Natural preservatives for superficial scald reduction and enhancement of protective phenolic-linked antioxidant responses in apple during post-harvest storage

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Abstract Superficial scald during post-harvest storage is a serious problem for long-term preservation and shelf-life of some apple and pear cultivars. Development of superficial scald and related physiological disorders such as enzymatic and non-enzymatic browning are associated in part with oxidative breakdown and redox imbalance. Therefore, targeting natural antioxidants from food-grade sources as post-harvest treatment to reduce superficial scald has merit. Such natural antioxidants can potentially counter oxidation-linked damages associated with superficial scald through stimulation of antioxidant enzyme responses and biosynthesis of less-oxidized phenolics involving protective redox-linked pathway such as proline-associated pentose phosphate pathway. Based on this rationale, bioprocessed food-grade oregano extract (OX) and soluble chitosan oligosaccharide (COS) were targeted as post-harvest treatment (2 and 4 g L⁻¹) and were compared with diphenylamine (DPA) (1 and 2 g L⁻¹) to reduce superficial scald and to improve protective phenolic-linked antioxidant responses in “Cortland” cultivar stored at 4 °C for 15 weeks. Overall, significant reduction of superficial scald and conjugated triene was observed with DPA and OX (2 g L⁻¹) post-harvest treatments. Furthermore, stimulation of antioxidant enzyme responses such as increases in

superoxide dismutase and guaiacol peroxidase activity was also observed, but was more evident with DPA and COS treatment. Overall, results of this study indicated that critical balance of less-oxidized phenolics and antioxidant enzymes and associated anabolic PPP-linked redox regulation is essential for improving post-harvest preservation and reduction of superficial scald in apple.

Keywords Antioxidants · Chitosan oligosaccharide · Natural preservatives · Phenolics · Redox-balance · Superficial scald

Introduction

Browning of skin after cold storage referred to as superficial scald is a physiological disorder of fruits at post-harvest stage, especially in some cultivars of apple and pear. Therefore managing superficial scald after cold storage is essential to improve overall post-harvest preservation and shelf-life of these fruits. Currently diphenylamine (DPA) or 1-methylcyclopropene (MCP) is used as post-harvest treatments to prevent superficial scald (Du et al. 2017). However, DPA used as post-harvest treatment is one of the major chemical residues detected in highest concentration in apple peel (around 20%) (Iñigo-Núñez et al. 2010). In animals, DPA has been shown to cause increase in organ weights and cause damage to liver, spleen, and kidney (Drzyzga 2003). Therefore finding food-grade and plant-based natural alternatives to improve overall post-harvest preservation quality and to reduce superficial scald of apple has significant relevance.

In general, development of superficial scald in apples involves oxidation of α -farnesene to conjugated triene (CT), which is a product of oxidative breakdown (Rowan

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et al. 2001). Since scald development involves oxidation, endogenous antioxidants present in the cuticle of fruits may also play a critical role in preventing superficial scald and related enzymatic browning. In this context, endogenous antioxidant enzymes and secondary metabolites such as phenolics can prevent oxidative breakdown associated with superficial scald and related enzymatic browning. However, phenolics have been reported to have a dual role in scald development (Treutter 2001). Higher concentration of water soluble phenolics and their subsequent oxidation may contribute to browning due to polymerization by polyphenol oxidase (PPO); whereas presence of less-oxidized phenolics with high antioxidant potentials may prevent superficial scald by directly preventing oxidation of α -farnesene (Lurie and Watkins 2012) and by indirectly stimulating other antioxidant enzyme responses through up-regulation of protective pathways such as pentose phosphate pathway (PPP) (Shetty 1997; Shetty and Wahlqvist 2004).

Therefore, right balance of redox protective phenolics from a reduced oxidative state dependent on PPP and improved antioxidants enzyme response can potentially counter enzymatic browning linked oxidative reactions and development of superficial scald. These protective redox dependent metabolic reactions modulated by key dehydrogenases associated with anabolic metabolism such as PPP can also potentially enhance post-harvest preservation of fruits through critical modulation of this defense related anabolic pathway and would maintain the balance between catabolic and anabolic pathway regulation (Sarkar and Shetty 2014). The need of NADPH from anabolic PPP is critical which supports less oxidative state where glutathione reductase linked antioxidant response is essential and which can be modulated through PPP regulation and associated proline metabolism (Kwon et al. 2006; Sarkar et al. 2011). Therefore redox-linked PPP regulation and its critical modulation and switch under high and less oxidative state has relevance for controlling oxidative breakdown and associated enzymatic browning and superficial scald development in apple during post-harvest storage.

Pentose phosphate pathway (PPP) through generation of NADPH and sugar phosphates could stimulate both the shikimate and phenylpropanoid pathways, and therefore, the modulation of this pathway could lead to the stimulation of protective phenolic biosynthesis and coupled with antioxidant enzyme responses (Shetty 1997; Shetty and Wahlqvist 2004). In general, proline synthesis in the cytosol takes place by the reduction of pyrroline-5-carboxylate (P5C) leading to the accumulation of NADP⁺ (Verbruggen and Hermans 2008) which is a co-factor for the enzyme glucose-6-phosphate dehydrogenase (G6PDH) that catalyzes the rate limiting step of the PPP (Shetty and Wahlqvist 2004). Proline/G6PDH correlations during

phenolic response were also associated with phenolic content, potential polymerization of phenolics by guaiacol peroxidase (GPX) and antioxidant activity based on free radical scavenging activity of phenolics and other antioxidant enzyme like superoxide dismutase (SOD) (Adyanthaya et al. 2009; Sarkar et al. 2011). Therefore activation of proline-associated pentose phosphate pathway (PAPPP) and subsequent stimulation of less-oxidized protective phenolics and antioxidant enzyme responses, especially favoring less oxidative state could potentially counter respiration-linked catabolic oxidative browning and related superficial scald development in fruits during post-harvest storage (Fig. 1).

Based on this rationale, two natural and bio-processed preservatives; water extracted food grade oregano (OX) rich in natural antioxidants such as rosmarinic acid, and water soluble chitosan oligosaccharide (COS) derived from deacylated chitin were rationally selected as post-harvest treatments in apple to counter superficial scald and related browning through up-regulation of anabolic PAPPP mediated redox balance. Protective role of rosmarinic acid as natural antioxidants and its anti-microbial properties were reported previously (Chun et al. 2005; Shetty 2001). Similarly previous studies have reported that chitosan coating delayed changes in contents of anthocyanins, flavonoids, and total phenolics as well as delayed the increase in PPO and peroxidase activity (Muzzarelli et al. 2012). Superficial scald development is linked to critical balance between oxidized and less-oxidized phenolics, endogenous antioxidant enzyme responses, ethylene production as well as ripening. Treatments with natural preservatives such as soluble chitin derivative and rosmarinic acid rich herbal extract have been reported to affect each of those intrinsic parameters (Meng et al. 2008; Synowiec et al. 2014). Therefore, the major aim of this study was to examine the efficacy of a highly soluble COS and rosmarinic acid enriched oregano extract (OX) as potential inhibitors of superficial scald in apple when compared to the more conventional DPA chemical treatment. Furthermore, the role of redox-linked and anabolic PAPPP to improve protective phenolic-linked antioxidant enzyme responses and its relevance to counter superficial scald and enzymatic browning was also evaluated.

Materials and methods

Apple cultivar and harvest

Apple cultivar ‘Cortland’ was selected to investigate the effect of natural preservative COS and OX treatments as potential inhibitors of superficial scald. Selection of cv. ‘Cortland’ was based on its susceptibility against

Fig. 1 Critical role of proline-associated pentose phosphate pathway (PAPPP) for redox regulation through biosynthesis of less oxidized phenolics and antioxidant enzymes under high and low oxidative states and its subsequent protective functions against oxidative breakdown and development of superficial scald during post-harvest storage of apple

development of superficial scald during storage. Harvesting of apple was done at Cold Spring Orchard (Belchertown, MA-01007, USA) of University of Massachusetts (UMASS) on September 19, 2011. Eighty to hundred fruits from one tree were collected in a bushel. Each bushel was considered as a replicate. For seven treatments a total of 42 bushel was harvested.

Materials

Dried food grade and bioprocessed oregano (OX) with minimum of 7% rosmarinic acid were supplied by Barrington Nutritionals (Harrison, NY, USA). Water soluble chitosan oligosaccharide with ascorbic acid as a side chain (COS), a bioprocessed derivative from crab shell, was obtained from Kung Pung Bio (Jeju, South Korea). Unless mentioned, all other enzymes and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Post-harvest treatment

A total of seven post-harvest treatments were selected for this study. The treatments are described as follows a) T1- Control Dipped for 1 min in water, b) T2- DPA1 Dipped for 1 min in DPA @ 1 g L^{-1} , c) T3-DPA2 Dipped for 1 min in DPA @ 2 g L^{-1} , d) T4- OX1 Dipped for 1 min in OX @ 2 g L^{-1} , e) T5-OX2 Dipped for 1 min in OX @ 4 g L^{-1} , f) T6- COS1 Dipped for 1 min in COS-C @ 2 g L^{-1} , g) T7- COS2 Dipped for 1 min in COS-C @ 4 g L^{-1} . After

dipping, the bushels were allowed to dry before being stacked on a pellet and kept in a cold storage (4°C). Samples (about 15 fruit) were taken out at 0, 1, 2, 3 (Sept., Oct., Nov., Dec., Jan.) months for biochemical analysis.

Evaluation of superficial scald

Scald evaluations was carried out with a leading expert in this field at University of Massachusetts Amherst. After taking the samples for analysis at 3 months of storage the remaining apples were stored in the cold room for another 3 weeks. Bushels were taken out and stored at room temperature for allowing superficial scald to develop. After about 1 week of storage at room temperature when it was determined that about 50% of the control group had scald development, a comprehensive scald evaluation was undertaken. Every apple was evaluated for the developed scald and it was further classified as 'low', 'moderate' and 'severe' depending on the degree and/or the area of the scald on the apple surface.

Conjugated triene (CT) in apple peel

Method for determination of conjugated trienes (CT) was adapted from Rowan et al. (2001). Briefly, heptane 20 mL was added to glass evaporating dish (90 mm inner diameter $\times$ 50 mm deep) and 6 apples from each treatment were placed separately in the dish such that a part of the apple was immersed in heptane for 20 min. Apples were

removed and the area immersed in heptane was calculated by measuring the diameter of the area exposed to heptane. The volume of the heptane remaining in the dish was measured. CT content was determined by measuring absorbance of the heptane recovered at $A_{281}-A_{292}$ with $\epsilon_{281}-\epsilon_{292} = 25,000$ using a UV–VIS Genesys spectrophotometer (Milton Roy, Inc., Rochester, NY). The results of CT were expressed as nanomoles per square meter.

Extraction of enzyme and other biochemical assays

For enzyme assays, 2.5 g of apple peel (6 apples was used as replicate for each treatment) was cut into small pieces and added to 5 mL of cold enzyme extraction buffer (0.5% polyvinylpyrrolidone (PVP), 3 mM EDTA, 0.1 M potassium phosphate buffer of pH 7.5). Tissue tearor (Biospec Products, Bartleville, OK, USA) was used to homogenize and blend the peel with the buffer. The sample was then centrifuged at $12,000\times g$ for 30 min at $2-5^{\circ}\text{C}$ and stored on ice. The collected supernatant was used for further analysis.

For total soluble phenolic content and total antioxidant activity assay, 100 mg of apple peel tissue (from same number of apples-6 from each treatment) was immersed in 2.5 mL of 95% ethanol and kept in the freezer for 48–72 h. Samples were then homogenized using a tissue tearor (Biospec Products, Bartleville, OK, USA) and centrifuged at $12,000\times g$ for 20 min. Supernatant was used for further analysis.

Malondialdehyde (MDA) assay

Method for measurement of MDA was adapted from Tamagnone et al. (1998). A volume of 200 μL of the apple peel extract was combined with 800 μL of water, 500 μL of 20% (w/v) trichloroacetic acid and 1 mL of 10 mM thiobarbutyric acid. The test tubes were incubated for 30 min at 100°C and then centrifuged at $12,000\times g$ for 10 min. The absorbance of the supernatant was then measured at 532, 600 and 440 nm and the concentration of MDA was calculated from its molar extinction coefficient (ϵ) 15.6 mmol m^{-1} .

Polyphenol oxidase (PPO) assay

Method for estimation of PPO activity was adapted from Dawson and Magee (1955). Briefly, 2.6 mL of 50 mM potassium phosphate buffer, pH 6.5, was taken in a cuvette. To that 100 μL of L-DOPA (5 mM), 100 μL ascorbic acid (2.1 mM), and 100 μL of EDTA (0.065 mM) was added and mixed. To this 100 μL of the apple peel extract was added and the change in absorbance was monitored at $A_{265\text{nm}}$ until constant. Delta (δ) $A_{265\text{ nm/min}}$ was

calculated using the maximum linear rate for both the test and the blank. Enzyme activity was calculated by using the definition of one unit of PPO which is the change in $A_{265\text{nm}}$ of 0.001 per min at pH 6.5 in a 3 mL reaction mixture containing L-DOPA and L-ascorbic acid.

Total soluble phenolic content

The total phenolic content in apple peel was analyzed by the Folin-Ciocalteu method (Shetty et al. 1995). One milliliter of supernatant was transferred into a test tube and mixed with 1 mL of 95% ethanol and 5 mL of distilled water. To each sample 0.5 mL of 50% (v/v) Folin–Ciocalteu reagent was added and mixed. After 5 min, 1 mL of 5% Na_2CO_3 was added to the reaction mixture and allowed to stand for 60 min. The absorbance was read at 725 nm. The absorbance values were expressed in gram equivalents of gallic acid per kilogram fresh weight (FW) of the sample. Standard curves were established using various concentrations of gallic acid in 95% ethanol.

Total antioxidant activity

The total antioxidant activity was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay modified from Kwon et al. (2006). A 250 μL of the sample extract was mixed with 1250 μL of DPPH (60 μM in ethanol). Absorbance was measured at 517 nm using the Genesys UV/Visible spectrophotometer. The percentage inhibition was calculated by:

$$\% \text{ inhibition} = \frac{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{extract}})}{\text{Absorbance}_{\text{control}}} \times 100$$

Total protein assay

Protein content was measured by the method described by Bradford (1976). The protein dye reagent concentrate (Bio-Rad protein assay kit II, Bio-Rad Laboratory, Hercules, CA) was diluted 1:4 with distilled water. A volume of 5 mL of diluted dye reagent was added to 50 μL of the extract. After vortexing and incubating for 3 min, the absorbance was measured at 595 nm against a 5 mL reagent blank and 50 μL buffer solutions using a UV–VIS Genesys spectrophotometer (Milton Roy, Inc., Rochester, NY).

Glucose-6-phosphate dehydrogenase (G6PDH) assay

A modified version of the assay described by Deutsch (1983) was followed. The enzyme reaction mixture containing 5.88 μmol β -NADP, 88.5 μmol MgCl_2 , 53.7 μmol

glucose-6-phosphate, and 77 μmol maleimide was prepared. This mixture was used to obtain baseline (zero) of the spectrophotometer reading at 340 nm wavelength. To 1 mL of this mixture, 100 μL of the extraction sample was added. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture using the extinction co-efficient of NADPH (622 mM m^{-1}).

Succinate dehydrogenase (SDH) assay

Modified method described by Bregman (1987) was used to measure activity of succinate dehydrogenase. The assay mixture consisted of the following: 1.01 mL of 0.4 M potassium phosphate buffer (pH 7.2); 40 μL of 0.15 M sodium succinate (pH 7.0); 40 μL of 0.2 M sodium azide; and 10 μL of 6.0 g L^{-1} DCPIP. This reaction mixture was then used to obtain baseline (zero) of the spectrophotometer reading at 600 nm wavelength. To 1.0 mL of this mixture, 200 μL of the sample was added. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture using the extinction co-efficient of DCPIP (1.91 M m^{-1}).

Total proline content

High performance liquid chromatography (HPLC) analysis was performed to measure total proline content using an Agilent 1100 series liquid chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a diode array detector (DAD 1100). The analytical column was reverse phase Nucleosil C18, 250 mm $\times$ 4.6 mm with a packing material of 5 μm particle size. The extract samples from enzyme extraction method were eluted out in an isocratic manner with a mobile phase consisting of 20 mM potassium phosphate (pH 2.5 by phosphoric acid) at a flow rate of 1 mL min^{-1} and detected at 210 nm. L-Proline dissolved in the 20 mM potassium phosphate solution was used to calibrate the standard curve. The amount of proline in the sample was calculated as mg of proline per milliliter and converted and reported as g kg^{-1} FW.

Proline dehydrogenase (PDH) assay

A modified method described by Costilow and Cooper (1978) was carried out to assay the activity of proline dehydrogenase. The enzyme reaction mixture containing 100 mM sodium carbonate buffer (pH 10.3), 20 mM L-proline solution and 10 mM NAD was used. To 1 mL of this reaction mixture, 200 μL of extracted enzyme sample was added. The increase in absorbance was measured at 340 nm for 3 min, at 32 °C. The absorbance was recorded at zero time and then after 3 min. In this spectrophotometric assay, one unit of enzyme activity is equal to the

amount causing an increase in absorbance of 0.01 unit per min at 340 nm (1.0 cm light path).

Superoxide dismutase (SOD) assay

A competitive inhibition assay was performed that used xanthine–xanthine oxidase-generated superoxide to reduce nitroblue tetrazolium (NBT) to blue formazan. Spectrophotometric assay of SOD activity was carried out by monitoring the reduction of NBT at 560 nm (Oberley and Spitz 1985). The reaction mixture contained 13.8 mL of 50 mM potassium phosphate buffer (pH 7.8) containing 1.33 mM DETEPAC; 0.5 mL of 2.45 mM NBT; 1.7 mL of 1.8 mM xanthine and 40,000 IU L^{-1} catalase. To 0.8 mL of reagent mixture 100 μL of phosphate buffer and 100 μL of xanthine oxidase was added. The change in absorbance at 560 nm was measured every 20 s for 2 min and the concentration of Xanthine oxidase was adjusted to obtain a linear curve with a slope of 0.025 absorbance per min. The phosphate buffer was then replaced by the enzyme sample and the change in absorbance was monitored every 20 s for 2 min. One unit of SOD was defined as the amount of protein that inhibits NBT reduction to 50% of the maximum.

Catalase (CAT) assay

A method originally described by Beers and Sizer (1952), was used to assay the activity of catalase. To 1.9 mL of distilled water 1 mL of 0.059 M hydrogen peroxide (Merck's Superoxol or equivalent grade) in 0.05 M potassium phosphate, pH 7.0 was added. This mixture was incubated in a spectrophotometer for 4–5 min to achieve temperature equilibration and to establish blank rate. To this mixture 0.1 mL of diluted enzyme sample was added and the disappearance of peroxide was followed using a spectrophotometer by recording the decrease in absorbance at 240 nm for 2–3 min. The change in absorbance $\Delta A_{240}/\text{min}$ from the initial (45 s) linear portion of the curve was calculated. One unit of catalase activity was defined as amount that decomposes one micromole of H_2O_2

$$\text{Units /mg} = \frac{(\Delta A_{240}/\text{min}) \times 1000}{43.6 \times \text{mg enzyme/mL of reaction mixture}}$$

Guaiacol peroxidase (GPX) assay

Modified version of assay developed by Laloue et al. (1997), was used. Briefly, the enzyme reaction mixture contained 0.1 M potassium phosphate buffer (pH 6.8), 56 mM guaiacol solution and 0.2 mM hydrogen peroxide. To 1 mL of this reaction mixture, 50 μL of enzyme sample

was added. The absorbance was noted at zero time and then after 5 min. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture using the extinction co-efficient of the oxidized product tetraguaiacol (2.66 M m^{-1}).

Statistical analysis

All experiments were performed with six replications. The effect of each treatment was determined by the analysis of variance (ANOVA) of SAS (version 8.2; SAS Institute, Cary, NC). Differences among different treatments were determined by the Fishers least significant difference (LSD) test at the 0.05 probability level. Standard error was calculated using Microsoft Excel 2010.

Results and discussion

Superficial scald, conjugated triene (CT), malondialdehyde (MDA), and PPO activity of “Cortland” apple

The major aim of this study was to reduce superficial scald development in apple through modulation of redox-linked PAPPP and its associated stimulation of less oxidized phenolics and antioxidant enzyme responses during post-harvest storage using natural food-grade preservatives (COS and OX). Results of superficial scald were expressed as percentage (%) and apple with scald was further grouped as ‘low’, ‘moderate’ and ‘severe’ based on the scald intensity (Table 1). Overall, both DPA treatments had lowest ($P < 0.05$) superficial scald development followed by OX (@ 2 g L^{-1}) which were significantly lower when compared to the control, OX (@ 4 g L^{-1}), and COS (@ 2 and 4 g L^{-1}) post-harvest treatments. Further, DPA and OX (@ 2 g L^{-1}) also had reduced ‘moderate’ and ‘severe’ scald development as compared ($P < 0.05$) to the control, OX (@ 4 g L^{-1}), and COS post-harvest treatment. Alpha-Farnesene is oxidized to conjugated triene (CT), an oxidation product which has been reported to be the primary cause of scald development in apple (Rowan et al. 2001). Lower production of α -farnesene leads to lower accumulation of its oxidation products consequently lowering the scald susceptibility of apples (Lurie and Watkins 2012). Du and Bramlage (1993) further hypothesized that the ratio of CT258:CT281, which reflect the different oxidation production of α -farnesene is more important. Overall, CT of “Cortland” apple peel increased in this study with post-harvest storage as highest CT was observed after 3 months storage (Table 1). After 3 months storage significantly ($P < 0.05$) lower CT was observed in apple treated with DPA (@ 1 and 2 g L^{-1}) followed by OX (@ 2 g L^{-1}). The

results of CT after 3 month storage correlated with superficial scald result, as lowest scald development was also observed in DPA and OX (@ 2 g L^{-1}) treated apple. Therefore low CT content and lower number of scald in OX (@ 2 g L^{-1}) has significant relevance and can be further targeted as a safe and natural post-harvest treatment to reduce superficial scald in apple.

The malondialdehyde (MDA) content reflects breakdown of membrane due to likely effects of reactive oxygen species (ROS) and was therefore evaluated to indicate the protective effect of post-harvest treatments on apple peel and its cellular breakdown. Immediately after harvest (0 month), significantly ($P < 0.05$) high MDA content was observed in apple treated with OX and COS, while no statistically significant differences in MDA content were observed after 3 months of storage (Table 1). However, after 3 months of storage lowest MDA content was observed in apple treated with OX (@ 2 g L^{-1}) followed by DPA. This result suggested that lower MDA content in OX (@ 2 g L^{-1}) and DPA treated apple may had relevance for having lower CT content and reduction of superficial scald in same post-harvest treatments. Polyphenol oxidase (PPO) activity was determined which has relevance in linking enzymatic browning, phenolic content, and development of superficial scald in apple. Interestingly, no statistically significant difference was observed in PPO activity between post-harvest treatments after 3 months of storage (Table 1). This result suggested that PPO activity may be more relevant during or just prior to developing scald when apples are stored at room temperature and depending on the extent of tissue breakdown and availability of phenolics for polymerization.

Total soluble phenolic content and total antioxidant activity

Total soluble phenolic content in apple peel was assayed using the Folin–Ciocalteu method. Total soluble phenolic content of apple peel was ranged from 2.4 to 5.5 g kg^{-1} FW at 0 month of storage (Fig. 2a). No statistically significant differences in total soluble phenolic content were observed at 0 and after 1 month of storage. After 2 months of storage, COS (@ 2 and 4 g L^{-1}) and OX (@ 4 g L^{-1}) had significantly higher ($P < 0.05$) total soluble phenolic content. Similarly, after 3 months of storage control had the lowest total soluble phenolic content, whereas OX (@ 4 g L^{-1}) and COS (@ 2 g L^{-1}) post-harvest treatment had the highest phenolics, which also resulted in higher scald development. An increase in the key enzyme, phenyl ammonia lyase (PAL) of the phenolic pathway with chitosan treatments was previously reported (Zhang et al. 2011). However this result indicated that higher soluble phenolic content after cold storage may lead to higher scald

Table 1 Effect of postharvest treatments (control, DPA, OX, and COS) on superficial scald (low, moderate, severe) development, on polyphenol oxidase (PPO) activity, conjugated triene (CT), and malondialdehyde (MDA) content of “Cortland” apple peel during and after 3 months of storage

Treatment	Superficial scald development (%) after 3 months storage			PPO activity(1000 units L ⁻¹) after 3 months storage	CT (nmol m ⁻²) from 0 to 3 months of storage				MDA (nmol L ⁻¹) content from 0 to 3 months of storage			
	Low	Moderate	Severe		0	1	2	3	0	1	2	3
Control	24.5 ± 1.35	14.6 ± 0.7	15.3 ± 1.22	682 ± 20.0	0.22 ± 0.006	0.48 ± 0.145	1.26 ± 0.11	1.32 ± 0.29	0.5 ± 0.05	0.49 ± 0.03	0.58 ± 0.06	0.53 ± 0.08
DPA1	3.3 ± 0.5	1.2 ± 0.15	1.33 ± 0.41	696 ± 26.0	0.2 ± 0.01	0.18 ± 0.01	0.32 ± 0.05	0.39 ± 0.026	0.38 ± 0.08	0.46 ± 0.04	0.43 ± 0.08	0.47 ± 0.07
DPA2	4.2 ± 0.33	0.5 ± 0.14	0.3 ± 0.12	752 ± 32.1	0.18 ± 0.09	0.19 ± 0.02	0.26 ± 0.027	0.39 ± 0.05	0.49 ± 0.05	0.48 ± 0.04	0.6 ± 0.06	0.49 ± 0.08
OX1	17.5 ± 1.6	8.0 ± 0.58	6.5 ± 1.57	762 ± 33.4	0.27 ± 0.07	0.28 ± 0.04	0.98 ± 0.22	0.94 ± 0.18	0.78 ± 0.11	0.53 ± 0.08	0.53 ± 0.03	0.44 ± 0.07
OX2	18.2 ± 0.76	17.8 ± 0.81	19.3 ± 2.0	846 ± 61.2	0.15 ± 0.013	0.38 ± 0.03	1.16 ± 0.15	1.89 ± 0.21	0.6 ± 0.08	0.37 ± 0.09	0.41 ± 0.04	0.55 ± 0.09
COS1	21.8 ± 1.2	17.2 ± 0.9	10.4 ± 0.71	832 ± 56.0	0.19 ± 0.012	0.35 ± 0.049	1.29 ± 0.025	1.31 ± 0.26	0.74 ± 0.04	0.39 ± 0.03	0.57 ± 0.06	0.53 ± 0.08
COS2	19.4 ± 0.98	15.7 ± 0.99	13.8 ± 1.72	720 ± 60.2	0.16 ± 0.018	0.84 ± 0.025	1.2 ± 0.01	1.82 ± 0.24	0.83 ± 0.07	0.63 ± 0.05	0.56 ± 0.08	0.56 ± 0.07

± SE standard error

Fig. 2 Effect of postharvest treatments (control, DPA, OX, and COS) on total soluble phenolic content (g Kg^{-1} FW) (a) and total antioxidant activity (DPPH % inhibition) (b) of “Cortland” apple peel during and after 3 months of storage. Bars represent standard error ($\pm SE$) between replications

development. Therefore more than total phenolic content, composition of phenolic profiles (critical balance between oxidized and less-oxidized phenolics) may have relevance in determining scald development and related enzymatic browning in apple.

Total antioxidant activity of apple peel based on DPPH free radical scavenging assay did not vary significantly between post-harvest treatments ($P < 0.05$) (Fig. 2b). Similar to the total soluble phenolic content, high antioxidant activity was also observed in DPA (@ 1 and 2 g L^{-1}) and OX (@ 2 g L^{-1}) treated apple at 0 month. Interestingly, same post-harvest treatments also resulted in reduced superficial scald development after 3 months of storage. Therefore this result indicated that initial antioxidant activity (immediately after harvest) may have protective role against development of superficial scald during storage. However further study with different apple cultivars and different doses of these post-harvest treatments are required to prove this concept. Other factors such as type of antioxidants; lipophilic or hydrophilic, the stage at which they are overexpressed and the time of storage may also have relevance.

Glucose-6-phosphate dehydrogenase (G6PDH) and succinate dehydrogenase (SDH) activity in apple peel

The oxidative phase of the pentose phosphate pathway generates NADPH by converting glucose-6-phosphate to ribose-5-phosphate as G6PDH catalyzing the first rate limiting step (Puskas et al. 2000). At 0 month, control had significantly ($P < 0.05$) higher G6PDH activity, however it reduced significantly after 1 month of storage (Fig. 3a). Post-harvest treatments with COS, OX, and DPA resulted in higher activity of this PPP associated enzyme after 1 month when compared to the control. This increase in G6PDH activity should activate the PPP to drive the carbon flux towards erythrose-4-phosphate for biosynthesis of shikimate and phenylpropanoid metabolites (Shetty and Wahlqvist 2004) and perhaps other intermediates. In this study, we observed that COS treatment had higher stimulation of the PPP than DPA treatments after 1 month of storage and resulted in higher total soluble phenolic content in later stages of post-harvest storage (2 and 3 months). After 2 and 3 months of storage no statistically significant

differences in G6PDH activity between treatments were observed.

In order to investigate the effect of the post-harvest treatments in modulating respiration-linked energy metabolism using the Tricarboxylic Acid Cycle (TCA), the activity of SDH a key enzyme of TCA cycle was determined (Selak et al. 2005). At 0 month, highest SDH activity was observed with DPA (@ 2 g L^{-1}) post-harvest treatment ($P < 0.05$) (Fig. 3b). However after 1 month storage, DPA treatments reduced SDH activity, while it slightly increased with COS post-harvest treatments. After 3 months of storage, COS had significantly higher ($P < 0.05$) SDH activity than DPA, OX, and control. This suggests that COS treated apples had a higher need for cellular energy and this was achieved by stimulating the TCA cycle. High SDH activity indicates a higher carbon flux through glycolytic pathways providing the required phosphoenolpyruvate needed for phenolic synthesis through shikimate pathway.

Total proline content and proline dehydrogenase (PDH) activity

Initial baseline value of proline content at 0 month was not significantly different between post-harvest treatments (Fig. 4A). Total proline content was in the range of $7.5\text{--}9.4 \text{ g kg}^{-1}$ of apple peel at 0 month. Overall, proline content decreased after 1 month of storage and no significant changes were observed between treatments at 1 and 2 months of storage. Adyanthaya et al. (2009) reported similar decrease in proline content in apple for 2 and 3 month storage period after an initial increase at 1 month. However, after 3 months of storage, OX (@ 2 g L^{-1}) had significantly higher ($P < 0.05$) proline content when compared to other post-harvest treatments and control.

Proline dehydrogenase (PDH) can mediate proline oxidation as a potential alternate energy source via oxidative phosphorylation in the mitochondria (Shetty and Wahlqvist 2004). Based on this rationale the PDH activity along with proline content was evaluated (Fig. 4b). Similar to total proline content, PDH activity was also decreased during storage of apple, as highest average PDH activity was observed at 0 month. After 1 month of storage, PDH activity for DPA, OX and control treatments decreased, while it remained the same for COS treatment. At same time point, PDH activity of COS treated apple was significantly higher ($P < 0.05$) than DPA, OX, and control treatments. After 2 months of storage, PDH activity decreased for all treatments and it also remained the same after 3 months of storage. Overall decrease in proline content and PDH activity over the 3 months period of storage were observed in this study, however no clear

Fig. 3 Effect of postharvest treatments (control, DPA, OX, and COS) on glucose-6-phosphate dehydrogenase (G6PDH) activity (mmol kg^{-1} protein) (a), and succinate dehydrogenase (SDH) activity (mmol kg^{-1} protein) (b) of “Cortland” apple peel during and after 3 months of storage. Bars represent standard error ($\pm SE$) between replications

relationship between proline metabolism and superficial scald development in apple was found.

Superoxide dismutase (SOD), catalase (CAT), and guaiacol peroxidase (GPX) activity

To investigate the effects of post-harvest treatments on inducing antioxidant enzyme responses, the activity of three key antioxidant enzymes; SOD, CAT and GPX were evaluated. At 0 time highest SOD activity was observed in DPA treated apple when compared to control, OX, and COS treatments (Table 2). This could be due to higher SDH activity in these same treatments producing ROS and it is possible that this high amount of ROS may have triggered a higher SOD response. However, this difference was not statistically significant ($P < 0.05$) at 0 month. After 1 month of storage, significant difference ($P < 0.05$) in SOD activity was observed between post-harvest treatments as control had the lowest activity while COS (@ 2 and 4 g L^{-1}) and OX (@ 4 g L^{-1}) had significantly higher SOD activity. After 2 and 3 months of storage all post-harvest treatments resulted in higher SOD activity than control, however no statistically significant differences were observed.

Similarly, no significant differences ($P < 0.05$) in CAT activity of apples were observed between post-harvest treatments during the post-harvest storage period of the study. Overall, CAT activity of apple peel remained same during post-harvest storage, however slightly higher CAT activity was observed with COS post-harvest treatment (Table 2). After 3 months of storage, DPA (@ 1 g L^{-1}) post-harvest treatment resulted in highest CAT activity. The result of CAT activity indicated that although scald development may be related to oxidative stress, however hydrogen peroxide may not be involved in the progression of this physiological disorder. It is also possible that other antioxidant enzymes such as glutathione peroxidase or ascorbate peroxidase may be involved. Contradictory results have been reported in the literature on the effect of antioxidant enzymes on scald susceptibility. It has been reported that hydrogen peroxide concentration increased during storage (Zubini et al. 2007) and H_2O_2 concentration was higher in scald susceptible cultivars (Rao et al. 1998). Du and Bramlage (1995) reported no correlation between ROS, H_2O_2 , and scald susceptibility. They also reported

higher CAT and peroxidase (POX) activity in scald resistant ‘Empire’ as compared to scald susceptible ‘Cortland’ and ‘Delicious’. Overall relation of scald susceptibility to antioxidant enzyme has been inconclusive. However, a general theory of free radical induced α -farnesene oxidation and scald development and the role of antioxidant enzyme in lowering free radicals may explain the biochemical rationale.

Guaiacol peroxidase (GPX), another important antioxidant enzyme is an isoenzyme of peroxidases that cross-links the phenolic moieties from the phenylpropanoid pathway for the biosynthesis of lignins and lignans (Morales and Ros Barceló 1997). At 0 month and after 1 month of storage COS post-harvest treatment had significantly high GPX activity ($P < 0.05$) when compared to DPA, OX, and control treatments (Table 2). After 2 months of storage, no significant differences were observed in GPX activity between DPA, OX, and COS treatments. However, a significant difference was observed between COS and control treatment ($P < 0.05$). After 3 months storage, higher GPX activity was observed with COS (@ 4 g L^{-1}) and DPA (@ 2 g L^{-1}) post-harvest treatments, however it was not statistically significant. An increase in GPX should inversely correlate to scald development since it may make individual phenolic moieties unavailable for polymerization by PPO. However in this study higher GPX activity was observed with COS post-harvest treatment and it did not result in scald reduction. At this point the correlation between GPX activity and scald development is not clear and warrants further investigation with more cultivars and different doses of post-harvest treatments with natural preservatives such as OX and COS.

Conclusion

The aim of this investigation was to understand how stimulation of less oxidized inducible phenolics and antioxidants enzymes by driving redox-linked PAPPP may have positive preventive role against superficial scald development in apple. Understanding this link based on metabolically driven and redox-linked PAPPP rationale will help to identify key bio-markers involved in the process of scalding which will subsequently help to select right cultivars with better scald prevention based on the activity of these markers. Although OX in lower doses reduced superficial scald in “Cortland” apple, however its relationship with PAPPP mediated protective functions involving phenolic biosynthesis and antioxidant enzyme responses was not clear in this study. On the contrary COS post-harvest treatments increased the activity of antioxidant enzyme responses and phenolic biosynthesis as compared to DPA treatment, however it did not translate into

◀**Fig. 4** Effect of postharvest treatments (control, DPA, OX, and COS) on proline content (g kg^{-1}) (**a**), and proline dehydrogenase (PDH) activity (10^6 units kg^{-1} protein) (**b**) of “Cortland” apple peel during and after 3 months of storage. Bars represent standard error ($\pm SE$) between replications

reduction of superficial scald in “Cortland” cultivar. Therefore, further studies with different treatments combinations of OX and DPA in different doses and with other apple cultivars are required for alternative use of food-grade OX as post-harvest treatments to counter superficial scald in apple.

Table 2 Changes in antioxidant enzyme responses (superoxide dismutase-SOD, catalase-CAT, and guaiacol peroxidase-GPX activity) of Cortland apple with 7 different treatments (control, DPA 1, DPA 2, OX1, OX2, COS 1, COS 2) during post-harvest storage (0, 1, 2, 3 months)

Storage period	Treatments	SOD (10^6 units kg^{-1} protein)	CAT (10^6 units kg^{-1} protein)	GPX (mmol kg^{-1} protein)
0 Month	Control	8.9 ± 0.84	21.9 ± 3.9	77.6 ± 12.0
	DPA1	13.4 ± 0.97	19.9 ± 4.5	53.7 ± 8.5
	DPA2	12.3 ± 0.42	15.9 ± 5.4	58.5 ± 10.3
	OX1	9.0 ± 0.54	20.9 ± 1.8	62.4 ± 8.4
	OX2	9.3 ± 0.91	17.2 ± 3.4	48.5 ± 9.8
	COS1	10.0 ± 0.55	33.5 ± 1.4	89.7 ± 12.1
	COS2	9.2 ± 0.34	24.0 ± 3.2	104.3 ± 9.2
1 Month	Control	4.5 ± 0.42	12.2 ± 4.9	36.3 ± 2.6
	DPA1	6.6 ± 0.75	19.1 ± 5.0	55.8 ± 7.8
	DPA2	5.4 ± 0.88	15.6 ± 5.0	59.5 ± 10.1
	OX1	6.8 ± 0.72	14.8 ± 2.3	77.2 ± 13.4
	OX2	7.3 ± 0.74	33.2 ± 5.6	85.2 ± 15.0
	COS1	8.0 ± 0.23	21.8 ± 6.9	91.2 ± 14.0
	COS2	7.8 ± 1.27	28.3 ± 6.8	108.3 ± 13.3
2 Month	Control	4.3 ± 0.54	24.1 ± 5.7	28.2 ± 4.0
	DPA1	3.8 ± 0.30	27.7 ± 4.6	40.9 ± 5.1
	DPA2	4.7 ± 0.53	21.6 ± 3.8	45.3 ± 8.2
	OX1	5.1 ± 0.64	28.5 ± 1.7	43.1 ± 7.6
	OX2	5.2 ± 0.84	30.7 ± 2.4	57.9 ± 9.4
	COS1	4.7 ± 0.16	34.4 ± 2.8	55.5 ± 8.1
	COS2	4.4 ± 0.56	28.1 ± 3.9	59.5 ± 9.0
3 Month	Control	4.8 ± 0.69	27.2 ± 3.9	54.6 ± 13.4
	DPA1	6.3 ± 0.44	43.4 ± 4.5	67.7 ± 12.0
	DPA2	6.5 ± 0.47	26.1 ± 3.6	83.5 ± 10.9
	OX1	5.7 ± 0.52	16.8 ± 2.4	71.8 ± 9.2
	OX2	5.8 ± 0.78	20.5 ± 2.8	60.2 ± 11.0
	COS1	5.9 ± 0.62	36.8 ± 6.5	61.0 ± 6.6
	COS2	6.2 ± 0.54	32.9 ± 4.2	85.9 ± 13.1

$\pm SE$ standard error

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