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Photosynthetic inhibition after long-term exposure to elevated levels of atmospheric carbon dioxide

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Abstract. The effect of long-term exposure to elevated levels of CO_2 on biomass partitioning, net photosynthesis and starch metabolism was examined in cotton. Plants were grown under controlled conditions at 350, 675 and 1000 $\mu l l^{-1}$ CO_2. Plants grown at 675 and 1000 $\mu l l^{-1}$ had 72% and 115% more dry weight respectively than plants grown at 350 $\mu l l^{-1}$. Increases in weight were partially due to corresponding increases in leaf starch. CO_2 enrichment also caused a decrease in chlorophyll concentration and a change in the chlorophyll a/b ratio. High CO_2 grown plants had lower photosynthetic capacity than 350 $\mu l l^{-1}$ grown plants when measured at each CO_2 concentration. Reduced photosynthetic rates were correlated with high internal (non-stomatal) resistances and higher starch levels. It is suggested that carbohydrate accumulation causes a decline in photosynthesis by feedback inhibition and/or physical damage at the chloroplast level.

Abbreviations

Ci, internal CO_2 concentration; Chl, chlorophyll; DMSO, dimethylsulfoxide; HSD, honestly significant difference (procedure); MCW, methanol-chloroform-water; Pi, inorganic phosphate; S.E.M., standard error of mean.

Introduction

The direct effects of CO_2 enrichment on plant growth and physiology are diverse and complex. Many agricultural and non-agricultural species, including woody plants, show a marked increase in biomass when grown under elevated CO_2 concentrations [12]. However, generalizations about the nature of the CO_2 response are difficult. There are many intra- and interspecific differences depending on growth form, stage in development and photosynthetic pathway [13, 29].

Short-term exposure (minutes to hours) of C_3 plants to elevated CO_2 typically causes an increase in the rate of net photosynthesis [23]. In contrast, several studies have shown that long-term exposure (days to weeks) can result in a subsequent decline in net carbon assimilation when measured on a leaf area basis [13]. Tobacco grown at 1000 $\mu l l^{-1}$ for a period of weeks showed a 20% decline in the rate of net photosynthesis [24]. A decrease in net photosynthesis and a reduction in the light level required to saturate photosynthesis were also observed in *Desmodium paniculatum* when grown under CO_2 enrichment [33].

CO_2 enrichment frequently results in partial stomatal closure and consequently, an increase in stomatal resistance to gas exchange [25, 26]. However, CO_2-induced stomatal closure alone is insufficient to explain the decrease in photosynthesis observed in most plants grown at elevated levels of CO_2. Madsen [15, 16] observed that CO_2 enrichment caused a high level of starch accumulation in tomato leaves, and it has been suggested that starch accumulation results in feedback inhibition of photosynthesis at the enzyme level [9, 20].

Many plants, such as cotton, tomato, clover, *Desmodium* and soybean, show visible chlorosis and subtle changes in leaf texture when grown at elevated levels of CO_2 (personal observations). These effects have been interpreted as symptomatic of excessive starch accumulation. In this investigation the effects of CO_2 enrichment on net photosynthesis and starch metabolism in cotton were examined in order to determine the contribution of stomatal and non-stomatal factors to the decline in net photosynthesis following long-term exposure to elevated CO_2.

Material and methods

Cotton (*Gossypium hirsutum* L. cv. Stoneville 213) seeds were sown in 1 liter plastic pots in a mixture of equal parts of gravel and vermiculite. The pots were watered to saturation each morning with a modified 1/2 strength Hoagland's solution [5], and each afternoon with deionized water.

Plants were grown from seed at three CO_2 concentrations: 350, 675 or $1000\,\mu l\,l^{-1}$, in growth chambers in the Duke University Phytotron [14]. CO_2 was injected automatically and continuously monitored [10]. Ethylene contamination of CO_2 supplies has been reported and suggested as a source of error in CO_2 treatments [19]. The CO_2 used in this study was produced by a process that does not produce ethylene, and measurement of the atmosphere in the chambers showed no abnormal ethylene concentrations. Plants were grown under a 12 hour photo- and thermal-period. The day/night temperature and photosynthetic photon flux density were 26/20 °C and $600\,\mu mol\,s^{-1}\,m^{-2}$ respectively. Relative humidity was maintained at 70% during the day. To minimize 'chamber effects', all pots were rotated within each chamber daily and between chambers weekly.

After four weeks, 15 plants from each CO_2 treatment were selected at random for biomass and leaf area determination. Leaves, stems and roots were separated and dried to constant weight at 80 °C. Prior to drying, the total leaf area per plant was measured with a LiCor 3100 leaf area meter.

Net photosynthesis was measured on five representative plants from each treatment with an Anarad AR-500R infrared gas analyzer in an open system. Photosynthesis was measured for each plant on the most recently expanded leaf at 350, 675 and $1000\,\mu l\,l^{-1}$ CO_2 on three consecutive days. All photosynthetic measurements were made under saturating irradiance, at 26 °C and

with a 1 kPa vapor pressure deficit. Stomatal conductance and internal CO_2 concentration were calculated based on transpiration measurements. Photosynthetic rate, conductance and internal CO_2 concentration were calculated using the equations of Sestak [27] and Nobel [22].

Samples for starch analysis were taken with a hole punch from four plants in each treatment. Four punches (0.34 cm^2/punch) of mesophyll tissue were taken from the leaf at each sampling time: 6:00 AM (lights-on), 9:00 AM, 2:00 PM, 6:00 PM (lights-off) and at 6:00 AM the following morning. The tissue was killed in a microwave oven for 15 seconds, oven dried for 24 hours at 80 °C and stored dessicated. The dried samples were homogenized in a 12:5:3 (v/v) mixture of methanol, chloroform and water (MCW). The starch fraction was separated from the soluble sugars and pigments by four sequential centrifugations and resuspensions in MCW [8]. The starch was digested to glucose for 30 min at 55 °C with amyloglucosidase from *Asperillus oryzae* (Sigma A-9268, grade V, dialyzed and diluted to 10 units ml^{-1} in 50 mM Na-acetate buffer, pH 4.5). Glucose concentrations were determined using a glucose oxidase-iodide assay [6].

Three punches from each of 10 plants from each treatment were taken at 9:00 AM for chlorophyll (Chl) determination. Chl was extracted in dimethyl sulphoxide (DMSO)[11] and the optical density of the extracts were measured at 645 and 663 nm against a DMSO blank. Chl content was calculated following the equation used by Arnon [1].

Mean values were compared by a single factor analysis of variance and the HSD multiple range test.

Results

CO_2 enrichment caused a significant increase in leaf area and total biomass, and a decline in Chl concentration. The largest increase was in leaf biomass (Figure 1). Plants grown at 675 and 1000 $\mu l l^{-1}$ CO_2 produced 1.72 and 2.15 times more dry weight than the 350 $\mu l l^{-1}$ controls. CO_2 enrichment caused an increase in leaf area, though not as great as the increase in leaf biomass. The differential increase in leaf area and biomass resulted in a greater than two-fold rise in specific leaf weight at the higher CO_2 levels (Table 1). Chl content, measured on a dry weight or leaf area basis, and the Chl a/b ratio were also affected by CO_2 enrichment (Table 1). The decline in Chl concentration in the 675 and 1000 $\mu l l^{-1}$ CO_2 plants when measured on a dry weight basis was largely a result of the increase in specific leaf weight. However, a decrease in Chl content in high CO_2 grown plants was also apparent when measured on a surface area basis.

Growth under elevated CO_2 concentrations also produced changes in photosynthetic capacity. Short-term exposure to 1000 $\mu l l^{-1}$ CO_2 resulted in an increase in the rate of net photosynthesis in plants from 350 and 675 $\mu l l^{-1}$ CO_2 (Figure 2A). No pattern of photosynthetic acclimation to

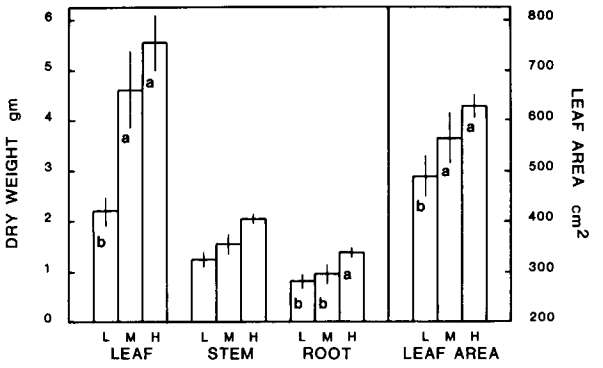

Figure 1. The effect of growth at 350 (L), 675 (M) or 1000 $\mu l l^{-1}$ (H) CO_2 on biomass production, biomass partitioning and leaf area production in cotton. The error bars are 2 × the standard error of the mean (S.E.M.). Bars designated by the same letter (a or b) are not different at the 0.05 level of significance. Stem weights were all significantly different at $p \leq 0.05$.

Table 1. Chlorophyll content and specific leaf weight in cotton grown at 350, 675 or 1000 $\mu l l^{-1}$ CO_2. Each value is the mean of 10 plants ± standard deviation. Values designated by the same letter (a or b) are not different at the 0.05 level of significance

	CO_2 Treatment $\mu l l^{-1}$		
	350	675	1000
Total chlorophyll (mg gm^{-1})	19.85 ± 2.28[a]	8.31 ± 2.12[b]	7.67 ± 1.40[b]
Total chlorophyll (mg cm^{-2})	5.20 ± 0.35[a]	4.68 ± 0.41[b]	4.87 ± 0.25[ab]
Chlorophyll a/b	1.94 ± 0.11[a]	1.76 ± 0.13[b]	1.82 ± 0.10[ab]
Specific leaf weight (mg cm^{-2})	2.64 ± 0.33[a]	6.04 ± 1.69[b]	6.51 ± 0.96[b]

the pretreatment CO_2 concentration was evident. The 350 $\mu l l^{-1}$ plants consistently had higher rates of photosynthesis than the 675 or 1000 $\mu l l^{-1}$ CO_2 plants when compared at any given measurement CO_2 concentration. A slight increase and then decline was observed in photosynthetic rates when 350 $\mu l l^{-1}$ grown plants measured at 350 $\mu l l^{-1}$ CO_2 were compared to 675 and 1000 $\mu l l^{-1}$ CO_2 grown plants measured at their respective growth CO_2 concentration.

The pattern of stomatal conductance in response to short-term exposure to elevated CO_2 was variable. Conductance in the 350 $\mu l l^{-1}$ CO_2 plants increased when measured at 675 $\mu l l^{-1}$ and then decreased to almost the 350 $\mu l l^{-1}$ level when measured at 1000 $\mu l l^{-1}$ CO_2 (Figure 2B). Conductance in the 675 $\mu l l^{-1}$ CO_2 plants increased slightly and the 1000 $\mu l l^{-1}$ grown plants showed a gradual decline with increasing CO_2 concentration. The percent reduction in maximum potential photosynthetic rate (assuming stomatal resistance = 0) between and within each CO_2 treatment was estimated using the Farquhar and Sharkey analysis [7]. No consistent differences were

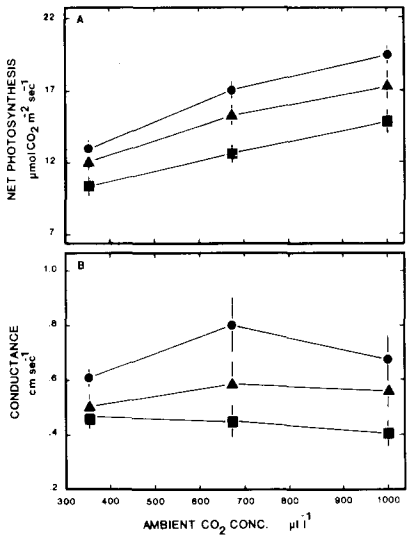

Figure 2. Net photosynthesis (A) and stomatal conductance (B) measured at 3 CO_2 concentrations in cotton grown at 350 (circles), 675 (triangles) or 1000 $\mu l l^{-1}$ (squares) CO_2. The error bars are 2 X S.E.M.

evident between CO_2 treatments (Table 2A). However, within a treatment an increase in the measurement CO_2 concentration resulted in a decrease in the percent stomatal limitation of net photosynthesis.

Non-stomatal limitations to photosynthesis in the high CO_2 grown plants became apparent when photosynthesis was plotted against internal CO_2 concentration (Ci). Internal CO_2 concentration ranged from 80 to 130 $\mu l l^{-1}$ below ambient for all CO_2 treatment and measurement CO_2 combinations. Photosynthetic rates increased with increasing Ci in all of the CO_2 treatments (Figure 3). However, at any given Ci the photosynthetic rate of 675 and 1000 $\mu l l^{-1}$ plants was substantially lower than those grown at 350 $\mu l l^{-1}$. CO_2 enrichment caused an 8.4 to 23.7% reduction in the maximum potential photosynthetic rate independently of CO_2 induced effects on stomatal conductance (Table 2B). The non-stomatal limitations to photosynthesis in the 675 $\mu l l^{-1}$ grown plants increased from 8.4% when measured at 350 $\mu l l^{-1}$ to 10.9% when measured at 1000 $\mu l l^{-1}$ CO_2. A 21 to 23.7% decrease in the potential photosynthetic rate was observed for the 1000 $\mu l l^{-1}$ CO_2 grown plants over the same range of measurement CO_2 concentrations.

CO_2 treatment had a profound effect on the diurnal pattern of leaf starch accumulation. In the 350 $\mu l l^{-1}$ plants, starch concentration increased gradually throughout the light period and declined to the previous morning's level by the end of the dark period (Figure 4). The rate of increase and maximum starch concentration during the light period was considerably greater in the 675 and 1000 $\mu l l^{-1}$ plants. Moreover, degradation and/or translocation of carbohydrates was insufficient in the high CO_2 grown plants to reduce

Table 2. The percent reduction of net photosynthesis caused by stomatal (A) and non-stomatal (B) factors in cotton grown at three CO_2 concentrations. The values were calculated from Figure 3

A. Reduction of potential net photosynthesis by stomatal resistance for a given CO_2 pretreatment and instantaneous CO_2 concentration ($\mu l\,l^{-1}$)

Growth concentration	Measurement concentration 350	675	1000
350	8.9%	5.3%	3.7%
675	6.4%	3.6%	3.8%
1000	7.3%	5.1%	4.5%

B. Reduction in potential net photosynthesis caused by non-stomatal factors (calculations assume stomatal conductance = infinity). Values are reported as a percent of the photosynthetic rate in the 350 $\mu l\,l^{-1}$ CO_2 grown plants

Growth concentration	Measurement concentration 350	675	1000
675	8.4%	10.3%	10.9%
1000	21.0%	22.8%	23.7%

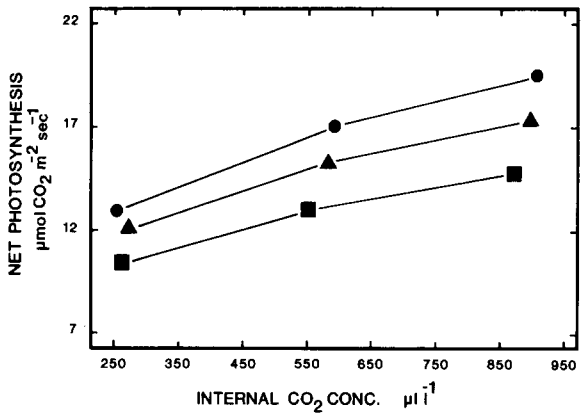

Figure 3. Net photosynthesis vs. internal CO_2 concentration (Ci) in cotton grown at 350 (circles), 675 (triangles) or 1000 $\mu l\,l^{-1}$ CO_2 (squares).

the starch pool to the previous morning's level by the end of the dark period (Figure 4).

Discussion

In contrast to the short-term response, long-term exposure of cotton to elevated CO_2 caused a decline in net photosynthesis at each measurement CO_2 concentration. Mauney *et al.* [18] reported a similar finding for cotton and three other species. Growth under elevated CO_2 also caused a reduction in stomatal conductance. This reduction, however, was not sufficiently large

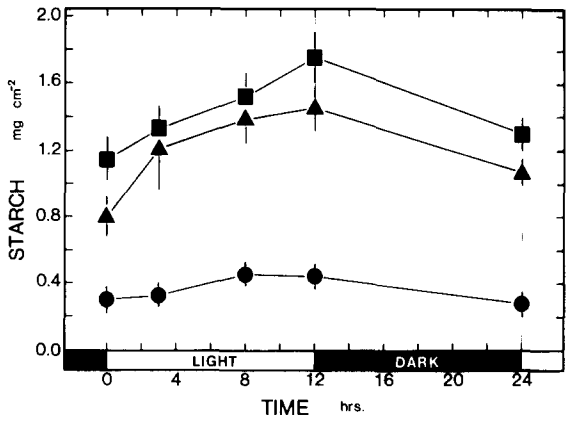

Figure 4. The diurnal pattern of starch accumulation for 350 (circles), 675 (traingles) or 1000 $\mu l l^{-1}$ (squares) CO_2 grown plants. The light and dark periods are indicated by the horizontal bar. The error bars are 2 × S.E.M.

to account for the observed decline in photosynthesis. The relationship between internal CO_2 concentration (Ci) and photosynthetic rate in the high CO_2 grown plants indicated a strong non-stomatal limitation to photosynthesis. The maximum potential photosynthetic rates in the 1000 $\mu l l^{-1}$ plants were reduced by more than 20% (Table 2B).

Despite similar photosynthetic rates, the high CO_2 grown plants accumulated greater biomass than the 350 $\mu l l^{-1}$ CO_2 grown controls. Part of this increase is attributable to starch accumulation. However, the development of greater total leaf area in the 1000 and 675 $\mu l l^{-1}$ CO_2 plants than in the 350 $\mu l l^{-1}$ plants is probably the predominant factor since photosynthesis on a per plant basis is increased. Mauney *et al.* [17] found that most of the increase in leaf area in cotton grown under CO_2 enrichment occurred in the first 20 days following emergence. Higher net assimilation rates were also measured in juvenile plants grown under CO_2 enrichment but steadily declined as the plants matured [17].

CO_2 enrichment also produced a large increase in leaf starch concentration and a disruption of equilibrium in the starch pool size on a diurnal basis. Degradation and mobilization of starch in the 350 $\mu l l^{-1}$ plants at the end of the dark period maintained the starch pool in a near equilibrium state (Figure 4). CO_2 enrichment produced a step-function in leaf starch accumulation as starch concentration in the high CO_2 plants did not return to the previous morning's level by the end of the dark period. Although we did not measure starch on successive days, the disequilibrium in pool size would presumably lead to a daily increase in leaf starch concentration in the high CO_2 plants until some equilibrium level was reached. An increase in leaf starch in response to elevated CO_2 has also been reported for tomato and several other species. However, CO_2 enrichment has been

found to have little or no effect on the concentration of soluble sugars [15, 18, 20].

A strong negative correlation between leaf starch concentration and photosynthesis was observed for cotton [18] and wheat [2], and it has been suggested that starch can limit the rate of photosynthesis by feedback inhibition [9, 12]. The mechanism of inhibition is not well understood. Azcon-Bieto [2] found the decline in photosynthesis and increase in starch concentration during the day in wheat was accompanied by a decrease in quantum yield of CO_2 fixation. A reduction in quantum yield indicates inhibition at the level of production and/or consumption of NADPH and ATP. Based on the interpretation of photosynthesis versus Ci curves [31], it was concluded that CO_2 enrichment inhibited carbon assimilation in bean plants by reducing carboxylase activity, regeneration of RUBP and the rate of photosynthetic electron transport [32]. Chang [4] found that CO_2 enrichment up to $850\,\mu l\,l^{-1}$ caused an increase in carbonic anhydrase activity and consequently, an increase in bicarbonate concentration *in vivo*. High bicarbonate concentration directly inhibited the rate of photophosphorylation and Hill reaction in isolated chloroplasts. It has also been suggested that photosynthesis can be limited at high Ci because of reduction in the inorganic phosphate (Pi) concentration in the chloroplasts [9, 28]. Pi concentration declines as it becomes sequestered into triose phosphates. There are many other possible mechanisms of feedback inhibition on photosynthesis including biochemical regulation of key anabolic and catabolic enzymes. It is not unreasonable to expect that several different mechanisms can operate simultaneously.

A possibility which has not been adequately explored is the potential for starch-induced physical damage at the subcellular level. Electron micrographs of chloroplasts from *Trifolium* leaves grown at $1000\,\mu l\,l^{-1}$ CO_2 showed the development of very large aberrant starch granules [3]. Large starch granules and reduced grana formation were also observed in *Desmodium* [33] and cotton (data not shown). The potential for damage at the thylakoid or photosynthetic unit level is further supported by the reduction in Chl content on a leaf area basis, and a reduction in the Chl a/b ratio (Table 1; [references 3, 33]). However, direct support for this hypothesis has not been demonstrated.

In conclusion, long-term CO_2 enrichment up to $1000\,\mu l\,l^{-1}$ produced a high level of starch accumulation and a decline in potential net photosynthesis in cotton plants. Leaf chlorosis, brittleness and leaf curling appear to be physical manifestations of excessive starch accumulation. The decline in photosynthesis in the high CO_2 plants was a result of non-stomatal limitations that contributed to higher internal resistances. It is suggested that carbohydrate induced feedback inhibition and possibly physical damage at the chloroplast level are responsible for limiting photosynthesis in the high CO_2 grown plants.

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References

1. Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenol-oxidase in *Beta vulgaris*. Plant Physiol 24:1–15
2. Azcon-Bieto J (1983) Inhibition of photosynthesis by carbohydrates in wheat leaves. Plant Physiol 73:681–686
3. Cave G, Tolley LC and Strain BR (1981) Effect of carbon dioxide enrichment on chlorophyll content, starch content and starch grain structure in *Trifolium subterraneum* leaves. Physiol Plant 51:171–174
4. Chang CW (1975) Carbon dioxide and senescence in cotton plants. Plant Physiol 55:515–519
5. Downs RJ and Hellmers H (1978) Controlled climate and plant research. World Meteorological Organization, Technical Note #148. Geneva
6. Ebell LF (1969) Specific total starch determinations in conifer tissues with glucose oxidase. Phytochemistry 8:25–36
7. Farquhar GD and Sharkey TD (1982) Stomatal conductance and photosynthesis. Ann Rev Plant Physiol 33:317–45
8. Haissig BE and Dickson RE (1979) Starch measurement in plant tissue using enzymatic hydrolysis. Physiol Plant 47:151–157
9. Herold A (1980) Regulation of photosynthesis by sink activity – the missing link. New Phytol. 86:131–144
10. Hellmers H and Giles LJ (1979) Carbon dioxide: critic I. *In* Tibbitts TW and Kozlowski TT (eds) Controlled Environment Guidelines for Plant Research. Academic Press, New York, pp. 229–234
11. Hiscox JD and Israelstam GF (1979) A method for the extraction of chlorophyll from leaf tissue without maceration. Can J Bot 57:1332–1334
12. Kimball BA (1983) Carbon Dioxide and Agricultural Yield: An Assemblage and Analysis of 770 Prior Observations. WCL Report. US Water Conservation Laboratory, Phoenix, Arizona
13. Kramer PJ (1981) Carbon dioxide concentration, photosynthesis and dry matter production. BioScience 31:29–33
14. Kramer PJ, Hellmers H and Downs RJ (1970) SEPEL: New phytotrons for environmental research. BioScience 20:1201–1208
15. Madsen E (1968) Effect of CO_2-concentration on accumulation of starch and sugar in tomato leaves. Physiol Plant 21:168–175
16. Madsen E (1975) Effect of CO_2-enrichment on growth, development, fruit production and fruit quality in tomato from a physiological point of view. *In* deBilderling N and Chouard P (eds) Phytotrons and Horticultural Research. Gauthier-Villars, Paris
17. Mauney JR, Fry KE and Guinn G (1978) Relationship of photosynthetic rate to growth and fruiting of cotton, soybean, sorghum and sunflower. Crop Sci 18: 259–263
18. Mauney JR, Guinn G, Fry KE and Hesketh JD (1979) Correlation of photosynthetic carbon dioxide uptake and carbohydrate accumulation in cotton, soybean, sunflower and sorghum. Photosynthetica 13:260–266
19. Morison JIL and Gifford RM (1984) Ethylene contamination of CO_2 cylinders. Plant Physiol 75:275–277
20. Nafziger ED and Koller HR (1976) Influence of leaf starch concentration on CO_2 assimilation in soybean. Plant Physiol 57:560–563

21. Neales TF and Incoll ID (1968) The control of leaf photosynthesis rate by the level of assimilate concentration in the leaf: a review of the hypothesis. Bot Rev 34: 107–125
22. Nobel PS (1983) Biophysical Plant Physiology and Ecology. W.H. Freeman and Co., NY 608 p
23. Pearcy RO and Bjorkman O (1983) Physiological effects. *In* Lemon ER (ed) CO_2 and Plants: The Response of Plants to Rising Levels of Atmospheric Carbon Dioxide. AAAS Selected Symposium #84. Westview Press, Inc. Boulder, 280 p
24. Raper DC and Peedin GF (1978) Photosynthetic rate during steady-state growth as influenced by carbon dioxide concentration. Bot Gaz 139:147–149
25. Raschke K (1975) Stomatal action. Ann Rev Plant Physiol 26:309–340
26. Raschke K (1979) Movements of stomata. Encyclopedia of Plant Physiology 7: 383–441
27. Sestak Z, Catsky J and Jarvis PG (eds)(1971) Plant Photosynthetic Production: A Manual of Methods. Dr W. Junk Publishers. The Hague, 818 p
28. Sharkey TD and Badger MR (1984) Factors limiting photosynthesis as determined from gas exchange characteristics and metabolite pool sizes. *In* Sybesma C (ed) Advances in Photosynthesis Research. Vol. 7. Martinus Nijhoff/Dr W. Junk Publishers, The Hague/Boston/Lancaster, pp. 325–328.
29. Strain BR and Bazzaz FA (1983) Terrestrial plant communities. *In* Lemon ER (ed) CO_2 and Plants: The Response of Plants to Rising Levels of Atmospheric Carbon Dioxide. AAAS Selected Symposium #84. Westview Press, Inc. Boulder, 280 p
30. Thomas JF, Raper CD Jr, Anderson CE and Downs RJ (1975) Growth of young tobacco plants as affected by carbon dioxide and nutrient variables. Agron J 67: 685–689
31. von Caemmerer S and Farquhar GD (1981) Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. Planta 153:376–387
32. von Caemmerer S and Farquhar GD (1984) Effects of partial defoliation, changes in irradiance during growth, short-term water stress and growth at enhanced p(CO_2) on photosynthetic capacity of leaves of *Phaseolus vulgaris*. Planta 160:320–329
33. Wulff RD and Strain BR (1981) Effects of CO_2 enrichment on growth and photosynthesis in *Desmodium paniculatum*. Can J Bot 60:1084–1091