Fruit Quality, Fermentation Products, and Activities of Associated Enzymes During Elevated CO₂ Treatment of Strawberry Fruit at High and Low Temperatures

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ABSTRACT. The effects of postharvest treatments of air and 20 kPa CO₂ (in air) at 2 or 20 °C on color, firmness, accumulations of acetaldehyde, ethanol, and ethyl acetate, activities of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) activity, and expression of an ADH gene were studied in strawberry fruit (*Fragaria xananassa* Duch. cv. Jewel). CO₂ treatment enhanced strawberry fruit firmness at 2 °C but not 20 °C, while the rate of color changes was affected by CO₂ treatment at 20 °C but not at 2 °C. Temperature also affected the accumulation of acetaldehyde, ethanol and ethyl acetate in CO₂-treated fruit. All three compounds accumulated in fruits at 2 °C. At 20 °C, ethanol accumulated slightly by day 6, although ethyl acetate accumulated in fruit from both atmospheres. PDC enzyme activity was higher in CO₂-treated fruit than their air-treated control at 2 °C but not at 20 °C. ADH activity and ADH mRNA accumulation of the CO₂-treated berries were higher than in air at 20 °C but not 2 °C. The results, overall, indicate that patterns of change among gene expression, enzyme activities, and fermentation product accumulation were not consistent.

The postharvest tolerances of most commercially important fruit and vegetables to low O₂ and elevated CO₂, and the optimum atmosphere combinations to achieve maximum product storability, have been established. However, wide variations in tolerances to storage atmospheres exist within and among species, cultivars, organ types, and developmental stages, these variations being a function of both metabolic and physical factors (Beaudry, 2000; Watkins, 2000). When a harvested product is subjected to atmospheres outside safe limits at any temperature/time combination, certain physiological disorders may develop. Off-flavor, usually associated with accumulation of the fermentation products, acetaldehyde and ethanol, is often detected before visible damage and sometimes results in rejection of produce in the marketplace.

Accumulation of acetaldehyde and ethanol is a major metabolic response of plant tissues to anoxia or severe hypoxia. When O₂ is limited, electron transport and oxidative phosphorylation are inhibited (Kennedy et al., 1992), and fermentation then provides a mechanism for pyruvate oxidation. In the fermentation pathway, acetaldehyde is produced by pyruvate decarboxylation via pyruvate decarboxylase (PDC), and is then converted to ethanol by alcohol dehydrogenase (ADH). In plant systems, such as fruit, esters also are produced by esterification of alcohols, e.g., ethyl acetate is formed from ethanol and acetyl CoA by the activity of alcohol acyltransferase (AAT) (Ke et al., 1994a; Perez et al., 1996).

Most research on atmosphere effects on plant metabolism has concentrated on metabolism of growing plants, often of roots, under anoxia (Geigenberger, 2003). In fruit and vegetables the focus has been on fermentation metabolism under low O₂ partial pressures (Chen and Chase, 1993; El-Mir et al., 2001; Imahori et al., 2002). Because most products respond optimally to low O₂, relatively

less is known about fermentative metabolism in harvested fruit and vegetables under elevated CO₂ conditions. The strawberry has received attention, however, because treatments with 15–20 kPa CO₂ are used commercially to maintain fruit firmness, slow red color development, and reduce decay incidence (Harvey, 1981). Moreover, CO₂ treatments have been shown to enhance rather than simply maintain firmness of strawberries (Larsen and Watkins, 1995a; Plochanski, 1982; Smith, 1992; Smith and Skog, 1992; Watkins et al., 1999). However, development of off-flavors can occur under elevated CO₂ conditions (Ke et al., 1991, 1994a; Larsen and Watkins, 1995a, 1995b). The extent of fermentation is affected greatly by cultivar, with some showing no accumulation of fermentation products while others accumulate large amounts under the same conditions (Fernandez-Trujillo et al., 1999; Pelayo et al., 2003; Watkins et al., 1999).

Ke et al. (1994a) found that acetaldehyde accumulation in strawberries exposed to CO₂ atmospheres was much greater in 21 kPa O₂ than in 0.25 kPa O₂ and that accumulation was similar in 0.25 kPa O₂ irrespective of CO₂ concentration. Ethanol accumulation, in contrast, was similar in all controlled atmosphere (CA) treatments. PDC activity was also similar in all CA treatments, whereas ADH activity was slightly elevated in 50 kPa CO₂ in 0.25 kPa and 21 kPa O₂, but markedly stimulated in 0.25 kPa O₂ without CO₂. Thus, low O₂ inhibited the increase of acetaldehyde caused by elevated CO₂, while elevated CO₂ inhibited the increased ADH activity caused by low O₂. Comparable results for fermentation product accumulation and enzyme activities also were shown by Ke et al. (1994b) for post- and pre-climacteric pear fruit, respectively. Fernandez-Trujillo et al. (1999) found that strawberry cultivars that did not accumulate fermentation products in response to 20 kPa CO₂ (in air) had higher activities of PDC and ADH than to air, while the cultivars that accumulated fermentation products had lower PDC activity or similar ADH activity in 20 kPa CO₂ than in air.

Information from other plant systems is limited. Acetaldehyde and ethanol concentrations increased in cherimoya (*Annona cherimola* Mill.) kept in 20 kPa CO₂, but activation of

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the fermentation pathway was due to greater increases in PDC activity than ADH activity compared with air storage (Muñoz et al., 1997). Increased ethanol accumulation in CO₂-treated sweet potato [*Ipomoea batatas* (L.) Lam.] roots also was associated with enhanced PDC activity rather than ADH activity (Chang et al., 1983). Even though some speculation on the cause of PDC or ADH enzyme activity increase under anaerobic conditions has been made (Ke et al., 1994a, 1995), there do not appear to be any published reports of CO₂ effects on fermentation pathway enzymes at the transcriptional level in the literature.

The objective of this study was to investigate the enzyme activities of PDC and ADH, and gene expression of ADH, in relation to changes in fermentation product accumulation, under 20 kPa CO₂ or air treatments at 2 and 20 °C.

Materials and Methods

PLANT MATERIAL. ‘Jewel’ strawberry fruit were harvested at the mature-orange stage of ripeness from strawberry plants growing at Cornell Orchards. The berries were sorted and 20-fruit samples were placed in 1-L glass jars. Three replicate samples of fruit were taken before any treatment as “at harvest” samples.

CO₂ TREATMENTS. Fruit were kept at 2 or 20 °C for 3 h before exposure to CO₂. Atmospheres were applied to the glass jars with inlet and outlet ports attached to a flow-through system (50 mL·min⁻¹) providing humidified air or 20 kPa CO₂ in air at both temperatures. The CO₂ and O₂ concentrations were checked daily by a Fisher gas partitioner (model 1200; Fisher Scientific, Springfield, N.J.). The 20-kPa CO₂ treatment was maintained within 1 kPa of the target partial pressure throughout the experiment. The berries were stored at 2 °C for up to 9 d, but at 20 °C for only 6 d because decay development in air-treated fruit samples became excessive after this time. Sample jars, in triplicate, were removed at 1, 2, 3, 6, and 9 d time points. From each replicate, 10 berries were used to measure fruit firmness and color, and three fruit were used for enzyme activity assays on days 1, 3, 6, and 9. Each replicate was processed immediately upon removal to minimize warming of the fruit. The remaining berries were sliced rapidly into liquid N₂. The frozen fruit were then kept at -80 °C for later RNA extraction and for measurements of fermentation products.

COLOR AND FIRMNESS MEASUREMENTS. Strawberry fruit skin color was measured three times on each fruit with a Minolta Chroma Meter (model CR-100; Minolta Corp., Ramsey, N.J.), using Illuminant C (6774K) attached to a DP-100 Data Processor. The measurements are expressed as chroma (intensity of color), hue angle (actual color, or redness), and L* value (color intensity changing from light to dark, or brightness).

Flesh firmness was measured on each fruit with intact skin using a Force Five Pressure Tester (model FDV-30; Wagner Instruments, Greenwich, Conn.) fitted with a 7-mm flathead (FDL/A2) probe (Watkins et al., 1999).

FERMENTATION PRODUCT MEASUREMENTS. Frozen samples were ground to a powder in liquid N₂ and a duplicate set of 5-g samples was weighed into 20-mL vials, which were sealed and placed into a water bath maintained at 40 °C for 20 min. Headspace samples of 0.5 mL were removed from the vials with a gas-tight syringe and injected immediately into a gas chromatograph (model 5890; Hewlett-Packard, Wilmington, Del.) fitted with a 15 m × 0.53 mm Stabilwax (Resteck, Bellefonte, Pa.) wide bore capillary column with a coating thickness of 1.0 μm and attached to a Hewlett-Packard 3396A integrator as described by Fernandez-

Trujillo et al. (1999). Acetaldehyde, ethanol, and ethyl acetate standards were prepared by weighing the respective compound into 20 mL vials to provide a range of concentrations 0–32, 0–357, and 0–148 mg·kg⁻¹, respectively. The detection limits for acetaldehyde, ethanol, and ethyl acetate were 0.2, 0.1, and 0.5 mg·kg⁻¹, respectively.

PDC AND ADH ACTIVITY MEASUREMENTS. Flesh tissue from three-fruit samples was bulked and 3 g of tissue homogenized in a cold mortar and pestle with 10 mL of 100 mM 2-(*N*-morpholino) ethane-sulfonic acid (MES) buffer (pH 6.5), containing 2% (w/v) soluble polyvinylpyrrolidone, 0.1 mM ZnSO₄, 10 mM β-mercaptoethanol, 1 mM benzamidine hydrochloride, and 5 mM ε-amino-*n*-caproic acid. The extract was centrifuged at 15,000 *g*_n at 4 °C for 20 min and the supernatant was used as the enzyme source. PDC and ADH activities were assayed by measuring NADH oxidation as indicated by the decrease in absorbance at 340 nm over time using a spectrophotometer (model DU-7400; Beckman Instruments, Fullerton, Calif.).

NORTHERN BLOT ASSAYS. RNA was extracted from 5-g samples according to Manning (1991). RNA gel electrophoresis and blotting procedures were based on Sambrook et al. (1989). Twenty micrograms of total RNA was used for each hybridization. The ADH probe (Z48234), a 1444-bp cDNA from apple (*Malus domestica* Borkh.) with 85% nucleotide sequence similarity to strawberry ADH gene (X15588) (Reid et al., 1996), was obtained from the Horticulture and Food Research Institute of New Zealand, Auckland. The insert was in a pAADH vector (pBluescript II SK). The cDNA fragments were cut out with restriction enzymes and labeled by using a random primer labeling kit from Boehringer Mannheim. Loading was determined using an 18S probe. Hybridization conditions were 15 min with 5× sodium chloride/sodium citrate (SCSC), 0.5% sodium dodecyl sulfate (SDS) at room temperature, 15 min with 1× sodium chloride/sodium citrate (SCSC), 0.5%–1.0% SDS at 42 °C, and 15 min with 0.1× SCSC, 1.0% SDS at 42 °C. The amount of the washing solution used was 0.5 mL·cm⁻² of the membrane area. Membranes were wrapped in plastic wrap and exposed to X-ray film for 25–60 h at -20 °C in a cassette fitted with an intensifying screen. Band intensity on the X-ray film was measured by using the “denso spoto” program of an AlphaImager (Alpha Innotech, Miami, Fla.) with version 5.5 software.

STATISTICAL ANALYSIS. The experiment, which was carried out once, consisted of four treatments, where fruit were exposed to air or CO₂ at 2 or 20 °C for 9 and 6 d, respectively. All means are the average of three replicates. Because exposure time was different, data were analyzed as a balanced design for both temperatures (6 d) or for 20 °C only (9 d) as appropriate. Data were subjected to General linear model (GLM) analysis of variance using Minitab software V 11.13 (Minitab, State College, Pa.).

Results

FRUIT COLOR. Color change during ripening of strawberry fruit in air at 20 °C was associated with decreasing brightness (L*), and decreasing red intensity (less vivid) and increasing redness as indicated by decreasing chroma (C*) and hue angle (h°), respectively (Fig. 1). These changes were slowed but not prevented by CO₂ treatment at 20 °C. The brightness and h° decreased slightly over time in fruit at 2 °C, but there was no significant effect of atmosphere on any color parameter (Fig. 1).

FRUIT FIRMNESS. Firmness of the berries increased during storage at 2 °C in air but to a much greater extent in CO₂ (Fig. 2).

Fig. 1. Color measurements ($\pm$ SE), expressed as (A) brightness (L^*), (B) chroma (C^*), and (C) hue angle (h°) of 'Jewel' strawberry fruit kept in air (open symbols) or 20 kPa CO₂ (closed symbols) at 2 °C (□, ■) or 20 °C (△, ▲) for up to 6 and 9 d, respectively. For air and CO₂ treatments at 2 °C for 9 d, only the effect of time was significant ($P \leq 0.001$) for L^* and h° . For air and CO₂ treatments at 2 and 20 °C for 6 d, all main effects and CO₂ $\times$ temperature and time $\times$ temperature interactions were significant ($P \leq 0.001$) for C^* , L^* , and h° . A CO₂ $\times$ day interaction was significant only for C^* , and no higher-order interaction was detected ($P \leq 0.05$).

The firmness of berries in CO₂ at this temperature averaged 9.6 N compared with 8.0 N in air. At 20 °C firmness in CO₂-treated fruit increased slightly compared with air on day 1, but there was no subsequent effect of atmosphere as fruit softened over time.

FERMENTATION PRODUCT ACCUMULATION. At 2 °C, acetaldehyde and ethanol accumulated in fruit treated with CO₂, with rapid increases in concentration occurring by day 1 of treatment, but then no additional increases occurred until after day 6 (Fig. 3A–B). Acetaldehyde and ethanol accumulations were not detected in fruit from any other treatment, except for a small but significant ethanol accumulation in CO₂-treated fruit at 20 °C on day 6 (Fig. 3B). Ethyl acetate accumulated in CO₂-treated fruit, but not air-treated fruit, at 2 °C (Fig. 3C). However, ethyl acetate

Fig. 2. Fruit firmness ($N \pm$ SE) of 'Jewel' strawberry fruit kept in air (open symbols) or 20 kPa CO₂ (closed symbols) at 2 °C (□, ■) or 20 °C (△, ▲) for up to 6 and 9 d, respectively. For air and CO₂ treatments at 2 °C for 9 d, effects of time ($P = 0.032$) and atmosphere ($P \leq 0.001$) were significant. For air and CO₂ treatments at 2 and 20 °C for 6 d, all main effects and a CO₂ $\times$ temperature interaction were significant ($P \leq 0.001$). No other interactions were significant.

accumulated in both air and CO₂-treated fruit kept at 20 °C after 2 to 3 d of treatment.

PDC AND ADH ENZYME ACTIVITIES. At 2 °C, the activity of PDC in CO₂-treated fruit was consistently higher than that of air-treated fruit, averaging 0.46 and 0.35 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ fresh weight, respectively. At 20 °C, PDC activities of fruit increased during storage, but were not affected by atmosphere (Fig. 4A).

ADH activity was not affected by atmosphere or storage period at 2 °C, but was higher in CO₂-treated fruit in air-treated berries at 20 °C, averaging 0.72 and 0.56 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ fresh weight (Fig. 4B).

ADH GENE EXPRESSION. ADH mRNA accumulation was higher in CO₂-treated than in air-treated fruit over all time points at 20 °C (Fig. 5). At 2 °C, the ADH transcript accumulation was slower over time and relative differences between hybridization for air-treated and CO₂-treated fruit were small.

Discussion

FRUIT COLOR. Red color development during ripening of strawberry fruit, which is largely a function of accumulation of water-soluble anthocyanin pigments, or pelargonidin 3-glucosides and cyanidin, is associated with decreasing brightness, chroma, and hue (Gil et al., 1997). Anthocyanin accumulation in strawberry fruit is related to increased activity of two enzymes: phenylalanine ammonia lyase (PAL), the first enzyme in the phenylpropanoid pathway, and uridine diphosphate glucose:flavonoid O3-glucosyl-transferase (GT), the final enzyme in the anthocyanin biosynthetic pathway (Given et al., 1988; Hyodo, 1971).

At 20 °C, L^* , C^* , and h° of the skin of strawberries decreased rapidly, but at a slower rate in fruit treated with CO₂ compared with air (Fig. 1). In contrast, at 2 °C, these variables changed slightly or remained constant over time, and they were not affected by CO₂ exposure. Little is known about treatments of fruit with CO₂ at higher storage temperatures, although Nuñez et al. (2002) also found that CO₂ delayed fruit coloring more effectively at 10 °C than at 4 °C. Effects of CO₂ on delaying color change of strawberries stored at low temperatures can be small or negligible (Smith and Skog, 1992; Watkins et al., 1999), or significant (Gil et al., 1997; Holcroft and Kader, 1999a; Ke et al., 1991; Nuñez et al., 2002). Differences among results may relate to cultivar, maturity of treated fruit, as well as methods used to determine

Fig. 3. Concentrations ($\mu\text{g}\cdot\text{kg}^{-1} \pm \text{SE}$) of (A) acetaldehyde, (B) ethanol, and (C) ethyl acetate of 'Jewel' strawberry fruit kept in air (open symbols) or 20 kPa CO_2 (closed symbols) at 2 °C ($\square$, $\blacksquare$) or 20 °C (Δ , $\blacktriangle$) for up to 6 and 9 d, respectively. Within 2 °C for 9 d, the higher-order interaction ($\text{CO}_2 \times \text{time}$) was significant ($P \leq 0.001$) for all fermentation products. Within 6 d, the highest-order interaction was significant ($P = 0.014$) for acetaldehyde. For ethanol, only CO_2 and temperature, and the $\text{CO}_2 \times \text{temperature}$ interaction were significant ($P \leq 0.001$). For ethyl acetate, CO_2 , temperature and time, and $\text{CO}_2 \times \text{temperature}$ interaction were significant ($P \leq 0.001$).

color changes. Although Smith and Skog (1992) and Watkins et al. (1999) did not detect cultivar effects on color responses to CO_2 atmospheres, Pelayo et al. (2003) found that CO_2 delayed color changes in one of three cultivars. The effects of CO_2 on delaying color change were greater in three-quarter colored than fully red fruit (Nuñez et al., 2002).

Delayed color change in CO_2 -treated strawberry fruit may result from inhibition of PAL and GT activities (Holcroft and Kader, 1999a). It has also been suggested that losses of anthocyanin may occur as a result of co-pigmentation and increases of pH in CO_2 -treated tissues (Gil et al., 1997; Holcroft and Kader, 1999b). Although CO_2 hydration and production of HCO_3^- and H^+ may reduce intracellular pH (Bown, 1985), higher rather than

Fig. 4. Enzyme activities ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ fresh weight $\pm \text{SE}$) of (A) pyruvate dehydrogenase (PDC) and (B) alcohol dehydrogenase (ADH) of 'Jewel' strawberry fruit kept in air (open symbols) or 20 kPa CO_2 (closed symbols) at 2 °C ($\square$, $\blacksquare$) or 20 °C (Δ , $\blacktriangle$) for up to 6 and 9 d, respectively. For PDC, effects of CO_2 ($P \leq 0.001$) and time ($P = 0.034$) within 9 d at 2 °C, and CO_2 ($P = 0.051$), temperature ($P = 0.006$), and time ($P = 0.001$), and time $\times$ temperature ($P = 0.037$) within 6 d were significant. For ADH, no main effects or interactions were significant within 9 d at 2 °C, while within 6 d, effects of CO_2 ($P = 0.002$), temperature ($P \leq 0.001$), and time ($P = 0.017$), $\text{CO}_2 \times \text{temperature}$ ($P = 0.029$) and time $\times$ temperature ($P = 0.001$) were significant.

lower pH has been observed in CO_2 -treated strawberry tissues (Holcroft and Kader, 1999a, 1999b). Responses to elevated CO_2 might be different in a fruit with a large, acidic vacuole such as strawberry than in fruit with a higher pH or in leafy tissues that do not accumulate high organic acid concentrations (Holcroft and Kader, 1999b).

FRUIT FIRMNESS. In contrast to color change of strawberries, the effects of CO_2 on firmness were more pronounced at 2 °C than at 20 °C (Fig. 2). Smith (1992) also found firmness enhancement at cold temperatures and none at 21 °C. Increased firmness of flesh in CO_2 -treated strawberry fruit rather than maintenance of harvest firmness is now recognized (Larsen and Watkins, 1995a; Plocharski, 1982; Smith, 1992; Smith and Skog, 1992; Watkins et al., 1999). Firmness of fruit is related to a number of cellular characteristics, including adhesion between neighboring cells, cell fragility, and internal turgor pressure (Harker et al., 1997). Cell wall analyses of air and CO_2 -treated strawberry fruit indicated that CO_2 treatment reduced the amount of pectin in the water-soluble fractions, and increased the pectin amount in other fractions (Plocharski, 1982; Siriphanich, 1998). However, no differences in pectin composition of cell wall fractions were detected by

Fig. 5. Expression of alcohol dehydrogenase (ADH) transcripts. (A) Hybridization of 20 μ g total RNA extracted from 'Jewel' strawberry fruit kept in air or 20 kPa CO₂ at 20 or 2 °C for up to 6 and 9 d, respectively, with an ADH cDNA probe. T₀ represents the d 0 or "at harvest" sample (B) Loading of RNA as indicated by an 18S probe.

Smith (1992). CO₂-induced firmness enhancement in strawberry may be due to changes in the pH of the apoplast that promote the precipitation of soluble pectins and thus enhance cell-to-cell bonding in the fruit (Harker et al., 2000). It is likely that the CO₂-induced firmness enhancement in strawberry is a physical phenomenon, although the involvement of cell wall disassembly enzymes is possible. Enzymes that may be involved in cell wall softening of strawberry fruit include polygalacturonase (PG) (Redondo-Nevado et al., 2001), pectin esterase (PE) (Castillejo et al., 2004), pectic lyase (Jimenez-Bermudez et al., 2002), and expansin (Civello et al., 1999). No information about the effect of elevated CO₂ on these enzymes is available for strawberry fruit, although PG gene expression is reduced by CO₂ treatment (unpublished data).

FERMENTATION PRODUCTS. Many studies have shown that acetaldehyde, ethanol and ethyl acetate are accumulated under low O₂ and/or high CO₂ conditions in strawberry fruit at low temperatures (Fernandez-Trujillo et al., 1999; Ke et al., 1991, 1994a; Larsen and Watkins, 1995b). These accumulations are associated with off-flavor development in the fruit (Ke et al., 1991; Larsen and Watkins, 1995b). Our study, which incorporated a higher temperature of 20 °C showed that temperature affected the fermentation product accumulation significantly. At 2 °C, acetaldehyde, ethanol and ethyl acetate accumulated in CO₂-treated fruit. However, at 20 °C, acetaldehyde accumulation was not detected, and only a small ethanol accumulation was found on day 6 for CO₂-treated fruit (Fig. 3B). The activities of PDC and ADH, the enzymes that catalyze the formation of acetaldehyde and ethanol, respectively, were higher at 20 °C than that of 2 °C. Moreover, ethyl acetate accumulated in CO₂-treated fruit kept at 20 °C after 2 to 3 d of treatment.

Studies on effects of elevated CO₂ or low O₂ on fermentation processes at warm temperature have been published (Imahori et al., 2002; Muñoz et al., 1997), but none have compared responses to CO₂ at temperatures such as 20 °C with those at lower temperatures. Woodward and Topping (1972) found less CO₂ injury in fruit kept at 3 °C than earlier reported for 0 °C, and suggested that tolerance of fruit to elevated CO₂ may be higher at higher temperatures. Although ethyl acetate concentrations are below those that are associated with off-flavor development (Larsen and Watkins, 1995b), it is doubtful that 20 °C would be a commercially useful temperature for strawberry storage. Faster respiration rates and loss of quality would be expected at higher storage temperatures, and Nuñez et al. (2002) found that fruit stored in CA at 4

°C were of higher quality than those stored at 10 °C.

Changes in the concentrations of metabolites are typically a function of the activity of enzymes that mediate production and utilization, but in the case of acetaldehyde, ethanol, and ethyl acetate, losses also may occur due to volatilization. Thus, the absence of acetaldehyde and ethanol accumulation in fruit in CO₂-treated fruit at 20 °C could be a function of their rapid metabolism to other end products and diffusion from the fruit. In root systems, ethanol leakage to the soil is associated with tolerance of plants to anaerobic conditions (Drew, 1997), and Fernandez-Trujillo et al. (1999) suggested that a similar mechanism could exist for strawberry fruit under elevated CO₂ conditions. Volatilization of fermentation products would be faster at higher than lower storage temperatures, but a full accounting of substrate flux through the fermentative pathways is necessary to clarify differences in metabolism of these products under elevated CO₂ conditions at the two storage temperatures.

PDC AND ADH ENZYME ACTIVITY AND ADH GENE EXPRESSION. The present study indicates that the involvement of PDC and ADH activities in strawberry fruit responses to elevated CO₂ is complex. PDC activity was higher overall at 20 °C than at 2 °C, but was higher in the CO₂ treated fruit only at the lower temperature (Fig. 4). In contrast, ADH activity was not affected by atmosphere at 2 °C, but was higher in the CO₂ treatment at 20 °C (Fig. 4). Overall, activity of this enzyme was highly correlated with abundance of ADH transcripts (Fig. 5).

Changes in PDC and ADH activities in response to CO₂ treatment at 2 °C (Fig. 4) are consistent with other reports on strawberry. PDC and ADH activities were only slightly increased by CO₂ treatments of 50 kPa (Ke et al., 1994a). Fernandez-Trujillo et al. (1999) found that PDC and ADH activities increased in cultivars that did not accumulate fermentation products, but decreased in cultivars that did accumulate these products. Changes of activities in vivo may be different, however, from those indicated by total enzyme assays of strawberry extracts, and isoenzymes were not investigated in this study. Low O₂ storage of avocado (*Persea americana* Mill.) results in induction of new ADH isoenzymes even though little change in total ADH activity is detected (El-Mir et al., 2001; Ke et al., 1995). Electrophoresis may remove an inhibitor of ADH and permit higher enzyme activity, and further experiments are required to elucidate these relationships for strawberry.

The activities of PDC and ADH did not correspond with the concentrations of their end products in strawberry fruit. Similar

discrepancies have been shown for strawberry (Fernandez-Trujillo et al., 1999; Ke et al., 1994a), sweet potato (Chang et al., 1983), bell pepper (*Capsicum annuum* L.) (Imahori et al., 2000; Imahori et al., 2002), and pear (*Pyrus communis* L.) fruit (Ke et al., 1994b), although Huang et al. (2002) found a strong correlation between PDC activity and acetaldehyde accumulation and a weak correlation between ADH activity and ethanol accumulation in sweet potato roots. Interestingly, ethanol production in hypoxic maize (*Zea mays* L.) root tips was correlated with ADH activity when the enzyme level was very low, but ethanol production was independent of ADH when its activity was high (Roberts et al., 1989).

In addition to molecular control of PDC and ADH, their activity is likely to be metabolically controlled by at least two major factors under low O₂ and elevated CO₂ conditions. The first of these is activation of PDC and ADH by a decrease in cytoplasmic pH. CO₂ treatment can decrease cytoplasmic pH (Bown, 1985; Ke et al., 1994b, 1995; Siriphanich and Kader, 1986) and often make it closer to the optimum pH for PDC and ADH of 6.0. Anaerobic conditions, for example, can reduce pH by 0.2–0.8 units (Bown, 1985). Another factor that will interact with effects of CO₂ on pH is temperature, as the solubility of CO₂ is higher at lower temperatures (Burton, 1974), and could further decrease the pH in treated fruit. However, proof of cytoplasmic pH decline in response to CO₂ has not been demonstrated in strawberries and the buffering capacity of the tissues may play an important role in homeostatic responses to imposed conditions. The second is via increased substrate concentration. PDC is an allosteric enzyme (Hubner et al., 1978) and its activity can be increased by a higher concentration of its substrate, pyruvate. The switch between oxidative phosphorylation and fermentation occurs at pyruvate where it can be metabolized by pyruvate dehydrogenase (PDH) or PDC. Glycolysis is induced under low O₂ conditions (Kennedy et al., 1992) and more pyruvate would therefore be produced. Activity of PDC is considered to be a key regulator of ethanolic fermentation under low O₂ stress (Huang et al., 2002; Tadege et al., 1999). The flux through the fermentation pathway is regulated by an interaction between PDH and PDC activities, and increased pyruvate might be a major driving force of ethanol fermentation (Imahori et al., 2002). High CO₂ causes pyruvate concentration increase in orange (Davis et al., 1973), but whether a similar mechanism whereby flux is controlled by a PDH and PDC activities under such conditions has not been demonstrated. In addition, CO₂ may affect NADH concentrations. NADH is a cofactor for the conversion of acetaldehyde to ethanol by ADH, as well as an activator of PDC and an inhibitor of PDH activity (Davies, 1980).

In conclusion, 20 kPa CO₂-treatment enhanced strawberry fruit firmness at 2 °C but not 20 °C, and delayed the more rapid rate of color change at 20 °C but not at 2 °C, where color changed little over the course of the experiment. Accumulations of acetaldehyde, ethanol and ethyl acetate in CO₂-treated fruit also were affected by temperature. At 2 °C, all three compounds accumulated, while at 20 °C, ethanol accumulated only slightly by day 6, and ethyl acetate accumulated in fruit at both atmospheres. PDC enzyme activity was higher, and acetaldehyde accumulated coincidentally, in CO₂-treated samples compared with those with air. Both ADH mRNA and ADH enzyme activity did not show differences in CO₂-treated samples from their air-treated control at 2 °C, but its product ethanol accumulated. At 20 °C, PDC enzyme activity of CO₂-treated berries was higher than at 2 °C, but acetaldehyde did not accumulate. Both ADH transcripts and ADH enzyme

activity were higher in CO₂-treated at 20 °C than that of 2 °C, but ethanol did not accumulate. These discrepancies may be related to differences in carbon flux that were not measured in this study, diffusion of the fermentation products, and as yet undescribed roles of isoenzymes.

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