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# Effects of $CO_2$ on Total Phenolics, Phenylalanine Ammonia Lyase, and Polyphenol Oxidase in Lettuce Tissue

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***Abstract.*** **An atmosphere of air + 15% $CO_2$ caused $CO_2$ injury in lettuce (*Lactuca sativa* L.) in about 10 days at 0°C. However, subsequent removal of $CO_2$ was necessary for the brown stain symptoms to develop. Under $CO_2$ treatment, phenylalanine ammonia lyase (PAL) was induced and its activity correlated well with the development of the injury. Nevertheless, PAL activity did not seem responsible for the differences in susceptibility to $CO_2$ injury among the 3 lettuce cultivars included in this study. Prevention of the development of brown stain symptoms by $CO_2$ probably was due to its inhibition of phenolics production and the inhibition of polyphenol oxidase activity.**

Atmospheres with reduced $O_2$ and/or elevated $CO_2$ levels are known to extend the storage life of fruit and vegetables (11, 23). Yet high levels of $CO_2$ also can be harmful. Some commodities, such as 'Anjou' pear and crisphead lettuce, could be injured by an atmosphere of only 1% $CO_2$, especially when combined with reduced $O_2$ levels (13). In most instances the symptoms of injury include a brown chlorotic area inside or on the surface of the commodity (11, 23). Brown stain is a $CO_2$-induced physiological disorder in lettuce (1, 2, 3).

Browning is common in plant tissues and is thought to be brought about through the metabolism of phenolic compounds (15, 17, 19). In short, the phenolic pathway begins with the deamination of phenylalanine by the enzyme phenylalanine ammonia lyase (PAL) to cinnamic acid. Cinnamic acid then is sequentially hydroxylated into various phenolic compounds. In the presence of $O_2$, the enzyme polyphenol oxidase (PPO) may oxidize these compounds to quinones which simultaneously polymerize into brown pigment (8, 15, 19, 20, 21). Murr and Morris (16) found that low concentrations of $CO_2$ inhibited the browning of mushrooms and that PPO was competitively inhibited by $CO_2$. At higher concentrations, however, $CO_2$ caused increased browning. Buescher (4) showed that 20% $CO_2$ in air prevented browning (due to mechanical wounding) of mechanically harvested snap-beans held at 27°C. In such conditions, total phenolic content and PPO activity were reduced considerably and, consequently, browning was inhibited. Hyodo and Yang (10) demonstrated that $C_2H_4$-induced PAL activity in pea seedlings could be inhibited by 5% or higher concentrations of $CO_2$. Thus, whereas $CO_2$ can cause injury in plant tissues, it also can prevent the development of injury symptoms. In order to understand the effects of $CO_2$ on plant tissues, we determined levels of PAL and PPO activity and total phenolics content in crisphead lettuce as influenced by high concentrations of $CO_2$ which could cause tissue injury.

## Materials and Methods

*Plant material.* Crisphead lettuce was obtained either from wholesale distributors in Sacramento or directly from the Univ. of California and USDA Experimental Stations in Salinas and El-Centro, Calif. Midrib segments (12–24 $cm^2$) were cut from the lower 3rd of the leaves which were in turn located one-third to two-thirds between the cap leaf and the center of the lettuce head. About 50 g of these sections were used per replicate. At least 3 replicates were used per treatment in all experiments.

The lettuce tissue segments were kept in closed containers ventilated with humidified air or humidified air plus 15% $CO_2$. Samples were taken for analysis at about one week intervals. At the end of each experiment, samples were transferred to 20°C for $CO_2$ injury evaluation. $CO_2$ injury was scored by the system described by Kader et al. (12), which is based on area of necrosis and intensity of browning, with a maximum score of 150.

*Chemical analysis.* Lettuce tissue was kept at −30°C until analyzed. For determining total phenolics content, the frozen tissue was homogenized with 2× volumes of ice cold 80% ethanol using a "Polytron" homogenizer (Brinkmann Instruments, New York). The homogenate was filtered through cheesecloth, centrifuged at 12,000 × g for 20 min, and the supernatant was used for the total phenolics analysis (22); gallic acid was used as the standard.

Enzyme extraction for PAL assay was done either by preparing an acetone powder which then was resuspended in 0.2 M borate buffer (pH 8.8), or by homogenizing the tissue directly with 3× weight borate buffer containing 5 mM mercaptoethanol and 0.1× weight polyvinyl-polypyrrolidone. The homogenate or the suspension of acetone powder was filtered through cheesecloth and centrifuged at 12,000 × g for 20 min. The supernatant was used to determine PAL activity with the method described by Zucker (27). During the preparation of the enzyme extract, the temperature was kept below 4°C at all times. The assay medium contained 0.3 mmoles concentrated borate buffer (pH 8.8), 60 μmoles phenylalanine, and the enzyme extract in the total volume of 6 ml. The mixture was incubated at 40°C for 1 hr. The reaction was stopped by adding 0.1 ml of conentrated HCl. PAL activity was determined by measuring the absorbance of the assay mixture at 290 nm for the production of cinnamic acid. One unit of PAL activity was defined as the amount of enzyme that produced one μmole of cinnamic acid in 1 hr under the specified conditions described.

Enzyme preparation for determining PPO was done in the same manner as with PAL except that 0.1 M citric acid-phosphate buffer (pH 6.5) was used instead of the borate buffer. The supernatant was used for PPO assay as described by Flurkey and Jen (6). The assay medium contained 0.5 mmoles citric acid-phosphate buffer (pH 6.5), 20 μmoles caffeic acid, and

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the enzyme extract. The activity of PPO was determined by the increase in absorbance of the mixture at 420 nm 1 min after adding the enzyme extract and continuously shaking the mixture. One unit of PPO activity was defined as the amount of enzyme that produced an increase in absorbance at 420 nm of 0.1 per min.

## Results and Discussion

*$CO_2$ effects on respiration, ethylene production, and injury symptoms.* Preliminary experiments showed that segments of midrib taken from leaves located about half way to the core are the most susceptible tissue to $CO_2$ injury. These midrib segments exhibited $CO_2$ injury symptoms identical to those found in intact lettuce heads, although the injury was somewhat reduced. Air + 15% $CO_2$ was chosen for all experiments because it was determined to be the lowest $CO_2$ concentration (added to air) that consistently caused $CO_2$ injury in lettuce. The respiration and $C_2H_4$ production rates of these midrib segments are shown in Fig. 1. A large increase in $CO_2$ and $C_2H_4$ production rates was noted upon transferring the lettuce tissue from the air + $CO_2$ atmosphere at 0°C to air either at the same temperature or at 20°, indicating that injury had occurred. In contrast, there was only a moderate increase in respiratory rate and no change in $C_2H_4$ production rate when the lettuce tissue kept in air at 0° (control) was transferred to 20°.

These data do not seem to support the notion that brown stain of lettuce may be a form of chilling injury that is aggravated by high $CO_2$ levels (2). Usually a burst of $C_2H_4$ production is seen upon transfer of chilled tissue to higher temperatures (25); however, no increase in $C_2H_4$ production was observed in our control tissue when transferred from 0° to 20°C, indicating no injury due to low temperature alone. In addition, we found that $CO_2$ injury could be induced at increased temperatures (10° and 20°C) with increased $CO_2$ concentrations; the high $CO_2$ level probably was required because of reduced solubility of $CO_2$ at high temperatures. At 20°C, the $CO_2$ injury occurred throughout the leaf, not limited just to the midrib, and was more severe on the inner than the outer part of the lettuce head.

After about 10 days under $CO_2$ treatment at 0°C, $CO_2$ injury was discernible as clear areas of collapsed cells; browning developed only when lettuce tissues were transferred to air. If the tissues were kept continuously at 0° upon transfer to air, appearance of the browning symptoms was delayed (Fig. 2). Browning also was reduced when the lettuce tissue was kept in $CO_2$ after transfer to 20°; both area of injury and browning severity were reduced and the injury score was about half of that kept in air. Thus, it is clear that $CO_2$ suppresses the development of $CO_2$ injury symptoms. No $CO_2$ injury symptoms were noted in lettuce tissue transferred from air at 0°C to air or $CO_2$ at either 0° or 20°.

*$CO_2$ effects on PAL activity.* PAL activity of the lettuce tissue kept in air at 0°C declined slightly or remained unchanged, but it increased gradually in tissue exposed to $CO_2$ (Fig. 3). Upon transfer from 0° to 20°C, PAL activity of $CO_2$-treated tissue increased several times, particularly if the tissue also was moved from the $CO_2$ atmosphere to air (Fig. 3, 4). When the tissue was kept at 20° longer than 24 hr, however, PAL activity declined to a trace level regardless of the atmospheric condition (data not shown). This drop could be the result of a lyase-inactivating system, such as the one illustrated by Zucker (27). The increase in PAL activity under $CO_2$ treatment varied among cultivars and experiments, but the trend was similar (Fig. 4). Although the increase in PAL activity of lettuce tissue treated with $CO_2$ correlated well with $CO_2$ injury (Fig. 5), we do not think that it was the primary cause of the injury. There are many reports of PAL induction in plant tissues by other kinds of environmental stress, such as wounding, infection, light, and growth modifiers (5). Thus, a high concentration of $CO_2$ could represent another form of stress probably by lowering the pH of plant tissues or by directly inhibiting respiratory enzymes (18, 26), and subsequently inducing higher PAL activity.

*$CO_2$ effects on PPO activity.* $CO_2$ did not have a very clear effect on PPO activity. Although the activity under $CO_2$ at-

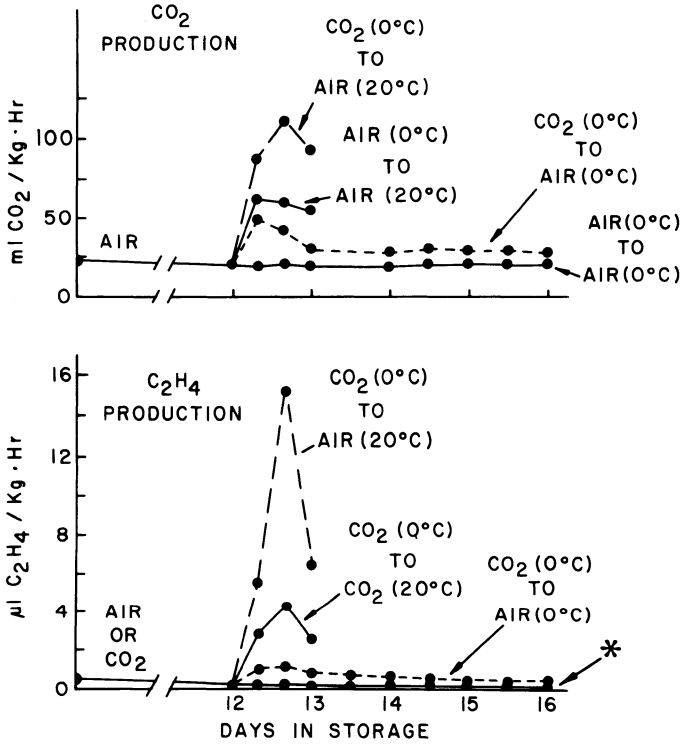


Fig. 1. Effects of $CO_2$ on $CO_2$ and $C_2H_4$ production at 0° or 20° by 'Climax' lettuce leaf midrib tissue previously kept at 0°C in air or air + 15% $CO_2$ for 12 days (* designates $C_2H_4$ production rate by tissue transferred from air at 0° to air or $CO_2$ at 0° or 20°).

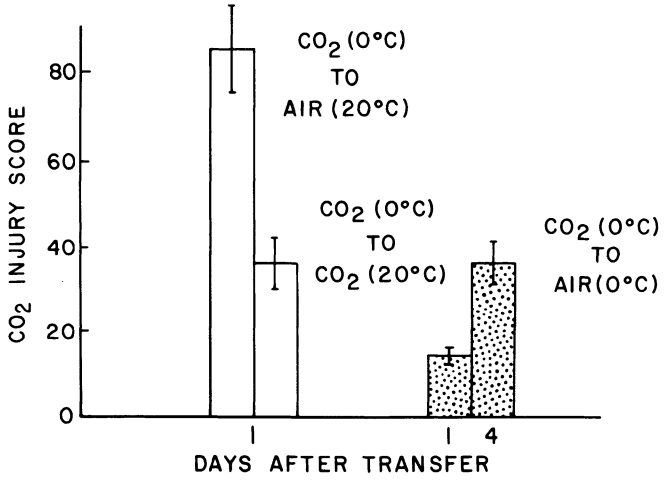


Fig. 2. $CO_2$ injury scores of 'Climax' lettuce leaf midrib tissue which was transferred to air or $CO_2$ at 0° or 20°C following 12 days of storage in air + 15% $CO_2$ at 0°C.

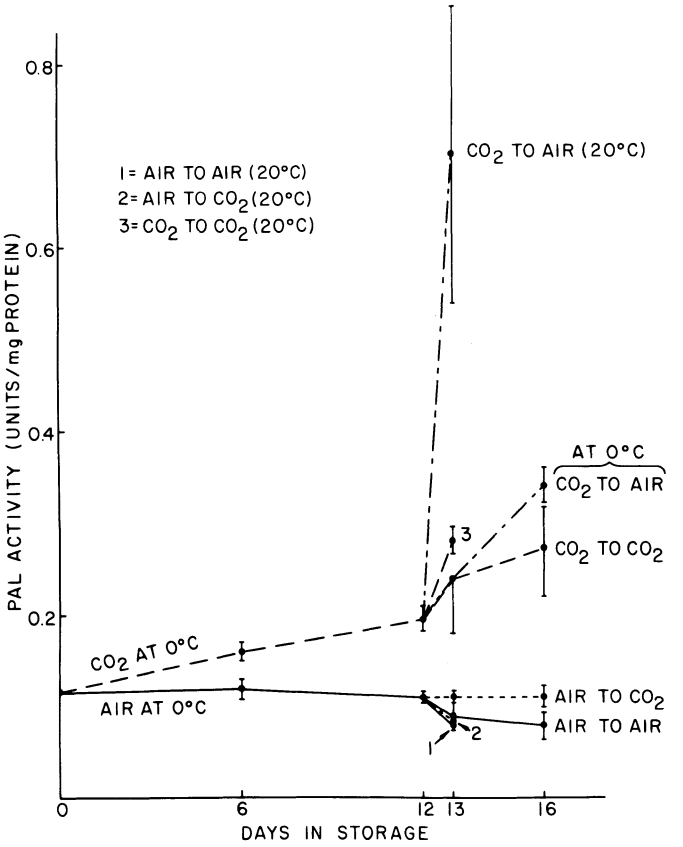


Fig. 3. Phenylalanine ammonia lyase (PAL) activity of 'Climax' lettuce leaf midrib tissue kept in air or air + 15% $CO_2$ for 12 days before transfer to various conditions.

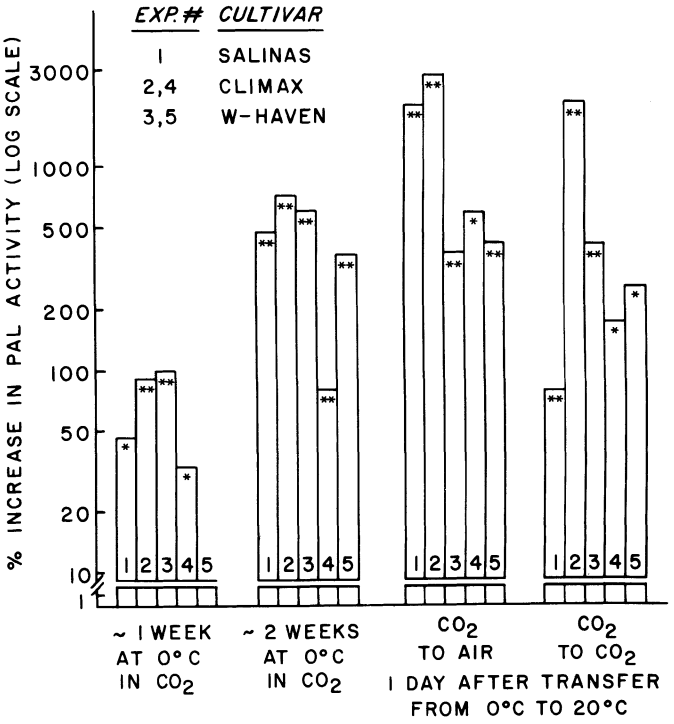


Fig. 4. Changes in PAL activity of 3 lettuce cultivars kept in air + 15% $CO_2$ for about 2 weeks at 0°C, then transferred to air or air + 15% $CO_2$ at 20° relative to control tissue (kept in air at 0° and 20°) in 5 experiments. (*, ** indicate significant differences from the control at the 5% and 1% level, respectively).

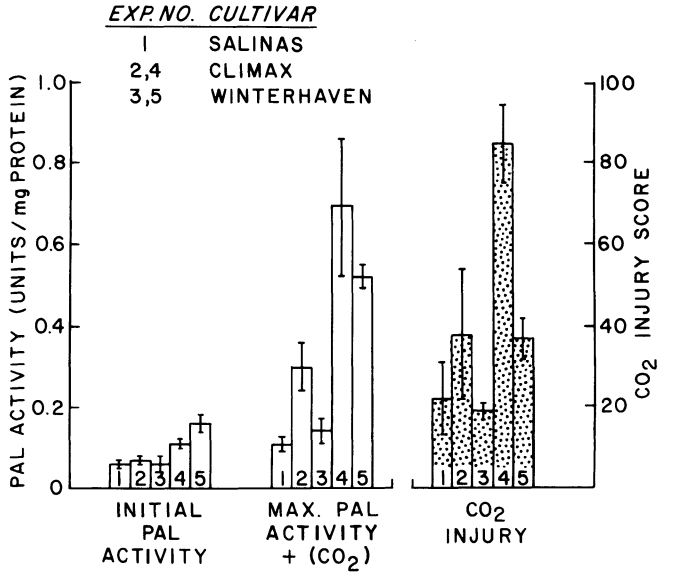


Fig. 5. Comparison between PAL activity and $CO_2$ injury scores for 3 lettuce cultivars kept in air + 15% $CO_2$ for about 2 weeks at 0°C, then transferred to air at 20° in 5 experiments.

mosphere often was lower than that under air control, particularly when lettuce tissues were transferred to 20°C (Fig. 6, 7), the difference was not always statistically significant. In no instance was browning of lettuce tissue observed even at the cut edge unless $CO_2$ was replaced by air or the temperature was raised to 20°. This prevention of browning by $CO_2$ probably was related to reduced PPO activity, since $CO_2$ is a competitive inhibitor of PPO as demonstrated by Murr and Morris (16). At 20°, $CO_2$ is about half as soluble in water as it is at 0° (14), hence its inhibitory effect on PPO could be reduced and browning could occur (Fig. 8).

*$CO_2$ effects on total phenolics content.* The total phenolics content of lettuce tissue in the air control increased by almost 50% from its original level, then remained relatively stable, whether or not the tissue was transferred to 20°C (Fig. 9). The increase may have been a response to the handling of the tissue, resembling the observed increase in phenolics in plants when wounded or attacked by insects or diseases (7, 19, 24). Under $CO_2$ treatment, phenolic content did not change significantly. If $CO_2$ was replaced after about 10 days or longer by air, phenolics content increased considerably; quickly at 20° and slowly at 0° (Fig. 10). It is clear that $CO_2$ suppresses the production of phenolic compounds. However, $CO_2$ injury scores did not correlate well with the level of total phenolic content. The symptoms could occur in the $CO_2$-treated tissue without higher total phenolic content than the control (Fig. 8).

*$CO_2$ effects on phenolic metabolism.* It is possible that the susceptibility of different lettuce cultivars to $CO_2$ injury, judging by the symptoms, could depend on the initial severity of $CO_2$ injury and on the development of the symptoms. Different cultivars may be equally injured by $CO_2$, but they may differ in the degree of symptom development due to a limiting step that controls changes associated with tissue browning. Since we found a good correlation between $CO_2$ injury symptoms and PAL (Fig. 5), PAL activity may be, at least in part, responsible for differences among cultivars in $CO_2$-injury symptom development.

Whereas high $CO_2$ concentrations caused injury to lettuce

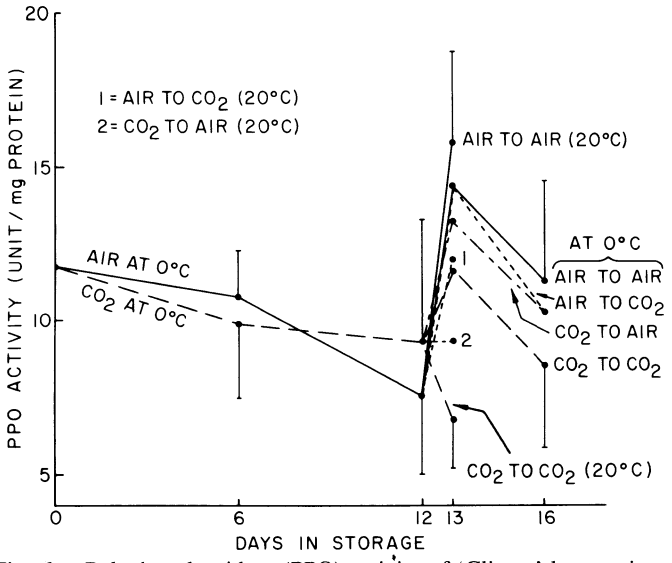


Fig. 6. Polyphenol oxidase (PPO) activity of 'Climax' lettuce tissue kept in air or air + 15% $CO_2$ for 12 days before transfer to various conditions.

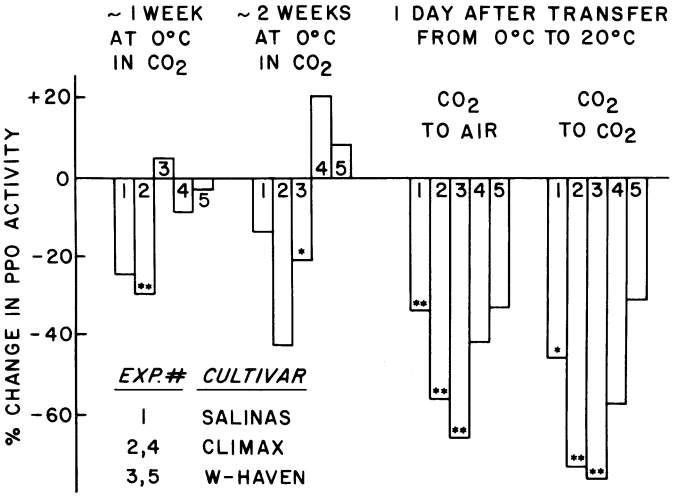


Fig. 7. Changes in PPO activity of 3 lettuce cultivars kept in air + 15% $CO_2$ for about 2 weeks at 0°C, then transferred to air or to air + 15% $CO_2$ at 20°, relative to control tissue (kept in air at 0° and 20°) in 5 experiments. (*, ** indicate significant differences from the control at the 5% and 1% level, respectively.)

tissue, our data indicate that $CO_2$ itself could prevent the symptom development. This phenomenon could be interpreted as follows:

•High $CO_2$ disrupted normal metabolic balance of plant cells, and consequently led to cell death. At the same time, PAL was induced as a natural defense mechanism of plants upon exposure to environmental stresses. However, the product(s) of PAL activity also could be toxic and may have contributed to the cause of injury. In the study on russet spotting, another disorder of lettuce caused by exposure to $C_2H_4$, Hyodo et al. (9) also found that PAL was induced, but they were not able to induce russet spotting by administering subsequent products of PAL, namely chlorogenic acid and isochlorogenic acid.

•When PAL was induced under $CO_2$ treatment, we might have seen some increase in phenolic content under $CO_2$ treatment. However, that was not the case. Thus, $CO_2$ must have inhibited some earlier step(s) in the pathway of the phenolic

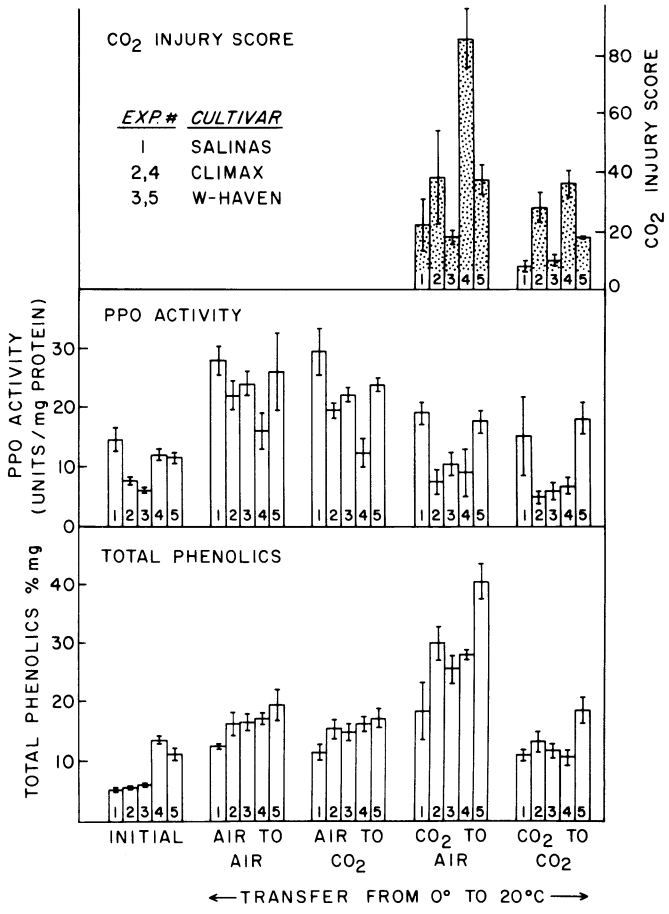


Fig. 8. Comparison among $CO_2$ injury scores, PPO activity, and total phenolics content of 3 lettuce cultivars kept in air or air + 15% $CO_2$ for about 2 weeks, then transferred to air or air + 15% $CO_2$ at 20°C, in 5 experiments.

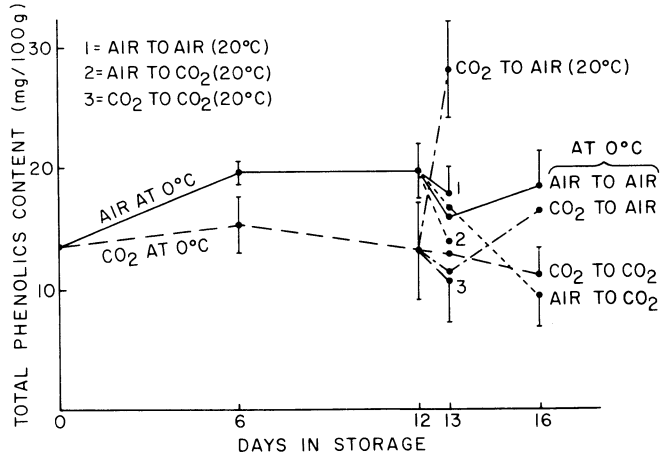


Fig. 9. Total phenolics content of 'Climax' lettuce kept in air or air + 15% $CO_2$ for 12 days before transfer to various conditions.

metabolism, where the hydroxyl group was not yet incorporated. The only possibility was the step where cinnamic acid was hydroxylated to p-coumaric acid (4-hydoxy cinnamic acid), which possessed phenol properties and gave positive response to the total phenolics determination procedure used in this study. If this was true, cinnamic acid, which gave negative response

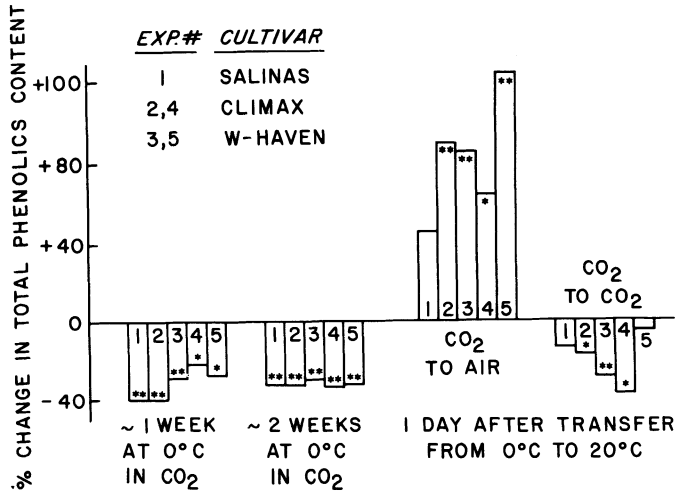


Fig. 10. Changes in total phenolics content of 3 lettuce cultivars kept in air + 15% $CO_2$ for about 2 weeks at 0°C, then transferred to air or to air + 15% $CO_2$ at 20°, relative to control tissue (kept in air at 0° and 20°) in 5 experiments (*, ** indicate significant differences from the control at the 5% and 1% level, respectively.)

to the determination procedure, could have accumulated to a level that may be toxic to the tissue.

•$CO_2$ prevented the browning of wounded plant tissues by both blocking the production of new phenolic compounds [described in (b) above] as well as inhibiting PPO activity. Although Murr and Morris (16) showed that 100% $CO_2$ could inhibit PPO by only 60% at 25°C in vitro, it should be noted that substrate concentrations for the exzyme in vivo are much lower than those in vitro. Furthermore, $CO_2$ is more water-soluble at low temperature, and the same concentration thus could have a stronger effect than at high temperature.

Our studies of the effects of $CO_2$ on phenolic metabolism in lettuce tissue are continuing with emphasis on the activity of the enzyme cinnamic acid-4-hydroxylase.

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