Department of Environment and Agriculture

Pre- and Post-Harvest Regulation of Fruit Quality in Sweet Orange

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Declaration

To the best of my knowledge and belief, this thesis contains no material previously published by any other person except where due acknowledgement has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature: 

Date: 2/03/2018

Dedication

To

My father (Late Abdul Rehman Babar),

My mother (Naheed Akhtar),

For

"A constant source of inspiration during the entire period of my PhD study and throughout my life…."

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In the name of Allah, the most Beneficent and the Merciful.

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Abstract

Poor rind colour development in early maturing cultivar M7 and extension of postharvest storage life in late maturing cultivars Midknight Valencia and Lane Late of sweet orange are major bottlenecks in extending the season of fresh orange fruit, and boosting profits to the growers. My research aimed at improving pre-harvest rind colour in early maturing cultivar M7 and extension of storage life and postharvest quality maintenance in late maturing cultivar Midknight Valencia and Lane Late sweet orange. The role of pre-harvest exogenous application of abscisic acid (S-ABA) and its biosynthesis inhibitor nordihydroguaiaretic acid (NDGA), gibberellin biosynthesis inhibitors such as prohexadione-calcium (Pro-Ca) or paclobutrazol (PBZ) and methyl jasmonate (MJ) at 6, 3 and 6 followed by 3 weeks before anticipated harvest (WBAH) on the rind colour development and quality in early maturing M7 Navel was investigated. Various postharvest factors such, storage temperature, exogenous application of MJ, salicylic acid (SA), hot water dip (HWD), thiabendazole (TBZ) and nitric oxide (NO) fumigation affecting storage life and maintaining fruit quality in a late maturing cultivar Midknight Valencia and Lane Late was also investigated.

Exogenous spray application of S-ABA regardless of the concentrations applied exhibited significantly lower hue angle (*h*°) with enhanced citrus colour index (CCI) and higher levels of total carotenoids in the rind during 2015 and 2016. The spray application of S-ABA (300 and 500 mg L^{-1}) resulted in higher level of total carotenoids (35.0 and 71.5 mg kg^{-1}) respectively, in the rind of M7 Navel during both years. The results also indicated that S-ABA treatments exhibited significantly reduced total organic acids in the juice, whilst total sugars were not affected by any of these treatments. Moreover, S-ABA treatments (200 and 300 mg L^{-1}) showed increased soluble solids concentration SSC/TA ratio (12.8 %) as a result of a reduction in titratable acidity (TA) (0.96 %).

Furthermore, the pre-harvest spray application of Pro-Ca (800, 1200 or 1600 mg L^{-1}) applied at 6 to 3 WBAH enhanced fruit colour by exhibiting reduces *h*°, increased CCI and levels of total carotenoids in the rind. A single spray application of PBZ (1000 or 1500 mg L^{-1}) applied at 6 or 3 WBAH respectively, improved fruit colour in M7 Navel.

The role of MJ in M7 rind colour development was also investigated by monitoring the changes in *h*°, CCI and levels of total carotenoids. The pre-harvest spray

application of MJ (5.0 or 7.5 mM) resulted in enhanced rind colour by employing reduced *h*° (55.7, 54.3), increased CCI (11.0, 12.0) and levels of total carotenoids (35.3, 58.3 mg kg^{-1}) respectively, in the rind of M7 Navel during 2015 and 2016. Furthermore, MJ treatments showed reduced SSC (%) in the juice. All the MJ treatments showed reduced levels of total sugars and organic acids in the juice during 2015.

Postharvest HWD treatment (50 ± 1°C) for 5 minute (min) alone or combined with TBZ (20 mg L^{-1}) or MJ (0.50 mM) alleviated chilling injury (CI) in Lane Late and Midknight Valencia during cold quarantine treatment (1°C for 21 d). The results also suggested that NO (5 µL L^{-1}) fumigation for 2 hours (h) significantly reduced weight loss (%) in Lane Late during cold quarantine treatment. However, NO (10 µL L^{-1}) resulted in the significantly highest level of vitamin C only in Midknight Valencia. Moreover, postharvest dip application of MJ (0.1, 0.25 or 0.50 mM) mitigates CI in Midknight Valencia stored at 4°C or 7°C for 90 days (d) followed by 10 d simulated shelf conditions in 2014 and 2015. MJ-treated fruit also showed higher SCC/TA ratio and reduced levels of vitamin C and total antioxidants as compared to the control. Furthermore, NO fumigation treatments (5, 10 or 20 µL L^{-1}) significantly reduced the CI irrespective of storage temperature (4 or 7°C) in Midknight Valencia and Lane Late stored for 90 d followed by 10 d simulated shelf condition. NO significantly reduced weight loss (%) in Lane Late only. The mean level of glucose, fructose, sucrose and total sugars in the juice of Midknight Valencia was reduced by all NO treatments.

In conclusion, the pre-harvest spray application of S-ABA (200-300 mg L^{-1}) or Pro-Ca (800 mg L^{-1}) or PBZ (1000 mg L^{-1}) applied at 6 WBAH or MJ (5.0 or 7.5 mM) applied at 3 WBAH promotes the rind colour of M7 Navel orange. However, the postharvest HWD at 50±1°C for 5 min alone or combined with TBZ reduced CI in Lane Late and Midknight Valencia during cold quarantine treatment (1°C for 21 d) without adversely affecting quality. Additionally, the postharvest MJ (0.1-0.50 mM) 1 min dip and NO (5-20 µL L^{-1}) 2 h fumigation reduced CI in Midknight Valencia and Lane Late stored at (4 or 7°C) for 90 d followed by 10 d simulated shelf conditions.

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%	per-cent
β	beta
µg	microgram(s)
µl	microliter (s)
/	divide
min	minute
=	equal to
>	greater than
±	plus minus
×	multiply/interaction between
≤	less than or equal to
°	degree
°C	degree celsius
13-HPOT	13 (S) - hydroperoxy linolenic acid
1-MCP	1-methylcyclopropene
a.i.	active ingredient
ABA	S- (+)-*cis*, *trans*-abscisic acid
ABS	Australian Bureau of Statistics
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	1-aminocyclopropane-1-carboxylic acid oxidase
ACS	1-aminocyclopropane-1-carboxylic acid synthase
ANOVA	analysis of variance
AOC	Allene oxidase cyclase
AOS	Allene oxide synthase
APX	Ascorbate peroxidase
b-CHX	b-carotene hydroxylase
C_2H_4	Ethylene
Ca	Calcium
CAB	chlorophyll a/b binding protein
CAT	Catalase
CIE	Commission Internationale de L'Eclairage
CO_2	Carbon dioxide
cPTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1- oxyl-3-oxide
cv	cultivar
d	day(s)
DAFWA	Department of Agriculture and Food, Western Australia
DAV	Days after veraison
DBH	Days before harvest
dH_2O	Distilled water
DMAPP	Dimethylallyl diphosphate
DPPH	2, 2-diphenyl-1-picryl-hydrazyl
DXR	1- deoxy-Dxylulose 5-phosphate reductoisomerase
DXS	1- deoxyxylulose-5-phosphate synthase
E	East
e-CHX	e-carotene hydroxylase
EDTA	Ethylene diamino tetra acetic acid
e-LCY	e-lycopene cyclase

ET	Ethylene
et al	et alia
Fig.	Figure
FJ	Fresh juice
FW	Fresh weight
g	gram(s)
GA_3	Gibberellic acid
GC	Gas chromatograph
GGPP	Geranylgeranyl diphosphate
h	Hour(s)
h°	Hue angle
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric Acid
HA	Hot air
HDR	1-hydroxy-2- methyl-2-(E)-butenyl 4-diphosphate reductase
HPLC	High-performance liquid chromatography
HSP	Heat shock proteins
HT	Heat treatment
HYb	*β*-ring hydroxylase
IPP	Isopentenyl diphosphate
IMZ	Imazalil
JAs	Jasmonates/ Jasmonic Acid
kg	kilogram(s)
L	Litre(s)
LCYe	lycopene cyclase
LHCP	light-harvesting chlorophyll a/b binding protein complex
LOX	Lipoxygenase
LSD	Least significant difference
Ltd.	Limited
LYb1, LYb2	lycopene cyclase
m	meter
MAP	modified atmosphere packaging
MCS	Metal chelating substance
MDA	Malondialdehyde
MeOH	Methanol
MEP	2-C-methyl-d-erythritol 4-phosphate
mg	milligram(s)
min	min(s)
ml	millilitre(s)
mm	Millimeter
mM	millimolar(s)
Mt	Metric tonnes
N	Newton
n	Number of replication
NCED	9-cis-epoxy carotenoid dioxygenase
NO	Nitric oxide
ns	Not significant
NSW	New South Wales
NSY	neoxanthin synthase
NYC	non-yellowing colour (chlorophyll b reductase)

O_2	Oxygen
POD	Peroxidase
OPDA	12-oxo phytodienoic acid
P	Probability
PA	Polyamines
PAL	Phenylalanine ammonia-lyase
PaO	Pheide a oxygenase
PBZ	Paclobutrazol
PDJ	n-propyl-dihydrojasmonate
PDS	Phytoene desaturase
PG	Polygalacturonase
PGRs	Plant growth regulators
pH	Symbol denoting hydrogen ion in a solution
PhQ	Phylloquinone
POD	Peroxidase
ppm	Part per million (10^{-6})
PPO	Polyphenol oxidase
PQ	Monoterpenes and Plastoquinone
Pro-Ca	Prohexadione-calcium
PSY	Phytoene synthase
PTOX	plastid terminal oxidase
PUT	Putrescine
r	Correlation coefficient
RCC	Chl catabolite
RCCR	red chl catabolite reductase
RH	Relative humidity
ROS	Reactive oxygen species
S	South
s	second(s)
SA	Salicylic acid
SE.	Standard error
SFA	Saturated fatty acid
SGR	stay-green protein
SOD	Superoxide dismutase
sp.	species
SSC	Soluble solids concentration
Std	Standard
T	Tonnes
TA	Titratable acidity
TBZ	Thiabendazole
Tm	Time
TP	Total Phenol
Tr	Treatment
Trolox	6-hydroxy-2, 5, 7, 8-tetramethychroman-2-carboxylic acid
USA	The United States of America
USFA	Unsaturated fatty acid
UV	Ultra-violet
v/v	volume by volume
VDE	violaxanthin de-epoxidase
VIC	Victoria

WA	Western Australia
WBAH	Weeks before anticipated harvest
ZDS	Zcarotene desaturase
ZDS	ζ-carotene desaturase
ZEP	zeaxanthin epoxidase
β-CHX	β-carotene hydroxylase
DAFWA	Department of Agriculture and Food WA
USDA	United States Department of Agriculture
O^{-2}	superoxide anion

CHAPTER 1

General introduction

Citrus fruit belong to the family *Rutaceae* and are one of the most important fruits of tropical and subtropical regions in the world. Citrus fresh fruit consumption and the demand for their processed products are very high around the world due to high vitamin C content and antioxidant properties (Gorinstein et al., 2001). Citrus is cultivated in the subtropical regions of the world between 40° North and South latitude in over 137 countries across the six continents of the world (Ismail and Zhang, 2004). Sweet orange (*Citrus sinensis* L. Osbeck) rank the first major commercial fruit crop among all the citrus producing countries. Australia ranks 12th in sweet orange production among all other producing countries of the world (USDA, 2017) with the total production of 398,610 tonnes (ABS, 2017). New South Wales (NSW) is the leading state of Australia for sweet orange production, which produces (48.6 %), followed by South Australia (SA) (31.5 %), Victoria (16.8 %), Queensland (0.6 %) and (2.2 %) by WA (ABS, 2017). The contribution of WA to the total citrus production of Australia is 15,000 tonnes, which includes 160 commercial citrus growers out of 2,800 total Australian growers (DAFWA, 2017). The suitable climate for citrus production in WA is found in the Gingin region in the north to the Bunbury region in the south. The citrus production regions have fertile soils and good quality irrigation water.

The WA citrus growers has been targeting the fresh market for the last few years. Early maturing M7 Navel and late maturing Midknight Valencia oranges are grown to extend the growing season in citrus growing regions of WA. M7 Navel matures in the month of May, three weeks earlier than Navelina Navel. Early maturing cultivar captures market and usually the produce is sold at higher price (DAFWA, 2017). M7 Navel may not colour well due to the warm winters in some years. Partially green fruit of M7 Navel with poor rind colour are difficult to sell in domestic or overseas markets. A late maturing Midknight Valencia selected from South Africa has excellent juice content, flavour and very few seeds. This variety is usually harvested between October and December in WA conditions (DAFWA, 2017). Valencia has the ability to store on the tree for longer durations; however, the fruit quality declines the longer the fruit are left on the trees. This cultivar is not widely grown in WA and growers have limited knowledge about this variety. There is no research knowledge

available about any growth regulator which can enhance rind colour in M7 Navel. In addition, cold storage potential of Midknight Valencia for export purpose needs to be investigated, as no knowledge is available about the effective storage of this variety.

Consumers buy citrus fruit for its taste, appearance and nutritional characteristics. Sweet orange fruit colour is an important fruit quality variable for consumers (Krajewski, 1996). Consumers are willing to pay a premium price for coloured citrus fruit; hence citrus fruit without its proper colour fetch a lower price in the market (Ladaniya, 2008). The most important and decisive factor for the consumer acceptance is the external quality of citrus fruit (Rodrigo et al., 2013a). In sub-tropical regions mature fruit are partially green due to lack of winter chill. During the development of citrus fruit, the rind colour changes from green to orange due to the degradation of chlorophyll and accumulation of carotenoid pigments (Goldschmidt, 1988). The transformation of chloroplast to chromoplast resulting in the colour break of the rind is a physiological event affected by hormonal factors, nutrition and environmental conditions (Goldschmidt, 1988). The transformation of chloroplast to chromoplast in early maturing cultivars is often limited because the environmental conditions are not suitable for colour development.

Abscisic acid (ABA) is a naturally occurring plant growth regulator and plays an important role in seed development, dormancy, environmental stress and fruit ripening (Zeevaart and Creelman, 1988; Kondo and Tomiyama, 1998). The application of ABA has been reported to be involved in improving colour by the accumulation of anthocyanins in strawberry (Jiang and Joyce, 2003) and grapes (Ban et al., 2003). The role of ABA in enhancing fruit colour development in apple and peaches has also been reported by Kondo et al. (1991) and Zhang et al. (2009). Increase in endogenous ethylene during maturation coincided with increased levels of endogenous ABA and anthocyanin accumulation in apple fruit (Kondo et al., 1991; Uthaibutra and Gemma, 1991). There is well-recognized evidence that increased ABA levels in mandarin (Lafuente et al., 1997), orange (Harris and Dugger, 1986) and sweet cherry (Kondo and Gemma, 1993) were responsible for the transition of chloroplast to chromoplast in the colour development process. However, no research has been reported on the effects of pre-harvest spray application of S-ABA on the fruit colour development and quality in early maturing M7 Navel orange grown under the Mediterranean climate of WA.

The high levels of gibberellic acid in the fruit rind during maturation inhibit the transformation of chloroplast to chromoplast (Goldschmidt, 1988), which leads to poor rind colour development at harvest (Gilfillan et al., 1974). A few commercial approaches have been taken to enhance citrus rind colour with growth regulators, inducing stress and nutritional applications. For example, foliar application of Pro-Ca at colour break stage (Barry and Le Roux, 2010), reduced late nitrogen application (Koo and Reese, 1977), deficit irrigation prior to maturation (Huff et al., 1981), increasing light intensity within tree canopy (Sites and Reitz, 1949), application of ethylene after colour break stage (Purvis, 1980), ethyclozate at the initiation of stage 2 of fruit development (Kamuro and Hirai, 1981), boric acid (Puzina, 2004), and PBZ application before summer flush (Gilfillan and Lowe, 1985). However, the rind colour is still the major problem for early maturing cultivars of citrus fruit. No research work has been reported on the effect of the pre-harvest foliar application of Pro-Ca and PBZ in promoting rind colour and regulating the quality of early maturing M7 Navel orange under WA conditions.

Methyl jasmonate (MJ) is an endogenous plant hormone. The pre- and postharvest applications of MJ have been well documented in the literature. Pre-harvest application of MJ stimulated chlorophyll deprivation and enhanced the development of anthocyanins in apple fruit (Rudell et al., 2002; Perez et al., 1993). Moreover, MJ stimulates β-carotene accumulation in the peel of tomato and apple fruit through the activation of ethylene (Czapski and Saniewski, 1992; Rudell et al., 2002). However, the postharvest application of MJ in reducing CI has been widely reported in fruit and vegetables for instance in zucchini squash (Wang and Buta, 1999), bell peppers, avocado (Meir et al., 1996), strawberry (Molin et al., 1997), mango (Gonzalez-Anguilar et al., 2000a, 2001), papaya (Gonzalez-Aguilar et al., 2003), guava (Gonzalez-Anguilar et al., 2004), pineapple (Nilprapruck et al., 2008), loquat (Cao et al., 2009, 2010; Cai et al., 2011), peach (Meng et al., 2009), pomegranate (Sayyari et al., 2011; Mirdehghan and Ghotbi, 2014), banana (He et al., 2014) and lemon (Siboza et al., 2014). No research work has been reported on the role of pre-harvest application of MJ in the accumulation of carotenoids in the peel of M7 Navel. Moreover, the role of postharvest application of MJ in reducing the incidence of CI and maintaining fruit quality induced by cold quarantine at (1°C for 21) in Lane Late and Midknight Valencia as well as in extending the cold storage life of Midknight Valencia at 4 or 7°C for 90 d warrants investigation.

Cold storage systems have been extensively used to extend the storage life of sweet oranges. Sweet orange fruit stored for extended period of time leads to CI. The symptoms of CI in citrus fruit are mainly exhibited as pitting, red blotches, scalding, rind staining, watery breakdown, soft glazed continuing lesion of mandarins, sunken tissues, damage to the styler end of lemons and necrosis on the rind (Sala and Lafuente, 1999; Reuther et al., 1989). To mitigate the problem of CI many non-chemical and chemical methods have been developed in the past.

Postharvest heat treatments (HT) are predominantly used to reduce the development of CI symptoms during cold storage and cold quarantine treatment (Schirra et al., 2004; Porat et al., 2000). Heat treatment (HT) includes HWD, hot air (HA) and hot vapours (HV). Hot water dip (HWD) to fruit and vegetables prior to storage at low-temperature have been reported to reduce CI in many fruit such as pepper (Gonzalez-Aguilar et al., 2000b), grapefruit (Rozenzvieg et al., 2004), pomegranate (Mirdehghan et al., 2007), lemon (Safizadeh et al., 2007), mandarin (Ghasemnezhad et al., 2008), orange (Bassal and El-Hamahmy, 2011) and banana (Wang et al., 2012). The positive effects of HT on the storage of citrus fruit are well documented (Hatton and Cubbedge, 1983; Rodov et al., 1995; Schirra and D'hallewin, 1997; Schirra et al., 1998; Rodov et al., 2000). Apart from HWD treatment alone, the combinations of HWD with TBZ have also been reported to reduce CI in grapefruit (Kokkalos, 1974; Schiffmann-Nadel et al., 1972; Chalutz et al., 1985; Wardowski et al., 1975). However, no research work has been reported on HWD treatment at 50±1°C alone or combined with TBZ on decreasing the incidence of CI and maintaining fruit quality induced by cold quarantine at (1°C for 21 d) in Lane Late and Midknight Valencia.

Nitric oxide (NO), a highly reactive free radical gas, which acts as a multifunctional signalling molecule involved in a large array of biochemical reactions in higher organisms (Wendehenne et al., 2001). It has been well recognized that NO enhanced chilling tolerance in many fruit and vegetable such as, Japanese plums (Singh et al., 2009), peach (Zhu et al., 2010), cucumber (Yang et al., 2011a), mango (Zaharah and Singh, 2011), loquat (Xu et al., 2012), banana (Wu et al., 2014; Wang et al., 2013), sweet orange Washington Navel (Ghorbani et al., 2017) and Hami melon (Zhang et al., 2017). However, no research work has been reported on NO gas fumigation on mitigating the incidence of CI induced by cold quarantine at 1°C for 21

d treatment and cold stored for 90 d at 4°C and 7°C whereby maintaining fruit quality in Lane Late and Midknight Valencia sweet orange.

The objectives of this research were:

1. To explicate the role of S-ABA in regulating fruit colour development from yellow to deep orange, levels of total carotenoids in the rind and fruit quality by pre-harvest spray application of S-ABA and its biosynthesis inhibitor NDGA at 6, 3 and 6 followed by 3 WBAH in M7 Navel.
2. To investigate the involvement of pre-harvest spray application of Pro-Ca and PBZ (gibberellic biosynthesis inhibitor) in enhancing fruit colour development by monitoring the changes in *h*°, CCI and levels of total carotenoids in the rind and fruit quality at 6, 3 and 6 followed by 3 WBAH in M7 Navel.
3. To elucidate the role of pre-harvest application of MJ in the regulation of fruit colour development, levels of total carotenoids in the rind and fruit quality at 6, 3 and 6 followed by 3 WBAH in M7 Navel.
4. To explicate the role of postharvest application of MJ, SA, NO, TBZ and HWD on mitigating the incidence of CI induced by cold quarantine treatment at 1°C for 21 d whereby maintaining fruit quality in Lane Late and Midknight Valencia sweet orange.
5. To underpin the role of postharvest application of MJ in reducing the incidence of CI and to maintain high quality in Midknight Valencia sweet oranges stored at 4°C or 7°C for 90 d followed by 10 d simulated shelf conditions.
6. To examine the role of postharvest NO fumigation in mitigating the incidence of CI and to maintain high quality in Lane Late and Midknight Valencia sweet oranges stored at 4°C or 7°C for 90 d followed by 10 d simulated shelf conditions.

CHAPTER 2

General literature review

2.1. Introduction

Citrus is one of the most important fruit of tropical and subtropical regions in the world. Citrus belongs to the family *Rutaceae*. Citrus fresh fruit consumption and the demand for their processed products are very high around the world. According to Gorinstein et al. (2001), citrus fruit global demand is due to its high vitamin C content and antioxidant properties. Sweet oranges possibly originated from adjacent portions of China, Myanmar and north-east India. Probably, Arab traders spread sweet oranges to the eastern Mediterranean basin through Africa, while in 1000 AD crusaders brought the fruit to Spain, Italy and Portugal (Scora, 1975). Sweet orange was introduced to the Western hemisphere by Columbus during 1493 and was introduced to South Africa by a Dutch merchant in 1654 (Oberholzer, 1969). Sweet orange is the most widely produced fruit, as a group of several species, it is grown in more than 80 countries (Chang, 1992). Sweet orange (*Citrus sinensis* L. Osbeck) rank the first major commercial fruit crop among all the citrus producing countries. Australia is one of the major citrus producing countries in the world, with the total production of 291,223 tonnes (ABS, 2017).

2.2. Production and trade of citrus in the world

Globally, sweet orange producing countries and their total production from 2014 to 2017 is shown in Fig 2.1. Among these countries, Brazil is the leading sweet orange producing country followed by China (USDA, 2017). Brazil sweet orange production is higher due to favourable weather and revived orchards recovering from an off-year, thereby resulting in good bloom and fruit set (USDA, 2017). The fresh sweet orange production in the European Union was down due to unfavourable dry weather and Citrus tristeza virus in parts of Italy (USDA, 2017). The Australian production of oranges increased from 430 metric tonnes (MT) in 2014/15 to 470 MT in 2016/17. The production of oranges in the United States of America (USA) is estimated to be down nearly 1.0 million tonnes to 4.6 million due to lower yields and reduced area (USDA, 2017). In addition, citrus greening also called Huanglongbing,

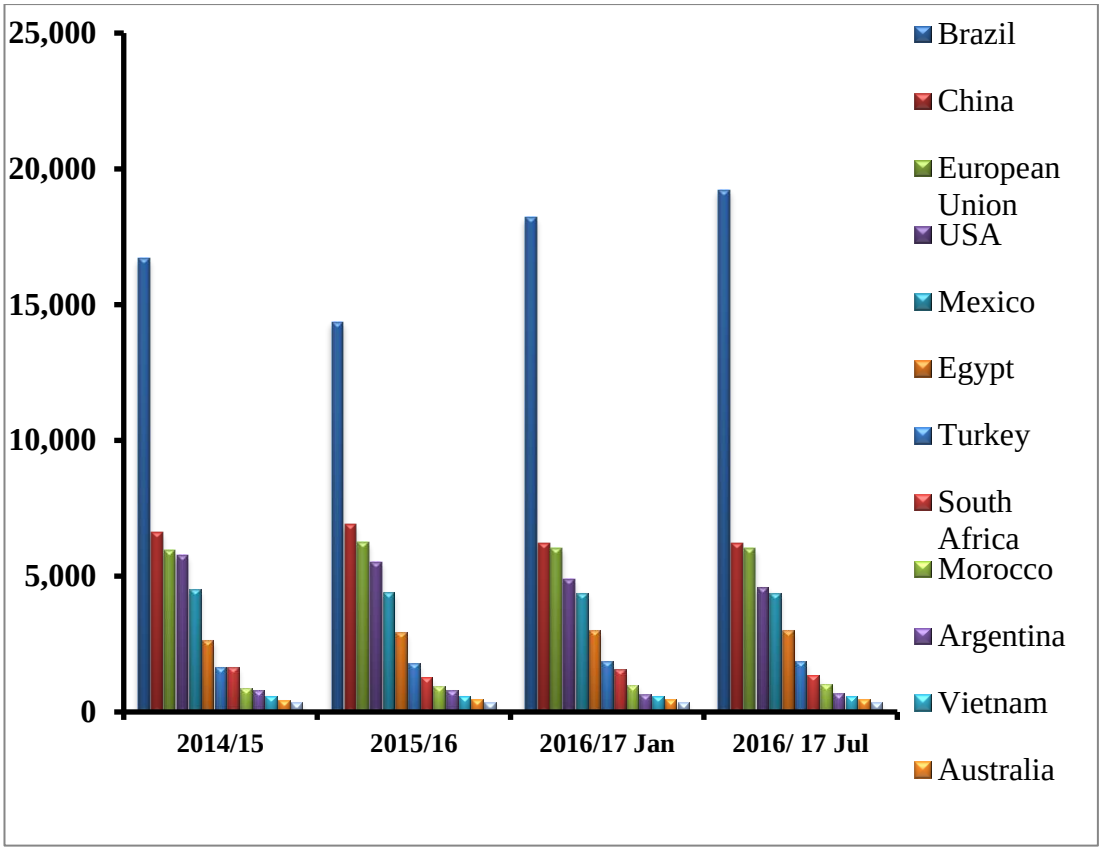


Fig: 2.1. Production of sweet oranges in selected countries (1,000 Metric Tonnes) (USDA, 2017).

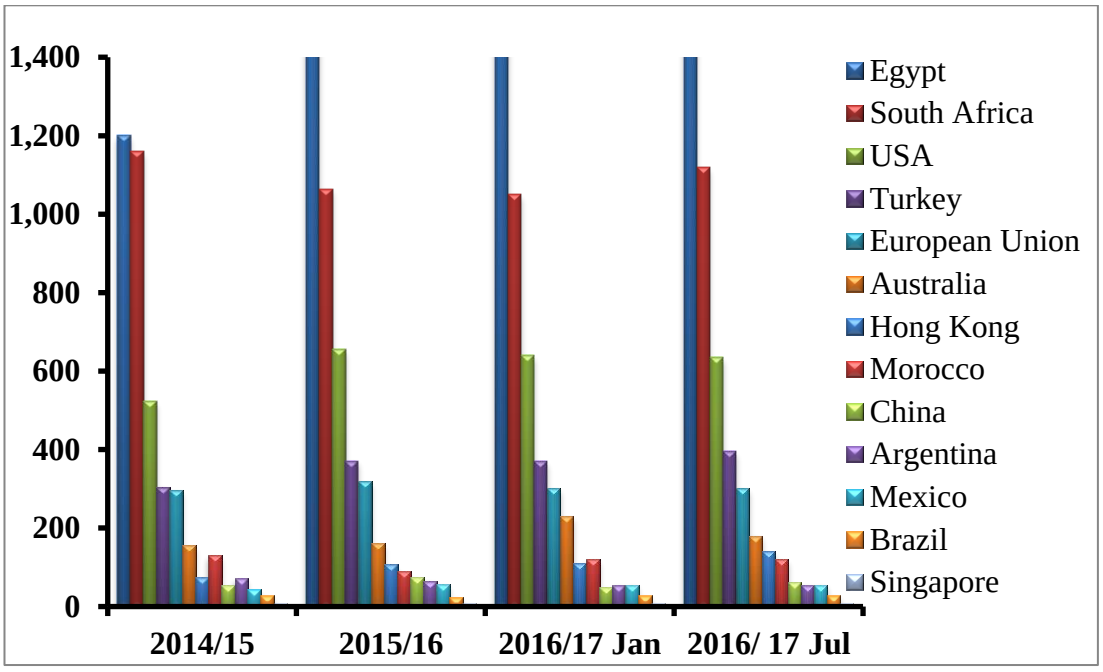


Fig: 2.2. Top twelve sweet orange exporting countries in the world (1,000 Metric Tonnes) (USDA, 2017).

which is one of the serious diseases in the USA caused a significant loss in citrus production. Egypt is a prominent country for sweet orange export in the world with the export quantity of 1,520 MT followed by South Africa (1,120 MT) during the year 2016/17 (July) (Fig 2.2) (USDA, 2017). Australia ranked sixth among the other sweet orange exporting countries with 180 MT during 2016/17 (July).

2.3. Citrus in Australia

Australian major citrus producing areas are New South Wales (NSW), South Australia (SA), Victoria, Queensland and WA. NSW is the leading state which produced 193,879.52 Tonnes of sweet oranges as compared to SA (125,797.94 Tonnes), WA (6,149.5 Tonnes) and Queensland (2,496.96 Tonnes) (Fig. 2.3, ABS, 2017).

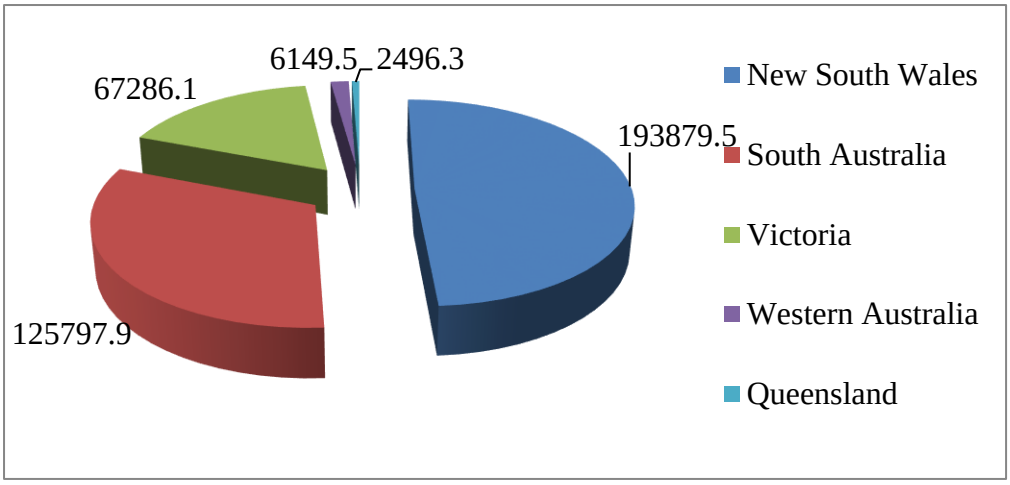


Fig: 2.3. Production of sweet orange in different states of Australia (Tonnes)

2.4. Top export destination for Australian orange

The Australian citrus industry usually exports sweet orange fruit to northern hemisphere markets such as China, Indonesia, Korea, Japan and the USA. As these exports are counter-seasonal, they do not compete with locally produced fruit. During the year 2015/16, exports are predicted to reach a record of 190,000 MT due to higher production, increasing demand and lower tariffs in key markets such as Japan and China (Citrus Australia, 2017). Recently, for Australian citrus exports, the USA markets are now less important as compared to Asian region markets. China is now Australia's third-largest citrus export destination.

2.5. Main sweet orange cultivars grown in WA

All sweet oranges grown in Australia are classified as commercial cultivars. In recent years, the plantation has been undertaken in WA of early and late maturing cultivars.

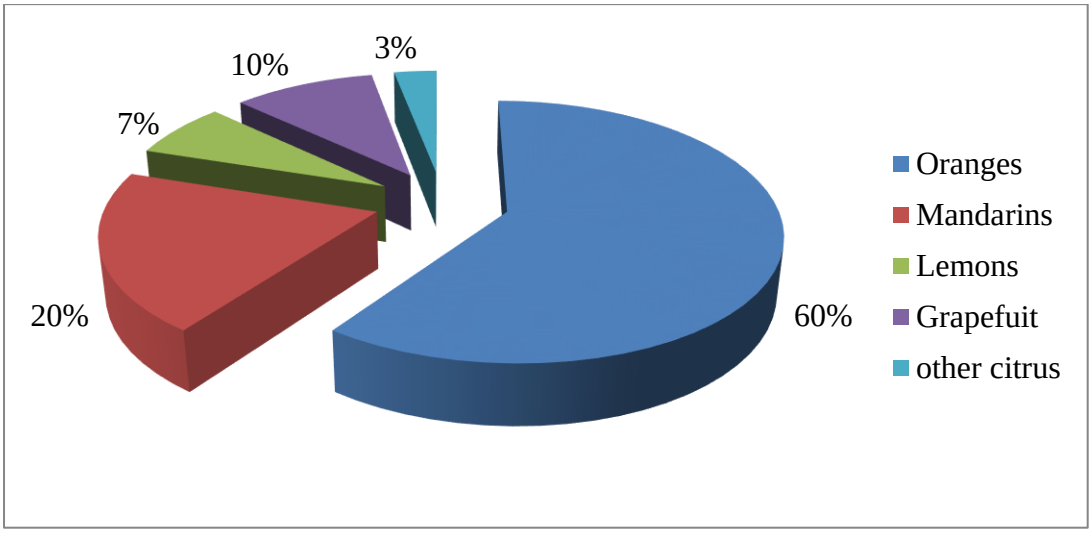


Fig: 2.4. Production of different citrus fruit crops in WA Department of Food and Agriculture WA (DAFWA, 2017).

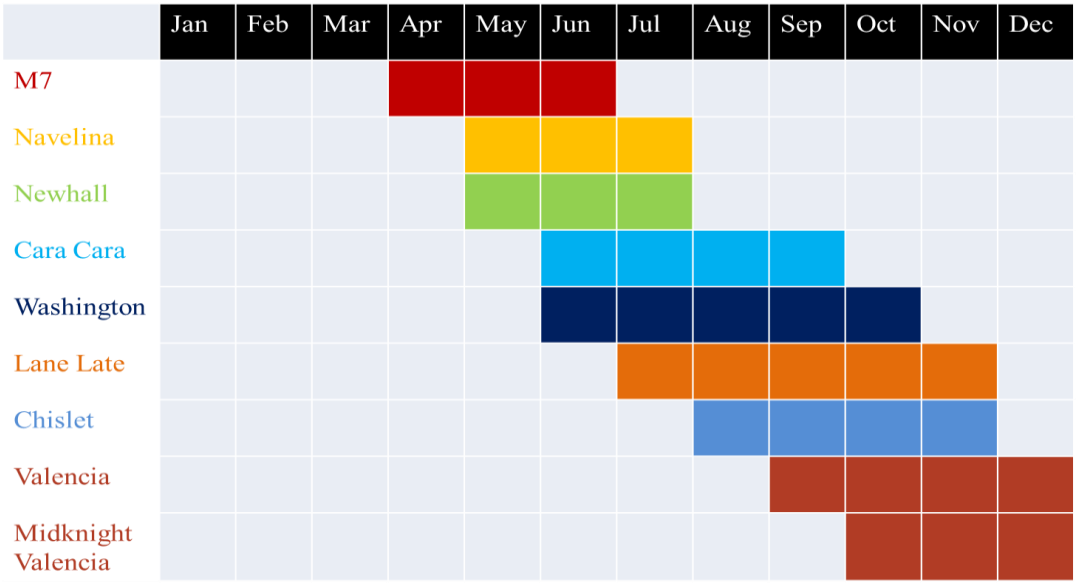


Fig: 2.5. Maturity timeline for some common sweet orange cultivars in WA (DAFWA, 2017).

An early maturing Navel sweet orange (M7) has been grown in WA. Sweet orange M7 is known to colour early and retain its acid level in the juice longer than standard Navelina; which gives a longer harvest period (DAFWA, 2017). The shape of M7 is rounder than Navelina and has good sugar and acid levels. Its performance under local conditions is still unclear. Poor rind colour of M7 sweet orange fruit has been reported at Moora citrus at the time of harvest in WA.

2.6. Citrus rind colour

Citrus fruit is still controversial in their taxonomical classification. The three-ancestral species recognised are: mandarin (*Citrus reticulata* Blanco), citron (*Citrus medica* L.) and pummelo (*Citrus maxima* (Burm.) Merrill): and the most commercial secondary species are: lime (*Citrus aurantifolia* (Cristm.) Swingle), (*Citrus latifolia* Tanaka) and (*Citrus limettioides* Tanaka), lemon (*Citrus limon* (L.) Osbeck), Clementines (*Citrus clementine* Hort. ex Tan), sweet orange (*Citrus sinensis* (L.) Osbeck) and grapefruit (*Citrus paradisi* Macfad) (Saunt, 2000). In this genus there is an outstanding difference in colour between distinct species and cultivars; high diversity in external and internal fruit colouration varies from the green of limes, red and pink of grapefruits, orange in sweet oranges and mandarin and yellow of lemons (Rodrigo et al., 2013a). Citrus fruit attracts the consumer due to its taste, appearance and nutritional characteristics. As far as citrus growers are concerned citrus fruit quality and its outer colour is the major concern for exporting this fruit to domestic and international markets. The desirability of this fruit is dependent on its deep rind colour. The rind of the citrus fruit is multilayered and formed by exocarp or rind tissue and the mesocarp or albedo tissue. The important preference by the citrus consumer is the rind colour (Krajewski, 1996; Ladaniya, 2008). Furthermore, Rodrigo et al., (2013a) stated that the most important and decisive factor for consumer acceptance is the external quality of citrus fruit. M7 is the early maturing cultivar of sweet orange in WA and has been reported to have poor rind colour at harvest. The effect of different plant growth regulators such as S-ABA, Pro-Ca, PBZ and MJ on improving the rind colour and quality is yet to be investigated.

2.7. De-greening

The major purpose behind the de-greening phenomena is to improve the visual colour of the citrus fruit. Most of the early season's citrus cultivars in sub-tropical regions usually become ready to harvest when the rind is still green. Furthermore, in tropical regions oranges and mandarin do not develop a cosmetic colour at the time of maturity and hence require de-greening (Ladaniya, 2008). Rodrigo et al. (2013a) reveal that diversity in citrus fruit colour is due to the accumulation of three main and important pigments: chlorophylls, carotenoids and anthocyanins. The green colour in immature fruit is chlorophyll; pink or red, orange and yellow colour is due to the accumulation of carotenoids; and anthocyanins specifically for blood oranges can display red or purple shades in the peel.

As citrus fruit mature, the rind colour starts changing from green to orange due to chlorophyll degradation and carotenoid accumulation (Goldschmidt, 1988). The degradation of chlorophyll in the citrus rind is as a result of deprivation of chlorophyllous tissues in the rind of citrus rind, and eventually the transfer of chloroplast into chromoplast. Goldschmidt, (1988) reveals that the transfer of chloroplast to chromoplast, resulting in the colour break of the rind is one of the important and major physiological responses affected by hormonal factors, nutrition and environment. "Colour break" is widely used terminology in citrus, which refers to a decline in chlorophyll concentration exposing the presence of carotenoid accumulation, followed by increased synthesis of carotenoids resulting in the first appearance of the orange colour of sweet orange and mandarin (El Zeftawi, 1978; Goldschmidt, 1988). The transformation of chloro-chromoplast in early maturing varieties is often limited because the environmental conditions are not suitable during the time of maturation.

2.8. Anatomy of citrus fruit

Botanically citrus fruit is classified as 'hesperidium' berry. The growth and development of the citrus fruit arise from an ovary. The core of the fruit is made up of 8-16 carpels clusters around and joined together to the floral axis (Ladaniya, 2008). The rind or peel (pericarp) is divided into exocarp (rind) and mesocarp (albedo). The outermost rind tissue has a cuticle covered with epidermis and parenchyma cell. The outer colour part of the citrus fruit is called flavedo, while the inner white (colourless)

or occasionally tinted part (as in red grapefruit and blood oranges) is referred to as albedo.

The rind consists of the epicarp, hypodermis, outer mesocarp and oil glands. A multilayer protective skin is present on the epicarp; the origin, structure and development of the cuticle is very complex (Ladaniya, 2008). The inner layer and outer layer of the cuticle consist of cutin - a heterogeneous polymer of cellulose and fatty acid (Baker et al., 1975). The deposition of the wax continues with the development of fruit and the wax hardens and develops breaks naturally. Lipids and waxes are synthesised by the epidermal cells and deposit on the cutin layer. Albrigo, (1972) reported that waxes are present in different forms such as platelets, rods and other structures surrounded with, or over, the epicuticular surface to prevent water loss from the peel epicuticular wax. The structure of epicuticular wax is quite complex in nature and is made up of ketones, aldehydes, paraffin and alcohols (Freeman, 1978). The cuticle plays a major role in preventing the water loss from the upper surface of fruit through evaporation. The high content of water is later used for normal metabolism. Plastids containing chlorophyll are present in epicarp, which slowly changes to chromoplast as fruit attain colour (Ladaniya, 2008).

2.9. Citrus growth and development

The citrus fruit growth pattern exhibits a single sigmoid curve, divided into three distinct stages (Bain, 1958; Subramanyam et al., 1965; Dhillon, 1986; Garcia-Luis et al., 2002). The stage 1 of the early citrus fruit growth is marked by cell division followed by cell expansion. The length of the growth stages is different among cultivars, climate and location, for instance, Washington Navel fruit completes growth from flowering to maturity within 35 weeks, while Valencia oranges take 59 weeks.

Generally, the time period for stage 1 (cell division) lasts for 30 to 40 (d), as compared to fruit cell enlargement which is negligible at this stage, but cell division is particularly rapid. The albedo layer of the citrus pericarp reaches up to 90% of the fruit volume at this development stage. Hutton et al. (2007) reported that the cell division stage starts approximately from October (full bloom) to about mid-December in Navel oranges. The small fruitlets are very prone to damage by biotic and abiotic factors for instance wind and insects due to the under-development of cuticle (Ladaniya, 2008). Though the peak of the cell division progression is completed in

stage 1 of the fruit growth and development process, some cell division can take place in the citrus peel, which makes the fruit prone to damage predominantly in Navel oranges. Many processes occur during citrus fruit development, for instance CO_2 is assimilated by chlorophyll in the presence of light (Bean and Todd, 1960; Todd et al., 1961), balance between the process of photosynthesis and respiration (no gain of energy) and the fruit photosynthesis process is reduced toward maturity (Bean et al., 1963). The transportation of sugar and organic acids is known to occur from leaves to fruit, but at the same time fruit are also known for their synthesis of compounds in their own tissue (Ladaniya, 2008). Furthermore, most of the time, leaves are effective suppliers of nutrients to the fruit.

Stages 2 is also known as the cell enlargement stage of the citrus fruit development process, a precarious stage of the overall growth period with rapid morphological and physiological changes in fruit. At this stage, the fruit increase in size by cell enlargement and accumulation of water. During this period the distinctive solutes are accumulated in their developing and enlarging juice sacs. At first, the solutes are low in sugars concentration while high in organic acid concentration, but this phenomenon is reversed while the fruit is attaining maturity (Ladaniya, 2008). Citrus fruit contains 85 % water which can vary from 80 to 90 % depending upon the species. In addition, the accumulation of reducing sugar, sucrose and citric acid is much more rapid in stage 2. Moreover, the citrus fruit flavour is significantly affected by sugar and acid content and this quality attribute is prominently dependent on the type of species. According to Hutton et al. (2007) stage 2 of citrus growth development in Australia lasts for approximately 29 weeks and starts from mid-December to mid-May in Navel oranges and up to mid-July in Valencia oranges (Bain, 1958).

Stage 3 is the final stage of the single sigmoid growth pattern of the citrus fruit development process. This stage constitutes changes related to maturation in which morphological, anatomical and physiological changes are decreased. Ladaniya, (2008) reported the single sigmoid growth pattern of hybrid Kinnow mandarin. Fruit set in hybrid Kinnow mandarin is completed in the month of April, while from July to November the fruit weight and diameter increase dramatically. When the fruit attains full size and volume by late November, a significant increase can be observed in carotenoids accumulation in the rind of the citrus peel. The level of chlorophyll '*a*'

and '*b*' in the peel of citrus fruit decreases toward maturity. Dhillon, (1986) reported that SSC increased while TA decreased until the end of January in hybrid Kinnow mandarin. During the entire period of citrus fruit growth and development, the SSC content increased as sugar concentration increased, while juice percentage and acids decreased. As the citrus fruit approached ripening the total carotenoids content showed a maximum increase while on the other hand the total chlorophyll content in the peel reduced to a minimum (Ladaniya, 2008). According to Hutton et al. (2007), stage III in the Navel oranges in WA starts from mid-May to November.

2.10. Citrus fruit maturation

The most important factor which determines the storage life and edible quality is the maturity level at harvest (Kader, 1997). Immature and overripe fruit are both prone to shrivelling and mechanical damage and are of inferior quality. In addition, fruit are more vulnerable to biological disorders when picked too early or too late in their harvesting seasons (Kader, 1997). Citrus fruit are non- climacteric in nature unlike apple, banana and persimmon which show a climacteric nature. The non-climacteric nature means that there is no sudden rise in the rate of respiration and ethylene production during the maturation/ripening phase. The rate of respiration and ethylene production is steady and in an equilibrium state throughout the process of growth and development. Citrus fruit do not show any major flavour and biochemical changes after harvest. In addition, citrus fruit don't ripen after harvest and the time taken to reach maturity depends on the species, cultivar, soil, climate and internal physiology.

2.10.1. Ripening

Throughout the growth and development process of fruits, ripening is the early stage of senescence and later stage of growth and development (Kader, 1997). The ripening process is characterised by aesthetic value, flavour, compositional changes, sensory attributes, texture and colour. Kader, (1997) reported two groups of fruits on the basis of their ethylene production such as group 1, berries (strawberry, raspberry, blackberry), citrus (oranges, mandarin, lime, grapefruit), lychee, pineapple, pomegranate, cherry; and group 2 includes apple, pear, plum, apricot, quince, persimmon, banana, mango, nectarine, papaya, avocado and guava. Group 1, which produces less ethylene, do not respond to ethylene treatment except in terms of de-greening (loss of chlorophyll). However, the fruit belonging to group 2 produce a much higher amount of ethylene than group 1 and ripen quickly. Citrus fruit maturity is related to the accumulation of sugars and loss of acidity through the process of biochemical changes. Reducing sugars in the rind, increasing sucrose in the juice and gradual sugar accumulation has been observed in all species during ripening (Ladaniya, 2008). Utsunomiya et al. (1982) reported that temperature also significantly influenced the maturation of citrus fruit. Higher carotenoid accumulation and early chlorophyll degradation are due to the lower temperature, whilst sugar content and SSC in the juice was highest at 23°C (Ladaniya, 2008).

2.10.2. Indices of maturity

The maturity of sweet orange, grapefruit and mandarin is considered on the basis of their juice content and the ratio of SSC, TA and SSC/TA. The citrus fruit which consumed as fresh or for processing purposes, maturity is usually determined on the basis of SSC/TA (Ladaniya, 2010). This ratio (SSC/TA) is used to determine the maturity index. The dependence on this ratio could be deceptive. Sites and Reitz (1949) reported that like colour break and juice content, minimum sugar or SSC content should be considered as part of maturity. According to Ladaniya (2008) determination of citrus fruit, maturity is done by different methods, for instance in rural areas the content of SSC is used by different growers to determine the maturity index; visual change of colour from light green to yellow-orange is another method for determining the maturity in the orchard. Goldweber et al. (1956) found that fruit size cannot be depended upon as criteria to determine the maturity index of citrus fruit. In high humidity (tropical) citrus growing areas where fruits do not develop their

acceptable colour, the standard basis of their SSC should be relied upon for citrus fruit. Arpaia and Kader (2000) reported a minimum of 10 % SSC with SSC/TA ratio of 8 % from the California standard of maturity indices in oranges. The SSC/TA ratio of 12.0 is a legally acceptable indicator for juice in the USA (Nordby and Nagy, 1977). In WA the standard SSC (8 %) and SSC/TA (8:1) is acceptable for Navel oranges. However, for all other sweet oranges, SSC (8) and SSC/TA (7:1) is required as standard (DAFWA, 2017).

2.10.3. SSC (%) and TA (%)

Soluble solids concentration (SSC) in the juice of sweet orange, grapefruit, mandarin and pummelo contains 80 to 85 % of sugars, while the remaining 15 % compositions of the SSC are citric acid, nitrogenous compounds, amino acids, ascorbic acid, pectins, glycosides, esters, oils and other water-soluble vitamins, which are relatively unstable (Sinclair, 1961). The main constituents of SSC in oranges are carbohydrate and organic acids. Davies and Albrigo (1994) reported that in oranges SSC varies from 10 to 20 % of the fruit on a fresh weight basis. SSC content is significantly affected by sweet orange fruit size. An increase in SSC was noticed with a decrease in the size of sweet orange fruit (Sinclair 1961). Deficient irrigation is reported to predominantly reduce fruit size and increase SSC, in oranges (Hutton et al., 2007; Treeby et al., 2007) and mandarins (Gonzalez-Altozano and Castel, 1999). Genetic factors also influence the fruit quality. Moreover, SSC was predominantly different among different citrus cultivars (Pretel et al., 2004). A significant increase in SSC of Feutrell's Early and Kinnow was reported between different harvesting maturities (Iqbal et al., 2012).

The taste of sweet orange fruit is greatly influenced by the TA. The main constituents of TA are citric acid, malic acid and to a lesser extent tartaric acid, fumaric and succinic acid (Davies and Albrigo, 1994; Ladaniya 2008). Monselise (1976) reported that TA was found to be at a maximum during the growth phase II and decreased in phase III due to catabolism of citric acid and an increase in the levels of sugars in the juice of a sweet orange. Reduction in TA is mainly due to the reduction in citric acid and both citric and malic acid also declined during cold storage (Ladaniya, 2008). Furthermore, TA in orange juice fruit is affected by irrigation (Treeby et al., 2007) and soil fertility (Koo and Reese, 1977); whilst, application of

ethylene does not affect SSC and TA (Al-Mughrabi et al., 1989). The SSC: TA ratio is commonly used to determine the maturity index.

2.10.4 Total antioxidants and Vitamin C

Sweet orange juice contains various main antioxidant components including phenolics, anthocyanins, flavanones, hydroxycinnamic acid, carotenoids and ascorbic acid. Total antioxidants are also known as anti-carcinogenic agents. However, the content of antioxidants varies in orange juice. Rapisarda et al. (1999) reported that the levels of antioxidants in fresh orange juice significantly depend on fruit maturity and type of cultivar. An increase in antioxidants capacity during storage caused mainly by phenolic accumulation was found in Tarocco Messina, Tarocco Meli and Moro and vitamin C increase in Ovale and Valencia Late (Rapisarda et al., 2008). The vitamin C content in fruits and vegetables can be influenced by various factors such as genotype, pre-harvest conditions, cultural practices, maturity, harvesting methods and postharvest handling procedures (Lee and Kader, 2000). Citrus fruit is known as a major source of ascorbic acid (Vitamin C) which is an important part of human nutrition. Ascorbic acid content decreases during the storage of citrus fruit and the loss is more rapid at higher temperatures (Ladaniya, 2008). However, at the early fruit development stage, the ascorbic acid content in citrus juice is higher and decreases at the ripening stage (Ladaniya, 2008). The effects of the pre-harvest application of various growth regulators such as Pro-Ca, PBZ, MJ and ABA on the regulation of levels of vitamin C and total antioxidants in M7 Navel fruit are yet to be investigated.

2.11. Carotenoids

The name of carotenoid comes from carrot (*Daucus carota* L.), a plant that accumulates high levels of carotenoids in their root. However, underground organs such as tubers and roots and their accumulation of carotenoids is an exception among plants. Most commonly, plant carotenoids colour flowers, fruits and seeds. Plant pigments have been studied broadly because of their important role in colouration of fruits, vegetables and ornamental plants. Colour of fruits and vegetables is now a major concern for horticulturists for determining both grade and quality of these commodities. The most important external quality parameter in citrus fruit is rind colour. This rind colour is key in determining consumer acceptance (Goldschmidt,

1988). However, a visual expression of the colour is affected by temperature, climate, cultivar and culture practices. Gross (1987) stated that one of the complex sources of carotenoids is in citrus fruit, with the largest number of carotenoids than in any other fruit. Some carotenoids serve as originators for vitamin A, which is essential to human and animal diets; and serve in reducing the risk of certain forms of cancer by providing antioxidants (Olson, 1989). Carotenoids accumulate in the chromoplasts of flowers and fruits and provide the bright yellow, orange and red colours (Kato et al., 2004). Matsumoto et al. (2007) reported several important citrus carotenoids (phytoene, ζ-carotene, R-carotene, lutein, β-carotene, β-cryptoxanthin, zeaxanthin, and violaxanthin). Gross, (1987) found that β-xanthophylls, violaxanthin and β-cryptoxanthin, and their esters, are particularly common to most citrus.

2.11.1. Biosynthesis of carotenoids

The biosynthetic pathway of carotenoids has been studied for many years and summarised in (Figure 2.6). An available source of isoprenoid substrates is essential for the biosynthesis of carotenoids, resulting from the plastid-localised 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Rodriguez-Concepcion, 2010). A different range of compounds such as GAs, ABA, tocopherols, chlorophylls, phylloquinone (PhQ), monoterpenes and plastoquinone (PQ) are derived from isoprenoids also known as terpenoids. Isoprenoids are naturally-occurring organic chemicals that serve as originators for the formation of these compounds. During the process of the Calvin cycle or glycolysis, glyceraldehyde- 3-phosphate and pyruvate are produced, and this glyceraldehyde- 3-phosphate and pyruvate act as initial substrates leading to five carbon isoprene isomers, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are then compressed to synthesise geranylgeranyl diphosphate (GGPP) (Cazzonelli, 2011). The first steps in the MEP pathway are catalysed by 1-deoxyxylulose-5-phosphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR). The 1-hydroxy-2- methyl-2-(E)-butenyl 4-diphosphate reductase (HDR) then catalyses the production of IPP and DMAPP (Cazzonelli, 2011).

2.11.2. Carotenoids and citrus fruit colour

Fruits colours have been studied for many years because of their important role in the visual appearance and their effect on grower income. Gross, (1987) reported that the colour development in citrus fruit occurs with the transformation of chloroplast to the chromoplast containing carotenoid. Rind of the fully coloured mature orange (*Citrus sinensis* L. Osbeck) fruit is full of carotenoid and it probably is this structure that is mostly studied. There are two important acyl esters of carotenoids (9Z)-violaxanthin and *β*-citraurin. Oberholster et al. (2001) studied the biochemical basis of colour in Navel and Valencia orange using high-performance liquid chromatography and found that rind colour of the mature colour fruit depends on the content and ratio of (9Z)-violaxanthin and β-citraurin (Fig 2.7). Furthermore, the quantitative data showed that colour intensity of rind is associated with an increase in (9Z)-violaxanthin and β-citraurin content, associated with the decline in the (9Z)-violaxanthin: β-citraurin ratio from more than 50% to 10% (Oberholster et al., 2001).

The most abundant carotenoid present in citrus rind is (9Z)-violaxanthin (Gross, 1987). The (9Z)-violaxanthin carotenoid works as a yellow pigment in the background of orange rind. Farin et al. (1983) reported that red-orange pigment and bright orange colour of tangerine rind is significantly affected by C-30 apocarotenal or β-citraurin. A very high level of chlorophyll, low level of (9Z)-violaxanthin and an undetectable amount of β-citraurin was present in poorly coloured fruits (Oberholster et al., 2001). In addition, the fruit of average colour had a relatively low amount of both pigments, while fruit with good colour grade contained increased levels of (9Z)-violaxanthin and β-citraurin. Furthermore, an increase in colour grade is due to the further accumulation of (9Z)-violaxanthin and less intense increase in β-citraurin in both Navel and Valencia oranges. Reeves et al. (1997) claimed that the colour intensity or chroma of the citrus rind is dependent on the amount of the (9Z)-violaxanthin and β-citraurin, whereas the ratio of (9Z)-violaxanthin: β-citraurin is responsible for hue angle in the quality assessment of the fruit.

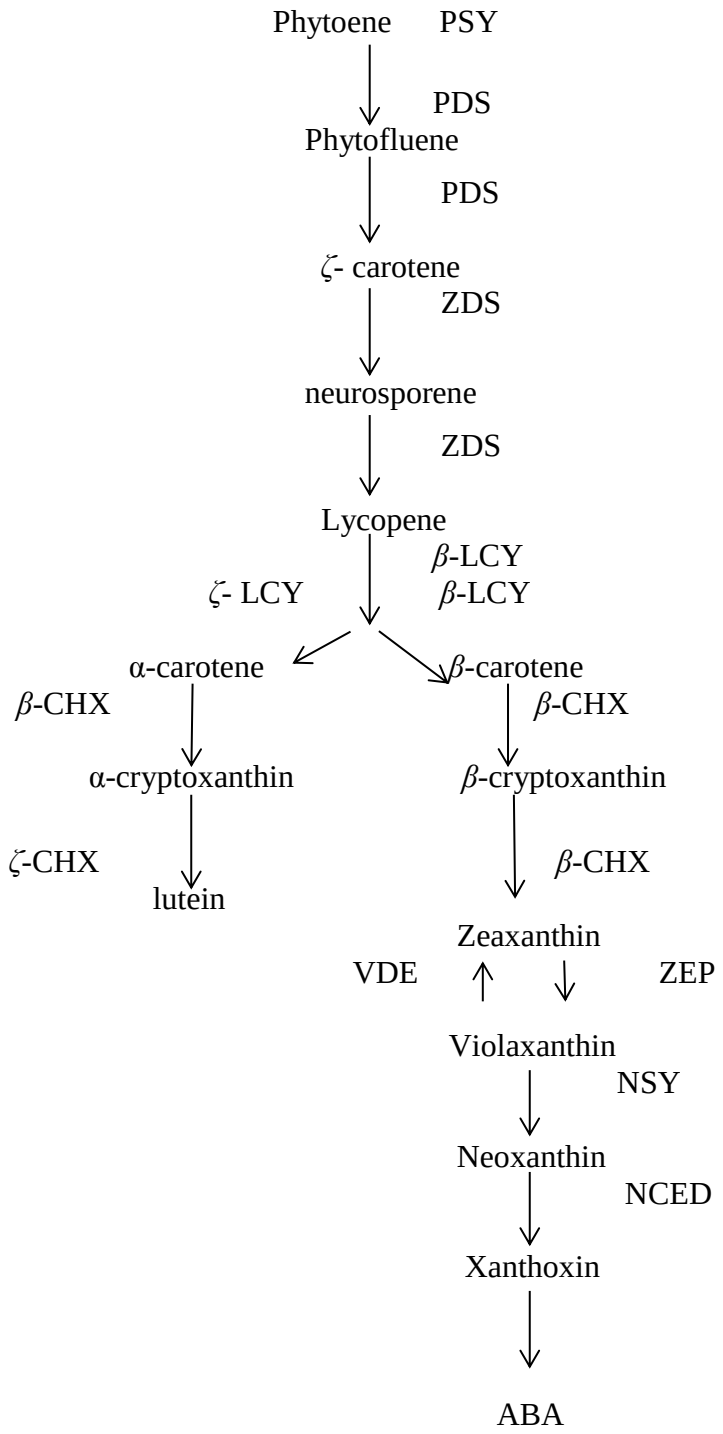
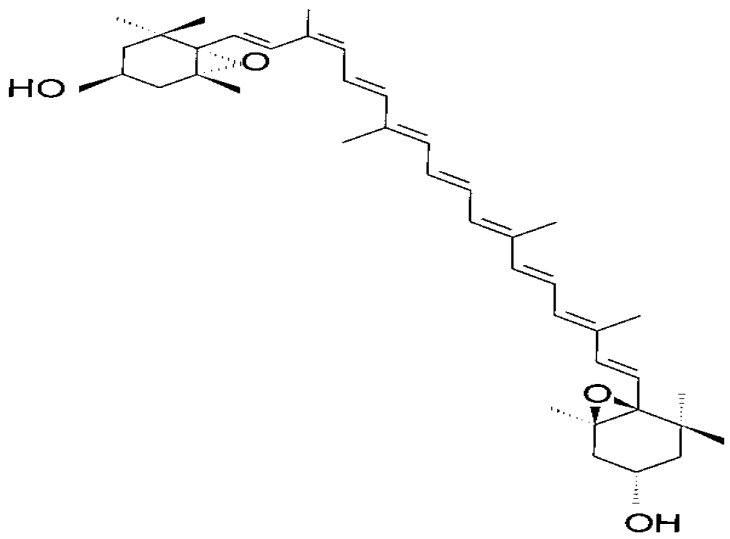


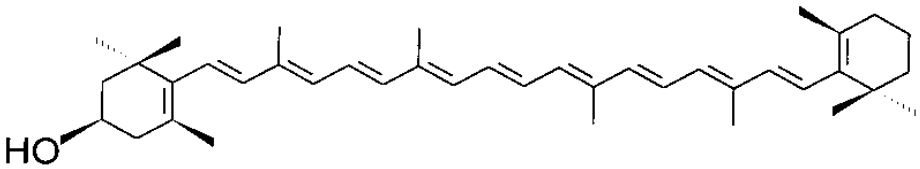
Fig: 2.6. Diagrammatic representation of carotenoid biosynthesis pathway in plants. PSY (phytoene synthase); PDS (phytoene desaturase); ZDS (zcarotene desaturase); *β*-LCY, *β*-lycopene cyclase; *ζ*-LCY, *ζ*-lycopene cyclase; *β*-CHX, *β*-carotene hydroxylase; *ζ*-CHX, *ζ*-carotene hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NSY, neoxanthin synthase; NCED, 9-cis-epoxycarotenoid dioxygenase (Rodrigo et al., 2003).

2.11.3 Function of Carotenoids

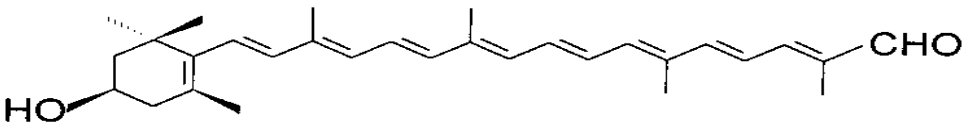
Carotenoids contain up to 15 conjugated double bonds and isoprenoid compounds are typically C40 with polyene chains. Naturally occurring, 700 carotenoids have been identified (Britton et al., 1995, 2004). Carotenoids differ from anthocyanins and betalains due to the following essential roles in plant life, for instance, provision of substrates for biosynthesis of the plant growth regulator ABA (Nambara and Marion-poll, 2005); photo protective functions during photosynthesis (Green and Durnford, 1996; Niyogi, 2000) and perhaps other hormones as well (Auldridge et al., 2006). One of the important roles of carotenoid in human nutrition and health is providing pro-vitamin A which has anti-cancer properties (Mayne, 1996). Some carotenoids are used as food colourants, cosmetics or pharmaceuticals.



9Z-violaxanthin



β-cryptoxanthin



β-citraurin

Fig: 2.7. Structures of major colour imparting carotenoids in citrus rind, (9Z) – violaxanthin, β-cryptoxanthin, and β-citraurin (Oberholster et al., 2001).

2.12. Chlorophylls

Chlorophylls are not present in the rind of a mature fruit or ripe fruit, while this is the main pigment present in the peel of immature or green fruit (Eilati et al., 1969a, 1975; Gross, 1987; Alos et al., 2006). Chlorophyll is responsible for the green colour of the fruits. But the degradation of this pigment is important during the ripening of the fruit for its cosmetic appearance. The highest value of this pigment reaches around 300-250μg g^{-1} during the colour break stage in the rind of green fruits, while its concentration starts to decrease predominantly as the fruit is advancing toward maturity (Yamauchi et al., 1997; Rodrigo et al., 2004; Alos et al., 2006, 2008; Alquezar et al., 2008, 2013). Chlorophyll is broken down into colourless linear tetrapyrroles, which accumulate in the vacuoles of de-greened cells during the process of leaf senescence and fruit ripening (Hortensteiner, 2006). Furthermore, the chlorophyll degradation pathway in the developmental process depends on the cell biology and biochemistry of that cell. The chlorophyll degradation pathway includes at least six enzymatic and one non- enzymatic reaction (Hortensteiner, 2006) (Fig 2.8). The major component of chlorophyll in terms of abundance is chlorophyll a, followed by chlorophyll b (Yamauchi et al., 1997; Alos et al., 2008; Srilaong et al., 2011). Yamauchi et al. (1997) reported that during the natural de-greening process of Wase Satsuma mandarin, some chlorophyll derivatives such as OH-chlorophyll a, chlorophyllide a, pheophorbide a, and pyropheophorbide a significantly decrease. It has been reported that (OH-chlorophyll a) and (pheophytin a) have been detected at a mature green stage in Washington Navel oranges instead of Chlorophyll a and Chlorophyll b, and eventually, (OH-chlorophyll a) and (pheophytin a) pigments decreased and disappeared, respectively (Alos et al., 2008).

The process of photosynthesis is significantly affected by chlorophyll and also participates in the colouration of fruit. One of the important indexes for fruit maturity is colouration; a colour change from green to yellow is associated with the degradation of chlorophyll and the accumulation of carotenoids (Peng et al., 2013). Chlorophyll biosynthesis and chlorophyll a/b interconversion, as well as degradation, significantly affect the amount of chlorophyll (Tanaka and Tanaka, 2006). Furthermore, it was reported previously by Mayfield and Huff (1986) that chlorophyll binding conditions predominantly influence chlorophyll degradation. Moreover, there are four main parts of chlorophyll metabolism: chlorophyll

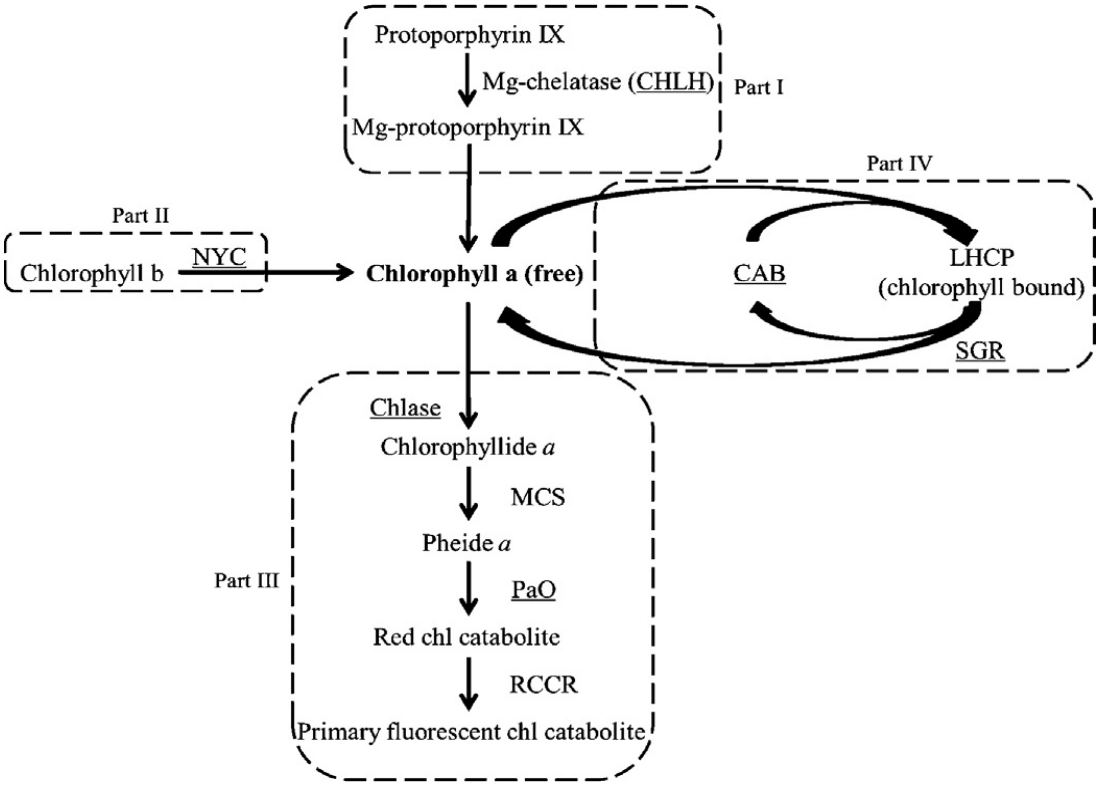


Fig 2.8 Systematic diagrammatic representation of chlorophyll metabolism and proteins (enzymes) involved. NYC, non-yellowing colour (chlorophyll b reductase); Chlase, chlorophyllase; MCS, metal chelating substance; PaO, pheophorbide a oxygenase; RCCR, red chlorophyll catabolite reductase; LHCP, light-harvesting chlorophyll a/b binding protein complex; SGR, stay-green protein; CAB, chlorophyll a/b binding protein. Part I, chlorophyll a synthesis; Part II, chlorophyll interconversion between chlorophyll a and chlorophyll b; Part III, chlorophyll degradation; Part IV, chlorophyll state: bound or free (Peng et al., 2013).

synthesis, chlorophyll a/b interconversion, chlorophyll binding and chlorophyll degradation. According to Peng et al. (2013) molecular study of the chlorophyll degradation reveals that down-regulation of chlorophyll a/b binding protein (CitCABs), is likely to be the main reason for chlorophyll reduction during natural and induced ethylene de-greening. The process of chlorophyll catabolism in citrus peel is associated with fruit ripening, and involves four basic steps: chlorophyllase (Chlase), producing chlorophyllide a; firstly, an unidentified metal-chelating substance called chlorophyllide a, removes magnesium and produces pheophorbide a; and finally, pheophorbide a oxygenase (PaO) and red chlorophyll catabolite reductase (RCCR) converts pheophorbide a to red chlorophyll catabolite (RCC) and then top FCCs (reviewed in Hortensteiner, 1999). Hortensteiner, (2006) reported about the degradation of chlorophyll pathway. The pathway is catalysed by four enzymes initiated by chlorophyllase (Chlase) enzymes and is to be considered the main enzyme involved in chlorophyll loss. Furthermore, chlorophyllase (Chlase) activity initiated by ethylene treatment and its association with the breakdown of chlorophyll in the citrus rind is well understood (Hirschfeld and Goldschmidt, 1983; Purvis and Barmore, 1981; Harpaz-Saad et al., 2007; Shemer et al., 2008).

2.13. Environmental factors affecting citrus rind colour

2.13.1. Light

The synthesis of the chlorophyll in the green part of the plant occurs under the light, while the degradation of chlorophyll during the fruit ripening occurs under dark conditions (Gross, 1987). The accumulation of carotenoid in the peel of citrus fruit requires light (Ruiz-Sola and Rodriguez Concepcion, 2012). The previous point is supported by Lewis and Coggins (1964) that light is required for chlorophyll degradation and carotenoid accumulation. However, in other parts of citrus fruit, as in the pulp, there might not be an essential requirement for light. Furthermore, light considerably influence carotenoid accumulation in the chloroplasts (Gross, 1987). The fruit position on the tree greatly influences the rind colour (Sites and Reitz, 1950; Iwagaki and Kudo, 1977; Tao et al., 2003). Casas and Mallent (1988) reported that mandarin and sweet orange fruit exposed to the light side develop better colour than the fruit on the darker side. In addition, fruit is found to be more intense in colour in a less dense orchard (Boswell et al., 1982). Furthermore, Tao et al. (2003) claimed that

a significantly reduced rind colour was found in citrus peel when the fruit was bagged with black polyethene film to avoid sunlight. Thus, the evidence showed that light greatly influences rind colour.

2.13.2. Temperature

Temperature plays a key role in developing rind colour. Citrus-producing countries where the temperature is high all around the year produce fruit with more chlorophyll content as compared to the countries where the night temperature less than 13°C (Caprio, 1956). Gross (1987) reported that peel colouration during ripening of citrus fruit requires special temperatures, as the transformation of chloroplast into chromoplast is prompted by low temperature. The night temperature below 13°C is required to initiate fruit colour break in different cultivars of sweet oranges (Stearns and Young, 1942).

Furthermore, rind colour depends upon the alteration between day and night temperatures. The degradation of chlorophyll and accumulation of carotenoid concurs with a below 13°C night temperature during the maturation phase. However, this chlorophyll degradation depends on cultivar and the duration of night temperature. Young (1961) reported a study regarding rind colouration and temperature regimes, pointing out that a day/night temperature regime of 20°C/7°C may be a possible range to induce bright orange coloured fruit in Valencia orange and the night temperature below 12°C is typical for orange colouration. Meredith and Young (1969) claimed that a day temperature of 16°C and night temperature of 5°C are required for the biosynthesis of carotenoid in sweet orange and red grapefruit; however, a maximum day temperature of 35°C and night temperature of 30^{o}C stimulates lycopene formation. Manera et al. (2012) pointed out the stimulatory effect of low temperature (6-15°C) in the peel colouration of different cultivars of lemon, whilst in Satsuma mandarin faster carotenoid accumulation was noted at 14-30°C (Sonnen et al., 1979) and in Clementine mandarin 20-23°C stimulated rind colour development (Mesejo et al., 2012).

The main reddish pigment in the peel of many orange and mandarin cultivars is C30 apocarotenoid β-citraurin. This C30 apocarotenoid β-citraurin is highly sensitive to temperature; its synthesis is highly stimulated at low temperature, while it

decreases when the temperature is above 30°C (Stewart and Wheaton, 1972). To maintain the postharvest life and quality of citrus fruit, a temperature range between 1°C and 4°C is practised to maintain quality, in addition, it also significantly affects rind colour. Carmona et al. (2012) claimed that storage temperature of 12°C significantly increased peel colouration and enhanced total carotenoid accumulation in both pulp and rind; on the contrary, fruit stored at 2°C showed no sign of change.

2.13.3. Fruit position and tree age

The rind colour of the citrus fruit is deliberately influenced by both the position of the fruit on the tree as well as tree age. The fruit position on the tree is very important to determine the peel colour characteristic. Stewart (1975) claimed that juice colour was brighter in the fruit growing on the north side of the tree than from the south side and the same result was reported for peel colour in six different cultivars of sweet orange. Tree age is also a predominant factor in citrus rind colour. Old trees are often less vigorous than young trees. The vigour of the tree negatively affects the peel pigmentation. The main reasons behind the peel colouration might be tree vegetative growth which limits light penetration into the tree canopy (Krajewski, 1996).

2.13.4. Climate

Citrus production is scattered over 30 countries in the world and these countries are mostly located at 40° north-south latitude. The citrus fruit quality, yield, growth and development are significantly affected by the climatic condition of these latitudes. Caprio (1956) reported that weather condition and temperature affect the rind colour, during stage 2 of fruit development. Davies and Albrigo (1994) reported that different climatic zones and their characteristics affect the citrus fruit quality, thus, the above evidence suggests that climatic conditions strongly influence fruit quality and rind colour.

2.14. Nutritional factors affecting citrus rind colour

Nitrogen, phosphorus and potassium along with other essential nutrients have been widely used to fertilize citrus trees over the years. These fertilizers affect the quality attributes both internally and externally. Citrus rind colour depends on a transition from chloroplast to chromoplast. Rodrigo et al. (2013b) reported that on the basis of past experiments nutrient availability is one of the main factors in stimulating

and controlling citrus rind colour. Nitrogenous fertilizer has been used for years in different fruits species, especially for the vegetative growth. Heavy application of nitrogen fertilizer results in an increased fruit set but decreases the average size of the fruit. Rodrigo et al. (2013b) reveal that nitrogen and sugar are recognized as essential effectors of reversible conversion from chloroplast to chromoplast. The delay in peel de-greening might be due to the high dose of nitrogen application (Jones, 1959). Sugar-induced chlorophyll degradation is negatively influenced by high nitrogen concentration in the rind of citrus fruit (Huff, 1983, 1984). In North Carolina, peach trees with deficient phosphorus nutrition produced poor colour, misshapen and unpleasant tasting fruit (Reuther et al., 1958). Available evidence showed that apple trees which showed the symptoms of potassium deficiency produce fruit that are not fully coloured (Reuther et al., 1958). This means that potassium could play a role in colour development.

2.15. Growth regulators and rind colour

2.15.1 Methyl Jasmonate (MJ)

MJ was first extracted from jasmine (*Jasminium grandiflorum* L.) flower as a sweet-smelling compound in 1962 (Demole et al., 1962). Jasmonates are a class of endogenous plant growth regulators and their cellular response occurs at low concentration distant from their sites of synthesis. Jasmonates include MJ, and jasmonic acid (JA).

2.15.2. Biosynthesis of MJ

According to Vick and Zimmerman (1984) jasmonate and its methyl ester (MeJA) was first synthesised from linolenic acid; this linolenic acid was initially oxygenated by lipoxygenase (LOX) to form 13 (S) - hydroperoxy linolenic acid (13-HPOT). The chemical structure of jasmonates is characterised by cyclopentanone rings, unevenly placed at positions C-3, C-6 and C-7 (Sembdner and Parthier, 1993). Two of them are biologically active in plants and fungi such as (-) JA and (+)-7-iso-JA. The fatty acid hydroperoxide can be cyclized to 12-oxo Phytodienoic acid (OPDA) (Zimmerman and Feng, 1978) and the activities of hydroperoxide cyclase was found to be present in many plant species (Vick and Zimmerman 1984). Two important enzymes specifically, allene oxide synthase (AOS) and allene oxide cyclase (AOC)

play vital roles in the cyclization process. Vick and Zimmerman (1984) demonstrated that OPDA further metabolised to JA. Vick and Zimmerman (1984) claimed that the metabolism of JA includes the reduction in the cyclopentenone ring of OPDA to form respective cyclopentanone (OPC 8:0) followed by three β-oxidation cycles resulting in the shortening of octanoic side chain and formation of JA.

2.15.3. Role of MJ

The application of JA causes the loss of chlorophyll from the leaves. MJ stimulates chlorophyll deprivation and the development of anthocyanins (Creelman and Mullet, 1997; Perez et al., 1993), aroma development (Olias et al., 1992) and ethylene production in mango and plum fruit (Lalel et al., 2003a; Khan and Singh, 2007). Jasmonate might be expected to play a role in flower and fruit development because it is present in comparatively higher concentrations in the reproductive tissues. JS regulates fruit ripening, carotenoid composition and expression of genes involved in seed and vegetative storage proteins. JA stimulates tomato and apple fruit ripening through the activation of ethylene (Shafiq et al., 2013; Czapski and Saniewski 1992). A possibility exists that ethylene production in fruit is stimulated by jasmonates which ultimately leads to ripening. MJ application to tomato fruit stimulates the accumulation of β-carotene and inhibits the accumulation of lycopene in tomato fruit (Saniewski and Czapski, 1983). The application of MJ showed a possible anthocyanin accumulation in *Vitus vinifera* cell culture (Curtin et al., 2003; Zhang et al., 2002). Pre-harvest spray application of MJ resulted in an enhanced level of anthocyanin (Rudell et al., 2002) and carotenoid (Perez et al., 1993) in apple. In addition, pre-harvest treatment of MJ at a concentration of 0.01mM or 0.001mM stimulates anthocyanin, anti-oxidant, flavonoid and phenolic content in raspberries (Wang and Zeng, 2005). The effects of exogenous application of MJ on sweet orange fruit colour development are yet to be investigated.

Methyl jasmonate (MJ) has been reported to reduce CI and extend storage life in grapefruit (Meir et al., 1996), papaya (Gonzalez-Aguilar et al., 2003), guava (Gonzalez-Aguilar et al., 2004), peach (Budde et al., 2004), mango (Gonzalez-Aguilar et al., 2000a), pineapple (Nilprapruck et al., 2008), loquat (Cai et al., 2011), pomegranate (Mirdehghan and Ghotbi, 2014) and lemon (Siboza et al., 2014). The

effects of MJ in reducing CI in Midknight Valencia and Lane Late sweet orange are yet to be investigated.

2.15.4. Application methods of MJ

There are various methods for the application of MJ for instance spray, fumigation, dip and in paste form. The application of MJ in a paste form mixed with lanolin paste and spread on plant tissue has been reported in apples (Miszczak et al., 1995). In addition, Baldwin and Schmelaz (1996) claimed that MJ has also been used by some researchers in liquid form as a hydroponic solution. Jasmonate spray contains small amounts of solvent such as methanol (MeOH) or acetone to dissolve the jasmonate and also contains a surfactant such as Triton X- 100®. Molin et al. (1997) applied MJ as fumigation to strawberry cultivars stored at 20°C which reduced decay. Later on, application of MJ as fumigation has also been reported in various fruits such as strawberry fruit (Ayala-Zavala et al., 2005), pineapple (Martinez-Ferrer and Harper, 2005) and apple (Perez et al., 1993). The pre-harvest spray application of MJ has also been reported in plum and apple (Kucuker et al., 2014; Rudell et al., 2005; Shafiq et al., 2013). Meir et al. (1996) reported that avocado, grapefruit and pepper fruit showed reduced CI when MJ was applied as a dip (< 25μM) and fumigation (<100μM) in gas form.

2.15.5 MJ and fruit quality

Fruit quality in general which includes external features such as appearance (shape, size, colour, injuries, diseases and blemish), texture (firmness, juiciness and maleness), flavour (sugar, sweetness, astringency and aroma), nutrient content (vitamin, mineral, dietary fibre carbohydrates) and phytochemical (Knee, 2002). Exogenous application of MJ has been found to improve fruit colour in plums (Khan and Singh 2007), apple (Fan and Mattheis, 1999; Rudell et al., 2002), raspberries (Wang and Zheng, 2003), mango (Lalel et al., 2003a) and aroma volatile production in mango (Lalel et al., 2003b). In addition, exogenous application of MJ enhances chlorophyll degradation and the biosynthesis of β-carotene and the level of antioxidant in apple (Rudell et al., 2002). According to Saniewski and Czapski (1983), exogenous application of MJ stimulates β-carotene accumulation and inhibits lycopene accumulation in the ripening tomatoes. Meanwhile, the effects of exogenous application of MJ on the accumulation of total carotenoids in the rind of M7 Navel are

yet to be investigated. Pre-harvest MJ application significantly affects the fruit quality attributes such as SSC, total sugars, fructose, glucose, sucrose and TA, malic acid and citric acid than in untreated fruits in black raspberries (Wang and Zheng 2005). Moreover, it has been reported that pre-harvest MJ treatment could enhance antioxidant capacity and improve flesh firmness of plum fruit by increasing phenolic content (Ozturk et al., 2015). Furthermore, MJ has also been used as an effective treatment to enhance colour in apple (Ozturk et al., 2013). Application of MJ (10^{-5}M) in combination with modified atmosphere packaging (MAP) enhances the postharvest quality of Sunrise papaya during 32 d of storage at 20°C (Gonzalez-Aguilar et al., 2003). Wang (2003) claimed that application of MJ (22.4 μL L^{-1}) at 20°C for 16 h exhibited a higher level of sugars, organic acid and oxygen radical absorbance capacity with higher intensity of red colour development as compared to untreated fruit in cultivar Heritage raspberries. The effect of pre-harvest spray application of MJ on improving the M7 rind colour and maintaining the quality is yet to be investigated. Moreover, postharvest dip application of MJ to mitigate CI in Midknight Valencia and Lane Late during cold quarantine treatment (1°C for 21 d) and extending storage life at 4°C or 7°C for 90 d followed by 10 d simulated shelf condition only in Midknight Valencia.

2.16. Abscisic acid (ABA)

S- (+)-*cis*, *trans*-Abscisic acid (ABA) is a naturally-occurring plant growth regulator. ABA plays a very important role in seed development, dormancy, environmental stress, fruit ripening and its role continues throughout the life cycle of plants. Many researchers believe that ABA has a role in fruit ripening, while others argue that it promotes the effect of ethylene on fruit ripening. Unlike other plant hormones, ABA has a unique ability to regulate its concentration in different tissues in response to developmental and environmental changes.

It is believed by many researchers that not only ethylene but ABA also plays an important role in the regulation of fruit ripening (Zhang et al., 2009). The levels of ABA in climacteric and non-climacteric fruits are different at different growth periods. The level of ABA in climacteric fruits such as apple increase from maturation to harvest; while in non-climacteric fruits such as sweet cherries the level of ABA increases before maturation and decreases until harvest (Setha, 2012). The varying

amount of ABA in different fruits suggests its role in ripening. To know the physiological role of ABA in regulating fruit ripening, it is important to find out its endogenous levels and the effect of exogenous application of ABA on fruit ripening (Setha, 2012).

2.16.1. Effect of ABA on pigment and colour changes

The levels of ABA in the fruits are closely correlated with colour changes and pigment content (Kondo et al. 1991; Sandhu et al., 2011). The effect of ABA on anthocyanins accumulation in grapes has been reported by many researchers. In addition, anthocyanin accumulation in grapes commences at the onset of maturation; its accumulation is regulated by a part of ABA (Ban et al., 2003). The exogenous application of ABA resulted in improved grape skin colour in different cultivars such as Cabernet Sauvignon (Jeong et al., 2004), Tempranillo (Delgado et al., 2002), Flame Seedless (Peppi et al., 2006) and Redglobe (Peppi et al., 2007). Roberto et al. (2013) reported that application of ABA (400 mg L^{-1}) applied twice at 7 d after veraison (DAV) followed by 15 days before harvest (DBH) improved the colour of Rubi table grapes.

It has been observed that the development of colour during ripening in strawberries is induced by ABA through up-regulation of ethylene (Jiang and Joyce, 2003). Moreover, ABA treatment hastens colour development in harvested strawberries by inducing anthocyanin, phenolic contents and phenylalanine ammonia-lyase (PAL) activity during storage. The role of ABA in apple and peaches colour development has also been previously reported (Zhang et al., 2009; Kondo et al., 1991). The role of ABA in citrus fruit ripening is well studied, and many researchers suggest that ABA plays an important role in fruit development and ripening (Goldschmidt et al., 1973; Nooden, 1988). According to Aung et al. (1991) the involvement of ABA cannot be ruled out in fruit colour development since the content of ABA in the citrus rind was increased during the process of fruit development. Valero et al. (1998) previously reported that lower ABA levels were accompanied by a delay in colour change in stage 1 of lemon fruit. Harris and Dugger (1986) reported that increased levels of ABA in the citrus fruit exocarp were associated with senescence of chlorophyll and development of chloroplast. Furthermore, during the transformation of chloroplast to chromoplast, the level of ABA increased 12.6- fold

which shows a possible association of ABA with carotenoids biosynthesis. Several authors have shown that ABA plays a significant role in the colour development of fruits. There is well-recognized evidence that increased ABA levels in mandarins (Lafuente et al., 1997), oranges (Harris and Dugger, 1986) and sweet cherry (Kondo and Gemma, 1993) were responsible for the transition of chloroplast to chromoplast in colour development. However, no research has been reported on the effect of S-ABA on the colouring and carotenoid accumulation in early maturing M7 Navel orange under the agro-climatic condition of WA and is yet to be investigated.

2.17. Gibberellins

The role of GAs in fruit and tree physiology has been well documented in the literature. Gibberellins are supposed to be an essential effector in citrus fruit responsible for ovary- fruit transition (Talon et al., 1992). In addition, cell division and cell enlargement is thought to be activated by GAs. Talon and Zeevaart (1992) reported that the role of GAs is generally associated with the initiation of growth. In citrus fruit the endogenous GAs found are the key members of the 13-hydroxylation pathway [GA_{35}, GA_{97}, GA_{44}, GA_{17}, GA_{19}, GA_{20}, GA_{29}, GA_1, epi-GA_1, and GA_8 (Goto et al., 1989; Turnbull, 1989; Talon et al., 1990)]. This pathway also works in the vegetative part of the citrus fruit (Vidal et al., 2003).

As discussed previously, citrus peel colour is one of the important fruit quality parameters and consumers pay a premium price for bright coloured fresh fruit. The colour development results from the degradation of chlorophyll (El-Zeftawi, 1978) and the accumulation of carotenoids (Stewart and Wheaton, 1972) with the changes in the plastids (Thomson et al., 1967; El-Zeftawi and Garrett, 1978; Ljubesic, 1984). The manipulation of fruit colour development by using the plant growth regulators is done by two ways (1) enhancing colour changes in early maturing varieties (2) retarding colour changes to delay peel softening and to decrease peel disorders (El-Otmani et al., 2000). The phenomenon of enhancing colour is called de-greening; however, the delay in colour changes in the peel of citrus fruit is called re-greening. El-Otmani et al. (2000) observed that de-greening is done by the grower and packers in early season varieties due to the fact that early in the season demand is high and supply is low. A pre-harvest spray of ethephon boosts colour development in oranges and mandarin (Young et al., 1974; Pons et al., 1992). Furthermore, postharvest ethylene application

is common practice to enhance fruit colour in the Mediterranean and other citrus-producing areas such as California (Coggins, 1992).

In contrast, GA_3 inhibit the peel colouration by delaying the loss of chlorophyll and reducing the accumulation of carotenoids (El-Zeftawi, 1978; EI-Zefatwi and Garrett, 1978). The onset of chlorophyll degradation and the initiation of carotenoids accumulation were retarded by the application of GA_3 and the response was maximum when GA_3 was applied during 10 d intervals (Garcia-Luis et al., 1992). Goldschmidt and Eilati (1970) investigated the inhibitory effect of gibberellins on colour changes associated with the maturation of Shamouti oranges. Furthermore, quantitative estimation showed that gibberellins had a similar effect on fruit detached or attached to the tree. GAs has a significant inhibitory role in the colouring of fruit by delaying chlorophyll degradation and the accumulation of carotenoids. However, the effect of inhibitors of GAs biosynthesis on the colouring of early maturing M7 under the agro-climatic condition of WA warrants investigation.

2.18. Pro-Ca and PBZ

Prohexadione-calcium (Pro-Ca) (3-oxido-4-propionyl-5-oxo-3-cyclohexene-carboxylate) is a plant growth regulator, predominantly used on fruit trees (Evans et al., 1999) and is also known for its anti-gibberellin activity in rice (*Oryza sativa* L.) (Nakayama et al., 1992). Presently, this growth inhibitor is known by the trade name Apogee® (27.5% Pro-Ca) in the USA and Regalis® (10% Pro-Ca) in some European countries (Prive et al., 2006). Pro-Ca retards the synthesis of gibberellins and reduces excessive vegetative growth and also the incidence of fire blight in apples (Buban et al., 2004; Evans et al., 1999; Roemmelt et al., 1998; Roemmelt et al., 2003). The action of Pro-Ca varies from other gibberellin biosynthesis inhibitors, it has been known to reduce levels of GA_1 (highly active) and cause accumulation of its immediate precursor, GA_{20} (inactive) (Fig. 2.10) as result blocking the conversion of inactive GA_{20} to active GA_1, thus reducing longitudinal vegetative growth (Evans et al., 1999). In addition, an estimated time of 8 hours is taken for the uptake of Pro-Ca in the plant and for movement to the growing points of individual shoots. Greene, (2008) reported that reduced vegetative growth was noticeable after 2 weeks of Pro-Ca spray and its activity lasted for 3 to 4 weeks (Unrath, 1999). It has been previously reported that the half-life of Pro-Ca is 2 weeks before changing to the naturally occurring propane-1, 2,

3-tricarboxylic acid and less than 24 h in the soil before decomposition to CO_2 (Evans et al., 1999). Moreover, Pro-Ca has no hazardous, mutagenic or tetragenic effects. Thus, Pro-Ca has a low toxicity and inadequate persistence in trees. The ring structure of Pro-Ca is similar to 2-oxoglutarate. Moreover, the ring structure is able to constrain dioxygenase enzymes (Rademacher, 2000), which involved in gibberellins and flavonoid biosynthesis (Forkmann and Heller, 1999).

Pro-Ca spray has been successfully used to inhibit vegetative growth in numerous horticulture and agronomic crops, for instance, pear (Elfving et al., 2003b; Elfving et al., 2000; Southwick et al., 2004), plum (*Prunus domestica* L.) (Basak and Rademacher, 2000), sweet cherry (*Prunus avium* L.) (Elfving et al., 2003a), tomato (*Lycopersicon esculentum* Mill.) (Yamaji et al., 1991) and apple (Miller and Tworkoski, 2003; Evans et al., 1997; Basak and Rademacher, 1998; Greene, 2008; Miller, 2002). The available literature showed that Pro-Ca significantly influenced the concentration of anthocyanins and carotenoids in apples but concentration is cultivar dependent. In addition, Pro-Ca has been revealed to enhance colour intensity, total anthocyanins and total

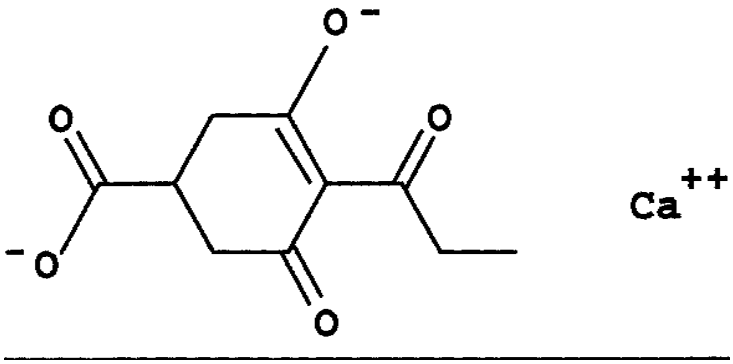


Fig.2.9 The ring structure of Pro-Ca (Evans et al., 1999)

phenols, although having a negligible effect on crop yield (Giudice et al., 2004; Pilar Mata et al., 2006). However, limited studies have been reported on the response of vegetative growth to different gibberellin biosynthesis inhibitors, and ultimately their effects on the rind colour initiation of citrus species. Barry and Le Roux (2010) reported that spray application of Pro-Ca (400 mg L^{-1}) 6+3 weeks before harvest significantly enhanced citrus rind colour in the early maturing citrus cultivar Navelina Navel orange [*C. sinensis* (L.) Osbeck] as a result of increased carotenoid to chlorophyll ratio.

Monselise et al. (1976) reported that chlorophyll degradation in the rind of sweet orange was significantly enhanced by the application of PBZ. Furthermore, more abrupt rind colour change was recorded in Topaz tangor (*C. reticulata* Blanco x *C. paradisi* Macf) after the application of PBZ. Gilfillan and Lowe (1985) reported that PBZ (1 gL^{-1}) when applied after physiological drop improved the rind colour of Satsuma mandarin (*Citrus unshiu* Marc) by one to two colour rating units. The available evidence showed that Pro-Ca significantly affects anthocyanin accumulation in apple fruit resulting in the initiation of red colour in apples. In addition, sporadic and inconclusive information is available on the effect of Pro-Ca and PBZ to stimulate chlorophyll degradation and enhance carotenoid accumulation in citrus spp. The effects of Pro-Ca and PBZ in inducing colouring in early maturing M7 sweet orange under the Mediterranean climate of WA are yet to be investigated.

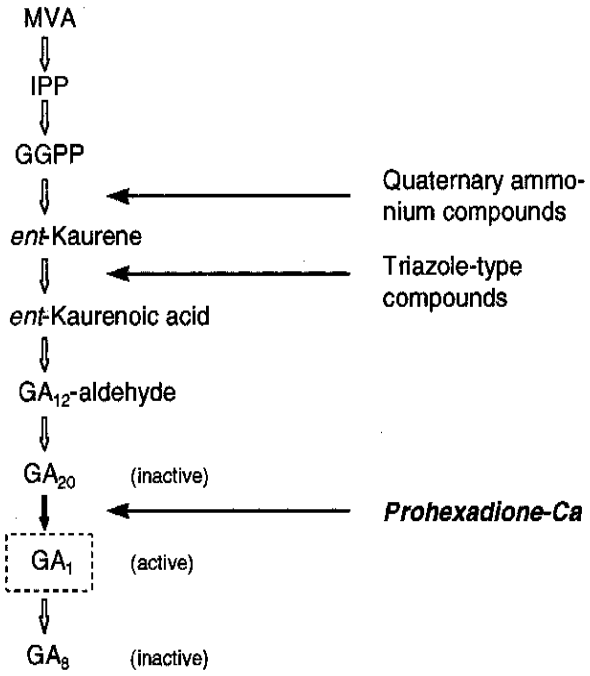


Fig.2.10 The process of GA biosynthesis and main point of inhibition by Pro-Ca (Evans et al., 1999)

2.19. Storage of sweet orange

2.19.1. Cold storage

Presently, cold storage systems are being used worldwide to extend the storage and shelf life of fruits and vegetables. In the 1880s, cold storage practices of citrus were started in the USA, but Britain started these practices even before (Ladaniya, 2008). The most important factors such as climate, temperature, humidity and pre- and postharvest factors significantly influence the success and failure during the storage life of the commodities. In addition, fruit maturity and handling procedures also affect the quality of the stored product. In citrus fruit, different cultivars of the same species or different species respond differently to the storage conditions (Ladaniya, 2008). One of the keys focuses of many researchers is to investigate the fruit quality and shelf life after removing the commodity from cold storage. Over the period of storage duration, the set temperature and relative humidity (RH) have to be uniform throughout the storage room. Internal CO_2 increases and O_2 decreases when the RH goes down (66-65 %), leading to a higher transpiration rate, earlier senescence and

rapid deterioration in the stored fruit (Ladaniya, 2008). The elevated RH (90-95 %) adequately protected the fruit from decay and the fruit can be stored at a relatively higher temperature, (5-10 °C) higher than normally used with the benefit of saving energy and reducing the risk of physiological changes. Cold cannot be exploited to its full potential in extending the storage life of various tropical and subtropical fruits because of their susceptibility of CI when stored at low temperature.

2.19.2 Chilling injury

Chilling injury (CI) is a major physiological disorder induced by low but non-freezing temperature which imposes limitations for extending the storage life of tropical and subtropical horticulture crops (Nilprapruck et al., 2008). CI disrupts normal cell metabolism and negatively affects fruit quality, one of the significant contributors to a postharvest loss in citrus fruit (Sala et al., 2005). In citrus fruit CI exhibits as rind staining, pitting, red blotches, scalding, watery breakdown, soft glazed continuing lesion of mandarins, sunken tissues, damage to the styler end of lemons and necrosis on the rind (Sala and Lafuente, 1999). Many biotic and abiotic factors influence the CI susceptibility of fruit including cultivar, harvest date, microclimate and management practices. In addition, other factors also influence the severity of CI such as fruit size, canopy position and rind colour. The severity of CI depends on the type of cultivar, mandarins are more susceptible as compared to Navel and Valencia orange (Lafuente et al., 2003).

Citrus fruit contains antioxidants such as phenolic compounds. These phenolic compounds significantly lessen the cell damage during CI (Grace and Logan, 2000). Cold storage negatively affects the concentration of phenolic antioxidants (Tomas-Barberán and Espin, 2001). As a result of the reduced concentration of antioxidants, a poor resistance to stress ultimately leads to membrane damage and CI. According to Tomas-Barberán and Espin (2001), phenolic compounds are plant secondary metabolites involved in fruit quality such as appearance, flavour and health-promoting compounds. These phenolic compounds are strong antioxidants capable of scavenging reactive oxygen species (ROS) (Grace and Logan, 2000; Gonzalez-Aguilar et al., 2004).

Rivero et al. (2001) claimed that PAL is a key enzyme at the start of the phenylpropanoid pathway. In addition, this enzyme is involved in defence against CI. Furthermore, the initiation of PAL activity in response to stress has been reflected as a defence mechanism of fruit to withstand cold stress (Martinez-Tellez and Lafuente, 1997). Therefore, PAL activity is predominantly affected by chilling stress (Sanchez-Ballesta et al., 2000). The production of phenolic compounds and PAL activity increases during chilling stress (Rivero et al., 2001). The initiation of PAL activity by chilling stress has been related with chilling tolerance in many citrus fruits (Sanchez-Ballesta et al., 2000; Martinez-Tellez and Lafuente, 1992). The higher PAL activity is related to reduce CI in cold storage (Lafuente et al., 2004). Moreover, Lafuente et al. (2003) showed that increased ethylene production and a PAL are both cold-induced responses and may occur in parallel with the development of CI in citrus cultivars. Martinez-Tellez and Lafuente (1997) investigated the effect of cold storage chilling temperatures (1.0, 2.5, and 5^{o}C) and non-chilling temperatures (10^{o}C) on the activation of three enzymes such as PAL, POD and polyphenol oxidase (PPO) in the rind tissue of two different citrus cultivars Fortune (*Citrus reticulata*) and Navelina (*Citrus sinensis* L. Osbeck). In addition, no correlation was found between peroxidase (POD), PPO and the development of CI in both citrus cultivars. The results showed that differences in susceptibility to CI are more likely related to PAL activity as compared to PPO and POD activity in both cultivars.

2.20. MJ and CI

MJ, as a natural plant regulator, plays a major role in plant growth, development, environmental stresses and fruit ripening (Creelman and Mullet, 1997). Exogenous application of MJ has been shown to reduce CI in several horticultural crops (Gonzalez-Aguilar et al., 2010).

The primary concern behind the storage of fruit at low temperature is to reduce the rate of fruit respiration, initiation of decay, water losses as well as physiological processes, ultimately to prolong the storage/shelf life of the fruit by maintaining its quality. The application of MJ has been reported to reduce CI and maintain quality in many fruit e.g. avocado (Meir et al. 1996), strawberry (Molin et al. 1997), tomato (Ding et al., 2002), papaya (Gonzalez-Aguilar et al., 2003), guava (Gonzalez-Aguilar et al. 2004), mango (Gonzalez-Aguilar et al., 2000a), peach (Budde et al., 2004; Meng

et al., 2009; Jin et al., 2009), pineapple (Nilpraprucк et al., 2008), lemon (Siboza et al., 2014) and loquat (Jin et al., 2014). Siboza et al. (2014) reported that 10uM MJ plus 2mM SA treatment were more effective in reducing chilling sensitivity in the lemon fruit.

It has been reported by Nilpraprucк et al., (2008) that pineapple treated with MJ significantly reduces the CI symptoms and the percentage of fruit weight loss as compared to the control treatment; while SSC, TA, total sugars and reducing sugars of the treated pineapple were not significantly different. Meng et al. (2009) revealed that MJ treatments before cold storage were beneficial for reducing the CI and to maintain the quality in peach fruit. Similarly, Jin et al. (2009) claimed that the combination of HA and MJ treatment might be a useful technique to delay CI and maintain the peach fruit quality during cold storage. MJ vapour treatment (10^{-4} M) for 24 h at 25°C in mango (*Mangifera indica* cv. Tommy Atkins) increased chilling tolerance and SSC but did not affect TA during subsequent storage for 21 d at 7^{o}C and after 5 d of shelf life at 20°C (Gonzalez-Aguilar et al., 2000a). Likewise, dips in n-propyl-dihydrojasmonate (PDJ) delay the development of CI in mango fruit stored at 6°C (Kondo et al., 2005). Gonzalez-Aguilar et al. (2004) reported that MJ increased sugar content, PAL, LOX enzymes activities while reducing CI index and the ion leakage percentage in guava fruit. Jin et al. (2014) reported that the activities of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) were enhanced while on the contrary PAL, POD, and PPO were inhibited with the combined treatment of hot air and MJ, resulting in lower lignin content. Similarly, Cao et al. (2009) reported that reduction in CI in loquat fruit with the application of MJ may be due to the enhanced antioxidant enzymes and higher unsaturated/saturated fatty acid. Moreover, MJ treated fruit showed significantly lower activates of PAL, POD, PPO and higher polygalacturonase (PG) activity than the control in the cold stored loquat fruit, which suggests that increase in chilling tolerance by MJ may be due to high polysaccharides solubilisation and inhibited lignin accumulation (Cao et al., 2010). Meir et al. (1996) reported that MJ dip for 30 seconds (s) in avocado (2.5μM), grapefruit (10 μM) and red bell pepper fruit (25 μM) significantly reduced the severity of CI and fruit weight loss following 4-10 weeks cold storage at 2°C. Mirdehghan and Ghotbi (2014) reported that 0.4mM MJ treated pomegranate fruit showed a lower CI index than the control. Similarly, Sayyari et al.

(2011) reported that the postharvest application of MJ and methyl salicylate induced chilling tolerance and maintained pomegranate fruit quality. As discussed previously, MJ plays a major role in inducing chilling tolerance and enhancing antioxidant capacity in many fruit crops. The postharvest application of MJ inducing chilling tolerance and improving fruit quality in sweet orange needs to be investigated.

2.20.1 MJ and fruit quality

Methyl jasmonate affects fruit quality including texture, colour, aroma, flavour and antioxidant properties. The exogenous application of MJ also affects physiological processes and the postharvest quality of fruit. MJ has been reported to maintain post-harvest quality in many fruits and vegetables, including loquat fruit (Cao et al., 2009) and papaya (Gonzalez- Aguilar et al., 2003). Furthermore, Wang and Buta (2003) reported that postharvest application of MJ maintained higher levels of sugars and organic acids in kiwi fruit. A class of pigments such as chlorophyll, carotenoids and anthocyanin are responsible for fruit colour. Shafiq et al. (2011) reported that pre-harvest spray application of MJ improved the colour of apple fruit through the enhanced accumulation of flavonoids and anthocyanins in the apple skin without adversely affecting the fruit quality.

2.21. Salicylic acid

Salicylic acid (SA) has been known to produce a wide range of metabolic and physiological responses in plants and as a result, significantly affecting their growth and development. SA as a natural and safe phenolic compound has a high impending effect in controlling postharvest losses in fruit and vegetables. Asghari and Aghdam (2010) reported that exogenous application of SA predominantly affects various physiological processes such as postharvest life, disease tolerance, fruit ripening, ethylene biosynthesis, oxidative stress, respiration, fruit firmness, antioxidant system and nutritional quality. SA is an endogenous signal molecule, playing a major role in regulating plant developmental processes and stress responses including stomata conductance, crop yield, glycolysis, seed germination, disease resistance, transpiration and photosynthesis (Klessig and Malamy, 1994). SA is a key signal molecule for the expression of multiple modes of plant biotic and abiotic stress, such as chilling stress, drought, salinity and heat shock (Ding and Wang, 2003). Srivastava and Dwivedi (2000) reported that SA might delay the ripening of fruit via the inhibition of ethylene

biosynthesis and as a result maintain the postharvest quality of the commodity. The pre-harvest application of SA (0.3, 0.6 and 0.9mM) significantly improved the TA, SSC, chroma, *h*° of skin and b* index of arils in pomegranate fruit (Mirdehghan et al., 2014).

2.21.1 SA and CI

Chilling injury is a physiological disorder occurring as a result of oxidative stress in low-temperature conditions disrupting normal cell metabolism. The applications of SA to various horticulture crops are easy to use and inexpensive (Ding et al., 2002). It has been previously reported that the exogenous application of SA both pre- and postharvest treatment induces chilling tolerance in pomegranate (Sayyari et al., 2009; Mirdehghan et al., 2014), mandarin (Zheng and Zhang, 2002), peach (Wang et al., 2006; Tareen et al., 2012), citrus (Huang et al., 2008; Ahmad et al., 2013b), pineapple (Hong et al., 2013), mango (Ding et al., 2007), strawberry (Karlidag et al., 2009), plum (Luo et al., 2011), cherry (Dokhanieh et al., 2013), lemon (Siboza et al., 2014) and sweet cherries (Gimenez et al., 2014). It has been reported that pre-harvest application of SA (8 mM and 9 mM) significantly reduced CI in Lane Late and Valencia Late (Ahmad et al., 2013b). Siboza et al. (2014) reported that combined treatment of 10 μM MJ and 2 mM SA resulted in the change of phenolic metabolism and reduced CI may be due to the increased production of total phenolic and the initiation of PAL activity and inhibition of POD. Sayyari et al. (2009) reported that SA (2 mM) treatment is effective in inducing chilling tolerance and reducing electrolyte leakage as well as ascorbic acid in pomegranate fruit. Mango fruit treated with SA (2 mM) resulted in reduced CI (Ding et al., 2007). Induction of chilling tolerance by SA in plum fruit was associated with the increased accumulation of polyamines (PA), and with reduced leakage, malondialdehyde (MDA) content and delayed activities of PPO and POD (Luo et al., 2011). In addition, treatment of SA inhibits respiration and ethylene production and also delays the onset of climacteric respiration peak (Luo et al., 2011). Wang et al. (2006) reported that 1mM SA alleviates CI of peaches during cold storage. It has been documented that low storage temperature and exogenous application of SA regulate the antioxidant system, as a result reducing lipid peroxidation and maintaining beneficial antioxidant activity during cold storage of navel oranges (Huang et al., 2008). The effect of SA in reducing

CI during cold quarantine treatment (1°C for 21 d) in Midknight Valencia and Lane Late sweet orange is yet to be investigated.

2.22 Nitric oxide (NO)

Nitric oxide, a highly reactive gaseous free radical molecule, acts as a multifunctional signalling molecule in many plant tissues (Besson-Bard et al., 2008). NO orchestrates a biochemical pathway, is known to play an important role in modulation of plant hormones and defence against abiotic and abiotic stress (Xu et al., 2012).

2.22.1 NO effects on postharvest quality

Horticultural produce such as fruit and vegetables is obstructed by a range of abiotic stress during storage resulting in enhanced ripening and senescence and thus reduced shelf life. Postharvest application of NO reduces abiotic stress, extends postharvest life and improves quality in a wide range of horticultural produce (Wills et al., 2015). It has been well recognized that NO reduced respiration rate and ion leakage subsequently maintain cellular integrity; reduction in oxidative stress through reduced lipid oxidation and enhanced activity of antioxidant enzymes; reduction in PPO activity related with reduced internal browning; and mitigation of CI through the enhanced natural antioxidant system (Wills et al., 2015). Postharvest application of NO has been reported to alleviate CI and maintain fruit quality in climacteric fruit such as kiwifruit (Zhu et al., 2008), peach (Zhu et al., 2010; Flores et al., 2008), Japanese plum cv Amber Jewel (Singh et al., 2009), peach (*Prunus persica* (L.) Batsch, cv. Feicheng) (Zhu et al., 2010), mango (*Mangifera indica* L. cv. Kensington Pride) (Zaharah and Singh, 2011), tomato (Zhao et al., 2011), yali pears (Liu et al., 2011), banana (*Musa* spp., AAA group cv. Brazil) (Wang et al., 2013), papaya (Li et al., 2014) and non-climacteric fruit longan (Duan et al., 2007), strawberry (Wills et al., 2000) Chinese bayberry (Wu et al., 2012) and loquat (Xu et al., 2012).

Leshem and Wills (1998) claimed 70 to 180 % of extension of postharvest life with NO application in strawberry, kiwi fruit, mushroom, broccoli, cucumber and Chinese broccoli. Moreover, NO exposure for 24 h (hours) to different fruits, vegetables and cut flowers showed 20 % reduced water loss (Ku et al., 2000). It has been reported that pear fruit fumigated with 10 µL L^{-1} NO gas exhibited reduced

ethylene production and delayed skin yellowing; while NO fumigation at 10 and 50 µL L^{-1} did not affect fruit softening (Sozzi et al., 2003). In addition, peach fruit treated with NO gas delayed ripening through reduced activity of LOX and suggested that decrease in LOX might be involved in reducing ethylene biosynthesis (Zhu et al., 2006). Wills et al. (2007) argued that NO application regulated ethylene (C_2H_4) biosynthesis, which is responsible for ripening of fruit. In another study, peach fruit fumigated with NO resulted in reduced respiration rate increased firmness and reduced electrolyte leakage as compared to control fruit (Flores et al., 2008). Furthermore, Singh et al. (2009) reported that NO fumigated Japanese plum fruit showed reduced respiration rate and ethylene production during ripening at 21°C. Zaharah and Singh (2011) reported that NO fumigation inhibited the activity of C_2H_4 biosynthetic enzymes such as 1- aminocyclopropane-1-carboxylic acid synthase (ACS) and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO), leading to the reduced level of immediate precursor (ACC) in fruit pulp.

2.22.2 NO effect on CI

Chilling injury is one of the major physiological disorders in fruit and vegetables, particularly from tropical and subtropical origins. NO has been known to reduce CI in climacteric and non-climacteric fruit. Postharvest NO fumigation (10 µL L^{-1}) reduced CI during storage at 0°C for 6 weeks in Japanese plum in the air (Singh et al., 2009). Later on, Zaharah and Singh (2011) reported that NO fumigation (10, 20 and 40 µL L^{-1}) not only delayed mango softening and colour changes but also reduced CI during storage at 5°C for 2 and 4 weeks. Xu et al. (2012) claimed that loquat fruit during cold storage (1°C) prompted a noticeable increase in endogenous NO. Meanwhile, the pre-treatment of loquat fruit with the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1- oxyl-3-oxide (cPTIO) not only eradicated endogenous NO accumulation but also intensified CI symptoms in the fruit stored at 1 °C and 95% RH, which confirms the role of endogenous NO production in alleviating CI. The role of NO in mitigating CI was also ascribed to stimulation of the antioxidant defence system in the fruit. NO fumigation of the climacteric Chinese bayberry (*Myrica rubra*) fruit with 20 µL L^{-1} gas for 2 h inhibited ethylene production, disease incidence and delayed the decrease in firmness, total phenolic as well as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. They also proposed that NO might maintain the balance between the formation and detoxification of ROS

and enhance the resistance of tissues to decay (Wu et al. 2012). Wu et al. (2014) reported that mature green banana treated with 60 µL L^{-1} NO gas for 3 h at 22 °C was effective in reducing CI.

Zhang et al. (2017) pre-treated Hami melon fruit with 60 µL L^{-1} NO for 3 h and showed effectively decreased CI index, minimised increase in membrane permeability and MDA content, inhibited superoxide anion (O^{-2}) production rates, reduced H_2O_2 content, and sustained higher activity of SOD, POD, CAT and APX in the rind of Hami melon. Additionally, Ghorbani et al. (2017) documented that NO successfully mitigated CI in Washington Navel, possibly through the elicitation of an antioxidant response, through maintaining the quality of the fruit by decreasing lipid peroxidation and the hydrogen peroxide (H_2O_2). The effect of NO fumigation on mitigating CI during cold quarantine treatment (1°C for 21 d) in Midknight Valencia and Lane Late, whilst extending cold storage life only in Midknight Valencia for 90 d followed by 10 d simulated self-condition is yet to be investigated.

2.23 Heat treatment

Postharvest handlings such as HT, HWD, rinses or brushing, vapour heat and HA are applied commercially in order to enhance chilling tolerance, reduce decay development, inhibit ripening and to eradicate insects (Lurie, 1998). The tolerance of fruits and vegetables to HT was determined by maturity at harvest, species and cultivar, pre-harvest factors such as season, growing location, soil type, and production practices (Fallik, 2004; Valero and Serrano, 2010). Postharvest HWD applied to fruit and vegetables prior to cold storage to mitigate CI have been commercially applied in pepper (Gonzales-Aguilar et al., 2000b), grapefruit (Rozenzvieg et al., 2004), lemon (Safizadeh et al., 2007), pomegranate (Mirdehghan et al., 2007), mandarin (Ghasemnezhad et al., 2008) and orange (Bassal and El-Hamahmy, 2011).

The enhancement of chilling tolerance in heat-treated fruit and vegetables could be accredited to (1) enrichment of membrane integrity by enhancing the unsaturated fatty acid (USFA)/saturated fatty acid (SFA) ratio; (2) enhancement of antioxidant system activity; (3) accumulation and improvement of heat shock proteins (HSP) gene expression; (4) enhancement of the arginine pathways which lead to the accumulation of signalling molecules with essential roles in reducing CI such as NO ,

polyamines, and proline; (5) modification in PAL and PPO enzyme activities; and (6) augmentation of sugar metabolism (Aghdam and Bodbodak, 2013). Ghasemnezhad et al. (2008) reported that HWD treatment of 50 °C for 2 min (min) mitigates CI in mandarin with reduced respiration rate, ethylene production and anaerobic metabolite content such as ethanol and acetaldehyde. Bassal and El-Hamahmy (2011) also found that HWD at 40°C for 20 min combined with pre-storage conditioning at 16-18°C for 6 d effectively reduced CI and maintained quality in Valencia and Navel oranges by way of enhancing the POX and CAT activities and total phenol (TP) content. Furthermore, $CaCl_2$ and hot water (53°C for 3 min) reduced CI in Lisbon lemon by aggregating SOD, CAT activities and lessening POD activity, which led to maintenance of membrane integrity reflected by a reduced e MDA (Safizadeh et al., 2007).

Schirra et al (2004) documented that HWD for 3 min at 50°C and HAT at 37°C for 48 h reduced CI and decay in Tarocco, Moro, Sanguinello and Doppio- sanguigno blood oranges during cold quarantine at 1°C for 16 d. HWD (52°C for 3 min) and short-term storage (7°C for 10 d) afterwards with delay (<6 h) showed reduced CI in banana fruit by the exhibited rise of APX gene expression and activity during cold storage. It was also reported that the short HWD at 62°C for the 20s, in grapefruit mitigated CI by increased expression of the various HSP cDNAs in grapefruit peel tissue (Rozenzvieg et al., 2004). Mirdehghan et al. (2007) claimed that HWD (45°C) for 4 min prior to cold storage (2°C) for 90 d showed reduced CI in pomegranate fruit through increased free putrescine and spermidine as well as maintenance of the USFA/SFA ratio for the maintenance of membrane integrity and fluidity. Moreover, HWD treatment (45°C for 4 min) exhibited higher levels of glucose and fructose and organic acids (malic, citric and oxalic acids) as well as total antioxidant activity (Mirdehghan et al., 2007).

McDonald et al. (1991) argued that certain fungicides such as TBZ, IMZ and HT reduced CI in citrus fruit and produced positive synergistic effects in reducing CI when IMZ or TBZ were used in combination with hot water. The efficacies of postharvest HWD combined with TBZ dip have been reported to reduce CI and maintain fruit quality in various fruit crops. Schirra and Mulas (1995) reported that HWD (52°C) of Tarocco oranges resulted in significant control of CI and decay

occurred both during cold storage and simulated shelf-life. Furthermore, the effectiveness of TBZ was enhanced when used with hot water solution and also reduced diseases and disorders. It has also been reported that Star Ruby grapefruit (*Citrus paradisi* Macf.) treated with HWD alone at 50°C or combined with 200 mg L^{-1} of imazalil (IMZ) reduced CI following six weeks cold (2°C) storage 90–95% RH (Schirra et al., 2000). Tang et al. (2017) reported that HT (55°C) for the 20s alone or combined with 25% of the preservative dosage used in production of iminoctadine tris (albesilate), 2, 4-dichlorophenoxyacetic acid, and IMZ significantly reduced the decay rate without affecting fruit quality in Ponkan fruit (*Citrus reticulata* Blanco cv. Ponkan) during storage. Palma et al. (2013) reported that treatments with 300 mg L^{-1} TBZ 53°C for the 60s or 56°C for 30s effectively reduced decay after quarantine in Tarocco oranges. Rodov et al. (1995) reported that HWD (53°C, 2-3 min) significantly reduced the sensitivity of CI in grapefruit (*Citrus paradisi* Macf., cv. Marsh), lemon (*Citrus limon*. Burm., cv. Eureka), oroblanco (*C. grandis* Osb. x *C. paradisi*, cv. Oroblanco, syn. Sweety) and kumquat (*Fortunella margarita* Swingle, cv. Nagami), but the addition of fungicides (IMZ or TBZ, 1000 mg L^{-1}) did not reduce CI, but prevented fruit decay. The effect of HWD alone or in combination with TBZ in reducing CI during cold quarantine treatment (1°C for 21 d) in Midknight Valencia and Lane Late is yet to be investigated.

CHAPTER 3

General materials and methods

3.1. Plant and fruit materials

A range of experiments to improve the fruit colour of early maturing M7 Navel orange were conducted at Moora Citrus, a commercial orchard located in Dandaragan, WA, during 2015 and 2016. Several experiments to extend the storage life and maintain the fruit quality in Midknight Valencia and Lane Late cultivars were also conducted during 2014-2016. All the experimental trees and fruit were sourced from a commercial orchard located at Dandaragan, WA (latitude 30° 41, South, longitude 115° 42, East), WA (Fig 3.1). Five-year old uniform M7 Navel trees were used for the pre-harvest spray to improve the rind colour. However, Midknight Valencia and Lane Late (nine and seven years old respectively) trees were selected from which to harvest the fruit for the postharvest experiments. All the cultivars used for the experiments were previously grafted to Carrizo citrange (*Citrus sinensis* (L.) Osbeck × *Poncirus trifoliata* Raf.). M7 trees were spaced at 5.0 m between rows and 2.5 m within rows; whilst Midknight Valencia and Lane Late were at 7.5 m between the rows and 2.7 within rows in the North-South orientation. All the experimental trees received similar cultural practices including fertilizer, irrigation, weed control and plant protection. The experimental site has a deep sandy loam soil. The climate is dominated by cool wet winter and hot dry summers.

Fig 3.1 Different citrus growing areas in Australia including the map of the experimental site at Moora, WA (*Source*: Citrus Australia, see: http://www.citrusaustralia.com.au/industry/our-industry.htm).

3.2. Pre-harvest spray application of different growth regulators for regulation of rind colour and fruit quality in M7 Navel.

Various experiments were conducted during 2015 and 2016 to improve the rind colour in M7 Navel particularly from yellow to deep orange and to regulate the quality. Growth regulators such as S-ABA, Pro-Ca, PBZ and MJ were sprayed at (6 or 3 weeks before anticipated harvest, WBAH) as single spray or 6 followed by 3 WBAH as double spray during both years. Data were collected on fruit peel colour ($h°$ and CCI), levels of total carotenoids in the rind and fruit firmness. Different quality variables such as SSC, TA, SSC/TA ratio, vitamin C, total antioxidants, individual sugars and organic acids were determined from the juice.

3.2.1. Abscisic acid (S-ABA)

Different concentrations of an aqueous solution of S-ABA and its biosynthesis inhibitor NDGA were sprayed in 2015 and 2016 at different spray timings on M7 Navel trees to improve rind colour and regulate the fruit quality. The details of the treatments are discussed in the methods and materials section of Chapter 4.

3.2.2. Pro-Ca and PBZ

An aqueous solution containing different concentrations of Pro-Ca and PBZ (GAs biosynthesis inhibitor) were sprayed on M7 Navel fruit to enhance rind colour and regulate the fruit quality during 2015 and 2016. The detailed experimental information is included in the methods and materials section of Chapter 5.

3.2.3. MJ

The efficacy of different concentrations of MJ spray applications applied at pre-harvest stage (6 or 3 WBAH) on rind colour development particularly from yellow to deep orange and on the fruit quality of M7 Navel was examined during 2015 and 2016. The detailed information of these experiments has been included in the methods and materials section of Chapter 6.

3.3. Postharvest treatment to extend storage life, reduce CI and maintain fruit quality in Midknight Valencia and Lane Late sweet orange

Different treatments have been evaluated such as HWD alone or combined with TBZ, MJ or SA 1 min dip and NO fumigation for 2 h to mitigate CI and regulate fruit quality in Midknight Valencia and Lane Late sweet orange stored at cold quarantine treatment (1°C 21 d). Moreover, different dip treatments of MJ have also been tested to reduce CI and regulate fruit quality in Midknight Valencia cold stored at 4°C or 7°C for 90 d followed by 10 d simulated shelf condition. Furthermore, the fruit of Midknight Valencia and Lane Late were fumigated for 2 h with different concentrations of NO to reduce CI and regulate fruit quality at 4°C or 7°C for 90 d of cold storage followed by 10 d simulated shelf condition.

3.3.1. Heat treatment

Midknight Valencia and Lane Late fruit were dipped in HWD (50±1°C for 5 min) alone and combined with TBZ (20 mg L^{-1}) (Fig 3.2). The temperature of the water was kept constant at 50±1°C throughout the treatment. Fruits were air dried at room temperature for 6 h. The effect of HWD alone or in combination with TBZ to alleviate CI and regulate the fruit quality has also been discussed in detail in Chapter 7.

(A) **(B)**

Figure 3.2 (A) HWD treatment of Midknight Valencia and Lane Late fruit at 50±1 °C for 5 min (B) HWD combined with TBZ at 50±1 °C for 5 min.

3.3.2. MJ dip application

Midknight Valencia was harvested at physiological maturity (SSC 9.0 % and juice content 38.0 %). Fruit were randomly harvested around the tree canopy. The fruit of Midknight Valencia were dipped for 1 min in an emulsion containing different concentrations of MJ in a 60 L plastic tub (Fig 3.3). The detailed procedure has also been explained in Chapter 8.

Figure 3.3 Sweet orange fruit dipped in MJ solution.

3.3.3. NO fumigation

Midknight Valencia and Lane Late fruit were fumigated in a sealed plastic container (67 L) with different concentrations of NO for 2 h. Following the treatments, the fruit were kept for 2 h at ambient temperature (21±1°C). NO was injected into the sealed plastic container through an airtight rubber septum on the lid with the help of a (50 mL) syringe (Fig 3.4). Soda lime (25 g) was placed inside the container in a petri dish to absorb excessive CO_2. The detailed process has also been described in Chapter 9.

Fig 3.4 NO fumigation of sweet orange fruit in plastic containers (67 L).

3.4. Determination of fruit rind colour

Ten sweet orange fruit were randomly selected from each replication for the colour determination. The colour coordinates such as L*, a* and b* of the fruit rind was recorded by using a colorflex EZ (45°/0° design) spectrocolorimeter (Hunter Lab, Hunter Associates Laboratory Inc., Reston, VA, 20190, USA) at three positions around the equatorial plane of the fruit. L* represents the lightness of the fruit colour (0 to 100, black to white), while a* specifies the redness (+a*) or greenness (-a*), and b* indicates the yellow (+b*) or blue (-b*) colour of fruit skin. The *h*° value was calculated as $h° = tan^{-1}\ b*/a*$ (Figure 3.4). *h*° represents actual apparent colour, i.e. orange or green, and is the primary variable of changes in orange fruit colour (Stearns and Young, 1942). For the elucidation of *h*°-values, it is to be expected that as the fruit matures, rind colour changes from green (180°) toward yellow (90°) and approaches orange, moving away from (yellow) 90° towards (red) 0°; orange is thus somewhere between yellow and red depending on the shade of orange, i.e. 60-70° (Fig. 3.5). CCI was also calculated by using the following formula (Jimenez-Cuesta et al., 1981).

$$\mathrm{CCI} = \frac{1000.a}{L.b}$$

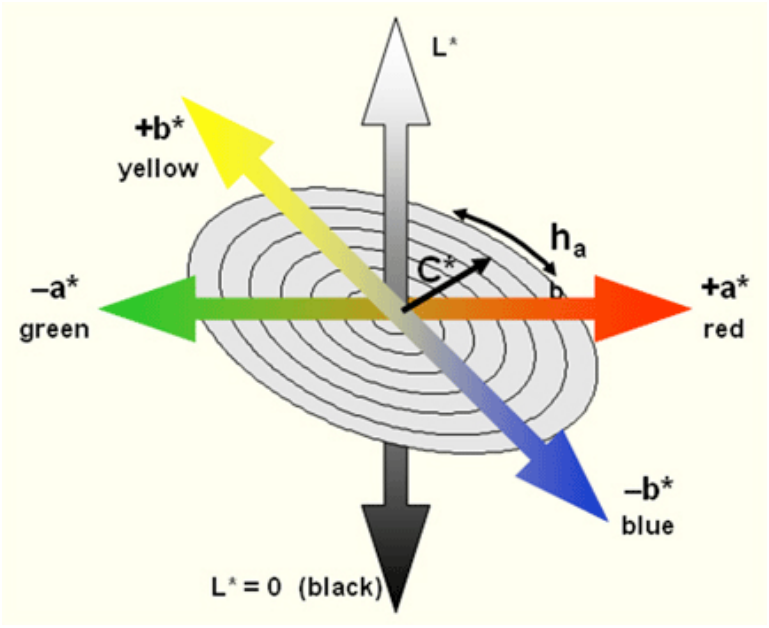


Figure 3.5 CIELAB colour scale (www. globalspec.com)

3.5. Determination of level of total carotenoids in the rind

The level of total carotenoids in the rind of M7 Navel fruit was determined following the method of Lee and Castle (2001) with some modifications (Fig 3.6). In brief, 0.25 g of rind and 25 mL of n-hexane- acetone-MeOH (v/v 50:25:25) were placed in a centrifuge tube, sample was homogenised using the homogenizer (DIAX 900, Heidolph Co., Ltd., Schwabach, Germany) and then centrifuged for 5 min at 4°C at 4732g. The supernatant was collected and then 10 ml NaCl (10 %) was added to separate the layers of dissolved layers of carotenoids. Samples were taken from the top coloured portion of separated layers with dissolved carotenoids and the absorbance was determined at 450nm wavelength using a UV/VIS spectrophotometer (Jenway spectrophotometer Model 6405, Dunmow, Essex, UK). The total carotenoid content was expressed as β-carotene equivalents. Standard β-carotene was purchased from Sigma-Aldrich Pty. Ltd. (12 Anella Avenue Castle Hill NSW 2154 Australia).

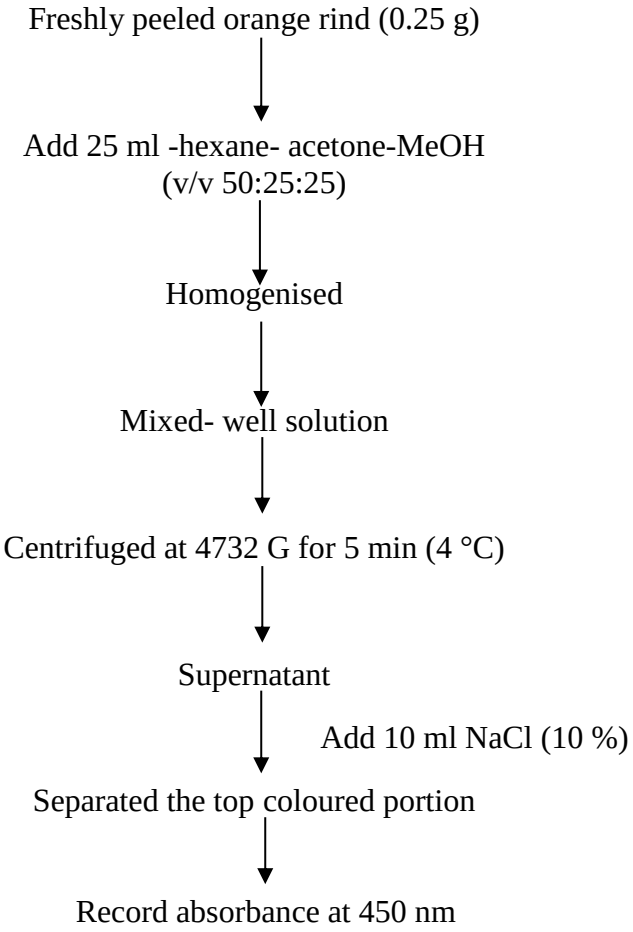


Fig 3.6 Flow chart for the determination of levels of total carotenoids in the rind of M7 Navel fruit.

3.6. CI incidence (%)

All the fruit were visually examined for the symptoms of CI following different cold storage periods and 10 d simulated shelf conditions (21 ± 1°C). Chill injured fruit (Fig 3.7) were counted from the total fruit in each replication. Percentage CI incidence (a percentage of chill injured fruit) was calculated using the following formula:

$$\text{CI incidence (\%)} = \frac{\text{Number of chill injured fruit}}{\text{Total number of fruit}} \times 100$$

(A) (B)

Fig 3.7 (A) Normal fruit (B) Chill injured fruit

3.7. Determination of physiological loss of fruit weight

Initial fruit weight was recorded at the commencement of storage and final fruit weight was recorded at the end of the storage period and the difference in fruit weight loss was calculated as below:

$$\text{Weight loss (\%)} = \frac{(\text{Initial weight} - \text{Final weight}) \times 100}{\text{Initial weight}}$$

3.8. Fruit firmness (N)

Fruit firmness of M7, Midknight Valencia and Lane Late sweet orange was determined using a texture profile analyser (TPA Plus, AMETEK Lloyd Instruments Ltd, Fareham, UK) and the data was processed by using Nexygen® 4.6 software. The individual fruit was positioned between two horizontal plates with the stem axis perpendicular to the plate. The crosshead speed was 200 mm min^{-1} and the test was completed at a strain of 50 % of fruit height. Ten fruit in each replication were used and the average was calculated. Fruit firmness was expressed in newtons (N).

3.9. Soluble solids concentration (SSC)

Ten fruit per replication were squeezed to extract the juice for the determination of SSC in M7, Midknight Valencia and Lane Late fruit. The fruits were randomly selected from each replication. A digital refractometer (Atago-Palette PR 101, Atago CO. Ltd, Itabashi-Ku, and Tokyo, Japan) was used to estimate the SSC in the juice and expressed as a percentage.

3.10. Titratable acidity (TA)

A 10 ml fresh juice was diluted with 20 ml dH_2O to determine the TA. An aliquot (5 ml) was titrated against 0.1 N NaOH solutions using 2-3 drops of phenolphthalein as an indicator to a pale pink colour end point. TA was expressed as percentage citric acid and calculated as follows:

$$\text{Citric acid \%} = \frac{(\text{milli equivalent factor } 0.0064)\text{x } (\text{Volume of Titrant})\text{x } (\text{Volume of NaoH})\text{x } 100}{(\text{ml of juice} \times \text{Volume of an aliquot})}$$

Where,

Milli-equivalent factor of citric acid	= 0.0064
Total volume (ml)	= 30 ml
Extract juice sample (ml)	= 10 ml
Volume of aliquot (ml)	= 5 ml

3.11. SSC/TA ratio

SSC/TA ratio was also calculated by dividing SSC (%) with the corresponding TA value (%).

3.12. Determination of individual sugars and organic acids

3.12.1. Chemicals used

The individual standard of sugars (sucrose, D-glucose anhydrous and D-(-)-fructose) and organic acid (citric, malic and tartaric) were purchased from Sigma-Aldrich, Australia of high-performance liquid chromatography (HPLC) grade.

3.12.2. Sample preparation

The fruit juice was extracted from ten randomly selected fruit from each replication. The juice (1 mL) was diluted with Milli-Q water (19 ml). The Milli-Q water was purified by Millipore system (Millipore, Bedford, MA, USA) and homogenised using a homogeniser (DIAX 900, Heidolph Co., Ltd., Schwabach, Germany) for 1 min. The diluted juice was centrifuged using a refrigerated centrifuge (Eppendorf Centrifuge 5810R, Hamburg, Germany) at 12857× g for 10 min at 4°C. Subsequent to centrifugation, a 0.22-µm nylon syringe filter (Altech Associates, Baulkham Hills, New South Wales, Australia) was used to filter 1 ml diluted juice for determination of individual sugars and organic acids by HPLC (Fig 3.8).

3.12.3. HPLC conditions

An HPLC system (Waters 1525, Milford Corp., MA, USA), which was fitted to Dual λ Absorbance Detector (Waters 2487, Milford Corp., MA, USA) was used for identification, separation and quantification of individual sugars and organic acids in the juice of a sweet orange. An auto sampler (Waters 717plus, Milford Corp., MA, USA) was used to inject an aliquot (20 µl) of the extract from the sample kept at 25°C. Individual sugars such as sucrose, glucose and fructose were analysed isocratically on the Bio-Rad Aminex® HPX-87C Fast Carbohydrate column (100 × 7.8 mm; particle size of 9 µm). Predominant individual organic acids (citric, malic and tartaric) were separated on Bio-Rad Aminex® HPX-87H column (300 × 7.8 mm; particle size of 9 µm) (Bio-Rad Laboratories, Inc., Hercules, USA). The column was headed by a Cation H Bio-Rad Micro-Guard® column (30 × 4.6 mm) (Bio-Rad Laboratories, Inc., Hercules, USA). Both the column and guard column were kept at 60°C (sugars) and 45°C (organic acids) during the analysis. The sulphuric acid (H_2SO_4) solution (0.05 mM) with the flow rate of 0.6 ml min^{-1} was used as a mobile phase for elution of organic acids. The degassed water only with the flow rate at 0.6 ml min^{-1} was used to elute sugars. Dual wavelength UV detector at 210 nm were used for the detection of all individual organic acids, whilst the individual sugars were detected using a Refractive Index (RI) Detector (Water 2414, Milford Corp., MA, USA). Chromatographic peaks were identified by comparing retention times with those of standards. The data were collected and processed with Breeze® 3.30 software (Waters,

Milford Corp., MA, USA). All the individual sugars and organic acids were expressed as g L^{-1} FJ.

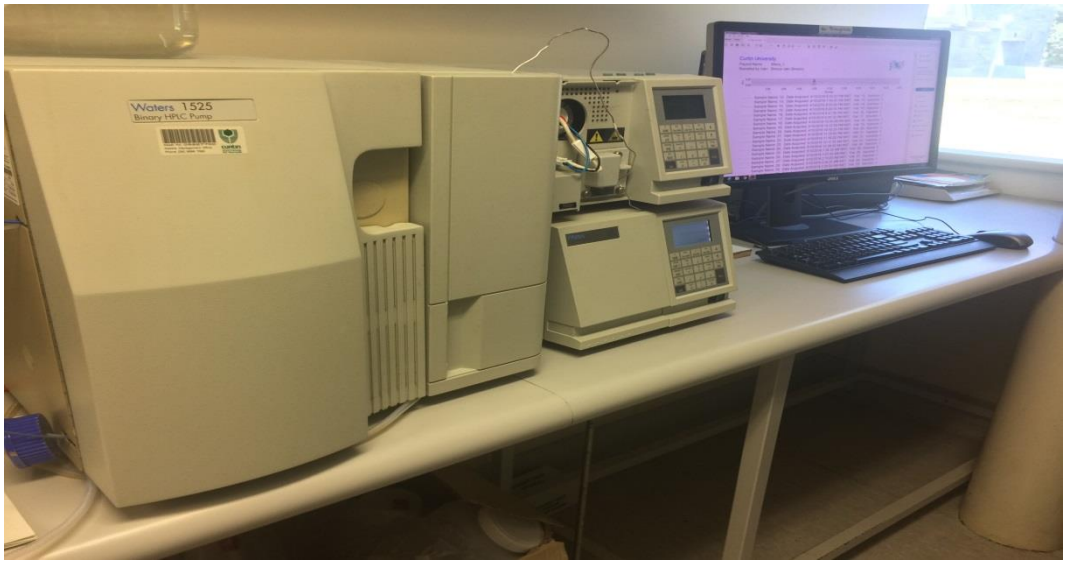


Fig 3.8 High-performance liquid chromatography (HPLC) used to determine individual sugars and organic acids

3.13. Estimation of ascorbic acid

The methods previously described by Hussain (2014) were used for the determination of ascorbic acid concentration. Firstly, fresh orange fruit juice (5 ml) was mixed with 25 ml 6 % metaphosphoric acid containing (0.18 %) disodium salt ethylenediaminetetraacetate acid (EDTA). Following the homogenisation it was centrifuged (Eppendorf Centrifuge 5810 R, Hamburg, Germany) at 3220x g for 15 min. The supernatant (400 µl) was mixed with (200 µl) of 3 % metaphosphoric acid, 1.4 ml dH_2O, and diluted with 200µl folin reagent (Folin: dH_2O, 1:5 v/v). The absorbance of the mixed sample was recorded at 760 nm wavelength using UV/VIS spectrophotometer (Jenway spectrophotometer Model 6405, Dunmow, Essex, UK). Finally, the concentration of ascorbic acid was calculated by employing L-ascorbic acid standard curve. The levels of vitamin C in fresh juice were expressed as g L^{-1}.

3.14. Determination of total antioxidants

Freshly extracted fruit juice of sweet orange M7, Midknight Valencia and Lane Late was used to determined total antioxidants by using the previously outlined methods of Brand-Williams et al. (1995) with some modifications. A stock solution

was prepared from (24 mg DPPH in 100 ml MeOH) of DPPH (1, 1diphenyl-2-picrylhydrazal) used as free radical using 80 % (v/v) MeOH as a solvent. An aliquot (50 µl) of the diluted extract was mixed with (950 µL) of the freshly prepared methanolic DPPH (12 µM). The mixture was vortexed for 5 seconds and kept in the dark for 15 min at 21±1 ^{o}C. By using a 6405 UV/VIS Spectrophotometer (Jenway Spectrophotometer Model 6405, Dunmow, Essex, UK), the decrease in absorbance of DPPH was measured at 515 nm. The standard curve of 6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid (Trolox) was used to calculate total antioxidants. Total antioxidants were expressed as µM trolox equivalent antioxidant activity (TEAC) L^{-1} fresh juice (FJ) basis.

3.15. Statistical analysis

Depending upon the experiment, the data were subjected to one and two-way analysis of variance (ANOVA), using GenStat 14th edition (Lawes Agricultural Trust, Rothamsted Experimental Station, Rothamsted, UK). Means were separated after significant analysis of variance with the Duncan Multiple Range test. All the assumptions of analysis were checked to ensure the validity of statistical analysis.

CHAPTER 4

Pre-harvest spray application of abscisic acid (S-ABA) regulates fruit colour development and quality in early maturing M7 Navel orange

Abstract

Poor fruit colour development at harvest in early maturing M7 sweet orange cultivar causes economic losses to the growers. The responses of fruit colour and quality to different concentrations (50, 100, 200, 300 or 500 mgL^{-1}) of S-ABA and its biosynthesis inhibitor NDGA (0.01, 0.02, 0.04 mM) at pre-harvest stage (6 or 3 weeks before anticipated harvest) on peel colour development particularly from yellow to deep orange and on the fruit quality of M7 were studied during 2015 and 2016 in WA. S-ABA treatments during both years irrespective of the concentrations applied exhibited significantly lower *h*° with enhanced CCI and higher levels of total carotenoids in the rind during 2015 and 2016. Spray application of S-ABA (300 and 500 mg L^{-1}) resulted in higher level of total carotenoids (35.0 and 71.5 mg kg^{-1}) in the rind during 2015 and 2016. A single spray application of S-ABA applied at 6 weeks before harvest (WBAH) showed higher mean CCI (10.1) and level of total carotenoids (37.6 mg kg^{-1}) as compared to its single application at 3 WBAH and double spray at 6 WBAH followed by 3 WBAH in 2015. However, nordihydroguaiaretic acid (NDGA) restricted colour development indicated by higher *h*° and reduced CCI and lower levels of total carotenoids in the rind during 2015. S-ABA treatments exhibited significantly reduced total organic acids in the juice, whilst total sugars were not affected by any of the treatments. S-ABA treatments (200 and 300 mg L^{-1}) showed increased SSC/TA ratio (12.8 %) as a result of a reduction in total acidity (TA) (0.96 %). In conclusion, pre-harvest spray application of S-ABA promoted fruit colour development from yellow to deep orange, indicated by reduced *h*° and increased CCI and the levels of total carotenoids in the rind M7 Navel orange. Promotion of fruit colour development (yellow to deep orange) with the pre-harvest application of S-ABA and its down-regulation with the application of ABA biosynthesis inhibitor NDGA suggested the involvement of S-ABA in rind colour development in M7 Navel orange fruit.

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4.1. Introduction

Sweet orange (*Citrus sinensis* L. Osbeck) is one of the important fruit crops grown in tropical and subtropical regions of the world. Most of the sweet orange fruit produced in Australia is sold in the domestic markets while the remaining is exported to South East Asian markets. Early and late maturing cultivars have been introduced in recent years to extend the availability of fresh sweet orange fruit in local markets. An early maturing M7 Navel sweet orange cultivar has been grown on a large scale in WA. M7 is a bud mutation of Navelina, known to colour early and has better internal fruit quality than Navelina (DAFWA, 2017). As a prelude, M7 has the advantage of colouring earlier, but in WA, poor peel colour development of M7 at harvest causes serious economic losses to the growers.

S-(+)-cis, *trans*-abscisic acid, is one of the naturally occurring plant growth regulators and plays an important role in seed development, dormancy, responses to environmental stresses and fruit ripening (Setha, 2012). Exogenous application of ABA increases levels of the anthocyanins in grape berry skin (Ban et al., 2003; Jeong et al., 2004) and also improves the skin colour and quality in grapes (Cantin et al., 2007; Peppi et al., 2006; Sandhu et al., 2011; Roberto et al., 2013).

The role of ABA and ethylene in skin colour development during maturation and ripening of non-climacteric fruits is well documented. Wang et al. (2007) reported that combined application of ABA (100 mg L^{-1}) and ethrel (400 mg L^{-1}) to *Litchi chinensis* Sonn at 3 weeks before harvest (WBAH) was more effective in enhancing both chlorophyll degradation and anthocyanin biosynthesis than the application of ABA alone. ABA-induced ethylene biosynthesis has been reported to up-regulate fruit colour development during ripening in strawberries (Jiang and Joyce, 2003). Moreover, ABA application also improves colour development in harvested strawberries by enhancing biosynthesis of anthocyanins during storage. The role of ABA in enhancing fruit colour development in apple and peaches has also been previously reported (Kondo et al., 1991; Zhang et al., 2009). It has been reported that Jonagold apple (*Malus domestica* Borkh.) showed increased accumulation of

anthocyanins with the parallel increase in ABA levels in the peel and pulp of the fruit at 160 d after full bloom (Uthaibutra and Gemma, 1991). ABA also plays an important role in citrus fruit colour development and ripening (Goldschmidt, 1976; Nooden, 1988; Aung et al., 1991; Valero et al., 1998). Previously, Valero et al. (1998) reported that lower levels of ABA were accompanied by a delay in colour change at stage 1 in lemon fruit. Harris and Dugger (1986) reported that increased level of ABA in the citrus fruit exocarp is associated with the natural colour transition from green to orange. There is well-recognized evidence that increased ABA levels are responsible for the transition of chloroplast to chromoplast during fruit colour development in oranges (Harris and Dugger, 1986), mandarins (Lafuente et al., 1997), and sweet cherry (Kondo and Gemma, 1993). Recently, Wang et al. (2016) reported that exogenous application of ABA (500 µM) before colour break stage improves colour in mandarin fruit (*Citrus reticulata* Blanco cv. Ponkan). However, no research has been reported on the effect of exogenous application of ABA and its inhibitor on regulating fruit colour development in M7 sweet orange. Therefore, the objective of the present investigation was to elucidate the role of S-ABA in regulating fruit colour development from yellow to deep orange, levels of total carotenoids in the rind and fruit quality by pre-harvest spray application of S-ABA and its biosynthesis inhibitor (NDGA) in M7 sweet orange grown under the Mediterranean climate of WA.

4.2. Materials and methods

4.2.1. Plant material

Three independent experiments were conducted in a commercial orchard located at Moora (latitude 30° 41, South, longitude 115° 42 East), WA in 2015-2016. Five-year old uniform sweet orange trees previously grafted to Carrizo citrange (*Citrus sinensis* (L.) Osbeck *x Poncirus trifoliata* Raf.) rootstock were used for the experiments. The trees were spaced 5.0 m between rows and 2.5 m within rows in the North-South orientation. These experiments were conducted on early maturing M7 Navel sweet orange over two consecutive years 2015 and 2016. The experimental trees received cultural practices including fertilisers, irrigation and plant protection (Moulds and Tugwell, 1999), except for the experimental treatments.

4.2.2. Experiment 1: Pre-harvest treatments of S-ABA (S- abscisic acid) at 6, 3 anticipated weeks before harvest (WBAH) single spray and double spray at 6 WBAH followed by 3 WBAH in M7 sweet orange during 2015

An aqueous solution containing different concentrations (50, 100, 200 and 300 mg L^{-1} S-ABA) using [Pro Tone® SG soluble granule containing active ingredients (a.i.) (200 g kg^{-1}) of S-Abscisic acid (S-ABA)] (Valent Bioscience Corporation 870 Technology Way Libertyville, IL 60048 , USA) and 'Tween 20' (0.05 %,v/v) as a surfactant were sprayed on whole trees until run off at 6 WBAH on 8 April 2015 or 3 WBAH on 30 April 2015 as a single spray application or as double sprays applied at 6 WBAH followed by 3 WBAH in 2015. Unsprayed trees were kept as control. The experimental layout was randomised block design with two-factor factorial including S-ABA treatments and times of application. A single tree was treated as an experimental unit and included three replicates. Twenty-five blemish-free fruit were randomly harvested around the tree canopy. The fruit were transported in an air-conditioned vehicle within three hours of harvest to Curtin Horticulture Laboratory. Fruit peel colour (*h*° and CCI), levels of total carotenoids in the rind and fruit firmness were recorded. Soluble solids concentration (SSC), titratable acidity (TA), SSC/TA ratio, vitamin C, total antioxidants, individual sugars and organic acids were determined from the juice.

4.2.3. Experiment 2: Pre-harvest treatment of nordihydroguaiaretic acid (NDGA) S-ABA biosynthesis inhibitor on colour of M7 sweet orange fruit at 6 WBAH in 2015

In 2015, an aqueous solution containing different concentrations of NDGA (0.01, 0.02 and 0.04 mM) with 'Tween 20' as a surfactant were sprayed onto the whole trees until run off at 6 WBAH (8 April 2015). Control trees were not treated. NDGA was obtained from Sigma-Aldrich Pty. Ltd. (12 Anella Avenue, Castle Hill, NSW, 2154 Australia). The experiment was laid out as a randomised block design and replicated four times. A single tree was kept as an experimental unit. Twenty-five blemish-free fruit per tree were randomly harvested around the tree canopy. Fruit peel colour (*h*° and CCI) and levels of total carotenoids in the rind were estimated.

4.2.4. Experiment 3: Pre-harvest spray treatments of different concentrations of S-ABA applied at 3 WBAH in M7 sweet orange fruit during 2016

In 2016, an aqueous solution comprising different concentrations of S-ABA (200, 300 and 500 mgL^{-1} S-ABA) [Pro Tone® SG soluble granule] were sprayed till run off onto the M7 sweet orange trees at 3 WBAH (30 April 2016) at Moora citrus, WA. The control trees were untreated. The experiment was laid out as a randomised block design, with single tree plot as an experimental unit replicated four times. Twenty-five blemish-free fruit per tree were randomly harvested around the tree canopy. The fruit peel colour (*h*° and CCI), levels of total carotenoids in the rind and fruit firmness were assessed. SSC, TA, SSC/TA ratio, vitamin C and total antioxidants were determined from the juice.

4.2.5. Determination of the fruit colour

Ten fruit were randomly selected from each replication for the colour determination by using a colorflex EZ (45°/0° design) spectrocolorimeter (Hunter Lab, Hunter Associates Laboratory Inc., Reston, VA, 20190, USA) as described in Chapter 3, Section 3.4.

4.2.6. Determination of level of total carotenoids

The level of total carotenoids in the rind of M7 Navel fruit was determined following the method of Lee and Castle (2001) with some modifications. The detailed method has also been detailed in Chapter 3, Section 3.5.

4.2.7. Fruit firmness

Ten fruit per replication were selected to determine the fruit firmness (N) by employing the fruit compression test as outlined in Chapter 3, Section 3.8. Fruit firmness was expressed in newtons (N).

4.2.8. SSC, TA and SSC/TA ratio

The juice was extracted from ten randomly selected fruit in each replication. The SSC content of the juice was determined using a digital refractometer (Atago-Palette PR 101, Atago CO. Ltd, Itabashi-Ku, and Tokyo, Japan) and expressed as a percentage. The titratable acidity was determined by titrating the juice against 0.1N NaOH using 2-3 drops of phenolphthalein as an indicator to a pink colour end point.

TA was expressed as percentage citric acid. SSC/TA ratio was calculated by using SCC and TA values. Detailed procedure has been explained in Chapter 3, Section 3.9, 3.10 and 3.11.

4.2.9. Determination of individual sugars and organic acids

The levels of individual sugars and organic acids in the juice were determined using reverse-phase high-performance liquid chromatography system (RP-HPLC; Waters, Milford, MA, USA) fitted with refractive index detector and dual wavelength UV detector respectively. The detailed method has been described in Chapter 3, Section 3.12. All the individual sugars and organic acids were expressed as (g L^{-1}).

4.2.10. Determination of vitamin C

The level of vitamin C from the fruit juice was determined using a UV/VIS spectrophotometer (Jenway spectrophotometer Model 6405, Dunmow, Essex, UK) outlined in Chapter 3, Section 3.13. The concentration of ascorbic acid was calculated by using the standard curve of L-ascorbic acid and expressed as (mg L^{-1}) of fresh juice.

4.2.11. Determination of total antioxidants

The total antioxidant levels were determined from the freshly extracted juice of M7 by methods outlined by Brand-Williams et al. (1995) with some modifications as detailed in Chapter 3, Section 3.14 by using UV/VIS spectrophotometer (Jenway spectrophotometer Model 6405, Dunmow, Essex, UK). Total antioxidant was calculated using a standard curve of 6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid (Trolox) and was expressed as µM Trolox equivalent antioxidant activity (TEAC) (L^{-1}) FJ basis.

4.2.12 Statistical analysis

The experimental data were analysed by one-way or two-way analysis of variance (ANOVA) using GenStat 14th edition (release 14.1; Lawes Agricultural Trust, Rothamsted Experimental Station, Rothamsted, UK). The effect of various S-ABA treatments, timings of application and their interactions were assessed within ANOVA. The least significant differences (Fisher's LSD) were calculated following

significant ($P \leq 0.05$) F test. The validity of statistical analysis was tested by checking all the assumptions of ANOVA.

4.3. Results

4.3.1. Hue angle (h°)

When averaged over different spray timings, mean *h*° was significantly ($P \leq 0.05$) reduced with the application of S-ABA irrespective of the concentrations applied as compared to control (58.9) in 2015 (Table 4.1). When averaged over different concentrations of S-ABA sprays applied, mean *h*° of the fruit was not significantly affected with the time of its spray applications. There was a significant interaction found between different concentrations of S-ABA applied and its different spray timings for *h*°. A single spray application of S-ABA (50 mg L^{-1}) applied at 6 WBAH showed decreased *h*° (56.3) as compared to the control in 2015 (Table 4.1). In 2016, single spray application of S-ABA irrespective of the concentration applied (200 to 500 mg L^{-1}) at 3 WBAH significantly reduced *h*° (55.3 to 55.2) as compared to the control (62.7) (Table 4.2).

4.3.2. CCI

In 2015, when averaged over different spray timings, mean CCI was significantly ($P \leq 0.05$) enhanced (10.0 and 10.2) with the application of S-ABA (200 and 300 mg L^{-1}) respectively as compared to control (9.4) (Table 4.1). When averaged over different concentrations of S-ABA sprays applied, mean CCI of the fruit were significantly affected by the time of its spray applications. Single spray application of S-ABA applied at 6 WBAH or 3 WBAH showed highest mean CCI (10.1, 9.9) respectively as compared to its double spray (9.7) applied at 6 WBAH followed by 3 WBAH. The interaction between different S-ABA treatments and spray timings were found to be non-significant for CCI in 2015. Single spray application of S-ABA regardless of concentrations applied (200-500 mg L^{-1}) at 3 WBAH resulted in significantly ($P \leq 0.05$) highest CCI (11.3) as compared to control (7.6) (Table 4.2) in 2016.

4.3.3 Level of total carotenoids in the rind

When averaged over different spray timings, the mean level of total carotenoids in the rind was significantly ($P \leq 0.05$) increased (35.0 mg kg^{-1}) with the spray application of an aqueous solution containing S-ABA (300 mg L^{-1}) as compared to all other treatments and control (20.7 mg kg^{-1}) during 2015 (Table 4.1). When averaged over different treatments of S-ABA sprays applied, levels of mean total carotenoids in the rind were significantly affected by the time of its spray applications in 2015. A single spray application of S-ABA applied at 6 WBAH showed the highest mean level of total carotenoids (40.5 mg kg^{-1}) followed by 3 WBAH (28.1 mg kg^{-1}) as compared to double spray at 6 WBAH followed by 3 WBAH (20.0 mg kg^{-1}) in the rind. There was a significant ($P \leq 0.05$) interaction between different concentrations of S-ABA applied and its different spray timings for total carotenoids in 2015.

(A) **(B)**

Fig 4.1 Effect of pre-harvest spray application of S-ABA on the rind colour of M7 Navel (A) Control fruit (B) S-ABA (500 mg L^{-1})

Single spray application of S-ABA (200 and 300 mg L^{-1}) applied at 6 WBAH showed highest levels of total carotenoids in the rind (44.8 - 48.4 mg kg^{-1}) respectively as compared to all the treatments and control during 2015 (Table 4.1). In 2016, a single spray application of an aqueous solution containing S-ABA (500 mg L^{-1}) applied at 3 WBAH significantly ($P \leq 0.05$) increased the level of total carotenoids (71.5 mg kg^{-1}) in the rind as compared to the control (41.0 mg kg^{-1}) and all other S-ABA treatments applied (Table 4.2).

Table 4.1 Hue angle (*h*°), CCI and level of total carotenoids in the rind of M7 sweet orange influenced by different concentrations of S-ABA applied at 6, 3 WBAH single spray or double spray at 6 WBAH followed by 3 WBAH in 2015.

Hue angle (*h*°)				
Treatments (mg L^{-1})	6 WBAH	3 WBAH	6 fb 3 WBAH	Mean (Tr)
Control	58.3±0.24 abc	59.4±0.31 a	59.1±0.07 a	58.9a
S-ABA (50)	56.3±0.11 d	58.9±0.46 ab	58.6±0.07 abc	57.9b
S-ABA (100)	58.0±0.17 abc	57.4±0.11 bcd	58.7±0.16 abc	58.0b
S-ABA (200)	57.9±0.03 abcd	57.1±0.08 cd	58.0±0.13 abc	57.7b
S-ABA (300)	57.1±0.20 cd	57.8±0.27 abcd	57.1±0.20 cd	57.4b
Mean (Tm)	57.3*	57.8*	58.1*	
CCI				
Control	9.8±0.12	9.2±0.13	9.3±0.04	9.4b
S-ABA (50)	10.6±0.06	9.5±0.22	9.5±0.02	9.9ab
S-ABA (100)	9.8±0.09	10.1±0.05	9.5±0.07	9.8ab
S-ABA (200)	9.9±0.02	10.3±0.03	9.8±0.06	10.0a
S-ABA (300)	10.3±0.10	10.0±0.15	10.3±0.10	10.2a
Mean	10.1*a	9.9*ab	9.7*b	
Total carotenoids (mg kg^{-1})				
Control	25.6±0.62 c	20.3±1.02 cd	16.3±0.79 d	20.7c
S-ABA (50)	35.5±1.17 b	20.7±0.87 cd	21.9±0.62 cd	26.0b
S-ABA (100)	33.6±0.81 b	35.5±1.85 b	16.7±0.30 d	28.6b
S-ABA (200)	44.8±1.22 a	22.3±0.50 cd	23.1±0.14 cd	30.1b
S-ABA (300)	48.4±1.02 a	34.1±1.08 b	22.5± 0.66 cd	35.0a
Mean	40.5*a	28.1*b	21.0*c	

Tr = treatments, Tm = times of spray application, fb = followed by. Data represent means of 3 replicate samples of 75 units for M7. Mean separation for significant analysis of variance within the columns and rows were tested by Duncan's multiple range tests at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns or rows. *Mean of S-ABA (50,100,200 and 300 mgL^{-1}). Standard error SD (±).

Table 4.2 Effect of spray application of different concentrations of S-ABA applied at 3 weeks before anticipated harvest on hue angle (*h*°), CCI and level of total carotenoids in the rind of M7 sweet orange fruit in 2016.

Treatments (mg L^{-1})	*h*°	CCI	Total carotenoids (mg kg^{-1})
Control	62.7±0.19 a	7.6±0.10 b	41.0±0.17 c
S-ABA (200)	55.3±0.12 b	11.3±0.06 a	46.5±1.07 c
S-ABA (300)	55.4±0.11 b	11.3±0.06 a	65.0±0.51 b
S-ABA (500)	55.2±0.19 b	11.3±0.11 a	71.5±0.92 a

Data represent means of 4 replicate samples of 100 units for M7. Mean separation for significant analysis of variance within the columns were tested by Duncan's multiple range tests at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns. Standard error SD (±).

4.3.4. Fruit firmness (N)

During 2015, when averaged over different spray timings, mean fruit firmness was significantly ($P \leq 0.05$) reduced (364.2 N) with the application of an aqueous solution containing S-ABA (100 and 200 mg L^{-1}) respectively as compared to control (399.4 N) and all other treatments (Table 4.3). When averaged over different treatments of S-ABA sprays applied, mean fruit firmness was significantly affected by the time of its spray applications in 2015. Application of single spray applied at 6 WBAH and 3 WBAH showed significantly increased fruit firmness (386.5, 390.0 N) respectively as compared to double spray (365.2 N) applied at 6 WBAH followed by 3 WBAH (Table 4.3). A non-significant ($P \leq 0.05$) interaction was found between different concentrations of S-ABA treatments applied and its different spray timings for fruit firmness. In 2016, S-ABA spray treatments applied at 3 WBAH did not significantly ($P \leq 0.05$) affect fruit firmness (Table 4.4).

4.3.5. SSC (%)

When averaged over different spray timings, all the S-ABA treatments did not significantly ($P \leq 0.05$) affect mean SSC in the juice except S-ABA (300 mg L^{-1}) as compared to control in 2015 (Table 4.3). When averaged over different spray timings of S-ABA applied, mean SSC in the juice was significantly ($P \leq 0.05$) higher (12.8 %) with single spray application at 6 WBAH and double sprays applied at 6 WBAH followed by 3 WBAH respectively as compared to single spray (12.1 %) at 3 WBAH (Table 4.3). The interactions between different concentrations of S-ABA treatments

applied and its different spray timings were found to be significant for SSC in 2015. Single spray application of S-ABA (50 and 200 mg L^{-1}) applied at 6 WBAH showed higher SSC (13.3 %) respectively as compared to control and all other treatments in 2015 (Table 4.3). During 2016, all the treatments of S-ABA spray application applied 3 WBAH did not significantly affect SCC in the fruit juice (Table 4.4).

4.3.6. TA (%)

When averaged over different spray timings, mean TA was significantly ($P \leq 0.05$) reduced in the juice (1.0 - 0.9 %) with the spray application of aqueous solution containing S-ABA (200 - 300 mg L^{-1}) respectively as compared to control (1.1 %) and all other treatments in 2015 (Table 4.3). Moreover, when averaged over different treatments of S-ABA sprays applied, mean TA in the juice was significantly ($P \leq 0.05$) higher (1.1 %) with single spray application applied at 6 WBAH than its application at 3 WBAH (0.94 %) (Table 4.3). Significant interactions between different concentrations of S-ABA treatments applied and its different spray timings were found to be significant for TA (%) in 2015. Single spray application of S-ABA (300 mg L^{-1}) resulted in lower TA (0.86 %) as compared to control and all other treatments in 2015 (Table 4.3). During 2016, single spray application of an aqueous solution containing S-ABA (200 mg L^{-1}) at 3 WBAH significantly ($P \leq 0.05$) increased TA (1.3 %) as compared to all other treatments (Table 4.4).

4.3.7. SSC/TA

When averaged over different spray timings, application of S-ABA (200 and 300 mg L^{-1}) significantly ($P \leq 0.05$) increased mean SSC/TA (12.8) as compared to the control (11.9) in 2015 (Table 4.3). However, when averaged over the different S-ABA treatments applied, mean SSC/TA in the juice was significantly ($P \leq 0.05$) higher (12.9) with single spray application applied at 3 WBAH and double spray applied at 6 WBAH followed by 3 WBAH respectively as compared to (11.5) at 6 WBAH (Table 4.3). There was a significant interaction between different concentrations of S-ABA applied and its different spray timings for SSC/TA. In 2016, a single spray application of an aqueous solution containing S-ABA applied (200 mg L^{-1}) at 3 WBAH showed significantly ($P \leq 0.05$) reduced SSC/TA (9.3) as compared to the control (10.6) and all other treatments (Table 4.4).

Table 4.3 Fruit firmness (N), SSC (%), TA (%) and SSC/TA in the peel of M7 sweet orange influenced by different concentrations of S-ABA applied at 6, 3 WBAH single spray or double spray at 6 WBAH followed by 3 WBAH in 2015.

Fruit firmness (N)				
Treatments (mg L^{-1})	6WBAH	3WBAH	6 fb 3 WBAH	Mean (Tr)
Control	402.4±5.7	410.6±9.2	385.0±4.0	399.4a
S-ABA (50)	398.7±3.0	436.7±4.8	393.3±1.5	409.6a
S-ABA (100)	377.1±4.8	370.8±7.1	344.7±4.6	364.2b
S-ABA (200)	379.4±8.2	359.1±3.6	354.0±4.5	364.2b
S-ABA (300)	391.1±10.0	393.4±9.3	369.1±4.4	384.5ab
Mean (Tm)	386.5*a	390.0*a	365.2*b	
SSC (%)				
Control	13.0±0.17 abcd	13.0±0.06 abcd	12.8±0.09 abcd	12.9a
S-ABA (50)	13.3±0.01 a	11.8±0.04 f	13.1±0.02 abc	12.7a
S-ABA (100)	13.1±0.07 ab	12.4±0.02 de	12.9±0.03 abcd	12.8a
S-ABA (200)	13.3±0.03 a	12.0±0.11 ef	12.7±0.11 abcd	12.7a
S-ABA (300)	11.6±0.17 f	12.4±0.10 cde	12.5±0.09 bcde	12.2b
Mean	12.8*a	12.1*b	12.8*a	
TA (%)				
Control	1.20±0.02 ab	0.92±0.02 efg	1.13±0.01 bcd	1.1a
S-ABA (50)	1.32±0.03 a	0.92±0.01 efg	1.0±0.01 def	1.0a
S-ABA (100)	1.22±0.02 ab	0.95±0.02 efg	1.02±0.01 de	1.0ab
S-ABA (200)	1.16±0.02 bc	0.87±0.03 fg	0.97±0.01 efg	1.0bc
S-ABA (300)	0.86±0.02 g	1.05±0.01cde	0.96±0.01 efg	0.96c
Mean	1.1*a	0.94*c	0.98*b	
SSC/TA				
Control	10.8±0.14 de	13.5±0.13 a	11.3±0.13 cde	11.9b
S-ABA (50)	10.2±0.22 e	12.8±0.13 abc	13.1±0.14 ab	12.0ab
S-ABA (100)	10.8±0.16 de	13.1±0.27 ab	12.6±0.06 abc	12.2ab
S-ABA (200)	11.4±0.20 cde	14.0±0.43 a	13.1±0.15 ab	12.8a
S-ABA (300)	13.6±0.22 a	11.8±0.12 bcd	13.0±0.11 ab	12.8a
Mean	11.5*b	12.9*a	12.9*a	

Tr = treatments, Tm = times of spray application, fb = followed by. Data represent means of 3 replicate samples of 75 units for M7. Mean separation for significant analysis of variance within the columns and rows were tested by Duncan's multiple range tests at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns or rows. *Mean of S-ABA (50,100,200 and 300 mgL^{-1}). Standard error SD (±).

Table 4.4 Effect of spray application of different concentrations of S-ABA applied at 3 weeks before anticipated harvest on fruit firmness (N), SSC (%), TA (%) and SSC/TA in M7 sweet orange fruit in 2016.

Treatment (mg L^{-1})	Firmness (N)	SSC (%)	TA (%)	SSC/TA ratio
Control	364.3±8.6	12.4±0.09	1.2±0.01b	10.6±0.12 a
S-ABA (200)	336.0±6.6	11.7±0.10	1.3±0.02a	9.3±0.20 b
S-ABA (300)	320.4±1.7	12.4±0.08	1.2±0.02b	10.5±0.20 a
S-ABA (500)	348.1±4.5	12.2±0.06	1.2±0.01b	10.4±0.06 a

Data represent means of 4 replicate samples of 100 units for M7. Mean separation for significant analysis of variance within the columns were tested by Duncan's multiple range tests at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns. Standard error SD (±).

4.3.8. Individual and total sugars

When averaged over different spray timings, the mean levels of glucose, fructose, sucrose and total sugars in the juice were not significantly ($P \leq 0.05$) affected by S-ABA treatments irrespective of the concentrations applied in 2015 (Table 4.5). Moreover, when averaged over the different treatments of S-ABA spray applied, mean glucose level in the juice was significantly higher (35.7 g L^{-1}) with the double spray applied at 6 WBAH followed by 3 WBAH as compared to the single spray at 6 WBAH and 3 WBAH. Meanwhile, mean sucrose level in the juice was significantly higher (68.2 g L^{-1}) with the single spray applied at 3 WBAH as compared to the other spray timings (Table 4.5). The interaction between spray timings and different concentrations of S-ABA applied were found to be significant ($P \leq 0.05$) for levels of glucose (40.7 g L^{-1}) as compared to all other treatments but non-significant for fructose, sucrose and total sugars (Table 4.5).

Table 4.5 Levels of glucose, fructose, sucrose, and total sugars in the juice of M7 sweet orange influenced by different concentrations of S-ABA applied at 6, 3 WBAH single spray or double spray at 6 WBAH followed by 3 WBAH in 2015.

Glucose (g L^{-1})				
Treatment (mg L^{-1})	6WBAH	3WBAH	6 fb 3WBAH	Mean (Tr)
Control	23.8±0.56 cde	22.1±0.61 de	22.8±0.50 cde	22.9
S-ABA (50)	23.5±0.36 cde	23.7±0.62 cde	35.6±2.8 ab	27.6
S-ABA (100)	21.0±0.27 de	19.3±0.26 e	40.7±1.62 a	27.0
S-ABA (200)	22.6±0.68 cde	21.7±0.26 de	35.9±0.69 ab	26.7
S-ABA (300)	20.5±0.18 de	28.5±1.4 bcd	30.9±0.66 bc	26.6
Mean (Tm)	21.9*b	23.3*b	35.7*a	
Fructose (g L^{-1})				
Control	31.4±1.7	28.0±0.6	28.4±0.9	29.3
S-ABA (50)	29.0±0.3	34.3±2.3	27.8±0.4	30.4
S-ABA (100)	28.9±0.4	38.7±3.0	26.4±0.3	31.3
S-ABA (200)	35.6±1.0	28.4±0.3	35.2±1.9	33.0
S-ABA (300)	32.5±0.8	35.0±0.5	36.9±2.6	34.8
Mean	31.5*	34.1*	31.6*	
Sucrose (g L^{-1})				
Control	67.3±0.56	66.9±0.49	62.1±0.82	65.4
S-ABA (50)	68.5±0.73	68.3±1.8	64.4±1.4	67.1
S-ABA (100)	71.3±0.78	69.1±1.94	62.6±0.88	67.7
S-ABA (200)	64.1±1.5	69.3±0.47	65.9±0.90	66.4
S-ABA (300)	60.9±0.87	65.9±1.5	59.9±1.3	62.2
Mean	66.2*ab	68.2*a	63.2*b	
Total sugars (g L^{-1})				
Control	122.5±1.0	116.9±1.6	113.2±1.2	117.6
S-ABA (50)	121.0±1.30	126.4±1.8	127.8±4.2	125.0
S-ABA (100)	121.2±0.57	127.1±4.1	129.6±1.4	126.0
S-ABA (200)	122.4±1.8	119.4±0.17	136.9±2.5	126.2
S-ABA (300)	114.0±0.6	129.4±2.2	127.7±3.1	123.7
Mean	119.7*	125.6*	130.5*	

Tr = treatments, Tm = times of spray application, fb = followed by. Data represent means of 3 replicate samples of 75 units for M7. Mean separation for significant analysis of variance within the columns and rows were tested by Duncan's multiple range tests at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns or rows. *Mean of S-ABA (50,100,200 and 300 mgL^{-1}). Standard error SD (±).

4.3.9. Individual and total organic acids

In 2015, when averaged over different spray timings, mean levels of citric acid and total organic acids were significantly reduced (12.1 and 18.8 g L^{-1}) with the spray application of an aqueous solution containing S-ABA (200 and 300 mg L^{-1}) respectively as compared to control and all other treatments (Table 4.6). Meanwhile, S-ABA treatments did not significantly affect mean levels of malic and tartaric acid in the fruit juice as compared to the control. When averaged over the different treatments of S-ABA spray applied, mean levels of malic acid (6.9 g L^{-1}), citric (13.8 g L^{-1}) and total organic acids (21.5 g L^{-1}) were significantly higher with single spray application applied at 6 WBAH as compared to other timings during 2015 (Table 4.6). The interactions between different S-ABA treatments and spray timings were found to be significant only for mean citric and total organic acids in the juice during 2015. Single spray application of S-ABA (50 mg L^{-1}) applied at 6 WBAH showed a higher level of citric acid (16.3 g L^{-1}) as compared to 200 mg L^{-1} S-ABA (11.4 g L^{-1}) applied as a single spray at 3 WBAH during 2015. However, single spray application of S-ABA (200 mg L^{-1}) at 3 WBAH showed lower levels of total organic acid (16.3 g L^{-1}) as compared to control and all other treatments (Table 4.6).

Table 4.6 Individual and total organic acids in the juice of M7 sweet orange influenced by different concentrations of S-ABA applied at 6, 3 WBAH single spray or double spray at 6 WBAH followed by 3 WBAH in 2015.

Citric (g L^{-1})				
Treatment (mg L^{-1})	6WBAH	3WBAH	6 fb 3WBAH	Mean (Tr)
Control	14.2±0.81 abc	11.9±0.26 cd	15.0±0.10 ab	13.7ab
S-ABA (50)	16.3±0.14 a	13.6±0.19 bcd	12.3±0.08 cd	14.0b
S-ABA (100)	14.0±0.16 bc	13.6±0.39 bcd	12.5±0.10 cd	13.4ab
S-ABA (200)	12.9±0.10 bcd	11.4±0.42 d	12.1±0.03 cd	12.1c
S-ABA (300)	12.0±0.19 cd	13.9±0.11 bc	11.9±0.16 cd	12.6bc
Mean (Tm)	13.8*a	13.1*b	12.2*b	
Malic (g L^{-1})				
Control	7.9±0.29	5.5±0.31	7.6±0.06	7.0
S-ABA (50)	6.7±0.02	5.4±0.07	6.8±0.08	6.3
S-ABA (100)	7.1±0.14	6.3±0.10	5.8±0.06	6.4
S-ABA (200)	7.2±0.07	5.1±0.40	6.5±0.16	6.3
S-ABA (300)	6.4±0.05	4.8±0.36	6.9±0.12	6.0
Mean	6.9*a	5.4*b	6.5*a	
Tartaric (g L^{-1})				
Control	0.19±0.0	0.23±0.01	0.21±0.01	0.21
S-ABA (50)	0.19±0.0	0.23±0.02	0.19±0.01	0.21
S-ABA (100)	0.21±0.01	0.19±0.0	0.21±0.01	0.20
S-ABA (200)	0.20±0.0	0.18±0.0	0.24±0.01	0.21
S-ABA (300)	0.19±0.0	0.18±0.0	0.19±0.0	0.19
Mean	0.20*	0.20*	0.21*	
Total organic acid (g L^{-1})				
Control	22.3±1.1 ab	17.6±0.56 de	22.9±0.05 a	20.9a
S-ABA (50)	23.2±0.13 a	19.2±0.17 bcde	19.3±0.15 bcde	20.6ab
S-ABA (100)	21.3±0.30 abc	20.1±0.44abcde	18.6±0.05 cde	20.0abc
S-ABA (200)	20.3±0.16 abcd	16.7±0.78 e	18.8±0.18 bcde	18.6bc
S-ABA (300)	21.1±0.25 cde	18.9±0.31 bcde	19.2±0.26 bcde	18.8c
Mean	21.5*a	18.7*b	19.0*b	

Tr = treatments, Tm = times of spray application, fb = followed by. Data represent means of 3 replicate samples of 75 units for M7. Mean separation for significant analysis of variance within the columns and rows were tested by Duncan's multiple range tests at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns or rows. *Mean of S-ABA (50,100,200 and 300 mgL^{-1}). Standard error SD (±).

4.3.10. Vitamin C and total antioxidants

When averaged over the different spray timings, mean vitamin C levels were significantly ($P \leq 0.05$) reduced with all S-ABA treatments irrespective of the concentrations applied as compared to control but these treatments did not significantly affect total antioxidants in 2015 (Table 4.7). Moreover, when averaged over the different treatments of S-ABA spray applied, mean vitamin C level in the fruit juice were significantly higher (554.2 and 548.3 mg L^{-1}) at 3 WBAH and 6 followed by 3 WBAH respectively, as compared to (475.6 mg L^{-1}) at 6 WBAH. Furthermore, mean total antioxidant in the juice were also significantly higher (548.4 and 532.8 µM L^{-1} Trolox) at 3 WBAH and 6 followed by 3 WBAH respectively, as compared to (488.9 µM L^{-1} Trolox) at 6 WBAH in 2015 (Table 4.7). The interactions between different treatments of S-ABA and spray timings were found to be significant for levels of vitamin C but non-significant for total antioxidants. Single spray application of S-ABA (100 and 300 mg L^{-1}) applied at 6 WBAH showed a reduced level of vitamin C in the juice (445.3 and 438.4 mg L^{-1}) respectively as compared to control and all other treatments during 2015 (Table 4.7). During 2016, vitamin C level and total antioxidants were not significantly ($P \leq 0.05$) affected by S-ABA treatments applied at 3 WBAH (Table 4.8).

Table 4.7 Levels of vitamin C and total antioxidants in the juice of M7 sweet orange influenced by different concentrations of S-ABA applied at 6, 3 WBAH single spray or double spray at 6 WBAH followed by 3 WBAH in 2015.

Vitamin C (mg L^{-1})				
Treatment (mg L^{-1})	6WBAH	3WBAH	6 fb 3WBAH	Mean (Tr)
Control	534.2±3.9 bcd	544.5±13.0 bcd	611.4±8.7 a	563.4a
S-ABA (50)	484.6±6.4 de	525.6±3.5 bcd	572.2±11.5 abc	527.4bc
S-ABA (100)	445.3±9.0 e	544.1±1.7 bcd	537.2±12.9 bcd	508.9c
S-ABA (200)	534.2±9.5 bcd	592.9±3.5 ab	523.8±9.1 cd	550.3ab
S-ABA (300)	438.4±0.5 e	554.0±6.7 abc	560.1±8.0 abc	517.5bc
Mean (Tm)	475.6*b	554.2*a	548.3*a	
Total antioxidants (µM L^{-1} Trolox)				
Control	482.3±11.0	469.5±6.2	507.9±12.0	486.5
S-ABA (50)	498.1±4.9	515.6±4.1	571.1±17.5	528.2
S-ABA (100)	470.3±22.1	551.4±3.5	548.9±1.8	523.5
S-ABA (200)	524.1±9.8	559.5±15.0	512.2±5.9	531.9
S-ABA (300)	463.1±15.7	567.2±14.7	498.9±11.3	509.7
Mean	488.9*b	548.4*a	532.8*a	

Tr = treatments, Tm = times of spray application, fb = followed by. Data represent means of 3 replicate samples of 75 units for M7. Mean separation for significant analysis of variance within the columns and rows were tested by Duncan's multiple range tests at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns or rows. *Mean of S-ABA (50,100,200 and 300 mgL^{-1}). Standard error SD (±).

Table 4.8 Effect of spray application of different concentrations of S-ABA applied at 3 WBAH on levels of vitamin C and total antioxidants in the juice of M7 sweet orange fruit in 2016.

Treatment (mg L^{-1})	Vitamin C (mg L^{-1})	Antioxidants (µM L^{-1} Trolox)
Control	552.4±3.2	655.8±18.1
S-ABA (200)	563.8±4.1	621.9±8.6
S-ABA (300)	563.8±9.3	603.0±14.5
S-ABA (500)	587.1±7.3	609.0±13.5

Data represent means of 4 replicate samples of 100 units for M7. Mean separation for significant analysis of variance within the columns were tested by Duncan's multiple range tests at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns. Standard error SD (±).

4.3.11. Hue angle, CCI and levels of total carotenoids

All the NDGA spray treatments applied at 6 WBAH showed significantly ($P \leq 0.05$) higher *h*° and decreased CCI and levels of total carotenoids in the peel of M7 sweet orange fruit during 2015 (Table 4.9). NDGA spray treatments applied at 6 WBAH irrespective of concentration resulted in significantly higher *h*° (59.6 - 61.4) as compared to the control (58.0) (Table 4.9). Meanwhile, CCI value declined significantly (9.2 to 8.1) with the application of NDGA single spray at 6 WBAH as compared to control (9.9). As expected, total carotenoids were also significantly decreased (14.9 to 12.2 mg kg^{-1}) with the application of NDGA spray treatments applied at 6 WBAH as compared to the control (25.6 mg kg^{-1}).

Table 4.9 Hue angle (*h*°), CCI and levels of total carotenoids in the peel of M7 sweet orange fruit influenced by different concentrations of NDGA applied at 6 WBAH in 2015.

Treatments (mM)	*h*°	CCI	Total carotenoids (mg kg^{-1})
Control	58.0±0.28 b	9.9±0.14 a	25.6±0.49 a
NDGA (0.01)	61.4±0.30 a	8.3±0.12 bc	12.4±0.39 c
NDGA (0.02)	59.6±0.17 ab	9.2±0.12 ab	14.9±0.43 b
NDGA (0.04)	61.3±0.21 a	8.1±0.15 c	12.2±0.28 c

Data represent means of 4 replicate samples of 100 units for M7. Mean separation for significant analysis of variance within the columns were tested by Duncan's multiple range tests at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns. Standard error SD (±).

4.4. Discussion

Sweet orange fruit colour is one of the important quality parameters for acceptance by consumers. The change in citrus fruit colour is the result of chlorophyll degradation and accumulation of carotenoids pigments in the rind of the fruit (Gross, 1987). Citrus fruit is a complex source of carotenoids and so far 115 different types of carotenoids have been reported (Stewart and Wheaton 1973; Goodner et al., 2001). These carotenoids pigments are responsible for the internal and external colouration of the fruit (Alquezar et al., 2008). Changes in the content and composition of carotenoids have been reported in Satsuma mandarin (*Citrus unshiu* Marc.), Valencia orange (*Citrus sinensis* L. Osbeck), Lisbon lemon (*Citrus limon* Burm.f.) (Kato et al., 2004) and Navelate (*Citrus sinensis* L. Osbeck) (Rodrigo et al., 2004).

Pre-harvest spray application of S-ABA has indicated reduced *h*° and enhanced CCI in cultivar M7 Navel in 2015 and 2016 growing seasons. However, nordihydroguaiaretic acid (NDGA), an ABA biosynthesis inhibitor, resulted in enhanced *h*° and reduced CCI, which confirms the possible role of S-ABA in colour development in M7 Navel fruit. Previously, NDGA-treated sweet oranges showed reduced expression levels of CsPP2C 25, CsPP2C 56 and CsSnRK2s leading to reduced levels of endogenous ABA (Wang et al., 2016). The improved fruit colour with the spray application of S-ABA in M7 Navel sweet orange may be ascribed to the increased level of total carotenoids in the rind. Moreover, pre-harvest spray application of NDGA reduced biosynthesis of carotenoids in the rind of the fruit consequently retarding fruit colour development in M7 Navel. Similarly, Wang et al. (2016) previously reported that exogenous application of ABA improved colour determined by reduced *h*° and increased CCI in Ponkan mandarin (*Citrus reticulata* Blanco). ABA is closely involved in the metabolism of carotenoids and plays an important role in the composition and regulation of carotenoid content in plants (Rodrigo and Zacarias, 2007). Furthermore, during the transformation of chloroplast to chromoplast, the level of ABA increased 12.6- fold in (*Citrus sinensis* [L.] Osbeck cv Washington navel) which shows a possible association of ABA with carotenoids biosynthesis (Harris and Dugger, 1986).

ABA accumulation in the peel during maturation is known to play an important role in chloroplast development during fruit colouration (Rodrigo et al., 2006; Kato et al., 2006 and Harris and Dugger, 1986). The ABA-deficient orange mutant has shown

a delay in peel de-greening process which confirms the involvement of ABA in peel colour development (Rodrigo et al., 2003). Therefore, the increased levels of carotenoids in the rind of M7 Navel fruit with the pre-harvest spray application of S-ABA may possibly be ascribed to the up-regulation of expression of phytoene synthase (*PSY)*, phytoene desaturase (*PDS*), *ζ*-carotene desaturase (*ZDS*), lycopene cyclase (*LYb1, LYb2*) and *β*-ring hydroxylase (*HYb*) as reported earlier in Satsuma mandarin, Valencia orange and Lisbon lemon (Zhang et al., 2012).

There are two possible ways through which S-ABA plays a role in carotenoids accumulation. Firstly, S-ABA might play a direct role through downregulation of *LCYe* transcript and upregulation of *LCYb*. Furthermore, lycopene cyclase (*LCYe*, *LCYb*) and (*LCYb1*, *LCYb2*) work as a catalyst during the cyclisation of lycopene change to *α*-carotene and *β*-carotene respectively (Ikoma et al., 2016). The change in the rind colour from green to orange in Valencia sweet orange and Lisbon lemon has been suggested to be associated with the down-regulation of *LCYe* transcripts and the increased *LCYb* transcripts (Kato et al., 2004). Down-regulation of gene expression *LCYe* in the rind of Navelate navel orange is predominantly responsible for colour changes from green to orange (Rodrigo et al., 2004). Increased expression of *PSY*, *PDS*, *ZDS* and *HYb* genes were observed in the rind of 'Navelate' during substantial accumulation of carotenoids (Rodrigo et al., 2004). During citrus fruit ripening, up-regulation of *LCYb* and down-regulation of *LCYe* genes have been previously reported (Rodrigo et al., 2004; Kato et al., 2004 and Fanciullino et al., 2008).

Secondly, S-ABA application might enhance colour development through the upregulation of ethylene biosynthesis. The role of ethylene has been known for decades to enhance colour during the process of de-greening. ABA has been reported to enhance the sensitivity of the tissue to ethylene in climacteric fruits (Alferez and Zacarias, 1999; Eveland and Jackson, 2011). Ethylene is also responsible for degradation of chlorophyll and stimulation of carotenoids biosynthesis in the rind of citrus fruit (Rodrigo et al., 2013b). Previously, the role of ethylene in enhancing fruit peel colour development in citrus has been ascribed to upregulation of red carotenoids such as *β*-cryptoxanthin and *β*-citraurin (Stewart and Wheaton, 1973). Higher levels of phytoene, *β*-cryptoxanthin and *β*-citraurin were noticed in ethylene-treated fruit as compared to control (Rodrigo and Zacarias 2007; Eilati et al., 1975). Surprisingly, ethylene also up-regulates the expression of *PSY*, *ZDS* and *β*-carotene hydroxylase (*β-CHX*) transcript, persistently or rapidly increased the expression of *PDS*, plastid

terminal oxidase (*PTOX*), lycopene *β*-cyclase (*LCYb*) and zeaxanthin epoxidase (ZEP) and decreased the expression of *LCYe* (Alquezar et al., 2008). Possibly, ABA either directly or through up-regulation of ethylene biosynthesis improves fruit colouring in M7 Navel.

Exogenous application of S-ABA reduced the total organic acids and increased SSC/TA in the juice of M7 Navel. Meanwhile, the application of S-ABA or NDGA did not significantly affect the levels of glucose, sucrose, fructose and total sugars in the juice of the M7 Navel orange. The increased SSC/TA ratio with the application of S-ABA may be ascribed to the reduced total acidity in the fruit juice. ABA content was found to be high in strawberry (Jiang and Joyce 2003) and sweet cherry (Kondo and Gemma, 1993) fruit which synchronised with sugar accumulation and decreased acidity in the later stage of fruit development. Wang et al. (2016) reported that exogenous application of ABA significantly reduced the levels of total organic acids in Ponkan mandarin (*Citrus reticulata* Blanco). ABA-treated sweet orange fruit exhibited higher transcript levels of (CsACO1) and (CsNADP-IDH) which could be involved in the degradation of organic acids (Wang et al., 2016). In conclusion, exogenous application of S-ABA enhanced rind colour in M7 Navel by reduced *h*° and increased CCI and levels of total carotenoids, whilst spray application of NDGA downregulates colour development which suggests a key role of ABA in enhancing fruit colour development in sweet orange. S-ABA significantly reduced total organic acids in the juice of M7 Navel and was able to increase SSC/TA.

CHAPTER 5

Pre-harvest spray application of prohexadione-calcium and paclobutrazol improves rind colour and regulates fruit quality in M7 Navel oranges

Abstract

Sweet orange (*Citrus sinensis* L. Osbeck) cv. M7 Navel exhibits poor rind colour at harvest in WA. Gibberellins are known to retard fruit colour development in citrus fruit, therefore the efficacy of different concentrations and timings of spray applications of two inhibitors of gibberellin biosynthesis such as Pro-Ca and PBZ on rind colour development and fruit quality was investigated. The effects of an aqueous solution containing different concentration of Pro-Ca (200, 400, 600, 800, 1200, 1600 or 2000 mg L^{-1}), and PBZ (100, 250, 500, 1000, 1500 and 2000 mg L^{-1}) sprayed at 6 or 3 weeks before anticipated harvest (WBAH) on rind colour development particularly from yellow to deep orange and on the fruit quality were investigated during 2015 and 2016. The spray application of Pro-Ca (800 mg L^{-1}) resulted in decreased *h*° (57.5), and increased CCI (10.2) and, total carotenoid levels (38.3 mg kg^{-1}) in the rind during 2015. Furthermore, Pro-Ca (600 or 800 mg L^{-1}) showed enhanced levels of total carotenoids (39.2 and 38.3 mg kg^{-1}) respectively, during 2015. In 2016, a single spray application Pro-Ca (1200 mg L^{-1}) at 3 WBAH exhibited reduced *h*° (56.6) and increased CCI (10.7), and levels of total carotenoids (36.8 mg kg^{-1}) as compared to the control. Additionally, a single spray application of Pro-Ca (800, 1200 or 1600 mg L^{-1}) was able to increase total carotenoids in the rind (32.7, 36.8 or 33.1 mg kg^{-1}) respectively. In 2016, Pro-Ca (800 mg L^{-1}) exhibited increased SSC (13.0 %) and reduced TA (1.08 %) in the fruit juice. A single spray application of PBZ (1000 mg L^{-1}) at 6 WBAH significantly reduced *h*° (56.7) and increased CCI (10.6) and levels of total carotenoids in the rind (42.6 mg kg^{-1}). However, a single spray application of PBZ (1500 mg L^{-1}) at 3 WBAH showed lower *h*° (55.4) and enhanced CCI (11.2) and level of total carotenoids (47.1 mg kg^{-1}) in the rind during 2016. In conclusion, pre-harvest spray application of Pro-Ca (800 and 1200 mg L^{-1}) applied at 6 and 3 WBAH respectively enhanced fruit colour. A single pre-harvest spay application of PBZ (1000 and 1500 mg L^{-1}) applied at 6 and 3 WBAH respectively improved fruit colour in early maturing M7 Navel.

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5.1. Introduction

Early maturing cultivars have been introduced in WA to capture the early market of Navel oranges. M7 Navel matures three weeks earlier than Navelina Navel and can be harvested in May to cater for the early market. Fruit is usually sold at high prices due to its early presence in the market. M7 also holds its acid levels within the fruit a bit longer than Navelina Navel (DAFWA, 2017). However, M7 does not colour properly due to the warm climate in Western Australian conditions. The poor and inconsistent rind colours reduce economic returns for M7 growers and partially green fruit are difficult to sell in domestic or overseas markets. The colour of the rind has a significant importance when consumers buy Navel oranges. Rind colour changes occur in citrus as a result of increased chlorophyll degradation and accumulation of carotenoids pigments (Eilati et al., 1969a; Goldschmidt, 1988). Colour break in citrus occurs when a decrease in chlorophyll concentration exposes the presence of carotenoids, resulting in the appearance of the first citrus colour of sweet orange (Goldschmidt, 1988; El-Zeftawi and Garrett, 1978). The rind colour of Navel oranges is due to the genetic makeup and is also influenced by growing location (Barry and Le Roux, 2010). Other factors, such as rootstocks (Rabe and Von Broembsen, 1995), nutrition (Reitz and Koo, 1960), light interception (Sites and Reitz, 1949), plant growth regulators (Garcia-Luis et al., 1986), canopy size (Krajewski, 1996) and deficient irrigation (Peng and Rabe, 1996) have been reported to affect rind colour. However, the cold temperatures in winter months have a pronounced effect on rind colour development (Young, 1961).

Gibberellic acid (GA_3) has been known to delay chlorophyll degradation and inhibit carotenoids accumulation in the rind of citrus fruit (Coggins, 1992; Goldschmidt and Eilati, 1970). The level of GA_3 was found to be lowest at the time of ripening in the rind of satsuma mandarin (Kuraoka et al., 1979). Furthermore, GA_3 applications before colour break reduced carotenoid accumulation and caused delayed colour development in Navel orange (Lewis and Coggins, 1964). Young fruit and leaves are the major sites of gibberellin biosynthesis (Spiegel-Roy and Goldshmidt,

1996). Delay in the rind colour in citrus is due to higher levels of endogenous gibberellins (Garcia-Luis et al., 1985).

Prohexadione-calcium (Pro-Ca) (3-oxide-4-propionyl-5-oxo-3-cyclohexene-carboxylate) also known as Apogee® (27.5 % Pro-Ca) and Regalis® (10% Pro-Ca) is a growth retardant used to inhibit vegetative growth in fruit trees (Evans et al., 1999 and Prive et al., 2006). Bizjak et al. (2013) reported that Pro-Ca application also improved anthocyanin accumulation in Braeburn apple fruit resulting in the initiation of red colour for short period and no effect was found during apple storage. Furthermore, Barry and Le Roux (2010) reported that foliar spray application of Pro-Ca (400 mg L^{-1}) applied as a double dosage 6 and 3 weeks before anticipated harvest has the potential to improve the rind colour of Nules Clementine mandarin and Navelina Navel oranges. Whilst, Barry and Le Roux (2010) tested only two concentrations (200 or 400 mg L^{-1}) of Pro-Ca in inducing colour in the rind of mandarin, sweet orange and lemon fruit, and suggested that higher concentrations are more consistent to accumulate carotenoids in the rind and proposed to optimise the application rate further. The effects of Pro-Ca application on sweet orange fruit quality are also yet to be investigated.

Paclobutrazol (PBZ) is another growth retardant which is known to inhibit gibberellin biosynthesis (Rademacher, 2000). Greenberg et al. (1992) reported that foliar or soil application of PBZ during the autumn increased a total number of spring flush shoots by 1.6 - 2.7 fold and reduced the percentage of vegetative shoots in Shamouti orange trees. Smeirat and Qrunfleh, (1988) also reported that PBZ reduced shoot and internode length and increased shoot diameter in Lisbon lemon. PBZ has been reported to reduce vegetative growth in Minneola tangelo (Monselise et al., 1976), Valencia sweet orange (Delgado et al., 1986). Gilfillan and Lowe (1985) reported that PBZ enhanced Satsuma mandarin rind colour by 1 to 2 units. Monselise (1985) reported that PBZ caused a rapid change in the rind colour in Topaz tangor. The effect of PBZ application in reducing vegetative growth in different fruit crops has been investigated in detail but its application on promoting the rind colour and fruit quality in M7 Navel need to be further investigated.

As a prelude, gibberellins are known to retard citrus fruit colour development whilst the role of inhibitors of gibberellins biosynthesis such as Pro-Ca and PBZ in regulating fruit colour development and quality in M7 Navel grown under a Mediterranean climate of WA warrants to be investigated. Therefore, the objective of

the present investigation was to explicate the role of gibberellins biosynthesis inhibitors including Pro-Ca and PBZ in promoting fruit colour development particularly from yellow to deep orange, levels of total carotenoids in the rind and fruit quality with the pre-harvest spray application in M7 sweet orange.

5.2. Materials and methods

5.2.1. Plant material

Various experiments were initiated on uniform size, 5-year-old early maturing M7 Navel grafted on Carrizo citrange (*Citrus sinensis* (L.) Osbeck *x Poncirus trifoliata* Raf.) rootstock in a commercial orchard located at Moora (latitude 30° 41, South, longitude 115° 42, East), WA in 2015 and 2016. The trees were spaced 5.0 x 2.5 m between and within rows. The row direction was North-South orientation. All the experimental trees received similar cultural practices including nutrition, plant protection and irrigation except excremental treatments.

5.2.2. Experiment 1: Pre-harvest treatments of Pro-Ca at 6, 3 WBAH single sprays or double spray at 6 WBAH followed by 3 WBAH on fruit colour and quality in M7 Navel

An aqueous solution containing different concentrations (200, 400, 600 or 800 mg L^{-1}) using [Regalis® soluble granule containing a.i. (100 g kg^{-1}) (Pro-Ca)] (Nufarm Australia Pty, Ltd, Laveston North, Victoria 3026) and 'Tween 20' (0.05 %, v/v) as a surfactant. The whole trees were sprayed until runoff at 6 WBAH on 8 April or 3 WBAH on 30 April as a single spray application or as double sprays applied at 6 WBAH followed by 3 WBAH in 2015. In 2016, higher concentrations of Pro-Ca (400, 800, 1200, 1600 or 2000 mg L^{-1}) were applied as a single spray at 3 WBAH on 30 April. Unsprayed trees were kept as a control. The layout of the experiment was randomised block design with two-factor factorial including Pro-Ca treatments and times of application in 2015. A single tree was treated as an experimental unit and included three replicates during 2015. In 2016, the experiment was designed by following one-factor factorial randomised block design including four replicates. Blemish-free twenty-five fruit were randomly harvested around the tree canopy. An air-conditioned vehicle was used to transport the fruit to Curtin Horticulture Laboratory. Fruit peel colour (*h*° and CCI), levels of total carotenoids in the rind and fruit firmness were recorded. Various quality parameters of the fruit, including soluble

solids concentration (SSC %), titratable acidity (TA %), SSC: TA ratio, ascorbic acid concentration and total antioxidants, in the juice were determined in both years.

5.2.3. Experiment 2: Pre-harvest treatments of PBZ single spray at 6 WBAH in 2015 and 3 WBAH on fruit colour and quality in M7 Navel during 2016

An aqueous emulsion containing different concentrations (100, 250, 500, or 1000 mg L^{-1}) of PBZ using [Payback® liquid containing a.i. (250 g L^{-1}) of PBZ] (Crop Care Australasia Pty. Ltd, Murarrie, Qld 4172) and 0.05 % 'Tween 20' as a surfactant were sprayed at 6 WBAH (8 April) onto whole trees until runoff in 'M7' by using a sprayer (The Selecta Trolleypak Mk II, Acacia Ridge, Australia) during 2015. In 2016, higher concentrations (500, 1000, 1500 or 2000 mg L^{-1}) of PBZ were applied 3 WBAH (30 April). Control trees were kept unsprayed during both years. The randomised block design was used in the experiment. Single tree plot was treated as an experimental unit and replicated four times during both years. A single tree was assigned as an experimental unit in 2015 and 2016. The fruit (25 per tree) were randomly picked around the tree canopy to assess colour and other quality parameters. The fruit peel colour (*h*° and CCI) and fruit firmness were assessed. The levels of total carotenoids in the rind SSC (%), TA (%), SSC/TA ratio, vitamin C and total antioxidants in the juice were determined in both years.

5.2.4. Determination of the fruit colour

The colour coordinates (L*, a* and b*) were determined using a Colorflex EZ (45°/0° design) spectrocolorimeter (Hunter Lab, Hunter Associates Laboratory Inc., Reston, VA, 20190, USA) at three positions around the equatorial plane of the fruit detailed in Chapter 3, Section 3.4.

5.2.5. Determination of level of total carotenoids

The level of total carotenoids in the rind tissue of M7 Navel fruit was estimated by using the method Lee and Castle (2001) as detailed outlined in Chapter 3, Section 3.5.

5.2.6. Fruit firmness

The fruit firmness of ten randomly selected fruit was estimated by using a texture profile analyser (TPA Plus, AMETEK Lloyd Instruments Ltd, Fareham, UK) interfaced with Nexygen® 4.6 software detailed in Chapter 3, Section 3.8. The firmness of fruit was expressed in newtons (N).

5.2.7. SSC, TA and SSC/TA ratio

Ten randomly selected fruit in each replication were squeezed to extract the juice for the determination of SSC and TA. SSC was determined using a digital refractometer (Atago-Palette PR 101, Atago CO. Ltd, Itabashi-Ku, and Tokyo, Japan) and expressed as a percent. To determine TA, the juice was titrated against 0.1N NaOH using 2-3 drops of phenolphthalein as an indicator to a pink colour end point. TA was expressed as percentage citric acid. SSC/TA ratio was calculated by dividing SCC and TA values. Detailed procedure has been outlined in Chapter 3, Section 3.9, 3.10 and 3.11.

5.2.8. Determination of vitamin C and total antioxidants

The levels of vitamin C and total antioxidants in the fresh juice of M7 were estimated by using a UV/VIS spectrophotometer (Jenway spectrophotometer Model 6405, Dunmow, Essex, UK). The concentration of vitamin C in the juice was expressed as mg L^{-1}, while total antioxidants was expressed as µM Trolox equivalent antioxidant activity (TEAC) (L^{-1}) FJ basis. The detailed method for estimation of vitamin C and total antioxidants in the fresh orange juice has been described in Chapter 3, Section 3.13 and 3.14.

5.2.9. Statistical analysis

The experimental data were analysed by one-way or two-way analysis of variance (ANOVA) using GenStat 14th edition (release 14.1; Lawes Agricultural Trust, Rothamsted Experimental Station, Rothamsted, UK). The effects of different treatments of inhibitors of gibberellins biosynthesis and their timings of application along with their interactions on various variables were assessed within ANOVA. The least significant differences (LSD) were calculated following Duncan multiple range test at probability ($P \leq 0.05$).

5.3. Results

5.3.1 Effect of pre-harvest spray application of Pro-Ca on fruit colour and quality

5.3.1.1 Hue angle (h°)

Pre-harvest spray application of Pro-Ca (800 mg L^{-1}) significantly ($P \leq 0.05$) reduced mean *h*° angle (57.5) as compared to the control (61.1) and all other treatments in 2015 (Table 5.1). However, mean *h*° angle of the fruit was significantly affected with the time of spray application. Single spray application of Pro-Ca applied at 3 WBAH or double spray at 6 WBAH followed by 3 WBAH showed the lowest mean *h*° angle (59.35, 59.37) respectively, as compared to a single spray (60.1) applied at 6 WBAH (Table 5.1). The interaction between different Pro-Ca treatments and spray timings were found to be non-significant for *h*° in 2015. In 2016, single spray applications of an aqueous solution containing Pro-Ca irrespective of the concentrations applied (400 to 2000 mg L^{-1}) at 3 WBAH significantly reduced *h*° as compared to the control (63.3) (Table 5.2). Additionally, spray application of Pro-Ca (1200 mg L^{-1}) at 3 WBAH significantly reduced *h*° (56.6) as compared to the control and all other treatments.

5.3.1.2 CCI

In 2015, mean CCI was significantly ($P \leq 0.05$) enhanced (10.2) with the application of Pro-Ca (800 mg L^{-1}) as compared to the control (8.5) and all other treatments (Table 5.1). However, time of spray application did not significantly affect mean CCI of the fruit. The interaction between different Pro-Ca treatments and spray timing was found to be significant for CCI in 2015. Single spray application of Pro-Ca (800 mg L^{-1}) applied at 3 WBAH or 6 WBAH showed highest (10.8 or 10.3) CCI as compared to all other treatments and the control in 2015 (Table 5.1). A single spray application of an aqueous solution containing Pro-Ca (1200 mg L^{-1}) applied at 3 WBAH significantly increased CCI (10.7) as compared to the control (7.6) and all other Pro-Ca treatments applied in 2016 (Table 5.2).

5.3.1.3 Levels of total carotenoids

Mean levels of total carotenoids in the rind of M7 were significantly ($P \leq 0.05$) increased (39.2 and 38.3 mg kg^{-1}) with the spray application of Pro-Ca (600 and 800 mg L^{-1}) respectively, as compared to all other treatments and the control (17.4 mg kg^{-1}) during 2015 (Table 5.1).

(A) **(B)**

Fig 5.1 Effect of pre-harvest spray application of Pro-Ca on the rind colour of M7 Navel (A) Control (B) Pro-Ca (800 mg L^{-1})

However, mean levels of total carotenoids in the rind were not significantly affected by the timing of spray application in 2015. The interaction between different concentrations of Pro-Ca applied and its different spray timings were found to be significant for total carotenoids in the rind during 2015. A single spray application of Pro-Ca (800 mg L^{-1}) applied at 6 WBAH showed the highest levels of total carotenoids in the rind (42.3 mg kg^{-1}) as compared to all the treatments during 2015 (Table 5.1). In 2016, single spray application of aqueous solution containing Pro-Ca (1200 mg L^{-1}) applied at 3 WBAH resulted in the significantly highest levels of total carotenoids in the rind (36.8 mg kg^{-1}), as compared to the control (20.2 mg kg^{-1}) and all other Pro-Ca treatments applied except (800 and 1600 mg L^{-1}) (Table 5.2).

Table 5.1 Hue angle (*h*°), CCI and levels of total carotenoid in the peel of M7 Navel influenced by different concentrations of Pro-Ca applied at 6, 3 weeks before anticipated harvest (WBAH) as a single spray; or double spray at 6 WBAH followed by 3 WBAH in 2015.

		h°		
Treatment (mg L^{-1})	6 WBAH	3 WBAH	6 fb 3 WBAH	Mean (Tr)
Control	62.7±0.43	61.3±0.25	59.1±0.18	61.1a
Pro-Ca (200)	60.2±0.62	60.5±0.40	58.6±0.36	59.7a
Pro-Ca (400)	60.0±0.38	59.6±0.13	59.8±0.27	59.8a
Pro-Ca (600)	60.2±0.23	58.9±0.20	60.2±0.24	59.8a
Pro-Ca (800)	57.4±0.45	56.2±0.24	58.9±0.05	57.5b
Mean (Tm)	60.14a	59.35b	59.37b	
		CCI		
Control	7.9±0.19e	8.3±0.12de	9.4±0.08bcd	8.59b
Pro-Ca (200)	8.9±0.29cde	8.7±0.17cde	9.8±0.20abc	9.20b
Pro-Ca (400)	9.0±0.16bcde	9.2±0.04bcd	9.2±0.11bcd	9.17b
Pro-Ca (600)	9.0±0.14bcde	9.4±0.11bcd	8.9c±0.11de	9.17b
Pro-Ca (800)	10.3±0.23ab	10.8±0.14a	9.5±0.0bcd	10.26a
Mean	9.08	9.34	9.41	
		Total carotenoids (mg kg^{-1})		
Control	13.4±0.72h	23.1±0.80g	15.6±0.46h	17.4d
Pro-Ca (200)	27.6±0.46fg	31.0±0.89ef	29.6±0.54ef	29.4c
Pro-Ca (400)	32.0±0.68def	34.7±0.57cde	30.6±0.44ef	32.4b
Pro-Ca (600)	41.9±0.62ab	36.9±0.22bcd	38.9±0.62abc	39.2a
Pro-Ca (800)	42.3±1.47a	30.8±0.87ef	41.7±0.30ab	38.3a
Mean	31.5	31.3	31.3	

Tr = treatments, Tm = time of spray application, fb = followed by. Data represent means of 3 replicates (20 fruit per replication) for M7. Mean separation for significant analysis of variance within the columns and rows was tested by Duncan's multiple range tests at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns or rows.

Table 5.2 Effect of spray application of different concentrations of Pro-Ca applied at 3 WBAH on *h*°, CCI and level of total carotenoids in the peel of M7 Navel in 2016.

Treatments (mg L^{-1})	*h*°	CCI	Total carotenoids (mg kg^{-1})
Control	63.3±0.12a	7.6±0.21c	20.2±0.56b
Pro-Ca (400)	61.5±0.26b	8.4±0.42c	22.2±1.1b
Pro-Ca (800)	58.4±0.23c	9.1±0.22b	32.7±1.9a
Pro-Ca (1200)	56.6±0.17d	10.7±0.34a	36.8±1.1a
Pro-Ca (1600)	59.4±0.23c	9.3±0.42b	33.1±0.67a
Pro-Ca (2000)	61.7±0.27b	8.3±0.46c	19.8±0.72b

Data represent means of 4 replicates (20 fruit per replication) for M7. Mean separation for significant analysis of variance within the columns was tested by Duncan's multiple range tests at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns.

5.3.1.4 Fruit firmness (N)

Pre-harvest spray application of Pro-Ca significantly ($P \leq 0.05$) reduced mean fruit firmness irrespective of the concentrations applied as compared to the control during 2015 (Table 5.3). A single spray application at 3 WBAH or double spray 6 followed by 3 WBAH showed significantly reduced (395.2 or 394.1 N) fruit firmness as compared to the single spray applied 6 WBAH (412.6 N) (Table 5.3). A non-significant interaction was found between different concentrations of Pro-Ca treatments applied and its different spray timings for fruit firmness. Pro-Ca spray treatments applied at 3 WBAH did not significantly affect fruit firmness in 2016 (Table 5.4).

5.3.1.5 SSC (%)

In 2015, all the Pro-Ca treatments except Pro-Ca (800 mg L^{-1}) significantly ($P \leq 0.05$) reduced mean SSC (%) in the juice as compared to the control (Table 5.3). However, mean SSC in the juice was significantly higher (13.4 %) with the single spray application applied at 6 WBAH as compared to the single spray at 3 WBAH and a double spray of 6 followed by 3 WBAH (Table 5.3). The interaction between different concentrations of Pro-Ca treatments applied and its different spray timings were found to be significant for SSC (%) in 2015. In 2016, all the Pro-Ca treatments except (1600 or 2000 mg L^{-1}) reduced SSC (%) in the juice of 'M7' applied as a single spray at 3 WBAH as compared to the control (Table 5.4).

5.3.1.6 TA (%)

All the spray applications of Pro-Ca treatment except (400 or 800 mg L^{-1}) reduced TA (%) as compared to the control during 2015 (Table 5.3). However, mean TA (%) in the juice was not significantly affected by the time of spray application (Table 5.3). The interaction between different concentrations of Pro-Ca treatments applied and its different spray timings were found to be significant for TA (%) in 2015. Application of Pro-Ca (400 mg L^{-1}) single spray at 6 WBAH resulted in higher TA (1.13 %) as compared to the control and all other treatments during 2015 (Table 5.3). In 2016, single spray applications of an aqueous solution containing Pro-Ca irrespective of the concentration applied at 3 WBAH did not significantly affect TA (%) in the fruit juice (Table 5.4).

5.3.1.7 SSC/TA ratio

In 2015, all the Pro-Ca spray treatments reduced mean SSC/TA ratio as compared to the control except Pro-Ca (600 mg L^{-1}) treatment (Table 5.3). Mean SSC/TA ratio was significantly higher with the single spray of Pro-Ca applied at 6 or 3 WBAH, as compared to the double spray applied at 6 followed by 3 WBAH (Table 5.3). A non-significant interaction was found between different concentrations of Pro-Ca applied and its different spray timings for SSC/TA ratio. In 2016, the single spray application of an aqueous solution containing Pro-Ca (1600 mg L^{-1}) applied at 3 WBAH resulted in the significantly highest SSC/TA ratio (11.2), as compared to the control (10.0) and all other treatments except (2000 mg L^{-1}) (Table 5.4).

Table 5.3 Fruit firmness (N), SSC (%), TA (%) and SSC/TA in M7 Navel influenced by different concentrations of Pro-Ca applied at 6, 3 WBAH single spray; or double spray at 6 WBAH followed by 3 WBAH in 2015.

Fruit firmness (N)				
Treatments (mg L^{-1})	6WBAH	3WBAH	6 fb 3 WBAH	Mean (Tr)
Control	436.4±1.4	411.5±9.3	423.9±5.4	423.9 a
Pro-Ca (200)	406.5±4.4	407.4±4.5	389.2±6.8	401.0 b
Pro-Ca (400)	384.1±4.4	394.2±1.4	400.4±1.0	392.9 b
Pro-Ca (600)	440.3±6.3	376.2±2.2	387.8±4.2	401.4 b
Pro-Ca (800)	395.6±4.7	386.6±3.4	369.5±3.9	383.9 b
Mean (Tm)	412.6a	395.2b	394.1b	
SSC (%)				
Control	13.7±4.0 a	13.2±3.8 abc	12.6±3.7 def	13.2 a
Pro-Ca (200)	13.0±3.8 bcd	12.8±3.7 cde	11.4±3.3 g	12.4 c
Pro-Ca (400)	13.6±4.0 ab	13.3±3.9 abc	11.6±3.4 g	12.8 b
Pro-Ca (600)	12.9±3.8 cde	13.1±3.8 abcd	12.4±3.6 ef	12.8 b
Pro-Ca (800)	13.6±4.0 ab	13.2±3.8 abc	12.2±3.6 f	13.0 ab
Mean	13.38a	13.16b	12.07c	
TA (%)				
Control	1.02±0.30 bcd	1.01±0.29 cd	1.04±0.30 bcd	1.02 b
Pro-Ca (200)	1.01±0.29 cd	1.06±0.31 abcd	1.0±0.29 d	1.02 b
Pro-Ca (400)	1.13±0.33 a	1.01±0.29 cd	1.0±0.29 d	1.05 ab
Pro-Ca (600)	1.0±0.29 d	1.02±0.30 bcd	1.01±0.29 cd	1.01 b
Pro-Ca (800)	1.09±0.32 abc	1.10±0.32 ab	1.05±0.31 abcd	1.08 a
Mean	1.05	1.04	1.02	
SSC/TA				
Control	13.4±3.9	13.1±3.8	12.2±3.6	12.9 a
Pro-Ca (200)	12.9±3.8	12.1±3.5	11.4±3.3	12.1 c
Pro-Ca (400)	12.0±3.5	13.1±3.8	11.6±3.5	12.2 bc
Pro-Ca (600)	12.9±3.8	12.8±3.7	12.3±3.6	12.7 ab
Pro-Ca (800)	12.5±3.6	12.0±3.5	11.6±3.4	12.0 c
Mean	12.7a	12.6a	11.8b	

Tr = treatments, Tm = time of spray application, fb = followed by. Data represent means of 3 replicates (20 fruit per replication) for M7. Mean separation for significant analysis of variance within the columns and rows was tested by Duncan's multiple range tests at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns or rows. Standard error SD (±).

Table 5.4 Effect of spray application of different concentrations of Pro-Ca applied at 3 weeks before anticipated harvest on fruit firmness (N), SSC (%), TA (%) and SSC/TA in 'M7' Navel during 2016.

Treatment (mg L^{-1})	Firmness (N)	SSC (%)	TA (%)	SSC/TA ratio
Control	351.9±6.0	12.6±0.08ab	1.3±0.02	10.0±0.11b
Pro-Ca (400)	336.6±8.8	12.2±0.03bc	1.3±0.01	9.7±0.11b
Pro-Ca (800)	360.8±5.0	12.2±0.08bc	1.2±0.01	10.0±0.15b
Pro-Ca (1200)	363.4±6.9	11.9±0.17c	1.2±0.02	9.7±0.21b
Pro-Ca (1600)	370.1±4.5	13.1±0.06a	1.2±0.01	11.2±0.07a
Pro-Ca (2000)	353.8±4.4	13.0±0.10a	1.2±0.01	10.7±0.12ab

Data represent means of 4 replicates (20 fruit per replication) for M7. Mean separation for significant analysis of variance within the columns was tested by Duncan's multiple range tests at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns. Standard error SD (±).

5.3.1.8 Vitamin C and total antioxidants

All Pro-Ca treatments irrespective of the concentrations applied and time of application did not significantly ($P \leq 0.05$) affect vitamin C levels in the juice as compared to the control in 2015 and 2016 (Table 5.5 and 5.6). In 2015, double spray application of Pro-Ca applied at 6 followed by 3 WBAH showed significantly higher mean total antioxidants (478.9 µM L^{-1} Trolox) as compared to the other two spray timings (Table 5.5). The interactions between different Pro-Ca treatments and spray timings were found to be significant for the levels of total antioxidants in 2015. During 2016, a single spray application of Pro-Ca (800 mg L^{-1}) resulted in the significantly highest total antioxidants (689.4 µM L^{-1} Trolox) as compared to the control and all other treatments except Pro-Ca (2000 mg L^{-1}) (Table 5.6).

Table 5.5 Levels of vitamin C and total antioxidants in the juice of M7 sweet orange fruit influenced by different concentrations of Pro-Ca applied at 6, 3 WBAH single spray or double spray at 6 WBAH followed by 3 WBAH in 2015

Vitamin C (mg L^{-1})				
Treatment (mg L^{-1})	6WBAH	3WBAH	6 fb 3WBAH	Mean
Control	478.9±8.6	498.4±19.2	459.1±20.6	478.8
Pro-Ca (200)	327.9±13.6	385.3±8.2	343.0±15.0	352.0
Pro-Ca (400)	461.7±9.3	345.1±21.9	346.0±30.4	384.3
Pro-Ca (600)	515.6±30.4	422.8±44.3	354.2±14.4	430.9
Pro-Ca (800)	390.9±32.1	294.2±23.1	496.6±33.4	393.9
Mean	435.0	389.1	399.7	
Total antioxidants (µM L^{-1} Trolox)				
Control	346.2±efgh	296.8±h	531.3±a	391.4
Pro-Ca (200)	337.9±fgh	316.7±gh	519.0±ab	391.2
Pro-Ca (400)	415.5±cdef	438.4±abcde	433.3±abcd	439.1
Pro-Ca (600)	412.5±cdefg	409.9±defg	431.8±bcdef	418.1
Pro-Ca (800)	379.7±defgh	510.0±abc	449.0±abcd	446.2
Mean	378.4b	394.4b	478.9a	

Tr = treatments, Tm = time of spray application, fb = followed by. Data represent means of 3 replicates (20 fruit per replication) for M7. Mean separation for significant analysis of variance within the columns and rows was tested by Duncan's multiple range tests at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns or rows. Standard error SD (±).

Table 5.6 Effect of spray application of different concentrations of Pro-Ca applied at 3 WBAH on levels of vitamin C and total antioxidants in the juice of 'M7' sweet orange fruit in 2016.

Treatment (mg L^{-1})	Vitamin C (mg L^{-1})	Antioxidants (µM L^{-1} Trolox)
Control	549.2±14.3	553.1±10.5 b
Pro-Ca (400)	554.4±12.1	486.5±10.8 b
Pro-Ca (800)	631.7±15.4	689.4±4.9 a
Pro-Ca (1200)	510.7±12.5	517.3±15.0 b
Pro-Ca (1600)	583.5±5.3	532.3±4.3 b
Pro-Ca (2000)	600.7±13.4	655.6±12.7 a

Data represent means of 4 replicates (20 fruit per replication) for M7. Mean separation for significant analysis of variance within the columns was tested by Duncan's multiple range tests at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns. Standard error SD (±).

5.3.2 Effect of pre-harvest spray application of PBZ on fruit colour and quality

5.3.2.1 Hue angle (h°), CCI and levels of total carotenoids

Single spray applications of an aqueous solution containing PBZ (1000 mg L^{-1}) applied at 6 WBAH showed reduced *h*° angle (56.5), increased CCI (10.6) as compared to the control and all other treatments except PBZ (250 and 500 mg L^{-1}) during 2015 (Table 5.7). In 2016, PBZ single sprays (1000 and 1500 mg L^{-1}) applied at 3 WBAH were effective in reducing *h*° and improving CCI as compared to the control and all other treatments (Table 5.7). Single spray applications of PBZ (1000 mg L^{-1}) applied at 6 WBAH resulted in the highest levels of total carotenoids in the rind (42.6 mg L^{-1}) as compared to the control and all other treatments in 2015 (Table 5.7). In 2016, a spray application of PBZ (1500 mg L^{-1}) at 3 WBAH showed the highest levels of total carotenoids in the rind (47.1 mg L^{-1}) as compared to the control and all other treatments except PBZ (1500 mg L^{-1}) (Table 5.7).

Table 5.7 Fruit colour (*h*°, CCI) and levels of total carotenoids in the peel of M7 Navel influenced by different concentrations of PBZ applied at 6 WBAH in 2015 and 3 WBAH in 2016

6 WBAH (2015)			
Treatment (mg L^{-1})	*h*°	CCI	Total carotenoids (mg kg^{-1})
Control	60.1±0.21 a	8.8±0.12 b	10.5±0.35 e
PBZ (100)	60.3±0.65 a	9.1±0.28 b	21.9±0.35 d
PBZ (250)	58.4±0.43 ab	9.8±0.21 ab	30.4±0.78 c
PBZ (500)	57.4±0.19 ab	10.2±0.09 ab	34.0±1.12 b
PBZ (1000)	56.5±0.29 b	10.6±0.16 a	42.6±0.33 a
3 WBAH (2016)			
Control	63.1±0.28 a	7.7±0.12 c	14.0±1.2 c
PBZ (500)	56.9±0.06 b	10.5±0.04 b	28.7±1.5 b
PBZ (1000)	54.9±0.07 c	11.6±0.04 a	32.2±0.9 b
PBZ (1500)	55.4±0.22 c	11.2±0.13 a	47.1±3.8 a
PBZ (2000)	56.7±0.27 b	10.5±0.13 b	40.0±0.9 ab

Data represent means of 4 replicate (20 fruit per replication) for M7. Mean separation for significant analysis of variance within the columns was tested by Duncan's multiple range tests at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns. Standard error SD (±).

5.3.2.2 Fruit firmness, SSC (%), TA (%) and SSC/TA ratio

In 2015, single spray applications of PBZ applied 6 WBAH irrespective of the concentrations did not significantly affect fruit firmness, SSC (%) and SSC/TA ratio (Table 5.8). Meanwhile, spray applications of PBZ (100 mg L^{-1}) resulted in lower TA (1.02 %) as compared to the control (1.10 %) and all other treatments except PBZ (250 and 100 mg L^{-1}). In 2016, fruit firmness and SSC/TA ratio were not significantly affected by PBZ treatments applied as a single spray at 3 WBAH (Table 5.8). All the Pro-Ca treatments exhibited reduced SSC (%) and TA (%) as compared to the control and all other treatments except PBZ (500 mg L^{-1}).

Table 5.8 Fruit firmness (N), SSC (%), TA (%) and SSC/TA in M7 Navel influenced by different concentrations of PBZ applied at 6 WBAH in 2015 and 3 WBAH in 2016

		6 WBAH (2015)		
Treatment (mg L^{-1})	Fruit firmness (N)	SSC (%)	TA (%)	SSC/TA
Control	390.3±6.7	13.4±0.06	1.10±0.01 a	12.1±0.13
PBZ (100)	397.0±9.4	13.1±0.09	1.02±0.01 c	12.9±0.09
PBZ (250)	388.8±3.0	13.3±0.05	1.04±0.01 abc	12.8±0.07
PBZ (500)	413.1±1.9	13.2±0.06	1.03±0.01 bc	12.9±0.22
PBZ (1000)	404.3±5.6	13.3±0.06	1.09±0.01 ab	12.2±0.15
		3 WBAH (2016)		
Control	358.1±2.9	12.8±0.12 a	1.2±0.01 ab	10.4±0.10
PBZ (500)	354.6±11.4	12.5±0.08 ab	1.3±0.01 a	9.7±0.12
PBZ (1000)	333.8±7.3	12.0±0.04 bc	1.1±0.01 c	10.8±0.09
PBZ (1500)	350.6±6.7	11.8±0.06 c	1.1±0.0 c	10.4±0.08
PBZ (2000)	370.7±4.9	12.2±0.04 bc	1.2±0.01 bc	10.3±0.04

Data represent means of 4 replicates (20 fruit per replication) for M7. Mean separation for significant analysis of variance within the columns was tested by Duncan's multiple range tests at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns. Standard error SD (±).

5.3.2.3 Vitamin C and total antioxidants

Single spray applications of an aqueous solution containing PBZ did not significantly affect vitamin C and total antioxidants at 6 WBAH and 3 WBAH in 2015 and 2016 respectively (Table 5.9).

Table 5.9 Vitamin C and total antioxidants in the juice of M7 sweet orange fruit influenced by different concentrations of PBZ applied at 6 WBAH in 2015 and 3 WBAH in 2016

6 WBAH (2015)		
Treatment (mg L^{-1})	Vitamin C (mg L^{-1})	Total antioxidants (µM L^{-1} Trolox)
Control	541.4±8.9	365.9±6.7
PBZ (100)	533.3±2.7	498.2±11.8
PBZ (250)	517.5±8.3	342.0±10.8
PBZ (500)	582.2±6.5	449.2±22.7
PBZ (1000)	555.3±9.5	408.4±24.3
3 WBAH (2016)		
Control	542.1±3.1	516.3±5.0
PBZ (500)	555.7±15.9	505.3±18.5
PBZ (1000)	563.1±14.1	463.1±10.5
PBZ (1500)	500.6±3.9	468.0±12.0
PBZ (2000)	543.7±15.0	519.8±12.4

Data represent means of 4 replicates (20 fruit per replication) for M7. Mean separation for significant analysis of variance within the columns was tested by Duncan's multiple range tests at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns. Standard error SD (±).

5.4. Discussion

Fruit colour is an important parameter of quality and influences the decision of consumers to purchase the fruit. The enhanced rind colour of the citrus fruit is due to the degradation of chlorophyll which unmasks the presence of carotenoids (Gambetta et al., 2012). Gibberellins are known to retard colour development during the colour break and also restrict the degradation of chlorophyll and accumulation of carotenoids (Le Roux, 2006). During citrus fruit maturation, an elevated level of GA resulted in green colour and delayed chloroplast to chromoplast transformation (Iglesias et al., 2001). Various inhibitors of gibberellins biosynthesis have been used for many years to down-regulate the level of GAs to hasten the process of fruit colour development. The gibberellin biosynthesis (GA) inhibitors are generally classified into two categories such as *ent*-kaurene synthetase or *ent*-kaurene oxidase, depending upon their site of action (Hedden and Kamiya, 1997) and acylcyclohexanediones (cyclohexanediones) (3, 5-dioxo-4-butyryl-cyclohexane carboxylic acid) represent a new class of growth retardant, which include Pro-Ca (Rademacher et al., 1992).

Pre-harvest spray application of Pro-Ca showed reduced *h*° and enhanced CCI depending upon concentration and time of application in M7 Navel during 2015 and 2016 (Table 5.1 and 5.2). The enhanced rind colour with the spray application of Pro-Ca in M7 Navel ascribed to the increased level of total carotenoids in the rind (Table 5.1 and 5.2). Possibly, the enhanced the accumulation of carotenoids in the rind with the spray application of Pro-Ca may be attributed to the reduced levels of highly active gibberellin (GA_1) and causing accumulation of its immediate precursor GA_{20} (inactive), consequently block the conversion of inactive (GA_{20}) to active (GA_1) as previously reported (Evans et al., 1999; Rademacher et al., 1992; Rademacher, 2000). Pro-Ca is similar to the structure of 2-oxoglutaric acid, co-enzymes of the involved dioxygenases, which are assumed to be responsible for blocking GA metabolism in the later stage of biosynthesis (Evans et al., 1999). It has also been reported by Rademacher et al. (1992) that Pro-Ca inhibits the biosynthesis of 3β-hydroxyl-GA_S and therefore supports the argument that 3β-hydroxylation is important for the formation of growth-active GA_S. Similarly, Barry and Le Roux (2010) previously reported that Pro-Ca (400 mg L^{-1}) applied at 6 followed by 3 weeks before harvest enhanced the rind colour of Nules Clementine mandarin and Navelina Navel as result of enhanced levels of carotenoids. In addition, Pilar Mata et al. (2006) also reported

that the application of Pro-Ca on the blushed side of the Fuji apple fruit (*Malus domestica* Borkh.) exhibited a deep red colour due to greater concentrations of anthocyanins and carotenoids.

Single pre-harvest spray application of PBZ applied at 6 WBAH in 2015 and 3 WBAH during 2016 also reduced *h*° and enhanced CCI in M7 Navel orange fruit (Table 5.7). The enhanced rind colour is attributed to the increased level of total carotenoids in the rind (Table 5.7) possibly by reducing the levels of gibberellins in the fruit rind by blocking the oxidation from *ent*-kaurene to *ent*-kaurenoic acid by hindering cytochrome P450-dependent monooxygenases in the early steps of gibberellin biosynthesis pathway (Rademacher, 2000; Rademacher et al., 1992). Similarly, Gilfillan and Lowe (1985) previously reported that PBZ application enhanced rind colour by 1 to 2 units in Satsuma mandarin. Earlier, Monselise (1985) previously also reported that PBZ application hastens the rind colour development in Topaz tangor. As a prelude, GA_3 application even at low concentration (0.1 mg L^{-1}) delays colour development in citrus fruit (Goldschmidt, 1988; Eilati et al., 1969b; Coggins and Henning, 1988).

The influence of Pro-Ca spray treatments on fruit quality variables such as fruit firmness, SSC (%), TA (%), SSC/TA, vitamin C and total antioxidants were not consistent during both years (Table 5.3, 5.4, 5.5 and 5.6). Pro-Ca treatments exhibited decreased fruit firmness in 2015 but did not significantly affected fruit firmness in 2016. However, Basak (2004) previously reported that double spray application of Pro-Ca (200 mg L^{-1}) did not affect apple firmness as compared to single spray application. In addition, Pro-Ca application exhibited higher fruit firmness in Jen-Ju Bar guava (*Psidium guajava* L.) in one year but the data was not significant in the second year (Chang, 2016). These contrasting results have been supported by Faust (1972) who argued that the effect of plant growth regulator on the fruit firmness are unexpected and cannot be elucidated by changes in the pectin content in the cell wall. Furthermore, Elfving et al. (2003b) reported that application of Pro-Ca did not affect quality Bartlett and D'Anjou pear. Higher concentration of Pro-Ca sprayed (800, 1200 or 1600 mg L^{-1}) exhibited increased SSC (%) in M7 orange during both years (Table 5.3 and 5.4). Our findings are in line with Basak (2004) claims that double spray application of Pro-Ca (200 mg L^{-1}) increased the SSC (%) in Elstar apple fruit. Furthermore, Chang (2016) also reported that Pro-Ca treatment exhibited higher SSC (%). On the other hand, Medjdoub et al. (2004) argued that spray application of Pro-

Ca reduced SSC (%) in Smoothee Golden Delicious apple. Additionally, Miller (2002) has reported that Pro-Ca has no effect on SSC (%). The possible mechanism of reducing fruit firmness and increasing SSC (%) in M7 Navel orange with the spray application of Pro-Ca warrants to be investigated. PBZ spray application did not significantly affect fruit firmness, SSC/TA, levels of vitamin C and total antioxidants in the juice. Whilst, spray application of PBZ reduced the juice acidity as compared to the control during both years but its exact mechanism in reducing juice acidity is yet to be investigated. Moreover, all the quality variables are within the acceptable range and no negative effects of the spray application of Pro-Ca or PBZ were noted.

In conclusion, a pre-harvest spray application of Pro-Ca (800 mg L^{-1}) applied at 6 WBAH or (1200 mg L^{-1}) at 3 WBAH as a single spray enhanced the rind colour of M7 Navel as depicted by reduced *h*° angle, enhanced CCI and levels of total carotenoids. Furthermore, a pre-harvest spray application of PBZ (1000 mg L^{-1}) at 6 WBAH or (1500 mg L^{-1}) at 3 WBAH showed improved rind colour in M7 sweet orange fruit illustrated by reduced *h*° and increased CCI and levels of total carotenoids. The quality variables were not consistent during both years. However, no negative effects on fruit quality were recorded.

CHAPTER 6

Pre-harvest spray application of methyl jasmonate promotes fruit colour and regulates quality in M7 Navel orange

Abstract

Poor rind colour in cv. M7 Navel (*Citrus sinensis* L. Osbeck) at harvest causes serious economic losses to the growers in WA. The efficacy of different concentrations (0, 1.25, 2.5, 5.0 and 7.5) of MJ spray application at pre-harvest stage (6 or 3 WBAH) on rind colour development particularly from yellow to deep orange and on the fruit quality of M7 was investigated during 2015 and 2016. The pre-harvest spray application of MJ (5.0 or 7.5 mM) resulted in enhanced rind colour, reduced *h*° angle (55.7, 54.3) and increased CCI (11.0, 12.0) and levels of total carotenoids in the rind (35.3, 58.3 mg kg^{-1}) respectively in M7 Navel, during both years. Single spray application applied at 3 WBAH resulted in higher level of total carotenoids (40.4 mg kg^{-1}) as compared to the other spray timings in the rind of M7 Navel in 2015. However, time of MJ application did not influence *h*° angle and CCI. In 2015, all the pre-harvest MJ treatments except (1.25 mM) exhibited reduced fruit firmness. Furthermore, MJ treatments exhibited reduced soluble solids concentration (SSC %) in the juice. All the MJ treatments showed reduced levels of total sugars and organic acid in the juice during 2015. In conclusion, pre-harvest spray application of MJ (5.0 or 7.0 mM) at 3 WBAH reduced *h*°, increased levels of total carotenoids and CCI in the rind of M7 Navel.

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6.1. Introduction

Citrus fruit colour is one of the factors which influence the decision making of the consumers to purchase the fruit. M7 is an early maturing cultivar of sweet orange (*Citrus sinensis* L. Osbeck) mainly harvested in the month of May in WA. M7 is a bud mutation of Navelina and has been introduced in recent years to extend the availability of sweet orange fruit. The fruit of this cultivar is rounder in shape and with higher soluble solids concentration and acid ratio than Navelina (DAFWA, 2017). M7 at fruit maturity attains excellent internal fruit quality but coupled with poor rind colour development in WA, consequently causing serious economic losses to the growers.

Citrus rind colour results from chlorophyll degradation and accumulation of carotenoids (Gross, 1987). Different types of carotenoids along with a large number of their isomers have earlier been reported in citrus fruit (Goodner et al., 2001; Stewart and Wheaton, 1973). These carotenoid pigments are responsible for the internal and external colouration of Cara Cara sweet orange fruit (Alquezar et al., 2008). Carotenoids are not only involved in enhancing rind colour, but are also originators of vitamin A, and have a substantial antioxidant activity which protects humans against cardiovascular diseases and carcinogenesis (Fraser and Bramley, 2004; Clinton, 1998; Sandmann, 2001).

Jasmonates (JA_S) [(jasmonic acid (JA) and MJ] are naturally occurring cyclopentanone compounds which modulate a wide range of plant responses (Creelman and Mullet, 1997; Sembdner and Parthier, 1993), including promotion of chlorophyll degradation (Abeles and Dunn 1989; Hung and Kao, 1996; Ueda and Kato, 1980); accumulation of anthocyanins (Shaifq et al., 2011); reduction of postharvest decay and incidence of green mould; enhancement of fruit colour and reduction of CI in cold stored fresh produce (Reyes-Díaz et al., 2016). MJ has been reported to promote chlorophyll degradation and biosynthesis of anthocyanins in apple (Rudell et al., 2002; Perez et al., 1993), tomato (Saniewski and Czapski, 1983), lychee (Yang et al., 2011b), papaya (Gonzalez-Aguilar et al., 2003) and mango (Gonzalez-Aguilar et al., 2000a). It has also been reported to regulate aroma development in apple (Olias et al., 1992) and mango fruit (Lalel et al., 2003a). Kondo et al. (2007) reported that La France pears (*Pyrus communis* L.) fruit dipped in 0.39 mm n-propyl dihydrojasmonate (PDJ), which is a jasmonic acid derivative, following storage at 4°C

for 15 d upregulated the expression of *ACC synthase* **(***ACS***)** *1* and *ACC oxidase* **(***ACO***)** *1* and ethylene production during fruit ripening.

The level of JA in the cell has been reported to change during fruit development. In '*Tsugaru*' apple [*Malus sylvestris* (L.) Mill. var. domestica (Borkh.) Mansf.], the endogenous MJ concentration has been reported to be high at maturity as compared to the early stages of fruit development (Kondo et al., 2000). Whereas, the concentration of MJ has been reported to be higher at the initial stage of fruit growth and gradually decreasing toward harvest in different fruit such as grapes (Kondo and Fukuda, 2001), strawberry (Gansser et al., 1997) and sweet cherry (Kondo et al., 2000). Saniewski and Czapski (1983) previously reported that exogenous application of MJs stimulates *β*-carotene accumulation and inhibits lycopene accumulation during the ripening of tomatoes. Pre-harvest spay application of MJ improved red blush and accumulation of flavonoids in apple fruit (Shafiq et al., 2011). Earlier, MJ application has been reported to enhance apple fruit colour development independent of ethylene action, signifying that its effect on colour development in apple may partly be independent of ethylene (Fan and Mattheis, 1999; Kondo et al., 2001). As a prelude, pre-harvest spray application of MJ enhanced chlorophyll degradation and anthocyanin accumulation in different fruit. Recently, pre-harvest spray application of S-ABA, Pro-Ca or PBZ applied 3-6 weeks prior to harvest has been reported to enhanced fruit colour development in M7 sweet orange (Rehman et al., 2018 a and b). Meanwhile, no research work has been reported on the effects of pre-harvest spray application of MJ on carotenoids accumulation in the rind, fruit colour development and quality in M7 sweet orange fruit. Therefore, the objective of the present investigation was to elucidate the role of pre-harvest spray application of MJ in regulating fruit colour development particularly from yellow to deep orange and levels of total carotenoids in the rind and fruit quality in M7 sweet orange grown under a Mediterranean climate in WA.

6.2. Materials and methods

6.2.1. Plant material

Sweet orange M7 (*Citrus sinensis* L. Osbeck) were hand harvested from five-year old sweet orange trees previously grafted on Carrizo citrange (*Citrus sinensis* (L.) Osbeck *x Poncirus trifoliata* Raf.) rootstock grown in a commercial orchard located at Moora (latitude 30° 41, South, longitude 115° 42, East), WA and transported to the laboratory within four hours. M7 trees were spaced 5.0 m between rows and 2.5 m within rows in the North-South direction and provided with similar cultural practices including nutrition, irrigation and plant protection. Two independent experiments were conducted on M7 Navel sweet orange during two consecutive years in 2015 and 2016.

6.2.2. Experiment 1: Pre-harvest spray application of MJ applied at 6 or 3 WBAH as single sprays and double spray at 6 followed by 3 WBAH in M7 Navel orange during 2015.

M7 trees were sprayed onto whole trees until runoff with an aqueous emulsion containing different concentrations of MJ (1.25, 2.5 or 5.0 mM) obtained from (Sigma-Aldrich, Saint Louis, USA) and Tween® 20 (0.25 %) as a surfactant at 6 WBAH (8 April) or 3 WBAH (30 April) as a single spray application and sprayed twice at 6 followed by 3 WBAH in 2015. Unsprayed trees were kept as control. The experiment was laid out by following a two-factor factorial (MJ treatments and times of application) randomised block design. A single tree was treated as an experimental unit and included three replicates. At harvest maturity, 25 fruit per tree free from blemishes, diseases and pests were randomly harvested around the tree canopy. An air-conditioned vehicle was used to transport the fruit to Curtin Horticulture Laboratory within four hours of harvest. The colour coordinates such as *h*°, CCI and levels of total carotenoids in the rind of the fruit were determined. Fruit firmness, soluble solids concentration, titratable acidity (TA), SSC/TA ratio, vitamin C, total antioxidants, individual sugars and organic acids were determined from the juice.

6.2.3. Experiment 2: Pre-harvest single spray application of MJ applied at 3 WBAH in M7 Navel orange during 2016.

In 2016, an aqueous emulsion containing different concentrations of MJ (1.25, 2.5, 5.0, 7.5 mM) as well as Tween® 20 (0.25 %) as a surfactant was sprayed onto the whole trees until runoff at 3 WBAH (30 April). The control trees were kept as unsprayed. The experimental design was one-factor factorial (MJ treatments) randomised block design and replicated four times. A single tree was kept as an experimental unit. At harvest maturity, 25 fruit per tree from around the tree canopy were randomly harvested. Following the harvest, the fruit rind colour (*h*° and CCI) and levels of total carotenoids in the rind were estimated. Quality variables (except sugars and organic acids) were recorded and analysed as per Experiment 1.

6.2.4. Determination of the fruit colour

By using a colorflex EZ (45°/0° design) spectrocolorimeter (Hunter Lab, Hunter Associates Laboratory Inc., Reston, VA, 20190, USA) colour coordinates (L*, a* and b*) were determined at three positions around the equatorial plane of the fruit described in detailed Chapter 3, Section 3.4.

6.2.5. Determination of level of total carotenoids

Rind tissue of M7 Navel fruit was excised from ten randomly selected fruit in each replication. The pooled rind tissue was used to determine the level of total carotenoids by following the method used by Lee and Castle (2001) outlined in Chapter 3, Section 3.5.

6.2.6. Fruit firmness

A texture profile analyser (TPA Plus, AMETEK Lloyd Instruments Ltd, Fareham, UK) interfaced with Nexygen® 4.6 software was used to estimate fruit firmness of ten randomly selected fruit in each replication detailed in Chapter 3, Section 3.8. The firmness of fruit was expressed in newtons (N).

6.2.7. SSC, TA and SSC/TA ratio

A digital refractometer (Atago-Palette PR 101, Atago CO. Ltd, Itabashi-Ku, and Tokyo, Japan) was used to determine SSC (%) in the pooled juice from ten randomly selected fruit in each replication and expressed as a percentage. Moreover,

the juice was titrated against 0.1N NaOH using 2-3 drops of phenolphthalein as an indicator to a pink colour endpoint to determine TA and expressed as percentage citric acid. SSC/TA ratio was calculated by dividing SCC and TA values. Thorough outlined method has been described in Chapter 3, Section 3.9, 3.10 and 3.11.

6.2.8 Determination of sugars and organic acids

The levels of individual sugars and organic acids in the juice of M7 Navel were determined by using reverse-phase high-performance liquid chromatography system (RP-HPLC; Waters, Milford, MA, USA) fitted with refractive index detector (sugars) and dual wavelength UV detector (organic acids) as outlined in Chapter 3, Section 3.12. All the individual sugars and organic acids were expressed as (g L^{-1}).

6.2.9. Determination of vitamin C and total antioxidants

A UV/VIS spectrophotometer (Jenway spectrophotometer Model 6405, Dunmow, Essex, UK) was used to determine the levels of vitamin C and total antioxidants in the pooled juice from ten M7 fruit in each replication. The detailed method for estimation of vitamin C and total antioxidants has been explained in Chapter 3, section 3.13 and 3.14.

6.2.10. Statistical analysis

The experimental data were subjected to one-way or two-way analysis of variance (ANOVA) using GenStat 14th edition (release 14.1; Lawes Agricultural Trust, Rothamsted Experimental Station, Rothamsted, UK). Following a significant ($P \leq 0.05$) F-test, Fisher's least significant differences (LSD) was calculated. Within ANOVA, the effects of various MJ treatments and their timings of the application as well as their interactions on various variables were evaluated.

6.3. Results

6.3.1. Hue angle (h°)

Mean *h*° was significantly ($P \leq 0.05$) reduced (55.7) with the pre-harvest spray application of MJ (5.0 mM) as compared to the control (61.1) and all other treatments during 2015 (Table 6.1). However, mean *h*° of the fruit was not significantly affected

with the time of spray application. The interaction between different treatments of MJ and time of spray application was found to be non-significant for *h*°. In 2016, single spray application of an aqueous emulsion containing MJ (7.5 mM) at 3 WBAH showed significantly reduced *h*° (54.3) as compared to the control (62.9) and all other treatments of MJ except 2.5 and 5.0 mM (Table 6.2).

6.3.2. CCI

In 2015, mean CCI was significantly ($P \leq 0.05$) enhanced (11.0) with the spray application of MJ (5.0 mM) as compared to the control (8.5) and all other treatments (Table 6.1). Moreover, mean CCI of the fruit was not significantly affected by the time of spray application. The interaction between different MJ treatments and spray timings was found to be non-significant for CCI in 2015. In 2016, a single spray application of MJ regardless of concentration applied (2.5 - 5.0 mM) at 3 WBAH resulted in significantly enhanced CCI (11.4 - 12.0) respectively as compared to the control (7.8) (Table 6.2).

6.3.3. Levels of total carotenoids

In 2015, the mean level of total carotenoids in the rind was significantly ($P \leq 0.05$) increased (35.3 mg kg^{-1}) with the spray application of an aqueous emulsion containing MJ (5.0 mM) as compared to the control (18.2 mg kg^{-1}) and all other treatments (Table 6.1).

(A) **(B)**

Fig 6.1 Effect of pre-harvest spray application of MJ on the rind colour of M7 Navel (A) Control (B) MJ (7.5 mM)

However, the MJ single spray application applied at 3 WBAH showed the highest mean level of total carotenoids (40.4 mg kg^{-1}) in the rind as compared to the other spray timings. A significant interaction was found between different concentrations of MJ applied and its different spray timings for total carotenoids in 2015. A single spray application of MJ (1.25 to 5.0 mM) applied at 3 WBAH showed the highest levels of total carotenoids in the rind (45.0 to 49.2 mg kg^{-1}) as compared to all other treatments during 2015 (Table 6.1). In 2016, a single spray application of an aqueous emulsion containing MJ (5.0 and 7.5) applied at 3 WBAH significantly increased the level of total carotenoids (47.6 and 58.9 mg kg^{-1}) respectively, in the rind as compared to the control (18.0 mg kg^{-1}) and all other treatments (Table 6.2).

Table 6.1 Hue angle (*h*°), CCI and levels of total carotenoid in rind of M7 Navel influenced by different treatments (Tr) of MJ and time of application (Tm) applied at 6, 3 WBAH (weeks before anticipated harvest) as single spray and 6 followed by (fb) 3 WBAH as double spray in 2015 growing seasons.

Treatments (MJ mM)	6 WBAH	3 WBAH	6 fb 3 WBAH	Mean (Tr)
		(*h*°)		
Control	61.2±0.49	61.7±0.26	60.5±0.36	61.1
MJ (1.25)	58.7±0.23	56.7±0.06	57.8±0.15	57.7
MJ (2.5)	57.7±0.25	56.0±0.19	57.2±0.21	57.0
MJ (5.0)	55.3±0.14	55.8±0.09	56.1±0.13	55.7
Mean (Tm)	58.2	57.5	57.9	
LSD ($P \leq 0.05$)	Tr = 0.98	Tm = ns	Tr xTm = ns	
		CCI		
Control	8.5±0.22	8.2±0.08	8.7±0.16	8.5
MJ (1.25)	9.6±0.10	10.3±0.07	10.1±0.09	10.1
MJ (2.5)	10.0±0.12	10.9±0.10	10.2±0.11	10.4
MJ (5.0)	11.3±0.07	10.9±0.04	10.8±0.08	11.0
Mean	9.9	10.2	10.0	
LSD ($P \leq 0.05$)	Tr = 0.46	Tm = ns	Tr xTm = ns	
		Total carotenoids (mg kg^{-1})		
Control	16.2 ±0.25	20.5±1.4	18.1±0.44	18.2
MJ (1.25)	22.3±0.50	45.0±0.62	19.0±0.24	28.8
MJ (2.5)	21.5±0.68	47.1±0.43	20.7±0.62	29.8
MJ (5.0)	38.9±0.25	49.2±0.52	17.7±0.38	35.3
Mean	24.7	40.4	18.8	
LSD ($P \leq 0.05$)	Tr = 0.46	Tm = 2.2	Tr xTm = 4.5	

Mean separation within the column (Mean Tr) was tested with LSD *(P<0.05*); while mean separation within the row (Mean Tm) was tested with LSD *(P<0.05*). Mean separation for the interaction effects (Tr x Tm) was tested with LSD *(P<0.05*) at the same level of Tm across Tr. ns = not significant, n = three replicates (20 fruit per replication).

Table 6.2 Effect of pre-harvest single spray application of different concentrations of MJ applied at 3 WBAH on (*h*°), (CCI) and level of total carotenoids in the rind of M7 Navel in 2016.

Treatments (MJ mM)	*h*°	CCI	Total carotenoids (mg kg^{-1})
Control	62.9±0.25	7.8±0.09	18.0±1.5
MJ (1.25)	57.0±0.33	10.5±0.18	38.1±1.0
MJ (2.5)	55.4±0.22	11.4±0.12	43.3±1.6
MJ (5.0)	55.4±0.18	11.4±0.10	47.6±2.5
MJ (7.5)	54.3±0.26	12.0±0.18	58.9±1.2
LSD ($P \leq 0.05$)	1.8	0.9	12.9

Mean separation within the column (Mean Tr) was tested with LSD *(P<0.05*). ns = not significant, n = four replicates (20 fruit per replication)

6.3.4. Fruit firmness (N)

During 2015, all MJ treatments except MJ (1.25 mM) showed significantly ($P \leq 0.05$) reduced fruit firmness as compared to the control (398.9 N) (Table 6.3). On the other hand, mean fruit firmness was also significantly affected by the time of spray application in 2015. Double spray application applied at 6 WBAH followed by 3 WBAH showed significantly higher fruit firmness (395.5 N) as compared to other spray timings (Table 6.3). A non-significant interaction was found between different concentrations of MJ treatments applied and its different spray timings for fruit firmness. In 2016, all the MJ spray treatments applied at 3 WBAH reduced fruit firmness as compared to the control (366.1 N) (Table 6.4).

6.3.5. SSC (%), titratable acidity (TA %) and SSC: TA

All MJ treatments showed significantly ($P \leq 0.05$) reduced mean SSC (%) in the juice as compared to the control, whilst mean titratable acidity (TA %) and SSC/TA was found to be non-significant in 2015 (Table 6.3). However, mean SSC and TA (%) in the juice were significantly reduced with the single spray application applied at 3 WBAH as compared to the other spray timings. Furthermore, mean SSC/TA in the juice was higher (13.5) with the single spray application at 3 WBAH as compared to other spray timings (Table 6.3). The interactions between different MJ treatments and its different spray timings were found to be significant for SSC but not for TA (%) and SSC/TA in 2015. During 2016, all the treatments of MJ spray applied 3 WBAH did not significantly affect SCC (%) in the fruit juice, but the application of

MJ (1.25 mM) exhibited higher TA (1.3 %) as compared to the control and all other treatments (Table 6.4). In addition, the spray application of MJ (5.0 mM) resulted in higher SSC/TA ratio (11.1) as compared to all other treatments.

Table 6.3 Fruit firmness (N), SSC (%), TA (%) and SSC/TA ratio in M7 Navel influenced by different treatments (Tr) of MJ and time of application (Tm) applied at 6, 3 WBAH (weeks before anticipated harvest) as single spray and 6 followed by (fb) 3 WBAH as double spray in 2015 growing seasons.

Treatments (MJ mM)	6 WBAH	3 WBAH	6 fb 3 WBAH	Mean (Tr)
		Fruit firmness (N)		
Control	389.3±4.4	389.3±5.4	418.0±3.2	398.9
MJ (1.25)	394.3±2.2	386.9±1.7	407.1±1.6	396.1
MJ (2.5)	356.6±2.2	354.3±5.9	364.1±2.4	358.3
MJ (5.0)	365.6±3.0	369.3±10.0	392.7±2.0	375.7
Mean (Tm)	376.3	374.9	395.5	
LSD ($P \leq 0.05$)	Tr = 18.6	Tm = 16.1	Tr xTm = ns	
		SSC (%)		
Control	12.8±0.11	13.4±0.11	13.2±0.02	13.1
MJ (1.25)	13.1±0.07	12.3±0.04	12.1±0.04	12.5
MJ (2.5)	12.8±0.11	11.5±0.01	13.2±0.04	12.5
MJ (5.0)	12.5±0.07	12.4±0.02	12.2±0.02	12.3
Mean	12.8	12.4	12.8	
LSD ($P \leq 0.05$)	Tr = 0.22	Tm = 0.19	Tr xTm = 0.39	
		TA (%)		
Control	1.01±0.02	0.93±0.04	1.15±0.01	1.03
MJ (1.25)	1.02±0.02	0.93±0.01	0.99±0.01	0.98
MJ (2.5)	1.02±0.02	0.88±0.02	0.96±0.01	0.96
MJ (5.0)	1.05±0.02	0.95±0.01	0.95±0.01	0.98
Mean	1.03	0.92	1.01	
LSD ($P \leq 0.05$)	Tr = ns	Tm = 0.06	Tr xTm = ns	
		SSC/TA		
Control	12.7±0.13	14.7±0.73	11.4±0.08	12.9
MJ (1.25)	12.8±0.16	13.2±0.12	12.2±0.06	12.7
MJ (2.5)	12.5±0.19	13.1±0.23	13.8±0.11	13.1
MJ (5.0)	12.0±0.21	13.1±0.08	12.8±0.09	12.6
Mean	12.5	13.5	12.6	
LSD ($P \leq 0.05$)	Tr = ns	Tm = 0.6	Tr xTm = ns	

Mean separation within the column (Mean Tr) was tested with LSD *(P<0.05*); while mean separation within the row (Mean Tm) was tested with LSD *(P<0.05*). Mean separation for the interaction effects (Tr x Tm) was tested with LSD *(P<0.05*) at the same level of Tm across Tr. ns = not significant, n = three replicates (20 fruit per replication).

Table 6.4 Effect of pre-harvest single spray application of different concentrations of MJ applied at 3 WBAH on fruit firmness (N), SSC (%), TA (%) and SSC/TA in M7 Navel during 2016.

Treatment (MJ mM)	Firmness (N)	SSC (%)	TA (%)	SSC/TA ratio
Control	366.1±5.0	11.9±0.15	1.1±0.0	10.5±0.13
MJ (1.25)	361.5±2.3	11.9±0.12	1.3±0.01	9.5±0.03
MJ (2.5)	358.3±3.8	12.2±0.04	1.2±0.02	10.2±0.13
MJ (5.0)	327.9±6.1	11.7±0.15	1.1±0.01	11.1±0.19
MJ (7.5)	323.8±3.7	11.7±0.07	1.2±0.01	9.9±0.11
LSD ($P \leq 0.05$)	33.20	ns	0.08	1.02

Mean separation within the column (Mean Tr) was tested with LSD *(P<0.05*). ns = not significant, n = four replicates (20 fruit per replication)

6.3.6. Individual and total sugars

The mean levels of sucrose and total sugars in the juice were significantly ($P \leq 0.05$) reduced by MJ treatments regardless of the concentrations applied in 2015. Meanwhile, the levels of glucose were not significantly affected with any of the MJ treatments (Table 6.5). Furthermore, all the MJ treatments except 2.5 mM, exhibited reduced levels of fructose in the juice as compared to the control. On the other hand, the effects of different spray timings on the mean level of glucose, fructose, sucrose and total sugars were found to be significant. Double spray application of MJ resulted in reduced levels of glucose and fructose as compared to other spray timings. Furthermore, a single spray application applied 3 WBAH resulted in a reduced level of sucrose as compared to other spray timings. Mean total sugars were higher (161.4 g L^{-1}) with the spray applied at 6 WBAH as compared to the other two spray timings. The interaction between spray timings and different concentrations of MJ applied was found to be significant ($P \leq 0.05$) for levels of glucose, fructose, sucrose and total sugars (Table 6.5).

Table 6.5 Levels of individual and total sugars in the juice of M7 Navel influenced by different treatments (Tr) of MJ and time of application (Tm) applied at 6, 3 WBAH (weeks before anticipated harvest) as single spray and 6 followed by (fb) 3 WBAH as double spray in 2015 growing seasons.

Treatment (MJ mM)	6 WBAH	3 WBAH	6 fb 3 WBAH	Mean (Tr)
		Glucose (g L^{-1})		
Control	26.5±0.71	25.5±0.32	16.5±0.45	22.8
MJ (1.25)	19.6±1.1	19.2±0.94	18.0±0.43	18.9
MJ (2.5)	24.6±1.0	14.8±0.37	17.7±0.95	19.0
MJ (5.0)	18.5±0.13	24.0±1.3	16.2±0.78	19.6
Mean (Tm)	22.3	20.9	17.1	
LSD ($P \leq 0.05$)	Tr = ns	Tm = 2.8	Tr xTm = 9.6	
		Fructose (g L^{-1})		
Control	26.7±0.23	28.8±0.26	23.8±0.27	26.5
MJ (1.25)	26.7±0.25	24.9±0.48	23.3±0.22	25.0
MJ (2.5)	27.4±0.37	23.5±0.13	29.0±0.75	26.6
MJ (5.0)	26.3±0.30	25.5±0.41	23.1±0.15	25.0
Mean	26.8	25.7	24.8	
LSD ($P \leq 0.05$)	Tr = 1.3	Tm = 1.1	Tr xTm = 2.3	
		Sucrose (g L^{-1})		
Control	111.7±0.46	112.8±1.6	112.6±1.6	112.4
MJ (1.25)	115.1±0.97	94.3±1.0	105.0±1.3	104.8
MJ (2.5)	113.7±2.0	93.4±1.0	104.6±0.16	103.9
MJ (5.0)	108.7±2.6	108.2±1.1	107.9±1.6	108.3
Mean	112.3	102.2	107.5	
LSD ($P \leq 0.05$)	Tr = 5.7	Tm = 4.9	Tr xTm = 9.8	
		Total sugars (g L^{-1})		
Control	165.0±1.2	167.2±2.1	153.0±2.0	161.7
MJ (1.25)	161.5±1.7	138.5±1.2	146.3±0.7	148.8
MJ (2.5)	165.7±2.6	131.7±1.1	151.3±1.6	149.6
MJ (5.0)	153.6±2.5	157.8±0.1	147.2±0.9	152.8
Mean	161.4	148.8	149.5	
LSD ($P \leq 0.05$)	Tr = 6.4	Tm = 5.6	Tr xTm = 11.2	

Mean separation within the column (Mean Tr) was tested with LSD *(P<0.05*); while mean separation within the row (Mean Tm) was tested with LSD *(P<0.05*). Mean separation for the interaction effects (Tr x Tm) was tested with LSD *(P<0.05*) at the same level of Tm across Tr. ns = not significant, n = three replicates (20 fruit per replication).

6.3.7. Individual and total organic acids

During 2015, the mean levels of citric, malic and total organic acid in the juice were reduced by MJ treatments as compared to the control, whilst the mean levels of tartaric acid in juice were found to be non-significant (Table 6.6).

Table 6.6 Levels of individual and total organic acids in the juice of M7 Navel influenced by different treatments (Tr) of MJ and time of application (Tm) applied at 6, 3 WBAH (weeks before anticipated harvest) as single spray and 6 followed by (fb) 3 WBAH as double spray in 2015 growing season.

Treatment (mM)	6 WBAH	3 WBAH	6 fb 3 WBAH	Mean
Citric acid (g L^{-1})				
Control	13.0±0.22	12.2±0.19	15.6±0.28	13.6
MJ (1.25)	13.7±0.08	12.6±0.19	12.2±0.20	12.8
MJ (2.5)	12.5±0.15	12.1±0.04	11.7±0.36	12.1
MJ (5.0)	14.2±0.39	13.0±0.42	12.4±0.25	13.2
Mean	13.4	12.5	13.0	
LSD ($P \leq 0.05$)	Tr = 0.5	Tm = 0.5	Tr xTm = 0.9	
Malic acid (g L^{-1})				
Control	4.8±0.12	4.5±0.17	4.9±0.21	4.7
MJ (1.25)	5.4±0.13	4.0±0.33	4.6±0.18	4.7
MJ (2.5)	4.6±0.15	3.7±0.0	4.1±0.16	4.1
MJ (5.0)	4.1±0.30	4.2±0.20	4.3±0.18	4.2
Mean	13.4	12.5	13.0	
LSD ($P \leq 0.05$)	Tr = 3.9	Tm = 3.4	Tr xTm = ns	
Tartaric acid (g L^{-1})				
Control	0.55±0.0	0.58±0.02	0.51±0.0	0.55
MJ (1.25)	0.57±0.01	0.62±0.02	0.54±0.01	0.58
MJ (2.5)	0.59±0.02	0.54±0.0	0.53±0.01	0.55
MJ (5.0)	0.53±0.01	0.54±0.01	0.51±0.0	0.52
Mean	13.4	12.5	13.0	
LSD ($P \leq 0.05$)	Tr = ns	Tm = ns	Tr xTm = ns	
Total organic acids (g L^{-1})				
Control	18.4±0.34	17.2±0.37	21.1±0.49	18.9
MJ (1.25)	19.7±0.19	17.3±0.50	17.4±0.37	18.1
MJ (2.5)	17.8±0.27	16.4±0.03	16.3±0.53	16.8
MJ (5.0)	19.0±0.68	17.7±0.62	17.3±0.41	18.0
Mean	13.4	12.5	13.0	
LSD ($P \leq 0.05$)	Tr = 0.8	Tm = 0.7	Tr xTm = 1.4	

Mean separation within the column (Mean Tr) was tested with LSD *(P<0.05)*; while mean separation within the row (Mean Tm) was tested with LSD *(P<0.05)*. Mean separation for the interaction effects (Tr x Tm) was tested with LSD *(P<0.05)* at the same level of Tm across Tr. ns = not significant, n = three replicates (20 fruit per replication).

However, the level of mean citric acid (13.4 - 13.0 g L^{-1}), malic (4.7- 4.5 g L^{-1}) and total organic acid (18.7 - 18.0 g L^{-1}) in the juice was significantly higher with the single spray application applied at 6 WBAH and double spray applied at 6 WBAH followed by 3 WBAH respectively, as compared to the single spray applied at 3 WBAH. The interaction between spray timings and different concentrations of MJ applied were found to be significant ($P \leq 0.05$) for levels of citric acid and total organic acid, whilst non-significant for malic and tartaric acid**.**

6.3.8. Vitamin C and total antioxidants

All MJ spray treatments regardless of the concentrations applied did not significantly ($P \leq 0.05$) affect mean vitamin C and total antioxidant levels in 2015 (Table 6.7). However, mean vitamin C level and total antioxidant in the juice were significantly higher (580.4 mg L^{-1} and 539.4 µM L^{-1} Trolox respectively) with the single spray applied at 3 WBAH, as compared to other spray timings. The interactions between different treatments of MJ and spray timings were found to be significant for levels of vitamin C but non-significant for total antioxidants. The single spray application of MJ (1.25 mM) at 6 WBAH exhibited a higher level of vitamin C (607.6 mg L^{-1}) in the juice as compared to all other treatments. During 2016, the single spray application of MJ (2.5 mM) showed a higher mean vitamin C level in the juice (577.7 mg L^{-1}) as compared to the control and other spray treatments (Table 6.8). In addition, all MJ treatments except MJ (2.5 mM) showed substantially higher levels of total antioxidants in the juice.

Table 6.7 Levels of vitamin C and total antioxidants in the juice of M7 Navel influenced by different treatments (Tr) of MJ and time of application (Tm) applied at 6, 3 WBAH (weeks before anticipated harvest) as single spray and 6 followed by (fb) 3 WBAH as double spray in 2015 growing season.

Treatment (MJ mM)	6 WBAH	3 WBAH	6 fb 3 WBAH	Mean
		Vitamin C (mg L^{-1})		
Control	541.1±4.8	564.8±5.6	570.9±8.1	558.9
MJ (1.25)	607.6±4.2	577.8±3.4	505.3±9.4	563.5
MJ (2.5)	547.6±10.0	586.0±5.6	560.1±11.6	564.5
MJ (5.0)	549.7±8.3	592.9±6.3	524.7±3.2	555.8
Mean	561.5	580.4	540.2	
LSD ($P \leq 0.05$)	Tr = ns	Tm = 25.0	Tr x Tm = 50.0	
		Total antioxidants (µM L^{-1} Trolox)		
Control	497.8±4.0	553.8±26.1	532.6±5.1	528.1
MJ (1.25)	450.4±8.2	493.4±18.7	496.4±13.9	480.0
MJ (2.5)	402.5±0.18	505.4±5.0	456.8±14.9	513.9
MJ (5.0)	452.6±11.1	604.8±15.6	503.2±12.9	520.2
Mean	450.8	539.4	497.2	
LSD ($P \leq 0.05$)	Tr = ns	Tm = 81.2	Tr x Tm = ns	

Mean separation within the column (Mean Tr) was tested with LSD *(P<0.05*); while mean separation within the row (Mean Tm) was tested with LSD *(P<0.05*). Mean separation for the interaction effects (Tr x Tm) was tested with LSD *(P<0.05*) at the same level of Tm across Tr. ns = not significant, n = three replicates (20 fruit per replication).

Table 6.8 Effect of spray application of different concentrations of MJ applied at 3 weeks before anticipated harvest on levels of vitamin C and total antioxidants in the juice of M7 Navel in 2016.

Treatment (MJ mM)	Vitamin C (mgL^{-1})	Antioxidants (µM L^{-1} Trolox)
Control	511.6±14.9	473.9±6.3
MJ (1.25)	504.5±14.3	590.6±8.3
MJ (2.5)	577.7±6.0	446.8±7.0
MJ (5.0)	496.4±9.1	590.7±6.1
MJ (7.5)	563.4±6.8	588.2±3.5
LSD ($P \leq 0.05$)	57.9	46.6

Mean separation within the column (Mean Tr) was tested with LSD *(P<0.05*). ns = not significant, n = four replicates (20 fruit per replication)

6.4. Discussion

The class of pigments such as carotenoids, anthocyanins, chlorophyll and flavonoids are responsible for the colour of the fruit. Sweet orange rind colour is the result of chlorophyll degradation and accumulation of carotenoids (Gross, 1987). Alquezar et al. (2008) reported that carotenoid pigments are responsible for the internal and external colouration of the orange fruit.

Pre-harvest spray application of MJ showed significantly reduced *h*° angle and enhanced CCI in two consecutive years 2015 and 2016 (Table 6.1). An improved fruit colour with a pre-harvest spray application of MJ attributed to enhanced levels of total carotenoids in the rind of M7 sweet orange fruit (Table 6.1). The mechanism by which MJ promotes rind colour through the accumulation of carotenoids and anthocyanin is still unclear. Possibly, MJ enhances the rind colour independently or through the upregulation of ethylene production in M7 Navel. The enhanced accumulation of carotenoids in the rind with the spray application of MJ may be attributed to the increased *b*-carotene in the rind of M7 Navel. Similarly, increased *b*-carotene in the peel of the apple (Perez et al., 1993) and tomato (Saniewski and Czapski., 1983) has been previously reported to enhance colour with the application of MJ. MJ stimulates chlorophyll degradation independently (Emery and Reid, 1996) or through enhanced ethylene production (Hung and Kao, 1996). Previously, the application of MJ decreased the content of chlorophyll in *Arabidopsis thaliana* plant (Jung, 2004). Furthermore, the chlorophyll a/b ratio was decreased with the application of MJ in Fuji apple fruit (Rudell and Mattheis, 2008). It has been reported by Jung et al. (2007) that MJ at a concentration of 100 μM or higher down-regulated genes involved in chlorophyll *a*/*b*-binding protein. Application of MJ degraded chlorophyll *a* more rapidly than chlorophyll *b* in barley (*Hordeum vulgare* L) as reported by (Cuello, 1997) and chlorophyll a/b ratio decreased more quickly in Golden Delicious apple peel with increased MJ exposure (Perez et al., 1993).

It has been well documented that ethylene enhanced chlorophyll degradation (Purvis and Barmore, 1981) as well as the synthesis of carotenoids (Steward and Wheaton, 1972). MJ vapour application to Golden Delicious apples have been shown to significantly accelerate ethylene production (2.5- and 4.6-fold in the cortical and peel tissues, respectively) (Olias et al., 1992). Moreover, it has been well acknowledged that exogenous application of MJ increases endogenous ethylene

production and colour development in non-climacteric fruit such as strawberry (Mukkun and Singh, 2009) and in climacteric fruit such as plum, mango and apple (Khan and Singh, 2007; Lalel et al., 2003a; Fan et al., 1997). Previously, pre-harvest applications of MJ showed enhanced red colour due to the accumulation of red pigments in Fuji apple fruit skin (Rudell et al., 2005; Rudell and Mattheis, 2008; Shafiq et., 2013).

In the present findings, MJ treatments showed reduced SSC (%) and TA (%); while SSC/TA ratio was unaffected with any of the MJ treatments (Table 6.3). Previously, pre-harvest MJ application showed increased SSC (%) and decreased TA (%) in Fortune and Friar (Ozturk et al., 2015), Amber Jewel, Angelino and Black Amber plums (Khan and Singh, 2007), Jewel (black blackberry) Autumn Bliss (red blackberry) (Wang and Zheng, 2005) and blackberry cultivars (Hull Thornless, Chester Thornless and Triple Crown (Wang et al., 2008) which is contrary to our results. Moreover, the effect of MJ on sugar content in the literature is debatable. All MJ treatments showed reduced level of total sugar in the juice as compared to control. However, MJ treatment 10 µL L^{-1} exhibited higher sugars in loquat fruit stored at 1 ºC for 35 d (Cao et al., 2009).

In the present study, pre-harvest spray application of MJ (1.25, 5.0 and 7.0 mM) exhibited enhanced antioxidant activity in the juice as compared to the control. Similarly, Khan and Singh (2007) reported increased antioxidant capacity in four different plum cultivars with MJ treatments. Furthermore, MJ treatments enhanced phenolic contents and subsequently the antioxidant capacity in various fruit (Karaman et al., 2013; Rudell et al., 2002; Wang and Zheng 2005; Cao et al., 2009). In conclusion, pre-harvest spray application of MJ (5.0 or 7.5 mM) applied at 3 WBAH promoted the colour from yellow toward deep orange exhibited by reduced *h*° angle and enhanced CCI and the total level of total carotenoids in the rind without any adverse effects on the fruit quality.

CHAPTER 7

Alleviation of chilling injury induced by cold quarantine treatment in Midknight Valencia and Lane Late sweet orange

Abstract

Cold quarantine treatment (1°C for 21 d) induces CI in sweet orange fruit. We investigated the effects of different treatments such as hot water dip (HWD, 50°C) alone or combined with TBZ five-min, different concentrations of SA, MJ one-min dip and fumigation of nitric oxide (NO) two-hour and ethylene (ET) (six-hour) on CI and fruit quality following cold quarantine treatment for 10-day at ambient temperature in Lane Late and Midknight Valencia fruit. HWD alone or combined with TBZ, or MJ significantly reduced CI in both cultivars. NO (5 μL L^{-1}) fumigation significantly reduced weight loss in Lane Late only as compared to all other treatments except SA (1, and 3 mM). SCC/TA ratio was significantly reduced with ethylene, HW alone or combined with TBZ or MJ (0.25 mM) as compared to all other treatments in Midknight Valencia, but not in Lane Late. The NO (10 μL L^{-1}) fumigation resulted in the significantly highest level of vitamin C only in Midknight Valencia. SA (3 mM) dip treatment resulted in the significantly highest levels of total antioxidants as compared to all other treatments in Lane Late but not in Midknight Valencia. In conclusion, HWD alone or in combination with TBZ (20 mg L^{-1}) or MJ (0.50 mM) effectively reduced CI caused by cold quarantine treatment without adversely affecting fruit quality.

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7.1. Introduction

Citrus is one of the most important fruit crops in Australia with a total production of 470 MT in 2016-17 (USDA, 2017). Australia exports high quality fresh sweet oranges fruit (180 MT) during the off-season to markets of the Northern Hemisphere (USDA, 2017). In Australia, cold quarantine treatment against Mediterranean fruit fly [MFF; *Ceratitis capitata* (*Diptera*: *Tephritidae*)] and Queensland fruit fly [QFF; *Bactrocera tryoni* (Froggatt)] in the Western and Eastern regions respectively, mandatory to comply with quarantine requirements of the importing countries (De Lima et al., 2007). Previously, fumigation with methyl bromide, irradiation and cold treatment were practised to disinfect the citrus fruit and currently the use of methyl bromide has been restricted in Australia because it shortens the storage life of citrus fruit due to its phytotoxic nature and also affects ozone depletion (De Lima et al., 2007). Consumer demands, environmental factors and government regulations have led to research on adopting non-chemical methods for the postharvest protection of horticulture commodities (Sharples, 1990). Cold quarantine treatment is a non-chemical, approved method for disinfestation of QFL and MFF. This involves the exposure of the sweet orange fruit to non-freezing temperatures (1.1 - 2.2°C) for a period of 14-18 d (Powell, 2003). However, the application of this treatment leads to the development of CI more or less in all citrus cultivars when kept to room temperature (Martinez-Téllez and Lafuente, 1997). The CI symptoms mainly manifest as scalding, rind pitting, watery breakdown, development of a woolly or leathery texture and decay (Reuther et al., 1989). CI negatively impacts the consumer preference and overall quality of the fruit. The sensitivity of the citrus fruit to low temperature causes serious economic implications for its export to the different fruit fly free zones of importing countries (US Department of Agriculture Animal and Plant Protection Service, 1976).

Various approaches have been tested to minimise CI in different fruit crops such as postharvest hot water dipping (HWD), application of MJ or NO fumigation. Previously, HWD alone or in combination with TBZ reduced CI in citrus fruit induced by cold quarantine in different cultivars of sweet oranges such as Washington Navel and Valencia Late (Bassal and El-Hamahmy, 2011), Tarocco, Moro, Sanguinello and Doppio Sanguigno (Schirra et al., 2004), and Tarocco (Palma et al., 2013).

Methyl jasmonate has been previously reported to reduce CI in different fruit crops such as lemon (*Citrus limon* Burm.) (Siboza et al., 2014), mangoes (*Mangifera indica* cv. Kent) (Gonzalez-Aguilar et al., 2000a), guava (*Psidium guajava*) (Gonzalez-Aguilar et al., 2004), peach (*Prunus persica* Batsch. cv. Baifeng) (Meng et al., 2009), loquat (Cao et al., 2009), pomegranate (Mirdehghan and Ghotbi, 2014), pineapple (Nilprapruck et al., 2008), bell pepper, avocado, and grapefruit (Meir et al., 1996). No research work has been reported on the efficacy of exogenously applied MJ in reducing CI induced by cold quarantine treatment in sweet orange fruit. The application of SA alone and combined application of MJ and SA enhanced chilling tolerance in cold-stored pomegranate (Mirdehghan and Ghotbi, 2014), lemon fruit (Siboza and Bertling, 2013) and tomato (Ding et al., 2002).

Nitric oxide is a free radical and highly reactive gas, acting as a multifunctional signalling molecule in various physiological responses (Wendehenne et al., 2001). NO has been reported to reduce CI and maintain fruit quality in climacteric fruits such as Japanese plum cv Amber Jewel (Singh et al., 2009), banana (*Musa* spp., AAA group cv. Brazil) (Wang et al., 2013), peach cv. Feicheng) (Zhu et al., 2010) and mango cv. Kensington Pride) (Zaharah and Singh, 2011). Recently, Ghorbani et al. (2017) reported that 0.5mM SNP (sodium nitroprusside) 5 min dip treatment reduce CI in Washington Navel orange stored for five months at 3°C. However, the efficacy of NO in reducing CI induced by cold quarantine treatment in sweet oranges yet to be investigated.

No research work has been reported on the effects of exogenous application of MJ, NO and SA on inducing chilling tolerance during cold quarantine treatment (1 °C for 21d) in Lane Late and Midknight Valencia sweet orange fruit. It was hypothesised that exogenous postharvest application of MJ, NO and SA may induce chilling tolerance in sweet orange fruit when exposed to cold quarantine at 1°C for 21d. These observations prompted to investigate the effects of exogenous application of MJ, SA, NO, TBZ, and HW on the incidence of CI induced by cold quarantine treatment (1°C for 21 d) and fruit quality in sweet orange.

7.2. Materials and methods

7.2.1. Plant material

Mature fruit of Lane Late and Midknight Valencia (*Citrus sinensis* (L.) Osbeck) were harvested randomly around the tree canopy from a commercial orchard at Moora (latitude 30° 41, South, longitude 115° 42, East), WA during 2015. Lane Late and Midknight Valencia sweet orange trees (seven and nine years old respectively) earlier grafted on Carrizo citrange (*Citrus sinensis* (L.) Osbeck × *Poncirus trifoliata* Raf.) rootstock. The trees were spaced at 2.7 x 7.5 m, row direction north-south. Following the harvest, the fruit were brought to the Horticulture Research Laboratory, Curtin University, Perth, WA. The fruit used in this experiment were free from symptoms of diseases, pest damage, blemishes and physical injuries.

7.2.2. Experiment 1: Effects of HW, MJ, TBZ and NO treatments on CI incidence and fruit quality in Lane Late sweet orange

Lane Late sweet orange fruit were dipped in HW (50°C for 5 min) alone and combined with TBZ (20 mg L^{-1}), MJ (0.10, 0.25 or 0.50 mM) and SA (1, 2 or 3 mM) one min dip and untreated fruit were kept as control. Fruit were fumigated with different concentrations of NO (5, 10 or 20 µL L^{-1}) for 2 h in a 60 L container. Following the treatments, the fruit were dried for 6 h at room temperature (20 ± 1 °C) and RH (60 ± 5%). The fruit were transferred to the cold storage (1°C for 21 d) with RH (85-90 %). The experiment was laid out by following completely randomised design and included three replications. Each replication included 30 fruit. The observations recorded were incidence of CI (%), colour coordinates [(*h*° and CCI], percentage weight loss, fruit firmness, SSC, TA, SSC: TA, vitamin C and total antioxidants, individual and total sugars and organic acids, were determined from the fruit stored at 1ºC for 21 d and followed by 10 d in simulated shelf conditions (21 ± 1°C). However, the fruit percentage weight loss was recorded only at 22 d after cold storage.

7.2.3. Experiment 2: Effects of HW, MJ, TBZ, ET and NO treatments on CI incidence and fruit quality in Midknight Valencia sweet orange

In this experiment, the Midknight Valencia sweet orange fruit were treated with HW (50±1°C for 5 min) dip alone and combined with TBZ (20 mg L^{-1}), MJ (0.10, 0.25 or 0.50 mM) one min dip as well as fruit fumigated with different concentrations of NO (5, 10 or 20 µL L^{-1}) for 2 h and ethylene (ET) (10 µL L^{-1}) for 6 h in 60 L containers. The fruit were dried for 6 h at room temperature (20 ± 1 °C) and RH (60 ± 5%) and then transferred to the cold storage (1°C for 21 d) with RH (85-90 %). The experiment was designed by following completely randomised with one factor including treatments with three replications. Each replication included 30 fruit. All the observations mentioned in Experiment 1 were also recorded in this experiment.

7.2.4. CI incidence (%)

All the fruit were visually examined for the symptoms of CI following 90 d cold storage and 10 d simulated shelf conditions (21 ± 1°C) and detailed in Chapter 3, Section 3.6.

7.2.5. Determination of the fruit colour

Citrus rind colour was recorded by using colour flex EZ (45°/0° design) spectrocolorimeter (Hunter Lab, Hunter Associates Laboratory Inc., Reston, VA, USA) on three positions around the equatorial plane of the fruit. Ten fruit were randomly selected from each replication as detailed in Chapter 3, Section 3.4.

7.2.6. Determination of loss of fruit weight

The weight of the fruit at the commencement of storage (initial fruit weight) and following the 22 d cold storage (final fruit weight) was recorded by using a digital weighing balance and previously described in detailed Chapter 3, Section 3.7.

7.2.7. Fruit firmness

Fruit firmness was determined using a texture profile analyser (TPA Plus, AMETEK Lloyd Instruments Ltd, Fareham, UK) interfaced with Nexygen® 4.6 software previously detailed in Chapter 3, Section 3.8. Fruit firmness was expressed in newtons (N).

7.2.8. Soluble solids concentration (SSC) and Titratable acidity (TA)

A digital refractometer (Atago-Palette PR 101, Atago CO. Ltd, Itabashi-Ku, and Tokyo, Japan) was used to estimate SSC in the fresh juice of Midknight Valencia and Lane Late. SSC was expressed as a percentage. The juice was titrated against 0.1N NaOH to a pink colour end point. Phenolphthalein (2-3 drops) was used as an indicator. TA was calculated as percentage equivalent of citric acid as described previously in Chapter 3, Section 3.9, 3.10 and 3.11.

7.2.9. Determination of sugars and organic acids

The levels of individual sugars and organic acids in the juice of Midknight Valencia (ten fruit) and Lane Late (ten fruit) were determined by following the method including conditions of analysis reported (Hussain, 2014) by using reverse-phase high-performance liquid chromatography system (RP-HPLC; Waters, Milford, MA, USA) fitted with refractive index detector (sugars) and dual wavelength UV detector (organic acids) as outlined in Chapter 3, Section 3.12. All the individual sugars and organic acids were expressed as (g L^{-1}).

7.2.10. Determination of vitamin C and total antioxidants

Ten randomly selected fruit from each cultivar were used to extract the juice, which was used for the determination of vitamin C and total antioxidants by using the method reported earlier by Hussain, (2014) and Brand-Williams et al. (1995) respectively, using a UV/VIS spectrometer (Jenway spectrophotometer Model 6405, Dunmow, Essex, UK). The standard curve of L-ascorbic acid was used to calculate vitamin C concentration in the juice and expressed as (mg L^{-1}) of fresh juice. However, a standard curve of 6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid (Trolox) was used to calculate total antioxidant and expressed as µM Trolox equivalent antioxidant activity (TEAC) (L^{-1}) FJ basis. The detailed method has been previously defined in Chapter 3, Section 3.13 and 3.14.

7.2.11. Statistical analysis

The experimental data were subjected to one-way analysis of variance (ANOVA) using GenStat 14th edition (release 14.1; Lawes Agricultural Trust, Rothamsted Experimental Station, Rothamsted, UK). The influences of treatments on different variables were evaluated by ANOVA. The least significant differences

(LSD) were gauged following the Duncan multiple range test method at probability ($P \leq 0.05$).

7.3. Results

7.3.1. CI percentage

Hot water dip (HWD) at 50±1°C alone and combined with TBZ (20 mg L^{-1}) five min dip and MJ (0.50 mM) one min dip have significantly ($P \leq 0.05$) reduced the incidence of CI (8.9, 8.9 and 15.6 % respectively) as compared to the control (40.5 %) in Lane Late sweet orange (Fig. 7.1). In Midknight Valencia, HWD at 50±1°C alone or combined with TBZ for 5 min dip and MJ (0.50 mM) significantly reduced CI (8.8 to 16.6 %) as compared to the control (28.8 %) (Fig 7.2). Fruit dipped in MJ (0.50 mM) for one min exhibited the lowest percentage of CI incidence as compared to all other treatments (Fig 7.2).

7.3.2. Fruit Colour

Citrus colour index (CCI) was significantly affected by all treatments in Lane Late sweet orange but not in Midknight Valencia. In Lane Late, MJ (0.10 mM) one-min dip treatment resulted in significantly reduced *h*° (60.7) and increased CCI (8.7) as compared to the control and all other treatments (Table 7.1). In Midknight Valencia, TBZ (20 mg L^{-1}) combined with HW five min dip treatment showed significantly reduced *h*° (61.2) as compared to the control and all other treatments (Table 7.1).

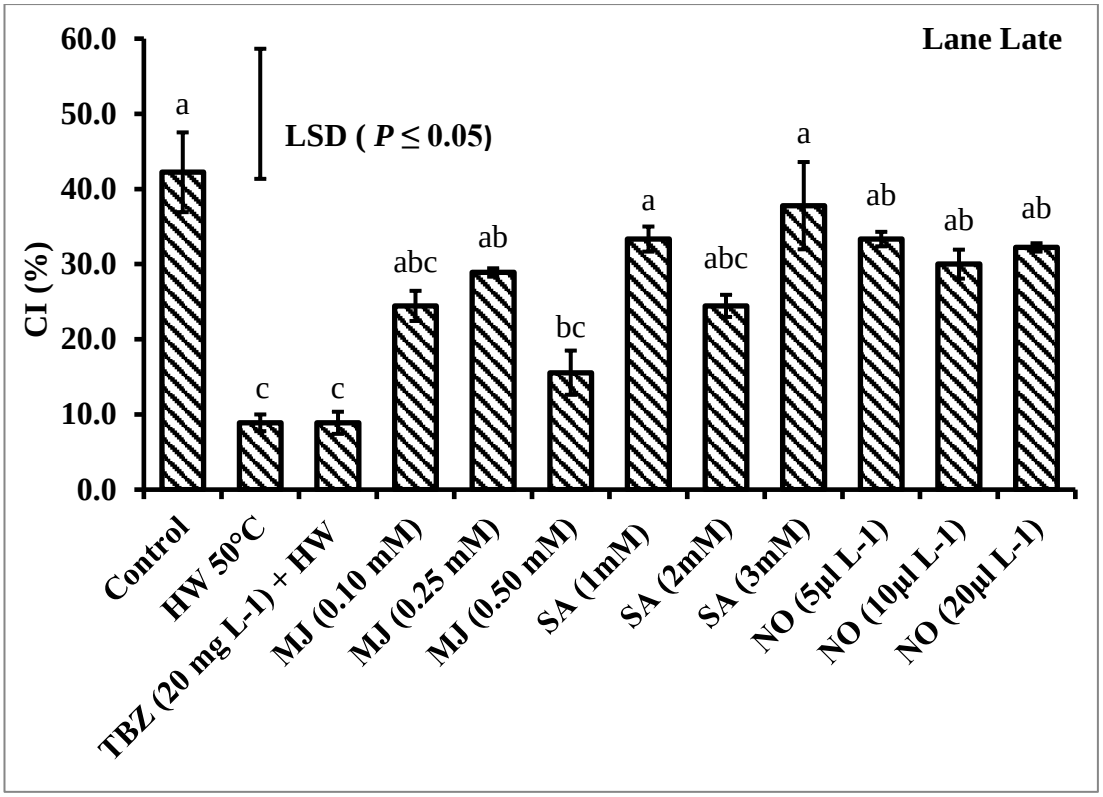

Fig 7.1 Incidence of CI (percentage chill injured fruit) affected by hot water alone and combined with TBZ and different concentrations of MJ, SA dips and fumigation of NO in sweet orange Lane Late following the cold quarantine treatment (1°C for 21 d) and 10 d at simulated shelf-life conditions. Vertical bars represent SE, n = three replicates, thirty fruit per replication. Any two means with different lower-case letters represent significant differences at ($P \leq 0.05$). HWD, TBZ combined with HW, MJ one min dip, SA one min dip and NO fumigation for 2 h.

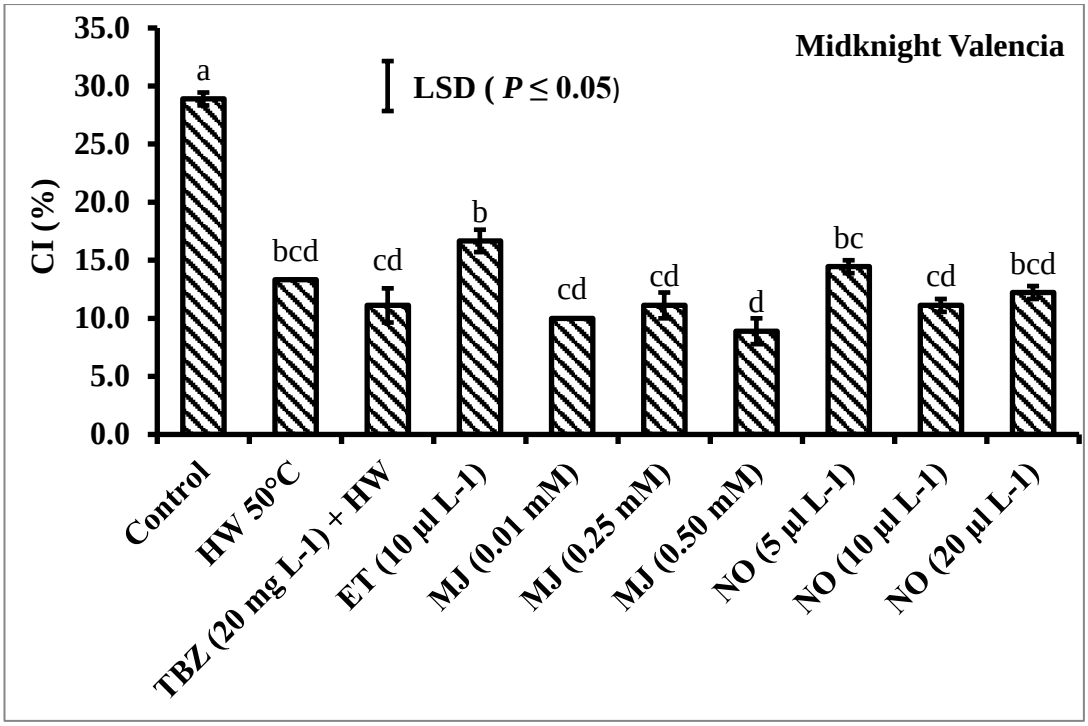

Fig 7.2 Incidence of CI (percentage chill injured fruit) affected by hot water alone and combined with TBZ and different concentrations of MJ, SA dips and fumigation of NO in sweet orange Midknight Valencia following the cold quarantine treatment (1°C for 21 d) and 10 d at simulated shelf-life conditions. Vertical bars represent SE, n = three replicates, thirty fruit per replication. Any two means with different lower-case letters represent significant differences at ($P \leq 0.05$). HWD 5-min dip, TBZ combined with HW 5 min dip, ET 6 h fumigation, MJ one min dip, SA one min dip and NO fumigation for 2 h.

Table 7.1 Effects of HWD alone and combined with TBZ, different concentrations of MJ, SA dips and NO fumigation on *h*° and CCI in sweet orange Lane Late and Midknight Valencia following the cold quarantine treatment (1°C for 21 d) and 10 d at simulated shelf-life conditions.

Fruit colour		
Lane Late		
Treatments	*h*°	CCI
Control	62.0a	8.1b
HWD 50°C	62.1a	8.0b
TBZ (20 mg L^{-1}) + HW	62.1a	8.1b
MJ (0.10 mM)	60.7b	8.7a
MJ (0.25 mM)	61.8a	8.2b
MJ (0.50 mM)	61.8a	8.2b
SA (1 mM)	62.2a	8.0b
SA (2 mM)	62.5a	7.9b
SA (3 mM)	62.4a	7.9b
NO (5 µL L^{-1})	62.6a	7.9b
NO (10 µL L^{-1})	62.5a	7.9b
NO (20 µL L^{-1})	62.5a	7.8b
Midknight Valencia		
Control	62.2ab	8.0
ET (10 µL L^{-1})	61.7abc	8.3
HWD 50°C	62.2ab	8.0
TBZ (20 mgL^{-1}) + HW	61.2c	8.4
MJ (0.10 mM)	61.6abc	8.3
MJ (0.25 mM)	62.4a	8.0
MJ (0.50 mM)	62.3a	8.1
NO (5 µL L^{-1})	62.0abc	8.1
NO (10 µL L^{-1})	62.1ab	8.2
NO (20 µL L^{-1})	61.4bc	8.5

Data represent means of 3 replicate samples of 90 units for Lane Late and Midknight Valencia. Mean separation for significant analysis of variance within the columns and rows was tested using Duncan's multiple range test at ($P < 0.05$). Mean followed by the same letter was not significantly different within the columns. HWD 5-min, TBZ combined with HW 5 min dip, ET 6 h fumigation, MJ one min dip, SA one min dip and NO fumigation for 2 h.

7.3.3. Weight loss (%) and fruit firmness (N)

All the treatments significantly ($P \leq 0.05$) affected percentage weight loss in Lane Late sweet orange fruit but not in Midknight Valencia. In Lane Late, NO fumigation (5 µL L^{-1}) for 2 h significantly reduced weight loss (2.9 %) as compared to the control (6.0 %) and all other treatments (Table 7.2). Fruit firmness (N) was not significantly affected by any of the treatments in both the cultivars (Table 7.2).

Table 7.2 Effect of HWD alone and combined with TBZ, different concentration of MJ, SA dips and fumigation of NO on weight loss (%) and fruit firmness in sweet orange Lane Late and Midknight Valencia following the cold quarantine treatment (1°C for 21 d) and 10 d at simulated shelf-life conditions.

Lane Late		
Treatment	Weight loss (%)	Firmness (N)
Control	6.0abc	262.2
HWD 50°C	7.6ab	302.4
TBZ (20 mgL^{-1}) + HW	7.1abc	286.7
MJ (0.10 mM)	6.3abc	291.8
MJ (0.25 mM)	7.6ab	274.1
MJ (0.50 mM)	4.3abc	269.0
SA (1 mM)	4.9bcd	266.5
SA (2 mM)	7.9ab	272.0
SA (3 mM)	5.1bcd	284.1
NO (5 µL L^{-1})	2.9d	277.4
NO (10 µL L^{-1})	8.3a	253.1
NO (20 µL L^{-1})	9.0a	267.9
Midknight Valencia		
Control	1.8	371.2
ET (10 µL L^{-1})	1.3	358.8
HWD 50°C	1.4	414.4
TBZ (20 mgL^{-1}) + HW	1.5	380.9
MJ (0.10 mM)	1.4	405.4
MJ (0.25 mM)	1.5	393.1
MJ (0.50 mM)	1.6	394.5
NO (5 µL L^{-1})	1.4	368.8
NO (10 µL L^{-1})	1.4	355.9
NO (20 µL L^{-1})	1.3	336.3

Data represent means of 3 replicate samples of 90 units for Lane Late and Midknight Valencia. Mean separation for significant analysis of variance within the columns and rows was tested using Duncan's multiple range test at ($P < 0.05$). Mean followed by the same letter was not significantly different within the columns. HWD 5-min, TBZ combined with HW 5 min dip, ET 6 h fumigation, MJ one min dip, SA one min dip and NO fumigation for 2 h.

7.3.4. SSC, TA and SCC/TA ratio

All the treatments except MJ (0.50 mM) one min dip resulted in significantly ($P \leq 0.05$) reduced SSC (%) in the juice of Lane Late sweet orange as compared to the control. However, in Midknight Valencia, SSC (%) was reduced as compared to the control and all the treatments applied except ethylene (10 µL L^{-1}) six h fumigation, TBZ combined with HW five min dip and NO (10 and 20 µL L^{-1}) two h fumigation (Table 7.3). In Lane Late, TA (%) and SSC/TA ratio were not significantly affected by any of the treatments applied (Table 7.3). Moreover, TA in Midknight Valencia juice was significantly highest (0.91 and 0.92 %) when the fruit were fumigated with ethylene (10 µL L^{-1}) for six h and MJ (0.25 mM) one min dip treatment, respectively. SSC/TA ratio in the juice of Midknight Valencia was significantly higher in all the treatments except ethylene (10 µL L^{-1}) six h fumigation, HWD alone five min dip, TBZ combined with HW five min dip and MJ (0.25 mM) one min dip.

7.3.5. Vitamin C and total antioxidants

The concentrations of vitamin C in the juice were significantly ($P \leq 0.05$) affected by all treatments in Midknight Valencia but not in Lane Late. In Midknight Valencia, all the treatments except ethylene (10 µL L^{-1}) six h fumigation, MJ (0.50 mM) one min dip and NO (5 and 10 µL L^{-1}) 2 h fumigation significantly reduced the concentrations of vitamin C in the juice as compared to the control (295.9 mg L^{-1}) (Table 7.3). Meanwhile, all the treatments significantly affected the level of total antioxidants in Lane Late sweet orange fruit but not in Midknight Valencia. Lane Late fruit treated with SA (3 mM) one min dip resulted in the significantly highest level of antioxidants (569.5 µM L^{-1} Trolox) as compared to the control and all other treatments (Table 7.3).

Table 7.3 Effect of HWD alone and combined with TBZ, different concentrations of MJ, SA dips and fumigation of NO on weight loss (%) and fruit firmness in sweet orange Lane Late and Midknight Valencia following the cold quarantine treatment (1°C for 21 d) and 10 d at simulated shelf-life conditions.

Lane Late					
Treatment	SSC (%)	TA (%)	SSC/TA ratio	Vitamin C (mg L^{-1})	Antioxidants (µM L^{-1} Trolox)
Control	12.1a	0.61	19.7	253.2	460.3cd
HWD 50°C	11.1b	0.61	18.2	261.4	476.0bc
TBZ (20 mgL^{-1}) + HW	11.4b	0.59	19.3	253.6	501.7b
MJ (0.10 mM)	11.4b	0.63	18.2	244.1	442.6d
MJ (0.25 mM)	11.0b	0.56	19.5	255.4	478.6bc
MJ (0.50 mM)	11.6ab	0.55	21.0	231.2	436.9d
SA (1 mM)	11.2b	0.60	18.7	225.1	439.5d
SA (2 mM)	10.4c	0.58	18.0	261.8	478.8bc
SA (3 mM)	9.9c	0.55	18.1	270.9	569.5a
NO (5 µL L^{-1})	11.3b	0.59	19.2	237.2	453.7cd
NO (10 µL L^{-1})	11.3b	0.56	20.1	225.6	406.7e
NO (20 µL L^{-1})	11.3b	0.58	19.6	240.7	463.4cd
Midknight Valencia					
Control	13.1a	0.78c	16.8a	295.9abc	482.3
ET (10 µL L^{-1})	13.0ab	0.91a	14.3d	324.0ab	490.0
HWD 50°C	12.7bcd	0.81bc	15.7bc	244.6cd	492.5
TBZ (20 mgL^{-1}) + HW	12.8abcd	0.83b	15.4c	215.2d	507.9
MJ (0.10 mM)	12.5cd	0.77c	16.3ab	247.6bcd	474.6
MJ (0.25 mM)	12.7bcd	0.92a	13.8d	222.6cd	454.9
MJ (0.50 mM)	12.5d	0.78c	16.0abc	297.2abc	466.0
NO (5 µL L^{-1})	12.6cd	0.78c	16.1abc	295.5abc	484.0
NO (10 µL L^{-1})	12.9abc	0.78c	16.5ab	359.4a	485.7
NO (20 µL L^{-1})	12.8abcd	0.78c	16.4ab	213.5d	465.7

Data represent means of 3 replicate samples of 90 units for Lane Late and Midknight Valencia. Mean separation for significant analysis of variance within the columns and rows was tested using Duncan's multiple range test at ($P < 0.05$). Mean followed by the same letter was not significantly different within the columns. HWD 5-min dip, TBZ combined with HW 5 min dip, ET 6 h fumigation, MJ one min dip, SA one min dip and NO fumigation for 2 h.

7.3.6. Individual and total sugars

All the treatments significantly affected the level of glucose in the juice of Lane Late and Midknight Valencia. In Lane Late, all the NO treatments, SA (3 mM) and MJ (0.10 and 0.50 mM) one min dip, showed a significantly ($P \leq 0.05$) reduced concentration of glucose in juice as compared to the control (19.3 g L^{-1}) and all other treatments (Table 7.4). Moreover, Midknight Valencia fruit fumigated with NO (20 µL L^{-1}) for 2 h and MJ (0.1 or 0.50 mM) one min dip exhibited a reduced level of glucose in the juice as compared to the control and all other treatments. In Lane Late, all the treatments except TBZ (20 mg L^{-1}) combined with HW five min dip show significantly reduced concentrations of fructose in the juice as compared to the control (25.4 g L^{-1}). On the other hand, in Midknight Valencia HWD alone for five min, MJ (0.1 and 0.50 mM) one min dip, and NO (20 µL L^{-1}) two h fumigation resulted in significantly reduced levels of fructose in the juice as compared to the control (26.0 g L^{-1}) and all other treatments (Table 7.4). The concentrations of sucrose in the juice of Lane Late were significantly reduced by all treatments except MJ (0.50 mM) one min dip and NO (20 µL L^{-1}) two h fumigation as compared to the control (59.8 g L^{-1}). In Midknight Valencia, all the treatments except ethylene (10 µL L^{-1}) six h fumigation and NO (10 µL L^{-1}) 2 h fumigation exhibited reduced concentrations of sucrose in the juice as compared to the control (53.4 g L^{-1}). The concentrations of total sugars in the juice of Lane Late were significantly reduced by all the treatments as compared to the control (104.5 g L^{-1}). Meanwhile, in Midknight Valencia, the concentrations of total sugars in the juice were significantly reduced with all the treatments except ethylene (10 µL L^{-1}) six h fumigation and NO (10 µL L^{-1}) 2 h fumigation as compared to the control (99.3 g L^{-1}).

Table 7.4 Effect of HWD alone and combined with TBZ, different concentrations of MJ, SA dips and fumigation of NO on the levels of individual and total sugars in sweet orange Lane Late and Midknight Valencia following the cold quarantine treatment (1°C for 21 d) and 10 d at simulated shelf-life conditions.

Lane Late				
	Individual and total sugars (gL^{-1})			
Treatment	Glucose	Fructose	Sucrose	Total Sugars
Control	19.3a	25.4a	59.8a	104.5a
HWD 50°C	17.9abcde	22.8bc	50.8bc	91.7bc
TBZ (20 mgL^{-1}) + HW	19.2ab	24.5ab	52.4bc	96.3b
MJ (0.10 mM)	17.7cde	22.7bc	51.6bc	92.1bc
MJ (0.25 mM)	18.4abcd	23.2bc	48.9cd	90.7bc
MJ (0.50 mM)	17.7cde	23.2bc	55.1ab	96.1b
SA (1 mM)	18.7abc	23.5bc	51.4bc	93.6b
SA (2 mM)	17.9abcde	22.5c	45.4d	85.5c
SA (3 mM)	17.9bcde	22.5c	44.9d	85.3c
NO (5 µL L^{-1})	17.5cde	22.7bc	51.8bc	92.1bc
NO (10 µL L^{-1})	17.0e	22.3c	51.0bc	90.4bc
NO (20 µL L^{-1})	17.3de	22.6c	54.6abc	94.6b
Midknight Valencia				
Control	19.9ab	26.0a	53.4a	99.3a
ET (10 µL L^{-1})	20.1a	26.1a	53.4a	99.7a
HWD 50°C	18.9bc	24.6bc	50.8c	94.4bcd
TBZ (20 mgL^{-1}) + HW	19.2abc	25.1ab	52.1b	96.5b
MJ (0.10 mM)	17.9d	23.8c	52.1b	93.9cd
MJ (0.25 mM)	19.2abc	25.1ab	51.7bc	96.0bc
MJ (0.50 mM)	18.4cd	24.3bc	51.1bc	93.8d
NO (5 µL L^{-1})	19.3abc	25.2ab	50.9c	95.4bcd
NO (10 µL L^{-1})	19.7ab	26.0a	54.2a	100.0a
NO (20 µL L^{-1})	18.5cd	24.4bc	51.3bc	94.1cd

Data represent means of 3 replicate samples of 90 units for Lane Late and Midknight Valencia. Mean separation for significant analysis of variance within the columns and rows was tested using Duncan's multiple range test at ($P < 0.05$). Mean followed by the same letter was not significantly different within the columns. HWD 5-min dip, TBZ combined with HW 5 min dip, ET 6 h fumigation, MJ one min dip, SA one min dip and NO fumigation for 2 h.

7.3.7. Individual and total organic acids

Amongst various organic acids, citric, malic, tartaric, fumaric and succinic acid were identified and quantified in the juice of both cultivars (Table 7.5). The concentrations of all individual and total organic acids in the juice of Lane Late and Midknight Valencia sweet orange fruit (except citric acid in Lane Late) were not significantly affected by any of the treatments applied (Table 7.5). The concentration of citric acid in the juice of Lane Late was significantly reduced when the fruit were

treated with MJ (0.1, 0.25 or 0.50 mM) one min dip and NO (5 and 10 µL L^{-1}) two h fumigation as compared to the control and all other treatments.

Table 7.5 Effect of HWD alone and combined with TBZ, different concentrations of MJ, SA dips and fumigation of NO on the levels of individual and total organic acids in sweet orange Lane Late and Midknight Valencia following the cold quarantine treatment (1°C for 21 d) and 10 d at simulated shelf-life conditions.

	Lane Late					
	Individual and total organic acids (g L^{-1})					
Treatment	Citric acid	Malic acid	Tartaric acid	Fumaric acid	Succinic acid	Total organic acids
Control	2.1abc	0.68	0.63	0.41	0.76	4.6
HWD 50°C	2.3a	0.59	0.63	0.39	0.79	4.7
TBZ (20 mgL^{-1}) + HW	2.0abc	0.53	0.64	0.39	0.83	4.4
MJ (0.10 mM)	1.7bc	0.61	0.63	0.39	0.69	4.1
MJ (0.25 mM)	1.6c	0.49	0.63	0.37	0.90	4.0
MJ (0.50 mM)	1.7bc	0.71	0.62	0.42	0.77	4.2
SA (1 mM)	2.2ab	0.61	0.63	0.39	0.72	4.6
SA (2 mM)	1.8abc	0.49	0.62	0.36	0.82	4.6
SA (3 mM)	2.1abc	0.48	0.63	0.36	0.80	4.1
NO (5 µL L^{-1})	1.7bc	0.57	0.63	0.37	0.79	4.1
NO (10 µL L^{-1})	1.7bc	0.53	0.62	0.38	0.73	4.0
NO (20 µL L^{-1})	1.8abc	0.51	0.63	0.37	0.75	4.1
	Midknight Valencia					
Control	1.8	0.51	0.63	0.36	0.64	4.0
ET (10 µL L^{-1})	2.7	0.76	0.63	0.43	0.67	5.2
HWD 50°C	2.1	0.58	0.63	0.39	0.61	4.3
TBZ (20 mgL^{-1}) + HW	2.7	0.75	0.63	0.43	0.63	5.1
MJ (0.10 mM)	2.1	0.76	0.63	0.42	0.66	4.5
MJ (0.25 mM)	2.2	0.82	0.62	0.39	0.66	4.3
MJ (0.50 mM)	2.0	0.60	0.62	0.44	0.64	4.7
NO (5 µL L^{-1})	2.3	0.64	0.63	0.40	0.70	4.7
NO (10 µL L^{-1})	2.3	0.80	0.63	0.43	0.67	4.8
NO (20 µL L^{-1})	2.1	0.52	0.64	0.37	0.66	4.3

Data represent means of 3 replicate samples of 90 units for Lane Late and Midknight Valencia. Mean separation for significant analysis of variance within the columns and rows was tested using Duncan's multiple range test at ($P < 0.05$). Mean followed by the same letter was not significantly different within the columns. HWD 5-min dip, TBZ combined with HW 5 min dip, ET 6 h fumigation, MJ one min dip, SA one min dip and NO fumigation for 2 h.

7.4. Discussion

Postharvest disinfection of citrus fruit by employing quarantine treatments is mandatory to meet the requirements outlined by importing countries. During the past few decades, HT has been used to control postharvest fungal disease and insect disinfestation (Barkai-Golan and Phillips 1991). The HT can also be commercially used to enhance chilling tolerance during cold storage (Wang, 1993). The symptoms of CI on citrus fruit is expressed as rind staining, pitting, red blotches, scalding, watery breakdown, sunken tissues, damage to the styler end of lemons and necrosis on the rind (Reuther et al., 1989).

The experimental results indicate that HWD at 50±1 °C for 5 min is effective in reducing CI caused by cold quarantine treatment (1°C for 21 d) in sweet orange cv. Lane Late and Midknight Valencia. Possibly, HWD treatment might enhance the natural defence system of the fruit against CI by changing the arrangement, morphology and assembly of epicuticular wax, known to play a role in CI development (McDonald et al., 1993a). Secondly, HT may alter the enzyme system responsible for tissue degradation during the development of CI (Parkin et al., 1989; Martinez-Tellez and Lafunte, 1997). Thirdly, HWD treatment probably enhanced chilling tolerance by the upregulation of POX, CAT and total phenolic content (TP) as reported earlier in Valencia and Navel oranges (Bassal and El- Hamahmy 2011). Previously, HWD (2-3 min) has been reported to enhance the chilling tolerance in Valencia sweet orange fruit (Wild and Hood, 1989) and HWD (53ºC for 6 min and 48ºC for 12 min) also reduced CI incidence in Satsuma mandarins (Ghasemnezhad et al., 2008).

The experimental results exhibit that TBZ (20 mg L^{-1}) combined with hot water (HW) (50°C for 5 min) dip treatment significantly reduced CI in Lane Late and Midknight Valencia sweet orange. Presently, the exact mode of action of TBZ in enhancing chilling tolerance in sweet orange fruit is unclear. However, it may be argued that TBZ may have induced chilling tolerance by acting indirectly to suppress latent infections that might develop due to low-temperature storage as reported earlier in grapefruit, as result weaken fruit resistance to CI (Schiffmann-Nadel et al., 1972). Regarding the efficacy of TBZ in controlling CI, it has been reported that due to increased fungicide concentrations and deposits in fruit; its physiological effect has been attributed to a decreased rate of peel senescence (Schiffmann-Nadel et al., 1972). It has also been speculated that the HW fungicide treatment enriched infiltration of the

fungicide through the epicuticular wax (Hordijk et al., 2013). The effectiveness of TBZ with HW to reduce CI in various citrus fruits has been reported in Marsh and Redblush grapefruit (*Citrus paradisi* Macf.) (McDonald et al., 1991), Tarocco oranges (Schirra and Mulas, 1995) and Valencia oranges (Wild and Hood, 1989). The synergistic effect of TBZ with HW on reducing CI in fruits has also been previously reported by Hordijk et al. (2013).

Methyl jasmonate (0.5 mM) one-min dip treatment reduced CI in both cultivars. However, MJ (0.1 or 0.25) treatments induced chilling tolerance only in Midknight Valencia. Possibly, MJ has reduced CI by the activation of defence mechanisms, such as heat shock proteins (HSPs) and phenolic compounds (Meir et al., 1996; Meng et al., 2009). MJ has also been reported to stimulate the build-up of HSPs which reduced CI in tomato fruit (Ding et al., 2002). Meir et al. (1996) suggested that MJ probably acts together with single transduction cascade of the chemical changes involved in the reduction of CI. Previously, MJ has been applied to control CI in many fruits such as guava, tomato, papaya, mango and pomegranate (González-Aguilar et al., 2004; Ding et al., 2002; Mirdehghan and Ghotbi, 2014).

All NO (5, 10 or 20 µL L^{-1}) fumigation treatments for two h showed significantly reduced CI only in Midknight Valencia, not Lane Late fruit. The mode of action through which NO induces chilling tolerance in citrus fruit is yet to be explored. Possibly, NO fumigation may have protected the membrane from damage through the reduction of ROS and enhanced levels of antioxidants in Midknight Valencia sweet orange. Earlier, Zhu et al. (2008) reported that treatments with (1µmol L^{-1}) NO aqueous solution could protect kiwi fruit from oxidative damage caused by ROS through an enhanced activity of antioxidant enzymes. It may also be argued that endogenous NO production plays an important role in alleviating CI by affecting the antioxidant defence system as reported earlier by Xu et al. (2012).

Fumigation with NO (5µL L^{-1}) exhibited reduced (2.9 %) water loss in Lane Late but not in Midknight Valencia after cold quarantine treatment (1°C for 22 d), which may be ascribed to the genetic difference between two cultivars. Possibly, the reduced water loss in horticultural commodities with NO treatment may be ascribed to a reduced transpiration rate as reported earlier (Ku et al., 2000).

In Lane Late, all the treatments except MJ (0.01 mM) showed significantly reduced SSC (%) and enhanced CCI as compared to the control but not in Midknight

Valencia. Previously, an increased SSC (%) in Tommy Atkin MJ treated mangoes has been reported by (Gonzalez-Aguilar et al. 2000a). Fruit firmness was not significantly affected by any of the treatments in both the cultivars. Significantly reduced total sugars were recorded in both cultivars except NO and ET (10 µL L^{-1}) in Midknight Valencia. Contrarily, Deng et al. (2013) reported earlier that pre-harvest treatment with (50 µM) SNP efficiently maintained a higher content of sucrose and lower content of glucose in Golden Delicious apples. Furthermore, Li et al. (2014) claimed the levels of glucose; fructose and sucrose during fruit ripening of papaya fruit were significantly influenced by NO fumigation. It appears that the effects of NO fumigation are not only limited to SSC but also influence postharvest sugar metabolism. In conclusion, HWD (50±1°C for 5 min) alone or combined with TBZ (20 mg L^{-1}) or MJ (0.05 mM) one min dip were effective in mitigating CI in Lane Late and Midknight Valencia caused during cold quarantine treatment (1°C for 21 d). NO (5 µL L^{-1}) fumigation for 2 h significantly reduces percentage weight loss in Lane Late only. MJ (0.1 mM) dip treatment significantly enhanced CCI only in Lane Late.

CHAPTER 8

Methyl jasmonate alleviate chilling injury and regulate fruit quality in Midknight Valencia orange

Abstract

Susceptibility of sweet oranges to CI restricts the utilisation of cold storage to its full potential in order to extend storage life and maintain fruit quality. The present investigation examined the role of postharvest dip application of MJ and different cold storage temperatures on the incidence of CI and fruit quality of Midknight Valencia. The fruit were dipped for 1 min in aqueous emulsions containing different concentrations 0.10, 0.25 or 0.50 mM of MJ and 'Tween 20' (0.01 %) as a surfactant and. The fruit treated with water (0 Mm MJ) were used as a control. The fruit were stored at 4°C or 7°C for 90 d followed by 10 d simulated shelf conditions in 2014 and 2015 growing seasons. All the MJ dip treatments, irrespective of the concentration applied reduced CI in the fruit during both years. The fruit treated with 0.25 mM MJ followed by 90 d cold storage and 10 d simulated shelf conditions were free from CI, irrespective of the cold storage temperatures during both years. Dip treatments of 0.25 or 0.50 mM MJ reduced soluble solids concentration (SSC) and titratable acidity (TA); however, the SCC/TA ratio was significantly higher when fruit was dipped in 0.25 mM MJ as compared to all other treatments. Dip treatments 0.25 or 0.50 mM MJ reduced concentrations of vitamin C and total antioxidants compared to all other treatments. In conclusion, the fruit dipped in 0.25 mM MJ for one min showed no CI, when stored at 4°C or 7°C for 90 d followed by 10 d simulated shelf conditions in both years. MJ-treated fruit also showed higher SCC/TA ratio and reduced levels of vitamin C and total antioxidants as compared to the control.

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8.1. Introduction

Chilling injury is a major physiological disorder, which mainly develops in tropical and subtropical fruit when stored below 10-15°C (Ladaniya, 2008). Postharvest CI deteriorates the overall quality and marketability of many tropical and subtropical fruits and vegetables (Cao et al., 2009). Citrus fruit requires moderately low temperatures, ranging from 6 to 10°C, depending on cultivar, species, and storage duration (Schirra et al., 1998). CI in citrus fruit exhibits as rind staining, pitting, red blotches, scalding and watery breakdown on the rind (Sala and Lafuente, 1999; Reuther et al., 1989). CI disrupts the normal cell metabolism and negatively affects the overall fruit quality (Lyons, 1973). Various factors affect CI susceptibility of the fruit such as cultivar, harvest date, fruit size, position of the fruit in the canopy, rind colour, microclimate and management practices (Paull, 1990). It has been reported by Cao et al. (2009) that MJ treatment (10 µmole L^{-1}) reduced CI symptoms in loquat fruit as a result of reduced LOX activity and maintenance of a high level of unsaturated/saturated fatty acids ratio stored at 1°C for 35 d. Furthermore, Martinez-Tellez and Lafuente (1992) associated the increased CI with the enhanced level of phenylalanine ammonia-lyase (PAL) in Fortune mandarin stored at 2.5, 5 and 10 °C for 25 d.

Methyl jasmonate regulates many aspects of plant growth and development including fruit ripening, flowering and senescence (Creelman and Mullet, 1995). MJ is known as a signalling molecule which plays a role in biotic and abiotic stress responses such as pathogen/insect attack, drought, mechanical and CI (Creelman and Mullet, 1995; Hayat et al., 2007). MJ has also been widely known to induce defence mechanisms against a wide range of pathogens in many plant species (Penninckx et al., 1998). Dysfunction of the cell membrane occurs when fruit commodities stored at low temperature eventually leads to the development of CI (Zhang and Tian, 2009). Jasmonoic acid is a final product of the enzymatic oxidation of unsaturated fatty acids, and LOX is a pivotal enzyme in this pathway (Vick and Zimmerman, 1984). MJ plays an integral role in the intracellular signal-transduction cascade that operates in the plant to induce stress responses (Sembdner and Parthier, 1993).

Application of MJ prior to low-temperature exposure has reduced the development of CI symptoms in various non-climacteric fruits such as lemon (2 °C) (Siboza et al., 2014), pomegranate (1.5°C) (Mirdehghan and Ghotbi, 2014), pineapple

(10°C) (Nilpraprucк et al., 2008), loquat (1°C) (Cai et al., 2011), grapefruit, avocado (2°C) (Meir et al., 1996) and guava (5°C) (González-Aguilar et al., 2004). MJ, when applied at the optimum concentration, induces chilling tolerance in different fruits and vegetables (Meir et al., 1996). Recently, Siboza and Bertling (2013) reported that postharvest treatment with 10 µM MJ alone or in combination with 2 mM SA significantly reduced CI and membrane lipid peroxidation; inhibited ROS production and enhanced antioxidant activity in the rind of Eureka lemon.

Therefore, it was surmised that the postharvest application of MJ may reduce CI and extend cold storage postharvest life of sweet oranges. No research work has been reported on the effect of the postharvest application of MJ on extending postharvest life, reducing CI and maintaining quality in cold-stored sweet oranges. Cultivation of a late maturing Midknight Valencia sweet orange (harvest season from September to December) has gained a great impetus in WA due to excellent juice content, flavour and very few seeds (DAFWA, 2017). This variety has had limited testing under WA conditions, although some significant plantings have been made in recent years. In addition, cold storage potential of Midknight Valencia for export purpose warrants to be considered. Therefore, the present study aimed at investigating the effects of MJ application on reducing the incidence of CI and maintaining fruit quality in Midknight Valencia sweet oranges stored at 4 °C or 7 °C for 90 d followed by 10 d simulated shelf conditions.

8.2. Materials and methods

8.2.1. Fruit material

Sweet orange cv. Midknight Valencia (*Citrus sinensis* L. Osbeck) was harvested at the physiological maturity (SSC 9.0 % and juice content 38.0 %) from commercial orchard Moora Citrus (latitude 30° 41, South, longitude 115° 42, East) Dandaragan, WA. Seven-year old uniform sweet orange trees previously grafted to Carrizo citrange (*Citrus sinensis* (L.) Osbeck *x Poncirus trifoliata* Raf.) rootstock were used for the experiments. The trees were spaced 2.7 tree to tree and 7.5 m between rows on a north-south orientation. The experiments were conducted on late-maturing Midknight Valencia sweet orange over two consecutive years 2014 and 2015. Fruit of uniform maturity, size and free from symptoms of disorders, diseases and blemishes

were randomly harvested around the tree canopy. Fruit were transported directly in a closed container to the Horticulture Research Laboratory, Curtin University, Perth, WA, within four hours of harvest for further treatments. All the experimental trees received cultural practices including fertilisers, irrigation and plant protection.

8.2.2. Experiment 1: Effects of different concentrations of MJ dip treatments and cold storage temperature on CI incidence during 2014.

Fruit were dipped for 1 min in an emulsion containing different concentrations 0.1, 0.25 or 0.50 mM MJ obtained from Sigma-Aldrich, (Saint Louis, USA). Tween® 20 (0.25 %) was used as a surfactant. The fruit treated with water (0 Mm MJ) were used as a control. Following the treatments, the fruit were held for 6 h at room temperature (20 ± 1 °C) and RH (60 ± 5 %). Following the drying, the fruit were packed in plastic crates (20 per crate) before being held in two cold storage temperatures 4 °C or 7 °C for 90 d with RH (85-90 %). There was no other fruit crop stored with Midknight Valencia at both storage temperatures. The experiment was designed as a completely randomised design with two factors including MJ dip treatments and storage temperatures with three replications, each including twenty-five fruit. CI incidence (%) was recorded following 90 d cold storage and 10 d simulated shelf conditions (21 ± 1 °C).

8.2.3. Experiment 2: Effects of different concentrations of MJ dip treatments and cold storage temperature on CI incidence and fruit quality during 2015.

In 2015, the first experiment was repeated having the same treatments of MJ, storage temperatures, time period and experimental design and had four replications with twenty-five fruit per replication. In addition to the CI incidence (%), various fruit quality variables such as fruit firmness, SSC, TA, SSC/TA, vitamin C and total antioxidants were also determined from the fruit juice stored at 4 °C and 7 °C for 90 d and followed by 10 d in simulated shelf conditions (21 ± 1 °C). Meanwhile, the fruit weight loss (%) was recorded only after 90 d cold storage in 2015.

8.2.4. CI incidence (%)

All the fruit were visually examined for the symptoms of CI following 90 d cold storage and 10 d simulated shelf conditions (21 ± 1 °C). The chill injured fruit was counted from the total fruit in each replication as detailed outlined in Chapter 3, Section 3.6.

8.2.5. Determination of fruit weight loss

Initial fruit weight was recorded at the start of cold storage time and final fruit weight was recorded after 90 d of cold storage by using a digital weigh balance as reported by Ahmad et al. (2013a) and also detailed outlined in Chapter 3, Section 3.7.

8.2.6. Fruit firmness

Fruit firmness was determined using a texture profile analyser (TPA Plus, AMETEK Lloyd Instruments Ltd, Fareham, UK) interfaced with Nexygen® 4.6 software previously detailed outlined in Chapter 3, Section 3.1.1. Fruit firmness was expressed in newtons (N).

8.2.7. Soluble solids concentration (SSC) and titratable acidity (TA)

Fresh juice of Midknight Valencia was squeezed from randomly selected 10-fruit in each replication to determine the SSC and expressed as a percentage by using a digital refractometer (Atago-Palette PR 101, Atago CO. Ltd, Itabashi-Ku, and Tokyo, Japan). The TA was determined by titrating the juice with 0.1 N NaOH using 2-3 drops of phenolphthalein as an indicator to a pink colour end point. TA was calculated as percentage citric acid. The detailed method has also been explained in Chapter 3, Section 3.8.

8.2.8. Determination of vitamin C

The levels of vitamin C from the fruit juice were estimated by employing the method described earlier by Hussain (2014). The standard curve of L-ascorbic acid was used to calculate the concentration of ascorbic acid. The concentration of ascorbic acid was expressed as mg L^{-1} of fresh juice. The detailed method has also been included in Chapter 3, Section 3.13.

8.2.9 Determination of total antioxidants

The total antioxidant levels in the juice of Midknight Valencia sweet orange were estimated by following methods of Brand-Williams et al. (1995) with some modifications. A standard curve of 6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid (Trolox) was used to calculate the levels of total antioxidants in the juice. The total antioxidant levels were expressed as µM Trolox equivalent antioxidant activity (TEAC) (L^{-1}) fresh juice basis. The detailed procedure has also been included in Chapter 3, Section 3.14.

8.2.10 Statistical analysis

The experimental data were subjected to two-way analysis of variance (ANOVA) using GenStat 14th edition (release 14.1; Lawes Agricultural Trust, Rothamsted Experimental Station, Rothamsted, UK). The effects of treatments and cold storage temperatures along with their interactions on various variables were assessed using ANOVA. The least significant differences (LSD) were calculated following Duncan multiple range test at probability ($P \leq 0.05$).

8.3. Results

8.3.1. CI

Dip treatments 0.1, 0.25 and 0.50 mM of MJ significantly reduced CI as compared to control stored for 90 d followed by 10 d of simulated shelf conditions during both years (Table 8.1). However, the mean CI % was significantly lower after fruit storage at 7 °C (0.6 %) for 90 d followed by 10 d of simulated shelf conditions as compared to those which were held at 4 °C (5.3 %) in 2014 and there was no effect in 2015. There was a significant interaction between treatments and storage temperatures for CI in 2015. All dip treatments 0.1, 0.2 or 0.50 MJ exhibited significantly lower CI as compared to control (7.5 %) stored at 4 °C; meanwhile, all the MJ treatment effect was found non-significant at 7 °C.

Table 8.1. CI (%) influenced by different concentrations of MJ treatments (Tr) and storage temperatures (ST) for 90 d followed by 10 d of simulated shelf conditions (21 ± 1 °C) for 2014 and 2015 growing seasons.

	CI (%)					
	2014			2015		
Treatments (MJ mM)	4 °C	7 °C	Mean (Tr)	4 °C	7 °C	Mean (Tr)
Control	5.3	2.6	4.0	7.5	2.5	5.0
0.1	1.3	0.0	0.6	0.0	0.0	0.0
0.25	0.0	0.0	0.0	0.0	0.0	0.0
0.50	2.6	0.0	1.3	1.2	1.2	1.2
Mean (ST)	5.3	0.6		2.1	0.9	
LSD ($P \leq 0.05$)	Tr = 2.2, ST = 1.5, Tr x ST = ns			Tr = 1.9, ST = ns, Tr x ST = 2.7		

Data represent means of 3 replicate samples of 75 units (2014) and means of 4 replicate samples of 100 units (2015). Mean separation for significant analysis of variance within the columns and rows were tested by LSD at ($P < 0.05$).

8.3.2. Weight loss

Mean percentage weight loss in the fruit was significantly reduced with all the dipping treatments of MJ irrespective of the concentrations as compared to the control in 2015 (Table 8.2). Moreover, mean percentage weight loss was significantly higher (9.1 %) when fruit was stored at 7 °C for 90 d than those kept at 4°C (6.0 %). The interaction between different treatments and cold storage temperatures for weight loss was found to be significant. All MJ treatments irrespective of the concentration applied showed significantly reduced weight loss as compared to the control (8.8 %) when stored at 4 °C. However, all the MJ treatments effect was non-significant at 7 °C.

8.3.3. Fruit Firmness

Methyl jasmonate dip treatments and cold storage temperatures did not significantly affect fruit firmness (N) after 90 d cold storage followed by 10 d simulated shelf conditions (Table 8.3). The interaction effect between MJ dip treatments and cold storage temperatures was non-significant.

Table 8.2. Percentage weight loss in the fruit influenced by different concentrations of MJ dipping and two cold storage temperatures (4 or 7 °C) for 90 d for 2015 growing season.

	Weight loss (%)		
Treatments (MJ mM)	4 °C	7 °C	Mean (Tr)
Control	8.8	8.8	8.8
0.1	4.6	10.0	7.9
0.25	5.8	8.4	7.1
0.50	4.8	9.1	7.0
Mean (ST)	6.0	9.1	
LSD ($P \leq 0.05$)	Tr =1.3, ST = 0.92, Tr x ST =1.84		

Data represent means of 3 replicate samples of 75 units (2014) and means of 4 replicate samples of 100 units (2015). Mean separation for significant analysis of variance within the columns and rows were tested by LSD at ($P < 0.05$).

Table 8.3. Fruit firmness in the fruit influenced by different concentrations of MJ dipping and two cold storage temperatures (4 or 7 °C) for 90 d followed by 10 d of simulated shelf conditions (21 ± 1 °C) for 2015 growing season.

	Firmness (N)		
Treatments (MJ mM)	4 °C	7 °C	Mean (Tr)
Control	340.8	335.0	337.9
0.1	341.6	342.0	341.8
0.25	338.1	357.6	347.9
0.50	356.6	335.7	346.1
Mean (ST)	344.3	342.6	
LSD ($P \leq 0.05$)	Tr =ns, ST = ns, Tr x ST =ns		

Data represent means of 3 replicate samples of 75 units (2014) and means of 4 replicate samples of 100 units (2015). Mean separation for significant analysis of variance within the columns and rows were tested by LSD at ($P < 0.05$).

8.3.4. SSC, TA and SSC/TA ratio

Methyl jasmonate dip treatments 0.25 or 0.50 mM significantly reduced mean SSC from 12.6 to 12.0 % and TA from 0.65 to 0.67 % respectively, as compared to the control in the juice of 90 d cold stored fruit followed by 10 d simulated shelf conditions (Table 8.4). Moreover, mean SCC/TA ratio was significantly higher (19.4) when fruit was dipped in 0.25 mM MJ as compared to the control (17.8) and all other treatments. However, SSC and SSC/TA was significantly higher in the fruit juice stored at 7°C (12.9 %) (18.9) than those kept at 4 °C (12.2 %) (17.7), respectively. TA was not affected by cold storage temperatures. There was a significant interaction between MJ treatments and storage temperatures for SSC, TA and SSC/TA ratio. MJ (0.25 or 0.50 mM) exhibited lower SSC as compared to control except MJ (0.1 mM) in the fruit juice stored at 4°C and 7°C. Furthermore, all the MJ concentrations showed significantly lower TA as compared to control (0.72 %) in the fruit juice stored at 4°C; whilst all the MJ treatments except (0.1 mM) exhibited lower TA as compared to control (0.72 %) in the fruit juice stored at 7 °C. SSC/TA was significantly higher (20.5) with the MJ treatment (0.25 mM) as compared to control and all other treatments when stored at 7°C.

Table 8.4. SCC, TA and SSC/TA ratio in the juice as influenced by different concentrations of MJ dipping and two cold storage temperatures (4 or 7 °C) for 90 d followed by 10 d of simulated shelf conditions (21 ± 1 °C) for 2015 growing season.

Treatments (MJ mM)	4 °C	7 °C	Mean (Tr)
		SSC (%)	
Control	12.6	13.1	12.8
0.1	12.6	13.1	12.8
0.25	12.3	12.8	12.6
0.50	11.4	12.7	12.0
Mean (ST)	12.2	12.9	
LSD ($P \leq 0.05$)	Tr =0.20, ST = 0.14, Tr x ST =0.28		
		TA (%)	
Control	0.72	0.72	0.72
0.1	0.69	0.74	0.72
0.25	0.67	0.62	0.65
0.50	0.67	0.66	0.67
	0.69	0.69	
LSD ($P \leq 0.05$)	Tr =0.02, ST = ns, Tr x ST =0.02		
		SSC/TA	
Control	17.8	18.2	17.8
0.1	18.2	17.7	18.0
0.25	18.4	20.5	19.4
0.50	16.9	19.1	18.0
	17.7	18.9	
LSD ($P \leq 0.05$)	Tr =0.62, ST = 0.44, Tr x ST =0.87		

Data represent means of 3 replicate samples of 75 units (2014) and means of 4 replicate samples of 100 units (2015). Mean separation for significant analysis of variance within the columns and rows were tested by LSD at ($P < 0.05$).

8.3.5. Vitamin C and total antioxidants

Methyl jasmonate treatments (0.25 and 0.50 mM) significantly reduced mean vitamin C (285.4 and 266.9 mg L^{-1}) and total antioxidants (406.7 and 396.7 µM Trolox L^{-1}) respectively, as compared to all other treatments in the juice of 90 d cold stored fruit followed by 10 d simulated shelf conditions (Table 8.5). However, the mean levels of vitamin C (301.4 mg L^{-1}) and total antioxidants (430.0 µM Trolox L^{-1}) were higher in the fruit juice which was stored at 4 °C as compared to those stored at 7 °C

(283.3 mg L^{-1} and 409.7 µM Trolox L^{-1}). The interaction effect was non-significant for vitamin C and total antioxidants.

Table 8.5. Vitamin C and total antioxidants in the juice influenced by different concentrations of MJ dipping and two cold storage temperatures (4 and 7 °C) for 90 d followed by 10 d of simulated shelf conditions (21 ± 1 °C) for 2015 growing seasons.

Treatments (MJ mM)	4 °C	7 °C	Mean (Tr)
	Vitamin C (mg L^{-1})		
Control	312.5	288.9	300.7
0.1	342.3	290.5	316.4
0.25	275.6	295.1	285.4
0.50	275.0	258.8	266.9
Mean (ST)	301.4	283.3	
LSD ($P \leq 0.05$)	Tr =25.5, ST = 18.0, Tr x ST =ns		
	Total Antioxidants (µM L^{-1} Trolox)		
Control	443.1	415.2	429.2
0.1	457.1	436.5	446.8
0.25	418.3	395.1	406.7
0.50	401.5	392.0	396.7
	430.0	409.7	
LSD ($P \leq 0.05$)	Tr =29.5, ST = 20.6, Tr x ST =ns		

Data represent means of 3 replicate samples of 75 units (2014) and means of 4 replicate samples of 100 units (2015). Mean separation for significant analysis of variance within the columns and rows were tested by LSD at ($P < 0.05$).

8.4. Discussion

As a prelude, citrus fruit are stored at tolerably low temperatures (6 to 10 °C), depending on cultivar, species, and storage duration (Schirra et al., 1998). In our study, 0.1-0.25 mM MJ dipped treatment for 1 min was most effective in reducing CI (%) in the fruit stored for 90 d at 4 °C or 7 °C followed by 10 d simulated shelf conditions during 2014 and 2015 growing seasons. The mechanism by which MJ induces chilling tolerance in sweet oranges is still unclear. The cell membrane is the main site for CI followed by membrane disruption and loss of membrane integrity (Li et al., 2012; Saltveit and Morris, 1990). Possibly, MJ application may have protected the fruit from membrane damage and enhanced chilling tolerance in Midknight Valencia fruit through the improved activity of PAL, total antioxidants and phenolic in the rind. Previously, Siboza and Bertling (2013) and Siboza et al. (2014) also reported that activation of PAL and increased production of phenolics and total antioxidants were involved in reducing CI in the rind of lemon during cold storage. Similarly, differences in susceptibility to CI in Fortune mandarins (*Citrus reticulata* Blanco) and Navelina (*Citrus sinensis* L. Osbeck) have been reported to be related to the activity of PAL than to PPO and POD (Martinez-Tellez and Lafuente, 1992). Additionally, no correlation was found between POD, PPO and the development of CI in Fortune mandarins and Navelina (Martinez-Tellez and Lafuente, 1992). The higher PAL activity is related to reduce CI in cold storage (Lafuente et al., 2003).

Conceivably, it may also be argued that MJ application may have protected the fruit from membrane damage, electrolyte leakage and membrane lipid peroxidation products, such as MDA leading to the development of CI. Similarly, Jin et al. (2013) also reported that MJ application inhibits ion leakage and MDA content consequently protecting fruit from membrane damage and reducing CI in peach fruit. Zhang and Tian (2009) reported that application of MJ at a concentration of (0.1 mM) showed reduced CI in peach fruit when stored at low temperature. In addition, MJ treatment reduced CI index and ion leakage percentage in guava fruit stored at 5 °C (González-Aguilar et al., 2003). MJ treatment showed an increased chilling tolerance in loquat fruit by reducing LOX activity and higher unsaturated/saturated fatty acid (Cao et al., 2009). The accumulation of ROS and oxidative stress from over production are also one of the major reasons behind the incidence of CI. Chilling tolerance in horticultural crops has also been improved by activation of antioxidants (Cao et al., 2009). The

antioxidant system, comprising enzymatic and non- enzymatic constituents, plays an important role in scavenging ROS and produces chilling tolerance in many fruits (Jimenez et al., 2002). The exact mechanism of MJ application in reducing CI in Midknight Valencia sweet orange fruit warrants investigation.

Methyl jasmonate (0.1, 0.25 and 0.50 mM) dipped treatment for 1 min irrespective of the concentrations applied significantly reduced mean weight loss as compared to the control after 90 d of storage in 2015. The reduction in weight loss could possibly be ascribed to the role of MJ in stomatal closure, which had reduced transpiration rate observed in other tissues (Ueda at al., 1991). Similarly, Nilprapruck et al. (2008) reported that exogenous application of MJ reduced the weight loss in pineapple fruit with enhanced chilling tolerance when stored at 10°C. In addition, González-Aguilar et al. (2001) revealed that MJ treated mango fruit also showed less weight loss when stored for 14 d at 10°C.

During both the years, the mean weight loss was significantly higher when fruit was stored at 7°C for 90 d (5.6 and 9.1 %) than those kept at 4°C (3.0 and 6.0 %) respectively. The reduced weight loss in the fruit when stored at a lower temperature (4°C) than the higher one (7°C) may be ascribed to the reduced rate of respiration and transpiration, also reported earlier in fruits (Wills et al., 2007). Similarly, Roongruangsri et al. (2013) reported two tangerine cultivars Sai Num Phueng and See Thong storage at 5°C showed reduced weight loss and moisture content of the peel as compared to storage at 25°C.

Fruit dipped in 0.1 mM MJ showed an increased level of total antioxidants (446.8 µM L^{-1} Trolox) in the juice as compared to other dipping treatments except for the control (429.2 µM L^{-1} Trolox) in 2015 and the trend was reversed when fruit was treated with higher concentrations. Similarly, Chanjirakul et al. (2006, 2007) have shown that MJ, through enhanced antioxidant activity, might improve functional properties of harvested fruit. Furthermore, Cao et al. (2009) found that postharvest application of MJ in loquat fruit exhibited higher levels of total phenolic and maintained higher antioxidant activity in the pulp as compared to the control fruit. The mode of action of dip application of MJ in regulating the levels of total antioxidants in cold stored fruit is yet to be investigated. In conclusion, postharvest dip application of MJ reduced CI in cv. Midknight Valencia oranges. 0.25 mM MJ dip for 1 min has shown no CI, irrespective of cold storage temperature in two consecutive years. All MJ treatments showed reduced weight loss after 90 d of cold stored fruit. MJ dip

treatments also exhibited higher SCC/TA ratio and reduced vitamin C and total antioxidants.

CHAPTER 9

Nitric oxide fumigation alleviates chilling injury and regulates fruit quality in sweet orange stored at different cold temperatures

Abstract

Cold storage of sweet oranges below 7°C causes CI and adversely affects fruit quality. Midknight Valencia and Lane Late sweet oranges were fumigated for 2 hours with different concentrations (5, 10 or 20 µL L^{-1}) of nitric oxide (NO) and stored at (4 or 7°C) to investigate the effect on CI incidence and fruit quality after 90 d storage followed by 10 d simulated shelf conditions. All NO fumigation treatments (5, 10 or 20 µL L^{-1}) significantly reduced the CI irrespective of storage temperature as compared to the control in both the cultivars. All the NO treatments significantly reduced percent weight loss as compared to control in Lane Late. Mean weight losses were higher (8.3 % and 5.5 %) when fruit were stored at 7 °C as compared to those stored at 4 °C (4.8 % and 3.5 %) in Midknight Valencia and Lane Late respectively. All the NO fumigation treatments significantly reduced the concentrations of glucose, fructose, sucrose and total sugars in the juice of Midknight Valencia only. All NO fumigation treatments significantly reduced mean concentration vitamin C in the fruit juice of Lane Late as compared to the control. Meanwhile, in Midknight Valencia, NO (10 or 20 µL L^{-1}) fumigated fruit showed a significant reduction in mean concentration of vitamin C as compared to NO (5 µL L^{-1}) fumigation and control. The juice of Midknight Valencia had higher mean total antioxidants when fumigated with NO (5 µL L^{-1}) as compared to the control, but not in Lane Late. In conclusion, all the NO fumigation treatments significantly reduced CI, but NO (10 µL L^{-1}) was most effective in both cultivars. NO fumigation treatments did not affect SCC/TA ratio, but reduced all the individual and total sugars as well as vitamin C in the fruit stored for 90 d followed by 10 d simulated shelf conditions.

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9.1. Introduction

Chilling injury, a major physiological disorder mainly develops in tropical and subtropical fruit when stored below 10-15°C for certain period of time (Ladaniya, 2008). Citrus is a non-climacteric fruit with a low level of ethylene and respiration rate (Kader, 2002). Low storage temperatures are used to extend the postharvest life of fruit and vegetables (Lyons, 1973). CI occurs due to temperature and time relationship, and the symptoms usually appear quite rapidly when the citrus fruit is kept at room temperature (Ladaniya, 2008). CI disrupts the normal cell metabolism and negatively affects the fruit quality, one of the significant contributors to the postharvest loss in citrus fruit (Sala et al., 2005). CI in citrus fruit exhibits as rind staining, pitting, red blotches, scalding, watery breakdown, sunken tissues, damage to the styler end of lemons and necrosis on the rind (Reuther et al., 1989). It has been reported that CI symptoms might be the result of oxidative stress from excess ROS that induces peroxidation and breakdown of unsaturated fatty acids in membrane lipids (Lyons, 1973).

Nitric oxide (NO), is a relatively stable, highly reactive free radical gas and acts as a multifunctional signalling molecule in many plant tissues (Wendehenne et al., 2001). It provides defence against biotic and abiotic stress and intricately involved in many physiological processes including ripening of climacteric and non-climacteric fruits (Besson-Bard et al., 2008; Xu et al., 2005). According to Wink and Mitchell (1998) NO probably act as an antioxidant that is capable to scavenge the ROS to protect plant cells from oxidative stress. In addition, Zhu at al. (2008) reported that ROS triggered oxidative stress, which was protected by the exogenous application of NO in kiwi fruit. It has been well documented that NO inhibits ethylene biosynthesis through the inhibition of ethylene biosynthesis enzymes and as a result delays ripening and senescence in many climacteric and non-climacteric fruit (Wills et al., 2000). On the contrary, Huque et al. (2013) reported that NO-induced the inhibition of browning in apple slices without affecting ethylene production. In addition, NO also reduced cell respiration by inhibiting the cytochrome pathway in mitochondria (Millar and Day, 1996; Zottini et al., 2002).

Furthermore, postharvest application of NO has been reported to alleviate CI and maintain fruit quality in climacteric fruit such as Japanese plum cv Amber Jewel (Singh et al., 2009), banana (*Musa* spp., AAA group cv. Brazil) (Wang et al., 2013),

peach (*Prunus persica* (L.) Batsch, cv. Feicheng) (Zhu et al., 2010), mango (*Mangifera indica* L. cv. Kensington Pride) (Zaharah and Singh, 2011), banana (Wang et al., 2013; Wu et al., 2014), papaya (Li et al., 2014), tomato (Zhao et al., 2011), peach (Zhu et al., 2010; Flores et al., 2008), kiwifruit (Zhu et al., 2008), Yali pears (Liu et al., 2011) and non-climacteric fruit Chinese bayberry (Wu et al., 2012), loquat (Xu et al., 2012), longan (Duan et al., 2007) and strawberry (Wills et al., 2000). Recently, Ghorbani et al. (2017) reported that a 0.5mM SNP (sodium nitroprusside) 5 min dip treatment increases antioxidant enzyme activity and reduced CI in Washington navel orange stored for five months at 3°C. However, the effect of NO fumigation on quality of cold stored sweet orange fruit is yet to be investigated. To our knowledge, no research work has been reported on the effect of NO fumigation to alleviate CI and maintain fruit quality in Midknight Valencia and Lane Late sweet orange cultivars. The aim of the present study was to investigate the effect of NO on regulating the incidence of CI and fruit quality in cold stored fruit (4°C and 7°C for 90 d followed by 10 d of simulated shelf conditions) in Midknight Valencia and Lane Late sweet orange.

9.2. Materials and methods

9.2.1. Fruit material

Mature fruit of Midknight Valencia and Lane Late (*Citrus sinensis* (L.) Osbeck) were harvested from a commercial orchard Moora Citrus (latitude 30° 41, South, longitude 115° 42, East) Dandaragan, WA during 2015. The trees of Midknight Valencia and Lane Late were nine and seven years old respectively. The Midknight Valencia and Lane Late both were on Carrizo citrange (*Citrus sinensis* (L.) Osbeck × *Poncirus trifoliata* Raf.) rootstock. The planting space was 2.7 m tree to tree and 7.5 m between rows and a north-south orientation was used in the experiments. The fruit were randomly harvested around the tree canopy. Fruit were transported directly in a closed container to the Horticulture Research Laboratory, Curtin University, Perth, WA, within four hours of harvest. All fruit were visually observed for diseases and blemishes.

9.2.2. Experiment 1: Effects of different concentrations of NO fumigation and cold storage temperature on CI incidence in Lane Late during 2015.

The first experimental trial was conducted on Lane Late. Different concentrations of NO (5, 10 or 20 µL L^{-1}) were used to fumigate the fruit in a sealed plastic container (67 L). NO was obtained from a cylinder containing 4810±100 µL L^{-1} NO in a nitrogen gas (BOC Gases Ltd., Sydney, NSW, Australia) and injected into the container through an injection port on the container lid by using (50 mL) of the syringe. Fruit were kept in a NO atmosphere for 2 hours at ambient temperature (21±1 °C). NO has been reported to be effectively stable at low concentrations and as a result oxygen gas (O_2) in the container was not depleted and short treatment times required for the fruit to be treated in normal air (Soegiarto et al., 2003). Untreated fruit were placed in the plastic container for the same period of time without any treatment. The experiment was designed as a completely randomised design with two factors including NO fumigation treatments and storage temperatures with four replications, each including twenty fruit. Following the NO fumigation treatments, fruit were weighed before being stored at two cold storage temperatures of 4°C and 7°C for 90 d with RH (85-90 %). CI incidence (%) was recorded following 90 d cold storage and 10 d simulated shelf conditions (21 ± 1°C) and fruit weight loss was recorded only at 90 d after cold storage. Additionally, various fruit quality variables such as fruit firmness, SSC (%), TA (%), SSC/TA, sugars, vitamin C and total antioxidants were also determined from the fruit stored at both 4°C and 7°C for 90 d and followed by 10 d in simulated shelf conditions (21 ± 1°C).

9.2.3. Experiment 2: Effects of different concentrations of NO fumigation and cold storage temperature on CI incidence in Midknight Valencia during 2015.

In the same year, the first experiment was repeated keeping the same treatments of NO, storage temperatures, time period and experimental design but using late maturing sweet orange Midknight Valencia. Twenty fruit were also included in each replication and replicated four times. In addition, all the parameters noted in experiment 1 were recorded for Midknight Valencia.

9.2.4. CI incidence (%)

Following 90 d cold storage and 10 d simulated shelf conditions (21 ± 1°C), the symptoms of CI on the surface of individual fruit in Midknight Valencia and Lane Late were recorded. The detailed method has been previousely explanined in Chapter 3, Section 3.6.

9.2.5. Determination of loss of fruit weight

Initial fruit weight of Midknight Valencia and Lane Late fruit was recorded using a digital weigh balance at the commencement of cold storage. Following 90 d of cold storage, the final weight of the fruit was noted. The detailed method has also been described in Chapter 3, Section 3.7.

9.2.6. Fruit firmness

A texture profile analyser (TPA Plus, AMETEK Lloyd Instruments Ltd, Fareham, UK) interfaced with Nexygen® 4.6 software was used to estimate the fruit firmness in Midknight Valencia and Lane Late by following the methods earlier described in Chapter 3, Section 3.8. Fruit firmness was expressed as newtons (N).

9.2.7. SSC and TA

The SSC in the fresh juice extracted from ten randomly selected fruit per replication in Midknight Valencia and Lane Late was assessed by utilising a digital refractometer (Atago-Palette PR 101, Atago CO. Ltd, Itabashi-Ku, and Tokyo, Japan) and expressed as a percentage, and the acidity percentage was determined by titrating the juice against 0.1N NaOH using 2-3 drops of phenolphthalein as an indicator to a pink colour end point. TA was expressed as percent citric acid. The detailed method has also been included in Chapter 3, Section 3.9, 3.10 and 3.11.

9.2.8. Determination of sugars

The levels of individual sugars in the juice of Midknight Valencia and Lane Late were quantified using reverse-phase high-performance liquid chromatography system (RP-HPLC; Waters, Milford, MA, USA) equipped with refractive index detector. The detailed conditions of analysis have been reported earlier in Chapter 3, Section 3.12. All the individual sugars were expressed as (g L^{-1}) fresh juice.

9.2.9. Determination of vitamin C and total antioxidants

The level of vitamin C and total antioxidants in the fresh juice of ten randomly selected fruit from each cultivar of Midknight Valencia and Lane Late were estimated by using a UV/VIS spectrometer (Jenway spectrophotometer Model 6405, Dunmow, Essex, UK) as per the previously reported method (Hussain, 2014; Brand-Williams et al., 1995 respectively). The concentration of vitamin C in the juice of both cultivars was calculated by using the standard curve of L-ascorbic acid and expressed as (mg L^{-1}) of fresh juice. On the other hand, the concentration of total antioxidants was calculated by using the standard curve of 6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid (Trolox) and expressed as a µM Trolox equivalent antioxidant activity (TEAC) (L^{-1}) fresh juice basis. The detailed procedure has also been incorporated in Chapter 3, Section 3.13 and 3.14.

9.2.10. Statistical analysis

The experimental data were analysed by two-way analysis of variance (ANOVA) using GenStat 14th edition (release 14.1; Lawes Agricultural Trust, Rothamsted Experimental Station, Rothamsted, UK). Mean separations were tested by Duncan's multiple range test ($P < 0.05$).

9.3. Results

9.3.1. CI (%)

Fumigation treatments of NO significantly ($P < 0.05$) reduced mean CI (%) on the fruit as compared to the control in Midknight Valencia and Lane Late stored for 90 d followed by 10 d simulated shelf conditions (Table 9.1). However, CI % was significantly lower when fruit were stored at 7°C (2.2, 4.2 %) as compared to those kept at 4°C (6.9, 11.2 %) in Midknight Valencia and Lane Late respectively. The interaction effects were not significant between treatments and storage temperatures for CI in both cultivars.

9.3.2. Weight loss (%)

Weight loss (%) in Lane Late was significantly ($P < 0.05$) reduced with all NO fumigation treatments irrespective of the concentrations applied; it was not affected in Midknight Valencia (Table 9.2). However, weight loss (%) was significantly higher

once fruit were stored at 7 °C (8.3 and 5.5 %) compared to those kept at 4°C (4.8 and 3.5 %) respectively, for both Midknight and Lane Late. The interaction effects between different treatments and cold storage temperatures were not significant for Midknight Valencia and Lane Late.

Table 9.1 CI (%) affected by different concentrations of NO fumigation and cold storage temperatures (4 or 7°C) for 90 d followed by 10 d of simulated shelf conditions (21 ± 1 °C) on Midknight Valencia and Lane Late.

CI (%)						
	Midknight Valencia			Lane Late		
Treatments (NO μL L^{-1})	4 °C	7 °C	Mean (Tr)	4 °C	7 °C	Mean (Tr)
Control	12.5±0.6	8.8±0.5	10.6a	18.3±1.4	15.0±1.4	16.7a
(5)	7.5±0.6	0.0±0	3.8b	11.7±1.8	1.7±0.7	6.7b
(10)	3.8±0.5	0.0±0	1.9b	6.7±1.2	0.0±0	3.3b
(20)	3.8±0.5	0.0±0	1.9b	8.3±1.4	0.0±0	4.2b
Mean (ST)	6.9a	2.2b		11.2a	4.2b	

Data represent means of 4 replicate samples of 80 units for Midknight Valencia and Lane Late. Mean separation for significant analysis of variance within the columns and rows was tested using Duncan's multiple range test at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns or rows. Standard error (SE ±). Treatments (Tr), Storage temperature (ST).

Table 9.2 Percent weight loss in Midknight Valencia and Lane Late sweet orange fruit influenced by different concentrations of NO fumigation and two cold storage temperatures (4 or 7°C) for 90 d.

Weight loss (%)						
	Midknight Valencia			Lane Late		
Treatments (μL L^{-1})	4 °C	7 °C	Mean (Tr)	4 °C	7 °C	Mean (Tr)
Control	4.5±0.11c	8.0±0.20a	6.2	5.3±0.3a	5.5±0a	5.4a
(5)	3.9±0.10c	9.0±0.20a	6.5	2.9±0.1b	5.9±0a	4.4b
(10)	5.6±0.17b	8.3±0.15a	7.0	2.8±0b	5.4±0a	4.1b
(20)	5.0±0.11bc	7.9±0.09a	6.4	3.1±0b	5.1±0.1a	4.1b
Mean (ST)	4.8b	8.3a		3.5b	5.5a	

Data represent means of 4 replicate samples of 80 units for Midknight Valencia and Lane Late. Mean separation for significant analysis of variance within the columns and rows was tested using Duncan's multiple range test at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns or rows. Standard error (SE ±). Treatments (Tr), Storage temperature (ST).

9.3.3. Fruit firmness

Nitric oxide fumigation treatments and cold storage temperatures did not significantly ($P < 0.05$) affect fruit firmness (N) in 90 d cold stored followed by 10 d simulated shelf conditions in Midknight Valencia and Lane Late oranges (Table 9.3). The interaction effects were also not significant for both cultivars.

Table 9.3 Compression (N) in Midknight Valencia and Lane Late influenced by different concentrations of NO fumigation and two cold storage temperatures (4 or 7 °C) for 90 d followed by 10 d of simulated shelf conditions (21 ± 1 °C).

Fruit firmness (N)						
	Midknight Valencia			Lane Late		
Treatments (µL L^{-1})	4 °C	7 °C	Mean (Tr)	4 °C	7 °C	Mean (Tr)
Control	286.3±2.0	288.0±7.6	287.2	286.3±2.0	288.0±7.6	287.2
(5)	303.4±6.8	292.0±3.0	297.7	303.4±6.8	292.0±3.0	297.7
(10)	288.5±4.8	280.8±3.7	284.7	288.5±4.8	280.8±3.7	284.7
(20)	283.4±4.6	285.9±2.3	284.6	283.4±4.6	285.9±2.3	284.6
Mean (ST)	290.4	286.7		290.4	286.7	

Data represent means of 4 replicate samples of 80 units for Midknight Valencia and Lane Late. Mean separation for significant analysis of variance within the columns and rows was tested using Duncan's multiple range test at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns or rows. Standard error (SE ±). Treatments (Tr), Storage temperature (ST).

9.3.4. SSC

All treatments except NO (5 µL L^{-1}) (12.2 %) significantly ($P < 0.05$) increased SSC % in the fruit juice of 90 d cold stored followed by 10 d simulated shelf conditions in Midknight Valencia and was not significant in Lane Late navel (Table 9.4). However, SSC (%) was significantly higher in the juice of fruit stored at 7 °C (12.8, 11.8 %) than those stored at 4°C (12.3, 11.4 %) in Midknight Valencia and Lane Late, respectively. The interaction was found to be significant for Midknight Valencia but not Lane Late. In Midknight Valencia, NO (10 or 20 µL L^{-1}) fumigation treatments increased SSC (%) (12.5 and 12.7 %) respectively, as compared to all other treatments.

9.3.5. TA

Nitric oxide fumigation treatments showed significantly ($P < 0.05$) reduced mean TA (%) regardless of the concentrations applied as compared to the control after 90 d of cold storage followed by 10 d simulated shelf conditions (Table 9.4). Moreover, mean TA (%) was significantly higher in the fruit juice stored at 4°C (0.66, 0.55 %) than those stored at 7°C (0.54, 0.53 %) in Midknight Valencia and Lane Late, respectively. The interaction between NO treatments and storage temperatures for TA (%) was found to be significant in Midknight Valencia but not in Lane Late. In Midknight Valencia, all NO fumigation treatments except NO (5µL L^{-1}) showed higher TA (%) in the juice.

Table 9.4 SCC, TA and SSC/TA ratio in Midknight Valencia and Lane Late sweet orange fruit influenced by different concentrations of NO fumigation and two cold storage temperatures (4 or 7 °C) for 90 d followed by 10 d of simulated shelf conditions (21 ± 1 °C).

SSC (%)						
	Midknight Valencia			Lane Late		
Treatments (µL L^{-1})	4 °C	7 °C	Mean (Tr)	4 °C	7 °C	Mean (Tr)
Control	12.1±0.03bc	12.8±0.11a	12.5ab	12.1±0.1	11.7±0.1	11.9
(5)	11.7±0.07c	12.7±0.13a	12.2b	10.9±0.1	11.6±0.2	11.3
(10)	12.5±0.05ab	12.8±0.02a	12.6a	11.4±0.1	12.0±0	11.7
(20)	12.7±0a	12.7±0.04a	12.7a	11.1±0.2	11.9±0.1	11.5
Mean (ST)	12.3b	12.8a		11.4b	11.8a	
TA (%)						
Control	0.70±0.01a	0.55±0c	0.62a	0.58±0	0.57±0	0.57a
(5)	0.61±0.01b	0.55±0c	0.58b	0.54±0	0.52±0	0.53b
(10)	0.67±0a	0.53±0c	0.60ab	0.55±0	0.51±0	0.53b
(20)	0.66±0a	0.55±0c	0.60ab	0.54±0	0.52±0	0.53b
Mean (ST)	0.66a	0.54b		0.55a	0.53b	
SSC/TA						
Control	17.4±0.2	23.4±0.2	20.4	21.1±0.1	20.7±0.3	20.9
(5)	19.1±0.3	23.3±0.2	21.2	20.3±0.1	22.5±0.4	21.4
(10)	18.6±0.1	24.2±0.2	21.4	20.8±0.4	23.7±0.2	22.2
(20)	19.1±0.1	23.2±0.1	21.2	20.6±0.4	23.0±0.4	21.8
Mean (ST)	18.6b	23.5a		20.7b	22.5a	

Data represent means of 4 replicate samples of 80 units for Midknight Valencia and Lane Late. Mean separation for significant analysis of variance within the columns and rows was tested using Duncan's multiple range test at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns or rows. Standard error (SE ±). Treatments (Tr), Storage temperature (ST).

9.3.6. SSC/TA ratio

Nitric oxide fumigation treatments did not significantly ($P < 0.05$) affect SSC/TA ratio in Midknight Valencia and Lane Late after 90 d of cold storage followed by 10 d simulated shelf conditions (Table 9.4). However, SSC/TA ratios were significantly higher in fruit juice stored at 7°C (23.5, 22.5 %) than those stored at 4°C (18.6, 20.7 %) for Midknight Valencia and Lane Late, respectively. The interaction effects were not significant for both cultivars.

9.3.7. Glucose

All NO fumigation treatments irrespective of the concentrations applied showed significantly ($P < 0.05$) reduced glucose levels in fruit juice of Midknight Valencia and did not affect Lane Late after 90 d of cold storage followed by 10 d simulated shelf conditions (Table 9.5).

9.3.8. Fructose

In Midknight Valencia, irrespective of the concentrations applied, all NO fumigation treatments reduced the fructose levels in fruit juice as compared to the control (24.6 g L^{-1}) after 90 d of cold storage followed by 10 d simulated shelf conditions and were found to be non-significant in Lane Late (Table 9.5). However, mean fructose levels was higher in the fruit juice stored at 7°C (23.7 g L^{-1}) compared to 4°C (22.2 g L^{-1}) in Midknight Valencia only. The interaction effects were significant for Midknight Valencia not for Lane Late. All the NO treatments except NO (20 µL L^{-1}) reduced fructose levels in fruit juice of Midknight Valencia as compared to the control stored at 4°C and 7°C.

9.3.9. Sucrose

Sucrose levels in fruit juice of Midknight Valencia were reduced by all NO fumigation treatments regardless of the concentrations applied as compared to the control (54.5 g L^{-1}) after 90 d of cold storage followed by 10 d simulated shelf condition but was not reduced in Lane Late navel (Table 9.5). However, sucrose levels in fruit juice were higher (53.6 g L^{-1}) when stored at 4°C compared to fruit stored at 7°C (51.1 g L^{-1}) in Midknight Valencia only. The interaction effects were not significant for both cultivars.

Table 9.5 Glucose, fructose, sucrose and total sugars in Midknight Valencia and Lane Late sweet orange fruit influenced by different concentrations of NO fumigation and two cold storage temperatures (4 or 7°C) for 90 d followed by 10 d of simulated shelf conditions (21 ± 1 °C).

Glucose (g L^{-1})						
	Midknight Valencia			Lane Late		
Treatments (μL L^{-1})	4 °C	7 °C	Mean (Tr)	4 °C	7 °C	Mean (Tr)
Control	14.8±0.1b	17.2±0.2a	16.0a	14.4±0.1	14.7±0.2	14.5
(5)	12.2±0.2c	15.5±0.2b	13.9b	12.1±0.3	15.3±0.4	13.7
(10)	12.2±0c	14.9±0b	13.5b	14.6±0.3	16.0±0.1	15.3
(20)	14.1±0.4b	14.8±0.2b	14.5b	12.9±0.4	15.7±0.3	14.3
Mean (ST)	13.3b	15.6a		13.5b	15.4a	
Fructose (g L^{-1})						
Control	23.4±0.3b	25.8±0.2a	24.6a	21.6±0.4	22.7±0.3	22.1
(5)	21.1±0.1cd	23.6±0.3b	22.4bc	20.4±0.4	23.1±0.6	21.8
(10)	20.8±0.1d	23.0±0b	21.9c	23.5±0.1	23.5±0.3	23.5
(20)	23.6±0.3b	22.5±0.3bc	23.1b	23.4±0.4	23.6±0.2	23.5
Mean (ST)	22.2b	23.7a		22.2	23.2	
Sucrose (g L^{-1})						
Control	57.5±0.4	51.5±0.1	54.5a	55.4±1.2	50.4±0.9	52.9
(5)	51.7±0.4	51.7±0.7	51.7b	50.6±0.4	51.6±1.3	51.1
(10)	52.7±0.4	51.5±0.6	52.7ab	53.6±0.4	51.3±0.3	52.5
(20)	50.5±0.4	49.8±0.1	50.5b	54.4±0.7	50.6±0.7	52.5
Mean (ST)	53.6a	51.1b		53.5	51.0	
Total sugars (g L^{-1})						
Control	95.7±0.2	94.5±0.2	95.1a	90.1±2.0	87.8±1.4	89.0
(5)	84.9±0.3	90.8±0.3	87.9b	83.1±0.9	84.7±0.4	83.9
(10)	87.0±0.4	89.4±0.4	88.2b	91.7±0.7	90.8±0.1	91.2
(20)	89.0±1.0	87.1±1.0	88.0b	90.7±1.1	89.9±1.1	90.3
Mean (ST)	89.1	90.4		88.9	88.3	

Data represent means of 4 replicate samples of 80 units for Midknight Valencia and Lane Late. Mean separation for significant analysis of variance within the columns and rows was tested using Duncan's multiple range test at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns or rows. Standard error (SE ±). Treatments (Tr), Storage temperature (ST).

Moreover, mean glucose levels were higher in fruit juice of Midknight Valencia and Lane Late navel stored at 7 °C (15.6, 15.4 %) than those stored at 4°C (13.3, 13.5 %) respectively. The interaction effects between different NO treatments and storage temperature were significant only for Midknight Valencia and the NO fumigation treatments reduced glucose levels in fruit juice as compared to the control at both storage temperatures.

9.3.10. Total sugars

In Midknight Valencia, all the fruit fumigated with NO irrespective of the concentrations reduced total sugars in fruit juice as compared to the control (95.1 g L^{-1}) after 90 d of cold storage followed by 10 d simulated shelf conditions (Table 9.5). The interaction effects between different treatments and cold storage temperature were not for both cultivars.

9.3.11. Vitamin C

Vitamin C levels were reduced in fruit juice of Midknight Valencia and Lane Late with NO fumigation treatment irrespective of concentrations applied except NO (5 µL L^{-1}) (357.4 mg L^{-1}) in Midknight Valencia as compared to control after 90 d of cold storage followed by 10 d simulated shelf conditions (Table 9.6). On the other hand, mean vitamin C levels were higher in fruit stored at 7°C (362.4 mg L^{-1}) than those stored at 4°C (282.0 mg L^{-1}) in Midknight Valencia only. The interaction effects for vitamin C levels were significant in Midknight Valencia only. NO treatments reduced the level of vitamin C except NO (5 µL L^{-1}) (416.1 mg L^{-1}) as compared to the control and all other treatments.

9.3.12. Total antioxidants

In Midknight Valencia, NO (5 or 20 µL L^{-1}) increased levels of total antioxidants (416.5 or 383.3 µM L^{-1} Trolox) as compared to the control (369.3 µM L^{-1} Trolox) and other treatments after 90 d of cold storage followed by 10 d simulated shelf conditions but not in Lane Late navel (Table 9.6). The interaction effects were significant in Midknight Valencia only.

Table 9.6 Vitamin C and total antioxidants in Midknight Valencia and Lane Late sweet orange fruit influenced by different concentrations of NO fumigation and two cold storage temperatures (4 or 7 °C) for 90 d followed by 10 d of simulated shelf conditions (21 ± 1 °C).

Vitamin C (mg L^{-1})						
	Midknight Valencia			Lane Late		
Treatments (NO μL L^{-1})	4 °C	7 °C	Mean (Tr)	4 °C	7 °C	Mean (Tr)
Control	379.2±4.6a	372.4±14.5ab	375.8a	317.4±2.7	334.9±7.3	326.1a
(5)	298.6±6.0c	416.1±1.9a	357.4a	290.2±5.4	307.4±9.8	298.8b
(10)	217.7±8.7d	307.0±8.0bc	262.4b	275.6±3.3	277.6±1.0	276.6b
(20)	232.6±13.4d	354.0±8.4abc	293.3b	284.7±6.6	308.0±8.4	296.4b
Mean (ST)	282.0b	362.4a		292.0	307.0	
Total Antioxidants (μM L^{-1} Trolox)						
Control	333.5±8.0c	405.1±8.8ab	369.3b	356.2±14.7	356.4±9.2	356.3
(5)	447.0±4.1a	385.9±8.2abc	416.5a	334.4±7.9	343.5±8.5	339.0
(10)	334.1±11.1c	365.1±11.0bc	349.6b	338.5±4.7	327.3±4.2	332.9
(20)	390.8±9.3abc	375.8±12.0bc	383.3ab	347.3±10.2	329.6±8.8	338.5
Mean (ST)	376.3	383.0		344.1	339.2	

Data represent means of 4 replicate samples of 80 units for Midknight Valencia and Lane Late. Mean separation for significant analysis of variance within the columns and rows was tested using Duncan's multiple range test at ($P < 0.05$). Mean followed by the same letter is not significantly different within the columns or rows. Standard error (SE ±). Treatments (Tr), Storage temperature (ST).

9.4. Discussion

Chilling injury (CI) symptoms adversely affect cosmetic appearance of the fruit and deteriorate the quality in tropical and subtropical fruit including sweet oranges and mandarins. Chilling temperatures has been reported to cause oxidative burst as a result of over-production of ROS, such as H_2O_2 and (O_2^-) consequently increases lipid peroxidation and ultimately leading to cellular membrane damage (Xie et al., 2008). Previously, Sala (1998) has reported that oxidative stress was involved in cold-induced rind damage in Nova and Fortune cultivars of mandarins. Postharvest NO fumigation reduced CI in Midknight Valencia and Lane Late after 90 d of cold storage followed by 10 d simulated shelf conditions (Table 9.1). Possibly, NO fumigation application may have prevented membrane damage and enhanced chilling tolerance in Midknight Valencia and Lane Late orange through the enhanced activity of antioxidants by detoxifying ROS. Similarly, Ghorbani et al. (2017) also reported that application of 0.5 mM sodium nitroprusside (SNP) as a NO donor was able to reduce CI in Washington navel orange stored at 3 °C through the increased response of antioxidants and reduced level of lipid peroxidation and H_2O_2 content. Additionally, NO treatment increased the activities of catalase (CAT), POD, SOD and APX under chilling stress in Washington navel orange (Ghorbani et al., 2017). Zhu et al. (2008) also reported related results that NO treated kiwi fruit had higher activities of SOD, CAT, APX and POD and higher DPPH-radical scavenging activity than control fruit during the storage. Previously, Manjunatha et al. (2010) also reported that NO has antioxidant properties and also plays an important role in the ROS metabolism and signalling network during normal and stress conditions. NO fumigation has been previously reported to reduced CI and maintain fruit quality in many non-climacteric horticulture crops such as strawberry (Zhu and Zhou, 2007; Wills et al., 2000), Chinese bayberry (Wu et al., 2012), cucumber (Yang et al., 2011a), Chinese winter jujube (Zhu et al., 2009), longan (Duan et al., 2007), loquat (Xu et al., 2012) and sweet potato (Yin et al., 2012). Furthermore, Japanese plums fumigated with NO showed reduced CI symptoms during storage at 0°C for 6 weeks (Singh et al., 2009).

Enhanced chilling tolerance by reduced ethylene production and respiration rate with the application of NO has earlier been reported in Kensington Pride mango (Zaharah and Singh, 2011). The development of CI in relation to ethylene production in various climacteric and non-climacteric fruit are multifarious, with no dependable

relationship (Watkins and Miller, 2004). Fumigation of the climacteric Chinese bayberry (*Myrica rubra*) fruit with NO gas in nitrogen for 2 h suppressed ethylene production, total phenolic and DPPH radical scavenging activity (Wu et al., 2012).

All NO fumigation treatments was able to reduce mean weight loss percentage only in Lane Late (Table 9.2), which may be ascribed to the genotypic differences between both cultivars. It may also be argued that reduction in water loss with NO fumigation seems to play some role in reducing CI. Previously, increased moisture loss was significantly correlated with an increased CI in Lanes Late navel orange (Henriod et al., 2005). Furthermore, Ku et al. (2000) also reported that NO fumigated fruit, vegetables and flowers exhibited 20 % reduced water loss due to reduced transpiration rate.

All the NO fumigation treatments significantly reduced the TA (%) in the juice of Midknight Valencia and Lane Late. On the other hand, Singh et al. (2009) reported that NO treatments (10 or 20 μL L^{-1}) increased TA (%) in Japanese plums cv. Amber Jewel. In addition, NO fumigation treatments irrespective of the concentrations applied did not affect the level of glucose, fructose, sucrose and total sugars in Lane Late only (Table 9.5). Whilst, in Midknight Valencia, NO fumigation reduced the levels of individual and total sugars which suggest that NO fumigation affect sugar metabolism in the orange fruit in a genotype dependent manner. Our findings are in line with Zaharah and Singh (2011) who reported that NO fumigation treatments did not influence the concentration of sucrose and glucose in the pulp of mango fruit as compared to non-fumigated fruit. In conclusion, all the NO fumigation treatments (5, 10 or 20 μL L^{-1}) significantly reduced the CI as compared to the control irrespective of storage temperature in Midknight Valencia and Lane Late, but NO (10 μL L^{-1}) treatment was most effective in both cultivars. All the NO fumigation treatments reduced percent weight loss in Lane Late only. NO fumigation treatments irrespective of the concentration applied reduced the concentrations of glucose, fructose, sucrose and total sugars in the juice of Midknight Valencia only.

CHAPTER 10

General discussion, conclusions and future research

10.1. Introduction

Poor rind colour of the sweet orange fruit is one of the major concerns for growers around the world. Sweet orange fails to develop a deep orange colour in subtropical regions at the time of harvest (Ladaniya, 2008). The rind colour of the sweet orange fruit is mainly associated with weather conditions during fruit maturation (Barry and Van Wyk, 2006). A favourable temperature is required for chlorophyll degradation; carotenoid accumulation eventually leads to a deep orange rind colour (Young and Erickson, 1961). The rind colour changes from a dark, solid green to a pale green colour, coinciding with the onset of maturity or 'colour break' in sweet orange fruit grown under tropical conditions (Reuther and Rios-Castano, 1969; Samson, 1980). M7 Navel an early maturing sweet orange cultivar has been planted in WA. The regulation of poor rind colour of M7 Navel fruit grown in the Mediterranean climate of WA warrants investigation. Therefore, the general aim of this research was to examine the role of S-ABA, MJ, Pro-Ca and PBZ (GA biosynthesis inhibitor) in enhancing the rind colour of M7 Navel whereby maintaining fruit quality.

Cold storage is used widely in extending storage life and maintaining the quality of fresh horticultural produce. Sweet orange fruit is prone to CI when stored below 10°C for a long period of time, hence cold storage cannot be utilised to its full potential in extending the storage life and maintaining the fruit quality of sweet orange fruit. In WA a late maturing sweet orange cultivar Midknight Valencia selected from South Africa is available through Auscitrus and local nurseries. Midknight Valencia has excellent juice content, flavour and very few seeds (DAFWA, 2017). It holds on the tree for as long as standard Valencia's but fruit quality can decline, although some plantations have been done in recent years. In addition, cold storage potential of Midknight Valencia for export purpose warrants investigation.

10.2. Pre-harvest spray application of S-ABA to regulate fruit colour development and quality in early maturing M7 Navel orange.

Coloured skin fruit is the major prerequisite to meet quality standards and consumer prospects. The role of ABA and ethylene in skin colour development of non-climacteric fruits such as grape (Lurie et al., 2009; Peppi et al., 2006; Coombe and Hale, 1973; El-Kereamy et al., 2003) and litchi (Wang et al., 2007; Wei et al., 2011) has been well documented in the literature. The exogenous application of S-ABA promotes accumulation of anthocyanins in the skin of table grape, without changing the berries maturation (Cantin et al., 2007; Peppi et al., 2008; Peppi et al., 2006). However, the role of S-ABA in the accumulation of carotenoids in the rind of sweet orange is not yet well understood. Sweet orange rind colour is due to the accumulation of different types of carotenoids. The deep orange colour of the sweet orange fruit is one of the important quality parameters which attract the consumers in the market. The development of the citrus rind colour is the result of the synchronized accumulation of carotenoids and chlorophyll degradation (Gross, 1987). Moreover, the concentration of carotenoids in the rind of citrus fruit varies significantly among different genotypes and depends on the growing conditions. My experimental results show that exogenous spray application of S-ABA applied 3-6 WBAH showed reduced *h*° and enhanced CCI in cultivar M7 Navel in 2015 and 2016 growing seasons. On the contrary, NDGA an ABA biosynthesis inhibitor resulted in enhanced *h*° and reduced CCI, which endorses the possible role of S-ABA in colour development in M7 Navel fruit.

Spray application of S-ABA exhibited enhanced rind colour in M7 Navel through an increased level of total carotenoids (Chapter 4 and Table 4.1). Meanwhile, the pre-harvest spray application of NDGA retarded fruit colour development in M7 Navel and reduced biosynthesis of carotenoids in the rind of the fruit. Previously, Wang et al. (2016) reported that NDGA-treated sweet oranges showed reduced expression levels of CsPP2C 25, CsPP2C 56 and CsSnRK2s prominent to reduced levels of endogenous ABA. Similarly, Wang et al. (2016) earlier reported that exogenous application of ABA improved colour in Ponkan mandarin (*Citrus reticulata* Blanco) noted by reduced *h*° and increased CCI. It has also been reported by Rodrigo and Zacarias (2007) that ABA is meticulously involved in the metabolism of carotenoids and plays an important role in the composition and regulation of

carotenoids in plants. A comparatively small upregulation of ABA was recorded in the rind during the natural colour changeover from green to orange (Harris and Dugger, 1986). Together, the ABA conjugate level increased approximately 12-fold in the rind tissue of sweet orange fruit (*Citrus sinensis* [L.] Osbeck cv Washington Navel). It may also be argued that ABA accumulates in the peel during maturation and is thought to play a pivotal role in chloroplast development during fruit colouration (Rodrigo et al., 2006; Kato et al., 2006 and Harris and Dugger, 1986). Furthermore, Rodrigo et al. (2003) also confirmed that an ABA-deficient orange mutant has shown a delay in the peel de-greening process. As discussed previously in (Chapter 4 section 4.4) pre-harvest spray application of S-ABA may possibly have increased levels of carotenoids in the rind of M7 Navel through the up-regulation of expression of *PSY*, *PDS*, *ZDS*, *LYb1*, *LYb2* and *HYb* as stated previously in Satsuma mandarin, Valencia orange and Lisbon lemon (Zhang et al., 2012).

Primarily, S-ABA probably plays a direct role in the down-regulation of *LCYe* transcript and upregulation of *LCYb*. It has been observed by Kato et al. (2004) the change in the rind colour from green to orange in Valencia sweet orange and Lisbon lemon has been suggested to be associated with the down-regulation of *LCYe* transcripts and the increased *LCYb* transcripts. In addition, Rodrigo et al. (2004) claimed that down-regulation of gene expression (*LCYe)* in the rind of Navelate navel orange is predominantly responsible for the colour change from green to orange. Furthermore, increased expression of *PSY*, *PDS*, *ZDS* and *HYb* genes has been observed in the rind of Navelate during substantial accumulation of carotenoids (Rodrigo et al., 2004). Many researchers have reported the up-regulation of *LCYb* and down-regulation of *LCYe* genes during citrus fruit ripening (Rodrigo et al., 2004; Kato et al., 2004 and Fanciullino et al., 2008).

It may also be argued that S-ABA application might enhance colour development through the upregulation of ethylene biosynthesis. In climacteric fruits, the enhanced sensitivity of the tissues to ethylene has been associated with the levels of ABA (Alferez and Zacarias, 1999; Eveland and Jackson, 2011). It has also been discussed in detail in (Chapter 4 Section 4.4) peel colour development in citrus has been ascribed to upregulation of red carotenoids such as *β*-cryptoxanthin and *β*-citraurin (Stewart and Wheaton, 1973). Moreover, Alquezar et al. (2008) reported that remarkably, ethylene also up-regulates the expression of *PSY*, *ZDS* and *β-CHX*

transcript, persistently or rapidly increased the expression of *PDS*, *PTOX*, *LCYb* and ZEP and decreased the expression of *LCYe* Conceivably, ABA either directly or through up-regulation of ethylene biosynthesis improves fruit colouring in M7 Navel.

10.3. Pre-harvest spray application of Pro-Ca and PBZ regulates fruit colour development and quality in early maturing M7 Navel orange.

As a prelude, poor rind colour of early maturating Navel orange fruit in warm climate causes serious economic losses to the growers. To improve rind colour, particularly from yellow to deep orange in an early maturating M7 Navel fruit various experiments were conducted in two consecutive years 2015-2016 growing seasons. Pro-Ca or PBZ were sprayed at 6, 3 and 6 followed by 3 WBAH on early-maturing M7 Navel to optimise the concentration and timing of Pro-Ca and PBZ application to improve rind colour by enhancing levels of total carotenoids, CCI and reduced *h*° angle. My experimental results showed that both Pro-Ca and PBZ exhibited improved rind colour in M7 Navel by enhanced CCI, total carotenoids and reduced *h*° angle (Chapter 5 Table 5.1). It has been reported that GA_3 appears to delay chloroplast to chromoplast transformation in citrus fruit rind (Thomson et al., 1967). Furthermore, Coggins et al. (1962) argued that GA applied at colour break resulted in unacceptably green fruit at harvest.

As discussed previously in (Chapter 5 section 5.4), there are two possible ways through which Pro-Ca inhibit the biosynthesis of GA_3 in plants. Firstly, Pro-Ca is similar to the structure of 2-oxoglutaric acid; co-enzymes of the involved dioxygenases are assumed to be responsible for blocking GA metabolism in the later stage of biosynthesis (Evans et al., 1999). Secondly, Pro-Ca seems to inhibit 3β-hydroxylation [(conversion of GA_{20} (inactive) to GA_1 (active)] as previously reported (Grigges et al., 1991; Nakayama et al., 1992). Moreover, Pro-Ca also delays the metabolic inactivation of GA_S by blocking their 2β-hydroxylation. Similarly, the pre-harvest spray application of Pro-Ca (400 mg L^{-1}) 6 plus 3 weeks before anticipated harvest applied on Navelina Navel orange improved rind colour development by enhancing chlorophyll degradation and improving biosynthesis of carotenoids (Barry and Le Roux, 2010).

Paclobutrazol (PBZ) is known to reduce the levels of gibberellins in plants and subsequently reduces vegetative vigour (Smeirat and Qrunfleh, 1988; Wheaton, 1989).

PBZ is an N-containing heterocycle compound acting as an inhibitor of early stages of GA biosynthesis process. Probably, PBZ blocks the oxidation from *ent*-kaurene to *ent*-kaurenoic acid by delaying cytochrome P450-dependent monooxygenases in the early steps of GA biosynthesis pathway as reported earlier (Rademacher, 2000; Dalziel and Lawrence, 1984). Moreover, monooxygenases catalysed the oxidative process from *ent*-kaurene to *ent*-kaurenoic acid located on endoplasmatic reticulum, which requires O_2 and NADPH for activity. In conclusion, GA_3 biosynthesis inhibition such as Pro-Ca and PBZ improved rind colour of M7 Navel by showing reduced *h*° angle and enhanced CCI and levels of total carotenoids.

10.4. Pre-harvest spray application of MJ modulates fruit colour development and quality in an early maturing M7 Navel orange.

Early-season sweet orange fruit in the Mediterranean area attain acceptable internal maturity standards, while the fruit is still green. A de-greening treatment is widely used as a pre- and postharvest practice to improve the external fruit colour. Jasmonic acid (JA), and MJ are known to promote chlorophyll degradation and accumulation of anthocyanins in a variety of plants (Abeles and Dunn 1989; Hung and Kao, 1996). My experimental results suggested that pre-harvest spray application of MJ exhibited enhanced rind colour of M7 Navel by showing reduced *h*° angle and enhanced CCI in two consecutive years 2015 and 2016 (Chapter 6 and Table 6.1). Probably, improved rind colour of M7 Navel is due to the accumulation of carotenoids in the peel. My experimental findings are in line with Perez et al. (1993) who reported that MJ stimulates β-carotene biosynthesis in Golden Delicious apple peel. Furthermore, Rudell et al. (2002) also claimed that MJ treatment enhanced β-carotene in Fuji peel. Moreover, MJ has been reported previously to stimulate β-carotene synthesis in the tomato fruit (Saniewski and Czapski, 1983). The exact mode of action of MJ to stimulate colour development is still unknown. However, it has been argued that possibly MJ stimulate colour independently of the activation of ethylene action. It is well known that ethylene accelerates the accumulation of carotenoids (Steward and Wheaton, 1972) and chlorophyll degradation (Purvis and Barmore, 1981) resulting in the unmasking of deep colour. Furthermore, Olias et al. (1992) claimed that MJ vapour application to Golden Delicious apples has been shown to significantly accelerate ethylene biosynthesis

(2.5- in the cortical and 4.6-fold in the peel tissues). It has also been stated that exogenous application of MJ accelerates endogenous ethylene production and as result enhanced colour development in climacteric fruit such as mango (Lalel et al., 2003a), plum (Khan and Singh, 2007), apple (Fan et al., 1997), and in non-climacteric fruit such as strawberry (Mukkun and Singh, 2009). Moreover, ethylene played a pivotal role in the development of the red blush on the surface of Pink lady apple fruit as reported by (Whale and Singh, 2007). It has been also reported by Rudell and Mattheis (2008) that Fuji apple fruit treated with an inhibitor of ethylene action (1-MCP) plus MJ reduced the red colouration in Fuji apple fruit, compared with MJ treatment alone. In conclusion, pre-harvest spray application of MJ showed enhanced rind colour of early maturing M7 Navel due to reduced *h*° angle and enhanced CCI and levels of total carotenoids in the rind.

10.5. Alleviation of CI induced by cold quarantine treatment in Midknight Valencia and Lane Late sweet orange.

Quarantine treatments are obligatory to meet the requirements outlined by importing countries to disinfect the fruit before exporting to the international market. CI in sweet orange fruit caused by cold quarantine treatment is one of the major bottleneck in extending the storage life and disinfestation of the fruit. Quarantine treatment involves the acquaintance of the fruit at 1°C for 21 d to disinfect the fruit against Medfly (Powell, 2003). This non-freezing temperature leads to the physiological disorder of CI. The symptoms of CI on citrus fruit are expressed as rind staining, pitting, red blotches, scalding, watery breakdown and sunken tissues on the rind (Reuther et al., 1989). Various non-chemicals methods such as hot water dips, vapour heat, and forced air have been used to control fungal rot and insect infestation in many fruits and vegetables (Couey, 1989; Barkai-Golan and Phillips 1991). The experimental results indicate that HWD at 50±1 °C for 5 min is effective in reducing CI caused by cold quarantine treatment (1°C for 21 d) in sweet orange Lane Late and Midknight Valencia. As explained in (Chapter 7, Fig 7.1 and 7.2), HWD treatment possibly, plays an important role in reducing CI by changing the structure of epicuticular wax (McDonald et al., 1993b). Secondly, the HT may alter the enzyme system responsible for the development of CI (Parkin et al., 1989; Martinez-Tellez and Lafuente, 1997). Thirdly, HWD treatment probably enhanced chilling tolerance by upregulation of POX, CAT and total phenolic content (TP) as reported earlier in

Valencia and Navel oranges (Bassal and El- Hamahmy, 2011). However, TBZ probably leads to reduced CI by acting indirectly to suppress latent infections that might develop due to low-temperature storage as reported earlier in grapefruit by Schiffmann-Nadel et al. (1972). In addition, increased fungicide concentrations and deposits in fruit decreased rate of peel senescence. Hordijk et al. (2013) reported that HW fungicide treatment deepened the penetration of the fungicide through the epicuticular wax. Furthermore, TBZ with HWD has a synergistic effect on reducing CI in different fruit. The effectiveness of TBZ with HWD to reduce CI in various citrus fruits has been reported in Marsh and Redblush grapefruit (*Citrus paradisi* Macf.) (McDonald et al., 1991), Tarocco oranges (Schirra and Mulas, 1995) and Valencia oranges (Wild and Hood, 1989).

My experimental data also showed that MJ (0.5 mM) one-min dip treatment reduced CI in both cultivars; meanwhile, MJ (0.1 or 0.25) induced chilling tolerance only in Midknight Valencia during cold quarantine treatment (1°C for 21 d). Possibly, MJ reduced CI by the triggering of heat shock proteins (HSPs) and phenolic compounds (Meir et al., 1996; Meng et al., 2009). It has also been reported that MJ stimulates the build-up of HSPs, which reduced CI in tomato fruit (Ding et al., 2002). MJ probably acts together with single transduction cascade of the chemical changes involved in the reduction of CI (Meir et al., 1996). Previously, MJ has been applied to control CI in many fruits such as guava, tomato, papaya, mango and pomegranate (González-Aguilar et al., 2004; Ding et al., 2002; Mirdehghan and Ghotbi, 2014). All the MJ treatments except MJ (0.01 mM) showed significantly reduced SSC (%) and enhanced CCI as compared to the control in Lane Lane but not in Midknight Valencia. Previously, an increased SSC (%) in Tommy Atkin MJ treated mangoes has been reported (Gonzalez-Anguilar et al., 2000a)

NO fumigation treatments (5, 10 or 20 µL L^{-1}) for two hours exhibited enhanced chilling tolerance only in Midknight Valencia fruit during cold quarantine treatment (1°C for 21 d). Possibly, NO fumigation may have protected the membrane from damage through the reduction of ROS and enhanced levels of antioxidants in the rind of Midknight Valencia sweet orange. Previously, NO aqueous solution (1µmol L^{-1}) has been reported to protect kiwi fruit from oxidative damage caused by ROS through an enhanced activity of antioxidant enzymes (Zhu et al., 2008). Fumigation with NO (5µL L^{-1}) exhibited reduced (2.9 %) water loss in Lane Late but not in

Midknight Valencia after cold quarantine treatment (1°C for 22 d), which may be ascribed to the genetic difference between two cultivars. Possibly, the reduced water loss in horticultural commodities with NO treatment may be ascribed to a reduced transpiration rate as reported earlier (Ku et al., 2000). In conclusion, HWD (50±1°C for 5 min) alone or when combined with TBZ (20 mg L^{-1}) was effective in reducing CI in Lane Late and Midknight Valencia caused during cold quarantine treatment (1°C for 21 d). Moreover, MJ (0.05 mM) 1 min dip was effective in CI in Lane Late and Midknight Valencia caused during cold quarantine treatment (1°C for 21 d).

10.6. Methyl jasmonate alleviates chilling injury and regulates quality in cold-stored Midknight Valencia orange.

MJ is considered as a signalling agent and plays a vital role in extending the postharvest life of many fruit and vegetables (Aghdam and Bodbodak, 2013). Among all the physiological and biochemical processes; MJ induces resistance in the fruit stored at low temperature. Citrus fruit develops the symptoms of CI when stored at non-freezing temperature. MJ has been reported to reduce CI in many climacteric and non-climacteric fruit. In my experimental results, one min dip treatment of MJ (0.1-0.25 mM) was the utmost effective in inducing chilling tolerance in Midknight Valencia fruit stored for 90 d at 4 or 7°C followed by 10 d simulated shelf conditions during 2014 and 2015 growing seasons (Chapter, 8 and Table 8.1). Earlier, it has been reported by Saltveit and Morris (1990) that the key area for CI is cell membrane followed by membrane disturbance and loss of membrane fluidity. The exact mechanism through which MJ reduces CI in sweet orange fruit is not yet explored. Perhaps, MJ dip application enhances chilling tolerance in Midknight Valencia through the improved activity of PAL, total antioxidants and phenolic in the rind and as a result protects the membrane from damage. Similarly, activation of PAL and increased production of phenolics and total antioxidants were involved in reducing CI in the rind of lemon during cold storage (Siboza and Bertling, 2013; Siboza et al., 2014). The activity of PAL in the rind of Fortune mandarins and Navelina were more related to the reduction of CI as compared to PPO and POD (Martinez-Tellez and Lafuente, 1992). Conceivably, it may also be argued that MJ application may have protected the fruit from membrane damage, electrolyte leakage and membrane lipid peroxidation products, such as MDA leading to the development of CI. Postharvest

MJ application also protected membrane damage in peach fruit by inhibiting ion leakage and MDA content (Jin et al., 2013). It has also been reported that MJ reduce CI in loquat fruit by reduces LOX activity and maintaining higher unsaturated/saturated fatty acid ratio (Cao et al., 2009). In conclusion, postharvest dip application of MJ 0.25 mM MJ dip for 1 min mitigates CI in Midknight Valencia oranges. Moreover, MJ 0.250.25 mM dip for 1 min has shown no CI symptoms on the fruit rind, irrespective of cold storage temperature in two years 2014-2015. MJ dip treatments also showed higher SCC/TA ratio and reduced vitamin C and total antioxidants.

10.7. Nitric oxide fumigation alleviates CI and regulates fruit quality in cold-stored sweet orange.

Nitric oxide (NO) is a free radical lipophilic diatomic gas, uncharged, scarcely polar molecule; thus, it can freely diffuse across membranes from one section to the other (Siddiqui et al., 2016). NO plays a pivotal role in various physiological responses in plants. For instance, postharvest application of NO has shown promising results in extending the postharvest life of fruit, vegetables and flowers. Cold storage of sweet orange fruit leads to the development of CI. It has been reported by Xie et al. (2008) that chilling temperature leads to oxidative burst due to over-production of ROS, for instance, H_2O_2 and $O_2^{\cdot -}$ therefore increases lipid peroxidation and ultimately leading to cellular membrane damage. Previously, oxidative stress has been reported to be involved in cold-induced rind damage in Nova and Fortune cultivars of mandarins (Sala, 1998). The experimental data showed that postharvest NO fumigation mitigates CI in Midknight Valencia and Lane Late after 90 d of cold storage followed by 10 d simulated shelf conditions (Chapter 9 Table 9.1). Probably, application of NO fumigation may have protected the membrane damage and reduced CI in Midknight Valencia and Lane Late orange through the higher activity of antioxidants in the peel by detoxifying ROS. Similarly, application of 0.5 mM SNP as a NO donor was able to reduce CI in Washington Navel orange stored at 3°C through the increased response of antioxidants and reduced level of lipid peroxidation and H_2O_2 content (Ghorbani et al., 2017). Furthermore, under chilling stress conditions in Washington Navel orange; NO treatment increased the activities of CAT, POD, SOD and APX (Ghorbani et al., 2017).

Nitric oxide (NO) fumigation reduced mean weight loss percentage only in Lane Late (Chapter 9 Table 9.2). The differential response of two cultivars may be ascribed to the genotypic differences. It may also be argued NO fumigation seems to play some role in reducing CI by reducing water loss. Previously, increased moisture loss was significantly correlated with an increased CI in Lane Late Navel orange (Henriod et al., 2005). Furthermore, NO fumigated fruit, vegetables and flowers exhibited 20 % reduced water loss due to reduced transpiration rate (Ku et al., 2000).

Fruit quality such as TA (%) was significantly reduced by all NO fumigation treatments in the juice of Midknight Valencia and Lane Late (Chapter 9 Table 9.4). The level of glucose, fructose, sucrose and total sugars in Lane Late was not affected by any of the NO treatments (Chapter 9 Table 9.5). Meanwhile, NO fumigation reduced the levels of individual and total sugars in Midknight Valencia, which suggest that NO fumigation affect sugar metabolism in the orange fruit in a genotype-dependent manner. In conclusion, NO fumigation treatments (5, 10 or 20 µL L^{-1}) considerably alleviate the CI irrespective of storage temperature in Midknight Valencia and Lane Late; however, NO (10 µL L^{-1}) treatment was utmost effective in both cultivars. All the NO fumigation treatments reduced percentage weight loss in Lane Late. Moreover, all NO treatments reduced the concentrations of glucose, fructose, sucrose and total sugars in the juice of Midknight Valencia.

10.8. Conclusions

- Pre-harvest spray application of S-ABA applied at 6 WBAH enhanced the rind colour, reduced *h*° angle; and increased CCI and levels of total carotenoids in the rind of M7 Navel orange. However, the spray application of NDGA (inhibitor of ABA biosynthesis) downregulated colour development in the rind which suggests a possible role of ABA in promoting fruit colour development in M7. S-ABA significantly reduced total organic acids; however, increased SSC/TA in the juice of M7 Navel.
- The exogenous spray application of Pro-Ca or PBZ (GA biosynthesis inhibitor) enhanced the rind colour of M7 Navel as depicted by reduced *h*° angle, enhanced CCI and levels of total carotenoids in the rind. However, no negative effects on fruit quality variables were recorded during both years.

- Pre-harvest spray application of MJ promoted the rind colour of M7 Navel from yellow toward deep orange by exhibiting reduced *h*° angle and increased CCI and the total level of total carotenoids in the rind without any adverse effects on the fruit quality.
- Postharvest HWD alone or combined with TBZ for 5 min at 50±1 °C alleviated the symptoms of CI in Lane Late and Midknight Valencia during cold quarantine storage (1°C for 21 d). NO fumigation (2 h) significantly reduced percentage weight loss and MJ dip treatment significantly enhanced CCI in Lane Late only.
- Postharvest dip application of MJ mitigates CI in Midknight Valencia stored at 7 or 4 °C for 90 d followed by 10 d simulated shelf condition during two consecutive years. All treatments of MJ exhibited reduced weight loss after 90 d of cold stored fruit. MJ dip treatments also exhibited higher SCC/TA ratio and reduced vitamin C and total antioxidants in the juice of Midknight Valencia stored for 90 d followed by 10 d simulated shelf condition.
- Postharvest NO fumigation alleviates CI in Lane Late and Midknight Valencia stored at 7 or 4 °C for 90 d followed by 10 d simulated shelf condition. All NO fumigation treatments reduced percentage weight loss in Lane Late only. NO fumigation treatments irrespective of the concentration applied reduced the concentrations of glucose, fructose, sucrose and total sugars in the juice of Midknight Valencia only.

10.9. Recommendations to the citrus industry

- Pre-harvest spray application of S-ABA (200-300 mg L^{-1}), when applied at 6 WBAH, enhanced the rind colour of M7 Navel.
- Pre-harvest spray application of Pro-Ca (800 mg L^{-1}) applied at 6 WBAH or (1200 mg L^{-1}) at 3 WBAH and spray application of PBZ (1000 mg L^{-1}) at 6 WBAH or (1500 mg L^{-1}) at 3 WBAH promoted the rind colour of M7 Navel.
- Exogenous spray application of MJ (5.0 or 7.5 mM) applied at 3 WBAH promoted the rind colour of M7 Navel.

- Postharvest HWD at 50±1°C for 5 min alone or combined with TBZ reduced CI in Lane Late and Midknight Valencia during cold quarantine treatment (1°C for 21 d) without adversely affecting quality.
- Postharvest MJ dip treatment (0.1, 0.25 or 0.50 mM) for 1 min reduced CI in Midknight Valencia stored at (4 or 7 °C) for 90 d followed by 10 d simulated shelf conditions.
- Postharvest NO fumigation (5, 10 or 20 µL L^{-1}) for 2 h alleviated the symptoms of CI in Midknight Valencia and Lane Late stored at (4 or 7 °C) for 90 d followed by 10 d simulated shelf conditions.

10.10. Disclaimer:

These recommendations are purely based upon two-year experiments conducted at one location at Moora Citrus, Dandaragan, WA. Inconsistencies may arise in the outcomes of these findings under different locations in WA. The project investigator Muneer Rehman and Curtin University accept no liability whatsoever for any reason such as negligence or otherwise arising from the reliance or use of these recommendations.

10.11. Future research

This research work focused on the role of pre-harvest spray application of S-ABA, Pro-Ca, PBZ and MJ on improving the rind colour of early maturing M7 Navel without adversely affecting the fruit quality. Meanwhile, the roles of HWD, TBZ, MJ and NO to mitigate CI during cold quarantine (1°C for 21 d) in Midknight Valencia and Lane Late were examined. Furthermore, dip treatment of MJ for 1 min to alleviate CI in Midknight Valencia and NO fumigation treatments for 2 h in Midknight Valencia and Lane Late were investigated. However, future research work on improving the rind colour and alleviation of CI in sweet orange fruit is required in the following areas.

The role of endogenous S-ABA in improving the rind colour of M7 Navel needs to be investigated.

- The exact mechanism through which MJ enhanced rind colour and its interaction with ethylene warrants to be investigated.
- The effect of S-ABA, Pro-Ca, PBZ and MJ on the accumulation of individual carotenoids such as β-xanthophylls, violaxanthin and β-cryptoxanthin in the rind of M7 Navel needs to be explored.
- The effect of S-ABA, Pro-Ca, PBZ and MJ on the expression of genes involved in citrus fruit colour development warrants to be investigated.
- The effect of S-ABA, Pro-Ca, PBZ and MJ on the regulation of sweet orange fruit colour development at different locations in WA is yet to be investigated.
- The effect of MJ on the activity of PAL, PPO, POD and total antioxidants in the rind of chill injured fruit warrants to be investigated.

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