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Ethanolic Fermentation of 'Bartlett' Pears as Influenced by Ripening Stage and Atmospheric Composition

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Abstract. **Changes in fermentation volatiles and enzymes were studied in preclimacteric and postclimacteric 'Bartlett' pears (*Pyrus communis* L.) kept in air, 0.25% O_2, 20% O_2 + 80% CO_2, or 0.25% O_2 + 80% CO_2 at 20C for 1, 2, or 3 days. All three atmospheres resulted in accumulation of acetaldehyde, ethanol, and ethyl acetate. The postclimacteric pears had higher activity of pyruvate decarboxylase (PDC) and higher concentrations of fermentation volatiles than those of the preclimacteric fruit. For the preclimacteric pears, the 0.25% O_2 treatment dramatically increased alcohol dehydrogenase (ADH) activity, which was largely due to the enhancement of one ADH isozyme. Exposure to 20% O_2 + 80% CO_2 slightly increased ADH activity, but the combination of 0.25% O_2 + 80% CO_2 resulted in lower ADH activity than 0.25% O_2 alone. For the postclimacteric pears, the three atmospheres resulted in higher PDC and ADH activities than those of air control fruit. Ethanolic fermentation in 'Bartlett' pears could be induced by low O_2 and/or high CO_2 via 1) increased amounts of PDC and ADH; 2) PDC and ADH activation caused by decreased cytoplasmic pH; or 3) PDC and ADH activation or more rapid fermentation due to increased concentrations of their substrates (pyruvate, acetaldehyde, or NADH).**

Exposing harvested fruit to low O_2 and/or high CO_2 can be beneficial or harmful, depending on concentrations of these gases, temperature, exposure duration, and commodity. Beneficial effects include disease control, insect disinfestation, and alleviation of chilling injury and other physiological disorders. We have investigated the tolerance limits of several fruit, including pears, to such fungicidal and insecticidal atmospheres and the capacity of the fruit tissue to recover from exposure to low O_2 and/or high CO_2 for short durations.

Ethanolic fermentation is a major pathway induced in plant tissues in response to very low O_2 and/or very high CO_2 concentrations. In this pathway, acetaldehyde is produced through pyruvate decarboxylation catalyzed by PDC. The enzyme ADH reduces acetaldehyde into ethanol using NADH. Ethanol is usually the major product of the pathway in low O_2-stressed fruit (Ke and Kader, 1992; Ke et al., 1991b), but in some fruit tissues, part of the ethanol is converted into ethyl acetate. In CO_2-zymasis, the ratio of ethanol to acetaldehyde was 2:1, contrasting strikingly with 50:1 obtained in anaerobic zymasis in apple (Thomas, 1929).

Oxygen levels <1% or CO_2 levels >20% caused acetaldehyde and ethanol accumulation in apples (Ke et al., 1991b; Knee, 1991; Nichols and Patterson, 1987; Thomas, 1929), pears (Ke et al., 1990, 1991b), stone fruit (Ke and Kader, 1992; Smilanick and Fouse, 1989), oranges (Ke and Kader, 1990), strawberries (Ke et al., 1991a), blueberries (Saltveit and Ballinger, 1983a), grapes (Saltveit and Ballinger, 1983b), carrots (Leshuk and Saltveit, 1991), and sweetpotatoes (Chang et al., 1982, 1983). Increased PDC and ADH activities were found in sweetpotatoes (Chang et al., 1982, 1983), tomatoes (Longhurst et al., 1990), avocadoes (Kanellis et al., 1991), and pears (Nanos et al., 1992) in response to low O_2 or high CO_2.

Although intensive research has been done on anaerobic metabolism in maize, barley, and rice (Davies, 1980; Gerlach et al., 1982; Good and Crosby, 1989; John and Greenway, 1976; Kelley, 1989; Kennedy et al., 1992; Roberts, 1989; Roberts et al., 1989), the mechanism for regulation of ethanolic fermentation in fruit is not clear. In this research, we studied the regulating ethanolic fermentation in 'Bartlett' pears kept in 0.25% O_2, 20% O_2 + 80% CO_2, or 0.25% O_2 + 80% CO_2 as influenced by ripening stage.

Materials and Methods

Materials and treatments. 'Bartlett' pears were obtained on the day of harvest (at the mature-green stage of maturity) from commercial shippers in Lake County, Calif., and stored in air at 0C until used for experiments. Ripening stage was estimated by measuring skin color using a color difference meter (CDM) calibrated with a white plate (x = 84.1, y = 81.7, z = 92.9) and respiration and ethylene production rates. Preclimacteric pears were stored in air at 0C for <2 weeks before being used for experiments; their skin color was green with an average CDM "a" value of –18.3. To obtain postclimacteric pears, the fruit were stored in air at 0C for >2 weeks and then, if necessary, held in air at 20C for a certain period to allow them to ripen. During this holding period, respiration and ethylene production rates were monitored and, immediately after the pears passed the climacteric peak, they were used for experiments. The postclimacteric pears had green-yellow skin color with an average CDM "a" value of –8.6. For each experiment, fruit were selected for freedom from defects and matched by color. Nine fruit were placed in a 4-liter glass jar and ventilated with humidified air or a desired 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 20C for 1, 2, or 3 days. The preclimacteric pears were subsequently transferred to air at 20C for another 3 days to observe the post-controlled atmosphere (CA)-stress changes.

Volatile measurement. Frozen pear juice was thawed and a 5-ml sample was put in a 10-ml screw-cap test tube, which was closed

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with a plastic cap and incubated in a water bath at 60C. After 60 min, a headspace sample was taken with a 1-ml glass syringe for determining acetaldehyde, ethanol, and ethyl acetate concentrations using a gas chromatograph (HP5890A; Hewlett Packard, Palo Alto, Calif.) with a flame ionization detector (at 250C) and a glass column (2 mm × 1.0 m) containing 5% Carbowax on 60/80 Carbopack as stationary phase (Supelco, Bellafonte, Pa.) at 85C.

Enzyme extraction and assay. For each replicate, 3 g tissue was obtained from three fruit and homogenized in 10 ml of 100 mM 2-(*N*-morpholino)ethane-sulfonic acid (MES) buffer (pH 6.5) containing 2 mM dithiothreitol and 1% (w/v) polyvinylpyrolidone (PVP). The homogenate was filtered through four layers of cheesecloth and centrifuged at 27,000× *g* for 10 min. The supernatant was retained as enzyme extract for measuring PDC and ADH activities. PDC was assayed through coupling with ADH reaction by mixing 0.45 ml of 100 mM MES buffer (pH 6.5), 0.1 ml of 5 mM thiamine pyrophosphate, 0.1 ml of 50 mM $MgCl_2$, 0.05 ml of 1.6 mM NADH, 0.1 ml of commercial ADH solution (containing 13.5 enzyme units), 0.1 ml of enzyme extract, and 0.1 ml of 50 mM pyruvate. ADH activity was measured by mixing 0.8 ml of 100 mM MES buffer (pH 6.5), 0.05 ml of 1.6 mM NADH, 0.1 ml of enzyme extract, and 0.05 ml of 80 mM acetaldehyde. For PDC and ADH, NADH oxidation was measured by recording the decrease in absorbance at 340 nm using a spectrophotometer. Enzyme activities were expressed as moles of substrate used per minute per gram of fresh weight. For in vitro studies of enzyme kinetics, several assay pH values and substrate

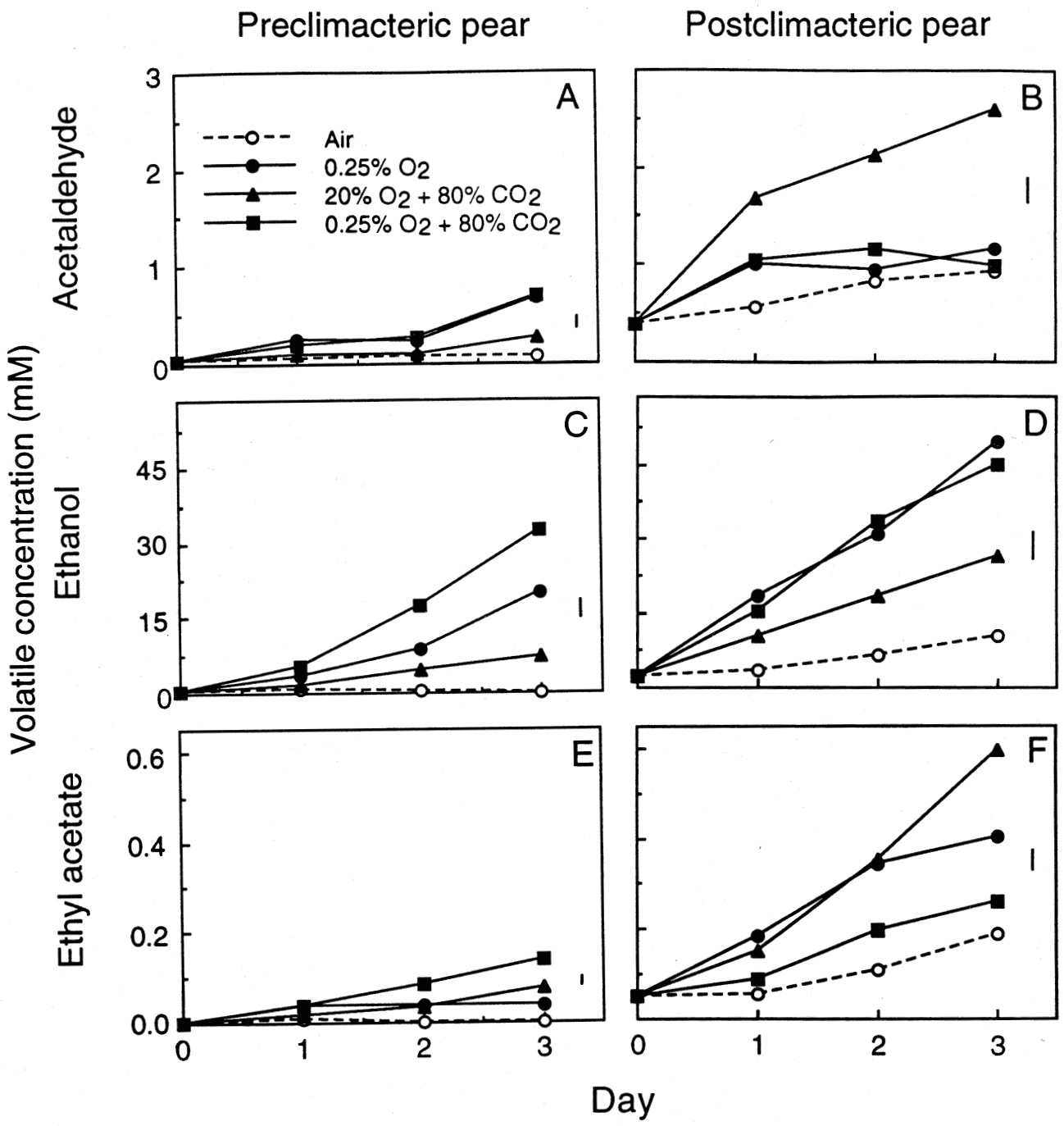


Fig. 1. Changes in acetaldehyde, ethanol, and ethyl acetate concentrations in preclimacteric and postclimacteric 'Bartlett' pears kept in air, 0.25% O_2, 20% O_2 + 80% CO_2, or 0.25% O_2 + 80% CO_2 at 20C for 1, 2, or 3 days. The vertical bars represent LSD at $P = 0.05$.

concentrations of pyruvate, acetaldehyde, and NADH were used to study the changes in PDC and ADH activities.

ADH isozyme extraction, electrophoresis, and staining. Nine grams of fruit tissue was homogenized in 12 ml extraction buffer and 1 g polyvinyl-polypyrrolidone (PVPP). The homogenate was filtered through four layers of cheesecloth and centrifuged at 27,000× *g* for 10 min. The supernatant was retained for isozyme analysis by starch gel electrophoresis. The gels were prepared and run, and ADH isozymes were stained according to Arulsekar and Parfitt (1986).

Results and Discussion

Changes in fermentation volatiles and enzymes. For the postclimacteric 'Bartlett' pears, acetaldehyde, ethanol, and ethyl acetate concentrations increased when the fruit were kept in air at 20C for 2 to 3 days (Fig. 1). For the preclimacteric pears, such increases in volatiles of the air control fruit occurred only after 6 days when the fruit were ripe (Table 1). Acetaldehyde, ethanol, and ethyl acetate production seems to be a natural process of pear ripening. At relatively low concentrations, these volatiles may be beneficial to flavor development or as substrates for biosynthesizing other characteristic aromas.

Preclimacteric pears exposed to 0.25% O_2, 20% O_2 + 80% CO_2, or 0.25% O_2 + 80% CO_2 at 20C for 1 to 3 days accumulated more acetaldehyde, ethanol, and ethyl acetate (Fig. 1 A, C, and E) than air control fruit. Higher concentrations of these fermentation volatiles were found in postclimacteric fruit (Fig. 1 B, D, and F). Boersig et al. (1988) found that ripe pears were less tolerant to low O_2 since a higher O_2 concentration was required to maintain aerobic respiration when the fruit became more mature.

The large increases in concentrations of fermentation volatiles, especially in the postclimacteric pears, by the very low O_2 and/or very high CO_2 atmospheres may be detrimental to the commodity since high concentrations of these volatiles may cause off-flavors (Ke and Kader, 1990; Ke et al., 1991b).

PDC extractable activity in postclimacteric pears was 2 to 3 times greater than that in preclimacteric fruit (Fig. 2 A and B). For postclimacteric pears, PDC activity was higher in fruit kept in 0.25% O_2, 20% O_2 + 80% CO_2, or 0.25% O_2 + 80% CO_2 than in the air control fruit (Fig. 2B); the CA effect on PDC activity was less obvious in preclimacteric pears (Fig. 2A).

After 2 to 3 days, ADH activity was slightly increased by 20% O_2 + 80% CO_2 in preclimacteric and postclimacteric pears (Fig. 2 C and D). For the preclimacteric pears, much higher ADH activity was induced by 0.25% O_2; however, 0.25% O_2 + 80% CO_2 resulted in lower ADH activity than 0.25% O_2 alone (Fig. 2C). For postclimacteric pears, ADH activity was similar for the fruit kept in 0.25% O_2 alone or in 0.25% O_2 + 80% CO_2 (Fig. 2D). The postclimacteric pears kept in 20% O_2 + 80% CO_2 had the highest acetaldehyde concentration (Fig. 1B) and a low ethanol concentration (Fig. 1D), which may be related to the low ADH activity in the high-CO_2-treated fruit (Fig. 2D) compared to the fruit kept in 0.25% O_2 or 0.25% O_2 + 80% CO_2.

There are three ADH isozymes (ADH1, ADH2, and ADH3) in plants (Hanson et al., 1984). The active ADH isozymes do not exist as a single subunit. Instead, they occur as homogeneous or heterogeneous dimers to have function. Theoretically, three ADH isozymes may result in six dimers (ADH1–ADH1, ADH1–ADH2, ADH1–ADH3, ADH2–ADH2, ADH2–ADH3, and ADH3–ADH3). However, not all six dimers could be found in each plant tissue, due to the limited expression of one or two specific ADH isozymes. Using starch gel electrophoresis, only four ADH dimers could be clearly and consistently identified in preclimacteric pears (Fig. 3). Occasionally, two additional ADH dimers appeared at very low intensities, as has been reported by Nanos et al. (1992). After 1 day, only one ADH dimer stained slightly more intensely in fruit kept in 0.25% O_2 than in air control fruit (Fig. 3A). After 3 days, this ADH dimer stained much more intensely in fruit kept in either 0.25% O_2 or 0.25% O_2 + 80% CO_2 (Fig. 3B); 20% O_2 + 80% CO_2 slightly increased the intensity of this ADH dimer. Another ADH dimer stained slightly more intensely in fruit kept in 0.25% O_2 or 0.25% O_2 + 80% CO_2 (Fig. 3B). Generally, the changes in ADH isozymes (Fig. 3) correlated well with changes in extractable ADH activity (Fig. 2C, Table 1) of the preclimacteric pears.

After transferring the low-O_2- and/or high-CO_2-treated preclimacteric pears to air for another 3 days, acetaldehyde and ethyl acetate concentrations slightly increased or did not change, while ethanol content decreased (Table 1). During this period, PDC activity in fruit treated with 0.25% O_2 or 0.25% O_2 + 80% CO_2 slightly decreased, while ADH activity in fruit treated with 0.25% O_2 + 80% CO_2 slightly increased (Table 1). The low-O_2- and/or high-CO_2-induced ADH isozymes (Fig. 3C) were similar to those observed at transfer (Fig. 3B).

PDC and ADH regulation. PDC and ADH induction is generally regarded as one of the reasons for anaerobic volatile accumulation (Kennedy et al., 1992). In several studies (Gerlach et al., 1982; Good and Crosby, 1989; Kelley, 1989), increased PDC and ADH activity has been found to be due to increased transcription and translation, resulting in new mRNA synthesis and *de novo* synthesis of the corresponding enzyme proteins.

Our data (Figs. 1 and 2, Table 1) indicate that, although low O_2 and high CO_2 generally increased PDC and ADH activities, the changes in the extractable activities of these two fermentation enzymes did not always correlate with the concentrations of their corresponding products. For example, 0.25% O_2 induced much higher ADH activity than 0.25% O_2 + 80% CO_2 in preclimacteric pears (Fig. 2C), but the combination treatment caused more ethanol to accumulate (Fig. 1C). The changes in ADH isozymes

Table 1. Concentrations of fermentation volatiles and activities of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) in preclimacteric 'Bartlett' pears kept in air, 0.25% O_2, 20% O_2 + 80% CO_2, or 0.25% O_2 + 80% CO_2 for 3 days (day 3) followed by storage in air for another 3 days (day 6) at 20C.

	Volatile concn (mM)						Enzyme activity (μmol·min^{-1}·g^{-1})			
	Acetaldehyde		Ethanol		Ethyl acetate		PDC		ADH	
Treatment	Day 3	Day 6	Day 3	Day 6	Day 3	Day 6	Day 3	Day 6	Day 3	Day 6
Air	0.07	0.32	0.2	1.5	0.00	0.02	0.27	0.28	0.25	0.29
0.25% O_2	0.61	0.85	22.9	11.8	0.04	0.14	0.32	0.27	0.84	0.78
20% O_2 + 80% CO_2	0.24	0.35	8.2	3.1	0.08	0.08	0.25	0.24	0.32	0.34
0.25% O_2 + 80% CO_2	0.62	0.76	37.2	23.9	0.14	0.15	0.32	0.23	0.42	0.55
LSD (P = 0.05)	0.13		4.4		0.03		0.04		0.07	

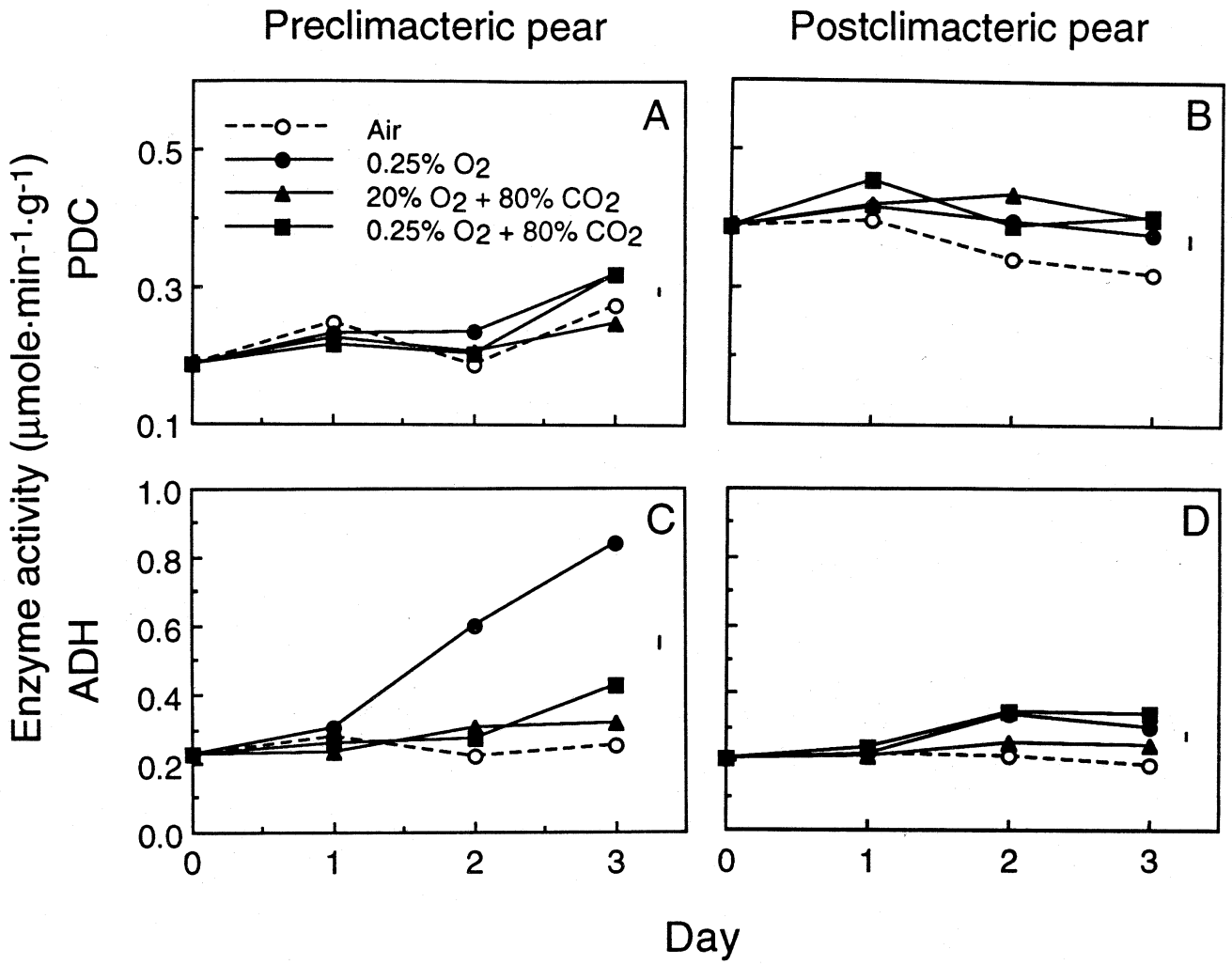


Fig. 2. Changes in pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) activities in preclimacteric and postclimacteric 'Bartlett' pears kept in air, 0.25% O_2, 20% O_2 + 80% CO_2, or 0.25% O_2 + 80% CO_2 at 20C for 1, 2, or 3 days. The vertical bars represent LSD at $P = 0.05$.

(Fig. 3B) also could not explain why 0.25% O_2 + 80% CO_2 induced higher ethanol accumulation than 0.25% O_2 alone. Similarly, although 0.25% O_2 caused more ethanol to accumulate in postclimacteric (Fig. 1D) than that in preclimacteric pears (Fig. 1C), this treatment induced much higher ADH activity in preclimacteric pears (Fig. 2C). On the other hand, postclimacteric pears had higher PDC activity (Fig. 2B) and greater acetaldehyde accumulation (Fig. 1B) compared to preclimacteric fruit. Chang et al. (1983) suggested that PDC was more likely to be a rate-limiting enzyme for ethanolic fermentation than ADH in sweetpotatoes. Roberts et al. (1989) reported that ethanol production rate was correlated with ADH activity when the enzyme level was very low, but, at high enzyme levels, ethanol production was independent of ADH activity. For 'Bartlett' pears, the extractable ADH activity of 0.55 mmol·min^{-1}·g^{-1} seemed to be high enough for ethanol accumulation (Fig. 1 C and D, Fig. 2 C and D, Table 1).

It seems that only measuring PDC and ADH activities does not adequately explain the differences in acetaldehyde, ethanol, and ethyl acetate concentrations in preclimacteric and postclimacteric pears exposed to the three atmospheres. Other metabolic factors may also be involved in regulating ethanolic fermentation.

One of these factors may be the change in cytoplasmic pH. Davies (1980) and Roberts (1989) proposed that, under limited O_2 supply, cytoplasmic pH decreased, which activated PDC and induced ethanolic fermentation. The cytoplasmic pH of 'Bartlett' pears kept in air was 7.4; exposure to 0.25% O_2 decreased cytoplasmic pH to 7.0 (Nanos and Kader, 1993) and exposure to elevated CO_2 atmospheres caused the cytoplasmic pH to drop to 6.6 (Chavez, 1991). The optimum pH for PDC and ADH was 6 (Fig. 4). The decreases in cytoplasmic pH by low O_2 and/or high CO_2 would significantly activate PDC and slightly activate ADH.

Another factor could be the changes in substrate concentrations. PDC was reported to be an allosteric enzyme (Hubner et al., 1978). This enzyme had a Km value of 0.84 mM for pyruvate (Fig. 5). PDC was not active at pyruvate concentrations <0.1 mM, but it became much more active if the substrate concentration was increased to 0.25 mM (Fig. 5). Davis et al. (1973) reported that pyruvate concentration in orange was 0.02 to 0.1 mM and that low O_2 and high CO_2 atmospheres increased pyruvate concentration. Such an increase in pyruvate concentration by stress atmospheres would, in turn, activate PDC due to a conformation change of the allosteric enzyme through the combination to its substrate.

ADH had a Km value of 0.86 mM for acetaldehyde (Fig. 6). For preclimacteric pears, air control fruit had an acetaldehyde concentration of 0.04 to 0.08 mM (Fig. 1, Table 1), which was much lower than the Km value. When the pears were ripe, acetaldehyde concentration of the air control fruit reached 0.3 to 0.8 mM. The CA treatments increased acetaldehyde concentration to near or above the Km value of ADH in preclimacteric and postclimacteric pears. The increase in acetaldehyde concentration could have partly

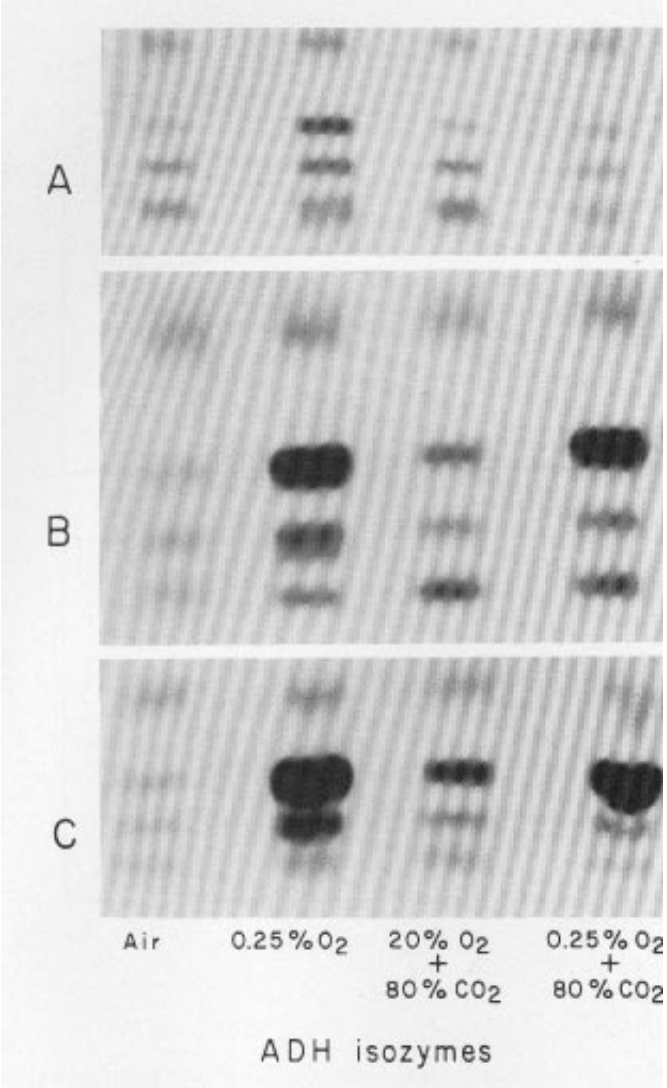


Fig. 3. Changes in alcohol dehydrogenase (ADH) isozymes in preclimacteric 'Bartlett' pears kept in air, 0.25% O_2, 20% O_2 + 80% CO_2, or 0.25% O_2 + 80% CO_2 for 1 (**A**) or 3 days (**B**) followed by storage in air for another 3 days (**C**) at 20C. Top is the anode and bottom is the cathode in each section.

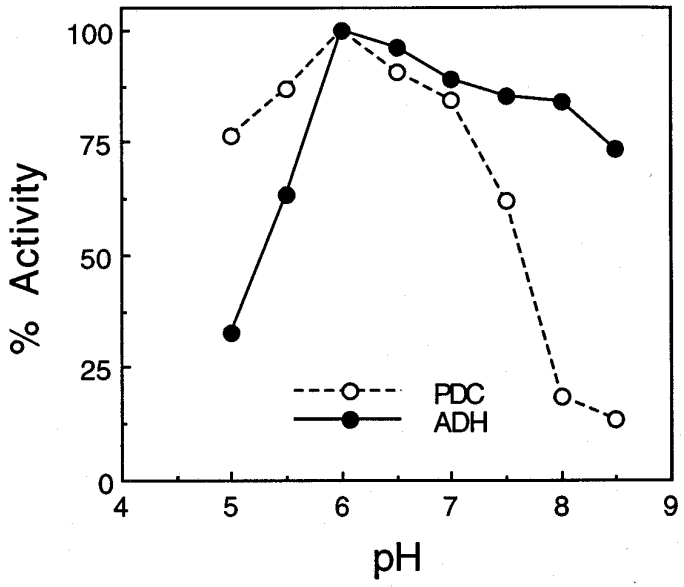


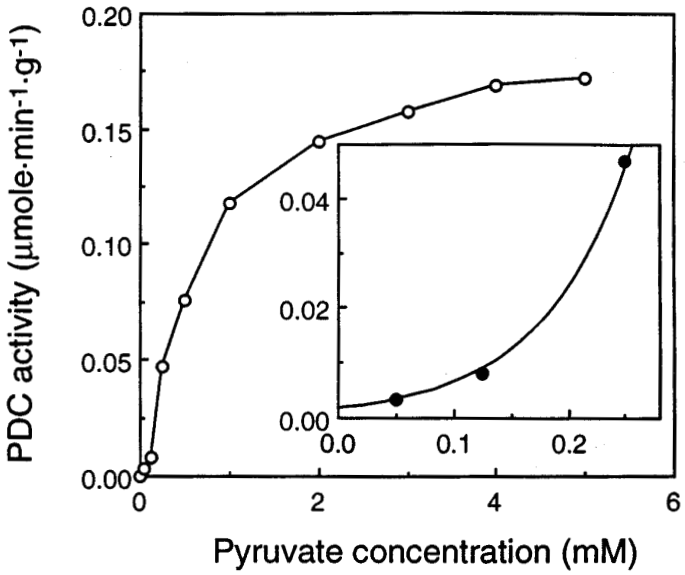
Fig. 4. Pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) activities in 'Bartlett' pears as influenced by pH.

contributed to ethanol accumulation in the fruit.

The Km value of ADH for NADH (Fig. 6) was much lower than that for acetaldehyde. If no NAD was added, ADH had a Km value of 0.023 mM for NADH; if 0.08 mM NAD was added, the Km value of ADH for NADH increased to 0.057 mM. Since oxidative phosphorylation of 'Bartlett' pears was strongly inhibited by low O_2 (Nanos and Kader, 1993) or high CO_2 (Chavez, 1991), as indicated by reduced ATP levels, electron transport through the cytochrome pathway could have been greatly reduced. This could increase NADH concentration and decrease NAD concentration, which in turn reduces the Km value of ADH for NADH and activates the enzyme. The induction of ethanol biosynthesis will use NADH and prevent its further accumulation in the tissue.

Conclusion

Low O_2 and/or high CO_2 concentrations may induce ethanolic fermentation (Fig. 7) by one or more of the following means: 1) increased amounts of PDC and ADH due to *de novo* biosynthesis;



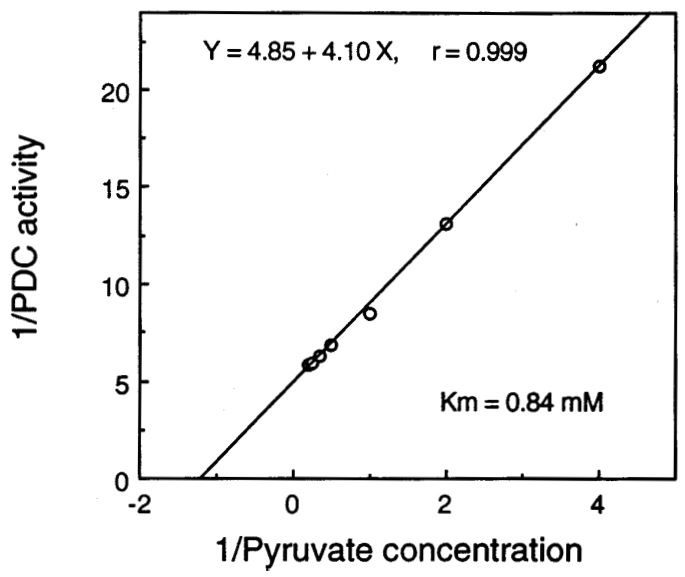


Fig. 5. Pyruvate decarboxylase (PDC) activity in 'Bartlett' pears as influenced by pyruvate concentration.

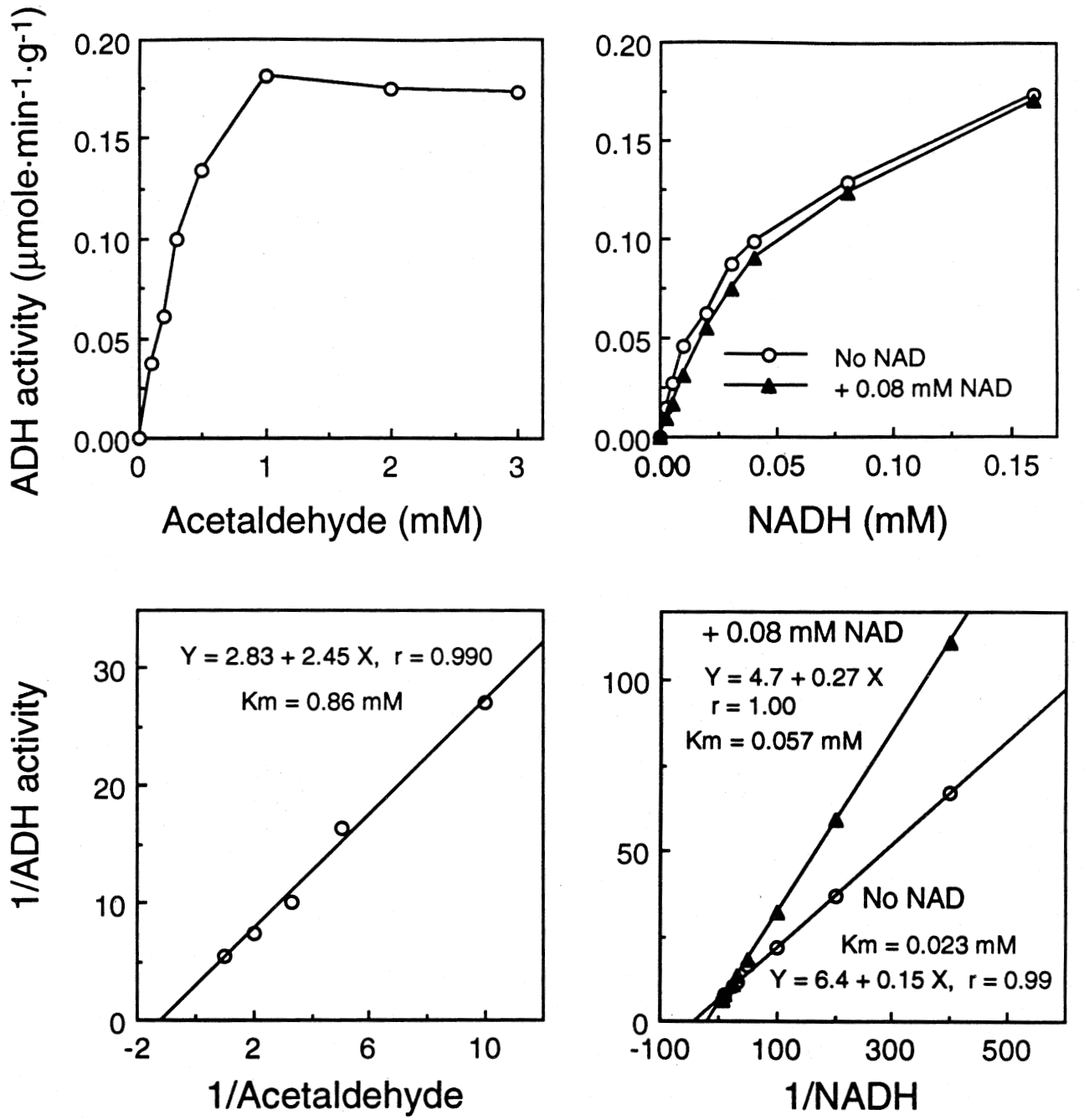


Fig. 6. Alcohol dehydrogenase (ADH) activity in 'Bartlett' pears as influenced by acetaldehyde and NADH concentrations with or without 0.08 mM NAD.

2) PDC and ADH activation by a decrease in cytoplasmic pH; and 3) PDC and ADH activation and more rapid fermentation due to increased pyruvate, acetaldehyde, and NADH concentrations and decreased NAD level. ADH is induced to a greater extent by low O_2 than high CO_2 atmospheres. On the other hand, high CO_2 decreases cytoplasmic pH to a greater degree, which has a stronger effect on PDC activation. The induction of ethanolic fermentation will use NADH and allow glycolysis to go on. A small amount of ATP can be produced through substrate phosphorylation to permit the plant tissue to survive temporarily. However, acetaldehyde, ethanol, and ethyl acetate accumulation may cause detrimental effects if their concentrations are beyond the tolerance limits of plant tissues. Postclimacteric pears were more sensitive to low O_2 and/or high CO_2, as indicated by greater acetaldehyde, ethanol, and ethyl acetate accumulation compared to preclimacteric fruit.

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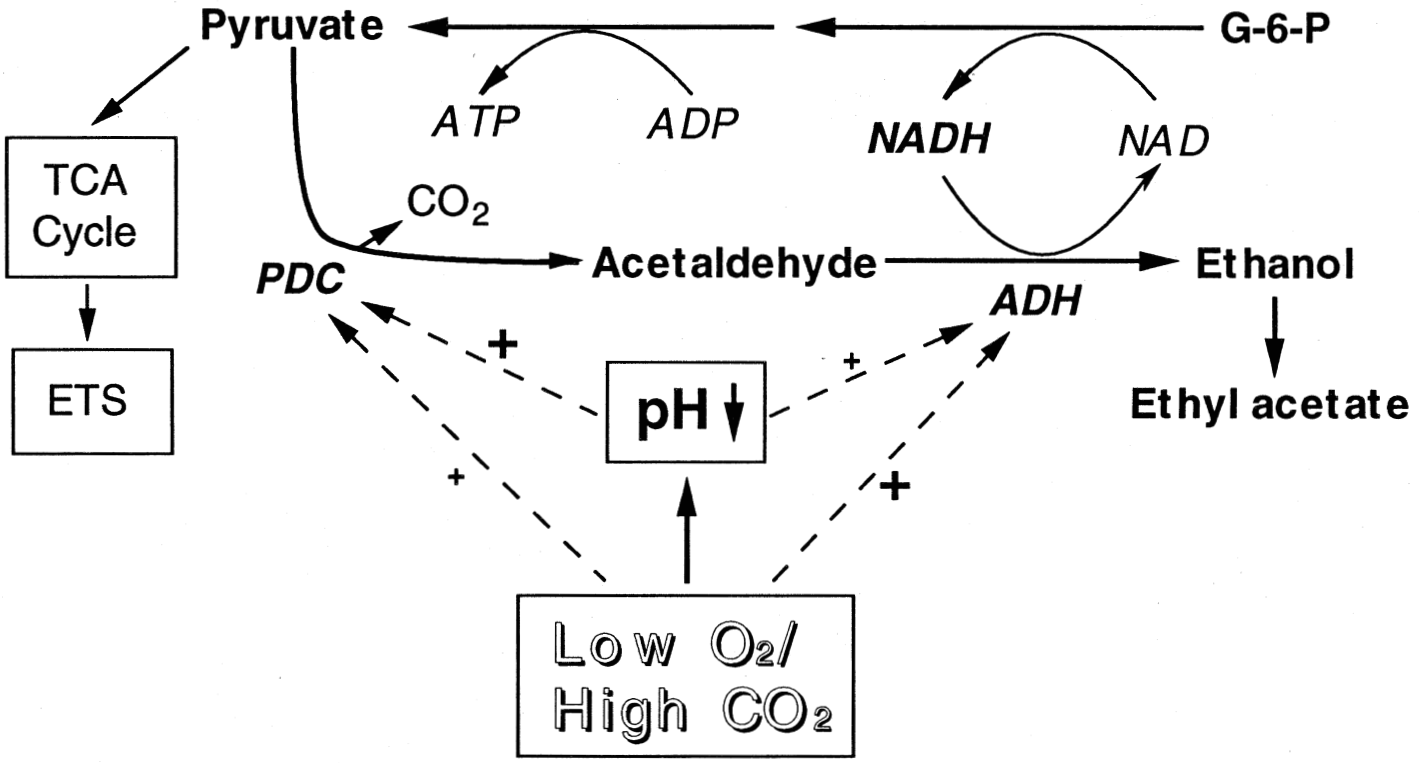

Fig. 7. Ethanolic fermentation pathway in 'Bartlett' pears as proposed to be regulated by low O_2 and/or high CO_2 atmospheres. G-6-P = glucose-6-phosphate, PDC = pyruvate decarboxylase, ADH = alcohol dehydrogenase, TCA = tricarboxylic acid, ETS = electron transport system, -----±-----> = induction and/or activation.

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