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Changes in Alternative Pathway and Mitochondrial Respiration in Avocado in Response to Elevated Carbon Dioxide Levels

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ABSTRACT. Partially ripened avocado [*Persea americana* (Mill.) cv. Hass] fruit harvested in either June or Aug. 1994 were kept at 10 °C in air (21% O_2), 20% CO_2(17% O_2, balance N_2), or 40% CO_2 (13% O_2, balance N_2) for 7 to 12 days and then were transferred to air at 10 °C for 2 to 3 days. Mitochondrial respiration was stimulated in response to elevated CO_2 treatments at 10 °C. A shift to alternative pathway (Alt) respiration occurred on day 4 in experiments using avocados from both harvest dates, with a return to initial levels in only the 20% CO_2-treated fruit (June-harvested fruit after return to air). Elevated CO_2 at 20 °C decreased the in vitro O_2 consumption of isolated mitochondria compared to mitochondria kept in air. The Alt pathway contributed less to the total O_2 uptake of CO_2-treated mitochondria compared to mitochondria kept in air. The respiratory control ratios of the CO_2-treated fruit and mitochondria were higher and lower, respectively than the air controls. Induction of 33 to 37 kD proteins (corresponding to the size of the alternative oxidase proteins) occurred in avocados after 4 days in 40% CO_2. These results indicate that elevated CO_2 has various effects depending on concentration, duration and temperature of exposure, and mitochondrial function of avocado fruit, such as increased and altered respiratory oxidation and up-regulation of alternative oxidase proteins.

The alternative respiratory (Alt) pathway, which is cyanide resistant, is present in a wide range of plant tissues (Laties, 1982; Solomos, 1977; Solomos and Laties, 1976), including avocados (Laties, 1982), cucumbers *(Cucumis sativus* L.) (Morohashi et al., 1991), oranges [*Citrus sinensis* (L.) Osbeck] (Bruemmer, 1989), apples *(Malus domestica* Borkh.), and peppers *(Capsicum annuum* L.) (Lurie and Klein, 1989). While the function of this nonphosphorylating pathway is apparent in thermogenic species, its role in nonthermogenic tissue is unclear (McIntosh, 1994). The Alt pathway may provide a way to disperse excess reducing power when cytochrome oxidase is inhibited

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(Lambers, 1982). Collier and Cummins (1991) reported that the Alt pathway may have been used during *Saxifraga cernua* L. petal unfolding as an inefficient energy source. During fruit ripening, the energy charge of the tissue is high and the rate of cytochrome (Cyt) pathway oxidation is coupled tightly to oxidative phosphorylation. The Alt pathway may provide a means of oxidizing the large electron flow that occurs during fruit ripening without producing as large amounts of ATP as occurs when the Cyt pathway is operating alone.

The ability of CO_2 to stimulate the Alt pathway has been reported previously, but most of these studies focused on the effects of CO_2 in combination with ethylene (Day et al., 1978; Laties, 1987). Laties (1982) concluded that CO_2 appeared to enhance ethylene-induced Alt respiration. Carbon dioxide alone has sustained the cyanide resistant pathway in wheat *(Triticum aestivum* L.) seedlings (McCaig and Hill, 1977), carnation *(Dianthus caryophyllus* L.) callus (Palet et al., 1991), and Jerusalem artichoke *(Helianthus tuberosus* L.) tubers (Stegink and Siedow, 1986). To our knowledge, the ability of CO_2 to stimulate the Alt pathway in fruit tissue has not been demonstrated. One of our objectives was to determine if, and to what degree, CO_2 stimulates the Alt pathway.

Elthon and Mclntosh (1987) identified alternative oxidase (AltOx) proteins (molecular weights = 35 to 37 kD) in the thermogenic spadix of *Sauromatum guttatum* Schott and later developed monoclonal antibodies to these proteins (Elthon et al., 1989). AltOx proteins subsequently have been identified in a number of non-thermogenic plant species, including avocado fruit, etiolated mung bean *(Vigna radiata* L.) hypocotyls, fresh potato *(Solanum tuberosum* L.) tubers, and tobacco *(Nicotiana tabacum* L.) callus (Elthon et al., 1989). Rhoads and Mclntosh (1993) reported that increased Alt pathway activity correlated with an accumulation of a 35-kD protein in tobacco suspension cultures and de novo transcription and translation were necessary to cause the maximum accumulation of the 35-kD protein. Yoshimoto et al. (1989) reported that the Alt pathway was induced simultaneously with the appearance of a 36-kD protein in yeast. Although the AltOx proteins are present in fruit tissue, there is still some question of whether or not they are constitutive and always present or synthesized *de novo* in coordination with developmental changes. AltOx in pear *(Pyrus communis* L.) was constitutive, and changes in its activity did not involve transcriptional or translational events (R. J. Romani, unpublished data).

Huang and Romani (1991) reported that avocado mitochondria have an intrinsic homeostatic capacity in which disrupted energy-linked functions are self-restored. In addition, the storage life of avocados can be extended by exposure to elevated CO_2 atmospheres (Spalding and Reeder, 1974; Trüter and Eksteen, 1987).Therefore, we used avocado fruit and their mitochondria to study the direct and indirect effects of CO_2 stress on enhancement and partitioning of respiration in fruit tissue.

Materials and Methods

PLANT MATERIAL AND TREATMENTS. 'Hass' avocado fruit were harvested at commercial maturity (≥24% dry mass) from an orchard in Santa Barbara County, Calif., and were transported to our laboratory in Davis, Calif., where they were stored at 10 °C for up to 1 week until initiation of the experiments. Fruit uniform in size and free from defects were

selected. Before initiating experiments, fruit were partially ripened at 20 °C for 4 d with a continuous flow of ethylene at 10 μL-L^{-1} in humidified air at a flow rate of 400 mL-min^{-1}.

Experiments were conducted at 10 °C for up to 15 d. Nine fruit were placed in a 4-L glass jar and ventilated with humidified air (21% O_2 + 0.3% CO_2 + balance N_2) or a specified gas mixture at a continuous flow rate of 100 mL-min^{-1}. The gas mixtures included 20% CO_2 mixed with air (20% CO_2+17% O_2+balance N_2) or 40% CO_2 mixed with air (40% CO_2+ 13% O_2+ balance N_2). Avocado fruit were kept in air or CO_2-enriched atmospheres at 10 °C for 12 (June 1994) or 7 (Aug. 1994) d and then transferred to air for 3 (June) or 2 (August) d.

Individual fruit were selected for each experiment based on screening for uniform C_2H_4 production rates. Partially ripened avocados were screened after 4 d of ripening. For every sampling date, treatments were replicated three times, each replicate having three fruit. All of the fruit for one sampling date for each treatment were contained in the same jar.

Intact avocados were exposed to air or CO_2-enriched atmospheres at 10 °C and then were used in mitochondrial assays unless otherwise stated.

MITOCHONDRIAL ISOLATION AND PARTIAL PURIFICATION. Avocado fruit mitochondria were isolated using the method of Moreau and Romani (1982b). All extraction procedures were performed over ice. Homogenization of the fruit tissue was conducted in a 10°C storage room. For the mitochondrial O_2 uptake assays, 100 g of tissue were homogenized in 300 mL isolation medium using a fine wire-mesh screen submersed in medium. The isolation medium consisted of 0.25 M sucrose, 50 mM potassium phosphate (pH =7.2), 5 mM EDTA, 5 mM ß-mercaptoethanol, 0.2% (w/v) soluble (40,000 MW) PVP, and 0.1% BSA. In all experiments, the homogenate was filtered through four layers of cheesecloth and centrifuged at 2000 x *g* for 10 min. The supernatant was filtered through four layers of cheesecloth and centrifuged at 10,000 x *g* for 15 min. This supernatant was discarded, and the pellet was resuspended in 3 mL wash medium and homogenized in the presence of 11 mL additional wash medium. The wash medium consisted of 0.25 M sucrose, 50 mM potassium phosphate (pH = 7.2), 5 mM ß-mercaptoethanol, and 0.1% BSA. This homogenate was recentrifuged at 2000 x *g* for 5 min to pelletize remaining chloroplasts out of the mitrochondrial supernatant.

Partial purification of the mitochondria was conducted in the following manner. An 8,000 x *g* centrifugation step was substituted with a 20,000 x *g* centrifugation step through 20 mL of 25% Percoll (Sigma Chemical Co., St. Louis) "pad" for 10 min [as described in Day and Hanson (1977) and modified by Romani (1994) personal communication]. This partial purification was necessary to eliminate most of the nonmitochondrial organelles that would interfere with the oxidase assays. A full Percoll purification system was not used (Moreau and Romani, 1982a) because a more rapid cleanup was necessary. The pad medium consisted of 25% Percoll, 0.25 M sucrose, and 50 mM potassium phosphate (pH = 7.2). The resulting mitochondrial pellet was washed with wash medium and centrifuged at 2000 x *g* for 5 min several times to remove the Percoll with the supernatant, and then the mitochondrial pellet was resuspended in 0.3 to 0.5 mL wash medium. Between the addition of wash medium and centrifugation steps, the supernatant was removed by vacuum aspiration and discarded. Protein content of the

purified mitochondrial preparation was determined by the Bradford (1976) method using BSA as the standard.

POLAROGRAPHIC MEASUREMENT OF O_2 UPTAKE AND RESPIRATORY CONTROL. Mitochondrial functions were monitored, including the measurement of O_2 uptake, the contributions of different oxidase pathways toward total O_2 consumption, and respiratory control ratios that indicate the degree of oxidative phosphorylation. One milliliter of reaction media [0.25 M sucrose, 0.1% BSA, 50 mM phosphate (pH 7.2), 1 mM $MgCl_2$, 10 µM CoA, 100 µM TPP (thiamin pyrophosphate), and 100 mM NAD^+] and an appropriate aliquot of mitochondria (0.2 to 0.4 mg protein) were placed in a 1-mL chamber fashioned from plexiglass and equipped with an O_2 electrode (Yellow Springs Instruments, Yellow Springs, Ohio).The electrode was maintained at a constant temperature of 25 °C. The reaction mixture was constantly stirred with a micro stir bar. The O_2 content of air-saturated water was estimated according to Estabrook (1987). Oxygen consumption was measured following the addition of 10 mM malate as substrate. The contribution of the Cyt and Alt pathways to total respiration was determined using 1mM KCN and 3 mM SHAM (salicylhydroxamic acid), respectively. KCN was always added first to avoid stimulation of O_2 uptake by SHAM through CN-sensitive peroxidases (Møller et al., 1988). Respiratory control ratios (RCR) were determined by adding stoichiometric amounts of ADP (≈0.15 mM) and measuring the state 3:state 4 O_2 consumption rates ratio.

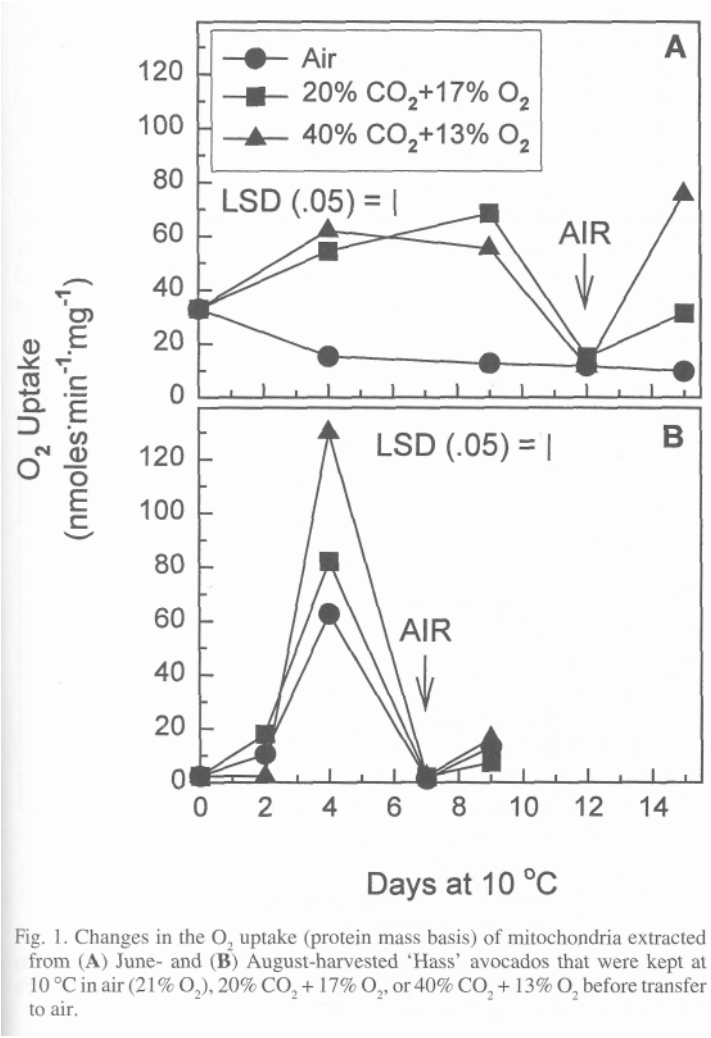

Fig. 1. Changes in the O_2 uptake (protein mass basis) of mitochondria extracted from (**A**) June- and (**B**) August-harvested 'Hass' avocados that were kept at 10 °C in air (21% O_2), 20% CO_2 + 17% O_2, or 40% CO_2 + 13% O_2 before transfer to air.

***IN VITRO* EXPOSURE OF ISOLATED MITOCHONDRIA TO ELEVATED CO_2 ATMOSPHERES.** To measure the effects of *in vitro* exposure to stress levels of CO_2, mitochondria were extracted as previously described from avocado fruit stored in air for <2 weeks at 10 °C and were ripened partially with a continuous flow of C_2H_4 at l0 µL-L^{-1} in humidified air at a flow rate of 400 mL-min^{-1}. The fruit were ripened partially to facilitate tissue homogenization. The mitochondria were suspended in reaction mixture and separated into three equal aliquots (one per treatment) of 10 mL each in a 50-mL Erlenmeyer flask. The mitochondrial suspensions were held at 20 °C on a rotary shaker on a low

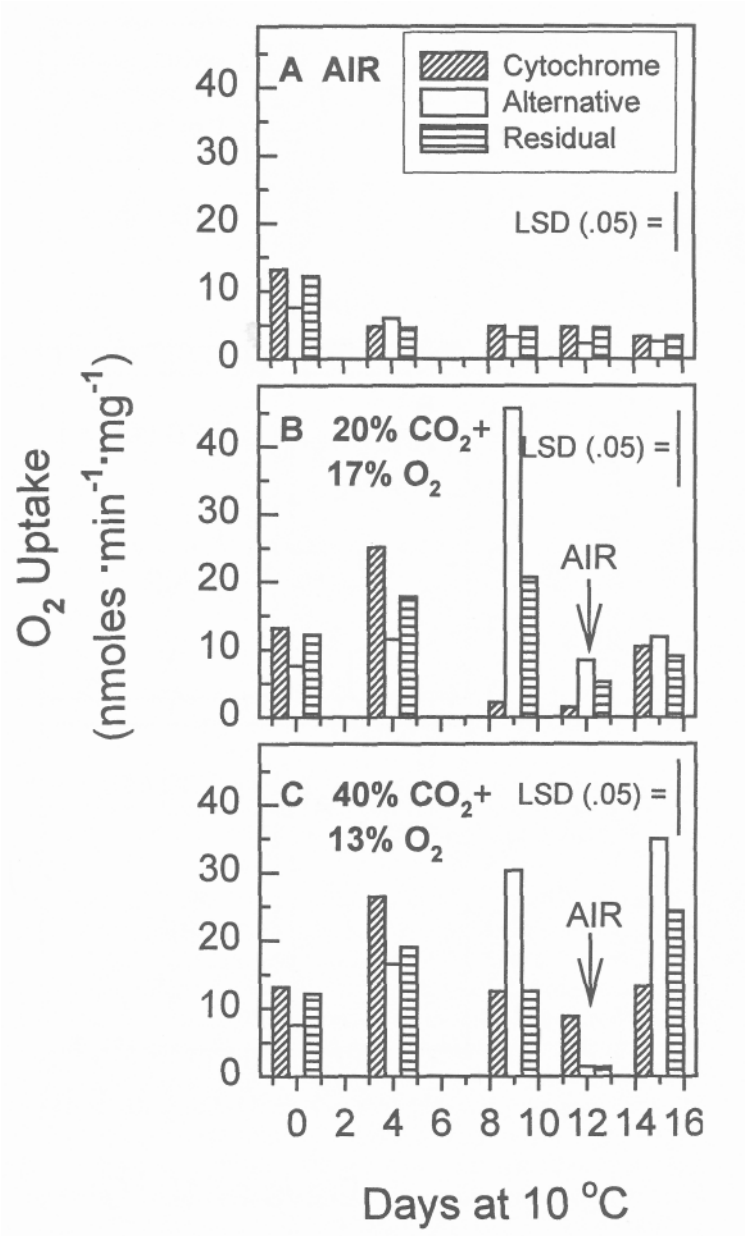

Fig. 2. Oxygen uptake (protein mass basis) due to the activities of the cytochrome alternative and residual oxidase pathways of mitochondria extracted from June-harvested 'Hass' avocados that were stored at 10 °C in (**A**) air, (**B**) 20% CO_2, or (**C**) 40% CO_2 before transfer to air on day 12.

setting. Each flask was sealed with a serum cap supplied with inlet and outlet flow lines to the headspace. Air or the CO_2-enriched atmosphere (20% CO_2 + 17% O_2 or 40% CO_2 + 13% O_2) was flushed through the headspace at 20 mL-min^{-1}. Before each assay, the headspace was aerated momentarily to provide necessary O_2 as a substrate for oxidase reactions. At the designated times, a 1-mL aliquot of the mitochondrial mixture was removed and tested for respiratory control and different oxidase pathway contributions.

DETERMINING ALTERNATIVE OXIDASE (ALTOX) PROTEIN ABUNDANCE. Mitochondria were isolated and partially purified as previously described with minor modifications. No BSA was used in the isolation, wash, or purification medium to avoid overestimation of protein in the samples. Partially purified samples were dialyzed overnight at 4 °C in 5 mM phosphate buffer (pH 7.2) and 10% glycerol to remove sucrose and salts, frozen in liquid N_2 and stored at -80 °C for ≤5 months. Samples were prepared as previously described by Elthon and McIntosh (1987) with the following modifications: ß-mercaptoethanol concentration was increased to10% (v/v), and the samples were boiled for 5 min before adding the tracking dye 0.04% bromophenol blue. Electrophoresis was conducted with the buffer system of Laemmli (1970) using a 5% stacking and a 15% polyacrylamide separating gel. The gels were stained with Coomassie Blue to verify equal loading of protein. Bio-Rad low molecular-mass protein standards, either unstained or biotinylated for gels or immunoblots, respectively, were used to estimate molecular mass. Protein blotting followed the protocol of Blake et al. (1984) except that antibody incubations were for 2 h at room temperature. In accordance with Elthon and McIntosh (1987), the AltOx proteins were

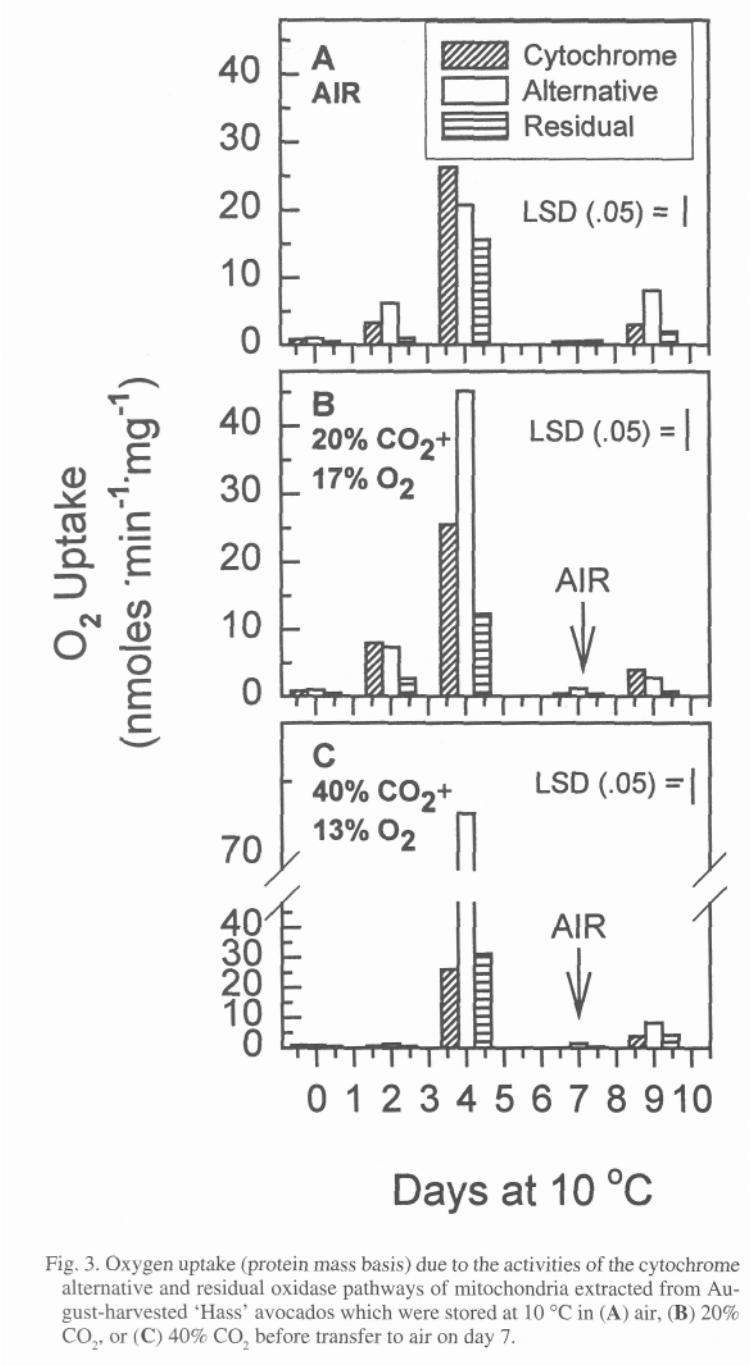

Fig. 3. Oxygen uptake (protein mass basis) due to the activities of the cytochrome alternative and residual oxidase pathways of mitochondria extracted from August-harvested 'Hass' avocados which were stored at 10 °C in (**A**) air, (**B**) 20% CO_2, or (**C**) 40% CO_2 before transfer to air on day 7.

detected with a 1 monoclonal antibody :10 AltOx dilution from *Sauromatum guttatum* (courtesy of T.Elthon).

Results and Discussion

The O_2 uptake of mitochondria extracted from partially ripe 'Hass' avocados that were kept at 10 °C in air (Fig. 1) followed a similar pattern to that of the intact fruit (Lange and Kader, 1996). Uptake of O_2 by mitochondria from June-harvested fruit kept in air gradually declined over the 15-d storage period at 10 °C, whereas O_2 uptake by mitochondria from August-harvested fruit peaked on day 4. Avocado fruit exposed to 20% CO_2 had an increase in the O_2 uptake of extracted mitochondria on day 4 (Fig. 1 A and B). The O_2 uptake of 40% CO_2-stored mitochondria from June-harvested avocado fruit was 3-fold greater than the air control on treatment days 4 and 9 (Fig. 1A), whereas with August-harvested fruit, mitochondrial O_2 uptake was 2-fold greater than the air control on day 4 (Fig. IB). After transfer to air, the mitochondrial O_2 uptake of 20% CO_2- (Fig. 1A) and 40% CO_2-treated (Fig. 1A and B) avocados increased again. Mathooko et al. (1995) also found that elevated CO_2 concentrations increased the mitochondrial activity of cucumber fruit exposed to 30% or 60% CO_2. Perez-Trejo et al. (1981) found that only 2 to 3 h of exposure to 10% to 30% CO_2 were required to cause a 6-fold rise in respiration of potato tubers. This increased whole potato tuber respiration may be a result of in-creased mitochondrial respiration. Lange and Kader (1997) found that 40% CO_2-stored avocado fruit had increased total fruit respiration, just as we observed in this fruit study with mitochondrial respiration.

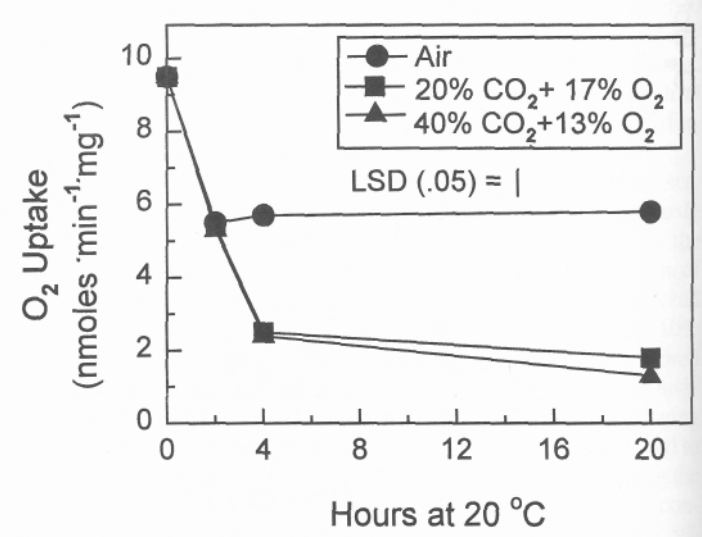

Fig. 4. Changes in the O_2 uptake (protein mass basis) of isolated 'Hass' avocado fruit mitochondria treated at 20 °C with air (21% O_2), 20% CO_2 + 17% O_2, or 40% CO_2 + 13% O_2 for up to 20 h.

Mitochondrial respiration of June-harvested, air-stored avocado fruit was relatively low and gradually declined, regardless of the pathway contributing to O_2 uptake (Fig. 2A). In 20% CO_2-stored avocado fruit, the primary terminal oxidase pathway was the cytochrome oxidase (Cyt) pathway on day 4. However, on day 9, the predominant terminal oxidase pathway was the alternative (Alt) pathway (Fig. 2B). Treatment of avocado fruit with 40% CO_2 elicited a similar effect as that of 20% CO_2, but after transfer to air or 2 d, the Alt pathway became predominant again (Fig. 2C). The August-harvested avocados had a similar pattern of response due to terminal oxidase pathways with most of the differences occurring on day 4 (Fig. 3). The Cyt pathway was favored in air-stored fruit (Fig. 3A), whereas the Alt pathway was favored in avocados exposed to either CO_2 concentration (Fig. 3 B and C). Residual respiration (i.e., respiration in the presence of KCN and SHAM) usually accounts for at most 10% to 20% of the total O_2 uptake in fruit (Romani, personal communication). In our study, residual respiration was substantial (often >30%) and appeared to best stimulated by elevated CO_2 exposure (Figs. 2 and 3).

Laties (1982) reported that exposure of potato tubers to 10% CO_2 for 72 h yielded CN-resistant tissue as well as a significant rise in residual respiration. Residual respiration, at least in part, may be due to monoxygenases, which probably contribute to the total O_2 consumed by plant tissues (Day et al., 1980). There also may be a contribution of α-oxidation to residual respiration.

The direct *in vitro* exposure to CO_2 caused partial inhibition of O_2 uptake of mitochondria extracted from 'Hass' avocados (Fig.4). Within 2 h, the rates of O_2 uptake of mitochondria kept in air, 20% CO_2, or 40% CO_2 were all reduced by ≈50%. Rates remained at a constant level in mitochondria kept in air, whereas the respiration rates of CO_2-treated mitochondria decreased to 20% of the initial rate after 20 h at 20 °C. Mathooko et al. (1995) also found that elevated CO_2 concentrations (up to 60%) inhibited the *in vitro* O_2 uptake rates of mitochondria from cucumber fruit, broccoli *(Brassica oleracea* var. *italica* Plenck) buds, and carrots *(Daucus carota* L.).

Most of the initial O_2 uptake of extracted avocado mitochondria was contributed by the Alt pathway (Fig. 5), but this decreased to 50%, 40%, and 20% of the initial activity after only 2 h in air, 20% CO_2, and 40% CO_2, respectively. For the rest of the experiment, the mitochondria kept in air maintained an Alt O_2 uptake that was 50% of the initial rate, whereas the CO_2-treated mitochondria had a continued decrease in O_2 uptake resulting from the Alt pathway. The relative contribution of the Cyt pathway was higher than the

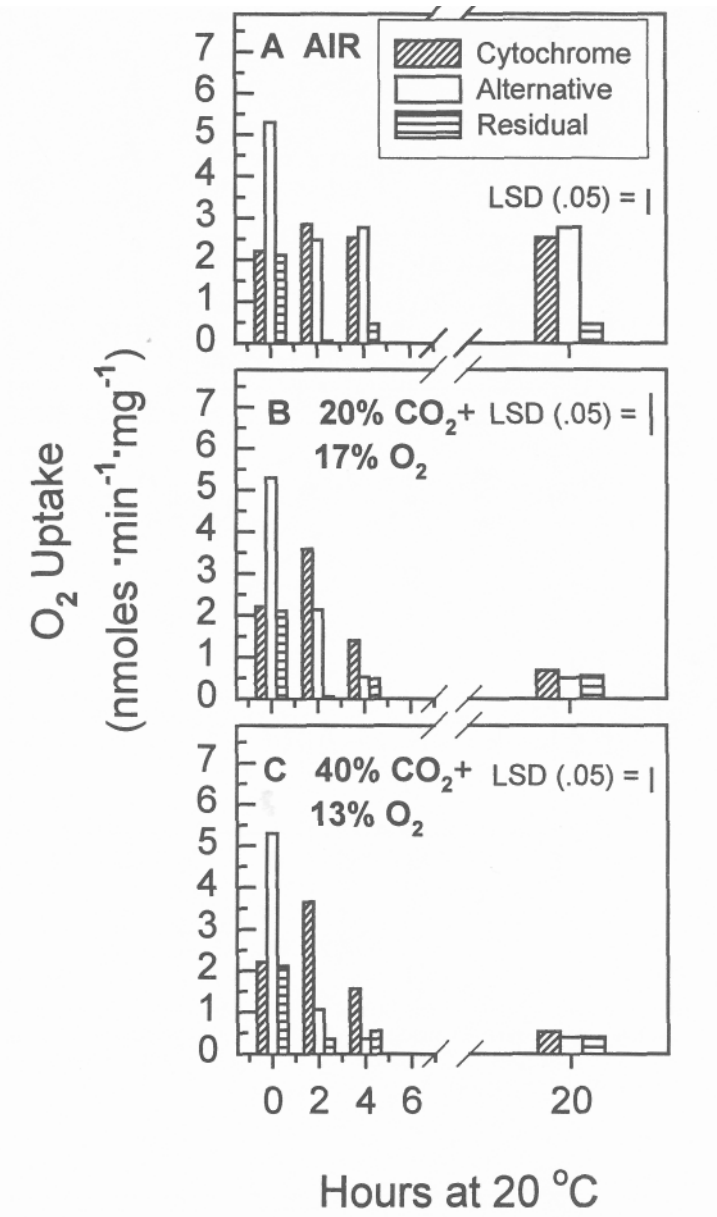

Fig. 5. Changes in the O_2 uptake (protein mass basis) of the cytochrome, alternative, and residual oxidase pathways of 'Hass' avocado fruit mitochondria treated at 20 °C with air (21% O_2), 20% CO_2 + 17% O_2, or 40% CO_2 + 13% O_2 for up to 20 h.

other pathways in the CO_2-treated mitochondria after 2 and 4 h at 20 °C (Figs. 5B and 5C).

Since the CO_2 treatments included slightly lower levels of 0_2 (17% for the 20% CO_2 treatment and 13% for the 40% CO_2 treatment) than the air treatment (21% O_2), partial inhibition of the Alt pathway by CO_2 may be due to the higher K_m of alternative oxidase for O_2, which is estimated to be 10-times higher than the K_m or cytochrome oxidase (Solomos, 1977). For example, Sherald and Sisler (1972) observed a K_m of 11 to 14 μM for AltOx compared with 1.2 to 1.4 μM for CytOx. However, the levels of O_2 in the $C0_2$ treatments are too high to have a major effect on the affinities of these terminal oxidase pathways for O_2. Other direct effects of $C0_2$ on the mitochondria, such as the acidification of the intracellular spaces or structural changes in proteins or lipid membranes, may have played a role as well (Romo-Parada et al., 1991; Shipway and Bramlage, 1973). Moriguchi and Romani (1995) concluded that exposure of avocado fruit to CO_2-rich atmospheres enhanced the capacity of their mitochondria to restore energy-linked functions.

The respiratory control ratio (RCR) is defined as the state 3 O_2 uptake rate (in the presence of added ADP) divided by the state 4 O_2 uptake rate (where ADP supply has been depleted). RCR values for freshly isolated avocado mitochondria are in the range of 2 to 5 (Moreau and Romani, 1982a). The indirect effect of CO_2 on the RCR of treated avocado fruit was to maintain or to protect the tissue from a loss of respiratory control (Fig. 6). After transfer to air for 2 to 3 d, the RCR levels dropped to near 1, regardless of the previous treatment. The effect of CO_2 on the RCR levels of either June- or August-harvested 'Hass' avocado fruit was similar. Elevated CO_2 atmospheres have slowed senescence of a wide range of horticultural commodities (Kader, 1986; Wang, 1990).This slowing may be due partially to the protective role that CO_2 may play in maintaining membrane integrity (Day et al., 1978).

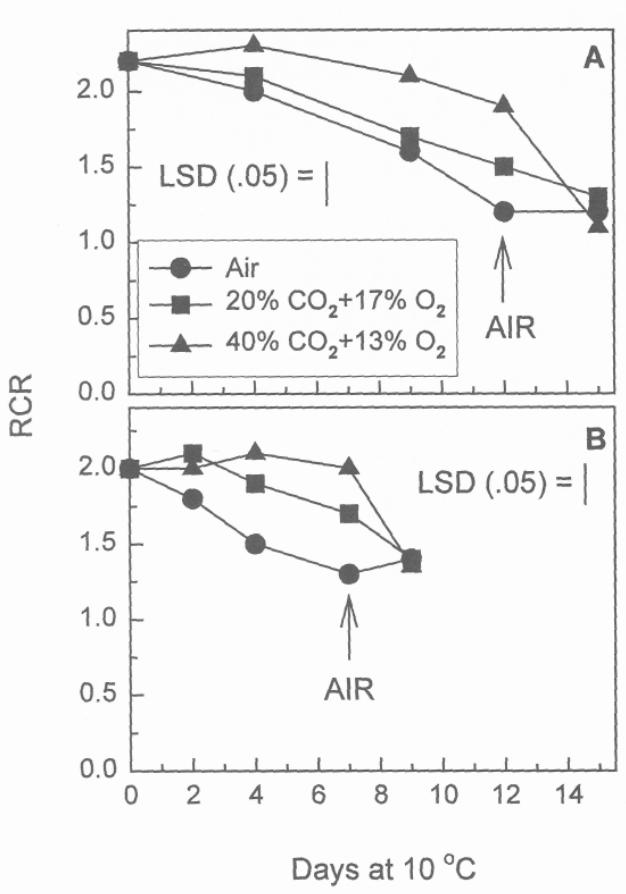

Fig. 6. Changes in respiratory control ratio (RCR) of mitochondria extracted from (**A**) June-harvested and (**B**) August-harvested, partially ripe 'Hass' avocados that were kept at 10 °C in air (21%), 20% CO_2 + 17% O_2, or 40% CO_2 + O_2, and transferred to air.

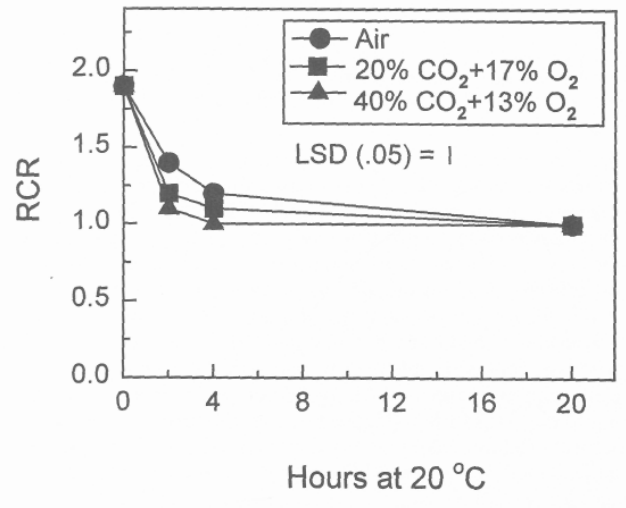

Fig. 7. Changes in the respiratory control ratio (RCR) of isolated 'Hass' avocado fruit mitochondria treated at 20 °C with air (21% O_2), 20% CO_2 + 17% O_2, or 40% CO_2 + 13% O_2 for up to 20 h.

The RCR levels of *in vitro* CO_2-treated avocado mitochondria were slightly lower than mitochondria kept in air after 2 and 4 h of CO, exposure (Fig. 7). RCR dropped to 1 in all treatments, indicating the total loss of respiratory control, after 20 h. (RCR =1). Although it was not a strong uncoupler, the direct effect of CO_2 appears to be through an uncoupling of oxidative phosphorylation, as was described previously by Shipway and Bramlage (1973).

Western blot (immunoblot) analysis, using a monoclonal anti-body to the AltOx proteins, revealed the presence of three or more proteins in the 33- to 41-kDa range (Figs. 8-10). Elthon et al. (1989) reported the presence of alternative oxidase (AltOx) proteins in avocado fruit (range = 35 to 37 kDa). The Alt pathway is stimulated by ethylene produced during climacteric fruit ripening (Laties, 1982). Since partially ripe avocado fruit tissue that was producing large amounts of ethylene was used in these experiments, there was already an abundance of AltOx proteins on day 0 (Figs. 8 and 9) (Lange and Kader, 1996). On days 9 and 15 (labeled AT-Air) of air storage of June-harvested avocado fruit at 10 °C, the AltOx proteins accumulated to higher than initial levels (Fig. 8). Exposure to CO_2-enriched atmospheres decreased the amount of AltOx proteins until after transfer to air for 3 d at 10 °C, after which there was no effect of previous treatment with CO_2 on the abundance of AltOx proteins. In August-harvested avocado fruit stored at 10 °C, an accumulation of AltOx proteins only occurred in fruit stored in 40% CO_2 for 4 d (Fig. 9). Again, after transfer to air for 2 d, there were no differences in the levels of AltOx proteins. In a second experiment to determine the effects of all treatments on August-harvested avocados on day 4

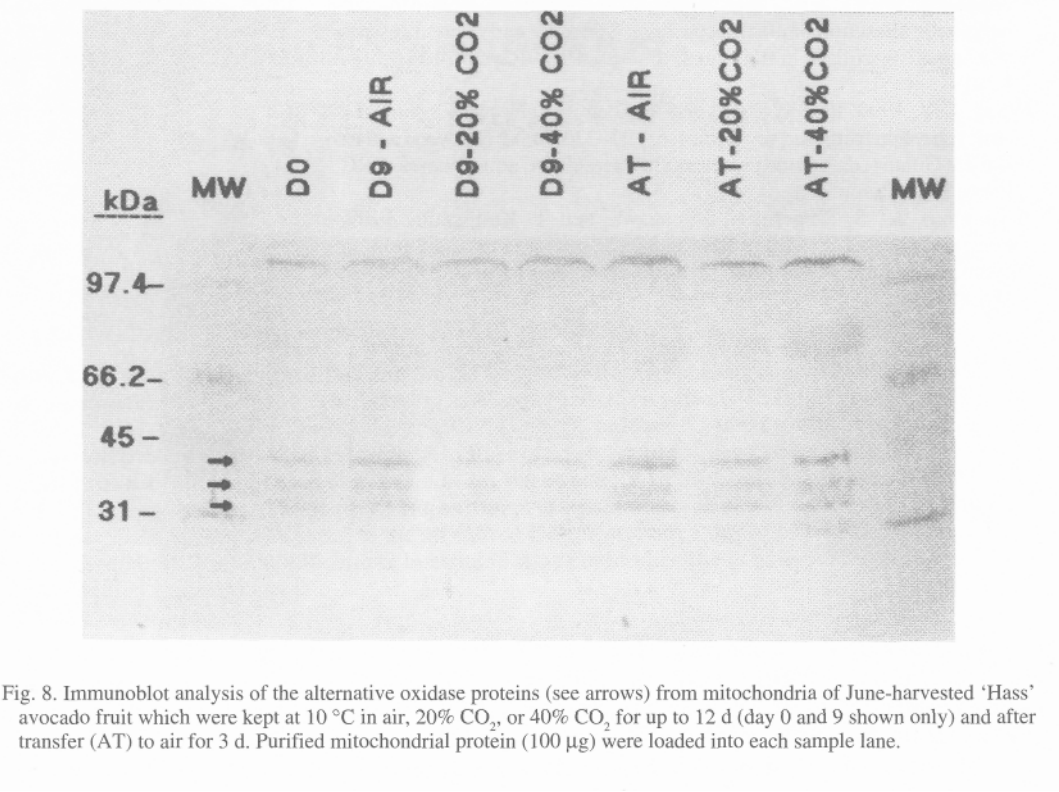

Fig. 8. Immunoblot analysis of the alternative oxidase proteins (see arrows) from mitochondria of June-harvested 'Hass' avocado fruit which were kept at 10 °C in air, 20% CO_2, or 40% CO_2 for up to 12 d (day 0 and 9 shown only) and after transfer (AT) to air for 3 d. Purified mitochondrial protein (100 µg) were loaded into each sample lane.

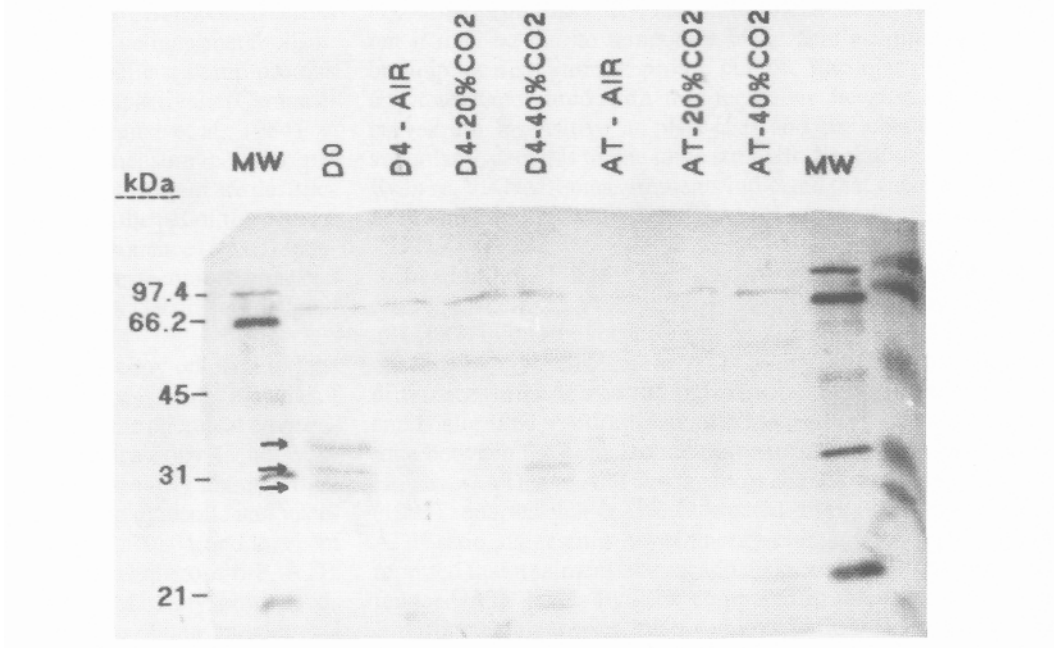

Fig. 9. Immunoblot analysis of the alternative oxidase proteins (see arrows) from mitochondria of August-harvested 'Hass' avocado fruit that were kept at 10 °C in air, 20% CO_2, or 40% CO_2 for 7 d (day 0 and 4 shown only) and after transfer (AT) to air for 2 d. Purified mitochondrial protein (60 µg) were loaded into each sample lane.

(Fig. 10), the air-stored fruit had small amounts of AltOx proteins, the 20% CO_2-stored fruit had intermediate levels, and the 40% CO_2-stored fruit had a high abundance of AltOx proteins.

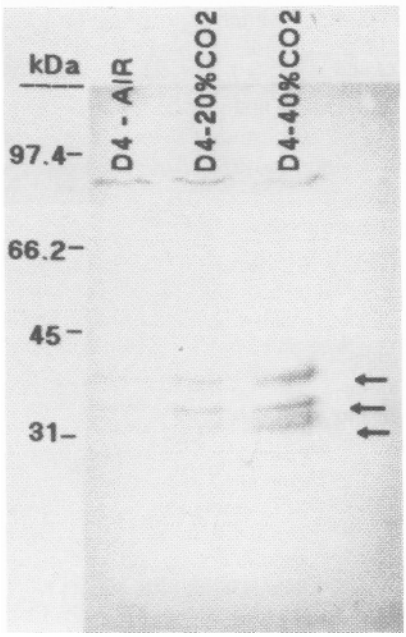

Fig. 10. Immunoblot analysis of the alternative oxidase proteins (see arrows) from mitochondria of August-harvested 'Hass' avocado fruit that were kept at 10 °C in air, 20% CO_2, or 40% CO_2 for 4 d. One-hundred micrograms of purified mitochondrial protein was loaded into each lane.

Expression of the Alt pathway in nonthermogenic tissues is often correlated with increased metabolic activity, such as that observed in fruit ripening or wound stress responses (Day et al., 1980; Laties, 1982; Palmer, 1976; Solomos, 1977). One reason for Alt pathway expression during fruit ripening or stress responses may be to allow for the continued generation of synthetic intermediates by the mitochondria when the energy charge is high (Lambers, 1982). In addition, expression of the Alt pathway allows for the recycling of mitochondrial matrix and cytoplasmic NADH by by-passing normal respiratory control. Possibly, the increased expression of the Alt pathway during elevated CO_2 exposure was due to a physiological response that triggered activation of the Alt path-way. During conditions of Cyt pathway inhibition (such as the use of stress levels of CO_2), the fermentative and Alt pathways are stimulated. Induction of the Alt pathway, compared to induction of the fermentative pathway, results in an 8-fold increase in ATP production, less substrate consumption, less disturbance to normal metabolism by maintaining the Krebs cycle, and, therefore, greater tolerance of the commodity to high CO_2 stress. Recently, the importance of the Alt pathway *in vivo* has become clearer with the advent of transgenic tobacco plants that have reduced or over expressed AltOx (Vanlerberghe et al., 1994). The antisense-AltOx tobacco suspension cells did not survive when they were grown under conditions that inhibited the Cyt pathway, while the wild type cells were able to grow due to the functioning of AltOx. Cells with over expressed AltOx did not have increased partitioning to the Alt pathway, suggesting that this partitioning may be subject to additional regulatory factors *in vivo*, such as post-translational modifications to AltOx protein that limits AltOx pathway activity.

Mitz (1979) suggested that the mechanism of CO_2 action in disrupting typical cellular function may be due to changes in protein configuration and membrane permeability. Taking into account these broad changes in plant cells, the use of stress levels of CO_2 to control disease, insects, or physiological disorders in plant tissues may be a feasible alternative to using chemicals, provided that specific plant tissues and treatment conditions (time, temperature, relative humidity, and O_2 and CO_2 concentrations) are thoroughly tested before applying these treatments.

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