School of Agriculture and Environment

Regulation of Fruit Colour Development, Quality and Storage Life of 'Cripps Pink' Apple with Deficit Irrigation and Plant Bioregulators

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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 the acknowledgement has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature: _____

Date: _____

Dedication

To

My mother *(Maznah Hassan)*
&
My father *(*Late *Wan Sembok Wan Ali)*

'A constant source of inspiration during the entire period of my PhD study and throughout my life'

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

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Abstract

Poor and erratic fruit colour development in ‘Cripps Pink’ apple causes serious economic losses to the growers and/or exporters of Western Australia and other parts of the world. Many internal and external factors such as genetic, light, temperature, irrigation, application of chemicals and also soil and tree factors affect the biosynthesis of anthocyanins consequently fruit colour. Some of the past approaches followed to improve fruit skin colour resulted in limited outcomes. The aim of my research was to evaluate the effects of water saving strategies and newly developed plant bioregulators in improving fruit colour development without adversely affecting fruit size and quality of ‘Cripps Pink’ apple at harvest, following cold and controlled atmosphere (CA) storage. I also investigated the individual polyphenolics profiles, their identification and confirmation in the skin of this apple cultivar. Nine polyphenolic compounds (cyanidin 3-*O*-galactoside, chlorogenic acid, phloridzin, quercetin 3-*O*-rutinoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-xyloside, quercetin 3-*O*-arabinoside and quercetin 3-*O*-rhamnoside) in the fruit skin of ‘Cripps Pink’ apple were identified, quantified and re-confirmed using high performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-ESI-MS). Increased concentration of cyanidin 3-*O*-galactoside in ‘Cripps Pink’ apple skin coincided with the increase in total anthocyanins concentrations.

Water saving strategies, regulated deficit irrigation (RDI) and withholding irrigation (WHI), have been carried out for two seasons (2005-06 and 2006-07, and 2006-07 and 2007-08, respectively) in a commercial apple orchard. The treatment (75% RDI applied for 72 days, commencing on 135 days after full bloom (DAFB) and WHI for 20 to 30 days, commencing on 135 and 145 DAFB) increased red skin colour, concentration of total anthocyanins and polyphenolic compounds such as cyanidin 3-*O*-galactoside and quercetin glycosides. These treatments also improved fruit firmness and soluble solids concentration (SSC) of ‘Cripps Pink’ apple at harvest without adversely affecting postharvest quality in cold and controlled atmosphere (CA) storage, and also saved the irrigation water. To the best of my knowledge, this may be the first report on the effects of water-deficit on accumulation of flavonoids and other phenolic compounds in red-skinned apple particularly ‘Cripps Pink’

cultivar and also its impact on postharvest storage performance in CA storage. Soil-plant water relations such as volumetric soil water content, stomatal conductance, leaf water potential and stem water potential was pronounced with the application of these water saving strategies applied in the middle of stage II of fruit development of 'Cripps Pink' apple. The sparse leaf abscission due to water-deficit has improved light penetration, consequently improved red skin colouration through increased accumulation of anthocyanins particularly cyanidin 3-*O*-galactoside. This highlighted the importance of water stress and light in regulating colour and biosynthesis of anthocyanins.

Newly developed plant growth regulator, Prohexadione-calcium (ProCa) improved fruit colour development of this apple cultivar by manipulating the light interception into the tree canopy and onto the fruit through reduction of vegetative growth. The reduction of shoot length was pronounced with three spray applications of ProCa (500 $mg \cdot L^{-1}$) on 3, 33 and 63 DAFB or two sprays of ProCa (500 $mg \cdot L^{-1}$) on 2 and 32 DAFB in combination with summer pruning (SP). The above mentioned treatments increased concentration of anthocyanins, cyanidin 3-*O*-galactoside, and all individual quercetin glycosides (quercetin 3-*O*-rutinoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-xyloside, quercetin 3-*O*-arabinoside and quercetin 3-*O*-rhamnoside) and also maintained other fruit quality attributes such as fruit firmness and SSC of this apple cultivar.

Lysophophatidylethanolamine (LPE) spray, 125 $mg \cdot L^{-1}$ (at two and four weeks prior to anticipated commercial harvest) or 250 $mg \cdot L^{-1}$ (at four weeks before harvest) appeared to be promising in improving fruit colour development, accumulation of anthocyanins and polyphenolic compounds (cyanidin 3-*O*-galactoside, quercetin glycosides and also individual quercetin glycosides such as quercetin 3-*O*-xyloside, quercetin 3-*O*-arabinoside and quercetin 3-*O*-rhamnoside), and other fruit quality attributes of 'Cripps Pink' apple. However, the mode of action of LPE in improving red colour in apple skin is possibly associated with enhanced ethylene production.

In conclusion, fruit colour development of 'Cripps Pink' apple can be improved by applications of water saving techniques in the middle of stage II of fruit development such as 75% RDI for 72 days commencing on 135 DAFB or WHI for 20 (135-155

DAFB) to 30 (145-175 DAFB) days, and also newly developed plant bioregulators such as ProCa (three spray applications of ProCa (500 $mg \cdot L^{-1}$) on 3, 33 and 63 DAFB or two sprays of ProCa (500 $mg \cdot L^{-1}$) on 2 and 32 DAFB in combination with SP) or LPE (two spray applications (125 $mg \cdot L^{-1}$) at two and four weeks prior to anticipated commercial harvest or single spray (250 $mg \cdot L^{-1}$) at four weeks before harvest) without adversely affecting other fruit quality attributes.

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List of Symbols and Abbreviations

°	Degree
%	Percent
°C	Degree Celsius
α	Alpha
β	Beta
θ	Volumetric soil water content
ψ	Water potential
µg	Microgram(s)
µL	Microliter(s)
µm	Micrometer
ABA	Abscisic acid
a.i.	Active ingredient
ANOVA	Analysis of variance
ANS	Anthocyanidin synthase
ACPI	Atmospheric pressure chemical ionization
AU	Absorbance units
AVG	Aminoethoxyvinylglycine
CI	Commercial irrigation/Control
C*	Chroma
CA	Controlled atmosphere
CHI	Chalcone isomerase
CHS	Chalcone synthase
CIE	Commission Internationale de L'Eclairage
cm	centimetre(s)
Co	Company
CO_2	Carbon dioxide
CoA	Coenzyme A
DAFB	Days after full bloom
DFR	Dihydroflavonol reductase
DI	Deficit irrigation
DPA	Diphenylamine
E	East

EDTA	Ethylenediamine tetraacetic acid
ESI-MS	Electrospray ionization mass spectrometry
et al.	et alia
F3H	Flavanone 3-hydroxylase
F3'H	Flavonoid 3'-hydroxylase
FAO	Food and Agriculture Organisation
FLS	Flavonol synthase
FXH	Flavanone 3-hydroxylase
g	Gram(s)
h°	Hue angle
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
i.d.	Internal diameter
kg	Kilogram(s)
MPa	Mega Pascal
L	Liter(s)
L*	Lightness
LPE	Lysophosphatidylethanolamine
LSD	Least significant difference
Ltd.	Limited
MA	Massachussets
MeOH	Methanol
mg	Milligram(s)
mL	Mililiter (s)
Mm	Milimeter
mM	Mili molar
Mt	Metric tonnes
min.	Minute
MS	Mass spectrometry
N	Newtons
NaF	Sodium fluoride
NaOH	Sodium hydroxide
nL	Nanoliter(s)
nm	Nanometer

NS	Not significant
O_2	Oxygen
Pa	Pascal
ProCa	Prohexadione-calcium
p.s.i.	Pounds per square inch
r	Correlation coefficient
RDI	Regulated deficit irrigation
RI	Retention index
rpm	Revolutions per min
RT	Retention time
S	South
SP	summer pruning
SSC	Soluble solids concentration
TA	Titratable acidity
UFGT	UDP glycosyl:flavonoid 3-*O*-glycosyltransferase
UFGalT	UDP galactose:flavonoid 3-*O*-galactosyltransferase
UFGluT	UDP glucose:flavonoid 3-*O*-glucosyltransferase
U.K.	United Kingdom
U.S.A	United States of America
UV-VIS	Ultraviolet-visible
v/v	Volume per volume
WHI	Withholding irrigation

CHAPTER 1

General Introduction

Apple [*Malus sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.] is the most popular and widely grown fruit crop throughout the world. Asia is the largest apple producer in the world, followed by Europe, North America, South America, Africa and Australia. In 2007, the Australian apple industry contributes only 0.3% of the world total production (FAO, 2008). Even though, apple production is still imperative to Australian apples industry, in which estimated gross value for export in 2007-08 was A$ 7.2 million (Department of Agriculture and Food Western Australia, 2008). In 2007-08, Australia produced about 265,500 thousand tonnes of apples, and 'Cripps Pink' apple contributed about 60,500 tonnes (Australian Bureau of Statistics, 2008). The most common apple cultivars grown in Australia in 2007-08 were 'Cripps Pink' (60,500 tonnes), 'Granny Smith' (58,600 tonnes) and 'Gala' (39,500 tonnes) (Australian Bureau of Statistics, 2008). 'Cripps Pink' apple under the trade brand name of 'Pink Lady^{TM}' is the leading cultivar of Australia. In 2008, Victoria was the major producer of 'Cripps Pink' apple (49.9%), followed by Western Australia (17.7%), New South Wales (10.9%), Queensland (9.4%), South Australia (8.4%) and Tasmania (3.6%) (Australian Bureau of Statistics, 2008). In Western Australia, most of the commercial orchards are concentrated in Donnybrook, the Perth Hills, Manjimup and Dwellingup. The major grown cultivars are 'Cripps Pink', contributing 34% of state's apple production, followed by 'Granny Smith' (17%) and 'Gala' (17%) (Apple and Pear Australia Limited, 2008).

'Cripps Pink' is an attractive apple cultivar with an oblong-conical shape and solid pinkish red skin, and is very popular among apple consumers. 'Cripps Pink' apple requires chilling conditions below 7°C for less than 400 hours (Mackay et al., 1994) and as resistant to sunburn, russet, surface cracking, bitter pit or internal disorders (Cripps et al., 1993; Mackay et al., 1994), which has made it possible to grow in the warmer areas such as Mediterranean climate of Western Australia. However, poor and erratic skin colour causes a reduction in grade and consumer acceptance, which results in serious marketing problems and economic losses to apple fruit growers. The reduction of exports quantity of 'Cripps Pink' apple is due to unfavourable

climatic conditions such as the incidence of high temperatures just before commercial harvest in 2003-04 (Whale et al., 2008). Red skin colour is one of the most important requirements for 'Cripps Pink' apple to be accepted in both domestic and export markets. To fulfil export quality standards, 'Cripps Pink' apple fruit surface should attain above 40% a bright pink-red blush, 7 to 9 $kg \cdot cm^{-2}$ of fruit firmness, soluble solids concentration (SSC) of 13 to >15 ^{o}Brix, 0.7 to 0.9% titratable acidity and minimum size of 65 mm (Cripps et al., 1993; Mackay et al., 1994). Iglesias et al. (2002) reported that poor fruit skin colour causes serious reduction in marketable grading standard even if the size of the fruit meets quality standards.

Apple skin colour is a combination of different amount of various pigments such as anthocyanins, chlorophylls and carotenoids. Anthocyanin is one of the important flavonoid compounds and found only in red-skinned apple, and is located in the vacuoles of skin cells. The major anthocyanins in apple skin are cyanidin glycosides, while cyanidin-3-*O*-galactoside (idaein) is the leading individual anthocyanin (Awad et al., 2000; Lancaster et al., 1994; Mazza and Velioglu, 1992). In the early season of apple fruit growth, concentration of anthocyanins is relatively high and gradually decreases to a very low steady level, but increases again near to maturation (Saure, 1990; Whale and Singh, 2007). In addition to anthocyanins, there are another three major classes of flavonoids present in apple fruit such as flavonols (quercetin glycosides), flavanols (catechin, epicatechin and procyanidins), and dihydrochalcones (phloridzin), and also a class of phenolic acid (chlorogenic acid) (Awad et al., 2000; Lancaster, 1992; Tsao et al., 2003; Whale and Singh, 2007). The biosynthesis of anthocyanins is influenced by environmental and endogenous plant factors such as genetic, light, temperature, ethylene, soil type, altitude, microbial interactions and preharvest practices including irrigation, plant growth regulators, fruit thinning, pruning, mulching and fertilization (Lancaster, 1992; Saure, 1990). The biosynthesis of anthocyanins is strongly light dependent. Some of preharvest practices such as pruning, reflective mulches and use of shoot growth retardants improve fruit skin colour through regulation of anthocyanins by light. These five major polyphenolic compounds are found in red-skinned apple and their concentration is mainly influenced by genotype, growth periods, geographical locations and growing seasons (Awad et al., 2000; Tsao et al., 2003). Some of the

preharvest practices tested to improve fruit skin colour resulted in successful and limited outcomes such as application of ethephon, paclobutrazol and seniphos, reflective mulches, bagging, potassium fertilizer, overhead sprinkler, combination of aminoethoxyvinylglycine (AVG) and ethephon, and AVG alone. The regulation of fruit colour development and polyphenolic compounds in 'Cripps Pink' apple using various exogenous spray applications under Mediterranean climate of Western Australia has been investigated by Whale et al. (2008) and Whale (2005). However, the effects of water saving techniques and other potential plant bioregulators on fruit colour development and polyphenolic compounds warrant further investigations as polyphenolic compounds identified in the skin of 'Cripps Pink' apple earlier were not confirmed. In addition, no information is available on mechanisms and the effects of water-deficit on the dynamics of the anthocyanins biosynthesis in red-skinned apple especially 'Cripps Pink' cultivar.

Water saving techniques such as regulated deficit irrigation (RDI) and withholding irrigation (WHI) on apple fruit tree have been explored in various cultivars under various climatic conditions such as 'Braeburn' and 'Delicious' in humid region (Ebel et al., 1995; Mills et al., 1996b) and 'Cripps Pink' apple in temperate (O'Connell and Goodwin, 2007) and in Mediterranean region (Talluto et al., 2008) with promising outcomes in red skin colour development (Kilili et al., 1996a; Mills et al., 1996a; Mills et al., 1994). It is well documented that RDI and WHI reduce leaching of nutrients and biocides into the underground water, decrease vegetative growth, reduce maintenance costs and enhance fruit quality (Behboudian and Mills, 1997), saved irrigation water (Behboudian et al., 1998; Leib et al., 2006; Mpelasoka et al., 2001a), and no reduction in apple fruit size (Kilili et al., 1996a; Mills et al., 1996b). However, the information on the effects of RDI and WHI on postharvest storage performance in cold and controlled atmosphere (CA) storage is scant. Time of application of RDI and WHI is also crucial for achieving acceptable fruit size that has been set by the apple industry. Therefore, the exact time of application of these water saving strategies is yet to be investigated.

The role of Prohexadione-calcium (ProCa) in improving red skin colour and concentration of anthocyanins in 'Fuji' apple in warm and dry climatic region of Spain has been reported (Mata et al., 2006a; Medjdoub et al., 2005). Increased

accumulation of anthocyanins in 'Fuji' apple treated with ProCa has been related to the reduction in shoots length, consequently allowing greater penetration of sunlight into the tree canopy and onto the fruit (Mata et al., 2006a). In addition, ProCa has also been known to be an effective shoot growth retardant in apple trees, without adversely affecting others major fruit quality attributes (Mata et al., 2006b). No information is available on the effects of different concentrations and number of sprays of ProCa in improving fruit colour of 'Cripps Pink' apple under Mediterranean climate of Western Australia. Moreover, no research work has been reported on the effects of ProCa on production of anthocyanins and polyphenolic compounds in 'Cripps Pink' apple fruit skin. Therefore, the effects of ProCa in improving fruit colour and accumulation of red skin pigmentation and other fruit quality parameters in 'Cripps Pink' apple in Western Australia conditions require investigation.

Lysophophatidylethanolamine (LPE) is a potential plant growth regulator that has been reported to improve fruit colour and concentration of anthocyanins in apples (Farag and Palta, 1991b), cranberries (Özgen et al., 2004; Özgen and Palta, 2003), table grapes (Hong, 2008) and red pepper (Kang et al., 2003). No information is available on the effects of LPE in improving fruit colour, polyphenolic profiles and other fruit quality attributes especially in 'Cripps Pink' apple cultivar. In addition, the optimum concentration of LPE and time of application seem to be critical in apple fruit particularly in improving apple skin colour and quality as the effects of LPE in other fruit crops are concentration dependant (Farag and Palta, 1993a; Kaur and Palta, 1997; Özgen et al., 2004; Ryu et al., 1997). Hence, this investigation aimed to evaluate the effects of different concentrations and number of sprays of LPE on fruit colour development, accumulation of anthocyanins and polyphenolic compounds and also on other fruit quality attributes of 'Cripps Pink' apple grown under Western Australia conditions.

My research was aimed to evaluate the effects of water saving strategies and also newly developed plant bioregulators in improving fruit colour development without adversely affecting fruit size and quality of 'Cripps Pink' apple at harvest, following cold and controlled atmosphere storage.

The main objectives of my research were:

1. To identify, quantify and confirm the individual polyphenolic profiles in the skin of 'Cripps Pink' apple fruit subjected to water-deficit (regulated deficit irrigation and withholding irrigation) and some newly developed plant bioregulators (prohexadione-calcium and lysophosphatidylethanolamine).

2. To investigate the role of regulated deficit irrigation (RDI) applied in the middle of stage II of fruit development on plant water relations, development of fruit colour and polyphenolic profiles in skin, fruit size and other major fruit quality attributes at harvest, following cold and controlled atmosphere storage.

3. To elucidate the impact of withholding irrigation (WHI) applied at later stages of fruit development and maturation (stage II and III) on plant water relations, development of fruit colour, profile of flavonoids and other phenolic compounds in skin and other major fruit quality attributes at harvest and following long-term cold storage.

4. To investigate the role of different concentrations and number of sprays of prohexadione-calcium (ProCa) on fruit colour development, concentration of anthocyanins, profile of flavonoids and other phenolic compounds in fruit skin, other fruit quality parameters, concentration of ascorbic acid and total antioxidants at commercial harvest.

5. To evaluate the effects of different concentrations and number of sprays of lysophosphatidylethanolamine (LPE) on fruit colour development, profile of flavonoids and other phenolic compounds in fruit skin, and other fruit quality parameters at commercial harvest.

CHAPTER 2

General Review of Literature

2.1. Introduction

Apple is one of the most widely cultivated fruit crops and produced commercially in over eighty countries around the world (FAO, 2008). Apple is the third most valuable fruit crop after bananas and grapes in fruit production and ranked second behind grapes in acreage (FAO, 2009). In 2007, the commercial production of world apple was 64.2 million tonnes from an area of 4.9 million hectares (FAO, 2008). Currently, global apple production is dominated by China, which produces about 27 million tonnes (42.8% of world total production), followed by United States of America (6.6%), Iran (4.1%) and Turkey (3.5%) (Table 2.1). In 2007, the production of Australian apple was 221,000 metric tonnes (FAO, 2008) which an estimated value for export was A$ 7.2 million (Department of Agriculture and Food Western Australia, 2008).

Table 2.1. Apple production in different countries of the world in 2007.

Country	Apple production (metric tonnes)
China	27,507,000
United States	4,237,730
Iran	2,660,000
Turkey	2,266,437
Russia	2,211,000
Italy	2,072,500
India	2,001,400
France	1,800,000
Australia	221,000

Source: FAO (2008).

The Australian apple production has fluctuated between 2002-03 and 2007-08 (Table 2.2). The seasonal fluctuations may be attributed to the global climatic change such as several growers leave the industry and some growers adjust orchards to new

cultivars that are in demand and replanting with newer cultivars. Australian apple industry faces difficult condition as returns from overseas export decline due to the increasing competition in the global markets especially from Southern hemisphere countries, a higher exchange rate, competitive domestic markets and increasing production costs.

Table 2.2. Quantity and value of Australian and Western Australian apple production during 2002-03 to 2007-08.

Year	Production (tonnes)	Export (A$ million)
2002-03	320,526	41.6
2003-04	326,072	20.3
2004-05	254,925	17.2
2005-06	326,584	12.1
2006-07	276,400	12.0
2007-08	221,000	7.2

Source: Department of Agriculture and Food Western Australia (2006; 2008).

The major commercial apple production areas in Australia are concentrated in Stanthorpe in Southern Queensland, Orange and Batlow in New South Wales, Goulburn Valley in Southern Victoria, Huon Valley in Tasmania, Adelaide Hills in South Australia and Perth Hills, Donnybrook and Manjimup in Western Australia (Figure 2.1).

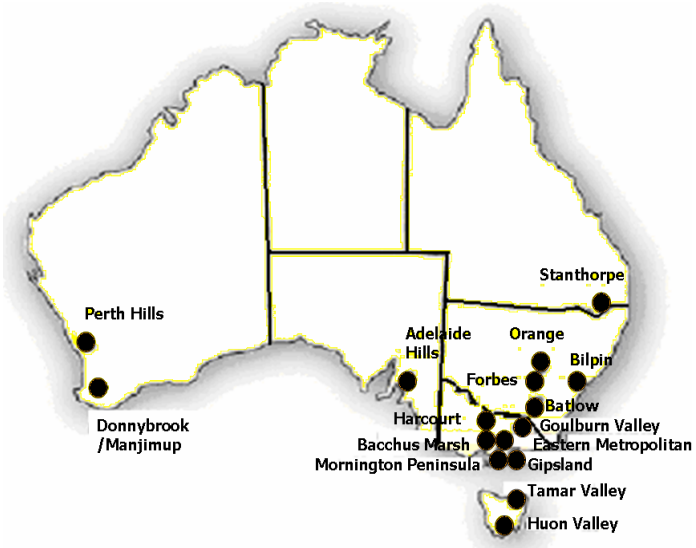

Figure 2.1. Map of major apple production areas in Australia. Source: Plant Health Australia (2008).

The three most common apple cultivars produced in Australia in 2007-08 were 'Cripps Pink' (60,500 tonnes), Granny Smith (58,600 tonnes) and 'Gala' (39,500 tonnes) (Australian Bureau of Statistics, 2008). In 2006-07, the production of 'Cripps Pink' apple in Australia was slightly decreased as compared to 2005-06 (Table 2.3). Similarly, Western Australian apple production has also fluctuated between 2002-03 and 2007-08 and the exports value declined from year to year (Table 2.4). As presented in Table 2.4, the exports value of Western Australian apple in 2007-08 declined 3-fold as compared to 2006-07. In addition, in 2005-06, exports value of 'Cripps Pink' apple in Western Australia dropped to A$ 1.8 million as compared to the previous year A$ 3.5 million in 2004-05 (Department of Agriculture and Food Western Australia, 2007). The unfavourable climatic conditions may have influenced the quantity of 'Cripps Pink' apple production and its fruit quality. Poor fruit colour development especially in warmer areas and sunburn in 'Cripps Pink' apple has been reported to reduce the amount of fruit suitable for export. The reduction in 'Cripps Pink' apple export quantity often causes serious economic losses to Western Australia apple growers.

Table 2.3. The production of 'Cripps Pink' apple in Australia during 2002-03 to 2007-08.

Year	Production (tonnes)
2002-03	48,200
2003-04	47,400
2004-05	61,100
2005-06	56,500
2006-07	55,900
2007-08	60,500

Source: Australian Bureau of Statistics (2006; 2007a; 2008)

Table 2.4. Apple fruit tree numbers, production and value of exports in Western Australia during 2002-03 to 2007-08.

Year	Tree numbers	Production (tonnes)	Export (A$ million)
2002-03	898,000	44,786	8.9
2003-04	887,000	38,869	7.0
2004-05	976,000	37,745	4.9
2005-06	874,000	37,871	3.3
2006-07	878,474	40,916	4.7
2007-08	854,000	31,932	1.5

Source: Department of Agriculture and Food Western Australia (2006; 2008)

The red colour in fruit skin surface in 'Cripps Pink' apple is the prime importance grading standards to be accepted in domestic and export markets. In both markets, red-skinned apples fetch higher prices than green-skinned apple due to its attractive appearance, higher sugar content and higher flavonoids and other phenolic compounds. The red colouration in 'Cripps Pink' apple skin is derived from pigment components called anthocyanins that belong to a class of flavonoids. These compounds occur twice in the fruit development stages, early in the life of fruit development and during maturation, about two to three weeks prior to commercial harvest (Chalmers et al., 1973; Iglesias et al., 2002; Whale and Singh, 2007). The latter occurrence is more economical as red skin colouration is an important factor in markets acceptance. Hence, the regulatory mechanism of biosynthesis of anthocyanins attracts interest and various approaches have been explored and developed to improve fruit colour development especially in 'Cripps Pink' apple. This review aims at providing a critical analysis of the research work previously reported on the regulation of fruit colour development in 'Cripps Pink' apple with deficit irrigation and newly developed plant bioregulators under Mediterranean climate of Western Australia which could be the best options toward satisfying the apple quality standards for domestic and global markets.

2.2. 'Cripps Pink' apple cultivar

'Cripps Pink' apple was originated from a cross bred between 'Lady Williams' and 'Golden Delicious' cultivars (Cripps et al., 1993). The hybrid cultivar was bred by

apple breeders at the Department of Agriculture in 1973, at Stoneville Research Station, Australia. It was first introduced to be commercially planted in 1985. The cross aimed to combine the sweet flavour and scald-free surface of 'Golden Delicious' with firmness and long shelf life of 'Lady Williams' cultivar (Cripps et al., 1993; López et al., 2007).

This cultivar is oblong-conical in shape with medium size (average 70 to 75 mm), smooth textured flesh and thin skin which it inherited from male parent. The male parent is 'Golden Delicious', which is renowned internationally as eating apple with its sweet, juicy and crisp flesh. While, female parent 'Lady Williams' has inherited its resistance to sunburn, vegetative vigour, high sugar to acid ratio, crispness and firmness (Mackay et al., 1994). 'Cripps Pink' apple is a late season maturing cultivar that able to adapt in various climatic conditions and less prone to sunburn, russet, surface cracking, bitter pit or internal disorders (Cripps et al., 1993; Mackay et al., 1994). This cultivar suitable to warmer climatic conditions of Australia and has been reported to produce crisp fruit, high sugar content and have an excellent retail shelf life (Cripps et al., 1993; Mackay et al., 1994).

This cultivar gained high popularity among consumers due to its fruit quality, increases concern to ensure ongoing consumer acceptance and not become 'just another new variety' (Gapper, 2004). This cultivar requires satisfying main export specifications such as red skin colour should not less than 40% on fruit skin surface. 'Cripps Pink' cultivar is under the trademark name of 'Pink Lady^{TM}' and owned by Apple and Pear Limited Australia. In order to maintain the 'Pink Lady^{TM}' trademark, only 'Cripps Pink' with a sufficient quality especially red blush on the skin surface could be sold as 'Pink Lady^{TM}'. In 2004, a royalty of US$ 1 (A$ 1.43) per 13 kg cartons of apple covers licensing and marketing cost which associates with the promotion, branding and development of this cultivar (Gapper, 2004).

2.2.1. Apple fruit colour

Apple fruit colour is due to the blending of various amounts of different pigments including plastid-based pigments such as chlorophylls and carotenoids and also vacuole-based pigments such as anthocyanins and flavonols (Lancaster et al., 1997; Lancaster, 1992). Chlorophylls are responsible for the green background colour and

located in chloroplast, while carotenoids responsible for the yellow background colour which is liposoluble and located in chromoplast (Lancaster, 1992). Whilst, vacuole-based pigments, anthocyanins responsible for the red colour and its production is genetically control (Jackson, 2003; Lancaster, 1992). These pigments change continuously during the maturation and ripening of apple fruit (Knee, 1972).

Immature apple fruit usually dark green in colour (Jackson, 2003) due to the abundant chlorophylls compounds. The reduction in chlorophyll during maturation was due to the increased activity of chlorophyllase, which coincides with increased carotenoid concentration results in yellow background colour (Knee, 1972). As mentioned earlier, anthocyanins biosynthesis peaked twice during fruit development; first occurs during cell division and the second appears during maturation of the fruit. The latter peak takes place only in the red-skinned apple (Ubi et al., 2006) and usually coincides with a reduction in chlorophyll and increased concentration of carotenoid (Lancaster, 1992; Reay et al., 1998). The accumulation of anthocyanins pigment also coincides with the ripening of red-skinned apple cultivars and may continue after harvest (Jackson, 2003). The production of anthocyanins in apple skin of immature fruit was lower than in mature fruit due the lack of factors that require in the process has been reported (Faust, 1965). Another vacuole-based pigment such as flavonols do not contribute directly to fruit skin colouration, but indirectly enhance anthocyanins expression by copigmentation (Lancaster, 1992).

Apart from contributing to the fruit colour, anthocyanins and other phenolic compounds have been reported to reduce chronic disease such as various cancer particularly lung, prostate, liver and colon cancer (Boyer and Liu, 2004; Eberhardt et al., 2000; Xing et al., 2001), cardiovascular diseases, asthma and type II diabetes (Boyer and Liu, 2004). These polyphenolic compounds are very strong antioxidants which act as anti-oxidative, anti-mutagenic, anti-microbial and anti-carcinogenic (Awad et al., 2000; Formica and Regelson, 1995; Robards and Antolovich, 1997), which inhibits cancer cell proliferation, decreases lipid oxidation and lowers cholesterol. However, the concentration of these polyphenolic compounds varies greatly among apple cultivars, maturation and fruit ripening (Boyer and Liu, 2004). These polyphenolic compounds are abundance in apple skin rather than in fruit pulp (Tsao et al., 2003), thus peeling an apple before eating will reduce the nutritional and

health value of the apple. Many attempts have been made to improve red colouration of 'Cripps Pink' apple through increasing concentration of anthocyanins. Anthocyanins are of importance in its market acceptability and also for human health. It will be discussed in more detail in Section 2.4 and 2.5.

2.3. Phenolic compounds

Phenolic compounds are ubiquitous in all plant tissues (Hamauzu, 2006). Apart from fruit colour, these compounds closely related to sensory qualities of fruits such as astringency, bitterness and aroma (Macheix et al., 1990). Phenolic compounds have one or more hydroxyl groups attached to an aromatic ring. While, polyphenolic compounds have more than one phenolic hydroxyl attached to one or more benzene ring (Vermerris and Nicholson, 2006). These phenolic compounds can be classified into different categories such as phenolic acid, flavonoids, stilbenes and lignans (Hamauzu, 2006). Amongst these phenolic compounds, flavonoids are the most common and widespread group, which virtually present particularly in the plant photosynthesis cells.

2.3.1. Flavonoids

Flavonoids are plant secondary metabolites and more than 5000 different compounds have been isolated from different plants (Prior et al., 2006). All flavonoids compounds are water soluble. Most of red-skinned apples contain five major groups of polyphenolic such as anthocyanins [primarily cyanidin 3-*O*-galactoside], flavonols [mainly quercetin derivatives], flavanols [(+)-catechin, (-)-epicatechin and procyanidins], dihydrochalcones [mainly phloridzin], and hydroxycinnamic acids [primarily chlorogenic acid] (Cook and Samman, 1996; Łata et al., 2009; Treutter, 2006; Tsao et al., 2005; Tsao et al., 2003). In red-skinned apple, the concentration of quercetin glycosides, phloridzin and chlorogenic acid has been reported to be highest early in the season, then decreased at different rates during fruit development and become stable during maturation or ripening (Awad et al., 2001a). However, the concentration of anthocyanins (cyanidin 3-*O*-galactoside) has been reported to relatively higher in the early season, later on start to increase near maturation (Hamauzu, 2006). Amongst polyphenolic compounds in apple skin, anthocyanins are the most important compounds. These compounds play an important role in apple skin reddening. Cyanidin 3-*O*-galactoside (~80%) is the major compounds in red

skin pigmentation, while cyanidin 3-*O*-arabinoside, cyanidin 3-*O*-rutinoside, cyanidin 3-*O*-xyloside and cyanidin 3-*O*-glucoside present in minor amounts in some apple cultivars (Gomez-Cardoves et al., 1996; Lancaster, 1992). There is a huge variety of anthocyanins spread in nature and more than 500 different anthocyanins has been reported in plants (Andersen and Jordheim, 2006; Mazza and Miniati, 1993). In addition, there also a variety of types and distribution of anthocyanins in different fruits such as apple, pear and peach, have compositionally simple anthocyanins, whereas blueberry has approximately 20 types of anthocyanins (Hamauzu, 2006). The anthocyanins compounds have been discussed in detail in Section 2.3.2.

Flavonols, flavanols and anthocyanins are the most abundant compounds found in fruit and are quantitatively dominant (Solovchenko and Schmitz-Eiberger, 2003). The other classes such as flavanones, chalcones, dihydrochalcones and dihydroflavonols are important only in a particular fruit. Flavonols and anthocyanins are present in glycosylated forms, whereas flavanols are present as monomers and in condensed forms (Robards and Antolovich, 1997). Flavonols present in many fruits, vegetables and beverages. More than 200 flavonols have been identified in plants and only four of these, quercetin, kaempferol, myricetin and isorhamnetin are common in fruits (Robards and Antolovich, 1997). Flavonols are found in plants, bound to sugars as *O*-glycosides (Hollman and Arts, 2000). Flavonols compounds, primarily quercetin derivatives has been reported abundant in the skin of apple fruit (McGhie et al., 2005) and in low concentrations in the pulp (van der Sluis et al., 2001). In addition, quercetin 3-*O*-glucoside (isoquercitrin) and quercetin 3-*O*-rutinoside (rutin) are typical flavonols that often observed in fruit skin (Hamauzu, 2006). Whilst, flavanols are present as monomers (catechins) and in oligomeric or polymeric as proanthocyanidins (condensed tannin) (Hamauzu, 2006). The monomeric and polymeric flavanols represent about 60% of the total phenolics concentration in apple skin, followed by flavonols (18%), hydroxycinnamic acids (9%), dihydrochalcones (8%) and anthocyanins (5%) (Łata et al., 2009). Most common flavanols found in nature are (+)-catechin, (-)-epicatechin, (+)-gallocatechin and (-)-epigallocatechin and gallic acid ester such as (-)-epigallocatechin gallate (Hollman and Arts, 2000). All these compounds are in monomers forms and simply distinguish from other flavonoids. The catechins compounds are known to play important role in browning

incident and its concentration increase from the seed towards the apple skin (Awad et al., 2000). Proanthocyanidins are polymeric flavanols mainly present in grape seeds. The concentration of dihydrochalcones such as phloridzin increases from the skin towards the seeds (Awad et al., 2000). The concentration of hydroxycinnamic acids such as chlorogenic acid has been reported highest near the core and the seeds of apple fruit than the skin (Awad et al., 2000). The concentration of phenolic in apple skin varies among cultivars (Napolitano et al., 2004) and demonstrate non-uniform behaviour during adaptation of plants to strong sunlight (Awad et al., 2000; Ryan et al., 2002; Wang et al., 2000). Although flavonoids and other phenolic compounds have been estimated in 'Cripps Pink' apple grown in Western Australia by Whale and Singh (2007) and Whale et al. (2008). No research work has been reported on the effects of water-deficit strategies and newly developed plant growth regulators on the concentrations of these compounds in the skin of 'Cripps Pink' apple.

2.3.2. Anthocyanins

Red skin colour in 'Cripps Pink' apple is due to the presences of anthocyanins compounds. The word anthocyanin, derived from Greek words which *Anthos* means flower and *kyanos* means blue (Mazza and Miniati, 1993). Anthocyanins are present in a wide range of tissues in various plants organs such as fruits, flowers, leaves and also storage organs, roots tubers and stems (Brouillard, 1982; Deroles, 2009). Anthocyanins are highly soluble in water and also alcoholic solutions (de Pascual-Teresa and Sanchez-Ballesta, 2008) which impart red, blue, purple, and intermediate hues, and sometimes appear 'black' in some commodities (Mazza and Miniati, 1993). The occurrence of anthocyanins is abundance in epidermal and subepidermal cells, dissolved in vacuoles or accumulated in vesicles called anthocyanoplast (Andersen, 2002) and do not participate in primary photosynthetic reactions in chloroplast (Harborne, 1976; Lancaster et al., 1994; Saure, 1990). Mature apple fruit skin consists of waxy cuticle and four to eight layers of cells beneath which containing the epidermal and subepidermal layers which are cultivar dependent (Dayton, 1959; Pratt et al., 1975). The anthocyanins pigment may be located in outer four to six or even eleven epidermal and subepidermal layers and the thickness of the skin depends on these layers of cells (Dayton, 1959; Pratt et al., 1975). Increased skin darkness in apple has been reported due to increased concentration of the

anthocyanins in darker red vacuoles, larger vacuoles and several layers of red cells (Lancaster et al., 1994).

Cyanidin 3-*O*-galactoside is a major anthocyanins compounds present in the fruit skin, which plays a vital role in apple skin reddening and formed from the glycosylation of cyanidin (Lancaster, 1992). Anthocyanins have been reported to have higher antioxidants activity than vitamin C and E (Bagchi et al., 1998), and these compounds are able to capture free radicals by donation of phenolic hydrogen atoms (Chen et al., 1996). This is the reason for its anticariogenic activity (Kamei et al., 1995). Many endo- and exogenous factors affect the concentration of anthocyanins such as genetic, intensity and types of light, temperatures and agronomic factors including irrigation, pruning, plant growth regulators and fertilization (Saure, 1990). It has been discussed in Section 2.3.2.4.

2.3.2.1. Anthocyanins structure

Anthocyanidins or also called aglycones are basic structure of the anthocyanins. These aglycones or anthocyanidins contains of an aromatic ring [A] bonded to an hetrocyclic ring [C] that consist of oxygen, which is also bonded by carbon-carbon bond to a third aromatic ring [B] (Figure 2.2) (Kong et al., 2003). There is seventeen known naturally occurring anthocyanidins, but only six are common such as cyanidin, pelargonidin, delphinidin, peonidin, petunidin and malvidin, which vary by the hydroxylation and methoxylation pattern on their B-rings (Andersen and Jordheim, 2006; Eder, 2000; Kong et al., 2003). These six common anthocyanidins can be differentiated by its chemical structure at 3' and 5' position of B-ring (Horbowicz et al., 2008). The distribution of these six common anthocyanidins in fruits and vegetables is 50% cyanidin, 12% delphinidin, 12% pelargonidin, 12% peonidin, 7% petunidin and 7% malvidin (Kong et al., 2003). Cyanidin, delphinidin and pelargonidin are the most widespread in nature, being found in 80% of pigmented leaves, 69% in fruits and 50% in flowers (Dey and Harborne, 1993). Amongst four classes of anthocyanidins glycosides, cyanidin 3-*O*-glucosides are the most widespread (Kong et al., 2003). The number of hydroxyl and methoxyl groups affects the intensity and type of anthocyanins colouration. For example, if more hydroxyl groups, then the colour go towards bluish shade; if more methoxyl, then redness is increased (Delgado-Vargas and Paredes-López, 2003; Heredia et al.,

1998). The anthocyanidins or aglycones present in trace amount in fresh plant materials because they are highly instable (Clifford, 2000; Prior, 2004) and easily susceptible to degradation.

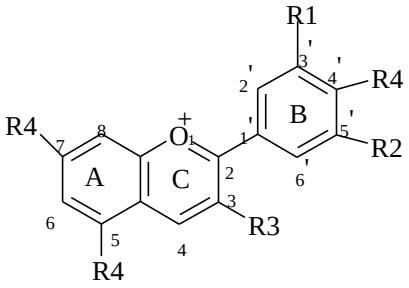

Figure 2.2. The flavylium cation. R1 and R2 are H, OH, or OCH_3; R3 is a glycosyl or H; R4 is OH or a glycosyl. Source: Kong et al. (2003).

The glycosylated anthocyanidins are called anthocyanins (Horbowicz et al., 2008) which more stable than anthocyanidins (Andersen, 2002), thereby these compounds are ubiquitous in nature. The individual anthocyanins can be identified by the number of hydroxyl groups, the number of sugars and aliphatic or aromatic groups attached to the molecule and the position of the attachment (Kong et al., 2003). More than 80 different sugars have been bound to flavonoids in plants including 10 monosaccharides, 39 disaccharides, 30 trisaccharides and 1 tetrasaccharides (Hollman and Arts, 2000). The most common sugars linked to anthocyanins are monosaccharides such as glucose, rhamnose, galactose, arabinose and xylose (Horbowicz et al., 2008). While, di and trisachharides sugars linked to anthocyanins are rutinoside, sophorose, sambubiose and glucorutinose (Figure 2.3). Glucose (90%) is the most abundant monosaccharide found in glycosylation, followed by rhamnose, galactose, xylose and arabinose (Andersen and Jordheim, 2006).

In many cases, anthocyanins can also be acylated with organic acids (Eder, 2000; Mazza and Miniati, 1993). Aliphatic dicarboxyl acid or aromatic phenolic acids or combination of both can be acylated to the anthocyanins glycosyl units. Acylation with phenolic aromatic acyl groups include various hydroxycinnamic acids derivatives such as *p*-coumaric acid, ferulic, caffeic, and sinapsic acids and hydrobenzoic acids (Andersen and Jordheim, 2006; Horbowicz et al., 2008). While, the most common acylation with aliphatic acids includes acetic, malic, succinic, tartaric and oxalic acids (Andersen and Jordheim, 2006; Horbowicz et al., 2008). The

stability of anthocyanins can be increased by co-pigmentation reactions (Horbowicz et al., 2008) and its stability can be influenced by several factors such as pH, light, oxygen, storage temperature, chemical structure, concentration of anthocyanins and the occurrence of other compounds such as other polyphenolic, enzymes, protein and metallic ions (Rein, 2005). Copigmentation is a phenomenon in which the anthocyanins molecule reacts with other natural plant components such as colourless organic compounds or metallic ions through weak interactions, thereby improved and stabilized red colour (Darias-Martin et al., 2002; Talcott et al., 2003). The copigmentation reactions involve two mechanisms such as intermolecular and intramolecular complex formations (Mazza and Miniati, 1993).

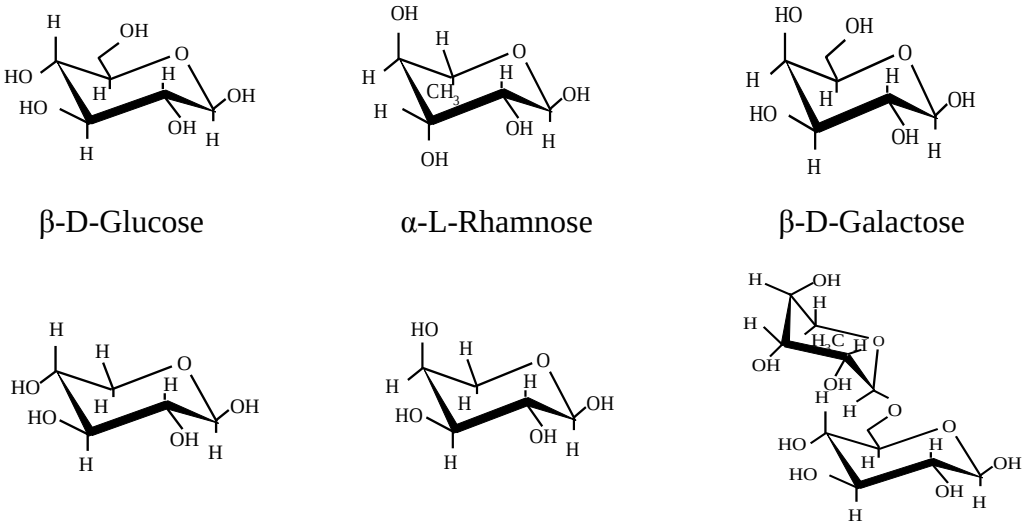

Figure 2.3. The most common glycosyl units of anthocyanins. Source: Rein (2005).

2.3.2.2. The biosynthesis of anthocyanins

Anthocyanins are synthesised via the flavonoid pathway; a branch of the phenylpropanoid pathway which consists of a number of enzymatic steps that are critical in regulating its biosynthesis (Takos et al., 2006a). Phenylalanine-ammonia lyase (PAL), chalcone isomerase (CHI), UDP galactose:flavonoid 3-*O*-galactosyltransferase (UFGalT) enzymes may have closed relationship with the anthocyanins synthesis in apple skin (Lister et al., 1994). As shown in Figure 2.4, PAL is the key enzyme in the deamination of phenylalanine, producing *trans*-cinnamic acid which then converted to 4-coumaric acid by cinnamate 4-hydroxylase

(Hamauzu, 2006). The biosynthetic pathway of anthocyanins start with the condensation of 4-coumarate coenzyme A (shikimic derived, B ring) with the malonyl coenzyme A molecules (polyketid origin, A ring) to give 2', 4', 6', 4-tetrahydroxychalcone, which is catalyzed by chalcone synthase (CHS) enzymes (Strack and Wray, 1994). Chalcone isomerase (CHI) enzyme catalyzes the isomerization of chalcone to produce naringenin chalcone (Ju et al., 1995b); then converted it to flavanone and naringenin (Holcroft and Kader, 1999). Dong et al. (1995) reported that naringenin is colourless, and the first molecule in this pathway. Flavanone 3-hydroxylase (F3H) enzymes catalyze the hydroxylation of flavanones to form dihydrokaempferol, which then undergoes the hydroxylation at 3' position to form dihydroquercetin or hydroflavonol (Ubi, 2007). The latter reaction is catalyzed by flavonoid 3'-hydroxylase (F3'H). The reduction of dihydroflavonol by dihydroflavonol 4-reductase (DFR) enzymes leads to the formation of leucoanthocyanidins, which are the immediate precursors of proanthocyanidins, catechins and anthocyanidins. Anthocyanidin synthase (ANS) enzymes then convert leucoanthocyanidins to anthocyanidin, which is the first coloured compound in the anthocyanins biosynthetic pathway. The end-products of the anthocyanins biosynthesis pathway is either the formation of UDP glucose:flavonoid 3-*O*-glucosyltransferase (UFGluT) (Ubi, 2007) and UDP galactose:flavonoid 3-*O*-galactosyltransferase (UFGalT). However, UFGluT catalysis reaction depends on the plant species that transfers a glycosyl groups such as glucosyl, galactosyl or other sugar groups to cyanidin (Lancaster, 1992; Ubi, 2007). Honda et al. (2002) reported that the key enzyme controlling red colouration is not UFGluT, but UFGalT as cyanidin 3-*O*-galactoside is the main anthocyanin identified in apple skin.

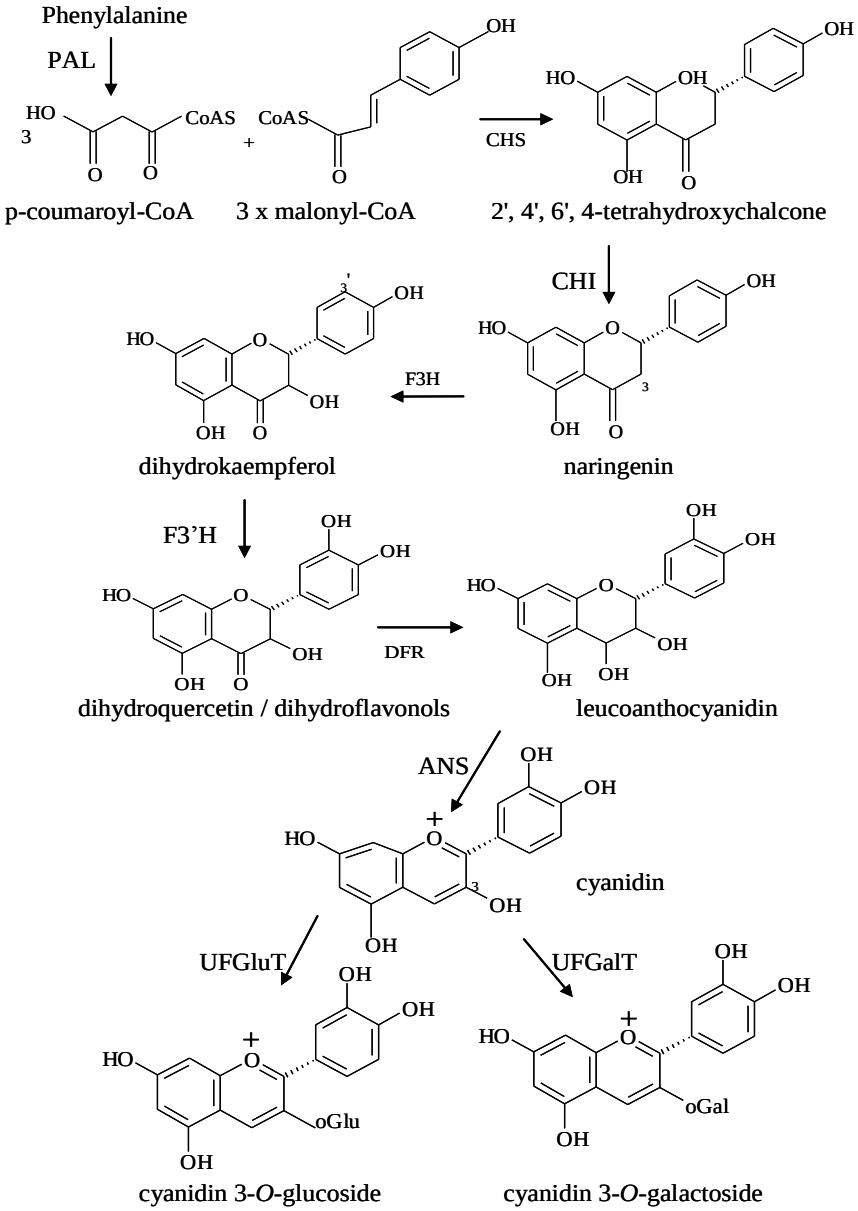

Figure 2.4. Scheme of anthocyanins biosynthesis pathway in apple skin. CHS = chalcone synthase, CHI = chalcone isomerase, F3H = flavanone 3-hydroxylase, F3'H = flavonoid 3'-hydroxylase, DFR = dihydroflavonol 4-reductase, ANS = anthocyanidin synthase; UFGluT = UDP glucose:flavonoid 3-*O*-glucosyltransferase, UFGalT = UDP galactose:flavonoid 3-*O*-galactosyltransferase, glu = glucose, gal = galactose. Source: Jordheim (2007) and Honda et al. (2002) .

2.3.2.3. Enzymes involved in biosynthesis of anthocyanins and gene expression

Phenylalanine-ammonia lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS) and UDP glucose:flavonoid 3-*O*-glucosyltransferase (UFGluT) and also UDP galactose:flavonoid 3-*O*-galactosyltransferase (UFGalT) exhibited individual key role in enhancing concentration of anthocyanins in apple

fruit skin. However, the relationships between these enzymes and anthocyanins biosynthesis in apple fruit are inconclusive (Ju et al., 1999b).

2.3.2.3.1 Phenylalanine-ammonia lyase (PAL)

PAL enzyme is the main precursor in conversion of phenylalanine to cinnamic acid. The activity of PAL enzyme was maximum in young fruits and then culminates during maturation (Macheix et al., 1990). These enzymes have been reported to be correlated well with the synthesis of anthocyanins in various fruit crops such as grapes (Hrazdina et al., 1984), strawberries (Given et al., 1988) and apples (Arakawa et al., 1986; Faragher and Brohier, 1984; Faragher and Chalmers, 1977; Tan, 1979). However, these enzymes play a minor role in biosynthetic pathway of anthocyanins in apple fruit and its involvement is dubious. PAL enzyme has been reported to be responsible in regulating the anthocyanins synthesis in apple skin (Aoki et al., 1970; Faragher and Chalmers, 1977; Li et al., 2004; Lister et al., 1996a; Lister et al., 1996b; Tan, 1979). Contrarily, Ju et al. (1995a); Ju et al. (1995b) and Lancaster, (1992) noticed that PAL enzyme did not correlate with the accumulation of anthocyanins in apple skin. Ju et al. (1995a) suggested that PAL is necessary in fruit colouration of apple to supply precursors, but when the quantity of precursors is adequate in mature fruit, PAL activity has no effect on the formation of anthocyanins. The inconsistent outcomes among investigators may be attributed to the different growing conditions and PAL itself is an inducible enzyme which could be affected by various factors (Lancaster, 1992; Saure, 1990). The activity of these enzymes has been reported to be regulated by light, temperature, wounding and chemicals (Saure, 1990). Even the occurrence of PAL is required in biosynthesis of anthocyanins, but it is not the crucial enzyme in controlling anthocyanins synthesis. In addition, Honda et al. (2002) reported that there are five other genes responsible in regulating biosynthesis of anthocyanins such as CHS, F3H, DFR, ANS and UFGT.

2.3.2.3.2 Chalcone synthase (CHS)

In anthocyanin biosynthetic pathway, chalcone synthase (CHS) is an initial step in catalysing the condensation of coumaroyl-CoA (three molecules of malonyl CoA) to produce the yellow coloured tetrahydroxy-chalcone (Dong et al., 1995; Lancaster, 1992). CHS is mainly responsible for the formation of C_{15} skeleton of flavonoid compounds, and also correlated well with the synthesis of anthocyanins in several

plant species (Heller and Forkmann, 1988; Ju et al., 1995a; Saleh et al., 1978). However, CHS activity has been shown not responsible in regulating apple colour. Heller and Forkmann (1994) noted that the activity of CHS remained stable during fruit development even there was higher or lower accumulation of anthocyanins. These findings have also been supported by Ju et al. (1995a); Ju et al. (1999b) and Treutter (2001) as they found that CHS activity did not play key role in regulating the anthocyanins formation. Therefore, CHS is not considered to regulate the synthesis of anthocyanins (Ju et al., 1995a; Ju et al., 1999b; Treutter, 2001).

2.3.2.3.3 Chalcone isomerase (CHI)

CHI has been closely associated with the accumulation of anthocyanins in apple skin (Li et al., 2002a) and the most important step in this pathway (Stafford, 1990). In addition, CHI activity increased due to the exposure of 'Royal Gala' apple fruit to UV light and white light (Dong et al., 1995) and suggested as one of the critical enzyme involved in red colouration of this cultivar. However, CHI did not regulate the accumulation of anthocyanins in apple skin as the activity of this enzyme in red apple cultivar 'Splendour' and a green cultivar 'Granny Smith' was high at all fruit development stages (Lister et al., 1996a). The contradictory outcomes may be due to the factors affecting the activities of these enzymes. The activity of CHI is induced by both white light and UV light (Dong et al., 1995) and also concentration of ethylene (Li et al., 2002a).

2.3.2.3.4 Flavanone 3-hydroxylase (F3H)

Flavanone 3-hydroxylase (F3H) is the third enzyme in anthocyanin biosynthetic pathway which catalyzed hydroxylates naringenin (4'-hydroxylated) or eriodyctiol (3', 4'-hydroxylated) to dihydrokaempferol or dihydroquercetin, respectively (Ubi, 2007). The activity of these enzymes is necessary for the production of both anthocyanins and flavonols.

2.3.2.3.5 Dihydroflavonol 4-reductase (DFR)

Dihydroflavonol 4-reductase (DFR) is the fourth enzyme which catalyzes dihydroquercetin into leucocyanidin, which is the precursor for anthocyanidin or procyandin synthesis (Ju et al., 1997; Ubi, 2007). Dihydroquercetin has been reported as one of the important intermediate in pathway of anthocyanins

biosynthesis (Ju et al., 1997). In addition, Murray and Hackett (1991) noticed that DFR is a critical enzyme in regulating anthocyanins biosynthesis in English Ivy only during juvenile phase, but not in mature phase. In contrast, Ju et al. (1997) claimed that the accumulation of anthocyanins in 'Delicious' apple skin during mature phase are closely correlated with the increased activity of DFR. However, the activity of DFR was also observed in non-red apple cultivars such as 'Golden Delicious' and 'Indo', but the activity was slightly lower than in 'Delicious' apple (Ju et al., 1997). The DFR enzyme is necessary for anthocyanins biosynthesis, but not considered as the critical enzyme in the pathway (Ju et al., 1997; Treutter, 2001).

2.3.2.3.6 Anthocyanidin synthase (ANS)

Anthocyanidin synthase (ANS) is the fifth enzyme in anthocyanin biosynthetic pathway which is required for the formation of anthocyanidins from leucoanthocyanidins (Ubi, 2007). This enzyme has been proposed to play a role in controlling biosynthesis of anthocyanins in apple fruit (Stafford, 1990). Kondo et al. (2002b) reported that the accumulation of anthocyanins in apple fruit is controlled by *MdANS* genes. Similarly, Ubi et al. (2006) noticed that the accumulation of anthocyanins in 'Sansa' and 'Tsuguru' apple was closely related to *MdANS* genes expression. Therefore, ANS is considered as one of the important enzymes in regulating red skin colour in apple fruit.

2.3.2.3.7 UDP glucose:flavonoid 3-*O*-glucosyltransferase (UFGluT) and UDP galactose:flavonoid 3-*O*-galactosyltransferase (UFGalT)

The last enzyme involved in anthocyanins biosynthesis is UFGluT as reported by Ubi (2007). However, Honda et al. (2002) suggested that UFGalT enzyme play more important role than UFGluT in apple skin, as the concentration of cyanidin 3-*O*-galactoside was higher than cyanidin 3-*O*-glucoside. This enzyme converts the unstable anthocyanidins to stable formation of anthocyanins. Anthocyanidin is unstable and glycosylation is the most important steps to stabilize the red pigment in the cells (Ju et al., 1995a; Lancaster, 1992; Stafford, 1990). This enzyme showed a positive correlation with the formation of anthocyanins in apple skin (Ju et al., 1999a; Ju et al., 1995a; Lister et al., 1996a; Stafford, 1990). However, the higher activity of UFGalT alone without the availability of precursor for anthocyanins biosynthesis may not increase the accumulation of anthocyanins (Ju et al., 1999b).

These enzymes also catalyzes quercetin glycosylation as its concentration increased in 'Fuji' apple skin and coincides with the enhancement of UFGalT activity (Li et al., 2002b). The accumulation of quercetin glycosides was noticeable in both red and non red apple cultivars as reported by Ju et al. (1995a); Ju et al.(1995b); Lancaster (1992); Lister et al. (1994). This may be attributed to the different apple cultivars, which showed different colouration mechanisms as reported by Li et al. (2002b). In addition, UFGalT enzyme have been reported as an important enzyme in regulating anthocyanins synthesis (Ju et al., 1999b). Hence, the key point for anthocyanins synthesis in apple has been suggested to be located between the reactions from leucocyanidin to cyanidin formation (Ju et al., 1997; Ju et al., 1995a; Lancaster, 1992; Lister et al., 1994; Lister et al., 1996a). In addition, the activity of UFGluT and UFGalT enzymes were regulated by light (Ju et al., 1999a) and temperature (Mori et al., 2005). Although, UFGalT activity increased the accumulation of anthocyanins in apple fruit skin, its warrant further investigations especially its role during apple skin colouration (Ju et al., 1995a).

2.3.2.3.8 Gene expression

As a prelude, red skin colouration is an important factor determining market value, thereby many investigations have been done in developing redder fruit in various apple cultivars (Iglesias et al., 2002; Ingle and Townsend, 1997; Kikuchi et al., 1997) including breeding programmes focussed on developing redder variants (Ben-Yehudah et al., 2005). In apple, genes encoding enzymes in the biosynthetic pathway of anthocyanins have been isolated and its expression has been analysed including chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanin synthase (ANS) and UDP glucose:flavonoid 3-*O*-glucosyltransferase (UFGT) as reported by Honda et al. (2002). The expression of apple anthocyanins biosynthetic genes occurs in two stages; during flowering (early expressing genes for synthesis of various flavonoids) (Dong et al., 1998) and during maturation (late expressing genes responsible for the formation of anthocyanins in the skin) (Honda et al., 2002; Kondo et al., 2002a; Kondo et al., 2002b). Ben-Yehudah et al. (2005) proposed that the pathway may be up-regulated by two different steps, in which first steps involve the production of various flavonoids and the second steps, promote the higher expression of the already active early genes especially in red cultivars.

Many investigations have been done on expression of the various genes in the later stage than in early stage of fruit development (Ben-Yehudah et al., 2005), which is commercially important. The early expressing genes were noticeable only in non-red apples as expression levels of various genes in the biosynthetic pathway of anthocyanins are genetic dependent (Kim et al., 2003; Kondo et al., 2002a). However, they claimed that if these fruit were bagged and then remove the bags before commercial harvest may induce red colouration and also expression of late genes such ANS and UFGT. The late expressing genes such as UFGT has been reported to be galactosylated (and not glucosylated) in most of red-skinned apples (Ben-Yehudah et al., 2005; Honda et al., 2002; Kondo et al., 2002a; Kondo et al., 2002b). Ben-Yehudah et al. (2005) reported that these genes belong to two gene families of transcription factors, carrying either a *MYB* domain or a basic helix-loop-helix (*bHLH*) domain. The accumulation of anthocyanins has been reported to positively correlated with the expression level of anthocyanins biosynthetic genes and their expression is believed to be controlled by *MYB* transcription factors (Ban et al., 2007). In grapes, the different types of *MYB* transcription factors showed different regulation in the biosynthetic pathway of anthocyanins either in the early stage or during ripening as reported by Ben-Yehudah et al. (2005). However, the MYB transcription factors in red-skinned apples may be up-regulated by different genes in the phenylpropanoid pathway (Ben-Yehudah et al., 2005). In addition, there are two loci of anthocyanin-related MYB transcription factors in apple; i) *MdMYB10*, genes encoding enzymes correspond to the colouration of the cortex of red-fleshed cultivars, and, ii) *MdMYB1* and *MdMYBA*, corresponding to white-fleshed cultivars as reported by Ban et al. (2007). Thus, different apple cultivars may have different expression of genes in regulating the biosynthetic pathway of anthocyanins (Ben-Yehudah et al., 2005). In addition, apple is not a true fruit and has been reported to have different genetic activities in the different part of the fruit (Awad et al., 2000).

The accumulation of anthocyanins and expression of various genes encoding enzymes in apples and grapes skin may be up-regulated by environmental stimuli such as light, temperature, ethylene and also water-deficit as reported by (Castellarin et al., 2007a; Dong et al., 1995; El-Kereamy et al., 2003; Kim et al., 2003; Mori et al., 2005). The expression of anthocyanins biosynthetic genes in apple skin such as *MdCHS*, *MdF3H*, *MdANS* and *MdUFGT* increased by UV-B irradiation and low

temperature treatment as reported by Ubi et al. (2006) and Ban et al. (2007). The expression of anthocyanins biosynthetic genes in grape berries has been noticed to decreased in high night temperature than lower night temperature (Mori et al., 2005). The effects of light and temperature in up regulation anthocyanins biosynthesis genes have been well documented. Takos et al. (2006b) reported that the re-exposure of 'Cripps Red' (a siblings of 'Cripps Pink') apple to the sunlight increased transcript levels of genes required in the biosynthesis of anthocyanins including *MdF3H, MdDFR, MdLDOX* and *MdUFGT*. However, the effect of temperature and light on levels of expression of these genes encoding enzymes in 'Cripps Pink' apple is yet to be confirmed.

The application of plant growth regulators such as ethephon (an ethylene releasing compounds) has been reported increased accumulation of anthocyanins in grape berries skin by up regulation of anthocyanins biosynthesis genes such as CHS, F3H and UFGT (El-Kereamy et al., 2003). However, the expression of genes encoding enzymes in biosynthetic pathway of anthocyanins attributed to the application of newly developed plant growth regulators such as ProCa and LPE is yet to be investigated as these chemicals enhanced the development of red skin colour of apple fruit.

The application of water-deficit early in the season (from fruit set till two days before end of veraison) increased the accumulation of anthocyanins in the 'Cabernet Sauvignon' grape berry skin via increasing the expression of F3H, DFR and UFGT (Castellarin et al., 2007a). In addition, they reported that increased concentration of anthocyanins in grape berry skin may be ascribed to the increased levels of trisubstituted anthocyanins by differential regulation of F3'H and F3'5'H genes. However, the expression of various genes encoding enzymes in 'Cripps Pink' apple subjected to water-deficit application is yet to be investigated as this water saving techniques showed promising outcomes in improving development of red skin colour.

2.3.2.4. Factors influencing production of anthocyanins

The development of fruit skin colouration in apple during ripening differs among cultivars (Arakawa, 1988). In 'Cripps Pink' apple, the development of fruit colour

commences close to the commercial harvest (Marais et al., 2001). While, the development of fruit skin colour in 'McIntosh' apple occurs 20 days anticipated to commercial harvest (Proctor and Lougheed, 1976). These differences may be related to the various factors affecting accumulation of anthocyanins including internal and external factors. External factors controlling anthocyanins formation include light, temperature, plant and soil factors, and cultural practices such as irrigation, pruning, plant growth regulators, fertilization and wounding (Lancaster, 1992; Saure, 1990). While, internal factors affecting anthocyanins formations such as genetic variations, enzymes activities and endogenous plant hormones. Light, temperatures, irrigation and plant regulators will be further discussed in Section 2.3.2.4.1., 2.3.2.4.2., 2.4. and 2.5.

2.3.2.4.1 Light

The development of fruit colour in apple is light dependent (Ju et al., 1995a; Saure, 1990; Siegelman and Hendricks, 1958). Apple fruit skin may not go red in the condition of low availability of light or under dark environment (Lancaster, 1992; Saure, 1990). The intensity and quality of light play a vital role in regulating the formation of anthocyanins in apple skin (Saure, 1990). A linear increase in the concentration of anthocyanins in pieces of apple skin with light intensity above a certain threshold value has been reported (Siegelman and Hendricks, 1958). The position of apple fruit on the tree may also stimulates the concentration of anthocyanins differently due to the different light intensity and quantity (Lister et al., 1994). Moreover, fruit at the top of the tree exposed to higher light intensity with relatively more UV than in other positions as reported by Looney (1968) and Proctor et al. (1975). Fruit received 70% full sunlight develop good colour, 40 to 70% sunlight is adequate in colour and below 40% sunlight is inadequate in colour (Heinecke, 1966; Westwood, 1995). Meanwhile, light quality involves the blue-violet and ultra-violet (UV) regions of spectrum that promotes the biosynthesis of anthocyanins, while the red light inhibits its synthesis (Saure, 1990). Rudell et al. (2002) reported increased accumulation of anthocyanins in 'Fuji' apple skin due to the application of artificial UV-Visible light. However, they also found that the production of catechin (-epicatechin) and quercetin was not affected by this artificial light. Similarly, increased accumulation of anthocyanins in pre-climacteric 'Royal Gala' apples treated with UV and white light for 3 days at 14°C has also been

reported (Dong et al., 1995). Siegelman and Hendricks (1958) noticed that in apple skin, the maximum accumulation of anthocyanins can be achieved with a high-energy photoreaction at wavebands between 650 and 670 nm, and later Downs et al. (1965) proposed a subsidiary range at 430 to 480 nm. Besides light intensity and quality, the energy of light also necessary for the accumulation of anthocyanins, 100 $J \cdot cm^{-2}$ has been suggested as the required energy (Proctor and Creasy, 1971).

Awad et al. (2001b) noted that apple fruit at the top of the tree canopy had the highest concentration of anthocyanins, followed by the fruit from outside of the canopy and those fruit from inside canopy. In addition, the sun-exposed skin of apple fruit increased concentration of cyanidin 3-*O*-galactoside (anthocyanin) and quercetin glycosides than those on the shaded side has been reported, which indicate that anthocyanin synthesis is a light dependent process (Awad et al., 2000). Increased light penetration into the tree canopy due to the reflective mulches has been reported to improve the concentration of anthocyanins in apple skin (Ju et al., 1999a). The formation of anthocyanins in apple was inhibited due to the fruit cover with the bags, and after removal of the bags and re-exposure to the sunlight increased the accumulation of anthocyanins (Ju, 1998). Similarly, 'Cripps Red' apple fruit bagged to exclude the light exposure, and the accumulation of anthocyanins and also levels of flavonols decreased in bagged fruit than unbagged (Takos et al., 2006b). All these trials highlighted the importance of light either its intensity or quality in enhancing fruit colour development via increasing the biosynthesis of anthocyanins.

The pertinent question to answer is how does light trigger the accumulation of anthocyanins in the skin of apple? Many investigators have reported that the promising outcomes and evidence by which light mediates the formation of anthocyanins. Light triggered the activity of various enzymes such as PAL, CHI and UFGalT in the biosynthetic pathway of anthocyanins as discussed earlier in Section 2.3.2.3.

2.3.2.4.2 Temperature

Temperature has a profound effect in promoting anthocyanins formation in apple fruit has been well documented (Arakawa, 1991; Creasy, 1968; Lancaster, 1992; Leng et al., 2000; Saure, 1990). The optimum temperature requirement for good

colour development varies among apple cultivars and also climatic conditions. For example, 'Cripps Pink' apple grown in South Africa showed that the alternate temperatures of 6°C and 20°C increased fruit skin colour as compared to the constant temperature at 6°C (Marais et al., 2001). Meanwhile, fruit colour development in 'Cripps Pink' apple grown in Australia determines by the temperatures throughout the growing season (Mackay et al., 1994). The concentration of anthocyanins in 'McIntosh' apple skin increase with the average temperatures below 18°C (Creasy, 1968). While, Curry (1997) suggested that the accumulation of anthocyanins in pre-climacteric stage of 'Gala', 'Delicious', 'Fuji' and 'Braeburn' apples were higher than in post-climacteric stage, and the optimum temperature for both stage ranged from 20°C to 25°C. In addition, the maximum accumulation of anthocyanins in unripe green apples of 'Jonathan' was noticeable at 12°C (Faragher, 1983).

The night temperatures (<20°C) promote biosynthesis of anthocyanins and the warm temperatures prevent its accumulation in apple fruit (Saure, 1990). Similarly, Li et al. (2004) reported that concentration of anthocyanins in 'Starkrimson' and 'Golden Delicious' apple increase rapidly in the cold weather than the warm weather conditions. Low night temperatures of 10°C improve fruit colour development than night temperatures of 21°C to 26°C has also been reported (Blankenship, 1987; Creasy, 1968). Furthermore, Reay (1999) reported that the accumulation of anthocyanins in the skin of 'Granny Smith' apple was also noticeable with the combination of 4°C and 20°C with UVB-Visible irradiation. It suggest that the cold treatment must followed by a higher temperature for the occurrence of anthocyanin formation. The differences between day and night temperatures in the apple orchard seem to play a vital role in improving colour development in apple fruit. Reay (1999) suggested that in the orchard conditions, the cold nights and clear warm days are more effective in improving accumulation of anthocyanins by allowing radiative cooling and direct UVB and visible light illumination of the cold apple fruit. The development of fruit colour in apple was noticeable at the optimum temperatures of 20°C to 25°C during day time and below 18°C at night (Chalmers et al., 1973). Furthermore, mature 'Jonathan' apple fruit exposed to 2°C nights and 24°C days exhibited slightly improve red skin colour than fruit exposed continuos 24°C (Diener and Naumann, 1981).

The increased in accumulation of anthocyanins in the apple skin is closely related to the activities of enzymes involved in its biosynthetic pathway. PAL activity increased at low temperature than high temperature, while high temperature decrease its activities (Faragher, 1983). In addition, the activity of these enzymes in the skin of apple fruit exposed to 6°C and 18°C alternately increased than the fruit exposed to 12°C and 18°C (Tan, 1979). Saure (1990) concluded that the accumulation of anthocyanins may be stimulated at low temperatures, but it also depends on the maturity of apple fruit. In addition, low night temperatures may not replaced light requirement, but they can increase the formation of anthocyanins to those fruit in the shaded side of tree canopy (Creasy, 1968). This highlighted that the availability of light is more important than temperatures in the orchard, even there is sufficient temperatures for red skin colouration.

2.3.2.4.3 Fruit maturity

Fruit colour has been reported as one of the fruit maturity indicators for apple (Bai et al., 2009). However, the red skin colour of 'Cripps Pink' apple on skin surface is not a good indicator of harvest maturity as development of fruit colour mainly concentration of anthocyanins is light dependent (Mackay et al., 1994). The development of red colouration in apple is thought to be dependent on the accumulation of anthocyanins during maturation period (Arakawa, 1988). Marais et al (2001) suggested that 'Cripps Pink' apple must be close to harvest maturity before anthocyanins synthesis occurred, even under inductive conditions. While, Chalmers et al. (1973) reported that fruit develop colour 14 to 21 days prior to harvest maturity. Later on, Proctor and Lougheed (1976) noticed that the onset of red colour pigmentation in 'McIntosh' apples was about 20 days before commercial harvest. Apple colour changes markedly as fruit mature and its changes are an important indicator of fruit ripeness. The ripening stage of different apple cultivars showed varied impact in enhancing fruit colour development (Faragher, 1983). Marais et al. (2001) claimed that the red pigmentation of apple fruit depends on the stage of fruit ripeness and closely related to the temperatures. In addition, the accumulation of anthocyanins during ripening has been reported to be correlated well with the temperature as the lower temperature increased red skin colour (Reay and Lancaster, 2001). Most red-skinned apple cultivars lose their chlorophylls (green background colour) and their red colour appears during maturation.

2.3.2.4.4 Ethylene

Ethylene is naturally occurring plant growth regulator involved in germination, leaf and flower senescence and abscission, cell elongation and fruit ripening (Abeles et al., 1994) and is referred to as ripening or stress hormone (Chang, 2007). The biosynthesis of ethylene commences with the conversion of amino acid, methionine into S-adenosylmethionine (SAM) via 1-aminocyclopropane-1-carboxylic acid (ACC), catalyzed by 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) (Oetiker and Yang, 1995). There are two systems involve in the production of ethylene; *System 1* is referred to the low level of ethylene present in early pre-climacteric stage of the fruit before the onset of ripening, while *System II* represents the high rate of production of ethylene recorded during climacteric (McMurchie et al., 1972). Thus, fruit have been grouped according to the ability to produce ethylene during maturation and ripening such as climacteric (i.e. banana and apple) and non-climacteric (i.e. grape, citrus and strawberry) (Barry and Giovannoni, 2007; Mailhac and Chervin, 2006). In climacteric fruit, the production of ethylene is restricted by the conversion of SAM into ACC, but not ACC to ethylene (Yu et al., 1979). Conversely, pre-climacteric fruit unable to convert both SAM to ACC and ACC to ethylene (Hoffman and Yang, 1980).

Apple is a climacteric fruit and ripening process continues after harvest. Increased accumulation of anthocyanins is associated with ripening closely related to increased production of ethylene (Faragher and Brohier, 1984). In addition, Blankenship and Unrath (1988) found that the development of red skin colour and accumulation of anthocyanins in fruit skin correlated well with the production of endogenous ethylene. The concentration of endogenous ethylene in ripe apples ranged between 25 and 2500 $\mu L \cdot L^{-1}$ (Burg and Burg, 1962). The development of fruit colour and accumulation of anthocyanins in apple skin has been widely reported to be up-regulated by ethylene production (Faragher and Brohier, 1984; Kondo et al., 1991; Lancaster, 1992; Saure, 1990; Whale et al., 2008). In support, Whale et al. (2008) found that the development of fruit colour and accumulation of anthocyanins in 'Cripps Pink' apple skin were enhanced with the exogenous application of ethephon (an ethylene-releasing compound) and were delayed by the aminoethoxyvinylglycine (an ethylene inhibitor) when applied at 4 to 5 weeks before commercial harvest.

Many investigators found that the production of ethylene directly affect the biosynthetic pathway of anthocyanins, whereas others reported the indirect effect on the biosynthesis of anthocyanins through advancing the fruit maturity (Saure, 1990). During maturation, the production of internal ethylene in 'Tsuguru', 'Senshu' and 'Fuji' apples paralleled with increased accumulation of anthocyanins as reported by Kondo et al. (1991). The production of ethylene in 'Delicious' and 'Braeburn' apples increased due to water-deficit application has also been reported (Ebel et al., 1993; Kilili et al., 1996b). This warrants further investigations on the effects of water-deficit on the production of ethylene which may have play a role in enhancing red skin colour especially in 'Cripps Pink' apple.

Ethylene production has also been reported to stimulate red skin colour in apple by triggering the activity of enzymes such as PAL and UFGalT. Faragher and Chalmers (1977) reported that low concentration of ethylene may stimulate the formation of anthocyanins by increasing the activity of PAL enzyme. Furthermore, the production of ethylene stimulate the activity of PAL has been noticed in 'Red Delicious' apple as the concentration of ethylene and PAL activity increased concurrently (Blankenship and Unrath, 1988). In addition, the exogenous application of ethephon (an ethylene-releasing compound) at 110 days after full bloom (DAFB) significantly increased concentration of anthocyanins and activity of UFGalT enzymes in the skin of 'Delicious' and 'Ralls' apples (Ju et al., 1995a).

2.3.2.4.5 Preharvest practices

Fruit colour development in apple skin is affected by various preharvest factors such as application of chemicals, pruning, reflective mulches, irrigation, fruit thinning and fertilization. Many findings on the application of irrigation techniques such as regulated deficit irrigation (RDI), withholding irrigation (WHI), partial root-zone drying (PRD) and evaporative cooling in apple trees in various climatic conditions have been explored with brilliant outcomes in improving fruit quality especially red skin colour development (Iglesias et al., 2005; Kilili et al., 1996a; Mills et al., 1996a; Mills et al., 1994). Plant growth regulators are also known to be the most effective technique in improving fruit skin colour such as daminocide (alar) and paclobutrazol (cultar) (Saure, 1990), senipos (Gomez-Cardoves et al., 1996; Larrigaudiere et al., 1996), vitamin E formulation (Schmitz and Noga, 1998), shikimic acid (Faust, 1965)

and ethephon (Gomez-Cardoves et al., 1996; Ju et al., 1995a; Whale et al., 2008). However, several of these chemicals are dangerous to the environment and human such as daminozide (alar) and paclobutrazol (cutlar) which has been banned due to its persistence in fruit. Besides that, fertilization practices such as calcium and potassium also has been known improve red skin colour. However, excessive application of nitrogen fertilizer has been reported to reduce red skin colouration (Raese and Drake, 1997). Therefore, the promising impact of RDI and WHI on SSC and fruit firmness without any reduction in apple fruit size (Kilili et al., 1996a; Mills et al., 1996b) and also saves irrigation water (Mpelasoka et al., 2001a) offer an attractive opportunity to investigate further on other fruit quality especially in enhancing red skin colouration of 'Cripps Pink' apple.

2.4. Irrigation and water saving strategies

Water scarcity and climatic change throughout the world increase concern to use every drop of water wisely mainly in agriculture industry. Irrigation is a major agriculture activity and its accounts for over 85% of water usage worldwide (Behboudian et al., 2005). Water plays a major role in all plant physiological processes; which can affect directly or indirectly. Approximately 80 to 95% of water is allocated in the biomass of non-woody plant tissues and over 50% in the fresh weight of woody plants (Gindaba, 2005a). The limited availability of water is a major threat to the sustainable apple production in Australia. In 2005-06, Australian agriculture sectors used 11,698 gigaliters of water for agriculture production, which 10,737 gigaliters used for the irrigation of crops and pastures (Australian Bureau of Statistics, 2007b). The average rate of water consumption for 250,000 hectares Australia's horticultural crops is approximately 8 megaliters per hectare per year (10% of Australia's water consumption) (Loveys, 2000). The increasing demand of water in urban area is compelling the diversion of water use from agriculture industry to municipalities. Due to the higher importance of water use in agriculture sectors such as apple industry, there is an urgent need to identify and adopt effective irrigation management strategies in improving apple production and also its quality. Deficit irrigation (DI) is defined as a system of managing soil water supply to impose periods of pre-determined plant or soil water-deficit that can result in some economic benefits (Behboudian et al., 2005). DI is designated to reduce or control vegetative growth and at the same time optimizing fruit size, fruitfulness and fruit

quality (Jackson, 2003). Regulated deficit irrigation (RDI), partial rootzone drying (PRD) and withholding irrigation (WHI) has been reported as effective water savings strategies in improving water use efficiency in apple industry and possibly, enhance fruit quality especially colour development in apple fruit. These irrigation approaches lead to less nutrient and biocides losses to underground water and also saved water use. These deficit irrigation strategies have been very effective for various fruit crops including peaches (*Prunus persica* L.) (Chalmers et al., 1981), European (*Pyrus communis* L.) and Asian pears (*Pyrus serotina* Rehd.) (Caspari et al., 1994; Mitchell et al., 1984; Mitchell et al., 1989), French prunes (*Prunus domestica* L.) (Lampinen et al., 1995), various plum cultivars (Intrigliolo and Castel, 2006; Naor, 2004; Naor et al., 2004) and also various apple cultivars (Ebel et al., 1993; Kilili et al., 1996a; Mpelasoka et al., 2001c).

2.4.1. Regulated deficit irrigation, withholding irrigation and its concept

Deficit irrigation (DI) supplies the entire root zone with an amount of water less than the potential evapotranspiration (English et al., 1990) and also sometimes lower the optimum level of commercial irrigation. Behboudian et al. (2005) reviewed that the term of regulated deficit irrigation (RDI) is normally used to indicate DI of trees in the early season. Whilst, some authors used the term of RDI as the application of DI in the late season before commercial harvest with specific purpose such as to control excessive shoot growth and minimise inputs for fruit production with some beneficial effects on fruit quality. The term DI also refers to 'non irrigation' by some authors (Behboudian et al., 2005), however, in the present study DI used to be synonymous with the term of RDI. Whilst, the term of withholding irrigation (WHI) is related to water-deficit induce with considering the baseline value of stem water potential (>-2.5 MPa) (Behboudian, M.H. pers. commun. 2006). RDI was first introduced in Australia for controlling vigour in high-density plantings of 'Golden Queen' peaches and 'Bartlett' pears (Chalmers et al., 1981; Mitchell and Chalmers, 1982; Mitchell et al., 1984). Vegetative and reproductive growth of peaches and pears occur in different periods, thereby allowing for control of shoots growth without any decrease in fruit size or yield (Chalmers, 1989). On the other hand, the vegetative and reproductive growth of apple occur concurrently (Forshey et al., 1983), thereby water-deficit may reduce fruit size and yield. However, the application of RDI did not affect fruit growth and yield has been reported (Behboudian et al., 2005). In

addition, RDI has been reported to reduce water and fertilizer use, decrease nutrients and biocides leaching into the underground water, decrease vegetative vigour, reduce maintenance costs and possibly improve fruit quality (Behboudian and Mills, 1997). This water-deficit technique has been explored in various apple cultivars in various climatic conditions i.e. 'Braeburn' and 'Delicious' in humid region (Ebel et al., 1995; Mills et al., 1996b) and 'Cripps Pink' apple in temperate (O'Connell and Goodwin, 2007) and in Mediterranean region (Talluto et al., 2008). However, the information of the effects of RDI on 'Cripps Pink' apple under Western Australian conditions in the Mediterranean climate is limited.

Apple tree are sensitive to water stress especially the stress applied during fruit development stages, which may reduce fruit size and total yield (Chalmers et al., 1984), thereby the exact time of application, degree and duration are critical. The application of DI in stage I and II of fruit development, did not affect yield (Li et al., 1989), whereas in stage III decreased fruit size (Berman and DeJong, 1996; Naor et al., 1999) and changed some fruit quality parameters (Li et al., 1989). In contrast, the application of water-deficit in pear trees during stage I and II increased numbers of fruit, but reduced fruit size (Mitchell and Chalmers, 1982). However, Boland et al. (1993) noticed that fruit crop acquire higher water consumption during stage III of fruit development as compared to stage II. In addition, the stage II of fruit development had the lowest sensitivity to water stress has been reported (Mitchell and Chalmers, 1982). The stage III of fruit development is highly sensitive to water-deficit particularly in reducing fruit size has also been reported in peach (Girona et al., 2002), nectarine (Naor et al., 2001; Naor et al., 1999), apricot (Torrecillas et al., 2000) and Japanese plum (Naor, 2004; Naor et al., 2004). Fruit development stages of 'Cripps Pink' apple grown in Western Australia has been grouped into four different stages (Whale and Singh, 2007). The stage I, from 0 to 35 DAFB when fruit development was characterized by cell division (fruitlet stage); stage II from 36 to 180 DAFB, the main fruit development stage characterized by cell elongation; stage III from 181 to 190 DAFB, the maturation phase; and stage IV from 191 and beyond corresponding to fruit ripening. The application of different level of irrigation affects soil water availability (Li et al., 1989; Naor et al., 1999), and consequently, plant water relation (Berman and DeJong, 1996; Naor et al., 1999), shoot growth (Chalmers et al., 1981), stomatal conductance (Naor, 1998) and fruit size (Berman

and DeJong, 1996; Boland et al., 1993; Naor et al., 1999). The availability of soil water during fruit development stages play a major role in influencing the development of cell division and cell expansion (Atkinson et al., 1998). Shoot growth and leaf expansion of fruit tree has been reported as the earliest part that shows negative respond to water-deficit (Bradford and Hsiao, 1982). Apple tree has also been reported as complex perennial tree that respond differently to water-deficit depending on their physiological stage of fruit development (Landsberg and Jones, 1981). However, apple tree has been shown to adjust well to soil water reduction through the maintenance of turgor by active osmotic adjustment (Lakso et al., 1984; Wang et al., 1995), in which enabling the apple tree to continue growth processes at very low leaf water potential (Einhorn and Caspari, 2004). Inadequate water supply to the apple trees will give a loss of turgor and wilting, cessation of growth, or even death of the plant or plant parts, closure of stomata, reduction in photosynthesis and interference of other metabolic processes (Gindaba, 2005b). Thus, the application of RDI in apple during fruit set and cell division stages of fruit development which are reportedly sensitive to water-deficit should be avoided. In addition, Behboudian et al. (2005) reported that the application of DI late in the season is more effective in realising the improvements of fruit quality rather than application earlier in the season.

2.4.1.1. Effects of RDI and WHI on soil-plant water relations

A decrease in soil moisture under water-deficit conditions often resulted in reduce stomatal conductance and leaf water potential (Mpelasoka et al., 2000b). Similarly, Naor (2001) reported the decreased in soil moisture content in the roots under water-deficit conditions may be ascribed to the decrease of soil hydraulic conductivity, thereby resulted in reduction midday stem water potential. Midday stem water potential has been proposed as an indicator of plant water stress (Garnier and Berger, 1985; McCutchan and Shackel, 1992; Naor et al., 1995; Naor et al., 1997a; Naor et al., 1999; Shackel et al., 1997; Stern et al., 1998) and more sensitive to water-deficit conditions as compared to leaf water potential (Garnier and Berger, 1985; McCutchan and Shackel, 1992; Naor et al., 1995; Stern et al., 1998) and soil water potential (Naor et al., 1995; Naor et al., 1997a; Naor et al., 1999). Stomatal closure has been known to be the main cause for the decrease in the photosynthetic rate under mild water-deficit conditions (Chaves, 1991; Chaves et al., 2002). A reduction

in stomatal conductance occurred only at stem water potential less than -2.1 MPa (Naor, 2001). The decreasing in stomatal opening under water-deficit conditions may be ascribed to the increased chemical signalling message, abscisic acid (ABA) in the dehydrated roots and then translocated it to the leaf through xylem (Raschke and Hedrich, 1985). ABA has been proposed to play a role in stomatal closure (Liang et al., 1996a; Zhang and Davies, 1990) due to its higher concentration in the leaf under water-deficit conditions and also stimulates rapid ion efflux from the guard cells (Raschke and Hedrich, 1985). Stomatal conductance has been reported had better correlation with stem water potential than leaf water potential in apple, grape and nectarine (Naor, 1998). Water uptake from the wetted roots has been reported to increased by two fold and compensated for the reduce uptake by the roots located in the dry soil (Green et al., 1997).

On the other hand, plants experienced water-deficit conditions can partially maintain cell turgor by reducing stomatal aperture, thereby decrease transpiration and may increase water-use efficiency (Talluto et al., 2008). Increased water-use efficiency by imposition of water-deficit such as RDI, WHI and PRD is well documented in various crops species (Dorji et al., 2005; Kang et al., 2002; Lo Bianco et al., 2008; Spreer et al., 2007; van Hooijdonk et al., 2007; Zegbe and Behboudian, 2008; Zegbe et al., 2004). Under water-deficit conditions, vegetative growth may be reduced more and at the same time may increase light penetration and reduce pruning cost (Talluto et al., 2008). Fruit growth has been noticed to be less sensitive to water-deficit than the above ground part of the tree (shoots) because fruit are stronger sinks and accumulate large quantities of soluble solids over the season (Chalmers, 1989). The reduction of plant water status in ‘Braeburn’ apple under DI conditions has been reported to increased some fruit qualities without affecting fruit size (Mills et al., 1994).

2.4.1.2. Effects of RDI and WHI on fruit colour development and quality

The export market demands at least 40% of fruit skin surface exhibiting a bright pink-red blush in ‘Cripps Pink apple is a prerequisite that has been set by apple industry (Department of Agriculture Western Australia, 2000). The DI technique in apple tree has been explored under various climatic conditions in various apple cultivars such as ‘Braeburn’ and ‘Golden Delicious’ with promising outcomes

especially in the development of fruit colour (Drake et al., 1981; Kilili et al., 1996a; Kilili et al., 1996b; Mills et al., 1996a; Mills et al., 1994). However, the application of DI did not significantly affect the development of skin colour in 'Braeburn', 'Delicious' and 'Pacific Rose^{TM}' apple (Ebel et al., 1993; Mpelasoka et al., 2001b; Proebsting et al., 1984; van Hooijdonk et al., 2004) and Asian pear (Caspari et al., 1996). A reduction in excessive shoot growth through applying RDI in deciduous trees may have improve light penetration (Naor, 2006), thereby increase fruit colour development (Li et al., 1989; Mitchell and Chalmers, 1982; Mitchell et al., 1984). The increased fruit colour development in apple skin under RDI or WHI could be due to several factors which regulate the concentration of anthocyanins such as light interception and the difference between day and night temperatures in the apple orchard (Lancaster, 1992; Saure, 1990). These inconclusive reports on the effects of RDI on fruit colour development in apple warrant further investigations especially in 'Cripps Pink' apple grown in the Mediterranean climate of Western Australia. Currently, no research work has been reported on the effect of RDI on the concentration of anthocyanins and other polyphenolic compounds in 'Cripps Pink' apple fruit skin.

Besides fruit colour, 'Cripps Pink' apple also needs to fulfil other standard requirements for export such as 7 to 9 kg·cm^{-2} of fruit firmness, 13 to ≥15 ^{o}Brix SSC, size ≥65 mm and 0.7 to 0.9% titratable acidity (TA) (Cripps et al., 1993; Department of Agriculture Western Australia, 2000). Effects of DI on fruit firmness in some cases are in agreement and sometimes contradictory. Increased fruit firmness in apple under DI application has been reported to be an indirect effect due to a reduction in fruit size, in which smaller fruit were firmer than larger fruit (Behboudian et al., 2005; Ebel et al., 1993; Guelfat' Reich et al., 1974; Mpelasoka et al., 2000a; Volz et al., 2003). However, the similar sizes of 'Braeburn' (Mpelasoka et al., 2000a; Mpelasoka et al., 2001b) and 'Fuji' apple (Leib et al., 2006) under DI remained firmer as compared to control fruit. The firmer fruit in DI than in control could be due to the reduction in cellular hydration and also higher density in DI fruit (Mpelasoka et al., 2000a). It is well documented that water-deficit increased SSC in 'Braeburn', 'Delicious', 'Golden Delicius', 'Fuji' and 'Cox Orange Pippin' apples (Behboudian et al., 1998; Irving and Drost, 1987; Kilili et al., 1996a; Leib et al., 2006; Mills et al., 1996a; Mills et al., 1996b; Mills et al., 1994; Mpelasoka and

Behboudian, 2002; Mpelasoka et al., 2000a; Mpelasoka et al., 2001a; Mpelasoka et al., 2001b; Proebsting et al., 1984), pear (Naor et al., 1997b) and peach (Crisosto et al., 1994). However, the application of DI early in the season followed by recovery from stress showed non-significant effects on SSC in Asian pear (Behboudian and Lawes, 1994; Caspari et al., 1996). Increased SSC in apple fruit under DI treatments has been reported due to the conversion of starch into sugars (Kramer, 1983) and also attributed to the advanced fruit maturity (Proebsting et al., 1984). Whilst, TA in apple fruit under water-deficit also resulted in inconsistent outcomes. The application of water-deficit increased TA in 'Braeburn' apple (Mills et al., 1996a; Mills et al., 1994), decreased TA in 'Golden Delicious' and 'Braeburn' (Drake et al., 1981; Mpelasoka and Behboudian, 2002) and no significant effect on TA in 'Braeburn', 'Fuji' and 'Cox's Orange Pippin' apples have been reported (Irving and Drost, 1987; Leib et al., 2006; Mpelasoka et al., 2001b). The application of water-deficit in various apple cultivars such as 'Braeburn', 'Delicious' and 'Pink Lady' consistently reduce final fruit size (Ebel et al., 1995; Ebel et al., 1993; Lotter et al., 1985; Mpelasoka et al., 2000a; Mpelasoka et al., 2001a; Talluto et al., 2008). However, no apparent effects of water-deficit on final fruit size in 'Fuji' and 'Braeburn' apple has also been reported (Kilili et al., 1996c; Leib et al., 2006; Mills et al., 1996b). These contradictory results may be associated to the weather conditions or cultivars used in the experimentation. Therefore, there is a need to investigate the effects of RDI on these fruit quality parameters particularly in 'Cripps Pink' apple grown in Western Australia.

Late-season DI and whole season DI application in apple advances fruit maturity, but early-season DI has not been reported (Mpelasoka et al., 2001b). The increased rate of ethylene production in apple fruit due to water-deficit has also been reported in 'Delicious' and 'Braeburn' apple at harvest and also during storage (Behboudian et al., 1998; Ebel et al., 1993; Kilili et al., 1996b; Mpelasoka and Behboudian, 2002; Mpelasoka et al., 2001a). Higher production of ethylene in 'Braeburn' apple under late-season DI as compared to early-season DI and control, which indicates advanced fruit maturity (Behboudian et al., 1998; Kilili et al., 1996b). The concentrations of ethylene in apple fruit has also been reported to play a key role in improving of fruit colour and accumulation of anthocyanins (Saure, 1990; Whale and Singh, 2007). These warrant further investigations whether ethylene concentration may be involved

in regulating fruit colour development and also anthocyanins concentration in 'Cripps Pink' apple under RDI and WHI irrigation techniques.

The effects of water-deficit on sugars concentration were noticeable, but its accumulation depends on the time of stress applied at fruit development stages. The higher concentration of sugars under early- and/ or late-season DI has been reported in 'Braeburn' apple (Mills et al., 1997b; Mpelasoka et al., 2001b) and Asian pear (Behboudian et al., 1994). Whilst, the increased levels of glucose, fructose, sucrose and sorbitol in Braeburn' apple under late-season DI (Mpelasoka et al., 2000a) and early-season DI has been reported (Mills et al., 1997b). The most important component of fruit osmotic potential in apple is soluble carbohydrates (Pavel and DeJong, 1995) and increased concentration in DI fruit could be the mechanism of osmotic adjustment (Mpelasoka et al., 2001b). Similarly, the elevated levels of organic acids such as malic acid may also contributed to the fruit osmotic adjustment (Ebel et al., 1993; Mills et al., 1997b).

The water-deficit irrigation strategies significantly reduce water usage in various fruit crops such as peach (Boland et al., 1993; Girona et al., 2005; Li et al., 1989; Mitchell and Chalmers, 1982), pear (Caspari et al., 1994; Marsal et al., 2002; Mitchell et al., 1989), tomato (Nakajima et al., 2004; Zegbe-Dominguez, 2003), grape (Nakajima et al., 2004) and olive (Nakajima et al., 2004). DI applied late in the season saved about 60% of water in 'Braeburn' apple grown in lysimeters (Mpelasoka et al., 2001a). While, water savings of 45 to 50% in 'Fuji' and 'Gala' apple under DI has also been reported (Einhorn and Caspari, 2004; Leib et al., 2006).

2.4.1.3. Effects of RDI and WHI on postharvest storage performance and quality

Little information is available on the effects of RDI in apple fruit on postharvest storage performance and its quality under cold and controlled atmosphere (CA). However, RDI and WHI have shown the promotive effects on postharvest performance of apple fruit during storage and shelf life. Early- and late-season DI increased fruit firmness and aroma volatile concentration in 'Braeburn' apple stored for 12 weeks at 0°C than control fruit (Behboudian et al., 1998). While, 'Braeburn' apple fruit kept in cold storage for 12 weeks under WHI treatment increased fruit

firmness and lower weight loss as compared to control has also been reported (Kilili et al., 1996b). Mpelasoka et al. (2001b) claimed that 'Braeburn' apple under WHI treatment, stored in cold at least for 10 weeks increased SSC and fruit firmness as compared to control fruit. In addition, 'Braeburn' apple fruit from WHI treatment had higher SSC and retained higher fruit firmness following 7 days shelf life (Mpelasoka et al., 2000a; Mpelasoka et al., 2001b). 'Braeburn' apple fruit under WHI treatment, cold-stored for 12 weeks had higher concentrations of fructose, sucrose and SSC as compared to control fruit (Mills et al., 1996a; Mpelasoka et al., 2000a). Due to the potential impact of RDI and WHI on postharvest performance of apple fruit during storage, thus warrants to be investigated on 'Cripps Pink' apple grown in Mediterranean climate of Western Australia.

2.4.2. Partial rootzone drying

Partial root drying (PRD) is one of the water savings techniques (Caspari et al., 2004b) that thought to be first introduced in viticulture (Dry and Loveys, 1998; Dry et al., 1996; Einhorn and Caspari, 2004) with the benefits of reducing vegetative vigour, improve light interception into canopies and maintain fruit yield (Dry and Loveys, 1998). PRD has also been reported to be successful water saving technique in wine grape (de Souza et al., 2003; Loveys et al., 2000) and pear (Kang et al., 2002) production. Mode of action of PRD substantially different with other water savings strategies as discussed in Section 2.4.1. PRD is defined as deficit irrigation strategy where part of root systems is dry out and another part is well-watered (Dry et al., 1996; Düring et al., 1997; Kang and Zhang, 2004; Loveys et al., 2000) and the quantity of water supplied sometimes lower than the optimum level. The dehydrated roots in the drying soil produce chemical signalling such as abscisic acid (ABA) and translocated it to the leaf via xylem, thereby reduce partial stomatal aperture (Dry et al., 1996; Kang and Zhang, 2004). The reduction in stomatal opening reduces transpiration and photosynthesis process and also increases water use efficiency (Dry and Loveys, 1998; Düring et al., 1997; Lo Bianco et al., 2008), thereby reduces shoot extension (Zegbe and Behboudian, 2008). On the well-watered rootzone, plant water relation and physiological processes related to water-deficit are not affected (Dry et al., 1996; Dry et al., 2000). PRD is usually alternate from wet and dry plant root system for every 10 to 15 days (Dry and Loveys, 1999). The important of alternating wet and dry part of plant roots system is to stimulates the new secondary root growth

(Liang et al., 1996b). These new roots are sensitive to soil drying condition, which contributes in enhancement of nutrient uptake in this particular soil zone (Liang et al., 1996b).

2.4.2.1. Effects of PRD on fruit colour development, quality and postharvest storage performance

The effects of PRD on vegetative growth in various fruit crops are variable. The information of the effects of PRD in apple fruit tree is inconsistent especially the yield and its fruit quality. No significant effects of PRD on yield, fruit size and quality of 'Pink lady', 'Pacific Rose^{TM}', 'Gala' and Fuji' apples have been reported (Caspari et al., 2004a; Caspari et al., 2004b; Talluto et al., 2008; van Hooijdonk et al., 2004; van Hooijdonk et al., 2007). Contrarily, Lombardini et al. (2004) noticed that PRD significantly reduced final fruit size of 'Fuji' apple. Little information is available on the effects of PRD on fruit colour development in apple. However, the effect of PRD on other fruit quality attributes such as fruit firmness, SSC and fruit size in various apples cultivars have been well notified. Increased fruit firmness under PRD has been reported in 'Fuji' and 'Pink Lady' apple (Leib et al., 2006; Lo Bianco et al., 2008). Higher SSC in 'Fuji' apple under PRD has been reported by Caspari et al.(2004b), whereas increased sugars concentration has also been reported (Dry and Loveys, 1998; Dry and Loveys, 1999; Dry et al., 2000; Loveys et al., 1997). The effects of PRD on postharvest storage performance and quality of apple are scant. Fruit quality of 'Gala' apple after storage was not affected with PRD application (Einhorn and Caspari, 2004). However, PRD application on 'Pacific Rose^{TM}' had higher SSC, fruit firmness and lower weight loss than control fruit during cold storage for 10 weeks has been reported (van Hooijdonk et al., 2007). PRD application could save water up to 30-50% in 'Gala', 'Fuji' and 'Pacific Rose^{TM}' apple, 50% in grapevine, 37% in potato (*Solanum tuberosum* L. cv. Flora) and 25-50% in tomato (*Lycorpersicom esculentum* Mill) as compared to control (Caspari et al., 2004b; Kang and Zhang, 2004; Liu et al., 2006; Loveys, 2000; Zegbe and Behboudian, 2008; Zegbe et al., 2004; Zegbe et al., 2006). Inconclusive results on the effects of PRD on apple fruit quality may be attributed to the different irrigation systems, delivery rates, timings, amounts, soil and climatic conditions in the various field trials which may have experienced different degree of water stress by the plants.

2.4.3. Comparison between RDI, WHI and PRD

The difference between RDI, WHI and PRD is on the purpose mode of action. All these water savings techniques are the example of manipulation of root signals to enhance the water use efficiency, improve or maintain yield and fruit quality of fruit crops. RDI and WHI application in various apple cultivars such as 'Braeburn', 'Pacific Rose^{TM}', 'Redspur Delicious', 'Delicious' have been reported to reduce vegetative growth, increase fruit yield and quality either at harvest or during storage and also saved irrigation water (Behboudian et al., 1998; Chalmers, 1989; Chalmers et al., 1981; Ebel et al., 1995; Ebel et al., 1993; Kilili et al., 1996a; Mills et al., 1996a; Mitchell et al., 1989; Mpelasoka et al., 2000a). van Hooijdonk et al (2007) claimed that the PRD and WHI are suitable for apple grown in humid climatic conditions, while PRD may also have potential for apple in semi-arid climates (Leib et al., 2006). PRD has been known to be successful in reducing shoot growth without affecting yield and grape quality (Dry et al., 1996). However, PRD application in 'Pink Lady' apple grown in the Mediterranean climate of Italy has been purposed as an irrigation technique to increase water use efficiency, but not in reducing excessive vegetative vigour and also improving fruit quality traits (Talluto et al., 2008). Thus, RDI and WHI could be the potential water-saving approaches suitable for 'Cripps Pink' apple grown in the Mediterranean climate of Western Australia.

2.5. Plant growth regulators

Plant growth regulators have been known to affect growth, development and maturation of vegetative and reproductive plants structures. Growth retardants are the biggest group of plant growth regulators that widely used in various crops including chlormequat chloride, mepiquat chloride, paclobutrazol, uniconazole and flurprimidol in different cultivar of pome and stone fruit, citrus, nut and grapes (Considine, 1983; Curry et al., 1987; Davis and Curry, 1991; Harty and van Staden, 1988; Miller, 1988; Rademacher, 1995). Most of the growth retardants act by inhibiting the last step in the gibberellin biosynthetic pathway (Rademacher, 1995). The reduction in shoot growth may leads to reduce the pruning cost and also promotes the process of flower initiation, thereby increasing yield of the crops (Rademacher, 1995) and possibly fruit quality particularly fruit colour development.

Various plant growth regulators and others chemicals have been tested in improving fruit colour development through increasing the accumulation of anthocyanins in various apple cultivars such as SADH (alar) in 'Rome' (Gianfagna et al., 1989), senipos (a phosphorus-calcium mixture) in 'Starking Delicious' (Larrigaudiere et al., 1996), vitamin E formulation (25% alpha-tocopherol) in 'Elstar' and 'Jonagold' (Schmitz and Noga, 1998), shikimic acid (a flavonoid precursor) in 'McIntosh' (Faust, 1965), ethephon in 'Delicious' and 'Ralls' (Ju et al., 1995a), 'Starking' (Gomez-Cardoves et al., 1996) and 'Cripps Pink' (Whale et al., 2008), methyl jasmonate in 'Fuji' (Rudell et al., 2002), prohexadine-calcium in 'Fuji' (Mata et al., 2006a), 5-aminolevulinic acid in 'Fuji" (LiangJu et al., 2004) and lysophosphatidylethanolamine in 'McIntosh' (Farag and Palta, 1991b). Spray application of galactose and glucose also enhanced the formation of anthocyanin in 'Fuji' apple (Bae and Lee, 1995). In contrast, the spray application of plant growth regulators such as alar (daminozide), cycocel (CCC), gibberellins (GA_{4+7}), prohexadione-calcium (ProCa), plantacur-E, shikimic acid, galactose and senipos did not significantly affect the formation of anthocyanins in 'Jonagold' apple skin (Awad and de Jager, 2002). They also noticed that the spray application of ethephon (S)-trans-2-amino-4(2-aminoethoxy)-3-butenoic acid hydrochloride (ABG-3168) and gibberellins (GA_3) retard the formation of anthocyanins, and chlorogenic acid in 'Jonagold' apple cultivar.

Other fruit quality attributes such as fruit firmness, SSC, TA and size of apple fruit affected by plant growth regulators in some cases were in agreement but some in contradictory. It is well documented that the application of daminozide affect numerous apple fruit quality attributes (Miller, 1988) and fruit size (Looney et al., 1967). ReTain™ an ethylene inhibitor delays fruit maturation and ripening in apple, allowing enhancement in fruit size has also been reported (Hewett, 2006). Meanwhile, the spray application of ProCa did not affect quality of apple fruit has been well documented (Miller, 1988). However, the sporadic and contradictory outcomes of these plant growth regulators on fruit quality need further investigations especially in improving fruit colour development of 'Cripps Pink' apple cultivar grown in Western Australia.

2.5.1. Prohexadione-calcium (ProCa)

Prohexadione-ca (ProCa) (3-oxido-4-propionyl-5-oxo-3-cyclohexene-carboxylate) is a new growth retardant with anti-gibberellin activity on rice (*Oryza sativa* L.) that has been reported in 1990 by Nakayama et al. (1990). Currently, this growth retardant is marketed by BASF (Carl-Bosch-Straße, Lamburgerhof, Germany) and registered as Apogee® (27.5% ProCa) in USA; Regalis® (10% ProCa) in some European countries (Prive et al., 2006). The mode of action of ProCa differs from other gibberellin biosynthesis inhibitors, it has been known to block the conversion of gibberellin A_{20} (inactive) to gibberellin A_1 (active), thereby reducing shoot elongation (Evans et al., 1999; Rademacher, 1993). The maximum uptake of ProCa by the plants is estimated for 8 hours and then moves acropetally to the growing points of individual shoots (Evans et al., 1999). The reduction of shoot growth was pronounced after 2 weeks of application of ProCa spray (Greene, 1999) and its activity lasting in 3 to 4 weeks (Unrath, 1999). The half life of ProCa has been reported only 14 days in plants before degrading to the naturally occurring propane-1, 2, 3-tricarboxylic acid and less than 1 day in soil before decomposition to the CO_2 (Evans et al., 1999), thereby it has a low toxicity and limited persistence in the trees. In addition, ProCa has no carcinogenic, mutagenic or tetragenic effects as reported by Evans et al. (1997).

Controlling vigour and shoot growth in apple trees have been explored using various techniques including spray application such as ethephon, daminozide, chlormequat and paclobutrazol, shoot and root pruning, dwarfing rootstock, girdling, fruit load and deficit irrigation (Autio and Greene, 1994; Behboudian and Mills, 1997; Goren et al., 2004; Miller and Tworkoski, 2003; Prive et al., 2006; Schupp and Ferree, 1988; Williams, 1984). However, these controlling techniques have the limitations such as ethephon stimulate preharvest drop and hasten ripening (Autio and Greene, 1994), daminozide and paclobutrazol has been banned from the market due to their toxicological risks to human and environment (Curry and Williams, 1983; Green, 1986; Miller and Tworkoski, 2003), chlormequat was not registered for use in fruit trees in United States (Miller and Tworkoski, 2003) and aggressive pruning accelerates even more shoot growth for the next season resulted in negative effects in fruit set, fruit size, fruit quality and yield (Prive et al., 2006). Meanwhile, selection of apple dwarfing rootstock is complex and girdling regards to long-term tree health by

affecting trees growth for one or more years after the treatment (Green and Lord, 1978; Green and Lord, 1983; Hoying and Robinson, 1992). Traditionally, pruning is a technique that widely used to reduce vegetative growth. However, pruning is most expensive, labour-intensive and time consuming practices (Felland, 1998; Forshey et al., 1992).

Poor management of vigorous shoot growth resulted in reduction in light penetration, reduce tree productivity, fruit quality, profit and negatively influence pest control in apple and pear trees (Aśin et al., 2007; Forshey et al., 1992; Greene, 1999; Miller, 1995). It is well documented that the spray application of ProCa inhibits vegetative growth in various crops such as apple (Basak and Rademacher, 2000; Byers and Yoder, 1999; Evans et al., 1997; Greene, 1999; Miller, 2002; Miller and Tworkoski, 2003), pear (Elfving et al., 2003b; Elfving et al., 2002; Southwick et al., 2004), plum (*Prunus domestica* L.) (Basak and Rademacher, 2000), sweet cherry (*Prunus avium* L.) (Elfving et al., 2003a), rice (*Oryza sativa* L) (Nakayama et al., 1992), tomato (*Lycopersicon esculentum* Mill.) (Yamaji et al., 1991), grain sorghum (*Sorghum bicolor* (L.) Moench) (Lee et al., 1998), wheat (*Triticum aestivum* L.) and oilseed rape (*Brassica napus* L.) (Grossman et al., 1994).

The pronounced effects of ProCa in reducing shoot elongation in apple trees were profitable towards reducing pruning cost and would be appealing for the growers to implement. However, the side effects on the fruit quality of apple must be taken into account. Numerous reports have been documented that the spray application of ProCa did not negatively effects the quality of apple fruit such as fruit firmness, SSC and TA (Mata et al., 2006a; Mata et al., 2006b; Medjdoub et al., 2005; Miller, 1988; Miller, 2002). The numbers of apple fruit may also increased with spray application of ProCa by enhancing flower bud formation as reported earlier by Prive and Stewart (2002). Fruit skin colour in red-skinned apple is prerequisite to be accepted in domestic and export markets. Intensification of fruit skin colouration in 'Fuji' apple was pronounced with the repeated spray applications of ProCa (125 or 250 $mg \cdot L^{-1}$) has been reported (Medjdoub et al., 2005). Similarly, Mata et al. (2006a) reported that higher concentration of anthocyanins in 'Fuji' apple skin with two sprays (250 $mg \cdot L^{-1}$ and 100 $mg \cdot L^{-1}$) and three sprays (100 $mg \cdot L^{-1}$, 100 $mg \cdot L^{-1}$ and 50 $mg \cdot L^{-1}$) application of ProCa. In addition, Lo Giudice et al. (2004) also found that increased

red colouration in 'Seyval' grape berries with three spray applications of ProCa (250 $mg \cdot L^{-1}$). The reduction in shoot elongation may be ascribed to improve light penetration into tree canopies and also the difference between day and night temperatures, thereby increased red skin pigmentation in 'Fuji' apple (Mata et al., 2006a; Medjdoub et al., 2005). Furthermore, the spray application of ProCa increased light interception into the canopies of fully grown apple trees has been reported (Basak, 2004; Prive and Stewart, 2002). Light play key role in regulating anthocyanins biosynthesis (Saure, 1990). However, Mata et al. (2006b) claimed that single spray application of ProCa (250 and 500 $mg \cdot L^{-1}$) did not significantly increase the concentration of anthocyanins in 'Royal Gala' apple. On the other hand, ProCa application has been reported to reduce anthocyanins formation by blocking flavanone-3-hydroxylase precursor, which play key role in flavonoid biosynthesis (Halbwirth et al., 2003; Heller and Forkmann, 1994; Rademacher, 2000; Roemmelt et al., 2003). Inhibition of flavanone-3-hydroxylase may also leads to reduced anthocyanins formation, but ProCa is easily decomposed and has a short-life either in plants or soils (Evans et al., 1999; Evans et al., 1997) and not translocated to into growing fruit (Halbwirth et al., 2003; Rademacher, 2000). Moreover, the spray application of ProCa at stage I and II fruit development, which is ahead of the onset of fruit colour development (Mata et al., 2006a). The sporadic outcomes of anthocyanins concentration subjected to the application of ProCa warrant further investigation especially in 'Cripps Pink' apple cultivar grown in Mediterranean climate of Western Australia.

2.5.2. Lysophosphatidylethanolamine (LPE)

Lysophophatidylethanolamine (LPE) is a naturally-occurring phospholipid which derived from natural sources such as egg yolk and soy lecithin (Özgen et al., 2004) and present in small amount in plant tissues (Palta and Farag, 1992). LPE present in all extra-chloroplastic membranes and is formed from parent phospholipid, phosphatidylethanolamine (PE) by the action of phospholipase A_2 (PLA_2) (Hong et al., 2009b). In plants, phosphoinositides, phosphatidic acid, diacyglycerol pyrophosphate, lysophospholipids, and phospholipase A_2, C and D are known as the key lipid signalling components (Munnik, 2001; Ryu, 2004; Testerink and Munnik, 2005; Wang, 2005).

The exogenous application LPE has been reported to accelerate ripening and prolong life of tomato fruit (Farag and Palta, 1993a), enhance ethylene production (Farag and Palta, 1989; Hong et al., 2001; Kang et al., 2003), retard senescence of tomato leaf and fruit (Farag and Palta, 1993b), prolong vase-life of snapdragon flowers (Kaur and Palta, 1997) and inhibit the activity of phospholipase D (PLD), a membrane degrading enzymes (Ryu et al., 1997). However, mode of action of LPE on plant growth is not fully elucidated. Possibly, exogenous phospholipids and lysophospholipid application affected the hypersensitive response and systemic acquired resistance in plant to improve crop performance and product quality has been suggested by Cowan (2006). The accurate concentrations of preharvest application LPE in various horticulture crops appear to be crucial as its effects are concentration dependant (Farag and Palta, 1993b; Kaur and Palta, 1997; Özgen et al., 2004; Ryu et al., 1997).

Improved fruit quality in terms of fruit colour development with the application of LPE has also been notified in cranberries (*Vaccinium macrocarpon* Ait. Steven) (Özgen et al., 2004; Özgen and Palta, 2003), 'McIntosh' apple (Farag and Palta, 1992), 'Crimson' and 'Red Globe' grapes (Hong, 2008), tomato (Farag and Palta, 1993a; Pinhero et al., 2003) and red pepper (*Capsicum annuum*) (Kang et al., 2003). The increased fruit colour may be coincided with the increase concentrations of anthocyanins which has been noticed in cranberry and apple with LPE treatments (Özgen et al., 2004; Özgen and Palta, 2003). However, the exact mechanism of LPE in mediating fruit colour development is not known. In addition, no research work has been reported on the effects of exogenous application of LPE on the poyphenolic compounds such as anthocyanins, flavonols, flavanols, hydroxycinnamic acid and dihydrochalcones in red-skinned apple. All these polyphenolic compounds are crucial important especially for colour development, taste, technological properties and also putative health promoting benefits as discussed earlier in Section 2.2.1. and 2.3. LPE application has also been reported to improve fruit firmness in 'McIntosh' apple, 'Thompson seedless' table grapes and tomato (Farag and Palta, 1993a; Farag and Palta, 1992; Hong et al., 2009a; Pinhero et al., 2003). Fruit treated with LPE had higher firmness due to its ability in protection of membrane integrity during senescence (Farag and Palta, 1993a) and also inhibit the activity of polygalacturonase (PE) (Farag and Palta, 1992) in which PE closely related to the

softening of fruit during ripening (Fisher and Bennett, 1991). In addition, exogenous application of LPE enhances postharvest storage performance of 'McIntosh', 'Golden Delicious' and 'Delicious' apples (Palta and Farag, 1992). Thus, this warrants further investigation on the effects of exogenous application of LPE on fruit colour development, polyphenolic compounds and also other quality attributes of 'Cripps Pink' apple grown in Western Australia.

2.6. Fruit quality during long term storage

The main focus of postharvest storage is to prolong the shelf life of the fruit tissues by slowing down the rate of metabolic processes, without altering fruit quality. Apple is a climacteric fruit in which the postharvest processes such as fruit softening, changes in background colour from green to yellow, loss of acidity, conversion of starch to sugars, formation of cuticular waxes and synthesis of aroma volatile compounds continues during maturation and ripening. Many of these changes will reduce the consumer acceptability quality, consequently, reduce net income of apple industries (Little and Holmes, 2000). Cold storage is the most common method used in extending storage and shelf life of various fruits, thereby making fruits available throughout the year. Low temperature storage reduce respiration, water loss, aging and breakdown processes (Paull, 1999). However, some apple cultivars are susceptible to low temperature storage, which causes chilling injury (Bai et al., 2009). The recommended cold storage temperature can be as high as 4°C, but depends on cultivar and growing region (Bai et al., 2009; Kupferman, 2003). 'Pink Lady' apple requires cooling down slowly to 0.5° to 1°C over 5 to 7 day period, while 'Braeburn' and 'Fuji' require cooling to 1°C for 2 to 3 weeks (Bai et al., 2009). Meheriuk (1993) reported that 'Cripps Pink' apple maintain its quality when stored for a period of 180 days under normal atmosphere storage. In addition, Gualanduzzi et al. (2005) reported that 'Pink Lady' apple suitable to be stored at 0°C for 60 to 120 days and also for 7 to 14 days shelf life at 20°C. Fruit quality of this cultivar decreased after 180 days in cold storage due to the loss of juiciness, crispness and also increased mealiness (Gualanduzzi et al., 2005). They also concluded that the best storability for 'Pink Lady' apple when fruit harvested with the following maturity indices such as light green yellow ground colour (69 to 71 L*; -9 to -11 a*; 40 to 43 b*), fruit firmness (7.5 to 8 kg_f), SSC (15%) and TA (1 to 1.3 $meq \cdot 10^{-1}$ mL).

The exogenous spray application of ethephon on 'Cripps Pink' apple did not cause deleterious effects on fruit quality such as fruit firmness, SSC, TA and SSC/TA ratio in long-term in cold storage as reported by Whale (2005). Similarly, no apparent effect of ethephon on postharvest storage performance of 'Tydeman Early' (Jones, 1979) and 'Jonathan' (Brohier and Faragher, 1984) apples have also been reported. On the other hand, under water-deficit condition, fruit firmness and SSC in 'Braeburn' apple improved in cold storage (Behboudian et al., 1998; Kilili et al., 1996b; Mpelasoka et al., 2000a) and TA decreased as cold storage prolonged (Mpelasoka et al., 2000a). The information on performance of 'Cripps Pink' apple under cold storage subjected to the water-deficit application is limited. Thus, warrants further investigations especially the impact of RDI and WHI on the storage performance of 'Cripps Pink' apple grown under the Mediterranean climate of Western Australia.

Controlled atmosphere (CA) storage is a technique involves altering the normal atmosphere composition of air around the fruit, generally by controlling the concentrations of certain gases such carbon dioxide (CO_2) and oxygen (O_2). The suppression of enzymes activity in respiratory metabolism and delays the rise of ethylene production in apple fruit stored in CA has been reported (Jobling and McGlasson, 1995). In general, CA storage conditions with 1.5% O_2 and up to 3% CO_2 at temperature of 1°C to 3°C have been reported as ideal conditions for most apple cultivars (Dilley et al., 1989; Meheriuk, 1990). However, the duration of storage has been reported to be dependent on cultivar, maturity stage at harvest, the composition of mineral in the fruit, storage temperature and humidity (Little and Holmes, 2000). CA storage has been reported to extend the shelf life and maintain the quality of 'Cripps Pink' apple (Cripps et al., 1993). The composition of CO_2 and O_2 for apple storage in CA varies among cultivar, growing conditions and years (Bai et al., 2009). The suitable CA condition for 'Cripps Pink' apple has been reported at 2% O_2 and 1% CO_2 (Jackson, 2003). Whereas, Meheriuk (1993) reported that 'Cripps Pink' apple respond well under CA storage using 1% O_2 and 1% to 3% CO_2. The effects of RDI and WHI on fruit quality and storage performance of 'Cripps Pink' apple under CA are scant. In addition, no research work has been reported on the effect of RDI on postharvest performance of apple fruit stored in CA.

CHAPTER 3

General Materials and Methods

3.1. Plants materials, experiments and climatic conditions

'Cripps Pink' apple [*Malus sylvestris* (L.) Mill var. *domestica* (Borkh.) Mansf.] trees grafted on MM.109 rootstock were used in various experiments conducted for three years. The experiments were carried out in two different commercial orchards at Karragullen and Carmel, Western Australia:

1. G. Casotti & Company, Karragullen (latitude 32°5'28"S; longitude 116°7'19"E), Perth Hills, Western Australia. Four experiments were conducted in the orchard during 2005-2008:

i) Effects of regulated deficit irrigation on water relations, fruit colour development, fruit quality and postharvest performance in 'Cripps Pink' apple during 2005-06 and 2006-07.

ii) Plant water relations, anthocyanins accumulation, fruit quality and post storage performance in 'Cripps Pink' apple in relation to withholding irrigation during 2006-07 and 2007-08.

iii) Effects of exogenous application of prohexadione-calcium on fruit colour development and quality of 'Cripps Pink' apple during 2007-08.

2. Giumelli and Son, Carmel (latitude 32°1'0"S; longitude 116°5'60"E), Perth Hill, Western Australia. One experiment was carried out in the orchard:

i) Promotive effects of lysophosphatidylethanolamine on fruit colour development and quality in 'Cripps Pink' apple during 2007-08.

Trees of uniform size free from pests and diseases were used in all the experiments. All trees in the orchards received similar cultural practices such as pruning, fertilization, thinning, irrigation, pesticides and fungicides sprays except experimental treatments. All locations were in a Mediterranean climate characterised by hot, dry summer and mild, wet winter.

3.2. Preharvest parameters

3.2.1. Volumetric soil water content

Volumetric soil water content (θ) was monitored using a Moisture Probe Meter (MPM 160, ICT International Pty. Ltd., Armidale, New South Wales, Australia) at a distance of between 0.5 m from tree trunk (Kilili et al., 1996a) in which 80% of root density was observed in the area (Sharma and Chauhan, 2005). The measurement was taken between 10:00 and 11:00 solar time at approximately seven and ten-day intervals. In regulated deficit irrigation experiments, θ was measured at two depths i.e. 200-300 mm and 300-400 mm in 2005-06, while 200-300 mm and 400-500 mm depths in 2006-07. Similarly, θ at 200-300 mm and 400-500 mm depths were measured for withholding irrigation experiments over two consecutive years, 2006-07 and 2007-08.

3.2.2. Leaf water potential

Leaf water potential (Ψ_{leaf}) was determined with a pressure chamber (Model 3000, Soil Moisture Equipment Corp., Santa Barbara CA, USA) using two fully expanded leaves (fifth or sixth leaf from the shoot tip) exposed to direct sunlight. The chosen leaf from each tree was from the middle height of the tree canopy. The targeted leaf, prior to excision, remain exposed to direct sunlight at least 1 hour before measurement. The petiole of the selected leaf was cut from the shoot with a surgical blade (Paramount Surgimed Ltd., Okhla Phase-II, New Delhi, India) and then quickly placed through the chamber lid with the cut edge of the petiole facing the top-outside surface and leaf blade inside the chamber. The chamber was sealed and then it was slowly pressurized with high purity nitrogen (concentration: 1.4 sm^3, U.N. No. 1066, BOC Gases Australia Ltd., Lismore, New South Wales, Australia). The positive pressure exerted on the leaf in the chamber equals the negative pressure inside the leaf which causes fluid in the leaf to force out of the cut edge of the leaf. During pressurization, sap droplet appeared in the exposed edge of the petiole, and the corresponding pressure was read from the chamber gauge. Leaf water potential values expressed in (-) mega Pascal (MPa). Measurement was taken on 11:00 and 14:00 solar time at approximately seven and ten-day intervals.

3.2.3. Stem water potential

Stem water potential (Ψ_{stem}) was determined using a pressure chamber (Model 3000,

Soil Moisture Equipment Corp., Santa Barbara, CA, USA) from two leaves (fifth or sixth leaf from the shoot tip) per tree. Stem water potential (Ψ_{stem}) was a reading of water potential within the xylem of the plant. Leaf in the shaded side of the canopy (near the trunk) was chosen to minimize any possible heating effects. To record $\Psi_{stem,}$ the leaf was enclosed in a plastic bag that was covered with aluminium foil to allow the water tension in the leaf to equilibrium with the water tension in the stem (Scholander et al., 1965). The bag was left for 90 to 120 minutes before measurement taken between 11:00 and 14:00 solar time at approximately seven and ten-day intervals. The excised leaf was then placed through the chamber as detailed in Section 3.2.2. Ψ_{stem} was expressed as in (-) mega Pascal (MPa).

3.2.4. Stomatal conductance

Stomatal conductance (g_s) was determined using a Steady State Diffusion Porometer (Model SC-1, Decagon Devices Inc., Pullman, WA, USA). Three randomly selected leaves from each tree were used to determine stomatal conductance. Two leaves from the exposed side and one leaf from the shaded side of the tree were selected and measured at 11:00 to 01:00 solar time. g_s was expressed as $mmol \cdot m^{-2}s^{-1}$.

3.2.5. Fruit drop

Five branches per tree were randomly tagged on the trees and total number of fruits on each branch was counted at the commencement of experiment until harvest. Every fruit from all the branches were counted on weekly basis. Fruit drop was expressed as percentage.

3.2.6. Shoot growth

Six current-season shoots were randomly selected and tagged at the outer and middle part of the canopy. The shoot length was measured at full bloom (0), 25, 50, 100 and 175 days after full bloom (DAFB). Cumulative shoot length was expressed in centimetre (cm).

3.3. Fruit quality: fruit colour

3.3.1. Surface skin colour: visual assessment

Percentage red blush of individual apples were visually assessed and blush score was on a percentile basis on a scale of 0 to 100%. Zero percent was allocated to the apple

with no red blush, while 100% was allocated to apple with a fully red skin surface (Whale and Singh, 2007). Twenty to twenty-five fruit were visually assessed and fruit blush was expressed in percentage (%).

3.3.2. Surface skin colour: HunterLab ColorFlex

The apple fruit colour was also recorded using a Hunter Lab ColorFlex $45^o/0^o$ Spectrophotometer (HunterLab ColorFlex, Hunter Associates Inc. Reston, Virginia, U.S.A.). Colour was measured at each of two equatorial opposite sides; ES (exposed side) and SS (shaded side). Fruit colour data were expressed in L*, a* and b* values (Figure 3.1) (Hunter, 1975). L* represented the lightness coefficient which ranges from 0 (black) to 100 (white). a* ranges from -60 to +60, which indicates red (+60) and green (-60) colours. Meanwhile b* ranges from -60 to +60, which indicates as yellow (+60) and blue (-60) colours. a* and b* were further used to calculate hue angle ($h^o = \tan^{-1}$ b*/a*) for colour interpretation. Chroma (C*) corresponded to the intensity or colour saturation, in which low values represent dull colour while high values represent vivid colour. Chroma was calculated from $(a^{*2}+b^{*2})^{½}$. Hue angle (h^o) represented red-purple (0^o), yellow (90^o), bluish green (180^o) and blue (270^o) (McGuire, 1992). Fruit colour data was expressed in L* (lightness), a* and b*, while hue angle and chroma using formula explained by (McGuire, 1992).

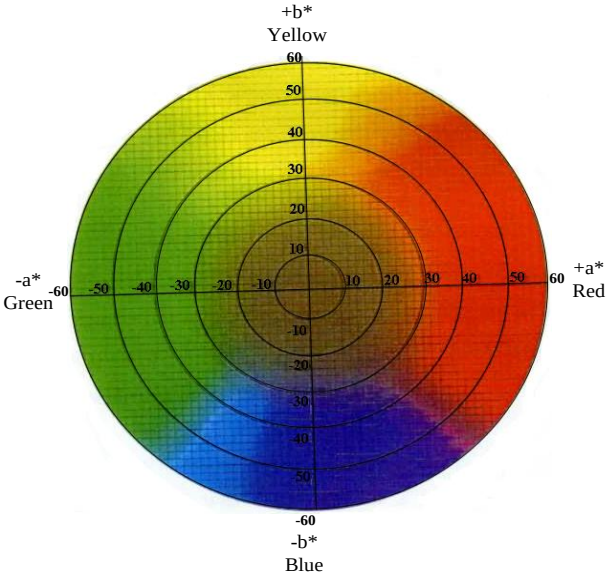

Figure 3.1. HunterLab colour chart-Commission International de L'Eclairage (CIE) L*, a* and b*. Source: (HunterLab, 1998).

3.3.3. Analysis of skin pigment

3.3.3.1. Total anthocyanins

Anthocyanin was extracted from apple skin and quantified following the method described by Bishop and Klein (1975) and Whale and Singh (2007). Apple skin (1 g) of fruit skin was cut into smaller pieces then soaked in 10 mL of methanol (95%): concentrated hydrochloric acid (HCl) in the ratio (97:3 v/v). The skin was soaked in the methanol: HCl solution ±18 hours at 2° to 4°C in the dark. The extract was then decanted and centrifuged at 5000 rpm for 20 min at 4°C (Eppendorf 5810R, Hamburg, Germany). The supernatant was then determined using an UV-VIS spectrophotometer (Jenway Spectrophotometers Model 6405, Dunmow, Essex, U.K.) at 530 nm. Total anthocyanins calculated using a molar co-efficient of 3.43×10^4 for idaein chloride (Siegelman and Hendricks, 1958) and expressed in $\mu g \cdot g^{-1}$ fresh weight.

$$\text{Total anthocyanins concentration} = \frac{\text{Absorbance at 530 nm}}{3.43 \times 10^4 \text{ L}}$$

Where L = length of light path (cm).

3.3.3.2. Flavonoids and other phenolic compounds

3.3.3.2.1 Chemicals

Phenolic standards for high performance liquid chromatography (HPLC) were purchased from different manufacturers. Chlorogenic acid was the product of Sigma (Castle Hills, New South Wales, Australia), phloridzin dihydrate, quercetin 3-*O*-rutinoside (Rutin), quercetin 3-*O*-glucoside (isoquercetin), quercetin 3-*O*-rhamnoside (quercitrin) and quercetin 3-*O*-galactoside (hyperoside) from Carl Roth (Karlrushe, Germany), quercetin 3-*O*-arabinoside (avicularin) and quercetin 3-*O*-xyloside (reynoutrin) (AApin Chemicals Ltd, Oxon, UK), cyanidin 3-*O*-galactoside (idean chloride) (Extrasynthase, Genay, France). Water used for HPLC was deionised water that has been purified in a Mili-Q system (Milipore, Bedford, MA, U.S.A). Acetic acid and water were filtered using 0.45 µm nylon filters (Alltech Associates, Baulkham Hills, New South Wales, Australia). While, acetonitrile was filtered through 0.45 µm filters (Fluoropore™ Membrane filter, Milipore, Ireland). All others solvents used were of HPLC grade including acetonitrile (Ajax Finechem, Pty Ltd, New South Wales, Australia) and formic acid (Merck, Darmstadt, Germany).

3.3.3.2.2 Extraction procedure of flavonoids and other phenolic compounds

Flavonoids and other phenolic compounds were extracted using a modified method outlined by Whale and Singh (2007). Apple skin (1.5 g) was ground in mortar and pestle in the presence of White Quartz Sand (-50+70 mesh, Sigma-Aldrich Chemie GMbh, Steinheim, Germany) using 10 mL solution of methanol: 10% acetic acid (85:15 v/v). The extract was centrifuged at 10000 x g for 20 min at 4 °C (Eppendorf 5810R, Hamburg, Germany). The supernatant was filtered through a 0.45 μm membrane filter (Fluoropore™ Membrane filter, Millipore, Ireland) and analysed by HPLC.

3.3.3.2.3 Identification of flavonoids and other phenolic compounds

Flavonoids and other phenolic compounds in apple skin were identified by comparing their retention times with authentic standards and re-confirmed using high performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-ESI-MS). The HPLC-ESI-MS was performed in MS analysis in negative and positive ion atmospheric pressure chemical ionization (APCI) mode on the Agilent 1100 LC-MSD. Trap with the instrument parameters set as dry gas flow 5 L per min, nebuliser pressure at 60 per square inch (p.s.i). Drying gas temperature and vaporizer temperature was set up at 350°C. Extracted samples (20 μL) were injected into the HPL-ESI-MS-system following the same conditions and gradient as detailed in Section 3.3.3.2.4.

3.3.3.2.4 Quantification of flavonoids and other phenolic compounds

For quantification, a 20 μL of extract as detailed in Section 3.3.3.2.2 was injected into the HPLC system (Waters 1525 Binary HPLC Pump fixed to Waters 2487 Dual Wavelength Absorbance Detector and Waters 717 plus Autosampler; Waters Corp., Milford, Mass., USA). Separation was performed on Alltima™ C18 column-W (250 mm x 4.6 mm i.d.; 5μm, Grace Davison Discovery Science, Baulkham Hills, New South Wales, Australia) that was preceded by a Symmetry C_{18} guard column (Waters Corp., Milford, Mass., USA) containing the same stationary phase. The column and guard column were maintained at 35°C. The mobile phase was composed of solvent A (5% formic acid) and solvent B (100% acetonitrile). The elution was achieved in 35 min using the following gradient: linear gradient from 5% B to 20% in 20 min, from 20% to 30% in 10 min, from 30% to 0% in 1 min, then isocratic at 0% for 4

min (Table 3.1). The system was equilibrated for 20 min before the next injection. The constant flow rate was 1.0 mL per min, and the runs were monitored at the following wavelength: hidroxycinnamic acid and dihydrochalcones at 280 nm, flavonols (quercetin glycosides) at 350 nm and anthocyanins (cyanidin 3-*O*-galactoside) at 530 nm. All analyses were done in duplicate. Flavonoids and other phenolic compounds in the apple skin were expressed as $\mu g \cdot g^{-1}$ fresh weight.

Table 3.1. Gradient elution used for separation of flavonoids and other phenolic compounds in the skin of 'Cripps Pink' apple.

Time (min)	Solvent A (%)	Solvent B (%)
	95	5
4	95	5
20	80	20
30	70	30
31	100	0
35	100	0

3.3.3.2.5 Preparation of standards

Standards solutions were prepared by dissolving 0.5 to 1.5 mg of accurately weighed standard (refer Section 3.3.3.2.1.) in 10 mL methanol: 10% acetic acid (85:15 v/v). Aliquots of 2.5, 5, 10, 15 and 25 µL were injected into the HPLC system following the same conditions and gradient as detailed in Section 3.3.3.2.4. Standard curves for individual standard were generated using Waters Breeze software (Version 3.30) by plotting each peak area against different amounts (mg) of the analyte. Calibrations for each standard were made at three different wavelengths as detailed in Section 3.3.3.2.4. The relationship between the peak area and amount is shown by slope *(a)*, intercept *(b)* and *r* values in Table 3.2. *a* and *b* represent coefficients of the regression equation $y = ax + b$, where *x* is amount of the phenolic compounds (mg), *y* is peak area and *r* is correlation coefficient of the equation. The *r* values for all polyphenolic compounds showed very high linearity ($r = 0.999$). The elution order and retention times of the polyphenolic compounds (of standards and extracts) are shown in Table 3.3., Figure 3.2 and Figure 3.3.

Table 3.2. Analytical characteristic of the standard curves.

Standard	Slope *(a)*	Intercept *(b)*	*r*
Chlorogenic acid	1.48e+006	4.06e+004	0.999
Cyanidin 3-*O*-galactoside	2.86e+006	6.12e+004	0.999
Quercetin 3-*O*-rutinoside	2.75e+006	2.30e+003	0.999
Quercetin 3-*O*-galactoside	2.32e+006	1.16e+005	0.999
Quercetin 3-*O*-glucoside	2.95e+006	1.35e+004	0.999
Quercetin 3-*O*-xyloside	1.48e+006	7.65e+001	0.999
Quercetin 3-*O*-arabinoside	1.68e+006	2.15e+004	0.999
Quercetin 3-*O*-rhamnoside	3.03e+006	4.19e+003	0.999
Phloridzin	2.31e+006	5.07e+004	0.999

Table 3.3. Elution order and retention times of different flavonoid standards used for identifying the polyphenolic profile in ‘Cripps Pink’ apple

Elution order	Standard	Retention time (min)	Detection wavelength (nm)
1	Chlorogenic acid	16.69	280
2	Cyanidin 3-*O*-galactoside	19.44	530
3	Quercetin 3-*O*-rutinoside	26.02	350
4	Quercetin 3-*O*-galactoside	26.46	350
5	Quercetin 3-*O*-glucoside	26.86	350
6	Quercetin 3-*O*-xyloside	27.95	350
7	Quercetin 3-*O*-arabinoside	28.83	350
8	Quercetin 3-*O*-rhamnoside	29.53	350
9	Phloridzin	29.98	280

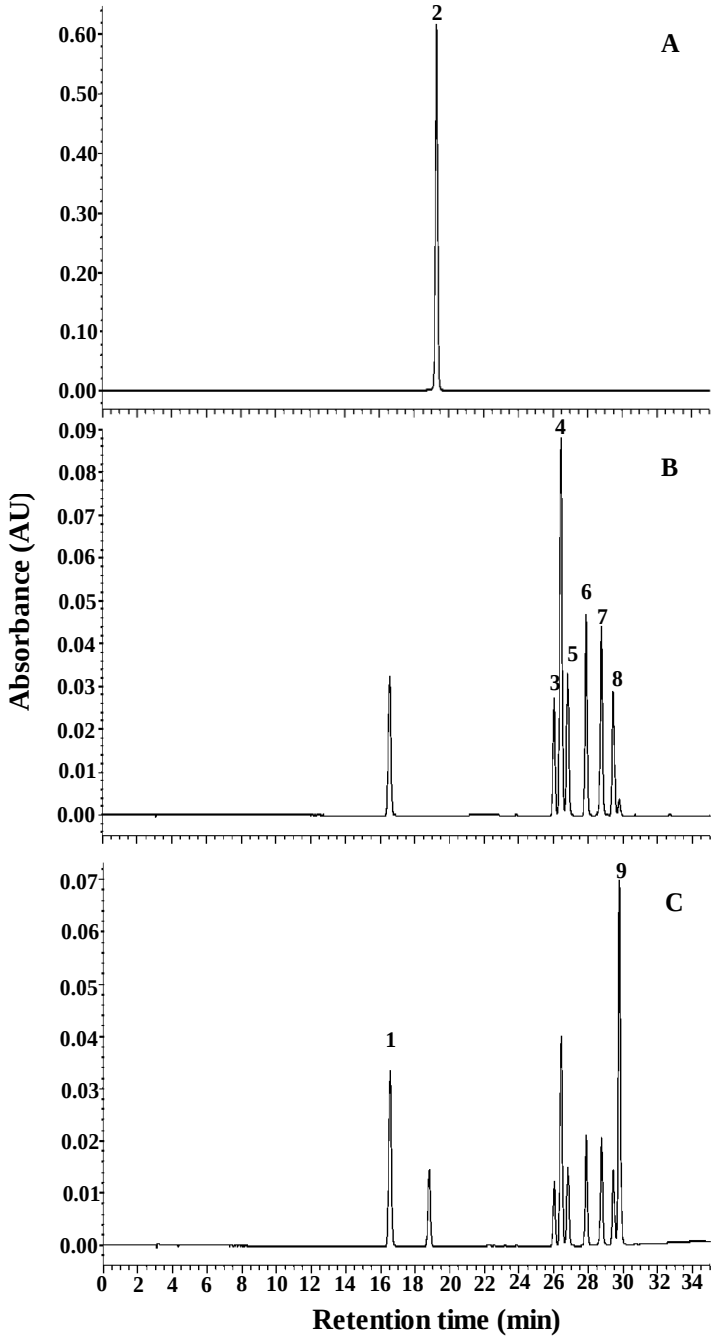

Figure 3.2. HPLC chromatograms of the standards used for identifying different flavonoids and other phenolic compounds in the skin of 'Cripps Pink' apple: (A) anthocyanin at 530 nm; (B) flavonols at 350 nm; (C) hydroxycinnamic acid and dihydrochalcone at 280 nm. Peak 1: chlorogenic acid; peak 2: cyanidin 3-*O*-galactoside; peak 3: quercetin 3-*O*-rutinoside; peak 4: quercetin 3-*O*-galactoside; peak 5: quercetin 3-*O*-glucoside; peak 6: quercetin 3-*O*-xyloside; peak 7: quercetin 3-*O*-arabinoside; peak 8: quercetin 3-*O*-rhamnoside; peak 9: phloridzin.

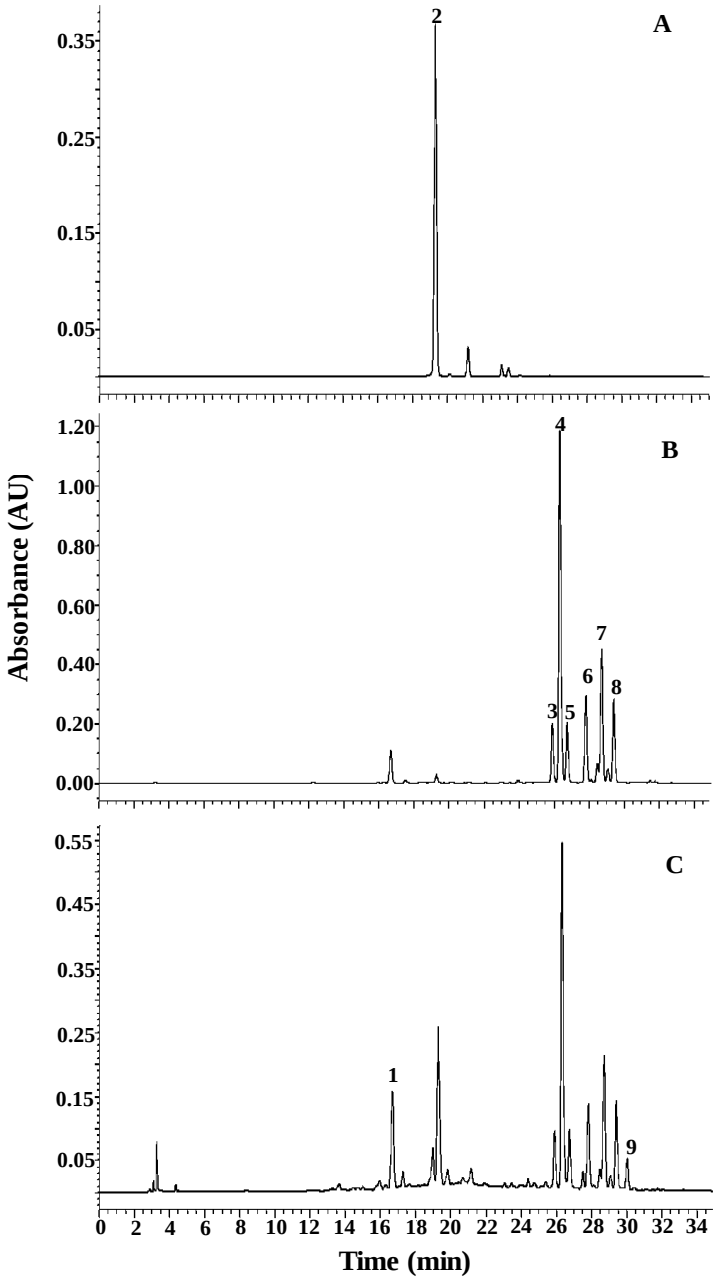

Figure 3.3. HPLC chromatograms of skin extracts of 'Cripps Pink' apple: (A) anthocyanin at 530 nm; (B) flavonols at 350 nm; (C) hydroxycinnamic acid and dihydrochalcone at 280 nm. Peak 1: chlorogenic acid; peak 2: cyanidin 3-*O*-galactoside; peak 3: quercetin 3-*O*-rutinoside; peak 4: quercetin 3-*O*-galactoside; peak 5: quercetin 3-*O*-glucoside; peak 6: quercetin 3-*O*-xyloside; peak 7: quercetin 3-*O*-arabinoside; peak 8: quercetin 3-*O*-rhamnoside; peak 9: phloridzin.

3.4. Other fruit quality parameters

3.4.1. Fruit diameter

Ten fruit were randomly selected and tagged in each experimental tree and fruit diameter was recorded using a digital vernier calliper at weekly intervals and expressed in mm.

3.4.2. Fruit firmness

Fruit firmness was determined using an electronic pressure tester (Model EPT-1 pressure tester, Lake City Technical products Inc., Kelowna, BC, Canada) fitted with 11 mm diameter plunger from two peeled apple surfaces opposite each other at the equator of each region. Fruit firmness was expressed as Newtons (N).

3.4.3. Titratable acidity

Twenty-five fruit were extracted using a juice extractor (Sunbeam, Model JE 8500, Botany, New South Wales, Australia). Extracted juice (10 mL) was diluted with 20 mL of distilled water. Five mL of this aliquot was titrated against 0.1N NaOH (sodium hydroxide) using phenolphthalein as an indicator of the end point by change in colour to pink. Titratable acidity (TA) was expressed as percent malic acid using the formula given below:

$$\text{Malic acid } (\%) = \frac{0.0067\,(\text{mL of NaOH}) \times (\text{volume made up})}{(\text{mL of juice taken}) \times (\text{Volume of aliquot taken})} \times 100$$

3.4.4. Soluble solids concentration

The soluble solids concentration (SSC) of freshly extracted juice was recorded using an infrared digital refractometer (Atago-Pallette PR 101, Atago Co. Ltd, Itabashi-Ku, Tokyo, Japan). A refractive index was measured at 20^{o}C and the results were expressed in percentage (%).

3.4.5. SSC/TA ratio

SSC/TA ratio was calculated by dividing soluble solids concentration with the titratable acidity values. The results expressed in percentage (%).

3.5. Determination of ascorbic acid, total antioxidants, individual sugars and organic acids

3.5.1. Ascorbic acid

Ascorbic acid was determined according to the method by Jagota and Dani (1982) and Malik and Singh (2005). Apple pulp (5 g) were homogenised in glass mortar and pestle using White Quartz Sand (-50+70 mesh, Sigma Aldrich, USA) with 25 mL of 6% metaphosphoric acid containing 0.18 g of ethylenediamine tetra-acetic acid disodium salt (EDTA). The homogenate was centrifuged at 5000 rpm for 20 min (Eppendorf Centriguge, Hamburg, Germany). The supernatant (500 µL) was mixed with 200 µL (3%) metaphosphoric acid, 1400 uL distilled water, and diluted 200 µL Folin reagent (Folin:distilled water, 1:5 v/v). Disposable cuvettes (2 mL) were used to record the absorbance of the mixed sample after 10 minutes at 760 nm wavelength using a UV-VIS spectrophotometer (Jenway Spectrophotometers Model 6405, Dunmow, Essex, U.K.). Ascorbic acid concentration was quantified using a standard curve of *L*- ascorbic acid and was expressed as mg·100 g^{-1} fresh weight.

3.5.2. Total antioxidants

Total antioxidants were determined following the method of Brand-Williams et al. (1995) and Khan et al. (2008). Apple pulp (15 g) and skin (0.5 g) were ground in a mortar and pestle using White Quartz Sand (-50+70 mesh, Sigma Aldrich, USA) in 10 mL extraction buffer (2 mM NaF dissolved in 200 mL distilled water and 800 mL methanol). The free radical was 2, 2-diphenyl-1-picrylhydrazyl (DPPH) as stock solution by dissolving 24 mg DPPH in 100 mL methanol. Diluted stock solution (1:4 v/v) was prepared as working solution. The homogenate was centrifuged at 10000 x g for 20 min (Eppendorf 5810R, Hamburg, Germany). The supernatant (50 µL) was mixed with 950 µL working solution. The absorbance was recorded using a UV-VIS spectrophotometer (Jenway Spectrophotometers Model 6405, Dunmow, Essex, U.K.) at 515 nm wavelength. Total antioxidants was estimated using a standard curve of 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and was expressed as mM Trolox Equivalent·g^{-1} fresh weight.

3.5.3. Determination of individual sugars and organic acids

3.5.3.1. Chemicals

Chemicals used for sugars were D-(+)-glucose, D-(-)-fructose, sucrose and sorbitol. All sugars were the product of Sigma (Sigma-Aldrich, St. Louis, U.S.A) excluding sorbitol the product of BDH-GPR, England. For individual organic acids, chemical used were citric acid, L-malic acid, fumaric acid, ammonium tartarate disodium salt and succinic acid. All organic acids were the product of Sigma (Sigma-Aldrich, St. Louis, U.S.A) excluding succinic acid which was purchased from Fluka, U.S.A.

3.5.3.2. Extraction and estimation of individual sugars and organic acids

Apple pulp (5 g) was homogenized in 50 mL of Mili-Q water (Mili-Q system, Milipore, Bedford, MA, U.S.A) using hand blender (Model HB95, Breville Pty. Ltd., New South Wales, Australia) for 1 minute. The homogenate was centrifuged at 10000 x g for 15 min (Eppendorf 5810R, Hamburg, Germany). The supernatant was filtered through 0.22 µm nylon filters (Alltech Associates Ltd., Baulkham Hills, New South Wales, Australia). Following filtration, 20 µL of supernatant was injected into the HPLC system. Chromatographic peaks of sugars and organic acids were identified by comparing retention times and peak area with the known individual sugars and organic acids standards as detailed in Section 3.5.3.1. The elution order and retention times of the individual sugars and organic acids are shown in Table 3.4 and Table 3.6. Individual sugars and organic acids were further calculated and expressed as $g \cdot kg^{-1}$ fresh weight excluding fumaric acid in $mg \cdot kg^{-1}$ fresh weight.

3.5.3.3. HPLC conditions

HPLC was used for separation, identification and quantification of individual sugars and organic acids in the apple juice. The HPLC system, Waters 1525 Binary HPLC Pump fixed to Waters 2487 Dual Wavelength Absorbance Detector, Waters 2414 Refractive index detector and Waters 717plus Autosampler (Waters Corp., Milford, Mass., USA) was used. Individual sugars were glucose, fructose, sucrose and sorbitol were analysed isocratically on the Fast Carbohydrate Analysis column (Aminex-HPX 87C, 100 x 7.8 mm) (Bio-Rad Laboratories, Hercules, CA, USA) was preceded with the same guard column as above (Bio-Rad Laboratories, Hercules, CA, USA) with the eluent flow rate of 1.2 mL per min at 60°C. Mili-Q water (Mili-Q system, Milipore, Bedford, MA, USA) was used as an eluant and vacuum degassed prior to

analysis. The elution was achieved in 10 min. Sugars were identified and quantified through a refractive index detector (Waters 2414, Waters Corp. Milford, MA, USA) (Figure 3.4).

Individual organic acids were malic, citric, fumaric, tartartic and succinic were separated on a Bio Rad Aminex-HPX 87H (300 x 7.8 mm; particle size 9 µm) (Bio-Rad Laboratories, Inc., Hercules, USA) with preceded with guard column; Cation H Bio Rad Micro-Guard® (30 x 4.6 mm) (Bio-Rad Laboratories, Inc., Hercules, USA). Organic acids were eluted isocratically with sulphuric acid (0.5 mM, solvent A) and Mili-Q water (solvent B) as mobile phase. Both columns were kept at 45°C. The elution was achieved in 30 min with the flow rate at 0.3 mL per min. Organic acids compounds were detected with absorbance detector at 210 nm wavelength (Figure 3.5).

3.5.3.4. Preparation of standards

Standard solutions for sugars were prepared by dissolving 0.5 g in 100 mL Milli-Q water. For organic acids, standard solutions were prepared by dissolving 0.01 g to 0.2 g in 100 mL Milli-Q water. Aliquots of 4, 8, 12, 16 and 20 µL of standard solutions were injected into the HPLC system following the same conditions and gradient as detailed in Section 3.5.3.3. Standards curves (Table 3.5 and 3.7) and quantification of sugars and organic acids concentration were done as detailed in Section 3.3.3.2.5.

Table 3.4. Elution order and retention times of different sugars standard used for identifiying the individual sugars concentration in ‘Cripps Pink’ apple

Elution order	Standard	Retention time (min)	Detection wavelength (nm)
1	Sucrose	2.02	RI
2	Glucose	2.37	RI
3	Fructose	3.31	RI
4	Sorbitol	7.28	RI

RI = refractive index

Table 3.5. Analytical characteristic of the standard curves of different sugars standard.

Standard	Slope *(a)*	Intercept *(b)*	*r*
Sucrose	5.59e+003	-6.25e+003	0.999
Glucose	5.59e+003	-6.25e+003	0.999
Fructose	5.55e+003	-6.60e+003	0.999
Sorbitol	5.61e+003	-1.20e+004	0.999

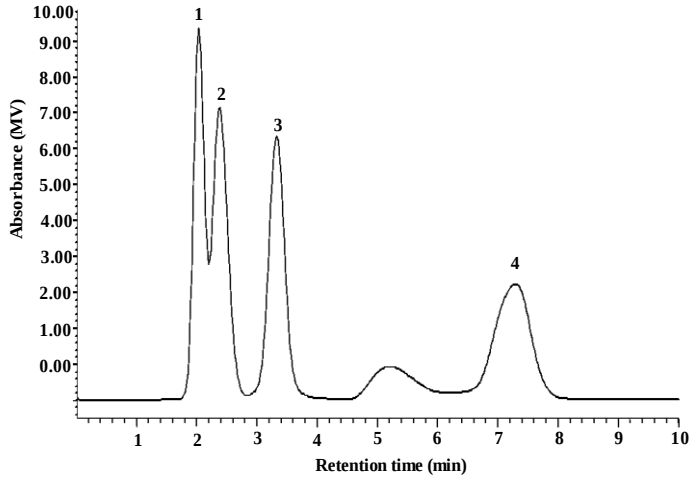

Figure 3.4. HPLC chromatograms of the standards used for identifying individual sugars in the 'Cripp's Pink' apple pulp. Peak 1: Sucrose; peak 2: Glucose; peak 3: Fructose; peak 4: Sorbitol.

Table 3.6. Elution order and retention times of different organic acids standard used for identifying individual organic acids concentration in 'Cripps Pink' apple

Elution order	Standard	Retention time (min)	Detection wavelength (nm)
1	Citric	15.88	210
2	Tartaric	17.09	210
3	Malic	18.85	210
4	Succinic	23.46	210
5	Fumaric	27.29	210

Table 3.7. Analytical characteristic of the standard curves of individual organic acids standards.

Standard	Slope *(a)*	Intercept *(b)*	*r*
Citric	2.13e+005	-1.46e+004	0.999
Tartaric	2.52e+005	-1.39e+005	0.999
Malic	1.63e+005	-4.71e+004	0.999
Succinic	1.13e+005	-4.30e+004	0.999
Fumaric	2.19e+007	-1.46e+005	0.999

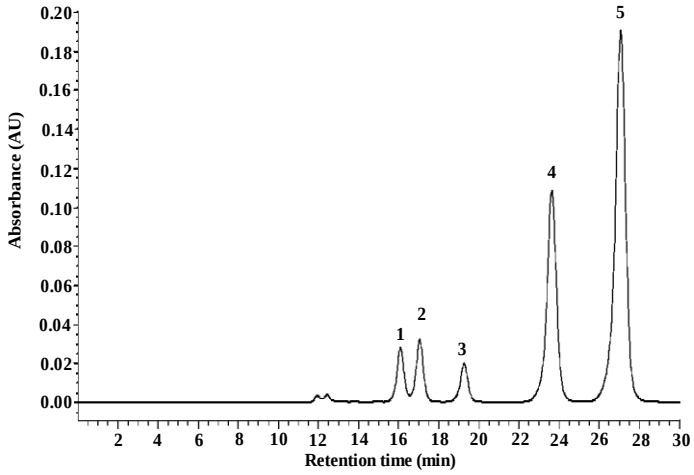

Figure 3.5. HPLC chromatograms of the standards used for identifying individual organic acids in the 'Cripps Pink' apple pulp at 210 nm. Peak 1: Citric acid; peak 2: Tartaric acid; peak 3: Malic acid; peak 4: Succinic; peak 5: Fumaric acid.

3.6. Statistical analysis

The data were subjected to the analysis of variance (ANOVA) using GLM (General Linear Models) procedures with SAS 9.1 software package, SAS Institute Inc, Cary, NC, USA. Treatments means were further separated by LSD for least significance at $P \leq 0.05$ (SAS Institute Inc., 1999). To ensure the validity of analysis, all the assumptions of analysis were checked. The statistical analyses are explained in detail in each chapter.

CHAPTER 4

Effects of Regulated Deficit Irrigation on Water Relations, Fruit Colour Development, Fruit Quality and Postharvest Performance in 'Cripps Pink' Apple

Summary

Poor fruit colour development in this cultivar and limited availability of water are major threats to the sustainable apple production in Australia. Apple trees (13-14 years old) were irrigated with (i) 100%, commercial irrigation (CI) (70 $L \cdot h^{-1}$); (ii) 25% RDI (50 $L \cdot h^{-1}$); (iii) 50% RDI (35 $L \cdot h^{-1}$); and (iv) 75% RDI (20 $L \cdot h^{-1}$) to evaluate the impact on plant water relations, fruit colour development and the accumulation of anthocyanins during two consecutive growing seasons. A decreasing trend of soil volumetric water content at two different soil depths was recorded in 75% RDI treatment during 2006-07. In both seasons, 75% RDI treatment exhibited a significant decrease of stem water potential (Ψ_{stem}) as compared to CI, approximately after 2 to 3 weeks application. Stomatal conductance (g_s) had the similar trends as Ψ_{stem}. Water-deficit fruit in 2006-07 had a higher percentage of visual colour blush, total anthocyanins concentration, lower hue angle and lightness on the shaded sides of apple skin. In addition, 75% RDI treatment during 2006-07 also increased soluble solids concentration (SSC) and fruit firmness; but decreased the fruit diameter marginally which still meet the export criteria (>65 mm). Nine polyphenolic compounds (cyanidin 3-*O*-galactoside, chlorogenic acid, phloridzin, quercetin 3-*O*-rutinoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-xyloside, quercetin 3-*O*-arabinoside and quercetin 3-*O*-rhamnoside) in the fruit skin of this apple cultivar subjected to water-deficit were identified and confirmed using HPLC-ESI-MS. The concentrations of cyanidin 3-*O*-galactoside and quercetin glycosides were higher in the skin of apple fruit with 75% RDI treatment during 2006-07. The RDI fruit (75%) stored for 135 days in cold storage (0 ±0.1°C, 90

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Impact of Regulated Deficit Irrigation on Fruit Quality and Postharvest Storage Performance of 'Cripps Pink' Apple (S2-0.3/PPH/PB 118-OR. pp: 21)

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±2.0%RH) remained firmer and had higher SSC as compared to CI fruit. Similarly, RDI fruit (75%) stored for 155 days in controlled atmosphere (2.7% O_2 + 1.9% CO_2) at 0°C had higher SSC and fruit firmness than in CI. In conclusion, RDI imposed late in the season for 72 days in 2006-07 enhanced red skin colour, total anthocyanins concentration, the levels of polyphenolic compounds and also fruit firmness and SSC of 'Cripps Pink' apple at harvest without adversely affecting postharvest quality in cold and CA storage and also saved the irrigation water.

4.1. Introduction

'Cripps Pink' is a commercially important apple cultivar grown in Australia, Brazil, China, Europe, North America, South Africa, South America and New Zealand. A distinctive pink blush on a pale green background coupled with crisp and juicy taste and high soluble solids concentration (SSC) to acid ratio are special trademark of this cultivar (Mackay et al., 1994). The export market demands at least 40% of fruit surface exhibiting a bright pink-red blush, 7 to 9 $kgcm^{-2}$ of fruit firmness, 13 to ≥15 ^{o}Brix SSC, size ≥65 mm, and 0.7 to 0.9% TA (Cripps et al., 1993; Mackay et al., 1994). However, poor and erratic colour development in 'Cripps Pink' apple depending upon weather conditions causes recurrent serious economic losses to the growers (Whale et al., 2008).

Apple skin colour results from the blend of chlorophyll, carotenoid and anthocyanins pigments (Lancaster, 1992). Most red-skinned apples contain five major groups of polyphenolic compounds such as anthocyanins, flavonols, flavanols, dihydrochalcones and hydroxycinnamic acids (Awad et al., 2000; D'Angelo et al., 2007; Lancaster, 1992). In Cripps Pink' apple, Whale and Singh (2007) identified five major polyhenolic groups include anthocyanins (cyanidin 3-*O*- galactoside), flavanols (catechin and epicatechin), flavonols (quercetin 3-*O*-galactoside, quercetin 3-*O*-xyloside, quercetin 3-*O*-rutinoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-arabinoside and quercetin 3-*O*-rhamnoside), hydroxycinnamic acid (chlorogenic acid) and dihydrochalcone (phloridzin) using HPLC. However, this identification was not further re-conrfimed using high performance liquid chromatography-electrospray impact mass spectrometric (HPLC-ESI-MS). Anthocyanins are the major determinant of apple skin reddening. Cyanidin 3-*O*-galactoside (idaein) is the dominant anthocyanin pigments in apple skin. Anthocyanin biosynthesis begins at

the fruit set stages and culminates at fruit ripening stages (Lancaster, 1992; Saure, 1990). Anthocyanins are very strong antioxidant that acts as anti-oxidative, anti-mutagenic, anti-microbial and anti-carcinogenic (Awad et al., 2000; Formica and Regelson, 1995; Robards and Antolovich, 1997), which inhibits cancer cell proliferation, decreases lipid oxidation and lower cholesterol (Boyer and Liu, 2004).

Many external factors influence the biosynthesis of anthocyanins. These include light irradiation, soil and tree factors, application of chemicals and irrigation. Some of the approaches tested to improve fruit skin colour resulted in successful and limited outcomes such as application of ethephon and seniphos (Gomez-Cardoves et al., 1996), reflective mulches (Layne et al., 2002), bagging (Fan and Mattheis, 1998), cultar (paclobutrazol) and potassium fertilizer (Saure, 1990), overhead sprinkler (Iglesias et al., 2002), combination of aminoethoxyvinylglycine (AVG) and ethephon (Whale et al., 2008) and AVG alone (Phan-Thien et al., 2004; Stover et al., 2003; Whale et al., 2008).

The application of regulated deficit irrigation (RDI) on apple fruit tree has been explored under various climatic conditions with promising outcomes in red skin colour development (Kilili et al., 1996b; Mills et al., 1996a; Mills et al., 1994). It is well documented that RDI reduced leaching of nutrients and biocides into the underground water, decreased vegetative growth, reduced maintenance costs (pruning), enhanced fruit quality and saved irrigation water (Behboudian and Mills, 1997). The consistent increase of polyphenolic concentration especially anthocyanins in grape berries subjected to water-deficit has been reported by Casterllarin et al. (2007a; 2007b). However, no information is available on mechanisms and the effects of water-deficit on the dynamics of the anthocyanins biosynthesis in red-skinned apple.

Increased SSC, fruit firmness (Kilili et al., 1996a) and saved irrigation water (Behboudian et al., 1998; Leib et al., 2006; Mpelasoka et al., 2001a; van Hooijdonk et al., 2004) in apple trees subjected to RDI application has been reported. No reduction in fruit size observed due to water-deficit application late in the season as reported in ‘Braeburn’ apple (Kilili et al., 1996a; Mills et al., 1996b; Mills et al., 1997a). Time of application of RDI is prerequisite to attain acceptable fruit size that

has been set by the industry. Stage II of fruit development had the lowest sensitivity to water-deficit (Mitchell and Chalmers, 1982). Little information is available on the effects of RDI on postharvest storage performance under cold and controlled atmosphere (CA) storage. Water-deficit fruit stored for 12 weeks in cold storage increased fruit firmness (Kilili et al., 1996b) and stored for 12 weeks following 7 days shelf life had higher firmness and SSC (Mpelasoka et al., 2000a). Water-deficit application has been explored in various apple cultivars in various climatic conditions i.e. 'Braeburn' and 'Delicious' in humid region (Ebel et al., 1995; Mills et al., 1996b) and 'Cripps Pink' apple in temperate (O'Connell and Goodwin, 2007) and in Mediterranean region (Talluto et al., 2008). However, the information on the effects of RDI on 'Cripps Pink' apple under Western Australian conditions in the Mediterranean climate is limited. These observations prompted to investigate the effects of RDI application during stage II of fruit development of 'Cripps Pink' apple on plant water relations, development of red skin colour and polyphenolic profiles, fruit size and other major fruit quality attributes at harvest, after cold and controlled atmosphere storage.

4.2. Materials and Methods

4.2.1. Location and climatic conditions

Two experiments were conducted over two consecutive years (2005-06 and 2006-07) at the commercial orchard in Karragullen (latitude 32°5'28"S; longitude 116°7'19"E), Perth Hills, Western Australia. The location was in a Mediterranean climate characterised by hot, dry summers and mild, wet winters.

4.2.2. Plant materials

'Cripps Pink' apple trees grafted on MM.109 rootstock were used in both experiments. Experiments 1 and 2 were performed on 13 and 14-year-old trees, respectively. The experimental trees were planted in the east-west direction, maintaining row distances of 4.5 m and plant distances of 2.4 m. The orchard soil is gravel in a sandy or loamy matrix. Full bloom (>80% of the buds are open) for 2005-06 and 2006-07 occurred on 20th October 2005 and 9th October 2006, correspondingly. All experimental trees were trained in central leader system and received similar cultural practices including fertilization, thinning, irrigation,

pesticides and fungicides sprays except experimental trees during the period of investigations.

4.2.3. Experiment 1: 2005-06

Various treatments comprising of four different regimes of irrigation: (i) 100%, (70 $L \cdot h^{-1}$, commercial irrigation (CI)), (ii) 25% RDI (50 $L \cdot h^{-1}$), (iii) 50% RDI (35 $L \cdot h^{-1}$), and (iv) 75% RDI (20 $L \cdot h^{-1}$) were laid out by following a randomized complete block design with four replicates. Two trees were treated as an experimental unit. Adjacent plots were separated by guard trees. Micro-sprinklers (Regulated Micro Sprinkler, Netafim Supernet, Tel Aviv, Israel) with a flow rate of 20 to 90 $L \cdot h^{-1}$ were used for irrigation and wetted diameter range from 3.0 to 6.0 m. Irrigation was applied for two hours daily at 14:30 to 15:30 hours. The duration of the irrigation treatments was 40 days commencing from 161 DAFB anticipated commercial harvest 201 DAFB. All the experimental trees were irrigated the same as CI treatment, outside the deficit periods. Stem water potential (Ψ_{stem}), volumetric soil water content (θ) and stomatal conductance (g_s) were recorded on 167, 174, 180 and 188 DAFB. Leaf water potential (Ψ_{leaf}) was measured on 173, 179 and 187 DAFB. Fruit drop and fruit diameter were recorded on 166, 173, 179 and 187 DAFB.

4.2.4. Experiment 2: 2006-07

Similar experimental design and irrigation treatments as explained in experiment 1 were followed in this experiment. However, the experiment duration was longer (72 days) compared to 2005-06. Irrigation was applied for two hours daily at 17:00 to 19:00 hours. Irrigation treatments commenced on 135 DAFB prior to commercial harvest. Stem water potential (Ψ_{stem}) and Ψ_{leaf} were recorded on 146, 156, 162, 169, 177, 183, 191, 201 DAFB as explained in experiment 1. Volumetric soil water content (θ), g_s, fruit drop and fruit diameter were recorded on 143, 149, 157, 163, 170, 178, 184, 192 and 195 DAFB. Outside the deficit periods, all the trees were irrigated the same as CI.

4.2.5. Temperature monitoring at experimental site

In both the growing seasons, all experimental trees were free from pests and diseases as detailed in Chapter 3, Section 3.1. Data logger (Tinytag*Plus* Gemini Data Logger, UK) was used for recording daily temperatures in the orchard. Daily temperatures

data were obtained using Gemini Logger Manager Software (Version 2.8). Rainfall and evapotranspiration (ET) data were obtained from Bureau of Meteorology, Perth, Western Australia. Summary of the monthly climates data for two years growing seasons presented in Figure 4.1A and 4.1B. Daily average day temperature was calculated between sunrise and sunset, while daily average night temperature was calculated between sunset and sunrise. Sunset and sunrise times during the experimental period were obtained from Geoscience Australia (2008).

4.2.6. Fruit sampling and storage conditions

4.2.6.1. Fruit sampling and parameter measurements

Fruit were randomly harvested from all parts of the tree canopy up to height of 2 m from the ground. In 2005-06 growing season, fruit were harvested at commercial fruit maturity (starch index 3 to 3.5) on 201 DAFB (8th May 2006), whilst 2006-07 growing season on 207 DAFB (3rd May 2007). Twenty-five fruit per replicate were used for fruit quality assessment following harvest such as percentage red blush >40 of fruit surface, fruit colour, total anthocyanins concentration, flavonoids and other phenolic compounds, fruit firmness, titratable acidity (TA), soluble solids concentration (SSC), ascorbic acid concentration, total antioxidants, sugars and organic acids concentration.

4.2.6.2. Cold storage

In 2006-07, fruit were harvested from various experimental trees and dipped in commercial diphenylamine (DPA) to prevent superficial scald. The DPA-dipped fruit for cold storage were packed in cardboard box with trays for individual apple. Fruit quality was assessed following 45, 70 and 135 days cold storage (0 ±0.1°C, 90 ±2.0%RH). Twenty fruit were included in each replication and replicated four times. Various fruit quality parameters including fruit firmness, TA, SSC, SSC/TA ratio, ascorbic acid concentration and total antioxidants were assessed following various cold storage periods.

4.2.6.3. Controlled atmosphere storage

In 2006-07, fruit harvested were further stored in controlled atmosphere (CA) storage for 155 days (approximately 5 months). DPA-dipped fruit from each treatment were packed in plastic container and stored in CA containing 2.7% O_2 + 1.9% CO_2 at 0°C

for 155 days and 155 days following 14 days at ambient (20 ±2.0 °C). Each replicate contains twenty fruit and replicated for four times. Fruit quality parameters assessed were fruit firmness, TA, SSC, ascorbic acid concentration and total antioxidants.

4.2.7. Preharvest parameters: soil-plant water relations

4.2.7.1. Volumetric soil water content

Volumetric soil water content (θ) was monitored using a Moisture Probe Meter (MPM 160, ICT International Pty. Ltd., Armidale, New South Wales, Australia) between 10:00 and 11:00 solar time at approximately seven-day intervals as detailed in Chapter 3, Section 3.2.1.

4.2.7.2. Leaf water potential and stem water potential

Leaf water potential (Ψ_{leaf}) and stem water potential (Ψ_{stem}) was recorded using a pressure chamber (Model 3000, Soil Moisture Equipment Corp., Santa Barbara CA, USA) between 11:00 and 14:00 solar time at approximately seven-day intervals as explained in Chapter 3, Section 3.2.2 and 3.2.3.

4.2.7.3. Stomatal conductance

Stomatal conductance (g_s) was recorded using a Steady State Diffusion Porometer (Model SC-1, Decagon Devices Inc., Pullman, WA, USA) as detailed in Chapter 3, Section 3.2.4.

4.2.7.4. Fruit drop

Total numbers of fruit on randomly selected branches were counted at the commencement of treatments until harvest at approximately seven-day intervals as outlined in Chapter 3, Section 3.2.5.

4.2.8. Fruit quality: fruit colour

4.2.8.1. Surface skin colour

Percentage red blush of individual apples were visually assessed as described in Chapter 3, Section 3.3.1. The apple fruit colour was also measured by a Hunter Lab ColorFlex $45^o/0^o$ Spectrophotometer including chromaticity value a*, b*, lightness (L*), chroma (C*) and hue angle (h°) as outlined in Chapter 3, section 3.3.2.

4.2.8.2. Analysis of skin pigment

4.2.8.2.1 Total anthocyanins

Anthocyanins were extracted and quantified according to the method outlined by Whale and Singh (2007) as described in Chapter 3, Section 3.3.3.1.

4.2.8.2.2 Flavonoids and other phenolic compounds

Chemicals used for flavonoids and other phenolic compounds determination were as mentioned in Chapter 3, Section 3.3.3.2.1. Flavonoids and phenolic compounds of apple skin were extracted according to the procedure outlined by Whale and Singh (2007) with some modifications as described in Chapter 3, Section 3.3.3.2.2. The flavonoids and other phenolics were identified by comparing their retention times with authentic standards and quantified using high performance liquid chromatography (HPLC) system (Waters 1525 Binary HPLC Pump fixed to Waters 2487 Dual Wavelength Absorbance Detector and Waters 717 plus Autosampler; Waters Corp., Milford, Mass., USA) as described in Chapter 3, Section 3.3.3.2.3 and 3.3.3.2.4. Flavonoids and other phenolic compounds were re-confirmed using HPLC-electrospray ionization mass spectrometry (HPLC-ESI-MS) as mentioned in Chapter 3, Section 3.3.3.2.3.

4.2.9. Other fruit quality parameters

4.2.9.1. Fruit diameter

Fruit diameter was recorded using a digital vernier calliper as described in Chapter 3, Section 3.4.1.

4.2.9.2. Fruit firmness

Fruit firmness was determined using an electronic pressure tester (Model EPT-1 pressure tester, Lake City Technical products Inc., Kelowna, BC, Canada) as described in Chapter 3, Section 3.4.2.

4.2.9.3. Titratable acidity, soluble solids concentration and SSC/TA ratio

Titratable acidity (TA) was determined using method outlined in Chapter 3, Section 3.4.3. A soluble solids concentration (SSC) was recorded using infrared digital refractometer as described in Chapter 3, Section 3.4.4. SSC/TA ratio was calculated by dividing SSC with the TA as described in Chapter 3, Section 3.4.5.

4.2.10. Determination of ascorbic acid, total antioxidants, individual sugars and organic acids

4.2.10.1. Ascorbic acid

Ascorbic acid was determined according to the outlined method by Jagota and Dani (1982) and Malik and Singh (2005) as described in Chapter 3, Section 3.5.1.

4.2.10.2. Total antioxidants

Total antioxidants of apple skin and pulp were determined according to the method outlined by Brand-Williams et al. (1995) and Khan et al. (2008) as described in Chapter 3, Section 3.5.2.

4.2.10.3. Individual sugars

Chemicals used for individual sugars determination as detailed in Chapter 3, Section 3.5.3.1. The homogenate apple pulp using a hand blender (Model HB95, Breville Pty. Ltd., New South Wales, Australia) were then centrifuged and filtered as described in Chapter 3, Section 3.5.3.2. Glucose, fructose, sucrose and sorbitol were separated, identified and quantified using the HPLC system (Waters Corp., Milford, Mass., USA) fixed with the Fast Carbohydrate Analysis column (Aminex-HPX 87C, 100 x 7.8 mm) (Bio-Rad Laboratories, Hercules, CA, USA) as outlined in Chapter 3, Section 3.5.3.3.

4.2.10.4. Individual organic acids

Chemicals used for individual organic acids determination as described in Chapter 3, Section 3.5.3.1. Apple pulp were homogenized using a hand blender (Model HB95, Breville Pty. Ltd., New South Wales, Australia) as described in Chapter 3, Section 3.5.3.2. Individual organic acids such as malic, citric, fumaric, shikimic, succinic and tartaric acid in apple juice were separated, identified and quantified using the HPLC system (Waters Corp., Milford, Mass., USA) fixed with a Bio Rad Aminex-HPX 87H (300 x 7.8 mm; particle size 9 µm) (Bio-Rad Laboratories, Inc., Hercules, USA) column as outlined in Chapter 3, Section 3.5.3.3.

4.2.11. Statistical analysis

Data were subjected to analysis of variance (ANOVA) using GLM (General Linear Models) procedures with SAS 9.1 software package, SAS Institute Inc, Cary, NC,

USA. The treatment effects on various parameters including the effects of treatment, storage period and their interaction were assessed within ANOVA. Least significance differences (LSD) were calculated at level $P \leq 0.05$ following significance F test (SAS Institute Inc., 1999). All the assumptions of analysis were checked to ensure validity of statistical analysis. The data of two years were not pooled because error means squares over years were heterogenous.

4.3. Results

4.3.1. Weather conditions

Rainfall in both years was higher during the two weeks period of anticipated commercial harvest. However, rainfall for 2005-06 was lower compared to 2006-07, while mean air temperature and evapotranspiration (ET) were greater compared to previous years. In 2006-07, mean air temperature and ET shows decreasing trends towards harvesting date, 207 DAFB (Figure 4.1).

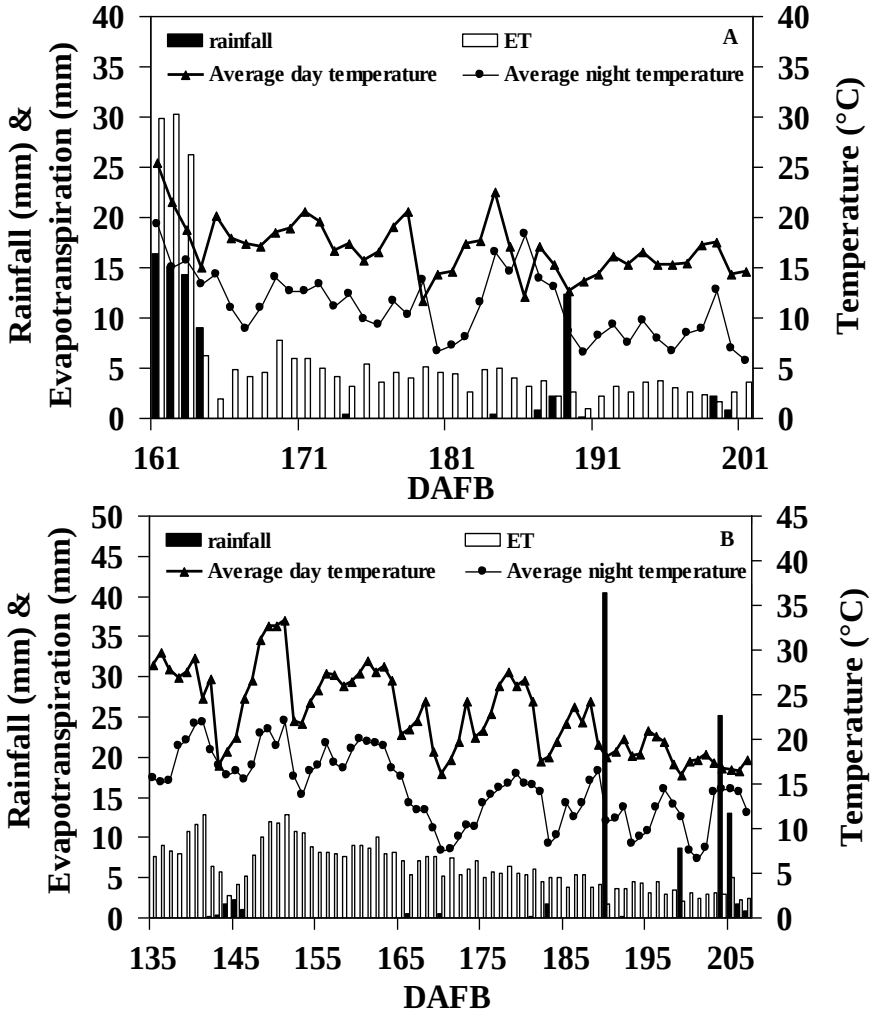

Figure 4.1. Daily average day and night temperatures, rainfall and evapotranspiration

(ET) during (A) 2005-06 and (B) 2006-07 growing season at commercial apple orchard, Karragullen, the Perth Hills, Western Australia.

4.3.2. Plant water relations

4.3.2.1. Volumetric soil water content

During 2005-06, all the RDI treatments applied on 161 DAFB resulted in lower θ at both soil depths than those in control starting from 167 DAFB until 180 DAFB (Figure 4.2). After 180 DAFB, θ at both soil depths gradually increased due to the rainfall two weeks prior to anticipated commercial harvest. Water-deficit treatment (75% RDI) applied on 161 DAFB resulted in significantly ($P \leq 0.05$) lowest θ at 200-300 mm (4.23%) and 300-400 mm depth (8.86%) on 167 and 174 DAFB, respectively.

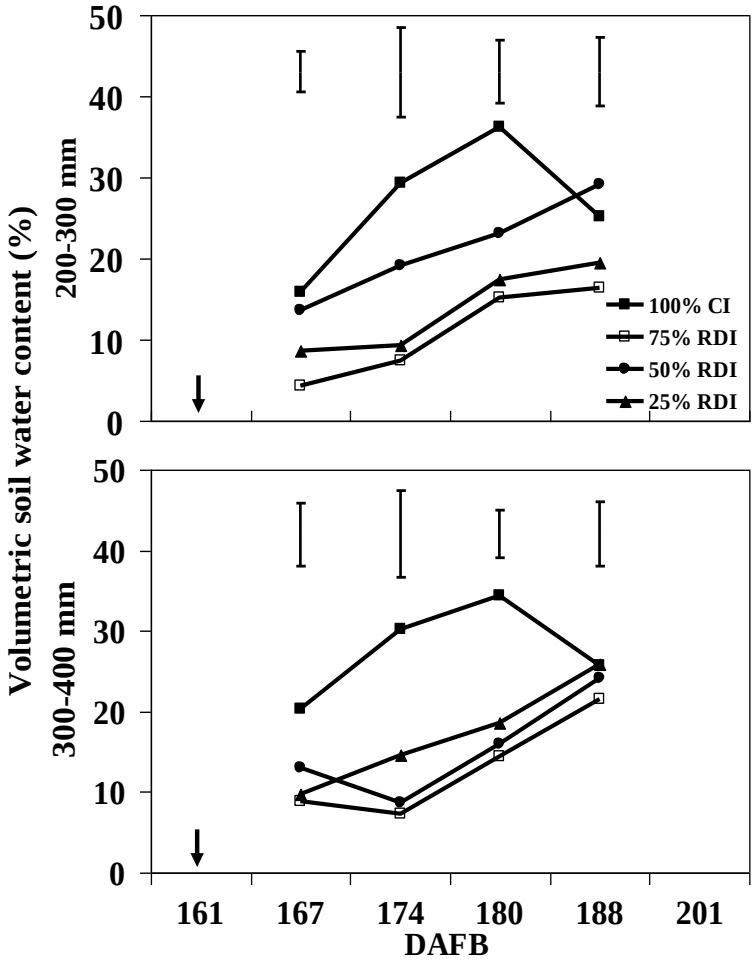

Figure 4.2. Changes in volumetric soil water content affected by different irrigation treatments during fruit development and maturation in 2005-06. Irrigation treatments were control (100% CI), 25% RDI, 50% RDI and 75% RDI. Vertical bars represent LSD ($P \leq 0.05$). Arrow indicates the commencement of RDI treatments on 161 days after full bloom (DAFB) in 2005-06. CI = commercial irrigation and RDI = regulated deficit irrigation.

In general, during 2006-07, θ at 200-300 mm and 400-500 mm depths were reduced up to 178 DAFB with water-deficit treatments (Figure 4.3). All RDI treatments resulted in decreased θ at both soil depths after two weeks of their application and rapid increased in the two weeks prior to anticipated commercial harvest. θ in CI trees were maintained at 21.4% and 22.0% in 200-300 mm and 400-500 mm depth, respectively.

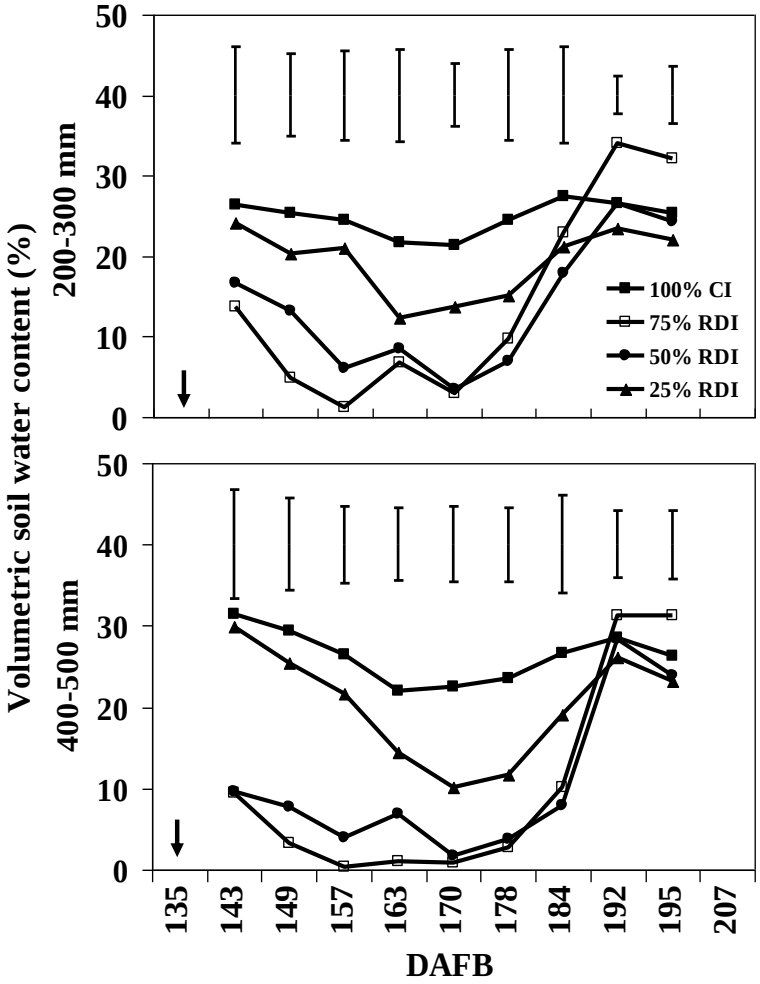

Figure 4.3. Changes in volumetric soil water content affected by different irrigation treatments during fruit development and maturation in 2006-07. Irrigation treatments were control (100% CI), 25% RDI, 50% RDI and 75% RDI. Vertical bars represent LSD ($P \leq 0.05$). Arrow indicates the commencement of RDI treatments on 135 days after full bloom (DAFB) in 2006-07. CI = commercial irrigation and RDI = regulated deficit irrigation.

4.3.2.2. Stem water potential and leaf water potential

During 2005-06, 75% RDI resulted in significantly ($P \leq 0.05$) lower Ψ_{stem} (below -0.71 MPa) throughout the season (Figure 4.4A). In 2006-07, Ψ_{stem} with 75% RDI began to decline from the initial values -1.65 MPa on 146 DAFB to -2.37 MPa on 162 DAFB, and then increased substantially to -2.14 on 177 DAFB, and then remain similar to CI until 201 DAFB (Figure 4.4B). Ψ_{stem} with 75% RDI was significantly ($P \leq 0.05$) reduced as compared to all other RDI treatments on 146 DAFB until 183 DAFB, however, no differences were recorded on 201 DAFB. During 2005-06, Ψ_{leaf} was significantly ($P \leq 0.05$) lowered (-1.94 MPa) with 75% RDI on 179 DAFB as compared to CI (Figure 4.5A). In 2006-07, Ψ_{leaf} with 75% RDI significantly ($P \leq 0.05$) reduced (-2.45 MPa on 156 DAFB) after three weeks application and remained lower (-3.03 MPa on 162 MPa) until reached normal baseline for water potential values in apple trees (-1.15 MPa) on 191 DAFB (Figure 4.5B).

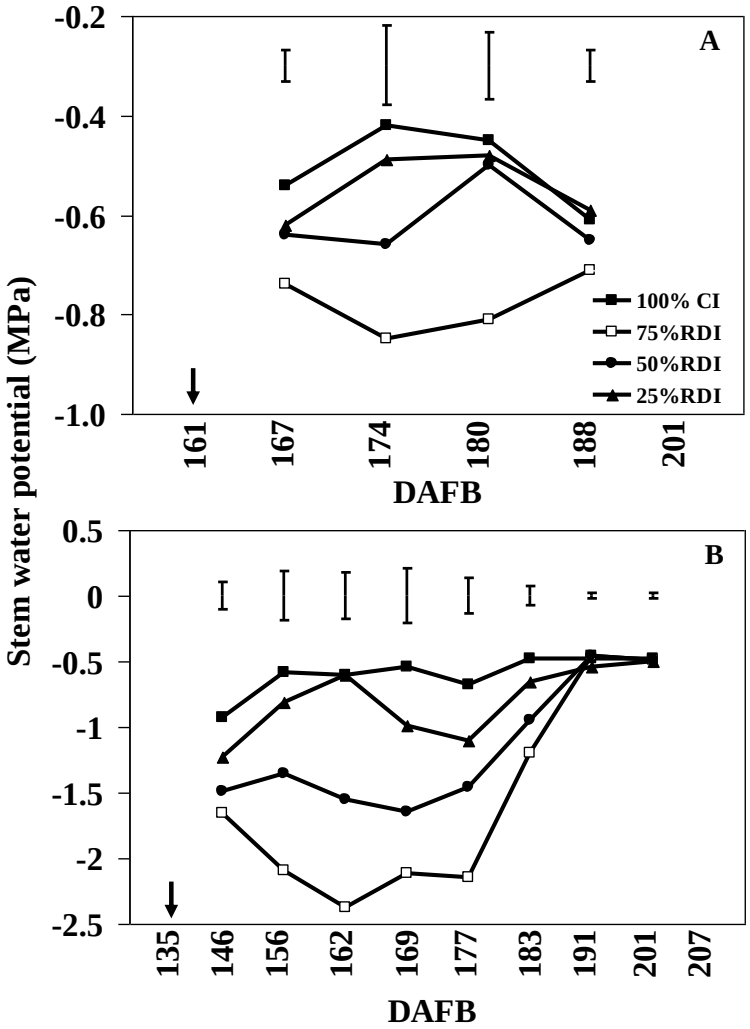

Figure 4.4. Changes in stem water potential in 'Cripps Pink' apple trees affected by different irrigation treatments during fruit development and maturation in 2005-06

(A) and 2006-07 (B). Irrigation treatments were control (100% CI), 25% RDI, 50% RDI and 75% RDI. Vertical bars represent LSD ($P \leq 0.05$). Arrow indicates the commencement of RDI treatments on 161 days after full bloom (DAFB) in 2005-06 and 135 DAFB in 2006-07. CI = commercial irrigation and RDI = regulated deficit irrigation.

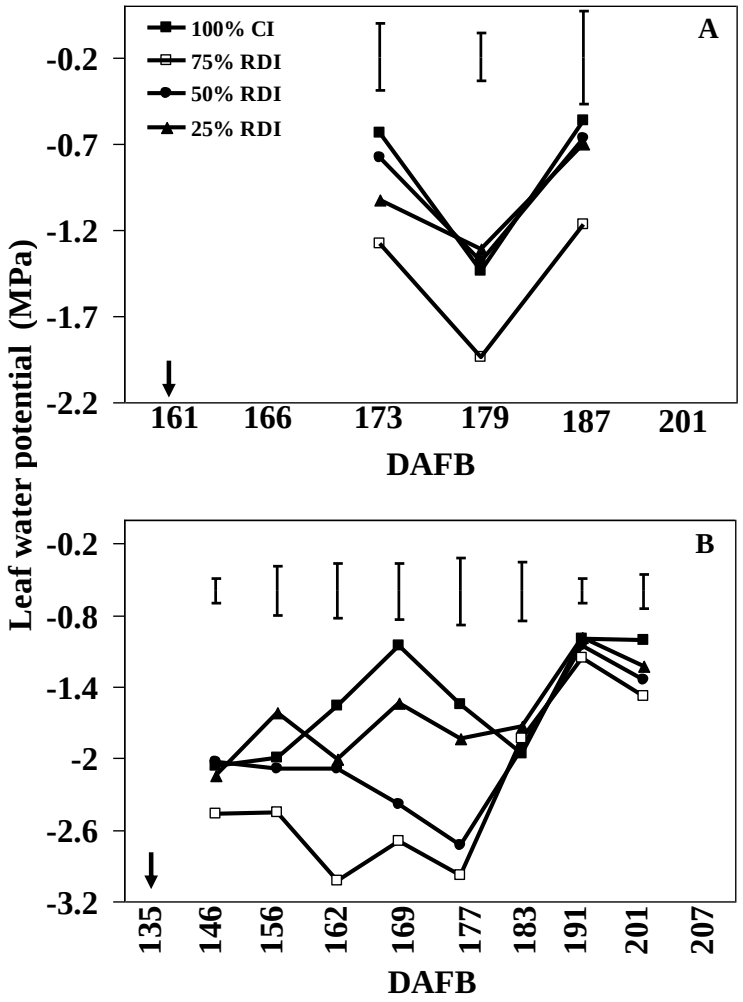

Figure 4.5. Changes in leaf water potential in 'Cripps Pink' apple trees affected by different irrigation treatments during fruit development and maturation in 2005-06 (A) and 2006-07 (B). Irrigation treatments were control (100% CI), 25% RDI, 50% RDI and 75% RDI. Vertical bars represent LSD ($P \leq 0.05$). Arrow indicates the commencement of RDI treatments on 161 days after full bloom (DAFB) in 2005-06 and 135 DAFB in 2006-07. CI = commercial irrigation and RDI = regulated deficit irrigation.

4.3.2.3. Stomatal conductance

In 2005-06, leaf stomatal conductance (g_s) reduced significantly ($P \leq 0.05$) with 75% and 50% RDI treatment (75.4 $mmol \cdot m^{-2}s^{-1}$ and 91.5 $mmol \cdot m^{-2}s^{-1}$, respectively) on 174 DAFB after two weeks application of water-deficit (Figure 4.6A). The g_s with control irrigated-trees remain stable, above 125.8 $mmol \cdot m^{-2}s^{-1}$. During 2006-07, 75% RDI treatment resulted in significantly ($P \leq 0.05$) lower g_s compared to control

irrigation except on 195 DAFB. In the descending order, 75% RDI had the lowest g_s, followed by 50% RDI, 25% RDI and 100 % irrigation (Figure 4.6B). Leaves of RDI trees exhibited lower g_s ranged between 38.4 and 178.7 $mmol \cdot m^{-2}s^{-1}$. The reduction of Ψ_{stem} with 75% RDI corresponds well with the decreased θ (Figure 4.3) and g_s (Figure 4.6). Reduced Ψ_{stem} and g_s indicates that RDI trees had experienced leaf water-deficit.

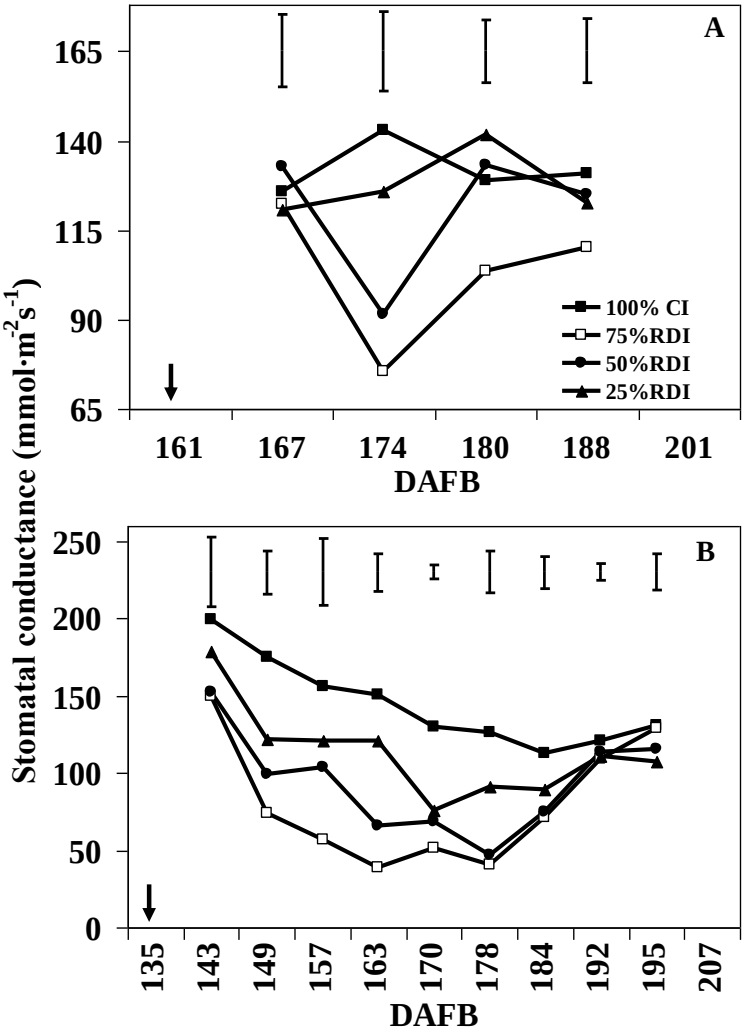

Figure 4.6. Changes in leaf stomatal conductance in 'Cripps Pink' apple trees affected by different irrigation treatments during fruit development and maturation in 2005-06 (A) and 2006-07 (B). Irrigation treatments were control (100% CI), 25% RDI, 50% RDI and 75% RDI. Vertical bars represent LSD ($P \leq 0.05$). Arrow indicates the commencement of RDI treatments on 161 days after full bloom (DAFB) in 2005-06 and 135 DAFB in 2006-07. CI = commercial irrigation and RDI = regulated deficit irrigation.

4.3.2.4. Fruit drop

In both seasons, fruit drop was not significantly affected with the application of irrigation treatments (Figure 4.7).

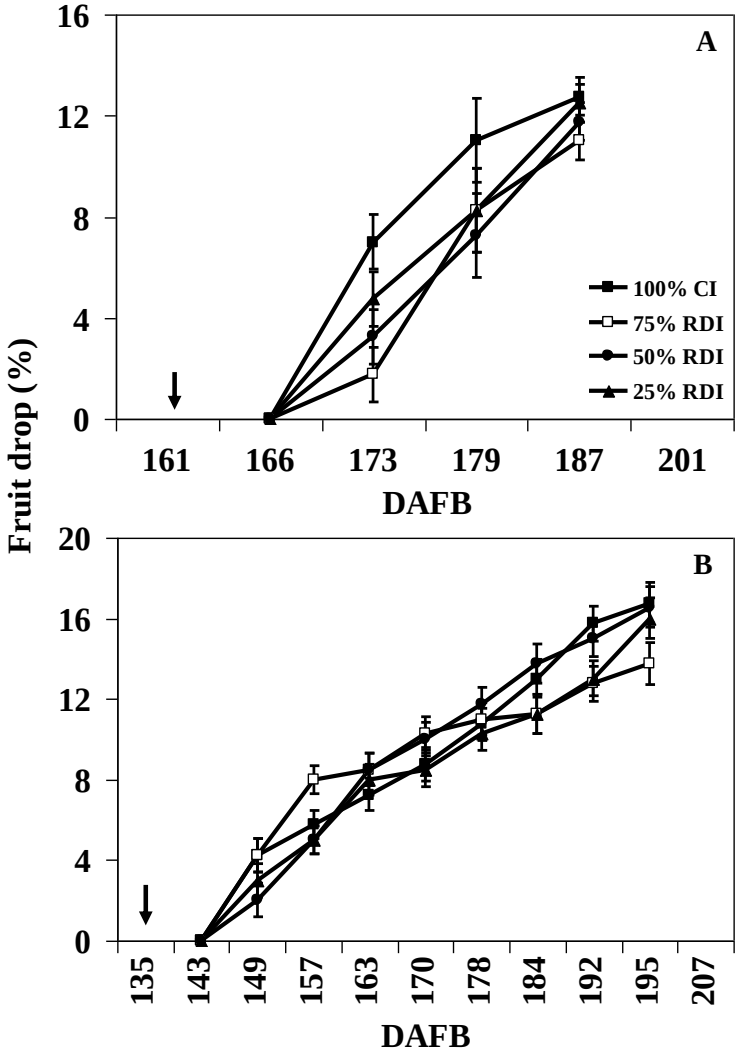

Figure 4.7. Changes in fruit drop in 'Cripps Pink' apple affected by different irrigation treatments during fruit development and maturation in 2005-06 (A) and 2006-07 (B). Irrigation treatments were control (100% CI), 25% RDI, 50% RDI and 75% RDI. In 2005-06, LSD ($P \leq 0.05$): 173 = NS (2.14), 179 = NS (3.32), 187 = NS (1.48). In 2006-07, LSD ($P \leq 0.05$): 149 = NS (1.63), 157 = NS (1.40), 163 = NS (1.56), 170 = NS (1.67), 178 = NS (1.61), 184 = NS (1.89), 192 = NS (1.74), 195 = NS (2.03). Vertical bars and values within brackets represent standard errors of means. Arrow indicates the commencement of RDI treatments on 161 days after full bloom (DAFB) in 2005-06 and 135 DAFB in 2006-07. CI = commercial irrigation and RDI = regulated deficit irrigation. NS = not significantly different at $P \leq 0.05$.

4.3.3. Fruit quality assessment at harvest

4.3.3.1. Fruit colour and total anthocyanins concentration

The irrigation treatments imposed did not significantly affect fruit colour development in 'Cripps Pink' apple during 2005-06. However, in 2006-07, fruit colour was significantly ($P \leq 0.05$) increased with the application of RDI treatments (Table 4.1). Fruit from 75% RDI treatment exhibited the higher fruit skin colour (80.0%) as compared to CI. During both seasons, total anthocyanins concentration on the exposed (ES) and shaded (SS) sides of the apple skin was significantly ($P \leq 0.05$) affected with the irrigation treatments (Table 4.1). In general, the higher total anthocyanins concentration on both sides of apple skin was recorded in 50% RDI and 75% RDI during 2005-06. While, in 2006-07, higher concentration of total anthocyanins on both sides of apple skin was in 75% RDI fruit as compared to CI. The concentration of anthocyanins in 50% RDI was comparable to 75% RDI.

Table 4.1. Effects of different irrigation treatments on visual fruit colour and total anthocyanins in the exposed (ES) and shaded (SS) sides in 'Cripps Pink' apple during 2005-06 and 2006-07.

Treatment	Visual colour (% red blush)		Total anthocyanins ($\mu g \cdot g^{-1}$ FW)			
			ES		SS	
	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07
100% CI	40.0	57.0 c	60.2 b	142.3 b	14.7 c	28.5 c
25% RDI	45.0	69.0 b	60.5 b	180.6 ab	17.4 bc	54.9 b
50% RDI	40.0	72.0 ab	80.0 ab	212.9 a	21.4 a	60.7 b
75% RDI	43.0	80.0 a	116.6 a	212.1 a	19.5 ab	154.0 a
LSD ($P \leq 0.05$)	NS (4.27)	10.1	42.4	52.4	3.69	23.9

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. Values within brackets represent standard error of means. NS indicates not significantly different at $P \leq 0.05$. CI denotes to commercial irrigation and RDI indicates regulated deficit irrigation.

4.3.3.2. Chromaticity value a*, b*, lightness, hue angle and chroma

A reduction in hue angle (h°), lightness (L*), chromaticity value b* and higher chromaticity value a* indicates redder fruit skin colour. As shown on Table 4.2, the chromaticity value a* and b* on the ES of apple skin was significantly ($P \leq 0.05$) affected with the irrigation treatments only in 2006-07 and 2005-06, respectively. On

the ES of apple skin, the highest chromaticity value a* (26.4 a*) was recorded in 75% RDI treatment as compared to CI. The chromaticity value a* on the SS of apple skin was significantly ($P \leq 0.05$) affected with the application of RDI treatments in both years. However, the chromaticity value b* was only affected with RDI treatments in 2006-07. During 2005-06, the highest chromaticity value a* (2.02 a*) on the SS of apple skin was recorded in 50% RDI treatment as compared to CI. However, in 2006-07, all RDI treatments resulted in the comparable chromaticity value a* on the SS of apple skin as compared to CI. The lowest chromaticity value b* (23.7 b*) on the SS of apple skin was observed in 75% RDI treatment as compared to CI. The RDI treatments did not significantly affect hue angle and lightness on the ES of apple skin in both consecutive years excluding chroma (Table 4.3). Even hue angle and lightness on the ES of apple skin during 2006-07 were non-significant, 75% RDI fruit skin tends to have the lower h° and L* as compared to CI. Chroma on the ES of apple skin was highest in 25% RDI (25.4 C*) during 2005-06 and in 75% RDI (30.8 C*) during 2006-07 as compared to CI. Hue angle and lightness on the SS of apple skin was significantly ($P \leq 0.05$) affected by irrigation treatments during both years except chroma in 2005-06. The RDI treatments (75% RDI) resulted in the lowest hue angle on the SS of apple skin during both years as compared to CI. Meanwhile, the lowest lightness on the SS of apple skin was found in 50% RDI (40.2 L*) during 2005-06 and in 75% RDI (34.2 L*) during 2006-07 seasons. Hue angle and lightness in 2006-07 was lowered as compared to in 2005-06. The highest chroma on the SS of apple skin was found in 75% RDI as compared to CI.

Table 4.2. Effects of different irrigation treatments on chromaticity value a* and b* on the exposed (ES) and shaded (SS) sides of 'Cripps Pink' apple during 2005-06 and 2006-07.

Treatment	Exposed side (ES)				Shaded side (SS)			
	a*		b*		a*		b*	
	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07
100% CI	16.6	23.4 b	16.4 b	16.8	-0.87 b	6.99 b	27.2	26.7 a
25% RDI	16.9	25.6 a	17.1 ab	15.6	0.59 ab	11.4 a	26.9	24.1 bc
50% RDI	13.4	25.3 ab	19.6 a	16.2	2.02 a	10.7 a	26.9	25.4 ab
75% RDI	15.0	26.4 a	18.4 ab	15.6	1.99 a	11.4 a	27.2	23.7 c
LSD ($P \leq 0.05$)	NS (1.46)	2.13	2.56	NS (0.55)	2.33	3.14	NS (0.44)	1.65

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. Values within brackets represent standard error of means. NS = not significantly different at $P \leq 0.05$. CI = commercial irrigation and RDI = regulated deficit irrigation.

Table 4.3. Effects of different irrigation treatments on hue angle and lightness on the exposed (ES) and shaded (SS) sides of 'Cripps Pink' apple during 2005-06 and 2006-07.

Treatment	Exposed side (ES)						Shaded side (SS)					
	Hue angle (^{o}h)		Lightness (L*)		Chroma (*C)		Hue angle (^{o}h)		Lightness (L*)		Chroma (*C)	
	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07
100% CI	45.5	36.1	29.8	25.4	24.2 b	29.2 b	91.4 a	74.8 a	41.8 a	39.1 a	27.4	28.3 a
25% RDI	46.2	31.6	30.1	24.6	25.4 a	30.1 a	87.8 ab	64.3 bc	41.7 a	36.5 b	27.5	27.4 b
50% RDI	56.2	32.8	32.1	24.5	24.7 ab	30.3 a	86.4 ab	67.1 b	40.2 b	37.5 ab	27.4	28.2 a
75% RDI	50.6	30.8	31.4	23.4	25.0 ab	30.8 a	85.3 b	59.9 c	40.3 b	34.2 c	27.4	28.2 a
LSD ($P \leq 0.05$)	NS (3.65)	NS (1.68)	NS (1.04)	NS (0.74)	0.98	0.73	6.02	7.02	0.71	2.17	NS (0.31)	0.71

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. Values within brackets represent standard error of means. NS = not significantly different at $P \leq 0.05$. CI = commercial irrigation and RDI = regulated deficit irrigation.

4.3.3.3. Flavonoids and other phenolic compounds

Nine compounds of flavonoids and other phenolic compounds in the apple skin of 'Cripps Pink' apple were identified and re-confirmed using HPLC-ESI-MS including cyanidin-3-*O*-galactoside, chlorogenic acid, phloridzin, quercetin 3-*O*-rutinoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-xyloside, quercetin 3-*O*-arabinoside and quercetin 3-*O*-rhamnoside (Table 4.4, Figure 4.8 and 4.9).

Table 4.4. HPLC-ESI-MS anthocyanins, hydroxycinnamic acid, dihydrochalcones and flavonol profile in the skin of 'Cripps Pink' apple.

HPLC peak	Compound	Retention time	$[M - H]^-$ (m/z)
1	Chlorogenic acid	16.69	353
2	Cyanidin 3-*O*-galactoside	19.44	447
3	Quercetin 3-*O*-rutinoside	26.02	609
4	Quercetin 3-*O*-galactoside	26.46	463
5	Quercetin 3-*O*-glucoside	26.86	463
6	Quercetin 3-*O*-xyloside	27.95	433
7	Quercetin 3-*O*-arabinoside	28.83	433
8	Quercetin 3-*O*-rhamnoside	29.53	447
9	Phloridzin	29.98	435

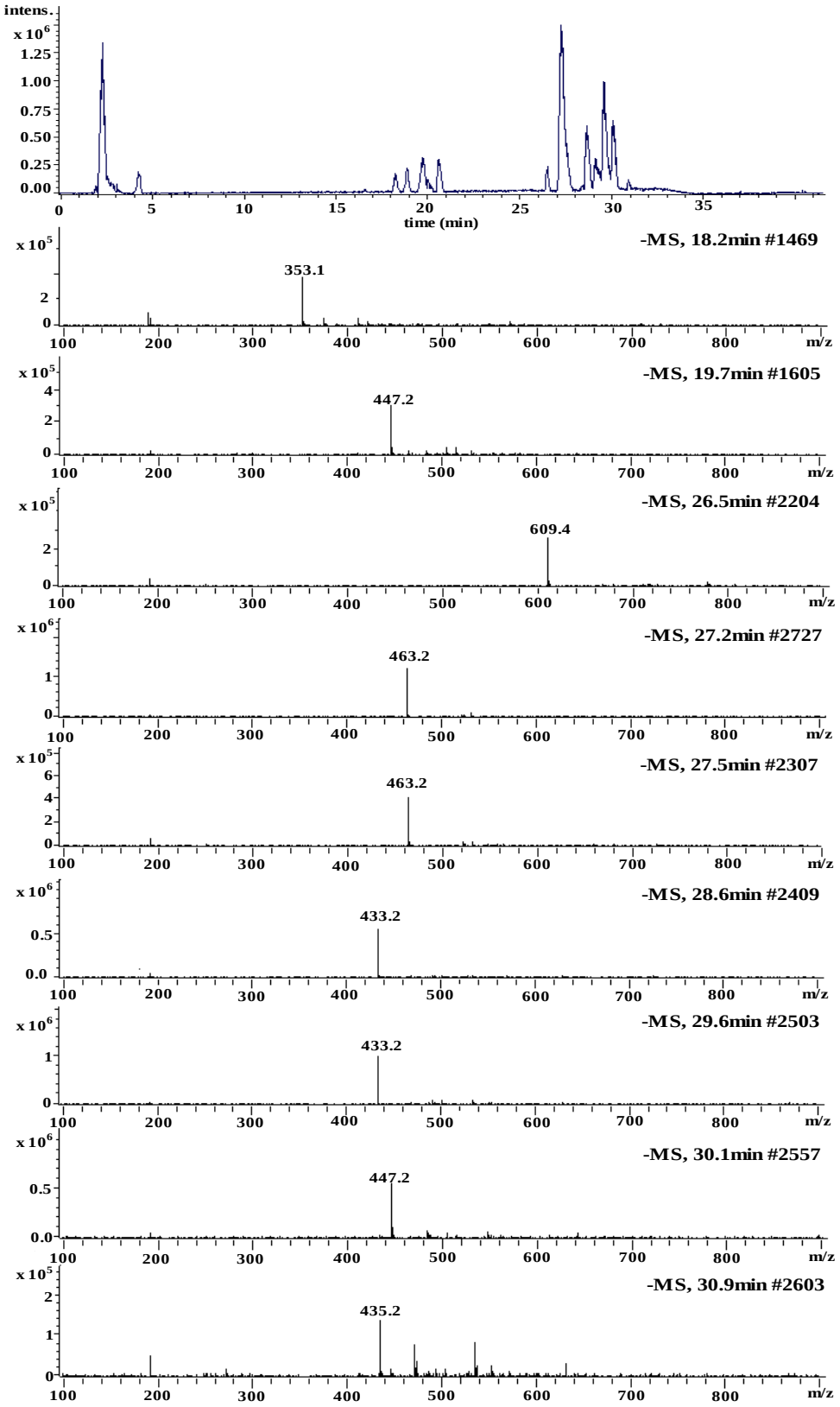

Figure 4.8. Negative mode ESI-MS spectra of chlorogenic acid; cyanidin 3-*O*-galactoside; quercetin 3-*O*-rutinoside; quercetin 3-*O*-galactoside; quercetin 3-*O*-glucoside; quercetin 3-*O*-xyloside; quercetin 3-*O*-arabinoside; quercetin 3-*O*-rhamnoside; phloridzin.

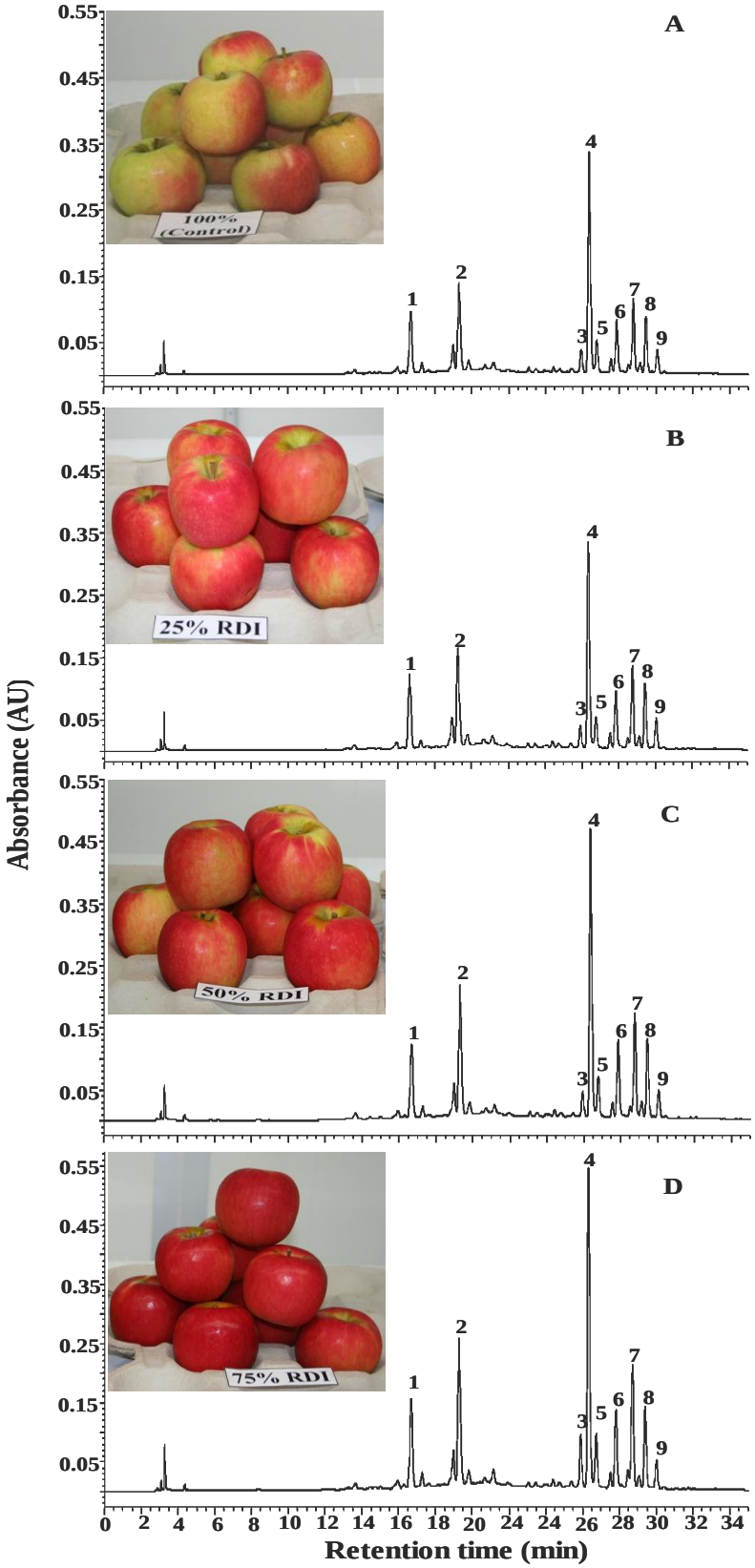

Figure 4.9. Typical HPLC chromatograms at 280 nm showing changes in flavonoid and other phenolic compounds in the skin of 'Cripps Pink' apple in CI (A), 25% RDI (B), 50% RDI (C) and 75% RDI (D). Panel from top to bottom shows HPLC chromatograms at 280 nm showing changes in flavonoid and phenolic compounds in the skin of 'Cripps Pink' apple in CI (A), 25% RDI (B), 50% RDI (C) and 75% RDI

(D) and also few fruit harvested from in CI (A), 25% RDI (B), 50% RDI (C) and 75% RDI (D). Peak 1: chlorogenic acid; peak 2: cyanidin 3-*O*-galactoside; peak 3: quercetin 3-*O*-rutinoside; peak 4: quercetin 3-*O*-galactoside; peak 5: quercetin 3-*O*-glucoside; peak 6: quercetin 3-*O*-xyloside; peak 7: quercetin 3-*O*-arabinoside; peak 8: quercetin 3-*O*-rhamnoside; peak 9: phloridzin.

The concentration of cyanidin 3-*O*-galactoside, chlorogenic acid and quercetin glycocides in fruit skin was not significantly affected with irrigation treatments during 2005-06 (Table 4.5). However, the RDI treatments significantly ($P \leq 0.05$) affected the concentration of cyanidin 3-*O*-galactoside, chlorogenic acid and quercetin glycosides in 2006-07 only. In both consecutive years, the concentration of phloridzin in fruit skin was significantly ($P \leq 0.05$) affected with the application of RDI treatments. However, no specific trends of phloridzin concentration could be deduced in regards to the RDI application. The RDI treatment (75% RDI) resulted in the highest concentration of cyanidin 3-*O*-galactoside (311.6 $\mu g \cdot g^{-1}$FW) and quercetin glycosides (2533.8 $\mu g \cdot g^{-1}$ FW) in fruit skin as compared CI. The highest concentration of chlorogenic acid was recorded in 50% RDI (282.3 $\mu g \cdot g^{-1}$FW).

The individual compounds of quercetin glycosides detected in 'Cripps Pink' apple skin were quercetin 3-*O*-rutinoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-xyloside, quercetin 3-*O*-arabinoside and quercetin 3-*O*-rhamnoside (Table 4.6). The concentration of quercetin 3-*O*-rutinoside and quercetin 3-*O*-glucoside was not significantly affected by the irrigation treatments during both consecutive years. However, the concentrations of quercetin 3-*O*-galactoside, quercetin 3-*O*-xyloside, quercetin 3-*O*-arabinoside and quercetin 3-*O*-rhamnoside were significantly ($P \leq 0.05$) affected by the RDI treatments during 2006-07, but during 2005-06 the concentration of these compounds were non-significant. The highest concentration of quercetin 3-*O*-galactoside (1055.7 $\mu g \cdot g^{-1}$ FW), quercetin 3-*O*-xyloside (449.0 $\mu g \cdot g^{-1}$ FW), quercetin 3-*O*-arabinoside (524.8 $\mu g \cdot g^{-1}$ FW) and quercetin3-*O*-rhamnoside (212.7 $\mu g \cdot g^{-1}$ FW) in fruit skin was recorded in 75% RDI treatments as compared to other treatments. In general, the concentration of individual total quercetin glycosides in the apple skin of this cultivar in descending order were quercetin 3-*O*-galactoside > quercetin 3-*O*-arabinoside > quercetin 3-*O*-xyloside> quercetin 3-*O*-glucoside> quercetin 3-*O*-rhamnoside>quercetin 3-*O*-rutinoside.

Table 4.5. Effects of different irrigation treatments on flavonoids and other phenolic compounds in 'Cripps Pink' apple skin during 2005-06 and 2006-07.

Treatment	Flavonoids and phenolic compounds (μg·g^{-1} FW)							
	Cyanidin 3-*O*-galactoside		Chlorogenic acid		Phloridzin		Quercetin glycosides	
	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07
100% CI	72.7	160.3 b	228.2	235.0 ab	65.8 a	47.9 a	1928.5	1796.5 b
25% RDI	114.7	173.7 b	227.3	223.5 b	49.9 b	38.2 b	1959.7	1467.6 b
50% RDI	66.6	289.3 a	249.7	282.3 a	53.9 ab	48.2 a	1988.3	2012.0 ab
75% RDI	100.9	311.6 a	237.0	277.6 a	58.3 ab	47.3 a	1566.2	2533.8 a
LSD ($P \leq 0.05$)	NS (32.9)	99.6	NS (33.6)	50.9	13.5	8.61	NS (407.7)	696.4

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. Values within brackets represent standard error of means. NS = not significantly different at $P \leq 0.05$. CI = commercial irrigation and RDI = regulated deficit irrigation.

Table 4.6. Effects of different irrigation treatments on individual quercetin glycosides compounds in fruit 'Cripps Pink' apple skin during 2005-06 and 2006-07.

Treatment	Quercetin glycosides (μg·g^{-1} FW)											
	Quercetin 3-*O*-rutinoside		Quercetin 3-*O*-galactoside		Quercetin 3-*O*-glucoside		Quercetin 3-*O*-xyloside		Quercetin 3-*O*-arabinoside		Quercetin 3-*O*-rhamnoside	
	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07
100% CI	43.6	53.6	808.3	695.5ab	101.7	206.6	375.3	325.6 b	410.91	349.6 b	188.7	164.8 b
25% RDI	46.7	36.1	855.2	536.7 b	101.7	182.8	361.8	281.8 b	402.58	286.5 b	191.7	143.6 b
50% RDI	48.8	80.0	842.5	764.7ab	105.3	214.3	369.4	369.7ab	435.66	404.5ab	186.6	176.8ab
75% RDI	22.8	93.0	635.1	1055.7a	72.7	198.6	322.0	449.0 a	344.41	524.8 a	169.1	212.7 a
LSD ($P \leq 0.05$)	NS (12.6)	NS (19.5)	NS (201.8)	367.32	NS (21.6)	NS (142.4)	NS (67.1)	111.4	NS (76.7)	120.4	NS (34.8)	41.7

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. Values within brackets represent standard error of means. NS = not significantly different at $P \leq 0.05$. CI = commercial irrigation and RDI = regulated deficit irrigation.

4.3.3.4. Fruit diameter

Fruit diameter was significantly ($P \leq 0.05$) affected with the RDI treatments applied in both seasons (Figure 4.10). The smallest fruit diameter was recorded in 50% RDI (70.5 mm) and 75% RDI (70.2 mm) treatments during 2005-06 and 2006-07, respectively as compared to CI and other treatments.

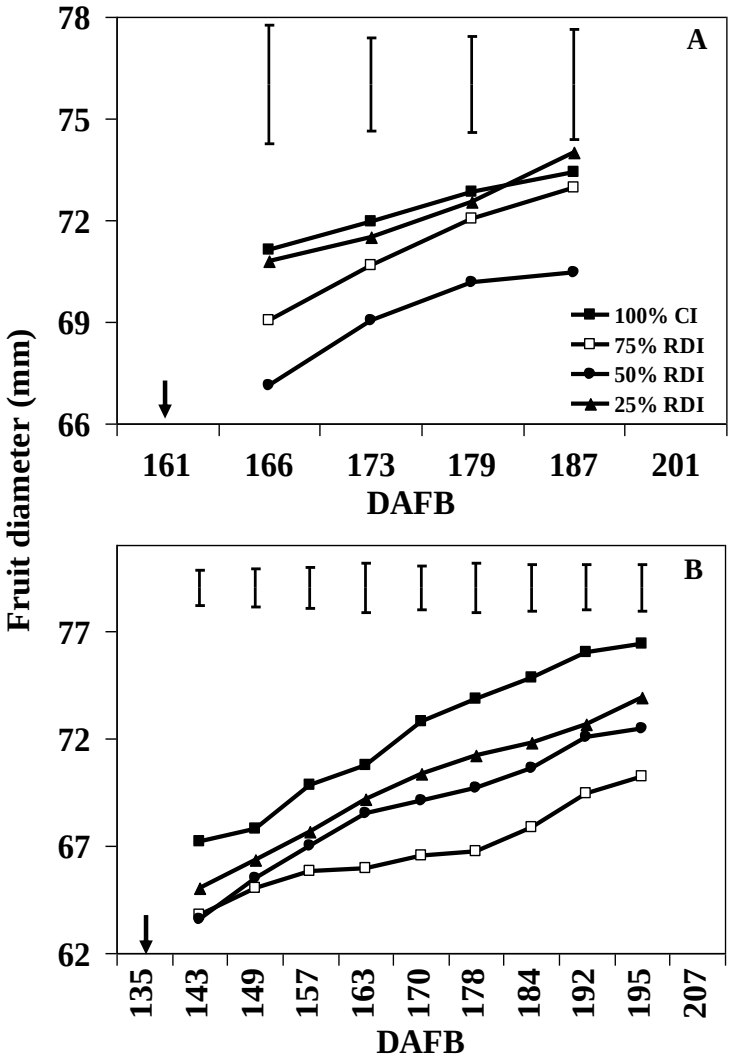

Figure 4.10. Changes in fruit diameter in 'Cripps Pink' apple affected by different irrigation treatments during fruit development and maturation in 2005-06 (A) and 2006-07 (B). Irrigation treatments were control (100% CI), 25% RDI, 50% RDI and 75% RDI. Vertical bars represent LSD ($P \leq 0.05$). Arrow indicates the commencement of RDI treatments on 161 days after full bloom (DAFB) in 2005-06 and 135 DAFB in 2006-07. CI = commercial irrigation and RDI = regulated deficit irrigation.

4.3.3.5. Firmness, titratable acidity, soluble solids concentration

The application of RDI did not significantly affect fruit firmness during 2005-06 (Table 4.7). However, during 2006-07, fruit firmness showed a significant increased

(90.2 N) with 75% RDI treatment as compared to CI. SSC was significantly ($P \leq 0.05$) affected with the application of RDI during both seasons. During 2005-06, the highest SSC (12.8%) was recorded in 25% RDI treatment as compared to CI. Whilst during 2006-07, 75% RDI treatment resulted in the highest SSC (17.2%) as compared to other treatments. The application of RDI treatments during 2005-06, resulted in a significant increased TA in all RDI treatments except 50% RDI. However, no differences between RDI treatments were recorded in TA during 2006-07.

4.3.3.6. Concentration of ascorbic acid and total antioxidants

The concentration of ascorbic acid was significantly ($P \leq 0.05$) affected with the application of RDI treatments in both consecutive years (Table 4.8). The RDI treatments, 50% RDI in 2005-06 and 75% RDI in 2006-07 resulted in significantly ($P \leq 0.05$) higher ascorbic acid concentration (10.4 and 12.4 $mg \cdot 100g^{-1}$ FW, respectively) as compared to other treatments. The RDI treatments did not significantly ($P \leq 0.05$) affect total antioxidants on the exposed side (ES) of apple skin in both growing seasons (Table 4.8). Total antioxidants also did not differ among RDI treatments on the shaded side (SS) of apple skin and fruit pulp during 2005-06 seasons. But in 2006-07, the RDI treatments significantly ($P \leq 0.05$) increased the capacity of total antioxidant on the SS of apple skin and fruit pulp. The highest levels of total antioxidants were recorded in 75% RDI (13.6 mM $TE \cdot g^{-1}$ FW) and 50% RDI (0.33 mM $TE \cdot g^{-1}$ FW) on the SS of apple skin and fruit pulp, respectively as compared to CI and other treatments.

4.3.3.7. Concentrations of sugars and organic acids

The RDI treatments did not significantly affect the concentrations of individual sugars (glucose, sucrose and fructose) and total sugars in fruit juice in both years excluding sorbitol concentration (2005-06) (Table 4.9). The effects of RDI treatments were significant during 2005-06 only. The RDI treatments (75% RDI) resulted in higher concentration of sorbitol (0.57 $g \cdot kg^{-1}$) than in CI fruit (0.33 $g \cdot kg^{-1}$). Even the effects of RDI treatments during 2006-07 were not significant, 75% RDI resulted in higher sorbitol concentration (11.2 $g \cdot kg^{-1}$) as compared to CI fruit (9.58 $g \cdot kg^{-1}$).

The RDI treatments did not significantly affect the concentrations of malic acid, citric acid and total acids in apple fruit in both consecutive years (Table 4.10). However, the RDI treatments were significantly ($P \leq 0.05$) affected the concentrations of fumaric and succinic during 2006-07 only. The higher concentrations of fumaric (5.50 $mg \cdot kg^{-1}$) and succinic (1.10 $g \cdot kg^{-1}$) were recorded in 25% RDI as compared to other treatments (Table 4.10). The RDI treatments were significantly ($P \leq 0.05$) affected the concentrations of tartaric acid during both seasons. The highest concentrations of tartaric acid were recorded in fruit from 50% RDI (0.15 $g \cdot kg^{-1}$) treatment during 2005-06 and in 50% and 75% RDI (0.14 $g \cdot kg^{-1}$) treatments in 2006-07.

Table 4.7. Effects of different RDI treatments on firmness, soluble solids concentration, titratable acidity in 'Cripps Pink' apple during 2005-06 and 2006-07.

	Firmness (N)		Soluble solids concentration (%)		Titratable acidity (% malic acid)	
Treatment	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07
100% CI	81.0	79.5 b	12.3 b	15.0 c	0.50 b	0.79
25% RDI	83.4	83.9 ab	12.8 a	15.8 b	0.67 a	0.97
50% RDI	85.2	85.2 ab	11.3 c	16.2 b	0.56 ab	0.77
75% RDI	83.7	90.2 a	11.4 c	17.2 a	0.66 a	0.97
LSD ($P \leq 0.05$)	NS (1.44)	6.71	0.31	0.70	0.12	NS (0.14)

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. Values within brackets represent standard error of means. NS = not significant ($P \leq 0.05$). CI = commercial irrigation and RDI = regulated deficit irrigation.

Table 4.8. Effects of different RDI treatments on concentrations of ascorbic acid and total antioxidants in 'Cripps Pink' apple during 2005-06 and 2006-07.

Treatment	Ascorbic acid ($mg \cdot 100g^{-1}$ FW)		Total antioxidants (mM TE$\cdot g^{-1}$ FW)					
			Skin (ES)		Skin (SS)		Pulp	
	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07
100% CI	8.10 c	9.03 b	35.5	20.3	24.1	7.09 b	0.29	0.19 b
25% RDI	8.45 bc	8.50 b	36.5	25.0	20.1	6.88 b	0.37	0.29 a
50% RDI	10.4 a	9.59 b	35.2	21.3	21.1	7.82 b	0.42	0.33 a
75% RDI	9.46 ab	12.4 a	36.6	24.4	20.8	13.6 a	0.37	0.27 ab
LSD ($P \leq 0.05$)	1.23	2.54	NS (4.71)	NS (3.75)	NS (2.54)	2.11	NS (0.07)	0.09

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. Values within brackets represent standard error of means. NS = not significant ($P \leq 0.05$). CI = commercial irrigation and RDI = regulated deficit irrigation.

Table 4.9. Effects of different RDI treatments on the concentrations of individual sugars in 'Cripps Pink' apple during 2005-06 and 2006-07.

Treatment/	Glucose ($g \cdot kg^{-1}$)		Sucrose ($g \cdot kg^{-1}$)		Fructose ($g \cdot kg^{-1}$)		Sorbitol ($g \cdot kg^{-1}$)		Total sugar ($g \cdot kg^{-1}$)	
Compound	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07
100% CI	1.61	5.69	43.4	54.6	43.2	57.3	0.33 c	9.58	88.6	127.3
25% RDI	1.97	1.85	43.9	59.7	40.8	54.4	0.51 b	9.32	87.2	125.2
50% RDI	2.88	10.2	45.0	46.5	41.8	61.4	0.52 b	10.6	90.1	128.7
75% RDI	1.67	8.42	40.3	52.0	38.3	61.0	0.57 a	11.2	80.8	132.7
LSD ($P \leq 0.05$)	NS (0.94)	NS (2.76)	NS (2.42)	NS (5.11)	NS (3.10)	NS (2.73)	0.05	NS (0.73)	NS (0.01)	NS (2.73)

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. Values within brackets represent standard error of means. NS = not significant ($P \leq 0.05$). CI = commercial irrigation and RDI = regulated deficit irrigation. Total acids = glucose ($g \cdot kg^{-1}$) + sucrose ($g \cdot kg^{-1}$) + fructose ($g \cdot kg^{-1}$) + sorbitol ($g \cdot kg^{-1}$).

Table 4.10. Effects of different RDI treatments on the concentrations of individual organic acids in 'Cripps Pink' apple during 2005-06 and 2006-07.

Treatment/ Compound	Malic acid ($g \cdot kg^{-1}$)		Citric acid ($g \cdot kg^{-1}$)		Tartaric acid ($g \cdot kg^{-1}$)		Fumaric acid ($mg \cdot kg^{-1}$)		Succinic acid ($g \cdot kg^{-1}$)		Total acids ($g \cdot kg^{-1}$)	
	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07	2005-06	2006-07
100% CI	7.39	8.49	0.17	0.18	0.13 ab	0.12 b	4.85	4.44 b	1.14	1.03 ab	8.85	9.84
25% RDI	7.33	9.52	0.17	0.19	0.12 b	0.13 ab	4.35	5.50 a	1.15	1.10 a	8.78	11.0
50% RDI	7.03	8.49	0.19	0.19	0.15 a	0.14 a	4.62	4.78 b	1.20	1.04 ab	8.58	9.87
75% RDI	7.03	7.40	0.16	0.19	0.12 b	0.14 a	4.42	4.54 b	1.01	0.99 b	8.34	8.73
LSD ($P \leq 0.05$)	NS (0.46)	NS (0.68)	NS (0.01)	NS (0.01)	0.02	0.01	NS (0.21)	0.66	NS (0.07)	0.09	NS (0.51)	NS (0.70)

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. Values within brackets represent standard error of means. NS = not significant ($P \leq 0.05$). CI = commercial irrigation and RDI = regulated deficit irrigation. Total acids value represent cumulative value of malic acid ($g \cdot kg^{-1}$), citric acid ($g \cdot kg^{-1}$), tartaric acid ($g \cdot kg^{-1}$), fumaric acid ($g \cdot kg^{-1}$) and succinic acid ($g \cdot kg^{-1}$).

4.3.4. Effects of irrigation treatments on fruit quality after long term cold storage

4.3.4.1. Firmness, TA, SSC and SSC/TA ratio

The interactions between the RDI treatments and cold storage periods in flesh firmness, SSC, TA and SSC/TA ratio were non-significant (Table 4.11). Firmness, TA and SSC/TA ratio was significantly ($P \leq 0.05$) affected with the RDI treatments and cold storage periods excluding SSC, which was only significant with the application of different RDI treatments. Fruit from 75% RDI had significantly ($P \leq 0.05$) higher firmness following cold storage for 45, 90 and 135 days as compared to other treatments. Cold-stored-fruit from all irrigation treatments showed a decreasing trend in firmness but still retain firmer above standard requirements set by the industry (68.0 N) as storage period prolonged to 135 days. Following cold storage for 45, 90 and 135 days, the SSC was significantly ($P \leq 0.05$) higher in 75% RDI stored-fruit as compared to CI. All irrigation treatments had significantly ($P \leq 0.05$) reduced TA as cold storage period prolonged to 135 days. Fruit from 75% RDI treatment stored for 135 days decreased 32% of TA as compared to stored-fruit for 45 days. The sugar acid ratio in all irrigation treatments was significantly ($P \leq 0.05$) increased as storage periods extended. SSC/TA ratio was higher in 75% RDI stored-fruit compared to CI stored-fruit as storage period prolonged to 90 days.

4.3.4.2. Concentration of ascorbic acid and total antioxidants

The concentration of ascorbic acid in the pulp and total antioxidants on the ES of apple skin exhibited a significant ($P \leq 0.05$) interaction between the RDI treatments and cold storage periods (Table 4.11). However, no interactions were recorded between the RDI treatments and storage periods for total antioxidants on the SS of fruit skin and pulp. All irrigation treatments showed the increased concentration of ascorbic acid as storage period prolonged to 135 days. Even there was a significant interaction recorded in total antioxidants on ES of apple skin, no specific trend can be deduced as the level of total antioxidant fluctuated from one storage period to another. Total antioxidants on SS of apple skin and pulp were significantly ($P \leq 0.05$) affected by the RDI treatments and duration of cold storage. Fruit from all irrigation treatments experienced the higher total antioxidants on SS during 90 days of cold storage, but the total antioxidants decreased as cold storage period prolonged to 135 days. Following 90 days cold storage, the total antioxidants on the SS of fruit

skin were higher in 75% RDI cold-stored-fruit as compared to CI stored-fruit. On the other hand, total antioxidants in the pulp in all irrigation treatments showed an increasing trend with extended cold storage period. The higher level of total antioxidants in pulp was recorded in 75% RDI stored-fruit during 90 days storage as compared to CI stored fruit.

4.3.5. Effect of irrigation treatments and controlled atmosphere storage on fruit quality

4.3.5.1. Firmness, TA and SSC

The RDI treatments significantly ($P \leq 0.05$) affected fruit firmness and SSC in CA-stored-fruit for 155 days and the CA-following 14 days shelf life excluding TA (Table 4.12). Flesh firmness in CA-stored-fruit for 155 days and CA-following 14 days shelf-life was significantly ($P \leq 0.05$) increased (89.0 N and 91.4 N, respectively) with 75% RDI treatment as compared to other treatments. The highest SSC was recorded in 75% RDI stored-fruit for 155 days in CA and CA-following 14 days shelf life (17.2% and 17.3%, respectively) as compared to CI treatment.

4.3.5.2. Concentration of ascorbic acid and total antioxidants

The RDI treatments significantly ($P \leq 0.05$) affected the levels of ascorbic acid in CA-stored-fruit for 155 days (Table 4.12), and the highest concentration was recorded in fruit from 75% RDI treatment (12.6 mg·100g^{-1} FW) as compared to CI. No significant effect on the concentration of ascorbic acid was observed among irrigation treatments in fruit stored in CA-following 14 days shelf life. However, fruit-stored in CA-following 14 days shelf life had gradually lowered the concentration of ascorbic acid than in CA-stored-fruit. Total antioxidants on the ES of fruit skin in CA stored fruit were significantly ($P \leq 0.05$) affected with the RDI treatments (Table 4.12). However, the levels of total antioxidants were not affected with RDI treatments in fruit stored in CA-following 14 days shelf life. The highest total antioxidants on the ES of fruit skin (34.7 mM TE·g^{-1} FW) in CA were recorded in fruit from 75% RDI treatment as compared to CI (23.2 mM TE·g^{-1} FW). Total antioxidants in CA-stored-fruit and CA-following 14 days shelf life in RDI treatment (75% RDI) significantly ($P \leq 0.05$) increased total antioxidants on the SS of fruit skin (24.4 and 25.0 mM TE·g^{-1} FW, respectively) and fruit pulp (0.62 and 0.72 mM TE·g^{-1} FW, respectively) as compared to CI (Table 4.12). Total antioxidants in CA-

Table 4.11. Fruit firmness, soluble solids concentration, titratable acidity, SSC/TA ratio, concentration of ascorbic acid and total antioxidants in 'Cripps Pink' apple affected by different RDI treatments and cold storage during 2006-07.

Treatment	Storage (days)	Firmness (N)	SSC (%)	TA (%malic acid)	SSC/TA ratio	AA ($mg \cdot 100g^{-1}$ FW)	Total antioxidants (mM TE·g^{-1} FW)		
							Skin (ES)	Skin (SS)	(pulp)
100% CI	45	82.1 a C	15.5 B	0.73 AB	21.7 b B	8.45 c A	13.6 b B	8.72 b B	0.11 c
	90	81.9 a AB	15.9 B	0.70 A	22.6 ab B	11.6 a A	25.6 ab	17.8 a C	0.35 b B
	135	75.3 bB	15.8 B	0.63	25.6 a	10.5 a B	31.6 a A	9.54 b	0.43 a
	Mean	79.6	15.7	0.69	23.1	10.2	23.6	12.0	0.29
25 % RDI	45	88.2 a AB	16.9 a A	0.77a A	21.9 c AB	6.46 c B	26.7 b AB	14.8 AB	0.11 b
	90	80.2 b B	16.5 b AB	0.62 b B	26.5 b A	9.46 b C	31.2 b	19.7 BC	0.48 a AB
	135	77.6 b B	16.1 c B	0.56 c	28.8 a	10.5 a B	33.8 a A	19.8	0.48 a
	Mean	82.0	16.5	0.65	25.7	10.7	27.3	20.3	0.41
50 % RDI	45	85.6 BC	16.4 A	0.70 a B	23.3 b A	6.13 b B	33.1 A	17.4 A	0.11 b
	90	84.1 AB	16.7 A	0.64 a AB	26.1 b A	10.5 a B	26.5 B	24.6 AB	0.48 a AB
	135	79.4 AB	16.4 AB	0.53 b	31.4 a	11.6 a A	24.8 B	16.8	0.53 a
	Mean	83.1	16.5	0.62	26.9	9.39	29.7	19.6	0.38
75 % RDI	45	91.8 a A	17.2 A	0.73 a AB	23.4 b A	9.13 b A	28.1 A	20.9 a A	0.13 b
	90	85.6 b A	17.2 A	0.60 ab B	28.4 ab A	11.7 a A	29.2	27.4 a A	0.53 a A
	135	84.5 b A	17.0 A	0.49 b	30.2 a	11.3 a AB	24.6 B	12.5 b	0.57 a
	Mean	87.3	17.1	0.61	27.3	8.86	29.0	18.1	0.35
LSD ($P \leq 0.05$)									
Irrigation (I)		2.54	0.38	0.06	2.16	0.63	NS (1.87)	4.99	0.07
Storage (S)		2.03	NS (0.11)	0.06	1.87	0.56	NS (1.74)	4.32	0.06
I x S		NS (1.53)	NS (0.23)	NS (0.04)	NS (1.30)	1.13	21.40	NS (3.01)	NS (0.04)

Means followed by the same letter within column in capital letter are not significantly different between the treatments and in small letter within column are not significantly different between storage periods at $P \leq 0.05$. Values within brackets represent standard error of means. NS = not significant ($P \leq 0.05$). CI = commercial irrigation and RDI = regulated deficit irrigation. SSC = soluble solids concentration, TA = titratable acidity, AA = ascorbic acid concentration.

Table 4.12. Fruit firmness, soluble solids concentration, titratable acidity, ascorbic acid concentration and total antioxidants in 'Cripps Pink' apple affected by different RDI treatments following controlled atmosphere storage and shelf life period during 2006-07.

Measurements	Storage period (days)	100%CI	25 % RDI	50 % RDI	75 % RDI	LSD $P \leq 0.05$
Firmness (N)	155 days CA	82.3 b	81.8 b	82.9 b	89.0 a	5.57
	155 days CA +14 days	84.5 b	85.2 b	86.3 b	91.4 a	4.16
SSC (%)	155 days CA	15.4 d	15.9 c	16.5 b	17.2 a	0.39
	155 days CA +14 days	15.6 c	16.0 c	16.8 b	17.3 a	0.44
TA (% malic acid)	155 days CA	0.65	0.65	0.65	0.63	NS (0.02)
	155 days CA +14 days	0.63	0.60	0.58	0.61	NS (0.01)
AA ($mg \cdot 100g^{-1}$ FW)	155 days CA	10.2 d	10.9 c	11.6 b	12.6 a	0.50
	155 days CA +14 days	9.89	8.99	10.2	9.73	NS (0.40)
Total antioxidants (mM TE$\cdot g^{-1}$ FW)						
Skin (ES)	155 days CA	23.2 b	26.0 ab	24.5 ab	34.7 a	11.5
	155 days CA +14 days	23.8	33.4	29.9	32.5	NS(3.11)
Skin (SS)	155 days CA	12.6 c	14.7 bc	19.5 ab	24.4 a	5.07
	155 days CA +14 days	16.5 b	19.8 ab	17.9 b	25.0 a	6.23
Pulp	155 days CA	0.38 b	0.48 b	0.45 b	0.62 a	0.10
	155 days CA +14 days	0.52 b	0.60 ab	0.72 a	0.72 a	0.14

Means followed by the same letter within row are not significantly different at $P \leq 0.05$. Values within brackets represent standard error of means. NS = not significant ($P \leq 0.05$). CI = commercial irrigation and RDI = regulated deficit irrigation.

following 14 days shelf-life in 50% RDI treatment showed a comparable level to 75% RDI stored-fruit. In general, total antioxidant levels in the skin were higher than in the pulp of CA-stored-fruit and also following 14 days shelf-life.

4.4. Discussion

In both experiments, RDI treatments resulted in a moderate stress according to the classification of Hsiao (1973), in which the difference between leaf water potential (Ψ_{leaf}) of RDI treatment and CI ($\Delta\Psi$) was between -0.5 and -1.5 MPa. The significantly lower θ with RDI treatments (Figure 4.3) during 2006-07 created soil water-deficit, which resulted in reduce Ψ_{stem} (Figure 4.4B), Ψ_{leaf} (Figure 4.5B) and g_s (Figure 4.6B). However, the implementation of RDI during 2006-07 was 35 days earlier than in 2005-06, which was more intense to influence plant water relations.

The pronounced effects of RDI during 2006-07 on plant water relations resulted in significantly increased fruit colour, concentration of total anthocyanins in the skin of both sides of fruit (Table 4.1), SSC and fruit firmness (Table 4.7) with slightly reduced final fruit size (range from 3% to 8%, Figure 4.10) as compared to CI. Increased fruit colour may be attributed to the higher concentration of total anthocyanins in the skin of both sides of the apple fruit. Possibly, it may be associated to the improved penetration of sunlight into tree canopy and onto the fruit due to sparse leaf abscission caused by the RDI treatments. As reported earlier, increased anthocyanins concentration in apple fruit skin depends on light (Reay and Lancaster, 2001; Saure, 1990). Light activates various enzymes in anthocyanins biosynthesis pathway such as phenylalanine ammonia-lyase (PAL) and UDPGal: flavonoid-3-*O*-galactosyltransferase (UFGalT), in which activity of UFGalT in 'Fuji' apple increased as fruit exposed to the reflective mulches (Ju et al., 1999a) and PAL activity in green 'Royal Gala' apple increased due to the light irradiation (Dong et al., 1995). The increase in apple fruit colour development with RDI treatments was evident from lower chromaticity value b*, hue angle, lightness and the increased in chromaticity value a* during 2006-07, which indicates redder skin colour (Table 4.2 and Table 4.3). Similarly, Whale and Singh (2007) reported that the reduction in hue angle and the increased red skin colour was coincided with the increased in total anthocyanins concentration in 'Cripps Pink' apples.

As a prelude, nine polyphenolic compounds in 'Cripps Pink' apple fruit skin were identified and confirmed by matching the mass spectra obtained using HPLC-ESI-MS (Table 4.4 and Figure 4.8 and 4.9). The separation and elution order of individual quercetin glycosides determined were in agreement with the reports of (Schieber et al., 2002). The increased concentrations of total anthocyanins in fruit skin with the RDI treatments during 2006-07 may also coincided with the increased concentrations of these polyphenolic compounds such as cyanidin 3-*O*-galactoside, chlorogenic acid, quercetin glycosides, quercetin 3-*O*-galactoside, quercetin 3-*O*-xyloside, quercetin 3-*O*-arabinoside and quercetin 3-*O*-rhamnoside (Table 4.5 and Table 4.6). The RDI treatment (75% RDI) exhibited the increased concentrations of 94% cyanidin 3-*O*-galactoside, 18% chlorogenic acid, 54% quercetin 3-*O*-galactoside, 33% quercetin 3-*O*-xyloside, 50% quercetin 3-*O*-arabinoside and 29% quercetin 3-*O*-rhamnoside as compared to CI fruit during 2006-07.

The improved fruit colour and increased concentration of anthocyanins in fruit skin with RDI treatments may be ascribed to the increased levels of abscissic acid (Zhang and Davies, 1990) and/or ABA induced ethylene production (Gomez-Cadenas et al., 1996) consequently up regulating expression of gene(s) involved in biosynthesis of anthocyanins. Earlier, the increased rate of ethylene production in 'Delicious' and 'Braeburn' apple fruit with the deficit irrigation has been reported (Behboudian et al., 1998; Ebel et al., 1993; Kilili et al., 1996b). The ethylene has also been reported to play key role in improving fruit colour and accumulation of total anthocyanins in the skin of 'Cripps Pink' apple fruit (Whale and Singh, 2007; Whale et al., 2008). To the best of my knowledge, this may be first report on the effects of water-deficit on production of flavonoids and other phenolic compounds in red-skinned apple particularly in 'Cripps Pink' apple. However, its exact mechanisms in increasing anthocyanins and other polyphenolic compounds in this apple cultivar warrant further investigations.

As a prelude, the RDI treatments have also enhanced exposure of apple fruit to sunlight due to sparse abscission of leaves. Earlier, Castellarin et al. (2007b) claimed that the anthocyanins biosynthesis has been partly affected by solar radiation, but primarily due to water-deficit application during intense phase of anthocyanins biosynthesis. They also reported that under water-deficit condition, concentration of

anthocyanins has been modified by stimulating hydroxylation and methylation of the flavonoid B-ring. The biosynthesis of flavonols, anthocyanins, proanthocyanin, flavanols in grape berries was increased due to the severe water-deficit applied from varaison to maturation has been reported (Ojeda et al., 2000). The anthocyanins-specific genes UDP glucose:flavonoid 3-*O*-glucosyltransferase (UFGT), chalcone synthase (CHS2 and CHS3) and flavanone 3-hydroxylase (FH3) were up-regulated in water-deficit grapes than control, which coincide with the increased anthocyanins concentration (Castellarin et al., 2007b). Furthermore, genes coding for flavonoid 3'5'-hydrxylase (*F3'5'H*) and *O*-methyltransferase (*OMT*) also up-regulated with water-deficit in dehydrated berries skin, in which anthocyanins concentration increased more hydroxylated (malvidin) and methylated (peonidin) derivatives (Castellarin et al., 2007b). Grimplet et al. (2007) reported that water-deficit enhanced mRNA expression accumulation specifically in the skin of grapes in which may attributed to the increase of anthocyanins concentration. A high PAL activity with the accumulation of anthocyanins and other phenolic compounds were also observed in water-deficit olives (*Oleo europaea* L. cv. Arbequina) as reported by Tovar et al. (2002). Possibly, water-deficit imposed in red skinned apple may also trigger the activity of enzymes and anthocyanins-specific genes in anthocyanins biosynthetic pathway. It warrants further investigations on genes expression profiling in apple skin especially in 'Cripps Pink' apple cultivar subjected to RDI treatments. In this study, 75% RDI imposed in the stage II of fruit development (135-207 DAFB) during 2006-07 increased the concentration of cyanidin 3-*O*-galactoside and other phenolic compounds, which was similar to the report of Whale and Singh (2007).

At harvest, a higher SSC in RDI fruit (Table 4.7) has also been reported earlier (Kilili et al., 1996a) and the increased of SSC may be due to the conversion of starch into sugars (Kramer, 1983; Landsberg and Jones, 1981). Firmer fruit with RDI treatments (Table 4.7) may be attributed to the reduction in cellular hydration and increased flesh compactness (Mpelasoka et al., 2000a). The slight reduction observed in fruit size with RDI treatments may be due to the sources limitation (DeJong and Grossman, 1995) of photosynthesis caused by water-deficit due to lower stomatal conductance (Naor, 1998). The RDI was implemented in stage II of fruit development, which had the lowest sensitivity to water-deficit (Mitchell and Chalmers, 1982). The short period of RDI application during 2005-06 unable to

create water-deficit condition and therefore the effects of RDI on fruit quality was not evident.

Increased concentration of ascorbic acid due to water-deficit treatments (Table 4.8) has been reported, but, in different crops such table grape (*Vitis vinifera* L. cv Rizamat) (Du et al., 2008), pear-jujube (*Zizyphus jujuba* Mill. cv. Lizao) (Cui et al., 2008), strawberry (*Fragaria ananassa* Duch.) (Liu et al., 2001), tomato (*Lycopersicon esculentum* Mill. cv. Vanessa) (Veit-Kohler et al., 1999) and hot pepper (*Capsicum annuum* L.) (Dorji et al., 2005). The increased concentration of ascorbic acid may be associated with the higher sugars accumulation in water-deficit fruit that promotes its production during fruit ripening (Veit-Kohler et al., 1999). In addition, the higher ascorbic acid concentration in fruit may be related to its increased concentration in apple leaves under moderate water stress (Sircelj et al., 2005; Sircelj et al., 2007).

The RDI treatments significantly increased total antioxidants on the SS of apple skin and pulp, possibly due to the increased total anthocyanins concentration on the SS of apple skin and also the increase of ascorbic acid concentration in the pulp (Table 4.8).

Amongst sugars, sorbitol concentrations were higher in RDI fruit than CI in both seasons (Table 4.9). This may be ascribed to the higher sorbitol concentration under moderate water stress and their concentration appeared as reliable biochemical indicators for moderate stress markers in 'Elstar' apple leaves (Sircelj et al., 2007). Similarly, the higher sorbitol concentration was recorded in 'Braeburn' apple under water-deficit (Mills et al., 1996a; Mills et al., 1996b; Mills et al., 1997b; Mills et al., 1994). The concentrations of tartaric, fumaric and succinic acid were significantly increased with RDI treatments, in which all these acids closely related to TA (Table 4.10). Higher TA in early DI indicates the contribution of organic acids to the fruit osmotic adjustment as reported by Mills et al. (1997a) and Pavel and DeJong (1995).

Improved fruit firmness in cold storage subjected to water-deficit (Table 4.11) has been reported (Behboudian et al., 1998; Kilili et al., 1996b; Mpelasoka et al., 2000a). As a prelude, the higher firmness may be ascribed to the reduction in cellular

hydration. Higher SSC following cold storage might be due to the conversion of starch into sugars during ripening (Brady, 1987). SSC increased with extended storage duration. Similar effects of cold-stored-fruit in 'Braeburn' apple under water-deficit have also been reported (Kilili et al., 1996b). As storage period prolonged, all RDI treatments experienced a decreased of TA. Similarly, the decreasing trends of TA was recorded in water-deficit 'Braeburn' apple following cold storage (Mpelasoka et al., 2000a), possibly due to the consumption of malic acid as a metabolite substrate in fruit respiration (Ackermann et al., 1992). The ascorbic acid concentration increased in cold-stored-fruit from 75% RDI treatment as storage duration prolonged (Table 4.11). In contrast, the ascorbic acid concentration in apple decreased during long-term storage has also been reported earlier (Meberg et al., 2000). Possibly, the increased concentration of ascorbic acid in 75% RDI cold-stored fruit may be reflected to the increased of ascorbic acid concentration at harvest and also might be due to the higher accumulation of sugars which stimulates its synthesis as explained earlier. Higher total antioxidants in cold-stored-fruit from 75% RDI fruit were recorded (Table 4.11). Similarly, it has been reported that total antioxidants activity in apple skin almost double during 4 months cold storage (Leja et al., 2003). Possibly, the ethylene action during storage may have stimulated the activity of PAL, a key enzyme in biosynthesis of phenolic compounds consequently contributed to the increased levels of antioxidants (Leja et al., 2003).

No research work has been reported on the effects of RDI on postharvest performance of apple fruit during CA storage. Higher firmness and SSC of RDI fruit in CA and CA-following 14 days shelf life was recorded (Table 4.12). As explained earlier, higher firmness may be due to the cellular hydration and the increased of SSC might be ascribed to the conversion of starch into sugars. The ascorbic acid concentration was significantly increased with RDI treatments in CA-stored fruit. However, fruit from all treatments in CA-following 14 days shelf life exhibited the decreasing trends in ascorbic acid concentration. Possibly, this may be related to the increased activity of cytochrome oxidase, ascorbic acid oxidase and peroxidase enzymes (Rocha et al., 1995). In general, total antioxidants in both sides of apple skin increased in CA and CA-following 14 days shelf life in 75% RDI treatment. As a prelude, the higher total antioxidants may be ascribed to the increased levels of

enzymes activity and ethylene action may be stimulated the activity of enzymes precursor.

In conclusion, the RDI (75%) at stage II of fruit development commencing from 135 DAFB continuously for 72 days until harvest (207 DAFB) effectively enhanced fruit colour development through increasing accumulation of anthocyanins and polyphenolic compounds of 'Cripps Pink' apple and also other fruit quality attributes such as firmness and SSC at harvest, cold and CA storage without adversely affecting the fruit size.

CHAPTER 5

Plant Water Relations, Anthocyanins Accumulation, Fruit Quality and Post Storage Performance in 'Cripps Pink' Apples in Relation to Withholding Irrigation

Summary

The present study was carried out to investigate the effects of withholding irrigation (WHI) at various stages of apple fruit development on fruit colour, fruit quality at harvest and following storage. WHI treatments were applied during phase II and III of fruit development commencing from 135, 145 and 155 days after full bloom (DAFB). The periods of WHI are related to the baseline value of stem water potential for water stress tree (>-2.5 MPa). The study was carried out for two consecutive years, treatments during 2006-07 were (i) commercial irrigation as control (CI), (ii) WHI-1, from 135 to 153 DAFB, (iii) WHI-2, from 145 to 175 DAFB, and (iv) WHI-3, from 155 to 200 DAFB. While, in 2007-08, treatments included were: (i) CI, (ii) WHI-1, from 135 to 155 DAFB, (iii) WHI-2, from 145 to 200 DAFB, (iv) WHI-3, from 155 to 200 DAFB. Withholding irrigation treatment (WHI-2) was imposed for 30 days, 2006-07 and WHI-1 (20 days, 2007-08) resulted in significantly reduced volumetric soil water content (θ), stem water potential (Ψ_{stem}), leaf water potential (Ψ_{leaf}), and stomatal conductance (g_s), 10 days after the commencement of the treatments. Irrigation withheld for 30 days (WHI-2) and 20 days (WHI-1) during 2006-07 and 2007-08 seasons respectively, significantly increased red skin colour, concentration of total anthocyanins, higher chromaticity value a*, lower hue angle and lightness on exposed and shaded sides of apple fruit skin as compared to CI. In both seasons, the WHI treatments (WHI-1 and WHI-2) significantly increased cyanidin 3-*O*-galactoside as compared to CI fruit. In conclusion, WHI applied in the middle of stage II for 20 to 30 days (commencement on 135 and 145 DAFB) improved fruit colour development and other major fruit quality parameters at

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harvest without adversely affecting postharvest quality in cold storage and also reduced water use.

5.1. Introduction

Poor fruit colour development and water use efficiency in 'Cripps Pink' apple are major constraints for Australian apple growers to boost profits. A bright pink-red blush ≥40%, 7-9 $kgcm^{-2}$ of fruit firmness, 13 to ≥15 °Brix SSC, size is ≥65 mm and 0.7-0.9% titratable acidity (TA) are the specific criteria for export markets of 'Cripps Pink' apples (Cripps et al., 1993; Department of Agriculture Western Australia, 2000). Western Australian apple export declined from year to year. In 2002-03, export values were A$ 8.9 million declined to A$ 1.5 million in 2007-08 (Department of Agriculture and Food Western Australia, 2006; Department of Agriculture and Food Western Australia, 2008) due to unfavourable climatic conditions and mainly poor fruit colour development. Water scarcity and climatic change throughout the world increase concern to use every drop of water wisely mainly in agriculture industry. Minimising water use efficiency in apple industry under various climatic conditions through regulated deficit irrigation (RDI) and partial root zone drying (PRD) have been explored with promising outcomes (Ebel et al., 1993; Kilili et al., 1996b; Mills et al., 1996b; Mills et al., 1994; Mpelasoka et al., 2001b; O'Connell and Goodwin, 2007; Talluto et al., 2008; van Hooijdonk et al., 2004). Withholding of irrigation (WHI) is one of the strategies on minimising water use that have been reported to improved red skin colour, fruit firmness, soluble solids concentration (SSC) and also advanced maturity and reduce weight loss in storage of 'Braeburn' apple in humid-temperate region (Kilili et al., 1996b; Mpelasoka et al., 2000a). Due to the potential impact of water-deficit in improving apple skin colour and increasing demand of irrigation water use, withholding irrigation (WHI) strategy may be another useful tools to enhance these traits.

Anthocyanins are pigment compounds that impart red colour to the apple skin. The accumulation of anthocyanins are regulated by many factors such as light, temperature (Awad et al., 2000; Lancaster, 1992; Lister et al., 1994; Saure, 1990), application of chemicals (Gomez-Cardoves et al., 1996; Saure, 1990; Whale et al., 2008) and irrigation (Iglesias et al., 2000; Iglesias et al., 2002). Water-deficit increased the accumulation of polyphenolic compounds especially anthocyanins in

grapevine (Matthews and Anderson, 1988; Ojeda et al., 2000; Roby et al., 2004) and had direct effect on flavonoid gene expression (Castellarin et al., 2007a; Castellarin et al., 2007b). However, limited information is available on the effects of water-deficit on anthocyanin biosynthesis in red-skinned apple. Besides that, these polyphenolic compounds possess a potential protective effect against chronic diseases such as cardiovascular disease, diabetes, asthma, arthritis and cancer (Boyer and Liu, 2004; Eberhardt et al., 2000; Formica and Regelson, 1995; Xing et al., 2001).

To date, no information is available on the specific time of WHI in improving fruit colour development and other fruit quality attributes at harvest and also following cold storage of 'Cripps Pink' apple under the Mediterranean climate of Western Australia. These observations prompted to evaluate the effects of WHI especially commencing at later stages of fruit development and maturation (stage II and III) on leaf hydraulic status, fruit colour and various fruit quality parameters at harvest and also after long term cold storage of 'Cripps Pink' apple.

5.2. Materials and Methods

5.2.1. Location and climatic conditions

Two experiments were conducted in 2006-07 and 2007-08 growing season on 'Cripps Pink' apple trees at commercial orchard in Karragullen, Perth Hills, Western Australia. The location was in a Mediterranean climate characterised by warm summers and cool winters.

5.2.2. Experiment 1: 2006-07

'Cripps Pink' apple trees of uniform size and age (14 years old), grafted on MM.109 rootstock were used in the experiment. Trees were planted in the east-west direction, maintaining row distances of 4.5 m and plant distances of 2.4 m. The orchard soil was gravel in a sandy or loamy matrix. Full bloom (>80% of the buds are open) occurred on 9th October 2006. The treatments were: (i) C1, commercial irrigation as control, (ii) WHI-1, from 135 to 153 DAFB, (iii) WHI-2, from 145 to 175 DAFB, and (iv) WHI-3, from 155 to 200 DAFB. The experiment was laid out by following a randomized complete block design with four replications. Single tree was treated as an experimental unit. Adjacent plots were separated by guard trees. Water stress in

trees through WHI was induced considering the baseline value of stem water potential (>-2.5 MPa) (Behboudian, M.H., pers. commun, 2006). The duration of the experiment was 65 days commencing from 135 days after full bloom (DAFB) until 200 DAFB. The commercial harvest date was on 207 DAFB (3rd May 2007). Commercial or full irrigation was applied for two hours daily at 17:30 to 19:30 hours. Leaf water potential (Ψ_{leaf}) and Ψ_{stem} were measured on 149, 155, 165, 176, 185 and 195 DAFB. Volumetric soil water content (θ), stomatal conductance (g_s), fruit diameter, and fruit drop were recorded on 146, 149, 155, 165, 176, 185 and 195 DAFB. Outside the deficit periods, all the experimental trees were irrigated the same as CI treatment.

5.2.3. Experiment 2: 2007-08

The experiment was performed in the same orchard as in Experiment 1, with twenty of 15-year-old apple trees on MM.109 rootstock. The full bloom for the season 2007-08 occurred on 21st October 2007. Similar experimental design as above mentioned was used in this experiment with some modifications of WHI treatments. The treatments tested were (i) CI, commercial irrigation as control, (ii) WHI-1, from 135 to 155 DAFB, (iii) WHI-2, from 145 to 200 DAFB, (iv) WHI-3, from 155 to 200 DAFB. Irrigation was applied for two hours daily at 19:00 to 21:00 hours. Irrigation treatments commenced on 135 DAFB (4th March 2008) until 200 DAFB (8th May 2008). The commercial harvest date was on 200 DAFB (8th May 2008). Ψ_{leaf}, Ψ_{stem}, and fruit drop were recorded on 135, 145, 155, 165, 175, 185 and 195 DAFB as explained in experiment 1. The fruit diameter, θ and g_s were recorded on 136, 146, 156, 166, 176, 186 and 195 DAFB. Outside the deficit periods, all the trees were irrigated the same as CI.

5.2.4. Temperature monitoring at experimental site

Data logger (Tinytag*Plus* Gemini Data Logger, UK) was used for recording daily temperatures in the orchard. Daily temperatures (average day and night temperatures) were obtained using Gemini Logger Manager Software (Version 2.8). Rainfall and evapotranspiration (ET) data were obtained from Bureau of Meteorology, Perth, Western Australia. Summary of the daily climates data for two years as presented in Figure 5.1A and 5.1B. Daily average day and night temperatures were calculated between sunset and sunrise times as detailed in Chapter 4, Section 4.2.5.

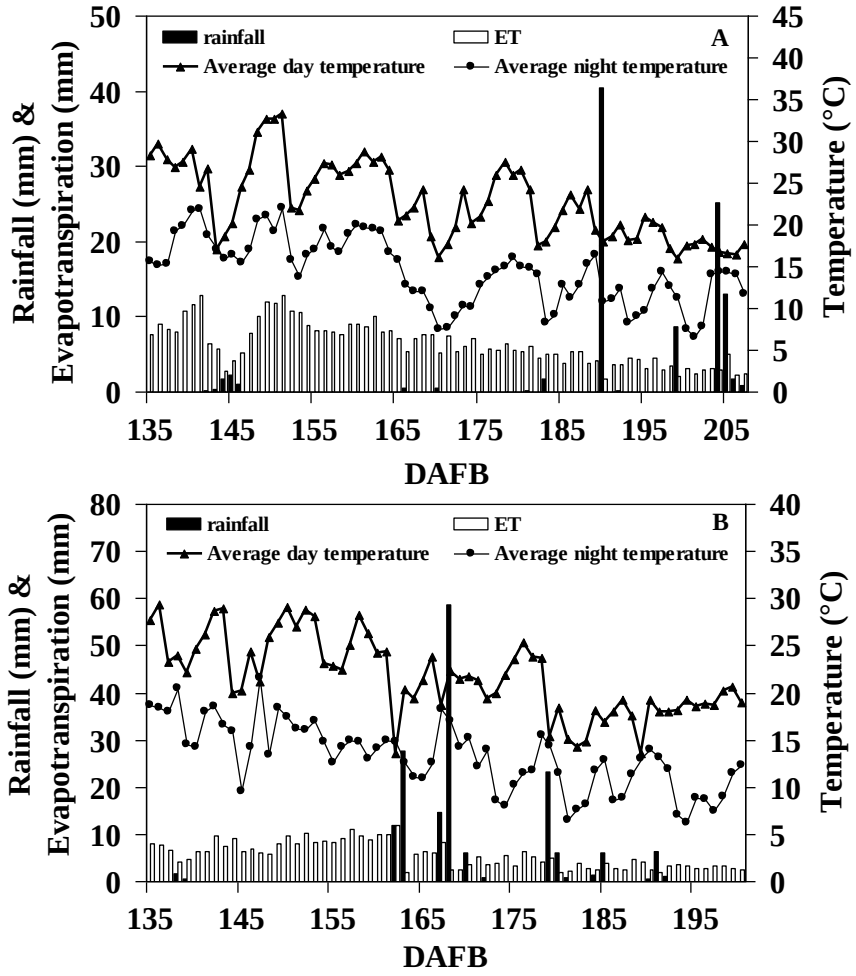

Figure 5.1. Daily average day and night temperatures, rainfall and evapotranspiration during (A) 2006-07 and (B) 2007-08 at the commercial apple orchard, Karragullen, Perth Hills, Western Australia.

5.2.5. Fruit sampling, observations recorded and storage conditions

Fruit were randomly selected from all parts of the tree canopy up to height of 2 m from the ground. Fruit were harvested on 207 DAFB (3rd May 2007) and 200 DAFB (8th May 2008) during 2006-07 and 2007-08, respectively. At harvest, twenty-five fruit were used for fruit quality assessment. The assessments consist of percentage red blush >40 fruit skin surface, fruit colour, total anthocyanins concentration, polyphenolic compounds, firmness, titratable acidity (TA), soluble solids concentration (SSC), SSC/TA ratio, ascorbic acid concentration, total antioxidants, sugars and organic acids concentration.

To evaluate the effects of WHI treatments on storage life of fruit, in 2006-07, the fruit were also kept in cold storage. The fruit were dipped in diphenylamine (DPA) to

avoid cold storage scald. The DPA-dipped fruit were packed in cartons which contain trays for individual apple. Each tray contains twenty-five fruit for each replicate. Two replicates per trays contain in one carton. All cartons were placed in cold storage at 0 ±0.1°C, 90 ±2% RH. Fruit quality was assessed following 70 and 140 days after cold storage. Fruit were placed at room temperature (20 ±1.0^{o}C) for 5-6 hours before quality assessment. Fruit quality parameters were firmness, TA, SSC, SSC/TA ratio, ascorbic acid concentration and total antioxidants.

5.2.6. Preharvest parameters: soil-plant water relations

5.2.6.1. Volumetric soil water content

Volumetric soil water content (θ) was recorded using a Moisture Probe Meter (MPM 160, ICT International Pty. Ltd., Armidale, New South Wales, Australia) between 10:00 and 11:00 am at approximately ten-day intervals commencing from 135 DAFB until 200 DAFB as outline in Chapter 3, Section 3.2.1.

5.2.6.2. Leaf water potential and stem water potential

Leaf water potential (Ψ_{leaf}) and stem water potential (Ψ_{stem}) was recorded with a pressure chamber between 11:00 and 14:00 am at approximately ten-day intervals as detailed in Chapter 3, Section 3.2.2 and 3.2.3, respectively.

5.2.6.3. Stomatal conductance

Stomatal conductance (g_s) was recorded using a Steady State Diffusion Porometer as described in Chapter 3, Section 3.2.4.

5.2.6.4. Fruit drop

Total numbers of fruit on randomly selected branches were counted at the commencement of treatments until harvest as explained in Chapter 3, Section 3.2.5.

5.2.7. Fruit quality: fruit colour

5.2.7.1. Surface skin colour

Percent red blush of individual apples were assessed visually as explained in Chapter 3, Section 3.3.1. The apple fruit colour was also recorded by a Hunter Lab ColorFlex 45^{o}/0^{o} Spectrophotometer including chromaticity value a*, b*, lightness (L*), chroma (C*) and hue angle (h°) as outlined in Chapter 3, section 3.3.2.

5.2.7.2. Analysis of skin pigment

5.2.7.2.1 Total anthocyanins

Anthocyanins of apple skin were extracted and quantified according to the procedure outlined by Whale and Singh (2007) using an UV-VIS spectrophotometer (Jenway Spectrophotometers Model 6405, Dunmow, Essex, UK) as detailed in Chapter 3, Section 3.3.3.1.

5.2.7.2.2 Flavonoids and other phenolic compounds

All standards used for flavonoids and other phenolic compounds determination were as mentioned in Chapter 3, Section 3.3.3.2.1. Flavonoids and phenolic compounds of apple skin were extracted according to the procedure described by Whale and Singh (2007) with some modifications as detailed in Chapter 3, Section 3.3.3.2.2. The extracted samples were identified by comparing their retention times with authentic standards and quantified using high performance liquid chromatography (HPLC) system (Waters Corp., Milford, Mass., USA) as described in Chapter 3, Section 3.3.3.2.3 and 3.3.3.2.4. Flavonoids and other phenolic compounds were re-confirmed using HPLC-electrospray ionization mass spectrometry (HPLC-ESI-MS) as outlined in Chapter 3, Section 3.3.3.2.3.

5.2.8. Other fruit quality parameters

5.2.8.1. Fruit diameter

Fruit diameter was determined using a digital vernier calliper as explained in Chapter 3, Section 3.4.1.

5.2.8.2. Fruit firmness

Fruit firmness was determined using an electronic pressure tester (Model EPT-1 pressure tester, Lake City Technical products Inc., Kelowna, BC, Canada) as outlined in Chapter 3, Section 3.4.2.

5.2.8.3. Titratable acidity, soluble solids concentration and SSC/TA ratio

Titratable acidity (TA) was determined according to the method outlined in Chapter 3, Section 3.4.3. A soluble solids concentration (SSC) was recorded as described in Chapter 3, Section 3.4.4. SSC/TA ratio was calculated as detailed in Chapter 3, Section 3.4.5.

5.2.9. Determination of ascorbic acid, total antioxidants, individual sugars and organic acids

5.2.9.1. Ascorbic acid

The concentration of ascorbic acid from pulp was determined following the method of Jagota and Dani (1982) and Malik and Singh. (2005) with some modifications as outlined in Chapter 3, Section 3.5.1.

5.2.9.2. Total antioxidants

Total antioxidants in apple skin and pulp were determined as according to the method detailed by Brand-Williams et al. (1995) and Khan et al. (2008) as outlined in Chapter 3, Section 3.5.2.

5.2.9.3. Individual sugars

Chemicals used for individual sugars determination as detailed in Chapter 3, Section 3.5.3.1. Apple pulp were homogenized using a hand blender (Model HB95, Breville Pty. Ltd., New South Wales, Australia) as detailed in Chapter 3, Section 3.5.3.2. Fructose, sucrose and sorbitol were separated, identified and quantified using the HPLC system (Waters Corp., Milford, Mass., USA) fixed with the Fast Carbohydrate Analysis column (Aminex-HPX 87C, 100 x 7.8 mm) (Bio-Rad Laboratories, Hercules, CA, USA) described in Chapter 3, Section 3.5.3.3. The concentration of total sugars was the cumulative of individual sugars such as concentration of fructose, sucrose and sorbitol.

5.2.9.4. Individual organic acids

Chemicals used for individual organic acids determination as described in Chapter 3, Section 3.5.3.1. The homogenate apple pulp using a hand blender (Model HB95, Breville Pty. Ltd., New South Wales, Australia) were then centrifuged and filtered as described in Chapter 3, Section 3.5.3.2. Individual organic acids such as malic, citric, fumaric, shikimic, succinic and tartaric acid in apple juice were separated, identified and quantified using the HPLC system (Waters Corp., Milford, Mass., USA) fixed with a Bio Rad Aminex-HPX 87H (300 x 7.8 mm; particle size 9 µm) (Bio-Rad Laboratories, Inc., Hercules, USA) column as detailed in Chapter 3, Section 3.5.3.3.

5.2.10. Statistical analysis

The data were subjected to the analysis of variance (ANOVA) using GLM (General Linear Models) procedures with SAS 9.1 software package, SAS Institute Inc, Cary, NC, USA. The treatment effects on various parameters including the effects of treatment, storage period and their interaction were assessed within ANOVA. Treatments means were further separated by LSD for least significance at $P \leq 0.05$ (SAS Institute Inc., 1999). To ensure the validity of analysis, all the assumptions of analysis were checked. The data of two years were not pooled because error means squares over years were heterogenous.

5.3. Results

5.3.1. Weather conditions

Weather during 2007-08 was wetter especially towards the commercial harvest date compared to the previous year (2006-07) (Figure 5.1). During 65 days experiment (February, March, April and May) rainfall total were 1.33 mm and 2.57 mm in 2006-07 and 2007-08 growing season respectively, whereas the corresponding cumulative evapotranspiration (ET) was 6.19 mm (2006-07) and 5.34 mm (2007-08). Cumulative day and night temperatures for 65 days experimental period were slightly higher (23.4°C and 14.7°C, respectively) in 2006-07 than in 2007-08 (21.7°C and 13.7°C, respectively) (Figure 5.1A and 5.1B).

5.3.2. Effects of WHI on plant water relations

In 2006-07, WHI treatments, WHI-1 and WHI-2 were resumed after 18 and 30 days, respectively due to Ψ_{stem} drop below -2.5 MPa whereas, in 2007-08, only WHI-1 was restored to full irrigation after 20 days without water.

In 2006-07, θ in CI trees at both depths remain constant throughout the season, above 24.1% (Figure 5.2). Withholding irrigation treatments (WHI-1 and WHI-2) resulted in significantly ($P \leq 0.05$) reduced θ (range from 0.41 to 9.27% and 0.47 to 13.5%, respectively) at both depths as compared to CI. WHI-1 treatment resulted in lower θ at the beginning of withholding irrigation; 0.41% (200-300 mm, on 146 DAFB) and 2.65% (400-500 mm, on 146 DAFB) and increased rapidly towards the end of experimental period after been restored full irrigation on 153 DAFB. Similar trend

was recorded for WHI-2 and WHI-3, but WHI-2 been restored to full irrigation on 175 DAFB.

In 2007-08, a higher rate of rainfall started at 163 DAFB and remains wet until commercial harvest compared to previous year. The WHI-1 exhibited significantly ($P \leq 0.05$) decreased of θ at both depths after 10 days without water and remained constant until 156 DAFB, and then increased rapidly after restored to full irrigation. Withheld irrigation, WHI-2 showed the decreasing trends of θ at both depths starting on 156 [11.5% (200-300 mm) and 6.30% (400-500 mm)] until 166 DAFB [10.6% (200-300 mm) and 4.00% (400-500 mm)] and then increased instantly due to the higher incidence of rainfall till commercial harvest. A rapid rise of θ for all treatments at both depths was recorded after 166 DAFB, above 19.6% (200-300 mm) and 11.9% (400-500 mm) (Figure 5.3).

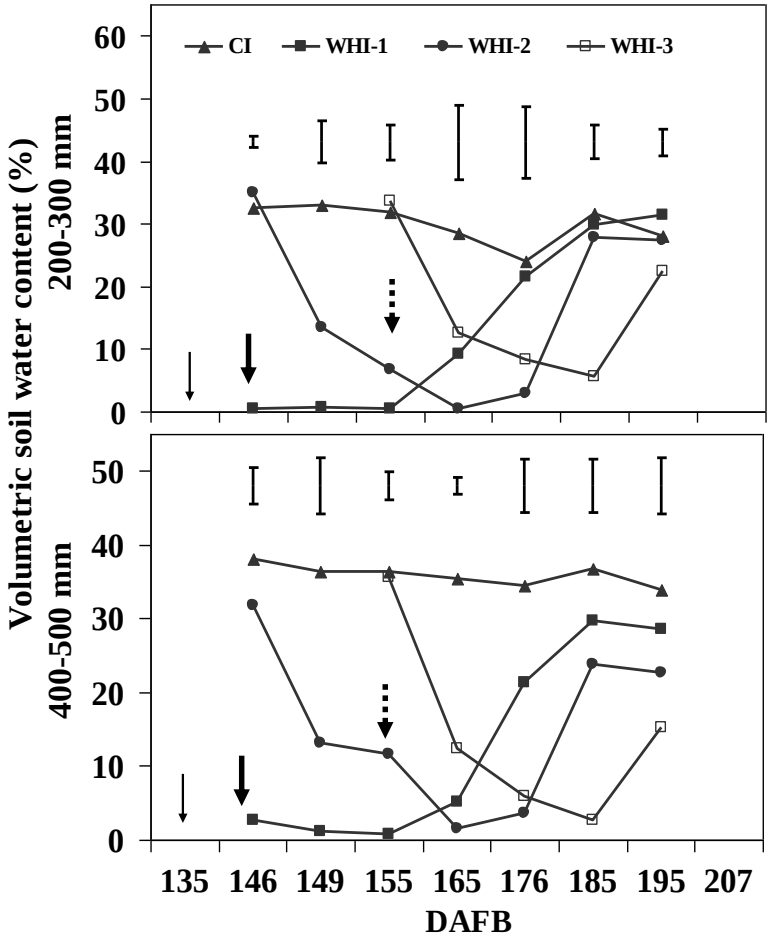

Figure 5.2. Changes in volumetric soil water content (θ) at different soil depths influenced by different WHI treatments during 2006-07 growing season. Vertical bar represent LSD at $P \leq 0.05$. The treatments were i) CI, commercial irrigation, ii) WHI-1, 135 - 153 DAFB, iii) WHI-2, 145 - 175 DAFB, iv) WHI-3, 155 - 200

DAFB. Arrows indicate the start of WHI. Thin arrow = WHI-1, thick arrow = WHI-2, dashed arrow = WHI-3.

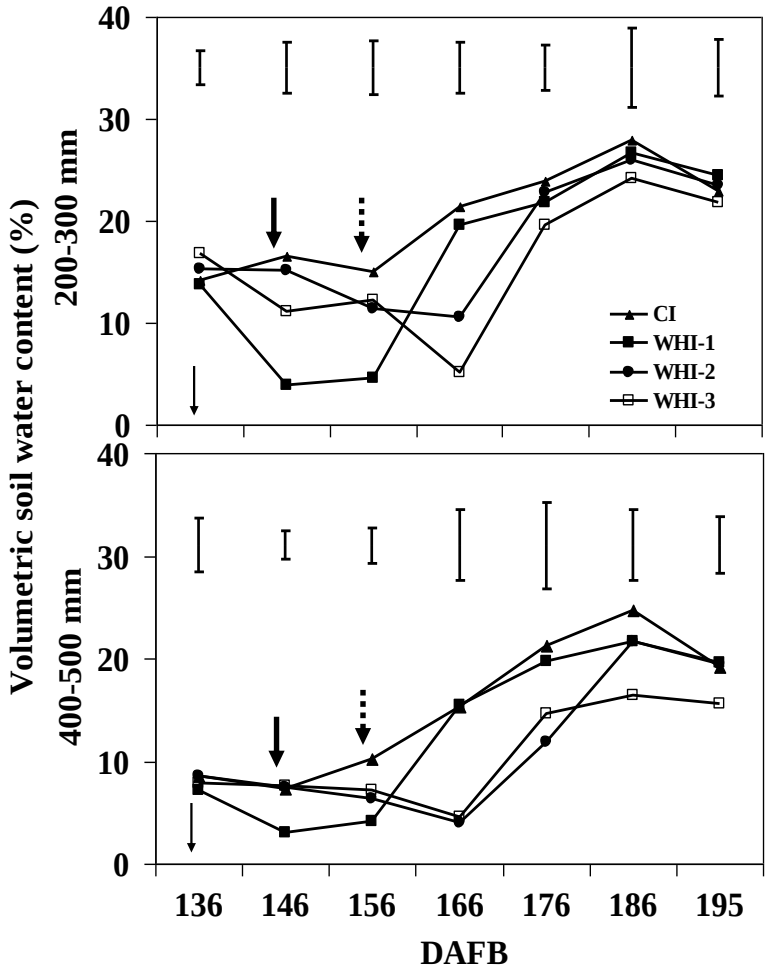

Figure 5.3. Changes in volumetric soil water content (θ) at different soil depths influenced by different WHI treatments during 2007-08 growing season. Vertical bar represent LSD at $P \leq 0.05$. The treatments were i) CI, commercial irrigation, ii) WHI-1, 135 - 155 DAFB, iii) WHI-2, 145 - 200 DAFB, iv) WHI-3, 155 - 200 DAFB. Arrows indicate the start of WHI. Thin arrow = WHI-1, thick arrow = WHI-2, dashed arrow = WHI-3.

In 2006-07, Ψ_{stem} and Ψ_{leaf} were recorded after two weeks of stopping irrigation due to the failure of pressure chambers. The lower Ψ_{stem} and Ψ_{leaf} (-3.18 and -2.48 MPa, respectively) recorded on 149 DAFB with the assumption both parameters were at the same level as CI at the beginning of withholding irrigation commenced (Figure 5.4A and 5.5A). The WHI-2 treatment (145 - 175 DAFB) resulted in a significantly ($P \leq 0.05$) reduced Ψ_{stem} and Ψ_{leaf} and reached the lowest values on 176 DAFB, -3.20 MPa and -3.63 MPa, respectively as compared to CI. After restored to full irrigation, Ψ_{stem} and Ψ_{leaf} increased rapidly on 185 DAFB and remained at similar level to CI. The consistent Ψ_{stem} and Ψ_{leaf} in CI trees were observed throughout the seasons.

During 2007-08, WHI-1 resulted in significantly ($P \leq 0.05$) decreased Ψ_{stem} and Ψ_{leaf} and reached the lowest values -3.32 MPa and 3.83 MPa, respectively on 155 DAFB as compared to CI (Figure 5.4B and 5.5B). Lower Ψ_{stem} (-2.58 MPa) and Ψ_{leaf} (-3.15 MPa) were also recorded in WHI-2 on 155 DAFB as compared to CI. The rapid increase Ψ_{stem} and Ψ_{leaf} in WHI-1 and WHI-2 on 165 DAFB were recorded and then remain above -0.89 and -1.70 MPa, respectively until 195 DAFB. However, Ψ_{stem} and Ψ_{leaf} in CI and WHI-3 had similar pattern throughout the season and were remained above -1.17 MPa and -2.75 MPa, correspondingly.

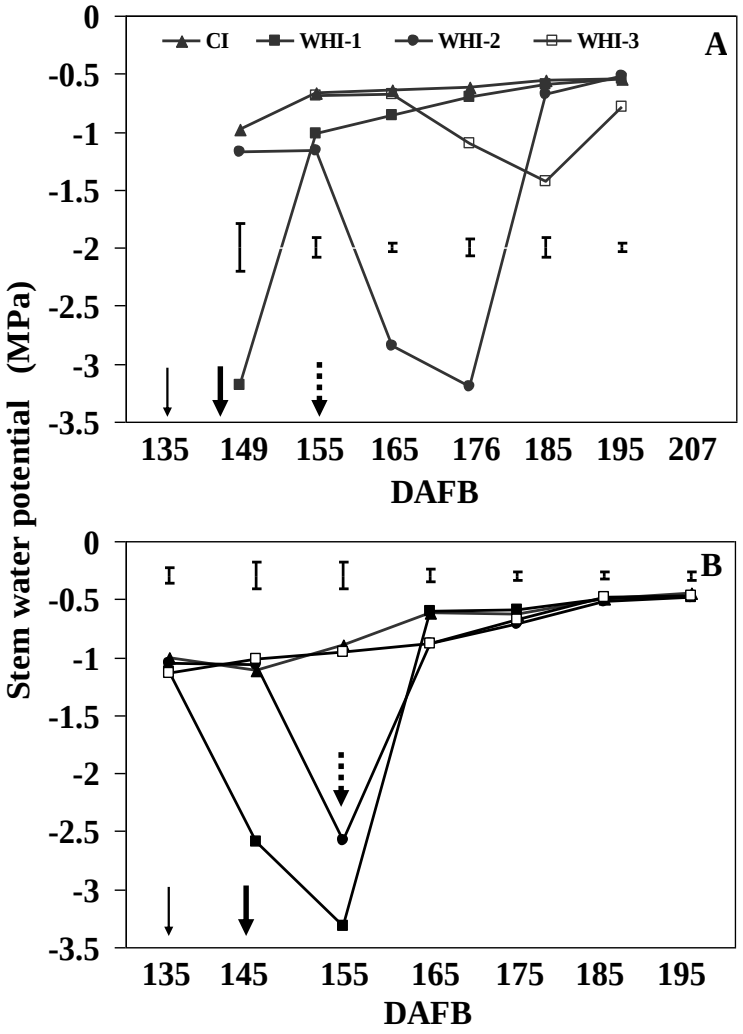

Figure 5.4. Changes in stem water potential in 'Cripps Pink' apple tree influenced by different WHI treatments during (A) 2006-07 and (B) 2007-08 growing seasons. Vertical bar represent LSD at $P \leq 0.05$. During 2006-07, the treatments were i) CI, commercial irrigation, ii) WHI-1, 135 - 153 DAFB, iii) WHI-2, 145 - 175 DAFB, iv) WHI-3, 155 - 200 DAFB. While, in 2007-08, the treatments were i) CI, ii) WHI-1, 135 - 155 DAFB, iii) WHI-2, 145 - 200 DAFB, iv) WHI-3, 155 - 200 DAFB. Arrows indicate the start of WHI. Thin arrow = WHI-1, thick arrow = WHI-2, dashed arrow = WHI-3.

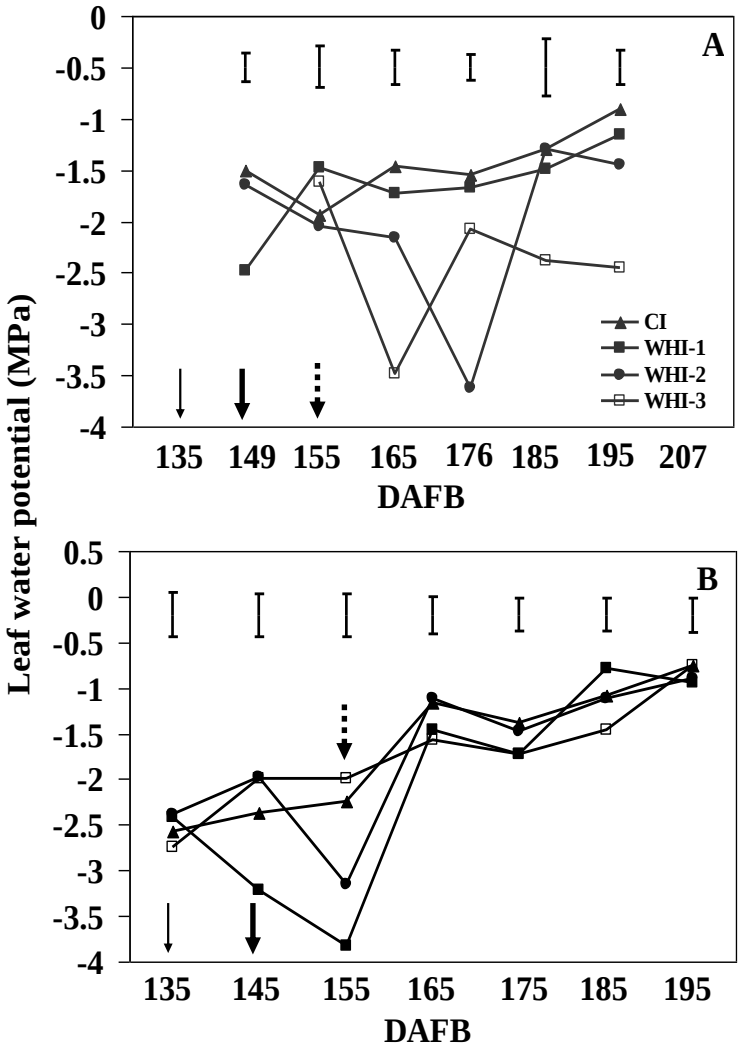

Figure 5.5. Changes in leaf water potential in 'Cripps Pink' apple tree influenced by different WHI treatments during (A) 2006-07 and (B) 2007-08 growing seasons. Vertical bar represent LSD at $P \leq 0.05$. During 2006-07, the treatments were i) CI, commercial irrigation, ii) WHI-1, 135 - 153 DAFB, iii) WHI-2, 145 - 175 DAFB, iv) WHI-3, 155 - 200 DAFB. While, in 2007-08, the treatments were i) CI, ii) WHI-1, 135 - 155 DAFB, iii) WHI-2, 145 - 200 DAFB, iv) WHI-3, 155 - 200 DAFB. Arrows indicate the start of WHI. Thin arrow = WHI-1, thick arrow = WHI-2, dashed arrow = WHI-3.

In both consecutive years, g_s in WHI treatments were lower (<200 mmol·m^{-2}s^{-1}) as compared to measurements by (Andersen, 1991), (≥200 mmol·m^{-2}s^{-1}). In 2006-07, a g_s reduction in WHI trees was closely related with the decrease in θ, Ψ_{stem}, Ψ_{leaf} (Figure 5.6A). The g_s was significantly ($P \leq 0.05$) reduced in WHI-1 and WHI-2 as compared CI. The lowest g_s recorded on 155 DAFB (58.0 mmol·m^{-2}s^{-1}) and 176 DAFB (38.4 mmol·m^{-2}s^{-1}) in WHI-1 and WHI-2, correspondingly. On restoring irrigation in WHI-1 and WHI-2, there was an increased in g_s reaching the same level as CI and WHI-3 on 165 DAFB and 185 DAFB, respectively. In the meantime, g_s in CI and WHI-3 were at the same pattern until the end of the experimental period. In

2007-08, WHI-1 and WHI-2 resulted in significant ($P \leq 0.05$) lower g_s as compared to in CI and WHI-3 on 146 and 156 DAFB (Figure 5.6B). Both treatments experienced a reduction of g_s after withheld irrigation and then gradually increased greater than CI. The fluctuation in g_s was recorded in CI and WHI-3 throughout the season.

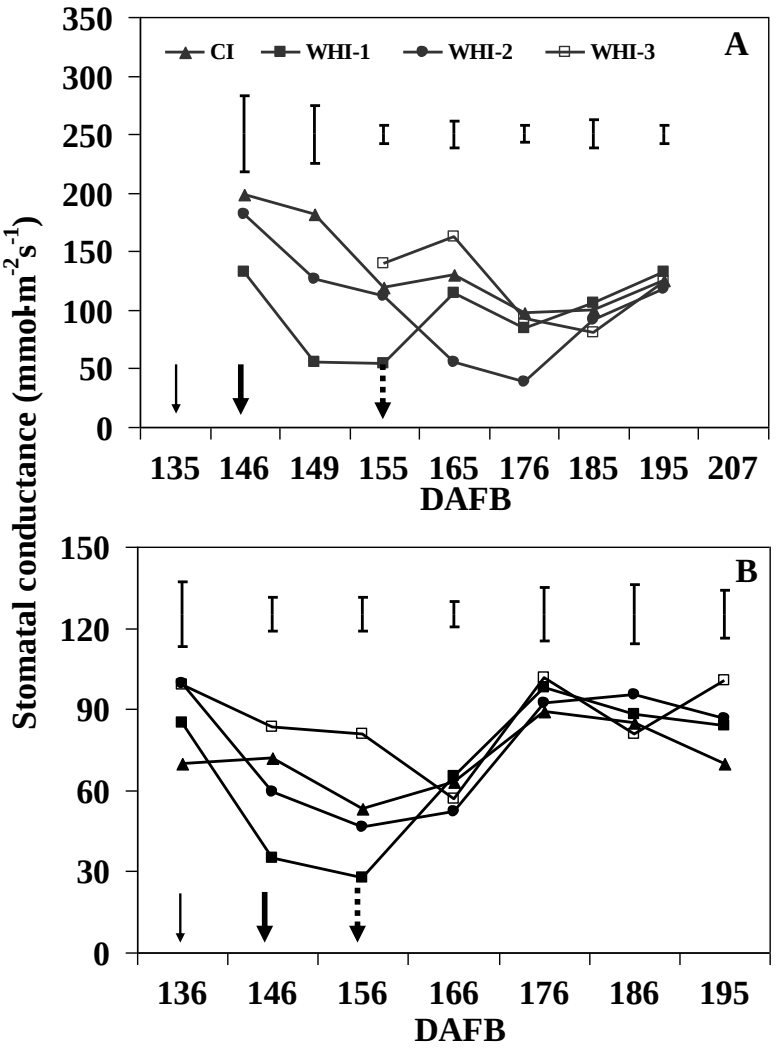

Figure 5.6. Changes in leaf stomatal conductance (g_s) in 'Cripps Pink' apple tree influenced by different WHI treatments during (A) 2006-07 and (B) 2007-08 growing seasons. Vertical bar represent LSD at $P \leq 0.05$. During 2006-07, the treatments were i) CI, commercial irrigation, ii) WHI-1, 135 - 153 DAFB, iii) WHI-2, 145 - 175 DAFB, iv) WHI-3, 155 - 200 DAFB. While, in 2007-08, the treatments were i) CI, ii) WHI-1, 135 - 155 DAFB, iii) WHI-2, 145 - 200 DAFB, iv) WHI-3, 155 - 200 DAFB. Arrows indicate the start of WHI. Thin arrow = WHI-1, thick arrow = WHI-2, dashed arrow = WHI-3.

Fruit drop in 2006-07 recorded after one week application of withholding irrigation. WHI treatments exhibited a significantly ($P \leq 0.05$) influence in fruit drop only on 176 and 185 DAFB (Figure 5.7A). But, in 2007-08, fruit drop was not differing among WHI treatments from time to time (Figure 5.7B).

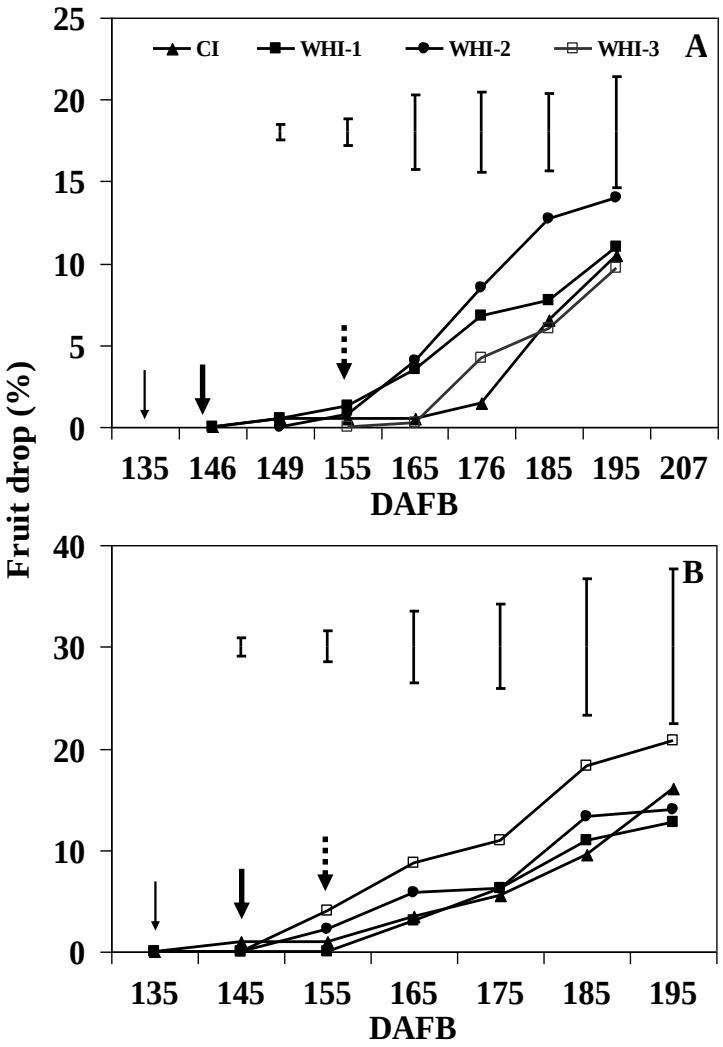

Figure 5.7. Changes in fruit drop in 'Cripps Pink' apple influenced by different WHI treatments during fruit development (A) 2006-07 and (B) 2007-08 growing seasons. Vertical bar represent LSD at $P \leq 0.05$. During 2006-07, the treatments were i) CI, commercial irrigation, ii) WHI-1, 135 - 153 DAFB, iii) WHI-2, 145 - 175 DAFB, iv) WHI-3, 155 - 200 DAFB. While, in 2007-08, the treatments were i) CI, ii) WHI-1, 135 - 155 DAFB, iii) WHI-2, 145 - 200 DAFB, iv) WHI-3, 155 - 200 DAFB. Arrows indicate the start of WHI. Thin arrow = WHI-1, thick arrow = WHI-2, dashed arrow = WHI-3.

5.3.3. Effects of WHI on fruit colour and total anthocyanins concentration

In 2006-07, average fruit surface and percentage fruit with more than 40% red blush on the surface were significantly ($P \leq 0.05$) higher with WHI-1 (135 - 153 DAFB, 18 days without water) and WHI-2 (145 - 175 DAFB, 30 days without water) as

compared to CI and WHI-3 (Table 5.1). Similarly, in 2007-08, WHI treatments (WHI-1, 20 days and WHI-2, approximately 10 days without water) significantly ($P \leq 0.05$) enhanced percentage red blush in fruit surface (60.8% and 54.3%, respectively) and percentage fruit for export (97.0% and 85.0%, respectively) as compared to the all other treatments (Table 5.1). In general, WHI treatments (WHI-1 and WHI-2) in both seasons resulted in significantly ($P \leq 0.05$) increased concentration of total anthocyanins in exposed and shaded sides of fruit skin as compared to all other treatments. However, in 2006-07, CI showed a comparable concentration of total anthocyanins in the ES of fruit skin to WHI-1 and WHI-2 treatments. WHI-3 in both seasons exhibited lower visual colour, percentage fruit meeting for export criteria and concentration of total anthocyanins in the SS of fruit skin.

5.3.4. Effects of WHI on chromaticity value a*, b*, lightness, hue angle and chroma

A reduction in chromaticity value b*, hue angle (h°) and lightness (L*) indicates redder fruit skin colour. While, higher chromaticity value a* and chroma (C*) denotes an increase in red skin colour. In both years, chromaticity value a* on both sides of the fruit skin in WHI-1 and WHI-2 were significantly ($P \leq 0.05$) higher as compared to all other treatments (Table 5.2). The highest chromaticity a* on the ES and SS of the fruit skin was recorded in WHI-2 (25.0 a* and 13.6 a*, respectively) in 2006-07 and WHI-1 (25.3 a* and 18.5 a*, respectively) in 2007-08. Chromaticity b* on the ES was not significantly affected with WHI treatments during both seasons. However, WHI-2 in 2006-07 and WHI-1 in 2007-08 resulted in significantly ($P \leq 0.05$) lower chromaticity value b* (23.5 b* and 21.9 b*, correspondingly) on the SS of apple skin. The WHI treatments significantly ($P \leq 0.05$) reduced hue angle (h°), lightness (L*) and increased chroma (C*) on the both sides of fruit skin as compared to other treatments (Table 5.3). The treatments, WHI-2 during 2006-07 and WHI-1 in 2007-08 exhibited the lowest h° (32.4 h° and 35.4 h°, respectively), L* (23.7 L* and 29.0 L*, respectively) and highest C* (29.8 C* and 31.2 C*, respectively) on the ES and SS (52.5 h° and 50.2 h°, respectively; 35.1 L* and 35.4 L*, respectively; 27.8 C* and 29.0 C*, respectively) on the fruit skin.

Table 5.1. Effects of different WHI treatments on visual colour, percentage fruit with red blush of more than 40% and concentration of total anthocyanins in the exposed (ES) and shaded (SS) sides of apple skin during 2006-07 and 2007-08.

Treatment	Visual colour (% red blush)		Percentage fruit with > 40% red blush		Total anthocyanins ($\mu g \cdot g^{-1}$ FW)			
					ES		SS	
	2006-07	2007-08	2006-07	2007-08	2006-07	2007-08	2006-07	2007-08
CI	56.8 bc	40.2 b	85.0 ab	55.0 b	194.6 a	185.1 b	57.1 bc	108.8 ab
WHI-1	67.1 ab	60.8 a	95.0 a	97.0 a	214.6 a	225.4 a	106.2 ab	144.9 a
WHI-2	74.3 a	54.3 a	97.0 a	85.0 a	224.7 a	226.4 a	156.8 a	144.9 a
WHI-3	45.7 c	41.8 b	66.0 b	52.0 b	136.3 b	208.8 a	31.9 c	100.9 b
LSD ($P \leq 0.05$)	12.6	8.24	24.6	18.3	41.4	17.9	51.7	41.5

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. During 2006-07, the treatments were i) CI, commercial irrigation, ii) WHI-1, 135 - 153 DAFB, iii) WHI-2, 145 -175 DAFB, iv) WHI-3, 155 - 200 DAFB. While, in 2007-08, the treatments were i) CI, ii) WHI-1, 135 - 155 DAFB, iii) WHI-2, 145 - 200 DAFB, iv) WHI-3, 155 - 200 DAFB.

Table 5.2. Effects of different WHI treatments on chromaticity value a* and b* on the exposed (ES) and shaded (SS) sides of fruit skin during 2006-07 and 2007-08.

Treatment	Exposed side (ES)				Shaded side (ES)			
	a*		b*		a*		b*	
	2006-07	2007-08	2006-07	2007-08	2006-07	2007-08	2006-07	2007-08
CI	23.4 a	19.3 b	16.8	18.6	6.99 c	7.34 c	26.7 a	26.0 a
WHI-1	24.0 a	25.3 a	16.9	17.9	10.4 b	18.5 a	25.6 a	21.9 b
WHI-2	25.0 a	23.4 a	15.7	17.7	13.6 a	15.4 b	23.5 b	22.1 b
WHI-3	18.2 b	19.6 b	17.5	18.9	5.03 c	8.47 c	26.2 a	25.0 a
LSD ($P \leq 0.05$)	3.71	2.33	NS (0.68)	NS (0.58)	3.15	2.85	1.97	1.32

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. NS = not significantly different at $P \leq 0.05$. Values within brackets represent standard error of means. During 2006-07, the treatments were i) CI, commercial irrigation, ii) WHI-1, 135 - 153 DAFB, iii) WHI-2, 145 -175 DAFB, iv) WHI-3, 155 - 200 DAFB. While, in 2007-08, the treatments were i) CI, ii) WHI-1, 135 - 155 DAFB, iii) WHI-2, 145 - 200 DAFB, iv) WHI-3, 155 - 200 DAFB.

Table 5.3. Effects of different WHI treatments on hue angle, lightness and chroma in the exposed (ES) and shaded (SS) sides of fruit skin during 2006-07 and 2007-08.

Treatment	Exposed side (ES)						Shaded side (SS)					
	Hue angle (^{o}h)		Lightness (L*)		Chroma (*C)		Hue angle (^{o}h)		Lightness (L*)		Chroma (*C)	
	2006-07	2007-08	2006-07	2007-08	2006-07	2007-08	2006-07	2007-08	2006-07	2007-08	2006-07	2007-08
CI	36.1 ab	46.1 a	25.4 ab	33.1 a	29.2 a	28.2 c	74.8 ab	73.9 a	39.1 a	42.50 a	28.3 a	27.6 b
WHI-1	35.4 b	35.4 b	25.4 ab	29.0 c	29.6 a	31.2 a	67.6 b	50.2 b	37.2 a	35.40 c	28.4 a	29.0 a
WHI-2	32.4 b	37.5 b	23.7 c	29.2 c	29.8 a	30.0 b	52.5 c	55.5 b	35.1 b	36.18 c	27.8 ab	27.5 b
WHI-3	44.6 a	44.6 a	28.1 a	31.2 b	26.1 b	27.9 c	81.2 a	70.9 a	39.1 a	39.08 b	27.1 b	27.1 b
LSD ($P \leq 0.05$)	8.66	5.46	3.15	1.70	1.13	0.98	11.9	6.31	1.85	1.63	1.08	0.91

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. During 2006-07, the treatments were i) CI, commercial irrigation, ii) WHI-1, 135 - 153 DAFB, iii) WHI-2, 145 - 175 DAFB, iv) WHI-3, 155 - 200 DAFB. While, in 2007-08, the treatments were i) CI, ii) WHI-1, 135 - 155 DAFB, iii) WHI-2, 145 - 200 DAFB, iv) WHI-3, 155 - 200 DAFB.

5.3.5. Effects of WHI on the concentrations of flavonoids and other phenolic compounds in fruit skin

As explained earlier, nine compounds of polyphenolics in 'Cripps Pink' apple skin were identified and confirmed using HPLC-ESI-MS as detailed in Chapter 4, Section 4.3.3.3. WHI treatments significantly ($P \leq 0.05$) affected the concentration of anthocyanins (cyanidin 3-*O*-galactoside) and hydroxycinnamic acid (chlorogenic acid) in the fruit skin in both consecutive years (Table 5.4). However, the concentration of hydrochalcones (phloridzin) and total quercetin glycosides were significantly ($P \leq 0.05$) affected with WHI treatments in 2006-07 only. The higher concentrations of cyanidin 3-*O*-galactoside were recorded in WHI-1 and WHI-2 treatments as compared to other treatments in both seasons. The highest concentrations of chlorogenic acid (321.0 $\mu g \cdot g^{-1}$ FW and 315.4 $\mu g \cdot g^{-1}$ FW, respectively) were recorded in WHI-2 in 2006-07 and WHI-1 during 2007-08. The WHI treatment (WHI-1, 2006-07) resulted in higher concentration of phloridzin (75.2 $\mu g \cdot g^{-1}$ FW) as compared to CI. While, the WHI treatments (WHI-1 and WHI-2, 2006-07) showed higher concentration of total quercetin glycosides (2682.0 $\mu g \cdot g^{-1}$ FW and 2471.3 $\mu g \cdot g^{-1}$ FW, respectively) as compared to other treatments. The individual quercetin glycosides identified in the fruit skin were quercetin 3-*O*-rutinoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-xyloside, quercetin 3-*O*-arabinoside and quercetin 3-*O*-rhamnoside. In 2006-07, WHI treatments significantly ($P \leq 0.05$) affected the concentrations of all individual quercetin glycosides excluding quercetin 3-*O*-glucoside (Table 5.5). WHI-1 and WHI-2 treatments resulted in higher concentrations of quercetin 3-*O*-rutinoside, quercetin 3-*O*-galactoside and quercetin 3-*O*-arabinoside in the fruit skin as compared to CI. While, WHI-1 resulted in the highest concentration of quercetin 3-*O*-xyloside (519.5 $\mu g \cdot g^{-1}$ FW) and quercetin 3-*O*-rhamnoside (234.6 $\mu g \cdot g^{-1}$ FW) as compared to CI. In contrast, during 2007-08, all individual compounds of quercetin glycosides were not significantly affected with WHI treatments.

Table 5.4. Effects of different WHI treatments on flavonoids and other phenolic compounds in the fruit skin during 2006-07 and 2007-08.

Treatment	Flavonoids and other phenolic compounds (μg·g^{-1} FW)							
	Cyanidin 3-*O*-galactoside		Chlorogenic acid		Phloridzin		Quercetin glycosides	
	2006-07	2007-08	2006-07	2007-08	2006-07	2007-08	2006-07	2007-08
CI	158.4 b	213.6 b	221.4 c	237.4 bc	43.0 c	40.5	1263.9 b	730.5
WHI-1	438.6 a	357.0 a	284.0 b	315.4 a	75.2 a	49.5	2682.0 a	806.8
WHI-2	534.8 a	378.4 a	321.0 a	281.9 ab	65.7 ab	55.1	2471.3 a	1029.0
WHI-3	167.5 b	149.5 b	195.7 c	220.1 c	53.1 bc	48.0	1651.3 b	962.9
LSD ($P \leq 0.05$)	142.1	142.5	33.5	58.4	13.9	NS (6.53)	780.0	NS (268.8)

Means followed by the same letter within column are not significantly different at $P \leq 0.05$.Values within brackets represent standard error of means. NS = not significantly different at $P \leq 0.05$. During 2006-07, the treatments were i) CI, commercial irrigation, ii) WHI-1, 135-153 DAFB, iii) WHI-2, 145 - 175 DAFB, iv) WHI-3, 155 - 200 DAFB. While, in 2007-08, the treatments were i) CI, ii) WHI-1, 135 - 155 DAFB, iii) WHI-2, 145 - 200 DAFB, iv) WHI-3, 155 - 200 DAFB.

Table 5.5. Effects of different WHI treatments on individual quercetin glycosides compound in the fruit skin during 2006-07 and 2007-08.

Treatment	Quercetin glycosides (μg·g^{-1} FW)											
	Quercetin 3-*O*-rutinoside		Quercetin 3-*O*-galactoside		Quercetin 3-*O*-glucoside		Quercetin 3-*O*-xyloside		Quercetin 3-*O*-arabinoside		Quercetin 3-*O*-rhamnoside	
	2006-07	2007-08	2006-07	2007-08	2006-07	2007-08	2006-07	2007-08	2006-07	2007-08	2006-07	2007-08
CI	39.5 b	12.0	317.0 b	266.9	80.3	32.8	341.4 b	159.8	317.0 b	151.2	168.8 b	107.9
WHI-1	89.8 a	10.5	1140.7 a	307.2	123.5	30.8	519.5 a	171.0	574.0 a	177.7	234.6 a	109.5
WHI-2	91.8 a	26.1	1029.6 a	406.9	109.5	45.0	451.9 ab	196.6	577.5 a	234.2	211.0 ab	120.2
WHI-3	53.3 ab	16.6	660.5 b	378.2	89.8	50.7	334.7 b	202.7	334.8 b	182.9	178.2 b	131.8
LSD ($P \leq 0.05$)	48.4	NS (9.81)	366.86	NS (130.1)	NS (16.6)	NS (13.5)	138.4	NS (44.7)	159.7	NS (51.9)	53.8	NS (20.3)

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. Values within brackets represent standard error of means. NS = not significantly different at $P \leq 0.05$. During 2006-07, the treatments were i) CI, commercial irrigation, ii) WHI-1, 135 - 153 DAFB, iii) WHI-2, 145 -175 DAFB, iv) WHI-3, 155 - 200 DAFB. While, in 2007-08, the treatments were i) CI, ii) WHI-1, 135 - 155 DAFB, iii) WHI-2, 145 - 200 DAFB, iv) WHI-3, 155 - 200 DAFB.

5.3.6. Effects of WHI fruit diameter, firmness, titratable acidity, soluble solids concentration and SSC/TA ratio

In 2006-07, the fruit size was not affected on 146 and 149 DAFB only with withholding irrigation treatments (WHI-1 and WHI-2) (Figure 5.8A). No apparent reduction was recorded in final fruit size with WHI-1 (70.4 mm) and WHI-2 (69.5 mm) treatments as compared to CI (71.7 mm). In 2007-08, WHI-1, WHI-2 and CI treatments resulted in the comparable final fruit size (71.9 mm, 72.3 mm and 73.8 mm, respectively) (Figure 5.8B). The smallest fruit diameter (71.9 mm) was observed in WHI-1 treatment.

In both seasons, the WHI treatments significantly ($P \leq 0.05$) affected firmness and SSC (Table 5.6). But, TA in WHI treatments was significantly ($P \leq 0.05$) affected only in 2007-08. The treatments, WHI-2 during 2006-07 and WHI-1 in 2007-08 were significantly ($P \leq 0.05$) increased fruit firmness (86.9 N and 96.5 N, respectively) as compared to CI and other treatments. CI fruit exhibited the lowest fruit firmness (range from 78.7 to 85.6 N) in both years. WHI for 30 days (WHI-2, 145 - 175 DAFB) showed the highest SSC (16.7%) as compared to CI (15.1%) fruit during 2006-07, while WHI-1 (20 days, 135-155 DAFB) in 2007-08 exhibited a significantly ($P \leq 0.05$) increased SSC (16.2%) as compared to other treatments. The lowest SSC found in CI (2006-07) and WHI-3 (2007-08) (15.1% and 14.2%, correspondingly). Withholding irrigation for 20 days (WHI-1, 135 - 155 DAFB) during 2007-08 significantly ($P \leq 0.05$) increased TA (0.91% malic acid) as compared other treatments. SSC/TA ratio was not significantly affected by WHI treatments in both years.

5.3.7. Effects of WHI on concentration of ascorbic acid and total antioxidants

WHI treatments significantly ($P \leq 0.05$) affected the concentrations of ascorbic acid in 2006-07, but no significant effects were recorded in 2007-08 (Table 5.7). WHI-1 (135 - 153 DAFB) and WHI-2 (145 - 175 DAFB) treatments significantly ($P \leq 0.05$) increased concentrations of ascorbic acid (7.21 and 7.09 $mg \cdot 100g^{-1}$ FW) as compared to CI and WHI-3. The WHI treatments significantly ($P \leq 0.05$) affected total antioxidants on the ES of apple skin during 2007-08 only, where WHI-1 (135 - 155 DAFB) showed the highest total antioxidants (32.5 mM $TE \cdot g^{-1}$ FW) as compared to all other treatments. No significant effects were observed in total antioxidants on the

SS of the fruit skin during both years. But, total antioxidants in the pulp were significantly ($P \leq 0.05$) affected with WHI treatments in both years. The higher total antioxidants were recorded in WHI-1 (range from 0.43 to 0.64 mM TE·g^{-1} FW) as compared to all other treatments during both seasons.

5.3.8. Effects of WHI on the concentrations of total sugars and organic acids

Total sugars changed significantly ($P \leq 0.05$) with WHI treatments in both years (Table 5.8). The treatments, WHI-2 in 2006-07 (129.2 g·kg-^{1}) and WHI-1 in 2007-08 (117.5 g·kg-^{1}) resulted in highest concentrations of total sugars as compared to all other treatments. Malic acid was the predominant single compounds in total organic acids, closely followed by succinic, citric and tartaric in the fruit of this cultivar. Individual organic acids and total acids were significantly ($P \leq 0.05$) affected with WHI treatments in both years excluding citric acid during 2007-08 (Table 5.8). Malic acid concentrations during both years were significantly ($P \leq 0.05$) changed with WHI-3 treatment. The higher malic acid concentrations were recorded in WHI-1 and WHI-2 during 2006-07 and WHI-1 (2007-08) as compared to WHI-3. In 2006-07, the treatments, WHI-1 and WHI-2 resulted in the higher citric acid concentration (0.20 g·kg^{-1} and 0.21 g·kg^{-1}, respectively) as compared to CI and WHI-3. In general, the higher tartaric acid concentrations were recorded in WHI-1 in both seasons. The concentration of succinic acid was higher in WHI-3 (2006-07) and WHI-1 (2007-08) as compared to all other treatments. Total acids concentration was higher in WHI-2 (2006-07, 10.0 g·kg^{-1}) and WHI-1 (2007-08, 6.90 g·kg^{-1}) as compared to WHI-3. In general, individual organic acids and total acids during 2006-07 experienced higher concentrations compared to 2007-08.

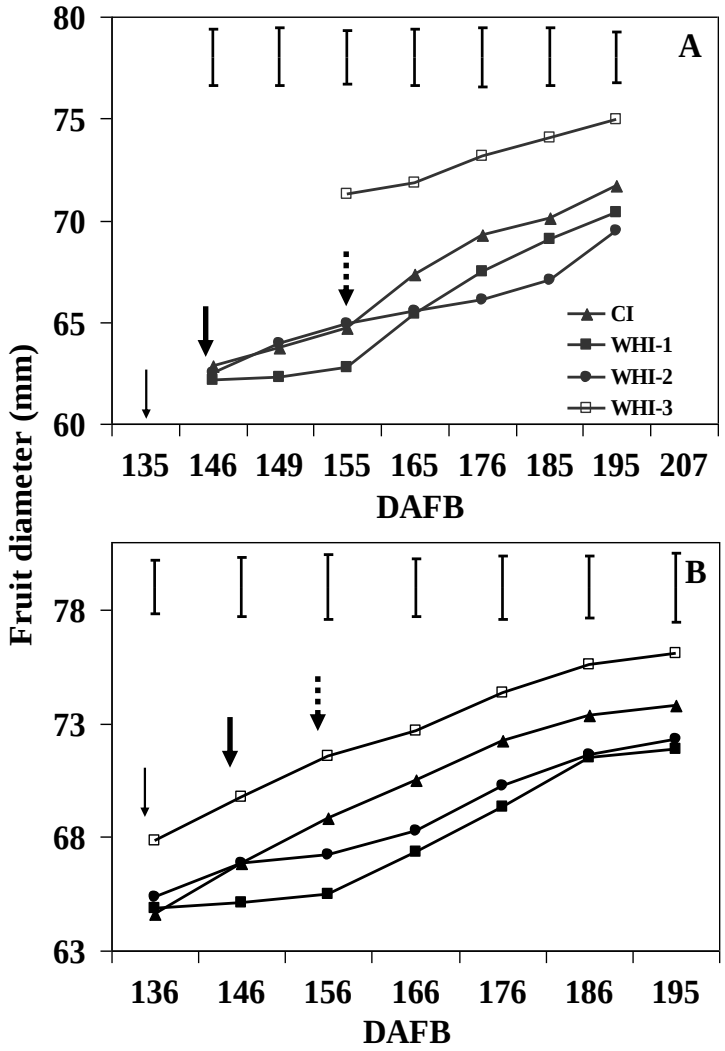

Figure 5.8. Changes in fruit diameter in 'Cripps Pink' apple tree influenced by different WHI treatments during fruit development (A) 2006-07 and (B) 2007-08 growing seasons. Vertical bar represent LSD at $P \leq 0.05$. During 2006-07, the treatments were i) CI, commercial irrigation, ii) WHI-1, 135 - 153 DAFB, iii) WHI-2, 145 - 175 DAFB, iv) WHI-3, 155 - 200 DAFB. While, in 2007-08, the treatments were i) CI, ii) WHI-1, 135 - 155 DAFB, iii) WHI-2, 145 - 200 DAFB, iv) WHI-3, 155 - 200 DAFB. Arrows indicate the start of WHI. Thin arrow = WHI-1, thick arrow = WHI-2, dashed arrow = WHI-3.

Table 5.6. Effects of different WHI treatments on fruit firmness, soluble solids concentration, titratable acidity and SSC/TA ratio during 2006-07 and 2007-08.

Treatment	Firmness (N)		SSC (%)		Titratable acidity (% malic acid)		SSC/TA ratio	
	2006-07	2007-08	2006-07	2007-08	2006-07	2007-08	2006-07	2007-08
CI	78.4 b	85.6 b	15.1 c	14.8 bc	0.67	0.83 b	24.8	17.8
WHI-1	82.8 ab	96.5 a	15.7 b	16.2 a	0.75	0.91 a	20.8	17.8
WHI-2	86.9 a	89.8 b	16.7 a	15.3 b	0.76	0.83 b	22.0	18.5
WHI-3	81.4 ab	88.0 b	14.2 d	14.6 c	0.66	0.78 b	21.7	18.7
LSD ($P \leq 0.05$)	8.47	4.81	0.62	0.62	NS (0.05)	0.06	NS (2.62)	NS (0.36)

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. NS = not significant at $P \leq 0.05$. Values within brackets represent standard error of means. During 2006-07, the treatments were i) CI, commercial irrigation, ii) WHI-1, 135 - 153 DAFB, iii) WHI-2, 145 - 175 DAFB, iv) WHI-3, 155 - 200 DAFB. While, in 2007-08, the treatments were i) CI, ii) WHI-1, 135 - 155 DAFB, iii) WHI-2, 145 - 200 DAFB, iv) WHI-3, 155 - 200 DAFB.

Table 5.7. Effects of different WHI treatments on concentration of ascorbic acid and total antioxidants in fruit skin and pulp during 2006-07 and 2007-08.

Treatment	Ascorbic acid ($mg \cdot 100g^{-1}$ FW)		Total antioxidants (mM TE.g^{-1} FW)					
			Skin (ES)		Skin (SS)		Pulp	
	2006-07	2007-08	2006-07	2007-08	2006-07	2007-08	2006-07	2007-08
CI	5.03 b	10.3	22.3	25.3 b	11.1	20.4	0.38 b	0.51 ab
WHI-1	7.21 a	11.6	27.5	32.5 a	11.5	22.7	0.43 a	0.64 a
WHI-2	7.09 a	11.2	27.3	30.9 ab	11.8	22.2	0.38 ab	0.56 ab
WHI-3	4.52 b	10.6	22.3	24.8 b	10.7	20.2	0.28 c	0.46 b
LSD ($P \leq 0.05$)	0.91	NS (0.42)	NS (3.78)	6.30	NS (0.80)	NS (1.28)	0.06	0.14

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. NS = not significant at $P \leq 0.05$. Values within brackets represent standard error of means. During 2006-07, the treatments were i) CI, commercial irrigation, ii) WHI-1, 135 - 153 DAFB, iii) WHI-2, 145 - 175 DAFB, iv) WHI-3, 155 - 200 DAFB. While, in 2007-08, the treatments were i) CI, ii) WHI-1, 135 - 155 DAFB, iii) WHI-2, 145 - 200 DAFB, iv) WHI-3, 155 - 200 DAFB.

Table 5.8. Effects of different WHI treatments on the concentration of total sugars, individual organic acids and total acids during 2006-07 and 2007-08.

Compound ($g \cdot kg^{-1}$)	Year	Treatment				LSD ($P \leq 0.05$)
		CI	WHI-1	WHI-2	WHI-3	
Total sugars	2006-07	119.2 b	119.2 b	129.2 a	111.3 c	5.03
	2007-08	111.8 b	117.5 a	113.2 b	112.1 b	3.70
Malic acid	2006-07	7.63 a	7.99 a	8.49 a	6.73 b	0.89
	2007-08	5.66 ab	6.17 a	5.73 ab	5.28 b	0.75
Citric acid	2006-07	0.18 b	0.20 a	0.21 a	0.17 b	0.017
	2007-08	0.05	0.06	0.06	0.05	NS (0.002)
Tartaric acid	2006-07	0.14 bc	0.18 a	0.16 ab	0.13 c	0.02
	2007-08	0.10 a	0.10 a	0.10 a	0.09 b	0.01
Succinic acid	2006-07	1.02 c	1.17 b	1.16 b	1.26 a	0.09
	2007-08	0.43 c	0.60 a	0.50 b	0.45 c	0.04
Total acids	2006-07	8.98 bc	9.53 ab	10.0 a	8.29 c	0.92
	2007-08	6.24 ab	6.90 a	6.39 ab	5.87 b	0.77

Means followed by the same letter within row are not significantly different at $P \leq 0.05$. NS = not significant at $P \leq 0.05$. Values within brackets represent standard error of means. During 2006-07, the treatments were i) CI, commercial irrigation, ii) WHI-1, 135 - 153 DAFB, iii) WHI-2, 145 -175 DAFB, iv) WHI-3, 155 - 200 DAFB. While, in 2007-08, the treatments were i) CI, ii) WHI-1, 135 - 155 DAFB, iii) WHI-2, 145 - 200 DAFB, iv) WHI-3, 155 - 200 DAFB.

5.3.9. Quality of cold stored fruit

5.3.9.1. Firmness, titratable acidity, soluble solids concentration and SSC/TA ratio

The interactions between WHI treatments and cold storage periods for SSC, TA and SSC/TA ratio were found to be non-significant (Table 5.9). Fruit from WHI-2 stored for 70 days showed higher firmness as compared to CI treatment. As storage period prolonged, fruit from all WHI treatments experienced the decreasing trends in fruit firmness (range from 5.60% to 12.4%), but remained firmer above the standard requirement for export >68 N. Following 140 days cold storage, the SSC was significantly ($P \leq 0.05$) higher in WHI-2 stored-fruit as compared to all other treatments. Fruit from WHI-1 and WHI-2 treatments significantly ($P \leq 0.05$) reduced TA as cold storage period prolonged to 140 days. In general, the SSC/TA ratio in all irrigation treatments showed an increasing trend as storage periods extended.

5.3.9.2. Concentration of ascorbic acid and total antioxidants

No significant interactions found between WHI treatments and cold storage periods for ascorbic acid concentration and total antioxidants in the apple skin and pulp (Table 5.9). All the WHI treatments resulted in significantly ($P \leq 0.05$) increased concentration of ascorbic acid with prolonged cold storage period up to 140 days. Fruit from WHI-2 treatment exhibited higher ascorbic acid concentration in stored-fruit following 140 days cold storage as compared to WHI-3 stored-fruit. Total antioxidants in both sides of fruit skin were not significantly affected with WHI treatments and storage periods. In general, all WHI treatments showed an increasing trend of total antioxidants in the apple pulp as storage period prolonged. Following 140 days cold storage, the higher total antioxidants in the apple pulp were 0.52 and 0.51 mM TE·g^{-1} FW recorded in WHI-1 and WHI-2 treatments, respectively.

Table 5.9. Fruit firmness, soluble solids concentration, titratable acidity, ascorbic acid concentration and total antioxidants in the fruit skin and pulp affected by different WHI treatments following cold storage during 2006-07.

Treatment	Storage (days)	Firmness (N)	SSC (%)	TA (%malic acid)	SSC/TA ratio	AA (mg·100g^{-1} FW)	Total antioxidants (mM TE·g^{-1} FW)		
							Skin (ES)	Skin (SS)	(pulp)
C1	70	83.8 a B	15.6 C	0.73 B	21.4 B	8.51 b B	19.1	11.8	0.33
	140	78.5 b B	15.6 B	0.63 A	24.5 B	13.4 a AB	20.6	14.0	0.44 AB
	Mean	81.0	15.6	0.68	23.0	10.9	19.8	12.9	0.38
WHI-1	70	85.7 AB	16.3 B	0.72 a B	22.6 b B	8.43 b B	23.3	11.2	0.37 b
	140	80.9 A	15.9 AB	0.57 b AB	27.8 a A	13.8 a A	21.8	13.7	0.52 a A
	Mean	83.3	16.9	0.64	25.2	11.1	22.6	12.4	0.45
WHI-2	70	90.7 a A	17.2 a A	0.78 a A	22.1 b B	10.4 b A	22.2	12.3	0.36 b
	140	78.9 b A	16.5 b A	0.62 b A	26.6 a AB	14.1 a A	23.6	18.1	0.51 a A
	Mean	84.8	16.1	0.70	24.3	12.2	22.9	15.2	0.44
WHI-3	70	82.3 a B	14.7 D	0.61 C	24.4 A	7.67 b B	22.9	15.3	0.31
	140	72.1 b B	14.1 C	0.52 B	27.0 AB	12.2 a B	16.8	12.2	0.37 B
	Mean	77.2	14.4	0.56	25.7	10.1	19.9	13.7	0.34
LSD ($P \leq 0.05$)									
Irrigation (I)		3.04	0.41	0.04	1.72	1.01	NS (2.58)	NS (1.43)	0.06
Storage (S)		2.15	0.29	0.03	1.22	0.72	NS (1.82)	NS (1.01)	0.04
I x S		4.30	NS (0.20)	NS (0.02)	NS (0.83)	NS (0.49)	NS (3.65)	NS (2.02)	NS (0.03)

Means followed by the same letter in capital letter (between irrigation treatments) and in small letter (between storage conditions) are not significantly different at $P \leq 0.05$. NS = not significant differences at $P \leq 0.05$. Values within brackets represent standard error of means. During 2006-07, the treatments were i) CI, commercial irrigation, ii) WHI-1, 135 - 153 DAFB, iii) WHI-2, 145 - 175 DAFB, iv) WHI-3, 155 - 200 DAFB. While, in 2007-08, the treatments were i) CI, ii) WHI-1, 135 - 155 DAFB, iii) WHI-2, 145 - 200 DAFB, iv) WHI-3, 155 - 200 DAFB. AA = concentration of ascorbic acid.

5.4. Discussion

Water-deficit condition in WHI trees was evident from significantly reduced θ (Figure 5.2 and 5.3) and hydraulic status of leaves (Ψ_{stem}, Ψ_{leaf} and g_s) as compared to CI trees (Figure 5.4, Figure 5.5 and Figure 5.6). A reduction of θ, Ψ_{stem}, Ψ_{leaf} and g_s in WHI-1 and WHI-2 (during 2006-07) and in WHI-1 (during 2007-08) trees experienced the wilting, yellowing and slight leaf abscission. This may be associated to the increased levels of endogenous abscissic acid (ABA) in the leaf due to the water-deficit as reported earlier by Zhang and Davies (1990). Consequently, elevated levels of endogenous ABA stimulate ethylene production which causes leaf abscissions (Gomez-Cadenas et al., 1996). The significant fruit drop recorded on 176 and 185 DAFB (Figure 5.7) may be attributed to the nature of fruit in reducing heavy crop load (Chalmers, 1989). On the other hand, the WHI-3 commenced on 155 DAFB during both years did not experience water-deficit conditions. This may be due to rainy and cloudy days, which may have masked the effects on hydraulic status of soil and leaf of apple trees which results in similar to CI trees. In addition, Ψ_{leaf} with WHI-3 from 176 to 195 DAFB was not sufficient to exhibit stomatal closure. Flore and Lakso (1989) reported that stomatal closure can occur when Ψ_{leaf} above -3.0 MPa.

The improved fruit skin colour and accumulation of anthocyanins in fruit skin with WHI-1 and WHI-2 treatments during 2006-07 (Table 5.1) may be ascribed to the improved light penetration directly onto the fruit due to the sparse leaf abscission. Earlier, the light has been reported to improve red skin colour and accumulation of anthocyanins in fruit skin (Saure, 1990). As a prelude, the increased levels of endogenous of ABA and/or ABA induced ethylene production due to water-deficit may have up regulated expression of gene(s) involved in biosynthesis of anthocyanins. Increased rate of ethylene production due to deficit irrigation has also been reported in 'Delicious' and 'Braeburn' apple (Ebel et al., 1993; Kilili et al., 1996b). Previously, Whale and Singh (2007) reported that ethylene increased fruit colour and total anthocyanins concentration in 'Cripps Pink' apple. Similarly, improved red skin colour due to water-deficit application has been reported in 'Braeburn' apple (Kilili et al., 1996a; Mills et al., 1996a; Mills et al., 1994).

Fruit from the trees with WHI (WHI-2 in 2006-07 and WHI-1 in 2007-08) treatments also exhibited the higher chromaticity value a*, lower hue angle and lightness and higher chroma as compared to CI and WHI-3, in which correspond to the increment of fruit colour development and total anthocyanins concentration in fruit skin (Table 5.2 and 5.3). Similarly, reduced hue angle and increased red skin colour coincided with the increased total anthocyanins concentration in fruit skin of 'Cripps Pink' apple has been reported (Whale and Singh, 2007). Nine polyphenolic compounds were identified and confirmed using HPLC-ESI-MS as detailed in Chapter 4, Section 4.3.3.3. As explained earlier, no recent investigation has been reported on identification of anthocyanins and other polyphenolic in subjected to WHI in 'Cripps Pink' apple cultivar. The higher total anthocyanins in WHI-1 and WHI-2 treatments in both seasons may be associated to the increase of polyphenolic compounds (Table 5.1, 5.4 and 5.5). To date, no research work has been reported on the effects of water-deficit on the production of flavonoids and other phenolic compounds in red-skinned apple. In 2006-07, the treatments (WHI-1 and WHI-2) increased concentration of cyanidin 3-*O*-galactoside (range from 176% to 237%), quercetin 3-*O*-rutinoside (range from 127% to 132%), quercetin 3-*O*-galactoside (range from 224% to 259%), quercetin 3-*O*-arabinoside (range from 81% to 82%) and total quercetin glycosides (range from 95.5% to 112.2%) as compared to CI fruit. During 2007-08, the treatments (WHI-1 and WHI-2) enhanced 67% to 77.1% concentration of cyanidin 3-*O*-galactoside as compared to CI. The increment of polyphenolic compounds during 2006-07 was more intense as compared to 2007-08. Possibly, the considerably increase of these polyphenolics compounds in 2006-07 may be attributed to the pronounced effect of WHI treatments applied. Another possible factors involved in improving these polyphenolic compounds in the fruit skin may be the differences between day and night temperatures in both years (Figure 5.1). Earlier, Saure (1990) reported that the cold night temperatures (>20°C) promote the formation of anthocyanins and the warms temperatures prevent its accumulation. However, low night temperatures may not replaced light requirement, but they can increase the accumulation of anthocyanins to those fruit in the shaded side of tree canopies (Creasy, 1968).

As argued earlier, the increased of total anthocyanins and other flavonoids in WHI fruit may be associated to the exposure of apple fruit to the sunlight. Contrarily,

Castellarin et al. (2007b) claimed that the anthocyanins synthesis was primarily affected due to water-deficit application during intense phase of anthocyanins biosynthesis and partly impacted with solar radiation. In addition, the accumulation of anthocyanins in grape berries was increased due to the water-deficit has also been reported (Matthews and Anderson, 1988; Ojeda et al., 2000; Roby et al., 2004) Possibly, the increased concentrations of these flavonoids compounds may be associated with the up-regulation of anthocyanins-specific genes such as UFGT, CHS2, CHS3 and FH3 due to water-deficit as reported in grapes (Castellarin et al., 2007b). In addition, mRNA expression specifically in the skin of grapes enhanced due to water-deficit (Grimplet et al., 2007). Water-deficit imposed may also triggered the anthocyanins-specific genes and it warrants further investigation on the effects of WHI on gene(s) expression involved in biosynthesis of anthocyanins in the skin of 'Cripps Pink' apple fruit.

In both seasons, final fruit size in WHI-1 and WHI-2 were comparable to CI fruit (Figure 5.8). Similarly, water-deficit applied late in the season did not affect apple fruit size in 'Braeburn' apple (Kilili et al., 1996a; Mills et al., 1996b). Higher fruit firmness and SSC with WHI-1 and WHI-2 treatments (Table 5.6) were recorded which similar to the reports of Ebel et al. (1993); Mpelasoka et al. (2001b), Kilili et al. (1996a) and Kilili et al. (1996b). Increased fruit firmness may be ascribed to the reduction in cellular hydration (Ebel et al., 1993; Kilili et al., 1996a; Mpelasoka et al., 2000a) with the WHI treatments. Fruit from WHI-1 and WHI-2 treatments exhibited higher concentrations of ascorbic acid (Table 5.7). Earlier, increased concentrations of ascorbic acid in fruit subjected to water-deficit has been reported in different crops such as hot pepper (Dorji et al., 2005), table grape (Du et al., 2008), tomato (Veit-Kohler et al., 1999), pear-jujube (Cui et al., 2008) and strawberry (Liu et al., 2001). Possibly, the increased concentrations of ascorbic acid may be due to the higher sugars accumulation that stimulated its production during fruit ripening (Veit-Kohler et al., 1999). Higher total antioxidants in the skin and pulp may be attributed to the higher concentrations of total anthocyanins in the skin and ascorbic acid in the pulp of the fruit (Table 5.7). As presented in Table 5.8, the increased concentrations of total sugars with WHI treatments may be ascribed to the conversion of starch into sugars (Kramer, 1983; Landsberg and Jones, 1981) and also might be attributed to the changes of fruit water potential that contributing to the

changes in fruit osmotic potential (Mills et al., 1996b). The most important component of fruit osmotic potential in apple is soluble carbohydrates (Pavel and DeJong, 1995) and increased its concentration in DI fruit could be the mechanism of osmotic adjustment (Mpelasoka et al., 2001c). In addition, the increased concentration of fruit sugar at the end of early-season DI has been reported in Asian pear (Behboudian et al., 1994) and 'Braeburn' apple (Mills et al., 1996b; Mills et al., 1997a; Mpelasoka et al., 2001c). In general, WHI treatments enhanced the concentrations of total acids during both years (Table 5.8) and its increased may be associated to the contribution of organic acids to the fruit osmotic adjustment (Mills et al., 1997a).

Fruit from WHI-1 and WHI-2 treatments kept in cold storage remained firmer as storage period prolonged (Table 5.9). In general, all WHI treatments exhibited the declining trends in fruit firmness following 140 days cold storage. However, fruit firmness met the required standards for export (>68N). Possibly, the slight reduction in fruit firmness may be ascribed to the occurrence of fruit softening during maturation and ripening due to the changes in cell wall (Hobson, 1981). Following 140 days in cold storage, WHI-1 and WHI-2 stored-fruit had higher SSC as compared to all other treatments. Similar effects of water-deficit on 'Braeburn' apple kept in cold storage has earlier been reported (Kilili et al., 1996b; Mills et al., 1996a; Mpelasoka et al., 2001a). The ascorbic acid concentration increased in cold-stored-fruit from all WHI treatments as storage duration prolonged (Table 5.9). Following 140 days cold storage, fruit from WHI-2 showed the highest concentrations of ascorbic acid as compared to other treatments. In contrast, during long-term cold storage a decrease in the concentrations of ascorbic acid in 'Aroma' apple has been reported (Meberg et al., 2000). As discussed earlier, the increased concentrations of ascorbic acid in WHI cold-stored fruit may be associated to the elevated concentrations of ascorbic acid in fruit at harvest and might also be ascribed to the higher sugars accumulation that stimulates its production during fruit ripening. The increased of total antioxidants in the pulp of WHI stored-fruit with extended storage period may also be attributed to the increased concentration of ascorbic acid.

In conclusion, WHI at stage II of fruit development (commencing from 135 and 145 DAFB) improved fruit colour fruit colour development and other fruit quality

attributes such as firmness and SSC at harvest and following cold storage without adversely affecting fruit size of ‘Cripps Pink’ apple.

CHAPTER 6

Effects of Exogenous Application of Prohexadione-calcium on Fruit Colour Development and Quality in 'Cripps Pink' Apple

Summary

The effects of various concentrations, number of sprays of Prohexadione-calcium (ProCa) and summer pruning alone or in combination were evaluated on fruit colour development, concentrations of polyphenolic compounds in fruit skin and fruit quality of 'Cripps Pink' apple. Two experiments were conducted during 2007-08 growing season. In Experiment 1, the treatments included were: (i) control, (ii) summer pruning (SP), (iii) 250 $mg \cdot L^{-1}$ two sprays (3 and 33 DAFB, respectively), (iv) 500 $mg \cdot L^{-1}$ two sprays (3 and 33 DAFB, respectively), (v) 750 $mg \cdot L^{-1}$ two sprays (3 and 33 DAFB, respectively), (vi) 250 $mg \cdot L^{-1}$ three sprays (3, 33 and 63 DAFB, respectively), (vii) 500 $mg \cdot L^{-1}$ three sprays (3, 33 and 63 DAFB, respectively), (viii) 750 $mg \cdot L^{-1}$ three sprays (3, 33 and 63 DAFB, respectively), with four replicates. While, in Experiment 2, treatments were: (i) control, (ii) SP, (iii) 500 $mg \cdot L^{-1}$ two sprays (2 and 32 DAFB, respectively), (iv) 500 $mg \cdot L^{-1}$ two sprays (2 and 32 DAFB, respectively) and SP, (v) 500 $mg \cdot L^{-1}$ three sprays (2, 32 and 62 DAFB, respectively), (vi) 500 $mg \cdot L^{-1}$ three sprays (2, 32 and 62 DAFB, respectively) and SP, with four replications. The reduction of shoot length was pronounced after the second spray application of ProCa in both experiments. In Experiment 1, the treatment of two sprays of ProCa (500 $mg \cdot L^{-1}$) and three sprays ProCa (500 and 750 $mg \cdot L^{-1}$) showed the highest reduction in the shoot length as compared to control. The SP alone, and three sprays of ProCa (500 $mg \cdot L^{-1}$ and 750 $mg \cdot L^{-1}$, on 3, 33 and 63 DAFB, respectively) significantly improved percent red blush, percent fruit for export, higher chromaticity value a*, lower chromaticity value b*, lightness and hue angle on the fruit surface compared to control. The treatment of three sprays of ProCa (750 $mg \cdot L^{-1}$) resulted the highest concentration of cyanidin 3-*O*-galactoside in apple skin than control. However, the treatment of three sprays (500 $mg \cdot L^{-1}$) had the comparable concentration of cyanidin 3-*O*-galactoside to the treatment of three sprays ProCa (750 $mg \cdot L^{-1}$). In Experiment 2, two and three sprays application of ProCa (500 $mg \cdot L^{-1}$) in combination with SP improved fruit colour via increased the accumulation of anthocyanins and polyphenolic compounds particularly cyanidin 3-

O-galactoside, higher chromaticity value a*, lower chromaticity value b*, lightness and hue angle on the fruit surface as compared to control. In both experiments, other fruit quality parameters such as fruit firmness, soluble solids concentration (SSC) and titratable acidity (TA) resulted in inconclusive outcomes, but still meet the standards requirements set by the industry. In conclusions, three spray applications of ProCa (500 $mg \cdot L^{-1}$) on 3, 33 and 63 DAFB or two sprays of ProCa (500 $mg \cdot L^{-1}$) on 2 and 32 DAFB in combination with SP reduced shoot growth, improved fruit colour, accumulation of anthocyanins and polyphenolic compounds such as cyanidin 3-*O*-galactoside, chlorogenic acid and quercetin glycosides and also maintained other fruit quality attributes in 'Cripps Pink' apple cultivar grown in the Mediterranean climate of Western Australia.

6.1. Introduction

The erratic and poor fruit colour development of 'Cripps Pink' apple at commercial harvest reduces its export. The red blush on the fruit surface of this cultivar is one of the important grading standards that have been set by the Australian apple industry. The reduction in export quantity of this apple cultivar often causes serious economic losses not only to Western Australian apple industry, but also to apple industry in other parts of the world especially in warm and humid climatic region. The red colour of apple is determined by the type and concentration of vacuole based phenolic compounds like anthocyanins which are water soluble and impart red, blue and also purple in some commodities (Mazza and Miniati, 1993). Many internal and external factors affect the concentration of anthocyanins such as genetic, intensity and types of light, orchard temperature, crop load, and agronomic factors including agrochemicals application, irrigation, pruning, training system and fertilization (Lancaster, 1992; Saure, 1990).

Poor management of vigorous shoot growth implies a diminution in light penetration, tree productivity, fruit quality, profit and negatively influence pest control in apple and pear trees (Aśin et al., 2007; Forshey et al., 1992; Greene, 1999; Miller, 1995; Miller and Tworkoski, 2003). Various techniques have been tested to reduce shoot growth in apple trees including ethephon (Autio and Greene, 1994), daminozide (2,2-dimethylhyrazide), chlormequat (2-chloro-N,N,N-trimethylethanamium chloride) and paclobutrazol (â-[(4-chlorophenyl)methyl}-á(1,1-dimethylethyl)-1H-1,2,4-

triazol-1-ethanol] (Miller and Tworkoski, 2003), shoot pruning (Prive et al., 2006), root pruning (Schupp and Ferree, 1988), dwarfing rootstocks, fruit load and return bloom (Williams, 1984), girdling (Goren et al., 2004; Miller and Tworkoski, 2003) and deficit irrigation (Behboudian and Mills, 1997). However, some of these techniques have the disadvantages such as daminozide and paclobutrazol has been withdrawn from the market due to their persistence residues in plants (Curry and Williams, 1983; Green, 1986; Miller and Tworkoski, 2003), ethephon stimulate preharvest drop and hasten ripening (Autio and Greene, 1994), chlormequat has limited activity in apple and was not registered for use in fruit trees in United States (Miller, 1988; Miller and Tworkoski, 2003) and aggressive pruning accelerates even more shoot growth for the next season, which negatively influence fruit set, fruit size, fruit quality and yield (Prive et al., 2006). The girdling may compromise long-term tree health by affecting trees growth for one or more years after the treatment (Green and Lord, 1978; Green and Lord, 1983; Hoying and Robinson, 1992). Pruning is a technique that generally used to reduce vegetative growth. However, pruning is most expensive, labour-intensive and time consuming practices (Byers and Yoder, 1999; Felland, 1998; Forshey et al., 1992).

Newly growth retardant with promising outcomes have been discovered namely Prohexadione-calcium (ProCa) which acts as inhibitor of gibberellin biosynthesis. ProCa an acyl-cyclohexadione (3-oxido-4-propionyl-5-oxo-3-cyclohexene-carboxylate) has been developed in collaboration of BASF (Limburgerhof, Germany) with Kumiai Chemical Industry (Tokyo) (Medjdoub et al., 2005). In USA, ProCa is registered as Apogee® (27.5% a.i. ProCa), while Regalis® (10% a.i. ProCa) in some European countries (Miller and Tworkoski, 2003; Prive et al., 2006). This growth retardant had low toxicity and limited persistence in the trees (Owens and Stover, 1999). Lo Giudice et al. (2004) reported that mode of action of ProCa differs from other gibberellin biosynthesis inhibitors, it has been known to interfere the 3-ß hydroxylation of GA_{20} to GA_1. Its application reduced the biologically active GA_1 and increased the concentrations of inactive GA_{20}. It is well documented that ProCa possess no apparent risk to human and the environment (Rademacher and Kober, 2003), and also had a very rapid metabolic catabolism (Evans et al., 1999; Evans et al., 1997). In addition, ProCa has a short-lived, and its application always been made in early season (Mata et al., 2006a), thus it is not translocated into the growing fruit

(Halbwirth et al., 2003; Rademacher, 2000). ProCa application has been reported to reduce vegetative growth of various crops including apple (Basak and Rademacher, 2000; Byers and Yoder, 1999; Evans et al., 1997; Miller, 2002; Miller and Tworkoski, 2003).

Two sprays (250 $mg \cdot L^{-1}$ and 100 $mg \cdot L^{-1}$, respectively) and three sprays (100 $mg \cdot L^{-1}$, 100 $mg \cdot L^{-1}$ and 50 $mg \cdot L^{-1}$, respectively), and also repeated spray application (125 or 250 $mg \cdot L^{-1}$) of ProCa has been reported to increase red skin pigmentation in 'Fuji' apple in warm and dry climatic region of Spain (Mata et al., 2006a; Medjdoub et al., 2005). The accumulation of anthocyanins in 'Fuji' apple treated with ProCa was higher due to the reduce shoots length and smaller leaves size consequently allowing greater penetration of sunlight onto the fruit and also due to the differences between day and night temperatures at the apple orchard (Mata et al., 2006a). On the other hand, higher dosage of ProCa has been reported to prevent the accumulation of novel flavonoids in apple leaf, by blocking flavanone-3-hydroxylase (F3H) precursor (Roemmelt et al., 2003); which F3H plays key role in flavonoid biosynthesis (Rademacher, 2000). These would also lead to reduced anthocyanins formation. However, their finding was subjected to the accumulation of anthocyanins in the leaf but not in the skin of apple fruit. To date, no information is available on the effects of ProCa on flavonoid biosynthesis in the skin of apple fruit.

ProCa is an effective apple shoot growth retardant in apple orchards, without detrimental effects on others major quality attributes has been well documented (Byers and Yoder, 1999; Miller, 1988). Currently, no information is available on the effects of different concentrations and number of sprays of ProCa application in improving fruit colour of 'Cripps Pink' apples under Mediterranean climate. Moreover, no research work has been reported on the effects of ProCa on production of anthocyanins and polyphenolic compounds in 'Cripps Pink' apple fruit skin. Therefore, these present investigations aimed to elucidate the effects of ProCa in improving fruit colour and accumulation of red skin pigmentation and other fruit quality parameters in 'Cripps Pink' apple in Western Australia conditions.

6.2. Materials and Methods

6.2.1. Experimental location and plant materials

Two experiments were carried out in a commercial orchard at Karragullen, Perth Hills, Western Australia (latitude 32°5'28"S; longitude 116°7'19"E) on 'Cripps Pink' apple trees during 2007-08 growing season. The location was in a Mediterranean climate characterised by warm summers and cool winters.

Thirty-two of 15-year-old and 24 of 14-year-old of 'Cripps Pink' apple trees, grafted on MM.109 rootstock were used in Experiment 1 and Experiment 2, respectively. All experimental trees used in both experiments were of uniform size and trained as a central leader (CL) system. The experimental trees in both experiments were planted in the east-west and north-south direction respectively, maintaining row distances of 4.5 m and plant distances of 2.4 m. Full bloom (>80% of the buds are open) occurred on 21st October 2007 at both locations.

6.2.2. Experiment 1: Effects of various concentrations and number of Prohexadione-calcium sprays on shoot growth, fruit colour development and quality of 'Cripps Pink' apple

Various concentrations and number of ProCa sprays were used as the treatments such as (i) control, (ii) summer pruning (SP), (iii) 250 mg·L^{-1} two sprays (3 and 33 DAFB, respectively) (iv) 500 mg·L^{-1} two sprays (3 and 33 DAFB, respectively), (v) 750 mg·L^{-1} two sprays (3 and 33 DAFB, respectively), (vi) 250 mg·L^{-1} three sprays (3, 33 and 63 DAFB, respectively), (vii) 500 mg·L^{-1} three sprays, (3, 33 and 63 DAFB, respectively), (viii) 750 mg·L^{-1} three sprays, (3, 33 and 63 DAFB, respectively). The experiment was laid out by following a randomized complete block design and included four replications. Single tree treated as an experimental unit. Prohexadione-calcium (Regalis 10% a.i., BASF, Carl-Bosch-Straße, Lamburgerhof, Germany) was a gift sample from Nufarm Australia Limited, Kwinana, Western Australia. An aqueous solution containing different concentrations of ProCa and a non-ionic surfactant Tween®20 (0.125% v/v, Sigma-Aldrich Chemie GMbh, Steinheim, Germany) were sprayed onto the fruit of whole tree till runoff. The spray treatments were applied first at 3-10 cm shoot length (3 DAFB), second on 33 DAFB and third on 63 DAFB. A sprayer (Selecta Trolleypak MKII, Model N TR25-P, Silvan Autralia Ltd, Wantirna, Australia) was used for

spraying an aqueous solution. Unsprayed trees served as control. Summer pruning was done on 156 DAFB by removing undesirable, excessively vigorous and upright growth of shoots of apple trees. Shoot length was recorded on 0, 28, 53, 103 and 178 DAFB. The commercial harvest date was 200 DAFB (8th May 2008). The observations recorded were percent fruit with >40% red blush on fruit surface, percentage fruit meeting export requirement for colour, fruit colour, concentration of total anthocyanins, polyphenolic compounds [cyanidin 3-*O*-galactoside, chlorogenic acid, phloridzin, quercetin glycosides (quercetin 3-*O*-rutinoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-xyloside, quercetin 3-*O*-arabinoside, quercetin 3-*O*-rhamnoside)], fruit firmness, titratable acidity (TA), soluble solids concentration (SSC), SSC/TA ratio, total antioxidants on the exposed (ES) and shaded (SS) sides of apple skin, ascorbic acid, sugars and organic acids concentration.

6.2.3. Experiment 2: Effects of various number of Prohexadione-calcium (500 mg·L^{-1}) sprays alone and in combination with summer pruning on shoot growth, fruit colour development and quality of 'Cripps Pink' apple

The product manufacturers recommend three sprays of ProCa (500 mg·L^{-1}) to reduce shoot growth in apple. The treatments included were: (i) control, (ii) SP, (iii) 500 mg·L^{-1} two sprays (2 and 32 DAFB, respectively), (iv) 500 mg·L^{-1} two sprays (2 and 32 DAFB, respectively) and SP, (v) 500 mg·L^{-1} three sprays (2, 32 and 62 DAFB, respectively), (vi) 500 mg·L^{-1} three sprays (2, 32 and 62 DAFB, respectively) and SP. The experiment was laid out by following a randomized complete block design. Single tree represented an experimental unit. All treatments were replicated four times. Aqueous solutions containing ProCa (500 mg·L^{-1}) was sprayed onto the fruit of whole tree till runoff using a sprayer (Selecta Trolleypak MKII, Model N TR25-P, Silvan Autralia Ltd, Wantirna, Australia). ProCa used in this experiment was the same as above mentioned in Experiment 1. Unsprayed trees served as control. Summer pruning was done on 156 DAFB as explained in Section 6.2.2. Shoot growth length was recorded on 0, 27, 52, 102 and 177 DAFB. The commercial harvest date was as mentioned in Experiment 1. All the parameters evaluation recorded were as detailed in Section 6.2.2.

6.2.4. Temperature monitoring at experimental site

Daily temperatures in the orchard were recorded using data loggers (Tinytag*Plus* Gemini Data Logger, UK) and all data were obtained using Gemini Logger Manager Software (Version 2.8). Daily average day and night temperatures (Figure 6.1) were calculated between sunset and sunrise times as outlined in Chapter 4, Section 4.2.5.

6.2.5. Fruit sampling

Twenty-five fruit were randomly chosen from all parts of the tree canopy up to height of 2 m from the ground for fruit quality assessment at harvest. Mature fruit (3.0 - 3.5 starch pattern index) were harvested on 200 DAFB (8th May 2008).

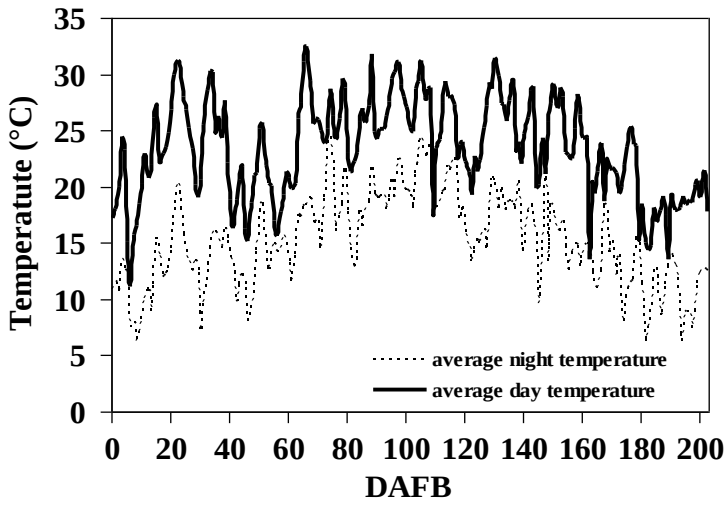

Figure 6.1. Average day and night temperatures during 2007-08 at commercial apple orchard in Karragullen, Perth Hills, Western Australia.

6.2.6. Shoot growth

Shoot length was recorded from randomly selected tagged shoots. Six current-season shoots were selected as detailed in Chapter 3, Section 3.2.6.

6.2.7. Fruit quality: fruit colour

6.2.7.1. Surface skin colour

Percentage red blush on individual fruit was visually assessed and scores was given ranging from 0 to 100% as described in Chapter 3, Section 3.3.1. Fruit colour was also determined on the fruit surface using a HunterLab ColorFlex $45^o/0^o$ Spectrophotometer including chromaticity value a*, b*, lightness (L*), chroma (C*), hue angle (h°) as detailed in Chapter 3, Section 3.3.2.

6.2.7.2. Analysis of skin pigment

6.2.7.2.1 Total anthocyanins

Total anthocyanins were extracted and quantified from apple skin according to the method outlined by Whale and Singh (2007) as described in Chapter 3, Section 3.3.3.1 using an UV-VIS spectrophotometer (Jenway Spectrophotometers Model 6405, Dunmow, Essex, UK).

6.2.7.2.2 Flavonoids and other phenolic compounds

All chemicals standards for polyphenolic compounds were purchased from various manufacturers as mentioned in Chapter 3, Section 3.3.3.2.1. Flavonoids and other phenolic compounds of apple skin were extracted following the method reported earlier by Whale and Singh (2007) with some modifications as detailed in Chapter 3, Section 3.3.3.2.2. These polyphenolic compounds were identified and quantified using a reversed-phase high performance liquid chromatography (RP-HPLC) as according to the procedure detailed in Chapter 3, Section 3.3.3.2.3 and 3.3.3.2.4. Flavonoids and other phenolic compounds were re-confirmed using HPLC-electrospray ionization mass spectrometry (HPLC-ESI-MS) as mentioned in Chapter 3, Section 3.3.3.2.3.

6.2.8. Other fruit quality parameters

6.2.8.1. Fruit firmness

An electronic pressure tester (Model EPT-1 pressure tester, Lake City Technical products Inc., Kelowna, BC, Canada) fitted with 11 mm tip was used to determined fruit firmness as detailed in Chapter 3, Section 3.4.2.

6.2.8.2. Titratable acidity, soluble solids concentration and SSC/TA ratio

Apple juice was titrated against 0.1N NaOH using phenolphthalein as an indicator to determine titratable acidity (TA) as detailed in Chapter 3, Section 3.4.3. An infrared digital refractometer (Atago-Palette PR 101, Atago Co. Ltd., Itabahi-Ku, Tokyo, Japan) was used to recorded soluble solids concentration (SSC) as described in Chapter 3, Section 3.4.4. SSC/TA ratio was calculated by dividing SSC with the TA.

6.2.9. Determination of ascorbic acid, total antioxidants, individual sugars and organic acids

6.2.9.1. Ascorbic acid

Ascorbic acid concentration from apple pulp was determined following the method outlined by Jagota and Dani (1982) and Malik and Singh (2005) as detailed in Chapter 3, Section 3.5.1.

6.2.9.2. Total antioxidants

The levels of total antioxidants of apple skin and pulp were determined as according to the method of Brand-Williams et al. (1995) and Khan et al. (2008) as detailed in Chapter 3, section 3.5.2.

6.2.9.3. Individual sugars

Chemicals used for individual sugars determination were purchased from different manufacturers as described in Chapter 3, Section 3.5.3.1. The procedures of extraction and quantification of individual sugar were described in Chapter 3, Section 3.5.3.2. Fructose, sucrose and sorbitol were separated, identified and quantified using the HPLC system (Waters Corp., Milford, Mass., USA) fixed with the Fast Carbohydrate Analysis column (Aminex-HPX 87C, 100 x 7.8 mm) (Bio-Rad Laboratories, Hercules, CA, USA) as detailed in Chapter 3, Section 3.5.3.3. The cumulative concentrations of individual sugars such as fructose, sucrose and sorbitol were calculated as total sugars.

6.2.9.4. Individual organic acids

The chemicals used for individual organic acids determination as detailed in Chapter 3, Section 3.5.3.1. A hand blender (Model HB95, Breville Pty. Ltd., New South Wales, Australia) was used to homogenized apple juice as detailed in Chapter 3, Section 3.5.3.2. The HPLC system (Waters Corp., Milford, Mass., USA) fixed with a Bio Rad Aminex-HPX 87H column (300 x 7.8 mm; particle size 9 µm) (Bio-Rad Laboratories, Inc., Hercules, USA) was used to separate, identify and quantify individual organic acids in apple juice as outlined in Chapter 3, Section 3.5.3.3.

6.2.10. Statistical analysis

Effects of different concentrations and number of ProCa sprays and also its combination with or without SP on various parameters were assessed within the analysis of variance (ANOVA) using GLM (General Linear Models) procedures with SAS 9.1 software package, SAS Institute Inc, Cary, NC, USA. Treatments means were further separated by least significant difference (LSD) at $P \leq 0.05$ following a significant F test (SAS Institute Inc., 1999). The validity of the analysis was ensured by checking all the assumptions of analysis.

6.3. Results

6.3.1. Experiment 1: Effects of various concentrations and number of Prohexadione-calcium sprays on shoot growth, fruit colour development and quality of 'Cripps Pink' apple

6.3.1.1. Shoot length

As shown in Figure 6.2, the application of different concentrations and number of ProCa sprays significantly ($P \leq 0.05$) inhibited shoot growth in 'Cripps Pink' apple trees. The reduction of shoot length was pronounced after two sprays of ProCa (750 $mg \cdot L^{-1}$) on the trees (Figure 6.2). A comparable level of inhibition of shoot growth after second spray also recorded in shoot treated with three sprays of ProCa (500 and 750 $mg \cdot L^{-1}$). After 100 (103 DAFB) and 175 days (178 DAFB) of first ProCa treatment, the trees with two sprays of ProCa (750 $mg \cdot L^{-1}$) continued to inhibit the shoot growth (22.3% and 33.6%, respectively) as compared to control. Following three sprays of ProCa (500 and 750 $mg \cdot L^{-1}$) the levels of inhibition were gradually decreased in shoot growth on 103 DAFB (17.1% and 19.3%, respectively) and 178 DAFB (29.6% and 28.4%, respectively).

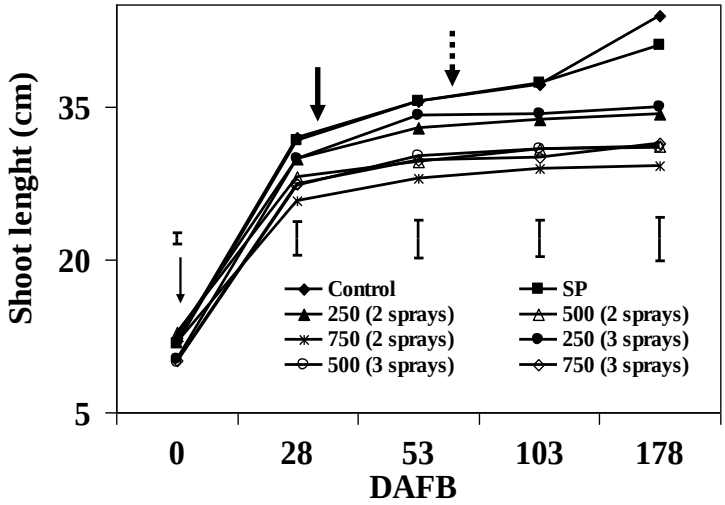

Figure 6.2. Effects of different concentrations and number of Prohexadione-calcium sprays as well as summer pruning (SP) on shoot growth length of 'Cripps Pink' apple. Vertical bars represent LSD. LSD ($P \leq 0.05$) on 0 DAFB = 0.55, 28 DAFB = 1.64, 53 DAFB = 1.82, 103 DAFB = 1.81, 178 DAFB = 2.09. LSD ($P \leq 0.05$) for treatments = 1.39, time = 1.10, treatment x time = 3.11. Thin arrow = first spray on 3 DAFB, thick arrow = second spray on 33 DAFB, dashed arrow = third spray on 63 DAFB. SP = summer pruning (156 DAFB).

6.3.1.2. Effects on fruit colour and total anthocyanins concentration

Visual fruit colour and total anthocyanins concentration were significantly ($P \leq 0.05$) affected with the application of different concentrations and different frequency of ProCa as well as SP (Table 6.1). Fruit from SP exhibited the highest fruit visual colour (55.4%) and percentage fruit meeting the export criterion (91.0%) as compared to control. Whilst, three spray applications of 500 $mg \cdot L^{-1}$ and 750 $mg \cdot L^{-1}$ ProCa showed the comparable improvements for visual fruit colour (53.5% and 54.9%, respectively) and percentage fruit for export (83.0% and 88.0%, respectively) to SP. Three sprays of ProCa (500 $mg \cdot L^{-1}$ and 750 $mg \cdot L^{-1}$) resulted in significantly ($P \leq 0.05$) higher total anthocyanins concentration in fruit skin (211.4 and 206.9 $\mu g \cdot g^{-1}$ FW, respectively) as compared to control (121.1 $\mu g \cdot g^{-1}$ FW) (Table 6.1). SP resulted in higher concentrations of total anthocyanins in fruit skin (193.4 $\mu g \cdot g^{-1}$ FW) than control fruit. There were no significant differences among treatments of three sprays of ProCa (500 $mg \cdot L^{-1}$ or 750 $mg \cdot L^{-1}$) and SP alone in increasing visual fruit colour, percentage fruit meeting export requirements for colour and enhancing concentrations of total anthocyanins in the fruit skin.

Table 6.1. Fruit colour and total anthocyanins of apple fruit affected with different concentrations, number of Prohexadione-calcium sprays and summer pruning.

Treatment	Visual colour (% red blush)	Percentage fruit with > 40% red blush	Total anthocyanins ($\mu g \cdot g^{-1}$ FW)
Control	40.2 d	55.0 d	121.1 d
Summer pruning (SP)	55.4 a	91.0 a	193.3 abc
ProCa 250 $mg \cdot L^{-1}$ (2 sprays)	43.8 cd	56.0 d	140.8 d
ProCa 500 $mg \cdot L^{-1}$ (2 sprays)	45.6 cd	66.0 cd	148.6 cd
ProCa 750 $mg \cdot L^{-1}$ (2 sprays)	47.2 bc	69.0 bcd	163.6 bcd
ProCa 250 $mg \cdot L^{-1}$ (3 sprays)	44.3 cd	63.0 d	153.4 cd
ProCa 500 $mg \cdot L^{-1}$ (3 sprays)	53.5 ab	83.0 abc	211.4 a
ProCa 750 $mg \cdot L^{-1}$ (3 sprays)	54.9 a	88.0 ab	206.9 ab
LSD ($P \leq 0.05$)	6.87	19.4	45.0

Means followed by the same letter within column are not significantly different at $P \leq 0.05$.

6.3.1.3. Effects on chromaticity value a*, b*, lightness, hue angle and chroma

The reduction in hue angle (h°), chromaticity value b*, lightness (L*) and higher chromaticity value a* indicates redder fruit skin colour. The application of different concentrations and number of ProCa sprays and SP significantly ($P \leq 0.05$) affected the chromaticity value a* on both sides of the fruit excluding chromaticity value b* on the ES side of apple skin (Figure 6.3). The application of three sprays of ProCa (500 and 750 $mg \cdot L^{-1}$) and also SP alone resulted in the higher chromaticity value a* on both sides of apple skin as compared to control. The lowest chromaticity value b* on the SS of apple skin (20.9 b*) was recorded with three sprays of ProCa (500 $mg \cdot L^{-1}$) than control fruit skin. The application of different concentrations and number of ProCa sprays and SP significantly ($P \leq 0.05$) affected L* and h° on the both sides of apple fruit skin excluding C* on the SS (Figure 6.4). The lowest L* on the ES (27.4 L*) and SS (34.8 L*) of apple fruit skin was recorded with three sprays of ProCa (500 and 750 $mg \cdot L^{-1}$, respectively) as compared to control. A significantly ($P \leq 0.05$) lower h° (33.7 h° on ES and 52.6 h° on SS) on the both sides of apple fruit skin was recorded with the three sprays application of ProCa (500 $mg \cdot L^{-1}$) as compared to control. The treatment of SP resulted in more saturated colour as

reflected by significantly ($P \leq 0.05$) higher C* on the ES of apple fruit skin than control. Three sprays application of ProCa (500 mg·L^{-1}) resulted in a comparable C* to the SP treatment.

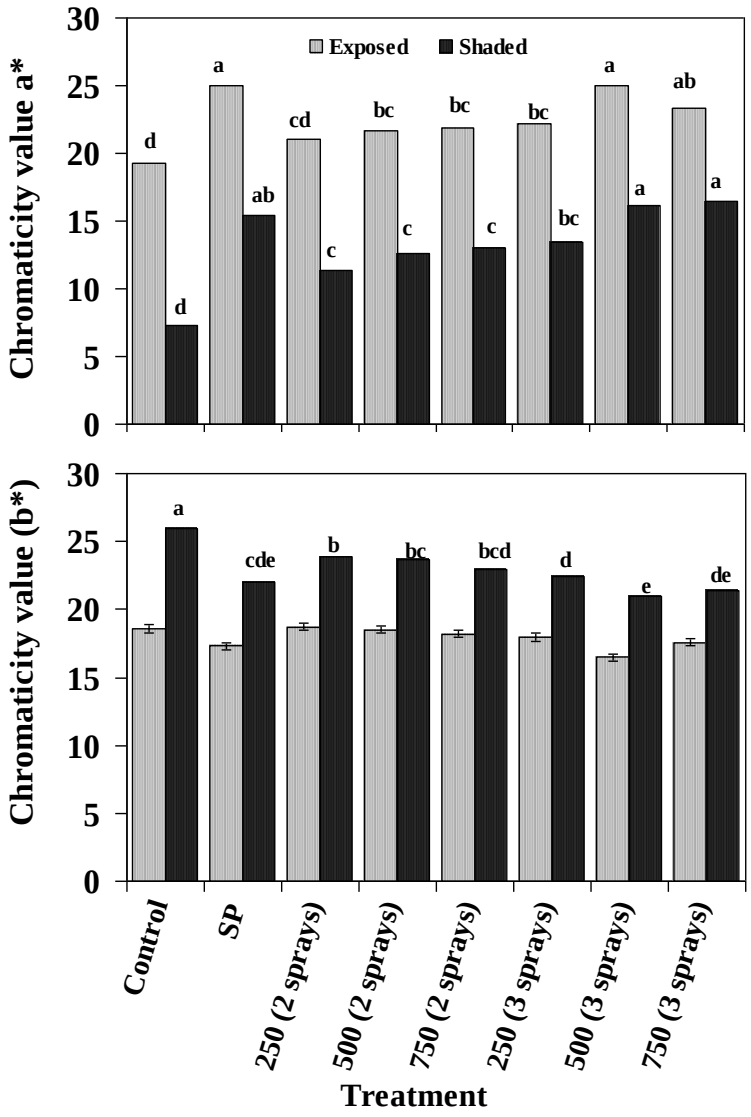

Figure 6.3. Effects of different concentrations, number of Prohexadione-calcium sprays and summer pruning on chromaticity value a* and b* in the exposed (ES) and shaded (SS) sides of 'Cripps Pink' apple. LSD ($P \leq 0.05$) for chromaticity value a* (ES) = 2.23, a* (SS) = 2.37, b* (ES) = NS (0.54), b* (SS) = 1.42. SP = summer pruning. Vertical bars represent standard error of means. NS = not significant ($P \leq 0.05$).

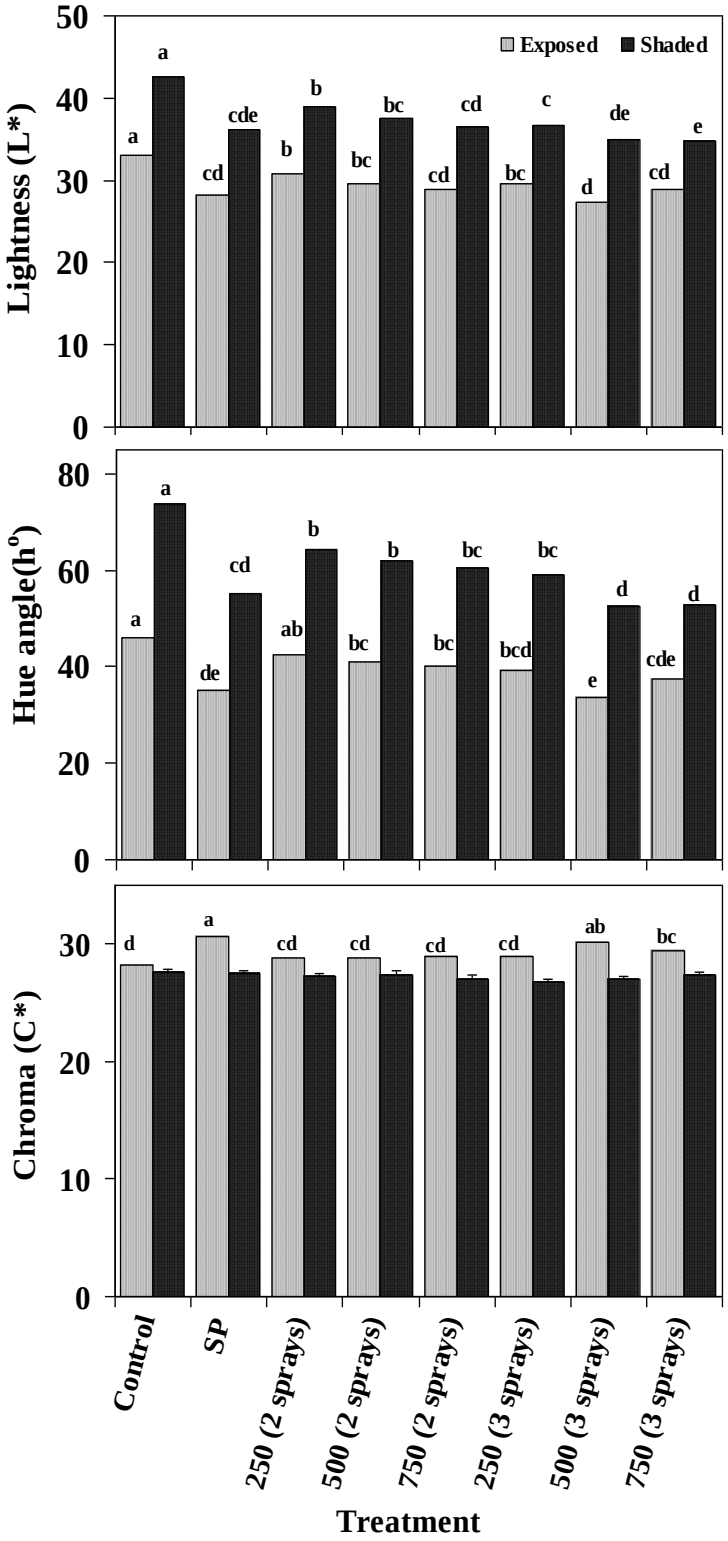

Figure 6.4. Effects of different concentrations, number of Prohexadione-calcium sprays and summer pruning on lightness (L*), hue angle (h°) and chroma (C*) in the exposed (ES) and shaded (SS) sides of 'Cripps Pink' apple. LSD ($P \leq 0.05$) for L* (ES) = 1.74, L* (SS) = 1.61, h° (ES) = 4.73, h° (SS) = 5.58, C* (ES) = 1.04, C* (SS) = NS (0.26). SP = summer pruning. Vertical bars represent standard error of means. NS = not significant ($P \leq 0.05$).

6.3.1.4. Flavonoids and other phenolic compounds

As a prelude, nine polyphenolic compounds in the fruit skin of this apple cultivar were confirmed using HPLC-ESI-MS (refer Chapter 4, section 4.3.3.3.). The application of different concentrations, number of ProCa sprays and SP significantly ($P \leq 0.05$) affected the concentration of flavonoids and other phenolic compounds in apple fruit skin (Table 6.2). Three sprays of ProCa (750 $mg \cdot L^{-1}$) resulted in a significant increased concentration of cyanidin 3-*O*-galactoside (478.1 $\mu g \cdot g^{-1}$ FW) in fruit skin than control. A comparable concentration of cyanidin 3-*O*-galactoside (417.5 $\mu g \cdot g^{-1}$ FW) was recorded with three sprays ProCa (500 $mg \cdot L^{-1}$) to the treatment of three sprays of ProCa (750 $mg \cdot L^{-1}$). The concentration of chlorogenic acid was significantly ($P \leq 0.05$) higher in the fruit skin (268.6 and 265.5 $\mu g \cdot g^{-1}$ FW) with SP and three sprays of ProCa (750 $mg \cdot L^{-1}$), respectively as compared to control. Whilst, the treatment of three spray applications of ProCa (500 $mg \cdot L^{-1}$) and two sprays of ProCa (250 $mg \cdot L^{-1}$) exhibited the higher concentrations of phloridzin (56.2 and 55.4 $\mu g \cdot g^{-1}$ FW, respectively) as compared to control. The treatment of three sprays of ProCa (750 $mg \cdot L^{-1}$) resulted in the highest concentration of total quercetin glycosides in fruit skin than in control (Table 6.3). The concentrations of individual compounds of quercetin glycosides namely quercetin 3-*O*-rutinoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-xyloside, quercetin 3-*O*-arabinoside and quercetin 3-*O*-rhamnoside were significantly ($P \leq 0.05$) affected with the application of various concentrations, number of ProCa sprays and SP. The treatment of three sprays of ProCa (750 $mg \cdot L^{-1}$) resulted in the highest concentrations of all individual compounds of quercetin glycosides in apple skin as compared to control. However, three sprays of ProCa (500 $mg \cdot L^{-1}$) resulted in a comparable concentration of total quercetin glycosides (1480.1 $\mu g \cdot g^{-1}$ FW), quercetin 3-*O*-glucoside (69.3 $\mu g \cdot g^{-1}$ FW), quercetin 3-*O*-xyloside (304.7 $\mu g \cdot g^{-1}$ FW), quercetin 3-*O*-arabinoside (295.5 $\mu g \cdot g^{-1}$ FW) and quercetin 3-*O*-rhamnoside (180.9 $\mu g \cdot g^{-1}$ FW) to three sprays of ProCa (750 $mg \cdot L^{-1}$). Amongst treatments tested, SP resulted in the lowest concentration of all individual quercetin glycosides in the fruit skin.

Table 6.2. Effects of different concentrations, number of Prohexadione-calcium sprays and summer pruning on the concentrations of flavonoids and other phenolic compounds in the fruit skin.

Treatment	Flavonoids and other phenolic compounds (μg·g^{-1} FW)		
	Cyanidin 3-*O*-galactoside	Chlorogenic acid	Phloridzin
Control	213.6 e	237.4 ab	40.5 b
Summer pruning (SP)	306.0 cde	265.5 a	51.7 ab
ProCa 250 mg·L^{-1} (2 sprays)	234.4 de	250.5 ab	55.4 a
ProCa 500 mg·L^{-1} (2 sprays)	324.6 bcd	226.6 b	51.4 ab
ProCa 750 mg·L^{-1} (2 sprays)	366.7 bc	226.9 b	39.1 b
ProCa 250 mg·L^{-1} (3 sprays)	265.7 cde	256.9 ab	49.6 ab
ProCa 500 mg·L^{-1} (3 sprays)	417.5 ab	260.1 ab	56.2 a
ProCa 750 mg·L^{-1} (3 sprays)	478.1 a	268.6 a	50.5 ab
LSD ($P \leq 0.05$)	101.6	38.2	12.8

Means followed by the same letter within column are not significantly different at $P \leq 0.05$.

Table 6.3. Effects of different concentrations, number of Prohexadione-calcium sprays and summer pruning on individual quercetin glycosides and total quercetin glycosides compounds in the fruit skin.

Quercetin glycosides (µg·g^{-1} FW)	Treatment								LSD ($P \leq 0.05$)
	Control	Summer pruning (SP)	ProCa (mg·L^{-1})						
			250 (2 sprays)	500 (2 sprays)	750 (2 sprays)	250 (3 sprays)	500 (3 sprays)	750 (3 sprays)	
Quercetin 3-*O*-rutinoside	12.0 bc	5.2 c	15.5 bc	33.2 b	18.6 bc	14.2 bc	36.7 b	65.7 a	25.6
Quercetin 3-*O*-galactoside	266.9 c	183.2 c	324.6 bc	493.5 bc	387.5 bc	354.7 bc	593.1 b	943.3 a	311.0
Quercetin 3-*O*-glucoside	32.8 cd	25.2 d	42.6 bcd	61.4 bc	40.4 bcd	40.9 bcd	69.3 ab	99.0 a	32.8
Quercetin 3-*O*-xyloside	159.8 c	138.6 c	202.7 c	231.9 bc	195.7 c	202.3 c	304.7 ab	367.3 a	99.8
Quercetin 3-*O*-arabinoside	151.2 c	134.9 c	188.6 bc	230.9 bc	192.6 bc	198.3 bc	295.5 ab	376.8 a	110.1
Quercetin 3-*O*-rhamnoside	107.9 bc	98.1 c	138.0 b	132.7 bc	109.0 bc	130.3 bc	180.9 a	188.7 a	36.1
Total quercetin glycosides	730.5 c	585.2 c	911.9 bc	1183.4 bc	943.8 bc	940.6 bc	1480.1 ab	2040.6 a	601.7

Means followed by the same letter within row are not significantly different at $P \leq 0.05$.

6.3.1.5. Fruit firmness, soluble solids concentration, tiratable acidity and SSC/TA ratio

The data presented in Table 6.4 show that SSC and TA were significantly ($P \leq 0.05$) affected with the application of various concentrations, number of sprays of ProCa and SP. Three sprays of ProCa (500 mg·L^{-1}) treatment significantly ($P \leq 0.05$) increased SSC (15.7%) than control. Whilst, the three sprays of ProCa (750 mg·L^{-1}) exhibited the highest TA than two sprays of ProCa (750 mg·L^{-1}). A comparable amount of TA (0.87% malic acid) was recorded with three sprays of ProCa (500 mg·L^{-1}) to the treatment of three sprays of ProCa (750 mg·L^{-1}). The ProCa and SP treatments showed no significant effects on the fruit firmness and SSC/TA ratio.

Table 6.4. Fruit firmness, soluble solids concentration, titratable acidity and SSC/TA ratio in apple fruit affected by different concentrations, number of Prohexadione-calcium sprays and summer pruning.

Treatment	Firmness (N)	SSC (%)	TA (% malic acid)	SSC/TA ratio
Control	85.6	14.8 bc	0.83 abc	17.8
Summer pruning (SP)	86.5	15.0 bc	0.84 ab	17.8
ProCa 250 mg·L^{-1} (2 sprays)	89.5	15.1 abc	0.81 abc	18.6
ProCa 500 mg·L^{-1} (2 sprays)	88.5	14.9 bc	0.84 ab	17.7
ProCa 750 mg·L^{-1} (2 sprays)	88.6	14.5 c	0.76 c	19.0
ProCa 250 mg·L^{-1} (3 sprays)	88.6	14.7 bc	0.80 bc	18.3
ProCa 500 mg·L^{-1} (3 sprays)	90.2	15.7 a	0.87 ab	18.0
ProCa 750 mg·L^{-1} (3 sprays)	87.1	15.3 ab	0.88 a	17.4
LSD ($P \leq 0.05$)	NS (1.28)	0.62	0.07	NS (0.41)

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. NS = not significant ($P \leq 0.05$). Values within brackets represent standard error of means.

6.3.1.6. Concentration of ascorbic acid and total antioxidants

As shown in Table 6.5, the concentration of ascorbic acid and total antioxidants in the pulp were not significantly affected with the application of various concentrations, number of ProCa sprays except total antioxidants in the skin. The highest total antioxidants in the fruit skin (34.5 mM TE·g^{-1} FW) were recorded in SP than in control.

Table 6.5. Ascorbic acid concentration and total antioxidants in apple skin and pulp affected by different concentrations, number of Prohexadione-calcium sprays and summer pruning.

Treatment	Ascorbic acid ($mg \cdot 100g^{-1}$ FW)	Total antioxidants ($mM\ TE \cdot g^{-1}$ FW)	
		skin	pulp
Control	10.3	18.5 c	0.51
Summer pruning (SP)	10.9	34.5 a	0.56
ProCa 250 $mg \cdot L^{-1}$ (2 sprays)	10.4	20.0 bc	0.64
ProCa 500 $mg \cdot L^{-1}$ (2 sprays)	9.83	18.3 c	0.51
ProCa 750 $mg \cdot L^{-1}$ (2 sprays)	9.80	19.0 bc	0.49
ProCa 250 $mg \cdot L^{-1}$ (3 sprays)	10.2	20.9 bc	0.55
ProCa 500 $mg \cdot L^{-1}$ (3 sprays)	10.7	23.9 b	0.55
ProCa 750 $mg \cdot L^{-1}$ (3 sprays)	10.2	21.8 bc	0.62
LSD ($P \leq 0.05$)	NS (0.32)	5.22	NS (0.04)

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. NS = not significant ($P \leq 0.05$). Values within brackets represent standard error of means.

6.3.1.7. Concentrations of total sugars and individual organic acids

The application of various concentrations, numbers of ProCa sprays and SP alone did not significantly affected concentration of total sugars in apple fruit (Table 6.6). In general, concentration of malic acid showed the biggest proportion of total acids, closely followed by tartaric, citric and succinic acid (Table 6.6). The concentrations of malic, succinic and total acids were significantly ($P \leq 0.05$) affected with the application of different concentrations, number of ProCa sprays and SP, but these treatments show non-significant effects on the concentrations of citric and tartaric acid. The higher concentration of malic acid (7.48 $g \cdot kg^{-1}$) was recorded in fruit-treated with three sprays of ProCa (250 $mg \cdot L^{-1}$) as compared to control. However, fruit-treated with three sprays of ProCa (500 $mg \cdot L^{-1}$) showed a comparable concentration of malic acid to the three sprays of ProCa (250 $mg \cdot L^{-1}$) treatment. The two sprays of ProCa (500 $mg \cdot L^{-1}$) resulted in significantly ($P \leq 0.05$) highest concentration of succinic acid (0.73 $g \cdot kg^{-1}$) as compared to all other treatments. Fruit-

Table 6.6. Individual organic acids and concentration of total acids in apple fruit affected by different concentrations, number of Prohexadione-calcium sprays and summer pruning.

Treatment	Total sugars ($g \cdot kg^{-1}$)	Organic acids ($g \cdot kg^{-1}$)				
		Malic	Citric	Tartaric	Succinic	Total acids
Control	114.3	5.40 c	0.06	0.10	0.45 d	5.99 b
Summer pruning (SP)	111.1	5.96 c	0.06	0.10	0.46 cd	6.59 b
ProCa 250 $mg \cdot L^{-1}$ (2 sprays)	106.3	5.62 c	0.06	0.10	0.48 cd	6.25 b
ProCa 500 $mg \cdot L^{-1}$ (2 sprays)	106.8	7.06 ab	0.07	0.10	0.73 a	7.96 a
ProCa 750 $mg \cdot L^{-1}$ (2 sprays)	112.1	6.14 bc	0.06	0.09	0.53 bc	6.83 b
ProCa 250 $mg \cdot L^{-1}$ (3 sprays)	110.9	7.48 a	0.07	0.10	0.60 b	8.25 a
ProCa 500 $mg \cdot L^{-1}$ (3 sprays)	117.6	7.40 a	0.07	0.10	0.57 b	8.13 a
ProCa 750 $mg \cdot L^{-1}$ (3 sprays)	101.4	6.21 bc	0.06	0.10	0.54 bc	6.91 b
LSD ($P \leq 0.05$)	NS (3.55)	0.97	NS (0.003)	NS (0.002)	0.08	1.10

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. NS = not significant ($P \leq 0.05$). Values within brackets represent standard error of means.

treated with two sprays of ProCa (500 mg·L^{-1}) and three sprays of ProCa (250 mg·L^{-1} and 500 mg·L^{-1}) resulted in the higher concentration of total acids (7.96, 8.25 and 8.13 g·kg^{-1}, respectively) as compared to all other treatments.

6.3.2. Experiment 2: Effects of various number of Prhexadione-calcium (500 mg·L^{-1}) sprays alone and in combination with summer pruning on shoot growth, fruit colour development and quality of 'Cripps Pink' apple

6.3.2.1. Shoot length

The shoot length was significantly ($P \leq 0.05$) affected with the application of different sprays of ProCa (500 mg·L^{-1}) alone and in combination with SP (Figure 6.5). The treatment of two sprays of ProCa (500 mg·L^{-1}) with SP and also shoot treated with three sprays of ProCa (500 mg·L^{-1}) with and without SP exhibited a significant shorter shoot length on 52, 102 and 177 DAFB as compared to control. Shoot treated with two sprays of ProCa (500 mg·L^{-1}) with SP and also shoot treated with three sprays of ProCa (500 mg·L^{-1}) with and without SP showed reduction in shoot length (24.4%, 17.7% and 24.4%, respectively) after second application as compared to control. While shoots treated with three sprays of ProCa (500 mg·L^{-1}) with and without SP showed reduction in shoot length (24.5% and 33.9%, respectively) as compared to control after the third application. The shortest shoot length (31.7 cm) was recorded with three sprays of ProCa (500 mg·L^{-1}) in combination with SP as compared to control (47.4 cm).

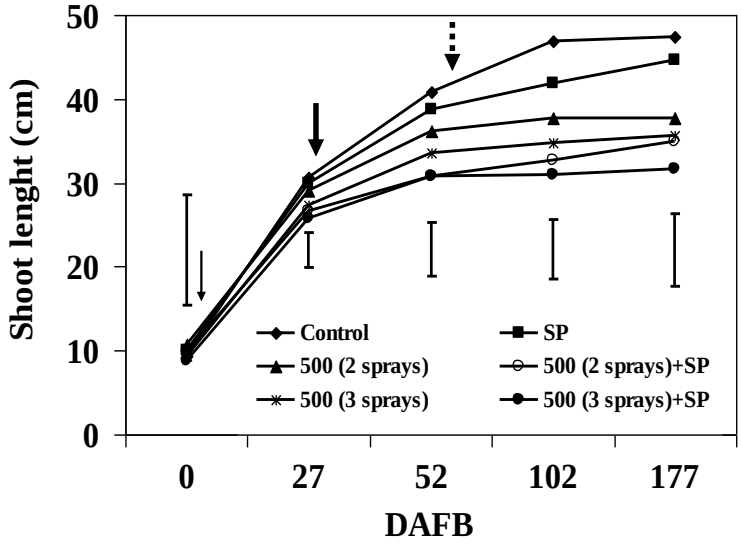

Figure 6.5. Effects of different number of Prohexadione-calcium (500 mg·L^{-1}) sprays with and without summer pruning on shoot length of 'Cripps Pink' apple tree. Vertical bars represent LSD value at $P \leq 0.05$. LSD ($P \leq 0.05$) on 0 DAFB = 13.11, 27 DAFB = 4.03, 52 DAFB = 6.44, 102 DAFB = 7.13, 177 DAFB = 8.74. LSD ($P \leq$

0.05) for treatments = 2.54, time = 2.32, treatment x time = 5.69. Thin arrow = first spray on 2 DAFB, thick arrow = second spray on 32 DAFB, dashed arrow = third spray on 62 DAFB. SP = summer pruning (156 DAFB).

6.3.2.2. Fruit colour and total anthocyanins concentration

The application of different number of ProCa (500 mg·L^{-1}) sprays with and without SP significantly ($P \leq 0.05$) affected visual fruit colour, percentage fruit for export and total anthocyanins concentration (Table 6.7). Fruit colour, percentage of fruit that met colour criteria for export and total anthocyanins concentration were significantly ($P \leq 0.05$) higher (57.5%, 88.0% and 195.9 µg·g^{-1} FW, respectively) with two sprays of ProCa (500 mg·L^{-1}) in combination with SP as compared to control. Three spray applications of ProCa (500 mg·L^{-1}) in combination with SP resulted in comparable visual fruit colour (51.3%) and percentage fruit for export (84.0%) with two sprays of ProCa (500 mg·L^{-1}) in combination with SP.

Table 6.7. Visual fruit colour and total anthocyanins concentration in apple skin affected by different number of Prohexadione-calcium sprays with and without summer pruning.

Treatment	Visual colour (% red blush)	Percentage fruit with > 40% red blush	Total anthocyanins (µg·g^{-1} FW)
Control	36.1 c	36.0 d	73.9 d
Summer pruning (SP)	49.1 ab	75.0 ab	142.3 bc
ProCa 500 mg·L^{-1} (2 sprays)	41.3 bc	54.0 cd	116.8 c
ProCa 500 mg·L^{-1} (2 sprays) +SP	57.5 a	88.0 a	195.9 a
ProCa 500 mg·L^{-1} (3 sprays)	48.9 ab	62.0 bc	162.7 ab
ProCa 500 mg·L^{-1} (3 sprays)+SP	51.3 a	84.0 a	163.9 ab
LSD ($P \leq 0.05$)	9.80	19.4	37.6

Means followed by the same letter within column are not significantly different at $P \leq 0.05$.

6.3.2.3. Chromaticity value a* and b*, lightness, hue angle and chroma

The reduction in chromaticity value b*, hue angle (h°), lightness (L*) and higher chromaticity value a* denotes to redder fruit skin colour. The application of two and three sprays of ProCa (500 mg·L^{-1}) with and without SP, showed a significant effect

on chromaticity value a*, b*, h°, L* and C* on the both sides of apple skin (Figure 6.6 and Table 6.8). The treatment of two sprays of ProCa (500 mg·L^{-1}) with SP showed the highest chromaticity a* values on the both sides of apple fruit skin (25.0 a* on ES and 15.9 a* on SS) as compared to control. Similarly, fruit treated with two sprays of ProCa (500 mg·L^{-1}) in combination with SP resulted in the lowest chromaticity value b* on the both sides of apple fruit skin (15.5 b* on ES and 20.2 b* on SS) than control.

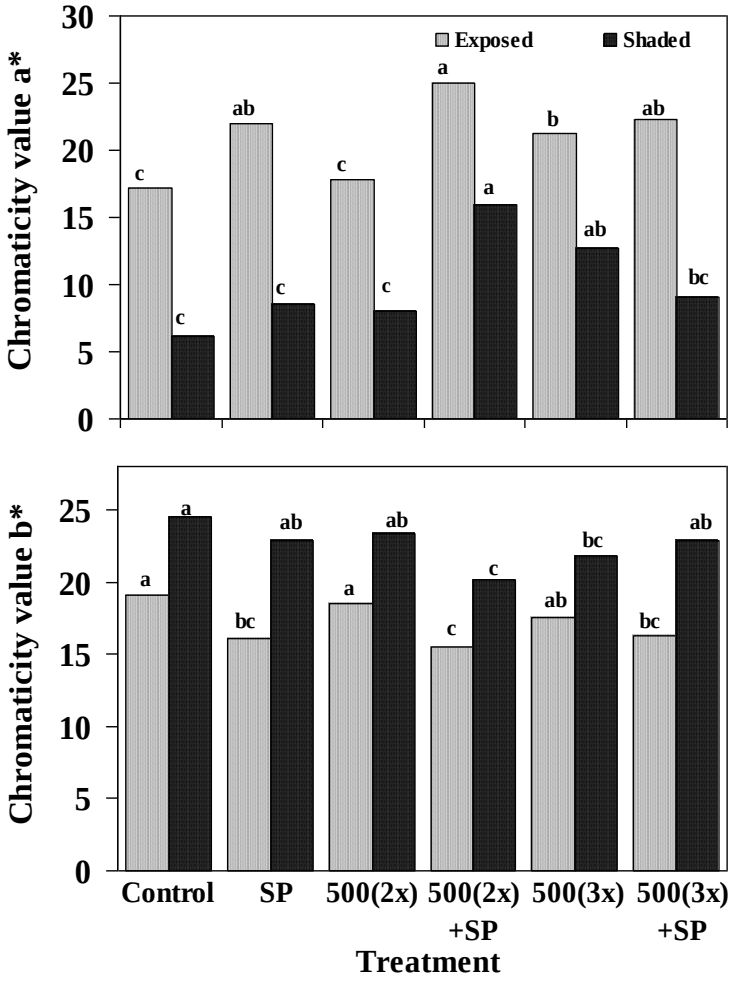

Figure 6.6. Effects of different number of Prohexadione-calcium sprays with and without summer pruning on chromaticity value a* and b* of apple fruit. LSD ($P \leq 0.05$) for a* ES = 3.32, a* SS = 3.99, b* ES = 1.58, b* SS = 1.76.

Fruit treated with two sprays of ProCa (500 mg·L^{-1}) in combination with SP exhibited the lowest L* and h° on the ES (26.3 *L and 32.4 h°) and the SS (34.9 L* and 52.5 h°) of apple fruit skin than control (Table 6.8). The application of three sprays of ProCa (500 mg·L^{-1}) in combination with SP and also SP alone showed a comparable L* (27.6 and 28.3 L*, respectively) and h° (36.8 and 37.3 h°, respectively) on the ES

Table 6.8. Effects of different number of Prohexadione-calcium sprays with and without summer pruning on lightness, hue angle and chroma in exposed and shaded sides of the fruit.

Treatment	Exposed side (ES)			Shaded side (SS)		
	Lightness (L*)	Hue angle (h^o)	Chroma (C*)	Lightness (L*)	Hue angle (h^o)	Chroma (C*)
Control	32.3 a	49.1 a	27.0 cd	40.5 a	75.4 a	25.8 abc
Summer pruning (SP)	28.3 cd	37.3 cd	28.1 bc	38.1 b	69.1 ab	25.3 c
ProCa 500 mg·L^{-1} (2 sprays)	31.6 ab	47.1 ab	26.6 d	36.9 ab	70.7 a	25.4 c
ProCa 500 mg·L^{-1} (2 sprays) +SP	26.3 d	32.4 d	29.9 a	34.9 c	52.5 c	26.6 a
ProCa 500 mg·L^{-1} (3 sprays)	29.6 bc	40.5 bc	28.5 ab	37.0 c	60.2 bc	26.4 ab
ProCa 500 mg·L^{-1} (3 sprays)+SP	27.6 cd	36.8 cd	28.2 bc	37.9 b	68.1 ab	25.4 bc
LSD ($P \leq 0.05$)	2.60	7.07	1.41	2.17	9.16	0.97

Means followed by the same letter within column are not significantly different at $P \leq 0.05$.

of apple fruit skin with two sprays of ProCa (500 mg·L^{-1}) in combination with SP. Chroma denotes to the degree of separation from grey towards a pure chromatic colour. Chroma or colour saturation on the ES and SS of the fruit skin was significantly ($P \leq 0.05$) higher (28.8 and 26.6 C*, respectively) with two sprays of ProCa (500 mg·L^{-1}) and SP as compared to other treatments

6.3.2.4. Flavonoids and other phenolic compounds

The concentration of cyanidin 3-*O*-galactoside, chlorogenic acid and total quercetin glycocides in fruit skin were significantly ($P \leq 0.05$) affected with the application of number of ProCa sprays with and without SP, excluding phloridzin (Table 6.9 and 6.10). The highest concentration of cyanidin 3-*O*-galactoside (569.4 µg·g^{-1} FW) was recorded in fruit skin with two sprays of ProCa (500 mg·L^{-1}) in combination with SP than control. However, the concentration of cyanidin 3-*O*-galactoside in fruit skin with three sprays of ProCa (500 mg·L^{-1}) with and without SP (525.3 and 535.1 µg·g^{-1} FW, respectively) were comparable to the amount of ProCa (500 mg·L^{-1}) two sprays with SP. The concentration of chlorogenic acid was highest (320.3 µg·g^{-1} FW) in fruit skin treated with three sprays of ProCa (500 mg·L^{-1}) than control.

The treatments of SP alone, three sprays of ProCa (500 mg·L^{-1}) with and without SP resulted in comparable concentrations of total quercetin glycosides (2987.3, 3013.5 and 2974.3 µg·g^{-1} FW, respectively) (Table 6.10). The individual compounds of quercetin glycosides such as quercetin 3-*O*-rutinoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-xyloside, quercetin 3-*O*-arabinoside and quercetin 3-*O*-rhamnoside were significantly ($P \leq 0.05$) affected with the application of number of ProCa sprays with and without SP. The treatments of SP alone and three sprays of ProCa (500 mg·L^{-1}) with and without SP resulted in highest concentrations of quercetin 3-*O*-rutinoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, and quercetin 3-*O*-xyloside in the fruit skin as compared to control, but the differences among these treatments were not significant. The treatment of three sprays of ProCa (500 mg·L^{-1}) with SP resulted in the highest concentration of quercetin 3-*O*-arabinoside (589.5 µg·g^{-1} FW) than control. However, SP alone, two sprays of ProCa (500 mg·L^{-1}) with SP and three sprays of ProCa (500 mg·L^{-1}) without SP showed a comparable amount of quercetin 3-*O*-arabinoside (569.0 µg·g^{-1} FW, 482.5 µg·g^{-1} FW and 584.6 µg·g^{-1} FW, respectively) to the three sprays with SP.

The highest concentrations of quercetin 3-*O*-rhamnoside (299.4 μg·g^{-1} FW) was recorded in fruit skin treated with three sprays of ProCa (500 mg·L^{-1}) than control. However, SP alone and three sprays of ProCa (500 mg·L^{-1}) in combination with SP resulted in a comparable concentration of quercetin 3-*O*-rhamnoside (287.5 μg·g^{-1} FW and 281.1 μg·g^{-1} FW, respectively) with three sprays of ProCa (500 mg·L^{-1}) treatment.

Table 6.9. Effects of different number of Prohexadione-calcium sprays with and without summer pruning on flavonoid and other phenolic compounds in apple fruit skin.

Treatment	Flavonoid and other phenolic compounds (μg·g^{-1} FW)		
	Cyanidin 3-*O*-galactoside	Chlorogenic acid	Phloridzin
Control	197.7 c	224.9 b	50.9
Summer pruning (SP)	456.8 ab	264.4 ab	59.9
ProCa 500 mg·L^{-1} (2 sprays)	307.6 bc	227.1 b	48.2
ProCa 500 mg·L^{-1} (2 sprays) +SP	569.4 a	264.3 ab	55.9
ProCa 500 mg·L^{-1} (3 sprays)	525.3 a	320.3 a	62.7
ProCa 500 mg·L^{-1} (3 sprays)+SP	535.1 a	270.0 ab	58.7
LSD ($P \leq 0.05$)	186.9	59.6	NS (5.52)

Means followed by the same letter within column are not significantly different at $P \leq 0.05$.Values within brackets represent standard error of means. NS = not significant ($P \leq 0.05$).

Table 6.10. Effects of different number of Prohexadione-calcium sprays with and without summer pruning on individual quercetin glycosides and total quercetin glycosides compounds in the fruit skin.

Quercetin glycosides (µg·g^{-1} FW)	Treatment						LSD ($P \leq 0.05$)
	Control	SP	ProCa (500 mg·L^{-1})				
			(2 sprays)	(2 sprays) + SP	(3 sprays)	(3 sprays) + SP	
Quercetin 3-*O*-rutinoside	22.7 b	106.5 a	45.0 ab	68.3 ab	90.7 a	90.88 a	66.9
Quercetin 3-*O*-galactoside	439.8 b	1335.7 a	793.5 ab	1012.1 ab	1315.8 a	1314.1 a	683.9
Quercetin 3-*O*-glucoside	61.7 b	150.0 a	93.6 ab	107.8 ab	160.7 a	148.7 a	84.1
Quercetin 3-*O*-xyloside	249.3 b	538.7 a	364.0 ab	440.2 ab	562.3 a	550.0 a	224.6
Quercetin 3-*O*-arabinoside	220.9 b	569.0 a	363.5 ab	482.5 a	584.6 a	589.5 a	256.4
Quercetin 3-*O*-rhamnoside	171.1 c	287.5 ab	194.9 bc	235.7 abc	299.4 a	281.1 ab	97.2
Total quercetin glycosides	1165.5 b	2987.3 a	1854.5 ab	2346.7 ab	3013.5 a	2974.3 a	1391.3

Means followed by the same letter within row are not significantly different at $P \leq 0.05$. SP = summer pruning.

6.3.2.5. Fruit firmness, soluble solids concentration, tiratable acidity and SSC/TA ratio

The application of two sprays of ProCa (500 mg·L^{-1}) with and without SP significantly ($P \leq 0.05$) affected fruit firmness and SSC, the effects of these treatments on TA and SSC/TA ratio were not significant (Table 6.11). Fruit treated with two sprays of ProCa (500 mg·L^{-1}) with SP and three sprays without SP exhibited highest fruit firmness (92.4 N) as compared to all other treatments. Even though, firmness in all treatments were above the standards requirements set by the industry. The highest SSC (14.6%) was recorded in fruit treated with three sprays of ProCa (500 mg·L^{-1}) without SP as compared to all other treatments.

Table 6.11. Effects of different number of Prohexadione-calcium sprays with and without summer pruning on firmness, soluble solids concentration, titratable acidity and SSC/TA in apple fruit.

Treatment	Firmness (N)	SSC (%)	TA (%malic acid)	SSC/TA ratio
Control	84.6 b	13.6 c	0.73	18.6
Summer pruning (SP)	85.4 b	13.8 bc	0.75	18.5
ProCa 500 mg·L^{-1} (2 sprays)	87.1 b	13.6 c	0.73	18.6
ProCa 500 mg·L^{-1} (2 sprays) +SP	92.4 a	14.1 b	0.76	18.6
ProCa 500 mg·L^{-1} (3 sprays)	92.4 a	14.6 a	0.79	18.4
ProCa 500 mg·L^{-1} (3 sprays)+SP	86.8 b	14.0 bc	0.76	18.3
LSD ($P \leq 0.05$)	4.84	0.44	NS (0.02)	NS (0.52)

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. NS = not significant ($P \leq 0.05$). Values within brackets represent standard error of means.

6.3.2.6. Concentration of ascorbic acid and total antioxidants

The data presented in Table 6.12 show that the concentrations of ascorbic acid and total antioxidants in apple pulp were not significantly affected with the application of different number of ProCa (500 mg·L^{-1}) sprays with and without SP except total antioxidants in the skin. Total antioxidants were significantly ($P \leq 0.05$) higher (35.2 mM TE·g^{-1} FW) in the fruit treated with three sprays of ProCa (500 mg·L^{-1}) in combination with SP than control. Fruit-treated with three sprays of ProCa (500 mg·L^{-1}) had a comparable level of total antioxidants (29.2 mM TE·g^{-1} FW) with three

sprays of ProCa (500 mg·L^{-1}) with SP. Total antioxidants in the skin were higher than in the pulp as observed in all treatments.

Table 6.12. Effects of different number of Prohexadione-calcium spray with and without summer pruning on ascorbic acids concentration and total antioxidants in apple skin and pulp.

Treatment	Ascorbic acid (mg·100g^{-1} FW)	Total antioxidants (mM TE·g^{-1} FW)	
		skin	Pulp
Control	10.1	23.1 b	0.54
Summer pruning (SP)	10.4	24.7 b	0.65
ProCa 500 mg·L^{-1} (2 sprays)	9.46	23.9 b	0.60
ProCa 500 mg·L^{-1} (2 sprays) +SP	10.4	27.6 b	0.65
ProCa 500 mg·L^{-1} (3 sprays)	9.72	29.2 ab	0.64
ProCa 500 mg·L^{-1} (3 sprays)+SP	9.87	35.2 a	0.61
LSD ($P \leq 0.05$)	NS (0.68)	7.19	NS (0.04)

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. NS = not significant $P \leq 0.05$. Values within brackets represent standard error of means.

6.3.2.7. Concentrations of total sugars and individual organic acids

Total sugars concentration in apple fruit was significantly ($P \leq 0.05$) affected with the treatments of spray application of ProCa (500 mg·L^{-1}) with and without SP (Table 6.13). The comparable amount of total sugars in apple fruit was recorded in fruit-treated with three sprays of ProCa (500 mg·L^{-1}) with and without SP and also in SP alone (109.9, 109.1 and 109.5 g·kg^{-1}, respectively). Individual organic acids such as malic, citric, succinic and also total organic acids were significantly ($P \leq 0.05$) affected with the application of different number of ProCa sprays with and without SP, excluding tartaric acid (Table 6.13). SP alone significantly ($P \leq 0.05$) increased malic (6.95 g·kg^{-1}), citric (0.07 g·kg^{-1}), succinic (0.56 g·kg^{-1}) and total organic acids (7.67 g·kg^{-1}) concentration as compared to control and other treatments.

Table 6.13. Effects of number of Prohexadione-calcium spray with and without summer pruning on total sugars and organic acids concentration in apple fruit.

Treatment	Total sugars	Organic acids concentration ($g \cdot kg^{-1}$)				
	($g \cdot kg^{-1}$)	Malic	Citric	Tartaric	Succinic	Total acids
Control	102.6 b	5.29 b	0.05 b	0.09	0.44 ab	5.88 b
Summer pruning (SP)	109.5 a	6.95 a	0.07 a	0.09	0.56 a	7.67 a
ProCa 500 $mg \cdot L^{-1}$ (2 sprays)	102.7 b	5.18 b	0.05 b	0.09	0.37 b	5.69 b
ProCa 500 $mg \cdot L^{-1}$ (2 sprays) +SP	103.5 b	5.39 b	0.05 b	0.09	0.43 ab	5.96 b
ProCa 500 $mg \cdot L^{-1}$ (3 sprays)	109.9 a	5.29 b	0.05 b	0.09	0.45 ab	5.88 b
ProCa 500 $mg \cdot L^{-1}$ (3 sprays)+SP	109.1 a	5.05 b	0.05 b	0.09	0.46 ab	5.66 b
LSD ($P \leq 0.05$)	4.57	1.32	0.01	NS (0.002)	0.14	1.45

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. NS = not significant $P \leq 0.05$. Values within brackets represent standard error of means.

6.4. Discussion

As expected, the spray application of ProCa reduced shoot length in both experiments during 2007-08 growing season. The efficacy of ProCa in reducing shoot length was dependant on the concentrations, times and number of sprays application. Possibly, the reduction in shoot length with the spray application of ProCa may be ascribed to the reduced of endogenous concentrations of the biologically active GA_1 and increased the concentrations of inactive GA_{20} via interfering the 3-ß hydroxylation of GA_{20} to GA_1 (Evans et al., 1999; Lo Giudice et al., 2004; Rademacher and Kober, 2003).

In the present study, the reduction of shoot length was pronounced after an initial spray at higher concentrations of ProCa ranged from 14% to 19% of control trees (Figure 6.2 and 6.5). Similarly, the reduction of shoot growth after the first spray has been reported (Byers and Yoder, 1999; Medjdoub et al., 2005; Medjdoub et al., 2004; Miller, 2002; Rademacher and Kober, 2003; Unrath, 1999). The gradual reduction in shoot length after two and more spray applications have also been reported (Miller, 2002). Similar observation on the relative increase in the reduction of shoot length recorded up to 33% in both experiments. Both experiments exhibited the single application of ProCa was not enough to prevent shoot growth of this cultivar. This may due to the rapid degradation of ProCa in the apple leaf (Evans et al., 1999; Evans et al., 1997; Rademacher and Kober, 2003) thereby, the second application of ProCa (Greene, 1999; Medjdoub et al., 2005; Unrath, 1999) or more were essential to retard the shoot regrowth (Elfving et al., 2003b; Rademacher and Kober, 2003).

In experiment 1, the higher concentrations of ProCa with three sprays and SP alone, increased percent red blush, percent fruit for export, higher chromaticity value a*, lower chromaticity value b*, lightness and hue angle on the fruit surface compared to control (Table 6.1, Figure 6.3 and 6.4). Similarly, in Experiment 2, multiple sprays of ProCa with SP improved fruit colour, higher chromaticity value a*, lower chromaticity value b*, lightness and hue angle on the fruit surface (Table 6.7, 6.8 and Figure 6.6). The intensification of red skin colouration on fruit skin has also been reported after ProCa treatment in apple (Byers and Yoder, 1999; Greene, 1999; Zadravec et al., 2008), 'Seyval' grape berries (Lo Giudice et al., 2004) and 'Forelle'

pear (Smit et al., 2005). The increased of fruit colour may be ascribed to the increased concentration of total anthocyanins and polyphenolics compounds due to the spray application of ProCa that reduced the shoot elongation. ProCa induced red skin colour (lower lightness) with more saturated (higher chroma) on the blush side of 'Fuji' apple and also higher total anthocyanins concentration (Medjdoub et al., 2005). Similarly, the higher concentration of total anthocyanins was found in 'Fuji' apple with the application of ProCa (Mata et al., 2006a). This may be attributed to the reduced vegetative growth, and consequently improves light penetration into tree canopy (Basak, 2004; Prive and Stewart, 2002). The increased light penetration into tree canopy has been reported to up regulate the activities of enzymes involved in biosynthesis of anthocyanins such as phenylalanine ammonia-lyase (PAL) and UDP galactose:flavonoid 3-*O*-galactosyltransferase (UFGalT). Earlier, light has been reported to up regulated the activity of UFGalT in 'Fuji' apple (Ju et al., 1999b) and PAL activity in 'Royal Gala' apple (Dong et al., 1995).

The increase of concentrations of polyphenolic compounds in apple skin due to the spray application of ProCa such as cyanidin 3-*O*-galactoside (range from 95% to 188%), chlorogenic acid (range from 10% to 42%), quercetin glycosides (range from 102% to 179%) and individual quercetin glycosides may also be coincided to the increase of total anthocyanins concentration (Table 6.2, 6.3 6.9 and 6.10). In contrast, Awad and de Jager (2002) claimed that the application of 125 $mg \cdot L^{-1}$ of three sprays application of ProCa did not affect the formation of anthocyanins (cyanidin 3-*O*-galactoside) and chlorogenic acid in 'Jonagold' apple skin grown in the humid-temperate climate of the Netherlands. The contradictory outcomes of cyanidin 3-*O*-galactoside and chlorogenic acid in apple skin of these two cultivars may be ascribed to the different environmental factors at both locations such as light and temperature (Saure, 1990), which play a major role in regulating those polyphenolic compounds. In addition, ProCa application has also been reported to inhibit the formation of anthocyanins in apple leaf (Rademacher, 2000; Rademacher et al., 1992) and carrot cell (Ilan and Dougall, 1992) by blocking flavanone 3-hydroxylase (FHX or F3H) (Rademacher, 2000). F3H is a key enzyme in the synthesis of dihydrokaempferol or dihydroquercetin and their activity is necessary for the production of both anthocyanins and flavonols (Ubi, 2007). However, a rapid metabolism of ProCa within a few weeks (Evans et al., 1997) and not persistent in the environment (Prive

et al., 2006) may not sufficient to block the accumulation of anthocyanins in apple skin. In addition, in the present study, ProCa has been sprayed on every 30 days. Moreover, Evans et al. (1999) reported that the half life of ProCa in the plant was about two weeks before degrading to the naturally occurring propane-1,2,3-tricarboxylic acid and take less than seven days in soil before decomposition to CO_2.

Summer pruning (SP) has also improved fruit colour and the concentration of total anthocyanins in both experiments (Table 6.1 and 6.7). Similarly, increased fruit colour with SP has been previously reported in 'McIntosh' (Autio and Greene, 1990) and 'Delicious' (Marini and Barden, 1982) and 'Cripps Pink' (Whale, 2005) apples. Similar mechanism as ProCa application may be involved in enhancing apple fruit colour in SP treatment by improving light penetration direct onto fruit surface as reported earlier by Saure (1990). However, SP has been reported to be very expensive and labour intensive for orchard management practices (Byers and Yoder, 1999; Cline et al., 2008) as compared to ProCa which was easily applied by spraying and safe to consumer and environment (Rademacher and Kober, 2003).

Fruit firmness, SSC and TA in both experiments was not consistent (Table 6.4 and 6.11). Increased fruit firmness in pear due to ProCa spray has been reported (Elfving et al., 2003b). In contrast, no apparent effects of ProCa spray have been reported on fruit firmness in apples (Byers and Yoder, 1999; Medjdoub et al., 2005; Medjdoub et al., 2004; Miller, 2002) and also in pears (Southwick et al., 2004). Faust (1972) reported that the changes of fruit firmness in fruit due to the plant growth regulators are sudden and cannot be explained by changes in pectin content in cell walls. In both experiments, no specific trend could be deduced in regards to ProCa spray application on SSC. ProCa treatments had no effect on SSC has been reported (Mata et al., 2006a; Miller, 2002). In contrast, Basak (2004) claimed that two sprays application of 200 $mg \cdot L^{-1}$ ProCa increased SSC in 'Elstar' apple fruit as compared to the single application. The possible mechanism subjected to the increased and also the reduced fruit firmness, SSC and also TA is not yet known. However, all these fruit quality parameters were within the range of standards set for export markets of 'Cripps Pink' apple. Fruit firmness was >68 N, SSC in between 13 and ≥ 15 ^{o}Brix and 0.7 to 0.9% TA (Cripps et al., 1993; Department of Agriculture Western Australia, 2000; Mackay et al., 1994). Higher total antioxidants in apple skin may

also be ascribed to the increase of total anthocyanins concentration, in which anthocyanins concentration is one of the important antioxidant in the red-skinned apple (Table 6.5 and 6.12). The effects of ProCa on total sugars concentration in both experiments are inconsistent (Table 6.6 and 6.13). However, ProCa spray application did not change the concentration of individual sugars in 'Royal Gala' apple has been reported (Mata et al., 2006b), which similar to the outcomes of total sugars concentration in apple fruit in the Experiment 1. It warrants further investigation. In addition, no specific trends could be deduced to the increase of organic acids concentration subjected to ProCa spray applications in both experiments (Table 6.6 and 6.13). The concentration of malic acid (>90%) dominates among concentrations of total acids in this cultivar followed by succinic, tartaric and citric acid. Similarly, Hulme and Rhodes (1971) reported that malic acid accounts for about 90% of total acids in apple fruit.

In conclusions, three sprays application of ProCa (500 $mg \cdot L^{-1}$) on 3, 33 and 63 DAFB or 500 $mg \cdot L^{-1}$ with two sprays (2 and 32 DAFB) in combination with SP are effective in reducing shoot growth, improving fruit colour development and also maintaining other fruit quality attributes in 'Cripps Pink' apple cultivar grown in Mediterranean climate of Western Australia

CHAPTER 7

Promotive Effects of Lysophosphatidylethanolamine on Fruit Colour Development and Quality in 'Cripps Pink' Apples

Summary

The effects of various concentrations and number of lysophophatidylethanolamine (LPE) sprays on colour development, accumulation of anthocyanins and polyphenolic compounds and other fruit quality attributes of 'Cripps Pink' apples were investigated. The trees were sprayed with an aqueous solution containing 125, 250 and 375 $mg \cdot L^{-1}$ of LPE at a commercial orchard in Carmel, Perth Hill, Western Australia. The treatments were applied approximately four and two weeks prior to anticipated commercial harvest. Fruit colour development, concentration of total anthocyanins and polyphenolic compounds in this cultivar were significantly enhanced with the application of two sprays of LPE (125 $mg \cdot L^{-1}$, at two and four weeks before commercial harvest) or single spray (250 $mg \cdot L^{-1}$, at four weeks anticipated to commercial harvest) as compared to control. Lower concentrations of LPE (125 and 250 $mg \cdot L^{-1}$) with double and single spray, respectively exhibited significantly lower chromaticity value b*, lightness, hue angle and higher chromaticity value a* on the both sides of apple fruit skin as compared to control. Polyphenolic compounds especially cyanidin 3-*O*-galactoside, quercetin glycosides and also individual quercetin glycosides such as quercetin 3-*O*-xyloside, quercetin 3-*O*-arabinoside and quercetin 3-*O*-rhamnoside were increased in fruit treated with the lower concentration of LPE (125 and 250 $mg \cdot L^{-1}$) with double and single spray, respectively. Fruit firmness was increased with the application of two sprays of LPE (250 $mg \cdot L^{-1}$) as compared to control. Meanwhile, soluble solids concentration (SSC), titratable acidity (TA), SSC/TA ratio, concentration of total sugars and ascorbic acid was maintained with the application LPE treatments. In conclusion, two sprays (at two and four weeks anticipated to commercial harvest) of LPE (125 $mg \cdot L^{-1}$) and 250 $mg \cdot L^{-1}$ (at four weeks before harvest) effectively enhanced fruit colour development through increased concentrations of total anthocyanins and polyphenolic compounds and also maintain other major fruit quality in 'Cripps Pink' apple.

7.1. Introduction

Red skin colouration in 'Cripps Pink' apple is an important criterion in domestic and international market acceptance. Optimum colour development at harvest is often a problem in this cultivar and also reduces profit to Western Australian apple growers. Red colour in apple skin is attributed to the presences of anthocyanin compounds, which belongs to a class of flavonoids. The accumulation of anthocyanins are influenced by light, temperature, ethylene and cultural practices such as pruning, chemicals application and irrigation (Curry, 1997; Lancaster, 1992; Saure, 1990). Cyanidin 3-*O*-galactoside is the most vital pigment responsible for red colouration in apple skin (Lancaster, 1992; Tsao et al., 2003). Various approaches have been tested to improve red skin colouration in apple through increased its accumulation of anthocyanins using preharvest application such as methyl jasmonate (Rudell et al., 2002), combination of aminoethoxyvinylglycine (AVG) and ethephon (Whale et al., 2008), paclobutrazol and ethepon alone (Saure, 1990) and the response was genotype dependent.

A newly developed plant growth regulators, lysophophatidylethanolamine (LPE) is a natural lipid which derived from natural sources such as egg yolk and soy lecithin (Özgen et al., 2004). In plants, phosphoinositides, phosphatidic acid, diacyglycerol pyrophosphate, lysophospholipids, and phospholipase A_2, C and D are known as the key lipid signalling components (Munnik, 2001; Ryu, 2004; Testerink and Munnik, 2005; Wang, 2005). In addition, LPE is formed from the parent phospholipid; phosphotidylethanolamine (PE) by action of the phospholipase A_2 (PLA_2) (Hong et al., 2009b).

Exogenous application of LPE has been reported to accelerate ripening and prolong life of tomato fruit (Farag and Palta, 1993a; Farag and Palta, 1991a; Farag and Palta, 1992), enhance ethylene production (Farag and Palta, 1989; Hong et al., 2001; Kang et al., 2003), retard senescence of tomato leaf and fruit (Farag and Palta, 1993b), prolonged vase-life of cut flowers (Kaur and Palta, 1997), inhibit the activity of phospholipase D (PLD), a membrane degrading enzymes (Kang et al., 2003; Ryu et al., 1997). It also improves fruit colour and anthocyanins concentration in cranberries (Özgen et al., 2004; Özgen and Palta, 2003), apples (Farag and Palta, 1991b), table grapes (Hong, 2008) and red pepper (Kang et al., 2001; Kang et al., 2003).

Time of preharvest spray seems to be critical in apple fruit particularly in improving apple skin colour and quality. The spray application of ethephon two to three weeks prior to commercial harvest enhanced red skin colour in 'Tydeman Early', 'Jonathan' and 'Delicious' apples (Brohier and Faragher, 1984; Jones, 1979). Whale et al. (2008) claimed that the preharvest spray application of AVG four to five weeks prior to commercial harvest followed by ethephon two to three weeks later enhanced red skin colour in 'Cripps Pink' apple. The accurate concentration of preharvest LPE also appears to be crucial as reported earlier that the promotive effects of LPE is concentration dependant in horticulture crops such as tomato, cranberry and snapdragon flowers (Farag and Palta, 1993b; Kaur and Palta, 1997; Özgen et al., 2004; Özgen et al., 2005; Ryu et al., 1997).

No information is available on the effects of LPE in improving fruit colour and other fruit quality attributes especially in 'Cripps Pink' apple cultivar. Hence, this investigation aimed to evaluate the effects of different concentrations and number of sprays of LPE on fruit colour development, accumulation of anthocyanins and polyphenolic compounds and also other fruit quality attributes of 'Cripps Pink' apples grown under Western Australia conditions.

7.2. Materials and Methods

7.2.1. Location and climatic conditions

The experiment was conducted in a commercial orchard at Carmel, Perth Hills (latitude 32°1'0"S; longitude 116°5'60"E) on 'Cripps Pink' apple trees during 2007-08. Rainfall and evapotranspiration were higher in the first week of trial period. Summary of climatic conditions at the commercial apple orchard (Figure 7.1).

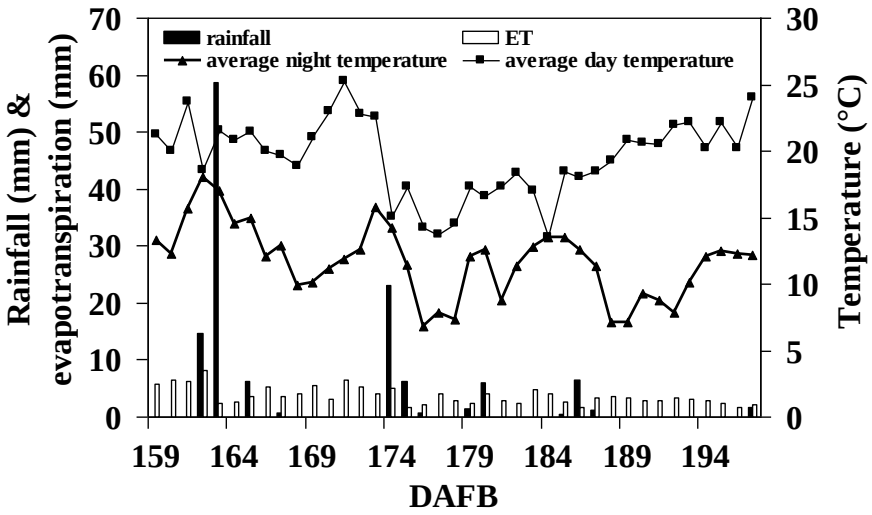

Figure 7.1. Daily average day and night temperatures, rainfall and evapotranspiration (ET) during 2007-08 growing season at a commercial orchard, Carmel, Perth Hills, Western Australia.

7.2.2. Treatments

Twenty-eight of 'Cripps Pink' apple trees, grafted on MM.109 rootstock were used in the experiment. Trees were 20-year-old and planted in the east-west direction, maintaining row distances of 4.2 m and plant distances of 2.4 m. Full bloom (>80% of the buds are open) occurred on 26th October 2007. The experiment was laid out following a randomized complete block design, single tree treated as an experimental unit and include four replications. The treatments were different concentrations and number of LPE sprays i.e. (i) control, (ii) 125 mg·L^{-1} (single spray at four weeks prior to commercial harvest), (iii) 125 mg·L^{-1} (double spray at four and two weeks prior to commercial harvest), (iv) 250 mg·L^{-1} (single spray at four weeks prior to commercial harvest), (v) 250 mg·L^{-1} (double spray at four and two weeks prior to commercial harvest) (vi) 375 mg·L^{-1} (single spray at four weeks prior to commercial harvest), and (vii) 375 mg·L^{-1} (double spray at four and two weeks prior to commercial harvest). LPE was obtained from Doosan Corporation Ltd., Chung-gu, Seoul, Korea. The spray solution of different concentrations of LPE and a non-ionic surfactant Tween®20 (0.05 % v/v, Sigma-Aldrich Chemie GMbh, Steinheim, Germany) were sprayed onto the fruit of whole tree until run off. These spray treatments were applied on 157 and 172 DAFB, approximately four and two weeks anticipated to commercial harvest (190 DAFB). A professional sprayer (Selecta Trolleypak MKII, Model N TR25-P, Silvan Autralia Ltd, Wantirna, Australia) was used for spraying the aqueous solutions. Unsprayed trees served as control.

Parameters evaluated were percentage red blush >40 of fruit surface, percentage fruit for export, fruit colour, fruit firmness, titratable acidity (TA), soluble solids concentration (SSC), SSC/TA ratio, concentration of anthocyanins, polyphenolic compounds, ascorbic acid, total antioxidants and concentrations of sugars and organic acids.

7.2.3. Temperature monitoring at experimental site

Daily temperatures in the orchard were recorded using data loggers (Tinytag*Plus* Gemini Data Logger, UK) and all temperatures data were obtained using Gemini Logger Manager Software (Version 2.8). Rainfall and evapotranspiration (ET) data were obtained from Bureau of Meteorology, Perth, Western Australia (Figure 7.1). The average of daily day and night temperatures was calculated between sunrise and sunset times as detailed in Chapter 4, Section 4.2.5.

7.2.4. Fruit sampling

Fruit were harvested on 190 DAFB (29th April 2008). Twenty-five fruit were randomly chosen from all parts of the tree canopy up to height of 2 m from the ground for fruit quality assessment.

7.2.5. Fruit quality: fruit colour

7.2.5.1. Surface skin colour

Individual apple was visually assessed for percentage red blush and scores were given ranging from 0 to 100% as detailed in Chapter 3, Section 3.3.1. A HunterLab ColorFlex 45^{o}/0^{o} Spectrophotometer was used to record fruit colour parameters icluding chromaticity value a*, b*, lightness (L*), chroma (C*), hue angle (h°) as described in Chapter 3, Section 3.3.2.

7.2.5.2. Analysis of skin pigment

7.2.5.2.1 Total anthocyanins

Underlying tissue was scrapped off from apple skin, and then weighed and extracted for analysis of anthocyanins. Total anthocyanins concentration of extracted apple skin was determined at 530 nm using an UV-VIS spectrophotometer (Jenway Spectrophotometers Model 6405, Dunmow, Essex, UK) and quantified following the

method outlined by Whale and Singh (2007) as detailed in Chapter 3, Section 3.3.3.1.

7.2.5.2.2 Flavonoids and other phenolic compounds

Chemicals standards used for flavonoids and other phenolic compounds were purchased from various manufacturers as detailed in Chapter 3, Section 3.3.3.2.1. Extraction, identification and quantification flavonoids and other phenolic compounds of apple skin were according to the procedure outlined by Whale and Singh (2007) with some modifications as detailed in Chapter 3, Section 3.3.3.2.2. These polyphenolic compounds were identified and quantified using a reversed-phase high performance liquid chromatography (RP-HPLC) following the procedure described in Chapter 3, Section 3.3.3.2.3 and 3.3.3.2.4. Flavonoids and other phenolic compounds were re-confirmed using HPLC-electrospray ionization mass spectrometry (HPLC-ESI-MS) as mentioned in Chapter 3, Section 3.3.3.2.3.

7.2.6. Other fruit quality parameters

7.2.6.1. Fruit firmness

Fruit firmness was determined using an electronic pressure tester (Model EPT-1 pressure tester, Lake City Technical products Inc., Kelowna, BC, Canada) fitted with 11 mm tip as described in Chapter 3, Section 3.4.2

7.2.6.2. Titratable acidity, soluble solids concentration and SSC/TA ratio

Titratable acidity (TA) was determined by titrating apple juice against 0.1N NaOH using phenolphthalein as an indicator as outlined in Chapter 3, Section 3.4.3. An infrared digital refractometer (Atago-Palette PR 101, Atago Co. Ltd., Itabahi-Ku, Tokyo, Japan) was used to measured soluble solids concentration (SSC) as described in Chapter 3, Section 3.4.4. SSC/TA ratio was calculated by dividing SSC with the TA.

7.2.7. Determination of concentration of ascorbic acid, total antioxidants, individual sugars and organic acids

7.2.7.1. Ascorbic acid

The concentration of ascorbic acid from apple pulp was determined following the

method outlined by Jagota and Dani (1982) and Malik and Singh (2005) as detailed in Chapter 3, Section 3.5.1.

7.2.7.2. Total antioxidants

The levels of total antioxidants of apple skin and pulp were determined following to the method of Brand-Williams et al. (1995) and Khan et al. (2008) as outlined in Chapter 3, section 3.5.2.

7.2.7.3. Individual sugars

Chemicals used for sucrose, fructose and sorbitol determination were purchased from different manufacturers as detailed in Chapter 3, Section 3.5.3.1. The procedures of extraction, centrifugation and filtration of sugars samples were as described in Chapter 3, Section 3.5.3.2. Individual sugars were separated, identified and quantified using the HPLC system (Waters Corp., Milford, Mass., USA) fixed with the Fast Carbohydrate Analysis column (Aminex-HPX 87C, 100 x 7.8 mm) (Bio-Rad Laboratories, Hercules, CA, USA) as described in Chapter 3, Section 3.5.3.3. The concentrations of total sugars were the cumulative of individual sugars as mentioned above.

7.2.7.4. Individual organic acids

The chemicals used for individual organic acids determination as explained in Chapter 3, Section 3.5.3.1. A hand blender (Model HB95, Breville Pty. Ltd., New South Wales, Australia) was used to homogenized apple juice as detailed in Chapter 3, Section 3.5.3.2. Separation, identification and quantification of individual organic acids in apple juice using the HPLC system (Waters Corp., Milford, Mass., USA) fixed with a Bio Rad Aminex-HPX 87H (300 x 7.8 mm; particle size 9 µm) (Bio-Rad Laboratories, Inc., Hercules, USA) column as detailed in Chapter 3, Section 3.5.3.3.

7.2.8. Statistical analysis

The influences of various concentrations and number of LPE sprays on various parameters were assessed within the analysis of variance (ANOVA) using GLM (General Linear Models) procedures with SAS 9.1 software package, SAS Institute Inc, Cary, NC, USA. Least significant difference (LSD) was calculated at level $P \leq$

0.05 following a significant F test (SAS Institute Inc., 1999). All the assumptions of analysis were checked to ensure validity of statistical analysis.

7.3. Results

7.3.1. Effects on fruit colour and total anthocyanins concentration

Fruit colour (visual fruit colour and percentage of fruit with more than 40% surface red blush) and concentration of total anthocyanins was significantly ($P \leq 0.05$) affected with the application of different concentrations and number of LPE sprays (Table 7.1). Double spray of LPE (125 $mg \cdot L^{-1}$) and single spray (250 $mg \cdot L^{-1}$) exhibited the higher visual fruit colour (52.0% and 51.9%, respectively) and also concentration of total anthocyanins (168.14 and 174.02 $\mu g \cdot g^{-1}$ FW, respectively) as compared to control. However, single spray of LPE (125 $mg \cdot L^{-1}$) showed the comparable percentage of red blush (50.7%) and also concentration of total anthocyanins (164.2 $\mu g \cdot g^{-1}$ FW) to the above mentioned treatments. Meanwhile, single spray of LPE (250 $mg \cdot L^{-1}$) resulted in the highest percentage of fruit with more than 40% surface red blush as compared to control.

Table 7.1. Effects of different concentrations and number of LPE spray on fruit colour and total anthocyanins concentration in apple.

LPE ($mg \cdot L^{-1}$)	Visual colour (% red blush)	Percentage fruit with > 40% red blush	Total anthocyanins ($\mu g \cdot g^{-1}$ FW)
Control	39.9 d	54.0 c	85.6 c
125 (single spray)	50.7 ab	84.0 ab	164.2 a
125 (double spray)	52.0 a	83.0 ab	168.1 a
250 (single spray)	51.9 a	92.0 a	174.0 a
250 (double spray)	50.4 abc	84.0 ab	142.5 b
375 (single spray)	42.6 bcd	64.0 bc	102.7 c
375 (double spray)	42.1 cd	60.0 c	102.3 c
LSD ($P \leq 0.05$)	8.42	22.9	21.1

Means followed by the same letter within column are not significantly different at $P \leq 0.05$

7.3.2. Effects on chromaticity value a*, b*, L*, hue angle and chroma

The reduction in chromaticity value b*, lightness (L*), hue angle (h°) and higher chromaticity value a* denote to redder fruit skin colour. The application of different concentrations and number of LPE sprays significantly ($P \leq 0.05$) affected the chromaticity value a*, b*, lightness and hue angle on both sides of apple skin (Table 7.2 and Figure 7.2). But, chroma on the ES of apple skin was not affected with the application of different concentrations and number of LPE sprays. The application of single spray of LPE (250 mg·L^{-1}) resulted in significantly ($P \leq 0.05$) increased chromaticity value a* (21.1 a*) on the ES of fruit skin as compared to control. Whilst, single and double spray of LPE (125 and 250 mg·L^{-1}) exhibited higher and comparable chromaticity value a* on SS of fruit skin as compared to all other treatments. The lowest and comparable chromaticity value b* on the ES (13.4 b* and 13.5 b*, respectively) of fruit skin were recorded with double spray of LPE (125 mg·L^{-1}) and single spray of LPE (250 mg·L^{-1}) than control. Meanwhile, single spray of LPE (250 mg·L^{-1}) exhibited the lowest chromaticity value b* on the SS (19.1 b*) of fruit skin than control.

Table 7.2. Effects of different concentrations and number of LPE sprays on chromaticity value a* and b* on the exposed (ES) and shaded (SS) sides of fruit skin.

LPE (mg·L^{-1})	Chromaticity value a*		Chromaticity value b*	
	ES	SS	ES	SS
Control	13.6 c	-0.67 b	18.1 a	25.9 a
125 (single spray)	20.2 ab	7.36 a	14.6 bc	21.1 c
125 (double spray)	20.8 ab	8.93 a	13.5 c	19.5 cd
250 (single spray)	21.1 a	10.31 a	13.4 c	19.1 d
250 (double spray)	19.3 ab	7.74 a	14.7 bc	21.0 c
375 (single spray)	17.3 bc	2.26 b	15.6 b	23.5 b
375 (double spray)	18.3 ab	1.74 b	15.8 b	24.8 ab
LSD ($P \leq 0.05$)	3.75	3.40	1.68	1.80

Means followed by the same letter within column are not significantly different at $P \leq 0.05$.

The application of two sprays of LPE (125 mg·L^{-1}) resulted in significantly ($P \leq 0.05$) lower L* on both sides of the fruit skin as compared to control (Figure 7.2).

The lowest hue angle on the both sides of fruit skin was recorded with the application of single spray of LPE (250 mg·L^{-1}) than those in control. Chroma on the SS of the fruit was significantly ($P \leq 0.05$) lower (vivid red colour) in fruit treated with double spray of LPE (125 mg·L^{-1}) and single spray (250 mg·L^{-1}) as compared to control.

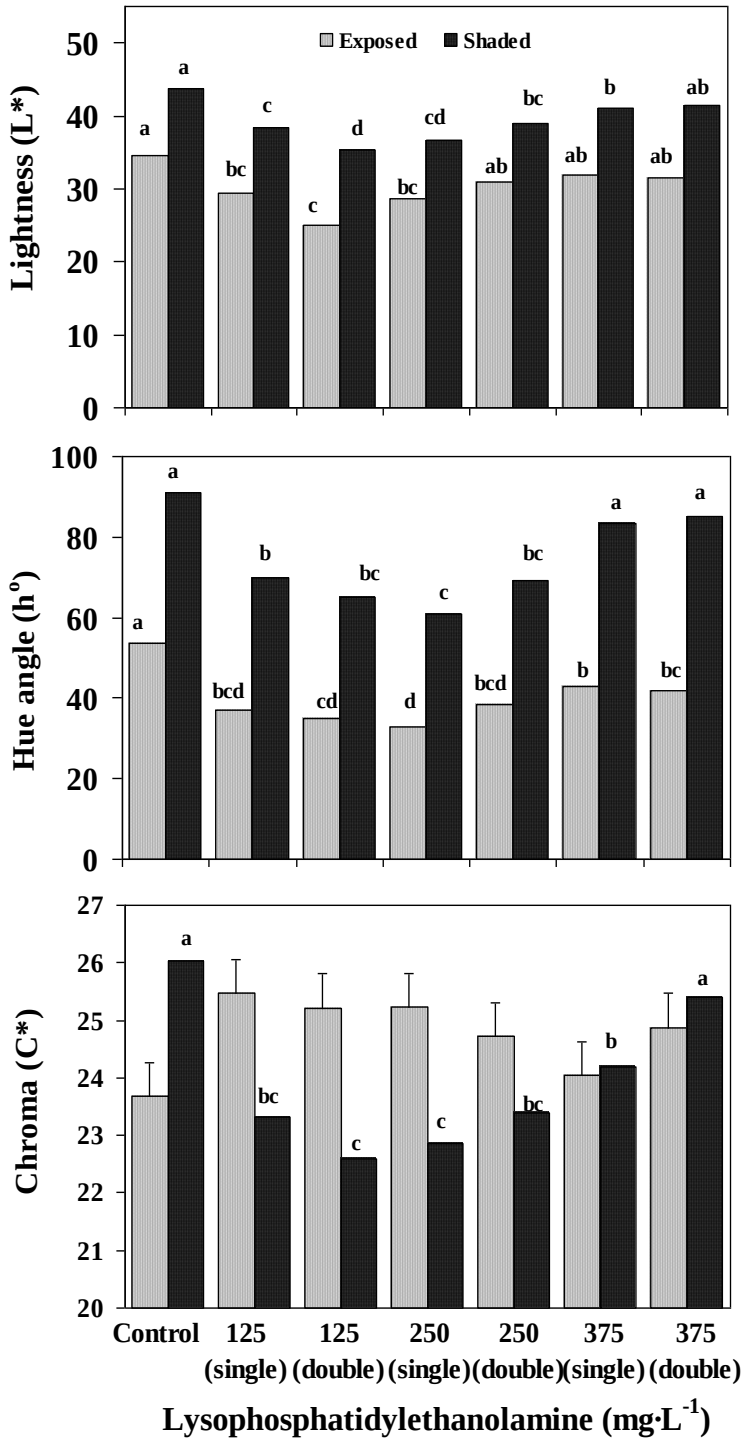

Figure 7.2. Effects of different concentrations and number of LPE sprays on lightness and hue angle in the exposed and shaded sides of 'Cripps Pink' apple. Vertical bars represent standard error of means. LSD ($P \leq 0.05$) for lightness (ES) =

5.00, lightness (SS) = 2.54, hue angle (ES) = 8.61, hue angle (SS) = 8.56, chroma (ES) = NS (0.59), chroma (SS) = 2.10. NS = not significantly different at $P \leq 0.05$.

7.3.3. Effects on flavonoids and other phenolic compounds

Nine flavonoids and other phenolic compounds were identified and re-confirmed using HPLC-ESI-MS in the apple skin of 'Cripps Pink' as mentioned in Chapter 4, Section 4.3.3.3. The concentration of anthocyanins (cyanidin 3-*O*-galactoside), hydroxycinnamic acids (chlorogenic acid) and dihydrochalcones (phloridzin) were significantly ($P \leq 0.05$) affected with the application of different concentrations and number of LPE sprays (Table 7.3). The application of double spray of LPE (125 $mg \cdot L^{-1}$) and single spray (250 $mg \cdot L^{-1}$) resulted in significantly ($P \leq 0.05$) increased cyanidin 3-*O*-galactoside (293.7 $\mu g \cdot g^{-1}$ FW and 287.7 $\mu g \cdot g^{-1}$ FW, respectively) in fruit skin as compared to control. The higher concentration of chlorogenic acid (188.1 $\mu g \cdot g^{-1}$ FW) and phloridzin (62.1 $\mu g \cdot g^{-1}$ FW) were recorded with double and single spray of LPE (125 $mg \cdot L^{-1}$), respectively as compared to control. However, fruit-treated with 375 $mg \cdot L^{-1}$ of LPE resulted in a comparable concentration of phloridzin (54.4 $\mu g \cdot g^{-1}$ FW) with the above mentioned treatments.

Table 7.3. Effects of different concentrations and number of LPE spray on flavonoids and other phenolic compounds of 'Cripps Pink' apple.

LPE ($mg \cdot L^{-1}$)	Flavonoids and other phenolic compounds ($\mu g \cdot g^{-1}$ FW)		
	Cyanidin 3-*O*- galactoside	Chlorogenic acid	Phloridzin
Control	168.9 c	148.7 bc	41.9 b
125 (single spray)	176.8 c	182.9 a	62.1 a
125 (double spray)	293.7 a	188.1 a	56.9 a
250 (single spray)	287.7 a	164.3 ab	26.7 c
250 (double spray)	244.6 ab	132.2 c	8.0 d
375 (single spray)	179.9 bc	123.5 c	31.6 bc
375 (double spray)	222.9 abc	181.5 a	54.4 a
LSD ($P \leq 0.05$)	74.7	27.9	11.4

Means followed by the same letter within column are not significantly different at $P \leq 0.05$.

Quercetin 3-*O*-rutinoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-xyloside, quercetin 3-*O*-arabinoside and quercetin 3-*O*-rhamnoside were the individual quercetin glycosides present in the fruit skin. The application of various concentrations and number of LPE sprays significantly ($P \leq 0.05$) affected the concentrations of only quercetin 3-*O*-xyloside, quercetin 3-*O*-arabinoside and quercetin 3-*O*-rhamnoside and also total quercetin glycosides in the fruit skin (Table 7.4). Amongst LPE treatments, double spray of LPE (125 $mg \cdot L^{-1}$) exhibited the highest concentrations of quercetin 3-*O*-xyloside (920.4 $\mu g \cdot g^{-1}$ FW), quercetin 3-*O*-arabinoside (930.5 $\mu g \cdot g^{-1}$ FW), quercetin 3-*O*-rhamnoside (391.1 $\mu g \cdot g^{-1}$ FW) and total quercetin glycosides (5225.5 $\mu g \cdot g^{-1}$ FW) as compared to control. However, double spray of LPE (125 $mg \cdot L^{-1}$) also tended to had higher concentrations of quercetin 3-*O*-rutinoside (266.2 $\mu g \cdot g^{-1}$ FW), quercetin 3-*O*-galactoside (2370.0 $\mu g \cdot g^{-1}$ FW) and quercetin 3-*O*-glucoside (347.3 $\mu g \cdot g^{-1}$ FW) in the apple skin.

Table 7.4. Effects of different concentrations and number of LPE spray on total quercetin glycosides and individual quercetin glycosides compounds of 'Cripps Pink' apple.

Quercetin glycosides ($\mu g \cdot g^{-1}$ FW)	LPE ($mg \cdot L^{-1}$)							
	Control	125 (single)	125 (double)	250 (single)	250 (double)	375 (single)	375 (double)	LSD ($P \leq 0.05$)
Quercetin 3-*O*-rutinoside	56.4	152.7	266.2	137.9	175.4	119.1	84.0	NS (37.4)
Quercetin 3-*O*-galactoside	764.7	1696.5	2370.0	1454	1627.7	1445.2	1057.6	NS (276.1)
Quercetin 3-*O*-glucoside	108.6	247.3	347.3	205.6	270.7	191.5	146.7	NS (46.6)
Quercetin 3-*O*-xyloside	412.4 c	704.3 ab	920.4 a	624.3 bc	618.2 bc	650.2 bc	494.4 bc	240.7
Quercetin 3-*O*-arabinoside	356.8 c	658.5 b	930.5 a	590.1 bc	574.7 bc	621.4 b	435.4 bc	240.2
Quercetin 3-*O*-rhamnoside	200.9 c	315.6 ab	391.2 a	269.7 bc	267.1 bc	283.0 bc	216.4 c	90.5
Total Quercetin glycosides	1899.8 c	3774.8 ab	5225.5 a	3533.8 b	3281.6 bc	3103.3 bc	2434.4 bc	1606.8

Means followed by the same letter within row are not significantly different at $P \leq 0.05$. Values within brackets represent standard error of means. NS = not significantly different at $P \leq 0.05$.

7.3.4. Effects on firmness, soluble solids concentration, tiratable acidity and SSC/TA ratio

The data presented in Table 7.5 show that the application of various concentrations and number of LPE sprays significantly ($P \leq 0.05$) affected fruit firmness, but SSC, TA and SSC/TA ratio were not significantly affected. The highest fruit firmness (87.0 N) was recorded with double spray of LPE (250 mg·L^{-1}) as compared to all other treatments. However, fruit firmness in all other LPE treatments were above the standards requirements set by the industry (> 68 N).

Table 7.5. Effects of different concentrations and number of LPE spray on firmness, soluble solids concentration, titratable acidity and SSC/TA in apple fruit.

LPE (mg·L^{-1})	Firmness (N)	SSC (%)	TA (% malic acid)	SSC/TA ratio
Control	81.4 bc	13.1	0.58	22.4
125 (single spray)	82.4 bc	13.4	0.59	22.5
125 (double spray)	81.2 c	13.2	0.56	23.3
250 (single spray)	82.9 bc	13.1	0.63	20.8
250 (double spray)	87.0 a	13.0	0.58	22.8
375 (single spray)	82.4 bc	13.0	0.58	22.1
375 (double spray)	83.8 b	13.2	0.62	21.1
LSD ($P \leq 0.05$)	2.39	NS (0.14)	NS (0.04)	NS (1.36)

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. Values within brackets represent standard error of means. NS = not significantly different at $P \leq 0.05$.

7.3.5. Effects on ascorbic acid concentration and total antioxidants

The ascorbic acid concentrations were not significantly affected with the application of different concentrations and number of LPE sprays, but these treatments showed significant effects on the total antioxidants in the fruit skin and apple pulp (Table 7.6). Total antioxidants in the fruit skin were higher (36.4 mM TE·g^{-1} FW and 36.1 mM TE·g^{-1} FW) which were sprayed once or twice with LPE (125 mg·L^{-1}), respectively as compared to all other treatments. Double spray of LPE (125 mg·L^{-1}) showed the highest (0.71 mM TE·g^{-1} FW) total antioxidants in the apple pulp as compared to other treatments.

Table 7.6. Effects of different concentrations and number of LPE spray on ascorbic acid concentration and total antioxidants in the apple skin and pulp.

LPE ($mg \cdot L^{-1}$)	Ascorbic acid ($mg \cdot 100g^{-1}$ FW)	Total antioxidants (mM TE$\cdot g^{-1}$ FW)	
		Skin	Pulp
Control	10.4	27.4 c	0.64 abc
125 (single spray)	10.2	36.4 a	0.62 abc
125 (double spray)	9.98	36.1 a	0.71 a
250 (single spray)	10.1	34.5 ab	0.60 abc
250 (double spray)	9.13	31.9 abc	0.54 c
375 (single spray)	10.3	30.5 bc	0.70 ab
375 (double spray)	10.6	30.5 bc	0.59 bc
LSD ($P \leq 0.05$)	NS (0.32)	5.06	0.12

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. Values within brackets represent standard error of means. NS = not significantly different at $P \leq 0.05$

7.3.6. Effects on concentration of total sugars and organic acids

The application of various concentrations and number of LPE sprays did not significantly affect the concentrations of total sugars, citric and succinic acid (Table 7.7). However, various LPE treatments significantly ($P \leq 0.05$) affected the concentrations of malic, tartaric and total acids (Table 7.7). The single spray of LPE (125 $mg \cdot L^{-1}$) significantly ($P \leq 0.05$) increased malic and total acids concentration (5.96 and 6.55 $g \cdot kg^{-1}$, respectively) as compared to all other treatments. While, single spray of LPE (375 $mg \cdot L^{-1}$) resulted in the highest concentration of tartaric (0.10 $g \cdot kg^{-1}$) as compared to all other treatments.

Table 7.7. Effects of different concentrations and number of LPE spray on concentrations of total sugars and individual organic acids in apple fruit.

LPE ($mg \cdot L^{-1}$)	Total sugars ($g \cdot kg^{-1}$)	Organic acids ($g \cdot kg^{-1}$)				
		Malic	Citric	Tartaric	Succinic	Total acids
Control	106.7	5.28 b	0.05	0.092 abc	0.39	5.81 b
125 (single spray)	105.6	5.96 a	0.05	0.097 ab	0.44	6.55 a
125 (double spray)	103.7	5.01 b	0.04	0.090 bc	0.36	5.50 b
250 (single spray)	104.2	4.92 b	0.04	0.086 c	0.43	5.48 b
250 (double spray)	103.9	4.88 b	0.04	0.086 c	0.41	5.43 b
375 (single spray)	104.4	4.81 b	0.14	0.100 a	0.47	5.42 b
375 (double spray)	104.8	4.79 b	0.04	0.097 ab	0.35	5.37 b
LSD ($P \leq 0.05$)	NS (2.13)	0.65	NS (0.03)	0.01	NS (0.02)	0.73

Means followed by the same letter within column are not significantly different at $P \leq 0.05$. Values within brackets represent standard error of means. NS = not significantly different at $P \leq 0.05$

7.4. Discussion

Fruit colour development in 'Cripps Pink' apple was enhanced with two sprays of LPE (125 $mg \cdot L^{-1}$) and single spray of LPE (250 $mg \cdot L^{-1}$) as compared to control (Table 7.1). The improved fruit colour development with the spray application of LPE has been reported earlier in cranberry (Özgen et al., 2004; Özgen and Palta, 2003), tomato (Farag and Palta, 1993a; Pinhero et al., 2003), red pepper (Kang et al., 2001; Kang et al., 2003), 'McIntosh' apple (Farag and Palta, 1992) and 'Crimson' and 'Red Globe' grapes (Hong, 2008). However, the exact mechanism by which LPE mediates fruit colour development is not known. Possibly, the increased of ethylene production in fruit with the application of LPE onset of fruit colour development (Abdallah and Palta, 1989; Hong et al., 2001; Kang et al., 2003). The increased of fruit colour may also associated to the higher chromaticity value a* and lower chromaticity value b*, lightness and hue angle on both sides of fruit skin which were sprayed with LPE (Table 7.2 and Figure 7.2). The higher chromaticity value a* and lower chromaticity value b*, hue angle and lightness were indicating to the redder and darker skin colour (Whale, 2005). The improved fruit colour may also be attributed to the increased accumulation of total anthocyanins in fruit treated with LPE. In the present study, the accumulation of total anthocyanins in LPE treated fruit

increased 92% to 103% as compared to control. Similarly, the preharvest application of LPE in cranberries increased 9% to 27% (Özgen et al., 2004) and 13% to 28% (Özgen and Palta, 2003) concentration of anthocyanins as compared to control fruit. In addition, Farag and Palta (1991b) reported that the concentration of anthocyanins was increased with the application of 100 ppm LPE in combination with 1% ethanol in 'McIntosh' apple. As a prelude, the increase in fruit skin colour closely related to the increased ethylene production consequently enhanced the accumulation of anthocyanins as reported earlier (Blankenship and Unrath, 1988; Faragher and Brohier, 1984; Kondo et al., 1991; Whale and Singh, 2007; Whale et al., 2008). LPE increased the production of ethylene has also been reported in tomato, cranberry, apple and red pepper (Farag and Palta, 1989; Farag and Palta, 1993a; Hong et al., 2001; Kang et al., 2003). Weather conditions such as cool nights and warm days at the apple orchard may also have increased the concentrations of anthocyanins. The optimum temperatures of 20°C to 25°C during day time and below 18°C at night improved the development of fruit skin colouration (Chalmers et al., 1973). In addition, the cold night temperatures (>20°C) promote the production of anthocyanins and the warms temperatures prevent its accumulation (Saure, 1990). In the present study, the average night and day temperatures was noticeable (11.73°C and 19.55°C, respectively) as presented in Figure 7.1.

No research work has been reported on the effects of exogenous application of LPE on the flavonoids and other phenolic compounds (especially five major groups of flavonoids) in red-skinned apple. In the present study, the lower concentrations of LPE spray treatments were more effective in improving fruit colour, accumulation of total anthocyanins, flavonoids and other phenolic compounds such as anthocyanins (cyanidin 3-*O*-galactoside), hydroxycinnamic acids (chlorogenic acid), flavonols (quercetin glycosides) in the fruit skin than the higher concentrations (Table 7.3 and 7.4). Fruit treated with LPE increased concentrations cyanidin 3-*O*-galactoside (range from 70% to 74%), chlorogenic acid (range from 23% to 26%), phloridzin (range from 36% to 48%), total quercetin glycosides (175%) and also individual quercetin glycosides such as quercetin 3-*O*-xyloside (123%), quercetin 3-*O*-arabinoside (160%) and quercetin 3-*O*-rhamnoside (94%) as compared to control. Possibly, increased ethylene production with the treatments of LPE may also have triggered the activity of anthocyanins biosynthetic enzymes. Earlier, the increased

activity of phenylalanine ammonia-lyase (PAL) in 'Red Delicious' apple with increased ethylene production has also been reported (Blankenship and Unrath, 1988). PAL activity was higher in radish cotyledons treated LPE and its application also can affect the physiology of plant tissues by changing this metabolite enzymes (Hong et al., 2009b). Amongst flavonoids compounds, quercetin glycosides showed the highest increment in LPE treated-fruit. Possibly, all these compounds were independently regulated and physically separated at the cellular level even they were formed from the same biosynthetic pathway as reported by Awad and de Jager (2002).

The applications of LPE at two and four weeks before commercial harvest have increased anthocyanins accumulation. The development of red skin colour in 'Cripps Pink' apple commenced close to the commercial harvest (Mackay et al., 1994; Marais et al., 2001), which may be attributed to the increased accumulation of anthocyanins and other phenolic compounds in LPE treated-fruit. In addition, the activity of PAL and CHI in anthocyanins biosynthetic pathway during fruitlet and maturation in apple skin has been reported to be closely associated with anthocyanin accumulation (Faragher and Brohier, 1984; Li et al., 2002b). Whale (2005) noticed that preharvest spray of ethephon application at four and five weeks prior to commercial harvest enhanced red blush in 'Cripps Pink' apple fruit. As argued earlier, this warrants further investigation to demonstrate the specific role of LPE in enhancing fruit colour and accumulation of anthocyanins.

Fruit firmness was affected with the LPE treatments, but still met the standards export markets set by the apple industry (>68N) (Table 7.5). Increased and delay loss of fruit firmness was also observed in 'McIntosh' apple, 'Thompson seedless' table grapes and tomato treated with LPE (Farag and Palta, 1993a; Farag and Palta, 1991b; Hong et al., 2009a; Pinhero et al., 2003). Possibly, the increased fruit firmness with spray treatments of LPE may be ascribed to its protection of membrane integrity during senescence (Farag and Palta, 1993a). It may also be argued that LPE act as a strong inhibitor of phospholipase D (Ryu et al., 1997), an enzyme known to cause membrane lipid degradation during senescence (Ryu and Wang, 1995). In addition, LPE application has also been reported to inhibit the activity of polygalacturonase (Farag and Palta, 1992), this enzymes were closely related to the softening of fruit

during ripening (Fisher and Bennett, 1991). SSC, TA and SSC/TA were not affected with LPE treatments (Table 7.5). These fruit quality parameters are important requirements in 'Cripps Pink' apple and meet the quality standard set by the industry; SSC were in the range of 13 to ≥ 15 ^{o}Brix and 0.4 to 0.6% TA (Department of Agriculture Western Australia, 2000).

The concentration of ascorbic acid was not affected with the spray application of LPE. While, higher total antioxidants in the apple skin in LPE treated-fruit may be attributed to the accumulation of total anthocyanins, which is one of the important antioxidant in the red-skinned apple (Table 7.6). Most of red-skinned apple possess five major groups of polyphenolic compounds namely anthocyanins, flavanols, flovonols, hydroxycinnamic acids and hydrochalcones, which have been reported as the major source of antioxidants (Eberhardt et al., 2000; Lee et al., 2003; Tsao et al., 2005; Tsao et al., 2003; Wang et al., 1996). No specific trends can be concluded on the effects of LPE on total antioxidants in the apple pulp. However, in general, total antioxidants in the skin of LPE treated-fruit were higher than in the pulp. Similarly, Tsao et al. (2003) reported that apple skin composed 90% of concentration of polyphenolic compounds (excluding hydroxycinnamic acid) than those in the pulp (60%). In the present study, the level of total antioxidants in the pulp was lower than in the skin which similar to the report of Escarpa and Gonzalez (1998) and Wolfe et al. (2003). The single spray application of LPE (125 mg·L^{-1}) resulted in higher malic and total acids concentration in apple fruit juice than untreated fruit (Table 7.7). The possible mechanism subjected to the increased of the concentration of organic acids is not yet known. This warrants further investigations to demonstrate the specific role of LPE in increasing concentrations of organic acids in apple fruit juice.

In conclusion, two preharvest sprays of LPE (125 mg·L^{-1}) applied at two and four weeks anticipated to commercial harvest or single spray of LPE (250 mg·L^{-1}) at four weeks before harvest effectively enhanced fruit colour development through increased accumulation of total anthocyanins and polyphenolic compounds and also maintain other major fruit quality in 'Cripps Pink' apple.

CHAPTER 8

General Discussion, Conclusions, Recommendations to the Industry and Future Research

8.1. Introduction

The red colour of apple skin is one of the important grading standards set by the industry for 'Cripps Pink apple. Poor skin colour at commercial harvest often causes serious economic losses to apple growers particularly in Western Australia. Red skin colour is mainly due to the occurrence of anthocyanins and their biosynthesis is genetically controlled, but it also influenced by various factors including environmental, soil-plant factors and endogenous plant factors (Saure, 1990). However, investigations on the effects of several factors such as regulated deficit irrigation (RDI), withholding irrigation (WHI) and newly developed plant growth regulators i.e. prohexadione-calcium (ProCa) and lysophosphatidylethanolamine (LPE) on the accumulation of anthocyanins in this apple cultivar under Mediterranean climate of Western Australia are scant. The aim of my research was to explore the influence of above mentioned factors on fruit colour development, accumulation of anthocyanins and other polyphenolic compounds and also other major fruit quality parameters such as firmness, SSC, TA and SSC/TA ratio at harvest and following cold as well as CA storage of 'Cripps Pink' apple.

8.2. Regulated deficit irrigation affects fruit colour development, accumulation of anthocyanins and polyphenolic compounds and also fruit quality at harvest, following cold and controlled atmosphere storage

Improved red skin colour and other major fruit quality parameters in apple fruit under various climatic conditions due to water deficit application has been documented (Kilili et al., 1996a; Mills et al., 1996a; Mills et al., 1994). However, no information on the effects of RDI on fruit colour development and quality of 'Cripps Pink' apple under Western Australian conditions in a Mediterranean climate. Hence, the present study has been carried out for two seasons (2005-06 and 2006-07) to evaluate the impact of RDI on soil-plant water relations, fruit colour development, accumulation of anthocyanins and also other fruit quality attributes without adversely affecting fruit size, fruit drop and storage life of 'Cripps Pink' apple. Nine

polyphenolic compounds in 'Cripps Pink' apple skin belongings to four major groups of polyphenolic were identified, quantified and confirmed using HPLC-ESI-MS as mentioned in Chapter 4, Section 4.3.3.3. As explained in Chapter 4, Section 4.4, individual quercetin glycosides determination exhibited the good separation and elution and in agreement with the reports of Schieber et al. (2002). However, the elution order of individual quercetin glycosides were not similar to the reports of Whale and Singh (2007). It may be ascribed to the different method of extraction, detection and identification of these polyphenolic compounds were used in both investigations.

The effects of RDI on the hydraulic status of soil and leaf of 'Cripps Pink' apple trees were evident in 2006-07 only (Figure 4.3, 4.4B, 4.5B and 4.6B). The RDI treatment (75%) exhibited a significant decreased θ, Ψ_{stem}, Ψ_{leaf} and g_s, which resulted in water stress, thereby significantly increased percentage of visual colour blush, concentration of anthocyanins (ES and SS of apple skin), concentration of cyanidin 3-*O*-galactosides and quercetin glycosides, lower hue angle and lightness on shaded side of apple skin (Table 4.1, 4.3 and 4.5). The application of RDI in 2006-07 was longer (72 days) than in 2005-06 which also contributed to the promising outcomes in improving fruit colour of this apple cultivar. Possibly, RDI application has increased levels of abscissic acid (ABA) (Zhang and Davies, 1990) and/or ABA induced ethylene production (Gomez-Cadenas et al., 1996) consequently up regulated of gene expression of anthocyanins biosynthesis. It may also be argued that improved penetration of light into canopy and onto the fruit due to the sparse leaf abscission has increased red skin colour of this apple cultivar. The application of deficit irrigation increased rate of ethylene production (Behboudian et al., 1998; Ebel et al., 1993; Kilili et al., 1996b), which has been reported to closely related with the increase fruit colour development and concentration of total anthocyanins in 'Cripps Pink' apple (Whale and Singh, 2007; Whale et al., 2008). It is well established that light exposure increases red skin pigmentation in apple fruit (Lancaster, 1992; Saure, 1990). However, the exact mechanisms involved in improving red skin colour via increased accumulation of anthocyanins and polyphenolic compounds particularly cyanidin 3-*O*-galactoside subjected to RDI is yet to be investigated. Castellarin et al. (2007b) claimed that the anthocyanins biosynthesis in grape berries under deficit irrigation was partly affected by solar radiation, but primarily due to water-deficit

application during intense phase of anthocyanins biosynthesis. Increased polyphenolic compounds were pronounced in grape berries due to the water-deficit application (Matthews and Anderson, 1988; Ojeda et al., 2000; Roby et al., 2004). Water-deficit application up-regulated the anthocyanins-specific genes (Castellarin et al., 2007b) and also enhanced the mRNA expression in the skin of grapes (Grimplet et al., 2007). Possibly, RDI application in 'Cripps Pink' apple may also have triggered the activity anthocyanins-specific genes involved in anthocyanin biosynthesis. However, it warrants further investigation on genes expression profiling in the skin of this apple cultivar.

The RDI was imposed at the stage II of fruit development (135 to 207 DAFB) of this apple cultivar and exhibited the non-significant effect on fruit drop and fruit size. However, a slight reduction in fruit size with RDI treatment (75% RDI) was noticed, but still met the standards prerequisite for export markets. The application of RDI late in the season has also been reported to increased SSC and fruit firmness at harvest (Table 4.7), following cold and CA storage (Table 4.11 and 4.12), in which firmer fruit were associated to the cellular hydration and increased flesh compactness (Mpelasoka et al., 2000a) and higher SSC was due to the conversion of starch into sugars (Kramer, 1983; Landsberg and Jones, 1981). Improved fruit firmness and SSC in cold storage subjected to water-deficit has also been reported (Behboudian et al., 1998; Kilili et al., 1996b; Mills et al., 1996a; Mpelasoka et al., 2000a; Mpelasoka et al., 2001a). In general, TA in stored-fruit in all treatments decreased as storage periods extended, possibly due to the consumption of malic acid as a metabolite substrate used in fruit respiration (Ackermann et al., 1992).

Ascorbic acid concentration in the pulp of apple fruit increased at harvest (Table 4.8), following cold and CA storage (Table 4.11 and 4.12) due to RDI application. The higher concentration of ascorbic acid may be ascribed to the higher sugars production due to water-deficit, which may have promoted its synthesis during fruit ripening (Veit-Kohler et al., 1999). However, fruit following 14 days shelf life exhibited the declining trends in the levels of ascorbic acid, which may be attributed to increased activity of cytochrome oxidase, ascorbic acid oxidase and peroxidase enzymes (Rocha et al., 1995). The levels of total antioxidants in apple skin and pulp increased at harvest, following cold and CA storage which may be related to the

increased concentration of total anthocyanins and ascorbic acid. In addition, the ethylene action during storage may have triggered the activity of phenolic biosynthesis enzymes, consequently contributed to the increased levels of antioxidants (Leja et al., 2003). This may be the first study reporting the effects of RDI on postharvest performance of apple fruit in CA storage.

8.3. Fruit quality and postharvest performance of 'Cripps Pink' apple in relation to withholding irrigation

Withholding irrigation (WHI) has been reported as one of the strategies for improving water use efficiency in apple industry particularly in humid-temperate region. However, no information is available on the specific time of WHI in improving fruit colour development and other fruit quality attributes at harvest and also following cold storage of 'Cripps Pink' apple under the Mediterranean climate of Western Australia. I investigated the effects of WHI in two seasons (2006-07 and 2007-08), commencing at later stages of fruit development and maturation (stage II and III) on soil and leaf hydraulic status and various fruit quality parameters at harvest and also after long term cold storage of 'Cripps Pink' apple.

A reduction in volumetric soil water content (θ) (Figure 5.2 and 5.3) and hydraulic status of leaves (Ψ_{stem}, Ψ_{leaf} and g_s) in WHI-1 and WHI-2 (during 2006-07) (Figure 5.4A, 5.5A and 5.6A) and in WHI-1 (during 2007-08) (Figure 5.4B, 5.5B and 5.6B) exhibited water stress. Consequently, these treatments have significantly enhanced red skin pigmentation, accumulation of anthocyanins and also the levels of polyphenolic compounds (Table 5.1, 5.4 and 5.5). The pronounced effects of these water-deficit indicators due to WHI application also increased other major fruit quality parameters such as firmness and SSC and also concentration of ascorbic acid at harvest and also following 70 and 140 days in cold storage (Table 5.6, 5.7 and 5.9). Higher firmness in water-deficit fruit at harvest may be due to reduction in cellular hydration (Ebel et al., 1993; Kilili et al., 1996a), increased SSC might be attributed to the conversion of starch into sugars (Kramer, 1983) and increased concentration of ascorbic acid may also due to the higher accumulation of sugars that stimulates its synthesis during fruit ripening (Veit-Kohler et al., 1999). In both seasons, final fruit size in WHI-1 and WHI-2 were similar as in CI fruit (Figure 5.8). Similarly, deficit irrigation imposed late in the season did not affect apple fruit size

of 'Braeburn' apple (Kilili et al., 1996a; Mills et al., 1996b). Conversely, the WHI-3 commenced on 155 DAFB during both years did not experience water-deficit conditions; consequently, fruit quality as above mentioned was not affected. Possibly, the incidence of rain and cloudy days have over ride the effect on water relations of apple trees which results in similar to CI trees.

As discussed earlier in Section 8.2, the mechanisms involved in the increased of fruit colour development through enhanced concentration of total anthocyanins and polyphenolic compounds due to WHI application may be attributed to the improved light penetration into tree canopy, and/or elevated levels of abscisic acid (ABA) and ethylene production in the leaf and fruit. The wilting, yellowing and slight leaf abscission due to WHI application may be related to the increased levels of ABA in the leaf due to the water-deficit (Zhang and Davies, 1990) and stimulate ethylene production consequently leaf abscission (Gomez-Cadenas et al., 1996). Thus, sparse abscission of leaves due to WHI treatments may have improved light penetration into tree canopy and onto the fruit. The presence of light induced red skin pigmentation has been well documented (Lancaster et al., 1994; Saure, 1990). In addition, elevated levels of ethylene in water-deficit trees has also been noticed in apple (Ebel et al., 1993; Kilili et al., 1996b) and enhanced fruit skin colour also pronounced due to the increased ethylene concentration (Blankenship and Unrath, 1988; Faragher and Brohier, 1984; Whale and Singh, 2007). In addition, Kilili et al. (1996a); Mills et al (1994) and Mills et al. (1996a) noted that water-deficit application improved red skin colour in 'Braeburn' apple. The levels of ABA increased due to water-deficit application may also related to the increased of ethylene production consequently up regulate expression of genes involved in anthocyanin biosynthesis.

As explained in Section 8.2, the investigation on the effects of water-deficit such as WHI on polyphenolic compounds in this cultivar is still limited. The concentrations of polyphenolic compounds in fruit skin from WHI treatments (WHI-1 and WHI-2) during 2006-07 were higher than in WHI treatment (WHI-1) in 2007-08 as detailed in Chapter 5, Section 5.4. The increment of polyphenolic compounds during 2006-07 was more intense than 2007-08, possibly, due to the pronounced effects of WHI treatments applied. The increased concentration of these polyphenolic compounds especially cyanidin 3-*O*-galactoside may be coincided with the increased of total

anthocyanins concentration in the apple skin. Possibly, the application of WHI for 20 to 30 days has triggered the expression of anthocyanins-specific genes such as UFGT, CHS2, CHS3 and FH3 as claimed by Castellarin et al. (2007b) and also mRNA expression (Grimplet et al., 2007). However, this warrants further investigation on the effects of WHI on genes expression particularly in the skin of 'Cripps Pink' apple cultivar.

In conclusion, WHI application for 20 to 30 days commencing from 135 and 145 DAFB effectively enhanced red skin pigmentation and other fruit quality at harvest and following storage without adversely affecting fruit size.

8.4. Various plant growth regulators affecting colour development and anthocyanins concentration and polyphenolic compounds

8.4.1. Prohexadione-calcium

The mode of action of Prohexadione-calcium (ProCa) is different from other gibberellin inhibitors as it reduces the biologically active GA_1 and increased the concentrations of inactive, GA_{20} by interfering the 3-β hydroxylation of GA_{20} to GA_1 (Lo Giudice et al., 2004). Two experiments of ProCa application have been conducted during 2007-08 growing season with promising outcomes. The application of ProCa at higher concentrations and multiple sprays effectively inhibited the shoot growth of 'Cripps Pink' apple trees. The effectiveness of ProCa in reducing shoot length in apple tree was dependant on the concentrations, times and numbers of application. In the present studies, shoot growth inhibition in this cultivar was evident after an initial spray at higher concentrations of ProCa ranged from 14% to 19% over control trees (Figure 6.2). This confirmed the previous reports on the reduction of shoot growth after the first application of ProCa (Byers and Yoder, 1999; Medjdoub et al., 2005; Medjdoub et al., 2004; Miller, 2002; Rademacher and Kober, 2003; Unrath, 1999). However, both experiments showed that the single application of ProCa was not enough to prevent shoot growth of this cultivar (Figure 6.2 and 6.5). This may be ascribed to the rapid degradation of ProCa in the apple leaf as reported by Rademacher and Kober (2003). Thus, the second application of ProCa or more were crucial to prevent the regrowth of shoots (Elfving et al., 2003b; Greene, 1999; Medjdoub et al., 2005; Rademacher and Kober, 2003; Unrath, 1999). Due to long growing season of this apple trees, the higher concentrations (500 and 750

$mg \cdot L^{-1}$ ProCa) with multiple applications (two or more sprays) may be required especially for 'Cripps Pink' cultivar grown under Western Australian conditions.

In the Experiment 1, three spray applications of ProCa (500 and 750 $mg \cdot L^{-1}$) and also summer pruning (SP) alone exhibited the higher percentage red blush, percentage fruit that met the export criteria, increased concentration of total anthocyanins and some polyphenolic compounds, higher chromaticity value a*, lower chromaticity value b*, lightness and hue angle on the fruit surface as compared to other treatments (Table 6.1, Figure 6.3 and 6.4). Whereas, in the Experiment 2, two spray applications of ProCa (500 $mg \cdot L^{-1}$) in combination with SP also influenced and/or increased the above mentioned parameters as compared to control (Table 6.7, 6.8 and Figure 6.6). Similarly, the redder skin colouration was noticeable after ProCa treatment in apples (Byers and Yoder, 1999; Greene, 1999; Zadravec et al., 2008), 'Seyval' grape (Lo Giudice et al., 2004) and 'Forelle' pear (Smit et al., 2005). Possibly, the increased in fruit colour development may be associated to the increased concentration of total anthocyanins and polyphenolic compounds (Table 6.2, 6.3, 6.9 and 6.10). In addition, Mata et al. (2006a) and Medjdoub et al. (2005) reported that the application of ProCa improved red skin colour on the blush side of 'Fuji' apple and also increased concentration of total anthocyanins. The reduced shoot growth allows greater light penetration into tree canopy and direct onto the fruit could be the precise mechanism involved (Basak, 2004; Prive and Stewart, 2002). Light regulates red skin pigmentation (Arakawa et al., 1986; Saure, 1990) and activates various enzymes involved in anthocyanin biosynthetic pathway such as PAL, CHI and UFGalT (Dong et al., 1995). It has been concluded that in the absence of light, anthocyanins biosynthesis may not exist (Lancaster, 1992). Fruit borne on the top and upper canopies usually obtain direct sunlight and often improve red skin colour at harvest compared to fruit from the inner and lower canopies (Awad et al., 2000). Conversely, the application of ProCa has been noticed to inhibit the formation of anthocyanins in apple leaf (Rademacher, 2000; Rademacher et al., 1992) and carrot cell (Ilan and Dougall, 1992) by blocking flavanone 3-hydroxylase (F3H or FHX) (Halbwirth et al., 2003; Rademacher, 2000), which is one of the important key enzymes in biosynthesis of anthocyanins (Ubi, 2007). However, these investigations were based on the accumulation of anthocyanins in the apple leaf, but not in the fruit skin. On the other hand, ProCa has been reported undergo a rapid metabolism within a few

weeks (Evans et al., 1997) and not persistent in the environment (Prive et al., 2006), which may not able to block the accumulation of anthocyanins in apple skin.

The increased concentration of anthocyanins and some phenolic compounds with SP may be ascribed to the increased light penetration into the canopy. Similarly, SP increased fruit colour in 'McIntosh' (Autio and Greene, 1990), 'Delicious' (Marini and Barden, 1987) and 'Cripps Pink' apple (Whale, 2005). However, SP has been known as one of the expensive and labour intensive for orchard management practices (Byers and Yoder, 1999; Cline et al., 2008) than ProCa which was easily applied by spraying and safe to consumer and environment (Rademacher and Kober, 2003).

In both experiments, fruit quality attributes such as fruit firmness and TA in ProCa treated fruit were in the range of standards for export markets for 'Cripps Pink' apple (Table 6.4 and 6.11). Therefore, it seems that three spray applications of ProCa (500 $mg \cdot L^{-1}$) on 3, 33 and 63 DAFB or two sprays of ProCa (500 $mg \cdot L^{-1}$) on 2 and 32 DAFB in combination with SP may be suited to commercially use in reducing shoot growth and also improving the development of fruit skin colour of 'Cripps Pink' apple without adversely affecting other major fruit quality.

8.4.2. Lysophosphatidylethanolamine

Lysophosphatidylethanolamine (LPE), a naturally occurring plant growth regulator enhances ethylene production (Farag and Palta, 1989; Hong et al., 2001; Kang et al., 2003), accelerates ripening and prolongs shelf life of tomato (Farag and Palta, 1993a), retards senescence of tomato leaf and fruit (Farag and Palta, 1993b), prolongs vase-life of snapdragon flowers (Kaur and Palta, 1997), inhibits the activity of a membrane degrading enzymes, phospholipase D (PLD) (Ryu et al., 1997), and also improves the development of fruit skin colour. In this investigation, the application of various concentrations and number of LPE sprays significantly affected the development of red blush in fruit surface of 'Cripps Pink' apple (Table 7.1). Fruit treated with two sprays of LPE (125 $mg \cdot L^{-1}$, at four and two weeks anticipated to commercial harvest) and single spray (250 $mg \cdot L^{-1}$, at four weeks before commercial harvest) enhanced red skin colour, concentration of total anthocyanins, higher chromaticity value a*, lower chromaticity value b*, lightness

and hue angle on both sides of apple skin (Table 7.1, 7.2 and Figure 7.2). Improved fruit skin colour has also been noticed in cranberry (Özgen et al., 2004; Özgen and Palta, 2003), tomato (Farag and Palta, 1993a), red pepper (Kang et al., 2003), 'McIntosh' apple (Farag and Palta, 1991b) and 'Crimson' and 'Red Globe' grapes (Hong, 2008). The exact mechanism could not be explained by which LPE mediates fruit colour development. Possibly, the application of LPE may stimulate ethylene production onset the fruit colour development (Abdallah and Palta, 1989; Kang et al., 2003). No research work has been reported on the effects of LPE on non-visual fruit colour parameters such as chromaticity value a*, b*, L*, hue angle and chroma. As a prelude, the increased fruit skin colour may be closely related to the ethylene production, in which ethylene enhanced accumulation of anthocyanins has been reported (Blankenship and Unrath, 1988; Faragher and Brohier, 1984; Kondo et al., 1991; Whale and Singh, 2007; Whale et al., 2008).

Increased concentrations of anthocyanins in fruit-treated LPE coincided with increased concentrations of flavonoids, cyanidin 3-*O*-galactoside ranged from 70% to 74% as compared to control (Table 7.3 and 7.4). The concentrations of other phenolic compounds in fruit treated LPE also increased such as chlorogenic acid (23% to 26%), phloridzin (36% to 48%), total quercetin glycosides (175%), and also individual quercetin glycosides [123% quercetin 3-*O*-xyloside, 160% quercetin 3-*O*-arabinoside and 94% quercetin 3-*O*-rhamnoside] as compared to control (Table 7.3 and 7.4). Amongst flavonoids compounds, quercetin glycosides showed the highest increment in LPE treated-fruit. Possibly, all these compounds were independently regulated and physically separated at the cellular level even they were formed from the same biosynthetic pathway as reported by Awad and de Jager (2002). The precise mode of LPE action is currently unknown and no information is available on the effects of the higher concentrations of LPE on crops particularly in improving apple skin colour. However, this may be postulated that the application of LPE may be improved in up regulation of activity of various enzymes in biosynthetic pathway of anthocyanins of apple fruit. Higher activity of PAL enzymes due to the application of LPE has been reported, but in other crops such as radish cotyledons (Hong et al., 2009b). In addition, the activity of PAL enzymes may be also associated to the increase of ethylene production as reported by Blankenship and Unrath (1988).

Nevertheless, this warrants further investigation to demonstrate the specific role of LPE in enhancing fruit colour and accumulation of anthocyanins.

Fruit quality such as SSC, TA and SSC/TA ratio was not affected with the application of various treatments of LPE (Table 7.5). However, all these fruit quality were maintained and still meets the requirements for export. Amongst LPE treatments, two spray applications of LPE (125 $mg \cdot L^{-1}$, four and two weeks prior to commercial harvest) and single spray (250 $mg \cdot L^{-1}$, four weeks before harvest) showed promising results as compared to other treatments.

8.5. Conclusions

The investigation of various approaches such as water savings techniques and also newly developed plant bioregulators in improving the development fruit skin colour and fruit quality of 'Cripps Pink' apple can be concluded as below:

- Nine polyphenolic compounds were identified and confirmed in the skin of 'Cripps Pink' apple grown in the Mediterranean climate of Western Australia.
- The treatment (75% RDI) at stage II of fruit development commencing from 135 DAFB effectively saved water, increased the development of fruit skin colour via increasing accumulation of anthocyanins and other polyphenolic compounds of 'Cripps Pink' apple and also other fruit quality attributes at harvest, following cold and CA storage without adversely affecting the fruit size.
- The application of WHI for 20 to 30 days (commenced from 135 and 145 DAFB) in the middle of stage II of fruit development of 'Cripps Pink' apple may be useful tool for saving irrigation water and also for improving fruit quality particularly red skin colour and also other quality attributes at harvest and following cold storage.
- Three spray applications of ProCa (500 $mg \cdot L^{-1}$) on 3, 33 and 63 DAFB or combination of two sprays ProCa (500 $mg \cdot L^{-1}$) on 2 and 32 DAFB in combination with SP is effective in improving fruit colour development and in maintaining other fruit quality attributes of this apple cultivar.

- Two spray applications of LPE (125 mg·L^{-1}) at four and two weeks prior to commercial harvest or single spray (250 mg·L^{-1}) at four weeks before harvest is effective in improving fruit colour development of 'Cripps Pink' apple. These LPE treatments also maintain other fruit quality attributes of this apple cultivar.

8.6. Recommendations to the industry

The experimental results have shown that red skin colouration of 'Cripps Pink' apple cultivated in Western Australia region increased with the application of water savings strategies and newly developed plant growth regulators. It can therefore be recommended that:

1. Regulated deficit irrigation (75% RDI) at stage II of fruit development commencing from 135 DAFB continuously for 72 days may be used for improving fruit colour development and other fruit quality attributes and also prolonging storage life (0 ±0.1 °C, 90±2.0%RH) and in CA (2.7% O_2 + 1.9% CO_2 at 0°C).
2. Withholding irrigation for 20 to 30 days at stage II and III of fruit development (commenced on 135 and 145 DAFB) may be used for enhancing the development of fruit colour without adversely affecting fruit size and other fruit quality attributes at harvest and following cold storage of 'Cripps Pink' apple.
3. Three spray applications of ProCa (500 mg·L^{-1}) applied on 3, 33 and 63 DAFB and/or two spray applications of ProCa (500 mg·L^{-1}) on 2 and 32 DAFB in combination with SP may be recommended to the apple growers to improve fruit colour and maintain other fruit quality attributes in 'Cripps Pink' apple.
4. Two spray applications of LPE (125 mg·L^{-1}) at four and two weeks prior to commercial harvest or single spray (250 mg·L^{-1}) at four weeks before harvest may be recommended to improve red skin pigmentation and also retain other fruit quality attributes of 'Cripps Pink' apple cultivar.

8.7. Future research

The present investigation contributes to the development of basic information on how water deficit and plant growth regulators can influence fruit quality parameters of 'Cripps Pink' apple particularly on fruit skin colour development and

anthocyanins biosynthesis and other classes of flavonoids. The findings also open up further research into the following areas:

1. In the present investigation, cyanidin 3-*O*-galactoside in the skin of this apple cultivar was identified, quantified and confirmed, but three other compounds in the groups of anthocyanins were not identified. Therefore, future research should focus on identification and confirmation of these compounds.
2. The effects of RDI and WHI on endogenous concentration of ABA and ethylene in fruit warrants further investigations.
3. Future research should also focus on genes expression profiling of anthocyanins biosynthesis and the specific enzymes involved such as PAL, CHI, ANS, DFR and UFGalT in improving apple skin subjected to RDI and WHI application.
4. The application of preharvest spray LPE needs further investigations particularly its mode of action in enhancing apple skin colour and regulation of activity of enzymes involved in anthocyanins biosynthesis.

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