hot water, molybdenum, water loss*

1. Introduction

Citrus fruit originate from a subtropical environment and are therefore chilling sensitive (Porat et al., 2000). According to the physiologically model of Lyons (1976), chilling conditions cause a phase transition in cellular membranes from the normal flexible liquid-crystalline to a solid-gel like structure. Such membrane transition results in contractions that cause cracks and channels and therefore resulting in an increased cell permeability and electrolyte leakage (Lyons, 1973). Pitting in cucumber fruit has been associated with cracks in the cuticle and sinking of epidermal cells (Hakim et al., 1999). In 'Fortune' mandarin, chilling symptoms are associated with microscopic cracks ad increased fruit weight loss (Schirra and D'hallewin, 1996). Furthermore, accelerated fruit weight loss has been found to be highly correlated to membrane damage, as well as to ion leakage in cold-stored peppers (Maalekuu et al., 2006). Fruit weight loss is, therefore, a sensitive indicator of chilling susceptibility and can be used as a non-destructive parameter as it determination is inexpensive and easy to carry out compared with destructive assays using wet chemistry (Cohen et al., 1994).

Previous work has only investigated fruit weight loss of different lemon cultivars ('Eureka' and 'Lisbon' lemon) during 42 days of cold storage at temperatures above 0°C (Underhill et al., 1999). To our knowledge, no information exists on the effect of HW plus Mo dips on fruit weight loss, and such weight loss has not been aligned with susceptibility to chilling injury. Therefore, the objective was to establish whether fruit weight loss can be used as a non-destructive, rapid and reliable parameter of chilling injury prediction in lemon fruit.

2. Materials and Methods

Mature lemon (c.v. Eureka) fruit were obtained from Ukulinga University Research Farm, Sun Valley Estates and Eston Estates Farm. Fruit were treated with a 30 min soak in 1 µM or 10 µM $Na_2MoO_4.H_2O$ followed by a 2 min hot water dips (HW 47 or 53°C). Thereafter, fruit were waxed (Citrashine Pty Ltd, Johannesburg, South Africa), weighed (Mettler SM 3000, Switzerland) and stored at -0.5°C and fruit were removed at weekly intervals (7, 14, 21 and 28 days) of storage for chilling injury evaluation. A second evaluation was carried out five days after removal from storage to determine shelf-life (5SL). The flavedo of the fruit was removed with a potato peeler and the peel stored at -21°C for further physiological analysis.

The method of Sala (1998) was used to evaluate the severity of chilling immediately after cold storage as well as after 5 days shelf-life. This method established a rating scale based on surface pitting intensity (0 - sound fruit, 1 -less than - 10%, 2 - 20% pitting, 3 - 30-40% and 4 - more than 50% pitting). The chilling injury index (CII), which expressed the severity of damage was calculated by adding the products of the number of fruit in each category by the value assigned to this category in the rating scale and dividing this sum by the total number of fruit evaluated.

Statistical comparison of mean values was performed by analysis of variance (ANOVA) using Genstat version 14. Means were separated using least significant difference (LSD) at the 5% level of significance.

3. Results and Discussion

Symptoms of chilling injury were observed only for lemon fruit from Sun Valley Estates (Table 1). However, fruit sourced from Ukulinga Farm and Eston Estates surprisingly did not show any chilling symptoms, despite lemon fruit being highly chilling susceptible (Chalutz at el., 1985). Hot water dips at 47°C were not as effective in mitigating chilling symptoms compared with HW 53°C with chilling indices of 0.18 and 0.09, respectively (Table 1). The ability of HW 53°C to reduce chilling injury confirms previous results in 'Valencia' oranges that HW 53°C for 3 min can reduce chilling injury to a chilling index of 0.45 (Erkan et al., 2005). In 'Torocco' bloody oranges, the same treatment reduced decay when fruit were stored at 3°C for up to 10 weeks (Schirra et al., 1997). Most previous reports have related the positive effectiveness of HW dips to production of heat shock proteins (Rozenzvieg et al., 2004). Molybdenum dips were effective at 1 μM Mo and 1 μM Mo plus HW 53°C dips with chilling indices of 0.04 and 0, respectively (Table 1). Therefore, an assumption can made that dipping lemon fruit in 1 μM Mo and 1 μMo Mo plus HW 53°C dips increased the activity of xanthine dehydrogenase (XDH). XDH is a molybdenum co-factor enzyme in plants and its activity produces reactive oxygen species (ROS) under stress condition. Reactive oxygen species below a damaging threshold may induce stress acclimation in plants (Foyer and Noctor, 2005; Foyer and Shigeoka, 2011). Previous work found that salinity increased the activity of XDH (Sagi et al., 1998). Additionally, Hesberg et al. (2003) found that cold and salinity stress decreased XDH activity. Moreover, whether the activity of XDH increases or decreases in response to Mo application has not been decisively proven yet. Furthermore, interaction of Mo with HW at 53°C dips (Table 1) has not yet been understood.

3.1 Effect of fruit origin on lemon fruit weight loss during cold storage

Chilling-sensitive lemons (cv Eureka) from Sun Valley Estates showed a higher weight loss after 5 days shelf-life, but this was not significantly different from Eston Estates and Ukulinga fruit (Figure 1). In the second year of the experiment Ukulinga fruit showed significantly high weight loss directly after storage as well as after 5 days shelf-life at ±21°C. The severity of chilling symptoms may increase when fruit are moved to warmer temperatures (Houch et al., 1990; Schirra et al., 1997); hence, all fruit showed additional fruit weight loss after 5 days shelf-life (Figure 1). As Sun Valley fruit had a similar percentage weight loss as fruit from other locations where no chilling injury occurred, fruit weight loss does not seem to be an appropriate indicator of chilling-susceptibility.

3.2 Effect of HWD and Mo dips on lemon fruit weight loss during cold storage

In accordance with Lyons (1976), chilling symptoms are accelerated after withdrawal from cold storage. There was a significant increase in fruit weight loss of Sun Valley Estates fruit after 28 days cold storage as well as a further accelerated loss following the 5 days shelf-life period. Therefore, theoretically an increase in chilling symptoms at shelf-life compared to non-chilled fruit onfirms that chilling injury is mainly appears at shelf-life not during cold storage (Table 1). Control fruit had a percentage weight loss of 8.3±0.7%, which indicates the highest chilling susceptibility in the Sun Valley Estates fruit at 28 days cold storage plus 5 days shelf-life. Hot water dips at 47°C as well as HW 53°C and 1 μM Mo plus HW at 53°C also reduced the percentage fruit weight loss to 7.0±0.6, 6.8±0.5, 7.2±0.3%, respectively for Sun Valley Estates fruit following 28 days cold storage plus 5 days shelf-life (Table 2).

Soaking Sun Valley fruit in a 1μM Mo solution was effective in reducing fruit weight loss to 5.8±0.8% compared with 8.3±0.7% of water-dipped fruit as determined after 28 days cold storage plus 5 days shelf-life (Table 2). Low Mo concentrations could increase the activity of XDH, thereby increasing the production of ROS (Sagi et al., 1998.). Increased ROS can act as stress aclimatory signiling molecules (Foyer and Noctor, 2011), ultimately leading to reduced membrane disintegration (Lyons, 1976; Wolfe, 1978) and reduced fruit weight loss (Table 2).

In general, HW dips seem enhance fruit weight loss after 7 days of cold storage plus 5 days shelf-life, especially for non-chilled consignments (Table 2), supporting findings by Schirra et al. (1997) that HW at 53°C mitigate chilling injury while resulting in increased fruit weight loss. However, it is evident that heat treatments, their efficacy to mitigate chilling injury, and their effect on fruit weight loss during cold storage, are strongly dependent on inherent susceptibility to chilling injury and pre-harvest orchard condition. Rozenzvieg et al. (2004) reported HSPs production to be the major response of fruit to intermittent warming. Moreover, membrane lipid composition also plays a role. Fruit that are resistant to chilling injury have more unsaturated and short-chained lipids (Wolfe, 1978). Heat treatments of tomatoes enhanced the conversion of membrane-phospholipids from saturated to unsaturated, a phenomenal not observed with non-heat treated tomato fruit (Lurie and Sabehat, 1995).

4. Conclusions

In conclusion, fruit weight loss in general has is not a reliable, non-destructive indicator of chilling injury appearance in lemon fruit. However, fruit source may contribute to fruit weight loss during cold storage, speculatively; such may be related to membrane fatty acid composition (Wolfe, 1978) and other physiological parameters such as membrane lipid peroxidation and electrolyte leakage.

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List of Figures

Figure 1: Effect of locality and harvest season on fruit weight loss during cold storage and after five days of withdrawal from storage. Means value with different lower-case letters were significantly different using the least significant difference ($LSD_{0.05}$ = 0.2107) test

List of Tables

Table 1: Effect of hot water and molybdenum post-harvest dips on chilling symptoms of lemon fruit cold stored (-0.5°C) for up to 28 days

Table 2: Effect of storage time, locality and harvest season, hot water and molybdenum dips on weight loss of lemon fruit during 28 days of cold storage at -0.5 °C. Results are presented as means ±S.E. for 5 replications

Table 1:

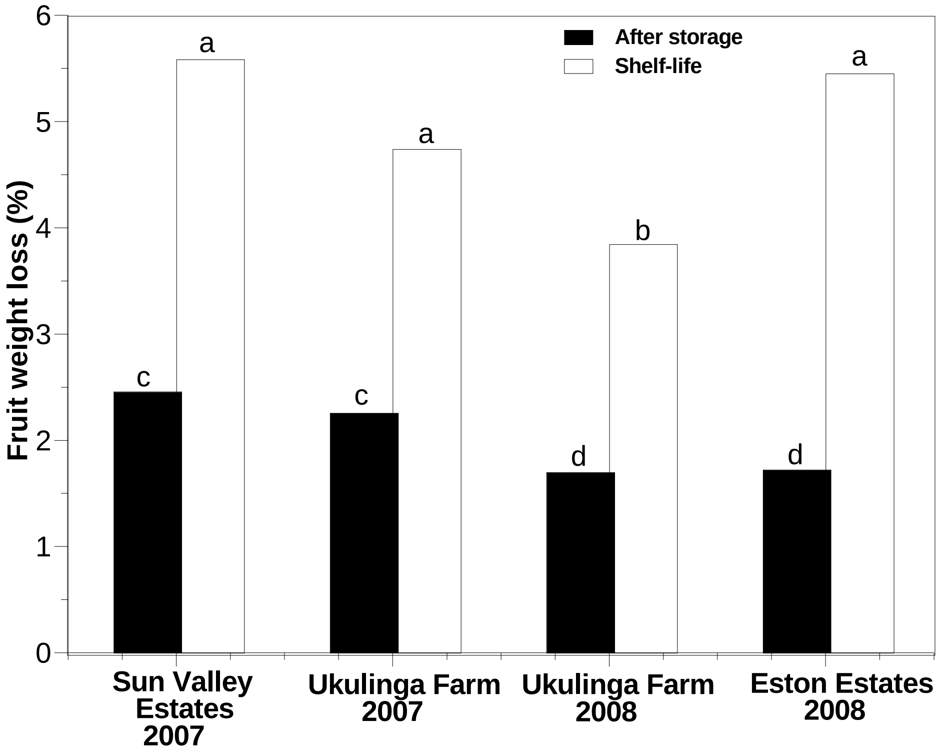
Treatments[a]	Ukulinga 2007[b]	Sun Valley Estates 2007[c]	Ukulinga 2008[b]	Eston Estates 2008[d]
Water dip (25°C)	No symptoms	0.13a	No symptoms	Below detection
HW 47°C	No symptoms	0.18a	No symptoms	Below detection
HW 53°C	No symptoms	0.09a	No symptoms	Below detection
1 µM Mo	No symptoms	0.04ab	No symptoms	Below detection
1 µM Mo + HW 53°C	No symptoms	0.02ab	No symptoms	Below detection
10 µM Mo + HW 53°C	No symptoms	0.00b	No symptoms	Below detection

[a] Treatment effect after 28 days cold storage plus 5 days shelf-life

[b] Ukulinga lemon fruit showed no chilling symptoms during 2007 and 2008 harvest seasons

[c] Sun Valley lemon fruit showed chilling symptoms expressible in chilling index, and means followed by the same letter were not significantly different at LSD(0.05) = 0.096

[d] Eston Estates lemon did show chilling symptoms but below detection and chilling index calculation



Fruit weight loss (%)
0
1
2
3
4
5
6
After storage
Shelf-life
c
a
c
a
d
b
d
a
Sun Valley Estates 2007
Ukulinga Farm 2007
Ukulinga Farm 2008
Eston Estates 2008

Figure 1:

Table 2:

		Locality and Harvest Season							
Storage Time (days)	Treatments	Sun Valley Estates 2007		Ukulinga 2007		Ukulinga 2008		Eston Estates 2008	
		AS* (%)	5SL** (%)	AS* (%)	5SL** (%)	AS* (%)	5SL** (%)	AS* (%)	5SL** (%)
7	Water dips	1.8±0.079	4.5±0.199	2.0±0.184	4.0±0.341	2.0±0.068	3.9±0.113	1.6±0.086	4.5±0.205
	HW 47°C	1.5±0.039	4.0±0.117	3.1±0.430	6.1±0.732	2.2±0.256	3.9±0.396	2.3±0.174	5.8±0.533
	HW 53°C	1.4±0.048	3.6±0.112	3.3±0.307	5.9±0.519	3.4±0.039	5.7±0.096	2.4±0.160	5.7±0.353
	1 µM Mo	1.4±0.046	3.9±0.134	0.9±0.046	3.7±0.152	1.4±0.128	3.9±0.259	0.9±0.067	4.6±0.301
	1 µM Mo + HW 53°C	1.2±0.058	4.3±0.145	1.3±0.427	3.2±0.621	1.9±0.220	3.8±0.347	1.9±0.168	5.9±0.636
	10 µM Mo + HW 53°C	1.2±0.054	4.1±0.198	2.1±0.105	4.6±0.209	1.0±0.084	3.7±0.255	0.8±0.065	6.8±0.288
	^{1}Mean	**1.4c**	**4.1c**	**2.1ab**	**4.6b**	**2.0a**	**4.1a**	**1.7a**	**5.6a**
14	Water dips	2.6±0.255	5.3±0.273	2.6±0.329	4.9±0.543	1.9±0.170	3.9±0.297	2.0±0.303	4.9±0.570
	HW 47°C	2.5±0.211	5.2±0.398	3.3±0.683	5.8±1.013	2.7±0.091	4.9±0.102	2.8±0.273	6.7±0.571
	HW 53°C	2.4±0.253	4.8±0.223	1.3±0.082	3.8±0.186	1.3±0.103	4.3±0.320	0.8±0.088	4.4±0.454
	1 µM Mo	2.4±0.171	5.1±0.374	2.2±0.297	5.0±0.485	1.5±0.128	3.4±0.212	1.5±0.164	5.2±0.450
	1 µM Mo + HW 53°C	2.2±0.187	4.8±0.280	4.0±0.281	7.2±0.467	2.2±0.114	4.3±0.208	2.6±0.173	6.1±0.318
	10 µM Mo + HW 53°C	1.8±0.133	3.9±0.223	1.9±0.210	4.2±0.505	1.3±0.083	3.0±0.153	1.8±0.113	5.4±0.325
	Mean	**2.3b**	**4.9b**	**2.5a**	**5.1a**	**1.8a**	**4.0a**	**1.9a**	**5.4ab**
21	Water dips	3.0±0.139	5.5±0.312	2.6±0.387	4.8±0.671	2.9±0.400	5.2±0.563	2.9±0.211	7.1±0.378
	HW 47°C	2.7±0.137	5.2±0.287	1.4±0.074	3.9±0.135	1.0±0.053	3.8±0.155	1.0±0.100	5.4±0.442
	HW 53°C	2.7±0.217	5.2±0.362	3.2±0.117	6.6±0.145	1.4±0.112	3.3±0.223	1.9±0.121	5.6±1.718
	1 µM Mo	2.7±0.189	5.3±0.382	2.3±0.224	4.7±0.302	1.6±0.145	3.4±0.230	2.2±0.126	5.7±0.305
	1 µM Mo + HW 53°C	2.5±0.078	4.9±0.133	1.8±0.194	5.0±0.493	0.8±0.041	3.3±0.151	0.6±0.043	4.7±0.257
	10 µM Mo + HW 53°C	2.5±0.210	5.0±0.344	3.3±0.644	6.3±1.060	2.2±0.155	4.3±0.268	2.0±0.226	5.7±0.427
	Mean	**2.7b**	**5.2b**	**2.4a**	**5.2a**	**1.6ab**	**3.9ab**	**1.8a**	**5.7a**
28	Water dips	4.0±0.489	8.3±0.735	1.5±0.141	4.2±0.307	0.6±0.028	2.9±0.117	0.7±0.036	4.8±0.262
	HW 47°C	3.4±0.359	7.0±0.547	1.8±0.230	3.9±0.398	1.0±0.047	2.6±0.127	1.3±0.137	4.8±0.501
	HW 53°C	3.1±0.299	6.8±0.493	2.2±0.150	4.4±0.315	1.3±0.051	3.2±0.063	1.9±0.173	5.8±0.294
	1 µM Mo	2.9±0.250	5.8±0.797	2.7±0.262	4.8±0.351	2.0±0.048	4.1±0.087	2.1±0.149	5.8±0.318
	1 µM Mo + HW 53°C	3.3±0.173	7.2±0.310	1.6±0.120	3.3±0.227	1.2±0.086	3.1±0.181	1.1±0.086	3.9±0.223
	10 µM Mo + HW 53°C	3.6±0.702	4.3±2.091	2.0±0.117	3.7±0.173	2.4±0.194	4.7±0.281	2.1±0.123	5.5±0.150
	Mean	**3.4a**	**8.2a**	**2.0b**	**4.0c**	**1.4b**	**3.4b**	**1.5a**	**5.1b**

* After storage

** 5 days shelf-life

^{1}Fruit source*Evaluation time*Cold storage time $LSD_{(0.05)} = 0.4$

^{2}Fruit source*Evaluation time*Cold storage time*Treatments $LSD_{(0.05)} = 0.9$

Values followed by a different lower-case letter within row means^1 are significantly different at P = 0.05

CHAPTER 3

Effect of Postharvest Hot Water and Molybdenum Dips on Soluble Sugars, Glucose-Ascorbic Relationship and Proline Accumulation in Lemon Rind during Cold Storage

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SUMMARY

Sugars play a significant role in plant cells during stress exposure. They are involved in different stress mitigating mechanisms in plants. Intensive stress can initiate the conversion of glucose to ascorbic acid (AA) thereby increasing the antioxidant capacity of plant cells. Coupled with the conversion of glucose is the initiation of proline synthesis from glutamate. Previous investigations have shown post-harvest hot water (HW) and molybdenum (Mo) treatments to mitigate chilling injury symptoms in lemon (cv. Eureka) flavedo during cold storage. Therefore this investigation aimed to investigate the potential role played by HW and Mo treatments in the conversion of flavedo glucose to AA as well as concomitant synthesis of proline from glutamate. Fruit from two sources were preconditioned for 2 min with HW (at 47°C or 53°C) in combination with a subsequent 1 or 10 μM Na_2MoO_4 soak for 30 min to test the efficacy of such treatments. Fruit were subsequently stored at -0.5°C for 7, 14, 21, 28 days and thereafter evaluated weekly for chilling injury. Chilling susceptible lemon fruit sourced from Sun Valley Estates and Eston Estates had lower glucose levels and therefore, speculatively; low ability to convert glucose to AA and proline leading to lower antioxidant capacity, ultimately resulting in the inability to resist chilling injury. Treatment with HW 53°C plus Mo seems to influence the conversion of glucose to AA and the synthesis of proline from glutamate. Reducing sugars are also involved in determining chilling susceptibility of lemon fruit, while, additionally pre-harvest and environmental factors seem to contribute significantly to chilling susceptibility.

Introduction

Cold storage has been found to be an ideal phytosanitary measure when shipping citrus fruits from areas harbouring undesired insect pests (Houch et al., 1990). However, cold sterilization, may result in chilling injury, particularly in lemons which are very susceptible to chilling injury. This susceptibility to chilling injury has been attributed to climatic differences, determined by the morphological and chemical composition of lemon fruit (Sinclair, 1984; Aung et al., 1998; 1999; 2001). Chilling injury manifests as dark sunken lesions and pitting on the flavedo surface (Porat et al., 2002) and negatively affecting post-harvest quality, hence, reducing marketability of fruit (Shellie and Mangan, 2000; Erkan et al., 2005). Currently, there are several methods employed to mitigate chilling injury. Hot water (HW) treatments have been found to possess positive synergy with certain fungicides to mitigate chilling injury in grapefruit (Schirra et al., 2000).

The physiological mechanisms underlying the success HW treatments remain unclear, except that they are capable of inducing heat shock proteins (HSPs) (Rozenzvieg et al., 2004). Preliminary results have shown molybdenum (Mo) treatments to reduce chilling symptoms; such applications alter the production of reactive oxygen species (ROS) (Datta et al., 1991; Hesberg et al., 2003; Yesbergenova et al., 2005).

As carbohydrates have been found to play a major role in enhancing resistance to chilling damage (Holland et al., 2002) and are abundant in citrus flavedo (Ingram and Bartel, 1996), they could play major role in the development of such injury. Carbohydrates can facilitate the maintenance of dehydrated cell membranes caused by chilling temperatures and thus maintain the labile membrane structure and securing protein position and membrane integrity (Crowe et al., 1998). Furthermore, carbohydrates counteract the effect of cold stress by replacing the polar residue of water situated within the membrane phospholipids and therefore, maintaining the position of membrane-bound proteins (Crowe et al., 1998). Carbohydrate can also stabilize membrane proteins, thereby, increasing the temperature at which protein denaturation occurs (Back et al., 1979), thus increasing membrane integrity under cold stress. Carbohydrates promote the formation of a metastable 'glassy' state Roos (1993), which is enhanced by high viscosity; therefore, providing greater protein and membrane stability during cold storage. The 'glass' state of amorphous carbohydrate also act as thermoplastics and therefore, stabilizing other compounds under stress (Roos, 1993).

Apart from being a strong water-soluble antioxidant (Smirnoff, 1996; Smirnoff and Wheeler; 2000; Smirnoff, 2000), ascorbic acid (AA) production has been associated with carbohydrates, especially glucose. Isherwood et al., (1954) proposed the first mechanism linking the synthesis of AA to hexoses, especially glucose. This biosynthetic pathway (Figure IB) involves the conversion of D-Glucose to GDP-mannose, GDP-L-galactose and L-galactose-1, 4-lactose as intermediates that leads to AA (Loewus, 1999; Smirnoff, 2000; Hancock and Viola, 2002). The final step in AA synthesis is determined by the need for AA in the plant cell (Loewus, 1999).

The presence of proline has been linked to stress tolerance (Ashraf and Fooland, 2007), particularly drought (Knipp and Honermeier 2006) and salinity resistance (Jaleel et al., 2007; Yazici et al., 2007). Furthermore, proline has been linked with freezing stress resistance in chickpea (*Cicer arietinum* L.) at the reproductive stage (Kaur et al., 2011), protecting folded proteins structure against denaturation (Claussen, 2005), stabilizing membranes by interacting with phospholipids and biomolecules, and functioning as free radical scavengers (Yazici et al., 2007). There is a proposed relationship between the concentration of glucose and proline (Figure 1A) during cell exposure to stress (Sarkat et al., 2009). There is a demand for NADPH during proline synthesis altering $NADP^+$/NADPH ratio, thereby, initiating the activity of glucose-6-phosphote dehydrogenase (G6PDH) leading to antioxidant synthesis especially of phenolics nature (Figure 1A) (Zheng et al., 2001; Shetty, 2004; Sarkat et al., 2009)

The aim of this research was to determine whether relatively high or low levels of glucose, AA and proline in lemon flavedo depending on fruit origin during postharvest cold storage and role of such in mitigating chilling injury. Secondly, the alterations of levels of such compounds due to HW and Mo treatments were investigated. Finally, determine whether such glucose-AA and proline levels are related to chilling injury.

Materials and Methods

Chemicals

Sodium molybdate, ethanol, ascorbic acid, oxalic acid, acetic acid, ammonium acetate, octylamine, sulfosalysic acid, acid ninhydrin, toluene, proline were obtained from Sigma-AIdrich Chemical Co. (St Louis, MO, USA).

Plant materials

Mature “Eureka” lemon fruit were obtained from Ukulinga Research Farm (29°39’48.82”S 30°24’19.89”E)and Sun Valley Estate (28°51’00”S 30°04’00”E) during the 2007 harvest season and from Ukulinga Research Farm (29°39’48.82”S 30°24’19.89”E) and Eston Estate (29°47’00”S 29°27’00”E) during the 2008 harvest season. Fruit were submerged in a 1 or 10 µM $Na_2MoO_4.2H_20$ solution for 30 min and in HW 47 or 53°C for 2 min. Treated fruit were waxed, weighed and stored at -0.5°C for up to 28 days and evaluated for chilling injury on a weekly basis. A second chilling injury evaluation was carried out five days after withdrawal from cold storage. Fruit were peeled and the peel freeze-dried, milled using a mortar and pestle and stored at -21°C for further physiological analysis.

Estimation of chilling injury

The method of Sala (1998) was used to evaluate chilling injury on a weekly basis five days after withdrawal from cold storage. This method establishes a rating scale based on surface browning/pitting intensity (0 - sound fruit, 1 - less than 10% pitting, 2 - 10 to 20% pitting, 3 - 30 to 40% pitting and 4 - more than 50% pitting). The chilling injury index (CII), which expressed the severity of damage, was calculated by adding the products of the number of fruit in each category by the value assigned to this category in the rating scale and dividing the sum by the total number of fruit evaluated.

$$\text{CII} = \Sigma\ (\text{number of fruit with chilling injury} \times \text{score of severity})\ /\ \text{Total number of fruit evaluated}$$

Soluble sugar extraction and HPLC determination

For sugar analysis the method of Aung et al., (1998, 1999) was followed, where 0.1 g of lyophilized flavedo was weighed in a test tube and 10 ml 80% ethanol was then added. The samples were homogenized for 30 s using an Ultra-Turrax (Model: T250, IKA-Mannheim, Germany) and thereafter incubated for 1 h in a shaking hot water bath (80°C). After removal from the hot water bath, test tubes were stored at 4°C for 24 h and afterwards centrifuged at 12, 000 g for 15 min in a refrigerated (4°C) centrifuge. Extracts were filtered through glass wool and dried down in vacuum. Samples were re-suspended in 2 ml ultra-pure water, centrifuged for 15 min and filtered through a 0.4 μm nylon filters. Soluble sugars were determined using an isocratic HPLC system (LC – 20AT, Shimadzu Corporation, Kyoto, Japan) equipped with a refractive index detector (RID-10A, Shimadzu Corporation, Kyoto, Japan). The sugar concentration of the sample was determined by comparison with of glucose, sucrose and fructose standard.

Determination of ascorbic acid

Ascorbic acid was extracted and determined with a modified method of Abeysinghe et al. (2007) with minor modifications. Ascorbic acid was extracted from 0.1 g lemon flavedo using 10 ml 0.1% (w/v) oxalic acid. After homogenising for 5 min using an Ultra-Turrax (Model: T250, IKA- Mannheim, Germany), the sample was centrifuged at 30 000g (2°C) for 10 min whereafter the resulting supernatant was filtered and injected into a reversed phase HPLC system Luna® C18 column to which a UV detector (PDA-100) was attached. Ascorbic acid was eluded at a flow rate of 1.0 ml min^{-1}; injection volume of 20 μl; detector wavelength of 251 nm. Ammonium acetate buffer (0.02 M, pH 5.4) with 1 mM octylamine was used as the mobile phase. The ascorbic acid concentration was expressed as mg 100g^{-1} DM.

Determination of proline

The flavedo proline concentration was determined according to the method of Bates et al. (1973), with modifications. A sample of 0.1 g freeze-dried lemon flavedo was homogenised in 10 ml 3% (w/v) sulphosalicylic acid. The homogenate was centrifuged at 12, 000 x g for 10 min at 4°C. Two milliliters of the supernatant were reacted with 2 ml 2.5% acid-ninhydrin and 2 ml glacial acetic acid in a thick boiling test tube for 1 h at 100°C. Thereafter, the reaction was terminated in an ice bath. The reaction mixture was combined with 4 ml toluene and vortexed for 15 to 20 s. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance read at 520 nm using toluene as a blank. The proline concentration was determined from a standard curve and calculated as follows:

$$[(\mu g / ml \text{ proline} / ml \text{ toluene}) / (115 \mu g / \mu mole)] / [(g \text{ sample}) / 5] = \mu moles \text{ proline} / g \text{ DM}$$

Statistical analysis

Statistical comparison of mean values was performed by analysis of variance (ANOVA) using Genstat Version 14 (VSN International, UK). Means were separated using Duncan's Multiple Range Test at the 5% level of significance. Furthermore, data were subjected to principal component analysis (PCA) using Unscrambler Version 9.8 (Camo Process AS, Oslo, Norway).

RESULTS

Effect of HW and Mo dips on chilling injury symptoms

Chilling injury symptoms were only observed in Sun Valley lemon fruit harvested during the 2007 season. Interestingly, Ukulinga and Eston Estate fruit did not show chilling injury symptoms up to 28 days into -0.5°C cold storage (Table I). Hot water treatment at 47°C was not as effective in mitigating chilling injury as HW 53°C, with chilling index of 0.18 and 0.09, respectively. The low concentration of Mo (1 µM Mo) reduced chilling injury significantly compared with the control (water dips), resulting in a chilling injury index of 0.04. The most effective combination treatments to mitigate chilling injury were; 1 µM Mo plus HW 53°C and 10 µM Mo plus HW 53°C resulting in a chilling injury index of 0.04 and 0.00, respectively (Table I).

Effect of fruit source on pre and post-harvest soluble sugars, ascorbic acid and proline on chilling susceptibility

Only fruit sourced from Sun Valley Estates showed significant chilling injury symptoms cold storage (Table I). Such fruit also contained significantly lower glucose and AA levels at pre and post-harvest compared with chilling-resistant lemon fruit from other sources (Figure IIA and B). In addition, lower AA concentration for Eston Estates fruit indicates chilling susceptibility (Figure IIA) which is shown by HPLC chromatography (Figure III). Furthermore, chilling susceptible lemon fruit from Sun Valley and Eston Estates had a lower flavedo AA concentration than chilling-resistant fruit from Ukulinga (Figure III).

Furthermore, chilling-susceptible lemon fruit from Sun Valley and Eston significant lower flavedo proline than chilling-resistant lemon fruit from Ukulinga farm at pre-harvest (Figure IIC). However, the proline concentration significantly increases for Sun Valley and Eston lemon after 28 days into cold storage (post-harvest) (Fig IIC).

With respect to reducing sugars (fructose and sucrose), Sun Valley had significantly lower reducing sugars during the 2007 harvest season (Figure IID and E). Chilling resistant lemon fruit from Ukulinga 2008 had significantly higher sucrose concentration (Figure IIE).

Principal component analysis

Principal component analysis (PCA) of all variables with respect to fruit source led to 100% variation being explained by PC1 because of differences in variable units (Figure IIIA). The variables that were major contributors to variation explained by PC1 were fructose, vitamin C and proline (Figure IIIA) and the principal score plot showed significant differences between chilling susceptible fruit (Sun Valley and Eston) and chilling-resistant lemon fruit from Ukulinga farm (Figure IIIB).

Effect of cold storage time on reducing sugars, and the glucose-ascorbic acid and glucose-proline relationship during cold storage

Cold storage induced an alteration in flavedo glucose-ascorbic acid for lemon fruit from all sources (Tables IIA and B). However, there was significant reduction in flavedo glucose levels 21 days into cold storage for both chilling-susceptible and chilling-resistant lemon fruit. A glucose-ascorbic synergism was observed in the flavedo of Ukulinga and Eston lemon fruit 21 to 28 days into cold storage. However, such a glucose-ascorbic acid relationship did not hold for chilling-susceptible lemon from Sun Valley 21 days into cold storage (Table IIA).

A critical cold storage period seems to at 21 days even for proline (Tables IIA and B). Fruit from all sources showed an increase in flavedo proline 21 days into cold storage with the exception of chilling-susceptible fruit from Sun Valley (Table IIA). This increase in proline concentration was associated with a significant decrease in glucose concentration (Tables IIA and B), indicating a possible glucose proline relationship induced by cold stress.

Reducing sugars significantly decreased 14 days into cold storage in the flavedo of Sun Valley fruit; whereas in the flavedo of chilling-resistant fruit from Ukulinga maintained the levels reducing sugars up to 28 days into cold storage (Tables IIA and B).

Effect of HW and Mo dips on lemon flavedo glucose-ascorbic acid, glucose-proline, and reducing sugar relationships during cold storage

The effect of HW and Mo dips on glucose-AA and glucose-proline relationship was not evident between 0 to 14 days for all fruit source. However, HW plus Mo showed positive glucose-AA and glucose-proline relationship between 21 to 28 days into cold storage condition (Tables III and IV).

Fruit treated with 1 µM Mo had an increased flavedo AA concentration with significant decreased glucose concentration 21 days into cold storage for Ukulinga, and Eston lemon fruit (Tables III and IV). In Sun Valley fruit, 1 µM Mo dip induced an early decrease (14 days cold storage) in AA and showing no clear glucose-AA relationship existed (Table III).

The 1 µM Mo plus HW 53°C treatment effect induced a glucose-AA relationship for Ukulinga fruit during the 2007 harvest season, whereas, 10 µM Mo plus HWD 53°C was effective for lemon fruit from all sources (Tables III and IV).

Furthermore, HW treatments without Mo did not show any effect on the glucose-AA relationship during cold storage except in Eston fruit (Table III). For Ukulinga and Sun Valley fruit, HW induced a significant decrease in glucose coupled with a decrease in AA concentration (Tables III and IV).

Interestingly, the glucose-proline relationship was influenced by HW treatments which the findings were contrary to glucose-AA relationship. A significant decrease in glucose was clearly coupled with significant increase in flavedo proline for Ukulinga, Sun Valley Estates fruit when dipped into HW 47 and 53°C between 21 and 28 days into cold storage (Tables III and IV).

Moreover, the 1 µM Mo treatment induced a significant increase in proline in the flavedo of lemon fruit from all sources during cold storage. However, 1 µM Mo and 10 µM Mo plus HW 53°C showed a significant increase in proline with a decrease in glucose in the chilling-susceptible fruit from Sun Valley (Table III), a feature aligned with reduced chilling symptoms (Table I).

The effect of HW and Mo dips on reducing sugars was not clear for chilling-resistant fruit as compared with chilled fruit. Flavedo reducing sugars were either increase or maintained for chilling-resistant lemon fruit during cold storage (Table III and IV). With Sun Valley fruit, reducing sugars significantly decreased 14 days into cold storage with all treatments except for 1 µM Mo dips (Table III).

Discussion

Only lemons from Sun Valley Estates showed quantifiable chilling symptoms (Table I). According to Chalutz et al. (1985), however, lemon fruit are the second most chilling-susceptible type within the citrus family, after grapefruit. Previously, it has been reported that chilling injury can be attributed to climatic differences, leading to chemical differences in the lemon flavedo (Aung et al., 1998, 1999, 2001). Furthermore, certain lemon cultivars are more chilling susceptibility than others; Underhill et al. (1999) found 'Lisbon' lemons to be more chilling-susceptible than 'Eureka' lemon fruit stored at 1°C for up 42 days.

Our results show that HW 53°C has a greater potential to mitigate chilling injury than compared with the control and HWD 47°C (Table I). A positive effect of HW 53°C to mitigate chilling injury has been reported by several researchers; in 'Valencia' oranges, showed to reduced chilling injury with chilling index of 0.45 following HWD 53°C for 3 min (Erkan et al., 2005); in 'Torroco' blood oranges, to reduced decay when fruit were stored at 3°C for up to 10 weeks (Schirra et al., 1997). So far, previous work pin pointed the induction of heat shock proteins (HSPs) by HWD 53°C as physiological role to increase chilling resistance in fruit (Rozenzvieg et al., 2004).

The ability of Mo to mitigate chilling injury, especially at low (1 µM Mo) concentration (Table I), can must be attributed to an increased in activity of xanthine dehydrogenase (XDH), an enzyme that produces reactive oxygen species (ROS) when plants are exposed to stress. Recently, ROS have been found to act as stress acclimation induce in plant cells when present below a critical threshold level (Foyer and Noctor, 2005; Foyer and Shigeoka, 2011). Salinity increased XDH activity (Sagi et al., 1998), while cold stress and salinity decreased Hesberg et al., (2003); hence it is not clear whether XDH activity is positively or negatively correlated to stress.

In general, lemons and any other citrus fruit contain three dominating flavedo sugars, with glucose present in the highest concentration, and reducing sugars (sucrose and fructose) in lower, but almost equal proportions (Aung et al., 2001). The accumulation of glucose in lemon flavedo is determined by pre-harvest conditions; including fruit source such that, coastal lemons accumulate more flavedo glucose than desert lemons (Aung et al., 1999). The accumulated glucose might have multiple physiological functions during stress exposure.

The conversion of glucose to AA was not clear with chilling-susceptible lemon fruit (Table IIA) but from chilling-resistant lemon fruit (Table IIIB). The conversion was speculatively reduced because of lower flavedo glucose accumulated at pre-harvest. Therefore, the conversion could be a secondary process which depends on available glucose concentration and signaled by reduction in cytoplasmic glucose level as oxidative stress intensifies, in this case evident 21 days into cold storage; primarily glucose support the pentose phosphate pathway (Zheng et al., 2001; Shetty 2004; Sarkar et al., 2009). Seemingly, hot water and molybdenum post-harvest dips have no ability to override the effect of low glucose concentration and possible influence on conversion of glucose to AA during post-harvest stress. However, when glucose concentration was higher, there was an increase in AA levels and hot water plus molybdenum dips seem to signal the conversion of glucose to AA and, therefore, increased antioxidant capacity. The conversion is important because AA is a major antioxidant in citrus (Rapisarda et al., 2008) and therefore low AA results in low glucose-AA conversion leading to chilling susceptibility of Sun Valley Estates fruit (Table IIA). In contrast, Eston fruit also contained low AA but less chilling-susceptible than Sun Valley Estates fruit (Tables IIA and B). It can be assumed that higher glucose concentration compensated for low AA in the flavedo of Eston fruit, which allowed for a glucose-AA conversion (Figure IB) (Loewus, 1999; Smirnoff, 2000; Hancock and Viala, 2002) and therefore, increased antioxidant capacity might have enhanced chilling resistance in such fruit (Table I).

The sysnthesis of proline is from glutamate (Figure IA) (Ruiz et al., 2002) coupled with an increase of G6PDH during stress exposure (Figure I) (Shetty, 2004; Ashraf and Foolad, 2007). Proline accumulation increases with exposure to environmental stress, such as drought (Dobra et al., 2010), heat stress (Dobra et al., 2010; Cvikrová et al., 2011), water (Grote et al., 2006) and salinity (Gzik, 1996; Kinipp and Honermeier, 2006; Yazici et al., 2007). The proline concentration of lemon flavedo increased during subzero (-0.5°C) storage (Tables IIA and B), however, this increase depended on fruit source. Proline has been reported to increase in *Phaseaolus vulgaris* cv. Strike seedlings following cold shock and storage at 4°C (Ruiz et al., 2002); and in Puma rye (*Secale vereale* L. cv. Puma) cold stored for up to three weeks (Koster and Lynch, 1992).

Post-harvest heat-conditioning of 'Fortune' mandarin fruit by exposure to 37°C for three days increased flavedo glucose breakdown coupled with increased cold stress resistance (Holland et al., 2002). Furthermore, decrease in glucose concurrently could have induced an increase in the activity of pyrroline-5-carboxylate (P-5-C) (Shetty, 2004; Ashraf and Foolad, 2007), an enzyme synthesizing proline from glutamate (Figure 1A). Hot water dips plus Mo further enhanced the breakdown of glucose, while a significant increase in proline was obesrved. The possible increase in XDH activity and increased production of ROS (below damaging critical threshold levels) (Datta et al., 1991; Hesberg et al., 2003; Yesbergenova et al., 2005) acted as secondary signal for increased activity of P-5-C therefore, increasing proline production an antioxidant.

Moreover, Aung et al. (2001) observed a significant decrease in glucose coupled with a significant increase in sucrose concentration in lemon flavedo during cold storage. Sucrose has been directly linked with plant stress tolerance (Holland et al., 2002) and synthesized from glucose and fructose by enzyme sucrose phosphate synthase (Komatsu et al., 1999). Bean (1985) proved that sucrose is produced from glucose by using a ^{14}C glucose. Seemingly, the activity of sucrose phosphate synthase is fruit source dependent as Aung et al. (2001) found different sucrose levels with desert lemons compared with coastal lemons and therefore, differences in chilling susceptibility.

The primary influence is on glucose and the breaking-down of glucose signals for increased activity of D-Glucose-P and all other enzymes involved (Fig IA and B) resulting in increased production of AA, proline and finally reduced oxidative stress.

In conclusion, the efficacy of any post-harvest treatment to mitigate chilling injury in citrus fruit is dependent on fruit source. Fruit source and pre-harvest growing conditions determines flavedo chemical composition of citrus fruit and therefore, chilling susceptibility. Furthermore, Mo treatments at lower concentration plus HWD can enhance chilling resistance in citrus fruit by increasing the speculative conversion of glucose to AA, increasing proline synthesis and possibly reducing sugars at critical cold storage time. The post-harvest concentration of glucose seems to regulate most plant cellular activities and therefore chilling susceptibility.

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List of Tables

TABLE I: Effect of hot water and molybdenum post-harvest dips on chilling symptoms of lemon fruit cold stored (-0.5°C) for up to 28 days.

TABLE II: Effect of cold storage time on lemon flavedo glucose, ascorbic acid, fructose, sucrose and proline of lemons from (A) Ukulinga and Sun Valley during 2007 harvest season (B) Ukulinga and Eston Estates during 2008 harvest season cold stored (-0.5°C) over 28 days

TABLE III: Effect of hot water and molybdenum post-harvest dips on lemon flavedo glucose, ascorbic acid, fructose, sucrose and proline over 28 days cold storage (-0.5°C) during 2007 harvest season

TABLE IV: Effect of hot water and molybdenum post-harvest dips on lemon flavedo glucose, ascorbic acid, fructose, sucrose and proline over 28 days cold storage (-0.5°C) during 2008 harvest season

List of Figures

FIGURE I: The proposed pathway illustrating the physiological role glucose in (A) proline synthesis from glutamate (Ashraf and Foolad, 2007) and (B) ascorbic in higher plants (Loewus, 1999; Smirnoff, 2000; Hancock and Viola, 2002). The enzymes are: (a) HK-hexokinase; (b) glucose phosphate isomerase; (c) PGI-phosphomannase isomerase; (d) PM-phosphomannase mutase; (e) GDPME-GDP-mannose pyrophosphorylase; (e) GDPME-GDP-mannose-3,5-epimerase; (f) ? – uncharacterized enzymes; (g) L-Gal DH-L-galactose dehydrogenase; and (h) L-Gal DH-L-galactono-1,4-lactone dehydrogenase

FIGURE II: Effect of lemon fruit source on flavedo (A) glucose (B) Ascorbic (C) proline (D) fructose and (E) sucrose concentration

FIGURE III: Chromatographic representation of lemon flavedo ascorbic acid before cold storage (A) Ukulinga 2007 (B) Eston Estates 2008 (C) Sun Valley Estates 2007 and (D) Ukulinga 2008

FIGURE IV: Principal component analysis (PCA) showing correlation loadings A. Score plot lemon flavedo glucose, ascorbic acid, fructose, sucrose and proline B. Score plot for the

groups of the glucose, ascorbic acid, fructose, sucrose and proline for lemons from different sources with differences in chilling susceptibility, PC1 explains 100% and PC2 explaining 0% total variation

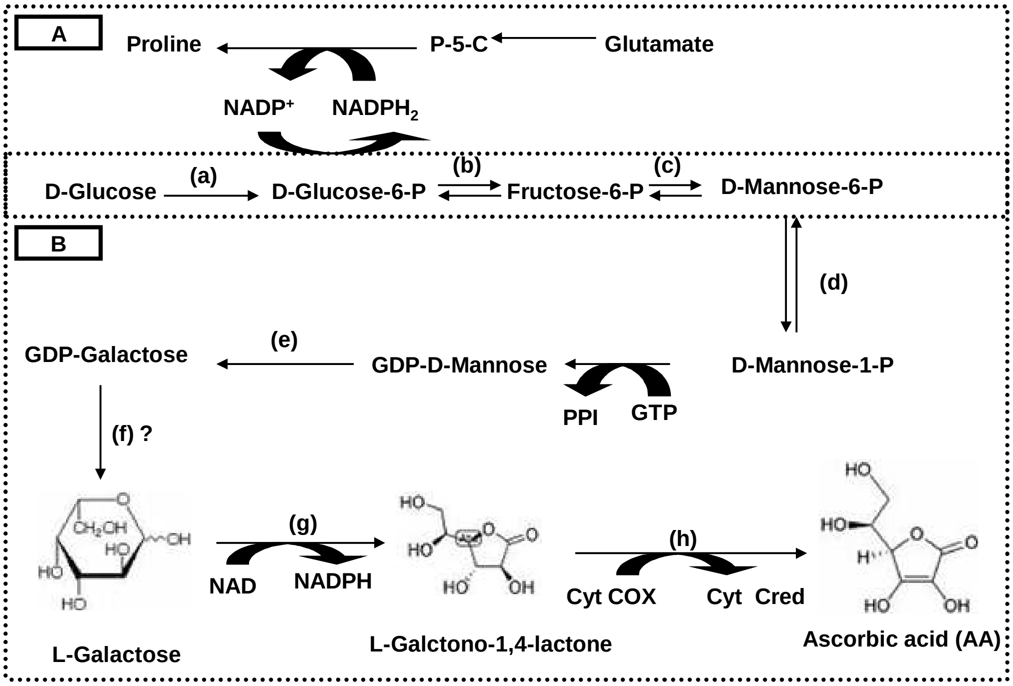
A
Proline
P-5-C
Glutamate
NADP+
NADPH2
D-Glucose
(a)
D-Glucose-6-P
(b)
Fructose-6-P
(c)
D-Mannose-6-P
B
(d)
GDP-Galactose
(e)
GDP-D-Mannose
D-Mannose-1-P
PPI
GTP
(f) ?
(g)
NAD
NADPH
(h)
Cyt COX
Cyt Cred
L-Galactose
L-Galctono-1,4-lactone
Ascorbic acid (AA)

FIG I:

TABLE I:

Treatments[a]	Ukulinga 2007[b]	Sun Valley Estates 2007[c]	Ukulinga 2008[b]	Eston Estates 2008[d]
Water dip (25°C)	No symptoms	0.13a	No symptoms	Below detection
HW 47°C	No symptoms	0.18a	No symptoms	Below detection
HW 53°C	No symptoms	0.09a	No symptoms	Below detection
1 µM Mo	No symptoms	0.04ab	No symptoms	Below detection
1 µM Mo + HW 53°C	No symptoms	0.02ab	No symptoms	Below detection
10 µM Mo + HW 53°C	No symptoms	0.00b	No symptoms	Below detection

[a] Treatment effect after 28 days cold storage plus 5 days shelf-life

[b] Ukulinga lemon fruit showed no chilling symptoms during 2007 and 2008 harvest seasons

[c] Sun Valley lemon fruit showed chilling symptom expressible in chilling index and means followed by the same letter were not significantly different at $P < 0.005$

[d] Eston Estates lemon did show chilling symptoms but below detection and chilling index calculation.

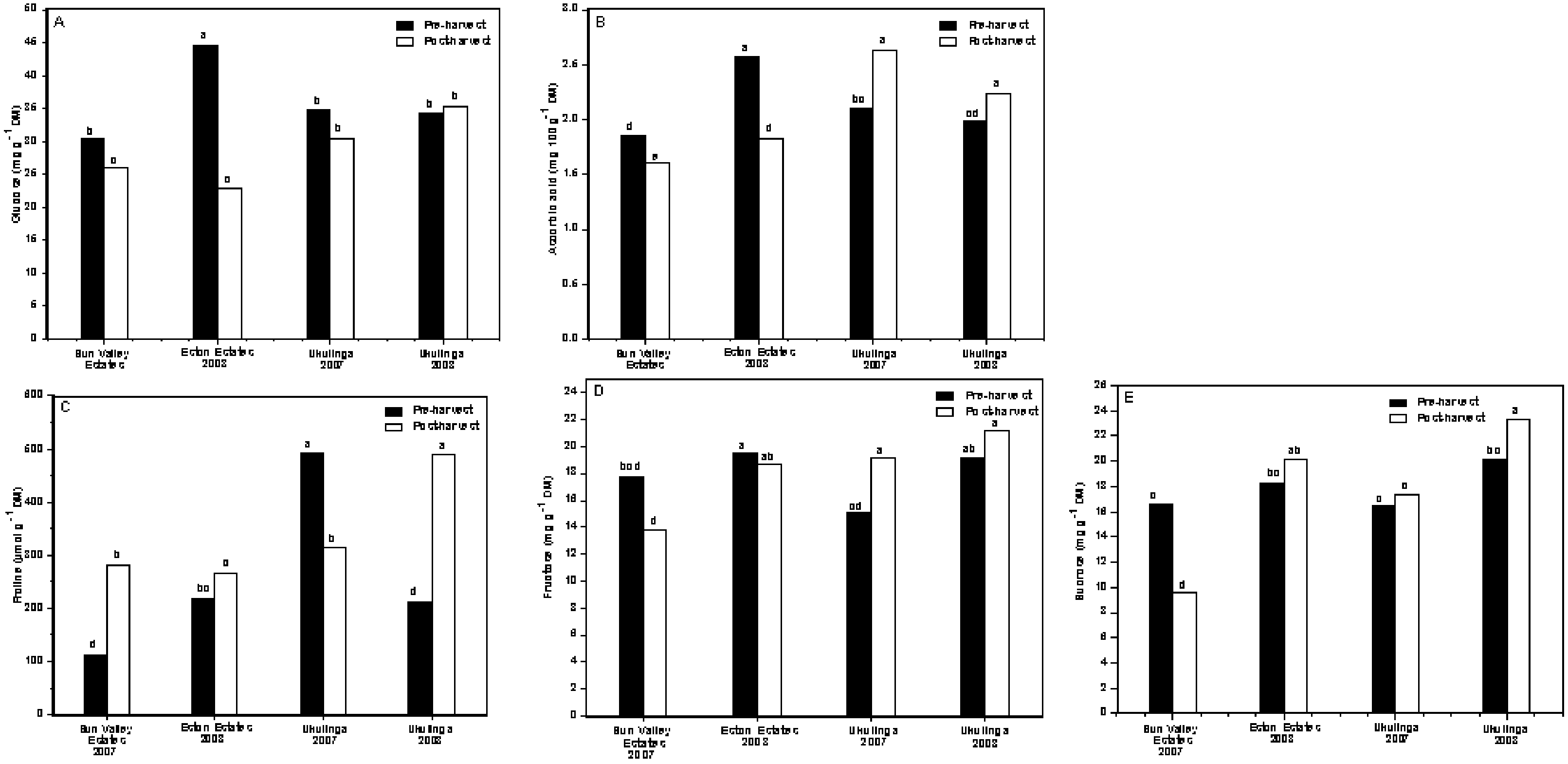
A
Glucose (mg g-1 DM)
0
5
10
15
20
25
30
35
40
45
50
Pre-harvest
Postharvest
b
c
a
c
b
b
b
b
Sun Valley Estates
Eston Estates 2008
Ukulinga 2007
Ukulinga 2008
B
Ascorbic acid (mg 100 g-1 DM)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Pre-harvest
Postharvest
d
e
a
d
bc
a
cd
a
Sun Valley Estates
Eston Estates 2008
Ukulinga 2007
Ukulinga 2008
C
Proline (µmol g-1 DM)
0
100
200
300
400
500
600
Pre-harvest
Postharvest
d
b
bc
c
a
b
d
a
Sun Valley Estates 2007
Eston Estates 2008
Ukulinga 2007
Ukulinga 2008
D
Fructose (mg g-1 DM)
0
2
4
6
8
10
12
14
16
18
20
22
24
Pre-harvest
Postharvest
bcd
d
a
ab
cd
a
ab
a
Sun Valley Estates 2007
Eston Estates 2008
Ukulinga 2007
Ukulinga 2008
E
Sucrose (mg g-1 DM)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Pre-harvest
Postharvest
c
d
bc
ab
c
c
bc
a
Sun Valley Estates 2007
Eston Estates 2008
Ukulinga 2007
Ukulinga 2008

FIG II:

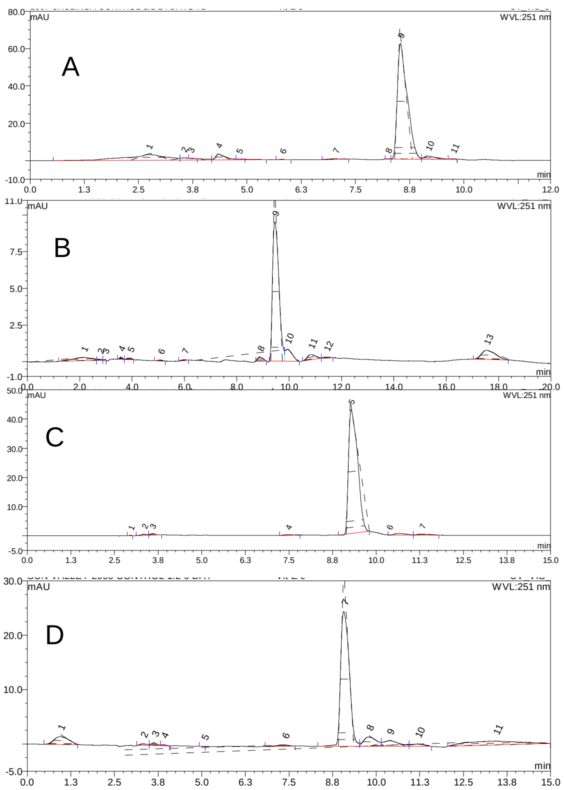
A
80.0
60.0
40.0
20.0
-10.0
mAU
WVL:251 nm
1
2
3
4
5
6
7
8
9
10
11
min
0.0
1.3
2.5
3.8
5.0
6.3
7.5
8.8
10.0
12.0
B
11.0
7.5
5.0
2.5
-1.0
mAU
WVL:251 nm
1
2
3
4
5
6
7
8
9
10
11
12
13
min
0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
20.0
C
50.0
40.0
30.0
20.0
10.0
-5.0
mAU
WVL:251 nm
1
2
3
4
5
6
7
min
0.0
1.3
2.5
3.8
5.0
6.3
7.5
8.8
10.0
11.3
12.5
13.8
15.0
D
30.0
20.0
10.0
-5.0
mAU
WVL:251 nm
1
2
3
4
5
6
7
8
9
10
11
min
0.0
1.3
2.5
3.8
5.0
6.3
7.5
8.8
10.0
11.3
12.5
13.8
15.0

FIG III:

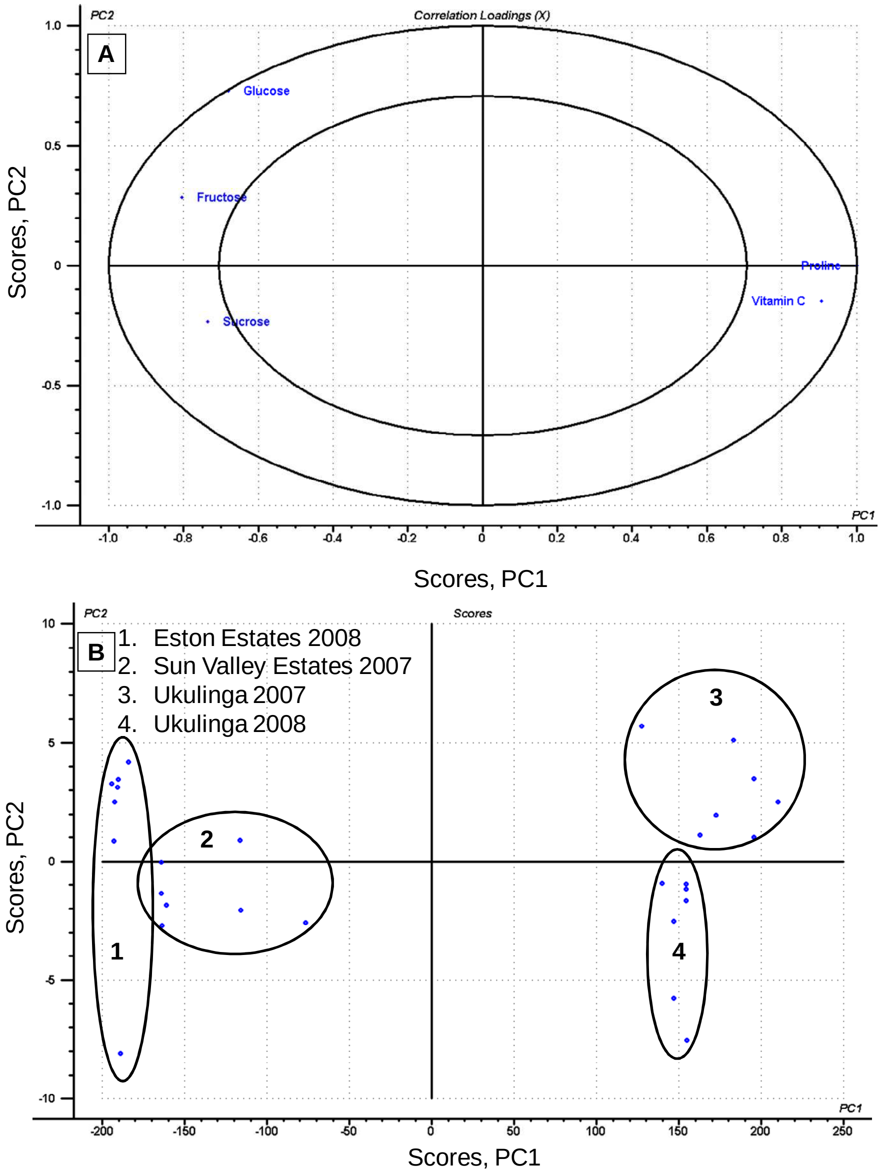
A
PC2
Correlation Loadings (X)
Glucose
Fructose
Proline
Vitamin C
Sucrose
1.0
0.5
0
-0.5
-1.0
PC1
-1.0
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1.0
Scores, PC2
Scores, PC1
B
PC2
Scores
1. Eston Estates 2008
2. Sun Valley Estates 2007
3. Ukulinga 2007
4. Ukulinga 2008
1
2
3
4
10
5
0
-5
-10
PC1
-200
-150
-100
-50
0
50
100
150
200
250
Scores, PC2
Scores, PC1

FIG IV:

Table IIA

Storage Time (day)	Ukulinga University farm 2007					Sun Valley Estates farm 2007				
	Sucrose[1]	Fructose[2]	Glucose[3]	AA[4]	Proline[5]	Sucrose[1]	Fructose[2]	Glucose[3]	AA[4]	Proline[5]
0	**18.19bcd**	**15.86def**	**34.24bcde**	**2.44d**	**381.0a**	**15.44fg**	**14.67ef**	**27.18f**	**2.19h**	**180.3gh**
7	**16.37def**	**16.21cde**	**31.90e**	**2.25g**	**323.1c**	**14.99fg**	**16.13cde**	**26.77f**	**1.99k**	**247.7f**
14	**16.94cdef**	**18.29ab**	**33.37cde**	**3.00a**	**272.9e**	**16.47def**	**18.48ab**	**32.18de**	**2.12i**	**244.3f**
21	**17.64bcde**	**19.01ab**	**37.91ab**	**2.49c**	**320.3c**	**13.85g**	**14.99ef**	**26.72f**	**1.92k**	**348.2b**
28	**17.53bcde**	**19.37ab**	**33.85cde**	**2.62b**	**307.1cd**	**9.81h**	**14.00f**	**24.67f**	**1.63m**	**272.4e**

Table IIB

Storage Time (day)	Ukulinga University farm 2008					Eston Estate farm 2008				
	Sucrose1	Fructose2	Glucose3	AA4	Proline5	Sucrose1	Fructose2	Glucose3	AA4	Proline5
0	**21.20a**	**17.96abc**	**34.19bcde**	**2.33f**	**198.7g**	**16.43def**	**18.64ab**	**37.09abc**	**2.12i**	**172.2h**
7	**20.93a**	**17.94abc**	**34.75bcde**	**2.18d**	**246.5f**	**18.14bcd**	**18.08abc**	**37.82ab**	**2.13i**	**197.9g**
14	**21.25a**	**18.36ab**	**33.45cde**	**2.59b**	**271.5de**	**18.97b**	**19.51a**	**35.93abcd**	**2.06j**	**199.6g**
21	**22.44a**	**18.90ab**	**35.20abcde**	**2.18h**	**365.7ab**	**18.57bc**	**18.82ab**	**38.68a**	**1.89l**	**165.0h**
28	**15.45fg**	**17.30bcd**	**24.18f**	**2.39e**	**381.9a**	**15.91ef**	**15.30ef**	**23.73f**	**2.06j**	**173.1h**

[1-3] Sugars mg g^{-1} DM

[4] AA-Ascorbic acid mg $100g^{-1}$DM

[5] Proline nmol g^{-1}DM

Means with different letters within same column represent significant difference at P=0.05.

TABLE III

Storage Time (day)	Treatments	Ukulinga University farm 2007					Sun Valley Estates farm 2007				
		Sucrose[1]	Fructose[2]	Glucose[3]	AA[4]	Proline[5]	Sucrose[1]	Fructose[2]	Glucose[3]	AA[4]	Proline[5]
0	Water dip (25°C)	16.42±0.85	15.09±1.32	30.37±3.31	2.11±0.05	49135±18.83	16.60±2.11	17.78±4.64	34.81±4.46	1.86±0.01	113.77±0.68
	HW 47°C	17.21±0.78	17.08±3.21	36.26±1.67	1.91±0.01	404.35±15.27	14.83±0.89	12.28±3.40	23.45±3.40	1.86±0.01	110.31±0.32
	HW 53°C	17.32±3.78	14.03±0.33	31.91±1.60	1.93±0.01	418.52±4.03	15.95±0.82	15.16±3.44	30.57±3.44	2.64±0.01	197.24±0.55
	1 µM Mo	18.03±2.26	15.70±1.50	34.95±4.65	1.88±0.01	248.62±1.77	16.51±3.36	16.03±0.37	26.99±0.37	1.82±0.03	157.62±0.60
	1 µM Mo + HW 53°C	21.25±1.75	17.10±2.02	38.15±3.88	3.28±0.02	284.88±0.78	15.78±1.75	12.85±3.74	26.55±3.74	2.73±0.03	243.61±1.77
	10 µM Mo + HW 53°C	18.92±3.82	16.15±0.62	33.80±2.13	3.56±0.03	438.35±18.21	12.96±2.45	13.91±2.15	20.70±2.15	2.24±0.01	259.42±2.57
7	Water dip (25°C)	15.87±0.22	16.41±0.98	30.14±2.13	2.49±0.03	249.54±4.35	13.74±5.20	17.63±5.31	31.72±4.15	1.62±0.03	216.81±0.61
	HW 47°C	16.20±2.17	16.08±2.72	32.36±2.26	1.88±0.01	184.96±0.59	15.04±4.04	16.42±7.19	28.87±4.13	2.03±0.01	283.57±1.39
	HW 53°C	17.59±3.70	17.06±1.39	33.27±6.45	2.61±0.02	342.93±2.78	15.00±3.59	16.32±6.59	25.89±6.97	2.56±0.02	214.00±0.59
	1 µM Mo	16.77±1.42	16.66±0.85	32.81±3.29	1.73±0.01	458.74±18.82	16.44±2.13	13.62±3.80	26.26±4.00	2.00±0.02	205.90±0.81
	1 µM Mo + HW 53°C	15.28±2.08	15.33±0.26	31.12±2.15	2.43±0.01	399.87±8.46	15.36±6.50	18.03±4.76	25.54±6.97	2.03±0.03	320.57±5.57
	10 µM Mo + HW 53°C	16.52±0.78	15.69±0.95	31.69±3.40	2.33±0.01	302.38±1.68	14.36±2.22	14.79±5.21	22.28±2.63	1.71±0.01	243.60±1.28
14	Water dip (25°C)	16.21±2.21	18.92±1.49	34.70±1.92	2.27±0.01	210.56±1.71	14.56±4.744	19.28±5.46	32.59±5.46	2.05±0.01	193.68±0.69
	HW 47°C	17.63±1.24	18.97±1.68	32.61±1.38	3.33±0.03	362.93±7.26	13.41±3.33	16.44±3.66	33.83±3.66	2.57±0.02	325.96±7.36
	HW 53°C	16.18±1.00	19.06±0.48	36.91±2.30	2.75±0.03	304.69±3.56	20.91±5.18	19.94±7.42	32.76±7.42	2.68±0.02	250.68±2.93
	1 µM Mo	18.31±0.76	18.71±0.58	35.04±1.41	4.27±0.03	195.63±0.44	17.02±1.55	20.13±8.89	31.24±8.89	1.61±0.01	224.65±1.69
	1 µM Mo + HW 53°C	15.66±2.50	16.27±1.91	28.82±4.02	3.17±0.04	229.58±1.41	16.10±4.69	20.10±3.56	31.11±3.56	2.10±0.01	241.66±1.71
	10 µM Mo + HW 53°C	17.64±2.40	17.83±1.84	32.13±1.92	2.21±0.01	334.26±5.25	16.84±1.55	15.03±3.42	31.54±3.42	1.69±0.03	229.45±1.08
21	Water dip (25°C)	16.67±0.68	17.93±1.63	37.30±6.11	3.80±0.04	238.65±0.75	15.91±1.54	15.75±4.32	32.48±4.32	1.99±0.03	270.43±3.74
	HW 47°C	17.02±1.36	17.29±0.82	34.10±1.86	2.19±0.01	377.41±10.89	12.32±1.50	14.25±4.33	29.18±4.33	1.83±0.01	388.98±5.82
	HW 53°C	16.97±0.74	18.13±0.30	36.81±3.87	3.05±0.17	437.33±8.98	13.72±1.10	15.23±1.94	28.42±1.94	1.89±0.01	228.06±1.99
	1 µM Mo	18.48±0.69	21.27±3.81	34.45±3.92	1.85±0.04	293.43±3.10	14.59±0.22	15.94±1.63	26.80±1.63	2.39±0.01	452.32±23.72
	1 µM Mo + HW 53°C	17.91±1.98	19.42±1.02	43.65±10.72	1.91±0.01	360.87±10.75	12.70±0.94	14.11±1.73	23.19±1.73	1.66±0.06	359.83±12.95
	10 µM Mo + HW 53°C	18.81±0.79	20.04±0.74	41.17±7.78	2.12±0.02	214.02±1.11	13.88±4.20	14.65±2.01	20.25±2.01	1.78±0.03	389.83±15.41
28	Water dip (25°C)	17.38±0.99	19.15±0.49	33.67±3.86	2.63±0.01	313.16±3.28	9.56±0.11	13.81±0.34	25.70±0.34	1.60±0.01	282.72±4.16
	HW 47°C	17.28±3.30	17.90±0.83	34.16±4.22	3.47±0.03	352.81±4.83	9.92±0.80	14.25±1.18	25.86±1.18	1.60±0.01	312.22±5.02
	HW 53°C	19.27±0.45	20.88±4.85	34.29±4.57	1.61±0.01	364.02±5.45	10.87±0.26	14.08±0.63	26.69±0.63	1.63±0.01	152.48±0.72
	1 µM Mo	16.27±1.59	17.97±2.89	32.87±6.56	2.13±0.01	286.23±0.79	9.42±0.22	14.24±0.60	22.10±0.60	1.65±0.01	325.84±5.31
	1 µM Mo + HW 53°C	17.64±0.82	20.22±0.99	34.54±1.72	2.51±0.01	378.65±8.79	9.50±0.07	14.03±0.78	24.06±0.78	1.65±0.01	347.44±9.54
	10 µM Mo + HW 53°C	17.33±0.84	20.13±1.46	33.56±0.86	3.39±0.02	147.48±0.28	9.60±0.15	13.57±0.26	23.60±0.26	1.63±0.01	213.85±1.01

[1-3] Sugars mg g^{-1} DM

[4] AA-Ascorbic acid mg 100g^{-1} DM

[5] Proline µmol g^{-1} DM

The values are means of 3 triplicates ± standard error (SE)

TABLE IV

Storage Time (day)	Treatments	Ukulinga University farm 2008					Eston Estate farm 2008				
		Sucrose[1]	Fructose[2]	Glucose[3]	AA[4]	Proline[5]	Sucrose[1]	Fructose[2]	Glucose[3]	AA[4]	Proline[5]
0	Water dip (25^{o}C)	20.21±0.65	18.72±1.24	34.24±1.24	1.98±0.14	212.46±15.82	18.28±3.08	19.46±1.22	44.62±2.36	2.57±0.19	217.89±0.92
	HW 47^{o}C	20.24±1.25	18.08±1.97	35.11±1.97	2.60±0.16	148.58±39.84	15.72±0.32	18.33±1.33	38.96±0.73	1.73±0.10	138.39±0.44
	HW 53^{o}C	22.23±1.14	16.08±1.21	35.57±1.21	2.90±0.11	218.85±18.68	16.13±0.55	19.63±0.96	33.98±3.74	2.06±0.19	158.07±1.08
	1 µM Mo	21.19±1.88	15.47±2.86	32.08±2.86	2.21±0.08	188.57±29.88	16.73±1.89	18.65±1.42	40.54±6.91	1.94±0.04	123.47±0.43
	1 µM Mo + HW 53^{o}C	20.29±1.61	18.52±0.75	32.18±0.75	1.73±0.06	244.49±26.02	15.75±0.39	16.10±2.18	28.15±0.67	2.81±0.11	140.99±0.48
	10 µM Mo + HW 53^{o}C	23.03±1.07	20.87±2.66	35.06±2.66	2.54±0.10	203.12±68.86	15.98±1.61	19.68±2.19	36.29±9.09	1.60±0.04	254.30±2.37
7	Water dip (25^{o}C)	23.43±5.04	19.41±0.65	33.86±0.65	2.52±0.18	339.68±77.94	15.96±1.24	16.87±1.79	41.36±0.88	1.82±0.06	182.93±0.67
	HW 47^{o}C	20.26±2.51	16.38±0.43	35.70±0.43	1.57±0.01	216.72±42.38	16.42±0.80	20.73±5.48	35.56±3.32	1.60±0.01	189.94±2.49
	HW 53^{o}C	21.58±0.57	19.14±1.69	38.94±1.69	2.31±0.08	296.02±84.56	21.67±2.35	21.53±1.34	37.71±1.83	3.10±0.19	214.00±0.87
	1 µM Mo	21.68±0.50	16.67±2.37	37.15±2.37	2.57±0.23	152.37±58.86	17.41±1.32	14.90±2.15	40.54±5.10	1.82±0.03	190.38±2.85
	1 µM Mo + HW 53^{o}C	20.26±0.54	18.15±1.86	32.48±1.86	2.02±0.03	257.75±8.69	18.40±1.70	17.70±4.60	34.99±2.66	1.66±0.03	276.99±4.99
	10 µM Mo + HW 53^{o}C	18.37±4.65	17.87±3.18	32.37±3.18	2.09±0.02	279.35±85.05	18.96±3.07	16.76±4.19	36.77±14.19	2.80±0.28	133.20±0.21
14	Water dip (25^{o}C)	22.82±5.06	16.87±0.86	31.65±6.71	2.46±0.07	134.68±10.88	18.17±1.75	17.16±2.03	31.21±3.89	1.93±0.05	91.27±2.20
	HW 47^{o}C	21.57±0.93	17.80±2.29	33.80±2.22	2.13±0.02	331.96±41.52	18.72±0.93	20.09±1.50	36.45±6.12	1.59±0.04	422.80±15.23
	HW 53^{o}C	20.62±0.69	18.27±1.47	32.15±4.66	3.49±0.08	267.25±41.29	21.03±0.42	20.68±0.74	39.46±2.78	2.64±0.09	107.70±0.45
	1 µM Mo	21.31±1.67	18.41±0.40	37.71±5.18	3.62±0.02	348.93±58.07	19.70±3.44	19.78±1.09	35.28±7.60	2.29±0.09	140.01±0.35
	1 µM Mo + HW 53^{o}C	20.59±1.12	17.61±0.19	30.94±3.26	2.16±0.02	257.15±55.18	17.90±2.12	20.08±3.81	39.82±7.90	1.63±0.02	159.04±2.03
	10 µM Mo + HW 53^{o}C	20.60±1.50	21.22±2.53	34.43±1.17	1.66±0.01	368.20±67.86	18.28±1.27	19.30±0.67	33.35±8.82	2.26±0.05	276.51±3.25
21	Water dip (25^{o}C)	23.46±0.61	19.67±0.34	35.11±5.08	2.13±0.05	261.72±73.27	19.90±6.88	20.86±2.16	47.47±8.51	1.99±0.01	192.49±0.50
	HW 47^{o}C	21.09±4.11	18.36±4.21	33.53±9.60	2.06±0.02	413.40±38.63	20.46±2.62	17.19±1.56	40.70±8.88	1.62±0.01	84.15±0.76
	HW 53^{o}C	23.84±1.32	18.53±1.14	37.50±1.25	2.46±0.06	373.65±32.43	19.27±3.40	18.76±1.36	37.50±6.87	1.76±0.04	138.06±0.33
	1 µM Mo	18.36±5.15	18.26±1.79	34.24±4.48	2.41±0.02	469.64±57.32	21.81±8.55	21.55±3.02	40.35±6.00	1.59±0.01	275.67±2.54
	1 µM Mo + HW 53^{o}C	23.29±0.51	18.39±1.48	36.22±4.41	2.10±0.04	415.96±33.43	15.31±3.73	18.00±2.17	38.09±12.84	2.78±0.20	94.40±0.15
	10 µM Mo + HW 53^{o}C	24.58±4.8	20.16±0.87	34.60±4.04	2.01±0.04	200.09±36.04	14.63±1.73	16.58±1.44	27.96±8.12	1.59±0.01	205.38±0.77
28	Water dip (25^{o}C)	23.37±2.11	21.16±1.42	35.25±1.23	2.24±0.04	488.22±90.62	15.03±2.29	14.49±1.33	22.94±4.48	1.83±0.06	267.52±2.29
	HW 47^{o}C	18.70±1.28	17.09±1.75	27.47±4.57	1.76±0.06	372.28±29.15	15.65±3.09	15.24±5.07	25.60±3.53	2.38±0.07	206.76±1.58
	HW 53^{o}C	11.58±0.72	13.21±1.63	18.25±1.71	1.83±0.01	465.72±36.61	16.77±2.51	14.55±1.52	23.13±2.72	2.03±0.02	81.54±0.38
	1 µM Mo	13.02±1.45	15.76±1.75	20.91±4.55	3.69±0.01	476.86±49.99	14.86±3.37	15.26±1.93	20.04±3.98	1.83±0.02	274.06±2.40
	1 µM Mo + HW 53^{o}C	14.10±0.63	18.52±0.96	22.60±0.95	1.87±0.02	251.00±19.10	16.85±4.40	14.84±2.41	26.93±2.79	2.01±0.012	80.75±0.47
	10 µM Mo + HW 53^{o}C	11.96±1.49	18.03±2.54	20.60±5.44	2.94±0.05	287.19±0.98	16.30±4.93	16.41±2.75	23.76±4.43	2.27±0.04	128.22±0.41

[1-3] Sugars mg g^{-1} DM

[4] AA-Ascorbic acid mg 100g^{-1} DM

[5] Proline nmol g^{-1} DM

The values are means of 3 triplicates ± standard error (SE)

CHAPTER 4

Hot Water and Molybdenum Dips: The Case of Antioxidant Assays in Lemon Flavedo during Cold Storage

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Abstract

Antioxidants are part of the plant's defence system, protecting tissue against oxidative stress. In citrus fruit, oxidative stress can occur during extended cold storage ultimately result in chilling injury. Citrus fruit contain certain rind antioxidants which occur in different forms (lipophilic and hydrophilic). Previous studies demonstrated a synergistic relationship between hot water (HW) and molybdenum (Mo) dips in mitigating chilling injury in citrus fruit. Therefore, the aim of this study was to investigate the potential of HW and Mo to alleviate chilling injury by enhancing antioxidant capacity in lemon flavedo of fruit stored at -0.5°C. Fruit from different sources, commonly displaying chilling injury and other seemingly resistant to the development of chilling symptoms, were preconditioned for 2 min with HWD (47°C or 53°C) and thereafter soaked in 1 or 10 μM Na_2MoO_4 solution for 30 min. Fruit were subsequently stored at -0.5°C for 7, 14, 21 or 28 days, moved to ambient temperature for a week and then evaluated for chilling injury symptoms. Chilling susceptible lemon fruit showed low Trolox equivalent total antioxidant capacity (TEAC), phenolic, and flavonoid concentrations and high lipid peroxidation. Hot water dip at 53°C, as well as 1 μM Mo and 10 μM Mo plus HWD 53°C treatments mitigated chilling injury by enhancing total antioxidant capacity, and total phenolic and flavonoid concentrations in the flavedo. However, the ability of these treatments to alter lipophilic and hydrophilic antioxidants was source of fruit dependent.

Keywords: Lemon (*Citrus limon*); chilling injury; antioxidant assays; hot water dips; molybdenum; cold storage

1. Introduction

A large portion of the world's citrus produce is transported over long distances and often requires cold sterilization as a phytosanitary requirement against fruit fly for importing countries such as Japan and the US. However, long-term cold storage may result in chilling injury (McLauchlan et al., 1997). Within the citrus fruit family, lemons are the second most chilling susceptible fruit after grapefruit (Chalutz et al., 1985). Chilling symptoms appear as sunken lesions, discolouration of peel and pitting, severely reducing fruit marketability (McLauchlan et al., 1997; Shellie and Mangan, 2002). Citrus fruit have a wide array of antioxidants, which play major roles in the defence against oxidative damage including chilling injury (Abeysinghe et al., 2007).

Oxidative stress results from damage to membrane lipids, DNA and protein caused by reactive oxygen species (ROS) ultimately leading to cell death (Huang et al., 2007). Reactive oxygen species such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH-) are unstable molecules and, therefore, rapidly attack bio-molecules; under stress the effect of ROS is even more severe (Tomizawa et al., 2005). Plants have developed a well-integrated antioxidant defence system to scavenge ROS and keep them below damaging levels (Huang et al., 2007). Recent research has revealed ROS as not only damaging molecules but also as stress acclamatory signaling molecules (Foyer and Shegeoka, 2011). Therefore, methods to mitigate oxidative stress need to consider ROS as both damaging and signaling molecules, in order to balance the cellular redox homeostasis and signaling (Foyer and Noctor, 2005, Foyer and Shegeoka, 2011).

Previous research has shown molybdenum (Mo) postharvest dips to also alleviate chilling symptoms in citrus fruit (Mathaba et al., 2008). Molybdenum, as a co-factor enzyme xanthine dehydrogenase (XDH), has been associated with oxidative stress (Hesberg et al., 2003, Yesbergenova et al., 2007) as the activity of XDH is increased by salinity (Sagi et al., 1998) and decreased by cold and salinity stress (Hesberg et al., 2003). Therefore, the effect of XDH activity during stress is still not well understood. However, Mo remains an essential element in plant metabolism and plays an important role in balancing redox homeostasis (Mendel and Schwarz, 1999). Hypothetically, addition of Mo would normalize activity of XDH and increase production of ROS related XDH activity which, in turn, would signal cold stress acclimation through increased antioxidants.

There is a growing need for non-chemical methods to mitigate postharvest disorders in citrus fruit and hot water dips have been found to be an effective alternative (Irtwange, 2006). Hot water treatments can enhance production and activation of heat shock protein (HSPs) (Rorat et al., 2002; Rozenzvieg et al., 2004) which function as molecular chaperones by assisting in protein folding, assembly and transport, as well as in directing damaged proteins towards proteolysis (Rozenzvieg et al., 2004). Furthermore, these HSPs prevent protein aggregation at higher temperatures (Pavoncello et al., 2001) and presumably also at low temperatures.

The counteraction of fruit to temperature stress can be affected by an array of antioxidants which make up a full defence mechanism in plant cells against oxidative damage (Huang et al., 2007). As the nature of antioxidants is diverse, various assays are used to estimate plant antioxidants (Re et al., 1999). The increase in the number of total antioxidant assays is due to the varying efficacy of assays to estimate antioxidants in different tissues. Several assays are commonly used to estimate total antioxidant capacity of a certain tissue; these include the 2, 2'-azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay (Pellegrini et al., 2003), the 1, 1'-diphenyl-2-picrylhydrazyl (DPPH) assay (Wang et al., 2005) and the ferric reducing power (FRAP) assay (Li et al., 2006). Antioxidant assays, such as DPPH and ABTS are classified as inhibition assays, as by donating a hydrogen or electron of a preformed free radical which expresses antioxidant capacity and some assays involve an antioxidant during the generation of a free radical (Re et al., 2008). The stable free radical DPPH contains an unpaired valence electron at one nitrogen atom bridge (Jin and Chen, 1998), the scavenging ability of this radical has increased the applicability of this antioxidant assay (Sharma and Bhat, 2009; Lee et al., 2007). The ABTS assay depends on the very stable ($ABTS^{\bullet+}$) radical which is produced by ABTS reacting with potassium persulfate to form the blue/green $ABTS^{\bullet+}$ chromophore. Numerous lipophilic and hydrophilic antioxidants reduce $ABTS^{\bullet+}$ to ABTS, with the time needed to form the ABTS depending on incubation time and the concentration of the antioxidant (Re et al., 2008).

The FRAP assay is based on the reduction of the ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex to ferrous tripyridyltriazine (Fe^{2+}-TPTZ) by a reductant at low pH, producing a deep blue colour when reacted with an antioxidant (Szőllősi and Varga, 2002).

The citrus flavedo contains an array of lipophilic and hydrophobic antioxidants which constitute a comprehensive defence system against oxidative stress. Therefore, an antioxidant assay that will be able to give a holistic antioxidant measure is required to fully understand the antioxidant strength of citrus flavedo.

The aim of this work was therefore to investigate the ability of HW and Mo dips to mitigate chilling injury and to identify the role played by antioxidants present in the flavedo during cold stress. Moreover, the efficacy of different antioxidant assays in quantifying the antioxidant capacity of lemon flavedo treated with hot water and molybdenum was studied.

2. Materials and Methods

2.1 Standards and chemicals

Naringin (naringenin-7-rhamnosidoglucosidose), hesperidin (hesperetin-7-rutinoside), Trolox® (6-hydroxy-2,5,7,8,-tetramethylchroman-2-carboxylic acid), 1,1'-diphenyl-2-picrylhydrazyl (DPPH), (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) ABTS, 2,4,6-tripyridyl-s-triazine (TPTZ), and α-tocopherol (vitamin E), β-carotene, ascorbic acid (vitamin C), rutin, chlorogenic acid, Folin-Ciocalteu reagent, ferric chloride ($FeCl_3.6H_2O$) were obtained from Sigma-AIdrich Chemical Co. (St. Louis, Mo, USA).

2.2 Plant materials

Lemon (c.v. Eureka) fruit were obtained from Ukulinga Research Farm (29°39'48.82''S; 30°24'19.89''E) and Sun Valley Estate Farm (28°51′00″S; 30°04′00″E) during the 2007 harvest season and from Ukulinga Research Farm and Eston Estate Farm (29°47'00''S; 29°27'00''E) during the 2008 harvest season. Fruit were soaked for 30 min in a 1 μM $Na_2MoO_4.2H_2O$ solution followed by a 2 min HW 53˚C. Treated fruit were waxed, left to air-dry, weighed and stored at -0.5˚C for up to 28 days, and sampled after 7, 14, 21 or 28 days for chilling injury evaluation. A second evaluation was carried out five days after withdrawal from cold storage, fruits were peeled and the peel freeze-dried, milled using mortar and pestle and stored at -21˚C for further physiological analysis.

2.3 Estimation of chilling injury

The method of Sala (1998) was used to evaluate chilling injury 5 days after fruit were removed from cold storage. The method is based on a rating scale of surface browning intensity (0 - sound fruit, 1 - less than 10%, 2 – 10 - 20%, 3 – 30 - 40% and 4 - more than 50% pitting). The chilling injury index (CII), which expresses the severity of cold damage, was calculated by adding the products of the number of fruit in each category by the value assigned to this category in the rating scale and dividing the sum by the total number of fruit evaluated.

> CII = Σ (number of fruit with chilling injury x score of severity) / Total number of fruit evaluated

2.4 **Quantification of total antioxidant capacity**

2.4.1 Extraction of total antioxidants

Total antioxidants were extracted according to Halvorsen et al. (2002) with minor modifications. Freeze-dried lemon flavedo (0.1 g) was mixed with 5 ml 1 N perchloric acid and homogenized using an Ultra-Turrax (Model: T25D, IKA-Germany) for 30 s. The homogenate was centrifuged at 10 000 g for 10 min at 4°C using a SORVALL® RC 5C PLUS centrifuge (Sorvall, Newtown, CT, USA), and the resulting supernatant collected and stored at 4°C until further analysis.

2.4.2 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out using a modified method of Li et al. (2006). The method is based on the reduction of the ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}-TPTZ) by a reductant, thereby determining the combined antioxidant power of lipophilic and lipophobic antioxidant molecules present in the tissue. The FRAP reagent was prepared freshly by mixing 300 mM sodium acetate buffer of pH 3.6, 10 mM Fe^{2+}-TPTZ in 40 mM HC*l* and 20 mM $FeCl_3\bullet6H_2O$ (10:1:1); 1000 µL FRAP reagent was mixed with 30 µL sample and the absorbance read at 593 nm after 10 min of reaction time.

2.4.3 2, 2'-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay

The ABTS assay was carried out according to Pellegrini et al. (2003), with slight modifications. The method is based on the ability of antioxidant molecules to quench the long-lived $\text{ABTS}^{\bullet+}$, a blue-green chromophore with a characteristic absorption at 734 nm, compared with that of Trolox, a vitamin E analog. The addition of antioxidant standard or sample to be the performed radical cation reduces $\text{ABTS}^{\bullet+}$ resulting in a decolorization. A stable stock solution of $\text{ABTS}^{\bullet+}$ was produced by reacting 7 mM aqueous ABTS solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark for 6 - 12 hours prior to use. The $\text{ABTS}^{\bullet+}$ stock solution was diluted daily before adjusting to an absorbance of 0.7±0.5 with ethanol. Immediately before conducting the assay, a standard sample (10 µl) was added to the diluted $\text{ABTS}^{\bullet+}$ working solution and the decrease in absorbance monitored at 734 nm; the antioxidant concentration was expressed as µmol Trolox equivalent per gram of tissue.

2.4.4 DPPH free radical scavenging capacity

The antioxidant capacity of lemon flavedo and standards was also determined using the 2, 2-diphenyl-1-picrylhydrazyl ($\text{DPPH}^{\bullet}$) free radical according to Wang et al. (2005). Briefly, a 0.1 mM DPPH solution in methanol was prepared. The initial absorbance of DPPH in methanol was measured at 515 nm. An aliquot (50 µl) of the extract was added to 3 ml of methanolic DPPH and the change in absorbance measured after 30 min. The antioxidant capacity based on the DPPH free radical scavenging ability of the extract was expressed as µmol Trolox equivalents per gram of lemon flavedo.

2.4.5 Total phenolics extraction

Phenolic compounds were extracted according to Abeysinghe et al. (2007) with minor modification. Briefly, 0.5 g ground lemon peel was weighed in a screw-capped test tube. Phytochemicals were extracted with 5 ml 50% DMSO, (50% 1.2 M HCl in 80% methanol/water) and vortexed for 1 min. Samples were then heated at 90°C for 3 hrs, with vortexing every 30 min. After samples had cooled to room temperature, they were diluted to 10 ml with methanol and centrifuged at 10, 000 g for 5 min to remove the solid fraction. The supernatant was used for determination of total phenolics and total flavonoids.

2.4.6 Determination of total phenolics

Total phenolics of the flavedo extract were measured using a modified Folin-Ciocalteu method (Abeysinghe et al., 2007). Distilled water (4 ml) and 0.5 ml diluted flavedo extract were placed in a glass test tube. Folin-Ciocalteu reagent (0.5 ml) was reacted with the sample for 3 min, the reaction neutralized with 1 ml saturated sodium carbonate and absorbance determined at 760 nm after 3 hrs. Chlorogenic acid was used as a standard; data were expressed as mg chlorogenic acid equivalent (CAE)/ 100 g DM.

2.4.6 Determination of total flavonoids

Total flavonoids were determined using a modified colorimetric method (Abeysinghe et al., 2007). Diluted flavedo extract (0.5 ml) was added to a glass test tube containing 3.5 ml of ethanol. After addition of 4 ml 90% diethylene glycol and thorough mixing, the reaction was initiated by adding 0.1 ml 4 M NaOH. The absorbance was read at 420 nm after 10 min incubation at 40°C. Rutin was used as a standard and the total flavonoids concentration was expressed as mg rutin equivalent (RE)/100 g DM.

2.4.7. Membrane lipid peroxidation

The malondialdehyde (MDA) concentration in flavedo tissue was determined by the thiobarbituric acid (TBA) reaction according to Heath and Packer (1968). Freeze-dried lemon flavedo tissue (0.5 g) were homogenised in a cooled mortar using a pestle with 10 ml of ice cold 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 20 000 g for 10 min at 4°C. An aliquot (1 ml) of the supernatant was thoroughly mixed with 4 ml 20% TCA containing 0.5% TBA. The mixture was incubated at 95°C for 30 min and then quickly cooled in an ice bath. After centrifugation at 20 000 g for 10 min at 4°C, the absorbance of the supernatant was read at 532 nm and corrected for non-specific absorbance at 600 nm. The MDA concentration was calculated using the formula below with an extinction coefficient of 155 mM cm^{-1}.

Total MDA (nmol 100 g^{-1} DM) = (Amount of extraction buffer (ml) x amount of supernant (ml) x [(Abs 532 – Abs 600)/155] x 103)* Amount of sample $(g)^{-1}$

2.5. Statistical analysis

Statistical comparison of mean values was performed by analysis of variance (ANOVA) using Genstat Version 14 (VSN International, UK). Means were separated using Duncan's Multiple Range Test at the 5% level of significance. Furthermore, data were subjected to principal component analysis (PCA) using an Unscrambler Version 9.8 (Camo Process AS, Oslo, Norway).

3. Results

3.1. Effect of HWD and Mo dips on chilling injury symptoms during cold storage

Fruit were sourced from different farms with different growing and climatic conditions. Symptoms of chilling injury were detected on lemon fruit sourced from Sun Valley Estates during the 2007 harvest season (Table 1). However, during the year 2007 and 2008 harvest seasons, respectively, fruit sourced from Ukulinga and Eston Estates did not show any chilling symptoms. Treating fruit with hot water dips at 47°C was not as effective in mitigating chilling injury symptoms for fruits harvested from Sun Valley Estates during 2007 compared with HW 53°C, resulting in chilling indices of 0.18 and 0.09 respectively (Table 1). Molybdenum dips were effective at 1 µM Mo (compared with 5 and 10 µM) reducing the chilling index to 0.04; 1 µM Mo plus HW 53°C reduced the chilling index to 0 (Table 1).

3.2. Effect of fruit origin on total antioxidant capacity, phenolics, flavanoids and lipid peroxidation in lemon flavedo

The total antioxidant assays used (FRAP, ABTS and DPPH) showed significantly lower antioxidant capacity for chilling-susceptible fruit from Sun Valley Estates compared with non-chilled fruit from Ukulinga and Eston Estates (Fig 1A, B and C). The DPPH assay also showed the antioxidant capacity of lemon fruit from Eston Estates to be not significantly different to those from Sun Valley (Fig 1A) with the ABTS assay providing similar trends (Fig 1B).

Total phenolics as well as total flavanoids showed a significantly lower concentration of these antioxidants in the flavedo of Sun Valley Estates lemon fruit compared with other sites (Fig 1E and F). However, total phenolics and flavanoids showed different results to the DPPH and ABTS assays (Fig 1A and B), respectively, with Eston Estates fruit containing the highest concentration of total phenolics and total flavonoids (Fig 1E and F).

The flavedo membrane damage (expressed as malondialdehyde (MDA)) of Sun Valley Estates lemons was significantly higher than that of Eston fruit, while Ukulinga fruit, in both seasons, displayed higher membrane damage than Eston fruit but less than Sun Valley fruit (Fig 1D).

The contribution of individual antioxidants to the "overall antioxidant strength" varied; specific antioxidants showed different activity in different assays. While, for all assays (FRAP, ABTS and DPPH), ascorbic acid was the most potent antioxidant (Fig 2B, C and A), the antioxidant strength with FRAP assay was vitamin C (ASE) > phenolics (CAE) > vitamin E (ATE) > flavonoids (RUE) > (hesperidin (HE) ≥ naringin (NE) ≥ β-carotene (BEC)) (Fig 1B). While using the ABTS assay, the order differs: vitamin C > β-carotene > phenolics > flavonoids > vitamin E > hesperidin > naringin (Fig 2C). With the DPPH assay a completely different result with antioxidant strength was obtained: vitamin C = flavonoids = vitamin E > phenolics > hesperidin > naringin > β-carotene (Fig 2A).

3.3. Principal component analysis

Subjecting data to principal component (PCA) on all data led to a reduction of variation by 68% with the variation explained by principal component one (PC1) and principal component two (PC3) (Fig 3A). PC1 explained the majority of variation (65%) associated with DPPH and FRAP; PC3 represented 3% of variation and was mainly associated with total phenolics (Fig 3A).

When grouping data, Eston Estates (cluster 2) and Ukulinga 2007 (cluster 3) fruit were similar and chilled fruit from Sun Valley showed a different cluster compared with Ukulinga 2008 fruit (Fig 3B).

3.4. Effect of cold storage on total antioxidants, phenolics, flavanoids and lipid peroxidation of lemon flavedo during cold storage

The FRAP and DPPH assays detected a significant increase in antioxidant capacity 7 days into cold storage in the flavedo of Ukulinga (2007), Sun Valley and Eston Estates fruit. However, ABTS showed a significant decrease in antioxidant capacity after 7 days of storage in flavedo of Ukulinga and Sun Valley Estates fruit (Tables 2A and B).

Total phenolics increased significantly in flavedo of Ukulinga fruit after 14 days of cold storage, whereas in flavedo of chilling-susceptible fruit from Sun Valley total phenolics decreased over the 28 day cold storage period. Total flavanoids significantly decreased in the rind of Ukulinga 2008 and Sun Valley Estates fruit while significantly increased in Ukulinga 2007 and Eston Estates fruit 28 days after cold storage (Tables 2A and B).

The flavedo membrane integrity (expressed as MDA) of lemon fruit sourced from Ukulinga significantly increased from pre-storage up to 14 days into cold storage (Tables 2A and B). Sun Valley chilling susceptible fruit showed increased flavedo membrane disruption after 21 days; a similar membrane disruption was visible in flavedo of Eston Estates lemon following 14 days of cold storage (Tables 2A and B).

3.5. Effect of HW and Mo dips on total antioxidants, total phenolics, total flavanoids and lipid peroxidation in lemon flavedo during cold storage

Postharvest HW at 53°C significantly increased total flavedo antioxidants, as shown by FRAP 7 days into cold storage, compared with control and HW at 47°C treatments for Ukulinga 2007 and Sun Valley 2007 fruit, respectively. However, total antioxidant capacity (ABTS assay) of HW 53°C decreased 7 days into storage for Ukulinga 2007 and Sun Valley Estates 2007, respectively (Table 3). Furthermore, HW 53°C significantly decreased total antioxidants (DPPH assay) of Sun Valley Estates fruit 7 days into cold storage (Table 3). Total phenolics in the fladevo of Sun Valley Estates HW 53°C treated fruit significantly decreased 21 days into cold storage. In addition, sole HW (47 or 53°C) significantly decreased total flavanoids for chilled fruit from Sun Valley Estates; HW 53°C significantly increased total flavanoids for all non-chilled fruit 21 days into cold storage (Tables 3 and 4). Ukulinga 2007 and Sun Valley Estates fruit showed decreased membrane integrity 7 days into cold storage due to HW 53°C treatment; however, membrane recovery significantly increased from 14 to 28 days (Tables 3 and 4).

Treatment with a low concentration Mo dip (1 μM Na_2MoO_4) significantly increased total antioxidant capacity (FRAP) in flavedo of Ukulinga (2007 and 2008) and Sun Valley Estates fruit 7 days into cold storage and maintained a high flavedo antioxidant capacity up to 28 days into cold storage (Tables 3 and 4). However, 1 μM Mo dips significantly increased the total antioxidant capacity (ABTS) of Sun Valley Estates and Ukulinga fruit 14 days into cold storage (Tables 2 and 3). In addition, 1 μM Mo dips significantly increased total antioxidant capacity (DPPH) as well as total phenolics and flavanoids of Sun Valley Estates fruit 21 days into cold storage, thus significantly increasing membrane integrity between 14 to 28 days (Tables 3 and 4).

The 10 μM Mo plus HW 53°C dips had a greater effect on total antioxidants (FRAP, ABTS and DPPH) after 14 to 28 days of cold storage compared with 1 μM Mo plus HW 53°C for Ukulinga and Sun Valley Estates fruit (Tables 3 and 4). The higher total antioxidants concentrations in Ukulinga and Sun Valley Estates flavedo observed between 14 to 28 days cold storage was aligned with decreased MDA levels in response to 10 μM Mo plus HW 53°C treatment; Mo plus HW 53°C did not significantly reduce MDA levels in Eston Estates fruit (Table 4).

4. Discussion

Bioactive compounds with antioxidant properties are potential markers for estimating the severity of oxidative stress; exposure to oxidative stress could affect post-harvest fruit quality, hence several assays have been developed to estimate total antioxidant capacity (Szőllősi and Varga, 2002). Recently, total antioxidant capacity has been expressed as Trolox (a vitamin E analogue) equivalent antioxidant capacity (TEAC) (Brezová et al., 2009; Art et al., 2001). Studies have shown that TEAC assays have drawbacks and can be biased towards either lipophilic (α-tocopherol, β-carotene and lycopene) or hydrophilic (ascorbic acid and phenolic groups) compounds (Sies and Stahl, 1995; Pérez-Jiménez et al., 2008). Our results confirmed that the $TEAC_{FRAP}$ assay mainly detected hydrophilic antioxidants (particularly ascorbic acid and phenolic groups), while the $TEAC_{ABTS}$ assay mainly identified lipophilic antioxidants (β-carotene). The $TEAC_{DPPH}$, however, detected both hydrophilic and lipophilic antioxidants (Fig 1). Brezová et al. (2009) found $TEAC_{DPPH}$ to detect the antioxidants strength of lipophilic as well as hydrophilic nature in different coffee cultivars.

None of the various TEAC assays has previously been correlated with chilling occurrence in citrus fruit. Previous investigations in other plant species have reported a strong positive correlation between the $TEAC_{ABTS}$ and the $TEAC_{DPPH}$ assay when using a Pearson correlation coefficient (r = 0.906) (Dudonné et al., 2009) in different exotic fruit and a Spearmans-Rho coefficient correlation (ρ = 0.949) in different fruit and vegetables (Floegel et al., 2011). In this study, chilling stress resistance was more related to $TEAC_{DPPH}$ and total phenolics than to $TEAC_{ABTS}$ or $TEAC_{FRAP}$ (Fig 3A); such correlation has been confirmed for different agricultural produce (Floegel et al., 2011).

Previous studies have reported differences in chilling susceptibility within citrus cultivars, with "Lisbon" lemons being more susceptible than "Eureka" lemons (Underhill et al., 1999); as well as coastal lemons being more susceptible than desert lemons (Aung et al., 1999); differences in chilling susceptibility have also been attributed to different micro-climates and farms practices (Mathaba et al., 2008). Differences in chilling susceptibility were further attributed to variations in antioxidant composition due to differences in fruit ripeness using $TEAC_{FRAP}$ assay (Huang et al., 2007). Our results further attributed differences in chilling injury to the low flavedo $TEAC_{DPPH,\ ABTS\ and\ FRAP}$ (Fig 2A, B, C) due to low phenolics (Fig 2F) and flavonoid concentrations (Fig 2E).

Antioxidant assays quantify the ability to protect a certain tissue against ROS membrane damage. The damage caused by ROS, particularly to polyunsaturated membrane fatty acids (MDA), makes membrane-bound proteins intercross and conjugate, leading to oxidative stress (Campos et al., 2003). A high TEAC combined with low membrane lipid peroxidation (Fig 2D) was previously observed in *Hypoxi rooperi* ("African potato") (Laporta et al., 2007) as well as in different parts of cucumber seedlings (Jian-young et al., 2008)

Cold storage appeared to have induced alterations in TEAC, phenolic and flavonoid concentrations. Such a phenomenon was previously observed in different orange segments stored after 12 days at 4°C ($TEAC_{DPPH}$) (Plaza et al., 2011); in broccoli florets stored at 2°C for up to 7 days ($TEAC_{DPPH}$ and total phenolics) (Cogo et al., 2011); in grapefruit juice stored at 4°C and 18°C for up to 2 months (total flavonoids) (Igual et al., 2011); in different cultivars of plums stored at 2°C for up to 35 days ($TEAC_{ABTS}$ and total phenolics) (Díaz-Mula et al., 2009). Alterations in total antioxidant concentrations further confirm that various antioxidants (lipophilics and hydrophilic) are responsible for deactivating ROS accumulating during cold stress thereby possibly creating a cascade of physiological events as a holistic protection mechanism against any form of stress.

The success of HW dips to maintain postharvest quality has been reported for various commodities (Fallik, 2004; Koukounaras et al., 2009); however, little research has successfully associated HWD with TEAC. In addition, HW dips have been proven to increase total phenolic and flavonoid concentrations thus reducing occurrence of chilling injury in different crops. In 'Fortune' mandarin, stored at 16°C for 32 days, heat conditioning at 37°C prior to storage reduced chilling injury by increasing $\text{TEAC}_{\text{DPPH}}$, $\text{TEAC}_{\text{ABTS}}$, and phenolic as well as flavonoid concentrations (Lafuente et al., 2011). However, in 'Navel' and 'Valencia' oranges, HW 50°C reduced free phenolics with an increase in chilling resistance compared with HW 41°C over 20 days cold storage (Bassal and El-Hamahmy, 2011); in rocket leaves, stored at 8°C for 5 to 10 days heat treatments (51°C) increased post-harvest shelf-life by increasing $\text{TEAC}_{\text{DPPH}}$ and significantly decreasing total phenolics (Koukounaras et al., 2009). The ability of HW dips to maintain fruit postharvest quality and to reduce postharvest physiological disorders and to extend shelf-life by increasing antioxidants (Tables 3 and 4), is supported by reduced lipid peroxidation in two banana cultivars stored at 4°C for 10 days (Promyou et al., 2008). According to Lamikanra and Watson (2007) hot water dips not only induce heat shock proteins but also alter the $\text{TEAC}_{\text{FRAP}}$, $\text{TEAC}_{\text{DPPH}}$, $\text{TEAC}_{\text{ABTS}}$, phenolics and flavonoids concentration depending on HW temperature, time of application, cultivar, and pre-harvest conditions.

Several chemical post-harvest dips have shown potential to mitigate post-harvest physiological disorders in different horticultural commodities. These chemicals have further shown positive, synergistic effect with different heat treatments; calcium plus post-harvest heat treatment reduced chilling disorders in plums stored at 2°C for up to 28 days (Valero et al., 2002). In peaches, hot air treatment plus methyl jasmonate alleviated internal browning when fruit were stored at 0°C for up to 5 weeks (Jin et al., 2008). Similarly, salicylic acid plus calcium chloride and heat treatment improved post-harvest quality of peaches and strawberries stored at 0°C and 2°C, respectively (Wang et al., 2006; Shafiee at al., 2010). Our results show molybdenum post-harvest dips to have a synergistic effect with hot water treatments in alleviating chilling injury in lemons (Table 1). Hot water dips have been found to activate HSPs, and molybdenum has been hypothesized to increase the production of ROS during cold storage (Yesbergenova et al., 2005; Hesberg et al., 2003; Sagi et al., 1998) probably to a threshold level that signals a cascade of defences resulting in production of antioxidants. Reactive oxygen species have also been found to induce acclimation to stress (Foyer and Shigeoka, 2011; Foyer and Noctor, 2005); and therefore it is proposed that ROS also increase both lipid and hydrophilic antioxidants. In addition, pre-exposure of ginseng root to methyl jasmonate or salicylic acid was shown to significantly increase $\text{TEAC}_{\text{DPPH}}$, phenolic and flavonoid concentrations which reduced oxidative stress during germination of roots (Ali et al., 2007).

In conclusion, antioxidants play an important role in mitigating oxidative stress during cold storage of lemon fruit. Furthermore, antioxidants differ in nature; the lipophilic component which is quantified mostly by using the $\text{TEAC}_{\text{ABTS}}$ assay and hydrophilic antioxidants are best quantified by using the $\text{TEAC}_{\text{FRAP}}$ assay, while the $\text{TEAC}_{\text{DPPH}}$ assay is sensitive to both lipophilic and hydrophilic antioxidants. It is therefore important to use several assays to attain a complete understanding of total antioxidant activity of a certain tissue. Any post-harvest treatment is unlikely to affect different antioxidant concentrations in a similar manner during cold storage.

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List of tables

Table 1: Chilling index of lemon fruit from different growing environment during the 2007 and 2008 harvest season with chilling index evaluated after 28 days cold storage plus 5 days shelf-life.

Table 2: Effect of cold storage time on total antioxidant capacity (FRAP, ABTS, DPPH), phenolics, flavonoids and lipid peroxidation of lemon flavedo of fruit harvested from (A) Ukulinga and Sun Valley Estates 2007 season (B) Ukulinga and Eston Estates 2008 season.

Table 3: Effect of fruit source, cold storage time, hot water and molybdenum dips on total antioxidant capacity (FRAP, ABTS, DPPH), phenolics, flavonoids and lipid peroxidation of lemon flavedo during 2007 harvest season.

Table 4: Effect of fruit source, cold storage time, hot water and molybdenum dips on total antioxidant capacity (FRAP, ABTS, DPPH), phenolics, flavonoids, and lipid peroxidation of lemon flavedo during 2008 harvest season.

List of figures

Figure 1: Effect of fruit source on lemon flavedo trolox equivalent total antioxidant capacity (TEAC), phenolics, flavonoids and lipid peroxidation. (A) DPPH, (B) ABTS, (C) FRAP, (D) MDA, (E) Total flavonoids and (F) Total phenolics

Figure 2: Evaluation of authentic equivalent bioactive compound standard over different trolox equivalent antioxidant (TEAC) assays. (A). FRAP, (B) ABTS, (C) DPPH,

Figure 3: Principal component analysis (PCA) showing correlation loadings. (A) Score plot lemon specific flavedo bioactive compounds, (B) Score plot for the groups of the bioactive compounds for lemons from different sources with differences in chilling susceptibility, PC1 explains 65% and PC3 explains 3% of total variation.

Table 1

Treatments[a]	Ukulinga 2007[b]	Sun Valley Estates 2007[c]	Ukulinga 2008[b]	Eston Estates 2008[d]
Water dip (25°C)	No symptoms	0.13a	No symptoms	Below detection
HW 47°C	No symptoms	0.18a	No symptoms	Below detection
HW 53°C	No symptoms	0.09a	No symptoms	Below detection
1 µM Mo	No symptoms	0.04ab	No symptoms	Below detection
1 µM Mo + HW 53°C	No symptoms	0.02ab	No symptoms	Below detection
10 µM Mo + HW 53°C	No symptoms	0.00b	No symptoms	Below detection

[a] Treatment effect after 28 days cold storage plus 5 days shelf-life

[b] Ukulinga lemon fruit showed no chilling symptoms during 2007 and 2008 harvest seasons

[c] Sun Valley lemon fruit showed chilling symptom expressible in chilling index and means followed by the same letter were not significantly different at LSD(0.05) = 0.096

[d] Eston Estates lemon did show chilling symptoms but below detection and chilling index calculation

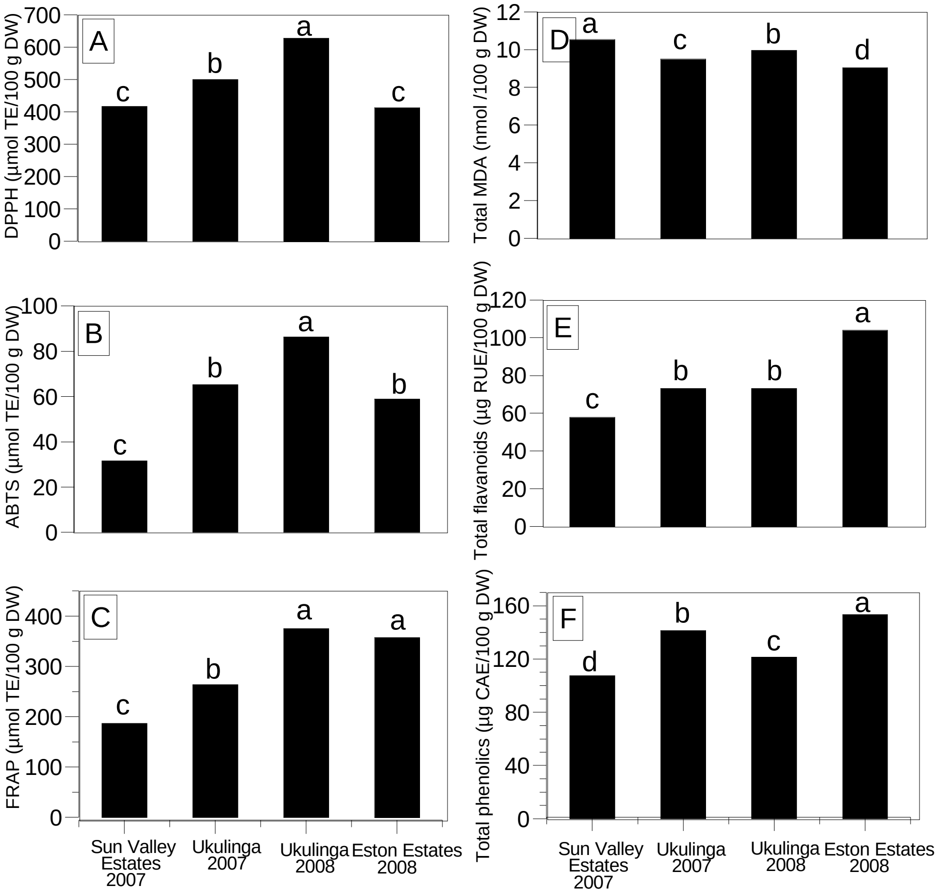

Fig 1:

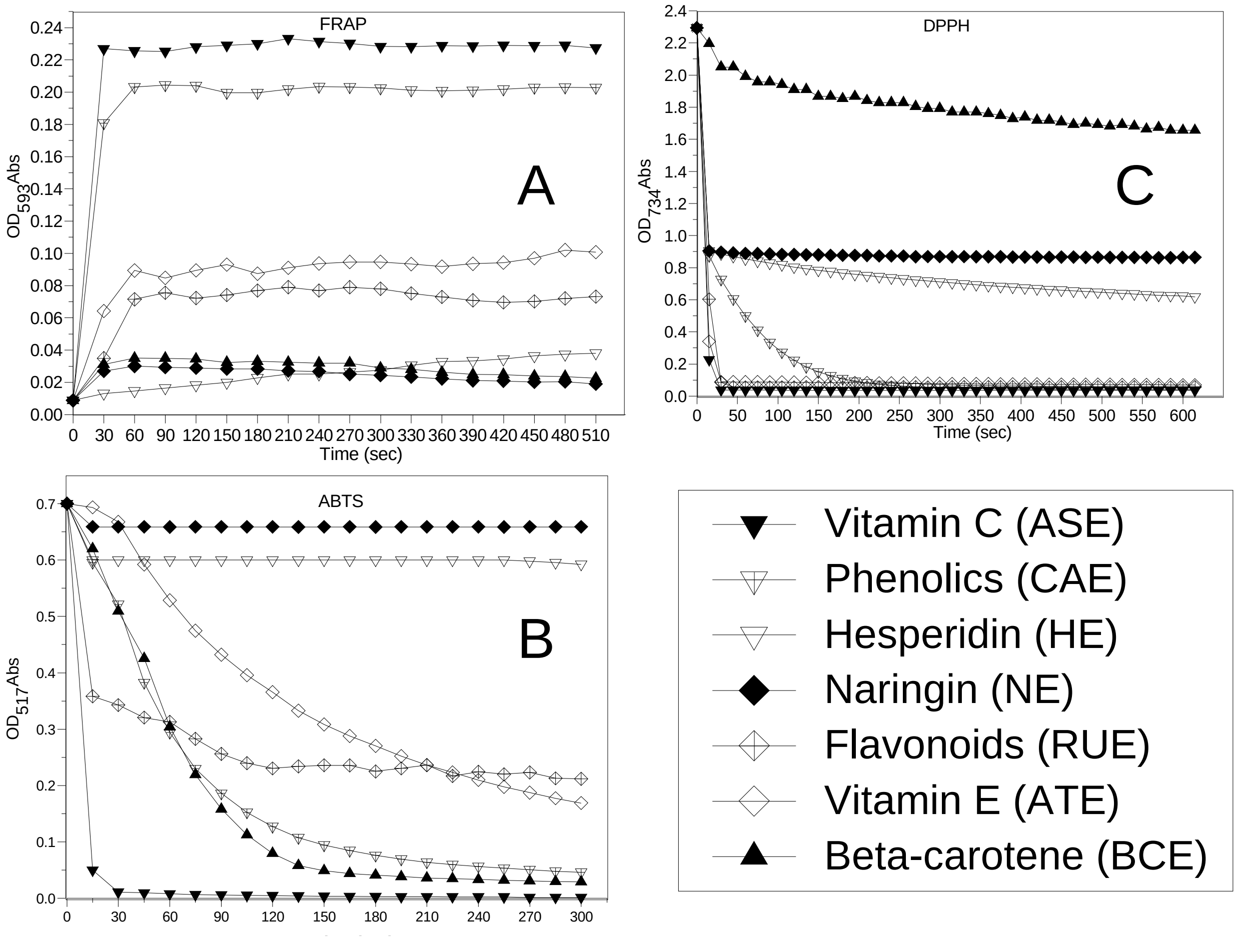
FRAP
A
OD593Abs
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
0.20
0.22
0.24
0 30 60 90 120 150 180 210 240 270 300 330 360 390 420 450 480 510
Time (sec)
DPPH
C
OD734Abs
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
0 50 100 150 200 250 300 350 400 450 500 550 600
Time (sec)
ABTS
B
OD517Abs
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0 30 60 90 120 150 180 210 240 270 300
Vitamin C (ASE)
Phenolics (CAE)
Hesperidin (HE)
Naringin (NE)
Flavonoids (RUE)
Vitamin E (ATE)
Beta-carotene (BCE)

Fig 2:

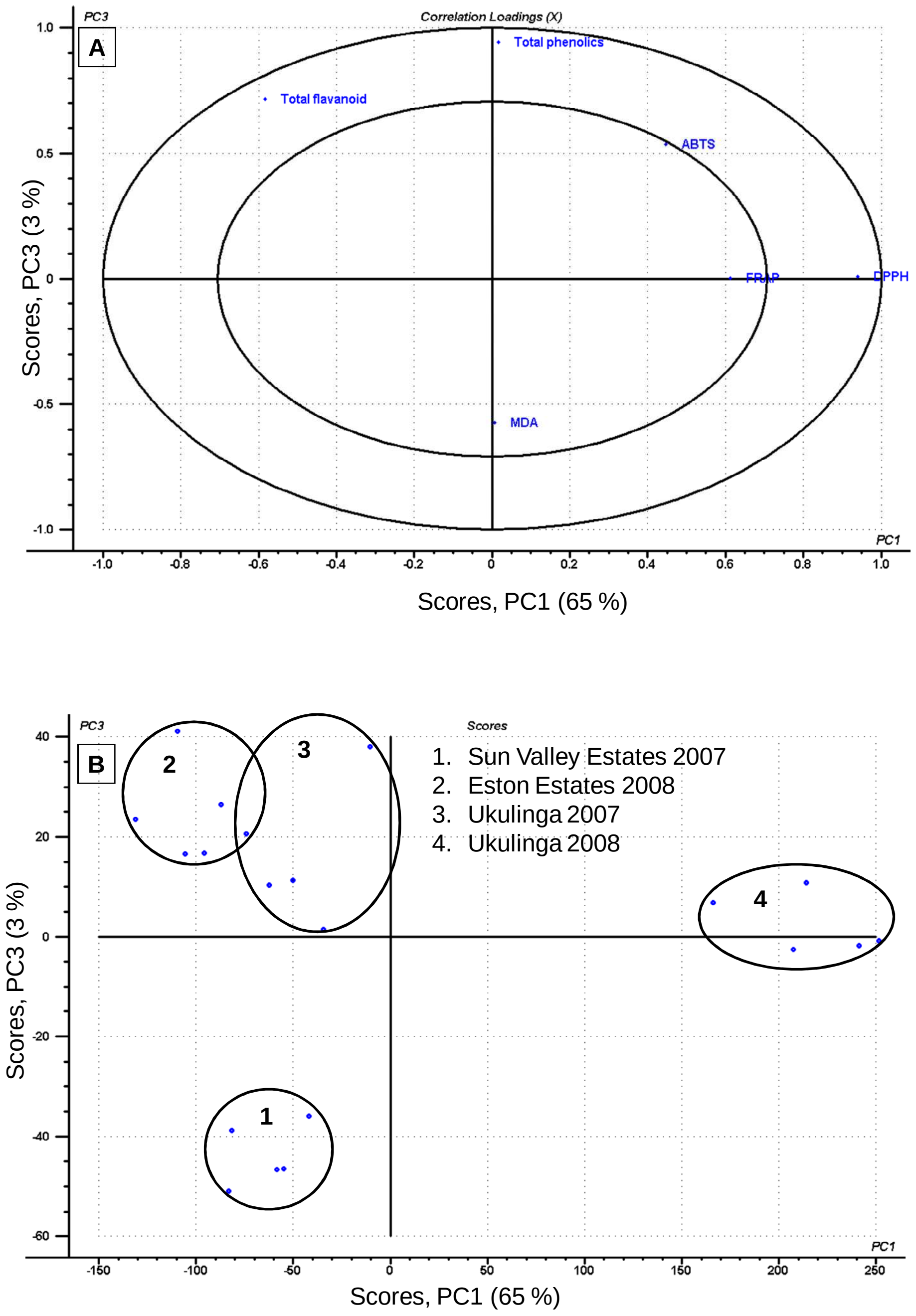
A
PC3
Correlation Loadings (X)
Total phenolics
Total flavanoid
ABTS
FRAP
DPPH
MDA
PC1
1.0
0.5
0
-0.5
-1.0
-0.8
-0.6
-0.4
-0.2
0.2
0.4
0.6
0.8
Scores, PC3 (3 %)
Scores, PC1 (65 %)
B
PC3
Scores
1. Sun Valley Estates 2007
2. Eston Estates 2008
3. Ukulinga 2007
4. Ukulinga 2008
1
2
3
4
40
20
0
-20
-40
-60
-150
-100
-50
0
50
100
150
200
250
PC1
Scores, PC3 (3 %)
Scores, PC1 (65 %)

Fig 3:

Table 2A

Cold storage time (days)	Ukulinga 2007						Sun Valley Estates 2007					
	TEAC						TEAC					
	FRAP[1]	ABTS[2]	FRAP[1]	TP[4]	TF[5]	MDA[6]	FRAP[1]	ABTS[2]	FRAP[1]	TP[4]	TF[5]	MDA[6]
0	**182.3hi**	**68.9efg**	**452.5ef**	**151.5c**	**74.9e**	**9.75g**	**205.9ghi**	**41.9bcd**	**452.0ef**	**96.8h**	**52.3h**	**11.70a**
7	**326.9bcd**	**25.5ab**	**543.6d**	**149.5c**	**68.4f**	**9.60h**	**240.2fgh**	**30.6abc**	**402.8gh**	**93.7h**	**61.6g**	**11.44b**
14	**278.7def**	**46.0bcde**	**536.9d**	**129.8de**	**68.9f**	**10.23de**	**158.9ij**	**14.0a**	**446.5ef**	**99.0h**	**60.2g**	**12.52d**
21	**259.8efg**	**71.6efg**	**467.2e**	**155.7bc**	**68.6f**	**8.74k**	**214.6ghi**	**32.1abc**	**379.8gh**	**155.7bc**	**65.4f**	**9.84g**
28	**276.8def**	**89.2gh**	**505.2d**	**122.2efg**	**85.9d**	**9.33i**	**117.5j**	**43.6bcd**	**412.1fg**	**90.6h**	**50.9h**	**9.57h**

Table 2B

Cold storage time (days)	Ukulinga 2008						Eston Estates 2008					
	TEAC						TEAC					
	FRAP[1]	ABTS[2]	DPPH[3]	TP[4]	TF[5]	MDA[6]	FRAP[1]	ABTS[2]	DPPH[3]	TP[4]	TF[5]	MDA[6]
0	**362.7bc**	**63.1def**	**603.3c**	**121.8efg**	**52.3h**	**10.50c**	**305.9cde**	**40.7bcd**	**381.gh**	**135.6d**	**95.1c**	**8.26l**
7	**372.8b**	**53.0cdef**	**664.5b**	**117.3fg**	**61.6g**	**9.02j**	**351.5bc**	**72.5fg**	**454.2ef**	**169.6a**	**95.1c**	**7.97m**
14	**370.7b**	**95.0gh**	**510.3d**	**131.8de**	**60.2g**	**9.72h**	**359.3bc**	**60.1def**	**447.2ef**	**150.1c**	**105.6b**	**9.78j**
21	**431.0a**	**102.2h**	**614.0c**	**112.4g**	**65.4f**	**8.95j**	**343.0bc**	**53.0cdef**	**417.9fg**	**164.5ab**	**106.1b**	**10.19def**
28	**342.7bc**	**98.8h**	**752.2a**	**125.4def**	**50.91**	**11.82a**	**431.7a**	**70.8efg**	**369.4h**	**148.6c**	**119.0a**	**10.09df**

[1,2,3] TEAC $_{\text{(FRAP, ABTS, DPPH)}}$- nmol/g DW

[4] Total phenolics- µg chlorogenic acid equivalent (CAE)/100 g DW

[5] Total flavanoids- µg rutin equivalent (RUE)/100 g DW

[6] Total MDA- nmol/g DW

Cold storage time mean within same column having different letters indicates significant difference (p<0.05)

Table 3

Cold storage time (days)	Treatments	Ukulinga 2007						Sun Valley Estates 2007					
		TEAC FRAP[1]	ABTS[2]	FRAP[1]	TP[4]	TF[5]	MDA[6]	TEAC FRAP[1]	ABTS[2]	FRAP[1]	TP[4]	TF[5]	MDA[6]
0	Water dip (25°C)	139.5±43.4	43.4±17.7	491.0±29.7	140.0±11.6	95.7±11.7	10.47±0.01	217.6±40.8	27.9±7.8	503.2±14.5	95.2±1.0	47.9±2.1	10.15±0.08
	HW 47°C	158.5±19.5	55.8±22.3	442.3±37.0	144.8±5.8	97.2±10.1	10.34±0.01	213.0±24.8	40.0±16.1	432.5±12.9	94.7±0.6	49.5±1.1	12.26±0.07
	HW 53°C	177.1±5.6	90.5±27.5	463.9±10.5	157.1±24.4	65.0±2.4	9.02±0.01	228.4±28.3	33.9±8.8	478.2±16.4	98.4±1.2	56.7±2.2	10.30±0.06
	1 µM Mo	128.7±17.1	105.2±23.5	412.8±44.7	157.1±24.4	65.6±0.9	8.20±0.01	200.0±15.2	26.8±8.1	442.6±18.4	95.8±1.2	46.8±0.7	12.15±0.09
	1 µM Mo + HW 53°C	258.9±33.7	116.1±36.0	507.7±19.1	174.9±24.3	61.7±1.5	8.85±0.02	209.6±33.8	51.1±26.8	413.2±10.7	100.2±1.3	55.1±1.1	13.35±0.16
	10 µM Mo + HW 53°C	231.0±30.1	73.5±18.3	319.5±14.1	135.3±10.1	63.9±3.5	11.65±0.02	166.7±18.4	33.5±17.1	424.5±27.8	96.3±1.6	58.1±0.7	11.99±0.02
7	Water dip (25°C)	329.2±27.5	16.1±4.0	607.2±15.5	157.0±24.4	70.0±2.1	9.17±0.09	213.6±6.0	62.6±20.1	410.2±16.2	92.0±1.3	49.0±1.4	11.72±0.07
	HW 47°C	298.4±33.6	21.0±7.0	555.9±17.4	157.0±24.4	68.2±1.0	8.17±0.01	197.2±62.2	10.9±5.1	377.5±29.5	92.9±1.1	59.6±1.8	9.82±0.13
	HW 53°C	339.5±28.6	24.7±9.2	566.8±16.7	172.1±27.6	67.4±1.0	15.24±0.08	260.0±20.0	27.1±11.8	397.7±13.1	93.3±1.1	67.6±1.0	12.18±0.02
	1 µM Mo	277.8±31.2	24.5±3.2	457.1±6.4	137.2±8.1	69.1±1.8	7.44±0.01	259.9±49.9	21.9±9.4	423.4±44.4	92.4±0.9	65.5±2.3	10.30±0.01
	1 µM Mo + HW 53°C	376.4±10.2	47.2±24.2	549.8±24.5	140.0±11.6	66.7±1.6	8.40±0.01	283.5±46.3	21.5±8.3	420.9±29.7	93.8±1.0	69.6±3.0	13.57±0.01
	10 µM Mo + HW 53°C	340.0±35.7	29.5±7.0	524.5±5.6	133.7±11.7	69.0±1.0	9.21±0.03	227.3±22.5	47.4±12.5	386.9±26.3	97.9±1.8	58.0±1.6	10.97±0.01
14	Water dip (25°C)	311.7±13.5	47.0±5.8	565.8±21.4	132.4±13.1	67.0±2.7	8.46±0.03	149.6±12.3	22.2±9.5	473.7±33.8	100.8±1.5	59.7±0.7	17.66±0.03
	HW 47°C	307.4±39.2	40.1±11.3	580.1±24.1	128.6±14.2	70.1±1.1	9.05±0.03	195.9±8.0	16.0±6.8	464.4±19.4	99.6±1.5	62.2±2.2	10.92±0.03
	HW 53°C	316.7±27.0	47.5±30.7	560.7±8.7	130.4±15.4	67.8±2.7	9.32±0.07	182.2±15.9	12.5±3.4	433.1±20.8	95.2±1.0	66.5±2.7	11.58±0.07
	1 µM Mo	265.8±19.1	34.5±8.7	537.8±13.8	130.9±12.0	72.4±3.4	11.24±0.01	125.4±14.9	17.4±8.9	419.7±21.4	97.8±0.7	53.3±2.2	11.12±0.01
	1 µM Mo + HW 53°C	227.0±22.7	55.7±12.7	526.0±20.6	124.9±8.1	69.2±1.0	11.24±0.01	184.4±5.5	12.3±5.8	451.4±27.0	101.7±3.1	66.6±3.3	12.40±0.01
	10 µM Mo + HW 53°C	243.6±23.3	39.2±3.0	451.1±24.0	131.4±14.3	66.8±1.0	12.06±0.01	115.5±46.6	10.3±4.4	436.9±32.0	99.0±1.4	52.8±1.3	11.47±0.01
21	Water dip (25°C)	262.0±12.4	48.9±4.3	535.9±10.5	140.0±11.6	76.0±1.5	8.15±0.02	207.1±42.7	14.8±4.4	415.0±31.8	140.0±11.6	58.0±3.1	9.29±0.08
	HW 47°C	218.4±16.6	40.9±3.2	425.2±23.1	153.5±27.8	71.6±1.0	8.27±0.01	215.9±11.1	5.6±3.1	422.4±19.4	153.5±27.8	66.0±3.9	8.59±0.05
	HW 53°C	249.7±9.5	49.7±10.0	464.3±5.4	153.5±27.8	72.7±1.3	9.62±0.01	237.7±27.8	34.9±14.8	370.3±27.5	153.5±27.8	62.5±3.4	10.32±0.09
	1 µM Mo	257.0±59.1	116.2±26.8	465.8±19.2	174.9±24.3	50.1±1.5	9.11±0.01	249.0±49.3	30.3±13.4	345.3±21.1	174.9±24.3	76.6±5.1	10.39±0.06
	1 µM Mo + HW 53°C	225.7±29.1	139.1±16.7	370.2±12.6	172.1±27.7	71.6±1.0	8.46±0.01	184.8±20.1	47.0±8.5	372.6±13.2	172.1±27.7	64.8±4.7	10.40±0.03
	10 µM Mo + HW 53°C	346.0±149	86.9±18.8	542.0±16.7	140.0±11.6	69.8±1.1	8.81±0.01	193.5±34.5	42.6±6.3	353.1±19.0	140.0±11.6	64.5±3.7	10.07±0.03
28	Water dip (25°C)	307.9±11.5	50.7±6.1	454.2±24.2	117.4±8.3	81.8±1.9	9.44±0.25	89.2±24.2	45.3±11.6	436.9±35.2	91.2±1.0	42.2±1.7	9.23±0.03
	HW 47°C	216.4±28.9	59.0±3.4	495.0±20.2	124.9±16.2	88.0±5.8	9.64±0.24	163.1±29.9	35.9±5.4	393.5±15.0	91.0±2.0	52.4±0.3	9.56±0.07
	HW 53°C	294.8±52.5	76.4±22.4	507.5±41.8	117.4±8.3	87.2±2.4	8.50±0.03	126.9±40.8	38.2±4.9	452.8±23.6	89.0±0.3	43.5±0.7	8.50±0.19
	1 µM Mo	255.2±17.5	129.1±25.8	481.0±19.8	120.2±10.9	87.2±2.4	8.28±0.01	88.2±31.8	52.6±14.1	412.2±42.4	91.7±2.4	48.8±2.2	9.56±0.03
	1 µM Mo + HW 53°C	295.4±36.9	118.8±12.9	519.9±15.0	123.0±10.4	87.2±2.4	8.51±0.12	109.8±3.4	39.8±13.2	373.6±9.6	91.5±0.7	57.0±3.7	10.23±0.02
	10 µM Mo + HW 53°C	245.9±21.0	135.7±24.7	501.5±18.6	130.5±15.4	83.7±3.7	11.73±0.02	127.9±8.8	69.1±11.8	403.5±23.4	127.7±5.1	61.4±1.0	10.34±0.05

[1,2,3] TEAC $_{\text{(FRAP, ABTS, DPPH)}}$- nmol/g DM

[4] Total phenolics- µg chlorogenic acid equivalent (CAE)/100 g DM

[5] Total flavanoids- µg rutin equivalent (RUE)/100 g DM

[6] Total MDA- nmol/g DW

Treatments mean (±SE) obtained from 3 replication for TEAC assays, total phenolics, flavonoid and lipid peroxidation

Table 4:

Cold storage time (days)	Treatments	Ukulinga 2008						Eston Estates 2008					
		TEAC FRAP[1]	ABTS[2]	DPPH[3]	TP[4]	TF[5]	MDA[6]	TEAC FRAP[1]	ABTS[2]	DPPH[3]	TP[4]	TF[5]	MDA[6]
0	Water dip (25°C)	312.2±74.4	47.2±7.8	628.8±46.3	122.8±2.5	95.7±11.7	13.51±0.04	249.2±52.0	40.2±4.5	395.0±19.0	127.7±5.1	104.5±9.2	8.67±0.02
	HW 47°C	408.2±36.8	45.2±11.9	658.5±38.9	129.5±3.1	97.2±10.1	10.49±0.03	345.7±46.8	54.7±11.6	366.2±35.4	135.2±1.0	104.5±9.2	8.13±0.03
	HW 53°C	356.0±46.8	44.1±13.5	563.7±19.9	116.2±1.0	65.0±2.4	10.23±0.01	318.0±96.8	59.8±17.9	354.2±25.7	130.5±5.8	87.5±2.4	9.88±0.03
	1 μM Mo	291.3±20.9	70.6±8.8	616.7±48.3	122.8±2.4	65.6±0.9	8.17±0.03	359.3±65.1	36.4±4.1	407.1±29.1	135.2±1.0	92.2±3.4	8.02±0.01
	1 μM Mo + HW 53°C	318.3±13.6	79.0±8.2	581.2±24.7	120.0±5.1	61.7±1.5	10.53±0.03	246.6±59.6	34.6±10.7	393.7±28.5	140.0±5.8	92.2±3.4	7.07±0.02
	10 μM Mo + HW 53°C	427.3±68.1	84.1±13.4	570.7±27.1	119.7±4.3	63.9±3.5	10.09±0.01	316.8±55.5	40.7±8.9	369.7±29.8	144.7±5.8	89.5±1.0	7.77±0.02
7	Water dip (25°C)	449.4±33.2	62.7±3.8	689.4±24.6	112.5±1.0	70.0±2.1	8.37±0.04	420.4±130	64.6±6.3	446.6±16.4	164.5±18.5	104.5±9.2	6.84±0.01
	HW 47°C	364.6±82.1	61.8±7.8	686.2±24.5	119.7±4.3	68.2±1.0	8.67±0.02	358.7±101	55.7±3.5	457.3±16.0	179.6±18.5	104.5±9.2	8.54±0.02
	HW 53°C	336.2±32.0	56.2±7.5	753.5±31.7	116.5±3.0	67.4±1.0	8.67±0.03	308.4±91.3	100.6±16.1	469.6±21.2	164.5±18.5	87.5±2.4	8.40±0.02
	1 μM Mo	499.8±157	49.5±10.3	622.5±41.4	113.7±1.5	69.1±1.8	9.01±0.03	319.0±39.2	73.3±4.9	433.9±106.3	164.5±18.5	92.2±3.4	9.37±0.02
	1 μM Mo + HW 53°C	297.2±38.0	59.5±8.2	672.8±33.0	116.5±3.0	66.7±1.6	10.01±0.02	382.9±28.8	68.7±8.1	466.8±19.5	179.6±18.5	92.2±3.4	5.24±0.02
	10 μM Mo + HW 53°C	289.5±24.0	70.4±13.7	562.6±27.7	124.8±2.4	69.0±1.0	8.84±0.02	319.7±34.6	64.0±3.7	451.0±28.4	164.5±18.5	89.5±1.0	9.40±0.02
14	Water dip (25°C)	423.0±97.5	147.9±47.3	524.2±27.5	124.7±2.4	67.0±2.7	9.17±0.03	220.7±55.3	100.4±23.0	435.1±27.2	149.5±1.0	104.6±9.2	8.71±0.15
	HW 47°C	280.6±54.6	102.7±10.2	451.6±86.3	137.0±8.1	70.1±1.1	9.46±0.01	227.5±74.0	54.6±12.5	472.4±27.4	164.5±18.5	107.3±5.8	9.54±0.03
	HW 53°C	313.9±62.1	77.8±8.3	535.4±23.3	130.3±5.8	67.8±2.7	10.96±0.02	395.8±68.6	46.8±4.6	450.2±5.4	144.7±5.8	99.8±8.1	9.26±0.03
	1 μM Mo	477.0±91.7	107.2±10.0	587.0±74.6	134.2±9.2	72.4±3.4	11.73±0.03	493.6±59.0	52.8±7.1	450.9±30.6	137.2±8.1	102.6±5.8	8.40±0.15
	1 μM Mo + HW 53°C	323.0±26.0	73.1±5.4	507.9±39.1	129.5±3.4	69.2±1.0	8.01±0.88	407.1±70.9	48.7±12.3	428.4±10.4	159.8±22.0	107.3±5.8	7.73±0.04
	10 μM Mo + HW 53°C	406.7±93.5	54.0±7.3	455.5±45.2	135.1±1.0	66.8±1.0	8.99±0.02	410.9±74.6	49.7±12.0	446.1±26.2	144.7±5.8	112.1±1.0	15.01±0.02
21	Water dip (25°C)	442.6±36.5	101.8±13.2	644.0±28.6	112.9±2.4	76.0±1.5	8.42±0.20	364.6±75.6	126.7±11.9	460.1±19.9	149.4±1.0	102.1±5.8	10.92±0.09
	HW 47°C	417.2±13.3	105.9±16.2	580.4±50.5	112.9±2.4	71.6±1.0	9.27±0.05	340.2±122	55.7±23.2	402.2±42.0	164.5±18.5	102.1±5.8	11.21±0.09
	HW 53°C	361.3±31.1	125.6±21.4	717.2±22.8	114.4±4.2	72.7±1.3	8.38±0.02	251.3±87.4	28.5±12.8	423.1±33.0	149.4±1.0	102.1±5.8	9.54±0.06
	1 μM Mo	442.8±28.7	119.2±16.1	559.0±40.7	109.5±1.0	50.1±1.5	7.44±0.01	364.2±12.7	25.8±8.6	424.0±40.0	164.5±18.5	111.6±1.0	9.79±0.16
	1 μM Mo + HW 53°C	434.4±72.8	113.1±12.7	589.9±40.9	110.6±1.3	71.6±1.0	10.90±0.03	359.0±113	62.9±15.9	408.7±51.4	164.5±18.5	106.9±5.8	9.78±0.06
	10 μM Mo + HW 53°C	487.7±14.4	113.8±10.0	593.9±17.6	114.0±15	69.8±1.1	9.32±0.01	378.8±51.3	35.8±16.4	389.2±22.5	194.7±1.0	111.6±1.0	10.20±0.05
28	Water dip (25°C)	322.6±76.7	29.0±7.0	777.7±33.0	127.0±1.0	81.8±1.9	14.61±0.11	292.4±27.8	97.6±10.5	360.9±88.5	144.7±5.8	114.7±4.9	8.52±0.24
	HW 47°C	377.3±7.1	214.6±40.1	769.4±25.7	123.5±4.3	88.0±5.8	11.81±0.27	368.2±93.4	74.4±25.1	394.9±27.2	149.4±1.0	119.7±4.9	11.27±0.21
	HW 53°C	224.9±71.2	80.2±10.4	748.6±28.0	131.5±4.3	87.2±2.4	9.66±0.025	511.9±106	37.1±12.7	386.3±24.9	149.4±1.0	123.7±1.0	10.45±0.14
	1 μM Mo	455.5±19.4	65.2±8.4	726.9±38.5	123.5±4.3	87.2±2.4	13.34±0.12	469.1±83.4	24.2±11.4	340.8±21.5	149.4±1.0	119.7±4.9	9.40±0.57
	1 μM Mo + HW 53°C	403.8±15.6	65.8±24.6	753.4±16.6	127.8±5.1	87.2±2.4	10.21±0.02	444.9±86.7	118.4±44.8	371.3±91.7	149.4±1.0	123.7±1.0	10.33±0.02
	10 μM Mo + HW 53°C	272.3±39.0	168.5±15.9	737.2±15.2	119.5±1.9	83.7±3.7	11.31±0.02	503.8±51.3	40.7±12.1	362.2±25.2	149.4±1.0	111.8±1.0	10.59±0.08

[1,2,3] TEAC $_{\text{(FRAP, ABTS, DPPH)}}$- nmol/g DW

[4] Total phenolics- µg chlorogenic acid equivalent (CAE)/100 g DW

[5] Total flavanoids- µg rutin equivalent (RUE)/100 g DW

[6] Total MDA- nmol/g DW

Treatments mean (±SE) obtained from 3 replication for TEAC assays, total phenolics, flavonoid and lipid peroxidation

Chapter 5

Effect of Hot Water and Molybdenum Dips on Bioactive Compounds with Antioxidant Properties in Lemon Flavedo during Cold Storage

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Abstract

Citrus fruit from certain destinations require cold sterilization against fruit fly, during or before shipping, to meet phytosanitary standards of exporting countries. However, such treatment may result in chilling injury, appearing as pitting or sunken lesions on the rind, thereby reducing the marketability of fruit. In order to counteract stress plants respond by synthesizing secondary metabolic compounds as a defense system. Certain bioactive compounds such as vitamin E (α-tocopherol), vitamin C (ascorbic acid), β-carotene, polyphenols (e.g. flavonoids and flavonones, viz hesperidin and naringin) have antioxidant proprieties and their present in the citrus flavedo play a significant role in mitigating oxidative stress such as chilling injury during cold storage. The combination of hot water (HWD) and molybdenum (Mo) dips has been shown to present a possible preconditioning means to alleviate chilling injury. The purpose of this investigating was, therefore, to alter the concentration of these bioactive compounds in lemon flavedo during cold storage to allow fruit to withstand cold storage symptoms of chilling injury. Fruit from two sources were treated for 2 min with HWD (47°C or 53°C) in combination with a subsequent 1 or 10µM Na_2MoO_4 soak for 30 min. Fruit were subsequently stored at -0.5°C for up to 7, 14, 21, or 28 days and thereafter moved to room temperature. Fruit were evaluated weekly for chilling injury symptoms. Lemon fruit from Sun Valley Estates were chilling susceptible and had the lowest concentration of bioactive compounds. Hot water dips 53°C, as well as 1 µM Mo in combination with HW 53°C enhanced the rind concentration of bioactive compounds. In general, HWD and Mo dips enhanced rind concentration of phenolics and vitamin C at specific storage times; however, the intensity of the alteration in such compounds depended on fruit origin, pointing towards an interaction of environmental conditions and preconditioning treatments as possible players to avoidance of chilling injury.

1. Introduction

The development of new markets is one of the major challenges of all fruit exporting countries. Potential new markets for African fruit, such as Japan and US, require cold sterilization against fruit fly as a phytosanitary measure; however, such treatment may alter imperative cellular pathways (El-Hilali et al., 2003), resulting in chilling injury.

Amongst citrus fruit, lemons very are chilling-susceptible with only grapefruit regarded more susceptible (Chalutz et al., 1985). Chilling symptoms in lemons generally appear as sunken lesions, discolouration of the peel, as well as pitting, which severely reduce fruit marketability (McLauchlan et al., 1997; Shellie and Mangan, 2000). Chilling damage is a result of oxidative stress, which occurs when reactive oxygen species (ROS), mainly superoxide radicals ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$), are produced at concentrations exceeding defined threshold levels (Huang et al., 2007). These ROS cause oxidative damage by rapidly attacking and altering bio-molecules, thus leading to membrane deterioration, lipid peroxidation, DNA mutation or oxidation of amino acid side chains (Tomizawa et al., 2005; Huang et al., 2007), finally resulting in cell death. In order to minimize the negative effect of ROS or oxidative damage, plant cells must develop a well-integrated defence system of antioxidants (Yesbergenova et al., 2007) to scavenge and maintain ROS levels below this critical threshold.

In response to stress, plants synthesize secondary metabolites, mainly bioactive compounds, to maintain cellular disruption (Cogo et al., 2011; Robles-Sánchez et al., 2009). Most of these bioactive compounds, such as phenolics, alkaloids, carotenoids and various nitrogen compounds, have antioxidant properties, acting as a defence system against oxidative stress (Cano et al., 2008; Abeysinghe et al., 2007). Antioxidants are divided into water-soluble (e.g. phenolics and vitamin C), and lipid-soluble (e.g. β-carotene and vitamin E), with α-tocopherol as the dominant antioxidant within the vitamin E group (Sies and Stahl, 1995). Phenolics are subdivided into flavonones and specific flavanoids, with naringin and hesperidin as dominant flavanoids in citrus (Igual et al., 2011).

Hot water dips has been proven to mitigate chilling injury in citrus fruit (Erkan et al., 2005) by enhancing heat-shock proteins (Lindquist, 1986; Porat et al., 2002; Rozenzvieg et al., 2004; Vierling, 1991). Low molecular weight chaperones assist in protein folding, as well as in directing damaged proteins towards proteolysis (Rozenzvieg et al., 2004).

Previous research has shown that postharvest Mo dips have the potential to mitigate chilling injury in citrus fruit, probably by increasing the activity of xanthine dehydrogenase (XDH), a Mo co-factor enzyme which is directly involved in stress response (Yesbergenova et al., 2005). Molybdenum is an essential element for most living organisms (Kisket et al., 1997); however, only when incorporated into apoprotein it assumes a pivotal role within a diverse range of redox active enzymes, including XDH (Mendel and Schwarz, 1999). Hesberg et al. (2003) found that

XDH activity during stress regulates ROS production and, therefore the occurrence of oxidative stress.

Most previous research has failed to recognise ROS as powerful and essential signaling molecules (Foyer and Shigeoka, 2011); provided production is maintained below a critical, damaging threshold level. Therefore, the addition of Mo can be used to balancing the cellular redox homeostasis during stress, leading to normal functioning of XDH and a low production of ROS as a cellular sign for stress acclimation (Foyer and Shigeoka, 2011), presumably through increased bioactive compound production.

Recent report has linked hot water dips and Mo to increase ROS production by signaling the production of bioactive compounds during cold storage of citrus fruit (Mathaba et al., 2008). Therefore, the aim of this experiment was to investigate if HW and Mo dips are treatments signaling an increase in specific bioactive compounds in lemon flavedo, if fruit are exposed to chilling injury conditions.

2. Materials and Methods

2.1. Plant materials

Mature 'Eureka' lemons were obtained from Ukulinga Research Farm (29°39'48.82''S, 30°24'19.89''E) and Sun Valley Estate (28°51′00″S, 30°04′00″E) in the first harvest season and from Ukulinga Research Farm as well as Eston Estate Farm (29°47'00''S, 29°27'00''E) in the second season of the experiment. Fruit were treated with a 30 min soak in 1µM $Na_2MoO_4.2H_20$ followed by a 2 min in hot water (HW) dips at 47 or 53°C. Thereafter, fifteen fruit per carton (3 replicates of carton per treatment) were waxed, weighed and stored at -0.5°C for up to 28 days and sampled on weekly basis for chilling injury evaluation. A second evaluation was conducted five days after withdrawal from cold storage. Fruit were peeled and the flavedo freeze dried, milled using a mortle and pestle and stored at -21°C for further analysis.

2.2. Chilling index determination

The method of Sala (1998) was used to evaluate chilling injury five days after fruit were withdrawn from cold storage. After 7, 14, 21 and 28 days of cold storage at -0.5°C plus 5 days shelf-life at room temperature, fruit were evaluated for chilling injury severity. Pitting and sunken lesions were evaluated based on a hedonic scale; 0 - sound fruit (no pitting), 1 –l ess than 10%, 2 – 10 - 20% pitting, 3 – 30 - 40% pitting and 4 - more than 50% pitting; and expressed as chilling injury index (CII) according to the following formula:

CII = Σ (number of fruit with chilling injury x score of severity) / Total number of fruit evaluated

2.3. Standard and chemicals

Naringin (naringenin-7-rhamnosidoglucosidose), hesperidin (hesperetin-7-rutinoside), α-tocopherol, β-carotene, ascorbic acid, rutin, chlorogenic acid, octylamine, tetrahydrofuran, butylated hydroxytoluene (BHT) and sodium molybdate dehydrate ($Na_2MoO_4.2H_20$) were purchased from Sigma-AIdrich Chemical Co. (St. Louis, Mo, US), citric acid, ethanol, acetronitrile, triethylamine, ammonium acetate, hydrochloric acid (HCL), acetic acid, potassium hydroxide, and sodium hydroxide were purchased from Saarchem (PTY) Ltd, Krugersdrop. Hexane and acetone purchased from Merk (PTY) Ltd South Africa. HPLC grade methanol purchased from Darmstad, (Germany), and dimethylsulphoxide (DMSO) purchased from Poch (South Africa), while oxalic acid was purchased from Analar, Johannesburg, South Africa.

2.4. Determination of vitamin C

Ascorbic acid was extracted and determined according to Abeysinghe et al. (2007), with minor modifications. Ascorbic acid was extracted from 0.1 g lemon flavedo using 10 ml 0.1% (w/v) oxalic acid and the resulting supernatant was injected into a reversed phase HPLC system. The ascorbic acid content was expressed as mg 100 g^{-1} DW.

2.5 Extraction of total phenolics, flavonoids, naringin and hesperidin

The two specific flavanones, naringin and hesperidin; total phenolics and flavanoids were extracted according to Abeysinghe et al. (2007) with slight modifications. Lemon peel (0.5 g DW) was accurately weighed in a screw-capped test tube. The phytochemicals were extracted with 5 ml DMSO: 1.2 M HCl (1:1 ratio) in 80% aqueous methanol and vortexed for 1 min. Samples were then heated at 90°C for 3 hrs, with vortexing every 30 min. After the samples cooled down to room temperature, the volume was adjusted to 10 ml with methanol and samples were centrifuged at 10, 000 g for 5 min to remove the solid fraction. The supernatant was used to determine total phenolics, total flavonoids and the two specific flavanones naringin and heperidin.

2.6 Determination of total phenolics

Total phenolics of the flavedo were measured using a modified Folin-Ciocalteu method (Abseysinghe et al., 2007). Chlorogenic acid was used as a standard; data were expressed as μg chlorogenic acid equivalents (CAE) 100 g^{-1} DW.

2.7 Determination of total flavanones, naringin and hesperidin

Total flavanoids of the samples were determined using a modified colorimetric method (Abseysinghe et al., 2007). Rutin was used as a standard and total flavanoid concentrations expressed as μg rutin equivalents (RUE) 100 g^{-1} DM.

Naringin and hesperidin content was determined using a reversed-phase HPLC coupled to a UV detector (PDA-100 Photodiode Array detector). The contents of naringin and hesperidin were expressed in mg 100 g^{-1} DW.

2.8 Extraction and determination of β-carotene and vitamin E (α-tocopherol)

The β-carotene and vitamin E were extracted according to method of Liu et al. (2009) and saponified according to Charoensiri et al. (2009). The β-carotene and α-tocopherol concentrations were analysed using HPLC coupled with a detector (PDA-100 Photodiode Array detector). The chromatographic separation was performed using an ODS C18 column (4.6 x 150 mm) for α-tocopherol and C18 (Vydac 20i TP 54, 25 x 0.46 cm) for β-carotene. The mobile phase for β-carotene consist of acetronitrile (CH_3CN):tretrahydrofuran (THF):methanol (CH_3OH):triethylamine (TEA), of 20:14:6:01 (v/v/v/v) containing 0.01% BTH, with 0.2% ammonium acetate at a flow rate of 1.0 ml min^{-1} and monitored at 450 nm. Methanol was mobile phase for vitamin E (α-tocopherol) at flow rate of 0.6 ml min^{-1} containing 0.01% BTH and monitored at 294 nm. The contents of β-carotene and α-tocopherol were expressed in µg 100 g^{-1} DW.

2.9 Membrane lipid peroxidation

The malondialdehyde (MDA) content was determined by the thiobarturic acid (TBA) reaction according to Dipierro and Leonardis (1997). The level of lipid peroxidation, estimated by production of MDA was calculated using an extinction coefficient of 155 mM cm^{-1} and expressed as nmol 100 g^{-1} DW.

$$\text{Total MDA (nmol 100 } g^{-1} \text{ DW)} = (\text{Amount of extraction buffer (ml)} \times \text{amount of supernant (ml)} \times [(\text{Abs } 532 - \text{Abs } 600)/155] \times 103)^{*} \text{ Amount of sample (g)}^{-1}$$

2.10 Statistical analysis

All data were analysed in triplicate and subjected to analysis of variance (ANOVA) using Genstat Version 14. Mean comparison separations were performed using the Duncan test ($P \leq 0.05$) to determine differences between harvest season, localities, cold storage duration and treatments. Furthermore, data was subjected to principal component analysis (PCA) and rotated according to Varimax using The Unscrambler Version 9.8. (Camo Process AS, Oslo, Norway).

3. Results

3.1 Effect of hot water and molybdenum dips on chilling symptoms

Fruit were sourced from different farms with different growing and climatic conditions. Occurrence of chilling injury was generally low but could be detected in lemon fruit sourced from

Sun Valley during the 2007 harvest season (Table 1). However, fruit sourced from Ukulinga during the same season did not show chilling symptoms in 2007 and 2008, while Eston fruit showed minor chilling injury symptoms. Exposure of Sun Valley fruit to HWD 47°C in the 2007 harvest season was not as effective in mitigating chilling injury symptoms as HWD 53°C which resulted in a chilling index of 0.18 and 0.09, respectively (Table 1). Molybdenum dips were effective at 1 μM Mo with a chilling index of 0.04 (compared with 5 and 10 μM Mo, preliminary results not shown) while the combination of 1 μM Mo plus HWD 53°C reduced the chilling index to 0.00 in Sun Valley fruit (Table 1).

3.2 Effect of fruit source on concentration of bioactive compounds in lemon flavedo

Lemon fruit from Sun Valley which showed chilling injury had significantly lower bioactive compounds compared with the non chilling sensitive lemon fruit from other sources (Fig. 1). The flavedo vitamin C concentration was significantly lower in Sun Valley fruit compared with that from other sources (Fig. 1A). The Vitamin E and β-carotene concentrations were also significantly lower in Sun Valley fruit than in the non-chilling susceptible lemons from Ukulinga (Fig. 1B). The β-carotene concentration of the flavedo was not significantly different between Ukulinga and Eston fruit. Furthermore, naringin and hesperidin concentrations were significantly lower in the rind of chilling susceptible Sun Valley fruit than in the flavedo of other fruit which did not show chilling injury symptoms (Fig. 1C). In addition, total phenolics and flavanones were significantly lower in the flavedo of chilling susceptible lemons from Sun Valley (Fig. 1D and E) compared with non-chilling susceptible fruit from Ukulinga and Eston. Finally, the MDA concentration was significantly higher in chilling susceptible Sun Valley fruit compared with non-chilling susceptible fruit (Fig. 1F).

3.3 Principal component analysis

Principal component analysis (PCA), use to determine the interrelationships between the measured bioactive compounds and reduce the total variation to 96% of the total variation. Principal component one (PC 1) explained 82% of the variation which was related to naringin and vitamin C while 14% variation was explained by PC 2 which was related to total phenolics

(Fig. 2A). All variable groups according to fruit source showed significant differences with chilling susceptible fruit (Sun Valley) compared with non-chilling susceptible fruit (Fig. 2B).

3.4 Effect of cold storage time on bioactive compounds in lemon flavedo

The concentration of bioactive compounds in the flavedo was altered in response to cold storage (Tables 2A, B and C). Vitamin C levels significantly decreased in the flavedo of Sun Valley lemons following 28 days of cold storage (Table 2A). Cold exposure increased vitamin E in both Sun Valley and Eston fruit during 28 days of cold storage (Tables 2A, B and C). Eston fruit had the lowest β-carotene concentration after 28 days cold storage (Table 2B). Total phenolics of the flavedo significantly increased in Ukulinga fruit after 14 days of cold storage; whereas decreased in chilling susceptible lemons from Sun Valley (Tables 2A, B and C). Total flavonoids significantly decreased in the flavedo of Ukulinga 2008 and Sun Valley fruit, while such compounds significantly increased in Ukulinga 2007 and Eston fruit 28 days after cold storage (Tables 2A, B and C). Furthermore, Sun Valley fruit had significantly lower naringin and hesperidin concentrations compared with non-chilled fruit from other sources these compounds; further decreased significantly for Sun Valley fruit during cold storage (Table 5).

The flavedo membrane integrity of lemon fruit sourced from Ukulinga decreased significantly from pre-storage (0 days cold storage) to 14 days into cold storage (Table 5). In contrast, chilling-susceptible fruit showed an increase in membrane disruption (detected as increase in MDA concentration) 21 days into cold storage of Sun Valley and 14 days into cold storage of Eston fruit (Table 5).

3.5 Effect of hot water and molybdenum on the concentration of certain bioactive compounds in lemon flavedo during cold storage

The effect of hot water and molybdenum on bioactive compounds is visible at three distinct stages: (i) immediately after treatment application (0 days cold storage), (ii) several days into storage (7 to 21 days of cold storage) and (iii) at the end of the storage period (28 days of cold storage). The combination of molybdenum and HWD 53°C enhanced vitamin C and vitamin E levels of fruit from most locations at stage (i), immediately after treatment application, while at stage (ii) no such increase was consistently visible. Possibly, vitamin C was used up fulfilling an antioxidant function in regenerating vitamin E (Smirnoff, 2000; Klimczak et al., 2007) leading to alterations in such vitamin C levels during stage (ii). The other bioactive compounds analysed (β-carotene, total phenolics, total flavonoids and naringin and hesperidin) did not show such alterations.

In the Sun Valley fruit, flavedo vitamin C levels doubled following HWD 53°C, 1 μM Mo plus HWD 53°C and 10 μM Mo plus HWD 53°C treatments compared with water dips (control) 0 days into cold storage (Table 3). The flavedo vitamin E concentration of these chilling-susceptible fruit increased when treated with HWD 53°C and 1 μM Mo plus HWD 53°C; however, these treatments could not significantly increase the flavedo β-carotene, naringin or hesperidin concentration (Table 5).

Furthermore, the HWD 53°C treatment was characterised by inducing a higher flavedo vitamin C concentration 7 and 14 days into the cold storage period in Sun Valley and Eston fruit (Tables 3 and 4). In addition, all treatments induced an increased in total phenolics and vitamin E concentration 7 to 21 days into the cold storage in these chilling-susceptible Sun Valley and Eston Estates fruit (Tables 3 and 4). However, HWD 53°C induced a reduction in total flavanoids and β-carotene during cold storage for both the chilling-resistant and chilling-susceptible fruit; however, Mo application plus HWD seemed to maintain β-carotene concentration during cold storage (Tables 3 and 4). Moreover, naringin and hesperidin concentrations were maintained by HWD 53°C, and all Mo containing treatments between 7 and 21 days of storage (Table 5).

The combination of 10 μM Mo plus HWD 53°C maintained higher vitamin C levels after 28 days storage plus 5 days shelf-life in the flavedo of Sun Valley Estates fruit (Tables 3 and 4). Furthermore, 1 or 10 μM Mo and HWD 53°C application resulted in significantly higher vitamin E and β-carotene concentrations in the flavedo of Sun Valley fruit, higher than in that of the control (water dips) and the chilling-resistant fruit from Ukulinga and Eston Estates (Tables 3 and 4). Naringin and hesperidin concentrations in Sun Valley Estates fruit were not affected by treatments 28 days into cold storage (Table 5).

After 7 days of cold storage MDA levels were significantly increased in Ukulinga 2007 and Sun Valley lemon flavedo following HWD 53°C; however, concentrations decreased from 14 to 28 days into cold storage (Table 5). The increase in total phenolics and total flavanoids of Sun Valley fruit at 21 days into cold storage corresponds with a significant decrease in MDA levels of fruit treated with 1 μM Mo dips between 14 to 28 days (Tables 3 and 5).

4. Discussion

Chilling susceptibility of lemon fruit is mainly attributed to climatic differences which determine the morphological and chemical composition of the rind (Sinclair 1984). Lemons are known to be the second most chilling susceptible citrus fruit, following grapefruit, which is more susceptible (Chalutz et al., 1985). Surprisingly, only lemon fruit sourced from Sun Valley Estates showed clear chilling symptoms (Table 1).

In Sun Valley fruit, HW 53°C was more effective in mitigating chilling injury than HWD 47°C and the control (water dips at ±25°C) (Table 1). This efficacy of HW 53°C was further improved when combined with Mo treatment. This positive effect of HW 53°C in mitigating chilling injury has been reported by several researchers; in 'Valencia' oranges, HW 53°C for 3 min reduced chilling injury to chilling index of 0.45 (Erkan et al., 2005), while in 'Torrocco' blood oranges the same treatment reduced decay when fruit were stored at 3°C for up to 19 weeks (Schirra et al., 1997).

The 1 μM Mo treatment also showed potential to mitigate chilling injury (Table 1); Mo is involved in the regulation of xanthine dehydrogenase (XDH) activity an enzyme linked to the production of reactive oxygen species (ROS) under stress conditions (Yesbergenova et al., 2005; Hesberg et al., 2003; Datta et al., 1991). The application of Mo might have triggered the production of ROS; however, such a level resulting in tissue damage below the critical threshold signal stress acclimation in plants (Foyer and Shigeoka, 2011). This probably resulted in the initial increase in production of bioactive compounds. Furthermore, it could be speculated that Mo plus HWD 53°C increased the activity of XDH during stress, thereby increasing the production of ROS which in turn triggered production of antioxidants (Mathaba et al., 2008).

Such compounds form part of the plant protection mechanism against ROS damage aiding in the reduction of membrane damage during stress (Campos et al., 2003). Bioactive compounds such as polyphenolics, flavonoids and some vitamins can act as antioxidants during stress exposure (Abseysinghe et al., 2007). Previous studies have indicated that the antioxidant capacity of citrus flavedo varies between cultivars (Huang et al., 2007); furthermore, significant differences in type of bioactive compounds which could serve as antioxidants are present in and between growing seasons (2007 and 2008) and conditions (fruit sourced from different farms) in lemon flavedo (Fig. 1). These differences may explain differences in the chilling susceptibility of fruit (Table 1).

Citrus fruit contain high concentration of vitamin C (Rapisarda et al., 2008), contributing more than 35% of the total antioxidants of citrus juice (Guimarães et al., 2009; Johnston and Hale, 2005) and between 26.9% to 45.9% in different citrus fruit tissues; including flavedo of different citrus cultivars (Abeysinghe et al., 2007). Similarly, in the lemon flavedo from different sources examined, vitamin C was the major antioxidant compound (Fig. 1A), a feature typical of fruit exocarp (Tesfay et al., 2010). Therefore, vitamin C can be assumed to be the first bioactive compound in the line of molecules able to reduce the onset of chilling injury. If concentrations of this bioactive compound in flavedo tissues are when fruit is exposed to chilling stress, chilling injury symptoms are likely to result (Fig. 1). Sun Valley fruit had the lowest vitamin C concentration followed by Eston Estates (Fig. 1A) and chilling injury was observed from both fruit localities (Table 1). Not surprisingly, principal component analysis revealed vitamin C as the major variable related to chilling-susceptibility of lemon fruit during cold storage (Fig 2A).

Vitamin C has been aligned to plant stress response such as cold stress. An alteration in vitamin C and other bioactive compounds was reported in curly kale leaves stored at 1°C for up to six weeks (Hagen et al., 2009); in ‘Green Star’ broccoli ascorbic acid increase or decrease was reduced by cold storage compared with room temperature storage (Cogo et al., 2011). In sweet orange (*Citrus sinensis*) β-carotene, vitamin C, naringin and hesperidin concentrations varied in segments and peel over 12 days at 4°C (Plaza et al., 2011); in tomato juice stored at 4°C for up to 100 days the decrease in vitamin C was a major factor contributing to quality reduction (Odriozola-Serrano et al., 2008). Similarly, alterations were observed with lemons stored at -0.5°C for up to 28 days (Tables 2 and 3). The activity of redox enzymes involved in the synthesis of vitamin C can explain such alterations during cold storage (Conklin et al., 1997; Hagen et al., 2009). Vitamin C has been proven to be synthesized from glucose as well as catabolised to glucose under stress condition; therefore, the transient change in vitamin C could be due to vitamin C storage or catabolism of ascorbic acid to glucose (Smirnoff, 2000).

Although hot water dips has have been shown to be beneficial as post-harvest practice to enhance stress resistance in plants (Rozenzvieg et al., 2004), their efficacy stimulation of bioactive compounds has not been fully explored. Furthermore, the potential of postharvest Mo solutions to alleviate chilling stress in citrus fruit have not been studied before. In the current study, the effect of hot water and Mo postharvest dips altered the concentration of bioactive compounds to levels probably above a certain critical threshold; thereby avoiding the occurrence of chilling symptoms (Tables 3 and 4). The HWD 53°C was effective in mitigating chilling symptoms a technique proven to stimulate HSPs (Rozenzvieg et al., 2004). Furthermore, HSPs could have signaled the up-regulation of enzymes synthesizing bioactive compounds just as Saffan (1998) found an increase in bioactive compounds, especially vitamin E, in heat-stressed peanut seedlings, which further indicated that HSPs might induce production of bioactive compounds.

It has not been clearly established how Mo is involved in the synthesis of bioactive compounds; however, the element has been reported to increase the production of ROS when plants are exposed to stress (Hesberg et al., 2003; Yesbergenova et al., 2005). A possible explanation might be that the supply of Mo increases the activity of XDH thereby triggering ROS production in lemon flavedo during cold stress as described by Mathaba et al. (2008). Foyer and Shigeoka (2011) proposed signalling of stress acclamation to be an essential function of ROS in plants. Therefore, the interaction between HWD and Mo dip could alleviate the stress in two ways; firstly, through the activation of HSPs which would signal the activation of enzymes involved in the production of bioactive compounds; secondly, Mo could increase the activity of XDH which enhance ROS production which signals the production of bioactive compounds measurable as reduced membrane damage (Table 4) and reduced chilling symptoms (Table 1).

The alteration in levels of bioactive compounds is influenced by their location within plant the cell (cytoplasm) or within the plasma membrane (membrane bound). Furthermore, these positions will determine whether a specific bioactive compound is water-soluble or lipid-soluble in nature (Sies and Stahl, 1995). Seemingly, bioactive compounds within specific locations of the cell further determine which compound acts as “first line defence” compound under stress. The potential for ROS to damage membrane lipids depends on their sub-cellular production site, as this determines the distance ROS have to be diffused to their site of action, mainly cellular organelles and cell membranes (Gill and Tuteja, 2010).

Ascorbic acid, a water-soluble compound, is located in the cytosol where it acts as a potent antioxidant (Gill and Tuteja, 2010); therefore, it possibly represents the “first line of defence” of the antioxidant system in lemon flavedo. Ascorbic acid is further involved in the regeneration of the lipid-soluble α-tocopherol from the tocopheroxy radical (Smirnoff, 2000; Klimczak et al., 2007), linking the function and recovery of water-soluble and lipid-soluble antioxidants.

The vitamin E concentration of the flavedo is relatively low compared with other bioactive compounds but the compound has a high antioxidant capacity exceeding that of most phenolics and carotenoids (Robles-Sánchez et al., 2009). Vitamin E is a lipid-soluble and membrane-bound compound with a function closely linked to vitamin C, playing a potent role in the defence against oxidative stress (Smirnoff, 2000; Klimczak et al., 2007; Gill and Tateja, 2010).

Total phenolics and flavonoids might be the "second line of defence" after ascorbic acid. Studies in broccoli leaves stored at 2°C for up to 7 days (Plaza et al., 2011) and grapefruit juice stored at 4°C for up to 2 months (Igual et al., 2011) displayed similar alterations in total phenolics and flavonoids during cold storage. Furthermore, total phenolics and flavonoids have been found to increase following heat treatments of other crops. In 'Fortune' mandarins, heat-conditioning at 34°C and subsequently stored at 16°C for 32 days (Lafauente et al., 2011) as well as in rocket heat-conditioned at 50°C and stored at 8°C for 10 days resulted in increased flavonoidlevels (Koukounaras et al., 2009).

Flavonoids form a large group within the phenolics family (Erlund, 2004) and are important contributors to the antioxidant capacity of lemon flavedo. Hesperidin is the main flavanone present in orange (Kanaze et al., 2004), mandarin (Erlund 2004) and lemon flavedo (González-Molina et al., 2010); which, naringin is dominant in grapefruit, giving it the characteristic bitter flavour (Kanaze et al., 2003). In general, naringin and hesperidin are present in small quantities (Klimczak et al., 2007) and, therefore, contributes less to the flavedo antioxidant capacity (Tables 3 and 4).

Another lipid-soluble bioactive compound with antioxidant properties is β-carotene. Previous research on orange juice stored at 4ºC has shown that β-carotene is not affected by cold storage (Plaza et al., 2011); however its presence is reduced in lemon flavedo following hot water dips (Tables 3 and 4).

Therefore, a cascade of bioactive compounds to mitigate oxidative stress in lemon flavedo is ascorbic acid > phenolics > flavanones (naringin and hesperidin) > vitamin E which might be triggered by ascorbic acid and finally β-carotene.

5. Conclusions

The susceptibility of lemon fruit to chilling stress is determined by the fruit source, which influences the bioactive compound composition and concentration. Therefore, chilling injury susceptibility during cold could be related to the concentration of bioactive antioxidants compounds in citrus flavedo. The triggering of the synthesis of these molecules following postharvest hot water and molybdenum treatments depends on the initial levels of such bioactive compounds. Hot water 53°C plus Mo showed potential to increase the concentration of bioactive compounds in the flavedo specifically vitamins C and E. Vitamin C seems to be the dominant flavedo antioxidant which concurrently regenerates vitamin E, an important membrane-bound antioxidant, providing protection for lipid-soluble as well as water-soluble flavedo compounds.

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List of Tables

Table 1: Effect of hot water and molybdenum postharvest dips on chilling symptoms of lemon fruit cold stored (-0.5°C) for up to 28 days.

Table 2: Effect of cold storage time on lemon flavedo specific bioactive compounds of fruit harvest from (A) Ukulinga and Sun Valley Estates 2007 season, (B) Ukulinga and Eston Estates 2008 season and (C) on specific flavanoids (naringin and hesperidin) and membrane lipid peroxidation from all fruit sources

Table 3: Effect of hot water and molybdenum postharvest dips on lemon flavedo specific bioactive compounds over 28 days cold storage (-0.5°C) during 2007 harvest season

Table 4: Effect of hot water and molybdenum postharvest dips on lemon flavedo specific bioactive compounds over 28 days cold storage (-0.5°C) during 2008 harvest season

Table 5: Effect of hot water and molybdenum postharvest dips on lemon flavedo specific flavanoids (naringin and hesperidin) and membrane lipid peroxidation over 28 days cold storage during 2007 to 2008 harvest seasons.

List of Figures

Figure 1: Effect of fruit source on lemon flavedo specific bioactive compounds on lemons harvested over 2007-2008 season

Figure 2: Principal component analysis (PCA) showing correlation loadings. A. Score plot lemon specific flavedo bioactive compounds B. Score plot for the groups of the bioactive compounds for lemons from different source with difference in chilling susceptibility, and PC1 explains 83% and PC2 explaining 14% of total variation.

Table 1:

Treatments[a]	Ukulinga 2007[b]	Sun Valley Estates 2007[c]	Ukulinga 2008[b]	Eston Estates 2008[d]
Water dip (25°C)	No symptoms	0.13a	No symptoms	Below detection
HW 47°C	No symptoms	0.18a	No symptoms	Below detection
HW 53°C	No symptoms	0.09a	No symptoms	Below detection
1 µM Mo	No symptoms	0.04ab	No symptoms	Below detection
1 µM Mo + HW 53°C	No symptoms	0.02ab	No symptoms	Below detection
10 µM Mo + HW 53°C	No symptoms	0.00b	No symptoms	Below detection

[a] Treatment effect after 28 days cold storage plus 5 days shelf-life

[b] Ukulinga lemon fruit showed no chilling symptoms during 2007 and 2008 harvest seasons

[c] Sun Valley lemon fruit showed chilling symptoms expressible in chilling index and means followed by the same letter were not significantly different at LSD(0.05) = 0.096

[d] Eston Estates lemons did show chilling symptoms but below detection and chilling index calculation

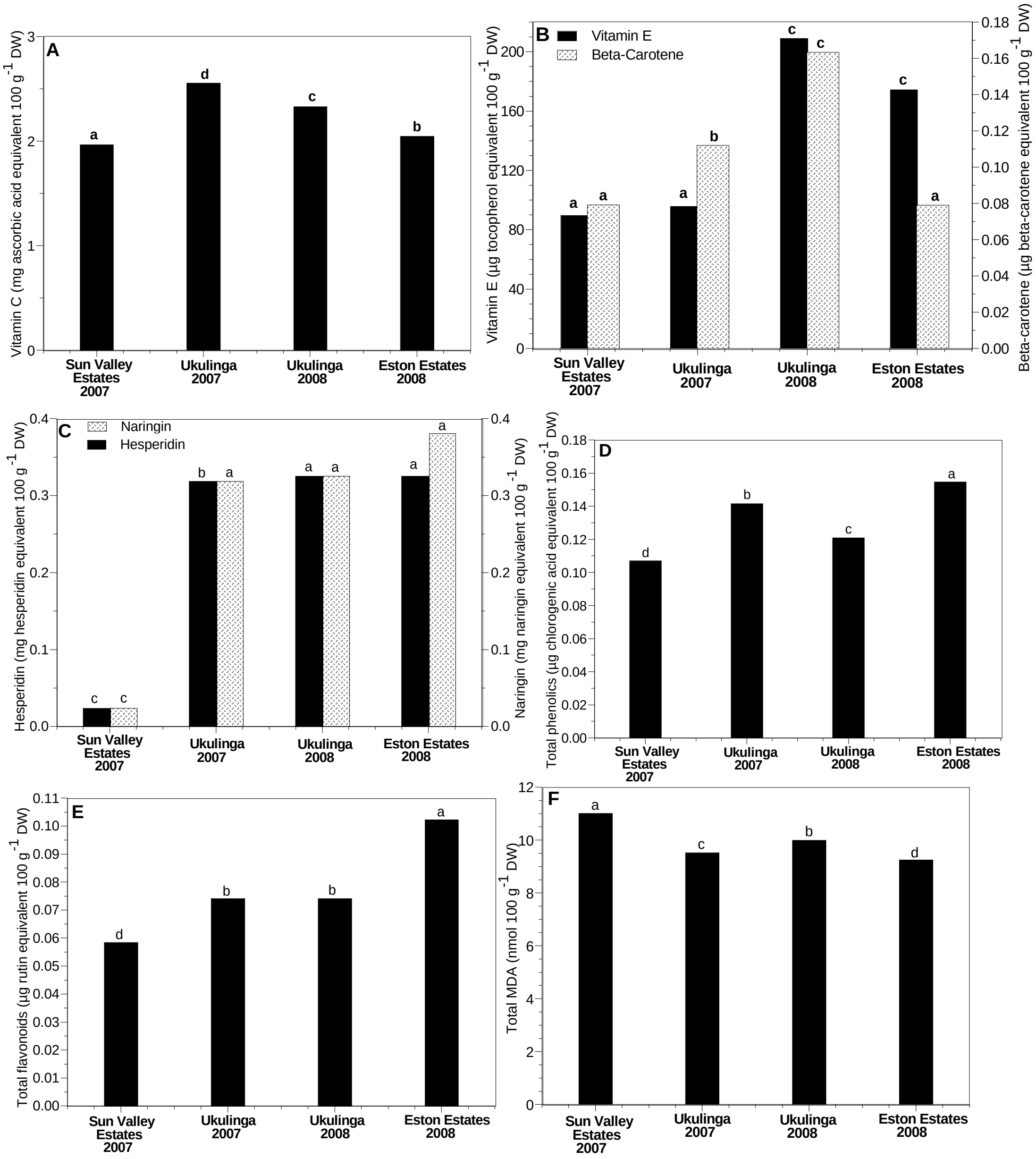
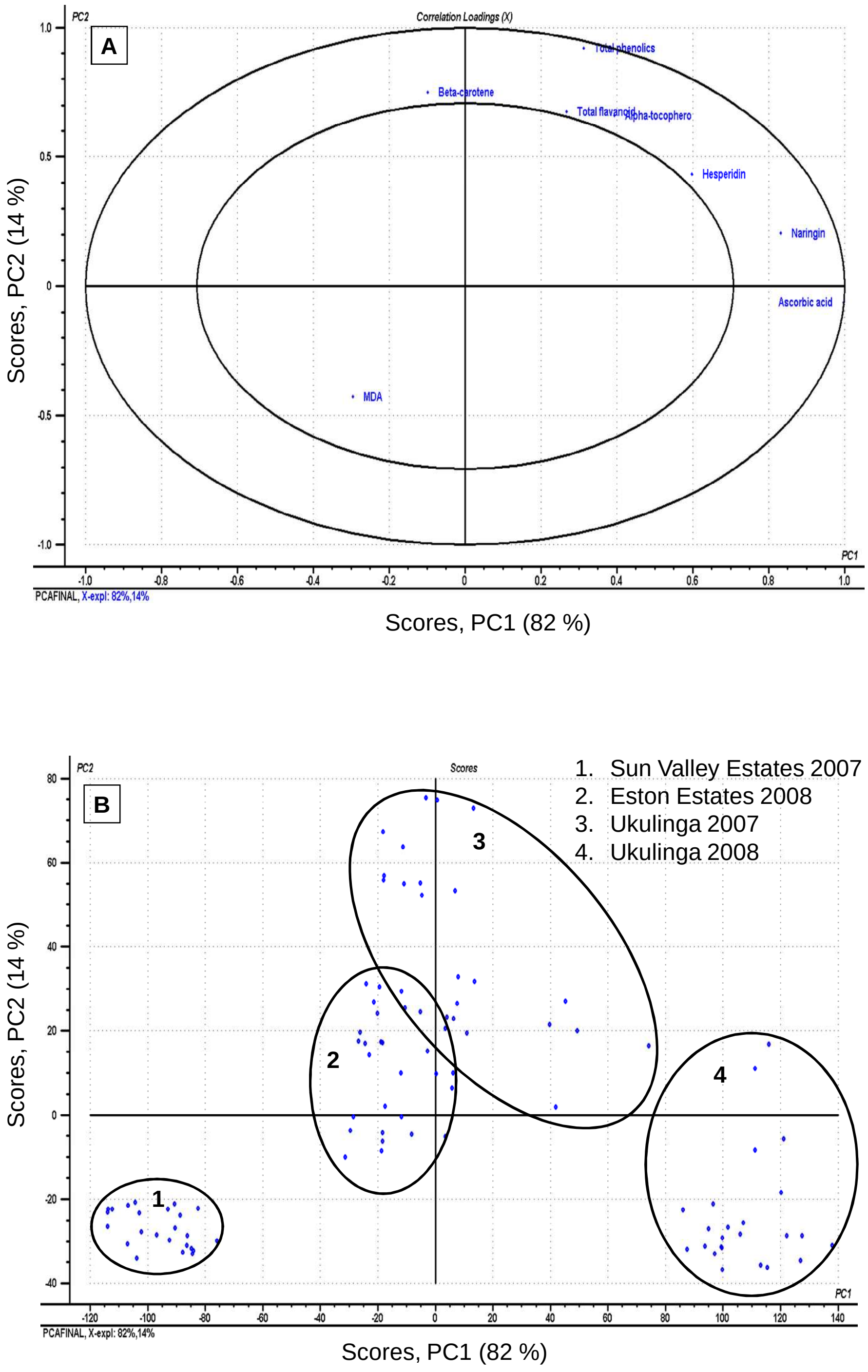

A
Vitamin C (mg ascorbic acid equivalent 100 g^{-1} DW)
0
1
2
3
a
d
c
b
Sun Valley Estates 2007
Ukulinga 2007
Ukulinga 2008
Eston Estates 2008
B
Vitamin E
Beta-Carotene
Vitamin E (µg tocopherol equivalent 100 g^{-1} DW)
Beta-carotene (µg beta-carotene equivalent 100 g^{-1} DW)
0
40
80
120
160
200
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
a
a
a
b
c
c
c
a
Sun Valley Estates 2007
Ukulinga 2007
Ukulinga 2008
Eston Estates 2008
C
Naringin
Hesperidin
Hesperidin (mg hesperidin equivalent 100 g^{-1} DW)
Naringin (mg naringin equivalent 100 g^{-1} DW)
0.0
0.1
0.2
0.3
0.4
c
c
b
a
a
a
a
a
Sun Valley Estates 2007
Ukulinga 2007
Ukulinga 2008
Eston Estates 2008
D
Total phenolics (µg chlorogenic acid equivalent 100 g^{-1} DW)
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
d
b
c
a
Sun Valley Estates 2007
Ukulinga 2007
Ukulinga 2008
Eston Estates 2008
E
Total flavonoids (µg rutin equivalent 100 g^{-1} DW)
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.10
0.11
d
b
b
a
Sun Valley Estates 2007
Ukulinga 2007
Ukulinga 2008
Eston Estates 2008
F
Total MDA (nmol 100 g^{-1} DW)
0
2
4
6
8
10
12
a
c
b
d
Sun Valley Estates 2007
Ukulinga 2007
Ukulinga 2008
Eston Estates 2008

Fig 1:



PC2
Correlation Loadings (X)
A
Total phenolics
Beta-carotene
Total flavanoid
Alpha-tocophero
Hesperidin
Naringin
Ascorbic acid
MDA
1.0
0.5
0
-0.5
-1.0
-1.0
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1.0
PC1
PCAFINAL, X-expl: 82%,14%
Scores, PC2 (14 %)
Scores, PC1 (82 %)
PC2
Scores
B
1. Sun Valley Estates 2007
2. Eston Estates 2008
3. Ukulinga 2007
4. Ukulinga 2008
1
2
3
4
80
60
40
20
0
-20
-40
-120
-100
-80
-60
-40
-20
0
20
40
60
80
100
120
140
PC1
PCAFINAL, X-expl: 82%,14%
Scores, PC2 (14 %)
Scores, PC1 (82 %)

Fig 2:

Table 2A

Cold storage time (days)	Ukulinga 2007					Sun Valley Estates 2007				
	Vitamin C[1]	Vitamin E[2]	B-carotene[3]	TP[4]	TF[5]	Vitamin C[1]	Vitamin E[2]	B-carotene[3]	TP[4]	TF[5]
0	**2.44c**	**200.8a**	**0.13b**	**151.5c**	**74.9e**	**2.19a**	**77.0b**	**0.02c**	**96.8h**	**52.3h**
7	**2.25d**	**74.8b**	**0.17a**	**149.5c**	**68.4f**	**1.99c**	**62.3b**	**0.09b**	**93.7h**	**61.6g**
14	**3.00a**	**85.8b**	**0.13b**	**129.8de**	**68.9f**	**2.12b**	**78.4b**	**0.12a**	**99.0h**	**60.2g**
21	**2.49c**	**50.4bc**	**0.09c**	**155.7bc**	**68.6f**	**1.92d**	**116.3a**	**0.09b**	**100.9h**	**65.4f**
28	**2.62b**	**67.7b**	**0.04d**	**122.2efg**	**85.9d**	**1.63e**	**115.1a**	**0.08b**	**90.6h**	**50.9h**

Table 2B

Cold storage time (days)	Ukulinga 2008					Eston Estates 2008				
	Vitamin C[1]	Vitamin E[2]	B-carotene[3]	TP[4]	TF[5]	Vitamin C[1]	Vitamin E[2]	B-carotene[3]	TP[4]	TF[5]
0	**2.33c**	**232.0b**	**0.122d**	**121.8efg**	**52.3h**	**2.12a**	**110.0c**	**0.20a**	**135.6d**	**95.1c**
7	**2.18d**	**183.2c**	**0.20a**	**117.3fg**	**61.6g**	**2.13a**	**126.3c**	**0.03c**	**169.6a**	**95.1c**
14	**2.59a**	**263.7a**	**0.14c**	**131.8de**	**60.2g**	**2.06b**	**128.4c**	**0.12b**	**150.1c**	**105.6b**
21	**2.18d**	**181.5c**	**0.17b**	**112.4g**	**65.4f**	**1.89d**	**323.3a**	**0.02c**	**164.5ab**	**106.1b**
28	**2.39b**	**186.3c**	**0.18b**	**125.4def**	**50.91**	**2.06b**	**185.7b**	**0.03c**	**148.6c**	**119.0a**

Table 2C

Cold storage time (days)	Ukulinga Research Farm 2007			Sun Valley Estates 2007			Ukulinga Research Farm 2008			Eston Estates 2008		
	Naringin[6]	Hesperidin[7]	MDA[8]	Naringin[6]	Hesperidin[7]	MDA[8]	Naringin[6]	Hesperidin[7]	MDA[8]	Naringin[6]	Hesperidin[7]	MDA[8]
0	**0.28b**	**0.28c**	**9.75g**	**0.04a**	**0.04a**	**11.70a**	**0.22d**	**0.22e**	**10.50c**	**0.26d**	**0.23e**	**8.26l**
7	**0.34a**	**0.34b**	**9.60h**	**0.03a**	**0.03a**	**11.44b**	**0.07e**	**0.07d**	**9.02j**	**0.45b**	**0.07f**	**7.97m**
14	**0.04c**	**0.34b**	**10.23de**	**0.02a**	**0.02a**	**12.52d**	**0.27c**	**0.27c**	**9.72h**	**0.61a**	**0.27c**	**9.78j**
21	**0.36a**	**0.36a**	**8.74g**	**0.02a**	**0.02a**	**9.84g**	**0.60a**	**0.60a**	**8.95j**	**0.20e**	**0.60a**	**10.19def**
se28	**0.28b**	**0.28c**	**9.33i**	**0.01ab**	**0.01ab**	**9.57**	**0.48b**	**0.48b**	**11.82a**	**0.38c**	**0.48b**	**10.09df**

Means with different letters within same column represent significant difference at P = 0.05.

Table 3:

		Ukulinga 2007					Sun Valley Estates 2007				
Cold storage time (days)	Treatements	Vitamin C[1]	Vitamin E[2]	B-carotene[3]	TP[4]	TF[5]	Vitamin C[1]	Vitamin E[2]	B-carotene[3]	TP[4]	TF[5]
0	Water dip (25°C)	2.11±0.05	140.36±59.69	0.13±0.03	140.0±11.6	95.7±11.7	1.86±0.01	59.16±4.17	0.02±0.00	95.2±1.0	47.9±2.1
	HW 47°C	1.91±0.01	227.57±15.21	0.18±0.01	144.8±5.8	97.2±10.1	1.86±0.01	48.79±1.50	0.02±0.00	94.7±0.6	49.5±1.1
	HW 53°C	1.93±0.01	169.05±2.94	0.15±0.02	157.1±24.4	65.0±2.4	2.64±0.01	136.82±9.38	0.02±0.00	98.4±1.2	56.7±2.2
	1 µM Mo	1.88±0.01	161.41±20.27	0.14±0.02	157.1±24.4	65.6±0.9	1.82±0.03	67.70±0.98	0.02±0.01	95.8±1.2	46.8±0.7
	1 µM Mo + HW 53°C	3.28±0.02	201.63±12.23	0.13±0.01	174.9±24.3	61.7±1.5	2.73±0.03	108.40±2.28	0.01±0.00	100.2±1.3	55.1±1.1
	10 µM Mo + HW 53°C	3.56±0.03	284.78±47.43	0.04±0.01	135.3±10.1	63.9±3.5	2.24±0.01	41.46±3.17	0.01±0.00	96.3±1.6	58.1±0.7
7	Water dip (25°C)	2.49±0.03	99.71±19.19	0.06±0.01	157.0±24.4	70.0±2.1	1.62±0.03	35.76±14.56	0.05±0.01	92.0±1.3	49.0±1.4
	HW 47°C	1.88±0.01	59.61±1.31	0.17±0.01	157.0±24.4	68.2±1.0	2.03±0.01	71.12±6.29	0.17±0.01	92.9±1.1	59.6±1.8
	HW 53°C	2.61±0.02	66.44±4.55	0.04±0.01	172.1±27.6	67.4±1.0	2.56±0.02	120.79±8.26	0.05±0.01	93.3±1.1	67.6±1.0
	1 µM Mo	1.73±0.01	56.62±3.73	0.38±0.01	137.2±8.1	69.1±1.8	2.00±0.02	20.46±7.98	0.16±0.01	92.4±0.9	65.5±2.3
	1 µM Mo + HW 53°C	2.43±0.01	64.07±1.21	0.33±0.01	140.0±11.6	66.7±1.6	2.03±0.03	102.28±19.56	0.13±0.02	93.8±1.0	69.6±3.0
	10 µM Mo + HW 53°C	2.33±0.01	102.62±1.58	0.03±0.01	133.7±11.7	69.0±1.0	1.71±0.01	23.33±8.60	0.01±0.00	97.9±1.8	58.0±1.6
14	Water dip (25°C)	2.27±0.01	28.76±2.92	0.13±0.01	132.4±13.1	67.0±2.7	2.05±0.01	11.56±1.46	0.05±0.02	100.8±1.5	59.7±0.7
	HW 47°C	3.33±0.03	24.54±3.98	0.07±0.01	128.6±14.2	70.1±1.1	2.57±0.02	137.28±24.33	0.19±0.01	99.6±1.5	62.2±2.2
	HW 53°C	2.75±0.03	29.87±1.50	0.11±0.01	130.4±15.4	67.8±2.7	2.68±0.02	140.33±41.25	0.17±0.01	95.2±1.0	66.5±2.7
	1 µM Mo	4.27±0.03	187.05±19.58	0.07±0.01	130.9±12.0	72.4±3.4	1.61±0.01	50.32±8.97	0.15±0.01	97.8±0.7	53.3±2.2
	1 µM Mo + HW 53°C	3.17±0.04	102.47±22.96	0.14±0.01	124.9±8.1	69.2±1.0	2.10±0.01	30.80±1.25	0.06±0.01	101.7±3.1	66.6±3.3
	10 µM Mo + HW 53°C	2.21±0.01	115.47±3.93	0.27±0.01	131.4±14.3	66.8±1.0	1.69±0.03	100.09±4.43	0.08±0.01	99.0±1.4	52.8±1.3
21	Water dip (25°C)	3.80±0.04	39.25±6.52	0.09±0.01	140.0±11.6	76.0±1.5	1.99±0.03	137.35±13.60	0.13±0.01	98.8±4.7	58.0±3.1
	HW 47°C	2.19±0.01	22.69±0.93	0.26±0.01	153.5±27.8	71.6±1.0	1.83±0.01	50.23±1.38	0.11±0.01	105.4±8.2	66.0±3.9
	HW 53°C	3.05±0.17	71.69±14.78	0.09±0.01	153.5±27.8	72.7±1.3	1.89±0.01	151.81±12.87	0.03±0.01	104.9±2.2	62.5±3.4
	1 µM Mo	1.85±0.04	72.65±19.87	0.05±0.01	174.9±24.3	50.1±1.5	2.39±0.01	172.28±18.74	0.09±0.01	104.7±7.4	76.6±5.1
	1 µM Mo + HW 53°C	1.91±0.01	3.33±0.90	0.06±0.01	172.1±27.7	71.6±1.0	1.66±0.06	112.88±7.26	0.04±0.01	97.0±10.8	64.8±4.7
	10 µM Mo + HW 53°C	2.12±0.02	92.82±17.45	0.02±0.01	140.0±11.6	69.8±1.1	1.78±0.03	73.50±2.95	0.12±0.01	93.7±10.8	64.5±3.7
28	Water dip (25°C)	2.63±0.01	124.85±11.31	0.03±0.01	117.4±8.3	81.8±1.9	1.60±0.01	99.17±1.43	0.09±0.01	91.2±1.0	42.2±1.7
	HW 47°C	3.47±0.03	31.06±3.12	0.01±0.01	124.9±16.2	88.0±5.8	1.60±0.01	22.43±3.85	0.03±0.01	91.0±2.0	52.4±0.3
	HW 53°C	1.61±0.01	11.40±0.60	nd	117.4±8.3	87.2±2.4	1.63±0.01	18.03±1.52	0.02±0.01	89.0±0.3	43.5±0.7
	1 µM Mo	2.13±0.01	10.01±1.25	0.09±0.01	120.2±10.9	87.2±2.4	1.65±0.01	104.47±3.45	0.06±0.01	91.7±2.4	48.8±2.2
	1 µM Mo + HW 53°C	2.51±0.01	117.53±17.00	0.06±0.01	123.0±10.4	87.2±2.4	1.65±0.01	197.21±11.15	0.14±0.02	91.5±0.7	57.0±3.7
	10 µM Mo + HW 53°C	3.39±0.02	111.59±9.47	0.06±0.01	130.5±15.4	83.7±3.7	1.63±0.01	255.81±3.49	0.14±0.01	127.7±5.1	61.4±1.0

[1] Vitanim C-mg ascorbic acid equivalent (ASE) 100 g^{-1} DW

[2] Vitamin E- µg α-tocopherol equivalent (ATE) 100 g^{-1} DW

[3] β-carotene – µg β-carotene equivalent (BE) 100 g^{-1} DW

[4] Total phenolics- µg chlorogenic acid equivalent (CAE) 100 g^{-1} DW

[5] Total flavonoids – µg rutin equivalent (RUE) 100 g^{-1} DW

The values are means of 3 triplicates ± standard error (SE)

Table 4:

		Ukulinga 2008					Eston Estates 2008				
Cold storage time (days)	Treatments	Vitamin C[1]	Vitamin E[2]	B-carotene[3]	TP[4]	TF[5]	Vitamin C[1]	Vitamin E[2]	B-carotene[3]	TP[4]	TF[5]
0	Water dip (25°C)	1.98±0.14	221.94±16.89	0.14±0.01	122.8±2.5	95.7±11.7	2.57±0.19	251.31±24.52	0.17±0.01	127.7±5.1	104.5±9.2
	HW 47°C	2.60±0.16	231.05±19.68	0.03±0.01	129.5±3.1	97.2±10.1	1.73±0.10	53.19±11.26	0.38±0.01	135.2±1.0	104.5±9.2
	HW 53°C	2.90±0.11	222.09±10.87	0.01±0.01	116.2±1.0	65.0±2.4	2.06±0.19	106.93± 8.03	0.01±0.01	130.5±5.8	87.5±2.4
	1 µM Mo	2.21±0.08	183.96±7.14	0.31±0.01	122.8±2.4	65.6±0.9	1.94±0.04	95.80± 4.86	0.06±0.01	135.2±1.0	92.2±3.4
	1 µM Mo + HW 53°C	1.73±0.06	209.12±22.60	0.01±0.01	120.0±5.1	61.7±1.5	2.81±0.11	53.77±10.94	0.25±0.03	140.0±5.8	92.2±3.4
	10 µM Mo + HW 53°C	2.54±0.10	323.99±140.93	0.06±0.01	119.7±4.3	63.9±3.5	1.60±0.04	98.88±31.17	0.36±0.03	144.7±5.8	89.5±1.0
7	Water dip (25°C)	2.52±0.18	164.97±4.37	0.03±0.01	112.5±1.0	70.0±2.1	1.82±0.06	131.01±14.26	0.02±0.01	164.5±18.5	104.5±9.2
	HW 47°C	1.57±0.01	210.47±8.90	0.14±0.01	119.7±4.3	68.2±1.0	1.60±0.01	99.13±5.00	0.04±0.01	179.6±18.5	104.5±9.2
	HW 53°C	2.31±0.08	268.75±13.46	0.07±0.03	116.5±3.0	67.4±1.0	3.10±0.19	202.10±19.77	0.01±0.00	164.5±18.5	87.5±2.4
	1 µM Mo	2.57±0.23	192.15±7.60	0.13±0.01	113.7±1.5	69.1±1.8	1.82±0.03	179.64±15.46	0.01±0.00	164.5±18.5	92.2±3.4
	1 µM Mo + HW 53°C	2.02±0.03	191.89±11.94	0.05±0.01	116.5±3.0	66.7±1.6	1.66±0.03	43.82±5.97	nd	179.6±18.5	92.2±3.4
	10 µM Mo + HW 53°C	2.09±0.02	71.06±4.66	0.19±0.01	124.8±2.4	69.0±1.0	2.80±0.28	101.85±6.53	0.04±0.02	164.5±18.5	89.5±1.0
14	Water dip (25°C)	2.46±0.07	203.45±15.94	0.09±0.01	124.7±2.4	67.0±2.7	1.93±0.05	127.90±6.16	0.35±0.01	149.5±1.0	104.6±9.2
	HW 47°C	2.13±0.02	389.77±26.23	0.05±0.01	137.0±8.1	70.1±1.1	1.59±0.04	193.66±22.72	0.12±0.01	164.5±18.5	107.3±5.8
	HW 53°C	3.49±0.08	241.68±25.08	0.13±0.01	130.3±5.8	67.8±2.7	2.64±0.09	107.45±10.25	0.01±0.00	144.7±5.8	99.8±8.1
	1 µM Mo	3.62±0.02	268.27±14.14	0.03±0.01	134.2±9.2	72.4±3.4	2.29±0.09	109.01±5.51	0.01±0.00	137.2±8.1	102.6±5.8
	1 µM Mo + HW 53°C	2.16±0.02	222.11±15.68	0.30±0.05	129.5±3.4	69.2±1.0	1.63±0.02	145.55±15.56	0.06±0.05	159.8±22.0	107.3±5.8
	10 µM Mo + HW 53°C	1.66±0.01	256.77±7.53	0.08±0.02	135.1±1.0	66.8±1.0	2.26±0.05	87.05±25.81	0.04±0.01	144.7±5.8	112.1±1.0
21	Water dip (25°C)	2.13±0.05	174.88±8.04	0.09±0.01	112.9±2.4	76.0±1.5	1.99±0.01	185.44±4.63	0.01±0.00	149.4±1.0	102.1±5.8
	HW 47°C	2.06±0.02	270.23±4.14	0.12±0.01	112.9±2.4	71.6±1.0	1.62±0.01	255.09±5.32	0.01±0.00	164.5±18.5	102.1±5.8
	HW 53°C	2.46±0.06	266.53±13.47	0.01±0.01	114.4±4.2	72.7±1.3	1.76±0.04	442.05±8.69	0.01±0.00	149.4±1.0	102.1±5.8
	1 µM Mo	2.41±0.02	176.29±13.13	0.06±0.01	109.5±1.0	50.1±1.5	1.59±0.01	172.47±12.20	0.03±0.00	164.5±18.5	111.6±1.0
	1 µM Mo + HW 53°C	2.10±0.04	94.24±15.64	0.10±0.01	110.6±1.3	71.6±1.0	2.78±0.20	606.45±44.34	0.03±0.00	164.5±18.5	106.9±5.8
	10 µM Mo + HW 53°C	2.01±0.04	212.52±81.07	0.11±0.02	114.0±15	69.8±1.1	1.59±0.01	278.56±15.97	0.01±0.00	194.7±1.0	111.6±1.0
28	Water dip (25°C)	2.24±0.04	232.86±4.80	0.15±0.01	127.0±1.0	81.8±1.9	1.83±0.06	130.25±5.11	nd	144.7±5.8	114.7±4.9
	HW 47°C	1.76±0.06	193.68±14.61	0.23±0.01	123.5±4.3	88.0±5.8	2.38±0.07	141.05±10.91	nd	149.4±1.0	119.7±4.9
	HW 53°C	1.83±0.01	118.22±8.05	0.16±0.01	131.5±4.3	87.2±2.4	2.03±0.02	180.52±11.12	0.16±0.06	149.4±1.0	123.7±1.0
	1 µM Mo	3.69±0.01	113.95±11.14	0.43±0.01	123.5±4.3	87.2±2.4	1.83±0.02	183.24±6.71	nd	149.4±1.0	119.7±4.9
	1 µM Mo + HW 53°C	1.87±0.02	254.86±23.83	0.13±0.01	127.8±5.1	87.2±2.4	2.01±0.012	280.83±9.07	0.01±0.00	149.4±1.0	123.7±1.0
	10 µM Mo + HW 53°C	2.94±0.05	204.00±7.57	0.11±0.01	119.5±1.9	83.7±3.7	2.27±0.04	198.19±5.86	nd	149.4±1.0	111.8±1.0

[1] Vitanim C-mg ascorbic acid equivalent (ASE) 100 g^{-1} DW

[2] Vitamin E- µg α-tocopherol equivalent (ATE) 100 g^{-1} DW

[3] β-carotene – µg β-carotene equivalent (BE) 100 g^{-1} DW

[4] Total phenolics- µg chlorogenic acid equivalent (CAE) 100 g^{-1} DW

[5] Total flavonoids – µg rutin equivalent (RUE) 100 g^{-1} DW

**The values are means of 3 triplicates ± standard error (SE)

Table 5:

Cold storage time (days)	Treatments**	Ukulinga Research Farm 2007			Sun Valley Estates 2007			Ukulinga Research Farm 2008			Eston Estates 2008		
		Naringin[6]	Hesperidin[7]	MDA[8]	Naringin[6]	Hesperidin[7]	MDA[8]	Naringin[6]	Hesperidin[7]	MDA[8]	Naringin[6]	Hesperidin[7]	MDA[8]
0	Water dip (25^{O}C)	0.42±0.03	0.42±0.03	10.47±0.01	0.03±0.00	0.03±0.00	10.15±0.08	0.42±0.02	0.42±0.02	13.51±0.04	0.48±0.02	0.42±0.02	8.67±0.02
	HW 47^{o}C	0.12±0.01	0.12±0.01	10.34±0.01	0.04±0.00	0.04±0.00	12.26±0.07	0.12±0.01	0.12±0.01	10.49±0.03	0.12±0.01	0.12±0.01	8.13±0.03
	HW 53^{o}C	0.12±0.01	0.12±0.01	9.02±0.01	0.04±0.00	0.04±0.00	10.30±0.06	0.12±0.01	0.12±0.01	10.23±0.01	0.15±0.01	0.12±0.01	9.88±0.03
	1 µM Mo	0.29±0.02	0.29±0.01	8.20±0.01	0.03±0.00	0.03±0.00	12.15±0.09	0.24±0.01	0.24±0.01	8.17±0.03	0.14±0.01	0.24±0.05	8.02±0.01
	1 µM Mo + HW 53^{o}C	0.28±0.01	0.28±0.02	8.85±0.02	0.04±0.00	0.04±0.00	13.35±0.16	0.28±0.01	0.28±0.01	10.53±0.03	0.09±0.01	0.28±0.01	7.07±0.02
	10 µM Mo + HW 53^{o}C	0.43±0.01	0.43±0.01	11.65±0.02	0.05±0.00	0.05±0.00	11.99±0.02	0.12±0.01	0.12±0.01	10.09±0.01	0.58±0.03	0.12±0.01	7.77±0.02
7	Water dip (25^{O}C)	0.39±0.01	0.39±0.01	9.17±0.09	0.03±0.00	0.03±0.00	11.72±0.07	0.05±0.00	0.05±0.00	8.37±0.04	0.11±0.01	0.05±0.00	6.84±0.01
	HW 47^{o}C	0.26±0.02	0.26±0.01	8.17±0.01	0.03±0.00	0.03±0.00	9.82±0.13	0.07±0.00	0.06±0.00	8.67±0.02	0.81±0.06	0.06±0.00	8.54±0.02
	HW 53^{o}C	0.38±0.01	0.38±0.01	15.24±0.08	0.04±0.00	0.04±0.00	12.18±0.02	0.05±0.00	0.05±0.00	8.67±0.03	0.06±0.01	0.05±0.00	8.40±0.02
	1 µM Mo	0.38±0.01	0.39±0.01	7.44±0.01	0.02±0.00	0.02±0.00	10.30±0.01	0.08±0.00	0.08±0.00	9.01±0.03	0.79±0.04	0.08±0.00	9.37±0.02
	1 µM Mo + HW 53^{o}C	0.31±0.01	0.31±0.02	8.40±0.01	0.04±0.00	0.03±0.00	13.57±0.01	0.10±0.00	0.10±0.00	10.01±0.02	0.42±0.02	0.10±0.00	5.24±0.02
	10 µM Mo + HW 53^{o}C	0.30±0.01	0.30±0.02	9.21±0.03	0.03±0.00	0.03±0.00	10.97±0.01	0.08±0.00	0.08±0.00	8.84±0.02	0.51±0.02	0.08±0.00	9.40±0.02
14	Water dip (25^{O}C)	0.21±0.01	0.21±0.02	8.46±0.03	0.03±0.00	0.03±0.00	17.66±0.03	0.09±0.01	0.10±0.02	9.17±0.03	0.91±0.02	0.10±0.01	8.71±0.15
	HW 47^{o}C	0.42±0.03	0.42±0.02	9.05±0.03	0.01±0.00	0.03±0.00	10.92±0.03	0.09±0.01	0.09±0.01	9.46±0.01	1.31±0.12	0.09±0.01	9.54±0.03
	HW 53^{o}C	0.49±0.01	0.49±0.01	9.32±0.07	0.03±0.00	0.03±0.00	11.58±0.07	0.11±0.01	0.11±0.01	10.96±0.02	1.08±0.10	0.11±0.01	9.26±0.03
	1 µM Mo	0.30±0.02	0.30±0.01	11.24±0.01	0.02±0.00	0.02±0.00	11.12±0.01	0.18±0.01	0.18±0.02	11.73±0.03	0.11±0.01	0.18±0.02	8.40±0.15
	1 µM Mo + HW 53^{o}C	0.30±0.02	0.30±0.02	11.24±0.01	0.02±0.00	0.02±0.00	12.40±0.01	0.60±0.02	0.60±0.04	8.01±0.88	0.19±0.02	0.60±0.09	7.73±0.04
	10 µM Mo + HW 53^{o}C	0.29±0.01	0.29±0.01	12.06±0.01	0.02±0.00	0.02±0.00	11.47±0.01	0.53±0.03	0.53±0.01	8.99±0.02	0.08±0.01	0.53±0.04	15.01±0.02
21	Water dip (25^{O}C)	0.37±0.01	0.37±0.01	8.15±0.02	0.01±0.00	0.01±0.00	9.29±0.08	0.40±0.01	0.40±0.01	8.42±0.20	0.29±0.07	0.40±0.03	10.92±0.09
	HW 47^{o}C	0.25±0.02	0.25±0.01	8.27±0.01	0.01±0.00	0.03±0.00	8.59±0.05	0.59±0.01	0.59±0.02	9.27±0.05	0.34±0.01	0.59±0.03	11.21±0.09
	HW 53^{o}C	0.45±0.01	0.45±0.03	9.62±0.01	0.02±0.00	0.02±0.00	10.32±0.09	0.87±0.06	0.87±0.01	8.38±0.02	0.07±0.01	0.87±0.01	9.54±0.06
	1 µM Mo	0.58±0.01	0.58±0.03	9.11±0.01	0.03±0.00	0.03±0.00	10.39±0.06	0.74±0.06	0.74±0.02	7.44±0.01	0.31±0.01	0.74±0.01	9.79±0.16
	1 µM Mo + HW 53^{o}C	0.23±0.01	0.23±0.01	8.46±0.01	0.02±0.00	0.02±0.00	10.40±0.03	0.66±0.02	0.66±0.01	10.90±0.03	0.19±0.02	0.66±0.02	9.78±0.06
	10 µM Mo + HW 53^{o}C	0.30±0.01	0.30±0.02	8.81±0.01	0.01±0.00	0.01±0.00	10.07±0.03	0.31±0.01	0.31±0.01	9.32±0.01	0.02±0.01	0.31±0.01	10.20±0.05
28	Water dip (25^{O}C)	0.27±0.01	0.27±0.01	9.44±0.25	0.01±0.00	0.01±0.00	9.23±0.03	0.69±0.03	0.69±0.02	14.61±0.11	0.32±0.02	0.67±0.04	8.52±0.24
	HW 47^{o}C	0.22±0.02	0.22±0.01	9.64±0.24	0.01±0.00	0.01±0.00	9.56±0.07	0.46±0.01	0.46±0.02	11.81±0.27	0.22±0.04	0.46±0.02	11.27±0.21
	HW 53^{o}C	0.33±0.02	0.33±0.01	8.50±0.03	0.01±0.00	0.01±0.00	8.50±0.19	0.32±0.01	0.32±0.01	9.66±0.025	0.66±0.02	0.32±0.02	10.45±0.14
	1 µM Mo	0.35±0.01	0.35±0.02	8.28±0.01	0.01±0.00	0.01±0.00	9.56±0.03	0.63±0.04	0.63±0.05	13.34±0.12	0.64±0.02	0.63±0.01	9.40±0.57
	1 µM Mo + HW 53^{o}C	0.26±0.01	0.26±0.01	8.51±0.12	0.01±0.00	0.01±0.00	10.23±0.02	0.27±0.01	0.27±0.01	10.21±0.02	0.31±0.01	0.27±0.01	10.33±0.02
	10 µM Mo + HW 53^{o}C	0.26±0.01	0.26±0.01	11.73±0.02	0.01±0.00	0.01±0.00	10.34±0.05	0.50±0.03	0.50±0.01	11.31±0.02	0.13±0.01	0.50±0.02	10.59±0.08

[6] Naringin- mg naringin equivalent (NE) 100 g^{-1} DW

[7] Hesperidin – mg hesperidin equivalent (HE) 100 g^{-1} DW

[8] Malondialdehyde (MDA) – nmol 100 g^{-1} DW .

**The values are means of 3 triplicates ± standard error (SE)

CHAPTER 6

Effect of Hot Water and Molybdenum Dips on Endogenous Polyamines and 70 kDa Protein (estimator of HSP70) in Lemon Flavedo and their Ability to Alleviate Chilling Injury during Cold Storage

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Abstract

Polyamines are cations which bind to negatively charged cellular compounds such as proteins, DNA and membrane phospholipids. The function of antioxidants, proteins (including heat shock proteins (HSPs)), and membranes is improved when conjugated with polyamines (PAs). As molecular charperones, HSPs re-direct damaged proteins towards proteolysis. Heat shock proteins also act synergistically with polyamines in mitigating plant stress. Furthermore, hot water (HW) treatment enhances HSP production and HW plus molybdenum (Mo) treatments have been reported to reduce chilling injury in citrus fruit. Therefore, the potential role of HW and Mo to mitigate chilling injury by enhancing heat shock protein activity was investigated. Fruit from different sources, commonly chilling-susceptible or resistant, were pre-conditioned for 2 min with HW 47 or 53°C in combination with a subsequent soak in 1 or 10 μM Na_2MoO_4 solution for 30 min. Fruit were subsequently stored at -0.5°C for 7, 14, 21 or 28 days, then moved to ambient temperature for a week and evaluated for chilling injury symptoms. The flavedo of lemon fruit treated with HW 53°C, 1 μM Mo, 10 μM Mo plus HW 53°C showed enhanced levels of expression of 70 kDa protein (estimator of HSP70) compared with control or HW 47°C fruit which correlated with increased soluble-conjugated polyamine and had reduced chilling injury. A protective function of PAs sustaining action of HSPs seems therefore, likely.

Keywords: *Lemon (Citrus limon); chilling injury; heat shock proteins; polyamines; hot water; molybdenum*

1. Introduction

The South African Citrus industry faces challenges of the competitive export market. Suppling high quality fruit and compliance with phytosanitary standards of importing countries requires cold sterilization of citrus fruit to eradicate various fruit flies (McLauchlan et al., 1997; Underhill et al., 1999). Such a treatment is a well established physanitary standard for most high paying, competitive markets (Houck et al., 1990; Porat et al., 2000). Lemons ('Eureka') like other citrus fruit, originate from subtropical regions and, are therefore, susceptible to chilling injury (CI) (Chalutz et al., 1985). Symptoms of CI in citrus fruit manifest as dark sunken lesions and pitting (Leguizamon et al., 2001). Furthermore, the chilling susceptibility of citrus fruit results in enormous economic losses, as fruit marketability is reduced (McLauchlan et al., 1997; Porat et al., 2000).

Postharvest hot water (HW) treatments has received much attention in the last decade as a potential technology to mitigate CI in 'Marsh' grapefruit (Ezz et al., 2004), 'Washington Navel' oranges (Erkan and Peckmezci, 2000), and 'Lisbon' and 'Eureka' lemons (McLaughlan et al., 1997; Underhill et al., 1999). The mechanism of action of such, treatments occurs through the activation of heat shock proteins (HSPs) (Lindquist, 1986; Vierling, 1991; Sabehat et al., 1996; Königshofer and Lechner, 2002; Rozenzvieg et al., 2004), acting as molecular chaperones that assist in the correct folding of newly synthesized proteins, their assembly, transport as well as in the repair of other proteins (Königshofer and Lechner, 2002; Rozenzvieg et al., 2004).

Previous results found postharvest molybdenum (Mo) dips to mitigate CI in lemons (Mathaba et al., 2008). However, how Mo is involved in mitigating chilling stress is not known. Xanthine dehydrogenase (XDH), a Mo co-factor enzyme, has been found to increase in senescent leaves of *Arabidopsis thaliana* under salinity and cold stress (Hesberg et al., 2003). Moreover, an increase in XDH activity correlates with the production of reactive oxygen species (ROS) in water-stressed leaves and roots of *A. thaliana* (Yesbergenova et al., 2005). Kesker et al. (1997) also found XDH activity to increase severalfold following pathogen attack. The production of ROS due to increased XDH activity under stress adds to the oxidative damage caused during stress. Furthermore, if present below critical damaging threshold, ROS molecules can signal stress acclimation in plant cells (Foyer and Noctor, 2005; Foyer and Shigeoka, 2011). It has been proposed that certain redox reactions produce ROS as acclimatory molecules to balance the cellular redox homeostasis (Foyer and Shgeoka, 2011); therefore, ROS produced through increased XDH activity could be important in signaling the production of other stress mitigating compounds, such as polyamines (PAs).

Polyamines are present in all compartments of living plant cells, including the nucleus (Wang et al., 1993; Messiaen et al., 1997; Valero et al., 2002; Königshofer and Lechner, 2002; Tassoni et al., 2004; Kuznetsov and Shevyakova, 2007; Wu et al., 2009; Naka et al., 2010). In plants putrescine (*put*), spermidine (*spd*) and spermine (*spm*) have been found to be the most abundant PAs (Kuznetsov et al., 2007) and involved in stress response in a variety of plant species. Exogenous application of *put* increases chilling resistance of plums when stored at 2°C for up to 28 days (Valero et al., 2002), of bell peppers when kept at 8°C for 28 days (González-Aguilar, 2000), of pomegranate fruit maintained at 2°C for up to 90 days (Mirdehghan et al., 2007), as well as zucchini squash stored at 2°C and 10°C for up to 12 days (Serrona et al., 1998; Martínez-Téllez et al., 2002), and Navel oranges and 'Ponkan' mandarins stored for three months (Tassoni et al., 2004; Zheng and Zhang, 2004). There are few proposed physiological mechanisms by which PAs counteract stress. At physiological cell pH, PAs are present as cations, binding to negatively charged molecules such as proteins, membrane phospholipids and DNA (Valero et al., 2002; Hönigshofer and Lechner, 2002; Tassoni et al., 2004; Kuznetsov and Shevyakova, 2007; Wu et al., 2009; Naka et al., 2010), thereby, protecting such molecules from oxidative damage.

Conjugated PAs have been found to be more important than free soluble PAs. When conjugated to non-enzymatic antioxidants, such as phenolics, PAs improve the ROS scavenging capacity (Kuznetsov and Sheyakova, 2007). The plant cell wall is primarily constituted by a cellulose-xyloglucan frame-work within the complex of specific pectic polysaccharides. These pectins make up about 30% of the total cell wall's dry mass. Polyanionic pectins form covalent bond with cationic PAs and the pectin-polyamine covalent linkage enhances cell-wall assembly, maintaining wall cohesion during cell expansion, and strengthening the wall during any form of stress (Messiaen et al., 1997; Lenecci et al., 2005).

In order to alleviate chilling injury in lemons, fruit underwent hot water and molybdenum postharvest treatments to enhance conjugation of the endogenous free-soluble, soluble-conjugated and membrane-conjugated PA concentrations. Furthermore, the effect of such treatments on heat shock proteins (particularly HSP70 which is aligned with chilling stress (Rozenzvieg et al., 2004)) and the potential correlation between soluble-conjugated and membrane-conjugated HSPs during cold storage of lemon fruit was investigated.

2. Methods and Material

2.1 Plant material

Mature lemon (“Eureka”) fruit were obtained from Ukulinga Research Farm (29°39’48.82”S, 30°24’19.89”E) and Sun Valley Estate (28°51’00”S, 30°04’00”E) in the first (2007) harvest season and from Ukulinga Research Farm (29°39’48.82”S, 30°24’19.89”E) and Eston Estates (29°47’00”S, 29°27’00”E) in the second (2008) harvest season. Fruit were treated by 30 min dip in 1 μM and 10 μM $Na_2MoO_4.2H_20$ followed by 2 min in HW at 53°C. Treated fruit were waxed, weighed and subsequently stored at -0.5°C for up to 28 days and evaluated on a weekly basis for chilling injury. In addition, fruit were evaluated five days after withdrawal from cold storage. Such fruit were peeled, the peel freeze-dried, milled using a mortar and pestle and stored at -21^{o}C for further physiological analysis.

2.2 Estimation of chilling injury

The method of Sala (1998) was used to evaluate for chilling injury on five days after withdrawal from cold storage. This method uses a rating scale based on surface browning intensity (0 - sound fruit, 1 - less than 10%, 2 - 10 to 20% pitting, 3 - 30 to 40% pitting and 4 - more than 50% pitting). The chilling injury index (CII), which expresses the severity of chilling damage was calculated by adding the products of the number of fruit in each category to the value assigned to this category in the rating scale and dividing this sum by the total number of fruit evaluated.

CII = Σ (number of fruit with chilling injury x score of severity) / Total number of fruit evaluated

2.3 Determination of free-soluble, soluble-conjugated and membrane-bound polyamines

The various PA fractions were extracted according to Valero et al. (2002). Polyamines were extracted twice; in the first procedure free-soluble and soluble-conjugated PAs were extracted, while in the second one, membrane-bound polyamines were released. One gram freeze-dried tissue was extracted with 10 ml 5% cold perchloric acid (PCA) and 1,6-hexanediamine (100 nmol g^{-1}) was added as an internal standard. The homogenate was then centrifuged for 30 min at 2000 x g. A 2 ml aliquot from the supernatant served to determine the free-soluble polyamines. For soluble-conjugated polyamine, a 2 ml aliquot was taken from the initial supernatant and then hydrolyzed with 6 N HCl at 110°C for16 hrs. Membrane-bound polyamines were determined by redissolving in 10 ml of 6 N HCl-5% containing PCA and then hydrolyzed as described above. Two ml of the free-soluble, the PCA soluble-conjugated and the membrane-bound hydrolyzed PAs supernatant was benzoylated with 10 µl benzoyl chloride after 2 ml 2 N NaOH were added. The benzoylated mixture was incubated at room temperature for 20 min; thereafter, 2 ml of saturated sodium chloride was added and benzoly-polyamines were partitioned with 2 ml of diethyl ether. One mL of the ether phase was dried under N_2, and dissolved in 100 µl methanol and analyzed by HPLC. The compounds were eluted with $MeOH/H_2O$ (64:36), at a flow rate of 1 ml min^{-1}. The following equations were used to determine the PA concentration: $y = 0.007x$ $r = 0.96$ (for *Put*), and $y = 0.0001 x – 0.0017$ $r = 0.97$ (for *Spm*), and $y = 0.0005 x – 0.0014$ $r = 0.99$ (for *Spd*).

2.4 SDS-PAGE analysis

2.4.1 Pre-treatment of citrus flavedo as recalcitrant material

Two grams of lemon flavedo was pulverized into fine powder using a cold mortar and pestle under liquid nitrogen. The fine powder was transferred into a test tube and filled with 30 ml 10% TCA/acetone containing 1% polyvinylpolypyrrolidone (PVPP). The mixture was vortexed and centrifuged at 16 000 x g for 10 min at 4°C. The supernatant was decanted carefully and the pellet washed until a white powder was obtained which was air-dried at room temperature.

2.3.2 Protein analysis

Proteins were extracted from lemon flavedo according to a modified method of Zhang et al. (2005). The extraction buffer consisted of a 150 mM Tris-HC*l* (pH 8.9), 2% (w/v) SDS, 10 mM

$MgCl_2$, 10 mM ascorbic acid, 2 mM EDTA-Na_2, 1 mM PMSF solution containing 0.2% (v/v) 2-mercaptoethanol and 2% (w/v) PVPP. The protein concentration was determined according to Bradford (1976) using bovine albumin (BSA) as standard. Extracted proteins were separated by SDS-PAGE as described by Laemmli (1970). Silver staining was used for protein band visualization.

2.5 Statistical analysis

Statistical comparison of mean values was performed by analysis of variance (ANOVA) using Genstat version 14. Means were separated using Duncan's multiple range test at a 5% significance level.

3. Results

3.1 Effect of HW and Mo treatments on chilling injury development during cold storage

Only lemon fruit sourced from Sun Valley Estates showed quantifiable chilling symptoms during the 2007 season, while Eston fruit only showed very few chilling symptoms, which were not quantifiable with the chilling index formula (Table 1). Fruit sourced from Ukulinga did not show any chilling symptoms during the two harvest seasons. Lower temperature hot water (HW 47°C) treatment was not as effective in reducing chilling injury development as HW 53°C. The chilling index of Sun Valley lemons was 0.18 and 0.09 for the HW 47°C and HW 53°C, respectively (Table 1). Furthermore, the 1 μM Mo treatment showed tendency to be more effective than both HW treatments with a chilling index of 0.04. The 10 μM Mo plus HW at 53 °C completely eliminated chilling symptoms (Table 1).

3.2 Separation of polyamines in lemon flavedo extracts

The benzoylation method clearly separated *put*, 1, 6-hexanediamine (internal standard), *spm* and *spd* which were elucidated at 7.07, 10.10, 11.41 and 18.50 min, respectively (Fig. 1A). The major polyamine was *put*, while *spd* and *spm* were present in lower but almost equal concentrations in the lemon flavedo. Furthermore, in all fractions (free-soluble, soluble-conjugates and membrane-conjugates) *put* was dominant (Fig. 1B-D). In addition, *spd* and *spm* mostly occurred in free soluble form (Fig. 1B).

3.3 Effect of fruit source and cold storage time on polyamines in lemon flavedo

Lemon fruit sourced from Sun Valley Estates were chilling susceptible (Table 1). However, they showed significantly ($P < 0.001$) higher free-soluble *put* compared with non-chilled lemon fruit from other sources (Fig. 2A). Soluble-conjugated *put* was significantly different ($p < 0.001$) for all fruit sources, with Ukulinga 2007 lemon showing higher soluble-conjugated *put* and Eston showing significantly lower soluble *put* (Fig. 2A). Fruit from non-chilling susceptible source had significantly ($p < 0.001$) lower membrane-conjugated *put* compared with chilling susceptible lemon fruit from Sun Valley Estates during the 2007 harvest season (Fig. 2A). There was no clear trend between chilling-resistant and chilling-susceptible free-soluble, soluble-conjugated and membrane-conjugated *spm* and *spd* in fruit from different sources (Fig 2B and C).

Sun Valley fruit showed higher free-soluble *put* during cold storage compared with chilling-resistant fruit from other sources (Fig. 3A). There were no clear differences in the soluble-conjugated *put* of chilling-resistant compared with chilling-susceptible fruit during 28 days into cold storage (Fig 3A). However, Sun Valley fruit showed a significant decrease in membrane-conjugated *put* compared with chilling-resistant fruit (Fig. 3A) and such was evident with membrane-conjugated *spm* (Fig. 3B). There was no clear trend in the *spd* concentration of chilling-susceptible and chilling-resistant lemons during cold storage (Fig. 3C).

3.4 Effect of HWD and Mo dips on 70 kDa proteins (estimator of HSP70) and total soluble-conjugated PA's before cold storage

The flavedo of chilling-resistant fruit from Ukulinga (Fig. 4A and C) had higher total protein (Fig. 5) and total soluble-conjugated PA concentrations than Sun Valley and Eston fruit (Fig. 6B and D). However, protein separation of flavedo of Ukulinga fruit was not clear (Fig. 4A and C).

The relationship between total soluble-conjugated PAs and 70 kDa protein (estimator of HSP70) was not clear in Ukulinga fruit flavedo (Fig. 6A and C); however, HW 53°C, and 10 μM Mo plus HW 53°C significantly increased total soluble-conjugated PAs which were associated with the strong presence of 70 kDa protein (estimator of HSP70) in chilling-susceptible fruit from Eston and Sun Valley (Fig. 6B and D).

3.5 Effect of HW and Mo dips on endogenous polyamines in lemon flavedo during cold storage

The HW 47°C significantly increased free lemon flavedo putrescine concentration 7 to 21 days into cold storage for fruit from all sources (Table. 2 and 3). However, the HW 53°C displays this during storage, only in flavedo of Ukulinga during 2007 (Table 2). Furthermore, the HW 53°C had no significant effect on soluble and membrane-conjugated *put* of chilling-susceptible Sun Valley fruit, while with Ukulinga flavedo treated with HW 47°C soluble and membrane-conjugated *put* significantly increased during cold storage . In addition, HW 53°C significantly increased soluble and membrane-conjugated *put* 7 and 14 days into cold storage for Eston fruit (Tables 2 and 3).

The HW 53°C was significantly ($p < 0.001$) increased the free and soluble-conjugated *spm* and *spd* for Sun Valley Estates fruit 7 days into cold storage (Table 2). However, *spd* and *spm* (free-soluble, soluble and membrane-conjugated) was significantly higher following HW 47°C as compared with HW 53°C for Ukulinga fruit during the 2007 and 2008 seasons (Tables 2 and 3). In addition, HW 53°C significantly reduced *spd* and *spm* (free, soluble and membrane conjugated) but increased in Ukulinga 2008 fruit during cold storage (Table 3).

The effect of molybdenum on flavedo PAs was significant on Ukulinga 2007 fruit, specifically with *put*, during cold storage (Table 2). In treatments of 1 μM Mo and 1 μM Mo + HW 53°C free-soluble *put* decreased after 7 days cold storage with a significant increase in soluble-conjugated and membrane-conjugated *put* (Table 2). Molybdenum plus HW dips significantly increased both *spd* and *spm* (soluble-conjugated and membrane-conjugated) during cold storage for chilled and non-chilling lemon fruit during 28 days into cold storage (Tables 2 and 3).

4. Discussion

Chilling susceptibility of citrus fruit has mainly been attributed to growing conditions which determine the fruit's flavedo chemical composition (Aung et al., 1998, 1999, 2001). Such differences have been proven with coastal and desert lemons with different harvest season (Aung et al., 2001). In addition, chilling susceptibility differs between lemon cultivars; 'Lisbon' lemons were found to be more chilling-susceptible compared with 'Eureka' lemons when stored at 1°C for up to 42 days (Underhill et al., 1999). The susceptibility of Sun Valley fruit compared with Ukulinga and Eston fruit in this study, further affirms differences in the chilling susceptibility with fruit source and harvest season (Table 1).

The success and mode of action of HW treatments have been associated with induction of heat shock proteins (Vierling, 1991). The potential of HW 53°C has previously been reported for 'Toccoro' blood oranges to reduce chilling injury and decay when fruit were stored at 3°C for up to 10 weeks compared with non-treated fruit (Schirra et al., 1997) and on 'Valencia' oranges, reduced chilling injury when fruit were stored at 4 °C for up to 6 months compared with HW 48°C and untreated fruit (Erkan et al., 2005). According to Schirra et al. (1997) HW also improved membrane damage recovery and therefore, improved membrane integrity (Lyons, 1976; Wolfe, 1978) during lemon cold storage (Mathaba et al., 1998).

Xanthine dehydrogenase is a Mo co-factor enzyme in plants; the increase in its activity under stress contributes to the production of reactive oxygen species. This research showed lower Mo (1 μM Mo dips) to significantly reduce the appearance of chilling symptoms during cold storage (Table 1). Hesberg et al. (2003) reported a decrease in XDH activity due to cold stress and salinity in *Arabidopsis thaliana* leaves stored at 4°C for up to 20 h. However, in annual ryegrass (*Lolium multiflorum* cv. Westerwoldicum) XDH root activity increased as a response to salinity (Sagi et al., 1998). Furthermore, there is a synergistic effect of Mo plus HW treatments in mitigating the appearance of chilling symptoms (Table 1). Hypothetically, the synergism could be the activation of HSPs by the HW treatments (Sabehat et al., 1996; Rozenzvieg et al., 2004) and Mo addition resulting in increased ROS production through increased activity of XDH (Sagi et al., 1998; Hesberg et al., 2003). On the other hand, ROS have recently been proposed as stress acclimatory signaling molecules if produced at a non-damaging critical thresh-hold level (Foyer and Shigeoka, 2011) and addition of Mo increases the activity of XDH, increase ROS production during stress exposure resulting in stress acclimation (Hesberg et al., 2003; Yesbergeniva et al., 2005).

Putrescine is the dominant PA in citrus flavedo with *spd* and *spm* in less, but almost equal concentrations (Fig. 1). These results concur with reports by Tassoni et al. (2004) and Velero et al. (1998). Furthermore, the PA concentration varies with citrus fruit segments (Tassoni et al., 2004) and also in the ripeness stage (Velero et al., 1998) and therefore, with different growing conditions (Fig 2). In addition, a study on tobacco showed higher soluble-conjugated PAs compared with free-soluble ones (Königshofer and Lechner, 2002). Our results further prove higher soluble-conjugated *put* than soluble-conjugated *spm* and *spd* in lemon flavedo (Fig. 1 and 2). Biochemically, *spd* and *spm* are synthesized from *put* (Handa and Mattoo 2010). Their power to attach to negatively charged cellular compounds (DNA, proteins, enzymes, membranes and pectins) depends on the valency of the specific PA ($spm^{4+} > spd^{3+} > put^{2+}$) (Messiaen et al., 1997; Lenucci et al., 2005). Chilling-resistant lemon fruit sourced from Ukulinga had higher soluble-conjugated *put* than the chilling-susceptible Sun Valley and Eston fruit; therefore, this low *put* pool could not contribute to the flavedo antioxidant capacity (Chapters 3 and 4). For an example, amides when conjugated to phenols form phenolamides, which are phenolics with an increased antioxidant capacity (Bassard et al., 2010). Furthermore, *put* is a substrate for *spd* and *spm* (Bassard et al., 2010; Handa and Mattoo, 2010); therefore, high put concentrations are likely to increase the production of *spd* and *spm*, important compounds rendering chilling resistance to lemon fruit. In addition, chilling-susceptible lemons (Eston and Sun Valley fruit) show increased conversion of *put* to *spd* and *spm* as a response to chilling stress (Fig. 2B and C) mainly in soluble and membrane-conjugated PA forms.

A similar trend as observed for the *put* concentration in lemon flavedo during cold storage (Fig. 3A) has also been reported for the mesocarp of peaches (*Prunus persica* L. Cv Maycrest) during storage at 5°C (Valero et al., 1997) and in the exocarp of pomegranate when stored at 2°C (Mirdehgan et al., 2007). Furthermore, a similar trend was also observed in Zucchini squash when stored at 2°C (Serrano et al., 1998). In lemons Valero et al. (1998) observed a decrease in flavedo *put* coupled to an increase in *spd;* while in Zucchini squash both *spd* and *spm* decreased during cold storage (Serrano et al., 1998). Our data (Fig. 3A-C) show a decrease in free soluble-put 14 days into cold storage and a further decrease in membrane-conjugated *put* (Fig. 2D). Spermidine and *spm* (free, soluble conjugates and membrane conjugated fractions) were altered by -0.5°C storage, and results similar to these were reported by Valero et al. (1998) in lemon (cv.

Verna) as confirmation of conversion of putrescine to high valency PA's (spm^{4+} > spd^{3+} > put^{2+}) (Messiaen et al., 1997; Lenucci et al., 2005) and therefore reduced cold stress.

Chilling-susceptible lemon fruit enhanced protein synthesis due to HW 53°C, 10 µM Mo plus HW 53°C (Fig. 4). This was particularly true for proteins with molecular weights close to 70 kDa protein (estimator of HSP70) (Plate 1). Similar results were reported in tobacco and alfalfa cells cultured at 38 and 40°C where an increase in HSP with a molecular weight of 92 and 80 kDa was observed (Königshofer et al., 2002). Furthermore, the increase in total soluble-conjugated PAs was associated with an increase in 70 kDa protein (estimator of HSP70), supporting results by Königshofer et al. (2002) that PAs directly affect HSP production at protein synthesis level while indirectly affecting cell membrane resistance to stress. In addition, reducing the effect of ROS on proteins, DNA, and membrane as PAs bound to these negatively charged compounds at cellular pH and therefore acting as antioxidants and increasing membrane stability (Li et al., 2004). Certain compounds, such as salicylic acid, were found to increase storage quality of 'Ponkan' mandarin (Zheng and Zhang. 2004) and maize drought tolerance by increasing the *put* and *spm* concentration, thereby reducing potential lipid peroxidation (Németh et al., 2001). Therefore, it is hypothesized that Mo acts by increasing ROS production during stress but below a damaging critical threshold level signalling protein synthesis, including HSPs due to the synergism between Mo and HWD, soluble-conjugated PAs resulting in stress mitigation.

5. Conclusion

The susceptibility of lemon fruit to chilling is influenced by fruit source and growing conditions. Putrescine is the dominant PA and a precursor for synthesis of *spm* and *spd*, which are higher valency PAs than *put*. Seemingly, the HW 53°C treatment enhances protein synthesis, including HSPs. Therefore, the more soluble-conjugated PAs can be conjugated to proteins (including 70 kDa proteins as estimated of HSP70), enzymes, bioactive compounds with antioxidant properties, to improved protection against oxidative stress. In addition, Mo treatments increased XDH activity and ROS production to levels below critical damaging threshold, which possibly signal cellular aclimation through synthesis of high affinity PAs (spermine and spermidine) via putrescine as precursor. Therefore, the combination of HW and Mo led to reduced chilling injury through enhanced protein synthesis (specifically 70 kDa protein as an estimator of HSP70)), increased ROS production and soluble-conjugate PAs to increase oxidative stress acclimation.

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List of Tables

Table 1: Effect of hot water and molybdenum postharvest dips on chilling symptoms of lemon fruit cold stored (-0.5°C) for up to 28 days

Table 2: Effect of hot water and molybdenum postharvest dips on lemon flavedo free, soluble-conjugated and membrane-conjugated polyamines over 28 days cold storage (-0.5°C) during 2007 harvest season

Table 3: Effect of hot water and molybdenum postharvest dips on lemon flavedo free, soluble-conjugated and membrane-conjugated polyamines over 28 days cold storage (-0.5°C) during 2008 harvest season

List of Figures

Figure 1: Confirmation of polyamines in lemon flavedo by chromatography. A. Polyamines authentic standards B. Flavedo free soluble polyamines C. Soluble-conjugated flavedo polyamines and D. Membrane-conjugated flavedo polyamines

Figure 2: Effect of fruit source (A-C) on free, soluble-conjugated and membrane-conjugated polyamines

Figure 3: Effect of cold storage time (A-C) on free-soluble, soluble-conjugated and membrane-conjugated PAs

Figure 4: SDS-PAGE of lemon flavedo from different growing condition over different harvest season pre-treated with hot water (47 or 53°C) and molybdenum.

Figure 5: Lemon flavedo total protein concentration over different fruit source and harvest season

Figure 5: Relationship between total soluble-conjugated polyamines and estimated HSP70 for lemon flavedo pre-treated with hot water and molybdenum before 28 days cold storage

Table 1

Treatments[a]	Ukulinga 2007[b]	Sun Valley Estates[c] 2007	Ukulinga 2008[b]	Eston Estates 2008[d]
Water dip (25°C)	No symptoms	0.13a	No symptoms	Below detection
HW 47°C	No symptoms	0.18a	No symptoms	Below detection
HW 53°C	No symptoms	0.09a	No symptoms	Below detection
1 µM Mo	No symptoms	0.04ab	No symptoms	Below detection
1 µM Mo + HW 53°C	No symptoms	0.02ab	No symptoms	Below detection
10 µM Mo + HW 53°C	No symptoms	0.00b	No symptoms	Below detection

[a] Treatment effect after 28 days cold storage plus 5 days shelf-life

[b] Ukulinga lemon fruit showed no chilling symptoms during 2007 and 2008 harvest seasonS

[c] Sun Valley lemon fruit showed chilling symptom expressible in chilling index ,and means followed by the same letter were not significantly different at LSD(0.05) = 0.096

[d] Eston Estates lemon did show chilling symptoms but below detection and chilling index calculation

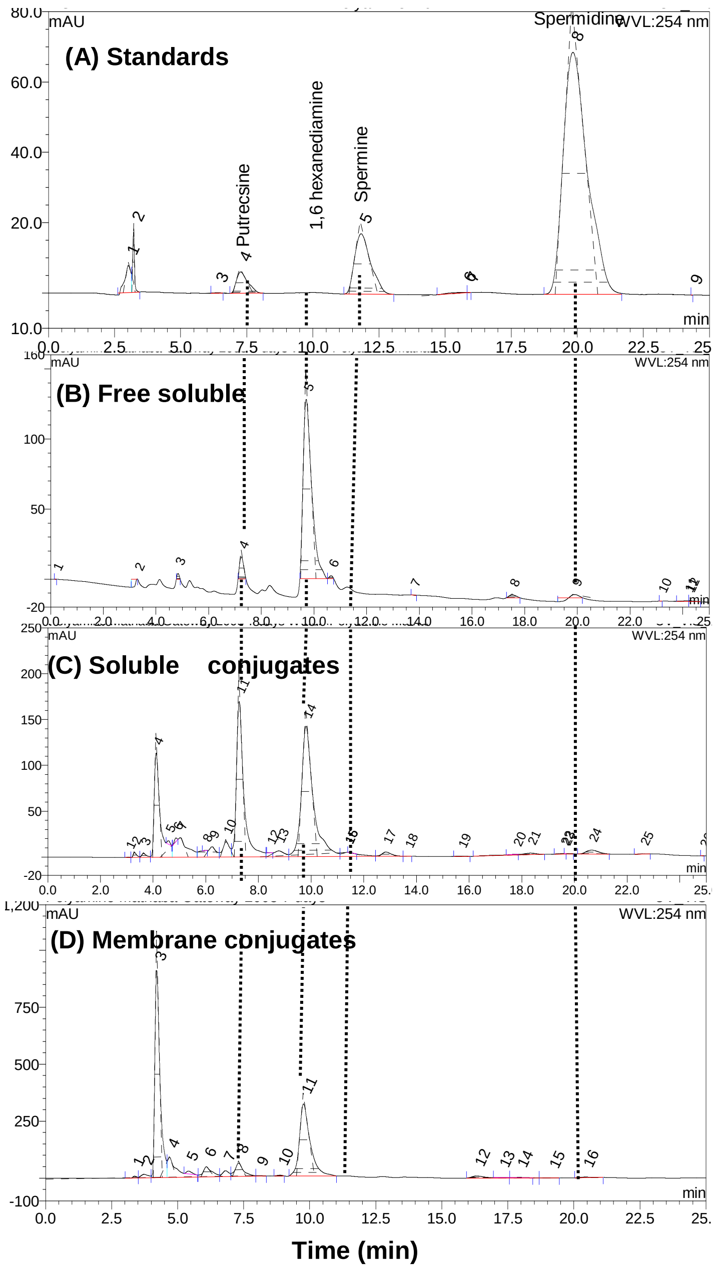
(A) Standards
mAU
WVL:254 nm
Spermidine
Putrecsine
1,6 hexanediamine
Spermine
80.0
60.0
40.0
20.0
10.0
min
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25
(B) Free soluble
mAU
WVL:254 nm
100
50
-20
min
(C) Soluble conjugates
mAU
WVL:254 nm
250
200
150
100
50
-20
min
(D) Membrane conjugates
mAU
WVL:254 nm
1,200
750
500
250
-100
min
Time (min)

Fig 1:

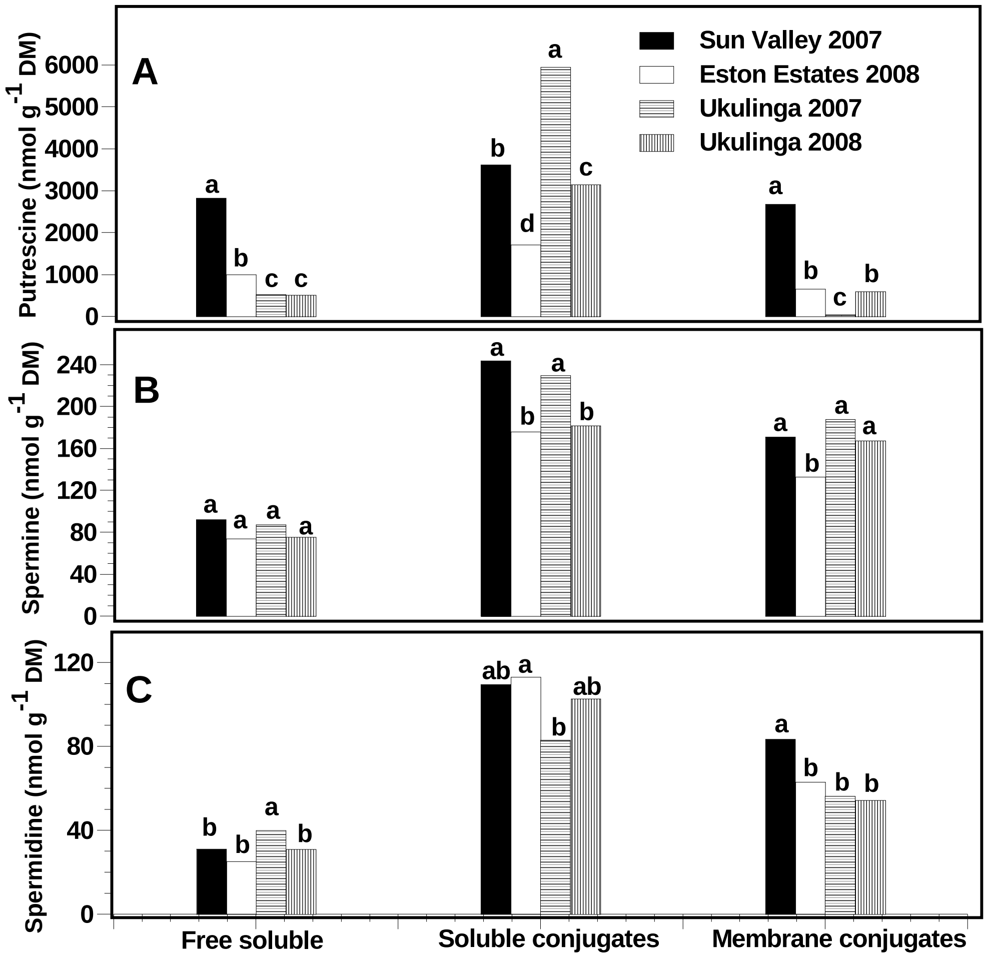
A
B
C
Putrescine (nmol g^{-1} DM)
Spermine (nmol g^{-1} DM)
Spermidine (nmol g^{-1} DM)
0
1000
2000
3000
4000
5000
6000
0
40
80
120
160
200
240
0
40
80
120
Sun Valley 2007
Eston Estates 2008
Ukulinga 2007
Ukulinga 2008
a
b
c
c
b
d
a
c
a
b
c
b
a
a
a
a
a
b
a
b
a
b
a
a
b
b
a
b
ab
a
b
ab
a
b
b
b
Free soluble
Soluble conjugates
Membrane conjugates

Fig 2:

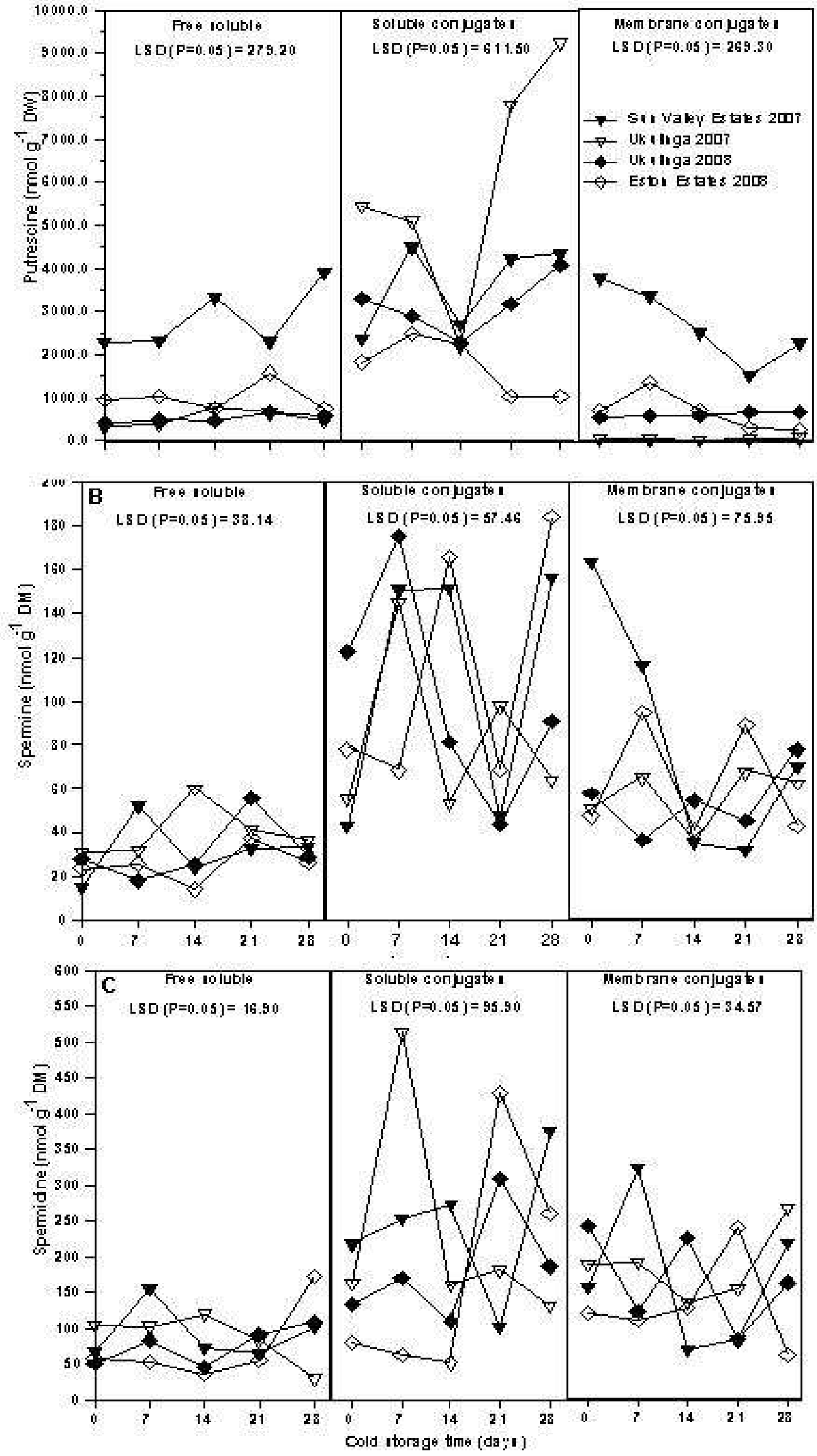
10000.0
9000.0
8000.0
7000.0
6000.0
5000.0
4000.0
3000.0
2000.0
1000.0
0.0
Putrescine (nmol g-1 DW)
Free soluble
LSD (P=0.05) = 279.20
Soluble conjugates
LSD (P=0.05) = 611.50
Membrane conjugates
LSD (P=0.05) = 269.30
Sun Valley Estates 2007
Ukulinga 2007
Ukulinga 2008
Eston Estates 2008
B
200
180
160
140
120
100
80
60
40
20
0
Spemine (nmol g-1 DM)
Free soluble
LSD (P=0.05) = 38.14
Soluble conjugates
LSD (P=0.05) = 57.46
Membrane conjugates
LSD (P=0.05) = 75.95
0
7
14
21
28
C
600
550
500
450
400
350
300
250
200
150
100
50
0
Spermidine (nmol g-1 DM)
Free soluble
LSD (P=0.05) = 16.90
Soluble conjugates
LSD (P=0.05) = 95.90
Membrane conjugates
LSD (P=0.05) = 34.57
0
7
14
21
28
Cold storage time (days)

Fig 3:

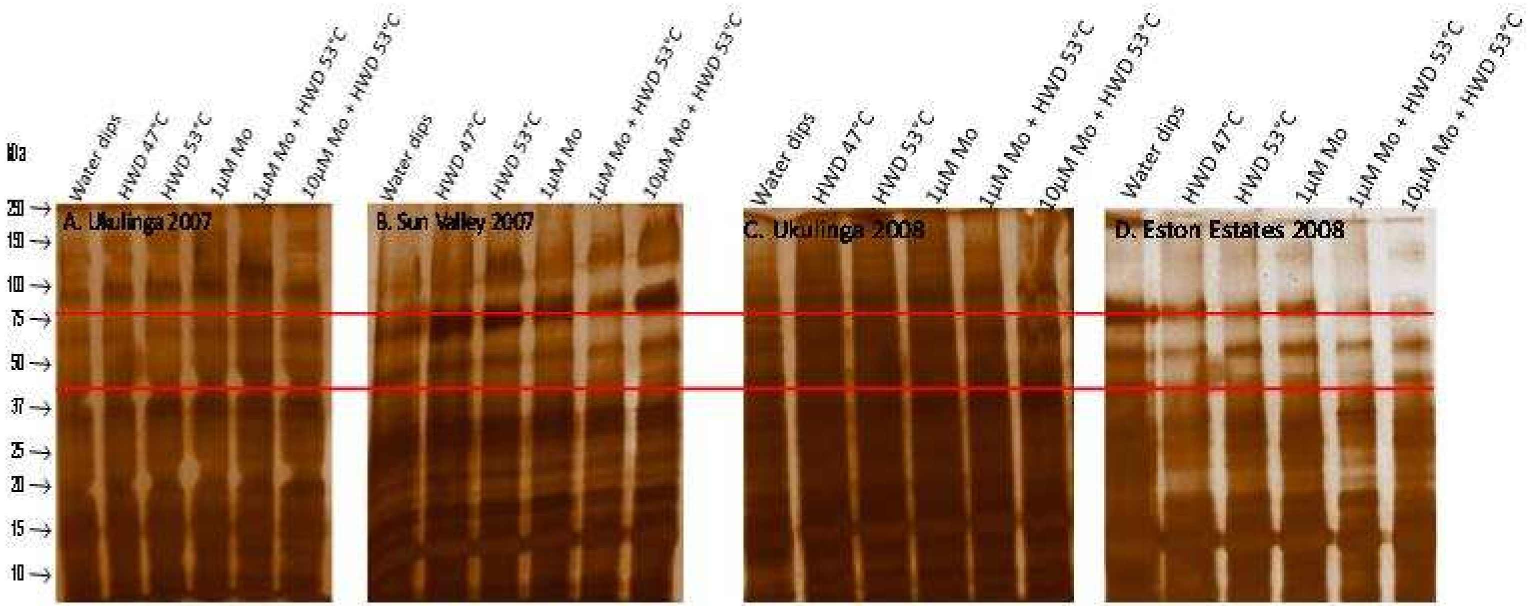
kDa
250
150
100
75
50
37
25
20
15
10
A. Ukulinga 2007
B. Sun Valley 2007
C. Ukulinga 2008
D. Eston Estates 2008
Water dips
HWD 47°C
HWD 53°C
1μM Mo
1μM Mo + HWD 53°C
10μM Mo + HWD 53°C

Fig 4:

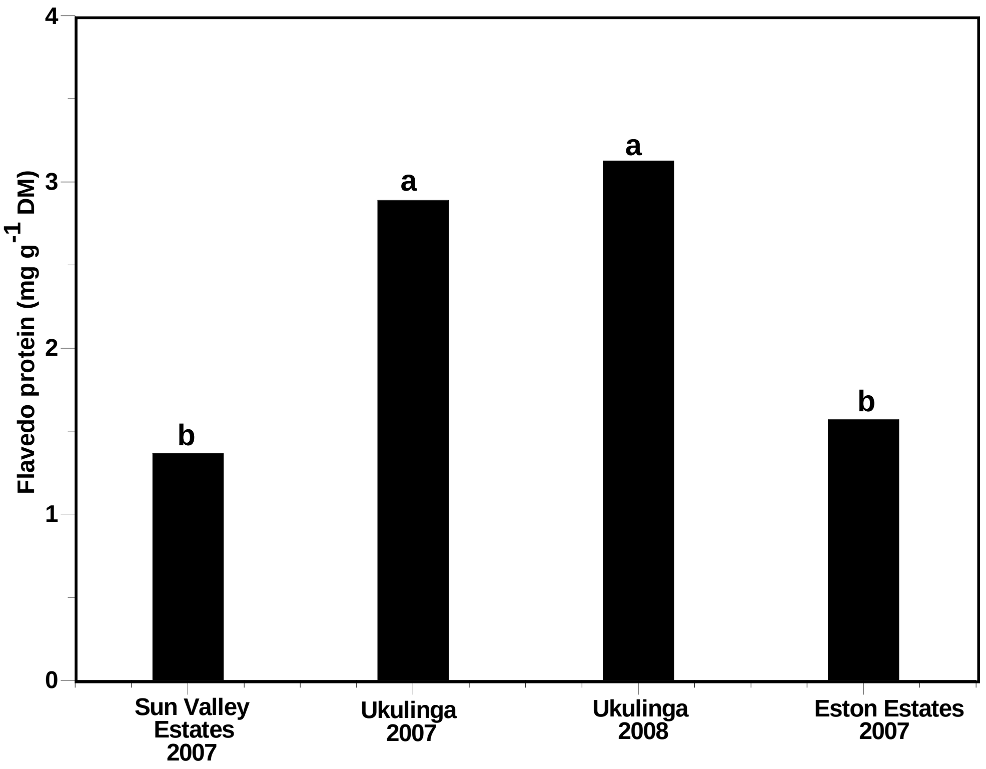
Flavedo protein (mg g^{-1} DM)
0
1
2
3
4
b
a
a
b
Sun Valley Estates 2007
Ukulinga 2007
Ukulinga 2008
Eston Estates 2007

Fig 5

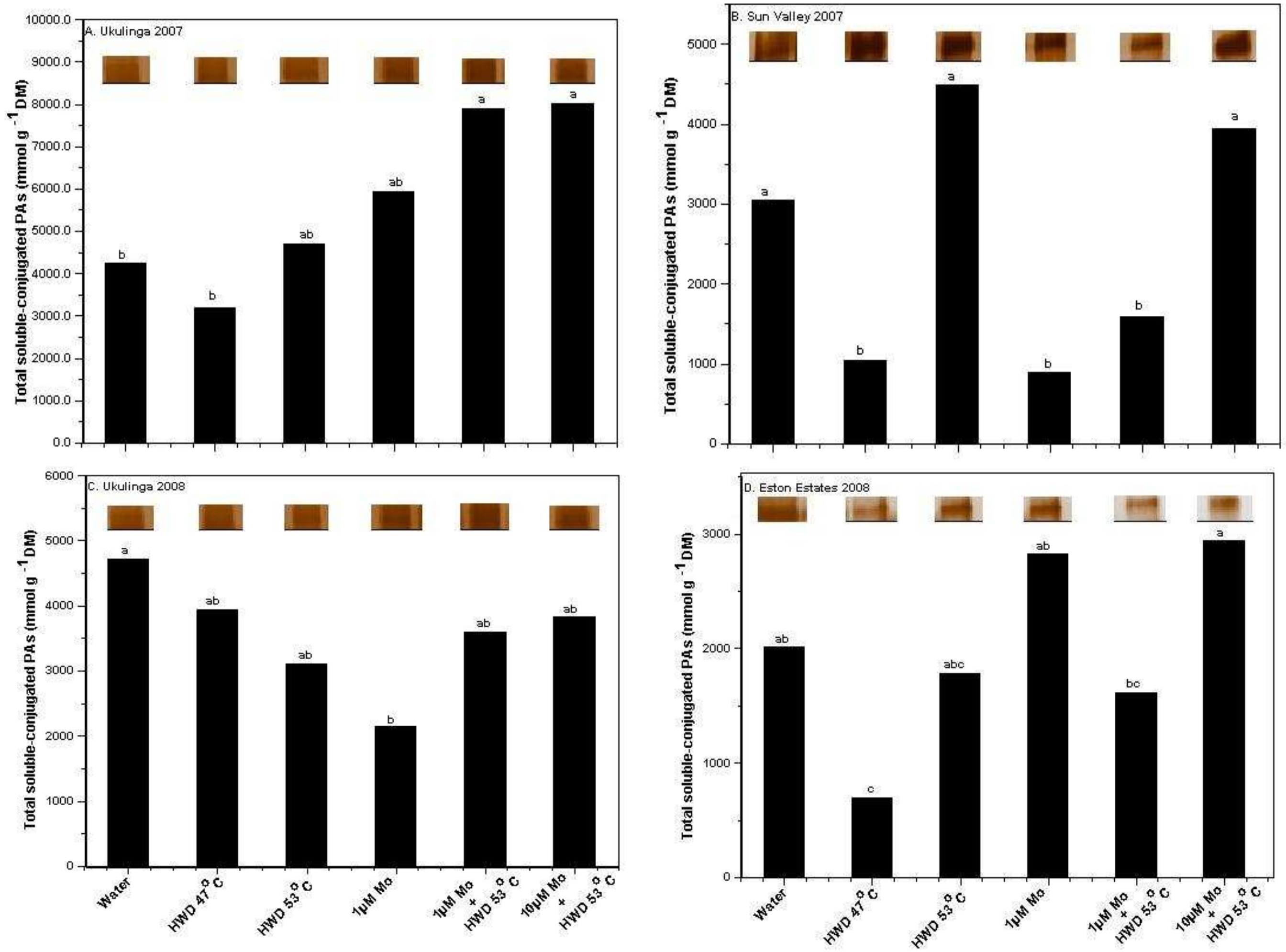
A. Ukulinga 2007
B. Sun Valley 2007
C. Ukulinga 2008
D. Eston Estates 2008
Total soluble-conjugated PAs (mmol g -1 DM)
10000.0
9000.0
8000.0
7000.0
6000.0
5000.0
4000.0
3000.0
2000.0
1000.0
0.0
b
b
ab
ab
a
a
5000
4000
3000
2000
1000
0
a
b
a
b
b
a
6000
5000
4000
3000
2000
1000
0
a
ab
ab
b
ab
ab
3000
2000
1000
0
ab
c
abc
ab
bc
a
Water
HWD 47° C
HWD 53° C
1µM Mo
1µM Mo + HWD 53° C
10µM Mo + HWD 53° C

Fig 6

Table 2.

Cold storage time (days)	Treatments	Ukulinga 2007									Sun Valley Estates 2007								
		Free-soluble*			Soluble-conjugates*			Membrane-conjugates*			Free soluble*			Soluble conjugates*			Membrane conjugates*		
		Put	Spd	Spm	Put	Spd	Spm	Put	Spd	Spm	Put	Spd	Spm	Put	Spd	Spm	Put	Spd	Spm
0	Water dip (25^{O}C)	446	27.7	31.8	4144	13.9	87.5	35	54.8	108.3	2853	3.8	87.3	3711	25.7	105.3	5200	206.3	19.9
	HW 47^{o}C	124	7.0	15.5	3135	12.4	33.4	25	34.8	252.2	2095	10.2	50.3	757	47.4	245.2	2793	199.8	281.0
	HW 53^{o}C	254	26.2	130.6	4573	58.1	64.4	37	52.8	152.9	501	2.1	4.5	4279	84.5	130.4	1828	108.5	152.9
	1 µM Mo	80	4.6	46.1	5596	33.6	300.7	44	13.7	101.4	3402	8.4	108.5	619	17.5	255.8	3337	341.9	152.1
	1 µM Mo + HW 53^{o}C	990	115.0	390.8	7399	143.4	357.2	65	75.1	448.2	2304	22.0	72.4	1404	16.2	196.1	3104	61.3	93.7
	10 µM Mo + HW 53^{o}C	60	2.9	3.3	7827	68.7	135.7	61	72.4	70.8	2549	41.5	74.1	3501	64.5	372.2	1462	89.7	236.2
7	Water dip (25^{O}C)	280	31.5	127.4	2083	7.1	262.9	18	39.8	127.5	2206	46.6	53.6	4495	41.2	201.1	4229	77.0	376.9
	HW 47^{o}C	114	7.7	5.2	7071	122.0	1028	55	13.3	108.8	503	143.8	47.7	10216	169.0	600.7	9158	157.8	665.5
	HW 53^{o}C	36	6.6	16.6	3400	92.6	488.6	26	39.9	62.5	2989	43.5	256.4	2242	242.6	388.8	803	67.6	62.5
	1 µM Mo	745	60.7	211.5	6505	18.7	525.6	56	54.8	246.1	2425	42.7	71.4	2780	165.4	94.0	2600	29.5	502.5
	1 µM Mo + HW 53^{o}C	812	52.0	163.9	1756	37.6	129.5	20	47.6	140.4	2600	28.9	222.3	323	15.2	101.1	1620	53.4	189.1
	10 µM Mo + HW 53^{o}C	375	32.6	90.7	9769	592.0	644.9	78	196.7	469.1	3254	6.6	284.9	6908	273.0	133.2	2150	333.7	57.3
14	Water dip (25^{O}C)	879	45.7	147.9	3862	63.6	191.0	37	14.0	34.0	4686	56.0	134.4	629	367.8	264.0	1519	15.2	11.4
	HW 47^{o}C	1131	194.7	371.8	1034	28.0	244.8	17	24.6	90.2	2595	32.0	187.6	6295	294.4	164.9	1700	8.10	83.2
	HW 53^{o}C	365	19.2	45.1	1822	87.6	227.7	19	91.7	162.1	4174	27.0	33.6	3074	151.9	159.9	1447	46.5	162.1
	1 µM Mo	470	33.0	25.3	701	91.2	88.6	9	17.4	281.7	4227	4.3	4.4	5869	29.0	328.0	4116	25.2	34.5
	1 µM Mo + HW 53^{o}C	527	40.0	65.9	5392	31.9	83.4	46	32.4	71.3	151	4.8	7.0	405	29.4	272.2	3982	92.0	47.8
	10 µM Mo + HW 53^{o}C	1073	27.7	62.2	331	15.1	121.5	11	34.2	175.3	4107	17.4	68.0	6785	35.9	444.5	331	27.0	132.6
21	Water dip (25^{O}C)	1516	15.8	80.6	11991	138.6	512.6	104	8.80	61.2	1917	66.0	94.9	15906	97.3	212.0	1060	96.4	5.8
	HW 47^{o}C	529	79.6	146.7	13277	83.0	152.2	106	37.0	78.0	3643	28.4	10.1	1974	17.7	25.3	47	5.8	11.9
	HW 53^{o}C	427	32.6	14.0	2466	27.9	45.1	22	50.1	477.0	1784	3.1	52.8	1343	24.7	79.1	42	4.6	477.0
	1 µM Mo	272	22.3	21.6	9295	214.5	106.6	74	207.7	139.0	1016	17.4	149.9	3117	10.9	114.2	2442	55.9	119.0
	1 µM Mo + HW 53^{o}C	489	61.0	84.5	4958	37.7	156.2	42	39.7	53.5	3401	20.3	19.4	767	82.2	73.8	2941	104.7	292.9
	10 µM Mo + HW 53^{o}C	637	37.3	144.9	4710	86.6	113.1	41	63.5	122.9	2052	58.9	63.5	2188	49.6	108.3	186	156.8	41.4
28	Water dip (25^{O}C)	255	44.6	68.6	12749	19.7	187.2	100	69.5	373.1	2648	6.9	24.6	9791	190.8	150.8	2925	54.8	188.2
	HW 47^{o}C	788	79.5	45.1	7152	20.6	124.9	61	101.3	370.5	4174	59.0	56.6	3963	370.3	646.1	2463	109.4	97.9
	HW 53^{o}C	895	32.1	7.5	5643	167.9	230.2	50	26.4	298.6	4244	20.5	74.3	4545	133.8	150.2	2453	21.7	298.6
	1 µM Mo	64	4.5	10.2	13753	54.5	73.9	106	54.3	252.0	5161	14.8	114.4	2937	154.1	120.9	44	60.6	459.9
	1 µM Mo + HW 53^{o}C	279	24.2	10.7	7014	37.8	69.2	56	72.9	223.5	2878	8.9	233.2	806	61.4	218.6	885	95.4	100.5
	10 µM Mo + HW 53^{o}C	583	33.0	34.7	9080	84.4	103.4	74	51.5	85.2	4408	90.1	105.7	3991	27.2	66.8	324	27.1	127.9
	F prob (5%)	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001

*PAs - mmol g^{-1} D

Table 3.

Cold storage time (days)	Treatments	Ukulinga 2008									Eston Estates 2008								
		Free-soluble*			Soluble-conjugates*			Membrane-conjugates*			Free-soluble*			Soluble-conjugates*			Membrane-conjugates*		
		Put	Spd	Spm	Put	Spd	Spm	Put	Spd	Spm	Put	Spd	Spm	Put	Spd	Spm	Put	Spd	Spm
0	Water dip (25^{O}C)	388	46.3	59.3	4629	14.2	84.6	738	29.4	198.0	961	11.7	67.9	1934	53.9	28.9	358	16.2	65.1
	HW 47^{o}C	411	20.2	147.7	3723	30.4	191.8	194	4.1	241.4	839	15.8	7.5	529	12.6	153.8	492	15.9	56.6
	HW 53^{o}C	521	55.9	17.1	2390	316.6	396.4	604	125.1	606.8	850	17.6	39.3	1578	67.2	133.9	984	61.0	111.6
	1 µM Mo	354	25.4	38.3	1820	265.4	59.1	509	27.5	166.4	924	50.0	102.9	2507	251.0	66.6	331	17.2	134.4
	1 µM Mo + HW 53^{o}C	652	10.2	24.3	3519	44.6	40.3	680	67.5	152.9	2097	40.9	107.8	1510	4.0	84.4	466	83.5	110.9
	10 µM Mo + HW 53^{o}C	71	8.2	9.0	3744	65.2	27.6	547	94.5	91.8	21.00	3.9	13.1	2846	80.7	11.4	1462	89.7	250.8
7	Water dip (25^{O}C)	590	31.0	151.8	1813	350.7	42.5	557	14.1	126.7	1038	36.6	121.3	4934	17.5	112.0	1621	49.4	110.2
	HW 47^{o}C	115	4.2	32.5	417	108.9	244.5	262	7.7	0.80	49	3.5	13.2	1451	12.6	20.6	72	2.6	0.90
	HW 53^{o}C	1275	12.4	177.5	2744	87.2	276.5	612	20.1	46.7	1317	9.6	82.0	3407	58.5	68.3	1914	100.2	16.5
	1 µM Mo	692	55.6	114.8	2058	293.2	183.0	570	55.1	319.9	2084	65.5	65.7	296	142.5	12.0	467	50.0	266.8
	1 µM Mo + HW 53^{o}C	81	1.2	2.6	6304	85.9	203.4	526	18.2	67.9	1331	9.6	26.0	3482	42.0	134.7	1871	33.9	226.0
	10 µM Mo + HW 53^{o}C	150	2.7	17.5	3980	126.6	69.8	941	102.8	177.8	355	29.1	2.8	1340	139.3	24.0	2150	333.7	49.8
14	Water dip (25^{O}C)	484	2.3	16.3	1845	7.8	84.0	381	55.5	314.7	644	3.0	10.3	1667	117.6	21.7	531	4.6	14.6
	HW 47^{o}C	403	37.8	31.2	2284	97.8	181.6	476	60.1	37.8	594	5.1	19.6	2621	79.0	30.1	663	55.5	188.9
	HW 53^{o}C	512	20.5	33.6	1344	103.4	132.6	855	95.4	457.9	515	5.4	19.5	4074	72.8	12.8	684	8.1	205.3
	1 µM Mo	30	13.9	1.3	1772	150.8	63.2	566	65.9	260.3	380	45.8	46.6	2482	309.7	127.0	1376	97.2	45.0
	1 µM Mo + HW 53^{o}C	911	41.8	47.2	1358	79.6	38.5	425	14.6	91.6	530	6.8	39.7	1705	44.8	34.4	575	55.6	99.2
	10 µM Mo + HW 53^{o}C	391	34.0	142.6	5064	49.6	158.2	727	34.5	192.6	1766	17.6	77.0	848	368.4	82.4	331	27.0	219.7
21	Water dip (25^{O}C)	200	33.1	28.9	1831	25.5	191.8	667	50.2	175.8	1816	15.8	29.6	674	147.1	27.6	477	197.4	317.3
	HW 47^{o}C	1023	45.8	290.1	4024	31.2	394.8	346	10.5	85.8	1912	89.0	24.9	739	56.6	414.1	353	40.6	335.0
	HW 53^{o}C	548	30.3	51.1	4599	78.5	463.5	597	60.4	9.2	767	34.8	15.9	654	29.6	651.3	292	41.7	151.0
	1 µM Mo	626	60.5	125.3	2887	32.0	310.6	1552	36.9	65.8	2361	44.2	194.4	444	41.8	532.4	315	47.7	123.7
	1 µM Mo + HW 53^{o}C	353	50.0	12.1	2594	5.0	88.9	280	60.5	123.8	787	27.3	49.9	3066	7.2	711.8	250	51.7	193.3
	10 µM Mo + HW 53^{o}C	1268	112.5	37.0	3097	88.3	407.3	532	52.2	39.3	1800	13.1	15.3	578	132.9	230.1	186	156.8	324.4
28	Water dip (25^{O}C)	564	9.0	8.8	1011	61.0	58.1	400	12.7	19.3	784	20.0	45.2	1549	32.8	330.0	249	23.7	32.1
	HW 47^{o}C	441	17.2	128.9	6855	231.8	385.4	1156	179.2	241.3	1196	24.0	445.2	1128	38.1	175.5	395	54.6	68.1
	HW 53^{o}C	152	37.2	145.2	5469	133.9	120.3	387	79.0	272.2	443	3.1	49.1	508	785.5	253.7	215	49.9	41.0
	1 µM Mo	890	57.6	121.8	4526	49.0	160.9	865	94.6	116.1	576	71.9	121.1	859	24.1	380.9	123	39.2	9.3
	1 µM Mo + HW 53^{o}C	332	39.4	151.1	3268	48.1	105.5	418	47.7	204.4	912	8.3	149.0	748	28.4	311.7	190	61.3	194.4
	10 µM Mo + HW 53^{o}C	1025	12.9	99.3	3385	22.7	285.9	709	56.3	127.4	528	28.6	225.1	1401	194.3	108.9	324	27.1	30.0
	F prob (5%)	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001	P<.001

*PAs - mmol g^{-1} DM

CHAPTER 7

A Holistic View: General discussion, conclusion and outlook

Pre-harvest, photosynthesis is the main physiological cellular mechanism for synthesis of carbohydrates, lipids, and, secondary metabolites. These latter compounds are subsequently utilized during development, maturity, after harvest, post-harvest storage, senescence, and respiration (Taiz and Zeiger, 2002; Ladaniya, 2008). Furthermore, secondary metabolites are diverse in chemical structure, cellular function, concentration, site of synthesis and function bringing about fruit development leading to the possibility for an extended post-harvest shelf-life. In addition, in citrus fruit development and post-harvest maintenance, proteins, amines, polyamines, complex carbohydrates, organic acids, lipids, phenols, flavonoids, mineral elements, hormones and vitamins can act as secondary metabolites and play a pivotal role in mitigating any form of stress (Ladaniya, 2008).

Citrus fruit originate from the subtropics and are, therefore, susceptible to chilling injury during cold storage (Porat et al., 2002; Erkan et al., 2005; Ladaniya, 2008). Reports have shown that carbohydrates are major metabolites in plant cold stress mitigation (Purvis and Yelenosky, 1982; Holland et al., 1999).

In citrus flavedo, glucose is a dominant sugar, a major source of energy and, therefore, proven to play a major role in mitigating stress through the production of secondary metabolites necessary in stress acclimation by allowing continued plant cell development and maintenance (Zheng et al., 2001). According to Smirnoff (2000) and, data presented in Chapter 3, glucose could be enzymatically converted to a potent antioxidant, ascorbic acid. However, conversion of glucose to ascorbic acid is dependent on cellular factors including that glucose must be present in high concentrations and the inherent low glucose level will result in chilling susceptibility (Chapter 3). In addition, the conversion of glucose to ascorbic acid could be affected by hot water and molybdenum post-harvest treatments provided the above cellular requirements are met (Bower et al., 2012). The role of hot water and molybdenum in the conversion of glucose to ascorbic acid is not understood but speculations would point towards activation of heat stress induced protein synthesis which is dependent on a pool of cellular energy (glucose). Molybdenum hypothetically increases the ROS production, which can act as a signal for the synthesis of stress acclimation proteins (Foyer and Shigeoka, 2011). Presumably, such metabolic processes require the use of glucose;

additionally a certain fraction of the glucose pool is used to be converted to ascorbic acid, which could reduce the occurrence of cold stress symptoms, like chilling injury.

Furthermore, glucose has been proven to be also a precursor to essential bioactive compounds with antioxidant properties, including polyphenolics, through the shikimate pathway of the pentose phosphate pathway (Fig 1; Zheng et al., 2001; Shetty, 2004; Ashraf and Foolad, 2007). Phenolics are the second major bioactive compounds in lemon flavedo following ascorbic acid, as determined in the ABTS, FRAP and DPPH total antioxidant assays (Chapter 4). Moreover, citrus peel is rich in unique and diverse phenolics, particulary flavonoids, including flavonones (mainly naringin and hesperidin), several polymethoxylated flavones (tangeretin, senensetin, nobiletin) (Interdonato et al., 2011). The cellular concentration of these phenolic bioactive compounds is dependent on the initial pre-harvest glucose concentration (Chapter 5). However, hot water and molybdenum treatments seem to maintain the phenolics pool but have no effect on flavedo naringin and hesperidin concentrations during cold storage (Chapter 5).

The usage of glucose in the production of phenolics is coupled to another essential physiological mechanism, the production of another important antioxidant, proline (Chapter 3, Fig. 1). Proline has antioxidant properties, also acts as an osmolyte adjuster and stabilizer of essential sub-cellular structures (membranes and other proteins), therefore, buffering cellular redox reactions under stress (Ashraf and Fooland, 2007). The involvement of glucose in the pentose phosphate pathway does not only increase phenolic concentration but can also induce an increase in proline concentration, thereby also increasing the antioxidant capacity and membrane stability mitigating oxidative stress (Chapter 3). Therefore, the effect of hot water and molybdenum on enhancing the use of glucose as a building block necessary for the synthesis of phenolics is observed in increased proline production, especially in chilling susceptible lemon fruit (Chapter 3).

The final role of glucose is to mitigate oxidative stress through its involvement in the synthesis of vitamin C (ascorbic acid). Vitamin C is involved in the regeneration of another potent membrane-bound antioxidant, vitamin E (α-tocopherol) (Smirnoff, 2000; Klimczak et al., 2007; Gill and Tuteja, 2010). Seemingly, hot water or molybdenum induce an up-regulation of ROS production, which signals stress protein synthesis (70 kDa protein as an estimator of HSP70), Chapter 6), and the conversion of glucose to ascorbic acid; ascorbic acid regenerating the α-tocopheroxy radical to α-tocopherol (Chapter 5). These cascades of cellular events link lipophilic and hydrophilic antioxidants to reduce oxidative stress through increased antioxidant capacity.

In many fruit, maturity is associated with change in colour. Chlorophyll is degraded to pigments such as carotenoids and anthocyanins (Will, et al., 2008), for many reasons. Firstly, photosynthesis is a high energy producing mechanism (Gill and Tuteja, 2010), after full maturity, citrus fruit converts flavedo chloroplast to pigments (Ladaniya, 2008) to reduce energy emitted during photosynthesis and thereby increasing antioxidant capacity against photo-excited radicals (Gill and Tuteja, 2010; Sies and Stahl, 1995). In citrus peel, the concentration of carotenoids such as β-carotene significantly increased with the reduction in chlorophyll concentration during fruit development (Gill and Tuteja, 2010). These carotenoids are specifically used to scavenge photo-excited singlet oxygen radicals (Sies and Stahl, 1995). However, post-harvest hot water dips or molybdenum soaks seem to have no effect on lemon flavedo β-carotene concentration (Chapter 5), possibly only bioactive compounds present in higher concentration are affected by such treatments (Chapter 5).

The efficacy of bioactive compounds to scavenge ROS can often be significantly improved by conjugation with polyamines (PAs) (Kuznetsov and Shevyakova, 2007). Following cold exposure chilling susceptible lemon fruit showed an increase in soluble-conjugated spermine and spermidine (Chapter 6 an expected response, as putrescine is converted to high valency PA's (Messiae et al., 1997; Lenucci et al., 2005)) as a stress acclimation response. In addition, hot water treatment enhanced protein synthesis in general and therefore, allows high valency PAs (Spm^{4+} and Spd^{3+}) to further conjugate with the newly synthesized proteins, such as HSP70 (Chaper 6). However, molybdenum soaks enhance ROS production, but seemingly to the level below the damaging critical threshold. The amount of ROS produced signals stress acclimation (Foyer and Shigeoka, 2011) and therefore, increases the conversion of Put to high affinity PAs (Chapter 6).

In general, this study has elucidated which compounds determine chilling resistance or susceptibility of lemon fruit. Furthermore, the action through which hot water dips and molybdenum soaks reduced the occurrence of chilling injury cold exposure has been elucidated. Such treatments alter the cellular bioactive compounds present in the flavedo, thereby acclimating the fruit to chilling exposure.

Future research and commercial implications

Although citrus fruit are known to be chilling susceptible to temperatures lower than 14.5°C, reduced chilling injury symptoms were identified at sub-zero storage temperatures. Future studies must be conducted to compare the physiology of sub-zero (-0.5°C) with higher storage temperatures (14.5, 7.5 or 2°C). This study has shown that a higher rind glucose concentration is a major determinant for chilling resistance and therefore, future studies need to investigate orchard practises to increase the lemon flavedo glucose concentration. Clearly, the flavedo glucose concentration also determines the concentrations of other compounds, including bioactive compounds with antioxidant properties which seem to play a significant role in mitigating oxidative stress.

Membrane composition can be affected by lipid composition. Cellular membranes which contain highly saturated, long-chained lipids are more chilling than those containing unsaturated long-chained lipids. Fruit sourced from chilling resistant micro-climates might have an altered membrane lipid composition compared with those from chilling-susceptible climates. Therefore, the role of micro-climate and orchard practises on membrane lipid composition must be investigated before application of any post-harvest treatments such as hot water or molybdenum is commercially recommended.

The activity of XDH and its contribution to total cellular ROS must be determined to be able to postulate and identifiy a clear role of XDH in chilling stress. Moreover, the assumed role of ROS to induce oxidative stress acclimation must be further studied. A variety of ROS assays must be used for this purpose. Means to enhancing bioactive compounds at pre-harvest must also must be investigated. In addition, a very complicated study is required to elude the contribution of specific ROS (O_2, H_2O_2, or $OH_{\cdot}$) on oxidative stress.

The antioxidant study did not include analysis of enzymatic antioxidants. However, there is no literature on the order of potency of enzymatic antioxidants as compared with bioactive compounds with antioxidant properties. Therefore, future research must aim at developing assays to quantify the potency of enzymatic antioxidants (SOD, CAT, POD) and develop “a holistic antioxidant capacity” that also includes bioactive compounds and enzymatic antioxidants.

The cascade of events leading to either chilling susceptibility or resistance is complex, especially at sub-zero storage temperature compared with higher chilling temperatures. Therefore, the cascade of physiological events that includes the role of sugars, bioactive compounds and enzymatic antioxidants must be studied.

This research identified glucose as a major driving component in chilling susceptibility of citrus fruit. Therefore, non-destructive methods to estimate the flavedo glucose concentration which are fast and reliable need to be identified to determine chilling susceptibility so that the need for post-harvest treatment with either hot water or molybdenum can be predicted and evaluated. Near infrared spectroscopy (NIRS) would be such a method as it has the potential to non-destructively determine flavedo glucose concentration pre- and post-harvest.

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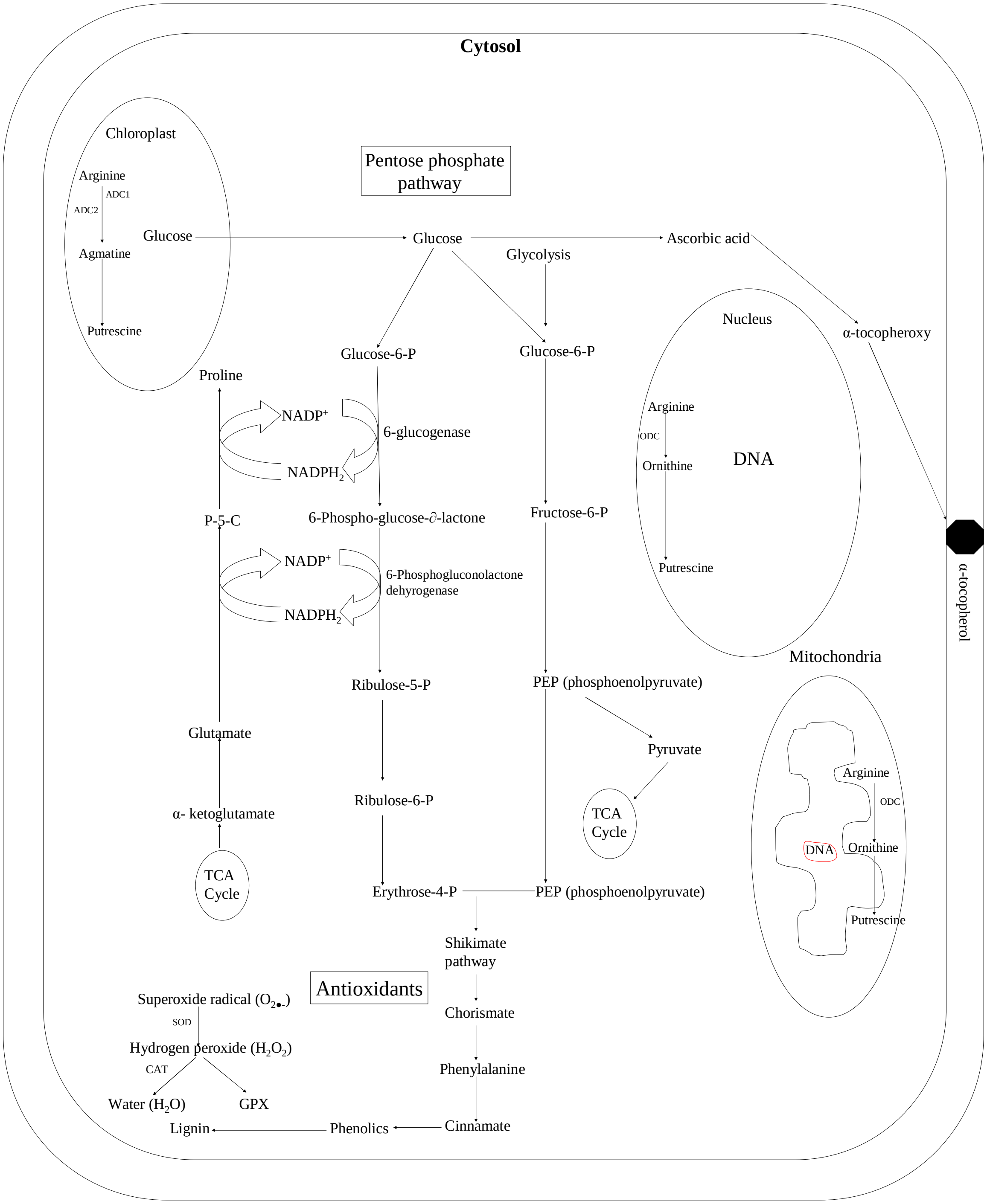
Cytosol
Chloroplast
Arginine
ADC1
ADC2
Agmatine
Putrescine
Glucose
Pentose phosphate pathway
Glucose
Glycolysis
Ascorbic acid
α-tocopheroxy
Glucose-6-P
Glucose-6-P
Nucleus
Proline
NADP+
6-glucogenase
NADPH2
Arginine
ODC
Ornithine
DNA
Putrescine
P-5-C
6-Phospho-glucose-∂-lactone
Fructose-6-P
NADP+
6-Phosphogluconolactone dehyrogenase
NADPH2
α-tocopherol
Mitochondria
Ribulose-5-P
PEP (phosphoenolpyruvate)
Glutamate
Pyruvate
Arginine
ODC
Ribulose-6-P
α- ketoglutamate
TCA Cycle
DNA
Ornithine
TCA Cycle
Erythrose-4-P
PEP (phosphoenolpyruvate)
Putrescine
Shikimate pathway
Antioxidants
Superoxide radical (O_2•-)
SOD
Chorismate
Hydrogen peroxide (H_2O_2)
CAT
Phenylalanine
Water (H_2O)
GPX
Lignin
Phenolics
Cinnamate

Figure 1: Proposed integrated cellular pathway and compounds involved in stress maintenance