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Photosynthetic acclimation of *Pinus taeda* (loblolly pine) to long-term growth in elevated pCO_2 (FACE)

A. ROGERS[1] & D. S. ELLSWORTH[2]

[1]*Environmental Sciences Department, Brookhaven National Laboratory, New York 11973-5000, USA and* [2]*School of Natural Resources and Environment, University of Michigan, Michigan 48109-1115, USA*

ABSTRACT

Growth in elevated pCO_2 generally leads to a stimulation of net CO_2 uptake rate. However, with long-term growth the magnitude of this stimulation is often reduced. This phenomenon, termed acclimation, has been largely attributed to a loss of Rubisco (ribulose 1,5 bisphosphate carboxylase). The mechanism by which Rubisco content declines with long-term growth is not certain. There is evidence for a sugar-mediated, selective down-regulation of Rubisco protein and also for a non-selective loss of total leaf nitrogen, which impacts Rubisco levels indirectly. Over a season, and including needles at different developmental stages, we investigated these two potential mechanisms in well-developed *Pinus taeda* grown for approximately 2·5 years in elevated (56 Pa) pCO_2 using free air CO_2 enrichment technology. Photosynthetic acclimation, as manifested by a decrease in the activity of Rubisco measured both *in vivo* (– 25%, via gas exchange) and *in vitro* (– 35%, via enzyme assays), was observed with growth in elevated pCO_2. This acclimation was observed in one-year-old needles but not in current-year needles. Needles exhibiting acclimation had reduced levels of Lsu Rubisco (– 25%) and an increased foliar carbohydrate content (+ 30%) but showed no evidence of a decrease in needle nitrogen or total protein content. These data support the concept that photosynthetic acclimation in elevated pCO_2 is caused by a selective down-regulation of Rubisco.

Key-words: *Pinus taeda*; gas exchange; phenology; Rubisco.

Abbreviations: A, net CO_2 uptake (μmol m^{-2} s^{-1}); FACE, free-air CO_2 enrichment; *Lsu*, large subunit of Rubisco; pCO_2, partial pressure of CO_2; $V_{c,max}$, maximum *in vivo* rate of ribulose 1,5 bisphosphate-saturated carboxylation (μmol m^{-2} s^{-1}).

INTRODUCTION

As plants acclimate to growth in elevated pCO_2 they often fail to sustain the initial, maximal stimulation of net CO_2 uptake rate under optimal microclimate conditions (Gunderson & Wullschleger 1994; Sage 1994; Curtis 1996; Drake, Gonzalez-Meler & Long 1997). Rogers & Humphries (2000) demonstrated that this phenomenon could be attributed almost entirely to a decrease in the carboxylation capacity of Rubisco. This decrease in carboxylation capacity with growth in elevated pCO_2 is well documented, and most commonly associated with a reduction in the amount of Rubisco (Drake *et al*. 1997; Moore *et al*. 1999; Stitt & Krapp 1999). A reduction in the amount of Rubisco following growth in elevated pCO_2 could have different causes and the mode by which this occurs is uncertain. There are two basic mechanisms by which Rubisco acclimation is thought to occur.

The first mechanism hypothesizes that the reduction in Rubisco content occurs via selective reduction in Rubisco resulting from a sugar-mediated feedback control on the amount of enzyme. Increased levels of hexose sugars produced as a consequence of growth in elevated pCO_2 lead, via a hexokinase-related signal, to the repression of Rubisco gene expression and a subsequent decrease in the levels of Rubisco protein (Koch 1996; Drake *et al*. 1997; Jang *et al*. 1997; Moore *et al*. 1999). There are problems with this proposed mechanism (Moore *et al*. 1999; Stitt & Krapp 1999), not least of which is the uncertainty as to the nature of the sugar signal and even whether acclimation is a uniquely sugar-mediated phenomenon (Stitt & Krapp 1999).

An alternative concept is that Rubisco acclimation is the result of a non-selective decrease in leaf nitrogen content (Jacob, Greitner & Drake 1995; Sicher & Bunce 1997; Nakano, Makino & Mae 1997; Theobald *et al*. 1998; Curtis *et al*. 2000). Under this hypothesis, decreases observed in Rubisco may reflect a general decrease of leaf protein, due to relocation of nitrogen within the plant (Makino *et al*. 1997; Nakano *et al*. 1997) or earlier leaf senescence in nitrogen-limited plants (Nie *et al*. 1995; van Oosten & Besford 1995; Pearson & Brooks 1995; Miller *et al*. 1997; Stitt & Krapp 1999). Under conditions of nitrogen limitation, acclimation may be accelerated in elevated pCO_2 because the plants are larger and therefore experience acute nitrogen limitation sooner, or to a greater extent. Farage, McKee & Long (1998) demonstrated that even when growth was restricted by a low-N relative addition rate, photosynthetic acclimation in elevated pCO_2 could be ameliorated if nitrogen was added in direct proportion to plant growth, supporting the concept that N dilution is the cause of Rubisco acclimation.

Correspondence: Alistair Rogers, Environmental Sciences Department, 490D, Bell Avenue, Brookhaven National Laboratory, Upton, NY 11973-5000, USA. Fax: + 1 631 344 2060; e-mail: arogers@bnl.gov

Although many studies have addressed the occurrence of acclimation in different species and growth conditions, very few have made sufficient measurements to draw conclusions about which of the two mechanisms may be operating. Changes in total leaf nitrogen and protein are difficult to interpret in isolation due to the large representation of Rubisco in leaf proteins (Evans & Seemann 1989) and the large diurnal changes in leaf nitrate content (Scheible *et al*. 1997). In this study the needle nitrogen and protein content were determined, in addition to the relative levels, activity and activation of Rubisco. Only by obtaining a suite of such measurements can inferences regarding the nature of the mechanism be drawn and further tested.

This study took place in well-developed *Pinus taeda* (loblolly pines) grown in elevated pCO_2 using *FACE* technology (Hendrey *et al*. 1999). Many observations of acclimation have been made in experiments where it is probable that growth in elevated pCO_2 resulted in the appearance or exacerbation of nitrogen limitation (Stitt & Krapp 1999). This study provides a unique opportunity to examine photosynthetic acclimation in well-developed trees growing in a forest ecosystem exposed to elevated pCO_2 where confounding factors such as those highlighted by Stitt & Krapp (1999) are absent. This study is especially relevant to mature forests, because other methods of CO_2 enrichment cannot be applied to such well-developed trees without significant microenvironmental effects (Hendrey *et al*. 1999; McLeod & Long 1999). We studied well-developed loblolly pines that had been exposed to a step increase of pCO_2 of + 20 Pa for approximately 2·5 years at three points in the 1999 growth season to test the following two hypotheses:

1 Rubisco acclimation is caused by a reduction in Rubisco protein content and is associated with increased levels of soluble sugars implicated in feedback control.
2 Rubisco acclimation is the consequence of a non-specific reduction in total needle N/protein content.

In order to test these hypotheses under different physiological conditions, sampling dates were chosen to include needles of different maturity and with different local source–sink relationships. The nearest source to a large carbohydrate sink will export a large proportion of its photosynthate to that sink (Dickson 1989). Based on previous work in perennial rye grass (Rogers *et al*. 1998) we further speculated that carbohydrate accumulation in source needles in elevated pCO_2 will be exacerbated when adjacent carbohydrate sinks are smallest. If so, carbohydrate-mediated acclimation should be most prevalent when local sinks for carbohydrate are absent.

MATERIALS AND METHODS

Plant material and site description

The study was conducted at the free-air CO_2 enrichment (FACE) site in the Blackwood Division of Duke Forest in Orange County, NC, USA. The site and the FACE facility are described by Ellsworth (1999). The forest site occurs on a nutrient-poor, ultic clay loam soil. Nitrogen supply is limiting plant growth on the site with an availability of approximately 3 g N m^{-2} $year^{-1}$ (Oren *et al*. 2001; Finzi *et al*. 2002). Three separate circular plots of 30 m diameter were exposed to elevated (targeted at current + 20 Pa) pCO_2 using the *FACE* approach (Hendrey *et al*. 1999). A daytime pCO_2 of 56·4 Pa ± 0·5 Pa over the entire year of 1999 was achieved in these plots. Three fully instrumented plots (including blowers) with no CO_2 injection served as controls (daytime pCO_2, 36·4 Pa).

We measured needles located in the upper crown of 16-year-old *Pinus taeda* L. (loblolly pine) at 95% of total tree height. The mid-sections of needles from trees grown for approximately 2·5 years in elevated and current pCO_2 were sampled on 12 May, 18 July and 15 October 1999 for the analysis described below. Four different ages of needles were sampled. In May, one-year-old needles that developed in 1998 were sampled, in July both one-year-old needles and current-year (1999) needles were sampled. In October it was only possible to sample current-year needles due to progressing senescence of the foliage from the previous year. The midday maximum temperatures were 30, 34 and 25 °C for May, July and October, respectively. The midday maximum photosynthetic quantum flux density was approximately 1800 μmol m^{-2} s^{-1} on all sampling days. Data were expressed on a total needle surface area basis, determined as described by Johnson (1984).

Phenological measurements were made by accessing the upper crown of the trees and making weekly measurements of shoot and needle length during elongation. These measurements were made throughout the growing season on three trees in each of the study plots.

Gas exchange

In situ measurements of the responses of A to pCO_2, frequently referred to as 'A–C_i curves', were measured with a portable photosynthesis system (Li-Cor model 6400; Li Cor, Lincoln, NE, USA). Sunlit pine needles at the top of the crown were sealed inside the chamber while ensuring that chamber conditions maintained growth pCO_2, light saturation and a constant seasonable temperature (leaf temperature was 29, 30 and 25 °C for May, July and October, respectively). After a short equilibration period to chamber conditions, the measurements of A, C_i, and stomatal conductance to water vapour were logged into memory along with environmental parameters. Chamber pCO_2 was then changed and stepped through seven different levels, starting close to the CO_2 compensation point and ending at elevated pCO_2. Measurements at each successive pCO_2 were made after complete flushing of the chamber with the desired pCO_2 level judged by stabilization of the CO_2 signal. We made frequent leak tests to minimize bias in the low pCO_2 measurements and used Teflon tape to seal the chamber for measurements. Measurements were made on needles from one tree in each separate experimental plot for the three replicate plots at current and elevated pCO_2, concurrently with the sampling for *in vitro* analysis.

Maximum rate of carboxylation ($V_{c,max}$) was calculated by fitting the equations of Farquhar, von Caemmerer & Berry (1980) and Evans & Farquhar (1991), following the procedure of Wullschleger (1993) with the temperature corrections of McMurtrie & Wang (1993).

Leaf nitrogen content, specific leaf mass

Samples were dried to a constant mass at 80 °C. Dry mass was determined for a subsample of known needle area. Each individual leaf sample was ground to a fine powder, and total leaf nitrogen was determined by a CHN analyser (NA-1500 elemental analyser; Carlo-Erba, Milan, Italy) after combustion.

Rubisco activity, activation state and content; protein content

Five subsamples were taken from each treatment replicate. The tip and base sections of each fascicle were discarded, the mid-section was immediately ground for 10 s in extraction buffer (Tissue, Thomas & Strain 1993) at 4 °C using a high-speed homogenizer (Polytron; Kinematica, Lucerne, Switzerland). Homogenized samples were frozen immediately and stored in liquid nitrogen until analysed. Sampling to freezing in liquid nitrogen took less than 2 min Samples were thawed and centrifuged at 13 000 × *g* for 30 s in a microcentrifuge tube. An aliquot of the supernatant was used immediately for determining the initial and total (fully activated) activity of Rubisco using the spectrophotometric, NADH, enzyme-coupled assay described by Tissue *et al*. (1993). Full activation of Rubisco was achieved by incubating an aliquot of the supernatant in the assay solution described by Tissue *et al*. (1993) at 25 °C for 15 min prior to addition of ribulose 1,5 bisphosphate and spectrophotometric analysis. The activation state was calculated as initial activity as a percentage of total activity.

For each treatment plot, three samples were taken for analysis of *Lsu* Rubisco levels. The relative levels of *Lsu* Rubisco at elevated and current pCO_2 were determined by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and subsequent laser densitometry as described by Rogers, Humphries & Ellsworth (2001). To allow cross comparison, a common sample was loaded in all gels for a given needle class. However, a common sample was not used for all needle classes and comparison of relative *Lsu* Rubisco levels between needle classes is not possible. The SDS-PAGE gels were loaded on an equal surface area basis (approximately 10 μg protein per well). Valid comparisons are only possible between current and elevated pCO_2 treatments for a given needle class. Total protein content was determined as described by Rogers *et al*. (2001).

Carbohydrate analysis

Ethanol-soluble carbohydrates were extracted from ground needles in four overnight incubations in 90% (v/v) ethanol; no starch was detected in this extract. Less rigorous extraction procedures failed to recover all the carbohydrates (data not shown). Bulk ethanol-soluble carbohydrate content was determined using the phenol-sulphuric acid assay described by Dubois *et al*. (1956). Following ethanol incubations, starch was extracted from the ground needles using 32% (v/v) perchloric acid as described by Farrar (1993) and assayed as described above.

Sampling and statistical analysis

Needles from three trees were sampled in each plot, except for *in vitro* Rubisco activity analysis in which five samples were taken from each plot and *in vivo* Rubisco measurements where only one tree was measured in each plot. Each FACE plot was treated as a replicate, and on each date we sampled and measured needles from all three replicate elevated and current pCO_2 plots, i.e. $n = 3$ in all cases. Because it was only the effect of CO_2 treatment that was tested, no *post hoc* comparisons of means were performed, and differences in biochemical parameters due to CO_2 treatment between the paired control and elevated plots were examined using a paired *t*-test (SYSTAT, SPSS Inc., Evanston, IL, USA).

RESULTS

Figure 1 shows the development of the current-year needles throughout the period of sampling and measurement. During May and June, current-year shoots and needles were developing. When we sampled in May, shoot development was nearing completion and needle development was just starting. In July, shoot development was complete and needle expansion was in progress. In October, both shoots and needles had completed their growth.

Growth in elevated pCO_2 resulted in a stimulation of A but this effect was only significant in October ($t_{(2),2}$, $P < 0{\cdot}05$; Fig. 2a). A 45% stimulation of A corrected to a constant pC_i/pCO_2 (e.g. the ratio of CO_2 in leaf intercellular air spaces to CO_2 of external air) was observed with growth in elevated pCO_2 in current-year needles in July and October ($t_{(2),2}$, $P < 0{\cdot}05$) but not in one-year-old needles ($P > 0{\cdot}1$, data not shown). A significant decrease in Rubisco activity measured both *in vivo* (–25%, $t_{(2),2}$, $P < 0{\cdot}1$, May; $t_{(2),2}$, $P < 0{\cdot}01$ July (old) and *in vitro* (–35%, $t_{(2),2}$, $P < 0{\cdot}05$) was observed in over-wintering pine needles grown in elevated pCO_2 (Fig. 2b & c). The discrepancy between the absolute values of *in vivo* and *in vitro* measurements of Rubisco activity was addressed by Rogers *et al*. (2001). They concluded that the *in vitro* values for Rubisco activity were too low to account for the observed A, and that this was due to an incomplete extraction of Rubisco prior to *in vitro* analysis (Rogers *et al*. 2001). This approximately 30% loss of carboxylation capacity in elevated pCO_2 was found in one-year-old needles but not in current-year needles. There was no effect of pCO_2 treatment on the percentage activation of Rubisco (Fig. 2d).

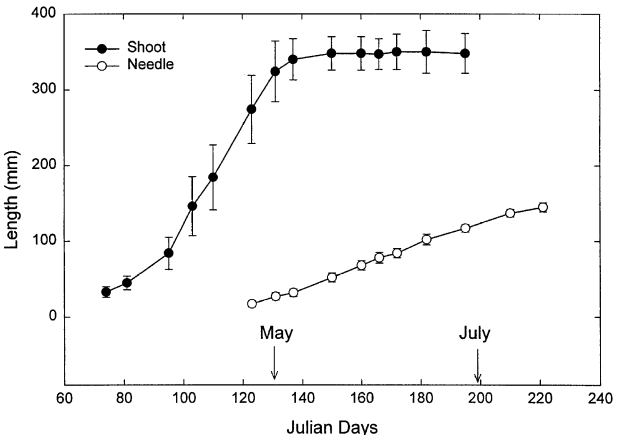

Figure 1. Mean shoot length (●) and needle length (○) ± SE from trees developed at both elevated and current pCO_2 (n = 6). Measurements are shown from emergence until full expansion. The arrows indicate the date of sampling in May and July.

SDS-PAGE of samples taken in parallel with those used for gas exchange and *in vitro* analyses revealed a significant 25% decrease in the levels of *Lsu* Rubisco in the one-year-old needles (May and July old, $t_{(2),2}$, $P < 0{\cdot}01$, Fig. 3). The decreases in the levels of *Lsu* Rubisco observed in May and July were not accompanied by concomitant decreases in either needle nitrogen or protein content (Fig. 2e & f). Interestingly, a significant increase in protein was observed in elevated pCO_2 in May ($t_{(2),2}$, $P < 0{\cdot}05$; Fig. 2f). Acclimation resulted in a decrease in the fraction of leaf N in the form of Rubisco, calculated as described by Tissue *et al.* (1993), in the one year needles from *c.* 9·5% in current pCO_2 to approximately 7·0% in elevated pCO_2.

There was a significant 20–40% increase in the ethanol-soluble carbohydrate fraction of one-year-old needles from trees grown in elevated pCO_2 ($t_{(2),2}$, $P < 0{\cdot}05$). A smaller but still significant increase in the content of this fraction that includes glucose, fructose and sucrose was also observed in July in current-year needles, but not in October (Fig. 4a). With the exception of the needles sampled in May, in which there was a considerable accumulation of starch (Fig. 4b), the starch content in all needles was low and there was no significant effect of CO_2 treatment. As a result, the levels of total non-structural carbohydrate shown in Fig. 4c largely reflect the level of ethanol-soluble carbohydrates. A significantly higher specific leaf mass was not detectable as a result of the carbohydrate accumulation in needles grown in elevated pCO_2 (Fig. 4d).

DISCUSSION

Acclimation of the photosynthetic apparatus of well-developed *Pinus taeda* was observed only in one-year-old needles in agreement with the findings of Turnbull *et al.* (1998), and Griffin *et al.* (2000) for *Pinus radiata*. Both current-year and one-year-old needles showed no significant decrease in needle nitrogen or protein content, with growth in elevated pCO_2 supporting the hypothesis that Rubisco acclimation is not the consequence of a non-selective reduction in leaf N content. Photosynthetic acclimation in pines without a significant decrease in needle N content has been reported previously (Tissue *et al.* 1993; Tissue, Thomas & Strain 1996; Turnbull *et al.* 1998; Tissue, Griffin & Ball 1999; Griffin *et al.*

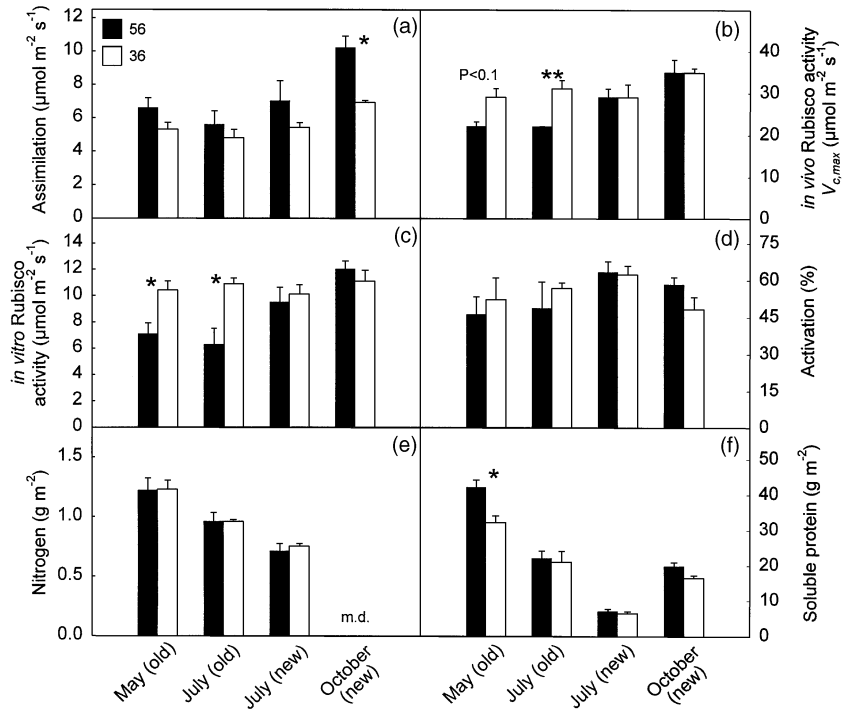

Figure 2. Mean ± SE (n = 3 replicate plots) of biochemical parameters measured in loblolly pines growing in approximately 56 Pa pCO_2 (elevated, 56) and approximately 36 Pa pCO_2 (current, 36). Measurements were made on one-year-old needles (*old)* and current-year needles (*new)* on three occasions *May*, *July* and *October* (m.d. = missing data). In May current-year needles had not yet developed and in October one-year-old needles had senesced, therefore only one cohort was sampled at these times. Where significant differences due to CO_2 treatment are present they are indicated above the bars (*$t_{(2),2}$, $P < 0{\cdot}05$; **$t_{(2),2}$, $P < 0{\cdot}01$).

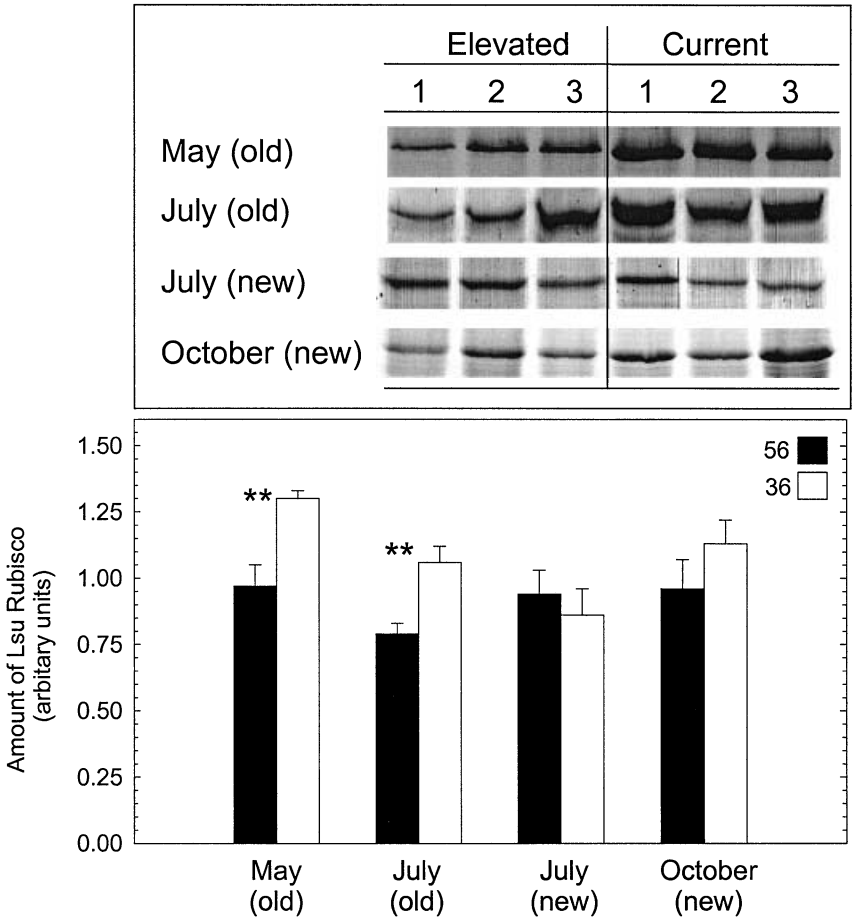

Figure 3. Sections of SDS-polyacrylamide gels showing levels of the *Lsu* Rubisco in needles sampled in parallel with those described in Fig. 2. The numbers 1, 2 and 3 refer to the three replicate treatment plots. Gels were loaded on an equal leaf area basis. SDS-PAGE was repeated on two additional samples from each treatment plot (gels not shown). Comparisons are only possible within a gel, i.e. it is not possible to compare Rubisco levels between different needle classes. The laser densitometric quantification includes the analysis of additional gels. The mean response for each treatment plot was treated as one replicate value, i.e. $n = 3$ replicated treatment plots. Comparisons are only valid between elevated pCO_2 (filled bars) and current pCO_2 (open bars) pairs, $^{**}t_{(2)}$, $P < 0{\cdot}01$.

2000). However, in contrast to our data, in only one of these studies was a loss of Rubisco activity in elevated pCO_2 linked with significant increases in needle sugar levels (Griffin *et al.* 2000).

With regard to *Pinus taeda*, Myers, Thomas & DeLucia (1999) made similar measurements at the same field site in its first year of CO_2 enrichment in this overall experiment. They reported a strong enhancement of A with growth in elevated pCO_2. Hymus *et al.* (1999) also made measurements at this field site during the first year of operation and in agreement with Myers *et al.* (1999) reported a stimulation in A with growth in elevated pCO_2. Myers *et al.* (1999) found no evidence of acclimation in their study as indicated by the lack of a decrease in $V_{c,max}$ with growth in elevated

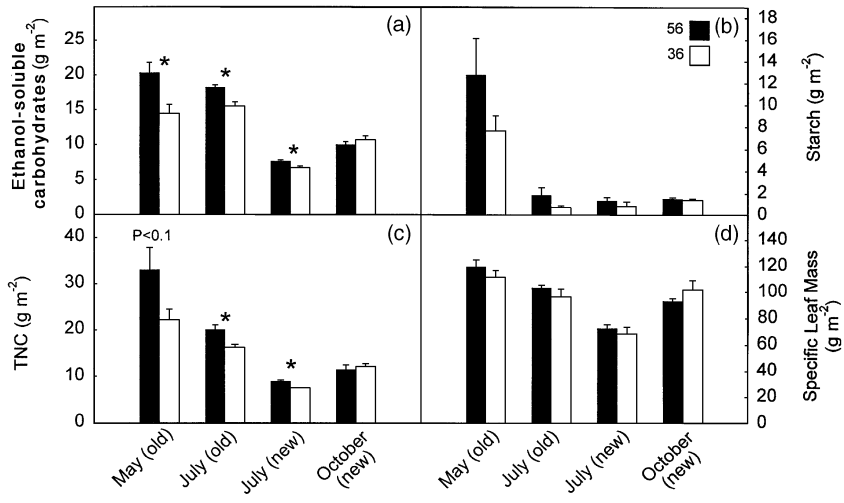

Figure 4. Mean ± SE ($n = 3$ replicate plots) of carbohydrate fractions and specific leaf mass measured in the loblolly pines described in Fig. 2. Total non-structural carbohydrate (TNC) content was calculated by addition of the value obtained for ethanol-soluble carbohydrates and starch. Where significant differences due to CO_2 treatment are present they are indicated above the bars ($^{*}t_{(2),2}$, $P < 0{\cdot}05$).

pCO_2. There was also no effect of growth in elevated pCO_2 on the nitrogen and sugar content of these needles (Myers *et al*. 1999). The large stimulation of A in elevated pCO_2 reported by Myers *et al*. (1999) translated into a 25% increase in the relative basal area increment of loblolly pines grown at elevated pCO_2 (DeLucia *et al*. 1999). The data presented in our study suggest that the enhancement observed in A and relative basal area increment to the step increase in pCO_2 reported by Myers *et al*. (1999) and DeLucia *et al*. (1999) may represent an early maximal response that may not be sustained in the long term. This is consistent with observations made over 7 years in the nearby, unreplicated FACE array (Ellsworth, LaRoche & Hendrey 1998; Oren *et al*. 2001) in the same parcel of forest. The forest site in our study and these previous studies is N-limited to an extent that represents a constraint on the magnitude of plant sinks for growth.

Earlier work on young *Pinus radiata* germinated in elevated pCO_2 and transferred to open top chambers showed no evidence of a decrease in Rubisco activity with growth in elevated pCO_2 at the start of the study (Hogan *et al*. 1996). However, after 4 years of growth in elevated pCO_2 no stimulation of A in one-year-old needles was evident and there was a significant reduction in $V_{c,max}$ (Griffin *et al*. 2000). The results of this open-top chamber experiment (Hogan *et al*. 1996; Turnbull *et al*. 1998; Griffin *et al*. 2000) and those of the Duke forest FACE experiment (Myers *et al*. 1999; and the data presented here) have a common pattern despite the differences in species, growth conditions, age and duration of CO_2 exposure. It remains to be seen whether the acclimation observed in one-year-old needles at key stages of development will become more prevalent with the continuation of this experiment. The results also suggest that species with greater leaf longevity may show a similar response, although few studies have examined species in other coniferous genera.

In some species Rubisco can account for up to 25% of the leaf nitrogen content (Webber, Nie & Long 1994). However, in pines Rubisco commonly comprises approximately 10% of needle nitrogen (Turnbull *et al*. 1998; Myers *et al*. 1999). Therefore, a 25% reduction in Rubisco content, as observed in this study, would result in a decrease in total leaf nitrogen of less than 3%. In this study we were unable to statistically detect the predicted 2·5% decrease in total needle nitrogen resulting from the approximately 25% decrease in Rubisco content. Stitt & Krapp (1999) have argued that leaf N content in isolation may not be a good indicator of the nitrogen status of a plant. In this study we measured total protein content and relative Rubisco content in order to address our hypotheses concerning the mechanism underlying photosynthetic acclimation. However, it is clear from the observed increase in total protein content in elevated pCO_2 in May (Fig. 2f) that analysis of nitrate and free amino acids will be necessary for a complete evaluation of the effects of elevated pCO_2 on leaf and plant nitrogen status.

The influence of sink development on photosynthetic acclimation is uncertain. We expected that acclimation would be absent when there was a developing proximal sink for carbohydrate (one-year-old needles, May and July) and prevalent when local sinks were absent (new needles, October). The opposite response was observed, suggesting that distal carbohydrate sinks, such as roots, may play a more significant role. However, developing needles are also sinks for nitrogen, and it is clear from Fig. 2e & f that the N/protein content in one-year-old needles is in decline during the period of shoot and needle development. We speculate that current-year needle development may be slowed by a whole plant N limitation that may lead to carbohydrate accumulation in one-year-old-needles. Because the supply of photoassimilate is greater in elevated pCO_2 than in current pCO_2 and accumulation was observed to be exacerbated in elevated pCO_2 (Fig. 4c), the observed photosynthetic acclimation is consistent with the sugar-signalling hypothesis of Moore *et al*. (1999). Starch accumulation in elevated pCO_2 appears to be uncoupled from acclimation (Figs 2b & 4b), but soluble sugar accumulation is linked to acclimation, providing indirect evidence that sugar signalling in pines may be analogous to that in higher plants.

CONCLUSIONS

Rubisco acclimation as manifested by reduced levels of Rubisco protein and activity is not caused by a non-specific decrease in leaf N or total protein content in ageing pine needles. Rubisco content was reduced significantly (by about 25%) in over-wintering pine foliage with long-term growth in elevated pCO_2 while significant changes in leaf N content were undetectable. This apparently selective reduction of Rubisco in older needles was associated with a significantly higher soluble carbohydrate content, and is consistent with the concept that acclimation to elevated pCO_2 involves a sugar-mediated response. However, the hypothesis that acclimation is driven purely by a carbohydrate-related signal may need revision.

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