Acclimation of Photosynthesis to Elevated CO$_2$ under Low-Nitrogen Nutrition Is Affected by the Capacity for Assimilate Utilization. Perennial Ryegrass under Free-Air CO$_2$ Enrichment$^1$

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Acclimation of photosynthesis to elevated CO$_2$ has previously been shown to be more pronounced when N supply is poor. Is this a direct effect of N or an indirect effect of N by limiting the development of sinks for photoassimilate? This question was tested by growing a perennial ryegrass (Lolium perenne) in the field under elevated (60 Pa) and current (36 Pa) partial pressures of CO$_2$ (pCO$_2$) at low and high levels of N fertilization. Cutting of this herbage crop at 4- to 8-week intervals removed about 80% of the canopy, therefore decreasing the ratio of photosynthetic area to sinks for photoassimilate. Leaf photosynthesis, in vivo carboxylation capacity, carbohydrate, N, ribulose-1,5-bisphosphate carboxylase/oxygenase, sedoheptulose-1,7-bisphosphatase, and chloroplastic fructose-1,6-bisphosphatase levels were determined for mature lamina during two consecutive summers. Just before the cut, when the canopy was relatively large, growth at elevated pCO$_2$ and low N resulted in significant decreases in carboxylation capacity and the amount of ribulose-1,5-bisphosphate carboxylase/oxygenase protein. In high N there were no significant decreases in carboxylation capacity or proteins, but chloroplastic fructose-1,6-bisphosphatase protein levels increased significantly. Elevated pCO$_2$ resulted in a marked and significant increase in leaf carbohydrate content at low N, but had no effect at high N. This acclimation at low N was absent after the harvest, when the canopy size was small. These results suggest that acclimation under low N is caused by limitation of sink development rather than being a direct effect of N supply on photosynthesis.

Acclimation of photosynthesis to growth in elevated pCO$_2$ has frequently been shown to be more marked under suboptimal N supply (Drake et al., 1997). Growth in low N limits the development of the shoot and root, and therefore the capacity for utilization of the additional photoassimilates formed under elevated pCO$_2$. Low N may therefore exacerbate the accumulation of carbohydrate observed under elevated pCO$_2$ (Webber et al., 1994; Drake et al., 1997). Alternatively, nitrate accumulation within the plant can alter gene expression (Paul and Driscoll, 1997; Scheible et al., 1997), and could lead to different patterns of acclimation to elevated pCO$_2$ depending on the N supply. Wheat grown under limiting N supply showed a greater loss of Rubisco in response to elevated pCO$_2$ than plants grown with free access to N (Rogers et al., 1996). This appeared to result from an accumulation of soluble carbohydrates in leaves, resulting in sugar repression of the expression of the genes encoding the LSU and the small subunit of Rubisco (rbc$L$ and rbc$S$, respectively) (Stitt, 1991; Sheen, 1994; Krapp and Stitt, 1995; Koch, 1996).

Most studies of acclimation to elevated pCO$_2$ under different levels of N nutrition have been conducted in containers in the laboratory. However, Arp (1991) demonstrated that such restriction of rooting volume might accentuate acclimation to elevated pCO$_2$. In addition to the physical constraint imposed by container walls, the initially enhanced growth under elevated pCO$_2$ will lead to a more rapid exhaustion of the N within the pot (Pettersson and MacDonald, 1994). In the field there is no restriction on rooting volume, and increased exploration of the soil with accelerated growth under elevated pCO$_2$ would allow the plant to utilize additional sources of N. Enclosures, including open-top chambers, allow the effects of elevated pCO$_2$ to be investigated in the field but impose significant changes in microclimate, which adds other uncertainties as to whether the same effects would be observed in the open air (Lewin et al., 1994). FACE overcomes the limitations imposed by open-top chambers and other field enclosures (Lewin et al., 1994).

Abbreviations: $A$, net rate of CO$_2$ uptake per unit leaf area ($\mu$mol m$^{-2}$ s$^{-1}$); $c$, pCO$_2$ in the substomatal cavity; FACE, free-air CO$_2$ enrichment; FBPase, chloroplastic Fru-1,6-bisphosphatase; LSU, large subunit of Rubisco; MSD, minimum significant difference; pCO$_2$, partial pressure of CO$_2$ in the atmosphere (Pa); RuBP, ribulose-1,5-bisphosphate; SBPase, sedoheptulose-1,7-bisphosphatase; $V_{\text{c,max}}$, maximum RuBP-saturated rate of carboxylation in vivo ($\mu$mol m$^{-2}$ s$^{-1}$); WSC, water-soluble carbohydrate.

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We have taken the unique opportunity provided by the FACE experiment on farmland at Eschikon, Switzerland (Zanetti et al., 1996) to examine in open-field conditions the hypotheses that acclimation to elevated pCO₂ is accentuated in low N, and that this in turn is an indirect effect resulting from N limitation of the development of sinks for photoassimilate. In this FACE experiment the perennial ryegrass *Lolium perenne* L. has been managed as a frequently cut herbage crop at low- and high-N supplies and test the effects of manipulating the source-sink balance on acclimation. This study is the first, to our knowledge, to p

ryegrass photoassimilate. In this FACE experiment the perennial resulting from N limitation of the development of sinks for 

ated in low N, and that this in turn is an indirect effect 

high levels of N application as NH₄NO₃; 140 and 560 kg 

vated design in three blocks, with each block including an ele-

Bastion) were planted according to a split plot, randomized 

conditions.

L. perenne by N supply, then in 

increased acclimation of photosynthesis to elevated 

demand for carbohydrate in shoot regrowth. Therefore, if 

photoassimilates, and in addition will lead to an increased 

the ratio of source, i.e. photosynthetic tissues, to sinks for 

atts provided by a water vapor generator (type WG-600, 

Analytical Development Co., Hoddesden, UK) and with 

CO₂ calibration gas at 60.5 Pa pCO₂ (27548-type 30L, Car-

bagas, Swiss Calibration, Zurich, Switzerland). The derived 
gas-exchange parameters A and cᵢ were calculated accord-


Projected leaf area was estimated by measuring leaf width and then calculating the area from the chamber diameter. Leaf CO₂ uptake in situ was measured for 2 h on either side of solar noon. Within each plot of each pCO₂ × N combination, measurements were made of five leaves starting at 11 AM, and the cycle was repeated three times until 3 PM. Mean rates of CO₂ uptake were therefore based on 45 measurements for each pCO₂ × N treatment.

The response of A to variation in cᵢ was determined for two to four leaves per treatment per ring. A stabilized quartz-iodide light source was clipped over the leaf cham-

ber to provide uniform, near-saturating PPFDs (750 μmol m⁻² s⁻¹). A stabilized 12-V direct current power supply ensured a constant photon flux for up to 8 h. Measurements were taken before 1 PM and/or were limited to overcast days to minimize the possibility of feedback inhibition of photosynthesis due to carbohydrate accumulation and cy-

tosolic Pi limitation. Vc,max and Jmax (light-saturated potential rate of electron transport [μmol m⁻² s⁻¹]) were the key 

variables determining in vivo Rubisco activity and maximum capacity for RuBP regeneration, respectively. 

These were calculated by fitting the equations of Farquhar et al. (1980) and Evans and Farquhar (1991), following the procedure of Wullschlieger (1993). Because temperature varied significantly between measurements, all estimates of Vc,max and Jmax were corrected to 25°C, following the equations of McMurtrie and Wang (1993).

**Carbohydrate Analysis**

WSCs were extracted as described in Fischer et al. (1997). The 1994 samples were analyzed using an anthrone/sulfu-

ric acid method modified from Deriaz (1961) and opti-

mized for the simultaneous determination of Glc and Fru. 

The 1995 samples were analyzed by the similar phenol-

sulfuric acid technique as described by Dubois et al. (1956).

**Protein Isolation, Western Blotting, Immunodetection, and Quantification**

Frozen leaf segments were powdered in liquid N₂ with a 
mortar and pestle. Total protein was extracted with 10% 
(w/v) TCA in aceton with 0.07% (v/v) β-mercaptoethanol 
as described by Damerval et al. (1986), followed by three 
washes in aceton with 0.07% (v/v) β-mercaptoethanol. 
The resulting dried protein pellet was solubilized in 62 mm 
Tris, 2% (w/v) SDS, 65 mm DTT, and 10% (v/v) glycerol, 
 pH 6.8. To avoid interference from this solubilization 
buffer, the protein in a subsample was precipitated with 5% (w/v) TCA, washed in aceton, and resuspended in 
0.1 m NaOH prior to determination of the protein content.
(detergent-compatible microplate protein assay, Bio-Rad). Samples were loaded on an equal-protein basis and resolved on 15% SDS-polyacrylamide gels and electroblotted onto a PVDF membrane (Immobion-P, Millipore; Trans-Blot, Bio-Rad). The western blots were blocked with 30 g L\(^{-1}\) fat-free, dried milk prior to probing with antibodies raised against Rubisco holoenzyme, FBPase, and SBPase, all from wheat. After washing in PBS in the presence of a mild detergent (0.0005% [v/v] Tween 20), blots were probed with the secondary antibody, a sheep anti-rabbit IgG, horseradish peroxidase conjugate (Serotec Ltd., Oxford, UK). Specific proteins were detected with enhanced chemiluminescence immunodetection (Amersham). Quantification of the individual enhanced chemiluminescence signals was performed from two-dimensional densitometric scanning of the film using a computer-controlled laser-scanning densitometer (model 300A, Molecular Dynamics, Sunnyvale, CA). Because of the nonproportional solubilization step in the preparation of protein samples for SDS-PAGE, determination of protein content per unit leaf area required a separate protein assay. A subsample of the lyophilized TCA protein precipitate was dissolved in 0.1 m NaOH prior to determination of protein content (detergent-compatible microplate protein assay, Bio-Rad).

**Leaf N Content**

On completion of gas-exchange measurements, leaf segments were cut and dried to constant weight at 80°C. Each individual leaf sample was ground to a fine powder, and total leaf N was determined by combustion and then thermal conductivity separation in an elemental analyzer (PE 2400 series II CHNS/O Analyzer, Perkin-Elmer). The analyzer was previously calibrated with acetonilide standards (Perkin-Elmer).

**Statistical Analyses**

Differences in \(V_{c,max}\), WSC, and N were examined by analysis of variance (version 5.04, Systat, Inc., Evanston, IL) using \(P = 0.05\) as the level of significance. The data were analyzed as a split-split block design with \(p\)CO\(_2\) and block as the main effects and N and cut as split-block factors (Mead et al., 1993). A post hoc Tukey’s test was used to examine significant pairwise comparisons. In 1995 no data were collected for the high-N-defoliated treatment. These data were therefore analyzed as two separate analysis of variances: first, analyzing \(p\)CO\(_2\) × N in the uncut subplots and second, analyzing \(p\)CO\(_2\) × cutting in the low-N plots. Data were treated as a split-block design, as for the 1994 data. Where significant effects were detected, pairwise comparison of means was by MSD based on Student’s \(t\)-distribution (Mead et al., 1993). For \(p\)CO\(_2\) comparisons of WSC levels and \(V_{c,max}\) critical values of Student’s \(t\)-distribution for a 1-tailed test were used, since our hypothesis predicts an increase in WSC content and a decrease in \(V_{c,max}\). All other comparisons used a two-tailed test, since the direction of change was not hypothesized. Comparisons of the absolute levels of Calvin cycle proteins are only possible within a blot, and not between, because of variation in exposure time, chemiluminescence, and the reaction of an individual protein with its antibody. The resulting ratio of a protein at elevated \(p\)CO\(_2\) to current \(p\)CO\(_2\) was determined within each block. Data were tested for heteroscedasticity, which, when found, was removed by log transformation (Zar, 1984). The means of the blocks, transformed where necessary, were then compared by Student’s \(t\) test.

**RESULTS**

Average midday leaf photosynthetic rates were about 35% higher in leaves growing under elevated \(p\)CO\(_2\), irrespective of N supply or cutting (Fig. 1). A similar increase was observed in 1995 (Hymus, 1995). In 1994 growth at elevated \(p\)CO\(_2\) resulted in a significantly higher WSC content (\(F_{1,2} = 50.7, P < 0.05\); Fig. 2A). There was a strong interaction between \(p\)CO\(_2\) and N (\(F_{1,4} = 39.75, P < 0.05\); at low N the soluble carbohydrate content of lamina grown at elevated \(p\)CO\(_2\) was almost double that of leaves grown at current \(p\)CO\(_2\) (\(P < 0.01\) post hoc Tukey’s test). Conversely, at high N there was no significant difference between the carbohydrate contents in leaves grown at current and elevated \(p\)CO\(_2\) (Fig. 2). Following cutting, carbohydrate concentration showed a highly significant decline (\(F_{1,9} = 299.8, P < 0.01\)), correlating to the large decrease in the source-to-sink ratio. The difference in WSC between elevated and current \(p\)CO\(_2\)-grown leaves before the cut at low N was absent after the cut (Fig. 2A). A similar pattern was observed when the measurements were repeated in 1995; growth at elevated \(p\)CO\(_2\) and low N resulted in a significantly higher WSC content (MSD, \(P < 0.05\); Fig. 2B).

The \(A/c_i\) response of photosynthesis implied acclimation to elevated \(p\)CO\(_2\). Before the cut, leaves grown at high \(p\)CO\(_2\) and low N showed a \(V_{c,max}\) that was 30% below that of controls (\(P < 0.05\), post hoc Tukey’s test; Fig. 3A), implying a marked decrease in the amount of active Rubisco. In contrast, \(V_{c,max}\) in leaves grown in high N was unaffected by \(p\)CO\(_2\) treatment, whereas \(p\)CO\(_2\) treatment

![Figure 1](image)

**Figure 1.** A measurements made for 2 h on either side of solar noon on days with clear skies in June, 1994. Measurements were taken on mature lamina of *L. perenne*. Plants were grown and measured at current (C; 36 Pa) and at elevated (E; 60 Pa) \(p\)CO\(_2\). There were two levels of N application; low N (LN; 140 kg hectare\(^{-1}\)) and high N (HN; 520 kg hectare\(^{-1}\)). Measurements were made before a harvest (UNCUT) and again 7 d after the canopy was partially defoliated (CUT); \(n = 3\) blocks.
had no effect on $V_{c,max}$ in leaves grown in either N treatment after the cut (Fig. 3A). In 1995 (Fig. 3B), the pattern was repeated, although the decrease in $V_{c,max}$ at elevated $pCO_2$ and low N was smaller (MSD, $P < 0.05$).

Leaf N content (Fig. 4) and total leaf-protein content ($F_{1,25} = 0.58, P > 0.05$) were not significantly decreased by growth at elevated $pCO_2$. In 1994, leaf N contents rose significantly both on a unit leaf-area basis ($F_{1,4} = 7.72, P < 0.05$) and a dry-mass basis ($F_{1,4} = 49.17, P < 0.01$) after the cut in both N treatments; this may have reflected the application of the fertilizer immediately after the cut. This was confirmed in 1995, when N-fertilizer application was withheld until completion of the measurements and there was no significant rise in leaf N content following cutting of plants grown at elevated $pCO_2$ ($F_{1,4} = 5.35, P > 0.05$) on total leaf-protein content. Rubisco large subunit showed a significant (Student’s $t$ test, $P < 0.05$) decrease in leaves grown at elevated $pCO_2$ prior to the cut (Figs. 5 and 6A) and corresponding to the significant increase in WSC. This appeared to be a selective effect upon Rubisco content, as there were no significant decreases in the amounts of two other Calvin cycle enzymes, FBPase and SBPase (Fig. 5). In high N before the cut and at high and low N after the cut, there were no significant decreases in Rubisco due to elevated $pCO_2$ (Figs. 5 and 6A and B). However, at high N before the cut there was a significant increase in the FBPase protein levels (Student’s $t$ test, $P < 0.05$) (Fig. 5 and 6A), a pattern repeated in 1995 (Fig. 5 and 6D).

**DISCUSSION**

Our results showed clearly that significant acclimation in *L. perenne* grown in open-field conditions at elevated $pCO_2$ was absent when N supply was high and when plants grown with a low-N supply were partially defoliated to lower the source-to-sink ratio. In addition, our results lend further support to the hypothesis that acclimation of photosynthesis to growth at elevated $pCO_2$ is an indirect effect resulting from N limitation of the development of sinks for photoassimilate.

Although acclimation decreased Rubisco content by about 25% (Figs. 5 and 6) and $V_{c,max}$ by 30% (Fig. 2) in the low-N treatment prior to cutting, the stimulation of leaf photosynthesis by elevated $pCO_2$ was similar to that of controls (Fig. 1). This may be explained by the shift in metabolic control away from Rubisco limitation as $pCO_2$ is increased. For leaves grown at the current ambient $pCO_2$ the $A/c_i$ response showed that the inflection between Rubisco and RuBP limitation occurred at a $pCO_2$ of about 30 Pa, just above the $c_i$ obtained in the current atmosphere (data not shown). This suggests that the amount of Rubisco was just sufficient to support the observed rate of light-saturated photosynthesis in the current atmosphere.
Figure 4. Leaf N content of the plants described in the legend for Figure 2 for 1994 (A and B) and 1995 (C and D) expressed on a dry-mass basis (A and C) and a leaf-area basis (B and D). Treatments and their abbreviations are as in the legend for Figure 1. Letters above bars are as described in the legend for Figure 2. n.d., No data.

Figure 5. Western blots showing levels of LSU, FBPase, and SBPase. Leaves sampled are as described in the legend for Figure 2. For each N and cutting treatment, proteins extracted from each pCO₂ treatment and for each replicate block were separated by SDS-PAGE and blotted. Because of variation in exposure time, chemiluminescence, and the reaction of a given protein with its antibody, comparisons are only possible within a blot. This allows for a comparison between pCO₂ treatments and any N or cutting treatment, but does not allow for a comparison between N and cutting treatments, except when standardized as a proportion of the control, as in Figure 6.
following the calculations of Woodrow (1994) these leaves growing under elevated \( pCO_2 \) at the mean measurement temperature of 27°C (Bryant, 1994; Hymus, 1995) would have about a 40% excess of Rubisco. This would mean that under elevated \( pCO_2 \) these leaves could lose 40% of their Rubisco, yet still maintain a photosynthetic rate at elevated \( pCO_2 \) equal to that of the leaves grown and measured at current \( pCO_2 \). In this study leaves lost about 25% of their Rubisco when grown at elevated \( pCO_2 \) and low N, yet still showed a stimulation of \( A_{max} \) in the field. These lower Rubisco levels may simply reflect a reallocation of resources away from Rubisco, which is in excess under the current growth conditions. In shifting control away from Rubisco, the rate of photosynthesis becomes limited by the regeneration of RuBP (Woodrow, 1994). FBPase and SBPase are considered to be two potential control points for the regeneration of RuBP (Bassham and Krause, 1969; Woodrow and Berry, 1988; Harrison et al., 1998). Neither of these proteins was decreased by growth at elevated \( pCO_2 \), even under low-N supply. Optimum use of resources would require a system that would allow a decrease in Rubisco without loss of capacity for RuBP regeneration (Drake et al., 1997).

Under high N no acclimatory loss of \( V_{max} \) or Rubisco was observed in elevated \( pCO_2 \), but FBPase protein increased by about 30%. Although this might imply an increase in capacity for RuBP regeneration, this was not evident in any significant increase in the \( CO_2 \)-saturated rate of photosynthesis in these leaves at elevated \( pCO_2 \) (data not shown). However, other studies have shown an increase in the maximum \( CO_2 \)-saturated rate of photosynthesis that would result from an increased capacity for regeneration of RuBP (Wong 1979; Sage et al., 1989; Ziska et al., 1991), which in turn implies increased FBPase activity, together with the other activity increases that would be necessary.

Acclimatory loss of Rubisco and carboxylation capacity with growth in elevated \( CO_2 \) have been phenomenologically linked to an increase in carbohydrate content (Webber et al., 1994; Drake et al., 1997). Our results are consistent with the idea that either an increase in carbohydrate content underlies acclimation or that acclimation and an increase in carbohydrate content at elevated \( CO_2 \) share a common cause. The absence of acclimation in plants grown at high N and elevated \( pCO_2 \), and in plants grown at low N and elevated \( pCO_2 \) when the source-to-sink ratio is low, show that acclimation is neither a direct effect of elevated \( pCO_2 \) nor directly modified by N supply. The results do show that under conditions that would exacerbate carbohydrate accumulation in the leaf, i.e. a high source-to-sink ratio and low N limiting the development of additional sink capacity, acclimation occurs. Carbohydrate repression of gene expression has been suggested as a cause of this pattern (Sheen, 1994). However, this simple model would not explain a loss of Rubisco without the loss of FBPase or SBPase, two other Calvin cycle enzymes whose expression is affected by carbohydrates (Jones et al., 1996). This might be explained if the threshold levels of carbohydrates required to affect expression differ or if amounts are affected posttranslationally. The relation of bulk carbohydrate content to acclimation is further complicated by compartmentalization, i.e. the fact that only a portion of the leaf carbohydrate could be in a compartment that could influence either gene expression or posttranslational processes.

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**Figure 6.** Levels of LSU, FBPase, and SBPase as a proportion of the quantity at current \( pCO_2 \) controls \((n = 3)\). The value for each replicate was the average of three to five repeated western blots. The broken line indicates no difference between elevated/current \( pCO_2 \), 1994 (A and B) and 1995 (C and D) at low N (□) and high N (■). There was no defoliation experiment at high N in the 1995 season. *, Statistically significant at the 0.05 level \( (t, P < 0.05) \); **, statistically significant at the 0.01 level \( (t, P < 0.01) \) two-tailed \( t \) test.
(Moore et al., 1997). Nevertheless, and consistent with our hypothesis, acclimation is correlated closely with conditions that induce accumulation of nonstructural carbohydrates in the leaf. In conclusion, this study has shown for the first time to our knowledge that in field conditions acclimation of photosynthesis to growth in elevated CO₂ conditions is an indirect effect of N and is dependent on the sink-source balance of the plant.

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**LITERATURE CITED**


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