Volatile Ester Suppression and Recovery following 1-Methylcyclopropene Application to Apple Fruit

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ABSTRACT. The effect of 1-methylcyclopropene (1-MCP) on biosynthesis of volatiles and fruit ripening in apple (*Malus domestica* Borkh.) was investigated using ‘Golden Delicious’, ‘Jonagold’, and ‘Redchief Delicious’ fruit. Application of 1-MCP to ‘Golden Delicious’ at the preclimacteric stage effectively inhibited ripening as determined by decreased expression of genes for 1-amino-1-cyclopropane carboxylic acid (ACC) oxidase (ACO), and ACC synthase, ACO protein content, climacteric ethylene production, respiration, and volatile ester biosynthesis. Exogenous ethylene applied after 1-MCP treatment did not induce ethylene production, respiration, or volatile production. Activity for alcohol acyltransferase, which catalyzes the final step in ester formation, was demonstrable for 1-MCP-treated fruit, indicating no strict limitation on ester formation is imposed by this enzyme and that ester formation in 1-MCP-treated apple fruit is at least partially limited by reduced substrate synthesis. Once volatile ester formation had been suppressed by 1-MCP, the recovery of volatile synthesis required ≈ 3 weeks for ‘Jonagold’ and 4 weeks for ‘Delicious’ when held in air at 22 °C. For the first 2 months of storage at 0 °C in air, ‘Jonagold’ and ‘Delicious’ required ≈ 3 weeks holding at 22 °C for volatile biosynthesis to initiate; after 5 months in refrigerated storage, volatile formation was evident at the time of removal from cold storage. For ‘Jonagold’ fruit held in controlled atmosphere (CA) storage for 2, 5, and 7 months at 0 °C, at least 3 weeks holding at 22 °C were required for volatile formation to begin to recover. The maximal amount of volatile formation was reduced 50% by 1-MCP relative to nontreated control fruit. CA storage had a similar impact on maximal volatile formation. The marketing of 1-MCP-treated fruit soon after treatment might result in the delivery of fruit to the consumer with little likelihood of recovery of volatile ester formation prior to consumption.

Apple aroma depends upon a complex mixture of organic compounds, some of which are produced upon the disruption of the tissue during mastication, but many of which are produced autonomously by the fruit during ripening (Fellman et al., 2000). Esters are quantitatively and qualitatively the most important autonomously produced volatile compounds contributing to apple aroma (Dixon and Hewett, 2000; Fellman et al., 2000). Ester production in apple fruit is an ethylene-dependent process and the majority of the esters are synthesized during the climacteric phase of ripening (Fan et al., 1998; Song and Bangerth, 1996).

Storage of apples in low oxygen atmospheres, which inhibits ethylene production and action, has been widely shown to sup-

press autonomous aroma volatile production (Brackmann et al., 1993; Fellman et al., 2000; Gorny and Kader, 1996; Mattheis et al., 1995; Patterson et al., 1974) and can reduce perceived aroma intensity and quality (Boylston et al., 1994; Plotto et al., 1999; Williams and Langron, 1983). Similarly, the ethylene action inhibitor, 1-MCP suppresses the formation of aroma-related volatiles (Abdi et al., 1998; Fan et al., 1999; Fan and Mattheis, 1999; Golding et al., 1998; Lurie et al., 2002) and suppresses the expression of genes associated with the pathways of ester formation, including alcohol acyl-transferase (AAT) (Defilippi et al., 2005), which transfers an acyl group from a CoA thioester to an alcohol in the final step of ester biosynthesis. It is believed that this enzyme imparts some limitation on the formation of esters in apple fruit in which ethylene action has been inhibited by growth regulators such as 1-MCP or in which ethylene perception has been impaired (Defilippi et al., 2004, 2005).

Like CA storage, 1-MCP has the potential to impact the perception of fruit aroma quality. Lurie et al. (2002) found that 1-MCP induced a moderate reduction in volatiles that qualitatively altered olfactory perception of diced ‘Anna’ fruit. Despite reduced production of total volatiles by the 1-MCP treated apple

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pieces, a reduction in the “ripe” aroma character was associated with higher acceptability. Nevertheless, a more severe reduction in odor-active compounds by 1-MCP than that achieved in the cited study may negatively impact aroma quality.

The process of recovery of the biosynthesis of esters and other ripening-related volatiles from the effects of 1-MCP has not been evaluated extensively. Mattheis et al. (2005) found that following removal from storage, volatiles from ‘Gala’ fruit tended to increase with duration of storage in air with and without 1-MCP treatment, although the absolute level of volatiles was reduced by 1-MCP. Under conditions of CA storage with and without 1-MCP, volatile production upon removal from storage declined as storage duration increased. Additional information regarding the ontogeny of ripening and volatile formation following 1-MCP treatment may help optimize storage regimes for the delivery of the highest quality fruit to the consumer. Our goal was to evaluate the timing of ripening events immediately following 1-MCP application and to detail the duration of 1-MCP suppression of the formation of esters and other volatiles for apple fruit stored in refrigerated air (RA) and CA storage and to more fully describe the extent and rapidity of the recovery of the capacity for volatile synthesis.

Materials and Methods

Two experiments were conducted. The objective of the first experiment was, using ‘Golden Delicious’ apple fruit, to evaluate the impact of 1-MCP on initial volatile suppression and the inhibition of two broadly accepted physiological measures of fruit ripening, ethylene formation and respiration. To further assess and verify the impact of 1-MCP on ripening, the effect of 1-MCP on gene expression for ACC synthase (ACS) and ACO as well as protein analysis of ACO were determined. To determine if volatile suppression by inhibition of ethylene action was by limiting AAT in the final reaction in the synthesis pathway of esters, immediate precursors to this step were applied exogenously. In the second experiment, we determined the time required for ‘Jonagold’ and ‘Redchief Delicious’ apple fruit to recover their capacity to produce esters and other volatiles (collectively termed “total volatiles”) either immediately after treatment or following storage in RA or CA at 0 °C. In both experiments, 1-MCP was applied to preclimacteric apples.

Expt. 1

PLANT MATERIAL. Sixty ‘Golden Delicious’ apple fruit were harvested in the orchard of the Horticultural Teaching and Research Center at Michigan State Univ. in early Oct. 1997. The internal ethylene concentration of 10 representative fruit was measured by analyzing a 1-mL gas sample withdrawn from the internal air space of the core of the fruit ovary as previously described (Mir et al., 2001) to determine if fruit ripening had initiated. At the time of harvest, the range of internal ethylene concentrations in the fruit was from 0.1 to 0.3 $\mu\text{L}\cdot\text{L}^{-1}$. The remaining fruit were used for the following studies.

1-MCP TREATMENT. To impose the 1-MCP treatment, 18 fruit were placed in a 9.5-L glass desiccator and held at 22 °C and 1-MCP gas was added directly to the container atmosphere. 1-MCP stock gas, contained in an amber glass bottle fitted with a crimp-top cap with Teflon septum (a gift from E.C. Sisler) was used as a source for 1-MCP. 1-MCP gas was removed from the stock gas using a glass syringe fitted with a Teflon-tipped plunger and injected into the jars to yield a final 1-MCP concentration of 0.5 $\mu\text{L}\cdot\text{L}^{-1}$. The containers were sealed for 14 h then ventilated

for 2 h to release the 1-MCP. Fifteen nontreated control fruits were enclosed in a similar container for the same period of time and also ventilated for 2 h. O_2 levels were not monitored while containers were sealed, but at typical respiratory rates measured, would be expected to deplete ≈ 600 mL of the ≈ 2000 mL of O_2 resident in the system upon sealing, yielding a decline of ≈ 6 kPa from the initial 21 kPa O_2 . After the treatment period, fruit were randomly placed into ventilated 1.9-L glass jars, three fruit per jar (≈ 500 – 650 g), and held at 22 °C for a total of six jars for the 1-MCP-treated fruit and five jars for the control fruit. Of these, three containers were used for monitoring respiration, ethylene production, and fruit volatile production for control and treated fruit for the first 11 d of the study. After 11 d, the three containers having 1-MCP-treated fruit were gassed with ethylene as described below and monitoring of respiration, ethylene production, and fruit volatile production was resumed on day 12 and concluded on day 14. The control and 1-MCP-treated fruit not being monitored were used for destructive analyses. Destructive analyses were performed after 0, 5, 11, and 14 d to measure ACO protein content and gene expression analysis as described below.

MEASUREMENTS OF RESPIRATION AND ETHYLENE PRODUCTION. The 1.9-L jars were ventilated with air at a flow rate of 30 $\text{mL}\cdot\text{min}^{-1}$, and held at 22 °C. A glass “tee” on the outlet line was fitted with a Teflon-lined half-hole septum (Alltech Assoc., Deerfield, Ill.) to create a port for withdrawal of gas samples. All the connecting gas lines were composed of Teflon-PFE (Cole-Parmer Instrument Co., Chicago). Fruit respiration was measured by withdrawing 0.1 mL of gas from the sampling port and injecting it into an infrared gas analyzer (model 225-MK3; Analytical Development Co., Hoddeson, England) operated in a flow-through mode with N_2 as the carrier gas and a flow rate of 100 $\text{mL}\cdot\text{min}^{-1}$. Ethylene production was determined using gas chromatography (GC) as previously described (Mir et al., 2001). Ethylene and CO_2 concentrations were calculated relative to a certified gas standard (Matheson Gas Products, Chicago) containing 0.979 $\mu\text{L}\cdot\text{L}^{-1}$ ethylene, 4.85% CO_2 , 1.95% O_2 balanced with N_2 as previously described (Mir et al., 2001). Respiration and ethylene production were monitored daily.

ETHYLENE TREATMENT. On the eleventh day, the 1-MCP-treated fruit used for monitoring CO_2 , ethylene, and fruit volatile production were exposed to an ethylene concentration of 36 $\mu\text{L}\cdot\text{L}^{-1}$ for 20 h by injecting 70 μL pure ethylene (Matheson Gas Products, Chicago) into the jar headspace; the 1-MCP-treated fruit being held for destructive analyses received no ethylene treatment. During the ethylene treatment period, the inlets and outlets of the jars were closed off and ventilation arrested. Ethylene levels were verified by GC as described previously. O_2 levels were not monitored during the treatment period, but at typical respiratory rates measured, would be expected to deplete ≈ 100 mL of the ≈ 380 mL of O_2 resident in the system upon sealing, yielding a decline of ≈ 6 kPa from the initial 21 kPa O_2 . After treatment, the jars were again ventilated to monitor respiration, ethylene evolution, and ester production. Respiration and ethylene production were monitored daily.

MEASUREMENT OF VOLATILE ESTERS OF WHOLE FRUIT. Ester measurement was by GC coupled with time-of-flight mass spectrometry (TOFMS). To sample volatile esters, solid phase micro-extraction (SPME) fibers (60- μm PDMS/DVB coating; Supelco Co., Bellefonte, Pa.) were inserted into the glass sampling “tee” in the outlet line and exposed to the effluent gas stream of the ventilated 1.9-L jars for 4 min and immediately transferred to the GC injection port and desorbed. Absorbed volatiles were

desorbed from the fiber coating by inserting the SPME fiber through a predrilled septum (Thermogreen LB-2; Supelco Co.) and into a glass-lined, splitless injector port (200 °C) of a gas chromatograph (HP-6890; Hewlett Packard Co., Wilmington, Del.) equipped with a mass selective detector (Pegasus II; Leco, St. Joseph, Mich.). Conditions of GC separation and TOFMS analysis were as previously described (Song et al., 1997). Identification of volatile components was confirmed by comparison of collected mass spectra with those of authenticated chemical standards and reference spectra in a mass spectral library (NIST MS Search 1.5; National Institute for Standard Technology, Gaithersburg, Md.). Data are presented for only three compounds: hexyl acetate, butyl acetate, and 2-methylbutyl acetate, which were emitted in the largest amounts relative to other esters and were considered to contribute significantly to apple fruit aroma (Plotto et al., 2000). Quantization of volatile compounds was performed by calibrating with a known amount of an authenticated and high purity standard mixture as previously described (Song et al., 1997). Ester measurements were made on days -1, 0, 1, 2, 5, 7, 9, 11, 12, and 14. All measurements were done in triplicate.

INCUBATION OF APPLE SLICES WITH ESTER PRECURSORS. 1-Butanol and butyric acid were purchased from a commercial source (Sigma-Aldrich, St. Louis). Five days after 1-MCP treatment, 1- to 2-mm-thick slices (0.9 g) of fruit skin and cortex from a pool of three fruit were cut and put on the surface of filter paper previously immersed in 1.0 mL of 0.1 M MES buffer (pH = 7.0) containing 10^{-9} mol 1-butanol or 10^{-8} mol butyric acid. Each slice was sealed in a 30-mL glass vial fitted with a cap housing a valved septum (Mininert; Supelco Co.) for 120 min at 22 °C. Volatile analysis was performed by using SPME/GC/TOFMS as described above. The effect of the addition of precursors was determined by calculating the ratio of ester concentration in vials without precursors to that of those with precursors. Measurements were conducted in triplicate.

ELECTROPHORESIS AND IMMUNOBLOTTING OF ACC-OXIDASE. Tissue samples were obtained on day 0, day 5, and day 11 for control and treated fruit. Total protein was extracted from apple tissue by using the method of Gorny and Kader (1996). The protein content of extracts for Western blot analysis was determined using the method of Bradford (1976). Twenty-five micrograms of total protein per lane were separated by SDS-PAGE as described by Laemmli (1970). Polyacrylamide gels (12%) were stained with Coomassie blue, and were electroblotted onto a nitrocellulose membrane (Bio-Rad, Hercules, Calif.). The membrane was probed with ACO antibody (Neogen, Lansing, Mich.). Immuno-detection with the alkaline phosphatase reaction was carried out as described by Gorny and Kader (1996).

RNA ISOLATION, ELECTROPHORESIS, AND IMMUNOBLOTTING. Fruit disks taken from skin and 2–3 mm of underlying cortex tissue were frozen in liquid nitrogen and stored at -80 °C. Five grams of tissue was ground into powder in liquid N₂ using a mortar and pestle. Total RNA was extracted using the hot borate/phenol technique essentially as described by Lopez-Gomez and Gomez-Lim (1992) and quantified spectrophotometrically. Northern analysis was essentially as described by Sambrook et al. (1989). Total RNA (25 µg per lane) was separated by electrophoresis in a 1.2% agarose gel containing 2.2 M formaldehyde in 20 mM MOPS running buffer. The 18S and 28S ribosomal bands were visualized using ethidium bromide fluorescence. After destaining, the gels were blotted onto nylon membranes (Hybond N; Amersham, Piscataway, N.J.), and fixed by exposure to ultraviolet radiation. *MdACO1* (AF030859) and *MdACS1* (L31347)

cDNA was labeled with [³²P]dCTP by using a random-primer DNA labeling kit (Boehringer Mannheim, Basel, Germany) and hybridized to the membranes using routine procedures. The blots on the membrane were visualized by autoradiography using Kodak XAR-5 film (Kodak, Rochester, N.Y.) with an intensifying screen at -80 °C.

Expt. 2

PLANT MATERIAL. ‘Jonagold’ and ‘Redchief Delicious’ fruit were harvested on 23 Sept. and 5 Oct., respectively, in 2002 from commercial orchards in the Fruit Ridge area of Michigan north of Grand Rapids. Fruit were harvested prior to the onset of autocatalytic ethylene production as determined by the average internal ethylene level, which was below $0.2 \mu\text{L}\cdot\text{L}^{-1}$ for 10 randomly selected fruit. Internal ethylene levels were determined as previously described (Mir et al., 2001).

1-MCP TREATMENT. For each cultivar, the harvested fruit were divided into two groups of ~220 fruit and each group was placed into 113-L plastic barrels fitted with airtight lids. O₂ levels were not monitored during the treatment period but typical respiratory activity for apple fruit would be expected to deplete ~6 L of the 22 L of O₂ resident in the system upon sealing, yielding a decline of ~5 to 6 kPa from the initial 21 kPa O₂. One barrel was used as a control and the second was used to treat fruit with 1-MCP. 1-MCP was generated using EthylBloc (Biotechnologies for Horticulture, Burr Ridge, Ill.) formulation (a.i. = 0.14%) in an amount sufficient to create an initial concentration of ~1 µL·L⁻¹. The EthylBloc was placed into four 1.5-mL microcentrifuge tubes and the 1-MCP released from the cyclodextrin matrix using 100 µL of water added to the well of the tube cap. The tubes were sealed, and shaken for 2 to 3 s to mix the water with the cyclodextrin. The 1-MCP was administered by opening the microcentrifuge tubes in the treatment chambers and immediately closing the lid. The exposure period was 20 h. 1-MCP concentrations were verified as previously described (Mir et al., 2001).

STORAGE TREATMENTS. For ‘Jonagold’, treated and nontreated fruit were divided into 10 lots of 20 fruit each. Each lot was placed in a 20-L plastic bucket fitted with an airtight lid having a 3-cm-diameter hole. The hole was left open for air-stored fruit, or plugged with a rubber stopper pierced by inlet and exit lines for CA-stored fruit. From treated and nontreated fruit, one lot was left to ripen at room temperature (22 °C), six lots were placed in RA storage at 0 °C for 1, 2, 3, 4, 5, and 7 months and the remaining three lots were placed in CA storage (1.5 kPa O₂ and ≤3 kPa CO₂) at 0 °C for 2, 4, and 7 months. CA conditions were established using N₂ gas from a nitrogen generator (CPA-1; Permea, St. Louis) and blending it with O₂ contained in compressed air and gaseous CO₂ from a liquified CO₂ (Linde Gas, Independence, Ohio) source. Gas concentrations were established manually. Pressure was maintained using two-stage regulators and flow was adjusted using needle valves. Blended gas was humidified by first bubbling the gas into water contained in a 20-L glass jar in the cold room where the fruit were stored. The humidified gas was then directed to a flow board external to the cold room to avoid problems with condensation and distributed to individual lines using a manifold outfitted with glass capillary tubes. Gas flow from the flow boards was returned to the cold room and into the 20-L plastic buckets containing fruit and was maintained at ~75 mL·min⁻¹. For ‘Redchief Delicious’, treated and nontreated fruit were divided into six lots of 20 fruit each. One lot was kept at 22 °C; the other lots were placed in RA storage and held at 0 °C for 2, 3, 4, 5, and 7 months as described previously for ‘Jonagold’.

VOLATILE ANALYSIS. Volatile analysis was performed on fruit immediately after harvest and after storage by holding the fruit at room temperature (22 °C) and monitoring volatile production until senescence was extensive based on external appearance (yellowing and greasiness). The volatile profile of 'Jonagold' fruit was analyzed after 0, 1, 2, 3, 4, 5, and 7 months of storage in RA and after 2, 4, and 7 months in CA. The volatile profile of 'Redchief Delicious' fruit was analyzed after 0, 2, 3, 4, 5, and 7 months in RA. On each analysis date, one lot each of control and 1-MCP-treated fruit was removed from the storage. From these, three fruits were placed singly into 1-L Teflon chambers (Saville Corp, Minnetonka, Minn.) fitted with gas sampling ports as in Expt. 1. The remaining fruit were held in a similar condition and used to replace fruit if any were lost to decay during the poststorage holding period. Chambers were ventilated at a rate of ≈ 35 mL·min⁻¹ with purified air and held at room temperature (22 °C) between 25 to 95 d. Volatile emissions were measured once or twice weekly, depending on the rate of fruit deterioration. Volatile analysis was by SPME/GC/TOFMS as described for Expt. 1. For each sample, volatile emissions were represented using the total ion count (TIC) for all volatiles combined excluding CO₂. The TIC is the sum of all ions detected between m/z 29 and m/z 600 with background noise subtracted. The time required for volatile production to begin was determined as the date after which the TIC was greater than 1×10^8 , which was subjectively chosen as a threshold since it is $\approx 20\%$ of the peak amount detected for the treatment with the lowest peak volatile production. In addition to volatile analysis, the internal ethylene concentration of 10 representative fruit was measured on each removal date after fruit had warmed to room temperature as previously described for Expt. 1.

STATISTICAL ANALYSIS. To objectively estimate the date of the peak in volatile formation and the relative amount of volatile formation, the data were fitted with an appropriate equation using commercial curve-fitting software (Table Curve 2D; Jandel Scientific, San Rafael, Calif.). A *t* test was performed using statistical software (SigmaStat 3.11; Systat Software, Point Richmond, Calif.) to compare nontreated and 1-MCP-treated fruit for RA- and CA-stored fruit using estimates of the peak in volatile synthesis provided by the fitted curves.

Results

Expt. 1

EFFECT OF 1-MCP ON RESPIRATION AND ETHYLENE BIOLOGY. The climacteric increase of respiration and ethylene production for nontreated fruit began ≈ 4 –5 d after harvest (Fig. 1 A and B). The initial increase in ethylene synthesis and CO₂ production was prevented by 1-MCP and, for the remainder of the 11-d monitoring period, respiration and ethylene production by fruit treated with 1-MCP remained at or below preclimacteric levels. Application of exogenous ethylene on day 11 to 1-MCP-treated fruit did not induce an increase in fruit respiration or ethylene production. Expression of *MdACO* and *MdACS* increased and the protein content of ACO increased on day 5 for control fruit, changing simultaneously with the increase in ethylene biosynthesis (Figs. 2 and 3). ACO content remained high at day 11. Relative to controls, 1-MCP treatment suppressed *MdACS1* and *MdACO1* expression measured on day 5 and ACO formation measured on day 5 and day 11. Exogenous ethylene applied 11 d after 1-MCP treatment did not induce an increase in expression of *MdACS1* or *MdACO1* relative to control fruit by day 14.

Fig. 1. Respiration (A) and ethylene production (B) of 'Golden Delicious' apple fruit treated with 1-MCP and held in air at 22 °C. Arrow indicates treatment of 1-MCP-treated fruit with ethylene. Vertical bars represent mean $\pm$ standard deviation, *n* = 3.

EFFECT OF 1-MCP ON FRUIT ESTER PRODUCTION AND BIOSYNTHESIS. In control fruit, volatile production of straight and branched chain esters started to increase ≈ 5 d after treatment (Fig. 4A–C). 1-MCP prevented this increase, with all volatiles declining from initial levels and maintaining extremely low production rates during the 11 d of the experiment and for 3 d following the application of exogenous ethylene on day 11. However, the exogenously applied precursors 1-butanol and butyric acid were preferentially converted into their corresponding volatile esters (i.e., 1-butanol into butyl acetate and butyric acid into ethyl butanoate) despite inhibition of ripening and volatile synthesis for 1-MCP-treated fruit on day 5 (Table 1). The incorporation of 1-butanol and butyric acid into butyl butanoate was much greater than into butyl acetate and ethyl butanoate.

Expt. 2

1-MCP and CA conditions reduced ethylene production of both 'Jonagold' and 'Redchief Delicious' apple fruit (data not shown). The internal ethylene content in 1-MCP-treated fruit was less than 1% that in nontreated fruit upon removal from storage for all assay dates. While more than 50 individual volatile compounds were detected in chromatograms of ripening fruit, most of these were esters. When the TIC was summed for all volatiles in the profile, excluding CO₂, esters and their acid and alcohol precursors accounted for $\approx 90\%$ to 95% of the total. The sesquiterpene α -farnesene accounted for nearly all the remainder, although several other compounds, including 6-methyl-5-hepten-2-one and 1-methoxy-4-(2-propenyl)-benzene were also detected. The sum of the TIC for all volatiles excluding CO₂ was used to evaluate the effect of 1-MCP on total volatile synthesis, recognizing that individual compounds may have different developmental patterns.

Fig. 2. Northern blot analysis for ACC oxidase (*MdACO*) and ACC synthase (*MdACS1*) of 'Golden Delicious' apple fruits treated with 1-MCP on day -1 and held in air at 22 °C. Assays were conducted 0, 5, and 14 d after 1-MCP treatment (sample on day 11 was lost); fruit assayed on d 14 were treated with 36 $\mu\text{L}\cdot\text{L}^{-1}$ ethylene for 20 h or were left untreated. Total RNA (25 $\mu\text{g}/\text{lane}$) was separated by formaldehyde agarose gel electrophoresis and blots were analyzed by hybridization to the [^{32}P]dCTP-labeled pAAS2 *MdACO* probe and *MdACS1* probe. The 18S rRNA was visualized using ethidium bromide staining as a measure of loading uniformity.

Fig. 3. Western blot analysis of ACO of 'Golden Delicious' apple fruit treated with 1-MCP on day -1 and held in air at 22 °C. Assays were conducted 0, 5, and 11 d after 1-MCP treatment. Proteins (25 $\mu\text{g}/\text{lane}$) were separated by sodium dodecyl (lauryl) sulfate-polyacrylamide gel (SDS-PAGE) (upper figure) and transferred to a nylon membrane and detected with ACC oxidase (ACO) antibody (lower figure). The ACO bands have an approximate molecular weight (Mol wt) of 39 kD as determined by biotinylated molecular weight markers (Mol wt mrkr); their approximate location is indicated on the SDS-PAGE by ellipses.

Generally, however, when ester content was low, other volatiles were also low or nondetectable (data not shown).

The data describing the pattern in volatile production for air- and CA-stored fruit, with and without 1-MCP treatment, could be empirically fit by several of the equations provided by the curve-fitting software. Of these, those equations having constants that could be used to gather physiologically relevant data such as the timing of the peak in volatile formation and its maximum were evaluated further. Of these, the equation providing the best fit for all 'Jonagold' and 'Redchief Delicious' profiles was: $\text{TIC} = a \exp(-\exp(-(x-b)/c)) - ((x-b)/c) + 1$, where x is the number of days holding at 22 °C after removal from RA or CA storage. The value of a was used to obtain an objective estimate of the maxi-

Fig. 4. Production of volatile aroma compounds of 'Golden Delicious' apple fruit treated with 1-MCP on day -1 and held in air at 22 °C. (A) butyl acetate, (B) hexyl acetate, and (C) 2-methylbutyl (2-MB) acetate. Arrow indicates treatment of 1-MCP-treated fruit with ethylene. Vertical bars represent mean $\pm$ standard deviation; $n = 3$.

Table 1. Ratio of esters formed by the application of aroma precursors 1-butanol and butyric acid relative to untreated tissues for tissue disks of 'Golden Delicious' apple fruit 5 d after fruit were treated with 1-MCP. Values represent the ratio between treatments and control fruit ($n = 3$) $\pm$ SD.

Ester	Ester precursor	
	1-butanol	butyric acid
ethyl butanoate	1.1 $\pm$ 0.2	1.8 $\pm$ 0.5
butyl acetate	2.1 $\pm$ 0.2	1.5 $\pm$ 0.3
butyl butanoate	147.3 $\pm$ 2.5	102.9 $\pm$ 4.7
hexyl acetate	4.1 $\pm$ 1.2	2.0 $\pm$ 1.1

imum TIC for fruit volatiles and that of b to estimate the day of the maximum; the relationship between the value of c and curve shape was not evident.

Nontreated 'Jonagold' fruit began synthesizing volatiles immediately after harvest while 1-MCP-treated fruit required ≈ 25 d (Fig. 5). Volatile production peaked after ≈ 14 d at room temperature for nontreated fruit, while for those treated with 1-MCP peak volatile synthesis required more than 50 d (Table 2). For 'Redchief Delicious', nontreated fruit began volatile formation after 5 d at room temperature, but 1-MCP delayed the onset of

Fig. 5. Total ion count (TIC) for volatiles produced by 'Jonagold' and 'Redchief Delicious' apple fruit treated with 1 mL·L⁻¹ 1-MCP (open triangles) or left nontreated as controls (solid circles) immediately after harvest while being held at 22 °C.

the increase in volatile synthesis until after 40 d. The peak in volatile biosynthesis took place after ≈30 and 75 d of holding for nontreated and treated fruit, respectively. The maximal level of volatile formation was reduced by 50% or more by 1-MCP for both cultivars (Table 2).

After 1 month in RA storage at 0 °C, nontreated 'Jonagold' fruit were already producing moderate amount of volatiles at the time of removal, while 1-MCP-treated fruit had essentially no volatile production (Fig. 6). Volatile synthesis increased for 1-MCP-treated fruit after 30 d poststorage holding at 22 °C. Aroma production peaked after ≈2 weeks for nontreated fruit and after 60 d for fruit treated with 1-MCP (Table 2). As the duration of cold storage increased, the poststorage delay in volatile production caused by 1-MCP declined (Fig. 6). After 5 months RA storage, 'Jonagold' fruit treated with 1-MCP were producing low levels of volatiles at the time of removal from storage. Similarly, the poststorage holding time until peak volatile synthesis occurred also declined (Fig. 6, Table 2). Results were similar for 'Redchief Delicious' fruit with the nontreated fruit producing significant quantities of volatiles upon removal from RA for all storage durations (1 month data are lacking) and treated fruit experiencing a poststorage delay in volatile synthesis that declined from 20 d at 2 months RA to 0 d after 5 months (Fig. 7). For nontreated fruit, the pattern of volatile formation during poststorage holding differed markedly for the two cultivars. For 'Jonagold' fruit, the initial level of volatile formation was relatively similar for the entire 7 months RA storage, however, the quantity of volatiles

Table 2. Coefficient of determination and constants *a*, *b*, and *c* for equation fitted to volatile production data of 'Jonagold' and 'Redchief Delicious' apple fruit treated with 1 μL·L⁻¹ 1-MCP (Y) or left nontreated (N) as controls following 0 to 7 months storage in refrigerated air (RA) or controlled atmosphere (CA) (1.5 kPa O₂, 3 kPa CO₂) storage at 0 °C. Equation: mass spectrometer total ion count = $a \times \exp(-\exp(-(x-b)/c)) - ((x-b)/c) + 1$, where *x* is the number of days holding at 22 °C after removal from RA or CA storage. The value of *a* represents the maximum and that of *b* depicts the day of the maximum; the relationship between the value of *c* and curve shape is unclear.

Treatments			Values for constants		
Month	1-MCP	R^2	a	b	c
<i>Jonagold (RA storage)</i>					
0	N	0.81	2.83E+09	14.24	9.09
0	Y	0.85	5.92E+08	54.43	16.49
1	N	0.14	2.73E+09	13.08	17.13
1	Y	0.96	9.84E+08	60.98	14.41
2	N	0.93	2.67E+09	12.48	12.99
2	Y	0.99	1.38E+09	39.56	8.31
3	N	0.90	2.21E+09	10.34	16.21
3	Y	0.84	9.24E+08	54.01	21.75
4	N	0.98	8.36E+10	-71.12	15.73
4	Y	0.92	7.75E+08	37.09	19.09
5	N	0.84	2.64E+09	3.86	13.85
5	Y	0.86	9.68E+08	27.23	15.02
7	N	0.98	2.39E+09	10.41	15.70
7	Y	0.89	1.34E+09	29.62	15.08
<i>Jonagold (CA storage)</i>					
2	N	0.72	1.36E+09	21.13	13.73
2	Y	0.70	5.01E+08	52.72	15.85
4	N	0.69	1.26E+09	16.49	12.46
4	Y	0.97	9.01E+08	47.23	10.88
7	N	0.85	8.43E+08	18.72	12.45
7	Y	0.99	5.43E+08	45.45	10.33
<i>Redchief Delicious (RA storage)</i>					
0	N	0.71	1.69E+09	30.70	19.85
0	Y	0.81	6.33E+08	76.34	28.33
2	N	0.89	2.30E+09	0.71	22.80
2	Y	0.76	5.18E+08	46.98	20.42
3	N	0.81	1.87E+09	6.62	22.37
3	Y	0.85	9.23E+08	48.07	21.62
4	N	0.63	9.72E+08	5.62	13.95
4	Y	0.71	1.17E+09	50.94	26.09
5	N	0.59	2.85E+10	-239	54.99
5	Y	0.72	1.59E+09	38.54	25.27
7	N	0.02	6.01E+08	-777.90	694.40
7	Y	0.59	1.09E+09	25.39	18.76

produced by 'Redchief Delicious' fruit declined by roughly 80% by 7 months.

In the case of 'Jonagold' fruit stored under CA conditions, nontreated fruit began to produce a moderate quantity of volatiles immediately after removal from storage for all three storage durations (Fig. 8). However, ≈30 d at room temperature passed before 1-MCP-treated fruit recovered the capacity to produce volatiles after 2 months in CA. After 4 and 7 months in CA, volatile formation began after ≈25 d at room temperature. Peak volatile formation required ≈50 d of poststorage holding for all CA storage durations (Table 2).

1-MCP reduced maximum volatile biosynthesis ($P < 0.05$) of RA-stored 'Jonagold' and 'Redchief Delicious' fruit (Figs. 6 and 7, Table 2). The average maximum TIC observed in nontreated

Discussion

The preclimacteric and climacteric rates of CO_2 production reported are similar to those reported previously (Hulme, 1954; Hulme et al., 1963; Jones et al., 1965; Looney, 1969). Ethylene production rates are similarly consistent with previous research (Saftner et al., 2003). The reduction in the biosynthesis of ethylene, CO_2 , and total volatiles by 1-MCP treatment is consistent with previous assertions that the climacteric rise in ethylene formation, CO_2 synthesis, and volatile ester production in climacteric tissues is an ethylene-dependent process (Burg and Burg, 1962; Defilippi et al., 2005; Fan et al., 1998; Golding et al., 1999; Saftner et al., 2003; Song and Bangerth, 1996). In the present study, effective inhibition of climacteric ethylene biology in 'Golden Delicious' fruit by 1-MCP and the consequent continued suppression of ethylene-related gene expression, and ethylene, CO_2 , and volatile production increases are consistent with previous reports (Abdi et al., 1998; Defilippi et al., 2005; Fan and Mattheis, 1999; Fan et al., 1999; Golding et al., 1998; Lurie et al., 2002). The effect of 1-MCP treatment on 'Golden Delicious' apple fruit in this experiment was not reversed in the short-term by exogenously applied ethylene, indicating that the effects on respiration and volatile synthesis were related to ethylene perception

and not lack of ethylene presence, again consistent with known climacteric responses to ethylene action (Burg and Burg, 1962; Defilippi et al., 2005).

The pathway for ester formation is partially described and the final step has been relatively well characterized (Defilippi et al., 2005; Fellman et al., 2000). The extensive incorporation of exogenously applied precursors into esters indicates that 1-MCP treatment did not completely arrest AAT activity, which is required for ester biosynthesis. This suggests that the final step in ester biosynthesis, which is carried out by a family of AAT enzymes, is not under the complete control of ethylene and that a significant portion of the control of ester formation by ethylene is at the level of precursor formation. This suggestion is consistent with the detection of significant AAT activity in preclimacteric and 1-MCP-treated 'Greensleeves' apple fruit that were not actively synthesizing esters (Defilippi et al., 2005). Defilippi et al. (2005) showed that gene expression of one member of the AAT family (*mdAAT2*) was detectable prior to ripening, although AAT activity and *mdAAT2* gene expression both increased following the onset of ripening in this study. Souleyre et al. (2005) demonstrated a clone of AAT in apple (*MpAAT1*), fully characterized at the protein level to confer ester-forming capacity, to be present throughout fruit development. The supply of ester precursors was also considered the limiting factor for apple fruit harvested at the

Fig. 6. Total ion count (TIC) for volatiles produced by 'Jonagold' apple fruit treated with $1 \text{ mL} \cdot \text{L}^{-1}$ 1-MCP (open triangles) or left nontreated as controls (solid circles) following refrigerated air storage (0°C) for 1, 2, 3, 4, 5, or 7 months while being held at 22°C .

'Jonagold' fruit stored in RA was 2.73×10^9 while for treated fruit, the average maximum was 1.04×10^9 ($\approx 62\%$ lower than nontreated fruit). In the case of fruit stored under CA conditions, there was no statistically significant effect of 1-MCP on the maximum level of volatile production. The average maximum GC/MS response observed in nontreated fruit under CA conditions was 1.54×10^9 counts, a value close to the one obtained in fruit treated with 1-MCP and stored under RA conditions. Treated fruit under CA conditions had an average peak of 9.20×10^8 counts.

The data from Table 2 for the delay to onset of volatile formation and the delay to peak production were fitted with the same equation used to describe volatile formation (Fig. 9). The data were insufficient for trend analysis, so values for the constants of the fitted curves are not presented. However, the curves were useful for visualizing the data. The delay to the onset of volatile formation declined in what appeared to be a sigmoidal fashion, remaining steady or increasing slightly initially in storage, then declining. The decline appeared to be slightly more rapid for 'Jonagold' than for 'Redchief Delicious' fruit. The pattern for the delay to the peak in volatile biosynthesis appeared to be more linear than that for the delay to onset for 1-MCP-treated fruit (Fig. 9). The delay to the peak in volatile formation for nontreated fruit declined for RA-stored fruit, but remained relatively constant for CA-stored fruit.

Fig. 7. Total ion count (TIC) for volatiles produced by 'Redchief Delicious' apple fruit treated with 1 mL·L⁻¹ 1-MCP (open triangles) or left nontreated as controls (solid circles) following refrigerated air storage (0 °C) for 2, 3, 4, 5, or 7 months while being held at 22 °C.

prelimacteric stage and stored under CA conditions (Bartley et al., 1985; Brackmann et al., 1993). The presence of prelimacteric ester forming capacity and substrate limitation is also found in banana (*Musa* L., AAA group, Cavendish subgroup, cv. Valery), for which one AAT clone (gi:10187178) in banana and in vivo AAT activity were readily detectable well in advance of the onset of fruit ripening although activity and gene expression both increased with ripening (Jayanty et al., 2004).

It is interesting that the application of either alcohol (1-butanol) or acid (butyric acid) enhanced ester formation in 1-MCP-treated fruit given that only one of two co-substrates was added at a time. Evidently, the capacity existed in the nonripening tissue to synthesize or otherwise make available a small amount of complementary two-carbon acid or alcohol co-substrates to make the esters ethyl butanoate and butyl acetate despite little to no active ester biosynthesis in the native tissue. The relatively high production rate of butyl butanoate in response to the addition of either butanol or butyric acid indicates that the enzymatic capacity to interconvert alcohol and acid precursors existed in the tissue, again suggesting that much of the machinery for ester biosynthesis (e.g., alcohol dehydrogenase, aldehyde dehydrogenase, and alcohol acyl transferase) is in place and active in nonripening tissue. Finally, the induction of hexyl acetate by the addition of butyl substrates of AAT is intriguing given that neither the acetate

or hexanol co-substrates needed for its formation were provided. Hexyl acetate induction by both butanol and butanoic acid may reflect a sensitivity of the ester synthesis pathway to pathway activity.

The duration of the effect of 1-MCP on delaying the initiation of ethylene-dependent ripening is a function of the crop species, ripening stage, storage temperature, the presence of ethylene or ethylene analogues, the duration of the 1-MCP treatment, and the concentration of the 1-MCP. Ripening inhibition by response-saturating levels of 1-MCP (or diazocyclopentadiene) has been reported to last 5–15 d for tomato (*Lycopersicon esculentum* Mill.) fruit (Mir et al., 2004; Nakatsuka et al., 1997; Sisler and Serek, 1997; Tian et al., 1997) and 12–25 d for banana fruit (Golding et al., 1998). In apple, Mir et al. (2001) demonstrated that the inhibition of fruit softening for 'Redchief Delicious' by a single dose of 1-MCP lasted ≈20 to 40 d at 20 °C and ≈100 d at 0 °C. The findings in the current study for the delay in volatile formation at 22 and 0 °C are consistent with these time scales. Repeated or continuous doses of 1-MCP at levels that saturated its response appeared to arrest indefinitely some aspects of ripening in tomato and apple (Jayanty et al., 2004; Mir et al., 2004). As the duration of the 1-MCP treatment increased, the delay in the onset of ripening increased for tomato (Mir et al., 2004). The impact of 1-MCP treatment duration on the recovery period for apple ripening is not known.

The effects of 1-MCP treatment on delaying the onset and reducing the rate of volatile production in apple fruit at 0 months RA storage were similar to the effects found for banana (Golding et al., 1998, 1999) and apple (Kondo et al., 2005) fruit. The delay in the recovery of volatile biosynthesis for 'Delicious' and 'Golden Delicious' apple held at 20 °C was reported to be ≈21 and 14 d, respectively (Kondo, et al., 2005). This agrees reasonably well with the delay of ≈25 and 40 d, respectively, for 'Jonagold' and 'Redchief Delicious' fruit in the current study. The greater duration of 1-MCP effectiveness in the current study may be related to the influence of cultivar or differences in fruit maturity at the time of treatment. In the cited study, internal ethylene levels at the time of treatment were ≈5- to 20-fold higher than in the current study.

The 1-MCP-induced delay in the recovery of volatile synthesis for apple fruit stored at low temperature (0 °C), then transferred to room temperature (22 °C) has not been previously reported to our knowledge. The delay in volatile synthesis for fruit stored in RA reached 0 d poststorage after about 5 months (150 d) storage, which roughly agrees with the delay in softening brought about by a single 1-MCP treatment of 'Redchief Delicious' fruit stored at 0 °C (Mir et al., 2001). Important among the findings in the

Fig. 8. Total ion count (TIC) for volatiles produced by 'Jonagold' and 'Redchief Delicious' apple fruit treated with $1 \text{ mL} \cdot \text{L}^{-1}$ 1-MCP (open triangles) or left non-treated as controls (solid circles) following controlled atmosphere storage (1.5 kPa O_2 , 3 kPa CO_2 , 0°C) for 2, 4, or 7 months while being held at 22°C .

current study is that the impact of 1-MCP on volatile synthesis does not appear to diminish at low temperature for at least the first 30 d in RA storage and for the first 60 d in CA. Afterwards, the decline in the delay in volatile synthesis suggests a decline in the 'resistance' to ripening initially imposed by 1-MCP. It is possible that 1-MCP may occupy all available ethylene receptors at the preclimacteric stage in apple fruit, initially suppressing expression of new receptors. However, as 1-MCP dissociates and/or new receptors form, the extent of metabolic changes required to bring the fruit to an ethylene responsive state apparently declines, thus reducing the time needed for the recovery of volatile synthesis.

The residual effects of 1-MCP on the initiation of volatile recovery may be a problem if fruit are marketed soon after treatment, especially for cultivars like 'Gala' for which aroma constitutes a significant quality component (White, 1991). It appears likely that given the current apple distribution system in the United States, most fruit emerging from RA or CA storage would be through the retail segment and in the consumer's hands well before volatile

Fig. 9. Number of days during post-storage holding at 22°C required for the onset (A) and peak (B) in volatile biosynthesis by 'Jonagold' apple following storage in refrigerated air (circles) and controlled atmosphere (1.5 kPa O_2 , 3 kPa CO_2) (squares) and by 'Redchief Delicious' apple fruit stored in refrigerated air (triangles). Fruit were treated with $1 \text{ mL} \cdot \text{L}^{-1}$ 1-MCP (solid symbols) or were left non-treated as controls (open symbols). The time required for onset of volatile biosynthesis for control fruit was 0 d for both cultivars following all storage durations with the exception of 0 months storage for 'Redchief Delicious', when 5 d passed before volatile production commenced.

recovery would commence for fruit held in RA 0 to 3 months and in CA for 7 months or less. However, it must be acknowledged that a significant quantity of aroma-related volatiles in apple is produced via cell disruption occurring during the process of mastication (Paillard, 1986). The fact that autonomous and disruption-dependent volatile synthesis are responsible for aroma perception may explain the relatively small impact of 1-MCP on aroma perception for 1-MCP-treated 'Anna' fruit (Lurie et al., 2002), which were cut prior to sensory assessment. The lack of a more significant impact in this study may also reflect a less complete inhibition of volatile formation by 1-MCP treatment than in the current study. The impact of 1-MCP on the capacity of apple fruit to produce volatiles requiring tissue disruption has received limited attention. Defilippi et al. (2004, 2005) found that 1-MCP treatment of 'Greensleeves' apple fruit did not markedly reduce saturated and unsaturated 6-carbon aldehydes resulting from lipoxygenase activity in disrupted cells.

The reduced maximum rate of volatile biosynthesis following 1-MCP and CA treatments is not easily explained based on ethylene biology alone given that, in the current study, ethylene biosynthesis recovered following CA and 1-MCP treatments (data not shown). The data suggest that permanent changes in gene expression and metabolism are induced by these treatments when applied prior to ripening. The shift in volatile forming capacity might reflect global shifts in metabolic behavior or might result from more specific effects on the substrates and enzymes

essential for formation of esters (Patterson et al., 1974). The importance of reduced maximal volatile formation in terms of sensory quality may be minimal. The peak in production does not necessarily correspond with the best eating quality and may, in fact, be more indicative of the onset of senescence. Saftner et al. (2005) found that softening of fruit of several apple cultivars held in RA storage preceded or was coincident with the peak in volatile synthesis. As the duration of storage increased, the number of days between the onset of volatile formation and the peak in volatile biosynthesis did not appear to increase or decrease for 1-MCP-treated fruit. The implication is that the processes associated with volatile recovery were relatively similar throughout storage and independent of the processes governing the delay to onset of volatile recovery.

The data here document that 1-MCP, through its action on ethylene perception, delays the onset of volatile production in a temperature-dependent manner and permanently suppresses the maximal amount of volatile synthesis in a manner similar to CA storage. For 'Jonagold' and 'Redchief Delicious' fruit, the delay in the recovery of volatile formation following removal from storage conditions exceeded 3 weeks for the first 2 or 3 months in RA and for the first 7 months in CA. The implication is that some components of fruit aroma character may be compromised when fruit are consumed during these marketing periods. A detailed study of the ontogeny of fruit quality perception or 1-MCP-treated fruit may be needed to assess whether the documented reductions in volatile production shown here result in important changes in sensory quality and/or product value.

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