Effect of delayed storage and continuous ethylene exposure on flesh reddening of 'Royal Diamond' plums

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Abstract

BACKGROUND: Flesh reddening has been described as one of the manifestations of plum fruits to low-temperature storage for prolonged periods. The influence of factors such as ethylene and delayed storage has not been studied to date. In order to assess that, plum cv. 'Royal Diamond' fruits were (a) stored at 5 °C (control), (b) held at 20 °C for 2 days before storage at 5 °C (delayed storage) or (c) maintained at 5 °C under 15 μL L⁻¹ ethylene. Fruits were then transferred to 20 °C and ethylene, respiration, weight loss, firmness, soluble solids content, acidity, flesh reddening, anthocyanin accumulation and phenylalanine ammonia-lyase (PAL) activity were determined.

RESULTS: Delayed storage fruits showed more extensive flesh reddening than control fruits, with increased PAL activity and higher anthocyanin accumulation. Symptoms were expressed more markedly when fruits were stored at 5 °C in ethylene.

CONCLUSION: Results indicated that the fruit ripening stage is a critical factor determining the susceptibility of 'Royal Diamond' plums to flesh reddening. Fruits continuously exposed to ethylene showed a dramatic increase in reddening, suggesting that ethylene contributes to the development of the disorder.
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Keywords: *Prunus salicina*; ripening stage; chilling injury; physiological disorder; market life

INTRODUCTION

Chilling injury (CI) is one of the main factors limiting refrigerated storage of fresh plums (*Prunus salicina* Lindell). The susceptibility to CI is variable and dependent on the genotype, but most plum cultivars are susceptible when stored at 5 °C.[1] In addition there is substantial variation in terms of the extent to which the disorder is manifested, and several different symptoms have been associated with plum CI. These include flesh translucency (gel breakdown), browning, failure to ripen and red pigment accumulation (reddening or bleeding).[2]

Several research groups have analysed the influence of ethylene and delayed storage on the incidence and severity of CI symptoms in other fruits.[3–5] In peaches, for instance, the underlying mechanisms of CI are well documented.[4–7] However, to date, only a few studies have been conducted with plums,[8] and the factors influencing the different manifestations of CI have not been thoroughly investigated.

The effect of ethylene on CI development has been controversial.[3,9–11] In some cases, increased ethylene production has been correlated with CI development; however, the interpretation of these results is usually limited, since it is difficult to determine whether ethylene increase is necessary for the manifestation of CI symptoms or whether it is just a consequence of tissue damage that is unrelated to symptom appearance. Ethylene has been demonstrated to be involved in the development of CI symptoms in some commodities, and reduction of the incidence and severity of symptoms by decreasing ethylene sensitivity and/or production has also been achieved in fruits such as avocado[10] and pineapple.[11] However, ethylene treatment alleviated CI symptoms (i.e., mealy texture and flesh reddening) in nectarines.[12] These differences in the impact of ethylene on CI development may be due to the distinct nature of the diverse CI manifestations in horticultural crops and suggest that the role of ethylene in CI should be studied on a case-by-case basis. In order to increase our understanding of flesh reddening (bleeding), one of the symptoms that has been associated with CI in plums, we evaluated the influence of delayed storage and continuous ethylene exposure on the development of the disorder in the susceptible cultivar 'Royal Diamond'.

MATERIALS AND METHODS
Fruit material and treatments
Plum fruits (cv. 'Royal Diamond') were harvested at the firm-ripe stage and immediately transported

to the laboratory. The average firmness, soluble solids content and titratable acidity at harvest were 31 ± 3 N, $11.9 \pm 1.3\%$ and $0.45 \pm 0.05\%$ (malic acid basis) respectively. Three lots of 96 plums each were put into cardboard boxes with individual plastic trays to prevent physical damage. The first group (control) was stored at $5\,^{\circ}$C for 2 or 4 weeks. The second group (delayed storage) was held at $20\,^{\circ}$C for 2 days and then stored at $5\,^{\circ}$C for 2 or 4 weeks. Fruits of the last group were stored for 2 or 4 weeks in 338 L sealed aluminium tanks connected to an ethylene flow-through system that had been set up in the cold storage room. Flow rates and mixtures of compressed air and ethylene were established using a main mixing board and secondary flow boards with micrometric needle valves to provide an ethylene concentration of $15\,\mu\text{L}\,\text{L}^{-1}$. Supply and exhaust ethylene concentrations were monitored periodically with a gas chromatograph equipped with a packed alumina column and a flame ionisation detector (Carle AGC-211, EG&G Chandler Engineering, Tulsa, OK, USA). Fruits from the control and delayed storage treatments were also put in sealed tanks but with circulating air. Total flow rates were adjusted to $23.3\,\text{mL}\,\text{s}^{-1}$ in order to get an adequate gas exchange rate and prevent CO_2 accumulation and excessive gas flow through the tanks. Flow rates were measured with a digital flowmeter (ADM-1000, J&W Scientific, Folsom, CA, USA).

Each 96-fruit lot for each treatment was divided into four 24-fruit sub-lots. Two sub-lots of each treatment were transferred to $20\,^{\circ}$C after 2 or 4 weeks of storage at $5\,^{\circ}$C and subsequently analysed after 1 and 5 days of shelf-life.

Tissue firmness

Fruit firmness was monitored non-destructively using a low-mass impact Sinclair IQ (Internal Quality) Firmness Tester (SIQ-FT Systems International, LLC, Fresno, CA, USA). The SIQ-FT pneumatically operated sensor has a head equipped with a piezoceramic generator, which is pushed out of the bellows end each time the device hits a fruit sample. The recorded signal is passed through a digital converter interfaced to a personal computer and is processed by proprietary software (Sinclair IQ Version PIQ01-v2.18.01) to return a measure of fruit firmness as a number indexed from 0 to 100 (arbitrary units). Data are reported in terms of this 'Sinclair firmness index' (SFI). Softer fruits are assigned a lower SFI value than firmer fruits.[13]

Tissue firmness was also monitored destructively with a Fruit Texture Analyser (QA Supplies, Norfolk, VA, USA) equipped with a 7.9 mm diameter, flat-tipped probe. Compression tests were performed at a speed of $0.17\,\text{mm}\,\text{s}^{-1}$.

Weight loss

Fruits were weighed at harvest and during the storage period. Weight loss was calculated for each fruit by the following equation: weight loss (%) = $[(W_{\text{o}} - W_{\text{f}})/W_{\text{o}}] \times 100$, where $W_{\text{o}} =$ initial weight and $W_{\text{f}} =$ final weight.

Soluble solids content (SSC) and titratable acidity (TA)

For SSC and TA measurements a longitudinal wedge (from stem end to calyx end) was removed from each fruit and pressed through cheesecloth. The juice from eight fruits was pooled and the SSC of the juice was measured with a temperature-compensated refractometer (ATC-1, Atago Co., Tokyo, Japan). TA was determined with an automatic titrator (Radiometer, Copenhagen, Denmark) according to the AOAC.[14]

Ethylene production and respiration rate

Ethylene production and respiration rate (i.e., not CO_2 production) were measured daily during storage at $20\,^{\circ}$C for each treatment. Individual fruits were weighed and placed for 1 h in a 0.705 L container, which was then sealed and held at $20\,^{\circ}$C and 90% relative humidity (RH). Carbon dioxide concentration was measured with an infrared gas analyser (Horiba PIR-2000R, Horiba Instruments Inc., Irvine, CA, USA) For ethylene measurements, samples were withdrawn with a 1 mL syringe through a septum fitted in the container lid, and gas analysis was performed using a gas chromatograph as described above. Five independent measurements were done daily for each treatment, up to 5 days after removal from the 2 or 4 week cold storage periods. Results were expressed as $\mu\text{L}\,\text{kg}^{-1}\,\text{h}^{-1}$ for ethylene production and $\text{mL}\,CO_2\,\text{kg}^{-1}\,\text{h}^{-1}$ for respiration rate.

Incidence of flesh reddening

Each fruit was cut longitudinally into two halves and classified in one of four distinct categories: (1) sound fruit; (2) fruit with slight disorder (less than 25% of the flesh showed reddening; (3) fruit with medium disorder (between 25 and 50% of the flesh was affected); (4) fruit with severe disorder (more than 50% of the flesh showed symptoms). Reddening severity for each lot of fruits was expressed as an index according to Dong et al.[12] as follows: [(% fruit with slight disorder $\times$ 1) + (% fruit with medium disorder $\times$ 2) + (% fruit with severe disorder $\times$ 4)]/4.

Anthocyanin content

Frozen fruit tissue was ground in an Ultraturrax (Kinematica, GmbH, Lucerne, Switzerland) with 10 mL of HCl-methanol ($10\,\text{mL}\,\text{L}^{-1}$) and held at $0\,^{\circ}$C for 10 min.[15] The slurry was centrifuged ($1500 \times g$, 10 min, $4\,^{\circ}$C) and the absorbance of the supernatant at 515 nm was measured. Results were calculated using $\varepsilon = 29\,600\,\text{L}\,\text{mol}^{-1}\,\text{cm}^{-1}$, permitting the calculation of anthocyanin content as μmol cyanidin 3-glucoside kg^{-1} fresh weight.[16]

Phenylalanine ammonia-lyase (PAL) activity

Fruit samples of 5 g were homogenised in an OmniMixer (OMNI Instruments Marletta, GA, USA) with 2

volumes of buffer containing $0.1 \, mol \, L^{-1} \, Na_2B_4O_7 \cdot 10H_2O$, $5 \, mmol \, L^{-1}$ 2-mercaptoethanol, $2 \, mmol \, L^{-1}$ ethylene diamine tetraacetic acid (EDTA) and $10 \, g \, L^{-1}$ poly(vinylpolypyrrolidone) (PVPP), pH 8.8. The mixture was stirred for 1 h at 4 °C and then centrifuged ($10\,000 \times g$, 20 min, 4 °C). The enzymatic activity was measured in the supernatant by the method reported by Zucker.[17] The reaction mixture was incubated at 30 °C and the reaction was evaluated based on the increase in optical density at 290 nm (ΔOD_{290}) caused by production of *trans*-cinnamic acid. Two independent extracts per treatment and storage condition were prepared and measurements were done in duplicate for each independent replication. Results were expressed as $\Delta OD_{290} \, kg^{-1} \, h^{-1}$.

Statistical analysis

The experimental design was a completely randomised block. A total of 288 fruits were used, with three replicates of eight fruits for each of the three treatments and four storage times analysed, unless stated otherwise. Data were subjected to analysis of variance (ANOVA) followed by the Waller–Duncan multiple range test at $P < 0.05$. Means were also compared by the least significant difference (LSD) test at $P \leq 0.05$.

RESULTS AND DISCUSSION
Fruit quality attributes

Slight fruit weight loss was found for all treatments (2.0–2.4%) after 4 weeks of cold storage plus 5 days at 20 °C (Table 1). As expected, weight loss increased during storage, but no differences among treatments were observed. Similarly, no differences were detected for TA or SSC (Table 1). However, ethylene-treated fruits showed increased softening relative to control fruits for all storage times analysed, as measured both destructively and non-destructively, showing that

Table 1. Weight loss (WL), soluble solids content (SSC) and titratable acidity (TA) of 'Royal Diamond' plum fruits after 2 or 4 weeks (w) of storage at 5 °C and additional ripening at 20 °C for 1 and 5 days (d). The least significant differences (LSD) at $P \leq 0.05$ are shown

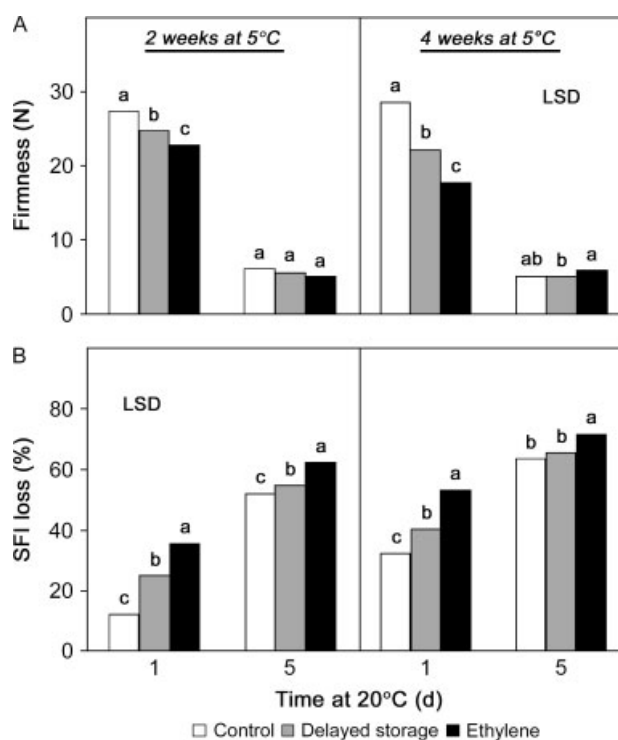|	Storage regime	WL (%)	SSC (%)	TA (%)
Control	2 w 5 °C + 1 d 20 °C	1.15	12.8	0.39
	2 w 5 °C + 5 d 20 °C	1.86	11.9	0.32
	4 w 5 °C + 1 d 20 °C	1.59	11.6	0.37
	4 w 5 °C + 5 d 20 °C	2.34	12.3	0.34
Delayed storage	2 w 5 °C + 1 d 20 °C	0.69	12.0	0.39
	2 w 5 °C + 5 d 20 °C	1.94	11.7	0.35
	4 w 5 °C + 1 d 20 °C	1.20	12.0	0.35
	4 w 5 °C + 5 d 20 °C	2.11	11.2	0.27
Ethylene	2 w 5 °C + 1 d 20 °C	0.92	11.7	0.38
	2 w 5 °C + 5 d 20 °C	1.87	11.7	0.43
	4 w 5 °C + 1 d 20 °C	1.29	11.8	0.35
	4 w 5 °C + 5 d 20 °C	2.35	11.7	0.34
LSD		0.51	1.2	0.10

Figure 1. (A) Destructive and (B) non-destructive (Sinclair firmness index loss) assessments of 'Royal Diamond' plum fruit firmness after 2 or 4 weeks of storage at 5 °C and additional ripening at 20 °C for 1 and 5 days (shelf-life). Letters on each column within each storage treatment show significant differences at the 5% level (Waller–Duncan multiple range test). The least significant differences (LSD) at $P \leq 0.05$ are shown.

exposure to the hormone even at 5 °C was sufficient to accelerate fruit ripening (Fig. 1).

Flesh reddening

Fruits refrigerated at a more advanced ripening stage (delayed storage) or exposed continuously to ethylene showed increased flesh reddening. Such symptoms are a cause of fruit rejection for cosmetic reasons. In peaches and nectarines the cells near the pit cavity contain anthocyanins, and extended cold storage leads to reddening of the entire area around the pit.[4] In 'Royal Diamond' plums, however, the symptoms were observed initially in the fruit flesh periphery, the discolouration later extending towards the pit cavity.[2] The manifestation of the disorder was similar to that previously reported for 'Blackamber' plums.[2] The severity of flesh reddening increased as ripening at 20 °C progressed, with differences among treatments. The reddening index after 2 and 4 weeks of cold storage and 5 days of shelf-life was highest (67 and 75% respectively) for ethylene-treated fruits, with intermediate values for delayed-stored fruits (23 and 35% respectively); only slight reddening was measured in control fruits (12 and 4% respectively) (Fig. 2).

To date, very little research to anlayse the factors influencing flesh reddening has been conducted and the focus of most studies has been limited to the description of symptom occurrence and the testing of some strategies to control the disorder. Flesh
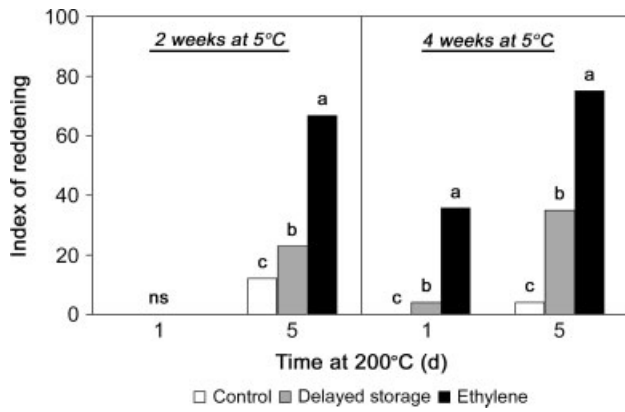
Figure 2. Index of reddening in 'Royal Diamond' plum fruits after 2 or 4 weeks of storage at 5 °C and additional ripening at 20 °C for 1 and 5 days (shelf-life). Letters on each column within each storage treatment show significant differences at the 5% level (Waller–Duncan multiple range test).

Figure 3. Anthocyanin content of 'Royal Diamond' plum fruits after 2 or 4 weeks of storage at 5 °C and additional ripening at 20 °C for 1 and 5 days (shelf-life). Letters on each column within each storage treatment show significant differences at the 5% level (Waller–Duncan multiple range test). The least significant difference (LSD) at $P \leq 0.05$ is shown.

Figure 4. PAL activity in 'Royal Diamond' plum fruits after 2 and 4 weeks of storage at 5 °C and additional ripening at 20 °C for 1 and 5 days (shelf-life). Letters on each column within each storage treatment show significant differences at the 5% level (Waller–Duncan multiple range test). The least significant difference (LSD) at $P \leq 0.05$ is shown.

reddening was first cited as a physiological disorder in nectarines,[12,18] but it is also described as one of the manifestations of CI in plums.[1,2]

Usually, chilling-injured fruits show higher ethylene production than healthy fruits.[19] However, the role of ethylene in CI symptom development has been controversial. This is probably due to the fact that CI includes a number of processes triggered by exposure of commodities to low temperatures. However, these processes may have quite different physiological and biochemical bases. Furthermore, in many of these cases, whether the increased ethylene production causes the disorder or is just a consequence of tissue disruption has not been determined. Ethylene seems to be involved in some but not all cases. CI-associated disorders are reduced by the ethylene inhibitor 1-methylcyclopropene (1-MCP) in some commodities such as avocado,[10] pineapple[11] and pear,[20] suggesting that ethylene induces some of the CI-associated symptoms. Increased ethylene production preceded the development of CI symptoms of mandarins.[21] However, in nectarines, 1-MCP treatments enhanced the appearance of the mealiness (dry texture) and reddening symptoms usually associated with CI,[5,12] and the application of ethylene during 0 °C storage for 30 days had no effect on the development of internal reddening or flesh bleeding.[22] The present study indicates that ethylene accelerates the development of plum fruit flesh reddening, suggesting that the hormone is involved in the development of the disorder in plums. This conclusion is also supported by a recent report which showed that 1-MCP treatments were effective in reducing reddening in plums.[23] In addition, fruits from the delayed storage treatment showed more extensive reddening than control fruits. These data suggest that the ripening stage is of critical importance in determining flesh susceptibility to reddening in 'Royal Diamond' plums. Despite the fact that reddening is usually exacerbated by chilling, the problem has occasionally been observed in non-refrigerated fruits (Ziosi V and
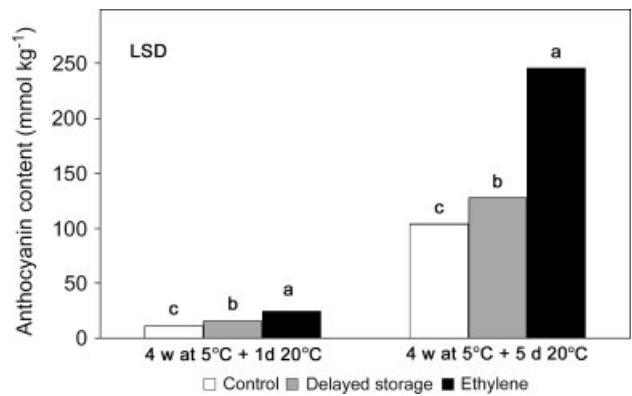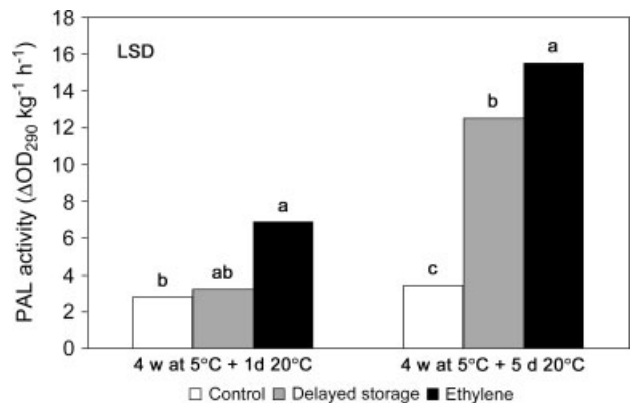
Costa G, personal communication; Crisosto CH et al., unpublished data), suggesting that it might be a more general response to stress conditions common in the postharvest environment, including, but not limited to, prolonged storage at chilling temperatures.

Anthocyanins and PAL activity

When fruits from all treatments were transferred to 20 °C after low-temperature storage, increased accumulation of anthocyanins was measured after 4 weeks of cold storage, being apparent after only 1 day at 20 °C (Fig. 3). In agreement with the data for tissue reddening, fruits continuously exposed to ethylene during low-temperature storage presented the highest anthocyanin accumulation. Delayed-stored fruits had slightly higher anthocyanin content than control fruits after both 1 and 5 days at 20 °C, but much lower than that observed in ethylene-treated fruits. The accumulation of anthocyanins in fruits from all treatments was paralleled by the extractable fruit PAL activity (Fig. 4). PAL is a key

enzyme in phenylpropanoid metabolism, catalysing the eliminative deamination of phenylalanine to produce *trans*-cinnamic acid. PAL expression has been reported to be activated by ethylene[24,25] and storage at chilling temperatures.[19,21]

Respiration rate and ethylene production

Respiration rates showed slight differences among treatments. After 2 weeks of cold storage plus 5 days of shelf-life, ethylene-treated fruits exhibited 30–50% greater CO_2 production than control and delayed-stored fruits (Fig. 5). Increased ethylene production was found in ethylene-treated fruits after 2 weeks of low-temperature storage, while after 4 weeks at 5 °C no differences among treatments were detected (Fig. 6). Intriguingly, control and delayed-stored fruits showed no significant differences in ethylene production throughout the 5 day ripening period after removal from 2 or 4 weeks of cold storage. This contradicts the idea that ethylene acts as a direct effector in PAL-mediated anthocyanin accumulation and reddening, unless the differences between symptoms of control and delayed-stored fruits are associated with increased
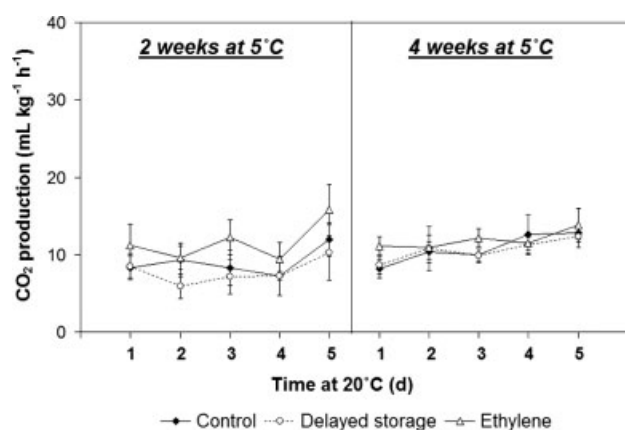
ethylene sensitivity of the more mature fruits from the delayed storage treatment. Alternatively, the ripening stage *per se* could be the primary determinant of fruit susceptibility to reddening; consequently, the more advanced the ripening stage of the fruit, the higher its susceptibility to flesh reddening. Thus the effect of continuous ethylene treatment in this scenario might be indirect, via its role in accelerating ripening (as suggested from the firmness data in Fig. 1). Another hypothesis is that exogenously applied ethylene accelerates fruit ripening. However, when symptoms of abnormal fruit ripening occur, such as flesh reddening, endogenous ethylene production may be blocked. Reduced levels of ethylene production by chilling-injured (dry-mealy) nectarine fruits have been associated with abnormal fruit ripening.[26] However, also taken into account should be the idea that low-temperature disorders, including flesh mealiness and flesh reddening, may be, to some extent, independent of each other.[27]

Overall, the results of our study indicate the complexity of plum fruit ripening, that ethylene production during postharvest storage is also affected by the thermal and developmental histories of the product and thus its direct or indirect roles are difficult to distinguish. Despite the potential direct or indirect roles of ethylene in flesh reddening, other, so far unidentified, ethylene-independent changes could be sufficient to trigger the increases in PAL and anthocyanin accumulation that ultimately lead to tissue reddening.

Figure 5. Respiration rate in 'Royal Diamond' plum fruits after removal from 2 and 4 weeks of cold storage and additional ripening at 20 °C for 1, 2, 3, 4 and 5 days (shelf-life). The standard deviations of the means ($\pm$SD, $n = 5$) are shown.
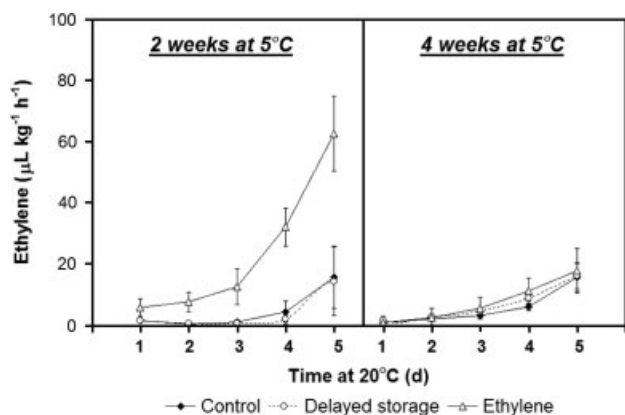
CONCLUSION

Delayed storage regimes and ethylene treatment during cold storage have been used to alleviate CI symptom development in nectarines, while 1-MCP treatment induced nectarine flesh reddening symptoms.[5,12] In 'Royal Diamond' plums, delayed storage increased the severity of flesh reddening, indicating that, as fruit ripening progresses, there is an increase in susceptibility to the disorder. In addition, continuous ethylene exposure led to the appearance of intense flesh reddening symptoms which were associated with higher extractable PAL activity and anthocyanin accumulation during post-storage ripening at 20 °C, suggesting a role for ethylene in plum flesh reddening. Determining whether or not ethylene acts directly by promoting pigment accumulation through the induction of PAL or indirectly by promoting fruit ripening in the cold storage environment and thus increasing susceptibility to reddening, which is ultimately triggered by an/other effector/s, would require further studies.

Figure 6. Ethylene production in 'Royal Diamond' plum fruits after removal from 2 and 4 weeks of cold storage and additional ripening at 20 °C for 1, 2, 3, 4 and 5 days (shelf-life). The standard deviations of the means ($\pm$SD, $n = 5$) are shown.

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