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Regulation of Fermentative Metabolism in Avocado Fruit under Oxygen and Carbon Dioxide Stresses

Dangyang Ke, Elhadi Yahia[1], Betty Hess, Lili Zhou, and Adel A. Kader[2]

Department of Pomology, University of California, Davis, CA 95616

ADDITIONAL INDEX WORDS. *Persea americana,* pyruvate decarboxylase, alcohol dehydrogenase, lactate dehydrogenase, pyruvate dehydrogenase, anaerobic products, pH, NADH, ATP

ABSTRACT. 'Hass' avocado *(Persea americana* Mill.) fruit were kept in air, 0.25% O_2 (balance N_2), 20% O_2 + 80% CO_2, or 0.25% O_2 + 80% CO_2 (balance N_2) at 20 °C for up to 3 days to study the regulation of fermentative metabolism. The 0.25% O_2 and 0.25% O_2 + 80% CO_2 treatments caused accumulations of acetaldehyde and ethanol and increased NADH concentration, but decreased NAD level. The 20% O_2 + 80% CO_2 treatment slightly increased acetaldehyde and ethanol concentrations without significant effects on NADH and NAD levels. Lactate accumulated in avocados kept in 0.25 % O_2. The 80% CO_2 (added to 0.25% O_2) did not increase lactate concentration and negated the 0.25% O_2-induced lactate accumulation. Activities of PDC and LDH were slightly enhanced and a new isozyme of ADH was induced by 0.25% O_2, 20% O_2 + 80% CO_2, or 0.25% O_2 + 80% CO_2; these treatments partly reduced the overall activity of the PDH complex. Fermentative metabolism can be regulated by changes in levels of PDC, ADH, LDH, and PDH enzymes and/or by metabolic control of the functions of these enzymes through changes in pH, ATP, pyruvate, acetaldehyde, NADH, or NAD. Chemical names used: alcohol dehydrogenase (ADH), adenosine triphosphate (ATP), lactate dehydrogenase (LDH), nicotinamide adenine dinucleotide (NAD), reduced NAD (NADH), pyruvate decarboxylase (PDC), pyruvate dehydrogenase (PDH).

Short-term exposure of fruit to very low O_2 and/or very high CO_2 concentrations may have potential benefits for insect disinfestation, disease control, and alleviation of some physiological disorders. However, some undesirable physiological changes may also occur in plant tissues under stress conditions of O_2 and CO_2. Yahia and Carrillo-López (1993) observed exocarp and mesocarp injury on 'Hass' avocado fruit exposed to 0.1% to 0.4% O_2 + 50% to 75% CO_2 at 20 °C for longer than one day before ripening in air.

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[1]Current address: Universidad Autónoma de Queretaro, Centro Universitario, Cerro de Las Campanas, Queretaro 76040, México.

[2]To whom reprint requests should be addressed.

Plant responses to very low O_2 and/or very high CO_2 concentrations include induction of fermentation pathways, accumulation of succinate and/or alanine, and decreases in intracellular pH and ATP levels. One pathway of fermentative metabolism results in accumulation of acetaldehyde and ethanol catalyzed by the enzymes PDC and ADH, respectively (Ke et al., 1994). In some plant tissues, lactate accumulation results from fermentation and this is catalyzed by the enzyme LDH. The major function of fermentative metabolism is to use NADH and pyruvate when electron transport and oxidative phosphorylation are inhibited so that glycolysis can proceed. This will allow for the production of some ATP through substrate phosphorylation, which permits the plant tissues to survive temporarily. Kanellis et al. (1991) found that ADH isozymes could be induced by exposure of avocado fruit to 2.5%, 3.5%, or 5.5% O_2. Increased activities of PDC and ADH were observed when sweet potato, 'Bartlett' pear, lettuce, and strawberry were kept in low O_2 or high CO_2 concentrations (Chang et al., 1983; Nanos et al., 1992; Ke et al., 1993, 1994).In maize, barley, and rice, the increases in activities of PDC, ADH, and LDH by low O_2 have been found to be due to increased transcription and translation of the related genes, resulting in new mRNA synthesis and *de novo* synthesis of the corresponding enzyme proteins (Gerlach et al.,1982; Good and Crosby, 1989; Kelley, 1989).

In this study, we investigated the regulation of ethanol and lactate fermentation in avocado fruit in response to low O_2 and/or high CO_2 stresses.

Materials and Methods

MATERIALS AND TREATMENTS. 'Hass' avocado fruit were harvested at commercial maturity from an orchard near Santa Barbara, Calif., and shipped to our laboratory in Davis, Calif., where they were stored in air at 5 °C for <1 week before use for experiments. Nine fruit were placed in a 4-liter glass jar and ventilated with humidified air or a specified gas mixture at a continuous 100 ml-min^{-1} flow rate. The gas mixtures included 0.25% O_2 (+ 99.75% N_2), 20% O_2 + 80% CO_2, and 0.25% O_2 + 80% CO_2 (+19.75% N_2). Three replicates were used for each treatment. The fruit samples were kept in air or the specified atmospheres at 20 °C for 1, 2, or 3 days followed by transfer to air at 20 °C for another 3 days to observe the post low O_2 and/or high CO_2 effects.

EXTRACTION AND MEASUREMENTS OF FERMENTATION PRODUCTS. Volatiles were extracted by homogenizing 10 g fruit tissue in 15 ml of 0.1 M HC1 solution. The homogenate was stored at -40 °C for <1 month before use for analysis of acetaldehyde and ethanol by a gas Chromatograph (HP5890A; Hewlett Packard, Palo Alto, Calif.). To extract lactate, 6 g fruit tissue was added to a 50-ml centrifuge tube containing 12 ml of 1.8% (w/v) $Ba(OH)_2$. The sample was homogenized and then 12 ml of 2% (w/v) aqueous $ZnSO_4$ solution was added and immediately shaken for 15 sec. The mixture was filtered through four layers of cheesecloth and centrifuged for 15 min at 27,000 x *g.* The supernatant was decanted, filtered through four layers of cheesecloth, and used for analysis of lactate by the enzymatic method of Leshuk and Saltveit (1991). Recovery rate of lactate was 78% from the extraction procedure.

EXTRACTION AND ASSAYS OF FERMENTATION ENZYMES. For each replicate, 6 g tissue (six discs) were obtained from three fruit and homogenized in 20 ml of 100 mM 2-(A-morpholino)ethane-sulfonic acid (MES) buffer (pH 6.5) containing 2 mM dithiothreitol and

1% (w/v) PVP. The homogenate was filtered through four layers of cheesecloth and centrifuged at 27,000 x *g* for 10 min. The supernatant was retained as enzyme extract for the measurements of ADH, LDH, and PDC activities. ADH activity was measured by mixing 0.88 ml of 100 mM MES buffer (pH 6.5), 0.06 ml of l.6 mM NADH, 0.01 ml of enzyme extract, and 0.05 ml of 80 mM acetaldehyde. LDH activity was measured by mixing 0.79 ml of 100 mM MES buffer (pH 6.5), 0.025 ml of 100 mM 4-methylpyrazol, 0.025 ml of 100 mM KCN, 0.05 ml of 1.6 mM NADH, 0.06 ml of 50 mM Na pyruvate, and 0.05 ml of enzyme extract. For ADH and LDH, oxidation of NADH was measured by recording the decrease in absorbance at 340 nm using a spectrophotometer. PDC activity was assayed by measuring acetaldehyde production of the enzyme reaction. The PDC reaction mixture was obtained by mixing 3.2 ml of 100 mM MES buffer (pH 6.5), 0.5 ml of 5 mM thiamine pyrophosphate (TPP), 0.5 ml of 50 mM $MgCl_2$, 0.5 ml of enzyme extract, and 0.3 ml of 50 mM Na pyruvate in a 15-ml screw-cap test tube. The sample was incubated at 30 °C for 60 min and a 1-ml headspace gas sample was taken for analysis of acetaldehyde concentration by a gas Chromatograph. The conventional method of measuring PDC by coupling PDC and ADH reactions through using pyruvate and NADH as substrates did not work with avocado since its high LDH activity competed and interfered with the coupled PDC and ADH reactions. Enzyme activities of PDC, ADH, and LDH were expressed as mmoles of product formed or substrate used per min per gram fresh weight. For *in vitro* studies of enzyme kinetics, several assay pH values and concentrations of pyruvate, acetaldehyde, NADH, NAD, and ATP were used to study the changes in activities of PDC, ADH, and LDH.

EXTRACTION, ELECTROPHRESIS, AND STAINING OF ADH ISOZYMES. Three grams of fruit tissue (three discs) were homogenized in 12 ml extraction buffer and l g PVPP. The homogenate was filtered through four layers of cheesecloth and centrifuged at 27,000 x *g* for 10 min. The supernatant was retained for isozyme analysis by starch gel electrophoresis. The gels were prepared, run, and ADH isozymes stained according to Arulsekar and Parfitt (1986).

ISOLATION AND MEASUREMENTS OF NAD AND NADH. The method of Greenbaum et al. (1965) was modified. Six grams of fruit tissue (12 half discs) were frozen in liquid nitrogen and then ground to powder. The powder was transferred to a 50-ml centrifuge tube that contained 12 ml of 0.l N HC1 for extraction of NAD. Similarly, the other 12 half discs (6 g) were frozen in liquid nitrogen, ground to powder, and then placed in another 50-ml centrifuge tube that contained 12 ml of 0.l N NaOH for extraction of NADH. After 20 min on ice with periodic stirring, the tubes were soaked in boiling water for 2 min and immediately transferred to ice and kept there for 15 min. The samples were filtered through four layers of cheesecloth and then 1 ml of 500 mM potassium phosphate buffer (pH 7.5) was added to each tube. The samples were neutralized to pH 7.3-7.5 while stirring on ice by drop-wise addition of l N NaOH to the acid extract and l N HC1 to the alkaline extract, respectively. The samples were centrifuged at 27,000 x *g* for 10 min at 4 °C. The supernatant was decanted, filtered through four layers of cheesecloth, and kept on ice for immediate assays of NAD and NADH. NAD or NADH concentration of each sample was measured in a very sensitive cyclic enzyme reaction system where NAD or NADH was used as a rate limiting component. The reaction mixture was obtained by mixing 0.63 ml of 100 mM potassium phosphate buffer (pH 7.5), 0.03 ml of 60 mM phenazine methosulfate (PMS), 0.04 ml of 0.6 mM dichlorophenol indolphenol

(DCPIP), 0.1 ml of 95% ethanol, 0.1 ml of commercial ADH solution (5 units), and 0.1 ml of sample extract. In this system, ethanol is oxidized to acetaldehyde by ADH with the reduction of NAD to NADH. NADH transfers protons and electrons to PMS and NAD is recycled for the ADH reaction. The reduced PMS transfers protons and electrons to DCPIP. Reduction of the blue-colored DCPIP to colorless $DCPIPH_2$ was measured by recording the decrease in absorbance at 600 nm. A standard curve for NAD or NADH over a range of 0.002-0.015 mM was obtained for calculating NAD or NADH concentration of the sample. Recovery rates of NAD and NADH from the extraction procedures were 83% and 78%, respectively.

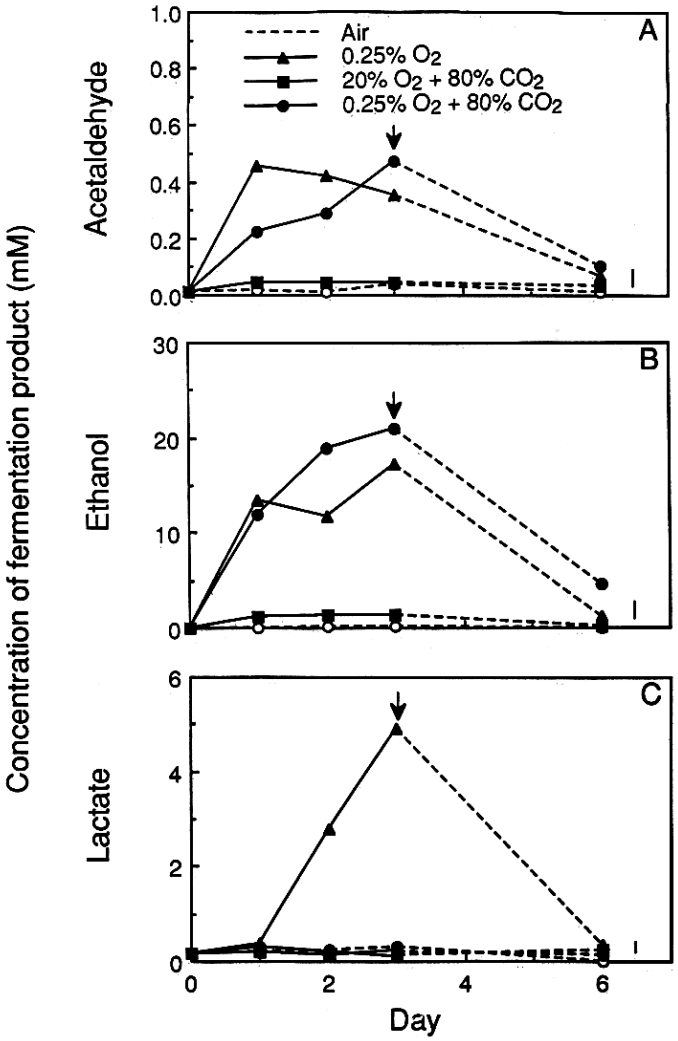

Fig. 1. Changes in concentrations of acetaldehyde, ethanol, and lactate of 'Hass' avocado fruit kept in air, 0.25% O_2, 20% O_2 + 80% CO_2, or 0.25%O_2 + 80% CO_2 at 20 °C for 1, 2, or 3 days followed by transfer (indicated by arrow) to air at 20 °C for 3 days. The vertical bars represent LSD at P = 0.05.

MITOCHONDRIA ISOLATION. Mitochondria were isolated from fruit tissue using the method of Morea and Romani (1982) with slight modification. Thirty grams of fruit tissue were macerated in 100 ml isolation medium which consisted of 50 mM potassium phosphate buffer (pH 7.2), 0.25 M sucrose, 5 mM EDTA, 4.5 mM ß-mercaptoethanol, 0.2% PVP (w/v) and 0.1% BSA (w/v). The homogenate was filtered through four layers of cheesecloth and centrifuged at 2,000 x *g* for 10 min. The supernatant was filtered

through four layers of cheesecloth and centrifuged at 10,000 x *g* for 5 min. This supernatant was discarded and the pellet was resuspended in 1 ml wash medium and homogenized with a glass microhomogenizer in the presence of an additional 3 ml wash medium. The wash medium contained 50 mM potassium phosphate buffer (pH 7.2), 0.25 M sucrose, and 0.1% BSA. The homogenate was centrifuged at 2000 x *g* for 5 min and the supernatant was recentrifuged at 8000 x *g* for 10 min. The pellet was resuspended in 0.5 ml wash medium and used as mitochondrial preparation. Protein content of mitochondrial preparation was determined by the standard Bradford (1976) method.

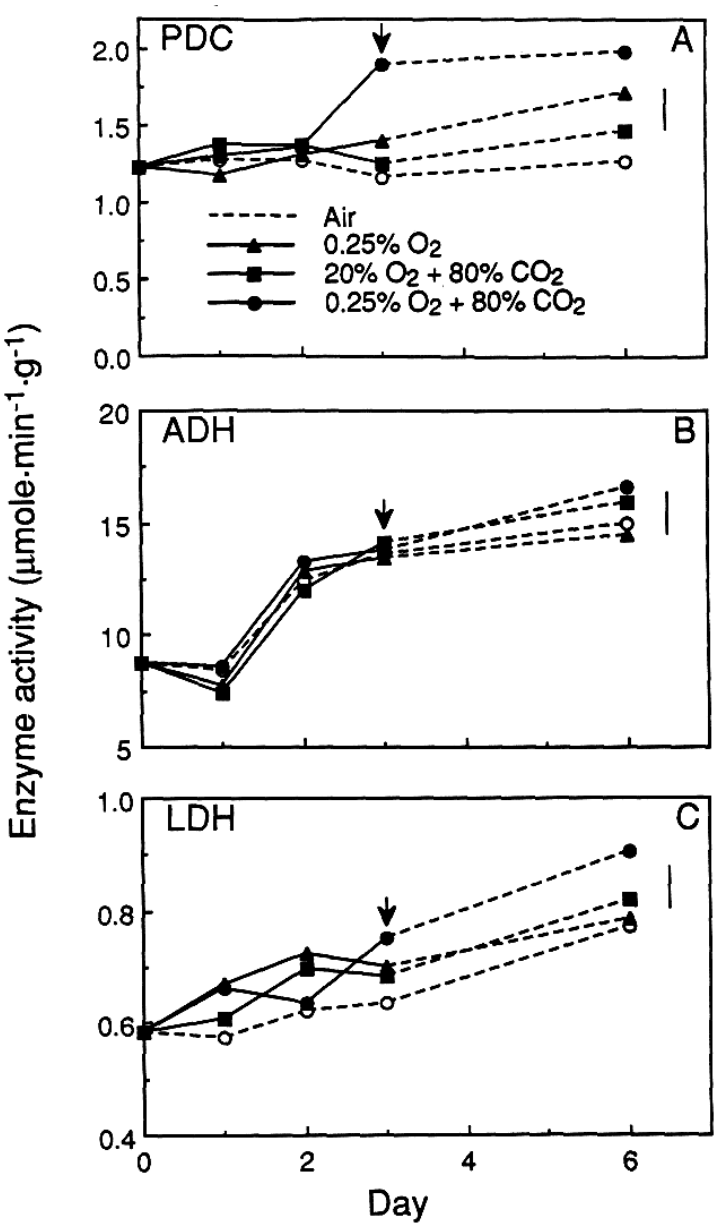

Fig. 2. Changes in activities of pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH), and lactate dehydrogenase (LDH) of 'Hass' avocado fruit kept in air, 0.25% O_2, 20% O_2 + 80% Co_2, or 0.25%O_2 + 80% CO_2 at 20 °C for 1, 2, or 3 days followed by transfer (indicated by arrow) to air at 20 °C for 3 days. The vertical bars represent LSD at *P* = 0.05.

ASSAY OF PDH. The overall activity of the PDH complex was assayed using the method of Budde and Randall (1987) with some modification. The reaction mixture was obtained

by mixing 0.55 ml of 200 mM potassium phosphate buffer (pH 8.0), 0.05 ml of 4% (v/v) Triton X-100, 0.05 ml of 50 mM $MgCl_2$, 0.05 ml of 40 mM NAD, 0.05 ml of 5 mM TPP, 0.05 ml of 2.5 mM LiCoA, 0.05 ml of 20 mM cysteine, 0.05 ml of 20 mM Na pyruvate, and 0.1 ml of mitochondrial preparation. Formation of NADH was measured by recording the increase in absorbance at 340 nm using a spectrophotometer. PDH activity was expressed as mmoles of product formed per min per mg protein.

Results and Discussion

CHANGES IN FERMENTATION PRODUCTS AND ENZYMES. Avocado fruit exposed to 0.25% O_2 or 0.25% O_2 + 80% CO_2 at 20 °C for 1 to 3 days accumulated acetaldehyde (Fig. 1A) and ethanol (Fig.1B). The fruit kept in 0.25% O_2 also accumulated lactate (Fig. 1C). After the fruit were transferred from 0.25% O_2 or 0.25% O_2 + 80% CO_2 to air for 3 days, the accumulated fermentation products largely disappeared, probably as a result of their metabolism to other compounds or due to diffusion of acetaldehyde and ethanol to outside the fruit tissue. The 20% O_2 + 80% CO_2 treatment did not significantly increase acetaldehyde and ethanol concentrations and it did not induce lactate accumulation at all. Also, the 80% CO_2 negated the effect of 0.25% O_2 on lactate accumulation (Fig. 1C).

After exposure of avocado fruit to 0.25% O_2, 20% O_2 + 80% CO_2, or 0.25% O_2 + 80% CO_2 for 2 to 3 days, extractable activities of PDC and LDH were slightly higher than those of air control fruit (Fig. 2 A and C). The low O_2 and/or high CO_2 treatments did not significantly influence extractable activity of ADH (Fig. 2B). However, isozyme analysis indicated that a new ADH isozyme appeared in the fruit exposed to 0.25% O_2, 20% O_2 + 80% CO_2, or 0.25% O_2 + 80% CO_2 for 2 days compared to the ADH isozyme profile of air control fruit (Fig. 3). The ADH activity in avocado fruit was about 10 times that of PDC activity and about 20 times that of LDH activity (Fig. 2). Furthermore, ADH activity of avocado was 40 to 80 times higher than that of 'Bartlett' pear (Ke et al., 1994), lettuce, and strawberry (Ke et al, 1993). The extremely high ADH activity in avocado fruit probably made it difficult to see any small change in the total enzyme level since the new ADH isozyme band had a very low intensity (Fig. 3).

Kennedy et al. (1992) reviewed studies of anaerobic metabolism in plants under low O_2 stress and concluded that the induction of PDC, ADH, and/or LDH was one of the mechanisms for accumulation of anaerobic products. Increases in activities of these enzymes by low O_2 have been found to be largely due to increased transcription and translation, resulting in new mRNA synthesis and *de novo* synthesis of the corresponding enzyme proteins (Gerlach et al., 1982; Good and Crosby, 1989; Kelley, 1989). Ke et al. (1993) found that 0.25% O_2 increased extractable activity of ADH several fold in 'Bartlett' pear, lettuce, and strawberry, largely due to the induction of one ADH isozyme. But 20% O_2 + 80% CO_2 had only slight effect on ADH activity of pears. The effects of 0.25% O_2 and/or 80% CO_2 on extractable activities of PDC and LDH of these commodities were also slight. Since exposure of avocado fruit to 0.25% O_2 for 3 days increased the concentrations of acetaldehyde, ethanol, and lactate over 8-, 172-, and 33-fold, respectively (Fig. 1), but had only slight effects on the levels of PDC, ADH and LDH (Figs. 2 and 3), it appeared that molecular induction of the expression of these fermentation enzymes was not the major mechanism for regulating fermentative metabolism in avocado fruit. Roberts et al. (1989) and Xia and Saglio (1992) also suggested that activities of ADH and LDH were not the rate limiting factors for

accumulations of ethanol and lactate in some plant tissues if the activities of these enzymes were high. We tested the possibility that other factors might be more important for induction of fermentation in avocado fruit.

CHANGES IN FERMENTATION SUBSTRATES AND COFACTORS. Avocado fruit kept in 0.25% O_2, 20% O_2 + 80% CO_2, or 0.25% O_2 + 80% CO_2 at 20 °C for 3 days had lower PDH activity than that of air control fruit (Table 1). This suggested that pyruvate flux through the tricarboxylic acid (TCA) cycle might have been reduced by the low O_2 and/or high CO_2 treatments. As a result pyruvate concentration in the treated fruit could have been increased. Davis et al. (1973) observed that pyruvate content was higher in low O_2 or high CO_2-treated citrus fruit than that of air control fruit. Recently, Good and Muench (1993) reported that levels of pyruvate as well as those of lactate and ethanol rapidly increased in barley root tissue exposed to anaerobic conditions.

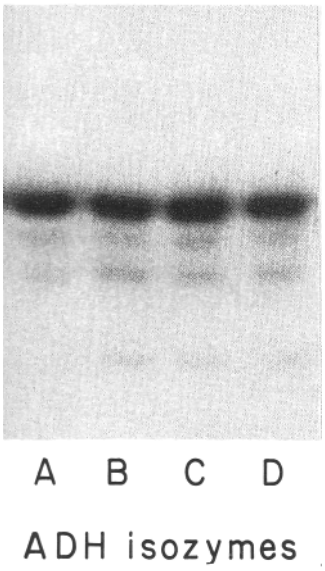

Fig. 3. Isozymes of alcohol dehydrogenase (ADH) of 'Hass' avocado fruit kept in air (lane A), 0.25% O_2 (lane B), 20% O_2 + 80% CO_2 (lane C) or 0.25% O_2 + 80% CO_2 (lane D) at 20C for 2 days.

NADH is the common substrate for ADH and LDH. Exposure of avocado fruit to 0.25% O_2 or 0.25% O_2 + 80% CO_2 increased NADH concentration but decreased NAD level (Table 1), which is correlated with the accumulation of ethanol in these fruit (Fig. 1B). The 20% O_2 + 80% CO_2 treatment had no significant effect on NADH and NAD contents, consistent with its slight effect on ethanol concentration. Similarly, the NADH:NAD ratio was greatly increased by the 0.25% O_2 or 0.25% O_2 + 80% CO_2 treatment but not by the 20% O_2 + 80% CO_2 treatment (Table 1). These results indicated that the decrease in O_2 concentration played a major role in increasing concentrations of fermentation substrates and in accumulations of fermentation products in avocado fruit. This is in contrast to some other fruit, such as 'Bartlett' pear and strawberry, where 80% CO_2 induced large accumulations of acetaldehyde and ethanol even under 20% O_2 (Ke et al., 1993).

Table 1. Effects of exposure to air, 0.25% O_2, 20% O_2 + 80% CO_2, or 0.25% O_2 + 80% CO_2 for 3 days at 20C on overall activity of pyruvate dehydrogenase (PDH) complex and concentrations of NAD and NADH of avocado fruit.

Treatment	PDH activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	NAD (mM)	NADH (mM)	NADH : NAD ratio
Air	0.015	0.064	0.002	0.03
0.25% O_2	0.007	0.020	0.008	0.38
20% O_2 + 80% CO_2	0.007	0.059	0.003	0.05
0.25% O_2 + 80% CO_2	0.009	0.023	0.011	0.50
LSD at $P = 0.05$	0.005	0.009	0.003	0.09

We have reported that avocado fruit had a cytoplasmic pH of 6.9 and that exposure to 0.25% O_2, 20% O_2 + 80% CO_2, or 0.25% O_2 + 80% CO_2 decreased the pH values to 6.7, 6.3, and 6.3, respectively (Hess et al., 1993). Reduced O_2 or elevated CO_2 concentrations also decreased cytoplasmic pH in 'Bartlett' pear (Nanos and Kader, 1993) and lettuce (Siriphanich and Kader, 1986). These decreases in cytoplasmic pH

may play an important role in regulating fermentative metabolism (Davies, 1980; Roberts, 1989). Under O_2 levels near 0% or high CO_2 concentrations, electron transport through the cytochrome pathway was inhibited (Kennedy et al., 1987). As a result, oxidative phosphorylation would be decreased. Nanos and Kader (1993) found that exposure of 'Bartlett' pear to 0.25% O_2 greatly reduced the ATP/ADP ratio. Exposure of avocado fruit to 0.25% O_2 or 20% O_2 + 80% CO_2 reduced ATP level by 20% and 22%, respectively (Hess et al„ 1993); the combination of 0.25% O_2 + 80% CO_2 had a synergistic effect on inhibiting ATP synthesis, resulting in a 63% reduction in ATP level. Kerbel et al. (1988) reported that exposure of 'Bartlett' pear to 10% CO_2 increased fructose-6-phosphate concentration but decreased fructose-1,6-bisphosphate level, consistent with a reduction in activities of ATP:phosphofructokinase and PPi:phosphofructokinase.

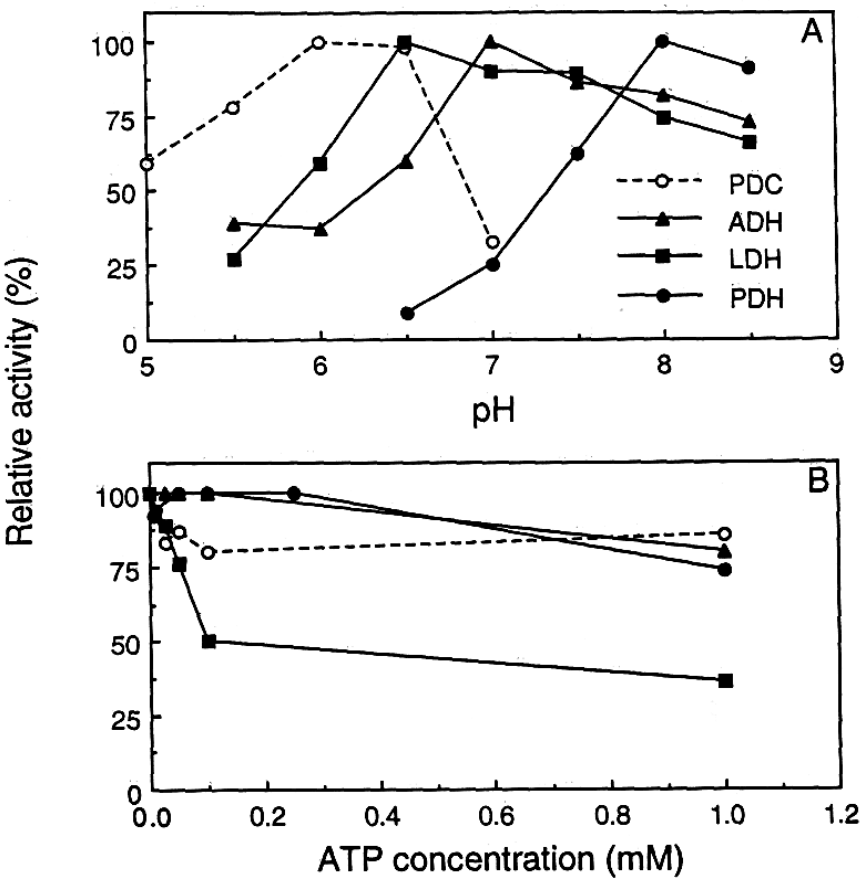

Fig. 4. Activities of pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH), and pyruvate dehydrogenase (PDH) in 'Hass' avocado fruit as influenced by pH and ATP concentration. PDH was incubated with specified ATP concentrations for 90 min at 20 °C before assay. All the other enzymes were assayed immediately following ATP addition.

METABOLIC REGULATION OF FERMENTATIVE METABOLISM. Fermentative metabolism can be regulated by two mechanisms: molecular control (also called coarse control) of the levels of PDC, ADH, and LDH and metabolic control (also called fine control) of the actual functions of these enzymes in plant tissue under O_2 and/or CO_2 stresses (John and Greenway, 1976; Chang et al., 1983; Ke et al., 1993). Roberts et al. (1989) found that ethanol production rate was correlated with ADH activity when the enzyme level

was very low; but at a high enzyme level, ethanol accumulation was independent of ADH activity. With limited enzyme level, the induction of a fermentation enzyme through molecular control (transcription and/or translation) is essential for the accumulation of fermentation products. If the enzyme level is already very high even under air control like the ADH activity in avocado fruit, then it may not be critical to induce more enzyme biosynthesis. On the other hand, metabolic control of the actual functions of the fermentation enzymes by changes in pH and concentrations of substrates, cofactors, and/or inhibitors may always be important in regulating fermentative metabolism.

The optimum pH for PDC of avocado fruit was about 6.0 to 6.5 (Fig. 4A). A decrease in cytoplasmic pH from 6.9 to 6.7 by 0.25% O_2 or to 6.3 by 80% CO_2 (Hess et al., 1993) would activate PDC. Davies (1980) and Roberts (1989) proposed that a decrease in cytoplasmic pH and the subsequent activation of PDC was the major reason to direct pyruvate to the production of acetaldehyde and ethanol in plant tissue. The optimum pH for ADH and LDH of avocado fruit was about 6.5-7.0. A decrease in cytoplasmic pH of 0.2 units by 0.25% O_2 would not significantly influence LDH activity but would slightly inhibit ADH (Fig. 4A). A decrease in cytoplasmic pH from 6.9 to 6.3 by 80% CO_2 would significantly inhibit ADH and LDH of avocado fruit (Fig. 4A). ADH activity (Fig. 2B) was 10 times higher than PDC activity of avocado fruit (Fig. 2A). Therefore, a partial inhibition of ADH by a decrease in pH would not limit the conversion of acetaldehyde into ethanol. On the other hand, since LDH activity (Fig. 2C) was only about half that of PDC activity (Fig. 2A) in avocado fruit, the inhibition of LDH by a decrease in pH from 6.9 to 6.3 due to 80% CO_2 might have a greater impact on LDH, which in turn favored PDC action. This may partly explain why 0.25% O_2 caused accumulations of acetaldehyde, ethanol, and lactate but the combination of 0.25% O_2 + 80% CO_2 resulted in accumulations of acetaldehyde and ethanol only (Fig. 1). In other words, 80% CO_2 decreased cytoplasmic pH to a level that significantly inhibited LDH and therefore negated the effect of 0.25% O_2 on lactate accumulation. In the range of pH 6.0-8.0, a decrease in pH would inhibit PDH (Fig. 4A) and therefore reduce the pyruvate flux through the TCA cycle.

Table 2. Km values of pyruvate decarboxylase (PDC), alcohol dehyrogenase (ADH), lactate dehydrogenase (LDH), and pyruvate dehydrogenase (PDH) of avocado fruit for their substrates.

	Km values (mM)			
			NADH	
Enzyme	Pyruvate	Acetaldehyde	–NAD	+0.08 mM NAD
PDC	1.10	---	---	---
ADH	---	1.22	0.019	0.034
LDH	0.08	---	0.030	0.091
PDH	0.15	---	---	---

LDH was inhibited at high ATP concentrations (Fig. 4B). ADH and PDH were slightly inhibited by 1 mM ATP but not by the other ATP concentrations used. High ATP concentrations slightly inhibited PDC. Oba et al. (1977) and Betsche (1981) also reported that LDH was inhibited at high ATP level. Exposure of avocado fruit to 0.25% O_2 reduced ATP level (Hess et al., 1993), which would have relieved the effect of ATP on inhibiting LDH and partly contributed to the accumulation of lactate in the tissue (Fig. 1C). It was reported that fructose-6-phosphate relieved the ATP inhibition of LDH in

lettuce (Betsche, 1981) and 10% CO_2 treatment caused an increase in concentration of fructose-6-phosphate (Kerbel et al., 1988)

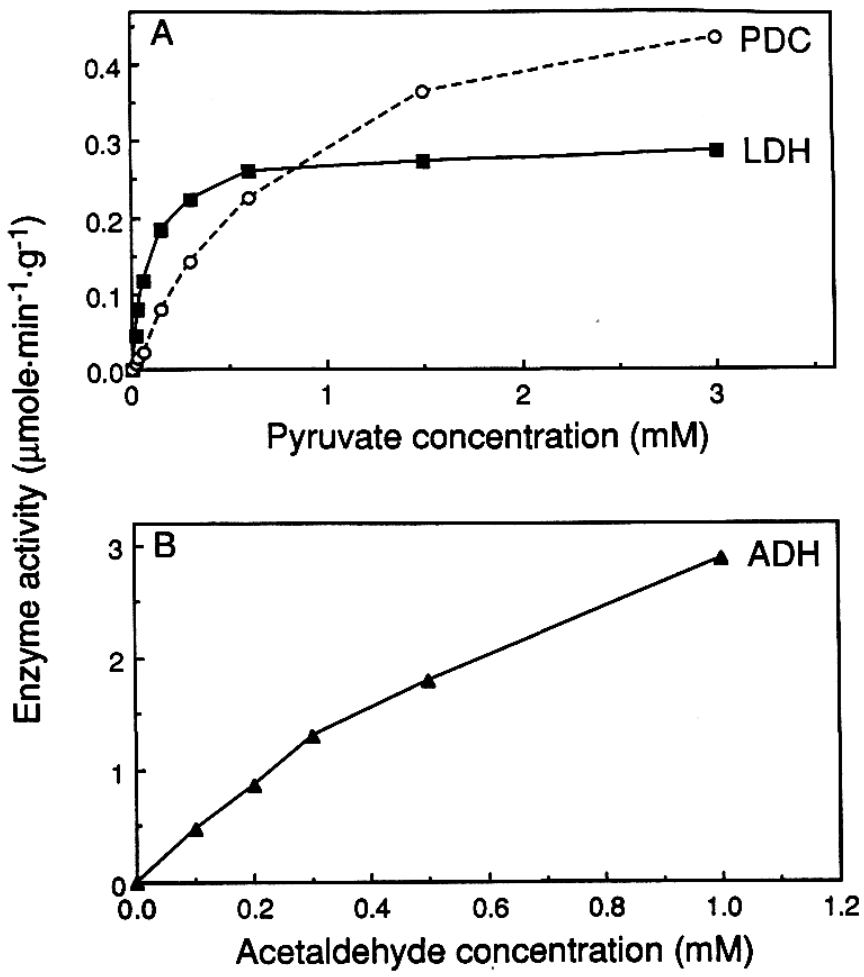

Fig. 5. Activitiesof pyruvate decarboxylase (PDC), lactate dehydrogenase (LDH), and alcohol dehydrogenase (ADH) in 'Hass' avocado fruit as influenced by pyruvate or acetaldehyde concentration.

Pyruvate is the common substrate for PDC, LDH, and PDH. The Km of LDH for pyruvate was 0.08 mM (Table 2). LDH was more preferred than PDC at lower pyruvate concentration (Fig. 5A). At higher pyruvate level, however, PDC would be more active than LDH. PDH also had a low Km for pyruvate (Table 2) and at normal physiological conditions of air control fruit pyruvate was expected to be largely directed to the PDH reaction and subsequently to TCA cycle. However, since 0.25% O_2 and/or 80% CO_2 treatment reduced PDH activity (Table 1) and a decrease in pH inhibited PDH (Fig. 4A), pyruvate flux through the PDH path could have been reduced, which in turn might have increased pyruvate concentration. This would activate PDC and LDH since both are allosteric enzymes that could be activated by their substrate (pyruvate) due to conformational changes (Betsche, 1981; Hubner et al, 1978).

ADH of avocado fruit had a Km of 1.22 mM for acetaldehyde (Table 2). In air control fruit, acetaldehyde concentration was much lower than the Km value (Fig. 1A). The 0.25% O_2 or 0.25% O_2 + 80% CO_2 treatment increased acetaldehyde concentration to

0.2-0.5 mM in 1 to 3 days, which would have greatly accelerated the ADH reaction and the accumulation of ethanol (Fig. 5B). Since ADH activity in avocado fruit was very high (Fig. 2B), ADH was not likely to be the factor that limited ethanol production. Instead, the increased acetaldehyde concentration was probably the major driving force for ethanol accumulation in avocado fruit kept in 0.25% O_2 or 0.25% O_2 + 80% CO_2 (Fig. 1 A and B and Fig. 5B).

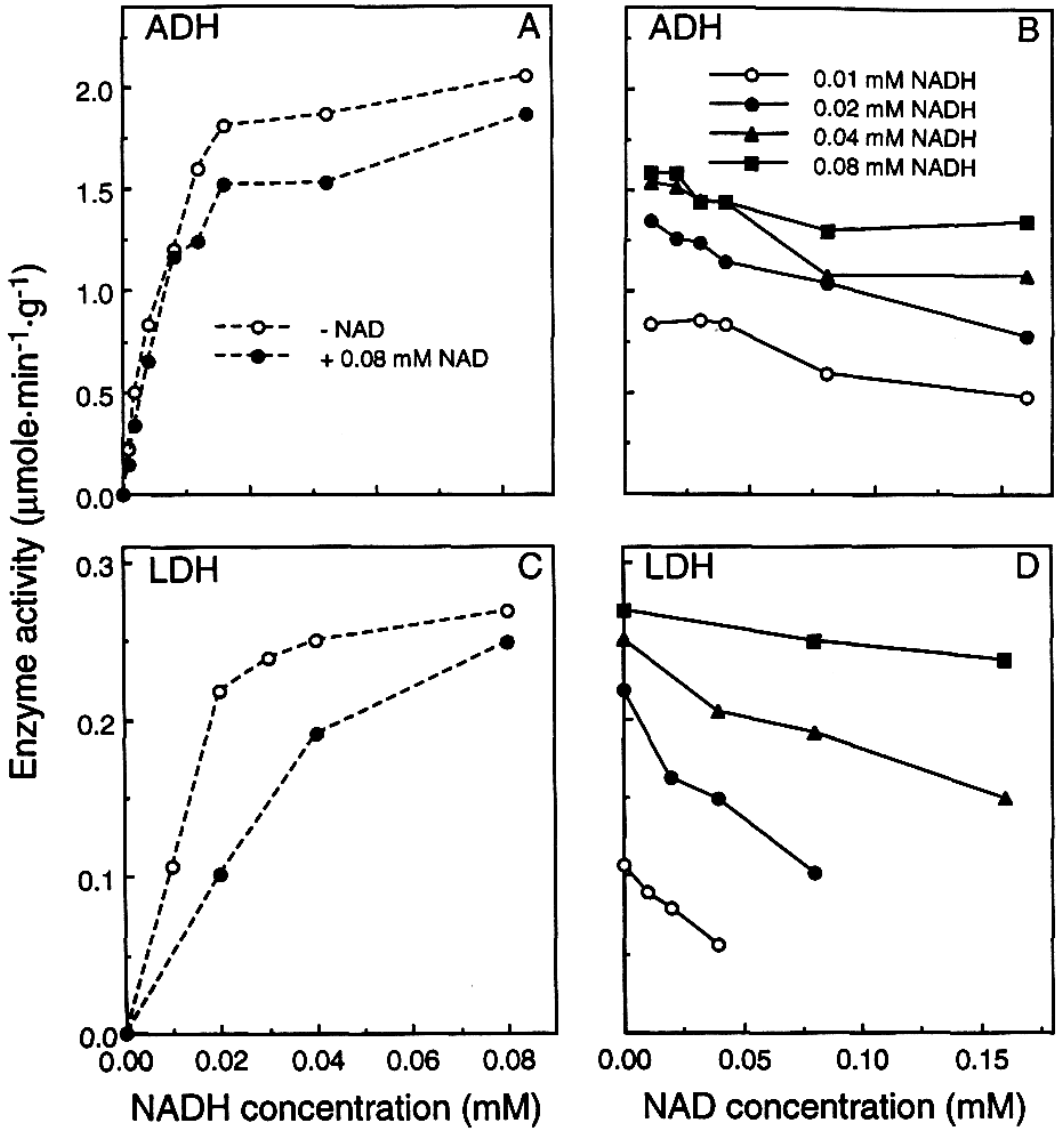

Fig. 6. Activities of alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH) in 'Hass' avocado fruit as influenced by concentrations of NADH and NAD.

NADH could be the substrate for ADH or LDH. Without the addition of NAD, the Km values of ADH and LDH for NADH were 0.019 and 0.030 mM, respectively (Table 2, Fig. 6A). This indicated that ADH of avocado fruit had a higher affinity for NADH than that of LDH. Addition of 0.08 mM NAD increased the Km values of ADH and LDH for NADH to 0.034 and 0.091 mM, respectively. The inhibition of NADH oxidation by NAD was more pronounced at lower NADH concentrations, such as 0.01 or 0.02 mM, than at higher NADH concentrations, such as 0.04 or 0.08 mM (Fig. 6 B and D). It was difficult to know

the actual NADH and NAD concentrations in cytoplasm and mitochondria of avocado fruit since the vacuole usually occupies the major volume of a mature fruit cell. However, the decrease in NAD concentration in avocado fruit kept in 0.25% O_2 or 0.25% O_2 + 80% CO_2 (Table 2) would have relieved the effect of NAD on inhibiting NADH oxidation by ADH or LDH. The increase in NADH concentration by 0.25% O_2 (Table 1) was probably an important driving force for the ADH or LDH reaction.

Lactate concentration was 6 times higher than acetaldehyde concentration in the avocado fruit kept in 0.25% O_2 for 2 to 3 days (Fig. 1). However, the total pyruvate flux to the ethanol pathway was 10 times higher than that to the lactate pathway. Although LDH had a higher affinity for pyruvate (Table 2), the PDC activity was twice that of LDH (Fig. 2). A decrease in cytoplasmic pH substantially activated PDC but not LDH (Fig. 4A). Furthermore, ADH had a higher affinity for NADH than that of LDH and the acetaldehyde produced from PDC reaction was easily converted into ethanol due to the high level of ADH activity. Therefore, the ethanol pathway was more preferred than the lactate pathway in avocado fruit as in other plant tissues such as 'Bartlett' pear, lettuce, and strawberry.

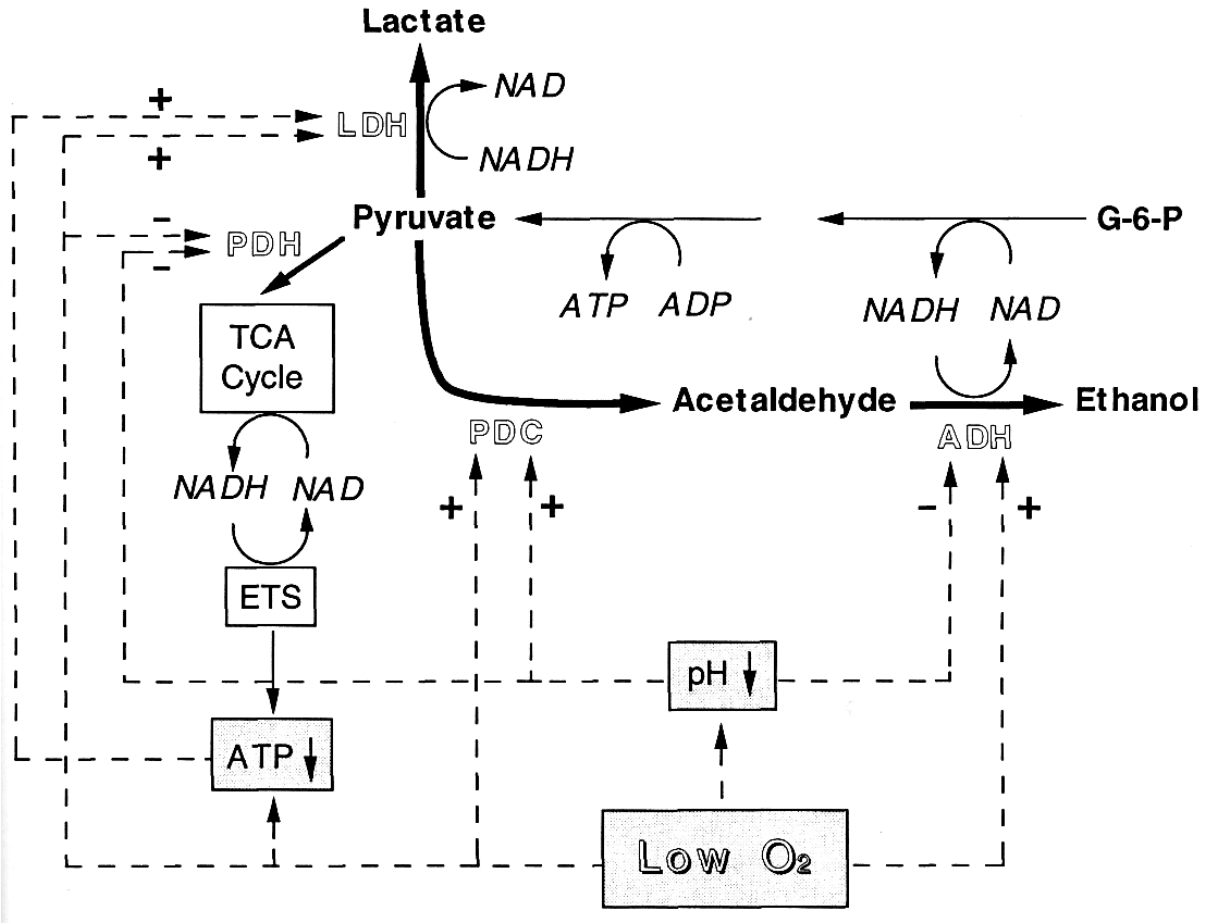

Fig. 7. Pathways of fermentative metabolism in 'Hass' avocado fruit as proposed to be regulated by low O2 stress. ADH = alcohol dehydrogenase; ETS = electron transport system; G-6-P = glucose-6-phosphate; LDH = lactate dehydrogenase; PDC = pyruvate dehydrogenase; TCA = tricarboxylic acid; --------$^{+}$----->, induction and/or activation; -------$^{-}$------>, reduction and/or inhibition.

From the results of this paper and cited references, a proposed mode of action of low O_2 stress on fermentative metabolism in avocado fruit is presented in Fig. 7. The low O_2 concentration substantially reduces NADH flux through the electron transport system (ETS). As a result, concentrations of NAD and ATP decrease while NADH level increases. Cytoplasmic pH is decreased, PDH activity is partly reduced and pyruvate flux through the TCA cycle decreases. PDC and LDH activities are enhanced and a new ADH isozyme is induced. PDC activity is enhanced by the decrease in pH and by an increase in pyruvate concentration. This directs pyruvate to the production of acetaldehyde. Although a decrease in pH slightly inhibits the very abundant ADH, the increased acetaldehyde and NADH concentrations and decreased NAD level drive ADH reaction, which causes ethanol to accumulate as the major fermentation product. The increase in pyruvate and NADH concentrations and decreased NAD and ATP levels activate LDH and drive pyruvate conversion into lactate as another end product. The induction of fermentative metabolism allows glycolysis to go on, NADH and pyruvate can be used, and a small amount of ATP can be produced through substrate phosphorylation to permit the plant tissue to temporarily survive. However, the accumulations of acetaldehyde, ethanol, and lactate and the disturbance of normal metabolism may be detrimental to the avocado fruit if it is exposed to low O_2 stress conditions beyond its limit. Although the 20% O_2 + 80% CO_2 treatment induced or activated PDC, ADH, and LDH (Figs. 2-4), it did not cause significant changes in NADH, NAD, and lactate (Table 1, Fig. 1C) and only slightly increased acetaldehyde and ethanol concentrations (Fig. 1A and B) in avocado fruit. The avocado responses to 20% O_2 + 80% CO_2 were different from those of pears and strawberries in which 20% O_2 + 80% CO_2 induced great accumulations of acetaldehyde and ethanol (Ke et al., 1993, 1994). This suggested that under 20% O_2 + 80% CO_2, electron transport in avocado fruit might be only partially inhibited and/or another pathway could have been induced to replace fermentative metabolism to use pyruvate and NADH and to produce ATP.

Literature Cited

Arulsekar, S. and D. E. Parfitt. 1986. Isozyme analysis procedures for stone fruits, almond, grape, walnut, pistachio and fig. *HortScience* 21:928-933.

Betsche, T. 1981. L-Lactate dehydrogenase from leaves of higher plants: Kinetics and regulation of the enzyme from lettuce *(Lactuca sativa* L.).*Biochem. J.* 195:615-622.

Bradford, M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. 72:248-254.

Budde, R. J. A. and D. D. Randall. 1987. Regulation of pea mitochondria! pyruvate dehydrogenase complex activity: Inhibition of ATP-dependent inactivation. *Arch. Biochem. Biophys.* 258:600-606.

Chang, L. A., L. K. Hammett, and D. M. Pharr. 1983. Carbon dioxide effects on ethanol production, pyruvate decarboxylase, and alcohol dehydrogenase activities in anaerobic sweet potato roots. *Plant Physiol*.71:59-62.

Davies, D. D. 1980. Anaerobic metabolism and the production of organic acids, p. 581-611. *In*: P. K. Stumpf and E. E. Conn (eds.). The biochemistry of plants: A comprehensive treatise, vol. 2. Academic Press, New York

Davis, P. L., B. Roe, and J. H. Bruemmer. 1973. Biochemical changes in citrus fruits during controlled-atmosphere storage. *J. Food Sci*. 38:225-229.

Gerlach, W. L., A. J. Pryor, E. S. Dennis, R. J. Ferl, M. M. Sachs, and W.J . Peacock. 1982. cDNA cloning and induction of alcohol dehydrogenase gene (Adh 1) of maize. *Proc. Natl. Acad. Sci.* USA. 79:2981-2985.

Good, A. G. and W. L. Crosby. 1989. Induction of alcohol dehydrogenase and lactate dehydrogenase in hypoxically induced barley. *Plant Physiol*. 90:860-866.

Good, A. G. and D. G. Muench. 1993. Long-term anaerobic metabolism in root tissue—Metabolic products of pyruvate metabolism. *Plant Physiol*. 101:1163-1168.

Greenbaum, M. A. L., J. B. Clark, and P. Mclean. 1965. The estimation of the oxidized and reduced forms of the nicotinamide nucleotides. *Biochem. J.* 95:161-166.

Hess, B., D. Ke, and A. A. Kader. 1993. Changes in intracellular pH, ATP, and glycolytic enzymes in 'Hass' avocado in response to low O_2 and high CO_2 stresses. Proc. 6th Intl. Controlled Atmosphere Res. Conf., Cornell Univ., Ithaca, N.Y. p. 1-9.

Hubner, G., R. Weidhase, and A. Schellenberger. 1978. The mechanism of substrate activation of pyruvate decarboxylase: A first approach. *Eur. J. Biochem*. 92:175-181.

John, C. D. and H. Greenway. 1976. Alcoholic fermentation and activity of some enzymes in rice roots under anaerobiosis. *Austral. J. Plant Physiol*. 3:325-336.

Kanellis, A. K., T. Solomos, and K. A. Roubelakis-Angelakis. 1991. Suppression of cellulase and polygalacturonase and induction of alcohol dehydrogenase isozymes in avocado fruit mesocarp subjected to low oxygen stress. *Plant Physiol*. 96:269-274.

Ke, D., M. Mateos, and A. A. Kader.1993. Regulation of fermentative metabolism in fruits and vegetables by controlled atmospheres. Proc. 6th Intl. Controlled Atmosphere Res. Conf., Cornell Univ., Ithaca, N.Y. p. 63-77.

Ke, D., E. Yahia, M. Mateos, and A. A. Kader. 1994. Ethanolic fermentation of 'Bartlett' pears as influenced by ripening stage and atmospheric composition. *J. Amer. Soc. Hort. Sci.* 119:976-982.

Kelley, P. M. 1989. Maize pyruvate decarboxylase mRNA is induced anaerobically. *Plant Mol. Biol.* 13:213-222.

Kennedy, R. A., T. C. Fox, and J. N. Siedon.1987. Activities of isolated mitochondria and mitochondrial enzymes from aerobically and anaerobically germinated barnyard grass (Echinochloa) seedlings. *Plant Physiol*. 85:474-80.

Kennedy, R. A., M. E. Rumpho, and T. C. Fox. 1992. Anaerobic metabolism in plants. *Plant Physiol.* 100:1-6.

Kerbel, E. L., A. A. Kader, and R. J. Romani.1988. Effects of elevated CO_2 concentrations on glycolysis in intact 'Bartlett' pear fruit. *Plant Physiol*. 86:1205-1209.

Leshuk, J. A. and M. E. Saltveit. 1991. Effects of rapid changes in oxygen concentration on respiration of carrot roots. *Physiol. Plant*. 82:559-568.

Morea, F. and R. J. Romani. 1982. Malate oxidation and cyanide-insensitive respiration in avocado mitochondria during the climacteric cycle. *Plant Physiol*. 70:1385-1390.

Nanos, G. D. and A. A. Kader, 1993. Low O_2-induced changes in pH and energy change in pear fruit tissue. *Postharvest Biol. Technol*. 3:285- 291.

Nanos, G. D., R. J. Romani, and A. A Kader. 1992. Metabolic and other responses of 'Bartlett' pear fruit and suspension-cultured 'Passe Crassane' pear fruit cells held in 0.25% O_2. *J. Amer. Soc. Hort. Sci.* 117:934-940.

Oba, K., S. Murakami, and I. Uritani. 1977. Partial purification and characterization of L-lactate dehydrogenase isozymes from sweet potato roots. *J. Biochem*. 81:1193-1201.

Roberts, J. K. M. 1989. Cytoplasmic acidosis and flooding tolerance in crop plants, p. 392-397. *In* D. D. Hook (ed.). The ecology and management of wetlands, vol 1. Croom-Helm Press, London.

Roberts, J. K. M., K. Chang, C. Webster, J. Callis, and V. Walbot. 1989. Dependence of ethanolic fermentation, cytoplasmic pH regulation, and viability on the activity of alcohol dehydrogenase in hypoxic maize root tips. *Plant Physiol*. 89:1275-1278.

Siriphanich, J. and A. A. Kader. 1986. Changes in cytoplasmic and vacuolar pH in harvested lettuce tissue as influenced by CO_2. *J. Amer. Soc. Hort. Sci*. 111:73-77.

Xia, J. H. And P. H. Saglio. 1992. Lactic acid efflux as a mechanism of hypoxic acclimation of maize root tips to anoxia. *Plant Physiol*. 100:40-46.

Yahia, E. and A. Carrillo-López. 1993. Responses of avocado fruit to insecticidal O_2 and CO_2 atmospheres. *Lebensm.-Wiss. u.-Technol*. 26:307-311.