

Respiratory Activity and Mitochondrial Oxidative Capacity of Bell Pepper Fruit following Storage under Low-oxygen Atmosphere

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Abstract. Bell pepper (*Capsicum annuum* L., var. 'Jupiter') fruit stored in 1.5%, 5%, or 10% O₂, or in air at 20C for 24 hours were compared to determine the residual effect of low-O₂ storage on respiration after transfer to air. The lowest O₂ concentration (1.5%) exerted the greatest residual effect on bell pepper fruit CO₂ production and O₂ uptake. No ethanol was detected in the headspace gas of fruit stored in 1.5% O₂. Carbon dioxide production continued to be suppressed for ≈ 24 hours after transfer from 1.5% O₂ to air. Exposure to 5% O₂ for 24 hours resulted in less suppression of CO₂ production and O₂ uptake upon transfer to air, while 10% O₂ exerted no residual effect. Extending the storage period in 1.5% O₂ to 72 hours extended the residual effect from 24 to 48 hours. Ethylene production was not affected by storage in 1.5% or 4% O₂ for 24 or 72 hours. The residual effect exhibited in whole fruit was not apparent in mitochondria isolated from bell pepper stored in 1.5% or 4% O₂.

The benefits of controlled-atmosphere (CA) storage of fresh fruits and vegetables include extended storage life and maintenance of quality. Reduced O₂ and elevated CO₂ in CA storage can reduce respiration and ethylene production rates, reduce tissue sensitivity to ethylene, delay ripening and softening, and slow down biochemical changes associated with ripening (Kader, 1986).

Concentrations of O₂ and CO₂, duration of exposure, and storage temperature are some important criteria for successful CA storage of fresh fruits and vegetables. Tolerance to low-O₂ levels varies with commodity. Kader (1986) indicated that, for most commodities, a minimum of 1% to 3% O₂ in the storage environment is required to avoid a shift to anaerobic metabolism. Oxygen concentrations as low as 0.2% within the plant cell may result in anaerobic respiration. Anaerobiosis normally results in the accumulation of ethanol and acetaldehyde (Patterson and Nichols, 1988), and increased activity of pyruvic decarboxylase (EC 4.1.1.1) and alcohol dehydrogenase (EC 1.1.1.1) (Davis et al., 1973).

Changes in physiological properties and quality attributes following short-term exposure to low O₂ have been elucidated for various commodities, including tree fruits (Ke and Kader, 1990; Ke et al., 1991) and strawberry (*Fragaria × ananassa* Duch.) fruits (Aharoni et al., 1979). In some commodities, the respiration and ethylene production rates continue to be suppressed even after transfer from a low-O₂ environment to air. Li and Kader (1989) have termed this phenomenon the "residual effect" of low-O₂ storage. These authors demonstrated that 'Selva' strawberry fruit stored in 0.5% to 2.0% O₂ at 2C for 7 days exhibited reduced respiration and ethylene production rates and maintained flesh firmness and color after removal to air. The residual effect was more pronounced following storage at lower O₂ concentrations.

Mitochondria are the cellular sites of oxidative phosphoryla-

tion, where respiratory enzymes are located. Low O₂ and high CO₂ may influence the activity of these enzymes, depending on the concentration and duration of exposure. Burton (1978) and Solomos (1982) have pointed out, however, that the decrease in respiration rate in response to low O₂ levels may not be due to a reduction in the activity of cytochrome oxidase (EC 1.9.3.1), a high-affinity, low-K_m oxidase, but rather to the diminished activities of low-affinity oxidases, such as polyphenol oxidase (EC 1.14.18.1), lipoxygenase (EC 1.13.11.12), and ascorbic acid oxidase (EC 1.10.3.3).

The primary objective of this study was to determine whether low O₂ exerts residual effects on respiration of bell pepper fruit. This included an evaluation of whether mitochondrial oxidative capacity was reduced after storage in low pO₂.

Materials and Methods

Plant material. Cauliflower (*Brassica oleracea*, Botrytis Group) florets, mature green tomato (*Lycopersicon esculentum* Mill.) fruit, and bell pepper fruit were examined in initial studies of low-O₂-imposed residual respiratory effects. For the reasons described in the Discussion section, this paper is limited to the results obtained with bell pepper.

Bell pepper fruit (var. 'Jupiter') were hand harvested at commercial maturity (green) and transported to the laboratory on the same day. Fruit were selected for uniform size and shape and freedom from defects. The fruit were wiped with paper towels moistened with 0.25% (v/v) sodium hypochlorite (1:20 dilution of 5% commercial bleach). Individual fruit were placed into 1.7-liter glass jars and kept in a 20C room for 24 h to allow handling-induced respiration to subside. Jars were sealed for 1 h, and 0.5 ml of headspace gas was removed for CO₂ measurement. About 80% of the total fruit samples having CO₂ production rates of 25 ± 0.5 ml·kg⁻¹·h⁻¹ and an average weight of 160 g were selected for the experiments.

Gas treatments. Fruit were sealed individually in 1.7-liter glass jars and exposed to 1.5%, 5%, or 10% O₂ in N₂ for 24 h at 20C. The control consisted of fruit exposed to air. Fruit also were stored in 1.5% O₂ for 72 h to determine the influence of prolonged exposure to low O₂ on the extent of the residual effect. There were three fruit

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for each treatment, and each fruit constituted a replicate. The humidified O₂ mixtures or air were introduced by means of a continuous flow-through system, and the flow rates were adjusted to keep CO₂ levels <0.3%. After treatment in low O₂ or air, all fruit were transferred to air and ventilated with humidified air at 20C. Gas measurements and ethanol content from the headspace gas were determined at various intervals during this subsequent storage in air.

In a separate experiment, bell pepper fruit were exposed to 4% O₂ or air for 24 or 72 h at 20C and then transferred to air at the same temperature.

Gas measurements. The rates of CO₂ and C₂H₄ production and O₂ uptake were measured within 10 min after 24 h of exposure to the various O₂ mixtures or air. Subsequent measurements were taken after 2 h and again at intervals following transfer of the fruit to air. For determining CO₂ production and O₂ uptake, 0.5 ml of the headspace gas (1.7-liter jars sealed for 1 h) was injected into a Fisher Model 1200 gas partitioner (Pittsburgh) equipped with a thermal conductivity detector, and Column Pak and molecular sieve columns at 60C. Ethylene in 0.5-ml samples of headspace gas was measured using a Photovac Model 10A 10 gas chromatograph (Thomhill, Ont., Canada) fitted with a photoionization detector and activated alumina column at ambient temperature.

Ethanol was determined using the Kentville Method (Lidster et al., 1985) following transfer to air by injecting 0.2 ml headspace gas into a gas chromatograph equipped with a flame ionization detector and 5% carbowax (80/120 mesh) column operated at 80C. The detector and injector settings were at 100C. Peak heights were compared to a standard curve prepared from the headspace of a series of ethanol dilutions in double-distilled water kept at 20C. This method was able to detect ethanol concentrations as low as 0.01% in the headspace gas.

Mitochondria isolation. Pericarp tissue (100 g fresh weight), obtained from the equatorial region of pepper fruit, was diced (≈ 2 × 2 mm) and the sections immersed in 150 ml of ice-cold grinding buffer containing 0.4 M sucrose, 0.05 M Trizma base (Sigma Chemical Co.), 1 mM ethylene glycol-bis (β-aminoethyl ether N,N,N',N'-tetraacetic acid (EGTA), 10 mM KH₂PO₄, 4 mM cysteine, and 1% (w/v) bovine serum albumin (BSA), adjusted to pH 7.6 with HCl. The tissue was disrupted with a Polytron homogenizer (Brinkmann Instruments, Rexdale, Ont., Canada) for 2 to 3 sec at a speed setting of seven, followed by filtration through two layers of Miracloth (Calbiochem, La Jolla, Calif.). The filtrate was centrifuged at 1 to 4C for 10 min at 1000× g to remove cell debris, followed by centrifugation of the supernatant for another 10 min at 1000× g to remove the remaining cell debris and other denser particles. Crude mitochondrial pellets were obtained by centrifugation of the supernatant at 16,000× g for 10 min. The pellets were resuspended in ice-cold medium containing 0.4 M mannitol, 10 mM KH₂PO₄, and 0.5% (w/v) BSA adjusted to pH 7.2 with KOH, and the crude mitochondrial suspension was brought to a volume of 8 ml.

Four milliliters of the mitochondrial suspension were layered onto a step gradient of Percoll (Sigma) following the methods of Moreau and Romani (1982), Duncan and Spencer (1987), and Phelps and McDonald (1990), with the following modifications: The gradient consisted of two steps of 20% and 45% Percoll with 0.25 M mannitol, 10 mM KH₂PO₄, and 0.5% (w/v) BSA, pH 7.2. Centrifugation of the gradients was at 26,000× g for 15 min. The mitochondria banded at a density of ≈ 1.06 as determined with density marker beads (Sigma), and were collected with a pasteur pipette. The mitochondria fraction was diluted to 20 ml and washed with resuspension buffer without BSA and centrifuged at

26,000× g for 20 min. Final washing was in resuspension buffer without BSA at 16,000× g for 20 min. The pellet was resuspended in a reaction medium containing 0.25 M sucrose, 10 mM KH₂PO₄, 10 mM Tris HCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 4 mM MgCl₂, and 0.05% (w/v) BSA, pH 7.2. All steps involved in isolating Percoll gradient-purified mitochondria were conducted at 1 to 4C.

Mitochondrial oxygen uptake. Oxygen consumption was measured at 20C with an oxygen electrode (Model 53, Yellow Springs Instruments Co., Yellow Springs, Ohio), standardized against air-saturated deionized water. Aliquots of 1.5 ml of the mitochondrial suspension were placed into a sample chamber containing 2 mM succinate and 150 μM adenosine 5'-diphosphate (ADP) to initiate state-3 respiration. The final volume of the solution in the reaction chamber was 3 ml. Oxygen consumption rate was expressed as nmol O₂/mg mitochondrial protein per min, and was calculated from steady state O₂ consumption values attained within 5 min after addition of succinate and ADP. Protein was determined following the method of Smith et al. (1985) using Pierce bicinchoninic acid (BCA) protein assay reagents (Sigma) and BSA as standard.

Enzyme assays. Cytochrome c oxidase (EC 1.9.3.1), a mitochondrial marker (Quail, 1979), was assayed by measuring the absorbance reduction of reduced cytochrome c at 550 nm (Storrie and Madden, 1990) using a Beckman spectrophotometer (Model DU-20, Irvine, Calif.). Catalase (EC 1.11.1.6), a peroxisome marker (Quail, 1979), was assayed by measuring the absorbance reduction of H₂O₂ at 240 nm (Luck, 1965).

Mitochondrial integrity. The intactness of purified mitochondria was evaluated according to Storrie and Madden (1990) by measuring the activity of cytochrome c oxidase in the presence or absence of nonionic detergent (Lubrol, Sigma).

Results

The magnitude of reduction in CO₂ production after low O₂ storage was greatest in fruit stored in 1.5% O₂ relative to 5% or 10% O₂, and air (Fig. 1). The CO₂ production rate of fruit measured after 24 h in 1.5% O₂ was 53% lower than that of the air-stored fruit when measured within 10 min of return to air. Preliminary experiments indicated that ≈ 2 h were required for the atmosphere inside the locules of fruit from the 1.5% O₂ treatment to equilibrate with an atmosphere of 21% (data not shown). Oxygen concentrations were measured in the fruit locule after 24 h in 1.5% O₂ and at 30 min intervals following transfer to air until an equilibrium was reached. The CO₂ production rate of fruit stored in 1.5% O₂ continued to be suppressed, and attained values similar to the air-stored fruit only after 24 h in air (Fig. 1). Treatment with 5% or 10% O₂ had no consistent effect on the CO₂ production rate upon transfer of bell pepper fruit to air. Within several hours in air, the CO₂ production rates of fruit previously stored in 5% or 10% O₂ reached values similar to those of fruit stored continuously in air. Extending the storage period of bell pepper fruit in 1.5% O₂ from 24 to 72 h suppressed CO₂ production for 48 h following transfer to air (Fig. 2). Ethanol was not detected in the headspace gas obtained from fruit after storage in 1.5% O₂ for 24 or 72 h.

The O₂ consumption rate of bell pepper fruit stored in 1.5% or 5% O₂ for 24 h was reduced by ≈ 80% or 40%, respectively, compared to the air-stored fruit (Fig. 3). While there was about a 35% recovery in the O₂ consumption rate 24 h after transfer from 1.5% O₂ to air, the O₂ consumption rate recovered by ≈ 50% following storage in 5% O₂. The O₂ consumption rates of fruit stored in 10% O₂ or after 24 h transfer to air were not significantly

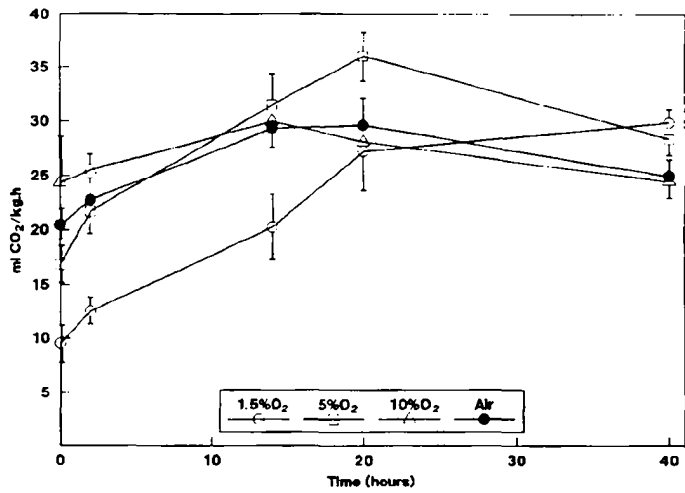


Fig. 1. Carbon dioxide production rates of bell pepper fruit in air following storage in 1.5%, 5%, or 10% O₂, or in air for 24 h at 20C. Vertical lines represent sd of the mean. Values are the means of three replications.

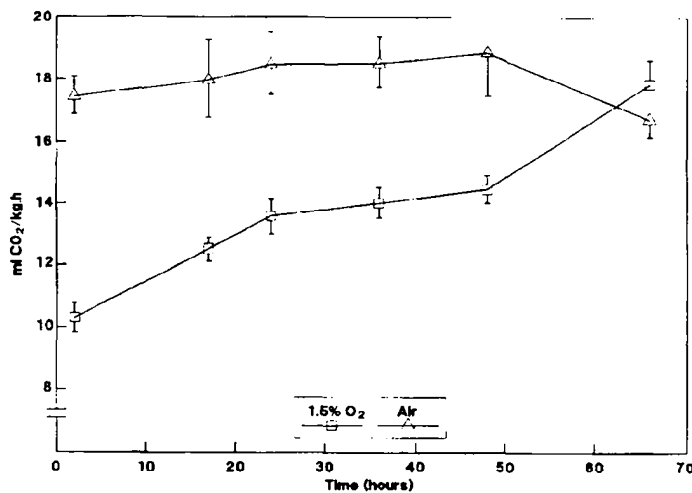


Fig. 2. Carbon dioxide production rates of bell pepper fruit in air after storage in 1.5% O₂, or in air for 72 h at 20C. Vertical lines represent sd of the mean. Values are the means of three replications.

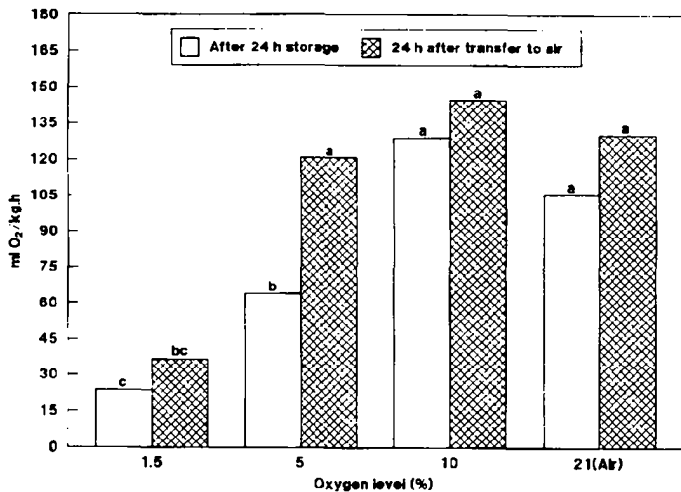


Fig. 3. Oxygen consumption rates of bell pepper fruit after storage in 1.5%, 5%, or 10% O₂, or in air for 24 h at 20C, and 24 h after transfer to air. Values are the means of three replications. Mean separation at $P = 0.01$ by Duncan's Multiple Range Test.

different ($P \leq 0.01$) from those of fruit stored continuously in air.

Fruit stored for 24 h in 1.5% O₂ had a respiratory quotient (RQ) of ≈ 1.15 , which had decreased to 0.95 24 h after transfer to air.

The CO₂ production rate of bell pepper fruit that had been stored in 4% O₂ for 24 h was $\approx 25\%$ lower than that of air-stored fruit measured within 10 min of transfer to air (Fig. 4A). The reduced CO₂ production was highly apparent during the first 6 h after transfer to air. After ≈ 24 h in air, the CO₂ production rate was similar to that of the air-stored fruit. There was no difference in the C₂H₄ production rates between fruit stored in 4% O₂ and air (Fig. 4B).

Carbon dioxide production rates measured within 10 min after 72 h of storage in 4% O₂ were 38% lower than the air-stored sample (Fig. 5A). Later, the values generally were not significantly different from fruit stored continuously in air. The C₂H₄ production rates of fruit previously stored in 4% O₂ for 72 h and then transferred to air were similar to those stored continuously in air (Fig. 5B).

Mitochondria were isolated from the fruit to determine whether the low O₂-induced residual respiratory effect could be explained by limitations in mitochondrial oxidative capacity. Purification of

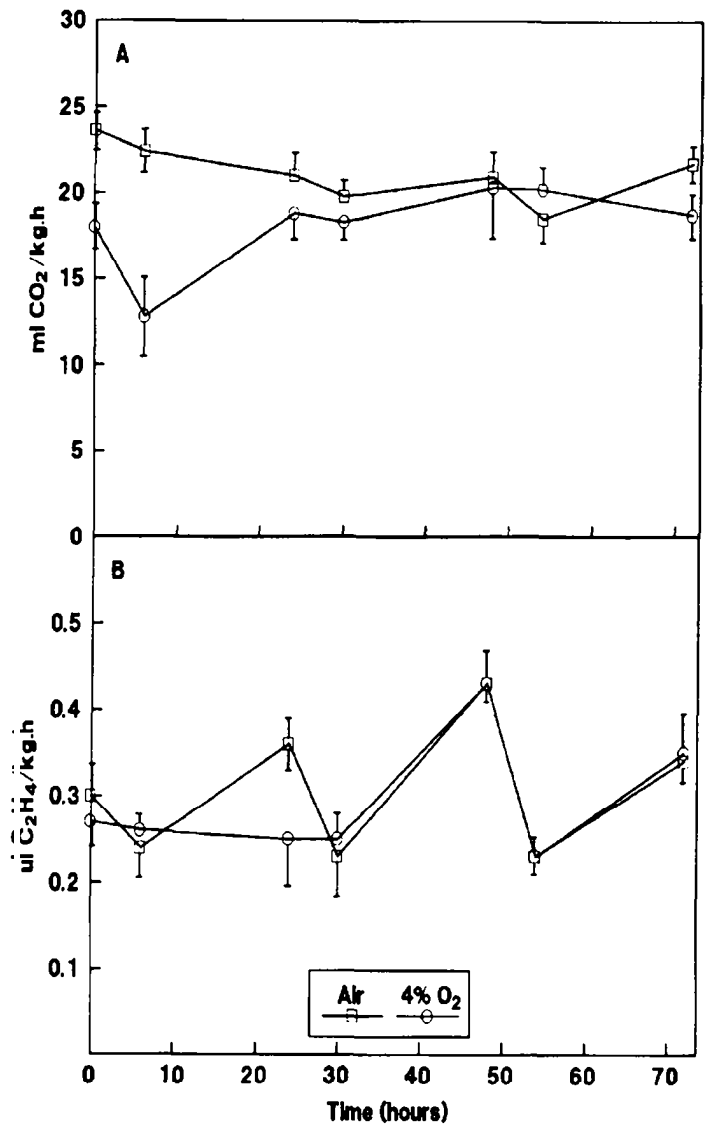


Fig. 4. Carbon dioxide (A) and ethylene (B) production rates of bell pepper fruit in air after 24 h of storage in 4% O₂, and of continuously air-stored samples at 20C. Vertical lines represent sd of the mean. Values are the means of three replications.

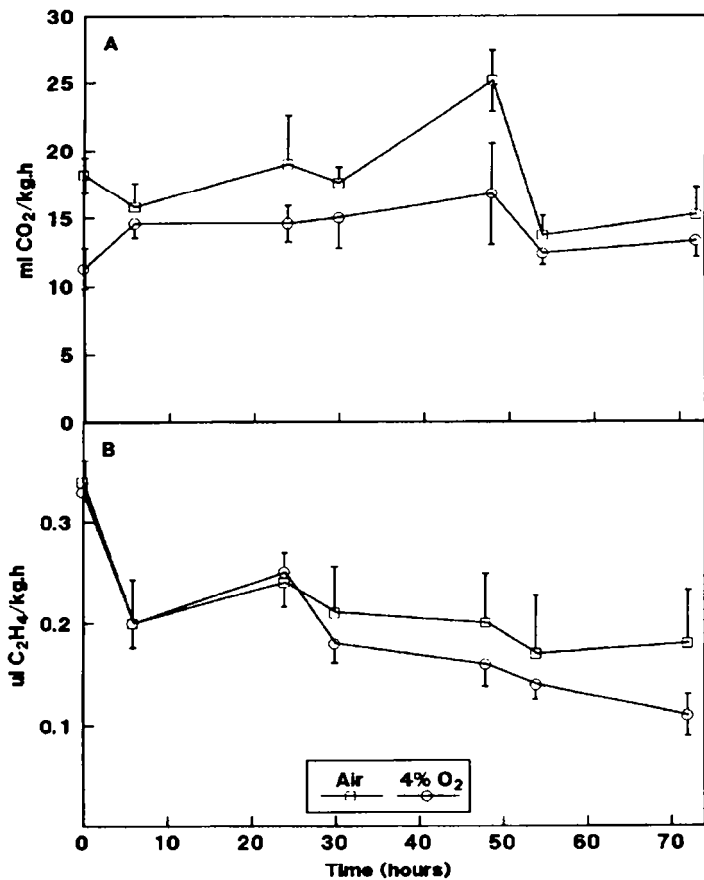


Fig. 5. Carbon dioxide (A) and ethylene (B) production rates of bell pepper fruit in air after 72 h of storage in 4% O₂, and of continuously air-stored samples at 20C. Vertical lines represent SD of the mean. Values are the means of three replications.

crude mitochondrial extracts on Percoll gradients yielded mitochondria free from contaminating peroxisomes, microsomal membranes, and thylakoid fragments, with ≈ 90% intact. The steady-state oxidative capacity of mitochondria isolated from bell pepper fruit stored in 4% O₂ for 3 days showed no marked difference from the mitochondria of air-stored fruit (Fig. 6). Steady state O₂ uptake rates were maintained for periods up to 20 min, at which time dissolved O₂ was nearly depleted. Mitochondria from fruit stored for 4 days in air or 3 days in 4% O₂ plus 1 day in air had higher rates of O₂ uptake than mitochondria from fruit stored only 3 days. The O₂ uptake capacity of mitochondrial isolates obtained from fruit stored in 1.5% O₂ for 1 day was also similar to those isolated from air-stored samples (Fig. 7).

Discussion

Several criteria were employed in selecting an appropriate commodity for the present study. The commodity 1) should exhibit a poststorage, residual respiratory response to low O₂, with "respiration" defined as the organ's net exchange of CO₂ or O₂ without regard to the cellular sites of production or consumption; 2) should not exhibit rapid developmental changes during the experimental period; and 3) should allow the isolation of relatively pure, high-integrity mitochondria. Among three commodities initially examined (bell pepper fruit, tomato fruit, and cauliflower florets), bell pepper best satisfied the above criteria. Comparisons of the residual effects of low O₂ imposed on tomato fruit respiration were confounded by the differential influence of low O₂ and air on the



Fig. 6. Oxygen uptake capacity of isolated mitochondria from bell pepper fruit stored for 3 days in 4% O₂, 3 days in air, 4 days in air, or 3 days in 4% O₂ plus 1 day in air. Values are the means of two replications.

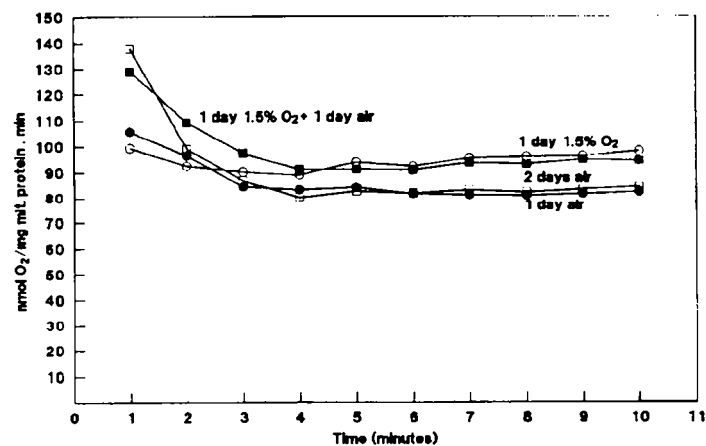


Fig. 7. Oxygen uptake capacity of isolated mitochondria from bell pepper fruit stored for 1 day in 1.5% O₂, 1 day in air, 2 days in air, or 1 day in 1.5% O₂ plus 1 day in air. Values are the means of two replications.

onset of ripening and the associated respiratory climacteric. Cauliflower florets, however, exhibited no apparent residual response of net gas exchange to low-O₂ storage.

The rationale for investigating the poststorage residual effect of low O₂ on respiratory activity is 2-fold. First, practical postharvest benefits may be derived from such a "plastic" response to low O₂. For example, Li and Kader (1989) indicated that exposing strawberries to an atmosphere containing 1% to 2% O₂ for at least 3 days at 2C could maintain quality during transport or temporary storage, and that such a low-O₂ exposure reduced deterioration during subsequent handling in air. Second, the maintenance of presumed low O₂-imposed metabolism upon transfer to air presents the opportunity for experimental elucidation, under ambient conditions, of the effects of low-O₂ storage.

These studies demonstrate that the respiration of bell pepper fruit is affected by prior storage under reduced O₂ levels. Kader (1992) indicated that the recommended level of O₂ for CA or MA storage of bell pepper is between 3% and 5%. The results of the present study demonstrate that 1.5% O₂ is more effective in reducing the respiration rate of bell pepper fruit than are higher O₂ levels. Additionally, the continued suppression of CO₂ production or O₂ consumption after return of the pepper fruit to air indicates

the presence of a residual effect as a consequence of prior exposure to low O₂. The residual effect exhibited by bell pepper fruit stored in 1.5% O₂ was apparent for almost 24 h after transfer to air. Thereafter, the pepper fruit recovered and attained respiration rates similar to those of continuously air-stored fruit. A residual effect of low O₂ on respiration and C₂H₄ production rates has been demonstrated in 'Selva' strawberries by Li and Kader (1989). They indicated that low O₂ (0.5% to 2%) reduced respiration and ethylene production rates during CA storage and after subsequent holding in air for 7 days at 2°C. Ke and Kader (1990) reported that 'Valencia' oranges (*Citrus sinensis* Osb.) tolerated up to 20 days of exposure to 0.5%, 0.25%, or 0.02% O₂ at SC or 10°C, followed by holding in air at 5°C for 7 days without any detrimental effects on external or internal appearance.

Storage of fresh fruits and vegetables under excessively low levels of O₂ may induce the accumulation of ethanol, which results in off-flavors (Ke et al., 1990). The absence of detectable levels (<0.01%) of ethanol in the headspace gas from bell pepper fruit stored in 1.5% O₂ indicates that the residual influence on respiration rate was not due to anaerobiosis induced by low-O₂ storage. While ethanol evaporates into the headspace gas, a considerable fraction may be retained in the fruit tissues. 'Bartlett' pears (*Pyrus communis* L.) stored in 0.5% or 1.0% O₂ for 10 days at 0, 5, or 10°C had almost the same levels of ethanol as the pears stored continuously in air, while 0.25% O₂ caused a slight increase (Ke et al., 1990).

The reduction in CO₂ production of bell pepper transferred from low O₂ to air persisted for ≈ 24 h, but was not significantly different after 72 h in 4% O₂. In contrast, storage of fruit in 1.5% O₂ for 72 h suppressed CO₂ production for ≈ 48 h. The residual effect likely is prolonged as the storage period under 1.5% O₂ is extended.

The RQ values of about one exhibited by fruit stored in 1.5% O₂ for 24 h or after transfer to air indicates that anaerobiosis is not occurring in the fruit tissues. According to Hole et al. (1992), the RQ under aerobic conditions is about one when glucose or other hexose sugars are used as the predominant C source in glycolysis. When a hypoxic condition was induced in highbush blueberry (*Vaccinium corymbosum* L.) fruit sealed in low-density polyethylene packages at 25°C, an RQ of ≈ 3 was measured (Beaudry et al., 1992).

In nonclimacteric bell pepper fruit, the C₂H₄ production rates were not affected by prior exposure to 1.5% (data not shown) or 4% O₂ (Saltveit, 1977). Therefore, there is no residual effect of low O₂ on the C₂H₄ production rate of bell peppers. In contrast, low O₂ often delays the onset of the rise in C₂H₄ and other changes associated with ripening in climacteric fruits (Hesselman and Freebaim, 1969).

The O₂ consumption rate was affected to a greater extent than the CO₂ production rate in fruit exposed to low O₂. An O₂ level of 1.5% exerted a greater residual effect than intermediate levels. The continued suppression of O₂ consumption rate upon transfer to air clearly demonstrates the pronounced residual effect from prior exposure to this low oxygen level. Earlier studies indicated that gas diffusion through fruit tissues is quite rapid. Hence, it is unlikely that the residual effect following storage of fruit in 1.5% O₂ results from resistance to gas diffusion through the fruit tissues.

The biochemical basis of the residual gas-exchange response is not known. In terms of cellular O₂ consumption, relevant enzymes have been categorized as low-affinity (high Km) or high-affinity (low Km) oxidases. Low-affinity oxidases include polyphenol oxidase, lipoxygenase, and ascorbic acid oxidase, whereas the primary high-affinity oxidase is cytochrome oxidase (Malmstrom,

1990). Studies by Burton (1982) on potato (*Solanum tuberosum* L.) tubers indicated that 1% gas phase O₂ would result in steady-state, cellular sap O₂ levels of 1.2 × 10⁻⁵ M. Under this condition, O₂ consumption by cytochrome oxidase was inhibited <1% relative to values observed in air. In agreement with these considerations, our studies revealed no residual influence of low O₂ (1.5% or 4.0%) on the oxidative capacity of mitochondria isolated from bell pepper fruit. While the residual respiratory response of intact bell pepper possibly is due, in part, to indirect effects on cytochrome oxidase (e.g., reduced carbon flux), our data reveal no residual impairment in the capacity of isolated mitochondria to oxidize exogenously added substrates.

We speculate that the residual respiratory responses may be explained as the suppression of extramitochondrial, low-affinity oxidases. Under gas phase O₂ of 1%, dissolved O₂ levels would result in a nearly 95% inhibition of these enzymes (Burton, 1982). Studies by Mapson and Burton (1962) indicated that ≈ 70% of the respiration of potato tuber in air occurred through cytochrome oxidase, and 30% through the low-affinity oxidases. The proportion of respiration passing through the low-affinity oxidases in 100% O₂ was increased to ≈ 50%.

In summary, reducing the O₂ concentration to 1.5% and prolonging the exposure period to 72 h resulted in a longer poststorage residual effect on the CO₂ production and O₂ consumption. Ethylene production rate was not affected by storage in 1.5% or 4% O₂ for 24 or 72 h. The absence of ethanol indicates that the residual effect induced by 1.5% O₂ is not injurious to the fruit tissue. Additionally, the residual effect of low O₂ on the respiratory activity of intact fruit is not apparent in isolated mitochondria.

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