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Elevated CO_2 in a Prototype Free-air CO_2 Enrichment Facility Affects Photosynthetic Nitrogen Relations in a Maturing Pine Forest

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Elevated CO_2 in a prototype free-air CO_2 enrichment facility affects photosynthetic nitrogen relations in a maturing pine forest

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Summary A maturing loblolly pine (*Pinus taeda* L.) forest was exposed to elevated CO_2 in the natural environment in a perturbation study conducted over three seasons using the free-air CO_2 enrichment (FACE) technique. At the time measurements were begun in this study, the pine canopy was comprised entirely of foliage which had developed under elevated CO_2 conditions (atmospheric $[CO_2] \approx 550$ μmol mol^{-1}). Measurements of leaf photosynthetic responses to CO_2 were taken to examine the effects of elevated CO_2 on photosynthetic N nutrition in a pine canopy under elevated CO_2. Photosynthetic CO_2 response curves ('*A*-c_i curves') were similar in FACE trees under elevated CO_2 compared with counterpart trees in ambient plots for the first foliage cohort produced in the second season of CO_2 exposure, with changes in curve form detected in the foliage cohorts subsequently produced under elevated CO_2. Differences in the functional relationship between carboxylation rate and N_a suggest that for a given N_a allocated among successive cohorts of foliage in the upper canopy, $V_{c\,max}$ was 17% lower in FACE versus Ambient trees. We also found that foliar Rubisco content per unit total protein derived from Western blot analysis was lower in late-season foliage in FACE foliage compared with ambient-grown foliage.

The results illustrate a potentially important mode of physiological adjustment to growth conditions that may operate in forest canopies. Our findings suggest that mature loblolly pine trees growing in the field may have the capacity for shifts in intrinsic nitrogen utilization for photosynthesis under elevated CO_2 that are not dependent on changes in leaf N. While carboxylation efficiency per unit N apparently decreased under elevated CO_2, photosynthetic rates in trees at elevated CO_2

concentrations ≈ 550 μmol mol^{-1} are still enhanced compared to trees grown and measured at the current ambient CO_2 concentration when compared at a common N status. The findings from this prototype study suggest a need for continued examination of internal feedbacks at the whole-tree and ecosystem level in forests that may influence long-term photosynthetic responses to elevated CO_2.

Keywords: Pinus taeda, elevated CO_2, free-air CO_2 enrichment (FACE), photosynthesis, Rubisco enzyme

Introduction

Numerous field studies over the past two decades have presented clear evidence that elevated atmospheric CO_2 concentrations will enhance the rate of photosynthesis in trees (Strain and Cure 1994). The degree of photosynthetic enhancement by long-term CO_2 enrichment has been shown to vary considerably depending on species physiology and growth conditions (Long and Drake 1992, Curtis 1996), and tissue type (Lee and Jarvis 1996). In some cases photosynthetic enhancements under long-term growth in elevated CO_2 are smaller than instantaneous or short-term enhancements, suggesting downward adjustments of leaf capacity for net CO_2 assimilation at light saturation (A) under long-term growth at elevated CO_2 (reviewed by Long and Drake 1992, Gunderson and Wullschleger 1994, Sage 1994). Often, this phenomenon is attributed to changes in leaf N concentration, which can result from dilution of nutrients by biomass accumulation (Luo et al. 1994). However, CO_2-induced changes in plant nutrition can also occur as a result of experimental artifacts due to soil disturbance (Johnson et al. 1995), or differential root binding and nutrient supply rate (Arp 1991, McConnaughay et al. 1993) between ambient and elevated CO_2-grown plants. Furthermore the interpretation of effects of elevated CO_2 on photosynthetic N nutrition are also complicated by the tendency to compare tree seedlings growing under different CO_2 regimes at a common age, despite plant size-dependent differences in morphology or nutrition that occur as a result of treatment effects on plant growth rate (Coleman et al. 1993, McConnaughay et al. 1996). While leaf N itself may change under elevated CO_2,

the evidence is mixed as to whether CO_2-induced changes in leaf photosynthetic N pools are a common response of trees in field situations (Amthor 1995, Curtis 1996).

A large majority of elevated CO_2 studies are conducted on very young seedlings of trees, while there is little information on the photosynthetic responses of intact forest vegetation at other life stages to elevated atmospheric CO_2 in the field (Körner 1995). Studies of physiological responses within the forest canopy of mature forests can provide much-needed data on the field responses to elevated CO_2 that can be used to test models of canopy processes and concepts developed from studies of short-statured, juvenile trees. Mature trees also exhibit more stable ontogeny and leaf nutrition than do small seedlings (Coleman et al. 1994), against which effects of elevated CO_2 on nutrient-use can be more easily evaluated than they can in small seedlings. With the advent of free-air CO_2 enrichment (FACE) systems such as have been applied to short-stature crop and seedling vegetation (Hendrey et al. 1993, McLeod 1995), there is now a means for studying the responses of mature forest vegetation to elevated CO_2 in the field. In a forest environment, the FACE approach offers a unique opportunity to investigate the photosynthetic responses to elevated CO_2 in trees near maturity and grown in the field under conditions of natural nutrient supply and without large-scale soil disturbances or alterations in canopy microclimate.

Previous studies on mature pine trees under elevated CO_2 (Teskey 1995, Murthy et al. 1996) used CO_2 exposures of single branches (e.g., < 1% of the tree crown exposed to elevated CO_2) or single-season exposures to elevated CO_2 (Ellsworth et al. 1995). These previous studies have not considered whole-plant sinks and within-canopy feedbacks

that can potentially affect CO_2 responses (Stitt 1991). Using the prototype forest FACE facility operated across multiple growing seasons, we measured foliage within a pine canopy comprised entirely of foliage which had developed under elevated CO_2 conditions (atmospheric $[CO_2] \approx 550$ μmol mol^{-1}). Since a characteristic of *Pinus taeda* in its native range is the production of multiple flushes of foliage in a growing season, we evaluated the photosynthetic N nutrition of maturing trees in two foliage cohorts produced during the second growing season of CO_2 exposure in FACE as well as the first cohort produced in the third exposure season.

The close relationship between CO_2 fixation capacity by foliage and leaf N content may be central to understanding process-based interactions between elevated CO_2 and soil resources (Field et al. 1992). While a close relationship between carboxylation capacity and leaf N is hypothesized as it is assumed that leaf N content is largely a reflection of N-rich leaf photosynthetic proteins (Field and Mooney 1986, Evans 1989), it is unclear if long-term elevated CO_2 will alter such relationships (Nijs et al. 1995). The carboxylation-N relationship is central to an ability to model CO_2 responses and biochemical limitations to photosynthesis at many scales (McMurtrie et al. 1992, Woodward et al. 1995). Changes in leaf N itself under elevated CO_2 are commonly observed (Drake et al. 1997), but few studies have examined the relationship between carboxylation capacity and leaf N particularly in canopies in the field environment. In this study we examined this key functional relationship in elevated CO_2-grown foliage compared to ambient foliage in the maturing pine forest canopy to test if prolonged

CO_2 exposure has an effect on N content or the partitioning of N to photosynthetic activity.

Materials and Methods

The study was conducted in a 14-year-old plantation of loblolly pine (*Pinus taeda*) of piedmont origin in the Blackwood Division of Duke Forest (35°58'N, 79°5'W) in Orange County, N.C., USA. Mean July maximum and minimum temperatures for this area are 31.5 °C and 18°C, respectively. The site and the free-air CO_2 enrichment (FACE) exposure system were described in Ellsworth et al. (1995). The stand is part of a 90 ha block of relatively uniform pine plantation that was established in early 1983 and is now approaching canopy closure. Density of co-dominant pines in the study portion of the stand was approximately 1600 trees ha^{-1} and total tree density of dominant and subcanopy hardwood trees was 3700 trees ha^{-1}.

In 1993 a 30-m diameter prototype FACE array was constructed in the loblolly pine forest to test the tractability of conducting an elevated CO_2 experiment in an intact forest plot. The CO_2 exposure was designed as a perturbation to study leaf and canopy responses rather than to mimic actual future conditions. The >10m tall forest plot was exposed to elevated CO_2 concentration during daylight hours for 82 days (6 June-31 August) in 1994 by injecting pre-diluted CO_2 into free-air emitted into the stand (Ellsworth et al. 1995). At the time measurements were begun in this study in 1995, the pine canopy was comprised entirely of foliage which had developed under an elevated

CO_2 atmosphere. In 1995, the prototype FACE system was operated from pre-dawn to civil twilight (when sun elevation angle exceeded 6° below the horizon) for 133 out of 145 days from 23 May to 14 October. The stand was similarly exposed to elevated CO_2 for 144 out of 153 days in the 1996 growing season (8 May-8 Oct. 1996). Over the operating season in 1995, the average daytime CO_2 concentration at 9m height at the center of the FACE plot was 553.9 ± 5.2 μmol mol^{-1} (mean ± s.d.), with a reliability of 97% (e.g., the system was in operation for 97% of scheduled hours). In 1996 average daytime CO_2 concentration in the upper canopy at the plot center was 551.0 ± 4.6 μmol mol^{-1} through early August when physiological measurements were completed. In the upper canopy (9-10m height) spatial variability in average daytime CO_2 concentration during the exposure season was 9% among 12 nodes sampled continuously in a grid located 2-9m from the plot center (Figure 1). For the portion of the exposure plot within 5m of the plot center, where the measurements were made, the season daytime CO_2 concentration was 560 ± 19 μmol mol^{-1} (mean ± s.d. of 5 continuously-monitored locations).

Ambient CO_2 concentration was monitored at two reference locations outside of the prevalent wind path at the site. The monitoring plots were located 85m NW and 80m due E of the FACE plot, respectively. At each of these plots, trees were also selected for physiological measurements (see below). The day-time CO_2 concentration measured at 9m height in the forest canopy at one of the Ambient plots was 356.5 ± 9.5 μmol mol^{-1} during the growing season in 1995. A more distant monitoring site was established ≈500 m upwind of the FACE plot. Transient deviations of the ambient CO_2 concentration in

the NW plot from the ambient CO_2 concentration at the more distant, upwind location were rarely observed and did not occur for more than 1-2 minutes in duration, indicating that there was little contamination of Ambient plots from FACE during the monitoring period. Night-time average CO_2 concentration at 9m in the canopy in both FACE and Ambient plots was 431 ± 44 μmol mol^{-1} in July and August.

Field photosynthesis measurements

Photosynthetic responses were evaluated for 3 subsequent foliage cohorts when each had reached full elongation. The first foliage cohort was approximately one-quarter elongated when exposure to elevated CO_2 was begun in 1995, and was measured in late July after 60d of development under elevated CO_2. Measurements on the second foliage cohort, which had developed entirely under elevated CO_2, were made over a 3-week period in late Sept.-early Oct. after foliage was fully elongated. The next foliage cohort (first foliage cohort of 1996) emerged in May of the following year, so measurements were made in late July or early August on foliage located on the same branches as previously. Since the seasonal CO_2 exposure was begun earlier in 1996, foliage was at a state of incipient emergence when CO_2 exposure for that season was begun. Measurements were generally made before solar noon to avoid afternoon declines in stomatal conductance and possible diurnal inhibition of photosynthesis by transient carbohydrate accumulation. Late in the sampling season, some measurements in one of the Ambient plots were made slightly later than solar noon when the sky had been cloudy for the day.

The response of photosynthetic CO_2 assimilation (A) to leaf intercellular CO_2 concentration (A- c_i curves) was measured for foliage near the tops of trees (generally 11-12m above the ground) in the FACE plot and in the two Ambient plots. The Ambient plots were comprised of trees in proximity to two towers constructed near the CO_2 monitoring locations in different cardinal directions from the FACE plot. The measurements were made on trees accessed by walk-up towers and extension booms in the center of the FACE plot and in both of the Ambient monitoring plots. Foliage located on the terminal shoot of major branches at the third whorl from the top of the tree in early 1995 was selected for the gas exchange measurements to standardize light environment and location within the tree crown. These branches had been produced under elevated CO_2 in the 1994 growing season.

A- c_i curve measurements of single fascicles of pine foliage were conducted with a portable infra-red gas analysis system for CO_2 and water vapor (CIRAS-1, PP-Systems, Hitchin, U.K.) with a leaf chamber operated in an open configuration. Photon flux density >1300 μmol m^{-2} s^{-1} at the leaf surface was provided by a 50W quartz-halogen lamp mounted perpendicular to the leaf chamber and powered by a 12V car battery. A Peltier-type cooling unit (Marlow Industries, Dallas, TX) was used to maintain foliage temperature at 30.0 ± 0.2°C during measurements. Measurements were begun after foliage had received saturating irradiance for >20 minutes to ensure that stomata were open. The foliage was shielded from direct radiation other than that provided by the artificial light source to prevent excess radiation loading. CO_2 concentration of air in the leaf chamber (c_a) was varied step-wise among 9 CO_2 levels ranging from the CO_2

compensation point to near-saturation using the gas exchange system's bank of internal solenoid valves and a miniature cylinder of compressed CO_2. At each c_a, A measurements were logged at 15 s intervals until stable (C.V. < 2%). Vapor pressure of the air stream entering the leaf chamber was adjusted by passing the airstream over saturated filter paper to maintain leaf-air vapor pressure difference in the range 1.2-1.4 Pa kPa^{-1} at all times. The conditions were chosen so as to be near-optimum for photosynthesis in *P. taeda*. Rates of upper canopy A were higher in this stand than previously reported (i.e., Ellsworth et al. 1995) largely because measurements were made at higher leaf temperatures and photon flux densities, lower leaf-air vapor pressure differences, and higher canopy positions than previously.

After measurements, the foliage was excised and measurements of needle diameter were made to the nearest 100 μm under a dissecting microscope with an ocular micrometer. All-sided needle surface area of foliage samples was estimated by a geometric approximation of each needle segment as a one-third sector of a cylinder. All area-based parameters are expressed on a needle surface area basis. After needle diameter was measured, foliage was then placed in a freezer until taken to the laboratory to be dried at 70°C. Foliage was then weighed, ground and a subsample was combusted for determination of total nitrogen (N) concentration by a CHN analyzer (Perkin-Elmer model 2400, Norwalk, CT). In Oct. 1995, the second cohort of current-year foliage from additional trees for which gas exchange was not measured was collected in the prototype FACE plot and Ambient plots from the same crown position as A- c_i curve foliage, and similarly analyzed for leaf N concentration.

Analysis of A- c_i *curves*

Data from field *A*- c_i curves were used to parameterize the biochemical model of C_3 photosynthesis described by Farquhar, et al. (1980) and Farquhar and von Caemmerer (1982) with recent modifications (see Harley et al. 1992, Wullschleger 1993). According to the model, light-saturated leaf CO_2 assimilation rate (*A*) is limited either by regeneration of ribulose 1,5-bisphosphate (RuBP) in the photosynthetic carbon reduction cycle or by the catalytic activity of Rubisco when the chloroplast RuBP concentration is saturating. Thus the initial slope of the relationship between *A* and c_i (here for $c_i < 280$ μmol mol^{-1}) is considered to be the region of limitation by Rubisco activity. Under these conditions, *A* is given by

$$A = V_{c\,max}\frac{c_i - \Gamma^*}{c_i + k_c\left(1+\frac{o_i}{k_o}\right)} - R_d$$

where $V_{c\,max}$ is the maximum catalytic activity of Rubisco with saturating RuBP, c_i and o_i are the intercellular CO_2 and O_2 concentrations, respectively, and k_c and k_o are the Michaelis coefficients of Rubisco for CO_2 and O_2. The temperature dependencies of the kinetic parameters k_c, k_o, and R_d are from Harley et al. (1992). When c_i is close to saturation for photosynthesis such that RuBP regeneration limits photosynthesis, *A* is given by

$$A = J\frac{c_i - \Gamma^*}{4\left(c_i + 2\Gamma^*\right)} - R_d$$

For the measurements at light and CO_2 saturation, the rate of electron transport $J=J_{max}$ where J_{max} is the theoretical maximum rate of electron transport at saturating PFD. In the calculations, Γ^* is assumed constant at the value reported in Villar et al. (1995) for sclerophyllous woody species with similar photosynthetic rates. Using this value, $V_{c\,max}$ and respiration in the light (R_d) were determined by least-squares regression fits to the data for $c_i < 280$ μmol mol^{-1}, and the above equations were then solved for the values of J_{max} that provided the best fit for the entire A- c_i curve for each tree. The current analysis assumes that the concentration of CO_2 at the chloroplast surface (c_{chl}) is equal to that at the intercellular air space (c_i). Recent data suggest that many tree species may have low internal conductances to CO_2 diffusion (Epron et al. 1995) so the estimated maximum rates of *in situ* carboxylation ($V_{c\,max}$) and electron transport (J_{max}) used here represent 'apparent' maximum values.

Treatment of the data

We initially tested for differences in A- c_i response curves among plots to determine if further analysis of derivative parameters such as A at a given c_a or $V_{c\,max}$ was warranted. Differences in A- c_i response curves between treatments are typically evaluated by testing for differences in individual component parameters of the response, which may lead to increased probability of statistical errors if the sample size is small relative to the number of parameters tested for differences (Sokal and Rohlf 1995). To avoid this problem, we used a statistical comparison of fitted curves (Potvin et al. 1990) to test for overall differences in fits to these response curves among plots. The entire field A- c_i response curve was fitted to a rectangular hyperbola model

(Ellsworth et al. 1995). Coefficients of determination (r^2) for the least-squares non-linear model fits to individual tree *A*- c_i response curves were 95-98%. Comparisons of the fitted response curves were made following Potvin et al. (1990) by fitting the complete data set to the model, and by fitting combined data for all trees within each plot to the model. Coefficients of determination (r^2) for the least-squares model fits for $V_{c\,max}$ were 94-99%. For linear regression relationships on $V_{c\,max}$ and leaf N, we tested for differences in regressions between Ambient and FACE trees using orthogonal contrasts. Analysis of covariance was carried out using foliar nitrogen per unit surface area (N_a) as a covariate. Linear and non-linear regression fits were carried out in SAS (version 6.08, SAS Institute, Cary, N.C.). The data were tested for homogeneity of variances and met the assumptions for regression analyses.

Regression analyses were conducted assuming that leaves from separate individual trees were approximately independent for the purpose of examining functional relationships between photosynthesis and leaf nitrogen content. The rationale for this treatment of the data is that the fundamental biological unit of response is the individual (see also Hättenschwiler and Körner 1996), bearing in mind that the prototype FACE array itself was not replicated and hence the possibility of unanticipated effects of the facility itself cannot be entirely excluded. However, no differences between physiological characteristics of foliage in the FACE plot compared to trees in Ambient plots were observed in a previous study (Ellsworth et al. 1995), and stand age and genetic stock, soil type, and management history were the same in the FACE and Ambient stands that we compared.

In the research forest, tree-to-tree variation in *A* and in foliar N (mass or area basis) among adjacent trees was generally large relative to local plot-to-plot variation within the stand. For N concentration of foliage from the same upper-canopy whorl, the tree-to-tree coefficient of variation was 11% (n=22 Ambient trees among 5 different plots at least 50m from one another) whereas the plot-to-plot coefficient of variation was 5% (n=5 plots). Therefore use of individual trees in the analysis provides information on an important component of overall variation of foliar N and photosynthesis within the forest stand. The spatial de-correlation observed for foliar N suggests that an appropriate experimental unit for leaf-level studies can be the individual tree since this may capture the largest source of variability in fine-scale physiological parameters (e.g., leaf-level photosynthetic parameters). Greater sample sizes will only be achieved as better access to forest tree canopies becomes available (Lowman and Nadkarni 1995).

Analysis of foliage carbohydrate and protein content

Since leaf N is only a proxy for leaf protein content in photosynthesis-N correlations, we also analyzed foliage for protein content using sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) and western blotting. For these analyses, approximately 5g of upper-canopy pine foliage was collected from sample branches for gas exchange on 6 trees in each plot (FACE plot and Ambient plot) on a sunny, warm day in early October and immediately immersed in liquid nitrogen. Foliage samples were taken from the terminal shoots corresponding to those selected for photosynthesis measurements and stored at -70°C until analysis. The samples were powdered in liquid

nitrogen with a mortar and pestle, and proteins were extracted using 10% trichloroacetic acid with 0.07% (v/v) 2-mercaptoethanol in cold 80% acetone (Nie et al. 1993). The extracted proteins were air-dried and solubilized in 0.1M Tris at pH 8.0, 10% sucrose, 2% SDS, 0.009% dithiothreitol. Total protein concentrations were measured using the bicinchoninic acid method (Smith et al. 1985) in aliquots precipitated with 72% trichloroacetic acid/ 0.075% deoxycholate and redissolved in 5% SDS/0.1M NaOH.

Equal amounts of proteins (25 μg) were separated according to their molecular masses by gel electrophoresis. Identical gels were either stained with Coomassie Brilliant Blue or transferred to 0.2 μm nitrocellulose membrane by electroblotting for 1 hour at 100V, 450 mA in Tris-glycine buffer containing 20% (v/v) methanol (Towbin et al. 1979). Blank and standard lanes were run on each gel. The blots were challenged sequentially with monoclonal antisera raised against either Rubisco from wheat or the light-harvesting protein complex (LHC) from pea, followed by the appropriate peroxidase conjugate. The colorimetric reaction was developed using 4-chloro-1-naphthol and scanned by laser densitometry (Model 300B Computing Densitometer, Molecular Dynamics, Sunnyvale, CA). Measures of optical density of immunoreactive bands of foliar samples relative to background values were obtained from volume integration of replicate samples.

Non-structural carbohydrates were assayed in the same foliage samples used for the protein analysis. Following a hot ethanol extraction, the starch fraction of the sample was digested by enzymatic reagents and analyzed colorimetrically. Soluble sugars

(sucrose and hexoses) in the extractant were assayed according to Hendrix (1993) using microtiter plates and plate reader.

Results

Photosynthesis of different foliage cohorts

Generally, two foliage cohorts are produced sequentially on upper crown branches of mature *Pinus taeda* during the growing season. The first cohort emerged in late April and was measured when it reached full elongation in July, and the second cohort was initiated in June and measured near the end of the growing season in late September-early October. There were no clear differences in phenology or foliage elongation between trees in the FACE plot compared to those in the Ambient plot, as was found by Teskey (1995).

For the 1995 first cohort of foliage (labeled '95-1' in Figures), photosynthetic capacity at a common c_a (either 350 μmol mol^{-1} or 550 μmol mol^{-1}) was similar in FACE trees and Ambient trees for this cohort (Fig. 2d). There was no significant difference between the overall A- c_i curve fits for FACE trees compared to Ambient trees ($F_{4,47}$=1.82, P>0.10; Figs. 2a, d). Leaf mass per unit surface area (M_a) was not different between upper canopy foliage in FACE and Ambient trees (Table 1), so differences in A at common c_a in FACE versus Ambient trees were maintained whether A was expressed on a mass or area basis.

Photosynthetic rates at a given c_a were similar for the first and second foliage cohorts (Fig. 2). However, for the 1995 second foliage cohort (hereafter labeled '95-2')

there was a significant difference between A- c_i curve fits for FACE versus Ambient trees (Figs. 2b, e; Table 2). For this cohort, the sample size (n=14 trees) was sufficient to evaluate plot-to-plot variation in A- c_i curves to examine if local-scale spatial variability in foliar traits within the upper canopy of the forest could entirely account for the observed differences in A- c_i curves rather than elevated CO_2. There was no significant difference between A- c_i curves for two different Ambient plots ($F_{4,79}$=2.01, P>0.10, Table 2), but the fitted curves were significantly different for the individual pairwise comparisons of FACE trees with those from either Ambient plot ($F_{4,81}$=16.07, P<0.0001; and $F_{4,88}$=11.36, P<0.0001 for FACE versus Ambient plot 1 trees and FACE versus Ambient plot 2 trees, respectively). Since A- c_i curves were similar between upper canopy foliage in the two Ambient plots, other photosynthetic parameters have been combined for these two plots in further analyses.

At a common C_a, A in the 95-2 foliage cohort was 14% lower in trees in the FACE plot compared to those in either Ambient plot (for c_a at either 350 μmol mol^{-1} or 550 μmol mol^{-1}; Fig. 2b,e). In order to eliminate possible confounding differences in gas exchange among plots owing to differences in stomatal conductance, we used the fitted A- c_i response curves in Fig. 2e to predict A at a common c_i. For calculations at a c_i of 230 μmol mol^{-1} (c_i/c_a ratio of 0.66), predicted A was 7.3 μmol m^{-2} s^{-1} for Ambient trees compared to A=6.1 μmol m^{-2} s^{-1} for FACE trees for the 95-2 foliage cohort. The instantaneous photosynthetic enhancement ratio (ratio of A_{550} to A_{350} for a given needle fascicle) was approximately 1.60 among all plots and was similar in FACE and Ambient trees despite differences in A at a common C_a. The net result is that when A at growth

c_a is compared for this foliage cohort, A_{550} in FACE trees was 39% higher than A_{350} in Ambient trees (Table 1).

For trees which had been measured in July in both 1995 and 1996, there was no significant year-year difference in fitted A- c_i curves (n=6, P>0.10) for the first cohort of foliage. However, the fitted curves for the first foliage cohort in 1996 were significantly different in FACE trees compared to Ambient trees ($F_{4,74}$=9.8, P<0.001). In 1996, greater sample size (n=4 trees per plot compared to n=3 trees in 1995) added statistical power. Differences in fitted A- c_i curves for FACE versus Ambient trees were found for this foliage cohort (Figs. 2c, f). The limited sample size of trees in some studies (i.e. Ellsworth et al. 1995, Murthy et al. 1996) should promote some caution in extrapolating inferences from such studies to larger populations, although at present there is a paucity of such data for actual forest canopies.

Comparing the 95-2 foliage cohort in FACE versus Ambient trees, $V_{c\,max}$ was 18% lower in elevated CO_2-grown trees (Table 1) and J_{max} was 16% lower (data not shown). Also, linear regressions using the initial slope of A- c_i response curves showed significant difference in slopes (P<0.01) between FACE and Ambient trees for this foliage cohort but not for 95-1 foliage, supporting results from analysis of the model parameter $V_{c\,max}$. Foliar N_a was similar between FACE and Ambient trees in all of the foliage cohorts measured, and there were also no differences in foliar N concentration (Table 1). Using a larger population of trees from 4 different reference plots located 80 to 200m from the FACE plot in different compass directions, upper-canopy foliar N

concentration was similar in Ambient trees (12.0 ± 0.3 mg g^{-1} for n=20 trees) and trees in the FACE plot (11.9 ± 0.5 mg g^{-1} for n=8 trees).

Leaf-level photosynthesis versus nitrogen

There was a weak but significant relationship between A_{350} and foliar nitrogen content (N_a) among the three successive cohorts of foliage ($P<0.0015$, $r^2 = 0.28$; data not shown). The low goodness of fit for this relationship can be attributed to variability in stomatal conductance such that A_{350} varied in different foliage cohorts and different trees. Hence a more robust relationship is that between $V_{c\,max}$ (as a measure of intrinsic foliar carboxylation capacity) and N_a. For the three foliage cohorts measured, $V_{c\,max}$ was significantly correlated with N_a among all trees although there was a tendency for lower $V_{c\,max}$ in FACE trees than Ambient trees for a given N_a (Fig. 3). There was no significant difference in the slope of the relationship between $V_{c\,max}$ and N_a for Ambient trees compared to FACE trees ($P>0.10$, separate slopes analysis). Using analysis of covariance with N_a as the covariate, a significant offset in the relationship between $V_{c\,max}$ and N_a ($P<0.0001$) was detected for FACE versus Ambient trees (Fig. 3). $V_{c\,max}$ was also significantly correlated with leaf N concentration among all trees and plots ($r^2=0.43$, $P<0.0001$). Based on this analysis, for a given N_a within the range of measured values, the least-squares estimate of $V_{c\,max}$ was 17% lower in FACE trees compared to Ambient trees. This is in agreement with the calculated difference in arithmetic means (15% among cohorts) between FACE and Ambient trees from data in Figure 2 b,c.

Leaf proteins

No difference in total soluble protein content in 95-2 foliage cohort was observed between Ambient and FACE trees (data not shown). Western blotting showed that Rubisco was lower as a proportion of leaf protein content for FACE compared to Ambient trees (Fig. 4). The integrated density of the whole specific band for each protein for elevated CO_2 samples was expressed as a percentage relative to the intensity of the lane from the ambient-grown trees for each specific gel run. Relative to samples from Ambient trees, FACE trees showed $26 \pm 4\%$ lower Rubisco content as a proportion of soluble proteins. Since there was no difference in M_a or in total protein content per unit area between FACE and Ambient trees, Rubisco content per unit leaf surface area was similarly 25% lower in FACE versus Ambient trees. Similar results were obtained when the 96-1 foliage was analyzed (data not shown). LHC content of FACE tree foliage was $17 \pm 9\%$ lower than Ambient foliage for the 95-2 foliage cohort.

Discussion

The canopy volume exposed to elevated CO_2 in this forest free-air CO_2 enrichment prototype study is two orders of magnitude larger than has been previously been attempted with open-top chambers, and nearly four orders of magnitude larger than chamber-enclosed branches. There has been relatively little experimentation with elevated CO_2 effects on entire mature trees despite recognition of the important differences between mature and juvenile stage trees that can affect plasticity of

physiological responses to elevated CO_2 and the overall sensitivity of tree growth and allocation (Ceulemans and Mousseau 1994, Lee and Jarvis 1996). Mature trees differ from juveniles in a number of respects such as carbon allocation patterns, magnitude of carbon sinks for growth and maintenance, and structural complexity (Hanson et al. 1994, Lee and Jarvis 1996). Elevated CO_2 responses of seedlings can in part be attributed to indirect CO_2 effects like shifts in allometry and size-dependent demands on nutrient and water availability (Coleman et al. 1993, Will and Teskey 1997). It is not yet clear to what extent data from tree seedlings can be 'scaled-up' and provide understanding of responses of older, more complex trees.

'Sun' leaf M_a values in mature trees in our study (Table 1) were more than 35% greater than values typically reported for fully-elongated foliage near the end of the growing season for saplings of this species in open-top chamber studies (Tissue et al. 1996). For *Pinus taeda*, Greenwood (1984) found that shoot growth and leaf M_a differed significantly in scions taken from one-year seedlings compared to those from 9- and 12-year individuals when grafted onto common root stock, indicating that this trait is developmentally fixed. M_a is an important leaf parameter related to leaf-level photosynthetic N relations (Reich and Walters 1994) and canopy carbon allocation, canopy leaf area ratio and net assimilation rate (Poorter and Remkes 1990, Reich et al. 1992). However in addition to the effect of juvenility, open-top chambers reduce radiation received by the plant and may alter the light regime sufficiently to influence M_a. Still, known differences in this and other key leaf and canopy traits between juvenile and mature trees (*e.g.*, see Dougherty et al. 1994, Hanson et al. 1994) could

affect the magnitude of tree responses to elevated CO_2. Hence caution must be taken by modelers in using appropriate data in efforts toward evaluating elevated CO_2 effects on tree canopy processes and carbon sequestration rates in forests beyond the establishment stage.

The relationship between leaf photosynthetic capacity at ambient CO_2 and leaf N content (either leaf N concentration or N_a) is considered to be a general functional relationship within and among C_3 plant species (Field and Mooney 1986, Evans 1989). Since N can be considered a proxy for leaf photosynthetic proteins, a close relationship between leaf carboxylation capacity ($V_{c\,max}$) as an indicator of the activity of Rubisco enzyme and N_a is predicted but has rarely been examined in native plants in the field. Long-term elevated CO_2 may not only affect leaf N but also the allocation of N among photosynthetic components within a species, although to date few studies have examined this relationship. For crops, intrinsic carboxylation capacity as a function of leaf N may (Harley et al. 1992) or may not (Nijs et al. 1995) be affected by long-term elevated CO_2. Our results suggest that for mature *Pinus taeda* this functional relationship does differ from ambient-grown foliage with CO_2 exposure in FACE across a range of N values common in this species in the field (Fig. 3). The reduction in carboxylation efficiency ($V_{c\,max}$) suggests changes in intrinsic nitrogen-use efficiency in elevated CO_2 foliage that could come about as a result of changes in internal partitioning of N to carboxylation functions. In support of this hypothesis, we have observed reductions in Rubisco protein per unit leaf total protein content (Fig. 4). However our study is inconclusive with respect to the absolute magnitude of CO_2-

induced adjustments of photosynthetic proteins because this depends on both the differences in N between these populations and the differences in underlying physiology as indicated by $V_{c\,max}$ per N_a. Still, at a common N_a status, the results indicate significant differences in caboxylation capacity at ambient c_a between leaves developed under ambient and elevated CO_2.

The observed differences in A at a common c_a for elevated CO_2-grown plants compared to counterparts in ambient CO_2, in A- c_i curve characteristics, and in photosynthetic proteins collectively represent evidence that changes in photosynthetic capacity (on the order of ≈15-20%) may be possible in mature loblolly pine trees growing under elevated CO_2 in the field. Since we did not observe overall differences in M_a or N_a (Table 1, Table 3) in FACE versus Ambient foliage, dilution of N by carbohydrate accumulation was not large enough to be detected in these parameters. The relative magnitude of photosynthetic adjustments in FACE trees versus Ambient trees is similar regardless of the nearby Ambient plot to which trees are compared (Fig. 2, Table 2) and of the N status of the foliage (Fig. 3).

While the mature trees in this study developed under ambient CO_2 conditions for 11 growing seasons prior to the 3 seasons of elevated CO_2 exposure, it is clear that the elevated CO_2 perturbation stimulates large photosynthetic enhancements but that these enhancements may be partly offset by internal physiological changes manifested after long-term seasonal CO_2 exposure. While it is unclear at what point such a system would function similarly to one continuously raised under elevated CO_2, achieving a full evergreen canopy of foliage produced under elevated CO_2 can be a reasonable first

approximation for examining functional responses of maturing forest trees in the absence of a full 14-year continuous CO_2 exposure of a forest (see Körner 1995). A very long-term FACE study (>10 years in duration) would be necessary to more carefully evaluate what ecophysiological feedbacks are not manifested in 2-3 growing seasons of CO_2 exposure. Despite the lack of continuous, all-year CO_2 exposure in this study, the effects of the CO_2 perturbation were examined only for current-year foliage exposed to elevated CO_2 since early needle intiation, and further work is also needed to understand possible seasonal differences in responses of evergreen foliage to elevated CO_2.

There are potentially confounding effects of the FACE facility and exposure to elevated CO_2 that must be considered when evaluating our study. Such possible effects on *Pinus taeda* foliage can be discounted for several reasons. While statistically significant effects of air injection into the FACE array on forest microenvironment have been observed under certain very stable atmospheric conditions (He et al. 1996), these effects are small in magnitude, relatively rare in terms of meteorological occurrences, and far removed from photosynthetic physiology. It is also possible that construction of the facility itself differentially affected foliar properties of the existing trees in the FACE plot relative to those of Ambient plots. However, this is unlikely since i) facility construction occurred in 1993 but afterward FACE and Ambient trees were found to be similar in terms of leaf photosynthetic and nutritional characteristics in this study and a previous one (Ellsworth et al. 1995, Ellsworth, *unpubl. data*), ii) possible effects of construction on the periphery of the FACE ring were at least 10m away from any of the

trees studied, and iii) site impacts such as localized digging and soil compaction typically result in changes in foliar nutrient status (Binkley 1986) although even at a common foliar N status, photosynthesis was different in FACE versus Ambient trees (Fig. 3). Considering the large and well-characterized effect that elevated CO_2 has on many components of tree physiological function (Bowes 1991, Amthor 1995), we suggest that the responses we observed may represent responses to elevated CO_2 manifested with increasing exposure duration. Future work needs to be directed at testing this hypothesis for non-chamber-grown field plants such as evergreen trees.

The occurrence, physiological mechanisms, and ecological determinants of photosynthetic adjustments to elevated CO_2 have been discussed extensively (see Sage et al. 1989, Bowes 1991, Bazzaz et al. 1993, Gunderson and Wullschleger 1994, Sage 1994) although an understanding of these adjustments with long-term CO_2 exposure remains incomplete. Alterations of N use and N partitioning among photosynthetic and non-photosynthetic components of leaf functioning are hypothesized to occur with long-term exposure of leaves to elevated CO_2 (Bowes 1991, Woodrow 1994). In a number of species grown under N limitation, Rubisco concentrations decrease in elevated CO_2 which may allow utilization of N in other photosynthetic proteins (Sage 1994, Webber et al. 1994, Epron et al. 1996, Lewis et al. 1996). Do the differences in $V_{c\,max}$ and photosynthesis per unit N observed for FACE vs. Ambient foliage in our study represent 'optimal' reallocation of leaf N between the $V_{c\,max}$ and J_{max} functions predicted by the Farquhar et al. (1980) photosynthesis model? This reallocation of N is hypothesized to permit greater efficiency in utilization of N-resources in leaves growing

under elevated CO_2 conditions (Sage 1994, Drake et al. 1997). Our data suggest that under optimal conditions for photosynthesis at c_a = 550 μmol mol^{-1} in *Pinus taeda*, leaf c_i remains near the responsive, initial carboxylation-limited portion of *A*- c_i curve (Fig. 3) such that photosynthesis is not ribulose bisphosphate (RuBP) regeneration-limited (sensu Farquhar and von Caemmerer 1982). While any change in operational c_i of leaves will shift *A* closer to this transition point, we calculate that c_i would need to reach nearly 500 μmol mol^{-1} for modeled RuBP regeneration limitations to predominate for *A*- c_i curves of the form shown (Fig. 3). This would require exposure to elevated CO_2 >700 μmol mol^{-1} at CO_2 supply rates characteristic of loblolly pine (Ellsworth et al. 1995, Ellsworth, unpubl. data). Foliar J_{max} and LHC proteins also decrease in elevated CO_2 (Ellsworth, unpubl. data, and Fig. 4) as has been commonly observed in other studies (Sage 1994). The gas exchange and protein content evidence suggests that leaf N resources are not reallocated from Rubisco to increase RuBP regeneration by enzymes in the photosynthetic carbon reduction cycle, as would be predicted if a functional balance of biochemical limitations to photosynthesis in upper canopy foliage was maintained. However, this does not preclude the possibility that N is reallocated from Rubisco to other key proteins or tissues within the tree.

Reduced *in situ* carboxylation capacity ($V_{c\,max}$; Fig. 3) coincident with decreased foliar Rubisco in trees in FACE versus Ambient plots (Fig. 4) indicates that photosynthetic adjustments occurred largely in response to changes in the amount of this photosynthetic protein and/or its activation state. Since all measurements were made in the morning or on cloudy days, possible short-term feedback inhibition effects

of carbohydrates on photosynthesis were minimized. The adjustments observed in the second foliage cohort were also maintained in the first cohort of foliage produced in the following growing season (Fig. 2c), suggesting that the observed adjustments in photosynthesis and Rubisco may not be transient and can occur during active growth. These findings are qualitatively consistent with data for saplings of the same species grown for 3 years under chamber conditions (Lewis et al. 1996), although recent chamber studies of single branches on maturing *Pinus taeda* did not find significant photosynthetic adjustments in a single or multiple growing seasons (Teskey 1995, 1997, Murthy et al. 1996). Our foliar gas exchange data suggest a possible mode by which photosynthetic adjustments may occur in maturing trees exposed to elevated CO_2 under actual forest conditions that is not explained by changes in foliar N content alone. The data suggest a decrease in intrinsic photosynthetic N use efficiency in elevated CO_2 that could have important implications for the sustainability of growth enhancements in forests limited by N availability in a future, high CO_2 atmosphere. While the capacity for adjustments in photosynthesis under elevated CO_2 are possibly exaggerated in a large perturbation study compared with those occurring under gradual rise in atmospheric CO_2 concentration, the perturbation approach in a mature forest can provide much-needed information relevant to this scale (Körner 1995, Carpenter 1996). Marshall and Monserud (1996) recently presented indirect evidence of physiological adjustments in conifer trees with the recorded rise in atmospheric CO_2 concentration in this century.

Since foliage properties can differ greatly from juvenile to mature stages, results from seedling studies do not entirely apply to mature tree crowns and canopies although many mechanistic relationships may remain similar through plant development. Our results should not be taken to indicate that chambered branch studies are not an appropriate experimental model for understanding canopy-level processes of mature trees under elevated CO_2. We instead argue that multiple scales of CO_2 exposure from < 0.5 m^3 branch chambers to the ~5000 m^3 canopy volume in FACE can yield useful information for understanding leaf to canopy-level processes in forests. Qualitative differences in CO_2 responses of a given tree species to differences in branch-level versus whole-tree exposures to CO_2 suggest that whole-canopy feedbacks can be important (i.e., this study; also Marek and Kalina 1996), but carefully-controlled studies are necessary to fully resolve this issue. Studies at appropriate scales for ecological feedbacks in forest ecosystems provide crucial checks for results at reduced scales.

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TABLES

Table 1. Summary of needle net photosynthesis (A), modeled carboxylation capacity ($V_{c\,max}$), modeled photosynthetic electron transport capacity (J_{max}) and related leaf traits of mature loblolly pine (*Pinus taeda*) measured at growth CO_2 concentration (c_a) for FACE and Ambient trees in 1995 and 1996. Data are means ± s.e. among trees within Ambient and FACE plots for the first foliage cohort in each year that were measured in a two-week period in late July-early August (95-1 cohort: N=3 for each plot; 96-1 cohort: N=4 for each plot).

Parameter	Ambient (95-1)	FACE (95-1)	Ambient (96-1)	FACE (96-1)
A (μmol m^{-2} s^{-1}) at growth c_a	6.8 ± 0.4	9.4 ± 0.4	6.5 ± 0.3	8.6 ± 0.6
$V_{c\,max}$ at 30°C (μmol m^{-2} s^{-1})	28.1 ± 0.5	25.8 ± 0.8	34.0 ± 3.0	29.4 ± 1.9
J_{max} at 30°C (μmol m^{-2} s^{-1})	61.5 ± 5.5	55.7 ± 1.3	63.0 ± 5.2	54.9 ± 3.4
M_a (g m^{-2})	70.2 ± 1.7	78.1 ± 3.1	76.0 ± 3.3	87.6 ± 3.5
N concentration (mg g^{-1})	10.6 ± 0.4	9.5 ± 0.3	12.2 ± 0.3	1.10 ± 0.6

Table 2. Summary of statistical results from non-linear curve fitting to A- c_i responses for the second foliage cohort of loblolly pine (95-2 foliage).

Source	d.f.	*F*-value	*P*
Among plots	8, 124	9.77	0.0001
Between FACE and Ambient	4, 128	16.88	0.0001
Between Ambient plots	4, 79	2.01	N.S.

Table 3. Average M_a, total leaf non-structural carbohydrate content (TNC), and M_a corrected for non-structural carbohydrates (M_a') for mature 96-1 foliage of loblolly pine under ambient CO_2 or elevated CO_2 in FACE (n=3 for each treatment). Data are means ± s.e.

	M_a ($g\ m^{-2}$)	TNC (%)	M_a' ($g\ m^{-2}$)
Ambient	81.7 ± 1.9	6.7 ± 0.2	76.2 ± 1.9
FACE	94.7 ± 10.9	8.1 ± 0.6	87.0 ± 10.8

FIGURES

Figure 1. Contour plot of spatial variability in season-long average daytime CO_2 concentration (units: μmol mol^{-1}) in the FACE prototype. The dark box in the center is the central tower used as the measurement platform and the square around it indicates the location of the trees that were measured near the ring center.

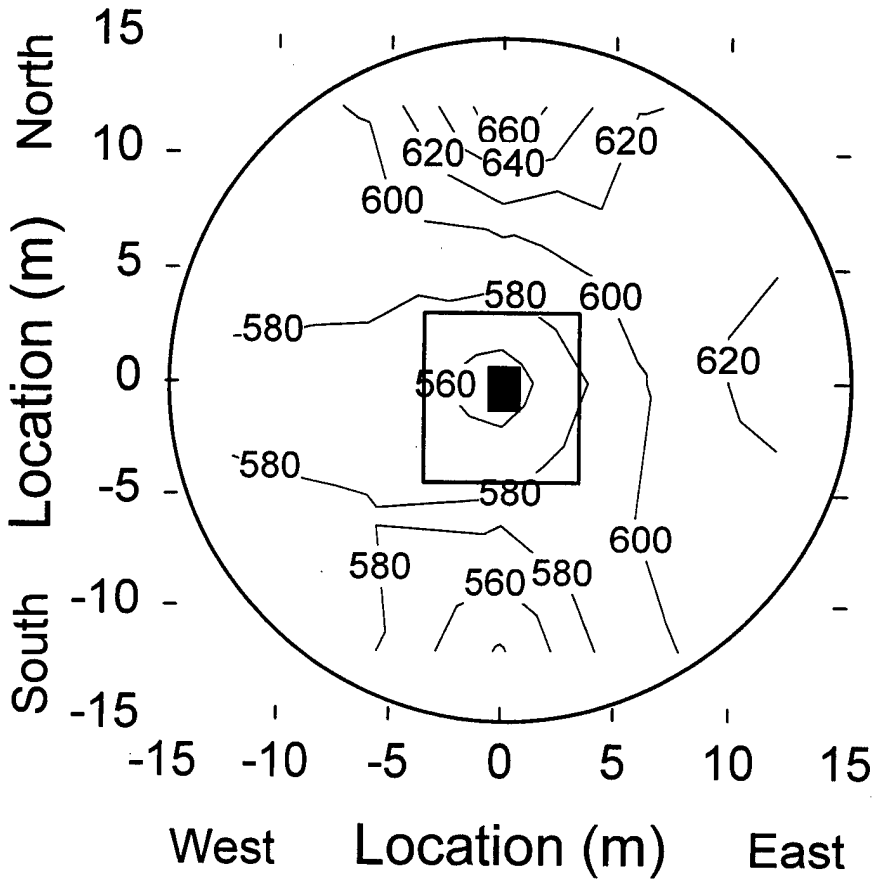


Figure 2. *A*- c_i response curves for upper canopy foliage cohorts of loblolly pine that developed under ambient and elevated CO_2 (FACE). All measurements were made at leaf temperature=30°C and VPD < 1.5 Pa kPa^{-1} on foliage at 10 m height in the canopy. (a) *A*- c_i curves from trees in Ambient plots (open symbols) and in the FACE plot (closed symbols) for 95-1 foliage. Different symbols denote individual trees. (b) *A*- c_i curves of foliage in Ambient plots versus the FACE plot for 95-2 foliage. (c) *A*- c_i curves of foliage in Ambient plots versus the FACE plot for the first cohort of 1996 (96-1 foliage). (d) Fitted *A*- c_i curves for the first cohort of 1995 foliage. Arrows indicate the mean operational c_i for Ambient and FACE trees at growth C_a. (e) Fitted *A*- c_i curves for the second cohort of 1995 foliage. Curves for two different Ambient plots are shown. Curve analyses are summarized in Table 1. (f) Fitted *A*- c_i curves for the second cohort of 1995 foliage.

Figure 2.

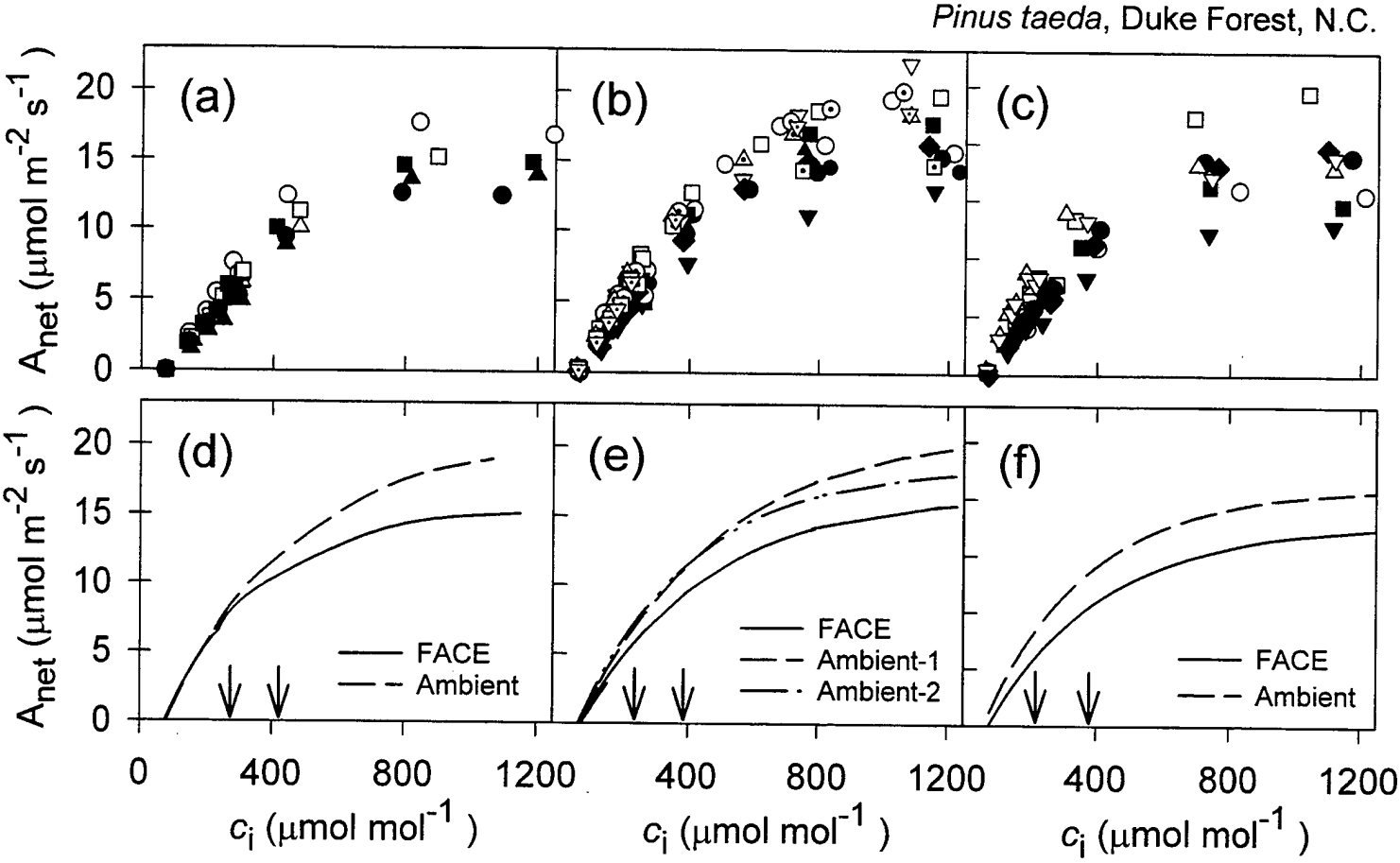

Pinus taeda, Duke Forest, N.C.
(a)
(b)
(c)
(d)
(e)
(f)
A_{net} (μmol m^{-2} s^{-1})
c_i (μmol mol^{-1})
FACE
Ambient
Ambient-1
Ambient-2
0
5
10
15
20
400
800
1200

Figure 3. Correlation between foliar nitrogen content (N_a) and modeled carboxylation rate based on gas exchange analysis ($V_{c\,max}$) based on data taken at a leaf temperature of 30°C. Data is shown for three successive cohorts of upper-canopy foliage measured when each was near full elongation at age 3-4 months. Open symbols indicate data from Ambient trees; closed symbols are for trees in the FACE plot. Different symbol shapes are used to denote different cohorts of foliage. The regression lines shown were significantly different with a 17% offset, but the slope of the lines for Ambient foliage versus FACE foliage was not significantly different (P>0.10). Lines shown are described as follows. Ambient: $Y = 9.2 + 0.29*X$, $r^2 = 0.31$, $P < 0.001$. FACE: $Y = 16.7 + 0.29*X$, $r^2 = 0.69$, $P < 0.0001$.

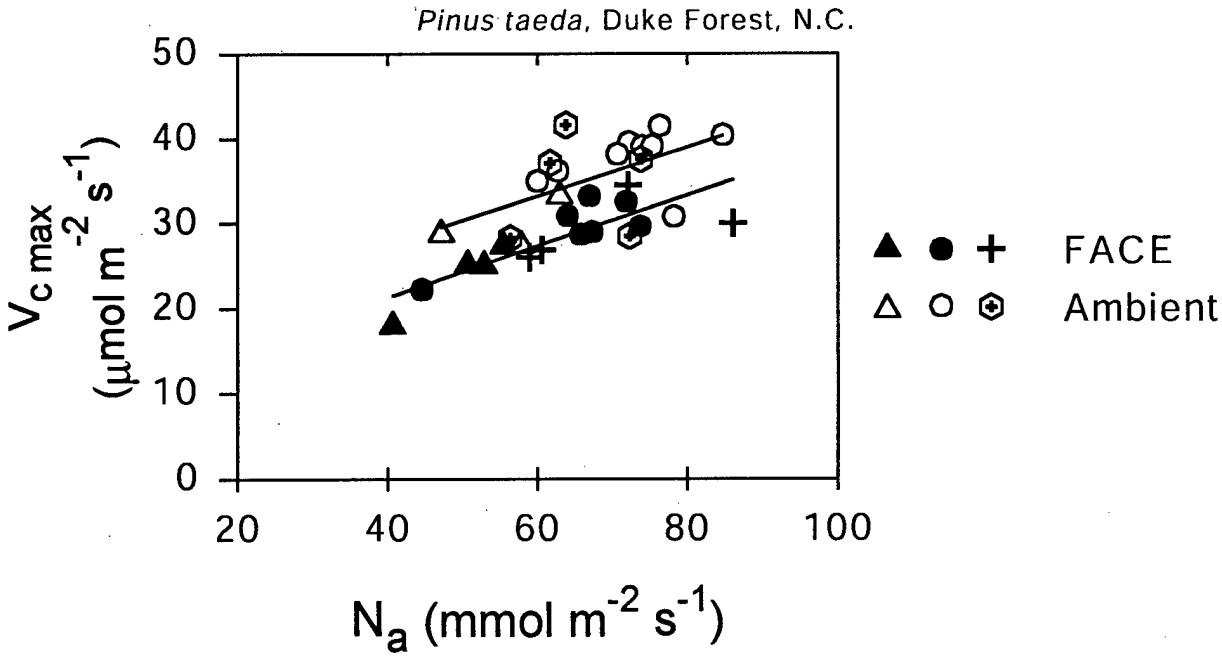
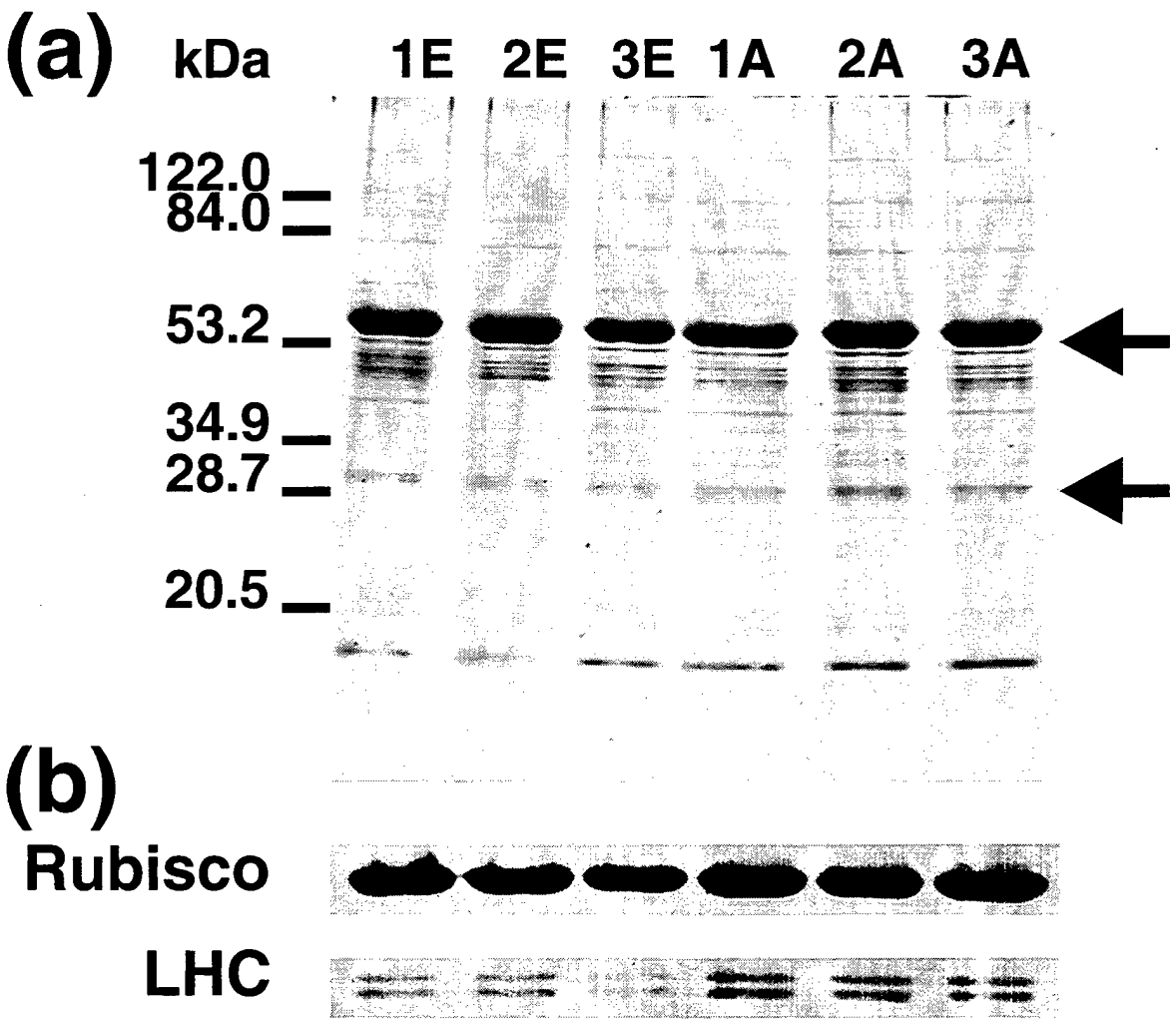


Figure 4. Immunodetection after SDS-PAGE and electroblotting of proteins extracted from *Pinus taeda* foliage grown in ambient CO_2 (A; trees from Ambient plot) or elevated CO_2 (E; trees from FACE plot). Shown are three of the six trees per treatment that were analyzed. (a) Coomassie stained polyacrylamide gels showing total protein profiles. Upper and lower arrows indicate Rubisco and LHC proteins, respectively. Samples were loaded on an equal unit area basis. Prestained molecular weight markers are shown on the left in kDa. (b) Immunodetection of Rubisco and LHC on Western blots loaded on a per unit protein basis. Lanes are as in (a).


(a)
kDa
1E
2E
3E
1A
2A
3A
122.0
84.0
53.2
34.9
28.7
20.5
(b)
Rubisco
LHC

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