Department of Environment and Agriculture

Role of Polyamines and Ethylene in Creasing of Sweet orange Fruit

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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 of diploma in any university.

Signature: _____

Date: _____

Dedication

To

My father (Late Manzoor Hussain),

My mother (Ayesha),

My sisters (Late Sakena, Robina and Zarenna)

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.

I have the part of my eyes to admire blessings of the compassionate and omnipotent because the words are bound, knowledge is limited and time is short to express his dignity. It is one of the infinite blessings of Allah that he bestowed me with potential and ability to complete the present research program in time and make a material contribution towards the deep oceans of knowledge. May peace and Blessings of Allah Almighty be upon His all Prophets including Muhammad (PBUH), His last messenger, who is the fountain of knowledge and guidance for the salvation of mankind in this world and the hereafter?

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Abstract

Creasing in sweet orange [*Citrus sinensis* (L.) Osbeck] fruit is a physiological disorder. It is also known as albedo breakdown, 'puffing', 'grooving', 'wrinkle skin' and 'crinkly skin'. Creasing affects different cultivars of sweet orange including Navelina, Washington Navel, Lane Late, Valencia and Nova Mandarins. It is often worse in 'Navel' orange and appears before and after the colour break stage of fruit development. Creasing was first reported from South Africa during 1938 and currently causes serious economic losses in the sweet orange industry in different parts of the world such as Australia, USA, Israel, Uruguay, Spain, South Africa and China.

In Australia, creasing is a major cause of fruit diversion from fresh markets to processing, and more than 50% of the sweet orange crop is affected to varying degrees (20-90%) due to this disorder. The involvement of polyamines in creasing and rind thickness of sweet orange fruit was investigated by monitoring the endogenous levels of free polyamines such as putrescine (PUT), spermidine (SPD), spermine (SPM) and total polyamines in the albedo and flavedo tissues of the fruit with the exogenous applications of PUT and a reversible inhibitor of polyamine biosynthesis (Guanylhydrazone; MGBG) at different fruit developmental stages. All the treatments of the PUT significantly improved the rheological properties of the fruit rind, physiological and biochemical properties of the fruit, irrespective of stage of application. In conclusion, higher levels of free PUT, SPD, SPM and total polyamines in the albedo and flavedo tissues of the fruit, a substantial reduction in creasing index with exogenous application of PUT, and its acceleration with application of MGBG, suggests the involvement of polyamines in creasing of sweet orange fruit.

The exogenous spray application of PUT (500-1000 µM) significantly reduces the incidence of creasing when applied at the golf ball stage in both cultivars of sweet orange. The PUT (1000 µM) applications also improve the textural properties of the fruit and rind including fruit firmness, rind hardness, rind tensile strength, fruit weight and diameter, individual sugar and total sugars, ascorbic acid and total antioxidants compared to the control in sweet orange cv. Washington Navel and Lane Late during 2010-11 and 2011-12.

The involvement of ethylene in creasing of sweet orange fruit was also investigated by monitoring the changes in endogenous levels of ethylene in the creased and normal fruit, effects of exogenous applications of ethrel and inhibitors of ethylene biosynthesis on creasing index. Endogenous levels of ethylene were significantly higher in the creased fruit than the normal ones in different cultivars of sweet orange including Navelina, Washington Navel, Lane Late and Valencia. Exogenous spray application of ethrel at the rate (250-750 mg L^{-1}) at mature fruit elevated the endogenous levels of ethylene in the fruit 30 to 120 days after spray (DAS) and increased the creasing index (CI) as compared to the control in cv. Washington Navel and Lane Late.

Similarly, the spray application with AVG (60 mg L^{-1}) or $CoSO_4$ (500 mg L^{-1}) improved the rind textural properties, physiological and chemical characteristics of both cultivars. The exogenous application of AVG (20-60 mg L^{-1}) or CoSO4 (125-250 mg L^{-1}) increased the fruit firmness, rind hardness and rind tensile strength compared to the control, irrespective of its application at the fruit set, the golf ball or at the colour break stage in both cultivars during 2011 and 2012. The results also indicated that the soluble solid concentration, contents of total antioxidants and total sugar were higher in cv. Lane Late than the cv. Washington Navel at higher concentration of AVG (60 mg L^{-1}) or $CoSO_4$ (500 mg L^{-1}) at the golf ball or fruit set stage; whilst total antioxidant levels were higher in cv. Washington Navel than the cv. Lane Late at the same concentration of AVG or $CoSO_4$. In conclusion, a substantial reduction in creasing with exogenous application of AVG or $CoSO_4$ suggests the involvement of ethylene in creasing of sweet orange fruit.

The higher levels of endogenous ethylene and lower levels of polyamines in the albedo tissues of the creased fruit than in the normal fruit in different cultivars of sweet orange and reduction in the incidence of creasing with the exogenous application of ethylene inhibitors (AVG, $CoSO_4$ or PUT), suggests the involvement of ethylene and polyamines in creasing of sweet orange fruit. I also investigated the mode of reduction in creasing with the exogenous application of PUT, AVG or $CoSO_4$ through determining the changes in the levels of the total, water soluble and water insoluble pectins as well as the activities of cell wall degrading enzymes such as PE, *exo*-PG, *endo*-PG, and EGase in the albedo and

flavedo tissues of the fruit at the various fruit developmental stages such as golf ball and ripe stage.

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List of symbols and abbrevations

×	multiply / interaction between
>	Greater than
≤	less than or equal to
±	plus minus
/	divide
=	equal to
´	minutes
°	degree
°C	degree celcious
%	percent
β	beta
P	picomole (s)
µg	microgram(s)
µL	Microliter (s)
µmol	micromole(s)
1-MCP	1-Methylcyclopropene
2-4-5-T	2, 4, 5-Trichorophenoxyacetic acid
2,4-D	2, 4-Dichlorophenoxyacetic acid
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
ADC	Arginine decarboxylase
a.i.	active ingredient
ANOVA	analysis of variance
AOA	Aminooxyacetic acid
AOC	Allene oxidase cyclise
AOS	Allen oxide synthase
APX	Ascorbate peroxidase
AVG	Aminoethoxyvinylglycine
BSTFA	*N,O*-Bis (trimethylsilyl) trifluoroacetamide
Ca	Calcium
$CaCl_2$	Calcium chloride
CAD	Cadaverine
CEPA	2-chlooethylphosphonic acid
C_2H_4	Ethylene
CHA	Cyclohexylamine
CI	Creasing Index
cm	Centimetre(s)
Co	Company
CO_2	Carbon dioxide / respiration
$CoSO_4$	Cobalt sulphate
conc.	concentration
$CoCl_2$	Cobalt chloride
CPPU	N-(2-chloro-4-pyridyl)-N′-phenylurea

cv	cultivar
d	day(s)
dH_2O	Disttle water
DAFB	Day after full bloom
DAFWA	Department of Agriculture and Food, Western Australia
DAFS	Days after fruit set
DFMO	Difluoromethylornithine
DFMA	Difluoromethylarginine
DAS	Day after Spray
DAH	Day after harvest
DAT	Day after treatment
DPH	Day prior harvest
DPPH	2, 2-diphenyl-1-picryl-hydrazyl
DW	Dry powder
E	East
EDTA	Ethylene diamino tetra acetic acid
EFE	Ethylene-forming enzyme
EGase	*Endo*-1,4-*β*-D-glucanase
EGTA	Ethyleneglycol-bis (beta -aminoethylether)-N,N'-tetraacetic acid
Endo-PG	*endo*-polygalacturonic acid
et al	et alia
Exo-PG	*Exo*-polygalacturonic acid
FAO	Food and Agriculture Organisation
FAOSTAT	Food and Agriculture Organisation Statistics
$FeSO_4$	Ferrous sulphate
FID	Flame ionization detector
Figure	Figure
FW	Fresh weight
g	gram(s)
MGBG	Guanylhydrazone
GC	Gas chromatograph
GA_3	Gibberellic acid
GR	Glutathione reductase
h	Hour(s)
h°	Hue angle
ha	hectare(s)
H_2SO_4	Sulphuric Acid
HCl	Hydrochloric acid
HgCl	Mercury chloride
HLA	13-hydroperoxylinolenic acid
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
J	Joules
JAs	Jasmonates
kg	kilogram(s)
KOH	Potassium hydroxide
kPa	kilo pascals
KMnO4	Potassium permanganate

L	Litre(s)
L*	Lightness
LSD	Least significant difference
Ltd.	Limited
LOX	Lipoxygenase
m	meter
mm	Milli meter
M	Molar
MA	Madison
MeOH	Methanol
mg	milligram(s)
$MgCO_3$	Magnesium carbonate
min	minute(s)
ml	millilitre(s)
mM	millimolar(s)
mmol	millimole(s)
Mt	Metric tonnes
N	Newton
n	Number of replication
NA	Not available
NAA	Naphthalene acetic acid
NaCl	Sodium chloride
NaF	Sodium fluoride
$NaHSO_3$	Sodium hydrogen sulphite
NaOH	Sodium hydroxide
NaOCl	Sodium hypochlorite
Na_2SO_4	Sodium sulphate
ng	nanogram(s)
nl	nanolitre(s)
nmol	nanomole(s)
NO	Nitric oxide
ns	Not significant
NSW	North South Wales
O_2	Oxygen
ODC	Ornithine decarboxylase
OA	Oxalic acid
P	Probability
PA	Pennsylvania
PAs	polyamines
Pa	Pascals
p.s.i.	Pounds per square inch
PAL	Phenylalanine ammonia-lyase
PCIB	α(p-Chlorophenoxy)isobutyric acid
PC	Pre-climacteric
PE	Pectin esterase
PG	Polygalacturonic acid
PGRs	Plant growth regulators
pH	Symbol denoting hydrogen ion in a solution
PL	Pectin lyase

POD	Peroxidase
ppb	Part per billion (10^{-9})
ppm	Part per million (10^{-6})
PPO	Polyphenol oxidase [catechol oxidase]
psi	Pounds per square inch
PUT	Putrescine
PRD	Partial root zone drying (PRD)
PVP	Polyvinylpyrolidone
r	Correlation coefficient
rcf	Relative centrifugation force
RH	Relative humidity
RDI	Regulated deficient irrigation
ROS	Radical oxygen species
rpm	Rounds per minute
S	South
s	second(s)
SA	Salicylic acid
SAM	S-adenosyl methionine
dSAM	Decarboxylated-SAM
SAMDC	S-adenosyl methionine decarboxylic acid
S.E.	Standard error
sp.	species
SPD	Spermidine
SPM	Spermine
SSC	Soluble solids concentration
Std	Standard
Treat	Treatment
TA	Titratable acidity
tan	Tangent
TAPP	Tanzania Agriculture Productivity Program
TBZ	Thiabendazole
Trolox	6-hydroxy-2, 5, 7, 8-tetramethychroman-2-carboxylic acid
UK	United Kingdom
USA	United States of America
UV	Ultra-violet
VIC	Victoria
VIS	visible
vs.	versus
v/v	volume by volume
WA	Western Australia
WAFB	Weeks after full bloom
WPH	Weeks prior harvest
w/v	weight by volume
w/w	weight by weight

CHAPTER 1

General introduction

Citrus is predominantly cultivated in the tropical and subtropical regions of the world between 40˚ north and south latitude, in more than 137 countries including six continents (Ismail and Zhang, 2004). Amongst citrus species, sweet orange (*Citrus sinensis* L. Osbeck) occupies a dominant position in the orange-producing countries in the world (FAOSTAT, 2013). Sweet orange is widely consumed both as fresh fruit and juice. Its global demand is attributed to higher vitamin C and total antioxidants (Goristein et al., 2001). Sweet oranges probably originated from central China and north-east India including Navel, Common, Acid less and Pigmented oranges. Navel oranges are mainly produced for the fresh market and are known for their larger size, seedlessness and early maturity compared to the other types of oranges (Horticulture Australia Limited, 2004). Australia is one of the major orange-producing countries with total production of 291,223 tonnes (FAOSTAT, 2013). New South Wales is the leading state of Australia, which produced (48%) compared to South Australia (30%), Queensland (3%), Victoria (17%) and Western Australia only 2% (Australian Bureau of Statistics, 2012). Western Australia contributes about 2% of the total sweet orange production of Australia which includes about 160 commercial citrus growers out of 2800 nationally (DAFWA, 2010).

Citrus fruit quality is a prime concern to growers; and domestic and international consumers. The appearance of citrus fruit is determined by rind characteristics such as colour, gloss, smoothness and firmness. The attractiveness of the citrus fruit depends on its rind. The rind of citrus fruit is multilayered and formed by the albedo (mesocarp) and flavedo (exocarp) tissues. The albedo tissue of sweet orange fruit rind is prone to fracturing leading to physiological disorders of the rind such as creasing in sweet oranges (Monselise et al., 1976) and puffiness in mandarins (Greenberg et al., 2006; García-Luis et al., 1985). It is also known as ‘albedo breakdown’, ‘puffing’, ‘grooving’, ‘wrinkle skin’ as well as ‘crinkly skin’ and usually detectable at fruit maturity (Gambetta et al., 2000; Jona et al., 1989; Jones et al., 1976; Erickson, 1968) or at the colour break stage (Storey et al., 2002). The incidence and severity of creasing increases with the advancement of fruit maturity (Embleton et al., 1973). Multiple cracking in the pitted peel occurs due to creasing (Li et al., 2009), separation of the albedo tissue and resulting channels formation in the fruit rind (Treeby et al., 1995). The severity of creasing varies from year to year, orchard to orchard and species to species

(Gilfillan et al., 1981). Creasing mostly affects cultivars of sweet oranges such as Washington Navel (Pham, 2009; Ali et al., 2000; Gambetta et al., 2000,), Valencia (Jones et al., 1967; Monselise et al., 1976), Navelina (Pham, 2009) and Nova mandarins (Greenberg et al., 2006). Creasing in sweet orange was first reported from South Africa during 1938 (Le Roux and Crous, 1938) and is currently a major problem in the citrus industry in different parts of the world including Australia (Treeby et al., 1995), USA (Jones et al., 1967), South Africa (Holtzhausen, 1981; Bower, 2004), Israel (Greenberg et al., 2006; Monselise et al., 1976;), Uruguay (Gambetta et al., 2000), Spain (Agustí et al., 2001) and China (Li et al., 2009). Creasing causes considerable economic losses to growers due to the substantial reduction in the price by the down-grading of orange value for the fresh market in Australia (Pellizo, 1997; Sneath, 1987; Storey and Treeby, 1994) and other orange-producing areas in the world (Li et al., 2009; Bower, 2004; Ali et al., 2000; Gambetta et al., 2000; Jones et al., 1967; Monselise et al., 1976). A huge proportion of fruit (from 50 to 90%) could be influenced by creasing in some localities in South Africa, Australia and Israel, respectively (Greenberg et al., 1996; Goldie, 1998). It is estimated that with each 1% reduction in creasing producers' income will increase by 1 to 2 million dollars in Australia (Treeby and Storey, 1994).

The incidence and severity of creasing disorder has been known to be influenced by multiple factors such as genotype (Agustí et al, 2003; Moulds et al., 1995; Treeby et al., 1995; Bevington et al., 1993), climate (Gambetta et al., 2000; Jones et al., 1967), rootstock (Storey et al., 2002), crop load (Jones et al., 1967), rind thickness (Holtzhausen, 1981), irrigation (Treeby et al., 2007; Agustí et al, 2004), mineral nutrition (Pham et al., 2012; Bower, 2004; Storey et al., 2002; Treeby and Storey, 2002; Ali et al, 2000; Jones et al., 1967) and plant growth regulators (Dick, 1995; Treeby and Storey, 1994; Tugwell et al., 1993; Jona et al., 1989; Embleton et al., 1973). Creasing mainly affects the fruit rind tissue and does not influence fruit quality (Goldie, 1998; Pham, 2009). However, the association between fruit quality and creasing has been reported by Sneath (1987), Jones and Embleton (1967), Jones et al. (1967) reporting that a higher percentage of juice and lower ascorbic acid content were linked with the incidence of creasing. Previously, Jones and Embleton (1967) reported higher soluble solids concentration and acid ratio in the creased fruit which indicated that fruit with creasing mature earlier than normal fruit on the same tree.

Several different approaches have been followed to control incidence of creasing in sweet orange. However, these control measures neither prevent creasing completely nor

reduce it substantially. However, the application of gibberellin (GA_3), a plant growth regulator; and calcium, an essential nutrient; have been used commercially to control creasing. The GA_3 application is being practiced to reduce the incidence and severity of creasing in sweet orange in different parts of the world (Coggins, 1969; Embleton et al., 1973; Jones et al., 1967). The spray application of GA_3 does not prevent creasing and only delays the onset of creasing (Bower, 2000). It also adversely impacts fruit rind colour development (Coggins, 1969, 1981), reduces fruit size (Jona et al., 1989) and slightly increases the juice content (Coggins, 1969). However, calcium spray has been used in Australia with limited success (25 to 30% reduction) in creasing of sweet orange as reported by Treeby et al. (2002) and Pham et al. (2012).

Creasing in sweet orange fruit has been reported to be associated with enhanced loss of pectin, starch in the cell walls of albedo, leading to cell wall loosening and formation of cracks consequently reducing hardness, stiffness and tensile force of the rind (Saleem et al., 2014; Monselise et al., 1976). Polyamines (PAs) act as an anti-senescence agent and are involved in cell division, strengthening of cell wall components and inhibition of both ethylene biosynthesis and cell wall degradation (Kumar et al., 1997; Tiburcio et al., 1993; Rastogi and Davies, 1991). PAs also restore cell wall thickness and are necessary for maintaining cell wall structure by strengthen the links between cell wall components (Berta et al., 1997). In different citrus species, the fruit growth and development are multiple processes and coordinated by the endogenous levels of PAs (El-Otmani et al., 1995). It has been reported that ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) had the greatest activity in fully developed but unripe mandarin fruit (Nathan et al., 1984). PAs have a physiological role in cell walls by interacting directly with wall components such as pectins (D'Orazi and Bagni, 1987). Polyamines are essential for strengthening the links between cell wall components by maintaining cell wall characteristics (Berta et al., 1997). However, no research work has been reported on the involvement of PAs in causation of creasing in sweet orange and their effects on rind and fruit quality.

Ethylene is a naturally-occurring gaseous plant hormone produced in all higher plants and is usually associated with various physiological processes commencing from seed germination to organ senescence (Kende and Zeevaart, 1997). Ethylene plays an important role as an inducer of fruit ripening (Bleecker, 2000) and accelerates softening in citrus fruit (Ladaniya, 2007). Creasing is similar to fruit softening caused by cell wall disassembly (Nishiyama et al., 2007; Brummel, 2006; Orfila et al., 2002). Softening is known as a

ripening process and associated with biochemical changes in cell wall fractions involving hydrolytic processes resulting in breakdown of cell wall polymers such as cellulose, hemicelluloses and pectins (Payasi et al., 2009). Liu et al. (2006) reported that creasing is closely associated with fruit softening in sweet orange fruit. Citrus is classified as a non-climacteric fruit, which produce only small amounts of ethylene, while ethylene plays an important role in changing fruit colour, flavour, chemical composition and texture in citrus fruit (Ladaniya, 2007). Ethylene has been found to enhance the chilling injury (Yuen et al., 1995), decay (Brown and Lee, 1993) and off-flavours in various fruit (Testoni et al., 1992). Some initial reports suggested that higher levels of endogenous ethylene were found in the albedo tissue of creased Valencia (Monselise et al., 1976) and Washington Navel sweet orange (Pham, 2009). However, the *in vivo* role of ethylene involving exogenous applications of ethylene and its inhibitors on creasing in sweet orange fruit is yet to be investigated.

Aminoethoxyvinylglycine (AVG) and cobalt sulphate ($CoSO_4$) are well known for the inhibition of ethylene biosynthesis, which suppresses the ethylene production in different plant tissues by inhibiting ACC synthase and also reduces the activities of cell wall degrading enzymes (Ladaniya, 2007; Even-Chen et al., 1982; Hyodo and Nishino, 1981; Yu and Yang, 1979; Boller et al., 1979; Yu et al., 1979). AVG and $CoSO_4$ act as anti-senescence agents regulate growth and differentiation as well as compete directly with the biosynthesis of ethylene (Bagni and Torrigiani, 1992). Exogenous application of AVG and $CoSO_4$ has been reported to maintain the fruit quality in mango (Wahdan, 2011; Singh and Agrez, 2002; Singh et al., 1994; Singh and Singh, 1993), pomegranate (Reddy et al., 2011), olive (Gad et al., 2006), peach (Kim et al., 2004; Kluge and Jacomino, 2002; Bregoli et al., 2002), apple (Greene, 2005; Benincore et al., 2000), fig (Teragishi et al., 2000) and sweet oranges (Al-Husseini, 2012). However, no research work has been reported on the regulation of ethylene biosynthesis by employing its inhibitors on creasing and fruit quality of sweet oranges, hence investigation is warranted. It was therefore hypothesised that PAs and ethylene play a role in causation of creasing; regulation of rheological properties of rind; and fruit quality in sweet oranges. The specific objectives of this research were:

- To investigate the involvement of polyamines in causation of creasing in sweet orange fruit by monitoring the changes in endogenous levels of free PAs (PUT, SPD, SPM and total polyamines) in the albedo and flavedo tissues of fruit with the exogenous applications of PUT and a reversible inhibitor of polyamine biosynthesis

guanylhydrazone (MGBG) at different fruit developmental stages and their effects on percent creasing index (CI) and rheological properties of the rind.

- To evaluate the efficacy of exogenous applications of different concentrations of PUT and inhibitor of PAs biosynthesis (MGBG) at various fruit developmental stages on incidence of creasing and fruit quality in sweet orange cv. Washington Navel and Lane Late.
- To investigate the role of ethylene in creasing of sweet orange fruit by determining the endogenous levels of ethylene in the creased and healthy fruit as well as the effects of up and down regulation of ethylene production with the exogenous application of ethylene and its inhibitor such as AVG or $CoSO_4$ on the incidence of creasing and fruit quality in sweet orange.
- To elucidate the mode of reduction of creasing with the exogenous application of PUT, AVG or $CoSO_4$ through determining the changes in the levels of total, water soluble and water insoluble pectins as well as the activities of cell wall degrading enzymes such as PE, *exo*-PG, *endo*-PG, and EGase in the albedo and flavedo tissues of the fruit at the golf ball and ripe stage in sweet orange cv. Washington Navel and Lane Late.

CHAPTER 2

General literature review

2.1. Introduction

Citrus fruit ranks as the largest tree crop in the world in terms of production (FAOSTAT, 2012). Sweet orange (*Citrus sinensis* L. Osbeck) is one of the major profitable fruit crops that is widely consumed both as fresh fruit and juice (Kalac and Krausová, 2005). Higher vitamin C content and antioxidants boost the global demand for sweet oranges (Goristein et al., 2001). Sweet oranges probably originated from north-east India and adjoining portions of China and Burma. Sweet oranges spread to the eastern Mediterranean basin through Africa by Arab traders, while crusaders brought the fruit to Spain, Italy and Portugal around 1000 AD (Scora, 1975). Columbus introduced sweet oranges to the western hemisphere during 1493 and was introduced to South Africa by a Dutch merchant in 1654 (Oberholzer, 1969). Sweet oranges are mostly produced for the fresh market and are well known for their large size, seedlessness and early maturity as compared to the other types of oranges (Horticulture Australia Limited, 2004). Presently, citrus is mainly cultivated in the tropical and subtropical regions of the world between 40˚ north and south latitude including six continents (Ismail and Zhang, 2004). Sweet oranges are grown in 144 countries; grape fruit (*Citrus paradise* Macf.) and pummelos (*Citrus maxima* Merr.) in 74 countries; and lemons/limes (*Citrus limon* [L.] Burm: *Citrus aurantifolia* [Christm] Swing.) in 94 different countries of the world (FAOSTAT, 2003).

2.2. Citrus production and trade in the world

Sweet orange producing countries of the world and their total production (%) in 2011-12 are presented in Figure 2.1. Among these countries, Brazil is one of the leading (36%) orange producers in the world followed by the USA and China contributing (16 and 13% respectively) (FAOSTAT, 2013).

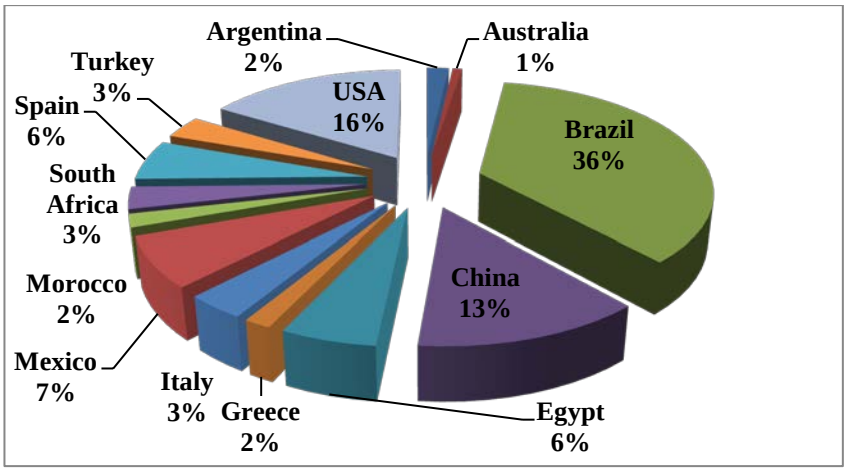


Figure 2.1. Major sweet orange producing countries in the world during 2011-12 (FAOSTAT, 2013).

Spain is a leading country for oranges export in the world with export quantity of 1526.6 thousand tonnes followed by South Africa (975.5, 000 tonnes) and USA (752.0, 000 tonnes) during 2012 (FAOSTAT, 2013). Australia ranked the eleven among the other orange exporting countries with (106.54, 000 tonnes) during 2012.

Table: 2.1. Major sweet orange exporting countries of the world during 2012.

Countries	Export Quantity (Tonnes)	Value 1000 USA$
Spain	1526.6	1325.6
South Africa	975.5	591.7
USA	752.0	649.6
Greece	400.1	203.0
Turkey	366.3	262.9
Netherlands	192.3	197.4
Morocco	171.6	123.5
China	157.1	128.2
Italy	135.4	119.2
Argentina	126.6	54.6
Australia	106.5	101.4
Portugal	37.7	37.7
Brazil	33.3	16.4
Mexico	16.5	7.0

(FAOSTAT, 2013)

2.3. Citrus production in Australia

Major citrus-producing areas in Australia are New South Wales, South Australia, Victoria, Western Australia, Queensland and the Northern Territory. New South Wales is the leading state which produced 31% followed by South Australia (29%) and Queensland (23%) of total sweet orange production in Australia (Figure 2.2).

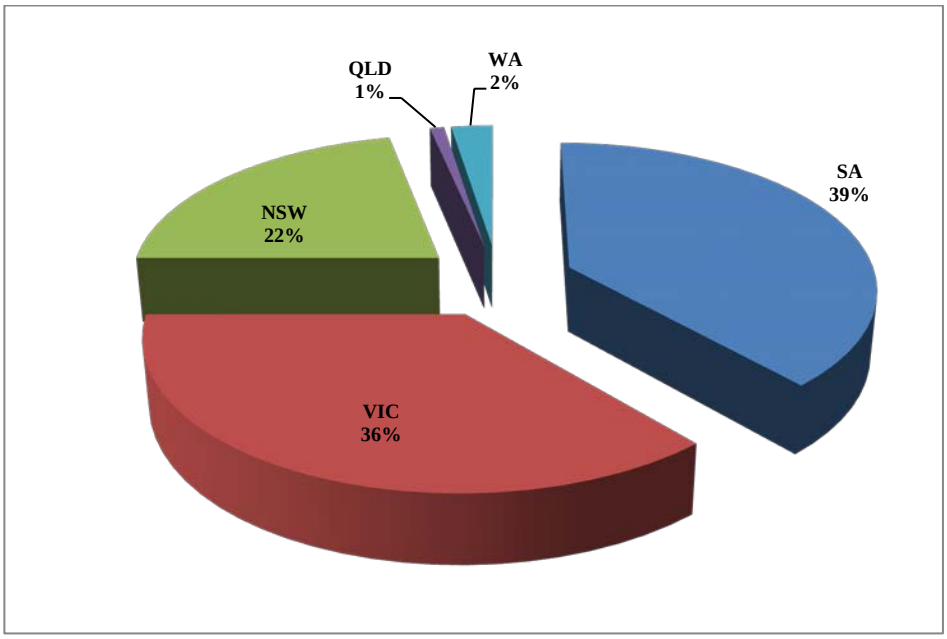


Figure 2.2. Sweet orange production in Australia (Citrus Australia, 2013)

In Australia, grapefruit, lemons, limes, mandarins and oranges are also grown, whilst orange production ranks first (78%) followed by mandarins (12%) and a smaller share of lemons, grapefruit and limes (Australian Bureau of Statistics, 2008) (Figure 2.3). However, the recurring incidence of creasing is a problem affecting the citrus industry of Australia and also the world.

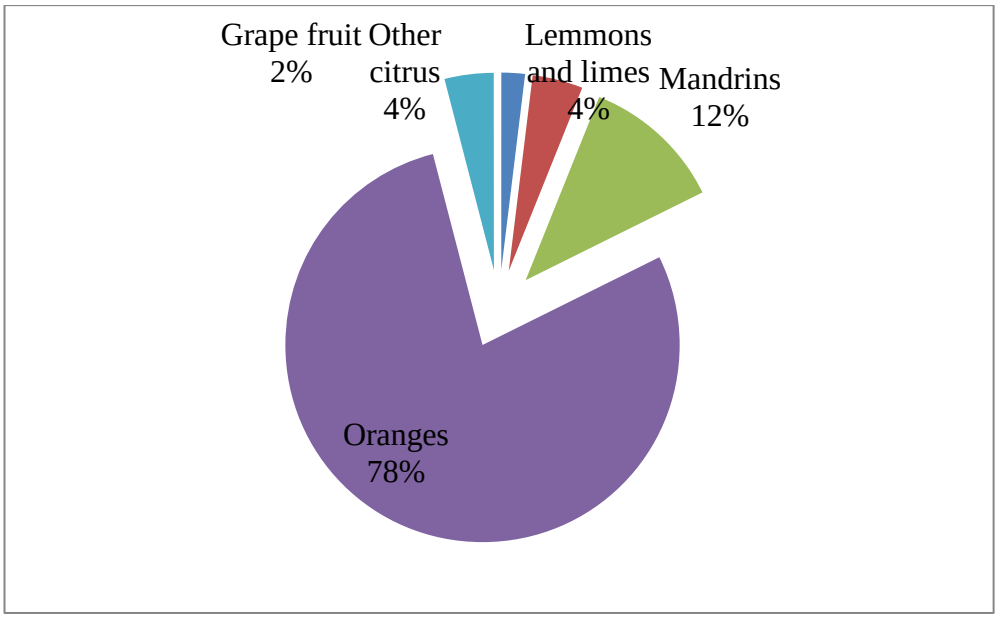


Figure 2.3. Production in different groups of citrus in Australia (Australian Bureau of Statistics, 2008).

2.4. Export of Australian oranges

Australia is the largest supplier of oranges to Japan in the southern hemisphere export season. In 2012, Australian sweet orange export accounted for 21 percent of all the oranges exported to Japanese markets, from a total of 83% of imports from the southern hemisphere. Similarly, Australia was a major orange exporting country to China, Malaysia, Singapore and the United States during 2012 (International Trade Centre, 2013). There was a significant reduction in the export of Australian oranges to the United States market during 2007 to 2012 (Table 2.2). Most oranges exported from Australia to the United States are sweet oranges, which are mainly exported during the non–producing months of May to September in the United States.

Table 2.2. Top five export destinations for Australia oranges (000'tonnes)

	2007	2008	2009	2010	2011	2012
China	27	18.6	22.5	17.4	18.8	30.9
Japan	15	13.4	18	27.4	24	29.8
Malaysia	12	13.4	15.1	4.2	15.5	11.6
Singapore	7	8.4	8.3	5.4	8.8	10.4
USA	31	21.8	23.6	15.7	8.1	11.2
Sub-total	91.1	75.6	87.5	70.1	75.2	93.9

(International Trade Centre, 2013)

2.5. Citrus fruit growth and quality

2.5.1. Citrus flowering and fruit set

Citrus species have comparatively long juvenility periods (two to five years) before the trees reach the mature stage to produce flowers. In citrus, the flower formation is related to deciduous trees (Monselise and Goldschmidt, 1982), but the fruit development is a long process and takes at least six months for early cultivars such as Marisol Clementine mandarin or Satsuma mandarin; and it takes at least 11 months for late cultivars such as Valencia and Lane Late oranges (El-Otmani et al., 2000). Flowering is necessary for production of citrus fruit and in citrus the inflorescence development is divided into leafless or leafy and these may carry a single flower or a bunch of citrus flowers (Goldschmidt and Huberman, 1974). Sweet orange flower are born on cymose inflorescence, which produces one to many flowers (Lovatt et al., 1988; Lord and Eckard, 1985; Monselise, 1985). Citrus usually produce a huge number of flowers depending on the citrus cultivar, tree age and environmental conditions (Monselise, 1986). Sweet oranges may produce 250 thousand flowers per tree in a season and less than 1% fruit set occurs (Erickson and Brannaman, 1960; Goldschmidt and Monselise, 1977). However, the fruit set is the critical phase in the transformation of the flower into a fruit for increasing grower's return (Lovatt, 1999). Fruit set is influenced by exogenous application of different plant growth regulators (Lovatt et al., 1992; Bower et al., 1990; Monselise and Goldschmidt, 1982). Polyamine spray application increases the fruit set in different fruit crops such as apple (Costa and Bangi, 1983), olive (Rugini et al., 1986), pear (Franco-Mora et al., 2005; Crisosto et al., 1988 ;), avocado (Loupassaki et al., 1995) and sweet orange (Saleem et al., 2008).

2.5.2. Fruit growth and rind thickness

Citrus is classified into a special type of berry known as a hesperidium, which is composed of pericarp (peel or rind) and the endocarp (pulp), which is the edible portion of the fruit (Iglesias et al., 2007). The pericarp is further divided into flavedo (exocarp), which is the external coloured portion and the albedo (mesocarp), the white layer of the citrus peel (Figure 2.4A). However, the fruit pulp consists of segments which are enclosed in a locular membrane and filled with the juice vesicles that are the ultimate sink organ of the citrus fruit (Iglesias et al., 2007; Ladaniya, 2007; Spiegel-Roy and Goldschmidt, 1996; Godden, 1988). Fruit growth is divided into three different stages (Iglesias et al., 2007; Godden, 1988; Bain, 1958). Stage 1 lasts between 4-9 weeks starting with cell division and continuing until full bloom is completed in all tissues except the outermost cell layers. In this fruit developmental stage albedo retains 90% of the fruit volume (Spiegel-Roy and Goldschmidt, 1996). However, this stage normally occurs from October to about mid December in Navel oranges in Australia (Hutton et al., 2007). At stage II, cell enlargement occurs with morphological and physiological changes in the fruit. At this stage the volume of albedo decreases (Godden, 1988). In Australia, this stage continues from 15 December to 15 July in Valencia oranges (Bain, 1958) and 15 December to 15 May in Navel oranges (Hutton et al., 2007). Stage III is known as the fruit maturation stage. This stage continues until harvesting of the fruit. In sweet oranges, stage III commences from mid May to November in Western Australia (Hutton et al., 2007). Stage II is most important for citrus fruit with growth and maximum increase in fruit weight and fruit diameter (Figure 2.4B) (Godden, 1988). However, the fruit weight, diameter and volume significantly reduce in stage III. The rind thickness increases and reaches its maximum at the end of the cell division stage. The rind becomes thinner during cell enlargement and rind thickness slightly increases during stage III with the expanding pulp (Figure 2.4a) (Spiegel-Roy and Goldschmidt, 1996; Bain, 1958). In Valencia oranges, rind reaches maximum thickness at the end of the cell division stage.

2.5.3 Fruit ripening

Citrus fruit growth and development consists of three different stages such as cell division, water accumulation and fruit ripening (Bain, 1958). During phase III, fruit growth ceases and the fruit undergo the process of ripening as well as colour change in fruit. The process of citrus fruit external colour development is of particular economic importance and is a critical quality parameter for the fresh market (Alós et al., 2006). However, the colour change is the transition of chloroplast to chromoplast which includes chlorophyll degradation and carotenoid accumulation in citrus and other fruit. The processes of colour change in fruit are under the control of environment; and nutritional and hormonal status (Alós et al., 2006). Environmental conditions influence chromoplast transformation and is expected when exposing plants to different environmental regimes, for example, low temperatures (Young and Erickson, 1961). The effects of nutrients and hormones are typically assessed by external chemical application. Therefore, it has been confirmed that sucrose accelerates the flavedo colour change in citrus fruit, whilst nitrogen application delays chromoplast formation (Iglesias et al., 2001, Huff, 1984). It has been previously reported that ethylene and gibberellins have contrary effects on the regulation of chlorophyll degradation in the flavedo (Jacob-Wilk et al., 1999). However, ethylene plays a key role in natural colour development in non climacteric fruit such as citrus; ethylene biosynthesis is minimal during the fruit development in growth phase III, when colour changes occur in citrus fruit (García-Luis et al., 1985). Whilst ethylene plays a role in regulating fruit ripening (Alba et al., 2005; Jacob-Wilk et al., 1999; Eilati and Goldschmidt. 1996), contrarily, gibberellin application is usually used to delay the colour development in several citrus species **(**Porat et al., 2001). In addition, an increase in sucrose levels has been shown to encourage colour development in citrus fruit (Iglesias et al., 2001; Huff, 1984). Colour change is the first step towards ripening. However, chlorophyll level is decreased, carotenoids and soluble solids concentrations are accumulated, while the organic acid level declines and the concentration of volatiles increases during fruit ripening **(**Katz et al., 2011; Yu et al., 2012). Plant hormones are very important for fruit development and ripening (Soto et al., 2013; Fan et al., 1998; Theologis, 1992). Abscisic acid (ABA) has been reported to promote fruit ripening in strawberry (*Fragaria ananassa*) and plays an important role in the regulation of fruit development and ripening in

strawberry (Ji et al., 2012; Jia et al., 2013 and 2011) and grapes (Koyama et al., 2010). A similar study was conducted on sweet orange, which showed that ABA was an important regulator for the onset of fruit degreening and carotenoid biosynthesis (Romero et al., 2012). Polyamines (PAs) have been linked with fruit ripening and the endogenous concentrations of PAs are involved in the ripening of fruit (Valero et al., 2002; Pandey et al., 2000). However, the exogenous application of PAs delays the fruit ripening process in apricot (Paksasorn et al., 1995), peaches (Bregoli et al., 2002), mango (Malik and Singh, 2006), nectarine (Torrigiani et al., 2004) and plum (Khan et al., 2007). The effect of PAs on sweet orange fruit ripening however, are not known and yet to be investigated.

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

In orange juice, SSC and TA significantly vary among different groups of sweet oranges and also vary between groups of the same variety, particularly in common oranges and navel orange groups (Hutton et al., 2007). However, in sweet orange, the SSC varies 10 to 20% and contains higher amounts of carbohydrates (70-80%) as well as minor quantities of organic acids, proteins, lipids and minerals (Davies and Albrigo, 1994). SSC is affected by fruit size and an increase of SSC is obtained with a decrease in fruit size (Sinclair, 1961). The levels of SSC are significantly increased due to deficit irrigation in mandarins (Gonzalez-altozano and Castel, 1999), oranges (Hutton et al., 2007; Treeby et al., 2007) and Marisol Clementine (Verreynne et al., 2001). The SSC is significantly different among orange varieties and the SSC:TA ratio is significantly higher in the fruit affected with creasing (Pretel et al., 2004; Jones et al., 1967).

TA is the main component affecting the taste of sweet orange fruit and it contains major amounts of citric acid, malic acid, oxalic acids and other related acids (Iglesias et al., 2007; Davies and Albrigo, 1994). It is reported that the TA in orange fruit is affected by soil fertility (Koo and Reese, 1977) and irrigation (Treeby et al., 2007). However, exogenous application of ethylene does not affect SSC and TA (Al-Mughrabi et al., 1989). Exogenous application of PAs is reported to increase SSC and TA in sweet orange cv. Blood Red (Saleem et al., 2008). TA is at a maximum during the growth phase II and decreases in the phase III (Figure 2.4E) due to catabolism of citric acid and an increase in the levels of sugars in the juice of

sweet orange (Monselise, 1986). The SSC:TA ratio is commonly used as the maturity index.

2.5.5. Total antioxidants and ascorbic acid

Total antioxidants are also known as anti-carcinogenic agents, which contain five main antioxidant components including phenolics, anthocyanin, flavanone, hydroxycinnamic acid and ascorbic acid in sweet orange juice. However, the antioxidant content varies in orange juice. The levels of antioxidant in fresh orange juice significantly alter depending on cultivar and fruit maturity (Rapisarda et al., 1999). In sweet orange cv. Valencia, the effect of storage time on the total antioxidant profile has a different trend, the flavanone concentration decreases with storage time, whilst ascorbic acid increases slightly (Rapisarda et al., 2008). Citrus fruit is known as a major source of ascorbic acid (Vitamin C) which is an important part of human nutrition. However, the ascorbic acid concentration in citrus juice is higher at the early fruit development stage and decreases at the ripening stage (Ladaniya, 2007). The levels of ascorbic acid vary among different sweet orange cultivars. Pretel et al. (2004) reported higher levels of ascorbic acid (78.0 mg $100ml^{-1}$) in Capuchina and lowest (29.5 mg $100ml^{-1}$) in Blanca sweet oranges. Ascorbic acid levels are significantly affected by creasing and lower levels are found in creased fruit than normal fruit (Jones et al., 1967). However, the ascorbic acid levels in sweet orange fruit are almost unchanged after harvest and during storage (Spiegel-Roy and Goldschmidt, 1996). The effects of exogenous application of PAs applied at different fruit developmental stages on the levels of total antioxidants in sweet orange fruit warrant investigation.

2.5.6. ***Organic acids and sugars***

The levels of organic acids and sugars are the major components in the juice of sweet orange fruit. The taste characteristics and organoleptic quality depend upon the nature and concentration of organic acid (Tucker, 1993). However, the organic acids and sugars vary among species and varieties, depending upon climate, rootstock and irrigation (Ting and Attaway, 1971). It has been previously reported that seven organic acids including citric, malic, quinic, tartaric, succinic, oxalic and ascorbic acids are found in the juice of sweet orange fruit. Amongst organic acids, citric acid

is a predominant organic acid in sweet orange fruit juice followed by malic acid, succinic, oxalic and tartaric acids (Iglesias et al., 2007; Albertini et al., 2006; Karadeniz, 2004; Pretel et al., 2004). However, organic acids are involved in fruit quality and regulation of acidity loss during fruit ripening is one of the main challenges for the citrus industry whereby warranting investigation. Citric acid accounts for 80-90% of the titratable acidity in fruit juice which also contains 9-15% malate and minor quantities of succinate and isocitrate (Baldwin, 1993).

Sweet orange fruit juice contains different sugars such as glucose, fructose and sucrose. However, the amount of sucrose is much higher than fructose and glucose. The sugar levels in sweet orange fruit juice are higher at the ripening stage than during the rapid growth period. The concentrations of sugars are higher in the orange juice (87.8–110.6 mg ml^{-1} juice) than in the orange rind (67.4–83.4 mg ml^{-1} fresh weight) (Ladaniya, 2007) and maximum levels occur at growth phase III (Figure 2.4D). The effects of exogenous application of PAs on the levels of total and individual organic acids as well as total sugars in the fruit juice of sweet orange are yet to be investigated.

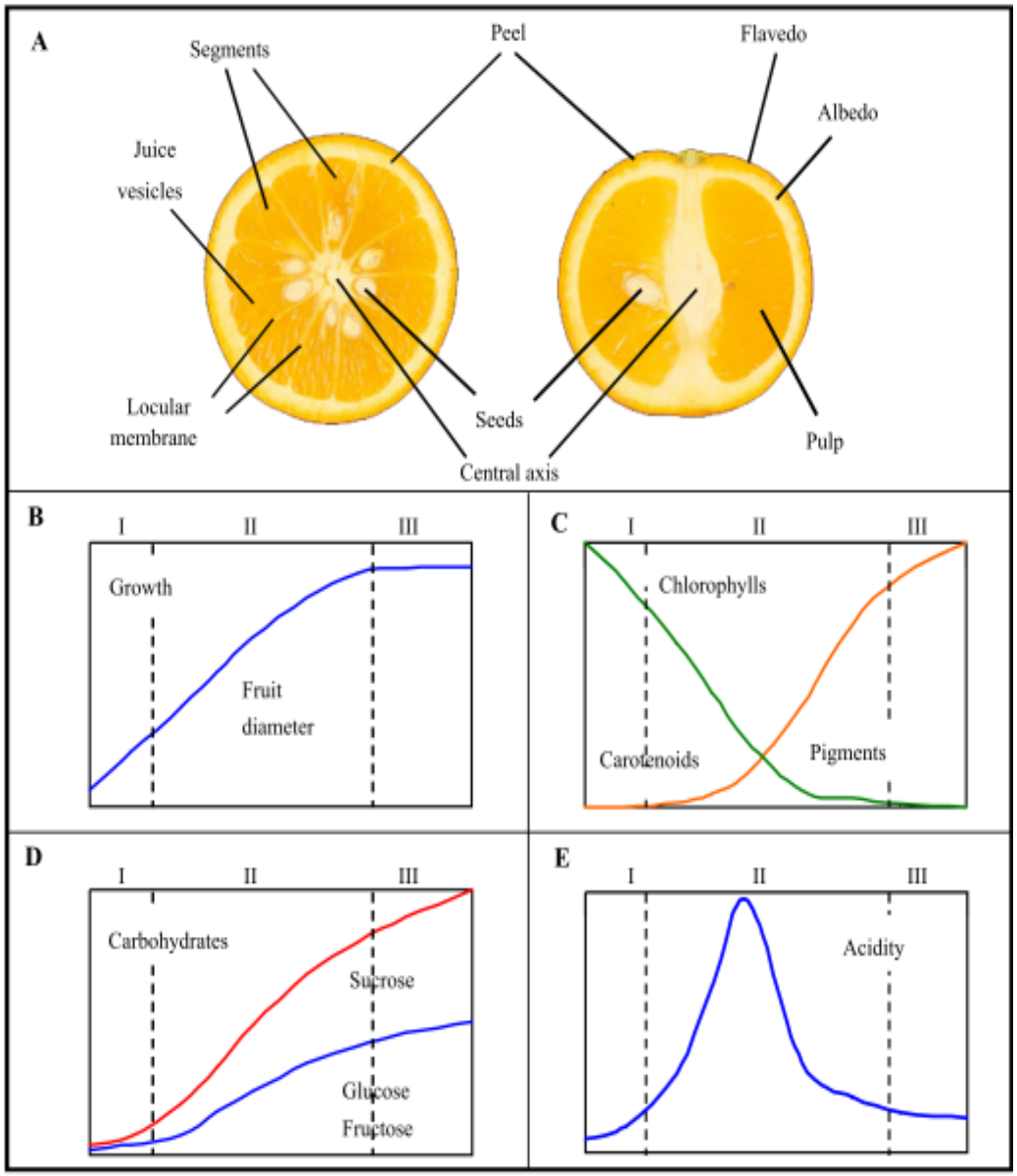


Figure 2.4. The structure and metabolic changes associated with the internal fruit development of citrus. (Iglesias et al., 2007). (A) Internal structure of sweet orange fruit. (B) Different fruit growth phases such as phase I; cell division and to water accumulation, during phase II; therefore cell enlargement, phase III; growth is arrested and fruit starts a ripening process. (C) Chlorophyll degradation and carotenoid biosynthesis in pulp. (D) Fruit growth turning toward maturation and ripening. The citrus fruit accumulates high amounts of sucrose, glucose and fructose in a 2:1:1 ratio. (E) The levels of acid accumulation in fruit pulp start during phase I and reaching a maximum around mid-phase II and decreased in the acidity of ripe fruit during phase III.

2.6. Creasing

The sweet orange fruit rind or peel is multilayered and formed by the combination of flavedo (exocarp) and albedo (mesocarp) tissues. The albedo white tissue of the citrus fruit rind is prone to fracturing and leads to rind disorders such as creasing (albedo breakdown) in sweet oranges (Monselise et al., 1976) and puffiness in mandarins (García-Luis et al., 1985). Creasing is a physiological disorder of sweet orange rind that occurs as the fruit reaches maturity and is characterized by irregular grooves running over the surface of the fruit (Figure 2.5). These grooves are caused by tissues or gaps in the inner, spongy portion of the albedo and the sinking of the overlying, oil-bearing layer (flavedo). Creasing was recognised and reported in the literature as an historic issue in sweet oranges (Jones and Embleton, 1967) and is now a serious issue in the citrus industry today.

(A) (B)

Figure 2.5. Symptoms of creasing on the flavedo (A) and albedo (B) tissue of the sweet orange fruit.

2.6.1. Symptoms of creasing

Creasing is a serious pre-harvest physiological rind disorder which is found in many citrus groves and the development of the creasing fruit varies in various cultivars of sweet oranges (Li et al., 2009). Although, creasing is usually detectable at the maturity or the colour break stage, however its initiation seems to be associated with fruit growth and development (du Plessis and Maritz, 2004; Gambetta et al., 2000; Storey et al., 2002; Storey and Treeby, 1994; Jona et al., 1989; Monselise et al., 1976). However, Abadalla et al. (1984) reported that creasing starts as early as at the

end of flowering. Visual symptoms of creasing development are separations of cells at the middle lamella of the white tissue of the albedo and fracture in the albedo and flavedo showing creases on the surface of the fruit (Treeby et al., 2000; Treeby et al., 1995; Storey and Treeby et al., 1994).

2.6.2. Effects of creasing on the citrus industry

Creasing is a major physiological rind disorder in the global citrus industry and renders large percentages of sweet orange fruit unsuitable for fresh consumption. Creasing affects different cultivars of sweet orange such as Washington Navel (Pham et al., 2012; Gambetta et al., 2000, Ali et al., 2000), Valencia (Jones et al., 1967; Monselise et al., 1976), Navelina (Pham, 2009) and Nova mandarins (Greenberg et al., 2006). However, Navel oranges are most susceptible to this physiological disorder. Creasing causes considerable economic losses, representing in some years a reduction of 50% or more of fresh fruit pack out. Creasing reduces profits to growers due to the dramatic reduction in price by the down-grading of orange value for the fresh market in Australia (Pellizo, 1997; Storey and Treeby, 1994; Sneath, 1987) and other citrus-producing areas in the world such as USA (Ali et al., 2000; Jones et al., 1967), Israel (Monselise et al., 1976; Greenberg et al., 2006), Uruguay (Gambetta et al., 2000), Spain (Augustí et al., 2001) and China (Li et al., 2009). In 1994, it was estimated that with each 1% reduction in albedo breakdown, producers' income will increase by 1 to 2 million dollars in Australia (Treeby and Storey, 1994).

2.6.3. Effects of creasing on fruit quality

Historically, a higher percentage of juice, a thin rind and lower ascorbic acid levels were linked with creasing in sweet oranges (Jones and Embleton, 1967; Jones et al., 1967; Sneath, 1987). Jones and Embleton (1967) claimed that fruit with creasing matured earlier than normal fruit on the same tree due to higher SSC and acid ratio. Contrarily, Goldie (1998) and Pham (2009) claimed that creasing mainly affects the albedo and flavedo tissues of fruit rind and did not influence fruit quality in sweet oranges. The reported research work on the effects of creasing on fruit quality is sporadic and inconclusive thus warranting further investigation.

2.7. Factors affecting incidence of creasing

2.7.1. Tree Factors

2.7.1.1. Effect of rootstock/scion on creasing

Rootstock and water relations are associated with fruit growth, development and the level of sugars accumulation of citrus fruit (Albrigo, 1977). Hence, the rootstock affects vegetative growth, crop load, fruit size and quality of sweet orange (Barry et al., 2004a; Syvertsen et al., 2000; Castle, 1995). However, rootstock has an indirect effect on creasing and tree health (Treeby et al., 1995; Treeby et al., 2000). In South Africa, the creasing incidence was higher on less vigorous rootstocks such as *Carrizo citrange* and *Swingle citrumelo* compared to the more vigorous rootstocks Volckameriana and Rough lemon (du Plessis and Martiz, 2004). A contrasting situation was found in Australia. According to Treeby et al., (2000; 1995) vigorous rootstock like Rough lemon and Rangpur lime have more chance of creasing than less vigorous rootstock such as sweet orange and Cleopatra mandarin. Treeby et al., (1995) rated from lowest to highest the creasing incidence of susceptible rootstocks to resistance as follows: Sweet orange < Cleopatra mandarin < Trifoliate orange < Carrizo citrange = Troyer citrange < Rough lemon < Rangpur lime. However, a lower incidence of creasing was observed on trees grafted on sour orange rootstock than on *Carrizo citrange* (Agustí et al., 2003). Rootstocks strongly affect fruit size and incidence of creasing varies according to fruit size (Treeby et al., 1995).

2.7.1.2. Crop load

It is a common observation that crop load and fruit size have a reciprocal relationship. A small crop load is associated with a larger fruit size. Many factors are associated with incidence and severity of creasing in sweet oranges. Generally, the tree with higher crop load and smaller fruit with thinner rinds are thought more likely to show symptoms of creasing than the larger fruit and fruit with thicker rinds (Jones et al., 1967; Le Roux and Crous, 1938). However, Treeby et al. (1995) and Gambetta et al. (2000) observed a negative correlation between crop load and creasing.

2.7.1.3. Fruit size

It has been reported that the incidence of creasing is related to fruit size (Treeby et al., 2000). It was reported that a higher incidence of creasing was observed in small fruit than in large fruit (McIntosh, 1998; Sneath, 1987; Jones et al. 1967). Treeby et al. (1995) found that smaller fruit (<62 mm in diameter) were less affected by creasing than large fruit and symptoms were less severe compared with symptoms in larger fruit. However, very large size fruit (> 79 mm) had a lower incidence of creasing than the medium sized (62-70 mm) and large fruit (71-79 mm). It was also found that creasing was worse when harvesting was delayed and fruit remained longer on the tree (Storey and Treeby, 2002).

2.7.1.4. Fruit position on the tree

It has been reported that creasing incidence was more severe on the shaded side of the tree than on the sunny side of the tree and creasing incidence was higher on the inside of the tree canopy (Treeby et al., 2000; Bevington et al., 1993; Jones et al., 1967). Gambetta et al. (2000) reported that creasing was usually greater in the shady part of the fruit as compared to the sunny part of the fruit. According to Chen et al. (2002) 86% of creased fruit were on the shaded part of the tree. The visual symptoms of creasing first occurred on the south side of the tree under southern Californian conditions (Jones et al., 1967).

2.7.1.5. Tree heredity

Jones et al. (1967) claimed that the genetic make-up of a tree has an influence on the severity of creasing. In Australia, Treeby et al. (2000) observed that early and mid-season cultivars have a higher incidence of creasing as compared to mid-late or late season cultivars. In South Africa, Bahianinha Navel oranges have a lower incidence of creasing than McClean Navels (du Plessis and Maritz, 2004).

2.7.1.6. Rind thickness

Rind thickness also plays an important role in creasing of sweet orange fruit and creasing incidence is greater in thinner fruit rind than in thicker rind (Holtzhausen, 1981). According to Jones et al. (1967), rootstock, nutrition and cultural practice improve the health of a tree and fruit with a thick rind consequently have a reduced

incidence of creasing compared with thinner rind fruit. In California, a negative correlation between creasing development and rind thickness was reported by Ali et al. (2000). However, du Plessis and Maritz (2004) could not find any relationship between rind thickness and the incidence of creasing in South Africa.

2.7.1.7. Harvesting time

It has been reported that incidence of creasing is related to climatic and nutritional factors. However it has not been reported that creasing is related to harvest maturity. Nagy et al., (1982) reported that creasing becomes more visible at late harvesting than early harvesting and mature fruit are more susceptible to incidence of creasing. Similarly, du Plessis and Maritz, (2004) reported that creasing was not visible at harvest, but the creasing developed within three weeks of storage.

2.7.2. Environmental factors

2.7.2.1. Temperature

Temperature is an important environmental factor affecting creasing incidence in sweet oranges. Temperature prior to flowering has an impact on incidence of creasing. Minimum and maximum temperatures during February have an impact on creasing of orange at harvest (Ali et al., 2000). Similarly, Treeby et al. (1995) also reported that summer temperature has an influence on the incidence of creasing. The temperature gradient across the fruit is responsible for creasing at the initial stage and creasing occurs on more on the shaded side than the sunny side of the tree (Jones et al., 1967). Creasing increased with a greater range of temperature between maximum and minimum temperature during the fruit season (Sneath, 1987; Shear, 1975). However, creasing has not been influenced by the mean temperature in Uruguay (Gambetta et al., 2000).

2.7.2.2 Light

Light is also an important environmental factor which plays an important role in creasing of sweet orange fruit. Previously, Treeby (1996) reported that creasing incidence was significantly higher on the shaded side rather than the sunny side of the sweet orange tree. Creasing severity was reported to be higher on the south side

than on the north side of the tree canopy in the northern hemisphere (Gilfillan et al., 1981; Jones et al., 1967). Verreynne (2006) claimed that fruit inside the canopy have a significantly higher incidence of creasing than fruit from the outside of the tree canopy. Similarly, more than 86% of creasing incidence on orange fruit occurs in the shaded half of fruit and mostly in heavy foggy and rainy days during fruit ripening (Chen et al., 2002).

2.7.2.3. Relative humidity

Relative humidity is one of the climatic factors which affect creasing in oranges. Gonzalez-altozano and Castel (1999) reported that creasing incidence is also due to sudden changes in relative humidity at fruit colour break stage. Similarly, Agustí et al., (2001) reported that sudden changes in relative humidity at fruit colour break stage seem to be responsible for the development of creasing. Contrarily, Gambetta et al. (2000) claimed that relative humidity and rainfall have no effect on the incidence of creasing in sweet oranges. Moisture stress is also responsible for incidence of creasing in sweet orange (McIntosh, 1998; Dick, 1995).

2.7.2.4. Water management

Fruit size, rind appearance and maturity are the major fruit quality parameters of citrus fruit and these variables are influenced by water management (Shalhevet and Levy, 1990). Direct effect of water stress on sweet orange fruit rind quality has not been investigated to any great extent. Creasing is the most prominent physiological rind disorder associated with water stress (Augustí et al., 2004). Water has indirect effect on creasing because it reduces the Ca level in rind during fruit development (Huang et al., 2000; Huang et al., 1986). According to Gonzàlez-Altozano and Castel (1999) regulated deficit irrigation during September and October resulted in significant reduction in fruit size, increased creasing and large proportions of fruit had decreased quality and price. Treeby et al. (2000) reported substantial reduction in creasing of sweet orange, but the application PRD (partial root zone drying) and RDI (regulated deficient irrigation) has negative effect on fruit size. Later on, Treeby et al. (2007) reported that both RDI and PRD, where half of the water volume was applied on both rows and half of the water volume was alternatively applied at each irrigation schedule respectively from June in the first year to July in the third year, resulted in lower incidence of creasing at harvest.

2.7.3. Nutritional factors and creasing

The deficiency of Nitrogen (N) in soil is also associated with fruit smoothness and a thin rind which is linked to incidence of creasing in sweet orange (Le Roux and Crous, 1938). However, Ali et al. (2000) observed that concentration of N was higher in creased fruit compared with normal fruit. Increased levels of phosphorous resulted in fruit with thinner rind and higher creasing compared to those fruit which had low phosphorous with thick and course rind (Haas, 1950). Gambetta et al. (2000) reported higher concentrations of phosphorous in creased fruit than healthy fruit at harvest. Potassium (K) is essential for fruit formation and to enhance fruit size, flavour and fruit rind colour (Obreza et al., 2003). Potassium plays a major role in enzyme activation, protein synthesis, stomata function, turgor-related processes and transport of metabolites. Higher levels of potassium resulted in large fruit size with thick and coarse rind (Alva et al., 2006) while K deficiency produced smaller fruit with thinner rind which were susceptible to creasing, fruit drop and also decreased SSC, organic acids and vitamin C content (Obreza et al., 2008; Alva et al., 2006). Creased fruit exhibited higher concentrations of potassium in the rind as compared to its levels in the rind of normal fruit (Storey et al., 2002; Gambetta et al., 2000).

Most physiological disorders in fruit and vegetables are related to calcium (Ca) deficiencies (Shear, 1975) such as bitter pit in apple (Ferguson and Watkins, 1989), fruit cracking of oranges (Jiankai et al., 1994), rind puffiness (Kawase, 1984), pitting in mandarin (Zaragoza et al., 1996; Jackson et al., 1992) and creasing in sweet orange (Pham et al., 2012; Treeby et al., 2002). Calcium is the second most abundant macronutrient and a high proportion of the total calcium in the plant tissue is located in the cell walls of citrus fruit (Marschner, 1995; Nagy et al., 1985). Calcium is essential for various structural developments of the cell wall and membranes and also acts as an intercellular messenger in the cytosol (Marshner, 1995). However, Calcium plays a critical role in strengthening the cell wall and providing cell wall rigidity by making a bound as pectate in the middle lamella (Easterwood, 2002) and 60% of calcium is stored in the cell membrane (Huang et al., 2008; Poovaiah, 1988). It is reported that calcium is associated with incidence of creasing and level of Ca was higher in albedo tissue of creased fruit compared to the albedo tissue of normal fruit under Australian conditions (Treeby and Storey, 1994). The development of

creasing is associated with the role of calcium in bonding of the pectin chains (Bower, 2004). Whilst a complex relationship was observed between calcium and incidence of creasing, the albedo tissue from properties with a higher incidence of creasing had higher calcium concentrations than the albedo tissue from properties with lower incidence of albedo creasing. The involvement of Ca in creasing development is associated with its role in bonding of the pectin chains (Bower, 2004). Creasing is a physiological disorder of peel; lower levels of calcium found in peel between 14-26 October were responsible for substantial reduction of creasing (Nagy et al., 1982). All the calcium spray treatments (amino or glucose-chelated Ca or Ca $(NO_3)_{2)}$ successfully reduced creasing in South Africa, when applied at mid December to the end of January (Verreynne and Phiri, 2008). However, these current control measures neither prevent creasing completely nor reduce it substantially. Calcium spray has been used in Australia, but with limited success (25 to 30% reduction) as reported by (Treeby et al., 2002). Similarly, Pham et al. (2012) also claimed that the five repeated foliar spray applications of calcium (2%) and Tween 20 (0.05%) as a surfactant starting from full bloom at 10 day intervals improved the calcium uptake in the leaf and pulp as well as reduced the incidence of creasing and maintained fruit quality in sweet orange cv. Washington Navel.

Boron (B) is also an essential micronutrient for normal growth and development of all plants including sweet oranges (Brown et al., 2002). It plays a crucial role in plant cell formation, integrity of plasma membranes, pollen tube growth and increases pollination and seed development (Oosterhuis, 2001). Boron is also important for carbohydrate metabolism and their translocation in different plant parts (Siddiky et al., 2007). Boron is closely associated with cell division and flower pollination and is therefore important for good seed set and fruit development (Havlin et al., 2005). In citrus, boron deficiency leads to lower sugar content, granulation and excessive fruit abortion as well as increased rind thickness (Reuther, et al., 1968). However, creasing is linked with thinner rind of sweet orange fruit than the fruit with thicker rind in California (Ali et al., 2000). It has been previously reported that boron is involved in building structures of the cell wall (Dong et al., 2009; Matoh, 1997). As a consequence, the integrity, elasticity and tensile strength of the cell wall are maintained with the application of boron (Dong, 2009; Goldbach and Wimmer, 2007). Whilst, Tariq et al. (2007) reported that foliar application of

boron resulted in a softer and thinner rind than in an untreated control. Contrarily, Maurer and Truman (2000) claimed that foliar application of boron did not affect rind thickness in sweet oranges.

2.7.4. Plant growth regulators (PGRs)

2.7.4.1. Gibberellins

Gibberellins (GA_3) are naturally-occurring plant hormones found in varying concentrations in various parts of a plant. Gibberellins promote cell growth and stimulation of cell elongation because of their involvement in increasing the cell wall plasticity. Gibberellins are used to regulate the conversion of chloroplasts to chromoplasts and vice versa (Coggins and Jones, 1977). The uptake of GA_3 is higher in attached fruit than detached fruit (Goldschmidt and Eilati, 1970). Enhancement of GA_3 uptake by acidifying the spray mixture suggests that GA_3 is more easily taken up in its lipophilic, non-dissociated form. Translocation of GA_3 in citrus fruit takes place by diffusion (Goldschmidt and Eilati, 1970). Uptake of GA_3 in citrus is more soluble in lipid and in humid hydrated environments (Greenberg and Goldschmidt, 1988). GA_3 applications are used to reduce the incidence and severity of creasing in orange-producing countries of the world such as America (Embleton et al., 1973; Coggins, 1969; Jones et al., 1967), South Africa (Gilfillan et al., 1981), Israel (Monselise et al., 1976) and South Africa (Tugwell et al., 1993; Bevington, 1973;). The effectiveness of GA_3 in controlling creasing incidence is associated with its role in stimulating cell elongation (Salisbury and Ross, 1992; Holtzhausen, 1981). The potential of the albedo tissue to increase and accommodate cell enlargement after cell division has ceased (Storey and Treeby, 1994; Holtzhausen, 1981) in the albedo is thought to be critical in creasing development. However, GA_3 treatment produced small size and compact fruit (Jona et al., 1989; Garcia-Luis et al., 1985. Creasing is also related to pectin and GA_3 are also used to reduce pectin methyl esterase activity (Jona et al., 1989). The effectiveness of GA_3 on creasing incidence depends upon optimum concentration (Bevington, 1973), the pH of spray solution (Tugwell et al., 1996; Greenberg and Goldschmidt, 1989) and the timing of application. GA_3 (20 mg L^{-1}) spray applied at 3 to 4 weeks prior to colour break stage significantly reduced the incidence of creasing in Navel orange (Gilfillan et al., 1981). Similarly,

gibberellic acid was used to control creasing in South Africa (Bower, 2000) and Uruguay (Gambetta et al., 2000). Greenberg et al. (2006) also reported that spray of GA_3 (20 mg L^{-1}) during July was more effective in reducing the incidence of creasing in sweet oranges in Israel. But the application of GA_3 does not prevent creasing and only delays the onset of creasing (Bower, 2000) and also adversely impacts the fruit rind colour development (Coggins, 1981, 1969). No substantial influence on the internal fruit quality has been reported apart from a slight increase in the juice content (Coggins, 1969) and reduced fruit size (Jona et al., 1989.

2.7.4.2. Polyamines

Polyamines (PAs) are biological compounds with low molecular weight and aliphatic nitrogen groups; and are present in all living organisms (Cohen, 1978). In plants, the most common PAs are putrescine (PUT) (butan-1, 4- diamine): H_3N+-CH_2-CH_2-CH_2-CH_2-N+H_3; triamine spermidine (SPD) [N- (3-amino propyl) butane-1, 4-diamine]; H_3N+-CH_2-CH2-CH2-CH2-CH2-N+H_2-CH_2-CH_2-CH_2-N+H_3 and tetra-amine spermine (SPM) [NN'- bis- (3-aminopropyl) butane-1, 4-diamine]: H_3N+-CH_2-CH2-CH2-CH2-CH2-N^+H_2-CH_2- CH_2- CH_2-N^+H_3. Their formulas are given below (Kalac and karusová, 2005).

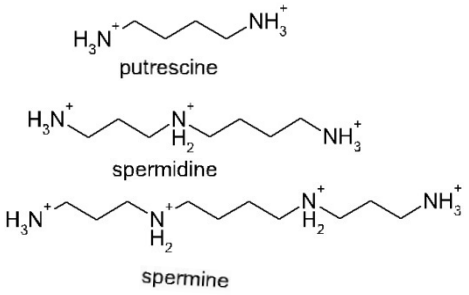


Figure 2.6. Polyamine formulas

PAs are involved in a large spectrum of physiological processes such as flower initiation and fruit set (Prista and Voyiatzis, 2004); cell division (Galston, 1983); growth and development (Malik and Singh, 2004); stress (Kakkar and Sawhney, 2002); fruit abscission (Malik and Singh, 2003); and ripening (Torrigiani et al., 2004). PAs play an important role in growth and developmental processes in plants

and animals including humans (Bradócz et al., 1993). Recent studies showed the average daily requirement of polyamines to be 350 μmol per day per person and the major source of PAs is fruit, non-green vegetables and cheese. SPD is also present in all kinds of food, although levels are higher in green leafy vegetables.PUT is present in citrus fruit, however meat is the richest source of SPM (Ellassen et al., 2002; Susan et al., 1995). The concentrations of PAs are significantly higher in cell rather than other plant hormones and required in millimoles (mM) rather than micromoles (μM) for biological processes (Evans and Malmberg, 1989); interact with (DNA) deoxyribose nucleic acid; (RNA) ribose nucleic acid; phospholipids and some proteins (Tabor and Tabor, 1984).

2.7.4.2.1 Role of PAs on cell division and cell wall

The interaction of PAs with anionic groups on membranes prevents leakage and causes stabilization under stress conditions (Smith, 1985). PAs also have potential radical scavenger properties. At a concentration range of 5-50 mM, PAs strongly inhibit the production of oxygen by senescing microsomal membranes and superoxide dependent conversion of ACC to ethylene (Drolet et al., 1986). The anti-senescence effects of PAs have been reported where by direct binding with the membrane occurs to maintain membrane integrity, by preventing lipid peroxidation and inhibition of ethylene synthesis; and by inhibiting ACC synthase and the terminal step of conversion to ethylene (Evans and Malmberg, 1989). PAs are used to restore cell wall thickness and other wall features and seem to be necessary for normal cell size and rigidity on the primary cell wall by strengthening cell wall components (Berta et al., 1997).

2.7.4.2.2 Role of polyamines in fruit set

PAs are naturally-occurring compounds which influence many plant growth and developmental processes; and are universally present throughout plant cells (Valero et al., 2002, Galston, 1983). It has been previously reported that PAs are one of the essential members of several internal components required for flower initiation and floral morphogenesis (Evans and Malmberg, 1989). Evidence is available to prove that PAs are involved in the flower induction, initiation and floral organ development (Huang et al. 2004; Applewhite et al. 2000; Tiburcio et al. 1990). Moreover, the use of some special types of mutants adds new avenues for investigating the relationship

between PAs biosynthesis and floral development. The impact of PAs on fruit set has been observed in different fruit crops such as apple and pear (Crisosto et al., 1988; Costa et al., 1986; Costa and Bagni, 1983**)** and in sweet orange (Saleem et al., 2007).

2.7.4.2.3 Role of polyamines in fruit development

Fruit development is regulated by plant growth regulators (PGRs) (Gillaspy et al., 1993). The development of parthenocarpy in peas and tomato fruit by GA_3 and other PGRs resulted in changes in the levels of PAs, and in the biosynthesis of PAs genes (Carbonell and Navarro, 1989, Pérez-Amador et al., 1995). It has been reported that PAs are involved in early fruit development in several species (Costa and Bagni, 1983; Ege-Cortines and Mizrahi, 1991; Evan and Malmber, 1989; Crisosto et al., 1988; Rugini and Mencuccini, 1985). Changes in the levels of PAs have been correlated with fruit growth and development processes such as PAs biosynthesis also related with post-fertilization growth and development of ovary tissues (Slocum and Galston, 1985). The levels of free PAs are relatively higher after the first week of full bloom, and gradually decrease in the fruit tissues of apple (Biasi et al., 1988), avocado (Kushad et al., 1988), peach (Kushad, 1998) and grape berry (Shiozaki et al., 2000). Similarly, changes in the levels of PAs followed the patterns of growth activity in fruit and were maximum at the early stage, at a minimum at mid-summer and intermediate at fruit development in the Murcot mandarin (Nathan et al., 1984).

2.7.4.2.4. Endogenous polyamines

Fruit growth and development phases in citrus are complex processes. To impose substantial effects of growth regulators like PA and ABA on these stages, their application must be in coordination with the appropriate stage of growth (El-Otmani et al., 1995). The endogenous levels of PAs are directly involved in the ripening process because PAs play an essential role in fruit growth and development (Valero et al., 2002). Information available on the role of endogenous levels of polyamines in citrus fruit ripening is partial and unconvincing, hence the need for investigation. However, lot of PAs research work has focused on their role in chilling injury of the different fruit. The level of putrescine increased in the cold storage of citrus crops such as mandarin, grapefruit, and sweet oranges and the magnitude of these responses was different in each cultivar (Yuen et al., 1995). The ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) have been involved in

different activities in unripe but fully developed mandarin fruit (Nathan et al., 1984). Since PAs are implicated in fruit growth, development and ripening. They also impact on the post-harvest physiology of fruit (Kramer et al., 1991).

2.7.4.2.5. Exogenous application of polyamines

PAs are small universal polycations which are involved in different processes of plant growth and development. They are also well known for their anti-senescence and anti-stress effects due to their acid neutralizing and antioxidant properties as well as cell membrane and cell wall stabilizing qualities (Zhao and Yang, 2008). It has been recommended that PAs play an important role in modulating the defence response of plants to diverse environmental stresses, (Bouchereau et al. 1999) which includes metal toxicity (Groppa et al., 2003), oxidative stress (Rider et al., 2007, drought (Yamaguchi et al., 2007), salinity (Duan et al., 2008) and chilling stresses (Cuevas et al., 2008, Groppa et al., 2003). It has been previously reported that the exogenous application of PAs is an effective approach for enhancing stress tolerance in crops for enhanced crop productivity. Exogenous application of PUT has been successfully used to enhance the tolerance in different process such as salinity (Ndayiragije and Lutts, 2006), cold (Nayyar and Chander, 2004; Nayyar, 2005), drought (Zeid and Shedeed, 2006), heavy metals (Wang al., 2008), osmotic stress (Liu et al., 2007), high temperature (Murkowski, 2001) and water logging (Arbona et al., 2008). However, it has been observed that genetic transformation with PAs biosynthetic genes encoding arginine decarboxylase (ADC), ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (SAMDC) or SPD synthase (SPDS) enhanced environmental stress tolerance in different plant species (Liu et al., 2007). It is informative to know that transgenic plants communicating ADC (Prabhavathi and Rajam, 2007), SPDS (Wang et al., 2008; Kasukabe et al., 2006) or SAMDC (Wi et al., 2006) tolerate many stress processes including salinity, drought and extremes of temperature. Such multiple abiotic stress tolerance is of practical importance since plants often suffer from several concurrent forms of environmental stress during their life cycle. PUT-enhanced pollen tube penetration into the ovule delays senescence without affecting flower ethylene production (Crisosto et al., 1988). PUT (1.0 mM) for *in vitro* pollination revealed higher pollen germination on the stigma of Japanese flowers compared to the control and PUT did not affect post-pollination ethylene production in the styles (Franco-Mora et al., 2005). Exogenous application of PAs

retarded softening of apple fruit during cold storage at 0ºC (Kramer et al., 1991) and strawberry fruit at 1ºC (Ponappa et al., 1993).

2.7.4.2.6. Polyamines inhibitors

Difluoromethylornithine (DFMO), difluoromethylarginine (DFMA), cyclohexylamine (CHA) and guanylhydrazone MGBG) are used to inhibit the biosynthesis of PAs. PUT is the precursor of PAs biosynthesis which is synthesized directly from ODC or indirectly from ADC. Both ways are present in higher plants. In mammals and fungi, the PUT synthesis occurs through ornithine decarboxylation (Galston and Sawhney, 1990). The inhibition of ODC has been used to inhibit pathogenic fungus growth in plants without damaging the host plants or other non-target organisms. DFMO is used as an inhibitor of ODC which is used to control powdery mildew and brown rust in cereals and chocolate spot in broad bean (Rajam et al., 1985). Application of DFMO had little effect on PUT, while DFMA depressed PUT by 72% against the control. MGBG, the irreversible inhibitor of S-adenosylmethionine decarboxylase, which converts SAM to decarboxylated-SAM (dSAM), thereby blocking biosynthesis of polyamine, decreased ethylene production and suppressed putrescine accumulation. Treatment with CHA increased putrescine while AOA depressed it as compared to the control.

2.7.4.3. Ethylene

Ethylene is a naturally-occurring gaseous plant hormone, is produced in all plants and is usually associated with different plant processes such as seed germination to organ senescence (Kende and Zeevaart, 1997). But particular economic important role of ethylene is a fruit ripening agent (Bleecker, 2000) and accelerator of fruit softening (Ladaniya, 2007). Citrus is known as a non-climacteric fruit and produces only small amounts of ethylene (Ladaniya, 2007). Ethylene plays an important role in changing fruit colour, flavour, chemical composition and texture in citrus fruit (Ladaniya, 2007). Ethylene has been used to enhance the chilling injury (Yuen et al., 1995), decay (Brown and Lee, 1993) and off-flavours in various fruit (Testoni et al., 1992).

2.7.4.3.1. Polyamine and ethylene biosynthesis

PAs and ethylene use a common precursor S-adenosylmethionine (SAM) for their biosynthesis. However, their function appears to be antagonistic in fruit ripening and senescence (Pandy et al., 2000). The PAs inhibit the ethylene production and ethylene inhibits the PAs biosynthesis (Icekson et al., 1986; Apelbaum et al., 1985; Apelbaum et al., 1981). However, PAs are biosynthesised through arginine and ornithine decarboxylase. The agmatine is synthesized from arginine and is then converted to PUT. PUT is then further converted to first SPD and then SPD is futher converted to SPM by successive transfers of aminopropyl groups from decarboxylated S-adenosylmethionine (dSAM) catalysed by specific SPD and SPM synthases. However, methionine is first converted to aminopropyl groups and is first converted to S-adenosylmethionine (SAM), and then decarboxylated into SAM decarboxylase (SAMDC). The resulting decarboxylated SAM is utilized as a donor of aminopropyl. The SAM is used as a common precursor for PAs and ethylene biosynthesis. However, SAMDC regulates biosynthetic pathways of PAs and ethylene as shown in Figure 2.7. Four different inhibitors of PA synthesis are: DFMO, DFMA, MGBG and CHA, (Bitonti et al., 1987; Bey et al., 1987; Hibasami et al., 1980; Williams-Ashman and Schenone, 1972). Common oxidases are diamine and polyamine oxidase (Smith and Marshall, 1988). Several research investigations have dealt with PAs localization and their biosynthesis through enzymes in plants (Slocum, 1991). The MGBG, which block the conversion of SAM to SPD and SPM, sharply increase ethylene evolution and ACC contents. The methionine is first converted to SAM through the SAM synthase enzyme and then SAM is converted to ethylene by the action of ACC synthase and ACC oxidase (Figure 2.7). Aminoethoxyvinylglycine (AVG) and cobalt sulphate ($CoSO_4$) are well-known ethylene biosynthesis inhibitors which block the conversion of methionine to ACC (Yu and Yang, 1979), by competitively inhibiting the activity of ACS enzyme in the ethylene biosynthesis pathway (McGlasson, 1985).

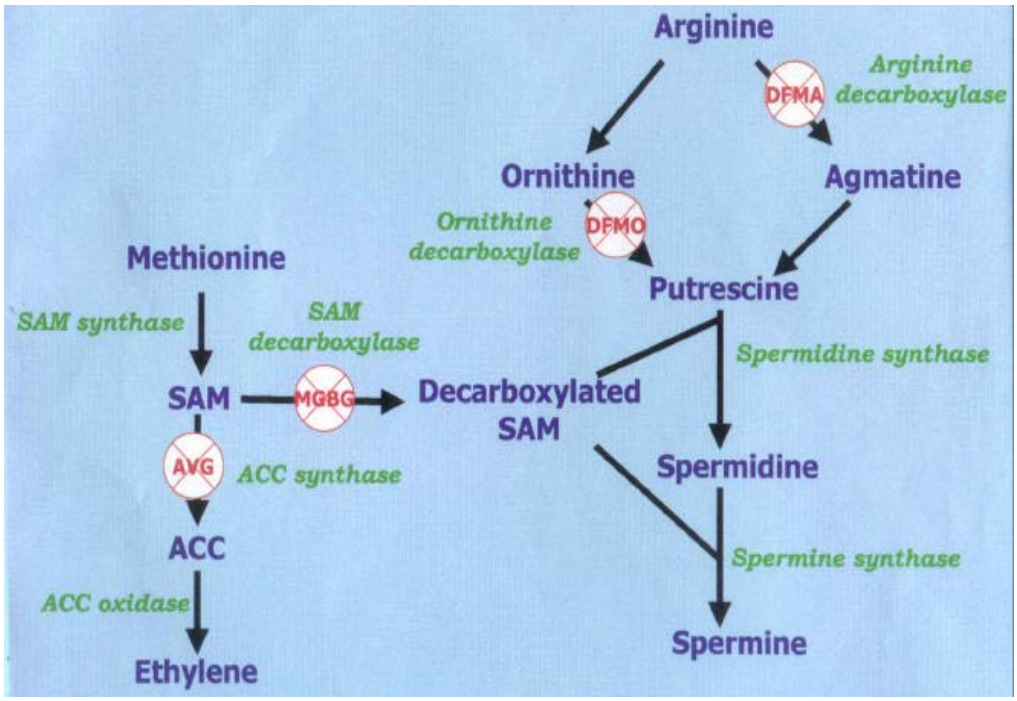


Figure 2.7. Polyamines and ethylene biosynthesis pathway (Pandy et al., 2000; Kaur-Sawhney et al., 2003).

2.7.4.3.2. Endogenous ethylene

Ethylene is very effective at lower concentrations ranging from a part-per-million (µL l^{-1}) to part-per-billion (µL l^{-1}) in regulating ripening and senescence in many fruit, vegetables and ornamentals (Saltveit, 1999). Ethylene accelerates softening in citrus fruit due to disintegrating cell membranes making them leakier (Ladaniya, 2007: Rath and Prentice, 2004). Ethylene plays a major role in regulating ripening and softening of fruit (Hadfield et al., 2000; Ayub et al., 1996), and consequently, the expression of some genes and activities of PGs are associated with ripening (Hiwasa et al., 2003; Sitrit and Bennett, 1998;). However, sweet orange fruit is non-climacteric, and produces only small amounts of ethylene (<0.1 µL. $Kg^{-1}h^{-1}$), inducing changes in fruit colour, flavour, chemical composition and texture in citrus fruit (Aharoni, 1968; Eaks, 1970; Ladaniya, 2007). The endogenous ethylene levels were higher (0.4, 0.3 and 0.2 µL.$kg^{-1}h^{-1}$) at fruit weight (50, 62 and 70 g) and decreased (less than 0.01 µL $kg^{-1}h^{-1}$) with the increase of fruit weight 120, 64 and 87 g for grapefruit, Valencia and Navel oranges, respectively (Eaks, 1970). Citrus fruit are generally classified as non-climacteric which produce only small amount of

endogenous ethylene and ethylene production does not occur after mature fruit are removed from the tree (Ables, 1973). When young fruit are cut into small pieces, the endogenous level of ethylene is produced at a higher rate with in a very short period; albedo tissues produced ethylene vigorously even though the tissue is taken from matured fruit, which not produced ethylene when intact from tree (Hyodo, 1977). Ethylene biosynthesis in the albedo tissue of Satsuma mandarin fruit has been reported by Hyodo, (1977). Some preliminary and sporadic reports suggested higher levels of endogenous ethylene in the albedo tissue of creased fruit than in normal fruit in Valencia orange (Monselise et al., 1976). Similarly, Pham, (2009) reported higher levels of endogenous ethylene in the rind of creased fruit than normal fruit in Washington Navel sweet orange.

2.7.4.3.3. Exogenous application of ethylene

The exogenous application of ethylene exerts a positive response on respiration and promotes ripening in citrus fruit although they produce very low levels of ethylene throughout development (Ladaniya, 2007; Aharoni, 1968; Eaks, 1970). Exogenous application of ethylene is involved in physiological and biochemical processes in citrus fruit, such as an increase in the activity of phenylalanine ammonia lyase (Riov et al., 1969), chlorophyllase (Trebitsh et al., 1993), cellulase (Kazokas and Burns, 1998), modification of protein (Alonso et al., 1995) and gene expression (Alonso and Granell, 1995; Jacob-Wilk et al., 1999; Kazokas and Burns, 1998). It has also been reported in previous research work that exogenous application of ethylene or ethephon significantly improved colour in citrus fruit (Ladaniya, 2007; Agustí et al. 2002; Burg, 2004; Porat et al., 2001; Al-Mughrabi et al., 1989; Monselise et al., 1976). However, Ladaniya (2007) reported that the application of ethephon (250 mg L^{-1}) significantly increased peel puffing in Satsuma mandarin when applied seven days before harvesting. It is well known that exogenous application of ethylene is used to enhance the colour development in early picked citrus fruit (Goldschmidt et al., 1993). The pre-harvest application of ethylene did not affect fruit quality parameters in citrus such as soluble solid concentration, juice percentage, fruit weight, rind thickness, acidity and ascorbic acid (Al-Mughrabi et al., 1989). Recently, Pham (2009) also claimed that exogenous application of ethrel substantially increased the incidence of creasing in Washington Navel sweet orange.

2.7.4.3.4. Inhibitor of ethylene biosynthesis

Ethylene plays a dominant role in modulating the process of ripening and softening in different fruit (Hadfield et al., 2000; Ayub et al., 1996), and ripening is associated with cell wall genes and activities, including PGRs (Hiwasa et al., 2003; Sitrit and Bennett, 1998;) and EGase (Lashbrook et al., 1994). Two different approaches have been reported to determine the exact role of ethylene in the fruit ripening and softening process such as transgenic suppression of endogenous ethylene production and the use of different inhibitors of ethylene production (Nishiyama et al., 2007). Many well-known compounds have been successfully used to suppress or inhibit ethylene production in climacteric and non-climacteric fruits, for instance, aminoethoxyvinylglycine (AVG), cobalt sulphate ($CoSO_4$), silver thiosulphate and 1-methylcyclopropene (1-MCP) (Adkins et al., 2005; Torrigiani et al., 2004; Mir et al., 2004).

2.7.4.3.4.1. Aminoethoxyvinylglycine (AVG)

AVG is a well-known reversible ethylene biosynthesis inhibitor which blocks the conversion of methionine to ACC (Yu and Yang, 1979), by competitively inhibiting the activity of ACS enzyme in the ethylene biosynthesis pathway (McGlasson, 1985). AVG (as ReTain®) is a well-known growth regulator which regulates the growth and differentiation and also competes directly with synthesis of ethylene (Bagni and Torrigiani, 1992). The effectiveness of AVG treatment depends upon cultivar, harvest maturity and age of the fruit (Jobling et al., 2003a,b). The effectiveness of the AVG application also depends on the endogenous level of ethylene and its time of application. Amarante et al. (2002) reported that fruit with high ethylene production required higher AVG concentration than fruit with low ethylene production to delay or inhibit fruit ripening and softening (Amarante et al., 2002). Similarly, the fruit with higher ACS activity requires higher AVG concentration to delay ripening (Autio and Bramlage, 1982). Pre-harvest application of AVG is used to inhibit ethylene activity, delay fruit maturity and retard fruit ripening (Hayama et al., 2008; Lu et al., 2007; McGlasson et al., 2005). Post-harvest application of AVG is used to slow down the maturity of peaches and nectarine (Byers, 1997) and in sweet orange fruit (Al-Husseini, 2012).

2.7.4.3.4.2. Cobalt Sulphate ($CoSO_4$)

Cobalt greatly inhibits ethylene production in different plants (Lau and Yang, 1976; Santana-Buzy et al., 2006). Cobalt is a plant nutrient which plays an important role in higher plants due to its direct role in plant metabolism and promotes stem coleoptile elongation, hypocotyl, leaf and bud development (Howell and Skoog, 1975). Cobalt is also involved in biosynthesis of vitamin B_{12} which is useful for both humans and animals (Smith, 1991). Reddy et al. (2011) observed a higher number of fruit, yield per tree and highest average fruit weight in pomegranate due to 2.5 ppm exogenous application of cobalt. It has been reported that foliar application of $CoSO_4$ increased the fruit set, retention, fruit yield, weight, size, fruit firmness, SSC and total sugar in different fruit such as mango (Wahdan, 2011; Singh and Agrez, 2002; Singh et al., 1994; Singh and Singh, 1993), pomegranate (Reddy et al., 2011), olive (Gad et al., 2006), peach and apple (Kim et al., 2004; Benincore et al., 2000) and fig (Teragishi et al., 2000). However, no research work has been described on the effects of ethylene biosynthesis inhibitors on incidence of creasing and fruit quality of sweet oranges.

2.7.4.3.5. Ethylene and cell wall loosening

The plant cell wall is a complex structure which determines cell size and shape; growth and development; intercellular communication; and interaction with the environment (Glickman, 1969). It is composed of cellulose; hemicelluloses and pectins; enzymes; and structural proteins. Pectins occur as complex polysaccharides in the cell walls of higher plants, acting as a hydrating agent and cementing material for the cellulosic network (Muralikrishna and Taranathan, 1994). The pectin is mostly produced during cell wall growth of both dicotyledonous and monocotyledonous plants and makes ~35% of the dry weight of their cell wall (Hoff and Castro, 1969). The higher concentration of pectin seems to occur in the cell wall of the middle lamella and decreases with the passage of time in plasma membrane. The concentration of pectin was higher in moisturised or in soft tissue of plants (Glickman, 1969). In mature fruit, pectin is bound with cellulose in the cell wall and is insoluble. However, during ripening the structure of pectin is changed by naturally-occurring cell wall degrading enzymes such as pectinesterase (PE), *exo*-polygalacturonase (*exo*-PG), *endo*-polygalacturonase (*endo*-PG) and spell out EGase

(EGase) (Kashyap et al., 2001). Ethylene plays a key role in regulating ripening and softening of fruit (Hadfield et al., 2000; Ayub et al., 1996). The ripening and softening process is associated with biochemical changes in breakdown of cell wall polymers such as cellulose, hemicelluloses and pectin (Payasi et al., 2009). Various hydrolytic reactions are brought about by polygalacturonases, pectin methylesterase, pectatelyase, rhamnogalacturonase, cellulase and galactosidase and are also involved in softening. Among these enzymes, the protein also plays a significant role in softening (Brummell and Harpster, 2001). It may be assumed that creasing may be similar to fruit softening caused by cell wall disassembly (Nishiyama et al., 2007; Brummel, 2006; Orfila et al., 2002). Creasing in sweet orange fruit is known to be associated with loss of pectin in the cell walls of the albedo, leading to cell wall loosening and formation of cracks on the rind of oranges (Saleem et al., 2014; Monselise et al., 1976). As cell wall loosening and degrading enzymes play a key role in creasing of sweet orange fruit. The activity of pectin methylesterase and concentration of water-soluble pectins of albedo tissue was associated with creasing due to loosening of cell wall connections between cells (Monselise et al., 1976) When the amount of pectin and hemicelluloses decreased to 70–80% of total polysaccharides, symptoms of creasing occurred on sweet oranges (Jona et al., 1989). Recently, Saleem et al. (2014) reported that creasing in sweet orange fruit is linked with enhanced loss of pectins in the cell walls of the albedo, leading to cell wall loosening, formation of cracks and consequently reduced hardness, stiffness and tensile force of the rind.

CHAPTER 3

General materials and methods

3.1. Plant and fruit materials

All the experiments were conducted on different cultivars of sweet orange [*Citrus sinensis* (L.) Osbeck] including Navelina, Washington Navel, Lane Late and Valencia grown in a commercial orchard located at Gingin (latitude 31° 21′ South, longitude 155° 55′ East), Western Australia (Figure 3.1). Twenty-five year old uniform sweet orange trees previously grafted onto trifoliate orange (*Poncirus trifoliate* Raf.) rootstock were used in different experiments. The trees were spaced 7.5 m between rows and 2.7 m within rows in the North-South orientation. All the experimental trees received similar cultural practices including fertilisers, irrigation, weed control and plant protection; except for the experimental treatments (Moulds and Tugwell, 1999). The experimental site has a sandy loam soil. The climate is dominated by cool wet winters and hot dry summers.

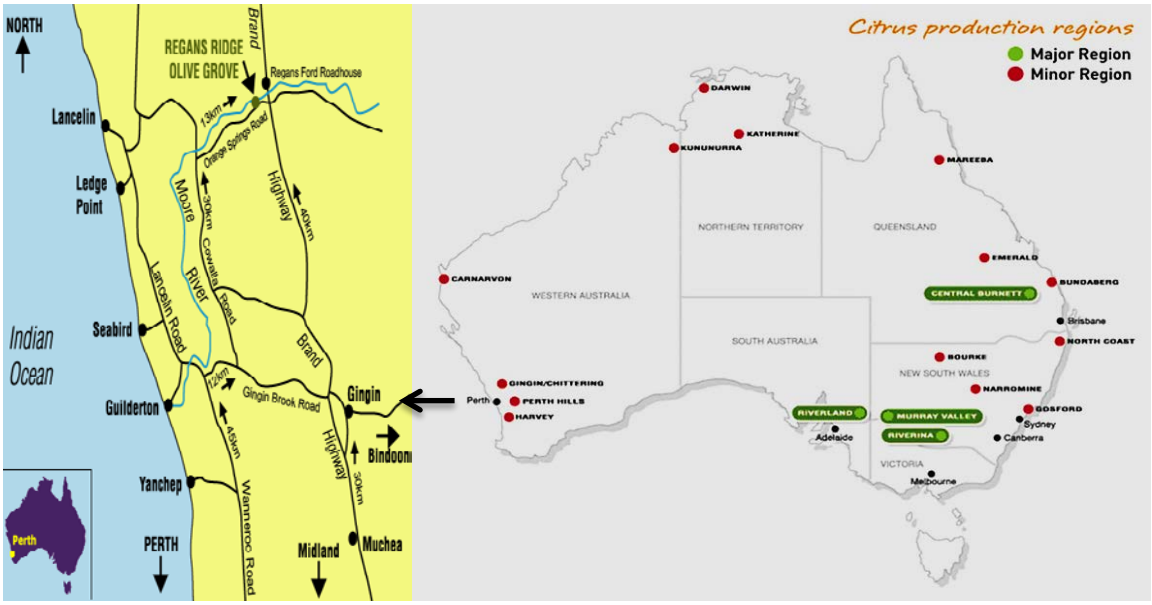


Figure 3.1. Major and minor citrus growing areas in Australia including the map of the experimental site at Gingin, Western Australia (http://www.ga.gov.au)

3.2. Determination of free polyamines in the albedo and flavedo tissues

3.2.1. Chemicals used

All of the standard polyamines such as putrescine (butan-1, 4-diamine), spermidine [N-(3-aminopropyl) – 1, 4-butane diamine] and spermine (N, N'-bis-[3-Aminopropyl]-1, 4-butane diamine) and internal standard (1, 6 hexane diamine) were purchased from Sigma-Aldrich, Australia. All the chemicals used were of High Performance Liquid Chromatography (HPLC) grade.

3.2.2. Collection of samples

In 2011-12, the effects of different concentrations of exogenous spray application of PUT on the changes of the endogenous levels of polyamines in the albedo and flavedo tissues were monitored in sweet orange cv. Washington Navel and Lane Late. Ten fruit per tree were harvested at the fruit set (67 days and 79 days after spraying, DAS), the golf ball (107 and 128 DAS) or at the colour break stage (56 and 75 DAS) in cv. Washington Navel and Lane Late, respectively. The concentrations of free polyamines putrescine (PUT), spermidine (SPD), spermine (SPM) and total polyamines were determined from the albedo and flavedo tissues of the fruit. At each sampling stage, harvested fruit were brought to the laboratory within 4 hours (h). Fruit were first washed with tap water and secondly with distilled water (dH_2O). The albedo and flavedo tissues were separated from the fruit, cut into small cubes, and frozen in liquid nitrogen for 2 to 3 minutes (min) prior to freeze drying using a freeze drier (Telstar, Model CRY0006-50; Barcelona, Spain). The dried samples were ground into powder using a small grinding mill (Schnitzler Pico, Schnitzler GmbH & Co. KG, Marlener, Germany), and stored at -20 °C prior to analysis.

3.2.3. Determination of free polyamines

The free PAs were determined in the albedo and flavedo tissues of sweet orange fruit by following the method of Morgan, (1998) with some modifications (Figure 3.2). The powdered tissues of the albedo and flavedo (1 g) were homogenised in cold per chloric acid (10 ml of 5%) with internal standard (1, 6 hexane diamine, 200 µl of 10 mM). The homogenate was kept on ice for one hour (h) prior to centrifugation at 12857 × g at 4℃ for 30 minutes (min) (Eppendorf 5810R Centrifuge, Hamburg, Germany) to obtain supernatant. Four ml NaOH (2M) was added to 1 ml of the supernatant containing free PAs, and then 80 µl of 50% benzoyl chloride solution in methanol was added. The mixture was homogenized vigorously for 1-2 minutes, and the reaction was allowed to proceed for 2 h at room temperature (22$\pm$2℃). Chloroform (2 ml) was also added to stop the reaction, and the solution was centrifuged at 3220 × g for 5 minutes (min).

The lower fraction was collected and 1 ml Milli Q water was added and the mixture again centrifuged. The lower layer was taken into a new sterile centrifuge tube and evaporated to dryness in air. After evaporation, 1ml methanol (65%) was added, the solution homogenised for 10 seconds and then filtered through 0.22 µm True ™ syringe filter (LabServ Filtration, Bio-Lab, Sydney, Australia) and loaded into the HPLC system with a 1 ml injection vial. An aliquot (50 µl) of free PAs extract was injected into the HPLC (Waters, Milford, MA, USA) system using an auto sampler (Water 717 plus, Milford, MA, USA). The samples of free PAs extracts were isocratically eluted through the Alltima C18 5u column-w (4.6 x 250 mm). Free PAs were detected using dual λ absorbance detector (Waters 2414, Milford, MA, USA) at 229 mm wave length. Chromatographic peaks were identified by comparing retention times with those of the authentic standard and by spiking samples with the pure compound. The peak areas were used to quantify the individual free PAs. Under these conditions, the retention time of putrescine, 1, 6 hexane diamine, spermidine, benzoyl chloride and spermine was 12.6, 15.2, 16.1, 18.1, and 20.9 (Figure 3.3). The data were collected and processed with Breeze® 3.30 software (Waters, Milford, MA, USA). All the free individual and total PAs were expressed as $nmol.g^{-1}$ DW.

Albedo or flavedo tissues of orange (1 g) DW

↓

Homogenised in 10 ml of 5% cold per-chloric acid (PCA)

↓

Added 1, 6 hexane diamine internal standard (200 µl of 10 mM)

↓

Vortex for 1-2 min

↓

Homogenate, kept on ice for 1 h

↓

Centrifuge at 12857 × g at 4℃ for 30 min

↓

Take supernatant (can be stored at 4℃ for few days)

↓

1ml polyamine extract

↓

Added 4 ml NaOH (2 M solution)

↓

40 µl of 50% Benzoyl chloride in the methanol added

↓

Mix vigorously (1-2 min)

↓

Allow reaction at (22±2℃) 30 min up to 2 h

↓

Added 1 ml chloroform and vortexes (1 to 2 min)

↓

Evaporate the solvent by using air

↓

Redissolve in 1 ml methanol (65%)

↓

Injected (50 µl) in to the column

Figure 3.2. Flow diagram of endogenous free polyamines analysis

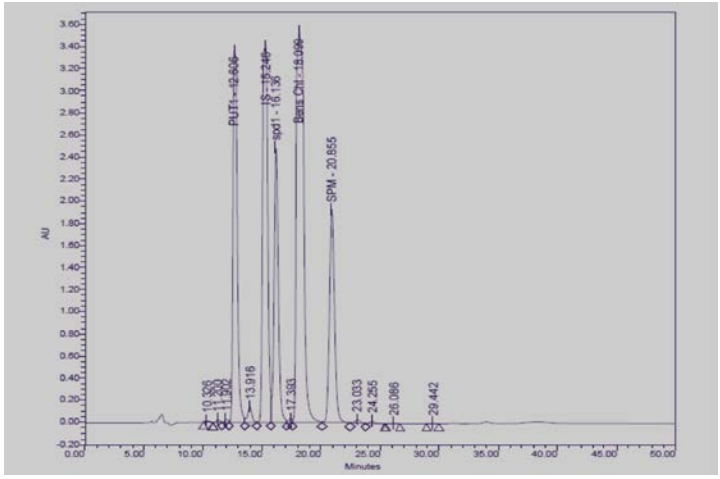


Figure 3.3. Chromatograph of PAs: PUT, SPD, SPM, Internal standard (IS) and Benzyl chloride.

3.3. Determination of creasing index percent

The severity of creasing was estimated by examining the degree of symptoms of creasing on the fruit surface using a subjective scale of 0 = no creasing; 1 = slightly creased (1 to 25% fruit surface with symptoms); 2 = moderately creased (26 to 50% fruit surface with symptoms); 3 = severely creased (> 51% fruit surface with symptoms) (Figure 3.4). The severity of creasing was assessed from 35 randomly harvested fruit from each tree. The creasing index was expressed as percentage (CI). The following formula was used to calculate the creasing severity as described by Treeby and Storey (2002).

$$\text{Creasing index \%} = \frac{[\sum (\text{Rating number x number of fruit in rating category})] \text{ x } 100}{\text{Highest rating value x Total number of fruit assessed}}$$

(0) (1) (2) (3)

Figure 3.4. Normal fruit (0), 1-25% creasing (1), 26 to 50 creasing (2), 51% or above (3)

3.3.1. Creasing (%)

In both years, 35 ripe fruit per tree were randomly harvested around the tree canopy. The incidence of creasing was examined on individual fruit, based on the appearance of fruit surface. Creasing incidence (%) was calculated by using the formula:

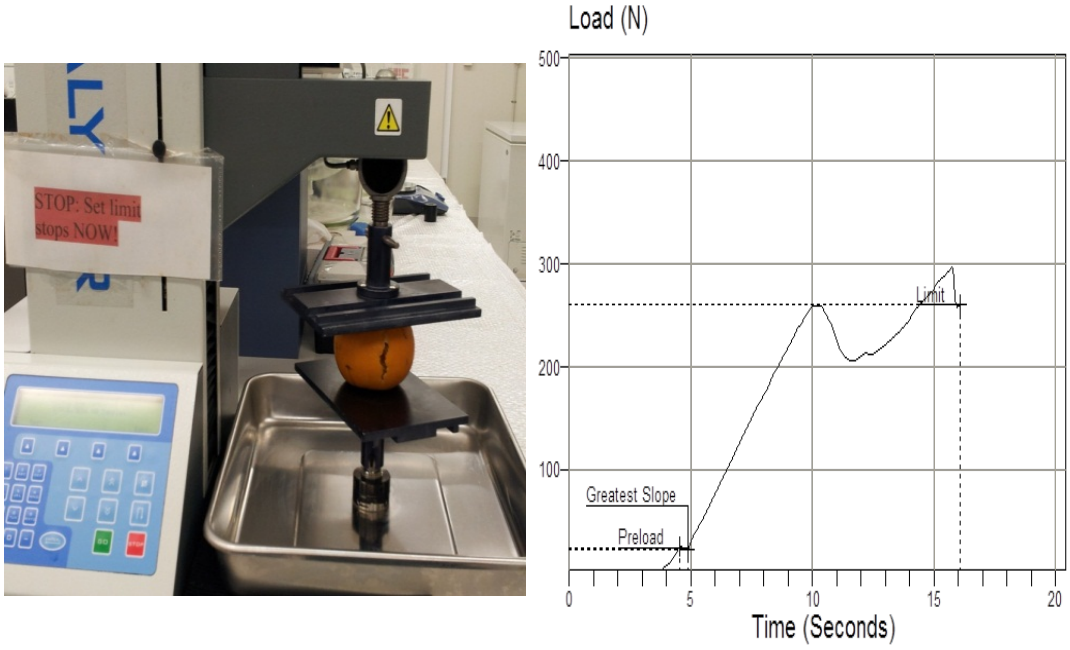
$$\text{Creasing}(\%) = \frac{(\text{Total number of creased fruit}) \text{ x } 100}{\text{Total number of fruit assessed}}$$

3.4. Rheological properties of fruit and rind

Rheological properties of fruit rind such as fruit firmness (N), rind hardness (N) and rind tensile strength (N) were determined using a textural analyzer (TA Plus, AMETEK Lloyd instruments Ltd., Hampshire, UK). The data were collected and processed by using Nexygen® 4.6 software.

3.4.1. Fruit firmness

The fruit firmness was determined by using a fruit compression test. Five fruit (75 mm high) were used for the fruit compression test using a textural analyzer (Figure 3.5). Each piece of fruit was placed between two flat plates with the stem axis perpendicular to the plate. The crosshead speed was 200 mm min^{-1} and test was completed at a strain of 50% of fruit height.

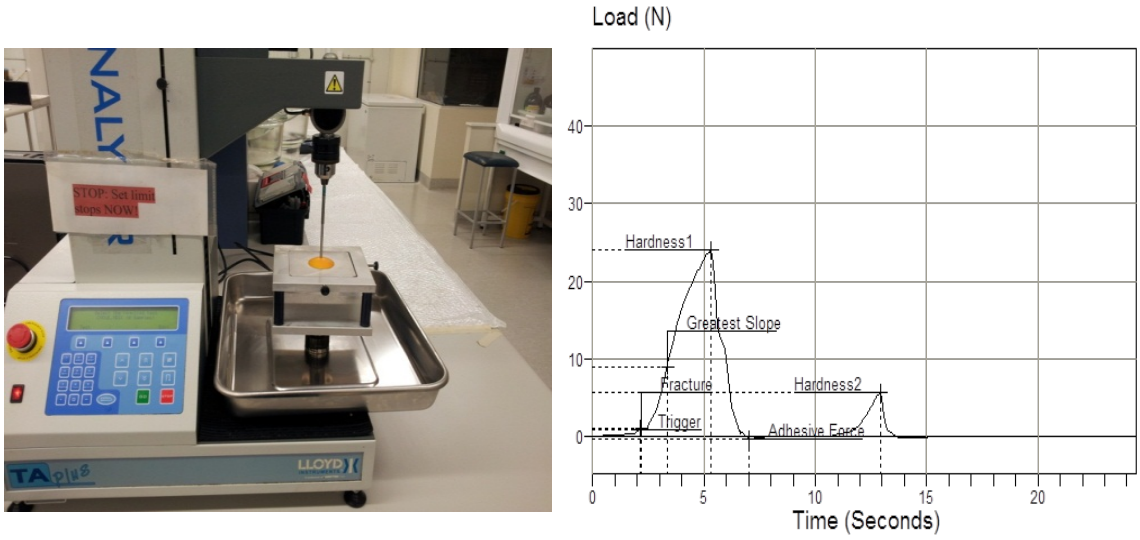


(A) (B)

Figure 3.5. A texture analyzer (TA Plus, AMETEK Lloyd instruments Ltd., Hampshire, UK) (A) for determining fruit firmness and a typical curve for fruit firmness (B).

3.4.2. Rind hardness

Rind hardness of sweet orange cv. Washington Navel and Lane Late was measured using the puncture test by employing a textural analyzer (Figure. 3.6). The fruit rind was cut in the size of 2.5 cm wide x 0.6 cm thick using a slicer (Zyliss Easy slice 2" folding Mandoline slicer, Swiss) to obtain uniform sections for determining rind hardness. Five fruit per tree were randomly selected to measure the fruit rind hardness. The fruit rind samples were placed on to the flat plate. A cylinder probe of 4 mm diameter attached to the load cell was used to measure rind thickness. The speed of probe was 50 mm min^{-1}. Hardness is the maximum force of the first penetration when the rind sample is contacted to the probe at 70% of rind compression. Rind hardness (N) is the force at the first significant peak during the first compression of fruit rind.

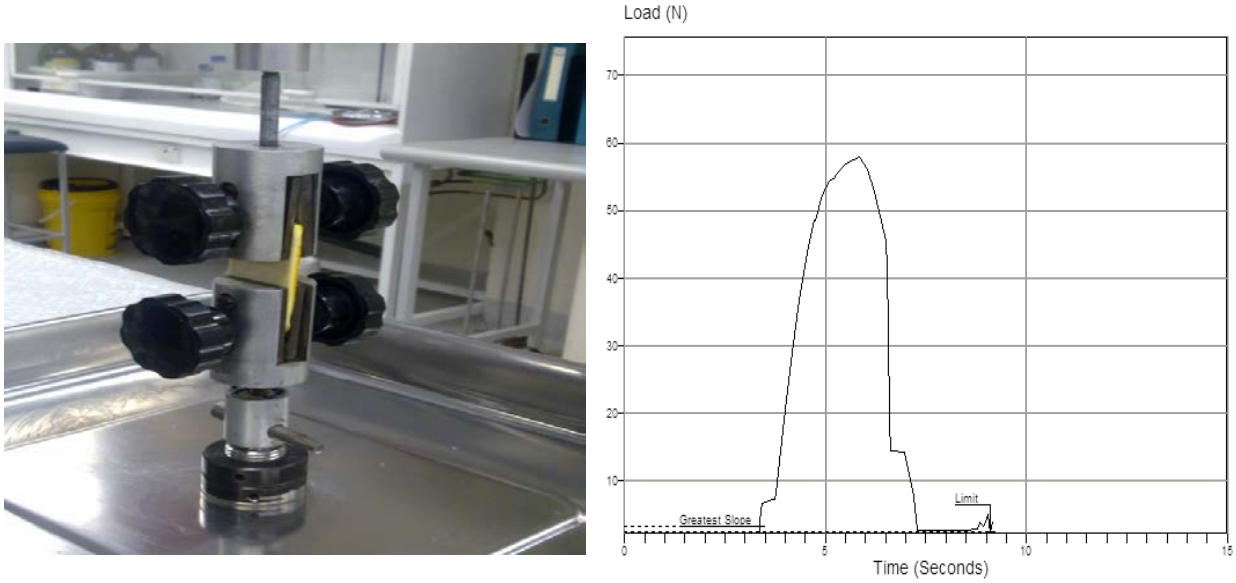


(A) (B)

Figure 3.6. A texture analyzer (TA Plus, AMETEK Lloyd instruments Ltd., Hampshire, UK) used for determining rind hardness (A) and a typical curve for puncture test (B).

3.4.3. Rind tensile strength

The rind tensile test was used to determine the rind tensile strength (Figure 3.7). Five fruit per replication was used for each test. Sweet orange fruit rind was carefully removed in the size 2.5 cm wide x 5.0 cm length x 0.6 cm thick using a slicer to give uniform sections. Then small rind portions were inserted longitudinally into two clamps of the texture analyzer. One clamp was fixed to the base of the machine while another one was attached to the moveable load cell. The rind sample was subjected to axial tensile loading until rind deflection of 10 mm at the crosshead speed of 100 mm min^{-1} and preload of 10 N. The rind tensile strength was calculated at the maximum load and limit points where the rind deflection occurred.



(A) (B)

Figure 3.7. A texture analyzer (TA Plus, AMETEK Lloyd instruments Ltd., Hampshire, UK) used for determining rind tensile strength (A) and a typical curve for rind tensile test (B).

3.5. Determination of fruit weight, diameter and rind thickness

The fruit weight was calculated by weighing ten fruit per replication by using a digital balance (A&D Limited, Tokyo, Japan) and average weight was calculated as grams (g) per fruit. Fruit diameter was also measured from ten fruit per replication using a digital vernier caliper and expressed in mm. Similarly, ten fruit per replication was selected and cut into two equal halves to measure the rind thickness from four equatorial regions of the fruit (Figure 3.8) using a digital vernier caliper and expressed as (mm).

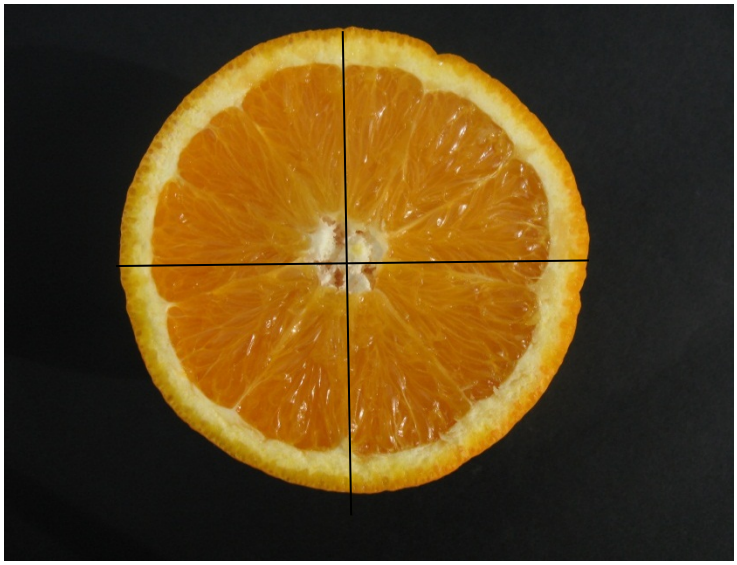


Figure 3.8. The equatorial region of sweet orange fruit used to measure rind thickness.

3.6. Soluble solids concentration (SSC)

The juice was extracted from ten randomly harvested sweet orange fruit to determine SSC, TA and SSC: TA ratio. The SSC was recorded by measuring refractive index using a digital refractometer (Atago-Palette PR 101, Atago CO. Ltd, Itabashi-Ku, Tokyo, Japan) and expressed as a percentage.

3.7. Titratable acidity (TA)

To determine TA, freshly extracted juice (10 ml) was diluted with 20 ml dH_2O. Aliquot (5 ml) was titrated against 0.1 N NaOH solutions using 2-3 drops of phenolphthalein as an indicator to a pink colour end point. TA was expressed as per cent citric acid and calculated by the following formula:

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

Where,

0.0064 = Milli-equivalent weight of citric acid

30 = Total volume (ml)

10 = Extract juice sample (ml)

5 = Volume of aliquot (ml)

3.8. SSC/TA ratio

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

3.9. Estimation of ascorbic acid

Ascorbic acid concentrations were estimated using the method previously described by Malik and Singh (2005) and Pham (2009) with some modifications as detailed in Figure 3.9. Freshly extracted orange juice (5 ml) was mixed with 25 ml 6% metaphosphoric acid containing (0.18%) disodium salt ethylenediaminetetraacetate acid (EDTA) then homogenised and centrifuged at 3220× g for 15 min using a centrifuge (Eppendorf Centrifuge 5810 R, Hamburg, Germany). The supernatant (400 µl) was mixed with 200 µl of 3% metaphosphoric acid, 1.4 ml dH_2O, and diluted with 200 µl folin reagent (Folin:dH_2O, 1:5 v/v). Disposable cuvettes (2 ml) were used to record the absorbance of the mixed sample after 15 min at 760 nm wavelength using a UV/VIS spectrophotometer (Jenway spectrophotometer Model 6405, Dunmow, Essex, UK). Ascorbic acid concentration was calculated by using standard curve of L-ascorbic acid and expressed as mg ascorbic acid per 100 ml fresh juice.

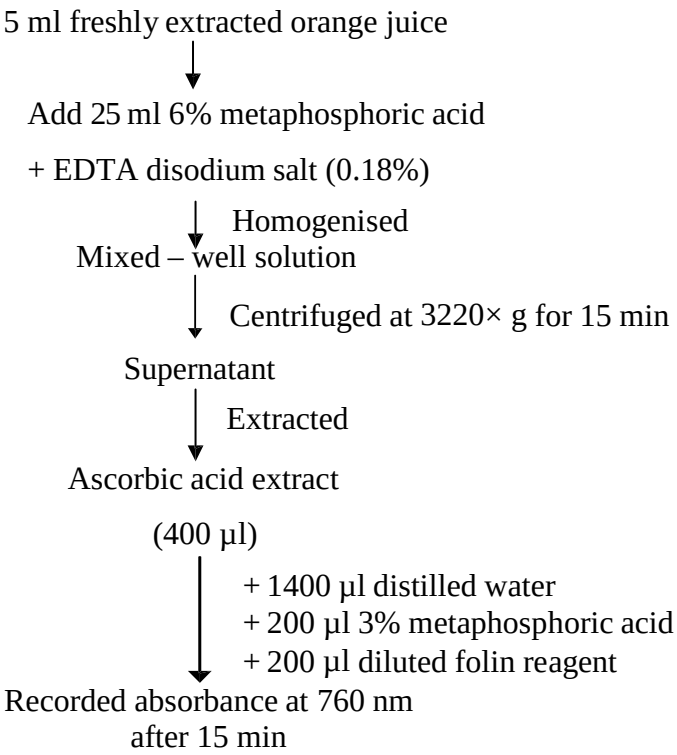


Figure 3.9. Flow chart for the estimation of concentration of ascorbic acid in sweet orange juice.

3.10. Determination of total antioxidants

The total antioxidants were determined by employing the modified method of Brand-Williams et al. (1995) and Pham (2009) from the freshly extracted juice of sweet orange cv. Washington Navel and Lane Late (Figure 3.10). A stock solution (24 mg DPPH in 100 ml MeOH) of DPPH (1, 1diphenyl-2-picrylhydrazl) was used as a source of free radical which was prepared in 80% methanol. An aliquot (50 µl) of the appropriately diluted extract was mixed with 950 µl of the freshly prepared methanolic DPPH (12 µM). The mixture was vortexed for 5 seconds and allowed to stand in the dark at 21±1℃ for 15 min. The decrease in absorbance of DPPH was measured at 515 nm by using 6405 UV/VIS Spectrophotometer (Jenway Spectrophotometers 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 mM Trolox equivalent antioxidant activity (TEAC) 100 ml^{-1} FJ basis.

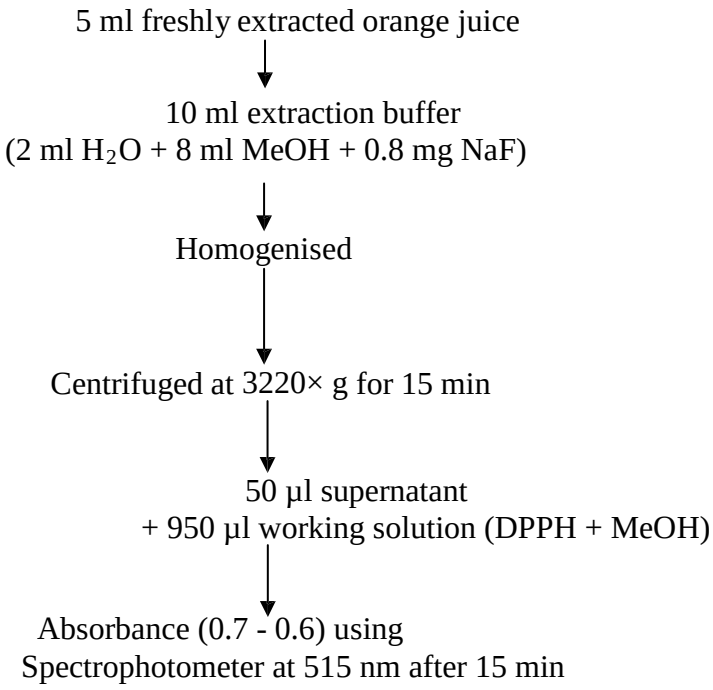


Figure 3.10. Flow chart for the determination of total antioxidants in juice of sweet orange.

3.11. Determination of individual sugars and organic acids

3.11.1. Chemicals used

The details of the individual standard used for determination of sugars (sucrose, D-glucose anhydrous and D-(-)-fructose) and organic acid (citric, tartrate, shikimic, fumaric and malic acid) were purchased from Sigma-Aldrich, Australia and all chemicals were of high-performance liquid chromatography (HPLC) grade.

3.11.2. Sample preparation

Sweet orange juice (1 ml) was homogenised in 19 ml of Milli-Q water, which was passed through a purification water system (Millipore, Bedford, MA, USA), using a mini-mixer (DIAX 900, Heidolph Co., Ltd., Schwabach, Germany) for 1 min to extract individual sugars and organic acids. The diluted juice was centrifuged at 12857× g for 10 minutes using a centrifuge (Eppendorf Centrifuge 5810R, Hamburg, Germany). Following centrifugation, 1 ml diluted juice mixture was filtered through a 0.22-µm nylon syringe filter (Altech Associates, Baulkham Hills, New South Wales, Australia) for determination of individual sugars and organic acids by HPLC.

3.11.3. HPLC conditions

The determination of individual sugars and organic acids was performed using reverse phase liquid chromatography with a HPLC system (Waters 1525, Milford Corp., MA, USA), which is fitted to Dual λ Absorbance Detector (Waters 2487, Milford Corp., MA, USA). An aliquot (20 µl) of the extract from orange juice was injected using an autosampler (Waters 717plus, Milford Corp., MA, USA) maintained at 25°C. The individual sugars and organic acids were separated on a Bio-Rad Aminex® HPX-87C Fast Carbohydrate column (100 × 7.8 mm) and Bio-Rad Aminex® HPX-87H column (300 × 7.8 mm) (Bio-Rad Laboratories, Inc., Hercules, USA) with a particle size of 9 µm, respectively. The column was preceded by 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 and 45°C for sugars and acids, respectively, during the analysis. The sulphuric acid solution (0.05 mM) was used as a mobile phase with the flow rate of 0.6 ml min^{-1} for elution of organic acids. The sugars were eluted with degassed water only and the flow rate was kept at 0.6 ml min^{-1}. All individual organic acids were detected at 210 nm with dual wavelength UV detector, whilst the individual sugars were detected using 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).

3.11.4 Elution orders and retention times

The elution order and retention times of the various sugars and organic acids wereidentified in sweet orange juice: samples are shown in Table 3.1 and 3.2, respectively. The chromatogram in Figures 3.11 and 3.12 show the different sugars and organic acids peak in the standard, respectively.

Table 3.1. Elution order, retention times and detective wavelength of different standards used for identifying the individual sugars in the juice of sweet orange cv. Washington Navel and Lane Late.

Elution order	Sugars standard compound	Retention Time (min)	Detector
1	Sucrose	3.75	RI
2	Glucose	4.00	RI
3	Fructose	5.75	RI

RI = refractive index

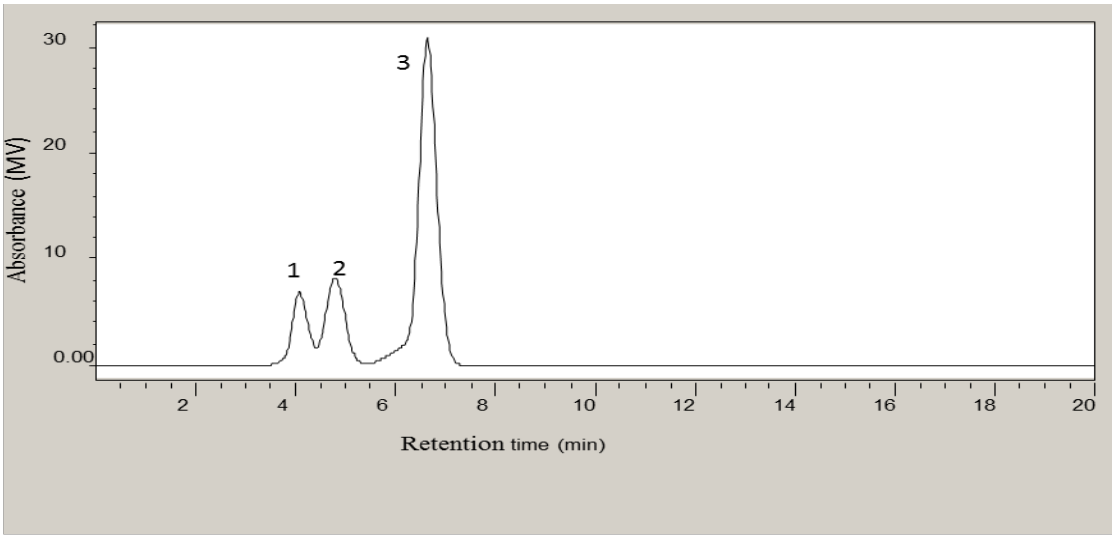


Figure 3.11. HPLC chromatographic profile of individual standard sugars (1) Sucrose, (2) Glucose and (3) Fructose; MV = Millivolt(s).

Table 3.2. Elution order and retention times of different organic acids used for identifying the organic acids in the juice of sweet orange cv. Washington Navel and Lane Late.

Elution order	Standard	Retention time (min)	Detection wavelength (nm)
1	Citric	15.75	210
2	Tartaric	16.50	210
3	Malic	18.50	210
4	Succinic	23.00	210
5	Fumaric	28.00	210

210= refractive index

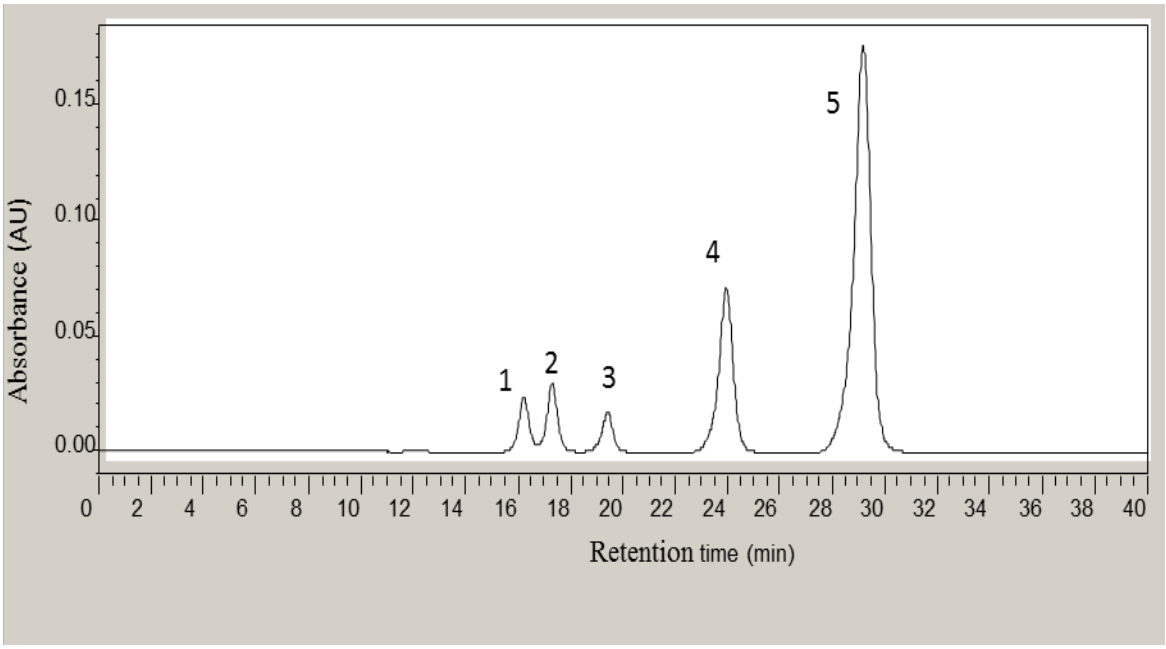


Figure 3.12. HPLC chromatographic profile of individual standard organic acids (1) Citric acid, (2) Tartaric acid, (3) Malic acid, (4) Shikimic acid and (5) Fumaric acid; AU = Absorbance units.

3.12. Ethylene estimation

The endogenous levels of ethylene were determined by following the method described earlier by Pranamornkith et al. (2012). After harvesting, the fruit were brought to the laboratory within three to four hours. The levels of ethylene were determined using an ETD 300 ethylene detector (Sensor sense B.V, Nijmegen, The Netherlands). The fruit were washed to remove the dust and other agro-chemicals. Fruit free from disease and injuries were used for the determination of ethylene. Each fruit sample was weighed before being transferred into cuvettes [i.e. 1.0 L air-tight jar, fitted with a rubber septum (SubaSeal®, Sigma-Aldrich Co., St. Louis, USA)]. All the cuvettes were kept tightly sealed to prevent leakage. Before connecting flow to the cuvette, it was ensured that the output of the cuvette was not blocked, in order to avoid pressure being built up in the cuvette. Each sample was run for 20 min and flow rate was 4 L hr^{-1}. The ethylene was expressed in ($\mu L.kg^{-1}.h^{-1}$).

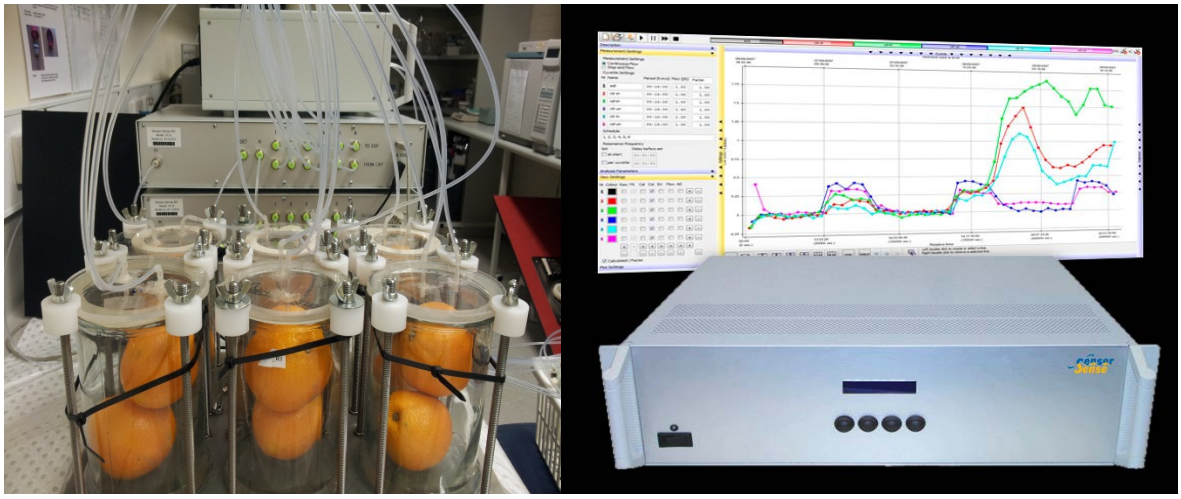


Figure 3.13. Ethylene estimation by using a Sensor Sense B.V (Nijmegen, The Netherlands) (http://www.sensor-sense.nl/).

3.13. Determination of pectins

Total, water soluble and water insoluble pectins were determined from the albedo and flavedo tissues of sweet orange cv. Washington Navel and Lane Late (Figure 3.14) following the method of Wang et al. (2008) by using an UV/VIS spectrophotometer (Model 6405; Jenway Limited, Gransmore Green, Felsted, Dunmow, Essex, UK).

3.13.1. Chemicals used

Different chemicals and acids such as sodium hydroxide, ethanol, sulphuric acid (H_2SO_4), 3 phenyl phenol, sodium tetraborate, dH_2O and galacturonic acid were purchased from Sigma-Aldrich, Australia to determine the total, water soluble and water insoluble pectins.

3.13.2. Sample preparation

A mixture of 2.5 g of albedo or flavedo tissues powder was added to 15 ml of hot absolute ethanol and heated in a 50 ml test tube for 10 min in a boiling water bath. Then samples were centrifuged at 12857 × g for 10 min at 4 ºC by using a centrifuge (Eppendorf 5810R Centrifuge, Hamburg, Germany). The residues/pallets were taken and dried for 24 h at 35 ºC, and alcohol insoluble solids (AIS) were obtained.

3.13.3. Total pectins

To determine total pectin, 5 mg of AIS was mixed with 2 ml of concentrated sulphuric acid in a test tube. Then one ml of dH_2O was added drop-by-drop whilst stirring for 20 min then left to stand for five hours until the AIS were completely dissolved (Figure 3.14). The AIS

mixture was transferred into a 25 ml volumetric flask and made up to volume with dH_2O for total pectin measurements.

3.13.4. Water soluble pectin

Similarly, for water soluble pectin, a mixture containing 80 mg of AIS and 20 ml of distilled water was stirred in a centrifuge tube (50 ml) for 5 minutes at room temperature (20± 2 ºC) and centrifuged at 12857 × g for 10 min at 4 ºC. The residue was extracted twice with 20 ml of dH_2O. Then all the supernatants were transferred into a 100 ml volumetric flask and made up to volume with dH_2O for water soluble pectin examination. One ml solution of total or water soluble pectins containing 6 ml of 0.0125 M sodium tetraborate (in concentrated sulphuric acid) were transferred on to an ice bath and then heated for 10 min in a boiling water bath. The solution was then transferred into a cold water bath for colour development followed by the addition of 0.1 ml of 0.15% m-hydroxydiphenyl and then incubation for 20 min at room temperature. The absorbance of total, water soluble and water insoluble pectins was measured at 525 nm by using UV/VIS Spectrophotometer (Jenway Spectrophotometers Model 6405, Dunmow, Essex, UK).

3.13.5. Water insoluble pectin

The water insoluble pectins were determined by subtracting water soluble pectins from the total pectins. Total, water soluble and water insoluble pectins were expressed as galacturonic acid equivalents and express as $mg.g^{-1}$.

2.5 g of the dried albedo and flavedo tissues of sweet orange

↓

+ 15 ml hot absolute ethanol (80 °C)
(In 50 ml centrifuge tube)

↓

Heated in boiling water (100 °C) bath for 10 minutes

↓

Centrifuged at 12857 × g at 4 °C for 10 minutes

↓

Take residue/pallet and let dry for 24 h at 35 °C
(AIS)

Total Pectin

5 mg AIS + 2 ml H_2SO_4 con in test tube
(Wait until complete dissolved)

↓

Add 1 ml dH_2O drop by drop with stirring for 20 min

↓

Transfer to 25 ml flask and made up to volume with dH_2O
(Sample ready for total pectin)

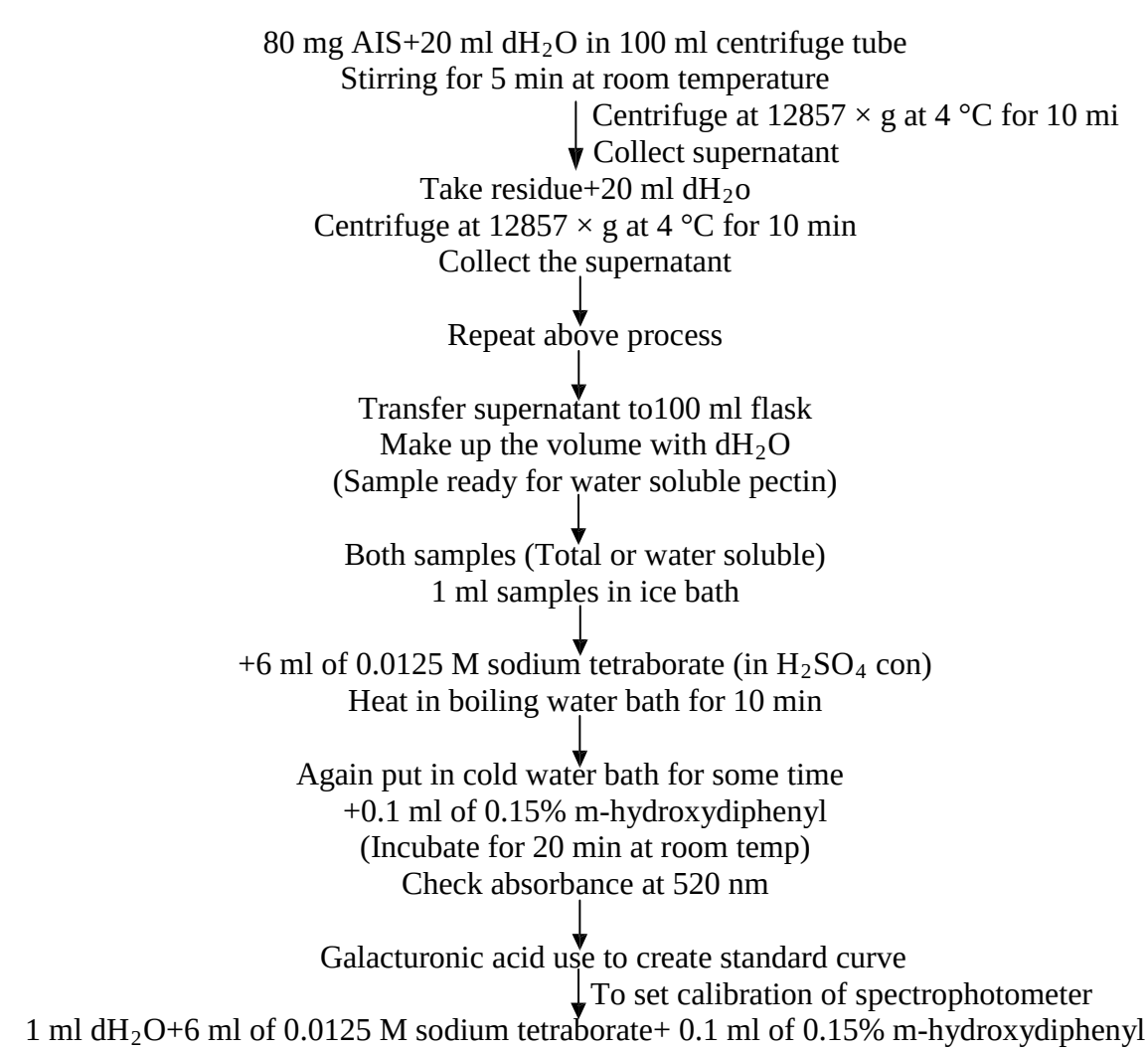


Figure 3.14. Flow chart of determination of pectins in the albedo and flavedo tissues of sweet orange fruit.

3.14. Determination of activities of cell wall degrading enzymes

Cell wall degrading/softening enzymes including *exo*-PG, *endo*-PG, PE and EGase in the albedo and flavedo tissues of sweet orange fruit were determined using the previously detailed methods (Zaharah and Singh, 2011; Khan and Singh, 2007) with some modifications.

3.14.1 Preparation of samples

The albedo and flavedo tissues of orange fruit (13 g) were homogenised with 13 ml cold aqueous solution containing 12% polyethylene-glycol and 0.025 sodium bisulphite ($NaHSO_3$) with glass pestle and mortar using ~200 mg white quartz sand (-50+70 mesh, Sigma Aldrich, USA). Following centrifugation at 12857 × g for 45 min at 4 °C, the pellet was washed with 13 ml cold aqueous solution of ($NaHSO_3$) (0.2%) and re-centrifuged at 12857 × g for 15 minutes at 4 °C; and stored at -80 °C before further analysis of *exo*- and *endo*-PG, PE and EGase. A flow chart of the enzyme extraction is shown in Figure 3.15.

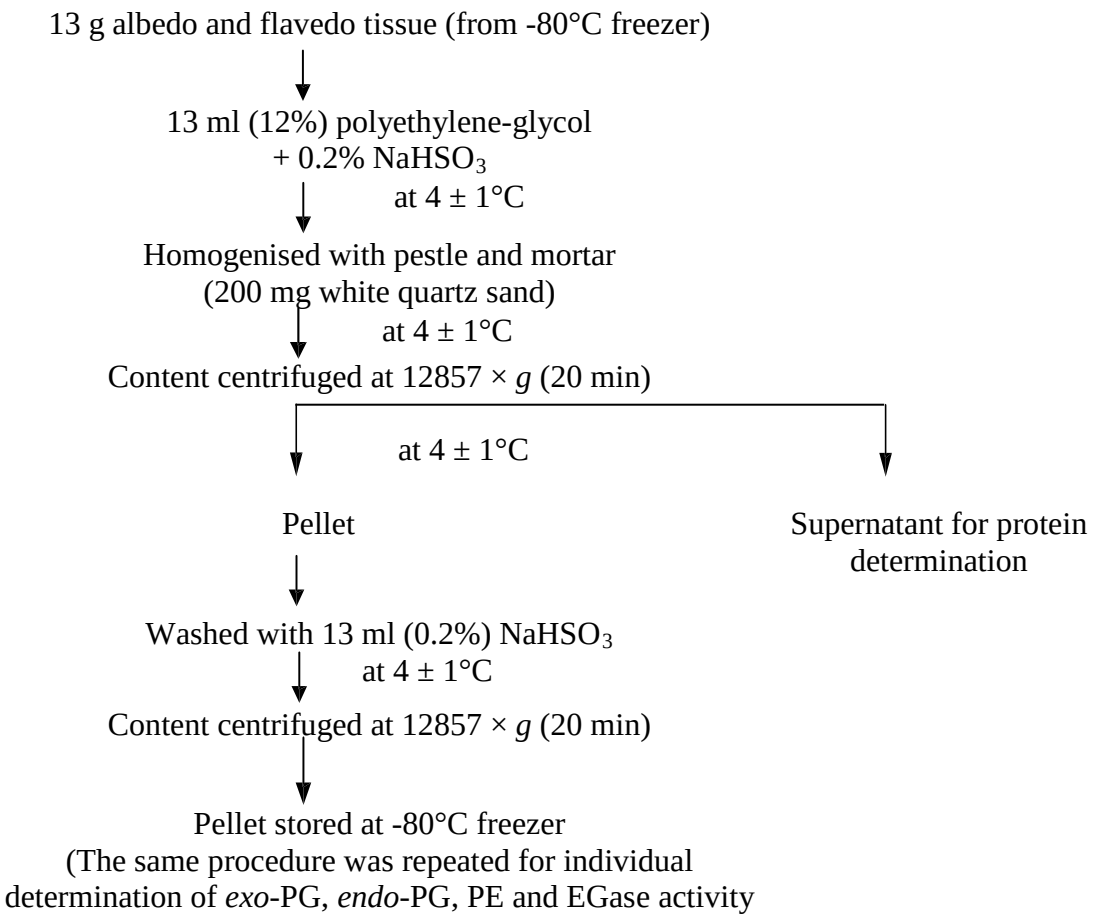


Figure 3.15. Flow chart for extraction and assay of *endo*-, *exo*-PG, PE and EGase activities in the albedo and the flavedo tissues of sweet orange fruit.

3.14.2. Preparation of crude enzyme extract for determination of exo- and endo-PG activity

The crude enzyme extracts for determination of *exo* and *endo*-PG were prepared by incubating the pellet in 15 ml cold 50 mM sodium acetate (CH_3COONa) buffer (pH 5) containing 0.5 M NaCl at 4 °C for one hour (Figure 3.16). Following the centrifugation at 12857 × g for 15 min at 4 °C, supernatant was diluted 1:1 with 50 mM sodium acetate buffer (pH 5) and used as crude enzyme extract for determination of *exo*- and *endo*-PG analysis.

Pellet incubated in cold 15 ml (50 mM) CH_3COONa, pH 5
+ 0.5 M NaCl for 1 h
↓ at 4 ± 1°C
Centrifuged at 12857 × *g* for 15 min
↓ at 4 ± 1°C
Supernatant diluted with 50 mM CH_3COONa buffer, pH 5 (1:1, v/v)
(for crude enzyme extract)

Figure. 3.16. Flow chart for preparation of crude enzyme extracts for determination of activities of *exo*- and *endo*-PG in the albedo and flavedo tissues of sweet orange fruit.

3.14.3 Determination of exo-PG activity

The enzyme extract (0.15 ml) was mixed with 0.15 ml polygalacturonic acid (0.5%) in 50 mM sodium acetate buffer (pH 4.4) and the contents were incubated at 30 °C for 18 h. To determine the amount of galacturonic acid released, 2 ml borate buffer (0.1 M) pH 9 and 0.3 ml cyanoacetamide (1 %) were added to the reaction mixture and boiled for 10 min. The absorbance of the cooled solution was recorded at 274 nm by using a UV-VIS spectrophotometer (Model 6405, Jenway Ltd., Felsted, Dunmow, Essex, England) and was calculated against a standard curve of D-galacturonic acid and expressed as µg galacturonic acid mg^{-1}protein h^{-1}(Figure 3.17).

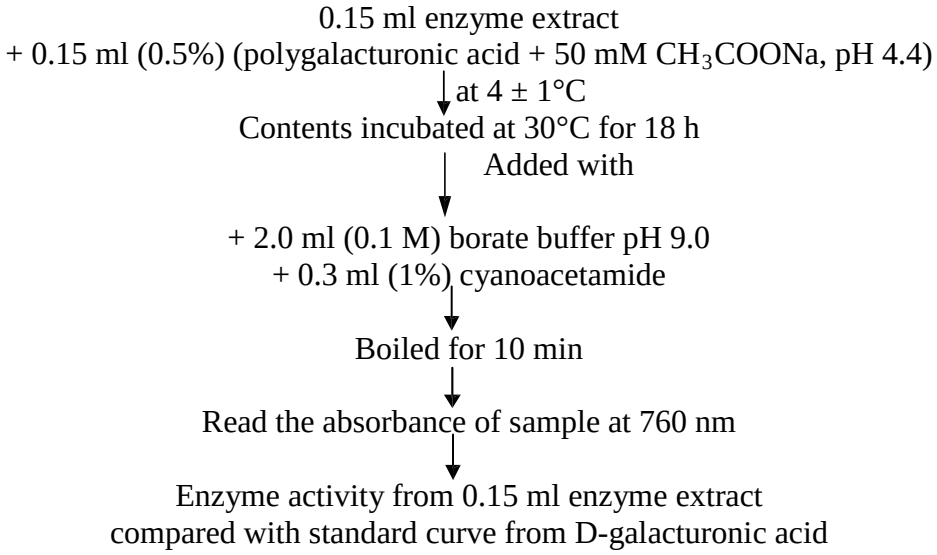


Figure. 3.17. Flow chart for the determination of *exo*-PG activity in the albedo and flavedo tissues of sweet orange fruit.

The activity of *exo*-PG (µg galacturonic acid mg^{-1}protein h^{-1}) was calculated by using the following formula:

$$\textit{Exo}\text{-PG activity} = \frac{\text{Galacturonic acid in sample (µg)} \times 2 \times 15}{0.15 \times 13 \times 18 \times \text{Protein amount (mg ml}^{-1})}$$

Where,

2 = Dilution factor

15 = Vol. of enzyme extract (ml)

0.15 = Vol. of extract used (ml)

13 = Vol. of extraction buffer (ml)

18 = Incubation time (h)

3.14.4 Determination of endo-PG activity

A Cannon-Fenske viscosity meter (Size-50, 2139 High Tech Road, State College, Pennsylvania, USA) was used to measure the activity of *endo*-PG. Three ml enzyme extract (as used for *exo*-PG) was mixed to a cold solution containing 4.5 ml polygalacturonic acid (2.0 %) in 50 mm sodium acetate buffer (pH 4.4). The viscosity was measured immediately following the addition of enzyme extract and after 18 h incubation at 30°C (Figure 3.18). The viscosity meter was cleaned with acetone after each run and calibrated with dH_2O. The activity of *endo*-PG enzyme was expressed as viscosity changes.mg.protein-$1h^{-1}$.

3 ml enzyme extract
+ 4.5 ml [(2%) polygalacturonic acid + 50 mM CH_3COONa, pH 4.4]
↓ at 2 ± 1°C
Contents incubated at 30°C for 18 h
↓
Viscosity measured immediately and after 18 h
↓
Enzyme activity from 3.0 ml enzyme extract

Figure 3.18. Flow chart for the determination of *endo*-PG activity in the albedo and flavedo tissues of sweet orange fruit.

The activity of *endo*-PG was expressed as viscosity mg protein^{-1} h^{-1} and calculated by using the following formula:

$$\textit{endo}\text{-PG activity} = \frac{[(V_{before} - VdH_2O) - (V_{after} - VdH_2O)] \times 2 \times 15}{3 \times 13 \times 18 \times \text{protein amount (mg ml}^{-1})}$$

Where,

V_{before} = Viscosity of sample before incubation (s)
V_{after} = Viscosity of sample after incubation (s)
VdH_2O = Viscosity of dH_2O (s)
2 = Dilution factor
15 = Vol. of enzyme extract solution (ml)
3 = Vol. of extract used (ml)
13 = Vol. of extraction buffer (ml)
18 = Incubation time (h)

3.14.5 Determination of pectin esterase (PE)

To determine the activity of PE from the albedo and flavedo tissues, the pellet was prepared as described in Section 3.14.1 and re-suspended in 15 ml (7.5% NaCl, w/v) and 0.75% (w/v) ethylene diamine tetra-acetic acid (EDTA) at 12857 × g at 4°C for 15 min (Figure 3.19). Five ml of supernatant (crude enzyme extract) was mixed with 20 ml (0.1 %) citrus pectin and titrated against 0.01 *N* NaOH, while incubating at 30°C to maintain pH 7.4 for 10 minutes. During the titration and incubation time, the reaction mixture was continuously and slowly shaken by hand. The total amount of 0.01 *N* NaOH to maintain pH 7.4 was used to calculate the PE activity following the formula as mentioned below and the activity was expressed as mM NaOH mg^{-1} protein h^{-1}.

$$\text{PE activity} = \frac{\text{(Vol. of 0.01 } N \text{ NaOH used} \times 0.01) \text{ mM NaOH} \times \text{Vol. of enzyme extracts (ml)}}{\text{Sample weight (g)} \times (20/60) \text{ h} \times \text{Protein content (mg mL}^{-1})}$$

Pellet suspended in 15.0 ml (7.5%) NaCl
+ 0.75% EDTA (pH 6.5)
↓
Content incubated at 4°C, (10 min)
↓
Content centrifuged at 12857× *g*, (15 min)
↓ at 4 ± 1°C
5 ml supernatant
+ 20 ml (1%) citrus pectin
↓
Contents titrated against 0.01 *N* NaOH to pH 7.4 at 30°C
↓
Reaction continued for 10 min
PE activity was calculated from 5.0 ml extract

Figure 3.19. Flow chart for determination of PE activity in the albedo and flavedo tissues of sweet orange fruit.

3.14.6. Determination of EGase activity

The albedo and flavedo tissue (13 g) was used to determine EGase activity. Pellet that formed through similar procedure as *endo* or *exo*-PG was stirred in 15 ml (0.1 M) citrate-phosphate buffer (pH 6) containing 1 M of NaCl for 1 h. Following centrifugation at 12857 × g for 15 min at 4°C, three ml of supernatant (crude enzyme extract) was mixed with 6.0 ml (0.2%, w/v) carboxymethyl cellulose in citrate phosphate buffer. The viscosity changes were measured immediately and after 18 h incubated at 30°C using a Cannon-Fenske viscometer (Size 50, Cannon Instrument Company, PA, USA). The enzyme activity from 3 ml enzyme extract was calculated following the same formula for calculating the activity of *endo*-PG as mentioned in Section 3.14.4 and expressed as viscosity-changes mg^{-1}protein h^{-1}.

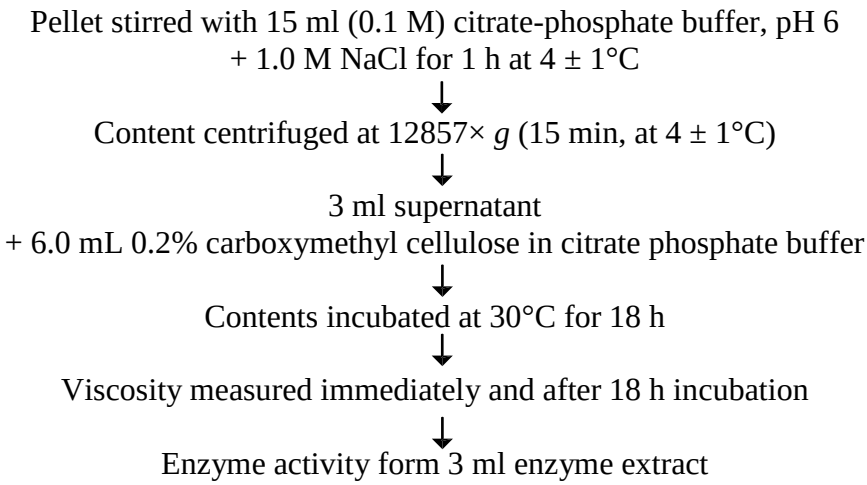


Figure 3.20. Flow chart for determination of EGase activity in the albedo and flavedo tissues of sweet orange fruit.

3.15. Protein determination

3.15.1 Protein reagent preparation

Total protein content of orange juice was determined following the Bradford method (Bradford, 1976). Coomassie brilliant blue dye G-250 (100 mg) was dissolved in 50 ml of 95% ethanol and 100 ml of 85% phosphoric acid (w/v). The volume was made up to 1 L with dH_2O. The dye was filtered through Whatman No.1 filter paper (Whatman Intl. Ltd., Maidstone, England) and stored in dark bottles at 4±1°C.

3.15.2. Determination of protein in orange juice

Protein contents from albedo or flavedo tissues of sweet orange fruit were estimated using the method of Bradford (1976). Bradford reagent solution (5 ml) was added into 100 µl of protein sample (see Section 3.14.1) and put in 12 × 100 mm reaction tube. The contents were mixed well using a vortex mixer (Heidolph, John Morris Scientific Pty. Ltd., Germany) and the absorbance was recorded at 595 nm in 3 ml plastic disposable cuvettes by using an UV/VIS spectrophotometer (Model 6405, Jenway Ltd., Felsted, Dunmow, Essex, England). The reagent blank was prepared by mixing 100 µl of the appropriate buffer with 5 ml of protein reagent. The protein concentration was determined by comparing the value with a standard graph prepared using bovine serum albumin and was expressed as $mg.g^{-1}$ FW.

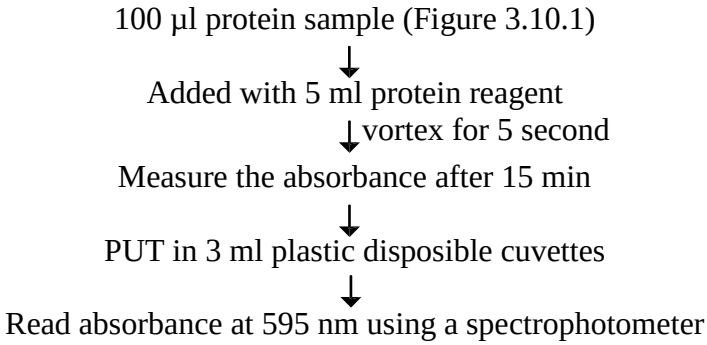


Figure 3.21. Flow chart for the determination of protein in the albedo and flavedo tissues of sweet orange fruit.

3.16. Statistical analysis

All the experimental data were subjected to one and two-way analysis of variance (ANOVA) depending upon experiment, using GenStat 14th edition (Lawes Agricultural Trust, Rothamsted Experimental Station, Rothamsted, UK). The effects of treatments and their interactions for various parameters were assessed within ANOVA and least significance differences (Duncan, LSD) were calculated following significant F-test at ($P \leq 0.05$). All the assumptions of analysis were checked to ensure validity of statistical analysis. Linear correlation was performed between rind thickness and creasing index using Microsoft excel 2010.

CHAPTER 4

Involvement of polyamines in creasing of sweet orange fruit

Summary

Creasing is a physiological disorder in the rind of sweet orange [*Citrus sinensis* (L.) Osbeck] fruit and causes serious economic losses globally. The involvement of polyamines (PAs) in creasing and rind thickness of sweet orange fruit was investigated by monitoring the endogenous levels of free putrescine (PUT), spermidine (SPD), spermine (SPM) and total polyamines in the albedo and flavedo tissues of the fruit rind following exogenous applications of PUT and a reversible inhibitor of PAs biosynthesis (guanylhydrazone, MGBG) at different fruit developmental stages, depending upon its concentration and time of application, spray application of PUT reduced creasing index percentage (CI) and increased rind thickness in cv. Washington Navel and Lane Late during 2011 and 2012. A spray application of PUT (500-1000 µM) at the fruit set or golf ball stage was more effective in reducing CI in both cultivars, as compared to all other treatments. PUT applied at the fruit set, golf ball or at the colour break stages resulted in increased levels of endogenous free PAs (PUT, SPD, SPM and total free polyamines) in the flavedo and albedo tissues of fruit in both cultivars. Spray application of MGBG (1000 µM) at the golf ball stage significantly increased CI in cv. Washington Navel and Lane Late sweet orange fruit. In conclusion, higher levels of free PUT, SPD, SPM and total polyamines in the albedo and flavedo tissues of the fruit with exogenous application of PUT, substantially reduced CI. CI was accelerated with applications of MGBG. These findings suggest the involvement of polyamines in creasing of sweet orange fruit.

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Hussain, Z and Z. Singh, Involvement of polyamines in creasing of sweet orange [Citrus sinensis (L.) Osbeck] fruit. Journal of Plant Growth Regulation (submitted).

4.1. Introduction

Creasing (albedo breakdown) is a peel-related disorder in sweet orange fruit. The symptoms of this disorder consist of small random cracks in the albedo tissue corresponding to sunken grooves on the fruit surface (Erickson, 1968). The fracturing of the albedo tissue is predominantly due to separation of adjacent cells rather than cleavage of individual cells (Storey and Treeby, 1994). Although creasing is usually detectable at fruit maturity, its initiation seems to be associated with earlier stages of fruit growth and development (Storey and Treeby, 1994).

Creasing affects different cultivars of sweet orange including Washington Navel (Gambetta et al., 2000; Ali et al., 2000), Valencia (Jones et al., 1967; Monselise et al., 1976) and Nova mandarins (Greenberg et al., 2006). Creasing was first reported from South Africa during 1938 (Le Roux et al., 1938) and is currently a major problem in the sweet orange industry in different parts of the world such as Australia (Storey and Treeby, 1994), USA (Ali et al., 2000; Jones et al., 1967), Israel (Monselise et al., 1976; Greenberg et al., 2006), Uruguay (Gambetta et al., 2000), Spain (Agustí et al., 2001) and China (Li et al., 2009). More than 50% losses in individual orchards have been reported from South Africa (Gilfillan et al., 1981). In the Australian citrus industry, creasing is a major cause of fruit diversion from fresh markets to processing and 50% of the Navel orange crop may be affected to varying degrees due to this disorder (Treeby et al., 2000).

Presently, the exact cause of this physiological disorder is not known. However, several factors have been associated with this disorder, such as genotype (Agusti et al., 2003); climate (Jones et al., 1967; Gambetta et al., 2000); rootstock (Storey et al., 2002); crop load (Jones et al, 1967; rind thickness (Holtzhausen, 1981); irrigation (Agustí et al., 2004) and mineral nutrition (Ali et al., 2000; Bower, 2004). Five repeated sprays of an aqueous solution containing calcium (0.33%) at 10 day intervals commencing at the golf ball stage reduced creasing (25-30%) in sweet orange fruit (Treeby et al., 2000; Pham et al., 2012). Similarly, GA_3 (20 mg L^{-1}) spray applied at 3 to 4 weeks prior to colour break or at colour break significantly reduced the incidence of creasing in Navel orange (Gilfillan et al., 1981), but resulted in poor rind colour development.

Citrus fruit growth and development is complex and is coordinated by changes in endogenous levels of one or more plant hormones, including polyamines (El-Otmani et al, 1995). Endogenous polyamines are involved in flower development in sweet orange (Sagee and Lovatt, 1991) and fruit growth of Murcott mandarin (Nathan et al., 1984). PAs are involved in many plant developmental processes, including cell division and morphogenesis (Cona et al., 2006; Kusano et al., 2007); fruit development and ripening (Kakkar and Rai, 1993); as well as fruitlet abscission and senescence (Bais and Ravishankar, 2002; Rastogi and Davies, 1991). Exogenous applications of PAs have been reported to reduce fruitlet abscission, (a cell separation process), depending upon type, concentration and time of application of PAs (Malik and Singh, 2003). The spray application of PAs at flowering markedly reduced fruitlet abscission in mandarin (Nathan et al, 1984) and sweet orange (Saleem et al., 2007).

PAs are biologically active compounds of low molecular weight with aliphatic nitrogen groups and are present in all living organisms (Cohen, 1978). The most common PAs are PUT, SPD, SPM and cadaverine (CAD). PAs exist in both free and conjugated forms in plants (Evans and Malmberg, 1989), however free PAs participate more actively in biological processes (Antognoni et al., 1998). Due to their cationic nature, PAs coordinate with anionic components of cell membranes, such as the phospholipids (Roberts et al., 1984). PAs have a physiological role in cell walls by interacting directly with pectins and cell wall components (D'Orazi and Bagni, 1987). PAs are essential for maintaining cell wall characteristics by strengthening the links between cell wall components (Berta et al., 1997). Creasing in sweet orange fruit is known to be associated with enhanced loss of pectins in the cell walls of the albedo, leading to cell wall loosening, formation of cracks and consequently reduced hardness, stiffness and tensile force of the rind (Saleem et al., 2014; Monselise et al., 1976).

However, no research work has been reported on the involvement of PAs in creasing of sweet orange. As creasing also involves cell separation, we hypothesised that PAs play a role in modulating creasing in sweet orange fruit. We investigated the changes in the concentrations of the endogenous free PAs such as PUT, SPD and SPM in the albedo and flavedo tissues of rind at various fruit developmental stages such as the golf ball, mature, and ripe stages. We also examined the effects of exogenous application of different concentrations of PUT at different fruit developmental stages including the fruit set, the golf ball or the colour break stage. The effects of the reversible inhibitor of PAs biosynthesis

(MGBG) in regulating the incidence of creasing in sweet oranges at the golf ball stage were also determined.

4.2. Materials and methods

4.2.1 Plant Material

Four different experiments were conducted during 2010-11 to 2011-12 in a commercial orchard located at Gingin (latitude 31° 21′ South, longitude 155° 55′ East), Western Australia. Twenty-five year old uniform sweet orange trees grafted on trifoliate orange (*Poncirus trifoliate* Raf.) rootstock were used in the experiments. The trees were spaced 7.5 m between rows and 2.7 m within rows in the North-South orientation. These experiments were conducted on Washington Navel and Lane Late sweet oranges. All the experimental trees received similar cultural practices including fertilisers, irrigation, weed control and plant protection, except for the experimental treatments. The experimental site has a sandy loam soil dominated by cool, wet winters and hot, dry summers.

4.2.2. Experiment I: Effects of exogenous application of PUT on the incidence of creasing and the endogenous levels of free polyamine in the albedo and flavedo tissues of rind in Washington Navel sweet orange

PUT (100, 250, 500 and 1000 μM) dissolved in water and 0.05% (v/v) 'Tween 20' as a surfactant were sprayed on whole trees until run off at the fruit set (fruit diameter: 15±5 mm), the golf ball (fruit diameter 40±5 mm) or at the colour break (fruit diameter: 80±5 mm) stages in cv. Washington Navel. Control trees were kept unsprayed. The experimental layout was randomised block design with two-factor factorial, treatments and stage of application, with a single tree as the experimental unit and four replicates. The experiment was conducted in two consecutive seasons during 2010-11 and 2011-12. The data of two years were not pooled because error mean squares over two years were heterogeneous. In both years, 35 ripe fruit per tree were randomly harvested around the tree canopy. The incidence of creasing was examined on individual fruit, based on the appearance of the fruit surface and a creasing index was calculated. Rind thickness was measured by using a Vernier caliper. In 2011-12, the effects of different concentrations of exogenously applied PUT on the changes in

endogenous levels of PAs in the albedo and flavedo tissues were monitored. Ten fruit per tree were harvested at the fruit set, the golf ball or at the colour break stage. The concentrations of free polyamines (PUT, SPD and SPM) were determined from the albedo and flavedo tissues of the fruit from the control and all the treatments.

4.2.3. Experiment II: Effects of exogenous application of PUT on the incidence of creasing and endogenous levels of free polyamine in the albedo and flavedo tissues of rind in Lane Late sweet orange

The **s**ame experiment was repeated employing the same PUT treatments and experimental design on the cultivar Lane Late during 2010-11 and 2011-12. In this experiment, the creasing index and rind thickness were recorded during both years however the effects of PUT treatments on levels of endogenous free polyamines in the albedo and flavedo tissues were also examined in 2011-12 only.

4.2.4. Experiment III: Effect of exogenous application of reversible inhibitor of PAs methylglyoxal-bis (guanylhydrazone) MGBG on the incidence of creasing in Washington Navel sweet orange

Uniform, 25 year old Washington Navel sweet orange trees were sprayed with an aqueous solution containing different concentrations (100, 250, 500 and 1000 µM) of (MGBG) and 0.05% 'Tween 20' as a surfactant at the golf ball stage (fruit diameter 40±5 mm) on whole trees until run off in 2010-11 . The untreated trees served as the controls. The experiment was laid out by following a randomised block design using single trees as an experimental unit and replicated four times. Creasing index was recorded from 35 randomly harvested fruit per tree.

4.2.5. Experiment IV: Effects of exogenous application of reversible inhibitor of PAs methylglyoxal-bis (guanylhydrazone) MGBG on the incidence of creasing in Lane Late sweet orange

Another experiment was conducted on cultivar Lane Late in 2010-11 . All the treatments and experimental design were similar to experiment III (4.2.4). At the ripe stage, 35 fruit per tree

were randomly harvested around the tree canopy. Creasing index (CI) was recorded from these harvested fruit.

4.2.6. Creasing index (%)

The thirty-five fruit from each experimental tree were randomly harvested around the tree canopy to determine the creasing index (%) by following the method of Treeby and Storey (2002) and the procedure for determination of creasing index percentage has been mentioned as detailed in Chapter 3, Section 3.3.

4.2.7. Measurement of rind thickness

Rind thickness was also measured from 10 randomly selected fruit (four equatorial regions of each fruit) using a digital Vernier caliper and expressed in mm as described in Chapter 3, Section 3.5.

4.2.8. Determination of free polyamines in the albedo and flavedo tissues

4.2.8.1. Collection of samples

In 2011-12, the effects of different concentrations of exogenous spray application of PUT on the changes of endogenous levels of free PAs in the albedo and flavedo tissues were monitored in sweet orange cv. Washington Navel and Lane Late. Ten fruit per tree were harvested 67 and 79 days after spraying (DAS) at the fruit set, 107 and 128 DAS at the golf ball stage, and 56 and 75 DAS at the colour break stage in cv. Washington Navel and Lane Late, respectively. The concentrations of free PAs (PUT, SPD, SPM and total polyamines) were determined from the albedo and flavedo tissues of the fruit from different treatments and the control.

4.2.8.2. Extraction and quantification of free PAs

The free PAs were estimated in the albedo and flavedo tissues of sweet orange fruit by following the method of Morgan (1998) with some modifications as explained in Chapter 3, Section 3.2. The data of free PAs were collected and processed with Breeze® 3.30 software (Waters, Milford, MA, USA) and expressed as $nmol.g^{-1}$ DW.

4.2.9. Statistical analysis

The experimental data were subjected to two-way analysis of variance (ANOVA), using GenStat 14th edition (Lawes Agricultural Trust, Rothamsted experimental station, U. K). The effects of treatments, time of application and their interactions on different parameters were assessed within ANOVA and the least significant differences were calculated following significance (Duncan LSD) at $P \leq 0.05$. All the assumptions of analysis were checked to ensure validity of statistical analysis.

4.2.10. Results

4.2.10.1. Effect of exogenous application of putrescine on CI (%) in cv. Washington Navel and Lane Late

The CI significantly ($P \leq 0.05$) decreased as the concentrations of PUT sprayed was increased in cv. Washington Navel and Lane Late during 2011 and 2012 (Figure 4.1). When averaged over different stages of PUT spray application, the treatment of PUT (1000 µM) significantly reduced mean CI as compared to the control and all other treatments except 500 µM PUT in cv. Washington Navel and Lane Late during both the years. The spray application of PUT at the fruit set or the golf ball stage was significantly more effective in reducing CI in cv. Washington Navel and Lane Late as compared to its application at the colour break stage. A spray application of PUT (1000 µM) applied at the golf ball stage resulted in a lower CI (14.1 and 17.0%) in cv. Washington Navel during 2011 and 2012, respectively as compared to the control (35.0 and 35.3%) and all other treatments. A spray application of PUT (1000 µM) at the golf ball stage resulted in lower CI (16.1 and 14.9%) as compared to the control (32.3 and 38.5%) in cv. Lane Late during 2011 and 2012, respectively. The interaction between treatments and their stages of application for CI was found to be non-significant ($P \leq 0.05$) in both cultivars during 2011 and 2012.

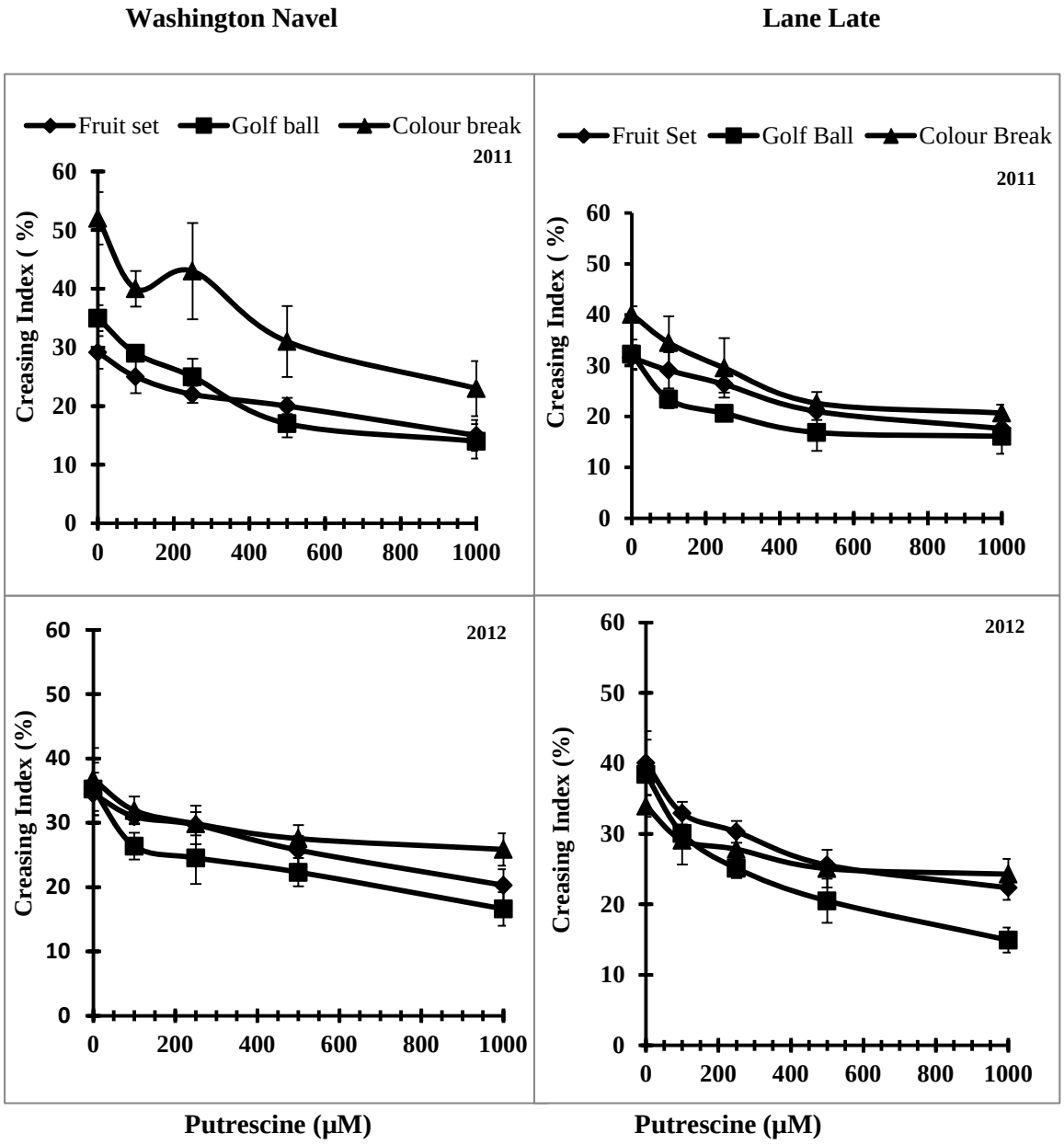

Figure 4.1 Effect of different concentrations of PUT sprayed at the fruit set, the golf ball or at the colour break stage on CI (%) on cv. Washington Navel and Lane Late sweet orange fruit during 2011 and 2012. n = 4 replications (35 fruit per replication). Vertical bars represent standard error means. LSD ($P \leq 0.05$) for Washington Navel 2011, Treatments = 6.1, Stage = 4.7, Treatments x stage = ns; Washington Navel 2012, Treatments = 4.71, Stage = 3.7, Treatments x stage = ns; LSD ($P \leq 0.05$) for Lane Late 2011, Treatments = 5.0, stage = 4.0, Treatments x stage = ns; ($P \leq 0.05$) for Lane Late 2012, Treatments = 4.3, stage = 3.3, Treatments x stage = ns; ns = not significant.

4.2.10.2 Effect of exogenous application of PUT on fruit rind thickness in cv. Washington Navel and Lane Late

All PUT treatments increased rind thickness of Washington Navel and Lane Late orange fruit with increased concentration, irrespective of its application at different stages during 2011 and 2012 seasons (Tables 4.1 and 4.2). When averaged over stage of spray application, the treatment of PUT (1000 µM) resulted in significantly ($P \leq 0.05$) higher mean fruit rind thickness (6.0 and 5.1 mm) as compared to the control (4.4 and 4.4 mm) during 2011 and all other treatments except PUT (250 µM) during 2012. In cv. Lane Late, the treatment of PUT (1000 µM) resulted in significantly ($P \leq 0.05$) higher mean fruit rind thickness (5.5 and 5.3 mm) as compared to the control (4.6 and 4.7 mm) and all other treatments except PUT (100 µM) during 2011 and 2012 (Table 4.2).

When averaged over treatments, the mean rind thickness was significantly increased (5.9 mm), when PUT was applied at the fruit set stage as compared to its application at the golf ball (5.4 mm) or at the fruit colour break stage (5.2 mm) in cv. Washington Navel. In 2012, mean rind thickness was significantly ($P \leq 0.05$) higher, when PUT was applied at the colour break stage (5.3 mm) as compared to its spray application at the fruit set (4.8 mm) or at the golf ball stages (4.2 mm) in cv. Washington Navel. In cultivar Lane Late, the stage of application of PUT affected rind thickness significantly only in 2012 (Table 4.2). The interaction between PUT spray treatments and their stage of application for rind thickness in cv. Washington Navel was non-significant ($P \leq 0.05$) during both years (Table 4.1). In cultivar Lane Late, significant interactions between the PUT treatments and its stages of application were also observed for fruit rind thickness during 2011, whilst it was found to be non-significant ($P \leq 0.05$) during 2012.

Table 4.1. Effect of different concentrations of PUT sprayed at the fruit set, the golf ball or at the colour break stage on fruit rind thickness of sweet orange cv. Washington Navel during 2011 and 2012.

	Rind thickness (mm)							
Treat (µM)	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	5.3	4.7	5.1	3.7	4.3	5.0	4.4 c	4.4 c
PUT 100	5.5	4.8	5.4	4.1	5.1	5.1	5.3 ab	4.7 bc
PUT 250	6.0	4.9	5.4	4.1	5.2	5.3	5.6 ab	4.6 ab
PUT 500	6.1	4.9	5.4	4.4	5.6	5.5	5.7 ab	4.9 ab
PUT 1000	6.4	4.9	5.9	4.4	5.6	5.8	6.0 a	5.1 a
Mean (stage)	5.9 b	4.8b	5.4 ab	4.2 c	5.2 a	5.3a		
LSD ($P < 0.05$)	2011	Treatments = 0.7, Stage = 0.5, Treatments x stage = ns						
	2012	Treatments = 0.3, Stage = 0.2, Treatments x stage = ns						

n = 4 replications (10 fruit per replication), means within a column and within a row followed by different letters are significantly different at $P < 0.05$, ns = not significant.

Table 4.2. Effect of different concentrations of PUT sprayed at the fruit set, the golf ball or at the colour break stage on fruit rind thickness of sweet orange cv. Lane Late during 2011 and 2012.

	Rind thickness (mm)							
Treat (µM)	Fruit set		Golf ball		Colour break		Means (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	4.5 de	4.6	4.2 e	4.5	5.1bcd	5.0	4.6 c	4.7 b
PUT 100	5.3 abcd	5.1	4.5 de	4.9	5.0 bcde	5.1	4.9 bc	5.0 a
PUT 250	5.4 abc	5.1	5.0 bcde	4.9	5.8 ab	5.2	5.4 a	5.1 a
PUT 500	5.3 abcd	5.2	5.3 abcd	5.0	5.1 bcde	5.3	5.2 ab	5.2 a
PUT 1000	6.1a	5.3	5.7 ab	5.1	4.7 cde	5.3	5.5 a	5.3 a
Mean (stage)	5.3	5.1 a	4.9	4.896 b	5.131	5.2 a		
LSD ($P < 0.05$)	2011	Treatments = 0.4, Stage = ns, Treatments x stage = 0.8						
	2012	Treatments = 0.3, Stage = 0.2, Treatments x stage = ns						

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different at $P < 0.05$, ns = not significant, Treat= Treatments.

4.2.10.3. Effect of exogenous application of PUT at various fruit developmental stages on comparative levels of endogenous free PAs in the flavedo and albedo tissues

All the treatments of PUT applied at the fruit set stage resulted in increased levels of endogenous free PAs (PUT, SPD, SPM and total free polyamines) in the flavedo and albedo tissues of fruit rind, when sample was collected at the golf ball stage (67 and 79 DAS) in cv. Washington Navel and Lane Late, respectively (Figure 4.2 A-H). Exogenous spray application of PUT (1000 µM) at fruit set stage was most effective in elevating the levels of PUT, SPD and SPM and total free polyamines in the albedo and flavedo tissues, when determined at the golf ball stage as compared to the control and all other treatments in both cultivars.

The levels of endogenous free polyamines (PUT, SPD, SPM and total free polyamines) in the flavedo and albedo tissues increased up to the mature fruit stage (107 and 128 DAS) in cv. Washington Navel and Lane Late, respectively, with the exogenous application of PUT at the golf ball stage (Figure 4.3 A-H). In general, the endogenous levels of PUT, SPD, SPM and total free polyamines were higher in the flavedo than albedo tissues of mature fruit in cv. Lane Late than Washington Navel. Similarly, the levels of endogenous free PAs (PUT, SPD, SPM and total free polyamines) in the flavedo and albedo tissues of ripe fruit (56 and 75 DAS) in both cultivars increased with the spray application of PUT at the colour break stage (Figure 4.4 A-H). In general, the endogenous levels of PUT, SPD, SPM and total free polyamines were higher in the flavedo than albedo tissue of the rind at the golf ball, the mature fruit and at the colour break stage in both the cultivars, irrespective of treatment and stage of application (Figure 4.2-4.4 A-H).

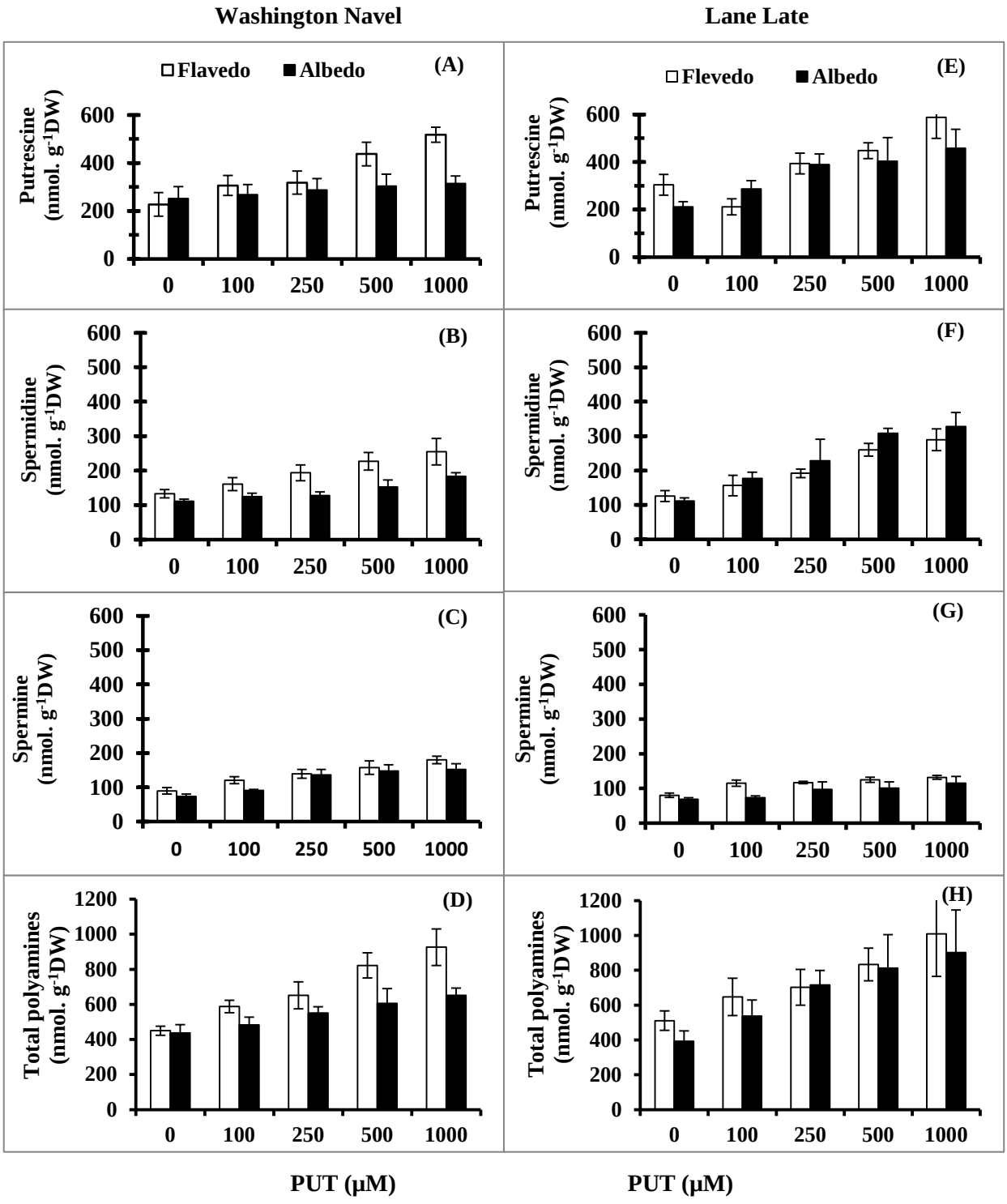

Figure 4.2. Effect of exogenous application of different concentrations of PUT on the levels of endogenous free PAs in the flavedo and albedo tissues at the fruit set stage of cv. Washington Navel and Lane Late fruit: n = 4 replications (10 fruit per replication). Vertical bars represent standard error means. LSD ($P \leq 0.05$) for Washington Navel at the fruit set stage for PUT, flavedo = 192.1, albedo = ns, for SPD: flavedo = 82; albedo = 38.5, for SPM: flavedo= 41.1, albedo = 42.1, for total PAs: flavedo = 223.7; albedo= ns; LSD ($P < 0.05$) for Lane Late at the fruit set stage for PUT flavedo = 146.1, albedo = ns, for SPD: flavedo = 67.8; albedo=119.6, for SPM: flavedo= 22.1, albedo = ns, for total PAs: flavedo = 81.7; albedo= 258.4; ns = not significant.

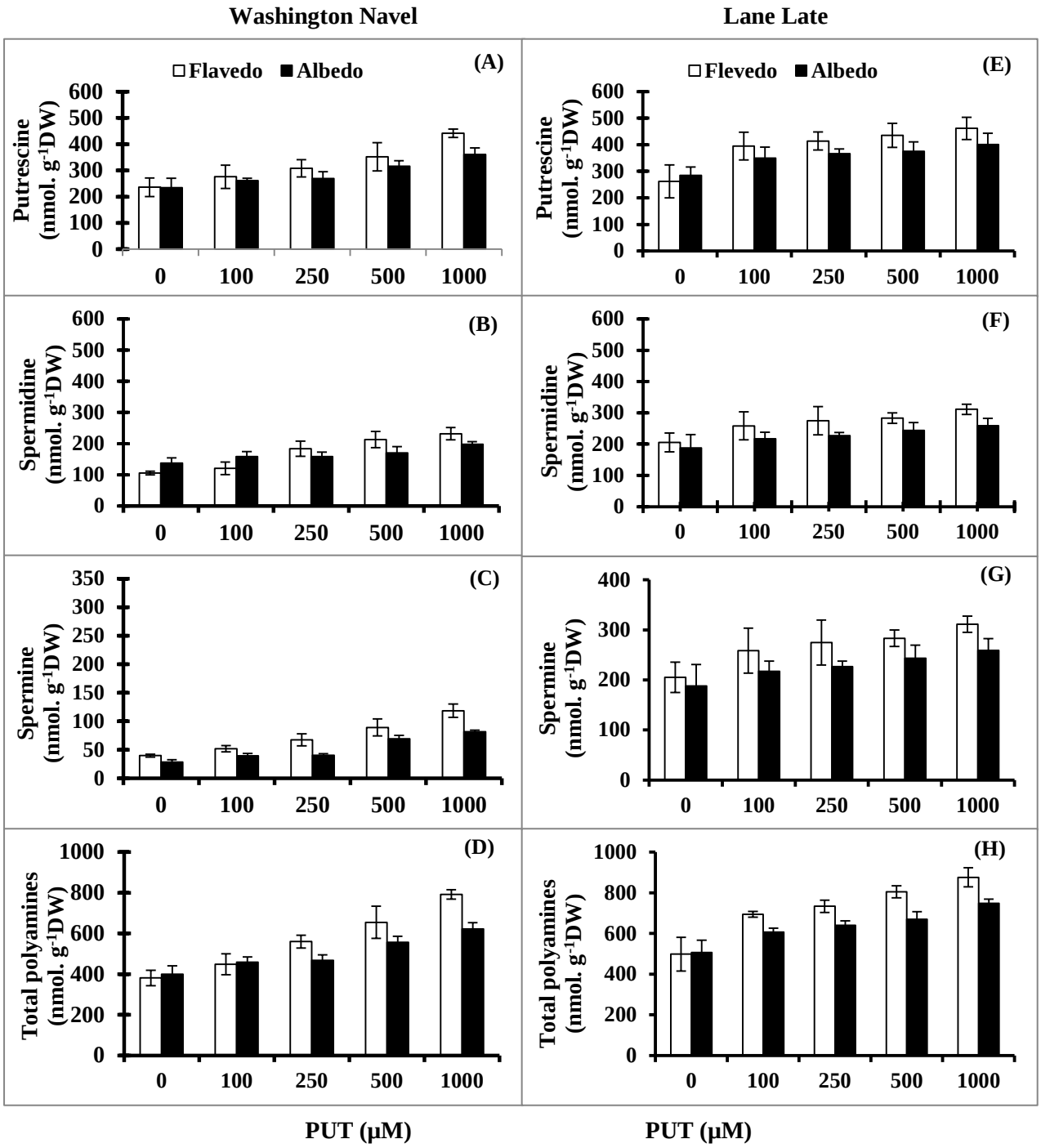

Figure 4.3. Effect of exogenous application of different concentrations of PUT on the levels of endogenous free PAs in the flavedo and albedo tissues at the golf ball stage of cv. Washington Navel and Lane Late fruit: n = 4 replications (10 fruit per replication). Vertical bars represent standard error means. LSD ($P \leq 0.05$) for Washington Navel at the golf ball stage for PUT, flavedo = ns, albedo = ns, for SPD: flavedo = 65.02; albedo = ns, for SPM: flavedo = 31.8, albedo = 11.2, for total PAs: flavedo = 164.4; albedo= 42; LSD ($P < 0.05$) for Lane Late at the golf ball stage for PUT flavedo = ns , albedo = ns, for SPD: flavedo = ns; albedo= ns, for SPM: flavedo= 19.14, albedo = 20.83, for total PAs: flavedo = 141.5; albedo = 106.4; ns = not significant.

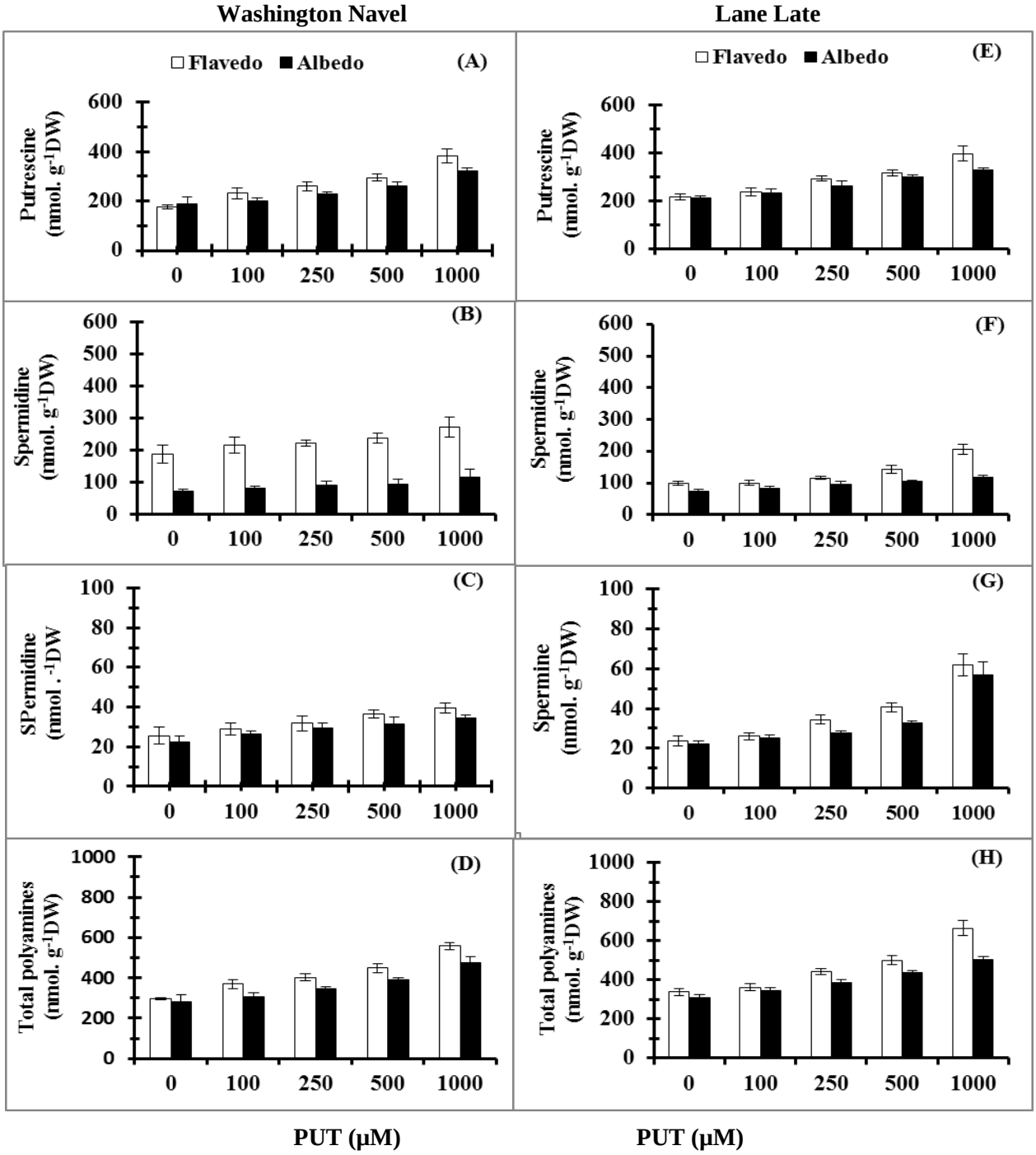

Figure 4.4. Effect of exogenous application of different concentrations of PUT on the levels of endogenous free polyamines in the flavedo and albedo tissues at the colour break stage of cv. Washington Navel and Lane Late fruit: n = 4 replications (10 fruit per replication). Vertical bars represent standard error means. LSD ($P \leq 0.05$) for Washington Navel at the colour break stage for PUT, flavedo = 65.6, albedo = 60.4, for SPD: flavedo = 71.0; albedo = ns, for SPM: flavedo= ns, albedo = ns, for total PAs: flavedo = 60.9; albedo= 72.9; LSD ($P < 0.05$) for Lane Late at the colour break stage for PUT flavedo = 101.6 , albedo = 69.2, for SPD: flavedo = 50.8; albedo = 27.3, for SPM: flavedo = 14.5, albedo =14.9, for total PAs: flavedo = 121.4; albedo= 70.5; ns = not significant.

4.2.10.4. Effect of exogenous application of reversible inhibitor of PAs biosynthesis MGBG on CI in Washington Navel and Lane Late sweet orange

All the spray treatments of MGBG applied at the golf ball stage significantly increased CI (%) in cv. Washington Navel and Lane Late, respectively (Figure 4.5). The spray application of MGBG (1000 µM) at the golf ball stage resulted in the highest CI (39.6 and 43.7%) as compared to the controls (25.5 and 23.2%) and all other treatments in cv. Washington Navel and Lane Late, respectively.

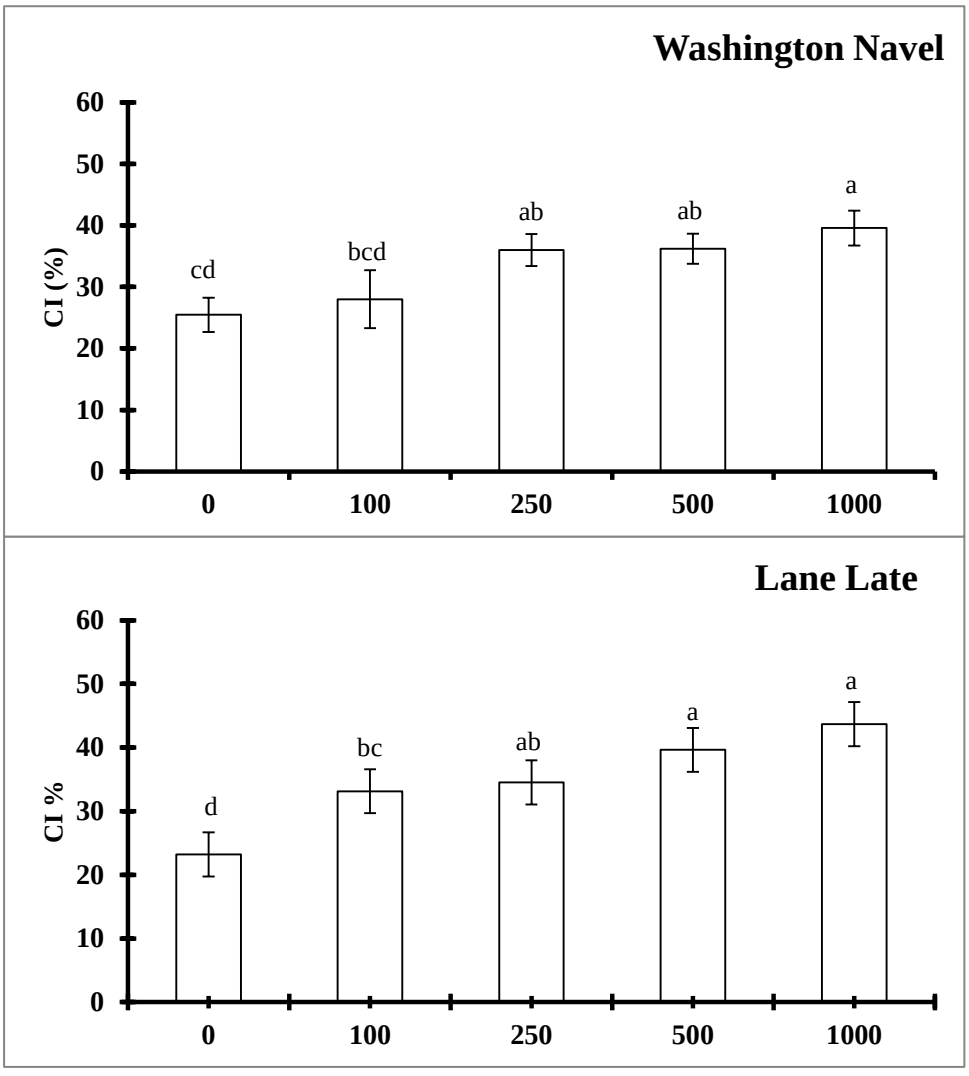

Figure 4.5. Effect of exogenous application of different concentrations of reversible inhibitor of polyamines biosynthesis (MGBG) on the incidence of creasing in sweet orange cv. Washington Navel and Lane Late: n = 4 replications (35-fruit per replication). Vertical bars represent standard error means. LSD ($P < 0.05$) for Treatments = 6.6, Cultivars = ns; Treatments × cultivars= ns. ns = not significant.

4.2.11. Discussion

Exogenous spray application of PUT resulted in a substantial reduction of CI depending upon its concentration and stage of application in cv. Washington Navel and Lane Late during 2010-11 and 2011-12. The spray applications of PUT (500-1000 µM) at the fruit set or the golf ball stage were more effective in reducing mean CI as compared to the control and all other treatments in cv. Washington Navel and Lane Late during both seasons (Figure 4.1). Exogenous spray application of PUT applied at the fruit set, the golf ball or at the mature fruit stage increased the levels of endogenous free PAs (PUT, SPD, SPM and total free polyamines) in the flavedo and albedo tissues of fruit (Figure 4.2 and 4.4 A-H). The reduction in CI in cv. Washington Navel and Lane Late sweet orange fruit with the exogenous application of PUT may be ascribed to the increased levels of endogenous free PAs (PUT, SPD, SPM and total free polyamines) in the flavedo and albedo tissues of the fruit. The increased levels may be attributed to the partial conversion of applied PUT to spermine by spermidine synthase and to spermidine by spermine synthase (Kumar et al., 1997). A considerable reduction of CI and elevation of endogenous levels of free PAs (PUT, SPD, SPM and total free polyamines) in the flavedo and albedo tissues of the fruit in cv. Washington Navel and Lane Late during two seasons with exogenous spray applications of PUT suggests the involvement of PAs in creasing of sweet orange fruit. It may also be argued that the increased levels of free polyamines in the albedo and flavedo tissue with the exogenous application of PUT may have inhibited ethylene production and consequently reduced the incidence of creasing. PAs are known to inhibit ethylene biosynthesis by inhibiting ACC synthase and the terminal step of conversion to ethylene (Liu et al., 2006; Evans and Malmberg, 1989). Similarly, the pre-harvest spray application PUT (1-2 mM) significantly suppressed ethylene biosynthesis and consequently reduced the activities of fruit softening enzymes such as *exo*-polygalacturonase (*exo*-PG), *endo*-polygalacturonase (*endo*-PG), pectin esterase (PE) and *endo*-1,4-*β*-D-glucanase (EGase) in skin and pulp tissues during storage of plum (*Prunus salicina* Lindl. cv. Angelino) fruit (Khan et al., 2007). Additionally, creasing is known to be associated with pectin degradation, loosening the connections between cells of the albedo tissue, higher activity of pectin methylesterase, and higher levels of water-soluble pectin similar to over-ripe fruit and is suggestive of early senescence (Monselise et al, 1976). Recently, Saleem et al. (2014) reported that higher activities of PE, *exo*-PG, *endo*-PG and EGase in the albedo of creased fruit at commercial harvest seems to enhance the loss of pectins in the cell walls of the albedo, leading to cell

wall loosening and formation of cracks and consequently reduced hardness and tensile strength of the rind. It may also be argued that the reduction in CI with exogenous spray application of PUT is possibly due to its direct effects on maintaining cell wall characteristics by strengthening the links between cell wall components. PAs are necessary for maintaining normal size and rigidity on the primary cell wall, by increasing their synthesis and strengthening cell wall components (Berta et al., 1997).

All PUT treatments have also increased rind thickness in cv. Washington Navel and Lane Late sweet orange fruit irrespective of different stages of its application during both years. When averaged over different stages of spray application, the treatments of PUT (1000 µM) resulted in significantly higher mean fruit rind thickness (6.0 and 5.1 mm) as compared to the controls (4.4 and 4.4 mm) (Table 4.1 and 4.2). Possibly, the increased levels of free PAs as a result of PUT spray application may have resulted in thicker rind of the fruit, and consequently reduced the CI. A direct significant negative correlation between rind thickness and CI ($r = 0.4$, $y = -0.03x + 5.9$, $r + - 0.50$, $y = -0.02x + 5.6$ in Lane Late during 2011 and 2012 respectively) and in Washington Navel during 2011 ($r = 0.3$, $y = -0.01x + 5.7$) supports the view that thicker rind due to spray application PUT has reduced CI. Similarly, earlier reports also indicate that sweet orange fruit with thinner rind were more prone to creasing than the fruit with thicker rind (Bevington et al., 1993; Jones et al., 1967; Ali et al., 2000; Moulds et al., 1995).

The exogenous spray application of MGBG (1000 µM) at the golf ball stage, a reversible inhibitor of PAs biosynthesis, significantly increased CI (%) in cv. Washington Navel and Lane Late sweet orange fruit (Figure 4.5). MGBG is a known inhibitor of S-adenosyl methionine-decarboxylase (Kumar et al., 1997). Possibly, inhibition of polyamine biosynthesis with the application of MGBG may have resulted in reduced levels of endogenous free polyamines and increased ethylene production in the albedo and flavedo tissues of fruit and consequent increases in CI (%). In contrast, elevated levels of endogenous free polyamines in the albedo and flavedo tissues of fruit with exogenous application of PUT may have reduced CI (Figure 4.1). In conclusion, higher levels of free PAs in the albedo and flavedo tissues of the fruit and the reduction in CI with exogenous application of PUT, as well as its acceleration with inhibitor of biosynthesis of PAs suggest the involvement of PAs in creasing of sweet orange fruit.

CHAPTER 5

Putrescine application alleviates creasing, improve rheological properties and fruit quality in sweet orange [*Citrus sinensis* (L.)Osbeck]

Summary

Creasing causes substantial economic losses to the citrus growers of the world. The effects of different concentrations (0, 100, 250, 500 and 1000 µM) of putrescine (PUT) applied at the fruit set, the golf ball or at the colour break stage on incidence of creasing, rheological properties of the rind and fruit quality in sweet orange cv. Washington Navel and Lane Late were investigated during 2010-11 and 2011-12. Thirty-five fruit per tree were harvested randomly around the tree canopy to determine the incidence of creasing, textural properties of the rind and fruit quality. The exogenous spray application of PUT (1000 µM) at the golf ball stage substantially reduced the incidence of creasing in cv. Washington Navel (22.1 and 24.3%) and in Lane Late (23.6 and 22.9%) during 2011 and 2012, respectively. All the treatments of the PUT improved the rheological properties of the rind and individual sugars, ascorbic acids as well as total antioxidants; in the juice of sweet orange fruit, irrespective of stage of PUT application. However, the treatment of PUT (1000 µM) was more effective in improving fruit firmness (379.9 N), rind hardness (30.8 N), rind tensile strength (76.6 N), fruit weight (319.8 g), diameter (91.0 mm) and total sugars (14.1 g $100ml^{-1}FJ$); when PUT was applied at the fruit set stage in cv. Lane Late. In cv. Washington Navel, fruit firmness (329.1 N), ascorbic acid (70.2 mg $ml100^{-1}$) and total antioxidants (54.4 mM Trolex $100ml^{-1}FJ$) were higher when PUT (1000 µM) was applied at the golf ball stage. Meanwhile, the highest fruit weight (301.5 g) and diameter (83.5 mm) was observed with PUT (1000 µM) when applied at the colour break stage in cv. Washington Navel. In conclusion, exogenous application of PUT (1000 µM) at the golf ball stage substantially reduced creasing incidence and improves fruit quality in sweet orange cv. Washington Navel and Lane Late.

5.1. Introduction

The white tissue of the rind (albedo) is prone to fracturing and is known as creasing (albedo breakdown) in sweet orange (Monselise et al., 1976) and puffiness in mandarins (García-Luis et al., 1985). The creasing also creates a weak point in the flavedo which cause cracks and fruit is ruptured when packed for fresh markets (Gilfillan et al., 1981). The symptoms of creasing also include multiple cracks in the pitted peel because of the albedo decomposition (Li et al., 2009) and separation of cells in the albedo tissue, resulting in channels in the fruit rind (Treeby et al., 1995). Creasing has been reported in different orange-producing countries of the world, including South Africa (Bower, 2004), Australia (Storey and Treeby, 1994), USA (Ali et al., 2000; Jones et al., 1967), Israel (Greenberg et al., 2006; Monselise et al., 1976), Uruguay (Gambetta et al., 2000), Spain (Augustí et al., 2001) and China (Li et al., 2009). Although creasing is visible at fruit maturity, losses due to creasing vary among seasons, location and cultivars, sometime exceeding more than 50% (Gilfillan et al., 1981).

Creasing incidence and severity depend upon various factors such as genotype (Agustí et al., 2003; Treeby et al., 1995), climate (Gambetta et al., 2000; Jones et al., 1967), rootstock (Treeby et al., 1995), crop load (Jones et al., 1967), rind thickness (Holtzhausen, 1981), irrigation (Agustí et al., 2004), tree age (Moulds et al., 1995), fruit position on the tree (Li et al., 2009; Bevington et al., 1993) and mineral nutrition (Pham et al., 2012; Bower, 2004; Ali et al., 2000; Treeby et al., 2002). Creasing is also associated with pectin degradation, loosening of the connection among cells of the albedo tissue, higher activity of pectin methylesterase and higher levels of water soluble pectin, which is associated with an earlier senescence of albedo tissue (Monselise et al., 1976). Recently, Saleem et al. (2014) reported higher activities of pectinesterase (PE), *exo*-polygalacturonase (*exo*-PG), *endo*-polygalacturonase (*endo*-PG), and Endo-1, 4-*ß* -D-glucanase (EGase) in the albedo tissues of creased fruit at commercial harvest seem to enhance the loss of pectins, loss of starch in the cell walls of the albedo, leading to cell wall loosening and formation of cracks on the rind surface of creased fruit of sweet oranges cv. Washington Navel and Navelina.

Creasing is the first step towards the puffiness characteristic of certain citrus genotype. It is undesirable for orange and creased fruit as they should be discarded has lost the main shipping advantage of sweet oranges (Monselise et al., 1976). Therefore, creasing is a major physiological rind disorder in the global citrus industry and large amounts of orange fruit become unsuitable for fresh fruit markets leading to considerable economic losses

(Treeby and Storey, 2002). In the Australian citrus industry, creasing is a major cause of fruit diversion from fresh markets to processing and more than 50% of the sweet orange crop is affected to varying degrees due to this disorder (Treeby et al., 2000). It is estimated that a 1% reduction in creasing will save 1 to 2 million AU$ to Australian citrus growers (Treeby and Storey 1994). The current suggested control measures involving the spray application of calcium has limited (25-30% reduction) success (Pham et al., 2012; Treeby et al., 2000). Similarly, gibberellic acid was used to control creasing in South Africa; it does not prevent creasing and only delays the onset of creasing (Bower, 2004).

Fruit colour, size, peel texture, juiciness, seedlessness, vitamin C, soluble solids concentration (SSC), titratable acidity (TA) and SSC: TA ratio is the important parameters of sweet orange fruit quality (Ahmad, 2006). The creasing did not adversely affect fruit quality including juice percent, SSC, TA, vitamin C, organic acids and sugars in Washington Navel sweet orange (Pham, 2009). Plant growth regulators are used to promote the citrus production and to improve internal fruit quality like SSC, acidity and external fruit parameters like rind colour and firmness (Saleem et al., 2007; El-Otmani et al., 2000). Polyamines (PAs) have been suggested to be a new class of plant growth regulators (Sharma and Dietz, 2006; Costa and Bagni, 1983). PAs are biologically active compounds of low molecular weight with aliphatic nitrogen groups, present in all living organisms (Cohen, 1978). The most common PAs are putrescine (butan-1, 4- diamine) (PUT), triamine spermidine (SPD), tetraamine spermine (SPM) and cadaverine (CAD). Spray application of PAs regulates the fruit set, yield and fruit quality in various fruit crops such as apple (Costa and Bagni, 1983), olive (Rugini and Mencuccini, 1985), litchi (Mitra and Sanyal, 1990), peaches (Bregoli et al., 2002), mango (Malik and Singh, 2003), nectarine (Torrigiani, et al., 2004), pear (Franco-Mora et al., 2005) and sweet orange (Saleem et al., 2007). However, the response of PAs depends upon its type, concentration and stage of application in different fruit crops (Malik and Singh, 2003; Bregoli et al., 2002; Costa and Bagni, 1983).

PAs are found in the cells of all living organisms and are involved in various processes; including regulation of gene expression, translation, cell proliferation and modulation of cell signalling; as well as membrane stabilization (Kusano et al., 2008). Therefore, PAs are used to sustain cell wall characteristics by strengthening the link between cell wall components (Berta et al., 1997). Some preliminary and sporadic research work suggests that the exogenous application of PUT, SPD and SPM (0.01 mM) at the full bloom improves the fruit weight, fruit diameter, peel thickness, SSC and TA in sweet orange cv.

Blood Red (Saleem et al., 2007). The majority of research work has reported the role of PAs in fruit set, development and post-harvest physiology of citrus (Liu et al., 2006; Sagee and Lovatt, 1991). The results verified in my chapter 4 suggest that the exogenous spray application of PUT may increase levels of free polyamines in the albedo and flavedo tissue resulting in inhibition of ethylene production and consequently reduced activity of cell wall degrading enzymes including PE, *endo*- and *exo*-PG and EGase as well as incidence of creasing in sweet orange. However, no research work has reported the effect of spray applications of different concentrations and the time of application of PUT on incidence of creasing; rheological properties of rind; and fruit quality in sweet orange. It was surmised that PUT application, depending upon its concentration and time of application, may reduce creasing and improve fruit quality. Therefore, the effects of different concentrations of PUT applied at the fruit set, the golf ball or at the colour break stage on incidence of creasing and fruit quality in Washington Navel and Lane Late sweet orange fruit were investigated.

5.2. Materials and methods

5.2.1. Plant material

Two different experiments were conducted during 2010-11and 2011-12, on twenty-five year old uniform cv. Washington Navel and Lane Late sweet oranges trees grafted on Trifoliate orange (*Poncirus trifoliata* Raf.) rootstock in a commercial orchard located at Gingin, Perth (latitude 31° 21′ South, longitude 155° 55′ East), Western Australia. The trees were spaced 7.5 m between rows and 2.7 m within rows from North-South row direction. The soil is sandy loam with a mild, wet winter and hot, dry summer. All the experimental trees received similar cultural practices including fertilisers, irrigation, weed control and plant protection (Moulds and Tugwell, 1999) except for experimental treatments.

5.2.2. Experiment I: Effects of exogenous application of different concentrations of PUT applied at the fruit set, the golf ball or at the colour break stage on incidence of creasing and fruit quality in sweet orange cv. Washington Navel

In the first experiment, an aqueous solution containing different concentrations of PUT (100, 250, 500 and 1000 µM) and 0.05% 'Tween 20' as a surfactant were applied at the fruit set (Fruit diameter: 15±5 mm; 07/11/2010 and 29/10/2011), the golf ball (Fruit diameter 40±5 mm; 28/12/2010 and 09/01/2012) or at the colour break stage (Fruit diameter: 80±5 mm;

06/06/2011and 27/04/2012) on to the whole tree until run off in cv. Washington Navel by using a sprayer (The Selecta Trolleypak Mk II, Acacia Ridge, Australia) during two consecutive years. Control trees were kept unsprayed. The experiment was laid out by following a two factor (treatments and stage of spray application) randomised block design with four replicates. Single trees were treated as one experimental unit. The experiment was repeated in two consecutive seasons (2010-11 and 2011-12). The data of two years were not pooled because error mean squares over years were heterogeneous. At the harvest, 35 ripe fruit per tree were randomly harvested around the tree canopy. The incidence of creasing was examined on individual fruit, based on the appearance of symptoms on the fruit surface and expressed as percent fruit with symptoms of creasing. The fruit firmness as fruit compression force, and textural properties of rind such as rind hardness and rind tensile strength were also determined. Fruit weight and diameter was also measured in both consecutive seasons. Similarly, SSC, TA, SSC: TA ratio, ascorbic acid concentration, total antioxidants, individual and total sugars as well as organic acid in the juice were determined during both years.

5.2.3. Experiment II: Effects of exogenous application of different concentrations of PUT applied at the fruit set, the golf ball or at the colour break stage on incidence of creasing and fruit quality in sweet orange cv. Lane Late

The second experiment was repeated for two consecutive years employing the same PUT treatments at the fruit set (Fruit diameter: 15±5 mm, dated: 09/11/2010 and 29/10/2011), the golf ball (Fruit diameter 40±5 mm, dated: 28/12/2010 and 10/01/2012) or at the colour break stage (Fruit diameter: 80±5 mm, dated: 06/07/2011and 28/07/2012) with the same experimental design on cultivar Lane Late during 2010-11 and 2011-12. The incidence of creasing was examined on individual fruit, based on the appearance of symptoms on the fruit surface and expressed as percent creased fruit. Fruit firmness, rind hardness and rind tensile strength were also determined. SSC, TA, SSC: TA ratio, ascorbic acid concentration, total antioxidants, individual and total sugars as well as organic acids were determined from the juice during 2010-11 and 2011-12.

5.2.4. Creasing (%)

In both years, 35 ripe fruit per tree were randomly harvested around the tree canopy from both cultivars. The incidence of creasing was examined on individual fruit, based on the appearance of fruit surface and expressed as percentage of creased fruit as described in Chapter 3, Section 3.3.1.

5.2.5. Rheological properties of fruit and rind

The effect of PUT on rheological properties of the fruit and rind such as fruit firmness (N), rind hardness (N) and rind tensile strength (N) was determined by using a textural analyzer (TA Plus, AMETEK Lloyd instruments Ltd., Hampshire, UK) as detailed in Chapter 3, Section 3.4. The data were collected and processed by using Nexygen® 4.6 software.

5.2.5.1. Fruit firmness

The fruit firmness (N) was measured with the compression test and five fruit per replication were selected with 75 mm height as mentioned in Chapter 3, Section 3.4.1.

5.2.5.2. Rind hardness

The fruit rind was cut 2.5 cm width having 6.0 mm thickness by using a slicer (Zyliss Easy slice, folding Mandoline slicer, Swiss) to give uniform sections for the determination of rind hardness as detailed given in Chapter 3, Section 3.4.2.

5.2.5.3. Rind tensile strength

The rind of sweet orange fruit was carefully cut in the size (2.5 x 5 cm area having 6 mm thickness). Then small rind portions were inserted longitudinally into two clamps of the texture analyzer to determine the rind tensile strength. The rind tensile strength was calculated at the maximum load and limit points, where the rind deflection occurred at 10 mm as reported in Chapter 3, Section 3.4.3.

5.2.6. Fruit weight and diameter

Fruit were harvested at commercial maturity. Average fruit weight (g) was calculated by weighing ten fruit per replication by using a digital electrical balance (A&D Limited, Tokyo, Japan). The fruit size was determined by measuring the fruit diameter of ten randomly chosen

fruit per replication with the help of a digital Vernier caliper and expressed in mm as described in Chapter 3, Section 3.5.

5.2.7. Soluble solids concentration (SSC), titratable acidity (TA) and SSC/TA ratio

Soluble solids concentration (SSC) of the juice was recorded by measuring the refractive index using an infrared digital refractometer (Atago-Palette PR 101, Atago CO. Ltd, Itabashi-Ku, Tokyo, Japan) as per the procedure outlined in Chapter 3, Section 3.6. TA was determined by titrating sweet orange fresh fruit juice against 0.1 N NaOH and expressed as percentage of citric acid as detailed in Chapter 3, Section 3.7. SSC: TA ratio was calculated by dividing the SSC (%) with the corresponding TA (%) as mentioned in Chapter 3, Section 3.8.

5.2.8. Determination of ascorbic acid concentration

Ascorbic acid concentration in the freshly extracted juice was determined using a UV/VIS spectrophotometer (Jenway spectrophotometer Model 6405, Dunmow, Essex, UK) according to the method previously explained by Pham (2009) and Malik and Singh (2005) with some modifications and as outlined in Chapter 3, Section 3.9. Ascorbic acid concentration was calculated by using a standard curve of L-ascorbic acid and expressed as mg ascorbic acid per 100 ml fresh juice.

5.2.9. Determination of total antioxidants

Total antioxidants was determined by using the method of Pham (2009) from the freshly extracted juice of sweet orange cv. Washington Navel and Lane Late and as detailed in Chapter 3, Section 3.10. Total antioxidants were estimated using a standard curve of 6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid (Trolox) and was expressed as mM Trolox Equivalent Antioxidant Activity (TEAC) $100ml^{-1}$ FJ basis.

5.2.10. Determination of individual and total sugars as well as organic acids

The individual sugars and organic acids were extracted from the fresh juice of sweet orange fruit (1 ml) and quantified using RP-HPLC system (Waters, Milford, MA, USA) as previously described in Chapter 3, Section 3.11. The concentration of individual, total sugar as well as individual organic acids was expressed as $g100\ ml^{-1}$ FJ.

5.2.11. Statistical analysis

The experimental data were subjected to a two–way analysis of variance (ANOVA) using GenStat 14th edition (VSN International Ltd., Hemel Hempstead, UK). The effects of treatments and stage of application, on different parameters were assessed within ANOVA and the least significant differences (LSD) were calculated following a significant F–test at ($P \leq 0.05$). The validity of statistical analysis was ensured by checking all the assumptions of analysis.

5.3. Results

5.3.1. Creasing (%)

The exogenous spray application of PUT substantially reduced the incidence of creasing (%) depending on its concentration and stage of its spray application in sweet orange cv. Washington Navel and Lane Late during two consecutive seasons during 2011 and 2012 (Figure 5.1). A spray application of PUT (1000 µM) applied at the golf ball stage was more effective in reducing creasing (22.1 and 24.3%) compared to the control (57.1 and 51.4%) and all other treatments in cv. Washington Navel during 2011 and 2012. Similar response to PUT in reducing creasing was also recorded in cv. Lane Late during 2011 and 2012. When averaged over stages of PUT application, the treatment of PUT (1000 µM) significantly ($P \leq 0.05$) reduced the mean creasing incidence (27.6 and 30.5%) compared to the control (56.9 and 53.6%) in sweet orange cv. Washington Navel during 2011 and 2012. A similar trend was observed in cv. Lane Late during both years. In general, spray application of PUT was more effective in reducing creasing when applied at the golf ball stage compared to its application at the fruit set or the colour break stage in cv. Washington Navel and Lane Late. The interaction between treatments and their stages of application were found to be non-significant ($P \leq 0.05$) for creasing in both the cultivars during 2011 and 2012.

5.3.2. Rheological properties of fruit and rind

The spray application of PUT (1000 µM) significantly ($P \leq 0.05$) improved the rheological properties (fruit firmness, rind hardness and tensile strength) in sweet orange cv. Washington Navel and Lane Late during 2011 and 2012 (Figure 5.2 A-F and 5.3 A-F).

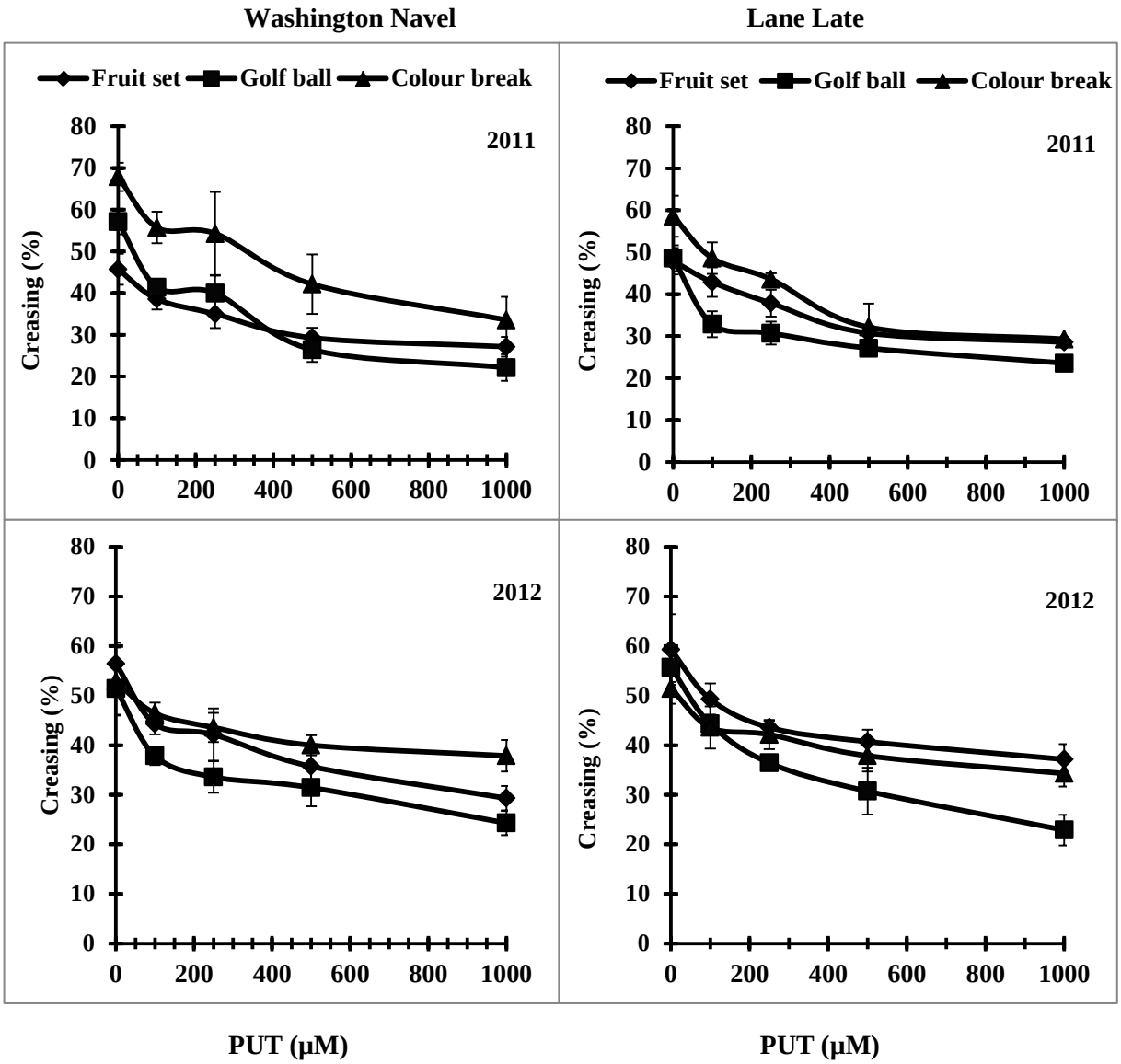

Figure 5.1. Effect of different concentrations of PUT sprayed at the fruit set, the golf ball or at the colour break stage on creasing (%) in sweet orange cv. Washington Navel and Lane Late during 2011 and 2012. n = 4 replications (35-fruit per replication). Vertical bars represent standard error means. LSD ($P \leq 0.05$) for Washington Navel 2011, Treatments = 7.4, Stage = 5.8, Treatments x stage = ns; Washington Navel 2012, Treatments = 6.2, Stage = 4.8, Treatments x stage = ns; LSD ($P < 0.05$) for Lane Late 2011, Treatments = 6.4, Stage = 4.9, Treatments x stage = ns; Lane Late 2012 = Treatments = 5.5, Stage = 4.2, Treatments x stage = ns; ns = not-significant.

When averaged over different stages of PUT application, the mean fruit firmness was higher with the treatments of PUT (500-1000 µM) compared to the control in cv. Washington Navel during both years. A similar trend was observed in cv. Lane Late during 2012 only. The spray application of PUT (250-1000 µM) was more effective in increasing fruit firmness compared to the control and all other treatments in cv. Lane Late during 2011. All the PUT treatments significantly ($P \leq 0.05$) increased the mean fruit firmness, when applied at the colour break stage (297.7 and 332.2 N) in cv. Washington Navel and Lane Late during 2011 and 2012, respectively. Exogenous spray application of PUT (1000 µM) was more effective in improving the fruit firmness as compared to the control and all other treatments in cv. Washington Navel and Lane Late during 2011 and 2012.

When averaged over different stages of PUT spray application, the treatment of 250-1000 µM significantly ($P \leq 0.05$) increased the mean rind hardness compared to the control and PUT (100 µM) in both cultivars during 2012. When averaged over all treatments of PUT, the mean rind hardness was significantly ($P \leq 0.05$) higher, when PUT was applied at the fruit set stage (21.93 and 28.81N) than the golf ball stage (19.95 and 25.27 N) or at the colour break stage (18.55 and 24.50 N) in cv. Lane Late during 2011 and 2012. Meanwhile, a non-significant ($P \leq 0.05$) effect was observed in cv. Washington Navel during 2011 and 2012. When averaged over different stages of PUT spray application, 500 and 1000 µM treatments resulted in significantly ($P \leq 0.05$) higher rind tensile strength than the control and all other treatments in cv. Washington Navel and Lane Late during 2011. In 2012, the mean rind tensile strength was significantly ($P \leq 0.05$) higher with PUT (250-1000 µM) treatments than the control and PUT (100 µM) in cv. Washington Navel and Lane Late. In 2011, mean rind tensile strength was significantly ($P \leq 0.05$) higher when PUT was applied at the fruit set compared to its application at the golf ball or at the colour break stage in cv. Washington Navel. When averaged over all the treatments in 2012, the spray application at the colour break stage or the fruit set significantly ($P \leq 0.05$) increased the mean rind tensile strength compared to spray application at the golf ball stage in cv. Washington Navel. In cv. Lane Late, the mean tensile strength was higher when PUT was applied at the colour break stage or the fruit set stage compared to its application at the golf ball stages during 2011 and 2012, respectively. The interaction between treatments and different stages of their application were found to be non-significant ($P \leq 0.05$) for fruit firmness, rind hardness and rind tensile strength in both cultivars during 2011 and 2012 except rind hardness in cv. Washington Navel during 2011.

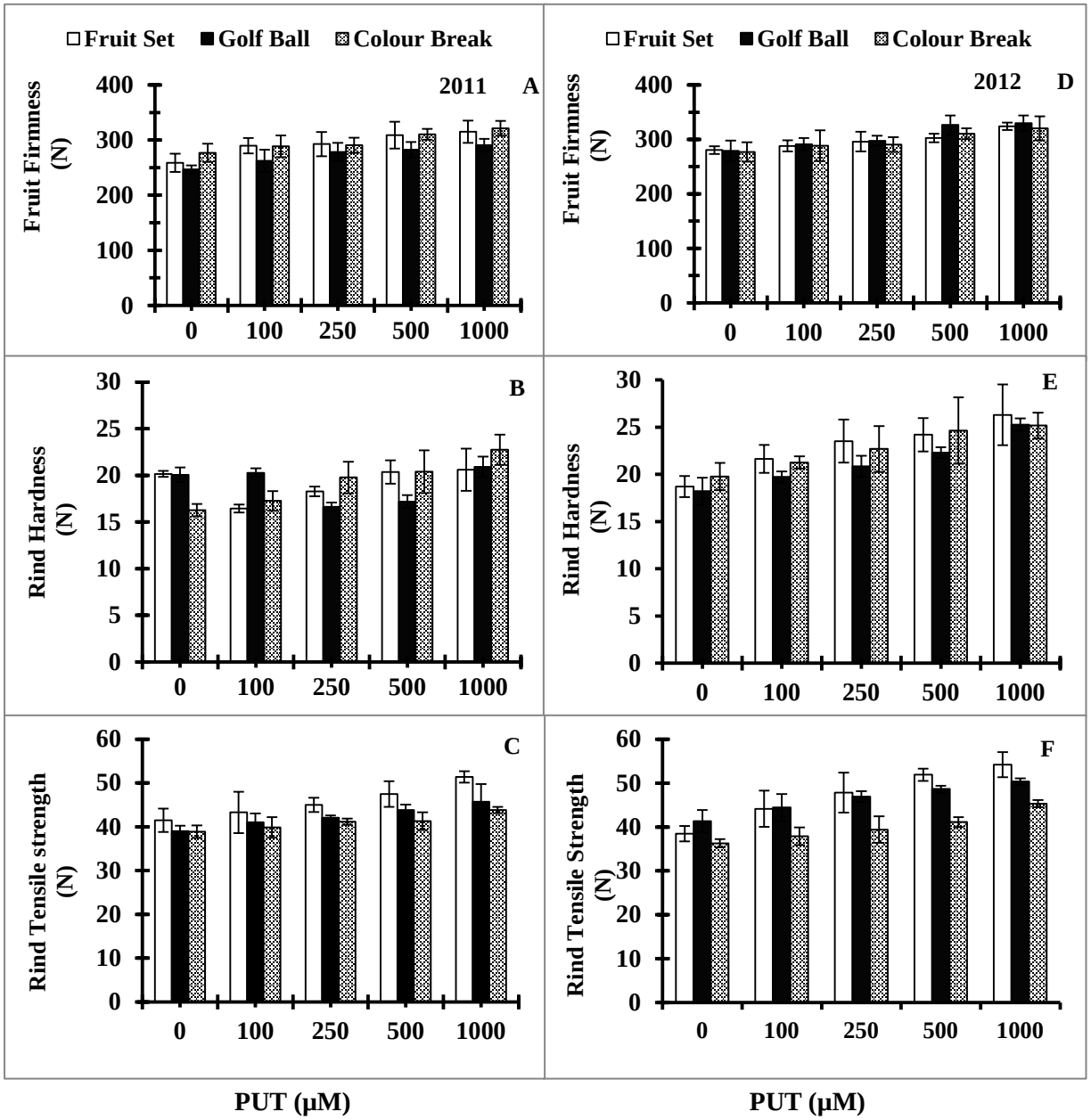

Figure 5.2. Effect of exogenous applications of different concentrations of PUT sprayed at the fruit set, the golf ball or at the colour break stage on rind textural properties of sweet orange cv. Washington Navel during 2011 and 2012. n = 4 replications (5-fruit per replication). Vertical bars represent standard error means. LSD ($P \leq 0.05$) for Washington Navel 2011, fruit firmness (N), Treatments = 27.6, Stage = 21.4, Treatments x stage = ns; for rind hardness, Treatments = 2.0, Stage = ns, Treatments x stage = 3.4; for rind tensile strength, Treatments = 3.8, Stage = 2.9, Treatments x stage = ns. LSD ($P < 0.05$) for Washington Navel 2012 fruit firmness, Treatments = 24.3, Stage = ns, Treatments x stage = ns; for rind hardness, Treatments = 3.1, stage = ns, treatments x stage = ns; for rind tensile strength, Treatments = 3.8, Stage = 2.9, Treatments x stage = ns; ns = not-significant.

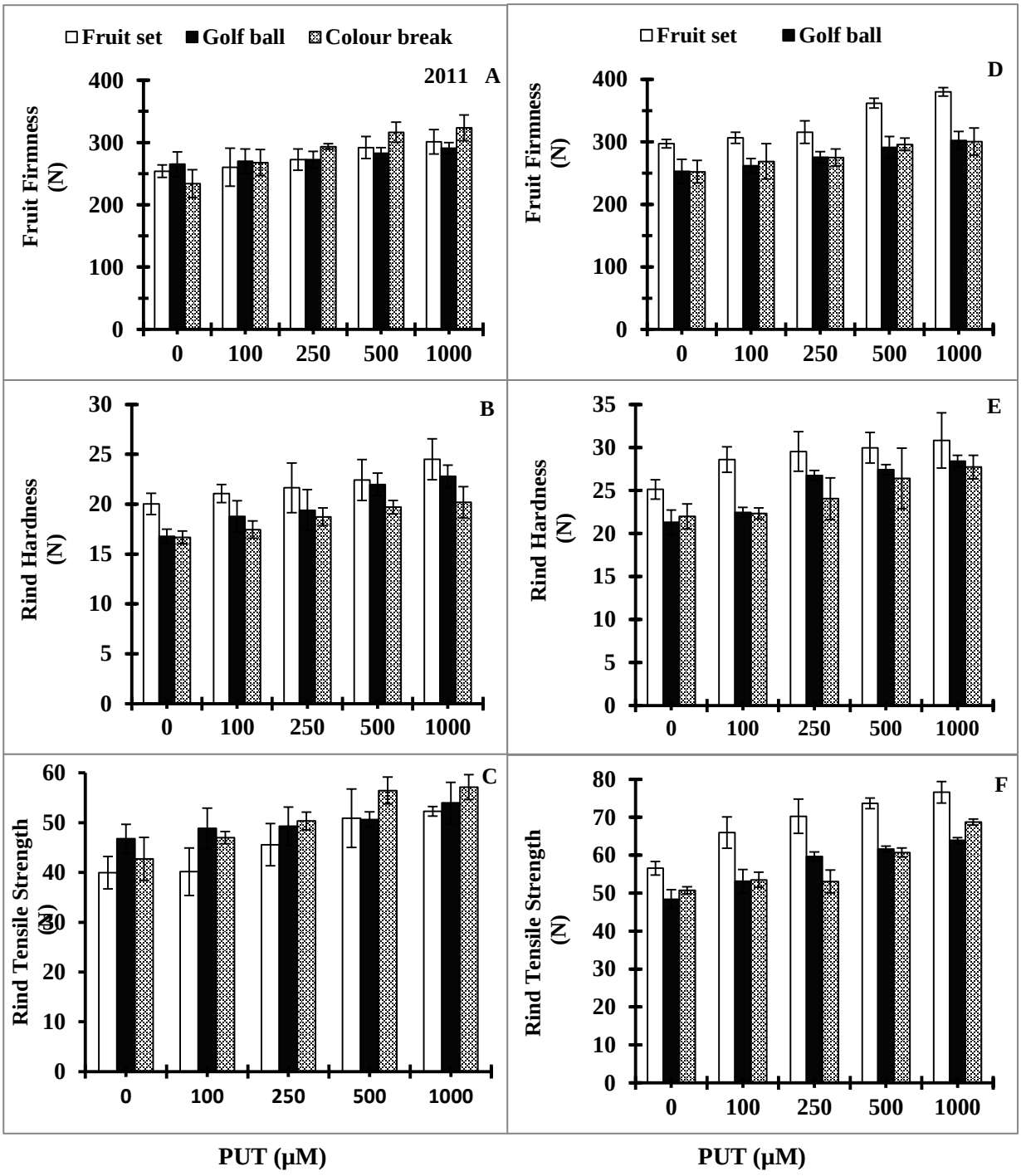

Figure 5.3. Effect of exogenous applications of different concentrations of PUT sprayed at the fruit set, the golf ball or at the colour break stage on the rind textural properties of sweet orange cv. Lane Late in 2011 and 2012. n = 4 replications (5-fruit per replication), LSD ($P \leq 0.05$) for Lane Late 2011 for fruit firmness, Treatments = 28.4, Stage = ns, Treatments x stage = ns; for rind hardness, Treatments = 2.2, Stage = 1.7, Treatments x stage = ns; for rind tensile strength, Treatments = 5.4, Stage = 4.2, Treatments x stage = ns, ns = not-significant. LSD ($P < 0.05$) for Lane Late 2012 for fruit firmness, Treatments = 28.2, Stage = 21.8, Treatments x stage = ns; for rind hardness, Treatments = 2.5, Stage = 2.0, Treatments x stage = ns; for rind tensile strength, treatments = 5.6, stage = 4.3, treatments x stage = ns; ns = not-significant.

5.3.3. Fruit weight

All the PUT treatments increased fruit weight compared to the control in cv. Washington Navel and Lane Late during 2011 and 2012 (Table 5.1). When averaged over different stages of spray application, the mean fruit weight was highest with the treatment of 1000 µM PUT compared to the control and all other treatments in cv. Washington Navel and Lane Late during 2011 and 2012, respectively. When averaged over all the treatments, the spray application of PUT resulted in significantly ($P \leq 0.05$) higher mean fruit weight (272.6 and 284.9 g), when applied at the colour break stage compared to its application at the fruit set or at the golf ball stage in cv. Washington Navel during 2011 and 2012. However in cv. Lane Late, the highest fruit weight (300.9 and 279.9 g) was recorded when PUT was sprayed at the fruit set stage compared to its application at the golf ball or at the colour break stage during 2011 and 2012, respectively. The interaction between treatments and their stages of spray application were found to be non-significant ($P \leq 0.05$) for fruit weight in both cultivars during 2011 and 2012.

5.3.4. Fruit diameter

The spray application of PUT at the fruit set, the golf ball or at the colour break stage increased fruit diameter compared to the control in both cultivars during 2011 and 2012 (Table 5.2). When averaged over different stages of spray application, the mean fruit diameter was increased with the highest concentration of PUT applied and largest fruit diameter (81.5 and 82.3 mm) was recorded with the treatment of PUT (1000 µM) compared to the control (69.5 and 74.5 mm) and 100 µM PUT (76.6 and 73.7 mm) in cv. Washington Navel during 2011 and 2012. In 2011, a similar trend was also observed in cv. Lane Late. In 2012, the mean fruit diameter was significantly ($P \leq 0.05$) higher with PUT (500 and 1000 µM) compared to the control and other treatments in cv. Lane Late. When averaged over all the treatments of PUT, the mean fruit diameter was significantly ($P \leq 0.05$) higher (87.4 and 82.0 mm) when PUT was applied at the fruit set stage compared to its application at the golf ball (79.5 and 79.9 mm) or at the colour break stage (79.9 and 81.7) in cv. Lane Late during 2011 and 2012, respectively. A similar trend was observed in 2012 in cv. Washington Navel, whilst non-significant ($P \leq 0.05$) effect was observed during 2011. The interaction between treatments and their different stages of spray application were found to be non-significant ($P \leq 0.05$) for fruit diameter in both cultivars during 2011 and 2012.

Table 5.1. Effect of different concentrations of PUT sprayed at the fruit set, the golf ball or at the colour break stage on fruit weight of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Fruit weight (g)								
Washington Navel								
Treat (µM)	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	248.7	250.0	247.5	249.7	262.2	267.5	252.8 c	255.7 b
PUT 100	250.7	265.2	251.2	258.8	263.2	282.7	255.1 bc	268.9 ab
PUT 250	264.0	267.5	258.7	263.5	274.0	282.5	265.6 ab	271.2 ab
PUT 500	270.8	270.7	260.7	265.8	278.0	290.2	269.8 a	275.6 a
PUT1000	272.0	278.5	263.5	271.0	285.5	301.5	273.7 a	283.7 a
Mean (stage)	261.2 b	266.3 b	256.3 b	261.8 b	272.6 a	284.9 a		
LSD ($P \leq 0.05$)								
Treatments			11.3	17.4				
Stage			8.8	13.5				
Treatments × stages			ns	ns				
Lane Late								
Treat (µM)	Fruit set		Golf ball		Colour Break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	265.2	270.2	263.2	247.2	269.2	265.7	265.9 b	261.1 b
PUT 100	297.0	276.2	277.1	252.0	265.7	275.0	279.9 ab	267.8 ab
PUT 250	311.0	279.3	279.9	255.7	268.1	276.0	286.3 ab	270.4 ab
PUT 500	311.8	284.7	280.3	266.2	288.2	283.2	293.4 a	278.1 a
PUT1000	319.8	289.1	283.8	273.8	285.6	283.0	296.4 a	281.9 a
Mean (stage)	300.9 a	279.9 a	276.9 b	259.0 b	275.4 b	276.6 a		
LSD ($P \leq 0.05$)								
Treatments			21.0	14.3				
Stage			16.3	11.1				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

Table 5.2. Effect of different concentrations of PUT sprayed at the fruit set, the golf ball or at the colour break stage on fruit diameter of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Fruit diameter (mm)								
Washington Navel								
Treat (µM)	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	71.2	67.7 d	75.5	74.0 c	61.8	81.7 ab	69.5 a	74.5 c
PUT 100	74.7	78.9 abc	77.8	75.3 bc	77.2	79.0 abc	76.6 ab	73.7 bc
PUT 250	79.9	80.1 abc	80.5	79.2 abc	78.1	79.2 abc	79.5 b	79.5 ab
PUT 500	81.3	81.2 ab	82.4	79.9 abc	79.3	81.2 ab	81.0 b	80.8 ab
PUT1000	81.5	82.0 ab	83.3	81.3 ab	79.6	83.5 a	81.5 b	82.3 a
Mean(stage)	77.7	77.96 b	79.9	78.0 b	75.2	80.5 a		
LSD ($P \leq 0.05$)								
Treatments			8.2	3.5				
Stage			ns	2.7				
Treatments × stages			ns	6.0				
Lane Late								
Treat (µM)	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	82.3	80.8	75.5	74.1	75.2	78.4	77.7 d	77.8 b
PUT 100	83.2	80.1	79.9	75. 6	79.4	79.6	80.8 acd	78.4 b
PUT 250	88.3	82.1	80.3	77.0	83.0	82.6	83.9 abc	80.6 ab
PUT 500	91.4	83.8	80.5	78.7	81.3	83.3	84.4 ab	81.9 a
PUT1000	91.7	82.2	81.3	80.8	80.6	84.6	84.5 a	82.9 a
Mean (stage)	87.4 a	82.0 a	79.5 b	77.2 b	79.9 b	81.7 a		
LSD ($P \leq 0.05$)								
Treatments			3.4	2.9				
Stages			2.6	2.3				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

5.3.5. SSC

The SSC was increased in sweet orange juice with the increase in concentration of PUT applied at the fruit set stage, the golf ball or at the colour break stage during both the years compared to the control in cv. Washington Navel and Lane Late (Table 5.3). When averaged over different stages of spray application, all the treatments of PUT were more effective in increasing mean SCC than the control in cv. Washington Navel and Lane Late during 2011 and 2012. A similar trend was observed in cv. Lane Late during both years.

When averaged over all the treatments of PUT, the mean SSC was significantly ($P \leq 0.05$) increased when PUT was applied at the fruit set stage (13.3%) compared to its application at the golf ball (12.7%) or at the colour break stage (12.4%) in cv. Washington Navel during 2012 only. In cv. Lane Late, SSC was significantly ($P \leq 0.05$) higher, when PUT was applied at the golf ball stage (13.4%) compared to its application at the colour break stage (12.5%) or at the fruit set stage (11.9%) during 2011, whilst non-significant ($P \leq 0.05$) effect was observed during 2012. The interaction between treatments and their different stages of application for SSC was found to be non-significant ($P \leq 0.05$) in cv. Washington Navel and Lane Late during both years, except in cv. Washington Navel during 2012.

5.3.6. SSC: TA ratio

The SSC: TA ratio was increased in sweet orange juice with the increased concentration of PUT applied during both years compared to the control in cv. Washington Navel only during 2011 and 2012 (Table 5.4). When averaged over different stages of spray application, the PUT treatments (250-1000 µM) were more effective in increasing SCC: TA ratio than the control and PUT (100 µM) during 2011. In 2012, all the PUT treatments resulted in significantly ($P \leq 0.05$) higher mean SSC: TA ratio compared to the control in cv. Washington. In cv. Lane Late, the treatments did not significantly ($P \leq 0.05$) affect SSC: TA ratio in 2011 and 2012. When averaged over all the treatments of PUT, the mean SSC: TA ratio was significantly ($P \leq 0.05$) increased when PUT was applied at the fruit set stage (10.1%) compared to its application at the golf ball (9.2%) or at the colour break stage (9.2%) in cv. Washington Navel during 2012. In 2011, the mean SSC: TA ratio was significantly ($P \leq 0.05$) higher when PUT was applied at the colour break stage (10.6%) or the golf ball stage (10.2%) than the fruit set stage (9.2%).

Table 5.3. Effect of different concentrations of PUT sprayed at the fruit set, the golf ball or at the colour break stage on SSC in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

SSC (%)								
Washington Navel								
Treat (µM)	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	11.9	12.0 c	12.0	11.8 c	12.0	10.2 d	11.9 b	11.3 c
PUT 100	12.6	13.0 b	12.6	12.8 b	12.4	12.8 b	12.5 a	12.9 b
PUT 250	12.4	13.5 ab	12.7	12.9 b	12.7	12.9 b	12.6 a	13.1 ab
PUT 500	12.5	13.7 ab	12.6	12.9 b	12.7	12.9 b	12.6a	13.2 ab
PUT1000	12.6	14.2 a	12.9	13.0 b	12.5	13.1 b	12.7 a	13.4 a
Mean (stage)	12.4 a	13.3 a	12.6 a	12. 7 b	12.5 a	12.4 b		
LSD ($P \leq 0.05$)								
Treatments			0. 5	0.5				
Stage			ns	0.3				
Treatments × stages			ns	0.03				
Lane Late								
Treat (µM)	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	11.3	11.6	12.0	11.2	11.8	10.9	11.7 c	11.2 c
PUT 100	11.8	12.0c	13.4	12.6	12.0	12.9	12.4 b	12.5 b
PUT 250	11.9	12.9	13.9	13.1	12.3	13.1	12.7 ab	13.0 a
PUT 500	12.2	13.0 a	14.0	13.2	13.2	13.4	13.1 a	13.2 a
PUT1000	12.3c	13.1 a	14.0	13.2	13.3	13.4	13.2 a	13.2 a
Mean (stage)	11.9 c	12.50	13.4 a	12.7	12.5 b	12.7		
LSD ($P \leq 0.05$)								
Treatments			0.5	0.5				
Stages			0.4	ns				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

Table 5.4. Effect of different concentrations of PUT sprayed at the fruit set, the golf ball or at the colour break stage on SSC: TA of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

SSC: TA ratio								
Washington Navel								
Treat (µM)	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	7.9	8.4	8.6	8.0	9.2	7.3	8.6 b	7.9 d
PUT 100	8.8	9.5	8.7	9.0	10.0	9.2	9.2 b	9.2 c
PUT 250	9.4	10.0	11.0	9.2	11.0	9.8	10.4 a	9.7 bc
PUT 500	9.6	10.9	11.2	9.2	11.1	10.0	10.7 a	10.0 ab
PUT1000	10.0	11.7	11.8	10.5	11.4	9.8	11.1 a	10.7 a
Mean (stage)	9.2 b	10.1 a	10.2 a	9.2 b	10.6 a	9.2 b		
LSD ($P \leq 0.05$)								
Treatments			1.0	0.7				
Stage			0.8	0.5				
Treatments × stages			ns	ns				
Lane Late								
Treat (µM)	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	10.3	10.6	10.1	10.4	13.0	9.8	11.1	10.2
PUT 100	10.1	10.6	10.1	10.9	11.9	11.2	10.7	10.9
PUT 250	10.1	10.5	11.3	10.9	12.2	11.2	11.2	10.9
PUT 500	11.1	10.5	11.2	10.9	12.5	11.3	11.6	10.9
PUT1000	10.6	10.5	12.1	10.9	12.8	11.3	11.8	10.9
Mean (stage)	10.4 b	10.5	10.9 b	10.8	12.5 a	11.0		
LSD ($P \leq 0.05$)								
Treatments			ns	ns				
Stages			0.7	ns				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

In cv. Lane Late, SSC: TA was significantly ($P \leq 0.05$) higher when PUT was applied at the colour break stage (12.5%) compared to its application at the golf ball stage (10.9%) or at the fruit set stage (10.4%) during 2011, whilst non-significant ($P \leq 0.05$) effect was observed during 2012. The interaction between treatments and their different stages of application for SSC: TA was found to be non-significant ($P \leq 0.05$) in cv. Washington Navel and Lane Late during both years.

5.3.7. Ascorbic acid

All the PUT treatments increased the levels of ascorbic acids in cv. Washington Navel and Lane Late fruit juice compared to the control during 2011 and 2012 (Table 5.4). When averaged over different stages of spray application, the treatments of PUT (250-1000 µM) significantly ($P \leq 0.05$) resulted in the highest mean levels of ascorbic acid compared to the control and PUT (100 µM) in cv. Washington Navel and Lane Late during 2011. In 2012, the spray application of PUT (500 and 1000 µM) resulted in significantly ($P \leq 0.05$) higher ascorbic acid compared to the control and all other treatments in cv. Washington Navel. Meanwhile, in cv. Lane Late mean ascorbic acid levels were significantly higher (66.8 mg $100ml^{-1}$ FJ) with the treatment of PUT (1000 µM) compared to the control (62.4 mg100 ml^{-1} FJ) and all other treatments during 2012. When averaged over all the PUT treatments, the mean ascorbic acid levels were significantly ($P \leq 0.05$) increased with PUT applied at the golf ball (66.1 mg $100ml^{-1}$ FJ) or the fruit set (65.2 mg $100ml^{-1}$ FJ) compared to its application at the colour break stage (61.7 mg $100ml^{-1}$ FJ) in cv. Washington Navel during 2011. However, in 2012 the mean ascorbic acid level was significantly ($P \leq 0.05$) higher when PUT was applied at the fruit set (65.3 mg $100ml^{-1}$ FJ) compared to its application at the golf ball (63.5 mg $100ml^{-1}$ FJ) or at the colour break stage (62.8 mg $100ml^{-1}$ FJ). In cv. Lane Late, the mean ascorbic acid was significantly ($P \leq 0.05$) higher when PUT was sprayed at the fruit set (69.5 mg $100ml^{-1}$ FJ) or the colour break (68.67 mg $100ml^{-1}$ FJ) compared to its application at the golf ball stage (62.4 mg $100ml^{-1}$ FJ) during 2011. In 2012, the mean ascorbic acid level was significantly ($P \leq 0.05$) higher when PUT was applied at the golf ball stage (68.8 mg $100ml^{-1}$ FJ) or at the colour break stage (63.7 mg $100ml^{-1}$ FJ) compared to its application at the fruit set (60.2 mg $100ml^{-1}$ FJ) in cv. Lane Late. In general, the ascorbic acid levels were higher in cv. Lane Late than the cv. Washington Navel during both years.

Table 5.5. Effect of different concentrations of PUT sprayed at the fruit set, the golf ball or at the colour break stage on the levels of ascorbic acids in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Ascorbic acids (mg 100ml^{-1} FJ)								
Washington Navel								
Treat (µM)	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	60.0	63.3	62.9	61.1	58.0	60.9	60.3 c	61.8 b
PUT 100	62.8	64.8	63.6	62.3	59.7	62.0	62.0 bc	63.0 ab
PUT 250	64.8	65.2	63.9	63.9	62.8	62.1	63.9 b	63.7 ab
PUT 500	68.9	65.8	69.8	64.5	63.7	64.8	67.5 a	65.0 a
PUT1000	69.2	67.2	70.2	65.6	64.2	64.2	67.9 a	65.7 a
Mean (stage)	65.2 a	65.3 a	66.1 a	63.5 ab	61.7 b			
LSD ($P \leq 0.05$)								
Treatments			2.3	2.5				
Stage			1.8	1.9				
Treatments × stages			ns	ns				
Lane Late								
Treat (µM)	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	65.7	58.5	60.0	66.3	66.4	62.3	64.0 c	62.4 b
PUT 100	67.3	59.5	60.6	67.8	68.2	63.0	65.3 bc	63.4 b
PUT 250	70.4	60.2	62.8	68.2	68.8	63.7	67.3 ab	64.0 ab
PUT 500	70.8	60.6	63.6	68.8	69.8	64.0	68.1 a	64.5 ab
PUT1000	73.3	62.2	65.0	72.7	70.2	65.5	69.5 a	66.8 a
Mean(stage)	69.5 a	60.2 c	62.4 b	68.8 a	68.7 a	63.7 b		
LSD ($P \leq 0.05$)								
Treatments			2.5	2.7				
Stages			2.0	2.1				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

The interaction between different treatments and their stages of spray application were found to be non-significant ($P \leq 0.05$) for levels of ascorbic acid in the juice of both cultivars during 2011 and 2012.

5.3.8. Total antioxidants

All the treatments of PUT increased total antioxidants in the juice compared to the control in sweet orange cv. Washington Navel and Lane Late during 2011 and 2012 (Table 5.5). When averaged over different stages of spray application, the mean total antioxidants were significantly higher with the treatment of PUT (100-1000 µM) compared to the control in cv. Washington Navel during 2011. In 2012, the mean total antioxidants significantly ($P \leq 0.05$) increased (47.4 mM Trolox $100ml^{-1}FJ$) with PUT (1000 µM) treatment compared to the control (42.0 mM Trolox $100ml^{-1}FJ$) in cv. Washington Navel. In cv. Lane Late, spray application of PUT (250-1000 µM) was more effective compared to the control and PUT (100 µM) during 2011. Whilst in 2012, the PUT (500-1000 µM) was more effective in increasing total antioxidants in the juice compared to the control and all other treatments. When averaged over all PUT treatments, mean ascorbic acid level was significantly ($P \leq 0.05$) higher when PUT was applied at the golf ball stage (45.5 and 46.1 mM Trolox $100ml^{-1}FJ$) compared to its application at the fruit set (43.4 and 44.0 mM Trolox $100ml^{-1}FJ$) or at the colour break stage (43.9 and 41.4 mM Trolox $100ml^{-1}FJ$) in cv. Washington Navel during 2011 and 2012, respectively. In cv. Lane Late total antioxidants were significantly ($P \leq 0.05$) higher when spray application of PUT was applied at the colour break stage (46.8 mM Trolox $100ml^{-1}FJ$) compared to its application at the fruit set stage (43.7 mM Trolox $100ml^{-1}FJ$) or at the golf ball stage (43.5 mM Trolox $100ml^{-1}FJ$) during 2011. In 2012, mean total antioxidants were significantly increased when PUT was applied at the colour break stage (46.1 mM Trolox $100ml^{-1}FJ$) or the golf ball (45.7 mM Trolox $100ml^{-1}FJ$) compared to its application at the fruit set stage (43.9 mM Trolox $100ml^{-1}FJ$). The interaction between treatments and their stages of spray application for total antioxidants was found to be non-significant ($P \leq 0.05$) in both cultivars during 2011 and 2012, except in cv. Washington Navel during 2012.

Table 5.6. Effect of different concentrations of PUT sprayed at fruit set, the golf ball or at the colour break stage on the levels of total antioxidants in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Total antioxidants (mM Trolex $100g^{-1}$ FW)								
Washington Navel								
Treat (µM)	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	39.8	41.9 bcd	42.0	43.7 bcd	41.3	40.5d	41.0 c	42.0 b
PUT 100	43.8	43.6 bcd	44.5	43.4 bcd	43.5	40.8 cd	43.9 b	42.6 b
PUT 250	44.2	44.8 bcd	44.9	43.8 bcd	44.1	41.0 bcd	44.4 b	43.2 b
PUT 500	44.4	44.9 bc	46.2	45.3 b	44.4	41.8 bcd	45.0 b	44.0 b
PUT1000	44.6	45.1 bc	50.2	54.4 a	46.2	42.6 bcd	47.0 a	47.4 a
Mean (stage)	43.4b	44.0 b	45.5a	46.1 a	43.9 b	41.4 c		
LSD ($P \leq 0.05$)								
Treatments			1.9	2.1				
Stage			1.5	1.6				
Treatments × stages			ns	3.6				
Lane Late								
Treat (µM)	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	42.1	42.0	43.0	44.7	44.2	44.4	43.1 b	42.1 c
PUT 100	43.1	43.6	42.5	44.5	45.5	45.7	43.7 b	43.1bc
PUT 250	43.5	43.9	43.4	45.0	46.6	46.0	44.5 b	43.5bc
PUT 500	44.5	44.4	43.7	46.9	46.8	47.2	45.0 ab	44.5bc
PUT1000	45.3	45.7	44.9	47.3	50.6	47.3	46.9 a	45.3bc
Mean (stage)	43.7 b	43.9 b	43.5 b	45.7 a	46.8 a	46.1 a		
LSD ($P \leq 0.05$)								
Treatments			2.1	1.5				
Stages			1.7	1.2				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

5.3.9. Fructose

The levels of fructose in the fruit juice increased with increased concentration of PUT applied as compared to the control in sweet orange cv. Washington Navel and Lane Late during 2011 and 2012 (Table 5.6). Averaged over different stages of PUT application, the PUT (500-1000 µM) resulted in significantly higher mean levels of fructose in the juice compared to the control during 2011 and 2012, respectively. A similar trend was observed in cv. Lane Late during 2012. Meanwhile, the PUT (250-1000 µM) was more effective than the control and the treatment of PUT (100 µM) in cv. Lane Late during 2011.The spray application of PUT applied at the fruit set stage resulted in significantly ($P \leq 0.05$) higher (1.0 and 1.4 fold) fructose levels in fruit juice compared to its application at the colour break or golf ball stage in cv. Washington Navel during 2011. A similar trend was observed during 2012 in cv. Washington Navel. In cv. Lane Late, the PUT application was more effective in increasing levels of fructose in the fruit juice, when applied at the fruit set stage (3.6 g $100ml^{-1}$ FJ) or at the golf ball stage (3.5 g $100ml^{-1}$ FJ) compared to its application at the colour break stage (3.1 g $100ml^{-1}$ FJ) in 2011. Whilst in 2012, the mean fructose levels were significantly ($P \leq 0.05$) higher (1.4 and 1.3 fold) when PUT was sprayed at the colour break stage compared to its application at the fruit set or the golf ball stage. The interaction between different treatments and their stages of spray application were found to be non-significant ($P \leq 0.05$) for levels of fructose in both cultivars during 2011 and 2012.

5.3.10. Glucose

The levels of glucose in the fruit juice increased with the increased concentration of PUT applied during both years in cv. Washington Navel and Lane Late. When averaged over different stages of PUT application, the treatment of PUT (250-1000 µM) was more effective in increasing the levels of glucose in the fruit juice compared to the control and PUT (100 µM) in cv. Lane Late during 2011. In cv. Washington Navel, all the treatments of PUT significantly ($P \leq 0.05$) increased the mean glucose levels in the fruit juice than the control during 2011. In 2012, the PUT (500-1000 µM) application resulted in significantly higher mean fructose levels in the fruit juice compared to the control and all other treatments in cv. Washington Navel during 2012. A similar trend was observed in cv. Lane Late during 2012. Whilst in 2011, averaged over all the PUT treatments, the mean glucose level was significantly higher when PUT was applied at the golf ball stage or the fruit set compared to its application at the colour break stage during 2011 in cv. Washington Navel.

Table 5.7. Effect of different concentrations of PUT sprayed at the fruit set, the golf ball or at the colour break stage on the levels of fructose in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Fructose (g 100ml^{-1} FJ)								
Washington Navel								
Treat (µM)	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	1.8	2.8	1.5	2.4	1.8	2.5	1.7 c	2.5 c
PUT 100	2.0	2.8	1.5	2.3	2.3	2.6	1.9 bc	2.6 c
PUT 250	1.2	3.0	1.5	2.7	2.3	2.7	2.0 bc	2.8 bc
PUT 500	2.9	3.2	1.8	3.0	2.5	2.8	2.4 ab	3.0 b
PUT1000	3.2	3.9	2.1	3.2	2.6	3.1	2.6 a	3.4 a
Mean (stage)	2.4 a	3.1 a	1.7 b	2.7 b	2.3 a	2.7 b		
LSD ($P \leq 0.05$)								
Treatments			0.4	0.4				
Stage			0.3	0.3				
Treatments × stages			ns	ns				
Lane Late								
Treat (µM)	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	3.3	2.8	3.1	2.7	2.8 e	4.1	3.1 c	3.2 b
PUT 100	3.4	2.9	3.5	3.3	3.0	4.3	3.3 bc	3.5 b
PUT 250	3.6	3.2	3.6	3.4	3.2	4.5	3.4 ab	3.7 ab
PUT 500	3.8	3.5	3.7	3.8	3.2	4.5	3.6 ab	3.9 ab
PUT1000	4.1	3.8	3.8	4.1	3.5	5.1	3.8 a	4.3 a
Mean (stage)	3.6 a	3.2 b	3.5 a	3.5 b	3.1 b	4.5		
LSD ($P \leq 0.05$)								
Treatments			0.3	0.7				
Stages			0.3	0.5				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

Table 5.8. Effect of different concentrations of PUT sprayed at the fruit set, the golf ball or at the colour break stage on the levels of glucose in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Glucose (g 100ml^{-1} FJ)								
Washington Navel								
Treat (µM)	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	1.1	1.4	1.7	1.5	0.7	1.2	1.2 d	1.4 c
PUT 100	1.4	1.4	1.9	1.7	1.0	1.4	1.4 c	1.5 bc
PUT 250	1.6	1.5	1.9	1.9	1.1	1.6	1.5 bc	1.6 bc
PUT 500	1.7	1.6	2.2	1.2	1.3	1.8	1.7 ab	1.8 ab
PUT1000	1.7	1.9	2.2	2.1	1.5	2.0	1.83 a	2.0 a
Mean (stage)	1.5 b	1.6 b	2.0 a	1.60 b	1.10 c	1.8 a		
LSD ($P \leq 0.05$)								
Treatments			0.2	0.3				
Stage			0.2	0.2				
Treatments × stages			ns	ns				
Lane Late								
Treat (µM)	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	1.1	1.4	1.8	1.3	1.6	1.7	1.5 c	1.5 d
PUT 100	1.2	1.6	1.9	1.9	1.6	2.0	1.6 bc	1.8 cd
PUT 250	1.2	1.9	1.8	2.1	1.6	2.1	1.6 bc	2.0 bc
PUT 500	1.3	2.1	1.9	2.6	1.7	2.4	1.6 ab	2.3 ab
PUT1000	1.3	2.4	2.0	3.2	1.8	2.4	1.0 a	2.7 a
Mean (stage)	1.2 c	1.9 a	1.9 a	2.2 a	1.7 b	2.1 a		
LSD ($P \leq 0.05$)								
Treatments			0.1	0.4				
Stages			0.1	ns				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

In. 2012, the mean fructose level in the fruit juice was significantly higher (0.9 fold) when PUT was applied at the colour break stage compared to its application at the golf ball stage or at the fruit set stage. In cv. Lane Late, the PUT application was more effective in increasing mean levels of glucose in fruit juice when applied at the golf ball or the colour break stage compared to its application at the fruit set stage during 2011 only. The interactions between different treatments and their spray application at different stages were found to be non-significant ($P \leq 0.05$) for glucose in both cultivars during 2011 and 2012.

5.3.11. Sucrose

All the treatments of PUT application increased the level of sucrose in the fruit juice compared to the control in both cultivars during 2011 and 2012 (Table 5.8). When averaged over different stages of PUT application, all the treatments of PUT resulted in significantly ($P \leq 0.05$) higher mean levels of sucrose in the fruit juice compared to the control in cv. Washington Navel during 2011. In 2012, the mean level of sucrose was significantly ($P \leq 0.05$) higher with the treatment of PUT (1000 µM) compared to the control and all other treatments in cv. Washington Navel. In cv. Lane Late, the mean level of sucrose in the fruit juice was significantly ($P \leq 0.05$) higher compared to the control and all other treatments during 2011. Whilst in 2012, the treatment of PUT (250-1000 µM) was more effective in increasing mean levels of sucrose compared to the control and treatment of PUT (100 µM) in cv. Lane Late. When average over all the treatments of PUT, the spray application of PUT at the fruit set stage significantly ($P \leq 0.05$) increased the mean level of sucrose (6.3 g $100ml^{-1}$ FJ) in fruit juice compared to its application at the colour break (5.6 g $100ml^{-1}$ FJ) or at the golf ball stage (5.4 g $100ml^{-1}$ FJ) during 2011 only in cv. Washington Navel. In cv. Lane Late, the application of PUT at the fruit set stage was more effective in increasing the levels of sucrose (7.4 g $100ml^{-1}$ FJ) in fruit juice compared to its application at the golf ball (7.5 g $100g^{-1}$ FJ) or at the colour break stage (6.5 g $100ml^{-1}$ FJ) during 2011. The mean sucrose level in fruit juice was significantly higher (6.2 g $100ml^{-1}$ FJ) when PUT was applied at the colour break stage compared to its application at the fruit set or at the golf ball stage in cv. Lane Late during 2012. The interaction between different treatments and their stages of spray application was found to be non-significant ($P \leq 0.05$) for the sucrose levels in cv. Washington Navel and Lane Late during 2011 and 2012.

Table 5.9. Effect of different concentrations of PUT sprayed at the fruit set, the golf ball or at the colour break stage on the levels of sucrose in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

	Sucrose (g 100ml^{-1} FJ)							
	Washington Navel							
Treat (µM)	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	5.5	5.8	4.4	5.3	4.0	5.3 d	4.7 c	5.5 c
PUT 100	5.9	6.2	5.2	5.7	5.7	5.8 cd	5.6 b	5.9 bc
PUT 250	6.6	6.5	5.6	5.6	5.8	5.9 bcd	6.0 ab	6.0 b
PUT 500	6.7b	6.6	5.8	6.0	6.0	6.1 bcd	6.2 ab	6.2 b
PUT1000	6.9	6.8	6.1	7.9	6.6	6.5 bc	6.5 a	7.1 a
Mean (stage)	6.3 a	6.4 a	5.4 b	6.1 ab	5.6 b	5.9 b		
LSD ($P \leq 0.05$)								
Treatments			0.7	0.5				
Stage			0.5	ns				
Treatments × stages			ns	ns				
Lane Late								
Treat (µM)	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	6.8	4.6	7.1	4.5	6.3	5.1	6.7 b	4.7 d
PUT 100	7.0	5.2	7.4	4.8	6.2	5.6	6.9 b	5.2 cd
PUT 250	7.1	5.4	7.5	4.9 d	6.3	6.5	7.0 b	5.6 bc
PUT 500	7.6	5.5	7.5	5.2	6.5	6.7	7.2b	5.9 ab
PUT1000	8.7	6.4c	7.7	5.5	7.0	7.1	7.8 a	6.3 a
Mean (stage)	7.4 a	5.5 b	7.5 a	5.0 c	6.5 b	6.2 a		
LSD ($P \leq 0.05$)								
Treatments			0.6	0.5				
Stages			0.5	0.5				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

5.3.12. Total sugars

The levels of total sugars were higher in cv. Lane Late (13.3 and 13.3 g $100g^{-1}$FJ) compared to Washington Navel (10.8 and 12.5 g $100ml^{-1}$ FJ) at a higher concentration of PUT (1000 µM) during 2011 and 2012, respectively (Table 5.9). When average over different stages of PUT application, all the treatments of PUT significantly ($P \leq 0.05$) increased the mean level of total sugars compared to the control in cv. Washington Navel during 2011. A similar trend was observed in cv. Lane Late during 2012. In 2011, the mean levels of total sugars were significantly ($P \leq 0.05$) higher with the spray application of PUT (500-1000 µM) than the control and all other treatments in cv. Lane Late. In cv. Washington Navel, the treatments of PUT (250-1000 µM) were more effective in increasing the mean levels of total sugars compared to the control and 100 µM PUT treatment in cv. Washington Navel during 2012. All the treatments of PUT significantly ($P \leq 0.05$) increased the mean levels of total sugars when PUT was applied at the fruit set stage (10.2 and 11.0 g $100ml^{-1}$ FJ) compared to its application at the golf ball (9.1 and 10.6 g $100ml^{-1}$ FJ) or at the colour break (9.0 and 10.3 g $100ml^{-1}$ FJ) in cv. Washington Navel in 2011 and 2012. In cv. Lane Late, the mean levels of total sugars were significantly ($P \leq 0.05$) higher when PUT was applied at the golf ball stage (12.9 g $100ml^{-1}$ FJ) compared to its application at the fruit set stage (12.3 g $100ml^{-1}$ FJ) or at the colour break stage (11.3 g $100ml^{-1}$ FJ) during 2011. Meanwhile in 2012, the mean levels of total sugars were significantly ($P \leq 0.05$) higher at the colour break stage (12.8 g $100ml^{-1}$ FJ) compared to its spray application at fruit set (10.7 g $100ml^{-1}$ FJ) or at the golf ball (10.6 g $100ml^{-1}$ FJ) in cv. Lane Late. The interactions between different treatments and their stages of spray application for total sugars were found to be non-significant ($P \leq 0.05$) in both cultivars during 2011 and 2012.

5.3.13. Organic acids

All the treatments of PUT irrespective of concentration and stage of application did not significantly affect the concentrations of citric acid, malic acid, succinic acid, fumaric and tartaric acid in the fruit juice during 2011 and 2012 in both cultivars (data not included).

Table 5.10. Effect of different concentrations of PUT sprayed at the fruit set, the golf ball or at the colour break stage on the levels of total sugars in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Total sugars (g 100ml^{-1} FJ)								
Washington Navel								
Treat (µM)	Fruit Set		Golf Ball		Colour Break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	8.4	9.9	7.7	9.1	6.5	9.0	7.5 c	9.4 d
PUT 100	10.0	10.4	8.5	9.8	8.9	9.8	9.1 b	10.0 cd
PUT 250	10.1	10.9	9.0	10.2	9.3	10.2	9.5 b	10.4 bc
PUT 500	11.3	11.4	9.9	10.9	9.7	10.7	10.3 a	11.0 b
PUT1000	11.4	12.6	10.3	13.2	10.8	11.6	10.8 a	12.5 a
Mean (stage)	10.2 a	11.0 a	9.1b	10.6 ab	9.0 b	10.3 b		
LSD ($P \leq 0.05$)								
Treatments			0.8	0.7				
Stage			0.7	0.6				
Treatments × stages			ns	ns				
Lane Late								
Treat (µM)	Fruit Set		Golf Ball		Colour Break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	11.2	8.8	12.1	8.5	10.7	10.9	11.3 c	9.4d
PUT 100	11.6	10.4	12.9	10.0	10.8	11.9	11.8 bc	10.8 c
PUT 250	11.9	10.5	12.9	10.4	11.2	13.0	12.0 bc	11.3 bc
PUT 500	12.6	11.4	13.1	11.5	11.4	13.5	12.4b	12.1 b
PUT1000	14.1	12.6	13.5	12.8	12.3	14.6	13.3 a	13.3 a
Mean (stage)	12.3 b	10.7 b	12.9 a	10.6 b	11.3 c	12.8 a		
LSD ($P \leq 0.05$)								
Treatments			0.7	1.1				
Stages			0.6	0.9				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

5.4. Discussion

All the treatments of PUT significantly reduced the incidence of creasing irrespective of its application at the fruit set, the golf ball or at the colour break stage in cv. Washington Navel and Lane Late during 2011 and 2012. However, the treatment of PUT (1000 µM) applied at the golf ball stage was more effective in reducing creasing compared to the control and all other treatments in cv. Washington Navel and Lane Late during 2011 and 2012 (Figure 5.1). Possibly, the exogenous application of PUT increased the levels of endogenous free PAs (PUT, SPD, SPM) in sweet orange fruit and its increased levels may be attribute to the conversion of PUT to SPD by spermidine synthase and to SPM by spermine synthase (Kumar et al.,1997). Exogenous spray application of PUT has increased the endogenous level of free PAs in the albedo and flavedo tissues of sweet orange fruit consequently reducing creasing (Chapter 4). The reduction in creasing incidence with exogenous application can also be ascribed to the direct effects of PAs on the cell wall and also their involvement in regulation of gene expression, translation, cell proliferation, modulation of cell signalling and membrane stabilization (Kusano et al., 2008). Similarly, in Cohen et al. (1983), polyamines (PUT, SPD and SPM) are used to maintain normal cell growth, but the inhibition of PAs biosynthesis blocks cell growth. Likewise, PAs have been reported to be involved in the cell wall characteristics by strengthening cell wall components (Berta et al., 1997).

It may also be argued that exogenous application of PAs has possibly reduced endogenous ethylene in sweet orange fruit. PAs are known to inhibit ethylene biosynthesis by inhibiting ACC synthase enzyme, which is involved in the conversion of SAM to ACC, eliminating the increase in ACC formation and ethylene production through the action of the ACC oxidase enzyme (Liu et al., 2006; Even and Melberg, 1989; Yu and Yang, 1979; Even-Chen et al., 1982; Hyodo and Nishino, 1981; Ladaniya, 2007). An increased level of endogenous ethylene in the albedo tissues of the creased fruit has been reported in sweet orange cv. Valencia and Washington Navel by Pham (2009); Monselise et al. (1976). Recently, the data presented in chapter 6 also show higher levels of endogenous ethylene in creased fruit of different cultivars of sweet orange including Navelina, Washington Navel, Lane Late and Valencia Late. The suppression of ethylene production may have led to lower activity of pectin methylesterase, *exo*-PG, *endo*-PG, and EGase leading to the reduction in pectin degradation, loosening the connection among cells of the albedo tissue of fruit.

The exogenous spray application of PUT significantly increased the rheological properties such as fruit firmness, rind hardness and rind tensile strength, irrespective of stage of application in cv. Washington Navel and Lane Late during 2011 and 2012 (Figure 5.2 and 5.3). It may also be argued that the increased levels of PUT application resulted in thicker and stronger rind of the fruit. Similarly, Berta et al. (1997) claimed that PAs are involved in strengthening the cell wall components. The fruit weight and diameter increased due to the increase in concentration of PUT (Table 5.2-5.3). PAs are reported to regulate fruit diameter, fruit weight and fruit quality in different fruit crops including apple (Costa and Bagni, 1983), olive (Rugini and Mencuccini, 1985), litchi (Mitra and Sanyal, 1990), mango (Malik and Singh, 2003), nectarine (Torrigiani, et al., 2004), pear (Franco-Mora et al., 2005), plum (Khan and Singh, 2010) and peaches (Bregoli et al., 2002).

PUT application increased the levels of glucose, fructose, sucrose and total sugars in the fruit juice as well as increased fruit size in both cultivars during 2011 and 2012; and may be ascribed to the reduced leaf abscission consequently attributed to higher supply of photosynthates to the fruit. Earlier, the reduction in leaf abscission with the application of PAs in sweet orange has been reported by Saleem et al. (2007). The effects of exogenous spray application of PAs on partitioning of photosynthates from leaf to the fruit in sweet orange warrants investigation. PAs are also used to improve fruit quality in different fruit crops such as the exogenous application of PUT (5×10^{-5} M) increased the SSC in apple (Costa and Bagni, 1983) and in litchi. PUT application also improved the SSC, SSC: TA ratio and ascorbic acid when applied at full bloom (Mitra and Senyal, 1990). Similarly, the favourable effect of PUT on fruit quality has been previously reported in grapes (Barry et al., 2004b), in mango (Malik and Singh, 2006), in olive (Ayad et al., 2011), in jujube (Kassem et al., 2011) and in plum (Khan et al., 2007).

PUT application also significantly ($P \leq 0.05$) increased the level of ascorbic acid and total antioxidants in both cultivars during 2011 and 2012. The increased level of ascorbic acid in the juice of sweet orange fruit treated with PUT at various stages can possibly be attributed to the suppression of ascorbate oxidase activity as a result of elevated levels of PAs in the fruit as also reported in mango fruit by Malik and Singh, (2006). The effects of exogenous application of PAs on the activities of ascorbate oxidase in sweet orange fruit warrant investigation. The increased levels of total antioxidants in the juice of sweet orange fruit treated with PUT at various stages of fruit growth and development can possibly be ascribed to the increased levels of ascorbic acid with PUT treatment, as ascorbic acid is an important

antioxidant (Davey et al, 2004). The exact mode of action of exogenously applied PAs in raising the levels of total antioxidants in the juice of sweet orange fruit is yet to be investigated. In conclusion, exogenous spray application of PUT significantly reduces the incidence of creasing; improves the rind textural properties including fruit firmness, rind hardness, rind tensile strength, fruit weight and diameter; individual and total sugars; ascorbic acid; and total antioxidants; in the juice compared to the control.

CHAPTER 6

Involvement of ethylene in causation of creasing in sweet orange [*Citrus sinensis* (L.) Osbeck] fruit

Summary

Creasing is a physiological disorder in the rind of sweet orange [*Citrus sinensis* (L.) Osbeck] fruit and causes serious economic losses. The involvement of ethylene in creasing of sweet orange fruit was investigated by monitoring the changes in endogenous levels of ethylene in the creased and normal fruit. The effects of exogenous applications of Ethrel® (2-chloroethylphosphonic acid) and inhibitors of ethylene biosynthesis on endogenous levels of ethylene in the fruit and creasing index (CI) were also examined. Endogenous levels of ethylene in the normal and the creased fruit of Navelina, Washington Navel, Lane Late and Valencia Late sweet orange were determined at harvest maturity. The effects of different concentrations of Ethrel® applied at fruit maturity (165 days after fruit set) on endogenous levels of ethylene in the fruit were determined 30 to 120 days after spray (DAS) and CI at fruit harvest. The efficacy of different concentrations of ethylene inhibitors such as aminoethoxyvinylglycine (AVG) or CoSO4 sprayed at the golf ball stage (fruit diameter 40±5 mm) on CI at harvest was also tested. Endogenous levels of ethylene were significantly higher in the creased fruit than the normal fruit in different cultivars of sweet orange including Navelina, Washington Navel, Lane Late and Valencia Late. Exogenous spray application of ethrel at the rate of 250 -750 mg L^{-1} on mature fruit elevated the endogenous levels of ethylene in the fruit 30 to 120 DAS and increased CI as compared to the control in Washington Navel and Lane Late. Meanwhile, the spray application of ethylene inhibitor AVG (20-60 mg L^{-1}) or cobalt sulphate ($CoSO_4$) (125-500 mg L^{-1}) at the golf ball stage was more effective in reducing CI compared to the control in all cultivars during 2011 and 2012. A spray application of AVG (60 mg L^{-1}) at the golf ball stage was more effective in reducing CI (16.14 % and 15.93%) compared to the control (34.18 and 35.25 %) in cv. Washington Navel during 2011 and 2012, respectively. Similarly, the application of $CoSO_4$ (500 mg L^{-1}) at the golf ball stage was more effective in reducing CI (19.32% and 19.32 %) compared to the control (32.11 and 38.46 %) in cv. Navelina during 2011 and Lane Late during 2012, respectively. In conclusion, higher levels of endogenous ethylene in the creased fruit and promotion of the CI with the exogenous application of ethrel and its reduction with the

application of ethylene inhibitors suggests the involvement of ethylene in the causation of creasing in sweet orange fruit.

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6.1. Introduction

Creasing (albedo breakdown) is a physiological disorder in the rind of sweet orange fruit. The symptoms of creasing include multiple cracking in the pitted peel because of albedo decomposition (Li et al., 2009) and separation of cells in the albedo tissue, resulting in channels in the fruit rind (Treeby et al., 1995). The creasing also creates a weak point in the flavedo, which causes cracks and fruit is ruptured following packaging for fresh markets (Gilfillan et al., 1981). Creasing has been reported in different orange-producing countries of the world, including Australia (Treeby et al., 1995), USA (Jones et al., 1976), South Africa (Holtzhausen, 1981), Spain (Agustí et al., 2003) and China (Li et al., 2009). The symptoms of creasing are visible at fruit maturity and losses due to creasing vary among seasons, locations and cultivars; sometimes exceeding more than 50% (Gilfillan et al., 1981).

Creasing has been reported to be associated with genotype (Agustí et al., 2003), climate (Jones et al., 1967; Gambetta et al., 2000), rootstock (Treeby et al., 1995), crop load (Jones et al., 1967), rind thickness (Holtzhausen, 1981), irrigation (Agustí et al., 2004) and mineral nutrition (Ali et al., 2000; Bower, 2004). Numerous attempts have been made to control the physiological disorder, but none appear to have conclusively identified the physiological variables leading to this disorder (Bower, 2004). It is well known that boron is a structural element of the plant cell wall and boron spray in early summer is effective in reducing creasing in sweet orange (Pham, 2009). In Australia, calcium spray has been used to control creasing resulting in a limited reduction in creasing (25 to 30 %) (Treeby et al., 2000; Pham et al., 2012). Similarly, gibberellic acid has been used to control creasing in South Africa; however, it does not prevent creasing but only delays the onset of creasing (Bower, 2000).

Ethylene acts as an inducer for fruit ripening (Bleecker, 2000). Ethylene is a key signal compound involved directly in regulation of fruit ripening, senescence and fruit quality (Manjunatha et al., 2012). Ethylene is very effective at lower concentrations ranging from a

part-per-million (μL L^{-1}) to part-per-billion (nL L^{-1}) in regulating ripening and senescence in many fruit, vegetables and ornamentals (Saltveit, 1999). Ethylene accelerates softening in citrus fruit due to disintegration of cell membranes making them leakier (Rath and Prentice, 2004; Ladaniya, 2007). The exogenous application of ethylene promotes respiration rate and ripening as well as improving colour development in citrus (Ladaniya, 2007; Burg, 2004; Agustí et al., 2002). However, the pre-harvest application of ethrel does not affect fruit quality parameters such as soluble solid concentration, juice percentage, fruit weight, rind thickness, acidity and ascorbic acid in the citrus (Al-Mughrabi et al., 1989).

As a prelude, the visible symptoms of creasing on sweet orange fruit appear mainly after fruit maturation and are coupled with ripening and/or over-ripening processes, which also involve cell separation (Monselise et al., 1976; Saleem et al., 2014). A substantial increase in the incidence of creasing with delayed harvesting in Washington Navel sweet orange fruit suggests that creasing is coupled with the ripening and/or over-ripening processes (unpublished data). Citrus is classified as a non-climacteric fruit, but produce only small amounts of ethylene, while ethylene plays an important role in changing fruit colour, flavour, chemical composition and texture in citrus fruit (Aharoni, 1968; Ekas, 1970; Ladaniya, 2007). The development of creasing in sweet oranges has been associated with increased water soluble pectins consequently leading to earlier senescence of albedo tissue (Monselise et al., 1976). Recently, Saleem et al. (2014) claimed that higher activities of pectinesterase (PE), *exo*-polygalacturonase (*exo*-PG), endo-polygalacturonase (*endo*-PG), and endo-1, 4-*ß* -D-glucanase (EGase) in the albedo of creased fruit at commercial harvest seem to be associated with enhanced loss of pectins and starch in the cell walls of albedo tissue, leading to cell wall loosening and formation of cracks which consequently reduce hardness, stiffness and tensile force of the rind.

Earlier, ethylene biosynthesis in the albedo tissue of 'Satsuma' mandarin fruit has been reported by Hyodo (1977). Some preliminary and sporadic reports suggested higher levels of endogenous ethylene in the albedo tissue of creased fruit than in normal fruit in Valencia orange (Monselise et al., 1976). Similarly, Pham (2009) reported higher levels of endogenous ethylene in the rind of creased fruit than normal fruit in Washington Navel sweet orange. However, the *in vivo* role of ethylene involving exogenous applications of ethylene and its inhibitors on creasing of sweet orange fruit is yet to be investigated. We hypothesised that ethylene plays a key role in modulating creasing in sweet orange fruit. We investigated the comparative changes in the concentrations of endogenous ethylene in the creased and normal fruit of four different cultivars of sweet orange. The effects of exogenous application of

different concentrations of ethrel at fruit maturation stage; and ethylene inhibitors such as aminoethoxyvinylglycine (AVG) and cobalt sulphate ($CoSO_4$) at golf ball stage in regulating the incidence of creasing in sweet oranges were also investigated.

6.2. Materials and methods

6.2.1. Plant material

Four different experiments were conducted in a commercial orchard located at Gingin (latitude 31° 21′ South, longitude 155° 55′ East), Western Australia. Twenty-five year old uniform sweet orange tree on trifoliate orange (*Poncirus trifoliate* Raf.) rootstock, planted at 2.7 x 7.5 m spacing on a north-south orientation were used in the experiments. These experiments were conducted on Navelina, Washington Navel, Lane Late and Valencia cultivars of sweet oranges. The orchard soil was sandy loam. All cultural practices, other than the experimental treatments, were similar to those practiced in commercial orchards (Mould and Tugwell, 1999).

6.2.2. Experiment I: Endogenous levels of ethylene in the normal and creased fruit of different cultivars of sweet orange

The experiment was conducted to determine the comparative endogenous levels of ethylene in normal and creased fruit of Navelina, Washington Navel, Lane Late and Valencia Late sweet orange. The experimental arrangement was a randomised complete block design with two-factor factorial (cultivars and type of fruit) with a single tree as an experimental unit and four replicates. Two fruit of each category (normal and creased) per tree were harvested at harvest maturity and endogenous levels of ethylene were determined.

6.2.3. Experiment II: Effects of exogenous application of CEPA on endogenous levels of ethylene and incidence of creasing in Washington Navel and Lane Late sweet orange

An aqueous solution containing different concentrations (250, 500 and 750 mg L^{-1}) of Ethrel® [2-chloroethylphosphonic acid (Rhone-Poulenc Rural Australia Pty Ltd, Baulkham Hills, NSW, Australia)] and 0.05% ‘Tween 20’ as a surfactant was sprayed on to the whole

tree until run off at fruit maturity (01 April, 2012: 165 Days after fruit set) in cv. Washington Navel using a sprayer (The Selecta Trolleypak Mk II, Acacia Ridge, Australia). Control trees were unsprayed. The experimental layout was a randomised complete block design with two-factor factorial (treatments and cultivars). A single tree was considered as an experimental unit and the experiments were replicated four times. At commercial harvest, 35 ripe fruit per tree were randomly harvested around the tree canopy. The endogenous levels of ethylene in the fruit were determined 30, 60, 90 and 120 DAFS. The creasing indexes in both cultivars were determined at fruit harvest.

6.2.4. Experiment III: Effect of exogenous application of AVG, an ethylene biosynthesis inhibitor, on the incidence of creasing in different cultivars of sweet orange during 2011 and 2012

The aim of this experiment was to down regulate the ethylene production using AVG, which is an inhibitor of ethylene biosynthesis and consequently shows its inhibition effects on creasing. An aqueous solution containing different concentrations (20, 40, 60 mg L^{-1}) of AVG (as ReTain® from Valent BioSciences®, Chatswood, NSW, Australia) and 'Tween 20' (0.05 %) as a surfactant was sprayed at the golf ball stage (fruit diameter 40±5 mm) onto the whole trees of Navelina, Washington Navel and Lane Late sweet oranges in 2011; and Washington Navel and Lane Late in 2012; using a sprayer (The Selecta Trolleypak Mk II, Acacia Ridge, Australia). Untreated trees served as a control. The experiment was laid out by following a two-factor (AVG treatments and cultivars) factorial randomised block design with four replicates. Single trees were treated as an experimental unit. At ripe stage, 35 fruit per tree were randomly harvested around the tree canopy. The incidence of creasing was recorded and expressed as a creasing index (CI, %).

6.2.5. Experiment IV: Effects of exogenous application of ethylene inhibitor ($CoSO_4$) on the incidence of creasing in different cultivars of sweet orange

In this experiment, an aqueous solution containing different concentrations (125, 250 and 500 mg L^{-1}) of CoSO4 and 'Tween 20' (0.05 %) as a surfactant was sprayed at golf ball stage on to the whole trees of Navelina, Washington Navel and Lane Late sweet oranges in 2011; and Washington Navel and Lane Late in 2012; using the same sprayer as mentioned in

experiment 4. The experiment used a completely randomized design with two factors including $CoSO_4$ treatments and cultivars. Single trees were treated as an experimental unit and the experiments included four replications. At ripe stage, 35 fruits per tree were randomly harvested around the tree canopy. Creasing index was recorded from these harvested fruits.

6.2.6. Determination of endogenous ethylene

Ethylene production was determined by following the method described earlier by Pranamornkith et al. (2012) and also described in Chapter 3, Section 3.12. The levels of ethylene were determined by using an ETD 300 ethylene detector (Sensor sense B.V, Nijmegen, The Netherlands) and were expressed in ($\mu L.Kg^{-1}.h^{-1}$).

6.2.7. Creasing index (%)

CI was determined using a 4-point hedonic scale based on the symptoms of creasing on the surface of individual fruit by following the method of Treeby and Storey (2002). The procedure for determination of creasing index percentage has been mentioned in more detail in Chapter 3, Section 3.3.

6.2.8 Statistical analysis

The experimental data were subjected to two-way analysis of variance (ANOVA) depending upon experimental design, using GenStat 14th edition (Lawes Agricultural Trust, Rothamsted experimental station, U.K). The effects of treatments, cultivars and their interactions on different parameters were assessed within ANOVA. The least significant differences (LSD) were calculated following a significant Duncan's test at $P \leq 0.05$. All the assumptions of analysis were checked to ensure validity of statistical analysis.

6.3. Results

6.3.1. Endogenous levels of ethylene in normal and creased fruit of different cultivars of sweet orange

The creased fruit of Navelina, Washington Navel, Lane Late and Valencia Late sweet orange exhibited significantly ($P \leq 0.05$) higher levels of endogenous ethylene (1.29, 1.18, 1.16, 0.86 $\mu L.\ kg^{-1}.hour^{-1}$) than normal fruit (1.06, 0.81, 0.98, 0.51 $\mu L.\ kg^{-1}.hour^{-1}$) respectively (Figure 6.1). When averaged over cultivars, the creased fruit exhibited

significantly higher mean endogenous levels of ethylene (1.12 µL. $kg^{-1}.hour^{-1}$) than normal fruit (0.84 µL. $kg^{-1}.hour^{-1}$). Mean endogenous levels of ethylene differed significantly ($P \leq 0.05$) among different cultivars of sweet orange. The interaction between type of fruit (creased and normal) and cultivar was found to be non-significant ($P \leq 0.05$) for endogenous levels of ethylene.

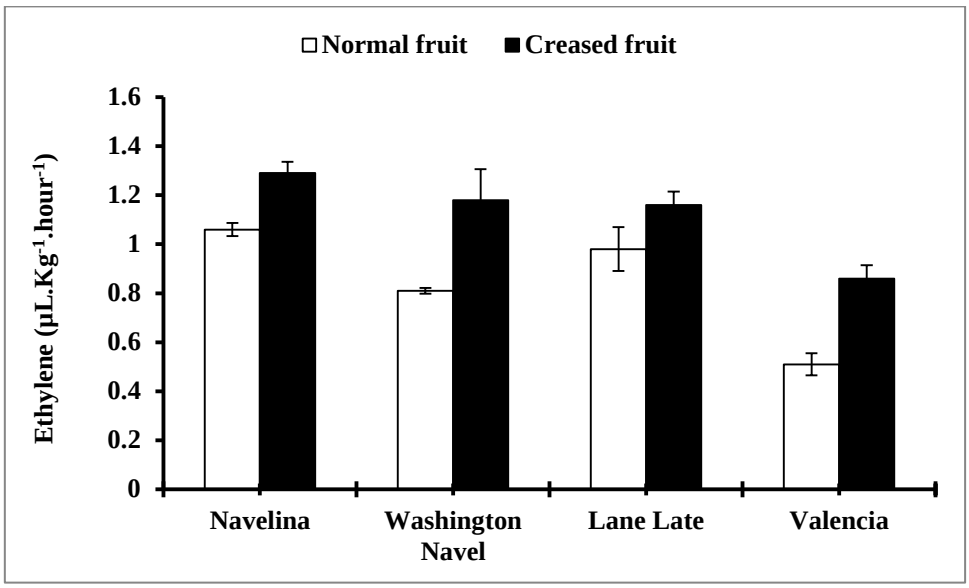

Figure 6.1. Comparative endogenous levels of ethylene in the creased and normal fruit of different cultivars of sweet orange. n = 4 replications (2 fruit per replication). Vertical bars represent standard error means. LSD ($P \leq 0.05$), Type of fruit = 0.1, Cultivars = 0.1, Cultivars x type of fruit = ns; ns = not-significant.

6.3.2. Effect of exogenous application of different concentrations of ethrel on endogenous level of ethylene in cv. Washington Navel and Lane Late fruit

All the ethrel treatments applied at 165 days after fruit set (DAFS) significantly ($P \leq 0.05$) increased endogenous level of ethylene in the fruit at 30 DAS in Washington Navel and Lane Late sweet orange as compared to the control (Table 6.1). When averaged over cultivars, the mean endogenous levels of ethylene 30 DAS in the fruit increased with increased concentrations of ethrel applied. However, the differences among treatments were not significant. Meanwhile, 60, 90 and 120 DAS, all the ethrel treatments significantly increased mean endogenous levels of ethylene in the fruit than in the untreated control, but the treatments of 750 mg L^{-1} ethrel resulted in highest levels of endogenous ethylene as compared to all other treatments and control. The effects of cultivars and interaction of cultivar with ethrel treatments were found to be non-significant ($P \leq 0.05$) for endogenous levels of ethylene in the fruit at 60, 90 and 120 DAS.

6.3.3. Effect of exogenous application of different concentrations of ethrel on creasing index at the mature stage in Washington Navel and Lane Late

All the ethrel treatments significantly ($P \leq 0.05$) increased mean CI as compared to the control and the creasing was more pronounced at higher concentration of ethrel applied. A similar trend of increased mean CI with exogenously applied ethrel was recorded in cv. Washington Navel and cv. Lane Late sweet oranges (Figure 6.2). In general, the CI was higher in cv. Washington Navel (50.71%) than Lane Late (43.52%) at higher concentration of ethrel (750 mg. L^{-1}) applied. The effects of cultivars and interaction of cultivar with treatments were found to be significant ($P \leq 0.05$) for creasing index.

Table 6.1. Effect of different concentrations of ethrel sprayed at mature stage on endogenous ethylene in cv. Washington Navel and Lane Late sweet orange fruit.

Ethylene (µL. $kg^{-1}.h^{-1}$) 30 DAS			
Treatments (mg L^{-1})	Washington Navel	Lane Late	Means (Treatment)
Control	0.6	0.9	0.8 b
Ethrel 250	1.0	1.2	1.1 a
Ethrel 500	1.1	1.2	1.1 a
Ethrel 750	1.2	1.1	1.1 a
Means (Cultivars)	0.9 b	1.1 a	
LSD ($P \leq 0.05$)	Treatments=0.2, Cultivars= 0.1, Treatments × cultivars=ns		
Ethylene (µL. $kg^{-1}.h^{-1}$) 60 DAS			
Control	0.6	0.7	0.7 c
Ethrel 250	0.9	1.0	0.9 b
Ethrel 500	0.9	1.0	1.0 b
Ethrel 750	1.2	1.1	1.1 a
Means (Cultivars)	0.9 a	1.0 a	
LSD ($P \leq 0.05$)	Treatments=0.1, Cultivars= ns, Treatments × cultivars=ns		
Ethylene (µL. $kg^{-1}.h^{-1}$) 90 (DAS)			
Control	0.7	0.8	0.7 c
Ethrel 250	0.8	0.8	0.8 b
Ethrel 500	0.9	1.0	0.9 b
Ethrel 750	1.0	1.1	1.1 a
Means (Cultivars)	0.8 b	0.9 a	
LSD ($P \leq 0.05$)	Treatments=0.1, Cultivars= ns, Treatments × cultivars=ns		
Ethylene (µL. $kg^{-1}.h^{-1}$) 120 (DAS)			
Control	0.8	0.7	0.7 c
Ethrel 250	0.8	0.8	0.8 b
Ethrel 500	0.9	0.9	0.9 b
Ethrel 750	1.1	1.2	1.1 a
Means (Cultivars)	0.9 a	0.9 a	
LSD ($P \leq 0.05$)	Treatments=0.1, Cultivars= ns, Treatments × cultivars=ns		

n = 4 replications (2 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different at $P \leq 0.05$ at 30, 60, 90 and 120 DAS. ns = non-significant

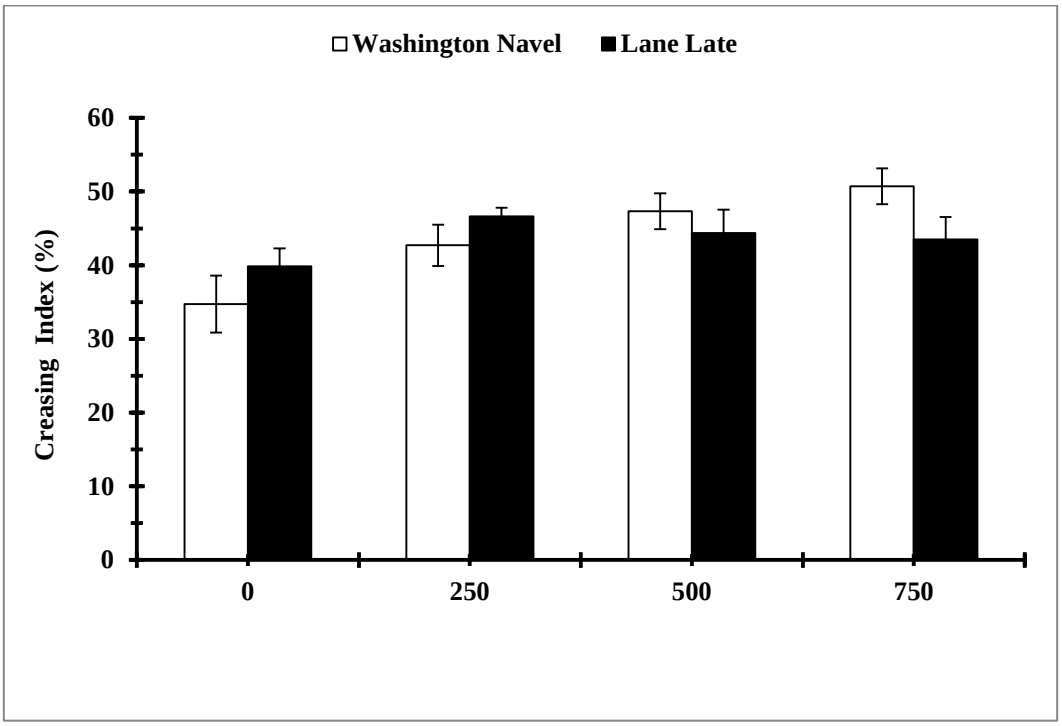

Figure 6.2. Effect of exogenous application of different concentrations of ethrel on CI at mature fruit in cv. Washington Navel and Lane Late fruit. n = 3 replications (35 fruit per replication). Vertical bars represent S.E. of means. LSD ($P \leq 0.05$) for creasing index, treatments = 4.3, Cultivars = ns, Treatments × cultivars = 6.1; ns = not-significant.

6.3.4. Effect of exogenous application of aminoethoxyvinylglycine (AVG) and $CoSO_4$ applied at the golf ball stage on CI of sweet orange

All the treatments of AVG applied at golf ball stage significantly ($P \leq 0.05$) reduced CI in sweet orange cv. Navelina, Washington Navel and Lane Late in 2011 and 2012 (Figure 6.3). When averaged over cultivars, the spray treatment of AVG (60 mg L^{-1}) resulted in a lower mean CI (18.29 and 18.45 %) than the control (34.08 and 36.86%) and all other treatments in 2011 and 2012. The interaction between the treatments and cultivars were found to be non-significant ($P \leq 0.05$) for CI during 2011 and 2012.

The CI was significantly ($P \leq 0.05$) reduced with increased concentration of $CoSO_4$ (125 to 500 mg L^{-1}) applied at golf ball stage compared to the control and all other treatments in 2011 and 2012 (Figure 6.4). The foliar application of $CoSO_4$ (500 mg L^{-1}) resulted in significantly lower mean CI (21.80 and 19.79 %) than the control (34.08 and 36.86%) during 2011 and 2012, respectively. When averaged over treatments, the mean CI was lowest in cv. Navelina (24.45 %) compared to cv. Washington Navel (27.06 %) and Lane Late (32.32 %) during 2011. The interactions between the treatments and the cultivars were also found to be non-significant ($P \leq 0.05$) for CI during 2011 and 2012.

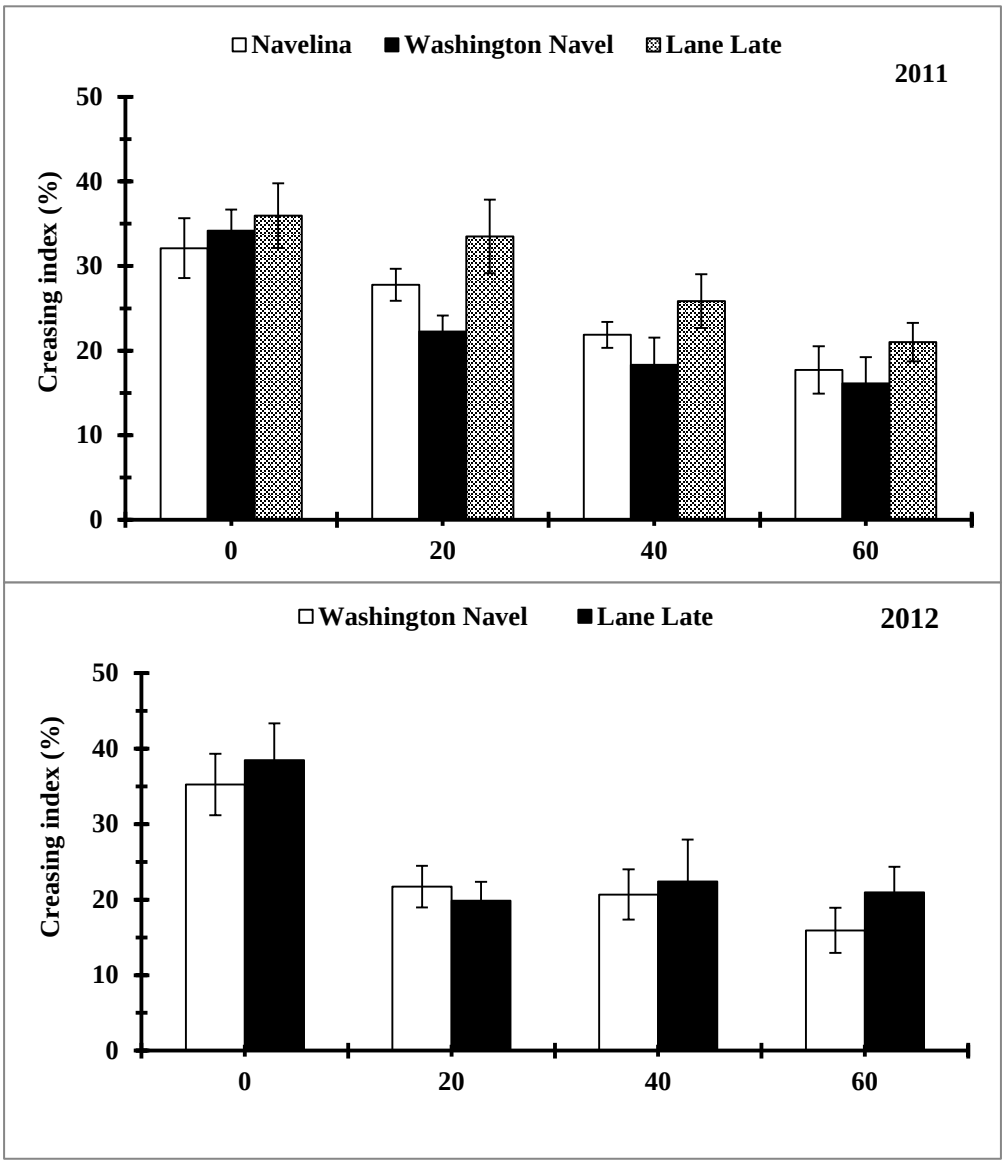

Figure 6.3. Effect of exogenous application of different concentrations of AVG applied at the golf ball stage on CI in different cultivars of sweet orange. n = 4 replications (35 fruit per replication). Vertical bars represent S.E. of means. LSD ($P \leq 0.05$) for 2011, Treatments = 7.4, Cultivars = 5.3, Treatments × cultivars = ns, LSD ($P \leq 0.05$) for 2012, Treatments = 8.1, Cultivars = ns, Treatments × cultivars = ns; ns = not-significant.

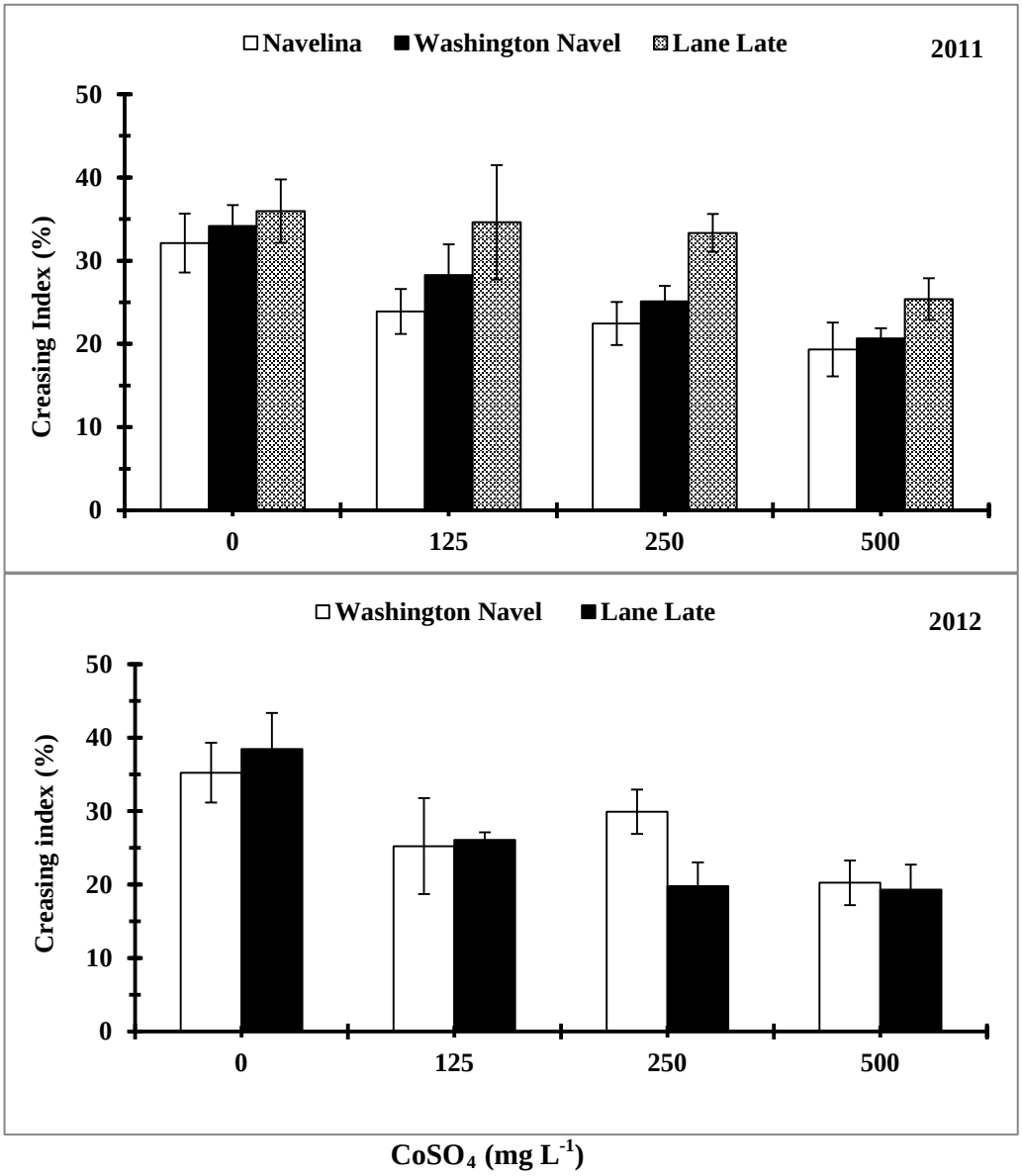

Figure 6.4. Effect of exogenous application of different concentrations of $CoSO_4$ applied at the golf ball stage on CI in different cultivars of sweet orange. n = 4 replications (35 fruit per replication). Vertical bars represent S.E. of means. LSD ($P \leq 0.05$) for 2011, Treatments = 6.8, Cultivars = 4.8, Treatments × cultivars = ns, LSD ($P \leq 0.05$) for 2012, Treatments = 8.6, Cultivars = ns, Treatments × cultivars = ns; ns = not-significant.

6.4. Discussion

Higher concentrations of endogenous ethylene in creased fruit than normal fruit in different cultivars of sweet oranges including Navelina, Washington Navel, Lane Late and Valencia Late (Figure 6.1) demonstrates the key role of endogenous ethylene in creasing of sweet orange fruit. Increased ethylene production in the creased fruit of sweet orange may possibly be ascribed to the increased activities of 1-aminocyclopro- pane-1-carboxylase synthase (ACC synthase, ACS) and ACC oxidase in the albedo and/or flavedo tissues of the rind. The activities of these enzymes in the albedo and/or flavedo tissues of the rind in sweet orange are yet to be investigated. Earlier, Hyodo (1977) reported active ethylene biosynthesis in the albedo tissue of 'Satsuma' mandarin fruit. Increased levels of endogenous ethylene in creased albedo tissue and fruit rind has been previously reported in Valencia and Washington Navel orange by Pham (2009) and Monselise et al. (1976) respectively. ACC is an intermediate pathway of ethylene biosynthesis in the albedo tissues. But 1-aminocyclopropane-1-carboxylic acid (ACC) formation and ACC conversion to ethylene are increased by aging and wounding in the albedo tissue of citrus fruit (Hyodo and Nishino, 1981).All the treatments of exogenous application of ethrel applied at 165 (DAFS) have significantly ($p \leq 0.05$) elevated the levels of endogenous ethylene in the fruit 30, 60, 90, 120 DAS compared to the control (Table. 6.1), consequently promoting creasing on the fruit in Washington Navel and Lane Late sweet orange (Figure 6.2). Promotion of creasing with exogenous application of ethrel due to increased ethylene production in the fruit of both cultivars also suggests the involvement of ethylene in creasing of sweet orange fruit. Exogenous application of ethylene biosynthesis inhibitors such as AVG or $CoSO_4$ at the golf ball stage significantly reduced CI in Navelina, Washington Navel and Lane Late cultivars of sweet orange during 2011 and 2012 (Figure 3 and 4), which further demonstrates that ethylene biosynthesis and/or its action is involved in creasing. The increased ethylene production was in parallel to the increase in ACC content, but the level of S-adenosylmethionine (SAM) was unaffected, suggesting that the conversion of SAM to ACC is a key reaction in the production of ethylene (Yu and Yang, 1980). It is well known that AVG and CoSO4 are inhibitors of ethylene biosynthesis which are involved in the conversion of SAM to ACC, eliminating the increase in ACC formation and ethylene production through the action of the ACC oxidase enzyme (Yu and Yang, 1980; Even-Chen et al., 1982; Hyodo and Nishino, 1981; Ladaniya, 2007). A substantial reduction in creasing of fruit with exogenous application of putrescine applied at the golf ball stage in sweet orange (data not included) also supports the involvement of ethylene in creasing.

Polyamines are known to inhibit ethylene biosynthesis by inhibiting ACC synthase enzyme (Liu et al., 2006; Even and Melberg, 1989). Similarly, the pre-harvest spray application of putrescine (1-2 mM) significantly suppressed ethylene biosynthesis and consequently reduced the activities of fruit softening-related enzymes such as *exo*-PG, *endo*-PG, PE and EGase in the skin and pulp tissues of plum (*Prunus salicina* Lindl. cv. Angelino) fruit during cold storage (Khan et al., 2007).

Higher endogenous levels of ethylene in creased fruit; acceleration of creasing with exogenous applications of ethrel; and reduced CI with ethylene inhibitors indicates the involvement of ethylene in creasing of sweet orange fruit. However, sweet orange fruit is non-climacteric, and produces only a small amount of ethylene (<0.1 µL. Kg^{-1}.$hour^{-1}$), which induces changes in fruit colour, flavour, chemical composition and texture in citrus fruit (Aharoni, 1968; Ekas, 1970; Ladaniya, 2007). Ethylene plays a major role in regulating ripening and softening of fruit (Ayub et al., 1996; Hadfield et al., 2000), and accordingly, the expression of some ripening-related and cell wall-associated genes and activities, including those of PGs (Hiwasa et al., 2003; Sitrit and Bennett, 1998), expansins (Rose et al., 1997) and EGases (Lashbrook et al., 1994). Possibly higher levels of ethylene in the fruit may have induced architectural changes in the structure of cell wall components in the albedo tissue including activities of various enzymes involved in cell wall degradation. Similarly, higher activities of PE, *exo*-PG, *endo*-PG, and EGase in the albedo and flavedo tissues of creased fruit than normal fruit have been reported to be associated with greater losses of pectins and starch in albedo cell walls, consequently leading to creasing (Saleem et al., 2014;Li et al., 2009). In conclusion, higher levels of endogenous ethylene in creased fruit than normal fruit in Navelina, Washington Navel, Lane Late and Valencia Late cultivars of sweet orange, acceleration of CI with exogenous application of ethrel, and its reduction with ethylene biosynthesis inhibitors indicate the involvement of ethylene in creasing of sweet orange fruit.

CHAPTER 7

Effects of ethylene inhibitor AVG on incidence of creasing and fruit quality in sweet orange cv. Washington Navel and Lane Late

Summary

Creasing is a physiological disorder of rind in sweet orange [*Citrus sinensis* (L.) Osbeck] fruit and causes serious economic losses in various countries of the world. Effects were investigated of different concentrations (0, 20, 40 and 60 mg L^{-1}) of aminoethoxyvinylglycine (AVG) with 0.05% 'Tween 20' as a surfactant applied at the fruit set, the golf ball or at the colour break stage on controlling creasing, rheological properties of fruit and rind as well as fruit quality in Washington Navel and Lane Late sweet orange. Creasing was substantially reduced and fruit quality was improved with the exogenous application of AVG depending upon its concentration and stage of application in both cultivars. The spray application of AVG (60 mg L^{-1}) at the golf ball stage was effective in reducing creasing (27.9 and 24.3%) compared to the control (52.1and 51.5%) in cv. Washington Navel during 2011 and 2012, respectively. Meanwhile, in cv. Lane Late the application of AVG at the fruit set stage resulted in the lowest creasing (22.9%) compared to the control (51.4%) during 2012. In cv. Washington Navel, AVG treatment (60 mg L^{-1}) was more effective in increasing fruit firmness (319.0 N) and rind hardness (25.9 N), when applied at the fruit set stage. However, rind tensile strength was higher when AVG was applied at the golf ball stage (54.1 N). In cv. Lane Late, rind hardness and rind tensile strength were also higher (28.6 and 78.8 N) when AVG was sprayed at fruit set stage. Meanwhile, the fruit compression force was higher (369.7 N) when AVG was applied at the golf ball stage. Similarly, the treatment AVG (60 mg L^{-1}) was more effective in improving fruit weight (281.0 and 298.5 g), fruit diameter (87.3 and 82.7 mm) and rind thickness (5.6 and 5.1 mm) when applied at the golf ball stage in cv. Washington Navel and Lane Late, respectively. Similarly, rind hardness (25.9 and 28.6 N), malic acid (0.4 and 0.6 g $100ml^{-1}$) and succinic acid (0.4 and 0.4 g $100ml^{-1}$) were also higher when AVG was applied at the fruit set stage in both cultivars. In conclusion, the exogenous applications of AVG (60 mg L^{-1}) substantially reduces the creasing incidence, improves rheological properties of fruit and rind as well as fruit quality in cv. Washington Navel and Lane Late sweet orange.

7.1. Introduction

Sweet orange (*Citrus sinensis* L. Osbeck) occupies a prominent position among citrus species in the world (FAOSTAT, 2011). Global demand for sweet orange is attributed to its higher vitamin C and antioxidants (Gorinstein et al., 2001). The albedo tissue of citrus fruit is prone to fracturing and leads to the physiological rind disorders such as creasing (albedo breakdown) in sweet oranges (Monselise et al., 1976) and puffiness in mandarins (García-Luis et al., 1985). Creasing also creates weak points in the flavedo tissues which cause grooves on the fruit surface and fruit is ruptured when packed for fresh markets (Gilfillan et al., 1981). The fracturing of the albedo tissue is predominantly due to separation of adjacent cells rather than cleavage of individual cells (Storey and Treeby, 1994). Creasing mainly affects different cultivars of sweet oranges such as Washington Navel (Gambetta et al., 2000, Ali et al., 2000), Valencia (Jones et al., 1967; Monselise et al., 1976) and Nova mandarins (Greenberg et al., 2006). Creasing was first reported from South Africa during 1938 (Le Roux and Crous, 1938) and currently is a major problem in sweet orange fruit grown in different parts of the world such as Australia (Treeby et al, 1994), USA (Ali et al., 2000; Jones et al., 1967), Israel (Greenberg et al., 2006; Monselise et al., 1976), Uruguay (Gambetta et al., 2000) and China (Li et al., 2009). More than 50% losses in individual orchards have been reported from South Africa (Gilfillan et al., 1981). Creasing is more prominent at fruit maturity; however its initiation seems to be associated with fruit growth and development (Storey and Treeby, 1994). Although no definite cause of creasing has been established, several factors have been associated with this disorder such as crop load, fruit position, peel thickness, climatic or nutritional factors, irrigation, rootstock and genotype (Treeby et al., 1995; Jones et al., 1967). Different techniques have been tested to control this disorder such as regulated deficient irrigation (Treeby et al., 2007), exogenous application of gibberellic acid (Greenberg et al., 2010; Jona et al., 1989; Monselise et al., 1976), potassium sulphate as well as phosphorous (Bevington et al., 1993; Jones et al., 1967) and repeated foliar application of calcium (Pham et al., 2012; Storey et al., 2002).

Citrus is a non-climacteric fruit, which produces only limited amounts of endogenous ethylene, while ethylene plays an important role in changing fruit colour, flavour, chemical composition and texture in citrus (Ladaniya, 2007). Ethylene plays an important role as an inducer of fruit ripening (Bleecker, 2000) and accelerates softening in citrus fruit due to disintegrating cell membranes making them leakier (Ladaniya, 2007; Rath and Prentice, 2004). The exogenous application of ethrel resulted in increased respiration rate, promoted

ripening and improved colour development in citrus (Ladaniya, 2007; Burg, 2004; Agustí et al., 2002). The pre-harvest application of ethylene did not affect fruit quality parameters of citrus such as soluble solid concentration, juice percentage, fruit weight, rind thickness, acidity and ascorbic acid (Al-Mughrabi et al., 1989). However, higher endogenous levels of ethylene in the albedo tissue of the creased fruit than in normal fruit in Valencia orange have been reported by Monselise et al. (1976) and in Washington Navel by Pham (2009). I also observed higher levels of endogenous ethylene in creased fruit compared to normal fruit in sweet orange including Navelina, Washington Navel, Lane Late and Valencia Late (Chapter 4). Ethylene biosynthesis and action has been known to be inhibited by various compounds such as AVG, cobalt sulphate ($CoSO_4$) and 1-Methylcyclopropene (1-MCP). AVG is known to be a reversible inhibitor of ethylene and defeats biosynthesis of ethylene in plant tissues through inhibiting 1-aminocyclopropane-1-carboxylic acid (ACC) (Rath et al., 2004). The conversion of S-adenosylmethionine (SAM into ACC eliminates the increase in ACC formation and ethylene production through the action of the ACC oxidase enzyme (Ladaniya, 2007; Even-Chen et al., 1982; Hyodo and Nishino, 1981). However, the effects of AVG depend upon its concentration and stage of application, cultivar and environmental conditions (Kim et al 2004; Matoo et al 1977). AVG is commercially available as 'ReTain'^{TM} and is an environmentally friendly organic product recommended for pre-harvest fruit drop of apple, pear, peach, plum and nectarine (Karaman et al., 2013). Recently, Al-Husseini (2012) applied different treatments of AVG (20-60 mg L^{-1}) at the fruit set or the golf ball stage to reduce the fruit drop and to improve the fruit weight, diameter, SSC as well as TA in sweet orange cv. Washington Navel and Lane Late.

Similarly, I also observed that reduction in the incidence of creasing in sweet orange fruit occurred with the exogenous application of ethylene inhibitor such as AVG, $CoSO_4$ or PUT through modifying the activities of cell wall degrading enzyme such as PE, *exo*-PG, *endo*-PG, and EGase in the albedo and flavedo tissues of the fruit (Chapter 9). However, no research work reported on the effect of different concentrations of AVG applied at the fruit set, the golf ball or at the colour break stage on incidence of creasing, rheological properties of fruit and rind as well as fruit quality in Washington Navel and Lane Late sweet orange. It was hypothesised that AVG applications at the fruit set, the golf ball or at the colour break stage, may reduce creasing and improve rheological properties of rind and fruit quality in sweet orange fruit. In the present study we investigated the role of AVG applied at the fruit set, the

golf ball or at the colour break stage on incidence of creasing, rheological properties of fruit and rind as well as fruit quality in sweet orange cv. Washington Navel and Lane Late.

7.2. Materials and methods

7.2.1. Plant material

Two experiments were conducted on Washington Navel and Lane Late sweet orange fruit at a commercial orchard located in Gingin (latitude 31° 21′ South, longitude 155° 55′ East), Western Australia during 2010-11 and 2011-12. Twenty-five year old uniform sweet oranges trees growing in sandy loam soil and grafted on trifoliate orange rootstock (*Poncirus trifoliate* Raf.) were used in both experiments. The trees were spaced 7.5 m between rows and 2.7 m within rows. Climate is characterised by a mild, wet winter and hot, dry summer. All the experimental trees received similar cultural practices including fertilisers, irrigation, weed control and plant protection except for experimental treatments.

7.2.2. Experiment I: Effects of exogenous application of different concentrations of AVG applied at the fruit set, the golf ball or at the colour break stage on incidence of creasing, rheological properties of fruit and rind as well as fruit quality of sweet orange cv. Washington Navel

An aqueous solution containing different concentrations (20, 40, 60 mg L^{-1}) of AVG (as Retain® from Valent BioSciences®, Chatswood, NSW, Australia) and 'Tween 20' (0.05 %) as a surfactant were sprayed at the fruit set (Fruit diameter: 15±5 mm), the golf ball (Fruit diameter 40±5 mm) or at the colour break stage (Fruit diameter: 80±5 mm) of sweet orange in cv. Washington Navel by using a sprayer (The Selecta Trolleypak Mk II, Acacia Ridge, Australia). The experimental layout was a completely randomised block with two-factor factorial including treatments and stages of application. Single trees were classed as an experimental unit and replicated four times. The experiment was repeated in two consecutive seasons (2010-11 and 2011-12). The data for two years were not pooled because error mean squares over years were heterogeneous. At harvest, 35 ripe fruit per tree were randomly harvested around the tree canopy. The incidence of creasing was examined on individual fruit, based on the appearance of symptoms on the fruit surface and rheological properties of

fruit and rind (fruit firmness, rind hardness and rind tensile strength). The physical characteristics of fruit, such as fruit weight, diameter and rind thickness were measured in both consecutive seasons. Soluble solids concentrations (SSC), titratable acidity (TA), SSC: TA ratio, ascorbic acid, total antioxidants, individual and total sugars as well as individual organic acid were determined from the juice during both years.

7.2.3. Experiment II. Effects of exogenous application of different concentrations of AVG applied at the fruit set, the golf ball or at the colour break stage on incidence of creasing, rheological properties of fruit and rind as well as fruit quality of sweet orange cv. Lane Late

The second experiment was conducted by employing the same AVG treatments at the same fruit developmental stages in cv. Lane Late during 2010-11 and 2011-12. The experimental arrangement was a two-factor factorial completely randomized design including different concentrations of AVG and stages of its application. Four single-tree replications were used per treatment. The incidence of creasing was examined on individual fruit, based on the appearance of symptoms on the fruit surface and rheological properties of fruit and rind. The fruit weight, diameter and rind thickness were measured in both consecutive seasons. Similarly, SSC, TA, SSC: TA ratio, ascorbic acid concentration, total antioxidants, individual and total sugars and organic acids were estimated in the juice during 2010-11 and 2011-12.

7.2.4. Creasing (%)

Thirty-five ripe fruit per tree were randomly harvested around the tree canopy in each cultivar to determine the creasing percentage as mentioned in Chapter 3, Section 3.3.1.

7.2.5 Rheological properties of fruit and rind

The effect of AVG on rheological properties of the fruit and rind such as fruit firmness (N), rind hardness (N) and rind tensile strength (N) was determined by using a textural analyzer (TA Plus, AMETEK Lloyd instruments Ltd., Hampshire, UK) as described in Chapter 3, Section 3.4. The data were collected and processed by using Nexygen® 4.6 software.

7.2.5.1. Fruit firmness

Five fruit per replication were selected with 75 mm height to measure the fruit firmness (N) by using the compression test as detailed in Chapter 3, Section 3.4.1.

7.2.5.2. Rind hardness

The sweet orange fruit rind hardness was measured with the help of a puncture test. The fruit rind was cut 2.5 cm width having 6.0 mm thickness by using a slicer (Zyliss Easy slice, folding Mandoline slicer, Swiss) to give uniform sections for the determination of rind hardness as reported in Chapter 3, Section 3.4.2.

7.2.5.3. Rind tensile strength

The fruit rind of sweet orange was carefully removed in the size (2.5 x 5 cm area having 6 mm thickness) to measure the rind tensile strength as described in Chapter 3, Section 3.4.3. The rind tensile strength was calculated at the maximum load and limit points, where the rind deflection occurred at 10 mm.

7.2.6. Fruit weight, diameter and rind thickness

The fruit weight was calculated by weighing ten randomly selected fruit per replication using a digital electrical balance (A&D Limited, Tokyo, Japan) and average weight was calculated as gram (g) per fruit. The fruit diameter and rind thickness of ten randomly selected fruit per replication was measured with a digital Vernier caliper and expressed in mm as detailed in Chapter 3, Section 3.5.

7.2.7. Soluble solids concentration (SSC)

The juice was extracted from freshly harvested orange fruit to determine SCC with the help of a digital refractometer (Atago-Palette PR 101, Atago CO. Ltd, Itabashi-Ku, Tokyo, Japan) and expressed in (%) as described in Chapter 3, Section 3.6.

7.2.8. Determination of ascorbic acid concentration

Ascorbic acid concentration was also determined by following the modified method of Malik and Singh (2005) and Pham (2009) as detailed in Chapter 3, Section 3.9. The ascorbic acid concentration was taken at 760 nm using an UV/VIS Spectrophotometer (Jenway 6405, Dunmow, Essex, UK) and expressed as mg ascorbic acid per 100 ml fresh juice.

7.2.9. Determination of total antioxidants

The total antioxidants were determined from the freshly extracted orange juice by the modified method of Brand-Williams et al. (1995) and Pham (2009) as explained in Chapter 3, Section 3.10. Total antioxidants were determined by using a UV/VIS spectrophotometer (Jenway 6405, Dunmow, Essex, UK) and expressed as mM Trolox equivalent antioxidant activity (TEAC) 100 ml^{-1} FJ basis.

7.2.10. Determination of individual sugar and total sugars as well as organic acids

The individual and total sugars as well as organic acids were extracted from the fresh juice of sweet orange fruit by using a RP-HPLC system (Waters, Milford, MA, USA) as previously described in Chapter 3, Section 3.11. The concentration of individual, total sugar as well as individual organic acids was expressed as g100 ml^{-1} FJ.

7.2.11. Statistical analysis

The experimental data were subjected to a two–way analysis of variance (ANOVA) using GenStat 14th edition (VSN International Ltd., Hemel Hempstead, UK). The effects of treatments of AVG and stages of application on different parameters were assessed within ANOVA and the least significant differences (LSD) were calculated following a significance F–test at ($P \leq 0.05$). To ensure the validity of statistical analysis, all the assumptions of ANOVA were checked.

7.3. Results

7.3.1. Creasing (%)

The incidence of creasing (%) decreased with increased concentrations of AVG applied, irrespective of its application at the fruit set, the golf ball or at the colour break stage in sweet orange cv. Washington Navel and Lane Late during 2011 and 2012 (Figure 7.1). When averaged over different stages of AVG spray application, all the treatments of AVG significantly ($P \leq 0.05$) reduced the mean creasing compared to the control in cv. Washington Navel during both years. In cv. Lane Late, the treatment of AVG (40-60 mg L^{-1}) significantly reduced creasing as compared to the control and AVG (20 mg L^{-1}) during 2011. However, all the treatments of AVG significantly ($P \leq 0.05$) reduced creasing compared to the control in

cv. Lane Late during 2012. A spray application of AVG (60 mg L^{-1}) applied at the golf ball stage resulted in lowest creasing (27.9 and 24.3%) compared to the control (52.1 and 51.5%) in cv. Washington Navel during 2011 and 2012. When averaged over all treatments, the mean creasing was significantly ($P \leq 0.05$) reduced when AVG was applied at the golf ball stage (39.5 and 34.1%) compared to its application at the fruit set or at the colour break stage in cv. Washington Navel during 2011 and 2012. In cv. Lane Late, different stages of AVG spray application did not significantly affect the incidence of creasing. The interaction between the treatments and their stages of application for creasing were found to be non-significant ($P \leq 0.05$) in both cultivars during 2011 and 2012.

7.3.2. Rheological properties of fruit and rind

7.3.2.1. Fruit firmness

Different concentrations of AVG applied at various stages improved the fruit firmness in sweet orange cv. Washington Navel and Lane Late during 2011and 2012 (Figure. 7.2 A-D and 7.3 A-D). When averaged over different stages of AVG spray application, the treatment of 40-60 mg L^{-1} AVG significantly ($P \leq 0.05$) improved fruit firmness compared to the control and AVG (20 mg L^{-1}) in cv. Washington Navel and Lane Late during 2011. In 2012, all treatments of AVG significantly ($P \leq 0.05$) increased mean fruit firmness as compared to the control in both cultivars.

When averaged over different treatments of AVG, mean fruit firmness was significantly ($P \leq 0.05$) increased when AVG was applied at the colour break (310.20 N) compared to its application at the fruit set (262.4 N) or at the golf ball stage (269.2 N) in cv. Washington Navel during 2011. Non-significant effects were observed during 2011 in cv. Lane Late. In 2012, the mean fruit firmness was significantly ($P \leq 0.05$) higher when AVG was applied at the colour break stage (330.8 N) compared to its application at the fruit set (309.3 N) or at the golf ball stage (319.1 N) in cv. Lane Late. However, in cv. Washington Navel non-significant effects were observed during 2012. The interaction between treatments and different stages of its application were found to be non-significant ($P \leq 0.05$) for fruit firmness in cv. Washington Navel and Lane Late during 2011 and 2012.

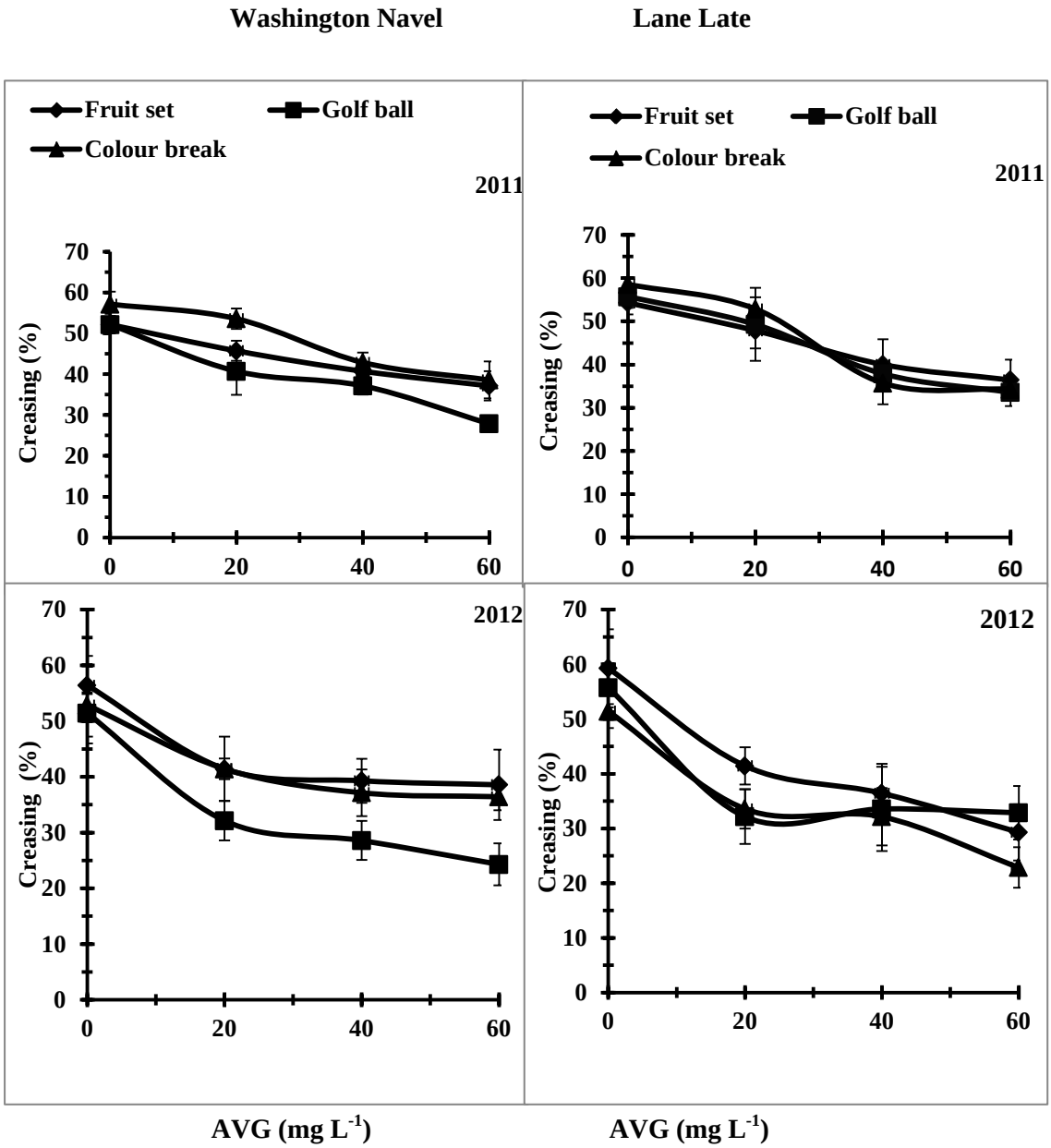

Figure 7.1. Effect of different concentrations of AVG sprayed at the fruit set, the golf ball or at the colour break stage on creasing (%) in sweet orange cv. Washington Navel and Lane Late during 2011 and 2012. n = 4 replications (35 fruit per replication), LSD ($P \leq 0.05$) for Washington Navel 2011, Treatments = 3.2, Stage = 2.79, Treatments x stage = ns; Washington Navel 2012, Treatments = 6.5, Stage = 5.7, Treatments x stage = ns; LSD ($P < 0.05$) for Lane Late 2011, Treatments = 7.3, Stage = ns, Treatments x stage = ns; Lane Late 2012 , Treatments = 8.4, Stage = ns, Treatments x stage = ns; ns = not-significant.

7.3.2.2. Rind hardness

Rind hardness (N) increased with increased concentrations of AVG applied, irrespective of spray application at the fruit set, the golf ball or at the colour break stage in cv. Washington Navel and Lane Late during 2011 and 2012 (Figure 7.2 B-E and 7.3 B-E). When averaged over different stages of AVG application, all treatments of AVG (20-60 mg L^{-1}) significantly ($P \leq 0.05$) improved the mean rind hardness compared to the control in cv. Lane Late during 2011. Meanwhile, the spray application of AVG (60 mg L^{-1}) significantly increased the rind hardness compared to the control and AVG (20-40 mg L^{-1}) in cv. Washington Navel during 2011. In 2012, the spray application of AVG (40-60 mg L^{-1}) significantly ($P \leq 0.05$) increased rind hardness compared to the control and AVG (20 mg L^{-1}) in both cultivars. The spray application of AVG exhibited non-significant effect on different stages of application in cv. Washington Navel and Lane Late during both years. The interaction between the treatments and their stages of application were found to be non-significant ($P \leq 0.05$) for rind hardness in both cultivars during 2011 and 2012.

7.3.2.3. Rind tensile strength

All treatments of AVG increased the rind tensile strength in sweet orange cv. Washington Navel and Lane Late during 2011 and 2012 (Figure 7.1C-F and 7.2 C-F). When averaged over different stages of spray application, the treatment of AVG (60 mg L^{-1}) resulted in significantly ($P \leq 0.05$) higher rind tensile strength (51.2 N) compared to the control (39.9 N) and all other treatments in cv. Washington Navel during 2011. However, in cv. Lane Late, all treatments of AVG (20-60 mg L^{-1}) significantly ($P \leq 0.05$) improved rind tensile strength compared to the control during 2011. Similarly, in 2012, all treatments of AVG significantly ($P \leq 0.05$) improved rind tensile strength compared to the control in both cultivars. When averaged over all the treatments, mean rind tensile strength was significantly ($P \leq 0.05$) higher when AVG was applied at the fruit set stage (71.4 N) compared to its application at the golf ball (57.9 N) or at the colour break stage (60.0 N) in cv. Lane Late during 2011 only. However, in cv. Washington Navel, non-significant ($P \leq 0.05$) effects were observed when AVG was applied at the fruit set, the golf ball or at the colour break stage during both years. The interaction between treatments and their stages of application for rind tensile strength was found to be non-significant ($P \leq 0.05$) in cv. Washington Navel and Lane Late during 2011 and 2012.

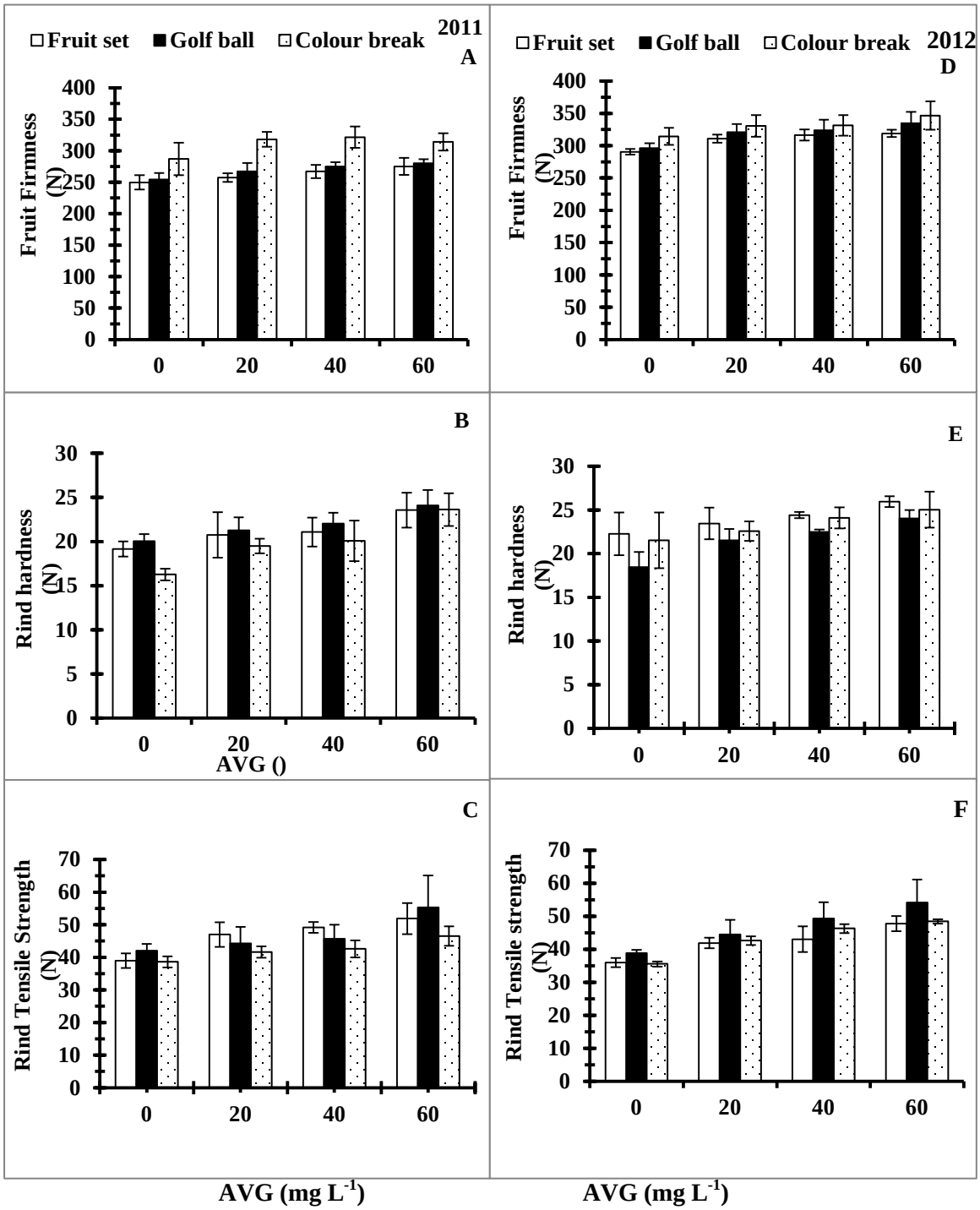

Figure 7.2. Effect of different concentrations of AVG sprayed at fruit set, the golf ball or at colour break stage on rheological properties of sweet orange cv. Washington Navel fruit during 2011 and 2012. n = 4 replications (5 fruit per replication). Vertical bars represent standard error means. LSD ($P \leq 0.05$) for 2011, Fruit firmness, Treatments = ns, Stage = 19.3, Treatments x stage = ns; for rind hardness, Treatments = 2.7, Stage = ns, treatments x stage = ns; for rind tensile strength, Treatments = 7.2, Stage = ns, Treatments x stage = ns; ($P \leq 0.05$) for year 2012; fruit compression force, Treatments = 20.2, Stage = ns, Treatments x stage = ns; for rind hardness, Treatments = 2.8, Stage = ns, treatments x stage = ns; for rind tensile strength, Treatments = 5.4, Stage = ns, Treatments x stage =; ns = not-significant.

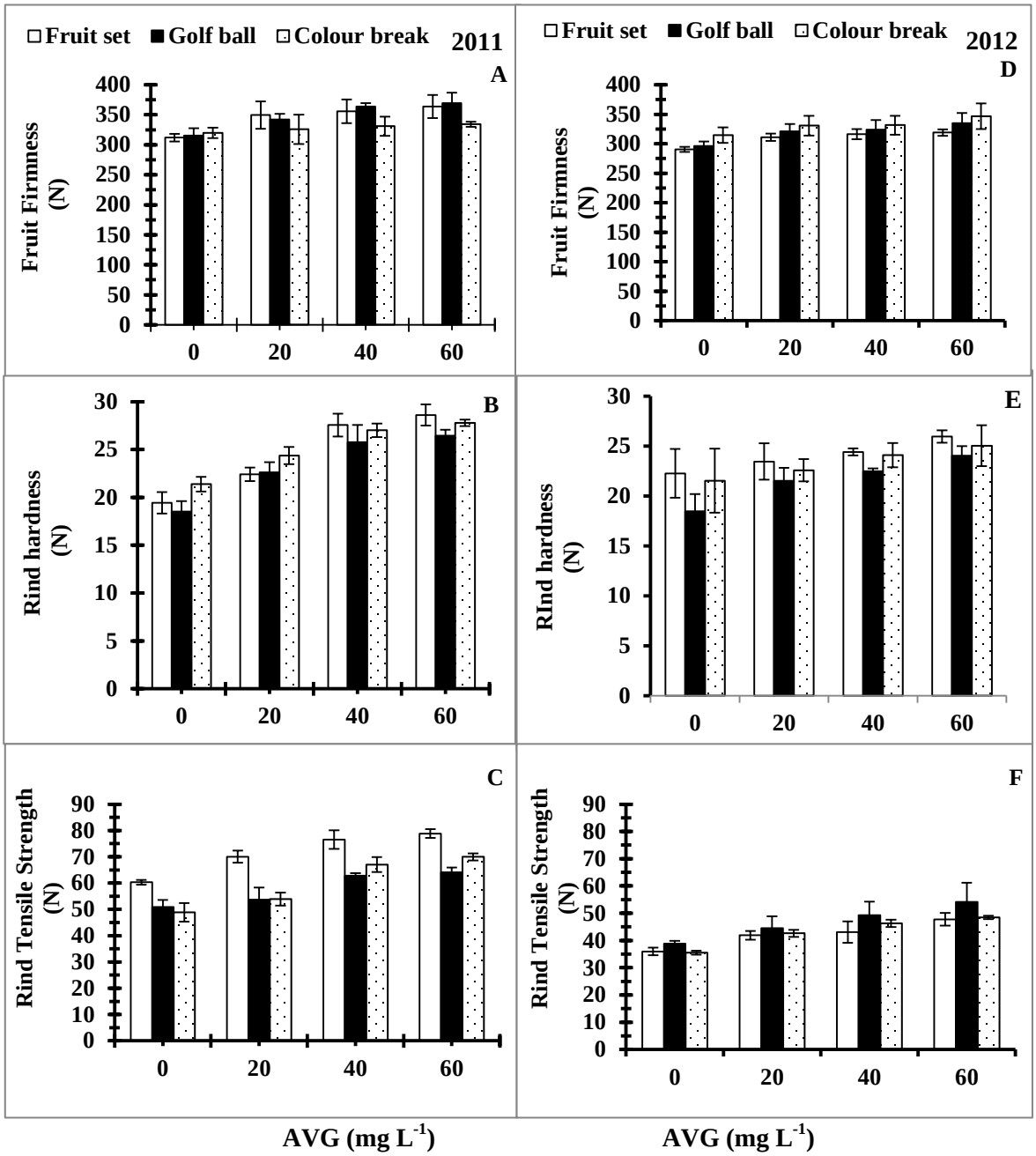

Figure 7.3. Effect of different concentrations of AVG sprayed at fruit set, the golf ball or at the colour break on rheological properties of sweet orange cv. Lane Late during 2011 and 2012. n = 4 replications (5 fruit per replication). Vertical bars represent standard error means. LSD ($P \leq 0.05$) for 2011; fruit firmness, Treatments = 26.1, Stage = ns, Treatments x stage = ns; for rind hardness, Treatments = 0.9, Stage = ns, treatments x stage = ns; for rind tensile strength, Treatments = 2.2, Stage = 1.9, Treatments x stage = ns; ($P \leq 0.05$) for year 2012; fruit firmness, Treatments = 20.2, Stage = 17.5, Treatments x stage = ns; for rind hardness, Treatments = 2.8, Stage = ns, treatments x stage = ns; for rind tensile strength, Treatments = 5.4, Stage = ns, Treatments x stage =; ns = not-significant.

7.3.3. Fruit weight

All the exogenous spray applications of different concentrations of AVG increased the fruit weight irrespective of different stages of application in both cultivars of sweet orange during 2011 and 2012 (Table 7.1). When averaged over different stages of spray application, the mean fruit weight significantly ($P \leq 0.05$) increased with the spray application of AVG (40-60 mg L^{-1}) compared to the control and AVG (20 mg L^{-1}) treatment in sweet orange cv. Washington Navel during 2011. In cv. Lane Late, the mean fruit weight was significantly ($P \leq 0.05$) highest (291.4 g) with the spray application of AVG (60 mg L^{-1}) compared to the control and all other treatments during 2011. A similar trend was noted in cv. Washington Navel during 2012. However, all the treatments of AVG significantly ($P \leq 0.05$) increased the mean fruit weight compared to the control in cv. Lane Late during 2012. When averaged over all treatments of AVG, the mean fruit weight was significantly ($P \leq 0.05$) higher when AVG was applied at the golf ball (253.5 g) compared to its application at the fruit set (239.2 g) or the colour break stage (237.0 g) in cv. Washington Navel during 2012. Meanwhile, in cv. Lane Late, spray application of AVG resulted in significantly ($P \leq 0.05$) higher fruit weight when applied at the fruit set stage (283.3 g) compared to its application at the colour break (279.0 g) or at the golf ball (266.6 g) in 2012. Interactions between treatments and different stages of application were also found to be non-significant ($P \leq 0.05$) for fruit weight in both cultivars during 2011 and 2012.

7.3.4. Fruit diameter

Fruit diameter increased with increased concentrations of AVG applied at the fruit set, the golf ball or at the colour break stage during 2011 and 2012 (Table 7.2). When averaged over different stages of application, treatment of AVG (40-60 mg L^{-1}) significantly ($P \leq 0.05$) increased mean fruit diameter compared to the control and AVG (20 mg L^{-1}) in cv. Washington Navel during 2011. In 2012, all treatments of AVG significantly improved mean fruit diameter compared to the control in cv. Washington Navel. In cv. Lane Late, the spray application of AVG (60 mg L^{-1}) significantly increased fruit diameter compared to the control and all other treatments. When averaged over different treatments of AVG, the mean fruit diameter significantly increased when AVG was applied at the colour break (82.9 mm), compared to its application at the fruit set (82.6 mm) or at the golf ball stage (77.9 mm) in cv. Lane Late during 2012.

Table 7.1 Effect of different concentrations of AVG sprayed at fruit set, the golf ball or at the colour break stage on fruit weight of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Fruit weight (g)

Washington Navel								
Treat (m gL^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	270.2	234.8	257.2	242.5	263.2	219.2	263.6 b	232.2 b
AVG 20	271.8	237.5	263.2	244.2	273.0	234.0	269.3 ab	238.6 ab
AVG 40	274.5	238.2	272.5	261.7	278.3	245.8	275.1 a	248.6 ab
AVG 60	279.2	246.2	281.0	265.5	279.0	249.0	279.8 a	253.6 a
Mean (stage)	273.9	239.2 ab	268.5	253.5 a	273.4	237.0 b		
LSD ($P \leq 0.05$)								
Treatments			10.8	17.0				
Stage			ns	14.7				
Treatments × stages			ns	ns				
Lane Late								
Treat (m gL^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	265.2	270.2	263.2	247.2	263.8	268.2	264.1 b	261.9 b
AVG 20	278.2	281.8	272.0	270.8	269.5	283.0	273.2 b	278.5 a
AVG 40	292.0	287.0	272.2	272.5	273.2	283.3	279.2 ab	280.9 a
AVG60	295.5	294.2	298.2	276.0	280.5	281.5	291.4 a	283.9 a
Mean (stage)	282.8	283.3 a	276.4	266.6 b	271.8	279.0 a		
LSD ($P \leq 0.05$)								
Treatments			16.9	12.9				
Stages			ns	22.4				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

Table 7.2. Effect of different concentrations of AVG sprayed at fruit set, the golf ball or at the colour break stage on fruit diameter of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Fruit diameter (mm)								
Washington Navel								
Treat (m gL^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	79.9	67.7 c	75.5	75.5 b	77.4	77.2 ab	77.6 b	73.5 b
AVG 20	79.9	81.8 ab	82.5	79.6 ab	80.8	78.6 ab	81.1 ab	80.0 a
AVG 40	81.4	82.1 a	85.6	80.9 ab	83.4	79.7 ab	83.4 a	80.9 a
AVG 60	82.8	83.4 a	87.3	82.2 a	84.0	80.9 ab	84.7 a	82.0 a
Mean (stage)	81.0	78.8 a	82.7	79.6 a	81.4	78.92 a		
LSD ($P \leq 0.05$)								
Treatments			3.6	1.6				
Stage			ns	ns				
Treatments × stages			ns	2.8				
Lane Late								
Treat (m gL^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	76.6	80.8	75.5	76.8	75.2	78.4	75.8 b	78.7 b
AVG 20	77.0	86.1	79.6	75.1	78.7	83.1	78.4 ab	81.4 ab
AVG 40	77.8	81.5	79.7	78.8	79.3	84.0	79.0 ab	81.4 ab
AVG 60	79.3	82.0	82.7	80.9	80.8	86.2	80.9 a	83.0 a
Mean (stage)	77.7	82.6 a	79.4	77.9 b	78.5	82.9 a		
LSD ($P \leq 0.05$)								
Treatments			3.19	2.89				
Stages			ns	2.51				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

AVG application at different stages of fruit growth did not significantly affect the fruit diameter in cv. Lane Late during 2011 and in Washington Navel during both years. The interaction between treatments and their stages of application for fruit diameter was found to be non-significant ($P \leq 0.05$) in cv. Lane Late during both years while significant ($P \leq 0.05$) effect was found in cv. Washington Navel during 2012.

7.3.5. Rind thickness

All the spray treatments of AVG increased rind thickness compared to the control in sweet orange cv. Washington Navel and Lane Late during 2011 and 2012 (Table 7.3). When averaged over different stages of application, all treatments of AVG resulted in significantly ($P \leq 0.05$) higher mean rind thickness compared to the control in cv. Washington Navel during 2011. Similarly, the treatments of AVG (40-60 mg L^{-1}) have significantly increased mean rind thickness compared to the control and treatment AVG (20 mg L^{-1}) in cv. Washington Navel during 2012. In cv. Lane Late, the treatments of AVG (60 mg L^{-1}) significantly ($P \leq 0.05$) improved mean rind thickness compared to the control and all other treatments during 2011. However, all the treatments of AVG resulted in significantly ($P \leq 0.05$) higher mean rind thickness compared to the control in cv. Lane Late during 2012. When averaged over different treatments of AVG, mean fruit rind thickness was significantly ($P \leq 0.05$) higher when AVG was applied at the colour break (5.3 mm) compared to its application at the golf ball (4.9 mm) or at the fruit set stage (4.9 mm) in cv. Washington Navel during 2011. In 2012, mean fruit rind thickness was significantly ($P \leq 0.05$) higher when AVG was applied at the golf ball (5.4 mm) compared to its application at the colour break (5.2 mm) or at the fruit set stage (5.1 mm) in cv. Washington Navel during 2012. However, in cv. Lane Late, the mean highest rind thickness was observed at the colour break (5.1 mm) compared to its application at the golf ball (5.0 mm) or at the fruit set stage (3.6 mm) during 2011. In 2012, the mean highest rind thickness was observed at the colour break (5.3 mm) compared to its application at the golf ball (4.9 mm) or at the fruit set stage (4.9 mm) in cv. Lane Late. Interaction between treatments and their stages of spray application for fruit rind thickness was found to be non-significant ($P \leq 0.05$) in both cultivars during 2011 and 2012.

Table 7.3. Effect of different concentrations of AVG sprayed at fruit set, golf ball or colour break stage on rind thickness of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Rind thickness (mm)								
Washington Navel								
Treat (m gL^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	4.6	4.7	4.5	5.1	5.0	5.0	4.69 b	4.92 b
AVG 20	4.9	5.1	5.0	5.3	5.2	5.0	5.05 a	5.14 ab
AVG 40	5.0	5.2	5.1	5.4	5.5	5.2	5.17 a	5.25 a
AVG 60	5.1	5.3	5.1	5.6	5.4	5.4	5.18 a	5.43 a
Mean (stage)	4.9 b	5.1 b	4.9 b	5.4 a	5.3 a	5.2 ab		
LSD ($P \leq 0.05$)								
Treatments			0.3	0.3				
Stage			0.2	0.2				
Treatments × stages			ns	ns				
Lane Late								
Treat (m gL^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	3.4	4.6	4.7 a	4.5	4.8	5.0	4.3 b	4.7 b
AVG 20	3.5	4.9	4.9 a	5.0	5.1	5.2	4.5 ab	5.1 a
AVG 40	3.6	5.0	5.0 a	5.1	5.2	5.5	4.6 ab	5.2 a
AVG 60	3.9	5.1	5.2 a	5.1	5.2	5.4	4.8 a	5.2 a
Mean (stage)	3.6 b	4.9 b	5.0 a	4.9 b	5.1a	5.3a		
LSD ($P \leq 0.05$)								
Treatments			0.3	0.2				
Stages			0.3	0.4				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

7.3.6. Soluble Solids Concentration (SSC %)

All the AVG spray treatments irrespective of stage of application have significantly ($P \leq 0.05$) increased SSC in the fruit juice as compared to the control in cv. Washington Navel and Lane Late during 2011 and 2012 (Table 7.4). When averaged over all treatments, the mean SSC was significantly ($P \leq 0.05$) higher when AVG was applied at the golf ball (13.3%) compared to its application at the colour break (12.6%) or at the fruit set stage (12.3%) in cv. Lane Late during 2011. Meanwhile, SSC was significantly ($P \leq 0.05$) higher, when AVG was sprayed at the colour break (12.5%) compared to its application at the golf ball (12.4%) or at the fruit set stage (12.4%) during 2012 in cv. Lane Late. However, in cv. Washington Navel, a non-significant ($P \leq 0.05$) effect was observed when AVG was applied at the fruit set, the golf ball or at the colour break stage during both years. The interactions between treatments and their stages of application were found to be non-significant ($P \leq 0.05$) for SSC in cv. Washington Navel and Lane Late during 2011 and 2012, except in cv. Washington Navel during 2012. All the AVG spray treatments irrespective of stage of application did not significantly ($P \leq 0.05$) affect TA and SSC: TA ratio in the juice of both cultivars during 2011 and 2012 (data not included).

7.3.7. Ascorbic acid

All the treatments of AVG increased the level of ascorbic acid in fruit juice compared to the control in cv. Washington Navel and Lane Late sweet orange, irrespective of stage of application of AVG treatment during 2011 and 2012 (Table 7.5). When averaged over different stages of spray application, the treatments of AVG (60 mg L^{-1}) resulted in significantly ($P \leq 0.05$) higher levels of mean ascorbic acid (69.5 and 65.4 mg $100ml^{-1}$ FJ) as compared to the control (66.2 and 61.0 mg $100ml^{-1}$ FJ) and other treatments of AVG (20-40 mg L^{-1}) in cv. Washington Navel during 2011 and 2012. In cv. Lane Late, all the treatments of AVG significantly ($P \leq 0.05$) increased mean levels of ascorbic acid compared to the control during 2011. However, in 2012, the treatments of AVG (60 mg L^{-1}) resulted in significantly ($P \leq 0.05$) higher levels of mean ascorbic acid (63.3 mg $100ml^{-1}$ FJ) compared to the control (61.0 mg $100ml^{-1}$ FJ) and other treatments in cv. Lane Late. When averaged over all the treatments, the mean ascorbic acid level was significantly ($P \leq 0.05$) increased, when AVG was applied at the golf ball (68.8 mg $100ml^{-1}$ FJ) as compared to its application at the colour break (68.6 mg $100ml^{-1}$ FJ) or at the fruit set stage (66.3 mg$100g^{-1}$ FJ) in cv. Washington Navel during 2011. In 2012, the levels of mean ascorbic acid were significantly

Table 7.4. Effect of different concentrations of AVG sprayed at fruit set, the golf ball or at the colour break stage on SSC in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

SSC (%)								
Washington Navel								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	10.9	11.7 d	11.2	11.8 d	11.4	10.2 e	11.2 b	11.2 c
AVG 20	12.0	12.4 cd	12.2	12.5 bcd	12.2	12.9 abc	12.1 a	12.6 b
AVG 40	12.3	12.9 abc	12.4	12.7 abc	12.4	13.4 ab	12.3 a	13.0 ab
AVG 60	12.4	13.1 abc	12.7	12.8 abc	12.6	13.4 a	12.5 a	13.1 a
Mean (stage)	11.9 a	12.5 a	12.1a	12.4 a	12.1	12.5 a		
LSD ($P \leq 0.05$)								
Treatments			0.3	0.5				
Stage			ns	ns				
Treatments × stages			ns	0.8				
Lane Late								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	11.3	11.6	12.0	11.2 cd	11.8	10.9	11.7 b	11.2 b
AVG 20	12.1	12.1	13.8	12.5 ab	12.6	12.9	12.8 a	12.5 a
AVG 40	12.8	12.9	13.7	12.8 a	13.1	12.9	13.2 a	12.9 a
AVG 60	13.0	12.9	13.8	13.1 a	13.1	13.2	13.3 a	13.1 a
Mean (stage)	12.3 b	12.4 a	13.3 a	12.4 a	12.6 b	12.5 a		
LSD ($P \leq 0.05$)								
Treatments			0.6	0.6				
Stages			0.6	0.5				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

Table 7.5. Effect of different concentrations of AVG sprayed at fruit set, golf ball or colour break stage on the levels of ascorbic acids in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Ascorbic acids (mg $100ml^{-1}$ FJ)								
Washington Navel								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	65.0	62.3	67.2	61.1	66.5	59.4	66.2 b	61.0 b
AVG 20	66.3	63.9	67.9	62.1	68.4	60.7	67.5 ab	62.3 b
AVG 40	66.8	64.0	68.7	64.8	69.5	61.6	68.3 ab	63.5 ab
AVG 60	67.1	64.1	71.3	68.6	70.1	63.5	69.5 a	65.4 a
Mean (stage)	66.3 b	63.6 a	68.8 a	64.2 a	68.6 a	61.3 b		
LSD ($P \leq 0.05$)								
Treatments			2.3	2.7				
Stage			2.0	2.3				
Treatments × stages			ns	ns				
Lane Late								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	61.9	61.2	62.7	59.8	63.4	62.3	62.7 b	61.0 b
AVG 20	66.8	62.2	66.3	62.1	67.4	61.9	66.8 a	61.7 ab
AVG 40	67.8	63.2	67.1	62.9	68.0	62.5	67.6 a	62.5 ab
AVG 60	70.2	61.9	69.1	63.4	68.5	63.4	69.3 a	63.3 a
Mean (stage)	66.7 a	62.1	66.3 a	62.0 a	66.8 a	62.5 a		
LSD ($P \leq 0.05$)								
Treatments			2.6	1.8				
Stages			ns	ns				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

($P \leq 0.05$) increased (64.2 mg $100ml^{-1}FJ$) when AVG was applied at the golf ball as compared to its application at the fruit set stage (63.6 mg $100ml^{-1}$ FJ) or at the colour break (61.3 mg $100ml^{-1}FJ$) in cv. Washington Navel. However, all the AVG treatments did not significantly affect the levels of ascorbic acid in the juice of cv. Lane Late during 2011 and 2012. The interaction between treatments and their stages of application for ascorbic acid was also found to be non-significant ($P \leq 0.05$) in both cultivars during 2011 and 2012.

7.3.8. Total antioxidants

All the AVG treatments increased total antioxidants in cv. Washington Navel and Lane Late sweet orange fruit juice during 2011 and 2012 (Table 7.6). When averaged over different stages of treatment application, the AVG (60 mg L^{-1}) treatment resulted in significantly ($P \leq 0.05$) higher mean total antioxidants compared to the control and all other treatments in cv. Washington Navel and Lane Late during 2011. Meanwhile, all the AVG (20-60 mg L^{-1}) treatments significantly ($P \leq 0.05$) increased the mean total antioxidants compared to the control in both cultivars. When averaged over all the treatments, mean levels of total antioxidants were significantly higher when AVG was applied at the colour break (47.4 mM Trolox $100ml^{-1}FJ$) compared to its application at the golf ball (45.3 mM Trolox $100ml^{-1}FJ$) or at the fruit set stage (45.0 mM Trolox $100ml^{-1}FJ$). However, in cv. Washington Navel, the application of AVG treatments did not significantly ($P \leq 0.05$) affect levels of mean total antioxidants during both years. The interaction between the treatments and their stages of application were found to be non-significant ($P \leq 0.05$) for total antioxidants in both cultivars during 2011 and 2012.

7.3.9. Fructose

The spray application of AVG, when applied at the fruit set, the golf ball or at the colour break stage increased the level of fructose in the fruit juice of sweet orange cv. Washington Navel and Lane Late compared to the control during 2011 and 2012 (Table 7.7). When averaged over different stages of spray application, the treatments of AVG (40-60 mg L^{-1}) resulted in significantly ($P \leq 0.05$) higher mean levels of fructose as compared to the control and treatment of AVG (20 mg L^{-1}) in cv. Washington Navel during 2011. However, all the spray applications of AVG resulted in significantly ($P \leq 0.05$) higher mean fructose levels as compared to the control in cv. Washington Navel during 2012.

Table 7.6. Effect of different concentrations of AVG sprayed at the fruit set, the golf ball or the colour break stage on the levels of total antioxidants in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Total antioxidants (mM Trolox 100ml^{-1} FJ)								
Washington Navel								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	42.1	41.9	42.0	43.7	42.1	42.7	42.0 b	42.8 b
AVG 20	42.9	44.6	42.4	44.0	43.9	46.3	43.1 ab	45.0 a
AVG 40	44.3	44.9	43.1	46.6	44.2	46.6	43.9 ab	45.5 a
AVG 60	45.1	45.3	43.9	45.4	45.2	46.9	44.7 a	45.9 a
Mean (stage)	43.6	44.2	42.9	44.5	43.8	44.5		
LSD ($P \leq 0.05$)								
Treatments			1.9	1.8				
Stage			ns	ns				
Treatments × stages			ns	ns				
Lane Late								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	42.0	42.0	43.0	44.7	43.6	45.5	42.9 b	44.0b
AVG 20	44.1	45.3	43.4	45.4	43.6	47.5	43.7 b	46.1a
AVG 40	45.7	46.2	43.7	45.2	43.9	48.3	44.4ab	46.6 a
AVG 60	46.9	46.4	45.2	46.1	45.7	48.3	45.8 a	46.9 a
Mean (stage)	44.6	45.0 b	43.8	45.3 b	44.2	47.4 a		
LSD ($P \leq 0.05$)								
Treatments			1.9	1.8				
Stages			ns	1.6				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

In cv. Lane Late, a similar trend was observed during 2011. In 2012, the treatments of AVG (42-60 mg L^{-1}) resulted in significantly ($P \leq 0.05$) higher mean levels of fructose as compared to the control and treatment of AVG (40 mg L^{-1}) in cv. Lane Late.

When averaged over all treatments, the mean levels of fructose were significantly higher when AVG was applied at the colour break stage (3.3 g $100ml^{-1}$ FJ) compared to its application at the golf ball (2.8 g $100ml^{-1}$ FJ) or at the fruit set stage (2.1 g $100ml^{-1}$ FJ) in cv. Washington Navel during 2011. In 2012, the mean levels of fructose were significantly ($P \leq 0.05$) higher when AVG was applied at the fruit set (3.3 g $100ml^{-1}$ FJ) compared to its application at the colour break (2.9 g $100ml^{-1}$ FJ) or at the golf ball stage (2.8 g $100ml^{-1}$ FJ) in cv. Washington Navel. Meanwhile, in cv. Lane Late, the mean level of fructose was significantly ($P \leq 0.05$) higher when AVG was applied at the golf ball (3.6 g $100ml^{-1}$ FJ) compared to its application at the colour break (3.0 g $100ml^{-1}$ FJ) or at the fruit set stage (2.9 g $100ml^{-1}$ FJ) during 2011. In 2012, the mean level of fructose was significantly ($P \leq 0.05$) higher when AVG was applied at the colour break (3.9 g $100ml^{-1}$ FJ) compared to its application at the golf ball stage (3.3 g $100ml^{-1}$ FJ) or at the fruit set stage (2.8 g $100ml^{-1}$ FJ) in cv. Lane Late. The interaction between different treatments and their stages of application were found to be non-significant ($P \leq 0.05$) for the levels of fructose in the juice of both cultivars during 2011 and 2012.

7.3.10. Glucose

All the treatments of AVG increased the level of glucose in the fruit juice compared to the control in cv. Washington Navel and Lane Late sweet orange fruit, irrespective of stage of application of AVG treatment in 2011 and 2012 (Table 7.8). When averaged over different stages of spray application, all the treatments of AVG significantly ($P \leq 0.05$) increased the mean level of glucose compared to the control in cv. Washington Navel during 2011. In 2012, the spray application of AVG (60 mg L^{-1}) significantly ($P \leq 0.05$) increased the mean level of glucose compared to the control and all other treatments. In cv. Lane Late, the spray application of AVG (40-60 mg L^{-1}) significantly ($P \leq 0.05$) increased the mean level of glucose as compared to the control and AVG (20 mg L^{-1}) treatment during 2011. In 2012, the spray application of AVG (60 mg L^{-1}) treatment significantly ($P \leq 0.05$) increased the level of glucose compared to the control in cv. Lane Late.

Table 7.7. Effect of different concentrations of AVG sprayed at fruit set, golf ball or colour break stage on the levels of fructose in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Fructose (g 100ml^{-1} FJ)								
Washington Navel								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	2.1	2.9	2.6	2.4	2.8	2.6	2.5 c	2.6 c
AVG 20	2.1	3.2	2.6	2.8	3.1	2.8	2.6 bc	3.0 b
AVG 40	2.1	3.5	2.8	2.9	3.5	2.9	2.8 ab	3.1ab
AVG 60	2.2	3.6	3.0	3.3	3.6	3.1	2.9 a	3.3 a
Mean (stage)	2.1c	3.3 a	2.8 b	2.8 b	3.3 a	2.9 b		
LSD ($P \leq 0.05$)								
Treatments			0.3	0.3				
Stage			0.2	0.3				
Treatments × stages			ns	ns				
Lane Late								
Treat (mg L$^{-1)}$	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	2.7	2.8	3.1	2.7	2.8	3.5	2.9 b	3.0 b
AVG 20	2.8	3.3	3.7	3.2	3.0	3.9	3.2 a	3.5 a
AVG 40	3.1	2.4	3.7	3.5	3.1	4.1	3.3 a	3.3 ab
AVG 60	3.1	2.8	3.7	3.8	3.2	4.1	3.3 a	3.6 a
Mean (stage)	2.9 b	2.8 c	3.6 a	3.3 b	3.0 b	3.9 a		
LSD ($P \leq 0.05$)								
Treatments			0.2	0.4				
Stages			0.2	0.4				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

Table 7.8. Effect of different concentrations of AVG sprayed at fruit set, golf ball or colour break stage on the levels of glucose in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Glucose (g $100ml^{-1}$ FJ)								
Washington Navel								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	1.1	1.4	1.9	1.4	1.7	1.3	1.6 b	1.4 b
AVG 20	1.4	1.6	2.0	1.5	2.2	1.5	1.9 a	1.5 ab
AVG 40	1.4	1.7	2.1	1.5	2.2	1.6	1.9 a	1.6 ab
AVG 60	1.7	1.9	2.2	1.6	2.2	1.8	2.0 a	1.8 a
Mean (stage)	1.4 b	1.7	2.0 a	1.5	2.1 a	1.6		
LSD ($P \leq 0.05$)								
Treatments			0.2	0.2				
Stage			0.2	ns				
Treatments × stages			ns	ns				
Lane Late								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	1.1	1.4	1.8	1.9	1.6	2.0	1.5 c	1.8 b
AVG 20	1.4	1.6	1.9	2.5	1.7	1.5	1.7 b	1.9 b
AVG 40	1.6	2.1	1.9	2.6	1.9	1.7	1.8 ab	2.1ab
AVG 60	1.6	2.2	2.2	2.7	2.0	2.1	1.9 a	2.3a
Mean (stage)	1.4 b	1.8 b	1.9 a	2.4 a	1.8 a	1.9 b		
LSD ($P \leq 0.05$)								
Treatments			0.2	0.4				
Stages			0.2	0.3				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

When averaged over all treatments, the mean levels of glucose in the juice were significantly ($P \leq 0.05$) higher when AVG was applied at the colour break stage (2.1 g100g^{-1} FJ) compared to its application at the golf ball (2.0 g 100ml^{-1} FJ) or at the fruit set stage (1. 4 g 100ml^{-1} FJ) in cv. Washington Navel during 2011 only. In cv. Lane Late, the mean levels of glucose were significantly ($P \leq 0.05$) higher when AVG was sprayed at the golf ball (1.9 g 100ml^{-1} FJ) compared to its application at the colour break (1.8 g 100ml^{-1} FJ) or fruit set stage (1.4 g 100ml^{-1} FJ) during 2011. In 2012, the mean glucose level in the juice was significantly ($P \leq 0.05$) higher with the application of AVG at the golf ball stage (2.4 g100g^{-1} FJ) compared to its application at the colour break (1.9 g 100ml^{-1} FJ) or fruit set stage (1.8 g 100ml^{-1} FJ) in cv. Lane Late. The interaction between treatments and their stages of spray application for glucose was found to be non-significant ($P \leq 0.05$) in both cultivars during 2011 and 2012 except in cv. Lane Late during 2012.

7.3.11. Sucrose

The sucrose level was increased with the exogenous application of AVG at the fruit set, the golf ball or at the colour break stage in cv. Washington Navel and Lane Late during 2011 and 2012 (Table 7.9). When averaged over different stages of spray application, treatments of AVG (60 mg L^{-1}) significantly ($P \leq 0.05$) increased the mean level of sucrose compared to the control and all other treatments in cv. Washington Navel during 2011. Whilst in 2012, all spray applications of AVG significantly ($P \leq 0.05$) increased the mean level of sucrose compared to the control in cv. Washington Navel. In cv. Lane Late, spray application of AVG (40-60 mg L^{-1}) significantly increased the level of sucrose compared to the control and treatment (20 mg L^{-1}). When averaged over all treatments, the mean glucose was significantly ($P \leq 0.05$) higher when AVG was applied at the colour break (5.5 g 100ml^{-1} FJ) compared to its application at the fruit set (5.3 g 100ml^{-1} FJ) or at the golf ball stage (4.5 g 100ml^{-1} FJ) in cv. Washington Navel during 2011 only. In cv. Lane Late, the mean level of glucose was significantly ($P \leq 0.05$) higher when AVG was applied at the fruit set (7.5 g 100ml^{-1} FJ) compared to its application at the colour break (6.7 g 100ml^{-1} FJ) or at the golf ball stage (6.1 g 100ml^{-1} FJ) during 2011. Meanwhile in 2012, the mean level of glucose was significantly ($P \leq 0.05$) higher when AVG was applied at the fruit set (6.4 g 100ml^{-1} FJ) compared to its application at the golf ball (5.6 g 100ml^{-1} FJ) or at the colour break stage (5.3 g 100ml^{-1} FJ). The interaction between treatments and stages of spray application for sucrose was found to be non-significant ($P \leq 0.05$) in cv. Washington Navel and Lane Late in both years.

Table 7.9. Effect of different concentrations of AVG sprayed at fruit set, golf ball or colour break stage on the levels of sucrose in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Sucrose (g 100ml^{-1} FJ)								
Washington Navel								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	4.8	6.1	4.0	5.3	5.0	5.3	4.6 b	5.5 b
AVG 20	5.1	6.1	4.3	5.9	5.6	6.0	5.0 ab	6.0 ab
AVG 40	5.4	6.3	4.8	6.0	5.6	6.2	5.3 ab	6.2 a
AVG 60	5.8	6.5	4.8	6.5	5.9	6.2	5.5 a	6.4 a
Mean (stage)	5.3 a	6.3	4.5 b	5.9	5.5a	5.9		
LSD ($P \leq 0.05$)								
Treatments			0.7	0.4				
Stage			0.8	ns				
Treatments × stages			ns	ns				
Lane Late								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	5.8	5.6	5.6	5.3	6.3	4.6	5.9 c	5.1 c
AVG 20	7.2	5.7	5.7	5.2	6.8c	4.9	6.6 b	5.2 c
AVG 40	8.5	6.8	6.6	5. 5	6.9	5.9	7.3 a	6.1 b
AVG 60	8.6	7.5	6.6	6.7	6.9	6.0	7.4 a	6.8 a
Mean (stage)	7.5 a	6.4 a	6.1 c	5.6 b	6.7 b	5.3 b		
LSD ($P \leq 0.05$)								
Treatments			0.7	0.6				
Stages			0.6	0.6				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

7.3.12. Total sugars

The levels of total sugars were significantly ($P \leq 0.05$) increased with increased concentration of AVG in cv. Washington Navel and Lane Late during both consecutive years (Table 7.10). Averaged over different stages of spray application, the levels of mean total sugars were significantly ($P \leq 0.05$) increased (11.0 and 11.6 g $100ml^{-1}$ FJ) with the application of AVG (60 mg L^{-1}) compared to the control (8.7 and 9.6 g $100ml^{-1}$ FJ) in cv. Washington Navel during 2011 and 2012. A similar trend was observed in cv. Lane Late during 2011. In 2012, the treatment of AVG (40-60 mg L^{-1}) significantly increased the levels of total sugars in cv. Lane Late. When averaged over all treatments, mean total sugars were significantly higher, when AVG was applied at the colour break stage (11.2 g $100ml^{-1}$ FJ) compared to its application at the fruit set stage (9.4 g $100ml^{-1}$ FJ) or at the golf ball stage (9.3 g $100ml^{-1}$ FJ) in cv. Washington Navel during 2011. In 2012, the mean levels of total sugars were significantly higher when AVG was applied at the fruit set (11.2 g $100ml^{-1}$ FJ) compared to its application at the golf ball (10.3 g $100ml^{-1}$ FJ) or at the colour break stage (10.3 g $100ml^{-1}$ FJ) in cv. Washington Navel. However, different stages of spray application of AVG did not affect the levels of total sugars in cv. Lane Late during 2011 and 2012. In general, levels of total sugars were higher in cv. Lane Late than in Washington Navel during both consecutive seasons duing 2011 and 2012. The interaction between treatments and their stages of spray application for total sugars in the juice of both cultivars was found to be non-significant ($P \leq 0.05$) during 2011 and 2012.

7.3.13. Citric acid

Exogenous spray application of AVG, irrespective of stages of application increased the levels of citric acid in cv. Washington Navel and Lane Late during 2011 and 2012 (Table 7.11). Averaged over different stages of AVG application, the treatments of AVG (40-60 mg L^{-1}) significantly ($P \leq 0.05$) increased the levels of mean citric acid in the juice compared to the control and treatment of AVG (20 mg L^{-1}) in cv. Washington Navel during 2011 and 2012. A similar trend was observed in cv. Lane Late during 2011. Meanwhile in 2012, all the treatments of AVG significantly ($P \leq 0.05$) increased the levels of citric acid compared to the control in cv. Lane Late. When averaged over all treatments, the levels of mean citric acid in the juice were significantly ($P \leq 0.05$) increased when AVG was applied at the colour break (1.6 g $100ml^{-1}$ FJ) compared to its application at the fruit set (1.5 g $100ml^{-1}$ FJ) or at the golf ball stage (1.4 g $100ml^{-1}$ FJ) in cv. Washington Navel during 2012 only.

Table 7.10. Effect of different concentrations of AVG sprayed at fruit set, golf ball or colour break stage on the levels of total sugars in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Total sugars (g 100ml^{-1} FJ)								
Washington Navel								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	8.0	10.3	8.4	9.1	9.8	9.4	8.7 c	9.6 c
AVG 20	9.2	11.0	8.9	10.2	11.3	10.0	9.8 b	10.4 b
AVG 40	9.8	11.6	9.8	10.3	11.6	10.6	10.4 ab	10.8 b
AVG 60	10.7	12.0	9.9	11.6	12.2	11.1	11.0 a	11.6 a
Mean (stage)	9.4 b	11.2 a	9.3 b	10.3 b	11.2a	10.3 b		
LSD ($P \leq 0.05$)								
Treatments			0.9	0.6				
Stage			0.8	0.5				
Treatments × stages			ns	ns				
Lane Late								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	9.6	9.8	10.6	9.9	10.7	10.0	10.3 c	9.9 c
AVG 20	11.3	10.5	11.3	10.8	11.6	10.3	11.4 b	10.5 c
AVG 40	13.2	11.4	12.2	11.5	11.7	11.7	12.4 a	11.5 b
AVG 60	13.3	12.5	12.5	13.2	12.0	12.3	12.6 a	12.7 a
Mean (stage)	11.8 a	11.1 a	11.6 a	11.3 a	11.5a	11.1a		
LSD ($P \leq 0.05$)								
Treatments			0.8	1.0				
Stages			ns	ns				
Treatments × stages			ns	0ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

Table 7.11. Effect of different concentrations of AVG sprayed at the fruit set, the golf ball or at the colour break stage on the levels of citric acid in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Citric acids (g $100ml^{-1}$ FJ)								
Washington Navel								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	1.4	1.3	1.4	1.4	1.2	1.3	1.3 b	1.4 c
AVG 20	1.5	1.4	1.5	1.4	1.3	1.5	1.4 ab	1.4 bc
AVG 40	1.6	1.6	1.5	1.4	1.6	1.6	1.6 a	1.5 ab
AVG 60	1.7	1.6	1.5	1.5	1.6	1.8	1.6 a	1.6 a
Mean (stage)	1.5	1.5	1.5	1.4	1.4	1.6		
LSD ($P \leq 0.05$)								
Treatments			0.2	0.1				
Stage			ns	0.1				
Treatments × stages			ns	ns				
Lane Late								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	1.1	1.1 de	1.0	1.1 cde	1.0	1.0 e	1.0 b	1.1 c
AVG 20	1.2	1.2 cd	1.2	1.3 b	1.1	1.2bcd	1.2 a	1.3 b
AVG 40	1.2	1.2bcd	1.2	1.6 a	1.2	1.2bcd	1.2 a	1.4 a
AVG 60	1.3	1.3 bc	1.2	1.2 cde	1.2	1.3 bc	1.2 a	1.2 b
Mean (stage)	1.2	1.2 b	1.2 a	1.3 a	1.1 a	1.2 b		
LSD ($P \leq 0.05$)								
Treatments			0.1	0.1				
Stages			ns	0.1				
Treatments × stages			ns	0.1				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

Meanwhile, in cv. Lane Late, levels of mean citric acid were significantly ($P \leq 0.05$) higher when AVG was applied at the golf ball (1.3 g $100ml^{-1}$ FJ) compared to its application at fruit set (1.2 g $100ml^{-1}$ FJ) or at the colour break stage (1.2 g $100ml^{-1}$ FJ) during 2012 only. The interaction between treatments and their stages of spray application for the levels of citric acid were found to be non-significant ($P \leq 0.05$) in both cultivars during 2011 and 2012, except in cv. Lane Late during 2012.

7.3.14. Malic Acid

All the treatments of AVG at the fruit set, the golf ball or the colour break stage have increased the levels of malic acid in the juice of cv. Washington Navel and Lane Late fruit during 2011 and 2012 (Table 7.12). When averaged over different stages of spray application, the treatment of AVG (60 mg L^{-1}) resulted in significantly ($P \leq 0.05$) higher levels of malic acid (0.3 $g100g^{-1}$ FW) compared to the control (0.3 g $100ml^{-1}$ FJ) and all other treatments in cv. Washington Navel during 2012 only. In cv. Lane Late, spray application of AVG (40-60 mg L^{-1}) significantly ($P \leq 0.05$) increased the mean levels of malic acid compared to the control and AVG (20 mg L^{-1}) treatment during 2011, whilst in 2012, the treatment AVG (60 mg L^{-1}) significantly increased the mean level of malic acid compared to the control and all other treatments of AVG during 2012. When averaged over all the treatments of AVG, the mean levels of malic acid significantly ($P \leq 0.05$) increased when AVG was applied at the fruit set (0.3 g $100ml^{-1}$ FJ) compared to its application at the golf ball (0.3 g $100ml^{-1}$ FJ) or at the colour break stage (0.2 g $100ml^{-1}$ FJ) in cv. Washington Navel during 2011 only. In cv. Lane Late, mean levels of malic acid were significantly ($P \leq 0.05$) higher when AVG was applied at the fruit set (0.6 g $100ml^{-1}$ FJ) compared to its application at colour break (0.4 g $100ml^{-1}$ FJ) or at the golf ball stage (0.4 g $100ml^{-1}$ FJ) during 2011. Meanwhile in 2012, the mean levels of malic acid were significantly ($P \leq 0.05$) higher when AVG was sprayed at the golf ball stage (0.3 g $100ml^{-1}$ FJ) compared to its application at the fruit set (0.3 g $100ml^{-1}$ FJ) or at the colour break stage (0.3 g $100ml^{-1}$ FJ). The interaction between treatments and their stages of spray application for malic acid were found to be non-significant ($P \leq 0.05$) in cv. Washington Navel and Lane Late during 2011 and 2012.

Table 7.12. Effect of different concentrations of AVG sprayed at fruit set, golf ball or colour break stage on the levels of malic acid in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Malic acid ($g100g^{-1}$ FJ)								
Washington Navel								
Treat ($mg\ L^{-1}$)	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	0.3	0.3	0.3	0.3	0.2	0.2	0.3	0.26 b
AVG 20	0.3	0.3	0.3	0.3	0.2	0.3	0.3	0.27 b
AVG 40	0.3	0.3	0.3	0.3	0.2	0.3	0.3	0.27 ab
AVG 60	0.4	0.3	0.3	0.3	0.2	0.3	0.3	0.30 a
Mean (stage)	0.3 a	0.3	0.3 a	0.3	0.2 b	0.3		
LSD ($P \leq 0.05$)								
Treatments			ns	0.03				
Stage			0.04	ns				
Treatments × stages			ns	ns				
Lane Late								
Treat ($mg\ L^{-1}$)	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	0.5	0.3	0.3	0.3	0.3	0.3	0.4 b	0.3 bc
AVG 20	0.6	0.3	0.3	0.3	0.3	0.3	0.4 ab	0.3 c
AVG 40	0.6	0.3	0.3	0.3	0.4	0.3	0. 5 a	0.3 b
AVG 60	0.6	0.3	0.4	0.4	0.4	0.3	0.5 a	0.4 a
Mean (stage)	0.6 a	0.3 b	0.4	0.3 a	0.4 b	0.3 b		
LSD ($P \leq 0.05$)								
Treatments			0.1	0.03				
Stages			0.1	0.02				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

7.3.15. Succinic acid

Exogenous spray applications of AVG at all three stages increased the level of succinic acid in the juice of cv. Washington Navel and Lane Late sweet orange fruit in both consecutive years (Table 7.13). When averaged over different stages of spray application, the treatment of AVG (60 mg L^{-1}) resulted significantly ($P \leq 0.05$) in the highest level of succinic acid (0.3 and 0.3 g.$100g^{-1}$ FW) as compared to the control (0.2 and 0.2 g $100ml^{-1}$ FJ) and all other treatments in cv. Washington Navel during 2011 and 2012. Meanwhile, in cv. Lane Late, spray application of AVG (40-60 mg L^{-1}) resulted in significantly higher mean succinic acid compared to the control and AVG (20 mg L^{-1}) treatment during 2012 only.

All spray applications of AVG significantly ($P \leq 0.05$) increased mean levels of succinic acid when applied at the fruit set (0.3 mg $100ml^{-1}$FJ) compared to its application at the golf ball (0.2 g $100ml^{-1}$FJ) or at the colour break stage (0.2 g $100ml^{-1}$FJ) in cv. Washington Navel 2011. Meanwhile in 2012, the mean level of succinic acid was significantly ($P \leq 0.05$) higher when AVG was applied at the golf ball stage (0.3 g $100ml^{-1}$FJ) compared to its application at the colour break stage (0.3 g $100ml^{-1}$FJ) or at the fruit set stage (0.2 g $100ml^{-1}$FJ) in cv. Washington Navel. In cv. Lane Late, mean levels of succinic acid were significantly ($P \leq 0.05$) higher when AVG was sprayed at the fruit set (0.3 and 0.4 g $100ml^{-1}$FJ) compared to its application at the golf ball (0.2 and 0.4 g $100ml^{-1}$FJ) or at the colour break stage (0.1 and 0.3 g $100ml^{-1}$FJ) during 2011 and 2012. The interactions between different treatments of AVG and different stages of spray application were found to be significant ($P \leq 0.05$) in cv. Lane Late during 2012 only, whilst non-significant ($P \leq 0.05$) effect was observed in cv. Washington Navel during both years.

7.4. Discussion

A substantial reduction in creasing incidence was recorded with exogenous application of ethylene inhibitor AVG at the fruit set, the golf ball or at the colour break stage in sweet orange cv. Washington Navel and Lane Late during 2011 and 2012. A spray application of AVG (60 mg L^{-1}) was more effective at the golf ball stage (27.9 and 24.3 %) compared to control (52.1 and 51.5 %) and all other treatments in cv. Washington Navel during 2011 and 2012 respectively. Similarly, in cv. Lane Late, a spray application of AVG (60 mg L^{-1}) was more effective at the colour break (22.9%) or fruit set stage (29.3%) compared to the control (51.4 and 59.3 %) and all other treatments during 2011 and 2012 (Figure7.1).

Table 7.13. Effect of different concentrations of AVG sprayed at fruit set, golf ball or colour break stage on the levels of succinic acid in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Succinic acid (g 100ml^{-1} FJ)								
Washington Navel								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	0.3	0.2	0.2	0.3	0.2	0.3	0.2 b	0.2 b
AVG 20	0.3	0.2	0.2	0.3	0.2	0.3	0.3 ab	0.3 b
AVG 40	0.3	0.2	0.2	0.3	0.2	0.3	0.3 ab	0.3 b
AVG 60	0.4	0.3	0.3	0.3	0.2	0.3	0.3 a	0.3 a
Mean (stage)	0.3 a	0.2 b	0.2 b	0.3 a	0.2 b	0.3 a		
LSD ($P \leq 0.05$)								
Treatments			0.03	0.02				
Stage			0.03	0.02				
Treatments × stages			ns	ns				
Lane Late								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	0.2	0.3 ef	0.2	0.3 cdef	0.1	0.3 gh	0.15 b	0.3 c
AVG 20	0.3	0.3 cdef	0.2	0.3 h	0.1	0.3 fg	0.17 ab	0.3 c
AVG 40	0.3	0.4bcd	0.2	0.4 bc	0.1	0.3 def	0.18 ab	0.4 b
AVG 60	0.3	0.4 ab	0.2	0.4 a	0.1	0.4 bcd	0.20 a	0.4 a
Mean (stage)	0.3 a	0.4 a	0.2 b	0.4 a	0.1 c	0.31 b		
LSD ($P \leq 0.05$)								
Treatments			ns	0.02				
Stages			0.03	0.02				
Treatments × stages			ns	0.04				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

Higher levels of endogenous ethylene are involved in creasing of sweet oranges cv. Valencia and Washington Navel (Monselise et al., 1976; Pham, 2009), due to increased ACC synthase and ACC oxidase activity (Hyodo and Nishino, 1981). Likewise recently, higher levels of endogenous ethylene in creased fruit than normal fruit among different cultivars of sweet orange including Navelina, Washington Navel, Lane Late and Valencia Late have been reported (Chapter 4). Hyodo (1977) reported ethylene biosynthesis in the albedo tissue of Satsuma mandarin. AVG is an inhibitor of ethylene biosynthesis, which suppresses the production of ethylene in plant tissues by inhibiting ACC synthase (Ladaniya, 2007; Even-Chen et al., 1982; Hyodo and Nishino, 1981; Yu and Yang, 1980; Boller et al., 1979; Yu et al., 1979). Similarly, substantial reduction in creasing with the exogenous application of putrescine, another ethylene inhibitor in sweet orange cv. Washington Navel and Lane Late also supports the involvement of ethylene in creasing (Chapter 6). Possibly, the reduction in ethylene production with the application of AVG may have reduced the activities of cell wall degrading enzymes such as PE, *exo*-PG, *endo*-PG, and EGase in the albedo tissues of the fruit consequently lessening the creasing incidence. In our previous study, the exogenous application of ethylene inhibitors such as putrescine, AVG or cobalt sulphate significantly reduced the activities of PE, *exo*-PG, *endo*-PG, and EGase as well as water soluble pectin in the albedo and the flavedo tissues of the fruit compared to the control in sweet orange (Chapter 9). Creasing is associated with higher activities of cell wall degrading enzymes such as PE, *exo*-PG, *endo*-PG, and EGase in the albedo tissues of the creased fruit. Saleem et al. (2014) and Li et al. (2009) ascribed that creasing has a linear correlation between the activities of cell wall degrading enzymes and cellulose in different cultivars of sweet orange.

All AVG treatments increased rind thickness as compared to the control when AVG was applied at the fruit set, the golf ball or at the colour break stage in Washington Navel and Lane Late orange during both years (Table 7.2) consequently reducing the incidence of creasing. Earlier reports claimed that sweet orange fruit with thinner rind were more prone to creasing than those with a thicker rind (Ali et al., 2000; Bevington et al., 1993; Jones et al., 1967).

The exogenous application of AVG (20-60 mg L^{-1}) increased the fruit firmness, rind hardness and rind tensile strength compared to the control, irrespective of its application at the fruit set, the golf ball or at the colour break stage in both cultivars during 2011 and 2012 (Figure 7.2 A-F and 7.3 A-F). The AVG inhibited ethylene emission and retained flesh firmness, rind hardness and rind tensile strength force while reducing S-adenos-ylmethionine

decarboxylase (SAMDC) activity (Bregoli, et al., 2002). AVG is a strong inhibitor of ethylene production and is used to reduce free to conjugate ratio of endogenous ethylene and transiently altered (SAMDC) activity, which is used to improve the growth, diameter, fruit weight and SSC (Byers et al., 2005; Greene, 2005; Bregoli et al., 2002).

The exogenous application of AVG (60 mg L^{-1}) at the fruit set, golf ball or colour break stage has significantly increased fruit weight, diameter and rind thickness compared to the control in cv. Washington Navel and Lane Late (Table 7.1-7.3). The spray application of AVG delayed fruit maturity on the tree and delayed harvest due to decreased ethylene production and respiration rate, while increasing fruit weight, diameter and rind thickness (Cetinbas and Koyuncu, 2011). Similarly, Byers (1997) stated that loss of fruit firmness and loss in ethylene emission were directly proportional to AVG concentration. Similarly, AVG application resulted in delayed maturity in various fruit crops such as peaches (Kim et al., 2004; Rath et al., 2004; Kluge and Jacomino, 2002; Bregoli et al., 2002), nectarine (Rath and Prentice, 2004), apples (Greene, 2005) and tomatoes (Jeong et al., 2002). Recently, Al-Husseini (2012) reported that AVG significantly reduced fruit drop and improved fruit weight, diameter and rind thickness in sweet oranges cv. Washington Navel and Lane Late. All the spray applications of AVG increased SSC, glucose levels, fructose, sucrose and total sugars in the fruit juice as well as increased citric, malic and succinic acid in both cultivars during 2011 and 2012. This may be ascribed to reduced leaf abscission and consequently higher supply of photosynthates into the fruit. Similarly, spray application of AVG improved the SSC in sweet orange (Al-Husseini, 2012), apple (Wargo et al., 2004; Greene, 2005) and peach (Singh, 2003; Rath et al., 2004). The reduction of leaf abscission in sweet orange cv. Blood Red with the exogenous application of polyamines and nitrogen resulted in improved SCC and other quality parameters (Saleem et al., 2007). In conclusion**,** the reduction of creasing in sweet orange fruit with the exogenous application of AVG can be attributed to the decrease in endogenous ethylene production whereby improving fruit firmness, rind hardness, rind tensile strength, fruit weight and diameter, ascorbic acid, total antioxidants, individual and total sugars as well as organic acid as compared to the control.

CHAPTER 8

Effects of ethylene inhibitor cobalt sulfate on incidence of creasing and fruit quality in sweet orange cv. Washington Navel and Lane Late

Summary

Creasing is a physiological rind disorder detectable at the fruit maturity or colour break stage. with Different concentrations (0,125, 250 and 500 mg L^{-1}) of cobalt sulphate ($CoSO_4$) were applied at the fruit set, the golf ball or at the colour break stage to control creasing and to improve rheological properties of fruit and rind as well as quality parameters of sweet oranges cv. Washington Navel and Lane Late during 2011 and 2012. Thirty-five ripe fruit per tree were harvested randomly to determine the incidence of creasing, rheological properties of fruit and rind as well as fruit quality. Creasing incidence reduced considerably when $CoSO_4$ (500 mg L^{-1}) treatment was applied at the golf ball stage (29.3 and 34.3 %) compared to the control (51.3 and 57.1 %) in cv. Washington Navel during 2011 and 2012. A similar trend was observed in cv. Lane Late during 2011 and 2012. The treatment of $CoSO_4$ (500 mg L^{-1}) was more effective when applied at the fruit set stage to improve the rind tensile strength (47.7 and 64.1 N), fruit diameter (83.5 and 83.0 mm), the levels of sucrose (8.2 and 9.7 g $100ml^{-1}$FJ) and total sugars (13.1 and 13.5 g $100ml^{-1}$ FJ) in both cultivars, respectively. Similarly, $CoSO_4$ (500 mg L^{-1}) was more effective when applied at the golf ball stage to increase the SSC, (12.3 and 13.5 %) and the levels of ascorbic acid (69.3 and 69.1 mg $100ml^{-1}$ FJ) in the juice of both cultivars. Whilst, the fruit weight (292.9 g), the levels of fructose (3.7 g $100ml^{-1}$ FJ) and glucose (2.7 g $100ml^{-1}$ FJ) were higher when $CoSO_4$ (500 mg L^{-1}) was applied at the colour break stage in cv. Washington Navel. Similarly, the rind hardness (25.8 N) and rind thickness (5.1 mm) were higher at the colour break stage in cv. Lane Late. In conclusion, exogenous applications of $CoSO_4$ substantially reduce the creasing incidence; improve rheological properties of fruit and rind as well as fruit quality in cv. Washington Navel and Lane Late sweet orange fruit.

8.1. Introduction

Creasing is a peel-related disorder in sweet orange fruit (Davies and Albrego, 1994; Treeby et al., 1996). Citrus fruit growth and development is a complex process. Cell division of albedo tissue ceases 8-9 weeks after full bloom and subsequent growth is due to cell expansion, while cell division in the flavedo continues until fruit maturity (Bain, 1958). Creasing is a physiological disorder which is usually visible at fruit maturity (Gambetta et al., 2000; Jones et al., 1976; Jona et al., 1989) or at the colour break stage (Storey et al., 2002). The incidence of creasing enhanced with the advancement of fruit maturity (Embleton et al., 1973). The visual symptoms of creasing are exhibited as cracks on the internal white tissue of the albedo (Jones et al., 1967; Monselise; 1976; Treeby e al., 2000) and irregular grooves or creases on the surface of the flavedo (Treeby et al., 1996; Davies and Albrego, 1994; Jona et al., 1989). The severity of creasing varies from year to year, orchard to orchard and specie to specie (Gilfillan et al., 1981). It was estimated by Treeby and Storey (1994) that a 1% decrease in creasing of sweet orange will save 1 to 2 million AU$ to Australian growers. In the Australian citrus industry, up to 90% of sweet orange fruit is affected due to this physiological disorder, however, more than 50% sweet orange crop affected is from South Africa (Goldie, 1998; Gilfillan et al., 1981) and 68% in Israel (Greenberg et al., 1996). Creasing is a physiological disorder and its development is associated with climatic, cultural and nutritional status (Monselise et al., 1976), but its physiology is poorly understood and different techniques have been tested to overcome this physiological disorder such as deficient irrigation (Pham, 2009; Treeby et al., 2007), application of gibberellic acid (Greenberg et al., 2006; Jona et al., 1989; Monselise et al., 1976), potassium sulphate as well as phosphorous (Bevington et al., 1993; Jones et al., 1967), boron (Pham, 2009) and calcium (Pham et al., 2012; Storey et al., 2002).

Citrus is a non-climacteric fruit which produces only small amounts of endogenous ethylene. Ethylene production does not occur when mature fruit have been removed from the tree (Abeles, 1973). The endogenous level of ethylene is produced at a higher rate when young fruit are cut into small pieces. Albedo tissues produce ethylene vigorously, even though the tissue is taken from matured fruit (Hyodo, 1977). Exogenous applications of ethylene on citrus play an important role in physiological and biochemical changes (Alonso et al., 1995; Trebitsh et al., 1993; Riov et al., 1969). The exogenous application of ethylene on citrus fruit also increased respiration (Eaks et al., 1970), induced abscission (Goren et al., 1992), loss of chlorophyll (Eilati and Goldschmidt, 1969) and degreening (Grierson et al..,

1986). Cobalt plays an important role in plant metabolism such as stem coleoptile elongation; hypocotyl opening and leaf and bud development (Howell and Skoog, 1975). Cobalt is an inhibitor of ethylene production; inhibits basal ethylene production (Oetiker and Yang, 1995), is involved in the conversion of S-adenosylmethionine (SAM) to 1-aminocyclopropane-l-carboxylic acid (ACC) and eliminates the increase in ACC formation and ethylene production through the action of the ACC oxidase enzyme on aged peel of Shamouti orange fruit (Even-Chen et al., 1982). Similarly, the exogenous application of $CoSO_4$ improves fruit set, retention, yield, weight, size, firmness, SSC and total sugars in mango (Wahdan, 2011; Singh and Agrez, 2002; Singh et al., 1994; Singh and Singh, 1993), pomegranate (Reddy et al., 2011), olive (Gad et al., 2006), peach (Kim et al., 2004), apple (Benincore et al., 2000) and Figure (Teragishi et al., 2000). Similarly, Saleem et al. (2008) reported the reduction of leaf abscission in sweet orange cv. Blood Red with the exogenous application of polyamines, an ethylene inhibitor, whereby improving the SCC, total sugars and other quality parameters. Recently, Al-Husseini (2012) reported that $CoSO_4$ application significantly reduced the fruit drop and increased the fruit weight, diameter, SSC, TA as well SSC: TA ratio in sweet orange cv. Washington Navel and Lane Late.

The endogenous level of ethylene was higher in the albedo tissues of creased fruit than in the normal fruit of sweet orange cv. Washington Navel and Valencia (Pham, 2009; Monselise et al., 1976). Recently, we observed higher levels of endogenous ethylene in creased fruit than in normal fruit in different cultivars of sweet orange including Navelina, Washington Navel, Lane Late and Valencia indicating the key role of endogenous ethylene in creasing (Chapter 6). I also observed (Chapter 9) that the exogenous application of ethylene inhibitors such as AVG, $CoSO_4$ or PUT at different fruit developmental stages significantly reduced the incidence of creasing in sweet orange through modifying the activities of cell wall degrading enzymes such as PE, *exo*-PG, *endo*-PG, and EGase in the albedo and flavedo tissues of the fruit. The aminoethoxyvinylglycine (AVG) and (aminoxy) acetic acid are known inhibitors of ethylene biosynthesis, though are costly and their availability and the large size of sweet orange trees further limit their use in regulating ethylene biosynthesis (Singh and Singh, 1993; Adams and Yang, 1979; Boller et al., 1979; Yu et al., 1979).

Although ethylene has a key role in creasing of sweet orange, the available information is sporadic and inconclusive. However, no research work has been reported on the efficacy of exogenous application of $CoSO_4$ on creasing, rheological properties of fruit and rind as well as fruit quality in sweet orange. Therefore, it was surmised that $CoSO_4$, an

inhibitor of ethylene biosynthesis, will reduce incidence of creasing and improve the rheological properties of fruit and rind as well as fruit quality parameters. In the present study, we investigated the role of $CoSO_4$ applied at the fruit set, the golf ball or at the colour break stage on creasing, rheological properties of the fruit and rind as well as fruit quality in different cultivars of sweet orange including cv. Washington Navel and Lane Late.

8.2. Materials and methods

8.2.1. Plant material

Two different experiments were conducted in two consecutive seasons (2010-11 and 2011-12) on Washington Navel and Lane Late sweet orange cultivars located at a commercial orchard in Gingin (latitude 31° 21′ South, longitude 155° 55′ East), Western Australia. The soil is sandy loam and climate is characterised by a mild, wet winter and hot, dry summer. All the experimental trees received similar cultural practices including fertilisers, irrigation, weed control and plant protection except for experimental treatments. Twenty-five year old uniform sweet orange plants grafted on trifoliate orange rootstock (*Poncirus trifoliate* Raf.) were used in these experiments. The trees were spaced 7.5 m between rows and 2.7 m within rows from North-South row direction.

8.2.2. Experiment I: Effects of exogenous application of different concentrations of $CoSO_4$ applied at the fruit set, the golf ball or at the colour break stage on incidence of creasing, rheological properties of fruit and rind as well as fruit quality of sweet orange cv. Washington Navel

Aqueous solutions containing different concentrations of $CoSO_4$ (125, 250 and 500 mg L^{-1}, respectively) with 0.05% 'Tween 20' as a surfactant were sprayed on to whole trees until run off at the fruit set (Fruit diameter: 15±5 mm), the golf ball (Fruit diameter 40±5 mm) or at the colour break stage (Fruit diameter: 80±5 mm) of sweet orange in cv. Washington Navel by using a sprayer (The Selecta Trolleypak Mk II, Acacia Ridge, Australia). Control trees were kept unsprayed. The experiment was laid out by following a two-factor (treatments and stage of spray application) factorial completely randomised block with four replications. Single trees were classed as an experimental unit. The experiment was repeated in two consecutive

seasons (2010-11 and 2011-12). The data of two years were not pooled because error mean squares over years were heterogeneous. Thirty-five ripe fruit per tree were randomly harvested around the tree canopy. The incidence of creasing was examined on individual fruit, based on the appearance of symptoms on the fruit surface and rheological properties of rind such as fruit firmness, rind hardness and rind tensile strength. Fruit weight, diameter, and rind thickness were measured in both consecutive seasons. The chemical characteristics of fruit, such as soluble solids concentrations (SSC), titratable acidity (TA), SSC: TA ratio, ascorbic acid concentration, total antioxidants, individual and total sugars in the juice were determined in both years.

8.2.3. Experiment II. Effects of exogenous application of different concentrations of $CoSO_4$ applied at the fruit set, the golf ball or at the colour break stage on incidence of creasing, rheological properties of fruit and rind as well as fruit quality of sweet orange cv. Lane Late

The same experiment was repeated employing the same $CoSO_4$ treatments and stages of spray application following a similar experimental design on cv. Lane Late during 2010-11 and 2011-12. The incidence of creasing was examined on individual fruit, based on the appearance of symptoms on the fruit surface and rheological properties of rind. The fruit weight, diameter, rind thickness, SSC, TA, SSC: TA ratio, ascorbic acid concentration, total antioxidants, individual and total sugars in the juice was determined in both year 2010-11 and 2011-12.

8.2.4. Creasing (%)

The incidence of creasing (%) was recorded on individual fruit, based on the appearance of symptoms on the fruit surface as described in Chapter 3, Section 3.3.1.

8.2.5. Rheological properties of fruit rind

The effect of AVG on rheological properties of the fruit and rind were determined using a textural analyzer (TA Plus, AMETEK Lloyd instruments Ltd., Hampshire, UK) as detailed in Chapter 3, Section 3.4. The data were collected and processed using a Nexygen® 4.6 software.

8.2.5.1. Fruit firmness

The fruit firmness (N) was determined with the help of a compression test by using a textural analyzer (TA Plus, AMETEK Lloyd instruments Ltd., Hampshire, UK) as outlined in Chapter 3, Section 3.4.1.

8.2.5.2. Rind hardness

The sweet orange fruit rind was cut to a 2.5 cm width having 6.0 mm thickness by using a slicer (Zyliss Easy slice, folding Mandoline slicer, Swiss) to give uniform sections for the determination of rind hardness as reported in Chapter 3, Section 3.4.2.

8.2.5.3. Rind tensile strength

The sweet orange fruit rind was carefully removed in the size 2.5 x 5 cm area having 6 mm thickness to measure the rind tensile strength as detailed in Chapter 3, Section 3.4.3. The rind tensile strength was calculated at the maximum load and limit points, where the rind deflection occurred at 10 mm.

8.2.6. Fruit weight, diameter and rind thickness

The fruit weight was calculated by weighing ten randomly selected fruit per replication using a digital electrical balance (A&D Limited, Tokyo, Japan) and average weight was calculated as gram (g) per fruit. The fruit diameter and rind thickness of the same fruit were also measured with a digital Vernier caliper and expressed in mm as detailed in Chapter 3, Section 3.5.

8.2.7. Determination of soluble solids concentration (SSC)

The juice was extracted from ten freshly harvested sweet orange fruit to determine SCC by using a digital refractometer (Atago-Palette PR 101, Atago CO. Ltd, Itabashi-Ku, Tokyo, Japan) and expressed in (%) as described in Chapter 3, Section 3.6.

8.2.8. Determination of ascorbic acid concentrations

Ascorbic acid concentrations in the juice were determined following the method of Malik and Singh (2005) and Pham (2009) with some modifications detailed in Chapter 3, Section 3.9. Ascorbic acid concentration was calculated by using a standard curve of L-ascorbic acid and expressed as mg ascorbic acid per $100ml^{-1}$ fresh juice.

8.2.9. Determination of total antioxidants

The total antioxidants were determined from the freshly extracted juice of sweet orange by using the modified method of Pham (2009) as previously reported in Chapter 3, Section 3.10. The levels of total antioxidants were measured at 515 nm by a 6405 UV/VIS Spectrophotometer (Jenway 6405, Dunmow, Essex, UK) and expressed as mM Trolox per 100ml fresh juice.

8.2.10. Determination of individual and total sugars as well as organic acids

The individual sugars and total sugars as well as organic acids were determined from the fresh juice of sweet orange fruit by using a RP-HPLC system (Waters, Milford, MA, USA) as reported in Chapter 3, Section 3.11. The concentration of individual, total sugar as well as individual organic acids was expressed as g 100 ml^{-1} fresh juice.

8.2.11. Statistical analysis

The experimental data were subjected to a two–way analysis of variance (ANOVA) using GenStat 14th edition (VSN International Ltd., Hemel Hempstead, UK). The least significant differences (LSD) were calculated following a significant F–test at ($P \leq 0.05$). The effects of all the treatments of $CoSO_4$ and stage of spray application on different parameters were assessed within ANOVA. All the assumptions of ANOVA were checked to ensure the validity of statistical analysis.

8.3. Results

8.3.1. Creasing (%)

All the treatments of $CoSO_4$ substantially decreased the incidence of creasing (%) depending on its concentration and stages of its spray application in sweet orange cv. Washington Navel and Lane Late during two consecutive seasons, 2011 and 2012 (Figure 8.1). When averaged over different stages of $CoSO_4$ spray applications, all the treatments of $CoSO_4$ significantly ($P \leq 0.05$) reduced the mean creasing incidence compared to the control in sweet orange cv. Washington Navel during 2011 and 2012. In cv. Lane Late, the treatment of $CoSO_4$ (500 mg L^{-1}) significantly reduced creasing compared to the control and all other treatments of $CoSO_4$ during 2011. In 2012, all the treatments of $CoSO_4$ significantly reduced the incidence of

creasing compared to the control in cv. Lane Late. In general, the spray application of $CoSO_4$ (500 mg L^{-1}) was more effective in reducing creasing when applied at the golf ball stage (22.9%) compared to its application at the colour break stage (27.1%) or at the fruit set (33.6%) in cv. Washington Navel during 2011 only. In cv. Lane Late, a substantial reduction in creasing was observed when the spray application of $CoSO_4$ (500 mg L^{-1}) was applied at the golf ball (40.0 and 28.6%) compared to its application at the colour break (42.9 and 38.6%) or at the fruit set stage (49.3 and 35.0%) during 2011 and 2012, respectively. The interaction between treatments and their stages of application for creasing was found to be non-significant ($P \leq 0.05$) in both cultivars during 2011 and 2012, except in cv. Lane Late during 2011.

8.3.2. Rheological properties of fruit and rind

8.3.2.1. Fruit firmness

The exogenous spray application of $CoSO_4$ increased the fruit firmness compared to the control in cv. Washington Navel and Lane Late (Figure 8.2 A-D and 8.3 A-D). When averaged over different stages of spray application, the treatment of $CoSO_4$ (500 mg L^{-1}) resulted significantly ($P \leq 0.05$) in the highest fruit firmness compared to the control and all other treatments during 2012 only. In cv. Lane Late, all the treatments of $CoSO_4$ significantly ($P \leq 0.05$) increased the mean fruit firmness compared to the control during 2011 only. All the $CoSO_4$ treatments significantly ($P \leq 0.05$) increased the mean fruit firmness when applied at the fruit set (316.1 N) compared to its application at the colour break (294.5 N) or its application at the golf ball stage (269.4 N) in cv. Washington Navel during 2011 only and non-significant effect was observed during 2012. In cv. Lane Late, the mean fruit firmness was significantly ($P \leq 0.05$) higher when $CoSO_4$ was applied at the colour break (310.4 N) followed by the fruit set (287.6 N) or at the golf ball stage (298.7 N) during 2012. The interaction between treatments and their stages of application were found to be non-significant ($P \leq 0.05$) for fruit firmness in both the cultivars during 2011 and 2012.

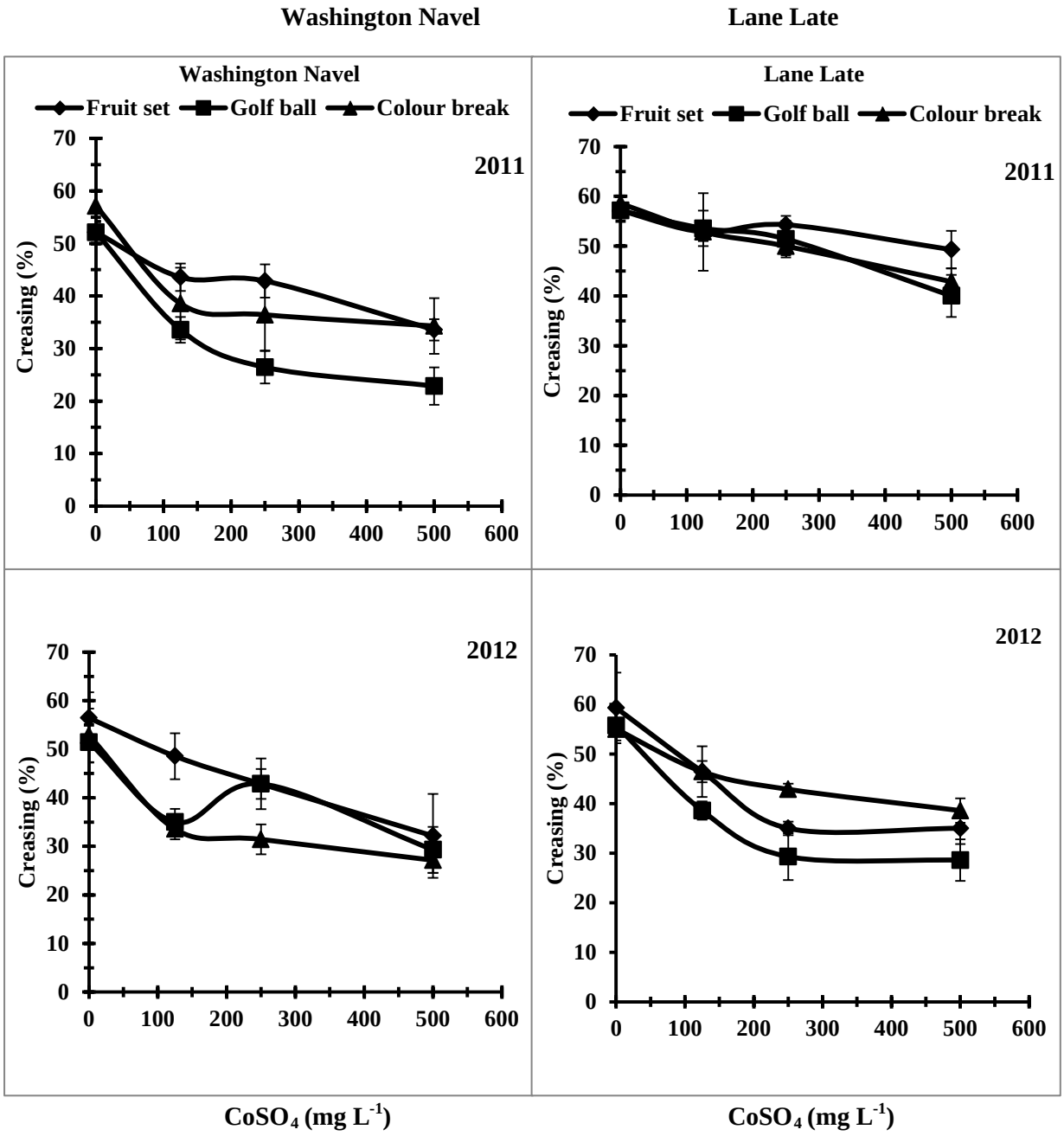

Figure 8.1. Effect of different concentrations of $CoSO_4$ at the fruit set, the golf ball or at the colour break stage on creasing (%) in cv. Washington Navel and Lane Late sweet orange fruit during 2011 and 2012. n = 4 replications (35 fruit per replication). Vertical bars represent standard error means. LSD ($P \leq 0.05$) for Washington Navel 2011 Treatments = 5.1, Stage = 4.5, Treatments x stage = ns; Washington Navel 2012 Treatments = 9.1, Stage = ns, treatments x stage = ns; LSD ($P \leq 0.05$) for Lane Late 2011, Treatments = 6.2, Stage = 5.3, Treatments x stage = 10.7; ($P \leq 0.05$) for Lane Late 2012 Treatments = 5.8, Stage = 5.1, Treatments x stage = ns; ns = not-significant.

8.3.2.2. Rind hardness

The rind hardness (N) increased with the increase in the concentrations of $CoSO_4$ compared to the control in sweet orange cv. Washington Navel and Lane Late (Figure 8.2 B-E and 8.3 B-E). Averaged over different stages of $CoSO_4$ application, all the spray applications of $CoSO_4$ significantly increased the mean rind tensile strength compared to the control in cv. Washington Navel during 2011. In 2012, the spray application of $CoSO_4$ (250-500 mg L^{-1}) resulted in significantly ($P \leq 0.05$) higher rind hardness compared to the control and treatment of $CoSO_4$ (125 mg L^{-1}) in cv. Washington Navel. In cv. Lane Late, the spray applications of $CoSO_4$ (250-500 mg L^{-1}) resulted in significantly ($P \leq 0.05$) higher rind hardness compared to the control and treatment of $CoSO_4$ (125 mg L^{-1}) during 2011 and 2012. When averaged over all the treatments of $CoSO_4$, the mean rind hardness was significantly ($P \leq 0.05$) higher when $CoSO_4$ was applied at the fruit set (22.0 N) compared to its application at the golf ball stage (21.6 N) or at the colour break stage (19.8 N) in cv. Washington Navel during 2011. In 2012, the mean rind hardness was significantly ($P \leq 0.05$) higher, when $CoSO_4$ was applied at the golf ball stage (24.4 N), compared to its application at the colour break stage (22.9 N) or at the fruit set stage (21.9 N) in cv. Washington Navel during 2012. In cv. Lane Late, the mean rind thickness was significantly ($P \leq 0.05$) higher at the colour break stage (25.7 N) compared to its application at the golf ball stage (23.3 N) or at the fruit set stage (21.9 N) during 2011. The stages of application of $CoSO_4$ did not significantly ($P \leq 0.05$) of $CoSO_4$ affect rind hardness in cv. Lane Late during 2012. The interactions between treatments and different stages of spray application for rind hardness were found to be non-significant ($P \leq 0.05$) in cv. Washington Navel during 2011 only and in cv. Lane Late during 2012 only.

8.3.2.3. Rind tensile strength

The rind tensile strength increased with increased concentrations of $CoSO_4$ compared to the control, irrespective of stage of its spray application in sweet orange cv. Washington Navel and Lane Late during 2011 and 2012 (Figure 8.2 C-F and 8.3 C-F). Spray applications of $CoSO_4$ (250-500 mg L^{-1}) significantly increased the mean rind tensile strength compared to the control and the treatment of $CoSO_4$ (125 mg L^{-1}) in cv. Washington Navel during 2011 and 2012. In cv. Lane Late, the spray applications of $CoSO_4$ (250-500 mg L^{-1}) significantly increased the mean rind tensile strength compared to the control and the treatment of $CoSO_4$ (125 mg L^{-1}) during 2011.

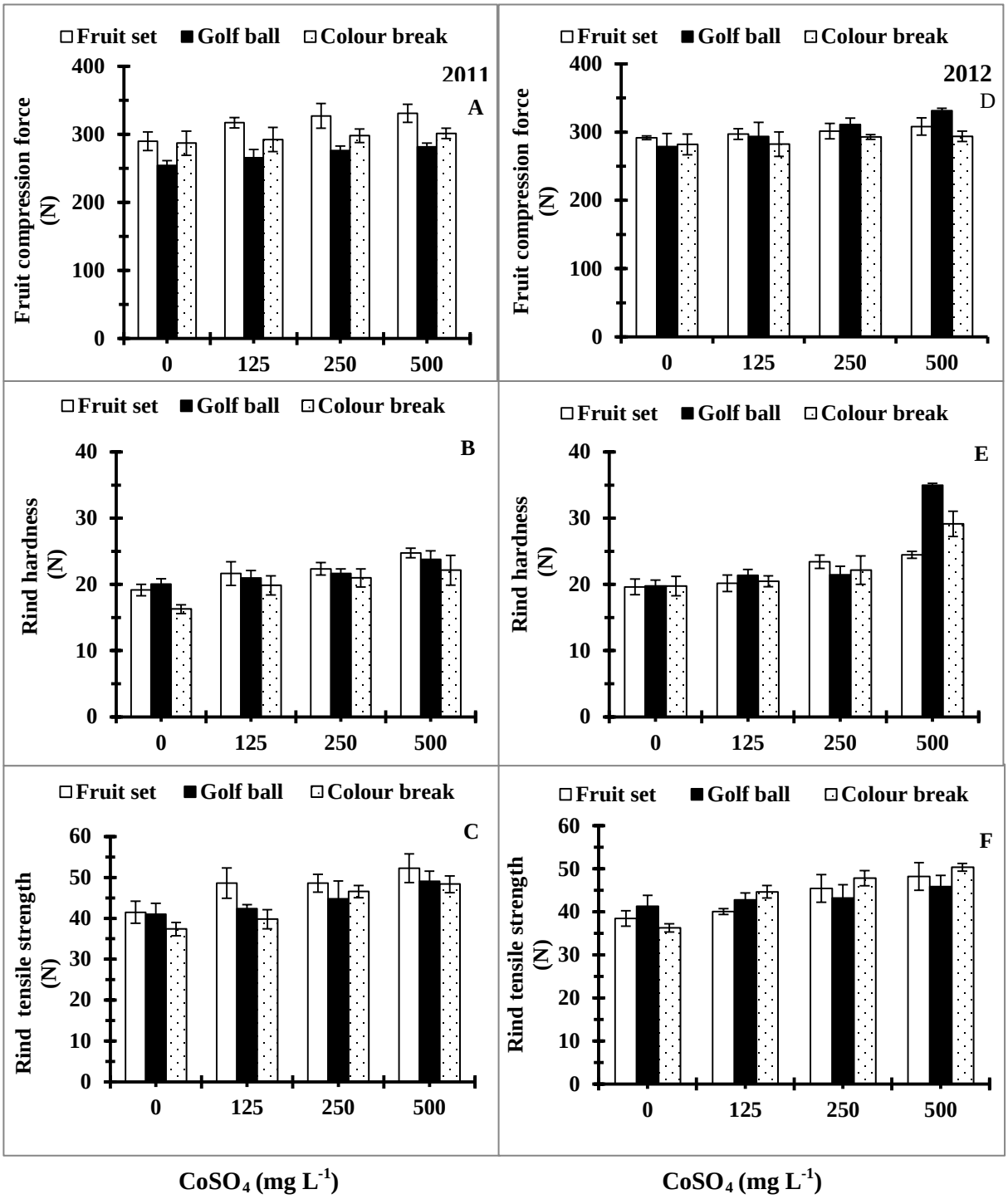

Figure 8.2. Effect of different concentrations of $CoSO_4$ applied at the fruit set, the golf ball or at the colour break on rheological properties of fruit and rind in sweet orange cv. on Washington Navel fruit during 2011 and 2012. n = 4 replications (5 fruit per replication). Vertical bars represent standard error means. LSD ($P \leq 0.05$) for fruit firmness in 2011, Treatments = 21.3, Stage = 18.4, Treatments x stage = ns; for rind hardness, Treatments = 2.2, Stage = 1.9, treatments x stage = ns; for rind tensile strength, Treatments = 4.7, Stage = ns, Treatments x stage = ns; ($P \leq 0.05$) for year 2012; fruit firmness, Treatments = 21.1, Stage = ns, Treatments x stage = ns; for rind hardness, Treatments = 2.0, Stage = 1.8, treatments x stage = 3.5; for rind tensile strength, Treatments = 4.3, Stage = ns, Treatments x stage =; ns = not-significant.

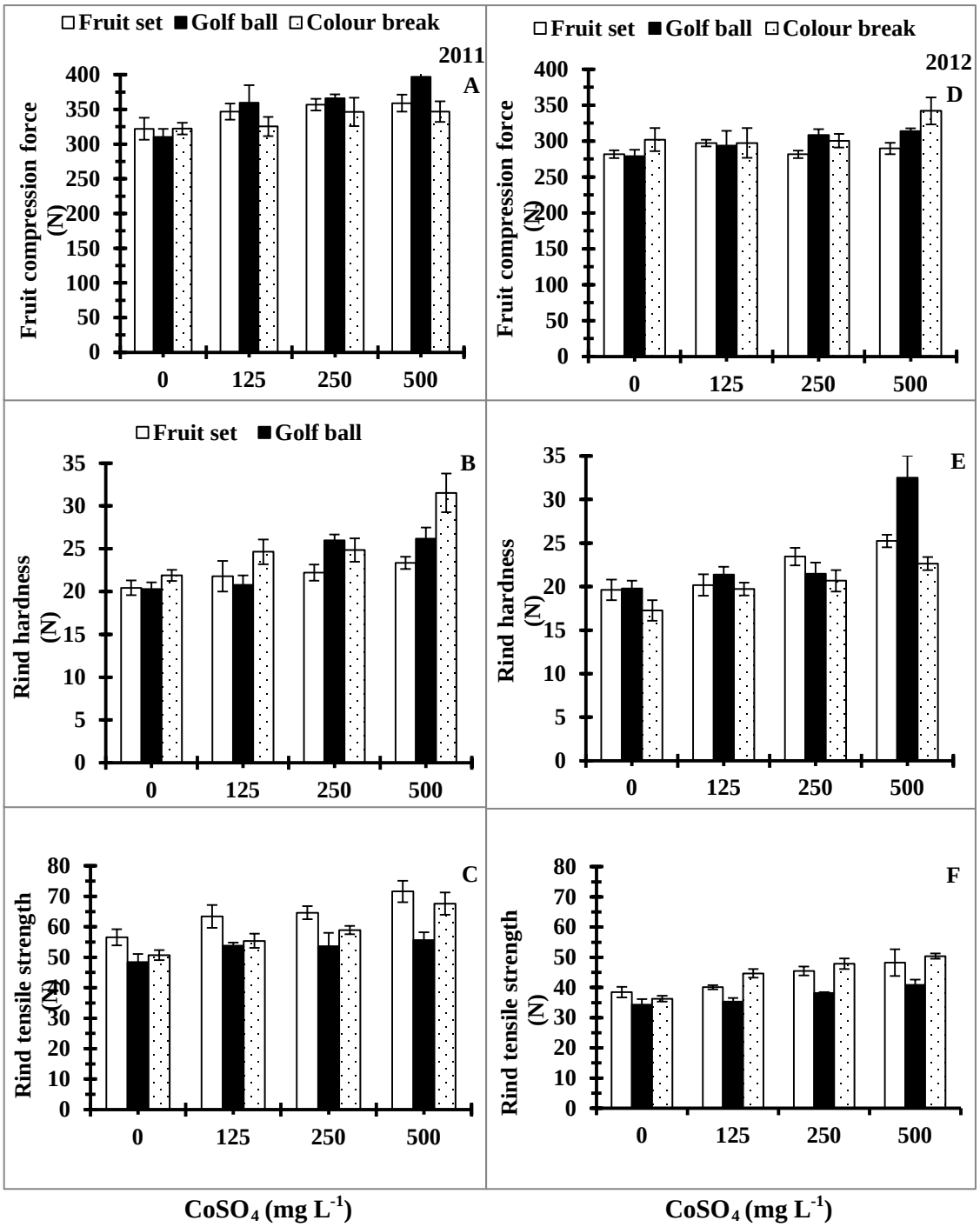

Figure 8.3. Effect of different concentrations of $CoSO_4$ applied at the fruit set, the golf ball or at the colour break on rheological properties of fruit and rind of sweet orange cv. Lane Late during 2011 and 2012. n = 4 replications (5 fruit per replication). Vertical bars represent standard error means. LSD ($P \leq 0.05$) for 2011; fruit firmness, Treatments = 25.1, Stage = ns, Treatments x stage = ns; for rind hardness, Treatments = 2.0, Stage = 1.8, Treatments x stage = 3.6; for rind tensile strength, Treatments = 6.2, Stage = 2.7, Treatments x stage = ns; ($P \leq 0.05$) for year 2012; fruit compression force Treatments = 20.5, Stage = 17.8, Treatments x stage = ns; for rind hardness, Treatments = 2.1, Stage = ns, treatments x stage = ns; for rind tensile strength, Treatments = 3.1, Stage = 2.7, Treatments x stage =; ns = not-significant.

Whilst in 2012, all the treatments significantly increased the mean rind tensile strength compared to the control in cv. Lane Late. When averaged over all the treatments, the mean rind hardness was significantly ($P \leq 0.05$) higher, when $CoSO_4$ was applied at the fruit set (64.1 N) compared to its applications at the golf ball (52.9 N) or at the colour break stage (58.2 N) in cv. Lane Late during 2011. In 2012, the mean rind hardness was significantly ($P \leq 0.05$) higher when $CoSO_4$ was applied at colour break stage (44.8 N) compared to its application at the fruit set (43.1 N) or at the golf ball stage (37.2 N) in cv. Lane Late during 2012. The interaction between treatments and their stages of application for rind tensile strength was found to be non-significant ($P \leq 0.05$) during 2011 and 2012 in cv. Washington Navel and Lane Late.

8.3.3. Fruit weight

The exogenous spray application of $CoSO_4$ (500 mg L^{-1}) significantly ($P \leq 0.05$) improved the fruit weight irrespective of its application at the fruit set, the golf ball or at the colour break stage in sweet orange cv. Washington Navel and Lane Late during 2011 and 2012 (Table 8.1). When averaged over different stages of spray applications, the application of $CoSO_4$ (500 mg L^{-1}) significantly ($P \leq 0.05$) increased the fruit weight compared to the control and other treatments of $CoSO_4$ in sweet orange cv. Washington Navel during 2011 and 2012. A similar trend was observed in cv. Lane Late during 2011 and 2012. Averaged over different treatments, the mean fruit weight was significantly ($P \leq 0.05$) increased when $CoSO_4$ was applied at the colour break (292.9 g) compared to its application at the golf ball (278.8 g) or at the fruit set stage (267.6 g) in cv. Washington Navel during 2011. A similar trend was observed in cv. Washington Navel during 2012. In cv. Lane Late, the mean fruit weight was significantly ($P \leq 0.05$) higher when $CoSO_4$ was applied at the colour break stage compared to its application at the golf ball or the fruit set stage during 2011 and 2012. The interaction between treatments and stages of spray application for fruit weight was found to be non-significant ($P \leq 0.05$) in both cultivars during 2011 and 2012.

Table 8.1. Effect of different concentrations of $CoSO_4$ sprayed at the fruit set, the golf ball or at the colour break stage on fruit weight of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Fruit weight (g)								
Washington Navel								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	258.5	255.8	265.0	262.2	279.5	278.2	267.7 b	265.4 b
$CoSO_4$ 125	263.5	257.8	269.5	272.0	282.8	281.0	271.9 b	270.2 ab
$CoSO_4$ 250	265.8	266.8	286.2	274.8	299.2	287.2	283.8 ab	276.2 ab
$CoSO_4$ 500	282.8	269.2	294.5	278.8	310.2	314.5	295.8 a	287.5 a
Mean (stage)	267.6 b	262.4 b	278.8 ab	271.9 b	292.9 a	290.2 a		
LSD ($P \leq 0.05$)								
Treatments			21.0	17.2				
Stage			18.2	14.9				
Treatments × stages			ns	ns				
Lane Late								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	260.2	270.2	263.2	247.2	263.8	248.2	262.4 b	255.2 b
$CoSO_4$ 125	263.8	271.8	269.5	252.5	285.2	276.0	272.8 ab	266.8 ab
$CoSO_4$ 250	265.0	275.5	280.5	254.8	293.8	277.2	279.8 ab	269.2 ab
$CoSO_4$ 500	266.5	278.2	292.0	258.8	313.2	322.0	290.6 a	286.3a
Mean (stage)	263.9 b	273.9 a	276.3 ab	253.3 b	289.0 a	280.9 a		
LSD ($P \leq 0.05$)								
Treatments			19.9	20.8				
Stages			17.3	18.0				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

8.3.4. Fruit diameter

All the treatments of $CoSO_4$ increased the fruit diameter when applied at the fruit set, the golf ball or at the colour break stage in both cultivars during 2011 and 2012 (Table 8.2). The treatments of $CoSO_4$ (250-500 mg L^{-1}) significantly ($P \leq 0.05$) resulted in higher fruit diameter (82.2 and 80.9 mm) compared to the control (77.6 mm) and treatment of 125mg L^{-1} $CoSO_4$ (79.9 mm) in cv. Washington Navel during 2011. In 2012, all the treatments of $CoSO_4$ significantly ($P \leq 0.05$) increased the fruit diameter compared to the control in cv. Washington Navel. A similar trend was observed in cv. Lane Late during 2011 and 2012. When averaged over different treatments, the mean fruit diameter was significantly ($P \leq 0.05$) higher when $CoSO_4$ was applied at the fruit set stage (81.8 mm) compared to its application at the golf ball stage (79.6 mm) or at the colour break (79.2 mm) in cv. Washington Navel during 2011. In 2012, the mean fruit diameter was higher when $CoSO_4$ was applied at the fruit set (83.5 mm) compared to its application at the colour break stage (81.4 mm) or at the golf ball stage (79.8 mm) in cv. Washington Navel. In cv. Lane Late, the mean fruit diameter was significantly ($P \leq 0.05$) higher when $CoSO_4$ was applied at the golf ball (81.0 mm) compared to its application at the fruit set (79.6 mm) or at the colour break stage (74.3 mm) during 2011. However in 2012, the mean fruit diameter was significantly ($P \leq 0.05$) higher when $CoSO_4$ was applied at the fruit set stage (83.5 mm) compared to its application at the colour break stage (81.4 mm) or at the golf ball stage (79.4 mm) in cv. Lane Late. The interaction between the treatments and their stages of application for fruit diameter was found to be non-significant ($P \leq 0.05$) in cv. Washington Navel and Lane Late during both years.

8.3.5. Rind thickness

All the spray applications of $CoSO_4$ increased the rind thickness in sweet orange cv. Washington Navel and Lane Late during 2011 and 2012 (Table 8.3). When averaged over different stages of spray application, a spray application of $CoSO_4$ (500 mg L^{-1}) resulted in significantly higher fruit rind thickness (5.9 and 5.0 mm) compared to the control (5.1 and 4.4 mm) in cv. Washington Navel and Lane Late during 2011. In 2012, all the treatments of $CoSO_4$ significantly increased the mean rind thickness as compared to the control in both cultivars.

Table 8.2. Effect of different concentrations of $CoSO_4$ sprayed at the fruit set, the golf ball or at the colour break stage on fruit diameter of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Fruit diameter (mm)								
Washington Navel								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	79.9	80.8	75.5	76.8	77.4	78.4	77.6 b	78.67c
$CoSO_4$ 125	81.1	82.6	79.6	78.9	79.2	81.6	79.9 ab	81.0 b
$CoSO_4$ 250	82.2	84.8	80.9	80.9	79.6	82.0	80.9 a	82.6 ab
$CoSO_4$ 500	83.9	85.9	82.2	80.9	80.6	83.6	82.2 a	83.5 a
Mean (stage)	81.8 a	83.5 a	79.6 b	79.4 c	79.2 b	81.4 b		
LSD ($P \leq 0.05$)								
Treatments			2.5	2.1				
Stage			2.2	1.8				
Treatments × stages			ns	ns				
Lane Late								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	75.6	80.8	75.5	76.8	66.5	78.4	72.5 c	78.7 c
$CoSO_4$ 125	79.0	82.6	79.0	78.9	74.8	81.6	77.6 b	81.0 b
$CoSO_4$ 250	80.1	84.8	79.2	80.9	76.5	82.0	78.6 b	82.6 ab
$CoSO_4$ 500	83.8	85.9	90.5	80.9	79.3	83.6	84.6 a	83.5 a
Mean (stage)	79.6 a	83.5 a	81.0 a	79.4 c	74.3 b	81.4 b		
LSD ($P \leq 0.05$)								
Treatments			3.2	2.1				
Stages			2.8	1.8				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

Table 8.3. Effect of different concentrations of $CoSO_4$ sprayed at the fruit set, the golf ball or at the colour break stage on fruit rind thickness of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Fruit rind thickness (mm)								
Washington Navel								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	5.0	4.6	5.1	4.5	5.1	5.0	5.1 b	4.7 b
$CoSO_4$ 125	5.1	5.0	5.6	5.1	5.8	5.1	5.5 ab	5.1 a
$CoSO_4$ 250	5.2	5.1	5.8	5.1	5.8	5.2	5.6 ab	5.1 a
$CoSO_4$ 500	5.5	5.1	6.0	5.2	6.2	5.4	5.9 a	5.3 a
Mean (stage)	5.2	5.0	6.0	5.4	5.7	5.2		
LSD ($P \leq 0.05$)								
Treatments			0.5	0.2				
Stage			ns	ns				
Treatments × stages			ns	ns				
Lane Late								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	3.8	4.6	4.6	4.5	4.9	5.0	4.4 b	4.7 b
$CoSO_4$ 125	4.0	5.0	4.7	5.1	5.1	5.1	4.6 ab	5.1 a
$CoSO_4$ 250	4.3	5.1	4.9	5.1	5.2	5.2	4.8 ab	5.1 a
$CoSO_4$ 500	4.5	5.1	5.1	5.2	5.3	5.4	5.0 a	5.3 a
Mean (stage)	4.2 b	5.0 b	4.8 a	5.0 b	5.1 a	5.2 a		
LSD ($P \leq 0.05$)								
Treatments			0.4	0.2				
Stages			0.3	0.2				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

Averaged over all the treatments, the fruit rind thickness was significantly ($P \leq 0.05$) higher when $CoSO_4$ was applied at the colour break (5.1 and 5.2 mm) compared to its application at the golf ball (4.8 and 5.0 mm) or at the fruit set stage (4.2 and 5.0 mm) in cv. Lane Late during 2011 and 2012. The stages of application of $CoSO_4$ did not significantly ($P \leq 0.05$) affect rind thickness in cv. Washington Navel during both years. The interaction between treatments and different stages of application of $CoSO_4$ for rind thickness was found to be non-significant ($P \leq 0.05$) in both cultivars during 2011 and 2012.

8.3.6. Soluble Solids Concentration (SSC %)

SSC in the juice of sweet orange increased with the increase in concentration of $CoSO_4$ applied, irrespective of stage of its application in cv. Washington Navel and Lane Late during 2011 and 2012 (Table 8.4). Averaged over different stages of spray application of $CoSO_4$, the treatment of $CoSO_4$ (500 mg L^{-1}) resulted in the significantly ($P \leq 0.05$) highest mean SSC compared to the control in cv. Washington Navel during 2011. In cv. Lane Late, all the treatments of $CoSO_4$ significantly ($P \leq 0.05$) increased the mean SSC compared to the control in both years. A similar trend was observed in cv. Washington Navel during 2012. When averaged over all the treatments, the mean SSC was significantly ($P \leq 0.05$) higher when $CoSO_4$ was applied at the golf ball (12.3%) compared to its application at the colour break (12.1 %) or the fruit set stage (11.6%) in cv. Washington Navel during 2011. A similar trend was observed in cv. Lane Late during 2011. In 2012, the mean SSC was significantly ($P \leq 0.05$) higher when $CoSO_4$ was applied at the fruit set (12.4%) compared to its application at the colour break (12.0%) or at the golf ball stage (11.8%) in cv. Washington Navel during 2012. However, in cv. Lane Late, the mean SSC (%) was significantly ($P \leq 0.05$) higher when $CoSO_4$ was applied at the golf ball stage (12.6%) compared to its application at the fruit set stage (12.5%) or at the colour break stage (11.9%) during 2012. The interaction between treatments and their different stages of application for SSC was found to be non-significant ($P \leq 0.05$) in cv. Lane Late and Washington Navel during 2011 and 2012.

Table 8.4. Effect of different concentrations of $CoSO_4$ sprayed at the fruit set, the golf ball or at the colour break stage on SSC in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

SSC (%)								
Washington Navel								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	10.9	12.0	11.2	10.5	11.4	10.2	11.2 c	10.9 c
$CoSO_4$ 125	11.7	12.3	12.4	11.7	12.2	12.0	11.9 b	12.0 b
$CoSO_4$ 250	11.8	12.4	12.8	12.2	12.4	12.8	12.0 b	12.4 ab
$CoSO_4$ 500	12.0	13.1	12.9	12.9	12.6	12.9	12.5 a	13.0 a
Mean (stage)	11.6 b	12.4 a	12.3 a	11.8 b	12.1 a	12.0 ab		
LSD ($P \leq 0.05$)								
Treatments			0.5	0.6				
Stage			0.4	0.5				
Treatments × stages			ns	ns				
Lane Late								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	11.2	11.6	12.0	11.2	11.8	10.9	11.6 c	11.2 c
$CoSO_4$ 125	12.0	12.8	13.7	12.6	12.9	11.4	12.9 b	12.2 b
$CoSO_4$ 250	12.6	12.9	14.1	13.1	13.2	12. 5	13.3 ab	12.8 ab
$CoSO_4$ 500	12.9	12.8	14.2	13.5	14.0	12.7	13.7 a	13.0 a
Mean (stage)	12.2 b	12.5 a	13.5 a	12.6 a	13.0 a	11.9 b		
LSD ($P \leq 0.05$)								
Treatments			0.6	0.7				
Stages			0.5	0.8				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

8.3.7. Ascorbic acid

All the treatments of $CoSO_4$ significantly increased the levels of ascorbic acid in the juice as compared to the control in cv. Washington Navel and Lane Late during 2011 and 2012 (Table 7.5). When averaged over different stages of spray application, the treatments of $CoSO_4$ (500 mg L^{-1}) resulted in significantly ($P \leq 0.05$) higher mean levels of ascorbic acid (69.3 and 69.1 mg $100ml^{-1}$ FJ) compared to the control (65.4 and 63.6 mg $100ml^{-1}$ FJ) in cv. Washington Navel and Lane Late respectively, during 2011. In 2012, all the treatments of $CoSO_4$ (125-500 mg L^{-1}) significantly increased the mean levels of ascorbic acid compared to the control in both cultivars. When averaged over all the treatments, the mean level of ascorbic acid in the juice was significantly higher when $CoSO_4$ was applied at the golf ball stage (69.5 mg $100ml^{-1}$ FJ) compared to its application at the colour break stage (68.3 mg $100ml^{-1}$ FJ) or at the fruit set stage (63.2 mg $100ml^{-1}$ FJ) in cv. Washington Navel during 2011 only. A similar trend was observed in cv. Lane Late during 2011 and 2012. The interaction between the treatments and their stages of application for ascorbic acid in the juice was also found to be non-significant ($P \leq 0.05$) in both cultivars during both years, except in cv. Lane Late 2011.

8.3.8. Total antioxidants

All the treatments of $CoSO_4$ resulted in significantly ($P \leq 0.05$) higher levels of total antioxidants in the juice, irrespective of its application at the fruit set, the golf ball or the colour break stage in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012 (Table 8.6). When averaged over different stages of spray application of $CoSO_4$, the mean levels of total antioxidants in the juice were significantly higher (45.3 mM Trolox $100ml^{-1}$FJ) with the treatment of $CoSO_4$ (500 mg L^{-1}) compared to the control (41.7 and 44.0 mM Trolox $100ml^{-1}$FJ) in cv. Washington Navel 2011. In 2012, all the treatments of $CoSO_4$ significantly increased the mean levels of total antioxidants in the juice compared to the control in cv. Washington Navel. In cv. Lane Late, the treatments of $CoSO_4$ (250-500 mg L^{-1}) significantly increased the mean SSC as compared to the control and $CoSO_4$ (125 mg L^{-1}) treatment during both years. When averaged over all the treatments, the mean levels of total antioxidants in the juice significantly ($P \leq 0.05$) increased when $CoSO_4$ was applied at the golf ball stage (44.3 mM Trolox $100ml^{-1}$FJ) compared to its application at the colour break (43.1 mM Trolox $100ml^{-1}$FJ) or at the fruit set stage (41.9 mM Trolox $100ml^{-1}$FJ) in cv. Washington Navel during 2011.

Table 8.5. Effect of different concentrations of $CoSO_4$ sprayed at the fruit set, the golf ball or at the colour break stage on the levels of ascorbic acids in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Ascorbic acid (mg 100ml^{-1} FJ)								
Washington Navel								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	62.5	62.1	67.2	61.1	66.5	65.4	65.4 b	61.7 c
$CoSO_4$ 125	62.5	62.7	67.9	63.6	68.4	66.3	66.3 ab	63.3 bc
$CoSO_4$ 250	63.0	64.3	68.7	64.8	69.5	67.1	67.1 a	64.5 ab
$CoSO_4$ 500	64.6	65.1	74.3	65.2	68.8	69.3	69.3 a	66.1 a
Mean (stage)	63.2 b	63.5 a	69.5 a	63.7 a	68.3 a	64.5 a		
LSD ($P \leq 0.05$)								
Treatments			2.7	2.4				
Stage			2.3	ns				
Treatments × stages			ns	ns				
Lane Late								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	59.67 d	55.8	66.0 bc	60.0	65.1 bc	62.3	63.6 b	59.3 b
$CoSO_4$ 125	62.8 cd	59.5	66.8 bc	64.8	65.7 bc	63.7	65.1 b	62.7 a
$CoSO_4$ 250	64.0 bc	59.5	67.2 bc	64.9	66.0 bc	64.2	65.7 b	62.9 a
$CoSO_4$ 500	64.0 bc	60.4	75.8 a	69.0	67.4 b	65.3	69.1 a	64.9 a
Mean (stage)	62.6 c	58.8 b	69.0 a	64.6 a	66.1 b	63.9 a		
LSD ($P \leq 0.05$)								
Treatments			2.2	2.8				
Stages			1.9	2.4				
Treatments × stages			3.8	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

Table 8.6. Effect of different concentrations of $CoSO_4$ sprayed at the fruit set, the golf ball or at the colour break stage on the levels of total antioxidants in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Total antioxidants (mM Trolox 100ml^{-1} FJ)								
Washington Navel								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	41.1c	41.9d	42.0 bc	43.7	42.1 bc	42.7	41.7 b	42.8 c
$CoSO_4$ 125	41.8 bc	44.5	42.6 bc	45.8	42.9 bc	43.0	42.4 b	44.4 bc
$CoSO_4$ 250	42.4 bc	45.3	43.5 bc	46.2	43.0 bc	43.3	43.0 b	44.9 b
$CoSO_4$ 500	42.5 bc	47.0	49.1 a	46.6	44.2 b	45.4	45.3 a	46.3 a
Mean (stage)	41.9 b	44.7 ab	44.3 a	45.5 a	43.1 ab	43.6 b		
LSD ($P \leq 0.05$)								
Treatments			1.5	1.8				
Stage			1.3	1.6				
Treatments × stages			2.5	ns				
Lane Late								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	42.1	42.0	43.0	44.7	38.9	45.5	41.3 c	44.0 c
$CoSO_4$ 125	43.6	42.9	44.2	45.4	41.2	45.5	43.0 bc	44.6 bc
$CoSO_4$ 250	45.6	45.2	44.4	46.9	42.9	46.6	44.3 b	46.2 ab
$CoSO_4$ 500	49.1	45.4	45.9	47.2	43.0	48.8	46.0 a	47.2 a
Mean (stage)	45.1 a	43.9 b	44.4 a	46.0 a	41.5 b	46.6 a		
LSD ($P \leq 0.05$)								
Treatments			2.5	2.0				
Stages			2.2	1.7				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

In 2012, the mean levels of total antioxidants in the juice were significantly ($P \leq 0.05$) higher, when $CoSO_4$ was sprayed at the golf ball stage (45.5 mM Trolox $100ml^{-1}FJ$) compared to its application at the fruit set stage (44.7 mM Trolox $100ml^{-1}FJ$) or at the colour break stage (43.6 mM Trolox $100ml^{-1}FJ$) in cv. Washington Navel. In cv. Lane Late, the mean levels of total antioxidants in the juice significantly ($P \leq 0.05$) increased when $CoSO_4$ was applied at the fruit set stage (45.1 mM Trolox $100ml^{-1}FJ$) compared to its application at the golf ball (44.4 mM Trolox $100ml^{-1}FJ$) or at the colour break stage (41.5 mM Trolox $100ml^{-1}FJ$) during 2011. A similar trend was observed in cv. Lane Late 2012. The interactions between the treatments and their stages of application for the levels of total antioxidants in the juice were found to be non-significant ($P \leq 0.05$) in cv. Washington Navel and Lane Late during 2011 and 2012, except in cv. Washington Navel during 2011.

8.3.9. Fructose

All the treatments of $CoSO_4$ significantly ($P \leq 0.05$) increased the levels of fructose in the juice, irrespective of different stages of its application in sweet orange cv. Washington Navel and Lane Late during 2011 and 2012 (Table 8.7). Averaged over different stages of spray applications all the treatments of $CoSO_4$ significantly ($P \leq 0.05$) increased the levels of fructose in the juice compared to the control during 2011 and 2012 in cv. Washington Navel during 2011. In 2012, the treatment of $CoSO_4$ (500 mg L^{-1}) significantly increased the mean levels of fructose in the juice compared to the control and the treatments of $CoSO_4$ (125-250 mg L^{-1}) in cv. Washington Navel. A similar trend was observed in cv. Lane Late during 2011. However, in cv. Lane Late, all the treatments of $CoSO_4$ significantly increased the mean levels of fructose in the juice compared to the control during 2012. When averaged over all the treatments, the mean levels of fructose in the juice significantly ($P \leq 0.05$) increased when $CoSO_4$ was applied at the colour break (3.3 g $100g^{-1}$ FJ) compared to its application at the golf ball (2.8 g $100g^{-1}$ FJ) or at the fruit set stage (2.4 g $100g^{-1}$ FJ) in cv. Washington Navel during 2011only. In cv. Lane Late, the mean levels of fructose in the juice were significantly increased when $CoSO_4$ was applied at the golf ball (3.4 g $100g^{-1}$ FJ) compared to its application at colour break (2.9 g $100g^{-1}$ FJ) or at the fruit set stage (2.9 g $100g^{-1}$ FJ) during 2011. In 2012, the mean levels of fructose in the juice were significantly higher at the colour break (3.9 g $100g^{-1}$ FJ), at the golf ball (3.4 g $100g^{-1}$ FJ) or at the fruit set stage (2.9 g $100g^{-1}$ FJ) in cv. Lane Late. The interactions between the $CoSO_4$ treatments and their stages of application for the levels of fructose in the juice were found to be non-significant ($P \leq 0.05$) in both cultivars during 2011 and 2012.

Table 8.7. Effect of different concentrations of $CoSO_4$ sprayed at the fruit set, the golf ball or at the colour break stage on the levels of fructose in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Fructose (g $100ml^{-1}$ FJ)								
Washington Navel								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	2.1 e	2.9	2.6 cd	2.4	2.8	2.6	2.5 c	2.6 b
$CoSO_4$ 125	2.4 de	3.0	2.7 cd	2.4	3.4	2.7	2.8 b	2.7 b
$CoSO_4$ 250	2.3 de	3.0	2.8 cd	2.7	3.5	2.8	2.9 ab	2.8 b
$CoSO_4$ 500	2.8 cd	3.2	3.0	3.3	3.7	3.3	3.1 a	3.3 a
Mean (stage)	2.4 c	3.0 a	2.8 b	2.7 a	3.3 a	2.9 a		
LSD ($P \leq 0.05$)								
Treatments			0.3	0.4				
Stage			0.3	ns				
Treatments × stages			ns	ns				
Lane Late								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	2.7	2.8	3.1	2.7	2.8	3.5	2.9 b	2.2 b
$CoSO_4$ 125	2.9	2.8	3.2	3.0	2.9	3.8	3.0 b	3.2 b
$CoSO_4$ 250	2.9	2.9	3.3	3.2	2.9	3.9	3.1 b	3.3 b
$CoSO_4$ 500	3.1	3.3	3.9	3.3	3.1	4.6	3.4 a	3.7 a
Mean (stage)	2.9 b	2.9 b	3.4 a	3.1b	2.9 b	3.9 a		
LSD ($P \leq 0.05$)								
Treatments			0.3	0.4				
Stages			0.2	0.3				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

8.3.10. Glucose

The levels of glucose in the juice were significantly ($P \leq 0.05$) increased with the increased concentration of $CoSO_4$, when applied at the fruit set, the golf ball or at the colour break stage in cv. Washington Navel and Lane Late during 2011 and 2012 (Table 8.8). When averaged over different stages of spray application, all the treatments of $CoSO_4$ significantly ($P \leq 0.05$) increased the levels of glucose in the juice compared to the control in cv. Washington Navel during 2011. In 2012, the treatments of $CoSO_4$ (250-500 mg L^{-1}) significantly increased the levels of glucose in the juice compared to the control and treatment of $CoSO_4$ (125 mg L^{-1}) in cv. Washington Navel. In cv. Lane Late, all the treatments of $CoSO_4$ significantly ($P \leq 0.05$) increased the mean levels of glucose in the juice compared to the control during 2011 and 2012. When averaged over all the treatments, the mean levels of glucose in the juice were significantly ($P \leq 0.05$) highest when applied at the colour break stage (2.4 g $100ml^{-1}$ FJ) compared to its application at the golf ball stage (2.0 g $100ml^{-1}$ FJ) or at the fruit set stage (1.8 g $100ml^{-1}$ FJ) in cv. Washington Navel during 2011 only. In cv. Lane Late, the mean levels of glucose in the juice were significantly ($P \leq 0.05$) highest when $CoSO_4$ was applied at the golf ball stage (1.9 g $100ml^{-1}$ FJ) compared to its application at the colour break (1.8 g $100ml^{-1}$ FJ) or at the fruit set stage (1.5 g $100ml^{-1}$ FJ) during 2011 only. The interactions between the treatments of $CoSO_4$ and their stages of spray application were found to be non-significant ($P \leq 0.05$) for glucose in the juice in both cultivars during 2011 and 2012, except in cv. Lane Late during 2011.

8.3.11. Sucrose

All the treatments of $CoSO_4$ (125-500 mg L^{-1}) significantly ($P \leq 0.05$) increased levels of sucrose in the juice compared to the control irrespective of stage of spray application in cv. Washington Navel and Lane Late during 2011 and 2012 (Table 8.9). When averaged over different stages of spray application, the treatments of $CoSO_4$ (250-500 mg L^{-1}) significantly increased the mean levels of sucrose in the juice compared to the control and the treatment of $CoSO_4$ (125 mg L^{-1}) in cv. Washington Navel during 2011 only. In cv. Lane Late, all the treatments significantly increased the mean levels of sucrose in the juice compared to the control during 2011. In 2012, the treatment of $CoSO_4$ (500 mg L^{-1}) significantly increased the mean levels of sucrose in the juice compared to the control and the $CoSO_4$ (125-250 mg L^{-1}) treatments in cv. Lane Late. When averaged over all the treatments, the mean level of sucrose was significantly ($P \leq 0.05$) higher when $CoSO_4$ was applied at the

Table 8.8. Effect of different concentrations of $CoSO_4$ sprayed at the fruit set, the golf ball or at the colour break stage on the levels of glucose in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Glucose (g $100ml^{-1}$ FJ)								
Washington Navel								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	1.1	1.4	1.9	1.5	2.0	1.5	1.7 c	1.5 c
$CoSO_4$ 125	1.7	1.6	2.0	1.7	2.3	1.6	2.0 b	1.6 bc
$CoSO_4$ 250	2.1	1.7	2.1	1.9	2.5	1.7	2.2 ab	1.7 b
$CoSO_4$ 500	2.1	2.0	2.1	2.1	2.6	2.1	2.3 a	2.1 a
Mean (stage)	1.8 c	1.7 a	2.0 b	1.8 a	2.4 a	1.7 a		
LSD ($P \leq 0.05$)								
Treatments			0.3	0.3				
Stage			0.2	ns				
Treatments × stages			ns	ns				
Lane Late								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	1.1	1.4	1.8	1.2	1.6	2.0	1.5 b	1.6 c
$CoSO_4$ 125	1.5	2.0	1.9 b	1.9	1.8	2.3	1.8 a	2.0 b
$CoSO_4$ 250	1.5	2.1	1.9 b	2.6	1.8	2.4	1.8 a	2.4 ab
$CoSO_4$ 500	1.6	2.2	2.1 a	2.7	1.8	2.6	1.8 a	2.5 a
Mean (stage)	1.5 c	2.0 b	1.9 a	2.1 ab	1.8 b	2.3 a		
LSD ($P \leq 0.05$)								
Treatments			0.1	0.4				
Stages			0.1	ns				
Treatments × stages			0.2	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

Table 8.9. Effect of different concentrations of $CoSO_4$ sprayed at the fruit set, the golf ball or at the colour break stage on the levels of sucrose in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Sucrose (g $100ml^{-1}$ FJ)								
Washington Navel								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	7.5	6.1	3.9	5.3	5.0	5.3	5.5	5.5c
$CoSO_4$ 125	7.4	6.2	4.3	5.8	5.6	6.0	5.8	6.0 bc
$CoSO_4$ 250	7.6	6.2	4.8	6.0	5.6	6.3	6.0	6.2 ab
$CoSO_4$ 500	8.2	6.4	4.8	6.5	5.9	7.1	6.3	6.7 a
Mean (stage)	7.7 a	6.2 a	4.5 c	6.0 a	5.5 b	6.2 a		5.5 c
LSD ($P \leq 0.05$)								
Treatments			ns	0.5				
Stage			0.7	ns				
Treatments × stages			ns	ns				
Lane Late								
Treat (mg L^{-1})	Fruit set		Golf ball		Colour break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	4.8	4.6	4.6	5.3	6.3	4. 6	5.3b	4.8b
$CoSO_4$ 125	6.5	4.8	5.1	5.3	6.5	5.1	6.0a	5.1b
$CoSO_4$ 250	6.6	5.0	5.3	5.6	6.6	5.4	6.2a	5.4b
$CoSO_4$ 500	6.7	5.9	5.6	6.9	6.6	5.6	6.3a	6.1a
Mean (stage)	6.2a	5.1b	5.1b	5.8a	6.5a	5.2b		
LSD ($P \leq 0.05$)								
Treatments			0.6	0.7				
Stages			0.5	ns				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

fruit set stage (7.7 g $100ml^{-1}$ FJ) compared to its application at the colour break (5.5 g $100ml^{-1}$ FJ) or at the golf ball stage (4.5 g $100ml^{-1}$ FJ) in cv. Washington Navel during 2011 only. In cv. Lane Late, the mean level of sucrose in the juice was significantly ($P \leq 0.05$) higher when $CoSO_4$ was applied at the colour break stage (6.5 g $100ml^{-1}$ FJ) compared to its application at the fruit set (6.2 g $100ml^{-1}$ FJ) or at the golf ball stage (5.1 g $100g^{-1}$ FJ) during 2011 only. The interaction between the treatments and their stages of spray application for sucrose in the juice was found to be non-significant ($P \leq 0.05$) in cv. Washington Navel and Lane Late during both years.

8.3.12. Total sugars

The levels of total sugars in the juice were significantly ($P \leq 0.05$) increased with the increased concentration of $CoSO_4$ applied irrespective of its application at the fruit set, at the golf ball or at the colour break stage in cv. Washington Navel and Lane Late during both consecutive years (Table 8.10). Averaged over different stages of spray application, the treatments of $CoSO_4$ (250-500 mg L^{-1}) significantly increased the mean level of total sugars in the juice compared to the control in cv. Washington Navel during 2011 and 2012. In cv. Lane Late, all the treatments of $CoSO_4$ (250-500 mg L^{-1}) significantly increased mean level of total sugars during 2011 and 2012. When averaged over different treatments, the mean levels of total sugars in the juice were significantly higher when $CoSO_4$ was applied at the fruit set stage (11.8 g $100ml^{-1}$ FJ) compared to its application at the colour break stage (11.1 g $100ml^{-1}$ FJ) or at the golf ball stage (9.3 g $100ml^{-1}$ FJ) in cv. Washington Navel during 2011 only. In cv. Lane Late, the mean levels of total sugars in the juice were significantly higher when $CoSO_4$ was applied at the colour break stage (11.2 g $100ml^{-1}$ FJ) compared to its application at the fruit set stage (10.5 g $100ml^{-1}$ FJ) or at golf ball stage (10.5 g $100ml^{-1}$ FJ) during 2011. In 2012, the mean levels of total sugars in the juice were significantly higher at colour break (11.4 g $100ml^{-1}$ FJ) compared to its application at the golf ball (10.9 g $100ml^{-1}$ FJ) or at the fruit set stage (10.0 g $100ml^{-1}$ FJ) in cv. Lane Late. The interactions between the treatments and their stages of spray application for total sugars in the juice were found to be non-significant ($P \leq 0.05$) in both cultivars during 2011 and 2012.

8.3.13. Organic Acids

All the treatments of $CoSO_4$ irrespective of its concentration and stage of application did not significantly affect the concentrations of citric, malic, succinic, fumaric and tartaric acid in the fruit juice during 2011 and 2012 in both cultivars (data not included).

Table 8.10. Effect of different concentrations of $CoSO_4$ sprayed at the fruit set, the golf ball or at the colour break stage on the levels of total sugars in the juice of sweet orange cv. Washington Navel and Lane Late during 2011 and 2012.

Total sugars (g $100ml^{-1}$ FJ)								
Washington Navel								
Treat (mg L^{-1})	Fruit Set		Golf Ball		Colour Break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	10.7	10.3	8.4	9.1	9.5	9.4	9.5c	9.6 c
$CoSO_4$ 125	11.4	10.7	8.9	10.0	11.3	10.4	10.5 b	10.3 b
$CoSO_4$ 250	12.0	10.9	9.8	10.5	11.6	10.9	11.1 ab	10.8 b
$CoSO_4$ 500	13.1	11.6	9.9	11.8	12.2	12.5	11.7 a	12.0 a
Mean (stage)	11.8 a	10.9	9.3 b	10.4	11.1a	10.8		
LSD ($P \leq 0.05$)								
Treatments			1.0	0.7				
Stage			0.8	ns				
Treatments × stages			ns	ns				
Lane Late								
Treat (mg L^{-1})	Fruit Set		Golf Ball		Colour Break		Mean (Treat)	
	2011	2012	2011	2012	2011	2012	2011	2012
Control	8.6	8.8	9.6	9.2	10.7	10.0	9.6 b	9.4 d
$CoSO_4$ 125	11.0	9.5	10.2	10.2	11.2	11.1	10.8a	10.3 b
$CoSO_4$ 250	11.1	10.1	10.5	11.4	11.3	11.8	11.0a	11.1 b
$CoSO_4$ 500	11.4	11.4	11.6	12.9	11.4	12.9	11.5a	12.4 a
Mean (stage)	10.5 b	10.0 b	10.5b	10.9 a	11.2a	11.4a		
LSD ($P \leq 0.05$)								
Treatments			0.7	0.8				
Stages			0.6	0.7				
Treatments × stages			ns	ns				

n = 4 replications (10 fruit per replication), any two mean within a column and within a row followed by different letters are significantly different; ns = not-significant; Treat = Treatments

8.4. Discussion

All the treatments of $CoSO_4$ have decreased the incidence of creasing (%) depending on its concentration and stages of spray application in sweet orange cv. Washington Navel and Lane Late during two consecutive seasons 2011 and 2012 (Figure 8.1). The substantial reduction in the incidence of creasing with the spray application of $CoSO_4$ may possibly be ascribed to the inhibition of ethylene biosynthesis. Earlier, the higher levels of endogenous ethylene in the creased fruit than in normal fruit in sweet oranges cv. Valencia Late (Monselise et al., 1976) and Washington Navel (Pham, 2009) have been reported. It is well documented that $CoSO_4$ blocks the conversion of ACC to ethylene by inhibiting the activity of ACC oxidase enzyme (Sisler and Yang, 1984; Even-Chen et al., 1982; Hyodo and Nishino, 1981). Earlier, Hyodo (1977) reported ethylene biosynthesis in the albedo tissues of Satsuma mandarin.

Loss of water soluble pectins and higher activities of the cell wall degrading enzymes such as PE, PG and cellulose in the albedo tissues of creased sweet orange fruit than healthy fruit have also been reported (Li et al., 2009; Monselise et al., 1976). Possibly, the spray application of $CoSO_4$ may have reduced the activities of cell wall degrading enzymes such as PE, *exo*-PG, *endo*-PG, and EGase in the albedo tissues of the fruit subsequently reducing the incidence of creasing in sweet orange. Previously, the spray application of ethylene inhibitors such as $CoSO_4$, AVG or PUT reduced the activities of PE, *exo*-PG, *endo*-PG, and EGase as well as water soluble pectin in the albedo and the flavedo tissues of the fruit compared to the control in sweet orange cv. Washington Navel and Lane Late (Chapter 9). Recently, Saleem et al. (2014) ascribed that creasing is associated with higher activities of cell wall degrading enzymes such as PE, *exo*-PG, *endo*-PG, and EGase in the albedo tissues of creased fruit.

The increased fruit firmness, rind hardness and rind tensile strength in cv. Washington Navel and Lane Late during both years (Figure 8.2 A-E and 8.3 A-E) with the exogenous application of $CoSO_4$ may also be attributed to reduced ethylene production. Spray application of $CoSO_4$ may also have reduced the activities of cell wall degrading enzymes such as PE, *exo*-PG, *endo*-PG, and EGase in the rind tissue of the fruit subsequently increasing the fruit firmness, rind hardness and rind tensile strength. Similarly, spray applications of $CoSO_4$ have lessened the activities of PE, *exo*-PG, *endo*-PG, and EGase as well as water soluble pectin in the albedo and the flavedo tissues of the rind of the fruit compared to the control in sweet orange cv. Washington Navel and Lane Late (Chapter 9).

Similar findings were observed in peach (Kim et al., 2004; Bregoli et al., 2002) and in apples (Benincore et al., 2000).

The rind thickness increased with increased concentrations of $CoSO_4$ applied, as compared to the control, irrespective of its application at the fruit set, the golf ball or at the colour break stage in cv. Washington Navel and Lane Late orange during 2011 and 2012 (Table 8.3). Increased rind thickness of the fruit with spray application of $CoSO_4$ may also possibly have contributed to the reduction in creasing. Earlier, it has been reported that creasing incidence was higher in sweet orange fruit with thinner rind compared to fruit with thicker rind (Ali et al., 2000; Bevington et al., 1993; Jones et al., 1967). Meanwhile, the exogenous application of ethylene inhibitor $CoSO_4$ (500 mg L^{-1}) significantly increased the fruit weight, diameter and rind thickness in both cultivars (Table 8.1-8.3). Similarly, Al-Husseini (2012) reported that $CoSO_4$ significantly improved the fruit weight, diameter and rind thickness in sweet oranges cv. Washington Navel and Lane Late and higher fruit weight in pomegranate (Reddy et al. 2011).

Similarly, exogenous spray application of $CoSO_4$ has also increased the SSC, ascorbic acid, total antioxidants, individual sugars and total sugars in the juice of both cultivars of sweet oranges during 2011 and 2012. This may be ascribed to the increased supply of photosynthates from the leaves to sweet orange fruit, as $CoSO_4$ application may also have reduced the premature leaf abscission in sweet orange trees. However, Saleem et al. (2007) reported leaf abscission in sweet orange cv. Blood Red with the exogenous application of polyamines - an inhibitor of ethylene, resulted in improved SCC and other quality parameters. Similarly, foliar applications of $CoSO_4$ have been reported to improve fruit weight, size, SSC, SSC: TA ratio and total sugars in mango (Singh and Singh, 1993 and Singh and Agrez, 2002), pomegranate (Reddy et al., 2011), olive (Gad et al., 2006), apples (Benincore et al., 2000), peaches (Kim et al., 2004), Figure (Teragishi et al,. 2000) and sweet orange (Al-Husseini, 2012; Saleem et al., 2007). In conclusion**,** the exogenous spray application of $CoSO_4$ (500 mg L^{-1}) significantly reduces the incidence of creasing when applied at the golf ball stage compared to the fruit set or at the colour break stage due to the inhibition of ethylene production and also improves the fruit firmness, rind hardness, rind tensile strength, fruit weight and diameter, ascorbic acid, total antioxidants, and individual and total sugars compared to the control in sweet orange cv. Washington Navel and Lane Late.

CHAPTER 9

Mode of reduction of creasing in sweet orange [*Citrus sinensis* (L.) Osbeck] fruit with exogenous application of ethylene inhibitors

Summary

Creasing (albedo breakdown) is a serious physiological disorder in many orange-producing countries of the world. We investigated how the exogenous application of putrescine (PUT), aminoethoxyvinylglycine (AVG) or cobalt sulphate ($CoSO_4$) reduces the incidence of creasing in sweet orange fruit. The effects of exogenous application of PUT, AVG or $CoSO_4$ on levels of total, water soluble and water insoluble pectins as well as on the activities of pectinesterase (PE), *exo*-polygalacturonase (*exo*-PG), *endo*-polygalacturonase (*endo*-PG) and endo-1,4-*β*-D-glucanase (EGase) in the albedo and flavedo tissues of the fruit at the golf ball and ripe stage were determined in sweet orange cv. Washington Navel and Lane Late. The level of total and water insoluble pectins significantly increased with the exogenous application of PUT, AVG and $CoSO_4$ at the golf ball and the ripe stage in both cultivars compared to the control. Meanwhile, the water soluble pectins significantly decreased in the treated fruit compared to the control, in both cultivars. The activities of PE, *exo*- and *endo*- PG and EGase were significantly reduced with the exogenous application of PUT, AVG or $CoSO_4$ than in the control fruit at the golf ball and ripe stage in both cultivars. The activities of PE, *exo*-PG, *endo*-PG, and EGase were higher in the albedo than flavedo tissues at the ripe stage in both cultivars. In conclusion, the application of PUT, AVG or CoSO4 significantly elevated the levels of total and water insoluble pectin, reduced water soluble pectin through decreased activity of cell wall degrading enzymes such as PE, *endo*- and *exo*-PG and EGase in the albedo and flavedo tissues of the fruit as compared to the control leading to alleviate creasing in sweet orange fruit.

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Hussain, Z and Z. Singh Mode of reduction of creasing in sweet orange [Citrus sinensis (L.) Osbeck] fruit with exogenous application of ethylene inhibitors. Plant Growth Regulation (submitted).

9.1. Introduction

Creasing in sweet orange [*Citrus sinensis* (L.) Osbeck] fruit is a physiological rind disorder with cracks in the albedo tissue causing puffiness of orange peel (Monselise et al., 1976; Treeby et al., 2002). It is also known as albedo breakdown 'puffing', 'grooving', 'wrinkle skin' and 'crinkly skin'. The development of creasing is connected to the degradation of pectins leading to the loosening of the connections among cells (Saleem et al., 2014). Creasing affects different cultivars of sweet orange including Washington Navel (Gambetta et al., 2000, Ali et al., 2000), Valencia (Jones et al., 1967; Monselise et al., 1976), Navelina (Pham, 2009) and Nova Mandarins (Greenberg et al., 2006). It is often worse in Navel orange and appears before and after colour break of fruit development (Storey et al., 2002).

Creasing was first reported from South Africa from 1938 (Le Roux and Crous, 1938) and currently causes serious economic losses in the sweet orange industry in different parts of the world such as Australia (Storey and Treeby, 1994), USA (Ali et al., 2000; Jones et al., 1967), Israel (Greenberg et al., 2006; Monselise et al., 1976), Uruguay (Gambetta et al., 2000), and China (Li et al., 2009). In Australia, creasing is a major cause of fruit diversion from fresh markets to processing and more than 50% of the Navel sweet orange crop may be affected to varying degrees (20-80%) due to this disorder (Treeby et al., 2000; 1995). Creasing has been reported to be associated with genotype (Agustí et al., 2003); climate (Gambetta et al., 2000; Jones et al., 1967); rootstock (Storey et al., 2002); crop load (Jones et al., 1967; rind thickness (Holtzhausen, 1981); irrigation (Agustí et al., 2004) and mineral nutrition (Bower, 2004; Ali et al., 2000). The creasing is usually detectable at maturity, however its initiation seems to be associated with pectins degradation, loosening of the connections between cells of the albedo tissue, higher activity of PE, higher contents of water soluble pectin similar to over-ripe fruit and is suggestive of early senescence (Saleem et al., 2014; Li et al., 2009; Jona et al., 1989; Monselise et al., 1976). Pectin is a complex class of polysaccharides which act as a hydrating and cementing material for the cellulosic network (Zareie et al., 2003; Thakur et al., 1997). Although pectins are widely present in plants, they are most abundant in citrus fruits (Baker, 1997). Mostly pectins are derived from citrus (lime, lemon, grapefruit, and orange) and apple (Thakur et al., 1997). Pectin is classified into water soluble, insoluble, and alcohol soluble (Van-Buren, 1991). The chemical structure of pectins varies from species to species and also during the fruit development (Fishman et al., 1991). In mature fruit, pectin is bound with cellulose in the cell wall and is insoluble. However, the structure of pectin is altered during ripening by naturally occurring pectic enzymes like PE,

endo-PG, *exo*-PG (Yoo et al., 2003; Kashyap et al., 2001) and EGase in the fruits (Brummell et al., 1997). This alteration involves the breakdown of the pectins chain, pectins become more soluble and its grip on the surrounding cell walls loosens and plant tissues turn soft.

Softening is also known as a ripening process, linked with biochemical changes in cell wall fractions which are involved in breakdown of cell-wall polymers such as cellulose, hemicelluloses and pectins (Payasi et al., 2009). Various hydrolytic reactions occur due to poly-galacturonase (PG), pectin methylesterase, pectatelyase, rhamnogalacturonase, cellulose and galactosidase. Besides these enzymes, protein also plays a significant role in softening (Brummell and Harpster, 2001). Creasing is similar to fruit softening caused by cell wall disassembly (Nishiyama et al., 2007; Brummel, 2006; Orfila et al., 2002). The activity of pectin methylesterase and concentration of water soluble pectins of the albedo tissue is also associated with creasing and the loosening of cell wall connections between cells (Monselise et al., 1976). When the pectins and hemicelluloses decrease to 70–80% of total polysaccharides, symptoms of creasing occurred (Jona et al., 1989). Similarly, Li et al. (2009) claimed that loss of pectins and higher activities of the cell wall degrading enzymes such as PE, PG and cellulose and expression of α–expansin genes in the albedo tissues is also associated with creasing in sweet orange. Recently, Saleem et al. (2014) reported the higher activities of PE, *exo*-PG, *endo*-PG, and EGase in the albedo tissues of creased Washington Navel and Navelina sweet orange fruit at commercial harvest seem to enhance the loss of pectins in the cell walls of the albedo, leading to cell wall loosening and formation of cracks and consequently reduced hardness, stiffness and tensile force of the rind. The higher levels of endogenous ethylene in the albedo tissues of creased fruit compared with normal fruit in sweet orange cv. Valencia (Monselise et al., 1976) and Washington Navel (Pham, 2009), indicate a key role of endogenous ethylene in creasing. Recently, higher levels of endogenous ethylene and lower levels of polyamines in the albedo tissues of the creased fruit than normal fruit in different cultivars of sweet orange coupled with a reduction in incidence of creasing with exogenous application of ethylene inhibitors (AVG, $CoSO_4$ or PUT), suggest the involvement of ethylene and polyamines in creasing of sweet orange. No information is available however, on how exogenous application of AVG, CoSO4 or PUT reduces the incidence of creasing in sweet orange fruit. We hypothesised that reduction in the incidence of creasing in sweet orange fruit with the exogenous application of AVG, $CoSO_4$ or PUT occurs through modulating the activities of cell wall degrading enzymes in the albedo and flavedo tissues of the fruit. We investigated the mode of reduction of creasing with the

exogenous application of PUT, AVG or $CoSO_4$ through determining the changes in the levels of the total, water soluble and water insoluble pectins as well as the activities of cell wall degrading enzymes such as PE, *exo*-PG, *endo*-PG, and EGase in the albedo and the flavedo tissues of the fruit at the golf ball and ripe stage.

9.2. Materials and methods

9.2.1. Plant material

Two different experiments were conducted on cv. Washington Navel and Lane Late sweet orange in a commercial citrus orchard located at Gingin (Latitude 31° 21′ South, Longitude 155° 55′ East), Western Australia. All the experimental trees received similar cultural practices including fertilisers, irrigation, weed control and plant protection except for experimental treatments. The row direction was from North-South, distance between row to row 7.5 m and plant to plant distance 2.7 m. The experimental tree was grown on sandy loam soil with mild, rainywinters and dry, hot summers.

9.2.2. Experiment I: Effects of exogenous spray application of PUT, AVG or $CoSO_4$ on the comparative levels of pectin and cell wall degradation enzymes in the albedo and flavedo tissues of Washington Navel sweet orange fruit

An aqueous solution containing PUT (1000 μM), AVG (60 mg L^{-1}) or $CoSO_4$ (500 mg L^{-1}) with 0.05% 'Tween 20' as a surfactant were sprayed on to the whole tree until run off at fruit set stage (fruit diameter: 15±5 mm) or colour break stage (fruit diameter: 80±5 mm) on sweet orange cv. Washington Navel by using a sprayer (The Selecta Trolleypak Mk II, Acacia Ridge, Australia). These selected concentrations of PUT, AVG or $CoSO_4$ were most effective in reducing creasing in sweet orange fruit based upon our previous experiments. Control trees were kept unsprayed. This experiment was conducted during 2012. The experimental lay out was randomised block design with two-factor factorial (treatments and stage of growth for spray application) with a single tree as the experimental unit which was replicated four times. Ten fruit per replication were randomly harvested at the golf ball (which were sprayed at fruit set) and ripe stage (sprayed at the colour break stage) to determine the levels of total pectin, water soluble pectin and insoluble pectin as well as the comparative changes in the activities

of the cell wall degrading enzymes including PE, *endo*-PG, *exo*-PG and EGase in the albedo and flavedo tissues of the fruit.

9.2.3. Experiment II: Effects of exogenous application of PUT, AVG or $CoSO_4$ on the comparative levels of pectin and cell wall degradation enzymes of sweet orange cv. Lane Late

A second experiment was conducted on cv. Lane Late employing the same treatments including PUT (1000 µM), AVG (60 mg L^{-1}) or $CoSO_4$ (500 mg L^{-1}) and experimental design. Ten fruit per replication were harvested randomly from each tree at the golf ball and ripe stage to determine the levels of total pectin, water soluble pectin and insoluble pectin as well as the comparative changes in activities of the cell wall degrading enzymes including PE, *endo*-PG, *exo*-PG and EGase in the albedo and flavedo tissues of the fruit.

9.2.4. Determination of total, water soluble and water insoluble pectins

Total and water soluble pectins from albedo and flavedo tissues of sweet orange cv. Washington Navel and Lane Late were determined by following the method detailed earlier by Wang et al. (2008). The water insoluble pectin was determined by subtracting total pectin from water soluble pectin. Total pectin, water soluble and water insoluble pectins were expressed as galacturonic acid equivalents and express as ($mg.g^{-1}$).

9.2.5. Protein determination

Protein contents from the albedo or flavedo tissues of fruit rind were estimated using the method of Bradford (1976). The protein concentration was determined by using a standard curve of bovine serum albumin and was expressed as $mg.g^{-1}$ FW.

9.2.6. Determination of exo-PG, endo-PG, EGase and PE activities in the albedo and flavedo tissue of the fruit

The activities of cell wall degrading enzymes including *exo*-PG, *endo*-PG, EGase and PE in the albedo and flavedo tissues of orange fruit were determined by following the method detailed earlier by Khan and Singh, (2007). The activity of *exo*-PG was expressed as µg galacturonic acid mg $protein^{-1}$ h^{-1} and *endo*-PG and EGase as viscosity changes mg $protein^{-1}$ h^{-1}. The activity of PE was expressed as mM NaOH mg $protein^{-1}$ h^{-1}.

9.2.7. Statistical analysis

The data were subjected to two-way analysis of variance (ANOVA), using GenStat 14th edition (Lawes Agricultural Trust, Rothamsted Experimental Station, Rothamsted, UK). The effects of various treatments, time of application and their interactions were assessed within ANOVA and least significant differences (Duncan, LSD) were calculated following significant ($P \leq 0.05$) F-test. All the assumptions of analysis were checked to ensure validity of statistical analysis.

9.3. Results

9.3.1. Effect of exogenous application of PUT, AVG and $CoSO_4$ on the levels of total pectin of sweet orange cv. Washington Navel and Lane Late

The spray application of PUT (1000 µM), AVG (60 mg L^{-1}) or $CoSO_4$ (500 mg L^{-1}) applied at the fruit set or at the colour break stage significantly ($P \leq 0.05$) increased the levels of total pectins in the albedo and flavedo tissues of the fruit at the golf ball and ripe stage as compared to the control in cv. Washington Navel and Lane Late (Table 9.1). When averaged over stage of application, the spray application of PUT, AVG or $CoSO_4$ significantly ($P \leq 0.05$) increased the mean levels of total pectins in the albedo and flavedo tissues of the fruit as compared to the control in cv. Washington Navel and Lane Late. When averaged over all the treatments, the application of spray treatments at the colour break stage was significantly ($P \leq 0.05$) more effective in increasing mean total pectins in the albedo and flavedo tissues at the ripe stage than golf ball in which fruit were treated at the fruit set stage, in both the cultivars. The interactions between treatments and stage of their application were found to be significant ($P \leq 0.05$) for total pectins in the albedo and flavedo tissues in both cultivars.

Table 9.1. Effect of exogenous application of PUT, AVG or $CoSO_4$ at fruit set or colour break stage on the levels of total pectin in the albedo and flavedo tissues of the fruit at the golf ball and ripe stage in cv. Washington Navel and Lane Late

Total Pectins ($mg.g^{-1}$)						
Washington Navel						
	Albedo tissues			Flavedo tissues		
Treatments	Golf ball	Ripe stage	Means (Treat)	Golf ball	Ripe stage	Means (Treat)
Control	87.9 f	125.2 c	106.6 c	84.5 e	114.0 c	99.2 d
PUT.1000 µM	96.2 ef	206.9 a	151.5 ab	118.0 cd	123.1 cd	120.6 c
AVG.60 mg L^{-1}	103.0 de	216.5 a	159.70 a	99.9 de	217.5 a	158.7 b
$CoSO_4$.500 mg L^{-1}	108.9 d	179.9 b	144.40 b	165.3b	181.7 b	173.5 a
Means (Stage)	99.0 b	182.1a		116.9 b	162.83 a	
LSD ($P \leq 0.05$)						
Treatments	8.3			14.8		
Stage	5.9			10.5		
Treatments × stage	11.8			21.0		
Lane Late						
	Albedo tissues			Flavedo tissues		
Treatments	Golf ball	Ripe stage	Means (Treat)	Golf ball	Ripe stage	Means (Treat)
Control	99.2 f	118.5 d	108.8 d	80.7 f	104.7 e	92.7 c
PUT.1000 µM	100.1 f	181.0 a	140.5 a	130.1 c	136.6 bc	133.3 b
AVG.60 mg L^{-1}	116.6 d	137.4 b	127.0 b	154.1 a	138.2 bc	146.1 a
CoSO4.500 mg L^{-1}	108.2 e	128.9 c	118.6 c	119.3 d	141.1 b	130.2 b
Means (Stage)	106.0 b	141.5 a		121.0 b	130.2 a	
LSD ($P \leq 0.05$)						
Treatments	4.8			6.4		
Stage	3.4			4.5		
Treatments × stage	6.8			9.1		

n = 4 replications (10 fruit per replication); any two mean within a column and within a row followed by different letters are significantly different at $P \leq 0.05$; Treat= Treatments; ns = non-significant

9.3.2. Effect of exogenous application of PUT, AVG and $CoSO_4$ on the levels of water soluble pectins of sweet orange cv. Washington Navel and Lane Late

The levels of water soluble pectins in the albedo and flavedo tissues of the fruit at the golf ball and ripe stage significantly ($P \leq 0.05$) decreased with the spray application of PUT (1000 µM), AVG (60 mg L^{-1}) or CoSO4 (500 mg L^{-1}) applied at the fruit set or colour beak stage as compared to the control in cv. Washington Navel and Lane Late (Table 9.2). When averaged over stage of spray application, the mean levels of the water soluble pectins were significantly ($P \leq 0.05$) reduced in the albedo and flavedo tissues of the fruit with all treatments as compared to the control in cv. Washington Navel and Lane Late. When averaged over treatments, mean levels of water soluble pectins were significantly lower in the albedo and flavedo tissues at the golf ball and ripe stage of the fruit which were treated at fruit set than at the colour break stage in both cultivars. The interaction between treatments and stage of their spray application was found to be significant ($P \leq 0.05$) for water soluble pectins in the albedo and flavedo tissue of fruit in cv. Washington Navel and Lane Late.

9.3.3. Effect of exogenous application of PUT, AVG and $CoSO_4$ on levels of water insoluble pectin of sweet orange cv. Washington Navel and Lane Late

All spray application treatments of PUT (1000 µM), AVG (60 mg L^{-1}) or $CoSO_4$ (500 mg L^{-1}) at the fruit set stage or the colour break stage significantly ($P \leq 0.05$) increased levels of water insoluble pectins in the albedo and flavedo tissues of the fruit compared to the control in cv. Washington Navel and Lane Late (Table 9.3). When averaged over all treatment stages, the mean levels of water insoluble pectins significantly ($P \leq 0.05$) increased in the albedo and flavedo tissues of fruit with the treatment of PUT (1000 µM), AVG (60 mg L^{-1}) or CoSO4 (500 mg L^{-1}) compared to the control in cv. Washington Navel and Lane Late, respectively. When averaged over all treatments, spray application at the colour break stage resulted in significantly ($P \leq 0.05$) higher levels of mean water insoluble pectin in the albedo and flavedo tissues of fruit at the ripe stage than at the golf ball stage, which were treated at the fruit set in cv. Washington Navel. In cv. Lane Late, mean levels of water insoluble pectins in the albedo tissue of the fruit at the ripe stage compared to the golf ball stage was significantly ($P \leq 0.05$) higher with spray application of treatments at the colour break stage than at the fruit set stage. In general, the water insoluble pectins were significantly ($P \leq 0.05$) higher in the albedo tissues than the flavedo tissues of the fruit in cv. Washington

Table 9.2. Effect of exogenous application of PUT, AVG or $CoSO_4$ at fruit set or colour break stage on the levels of water soluble pectin in the albedo and flavedo tissues of the fruit at the golf ball and ripe stage in cv. Washington Navel and Lane Late.

Water soluble pectins ($mg.g^{-1}$)						
Washington Navel						
	Albedo tissues			Flavedo tissues		
Treatments	Golf ball	Ripe stage	Means (Treat)	Golf ball	Ripe stage	Means (Treat)
Control	14.4 e	43.2 a	28.8 a	13.2 de	34.5 a	23.9 a
PUT.1000 µM	6.9 g	18.1 d	12.5 d	8.6 f	14.7 d	11.7 d
AVG.60 mg L^{-1}	12.1f	33.1 b	22.6 b	10.6 ef	29.8 b	20.2 b
$CoSO_4$.500 mg L^{-1}	11.4 f	29.7 c	20.6 c	8.6 f	22.8 c	15.7 c
Means (Stage)	11.2 b	31.0 a		10.3 b	25.5a	
LSD ($P \leq 0.05$)						
Treatments	1.0			2.1		
Stage	0.7			1.5		
Treatments × stage	1.4			3.0		
Lane Late						
	Albedo tissues			Flavedo tissues		
Treatments	Golf ball	Ripe stage	Means (Treat)	Golf ball	Ripe stage	Means (Treat)
Control	16.5 d	28.0 a	22.2 a	14.8 c	22.2 a	18.5 a
PUT.1000 µM	16.0 d	23.9 bc	20.0 b	8.7 e	16.6 b	12.7 d
AVG.60 mg L^{-1}	15.1 de	23.4 c	19.3 b	11.6 d	17.1 b	14.3 c
CoSO4.500 mg L^{-1}	13.2 e	26.4 ab	19.8 b	12.7 d	21.6 a	17.2 b
Means (Stage)	15.2 b	25.4 a		11.2 b	19.4 a	
LSD ($P \leq 0.05$)						
Treatments	1.9			1.3		
Stage	1.3			0.9		
Treatments × stage	2.6			1.9		

n = 4 replications (10 fruit per replication); any two mean within a column and within a row followed by different letters are significantly different at $P \leq 0.05$; Treat= Treatments; ns = non-significant

Table 9.3. Effect of exogenous application of PUT, AVG or $CoSO_4$ at fruit set or colour break stage on the levels of water soluble pectin in the albedo and flavedo tissues of the fruit at the golf ball and ripe stage in cv. Washington Navel and Lane Late.

Water insoluble pectins ($mg.g^{-1}$)						
Washington Navel						
	Albedo tissues			Flavedo tissues		
Treatments	Golf ball	Ripe stage	Means (Treat)	Golf ball	Ripe stage	Means (Treat)
Control	73.5 g	107.1d	90.3 c	71.3e	79.5 d	79.9 c
PUT.1000 µM	89.3 f	163.7 b	126.5 b	109.4 c	108.4 c	104.3 b
AVG.60 mg L^{-1}	90.9 f	183.4 a	137.1 a	89.3d e	187.6 a	146.0 a
$CoSO_4$.500 mg L^{-1}	97.6 e	150.2 c	123.9 b	156.7 b	159.0b	157.8 a
Means (Stage)	87.8 b	151.1 a		106.7 b	133.6 a	
LSD ($P \leq 0.05$)						
Treatments	8.0			12.3		
Stage	5.7			8.7		
Treatments × stage	11.4			17.4		
Lane Late						
	Albedo tissues			Flavedo tissues		
Treatments	Golf ball	Ripe stage	Means (Treat)	Golf ball	Ripe stage	Means (Treat)
Control	82.7 e	90.5 d	86.6 d	66.0 e	82.5 d	74.2 d
PUT.1000 µM	84.1 e	157.1 a	120.6 a	121.4 b	120.0 b	120.7 b
AVG.60 mg L^{-1}	101.4 c	114.0 b	107.7 b	142.5 a	121.1 b	131.8 a
CoSO4.500 mg L^{-1}	95.0 d	102.6 c	98.8 c	106.6 c	119.4 b	113.0 c
Means (Stage)	90.8 b	116.0 a		109.1 a	110.8 a	
LSD ($P \leq 0.05$)						
Treatments	4.2			6.8		
Stage	3.0			ns		
Treatments × stage	6.0			9.6		

n = 4 replications (10 fruit per replication); any two mean within a column and within a row followed by different letters are significantly different at $P \leq 0.05$; Treat= Treatments; ns = non-significant

Navel and Lane Late. The interaction between treatments and stages of their application were found to be significant ($P \leq 0.05$) for water insoluble pectins in the albedo and flavedo tissue of fruit in cv. Washington Navel and Lane Late.

9.3.4. Effect of exogenous application of PUT, AVG and $CoSO_4$ on activities of the EGase enzyme at the golf ball and ripe stage in cv. Washington Navel and. Lane Late

The activity of EGase was reduced in the albedo and the flavedo tissues of fruit at golf ball and ripe stage with spray application of PUT (1000 µM), AVG (60 mg L^{-1}) or $CoSO_4$ (500 mg L^{-1}) at the fruit set or colour beak stage in cv. Washington Navel and cv. Lane Late (Table 9.4). When averaged over stage of treatment application, the mean activity of EGase was significantly ($P \leq 0.05$) reduced in the albedo and the flavedo tissues of fruit with spray application of PUT (1000 µM), AVG (60 mg L^{-1}) or $CoSO_4$ (500 mg L^{-1}) in cv. Washington Navel and Lane Late (Table 9.4). Averaged over all treatments, spray application at the colour break stage significantly ($P \leq 0.05$) reduced the mean activity of EGase in the albedo tissues of fruit at the ripe stage than the golf ball stage which is treated at the fruit set stage, in both cultivars. The interaction between treatments and their stage of application was found to be significant ($P \leq 0.05$) for the activity of EGase in the albedo tissues in cv. Washington Navel and the flavedo tissues of fruit in cv. Lane Late.

9.3.5 Effect of exogenous application of PUT, AVG and $CoSO_4$ on the activities of endo-PG and exo-PG at the golf ball and the ripe stage of cv. Washington Navel

Treatment of PUT (1000 µM), AVG (60 mg L^{-1}) or $CoSO_4$ (500 mg L^{-1}) applied at fruit set or colour beak stage significantly ($P \leq 0.05$) reduced the activity of *endo*-PG and *exo*-PG in the albedo and flavedo tissues of fruit at golf ball and ripe stage in cv. Washington Navel and Lane Late (Table 9.5 and 9.6). When averaged over stage of spray application, all treatments significantly ($P \leq 0.05$) reduced the mean activity of *endo*-PG than in the control in the albedo and flavedo tissues of Washington Navel fruit. The mean activity of *endo*-PG was significantly ($P \leq 0.05$) reduced with spray application of all treatments than in the control in the albedo tissues of Lane Late fruit. The spray application of PUT significantly ($P \leq 0.05$) reduced the mean activity of *endo*-PG as compared to the control and all other treatments in the flavedo tissues of Lane Late fruit. However, the spray application of PUT or AVG significantly ($P \leq 0.05$) reduced the mean activity of *endo*-PG than the spray application of $CoSO_4$ and in the control in the albedo tissues of Washington Navel fruit. When averaged over all

Table 9.4. Effect of exogenous application of PUT, AVG or $CoSO_4$ at the fruit set or the colour break stage on the activity of E-Gase in the albedo and flavedo tissues of the fruit at golf ball and ripe stage in cv. Washington Navel and Lane Late.

E-Gase (Viscosity changes mg^{-1}protein h^{-1})						
Washington Navel						
	Albedo tissues			Flavedo tissues		
Treatments	Golf ball	Ripe stage	Means (Treat)	Golf ball	Ripe stage	Means (Treat)
Control	27.3 bc	41.6 a	34.5 a	17.4 ab	22.0 a	19.7 a
PUT.1000 µM	15.6 e	27.6 bc	21.6 b	15.5 b	13.9 b	14.7 b
AVG.60 mg L^{-1}	20.1 de	31.9 b	26.0 b	14.0 b	15.2 b	14.6 b
$CoSO_4$.500 mg L^{-1}	24.5 cd	27.8 bc	26.2 b	11.2 b	14.0 b	12.6 b
Means (Stage)	21.9 b	32.2 a		14.5 a	16.3 a	
LSD ($P \leq 0.05$)						
Treatments	4.70			3.92		
Stage	3.32			ns		
Treatments × stage	6.65			ns		
Lane Late						
	Albedo tissues			Flavedo tissues		
Treatments	Golf ball	Ripe stage	Means (Treat)	Golf ball	Ripe stage	Means (Treat)
Control	34.3 b	39.4 a	36.8 a	17.8 b	23.4 a	20.6 a
PUT.1000 µM	16.6 de	20.8 cd	18.7 c	13.4 bcd	11.5 cd	12.5 b
AVG.60 mg L^{-1}	13.0 e	19.5 cd	16.3 c	16.6 bc	15.4 bcd	16.0 b
CoSO4.500 mg L^{-1}	21.3 c	23.6 c	22.4 b	10.7 d	17.4 b	14.1 b
Means (Stage)	21.3 b	25.8 a		14.6 a	16.9 a	
LSD ($P \leq 0.05$)						
Treatments	4.7			3.9		
Stage	3.3			ns		
Treatments × stage	6.7			ns		

n = 4 replications (10 fruit per replication); any two mean within a column and within a row followed by different letters are significantly different at $P \leq 0.05$; Treat= treatments; ns = non-significant

Table 9.5 Effect of exogenous application of PUT, AVG or $CoSO_4$ at the fruit set or the colour break stage on the activity of endo-PG in the albedo and flavedo tissues of fruit at the golf ball and ripe stage in cv. Washington Navel and Lane Late.

Endo-PG (Viscosity changes mg^{-1}protein h^{-1})						
Washington Navel						
	Albedo tissues			Flavedo tissues		
Treatments	Golf ball	Ripe stage	Means (Treat)	Golf ball	Ripe stage	Means (Treat)
Control	25.1 b	35.3 a	30.2 a	18.2 ab	21.8a	20.0 a
PUT.1000 µM	16.7 b	17.2 b	17.0 c	10.2 d	12.1 cd	11.2 b
AVG.60 mg L^{-1}	20.1 b	17.5b	18.8 b c	10.6 d	17.1 abc	13.9 b
$CoSO_4$.500 mg L^{-1}	22.7 b	25.8 b	24.3 ab	14.5 bcd	15.3 bcd	14. 9 b
Means (Stage)	21.2 a	24.0 a		13.4 b	16.6 a	
LSD ($P \leq 0.05$)						
Treatments	6.5			3.8		
Stage	ns			2.6		
Treatments × stage	ns			ns		
Lane Late						
	Albedo tissues			Flavedo tissues		
Treatments	Golf ball	Ripe stage	Means (Treat)	Golf ball	Ripe stage	Means (Treat)
Control	21.6 bc	38.4 a	30.0 a	20.9 a	19.8 ab	20.3 a
PUT.1000 µM	10.8 d	20.0 bc	15.4 b	14.0 ab	12.9 b	13.5 b
AVG.60 mg L^{-1}	17.3 bcd	24.6 b	20.9 b	16.7 ab	14.9 ab	15.8 ab
CoSO4.500 mg L^{-1}	15.1 cd	21.9 bc	18.5 b	18.8 ab	15.3 ab	17.1 ab
Means (Stage)	16.2 b	26.2 a		17.6 a	15.7 a	
LSD ($P \leq 0.05$)						
Treatments	5.5			4.8		
Stage	3.9			ns		
Treatments × stage	ns			ns		

n = 4 replications (10 fruit per replication); any two mean within a column and within a row followed by different letters are significantly different at $P \leq 0.05$; Treat= Treatments; ns = non-significant

Table 9.6. Effect of exogenous application of PUT, AVG or $CoSO_4$ at fruit set or colour break stage on the activity of Exo-PG in the albedo and flavedo tissues of the fruit at golf ball and ripe stage in cv. Washington Navel and cv. Lane Late.

Exo-PG (µg galacturonic acids mg^{-1}preoteinh^{-1})						
Washington Navel						
	Albedo tissues			Flavedo tissues		
Treatments	Golf ball	Ripe stage	Means (Treat)	Golf ball	Ripe stage	Means (Treat)
Control	28.8 c	69.6 a	49.2 a	27.9 a	17.4 cd	22.7 a
PUT.1000 µM	19.1 c	50.2 b	34.7 b	19.2 c	14.1d	16.6 c
AVG.60 mg L^{-1}	20.7 c	51.5 b	36.1 b	21.4 bc	14.4 d	17.9 bc
$CoSO_4$.500 mg L^{-1}	24.0 c	56.5 b	40.3 b	25.3 ab	16.7 cd	21.0 ab
Means (Stage)	23.2 b	57.0 a		23.5 a	15.6 b	
LSD ($P \leq 0.05$)						
Treatments	6.77			3.15		
Stage	4.79			2.22		
Treatments × stage	ns			ns		
Lane Late						
	Albedo tissues			Flavedo tissues		
Treatments	Golf ball	Ripe stage	Means (Treat)	Golf ball	Ripe stage	Means (Treat)
Control	57.3 de	109.8 a	83.5 a	30.9 b	44.5 a	37.7 a
PUT.1000 µM	38.1 f	63.4 cd	50.8 c	23.0 bcd	19.3 d	21.2 c
AVG.60 mg L^{-1}	42.6 f	72.0 c	57.3 c	20.7 cd	23.5 bcd	22.1 c
CoSO4.500 mg L^{-1}	47.3 ef	90.0 b	68.6 b	28.7bc	28.9 bc	28.8 b
Means (Stage)	46.3 b	83.8 a		25.8	29.1	
LSD ($P \leq 0.05$)						
Treatments	7.02			5.31		
Stage	4.97			ns		
Treatments × stage	9.93			7.512		

n = 4 replications (10 fruit per replication); any two mean within a column and within a row followed by different letters are significantly different at $P \leq 0.05$; Treat= Treatments; ns = non-significant

the treatments, all spray applications at the colour break stage significantly ($P \leq 0.05$) reduced the mean activity of *endo*-PG in the albedo and flavedo tissues of fruit at the ripe stage than the golf ball stage which were treated at the fruit set stage in cv. Lane Late and Washington Navel, respectively. The interaction between treatments and their stage of application was found to be non-significant ($P \leq 0.05$) for *endo*-PG activity in the albedo and flavedo tissue of fruit at the golf ball and ripe stage in cv. Washington Navel and Lane Late. In the flavedo tissue, spray application of PUT and AVG significantly reduced the mean activity of *exo*-PG as compared to the control and $CoSO_4$ in cv. Washington Navel. In cultivar Lane Late, spray application of PUT, AVG or $CoSO_4$ significantly ($P \leq 0.05$) reduced the mean activity of *exo*-PG as compared to the control in the albedo and flavedo tissues of the fruit. When averaged over treatments, the mean activity of *exo*-PG was significantly ($P \leq 0.05$) higher in the albedo and flavedo tissues at the ripe stage than the golf ball stage which were sprayed at the colour break stage than at the fruit set stage in cv. Washington Navel. In cv. Lane Late, the mean activity of *exo*-PG was significantly ($P \leq 0.05$) higher (37.5 fold) at the ripe stage than golf ball stage in the albedo tissues of the fruit, which were sprayed at the colour break stage than at the fruit set stage. The interaction between treatments and stage of their spray application was found to be significant ($P \leq 0.05$) for the activity of *exo*-PG in the albedo and flavedo tissues in cv. Lane Late and non-significant ($P \leq 0.05$) in the albedo and flavedo tissues of fruit in cv. Washington Navel.

9.3.6. Effect of exogenous application of PUT, AVG and $CoSO_4$ onactivities of PE at the golf ball and the ripe stage of sweet orange cv. Washington Navel and Lane Late

The activity of PE in the albedo and flavedo tissues of fruit at the golf ball and ripe stage significantly ($P \leq 0.05$) reduced with spray application of PUT (1000 µM), AVG (60 mg L^{-1}) or $CoSO_4$ (500 mg L^{-1}) at the fruit set or the colour break stage in cv. Washington Navel and Lane Late (Table 9.7). When averaged over stages of treatments application, the treatments of PUT, AVG or $CoSO_4$ significantly ($P \leq 0.05$) reduced the mean activity of PE than for the control in the albedo and flavedo tissue of cv. Washington Navel fruit and flavedo tissue of Lane Late fruit. In the albedo tissues of Lane Late fruit, spray application of PUT or AVG significantly ($P \leq 0.05$) reduced the mean activity of PE as compared to the control and $CoSO_4$ treatment. All the spray applications at the colour break stage resulted in significantly ($P \leq 0.05$) higher mean activity of PE in the albedo and flavedo tissues of the fruit at the ripe stage in cv. Washington Navel and in the albedo tissue of Lane Late fruit compared to the golf ball stage, which were treated at fruit set stage.

Table 9.7. Effect of exogenous application of PUT, AVG or $CoSO_4$ at fruit set or colour break stage on the activity of PE in the albedo and flavedo tissues of the fruit at the golf ball and ripe stage in cv. Washington Navel and Lane Late.

PE (mM NaOH mg^{-1} protein h^{-1})						
Washington Navel						
	Albedo tissues			Flavedo tissues		
Treatments	Golf ball	Ripe stage	Means (Treat)	Golf ball	Ripe stage	Means (Treat)
Control	0.27 bc	0.35 a	0.31 a	0.21 b	0.27 a	0.24 a
PUT.1000 µM	0.20 e	0.22 de	0.21 c	0.13 e	0.17 cd	0.15 d
AVG.60 mg L^{-1}	0.25 cd	0.26 bc	0.25 b	0.16 d	0.18 cd	0.17 c
$CoSO_4$.500 mg L^{-1}	0.23 cde	0.28 b	0.26 b	0.18 c	0.18 c	0.18 b
Means (Stage)	0.24 b	0.28 a		0.17 b	0.20 a	0.24 b
LSD ($P \leq 0.05$)						
Treatments	0.03			0.01		
Stage	0.02			0.01		
Treatments × stage	ns			0.02		
Lane Late						
	Albedo tissues			Flavedo tissues		
Treatments	Golf ball	Ripe stage	Means (Treat)	Golf ball	Ripe stage	Means (Treat)
Control	0.31 ab	0.32 a	0.32 a	0.21 b	0.2534a	0.23 a
PUT.1000 µM	0.19 d	0.26 c	0.22 c	0.18 c	0.13 d	0.15 c
AVG.60 mg L^{-1}	0.24 c	0.29 b	0.26 b	0.20 b	0.16 c	0.18 b
CoSO4.500 mg L^{-1}	0.34 a	0.32 a	0.33 a	0.17c	0.18 c	0.17 b
Means (Stage)	0.27 b	0.30 a		0.19	0.18	
LSD ($P \leq 0.05$)						
Treatments	0.02			0.02		
Stage	0.02			ns		
Treatments × stage	0.03			0.02		

n = 4 replications (10 fruit per replication); any two mean within a column and within a row followed by different letters are significantly different at $P \leq 0.05$; Treat= Treatments; ns = non-significant

The interaction between treatments and the stage of spray application was found to be significant ($P \leq 0.05$) for the activity of PE in the albedo and flavedo tissues of Lane Late fruit and in the flavedo tissue of Washington Navel fruit.

9.4. Discussion

The development of creasing in sweet oranges has been associated with earlier senescence of the albedo tissue in which the albedo tissue becomes gradually spongier with the progress of fruit maturation and ripening and is the site of rupture characteristics of the creasing disorder (Monselise et al., 1976). The incidence of creasing has been reported to be partially controlled at an early stage of orange fruitlet growth with the exogenous application of gibberellins and mineral nutrients. Recently, Saleem et al. (2014) claimed that higher activities of PE, *exo*- PG, *endo*- PG, and EGase in the albedo of creased fruit at commercial harvest seem to be associated with enhanced loss of pectins and starch in the cell walls of albedo tissue, leading to cell wall loosening and formation of cracks consequently reducing hardness, stiffness and tensile strength of the rind. Our recent research work showed a substantial reduction in the incidence of creasing with the exogenous application of PUT, AVG or $CoSO_4$ at the golf ball stage or at the colour break stage and the present paper focuses on how these treatments have reduced the incidence of creasing in sweet orange.

All the treatments of PUT, AVG or $CoSO_4$ significantly increased the total and water insoluble pectin and decreased water soluble pectin in the albedo and flavedo tissues of fruit at the golf ball and ripe stage than for the control in cv. Washington Navel and Lane Late. The total pectin and insoluble pectin was higher in the flavedo tissue than the albedo tissues except water soluble pectins which were higher in the albedo tissues at the golf ball stage in cv. Washington Navel (Table 9.1-9.3). The plant cell wall is composed of polysaccharides such as cellulose, hemicelluloses and pectins, enzymes and structural proteins. The pectin is mostly produced during cell wall growth of both dicotyledonous and monocotyledonous plants and makes 35% of the dry weight of their cell wall (Hoff and Castro, 1969). The plant cell wall is a complex structure involved in cell size and shape, growth and development, intercellular communication, and interaction with the environment (Glickman et al., 1969). In mature fruit, pectins are bound with cellulose in the cell wall and are insoluble. However, during ripening the structure of pectin is changed by naturally occurring enzymes like PE, *endo*- and *exo*-PG (Yoo et al., 2003; Kashyap et al., 2001). Similarly, the increased levels of

water soluble pectins in the rind of creased sweet orange fruit have also been reported previously by Saleem et al. (2014); Li et al. (2009), Pham, (2009) and Monselise et al. (1976).

The exogenous spray application of PUT, AVG or $CoSO_4$ at the fruit set or colour break stage significantly reduced the activity of cell wall degrading enzymes such as PE, *endo*- and *exo*-PG and PG as compared to the control (Table 9.5-9.7). The reduction in the activity of cell wall degrading enzymes in albedo and flavedo tissue of the fruit with the exogenous application of PUT, AVG or $CoSO_4$ may be ascribed to the direct effects of these compounds. It may also be argued that exogenous application of these compounds has inhibited the ethylene biosynthesis consequently reducing the activity of cell wall degrading enzymes including PE, *endo*- and *exo*-PG and PG as compared to the control. Recently, we have also reported higher levels of endogenous ethylene in creased fruit. Promotion of the creasing incidence with the exogenous application of ethrel and its reduction with the application of ethylene inhibitors suggests the involvement of ethylene in causation of creasing in sweet orange fruit (Pham, 2009). Possibly, the exogenous spray application of PUT may increase levels of free polyamines in the albedo and flavedo tissue resulting in inhibition of ethylene production and consequently reduced activity of cell wall degrading enzymes including PE, *endo*- and *exo*-PG, EGase and incidence of creasing (Chapter 6). Ethylene plays a key role in accelerating ripening and softening of fruit (Hadfield et al., 2000; Ayub et al., 1996) and accordingly, the expression of some ripening-related cell wall-associated genes and their activities, including PGs (Hiwasa et al., 2003; Sitrit and Bennett, 1998), expansins (Rose et al., 1997) and EGase (Lashbrook et al., 1994).

It has been previously reported that cell wall degrading enzymes such as PG, PE and EGase are mainly responsible for ripening, pectin degradation and fruit softening (Brady, 1987; Huber, 1983). PE catalyses the softening process through desertification of pectin by PG (Roe and Bruemmer, 1981). The PE plays an important role in the breakdown of the pectin chain; in other cases pectin becomes more soluble during the softening process and its grip on the surrounding cell walls loosens and plant tissues soften (Yang et al 2008; Kesta et al., 1999) and are considered to be the basic cause of fruit softening during ripening. When the amount of pectins and hemicelluloses decreased to 70–80% of total polysaccharides, symptoms of creasing occurred (Jona et al., 1989). Similarly, Li et al. (2009) stated that loss of pectins and higher activities of cell wall degrading enzymes such as PE, PG and cellulose and expression of α–expansin genes in the albedo tissues is also associated with creasing of

sweet orange. In plants, PE plays an important role in cell wall metabolism through changes in the cell wall composition (Yang et al., 2008) with solubilisation of pectin (Kesta et al., 1999).

During fruit softening, pectin and hemicellulose typically undergo solubilisation and depolymerisation, which are involved in cell wall loosening and disintegration (Payasi et al., 2009; Wakabayashi, 2000; Fisher and Bennett, 1991). The cell wall hydrolase enzymes cellulose, *endo*- and *exo*-PG have been reported to play an important role in fruit softening (Brummell and Harpster, 2001). Creasing is similar to fruit softening caused by cell wall disassembly (Nishiyama et al., 2007; Brummel, 2006; Orfila et al., 2002). Higher activities of PE, PG and EGase may be involved in loss of pectins and cellulose in the cell wall of sweet orange, which cause cell separation in the albedo tissue resulting in grooves on the surface of the flavedo, which is a typical symptom of creasing. Similarly, Li et al. (2009) also confirmed that creasing has a linear correlation between the activities of PG and cellulose in sweet orange cv. An Liu. Recently, Saleem et al. (2014) reported that higher activities of PE, *exo*-PG, *endo*-PG, and EGase in the albedo of creased fruit at commercial harvest seem to enhance the loss of pectin in the cell walls of the albedo, leading to cell wall loosening and formation of cracks on the rind of sweet orange cv. Navelina and Washington Navel. In conclusion, the exogenous spray application of PUT, AVG or CoSO4 significantly increased the total and water insoluble pectin, decreased water soluble pectin through decreased activity of cell wall degrading enzymes such as PE, *endo* and*exo*-PG and PG in the albedo and flavedo tissues of fruit as compared to the control leading to reduction in the incidence of creasing in sweet orange.

CHAPTER 10

General discussion, conclusion and future research

10.1. Introduction

The albedo tissue of citrus fruit is prone to fracturing and leads to rind disorders such as creasing (albedo breakdown) in sweet oranges (Monselise et al., 1976) and puffiness in mandarins (García-Luis et al., 1985). The recurring incidence of creasing causes considerable losses to the sweet orange growers of the world including Australia (Li et al., 2009; Bower, 2004; Ali et al., 2000; Pellizo, 1997). Spray applications of GA_3 (20 mg L^{-1}) and calcium (0.33%) at the golf ball stage have been reported to reduce the incidence of creasing in sweet orange with variable success (Pham et al., 2012; Greenberg et al., 2006; Treeby and Storey et al., 2002; Gilfillan et al., 1981). Genotype, climate, rootstock, crop load, rind thickness, irrigation and mineral nutrition have been implicated as its causal factors (Monselise et al., 1976; Jones et al. 1967). Recently, higher activities of pectinesterase (PE), *exo*-polygalacturonase (*exo*-PG), *endo*-polygalacturonase (*endo*-PG), and Endo-1, 4-*ß* -D-glucanase (EGase) in the albedo of creased sweet orange fruit at harvest resulted in enhanced loss of pectins and starch in the cell walls of albedo tissues, leading to cell wall loosening and formation of cracks and consequently reduced hardness and tensile strength of the rind have been reported (Saleem et al., 2014). Visible symptoms of creasing on the rind of sweet orange fruit appears mainly after fruit maturation and is coupled with the ripening and/or over ripening process, which also involves cell separation (Monselise et al., 1976; Embleton et al., 1973; Jones et al., 1967). Some preliminary and sporadic reports suggested elevated levels of endogenous ethylene are associated with creasing in Valencia and Washington Navel sweet orange (Pham, 2009; Monselise et al., 1976). However, the role of ethylene and polyamines (PAs) in causation of creasing in sweet orange has not been investigated. Therefore, the general aim of this research was to investigate the role of PAs and ethylene in causation of creasing and to develop an effective method for controlling this physiological rind disorder whereby maintaining fruit quality in sweet orange.

10.2. Involvement of polyamines in creasing and quality of sweet orange [*Citrus sinensis* (L.) Osbeck] fruit

PAs are involved in many plant developmental processes including cell division and morphogenesis (Kusano et al., 2007; Cona et al., 2006); fruit development and ripening (Kakkar and Rai, 1993); as well as fruitlet abscission and senescence (Bais and Ravishankar, 2002; Rastogi and Davies, 1991). PAs also restore cell wall thickness and are essential for maintaining cell wall components (Berta et al., 1997). Creasing in sweet orange fruit is known to be associated with enhanced loss of pectins, starch in the cell walls of albedo, leading to cell wall loosening and formation of cracks consequently reducing hardness and tensile strength of the rind (Saleem et al., 2014; Monselise et al., 1976). My experimental data show that exogenous spray application of PUT at the fruit set, the golf ball or the mature stage significantly increased the endogenous levels of total free PAs, putrescine (PUT), spermidine (SPD) and spermine (SPM) in the albedo and flavedo tissues of sweet orange fruit (Figure 4.2-4.4) and consequently reduced incidence of creasing index (CI) in Washington Navel and Lane Late sweet oranges (Figure 4.1). Creasing has been reported to be associated with higher activities of cell wall degrading enzymes such as *exo*-PG, *endo*-PG, PE and Endo-1, 4-*β*-D-glucanase as well as water soluble pectins (Saleem et al., 2014; Li et al., 2009). The PAs are known as anti-senescence agents and inhibitors of ethylene biosynthesis which inhibit the ACC synthase and consequently reduce the activities of cell wall degrading enzymes such as PE, *exo*-PG, *endo*-PG, and EGase in sweet orange cv. Washington Navel and Lane Late (Chapter 8), plum (Khan et al., 2007) and mango (Zaharah and Singh, 2011). It may also be argued that the reduction of creasing with the exogenous application of PUT may be ascribed to the role of PAs in strengthening and maintaining cell wall components as reported earlier by Berta et al. (1997).

All the spray applications of PUT also increased the fruit rind thickness when applied at the fruit set, the golf ball or at the colour break stage in both cultivars during both years (Table 4.1 and 4.2). Possibly, the increased rind thickness with spray application of PUT is another important factor in reducing the CI in both cultivars. Similarly, it has been reported that incidence of creasing was greater on fruit with thinner rind compared to fruit with thicker rind in sweet orange fruit (Ali et al., 2000; Moulds et al., 1995; Bevington et al., 1993). Exogenous spray application of 1000 µM MGBG (an inhibitor of biosynthesis of PAs) when applied at the golf ball stage significantly increased the CI in cv. Washington Navel and Lane Late (Figure 4.5). Perhaps, the inhibition of PAs biosynthesis caused by MGBG spray may

have resulted in reduced levels of endogenous free PAs in the albedo and flavedo tissues of fruit and consequently increased the CI in both the cultivars. In conclusion, higher levels of free PAs in the albedo and flavedo tissues of fruit and reduction in CI with exogenous application of PUT, as well as the acceleration of creasing with MGBG (an inhibitor of biosynthesis of PAs) suggest the involvement of polyamines in creasing of sweet orange fruit. Exogenous spray application of PUT increased fruit firmness, rind hardness and tensile strength. This may be ascribed to the role of PAs in strengthening the cell wall components as reported earlier by Berta et al. (1997) and also acts as an anti-senescence agent (Kumar et al., 1996; Rastogi and Davies, 1991; Tiburcio et al., 1990). It may also be attributed to reduced ethylene production with the exogenous application of PUT consequently reducing fruit firmness, improved rind hardness and tensile strength in both cultivars. PAs are known as inhibitors of ethylene biosynthesis which are involved in the conversion of SAM to ACC, eliminating the increase in ACC formation and ethylene production through the action of the ACC oxidase enzyme (Ladaniya, 2007; Liu et al., 2006; Even and Melberg, 1989; Even-Chen et al., 1982; Hyodo and Nishino, 1981; Yu and Yang, 1979). The exogenous application of PUT significantly increased the fruit weight and diameter (Table 5.1 and 5.2). Similarly, PAs are known to increase fruit diameter, weight and improve fruit quality parameters in different fruit crops including apple (Costa and Bagni, 1983), olive (Rugini and Mencuccini, 1985), litchi (Mitra and Sanyal, 1990), mango (Malik and Singh, 2003), nectarine (Torrigiani, et al., 2004), pear (Franco-Mora et al., 2005), plum (Khan and Singh, 2010) and peaches (Bregoli et al., 2002). The exogenous application of PUT significantly increased soluble solids concentration (SSC), ascorbic acid, total antioxidants, individual and total sugars in both the cultivars of sweet orange (Chapter 5). Similarly, improved fruit quality with the exogenous application of PAs has been reported in different fruit crops such as apple (Costa and Bagni, 1983), litchi (Mitra and Senyal, 1990), mango (Malik and Singh, 2006), olive (Ayad et al., 2011), jujube (Kassem et al., 2011), plum (Khan et al., 2007) and sweet orange (Saleem et al., 2008). How the exogenous application of polyamines improves fruit quality in sweet orange fruit crops, is yet to be investigated in detail.

10.3. Creasing of sweet orange [*Citrus sinensis* (L.) Osbeck] fruit in relation to ethylene

Ethylene is a key signaling compound involved directly in the regulation of the ripening process in all fruit (Manjunatha et al., 2012). However, fruit softening is a ripening process associated with breakdown of cell wall polymers such as cellulose, hemicelluloses and pectins (Payasi et al., 2009). Ethylene accelerates softening in citrus fruit due to collapsing cell membranes making them leakier (Ladaniya, 2007; Rath and Prentice, 2004). Creasing is related to fruit softening caused by cell wall disassembly (Nishiyama et al., 2007; Brummel, 2006; Orfila et al., 2002). The levels of endogenous ethylene were higher in creased fruit compared to normal fruit in different cultivars of sweet orange including Navelina, Washington Navel, Lane Late and Valencia Late (Figure 6.1). Similarly, Monselise et al. (1976) and Pham (2009) also found higher levels of endogenous ethylene in the albedo tissues of creased fruit of sweet orange cv. Valencia and Washington Navel. The exogenous application of ethrel (an ethylene-releasing compound) significantly stimulated the levels of endogenous ethylene in the fruit consequently promoting the incidence of creasing in sweet orange cv. Washington Navel and Lane Late (Figure 6.2). Creasing incidence was increased due to elevated levels of endogenous ethylene in both cultivars. Possibly, the higher level of ethylene promoted the activities of cell wall degradation enzymes such as PE, *exo*-PG, *endo*-PG, and Endo-1, 4-*ß*-D-glucanase. Similarly, Saleem et al. (2014) and Li et al. (2009) also confirmed this thesis hypothesis that creasing is associated with higher activities of PE, *exo*-PG, *endo*-PG, and Endo-1, 4-*ß*-D-glucanase in the albedo and flavedo tissues of creased fruit than normal fruit in sweet orange. Moreover, reduction of creasing with the exogenous application of anti-ethylene compounds such as AVG and $CoSO_4$ (Figure 6.3 and 6.4) in sweet orange also supports the aforementioned hypothesis. In conclusion, higher endogenous levels of ethylene in creased fruit; promotion of incidence of creasing with exogenous applications of ethrel; and its reduction with the spray application of anti-ethylene compounds indicate the involvement of ethylene in creasing of sweet orange fruit. Higher levels of ethylene in the albedo and flavedo tissues of creased fruit than in healthy fruit; and elevated CI with exogenous application of ethrel in Washington Navel and Lane Late sweet orange; suggests the key role of ethylene in creasing. Exogenous spray application of PUT substantially reduced CI through raising the endogenous levels of free PAs (SPM, SPD and PUT) in the albedo and flavedo tissues and increased CI with exogenously applied MGBG (an inhibitor of PAs biosynthesis) in cv. Washington Navel and Lane Late sweet orange fruit also suggest involvement of PAs in creasing. Both ethylene and PAs share a common

precursor, S-adenosylmethionine (SAM) for their biosynthesis, but functions seem to be antagonistic in creasing of sweet orange fruit. This suggests a competitive relationship between PAs and ethylene in creasing of sweet orange fruit as reported earlier in fruit ripening and senescence (Saftner and Baldi, 1990).

10.4. Effect of AVG on fruit quality

AVG is a known inhibitor of ethylene biosynthesis, which suppresses production of ethylene in plant tissues by inhibiting ACC synthase, ACC oxidase and also reduces the activities of cell wall enzymes (Ladaniya, 2007; Even-Chen et al., 1982; Hyodo and Nishino, 1981; Yu and Yang, 1980; Boller et al., 1979; Yu et al., 1979). Spray application of AVG increased fruit firmness, rind hardness, rind tensile strength, weight, diameter and rind thickness in cv. Washington Navel and Lane Late during both years (Chapter 7). Improved rind fruit firmness, rind hardness, rind tensile strength with spray application of AVG can be ascribed to reduced ethylene production and reduced levels of water soluble pectins with the reduction in the activities of cell wall degrading enzymes such as PE, *exo*-PG, *endo*-PG, and Endo-1, 4-*ß*-D-glucanase in the albedo and flavedo tissues of creased fruit than normal fruit in sweet orange (Chapter 7). Earlier, spray application of AVG had been reported to inhibit ethylene production, consequently delaying fruit maturity leading to increased fruit weight, diameter and rind thickness as well as rheological properties of fruit and rind (Cetinbas and Koyuncu, 2011; Byers, 1977). Recently, Al-Husseini (2012) reported that AVG significantly improved fruit weight, diameter and rind thickness in sweet orange cv. Washington Navel and Lane Late. Similar effects of AVG application have also been reported on various fruit crops such as peaches (Kim et al., 2004; Rath et al., 2004; Bregoli et al., 2002), nectarine (Rath and Prentice, 2004), apples (Greene, 2005) and tomatoes (Jeong et al., 2002). All the spray treatments of AVG (20-60 mg L^{-1}) improved SSC, ascorbic acid, total antioxidants, individual sugars and total sugars as well as individual organic acid in the fruit juice in both cultivars of sweet orange during 2011 and 2012. The increase in SSC, ascorbic acid, total antioxidants, individual sugars and total sugars as well as organic acid may possibly be ascribed to reduced leaf abscission and consequently higher supply of photosynthates into the fruit. Similarly, spray application of AVG improved internal fruit quality parameters such as SSC, ascorbic acid, total antioxidants, individual sugars and total sugars as well as individual and total organic acid in different fruit crops such as apple (Wargo et al., 2004; Greene, 2005), peach (Singh, 2003; Rath et al., 2004) and sweet orange (Al-Husseini, 2012). In conclusion, exogenous spray application of AVG significantly reduced the incidence of

creasing and improved the rheological properties of fruit and rind, fruit weight, diameter, rind thickness as well as SSC, individual sugars, total sugars and organic acid as compared to the control.

10.5. Effect of cobalt sulphate on fruit quality

$CoSO_4$ is another inhibitor of ethylene biosynthesis, which blocks the conversion of ACC into ethylene by inhibiting the activity of ACC oxidase enzyme (Sisler and Yang, 1984; Even-Chen et al., 1982; Hyodo and Nishino, 1981).The exogenous application of $CoSO_4$ blocks the conversion of ACC to ethylene by inhibiting the activity of ACC oxidase enzyme (Sisler and Yang, 1984; Even-Chen et al., 1982; Hyodo and Nishino, 1981) in sweet orange cv. Washington Navel and Lane Late. Spray application of $CoSO_4$ improved fruit firmness, rind hardness, rind tensile strength as well as fruit weight, diameter and rind thickness in both cultivars during both years. Improved fruit firmness, rind hardness and rind tensile strength may be ascribed to reduced ethylene production through blocking the conversion of ACC to ethylene by inhibiting the activity of ACC oxidase enzyme (Sisler and Yang, 1984; Even-Chen et al., 1982; Hyodo and Nishino, 1981). All spray treatments of $CoSO_4$ (125-500 mg L^{-1}) improved SSC, ascorbic acid, total antioxidants, individual sugars and total sugars as well as organic acid in the fruit juice in both cultivars of sweet orange during 2011 and 2012.

$CoSO_4$ application may have reduced the premature leaf abscission due to inhibition of ethylene production in sweet orange and consequently higher supply of photosynthates to the fruit leading to improved SSC, ascorbic acid, total antioxidants, individual and total sugars. Historically, application of $CoSO_4$ promoted the internal fruit quality parameters such as SSC, ascorbic acid, total antioxidants, individual and total sugars in different fruit crops such as mango (Singh and Singh, 1993; Singh and Agrez, 2002), pomegranate (Reddy et al., 2011), olive (Gad et al., 2006), apples (Benincore, et al., 2000), peaches (Kim, et al., 2004), Figure (Teragishi, et al,. 2000) and sweet orange (Al-Husseini, 2012). However, all the treatments of $CoSO_4$ (125-500 mg L^{-1}) did not significantly affect the levels of individual and total organic acids in the juice of Washington Navel and Lane Late sweet orange fruit suggesting that cobalt does not play any significant role in the organic acid metabolism in sweet orange fruit and warrants investigation. In conclusion, the exogenous spray application of $CoSO_4$ (500 mg L^{-1}) was more effective in reducing creasing when applied at the golf ball stage compared to the fruit set or at the colour break stage and also improved fruit firmness, rind hardness, rind tensile strength, fruit weight and diameter, ascorbic acid, total

antioxidants, individual and total sugars as compared to the control in sweet orange cv. Washington Navel and Lane Late.

10.6. Mode of reduction of creasing in sweet orange [*Citrus sinensis* (L.) Osbeck] fruit with exogenous spray application of putrescine, aminoethoxyvinylglycine or cobalt sulphate

As a prelude, ethylene is a gaseous hormone involved in the softening and senescence of citrus fruit (Ladaniya, 2007; Rath and Prentice, 2004). Meanwhile, softening is known as a ripening process linked with biochemical changes in cell wall components such as cellulose, hemicelluloses and pectins (Payasi et al., 2009). The creasing phenomenon is similar to fruit softening and senescence caused by cell wall disassembly in sweet oranges (Nishiyama et al., 2007; Brummel, 2006; Orfila et al., 2002). Similarly, Monselise et al., (1976) claimed that creasing is associated with earlier senescence of albedo tissue during fruit maturation and ripening. It has been reported that creasing is also linked with loss of pectins and hemicelluloses in sweet oranges (Jona et al., 1989). However, Saleem et al. (2014) and Li et al. (2009) further described that incidence of creasing is linked with loss of pectins and higher activities of cell wall degrading enzymes such as PE, *exo*-PG and *endo*-PG and EGase in the albedo tissues of creased fruit. The exogenous application of PUT, AVG or $CoSO_4$ significantly reduced the incidence of creasing when applied at the golf ball or at the colour break stage. The exogenous application of PUT, AVG and $CoSO_4$ significantly increased the levels of total pectin and insoluble pectin in the albedo and flavedo tissues whilst decreasing water soluble pectins at the golf ball and ripe stage in cv. Washington Navel and Lane Late (Table 8.1-8.3). The exogenous application of PUT, AVG or CoSO4 significantly reduced the incidence of creasing due to reduction in the activities of cell wall degrading enzymes such as PE, *exo*-PG and *endo*-PG and EGase in the albedo and flavedo tissues at the golf ball and ripe stage of sweet orange cv. Washington Navel and Lane Late. Possibly, the exogenously applied PUT, AVG or $CoSO_4$ inhibited ethylene biosynthesis and consequently reduced the activity of cell wall degrading enzymes including PE, *endo*- and *exo*-PG and PG and consequently maintained higher levels of water insoluble and total pectins and lower levels of water soluble pectin in the albedo and flavedo tissue of the rind as compared to the control. Higher levels of endogenous ethylene were found in creased fruit compared to healthy fruit in different cultivars of sweet oranges including Navelina, Washington Navel, Lane Late and Valencia Late (Chapter 6), Pham (2009) and Monselise et al. (1976). In mature fruit, pectins are bound with cellulose in the cell wall and are insoluble; during ripening the structure of pectin is altered by cell wall degrading enzymes like PE, *endo*-PG and *exo*-PG (Yoo et al.,

2003; Kashyap et al., 2001). Similarly, higher levels of water soluble pectins in creased fruit of sweet orange have also been reported (Saleem et al., 2014; Li et al., 2009; Pham, 2009; Jona et al., 1989; Monselise et al., 1976). The higher activities of PE, *exo*-PG, *endo*-PG, and Endo-1, 4-ß -D-glucanase in albedo and flavedo of creased fruit than normal fruit have also been reported to be associated with greater loss of pectins and starch in the albedo cell walls, consequently leading to creasing (Saleem et al., 2014; Li et al., 2009). In conclusion, the exogenous spray application of PUT, AVG or $CoSO_4$ significantly decreased activities of cell wall degrading enzymes such as PE, *endo*- and *exo*-PG and EGase as well as levels of water soluble pectins in the albedo and flavedo tissues of the fruit as compared to the control leading consequently to reduced creasing in sweet orange.

10.7. Conclusions

- The incidence and severity of creasing significantly reduced with the exogenous application of PUT due to the increase in endogenous levels of free polyamines such as PUT, SPD and SPM.
- The exogenous spray application of PUT significantly improved the rheological properties of the fruit and rind such as fruit firmness, rind hardness and rind tensile strength as well as fruit weight, diameter and rind thickness. Similarly, the exogenous application of PUT improved the internal fruit quality parameters such as SSC, ascorbic acid concentrations, total antioxidants, individual and total sugars in sweet orange cv. Washington Navel and Lane Late.
- The endogenous levels of ethylene were higher in the creased fruit compared to normal fruit in different cultivars of sweet orange including Navelina, Washington Navel, Lane Late and Valencia. The incidence and severity of creasing significantly increased with the exogenous application of ethylene due to the increase in the levels of ethylene in different cultivars of sweet orange including Washington Navel and Lane Late. However, creasing was significantly reduced with the exogenous application of AVG and $CoSO_4$ due to inhibition of ethylene production.
- The exogenous application of AVG and $CoSO_4$ improved the rheological properties of fruit and rind such as fruit firmness, rind hardness and rind tensile strength as well as fruit weight, diameter and rind thickness. Similarly, the AVG and $CoSO_4$ also improved the internal fruit quality parameters such as SSC, TA, SSC: TA ratio,

ascorbic acid, total antioxidants, individual and total sugars as well as individual organic acid in sweet orange cv.Washington Navel and Lane Late during both the years.

- The incidence and severity of creasing significantly reduced with exogenous application of ethylene inhibitors such as PUT, AVG and $CoSO_4$ due to inhibition of ethylene biosynthesis by reduction in the activities of cell wall degrading enzymes such as PE, *exo*-PG and *endo*-PG and EGase as well as reduction in water soluble pectins in the albedo and flavedo tissues of sweet orange cv. Washington Navel and Lane Late.

10.8. Recommendations to the citrus industry

- The exogenous spray application of PUT (500-1000 µM) when applied at the golf ball stage reduced the incidence of creasing, improved rheological properties of the fruit and rind as well as other quality parameters of sweet orange fruit.
- The exogenous spray application of AVG (60 mg L^{-1}) or $CoSO_4$ (500 mg L^{-1}) at the golf ball stage reduced the incidence of creasing, improved the rheological properties of the fruit and rind such as fruit firmness, rind hardness and rind tensile strength as well as increased fruit weight, diameter, rind thickness, SSC, ascorbic acid concentrations, total antioxidants, individual and total sugars in sweet orange cv. Washington Navel and Lane Late.

10.9. Disclaimer

These recommendations to the citrus industry are purely based on the experiments conducted at only one location, Gingin, Western Australia. Inconsistencies may arise in outcomes under different locations. The project investigator Zahoor Hussain and Curtin University accept no liability whatsoever for any reason such as negligence or otherwise arising from the reliance or use of these recommendations.

10.10 Future research

This research work focused on the role of PAs and ethylene in creasing and quality of sweet orange fruit by determining the endogenous levels of PAs and ethylene through exogenous application of PUT, MGBG, ethrel, AVG and $CoSO_4$ on the creasing and fruit quality in sweet orange. However, future research work on creasing and fruit quality is required in the following areas:

- The interaction between PAs and ethylene biosynthesis in the albedo and flavedo tissue of sweet orange fruit and its implications for creasing.
- The exogenous spray application of PUT, AVG or $CoSO_4$ improved the fruit quality in sweet orange fruit, but the exact mechanism of these compounds in improving fruit quality warrants detailed investigation.
- Comparative transcriptomics, proteomics, metabolomics profiling of albedo and flavedo tissues of creased and healthy sweet orange fruit at various developmental stages is yet to be investigated.

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