Interactive Effect of Ethephon and Shading on the Anthocyanin Composition of *Vitis vinifera* L. cv. Crimson Seedless

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Abbreviations: Peonidin – Pn; Malvidin – Mv; Cyanidin – Cn; Delphinidin – Dn; Petunidin – Pt; glucoside – gluc

The *Vitis vinifera* cultivar Crimson Seedless primarily accumulates the anthocyanin peonidin-3-glucoside. The research undertook the study of two factors which could influence the accumulation of anthocyanin in grape berry skins: ethephon application and shade. Ethephon treatment at 200ppm applied one week post-véraison significantly increased the concentration of all anthocyanins in berry skins. Peonidin-3-glucoside was found to increase most significantly in response to ethephon application, and was increased 150% compared with an untreated control. The proportion of 3-monoglucoside anthocyanins increased in response to ethephon application. A shading treatment did not affect total anthocyanin concentration in berry skins, but the anthocyanin cyanidin-3-glucoside was decreased significantly by shade. Its content was 50% of a sun-exposed control. The observed effects were found to occur at two sites at which the experiment was performed in the Hex River and Paarl regions. Colour development in the *Vitis vinifera* cultivar Crimson Seedless does not appear to be influenced significantly by bunch shading. The use of commercial growth regulators like ethephon exert a strong influence on anthocyanin production in grape skins of this cultivar, and are therefore a more likely solution to overcome poor colour development in its production.

Vitis vinifera L. cv. Crimson Seedless is a late ripening, red seedless cultivar which can be highly profitable as it fills a niche gap in the market, as it is a seedless alternative for the red seeded grape, 'Emperor'. It is one of the most important table grape cultivars currently produced in South Africa and is widely cultivated in table grape producing regions, such as the Berg River and Hex River Valleys. However, a concern in the commercial production of this cultivar is that it has been observed to lack adequate size and colour required for export, and that practices which improve size, such as girdling and gibberellic acid application, reduce the colour even more (Jensen *et al.,* 1975; Carreno *et al*., 1997; Cantos *et al*., 2002; Peppi & Dokoozlian, 2003; Avenant & Avenant, 2006; Peppi *et al*., 2006; Yahuaca *et al*., 2006; Cantín *et al*., 2007; Peppi *et al*., 2007). Various reasons for inferior colour development in wine and table grapes have been reported for the conditions prevalent in South Africa, such as high temperatures (Kliewer & Torres, 1972; Kliewer 1977; Mori *et al*., 2005; Yahuaca *et al*., 2006) and vigorous growth with dense, shaded canopies (Smart *et al*. 1988; Hunter *et al*., 1991).

Apart from environmental factors which influence colour development in grapes, genetic factors also pre-dispose certain cultivars to accumulate lower levels of anthocyanin. Cantos *et al*. (2002) investigated the polyphenol profiles of seven table grape cultivars, and of the four red cultivars examined Crimson Seedless was found to have the lowest anthocyanin content. The most abundant anthocyanin in most table grape varieties studied was peonidin-3-glucoside (Pn-gluc), followed by cyanidin-3-glucoside (Cn-gluc), which contrasts with *V. vinifera* winegrape cultivars in which the most abundant anthocyanin has been reported to be malvidin-3-glucoside (Mv-gluc) (Mazza, 1995; Cantos *et al*., 2002; Peppi & Dokoozlian, 2003).

In an effort to increase colour and colour uniformity of Crimson Seedless, it has become common practice for producers to apply plant bio-regulators (Avenant & Avenant, 2006; Cantin *et al*., 2007). Ethylene-releasing compounds like ethephon, applied at véraison, have been used successfully in many *Vitis vinifera* L. cultivars to improve the colour of red grapes (Jensen *et al.,* 1975; Szyjewicz *et al*., 1984; Roubelakis-Angelakis & Kliewer, 1986; Fitzgerald & Patterson, 1994; El-Kereamy *et al*., 2000; Delgado *et al*., 2004; Gallegos *et al*., 2006; Yahuaca *et al*., 2006). Earlier work by Steenkamp *et al*. (1977) also showed that ethephon increased phenylalanine-ammonia-lyase (PAL) activity in table grapes which was accompanied by increased colour development. Ethephon treatments have also been shown to enhance gene expression for enzymes involved in anthocyanin biosynthesis such as UDP glucose-flavonoid 3-o-glucosyl transferase (UFGT) with concomitant increases in anthocyanin accumulation in *Vitis vinifera* cv. Cabernet Sauvignon (El-Kereamy *et al*., 2002; El-Kereamy *et al*., 2003). Higher anthocyanin levels at harvest in ethylene-treated Cabernet Sauvignon grapes were due to increased synthesis of anthocyanins, namely Mv-gluc (El-Kereamy *et al*., 2002; El-Kereamy *et al*., 2003).

The effect of cluster shading and/or exposure to sunlight is a subject which has been extensively documented for both table grapes (Kliewer & Antcliff, 1970; Wicks & Kliewer, 1983) and wine grapes (Crippen & Morrison, 1986a, b, Bledsoe *et al*.,

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1988; Smart *et al*., 1988; Morrison & Noble, 1990; Price *et al*., 1995; Bureau *et al*., 2000; Haselgrove *et al*., 2000; Bergqvist *et al*., 2001; Downey *et al*., 2004; Cortell & Kennedy, 2006). The effect of cluster exposure on anthocyanin accumulation is variable, and has been shown to either enhance (Morrison & Noble, 1990; Hunter *et al*., 1991; Price *et al*., 1995), maintain (Haselgrove *et al*., 2000; Downey *et al*., 2004; Ristic *et al*., 2007), or reduce (Kliewer 1977; Crippen & Morrison, 1986b; Fitzgerald & Patterson, 1994) anthocyanin concentration in grapes. The interactive effect of increased solar radiation resulting in increased temperature in sun-exposed clusters may account for this variability, in that increased temperature decreases anthocyanin synthesis (Bergqvist *et al*., 2001; Spayd *et al*., 2002; Downey *et al*., 2004; Mori *et al*., 2005).

A single study exists for table grape cultivars where the combined effect of ethephon application and variation in bunch-exposure on anthocyanin accumulation was studied (Wicks & Kliewer, 1983). In that study, variable responses were found for two table grape cultivars, Ribier and Emperor. In Emperor, shading significantly reduced anthocyanin concentration in grapes, and ethephon application only minimally enhanced anthocyanin accumulation, but under sun-exposed conditions the effect of ethephon was marked, increasing to 350% of the concentration found in the sun-exposed control. Conversely, the same treatment in Ribier was found to have a negligible effect on anthocyanin concentration. This early work may indicate that the response of anthocyanin accumulation to either shade or ethephon application is highly cultivar-specific. Hence, the study aimed to address two key questions: firstly whether shading affects the anthocyanin composition of Crimson Seedless and secondly to observe an interactive effect, if any, between ethephon application and bunch shading on anthocyanin composition. For the production of Crimson Seedless in South Africa, neither the timing nor concentration of ethephon application has been shown to significantly influence colour accumulation (Avenant & Avenant, 2006). Also, the effect of ethephon on fruit composition varies between cultivars; as well as timing, concentration and method of application, as contradictory results have been noted in its effects on soluble solids, titratable acidity and pH (Szyjewicz *et al*., 1984). Thus, for the purpose of the current study, ethephon was applied at one time point and concentration, a single application of 200 ppm ethephon at véraison.

MATERIALS AND METHODS

Site description

Experiments were conducted over a single season, 2005/2006, at two sites located in Paarl (33°08'S, 18°59'E, January-February temperature min. 20°C max. 32°C, Alt. 138 m), in the Berg River Valley and De Doorns (33°47'S, 19°67'E, January-February temperature min. 15°C max. 30°C, Alt. 457 m) in the Hex River Valley. Vineyards were selected for their comparability, since the experiments were performed in a single season. Both sites were located in 5-year-old commercial *V. vinifera* L. cv. Crimson Seedless (C102-26) vineyards, grafted on 'Richter 110' (*V. Berlandieri* x *V. rupestris* var. 'Martin') rootstock. For the Paarl site, vine spacing was 1.5 m in east/west orientated rows, with 3.5 m between rows (~1905vines/ha); and for the De Doorns site, vine spacing was 1.8 m in east/west orientated rows, with 2.8 m between rows (~1985vines/ha). For both sites, a Gable trellis system with split cordon was used, as described by Avenant (1991). Vineyard management and fertilisation for the sites was similar to the practices described by Avenant & Avenant (2006). Climatic data for both locations of the study were obtained from weather stations located close to the experimental site. This data was provided by ARC Infruitec Nietvoorbij.

Treatments

For both of the experimental sites, a single vine was used per treatment replicate with two adjacent vines in-row, between replicates, used as buffer vines. At the Paarl and De Doorns sites, there were four and eight treatment replicates respectively. A completely randomized design was applied. Four similar bunches were selected on alternate sides of each treatment vine and random numbers were used to assign the different treatments to each bunch. Treatments were applied on single bunches within a single vine for each replicate: control (no treatment); E (ethephon application only); S (shade application only) and E + S (ethephon and shade application). E and E + S treatments were applied, one week after véraison (January 2006), by dipping bunches for 20 s into a plant bio-regulator solution (200ppm Ethrel; 48% w/v ethephon) with a standard buffering wetting agent (Breakthru; at 40 mL/100L H_2O). The S and E + S treatments were applied through use of shade boxes which were modelled on the design used by Downey *et al*. (2004), to cover bunches immediately after berry set (November 2005) when berry diameter was ~2 mm. All bunches were trimmed to a length of ~13 cm before the shade boxes were put into place, and secured to the shoots with cable ties, over the selected bunches. The shaded bunches remained enclosed until harvest. Temperature within the shade boxes was compared with ambient conditions within the canopy by insertion of Tinytag (TGP-4017, Gemini Technologies, UK) data-loggers, both with and without shade boxes, in the vineyard canopy. Temperature measurements were logged at 5 minute intervals. Comparison of temperature showed no significant differences between air temperature within the shade box interior and ambient temperature in the canopy.

Grape sampling

Treatment bunches were collected and weighed separately at harvest. For analysis, 40 berries were collected at random from each bunch, 20 berries were frozen at -20ºC for later analysis of anthocyanins and the remaining 20 berries were weighed and then crushed by hand to extract the juice. The juice was used to determine the total soluble solids (TSS), the titratable acidity (TA), the pH and the maturity index (MI) defined as the ratio of TSS:TA (Boulton *et al*. 1996).

Extraction and quantification of anthocyanins

The berries collected at harvest were weighed and kept frozen at -20ºC for reverse phase-HPLC analysis of anthocyanins, which was done within 3 months of harvesting the grapes. The skins of these berries were removed from the flesh with a scalpel, after which it was freeze-dried and then finely ground under liquid nitrogen using a mortar and pestle. Ten mL of an acidified hydro-alcoholic solution (50% methanol:water; pH 2 with HCl) was added to 500 mg of the ground skins. The skins were extracted at room temperature (18°C), shaking for 2 hours. The extract was centrifuged (13000 rpm, 5 min) and the supernatant retained. Total phenolics in the berry skins was determined according to Iland *et al*. (2000). One mL of the supernatant was acidified with

4 mL of 1M HCl, and left to stand for 3 hours. The absorbance of the acidified methanolic skin extracts were measured at 280 nm. Another aliquot of the centrifuged supernatant was transferred to HPLC vials.

Extracts were separated and quantified by reverse phase-HPLC (Agilent model 1100) using a Supelcosil 3 µm Opti-guard column with a Supelcosil LC-18-DB (15 x 4.6 mm, 3 µm) column. A ramped gradient of 10% formic acid and 80% acetonitrile was used. The final run time was 55 min. All anthocyanins were quantified at 520 nm against a malvidin-3-monoglucoside (Extrasynthase, Germany) standard curve, which had a linear response within the range of concentrations injected onto the column. Anthocyanins were identified by their elution order in comparison to the Mv-gluc standard, according to the pattern described by Wulf and Nagel (1978). HP Chemstation software was used for the chromatographic analysis and integration.

Anthocyanin ratios

The ratios of the different anthocyanin derivatives were calculated according to the equations described by Mattivi *et al.* (2006). These values do not account for degradation of anthocyanins or removal of precursors to form other products, but broadly reflect enzyme activity at branch points within the anthocyanin pathway.

$$\text{Ratio 1: } \frac{\text{3', 5'-dihydroxy}}{\text{3'-hydroxy}} = \frac{\text{sum of Dn-, Pt-, and Mv-3-glucosides}}{\text{sum of Cn- and Pn-3-glucosides}}$$

$$\text{Ratio 2: } \frac{\text{3'-methoxy}}{\text{3'-hydroxy}} = \frac{\text{Pn-3-glucosides}}{\text{Cn-3-glucosides}}$$

$$\text{Ratio 3: } \frac{\text{3', 5'-methoxy}}{\text{3', 5'-hydroxy}} = \frac{\text{Mv-3-glucosides}}{\text{Dn-3-glucosides}}$$

Statistical analysis

Statistical analysis was carried out using STATISTICA software (data analysis software system), version 7.1 (StatSoft, Tulsa, OK). A repeated measures ANOVA (RMA) technique was applied to the data and the mean values were separated using Duncan's range test for significant differences. The RMA is used to analyze designs in which responses on multiple dependent variables correspond to measurements at the different levels of one or more varying factor, as each vine served as the replicate for all treatments the RMA was able to separate treatment effects statistically and also discern the interactive effects between treatments. This analysis allowed for the comparison of the treatment means at three levels: E, S and the interactive effect of ethephon in conjunction with shading E x S.

RESULTS

Regional temperature

For the season of the study, the mean, maximum and minimum monthly averages for temperature together with monthly rainfall averages for De Doorns and Paarl are shown in Tables 1 and 2 respectively. For the 2005-2006 growing season, from September to March, De Doorns had a cooler average temperature than Paarl, approximately 6% cooler. However, when the mean minimum and maximum temperatures are compared, it is evident that the cooler average temperature for De Doorns is largely due to cooler overnight temperatures, with the minimum temperature at De Doorns for the growing season being 20 – 37% cooler than Paarl. On the other hand, daytime maximum temperatures at De Doorns were on average 5% higher than at Paarl for the growing season.

Fruit analysis

In the two regions where this study was conducted, Paarl was the earlier ripening region compared to De Doorns, and in the 2005-2006 season this was evident as the grapes were harvested on the 24th of February in Paarl and on the 15th of March in De Doorns**.** At both sites where the experiment was performed, the data indicate that the E-treatment did not significantly influence any of the ripeness parameters measured (Table 3). For Paarl, the S-treatment influenced the average berry weight and maturity index significantly, decreasing the average berry weight and the both skin fresh and dry weights by ~20%. For Paarl, the maturity index was 10% greater for shaded berries compared to the sun-exposed control. At De Doorns the S-treatment did not influence the average berry weight, skin weight or the maturity index significantly. Conversely, the E-treatment, significantly increased the skin weights of treated grapes compared to the control treatment. There was an average increase of ~48% in the skin weights of

TABLE 1

Mean monthly average, minimum and maximum temperatures and rainfall for the experimental site at De Doorns (33°47'S, 19°67'E, Altitude 138 m) for the growing season in 2005-2006.

Year	Month	Average (°C)	Maximum (°C)	Minimum (°C)	Rain (mm)
2005	July	13.3	21.4	5.1	44.2
	August	11.4	17.7	5.1	69.6
	September	15.7	23.6	7.7	16.3
	October	17.6	25.9	9.2	0.0
	November	19.8	27.8	11.8	20.4
	December	20.3	29.7	10.8	0.0
2006	January	23.2	32.0	14.3	1.4
	February	24.1	32.6	15.6	0.4
	March	20.0	29.1	10.8	1.9
	April	17.8	25.5	10.0	49.2
	May	13.2	20.2	6.2	72.7
	June	12.6	20.6	4.6	55.3

TABLE 2

Mean monthly average, minimum and maximum temperatures and rainfall for the experimental site at Paarl (33°08'S, 18°59'E, Altitude 457 m) for the growing season in 2005-2006.

Year	Month	Average (°C)	Maximum (°C)	Minimum (°C)	Rain (mm)
	July	15.0	21.0	9.0	62.0
	August	12.0	16.1	7.9	141.0
2005	September	15.7	21.3	10.2	39.6
	October	17.5	23.1	12.0	17.5
	November	20.6	26.3	14.9	32.0
	December	21.4	28.2	14.6	0.0
	January	24.0	30.6	17.3	0.0
	February	26.1	30.8	20.7	8.1
2006	March	21.5	28.4	14.8	1.9
	April	19.4	25.3	13.6	52.6
	May	13.7	18.6	9.1	243.4
	June	14.5	20.6	8.5	76.4

TABLE 3

Fruit composition for Crimson Seedless treated with either shade boxes, ethephon (200 ppm) or a combination of both in 2005-2006. Significance (P) determined for three factors S = shade treatment; E = ethephon application and S x E = interactive effect of shade and ethephon application. P was calculated using repeated measures ANOVA where * indicates P<0.05; ** P<0.01; *** P<0.001 and ns is not significant (De Doorns: n = 8; Paarl: n = 4).

De Doorns

Parameter	Sun-exposed clusters		Shaded clusters		Significance (P)		
	Control	Ethephon	Control	Ethephon	S	E	S x E
Bunch weight (g)	356.4	595.2	555.9	536.6	ns	ns	ns
Berry weight (g)	4.0	4.2	4.2	4.3	ns	ns	ns
Skin fresh weight (g/berry)	0.37	0.58	0.41	0.58	ns	***	ns
Skin dry weight (g/berry)	0.13	0.20	0.13	0.17	ns	**	ns
TSS (°Brix)	19.9	20.3	18.9	18.9	***	ns	ns
pH (20 °C)	3.8	3.8	3.9	3.8	**	ns	ns
TA (g/L)	4.4	4.2	4.0	4.1	*	ns	ns
Maturity index	3.5	3.7	3.6	3.6	ns	ns	ns

Paarl

Parameter	Sun-exposed clusters		Shaded clusters		Significance (P)		
	Control	Ethephon	Control	Ethephon	S	E	S x E
Bunch weight (g)	706.5	756.3	645.5	621.0	ns	ns	ns
Berry weight (g)	7.2	6.6	5.0	5.8	**	ns	ns
Skin fresh weight (g/berry)	0.73	0.72	0.56	0.61	**	ns	ns
Skin dry weight (g/berry)	0.22	0.21	0.15	0.18	*	ns	ns
TSS (°Brix)	20.1	20.1	20.6	20.3	ns	ns	ns
pH (20 °C)	3.7	3.7	3.8	3.7	ns	ns	ns
TA (g/L)	3.6	3.4	3.1	3.2	ns	ns	ns
Maturity index	4.2	4.4	4.7	4.9	**	ns	ns

the ethephon-treated grape berries compared to the control berries. At De Doorns, the S-treatment decreased TSS and TA by approximately 6%, with a small increase in pH relative to the sun-exposed clusters.

Response of the anthocyanin profile to viticultural treatments

The major anthocyanin types detected were the 3-monoglucosides (gluc) and 3-p-coumarylglucosides (coum), which were represented by the five anthocyanidins commonly found in *Vitis vinifera* grape species. By proportion, the most abundant anthocyanin group in Crimson Seedless was the 3-monoglucosides, followed by the 3-p-coumarylglucosides. The 3-acetylglucosides of Crimson Seedless were also distinguished, but depending on the treatment, were not present in sufficiently quantifiable amounts to report using HPLC analysis. The most abundant anthocyanin present in Crimson Seedless grapes at both sites of the study,

based on the quantity present in control treatment berries, was Pn-gluc (Tables 4 and 5). For the experiment De Doorns Mv-gluc followed Pn-glc in order of abundance, but for the Paarl experiment, this was Cn-gluc. Between the two sites, there were found to be small differences in anthocyanin composition, but statistically these differences were not significant.

For both sites the E-treatment had the most significant effect on total anthocyanin concentration, and was 160 and 105% greater than the control treatment for Paarl and De Doorns respectively. Ethephon was found to increase the concentration of all anthocyanin types quantified. At Paarl, Pn-gluc was the anthocyanin type most significantly increased by the ethephon application (~240%), followed by Cn-gluc (~200%). The result at De Doorns was similar where Pn-gluc was increased ~160% by the E treatment, but was followed by Mv-gluc (~110%). Overall, changes in the anthocyanin profile were observed in response to ethephon application, such that the ethephon treatment increased the proportion of the 3-monoglucosides to total anthocyanins by ~70-80% in Paarl and ~70% at De Doorns relative to the control treatment. Thus, the primary fraction of anthocyanins affected by ethephon were the monoglucoside anthocyanins.

The S treatment was found to have a negligible effect on total anthocyanin concentration for both of the experimental sites. However, the individual anthocyanins were differentially affected by shade at the different sites. The only anthocyanin that was significantly influenced by the shade treatment at both sites, was Cn-gluc, being reduced ~50% and ~34% relative to the sun-exposed control, at De Doorns and Paarl respectively. At De Doorns, Dn-gluc and Pt-gluc were also significantly reduced by the S treatment. A significant interactive effect E x S was observed between the treatments at Paarl for Dn-, Cn- and Pt-gluc, such that sun-exposed clusters with the ethephon application had significantly higher concentrations of these anthocyanins compared to the other treatments. However, no significant interactive effect was observed for total anthocyanins or the most abundant anthocyanin, Pn-gluc.

Ratios of methylated and hydroxylated forms of anthocyanins

The ratios of the different derivatives of methylated and hydroxylated forms of the anthocyanins have been used as an estimate of the degree of enzyme activity of the enzymes flavonoid-3’**,** 5’-hydroxylase (F3’5’H), 3’O-methyltrans-ferase (3’OMT) and 5’O-methyltransferase (5’OMT) (Mattivi *et al*., 2006). Since changes in the composition of anthocyanin derivatives can be associated with the activity of these enzymes (Ageorges *et al.,* 2006; Bogs *et al.,* 2006; Jeong *et al.,* 2006) it is a useful indicator of where the applied treatments may have influenced flux within the anthocyanin pathway, but this technique does not account for the possible degradation of anthocyanins. The ratios for the anthocyanin derivatives are given in Table 6. At the De Doorns site, F3’5’H activity as estimated by the ratio of 3’5’-dihydroxy/3’-hydroxy anthocyanins was lowered in response to the ethephon treatment, thus indicating a potentially greater flux within the anthocyanin pathway was towards the F3’H branch. However, for the Paarl site, there was no significant effect of ethephon application on this ratio. For both sites, the ratio of 3’methoxy/3’-hydroxy anthocyanins was increased in response to both E and S. This ratio gives an indication of the potential 3’OMT activity within the pathway, i.e. the methylation of cyanidin to form peonidin. The value of ratio of 3’5’methoxy/3’5’-hydroxy anthocyanins was not significantly affected by either of the treatments, which may indicate that the conversion of delphinidin to malvidin was not altered in this study.

TABLE 4

Anthocyanin composition in skins of Crimson Seedless treated with either shade boxes, ethephon (200 ppm) or a combination of both in 2005-2006 at De Doorns. Significance (P) determined for three factors S = shade treatment; E = ethephon application and S x E = interactive effect of shade and ethephon application. P was calculated using repeated measures ANOVA where * indicates P<0.05; ** P<0.01 and ns is not significant (n = 8).

Parameter	Sun-exposed clusters		Shaded clusters		Significance (P)		
	Control	Ethephon	Control	Ethephon	S	E	S x E
Total anthocyanin (mg/kg)	1354.6	3614.4	1752.7	3139.3	ns	**	ns
Total phenolics (A280 units)	192.4	200.0	166.3	190.6	*	*	ns
3-monoglucoside anthcyanins (mg/kg)	955.4	2873.7	1136.8	2576.7	ns	**	ns
Delphinidin	65.7	127.1	73.3	64.7	ns	*	*
Cyanidin	149.0	483.0	98.6	148.6	*	*	*
Petunidin	68.2	129.9	75.6	75.3	ns	*	*
Peonidin	586.1	1853.0	777.6	2105.7	ns	**	ns
Malvidin	104.4	280.7	111.5	182.4	ns	**	ns
3-p-coumaroyl anthocyanins (mg/kg)	315.4	590.7	362.8	489.2	ns	*	ns
Delphinidin	22.1	74.5	18.2	41.2	ns	ns	ns
Cyanidin	75.2	148.3	87.9	100.4	ns	**	*
Petunidin	61.9	65.0	55.8	62.3	ns	ns	ns
Peonidin	92.6	207.7	121.7	206.4	ns	**	ns
Malvidin	63.5	95.3	79.2	79.0	ns	ns	ns

TABLE 5

Anthocyanin composition in skins of Crimson Seedless treated with either shade boxes, ethephon (200 ppm) or a combination of both in 2005-2006 at Paarl. Significance (P) determined for three factors S = shade treatment; E = ethephon application and S x E = interactive effect of shade and ethephon application. P was calculated using repeated measures ANOVA where * indicates P<0.05; ** P<0.01; *** P<0.001 and ns is not significant (n = 4).

Parameter	Sun-exposed clusters		Shaded clusters		Significance (P)		
	Control	Ethephon	Control	Ethephon	S	E	S x E
Total anthocyanin (mg/kg)	1607.0	2317.3	1414.6	2154.2	ns	***	ns
Total phenolics (A280 units)	153.8	171.9	160.3	159.3	ns	ns	ns
3-monoglucoside anthcyanins (mg/kg)	1078.2	1682.7	901.9	1526.6	ns	***	ns
Delphinidin	82.2	98.6	39.5	74.4	***	*	ns
Cyanidin	158.3	162.4	75.7	94.8	**	ns	ns
Petunidin	83.7	105.0	65.4	84.1	*	***	ns
Peonidin	523.4	933.4	538.2	1028.8	ns	***	ns
Malvidin	230.7	383.4	192.1	244.5	ns	***	ns
3-p-coumaroyl anthocyanins (mg/kg)	437.0	521.7	424.7	530.7	ns	***	ns
Delphinidin	53.0	66.8	53.2	71.2	ns	*	ns
Cyanidin	90.7	100.8	88.1	105.4	ns	**	ns
Petunidin	78.4	84.1	80.2	84.4	ns	***	ns
Peonidin	122.1	159.7	110.5	164.5	ns	**	ns
Malvidin	92.8	110.7	92.7	105.1	ns	***	ns

TABLE 6

Ratios of anthocyanin classes in skins of Crimson Seedless treated with either shade boxes, ethephon (200 ppm) or a combination of both in 2005-2006. Significance (P) determined for three factors S = shade treatment; E = ethephon application and S x E = interactive effect of shade and ethephon application. P was calculated using repeated measures ANOVA where * indicates P<0.05; ** P<0.01 and ns is not significant (De Doorns: n = 8; Paarl: n=4).

Ratio	Sun-exposed clusters		Shaded clusters		Significance (P)		
	Control	Ethephon	Control	Ethephon	S	E	S x E
De Doorns							
3'5'-dihydroxy / 3'-hydroxy	0.63	0.54	0.51	0.38	**	**	ns
3'-methoxy / 3'-hydroxy	3.35	6.48	6.96	10.74	**	*	ns
3'5'-methoxy / 3'5'-hydroxy	2.83	3.93	3.69	3.42	ns	ns	ns
Paarl							
3'5'-dihydroxy / 3'-hydroxy	0.42	0.26	0.34	0.15	ns	ns	ns
3'-methoxy / 3'-hydroxy	3.75	4.38	7.76	14.13	**	*	*
3'5'-methoxy / 3'5'-hydroxy	1.85	2.26	1.52	3.10	ns	ns	ns

DISCUSSION

Effect of shade and ethephon on fruit composition

The effects of ethephon on fruit maturity and composition are well documented in literature but the results are variable. For numerous cultivars generally no changes in TSS or acidity have been noted, and no or little change in pH, as well as total yield and weight per berry have been found due to its application (Szyjewicz *et al.,* 1984). The ripening response observed in this study is therefore in agreement with literature to date. On the other hand, grape developmental responses to natural or artificial shading are variable. Natural cluster shading has been noted to increase berry weight and either increased or maintained berry TA with negligible differences in TSS accumulation (Kliewer & Antcliff, 1970; Reynolds *et al*., 1986; Crippen & Morrison, 1986a; Morrison & Noble, 1990; Price *et al*., 1995). Increased TA was proposed to be due to reduced malate respiration under shaded conditions (Kliewer & Lider, 1968; Bledsoe *et al*., 1988; Smart *et al*., 1988; Price *et al*., 1995). Artificial shading of grape clusters from flowering or berry set has been shown to produce either no change in fruit composition, or decreased berry weight, increased pH due to accumulation of K^+ and increased TA due to increased malate while TSS was unchanged (Bindon, 2004; Downey *et al*., 2004;

Cortell & Kennedy, 2006; Ristic *et al*., 2007). The response to artificial shading in the experiments was variable between sites. In the case of the De Doorns experiment it delayed ripening. The reduced TSS and TA associated with this experiment was therefore probably not due to increased respiration of malic acid in the berries, but rather delayed maturity. However, in the case of the Paarl experiment, the results are in agreement with Ristic *et al*. (2007), where shade decreased berry weight while not altering TSS accumulation in Shiraz grapes. The reason for this reduced berry weight under artificial shade conditions has not been ascertained through research, but may be due to reduced dry weight accumulation pre-véraison, where the berry is unable to directly fix carbon via photosynthesis due to extreme darkened conditions and berry chlorosis (Downey *et al*., 2004).

Anthocyanin profile

Various researchers have shown that low light environments reduced the colour of grapes (Crippen & Morrison, 1986b; Smart *et al*., 1988; Morrison & Noble, 1990; Price *et al*., 1995; Bergqvist *et al*., 2001). However, as investigations into the effects of exposure on colour continued, a growing body of contradictory data began to appear (Downey *et al*., 2006). It was found in some studies that no change in total anthocyanins was observed with artificial shading (Downey *et al*., 2004; Ristic *et al*., 2007), while others have reported that increased exposure to sunlight resulted in decreased anthocyanin levels in berries, most likely due to decreased anthocyanin synthesis at the higher berry temperatures under these conditions (Hunter *et al*., 1995; Bergqvist *et al*., 2001; Spayd *et al*., 2002). In some cases, there was no difference in total anthocyanin levels, but alteration in anthocyanin composition was observed in response to altered light conditions within the bunch zone (Price *et al*., 1995; Haselgrove *et al*., 2000; Spayd *et al*., 2002; Downey *et al*., 2004). In studies on Shiraz grapes, shaded fruit generally did not have altered total anthocyanin levels, but was shown to have an increased proportion of dioxygenated anthocyanins, namely glucosides of Cn and Pn (Downey *et al*., 2004; Ristic *et al*., 2007). In another study, Keller & Hrazdina (1998) also found Cn to be the most strongly influenced by prevailing environmental conditions, while Mv was the least affected. In the current study the anthocyanin Cn in Crimson Seedless reflected the sensitivity to the shade treatment shown in other literature, and was the anthocyanin which responded most significantly to shaded conditions. However, it was decreased as a proportion of total anthocyanins in both sites where the experiment was performed rather than increased as shown in other studies (Downey *et al*., 2004; Ristic *et al*., 2007). In this research, Crimson Seedless was shown to accumulate primarily glucosides of Pn, in agreement with Cantos *et al.* (2002). This indicates that the biosynthetic pathway for anthocyanin in this cultivar is genetically pre-disposed to favour the F3'H branch of pathway toward Pn, rather than the F3'5'H branch of the pathway toward Mv as for winegrapes (Boss *et al*., 1996; Castellarin *et al*., 2006). Unlike the studies on Shiraz (Downey *et al*., 2004; Ristic *et al*., 2007) shading of Crimson Seedless berries did not alter the proportion of Pn, most likely because synthesis of this anthocyanin from Cn is already favoured in this cultivar at a genetic level. Analysis of the ratios of anthocyanins showed that conversion of Cn to Pn via the enzyme 3'OMT was most likely affected by the shade treatment, such that synthesis of Pn was favoured under shade conditions. However, only gene expression studies or radiolabelling experiments will verify this hypothesis.

The overriding effect of ethephon application to increase anthocyanin production in Crimson Seedless was an expected result of the research. Ethephon is well-known to increase the red colour of grapes of multiple cultivars (Takeda & Badr, 1977; Wicks, 1979; Powers *et al*., 1980; Wicks & Kliewer, 1983; Keller & Hrazdina, 1998; El-Kereamy *et al*., 2003; Lombard *et al*., 2004; Gallegos *et al*., 2006). There is speculation that the increase in anthocyanin is associated with increases in the presence of the monoglucoside pigments Pn and Mv (Takeda & Badr, 1977; Wicks, 1979; Powers *et al*., 1980; Wicks & Kliewer, 1983; El-Kereamy *et al*., 2003) indicating increased production of terminal anthocyanins within the biosynthetic pathway. This was confirmed through gene expression studies on Cabernet Sauvignon, which showed upregulation of the gene for UFGT (El Kereamy *et al*., 2002; El-Kereamy *et al*., 2003). In the current study, it is interesting to note the synergistic enhancement of Dn, Cn and Pt by ethephon in sun-exposed fruit at the Paarl trial site. This is unexpected, since ethephon application was generally found to promote the accumulation of highly methoxylated monoglucosides of Pn and Mv in the berry skin during ripening (Takeda & Badr, 1977; Wicks, 1979; Powers *et al*., 1980; Wicks & Kliewer, 1983; El-Kereamy *et al*., 2003; Gallegos *et al*., 2006). This may reflect a synergistic decrease in methoxylation of anthocyanins synthesised in response to ethephon under higher light conditions. The significant interactive effect of light and ethephon on the ratio of Pn/Cn may point to the involvement of a methyltransferase in the observed response.

CONCLUSIONS

This research has shown a strong cultivar-dependent effect on the response of anthocyanin accumulation to environmental conditions, in this case shading. For red table grapes, the response of anthocyanin accumulation have been shown to be either highly sensitive or insensitive to bunch shading. Crimson Seedless was shown to be insensitive to shade in terms of accumulation of its primary anthocyanin, Pn-glucoside. On the other hand, Crimson Seedless showed a strong positive response to ethephon application in terms of anthocyanin accumulation. This indicates that it is sensitive to the application of growth regulators, such as ethephon and potentially ABA (Cantín *et al.,* 2007) which can be used for the commercial enhancement of skin colour properties.

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