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Pre-harvest ethephon application and training systems affect colour development, accumulation of flavonoids and fruit quality of 'Cripps Pink' apple

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Abstract

Poor red blush development on the surface of apple (*Malus domestica* Borkh.) fruit at commercial harvest causes serious economic losses to apple growers. The effects of pre-harvest spray application of ethephon and different training systems (TS) on development of red blush, accumulation of flavonoids in the fruit skin and quality of 'Cripps Pink' apple were investigated for two consecutive years (2004-05). 'Cripps Pink' apple trees trained as vase (VS), spindle bush (SB), central leader (CL), and double row (DR) TS were sprayed with single dose of ethephon (480 g L^{-1}) 18 days prior to anticipated commercial maturity (CM). Spray application of ethephon significantly improved percent red blush, concentrations of total anthocyanin and cyanidin 3-galactoside in the fruit skin, irrespective of TS in both years. Chroma value and hue angle of the fruit colour were also significantly improved with pre-harvest spray application of ethephon. Concentrations of chlorogenic acid, phloridzin, quercetin glycosides, and flavanols in the skin both from exposed sides (ES) and shaded sides (SS) of the fruit, harvested from any of the TS were also significantly modified with pre-harvest spray application of ethephon in both years. At commercial maturity (CM), fruit firmness, soluble solids content (SSC) and titratable acidity (TA) of apple fruit from ethephon-treated trees exceeded the minimum standards for their export. Conclusively, TS and pre-harvest spray application of ethephon, applied about 18 days prior to the anticipated CM, substantially affected the development of red blush and modified the concentrations of chlorogenic acid and flavonoids in the fruit skin without substantially compromising fruit quality at CM in 'Cripps Pink' apple.

Keywords: Anthocyanin; Chlorogenic acid; Cyanidin 3-galactoside; Flavonoids; Phloridzin; Quercetin glycosides.
Abbreviations: a^*_colour coordinate from green to red on colour grid; b^*_colour coordinate from yellow to blue on colour grid; C^*_chroma (vividness/strength of colour); CL_central leader; CM_commercial maturity; DR_double row; ES_exposed side; ET_Ethephon®; h^o– hue angle (saturation of colour); L^*_lightness (of colour); SB_spindle bush; SS_shaded side; TS_training system (s); VS_vase.

Introduction

Poor and erratic development of red blush on the surface of 'Cripps Pink' apple fruit causes serious economic losses to the growers, particularly in warmer Mediterranean regions of the world such as California, South Africa and Australia (Whale and Singh, 2007). At least 40% surface area of 'Cripps Pink' apple fruit with pink blush over the cream-pale green background is required for its export. Poor development of red blush on the apple fruit surface may cause a reduction of exports and revenue (Whale et al., 2008). For example, the value of apple fruit exported from Australia declined by 30 % from 2003-04 to 2008-09 mainly because of poor development of red blush (Whale et al., 2008). The amount, nature and composition of anthocyanins in the skin of apple determine the intensity of red blush on the fruit surface. Cyanidin-3-galactoside (Idaein) is a main anthocyanin responsible for red blush in 'Cripps Pink' apple (Whale and Singh, 2007), which is derived from cinnamic acid through de-amination of *L*-Phenylalanine (Lancaster, 1992). Anthocyanin biosynthesis in apple fruit is regulated by genetic, environmental and cultural factors (Saure, 1990). Light distribution and utilization in an orchard and within the tree canopy is critical for the development of red blush on the surface of apple fruit. Light irradiation regulates accumulation of anthocyanin probably by promoting photosynthetic activities during fruit maturation, and reducing the temporary repression of anthocyanin formation during cell division phase in apple fruit (Saure, 1990). Similarly, biosynthesis of phenolic compounds is regulated by the availability of light during fruit growth and development (Montanaro et al., 2007). Light factor is manageable through different cultural techniques such as fruit bagging, thinning, late summer pruning, spreading reflective mulches over the orchard floor, and training systems (Saure, 1987; Fan and Mattheis, 1998; Hampson et al., 2004; Mika et al., 2007). Anthocyanin biosynthesis in apple has also been reported to be influenced by the concentrations of endogenous ethylene (Whale and Singh, 2007). During apple fruit maturation, the ripening-associated rise in red blush is closely related with concomitant rise in ethylene concentration (Chalmers and Faragher, 1977). Therefore, ethylene-releasing compounds such as ethephon (2-chloroethyl phosphonic acid), have been successfully incorporated in the production management of apple orchards, close to the CM to promote anthocyanin bio-syn-

Table 1. Effect of pre-harvest spray application of ethephon and different training systems on the development of red blush on fruit surface at commercial maturity in 2004 and 2005

Ethephon (g ha^{-1})	Training system	Red blush (%)	
		2004	2005
0	Vase	80.8	48.7
280	Vase	83.6	76.6
	Mean	82.2	62.7
0	Central Leader	68.1	58.6
280	Central Leader	70.9	72.5
	Mean	69.5	65.6
0	Spindle Bush	60.8	49.0
280	Spindle Bush	67.3	64.4
	Mean	64.1	56.7
0	Double Row	58.2	57.6
280	Double Row	59.5	65.3
	Mean	58.9	61.5
LSD $P \leq 0.05$			
Ethephon		1.2	4.8
Training system		0.8	3.4
Ethephon x Training system		1.6	6.8

n = 80 (20 fruit x 4 replications).

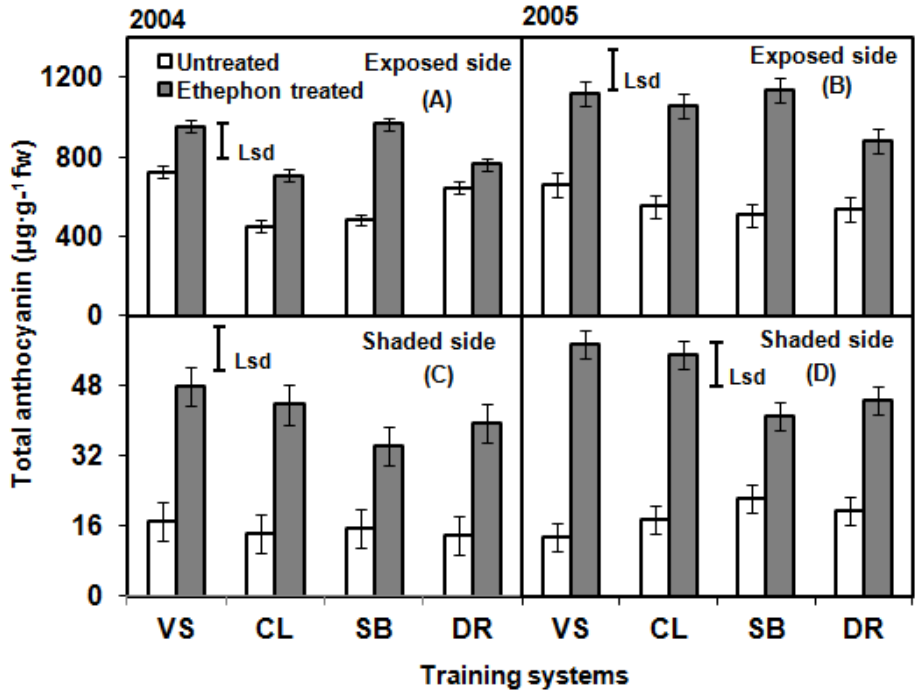

Fig 1. Effects of pre-harvest spray application of ethephon and different training systems on concentration of total anthocyanin in the exposed (A and B) and shaded (C and D) sides of fruit skin at commercial harvest in 2004 and 2005. n = 4 (4 replications). Vertical bars represent S.E. of means.

thesis in many apple cultivars (Li et al., 2002). For being a powerful ripening agent on apples, ethephon should judiciously be used during high temperature weather conditions for red colour development (Greene et al., 1974).Training systems (TS) are generally designed to ensure adequate light interception and penetration through the tree canopy consequently to influence yield and fruit quality (Hampson et al., 2004; Li and Lakso, 2004). The effects of different training systems on light interception, penetration into tree canopy, yield and fruit quality have been reported in various cultivars of apple (Robinson, 2000; Wertheim et al., 2001; Hampson et al., 2004; Ritenour and Khemira, 2007; Hassan et al., 2010). The influence of various training system such as vase, central leader, spindle bush and double-row on accumulation of individual flavonoids in the skin of apple warrants to be investigated.Earlier reports clearly indicate that light, harvest maturity and evolution of ethylene are the major factors affecting red colour development in apple fruit, and are interdependent to one another for anthocyanin biosynthesis. However, to our knowledge, no research work has been reported on the combined effects of training systems and pre-harvest application of ethephon (as an ethylene releasing ripening agent) on colour development, accumulation of individual flavonoids and important quality parameters of 'Cripps Pink' apple fruit at CM. The objective of this study was to examine the effects of pre-harvest spray application of Ethephon® and different training systems

Table 2. Effects of pre-harvest spray application of ethephon and different training systems on chromaticity values of skin colour on exposed sides of fruit at commercial maturity in 2004 and 2005.

Ethephon (g ha^{-1})	Training system (TS)	CIE L^{*} [a]		CIE a^{*} [b]		CIE b^{*} [c]		C^{*} [d]		h^{o} [e]	
		2004	2005	2004	2005	2004	2005	2004	2005	2004	2005
0	Vase	24.4	23.5	27.6	28.4	12.4	14.6	30.2	31.0	24.2	27.7
280	Vase	24.2	23.7	28.2	29.4	12.3	14.3	30.8	33.7	23.7	25.7
	Mean	24.3	23.6	27.9	28.9	12.4	14.45	30.5	32.35	23.95	26.7
0	Central Leader	26.6	23.9	23.3	27.0	13.2	13.3	26.2	28.4	31.4	28.4
280	Central Leader	26.4	21.4	24.3	27.4	13.2	13.2	28.8	32.3	28.0	24.2
	Mean	26.5	23.7	23.8	27.2	13.2	13.25	27.5	30.35	29.7	26.3
0	Spindle Bush	27.6	22.7	20.9	26.2	13.9	13.8	24.4	27.7	34.3	27.8
280	Spindle Bush	25.0	20.5	23.0	27.2	13.0	12.5	27.6	30.0	29.9	24.8
	Mean	26.3	21.6	21.95	26.7	13.45	13.15	26	28.85	32.1	26.3
0	Double Row	27.3	25.8	21.0	25.8	14.5	14.2	25.4	28.9	38.0	30.8
280	Double Row	26.2	24.1	22.1	26.7	14.5	13.8	26.9	30.8	30.6	25.8
	Mean	26.8	25.0	21.6	26.3	14.4	14.0	26.2	29.9	34.3	28.3

LSD $P \leq 0.05$	2004			2005		
	Ethephon	TS	Ethephon x TS	Ethephon	TS	Ethephon x TS
CIE L^{*}	1.0	1.5	NS	0.9	1.2	NS
CIE a^{*}	1.0	1.4	NS	0.6	0.9	1.3
CIE b^{*}	0.4	0.6	0.8	0.4	0.6	0.8
C^{*}	0.7	1.0	NS	0.7	0.7	0.9
h^{o}	2.7	1.9	NS	1.2	NS	NS

n = 80 (20 fruit x 4 replications). [a] Decrease in L^{*} indicate that colour is becoming darker. [b] Increase in a^{*} indicate shift from green to red. [c] Decrease in b^{o} indicate shift from blue to yellow. [d] Increase in C^{*} indicate increased colour saturation. Decrease in h^{o} indicate shift from orange to red.

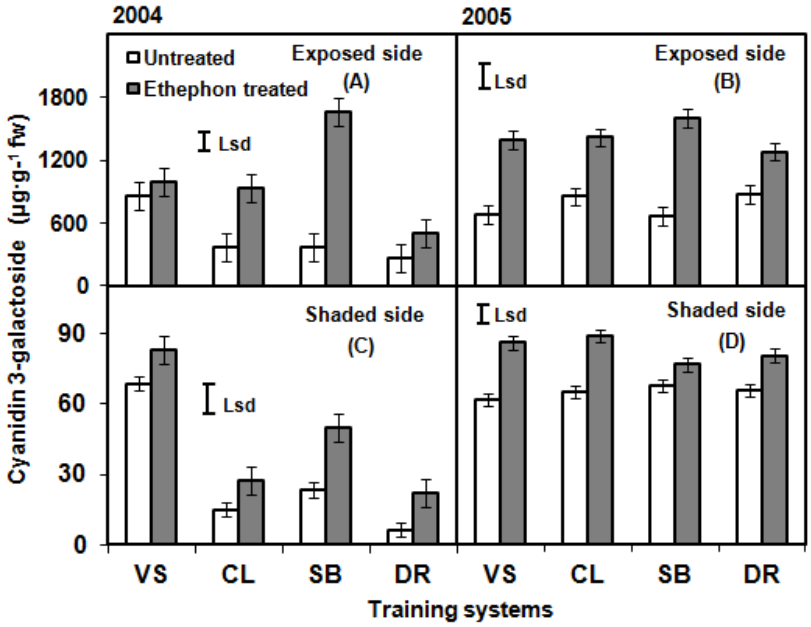

Fig 2. Effects of pre-harvest spray application of ethephon and different training systems on concentration of cyanin 3-galactotoside in the exposed (A and B) and shaded (C and D) sides of fruit skin at commercial harvest in 2004 and 2005. n = 4 (4 replications). Vertical bars represent S.E. of means.

commonly used in Australia on red blush development and accumulation of flavonoids in the fruit skin, and on fruit quality of 'Cripps Pink' apple at CM.

Results and Discussion

Fruit colour

Development of red blush on the fruit surface was significantly ($P \leq 0.05$) improved with ethephon application in all TS, in 2004 and 2005 (Table 1). Apple fruit harvested from the trees trained as VS system had the highest percent red blush on their surface, compared to those harvested from any other TS, irrespective of ethephon treatment. Similarly, the fruit harvested from ethephon-treated trees trained as VS system also exhibited the highest percentage of red blush on their surface as compared to those harvested from Ethephon®-treated or untreated trees trained as CL, SB or DR systems (Table 1). The spray application of ethephon significantly ($P \leq 0.05$) reduced the lightness value (CIE L^*) on ES of the fruit in both year (Table 2), indicating redder fruit. Whereas, the effect of ethephon on CIE L^* was not significant ($P \leq 0.05$) on the SS of the fruit in both year (Table 3). Effect of TS on the reduction of CIE L^* was also significant ($P \leq 0.05$), and fruit harvested from trees trained as SB system exhibited maximum reduction in CIE L^* as compared to all other TS. The interaction between ethephon spray application and TS was not significant ($P \leq 0.05$) on either of side of the fruit. CIE a^* value significantly ($P \leq 0.05$) increased with ethephon application on both ES and SS of the fruit in 2004 and 2005 (Table 2 and 3). The maximum increase in CIE a^* on ES was exhibited by the fruit harvested from the trees trained as SB, whilst SS of the fruit harvested from the trees trained as VS showed the highest increase in CIE a^* value. The interactions for ethephon application and TS were significant ($P \leq 0.05$) only in 2005 for both ES and SS of the fruit. The ES and SS of the fruit exhibited significant ($P \leq 0.05$) reduction in the CIE b^* value in response to ethephon spray application during both years, as compared to control (Table 2 and 3). The maximum reduction in CIE b^* was recorded in fruit harvested from trees trained on SB system irrespective of year of production in ES and SS of the fruit. The interactions between ethephon application and TS were significant ($P \leq 0.05$) for CIE b^* on ES in year 2004 and 2005, and on SS only in 2005. The C^* significantly increased with ethephon application on ES and SS of the fruit in both years (Table 2 and 3). Fruit harvested from trees trained as SB system exhibited highest increase in the C^* on ES and SS of the fruit. Interactive effects of ethephon application and TS on C^* were significant in 2005 for ES and SS of the fruit. Ethephon application significantly reduced h^o value in both years on both ES and SS of the fruit (Table 2 and 3). Maximum reduction in h^o was recorded in fruit harvested from the trees trained as DR system in both years on ES and SS of the fruit. The interactions between ethephon application and TS was significant ($P \leq 0.05$) for h^o on SS of fruit in 2005.

Total anthocyanins

Pre-harvest spray application of ethephon significantly ($P \leq 0.05$) increased the accumulation of total anthocyanins in the skin of both ES and SS of the fruit skin at CM in both years in all TS (Fig. 1). The trees trained as SB system showed the highest increase in the concentration of total anthocyanins (968 µg·g^{-1} and 977 µg·g^{-1} fw) in ES of fruit skin in both year as compared to all other TS (Fig. 2A and 2B). ES of the fruit harvested from SB trained trees sprayed with ethephon exhibited 2-fold and 2.2-fold increase in concentration of total anthocyanins in 2004 and 2005 as compared to control respectively. Whilst, in 2004 and 2005, among different TS, highest (3-fold and 4.3-fold) concentration of total anthocyanins were accumulated on the SS of the skin of the fruit harvested from trees trained as VS system in contrast to control respectively (Fig. 1C and 2D). The interactions

Table 2. Effects of pre-harvest spray application of ethephon and different training systems on chromaticity values of skin colour on exposed sides of fruit at commercial maturity in 2004 and 2005.

Ethephon (g ha^{-1})	Training system (TS)	CIE L^{*} [a]		CIE a^{*} [b]		CIE b^{*} [c]		C^{*} [d]		h^{o} [e]	
		2004	2005	2004	2005	2004	2005	2004	2005	2004	2005
0	Vase	24.4	23.5	27.6	28.4	12.4	14.6	30.2	31.0	24.2	27.7
280	Vase	24.2	23.7	28.2	29.4	12.3	14.3	30.8	33.7	23.7	25.7
	Mean	24.3	23.6	27.9	28.9	12.4	14.45	30.5	32.35	23.95	26.7
0	Central Leader	26.6	23.9	23.3	27.0	13.2	13.3	26.2	28.4	31.4	28.4
280	Central Leader	26.4	21.4	24.3	27.4	13.2	13.2	28.8	32.3	28.0	24.2
	Mean	26.5	23.7	23.8	27.2	13.2	13.25	27.5	30.35	29.7	26.3
0	Spindle Bush	27.6	22.7	20.9	26.2	13.9	13.8	24.4	27.7	34.3	27.8
280	Spindle Bush	25.0	20.5	23.0	27.2	13.0	12.5	27.6	30.0	29.9	24.8
	Mean	26.3	21.6	21.95	26.7	13.45	13.15	26	28.85	32.1	26.3
0	Double Row	27.3	25.8	21.0	25.8	14.5	14.2	25.4	28.9	38.0	30.8
280	Double Row	26.2	24.1	22.1	26.7	14.5	13.8	26.9	30.8	30.6	25.8
	Mean	26.8	25.0	21.6	26.3	14.4	14.0	26.2	29.9	34.3	28.3

LSD $P \leq 0.05$	2004			2005		
	Ethephon	TS	Ethephon x TS	Ethephon	TS	Ethephon x TS
CIE L^{*}	1.0	1.5	NS	0.9	1.2	NS
CIE a^{*}	1.0	1.4	NS	0.6	0.9	1.3
CIE b^{*}	0.4	0.6	0.8	0.4	0.6	0.8
C^{*}	0.7	1.0	NS	0.7	0.7	0.9
h^{o}	2.7	1.9	NS	1.2	NS	NS

n = 80 (20 fruit x 4 replications). [a] Decrease in L^{*} indicate that colour is becoming darker. [b] Increase in a^{*} indicate shift from green to red. [c] Decrease in b^{o} indicate shift from blue to yellow. [d] Increase in C^{*} indicate increased colour saturation. Decrease in h^{o} indicate shift from orange to red .

Table 3. Effects of pre-harvest spray application of ethephon and different training systems on chromaticity values of skin colour on shaded sides of fruit at commercial maturity in 2004 and 2005.

Ethephon ($g\ ha^{-1}$)	Training system (TS)	CIE L^{*} [a]		CIE a^{*} [b]		CIE b^{*} [c]		C^{*} [d]		h^{o} [e]	
		2004	2005	2004	2005	2004	2005	2004	2005	2004	2005
0	Vase	41.4	47.4	8.3	-1.9	22.1	24.0	24.6	26.1	68.7	83.6
280	Vase	41.4	43.6	8.6	8.2	22.1	23.8	24.8	29.2	68.2	70.6
	Mean	41.4	45.5	8.5	3.2	22.1	23.9	24.7	27.7	68.5	77.1
0	Central Leader	43.8	47.0	0.3	-2.8	25.4	27.4	25.2	26.4	88.8	85.4
280	Central Leader	44.0	45.4	3.1	5.8	24.3	27.1	25.7	29.1	81.8	76.7
	Mean	43.9	46.2	l1.7	1.5	24.9	27.3	25.5	27.8	85.3	81.1
0	Spindle Bush	43.6	48.7	-1.0	-0.5	27.1	29.0	26.4	27.0	91.6	90.8
280	Spindle Bush	41.6	43.1	3.0	1.6	25.5	27.7	27.4	29.9	82.3	86.3
	Mean	42.6	45.9	1.0	0.6	26.3	28.4	26.9	28.5	87.0	88.6
0	Double Row	46.9	46.9	-3.3	-2.6	30.6	30.5	30.5	29.6	96.2	94.9
280	Double Row	46.5	45.4	-2.6	1.7	30.3	29.2	30.8	30.3	74.8	76.5
	Mean	46.7	46.2	-3.0	-0.5	30.5	29.9	30.7	30.0	85.5	85.7

LSD $P \leq 0.05$	2004			2005		
	Ethephon	TS	Ethephon x TS	Ethephon	TS	Ethephon x TS
CIE L^{*}	NS	1.4	NS	NS	0.6	NS
CIE a^{*}	1.5	2.1	NS	0.9	1.2	1.7
CIE b^{*}	0.7	1.1	NS	0.6	0.9	1.2
C^{*}	0.5	0.6	NS	0.4	0.6	0.8
h^{o}	3.7	5.2	NS	2.1	2.9	4.1

n = 80 (20 fruit x 4 replications). [a] Decrease in L^{*} indicate that colour is becoming darker. [b] Increase in a^{*} indicate shift from green to red. [c] Decrease in b^{o} indicate shift from blue to yellow. [d] Increase in C^{*} indicate increased colour saturation. Decrease in h^{o} indicate shift from orange to red.

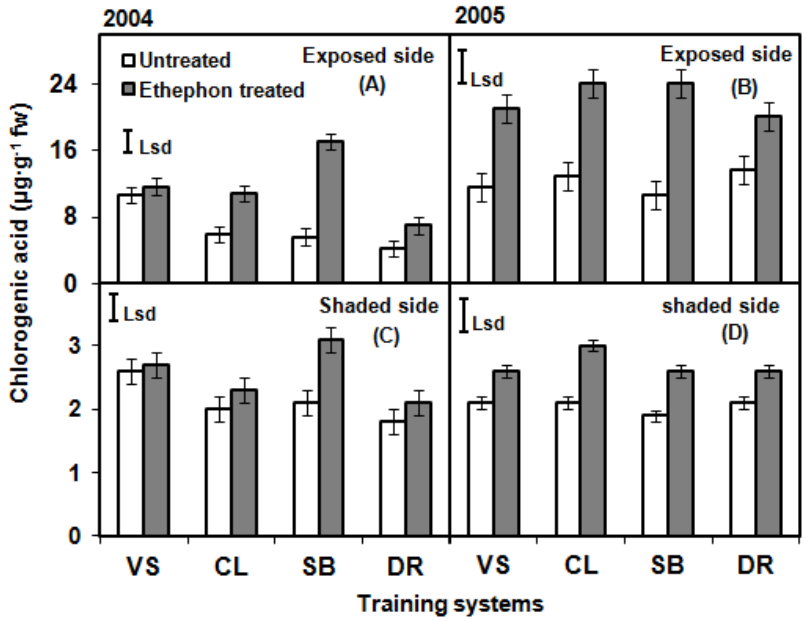

Fig 3. Effects of pre-harvest spray application of ethephon and different training systems on concentration of chlorogenic acid in the exposed (A and B) and shaded (C and D) sides of fruit skin at commercial harvest in 2004 and 2005. n = 4 (4 replications). Vertical bars represent S.E. of means.

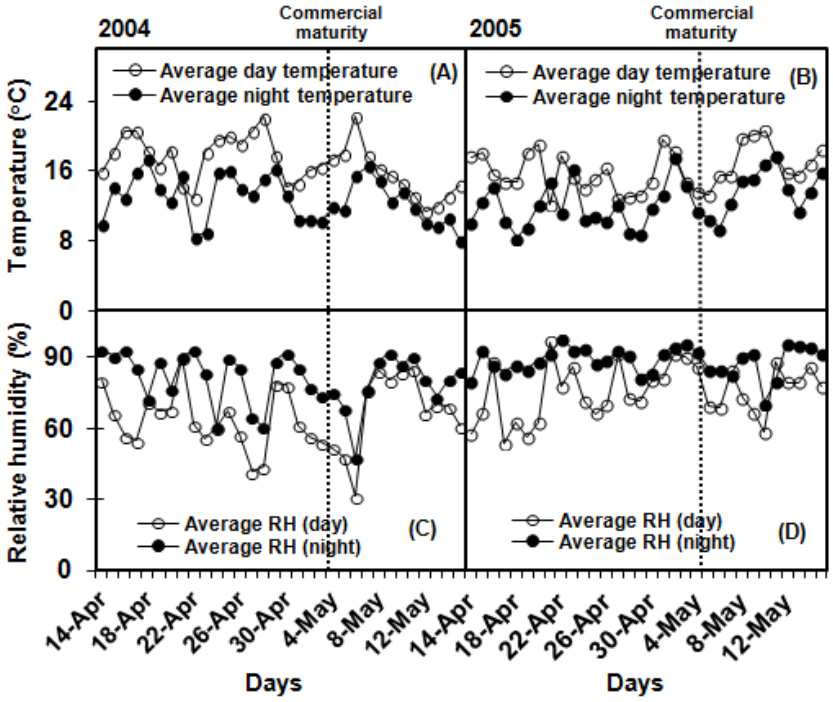

Fig 4. Average day and night temperature (A and B) and relative humidity (C and D) during fruit maturation recorded at Manjimup, Western Australia, in 2004 and 2005 using TinytagPlus Gemini Data loggers. Doted lines represent the date of commercial fruit maturity.

between ethephon application and TS were significant irrespective of fruit sides in both years.

Cyanidin 3-galactoside

Cyanidin 3-galactoside was the only anthocyanin identified in the skin of 'Cripps Pink' apple fruit. Pre-harvest spray application of ethephon significantly ($P \leq 0.05$) increased the concentration of cyanidin 3-galactoside in ES and SS of fruit skin the in all TS during both years (Fig. 2). In, 2004 and 2005, among different TS, the fruit harvested from ethephon-treated trees trained as SB system exhibited highest increase in cyanidin 3-galactoside (4.5-fold and 2.4-fold) in the skin of ES of the fruit as compared control respectively (Fig. 2A and 2B). Fruit harvested from ethephon-treated trees trained as VS and CL systems showed higher increase in concentrations of cyanidin 3-galactoside in SS of the fruit skin as compared with other TS in 2004 and 2005 respectively (Fig. 2C and D).

Chlorogenic acid

Pre-harvest spray application of ethephon significantly ($P \leq 0.05$) increased the concentrations of chlorogenic acid in the ES and SS of the fruit skin irrespective of the TS in both years (Fig. 3). In both years, amongst TS, , fruit harvested from ethephon-treated trees trained as SB system exhibited highest increase in the concentration of chlorogenic acid in ES and SS of the fruit skin as compared to control.

Phloridzin

The concentrations of phloridzin were significantly increased ($P \leq 0.05$) with the pre-harvest spray application of ethephon on the ES and SS of the fruit skin in all TS in 2004 and 2005 (Table 4). Among different TS systems, highest increases in the concentrations of phloridzin were noticed on the ES and SS of skin of the fruit harvested from ethephon-treated trees trained as SB and VS systems as compared to control in 2004 and 2005 respectively.

Catechin and epicatechin

Pre-harvest spray application of ethephon significantly ($P \leq 0.05$) increased the concentrations of catechin and epicatechin on ES and SS of the fruit skin in all TS in both years (Table 4). In 2004, among different TS, highest increase in the concentrations of catechin and epicatechin were exhibited in the ES of the fruit skin of ethephon-treated trees trained as VS system as compared control (Table 4). The fruit harvested from ethephon-treated trees trained as DR and SB systems exhibited highest increase in the concentrations of catechin and epicatechin in the ES and SS of the fruit skin as compared to all other TS and control in 2005. Interactions between catechin and epicatechin for SS side of the fruit in 2004, and ES and SS of the fruit in 2005 were significant.

Quercetin glycosides

Pre-harvest spray application of ethephon significantly ($P \leq 0.05$) increased the concentrations of quercetin 3-glycosides on ES of the fruit skin in all TS in both years (Table 5). The quercetin 3-galactoside was a major flavonol found in the ES of fruit skin of 'Cripps Pink' apple. Pre-harvest spray application of ethephon significantly ($P \leq 0.05$) increased the concentrations of quercetin 3-glycosides (quercetin 3-galactoside, quercetin 3-arabinoside, quercetin 3-rutinoside, quercetin 3-rhamnoside, quercetin 3-glucoside) on ES of skin of the skin harvested from trees trained as VS and DR systems in 2004 and 2005, respectively (Table 5). SS of skin of the fruit harvested from ethephon -treated trees in all TS exhibited significant ($P \leq 0.05$) increase in the concentrations of quercetin 3-arabinoside, quercetin 3-rutinoside in 2004, and quercetin 3-galactoside, quercetin 3-arabinoside, quercetin 3-rutinoside, quercetin 3-rhamnoside, quercetin 3-glucoside in 2005 (Table 6). Highest increase in the concentration of quercetin 3-glycosides (quercetin 3-galactoside, quercetin 3-arabinoside, quercetin 3-rutinoside, quercetin 3-rhamnoside, quercetin 3-glucoside) were exhibited in the skin of SS of the fruit harvested from ethephon-treated trees trained as VS system in 2004, and DR system in 2005, respectively (Table 6). The interactions between spray application of ethephon and TS for concentrations of all quercetin 3-glycosides were significant for SS of the fruit.

Fruit firmness, SSC, TA, and SSC: TA ratio

A significant ($P \leq 0.05$) reduction in fruit firmness was observed with pre-harvest spray application of ethephon in all TS in 2004 as compared to control (Supplementary data; Table 1). Fruit harvested from trees trained as VS system had significantly ($P \leq 0.05$) higher fruit firmness at harvest as compared with all other TS. However, the effect of ethephon spray application on the fruit firmness was not significant during 2005. The SSC, TA and SSC:TA ratio of the fruit was not significantly ($P \leq 0.05$) influenced by spray application of ethephon and TS except SSC in the year 2004 that was significantly influenced by TS. The fruit harvested from trees trained as VS system exhibited higher SSC as compared to all other TS (Supplementary data; Table 1).

Discussion

The results from present studies indicate that most of the colour parameters of apple fruit at harvest were significantly improved by a pre-harvest spray application of ethephon irrespective of TS (Table 1-3). At CM, the higher accumulation of total anthocyanin in the fruit skin (Fig. 1) or better red blush development on the fruit surface (Table 1) compared to control, in response to a pre-harvest spray application of ethephon might be associated with increased level of endogenous ethylene. Previously, ethephon treatment has been reported to enhance internal ethylene concentration in 'Cripps Pink' apple fruit (Whale et al., 2008).

It may also be argued that an exposure of ethylene to maturing apples through pre-harvest spray application of ethephon might have stimulated the activity of UDP-glucose:flavonoid 3-O-glucosyl transferase that catalyses the attachment of a sugar molecule to the anthocyanin aglycone, during the final stages of anthocyanin biosynthesis thereby making the anthocyanin molecules more stable in the cell vacuoles (Ju et al., 1995). Generally the development of red colour in apple fruit shows two peaks, one during the cell division phase and the second peak commences to develop a few weeks (generally 3-5) before CM under normal growing conditions (Mackay et al., 1994; Marais et al., 2001). Both peaks of colour development in 'Cripps Pink' apple coincides with simultaneous increase in the concentrations of total anthocyanins and cyanidin 3-galactoside in the fruit skin and parallels the increase in endogenous ethylene concentration (Whale and Singh, 2007) indicating that formation of red

colour in apple fruit both during cell division and cell enlargement appears to be regulated or induced by increased concentrations of endogenous ethylene.

The SS of the fruit were more responsive to pre-harvest ethephon treatment for the development of red pigmentation compared to the ES. Murphey and Dilley (1988) and Whale et al. (2008) suggested that only a brief exposure of ethylene is required to stimulate the biosynthesis of anthocyanins in apple fruit. The compound, if applied shortly before maturation, has extensively been reported to improve the development of red colour in apple fruit (Saure, 1990; Li et al., 2002).

Fruit colour development was also significantly influenced by the TS. Fruit harvested from trees trained as VS system exhibited higher concentrations of total anthocyanins in their skin on ES as compared to other TS studied. The performances of CL and SB systems for the development of red blush in the apple skin as shown by red blush percentage, anthocyanin contents and chromaticity values (a^*, C^*, h^o) were almost similar to one another probably due to their structural similarities. Higher red blush development on the fruit from VS may be attributed to more light interception or entrapment between the scaffolds (within the open centres) of the trees or lesser shading problem as compared to other TS. Fruit colour is generally improved in open trees of moderate vigour (Marsh et al., 1996). Corelli and Sansavini (1989) concluded that TS influence fruit quality more than their productivity under excessive canopy density or insufficient spacing between rows.

Chromaticity values have long been used to interpret colour parameters in fruit crops. Augmentation of a^* and C^* values is generally considered as an instrumental tool to explain the transition of green colour towards the red one and of dull colour towards the vivid one respectively. On the other hand, diminution of h^o is referred as development of more red/pink and less yellow colour on the fruit surface (Drake et al., 2002).

Pre-harvest spray application of ethephon substantially modified the concentrations of almost all the biosynthetic intermediates of anthocyanins (chlorogenic acid, phloridzin and flavanols and flavonol) determined both in ES and SS of the fruit in all TS (Tables 4-6). Compositional changes in the concentrations of flavonoids and anthocyanins in response to pre-harvest ethephon application indicate that the regulation of final red blush in apple skin is not just controlled by the final step(s) in the biosynthetic pathway between the leucocyanidin and cyanidin as suggested by Lancaster (1992). The compositional changes in the concentrations of biosynthetic intermediates of anthocyanins, particularly chlorogenic acid due to ethephon application may be attributed to the release of ethylene from the compound which consequently increased the levels of endogenous ethylene. It indicates that ethylene may be required to initiate and/or maintain the initial biosynthetic steps for the formation of flavonoid and anthocyanins. Earlier, it has been reported that anthocyanins formation is directly regulated by ethylene (Saure, 1990). Significant increases in the concentration of chlorogenic acid particularly in ES of the fruit with pre-harvest spray application of ethephon along with simultaneous increase in the concentrations of flavonoids and cyanidin 3-galactoside (Table 4) show that ethylene gas released from ethephon might have played a role in producing more cinnamates by promoting the activity of phenylalanine ammonia-lyase in phenylpropanoid pathway. It resulted in increased levels of chlorogenic acid and 4-coumaroyl-CoA that consequently produced more number of chalcone structures by the condensation of the co-enzyme involved. Possibly, production of more number of chlacone structures by the condensation of 4-coumaroyl-CoA, resulted in increased levels of biosynthetic intermediates of anthocyanins and finally the concentration of cyanidin 3-galactoside in the fruit skin. Further investigation on the pattern of changes in endogenous levels of chlorogenic acid in epidermal and hypodermal layers of fruit cells during fruit development and ripening may be feasible to conclude the exact role of chlorogenic acid in biosynthesis of flavonoids and anthocyanins. Increased levels of other biosynthetic intermediates of anthocyanins (phloridzin, flavonol, flavanols) and finally the concentration of anthocyanins in the fruit skin by ethephon application may also be attributed to the increased concentration of internal ethylene (Whale and Singh, 2007) that might have triggered the expression of biosynthetic genes encoding anthocyanins in the fruit skin. A similar suggestion for anthocyanin formation has been proposed by Wang and Dilley (2001) in apple fruit and by El-Kereamy et al. (2003) in grape berries. Increased levels of both the strong (quercetin glycosides; Table 5 and 6) and relatively weaker co-pigments (phloridzin and flavonols; Table 4) particularly in ES with spray application of ethephon in conjunction with concomitant increase in cyanidin 3-galactoside in our study indicate that both intra- and intermolecular co-pigmentation of anthocyanin with its biosynthetic intermediates may be involved in red colour intensification of apple fruit. However, a structural study involving inter- and intra-molecular co-pigmentation using conditions similar to our experiments would be beneficial to reach the final conclusion. Co-pigmentation involves the aggregation of biosynthetic intermediates in flavonoid pathway around anthocyanin molecules providing a shield against its hydration, thus making the molecule more stable as a coloured flavyllium cation (Broulliard et al., 1989). Compositional comparisons of chlorogenic acid, flavonol, phoridzin and flavanol in the skin of apple fruit from ethephon-treated and control apple trees may be informative to elucidate their contribution in the biosynthesis of anthocyanins and their roles in the biosynthetic pathway from deamination of phenylalanine to the final colour development in apple fruit. Large variations in the concentrations of flavonoids and anthocyanins between ES and SS from different training systems and between the two seasons (Tables 4-6) may be attributed to prevailing day and night temperature during fruit ripening (Fig. 4) and to the varying levels of light interception. These variations show that light and other climatic conditions not only play their roles in the accumulation of anthocyanins but may also be involved in other steps of flavonoid biosynthetic pathway. Further investigation on the biosynthesis of flavonoids as influenced by light and other climatic conditions such as temperature and relative humidity may be appropriate to confirm their roles in the process.

Materials and Methods

Plant and fruit materials

'Cripps Pink' apple trees grafted on M.M. 109 root stock from a commercial Orchard located at Manjimup (lat. 34°15´S: long. 116°09´E), in the South West region of Western Australia were used for the experiment in two consecutive years. The experimental trees, uniform and free from pests as well as diseases, trained as vase (VS), spindle bush (SB), central leader (CL), and double row (DR) TS with spacing of 6 m x 3 m, 5 m x 1.5 m, 6 m x 2 m and 5 m x 1 m, were planted in 1990, 1994, 1996 and 1998, respectively. All

the trees received similar cultural practices including, nutrition, irrigation, pruning and plant protection.

Treatments and experimental design

A single dose (280 g a.i. ha^{-1}) of Ethrel® (480 g L^{-1} ethephon; Bayer CropScience Pty. Ltd. East Hawthorn, Victoria, Australia) was sprayed as an aqueous solution containing 'Tween' 20 as a surfactant (0.02%) onto the whole experimental trees on 14 April 2004 (18 days prior to anticipated CM). Temperature and relative humidity within the tree canopy during both years were recorded using a data logger (TinyTag*Plus* Gemini Data Logger, UK). Data were obtained using Gemini Data Logger Manager Software (version 2.8) (Fig. 4). The experiment used randomised complete block design (RCBD) with four replicates using two trees as an experimental unit. Unsprayed trees were treated as control. Samples of 50 fruit were randomly harvested around the trees from each replication at the CM (3 May, 2004) to record the development of red blush, and determine the concentrations of total anthocyanin, cyanidin 3-galactoside, chlorogenic acid, phloridzin, catechin and epicatechin, and quercetin 3-glycosides in the fruit skin, as well as fruit quality. The experiment was repeated with same training systems selected from the same orchard but using different trees in the year 2005. All the treatments were similar to previous year. The samples of 50 fruit were harvested from each replication at the CM (6 May, 2005) and were analysed for fruit colour and quality parameters as described during 2004.

Measurement of red blush and chromaticity values of fruit skin colour

Red blush on the fruit surface was assessed visually on individual fruit and scores were given from 0% to 100%. Zero percent represented no red blush, whereas 100% represented a fully red apple. Instrumental measurements of fruit colour (CIE L^*, a^* and b^*) were recorded for ES or SS of fruit by averaging four measurements taken around the fruit's equator, using a Hunterlab ColorFlex $45^o/0^o$ Spectrophotometer (HunterLab ColorFlex, Hunter Associates Inc., Reston, Virginia, USA) equipped with a window-based 'Universal Software (version 5.1)' computer programme. The chroma value (C^*) and hue angle (h^o) were calculated from chromaticity CIE a^* and b^* values as reported earlier by Whale and Singh (2007) . The CIE L^* value represent whiteness of the colour ranges from black = 0 to white = 100; a^* and b^* represent green-red and yellow-blue colours respectively; C^* represents dull-vividness; h^o represent tint of the colour.

Determination of total anthocyanins and flavonoids

UV-Vis Spectrophotometer (Model 6405; Jenway Limited, Gransmore Green, Felsted, Dunmow, Essex, UK) was used to determine the concentration of total anthocyanin in the fruit skin by using the method as outlined earlier by Bishop and Klein (1975), and modified by Whales and Singh (2007). The absorbance was taken at 530 nm and molar extinction co-efficient (3.43×10^4) of idaein chloride was used to calculate concentrations of total anthocyanin in the fruit skin samples (Siegelman and Hendricks, 1958). Flavonols, flavanols, and chlorogenic acid were extracted from the skin of ES or SS of the fruit, following the method as described by Awad and de Jager (2000) with some modifications reported earlier by Whale and Singh (2007) . Reversed-phase high performance liquid chromatography (RP-HPLC) system (Waters 1525 binary HPLC Pump fitted to Waters 2487 Bual Wavelength Absorbance Detector and Waters 717plus Auto sampler; Waters Crop., Milford, Mass.) equipped with a C_{18} column (Waters Symmetry-$C_{18,}$ 159 x 4.6 mm i.d.; 5 µm packaging) was used for estimation. During analysis same system conditions were used as previously detailed previously by Whale and Singh (2007) and all the analyses were done in duplicate**.**

Determination of fruit firmness, soluble solids concentration and titratable acidity

Fruit firmness was determined from the two opposite surfaces of each fruit using an electric pressure tester (Model EPT-1 pressure tester, Lake City Technical Products Inc., Kelowna, BC, Canada) fitted with an 11 mm tip and were expressed as newtons (N). Fresh juice from the blended composite of apple slices was extracted by using a Panasonic juice extractor (Model MJ-66PR, Matsushita Electric Ind. Co. Ltd., Kadoma, Osaka, Japan). SSC was determined by measuring the refractive index of juice using an infrared digital refractometer (Atago-Palette PR-101, Atago Co., Ltd., Itabashi-Ku, Tokyo, Japan). TA was determined by titrating juice against 0.1 N NaOH, using phenolphthalein as an indicator. SSC: TA ratio was determined by dividing SSC with corresponding TA value.

Statistical analysis

The Experimental data were subjected to two-way analysis of variance (ANOVA), using Genstat 13 (release 13.1; Lawes Agricultural Trust, Rothamsted Experimental Station, Rothamsted, UK) including ethephon application and training systems as factors. The effects of various treatments and their interactions were assessed within ANOVA and least significant differences (Fisher's LSD) were calculated following significant ($P \leq 0.05$) F test. All the assumptions of analysis were checked to ensure validity of statistical analysis. The data of both years were not pooled because error mean squares over years were heterogeneous.

Conclusions

Pre-harvest spray application of ethephon 18 days prior to anticipated CM improved the development of red blush, due to ethylene signalling consequently accelerated accumulation of anthocyanins, and flavonoids in the skin of 'Pink Cripps' apple fruit during ripening. The effects were more pronounced with different systems of training suggesting that light and ethylene (endogenous or exogenous) are involved in the development of red blush on apple fruit skin indirectly through their modifying effects on the biosynthetic intermediates of anthocyanins.

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