

Short anaerobiosis period prior to cold storage alleviates bitter pit and superficial scald in Granny Smith apples

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Abstract

BACKGROUND: Californian Granny Smith apples are very susceptible to bitter pit (BP) and superficial scald symptoms that develop during cold storage. The main preventive means are diphenylamine dipping and/or gaseous application of the ethylene inhibitor 1-methylcyclopropene (1-MCP), which is effective against superficial scald but not against BP. This study investigated the efficacy of a non-chemical alternative, low-O₂ (LO2) stress, in preventing these two physiological disorders.

RESULTS: Application of LO2 stress at 20 °C for 10 days prior to cold storage of Granny Smith apples reduced superficial scald and BP incidence and severity during 8 months at 0 °C. LO2 treatments induced volatile alcohols and reduced ethylene and 6-methyl-5-hepten-2-one (MHO-on) production, thereby reducing superficial scald development after 4 months at 0 °C. In addition, LO2-treated fruits had higher pectin methyl esterase (*MdPME*) gene expression, similar to that of 1-MCP-treated fruits, associated with their higher firmness. Conversion of MHO-on to 6-methyl-5-hepten-2-ol (MHO-ol) in LO2-treated fruits may explain the lower scald development.

CONCLUSION: The ratio between MHO-on and MHO-ol might serve as an index of superficial scald severity. Reduction of BP symptoms in LO2-treated fruits could be due to accumulation of volatile alcohols in the peel tissue.

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Keywords: *Malus × domestica*; ethylene; fruit ripening; α -farnesene; 6-methyl-5-hepten-2-one; pectin methyl esterase

INTRODUCTION

Granny Smith apples are very susceptible to superficial scald, a chilling injury symptom, when stored in regular air at 0 °C for several months.¹ Over the years, many methods have been developed to control scald, of which the most common is treatment with the antioxidant diphenylamine (DPA).^{2,3} Storage under controlled atmosphere (CA) or with initial low-O₂ (LO2) stress prior to CA or dynamic CA was also effective in controlling scald.^{4,5}

The development of superficial scald has been attributed to autoxidation products of α -farnesene – identified as conjugated trienols and 6-methyl-5-hepten-2-one (MHO-on) – in the apple skin.⁶ Many studies have shown that synthesis of α -farnesene is mediated by ethylene,^{7,8} and recently it has been demonstrated that inhibition of ethylene action by 1-methylcyclopropene (1-MCP) is very effective in decreasing scald in apples, confirming that ethylene is the main scald inducer.^{4,9}

LO2 pretreatment or application of acetaldehyde or ethanol vapour to various fruits was proposed as a means to reduce chilling injury via reduction of ethylene production.¹⁰ Application of controlled LO2 stress (<1% O₂) for up to 10 days at 20 °C reduced scald development in Granny Smith apples after 16 weeks at 0 °C, and this reduction was correlated with the induction of ethanol production during LO2 stress.^{11,12} Initial LO2 stress followed by CA storage also controlled scald in apples and was associated with

higher ethanol production.^{13,14} Application of ethanol vapour at 0 or 20 °C reduced superficial scald in Granny Smith apples after a subsequent 16 weeks at 0 °C.¹⁵ In other work, ethanol treatments (10 days at 20 °C) were also effective in preventing superficial scald but led to a high incidence of internal browning after 2 months of cold storage.¹⁶

Application of hypoxic conditions to apples resulted in changes in alcohol acyl CoA transferase and alcohol dehydrogenase (ADH) activity, which affected volatile production.¹⁷ During pear ripening, ADH has been suggested to act on several substrates and not to be limited to ethanol production.¹⁸ In pears stored under LO2 conditions, acetaldehyde and ethanol production were observed to peak after 5 months of cold storage and to fall later, probably because of feedback inhibition of ADH by product accumulation.¹⁹

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Bitter pit (BP) is another major physiological disorder that develops during storage of apple fruits. Although lower levels of total calcium (Ca) content in fruit tissue have been correlated with higher probability of BP incidence, many reports have shown that total Ca content is not a reliable predictor of BP development in fruits, nor a reliable basis for prevention.^{20,21} There is suggestive evidence that BP development is dependent not only on low Ca concentration but also on abnormal Ca partitioning and distribution in the cell.²² At the same time, higher pectin methyl esterase (PME) activity would enable available Ca to interact strongly with pectins, thereby increasing cell wall strength and fruit firmness.²³ Colgan *et al.*²⁴ showed that delaying the establishment of CA conditions increased BP incidence. In a previous study we showed that LO₂ pretreatment was effective in reducing superficial scald and BP appearance in Californian Granny Smith apples.²⁵ Recently, LO₂ pretreatment was also shown to be effective against BP development in Golden Reinders apples.²⁶

In the present study we looked for effective LO₂ treatments prior to cold storage as a means to reduce superficial scald and BP development that would be suitable even for organically grown apples. We previously showed that short-term LO₂ pretreatment (7 days at 20 °C) reduced superficial scald symptoms in Israeli Granny Smith apples.¹² In the present study we observed that extended pretreatment of Californian Granny Smith apples with LO₂ (10 days at 20 °C) was effective in the reduction of BP symptoms also.

MATERIALS AND METHODS

Plant material and postharvest treatments

Granny Smith apples were brought on the day of harvest to the postharvest laboratory at the University of California, Davis from a commercial packinghouse in Stockton, CA. The fruits were taken from commercial bins before being drenched with DPA. The various treatments are summarised in Table 1. All pretreatments except LO₂late were initiated on the day of harvest.

For DPA treatment, fruits were dipped for 5 min in a 2.2 mL L⁻¹ solution of Shield Bright DPA 15% (Pace International, Seattle, WA, USA). 1-MCP was applied in 300 L stainless steel chambers at 20 °C for 24 h using SmartFresh tablets and activator solution (Agrofresh, Springhouse, PA, USA), which release 1-MCP at 1 mL L⁻¹ within that chamber volume according to the manufacturer.

The LO₂ treatments were applied in 300 L stainless steel chambers. Fruits were placed in the chambers in four plastic totes each containing 60 fruits, i.e. 240 fruits per chamber. Each treatment was applied in duplicate chambers. Variations of the

LO₂ treatment involved: (1) flushing the chamber continuously at 700 mL min⁻¹ for 10 days at 20 °C with N₂ (from a liquid N₂ cylinder) that had been humidified by bubbling through a bubbling stone in a water column (LO₂F); (2) flushing as in (1) but at 6 L min⁻¹ for about 2 h until the O₂ partial pressure fell below 3 kPa, then sealing the chambers for 10 days at 20 °C (LO₂C); (3) loading the chambers with 50% more fruits, i.e. 360 fruits per chamber, removing the fruits after 1 week at 0 °C and sealing them under an LO₂ atmosphere as in (2) at 20 °C for 10 days (LO₂late) (Table 1).

During the treatments, CO₂, O₂, acetaldehyde and ethanol levels in the chamber headspace were measured daily. CO₂ and O₂ were analysed with a PIR-2000R infrared gas analyser (Horiba Instruments, Irvine, CA, USA). Acetaldehyde and ethanol were measured with a GC-9AM gas chromatograph (Shimadzu Scientific Instruments, Columbia, MD, USA) fitted with a 60/80 Carbowax column filled with 5% Carbowax (Supelco, Bellefonte, PA, USA), with a flame ionisation detector (FID) at 250 °C, injection port at 250 °C and oven at 85 °C, and N₂ as the carrier gas. Headspace samples were quantified by comparison with known CO₂, O₂, acetaldehyde and ethanol standards.

After the various pretreatments at 20 °C, fruits were transferred from the plastic totes to cardboard apple boxes and stored in a cold room at 0 °C for up to 8 months.

Fruit quality: colour, firmness, total soluble solids and acidity

Fruits were stored in cardboard boxes in regular air at 0 °C for up to 8 months. Three trays (each containing 20 fruits) from each treatment were transferred to 20 °C after 4 and 8 months of storage. Fruit quality was evaluated immediately after removal from cold storage and after 1 and 2 weeks at 20 °C. Peel colour indices were recorded at two diametrically opposite sides of each apple fruit by means of a Chroma Meter CR-310 (Minolta, Osaka, Japan). Results were expressed as hue angle (H° , where 90° = full yellow and 180° = full green), lightness (L^* , where 0 = black and 100 = white) and chroma (C^* , colour saturation). Firmness was measured on opposite sides of the fruit, as resistance to penetration (5 mm depth) with an 11 mm probe mounted in a fruit texture analyser (Guss Manufacturing, Strand, South Africa), after removing a small area of peel. Total soluble solids (TSS) and titratable acidity (TA) were assayed in juice extracted from a composite sample of three wedges cut from stem end to blossom end of three separate fruits. Percentage TSS was determined with an Abbe 10450 digital refractometer (American Optical, Buffalo, NY, USA). TA (expressed as malic acid equivalents) was determined with a TIM 850 automatic titration system (Radiometer, Copenhagen, Denmark).

Table 1. Treatments applied to Granny Smith apples at same day of harvest (nos 1–6) or 1 week later (no. 7)

No.	Abbreviation	Treatment	Time at 20 °C	Time into 0 °C storage
1	Cont	Control	<6 h	Day of harvest
2	DPA	2200 ppm	<6 h	Day of harvest
3	1-MCP	1000 ppb	>24 h	1 day later
4	Cond	Conditioned, stayed at 20 °C in air	10 days in air	10 days later
5	LO ₂ F	Continuous flushing with N ₂	10 days in LO ₂	10 days later
6	LO ₂ C	Flush then seal in N ₂	10 days in LO ₂	10 days later
7	LO ₂ late	Flush then seal in N ₂ after 1 week at 0 °C	10 days in LO ₂ after 1 week at 0 °C	Day of harvest, stored for 1 week, then treated at 20 °C and stored again at 0 °C

Juice browning score

Juice extracted for TSS and TA determination after 4 months at 0 °C plus 14 days at 20 °C was also used for browning measurements. A 3 mL sample of crude apple juice (extracted with a hand squeezer) was mixed with 2 mL of 50 mL L⁻¹ trichloroacetic acid (TCA) at time 0 and a second 3 mL sample was incubated for 2 h at 20 °C before adding the TCA solution to stop the browning reactions. The stopped-reaction mixtures were centrifuged at 6000 × *g* for 20 min, the absorbance of the supernatants was measured at 420 nm and the difference in optical density (Δ OD) between the two supernatants (2 h–0 h) was designated as the browning score. The method was a modified and simplified version of that described by Murata *et al.*²⁷

Ethylene and respiration measurements

Three weeks after harvest, five individual fruits of similar weight and colour from each treatment were selected from the cold-stored boxes and transferred to 1 L plastic jars held at 0 °C. The jars were periodically sealed for 2 h to monitor respiration and ethylene production during 65 days of cold storage at 0 °C. The jars were then transferred to 20 °C for 1 week of shelf life, where they were sealed daily for 1 h and headspace samples were analysed for CO₂ with a PIR-2000R infrared gas analyser (Horiba Instruments) and for ethylene with a gas chromatograph fitted with an FID (GC/FID; Hach Carle, Loveland, CO, USA). Data from the three LO2 pretreatments were similar, so they were averaged together.

Headspace volatile analysis

Volatile production *in situ* was checked several times during cold storage and after transfer to 20 °C by GC/FID and GC/mass spectrometer (MS). Pieces (1 g) of apple peel plus 0.4 g of NaCl were placed in 20 mL amber-coloured vials with 2 mL of 200 g L⁻¹ NaCl solution. The high salt presence was to prevent enzymatic activity associated with biosynthesis of volatiles²⁸ and to enhance volatile partitioning.²⁹ The vials were sealed with screw caps and incubated for 1 h at 30 °C, then 1 mL headspace samples were taken and injected into the GC/FID. Acetaldehyde, ethanol and methanol were measured with the GC as described previously for the chamber measurements. Headspace samples were quantified by comparison with known acetaldehyde, ethanol and methanol standards.

For determining other volatiles, GC/MS and solid phase microextraction (SPME) techniques were used. Internal standards of 500 nL L⁻¹ 1-octanol were added to a second set of vials containing apple peel as described above³⁰ and the vials were incubated at 30 °C for 2 h. After incubation, volatiles were absorbed from the headspace for 20 min onto an SPME fibre (1 cm long, 100 μ m thick) coated with polydimethylsiloxane (Sigma-Aldrich/Supelco, Bellefonte, PA, USA). The fibre was desorbed at 260 °C for 10 min in the splitless injection port of a model 6890 GC/MS (Agilent, Santa Clara, CA) with He as the carrier gas. Volatiles were separated on a DB-Wax capillary column (31 m, 0.26 mm i.d., 0.26 μ m film thickness; Agilent). Chromatography conditions were an initial temperature of 40 °C for 1 min, then ramping up to 150 °C at 10 °C min⁻¹ and to 260 °C at 15 °C min⁻¹. α -Farnesene isomers and MHO were identified by comparison of their mass spectra with those of commercial standards and with spectra published in the US National Institute for Standards and Technology (NIST) Mass Spectral Library. Farnesene isomers were confirmed using a mixed farnesene standard (Cat. F0287, TCI America, Portland, OR, USA) and by comparison of retention times and spectra with

published values. MHO was confirmed using an external standard (Cat. M48805, Sigma-Aldrich, St. Louis, MO, USA) and quantified by comparison with the peak of the 1-octanol internal standard.³⁰

Scald and bitter pit evaluation

The superficial scald or BP index was assessed in Granny Smith apples according to a ten-point visual peel damage scale (0 = no injury, 1 = slight injury, 5 = moderate injury and 10 = severe injury)³¹ and calculated according to the following formula:

$$\text{Scald or BP index} = \frac{\sum_0^{10} (\text{index level}) \times (\text{fruits at this level})}{\text{total no. of fruits}}$$

Three boxes (each containing 60 fruits) from each treatment were examined after cold storage for 4 months at 0 °C plus 6 days of shelf life at 20 °C. Another examination was done after 8 months of cold storage at 0 °C plus 3 days of shelf life at 20 °C.

Gene expression analysis

Peel samples were frozen in liquid N₂ after 4 months at 0 °C plus 3 days of shelf life at 20 °C and stored at -80 °C. Frozen peel samples (three samples per treatment) were ground under liquid N₂ with a mortar and pestle. Total RNA was extracted from skin tissue by the hot borate method according to Wan and Wilkins.³² The RNA concentration and purity were determined at 260 and 260/280 nm respectively with a NanoDrop 2000 UV spectrophotometer (Thermo Scientific, Wilmington, DE, USA). For all samples, equal aliquots of 3 μ g of total RNA were reverse transcribed with SuperScript III (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. Quantitative real-time polymerase chain reaction was performed with the addition of 1 × SYBR green (Applied Biosystems, Foster City, CA, USA) to each sample, which contained about 100 ng of the synthesised cDNA. The data obtained were normalised with respect to expression of the housekeeping apple 18S rRNA.³⁰ Apple (*Malus domestica*) unigenes obtained via the NCBI database (<http://www.ncbi.nlm.nih.gov/Genbank/>) with high homology to gene sequences in other plants were used for the expression analysis. The putative sequence of *PME1* (NCBI Accession CO168183) had 98% identity to *pectin methyl esterase* (NCBI Accession AB067684.1), that of *PME2* (Accession CO415488) had 83% identity to *pectin methyl esterase* (NCBI Accession XP002278061.1), that of *PME3* (Accession CN994362) had 84.2% identity to *pectin methyl esterase* (NCBI Accession XP002317777) and that of *PME4* (Accession CN994197) had 88% identity to *pectin methyl esterase* (NCBI Accession X95991.1). All designed primers were 20 nucleotides long and had melting points of 58 ± 3 °C

Statistics

The gas concentrations produced inside the chambers during the treatments are means of four measurements, with least significant difference (LSD) at the 5% level. Firmness and colour values are means of 18 measurements on nine fruits, while fruit scald and BP index values are means of measurements on three trays each containing 20 fruits. TSS, TA and juice browning values are means of five measurements, each juice sample being a mixture from three fruits. Respiration and ethylene production rates are means of measurements on five individual fruits, with LSD at the 5% level. Volatile and gene expression data are means of three samples from three different fruits. Data were analysed with JMP 5.0 software

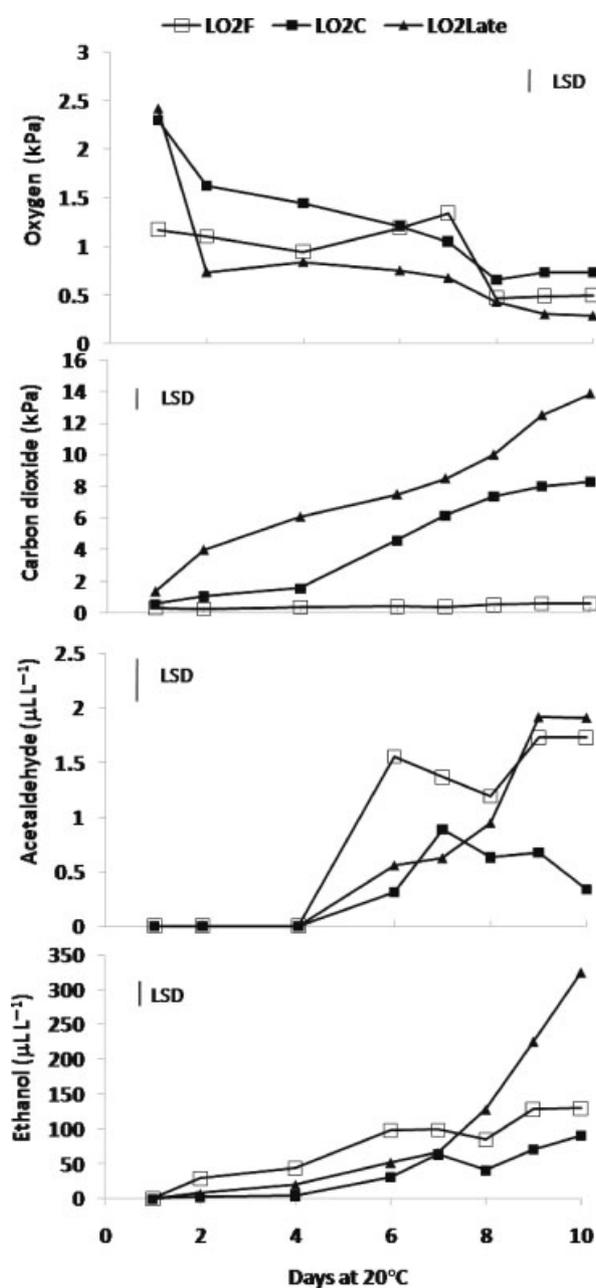


Figure 1. Headspace gas concentrations in various LO₂ pretreatments during 10 days at 20 °C. Headspace concentrations of oxygen, carbon dioxide, acetaldehyde and ethanol in the treatment chambers were checked via latex pipe. Results are means of four measurements in two chambers per treatment. LSD values at 5% confidence level: oxygen, 0.25 kPa; carbon dioxide, 1.28 kPa; acetaldehyde, 0.53 $\mu\text{L L}^{-1}$; ethanol, 20.14 $\mu\text{L L}^{-1}$.

(SAS, Cary, NC, USA) and separated according to Tukey's highest significant difference test at $P < 0.05$. Percentage data were \sin^{-1} transformed prior to statistical analysis.

RESULTS

Gas concentrations during treatments

CO₂, acetaldehyde and ethanol accumulated during treatments, whereas O₂ levels declined. At the end of the 10 days of treatment at 20 °C the O₂ levels in the various chambers ranged from 0.3 to

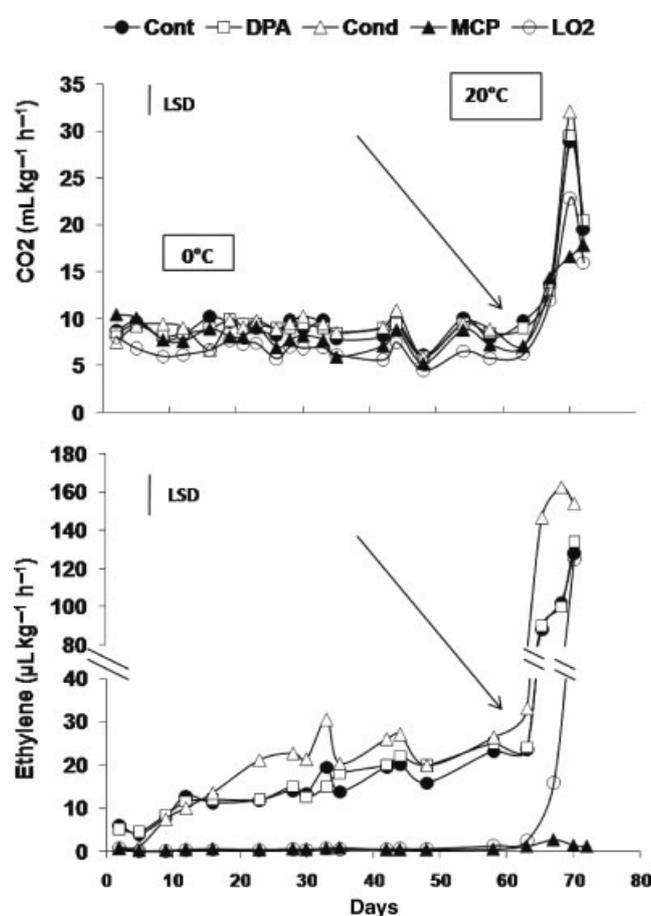


Figure 2. Effects of various pretreatments on ethylene and carbon dioxide production in Granny Smith apples. Measurements began 3 weeks after start of fruit storage at 0 °C, during 2 months of cold storage at 0 °C followed by 1 week at 20 °C. LO₂ data are means of three LO₂ treatments. Results are means of measurements on five individual fruits. LSD values at 5% confidence level: carbon dioxide, 2.93 $\text{mL kg}^{-1} \text{h}^{-1}$; ethylene, 24.42 $\mu\text{L kg}^{-1} \text{h}^{-1}$. Arrow indicates removal to shelf life.

0.7 kPa (Fig. 1). The levels of CO₂ were very low (at 0.5 kPa) only in the flushed system (LO₂F), which was flushed with N₂ during all 10 days; in the closed systems of LO₂C and LO₂late there was gradual accumulation of CO₂ up to 8 and 14 kPa respectively (Fig. 1). However, the levels of acetaldehyde and ethanol in the LO₂F chambers were significantly higher than those in the closed system (LO₂C) (Fig. 1). In the LO₂late treatment the amount of fruit per chamber (60 kg) was 50% greater than in the other treatments, the O₂ level decreased more than in the LO₂F and LO₂C treatments, and higher amounts of CO₂ and ethanol accumulated (Fig. 1).

Respiration and ethylene production

The LO₂ pretreatments greatly reduced ethylene production during 65 days of storage at 0 °C, similarly to the effect of 1-MCP (Fig. 2). However, the moment the fruits were transferred to 20 °C, there was a burst of ethylene in LO₂-treated fruits but not in 1-MCP-treated fruits (Fig. 2). DPA-treated fruits had the same ethylene levels as control fruits. Cond fruits that were held for 10 days at 20 °C prior to cold storage produced higher levels of ethylene during cold storage than Cont and DPA-treated fruits that were placed immediately at 0 °C (Fig. 2).

Table 2. Effects of various pretreatments of Granny Smith apples on: superficial scald and bitter pit indices after 4 months at 0 °C plus 6 days at 20 °C; headspace accumulation of total volatiles and (*E,E*)- α -farnesene (α -Far); α -Far as percentage of total volatiles; and headspace accumulation of 6-methyl-5-hepten-2-one (MHO-on) after 4 months at 0 °C plus 3 days at 20 °C

Treatment	Superficial scald (0–10)	Bitter pit (0–10)	Total volatiles (nL L ⁻¹)	α -Far (nL L ⁻¹)	α -Far (% of total)	MHO-on (nL L ⁻¹)
Cont	4.81a	1.98a	184.7a	145.7a	78.9a	11.50a
DPA	0.00c	0.11d	38.3cd	24.7cd	64.4ab	–
1-MCP	0.00c	0.90c	33.7d	14.1d	41.8d	–
Cond	2.63b	1.52b	63.7bc	38.3c	60.1b	2.12b
LO2F	0.00c	0.02d	53.2bc	37.4c	70.7a	–
LO2C	0.02c	0.17d	99.4b	67.0b	67.4ab	–
LO2late	0.00c	0.04d	40.6c	21.6cd	53.1c	–

Values followed by the same letter within a column do not differ significantly according to Tukey's test ($P < 0.05$, $n = 3$). Superficial scald and bitter pit index values are means of measurements on three trays each containing 20 fruits. Percentage data were transformed to \sin^{-1} prior to statistical analysis.

The CO₂ production pattern was similar for all treatments, although LO2-pretreated fruits produced lower amounts of CO₂ (Fig. 2). Removal to shelf life caused a burst of CO₂ production that was significantly higher in Cont, DPA-treated and Cond fruits compared with LO2- and 1-MCP-treated fruits (Fig. 2). Fruits were removed to shelf life after 65 days of measurements owing to the fact that Cont and Cond fruits already showed extensive BP symptoms.

Superficial scald and bitter pit symptoms

Upon removal from storage at 0 °C after 4 months, control fruits had no visible scald symptoms; however, after 6 days of shelf life at 20 °C, considerable amounts of scald were observed (Table 2). Untreated Cont fruits developed the highest levels of superficial scald (4.81), and Cond fruits also showed a significant amount of scald, but the levels were significantly lower (2.63) than in control fruits (Table 2). In all other treatments (DPA, 1-MCP, LO2F, LO2C and LO2late), no superficial scald developed during shelf life at 20 °C (Table 2).

Incidence of BP was already observed in Cont and Cond fruits after 2 and 3 months of cold storage at 0 °C (data not shown). After 4 months of cold storage plus 6 days of shelf life, BP incidence was mainly in Cont, Cond and 1-MCP-treated fruits, with the severity of BP symptoms being highest in Cont fruits (Table 2). DPA- and LO2-treated fruits showed significantly lower levels of BP than Cont, Cond and 1-MCP-treated fruits. The incidence of superficial scald in Cont fruits after 8 months of cold storage plus 3 days at 20 °C was more severe than that after 4 months plus 6 days at 20 °C (Table 3 vs Table 2) – so great that it masked the BP symptoms. However, in fruits such as 1-MCP-treated ones that did not have superficial scald, the BP incidence was very obvious, and it was greater after 8 months than after 4 months of cold storage (Table 3 vs Table 2). Again, Cont, Cond and 1-MCP-treated fruits showed significantly more BP incidence than DPA- and LO2-treated fruits after 8 months of cold storage, whereas LO2-treated fruits had the least BP incidence, similar to that after 4 months of cold storage (Table 3 vs Table 2).

Acetaldehyde, ethanol and methanol production

After removal from 4 months of cold storage at 0 °C, fruits from the three LO2 pretreatments (LO2F, LO2C and LO2late) produced much higher levels of acetaldehyde, ethanol and methanol than control fruits (Fig. 3). The acetaldehyde levels diminished during shelf life in fruits from all treatments, whereas the ethanol levels fell in LO2-treated fruits but increased slightly in control and 1-MCP-treated fruits. The highest levels of ethanol in the peel tissue were found in LO2F-treated fruits; this was also associated with the best quality fruits that had no visible scald or BP (Fig. 3 vs Table 2). Interestingly, the LO2 pretreatment induced methanol production, which, on the day of removal from cold storage, was higher in LO2-treated fruits than in fruits from all other treatments (Fig. 3). LO2C-treated fruits had significantly higher methanol levels but lower ethanol levels than LO2F- and LO2late-treated fruits (Fig. 3). After 1 week of shelf life, methanol levels increased in fruits from all treatments, except for LO2-treated apples, in which they remained constant or decreased (Fig. 3).

Colour, firmness, TSS, TA and juice browning

Fruits from all three LO2 treatments remained greener (as measured by H°) than Cont and Cond fruits during 14 days of shelf life following 4 months at 0 °C (Fig. 4). 1-MCP-, DPA- and LO2-treated fruits all maintained their green peel colour during

Table 3. Effects of various pretreatments of Granny Smith apples on: superficial scald and bitter pit indices; headspace accumulation of total volatiles and (*E,E*)- α -farnesene (α -Far); α -Far content as percentage of total volatiles; headspace accumulation of 6-methyl-5-hepten-2-one (MHO-on) and 6-methyl-5-hepten-2-ol (MHO-ol); and ratio of MHO-on to MHO-ol. Measurements were done following 8 months at 0 °C plus 3 days at 20 °C

Treatment	Superficial scald (0–10)	Bitter pit (0–10)	Total volatiles (nL L ⁻¹)	α -Far (nL L ⁻¹)	α -Far (% of total)	MHO-on (nL L ⁻¹)	MHO-ol (nL L ⁻¹)	Ratio MHO on/ol
Cont	6.82a	1.45a	29.3d	18.4c	62.8bc	12.52a	1.29c	9.71a
DPA	0.03c	1.06b	91.1a	50.6b	55.5c	1.63b	–	–
1-MCP	0.06c	1.79a	29.2d	21.5c	73.5b	–	–	–
Cond	2.78b	1.67a	79.0b	68.0a	86.1a	11.04a	5.49a	2.01b
LO2F	0.00c	0.23c	59.2c	45.7b	77.1a	1.33b	1.20c	1.11c
LO2C	0.09c	0.66bc	61.3c	49.2b	80.3a	2.43b	2.30b	1.05c
LO2late	0.05c	0.51c	47.3cd	38.7bc	81.8a	1.93b	2.36b	0.82c

Values followed by the same letter within a column do not differ significantly according to Tukey's test ($P < 0.05$, $n = 3$). Superficial scald and bitter pit index values are means of measurements on three trays each containing 20 fruits. Percentage data were transformed to \sin^{-1} prior to statistical analysis.

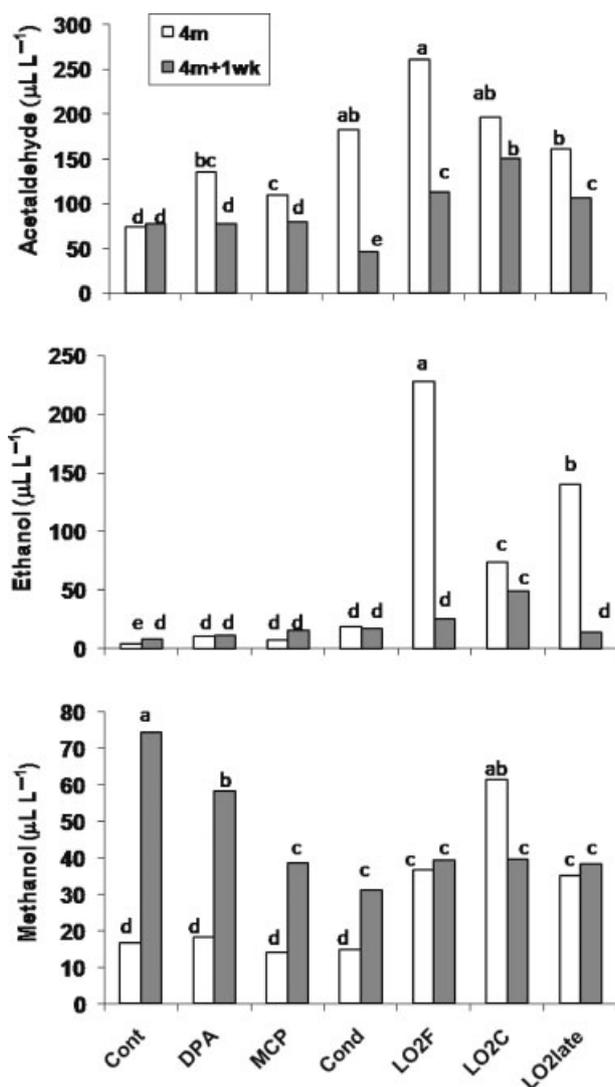


Figure 3. Effects of various pretreatments on headspace concentrations of acetaldehyde, ethanol and methanol produced by Granny Smith apple peel after 4 months in air at 0 °C followed by 1 week at 20 °C. Values marked with the same letter do not differ significantly according to Tukey's test ($P < 0.05$, $n = 3$).

the 14 day shelf life, with 1-MCP-treated fruits being greenest. Cont and Cond fruits suffered gradual degradation of their green colour, with Cont fruits showing the sharpest change from green (108°) to yellow (89°) (Fig. 4).

Fruits from all three LO2 treatments maintained similar but slightly lower fruit firmness than 1-MCP-treated fruits during 4 months at 0 °C followed by 6 or 14 days at 20 °C (Fig. 4). However, DPA-treated fruits had much lower firmness after 4 months at 0 °C followed by 14 days of shelf life; firmness of DPA-treated fruits was similar to that of Cont and Cond fruits (Fig. 4).

Cont apples had the lowest levels of TSS of all treated fruits when removed from cold storage in regular air after 4 months (Fig. 5). During the subsequent 14 days of shelf life at 20 °C there were additional reductions in TSS levels in all treated fruits except 1-MCP-treated ones (Fig. 5): Cont, DPA-treated and Cond fruits had the lowest TSS levels, LO2F and LO2late fruits showed intermediate levels and 1-MCP-treated fruits maintained the highest levels (Fig. 5).

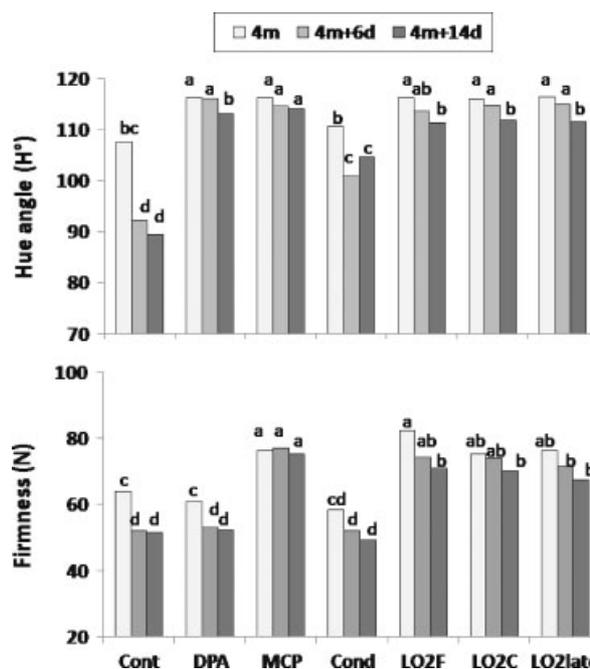


Figure 4. Effects of various pretreatments on fruit colour (expressed as hue angle H°) and firmness in Granny Smith apples after 4 months in air at 0 °C plus 6 or 14 days at 20 °C. Values marked with the same letter do not differ significantly according to Tukey's test ($P < 0.05$, $n = 18$).

Upon removal from cold storage after 4 months, LO2C- and LO2late-treated fruits showed the highest TA of all treatments (Fig. 5). Reductions in TA during shelf life were observed in all treatments; however, after 14 days at 20 °C, 1-MCP-treated fruits maintained the highest levels of TA, while Cont fruits had the lowest levels (Fig. 5). All LO2-treated fruits had higher TA than Cont and DPA-treated fruits after 6 days at 20 °C (Fig. 5).

After 4 months at 0 °C plus 14 days of shelf life at 20 °C the juice extracted from Granny Smith apples was evaluated for browning potential. Juice colour after 2 h of incubation at 20 °C for Cont, DPA-treated and Cond fruits was five times darker than for 1-MCP- and LO2-treated fruits (Fig. 5). There was a negative association between fruit firmness and juice browning: juice from firmer fruits showed less browning, while that from the softest (Cond) fruits showed the greatest browning (Figs 4 and 5).

Expression of PMEs

Putative *MdPME* genes were differentially expressed in response to the various treatments after 4 months at 0 °C plus 3 days at 20 °C. The 1-MCP treatment induced an approximately 17-fold increase in *MdPME1* expression levels and an approximately 20-fold increase in *MdPME3* and *MdPME4* expression levels (Fig. 6). Apple fruits exposed to LO2 in a closed system (treatment LO2C) also showed increased expression levels of *MdPME1*, *MdPME3* and *MdPME4*, by 3.9-, 9.7- and 5.5-fold respectively (Fig. 6). The expression levels of *MdPME4* in 1-MCP- and LO2-treated fruits were significantly higher than those in Cont, DPA-treated and Cond fruits, which may explain their higher firmness (Fig. 6 vs Fig. 4).

α -Farnesene and MHO production

The main volatile found by SPME/GC/MS measurement in the headspace over Granny Smith apple peel tissue after 4 months at 0 °C followed by 3 days at 20 °C was (*E,E*)- α -farnesene, which

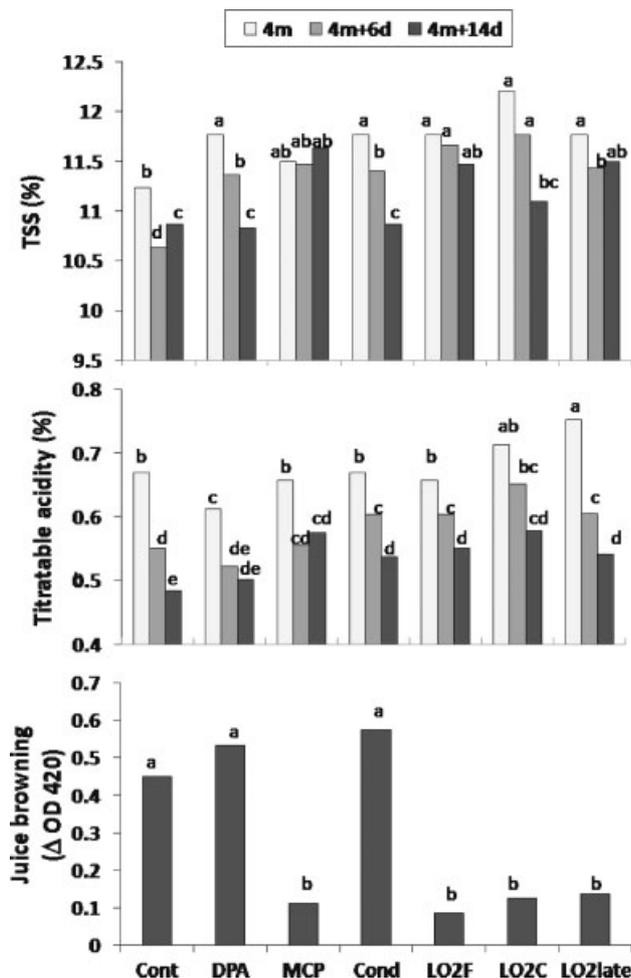


Figure 5. Effects of various pretreatments on total soluble solids (TSS) and titratable acidity (calculated as malic acid equivalents) in Granny Smith apples after 4 months in air at 0 °C plus 6 or 14 days at 20 °C. Effects of various treatments on browning (expressed as ΔOD at 420 nm) of juice extracted from Granny Smith apples after 4 months at 0 °C plus 14 days of shelf life. Values marked with the same letter do not differ significantly according to Tukey's test ($P < 0.05$, $n = 5$).

represented 50% of total volatiles in all treatments except 1-MCP (Table 2). 1-MCP-treated fruits had the lowest total peel volatiles and the lowest percentage represented by (*E,E*)- α -farnesene. The highest outputs of total volatiles and (*E,E*)- α -farnesene were found in Cont fruits that showed extensive scalding (Table 2). However, Cond fruits produced relatively small amounts of (*E,E*)- α -farnesene but nevertheless had visible scald, whereas LO2C-treated fruits produced larger amounts of (*E,E*)- α -farnesene but did not have any scalding (Table 2). The oxidation product of (*E,E*)- α -farnesene, the ketone MHO-on, was found only in the scalded Cont and Cond fruits (Table 2). The molecule MHO-on was five times more abundant in Cont fruits than in Cond fruits (11.5 vs 2.1 nL L⁻¹), which correlates with the higher level of superficial scald in Cont fruits than in Cond fruits after 4 months at 0 °C (Table 2).

After 8 months of cold storage followed by 3 days at 20 °C the incidence of superficial scald was similar in Cond and Cont fruits; however, its severity was lower in the former, with indices of 2.78 and 6.82 respectively (Table 3). In all other treated fruits, scald levels were low, with no scald being found in LO2F-treated fruits (Table 3). After storage at 0 °C for 8 months followed by 3 days at

20 °C the main volatile (accounting for over 50%) emitted from the peel of all treated fruits was still (*E,E*)- α -farnesene (Table 3). Severe superficial scald was associated with higher levels of MHO-on emission, similar to the situation after 4 months cold of storage (Table 3 vs Table 2), although, in addition to MHO-on, there was also accumulation of the alcohol 6-methyl-5-hepten-2-ol (MHO-ol) (Table 3). The ratio between MHO-on and MHO-ol was highest in Cont fruits (9.7), lower in Cond fruits (2.0) and lowest in LO2-treated fruits (~1.0) (Table 3). 1-MCP-treated fruits did not produce any MHO after 8 months in cold storage, while DPA-treated fruits produced only low levels of MHO-on (Table 3).

DISCUSSION

During the 10 days of anaerobic treatment at 20 °C, Granny Smith fruits continued to respire. In the LO2F system this was mainly through anaerobic respiration, whereas in the closed systems (LO2C and LO2late) the fruits consumed all available O₂ and then switched to anaerobic respiration. The reason for higher accumulation of acetaldehyde and ethanol in the LO2F treatment than in the LO2C treatment (Fig. 1) was probably occurrence of some feedback in the closed system, which would reduce the activity of pyruvate decarboxylase and ADH and thereby lead to lower acetaldehyde and ethanol levels. This phenomenon would be similar to the inhibition caused by feedback found in pears under LO2 storage.¹⁹ However, in the LO2C and LO2 treatments there was accumulation of CO₂ during treatment (Fig. 1), and this might have influenced several changes observed in the fruits after 4 months of storage, as manifested in enhanced TA (Fig. 5) and expression of PMEs (Fig. 6).

Superficial scald in apple, which is a result of chilling injury,¹ was controlled on highly susceptible Granny Smith apples by exposing them to LO2 stress for 10 days at 20 °C prior to cold storage in regular air. The LO2 stress was applied in three different ways, all of which were effective in controlling scald and BP. Our results are in agreement with those of Ghahramani and Scott,¹¹ who also showed that increased endogenous ethanol that appeared after O₂ stress at 20 °C reduced scald development in Granny Smith apples after 4 months of storage at 0 °C. In the present study the LO2 treatment was effective in controlling scald as well as BP in Granny Smith apples during 4–8 months of storage at 0 °C (Tables 2 and 3).

Reduced ethylene production during cold storage of LO2-treated fruits resulted in reduced scald development (Fig. 2 vs Table 2), which is consistent with the findings of many other studies that showed that ethylene stimulates superficial scald development.^{6–8,33} The very low ethylene production in LO2-treated fruits during the first 3 months of cold storage at 0 °C is consistent with results obtained with avocados treated with LO2 for 24 h prior to cold storage,³⁴ and with our previous results with Granny Smith apples that were pretreated with LO2 for 7 days at 20 °C.¹² The inhibition by LO2 treatment of ethylene production during subsequent cold storage was similar to the inhibition by 1-MCP that was described in previous reports.^{4,9,35} However, in LO2-treated fruits there was a burst of ethylene production when the fruits were transferred to 20 °C, whereas this did not occur in 1-MCP-treated fruits (Fig. 2). 1-MCP-treated fruits showed extremely low ethylene production after removal to shelf life – behaviour similar to that of transgenic apples with suppressed ethylene biosynthesis.³¹

The reduced ethylene production in LO2-treated fruits is probably the reason for their higher firmness, greener peel colour

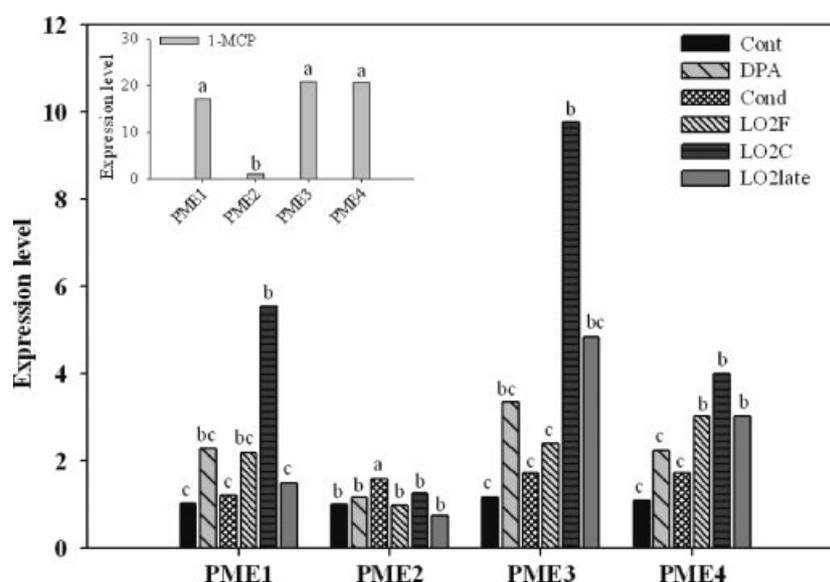


Figure 6. Expression levels of pectin methyl esterase (PME) genes in Granny Smith apples following various pretreatments and storage for 4 months at 0 °C plus 3 days of shelf life at 20 °C. Data for 1-MCP-pretreated fruits are shown in a separate graph in the upper left corner. Mean values for a given gene marked with different letters differ significantly according to Tukey's test ($P < 0.05$, $n = 3$).

and higher TA than those of fruits from the other treatments observed after 4 months in cold storage (Figs 4 and 5). However, during 14 days of shelf life there was a gradual decrease in TA in all treatments, in accordance with the well-known role of malic acid as the primary substrate for respiration and its decrease during apple ripening.³⁶ When Granny Smith apples were removed from 4 months of storage at 0 °C, the peel tissue of LO2-treated fruits generated higher headspace levels of acetaldehyde, ethanol and methanol than that of all other treated fruits (Fig. 3). The high levels of endogenous ethanol that were found in LO2F-treated fruits and the ability of these fruits to metabolise the ethanol during 1 week of shelf life (i.e. reduction from 250 to 25 $\mu\text{L L}^{-1}$) indicate that the protection by ethanol lasts a long time during cold storage and that the fruits can overcome the anaerobic stress. The reductions in acetaldehyde and ethanol contents during shelf life were probably due to metabolism and evaporation of these volatiles at room temperature.³⁷ The present finding that 1-MCP-treated fruits, followed by LO2-treated fruits, had the highest levels of PME expression could also account for the higher fruit firmness observed in these treatments after 4 months of cold storage. This is in accord with the results obtained in apples, whose PME activity decreased during ripening and softening,³⁸ and in pears, in which expression of *PcPME3* and *PcPME4* genes diminished during ripening and softening.³⁹ Moreover, in apple slices, application of calcium lactate induced PME activity and increased fruit firmness during 4 weeks of storage at 4 °C,⁴⁰ and infiltration of PME and calcium chloride into fresh-cut apples increased their firmness.⁴¹ Ralet *et al.*²³ showed that enhanced PME activity would enable available Ca to interact strongly with pectins, thereby increasing cell wall strength and fruit firmness.

The fact that juice extracted from 1-MCP- and LO2-treated fruits after 4 months of storage at 0 °C followed by 14 days at 20 °C was less oxidised than that from other treatments during 2 h of incubation (Fig. 5) is consistent with our previous finding that 1-MCP- and LO2-treated Granny Smith apples had reduced polyphenol oxidase (PPO) activity.¹² Murata *et al.*²⁷ showed that transgenic apples that lacked the PPO enzyme had diminished

browning potential. It was shown that, to reduce apple juice browning, it is important to abolish PPO activity.⁴²

The variation in α -farnesene levels observed in the present study (Tables 2 and 3) is in agreement with previous works that showed variation in (*E,E*)- α -farnesene during cold storage.^{9,33,43} Although the free radicals produced during oxidation of α -farnesene are believed to actually cause the scald damage, these products are highly unstable and are not measured by the GC/MS technique used here. However, the volatile MHO-on is a product of decomposition of a farnesyl oxyradical, which in turn is derived from the hydro- and endo-peroxide oxidation products of α -farnesene.³³ Thus MHO-on should be a good marker of free radical degradation of α -farnesene, and it has previously been shown to be associated with superficial scald symptoms.^{6,44} After 4 months at 0 °C, only Cont and Cond fruits that were already scalded contained MHO-on, while, after 8 months, MHO-on was also found in DPA- and LO2-treated fruits but not in 1-MCP-treated fruits (Table 3). However, in addition to MHO-on we also found the MHO alcohol (MHO-ol), which probably was produced from MHO-on by the enzyme ADH. It was shown in the past that LO2 stress induced ADH in apple fruits.^{17,45} In ripening pears, ADH was shown to utilise several substrates and was not limited to ethanol production.¹⁸ Moreover, Chervin *et al.*¹⁸ showed that expression of ADH included post-transcriptional regulation. The present finding that in all LO2-treated fruits the ratio between MHO-on and MHO-ol was around unity (Table 3) may indicate that the induction of ADH resulted in detoxification of MHO-on by converting it to alcohol, thereby moderating the damage. In Cont fruits the MHO-on to MHO-ol ratio was highest (9.7) and was correlated with the highest degree of scald symptoms, whereas in Cond fruits there were fewer scald symptoms and the ratio was lower (2.01) (Table 3).

The reductions in BP incidence in LO2-treated fruits after 4 and 8 months of cold storage (Tables 2 and 3) are consistent with results obtained with Golden Reinders apples, in which the same LO2 pretreatment reduced BP incidence during the subsequent 4 months of cold storage.²⁶ This reduction in BP could not be explained by changes in mineral content; there were no

changes in mineral content in the peel, and the Ca content in the pulp was lower.²⁶ In the present work, DPA-treated fruits showed significantly lower BP symptoms (Tables 2 and 3), which indicates that DPA has further capabilities besides inhibiting scald development, e.g. reduction of senescence breakdown in Cortland and Law Rome apples.⁴³ Our result is in agreement with Purvis,⁴⁶ who showed that DPA reduced the chilling-induced pitting of green bell peppers. In contrast, it was found that 1-MCP-treated fruits had enhanced incidence of BP, which indicates that ethylene is not a cause of BP. Recently, Kupferman⁴⁷ showed that, in Gala apples, late application of 1-MCP, 14 days after harvest, induced lenticel breakdown, which has similar but not the same symptoms as BP, and it was correlated with lower Ca levels in the peel tissue. Recently, it was suggested that BP development is dependent not only on low Ca concentration but also on abnormal Ca partitioning and distribution in the cell.²² One explanation for reduced BP symptoms in LO2-treated fruits could be that the accumulation of volatile alcohols in the peel tissue leads to this reduction. In pharmacology research, Seeman⁴⁸ suggested that alcohols may fluidise biomembranes by disrupting the packaging of membrane lipids. Subsequent studies indicated that ethanol and other alcohols induced membrane lipid disordering, which in turn resulted in reduction of membrane viscosity.⁴⁹ It is possible that the LO2 stress in Granny Smith apples, which induced production of various alcohols, including ethanol and methanol (Fig. 3), but reduced ethylene production (Fig. 2), was effective in prevention of both superficial scald and BP, whereas 1-MCP, which blocks the effects of ethylene, was effective mainly against superficial scald (Tables 2 and 3).

CONCLUSIONS

In this study we showed that a short LO2 pretreatment at 20 °C reduced superficial scald as well as BP development in Granny Smith apples during up to 8 months in cold storage. The major effect of LO2 treatment was reduction of ethylene production during cold storage, which led in turn to reduced superficial scald development. In addition, LO2 treatment maintained Granny Smith quality with respect to firmness, colour, sugar content and acidity and was also effective in diminishing juice oxidation. MHO-on levels were associated with scald development, and the ratio between MHO-on and MHO-ol might serve as an index of scald severity. All LO2 pretreatments examined were found to be effective against superficial scald and BP, indicating good potential to serve as alternatives to DPA and 1-MCP treatments, especially for organic fruits.

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