Journal of Experimental Botany, Vol. 52, No. 362, pp. 1769–1777, September 2001

Journal of Experimental Botany

Comparative study of the O_2, CO_2 and temperature effect on respiration between 'Conference' pear cell protoplasts in suspension and intact pears

J. Lammertyn[1], C. Franck, B.E. Verlinden and B.M. Nicolaï

Flanders Centre/Laboratorium of Postharvest Technology, Katholieke Universiteit Leuven, Willem de Croylaan 42, B-3001, Leuven, Belgium

Received 8 February 2001; Accepted 21 May 2001

Abstract

The influence of the O_2 and CO_2 concentration and the temperature on the O_2 uptake rate of cool-stored intact pears and pear cell protoplasts in suspension was compared. Protocols to isolate pear cell protoplasts from pear tissue and two methods to measure protoplast respiration have been developed. Modified Michaelis–Menten kinetics were applied to describe the effect of the O_2 and the CO_2 concentration on the O_2 uptake rate and temperature dependence was analysed with an Arrhenius equation. Both systems were described with a non-competitive type of CO_2 inhibition. Due to the inclusion of gas diffusion properties, the Michaelis–Menten constant for intact pears (2.5 mM) was significantly larger than the one for protoplasts in suspension (3 μM), which was in turn larger than the Michaelis–Menten constant obtained in mitochondrial respiration measurements described in the literature. It was calculated that only 3.6% of the total diffusion effect absorbed in the Michaelis–Menten constant for intact pears, could be attributed to intracellular gas diffusion. The number of cells per volume of tissue was counted microscopically to establish a relationship between the pear cell protoplast and intact pear O_2 uptake rate. A remarkable similarity was observed: values of 61.8 nmol kg^{-1} s^{-1} for protoplasts and 87.1 nmol kg^{-1} s^{-1} for intact pears were obtained. Also, the inhibitory effect of CO_2 on the respiration rate was almost identical for protoplasts and intact pears, suggesting that protoplast suspensions are useful for the study of other aspects of the respiration metabolism.

Key words: *Pyrus communis* L., protoplast isolation, respiration, metabolism, modelling.

Introduction

Respiration plays a central role in the overall metabolism of a plant, and it is, therefore, often used as a general measure of metabolic rate (Kays, 1991). Controlled atmospheres are applied to slow down the metabolic rate, and to prolong the storage life of fruits. Reduced O_2 and increased CO_2 levels have been thought of as the primary reason for the beneficial effects on the fruits (Mathooko, 1996). Michaelis–Menten kinetics are widely used to describe the relationship between the O_2 concentration and the O_2 consumption rate: the whole respiration pathway is assumed to be determined by one rate-limiting enzymatic reaction (Chevillotte, 1973).

However, modern studies of respiration by plant mitochondria have shown that the electron transport system is branched, terminating in two different terminal oxidase systems: a cytochrome oxidase and an alternative cyanide-resistant oxidase, each with its own affinity for O_2 (Millar *et al.*, 1994). In such a case, the Michaelis–Menten model can still be used as a semi-empirical model to describe the respiration characteristics, although its parameters should then be interpreted with caution. Since it is impossible to separate the activities of the two terminal oxidase systems in intact pears, and the same model structure should be taken for comparison purposes, the Michaelis–Menten model was used to describe the respiration of pear cell protoplasts in suspension.

[1] To whom correspondence should be addressed. Fax: +32 16 32 29 55. E-mail: jeroen.lammertyn@agr.kuleuven.ac.be

© Society for Experimental Biology 2001

For some commodities, the CO_2 concentration influences the O_2 consumption as well (Kerbel *et al*., 1990; Peppelenbos and van't Leven, 1996). However, the mechanism by which elevated CO_2 influences the regulation of the respiratory metabolism is still obscure and several hypotheses have been proposed for its mode of action (Mathooko, 1996). Chang distinguished three types of CO_2 inhibition on the reaction rate of an enzyme: competitive, uncompetitive and non-competitive inhibition (Chang, 1981). More recently, a mixed type of inhibition has been used (Peppelenbos and van't Leven, 1996). However, those models describe a combined effect of respiration and gas diffusion: the cytochrome *c* oxidase, which is believed to be the rate-determining enzymatic reaction, is saturated at O_2 levels lower than 5% (Cameron *et al*., 1995). Nevertheless, the respiration rate of some commodities still increases with increasing O_2 levels above 5%. This phenomenon is due to diffusion limitations, caused by the fruit skin and/or the tissue (Peppelenbos and van't Leven, 1996). Diffusion barriers, between the external atmosphere and mitochondria, the actual place were respiration takes place, should be taken into account (Solomos, 1987; Rajapakse *et al*., 1990; Knee, 1991) or eliminated. Boersig *et al*. studied the similarities between the responses of intact fruits and suspensions of cultured cells to limiting O_2 concentrations (Boersig *et al*., 1988). Brady and Romani used cell cultures of 'Passe Crassane' pear to study the respiration in non-growing cultured pear fruit cells in response to ethylene and modified atmospheres (Brady and Romani, 1988). However, in the literature so far, no quantitative description is available for the O_2 uptake rate of protoplasts, isolated from intact pear tissue, as a function of temperature, CO_2 and O_2 concentration. The protoplast suspension was preferred above measurements on mitochondria for several reasons. Cell protoplasts are the smallest building blocks of tissue. They can be isolated easily and their viability can be assessed microscopically. Moreover, the cell can be seen as a small bioreactor in which the mitochondria, the centres of respiration, 'live' in a natural and physiologically optimal environment. Finally, the cell protoplast respiration parameters can be coupled back to those of intact pears, to compare the respiration characteristics, which is difficult to do for mitochondria.

The constructed protoplast respiration model could serve as a building block for a respiration–diffusion model to describe the distribution of the O_2 and CO_2 concentration in an intact pear. This respiration–diffusion model can then be used to study the physiological mechanism of core breakdown, a disorder that occurs during storage of 'Conference' pears (*Pyrus communis* L.) (Lammertyn *et al*., 2000).

There are three objectives in this paper. The first objective was to develop a protocol to isolate pear cell protoplasts from intact pear tissue. The respiration rate was measured on these protoplasts instead of on cultured cells which might have a different metabolism and might be ripeness stage independent. The second objective was to study the influence of O_2 and CO_2 concentrations and temperature on the respiration rate of intact pears and pear cell protoplasts in suspension, quantitatively with modified Michaelis–Menten kinetics. Different techniques to monitor O_2 consumption were developed and their potential to provide accurate and reliable information on the protoplast O_2 uptake rate will be discussed. Thirdly, the respiration characteristics of intact pears and protoplasts in suspension were compared, to address the effect of macroscopic gas diffusion. For modelling purposes, it was assumed that there were no gas gradients within the protoplast.

Materials and methods

Fruit material

Pears (*Pyrus communis* L.) were harvested in September 1999 at a pre-climacteric stage at the Proeftuin voor Pit- en Steenfruit van het Proefcentrum voor de Fruitteelt in Velm (Belgium), and were cooled (−0.5 °C) for a period of 21 d preceding controlled atmosphere (CA) storage (2% O_2, 0.7% CO_2 and −0.5 °C) until they were used for the experiments, approximately 4 months after harvesting.

Intact pear respiration measurements

Respiration measurements on intact pears were carried out in a similar way to that described earlier (Peppelenbos and van't Leven, 1996; de Wild *et al*., 1999). The pears were placed separately in 1.2 l glass jars. After an adaptation period of 40 h, during which the jar headspace was flushed at 20 l h^{-1} with the applied humidified gas mixture, the jars were closed and the initial headspace (O_2, N_2 and CO_2) was measured with a gas chromatograph (Chrompack CP 2002, Bergen op Zoom, The Netherlands). Depending on the temperature (7, 15 or 23 °C), the headspace was analysed again after 8, 6 or 4 h. The total pressure was measured before and after each measurement (PTX 1400, Druck, Germany). The difference in gas partial pressure was converted to molar concentration according to the ideal gas law. The O_2 consumption rate was expressed in nmol per unit weight (kg fresh weight) and per unit time (s).

The respiration rate was measured for a full factorial design of 8 O_2 partial pressures (0, 1, 2, 4, 8, 10, 15, 20 kPa) and 3 CO_2 partial pressures (0, 5, 15 kPa) at 23 °C. To include the temperature effect in the gas exchange model, it was sufficient to measure the respiration rate at 20 kPa O_2 for the temperatures 7 °C and 15 °C (Hertog *et al*., 1998). Eight replicates were measured per treatment.

Protoplast isolation, protoplast viability and microscopy

Pear slices (20 g) of 1 mm thickness were gently macerated in a flask (500 ml) with 200 ml maceration medium containing 0.5 g pectinase (Merck, Germany), 1 ml cellulase Rohament-PL (Röhm Enzyme GmbH, Germany), 0.1 M phosphate buffer (pH = 7.4), 0.38 M mannitol, 0.08 M sucrose, 5 mM $MgCl_2$, and 0.4 g l^{-1} polyvinylpyrrolidone. The flasks were placed in

a shaking water bath (SWB-20, Haake, Germany) at 26 °C and 90 strokes min^{-1} for 12 h. The suspension was filtered on a nylon filter (250 μm) and centrifuged (Unicen 15 DR, Herolab, The Netherlands) for 5 min at 500 *g*. The pellet was resuspended in 40 ml of reaction medium, containing the same components as the maceration medium except the enzymes, and centrifuged for 5 min at 500 *g*. Finally, the pellet was resuspended again in 40 ml reaction medium. This procedure was repeated four times to obtain 160 ml of cell protoplast suspension, which was then conditioned in a large stirred bioreactor (250 ml) through which a gas mixture was bubbled with the same composition as applied later on during the measurements. The temperature was controlled with a waterjacket. Samples of 4 ml were taken from this reactor for O_2 consumption measurements. Before each measurement, a sample was also taken to determine the protoplast viability, because time and shear effects of the magnetic mixing bar could have an effect on the viability (Zhong *et al.*, 1994; Takeda *et al.*, 1994). The protoplast viability (*c.* 90%) was estimated by microscopic counting (BX40, Olympus Optical CO. Ltd., Tokyo, Japan) after selective staining with Evan's blue (0.5% w/v) (Shipway and Bramlage, 1973; Pushmann and Romani, 1983). Ten frozen sections of 120 μm, taken at three perpendicular directions in the pear tissue, were evaluated microscopically to calculate the volumetric cell density. The method of Baumann and Henze was used to measure the volume and the density of pear tissue (Baumann and Henze, 1983). These quantities were used to determine the number of protoplasts per unit weight.

Protoplast respiration measurements

The protoplast O_2 uptake rate was determined polarographically using a Clark type polarographic O_2-electrode, which was built according to Inoue (Inoue, 1989). The electrode was placed at the bottom of a small bioreactor with a maximal volume of 5 ml. After the addition of the reaction medium and the protoplasts, the headspace in the bioreactor was reduced with a screw top. A FieldPoint I/O module (National Instruments, Zaventem, Belgium) was used to establish communication between reactor and computer. The user interface was programmed in LabVIEW 5.0 (National Instruments, Zaventem, Belgium). Corrections were made for temperature, pressure and salt concentration on O_2 solubility. All assays were performed on 4 ml protoplast suspension in the small bioreactor.

Two different O_2 concentration versus time profiles were recorded to measure the protoplast O_2 uptake rate, namely re-aeration curves and O_2 depletion curves. For the re-aeration curve (Fig. 2a), the pear protoplasts started to consume O_2 until a certain O_2 level had been reached. Injection of O_2 or N_2 made it possible, respectively, to increase or decrease the O_2 concentration in the bioreactor and, hence, to measure the O_2 uptake rate at different O_2 concentrations or to measure the O_2 uptake rate repeatedly at the same O_2 concentration. Linear regression was used to calculate the slopes of the re-aeration curves and this value was assigned to the average O_2 concentration of the corresponding time interval (Fig. 2b). In the case of an O_2 depletion curve (Fig. 3a), the O_2 concentration in the reactor was recorded in time from 7 mg l^{-1} to complete O_2 depletion of the reaction medium, without any intervention by the operator.

Using re-aeration curves, protoplast respiration experiments in the presence of 1 mM KCN and/or 20 mM salicylhydroxamic acid (SHAM) (Lancaster, UK) were performed, to eliminate selectively the cytochrome and the cyanide-resistant respiration, respectively. The experiments were performed at 8 mg l^{-1} dissolved O_2 in the bioreactor, in the absence of CO_2 and at 23 °C. According to Lambers, the total O_2 uptake, v_{tot} (mg l^{-1} s^{-1} 10^{-6} cells), can be written as the sum of the respiration rate of the cytochrome pathway, v_{cyt} (mg l^{-1} s^{-1} 10^{-6} cells), the alternative respiration rate, v_{alt} (mg l^{-1} s^{-1} 10^{-6} cells) and the residual O_2 consumption, v_{res} (mg l^{-1} s^{-1} 10^{-6} cells) (Lambers, 1985). The alternative respiration rate was defined as the product of the maximum capacity of the alternative oxidase, V_{alt} (mg l^{-1} s^{-1} 10^{-6} cells) in the cell and ρ, the fraction of V_{alt} used by the cell (Equation 1).

$$V_{tot} = v_{cyt} + \rho V_{alt} + v_{res} \quad (1)$$

ρ was calculated according to Laties (Laties, 1982) and Lambers (Lambers, 1985).

Carbon dioxide concentrations

To determine the effects of CO_2 on the protoplast respiration, aqueous solutions of sodium bicarbonate were prepared in molar concentrations calculated by the Henderson–Hasselbalch equation to produce a pH = 7.4 at 23 °C, 15 °C and 7 °C in equilibrium with 0, 15 and 30% CO_2 (Umbreit *et al.*, 1964). A gas mixture with the corresponding CO_2 concentration was bubbled through the solution until a constant pH of 7.4 was obtained. These solutions were used to prepare the reaction media. Changes in pH due to loss of CO_2 from the medium during preparation and assay were minimal (less than 0.05 pH unit) (Shipway and Bramlage, 1973). Protoplast respiration measurements were performed at three temperatures: 7, 15 and 23 °C and at three CO_2-levels: 0, 15 and 30% CO_2. A full factorial design was applied with at least four repetitions for each temperature-CO_2 combination.

Model structure

Michaelis–Menten kinetics are widely used to describe the relationship between the O_2 concentration and the O_2 consumption rate: the whole respiration pathway is assumed to be determined by one rate-limiting enzymatic reaction (Chevillotte, 1973). Three types of CO_2 inhibition on O_2 uptake rate are distinguished: competitive, uncompetitive and non-competitive inhibition (Chang, 1981). In this paper the non-competitive type of inhibition was chosen: the inhibitor (CO_2) reacts both with the enzyme and the enzyme–substrate complex. The non-competitive inhibition model for protoplast O_2 uptake is described by Equation 2. The maximal O_2 uptake rate was made temperature-dependent by Arrhenius' law (Equation 3).

$$\frac{d[O_2]}{dt} = \frac{-n_{cell} V_{m,O_2}[O_2]}{(K_{m,O_2} + [O_2])\left(1 + \frac{[CO_2]}{K_{mn,CO_2}}\right)} \quad (2)$$

$$V_{m,O_2} = V_{m,O_2,ref} \exp\left[\frac{E_{a,vm,O_2}}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right] \quad (3)$$

with V_{m,O_2} the maximal O_2 uptake rate (mg l^{-1} s^{-1} 10^{-6} cells), $[O_2]$ the O_2 concentration (mg l^{-1}), $[CO_2]$ the CO_2 level (%), K_{m,O_2} (mg l^{-1}), and K_{mn,CO_2} (%) the Michaëlis constant for O_2 consumption (mg l^{-1}) and for the non-competitive CO_2 inhibition, respectively, n_{cell}, the number of cells (10^6), and $V_{m,O_2,ref}$ the maximal O_2 consumption rate at $T_{ref} = 283$ K (mg l^{-1} s^{-1} 10^{-6} cells), E_{a,vm,O_2} the activation energy (J mol^{-1}), T_{ref} the reference temperature (283 K), and R the universal gas constant (8.314 J mol^{-1} K). With small adaptations in units the equations were suited to model the respiration of

intact pears as well: V_{m,O_2} and $V_{m,O_2,ref}$ were expressed in nmol kg^{-1} s^{-1}, $[O_2]$ and $[CO_2]$ in kPa, K_{m,O_2} and K_{mn,CO_2} in kPa, n_{cell} was set to 1.

Parameter estimation

The respiration parameters of intact pears were estimated with the non-linear regression algorithm of SAS/STAT, version 6.12 (SAS Institute Inc., Cary NC. USA). The protoplast respiration model parameters were estimated by fitting experimental data on O_2 concentration profiles using non-linear regression. The numerical integration was performed using a variable order, variable step Adams method, NAG (Mark XIV), integration routine DO2CBF (NAG, 1986). The parameters were estimated, first by the described algorithm, with the cell counts fixed. Afterwards the cell counts were adjusted manually to improve the fit. The measurement error on the cell counts equalled 3.2×10^5 ($\pm5\%$ of total) cells and, hence, it was reasonable to adjust the cell counts within this range. For all curves an even smaller range was sufficient to obtain a good fit. Finally the model parameters were estimated, again based on the 'adjusted' cell counts.

Results and discussion

Intact pear respiration

Table 1 summarizes the results of the non-linear regression analysis for O_2 uptake for different types of inhibition by CO_2. No differences in fit were observed between the three inhibition models. This finding is consistent with results previously reported for pears (de Wild *et al.*, 1999). For reasons of simplicity the non-competitive type of inhibition was preferred. The model describes the measured values well (Fig. 1). Three groups of three curves can be distinguished from top to bottom, corresponding to 23, 15 and 7 °C. Within each temperature the effect of CO_2 on the respiration rate although small, is clearly visible. The higher the CO_2 level in the atmosphere the lower the respiration rate: the upper, middle and lower curve represent, respectively, the O_2 uptake rate at 0, 5 and 15% CO_2.

The maximum O_2 consumption rate at the reference temperature, $V_{m,O_2,ref}$ is comparable to that of other produce mentioned in literature. Hertog *et al.* found values of 106, 112 and 122 nmol kg^{-1} s^{-1} for, respectively, apple, chicory and tomato at the same reference temperature of 10 °C (Hertog *et al.*, 1998). Results of respiration measurements at 2, 7, 12, 15, and 23 °C on 'Conference' pears harvested in 1998, show a remarkable similarity in respiration parameters (Table 1). No year effect on respiration characteristics of intact pears was observed. All experiments were carried out at 0% CO_2, and, hence, no inhibitory CO_2 effect was included in the model. de Wild *et al.* measured a maximum respiration rate for 'Conference' pears at 2 °C of 21.7 nmol kg^{-1} s^{-1} (de Wild *et al.*, 1999). For both presented models the maximum O_2 uptakes at 2 °C were calculated/extrapolated, from Equation 3, to be around 39 nmol kg^{-1} s^{-1}, which is considerably higher than the value found by de Wild *et al.* (de Wild *et al.*, 1999). A possible explanation is that de Wild *et al.* described the effect of only two CO_2 concentrations at one temperature

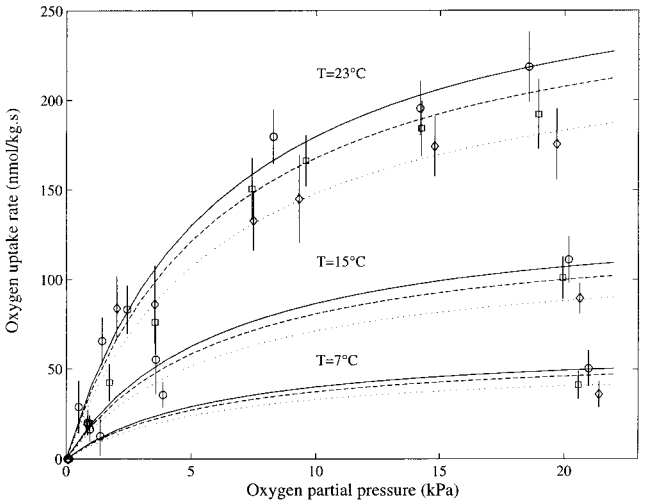

Fig. 1. Oxygen uptake rate for intact pears as a function of the temperature, O_2 and CO_2 partial pressure. Within one temperature, the upper, middle and lower curve represent the modelled O_2 uptake rate at, respectively, 0, 5 and 15% CO_2. Values represent means ($n=8$) at, respectively, 0 (○), 5 (□) and 15% (◇) CO_2. The vertical bars indicate the 95% confidence limits.

Table 1. *Results of the non-linear regression analysis for O_2 uptake by intact pears for different types of inhibition by CO_2*

Values represent estimates $\pm95\%$ confidence limits for $V_{m,O_2,ref}$, the maximal O_2 consumption rate at T_{ref}, E_{a,vm,O_2} the activation energy, K_{m,O_2} and K_{m,CO_2} the Michaëlis constant for O_2 consumption and for the CO_2 inhibition, respectively. T_{ref} (the reference temperature) = 283 K and R^2_{adj} is the percentage variance accounted for (a measure of the goodness of fit of the model). The models with and without inhibition are based on data of 1999 and 1998, respectively.

Parameter	Competitive inhibition $R^2_{adj}=96\%$	Uncompetitive inhibition $R^2_{adj}=97\%$	Non-competitive inhibition $R^2_{adj}=97\%$	No inhibition (data 1998) $R^2_{adj}=97\%$	Units
$V_{m,O_2,ref}$	80.8 ± 7.3	91.1 ± 8.7	87.1 ± 7.9	86.9 ± 6.9	nmol kg^{-1} s^{-1}
E_{a,vm,O_2}	64.6 ± 4.8	64.6 ± 4.7	64.6 ± 4.7	67.9 ± 3.7	kJ mol^{-1}
K_{m,O_2}	5.2 ± 0.9	7.0 ± 1.1	6.2 ± 0.9	5.9 ± 0.79	kPa
K_{m,CO_2}	26.4 ± 10.1	44.8 ± 14.5	70.7 ± 21.6	–	kPa

on the O_2 uptake rate (de Wild *et al.*, 1999), whereas in the present study, the same model structure was used to describe a full factorial design with three temperatures and three CO_2 concentrations and thus the presented model is more robust and can be applied in a broader range, but is somewhat less accurate. This statement was confirmed when only the data at 2 °C, from the data set of 1998, were used to identify the maximum respiration rate and the Michaelis–Menten constant of, respectively, 23.3 nmol kg^{-1} s^{-1} and 2.55 kPa. Those values were very close to the ones obtained previously (de Wild *et al.*, 1999).

Temperature is the most important factor to slow down the fruit metabolism (Wills *et al.*, 1998). The influence of temperature on the O_2 uptake is given by the activation energy E_{a,vm,O_2}. The estimated value equals 64.6 kJ mol^{-1} and is the same order of magnitude as the values 52.9, 67.1 and 67.3 kJ mol^{-1} found previously (Hertog *et al.*, 1998) for, respectively apple, chicory and tomato. Andrich *et al.* estimated an activation energy of 44.2 kJ mol^{-1} for 'Golden Delicious' apples (Andrich *et al.*, 1998). Again a high similarity was observed between the activation energies measured on the authors' data of 1998 and 1999. No other data on activation energies for pears were found in the literature.

An inhibitory effect of CO_2 on O_2 consumption was found for pears (Fig. 1). Increased CO_2 partial pressures slowed down the respiration. Respiration measurements (de Wild *et al.*, 1999) confirm the results obtained here for 'Conference' pears. No inhibitory effect of CO_2 on respiration was found earlier (Peppelenbos and van't Leven, 1996) for 'Golden Delicious' and 'Elstar' apples. However, Yearsley *et al.* reported a significant decrease in O_2 uptake at higher CO_2 partial pressures for 'Braeburn' apples (Yearsley *et al.*, 1997). This difference in CO_2 sensitivity between on the one hand 'Conference' pears and 'Braeburn' apples and on the other hand 'Golden Delicious' and 'Elstar' apples could explain the higher sensitivity of the former to develop core breakdown, a CO_2-related disorder, during storage (Veltman *et al.*, 1999; Lammertyn *et al.*, 2000).

Protoplast respiration

Figure 2a shows a re-aeration curve measured at 23 °C and 0% CO_2. The protoplast suspension was put in the reactor at time zero and, due to respiration, the O_2 concentration decreased during the first 3 min. At that time the screw top of the reactor was removed and the reaction medium was flushed with a gas mixture composed of 20% O_2, 0% CO_2 and 80% N_2 at 23 °C, for 5–10 s, resulting in an increasing O_2 concentration. After a period of 1–2 min, during which the equilibrium between the injected gas and the liquid phase was restored, the respiration continued at the initial rate. This was repeated twice. Around 900 s pure N_2 was injected to decrease the O_2 level in the medium. In this way the O_2 uptake rate at lower O_2 concentrations could be monitored. Three re-aeration steps were used at this low O_2 concentration. The slopes of these curves were calculated by linear regression and assigned to the average O_2 concentration during that time interval. The O_2 uptakes rates, measured at 23 °C and at 0, 5 and 15% CO_2, corrected for the number of viable protoplasts, are plotted in Fig. 2b as function of the O_2 concentration. No clear difference in O_2 uptake rate could be observed between high and low O_2 concentrations, but the influence of the CO_2 concentration was significant: a high CO_2 markedly reduced the O_2 uptake rate. Similar results were obtained when the experiments were repeated for 7 °C and 15 °C (data not shown). A serious drawback of this method is the lack of information on O_2 uptake rates at very low O_2 concentrations. It is technically impossible to repeat the re-aeration steps for O_2 concentrations lower than 0.5 mg l^{-1}. This implies that the Michaelis–Menten constant for CO_2 inhibition of O_2 consumption could not be identified accurately. However, this method will be used later on to validate the results obtained with the O_2 depletion curve.

The O_2 depletion curve offered an alternative to study the respiration characteristics of pear protoplasts in suspension as a function of the temperature and the gas atmosphere. Figure 3a shows a typical O_2 depletion

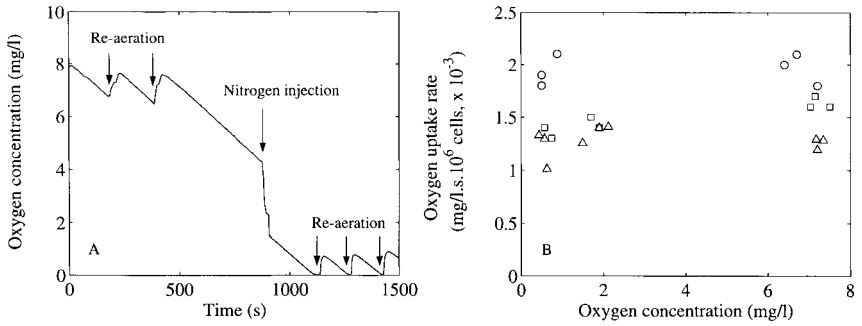

Fig. 2. Respiration of pear protoplasts. (A) Re-aeration curve, (B) O_2 uptake rate as a function of the O_2 concentration at 23 °C and 0% CO_2 (○), 5% CO_2 (□) and 15% CO_2 (△). Data obtained from re-aeration curves.

curve. The protoplasts were put in the bioreactor at time zero and the O_2 concentration was monitored until all O_2 had been consumed. During these measurements it was assumed that the CO_2 produced by the protoplasts did not inhibit its own respiration rate, which is a reasonable assumption as will be demonstrated later on.

The O_2 concentration versus time profile was processed in two different ways. The first was based on numerical differentiation of the O_2 concentration versus time curve. Since numerical differentiation required prior smoothing of these curves, it obscured the fast dynamics at low O_2 concentrations and the K_m values would, therefore, be overestimated. At higher O_2 concentrations this problem did not occur. Figure 3b, c and d show the O_2 uptake rate as a function of the O_2 concentration for different interval sizes used for differentiation. An increasing interval size reduced the noise on the signal, and resulted in more accurate parameter estimates, but increased the K_m value. Therefore, the estimated K_m depended on the processing technique. To avoid these problems it was chosen to fit the data immediately on the originally measured O_2 concentration curves by means of differential Equation 2 rather than taking the derivative and identifying the parameters on the modified Michaelis–Menten plot. Estimates of the respiration parameters and their 95% confidence intervals are listed in Table 2.

The model fit and the experimental data for four repetitions at 23 °C and 0% CO_2 are given in Fig. 4. Since the number of viable protoplasts was different for all repetitions, the O_2 concentration versus time curves were slightly separated. Figure 5 shows the inhibitory CO_2 and temperature effect on O_2 consumption of pear protoplasts in suspension. Three groups of three curves were calculated using the estimated parameters in Table 2. The upper three correspond to a temperature of 23 °C, the middle and lower group show the simulated values for, respectively, 15 °C and 7 °C. Within one group of curves the inhibitory effect of CO_2 is clear. The upper, middle and lower curve represent, respectively, 0, 15 and 30% CO_2.

The influence of the O_2 concentration on the O_2 uptake rate is described by the parameter K_m, the O_2 concentration at which half of the maximal O_2 uptake rate is reached. The estimate for K_m was 3 μM. As indicated above this value should be interpreted carefully, since it describes an average O_2 affinity for both the cytochrome and alternative oxidase. Taiz and Zeiger found a K_m value of 1 μM for the cytochrome *c* oxidase in plant tissue (Taiz and Zeiger, 1993). Solomos experimentally obtained a K_m value of 0.1 μM for the isolated cytochrome *c* oxidase of apples (Solomos, 1982). Millar *et al*. calculated K_m values of 0.14 μM and 1.7 μM for cytochrome oxidase and the

Table 2. *Results of the non-linear regression analysis for protoplast O_2 uptake for the non-competitive type of inhibition by CO_2*

Values represent estimates ±95% confidence limits for $V_{m,O_2,ref}$, the maximal O_2 consumption rate at $T_{ref} = 283$ K, K_{m,O_2} and K_{mn,CO_2} the Michaëlis constant for O_2 consumption and for the non-competitive CO_2 inhibition, respectively, and E_{a,vm,O_2} the activation-energy.

Parameter	Estimated value	Units
$V_{m,O_2,ref}$	$(0.658 \pm 0.002) \times 10^{-3}$	mg l^{-1} s^{-1} 10^{-6} cells
E_{a,vm,O_2}	91.7 ± 1.7	kJ mol^{-1}
K_{m,O_2}	0.098 ± 0.008	mg l^{-1}
K_{mn,CO_2}	95 ± 0.97	%

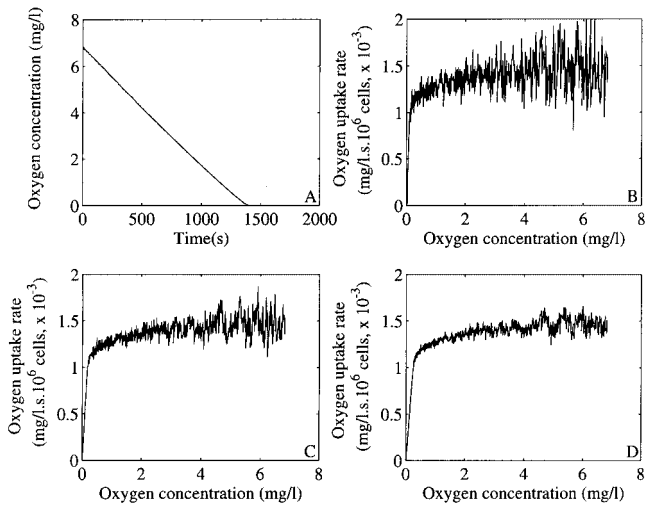

Fig. 3. (A) O_2 depletion curve for protoplasts at 23 °C and 15% CO_2. First derivative of depletion curve plotted as a function of the O_2 concentration. Interval size used to differentiate: 10 s (B), 20 s (C) and 30 s (D).

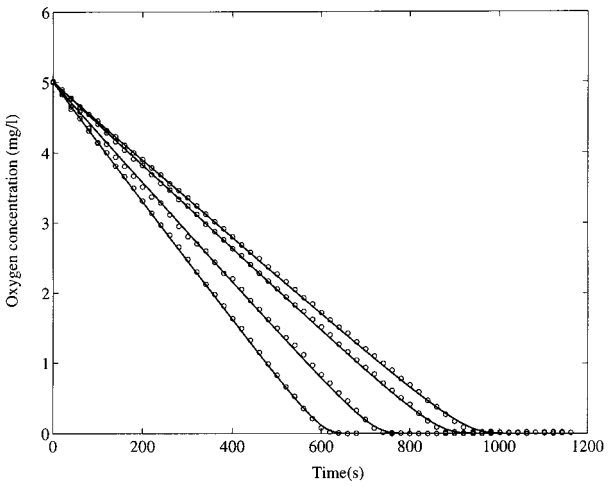

Fig. 4. Four measurements of O_2 uptake by four different densities of protoplasts at 23 °C and 0% CO_2 (○) and the corresponding modelled values (lines). The protoplast densities were from the left to the right curve, respectively, 4.26×10^6, 4.02×10^6, 3.70×10^6, and 3.59×10^6 protoplasts ml^{-1}.

alternative oxidase, respectively, in soybean shoot mitochondria (Millar *et al.*, 1994). The parameter estimate for K_m obtained in this study was somewhat higher, indicating that there is probably intracellular gas diffusion which was not accounted for in this experiment. This confirms the earlier stated hypothesis that when O_2 uptake is limited by diffusion, in this case by cellular membranes, the K_m value increases. There is considerable interest in the hypothesis that the cellular membrane forms a barrier for O_2. Grinsberg *et al.* found O_2 gradients up to 48 μM between intra- and extracellular compartments (Grinsberg *et al.*, 1998). This was contrary to Subczynski *et al.*, who concluded that the O_2 gradients over a (animal) plasmalemma have an order of magnitude of nM and thus cannot be rate determining for cellular respiration (Subczynski *et al.*, 1992). Uchida *et al.* obtained a diffusion coefficient for a (animal) plasmalemma which was much higher than that for mitochondrial membranes (Uchida *et al.*, 1992). Moreover, the O_2 concentration is not uniformly distributed in the cell. Clustering of mitochondria determines the magnitude and the location of the concentration gradients. Microheterogeneity can occur without membrane compartmentalization (Jones, 1986).

The V_{max} parameter in the Michaelis–Menten model is equal to the sum of the maximal respiration rate of both terminal oxidases. Respiration experiments on protoplasts in the presence of KCN and/or SHAM were performed selectively to eliminate, respectively, the cytochrome and the cyanide-resistant respiration. After subtraction of the residual respiration, the cytochrome and alternative respiration accounted for respectively, 88% and 12% of the V_{max}. A value of 54% was calculated for ρ, indicating that under the measurement circumstances, 54% of the maximal capacity of the alternative oxidase was used. These results should be interpreted carefully, since stress is a very important factor influencing the distribution between the two respiration pathways. Moreover, this distribution also fluctuates during postharvest storage as indicated previously (Duque and Arrabaça, 1999).

Model validation

As mentioned earlier, the re-aeration curve was not suited to identify accurately the Michaelis–Menten constant for O_2 consumption. However, this method serves well as an independent technique to validate the model based on O_2 depletion curve measurements. Figure 5 shows the O_2 uptake rates measured with the re-aeration curve at 23 °C for 0, 15 and 30% CO_2. Each validation point represents the average O_2 uptake rate based on three replicate measurements. A reasonable correspondence exists between the measurements done with both techniques.

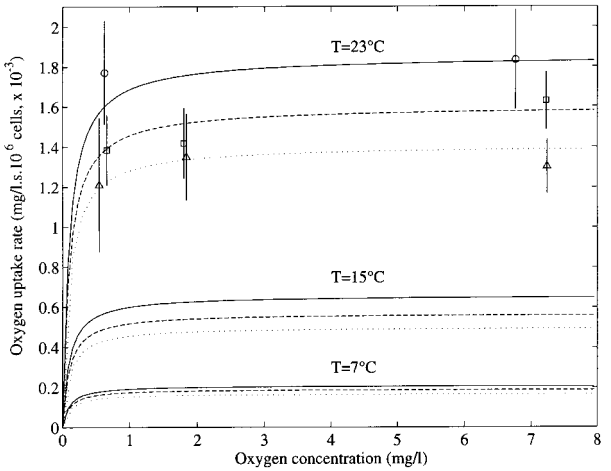

Fig. 5. Pear protoplast O_2 uptake rate as function of temperature, O_2 and CO_2 concentration. Within one temperature, the upper, middle and lower curve represent the modelled O_2 uptake rate at, respectively, 0, 15 and 30% CO_2. Values represent means ($n = 3$) at 0 (○), 15 (□) and 30% (△) CO_2, respectively, obtained with re-aeration curves. The vertical bars indicate the 95% confidence limits.

For the O_2 depletion curve, CO_2 accumulating during the measurement could influence (inhibit) the O_2 uptake rate. However, the validation results obtained with the re-aeration curve indicate this is unlikely to have occurred. In the case of a re-aeration curve, after each short respiration period, the gas concentration in the medium is set to the initial one by stripping the medium with the gas mixture of interest, and, hence, no long term CO_2 accumulation or inhibition could occur.

Comparison of intact pear and protoplast respiration

The Michaelis–Menten constant for the intact pear respiration (2.5 mmol l^{-1} air or according to Henry's Law 0.082 mmol l^{-1} H_2O) is clearly higher than for protoplast respiration. O_2 uptake measurements on small pear discs (6 mm diameter and 1 mm thick) by means of re-aeration curves resulted in a K_m value of 1.97 mmol l^{-1} air or 0.062 mmol l^{-1} H_2O (Lammertyn *et al.*, 2001). This illustrates that the K_m value measured on intact pears not only contains information about respiration but also about macroscopic gas diffusion through pear tissue. By using well-stirred protoplast suspensions, it is assumed that all but the intracellular diffusion properties are eliminated. Assuming that the K_m values for cytochrome *c* oxidase and the alternative oxidase in pear mitochondria are similar to those found earlier, in soybean mitochondria (Millar *et al.*, 1994), the ratio of K_m for intact pears to that for cytochrome oxidase in mitochondria ($= 82/0.14$) is used as a measure to quantify the total diffusion effect absorbed in the intact pear K_m value. Since for protoplasts a K_m value of 3 μM was found, the macroscopic ($=$ tissue) diffusion effect was equal to 96.4% of the total diffusion effect and the

intracellular diffusion accounted for only 3.6% of the total diffusion effect. This illustrates that the use of protoplast suspensions, instead of intact pears, eliminates nearly all the diffusion effect absorbed in the K_m for intact pears and that the intracellular diffusion is of minor importance from a modelling point of view. In further research, these parameters will be used to construct a respiration–diffusion model for intact pears, which can be used to simulate the O_2 and CO_2 concentrations inside the pear during storage under controlled atmosphere conditions.

A comparison of the effect of different CO_2 levels on the O_2 uptake rate of intact fruit and protoplasts in suspension is shown in Fig. 6. The percentage of the uninhibited respiration is given as function of the CO_2 partial pressure. Some small differences in magnitude aside, the trend and the direction of the respiration response to CO_2 by intact fruit and protoplast suspensions were essentially the same. This similarity suggests that the latter are useful for the study of other aspects of the respiration metabolism. Kerbel *et al.* came to a similar conclusion studying the effect of suspension cultured 'Passe Crassane' pear fruit cells to elevated CO_2 concentrations (Kerbel *et al.*, 1990).

The activation energy of the maximum respiration rate for intact pears (64.6 kJ mol^{-1}) is lower than the one for protoplasts in suspension (91.7 J mol^{-1}). This might be diffusion related as the activation energy of physical processes is, in general, lower than that for biochemical reactions (Toledo, 1991). For intact pears the activation energy is composed of a physical (the gas diffusion) and a biochemical component (the respiration), and will be lower than that for a mainly biochemical process, in the case of protoplast respiration.

Although the respiration rates for both systems are expressed in different units, they can be converted based on the number of cells per unit weight (kg fresh pear). An average number of 729×10^6 cells dm^{-3} was counted and a density of 0.970 kg dm^{-3} was used to estimate the number of cells kg^{-1} fresh weight. This resulted in a value of 751×10^6 cells kg^{-1}, which when multiplied by the maximal cellular respiration rate of 0.658 10^{-3} mg l^{-1} s^{-1} 10^{-6} cells at 10 °C and 0% CO_2, resulted in a value of 61.8 nmol kg^{-1} s^{-1}. This is reasonably close to the maximal respiration rate of 87.1 nmol kg^{-1} s^{-1}, measured at 10 °C and 0% CO_2 for intact pears. The difference could be attributed to errors on cell counts, natural variability, gas diffusion through tissue of intact pears, and other factors.

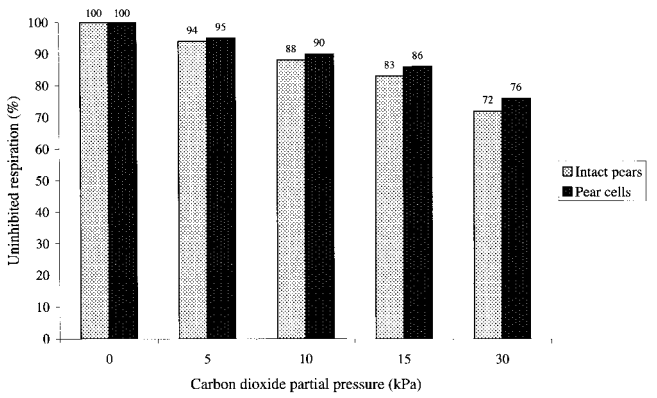

Fig. 6. Percentage of uninhibited respiration as a function of the CO_2 partial pressure for intact pear and pear protoplast respiration at 23 °C.

Conclusion

The respiration of intact pears and pear protoplasts in suspensions was compared with regard to the temperature and CO_2 effect on the O_2 uptake rate. Of the two measurement techniques to measure the protoplast O_2 uptake, the O_2 depletion curve identified the protoplast respiration parameters accurately, whereas the re-aeration curve was not suited for this purpose. However, the re-aeration curve was a valuable tool to validate the respiration models. A modified Michaelis–Menten model was used to describe the effects of the O_2 and CO_2 concentrations and temperature on the O_2 consumption rate of intact pears and pear cell protoplasts in suspension. For both systems a non-competitive type of CO_2 inhibition was assumed in which the inhibitor interacts both with the enzyme and the enzyme–substrate complex. The models presented described the experimental data well. Due to inclusion of diffusion properties, the Michaelis–Menten constant for intact pears was significantly larger (2.5 mM) than the one for protoplasts in suspension (3 μM), which was in turn an order of magnitude higher than the values reported in the literature for the apparent K_m values for cytochrome oxidase and the alternative oxidase measured on isolated mitochondria or on the purified enzymes. It was found that only a very small part of the total diffusion effect absorbed in the Michaelis–Menten constant for intact pears, could be attributed to intracellular gas diffusion. The major part was due to macroscopic diffusion through the fruit tissue. The maximal respiration rates of both systems were similar and the influence of CO_2 on the respiration rate was almost identical for protoplasts and intact pears, suggesting that protoplast suspensions may be useful for the study of other aspects of respiratory metabolism. In future work, the estimated respiration parameters will be used in a reaction–diffusion model, to describe changes in internal gas concentrations in pears during storage, and to study the mechanism of the development of core breakdown.

Acknowledgements

The Belgian Ministry of Small Enterprises, Traders and Agriculture and the Flemish Government are gratefully

acknowledged for financial support (project S-5901). This research was also financially supported by EC-FAIR1-CT96-1803. Jeroen Lammertyn is Research Assistant of the Fund for Scientific Research-Flanders (Belgium) (F.W.O-Vlaanderen).

References

Andrich G, Zinnai A, Balzini S, Silvestri S, Fiorentini R. 1998. Aerobic respiration rate of Golden Delicious apples as a function of temperature and pO_2. *Postharvest Biology and Technology* **14,** 1–9.

Baumann H, Henze J. 1983. Intercellular space volume of fruit. *Acta Horticulturae* **138,** 107–111.

Boersig MR, Kader AA, Romani RJ. 1988. Aerobic–anaerobic respiratory transition in pear fruit and cultured pear fruit cells. *Journal of the American Society for Horticultural Science* **113,** 869–873.

Brady CJ, Romani RJ. 1988. Respiration and protein synthesis in non-growing cultured pear fruit cells in response to ethylene and modified atmospheres. *Plant Physiology* **87,** 571–576.

Cameron AC, Talasila PC, Joles DW. 1995. Predicting film permeability needs for modified atmosphere packaging of lightly processed fruits and vegetables. *Hortscience* **30,** 25–34.

Chang R. 1981. *Physical chemistry with applications to biological systems*. New York: MacMillan Publishers.

Chevillotte P. 1973. Relation between the reaction cytochrome oxidase–oxygen and oxygen uptake in cells *in vivo*. *Journal of Theoretical Biology* **39,** 277–295.

de Wild HP, Woltering EJ, Peppelenbos HW. 1999. Carbon dioxide and 1-MCP inhibit ethylene production and respiration of pear fruit by different mechanisms. *Journal of Experimental Botany* **50,** 837–844.

Duque P, Arrabaça JD. 1999. Respiratory metabolism during cold storage of apple fruit. II. Alternative oxidase is induced at the climacteric. *Physiologia Plantarum* **107,** 24–31.

Grinsberg OY, James PE, Swartz HM. 1998. Are there significant gradients of pO_2 in cells? *Advanced Experimental Medical Biology* **454,** 415–423.

Hertog ML, Peppelenbos HW, Evelo RG, Tijskens LM. 1998. A dynamic and generic model on the gas exchange of respiring produce: the effects of oxygen, carbon dioxide and temperature. *Postharvest Biology and Technology* **14,** 335–349.

Inoue Y. 1989. Measurement of O_2 evolution in chloroplasts. In: Linskens HF, Jackson JF, eds. *Gases in plant and microbial cells*. Berlin: Springer-Verlag, 63–70.

Jones DP. 1986. Intracellular diffusion gradients of O_2 and ATP. *American Journal of Physiology* **250,** 663–675.

Kays SJ. 1991. *Postharvest physiology of perishable plant products*. New York: Van Nostrand Reinhold.

Kerbel EL, Kader AA, Romani RJ. 1990. Respiratory and glycolytic response of suspension-cultured 'Passe Crassane' pear fruit cells to elevated CO_2 concentrations. *Journal of the American Society for Horticultural Science* **115,** 111–114.

Knee M. 1991. Rapid measurement of diffusion of gas through the skin of apple fruits. *HortScience* **26,** 885–887.

Lambers H. 1985. Respiration in intact plant tissues: its regulation and dependence on environmental factors, metabolism and invaded organisms. In: Douce R, Day DA, eds. *Higher plant cell respiration*. Berlin: Springer-Verlag.

Lammertyn J, Aerts M, Verlinden BE, Schotmans W, Nicolaï BM. 2000. Logistic regression analysis of factors influencing core breakdown in 'Conference' pears. *Postharvest Biology and Technology* **20,** 25–37.

Lammertyn J, Scheerlinck N, Verlinden BE, Nicolaï BM. 2001. Core breakdown in 'Conference' pears: a respiration–diffusion model for disks and intact pears. *Acta Horticulturae* (in press).

Laties GG. 1982. The cyanide-resistant, alternative path in plant mitochondria. *Annual Reviews in Plant Physiology* **33,** 519–555.

Mathooko FM. 1996. Regulation of respiratory metabolism in fruits and vegetables by carbon dioxide. *Postharvest Biology and Technology* **9,** 247–264.

Millar AH, Bergersen FJ, Day DA. 1994. Oxygen affinity of terminal oxydases in soybean mtochondria. *Plant Physiology and Biochemistry* **32,** 847–852.

NAG. 1986. *The NAG Fortran workstation library handbook*. Oxford: Numerical Algorithms Group Ltd.

Peppelenbos HW, van't Leven J. 1996. Evaluation of four types of inhibition for modelling the influence of carbon dioxide on oxygen consumption of fruits and vegetables. *Postharvest Biology and Technology* **7,** 27–40.

Pushmann R, Romani R. 1983. Ethylene production by auxin-deprived, suspension-cultured pear fruit cells in response to auxins, stress, or precursor. *Plantphysiology* **73,** 1013–1019.

Rajapakse NC, Banks NH, Hewett EW, Cleland DJ. 1990. Development of oxygen concentration gradients in flesh tissues of bulky plant organs. *Journal of the American Society for Horticultural Science* **115,** 793–797.

Shipway MR, Bramlage WJ. 1973. Effects of carbon dioxide on activity of apple mitochondria. *Plant Physiology* **51,** 1095–1098.

Solomos T. 1982. Effects of low oxygen concentration on fruit respiration: nature of respiratory diminution. In: Richardson DG, Meheriuk M, eds. *Controlled atmosphere for storage and transport of perishable agricultural commodities*. Beaverton: Timber Press, 171–178.

Solomos T. 1987. Principles of gas exchange in bulky plant tissues. *HortScience* **22,** 766–771.

Subczynski WK, Hopwood LE, Hyde JS. 1992. Is the mammalian cell plasma membrane a barrier to oxygen transport? *Journal of General Physiology* **100,** 69–87.

Taiz L, Zeiger E. 1993. *Plant physiology*. California: The Benjamin/Cummings Publishing Company Inc.

Takeda T, Seki M, Furusaki S. 1994. Hydrodynamic damage of cultured cells of *Carthamus tinctorius* in a stirred tank reactor. *Journal of Chemical Engineering of Japan* **27,** 466–471.

Toledo RT. 1991. *Fundamentals of food process engineering*. New York: Van Nostrand Reinhold.

Uchida K, Matsuyama K, Tanaka K, Doi K. 1992. Diffusion coefficient for O_2 in plasma and mitochondrial membranes of rat cardiomyocytes. *Respiratory Physiology* **90,** 351–362.

Umbreit WW, Burris RH, Stauffer JF. 1964. *Manometric techniques*. Minneapolis: Burgess Publishing Co.

Veltman RH, Sanders MG, Persijn ST, Peppelenbos HW, Oosterhaven J. 1999. Decreased ascorbic acid levels and brown core development in pears (*Pyrus communis* L. cv. Conference). *Physiologia Plantarum* **107,** 39–45.

Wills R, McGlasson B, Graham D, Joyce D. 1998. *Postharvest, an introduction to the physiology and handling of fruit, vegetables and ornamentals*. Wallingford, Oxon: CAB International.

Yearsley CW, Banks NH, Ganesh S. 1997. Effect of carbon dioxide on the internal lower oxygen limits of apple fruit. *Postharvest Biology and Technology* **12,** 1–13.

Zhong JJ, Fujiyama K, Seki T, Yoshida T. 1994. A quantitative analysis of shear effects in cell suspension and cell culture of *Perilla frutescens* in bioreactors. *Biotechnology and Bioengineering* **44,** 649–654.