

# Zinc Deficiency, Carbonic Anhydrase, and Photosynthesis in Leaves of Spinach

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## ABSTRACT

A shortage in the zinc supply to spinach (*Spinacia oleracea* L.) drastically reduced carbonic anhydrase levels with little effect on net CO<sub>2</sub> uptake per unit leaf area, except with the most severe zinc stresses. Under these conditions, carbonic anhydrase was below 10% and photosynthesis 60 to 70% of the control levels. When photosynthesis was measured at a range of CO<sub>2</sub> supply levels, zinc-deficient leaves were less efficient at 300 to 350 microliters per liter CO<sub>2</sub> and above, but the same as controls at lower CO<sub>2</sub> levels. This suggests that carbonic anhydrase does not affect the diffusion of CO<sub>2</sub>, and that the effect of zinc deficiency was on the photosynthetic process itself. Our evidence does not support the hypothesis that carbonic anhydrase has some role in facilitating the supply of CO<sub>2</sub> to the sites of carboxylation within the chloroplast.

Carbonic anhydrase is widespread in plants, but its exact physiological role is still uncertain. It may be directly involved in photosynthesis, facilitating the diffusion of CO<sub>2</sub> through the liquid phase of the cell to the chloroplast (9, 13, 20). In algae grown at high CO<sub>2</sub>, carbonic anhydrase is absent. After transfer to low CO<sub>2</sub>, photosynthesis is at first negligible, but later increases in step with increasing carbonic anhydrase levels (8). In higher plants, acetazolamide, an inhibitor of carbonic anhydrase, has been shown to reduce photosynthesis by intact chloroplasts. The inhibition can be overcome by raising the bicarbonate level (4). Acetazolamide also inhibits the photosynthesis of detached leaves of spinach and maize (5).

Wood and Sibly (19) found that zinc-deficient plants contained low levels of carbonic anhydrase. In the experiments reported in this paper, we have studied the relationship between carbonic anhydrase and photosynthesis by manipulating the zinc supply to the plant. By this means, we have obtained large changes in carbonic anhydrase. However, these were accompanied by small effects on photosynthesis. The results reported do not support the hypothesis that carbonic anhydrase is directly involved in photosynthesis.

## MATERIALS AND METHODS

**Cultural Methods.** Seeds of spinach (*Spinacia oleracea* L. cv. True Hybrid 102) were germinated in vermiculite, irrigated with dilute nutrient solution. Fourteen days later seedlings were transferred to aerated nutrient solution. When established, they were thinned to leave six plants in each 3-liter pot. Plants were grown either with different levels of zinc or with adequate zinc

and later transferred to solutions without zinc or with growth-limiting levels of the element. The exact treatments are specified under "Results" for each experiment.

The nutrient solutions had the following composition (mg/l): nitrogen, 168 (as nitrate); phosphorus, 16; sulfur, 16; chloride, 85; calcium, 160; potassium, 156; magnesium, 40; iron, 2.8 (as iron EDTA); manganese, 0.25; copper, 0.025; molybdenum, 0.06; boron, 0.1; iodine, 0.002; cobalt, 0.01. Renewal of the solution was carried out as necessary, about once a week.

Salts, except those supplying iron and the other minor elements, were freed of zinc contamination by co-precipitation with magnesium hydroxide (12). Zinc contamination of the cultures was avoided by following the procedures of Hewitt (10).

The plants were grown in an air-conditioned room under mixed fluorescent and incandescent lights, supplying 17.5 cal·cm<sup>-2</sup>·hr<sup>-1</sup> at plant height during a 12-hr day. Temperatures were 26 C during the light and 22 C during the dark period.

**Photosynthesis Measurements.** Leaves were cut from the plant under water and petioles placed in distilled water in vials covered with Parafilm. Alternatively, the leaves were cut in air, quickly placed in water, and subjected to vacuum to remove any air which had entered the cut end of the petiole. Either method was satisfactory with this species. Three to six leaves were placed in an assimilation chamber under a bank of Philips HPL 1000 w lamps supplying 17 cal·cm<sup>-2</sup>·hr<sup>-1</sup>. An open system was used. The desired concentration of CO<sub>2</sub> in the air entering the chamber was obtained by mixing CO<sub>2</sub> with CO<sub>2</sub>-free air. This concentration was in the range of 300 to 330 μl/l, except where stated. To avoid depletion of CO<sub>2</sub> below a level necessary for the measurement of CO<sub>2</sub> uptake, flow rates were adjusted according to the area of leaves in the cabinet. The CO<sub>2</sub> concentration of the air leaving the cabinet was never less than 250 μl/l and usually around 270 μl/l.

Results are expressed as net CO<sub>2</sub> uptake·dm<sup>-2</sup> leaf area·hr<sup>-1</sup>. Leaf areas were estimated by comparing individual leaves with a set of standards of known leaf area, as outlined by Williams (18) for tomatoes.

**Carbonic Anhydrase Measurements.** Leaves were ground in a chilled glass mortar with ice-cold 10 mM tris-HCl buffer, pH 8.2, containing 5 mM 2-mercapto-ethanol, and centrifuged at 12,000g for 10 min in a refrigerated centrifuge at 0 C. The pale green supernatant had the same activity as the crude homogenate but was more convenient to use. Assays were completed within 45 min of macerating the tissue. Tests showed no loss of enzyme activity for up to 2 hr after grinding.

Carbonic anhydrase was measured by the veronal buffer-indicator method as described by Rickli *et al.* (15). Units of enzyme were calculated according to the formula  $u = 10 [(tb/te) - 1]$ , where *tb* is the time for the uncatalysed reaction, and *te* is the time for the reaction with active enzyme added.

For purposes of comparison, we have expressed both photosynthesis and enzyme activity on a leaf area basis. A number of measurements of protein (boiling 70% ethanol-insoluble nitrogen) and chlorophyll in leaves of the age and physiological status used in the present work showed no significant differences in content per unit area between zinc-deficient leaves and the appropriate controls.

**Zinc Analysis.** Leaf material was digested with a mixture of nitric, perchloric, and sulfuric acids, and zinc was measured by atomic absorption spectroscopy.

## RESULTS

**Effects of Zinc Deficiency on Dry Weight, Photosynthesis, and Carbonic Anhydrase.** Altering the zinc nutrition of the plant had considerable effects on yield, zinc content, and carbonic anhydrase, but comparatively small effects on net photosynthesis.

The effects on plant growth in a typical experiment are shown in Table I. Low zinc plants were considerably smaller than the controls throughout the experiment. Transfer of control plants to no zinc solutions at day 27 did not affect dry weight until the last harvest. Zinc-deficiency symptoms in these plants appeared soon after day 31 but were present in the low zinc treatment throughout the experimental period.

Total zinc per unit leaf area is shown in Figure 1A. The values in the low zinc plants were about one-half those for the control plants. In the no zinc treatment, concentrations fell from the control value at the first measurement to the value in the low zinc treatment at the last.

Zinc deficiency affected photosynthesis and carbonic anhydrase differently. Similar results were obtained in a number of experiments and for leaves of different ages. Data for the fourth leaf (not counting cotyledons) in the experiment for

Table I. *Effect of Zinc Deficiency on Whole Plant Dry Weight*

Data are means of three plants. For details of treatments see Figure 1.

Treatment	Days from Germination		
	30	31	34
	<i>g/plant</i>		
Control solution	0.76	0.86	2.60
No zinc after 27 days	0.77	0.86	1.31
Low zinc	0.48	0.59	0.94

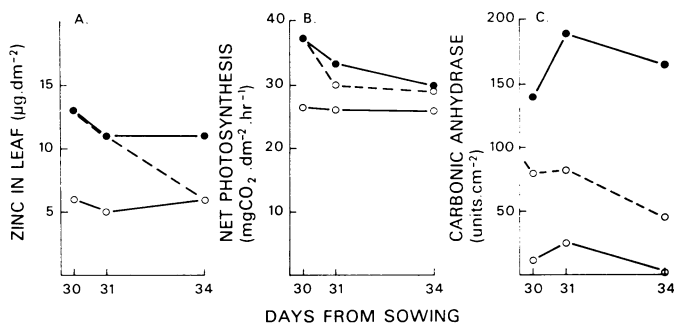


FIG. 1. Some effects of zinc nutrition on the fourth leaf of spinach. A: Zinc content; B: photosynthesis; C: carbonic anhydrase. —●—: Control (20  $\mu\text{g}$  of Zn supplied per plant); —○—: low zinc (3  $\mu\text{g}$  of Zn supplied per plant); ---○---: no zinc (transferred from control to zinc-free solution on day 27).

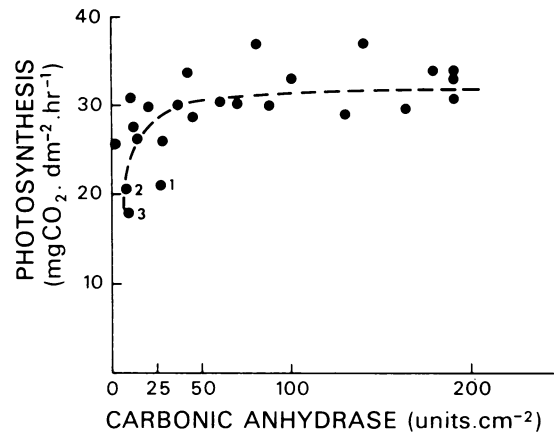


FIG. 2. Plot of net photosynthesis against carbonic anhydrase activity in spinach. Curve was plotted using the function  $y = 163.1 \times 10^{-1.4/x}$ . For the significance of points marked 1, 2, and 3 see text.

which yields were presented above are shown in Figure 1, B and C, respectively.

Photosynthesis in the low zinc leaves was about 70% of the control rate at the first measurement (Fig. 1B). By the last measurement, 4 days later, the control plant rate had declined, so that the rate in low zinc plants was 85% of the control. In marked contrast, the carbonic anhydrase activity of the low zinc plants was only 1 to 13% of the control rate during the period (Fig. 1C).

Three days after transfer of control plants to zinc-free solution there was little effect of treatment on rates of photosynthesis. Carbonic anhydrase activity declined rapidly, being 57% of that for the control at the first measurement and 27% 4 days later.

In Figure 2 photosynthetic rate is plotted against carbonic anhydrase activity in corresponding leaves. Data from Figure 1 are included together with values for leaves 5 and 6 from the same experiment and for leaf 4 from another experiment. Carbonic anhydrase activity varied over a wide range in leaves with normal levels of photosynthesis. It was only in leaves with very low levels of carbonic anhydrase that photosynthesis was reduced appreciably. The apparent relationship indicated by the fitted curve may be misleading. Much of the decline in photosynthesis at lower carbonic anhydrase levels was due to points marked 1, 2, and 3 in Figure 2. These represent low zinc leaves 5 and 6 at a young stage. Photosynthesis tends to be lower in younger leaves.

The data show that carbonic anhydrase was not directly involved in photosynthesis under the conditions used (320–330  $\mu\text{l/l}$   $\text{CO}_2$  and saturating light intensity), or, alternatively, that only a very low level of activity was adequate for maximum rates of photosynthesis. To test this possibility further, we measured photosynthesis in zinc-deficient and control plants over a range of  $\text{CO}_2$  levels.

**Photosynthesis at Different  $\text{CO}_2$  Concentrations in Relation to Carbonic Anhydrase Level.** If carbonic anhydrase has the direct physiological role in photosynthesis of increasing the rate of diffusion of  $\text{CO}_2$  towards the sites of photosynthesis in the chloroplast, it would be expected that its functional significance would become smaller at high external  $\text{CO}_2$  concentrations and least in the region of  $\text{CO}_2$  saturation.

Comparisons were made of photosynthetic rates of zinc-deficient and control plants at a range of  $\text{CO}_2$  supply levels. With the apparatus available, 500  $\mu\text{l/l}$   $\text{CO}_2$  in the air stream was the highest level obtainable. This was approaching the level of  $\text{CO}_2$  saturation as shown by Gaastra (7) for spinach,

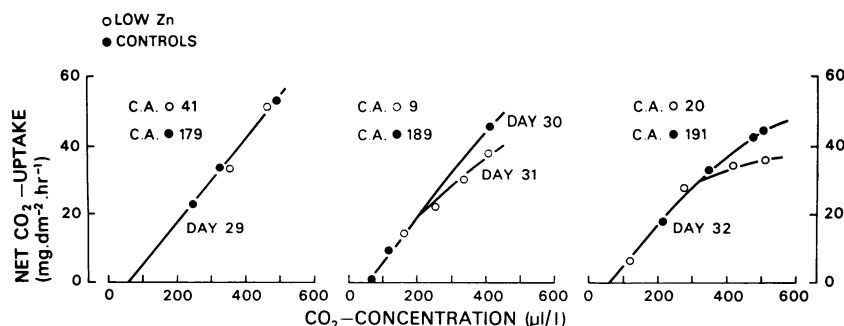


FIG. 3. Photosynthesis at different CO<sub>2</sub> concentrations in plants of varying zinc status. The third and fourth oldest true leaves from three plants were used. Photosynthesis was measured on each set of leaves at each CO<sub>2</sub> concentration and they were afterwards assayed for carbonic anhydrase. Values for each day are shown (C.A.: carbonic anhydrase units·cm<sup>-2</sup>). Plants were raised with adequate zinc (0.01 mg/l in the solution) and on day 25 were transferred to low zinc, —○— (0.001 mg/l) or to control solutions, —●— (0.03 mg/l).

and by Bouma (3) using another C<sub>3</sub> species, subterranean clover, in the apparatus used for the present work. Measurements were made on three occasions over a 4-day period (Fig. 3). During this time, low zinc plants became increasingly zinc-deficient, as judged visually.

In the first comparison, photosynthesis of both groups of leaves responded linearly to increasing CO<sub>2</sub> concentrations up to the highest level used (490 µl/l). Subsequently, the response of the control leaves was linear up to about 400 µl/l CO<sub>2</sub> suggesting a diminishing CO<sub>2</sub> response for these leaves. At low CO<sub>2</sub> concentrations, there was no evidence of a difference in photosynthesis between the low zinc (low carbonic anhydrase) plants and the controls. At about 300 µl/l CO<sub>2</sub> and above, zinc deficiency began to limit photosynthesis. This is strong evidence that mechanisms affecting the rate of CO<sub>2</sub> supply to the sites of photosynthesis are not impaired by zinc deficiency. In view of the greatly reduced carbonic anhydrase levels in the zinc-deficient plants (23, 5, and 10% of the respective controls at the three measurement occasions), it seems unlikely that this enzyme is involved in facilitating the movement of CO<sub>2</sub> to the sites of CO<sub>2</sub> fixation, at least at the activity levels found in our experiments.

## DISCUSSION

This work confirms published findings that carbonic anhydrase is depressed by zinc deficiency (2, 19). In plants, the enzyme is generally thought to contain tightly bound zinc (1, 17). Spinach, however, seems to be exceptional, in that the enzyme purified from this species does not appear to contain zinc (6). In spite of this, we were able to alter the carbonic anhydrase levels of spinach over a wide range by manipulating the zinc supply to the plant, without affecting rates of net CO<sub>2</sub> uptake. Only in severely zinc-deficient plants was photosynthesis affected, and this effect was relatively small (60–70% of the control values), while carbonic anhydrase activities of these leaves fell to 10% or less of the controls. This was observed in a number of experiments (Figs. 1 and 2) and casts doubt on the importance of carbonic anhydrase in maintaining high rates of photosynthesis.

A second reason why this relationship appears doubtful was found in the fact that zinc-deficient, low carbonic anhydrase plants were as efficient as high zinc, high carbonic anhydrase plants in their ability to take up CO<sub>2</sub> at external CO<sub>2</sub> levels below normal atmospheric concentrations, but less efficient at concentrations approaching CO<sub>2</sub> saturation (Fig. 3). This would not be expected if carbonic anhydrase was involved in the transport of CO<sub>2</sub> to the sites of assimilation. It also suggests that zinc deficiency impairs the biochemical capacity of the

plant to fix CO<sub>2</sub>. This would be in agreement with the results of Spencer and Possingham (16), who established a depressed Hill reaction activity in chloroplasts isolated from zinc-deficient spinach plants, and with the demonstration that zinc-deficient soybeans contain reduced levels of fraction I protein (11).

Much of the evidence for the involvement of carbonic anhydrase in photosynthesis has come from the use of inhibitors, in particular, acetazolamide. This substance inhibits carbonic anhydrase and photosynthesis in algae (8) and isolated spinach chloroplasts (4) and rapidly reduces photosynthesis in detached leaves (5). In several experiments, we have confirmed the drastic effects of acetazolamide on photosynthesis in detached leaves. However, the marked and rapid decline in photosynthesis upon addition of acetazolamide was substantially the same in leaves from zinc-deficient, low carbonic anhydrase plants as in the controls. For example, in a typical experiment, we added acetazolamide to the water supply of leaves detached from control and zinc-deficient plants. Ninety minutes later, net CO<sub>2</sub> uptake had decreased from initial rates of 28.0 and 25.5 mg·dm<sup>-2</sup>·hr<sup>-1</sup> to 12.5 and 11.0 mg·dm<sup>-2</sup>·hr<sup>-1</sup> respectively. Over the same period, the corresponding changes in carbonic anhydrase activity were from 46.5 to 6.0 units·cm<sup>-2</sup> in control leaves and from 13.0 to 11.0 units·cm<sup>-2</sup> in zinc-deficient leaves. The markedly different effects of the inhibitor on carbonic anhydrase activity of leaves differing in zinc status, compared with the virtually similar effects of the inhibitor on photosynthesis of these leaves, is difficult to reconcile with the view that the enzyme is of importance in photosynthesis, at least at the levels encountered in these experiments.

Transpiration was unaffected by acetazolamide (unpublished data), showing that the inhibitor did not cause stomatal closure, an effect which might have accounted for its drastic effect on rates of CO<sub>2</sub> uptake.

These results provide evidence against a close relationship between carbonic anhydrase and photosynthesis. They do not exclude the possibility that very small amounts may be adequate to facilitate the diffusion of CO<sub>2</sub> through the liquid phase to the photosynthetic sites in the chloroplasts. This would assume that in our comparisons (*e.g.*, Fig. 3) the low levels of carbonic anhydrase activity found in zinc-deficient plants would still be sufficient to perform such a function, even at low CO<sub>2</sub> supply levels. Such an argument would imply that plants normally contain a considerable excess of carbonic anhydrase, a conclusion reached by Poincelot (14). Alternatively, the enzyme may have more than one function.

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