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Changes in Cytoplasmic and Vacuolar pH in Harvested Lettuce Tissue as Influenced by CO_2

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Abstract. **'Climax' lettuce (*Lactuca sativa* L.) exhibited more severe CO_2 injury symptoms than 'Salinas' and 'Winterhaven' lettuce when exposed at 20°C to air for 1 day following treatment for 6 days at 0° with 15% CO_2 in air. All 3 cultivars, however, had similar decreases as revealed by NMR analysis, of about 0.4 and 0.1 pH units in the cytoplasm and vacuole, respectively. This result indicates that variation in the buffering capacity was not related to differences in susceptibility to CO_2 injury among these cultivars. Although CO_2 reduced pH, it also reduced titratable acidity of lettuce tissue. This change resulted in a higher pH when the lettuce was moved to air. Exposure of lettuce at 0° to light reduced CO_2 injury by about 50% relative to tissue kept in the dark. Lettuce tissue kept in air had a higher glucose-6-phosphate content than the CO_2-treated lettuce. A hypothesis regarding alternate energy supply mechanisms for resistance of lettuce tissue to elevated CO_2 injury is discussed.**

Differences in susceptibility to CO_2 injury among fresh horticultural commodities and cultivars within a given commodity are well known (12); but the physiological and biochemical basis for such differences and the mechanism of CO_2 injury are not understood. It has been suggested (2, 10, 28) that elevated CO_2 concentrations inhibit the activity of succinic dehydrogenase and thus result in accumulation of succinic acid, a toxicant to plant tissues. This mechanism, however, does not provide an adequate explanation for CO_2 injury in lettuce tissue, because Brecht (3) found the accumulation of succinic acid in lettuce stored under air + 5% CO_2 to be greater at 10° and 15°C than at lower temperatures, whereas CO_2 injury was much more severe at temperatures below 10° than at 10° or 15° (4).

Exposing plant tissues to elevated CO_2 concentrations inhibits the activity of various enzymes involved in respiratory metabolism (1, 2, 17, 22, 26). Also, CO_2 has been found to have an uncoupling effect on oxidative phosphorylation (8). Thus, CO_2 could limit the energy supply needed for survival of the tissue. We report in this paper on the effects of elevated CO_2 concentrations on the energy status (ATP and other phosphate compounds) in lettuce tissue.

Another important effect of CO_2 that has not been investigated fully is the increased acidity in plant tissues. Upon exposure to elevated CO_2, pH of living cells could drop (due to the dissociation of carbonic acid to bicarbonate and hydrogen ion) to a point at which normal physiological functions might not be sustained. Unless plant cells have a mechanism to exclude CO_2 or to maintain their pH within the physiological range, elevated CO_2 eventually could lead to their death. Previous reports on CO_2 effects on pH have been contradictory, with some (e.g., 14) reporting an increase in pH while others (e.g., 13) finding the opposite. It is possible that plant tissues that have a greater buffering capacity than others could maintain higher pH and survive under moderately elevated CO_2 atmospheres.

Consequently, our research objective was the determination of changes in pH in relation to the buffering capacity of 3 lettuce cultivars in response to CO_2. We used ^{31}P-Nuclear Magnetic Resonance (NMR) methodology (9, 18, 19, 20) for estimating cytoplasmic and vacuolar pH and for detecting energy-rich phosphate compounds in lettuce tissue as influenced by elevated CO_2 concentrations.

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Materials and Methods

Plant material. We obtained the lettuce cultivars 'Climax', 'Winterhaven', and 'Salinas' from the Univ. of California Experimental Station in El Centro, Calif. When cultivar differences were not at issue, lettuce was purchased from a local wholesale distributor. Leaf disks of about 27 mm in diameter were taken from the midrib area of leaves located one-third to two-thirds the distance from the cap leaf to the core. Tissue from this area is the most sensitive to CO_2 injury (3, 24). Green leaf disks were taken from wrapper leaves. Only disks which were relatively flat in shape were used in the NMR experiments. Leaf disks were kept at 0°C in closed glass containers, covered with aluminum foil to exclude light and ventilated with a continuous flow of humidified air or air + CO_2 at the desired concentration. At the end of each treatment, leaf disks were placed quickly into distilled water (0°) saturated with air or air + CO_2. About 20 g of disks were placed into a 30-mm NMR tube that was under water to minimize formation of air pockets in the tube. A capillary containing 0.5 M of methylenediphosphonic acid (MDP) in Tris buffer (pH 8.9) was used as an internal reference. The capillary was positioned either on the side or in the middle of the NMR tube. Since the ^{31}P signal from MDP was not stable (± 0.1 ppm), but those of other compounds from lettuce tissue were highly reproducible, the chemical shifts (δ) of various compounds were reported in relation to δ of 85% phosphoric acid, an external reference.

NMR analysis. ^{31}P and other nuclei possess magnetic moments due to their own spinning. Magnetic moments will arrange themselves either along or against an applied magnetic field which has a lower or higher energy state, respectively. If the nuclei are irradiated with a proper frequency range of radio waves (scanning), then the nuclei from a lower energy state will be excited to the higher state. But, the electron cloud surrounding these nuclei alters the applied magnetic field which they are experiencing. Thus, ^{31}P nuclei in different electronic environments (e.g., in various compounds) will be excited at different irradiation frequencies. The excited nuclei can be detected by measuring the voltage induced in a wire coil placed around the sample when the excited nuclei relax to the lower energy state. With repeated irradiation pulses, signals are accumulated and

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transformed to a conventional spectrum by plotting the intensity vs. frequency, or, more often, intensity vs. chemical shift (in ppm) in relation to the reference compound (9).

All scanning was done at 0°C in a Nicolet WB200 spectrometer operating at 80.99 MHz. For lettuce tissue, 60° pulses and 102.5 msec. acquisition time were used; for extracts, 30° pulses and 204.9 msec. acquisition time were used. A signal from water in a tube that was placed horizontally was used for optimizing magnetic field homogeneity. Proton decoupling was not used.

Extraction procedure. A modified method of Burt et al. (5) was used. Fifty grams of leaf disks were frozen in liquid N_2, then ground to powder with mortar and pestle. Cold 60% perchloric acid (2.5 ml) was added to the powder and the mixture warmed to 0°C, stirred for 20 min at 0°, filtered through cheese cloth and centrifuged at 10,000× *g* for 20 min. The supernatant was neutralized to pH 6.5–7.0 with KOH and KH_2CO_3, and centrifuged at 10,000× *g* for 20 min. The resultant supernatant was passed through a 1 × 10 cm column of Chelex 100, concentrated by freeze drying, redissolved into 25 ml of distilled water and adjusted to pH 9.

Light treatment. Lettuce disks in glass containers were exposed to light intensity of about 30,000 Lux using a GE 60 W 'Gro & Sho' light bulb.

Results and Discussion

In a preliminary experiment, lettuce disks were packed into an NMR tube under air or air + 15% CO_2. The NMR signals obtained were very broad and the cytoplasmic inorganic phosphate (P_i) peak could not be identified. In subsequent experiments, distilled water saturated with air or air + 15% CO_2 was added to the NMR tube and good signals were obtained (Fig. 1). There were still air pockets in lettuce disks inside the tube which caused nonuniformity of the magnetic field and, consequently, resulted in broad NMR signals. It would have been possible to obtain a better signal by infiltrating the tissue with water, but this was not attempted because it would have disturbed the atmosphere surrounding the cell. From packing to completion of scanning required 45 to 60 min, of which scanning itself took about 26 min. Lettuce tissue kept under these conditions appeared to be in a relatively good physiological condition (as indicated by respiration). Additionally, only 1% CO_2 had accumulated in the control tissue during scanning.

Figure 1A shows a typical ^{31}P-NMR spectrum of lettuce disks previously kept in air. By using a concentrated $HC10_4$-extract of lettuce tissue (Fig. 1C), together with addition of external standards, peaks in the spectrum were identified (Fig. 1). Two P_i peaks were observed. The stronger and upfield (to the right) peak that indicates an acid environment was designated the vacuolar P_i; the smaller downfield peak was designated the cytoplasmic P_i (19). When lettuce disks were kept under anaerobic conditions, i.e., 100% N_2 (Fig. 1B), the smaller P_i peak increased in intensity (presumably as a result of decrease in ATP level), which confirmed its identity as cytoplasmic P_i (21). The ATP peaks, particulary the γ and β peaks, diminished somewhat under anaerobic conditions (Fig. 1B).

Accurate estimation of cytoplasmic pH requires knowledge of chemical constituents (such as magnesium ion and ionic strength) which could influence the chemical shift of P_i, as demonstrated by Roberts et al. (20). We only attempted to determine the change in pH as influenced by CO_2; therefore, we refer to cytoplasmic pH values in this report as chemical shift of P_i. An estimation, however, of cytoplasmic pH values was

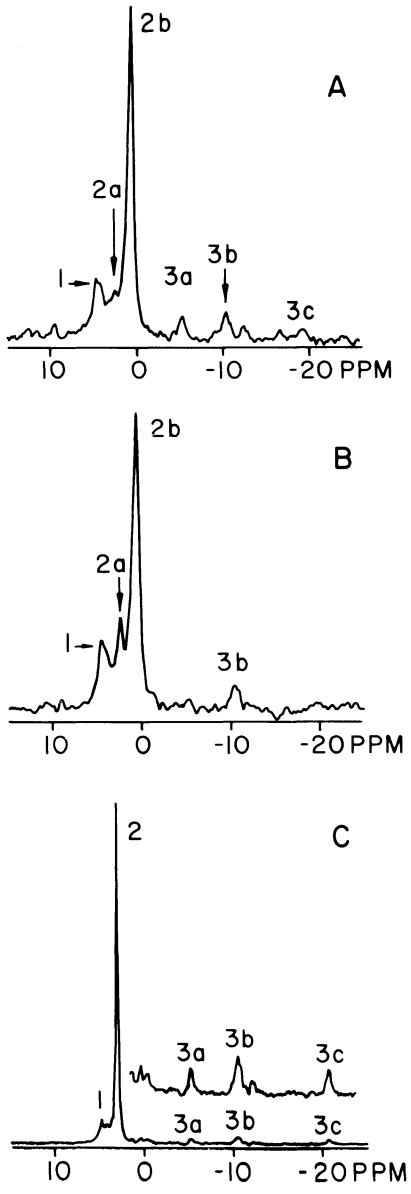

Fig. 1. Typical ^{31}P-NMR spectra (4000 scans) of lettuce leaf disks = (**A**) packed in water into a 30-mm NMR tube, (**B**) same as (A) but previously kept in 100% N_2 for 4 days, and (**C**) $HClO_4$-extract adjusted to pH 9 in a 20-mm NMR tube (1500 scans). Phosphate peaks were identified as 1 = glucose-6-phosphate, 2a = cytoplasmic P_i, 2b = vacuolar P_i, 3a = γ ATP, 3b = α ATP, 3c = β ATP.

made by using the titration curve of 5 mM KPO_4 reported by Roberts et al. (20). For vacuolar pH, a titration curve was constructed from the δ of P_i using lettuce tissue homogenate (Fig. 2).

In the control tissues, the δ of cytoplasmic and vacuolar P_i contents were found to be 2.5 ± .07 and 0.6 ± .03, respectively (Table 1). These δ values are equivalent to pH 6.7 for the cytoplasm and 5.7 for the vacuole. When lettuce disks were treated with air + 5% CO_2, the δ values of cytoplasmic and vacuolar P_i dropped to 2.2 ± 0.05 and 0.5 ± 0.01, respectively. With higher CO_2 concentrations (15% and 20%) the δ values are lower (Tables 1 and 2). While there was a distinct difference in the severity of CO_2 injury, all 3 lettuce cultivars used in the experiment behaved similarly under CO_2 treatment

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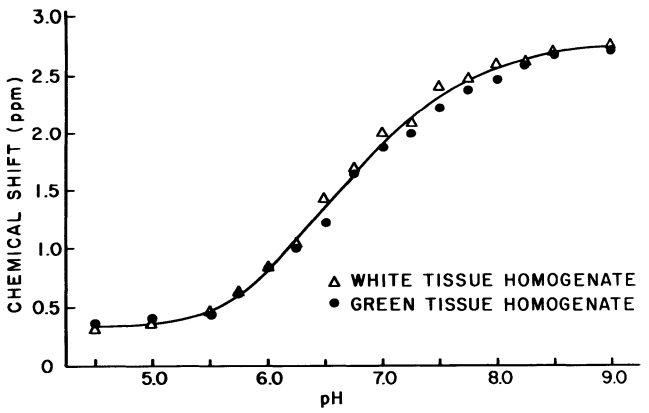

Fig. 2. Titration curve constructed from the chemical shifts of vacuolar P_i in green and white lettuce tissue homogenates.

(Table 1). There was no significant difference among cultivars in the drop of cytoplasmic or vacuolar pH, which was about 0.4 and 0.1 pH unit, respectively. To confirm this finding, green and white disks of 'Salinas' lettuce were compared. It is generally known that green tissues are more tolerant to high levels of CO_2 than the white tissues (15). Again, no difference was found in pH change, while there was moderate CO_2 injury in white but none in green tissue (Table 2). The δ values of P_i from green tissue in both cytoplasm and vacuole were higher than those from white tissue, but the upfield increase (pH drop) due to CO_2 appeared to be similar in magnitude. These data do not support our hypothesis that CO_2-susceptible tissue has a reduced buffering capacity. All tissues with different degrees of susceptibility to CO_2 appeared to have the same buffering capacity.

When pH of the tissue homogenate was determined by a glass-electrode pH meter, it was found that white tissues have a consistently higher pH than the green tissues. Data from NMR scanning of tissue homogenates also confirm this result. There was only a small difference between the titration curve of white and green tissue homogenates (Fig. 2). In contrast, the NMR study of intact tissue gave the opposite result (Table 2). Both cytoplasmic and vacuolar pH of white tissue had a lower P_i chemical shift indicating lower pH than that of green tissue. Estimation of pH by NMR technique could be influenced by ionic strength as well as Mg^{++} concentration of the sample (20). Green tissue might have increased free Mg^{++} concentration which could increase the chemical shift of P_i, hence giving a higher pH estimate. This effect of Mg^{++} might be abolished when compartmentation is destroyed during tissue homogenation. Roberts et al. (20) showed that ATP and citrate could reverse the effect of Mg^{++} on the chemical shift of P_i. The opposite also is possible, i.e., the 2 tissues might have similar Mg^{++} content but have different ATP and citrate concentrations.

When lettuce tissue was removed from the CO_2 atmosphere, an increase in δ of P_i was found in the vacuole (Table 1). 'Winterhaven' and 'Salinas' had a significant increase while 'Climax' did not. This increase in δ of P_i was sustained for at least 18 hr after the tissue was transferred from CO_2 to air. When we compared green and white tissues and exposed them to 20% CO_2, we found only a slight increase in δ of P_i (Table 2). When pH of the tissue homogenates was determined by glass-electrode pH meter, the increase in pH was found only in tissue samples immediately following removal from the CO_2 treatment. The pH of tissue homogenates increased as soon as CO_2 was applied and reached a stable level in about 24 hr (Fig. 3). If CO_2 was removed (even for only 1 hour) before sampling, pH was found to be the same as the original value, or slightly lower (Tables 1 and 2).

Although there are some discrepancies in the results between the 2 methods of pH determination, both showed that CO_2 induced an increase in tissue pH. Lebermann et al. (14) found a proportional increase in pH of broccoli relative to CO_2 concentrations; however, our data show that at 15% and 20% CO_2, the pH of the tissue homogenate was not higher than that at 10% CO_2 (Fig. 3).

We do not know whether or not this pH increase was just a consequence of CO_2 effect on normal metabolism, or a direct reaction by plant tissues to counteract the acidic effect of CO_2. By titrating tissue homogenates with a base, we found that there was less titratable acid (or more OH^-) in CO_2-treated tissue than in control tissue. Titration with acid gave inconsistent results (Table 3). The drop in titratable acid mirrored the rise in pH (Fig. 3) which suggests that a mechanism regulating pH might be operating under high CO_2 concentration. Davies (6) pointed out that malic enzyme was activated under acidic conditions and that it catalyzed the decarboxylation of malate into pyruvate, CO_2 and OH^-; such a reaction would prevent a drop in pH.

A study of changes in amino and other organic acids content

Table 1. Effect of CO_2 treatment for 6 days at 0°C on chemical shift of inorganic phosphate (P_i) in the cytoplasm and vacuole, on pH of tissue homogenate (as measured by glass electrode pH meter), and on CO_2 injury or 3 lettuce cultivars.

		P_i chemical shift (ppm) ± SD			
Treatment	Cultivar	Cytoplastmic	Vacuolar	pH ± (SD)	CO_2 injury[z]
Air	Climax	2.45 ± 0.09	0.58 ± 0.00	5.79 ± 0.03	---
	Winterhaven	2.52 ± 0.06	0.58 ± 0.00	5.85 ± 0.03	---
	Salinas	2.57 ± 0.13	0.62 ± 0.06	5.82 ± 0.02	---
CO_2	Climax	1.99 ± 0.13	0.47 ± 0.06	6.42 ± 0.02	---
	Winterhaven	2.05 ± 0.00	0.46 ± 0.04	6.34 ± 0.03	---
	Salinas	2.15 ± 0.05	0.50 ± 0.02	6.42 ± 0.03	---
CO_2 to air	Climax	2.45 ± 0.04	0.53 ± 0.02	5.48 ± 0.22	Severe
	Winterhaven	2.46 ± 0.02	0.73 ± 0.00	5.62 ± 0.16	Slight
	Salinas	2.48 ± [y]	0.83 ± 0.02	5.78 ± 0.12	Slight

[z]Evaluations were made 1 day after transfer from CO_2 to air.
[y]SD missing due to broad P_i signal.

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Table 2. Effect of CO_2 treatment for 6 days at 0°C on chemical shift of cytoplasmic and vauolar inorganic phosphate (P_i), on tissue homogenate pH (as measured by glass electrode pH meter), and on CO_2 injury of 'Salinas' lettuce.

		P_i chemical shift (ppm) (± SD)			
Treatment	Tissues	Cytoplasmic	Vacuolar	pH ± (SD)	CO_2 injury[z]
Air	White	2.54 ± 0.01	0.63 ± 0.03	6.03 ± 0.05	---
	Green	2.71 ± 0.04	0.70 ± 0.03	5.74 ± 0.12	---
CO_2	White	1.89 ± 0.08	0.38 ± 0.03	6.49 ± 0.03	---
	Green	2.09 ± 0.04	0.46 ± 0.01	6.21 ± 0.11	---
CO_2 to air	White	2.36 ± 0.04	0.67 ± 0.05	6.09 ± 0.05	Moderate
	Green	2.58 ± 0.02	0.78 ± 0.02	5.89 ± 0.09	None

[z]Evaluations were made 1 day after transfer from CO_2 to air.

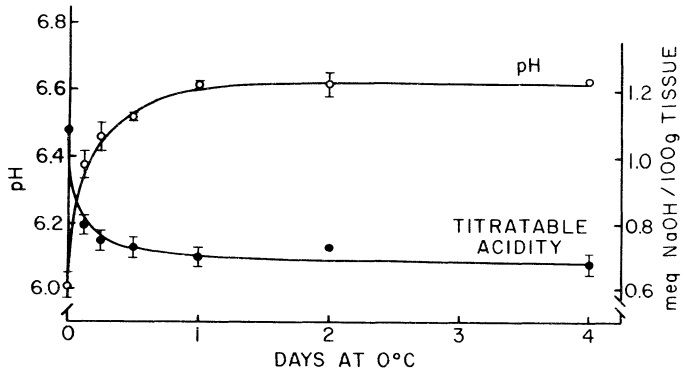

Fig. 3. pH and titratable acidity of lettuce tissue homogenate determined by glass-electrode pH meter immediately after removal from air + 15% CO_2 treatment for indicated durations at 0°C.

Table 3. Effect of CO_2 on pH and acidity of 'Salinas' lettuce tissue kept at 0°C (data shown are means ± SD).

CO_2 concn. (%)	Duration of treatment (days)	pH of tissue homogenate	Meq. acid/100 g tissue (titrated to pH 3)	Meq. base/100 g tissue (titrated to pH 8.3)
0	0	6.01 ± 0.04	2.80 ± 0.08	1.08 ± 0.00
0	5	6.10 ± 0.02	2.63 ± 0.03	1.00 ± 0.13
5	5	6.51 ± 0.03	2.60 ± 0.05	0.78 ± 0.03
10	5	6.65 ± 0.04	2.45 ± 0.05	0.73 ± 0.00
15	5	6.67 ± 0.05	2.58 ± 0.03	0.68 ± 0.04
20	5	6.65 ± 0.01	2.75 ± 0.03	0.65 ± 0.00

in response to elevated CO_2 might clear up the relationship between exposure to CO_2 and pH regulation. Previous reports (3, 7, 16, 22, 23, 25, 27, 28) showed a consistent accumulation of succinic acid (which is a minor acid in plant tissues), while other acids did not show a consistent response to CO_2; therefore, these reports do not help explain the observed pH increase.

High concentrations of CO_2 could reduce the energy supply of plant tissue by inhibiting various respiratory enzymes (1, 2, 17, 22, 26), and thus could eventually lead to cell death. In addition, Lipton (15) pointed out that green tissues were often more tolerant to CO_2 than nongreen tissues. Lipton also suggested that soluble solids content in lettuce might be related to the susceptibility to high CO_2, because it was observed that lettuce harvested in the afternoon suffer less CO_2 injury than that harvested in the morning; but no conclusive data have been reported (11). We tested whether using light as an external energy source for white (nongreen) lettuce tissue kept under air + 15% CO_2 would influence CO_2 injury. The light treatment reduced CO_2 injury by about 50% (Table 4).

Table 4. CO_2 injury scores of 'Salinas' lettuce tissue kept 2 weeks in air + CO_2 under light or dark at indicated tempertures.

Expt. no.	CO_2 (%)	CO_2 injury scores[z] ± SD (tissue temperature, °C) Light	Dark
1	10	18 ± 1 (0.5°)	56 ± 23 (0.5°)
2	15	21 ± 6 (0.5°)	39 ± 14 (0.5°)
3	15	38 ± 8 (0.5°)	56 ± 8 (1.0°)
4	15	23 ± 7 (1.0°)	42 ± 8 (2.0°)

[z]Based on number, size, and intensity of browning of the "brown stain" lesions.

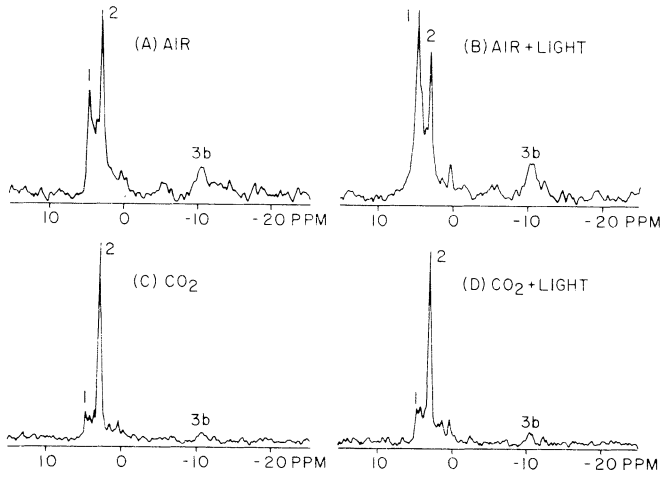

Fig. 4. ^{31}P-NMR spectra of $HClO_4$-extracts (without passing through a chelating column) of lettuce leaf disks previously kept at 2.5°C for 6 days in: (**A**) air, (**B**) air + light, (**C**) air + 15% CO_2, or (**D**) air + 15% CO_2 + light. Spectra were derived from ~3000 scans.

Figure 4 shows the NMR analysis of $HClO_4$-extracts of lettuce tissue from these experiments. In some experiments, the tissue in air under light treatment had a stronger signal for glucose-6-phosphate (G6P) and a weaker P_i than that which was kept in the dark (Fig. 4 A, B). This result could not be reproduced in 1 of 3 experiments; however, with CO_2 treatment (Fig. 4 C, D) there were consistently lower G6P concentrations than the air control, whether light was applied or not. Green tissue had a slightly stronger G6P signal but responded to CO_2 in the same manner as white tissue (data not shown). Lower ATP signals were detected in tissue treated with CO_2 than in tissue kept in air (Fig. 4), but the magnitude of this difference varied among experiments. The reduction of G6P level under CO_2 treatments as compared to air control provides additional evi-

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dence that glycolysis is enhanced by elevated CO_2 atmospheres. This enhancement of glycolysis indicates a need for extra energy supply by the tissue to sustain an adequate energy status. Light could satisfy such a need, but the mechanism by which light reduced CO_2 injury to lettuce tissue merits further investigation.

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