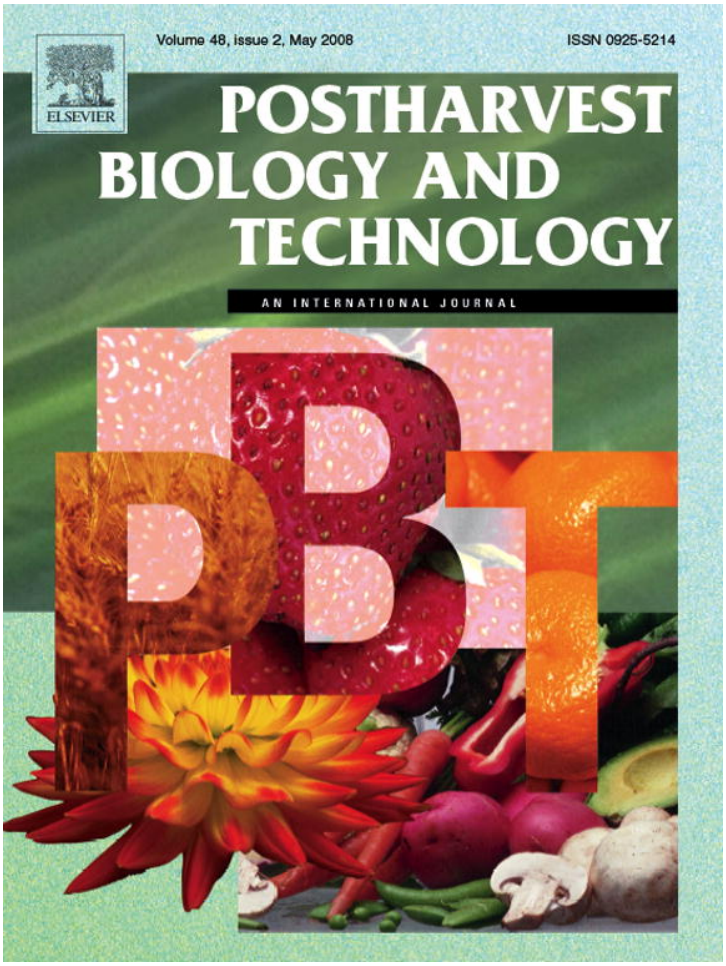

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Biochemical factors associated with a CO_2-induced flesh browning disorder of Pink Lady apples

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Received 29 November 2006; accepted 27 September 2007

Abstract

The underlying biochemical factors associated with a CO_2-induced internal flesh browning (FB) disorder of Pink Lady apples (*Malus domestica* Borkh 'Cripps Pink') are poorly understood. To investigate this disorder, Pink Lady apples were stored in air or controlled atmosphere (CA) with 1.5 kPa O_2 and 5 kPa CO_2 at 0.5 °C for 2 and 4 months in 2004 and 2005. Following CA storage, fruit were separated into two categories, damaged (FB) and undamaged tissue from each of the categories was studied separately. Cell viability studies revealed that the cells were dead in the brown tissue of damaged apples. All healthy tissue in the same apples contained viable cells. Both brown and surrounding healthy tissues in apples with FB showed a decrease in ascorbic acid and an increase in dehydroascorbic acid during the first 2 months of storage in CA, the time period when FB developed. Undamaged, CA-stored apples retained a higher concentration of ascorbic acid after 2 months in storage. The level of hydrogen peroxide (H_2O_2) increased more in the flesh of CA-stored apples than in air-stored apples, an indication of tissue stress. In addition, concentrations of H_2O_2 were significantly lower in diphenylamine (DPA)-treated apples. Treatment with DPA also inhibited FB completely compared to untreated apples. Polyphenol oxidase (PPO) activity was similar for apples kept in air or CA storage and between undamaged and damaged fruit. The results showed a closer association between FB and the oxidant–antioxidant mechanisms such as ascorbic acid, H_2O_2 and DPA, than to the activity of specific browning enzymes like PPO. Further investigation of the protective effect of ascorbic acid is warranted as is further research on the underlying causes of apple fruit susceptibility to FB.

Keywords: Ascorbic acid; Calcium; Cell viability; CO_2 injury; Hydrogen peroxide; *Malus domestica*; Polyphenol oxidase

1. Introduction

It is well known that the addition of controlled atmospheres (CA) during low-temperature storage can preserve the quality of apple fruit for extended periods. However, CA can also cause physiological disorders, such as CO_2-induced internal flesh browning (FB) in susceptible apple cultivars (Lau, 1998; Volz et al., 1998). Flesh browning in Pink Lady ('Cripps Pink' apples of an appropriate quality may be sold using the trademarked brand name Pink Lady® brand) apples generally occurs intermittently and in unpredictable patterns which makes it a difficult disorder for growers to manage. There are at least three different manifestations of this physiological disorder, one of them related to CA. One is diffuse FB, which appears to be related to chilling injury (Bramlage et al., 1980; James et al., 2005); the second is radial browning which is related to senescent breakdown (Wilkinson and Fidler, 1973) and the third is CO_2 injury (brown spots that may develop into cavities), associated with CA storage (Lau, 1998).

CO_2-induced FB disorders, either in apples or pears, are thought to be the consequence of oxidative damage caused by high concentrations of CO_2 that can be aggravated by low concentrations of O_2 (Lau, 1998; Larrigaudiere et al., 2001a; Veltman et al., 1999a). Research has shown that hydrogen peroxide (H_2O_2) production is related with stress conditions (Rea et al., 2004) and in pears, high concentrations of CO_2 inside the cells caused oxidative stress and accumulation of H_2O_2, which may enhance cell membrane damage (Larrigaudiere et al., 2001b).

Stress and high-peroxide levels can result in membrane dysfunction (Sears and Eisember, 1961; Frenkel and Patterson, 1973). Leakage of membranes could potentially lead to an

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doi:10.1016/j.postharvbio.2007.09.027

accumulation in the cytosol of plastid enzymes like polyphenol oxidase (PPO) in apples and its vacuole-stored substrates, the phenolic compounds. The oxidative reaction between enzyme and substrate would result in the formation of quinone compounds, leading to brown discoloration of the flesh. Eventually, leakage of membranes can lead to cell death.

Membrane integrity depends on several interacting factors. For example the availability and concentration of certain minerals, such as calcium and boron, have been shown to have an influence on membrane integrity. Boron is an important stabilizer of cell wall structure (O'Neill and York, 2003) and also has a role in the plasma membrane (Parr and Loughman, 1983). Calcium has also been shown to play an important role in cell membrane structure to stabilize phospholipids (Marinos, 1962). Picchioni et al. (1995) concluded that calcium infiltrated into fruit after harvest improved membrane organization and function during postharvest life. Additionally, De Castro et al. (2007b) found a relationship between high calcium and reduced susceptibility of Pink Lady apples to FB.

Another parameter that may be related to the development of FB is the level of antioxidants in the tissue. Antioxidants protect the cell membrane from oxidative damage. The antioxidants, ascorbic acid and glutathione, which are found in high concentrations in chloroplasts and other cellular compartments, are crucial for plant defense against oxidative stress (Noctor and Foyer, 1998). Ascorbic acid functions as the main defense against oxidative stress damage and reactive oxygen species (ROS). Ascorbic acid and calcium work together preserving the stability of the membrane. Veltman et al. (1999a) related decreasing ascorbic acid concentrations in high-CO_2-stored pears with the appearance of brown core. This may also be the case for the expression of FB in Pink Lady apples. Drake and Spayd (1983) showed that apples treated with $CaCl_2$ had less decrease in ascorbic acid content than untreated apples during storage.

In relation to CO_2 injury, Maguire and MacKay (2003) suggested that high concentrations of CO_2 inside the apple may result in production of stress-related free radicals, leading to membrane degradation, loss of compartmentalization and internal browning development as it has been previously described for brown core in pears (Veltman et al., 1999a,b). The goal of our research was to determine if the mechanisms of susceptibility and development of FB in Pink Lady apple are similar to those described for core browning in pear fruit. We want to identify the biochemical processes that contribute to the development of FB in individual apples and account for populations of damaged and undamaged apples in representative subsamples.

2. Materials and methods

2.1. Fruit material

Pink Lady apples were harvested three times in 2004 (21 September, 5 and 22 October) and twice in 2005 (28 September and 27 October) in the early morning from five 40-tree plots in a single orchard near Stockton, CA, USA. Approximately 50 apples were harvested from all parts of each tree, selecting the most mature fruit on the tree at each harvest. On the day of harvest, 30 fruit were selected randomly among the plots to determine starch content. The apples were cut in half equatorially, dipped for 1 min in iodine–potassium iodide (3%) and rinsed in fresh water. The starch level was scored using a 10-point scale CTIFL (Centre technique interprofessionnel des fruits et legumes, Paris, France).

2.2. Storage and internal browning evaluation

In 2004, all fruit were stored in air or 1.5 kPa O_2 in a factorial design with 1.0, 3.0 and 5.0 kPa CO_2 at 0.5 °C for 2, 4 or 6 months. To simulate industry conditions, fruit were sorted and cooled at 0.5 °C for 24 h before being placed into CA storage. In 2005, all fruit were stored in air or 1.5 kPa O_2 + 5.0 kPa CO_2, 1.5 kPa O_2 + <0.5 kPa CO_2 and 19 kPa O_2 + 5 kPa CO_2 at 0.5 °C for 2 or 4 months. At harvest 2 in 2004 and harvest 1 in 2005, one subset of fruit was immersed for 5 min in 2200 $\mu L\,L^{-1}$ diphenylamine (DPA, Pace International, Seattle, WA) in water, air dried at 20 °C, pre-cooled overnight at 0.5 °C and then placed into air or CA storage at 0.5 °C. A second subset of fruit was placed into CA storage for 2 or 4 months after a 4-week delay in air at 0.5 °C.

For FB evaluation after storage, 125 fruit per treatment were cut equatorially in three equally spaced locations resulting in ~2-cm thick slices, and the percentage area of flesh browning was visually estimated (Fig. 1A).

2.3. Cell viability

The viability of cells in the brown tissue was determined using intracellular enzymatic hydrolysis of fluorescein diacetate (Heslop-Harrison and Heslop Harrison, 1970). Fluorescein diacetate (Sigma chemical, St. Louis, MO) was dissolved in acetone (Fisher scientific, Fairlawn, NJ) (2 $g\,L^{-1}$) and diluted 1:500 with a 0.4 $mol\,L^{-1}$ sucrose solution. Observations of the fluorescein chromatic reaction were made with a Leica MZ 12 fluorescence microscope (Wetzlar, Germany) using mercury arc source (HBO 100 W, Osram lamp, Chicago, IL) exciting and T-2 barrier filters. The fluorescent light emission of individual cells was observed with an IP 28 photomultiplier tube and recorded with a Leica DC 300F digital camera system (Wetzlar, Germany). Viable cells dissociate fluorescein diacetate and can be seen easily with the fluorescent microscope. When cells are non-viable, they are not able to dissociate fluorescein diacetate and a dark area is observed.

2.4. Polyphenol oxidase

2.4.1. Subcellular fractionation

A plastid fraction was separated from a soluble fraction using a modified method of Mayer et al. (1964). Fresh apple flesh (6 g) was homogenized with 6 mL of cold extraction buffer (0.1 $mol\,L^{-1}$ NaK phosphate buffer (pH 7.2) containing 0.4 $mol\,L^{-1}$ sucrose and 0.001 $mol\,L^{-1}$ ascorbic acid). The homogenate was filtered with four layers of gauze. The filtrate was kept on ice and designated as the crude fraction.

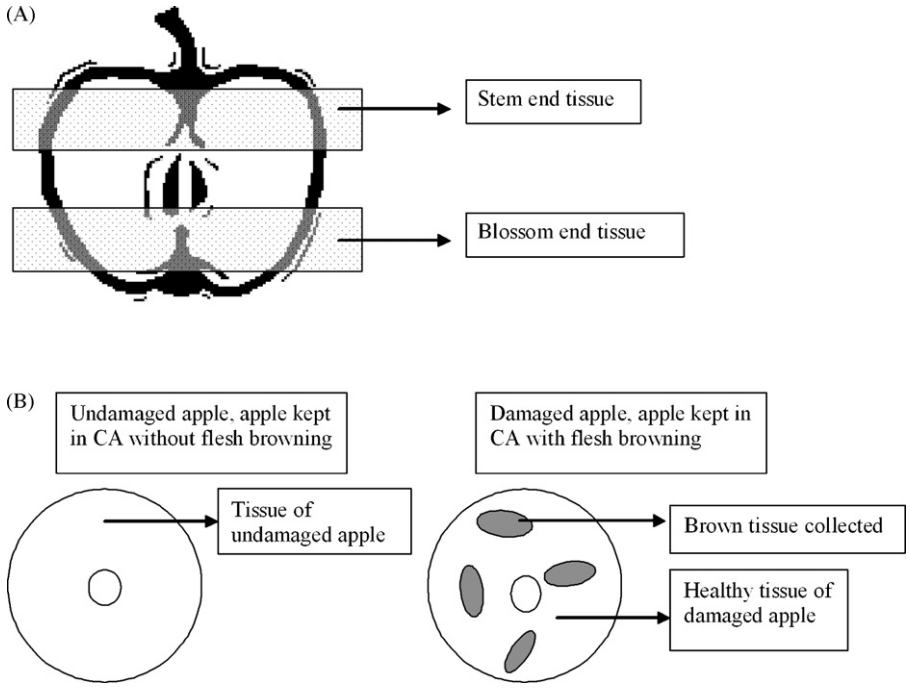

Fig. 1. Schemes of how tissue was selected, cut and denominated. (A) Transverse cuts were made to collect tissue from the region of the apple that was affected by FB and also sample tissue from different regions of the fruit (stem end and blossom end). (B) Tissue categories after cutting and evaluation, and denomination of the tissues that were collected for biochemical analysis.

Two replications of 1 mL each were centrifuged (4000 × *g*) for 5 min to precipitate cell plastids. The precipitate formed was washed and resuspended in 1 mL of cold buffer (0.1 mol L^{-1} NaK phosphate buffer containing 0.4 mol L^{-1} sucrose) and designated as the 'plastid' fraction, which included the cell wall fraction. The supernatant was kept on ice and designated as the 'soluble' (remaining soluble and mitochondrial) fraction. It is possible that some of the 'plastid' fraction may be from ionic or covalent bonding between PPO and other insoluble materials.

2.4.2. Enzyme activity

Polyphenol oxidase activity was measured spectrophotometrically as described by Espin et al. (1995). Assays were performed at 20 °C in 1.0 mL of a medium containing dihydroxycinnamic acid, 3-methyl-2-benzothiazolidone hydrozone (MBTH) and acetic acid buffer. An aliquot (10 μL) of the fraction (crude, plastid or soluble) was added and the increase in absorbance at 500 nm was monitored for 1 min. The rate of absorbance increase per minute was calculated on the interval between the 10th and the 20th second. One unit of enzyme activity was defined as the quantity of enzyme responsible for an absorbance increase of 1.6×10^{-5} s^{-1}. Results are expressed as enzyme units per kilogram of fresh weight.

2.5. Ascorbic acid

Ascorbic acid was determined in apple fruit at harvest and after each period of storage in each atmosphere. After storage, the apples were divided into two categories; undamaged, those without FB symptoms, and damaged, those with some flesh browning (Fig. 1A). Four apples of each category, damaged and undamaged were selected following storage in 5 kPa CO_2. For air and 3 kPa CO_2-stored fruit, only four undamaged apples were selected for analysis. Skinless tissue samples (10 g of fresh weight) were collected from the stem-end and the blossom-end halves of each apple (Fig. 1B). A whole transverse section (2 cm thick) from the stem or blossom end, respectively, was obtained from each fruit. Ten grams of tissue was collected from undamaged apples and 10 g of healthy tissue was collected from damaged apples for analysis. Brown tissue in the case of the damaged apples was collected from any location inside the apple, wherever it appeared or was more abundant.

All samples were evaluated for reduced L-ascorbate and dehydroascorbate content based on the method of Zapata and Dufour (1992). Samples were frozen with liquid N_2, crushed with a mortar and pestle and then homogenized with 10 mL of an extraction solution (0.1 mol L^{-1} citric acid, 0.05% EDTA, 0.004 mol L^{-1} sodium fluoride, 5% methanol) for 1 min at high speed in a blender. The homogenate was filtered through cheesecloth and then centrifuged for 5 min at 12,000 × *g* at 2 °C in a Sorvall RC-SB centrifuge (Sorvall Dupont Instruments, Wilmington, DE) using a SS-34 rotor. After adjusting the pH of the supernatant to 2.35–2.40, the sample was passed through a Sep-Pak Cl8 cartridge (Waters Assoc., Milford, MA) which had been preconditioned with 10 mL HPLC-grade methanol and 10 mL ddH_2O. The first 5 mL of eluent was discarded and the next 3 mL retained for analysis. As specified by Zapata and Dufour (1992), 37 min before injection onto the HPLC system, 1 mL

of 1,2-phenylenediamine (3.33 g L^{-1}) in methanol/water (5:95, v/v) was added. The mixture was immediately passed through a 0.45-mm filter (Acrodisc, Gelman Sciences, Ann Arbor, MI) into an amber sample vial and sealed.

The HPLC system consisted of a Hewlett Packard Series 1050 auto sampler, Series 1050 pump, and a Series 1040M diode array detector, operated by HP ChemStation software (Hewlett Packard, Waldbronn, Germany). A Waters pBondapak Cl8 reversed-phase column (Waters Assoc., Milford, MA), 30 cm × 3.9 mm i.d., was used for separation with a Bio-Rad Bio-sil Micro-Guard column (ODS-5S 4.6 mm × 3 cm i.d.) (Beckman Coulter Inc., Fullerton, CA). The eluent was methanol/water (5:95, v/v) containing 0.005 mol L^{-1} hexadecyltrimethyl-ammonium bromide and 0.05 mol L^{-1} potassium dihydrogen phosphate, with the pH adjusted to 4.59. The flow rate was 16.7 μL s^{-1}. Detection was at 261 nm for reduced L-ascorbate and at 348 nm for dehydroascorbate. Retention times were 4.3 and 6.3 min for dehydroascorbate and reduced L-ascorbate, respectively. L-Ascorbate and dehydroascorbate standards were supplied by Sigma–Aldrich, Inc. (St. Louis, MO). Results are expressed as mg of ascorbic acid or dehydroascorbic acid per kilogram of fresh weight.

2.6. Electrolyte leakage

Prior to electrolyte leakage studies, the isotonic concentration was determined based on the method of Saltveit (2002). Samples of apples randomly selected at harvest were monitored to study the weight loss or gain in different mannitol solutions (0.0–0.5 mol L^{-1} for 210 min). The samples consisted of 20 discs of 1 cm diameter and 0.5 cm thickness. A 0.5 cm slice was cut along the equator of the apple, 0.25 cm on each side of the equator. With a stainless steel 1 cm-diameter cork borer, discs were cut from the flesh halfway between the skin and the core. The discs were washed three times for about 1 min each in distilled H_2O, blotted dry and weighed (9–12 g total). Each sample was placed in a Petri dish with 20 mL of the corresponding mannitol solution and gently shaken on a rotary shaker at a speed of 1 s^{-1}. The solution was vacuum aspirated after 35, 75, 150 and 210 min and at each time the discs were weighed and fresh solution added.

Electrolyte leakage was determined in stem and blossom ends (Fig. 1A) within the apples at harvest and after 2 and 4 months of storage. After storage, apples were divided into undamaged and damaged apples. Eight apples of each category from each storage atmosphere were selected. Samples of approximately 3 g, consisting of 5 discs of 1 cm diameter and 0.5 cm thickness, were collected from the stem or blossom end slices as previously described. Each sample of discs was put into a 50-mL centrifuge tube with 20 mL of 0.35 mol L^{-1} mannitol, and the conductivity recorded at 0, 15, 30, 45, 60, 80, 140, 200 and 250 min. The tubes with the discs were frozen and thawed twice before the final conductivity was measured as total conductivity. The pre-frozen conductivity readings were divided by the total conductivity for that sample and the product multiplied by 100 for percent conductivity. The slope of the line defined by the percentage of electrolytes measured at each time (80, 140 and 200 min) was calculated to define the slow rate of leakage through the membrane (Saltveit, 2002).

2.7. Determination of hydrogen peroxide

Five apples per treatment and category were selected and samples of 6 g fresh weight were collected from the stem-end and the blossom-end halves of each fruit as described for the ascorbic acid analysis. Samples were frozen with liquid N_2, crushed with a mortar and pestle and homogenized with 5 mL of 5% trichloroacetic acid for 1 min at high speed in a blender. The homogenate was filtered through four layers of cheesecloth and centrifuged for 20 min at 20,000 × g at 2 °C in a Sorvall RC-SB centrifuge (Sorvall Dupont Instruments, Wilmington, DE) using a SS-34 rotor. Hydrogen peroxide concentration was determined using the Bioxytech H_2O_2-560 colorimetric assay (OXIS International Inc., Portland, OR) based on the oxidation of ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) by H_2O_2 under acidic conditions. The ferric ions bind with the indicator dye, xylenol orange, to form a stable, color complex which can be measured at 560 nm. The standards were prepared by diluting 30% H_2O_2 (Fisher Sci., Fair Lawn, NJ) in water and were measured at the same time as the samples.

2.8. Mineral analysis

Individual apple mineral composition was measured from the stem-end and the blossom-end. Tissue from undamaged apples and healthy tissue from FB apples was collected after 2, 4 and 6 months of CA storage. A total of 60 apples were analysed per year, 30 undamaged and 30 damaged. A whole transverse section (2 cm thick) from the stem or blossom end, respectively, was obtained from each fruit (Fig. 1A). Skinless flesh tissue samples (5 g fresh weight) were collected from the area half way between the skin and core. Samples were freeze-dried and sent to a laboratory for analysis (UC-DANR Analytical Laboratory, Davis, CA) of calcium and boron by inductively coupled plasma atomic emission spectrometry (ICP-AES) as described by Meyer and Keliher (1992).

2.9. Statistical analysis

The experiment had a factorial design with two storage times (2 and 4 months), two storage atmospheres (air and CA), one or two maturities and two locations within the fruit, stem and blossom-end. Fruit stored in 5 kPa CO_2 were further divided into two categories undamaged and damaged fruit. From damaged fruit we further analysed healthy and brown tissue. Data were analysed by storage atmosphere, location within the fruit and harvest maturity. For ascorbic acid there were four apples per treatment in 2004, and 10 apples per treatment for the first harvest and 5 apples per treatment for the later harvest in 2005. There were 8, 5, 5 and 7 apples per treatment for electrolyte leakage, mineral analysis, H_2O_2 and PPO activity, respectively. Analysis of variance was computed by SAS Version 8.02 (SAS

Table 1
Percentage flesh browning incidence in 2004 and 2005 after 2 months storage in controlled atmosphere (CO_2 with 1.5 kPa O_2) at 0.5 °C

Year	Harvest[a]	Additional treatment	CO_2 (kPa)		
			1	3	5
2004	1	–	13[b]	20	31
	2	–	2	10	16
	2	DPA[c]	–	–	0
	3	–	1	5	15
	3	4-week delay[d]	0	4	3
2005	1	–	–	–	5 a[e]
	1	DPA	–	–	0 b
	3	–	–	–	5 a

[a] Early (1), intermediate (2) and late (3) harvests correspond to starch indices of 3.5, 6.5 and 8.5, respectively.
[b] Minimum significant difference, within 2004, according to Tukey's test 15 % ($\alpha = 0.05$).
[c] 2200 μL L^{-1} diphenylamine.
[d] Four-week delay at 0.5 °C before CA storage.
[e] Different letters indicate significant differences ($P < 0.05$) according to Tukey's test. No significant differences in 2005 within harvests ($\alpha = 0.05$). DPA completely inhibited the disorder.

Institute Inc., Cary, NC). Multiple mean comparisons were performed using LSD and Tukey–Kramer adjustment ($\alpha = 0.05$).

3. Results

3.1. Flesh browning

In 2004, the incidence of CO_2-induced FB after 2 months storage was higher in apples stored in 5 kPa CO_2 + 1.5 kPa O_2 than in 1 and 3 kPa CO_2 + 1.5 kPa O_2 (Table 1). Air-stored apples did not show FB. In 2005, the incidence of FB in apples was only 5% in 5 kPa CO_2 + 1.5 kPa O_2 compared to 15% in 2004. The percentage of FB in each atmosphere remained constant between 2, 4 and 6 months of storage in any season (data not shown). DPA inhibited FB completely in 2004 and 2005 and the delayed CA storage treatment significantly reduced FB incidence, but did not eliminate the disorder (Table 1). In 2005, apples stored in 1.5 kPa O_2 + <0.5 kPa CO_2 never developed FB symptoms. Storage in 5 kPa CO_2 with 19 kPa O_2 reduced FB to 1% compared with 5% FB in 5 kPa CO_2 with 1.5 kPa O_2.

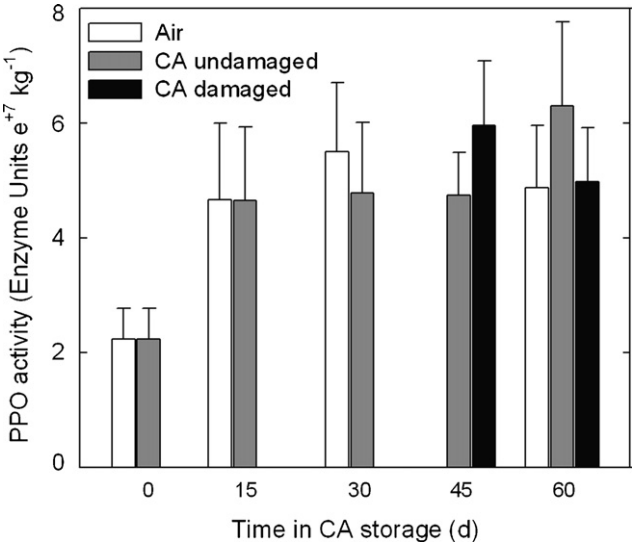

Fig. 3. Total (plastid plus soluble fractions) polyphenol oxidase activity (enzyme unit kg^{-1}) in apples stored for 2 months at 0.5 °C in air or CA storage (1.5 kPa O_2 + 5 kPa CO_2). Damaged apples showed flesh browning beginning at 45 d. Healthy tissue of damaged apples was selected for analysis. *No analysis was done in air at 45 d.

3.2. Cell viability

After 2 and 4 months of storage, the analysis of cell viability with fluorescein diacetate revealed that the brown tissue was a group of dead cells surrounded by healthy, viable cells that were not brown (Fig. 2). Apples that did not have brown tissue did not have any non-viable cells after 2 or 4 months of CA or air storage.

3.3. Polyphenol oxidase activity

Total PPO activity increased during the first 15 d of storage in air and CA (1.5 kPa O_2 + 5 kPa CO_2) in 2005 (Fig. 3),

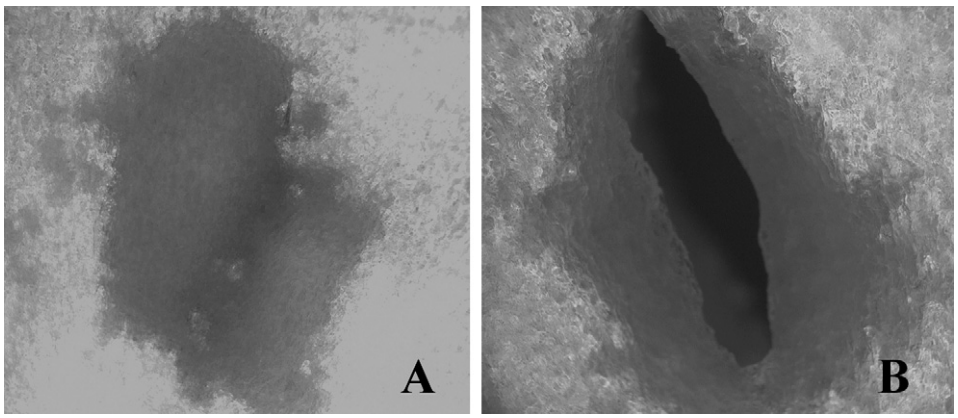

Fig. 2. Cell viability by induced fluorescence analysis in apples stored in 1.5 kPa O_2 + 5 kPa CO_2 for 4 months at 0.5 °C and showing flesh-browning injury. Cells without fluorescence were dead and cells with fluorescence were viable. The pattern matched with the pattern of flesh browning, e.g. all brown cells were non-viable, surrounding cells were viable. (A) Flesh of a damaged apple affected by flesh browning. (B) Flesh of a damaged apple affected by flesh browning with CO_2-induced cavity.

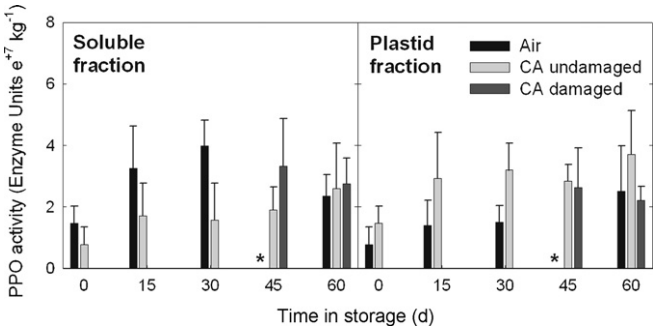

Fig. 4. Polyphenol oxidase activity (enzyme units kg^{-1}) from soluble and plastid fractions in apples stored for 2 months at 0.5 °C in air or CA (1.5 kPa O_2 + 5 kPa CO_2). Damaged apples showed flesh browning in CA (CA damaged). Healthy tissue of damaged apples was selected for analysis. *No analysis was done in air at 45 d.

and increases occurred in both the plastid and soluble fractions, although the increase in the plastid fraction was larger than that in the soluble fraction (Fig. 4). The PPO activity was similar in air and CA at 30 d after harvest. After 60 d the PPO activity in the plastid fraction decreased in air-stored fruit, but was unchanged or slightly higher in CA-stored fruit, and in both storage conditions the soluble fraction slightly increased after 30 d. There were also no other significant differences in PPO activity between the plastid and soluble fractions, among storage times, or between damaged and undamaged CA stored fruit (Figs. 3 and 4).

3.4. Ascorbic acid

Ascorbic acid levels decreased significantly with time in all storage conditions in fruit from the last harvest in 2004 and 2005 (starch index 8.5 and 8.0 for 2004 and 2005, respectively) (Fig. 5A and C). Apples stored in air had the lowest levels of ascorbic acid. After 4 months of storage in 2004, the initial value decreased by 70% (Fig. 3A). Apples stored in 3 or 5 kPa CO_2 that were not damaged by FB only lost 30% of the initial amount of ascorbic acid after 4 months.

Although 5 kPa CO_2 conserved ascorbic acid, once the apple was damaged by FB, the amount of ascorbic acid decreased to very low levels in the healthy tissue and in the brown tissue (Figs. 5A and 6A). After 2 months, ascorbic acid concentration in the healthy tissue surrounding the brown tissue in FB apples decreased to 35% of the initial amount and after 4 months to trace levels (Fig. 5A). Ascorbic acid concentration in the brown tissue was very low after 2 months of storage and after this time, the concentrations were very close to 0 mg kg^{-1} of fresh weight. In 2004, the loss of ascorbic acid in CA occurred sometime between 2 and 4 months (Fig. 5A), however, in 2005, the loss occurred within the first 2 months of storage and after that it did not decrease significantly (Fig. 5C).

In 2005, ascorbic acid was also monitored in early maturity fruit (starch index at harvest 3.5). Ascorbic acid decreased significantly during the first 2 months in all storage conditions (Fig. 6A). Ascorbic acid concentration after 2 months was similar for undamaged apples stored in 5 kPa CO_2 + 1.5 kPa O_2

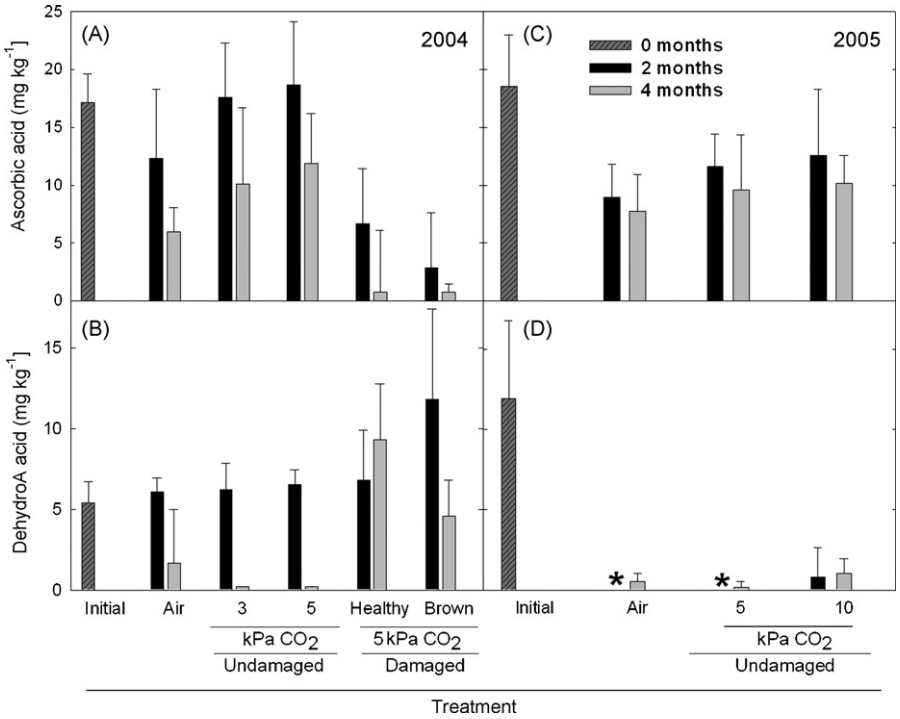

Fig. 5. Changes in ascorbic acid (A, C) and dehydroascorbic (dehydroA) acid (B, D) (mg kg^{-1}) concentrations in relation to storage regime and symptoms in year 2004 (A, B) and year 2005 (C, D), in apples at harvest (initial) and after storage at 0.5 °C in air or 1.5 kPa O_2 with 3 kPa or 5 kPa CO_2 in 2004 (A, B), and in air or 1.5 kPa O_2 with 5 kPa or 10 kPa CO_2 in 2005 (C, D) for 2 and 4 months. Apples stored in 5 kPa CO_2 were divided into undamaged and damaged fruit. Damaged fruit were further divided into brown (brown tissue from damaged apples) and healthy tissue (healthy tissue from damaged apples). Bars correspond to the average value of four apples in 2004 and five apples in 2005 and their standard deviation. Starch score at harvest = 8.5 in 2004 and 8.0 in 2005. *DehydroA acid not detected.

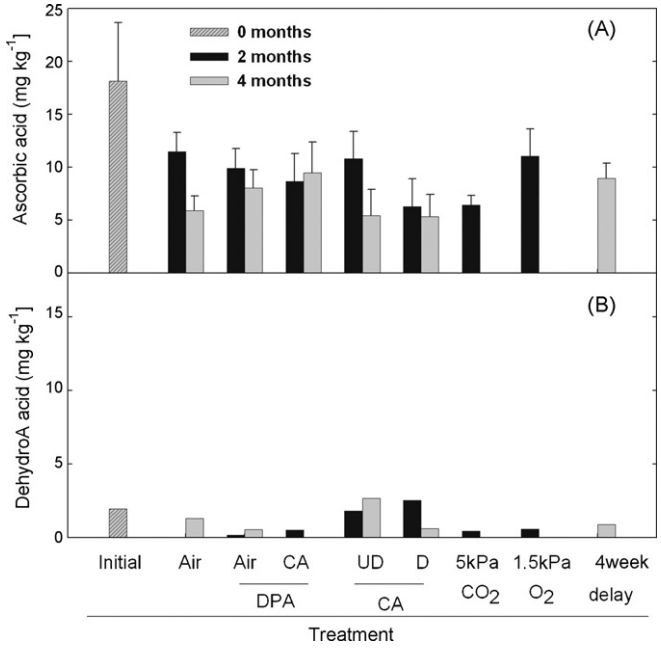

Fig. 6. Ascorbic acid (A) and dehydroascorbic (dehydroA) acid (B) (mg kg^{-1}) concentrations in relation to storage regime and symptoms in year 2005, in apples at harvest (initial) and after 2 and 4 months storage at 0.5 °C in air, CA (1.5 kPa O_2 + 5 kPa CO_2), 19 kPa O_2 + 5 kPa CO_2 (5 kPa CO_2), or 1.5 kPa O_2 + 0.5 kPa CO_2 (1.5 kPa O_2). Additional fruit treated with 2200 μL L^{-1} diphenylamine (DPA) were stored in air or CA. Apples stored in CA were divided into undamaged (UD) and damaged (D) fruit. One set of fruit was held 4 weeks at 0.5 °C in air before CA (4-week delay). Bars correspond to standard deviation. Starch index at harvest 1–3.5.

or <0.5 kPa CO_2 + 1.5 kPa O_2, DPA-treated apples stored in 5 kPa CO_2 + 1.5 kPa O_2 or air, and untreated air stored apples. Healthy tissue from damaged apples and apples stored in 19 kPa O_2 + 5 kPa CO_2 showed a significant decrease in ascorbic acid after 2 months. After 4 months, the DPA-treated apples stored in CA or air and apples subjected to delayed CA showed greater conservation of ascorbic acid.

Dehydroascorbic acid (DHA) concentrations followed a different pattern, remaining unchanged during the first 2 months in storage in 2004, and except for healthy tissue in damaged apples in 5 kPa CO_2, decreasing largely after that (Fig. 5B). The surrounding healthy tissue of FB fruit stored in 5 kPa CO_2 showed the highest DHA concentrations after 4 months followed by the brown tissue in the same apples (Fig. 5B). DHA levels were similar among the storage conditions after 2 months except for the brown tissue, where it was higher, and then decreased to very low levels in all samples except in damaged apples at 4 months, where DHA increased or decreased slightly in healthy and brown tissue, respectively. In 2005, the dehydroascorbic acid concentrations were very low in both early and late maturity apples from all storage conditions after 2 and 4 months in storage (Figs. 5D and 6B).

The total ascorbic acid concentration (ascorbic acid plus dehydroascorbic acid) in fruit at harvest was significantly higher in 2005 than in 2004 (30.4 vs. 22.5 mg kg^{-1}) and the fruit had lower CO_2-induced flesh browning incidence in 2005.

The percentage of fruit with damage at the stem-end was higher (66%) than fruit that only had symptoms at the blossom-end (13%) (Table 2), and 21% of the fruit showed FB symptoms that were equally distributed in the entire apple. The concentration of ascorbic acid in the blossom-end of the apple was slightly higher in air and CA-stored apples not affected by FB (Table 2). Once the apple showed FB, ascorbic acid concentration was lower than in undamaged apples and similar in the stem and blossom-ends of the apple (data not shown).

3.5. Electrolyte leakage

There was an increase in electrolyte leakage rate with time in storage in all storage conditions (data not shown). The highest leakage rate values were found in the brown tissue of damaged apples stored at 1.5 kPa O_2 + 5 kPa CO_2. There were no differences in electrolyte leakage rates between apples stored in air and undamaged apples or healthy tissue of FB apples stored in 1.5 kPa O_2 + 5 kPa CO_2.

Healthy tissue from the stem-end of CA-stored FB apples had a higher leakage rate than the blossom-end. FB incidence was also higher in the stem end of these apples (Table 2). Samples from undamaged apples in 5 kPa CO_2 or from other storage conditions showed similar electrolyte leakage rates in the stem and blossom-ends.

3.6. Hydrogen peroxide concentration

The concentration of H_2O_2 in the apple flesh increased significantly during 2 months of storage in all storage conditions and treatments (Table 3). However, apples stored in 5 kPa CO_2

Table 2
Percentage of apples showing flesh browning more in the stem or blossom end of the apple in relationship with ascorbic acid, calcium and boron concentrations, and electrolyte leakage rate for tissues in those locations

Location	Flesh browning incidence (%)[a]	Ascorbic acid[b] (mg kg^{-1})	Hourly electrolyte leakage[c] rate (%)	Flesh calcium[c] (mg kg^{-1})	Flesh boron[c] (mg kg^{-1})
Stem	66	13 a[d]	1.9[e]	33.6 a[d]	5.2 a[d]
Blossom	13	15 b	1.4[e]	31.1 b	5.8 b

[a] Percentage of total damaged fruit that showed more severity on the stem-end or on the blossom-end. Twenty-one percentages of fruit showed FB equally in both ends of the apple.
[b] Data pooled across storage conditions from air and undamaged CA-stored fruit.
[c] Data pooled from healthy tissue of undamaged apples and damaged apples stored in 1.5 kPa O_2 + 5 kPa CO_2.
[d] Different letters indicate significant differences ($P < 0.05$) according to Tukey's test.
[e] Corresponding to a difference $P > |t| = 0.06$.

Table 3
Hydrogen peroxide concentration in apples at harvest and after 2 months storage in air or in controlled atmosphere (CA) with 1.5 kPa O_2 + 5 kPa CO_2 in 2005

Evaluation time (month)	Storage atmosphere/ treatment	Damaged apples with FB	Hydrogen peroxide μmol kg^{-1}
0	–	–	8.61a[a]
2	Air	–	15.32 b
2	CA	–	20.81 d
2	CA	+[b]	19.88 cd
2	CA/DPA[c]	–	17.56 c

[a] Letters correspond to LSD mean separation ($\alpha = 0.05$), $n = 5$ apples.
[b] Healthy tissue was sampled from flesh browning damaged apples (+).
[c] Two thousand two hundred microliters per liter diphenylamine at harvest.

showed statistically higher concentrations of H_2O_2 than apples stored in air. DPA treatment significantly reduced the accumulation of H_2O_2, but levels remained significantly higher than in air-stored fruit. Healthy tissue from damaged apples had similar H_2O_2 levels as undamaged apples kept under the same CA. Hydrogen peroxide levels in stem and blossom ends of the apples were similar in 2005 (data not shown).

3.7. Mineral analysis

Calcium concentrations in the stem-end of the apple were significantly higher than the blossom-end (Table 2), however; the concentration of boron was significantly higher in the blossom-end than in the stem-end of the fruit.

4. Discussion

It has been hypothesized that brown core in pear fruit is a consequence of mixing of cellular compartments caused by membrane disintegration (Veltman et al., 1999b). After loss of compartmentation, the PPO enzyme solubilizes from the plastid to the cytoplasm at the same time the phenolic compounds leak from the vacuole leading to quinone formation and polymerization of brown pigments.

To check this hypothesis in Pink Lady, we analysed the relative changes in enzyme activity and solubilisation from the plastid form to the soluble form. According to our results, no increase in PPO was found in apples stored in CA when compared to air storage. Furthermore, no significant increase in the soluble form was found. These results showed the difficulty to correlate browning directly to PPO activity as described previously by Nicolas et al. (1994).

Our lack of relationship between PPO activity and FB agrees with Veltman et al. (1999b) who concluded that PPO activity was not a limiting factor for the development of brown core disorder in pears.

There was a large difference in the ascorbic acid content between apples stored in CA which had developed FB and healthy, undamaged apples stored under the same CA conditions. Our results showed that when low O_2 was a component of a high-CO_2 atmosphere, the loss of ascorbic acid was reduced. For example, apples stored in CA with 19 kPa O_2 and 5 kPa CO_2 lost ascorbic acid much faster during storage than undamaged apples stored in CA with 1.5 kPa O_2 + 5 kPa CO_2, unless these apples showed FB, and then the ascorbic acid level was similar to the apples stored under 19 kPa O_2 and 5 kPa CO_2. It appears that the low O_2 component of the CA preserves the ascorbic acid concentration while the high CO_2 component of the mixture creates oxidative stress in susceptible apples, leading to a loss of ascorbic acid which allows FB symptoms to be expressed. Ascorbic acid oxidation is strongly related to tissue browning. Browning generally does not occur until all ascorbic acid is oxidized (Ponting and Joslyn, 1948). In pears, Veltman et al. (1999a) also reported that there was no ascorbic acid left in the browned tissue once pears presented core browning.

DPA did not affect the loss of ascorbic acid during the first 2 months of storage. However, after 4 months of either air or CA storage, apples treated with DPA had higher levels of ascorbic acid. Apples that were held in air storage for 4 weeks before CA storage also conserved higher ascorbic acid concentration. Because delayed CA also partially reduced the incidence of FB in some seasons, this supports a relationship between ascorbic acid levels and the development of FB.

Veltman et al. (1999a) concluded that there was a threshold level for ascorbic acid in pears below which core browning develops. We had similar results with the brown tissue in our study, where we only found traces of ascorbic acid and our results also showed a decrease in the concentration of ascorbic acid in the healthy areas surrounding the brown tissue in FB-damaged apples. Ascorbic acid decreased to trace levels in the brown tissue, in relation to cell death, but ascorbic acid also decreased in tissue distant from the affected tissue, not dead or brown, within the same apple. We could not define an ascorbic acid threshold in Pink Lady apples, as the incidence of FB was much lower than in pears. Flesh browning in Pink Lady apples appears to occur in a small percentage of susceptible apples rather than being a general disorder of all stored fruit.

Several studies propose a role for H_2O_2 as a signal for the activation of stress-response and defense pathways. H_2O_2 produced during a stress-response is thought to diffuse into cells (Mittler, 2002; Love et al., 2005) together with other plant signals and activate many of the plant defenses, including programmed cell death. Ascorbic acid may decrease under the systemic response of the whole fruit.

Apples held in CA accumulated more H_2O_2 than apples stored in air, indicating stress from the high-CO_2 concentrations in storage. Apples stored in high-CO_2 atmospheres also produced more ethylene than apples kept in low CO_2 atmospheres (De Castro et al., 2007a), this could also be an indication that apples stored under high CO_2 were under stress. The fact that H_2O_2 was not higher in the healthy tissue of damaged apples may indicate that the greater stress leading to browning in some tissues of the apple may be due to other factors specific to that tissue. It is interesting to note that while DPA did not affect the loss of ascorbic acid during the first 2 months of storage, it did reduce the accumulation of H_2O_2.

Frenkel and Patterson (1973) found that pears in CA containing high concentrations of CO_2 showed alterations in different organelles including plastids and other membrane systems such

as the tonoplast and plasma membrane. However, our electrolyte leakage analysis of Pink Lady apples did not show that the rate of leakage due to membrane degradation was the main difference between apples stored in air or CA or between damaged and undamaged apples stored in high-CO_2 concentrations. Our method of electrolyte leakage analysis may not be sensitive enough to pick up very small differences in levels of electrolyte leakage between the treatments, and does not distinguish membrane leakiness within the various cellular membranes. The effect of CO_2 may be greater on plastid or vacuolar membranes, and our method may be only measuring plasma membrane leakage.

The non-viability of cells affected by FB explains the high rate of electrolyte leakage and the low concentration of ascorbic acid in these tissues. We were first able to detect FB after 45 d in storage and cells affected by FB were already dead at that time. It is hypothesized that the ascorbic acid was oxidized to DHA and the non-viable cells were unable to reduce the latter back to the former because of a lack of energy and resources.

4.1. Comparison of stem and blossom end

The stem-end of the apples had a higher incidence of FB than the blossom-end. The difference in FB susceptibility between stem and blossom end tissues of the apple fruit was related to higher concentrations of ascorbic acid in the blossom-end tissue, except in apples affected by FB. In the apples with FB, the difference between stem and blossom ends was slight, perhaps because the ascorbic acid was used to prevent oxidation and degradation caused by the high CO_2.

Although there is strong evidence of a role for calcium in maintaining the stability of membranes (Marinos, 1962), we observed higher FB incidence in the stem-end of the fruit where the concentration of calcium was statistically higher. However, the boron concentration was lower in the stem-end, and it has also been shown to maintain membrane stability and cell wall strength (Parr and Loughman, 1983; O'Neill and York, 2003). The rate of electrolyte leakage was significantly higher in the stem-end of the fruit in agreement with the higher incidence of FB in that part of the fruit. Factors like higher ascorbic acid concentration, higher boron concentration and lower electrolyte leakage rate at the blossom-end of the apple may be related to the fivefold lower percent incidence of FB in the blossom-end compared to the stem-end of the fruit.

4.2. Conclusion

It is clear that CO_2-induced flesh browning cannot be related to a single biochemical parameter at harvest or during storage. High CO_2 in storage can cause development of FB and can be further aggravated by low O_2 concentrations (De Castro et al., 2007b). Ascorbic acid concentration appears to be the most highly correlated factor associated with tissue susceptibility to the disorder, but there is some uncertainty about the mechanism of its involvement. Ascorbic acid may prevent the oxidative effect of high-CO_2 stress on membranes, or may reduce lipid peroxidation as Shalata and Neumann (2001) concluded. We do not know if it is the concentration of ascorbic acid at harvest or its maintenance during storage which determines the susceptibility of apples to FB, or if there is another biochemical factor that defines tissue susceptibility. It appears that CO_2 stress results in elevated oxidative levels in apple cells, probably more so in areas of susceptible tissues. When ascorbic acid is consumed in this oxidative environment, membranes are damaged, enzymes and substrates mix to form brown pigments, and eventually the cells die. DPA, with its antioxidant properties, reduces the peroxidation and the disruption of the membranes and delays the expression of browning symptoms.

Our results show that high-CO_2 concentration in CA storage does not damage all the apples in a storage lot. It seems that only a certain percentage of the apples are susceptible to FB and the percentage varies between years. Even further, only a portion of the apple is affected by FB, the brown area does not extend to the entire flesh, even though the healthy tissue in the same apple has very low ascorbic acid concentration and high-DHA concentration. Among the biochemical factors, it appears that ascorbic acid and H_2O_2 concentrations are the most highly correlated to the expression of FB of the factors investigated in this study, but further work is required to better understand the biochemical mechanism of CO_2-induced flesh browning susceptibility in Pink Lady apples.

Acknowledgements

The authors would like to thank Horticulture Australia LTD and Tim Sambado of A. Sambado and Sons for partial support of this project and Hamid Ahmadi for all his help with the cell viability studies.

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