Postharvest Biology and Technology 65 (2012) 79–91

Contents lists available at SciVerse ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Antioxidant metabolism of 1-methylcyclopropene (1-MCP) treated 'Empire' apples during controlled atmosphere storage

Jinwook Lee[a,b], Lailiang Cheng[a], David R. Rudell[b], Christopher B. Watkins[a,*]

[a] Department of Horticulture, Cornell University, Ithaca, NY 14853, USA
[b] USDA-ARS, Tree Fruit Research Laboratory, 1104 N. Western Avenue, Wenatchee, WA 98801, USA

ARTICLE INFO

Article history:
Received 11 October 2011
Accepted 19 November 2011

Keywords:
Apple
Malus x *domestica*
Reactive oxygen species
Enzymatic antioxidant system
Non-enzymatic antioxidant system
Partial least square regression analysis

ABSTRACT

'Empire' apples [*Malus sylvestris* (L.) Mill var. *domestica* (Borkh.) Mansf.] are susceptible to development of firm flesh browning during controlled atmosphere storage. The browning is thought to be a chilling injury and therefore fruit are typically stored at 2–3 °C to avoid fruit damage. However, flesh browning can be enhanced by 1-methylcyclopropene (1-MCP) treatment at these warmer temperatures. The objective of this work was to investigate the effect of 1-MCP on the antioxidant systems of 'Empire' apple fruit stored at 2 kPa O_2/2 kPa CO_2 at either 0.5 °C or 3.3 °C for up to 40 weeks. Lightness (L^*) and hue angle (h°) changes of the flesh tissues were correlated with development of flesh browning. Nitroblue tetrazolium reducing activity was lower in 1-MCP treated fruit, but while changes in H_2O_2, MDA, ascorbic acid and dehydroascorbate, reduced and oxidized glutathione concentrations, and the activities of associated enzymes were affected by storage temperature and 1-MCP treatment, no consistent patterns of change were detected. Partial least squares regression analysis revealed that the activity of ascorbate peroxidase might be the candidate metabolite in flesh browning development at 3.3 °C, but overall, the results do not reveal a direct role of antioxidant metabolism during development of flesh browning in 'Empire' apples.

1. Introduction

'Empire' is a major apple cultivar in the northeastern United States that is grown for both domestic and export markets. However, a number of physiological disorders limit its quality in controlled atmosphere (CA) storage, including external CO_2 injury, flesh browning and core browning (Watkins and Liu, 2010). While external CO_2 injury can be controlled by controlling CO_2 concentration during storage or by treatment of fruit with the antioxidant diphenylamine (DPA) (Watkins et al., 1997; DeEll et al., 2007; Fawbush et al., 2008), 'Empire' firm flesh browning disorder can also cause serious fruit losses and is more difficult to control (DeEll et al., 2005, 2007; Watkins and Nock, 2005; Fawbush et al., 2009). Firm flesh browning is similar to the flesh browning described by Meheriuk et al. (1994) in that affected tissues remain firm and juicy and therefore it is distinct from senescent breakdown. Firm flesh browning in 'Empire' apples typically becomes apparent in May/June, depending on the season, and is usually associated with storage temperatures of 0 °C. Therefore, warmer storage temperatures (2–3 °C) are used during CA storage to avoid injury.

Recently, the adoption of 1-methylcyclopropene (1-MCP)-based technology by apple industries has impacted 'Empire' fruit storage. Although this cultivar responds well to 1-MCP treatment resulting in firmer fruit and better quality characteristics after both air or CA storage (Watkins et al., 2000; DeEll et al., 2005), 1-MCP treated fruit can have much higher flesh browning incidence than untreated fruit after long term CA storage at warmer storage temperatures (Watkins, 2008; Jung et al., 2010; Jung and Watkins, 2011). Although the reasons for increased browning in 1-MCP treated fruit are unknown, Jung and Watkins (2011) suggested that inhibition of ethylene production at 4 °C by 1-MCP may induce stress-like conditions comparable with fruit stored at 0.5 °C, with or without 1-MCP.

Flesh browning in plant tissues is usually associated with enzymatic oxidation of phenolic compounds by polyphenol oxidase (PPO) to *o*-quinones, which polymerize non-enzymatically to produce heterogeneous black, brown or red pigments commonly called melanins (Tomas-Barberan and Espin, 2001). Under extended oxidative stress, phenolic compounds in vacuoles can leak into the cytosol, and subsequently, enzymatic browning reactions occur (Nicolas et al., 1994). Jung and Watkins (2011) found that PPO activity was higher in 1-MCP treated fruit at both 0.5 and 4 °C, but lowest in untreated fruit stored at 4 °C, where the least browning was observed.

Research into the origin of storage-related browning disorders has focused on catabolic or anabolic processes, but ultimately injury is reflected in the balance between oxidative and reductive processes (Franck et al., 2007). Damage to cellular processes can

* Corresponding author.
E-mail address: cbw3@cornell.edu (C.B. Watkins).

0925-5214/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.postharvbio.2011.11.003

occur if reactive oxygen species (ROS), such as superoxide anion ($O_2^{\bullet -}$) radicals, H_2O_2, hydroxyl ($HO^{\bullet}$) radicals, perhydroxyl ($^{\bullet}O_2H$), nitric oxide ($NO^{\bullet}$) and peroxynitrile ($ONOO^{\bullet}$), are not controlled by antioxidant scavenging systems. Lipid peroxidation, cell membrane deterioration, and DNA and RNA damage, followed by biochemical and metabolic malfunction, will ultimately cause cell death (Hodges et al., 2004; Toivonen, 2004).

Enzymes associated with enzymatic antioxidant scavenging systems include superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and ascorbate peroxidase (APX). SOD enzymes play a pivotal role in decomposing superoxide anion radicals into O_2 and H_2O_2, and then, APX, POX and CAT enzymes detoxify H_2O_2 into either H_2O or O_2 (Mittler, 2002). In addition, non-enzymatic antioxidant scavenging systems include ascorbic acid, glutathione, tocopherol, carotenoid and phenolic compounds (Larson, 1988; Noctor and Foyer, 1998; Apel and Hirt, 2004). The ascorbic acid and glutathione cycles play a fundamental role in a ROS scavenging system with APX, monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) activities that generate reduced forms of ascorbic acid and glutathione by using electrons derived from NAD(P)H, via the pentose phosphate pathway (Noctor and Foyer, 1998; Apel and Hirt, 2004).

Much research focus about flesh browning of apples and pears has been on CO_2-related injuries. A higher incidence of flesh browning was associated with lower ascorbic acid concentrations in 'Pink Lady' flesh tissues stored at 1.5 kPa O_2/5 kPa CO_2 (de Castro et al., 2008). These partial pressures were associated with reduced ascorbic acid concentrations but slightly increased dehydroascorbic acid concentrations in 'Pink Lady' apples over 4 months (de Castro et al., 2008). Storage at 1.5 kPa O_2/5 kPa CO_2 also resulted in higher H_2O_2 concentrations. Flesh browning was associated with the accumulation of malondialdehyde (MDA) and 4-hydroalkenals (4HNE) levels in 'Blanquilla' pear stored at 2 kPa O_2/5 kPa CO_2 (Larrigaudière et al., 2001b), and loss of ascorbic acid levels in CA stored 'Conference' and 'Rocha' pears (Veltman et al., 1999, 2000). Also, the levels of ascorbic acid were not evenly distributed and were lower within brown flesh tissues than healthy pear flesh tissues (Franck et al., 2003). Higher pCO_2 (5 kPa) in CA may provoke development of high CO_2-induced flesh browning in 'Empire' apples at 0 °C, but the browning is not a classic CO_2-related injury (Watkins and Liu, 2010). An atmosphere of 2 kPa CO_2 with 2 or 3 kPa O_2 inconsistently affected dehydroascorbic acid and total ascorbic acid concentrations, but increased total antioxidant activity in 1-MCP treated 'Empire' apple flesh tissues stored at 0.5 °C for 9 months (Fawbush et al., 2009).

Little is known about the effects of 1-MCP on antioxidant systems in apple fruit, and especially in relation to non-CO_2 related internal browning disorders. The objective of this study was to investigate the influence of two storage temperatures on the activities of enzymatic and non-enzymatic antioxidant scavenging systems of 1-MCP treated 'Empire' apple fruit during CA storage for up to 40 weeks.

2. Materials and methods

2.1. Fruit source, treatments and storage conditions

'Empire' apple [*Malus sylvestris* (L.) Mill var. *domestica* (Borkh.) Mansf.] fruit used in these experiments were harvested on October 9, 2007 from mature trees at the Cornell University orchards at Lansing, NY. Fruit were selected for uniform size (approximately 7 cm in length and 8 cm in diameter) and randomly sorted into experimental units of five fruit. Five fruit were used for the harvest assessment as described below, and the remaining fruit were precooled overnight at 3.3 °C. Half of the fruit units were then either untreated or treated with 1 μL L^{-1} 1-MCP (SmartFresh tablets, 0.36% a.i., AgroFresh Co., Spring House, PA) for 24 h in a 4000 L plastic tent using a release and fan system supplied by the manufacturers. Fruit were vented overnight at either 0.5 or 3.3 °C and then placed into 1 m^3 stainless steel chambers (Storage Control Systems, Inc., Spartan, MI) at 2 kPa O_2/2 kPa CO_2 balanced with N_2. Final atmosphere regimes were established within 48 h. Atmospheres were checked hourly and maintained within 0.2 kPa of target values with a ICA 61/CGS 610 CA Control System (International Controlled Atmosphere Ltd., Kent, UK), modified with flow controllers for the experimental chambers (Storage Control Systems, Sparta, MI).

2.2. Fruit sampling

Five fruit were taken at harvest, and from each atmosphere and temperature regime at 5 week intervals for 40 weeks, maintaining each fruit separately for all measurements and flesh sampling. Each fruit was used for assessment of internal ethylene concentration (IEC), and flesh color. For storage samples, the IEC of each fruit was taken immediately after removal from CA, ensuring that the fruit remained cold. Sub-samples of cortical tissue from each fruit were sliced into liquid nitrogen and stored at −80 °C for metabolite and enzyme activity analyses. The tissues were cryogenically milled to a fine powder with IKA® A11 basic (IKA® Works, Inc. Wilmington, NC) prior to analysis. Selected time points of 0, 5, 15, 30 and 40 weeks were used for metabolite measurements and enzyme assays.

2.3. Internal ethylene concentration (IEC), flesh color and browning severity assessment

The IEC of each fruit was measured on 1 mL samples of internal gas from the core cavity using a Hewlett-Packard 5890 series II gas chromatograph (Hewlett-Packard, Wilmington, DE) equipped with a flame ionization detector and fitted with a stainless steel column packed with 60/80 mesh alumina F-1 (Watkins et al., 2000).

Flesh color was measured at the calyx-end region (1.5 cm from the equatorial region), the equatorial region and the stem-end region (1.5 cm from equatorial region) at six sites with a Minolta chromameter CR-300 (Osaka, Japan). The color measurements were expressed as chroma (C^*, intensity of color), hue angle (h°, actual color) and lightness value (L^*, dark to light on a scale of 0–100) (McGuire, 1992). All fruit were then assessed for internal browning injury. Each fruit was cut at least three times equatorially and the incidence of any internal browning disorder assessed. Browning severity was determined using a subjective five-grade scoring system where 0 = 0%, 1 = 1–10%, 2 = 11–25%, 3 = 26–50%, and 4 = 51–100% browning.

2.4. Superoxide anion radical activity, hydrogen peroxide, and lipid peroxidation

The production of superoxide anion radical was determined according to the method of Doke (1983), based on its ability to reduce nitroblue tetrazolium (NBT) to formazan. NBT is a yellowish compound in its oxidized state and is converted to formazan (blue color) upon reduction by superoxide. Frozen ground tissue (0.3 g) was added into 3 mL reaction mixture of 10 mM Na_2HPO_4–KH_2PO_4 (pH 7.8), 0.05% (w/v) NBT and 10 mM NaN_3. After incubation at room temperature for 1 h, the reaction mixtures were heated to 85 °C for 15 min and then cooled rapidly on ice. The reaction mixture was filtered through a 0.2 μm syringe filter and then the absorbance at 580 nm was recorded using a Genesys 5 spectrophotometer (Spectronic Instruments, Waltham, MA). The ability of the extract to reduce NBT was expressed as OD_{580} g^{-1} h^{-1} (Chen and Cheng, 2004).

The extraction and determination of H_2O_2 was carried out by homogenizing 1 g of frozen ground tissue with a pre-cooled mortar and pestle in 2 mL 5% (w/v) trichloroacetic acid (TCA) and 30 mg of activated charcoal washed with HCl, then centrifuged at 16,000 × *g* at 4 °C for 20 min in Microfuge® 18 Centrifuge (Beckman Coulter Inc., Palo Alto, CA). The supernatant was neutralized to pH 8.5 with 17 M NH_4OH and then centrifuged at 16,000 × *g* at 4 °C for 10 min. The supernatant was immediately used for the H_2O_2 assay (Patterson et al., 1984). Each extract was divided into two aliquots of 0.6 mL. Four units of catalase (CAT, EC 1.11.1.6) were only added to the blank aliquot. Both the blank and the other aliquot (sample tube) without adding catalase were incubated at room temperature for 10 min and then 0.6 mL colorimetric reagent was added to both aliquots. The colorimetric reagent was daily made by mixing 1:1 (v/v) 0.6 mM potassium titanium oxalate and 0.6 mM 4-(2-pyridylazo) resorcinol monosodium salt. The assay mixture (1.2 mL) was incubated at 45 °C for 1 h, and then the absorbance was recorded at 508 nm using a Genesys 5 spectrophotometer by using the blank as a blank for each sample. The quantification of H_2O_2 was calculated based on the H_2O_2 standard regression curve, where H_2O_2 levels ranged from 0 to 100 μM.

Lipid peroxidation was determined by measuring the malondialdehyde (MDA) content in frozen ground tissue according to the method of Hodges et al. (1999). Frozen ground apple flesh tissues (0.25 g) were homogenized with a pre-cooled mortar and pestle in 2 mL of 80% (v/v) ice cold ethanol and 5% (w/v) insoluble polyvinylpolypyrrolidone (PVPP), then centrifuged at 3000 × *g* at 4 °C for 10 min in Microfuge® 18 Centrifuge. After appropriate dilution, the supernatant was divided into two aliquots of 0.6 mL. One aliquot was mixed with 0.6 mL of without thiobarbituric acid (TBA) solution (−TBA), which consisted of 20% trichloroacetic acid (TCA) and 0.01% butylated hydroxyltoluene (BHT), while the other aliquot was mixed with 0.6 mL of with TBA solution (+TBA), which is comprised of the above with 0.65% TBA. After vigorous mixing, the sample was incubated at 95 °C for 25 min, cooled down quickly on ice and then centrifuged at 3000 × *g* at 4 °C for 10 min. The absorbance of the sample was recorded at 440, 532 and 600 nm using a Genesys 5 spectrophotometer. MDA equivalents were calculated in the following manner:

(1) $[(\text{Abs } 532_{+TBA}) - (\text{Abs } 600_{+TBA}) - (\text{Abs } 532_{-TBA} - \text{Abs } 600_{-TBA})] = A$
(2) $[(\text{Abs } 440_{+TBA} - \text{Abs } 600_{+TBA}) \times 0.0571] = B$
(3) $\text{MDA equivalent } (\text{nmol g}^{-1}\text{ FW}) = [(A - B/157{,}000) \times 10^6 \times (\text{adjusted sample FW}) \times (\text{buffer volume}) \times (\text{dilution factor})]$

2.5. Antioxidant metabolites

Ascorbic acid (AsA) and dehydroascorbate (DHA) were measured according to Logan et al. (1998). One gram of frozen apple flesh tissue was homogenized with a pre-cooled mortar and pestle in 1.5 mL of 6% (v/v) ice cold perchloric acid ($HClO_4$) with 5% insoluble PVPP. The extract was centrifuged at 10,000 × *g* at 4 °C for 10 min in a Microfuge® 18 Centrifuge. The supernatant was immediately used for the assay. For the AsA assay, 175 μL of supernatant was neutralized with 27 μL of 1.5 M sodium carbonate to raise the pH to 1–2. AsA was assayed at 265 nm in 200 mM sodium acetate buffer (pH 5.6), before and after a 15 min with 1.5 units of ascorbic oxidase. For the total ascorbate assay, 185 μL extract was neutralized with 31 μL of 1.82 M sodium carbonate to raise the pH to 6–7 and incubated at room temperature for 30 min with an equal volume (216 μL) of 20 mM glutathione in 100 mM Tricine–KOH (pH 8.5). Total AsA was assayed as above. DHA content was obtained by calculating the difference between total ascorbate and AsA.

Reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined using the enzymatic cycling method described by Griffith (1980). Frozen ground flesh tissue (0.5 g) was homogenized with a pre-cooled mortar and pestle in 1.5 mL of 5% (w/v) sulfosalicylic acid with 5% insoluble PVPP. The extract was centrifuged at 14,000 × *g* at 4 °C for 10 min in a Microfuge® 18 Centrifuge. For total glutathione, 20 μL of the supernatant was mixed with 200 μL of buffer A, which consisted of 0.5 M sodium phosphate buffer (pH 7.5) and 6.3 mM EDTA, 560 μL of 10 mM EDTA, 100 μL of 6 mM DTNB dissolved in buffer A and 100 μL of 2.1 mM NADPH. The reaction was initiated by adding 10 μL of 1 unit of glutathione reductase and then the increase in absorbance at 412 nm was monitored over 3 min at room temperature, using a Beckman diode array spectrophotometer (Model DU 7400, Beckman Instruments, Columbia, MD). For GSSG, 20 μL of the supernatant was mixed with 206 μL buffer A, which was consisted of 0.5 M sodium phosphate buffer with pH 7.5 and 6.3 mM EDTA. After adding 4 μL of 2-vinylpyridine, the mixture was incubated at room temperature for 30 min to remove GSH by derivatization. The sample mixture was then mixed with 200 μL buffer A, 360 μL 10 mM EDTA, 100 μL 6 mM DTNB, and 100 μL 2.1 mM NADPH. The reaction was initiated by adding 10 μL of 1 unit of glutathione reductase and then the increase in absorbance at 412 nm was monitored over 3 min at room temperature, using a Beckman diode array spectrophotometer (Model DU 7400, Beckman Instruments, Columbia, MD). Total GSH and GSSG contents were quantified by using standard regression curves, which ranged from 0 μM to 50 μM for total GSH and from 0 μM to 10 μM for GSSG. GSH was obtained by calculating the difference between total GSH and GSSG.

2.6. Antioxidant enzyme activities

Enzyme extraction was carried out in an extraction buffer containing 0.2 M sodium phosphate buffer (pH 7.8), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM EDTA and 5% insoluble PVPP. One gram of frozen ground tissue was homogenized with a pre-cooled mortar and pestle in 1.5 mL extraction buffer. For extraction of APX, 5 mM ascorbate was added to the extraction buffer. After homogenization, the extract was centrifuged at 14,000 × *g* at 4 °C for 30 min in Microfuge® 18 Centrifuge. The supernatant was collected and immediately used for enzyme assay, or aliquoted and stored at −80 °C for subsequent protein quantification (Kochhar et al., 2003). The supernatant was used for the enzyme assays of SOD (EC 1.15.1.1), Cu/Zn-SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), POX (EC 1.11.1.7), MDHAR (EC 1.6.5.4), DHAR (EC 1.8.5.1) and GR (EC 1.6.4.2). The supernatant using the extraction buffer containing 1 mM ascorbate was only used for APX (EC 1.11.1.11) activity. All enzyme assays were carried out at 25 °C in a total volume of 1 mL, using a Beckman diode array spectrophotometer.

Total and Cu/Zn-SOD activity was assayed by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT), according to the method of Giannopolitis and Ries (1977) and Frei et al. (2010). One milliliter of reaction mixture contained 0.1 M sodium phosphate buffer (pH 7.8), 10 mM methionine, 57 μM NBT, 1.3 μM riboflavin, 0.025% (v/v) Triton® X-100, and 0.11 μM EDTA and 20 μL enzyme extract. For Cu/Zn-SOD activity, the reaction mixture also contained 2 mM KCN. The reaction mixtures were freshly made before the reaction. The reaction was initiated by illuminating the reaction mixture with 2 of 15 W fluorescent lights. After 10 min illimination, the reaction was stopped by removing the light source. A blank tube with reaction mixture and extraction buffer was kept in the dark as a blank, while another tube with reaction mixture and extraction buffer was kept in light to serve as a control tube but sample tube with reaction mixture and enzyme extraction was kept in light. Activity of SOD was reported as NBT reduction in light with extraction buffer (control tube) minus NBT reduction with sample extraction (sample tube). One unit of SOD

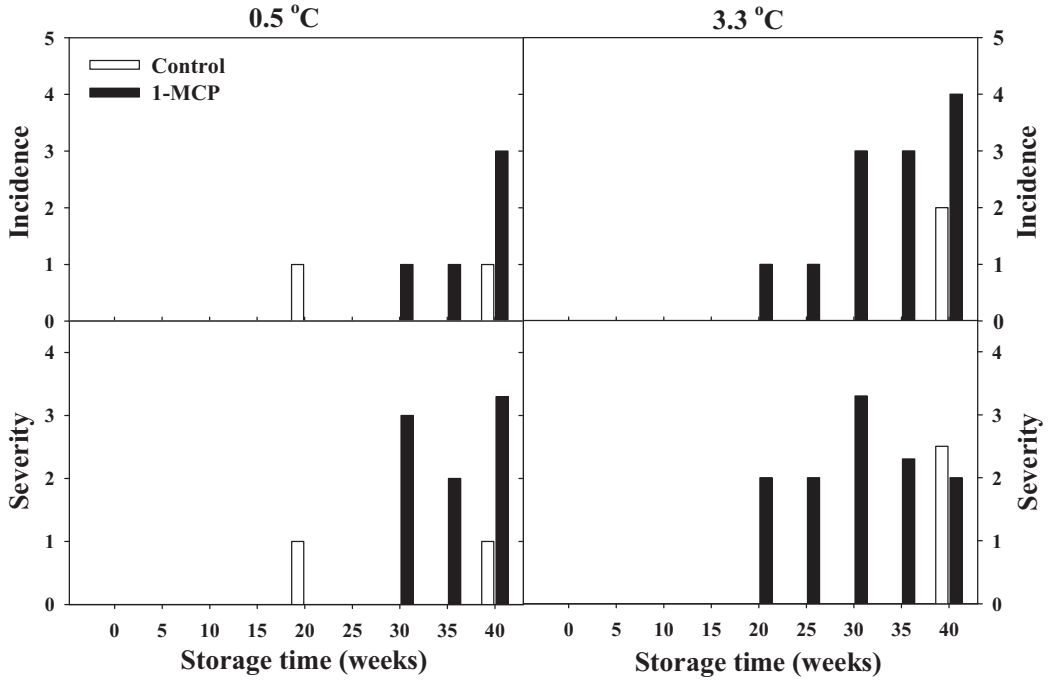

Fig. 1. Internal browning incidence and browning severity of 'Empire' apple fruit untreated, or treated with 1 μL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/CO_2 for up to 40 weeks at 0.5 °C or 3.3 °C, respectively. The open and closed bars represent untreated and 1-MCP treated apples, respectively. Incidence represents number out of five single fruit replicates that had detectable browning. Browning severity rating was subjectively evaluated where 0 = 0% browning, 1 = 1–10% browning, 2 = 11–25% browning, 3 = 26–50% browning and 4 = 51–100% browning coverage of fruit.

activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm.

APX activity was determined according to Grace and Logan (1996). The reaction mixture contained 50 mM Hepes-KOH (pH 7.6), 0.1 mM EDTA-Na_2, 0.5 mM ascorbate and 50 μL supernatant. The reaction was initiated by adding 0.4 mM H_2O_2. The absorbance rate was monitored at 290 nm over 3 min. APX activity was determined by monitoring the decrease in absorbance at 290 nm as ascorbate was oxidized. A molar extinction coefficient of 2.8 mM^{-1} cm^{-1} was used to calculate activity, which was expressed as μmol ascorbate min^{-1} mg^{-1} protein.

CAT activity was determined using the method described by Aebi (1984). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0) and 10 μL extracted sample. The reaction was initiated by adding 10 mM H_2O_2. The absorbance was monitored at 240 nm over 5 min. A molar extinction coefficient of 39.4 mM^{-1} cm^{-1} was used to calculate CAT activity, which was expressed as μmol H_2O_2 min^{-1} mg^{-1} protein.

MDHAR activity was assayed by monitoring the decrease in absorbance at 340 nm over 3 min due to NADH oxidation (Miyake and Asada, 1992). The reaction mixture contained 50 mM Hepes-KOH (pH 7.6), 0.1 mM NADH, 20 μL extracted sample and 2.5 mM ascorbate. The reaction was initiated by adding 0.5 unit of ascorbate oxidase. A molar extinction coefficient of 6.2 mM^{-1} cm^{-1} was used to calculate activity, which was expressed as μmol NADH min^{-1} mg^{-1} protein.

DHAR activity was measured using the method of Nakano and Asada (1981). The reaction mixture contained 50 mM Hepes-KOH (pH 7.6), 0.1 mM EDTA-Na_2, 100 μL extracted sample and 2.5 mM glutathione. The reaction was initiated by adding 0.2 mM dehydroascorbate. The absorbance was read by monitoring the increase in absorbance at 265 nm over 3 min due to ascorbate formation. A molar extinction coefficient of 14.0 mM^{-1} cm^{-1} was used to calculate activity, which was expressed as μmol DHA min^{-1} mg^{-1} protein.

GR activity was determined by the method described by Grace and Logan (1996) with some modification. The reaction mixture contained 100 mM Tris–HCl (pH 8.0), 1 mM EDTA-Na_2, 100 μL crude extracted sample and 1 mM GSSG (oxidized glutathione). The reaction was initiated by adding 0.2 mM NADPH. The absorbance was read by monitoring the decrease in absorbance at 340 nm over 3 min due to NADPH oxidation. A molar extinction coefficient of 6.2 mM^{-1} cm^{-1} was used to calculate activity, which was expressed as μmol NADPH min^{-1} mg^{-1} protein.

POX activity was measured by the method described by Hammerschmidt et al. (1982) using guaiacol as the hydrogen donor. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 10 mM guaiacol. The reaction was initiated by adding 10 μL crude extracted sample. The absorbance was recorded by monitoring the increase in absorbance at 470 mm over 3 min due to guaiacol oxidation. A molar extinction coefficient of 26.8 mM^{-1} cm^{-1} was used to calculate activity, which was expressed as μmol guaiacol min^{-1} mg^{-1} protein.

Protein concentrations were determined using the Bradford assay (Sigma–Aldrich Co., St. Louis, MO) according to the manufacturer's micro-assay protocol. Protein concentration was measured by comparison to the bovine serum albumin (BSA) standard curve from 0 to 10 mg L^{-1} (Bradford, 1976).

2.7. Statistical analysis

The MINITAB software release 15 (Minitab Inc., State College, PA) was used for analysis of the data. All data were analyzed using General Linear Model (GLM). Experimental data were represented as means ± standard error. Pearson correlations were used to determine the relationship between browning severity and flesh color responses [lightness (L^*), hue angle (h°) and chroma (C^*)], and between browning and antioxidant metabolism.

All antioxidant metabolite data were analyzed by using partial least-squares regression analysis (PLS) (Pérez-Enciso and

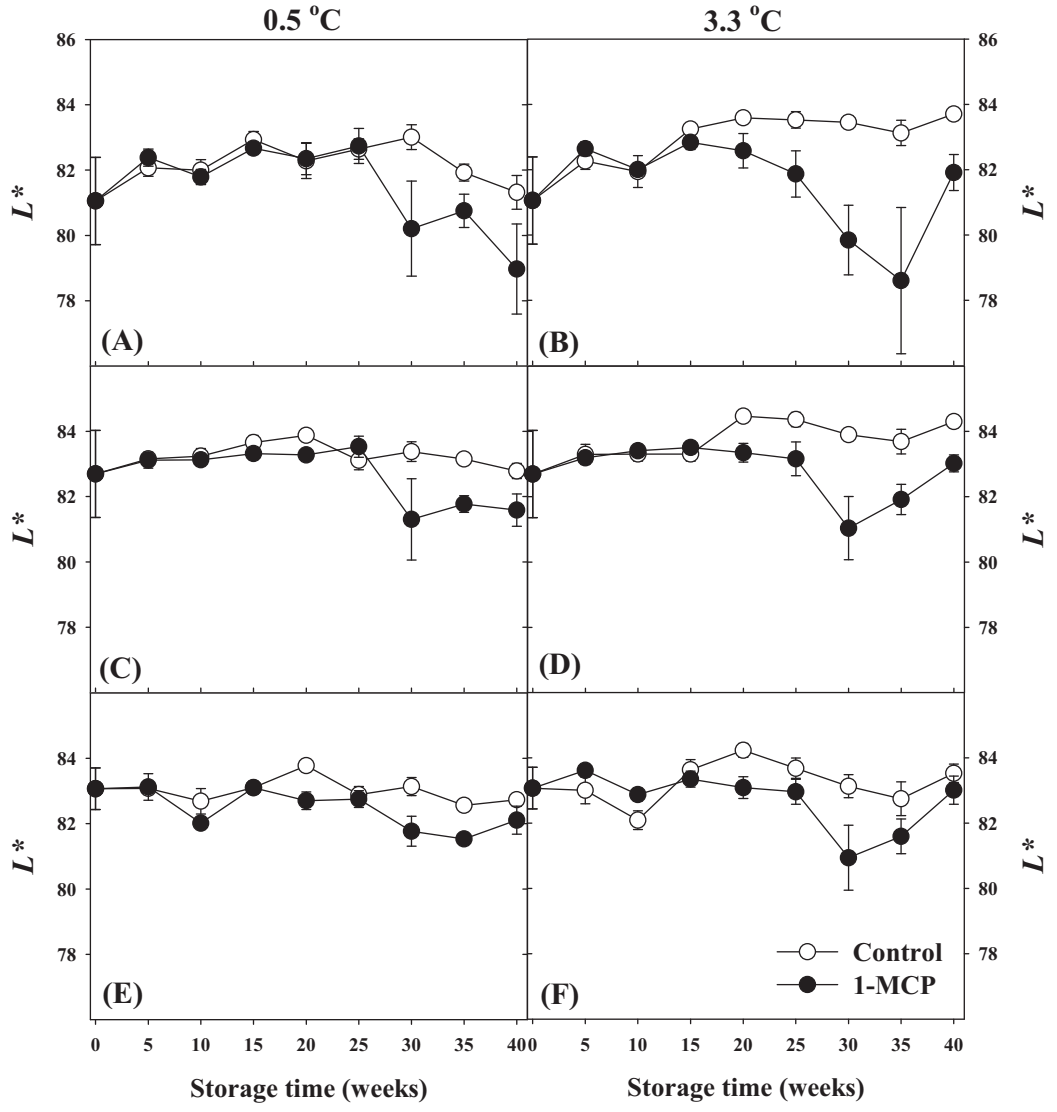

Fig. 2. Lightness (L^*) of 'Empire' apple fruit untreated, or treated with 1 $\mu L\,L^{-1}$ 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 °C or 3.3 °C, respectively. The open and closed bars represent untreated and 1-MCP treated apples, respectively. The lightness was assessed at the stem-end (A and B), equatorial (C and D) and calyx-end (E and F) region. Each data point is the mean of five single fruit replicates ± standard error, where larger than the symbols.

Tenenhaus, 2003). PLS relates variations in a limited number of predictor variables (*Y*-variables) to the variations of a large number of predictor variables (*X*-variables). PLS is a regression analysis technique in which the original X-data are projected onto a small number of underlying latent variables (LVs), which are concurrently used for regression of the Y-data in such a way that the first LVs are most relevant for predicting the *Y*-variables.

The antioxidant metabolites were considered to be predictor variables, whereas treatment factors (untreated or 1-MCP-treated), storage time (duration, 0–40 weeks), flesh browning rating and lightness (flesh tissue color) were considered to be response variables. The treatment factors were introduced as separate categorical variables (reading either −1 or 1), whereas storage time, flesh browning rating and lightness values were included as continuous variables. Both X- and Y-data were mean centered and scaled to unit variance to give all variables an equal chance to influence the model. PLS was performed using The Unscrambler version 10.0 (Camo A/S, Trondheim, Norway).

3. Results

3.1. Internal ethylene concentration (IEC), flesh color and browning severity assessment

The IEC of fruit at harvest was 46.4 $\mu L\,L^{-1}$, decreased when fruit were stored at either 0.5 or 3.3 °C, and was lower in 1-MCP treated than untreated fruit (data not shown).

At 0.5 °C, only a single fruit had flesh browning (slight severity) at weeks 20 and 30, but by week 40, three of five 1-MCP treated fruit had detectable browning (Fig. 1). Flesh browning in untreated fruit remained low, occurring only at week 40. At 3.3 °C, flesh browning was detected in one 1-MCP treated fruit at week 20, but the incidence of the disorder increased at week 30 (Fig. 1). At this temperature, flesh browning was detected in untreated fruit only at week 40.

The L^* values were relatively stable for 25 weeks of storage at 0.5 °C, but then declined to a greater extent in the stem-end tissues than the equatorial and calyx-end tissues (Fig. 2); during this time, the L^* values of tissues from the 1-MCP treated

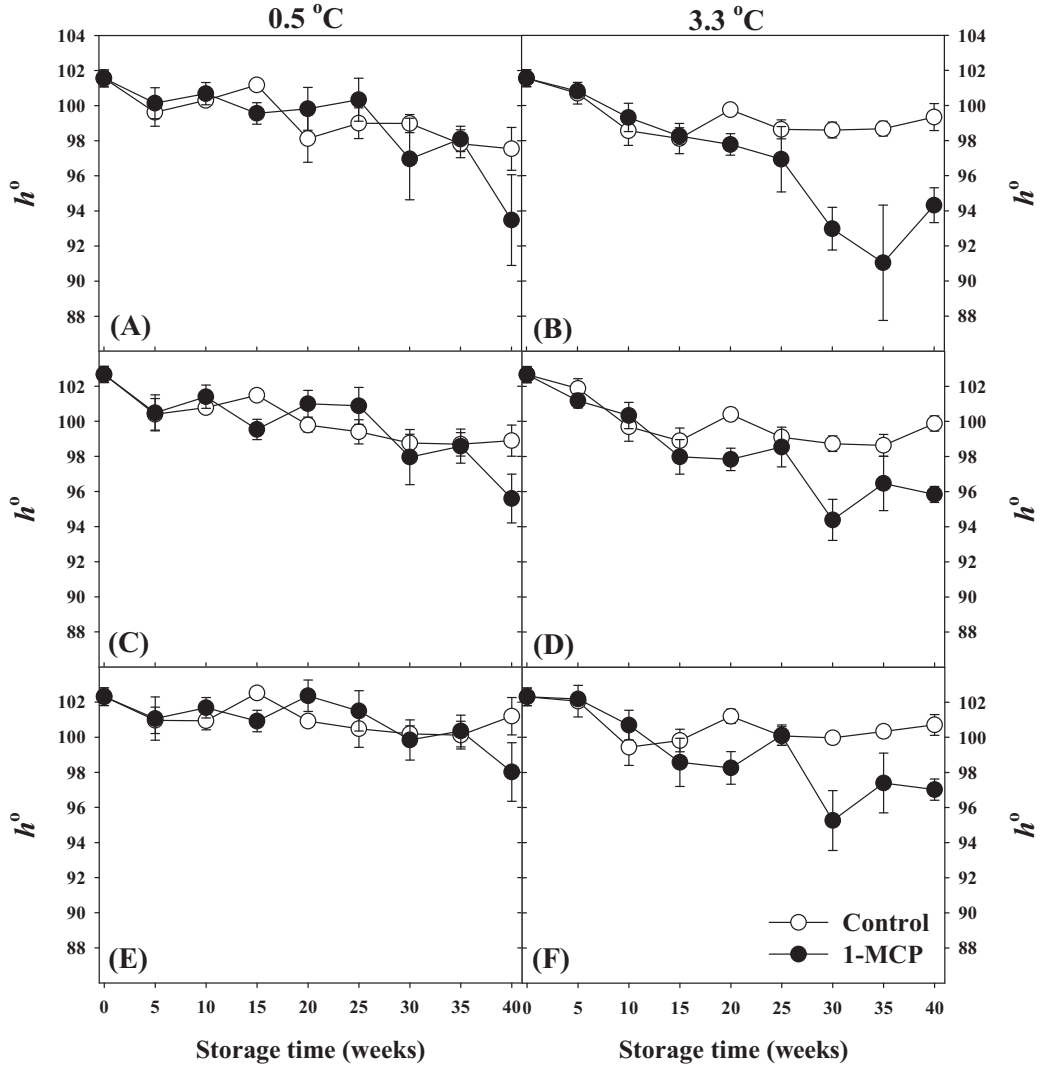

Fig. 3. Hue angle (h°) values of 'Empire' apple fruit untreated, or treated with 1 $\mu L\,L^{-1}$ 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 °C or 3.3 °C, respectively. The open and closed bars represent untreated and 1-MCP treated apples, respectively. The h° was assessed at the stem-end (A and B), equatorial (C and D) and calyx-end (E and F) region. Each data point is the mean of five single fruit replicates ± standard error, where larger than the symbols.

fruit were generally lower than those of the untreated fruit. At 3.3 °C, differences between untreated and 1-MCP treated fruit were more pronounced than at the colder storage temperature (Fig. 2), the L^* value in all tissue zones of untreated fruit remaining stable with increasing storage, while values decreased in similar patterns in the tissues of 1-MCP treated fruit as found in untreated fruit. The h° values showed similar patterns as those found for the L^* values, except that there was little difference between the untreated and 1-MCP treated fruit tissues at 0.5 °C (Fig. 3). The C^* values, while higher at 3.3 °C than at 0.5 °C in stem, equatorial and calyx end tissues ($P = 0.017$, 0.004 and 0.217, respectively), were not affected by 1-MCP treatment (data not shown).

Pearson correlation coefficients for all sampling points found that the browning severity of each fruit was strongly correlated with L^* for stem, equatorial and calyx regions ($r = -0.665^{***}$, -0.603^{***} and -0.507^{***}, respectively), and with h° ($r = -0.759^{***}$, -0.688^{***} and -0.691^{***}, respectively), but not with C^* values.

3.2. Superoxide anion generation, H_2O_2 concentrations and lipid peroxidation

NBT reducing activity, an indication of superoxide anion radical generation, increased over time ($P < 0.001$) but was lower in 1-MCP treated fruit than untreated fruit (Fig. 4), averaging 0.13 and 0.28 $OD_{580}\,g^{-1}\,h^{-1}$ ($P < 0.001$), respectively. Also, NBT reducing activity was higher at 3.3 °C than at 0.5 °C ($P = 0.017$), averaging 0.23 and 0.18 $OD_{580}\,g^{-1}\,h^{-1}$, respectively. However, the increase of NBT activity was greater in untreated fruit than 1-MCP treated fruit at 3.3 °C than at 0.5 °C ($P = 0.014$).

The accumulation of H_2O_2 was not affected by 1-MCP treatment as a main effect, but the effects of 1-MCP interacted with both storage temperature and time ($P < 0.001$). A large increase of H_2O_2 concentration occurred in untreated fruit stored at 3.3 °C at week 5, but patterns of change during storage were inconsistent (Fig. 4).

Lipid peroxidation, as indicated by MDA concentrations, was higher in 1-MCP treated fruit stored at 0.5 °C, but not at 3.3 °C

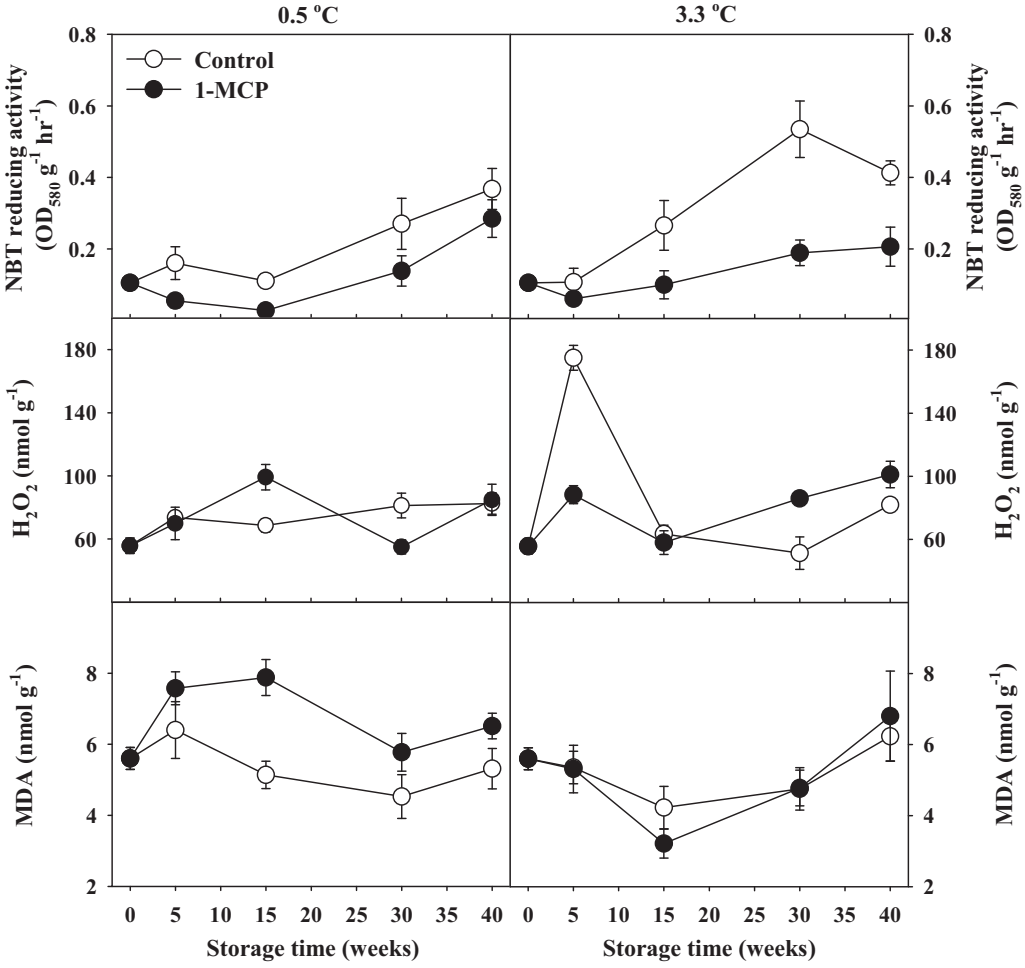

Fig. 4. Nitroblue tetrazolium (NBT) reducing activity, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents of 'Empire' apple fruit untreated, or treated with 1 $\mu L L^{-1}$ 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 °C or 3.3 °C, respectively. The open and closed bars represent untreated and 1-MCP treated apples, respectively. Each data point is the mean of five single fruit replicates ± standard error, where larger than the symbols.

($P = 0.008$; Fig. 4). Overall, MDA concentrations were lower at 3.3 °C than at 0.5 °C during the first 15 weeks of storage, but then increased ($P = 0.002$).

3.3. Antioxidant metabolites

Overall, AsA concentrations were higher in untreated than 1-MCP treated fruit ($P = 0.035$) and were higher at 0.5 °C than at 3.3 °C ($P = 0.002$). However, the effects of 1-MCP were detected only at week 40 in fruit stored at 0.5 °C, and weeks 30 and 40 in fruit stored at 3.3 °C ($P = 0.028$; Fig. 5). DHA concentrations were affected by an interaction among 1-MCP treatment, storage temperature and storage time ($P = 0.002$; Fig. 5); an effect of 1-MCP was detected only at weeks 15 and 30, when levels were higher in untreated than 1-MCP treated fruit at 3.3 °C. The AsA/DHA ratio was affected only by storage time (data not shown).

GSH concentrations were higher in 1-MCP treated fruit than in untreated fruit at week 5 when stored at 0.5 °C, and lower in 1-MCP treated fruit at week 40 when stored at 3.3 °C ($P = 0.006$; Fig. 6). GSSG concentrations were unaffected by 1-MCP treatment at 0.5 °C but at 3.3 °C, initially increased in both untreated and 1-MCP treated fruit, and decreased, but were then higher in 1-MCP treated fruit than untreated fruit at weeks 30 and 40. The GSH/GSSG ratio was unaffected by any treatment (data not shown).

3.4. The activities of antioxidant enzymes

APX activity was affected only by an interaction between storage time and storage temperature ($P = 0.019$), an increase in activity occurring at week 40 at 3.3 °C, but not at 0.5 °C (Fig. 7). MDHAR activity was affected by an interaction between 1-MCP treatment, storage temperature and storage time ($P = 0.037$), but no clear patterns of change are discernable (Fig. 7). DHAR activity was lower at 0.5 °C than at 3.3 °C ($P = 0.003$), but not affected by 1-MCP as a main effect; however, activity was lower in 1-MCP treated fruit at week 30 at 3.3 °C (Fig. 7). GR activity was affected by an interaction among 1-MCP treatment, storage temperature and storage time ($P = 0.001$), but changes over time were inconsistent (Fig. 7).

Both SOD and Cu/Zn-SOD activities were affected by an interaction among 1-MCP, storage temperature and storage time ($P = 0.014$ and 0.011, respectively; Fig. 8). At 0.5 °C, activity in 1-MCP treated fruit remained relatively unchanged, while activity in untreated fruit decreased at week 30 before increasing at week 40 to levels above those of 1-MCP treated fruit. At 3.3 °C, there was little

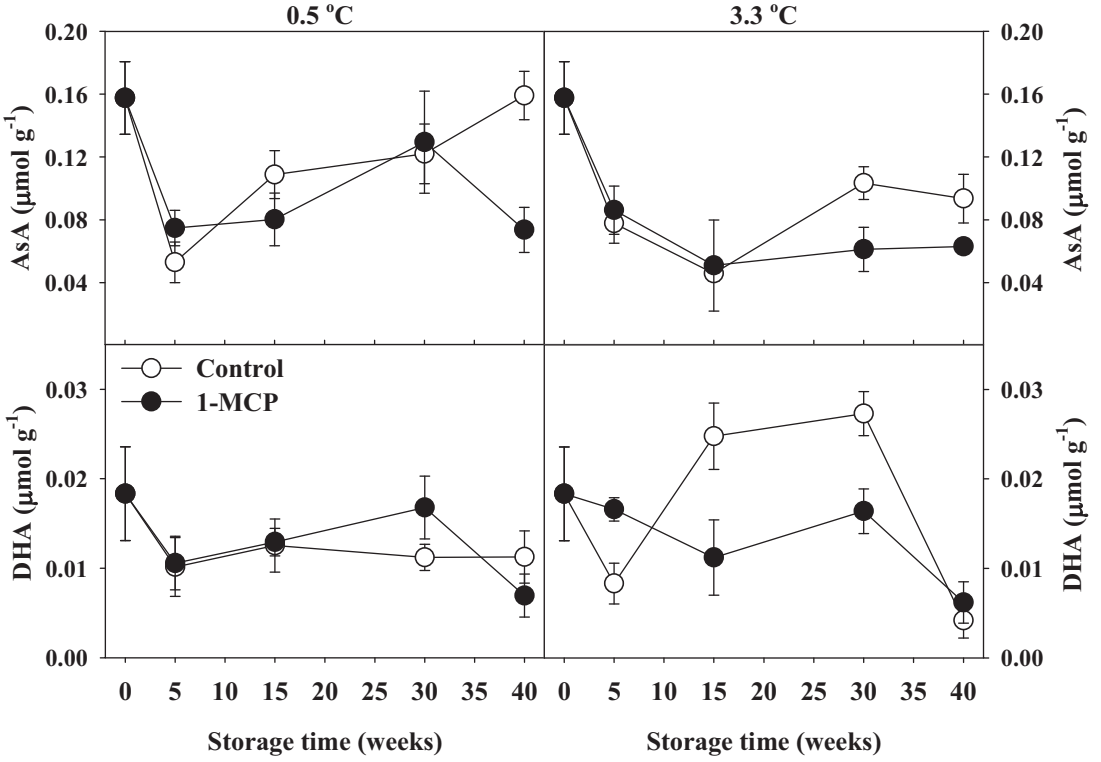

Fig. 5. Ascorbic acid (AsA) and dehydroascorbate (DHA) of 'Empire' apple fruit untreated, or treated with 1 μL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 °C or 3.3 °C, respectively. The open and closed bars represent untreated and 1-MCP treated apples, respectively. Each data point is the mean of five single fruit replicates ± standard error, where larger than the symbols.

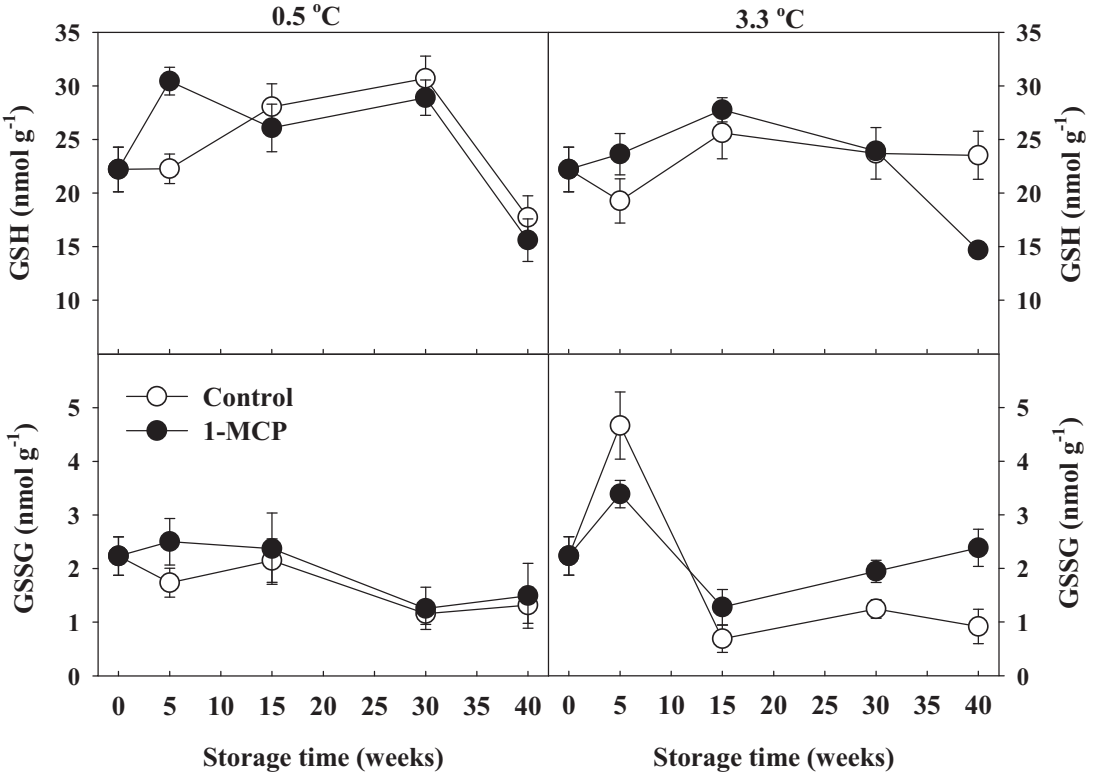

Fig. 6. Reduced glutathione (GSH) and oxidized glutathione (GSSG) of 'Empire' apple fruit untreated, or treated with 1 μL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 °C or 3.3 °C, respectively. The open and closed bars represent untreated and 1-MCP treated apples, respectively. Each data point is the mean of five single fruit replicates ± standard error, where larger than the symbols.

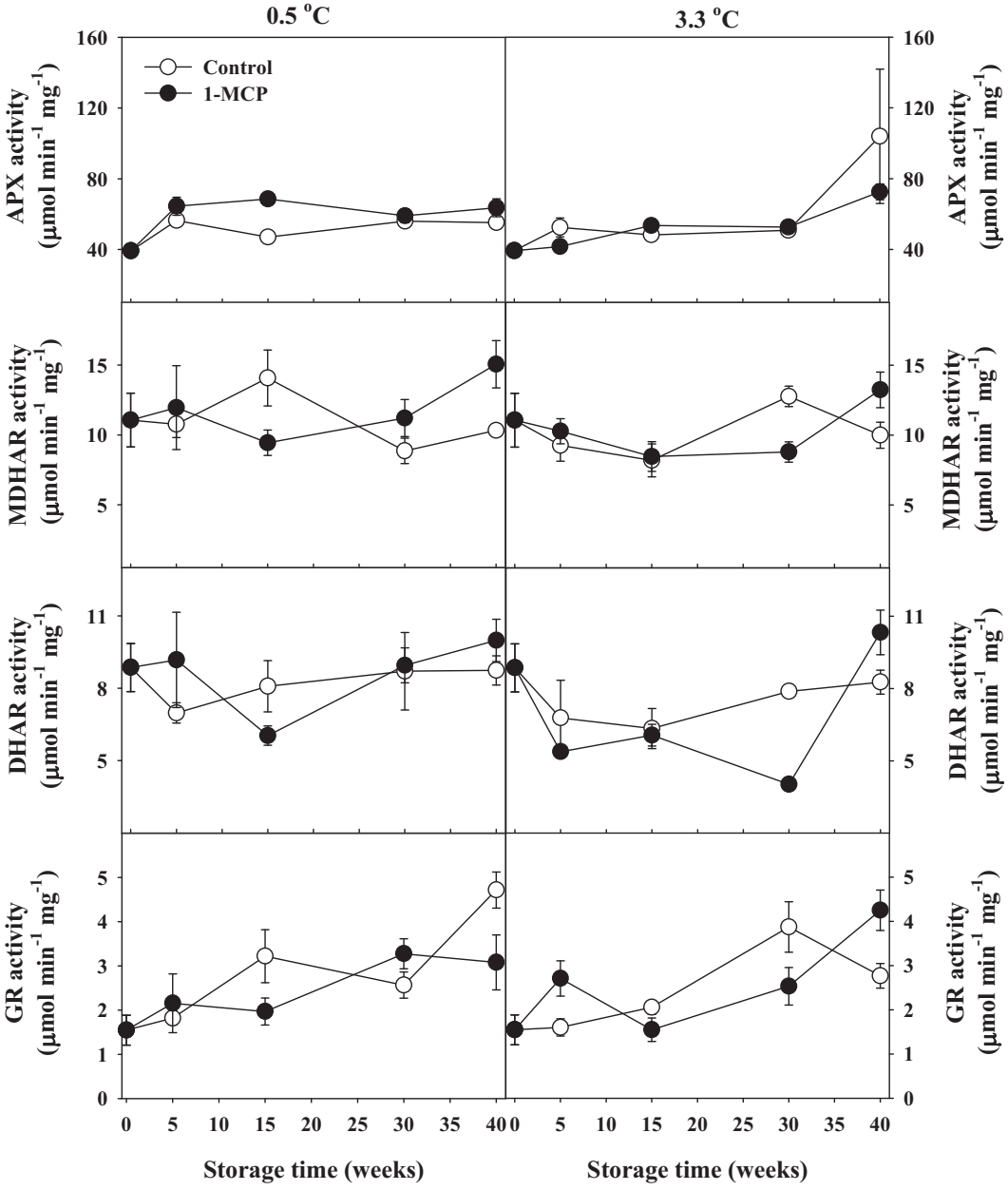

Fig. 7. Ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) activities on a protein basis of 'Empire' apple fruit untreated, or treated with 1 μL L^{-1} 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 °C or 3.3 °C, respectively. The open and closed bars represent untreated and 1-MCP treated apples, respectively. Each data point is the mean of five single fruit replicates ± standard error, where larger than the symbols.

difference in SOD activity between untreated and 1-MCP treated fruit with activity remaining similar and then increasing markedly after week 30. However, activity of Cu/Zn-SOD was lower in 1-MCP treated fruit than in untreated fruit at weeks 15 and 30.

CAT activity was initially lower in 1-MCP treated fruit stored at 0.5 °C, and then activity increased by week 40 to higher rates than in untreated fruit, but there was no treatment effect in fruit stored at 3.3 °C ($P = 0.034$; Fig. 8).

POX activity was affected by an interaction among 1-MCP, storage temperature and storage length ($P = 0.014$; Fig. 8), with a greater increase in activity in untreated than 1-MCP treated fruit at 0.5 °C, but a smaller increase in 1-MCP treated fruit at 3.3 °C and no difference between untreated and 1-MCP treated fruit until week 40 at 3.3 °C.

Pearson correlation coefficients were calculated for browning of each apple at weeks 30 and 40 of storage with antioxidant metabolites and enzyme activities. Only, AsA, DHA, GSH and Cu/Zn-SOD were correlated, being $r = -0.604^{***}$, -0.354^{*}, -0.357^{*} and 0.408^{**}, respectively.

3.5. PLS models of antioxidant metabolites

On the PLS loading plots of oxidative stress metabolites, 1-MCP was associated with MDA and APX at 0.5 °C but not at 3.3 °C (Fig. 9). The lightness (L^*) variable was not associated with flesh browning variable on the loading plots. Lightness was more closely associated with GSH, DHA, and GSH/(GSH + GSSG) at both storage temperature but CAT was linked with lightness only at 3.3 °C. Furthermore, flesh browning and storage duration variables were associated plotted at

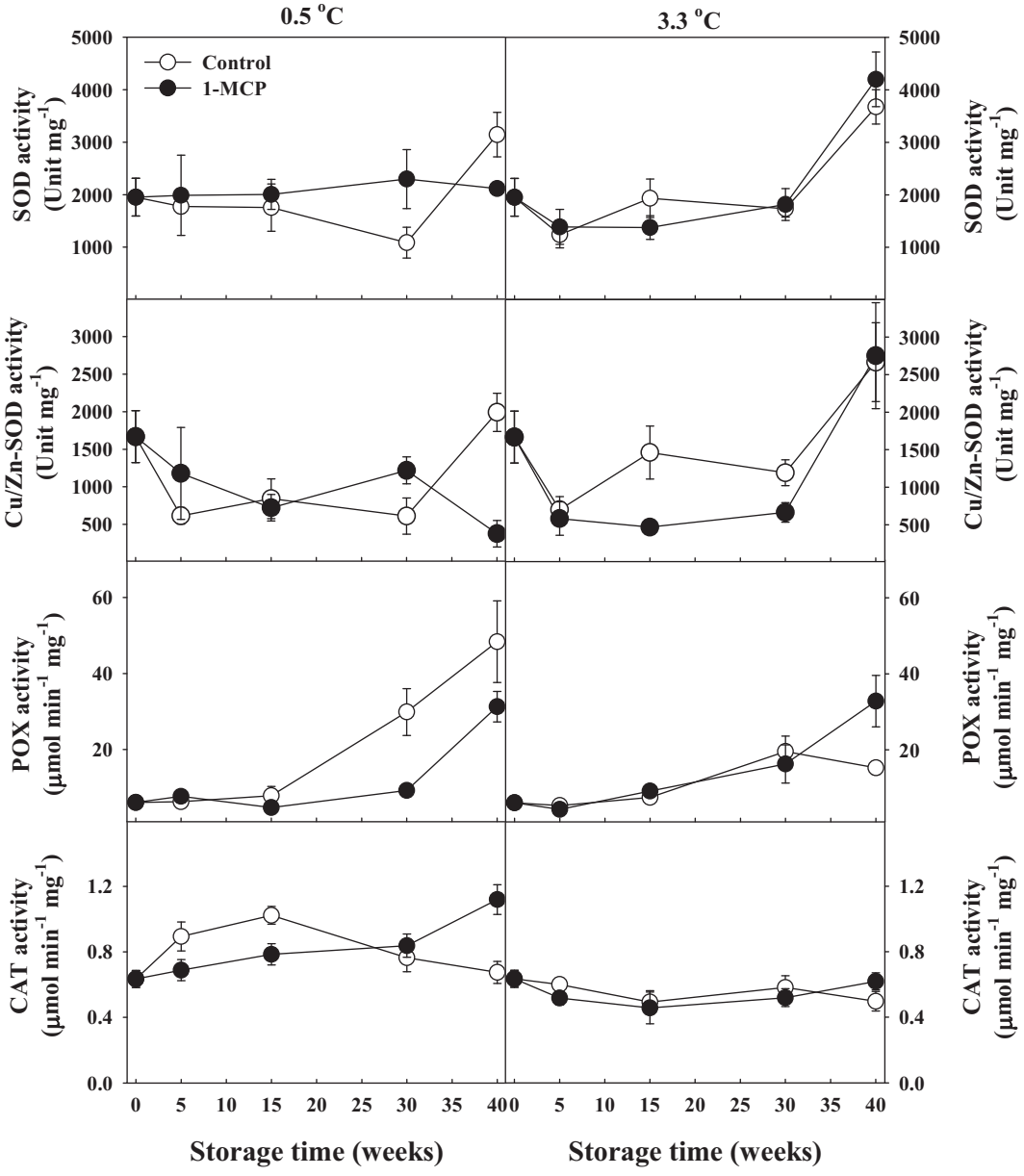

Fig. 8. Superoxide dismutase (SOD), copper/zinc-superoxide dismutase (Cu/Zn-SOD), catalase (CAT) and guaiacol peroxidase (POX) activities on a protein basis of 'Empire' apple fruit untreated, or treated with 1 $\mu L\,L^{-1}$ 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 °C or 3.3 °C, respectively. The open and closed bars represent untreated and 1-MCP treated apples, respectively. Each data point is the mean of five single fruit replicates ± standard error, where larger than the symbols.

either storage temperature. GR was strongly associated with both variables but POX was only closely associated with flesh browning and storage duration variables at 3.3 °C. Furthermore, APX was strongly associated with browning at 3.3 °C, but with 1-MCP at 0.5 °C (Fig. 9).

4. Discussion

1-MCP treated fruit had higher flesh browning incidence and severity than untreated fruit at 3.3 °C, and sometimes also at 0 °C (Fig. 1), as reported earlier (Watkins, 2008; Jung and Watkins, 2011). The L^* and h° values indicated that greater color changes of tissues occurred in fruit kept at 0.5 °C, regardless of 1-MCP treatment, suggesting that changes were taking place that the naked eye could not detect. However, a clear separation between untreated and treated tissues was detected at later stages of storage at 3.3 °C. Flesh color was strongly correlated with flesh browning incidence, which was more severe at 0.5 °C than at 3.3 °C. Symptoms identical to those observed at 0.5 °C were found following 1-MCP treatment in fruit stored at 3.3 °C.

Browning is a result of loss of membrane integrity that results in enzymatic oxidation of phenolic compounds by PPO (Nicolas et al., 1994; Tomas-Barberan and Espin, 2001). It is commonly assumed that changes to membrane integrity occur as a result of impairment of the cellular antioxidant system. However, the antioxidant metabolite and enzyme activity data obtained in this study were inconsistent with changes in flesh browning. The NBT reducing activity, an indication of superoxide anion radical generation, increased during storage and was higher at 3.3 °C than 0.5 °C, but was lower in 1-MCP treated than untreated fruit at both temperatures (Fig. 4). Superoxide anion radicals are typically generated by electron transport systems of photosynthesis and respiration, and NADPH oxidase and NADH peroxidase at the plasma membrane (Mittler, 2002). CA storage of 1-MCP treated fruit resulted

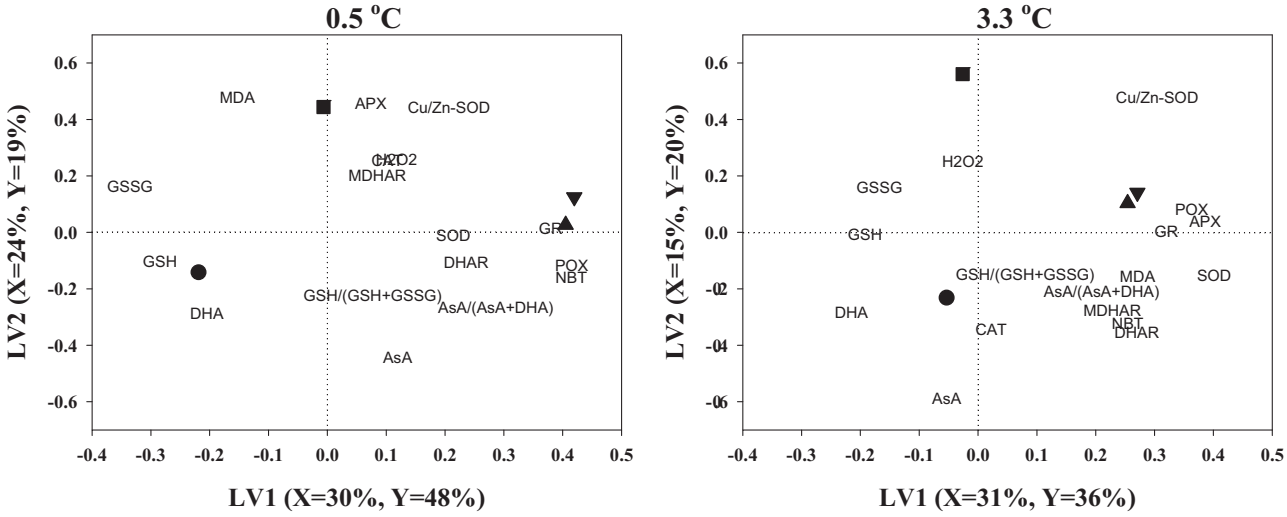

Fig. 9. PLS loading plots from models containing X-variables (antioxidant metabolites) and Y-variables (experimental factors: storage duration, ▲; flesh browning, ▼; flesh lightness, ●; and 1-MCP treatment, ■;) within the antioxidant metabolites of 'Empire' apple fruit untreated, or treated with 1 $\mu L\,L^{-1}$ 1-MCP at harvest, and then stored at 2 kPa O_2/2 kPa CO_2 for up to 40 weeks at 0.5 or 3.3 °C.

in lower respiration rates of 'Empire' apples than untreated ones (DeEll et al., 2005). Such treatments could result in suppression of alternative oxidases in electron transport systems of mitochondria, thereby reducing the generation of superoxide anion radicals. No other reports on superoxide anion radical production in apples under CA conditions appear to be available, although postharvest treatments that result in storage life extension of several crops are associated with lower rates of production of these radicals (Gao et al., 2009; Song et al., 2009).

As ROS, high H_2O_2 concentrations are often regarded as an indication of oxidative stress, although they are also important in signal transduction involved in various processes (Apel and Hirt, 2004). 1-MCP treatment did not affect H_2O_2 concentrations consistently (Fig. 4). Vilaplana et al. (2006) found that H_2O_2 concentrations were slightly lower at most time points in air stored 'Golden Smoothee' apples, but there was no flesh browning reported in the fruit used in that study. The H_2O_2 concentration was higher in CA- than air-stored 'Pink Lady' apples suggesting a response to stress, but concentrations were similar in undamaged and damaged tissues associated with CO_2-induced injury (de Castro et al., 2008). Interestingly, there was a sharp increase of H_2O_2 concentrations in untreated fruit kept at 3.3 °C for 5 weeks (Fig. 4), which was a similar response to that in CA-stored 'Conference' pear (Larrigaudière et al., 2001a). It is possible that H_2O_2 might play a role in a signaling molecule rather than toxic metabolite at this storage temperature, but further research is required.

As the final products of lipid peroxidation, MDA concentrations are often used as indicators of oxidative damage. However, in this study, the accumulation of MDA was not directly associated with the development of flesh browning. MDA concentrations were higher in 1-MCP treated fruit than untreated ones at 0.5 °C but there was no difference shown at 3.3 °C (Fig. 4). MDA results were not consistent with NBT reducing activity and H_2O_2 concentrations at 0.5 °C. In 'Golden Smoothee' apples, the levels of MDA and 4-hydroxyalkenals (4-HNE) were unaffected by 1-MCP treatment for the first 15 d of air storage, but were then lower in the 1-MCP treated fruit during subsequent storage (Vilaplana et al., 2006). While not all ROS were assessed, superoxide anion radical production and H_2O_2 concentrations are at best distinct from each other, and not indicative of higher ROS activity that would result in membrane damage.

Activity of the ascorbic acid and glutathione cycle is regarded as critical to scavenge ROS produced by stress (Noctor and Foyer, 1998). In this study, AsA, DHA, GSH and GSSG were assessed as the responses of antioxidant metabolites to 1-MCP treatment during CA storage (Figs. 5 and 6). 1-MCP treatment reduced AsA concentrations at both temperatures but reduced DHA concentrations at 3.3 °C. AsA concentrations decreased immediately after storage at both temperatures but then they recovered to previous concentrations at 0.5 °C, and to a slower extent at 3.3 °C in untreated fruit. AsA concentrations were lower in 1-MCP treated tissues towards the end of storage at both storage temperatures. DHA concentrations did not change greatly during storage at 0.5 °C but they increased in untreated fruit during storage at 3.3 °C at weeks 15 and 30. A reduction of AsA concentrations may be associated with the development of flesh browning in apples (de Castro et al., 2008) and pears (Veltman et al., 1999, 2000; Zerbini et al., 2002; Franck et al., 2003). However, it is uncertain if these relationships are a result, or a cause of injury.

SOD functions to catalyze the dismutation of superoxide anion radical into O_2 and H_2O_2 molecules (Apel and Hirt, 2004). 1-MCP did not affect SOD activity but reduced Cu/Zn-SOD activity at 3.3 °C, while effects were inconsistent at 0.5 °C (Fig. 8). Also, PLS models revealed that 1-MCP was not closely associated with SOD activity at either storage temperature but more closely associated with Cu/Zn-SOD activity at 0.5 °C than at 3.3 °C. Du and Bramlage (1994) reported that senescent apple peel had much greater activities of total SOD, Cu/Zn-SOD, and Mn-SOD than healthy apple peel in 'Empire' apple stored at 0 °C for 24 weeks. Total SOD activity represents all activities of iron-SOD (Fe-SOD), manganese-SOD (Mn-SOD) and Cu/Zn-SOD. Fe-SOD is localized in the chloroplast, Mn-SOD in the mitochondria and peroxisome, and Cu/Zn-SOD in the cytosol, chloroplast, peroxisome and cell wall (Alscher et al., 2002). Because superoxide anion radicals cannot diffuse across phospholipid membranes, they should decompose within the subcellular compartments (Takahashi and Asada, 1983). As another ROS, H_2O_2 can be detoxified by APX, CAT, POX and glutathione peroxidase (GPX) (Mittler, 2002). The relationship between H_2O_2 concentration and any specific H_2O_2-detoxifying enzyme was not

consistent in this study. Therefore, H_2O_2 detoxifying enzymes might play a cooperative role in scavenging H_2O_2 because H_2O_2 can be produced in several subcellular organs by numerous biochemical reactions such as the conversion of superoxide anion radicals into H_2O_2 in chloroplast and mitochondria, or glycolate oxidase and fatty acid β-oxidation in peroxisome, oxalate oxidase and amine oxidase in apoplast, and NADH peroxidases in cell wall (Mittler, 2002). Although H_2O_2 detoxifying enzyme activities were not affected by 1-MCP at 3.3 °C except at week 40, APX activity was strongly associated with browning variable at 3.3 °C, suggesting that APX activity might be associated with the development of flesh browning at 3.3 °C. POX activity was highest only at untreated fruit stored at 0.5 °C at week 40, where flesh browning was lowest. Interestingly, POX activity was more closely associated with the browning variable at 3.3 °C at PLS loading plots. As the indirect antioxidant scavenging enzymes, MDHAR, DHAR and GR were considered because they reduce DHA and GSSG back to AsA and GSH, respectively. These enzyme activities were not consistent with 1-MCP treatment or storage temperatures.

In conclusion, flesh browning was enhanced by 1-MCP treatment, and to a greater extent at 3.3 °C than at 0.5 °C, as indicated visually and by decreasing lightness and hue angle values over time. The study did not reveal associations among flesh browning and superoxide production, H_2O_2 concentrations, and lipid peroxidation. Associations with ascorbate and glutathione metabolism were also inconsistent, although the PLS models demonstrated an association of the browning variable with APX suggesting that it might be a candidate metabolite in flesh browning development at 3.3 °C.

Acknowledgements

The authors thank Jacqueline Nock and Jeanne Grace for their helpful assistance and Dr. Pengmin Li and Dr. Li-Song Chen for their technical support. This research was partly supported by Federal Formula Funds NE1036, the New York Apple Research and Development Program, and AgroFresh, Inc. Jinwook Lee was supported by a Department of Horticulture assistantship.

References

Aebi, H., 1984. Catalase in vitro. In: Lester, P. (Ed.), Methods Enzymol, vol. 105. Academic Press, pp. 121–126.
Alscher, R.G., Erturk, N., Heath, L.S., 2002. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J. Exp. Bot. 53, 1331–1341.
Apel, K., Hirt, H., 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Physiol. Plant Mol. Biol. 55, 373–399.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248–254.
Chen, L.-S., Cheng, L., 2004. CO_2 assimilation, carbohydrate metabolism, xanthophyll cycle, and the antioxidant system of 'Honeycrisp' apple leaves with zonal chlorosis. J. Am. Soc. Hortic. Sci. 129, 729–737.
de Castro, E., Barrett, D.M., Jobling, J., Mitcham, E.J., 2008. Biochemical factors associated with a CO_2-induced flesh browning disorder of Pink Lady apples. Postharvest Biol. Technol. 48, 182–191.
DeEll, J.R., Murr, D.P., Mueller, R., Wiley, L., Porteous, M.D., 2005. Influence of 1-methylcyclopropene (1-MCP), diphenylamine (DPA), and CO_2 concentration during storage on 'Empire' apple quality. Postharvest Biol. Technol. 38, 1–8.
DeEll, J.R., Ayres, J.T., Murr, D.P., 2007. 1-Methylcyclopropene influences 'Empire' and 'Delicious' apple quality during long-term commercial storage. HortTechnology 17, 46–51.
Doke, N., 1983. Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. Physiol. Plant Pathol. 23, 345–357.
Du, Z., Bramlage, W.J., 1994. Superoxide dismutase activities in senescing apple fruit (*Malus domestica* Borkh.). J. Food Sci. 59, 581–584.
Fawbush, F., Nock, J.F., Watkins, C.B., 2008. External carbon dioxide injury and 1-methylcyclopropene (1-MCP) in the 'Empire' apple. Postharvest Biol. Technol. 48, 92–98.
Fawbush, F., Nock, J.F., Watkins, C.B., 2009. Antioxidant contents and activity of 1-methylcyclopropene (1-MCP)-treated 'Empire' apples in air and controlled atmosphere storage. Postharvest Biol. Technol. 52, 30–37.
Franck, C., Baetens, M., Lammertyn, J., Scheerlinck, N., Davey, M.W., Nicolaï, B.M., 2003. Ascorbic acid mapping to study core breakdown development in 'Conference' pears. Postharvest Biol. Technol. 30, 133–142.
Franck, C., Lammertyn, J., Ho, Q.T., Verboven, P., Verlinden, B., Nicolaï, B.M., 2007. Browning disorders in pear fruit. Postharvest Biol. Technol. 43, 1–13.
Frei, M., Wang, Y., Ismail, A.M., Wissuwa, M., 2010. Biochemical factors conferring shoot tolerance to oxidative stress in rice grown in low zinc soil. Funct. Plant Biol. 37, 74–84.
Gao, H., Tao, F., Song, L., Chen, H., Chen, W., Zhou, Y., Mao, J., Zheng, Y., 2009. Effects of short-term N_2 treatment on quality and antioxidant ability of loquat fruit during cold storage. J. Sci. Food Agric. 89, 1159–1163.
Giannopolitis, C.N., Ries, S.K., 1977. Superoxide dismutases: I. Occurrence in higher plants. Plant Physiol. 59, 309–314.
Grace, S.C., Logan, B.A., 1996. Acclimation of foliar antioxidant systems to growth irradiance in three broad-leaved evergreen species. Plant Physiol. 112, 1631–1640.
Griffith, O.W., 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal. Biochem. 106, 207–212.
Hammerschmidt, R., Nuckles, E.M., Kuć, J., 1982. Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. Physiol. Plant Pathol. 20, 73–82.
Hodges, D.M., DeLong, J.M., Forney, C.F., Prange, R.K., 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207, 604–611.
Hodges, D.M., Lester, G.E., Munro, K.D., Toivonen, P.M.A., 2004. Oxidative stress: importance for postharvest quality. HortScience 39, 924–929.
Jung, S.-K., Watkins, C.B., 2011. Involvement of ethylene in browning development of controlled atmosphere-stored 'Empire' apple fruit. Postharvest Biol. Technol. 59, 219–226.
Jung, S.K., James, H., Lee, J., Nock, J.F., Watkins, C.B., 2010. Effects of ethylene inhibition on development of flesh browning in apple fruit. Acta Hortic. 877, 549–554.
Kochhar, S., Watkins, C.B., Conklin, P.L., Brown, S.K., 2003. A quantitative and qualitative analysis of antioxidant enzymes in relation to susceptibility of apples to superficial scald. J. Am. Soc. Hortic. Sci. 128, 910–916.
Larrigaudière, C., Lentheric, I., Pintó, E., Vendrell, M., 2001a. Short-term effects of air and controlled atmosphere storage on antioxidant metabolism in conference pears. J. Plant Physiol. 158, 1015–1022.
Larrigaudière, C., Pinto, E., Lentheric, L., Vendrell, M., 2001b. Involvement of oxidative processes in the development of core browning in controlled-atmosphere stored pears. J. Hortic. Sci. Biotechnol. 76, 157–162.
Larson, R.A., 1988. The antioxidants of higher plants. Phytochemistry 27, 969–978.
Logan, B.A., Grace, S.C., Adams III, W.W., Demmig-Adams, B., 1998. Seasonal differences in xanthophyll cycle characteristics and antioxidants in *Mahonia repens* growing in different light environments. Oecologia 116, 9–17.
McGuire, R.G., 1992. Reporting of objective color measurements. HortScience 27, 1254–1255.
Meheriuk, M., Prange, R.K., Lidster, P.D., Porritt, S.W., 1994. Postharvest Disorders of Apples and Pears. Agriculture Canada Publication, 1737/E.
Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7, 405–410.
Miyake, C., Asada, K., 1992. Thylakoid-bound ascorbate peroxidase in spinach chloroplasts and photoreduction of its primary oxidation product monodehydroascorbate radicals in thylakoids. Plant Cell Physiol. 33, 541–553.
Nakano, Y., Asada, K., 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22, 867–880.
Nicolas, J.J., Richard-Forget, F.C., Goupy, P.M., Amiot, M.J., Aubert, S.Y., 1994. Enzymatic browning reactions in apple and apple products. Crit. Rev. Food Sci. Nutr. 34, 109–157.
Noctor, G., Foyer, C.H., 1998. Ascorbate and glutathione: keeping active oxygen under control. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 249–279.
Patterson, B.D., MacRae, E.A., Ferguson, I.B., 1984. Estimation of hydrogen peroxide in plant extracts using titanium(IV). Anal. Biochem. 139, 487–492.
Pérez-Enciso, M., Tenenhaus, M., 2003. Prediction of clinical outcome with microarray data: a partial least squares discriminant analysis (PLS-DA) approach. Hum. Genet. 112, 581–592.
Song, L., Gao, H., Chen, H., Mao, J., Zhou, Y., Chen, W., Jiang, Y., 2009. Effects of short-term anoxic treatment on antioxidant ability and membrane integrity of postharvest kiwifruit during storage. Food Chem. 114, 1216–1221.
Takahashi, M.A., Asada, K., 1983. Superoxide anion permeability of phospholipid-membranes and chloroplast thylakoids. Arch. Biochem. Biophys. 226, 558–566.
Toivonen, P.M.A., 2004. Postharvest storage procedures and oxidative stress. HortScience 39, 938–942.
Tomas-Barberan, F.A., Espin, J.C., 2001. Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. J. Sci. Food Agric. 81, 853–876.
Veltman, R.H., Sanders, M.G., Persijn, S.T., Pemppelenbos, H., Oosterhaven, J., 1999. Decreased ascorbic acid levels and brown core development in pears (*Pyrus communis* L. cv. Conference). Physiol. Plant. 107, 39–45.
Veltman, R.H., Kho, R.M., van Schaik, A.C.R., Sanders, M.G., Oosterhaven, J., 2000. Ascorbic acid and tissue browning in pears (*Pyrus communis* L. cvs Rocha and Conference) under controlled atmosphere conditions. Postharvest Biol. Technol. 19, 129–137.
Vilaplana, R., Valentines, M.C., Toivonen, P., Larrigaudière, C., 2006. Antioxidant potential and peroxidative state of 'Golden Smoothee' apples treated with 1-methylcyclopropene. J. Am. Soc. Hortic. Sci. 131, 104–109.

Watkins, C.B., Silsby, K.J., Goffinet, M.C., 1997. Controlled atmosphere and antioxidant effects on external CO_2 injury of 'Empire' apples. HortScience 32, 1242–1246.
Watkins, C.B., Nock, J.F., Whitaker, B.D., 2000. Responses of early, mid and late season apple cultivars to postharvest application of 1-methylcyclopropene (1-MCP) under air and controlled atmosphere storage conditions. Postharvest Biol. Technol. 19, 17–32.
Watkins, C.B., Nock, J.F., 2005. Effects of delays between harvest and 1-methylcyclopropene treatment, and temperature during treatment, on ripening of air-stored and controlled-atmosphere-stored apples. HortScience 40, 2096–2101.
Watkins, C.B., 2008. Overview of 1-methylcyclopropene trials and uses for edible horticultural crops. HortScience 43, 86–94.
Watkins, C.B., Liu, F.W., 2010. Temperature and carbon dioxide interactions on quality of controlled atmosphere-stored 'Empire' apples. HortScience 45, 1708–1712.
Zerbini, P.E., Rizzolo, A., Brambilla, A., Cambiaghi, P., Grassi, M., 2002. Loss of ascorbic acid during storage of Conference pears in relation to the appearance of brown heart. J. Sci. Food Agric. 82, 1007–1013.