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1-Methylcyclopropene Interactions with Diphenylamine on Diphenylamine Degradation, α-Farnesene and Conjugated Trienol Concentrations, and Polyphenol Oxidase and Peroxidase Activities in Apple Fruit

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1-Methylcyclopropene (1-MCP) is a new technology that is applied commercially to inhibit ethylene action in apple fruit, but its interactions with existing technologies such as diphenylamine (DPA) for control of superficial scald development in fruit during and after storage is unknown. To investigate possible interactions between 1-MCP and DPA, Delicious apples were untreated or treated with 2 g L^{-1} DPA, and then with or without 1 μL L^{-1} 1-MCP. Ethylene production and respiration rates of fruit were measured immediately following treatment, and fruit was stored at 0.5 °C for 12 weeks. Internal ethylene concentrations (IEC), α-farnesene and conjugated trienol (CTol) concentrations, activities of peroxidase and polyphenol oxidase (PPO), and DPA levels in the skin of the fruit were measured at intervals during storage. 1-MCP reduced the rate of DPA loss from peel tissue so that by 12 weeks of storage concentrations of the chemical were 25% higher than in untreated fruit. 1-MCP, with and without DPA, markedly inhibited ethylene production and respiration rates, maintained low IEC and α-farnesene and CTol concentrations, while DPA had little effect on these factors except inhibition of CTol accumulation. Treatment effects on peroxidase and PPO activities were inconsistent.

KEYWORDS: Malus domestica; apples; superficial scald; storage; 1-methylcyclopropene; 1-MCP; diphenylamine; DPA; α-farnesene; peroxidase; polyphenol oxidase

INTRODUCTION

1-Methylcyclopropene (1-MCP) is an inhibitor of ethylene action that has been the subject of intensive research effort in fruits, vegetables, and ornamental products (*1*, *2*). Most commercialization of 1-MCP has occurred for the apple fruit. Apples can respond extremely well to 1-MCP application, showing inhibition of ethylene production and respiration rates and the associated maintenance of firmness and other quality aspects both during and after storage (*3*−*5*). While data on the extent of 1-MCP use are proprietary, the majority of some apple cultivars such as Delicious and Empire stored in controlled atmosphere (CA) storage are now treated with this technology in Washington and New York states. However, relatively little is known about the interactions of 1-MCP with other postharvest technologies.

One especially important postharvest chemical used routinely is diphenylamine (DPA), which controls development of superficial scald. Scald is a disfiguring physiological disorder, manifested as brown or black patches on the fruit skin, which develops during cold storage of susceptible cultivars and may be a chilling injury (*6*). It is generally accepted that scald development is caused by oxidation products of the sesquiterpene (*E*,*E*)-α-farnesene, probably the conjugated trienols (CTols), 9*E* and 9*Z* isomers of 2,6,10-trimethyldodeca-2,7,9,11-tetraen-6-ol (*7*, *8*). The factors that influence α-farnesene oxidation in vivo are not well understood but may involve antioxidant enzyme activities (*9*−*13*).

DPA does not always inhibit α-farnesene production, but consistently inhibits α-farnesene oxidation and development of scald during storage (*14*, *15*). DPA has also been reported to affect several aspects of fruit metabolism including reductions of respiration rate, color change, softening, activities of polyphenoloxidase (PPO), peroxidase, and lipoxygenase (*16*), and production of ester volatiles (*17*).

1-MCP also reduces scald development in apple fruit, but the mechanism of action appears primarily via inhibition of α-farnesene production and, therefore, CTol accumulation (*3*, *4*, *13*, *18*−*20*). Activities of several antioxidant enzymes including peroxidase were lower in 1-MCP-treated fruit during the first month of storage (*13*).

Because 1-MCP can inhibit scald development, some storage operators have used it in place of DPA. However, DPA use is still common for reasons that include lack of confidence in the

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new 1-MCP technology and because control of scald by 1-MCP in some cultivars is limited. In addition, some cultivars are susceptible to carbon dioxide injury in CA storage, and this injury is prevented by DPA (*21*, *22*).

Tolerances for DPA residues on marketed apple fruit vary by country, but the U. S. Food and Drug Administration level is 10 μg g^{-1} of fresh weight of fruit. Most of the DPA residues are located in the outer 2−4 mm of the fruit (*23*, *24*), and 60% of it is located in the waxy cuticle (*25*). DPA concentrations decline during storage (*15*, *25*, *26*), but the mechanisms of DPA loss are not well described. Huelin (*25*) concluded that volatilization accounted for only a small proportion of DPA loss from the fruit. Kim-Kang et al. (*24*) found that intact DPA remained the major contributor to the total residues during storage but that both peel and pulp tissues contained a variety of identifiable oxidative (hydroxylated) and conjugated transformation products.

No information exists about the possible effects of 1-MCP on the degradation of DPA, but anecdotal evidence from one country importing U.S. apples suggested that DPA concentrations might be higher in fruit also treated with 1-MCP than in those treated with DPA alone (personal communication).

The objectives of this study were to determine if the level of DPA in Delicious peel tissues was affected by 1-MCP treatment. In addition, we determined the effects of 1-MCP and DPA, alone and together, on the physiology of Delicious apples with respect to α-farnesene and CTol accumulation, and peroxidase and PPO activities.

MATERIALS AND METHODS

Plant Material and Treatments. Delicious apples were harvested from mature trees growing at the Cornell University Orchards at Lansing, NY. Sixteen boxes of approximately 100 fruit were harvested to provide four replicates for each of four treatments: untreated control, DPA, 1-MCP, and 1-MCP + DPA. DPA treatments were applied by immersing each box of fruit within 2 h of harvest for 1 min in 2 g L^{-1} DPA (Shield Brite DPA 15%, Pace International LP, Kirkland, WA). Fruit were allowed to dry and then, together with the non-DPA treated fruit, cooled overnight in a cold room maintained at 0.5 °C. 1-MCP was applied the following day using sealed 135 L plastic containers. 1-MCP as a powder (SmartFresh, AgroFresh, Springhouse, PA; 0.14 % active ingredient by weight) was weighed into Erlenmeyer flasks to provide a final gas concentration of 1 μL L^{-1}. Water was added to liberate the 1-MCP from the powder. After 24 h the containers were vented. Untreated and DPA treatment fruit were kept under the same conditions but without 1-MCP treatment. All fruit were stored at 0.5 °C for the duration of the experiment.

Ethylene and Respiration Rate. After treatments, 10 fruit per treatment were removed from cold storage and placed individually into sealed 1 L plastic containers with inlet and outlet ports and septa for gas sampling. The flow rates of air over the fruit were maintained at 25 mL min^{-1}. Ethylene and carbon dioxide concentrations in 1 mL gas samples of the air stream were measured daily for 10 days. Ethylene was measured using a Hewlett-Packard 5890 series II gas chromatograph (Hewlett-Packard, Wilmington, DE) equipped with a stainless steel column packed with 60/80 mesh alumina F-1 (2 m × 2 mm, i.d.) and fitted with a flame ionization detector. Analyses were run isothermally with an oven temperature of 200 °C and injector and detector temperatures of 220 and 250 °C, respectively. The flow rates for nitrogen, hydrogen, and compressed air were 30, 30, and 230 mL min^{-1}. Carbon dioxide was measured using a Buck model 910 gas chromatograph (Buck Scientific Inc., East Norwalk, CT) equipped with a stainless steel column packed with silica gel (0.92 m × 2 mm i.d.) and fitted with a thermal conductivity detector. The oven temperature was 100 °C, and the TCD current was set to low. The flow rate of helium was 10 mL min^{-1}. For both ethylene and carbon dioxide analyses, samples were directly injected into the gas chromatograph. Components were quantified by peak area, and external standards were used for calibration.

Internal Ethylene Concentration. Ten fruit from each of the four replicates per treatment were used for analysis. The internal ethylene concentration (IEC) of each fruit was measured on 1 mL samples of internal gas from the core cavity (*27*) by gas chromatography. Fruit from each replicate were sampled in the cold to avoid effects of warming the fruit.

Preparation of Samples for HPLC Analysis. DPA, α-farnesene, and CTols were extracted using the method described by Whitaker (*15*) but with the addition of biphenyl as a surrogate standard. Frozen peels from each replicate were ground under liquid nitrogen, and 5 g of the powdered sample was placed in 40 mL screw-cap Oakridge Teflon-lined tubes. The tubes were kept at room temperature for 10 min before biphenyl dissolved in methanol was added to the peels to give a concentration of 30 μg g^{-1} peel. After 10 min more, 20 mL of HPLC grade hexane (J. T. Baker, Phillipsburg, NJ) was poured into each tube. The tubes were sealed and shaken for 1.5 h at 4 °C. The resulting mixtures were vacuum-filtered through glass fiber disks and adjusted to 25 mL total volume with hexane. Aliquots of 15 mL each of the extracts were transferred into 40 mL vials. To avoid producing a solid residue that may be hard to redissolve upon evaporation of the hexane, 2 mL of HPLC grade 1-butanol (Sigma-Aldrich Co., St. Louis, MO) was added (*28*) before placing the vials in a nitrogen evaporator with a 30 °C water bath. After concentrating to 2 mL, the total volume was made-up to 10 mL with HPLC grade methanol (Sigma-Aldrich). The extracts were filtered through a 0.45 μm PTFE membrane (Whatman, Florham Park, NJ) to give HPLC ready samples.

HPLC Analyses. Diphenylamine, α-farnesene, conjugated trienols, and biphenyl in the methanol solution were separated using an HPLC system consisting of a Shimadzu SIL-10 ADVP automatic injector (Shimadzu Corp., Kyoto, Japan) and a Spectra Physics SP8800 HPLC system (Spectra Physics, San Jose, CA) fitted with a 4.6 × 250 mm Microsorb-MW C18 column. The mobile phase was methanol/acetonitrile/water (90:5:5) flowing at 0.8 mL min^{-1} (*15*). Methanol (Sigma-Aldrich) and acetonitrile (Fisher Chemical Co., Pittsburgh, PA) were HPLC grade. Detection of α-farnesene and CTols was accomplished using a Shimadzu SPD-10A VP UV−vis detector at 232 and 269 nm, respectively (*7*). DPA and biphenyl concentrations were measured using a Shimadzu RF-10 AXL fluorescence detector (Shimadzu) with an excitation wavelength of 285 nm and an emission wavelength of 340 nm (*28*). The retention times of these compounds were 5 min for DPA, 6.4 min for biphenyl, 6.6 min for CTols, and 14 min for α-farnesene. Since the residence times for the biphenyl and conjugated trienols did not allow good separation of their peaks, the samples with DPA were also analyzed without the addition of biphenyl. The concentrations of the analytes were obtained from their respective peak areas as measured by PeakSimple 3.29 software (SRI Instruments, Torrance, CA). The reported DPA concentrations in the peels were calculated by correcting for extraction efficiency of the surrogate standard, which ranged from 75 to 85%. The fluorescence detector was highly sensitive; therefore in some cases extracts were diluted to keep them within range of the standard curve. Standard curves were generated from authentic DPA (Sigma-Aldrich), biphenyl (ACROS, Fisher), and α-farnesene (Tokyo Chemical Industry, Japan). The α-farnesene was a mixture of isomers; however, apple fruit contain (*E*,*E*)-α-farnesene almost exclusively (*29*). CTols were quantified using published extinction coefficients (*30*). Whitaker et al. (*7*) have shown that the compounds with absorbances at 232 and 269 nm by HPLC with diode array detection represent α-farnesene and CTols as confirmed by UV spectrometry and GC-MS.

Enzyme Extraction and Assay. Frozen peels (5 g) were ground under liquid nitrogen and placed in 40 mL screw-cap tubes containing 15 mL of extract buffer (200 mM phosphate buffer with pH 7.8, 2 mM ethylenediaminetetraacetic acid (EDTA), 5% polyvinylpolypyrrolidone, and 1 mM phenylmethanesulfonyl fluoride). The mixture was homogenized by a vortex mixer and then centrifuged at 14 000 g for 30 min at 4 °C. The resulting supernatant was divided into aliquots and kept at −80 °C for protein and enzyme assays (*11*). Total soluble proteins were measured by the Bradford method using BioRad protein assay dye with bovine serum albumin as the standard (*31*).

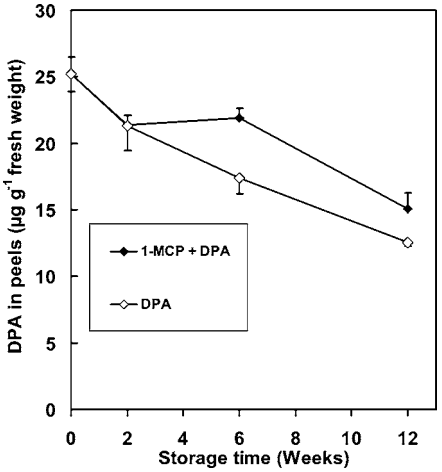

Figure 1. Diphenylamine (DPA) concentrations in peel tissues of Delicious apples treated with 2 g L^{-1} DPA and then treated with either air or 1 μL L^{-1} 1-MCP for 24 h and stored in air for 12 weeks at 0.5 °C. Each data point represents the mean of four replicates of bulked samples of 10 peeled fruit + 1 SE. Differences between non-1-MCP and 1-MCP treatments at weeks 6 and 12 are significant ($P < 0.001$).

Total soluble peroxidase activity was measured as described by Kochhar et al. (*11*). A 50 μL aliquot of the enzyme extract was added to 2.95 mL of assay solution (100 mM phosphate buffer with pH 6.8, 2.7 mM guaiacol, and 4 mM H_2O_2), and the increase in absorbance at 470 nm was monitored at 24 °C on a spectrophotometer (Beckman-Coulter, DU 7400, Fullerton, CA). Activity is reported as change in optical density (OD) min^{-1} (g of fresh weight)$^{-1}$.

Polyphenol oxidase (PPO) activity was assayed as reported by Zhou et al. (*32*). A 200 μL aliquot of the enzyme extract was added to 2.8 mL of assay solution (50 mM catechol in 100 mM citrate−200 mM phosphate buffer, pH 5.0), and the increase in absorbance at 420 nm was measured at 24 °C on a spectrophotometer (Beckman-Coulter, DU 7400). Activity is reported as the change in OD min^{-1} (g of fresh weight)$^{-1}$.

Statistical Analysis. All data were subjected to ANOVA using Minitab software v. 14.1 (Minitab, Inc., State College, PA). One-way analyses for each sampling point were performed for the ethylene production and respiration data, and two-way analyses, for all other factors, mean separation being by least significant differences (LSD) at $P = 0.05$. Because of the treatment effect on variances, however, data are presented as means and standard errors (SEs).

RESULTS AND DISCUSSION

DPA concentrations in peel tissues decreased over time in both DPA and DPA + 1-MCP treatments, and at 2 weeks of storage plus and minus 1-MCP treatments were not significantly different (**Figure 1**). However, at weeks 6 and 12, DPA concentrations were on average 25% higher in the 1-MCP treated fruit.

The effects of each treatment on metabolic rates of Delicious apples were assessed by measuring ethylene production and respiration rates of fruit treated and kept at 20 °C for 10 days. Ethylene production rates of fruit treated with 1-MCP or 1-MCP + DPA remained consistently low and never exceeded 1.3 μL kg^{-1} h^{-1} (**Figure 2a**). In contrast, the ethylene production rates of the untreated and DPA-treated fruit increased over time to reach 36.6 μL kg^{-1} h^{-1} in the untreated fruit. Although ethylene production rates of the DPA-treated fruit tended to be lower than those of untreated fruit, no significant differences between these treatments were detected. Respiration rates were lower in the 1-MCP- and (1-MCP + DPA)-treated fruit by day 2 (**Figure 2b**), being on average 42% lower than those of the untreated and DPA-treated fruit.

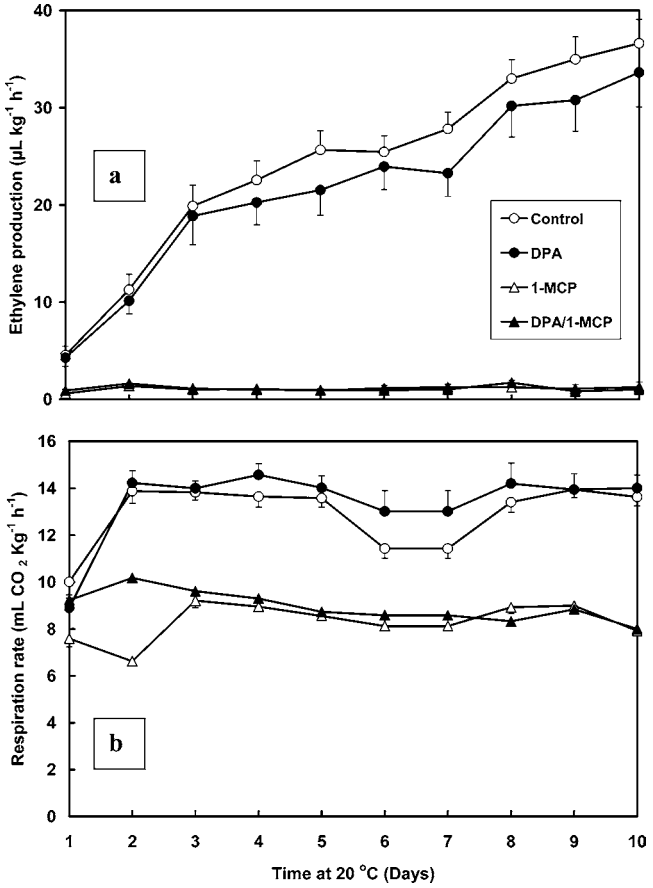

Figure 2. Ethylene production (**a**) and respiration rate (**b**) of Delicious apple fruit treated with 2 g L^{-1} DPA and then treated with either air or 1 μL L^{-1} 1-MCP for 24 h, and kept at 20 °C for 10 days. Each data point represents the mean of 10 fruit + 1 SE. Except for day 1, all differences between non-1-MCP treatments and 1-MCP treatments for ethylene production and respiration rate are significant ($P < 0.001$), as is the difference between 1-MCP with and without DPA for respiration rate on day 2.

The IEC of fruit from each treatment was also measured during the 12 week experimental storage period at 0.5 °C. In fruit treated with 1-MCP, with or without DPA, IECs showed a rapid decline within a week of storage and remained low. The IECs in untreated and DPA-treated fruit increased over time, after an initial decline in response to cold temperatures (**Figure 3**). No statistical differences between either pair of with or without 1-MCP treatments were detected.

The dramatic effect of 1-MCP on suppressing ethylene production and respiration rates, and the IEC, of apple fruit is similar to other reports for apples (*3*−*5*). In contrast, we found DPA did not suppress these factors, although inhibition of IEC, ethylene production, and/or respiration rates by DPA have been shown by others (*15*, *16*, *33*). A possible reason for the difference among studies is that some fruit used in our study had already entered the climacteric at the time of harvest and treatment, in which case increases in respiration and ethylene production had already commenced. All previous reports of DPA effects have used fruit at the preclimacteric stage of ripening.

α-Farnesene accumulation in the skin was dramatically inhibited in 1-MCP- and (1-MCP + DPA)-treated fruit, and lower for DPA-treated than for untreated fruit at week 12 (**Figure 4a**). Accumulation of CTols was inhibited by DPA and/or 1-MCP, with the maximum inhibition occurring in fruit with the combined treatment (**Figure 4b**). These data illustrate the

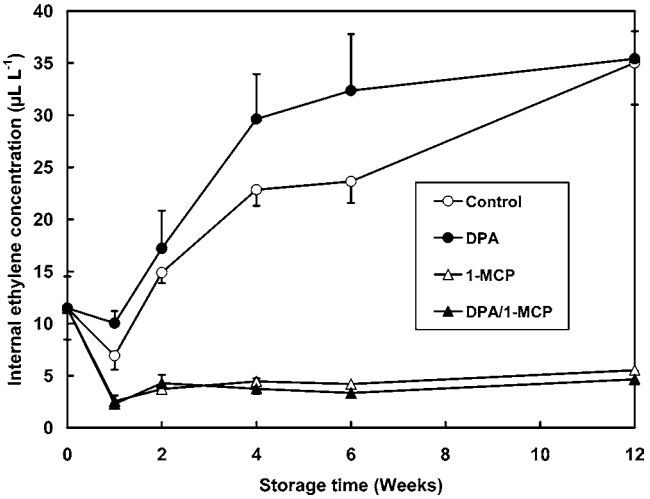

Figure 3. Internal ethylene concentrations in Delicious apple fruit untreated or treated with 2 g L^{-1} DPA and then treated with either air or 1 μL L^{-1} 1-MCP for 24 h, and kept at 0.5 °C for 12 weeks. Each data point represents the mean of four replicates of 10 individual fruit + 1 SE. All differences between non-1-MCP treatments and 1-MCP treatments are significant ($P < 0.001$).

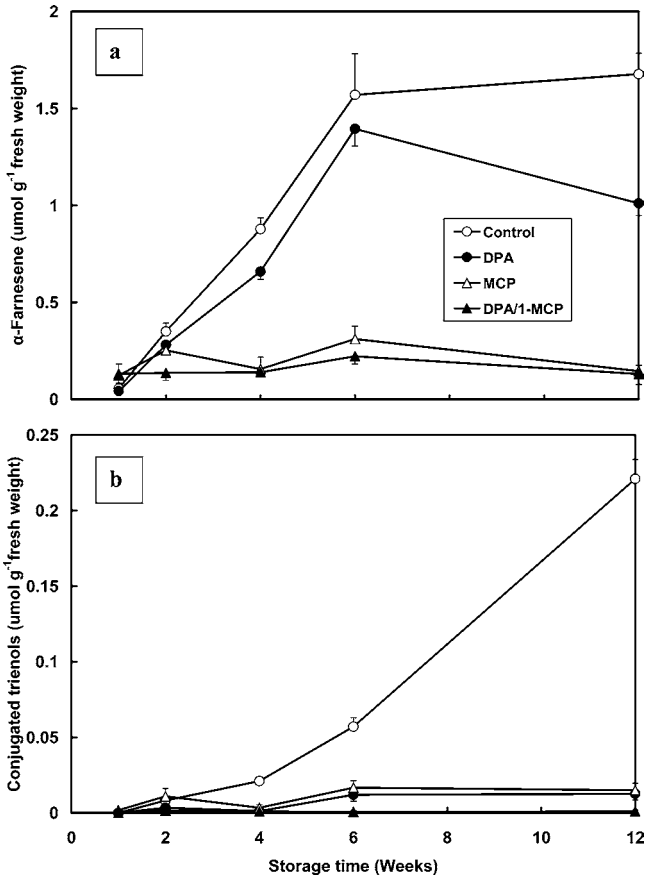

Figure 4. α-Farnesene (**a**) and conjugated trienol (**b**) concentrations in peel tissue of Delicious apple fruit untreated or treated with 2 g L^{-1} DPA and then treated with either air or 1 μL L^{-1} 1-MCP for 24 h, and kept at 0.5 °C for 12 weeks. Each data point represents the mean of four replicates of bulked samples of 10 peeled fruit + 1 SE. For α-farnesene, the differences between the non-1-MCP treatments and 1-MCP treatments are significant ($P < 0.001$), and for conjugated trienols, differences between the untreated control and other treatments are significant ($P < 0.001$).

differences in mechanism of scald control by 1-MCP and DPA. α-Farnesene accumulation in the skin of apples is closely

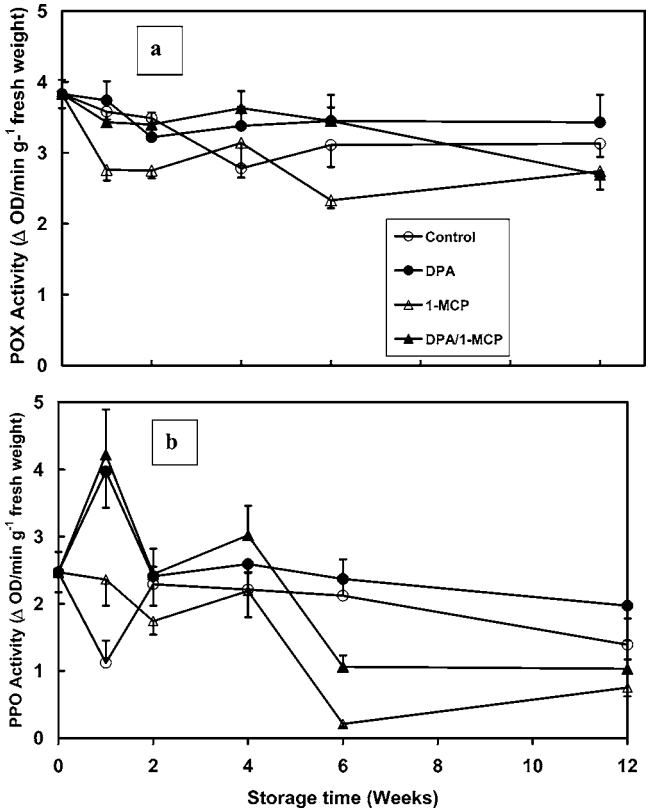

Figure 5. Peroxidase (**a**) and polyphenol oxidase (**b**) activities extracted from peel tissue of Delicious apple fruit untreated or treated with 2 g L^{-1} DPA and then treated with either air or 1 μL L^{-1} 1-MCP for 24 h, and kept at 0.5 °C for 12 weeks. Each data point represents the mean of four replicates of bulked samples of 10 peeled fruit + 1 SE.

associated with ethylene production by fruit, with patterns of change of α-farnesene generally reflecting those for IEC (*6*, *34*). 1-MCP inhibits α-farnesene production by inhibiting ethylene production, and as a result also prevents the accumulation of CTols (*3*, *13*, *18*−*20*). DPA did not affect the accumulation of α-farnesene in our study, probably because of its minor effects on fruit IECs, but it did prevent its oxidation to CTols. Inhibition of α-farnesene accumulation by DPA is usually associated with inhibited ethylene production (*33*). However, the effects of DPA on α-farnesene accumulation have been reported to be inconsistent in different years and seasons (*14*, *35*). Unfortunately no ethylene data were provided in those studies, so the link between ethylene production and α-farnesene accumulation remains unclear.

Recently, there has been interest in the possible roles of antioxidant enzymes in susceptibility of fruit to scald (*9*−*13*). This interest is based in part on early observations by Lurie et al. (*16*) who noted that peroxidase and PPO activities were lower in DPA-treated fruit than in untreated fruit. However, in our study, neither peroxidase nor PPO activities showed consistent patterns during storage in response to DPA treatment, even though statistical differences were detected. For peroxidase, skin samples from the 1-MCP treatment tended to have lower activities than those from the other treatments (**Figure 5a**), as also found by Shaham et al. (*13*). Interestingly, this effect was usually absent in 1-MCP-treated fruit that had also been treated with DPA.

At 1 week of storage, PPO activity was higher in DPA-treated fruit than those not treated with DPA, and higher in the 1-MCP-treated than in untreated fruit, but at weeks 2 and 4 treatment effects were minor (**Figure 5b**). At weeks 6 and 12, however,

the lowest PPO activity occurred in the 1-MCP and 1-MCP + DPA treatment. No effects of 1-MCP on the PPO activity of apples have been reported, but 1-MCP decreased PPO activity in avocado (*36*). The results for peroxidase and PPO as specific activity were similar to those expressed on a fresh weight basis (data not shown).

While it appears that changes in fruit metabolism affected DPA degradation, the mechanism whereby 1-MCP treatment retards DPA degradation is not known. Kim-Kang et al. (*24*) have shown that degradation of DPA in apples involves oxidative processes (e.g. hydroxylation). It is tempting to speculate that reduced oxidative activity such as that indicated by the lower PPO activity in 1-MCP-treated fruit contributes to slower degradation of DPA. Further research is required.

In this study, DPA concentrations were measured in peel tissues, which represent about 10% of the total mass, rather than whole fruit as required for residue analysis by regulatory authorities. Nevertheless, from a practical point of view, the finding that 1-MCP can maintain higher DPA concentrations in Delicious fruit may be important in meeting regulatory requirements for fruit residues, especially in countries that have lower tolerances than the 10 μg g^{-1} of fresh weight of fruit in the U.S.

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