THE EXPLORATION OF FACTORS ASSOCIATED WITH CITRUS FRUIT NON-CHILLING RIND PITTING: THE CASE STUDY OF HIGHLY PRONE 'BENNY' VALENCIA VARIETY

by

MATHOLO JOYCE MOTHAPO

THESIS

Submitted in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

AGRICULTURE

in the

FACULTY OF SCIENCE AND AGRICULTURE

(School of Agricultural and Environmental Sciences)

at the

UNIVERSITY OF LIMPOPO

SUPERVISOR: DR N MATHABA (UMP)

CO-SUPERVISOR: PROF TP MAFEO (UL)

2021

DECLARATION

I declare that the thesis submitted to the University of Limpopo, for the degree of doctor of philosophy in Agriculture has not previously been submitted by me for a degree at this or any other university; that it is my work in design and in execution, and that all material contained herein has been dully acknowledged.

Ms Mothapo MJ___ 05/11/2021

Surname and initials (title) **Date**

ACKNOWLEDGEMENTS

I would like to express my gratitude and appreciation to the following people and institutions:

1. Dr. Nhlanhla Mathaba and Prof. Tieho Paulus Mafeo, my academic supervisors, for invaluable guidance and advice during this study, which helped greatly in the preparation of this work. Your positive criticism and help in proofreading all the manuscripts and chapters improved my skills both as a scientist as well as an academic author.
2. Dr. Rebogile Mphahlele, for her tireless effort, encouragement, advice and support during this study.
3. A special thanks to all the students of the Postharvest Laboratory at Agricultural Research Council Tropical and Subtropical Fruit Crops (ARC-TSC) that I had the pleasure to work with, for their constant support and help with data collection: Rhulani Shibambu, Rhivhalani Baloyi, Maureen Nthai, Kholofelo Manase, Fezeka Vukeya and Khutjo Mmako.
4. My gratitude also goes to Jan Ndandani, Justice Mlimi, Fumani Shikwambana and Shakile Mathe for assisting with field trips during the harvesting of fruits.
5. The National Research Fund, Agricultural Research Council Tropical and Subtropical Fruit Crops and Citrus Academy for financial support which made it possible to undertake the study.
6. I am also grateful to Mahela Boerdery, Schoeman Boerdery and Noordgrens Landgoed for willingly supplying fruits to conduct this study.
7. I am indebted to my family: my mother (Makotseng Mothapo), my father (Mangene Mothapo) and my brothers (Mabua, Mokibelo and Ntasa Mothapo). All I can say is thank you for your kind words and support when I needed it the

most. My deepest appreciation and thanks to my sons (Masobe and Mofenyi) and daughter (Lethabo) for giving mommy space to work.

8. Last but not least, I would like to thank the special man in my life: my fiancé Ignatius Mojapelo for his continued support. And for always making me smile even when things got really tough and I was on the verge of quitting.
9. Importantly, I give praise to the almighty God for keeping me throughout the entire time, for giving me wisdom, being my source of strength and blessing me abundantly.

DEDICATION

I would like to dedicate this thesis to my sons Masobe and Mofenyi Mothapo and daughter Lethabo Mothapo.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

PUBLICATIONS AND CONFERENCE/S ATTENDED DURING CANDIDATURE

Already published article from the thesis

1. **Mothapo, M.J.,** Mathaba N., Mafeo, T.P. and Mphahlele, R.R., 2018. Effect of production site and postharvest treatments on the incidence of rind pitting and chemical properties of Benny sweet orange. *Acta Horticulture*. 15: 107-114.

Anticipated research articles from the thesis:

1. **Mothapo, M.J.,** Mathaba N. and Mafeo, T.P. Role of production site, postharvest treatments and cold storage temperature on rind biochemical concentrations associated with 'Benny' valencia fruit non-chilling rind pitting.
2. **Mothapo, M.J.,** Mathaba N. and Mafeo, T.P. The role of production site, wax plus dehydration and cold storage to regulate total antioxidants and their ability to mitigate 'Benny' valencia fruit non-chilling rind pitting.
3. **Mothapo, M.J.,** Mathaba N. and Mafeo, T.P. The contribution of production sites, postharvest treatments and cold storage temperature on non-chilling rind pitting and soluble sugars of 'Benny' valencia fruit.
4. **Mothapo, M.J.,** Mathaba N. and Mafeo, T.P. Expression of differentially expressed genes associated with 'Benny' valencia non-chilling rind pitting disorder.

National conference (s) attended and presented as part of the thesis:

1. **Mothapo, M.J.,** Mathaba N. and Mafeo, T.P. Investigating citrus rind pitting susceptibility on selected sweet orange (*Citrus sinensis*) cultivars in South Africa. Combined Congress. 23-26 January 2016. Klein Kariba Resort - Bela Bela.

2. **Mothapo, M.J.,** Mathaba N. and Mafeo, T.P. Role of production sites and postharvest treatments on rind biochemical concentrations in relation to rind pitting of ‘Benny’ sweet orange. Agricultural Research Council Conference. 27-29 August 2018. Roodeplaat - Pretoria.

International conference(s) attended and presented as part of the thesis:

1. **Mothapo, M.J.,** Mathaba N. and Mafeo, T.P. Investigating the effect of production region on non-chilling rind pitting of sweet orange (*Citrus sinensis*) cultivars in South Africa. 9th Citrus Research Symposium. 21-25 August 2016. Champagne Resort- Drakensburg.
2. **Mothapo, M.J.,** Mathaba N. and Mafeo, T.P. Effect of production site and postharvest treatments on the incidence of rind pitting and chemical properties of ‘Benny’ sweet orange. The International Society of Horticultural Science Conference. 4-7 September 2017. University of Stellenbosch - Stellenbosch.

THESIS STRUCTURE

The subsequent chapters in the thesis are presented as follows:

CHAPTER 1: This chapter addresses the background of rind pitting and its effect on fruit quality. Furthermore, the section presents the problem statement, motivation, aim, objectives and hypotheses of the study.

CHAPTER 2: This chapter focuses on the review of studies relating to factors that have been reported to be associated with citrus rind pitting. The factors include pre-harvest (production site and cultivar choice), postharvest (water status, wax and dehydration and cold storage temperature), genes expression in relation to rind pitting, membrane lips, rind soluble sugars and antioxidants activity.

CHAPTER 3: This chapter serves as the preliminary study to investigate the role of production sites, postharvest treatments and cold storage temperature on non-chilling rind pitting and physico-chemical properties of ‘Benny’ valencia citrus fruit.

CHAPTER 4: This chapter concerns the role of production sites, postharvest treatments and cold storage temperature on rind biochemical concentrations in relation to rind pitting of ‘Benny’ valencia citrus fruit.

CHAPTER 5: This chapter concerns the role of production sites, postharvest treatments and cold storage temperature on total antioxidants assays in relation to rind pitting of ‘Benny’ valencia citrus fruit.

CHAPTER 6: This chapter concerns the contribution of production sites, postharvest treatments and cold storage temperature on development of ‘Benny’ valencia citrus fruit rind pitting and their relationship with soluble sugars.

CHAPTER 7: This chapter investigates whether genes expressed in citrus rind pitting would be an effective tool to protect citrus fruit quality.

CHAPTER 8: In this chapter, the overall conclusion of the study was presented mainly focusing on chapter 3 to 7 and recommendations for future research were made.

GENERAL ABSTRACT

The rind physiological disorders incidence such as rind pitting is a challenge to the citrus industry as it affects appearance; and ultimately, acceptability and purchase in both local and international markets. Although the internal quality is not directly affected by rind pitting, fruit damaged by this disorder are rejected in the fresh fruit market. The susceptibility to this disorder varies among citrus fruit cultivars. Other factors impacting rind physiological disorders include; pre-harvest environmental conditions and postharvest storage conditions. However, the main cause of this disorder is still unknown. In South Africa, 'Benny' valencias are the most prone orange cultivars to rind pitting disorder within the sweet-orange-type. Therefore, the aim of this study was to investigate production site and postharvest treatments' effect on physico-chemical, biochemical properties, antioxidants, rind soluble sugars and gene expression in relation to rind pitting development of 'Benny' valencia citrus fruit. During 2016 and 2017 seasons, the study was conducted, whereby, 'Benny' valencia citrus fruit were harvested from Tzaneen, Groblersdal and Musina in South Africa. After harvesting, the fruits were transported to the Agricultural Research Council-Tropical and Subtropical Crops (ARC-TSC) postharvest laboratory in Nelspruit for sorting, grading treatment, cold storage and post-storage quality evaluation. After sorting and grading, fruits were subjected to the following treatments: T_1 = no wax plus dehydration, T_2 = wax plus dehydration and T_3 = wax plus no dehydration. Dehydrated treatments were applied for 3 days at relative humidity of ±45%, thereafter, fruit were stored at -0.6 and 4.5°C for 28 days plus 7 days shelf-life. After removal from cold storage plus 7 days shelf-life, fruit were analysed for rind pitting incidence (RPI), weight loss percentage (WL), firmness, total electrolyte leakage (TEL), total soluble solids (TSS), titratable acidity (TA) and TSS: TA ratio.

Afterwards, fruit were peeled to remove flavedo, thereafter; the flavedo peels were freeze-dried, milled and stored at -21°C for further physiological analysis. Freeze-dried flavedo peel was analysed for total flavonoids, total phenolics, vitamin C, soluble sugars (glucose, fructose and sucrose), antioxidant assays (FRAP, DPPH, ABST and ORAC) and genes.

The results showed that rind pitting incidence was high on fruit subjected to wax plus no dehydration across all storage temperatures and production sites. Furthermore, results showed that fruit harvested from Musina exposed to T_1 had higher incidence of rind pitting than those from Groblersdal and Tzaneen, irrespective of storage temperature. Meanwhile, fruit harvested from Musina had the highest TEL when compared with Groblersdal and Tzaneen irrespective of treatments and storage temperatures. A significantly higher ($P<0.05$) WL was observed in Musina fruit harvested from Tzaneen exposed to T_3 at both storage temperatures. Moreover, increased TSS was observed after storage across all production site and postharvest treatments. The study showed that production site and postharvest treatments had a significant influence on rind pitting and total electrolyte leakage. Additionally, fruit treated with no wax + dehydration was found to be more susceptible to rind pitting. However, fruit sourced from Tzaneen had significantly ($P<0.0001$) high TPC and TFC, irrespective of postharvest treatments therefore, low rind pitting incidence. While rind vitamin C was higher in fruit from Groblersdal when compared with Tzaneen and Musina. However, low RPI was also observed in fruit sourced from Groblersdal. Fruit from Musina subjected to wax plus dehydration had higher RSA and low RPI at both temperatures when compared with fruit sourced from Groblersdal and Tzaneen. Therefore, wax plus dehydration resulted in low rind pitting with an increased accumulation of rind biochemical concentrations,

irrespective of cold storage temperatures. These results suggested that there is a link between rind pitting and rind biochemical concentrations in the ‘Benny’ valencia citrus fruit. Moreover, fruit from Musina subjected to wax plus dehydration had higher antioxidant measured by DPPH and low RPI at both low storage temperatures when compared with fruit harvested from Groblersdal and Tzaneen. Furthermore, fruit harvested from Musina and treated with no wax plus dehydration, thereafter, stored at -0.6°C had low RPI with high antioxidant activity measured by FRAP than Tzaneen and Groblersdal regions in both seasons.

With respect to sugars, fructose was not significantly ($P<0.05$) affected by production sites, postharvest treatments and cold storage temperature, hence, the low pitting incidence. The highest glucose was observed in fruit harvested from Groblersdal, irrespective of treatments and cold storage temperatures when compared with those from Tzaneen and Musina, low RPI was also observed in fruit harvested from Groblersdal. However, fruit harvested from Groblersdal treated with wax plus dehydration and stored at 4.5°C had higher sucrose and low RPI when compared with Tzaneen and Musina. Moreover, this study suggested that soluble sugars in ‘Benny’ valencia flavedo during cold storage is involved in rind pitting tolerance mediated by wax plus dehydration treatment.

Three homologic genes: CsCP gene; CsNAC-domain protein gene; CsCP-F gene; were chosen to examine the relationship between their expression and citrus rind pitting through quantitative RT-PCR analysis in pitting and no-pitting fruits. Results showed that the expression of CsCP, CsNAC and CsCP-F genes were all higher in the pitting rind fruit harvested from Tzaneen and low in fruit with low pitting. Groblersdal and Musina fruit had low expression of genes and low rind pitting was observed. Therefore, findings suggested that CsCP, CsNAC and CsCP-F genes may

be linked to non-chilling rind pitting and could serve as targets for future investigation.

Generally, the overall results obtained in this study provided an understanding into the previous unknown complexities of citrus non-chilling rind pitting. Moreover, the study revealed that the studied factors had an influence on non-chilling rind pitting and physico-chemical properties of ‘Benny’ valencia citrus fruit. In addition, postharvest treatments resulted in low non-chilling rind pitting with an increased accumulation of rind biochemical concentrations. The fruit with high antioxidant capacity were found to be tolerant to rind pitting, whereas, fruit with low antioxidant capacity were found to be susceptible to rind pitting. Furthermore, soluble sugars are believed to be involved in the defence mechanisms against non-chilling rind pitting in the fruit. Gene expression changes also provided clues about the possible mechanisms involved in non-chilling rind pitting development.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background of the study

Citrus fruit is an important crop produced in large parts of the world including South Africa (FAO, 2013). In South Africa, the industry is predominantly focused on export markets and exports over 70% of fresh citrus fruit (Citrus Growers Association of Southern Africa, 2019). Therefore, the South African citrus industry is the second largest fresh citrus fruit exporter in the world after Spain (Citrus Growers Association of Southern Africa, 2019). The industry priorities production of high quality citrus fruits, as quality determines the market price. However, the industry encounters some challenges in maintaining quality and continues the pursue of high paying markets.

In citrus fruit, non-chilling rind pitting physiological disorder is frequently observed before or after harvesting (Cronje, 2007; Magwaza, 2008). The disorder starts with the collapse of the sub-epidermal rind cells with no discolouration taking place. As the disorder progresses, new depressed clustered spots appear randomly over the fruit surface. Ultimately, collapsed areas change colourless lesions to brown colour (Agusti *et al*., 2001). As the disorder becomes more severe, flavedo cell layers at oil glands bottom and adjacent areas appear twisted and wrinkled (Alférez *et al*., 2005). However, the susceptibility of these disorders vary among citrus fruit cultivars. Several citrus cultivars including ‘Navelina’ and ‘Navelate’ oranges from Spain, ‘Marsh’ grapefruit and ‘Benny’ valencia citrus fruit are highly susceptible to non-chilling rind pitting (Alférez *et al.,* 2005; Ehlers, 2016).

Non-chilling rind pitting incidence could manifest symptoms very early in the season, especially in the northern direction of the tree (Karim and Neven, 2012). Rainfall in some areas in the last few weeks before harvest may present another factor associated with the increase of pitting since it causes the breakdown of some volatile oil cells (Cronje *et al.,* 2011). In general, the cause of rind pitting, whether in the field or during the harvest process or after harvest in the storage room is not well defined. Several factors have been reported to be involved in the development of rind pitting such as climate, postharvest chilling, mechanical injury or chemical treatments (Almela *et al.,* 1992).

Citrus fruit's higher susceptibility to rind physiological disorders has been reported to be associated with various biochemical concentrations, such as lower total flavonoids, lower phenolic compounds and antioxidants (Sevillano *et al.,* 2009). However, phenolics are one of the important groups of compounds which are the major constituents responsible for antioxidant capacity and the level of fruit tolerance to rind physiological disorders (Lee *et al.,* 2003). Furthermore, the influence of vitamin C content in citrus fruit has been reported for instance, Bassal and El-Hamahmy (2011) reported high vitamin C content in 'Navel' oranges in fruit with rind disorder such as chilling injury. Moreover, there has been vast research on vitamin C and plant stress response such as non-chilling rind pitting (Magwaza *et al.,* 2013).

Another area that still needs further research is the relationship between the biochemical profile (total flavonoids and phenolics) of the rind, antioxidant system and development of citrus non-chilling rind pitting physiological disorder. Moreover, previous studies reported that fruit inside canopy rind soluble sugars were significantly lower than fruit from outside canopy, therefore, associated with high rind

break down for inside canopy when compared with outside canopy (Cronje *et al.,* 2011; Magwaza *et al.,* 2013). In addition, high soluble sugar concentrations have been reported to serve as energy source reserves and also contribute to rind cell structure sustenance (Zhu *et al.,* 2011). However, rind physiological disorders development has been associated with rind soluble sugars (Olarewaju *et al.,* 2017).

A study was done on cDNA library to screen differentially expressed genes in citrus peel pitting, in which the pitting rind and rind with no pitting were used as tester and driver, respectively (Gao *et al.,* 2006). By screening this library, it was expected that some genes which may play significant roles in non-chilling rind pitting development would be discovered. Therefore, understanding the possible factors that led to non-chilling rind pitting may generate knowledge that may contribute to the development of new postharvest management practises which may reduce losses and improve the standard of the South African Citrus Industry (SACI) and increase exports.

1.2 Problem statement

Non-chilling rind pitting is among the most important physiological rind disorders affecting the South African citrus industry. The wastage is so high that in some instances between 10 to 50% of export revenue is lost due to rind pitting that develops between the field and the consumer (Kaiser *et al.,* 2004). The mode of action is poorly defined, but evidence increasingly indicates that fluctuations in relative humidity during postharvest period could contribute to the disorder development (Alférez *et al.,* 2003; Agusti *et al.,* 2001). Poor understanding of the exact cause of the disorder affects both supply and profits.

Citrus non-chilling rind pitting is associated with a multitude of pre-harvest factors and post-harvest treatments (Alférez and Zacarías, 2001; Alférez *et al.,* 2005). South African citrus production occurs mainly in three climatically different production regions, the Mediterranean (Western Cape-Citrusdal); Coastal Warmer (Eastern Cape and KwaZulu-Natal) and Tropical/Subtropical (Mpumalanga and Limpopo). However, there is no research work on factors (environmental factors and production sites, postharvest treatments and cold storage temperature) as the contributing factors to non-chilling rind pitting on 'Benny' valencia citrus fruit and its relationship with biochemical properties, antioxidants, rind soluble sugars and genes expressed.

1.3 Motivation of the study

Several studies have been conducted on citrus rind pitting to investigate the causal factors. The focus of these studies was on post-harvest treatment factors such as waxing (Agusti *et al.,* 2001), relative humidity (Alférez *et al.,* 2005; Cronje, 2007) rind water status (Alférez, and Burns, 2004) and mineral nutrition (Kaiser *et al.,* 2004). According to Cronje (2007), there is increasing evidence, which indicates that fluctuations in relative humidity during postharvest period could contribute to the disorder development. However, there is limited information on the effect of pre-harvest factors or combination with post-harvest factors on development of this disorder. Therefore, the current study will generate information on pre-harvest production factors that may be relevant in understanding and reducing the susceptibility of the fruit to this subsequent disorder. Furthermore, this research will improve information in relation to aspects of the disorder that can be of practical use in identifying the exact cause of the disorder.

1.4 Aim of the study

The aim of the study was to generate knowledge on ‘Benny’ valencia citrus fruit non-chilling rind pitting disorder development as influenced by production sites, postharvest treatments and cold storage temperatures.

1.5 Objectives of the study

The objectives of this study were therefore:

i. To investigate the effect of production site, wax plus dehydration treatments and cold storage temperatures on non-chilling rind pitting development and physico-chemical properties of ‘Benny’ valencia citrus fruit.

ii. To assess the role of production sites, postharvest treatments and cold storage temperatures on rind biochemical concentrations associated with ‘Benny’ valencia citrus fruit non-chilling rind pitting.

iii. To evaluate the effect production sites, wax plus dehydration and cold storage temperatures on total antioxidants and their ability to mitigate ‘Benny’ valencia citrus fruit non-chilling rind pitting.

iv. To investigate production sites, postharvest treatments and cold storage temperatures as the contributing factors to non-chilling rind pitting on ‘Benny’ valencia citrus fruit and its relationship with rind soluble sugars.

v. To investigate genes expression in citrus rind showing pitting with quantitative write in full first (RT-PCR), in which the pitting rind and no-pitting rind were respectively used as tester and driver.

1.6 Hypotheses

i. Production site, wax plus dehydration treatments and cold storage temperatures have no effect on non-chilling rind pitting development and physico-chemical properties of ‘Benny’ valencia citrus fruit.

ii. Production sites, postharvest treatments and cold storage temperatures have no role in rind biochemical concentrations associated with ‘Benny’ valencia citrus fruit non-chilling rind pitting.

iii. Production sites, postharvest treatments and cold storage temperatures have no effect on total antioxidants and their ability to mitigate ‘Benny’ valencia citrus fruit non-chilling rind pitting.

iv. Production sites, postharvest treatments and cold storage temperatures have no contribution to Benny’ valencia citrus fruit non-chilling rind pitting and its relationship with rind soluble sugars.

v. Gene differentially expressed cannot be associated with ‘Benny’ valencia citrus fruit non-chilling rind pitting.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Worldwide, citrus fruit production was approximately 117,631 thousand tons in 2019 (Citrus Growers Association of Southern Africa, 2019). The highest producers were China (36 041 thousand tons), Brazil (19 274 thousand tons), India (11 773 thousand tons) and Mexico (8,253 thousand tons) with South Africa ranking 10th at 2 889 thousand tons production (Citrus Growers Association of Southern Africa, 2019). However, Spain exported the highest citrus fruit volume, thereby, contributing approximately 44% market share in 2019. The Spanish and South African citrus industry are export-oriented, primarily aiming at the European market. Currently, the South African citrus industry ranks 2nd in terms of world export and contributes 2 131 thousand tons citrus production (Table 2.1) (Citrus Growers Association of Southern Africa, 2019).

Table 2.1 World fresh citrus exports (thousand tons) from 2010 to 2019

Country	10/11	11/12	12/13	13/14	14/15	15/16	16/17	17/18	18/19	Rank
Spain	3 644	3 760	3 862	3 761	3 925	3 947	3 622	3 699	2 658	1
South Africa	**1 367**	**1 453**	**1 541**	**1 649**	**1 675**	**1 702**	**1 692**	**1 929**	**2 131**	**2**
Turkey	1 389	1 441	1 144	1 497	1 542	1 708	1 724	1 913	2 009	3
China	783	752	1 030	1 030	913	934	872	936	939	4
Mexico	491	470	550	550	663	750	744	823	805	5
Egypt	924	924	1 182	1 182	1 182	1 520	819	1 594	792	6
USA	1 134	1 025	951	951	833	990	1 350	808	712	7
Argentina	552	502	454	406	341	394	395	379	353	8
Morocco	592	490	385	584	500	640	610	706	341	9
Greece	466	372	445	451	433	613	589	416	339	10

Sourced from Citrus Growers Association of Southern Africa: Annual Report 2019

In South Africa, 65% of citrus total production went into export, 29% into the processing sectors and 6% into local markets (Figure 2.1) (Citrus Growers Association of Southern Africa 2019). The leading exported citrus types were Valencia oranges – 35%, navel oranges – 20%, lemons – 18%, soft citrus – 15% and grapefruits – 12% (Figure 2.2) (Citrus Growers Association of Southern Africa, 2019). While, the major export destinations include; Europe (33%), Middle east (19%), South East Asia (13%), United Kingdom (9%), Russian Federation (8%), Asia (7%), North America (7%) and other (1%) (Figure 2.2) (Citrus Growers Association of Southern Africa, 2019). However, the industry has been encountering some challenges in maintaining citrus fruit quality and continue to pursue high paying markets (Citrus Growers Association of Southern Africa, 2019).

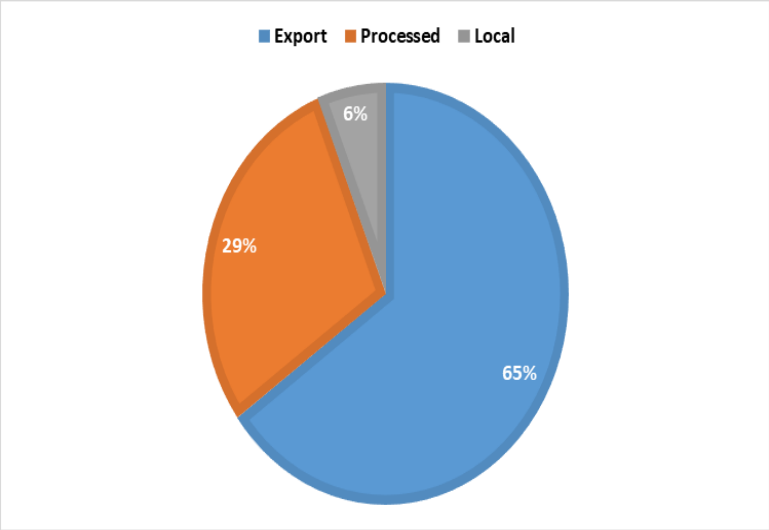

Figure 2.1: Percentage production sector volume (Citrus Growers Association of Southern Africa, 2019)

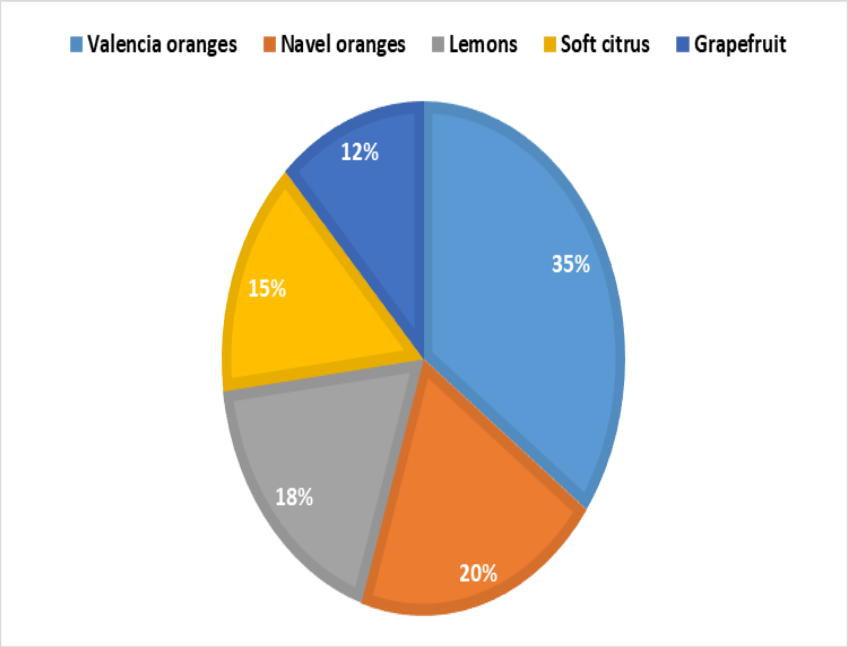

Figure 2.2: Export products (Citrus Growers Association of Southern Africa, 2019)

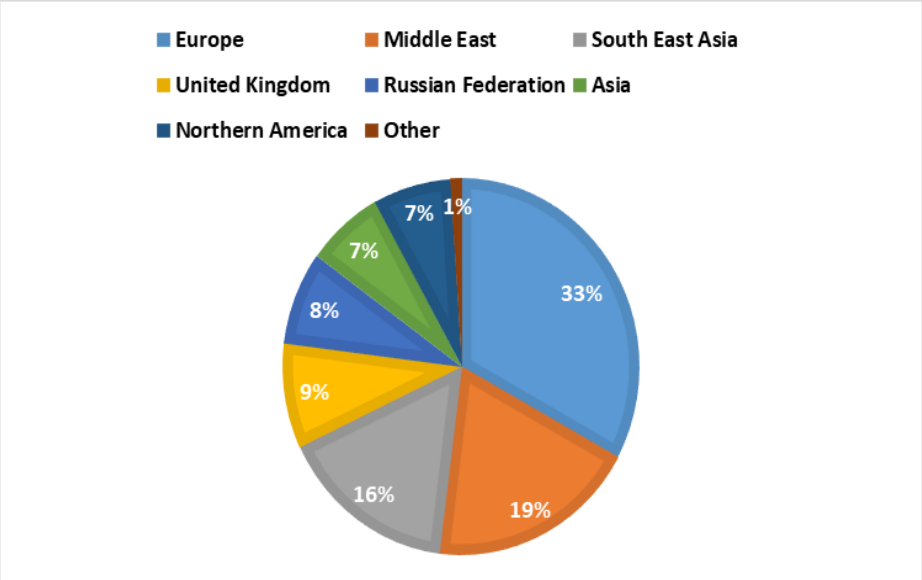

Figure 2.3: South African citrus industry major export destinations during 2019 harvest season (Citrus Growers Association of Southern Africa, 2019)

In citrus fruit, quality is based on internal parameters (unique flavour, nutritional value, juiciness and total soluble solids (TSS: Acid ratio) as well as external parameters including; good rind colour and no manifestation of postharvest diseases

(Ladaniya, 2008) or insect damage. Internal quality parameters can improve due to postharvest cold storage temperature due to reduced metabolic rate. However, the external quality declines as physiological disorders manifest, especially non-chilling rind pitting. In citrus fruit rind pitting is a disorder that starts with sub-epidermal rind cells collapse with no discoloration taking place (Agusti *et al*., 2001). As the disorder progresses, new depressed clustered spots appear randomly over the fruit surface (Figure 2.4). Finally, collapsed areas change colourless lesions to brown colour (Agusti *et al*., 2001). As the disorder becomes more severe, flavedo cells layers at the bottom of oil glands and adjacent areas appear twisted and wrinkled (Alférez *et al.,* 2005).

Figure 2.4: Rind pitting symptoms in ‘Benny’ valencia citrus fruit

Therefore, this chapter focused on the review of studies relating to factors that has been reported to be associated with citrus non-chilling rind pitting. These factors include pre-harvest (environmental factors and production sites, cultivar choice and mineral nutrition), postharvest (water status, wax and dehydration and cold storage

temperature), expression of genes, membrane lips, soluble sugars and antioxidants activity.

2.2 Factors affecting rind pitting

2.2.1 Pre-harvest factors

In recent years, efforts have been made to elucidate the primary factors triggering postharvest citrus rind pitting, but the fundamental causes remain unknown. In citrus fruit, several researchers reported that fruit position within a canopy (Kaiser *et al.,* 2004), mineral nutrition (Kruger *et al.,* 2005; Magwaza *et al.,* 2008), fruit maturity and crop load (Leguizamon *et al.,* 2001), adverse climatic conditions and water stress during critical fruit development stages (Kruger *et al.,* 2005) are the important pre-harvest rind pitting causative factors. Table 2.2 shows an overview of previous studies on different sweet orange rind pitting symptomology, time occurrence and rind pitting physiological disorder causal factors.

2.2.2 Effect of environmental factors and production site on non-chilling rind pitting

In citrus fruit, environmental conditions such as temperature, humidity and rainfall are critical in controlling rind pitting rate (Gonzalez-Aguilar *et al.*, 2000). The most important geographical aspects that could influence temperature in a specific area are latitude and altitude (Cronje, 2007). However, it is important to understand that physiological disorders are always associated with a particular symptomology as the fruit is subjected to a set of specific environmental conditions. In general, it is considered that citrus species whose natural habitat is closer to the equator are more sensitive to rind disorders when compared with those from higher latitudes due to high temperature (Patterson and Reid, 1990). However, the growing site is

considered one of the main factors affecting fruit susceptibility to rind pitting (Olarewaju *et al.*, 2017).

In ‘Navel’ orange citrus, non-chilling rind pitting differences were observed due to growing site when fruit were grown at various Australia sites (Lindhout, 2007). The sites were in Sunraysia district in New South Wales, whereby, peak flowering occurred mostly uniform and the observed difference in maturity and colour indices were related to environmental factors. Therefore, cultivar and site differences must be considered prior to orchard planting in order to avoid high rind pitting incidence in exported fruit. In ‘Marsh’ grapefruit, late season high temperatures resulted in a high non-chilling rind pitting incidence but the physiological reasons were not understood (Bramlage and Weiss, 1997). However, Gonzalez-Aguilar *et al.* (2000) found that mid-season ‘Fortune’ mandarin fruit harvested during cooler months of the year had high non-chilling rind pitting incidence when compared with those harvested during warmer months.

Table 2.2: An overview of symptomology, occurrence time and rind pitting causal factors physiological disorders on oranges

Cultivar	Symptoms	Causal factors	Time to occurrence	Pre- or post-harvest	Reference
'Navel'	Collapsed areas of flavedo and albedo part that becomes brown with time.	Wide array of pre-harvest and postharvest factors.	ND	Pre- and postharvest	Gao *et al.* (2006)
	Scattered cluster pits over the fruit surface that turn dark brown after several weeks.	Water loss and sudden changes from low to high RH and waxing.	1 to 2 weeks	Postharvest	Alférez and Zacarìas (2001)
	Collapsed flavedo areas and albedo part that becomes brown with time.	Wide array of pre-harvest and postharvest factors.	1 week	Postharvest	Li *et al.* (2009)
'Navelate'	Collapse, drying and flavedo browning.	Water loss and sudden changes from low to high RH.	7 to 28 days	Postharvest	Alquezar *et al.* (2010)
	ND	Ethylene inhibition by 1-MCP (reduced by external ethylene application).	2 to 14 days	Pre- and postharvest	Estables-Ortiz *et al.* (2009)
	Irregular depressed areas on the flavedo that may turn brown with time.	Lack of epicuticular wax and dehydration.	7 to 22 days	Postharvest	Cajuste and Lafuente (2007)
	ND	Dehydration	14 to 56 days	Postharvest	Alférez *et al.* (2005)
	ND	Lack of epicuticular wax and dehydration. Increased internal	14 days	Postharvest	Sala *et al.* (2005)

		ethylene production.			
‘Navelina’	ND	Water loss and sudden changes from low to high RH and increase in ethylene.	7 to 28 days	Postharvest	Sanchez-Ballesta *et al.* (2008)
	Flavedo collapse and albedo tissues.	Fruit maturity	ND	Pre-harvest	Alférez and Zacarìas (2012)
‘Shamouti’	Flavedo collapse and necrosis.	Water loss	3 to 5 weeks	Pre- and postharvest	Tamin *et al.* (2001)
‘Valencia’	Sunken-necrotic areas on the stylar fruit end.	Tree water stress at maturity.	ND	Pre-and postharvest	Albrigo *et al.* (1970)

ND = Not documented

Rainfall amount and distribution over a season is also important in rind disorder development in fruit production; therefore, affecting postharvest quality. According to Kruger and Classens (1997), high rainfall during fruit maturation increased internal disorders in ‘Fuerte’ and ‘Hass’ avocado fruit. In ‘Lisbon’ lemon fruit, Undurraga *et al.* (2006) found that extending harvest days after rainfall reduced rind pitting intensity. Moreover, water potential effect on ‘Navelate’ orange citrus fruit rind disorders has been confirmed to alter citrus fruit peel water potential and may be a triggering factor related to rind pitting (Alquezar *et al.,* 2010). However, the role of environmental and production sites in relation to rind pitting incidence in citrus fruit has not been clearly discussed and understood yet.

2.2.3 Cultivar choice

Citrus fruit sensitivity to rind physiological disorder varies significantly among different citrus types and cultivars (Alférez and Burns, 2004). Certain citrus types and cultivars are more susceptible to non-chilling rind pitting (Lafuente *et al.,* 2003). Although non-chilling rind pitting occurs on most orange and grapefruit cultivars, however, ‘Benny’ valencia citrus fruit are the most susceptible cultivar species within the sweet orange type (Kruger *et al*., 2005). Therefore, cultivar choice depends on different factors (environmental factors) in a growing area as well as differences in cultivar susceptibility to physiological disorder. In general, citrus fruit’s sensitivity to non-chilling rind pitting follow this pattern: ‘Benny’ orange > ‘Turkey’ orange > ‘Midknight’ orange > ‘Lavelle’ orange > Navel orange > ‘Marsh’ grapefruit > tangerines > mandarin (Cronje, 2007). However, a large variation can still occur within cultivar group (Ladaniya, 2008). In addition to variations in the susceptibility and intensity degree, non-chilling rind pitting disorder also varied from season to season and among fruits within the same tree (Alférez *et al.,* 2003). In citrus fruit,

challenges associated with susceptible non-chilling rind pitting cultivars is the unpredictability and erratic occurrence. Although the disorder may not be clearly apparent at harvest on some citrus cultivars, it may however, develop sharply after harvest on other cultivars, particularly when fruit were held at low relative humidity (Agusti *et al.,* 2001). In 'Nova' tangerines fruit, manifestation of non-chilling symptoms developed early before fruit reach full maturity and appeared at fruit distal-end (Lafuente *et al.,* 2003).

2.3. Postharvest factors

2.3.1. Effect of wax and dehydration on rind pitting

Waxing is the process of covering fruit with artificial waxing material (Petracek, 1998). In citrus fruit, waxing objectives are to prevent water loss and improve fruit appearance (Verma and Joshi, 2000). In addition, wax delay shrinkage and spoilage, thereby, improving fruit appearance (Verma and Joshi, 2000). In citrus fruit, different types and formulations of wax have been widely used. In most cases, carnauba, shellac and polyethylene coatings are wax coatings that contribute to fruit shining; thereby, maintaining gaseous exchange and water retention (Njobolwana *et al.,* 2013). Furthermore, carnauba wax is a natural vegetable wax that occurs as a protective coating on *Copernica cerifera* leaves (Brazilian palm tree) (Dou, 2004). Shellac is obtained from Thailand and India, by the raw lac refinement which is secreted by *Laccifer lacca* insect (Bourtoom, 2008; Dou, 2004). While, polyethylene is a synthetic wax coating with high shine and highly permeability to gases development (Cohen *et al*., 1990).

Waxing fruit with less permeable shellac-based waxes stimulate rind pitting (Petracek *et al.,* 1998). According to Alférez and Burns (2004), using more gas-

permeable fruit wax as well as cold temperature storage on fruit coated with less permeable wax was shown to alleviate postharvest rind pitting in ‘Marsh’ grapefruit and ‘Fallglo’ tangerine. Moreover, coating fruit with commercial waxes, coupled with warm temperature (25°C) storage has been reported to promote rind pitting in ‘Marsh’ grapefruit (Alférez *et al.,* 2003). In addition, Alférez and Burns (2004) found that rind pitting was higher on fruit exposed to dehydration at lower relative humidity (30%) for prolonged periods (three days). These authors concluded that waxing fruit enhanced the rind pitting severity and damage if there was a previous dehydration period. Alférez and Zacarìas (2012), also concluded that rind pitting incidence was triggered by transferring dehydrated waxed ‘Navelina’ orange fruit from 45 to 95% relative humidity (RH).

2.3.2 Water status

Fruit rind water status could be a major pitting incidence determinant when the postharvest handling has been delayed during the first three days after harvest, under low RH conditions (Alférez *et al.,* 2005). According to Alférez *et al.* (2003), when fruit were again transferred to high RH, vapour pressure deficit (VPD) was suddenly reduced and water potential recovered faster in the outer fruit layer (flavedo) than in the subtending albedo cells. Furthermore, dehydrated ‘Fallglo’ tangerine and ‘Marsh’ grapefruit flavedo can draw water from albedo cells, which then become water deficient at short durations of low RH (30%) storage (Alférez *et al.,* 2005).

The increased albedo water demand which result in a suction force, may subsequently cause internal flavedo and external albedo cell layer collapse due to reduced ability to rehydrate (Alferéz *et al.,* 2003; Alferéz and Burns, 2004). In

advanced rind pitting stages, oil glands in affected areas can become compressed and rupture, causing tissue browning. If dehydration continues for prolonged periods, albedo cell layers may collapse and lose their cellular contents before rehydration treatments commence (Figure 2.5) (Alférez *et al.,* 2005).

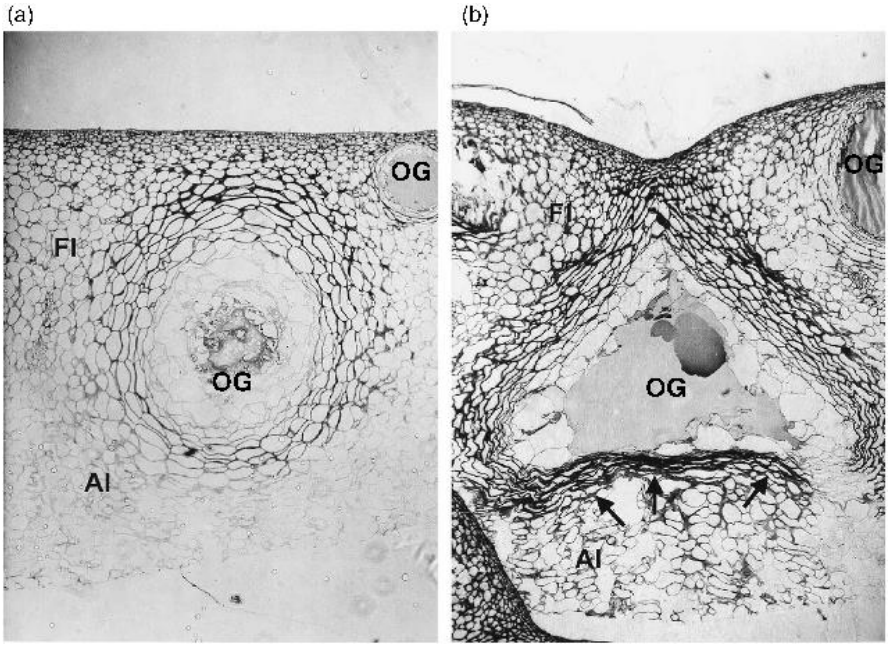

Figure 2.5: Light micrographs of cross-sections of 'Marsh' grapefruit peel from healthy (a) and pitted (b) fruit. Arrows shows layers of collapsed flavedo cells at the bottom of oil gland (b). AL: albedo; FL: flavedo; OG: oil gland; (Alférez *et al*., 2005)

2.3.3 Effect of cold storage on citrus non-chilling rind disorders

Storage conditions have been shown to affect postharvest quality and nutritional properties of citrus fruit (Lee and Kader, 2002). Citrus fruit quality is significantly influenced by storage and relative humidity (Alférez and Zacarìas, 2012). Lee and Kader (2002) indicate that temperature management is the most critical tool in maintaining quality and prolonging shelf-life of fresh produce.

Several citrus rind disorders have been related to suboptimal postharvest handling and ambient environmental conditions, especially temperature and humidity. These

disorders include; stem-end rind breakdown (SERB), chilling injury, non-chilling rind pitting and staining, as well as zebra skin of ‘Satsuma’ mandarin fruit (Petracek *et al.,* 2006). According to Lee and Kader (2002), citrus fruit require optimal temperature and RH (95%) for long-term storage. The influence of cold storage temperature on citrus fruit rind pitting has been reported by several researchers (Alférez *et al.,* 2003; Alférez and Burns, 2004).

In ‘Shamouti’ sweet orange, peel blemish known as noxan has been shown to be reduced by different postharvest treatments at high RH and by reducing storage temperature to 5°C (Ben-Yehoshua *et al.,* 2001). While, in ‘Navelina’ and ‘Navelate’ oranges and ‘Marsh’ grapefruit, increasing evidence indicated that RH variations during postharvest handling and storage changes the fruit water status, and thereby, causing rind pitting (Alférez *et al.,* 2003; Alférez and Burns, 2004). Furthermore, Alférez and Burns (2004) found that 3 hours of storage at low relative humidity induced ‘Marsh’ grapefruit and ‘Fallglo’ tangerine rind pitting.

2.4. Gene expression in relation to rind pitting

In citrus fruit, biochemical and molecular mechanisms involved in rind pitting disorder have been widely studied. Sanchez-Ballesta *et al.* (2001) studied the correlation between rind pitting and rind molecular characteristics. These researchers investigated the expression of cDNAs by screening cDNA library from non-chilling pitting sensitive citrus cultivars (‘Navelate’ and ‘Fortune’) and non-chilling pitting tolerant (‘Pinalate’) fruit. Results revealed that CrglcQ gene increased rapidly in fruit exposed to postharvest conditions favouring both non-chilling rind pitting and chilling injury disorders (Sanchez-Ballesta *et al.,* 2001). Furthermore, a full-length cDNA named 3c1, was isolated, which was differentially expressed by different postharvest stress conditions in ‘Navelate’ and ‘Fortune’ fruit (Sanchez-Ballesta *et al*., 2006).

Despite the disorders being induced by different environmental conditions and gene expression patterns, different disorders indicate that patterns may still be used as molecular markers for citrus fruit susceptibility to develop pitting (Sanchez-Ballesta *et al.,* 2008).

Gao *et al.* (2006) and Li *et al.* (2009) identified differentially expressed genes and showed that genes differently expressed in stressed fruit had significant similarity to known genes in 'Navel' oranges. For instance, the sequence analysis revealed a full-length cDNA of isolated clones had significant similarities to a Ca^{2+} binding protein, named CsCAB (Li *et al.*, 2009). Other isolated sequences were similar to cysteine protease, named CsCP and to an NAC domain protein known as CsNAC (Gao *et al.*, 2006). Quantitative analysis performed to determine cDNAs expression pattern during the wounding revealed that genes were involved in the process of rind pitting development (Gao *et al.*, 2006). Therefore, the conclusion to be drawn is that genes are involved in stress response related to physical damage and possibly to rind disorders.

2.5. Membrane lipid

The most significant changes in lipid composition are due to lipid peroxidation, phospholipids degradation and increase sterol-phospholipid ratio, which changes the membranes fluidity (Lee *et al.,* 2005). In 'Lisbon' lemon, membrane lipid characteristics were found to change at a temperature range of 7 to 15°C (Cohen *et al.*, 1994). According to Lee *et al.* (2005), about 10% or less of the membrane lipid undergoes a physical change, which is probably a separation phase in 'Eureka' lemon.

In 'Fortune' mandarin fruit, rind pitting development by light and scanning electron microscopy was observed and found that most external rind broke down in cellular

strata (Vercher *et al.*, 1994). While, low temperature impaired the physiological function of the cuticle, therefore, increased water permeability in 'Encore' mandarin fruit (Kaiser *et al.,* 2004). This was due to d-limonene, essential oils and other lipids in flavedo parenchyma released from their compartments, therefore, being toxic to the cells (Kaiser *et al.,* 2004). However, membrane lipid role during postharvest citrus rind pitting development has not been investigated to date.

2.6 Rind soluble sugars and antioxidants activity

In plants, sugars prevent desiccation by protecting membranes; and thereby, modifying the physical membrane properties to be resistant to any stress form (Crowe, 2002). Soluble sugars can stabilize membranes and phospholipid vesicles, with trehalose being the most effective (Crowe, 2002). The *D*-Glucose and *D*-Fructose (Figure 2.6 A and B) are the dominant monosaccharide sugars in citrus (Ladaniya, 2008). According to Ladaniya (2008), the combination of these two monosaccharides form a reducing disaccharide sugar (sucrose), which is the main translocation of sugars in plants and also in citrus fruit (Figure 2.7).

Carbohydrate metabolism has been linked with plant stress responses, especially in citrus flavedo (Tognetti *et al*., 1990). In 'Fortune' 'fruit mandarin sucrose has been found to be involved chilling resistance when stored at 2°C for 28 days (Holland *et al*., 2002). However, glucose, fructose and sucrose involvement in fruit affected by rind pitting has not been documented.

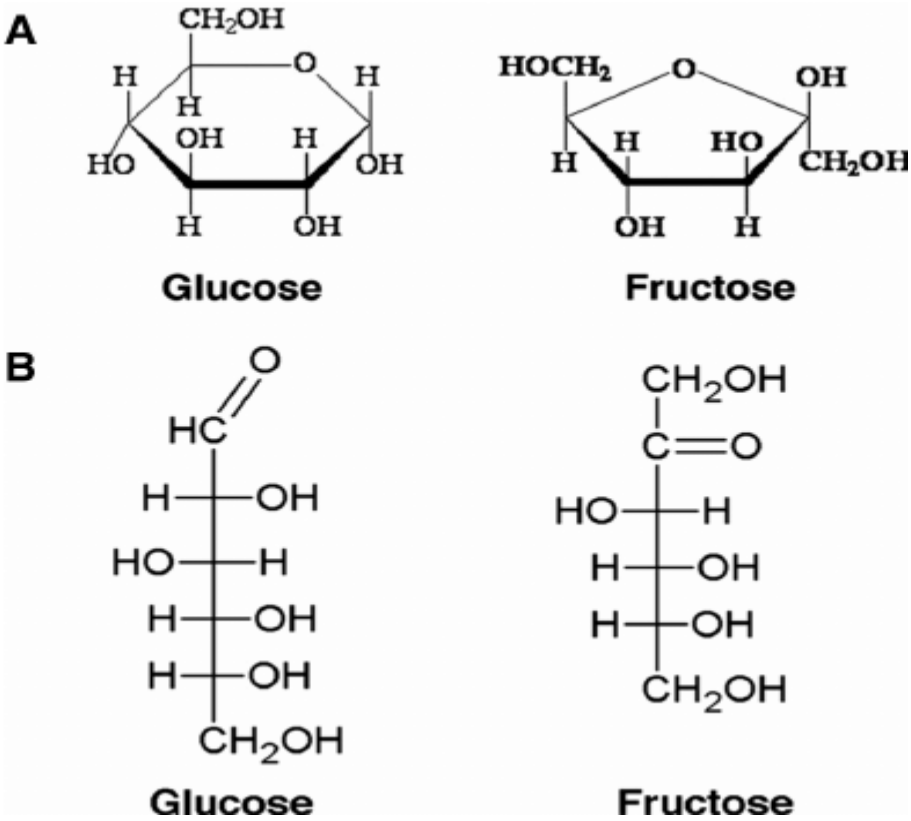

Figure 2.6: Chemical structure of sugars. A) Close chain structures of glucose and fructose. B) Open chain structures of glucose and fructose (Miller, 2009)

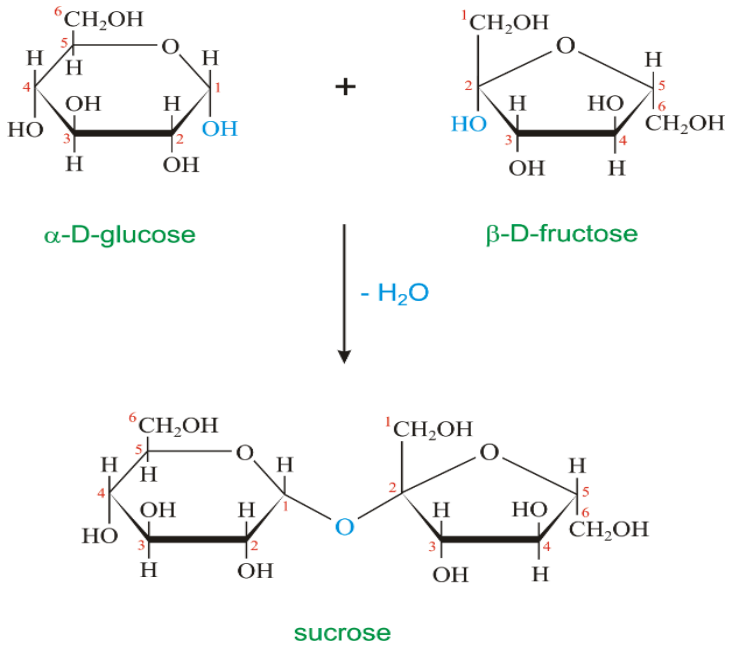

Figure 2.7: Sucrose chemical structure (Miller, 2009)

Glucose has been found to be the dominant carbohydrate in citrus flavedo and acts through a sequence of enzymatic and rate-limited reactions as the fundamental substrate in vitamin C synthesis (Aung *et al.,* 1999). Vitamin C is a water-soluble and potent antioxidant (Smirnoff, 2000) which contribute to the different citrus segments antioxidant capacity (Abeysinghe *et al.,* 2007). According to Imeh and Khokhar (2002), an antioxidant is a molecule responsible for slowing or preventing the oxidation of other molecules. Furthermore, reactive oxygen species (ROS) are detoxified by antioxidant defence systems consisting of non-enzymatic and enzymatic antioxidants.

Vitamin C plays a crucial role in plant stress physiology (Erkan *et al.,* 2005). According to Chen and Gallie (2004), ascorbate peroxidase use vitamin C to make monodehydroascorbate (MDA) in the detoxification of hydrogen peroxide by decreasing water, while ascorbate peroxidase can eliminate hydrogen peroxide. Moreover, there has been vast research on vitamin C and plant stress response such as non-chilling rind pitting (Magwaza *et al.,* 2013). The influence of citrus fruit vitamin C content has been reported. For instance, Bassal and El-Hamahmy (2011) found high vitamin C content in 'Navel' oranges in fruit with rind disorders. Similarly, vitamin C content had no effect on 'Star Ruby' grapefruit chilling injury (Chaudhary *et al.,* 2014). In contrary, Erkan *et al.* (2005) reported that 'Nules Clementine' mandarin fruit vitamin C significantly reduced as the chilling injury incidence and intensity increased.

In 'Green Star' broccoli, vitamin C increase was reduced by cold storage compared to room temperature storage (Cogo *et al*., 2011). Furthermore, in 'Navelina' orange, vitamin C, β- carotene, naringin and hesperidin concentrations varied in segments and peel over 12 days at 4°C (Plaza *et al.,* 2011). However, understanding the role

of vitamin C in fruit physiology can provide opportunities to alter its concentration in fruit; and thereby, potentially minimise rind disorders.

In response to stress, plants synthesise secondary metabolites, mainly bioactive compounds to maintain cellular disruption (Cogo *et al.,* 2011). 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. Generally, 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 (Figure 2.8). Phenolics are subdivided into flavonones and specific flavonoids, with naringin and hesperidin as dominant flavonoids in citrus (Igual *et al.,* 2011).

In citrus peel, the presence of bioactive compounds, such as phenolic compounds, and carotenoids in citrus peels have been reported by Chaudhary *et al.* (2014). These compounds are naturally occurring antioxidants that can scavenge reactive oxygen and nitrogen species, thereby, preventing oxidative damage to important biological macromolecules (Kanaze *et al.,* 2004).

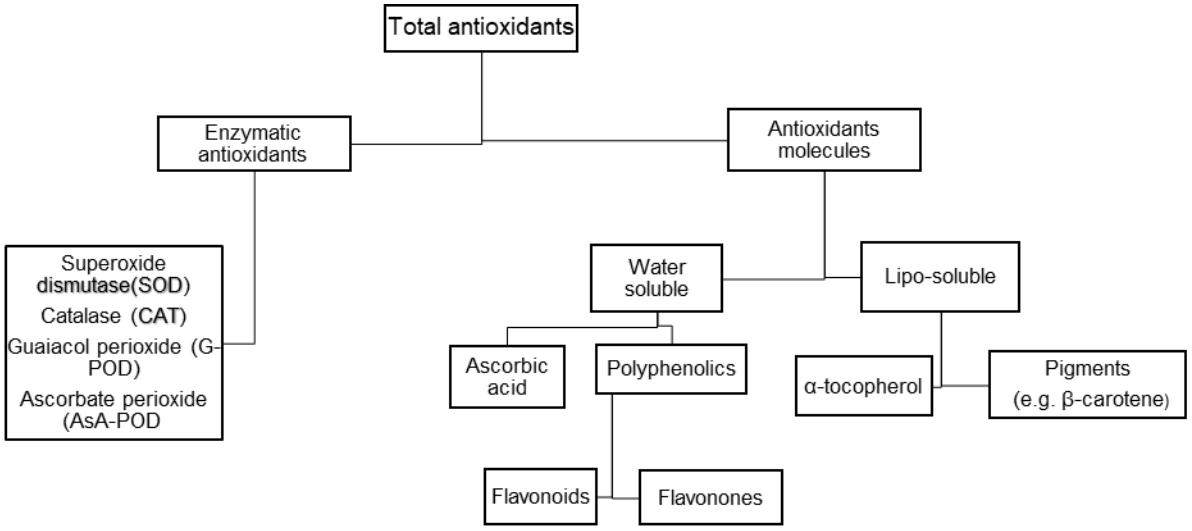

Figure 2.8: Schematic presentation of antioxidant systems in citrus fruit (Lee *et al.,* 2003)

In plants and fruits, phenolic compounds are of considerable interest and have received tremendous attention in recent years due to their bioactive functions (Lee *et al.,* 2003). There is also an increasing trend towards research in phenolic compounds present in citrus. According to Rapisarda *et al.* (2001), phenolic acids and flavonoids determine a major part of bioactive compounds for citrus fruits. However, flavonoids form a large group within phenolics family and are important contributors to antioxidant capacity. Hesperidin is the main flavonone that is present in mandarin (Erlund, 2004), orange (Kanaze *et al.,* 2004) and lemon (Gonzalez-Molina, 2010). Kanaze *et al.* (2003) found that naringin is dominant in grapefruit, giving it is the bitter flavour characteristic. Generally, naringin and hesperidin are present in small quantities (Klimczak *et al.,* 2007). However, fruits have an increased ability to induce flavonoid accumulation during cold storage (Chaudhary *et al.,* 2014). Rapisarda *et al.* (2008) found flavonoids increase under low temperatures storage in blood oranges.

Citrus fruit higher susceptibility to rind physiological disorders has been reported to be associated with various biochemical concentrations, such as lower total flavonoids, lower phenolic compounds and antioxidants (Sevillano *et al.,* 2009). In addition, phenolics are important groups of compounds which are the major constituents responsible for antioxidant capacity (Lee *et al.,* 2003). According to Lee *et al.* (2003), phenolics also have the level of fruit tolerance to rind physiological disorders.

Vitamin E is a lipid-soluble and membrane-bound compound with the role closely linked to vitamin C, playing a potent role in the defence against oxidative stress (Smirnoff, 2000). Furthermore, vitamin E concentration in the flavedo is relatively very low compared with other bioactive compounds but the compound has a high antioxidant capacity exceeding that of most phenolics and carotenoids (Robles-Sanchez *et al.,* 2009). Another lipid-soluble bioactive compound with antioxidant properties is β-carotene (Plaza *et al*., 2011). Beta-carotene is a strongly coloured red-orange pigment abundant in plants and fruits. Plaza *et al.* (2011) has previously shown that β-carotene on orange juice stored at 4°C was not affected by cold storage. However, less information has been reported on environmental factors and production site, postharvest treatments, cold storage temperature and their influence on bioactive compounds associated with 'Benny' valencia fruit rind pitting.

2.7 Quantification of antioxidant

In plants, antioxidants are part of the defence system that protects tissues against oxidative stress (Villano *et al.,* 2007). However, citrus fruit contain certain rind antioxidants that occur in different forms e.g. lipophilic and hydrophilic (Re *et al.,* 1999). In citrus fruit, oxidative stress can occur during postharvest, ultimately

resulting in rind pitting physiological disorder. However, in plant cell, the counteraction of rind physiological disorders can be enhanced by an array of antioxidants, making up a full defence mechanism against oxidative damage (Hung *et al*., 2007).

According to Mathaba *et al.* (2014), the nature of antioxidants is diverse, several assays are thereby required to quantify and estimate plant antioxidants capacity. The increase in total antioxidants assay number is due to the varying assay efficacy to estimate antioxidants in different plant tissues (Wang *et al*., 2005). In general, these various assays are used to estimate total antioxidant capacity of a certain tissue; these include the 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) assay (Pellegrini *et al*., 2003), 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Wang *et al*., 2005), ferric reducing ability of plasma (FRAP) assay (Li *et al*., 2006) and oxygen radical absorption capacity (ORAC) assay (Wang, 2003).

According to Re *et al.* (1999), antioxidant assays such as DPPH and ABTS are grouped as inhibition assays, because they donate a hydrogen or electron of a preformed free radical. They can express antioxidant capacity and some assays involve an antioxidant during generation of a radical (Re *et al*., 1999). While DPPH is a reliable assay for measuring antioxidant activities of fruits (Villano *et al*., 2007; Olarewaju *et al.,* 2017). It can donate hydrogen which is an established mechanism for anti-oxidation (Babbar *et al.,* 2011).

The ABTS assay depends on the very stable ($ABTS^{\bullet+}$) radical which is formed when ABTS is reacting with potassium persulfate to form the blue or green $ABTS^{\bullet+}$ chromophore (Pellegrini *et al.,* 2003). Several lipophilic and hydrophilic antioxidants decrease $ABTS^{\bullet+}$ to ABTS, during the time required to form ABTS depending on

incubation period and antioxidant concentration (Re *et al.,* 1999; Mathaba *et al.,* 2014). The ORAC assay is an example of antioxidant assay involving the production of a free radical (Wang *et al*., 2005). In addition, ORAC assay measures the effect of antioxidant components in fruit extracts on the decline in R phycoerythrin (R-PE) fluorescence (Wang, 2003). Common total antioxidant assays drawbacks have been summarised in Table 2.3.

Table 2.3: Main drawbacks associated with common total 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 ion. It is performed at non-physiological pH
DPPH	Other compounds may absorb at 515 nm. There may be steric hindrance for molecules with higher molecular weight. The free radical used is not present *in vivo* and is quite stable, unlike radicals present in living organisms.
ABST	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*
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 radical, present *in vivo*; however, the procedure to generate these peroxyl radicals is not physiological. Protein may have an interfering effect.

2.8 Conclusion

The development of rind pitting in citrus fruit has been under investigation for many decades, with the main aim of finding exact cause and methods of alleviating this physiological disorder at postharvest stage. It is clear from literature that 'Benny'

valencia citrus fruit are sensitive to rind pitting. In addition, ‘Benny’ valencia develop rind pitting symptoms which negatively affect fruit marketability and cause huge economic losses to the South African citrus industry. Currently, no reliable commercial method to lessen the development of rind pitting in citrus fruit exists, let alone on grapefruit and lemon fruit. Therefore, a basic understanding of the environmental factors and production sites, postharvest treatments, biochemical and molecular mechanisms involved in ‘Benny’ valencia fruit rind pitting tolerance would allow appropriate strategies to mitigate non-chilling rind pitting disorder.

CHAPTER 3

A PRELIMINARY STUDY ON THE EFFECT OF PRODUCTION SITES, POSTHARVEST TREATMENTS AND COLD STORAGE TEMPERATURES ON NON-CHILLING RIND PITTING AND PHYSICO-CHEMICAL PROPERTIES OF 'BENNY' VALENCIA CITRUS FRUIT

Abstract

In valencia citrus oranges, non-chilling rind pitting disorder has been shown to negatively affect fruit quality, however, the causes are still unknown. The study aimed to investigate effect of production sites, postharvest treatments and cold storage temperatures on non-chilling rind pitting development and physico-chemical properties of 'Benny' valencia citrus fruit. 'Benny' valencia citrus fruit were harvested from Tzaneen, Groblersdal and Musina, during the 2016 season in South Africa. Thereafter, fruit were subjected to the following treatments: T_1 = no wax and dehydration, T_2 = wax and dehydration and T_3 = wax and no dehydration; and dehydration was for 3 days at 25°C and stored at -0.6 and 4.5°C for 28 days. The treatments were replicated three times. The fruit were evaluated for rind pitting index (RPI), weight loss (WL), firmness, total electrolyte leakage (TEL), total soluble solids (TSS), titratable acidity (TA) and TSS: TA ratio. The results showed that fruit harvested from Groblersdal and exposed to T_2, had a higher RPI followed by Musina and Tzaneen with similar postharvest treatment stored -0.6 and 4.5°C. Meanwhile, fruit harvested from Groblersdal and Musina exposed to T_2 had the highest WL together with high RPI at 4.5°C whereas those from Tzaneen exposed to T_1 had the lowest WL and low RPI at -0.6°C. Storage at 4.5°C resulted in a significantly higher TEL but low RPI on fruit harvested from Tzaneen exposed to T_2. Furthermore, an increase in TSS and decreased in TA was observed after storage across all

production site, postharvest treatments and storage duration. In conclusion, production sites affected rind pitting incidence and physico-chemical properties of ‘Benny’ valenica fruit. Therefore, production site should be considered as it was shown to be responsible for the development of rind pitting.

Keywords: Electrolyte leakage, firmness, rind pitting, valencia orange, weight loss

3.1 Introduction

Citrus rind pitting is among the most important physiological disorders affecting the South African citrus industry and its profitability (Citrus Growers Association of Southern Africa, 2019). The disorder has been shown to negatively affect sweet orange (*Citrus sinensis*) rind, in turn, affecting the postharvest market value. Since the external appearance is the primary specification used to evaluate the quality of fresh citrus fruit (Alquezar *et al.,* 2010). Moreover, citrus rind pitting is responsible for heavy crop losses, in some seasons, approximately 60% of export 'Benny' valencia fruit is rejected due to rind pitting (Cronje, 2007).

The disorder is characterised by groups of collapsed oil glands forming a sunken pit in the rind (Alférez and Burns, 2004). In severe cases, pits may turn bronze in colour and later become necrotic (Cronje, 2007). The mode of action is not clear, but evidence increasingly indicates that fluctuations in relative humidity during postharvest period could contribute to disorder development (Agustí *et al.,* 2001; Alférez *et al.,* 2003). Furthermore, subsequent changes in rind water status seem to be involved in susceptibility to develop this disorder (Agusti *et al*., 2001). According to Alferez *et al.* (2005), citrus rind pitting also occurs before ripening time when fruit are exposed to low temperatures and air relative humidity.

The focus of these studies was on postharvest treatment factors such as waxing (Agustí *et al.,* 2001), relative humidity (Alférez *et al.,* 2005; Cronje, 2007), rind water status (Alférez and Burns, 2004) and mineral nutrition (Kaiser *et al.,* 2004). However, there is limited information on production site and specific postharvest treatments such as waxing, dehydration stress and cold storage on 'Benny' valencia orange susceptibility to non-rind pitting. Therefore, this study aimed to investigate production

sites, postharvest treatments and cold storage temperatures effect on non-rind pitting incidence and ‘Benny’ valencia citrus fruit physico-chemical attributes.

3.2 Materials and Methods

3.2.1 Fruit sources

During the 2016 season, ‘Benny’ valencia citrus fruit were harvested from commercial farms in Tzaneen (23°88′36″S, 30°82′34″E), Groblersdal (25°02′05″S, 29°21′56″E) and Musina (22°20′17″S, 30°02′30″E), in Limpopo Province, South Africa. **One season data was used in this study to preliminarily profile the susceptible of ‘Benny’ valencia citrus fruit to non-chilling rind pitting as explained in the literature review.** Fruit were harvested from June to July at commercial maturity. The rainfall, minimum and maximum relative humidity (RH), minimum and maximum temperature and vapour pressure deficit (VPD) registered during the growing seasons were recorded and presented in Tables 3.1, 3.2 and 3.3 for Tzaneen, Musina and Groblersdal, respectively.

3.2.2 Postharvest treatments

After harvesting, ‘Benny’ valencia fruit were transported at ambient temperature in ventilated crates to the Agricultural Research Council - Tropical and Subtropical Crops postharvest laboratory in Nelspruit (25°45′18″S, 30°96′97″E) for treatment, storage and analysis. Upon arrival, fruit were washed and subjected to the following treatments: T_1 = no wax and dehydration, T_2 = wax and dehydration and T_3 = wax and no dehydration (control); 30 fruit were used in each treatment and replicated three times. Thereafter, fruit were waxed manually with Citrashine and dehydrated for 3 days at 25°C using air conditioner (Model: GZ-50GB-E1, Maclaren, South Africa) before storage (Alférez *et al.,* 2003). After dehydration, fruit were stored at -0.6 and 4.5°C, 90 ± 3% RH for 28 days.

Table 3.1: Climatic data during the growing seasons in Musina

2016 season	Jan	Feb	Mar	Apr	May	June	July
Total Rainfall (mm)	400	340	270	200	100	0.0	0.0
Daily minimum temperature (ºC)	20.1	18.3	19.2	17.1	18.2	17.1	16.1
Daily maximum temperature (ºC)	33.8	34.2	33.1	30.7	30.1	28.3	29.4
Daily minimum relative humidity (%)	41.8	39.2	42.1	32.0	36.7	56.9	50.2
Daily maximum relative humidity (%)	91.6	72.3	83.1	83.5	82.2	82.4	83.2
Vapour pressure deficit (kpa)	0.3	0.6	0.3	0.5	0.4	0.4	0.3

Table 3.2: Climatic data during the growing seasons in Tzaneen

2016 season	Jan	Feb	Mar	Apr	May	June	July
Total Rainfall (mm)	378	280	256	180	0.0	0.0	0.0
Daily minimum temperature (ºC)	20.2	20.5	19.1	15.9	11.2	9.1	7.2
Daily maximum temperature (ºC)	30.1	31.2	31.4	30.2	25.9	25.8	24.9
Daily minimum relative humidity (%)	60.8	38.8	41.6	39.8	39.5	33.8	31.6
Daily maximum relative humidity (%)	92.2	84.9	89.6	90.2	92.8	91.1	91.5
Vapour pressure deficit (kpa)	0.1	0.5	0.2	0.8	0.8	0.4	0.3

Table 3.3: Climatic data during the growing seasons in Groblersdal

2016 season	Jan	Feb	Mar	Apr	May	June	July
Total Rainfall (mm)	278	250	269	240	0.0	0.0	0.0
Daily minimum temperature (ºC)	18.7	19.1	17.2	13.3	8.2	4.5	5.8
Daily maximum temperature (ºC)	32.1	32.1	31.1	30.8	25.4	23.7	23.1
Daily minimum relative humidity (%)	32.7	33.9	37.5	33.3	35.3	32.7	27.1
Daily maximum relative humidity (%)	91.6	82.9	84.6	85.4	87.3	84.9	80.8
Vapour pressure deficit (kpa)	0.7	0.7	0.8	0.7	0.5	0.5	0.2

3.2.3 Determination of rind pitting incidence (RPI)

Fruit were evaluated for pitting after 28 days of cold storage plus 7 days shelf-life. Briefly, fruit were rated on a 4-point hedonic scale scale: 0 (no pits), 1 (1 - 10% = less pitting), 2 (10 - 20% = moderate pitting) and 3 (20 - 50% = severe pitting). The rind pitting incidence (RPI) was calculated according to the formula previously reported by Alférez *et al.* (2003):

$$RPI = \frac{\sum \text{rind pitting scale (0-3) x number of fruit in each class}}{\text{total number of fruit}}$$

3.2.4 Weight loss

Fruit weight was measured using a weighing balance (Model: SBA 16, Scaltec instruments, Germany). Fruit weight loss was expressed as the difference in fruit initial weight before (W_0) and after storage (W_1). After storage, fruit of known weight before storage were reweighed to calculate weight loss using the equation: $WL = \frac{W0-W1}{W0}$ x 100. Where *WL* is the weight loss percentage.

3.2.5 Firmness

Firmness was determined before (at harvest) and after 28 days of storage using Sinclair firmness meter (Model: IQTM, Sinclair International, Norwich, United Kingdom). Ten fruits from each treatment were sampled and used for determination of firmness. From each fruit, firmness was measured from two sides with Sinclair firmness meter and the average was calculated, afterwards the results were recorded in mm units.

3.2.6 Electrolyte leakage

Membrane permeability was measured using electrolyte leakage according to Cohen *et al.* (1994). Three fruit from each treatment were sampled and used for determination of electrolyte leakage. Three flavedo discs from each fruit, with a diameter of one centimetre were cut and immersed in a test tube containing 10 ml de-ionized water. Prior to this, the peels were washed three times to eliminate the electrolyte leakage at the cut surface and prevent surface contamination. The first electrical conductivity (EC_1) was measured after shaking the sample for 3 hours, with an electrical conductivity meter (Model: HI 9033, Hanna instruments, Berlin, Germany). The second electrical conductivity (EC_2) was measured after the samples were placed in a hot water bath controlled at 100°C for 1 hour and allowed to cool at

room temperature. Electrolyte leakage was expressed in percentage using the formula:

Total electrolyte leakage (%) = (EC_1/ EC_2) x 100

3.2.7 Biochemical attributes

Total soluble solids (TSS) and titratable acidity (TA)

The TSS and TA were measured from 10 fruit per treatment before (at harvest) and after 28 days of cold storage. The TSS was measured using digital refractometer (Model: Atago PR-1, Atago Co, Ltd., Tokyo, Japan) and recorded as °Brix. Titratable acidity content was determined by titrating 0.1 N NaOH solution into 10 ml of fruit juice mixed with 3 drops of Phenolphthalein indicator. Titration was complete when the liquid turned pink in colour. The result was therefore converted into g/100 g of citric acid by the equation: Titratable acid content = (ml NaOH/ 10 ml) x (0.1 N NaOH/ 0.1562). TSS: TA was calculated as a ratio between TSS and TA (Mphahlele *et al*., 2016).

3.2.8 Statistical analysis

Experiment was laid out as a factorial design arrangement with the following factors: production sites (Tzaneen, Musina and Groblersdal); postharvest treatments (no wax plus dehydration, wax plus dehydration and wax plus no dehydration) and cold storage temperatures (-0.6 and 4.5°C). The data was subjected to analysis of variance by using Genstat version 18th software (VSN International, Hemel Hempstead, UK). The effects of production sites, postharvest treatments, storage duration and their interaction effects were determined using analysis of variance (ANOVA). Where significant differences were detected, means were separated using Duncan New Multiple Range Test (DNMRT) at 5% level of significance.

3.3 Results

3.3.1 Rind pitting incidence (RPI)

'Benny' valencia RPI of fruit harvested from different production sites, which were exposed to different wax plus dehydration postharvest treatments and stored at various temperatures, is presented in Figure 3.1. In this study, production site, wax plus dehydration treatments and storage temperature had a significant (P=0.0307) effect on the rind pitting incidence. Fruit harvested from Groblersdal and exposed to T_2 and stored at -0.6° had high (1.8) RPI. Low RPI was observed in fruit harvested from Tzaneen exposed to T_3 (0) and stored at both 4.5 and -0.6°C. In addition, RPI was low on fruit harvested from Musina exposed to T_1 (0) and T_3 (0) and stored at 4.5°C.

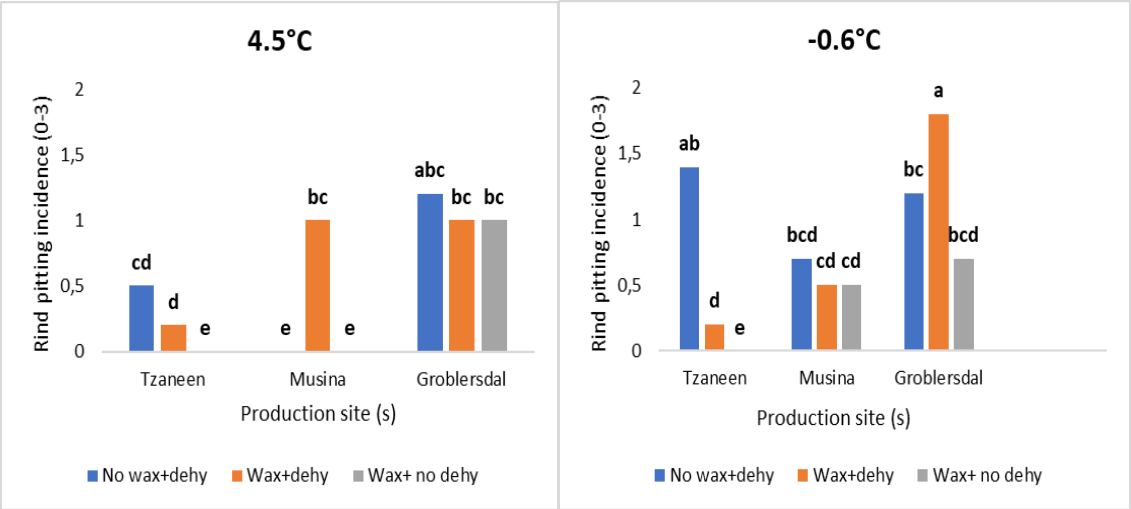

Figure 3.1: Effect of production site and postharvest treatments on rind pitting incidence of 'Benny' valencia fruit stored at 4.5 and -0.6°C. Mean values followed by different letter(s) within same graph are significantly different (P<0.05)

3.3.2 Total electrolyte leakage percentage (TEL)

In this study, the effect of production site and wax plus dehydration treatments on TEL (P=0.0066) is shown (Table 3.4). Low TEL was observed on fruit harvested from Tzaneen (29.5%) and Musina (32.9%) exposed to T_2 and stored at 4.5°C. In addition, low (32.9%) TEL was also observed in fruit harvested from Tzaneen exposed to T_2 and stored at -0.6°C. While, high TEL was observed in fruit harvested from Tzaneen exposed to T_3 (71.4%) and stored at 4.5°C.

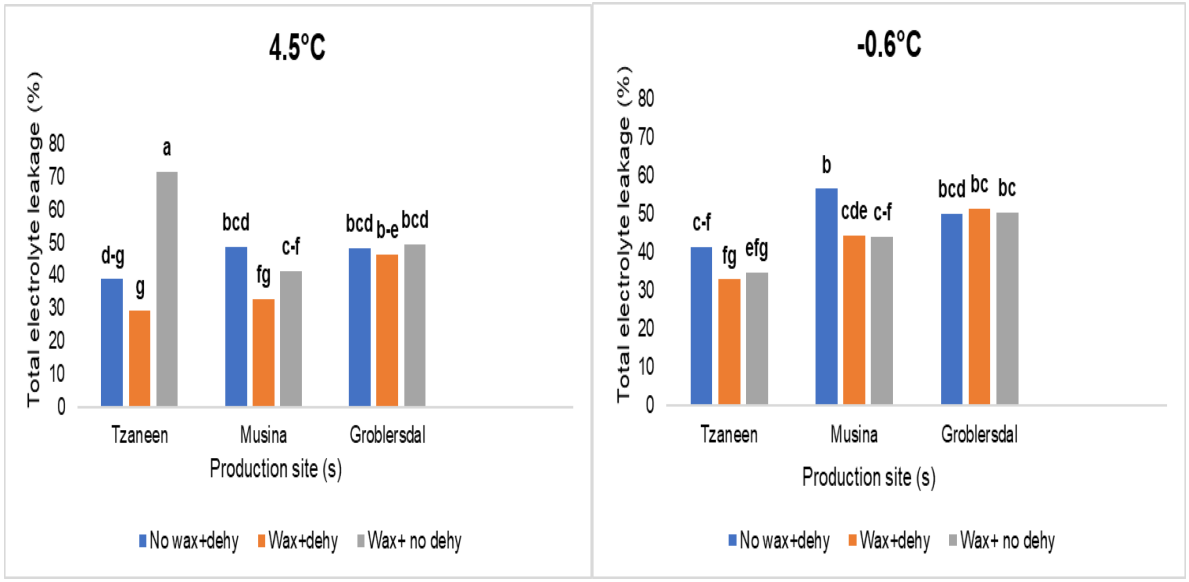

Figure 3.2: Effect of production site and postharvest treatments on total electrolyte leakage of 'Benny' valencia fruit stored at 4.5 and -0.6°C. Mean values followed by different letter(s) within same graph are significantly different (P<0.05)

3.3.3 Weight loss (WL)

Effect of production site, postharvest treatments were significant on WL but their interaction was not significant (P=0.0272) (Figure 3.4). However, weight loss was higher (5.2 and 4.2%) in fruit harvested from Musina exposed to T_1 at both storage temperatures. Low WL (0%) was observed in fruit exposed to T_3 in all production sites irrespective of storage temperature.

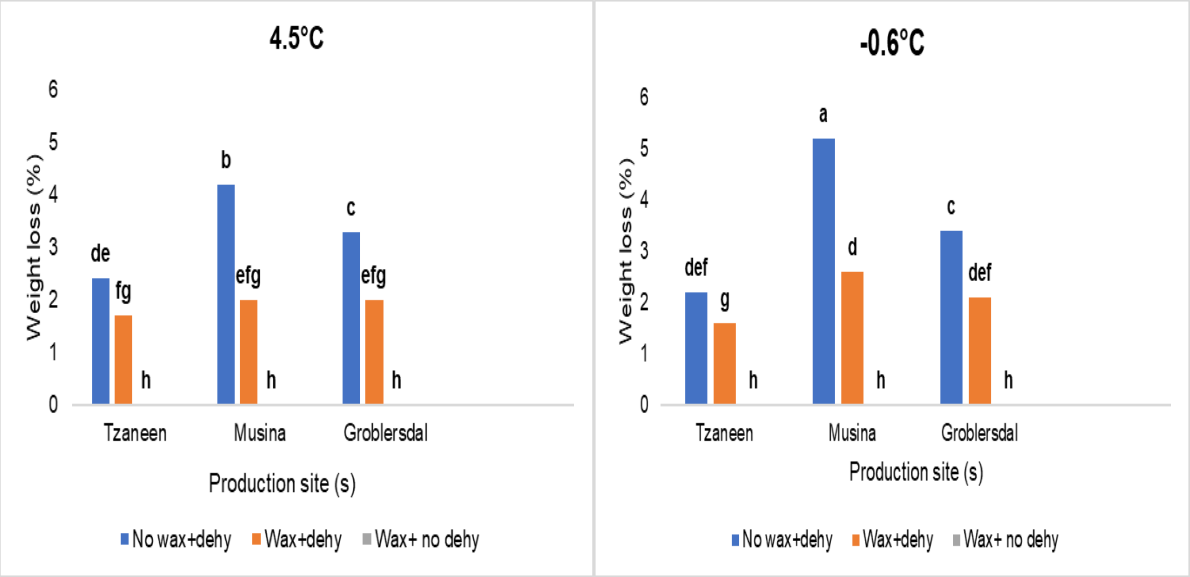

Figure 3.3: Effect of production site and postharvest treatments on weight loss of 'Benny' valencia fruit stored at 4.5 and -0.6°C. Mean values followed by different letter(s) within same graph are significantly different (P<0.05)

3.3.4 Firmness

Interactive effect of production sites, postharvest treatments, storage temperature and duration was not significant (P=0.5530). However, production sites and postharvest treatments had high significant effect (P=0.0002) on firmness. Fruit harvested from Groblersdal (20.1, 19.9 and 19.6%) showed high firmness, irrespective of postharvest treatments (Figure 3.4). Low firmness was observed in fruit harvested from Tzaneen (17.3%) and Musina (17.6%) treated with T_1. Furthermore, production sites and storage duration interaction was highly significant (P<0.0001) on firmness. High firmness was detected in fruit harvested from Groblersdal (24.1%) before storage (Figure 3.5). However, fruit harvested from Musina (15.0%) and Groblersdal (15.6%) had low firmness after storage. In addition, duration and postharvest treatments interaction also had a significant effect (P=0.0018) on firmness. Firmness was high before storage whereas low firmness

was observed after 28 days of storage irrespective of postharvest treatments (Figure 3.6).

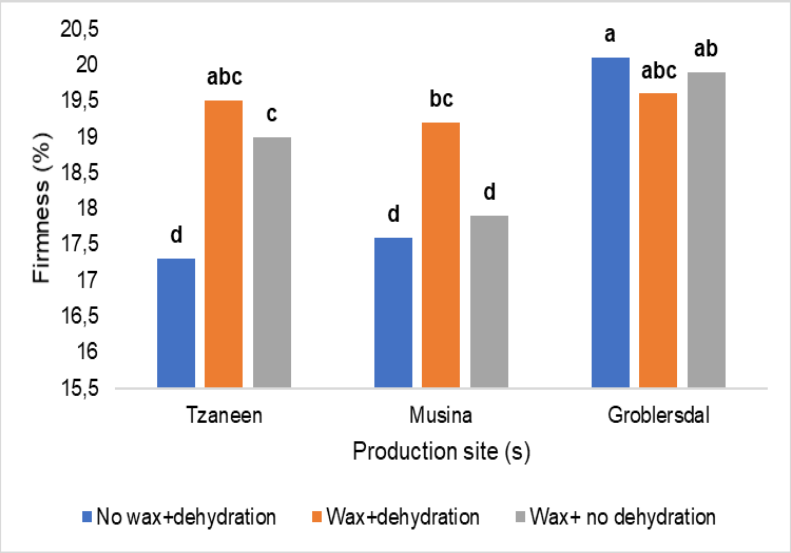

Figure 3.4: Effect of production sites and postharvest treatments on firmness of 'Benny' valencia fruit. Mean values followed by different letter(s) within same graph are significantly different (P<0.05)

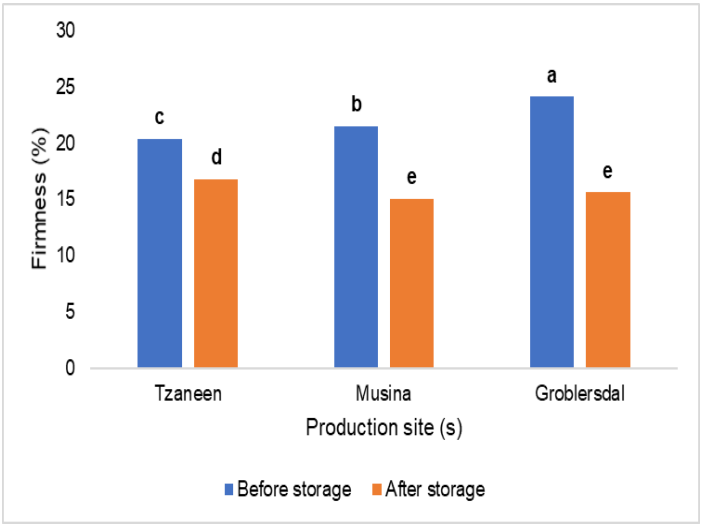

Figure 3.5: Effect of production sites and storage duration on firmness of 'Benny' valencia fruit. Mean values followed by different letter(s) within same graph are significantly different (P<0.05)

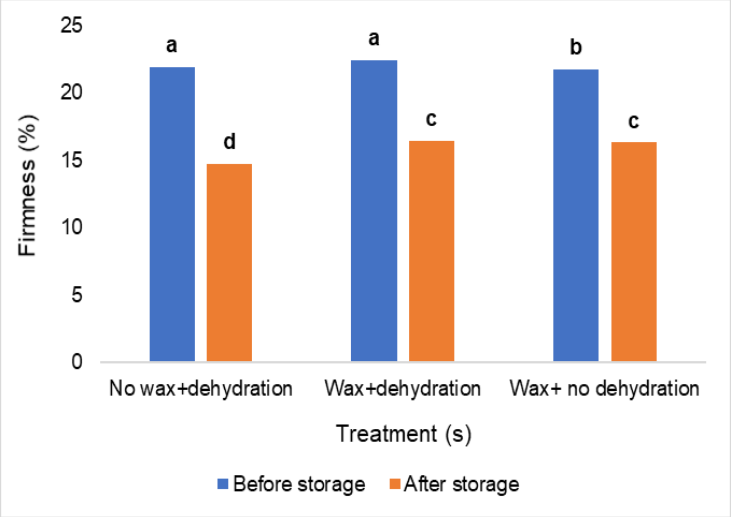

Figure 3.6: Effect of postharvest treatments and storage duration on firmness of 'Benny' valencia fruit. Mean values followed by different letter(s) within same graph are significantly different (P<0.05)

3.3.5 Biochemical properties (TSS, TA and TSS: TA ratio)

Changes in biochemical properties of fruit harvested from different production sites as influenced by various treatments and cold storage temperature are presented in Table 3.6. The production site, postharvest treatments and storage duration interaction had no a significant effect (P=0.7382) on the TSS content. However, the interaction between production sites and cold storage temperature was significant (P=0.0282) on TSS content. It is worth noting that fruit harvested from Tzaneen (11.1°Brix) and stored at -0.6°C were characterised by the highest TSS content compared to Groblersdal and Musina fruit (Figure 3.7). However, fruit harvested from Musina (9.1°Brix) and stored at 4.5°C were characterised by low TSS content. In addition, production sites and duration interaction was significant (P=0.0399) on TSS

content. High TSS content was observed in fruit harvested from Tzaneen (12.1°Brix) after storage whereas low TSS was observed in fruit harvested from Musina (9.6°Brix) before storage (Figure 3.8).

Postharvest treatments and storage duration also had a significant effect (P=0.0026) on TSS content. Fruit treated with T_1 h (10.4°Brix) had higher TSS content after storage while low TSS content was observed in fruit treated with T_3 (7.8°Brix) before storage (Figure 3.9).

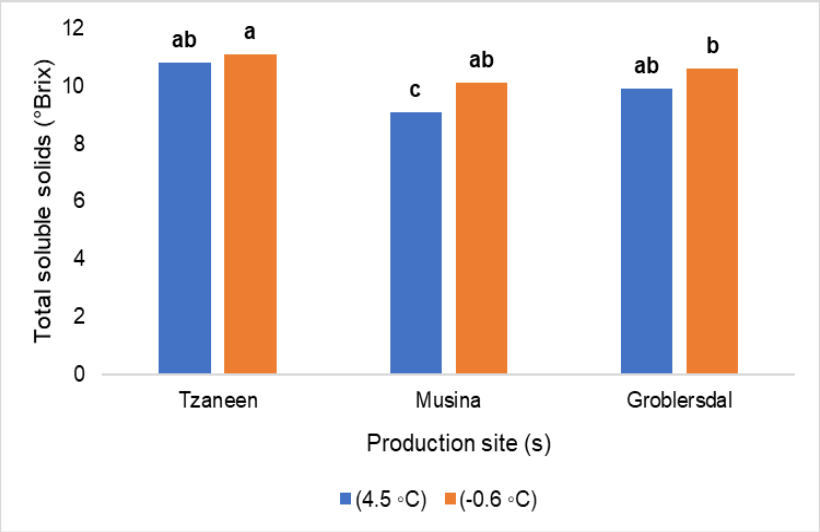

Figure 3.7: Effect of production sites and cold storage temperature on TSS of 'Benny' valencia fruit. Mean values followed by different letter(s) within same graph are significantly different (P<0.05)

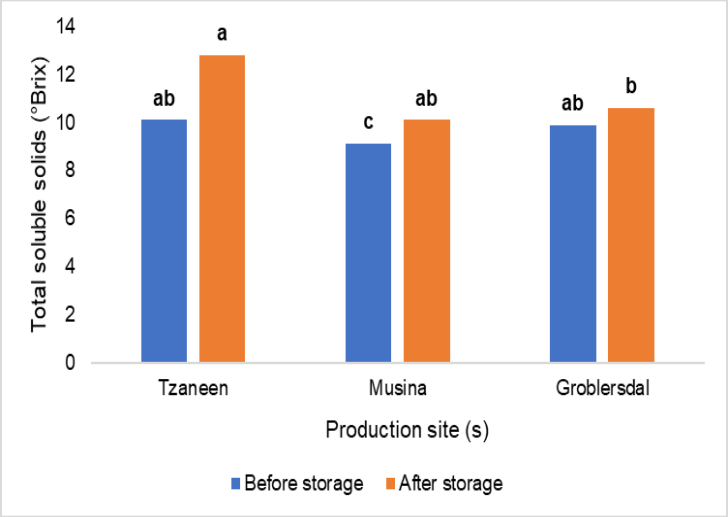

Figure 3.8: Effect of production sites and storage duration on TSS of 'Benny' valencia fruit. Mean values followed by different letter(s) within same graph are significantly different (P<0.05)

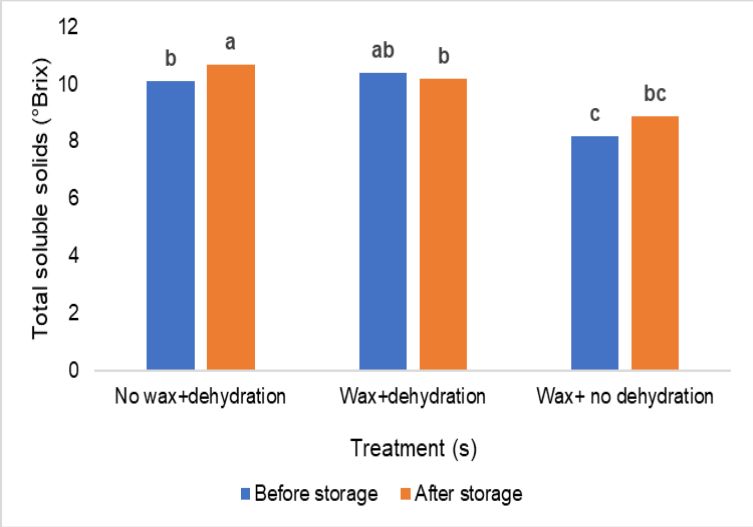

Figure 3.9: Effect of postharvest treatments and storage duration on TSS of 'Benny' valencia fruit. Mean values followed by different letter(s) within same graph are significantly different (P<0.05)

The effects of the production site, postharvest treatments cold storage temperature and storage duration interaction was not significant (P=0.7151) on TA. However, the interaction between production sites, storage duration and postharvest treatments

had a significant effect (P=0.0005) on TA. A higher TA was observed in fruit harvested from Groblersdal than fruit from Musina and Tzaneen regardless of postharvest treatments and storage duration (Figure 3.10). Before storage, low TA was observed in fruit harvested from Tzaneen, regardless of postharvest treatments. However, after storage; low TA was observed in Tzaneen and Musina fruit regardless of postharvest treatments. In addition, postharvest treatments and storage duration on had high significant (P<0.0001) effect on TA. In general, fruit harvested from Groblersdal had high TA irrespective of storage duration (Figure 3.11). Low TA was observed in fruit harvested from Tzaneen and Musina after storage.

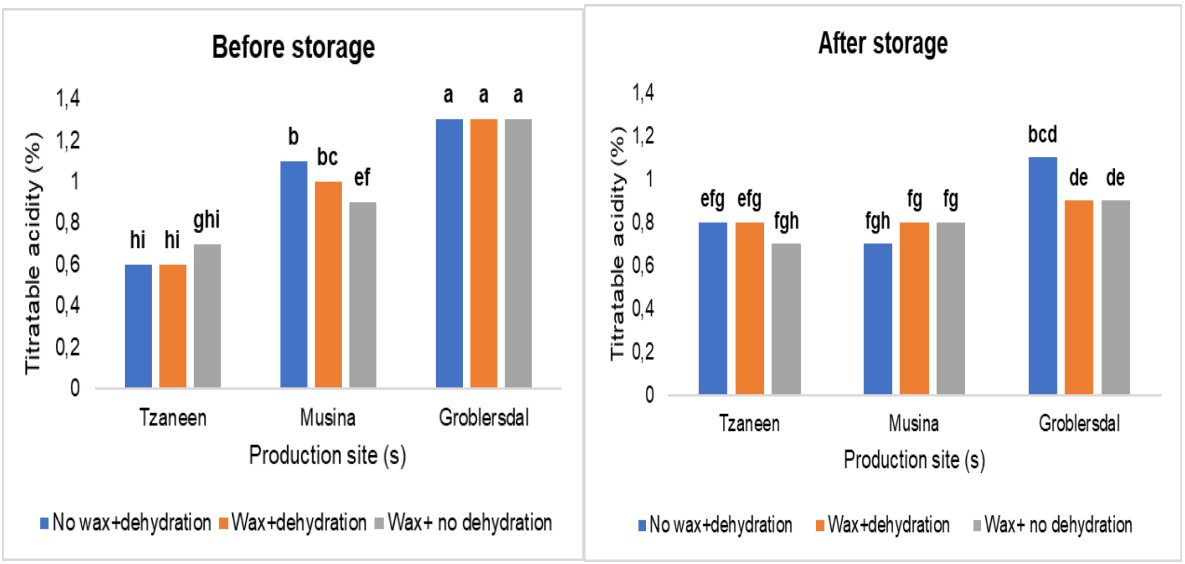

Figure 3.10: Effect of production sites, postharvest treatments and duration on TA of 'Benny' valencia fruit. Mean values followed by different letter(s) within same graph are significantly different (P<0.05)

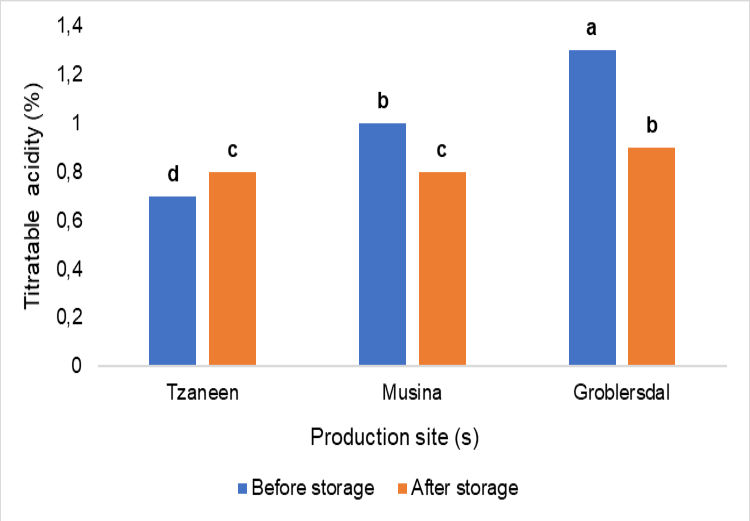

Figure 3.11: Effect of postharvest treatments and storage duration on TA of 'Benny' valencia fruit. Mean values followed by different letter(s) within same graph are significantly different (P<0.05)

The interaction effect of the production site, postharvest treatments and storage temperature and duration was not significant (P=0.1323) on TSS: TA ratio. However, production sites and storage duration had a high significant effect (P<0.0001) on TSS: TA ratio. In general, fruit harvested from Tzaneen had higher TSS: TA ratio before and after storage than fruit from Musina and Groblersdal (Figure 3.12). Low TSS: TA ratio was observed in fruit harvested from Groblersdal at both storage durations. Additionally, the interactive effect between production sites, postharvest treatments and storage duration was highly significant (P<0.0001) on TSS: TA ratio. Fruit harvested from Tzaneen had higher TSS: TA ratio before storage when compared with fruit from Musina and Groblersdal (Figure 3.13). Low TSS: TA ratio was observed in fruit harvested from Groblersdal. After storage, high TSS: TA ratio was observed in fruit harvested from Tzaneen and Groblersdal. However, low TSS: TA ratio was observed in fruit harvested from.

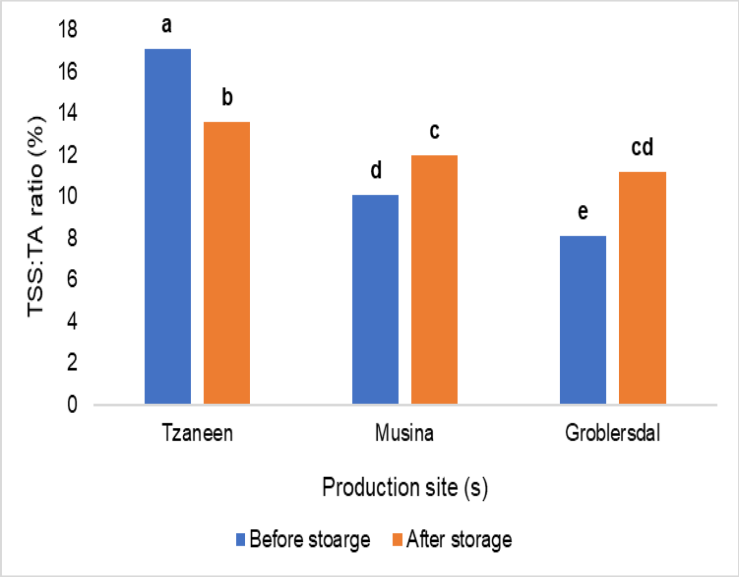

Figure 3.12: Effect of production sites and storage duration on TSS:TA ratio of 'Benny' valencia fruit. Mean values followed by different letter(s) within same graph are significantly different (P<0.05)

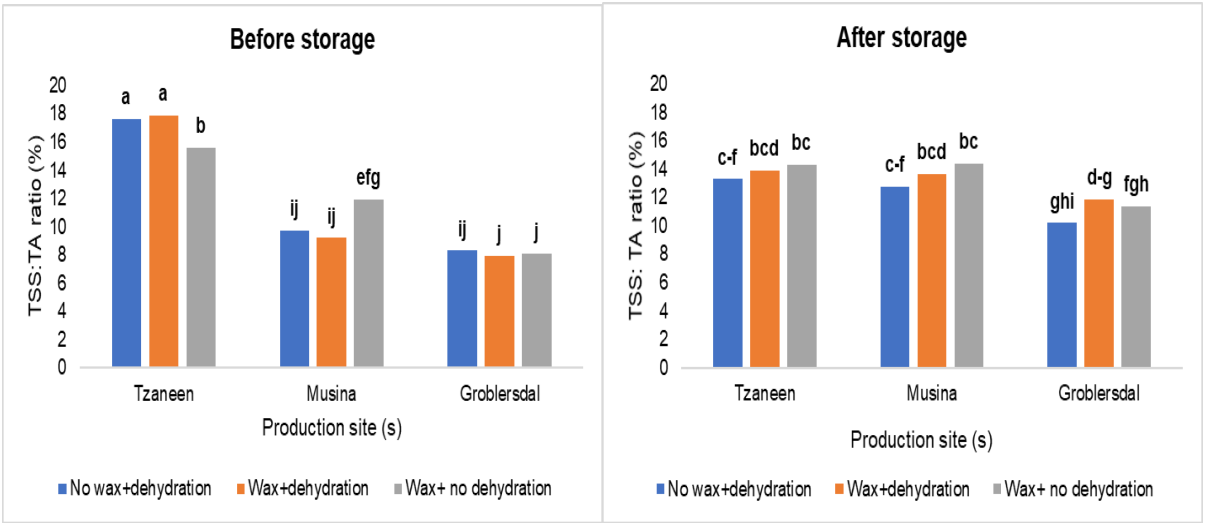

Figure 3.13: Effect of production sites, postharvest treatments and storage duration on TSS: TA ratio of 'Benny' valencia fruit. Mean values followed by different letter(s) within same graph are significantly different (P<0.05)

3.4 Discussion

3.4.1 Effect production site, postharvest treatments and cold storage temperature on 'Benny' valencia citrus fruit rind pitting incidence

In citrus fruit, rind disorders have been related to ambient environmental conditions, especially temperature and humidity (Table 3.1, 3.2 and 3.3). Furthermore, environmental factors such as temperature, humidity and rainfall are critical in controlling rind pitting rate (Gonzalez-Aguilar *et al.*, 2000). However, the growing site is considered as the main factor affecting fruit susceptibility to rind pitting (Olarewaju *et al*., 2017). In this study, fruit harvested from Groblersdal exposed to T_2 and stored at -0.6° had high RPI. Highest rind pitting index observed could be attributed to dehydration stress coupled with low relative humidity, subsequently causing flavedo and albedo cell layers collapse, which resulted in rind pitting development as reported by Agusti *et al.* (2001) and Alférez *et al. (*2005). Fruit resistance to develop peel damage by fluctuation of storage relative humidity depends on peel flavedo and albedo cells morphology and structure features (Dou, 2005).

Low RPI was observed in fruit harvested from Tzaneen exposed to T_3 and stored at both 4.5 and -0.6°C. In addition, RPI was low on fruit harvested from Musina exposed to T_1 and T_3 and stored at 4.5°C. The results from this study were in contrast with Alférez *et al.* (2005), whereby, wax application did not significantly increased rind-pitting incidence. Therefore, wax could not be regarded as the primary cause of rind pitting, however it can enhance the disorder.

3.4.2 Effect production site, postharvest treatments and cold storage temperature on 'Benny' valencia citrus fruit total electrolyte leakage

In this study, fruit harvested from Musina treated with T_2 had low TEL. This could be attributed to high environmental temperature in Musina, which resulted in low cell membrane damage (Table 3.2). Similar results were observed by Siboza and Bertling (2013), who found that electrolyte leakage in 'Eureka' lemon flavedo was influenced by treatments, cold storage temperature and different farm locations. Generally, low storage temperature is a major factor that increases fruit membrane permeability and the subsequent ion leakage rate (Saltveit, 2000). In addition, Gonzalez-Aguilar *et al.* (2000) reported that electrolyte leakage was an excellent indicator of cell membrane damage resulting from postharvest storage temperature stress. However, in this study, high TEL was observed in fruit harvested from Tzaneen exposed to T_2 and stored at 4.5°C for 28 days. In addition, high electrolyte leakage on 'Blood Red' sweet orange stored for 30 days at low temperature was associated with increased membrane permeability (Hassan *et al*., 2014). Cold storage temperature induces lipid phase transition from a more flexible liquid to crystalline structure to a solid gel (Hordjik, 2013). According to Lyons (1973), when gel-phase coexists, the lipids do not pack well, causing cracks and result into high solutes observed in fruit stored at -0.6°C.

3.4.3 Effect of production site, postharvest treatments and cold storage temperature on 'Benny' valencia citrus fruit weight loss percentage

After storage, fruit continue to respire and loss water to the surroundings environment due to transpiration (Rab *et al.,* 2015). According to Hung *et al.* (2011) 5-6% of weight loss during long-term storage of oranges and mandarin could result in fruit having a shrivelled appearance rendering them unmarketable. The primary factors affecting weight loss are relative humidity and storage temperature (Maguire *et al*., 2001; Jourbert, 2016). For instance, in this study, weight loss was higher in fruit harvested from Musina exposed to T_1 at both storage temperatures. Similar results were found by Gonzalez-Aguilar *et al.* (2000) whereby, 'Fortune' mandarin fruit cold stored at 12°C with 80-90% RH for 45 days showed higher weight loss of 2-15% compared to 2-5% lost at 2°C. This could be to attributed to high respiration and transpiration rate as a result of storing fruit under high temperatures (Alférez *et al*., 2005; Rab *et al*., 2015). Furthermore, high weight loss was due to high field temperature at harvest in Musina (Table 3.1) than Tzaneen (Table 3.2) and Groblersdal (Table 3.3). The ability of citrus fruit to continue losing water during postharvest storage depends on the amount of water present in the fruit at harvest (Hung *et al*., 2011).

3.4.4 Effect production site, postharvest treatments and cold storage temperature on 'Benny' valencia citrus fruit firmness

According to Ladaniya (2008), firmness has been used widely as a quality index for fruit as it relates to products physiological maturity, freshness and bruising extent, texture, compression and damage. Production site was also a primary factor that influenced fruit firmness. For instance, fruit harvested from Groblersdal showed high

firmness before storage and firmness declined after storage. Mothapo *et al*. (2018) found similar results whereby, firmness declined after 28 days of storage in 'Turkey' sweet orange fruit harvested from Tzaneen, Musina and Groblersdal. This was attributed to environmental factors, especially, temperature (Table 3.1, 3.2 and 3.3) which reduced fruit firmness. In addition, firmness declined due to pectin depolymerization in the cell wall, which culminates in dissolution of pectins (Silva *et al.,* 2009).

Several studies have previously shown that changes in fruit firmness properties were primarily due to storage temperature and duration (El-Hilali *et al.,* 2003; Ali *et al*., 2004; Khorshidi *et al.,* 2010) and to a minor extent, due to relatively humidity. In this study, postharvest treatments and duration interaction also had a significant effect on firmness. The decline in fruit firmness after storage was due to high weight loss, which was enhanced by high water loss and cell wall degradation (Ali *et al.,* 2004). In this study, results obtained were in agreement with those of Shin *et al.* (2008), whereby, a decreased firmness in 'Jewel' strawberry fruit stored at 10°C was also recorded. Furthermore, decreased firmness was observed on untreated fruit when compared to coated fruit in 'Giant Kew' pineapple fruit stored at 5°C (Hu *et al.,* 2011).

3.4.5 Effect of production site, postharvest treatments and cold storage temperature on 'Benny' valencia citrus fruit biochemical properties (TSS, TA and TSS: TA ratio)

Total soluble solids and titratable acids chemical parameters had been used to describe taste with regards to the sweetness and acidity (Ladaniya, 2008). According to Lado *et al.* (2014), TSS and TA are likely influenced by growing regions. In this study, production site was a major factor influencing TSS, TA and TSS: TA ratio. For

instance, high TSS, low TA and high TSS: TA ratio was observed in fruit harvested from Tzaneen when compared with Groblersdal and Musina. These results are in agreement with the findings of Mothapo *et al.* (2018) who found high TSS, low TA and high TSS: TA ratio in 'Turkey' fruit harvested from Tzaneen than Groblersdal and Musina. This was attributed to high relative humidity in Tzaneen production site (Table 3.2). The increased TSS, decreased TA and increased TSS: TA ratio might be possible due to conversion of complex starch or carbohydrate into simple compound at high relative humidity.

Cold storage temperature reduces the rate of metabolic activities such as, respiration; and therefore, making the fruit less acidic and acceptable for consumption (Hussain *et al*., 2015). In this study, cold storage temperature and duration also affected TSS content. Fruit stored at a higher temperature (4.5°C) had the highest TSS and TSS: TA ratio, while TA was low when compared to those stored at lower temperature (-0.6°C). Similar findings were reported found by Tietel *et al*., (2012), whereby, TSS was high in 'Or' and 'Odem' mandarin cultivars after storage at 2, 5 and 8°C for 4 weeks while acidity significantly decreased resulting in a higher TSS: TA ratio. The TSS: TA ratio is one of the most important factors influencing the taste, and determining fruit harvest time (Chaudhary *et al.,* 2017). During citrus fruit growth and development, sugars accumulated meanwhile acidity levels declined in the flesh (El-otmani *et al.,* 2011). Hence, determining different sugar/acidity ratios during ripening (El-otmani *et al.,* 2011). Furthermore, TSS levels have been found to increase and acidity to decline in citrus fruits during cold storage (Grierson and Ben-Yehoshua, 1986).

Citric acid has been previously reported to decrease during storage, therefore, resulting in decreased TA in stored citrus fruits (Grierson and Ben-Yehoshua, 1986).

The decline in TA was due to organic acids being used for energy production and alcoholic fermentation (Echeverria and Valich, 1989). However, TSS increase due to the *De novo* synthesis of organic acids, this mechanism is involved in the increase of sugar levels in citrus fruits during cold storage (Echeverria and Valich, 1989). In this study, increased TSS and lower TA could be attributed to a mechanism known as *De novo* synthesis of organic acids as previously suggested by Echeverria and Valich (1989).

3.5 Conclusion

The study showed that production site had an influence on the physico-chemical properties and rind pitting. Fruit harvested from Groblersdal exposed to (T_2) had the highest incidence of rind pitting compared to those from Tzaneen and Musina, irrespective of postharvest treatments when stored at chilling temperature (-0.6°C). The results from the present study suggest that the production site should be considered as it was shown to be responsible for the development of rind pitting. Moreover, exposing fruit to dehydration stress at low RH (45%) is a key factor leading to the occurrence of rind pitting incidence during storage. Therefore, during postharvest handling, waxing fruit could be important as it reduces water loss and occurrence of rind disorders, hence maintaining the fruit quality.

CHAPTER 4

ROLE OF PRODUCTION SITES, POSTHARVEST TREATMENTS AND COLD STORAGE TEMPERATURES ON RIND BIOCHEMICAL CONCENTRATIONS ASSOCIATED WITH 'BENNY' VALENCIA CITRUS FRUIT NON-CHILLING RIND PITTING

Abstract

In citrus fruits, non-chilling rind pitting remains an important physiological disorder as it affects external quality; and, therefore, the value of fresh market fruit. Although the cause of this disorder is unknown, rind biochemical concentrations have been hypothesised to play a significant role in the susceptibility of citrus fruit to various forms of rind disorders. Therefore, this study aimed at investigating the role production sites and postharvest treatments and cold storage temperatures on rind biochemical concentrations in relation to non-chilling rind pitting of 'Benny' valencia citrus fruit. During 2016 and 2017 seasons, 'Benny' valencia citrus fruit were harvested, sorted and graded, thereafter subjected to the following treatments: T_1 = no wax plus dehydration, T_2 = wax plus dehydration and T_3 = wax plus no dehydration. Dehydrated treatments were applied for 3 days at relative humidity of ±45%, thereafter, fruit were stored at -0.6 and 4.5°C for 28 days plus 7 days shelf-life. After withdrawal from cold storage plus 7 days shelf-life, fruit were analysed for rind pitting incidence (RPI). After RPI analysis, the flavedo was removed, freeze dried, thereafter, analysed for total phenolic concentrations (TPC), total flavonoid concentrations (TFC), vitamin C and radical scavenging activity (RSA). The results showed that rind pitting incidence was high on fruit subjected to wax plus no dehydration across all storage temperatures and production sites. However, fruit

sourced from Tzaneen had significantly ($p < 0.001$) high TPC, therefore, low rind pitting incidence TFC, irrespective of postharvest treatments. While rind vitamin C was higher in fruit from Groblersdal when compared with Tzaneen and Musina, however, low RPI was also observed in fruit sourced from Groblersdal. Moreover, fruit from Musina subjected to wax plus dehydration treatment had higher RSA and low RPI at both temperatures when compared with fruit sourced from Groblersdal and Tzaneen. Therefore, wax plus dehydration treatment resulted in low rind pitting with an increased accumulation of rind biochemical concentrations, irrespective of cold storage temperature. The results suggested that there is a link between rind pitting and rind biochemical concentrations in 'Benny' valencia citrus fruit.

Keywords: Antioxidants, citrus fruit, flavonoids, phenolics, rind pitting, vitamin C

4.1 Introduction

Other factors affecting rind physiological disorders and associated antioxidant activity include pre-harvest environmental conditions and postharvest storage conditions (Connor *et al.,* 2002). Previous studies have shown that micro-climates, such as vapor pressure deficit (VPD) and photosynthetically active radiation (PAR) affect the physiological activities and biochemical composition of fruit rind (Cronje *et al.,* 2011; Magwaza *et al.,* 2014). For instance, inadequate PAR resulted in lower rind biochemical concentrations in 'Nules Clementines' mandarin fruit, which could encourage the development of rind physiological disorders (Magwaza *et al.,* 2014). High VPD affects water that leads to lower rind biochemical composition, which could cause the incidence of rind physiological disorders (Sevillano *et al.,* 2009).

Moreover, citrus fruit are highly susceptible to rind physiological disorders and assumed to be associated with various biochemical attributes, such as total flavonoids, lower phenolic compounds and antioxidants (Sevillano *et al.,* 2009). In addition, phenolics are important compound groups increasing antioxidant capacity and fruit level tolerance to rind physiological disorders (Lee *et al.,* 2003). Furthermore, rind biochemical concentrations and radical scavenging activities of citrus rind have been reported to play an important role regarding the susceptibility to rind physiological disorders (Olarewaju *et al*., 2017).

There has been vast research on vitamin C aligned to plant stress response such as non-chilling rind pitting (Magwaza *et al.,* 2013). However, less information has been reported on production site, postharvest treatments, cold storage and their influence on vitamin C in relation to non-chilling rind pitting. Another area that still needs further research is the relationship between the biochemical profile (total flavonoids

and phenolics) of the rind, antioxidant system and development of citrus rind pitting physiological disorder. Therefore, this study was conducted to investigate the role of production sites, postharvest treatments and cold storage temperatures on rind biochemical concentrations associated with Benny' valencia citrus fruit non-chilling rind pitting.

4.2 Materials and methods

4.2.1 Study sites, treatments and storage

During 2016 and 2017 seasons, 'Benny' valencia citrus fruit were harvested from commercial farms in Tzaneen-Mahela Boerdery (23°88′36″S, 30°82′34″E), Groblersdal-Schoeman Boerdery (25°02′05″S, 29°21′56″E) and Musina-Noordgrens Landgoed (22°20′17″S, 30°02′30″E) in Limpopo Province, South Africa. Fruit were harvested from June to August at commercial maturity. The rainfall, minimum and maximum relative humidity (RH), minimum and maximum temperature and vapour pressure deficit (VPD) registered during the growing seasons were recorded and presented in Tables 4.1, 4.2 and 4.3 for Tzaneen, Musina and Groblersdal, respectively. After harvesting, fruit were transported at ambient temperature to the Agricultural Research Council-Tropical and Subtropical Crops (ARC-TSC) postharvest laboratory in Nelspruit (25°45′18″S, 30°96′97″E) for sorting, grading, treatment, cold storage, external quality evaluation and analysis. Upon arrival at the ARC-TSC laboratory, fruit were washed with 20 litres of water mixed with 10 ml of Sporekill® solution; and subjected to the following treatments: T_1 = no wax plus dehydration, T_2 = wax plus dehydration and T_3 = wax plus no dehydration; treatments were replicated three times. Thereafter, fruit were waxed manually with Citrashine and dehydrated for 3 days at 25°C using air conditioner (Model: GZ-50GB-E1,

Maclaren, Johannesburg, South Africa) before storage (Alférez *et al.,* 2003). After dehydration, fruit were stored at -0.6 and 4.5°C, 90±3% RH for 28 days.

Table 4.1: Climatic data during the growing seasons in Musina

2016 season	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**June**	**July**
Total Rainfall (mm)	400	340	270	200	100	0.0	0.0
Daily minimum temperature (ºC)	20.1	18.3	19.2	17.1	18.2	17.1	16.1
Daily maximum temperature (ºC)	33.8	34.2	33.1	30.7	30.1	28.3	29.4
Daily minimum relative humidity (%)	41.8	39.2	42.1	32.0	36.7	56.9	50.2
Daily maximum relative humidity (%)	91.6	92.3	93.1	93.5	92.2	92.4	93.2
Vapour pressure deficit (kpa)	0.3	0.6	0.3	0.5	0.4	0.4	0.3

Table 4.2: Climatic data during the growing seasons in Tzaneen

2016 season	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**June**	**July**
Total Rainfall (mm)	378	280	256	180	0.0	0.0	0.0
Daily minimum temperature (ºC)	20.2	20.5	19.1	15.9	11.2	9.1	7.2
Daily maximum temperature (ºC)	30.1	31.2	31.4	30.2	25.9	25.8	24.9
Daily minimum relative humidity (%)	60.8	38.8	41.6	39.8	39.5	33.8	31.6
Daily maximum relative humidity (%)	92.2	84.9	89.6	90.2	92.8	91.1	91.5
Vapour pressure deficit (kpa)	0.1	0.5	0.2	0.8	0.8	0.4	0.3

Table 4.3: Climatic data during the growing seasons in Groblersdal

2016 season	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**June**	**July**
Total Rainfall (mm)	278	250	269	240	0.0	0.0	0.0
Daily minimum temperature (ºC)	18.7	19.1	17.2	13.3	8.2	4.5	5.8
Daily maximum temperature (ºC)	32.1	32.1	31.1	30.8	25.4	23.7	23.1
Daily minimum relative humidity (%)	32.7	33.9	37.5	33.3	35.3	32.7	27.1
Daily maximum relative humidity (%)	91.6	92.9	94.6	95.4	97.3	94.9	90.8
Vapour pressure deficit (kpa)	0.7	0.7	0.8	0.7	0.5	0.5	0.2

4.2.2 Determination of non-chilling rind pitting incidence

Rind pitting incidence was determined as previously explained in Chapter 3 (**page 34**).

4.2.3 Reagents and standards

All chemicals including sodium hydroxide (NaOH), methanol, Folin-Ciocalteu reagent, DMSO, diethylene glycol, sulphosalicylic acid, 2,2-diphenyl-1-picrylhydrazyl

(DPPH), acid-ninhydrin, glacial acetic acid, gallic acid, metaphosphoric acid, sulphuric acid, perchloric acid, trichloroacetic acid, L-ascorbic acid and rutin were purchased from Sigma-Aldrich Company Ltd, (Dorset, UK).

4.2.4 Sample preparation

Ten (10) fruit per treatment were manually peeled to remove flavedo, with a fruit peeler (Model: 80SPE, Verimark Shogun, Johannesburg, South Africa). The peels (flavedo) were frozen in liquid nitrogen and stored at -21°C before being freeze-dried using Vitris Benchtop freeze drier system (Model: ES, SP Industries Inc., Warmister, USA) at 0.015 kPa and -54°C. Afterwards, freeze dried samples were milled into fine powder using pestle and mortar and stored in -21°C for further physiological analysis.

4.2.5 Total phenolic extraction

The extraction procedure of total phenolic compounds was determined according to the method described by Abeysinghe *et al.* (2007). A sample of 0.5 g milled 'Benny' valencia flavedo was weighed in a screw-capped tube. Phytochemicals were then extracted with 5 ml of 50% DMSO (5 ml of 1.2 M HCL in 80% methanol\water) and vortexed with vortex mixer for 1 min. Thereafter, samples were heated at 90°C for 3 hours and vortexed every 30 minutes. After heating, samples were allowed to cool to room temperature, thereafter diluted with methanol to make up 10 ml. The diluted samples were then centrifuged at 10,000 x g for 5 min at 4°C. The supernatant was used for determination of total phenolic content and flavonoids.

4.2.6 Determination of total phenolic content

Total phenolic content of the flavedo extract was measured using a modified Folin-Ciocalteu method (Abeysinghe *et al.,* 2007). A sample of 0.5 ml of diluted flavedo extract was added into a glass test tube containing 4 ml of distilled water. Folin-Ciocalteu reagent (0.5ml) was then added to the solution and allowed to react with the sample for 3 minutes followed by (a blank was prepared using 0.25 ml of methanol instead of plant extract) an addition of 1 ml 20% sodium carbonate. The solution was vortexed and allowed to stand for 3 hours before filtered in a Whatman® 0.45 µm poly filter prior to determination of the absorbance at 760 nm, using Spectrophotometer (Model: UV-1900i, Shimadzu Scientific Instruments, Inc., Washington, USA). Gallic acid was used as standard to prepare the calibration curve. The results were expressed as mg garlic acid equivalent (GAE)/ 100 g DM.

4.2.7 Determination of total flavonoids content

A colorimetric method previously described by Abeysinghe *et al.* (2007) was used to determine total flavonoids. Briefly, 0.5 ml of diluted flavedo extracts was added to a glass test tube containing 3.5 ml of ethanol. Afterwards, 4 ml of 90% diethylene glycol was added with thorough mixing followed by an addition of 0.1ml of 4M NaOH for the initiation of the reaction. The solution was again thoroughly mixed, and the absorbance was read at 420 nm after 10 min incubation at 40°C using Spectrophotometer (Model: UV-1900i, Shimadzu Scientific Instruments, Inc., Washington, USA). Rutin was used as a standard and the total flavonoids concentration was expressed as mg rutin equivalent (RE)/100 g DM).

4.2.8 Determination of vitamin C

Vitamin C content in the flavedo was determined using the method previously described by Böhm *et al.* (2006). One gram (DW) of the rind sample was mixed with 5ml 0.56 M metaphosphoric acid, vigorously shaken with a shaker and centrifuged at 2988 x g and the supernatant transferred into a volumetric flask. This procedure was repeated twice and thereafter the combined extracts made up to 20 ml using 0.56 M meta-phosphoric acid. Subsequently, 200 µl of the extract was mixed with 300 µl 0.3 M trichloroacetic acid and centrifuged at 17212 x g for 10 min at 4°C. Subsamples of the supernatant (300 µl aliquots) were mixed with 100 µl 2,4-dinitrophenylhydrazine reagent (0.013 M in 30% perchloric acid) and heated to 60°C for 1h and subsequently cooled in an ice bath for 5 min. Thereafter, 400 µl 15.75 M sulphuric acid was added to the sample and the absorbance read at 520 nm using Spectrophotometer (Model: UV-1900i, Shimadzu Scientific Instruments, Inc., Washington, USA). The ascorbic acid concentration was calculated by comparison to the values obtained with an L-ascorbic acid standard curve. Results were expressed as 100g DM.

4.2.9 Determination of antioxidants assay by DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

Total antioxidants were determined using antioxidant assay according to the method previously described by Karioti *et al.* (2004). Two (2) grams of powered ‘Benny’ valencia peel were accurately weighed into centrifuge tubes (Model: 309, Hermile labortechnik, Berlin, Germany). Afterwards 10 ml of 100% methanol was added and vortexed with vortex mixer (Model: SBB 62, Lasec, Germany) at room temperature for 30 sec. Subsequently centrifuged at 1000 rpm for 5 min at 4°C (to prevent the

interference of particles). Thereafter, 15 µl of sample was added into the eppendorf tubes and 735µl of 100% methanol was added into each sample. Thereafter, 750 µl of DPPH solution was added to each sample. The lids of the tubes were closed and incubate at room temperature for 30 min in the dark. A 100% methanol was used as blank and the absorbance was read at 517nm under the light using Spectrophotometer (Model: UV-1900i, Shimadzu Scientific Instruments, Inc., City, Washington, USA). Radical scavenging activity was calculated by the percentage of DPPH that was scavenged using the following equation:

Radical scavenging activities (%) = $(1 - A_E / A_D) \times 100$

Where A_E is the absorbance of the reaction mixture containing the standard antioxidant, or extract and A_D is absorbance of the DPPH solution only.

4.2.10 Statistical analysis

Experiment was laid out as a factorial design arrangement with the following factors: seasons (2016 and 2017), production sites (Tzaneen, Musina and Groblersdal); postharvest treatments (no wax plus dehydration, wax plus dehydration and wax plus no dehydration) and cold storage temperatures (-0.6 and 4.5°C). All statistical analyses were performed using GenStat 18th (VSN International, Hemel Hempstead, UK). Data were subjected to analysis of variance (ANOVA) with production site and postharvest treatments and cold storage temperature as factors. Where significant differences were detected, means were separated using Duncan's New Multiple Range Test (DNMRT) at the 5% level of significance.

4.3 Results

4.3.1. The effect production sites, treatments and cold storage temperatures on non-chilling rind pitting

The interaction between effect production site, treatments and cold storage temperature was significant (P=0.0006) on non-chilling rind pitting. ‘Benny’ valencia fruit harvested from Groblersdal had the highest rind pitting incidence, regardless of postharvest treatments and cold storage temperature. However, low rind pitting incidence was observed in fruit harvested from Tzaneen treated with wax plus dehydration and stored at 4.5°C cold storage temperature when compared with fruit from Musina and Groblersdal.

4.3.2 The of production sites, postharvest treatments and cold storage temperatures on rind total phenolics

The effect of production site, postharvest treatments and cold storage temperature on rind total phenolics are presented in Table 4.4. The interaction between production sites, postharvest treatments and cold storage had a highly significant effect (P<0.0001) on rind total phenolics. However, rind total phenolics were high in ‘Benny’ valencia citrus fruit sourced from Tzaneen when compared with Musina and Groblersdal, irrespective of postharvest treatments and storage temperatures. In terms of cold storage temperature, ‘Benny’ valencia citrus fruit sources from Musina and stored at 4.5°C had the lowest total phenolics, however, high rind pitting incidence was observed. Moreover, both Musina and Groblersdal fruit stored at -0.6°C had the lowest total phenolics, irrespective of wax plus dehydration treatment.

Table 4.4: Effect of production sites, treatments and cold storage on rind pitting, total phenolics and flavonoids, vitamin C and total antioxidant activity of 'Benny' valencia citrus fruit

Production site	Treatment	Cold storage treatment (˚C)	Rind pitting incidence (0-3)	Total phenolics (mg GAE/g)	Total flavonoids (mg RUE/g)	Vitamin C (mg AA/g)	DPPH (%)
Musina	No wax + dehydration	-0.6	0.4±0.1def	1136.1±218.3de	60.1±0.3def	760.5±64.3ab	15.4±3.5fgh
	Wax + dehydration		0.8±0.1c-f	1524.6±196.2c	60.4±0.3cde	654.7±63.9c	22.3±3.6efg
	Wax + No dehydration		0.8±0.1f	1236.0±220.3d	59.0.0±0.3f	256.4±65.3e	16.6±3.8fgh
	No wax + dehydration	4.5	0.5±0.1def	345.2±61.9h	60.9±0.3cd	175.9±69.1f	9.9±3.9h
	Wax + dehydration		0.4±0.1def	473.5±62.h	60.4±0.3cde	230.0±73.4ef	38.0±3.9cd
	Wax + No dehydration		0.5±0.2def	1234.0±220.1d	60.5±0.3cde	343.2±78.6d	29.9±4.2de
Tzaneen	No wax + dehydration	-0.6	0.9±0.2cde	886.9±51.5fg	64.0±0.2b	807.0±78.4a	34.8±4.4d
	Wax + dehydration		0.5±0.2def	196.5±46.0i	63.5±0.3b	184.6±85.4f	38.1±4.9cd
	Wax + No dehydration		0.3±0.2ef	107.0±39.6i	63.4±0.3fb	191.5±82.7ef	47.8±5.4ab
	No wax + dehydration	4.5	0.3±0.2ef	315.2±62.95ef	65.7±0.2a	694.9±90.1abc	53.4±5.7a
	Wax + dehydration		0.1±0.2f	356.9±61.8e	63.5±0.3b	678.3±98.9c	51.6±5.6a
	Wax + No dehydration		0.5±0.2fdef	300.6±60.3f	63.4±0.3b	828.9±101.6a	47.1±5.2abc
Groblersdal	No wax + dehydration	-0.6	1.8±0.2ab	1979.8±227.5a	60.5±0.3cde	190.3±45.2ef	17.4±4.1fgh
	Wax + dehydration		2.3±0.1a	1890.2±227.8ab	60.3±0.3cde	28.9±55.2i	12.8±4.9gh
	Wax + No dehydration		1.7±0.1ab	1267.3±220.3d	59.4±0.3ef	175.9±54.9g	24.2±5.9ef
	No wax + dehydration	4.5	1.4±0.2bc	956.0±52.5efg	61.1±0.3c	191.5±76.4ef	17.4±8.2fgh
	Wax + dehydration		1.3±0.2bc	1547.3±196.5c	60.1±0.3def	94.4±65.4g	11.1±3.6g
	Wax + No dehydration		1.1±0.2bcd	1696.5±199.4bc	61.0±0.3c	356.4±77.6d	38.4±5.6bcd

DPPH: 2,2-diphenyl-1-picrylhydrazyl. Mean same letter shows no significant different at <0.05% according to Duncan's new multiple range test (DNMRT). Each value in the table is presented as mean ± standard error

4.3.3 The effect of production sites, postharvest treatments and cold storage temperatures on rind total flavonoids

The analysis of variance revealed that canopy position had high significant (P=0.0015) effect on rind total flavonoids. Rind total flavonoids were high in 'Benny' valencia citrus fruit sourced from Tzaneen and Groblersdal treated with no wax plus dehydration and stored at 4.5°C (Table 4.4). Furthermore, rind total flavonoids were also high in fruit sourced from Tzaneen than Musina and Groblersdal, regardless of treatments and cold storage temperature. Moreover, the lowest rind total flavonoids were observed in fruit sourced from Musina treated with wax plus no dehydration and stored -0.6°C. In addition, low rind total flavonoids were observed in 'Benny' valencia citrus fruit sourced from Musina and Groblersdal, irrespective of treatments at both storage temperatures.

4.3.4 The effect of production sites, postharvest treatments and cold storage temperatures on vitamin C

In the current study, vitamin C fluctuated with production sites and cold storage temperature in all postharvest treatments (Table 4.4). However, there was a highly significant effect (P<0.0001) between production sites, postharvest treatments and cold storage temperatures interaction on vitamin C. Furthermore, 'Benny' valencia citrus fruit sourced from Tzaneen and stored at 4.5°C had high vitamin C than Musina and Groblersdal, irrespective of wax plus dehydration treatment. Moreover, a similar trend was observed in 'Benny' valencia fruit sourced from Tzaneen and Musina, treated with no wax plus dehydration and at -0.6°C. However, 'Benny' valencia citrus fruit sourced from Musina had the lowest vitamin C in all treatments when compared with Tzaneen and Groblersdal at -0.6°C. Additionally, 'Benny'

valencia citrus fruit sourced from Musina, treated with no wax plus dehydration had the lowest vitamin C followed by Tzaneen and Groblersdal at 4.5°C.

4.3.5 The effect of production sites, postharvest treatments and cold storage temperatures on radical scavenging activity

Production sites, postharvest treatments and cold storage temperatures interaction had a highly significant effect ($P<0.0001$) on radical scavenging activity. Furthermore, 'Benny' valencia citrus fruit sourced from Tzaneen had higher radical scavenging activity than Musina and Groblersdal, irrespective of postharvest treatments and cold storage temperatures (Table 4.4). However, Musina fruit treated with no wax plus dehydration had the lowest radical scavenging activity regardless of cold storage temperature.

4.4 Discussion

4.4.1 The effect of production sites, treatments and cold storage temperatures on rind pitting incidence

The environmental conditions under which fruit are grown have great influence on rind pitting susceptibility (Wang, 2010). In this study, the lowest rind pitting incidence was observed in 'Benny' valencia fruit sourced from Tzaneen treated with wax plus dehydration and stored at 4.5°C cold storage temperature. According to Ehlers (2016), when fruit is transferred to water saturated atmosphere (95% RH), water moves from the surroundings to the epidermal cells, resulting in cells rehydration. This could be the reason for low rind pitting observed in 'Benny' valencia sourced from Tzaneen due to high environmental vapour pressure deficit (VPD). Moreover, when VPD was high, flavedo and outer albedo cells rehydrate faster than subtending albedo cells, creating variation in water potential between cells (Alférez and Burns,

2004). This variation creates a suction force between cells; and subsequently cause of internal flavedo and external albedo cell layers collapse, leading to rind pitting (Aquezer *et al*., 2010).

‘Benny’ valencia fruit sourced from Groblersdal had the highest rind pitting incidence, regardless of postharvest treatments and cold storage temperature. Similarly, Olarewaju *et al.* (2017) found that production regions affected rind breakdown in ‘Nules Clementine’ mandarin fruit. However, these results were in agreement with those of Alférez *et al.* (2005) whereby, fruit exposed to dehydration stress at low relative humidity (RH) experiences higher moisture loss, therefore, resulting in high rind pitting. Furthermore, Mothapo *et al.* (2018) found that ‘Benny’ valencia sweet orange from different production sites were affected by dehydration and failed to recover after being transferred to higher RH. This could be the reason for higher rind pitting in no wax plus dehydration fruit observed in all production sites.

4.4.2 The effect of production sites, postharvest treatments and cold storage temperatures on total phenolics

Hagen *et al*. (2007) previously suggested that production of total phenolics in ‘Aroma’ apple fruit increased due to higher field temperatures. In citrus fruit, rind phenolic compounds are important in increasing antioxidant capacity and fruit tolerance level to rind physiological disorders (Lee *et al.,* 2003; Magwaza *et al.,* 2013). In this study, total phenolics were high in ‘Benny’ valencia citrus fruit sourced from Tzaneen when compared with Musina and Groblersdal, irrespective of treatments and storage temperatures. These results were contradicting with Olarewaju *et al.* (2017), whereby, ‘Nules Clementine’ mandarin fruit total rind phenolics from both production regions (Unifruit and Swartvelei farms) was

significantly higher in relation to rind breakdown. In Tzaneen fruit, high rind total phenolics observed was due to high environmental temperature, which increased rind total phenolics (Table 4.2).

In terms of cold storage temperature, ‘Benny’ valencia citrus fruit sourced from Musina and stored at 4.5°C had the lowest total phenolics, however, high rind pitting incidence was observed. Igual *et al.* (2011) found similar results whereby, low rind total phenolics were displayed during cold storage on ‘Star Ruby’ grapefruit juice stored at 4°C for up to two months. In ‘Golden Delicious’ apple, cold storage clearly reduced total phenolic concentrations in fruit stored at 0°C for 3 months (Tarozzi *et al.,* 2004). Another probable explanation is that cold storage temperature results in higher rind total phenolics degradation (Ncama, 2016). However, this statement does not agree with the results obtained in the current study. This could be due to storing ‘Benny’ valencia fruit at -0.6°C cold storage, which caused unsuitable conditions for phenolic production. During cold storage, rind total phenolics declined and it could be implicated in mitigation of rind physiological disorder development as low rind pitting incidence was recorded.

4.4.3 The effect of production sites, postharvest treatments and cold storage temperatures on total flavonoids

According to Treutter (2001), most enzymes involved in flavonoids production are stimulated by high environmental temperature in each growing region. In this study, rind total flavonoids were high in ‘Benny’ valencia citrus fruit sourced from Tzaneen and Groblersdal treated with no wax plus dehydration and stored at 4.5°C. Similar findings were reported by Olarewaju *et al.* (2017) who found significantly higher rind total flavonoids in ‘Nules Clementine’ mandarin fruit from Eastern Cape production

region when compared with fruit from Western Cape production region in relation to rind breakdown. Furthermore, citrus fruit has an increased ability to encourage flavonoids during cold storage in relation to rind physiological disorders (Chaudhary *et al.,* 2014).

Low rind total flavonoids were observed in ‘Benny’ valencia citrus fruit sourced from Musina and Groblersdal, irrespective of treatments at both storage temperatures. These findings were inconsistent with those of Cogo *et al.* (2011) who reported higher rind total flavonoids on ‘Green Star’ broccoli florets stored at 1°C for up to 7 days. Generally, high rind total flavonoid concentration could be implicated in the ability of fruit to repel the development of rind pitting associated with citrus fruit (Magwaza *et al.,* 2014). In general, this could be the reason why less rind pitting was observed on the rind of the fruit used in this study.

4.4.4 The effect of production sites, postharvest treatments and cold storage temperatures on vitamin C

Regulating storage temperature during postharvest processes is one of the most important factor in maintaining fruit quality and extending the shelf life. According to Chaudhary et *al.* (2017) vitamin C is significantly affected by storage temperature in fruits and vegetables. In this study, ‘Benny’ valencia fruit sourced from Tzaneen and stored at 4.5°C had high vitamin C than Musina and Groblersdal, irrespective of wax plus dehydration treatment. In ‘Golden Delicious’ apple, vitamin C content was increased by cold storage in fruit stored at 0°C for 3 months (Tarozzi *et al.*, 2004). Moreover, Siboza (2013) reported that vitamin C in ‘Eureka’ lemon flavedo was significantly affected by farm location and cold treatments. In ‘Marsh’ grapefruit, late season high field temperature resulted in high vitamin C content (Bramlage and

Weiss, 1997). In the current study, high vitamin C observed was due to high temperatures within Tzaneen production site.

'Benny' valencia citrus fruit sourced from Musina had the lowest vitamin C in all treatments when compared with Tzaneen and Groblersdal at -0.6°C. Similar results were found by Hagen *et al.* (2007) who reported low vitamin C in 'Curly' kale leaves stored at 1°C for up to six weeks. Furthermore, vitamin C content in 'Navel' oranges was higher in fruit with chilling injury stored at -0.6°C (Bassal and El-Hamahmy, 2011). This was due to low storage temperature, which significantly triggered plant defence compounds in 'Benny' valencia flavedo, which could have played a role in increasing rind pitting tolerance in 'Benny' valencia citrus fruit in this study.

4.4.5. The effect of production sites, postharvest treatments and cold storage temperatures on radical scavenging activity

Radical scavenging activity quantifies the ability to protect a certain tissue against cell membrane damage by reactive oxygen species (Villano *et al.,* 2007). In this study, 'Benny' valencia citrus fruit sourced from Tzaneen had higher radical scavenging activity than Musina and Groblersdal, irrespective of postharvest treatments and cold storage temperatures in both seasons. Results in these study were similar to those observed by Olarewaju *et al.* (2017) whereby, higher radical scavenging activity in 'Nules Clementine' mandarin fruit from Eastern Cape production region than fruit from Western Cape in both seasons. Moreover, Drogoudi and Pantelidis (2011) reported higher radical-scavenging activity from different production sites in different apple fruit cultivars ('Granny Smith', 'Golden Delicious' and 'Imperial Double Red'). It could be suggested that low temperature positively affected radical scavenging activity, which consistently inhibited the development of

rind pitting. In addition, low relative humidity could be another factor that is responsible for high radical scavenging activity observed in this study. However, Musina fruit treated with no wax plus dehydration had the lowest radical scavenging activity, regardless of cold storage temperature and seasons. Findings in this study were inconsistent with those found by Ncama (2016), whereby, growing regions had no significant effect on radical scavenging activity of 'Marsh' grapefruit treated with wax. It has been reported that plants synthesise compounds such as antioxidants in order to reduce oxidative damaging effects and other stresses (Posmyk *et al.,* 2005).

4.5 Conclusion

The study showed that production sites and postharvest treatments, cold storage temperature had an influence on rind biochemical concentrations and non-chilling rind pitting incidence. As observed, postharvest treatments resulted in low non-chilling rind pitting with an increased accumulation of rind biochemical concentrations. These results suggest that there is a link between non-chilling rind pitting and rind biochemical concentrations of 'Benny' valencia citrus fruit.

CHAPTER 5

THE ROLE OF PRODUCTION SITES, POSTHARVEST TREATMENTS AND COLD STORAGE TEMPERATURES IN REGULATING TOTAL ANTIOXIDANTS AND THEIR ABILITY TO MITIGATE 'BENNY' VALENCIA CITRUS FRUIT NON-CHILLING RIND PITTING

Abstract

In citrus fruit, antioxidant's defence system protects tissues against oxidative stress, ultimately, mitigating against rind pitting physiological disorders. However, citrus fruit contain certain rind antioxidants, which occur in different forms e.g. lipophilic and hydrophilic. Therefore, this study aimed to investigate the effect of production sites, postharvest treatments and cold storage temperatures on total antioxidants and their ability to mitigate 'Benny' valencia citrus fruit non-chilling rind pitting. During 2016 and 2017 seasons, fruit were harvested from Tzaneen, Groblersdal and Musina in South Africa. Afterwards, fruit were subjected to the following treatments: T_1 = no wax and dehydration, T_2 = wax and dehydration and T_3 = wax and no dehydration. Dehydrated treatments were applied for 3 days at ±45% relative humidity, thereafter, fruit were stored at -0.6 and 4.5°C for 28 days plus 7 days of shelf-life. After rind pitting incidence (RPI) analysis, the flavedo was removed, freeze dried, thereafter, antioxidant activities were measured using the ferric reducing ability of plasma (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) and the oxygen radical absorption capacity (ORAC) assays. The results showed that RPI was higher on fruit harvested in Groblersdal when compared with Tzaneen and Musina fruit, irrespective of postharvest treatments and

storage temperatures. These findings were supported by high rind total antioxidants quantified by ABTS for Groblersdal fruit when compared with Tzaneen and Musina fruit. However, fruit from Musina subjected to wax plus dehydration treatment had higher antioxidant measured by DPPH and low RPI at both low storage temperatures when compared with fruit harvested from Groblersdal and Tzaneen. Furthermore, fruit harvested from Musina treated with no wax plus dehydration and stored at -0.6°C had low RPI with high antioxidant activity measured by FRAP than Tzaneen and Groblersdal regions in both seasons. In conclusion, production sites affected RPI, with high vapour pressure deficit leading to high RPI in 'Benny' valencia citrus fruit. Seemingly, high orchard temperatures stimulate rind total antioxidant, which proved to be a defence mechanism against RPI in fruit sourced from Groblersdal, with wax plus dehydration treatments also stimulating total antioxidants.

Keywords: ABST, DPPH, FRAP, ORAC, Flavedo, Storage temperature

5.1 Introduction

In fruit and vegetables, antioxidant compounds are defence mechanisms that manage reactive oxygen species (ROS) at pre- and postharvest (Siboza 2013) and thereby, preventing damage against oxidative stress (Villano *et al.,* 2007). According to Rivera *et al.* (2007), antioxidant defence mechanism enhances tolerance to rind physiological disorders in fruits and vegetables. In citrus fruits, non-chilling rind pitting remains an important physiological disorder that is characterised by groups of collapsed oil glands forming a sunken pit in the rind (Alférez and Burns, 2004). This is an economically important postharvest problem that decreases the overall quality and marketability of citrus fruits (Dong *et al.,* 2012).

In a plant cell, the counteraction of rind physiological disorders can be enhanced by an array of antioxidants, making up a full defense mechanism against oxidative damage (Hung *et al.,* 2011). An antioxidant is a molecule responsible for slowing or preventing the oxidation of other molecules (Imeh and Khokhar, 2002). According to Mathaba *et al.* (2014), antioxidants nature is diverse, several assays are thereby required to quantify and estimate plant antioxidants capacity. The increase in total antioxidants assay number is due to the varying assay efficacy to estimate antioxidants in different plant tissues (Wang *et al.,* 2005). In general, these various assays are used to estimate total antioxidant capacity of a certain tissue and these include; the 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) assay (Pellegrini *et al.,* 2003), 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Wang *et al.,* 2005), ferric reducing ability of plasma (FRAP) assay (Li *et al.,* 2006) and oxygen radical absorption capacity (ORAC) assay (Wang, 2003).

According to Re *et al.* (1999), antioxidant assays such as DPPH and ABTS are grouped as inhibition assays, because they donate hydrogen or an electron of a preformed free radical. Furthermore, antioxidant assays express capacity and involve an antioxidant during generation of a radical, while DPPH is a reliable assay for measuring antioxidant activities of fruits (Villano et *al.,* 2007; Olarewaju *et al.,* 2017). According to Babbar *et al.* (2011), DPPH can donate hydrogen which is an established mechanism for anti-oxidation. The ABTS assay depends on the very stable ($ABTS^{\bullet+}$) radical which is formed when ABTS is reacting with potassium persulfate to form the blue or green $ABTS^{\bullet+}$ chromophore (Pellegrini *et al.,* 2003). Several lipophilic and hydrophilic antioxidants decrease $ABTS^{\bullet+}$ to ABTS, during the time required to form ABTS depending on incubation period and concentration of the antioxidant (Re *et al.,* 1999; Mathaba *et al.,* 2014). The ORAC assay measures the effect of antioxidant components in fruit extracts on the decline in R phycoerythrin (R-PE) fluorescence (Wang, 2003).

In plants, antioxidant capacity can be affected mainly by climate conditions that occur during development stage and postharvest storage (Dong *et al.,* 2012). In addition, pre-harvest environmental factors such as relative humidity and postharvest storage temperature, treatments and storage time influence citrus fruit antioxidant activity (Siboza 2013). In ‘Golden Delicious’ apple fruit, cold storage temperature influences antioxidant concentration and activity (Van der Sluis *et al.,* 2001). While in ‘Mexican’ lime citrus fruit, was found to contain more efficient antioxidant defence system (Rivera *et al.,* 2007).

The ability of a citrus rind radical scavenging capacity determines the susceptibility to rind break down and overall quality (Magwaza *et al.,* 2013). Antioxidant capacity increases fruit tolerance to rind physiological disorders (Lee *et al.,* 2003). However,

the mechanisms by which total antioxidant triggers defence rind pitting is unknown. Therefore, this study aimed to investigate the effect of production sites, postharvest treatments and cold storage temperatures on total antioxidants and their ability to mitigate ‘Benny’ valencia non-chilling rind pitting.

5.2 Materials and methods

5.2.1 Fruit sampling, treatments and storage

Fruit sampling, treatments and storage were determined as previously described in Chapter 4 (**page 56**).

5.2.2 Determination of rind pitting incidence

Rind pitting incidence was determined as described in Chapter 3 (**page 34**).

5.2.3 Reagents and standards

All chemicals including ethanol, sodium hydroxide, methanol, sodium acetate, hydrochloric acid, sulphosalicylic acid, 2,2-diphenyl-1-picrylhydrazyl, 2,2'-azobis (2-amidinopropane) dihydrochloride, FRAP reagent, metaphosphoric acid, trichloroacetic acid, trolox and potassium persulphate were purchased from Sigma-Aldrich Company Ltd. (Dorset, UK).

5.2.4 Sample preparation

Ten (10) fruit per treatment were manually peeled to remove flavedo with a fruit peeler (Model: 80SPE, Verimark Shogun, Johannesburg, South Africa). The peels were frozen in liquid nitrogen and stored at -21°C before being freeze-dried using Vitris Benchtop freeze drier system (Model: ES, SP Industries Inc., Warmister, USA) at 0.015 kPa and -54°C. Afterwards, freeze dried samples were milled into fine powder using pestle and mortar and stored in -21°C for further analysis.

5.2.5 Determination of antioxidant activity by Ferric Reducing 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-trripyridyl-s-triazine complex (Fe^{3+}- TPTZ) to the ferrous form (Fe^{2+}- TPTZ) by a reductant, thereby, determining the combined antioxidant power of 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 HCL and mM $FeCL_3$•$6H_2O$ (10:11); 1000 µL FRAP reagent was mixed with 30 µL sample and the absorbance read at 593 nm after 10 min of reaction time using Spectrophotometer (Model: UV-1900i, Shimadzu Scientific Instruments, Inc., Washington, USA).

5.2.6 Determination of antioxidant activity measured by ABTS

The antioxidant activity was measured using the capacity of the extract to scavenge $ABTS^{\bullet+}$ radicals (Liyana-Pathirana and Shahidi, 2006). In short, a 7 mM solution of ABTS in water was prepared and $ABTS^{\bullet+}$ was formed after the addition of potassium persulphate (2.45 mM) to the solution. After 16 h incubation in darkness at room temperature, the stock solution was diluted with ethanol until the absorbance reached 0.7 ± 0.02 at 734 nm. After mixing of 10 µl sample to 200 µL of diluted $ABTS^{\bullet+}$ solution, the reaction mixture was incubated for 5 min at 30°C. The decrease in the absorbance reflected the $ABTS^{+\bullet}$ radical scavenging capacity of the antioxidant. The absorbance of $ABTS^{\bullet+}$ without sample was measured as the control using Spectrophotometer (Model: UV-1900i, Shimadzu Scientific Instruments, Inc., Washington, USA). The inhibition percent was calculated using the following formulae: Inhibition % = [(AC – AS)/AC] ×100, where AC is the absorbance of the control and AS is the absorbance of the sample plus ABTS radical at t =5 min.

5.2.7 Determination of antioxidants assay by ORAC assay

The ORAC assay was performed according to Wang (2003) using microplate readers (Model: 384B, MG LABTECH, Berlin, Germany) with some modifications. Frozen flavedo tissue (5 g DM) was extracted in 45 mL phosphate buffer (75 mM, pH 7.0) using the Ultra-Turrax homogenizer (Model: KRH-I, Ultra-Turrax, Shangai, China) at 4°C for 2 min. The mixture was then centrifuged at 20, 000 rpm for 20 min at 4°C. The supernatant was used for the ORAC assay. The ORAC assay measures the effect of antioxidant components in fruit extracts on the decline in R phycoerythrin (R-PE) fluorescence. The R-PE fluorescence is induced by a peroxyl radical generator, which was prepared fresh all the time. The reaction mixture contai 3.4 mg L^{-1} of R-PE (100 µL), 75 mM phosphate buffer (pH 7.0) (1.7 mL) and sample (100 µL). The reaction mixture was incubated at 37°C for 15 min and the reaction was started by the addition of 320 mM 2,2'-azobis (2-amidinopropane) dihydrochloride (100 µL). Using a fully automated microplate-based multi-detection, fluorescence was measured and recorded every 5 min using microplate readers (Model: 384B, MG LABTECH, Berlin, Germany) excitation at 485 nm and emission at 520 nm. The phosphate buffer was used as a blank and 1 µM Trolox was used as a standard during each run. The results were expressed as µM TE g^{-1} DM.

5.2.8 Determination of antioxidants assay by DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity

Total antioxidants were determined using antioxidant assay according to the method previously described by Karioti *et al.* (2004). A 2 g of powered ‘Benny’ valencia peel was accurately weighed into centrifuge tubes. Afterwards 10 mL of 100% methanol was added and vortexed with vortex mixer at room temperature for 30 sec.

Subsequently, centrifuged at 1000 rpm for 5 min at 4°C. Thereafter, 15 µL of sample was added into the Eppendorf tubes and 735 µL of 100% methanol was added into each sample. Thereafter, 750 µL of DPPH solution was added to each sample. The lids of the tubes were closed and incubated at room temperature for 30 min in the dark. A 100% methanol was used as blank and the absorbance was read at 517 nm under the light using Spectrophotometer (Model: UV-1900i, Shimadzu Scientific Instruments, Inc., Washington, USA). Radical-scavenging activity was calculated by the percentage of DPPH that was scavenged using the following equation:

Radical-scavenging activities (%) = $(1 - A_E / A_D) \times 100$

Where A_E is the absorbance of the reaction mixture containing the standard antioxidant, or extract and A_D is absorbance of the DPPH solution only.

5.2.9. Statistical analysis

Experiment was laid out as a factorial design arrangement with the following factors: seasons (2016 and 2017), production sites (Tzaneen, Musina and Groblersdal); postharvest treatments (no wax plus dehydration, wax plus dehydration and wax plus no dehydration) and cold storage temperatures (-0.6 and 4.5°C). All statistical analyses were performed using GenStat 18th (VSN International, Hemel Hempstead, UK). Data were subjected to analysis of variance (ANOVA) with production site and postharvest treatments and cold storage temperature as factors. Where significant differences were detected, means were separated using Duncan's New Multiple Range Test (DNMRT) at the 5% level of significance.

5.3 Results

5.3.1 The effect of production sites, treatments and cold storage temperatures on rind pitting incidence

The results showed a significant interaction (B*C*D; P<0.0006) between production sites, postharvest treatments and storage temperatures on ‘Benny’ valencia rind pitting. With respect to wax plus dehydration treatment, unwaxed but dehydrated fruit stored at -0.6°C showed reduced rind pitting incidence in fruit harvested from Musina followed by fruit from Tzaneen and Groblersdal (Figure 5.1). Moreover, wax plus dehydration treatment effectively reduced rind pitting incidence in Tzaneen fruit stored at -0.6°C when compared to those harvested from Groblersdal and Musina fruit. In the present study, no significant difference was found between wax plus no dehydration and wax plus dehydration treatment on reduced rind pitting incidence of fruit harvested from Musina and stored at 4.5°C. However, wax plus no dehydration and wax plus dehydration appeared to have reduced rind pitting incidence in fruit harvested from Tzaneen; and subsequently, stored at 4.5°C.

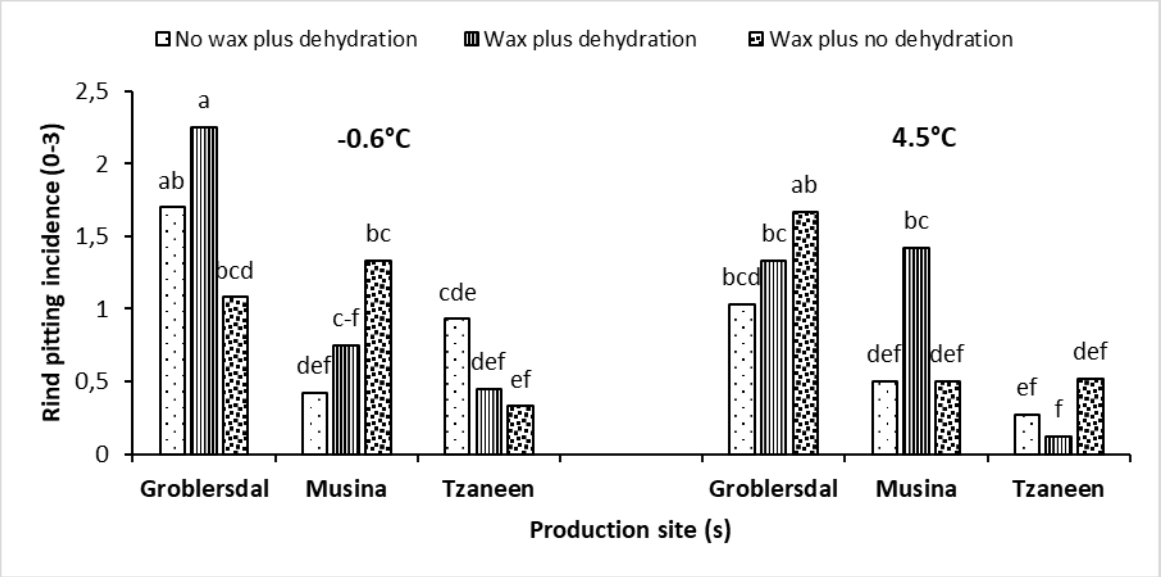

Figure 5.1: Rind pitting incidence (RPI) of 'Benny' valencia fruit influenced by an interaction between production sites (B), treatments (C) and cold storage temperatures (D). Mean values followed by different letter (s) within the same graph are significantly different (P<0.05). B*C*D (P<0.0006)

5.3.2 The effect of production sites, treatments and cold storage temperatures on total antioxidant activity quantified by DPPH assay

Production sites (B; P<0.0001), treatments (C; P<0.0001) and cold storage temperatures (D; P<0.0001) as well as their interaction had a significant effect (B*C*D; P<0.0001) on total antioxidant activity quantified by DPPH assay (Figure 5.2). In general, fruit harvest from Tzaneen recorded that highest total antioxidant activity quantified by DPPH assay than those harvested from Groblersdal and Musina, irrespective of treatment and storage condition (Figure 5.2). Moreover, fruit harvested from Tzaneen treated with wax plus no dehydration had the highest total antioxidant activity quantified by DPPH assay (46.2 µm TE g^{-1} DM) followed by fruit from Groblersdal (29.23 µm TE g^{-1} DM), with the lowest total antioxidant activity quantified by DPPH assay recorded in fruit from Musina (19.97 µm TE g^{-1} DM).

However, a different trend was observed at 4.5°C, where waxed plus dehydrated fruit from Tzaneen showed the highest total antioxidant activity quantified by DPPH assay (49.97 µm TE g^{-1} DM) followed by Musina (37.37 µm TE g^{-1} DM) with the lowest DPPH activity found in fruit from Groblersdal (14.63 µm TE g^{-1} DM) (Figure 5.2).

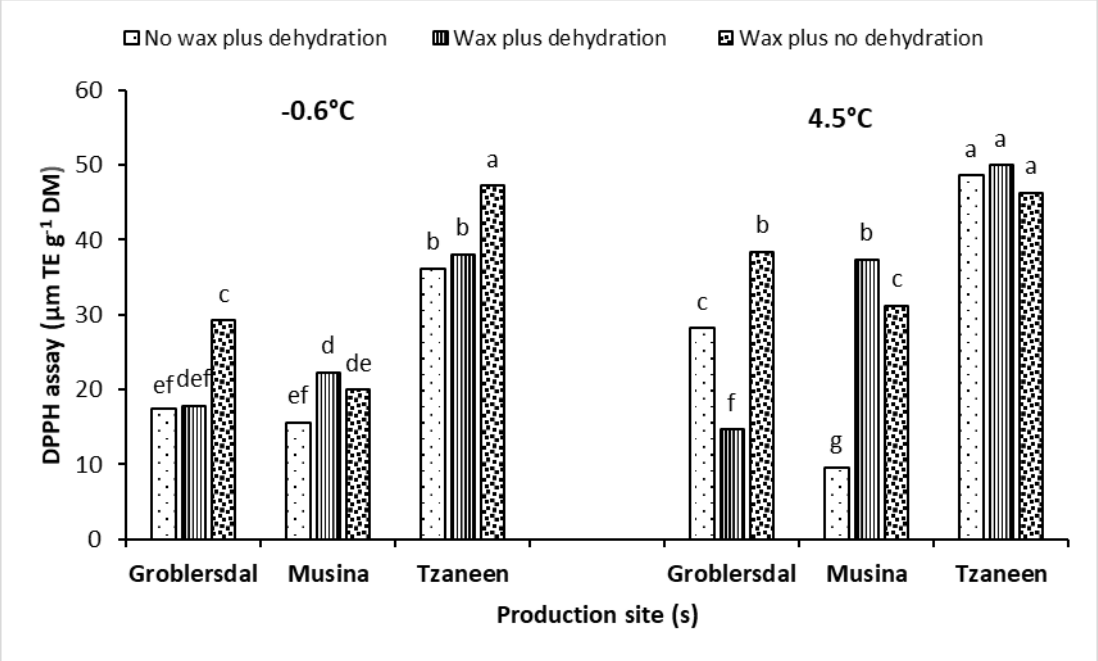

Figure 5.2: Total antioxidant activity quantified by DPPH assay of 'Benny' valencia fruit influenced by an interaction between production sites (B), treatments (C) and storage temperatures (D). Mean values followed by different letter (s) within same graph are significantly different (P<0.05). B*C*D (P<0.0001)

5.3.3 The effect of production sites, treatments and cold storage temperatures on total antioxidant activity quantified by FRAP assay

The results revealed that total antioxidant activity quantified by FRAP assay was significantly (P<0.0001) affected by an interaction between production site, postharvest treatment and storage temperatures (B*C*D) (Figure 5.3). In a treatment where no wax plus dehydration was applied, the total antioxidant activity quantified by FRAP assay increased in fruit harvested from all three production sites. Wax plus

dehydration resulted in higher total antioxidant activity quantified by FRAP assay in fruit harvested from Musina and Groblersdal stored at 4.5°C. Furthermore, wax plus no dehydration showed insignificant differences total antioxidant activity quantified by FRAP assay between fruit harvested from Groblersdal (36.3 µm TE g^{-1} DM) and Tzaneen (34.57 µm TE g^{-1} DM). However, fruit harvested from Musina and stored at -0.6°C exhibited significantly higher total antioxidant activity quantified by FRAP assay than Groblersdal and Tzaneen production sites, regardless of postharvest treatment. At 4.5°C, the highest FRAP activity was found in fruit harvested from Tzaneen (55.33 µm TE g^{-1} DM) treated with no wax plus dehydration, followed by fruit from Groblersdal (52.17 µm TE g^{-1} DM) and Musina (24.03 µm TE g^{-1} DM).

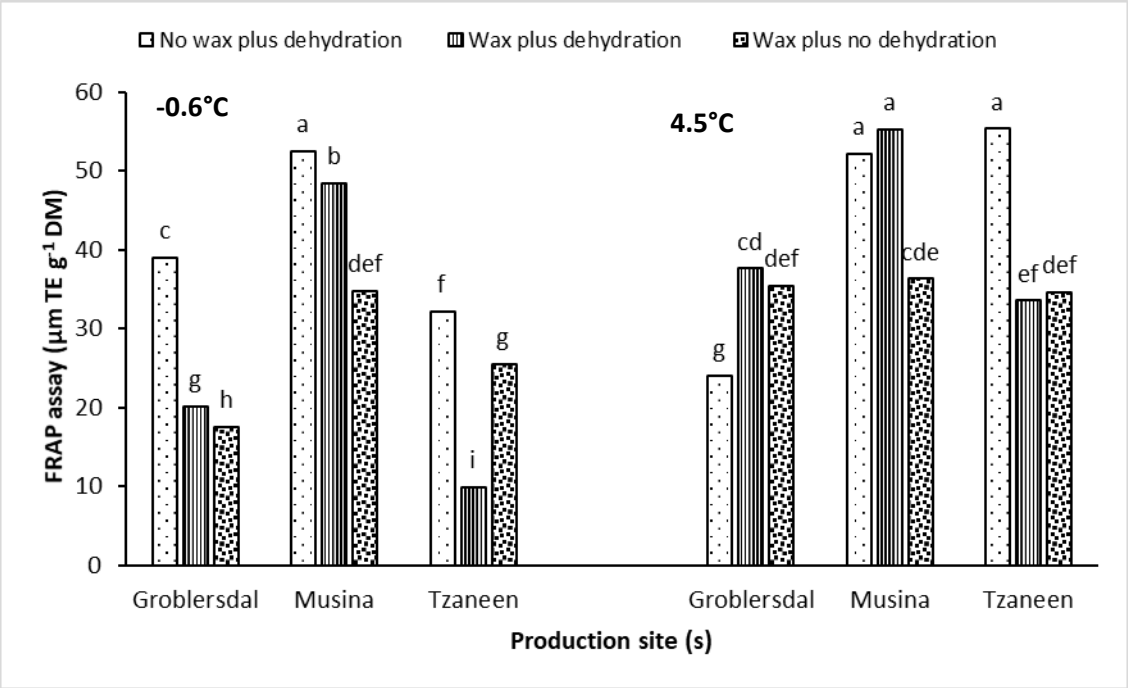

Figure 5.3: Total antioxidant activity quantified by FRAP assay of 'Benny' valencia fruit influenced by an interaction between production sites (B), postharvest treatments (C) and storage temperatures (D). Mean values followed by different letter (s) within same graph are significantly different (P<0.05). B*C*D (P<0.0001)

5.3.4 The effect of production sites, treatments and cold storage temperatures on total antioxidant activity quantified by ABST assay

The results showed a combined significant effect of production sites, postharvest treatments and storage temperatures (B*C*D; P=0.0064) on ABTS activity (Figure 5.4). The trend of total antioxidant activity quantified by ABTS assay was similar in fruit stored at -0.6 and 4.5°C for the three production sites, however, there were significant differences with postharvest treatment. Groblersdal fruit treated with wax plus dehydration and stored at -0.6°C had higher (74.21 µm TE g^{-1} DM) ABTS activity compared with those harvested from Musina (67.58 µm TE g^{-1} DM) and Tzaneen (63.49 µm TE g^{-1} DM). There was no significant postharvest treatment difference in fruit harvested from Musina stored at -0.6°C (Figure 5.4). Wax plus no dehydration treatment resulted in higher total antioxidant activity quantified by ABTS assay in fruit from Tzaneen stored at -0.6°C compared with other postharvest treatments. Groblersdal fruit stored at 4.5°C exhibited higher ABTS activity across all postharvest treatments compared with those from Musina and Tzaneen (Figure 5.4). In the case of Musina, fruit stored at 4.5°C, the influence of wax plus dehydration and wax plus no dehydration treatment did not differ on total antioxidant activity quantified by ABTS assay. In this study, Tzaneen fruit stored at 4.5°C, no postharvest treatment variation was observed on total antioxidant activity quantified by ABTS assay.

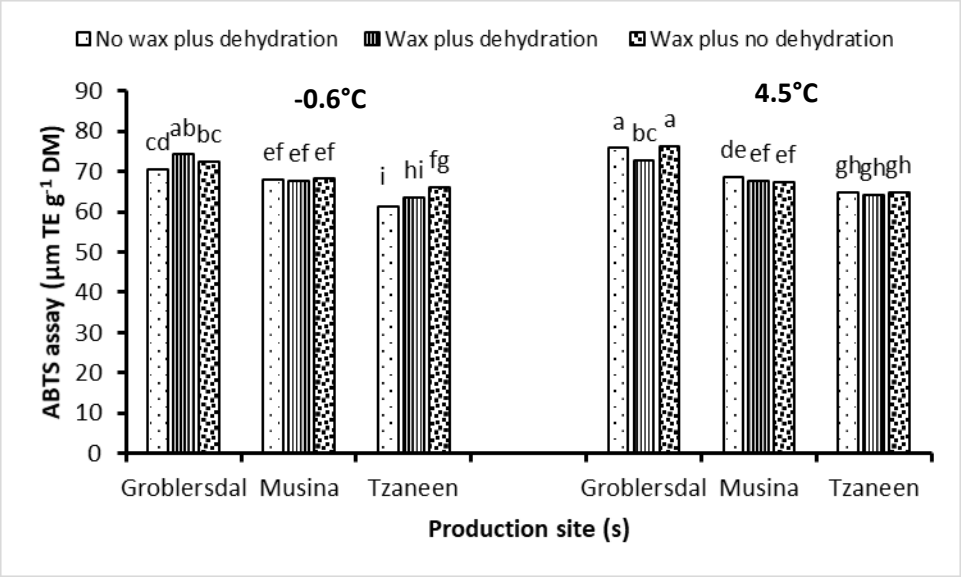

Figure 5.4: Total antioxidant activity quantified by ABTS assay of 'Benny' valencia fruit influenced by an interaction between production sites (B), treatments (C) and storage temperatures (D). Mean values followed by different letter (s) within same graph are significantly different (P<0.05). B*C*D (P=0.0064)

5.3.5 The effect of production sites, treatments and cold storage temperatures on total antioxidant quantified by ORAC assay

An interaction between production sites, treatments and storage temperatures (B*C*D; P<0.0001) had a significant effect on total antioxidant quantified by ORAC assay. Fruit from Groblersdal treated with wax plus no dehydration and stored at -0.6°C showed higher (48.70 µm TE g^{-1} DM) ORAC activity compared to those from Musina (17.23 µm TE g^{-1} DM) and Tzaneen (33.13 µm TE g^{-1} DM). Moreover, no wax plus dehydration treatment resulted in higher (46.57 µm TE g^{-1} DM) total antioxidant quantified by ORAC assay for fruit from Tzaneen followed by Musina (38.00 µm TE g^{-1} DM) while the lowest was recorded for Groblersdal (15.73 µm TE g^{-1} DM) fruit. Our results revealed that wax plus dehydration and wax plus no dehydration treatment had a positive influence on total antioxidant quantified by

ORAC assay of fruit from Musina and Tzaneen and stored at 4.5°C, respectively (Figure 5.5).

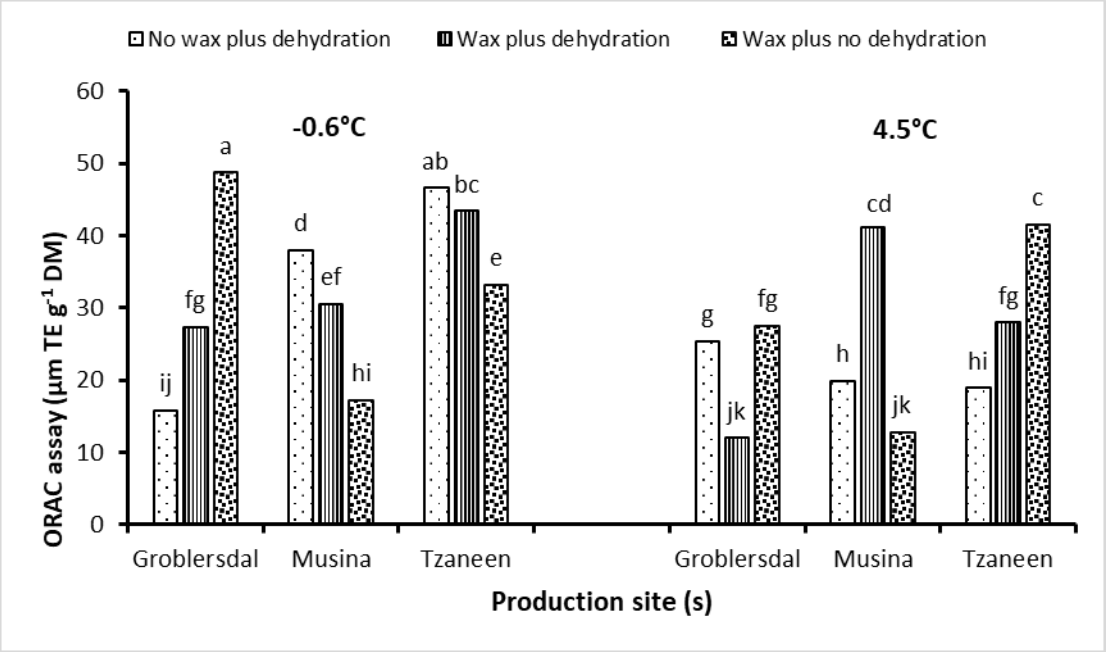

Figure 5.5: Total antioxidant activity quantified by ORAC assay of 'Benny' valencia fruit influenced by an interaction between production sites (B), treatments (C) and storage temperatures (D). Mean values followed by different letter (s) within same graph are significantly different (P<0.05). B*C*D (P<0.0001)

5.4 Discussion

5.4.1 The effect of production sites, treatments and cold storage temperatures on the incidence of rind pitting

The results also revealed that fruit harvested from Groblersdal had significantly higher rind pitting symptoms followed by fruit from Musina with the lowest incidence recorded in fruit harvested from Tzaneen in both seasons. These findings suggested that environmental conditions such as relative humidity (RH) and vapour pressure deficit (VPD) from Musina (Table 4.1, **page 57**) and Tzaneen (Table 4.3, **page 57**)

were more favourable for rind pitting resistance than the environmental conditions from Groblersdal (Table 4.3, **page 57**). Moreover, climatic conditions, especially vapour pressure deficit and relative humidity within production region promote rind pitting development (Lafuente and Zacarías, 2006). Therefore, low relative humidity from Groblersdal played a significant role in influencing rind pitting development in 'Benny' valencia citrus fruit.

The results also showed that wax plus dehydration treatment reduced rind pitting incidence in 'Benny' valencia citrus fruit, by increasing antioxidant activity in the flavedo during cold storage. In 'Fortune' mandarin fruit, the increased antioxidant activities could have contributed to rind pitting tolerance (Duarte and Guardiola, 1995). Ncama (2016) also explained the effect of wax plus dehydration in reducing rind pitting incidence effectively in 'Marsh' grapefruit stored at -0.6°C for up to 28 days plus 7 days.

5.4.2 The effect of production sites, treatments and cold storage temperatures on total antioxidant activity quantified by DPPH assay

In general, fruit harvest from Tzaneen recorded the highest total antioxidant activity quantified by DPPH assay than those harvested from Groblersdal and Musina, irrespective of postharvest treatment and storage condition (Figure 5.2). This was due to environmental conditions especially temperature from Tzaneen were favourable for total antioxidant activity quantified by DPPH assay than Musina and Groblersdal production sites. Generally, high temperature increased antioxidants synthesise quantified by DPPH assay (Drogoudi and Pantelidis, 2011).

A different trend was observed at 4.5°C, where waxed plus dehydrated fruit from Tzaneen showed the highest total antioxidant activity quantified by DPPH assay

followed by Musina with the lowest total antioxidant activity quantified by DPPH assay found in fruit from Groblersdal. The results suggested that the treatment combination (wax plus dehydration) can be used to enhance the total antioxidant activity quantified by DPPH assay at suitable storage temperature (Shahidi *et al.,* 2006). 'Benny' valencia citrus fruit with low total antioxidant activity quantified by DPPH assay were followed by severe rind pitting development compared to fruit with high accumulation of total antioxidant activity quantified by DPPH assay (waxed plus dehydrated treatment). However, these findings were inconsistent with those observed by Ncama (2016) who found that 'Marsh' grapefruit treated with wax had no significant effect on the total antioxidant activity quantified by DPPH assay. It has been reported that plants synthesise compounds such as antioxidants in order to reduce the damaging effects of oxidative and other stresses (Posmyk *et al.,* 2005). According to Connor *et al.* (2002), wax plus dehydration treatment could have influenced total antioxidant quantified by DPPH. In addition, cold storage temperature plays an important role at postharvest storage of citrus fruit (Magwaza *et al.,* 2014). In this study, the total antioxidant activity quantified by DPPH assay was affected by storage temperature. High accumulation of total antioxidant activity quantified by DPPH assay in 'Benny' valencia fruit stored 4.5°C, could have contributed in enhancing rind pitting tolerance in the fruit (Porat *et al.,* 2004).

5.4.3 The effect of production sites, treatments and cold storage temperatures on total antioxidant activity quantified by FRAP assay

In this study, high total antioxidant activity quantified by FRAP assay was accompanied by minimal manifestation of rind pitting symptoms. This result also suggested that environmental conditions such as temperature and rainfall from

Groblersdal (Table 4.3, **page 57**) were not favourable for maintaining antioxidant capacity in 'Benny' valencia flavedo (Drogoudi and Pantelidis, 2011). However, low antioxidant capacity in 'Benny' valencia citrus fruit was accompanied by severe manifestation of rind pitting symptoms. The results of this study showed that environmental conditions (vapour pressure deficit) from Musina (Table 4.1, **page 57**) were favourable for maintaining total antioxidant activity quantified by FRAP assay followed by the environmental conditions from Tzaneen (Table 4.2, **page 57**).

In a treatment where no wax plus dehydration was applied, total antioxidant activity quantified by FRAP assay increased in fruit harvested from all three production sites. This explains that no wax plus dehydration affected total antioxidant activity quantified by FRAP assay (Connor *et al.,* 2002). However, wax plus dehydration resulted in higher total antioxidant activity quantified by FRAP assay in fruit harvested from Musina and Groblersdal stored at 4.5°C. Wax plus dehydration treatment could have influenced total antioxidant quantified by DPPH (Yesbergenova *et al.*, 2005).

Storage temperature also plays an important role at postharvest of citrus fruit (Magwaza *et al.,* 2014). The effect of storage temperature on influencing total antioxidant activity quantified by FRAP was profound. In 'Benny' valencia citrus fruit, total antioxidant activity quantified by FRAP assay was maintained and enhanced by low temperature (-0.6°C) than 4.5°C temperature regime. This suggested that low storage temperature slow fruit metabolism, biochemical and physiological activities leading to rind pitting tolerance and high antioxidant capacity (Porat *et al.,* 2004). These results were similar to those of Mditshwa *et al.* (2013) who reported that storage temperature increased total antioxidants activity quantified by FRAP assay in

‘Eureka’ lemon fruit stored at -0.5°C. However, high temperature (4.5°C) accelerates biochemical, physiological and metabolic activities in the fruit, resulting in high depletion of defence systems such as antioxidants (Siboza, 2013). This was supported by the low manifestation of rind pitting in fruit stored at -0.6°C compared to those stored at 4.5°C.

5.4.4 The effect of production sites, treatments and cold storage temperatures on total antioxidant activity quantified by ABTS assay

Production site played a significant role in changes of total antioxidant activity quantified by ABTS assay. However, ‘Benny’ valencia fruit harvested from Groblersdal were associated with high rind pitting than fruit harvested from Tzaneen and Musina production sites. In addition, ‘Benny’ valencia fruit from Musina and Tzaneen were found to have low total antioxidant activity quantified by ABTS assay and had less rind pitting damage. Environmental conditions such as rainfall and relative humidity prevailing at production sites may have an influence on fruit quality at postharvest (Re *et al.,* 1999).

The low total antioxidant activity quantified by ABTS assay in ‘Benny’ valencia citrus fruit from Musina and Tzaneen was associated with defence mechanisms against rind pitting (Leong and Shui, 2002). Whereas, high total antioxidant activity quantified by ABTS assay in fruit from Groblersdal was associated with poor defence mechanism against rind pitting. These results were similar to those of Siboza (2013), whereby, environmental conditions prevailing at farm location had increased total antioxidant activity quantified by ABTS assay in ‘Eureka’ lemon fruit.

Wax plus no dehydration treatment resulted in higher total antioxidant activity quantified by ABTS assay in fruit from Tzaneen stored at -0.6°C compared with other

postharvest treatments. Ncama *et al.* (2017) reported similar findings, whereby, high antioxidant activity was observed in ‘Marsh’ grapefruit citrus treated with wax and stored at -0.6°C. The results suggested that wax plus no dehydration treatment can be used to increase total antioxidant activity measured by ABST.

5.4.5 The effect of production sites, treatments and cold storage temperatures on total antioxidant activity quantified by ORAC assay

In Musina production site, environmental factors were found to be unfavourable for antioxidant capacity measured by ORAC assay. Wang (2010) found similar findings whereby, the level and activity of antioxidants in ‘Navel’ orange fruit were affected by pre-harvest factors (environmental conditions). This was also observed in antioxidant capacities measured by other assays and by the development of rind pitting symptoms (Wang, 2003). In addition, the accumulation total antioxidant activity quantified by ORAC assay in ‘Benny’ valencia citrus fruit was depended on production sites. The difference of this accumulation was associated with the environmental factors from production sites (Lee *et al.,* 2006). This study suggested that environmental conditions from Groblersdal were favourable for triggering defence mechanisms in the fruit followed by Tzaneen conditions (Wang, 2003).

Combination of wax plus dehydration enhanced total antioxidant activity quantified by ORAC assay (Wang, 2003). The low total antioxidant activity quantified by ORAC assay in ‘Benny’ valencia fruit flavedo were associated with the low contribution of total antioxidant activity quantified by ORAC assay in defence against rind pitting (Srivastava *et al.,* 2007). The results of this study also provide evidence that postharvest storage temperatures may influence the antioxidant capacity in ‘Benny’ valencia fruit total antioxidant activity quantified by ORAC assay (Thaipong *et al.,*

2006). ‘Benny’ valencia fruit that were stored at -0.6°C were rind pitting tolerant with high total antioxidant activity quantified by ORAC assay than those stored at 4.5°C which were rind pitting susceptible. These findings suggested that storage of ‘Benny’ valencia fruit at -0.6°C reduced rind pitting by increasing antioxidant capacity in the fruit (Pérez-Jiménez *et al.,* 2008).

The increased total antioxidant activity quantified by ORAC assay could have been involved in defence mechanisms against the manifestations of rind pitting in ‘Benny’ valencia fruit. Dávila-Aviña *et al.* (2014) observed high total antioxidant activity quantified by ORAC assay in ‘Grandela’ tomato fruit treated with carnauba wax over 28 days of storage at 10°C, which agree with findings from this study. It has been explained that as a secondary response, some post-harvest treatments could encourage mechanisms that affect the metabolic activity of the treated fruit, such as triggering the fruit antioxidant mechanism (Gonzalez-Aguilar *et al.,* 2010).

5.5 Conclusion

Production sites and treatments and storage temperatures affected antioxidant activity measured by FRAP, ORAC and DPPH. The high increase in total antioxidant activity in wax plus dehydration treated fruit was associated with the defence response against rind pitting stress triggered by wax plus dehydration treatment. Cold storage temperature played an important role in the development of rind pitting. ‘Benny’ valencia citrus fruit with high antioxidant capacity (wax plus dehydration treated) were found to be tolerant to rind pitting, whereas, fruit with low antioxidant capacity were found to be susceptible to rind pitting.

CHAPTER 6

THE CONTRIBUTION OF PRODUCTION SITES, POSTHARVEST TREATMENTS AND COLD STORAGE TEMPERATURES ON NON-CHILLING RIND PITTING AND SOLUBLE SUGARS OF 'BENNY' VALENCIA CITRUS FRUIT

Abstract

Non-chilling rind pitting occurrence on 'Benny' valencia citrus fruit in relation to soluble sugars influenced by production sites, postharvest treatments and cold storage temperatures is not documented. Therefore, this study evaluated the contribution of these factors on 'Benny' valencia fruit non-chilling rind pitting development and the relationship with soluble sugars. During 2016 and 2017 seasons, fruit were harvested from Tzaneen, Groblersdal and Musina in South Africa. Afterwards, fruit were subjected to the following treatments: T_1 = no wax and dehydration, T_2 = wax and dehydration and T_3 = wax and no dehydration. Dehydrated treatments were applied for 3 days at relative humidity of ±45%; thereafter, fruit were stored at -0.6 and 4.5°C for 28 days plus 7 days of shelf-life. After rind pitting incidence (RPI) analysis, the flavedo was removed, freeze dried, thereafter, soluble sugars were analysed using a High Pressure Liquid Chromatography (HPLC). Results showed 'Benny' valencia fruit fructose was highly affected by season, production sites, postharvest treatments and cold storage temperature, therefore, low rind pitting. The highest glucose and low rind pitting incidence was observed in fruit harvested from Groblersdal irrespective of treatments and cold storage temperatures when compared with those from Tzaneen and Musina. However, fruit harvested from Groblersdal subjected to wax plus dehydration treatments and stored at 4.5°C had higher sucrose and low rind pitting

when compared with Tzaneen and Musina. Production of rind soluble sugars could have probably been part of a defence system against rind pitting damage, and maintaining fruit quality. This study suggested that soluble sugars in ‘Benny’ valencia flavedo during cold storage are involved in rind pitting tolerance mediated by wax plus dehydration treatment.

Keywords: Cold storage temperature, fructose, glucose, rind pitting, quality, sucrose

6.1 Introduction

Rind pitting is a non-chilling temperature physiological disorder affecting several citrus fruit cultivars (Cronje *et al.,* 2017; Alférez *et al.,* 2005). This disorder manifests as collapsed areas of the sub-epidermal rind cells with no discoloration-taking place at the early stages. Later, oil gland clusters are affected, thereby, releasing an intercellular content, ultimately, changing colourless lesions to brown colour. Several studies have reported non-chilling rind pitting disorder mostly in 'Nules Clementine' mandarin fruit (Olarewaju *et al.,* 2017), 'Turkey' orange fruit (Mothapo *et al.,* 2018) and 'Marsh' grapefruit (Ncama *et al.,* 2017). However, its occurrence on 'Benny' valencia citrus fruit has not been documented. Ehlers (2016) reported that it was highly susceptible. 'Benny' valencia citrus fruit are known as the non-chilling injury sensitive cultivar, however, highly susceptible to non-chilling related disorders such as; rind pitting and staining (Cronje *et al.,* 2017). Thus, Cronje *et al.* (2017) evaluated various plant growth regulators in reducing pitting incidence in 'Benny' valencia. However, other known contributing factors such as production sites, wax application plus dehydration treatment and cold storage temperature have not been evaluated on this citrus cultivar.

In 'Marsh' grapefruit, waxing has been shown to significantly promote rind pitting (Wild, 1991). Whereas, storage temperature below the cultivar specific temperature threshold may lead to chilling injury damage (Dou, 2005). Exposure to low relative humidity followed by rehydration at high relative humidity has been reported to result into rind pitting (Alférez *et al.,* 2003). In 2018, Mothapo *et al.* (2018) demonstrated that production sites influenced rind pitting occurrence and quality of 'Turkey' sweet orange fruit. This was mainly attributed to differing environmental conditions in different study areas. Apart from the environmental factors, rind dry matter, soluble

sugars, chlorophylls and carotenoids are also among physico-chemical properties related to difference in citrus fruit susceptibility to rind physiological disorders (Assimakopoulou *et al*., 2009; Ncama *et al.,* 2017).

Previous studies found that inside canopy rind soluble sugars were significantly lower than fruit from outside canopy, therefore, high rind break down was observed (Magwaza *et al.,* 2013). Huang *et al.* (2000) found that high rind sucrose concentration in orange cultivars, which is an osmoregulatory compound in cells, has been associated with decreased osmotic potential in plant cells. In addition, high sugar concentration has been reported to serve as energy reserves and also contribute to rind cell structure sustenance (Zhu *et al.,* 2011). However, rind physiological disorders development has been associated with inadequate supply of rind soluble sugars in 'Nules Clementine' mandarin fruit (Olarewaju *et al.,* 2017). Therefore, the objective of this study was to evaluate the production sites, postharvest treatments and cold storage temperatures as the contributing factors to non-chilling rind pitting on 'Benny' valencia fruit and its relationship with rind soluble sugars.

6.2 Materials and methods

6.2.1 Fruit source, postharvest treatments and storage

Fruit source, postharvest treatments and storage were determined as previously explained in Chapter 4 (**page 56**).

6.2.2 Determination of rind pitting incidence

Rind pitting incidence was determined as explained on Chapter 3 (**page 34**).

6.2.3 Reagents and standards

All chemicals were purchased from Sigma-Aldrich Company Ltd. (Dorset, UK).

6.2.4 Sample preparation

Ten (10) fruit per treatment were manually peeled to remove the flavedo with a fruit peeler (Model: 80SPE, Verimark Shogun, Johannesburg, South Africa). The flavedo were frozen in liquid nitrogen and stored at -21°C before being freeze-dried using Vitris Benchtop freeze drier system (Model: ES, SP Industries Inc., Warmister, USA) at 0.015 kPa and -54°C. Afterwards, freeze dried samples were milled into fine powder using pestle and mortar and stored in -21°C for further analysis.

6.2.5 Extraction and determination of rind soluble sugars

The extraction and determination of rind soluble sugars were carried out according to Patton *et al.* (2007). Briefly, soluble sugars were extracted from 0.5 g of dried flavedo powder using 10 mL of 80% (v/v) methanol. The samples were left for 1 h in warm water (80°C) with occasional agitation. Afterwards, filtered through Whatman™ filter paper (Model: WHA1450070, Whatman™, Darmstadt, Germany) to obtain liquid extract and evaporated in a Genvac evaporator (Model: EZ 2.3, IPSWICH, Winchester, UK) to remove alcohol. Before samples were filtered, the alcohol was replaced with 10 mL of distilled water through 0.25 µm syringe nylon filter and put in glass vials for HPLC analysis. Concentrations of glucose, sucrose and fructose were determined using isocratic HPLC system (Model: LC-10A, Shimadzu, Tokyo, Japan) equipped with a refractive index detector. Sample extracts were injected into a Rezex RCM monosaccharide Ca^{+} (8%) column of 7.8 mm diameter x 300 mm length. The column temperature was set at 80°C using a thermoregulated column compartment. The mobile phase used was HPLC-grade water at a flow fi 0.6

mL/min. The presence and concentration of the individual soluble sugars were determined by comparing peak area of samples against peak area of known standard concentrations using formulae from known curves (0.05-1.25 mg/ml; R^2 = 0.97-0.99).

6.2.6 Statistical analysis

Experiment was laid out as a factorial design arrangement with the following factors: seasons (2016 and 2017), production sites (Tzaneen, Musina and Groblersdal); postharvest treatments (no wax plus dehydration, wax plus dehydration and wax plus no dehydration) and cold storage temperatures (-0.6 and 4.5°C). All statistical analyses were performed using GenStat 18th (VSN International, Hemel Hempstead, UK). Data were subjected to analysis of variance (ANOVA) with season, production site, postharvest treatment and cold storage temperature as factors. Where significant differences were detected, means were separated using Duncan's New Multiple Range Test (DNMRT) at the 5% level of significance.

6.3 Results

6.3.1 The effect of production sites, treatments and cold storage temperatures on rind pitting

Season, production sites, treatments and cold storage temperatures interaction had a significant effect (P=0.0006) on rind pitting. 'Benny' valencia citrus fruit treated with no wax plus dehydration had the highest rind pitting incidence in all production sites, regardless of cold storage temperature during the 2016 season (Figure 6.1). However, Musina fruit treated with no wax plus dehydration had high rind pitting incidence when compared with fruit from Tzaneen and Groblersdal at both cold storage temperature during 2017 season (Figure 6.1). The lowest rind pitting was

detected in ‘Benny’ valencia citrus fruit harvested from Groblersdal, irrespective of wax plus dehydration treatments at both cold storage temperatures.

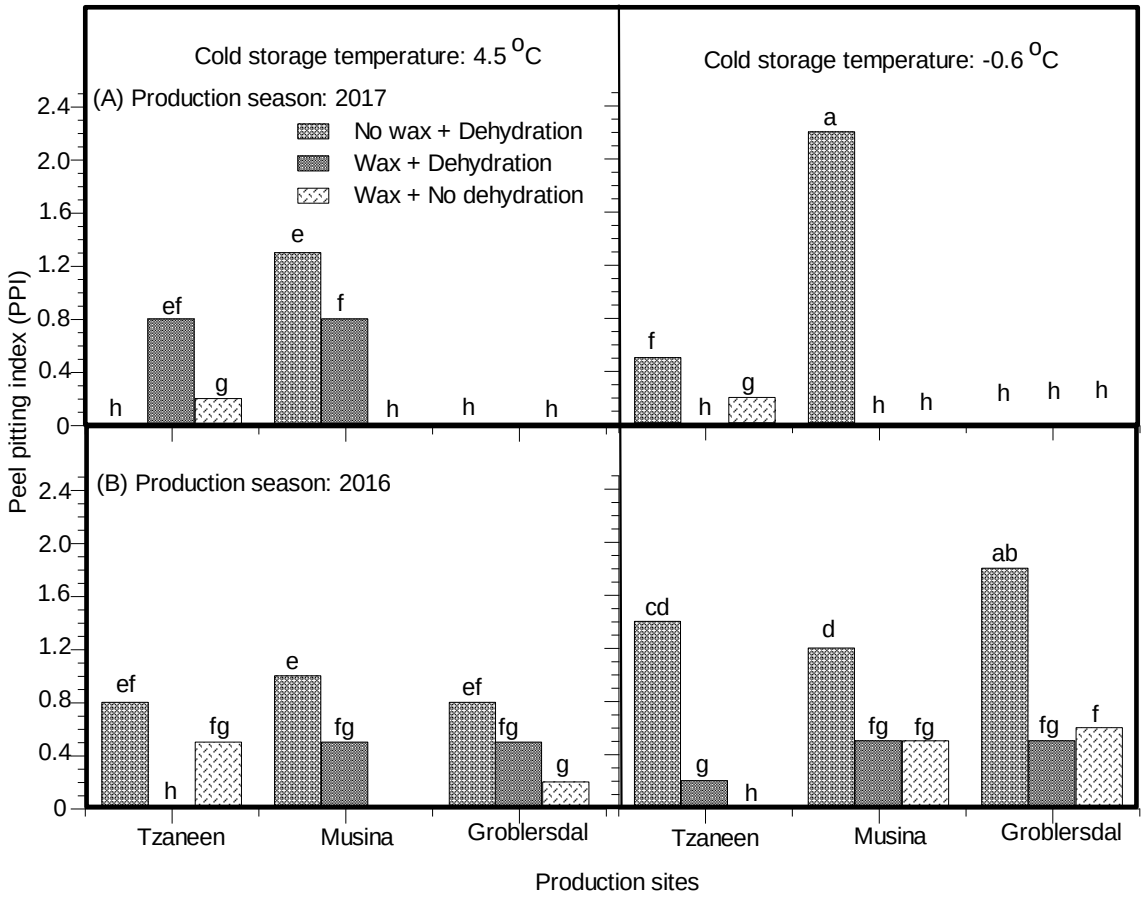

Figure 6.1: Effect of production sites, postharvest treatments and cold storage temperatures on rind pitting incidence of ‘Benny’ valencia citrus fruit in 2016 and 2017 seasons. Mean values followed by different letter (s) within same graph are significantly different ($p<0.05$)

6.3.2 Effect of production sites, postharvest treatments and cold storage temperature on fructose

The interaction between season, production sites, postharvest treatments and cold storage temperature had a high significant effect ($P<0.0001$) on ‘Benny’ valencia fructose (Figures 6.2). However, fruit harvested from Groblersdal treated with no wax plus dehydration and wax plus dehydration; and stored at both storage

temperatures, had high concentrations of fructose compared to those from Tzaneen and Musina during 2016 season. During 2017 season, fruit harvested from Musina fruit treated with wax plus no dehydration had the highest fructose concentrations at both storage temperatures than Tzaneen and Groblersdal. Moreover, fruit harvested from Tzaneen treated with no wax plus dehydration treatment and wax plus dehydration had low concentrations of fructose, irrespective of cold storage temperature in 2016 season. The lowest fructose concentrations were also observed in fruit harvested from Groblersdal treated with no wax plus dehydration regardless of cold storage temperature in 2017 season.

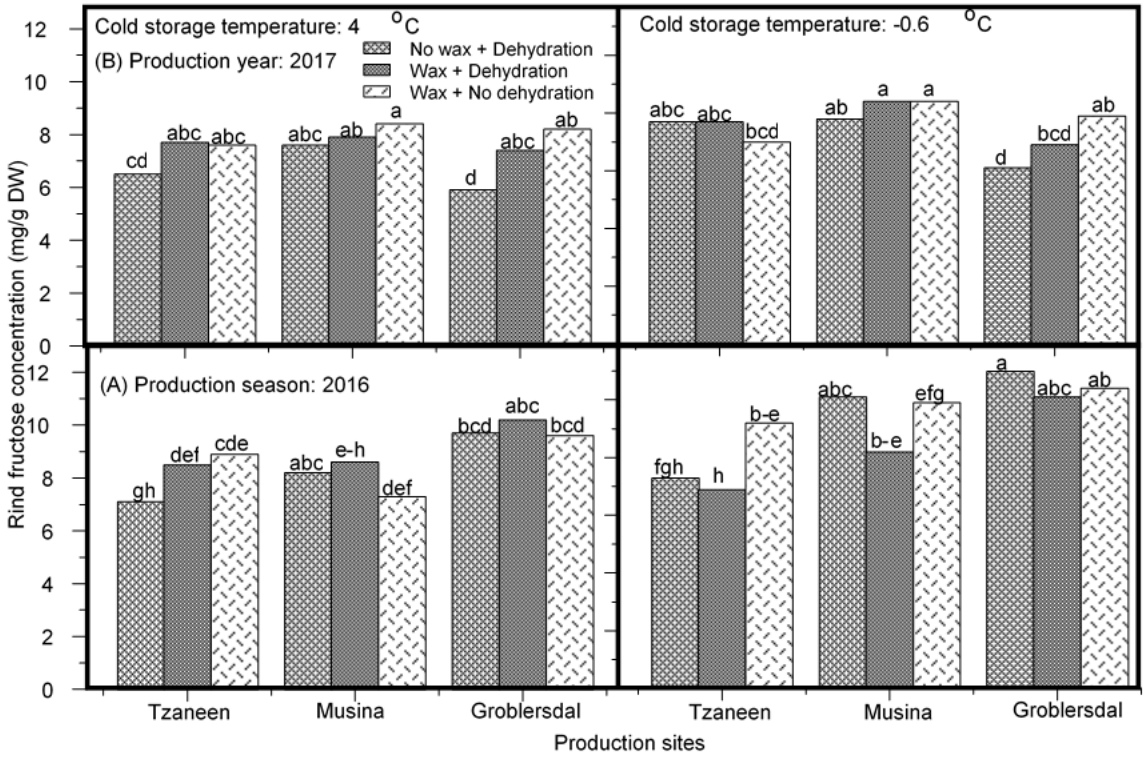

Figure 6.2: Effect of production site, postharvest treatments and cold storage temperatures on rind fructose concentration of 'Benny' valencia citrus fruit in 2016 and 2017 seasons. Mean values followed by different letter (s) within the same graph are significantly different ($p<0.05$)

6.3.3 Effect of production sites, postharvest treatments and cold storage temperatures on glucose

Season, production site, and treatments cold storage temperatures interaction had a significant effect (P=0.0037) on 'Benny' valencia fruit glucose. The highest glucose was observed in fruit harvested from Groblersdal irrespective of treatments and cold storage temperatures when compared with those from Tzaneen and Musina in 2016 season (Figure 6.3). During 2017 season, the highest glucose was observed in fruit harvested from Musina and Tzaneen treated with no wax plus dehydration treatment at both storage temperatures (Figure 6.3). Moreover, fruit harvested from Tzaneen treated with no wax plus dehydration had the lowest glucose than Groblersdal and Musina, regardless of cold storage temperature in 2016 season. Low glucose was detected in fruit harvested from Groblersdal treated with wax plus no dehydration irrespective of cold storage temperature, during 2017 season.

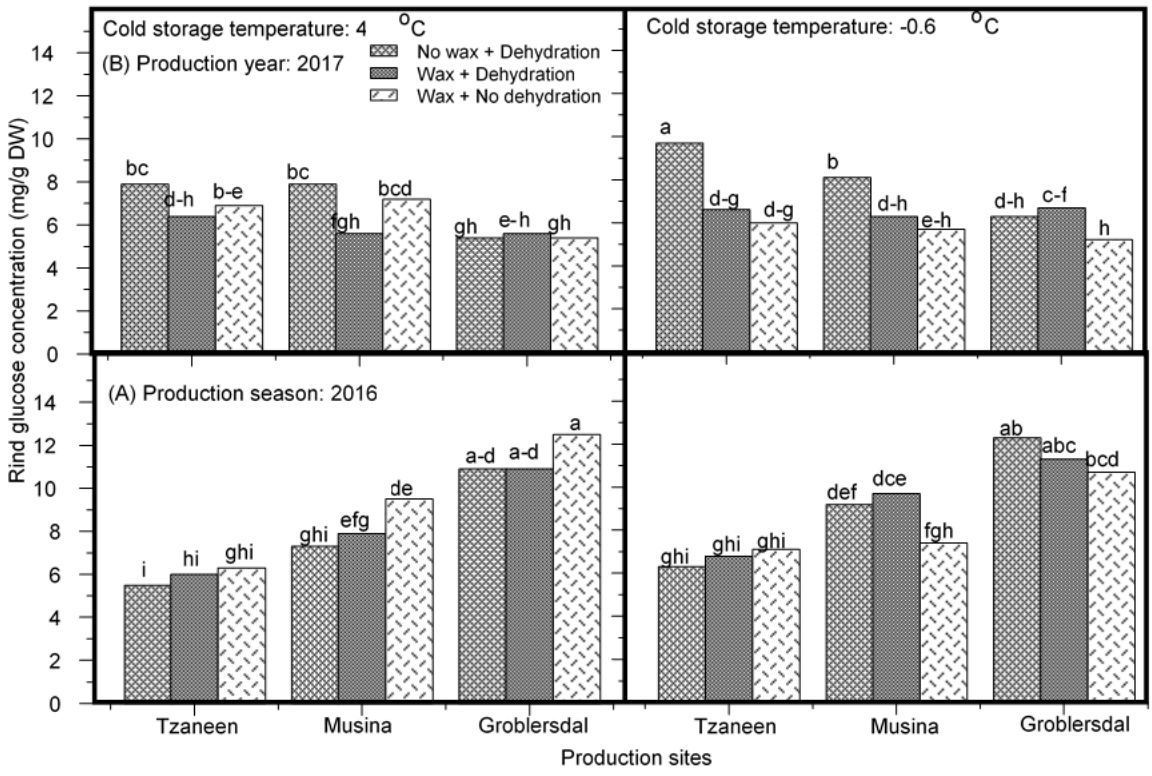

Figure 6.3: Effect of production sites, postharvest treatments and cold storage temperatures on rind glucose concentration of ‘Benny’ valencia citrus fruit in 2016 and 2017 seasons. Mean values followed by different letter (s) within the same graph are significantly different ($p<0.05$).

6.3.4 Effect production sites, postharvest treatments and cold storage temperatures

The effect of season, production site, treatments and cold storage temperatures on sucrose is shown in Figures 6.4. Season, production site, treatments and cold storage temperature had a significant effect (P=0.0134) on ‘Benny’ valencia fruit sucrose. However, sucrose was high in fruit harvested from Groblersdal treated with wax plus dehydration and stored at 4.5°C when compared with Tzaneen and Musina in 2016 season. Furthermore, sucrose was also high in fruit harvested from Musina treated with wax plus no dehydration and stored at -0.6°C. In 2017 season, fruit harvested from Tzaneen had the highest sucrose, irrespective of treatments and cold storage temperature. However, the lowest sucrose was observed in Musina fruit treated with no wax plus dehydration than those from Groblersdal and Tzaneen, regardless of cold storage temperature in both seasons.

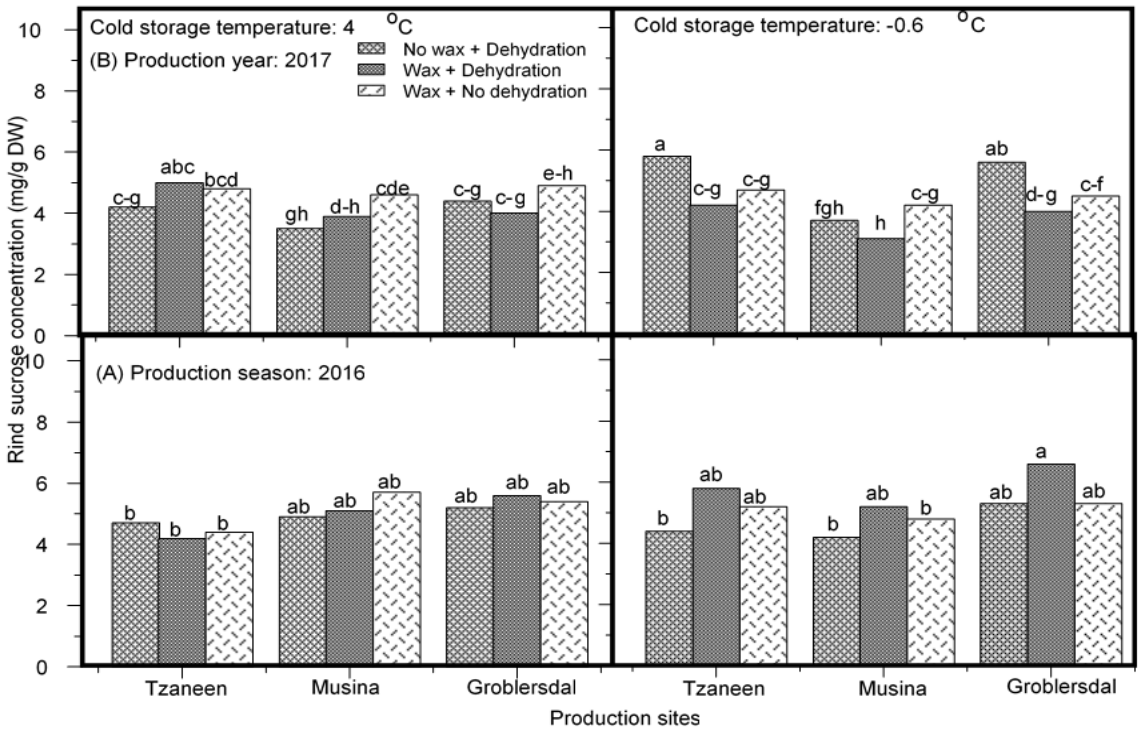

Figure 6.4: Effect of production sites, postharvest treatments and cold storage temperatures on rind sucrose concentration of 'Benny' valencia citrus fruit in 2016 and 2017 seasons. Mean values followed by different letter (s) within the same graph are significantly different ($p<0.05$)

6.4 Discussion

6.4.1 The effect of production sites, postharvest treatments and cold storage temperatures on rind pitting

In this study, environmental conditions such as temperature, relative humidity and rainfall at Musina and Tzaneen were more favourable for rind pitting resistance than environmental conditions from Groblersdal. Therefore, low relative humidity from Groblersdal played a significant role in influencing the development of rind pitting in 'Benny' valencia fruit. However, Musina fruit treated with no wax plus dehydration had high rind pitting incidence when compared with fruit from Tzaneen and Groblersdal at both cold storage temperature during 2017 season. These results

were supported by Alférez *et al.* (2005), whereby, fruit exposed to dehydration stress at low relative humidity (RH) experienced higher moisture loss which, ultimately led to rind damage and therefore, resulting in high rind pitting. Additionally, Mothapo *et al.* (2018) found that 'Benny' valencia fruit from different production sites were significantly affected by dehydration and failed to recover after being transferred to higher RH. This could be associated with higher rind pitting observed in no wax plus dehydration treatment across all production sites.

The lowest rind pitting incidence was detected in 'Benny' valencia citrus fruit harvested from Groblersdal, irrespective of wax plus dehydration treatments and cold storage temperatures. Olarewaju *et al.* (2017) reported comparable results, whereby, production regions resulted in low rind breakdown in 'Nules Clementine' mandarin fruit. Groblersdal had low minimum RH than Musina and Tzaneen; this could be the reason for low rind pitting observed in 'Benny' valencia harvested from Groblersdal.

6.4.2 Effect of production sites, postharvest treatments and cold storage temperatures on fructose

In this study, Musina (Table 4.1, **page 57**) production region is characterised by high environmental temperature than Tzaneen (Table 4.2, **page 57**) and Groblersdal (Table 4.3, **page 57**), this also contributed to the increased fructose in fruit rind from Musina due to high temperature exposure. These results were in agreement with those of Olarewaju *et al.* (2017) who found higher fructose in 'Nules Clementine' mandarin fruit from Western Cape than those from Eastern Cape stored at 7.5± 0.5°C. However, fructose high concentration is known to serve as sources of energy

reserves and contribute to rind cell structures substance. This could be the reason for high fructose concentration observed in this study.

Fruit harvested from Tzaneen treated with no wax plus dehydration and wax plus dehydration had low concentrations of fructose, irrespective of cold storage temperatures. Comparative results were found by Siboza (2013), whereby, ‘Eureka’ lemon fruit from Sun Valley Estate had the lowest fructose concentration compared to those from New Venture Farm, irrespective of cold storage temperature. This was attributed to different environmental conditions from both production sites. In this study, the abundance of soluble sugars in rind pitting tolerant fruit could have played a role in retarding or delaying the onset of rind pitting symptoms. In citrus fruit, soluble sugars role in increasing rind pitting tolerance has been associated with its ability to protect fruit against oxidative damage (Holland *et al.,* 2005).

6.4.3 Effect of production sites, postharvest treatments and cold storage temperatures on glucose

The results of this study showed that season and production sites influenced Benny valencia fruit glucose. This was due to seasonal environmental factors prevailing on production sites such temperature, rainfall and relative humidity. Furthermore, the highest glucose was observed in fruit harvested from Musina and Tzaneen treated with no wax plus dehydration at both storage temperatures. Siboza (2013) found high glucose in ‘Eureka’ lemon fruit treated with wax than untreated fruit stored at -0.5°C. High glucose accumulation was associated with the cold acclimation, which in turn enhanced chilling tolerance in the ‘Benny’ valencia fruit (Holland *et al.,* 2005). In addition, Patton *et al.* (2007) reported that glucose and sucrose play significant roles in maintaining membrane integrity during chilling stress by protecting the physical

characteristics of the membrane. Therefore, low glucose observed could be associated with multiple physiological functions during stress exposure. In general, citrus fruit contain three dominating flavedo sugars, with glucose present in the highest concentration and reducing sugars (fructose and sucrose) in lower concentration (Aung *et al*., 2001).

6.4.4 Effect of production sites, treatments and cold storage temperatures on sucrose

Magwaza *et al.* (2014) found carbohydrates use as a global biochemical marker of rind physiological disorder impossible due to citrus fruit's exposure to different pre-harvest and postharvest factors. Findings obtained in the current study support this statement as fruit sucrose responded differently in both seasons due to different environmental factors. However, the lowest sucrose was observed in Musina fruit treated with no wax plus dehydration than fruit from Groblersdal and Tzaneen, regardless of cold storage temperature in both seasons. Cronje *et al.* (2011) reported similar results, whereby, rind sucrose in 'Nules Clementine' mandarin fruit from Eastern Cape were lower than those from Western Cape. This could be attributed to high environmental temperature exposure that resulted in reduced sink strength effect on fruit (Cronje *et al*., 2011; Magwaza *et al*., 2013). In addition, sucrose has been directly linked with plant stress tolerance to physiological rind disorders (Holland *et al*., 2002).

6.5 Conclusion

Production site determines flavedo soluble sugars in citrus fruit; and therefore, rind pitting susceptibility. However, effect of wax plus dehydration treatment on 'Benny' valencia rind pitting tolerance was associated with treatment's ability to induce

soluble sugars accumulation. Furthermore, soluble sugars are believed to be involved in the defence mechanisms against rind pitting in the fruit. These results indicate that wax plus dehydration treatment in ‘Benny’ valencia citrus fruit make tissue more resistant to rind pitting by increasing biochemical compounds such as soluble sugars and other compounds that may be linked to play a role or function in the defence mechanisms.

CHAPTER 7

EXPRESSION OF GENES ASSOCIATED WITH 'BENNY' VALENCIA CITRUS FRUIT NON-CHILLING RIND PITTING DISORDER

Abstract

Investigating the genes expressed in citrus rind during pitting would be an effective tool to protect citrus fruit quality. Therefore, this study was conducted to investigate genes associated with the rind pitting of 'Benny' valencia citrus fruit. During 2017 season, fruits were harvested from Tzaneen, Groblersdal and Musina in South Africa. Afterwards, fruit were subjected to the following treatments: T_1 = no wax and dehydration, T_2 = wax and dehydration and T_3 = wax and no dehydration. Furthermore, treatments were applied for 3 days at relative humidity of ±45%, thereafter; fruit were stored at -0.6 and 4.5°C for 28 days plus 7 days of shelf-life. After rind pitting analysis, the flavedo was removed, stored at -80°C and used to analyse differentially expressed genes in citrus rind by quantitative reverse transcription polymerase chain reaction (RT-PCR) method. The pitting rind and no-pitting rind were selected as the tester and driver, respectively. Three homologic genes: CsCP gene; CsNAC-domain protein gene; CsCP-F gene; were chosen to examine the relationship between their expression and citrus rind pitting through quantitative RT-PCR analysis in pitting and non-pitting fruits. Results showed that the expression of CsCP, CsNAC and CsCP-F genes were relatively higher in fruit harvested from Tzaneen and low rind pitting was observed. Groblersdal and Musina fruit had low gene expression and low rind pitting was also observed. In conclusion, gene expression changes provided clues about the possible mechanisms involved in rind pitting disorder development. Therefore, these findings suggested that CsCP,

CsNAC and CsCP-F genes may be linked with rind pitting and could serve as targets for future investigation.

Keywords: Cold storage temperature, CsCP gene, CsCP-F gene, CsNAC gene, rind pitting

7.1 Introduction

In the past, efforts have been made to investigate factors triggering non-chilling rind pitting, but little is known about the molecular basis of non-chilling rind pitting development (Fan *et al.,* 2007). Previously, a study was done on suppression subtractive cDNA library to screen differentially expressed genes in citrus fruit with rind pitting, in which the pitting rind and rind with no pitting were used as tester and driver (Gao *et al.,* 2006). By screening, it was expected that some genes which may play significant roles in non-chilling rind pitting development would be found. Classification of these genes could provide some simplified strategies to reduce the citrus non-chilling rind pitting by altering their expression levels, e.g. treating with or preventing from specific biotic or abiotic stimulus. Fan *et al.* (2009) reported that the screening of differentially expressed sequences in citrus rind pitting area led to the isolation of a cDNA fragment showing significant similarities to cysteine protease genes from other species. Therefore, the objective of this study was to determine genes expressed in citrus rind showing non-chilling pitting with quantitative write in full first (RT-PCR), in which the pitting rind and no-pitting rind were respectively used as tester and driver.

7.2 Materials and methods

7.2.1 Fruit sampling, treatments and storage

Fruit source, postharvest treatments and storage were determined as previously explained in Chapter 4 (**page 56**). **Importantly, only one season (2017) data was used for quantitative RT-PCR due to the expensive nature of this analysis**.

7.2.2 Determination of non-chilling rind pitting incidence

Rind pitting incidence was determined as explained in Chapter 3 (**page 34**).

7.2.3 Sample preparation

Ten (10) fruit per treatment were manually peeled with a fruit peeler (Model: 80SPE, Verimark Shogun, Johannesburg, South Africa). The peels were frozen in liquid nitrogen and stored at -21°C before being freeze-dried using Vitris Benchtop freeze drier system (Model: ES, SP Industries Inc., Warmister, USA) at 0.015 kPa and -54°C. Afterwards, freeze dried samples were milled into fine powder using pestle and mortar and stored in -21°C for further analysis.

7.2.4 Quantitative RT-PCR

Total RNA was extracted from tested tissues of 'Benny' orange using Trizol reagent (Dingguo, China), and then treated with DNase I. Reversed transcription (RT) was performed on lg DNase-treated total RNA using M-MLV Reverse Transcriptase (Model: M-MLV, Promega Darmstadt, Germany) according to the manufacturer. Quantitative RT-PCR (qPCR) was carried out using iCycler™ Real Time PCR System (Model: 71842, Bio-Rad, Darmstadt, Germany). The citrus RNA polymerase II gene (GenBank accession no. EF17442 (Liu *et al.,* 2008) was amplified as reference gene in parallel with the target gene allowing gene expression normalisation and to provide quantification. Primer sequences were as follows: CRPII-F50-TAACAACAAATGCTGATGG-30 and CRPIIR5 0-CGAGATGGAATAGCGTGTG-GAT-30 for reference gene; CsCP-F 50-TCATCTGAAAATGTTGGCG-30 and CsCP-R 50-AACAGCAACGACGGCAT-30 for target gene. The qPCR was done using the SYBR® *Premix Ex Taq*™ Master mix kit (Model, T3073, Takara, Tokyo, Japan) following the manufacture's

recommendations. Reaction mixtures (25 µL) consisted of 29 SYBR® *Premix Ex Taq*™ Master mix (Model, T3073, Takara, Tokyo, Japan), 0.5 µM of each primer of CRPII or 0.2 µM of each primer of CsCP, 1 µL template (the equivalent of 50 ng total RNA). The qPCR conditions comprised of one cycle at 95°C for 30 s, followed by 42 cycles at 95°C for 10 s, 55°C for 30 s and 72°C for 20 s. For each sample, reactions were set up in triplicate to ensure the reproducibility of the results. At the end of qPCR, melting curves were performed to verify the specificity of amplification products. The PCR efficiency of reference and target genes were determined by generating standard curves based on serial dilutions of plasmids containing reference gene and target gene, respectively. The quantification of the relative expression levels was performed using the comparative CT method (Livak and Schmittgen, 2001). The relative expression of CsCP gene in each sample was normalised against citrus RNA polymerase II gene and calculated according to 2-DDCT, where DDCT = (CT,CsCP - CT,CRP) stressed - (CT,CsCP - CT,CRP) control. The calibrator whose relative expression was arbitrarily set as equal to one was defined as the sample giving the highest DCT (DCT = CT,CsCP - CT,CRP) value which corresponded to the lowest level of expression. The mean and standard error were calculated from the triplicate samples from each time point/tissue.

7.2.5 RNA Gel Blot Analysis

For RNA gel blot analysis, total RNA was extracted from flavedo tissues and then denatured at 65°C and separated on a 1% (w/v) formaldehyde denatured agarose gel (10 µg per lane). The RNA was transferred to a Hybond-N+ membrane (Amersham Biosciences, Darmstadt, Germany) for at least 15 h and fixed on the membrane using a UV Crosslinker. Blots were prehybridized in Church buffer (Church and Gilbert, 1984) [7% sodium dodecyl sulfate (SDS), 300 mM sodium

phosphate pH 7.4, 1 mM EDTA] at 65°C for at least 1 h. The DNA probe consisted of a PCR-amplified fragment corresponding to a conserved amino-terminal region of CsNAC and labeled to high specific activity by random priming at 37°C with [32P]-dCTP according to Random Primer Labeling Kit in Church buffer. After a 20-h hybridisation period, the membrane was washed three times with 0.5×SSC and 0.1% SDS at 65°C and exposed to autoradiography film at -80°C.

7.2.6 Statistical analysis

Experiment was laid out as a factorial design arrangement with the following factors: production sites (Tzaneen, Musina and Groblersdal); postharvest treatments (no wax plus dehydration, wax plus dehydration and wax plus no dehydration) and cold storage temperatures (-0.6 and 4.5°C). All statistical analyses were performed using GenStat 18th (VSN International, Hemel Hempstead, UK). Data were subjected to analysis of variance (ANOVA) with production sites, cold storage temperatures and treatments as factors. Where significant differences were detected, means were separated using Duncan's New Multiple Range Test (DNMRT) at 5% level of significance.

7.3 Results

7.3.1 Effect of production site, cold storage temperature and treatments on RPI of 'Benny' valencia fruit

The interaction between production sites, cold storage temperatures and treatments had a significant effect (P=0.0060) on rind pitting incidence. 'Benny' valencia fruit harvested from Tzaneen had the highest rind pitting incidence, regardless of treatments and cold storage temperatures (Figure 7.1). However, low rind pitting

incidence was observed in fruit harvested from Groblersdal and Musina, irrespective of treatments and storage temperatures.

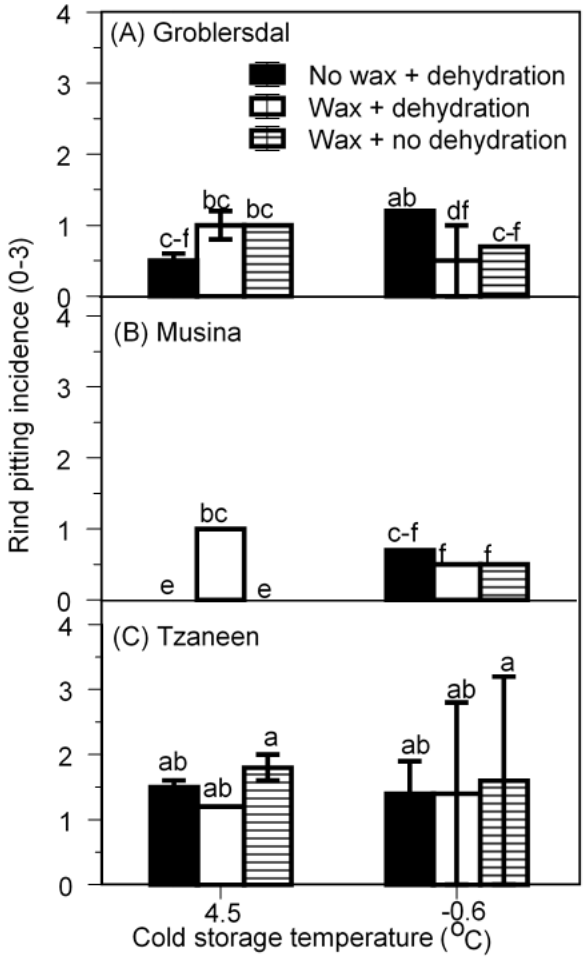

Figure 7.1: Rind pitting incidence of ‘Benny’ valencia fruit influenced by an interaction between production site (A), postharvest treatments (B) and cold storage temperature (C), (P=0.0006). Means values followed by different letter (s) are significantly different

7.3.2 Effect of production site, treatments and cold storage temperature on CsCP, CsNAC and CsCP-F genes in relation to rind pitting incidence

Production sites, cold storage temperature and treatments interaction was significant (P=0.0006) on CsCP gene. Fruit harvested from Tzaneen showed high CsCP gene when compared with fruit from Groblersdal and Musina, irrespective of treatments and cold storage temperatures (Figure 7.2). Low CsCP gene was observed in fruit harvested from Groblersdal at both cold storage temperature and treatments. However, low rind pitting incidence was detected in fruit harvested from Musina, irrespective of cold storage temperatures and treatments. Furthermore, production sites, cold storage temperature and treatments interaction was significant (P=0.0002) on CsNAC gene. High CsNAC gene was dectected in fruit harvested from Tzaneen, regardless of cold storage temperature and treatments (Figure 7.2). Fruit harvested from Groblersdal and Musina had low CsNAC gene in both cold storage temperatures and treatments. Low rind pitting incidence was also observed in fruit harvested from Musina, regardless of cold storage temperatures and treatments. In addition, production sites, cold storage temperature and treatments interaction was significant (P<0.0001) on CsCP-F gene. In all treatments and storage temperatures, fruit harvested from Tzaneen expressed high CsCP-F gene with high rind pitting incidence (Figure 7.2). However, fruit harvested from Groblersdal and Musina showed low CsCP-F gene, irrespective of cold storage treatments and treatments.

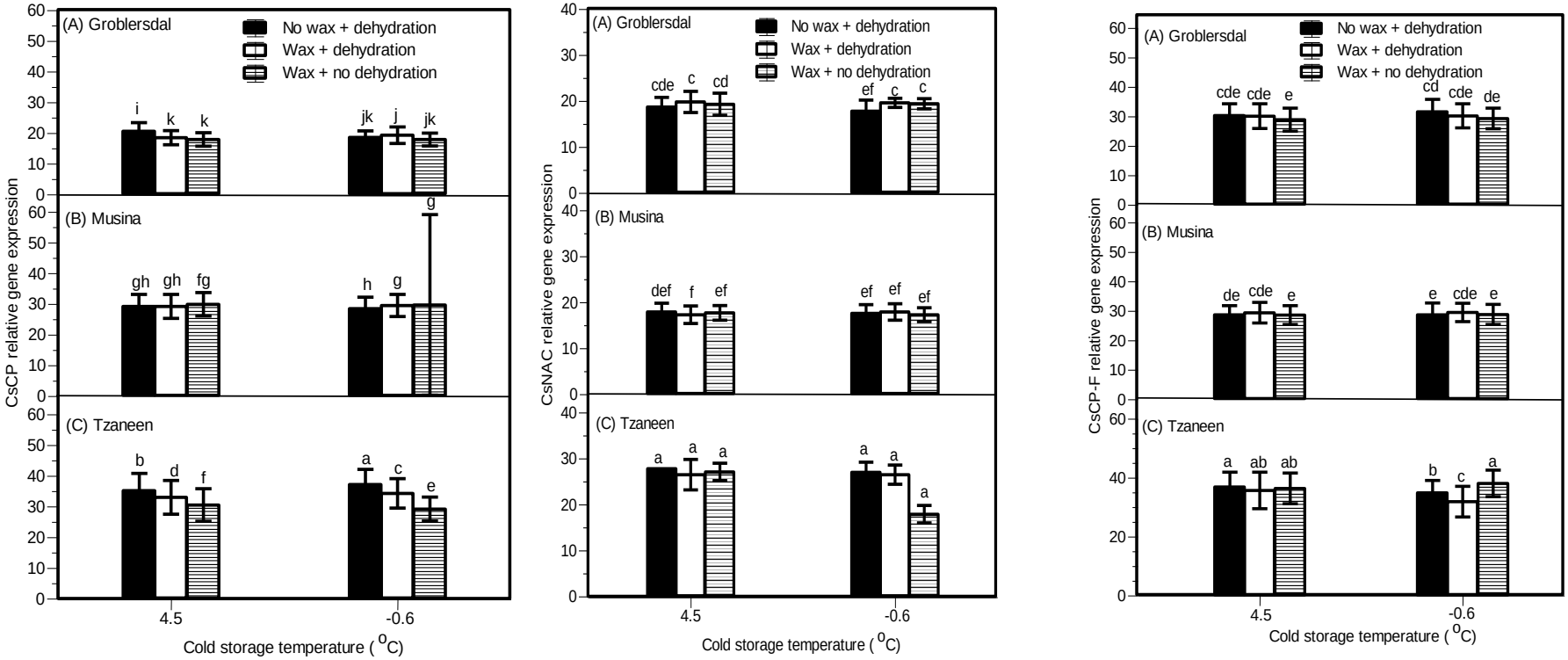

Figure 7.2: CsCP, CsNAC and CsCP-F genes expression of 'Benny' valencia fruit influenced by an interaction between production site (A), postharvest treatments (B) and cold storage temperature (C), CsCP gene (P=0.0006), CsNAC gene (P=0.0002) and CsCP-F gene (P<0.0001). Means values followed by different letter (s) within same graph are significantly different

7.4 Discussion

7.4.1 Effect of production site, treatments and cold storage temperature on CsCP, CsNAC and CsCP-F genes in relation to rind pitting incidence.

According to Fan *et al.* (2009), CsCP gene expression in 'Navel' orange was rapidly and strongly affected by hypoxia (3% O_2), which suggested its role at early stage of rind pitting induced by low O_2. It is possible that the hypoxia stress results in increasing of alcohols and aldehydes which are toxic compounds to plant cells; in response to CsCP gene, the might be up-regulated in order to remobilise the cellular materials (cell wall, cell membrane, etc.) out of the damaged cells (Li *et al.,* 2000). In spite of physiological disorders being induced by different environmental conditions, different gene expression in different disorders indicates that patterns may still be used as good molecular markers for the susceptibility of citrus fruit to develop flavedo pitting under different postharvest conditions (Sanchez-Ballesta *et al.,* 2008). In addition to its rind pitting-associated function, CsCP gene was also developmentally regulated and for that, it can be related to senescence associated phenomena.

Previously, Gao *et al.* (2006) used 'Navel' orange as experimental material to screen differentially expressed genes in citrus rind pitting by suppression subtractive hybridisation (S SH), in which the pitting and non-pitting rinds were performed as tester and driver, respectively. In this study, the expression of CsNAC gene was induced rapidly and strongly by wounding or rind pitting incidence. It is indicated that the CsNAC is not only a wound-inducible gene but also an anoxia-related gene, which may play an important role in citrus postharvest rind pitting caused by abiotic stresses (Singh *et al.,* 2002).

Furthermore, CsNAC genes have been shown to be involved in many processes of plant development, such as lateral root formation in auxin signalling (Xie *et al.,* 2000), leaf senescence (John *et al.,* 1997), formation of flower organ promordia (Sablowski and Meyerowitz, 1998), and responses to biotic or abiotic stresses (Hegedus *et al.,* 2003). Fan *et al.* (2007) reported that low temperature (4°C) and exposure to ethylene also increased the expression level of CsNAC gene. In this study, results showed that low temperature increased expression of CsNAC gene as it was highly expressed at both 4.5 and -0.6°C storage temperatures.

About two fold increase of CsCP-F transcript level was observed in 'Marsh' grapefruit at 35-day of storage, and was followed by a slightly lower expression at 55-day of storage (Livak and Schmittgen, 2001). In this study, the high CsCP-F was observed at 28 day of storage in fruit harvested from Tzaneen production site. This could be due to environmental conditions such as high temperature at Tzaneen, which might have resulted in high expression of CsCP-F. According to Li *et al.* (2006) high temperature (40°C) did not reduce CsCP-F gene expression level until 24 h after treatment in *Lolium multiflorum* leaves. Additionally, CsCP-F gene expression was rapidly reduced by low rind petting index in this study. In the studies by Fan *et al.* (2007, 2009) and Gao *et al.* (2006, 2009), the physiological and molecular response was studied by wounding 'Navel' orange fruit. Therefore, the conclusion to be drawn from these observations is that these genes are involved in stress response related to physical damage and possibly to the disorders.

7.5 Conclusion

Overall, the results obtained in this study provided an understanding into the previous unknown complexities of 'Benny' valencia fruit rind pitting. The discovery of

differential expression of genes in rind pitting allowed the visualisation of mechanisms that may play a role in this disorder. Gene expression changes also provided clues about the possible mechanisms involved in the development of this disorder. Taken together, the present investigation demonstrates the application of a powerful approach of combining the high throughput technology of SSH and RT-PCR analysis to study differential expression of genes in citrus rind pitting. Our results may provide the basis for future research of NAC-like gene's role in stress-induced citrus rind pitting.

CHAPTER 8

GENERAL CONCLUSIONS, COMMERCIAL RECOMMENDATIONS AND FUTURE RESEARCH

8.1 General conclusion

The study demonstrated that production sites, postharvest treatments had an influence on the physico-chemical properties of citrus fruit. Furthermore, non-chilling rind pitting incidence was influenced by the production site and postharvest treatments. Fruit harvested from Groblersdal which was waxed and dehydrated had the highest incidence of rind pitting compared to fruit from Tzaneen and Musina, irrespective of postharvest treatments when stored at chilling temperature (-0.6°C).

Rind biochemical concentrations (vitamin C, total phenolics and flavonoids) in citrus differs from one another and mostly influenced by environmental factors. Moreover, production sites and postharvest treatments had an influence on rind biochemical concentrations associated with non-chilling rind pitting incidence. In addition, postharvest treatments resulted in low non-chilling rind pitting with an increased accumulation of rind biochemical concentrations. These results suggested that there was a systemic link between non-chilling rind pitting and rind biochemical concentrations of ‘Benny’ valencia citrus fruit.

Postharvest treatments (wax plus dehydration) affected antioxidant activity measured by FRAP, ORAC and DPPH assays. However, high induction of antioxidant capacities in wax plus dehydration treated fruit was associated with the defence response against non-chilling rind pitting. In addition, cold storage temperature played a significant role in non-chilling rind pitting development. ‘Benny’ valencia fruit with high antioxidant capacity were found to be tolerant to rind pitting,

whereas, fruit with low antioxidant capacity were found to be susceptible to rind pitting. However, the effect of wax plus dehydration treatment on non-chilling rind pitting tolerance of ‘Benny’ valencia citrus fruit was associated with the treatment’s ability to induce the accumulation of soluble sugars. Furthermore, soluble sugars are believed to be involved in the defence mechanisms against non-chilling rind pitting in the fruit. These results indicated that wax plus dehydration treatment in ‘Benny’ valencia citrus fruit made tissue more resistant to rind pitting by increasing soluble sugars and other compounds that may be linked to play a role or function in the defence mechanisms.

Overall results obtained in this study provided an understanding into the previous unknown complexities of citrus non-chilling rind pitting. The discovery of differentialexpression of genes in non-chilling rind pitting allowed the visualisation of mechanisms that may play a role in this fruit disorder. Gene expression changes also provided clues about the possible mechanisms involved in non-chilling rind pitting development.

8.2 Commercial recommendations

The results from this study suggested that production sites should be considered as it was shown to be responsible for ‘Benny’ valencia fruit non-chilling rind pitting development. Moreover, exposing fruit to dehydration stress at low RH (45%) is a key factor leading to non-chilling rind pitting occurrence. Therefore, during postharvest handling, waxing fruit could be important as it reduces water loss and rind disorders occurrence, therefore, maintaining fruit quality. In addition, for successful rind disorders reduction during ‘Benny’ valencia orange fruit commercial

shipment, focus must not be on the single factor but rather a strategy that includes multiple factors to maintain fruit quality.

The study further demonstrated the importance of total phenolics and flavonoids, vitamin C, soluble sugars, total antioxidants capacity and genes on ‘Benny’ valencia rind and how these compounds vary across production sites. Furthermore, rind soluble sugars and total antioxidants capacity could be possible biochemical indicators of fruit susceptibility to non-chilling rind pitting.

8.3 Future research

This study has shown that higher rind soluble sugars concentration is the major determinant for non-chilling rind pitting resistance. Therefore, future studies need to investigate orchard practises to increase flavedo soluble sugars concentration. Clearly, flavedo soluble sugars concentration also determines other compounds concentrations, including bioactive compounds with antioxidant properties, which seem to play a significant role in mitigating oxidative stress. Furthermore, the present investigation demonstrated the application of a powerful approach of combining the high throughput technology of SSH and RT-PCR analysis to study differential gene expression in citrus non-chilling rind pitting. The results may provide the basis for future research of NAC-like gene’s role in stress-induced citrus rind pitting. In addition, the antioxidant chapter did not include enzymatic antioxidants analysis. Therefore, future research is required to use assays to estimate the strength of enzymatic antioxidants and their ability to mitigate non-chilling rind pitting. Moreover, future research should involve non-destructive measurement by the use of near infrared spectroscopy (NIR) to evaluate and predict internal and external fruit quality in relation to non-chilling rind pitting.

REFERENCES

Abeysinghe, D.C., Li, X., Sun, C., Zhang, W., Zhou, C. and Chen, K. 2007. Bioactive compounds and antioxidant capacities in different edible tissues of citrus fruit of four species. *Food Chemistry*, 104: 1338-1344.

Agusti, M., Almela, V., Juan, M., Alférez, F., Tadeo, F.R. and Zacarías, L. 2001. Histological and physiological characterization of rind breakdown of 'Navelate' sweet orange. *Annual Botany*, 88: 415-422.

Albrigo, L.G. 1970. Distribution of stomata and epicular wax on oranges as related to stem-end rind breakdown and water loss. *Journal of the American Society for Horticultural Science*, 97: 220-223.

Alférez, F., Agusti, M. and Zacarías, L. 2003. Postharvest rind staining in 'Navel' oranges is aggravated by changes in storage relative humidity: Effect on respiration, ethylene production and water potential. *Postharvest Biology and Technology*, 28: 143-152.

Alférez, F. and Burns, J.K. 2004. Postharvest rind pitting at non-chilling temperatures is promoted by changes from low to high relative humidity during storage. *Postharvest Biology and Technology*, 32: 79-87.

Alférez, F. and Zacarías, L. 2001. Postharvest pitting in 'Navel' oranges at non-chilling temperature: Influence of relative humidity. *Acta Horticulturae*, 553: 307-308.

Alférez, F. and Zacarías, L. 2012. Influence of fruit maturity in the susceptibility of 'Navelina' oranges to develop postharvest non-chilling rind pitting. *Food Science and Technology International*, 20: 183-191.

Alférez, F., Zacarías, L. and Burns, J. K. 2005. Low relative humidity at harvest and before storage at high humidity influence the severity of postharvest rind pitting in citrus. *Journal of American Society and Horticultural Science*, 130: 225-231.

Almela, V., Agustí, M. and Juan, M. 1992. El "picado" del fruto de la mandarina Fortune. *Descripción de la alteración y factores que lo influyen. Levante Agricola*, 319:80-86.

Ali, M., Chernova, T.A., Newnam, G.P., Yin, L., Shanks, J., Karpova, T.S., Andrew Lee, A., Laur, O., Subramanian, S., Kim, D., McNally, J, Seyfried, N.T., Yury O Chernoff, Y.O., Wilkinson K.D. 2004. Stress-dependent proteolytic processing of the actin assembly protein Lsb1 modulates a yeast prion. *Journal of Biology and Chemistry*, 40: 27625-39.

Alquezar, B., Mesejo, C., Alférez, A., Agusti, M. and Zacarías, L. 2010. Morphological and ultrastructural changes in rind of 'Navelate' oranges in relation to variations in relative humidity during postharvest storage and development of rind pitting. *Postharvest Biology and Technology*, 56: 163-170.

Assimakopoulou, A., Tsougrianis, C., Elena, K., Fasseas, C. and Karabourniotis, G. 2009. Pre-harvest rind spotting in 'Clementine' mandarin. *Journal of Plant Nutrition*, 32: 1486-1497.

Aung, L.H., Jenner, J.F., Fouse, D.C. and Houck, L.G. 1999. Postharvest conditioning temperatures and fruit maturity on flavedo soluble sugars of coastal and desert lemons. *Journal of Horticultural Science and Biotechnology*, 3: 288-292.

Aung, L.H., Obenland, D.M. and Houck, L.G. 2001. Conditioning and heat treatments influence flavedo soluble sugars of lemon. *Journal of Horticultural Science and Biotechnology*, 73: 399-402.

Babbar, N., Oberoi, H.S., Uppal, D.S. and Patil, R.T. 2011. Total phenolic content and antioxidant capacity of extracts obtained from six important fruit residues. *Food Research International*, 44: 391-396.

Bassal, M. and El-Hamahmy, M. 2011. Hot water dip and preconditioning treatments to reduce chilling injury and maintain postharvest quality of Navel and Valencia oranges during cold quarantine. *Postharvest Biology and Technology*, 60: 186-191.

Bramlage, W.J. and Weiss, S.A. 1997. Effects of temperature, light and rainfall on superficial scald susceptibility in apples. *Horticultural Science*, 32: 808-811.

Ben-Yehoshua, S., J. Peretz, R. Moran, B. Lavie, and Kim, J.J. 2001. Reducing the incidence of superficial flavedo necrosis (noxan) of 'Shamouti' oranges (*Citrus sinensis*, Osbeck). *Postharvest Biology and Technology*, 22: 19-27.

Böhm, V., Kuhnert, S., Rohm, H. and Scholze, G. 2006. Improving the nutritional quality of microwave-vacuum dried strawberries: A preliminary study. *International Journal of Food Science and Technology*, 12: 67-75.

Bourtoom, T., 2008. Edible films and coatings, characteristics and properties. *International Food Research Journal*, 15: 1-12.

Cajuste, J.F., Lafuente, M.T., 2007. Ethylene-induced tolerance to non-chilling rind pitting as related to phenolic metabolism and lignin content in 'Navelate' fruit. *Postharvest Biology and Technology*, 45: 193-203.

Chaudhary, P.R., Jayaprakasha, G.K., Porat R, Patil and B.S. 2014. Low temperature conditioning reduces chilling injury while maintaining quality and certain bioactive compounds of 'Star Ruby' grapefruit. *Food Chemistry*, 153: 243-249.

Chaudhary, P.R., Yu, Y., Jayaprakasha, G.K., Patil and B.S. 2017. Influence of storage temperature and low-temperature conditioning on the levels of heath-promoting compounds in 'Rio Red' grapefruit. *Food Science and Nutrition*, 5: 545-553.

Chen, Z. and Gallie, D.R. 2004. The ascorbic acid redox state controls guard cell signaling and stomatal movement. *Plant Cell*, 16: 1143-1162.

Citrus Growers Association of Southern Africa. 2019. Annual Research Report. www.cga.co.za.

Cogo, S.L.P., Chaves, C.F., Schirmer, M.A., Zambiazi, R.C. and Nora, L. 2011. Low soil water content during growth contributes to preservation of green colour and bioactive compounds of cold stored broccoli (*Brassica oleracea* L.*). Postharvest Biology and Technology*, 60: 158-163.

Cohen, E., Shalom, Y., Rosenberger, I., 1990. Postharvest ethanol builds up and off flavor in 'Murcott' tangerine fruit. *Journal of American Society of Horticultural Science*, 115: 775-778.

Cohen, E., Shapiro, B., Shalom, Y. and Klein, J. D. 1994. Water loss: a non-destructive indicator of enhanced cell membrane permeability of chilling-injured citrus fruit. *Journal of American Society of Horticultural Science*, 119: 983-986.

Connor, A.M., Luby, J.J. and Hancock, J.F. 2002. Changes in fruit antioxidant activity among blueberry cultivars during cold-temperature storage. *Journal of Agriculture and Food Chemistry*, 50: 893-898.

Cronje, P.J.R., 2007. *Postharvest rind disorders of citrus fruit*. Citrus Research International. Nelspruit, South Africa.

Cronje, P.J.R., Barry, G.H. and Huysamer, M. 2011. Postharvest rind breakdown of 'Nules Clementine' mandarin is influenced by ethylene application, storage

temperature and storage duration. *Postharvest Biology and Technology*, 60: 192-201.

Cronje, P.J.R., Ehlers, J.L., Alférez, F. and Theron, K.I. 2017. Efficacy of pre-harvest sprays to reduce rind pitting on 'Benny' valencia citrus fruit. *Citrus Research* and *Technology*, 38: 160-168.

Crowe, L.M. 2002. Lessons from nature: the role of sugars in anhydrobiosis. Comparative Biochemistry and Physiology, Part A: *Molecular Integrative Physiology*, 131: 505-513.

Dávila-Aviña, J.E., Villa-Rodríguez, J.A., Villegas-Ochoa, M.A., Tortoledo-Ortiz, O., Olivas, G.I., Ayala-Zavala, J. F. and González-Aguilar, G.A. 2014. Effect of edible coatings on bioactive compounds and antioxidant capacity of tomatoes at different maturity stages. *Journal of Food Science and Technology*, 51: 2706-2712.

Dong, J., Yu, Q., Lu, L. and Xu, M. 2012. Effect of yeast saccharide treatment on nitric oxide accumulation and chilling injury in cucumber fruit during cold storage. *Postharvest Biology and Technology*, 68: 1-7.

Dou, H. 2004. Effect of coating application on chilling injury of grapefruit cultivars. *Postharvest Biology and Technology*, 39: 558-561.

Dou, H. 2005. The influence of harvesting time and geographical location susceptibility to physiological rind disorders associated with four Florida grapefruit cultivars. *Journal of Horticultural Science and Biotechnology*, 80: 466-470.

Drogoudi, P.D. and Pantelidis, G. 2011. Effects of position on canopy and harvest time on fruit physico-chemical and antioxidant properties in different apple cultivars. *Sciencia Horticulturae* 129: 752–760.

Duarte, A.M.M. and Guardiola, J.L. 1995. Factors affecting rind pitting in the mandarin hybrids ‘Fortune’ and ‘Nova’. The influence of exogenous growth regulators. *Acta Horticulturae*, 379: 59-67.

Echeverria, E. and Valich, J. 1989. Enzymes of sugar and acid metabolism in stored valencia orange. *Journal of American Society of Horticultural Science*,114: 445-449.

Ehlers, J.L. 2016. Postharvest rind pitting studies on valencia oranges. MSc. Agriculture thesis. University of Stellenbosch, Stellenbosch, South Africa.

El-Hilali, F., Ait–Oubahou, A., Remah, A. and Akhayat, O. 2003. Chilling injury and peroxidase activity changes in ‘Fortune’ mandarin fruit during cold storage. *Bulgarian Journal of Plant Physiology*, 29: 44-54.

El-Otmani, M., Ait-Oubahou, A., and Zacarías, L. 2011. “*Citrus spp*. orange, mandarin, tangerine, clementine, grapefruit, pomelo, lemon and lime,” in Postharvest Biology and Technology of Tropical and Subtropical Fruits, ed. E. M. Yah. 437-516

Erkan, M., Pekmezci, M. and Wang, Y.C. 2005. Hot water and curing treatments reduce chilling injury and maintain postharvest quality of valencia oranges. *International Journal of Food Science and Technology*, 40: 91-96.

Erlund, I. 2004. Review of the flavonoids quercetin, hesperetin, and naringenin. Dietary sources, bioactivities, bioavailability and epidemiology. *Nutrition Research,* 24: 851-874.

Estables-Ortiz, B., Lafuente, M.T., Forment, J. and Gadea, J. 2009. Transcriptomic analysis of ethylene-induced tolerance to non-chilling rind pitting in citrus fruit. *Acta Horticulturae*, 839: 555-560.

Fan, J., Gao, X., Yang, Y.W., Deng, W. and Li, Z.G. 2007. Molecular cloning and characterization of a NAC-like gene in 'Navel' orange fruit response to postharvest stresses. *Plant Molecular Biology Reporter*, 25: 145-153.

Fan, J., Yang, W.Y., Gao, X., Deng, W., Falara, V., Kanellis, A.K. and Li, Z.G. 2009. Expression of a senescence-associated cysteine protease gene related to rind pitting of 'Navel' orange (*Citrus sinensis* L. Osbeck*). Plant Cell Tissue Organ Culture*, 98: 281-289.

FAO, 2013. Food and Agriculture Organization of the United Nations, Rome, Italy, www.faostat.org.

Gao, X., Li, Z.G., Fan, J. and Yang, Y.W. 2006. Screening and expression of differentially expressed genes for rind pitting of citrus fruit. *Acta Horticulturae*, 712: 473-479.

Gao, X., Li, Z.G., Fan, J., Yang, Y.W., Falara, V., Kanelis and A.K. 2009. Molecular cloning and expression of an expasin-like gene in 'Navel' fruit during postharvest stresses. *Plant Growth Regulation*, 59: 13-19.

Gonzalez-Aguilar, G.A., Fortiz, J., Cruz, R., Baez R. and Wang, C.Y. 2010. Methyl jasmonate reduces chilling injury and maintains postharvest quality of mango fruit. *Journal of Agriculture and Food Chemistry*, 48: 515-519.

Gonzalez-Aguilar, G.A., Zacarías, L., Perez-Amador, M.A., Carbonell, J. and Lafuente, M.T. 2000. Polyamine content and chilling susceptibility are affected by seasonal changes in temperature and by conditioning temperature in cold-stored 'Fortune' mandarin fruit. *Plant Physiology*, 108: 140-146.

Gonzalez-Molina, E. 2010. Natural bioactive compounds of *Citrus limon* for food and health. *Journal of Pharmaceutical and Biomedical analysis*, 51: 327-345.

Grierson, W. and Ben-yehoshua, S. 1986. Storage of citrus fruits. In Fresh Citrus Fruits; Wardowski, V. F., Nagy, S., Grierson, W., Eds.; Avi Publishing Co. Inc.: Westport, CT, pp 479-507.

Hagen, S.F., Borge, G.I.A., Solhaug, K.A., Bengtsson, G.B., Bilger, W., Berge, A., Haffner, K. and Solhaug, K.A. 2007. Phenolics contents and other health and sensory related properties of apple fruit (*Malus domestica* Borkh., cv. Aroma). *Postharvest Biology and Technology*, 45: 1-10.

Hassan, Z.H., Lesmayati, S., Qomariah, R. and Hasbianto, A. 2014. Effects of wax coating applications and storage temperatures on the quality of Tangerine citrus (*Citrus reticulata*) var. Siam Banjar. *International Food Research Journal*, 21: 641-648.

Hegedus, D., Yu, M., Baldwin, D., Gruber, M., Sharpe, A. and Parkin, I. 2003. Molecular characterization of *Brassica napus* NAC domain transcriptional activators induced in response to biotic and abiotic stress. *Plant Molecular and Biology*, 53: 383-97.

Holland, N., Menezes, H.C. and Lafuente, M.T. 2002. Carbohydrates as related to the heat-induced chilling tolerance and respiratory rate of 'Fortune' mandarin fruit harvested at different maturity stages. *Postharvest Biology and Technology*, 25, 181-191.

Holland, N., Menezes, H.C. and Lafuente, M.T. 2005. Carbohydrate metabolism as related to high-temperature conditioning and rind disorders occurring during storage of citrus fruit. *Journal of Agriculture and Food Chemistry*, 53: 8790-8796.

Hordjik, J., 2013. Studies to reduce the incidence of chilling injury in ‘Navel’ orange fruit. PhD Agric, thesis. University of Stellenbosch, Stellenbosch, South Africa.

Hu, H., Li, X., Dong, C. and Chen, W. 2011. Effects of wax treatment on quality and postharvest of pineapple fruit in cold storage. *Biotechnology*, 39: 7592-7603.

Huang, R., Xia, R., Hu, L., Lu, Y. and Wang M. 2000. Antioxidant activity and oxygen-scavenging system in orange pulp during ripening and maturation. *Scientia Horticulturae*, 113:166-172.

Hung, D.V., Tong, S., Tanaka, F., Yasunaga, E., Hamanaka, D., Hiruma, N. and Uchino, T. 2011. Controlling the weight loss of fresh produce during postharvest storage under a nano size mist environment. *Journal of Food Engineering*, 106: 325-330.

Hussain, P., Suradkar, P., Wani, A., and Dar, M. 2015. Retention of storage quality and post-refrigeration shelf-life extension of plum (*Prunus domestica* L.) cv. 'Santa Rosa' using combination of carboxymethyl cellulose (CMC) coating and gamma irradiation. *Radiation Physics and Chemistry*, 107: 136-148.

Igual, M., García-Martínez, E., Camacho, M.M. and Martínez-Navarrete, N. 2011. Changes in flavonoids content of grapefruit juice caused by thermal treatment and storage. *Innovative Food Science and Emerging Technologies*, 12: 153-162.

Imeh U. and Khokhar, S. 2002. Distribution of conjugated and free phenols in fruits: Antioxidant activity and cultivar variations. *Journal of Agricultural and Food Chemistry*, 50: 6301-6306.

John, I., Hackett, R., Cooper, W., Drake, R., Farrell, A. and Grierson, D. 1997. Cloning and characterization of tomato leaf senescence-related cDNAs. *Plant Molecular and Biology*, 33: 641-51.

Jourbert, J. 2016. Influence of rind water content on mandarin citrus fruit quality. PhD thesis. Stellenbosch of University. Stellenbosch, South Africa.

Kanaze, F.I., Gabrieli, C., Kokkalou, E., Georgarakis, M. and Niopas, I. 2003. Simultaneous reversed-phase high-performance liquid chromatographic method for the determination of diosmin, hesperidin and naringin in different citrus fruit juices and pharmaceutical formulations. *Journal of Pharmaceutical and Biomedical Analysis*, 33: 243-249.

Kanaze, F.I., Kokkalou, E., Georgarakis, M. and Niopas, I. 2004. A validated solid-phase extraction HPLC method for the simultaneous determination of citrus flavanone aglycones hesperetin and naringenin in urine. *Journal of Pharmaceutical and Biomedical Analysis*, 36: 175-181.

Karim, M. F. and Neven M.N.N. 2012. Mitigating the incidence of preharvest rind pitting of 'Nova' tangerines by early application of GA3, the cytokinin derivative, CPPU, and potassium. *Journal of Applied Sciences Research*, 8: 4724-4732.

Karioti, A., Hadjipavlou-Litina, D., Mensah, M.L.K., Fleischer, T.C. and Skaltsa, H. 2004. Composition and antioxidant activity of the essential oils of *Xylopia aethiopica* (Dun) A. Rich. (Annonaceae) leaves stem bark, root bark and fresh and dried fruits, growing in Ghana. *Journal of Agriculture and Food Chemistry*, 52: 8094-8098.

Kaiser, C., Westgate, P.E.C. and Helm, G. 2004. Effect of mineral element concentrations on lenticel damage of mango (*Mangifera indica* L.) fruit in Mpumalanga Province, South Africa. *South African Mango Growers' Association Research Journal*, 24: 47-49.

Khorshidi, J., Tabatsbaei, M.F. and Ahmedi, F.M. 2010. Storage temperature effects on the postharvest quality of Apple (*Malus domestica* Borkh. Cv. Red Delicious). *New York Science Journal*, 3: 67-70.

Klimczak, I., Malecka, M., Szlachta, M. and Gliszezynska-Swiglo, A. 2007. Effect of storage on the content of polyphenols, vitamin C and the antioxidant activity of orange juices. *Journal of Food Composition and Analysis*, 20: 313-322.

Kruger, F.J. and Claassens, V. 1997. What went wrong with export avocado physiology during the 1996 season? *South African Avocado Growers' Association Yearbook*, 20: 88-92.

Kruger, F.J, Penter, M.G., Masevhe, M.R. and Combrink, N.K. 2005. The use of fruit mineral content as a tool to investigate the epidemiology of citrus rind disorders. *South African Fruit Journal*, 23: 54-59.

Ladaniya, M.S. 2008. Citrus fruit Biology, technology and evaluation. Elsevier Academic Press, New York, USA. pp 125-190.

Lado, J. Rodrigo, M.J. and Zacarías, L. 2014. Maturity indicators and citrus fruit quality. *Stewart Postharvest Review*, 2: 1-6.

Lafuente, M.T., Zacarìas, L., Martìnez-Téllez, M.A., Sanchez-Ballesta, M.T. and Granell, A. 2003. Phenylalanine ammonia-lyase and ethylene in relation to chilling injury as affected by fruit age in citrus. *Postharvest Biology and Technology*, 29: 308-317.

Lafuente, M.T. and Zacarias, L. 2006. Postharvest physiological disorders in citrus fruit. *Stewart Postharvest Review*, 1: 1-9.

Lee, S.K. and Kader, A.A. 2002. Pre-harvest and postharvest factors influencing vitamin C content of horticultural crops. *Postharvest Biology and Technology*, 20: 207-220.

Lee, K.W., Kim, Y.J., Kim, D.O., Lee, H.J. and Lee, C.Y. 2003. Major phenolics in apple and their contribution to the total antioxidant capacity. *Journal of Agriculture and Food Chemistry*, 51: 6516-6520.

Lee, S.H., Ahn, S.J., Im, Y.J., Cho, K., Chung, G.C., Cho, B.H. and Han, O. 2005. Differential impact of low temperature on fatty acid unsaturation and lipoxygenase activity in fig leaf gourd and cucumber roots. *Biochemical and Biophysical Research Communications*, 330: 1194-1198.

Leguizamon, L., Luchsinger, L. and Razeto, B. 2001. Influence of harvest season, fruit maturity and storage temperature on the development of chilling injury in 'Eureka' lemon fruit. *Acta Horticulturae*, 533: 299-300.

Leong, L.P. and Shui, G. 2002. An investigation of antioxidant capacity of fruits in Singapore markets. *Food Chemistry*, 1: 69-75.

Li, Q., Bettany, A.J., Donnison, I., Griffiths, C.M., Thomas, H. and Scott, I.M. 2000. Characterisation of a cysteine protease cDNA from *Lolium multiflorum* leaves and its expression during senescence and cytokinin treatment. *Biochimica et Biophysica Acta*, 1492: 233-236.

Li, B.B., Smith, B. and Hossain, M.M. 2006. Extraction of phenolic from citrus rinds II. Enzyme-assisted extraction method. *Separation and Purification Technology*, 48: 189-196.

Li, Z.G., Gao, X., Fan, J., Yang, Y.W., Li, D.G. and Kanellis, A.K. 2006. The relationship between activity and gene expression of phenylalanine ammonia-lyase and rind pitting in 'Fengjie' navel orange fruits. *Journal of Plant Physiology and Molecular Biology*, 32: 381-386.

Li, Z.G., Fan, J., Gao, X., Yang, Y., Deng, W., Yuan, L. 2009. The mechanism and the control of rind pitting of citrus fruits. *Acta Horticulturae*, 837: 287-292.

Lindhout, K., 2007. Physiology of chilling related postharvest rind breakdown of Navel oranges (Citrus sinensis (L.) Osbeck). PhD.Thesis, La Trobe University, Bundoora, Australia.

Liu, H.G., Wang, Z.K., Cao, Y.Q., Xia, Y.X. and Ying, Y.P. 2008. Detection of Citrus tristeza virus using conventional and fluorescence quantitative RT-PCR assays. *Acta Phytopathologica Sinica*, 38: 24-30.

Livak, K.J. and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C(T) method. *Methods*, 25: 402-408.

Liyana-Pathirana, C.M. and F. Shahidi. 2006. Antioxidant activity of cherry laurel fruit (*Laurocerasus officinalis* Roem.) and its concentrated juice. *Journal of Food Chemistry*, 99: 121-128.

Lyons, M. J. 1973. Chilling injury in plants. *Annual Review of Plant Physiology*, 24: 445-466.

Maguire, K., Banks, N. and Opara, L. 2001. Factors affecting weight loss in apples. *Horticultural Reviews*, 25: 197-234.

Magwaza, L.S. 2008. A preliminary study on the effect of climatic conditions and fruit mineral concentration on the development of lenticel damage in 'Tommy Atkins' and 'Keitt' mangos and rind pitting in 'Benny' valencia citrus fruit. MSc thesis, University of KwaZulu-Natal, Pietermaritzburg, South Africa.

Magwaza, L.S., U.L. Opara, Cronje, P.J., Landahl, S., Nicolai, B.M. and Terry, L.M. 2013. Non-chilling physiological rind disorders in citrus fruit. *Horticulture Review*, 41: 131-175.

Magwaza, L.S., U.L. Opara, Cronje, P.J., Landahl, S., Nieuwoudt, H.H., Mouazen, A.M., Nicolaii, B.M. and Terry, L.M. 2014. Assesment of rind quality of 'Nules Clementine' mandarin during postharvest storage: 1 Vis/NIRS PCA models and relationship with canopy position. *Scientia Horticulturae*, 165: 410-420.

Mathaba, N., Bower, J.P. and Bertling, I. 2014. Effect of production site, storage duration, hot water and molybdenum dips on bioactive compounds with antioxidant properties in lemon flavedo during cold storage. *South African Journal of Plant and Soil*, 4: 203-212.

Mditshwa, A., Fawole, O.A., Al-Said, F., Al-Yahyai, R. and Opara, U.L. 2013. Phytochemical content, antioxidant capacity and physicochemical properties of pomegranate grown in different microclimates in South Africa. *South African Journal Plant and Soil*, 30: 81-90.

Miller, J. N. 2009. *Starch: Chemistry and Technology*. Food Science and Technology (3rd ed.). Academic Press, New York, USA. pp 52-54.

Mothapo, M. J., Mathaba N., Mafeo, T.P. and Mphahlele, R.R. 2018. Effect of production site and postharvest treatments on the incidence of rind pitting and chemical properties of 'Benny' sweet orange. *Acta Horticulturae*, 15: 107-114.

Mothapo, M. J., Mathaba N. and Mafeo, T.P. 2018. Effect of production site and postharvest treatments on rind pitting and physico-chemical properties of 'Turkey' sweet orange. *Transylvanian Review*, 34: 8729-8740.

Mphahlele, R.R., Fawole, O.N., Opara, U.L. 2016. Influence of packaging system and long- term storage on physiological attributes, biochemical quality, volatile composition and antioxidant properties of pomegranate fruit. *Scientia Horticulturae*, 211: 140-151.

Ncama, K. 2016. Non-destructive prediction of 'Marsh' grapefruit (Citrus x paradisi MacFad) postharvest quality and physiological rind disorders using visible to near infrared spectroscopy. MSc thesis. University of KwaZulu-Natal, Pietermaritzburg City, South Africa.

Ncama, K., Samson, Z.T., Fawole, O.A., Opara, U.L. and Magwaza, L.M. 2017. Non-destructive prediction of 'Marsh' grapefruit susceptibility to postharvest rind pitting disorder using reflectance Vis/NIR spectroscopy. *Scientia Horticulturae*, 231: 265-271.

Njombolwana, N.S., Erasmus, A., Van Zyl, J.G du Plooy, W., Cronje, P.J. and Fourie, P.H. 2013. Effects of citrus wax coating and brush type on imazalil residue loading, green mould control and fruit quality retention of sweet oranges. *Postharvest Biology and Technology*, 86: 362-371.

Olarewaju, O.O., Magwaza, L.M., Fawole, O.A., Samson Z.T. and, Opara, U.L. 2017. Role of canopy positions on rind biochemical concentrations and radical scavenging activities in relation to rind breakdown of 'Nules Clementine' mandarins stored at non-chilling temperature. *Scientia Horticulturae*, 226: 231-240.

Patterson, B.D. and Reid, M.S. 1990. Genetic and environmental influences on the expression of chilling injury. In, Wang, C.Y. (eds.) Chilling injury of horticultural crops. CRC Press, Florida, USA. pp 87-112.

Patton, A.J., Cunningham, S.M., Volenec, J.J. and Reicher, Z.J. 2007. Differences in freeze tolerance of zoysiagrasses: II. carbohydrate and proline accumulation. *Crop Science*, 47: 2170-2181.

Pellegrini, N., Serafini, M., Colombi, B., Del, Rio, D., Salvatore, S., Bianchi, M. and Brighenti, F. 2003. Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different in vitro assays. *Journal of Nutrition*, 133: 2812-2819.

Pérez-Jiménez, J., Arranz, S., Tabernero, M., Díaz- Rubio, M.E., Serrano, J., Goñi, I. and SauraCalixto, F. 2008. Updated methodology to determine antioxidant

capacity in plant foods, oils and beverages: extraction, measurement and expression of results. *Food Research International*, 41: 274-285.

Petracek, P.D., Kelsey, D.F.and Grierson, W., 2006. Physiological rind disorders. In: Wardowski, W., Miller, W.M., Hall, D.J., Grierson, W. (Eds.), Fresh Citrus Fruit. 2nd ed. Florida Science Source, Inc., Florida, USA, pp. 397-442.

Petracek, P.P., Dou, H. and Pao, S. 1998. The influence of applied waxes on postharvest physiology behaviour and pitting of grape fruit. *Postharvest Biology and Technology*, 14:99-106.

Plaza, L., Crespo, I., de Pascual-Teresa, S., de Ancos, B., Sánchez-Moreno, C., Muñoz, M. and Cano, M.P. 2011. Impact of minimal processing on orange bioactive compounds during refrigerated storage. *Food Chemistry*, 124:646-651.

Porat, R., Weiss, B., Cohen, L., Daus, A. and Aharoni, N. 2004. Reduction of postharvest rind disorders citrus fruit by modified atmosphere packaging. *Postharvest Biology Technology*, 33: 35-43.

Posmyk, M.M., Bailly, C., Szafrańska, K. and Corbineau, F. 2005. Antioxidant enzymes and isoflavonoids in chilled soybean (*Glycine max* (*L*.) Merr.) seedling. *Journal of Plant Physiology*, 162: 403-412.

Rab, A., Naija, M., Sajid, F, Bibi, I., Nabi, G. and Nawab, K. 2015. Quality changes in heat-treated sweet orange fruit during storage at low temperature. *Journal of Animal and Plant Sciences,* 25: 661-668.

Rapisarda, S.E., Bellomo, S. and Intelisano, S. 2001. Storage temperature effects on blood orange fruit quality. *Journal of Agricultural and Food Chemistry*, 49:3230-3235.

Rapisarda, P., Lo Bianco, M., Pannuzzo, P. and Timpanaro, N. 2008. Effect of cold storage on vitamin C, phenolics and antioxidant activity of five orange genotypes [*Citrus sinensis* (L.) Osbeck]. *Postharvest Biology Technology*, 49: 348-354.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C. 1999. Antioxidant activity applying an improved ABTS•+ radical cation decolarization assay. *Free Radical Biology and medicine*, 26: 1231-1237.

Rivera, F., Pelayo-Zaldivar, C., De León, F.D., Buentello, B., Castillo-River, M. and Pérez-Flores, L.J. 2007. Cold-conditioning treatment reduces chilling injury in Mexican limes (*Citrus aurantifolia S*.) stored at different temperatures. *Journal of Food Quality*, 30: 121-134.

Robles-Sánchez, M.R., Rojas-Graü, M.A., Odriazola-Serrano, I., González-Aguilar, G.A. and Martín-Belloso, O. 2009. Effect of minimal processing on bioactive compounds and antioxidant activity of fresh-cut 'Kent' mango (*Mangifera indica* L.). *Postharvest Biology and Technology*, 51: 384-390.

Tamim, M., Goldschmidt, E.E., Goren, R. and Shachnai, A. 2001. Cold-storage affects antioxidant properties of apples in caco-2 cells. *American Society for Nutritional Sciences,* 4: 1105-1109.

Tarozzi, A., Marchesi, A., Cantelli-Forti, G. and Hrelia, P. 2004. Potassium reduces the incidence of superficial rind-pitting (nuxan) in 'Shamouti' orange. *Acta Horticulturae*, 553: 303-306.

Thaipong, K., Boonprakoba,U., Crosbyb, K., Cisneros-Zevallosc, B., Byrne, D.H. 2006. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *Journal of Food Composition and Analysis*, 19: 669-675.

Tietel. T., Lewinsohn. E., Fallik. E. and Porat. R. 2012. Importance of storage temperature in maintaining flavour and quality of mandarins. *Postharvest Biology and Technology*, 64: 175-182.

Tognetti, J. A.; Salerno, G. L.; Crespi, M. D.; Pontis, H. G. 1990. Sucrose and fructan metabolism of different wheat cultivars at chilling temperatures. *Plant Physiology*, 78: 554-559.

Treutter, D., 2001. Biosynthesis of phenolic compounds and its regulation in apple. *Plant Growth Regulation*, 34: 71-89.

Sablowski, R.W. and Meyerowitz, E.M. 1998. A homolog of no apical meristem is an immediate target of the floral homeotic genes apetala/pistillata. *Cell Journal*, 92: 93-103.

Sala, J.M., Sanchez-Ballesta, M.T., Alférez, F., Mulas, M., Zacarìas, L., Lafuente and M.T. 2005. A comparative study of the postharvest performance of an ABA deficient mutant of oranges II. Antioxidant enzymatic system and phenylalanine ammonia-lyase in non-chilling and chilling rind disorders of citrus fruit. *Postharvest Biology Technology*, 37: 232-240.

Saltveit, M.E. 2000. Discovery of chilling injury in: Kung, S.D., Yung, S. F., (Eds) Discoveries in plant biology Elsevier Academic Press, New York, USA. pp. 690-697.

Sanchez-Ballesta, M.T., Lafuente, M.T., Granell, A. and Zacarìas, L. 2001. Isolation and expression of a citrus cDNA related to rind damage caused by postharvest stress conditions. *Acta Horticulturae*, 553: 293-296.

Sanchez-Ballesta, M.T., Gosalbes, M.J., Rodrigo, M.J., Granell, A., Zacarìas, L., Lafuente, L.T., 2006. Characterization of a β-1,3-glucanase from citrus fruit as

related to chilling-induced injury and ethylene production. *Postharvest Biology and Technology*, 40: 133-140.

Sanchez-Ballesta, M.T., Zacarìas, L., Granell, A. and Lafuente, M.T. 2008. β-1,3-Glucanase gene expression as a molecular marker for postharvest physiological disorders in citrus fruit and its hormonal regulation. *Postharvest Biology Technology*, 48: 146-149.

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

Shahidi, F., Liyana-Pathirana, C.M. and Wall, D.S. 2006. Antioxidant activity of white andblack sesame seeds and their hull fractions. *Food Chemistry*, 99: 478-483.

Shin, Y., Ryu, J.A., Liu, R.H., Nock, J.F. and Watkins, C.B. 2008. Harvesting maturity, storage temperature and relative humidity affect fruit quality, antioxidant contents and inhibition of cell proliferation of strawberry fruit. *Postharvest Biology Technology*, 49: 201-209.

Siboza, X.I. 2013. *Methyl jasmonate and salicylic acid enhance chilling tolerance in lemon (Citrus limon) fruit*. PhD thesis. University of KwaZulu-Natal, Pietermaritzburg, South Africa.

Siboza, X.I. and Bertling I. 2013. The effects of methyljasmonate and salicylic acid on suppressing the production of reactive oxygen species and increasing chilling tolerance in 'Eureka' lemon (*Citrus limon*). *Journal of Horticultural Science and Biotechnology*, 88: 269-276.

Silva, E.P., Vilas Boas, E.V.B., Rodrigues, L.J. and Siqueira, H.H. 2009. Physical, chemical and physiological characterization of gabiroba fruit (*Compomanesia*

pubescens) during its development. *Food Science and Technology*, 29: 803-809.

Singh, K., Foley, R.C. and Onate-Sanchez, L. 2002. Transcription factors in plant defence and stress responses. *Current Opinion in Plant Biology*, 5: 430-436.

Smirnoff, N., 2000. Ascorbic acid: metabolism and functions of a multi-facetted molecule. *Current Opinion in Plant Biology*, 3: 229-235.

Srivastava, A., Akoh, C.C., Yi, W., Fischer, J. and Krewer, G. 2007. Effect of storage conditions on the biological activity of phenolic compounds of blueberry extract packed in glass bottles. *Journal of Agriculture and Food Chemistry*, 55: 2705-2713.

Undurraga, M.P., Olaeta, C., Retamales, A.J. and Brito, P.A. 2006. Effect of dipping in calcium solutions on peteca (rind pitting) in lemons and limes, harvested after a rainfall and cold stored. *Agricultura Tecnica. Instituto de Investigaciones Agropecuarias, Chillan*, 66: 3-12.

Van der Sluis, A. A., Dekker, M., de Jager, A. and Jongen, W. M. 2001. Activity and concentration of polyphenolic antioxidants in apple: effect of cultivar, harvest year, and storage conditions. *Journal of Agriculture and Food Chemistry*, 49: 3606-3613.

Vercher R., Tadeo, F.R., Almela, V., Zaragoza, S., Primo-Millo, E. and Agustí M. 1994. Rind structure, epicuticular wax morphology and water permeability of 'Fortune' mandarin fruits affected by rind pitting. *Annual Botany*, 74: 619-625.

Verma, L.R. and Joshi, V.K. 2000. Postharvest Technology of Fruits and Vegetables: Handling, Processing, Fermentation, and Waste Management, Volume 1 General Concepts and Principles, New Delhi: Indus. p. 120.

Villano, D., Fernandez-Pachon, M., Moya M., Troncoso, A. and Garcia-Parrilla, M. 2007. Radical scavenging ability of polyphenolic compounds towards DPPH free radical. *Talanta*, 71: 230-235.

Wang, C.Y. 2003. Maintaining postharvest quality of raspberries with natural volatile compounds. *International Journal of Food Science and Technology*, 38: 869-875.

Wang, C.Y. 2010. Alleviation of chilling injury in tropical and subtropical fruits. *Acta Horticulturae*, 864: 267-274.

Wang, H., Hu, S.H., Liang, Z.C. and Lee, M.Y. 2005. Antigenotoxicity of extracts from *Pleurotus citrinopileatus*. *Journal of Science and Food Agriculture*, 85: 770-778.

Wild, B.L. 1991. Postharvest factors governing the development of peteca rind pitting on 'Meyer' lemons. *Horticultural Science*, 26: 287-28.

Xie, Q., Frugis, G., Colgan, D. and Chua, N.H. 2000. Arabidopsis NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development. *Genes Development*, 14: 3024-36.

Yesbergenova, Z., Yang, G., Oron, E., Soffer, D., Fluhr, R. and Sagi M. 2005. The plant Mo-hydroxylases aldehyde oxidase and xanthine dehydrogenase has distinct reactive oxygen species signatures and are induces by drought and abscisic acid. *Plant Journal*, 42: 862-876.

Zhu, A., Li, W., Ye, J., Sun, X., Ding, Y., Cheng, Y. and Deng, X. 2011. Microarray expression profiling of postharvest ponkan mandarin (*Citrus reticulata*) fruit under cold storage reveals regulatory gene candidates and implications on soluble sugars metabolism. *Journal of Integrative Plant Biology*, 53: 358-374.

APPENDICES

Appendix 3.1: Analysis of variance (ANOVA) for RPI of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	0,1937	0,09685		
Production site (A)	2	6,5693	3,28463	16,20	0,0000
Treatment (B)	1	0,6446	0,64463	3,18	0,0835
Storage (C)	2	2,4737	1,23685	6,10	0,0054
A*B	2	0,0226	0,01130	0,06	0,9459
A*C	4	2,3185	0,57963	2,86	0,0382
B*C	2	0,5448	0,27241	1,34	0,2744
A*B*C	4	2,4563	0,61407	3,03	0,0307
Error	34	6,8930	0,20273		
Total	53	22,1165			

Appendix 3.2: Analysis of variance (ANOVA) for weight loss of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	0,014	0,0072		
Production site (A)	2	9,003	4,5017	54,68	0,0000
Treatment (B)	1	0,375	0,3750	4,56	0,0401
Storage (C)	2	107,741	53,8706	654,40	0,0000
A*B	2	0,901	0,4506	5,47	0,0087
A*C	4	9,076	2,2689	27,56	0,0000
B*C	2	0,221	0,1106	1,34	0,2746
A*B*C	4	0,584	0,1461	1,77	0,0272
Error	34	2,799	0,0823		
Total	53	130,715			

Appendix 3.3: Analysis of variance (ANOVA) for electrolyte leakage of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	69,55	34,775		
Production site (A)	2	574,65	287,325	6,05	0,0057
Treatment (B)	1	0,21	0,214	0,00	0,9469
Storage (C)	2	847,96	423,979	8,92	0,0008
A*B	2	755,61	377,806	7,95	0,0015
A*C	4	1223,26	305,814	6,44	0,0006
B*C	2	821,23	410,616	8,64	0,0009
A*B*C	4	811,73	202,932	4,27	0,0066
Error	34	1615,58	47,517		
Total	53	6719,78			

Appendix 3.4: Analysis of variance (ANOVA) for firmness of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	7,67	3,84		
Production site (A)	2	51,12	25,56	24,10	0,0000
Duration (B)	1	1037,26	1037,26	978,17	0,0000
Storage (C)	1	14,16	14,16	13,35	0,0005
Treatment (D)	2	21,70	10,85	10,23	0,0001
A*B	2	103,78	51,89	48,93	0,0000
A*C	2	1,92	0,96	0,90	0,4093
A*D	4	27,11	6,78	6,39	0,0002
B*C	1	0,77	0,77	0,72	0,3980
B*D	2	14,72	7,36	6,94	0,0018
C*D	2	1,02	0,51	0,48	0,6213
A*B*C	2	1,23	0,61	0,58	0,5631
A*B*D	4	4,68	1,17	1,10	0,3624
A*C*D	4	9,10	2,27	2,14	0,0843
B*C*D	2	5,99	3,00	2,83	0,0661
A*B*C*D	4	3,24	0,81	0,76	0,5530
Error	70	74,23	1,06		
Total	107	1379,68			

Appendix 3.5: Analysis of variance (ANOVA) for TSS of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	0,8674	0,4337		
Production site (A)	2	21,2385	10,6193	20,56	0,0000
Duration (B)	1	1,5408	1,5408	2,98	0,0885
Storage (C)	1	0,3008	0,3008	0,58	0,4479
Treatment (D)	2	1,4746	0,7373	1,43	0,2468
A*B	2	3,4867	1,7433	3,38	0,0399
A*C	2	3,8822	1,9411	3,76	0,0282
A*D	4	1,2681	0,3170	0,61	0,6541
B*C	1	5,0268	5,0268	9,73	0,0968
B*D	2	2,4939	1,2469	2,41	0,0026
C*D	2	0,0372	0,0186	0,04	0,9646
A*B*C	2	0,3607	0,1804	0,35	0,7064
A*B*D	4	1,2644	0,3161	0,61	0,6553
A*C*D	4	2,4756	0,6189	1,20	0,3193
B*C*D	2	1,4424	0,7212	1,40	0,2543
A*B*C*D	4	1,0259	0,2565	0,50	0,7382
Error	70	36,1526	0,5165		
Total	107	84,3388			

Appendix 3.6: Analysis of variance (ANOVA) for TA of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	0,04463	0,02231		
Production site (A)	2	2,72519	1,36259	85,01	0,0000
Duration (B)	1	0,37926	0,37926	23,66	0,0000
Storage (C)	1	0,00593	0,00593	0,37	0,5451
Treatment (D)	2	0,02796	0,01398	0,87	0,4225
A*B	2	1,06963	0,53481	33,37	0,0000
A*C	2	0,00519	0,00259	0,16	0,8510
A*D	4	0,02759	0,00690	0,43	0,7862
B*C	1	0,01333	0,01333	0,83	0,3649
B*D	2	0,03130	0,01565	0,98	0,3818
C*D	2	0,01352	0,00676	0,42	0,6576
A*B*C	2	0,00222	0,00111	0,07	0,9331
A*B*D	4	0,36648	0,09162	5,72	0,0005
A*C*D	4	0,09870	0,02468	1,54	0,2003
B*C*D	2	0,02056	0,01028	0,64	0,5297
A*B*C*D	4	0,03389	0,00847	0,53	0,7151
Error	70	1,12204	0,01603		
Total	107	5,98741			

Appendix 3.7: Analysis of variance (ANOVA) for TSS: TA of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	8,71	4,353		
Production site (A)	2	648,94	324,471	110,78	0,0000
Duration (B)	1	9,66	9,660	3,30	0,0736
Storage (C)	1	0,84	0,836	0,29	0,5949
Treatment (D)	2	7,52	3,760	1,28	0,2834
A*B	2	206,73	103,367	35,29	0,0000
A*C	2	12,15	6,075	2,07	0,1333
A*D	4	2,70	0,675	0,23	0,9204
B*C	1	0,22	0,222	0,08	0,7837
B*D	2	10,63	5,317	1,82	0,1704
C*D	2	6,08	3,040	1,04	0,3596
A*B*C	2	3,18	1,590	0,54	0,5835
A*B*D	4	91,72	22,931	7,83	0,0000
A*C*D	4	28,87	7,217	2,46	0,0529
B*C*D	2	20,20	10,102	3,45	0,3730
A*B*C*D	4	21,47	5,367	1,83	0,1323
Error	70	205,03	2,929		
Total	107	1284,66			

Appendix 4.1: Analysis of variance (ANOVA) for RPI of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	0,6630	0,3315		
Season (A)	1	6,9312	6,9312	20,13	0,0000
Production site (B)	2	21,3328	10,6664	30,97	0,0000
Storage (C)	1	1,2118	1,2118	3,52	0,0649
Treatment (D)	2	1,0912	0,5456	1,58	0,2124
A*B	2	2,5854	1,2927	3,75	0,0283
A*C	1	0,0018	0,0018	0,01	0,9427
A*D	2	5,8483	2,9241	8,49	0,0005
B*C	2	0,4730	0,2365	0,69	0,5066
B*D	4	3,4442	0,8611	2,50	0,0502
C*D	2	0,7077	0,3538	1,03	0,3633
A*B*C	2	0,5886	0,2943	0,85	0,4299
A*B*D	4	0,4239	0,1060	0,31	0,8719
A*C*D	2	0,1277	0,0638	0,19	0,8312
B*C*D	4	7,7076	1,9269	5,59	0,0006
A*B*C*D	4	3,4087	0,8522	2,47	0,0521
Error	70	24,1084	0,3444		
Total	107	80,6553			

Appendix 4.2: Analysis of variance (ANOVA) for total phenolics of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	680,925	340		
Season (A)	1	43040,1	43040	1,42	0,2375
Production site (B)	2	6868605	3434302	113,26	0,0000
Storage (C)	1	2839409	2839409	93,64	0,0000
Treatment (D)	2	3317,56	1659	0,05	0,9468
A*B	2	164130	82065	2,71	0,0738
A*C	1	645,333	645	0,02	0,8844
A*D	2	68759,2	34380	1,13	0,3276
B*C	2	1636987	818493	26,99	0,0000
B*D	4	1,168E+07	2920126	96,30	0,0000
C*D	2	7266864	3633432	119,83	0,0000
A*B*C	2	92516,2	46258	1,53	0,2246
A*B*D	4	115135	28784	0,95	0,4409
A*C*D	2	42842,9	21421	0,71	0,4969
B*C*D	4	1559131	389783	12,85	0,0000
A*B*C*D	4	97593,6	24398	0,80	0,5263
Error	70	2122521	30322		
Total	107	3,460E+07			

Appendix 4.3: Analysis of variance (ANOVA) for total flavonoids of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	6,63185	3,31593		
Season (A)	1	1437,37	1437,37	1408,24	0,0000
Production site (B)	2	309,579	154,789	151,65	0,0000
Storage (C)	1	12,8133	12,8133	12,55	0,0007
Treatment (D)	2	16,0719	8,03593	7,87	0,0008
A*B	2	394,741	197,370	193,37	0,0000
A*C	1	3,029E-28	3,029E-28	0,00	1,0000
A*D	2	1,40741	0,70370	0,69	0,5052
B*C	2	0,20667	0,10333	0,10	0,9038
B*D	4	6,61037	1,65259	1,62	0,1791
C*D	2	8,00222	4,00111	3,92	0,0243
A*B*C	2	5,609E-28	2,804E-28	0,00	1,0000
A*B*D	4	2,81481	0,70370	0,69	0,6017
A*C*D	2	1,55556	0,77778	0,76	0,4706
B*C*D	4	7,56444	1,89111	1,85	0,0015
A*B*C*D	4	3,11111	0,77778	0,76	0,5535
Error	70	71,4481	1,02069		
Total	107	2279,93			

Appendix 4.4: Analysis of variance (ANOVA) for vitamin C of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	321	160		
Season (A)	1	9226	9226	8,65	0,0044
Production site (B)	2	2046162	1023081	959,07	0,0000
Storage (C)	1	44376	44376	41,60	0,0000
Treatment (D)	2	279863	139932	131,18	0,0000
A*B	2	57249	28624	26,83	0,0000
A*C	1	151	151	0,14	0,7077
A*D	2	54305	27152	25,45	0,0000
B*C	2	1997589	998795	936,30	0,0000
B*D	4	547871	136968	128,40	0,0000
C*D	2	1117078	558539	523,59	0,0000
A*B*C	2	18966	9483	8,89	0,0004
A*B*D	4	78943	19736	18,50	0,0000
A*C*D	2	23994	11997	11,25	0,0001
B*C*D	4	380404	95101	89,15	0,0000
A*B*C*D	4	32476	8119	7,61	0,0621
Error	70	74672	1067		
Total	107	6763647			

Appendix 4.5: Analysis of variance (ANOVA) for DPPH of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	4,6	2,31		
Season (A)	1	0,3	0,33	0,02	0,8968
Production site (B)	2	10925,5	5462,75	277,55	0,0000
Storage (C)	1	1286,9	1286,85	65,38	0,0000
Treatment (D)	2	1659,8	829,90	42,17	0,0000
A*B	2	62,2	31,08	1,58	0,2134
A*C	1	171,3	171,26	8,70	0,0043
A*D	2	174,5	87,25	4,43	0,0154
B*C	2	12,5	6,26	0,32	0,7288
B*D	4	2818,9	704,72	35,81	0,0000
C*D	2	36,2	18,10	0,92	0,4034
A*B*C	2	35,0	17,51	0,89	0,4154
A*B*D	4	126,7	31,67	1,61	0,1817
A*C*D	2	62,6	31,29	1,59	0,2113
B*C*D	4	1326,4	331,59	16,85	0,0000
A*B*C*D	4	76,1	19,04	0,97	0,4311
Error	70	1377,7	19,68		
Total	107	20157,1			

Appendix 5.1: Analysis of variance (ANOVA) for RPI of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	0,6672	0,3336		
Season (A)	1	6,9515	6,9515	20,20	0,0000
Production site (B)	2	21,3717	10,6858	31,06	0,0000
Storage (C)	2	1,0906	0,5453	1,58	0,2123
Treatment (D)	1	1,2033	1,2033	3,50	0,0657
A*B	2	2,5680	1,2840	3,73	0,0288
A*C	2	5,8369	2,9184	8,48	0,0005
A*D	1	0,0015	0,0015	0,00	0,9479
B*C	4	3,4444	0,8611	2,50	0,0500
B*D	2	0,4706	0,2353	0,68	0,5080
C*D	2	0,7039	0,3519	1,02	0,3649
A*B*C	4	0,4204	0,1051	0,31	0,8734
A*B*D	2	0,5891	0,2945	0,86	0,4293
A*C*D	2	0,1280	0,0640	0,19	0,8307
B*C*D	4	7,7022	1,9256	5,60	0,0006
A*B*C*D	4	3,4115	0,8529	2,48	0,0518
Error	70	24,0861	0,3441		
Total	107	80,6467			

Appendix 5.2: Analysis of variance (ANOVA) for DPPH activity of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	14,7	7,36		
Season (A)	1	12,0	12,00	0,70	0,4050
Production site (B)	2	10539,5	5269,75	308,18	0,0000
Storage (C)	2	1618,5	809,23	47,33	0,0000
Treatment (D)	1	1218,7	1218,74	71,27	0,0000
A*B	2	57,2	28,58	1,67	0,1954
A*C	2	176,2	88,08	5,15	0,0082
A*D	1	147,0	147,00	8,60	0,0045
B*C	4	2327,6	581,89	34,03	0,0000
B*D	2	22,1	11,03	0,65	0,5276
C*D	2	22,2	11,10	0,65	0,5256
A*B*C	4	88,3	22,08	1,29	0,2818
A*B*D	2	23,7	11,86	0,69	0,5031
A*C*D	2	82,4	41,19	2,41	0,0973
B*C*D	4	1422,6	355,64	20,80	0,0000
A*B*C*D	4	101,9	25,47	1,49	0,2147
Error	70	1197,0	17,10		
Total	107	19071,4			

Appendix 5.3: Analysis of variance (ANOVA) for FRAP activity of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	1,2	0,60		
Season (A)	1	60,7	60,75	7,52	0,0078
Production site (B)	2	6434,2	3217,12	398,02	0,0000
Storage (C)	2	2678,7	1339,35	165,70	0,0000
Treatment (D)	1	2372,6	2372,58	293,53	0,0000
A*B	2	4,5	2,25	0,28	0,7579
A*C	2	13,7	6,86	0,85	0,4323
A*D	1	144,7	144,68	17,90	0,0001
B*C	4	2648,3	662,08	81,91	0,0000
B*D	2	1232,8	616,38	76,26	0,0000
C*D	2	803,0	401,48	49,67	0,0000
A*B*C	4	48,1	12,03	1,49	0,2151
A*B*D	2	30,1	15,06	1,86	0,1627
A*C*D	2	28,6	14,29	1,77	0,1783
B*C*D	4	1826,1	456,52	56,48	0,0000
A*B*C*D	4	143,7	35,93	4,44	0,1229
Error	70	565,8	8,08		
Total	107	19036,8			

Appendix 5.4: Analysis of variance (ANOVA) for ABTS activity of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	3,89	1,943		
Season (A)	1	6,61	6,606	1,80	0,1842
Production site (B)	2	1672,56	836,281	227,73	0,0000
Storage (C)	2	21,17	10,587	2,88	0,0626
Treatment (D)	1	32,99	32,990	8,98	0,0038
A*B	2	1,82	0,911	0,25	0,7810
A*C	2	0,04	0,022	0,01	0,9941
A*D	1	0,83	0,834	0,23	0,6352
B*C	4	27,39	6,849	1,87	0,1263
B*D	2	29,00	14,498	3,95	0,0237
C*D	2	49,84	24,922	6,79	0,0020
A*B*C	4	3,30	0,825	0,22	0,9237
A*B*D	2	4,13	2,065	0,56	0,5724
A*C*D	2	1,37	0,687	0,19	0,8298
B*C*D	4	57,33	14,332	3,90	0,0064
A*B*C*D	4	11,52	2,881	0,78	0,5390
Error	70	257,06	3,672		
Total	107	2180,87			

Appendix 5.5: Analysis of variance (ANOVA) for ORAC activity of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	33,9	16,94		
Season (A)	1	6,3	6,26	0,69	0,4094
Production site (B)	2	1884,0	942,02	103,67	0,0000
Storage (C)	2	200,4	100,21	11,03	0,0001
Treatment (D)	1	1808,9	1808,93	199,08	0,0000
A*B	2	30,5	15,26	1,68	0,1939
A*C	2	13,7	6,84	0,75	0,4747
A*D	1	100,1	100,15	11,02	0,0014
B*C	4	5234,6	1308,65	144,02	0,0000
B*D	2	271,1	135,53	14,92	0,0000
C*D	2	210,4	105,21	11,58	0,0000
A*B*C	4	314,0	78,51	8,64	0,0000
A*B*D	2	12,7	6,37	0,70	0,4995
A*C*D	2	175,0	87,51	9,63	0,0002
B*C*D	4	4665,0	1166,25	128,35	0,0000
A*B*C*D	4	498,6	124,65	13,72	0,1321
Error	70	636,1	9,09		
Total	107	16095,4			

Appendix 6.1: Analysis of variance (ANOVA) for RPI of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	0,6672	0,3336		
Season (A)	1	6,9515	6,9515	20,20	0,0000
Production site (B)	2	21,3717	10,6858	31,06	0,0000
Storage (C)	1	1,2033	1,2033	3,50	0,0657
Treatment (D)	2	1,0906	0,5453	1,58	0,2123
A*B	2	2,5680	1,2840	3,73	0,0288
A*C	1	0,0015	0,0015	0,00	0,9479
A*D	2	5,8369	2,9184	8,48	0,0005
B*C	2	0,4706	0,2353	0,68	0,5080
B*D	4	3,4444	0,8611	2,50	0,0500
C*D	2	0,7039	0,3519	1,02	0,3649
A*B*C	2	0,5891	0,2945	0,86	0,4293
A*B*D	4	0,4204	0,1051	0,31	0,8734
A*C*D	2	0,1280	0,0640	0,19	0,8307
B*C*D	4	7,7022	1,9256	5,60	0,0516
A*B*C*D	4	3,4115	0,8529	2,48	0,0006
Error	70	24,0861	0,3441		
Total	107	80,6467			

Appendix 6.2: Analysis of variance (ANOVA) for rind fructose of ‘Benny’ valencia

Source	DF	SS	MS	F	P
Rep	2	1,386	0,6929		
Season (A)	1	64,868	64,8675	111,27	0,0000
Production site (B)	2	19,902	9,9512	17,07	0,0000
Storage (C)	1	0,549	0,5490	0,94	0,3352
Treatment (D)	2	4,396	2,1979	3,77	0,0279
A*B	2	32,917	16,4586	28,23	0,0000
A*C	1	0,058	0,0579	0,10	0,7536
A*D	2	6,434	3,2169	5,52	0,0060
B*C	2	0,795	0,3973	0,68	0,5092
B*D	4	8,743	2,1858	3,75	0,0080
C*D	2	2,156	1,0779	1,85	0,1650
A*B*C	2	2,079	1,0395	1,78	0,1757
A*B*D	4	16,967	4,2418	7,28	0,0001
A*C*D	2	0,772	0,3862	0,66	0,5188
B*C*D	4	3,840	0,9600	1,65	0,1723
A*B*C*D	4	2,410	0,6025	1,03	0,0001
Error	70	40,808	0,5830		
Total	107	209,079			

Appendix 6.3: Analysis of variance (ANOVA) for rind glucose of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	3,846	1,923		
Season (A)	1	126,533	126,533	146,32	0,0000
Production site (B)	2	59,644	29,822	34,48	0,0000
Storage (C)	1	3,378	3,378	3,91	0,0521
Treatment (D)	2	8,209	4,105	4,75	0,0117
A*B	2	198,089	99,045	114,53	0,0000
A*C	1	0,280	0,280	0,32	0,5711
A*D	2	16,845	8,422	9,74	0,0002
B*C	2	0,920	0,460	0,53	0,5898
B*D	4	2,138	0,535	0,62	0,6511
C*D	2	22,814	11,407	13,19	0,0000
A*B*C	2	2,366	1,183	1,37	0,2614
A*B*D	4	14,840	3,710	4,29	0,1575
A*C*D	2	0,502	0,251	0,29	0,7488
B*C*D	4	4,823	1,206	1,39	0,2449
A*B*C*D	4	5,916	1,479	1,71	0,0037
Error	70	60,534	0,865		
Total	107	531,677			

Appendix 6.4: Analysis of variance (ANOVA) for rind sucrose of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	1,076	0,5378		
Season (A)	1	16,101	16,1008	25,27	0,0000
Production site (B)	2	5,705	2,8525	4,48	0,0148
Storage (C)	1	0,871	0,8712	1,37	0,2463
Treatment (D)	2	0,296	0,1478	0,23	0,7936
A*B	2	7,821	3,9103	6,14	0,0035
A*C	1	0,001	0,0008	0,00	0,9713
A*D	2	5,227	2,6133	4,10	0,0207
B*C	2	4,802	2,4012	3,77	0,0279
B*D	4	3,983	0,9957	1,56	0,1939
C*D	2	0,534	0,2670	0,42	0,6593
A*B*C	2	0,724	0,3619	0,57	0,5692
A*B*D	4	1,486	0,3715	0,58	0,6759
A*C*D	2	8,329	4,1644	6,54	0,0025
B*C*D	4	0,080	0,0200	0,03	0,9981
A*B*C*D	4	2,244	0,5610	0,88	0,0134
Error	70	44,604	0,6372		
Total	107	103,883			

Appendix 7.1: Analysis of variance (ANOVA) for RPI of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	0,7315	0,36574		
Production site (A)	2	17,3704	8,68519	17,44	0,0000
Treatment (B)	1	0,5602	0,56019	1,12	0,2964
Storage (C)	2	4,4537	2,22685	4,47	0,0189
A*B	2	1,0370	0,51852	1,04	0,3641
A*C	4	1,5463	0,38657	0,78	0,5485
B*C	2	0,2870	0,14352	0,29	0,7515
A*B*C	4	8,6574	2,16435	4,35	0,0006
Error	34	16,9352	0,49809		
Total	53	51,5787			

Appendix 7.2: Analysis of variance (ANOVA) for CsCP gene of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	0,53	0,27		
Production site (A)	2	2096,72	1048,36	4990,45	0,0000
Treatment (B)	1	1,32	1,32	6,26	0,0173
Storage (C)	2	34,01	17,00	80,94	0,0000
A*B	2	10,08	5,04	24,00	0,0000
A*C	4	54,54	13,63	64,90	0,0000
B*C	2	2,24	1,12	5,33	0,0971
A*B*C	4	5,34	1,33	6,35	0,0006
Error	34	7,14	0,21		
Total	53	2211,92			

Appendix 7.3: Analysis of variance (ANOVA) for CsNAC gene of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	3,849	1,924		
Production site (A)	2	770,607	385,303	550,10	0,0000
Treatment (B)	1	6,229	6,229	8,89	0,0053
Storage (C)	2	8,829	4,413	6,30	0,0047
A*B	2	6,684	3,342	4,77	0,0149
A*C	4	43,497	10,342	15,53	0,0000
B*C	2	6,372	3,186	4,55	0,0178
A*B*C	4	20,774	5,194	7,41	0,0002
Error	34	23,814	0,700		
Total	890,653				

Appendix 7.4: Analysis of variance (ANOVA) for CsCP-F gene of ‘Benny’ valencia fruit

Source	DF	SS	MS	F	P
Rep	2	0,537	0,256		
Production site (A)	2	462,006	231,003	101,65	0,0000
Treatment (B)	1	0,834	0,834	0,37	0,5487
Storage (C)	2	5,207	2,603	1,15	0,3300
A*B	2	9,358	4,679	2,06	0,1432
A*C	4	43,192	10,798	4,75	0,0037
B*C	2	8,802	4,401	1,94	0,1597
A*B*C	4	16,633	4,158	1,83	0,0001
Error	34	77,263	2,272		
Total	53	623,834			