Growth Kinetics, Carbohydrate, and Leaf Phosphate Content of Clover (Trifolium subterraneum L.) after Transfer to a High CO₂ Atmosphere or to High Light and Ambient Air¹

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ABSTRACT

Intact air-grown (photosynthetic photon flux density, 400 microeinsteins per square meter per second) clover plants (Trifolium subterraneum L.) were transferred to high CO₂ (4000 microliters CO₂ per liter; photosynthetic photon flux density, 400 microeinsteins per square meter per second) or to high light (340 microliters CO₂ per liter; photosynthetic photon flux density, 800 microeinsteins per square meter per second) to similarly stimulate photosynthetic net CO₂ uptake. The daily increment of net CO₂ uptake declined transiently in high CO₂ but not in high light, below the values in air/standard light. After about 3 days in high CO₂, the daily increment of net CO₂ uptake increased but did not reach the high light values. Nightly CO₂ release increased immediately in high light, whereas there was a 3-day lag phase in high CO₂. During this time, starch accumulated to a high level, and leaf deterioration was observed only in high CO₂. After 12 days, starch was two- to threefold higher in high CO₂ than in high light, whereas sucrose was similar. Leaf carbohydrates were determined during the first and fourth day in high CO₂. Starch increased rapidly throughout the day. Early in the day, sucrose was low and similar in high CO₂ and ambient light (same light). Later, sucrose increased considerably in high CO₂. The findings that (a) much more photosynthetic carbon was partitioned into the leaf starch pool in high CO₂ than in high light, although net CO₂ uptake was similar, and that (b) rapid starch formation occurred in high CO₂ even when leaf sucrose was only slightly elevated suggest that low sink capacity was not the main constraint in high CO₂. It is proposed that carbon partitioning between starch (chloroplast) and sucrose (cytosol) was perturbed by high CO₂ because of the lack of photospiration. Total phosphate pools were determined in leaves. Concentrations based on fresh weight of orthophosphate, soluble esterified phosphate, and total phosphate markedly declined during 13 days of exposure of the plants to high CO₂ but changed little in high light/ambient air. During this time, the ratio of orthophosphate to soluble esterified phosphate decreased considerably in high CO₂ and increased slightly in high light/ambient air. It appears that phosphate uptake and growth were similarly stimulated by high light, whereas the coordination was weak in high CO₂.

Plants have the competence to acclimate, within species-dependent limits, to changes in irradiance. It is not clear, however, how and to which extent plants acclimate to variations in the atmospheric CO₂ concentration. Short-term responses (minutes or hours) of photosynthetic CO₂ uptake to atmospheric CO₂ enrichment are strongly positive with perhaps all C₃ plants. If one considers long-term effects of CO₂ enrichment (days or entire vegetation periods), it appears that the enhancements by CO₂ enrichment in growth and yield can vary considerably among species (6).¹ Can limit photosynthesis in isolated chloroplasts and intact plants (9, 10, 31), and in potato (13), pine seedlings (5), and clover (3), soil with a low P content negatively affects growth in high CO₂. This suggests that there is a relation between atmospheric CO₂ enrichment and the P status of the plant.

Large amounts of starch accumulate in leaves from C₃ plants exposed to high levels of CO₂ (4, 7, 8, 32). Rapid formation of leaf starch also occurs in air when photosynthesis is limited by sink demand (9, 23, 26) or when the leaf P status is low (10, 30). When sink demand is limiting, the rate of Pi recycling from Pe by sucrose formation declines because of feedback regulation (9, 31), and the rate of assimilate utilization determines the rate of photosynthetic CO₂ fixation (27, 29). In these conditions, Pe accumulates and the chloroplastic Pi concentration is low, which favors starch formation (22, 28). By contrast, P deficiency induced by a low level of P nutrition results in low concentrations of both Pi and Pe, which do not allow high rates of photosynthetic CO₂ fixation (10, 25). It is reasonable to propose based on the high level of starch formation in high CO₂ that the sink capacity of the plant would be too low to use the additionally fixed carbon. If this were so, stimulating net CO₂ uptake by high CO₂ or high light in ambient air should have the same effect on the carbohydrate status of the leaves and plant growth.

To investigate this, sets of intact clover plants grown in air and moderate light were exposed to high atmospheric CO₂ or high irradiance to provide similar stimulation of net photosynthetic CO₂ uptake in both environmental conditions. Detailed growth kinetics were recorded by gas exchange measurements after the change of the environmental conditions, and leaf carbohydrate concentrations were monitored. Nightly CO₂ release was also determined. Total pools of soluble Pi and Pe, total P, and the concentrations of various carbohydrates were determined in leaves that experienced the

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² Abbreviations: P, phosphate(s); Pe, esterified acid-soluble P; DICU, daily increment of the daily rate of net CO₂-uptake.
change in the environmental conditions and in other leaves that had expanded and matured in high CO₂ or high light and ambient air. The results are discussed with respect to the limitations for plant growth in a highly CO₂-enriched atmosphere.

**MATERIALS AND METHODS**

**Plants**

Clover seeds (*Trifolium subterraneum* L.) were sown in sand and germinated in the greenhouse. The seedlings were replanted in pots with perlite as the substrate and were then transferred to environmental chambers. The pots were covered with punctured black plastic foil to reduce algae growth. Plants were spaced widely to facilitate free expansion of individual leaf canopies. Irradiation was 400 μE m⁻² s⁻¹ and light/dark periods and temperatures were 14/10 h and 24/20°C, respectively. Irrigation was automatic with half-strength Hoagland solution (14) containing 0.5 mM NH₄H₂PO₄, 2 mM Ca(NO₃)₂, 3.2 mM KNO₃, and 1 mM MgSO₄ (pH 5.6). Trace elements were full strength.

**Gas Exchange**

Three-week-old plants of the same size were selected and transferred to two identical chambers (720-L volume). Net CO₂ uptake was measured in an atmosphere similar to ambient air (340 μL CO₂ L⁻¹, 20% O₂; 80% N₂, v/v; PPFD 400 μE m⁻² s⁻¹) for 12 d. RH was 75%. Water condensed at the cooling unit was weighed to estimate transpiration. Temperature and nutrient conditions were as before. Then, the CO₂ concentration was increased to 4000 μL CO₂ L⁻¹ in one chamber, and irradiance was increased to 800 μE m⁻² s⁻¹ in the other. Net CO₂ uptake was determined for 12 d (only 10 d are shown in Figs. 1 and 2). No plant material was removed during these experiments. The environmental chambers were connected to closed gas circuits (for detailed technical description, see ref. 1), and net CO₂ uptake, or net CO₂ release, was determined from the frequency of calibrated CO₂ injections (mixture of 20% CO₂ and 80% N₂, v/v) or from the duration of CO₂ trapping, respectively, required to maintain the desired CO₂ concentration. Irrigation was continually adjusted so as to twice exceed transpiration in high light/air. Regulation of environmental parameters and data acquisition were done by a computer. To detect possible leaks, krypton was injected into the chambers. The loss of krypton into the atmosphere was continually measured by MS. Less than 1% (v/v) of the injected krypton escaped during 24 h, and a correction was made for net CO₂ exchange. Volatile organic compounds such as ethylene in the closed circuit were removed by a trap consisting of KMnO₄-soaked perlite.

**Biochemical Analysis**

Leaves were sampled from two plants (for carbohydrates) or one plant (for P) after 5 h of illumination and at time intervals as indicated in the text. Because the total pool sizes of Pi vary considerably from one clover leaf to the other, all leaves from one plant were harvested at each time, and each individual leaf or leaflet was analyzed separately to improve the statistical significance of the results.

The individual leaves were weighed and frozen in liquid nitrogen. Two leaflets of the trifoliolate leaves were frozen separately for the subsequent P determinations. About 20 s elapsed between excision of the leaves and freezing in all experiments. The frozen leaves were ground in a mortar for P determinations or freeze-dried and then ground for carbohydrate determination. The third leaflet was used to determine fresh weight, dry weight (determined after 48 h at 60°C), and leaf area.

**Carbohydrates**

The extraction and determination of starch was done essentially as described in ref. 26. Leaf powder (10 mg) was resuspended in hot 80% (v/v) ethanol and centrifuged (5000g, 5 min) to remove pigments. The pellet was resuspended in 1.0 mL of 0.2 N KOH and heated in boiling water for 30 min. Thereafter, the pH was adjusted to 5.5 with acetic acid. To 200-μL aliquots, 250 nkat (15 units) of amyloglucosidase in 1 mL of 50 mM sodium acetate buffer, pH 4.5, was added. After the samples were heated to 55°C for 1 h followed by 1 min in boiling water, they were centrifuged at 12,000g for 3 min. Glucose formed from starch was determined enzymically (17). Sucrose was determined by a method adopted from ref. 17. Leaf powder (10 mg) was resuspended in 1.0 mL of 0.1 N NaOH at 0°C for 15 min. After centrifugation at 12,000g for 3 min, the supernatant was neutralized with HCl. Aliquots were analyzed for hexoses. Then, 230 nkat (13.8 units) invertase was added to 100 μL of supernatant. After 15 min of incubation at 37°C, the samples were assayed for hexoses. Sucrose contents were calculated from the invertase effect on hexose content.

**P**

The frozen powder from one leaflet was resuspended in 1 mL of 10% (w/v) TCA and incubated for 30 min in the cold. An aliquot from the supernatant obtained by centrifugation at 12,000g for 2 min was used to determine Pi by a colorimetric procedure (16). To determine total (acid) soluble P (Pi + Pe), other aliquots were combusted at 580°C (3.5 h) to convert Pe to Pi. The ash was extracted as described in ref. 16, except that 10% (v/v) H₂SO₄ was used. These extracts were assayed for Pi as described above, and Pe was calculated from the difference in the Pi content with and without combustion. To determine total P (soluble and insoluble P), another leaflet was combusted to convert all P to Pi, and the residue was extracted and further treated as described above.

**Statistics**

Calculations of SE and Student’s *t* tests were done using the computer program “Voyons” written by Jean Thiery (CEA, Centre de Cadarache, St. Paul les Durance, France). The term “significance” is used in its statistical sense in this paper.
RESULTS

Net CO₂ Uptake

Whole clover plants were grown in an atmosphere similar to ambient air at 400 μE m⁻² s⁻¹ for 12 d before the ambient CO₂ concentration or irradiation was increased to 4000 μL CO₂ L⁻¹ or 800 μE m⁻² s⁻¹, respectively. To analyze the growth response of clover after transfer to the new environmental conditions, daily net CO₂ uptake and the DICU were calculated. Net photosynthetic CO₂ uptake during the first day (day 0 in Fig. 1) in high CO₂ or in high light increased by a factor of 1.54 or 1.37, respectively. In high light, CO₂ uptake continued to be stimulated, i.e., DICU was high (Fig. 2), during another 2 d. Thereafter, DICU was the same as before the change of the light regimen during about 3 d (3.0 mmol CO₂ uptake d⁻¹, see Fig. 2) and then increased to a value of 5.0 mmol CO₂ uptake d⁻¹ (average value during 5 d; Fig. 2). In high CO₂, by contrast, DICU was below the control value (ambient air) during 3 consecutive d (Fig. 2) and then increased after day 4 to a value of 3.6 mmol CO₂ uptake d⁻¹ (average value during 5 d; Fig. 2). A decline of DICU was also observed in another four experiments with intact clover plants after atmospheric CO₂ enrichment to 4000 μL CO₂ L⁻¹ or to 1000 μL CO₂ L⁻¹. The effect was correlated with the level of P nutrition (3, 4; our unpublished results).

Nightly Net CO₂ Release

Net CO₂ release was 18% of daily net CO₂ uptake and was not at all stimulated by atmospheric CO₂ enrichment during the initial 3 or 4 d. This means that the CO₂ uptake to CO₂ release ratio declined in high CO₂. After 3 to 4 d (when DICU recovered), nightly net CO₂ release increased and the CO₂ uptake to CO₂ release ratio approached the value determined in ambient air (control). By contrast, nightly net CO₂ release and daily net CO₂ uptake were similarly stimulated by high light, i.e., the CO₂ uptake to CO₂ release ratio remained constant. These results demonstrate that the similar stimulation of photosynthetic net CO₂ uptake provided by either high CO₂ or high light affected differently the respiration rate (net CO₂ release) in the night.

Leaf Carbohydrate Content

After 12 d of exposure of plants to high CO₂ or high light, young, mature, and old leaves were harvested and analyzed for carbohydrate content. Leaf age was estimated from the position of insertion to the stem and from the morphological appearance. The young and mature leaves had expanded in the respective environmental conditions and were thus presumably acclimated. In high CO₂, many old leaves had yellowed and looked unhealthy. Figure 3 shows that, independ-
ently of leaf age, the concentrations of soluble carbohydrates based on dry weight were similar in high CO₂ and in high light. In contrast, starch concentrations were substantially elevated in high CO₂, particularly in old leaves. The difference in the starch content between high CO₂/standard light and ambient air/high light was least in mature leaves (Fig. 3). These results show that, in high CO₂, a large portion of the additionally fixed carbon was deposited in the leaves as starch, whereas it was exported from the leaves in high light (8, 15).

To further investigate carbon partitioning in high CO₂, kinetics of leaf carbohydrate content were recorded during the first day of CO₂ enrichment and 3 d later, and the results were compared with a control in air at the same irradiance (Fig. 4). Starch content increased more rapidly in high CO₂ than in air throughout the day, and nightly degradation was less than daily formation, leading to a high level of starch accumulation by the fourth day (Fig. 4, A and B). When measured after 5 h of illumination, during the first and fourth days of CO₂ enrichment, leaf sucrose was similar in high CO₂ and in ambient air (Fig. 4, C and D). During the rest of the day, sucrose remained at this level in air, whereas it nearly doubled in high CO₂. Nightly sucrose degradation and export balanced daily sucrose accumulation in ambient air. By contrast, the sucrose level increased to some extent during 3 d in high CO₂ (Fig. 4, C and D).

**Leaf P Content**

Pi plays an important role in the regulation of carbon distribution between starch and sucrose during photosynthesis (10, 31). Therefore, concentrations of Pi, Pe, and total P and Pi/Pe ratios (soluble pools) were determined during the first day and after prolonged exposure of the plants to the high CO₂ atmosphere or to ambient air and high light (Table 1). Pe based on fresh weight initially increased slightly and then decreased by 27% between day 1 and day 13 (average values for all leaves were calculated). Total soluble Pi/fresh weight decreased by 50% and total soluble P (Pi + Pe) decreased by about 40% between day 1 and day 13 in high CO₂ (Table 1). Total P followed the changes of total soluble P. Contrary to the effect of high CO₂, the high light environment did not lead to a significant decline of P pools based on fresh weight.

**Figure 3.** Carbohydrate concentrations expressed on a dry weight basis in young leaves (A), mature leaves (B), and old leaves (C) after 12 d of exposure to high CO₂ or high light. See legend of Figure 1 for experimental details. The leaves (between 10 and 18 leaves per sample) were pooled before the determinations. For variance tests, each leaf was analyzed separately in a previous experiment. SE = 6% for starch and sucrose. Suc, Sucrose; Glc, glucose; Fru, fructose; Dw, dry weight; equiv., equivalents.

**Figure 4.** Diurnal variations in the starch and sucrose content expressed on a dry weight (Dw) basis in clover leaves exposed to high CO₂ (A) or ambient air as a control (Δ). Kinetics were recorded during the first day with CO₂ enrichment and 3 d later. Note that in this experiment irradiance was identical in both environmental conditions (400 μE m⁻² s⁻¹). SE = 6% for starch and sucrose (see Fig. 3). A, Starch content during the first day in high CO₂; B, starch content during the fourth day in high CO₂; C, sucrose content during the first day in high CO₂; D, sucrose content during the fourth day in high CO₂. Glc, Glucose; equiv., equivalents.
Statistically significant differences in the effects of high CO₂ and high light/ambient air on the Pi/Pe ratio were encountered (Table I). The Pi/Pe ratio declined immediately and persistently in high CO₂ but not in high light, and after 13 d of exposure to high CO₂, the Pi/Pe ratio was only about one-third the value determined with high light leaves (Table I). During 13 d of exposure to high light, the Pi/Pe ratio increased slightly.

**DISCUSSION**

The photosynthetic carbon metabolism in leaves is qualitatively the same at different light levels, whereas the proportion of carbon that enters the photorespiratory pathway declines by atmospheric CO₂ enrichment. Because of this fundamental difference, little or no photorespiratory glucose, depending upon the CO₂ concentration, is available for mitochondrial glycine oxidase when photosynthetic CO₂ uptake is stimulated by high CO₂, whereas glycine formation increases when CO₂ fixation increases because of increased light levels. Leaf mitochondria supply much of the ATP for energy-dependent reaction in the cytosol, e.g. sucrose formation (18), and it has been reported that suppression of photorespiration (glycine formation) by high CO₂ can lower the cytosolic ATP concentrations in protoplasts and leaves (4, 12). It is not clear, however, whether the lack of photorespiratory glycine in leaves exposed to high CO₂ slows down sucrose formation. Another differential effect is that atmospheric CO₂ enrichment alters the metabolism on the chloroplast level because, in nonphotorespiratory conditions (very high CO₂ as in our experiments), no Pi is regenerated from phosphoglycolate within the chloroplast (20). This could be compensated for by Pi regeneration during starch formation.

How did the photosynthetic carbon metabolism and growth of clover respond to atmospheric CO₂ enrichment as compared to high light/ambient air? We observed that large amounts of leaf starch accumulated in high CO₂, whereas the starch levels were much less elevated in high light/ambient air. To date, there is no straightforward explanation for the generally high starch levels in leaves in high CO₂-enriched atmospheres (4, 7, 8, 32). There is the notion, however, that the high level of starch accumulation would come from sink limitation of photosynthesis. If this were the case, leaf sucrose should also accumulate. In cotton, however, leaf sucrose content did not increase in high CO₂ (7), whereas it increased to the same extent in soybean (15). Our kinetics of leaf carbohydrate content show that the high CO₂ effect on the sucrose content of clover leaves varies during the course of the day (Fig. 4). Another conclusion that can be drawn from these kinetics is that rapid starch formation in leaves occurs in high CO₂ even when the sucrose concentration is only slightly elevated (Fig. 4). Moreover, in plants having the same net CO₂ uptake rate after photosynthetic stimulation provided by high CO₂ or high light/ambient air, only high CO₂ caused extremely elevated levels of leaf starch accumulation (Fig. 3).

We conclude from the latter finding that the investigated clover plants had enough sink capacity to accommodate the surplus of photosynthetic. It is possible, however, that the level of sink activation is lower in CO₂ for some reason. The finding that the concentration of the sucrose precursor glucose 6-P was higher in high light than in high CO₂ (4) does not support this suggestion. In our view, these results support the proposal that atmospheric CO₂ enrichment perturbs the partitioning of photosynthetically fixed carbon between the starch pool (chloroplast) and sucrose (cytosol), presumably because of the suppression of photorespiration. It is worth noting in this context that both P starvation in ambient air and atmospheric CO₂ enrichment induce high levels of starch accumulation (4, 10, 30), low ATP to ADP and triose P to 3-phosphoglycerate ratios (4, 9, 11), and high levels of nonphotochemical Chl fluorescence quenching (4, 11, 21). Moreover, the ribulose biphosphate concentration was shown to increase and the activation state of Rubisco to decline in both conditions (2, 3, 11; our unpublished results). Nevertheless, no final conclusion can be drawn until in vivo data about stromal Pi concentrations in high CO₂ become available.

DICU represents the daily changes in the plant's growth rate. In spite of the strong initial stimulation of daily net CO₂ uptake by high CO₂, DICU was considerably repressed after atmospheric CO₂ enrichment during several days. The low
DICU values could have been caused by increased nightly carbon loss by respiration. However, the lack of stimulation by high CO$_2$ of nightly dark respiration of the whole plants refutes this proposal, and it appears that much of the additionally fixed carbon was not available for investment in the photosynthetic (growth) capacity because of sequestration to the leaf starch pool (see carbohydrate results). The higher DICU values after extended exposure to high CO$_2$ indicate that, during acclimation, more photosynthetically fixed carbon is available, or used, for investment in the growth capacity of the plant.

Photosynthetic inhibition and leaf yellowing because of oversized starch granules have been observed in air-acclimated leaves from clover (4, 7) and other species (32) that were exposed to high CO$_2$. No leaf yellowing was observed in acclimated clover leaves that were formed in high CO$_2$, but the starch content was nonetheless high (Fig. 3). This shows that the high CO$_2$-induced metabolic perturbation was only alleviated by acclimation to high CO$_2$ on the leaf level.

The small size of the total Pi pool in high CO$_2$ in comparison to high light suggests that high CO$_2$ can reduce the P status of clover leaves (Table 1). It is clear, however, that only a limited amount of information is available from the measurement of the total Pi pool because it takes no account of subcellular pools. Nevertheless, the finding that not only Pi but also Pe and the total P fraction consisting of soluble and insoluble P decline in high CO$_2$ but not in high CO$_2$ supports the suggestion that high CO$_2$ can provoke low P conditions in clover leaves.

It is an open question why the fresh weight-based P content of clover leaves declined in high CO$_2$. There are three possible mechanisms: (a) P was sequestered into the insoluble fraction, e.g. phospholipids. The finding that total P (soluble and insoluble) also declined does not support this suggestion; (b) high CO$_2$-exposed leaves accumulated structural material or starch that “diluted” leaf P. This effect has been reported to occur in Chrysanthemum (19) and bean leaves (24) when P content was expressed on a dry weight basis. In clover, the dry weight to fresh weight ratio of about 7 (control leaves in ambient air and standard light) increased by only 8% in the CO$_2$-enriched atmosphere (dry weight to fresh weight ratio in high light was not determined), and the fresh weight to leaf area ratio increased by 6% in high CO$_2$ and by 19% in high light. These values exclude dilution by starch or cellulose as the main reason for the low fresh weight-based leaf P concentrations in high CO$_2$; (c) reduced transpiration limited P uptake or P translocation. To estimate the capacity of the clover plants for P translocation in the transpiration stream, it was assumed that the P concentration in the xylem sap of clover and barley was similar (0.2 mM Pi, ref. 22), and the capacity for Pi transport was calculated from the transpiration values. Setting the carbon gain equivalent to the gain in dry weight, these calculations showed that, in high CO$_2$, the transpiration stream could have translocated fivefold of the amount of P required to maintain the leaf P concentration at the level of control leaves (ambient air). Thus, it is not probable that the transpiration stream limited the P supply to the leaves in high CO$_2$. Rather, it appears that P uptake and growth of clover plants were similarly stimulated by high light, whereas this coordination was weak in high CO$_2$.

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LITERATURE CITED