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## Low O<sub>2</sub>-induced changes in pH and energy charge in pear fruit tissue \*

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### ABSTRACT

Pear (*Pyrus communis* L. cv. 'Bartlett') fruit were stored at 0°C for 2 or 4 weeks and subsequently ripened in air at 20°C or treated for 4 days with 0.25% O<sub>2</sub> at 20°C before transfer to air for ripening. The juice pH (indicator of vacuolar pH) of air-treated fruit decreased slightly with ripening. Exposure to 0.25% O<sub>2</sub> resulted in higher juice pH during the treatment and after transfer to air. Pear fruit discs, after 10 h aging, were also kept in air or 0.25% O<sub>2</sub> at 20°C for 2 days and their <sup>31</sup>P-NMR spectra obtained. The 0.25% O<sub>2</sub>-treated discs had higher cytoplasmic Pi (relative to the vacuolar Pi), had about 0.4 pH units lower cytosolic pH and lower ATP/ADP ratio indicating lower energy charge. The above changes could alter the in vivo activity of a number of respiratory enzymes in fruit subjected to hypoxic treatment.

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Key words: *Pyrus communis*; Anaerobic respiration; Controlled atmosphere; Nuclear magnetic resonance

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### INTRODUCTION

Cellular pH is very important in the regulation of metabolism. In fruit, more than 90% of the cellular volume is occupied by the vacuole, which is usually very acidic (pH < 5). The cellular functions that take place in the cytosol are optimal at a pH near neutral and the cytosolic pH is usually found to be around 7.4. To maintain cytoplasmic pH, metabolic processes consume or produce protons and H<sup>+</sup>-pumps operate in the plasmalemma and in the tonoplast (Torimitsu et al., 1984; Cerana et al., 1989; Kurkdjian and Guern, 1989). These pumps use large

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amounts of energy to maintain proton gradients and counteract leakage through the plasmalemma or tonoplast.

Various methods have been used to measure cellular pH and all have certain disadvantages (Kurkdjian and Guern, 1989). The easiest method involves tissue maceration and measuring the pH of the resultant slurry with a glass electrode. In fruit this pH value is close to vacuolar pH, because the vacuole is large and high in acid content.

Nuclear Magnetic Resonance (NMR) spectroscopy has proven valuable as a nondestructive technique to study aspects of plant metabolism during the last decade (Roberts, 1987). This technique separates and detects phosphorus-containing metabolites of ample concentration according to the chemical environment around each P nucleus.  $^{31}\text{P}$ -NMR is useful in the nondestructive evaluation of cytoplasmic and vacuolar Pi content, and levels of some phosphorylated metabolites, such as adenosine triphosphate (ATP), adenosine diphosphate (ADP), glucose-6-P and nicotinamide adenine dinucleotide phosphate (NADP).

The levels of Pi in the vacuole and cytoplasm can be estimated relatively accurately from the intensity and area of the respective peaks that appear in NMR spectra. Using this technique cytoplasmic Pi was found to increase with anoxia and attributed to either ATP dephosphorylation or leakage of vacuolar Pi (Martin et al., 1982; Roberts et al., 1982; Lee and Ratcliffe, 1983; Siriphanich and Kader, 1986).

$^{31}\text{P}$ -NMR measurements have also shown that cytoplasmic acidosis occurs under anoxia. The lowering of cytoplasmic pH has been attributed to temporal lactate accumulation, dysfunction of  $\text{H}^+$ -pumps or  $\text{CO}_2$  accumulation (Roberts et al., 1982; Wray et al., 1985). Unfortunately, vacuolar pH can not be measured accurately with  $^{31}\text{P}$ -NMR because the titration curves are insensitive at pHs below 5 (Kurkdjian et al., 1985). Nevertheless,  $^{31}\text{P}$ -NMR measurements have indicated that vacuolar pH does not change in hypoxic maize roots (Roberts et al., 1982).

$^{31}\text{P}$ -NMR spectroscopy can also be used to evaluate energy-charge or the levels of ATP and ADP in the cell. It has been found that the energy charge decreases (and later stabilizes) during hypoxia from an initial value of 0.9 to 0.8 for anoxia-tolerant species and to 0.6 for anoxia-intolerant species (Raymond and Pradet, 1980; Saglio et al., 1980; Mocquot et al., 1981; Davies et al., 1985; Vanlerberghe et al., 1990). Upon transfer to air, energy charge returned to its initial value.

The purpose of this study was to evaluate the effect of 0.25%  $\text{O}_2$  treatment on pear fruit vacuolar pH, cytosolic pH, Pi concentration and energy charge. These alterations are discussed as possible regulators of biochemical changes associated with low  $\text{O}_2$  treatment and ripening of pear fruit.

## MATERIALS AND METHODS

*Plant material and treatment.* Mature-green 'Bartlett' pear fruit (*Pyrus communis* L.) were obtained on the day of harvest from Sacramento and Lake counties, CA and stored at  $0^\circ\text{C}$  until the experiments were conducted two and four weeks

after harvest. Fruit were selected for uniformity of size and freedom from defects. All experiments were conducted at 20°C. Individual fruit were put in 450 ml jars and ventilated with air (controls) or 0.25% O<sub>2</sub> (balance N<sub>2</sub>), using a continuous flow-through system at 30 ml/min. After 4 days the fruit were transferred to air and monitored for six additional days. Measurements of the air-treated fruit were taken initially and after 1, 2, 4, 6 and 8 days. For low O<sub>2</sub>-treated fruit, measurements were taken after 1, 2 and 4 days under 0.25% O<sub>2</sub> and after 1, 2, 4 and 6 days upon transfer to air.

**Juice pH.** Initially and for every sampling period thereafter, three individual fruit per treatment were sampled (2 slices per fruit). The slices were chopped into small pieces, put in cheesecloth and juice was extracted with a hand-press. The juice pH was evaluated immediately with a glass electrode and a Corning pH meter (Model 140).

**NMR measurements.** Fruit discs (1 cm thick and 1.5 cm diameter) were cut and put in test tubes (8 discs per tube) under continuous air flow of 30 ml/min for 10 h to overcome the injury response. Half of the discs were subsequently kept under 0.25% O<sub>2</sub> (balance N<sub>2</sub>) for 2 days, then used for the NMR measurements. Four discs were placed in the NMR tube, 5 cm long, 1.8 cm internal diameter (Wilmad Glass Co), the tube was capped and capillary tubing was used to circulate 30 ml/min of air or 0.25% O<sub>2</sub> around the discs while in the spectrometer. The spectrum was recorded using a General Electric NMR Omega 7 Tesla spectrometer at 121.6 MHz, 6000 transients were acquired using a 60° pulse, a 0.41s pulse repetition time, 4096 time domain points and a 10 kHz spectral width. Line broadening of 20 kHz was applied. Total acquisition time was 2 h. The experiment was repeated 3 times.

## RESULTS AND DISCUSSION

The juice pH at the beginning of the storage period was between 4.1 and 4.2. Fruit that were kept in air showed a slow decline in juice pH with ripening and senescence (Fig. 1). Fruit treated with 0.25% O<sub>2</sub> retained their pH near initial

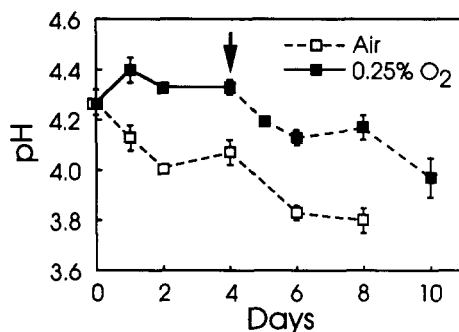


Fig. 1. Changes in the juice pH of 'Bartlett' pears during ripening at 20°C in air or during treatment with 0.25% O<sub>2</sub> and subsequent transfer to air.

values during 4 days at 20°C. Upon transfer to air, low O<sub>2</sub>-treated fruit showed a decline in juice pH but it remained higher than pH of the air-control pears. An analogous 0.2 unit higher cell sap pH in anoxic rice seedlings compared to air-treated ones was found by Menegus et al., (1989), who speculated that the alkalization in anoxic rice can be due to the production of  $\gamma$ -aminobutyrate from glutamate, high succinate production and low lactate production.

<sup>31</sup>P-NMR measurements on pear fruit discs showed a shift in the resonance of cytosolic Pi due to the hypoxic treatment (Fig. 2). The shift was in the order of 0.3 ppm (Table 1). This amounts to about 0.36 pH units drop in cytosolic pH. Using the standard curves of Roberts et al. (1981) obtained in the presence of 5 mM MgCl<sub>2</sub> and 100 mM KCl, we estimated that the cytoplasmic pH of air-treated discs was 7.4, while 0.25%-O<sub>2</sub> treatment reduced the pH to about 7. Use of the standard curve for the cytoplasmic pH estimation is justified, since potassium and magnesium contents of pears are comparable to the above-mentioned amounts. The results agree with work previously reported on maize roots and *Nicotiana* cells (Roberts et al., 1982; Lee and Radcliffe, 1983; Wray et al., 1985). The reasons for acidification of cytoplasm are not fully understood. In static systems, CO<sub>2</sub> accumulation must be responsible for the extensive continuous pH drop (Wray et al., 1985). In a flow-through system (flushed with nitrogen), the initial drop in pH may be attributed to lactate accumulation or H<sup>+</sup>-pump dysfunction. Measurements taken under continuous flow of nitrogen showed a stabilization of pH at 0.3–0.6 units below normal in maize root tips (Roberts et al., 1982). This stabilization is unexplainable and pH could recover to normal values after transfer to air. It is also known that metabolism is widely affected by anoxia and could be either the result or cause of cytoplasmic acidosis. The vacuolar pH changes can not be measured with <sup>31</sup>P-NMR because the titration curves are insensitive at pHs below 5 (Kurkdjian et al., 1985).

Cytosolic Pi increased substantially relative to vacuolar Pi (Table 1). The ratio cyt. Pi/vac. Pi was 0.22 for air-treated discs and 0.78 for 0.25% O<sub>2</sub>-treated ones. The increase in cytosolic Pi can be the result of several events, including partial ATP breakdown or vacuolar leakage. An increase in cytosolic Pi has also been found in anoxic maize roots and *Acer* cells (Martin et al., 1982; Roberts et al., 1982; Lee and Radcliffe, 1983).

The ratio of estimated nucleotide triphosphate (NTP)/nucleotide diphosphate (NDP) content was around 5 in aerated pear fruit discs but dropped to 0.9 when pear discs were treated with 0.25% O<sub>2</sub> (Table 1). The change in ratio and drop in energy charge of the cell during the 2-day hypoxic treatment is largely the result of reduced NTP levels, that can be attributed to low phosphorylation and increased nucleotide breakdown (Davies et al., 1985; Raymond et al., 1987). A decrease in energy charge in tissues subjected to anoxia or hypoxia has also been observed in maize roots, rice seedlings, germinating lettuce seeds and the alga *Selenastrum* (Raymond and Pradet, 1980; Saglio et al., 1980; Mocquot et al., 1981; Lee and Ratcliffe, 1983; Roberts et al., 1985; Vanlerberghe et al., 1990). The drop in NTP levels will generally result in increased Pi levels and this agrees with the elevated cytoplasmic Pi levels found in these hypoxic tissues.

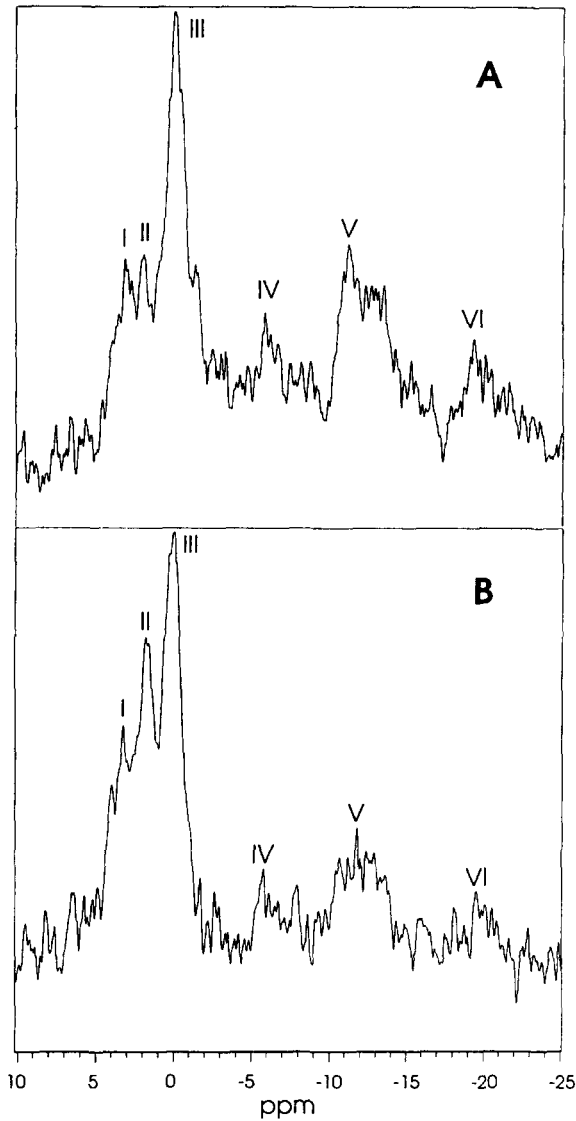


Fig. 2. <sup>31</sup>P-NMR spectra of pear fruit discs. Spectra were obtained at 121.6 MHz and 20°C and are the results of 6000 transients. The pear discs were perfused during the measurements with air (A) or 0.25% O<sub>2</sub> (B) at a flow rate of 30 ml/min. Peak identification: I. Glucose-6-P, II. Cytoplasmic Pi, III. Vacuolar Pi, IV.  $\gamma$ -NTP +  $\beta$ -NDP, V.  $\alpha$ -NTP +  $\alpha$ -NDP and VI.  $\beta$ -NTP.

Hypoxic treatment caused a number of changes in pear fruit similar to changes observed previously with other plant tissues. There was an increase in cytoplasmic Pi concentration (relative to vacuolar Pi), a decrease of about 0.4 pH units in cytosolic pH and the NTP (mainly ATP) level dropped resulting in an NDP (mainly ADP) increase. Consequently, it is expected that Mg<sup>2+</sup> levels will rise. It is

TABLE 1

Amounts of various metabolites derived from NMR spectra (see Fig. 2) of pear fruit discs perfused with air or 0.25% O<sub>2</sub>

	Air	0.25% O <sub>2</sub>
Glu-6-P	178	95
Cytoplasmic Pi	100	100
Vacuolar Pi	452	129
$\gamma$ -NTP + $\beta$ -NDP	126	58
$\alpha$ -NTP + $\alpha$ -NDP	379	105
$\beta$ -NTP	105	28
Cyt. Pi shift (ppm)	2.0	1.7
NTP/NDP	5.00	0.93
Cyt. Pi/Vac. Pi	0.228	0.78

The values are relative to the area of the cytoplasmic Pi peak which is taken as 100. Changes in the chemical shift of cytoplasmic Pi and some ratios are also shown.

unknown how Mg<sup>2+</sup> will be distributed in the vacuole, cytosol or other organelles, but, it is known to interfere with the NMR measurements (Roberts et al., 1981).

ATP, ADP, Pi and Mg<sup>2+</sup> have been found to regulate an array of respiratory enzymes and the changes of these metabolites resulting from hypoxic treatment can play an important role in the regulation of respiration (Raymond et al., 1987). The decrease in cytosolic pH in hypoxic pear fruit discs can also affect the activity of a number of respiratory enzymes; their activity may be altered only partially but enough to have significant metabolic effects. In vitro studies have shown that hypoxia causes a 20 to 40% change in the activity of some respiratory enzymes in pears (Nanos, 1991; Nanos et al., 1992).

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