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Elevated atmospheric CO_2 differentially affects needle chloroplast ultrastructure and phloem anatomy in *Pinus palustris*: interactions with soil resource availability

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ABSTRACT

The response of forest species to increasing atmospheric CO_2, particularly under resource limitations, will require study in order to predict probable changes which may occur at the plant, community and ecosystem levels. Longleaf pine (*Pinus palustris* Mill.) seedlings were grown for 20 months at two levels of CO_2 (365 and 720 μmol mol^{-1}) in two levels of soil nitrogen (4 and 40 g m^{-2}), and with two levels of soil moisture (–0·5 and –1·5 MPa xylem pressure potential). Leaf tissue was collected in the spring (12 months exposure) and autumn (20 months exposure) and examined using transmission electron microscopy (TEM) and light microscopy. During early spring, elevated CO_2 magnified effects of N and water treatment on starch accumulation and in some cases contributed to altered organization of mesophyll chloroplasts. Disruption of chloroplast integrity was pronounced under elevated CO_2, low N and water stress. In autumn, needles contained little starch; however, chloroplasts grown under high CO_2 exhibited stress symptoms including increased plastoglobuli and shorter grana. A trend for reduced needle phloem cross-sectional area resulting from fewer sieve cells was also observed under elevated CO_2. These results suggest that, in nature, longleaf pine seedlings may not benefit from a doubling of CO_2, especially when soil resources are limiting.

Key-words: *Pinus palustris*; Pinaceae; longleaf pine; acclimation; chloroplast ultrastructure; CO_2; resource limitations.

INTRODUCTION

Carbon dioxide is a by-product of more than 80% of the energy used by the world's population (Starr, Searl & Alpert 1992). This, in concert with deforestation practices, is causing atmospheric CO_2 concentrations to increase at a rate of geological proportions (Sundquist 1993). Current levels are expected to double within the next century (Keeling *et al.* 1989).

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Doubling atmospheric CO_2 levels potentially doubles the carbon available for carbohydrate production during photosynthesis. However, growth responses may be mediated by many factors including the photosynthetic pathway, nutrient conditions, phenology, phenotypic plasticity and, in general, plant life history strategies. Poorter (1993) surveyed the literature (156 plant species) and found the average stimulation of vegetative whole-plant growth to be 37%. However, representatives of C_3, C_4 and CAM photosynthetic pathways did not respond equally, exhibiting increases of 41, 22 and 15%, respectively. Differential responses to CO_2 by different species and intraspecific variation among members of the same species threaten to change the physiognomy, genetic make-up, and function of plant communities (Bazzaz 1990; Griffin, Thomas & Strain 1993; Poorter 1993; Mitchell *et al.* 1995). In order to predict the direction and magnitude of changes which may occur in ecosystems as a direct result of rising [CO_2], we must first understand how the physiology of carbon fixation affects partitioning of assimilates throughout the plant in species representative of a wide range of life history patterns.

In some instances, increased growth in response to elevated [CO_2] is temporary, and some species are unable to realize a long-term benefit from the extra carbon supply (Yelle *et al.* 1989; Bazzaz 1990). The mechanisms behind the down-regulation in photosynthetic rates over time are not well understood. However, this acclimation phenomenon is often attributed to end-product inhibition caused by the overproduction of carbohydrates, and consequently a whole-plant source–sink imbalance. The inability of sinks to metabolize sugars at the same rate at which they are produced is known to increase leaf starch in crop and tree species (Madson 1968; Cave, Tolley & Strain 1981; Clough, Peet & Kramer 1981; Vu, Allen & Bowes 1989; Mitchell *et al.* 1995). It has been suggested that this excessive accumulation of starch may negatively affect the internal organization of chloroplasts and thus contribute to the acclimation phenomenon (Yelle *et al.* 1989). For example, Cave *et al.* (1981) observed that *Trifolium subterraneum* grown in elevated [CO_2] exhibited a build-up in chloroplast starch that appeared to alter normal chloroplast structure and disturbed the configuration of granal stacks. They also observed a reduction in chlorophyll *a* content

which contributed to leaf chlorosis. Wulff & Strain (1981) observed a reduction in photosynthetic rates of *Desmodium paniculatum* exposed to elevated [CO_2] which they correlated with increased starch accumulation and reduced grana formation.

To date, most studies on the effect of elevated [CO_2] on ultrastructure and anatomy have been conducted on annual crop species under non-limiting water and nutrient conditions at a single harvest. While this approach may be useful in the elucidation of crop responses to elevated CO_2, it is of little value in assessing the effects on perennial forest trees such as pine species, which typically grow under nutrient and water limitations (Conroy *et al.* 1990). Furthermore, because of differences inherent in gymnosperm anatomy, assimilate transport and thus the capacity to exploit extra carbon may not be analogous to that found in flowering plants. For example, flowering plants have comparatively large open pores which are typically aggregated on end walls between adjacent sieve tube members. Sieve cell pore diameters in angiosperms range from 0·5 μm in grass species to 15 μm in woody plants (Sheehy *et al.* 1995). In contrast, Murmanis & Evert (1966) reported the diameter of the openings of sieve cell pores of a representative pine species to be 0·07 μm. Furthermore, the sieve areas of gymnosperms are blocked by large aggregations of continuous tubular endoplasmic reticulum which meets between contiguous cells in the median cavity (Neuberger & Evert 1974). Dicotyledonous and some monocotyledonous species also contain large aggregations of P-protein within sieve tube members which are markedly absent in gymnosperms (Neuberger & Evert 1974). The possible functional differences reflected by the absence of P-protein and the presence of extensive ER in gymnosperms are not understood. Finally, it is generally thought that, in most species, solute movement between mesophyll cells is largely symplastic, and the extent to which symplastic transport occurs during short-distance transport may be reflected by the number of plasmodesmata connecting contiguous cells (Madore & Lucas 1989). However, mesophyll cells of pine species share very few plasmodesmata (Campbell 1972) and this implies, on structural grounds, that assimilates are transported from the sites of photosynthesis to the endodermis apoplastically. Subsequent transport through the endodermis and transfusion parenchyma and phloem loading from albuminous cells into the sieve cells proper are probably symplastic due to the high degree of plasmodesmatal connectivity (see Gamalei 1989). Körner, Pelaez-Riedl & van Bel (1995) found that, following exposure to elevated CO_2 (600 μmol mol^{-1}), species which load phloem symplastically accumulated 41% total non-structural carbohydrates compared to 25% in species which exhibit apoplastic phloem loading. Such differences in form which reflect differences in strategies for short-distance assimilate transport, phloem loading, and long-distance phloem transport may in part explain the differences in response patterns of pine species to elevated [CO_2] compared to broadleaved species. Ceulemans & Mousseau (1994) reported increases in biomass and photosynthesis of pine species to be 38 and 40% compared to increases of 63 and 61% in broadleaved species.

Longleaf pine is the keystone species in a dwinding south-eastern United States ecosystem. This ecosystem, once dominant, has shrunk from 92 million acres to 3·2 million acres (Landers *et al.* 1995) due to the suppression of fire and exploitation for timber, turpentine and resin (Peet & Allard 1994). Longleaf pine savannas are a fire subclimax ecosystem, and typically occupy xeric, N-limited sites. Longleaf pine is able to outcompete broadleaved species because of its adaptations to fire and its ability to maintain productivity when soil resource availability is unfavourable. The ability of longleaf and other pine species to compete and persist as [CO_2] continues to increase is of concern.

While several studies have looked at pine leaf anatomy (Thomas & Harvey 1983; Conroy *et al.* 1986), few studies have examined leaf anatomy under nitrogen and water limitations (Mosjidis *et al.*, Auburn University, unpublished results), and to date no study has examined the leaf ultrastructure of pine grown in elevated CO_2 in the presence of varying soil moisture and nitrogen supply. Thus, the purpose of this study was to examine the anatomy and ultrastructure of longleaf pine (*Pinus palustris* Mill.) needles grown in ambient (365 μmol mol^{-1}) and twice-ambient (720 μmol mol^{-1}) concentrations of CO_2, and with two rates of soil N and water supply. Anatomical and ultrastructural characteristics which may reflect whole-plant source–sink relationships were observed and quantified in order to predict the response of longleaf pine seedlings to future elevated concentrations of CO_2 as influenced by nutrient and water availability. Autumn and spring harvests were compared in order to determine seasonal response to CO_2 enrichment.

METHODS AND MATERIALS

Plant exposure system

Longleaf pine seedlings (mean root collar diameter = 13 mm, one standard deviation = 2) from a wild seed source were exposed to elevated (≈720 μmol mol^{-1}) or ambient CO_2 (≈365 μmol mol^{-1}) conditions beginning on 30 March 1993 and were maintained until the final harvest on 28 November 1994 in open-top chambers (Rogers, Heck & Heagle 1983). The chambers, CO_2 supply and CO_2 monitoring/dispensing systems have been previously described for this study site (Mitchell *et al.* 1995). Treatments were arranged in a split-plot design with three replications. Six open-top chambers (OTC) were used; three were maintained at ambient CO_2 levels (≈365 μmol mol^{-1}) and three at twice-ambient levels (≈720 μmol mol^{-1}). Carbon dioxide treatments (main plots) were randomly assigned to OTCs within replicates. Nitrogen and water treatments (subplots) were randomly assigned to a total of 16 containers within each OTC. Pots were randomized each month to eliminate within-chamber effects.

Seedlings were planted in a coarse sandy medium (pH 5·1) in 45 dm^3 pots. Two rates of supply of both soil N and water were employed. Nitrogen treatments (applied as sulphur coated urea, 38-0-0) consisted of 4 g m^{-2} for the low treatment and 40 g m^{-2} for the high treatment (applied at 3 month intervals) and were administered according to Mitchell *et al.* (1995). Other nutrients were maintained in non-limiting amounts by application of sulphur-coated potassium (0·04 mg K g^{-1} soil) and MicroMax™ Plus (P = 0·14, Ca = 0·57, Mg = 0·28 and S = 0·05 mg g^{-1} soil, plus a complete complement of micronutrients) at the beginning of the experiment. In April 1993, a single application of iron chelate (0·007 mg Fe g^{-1} soil) was made.

Teflon caps were fitted to the chambers in order to exclude rainfall so that soil moisture levels could be experimentally controlled. After seedling initiation (19 weeks after initiating study) water treatments were implemented. Target values for soil moisture treatments were –0·5 MPa for the well-watered treatment, and –1·5 MPa pre-dawn xylem pressure potential for the water-stress treatment. Xylem pressure potentials were determined periodically from one needle for each pot for each chamber throughout the study with a pressure bomb. Water status determined from the pressure bomb was converted into gravimetric standards so that appropriate water regimes could be maintained on a short-term basis using a pneumatic weighing device.

Electron microscopy procedures

Needles selected for the spring (12 month) harvest were chosen from a previously delineated cohort of needles which were known to have expanded entirely under experimental conditions from buds set just prior to initiation of treatments. Needles of longleaf pine abscise in their second year (Chamberlain 1941); thus, considering the length of this study, needle ontogeny and development to maturity for needles selected at the final 20 month harvest occurred entirely under imposed experimental conditions. Leaves sampled were clearly second-year needles because of the presence of secondary needle phloem.

Needle tissue was collected twice; the first harvest was on the morning of 21 March 1994 (12 months) and the second was on the morning of 28 November 1994 (20 months). Needles were selected from three needle fascicles from seedlings representing all possible combinations of N and water treatments from three chambers supplied with ambient CO_2 and three amended with twice-ambient CO_2. Segments (4 mm) were excised from the centre portion of each needle. Tissue was fixed for 2 h in 5% glutaraldehyde in a phosphate buffer (pH 6·8). Portions were then trimmed from both ends (1 mm) of each needle segment under a drop of fixative, and the remaining 2 mm was then transferred to fresh fixative for an additional 2 h. Tissues were washed three times in a phosphate buffer and postfixed in 2% osmium tetroxide at 4 °C overnight. Following postfixation, tissues were washed twice in phosphate buffer, passed through an ethanol and propylene oxide dehydration series, and then embedded in resin (Spurr 1969). Ultrathin sections from needle cross-sections were cut on an ultramicrotome (MT2-B, Sorvall Inc., Newtown, Connecticut) with a diamond knife and stained with uranyl acetate and lead citrate using standard procedures. Sections were observed using a transmission electron microscope (EM 10, Carl Zeiss, West Germany). Between six and eight chloroplasts representing different mesophyll cells within a needle segment from each replicate OTC were photographed at a primary magnification of 12 700×. Contact prints (7·6 × 12·7 cm) were used to complete counts of plastoglobuli per chloroplast, thylakoids per grana and starch grains per plastid, and starch grain and chloroplast profile (visible cross-sectional) areas per chloroplast were quantified with an image analysis system (Optimas, Bioscan Inc., Edmonds, Washington).

The extent of thylakoid stacking was quantified by counting the number of thylakoid lamellae in the highest granum per chloroplast profile. Inconsistent fixation, sectioning and embedding difficulties typical for studies on pine ultrastructure (Carde 1978; Ewers 1982) made it impractical to count the heights of all grana. However, because of the relative consistency in heights of high grana within each plastid, the comparison of the tallest granum per chloroplast provided a useful feature for assessing differences in membrane stacking characteristics between treatments. Comparison of tall grana to assess membrane stacking characteristics has been reported previously (Schiffgens-Gruber & Lütz 1992).

Preparations for light microscopy

Fully expanded needles from the autumn harvest were immersed in chloroform for 30 s. Segments 4 mm in length were excised and fixed in 2·5% glutaraldehyde and 1% acrolein in a phosphate buffer (pH 6·8) for 2 h at 4 °C. Tissues were then trimmed as described above and fixed for an additional 2 h in fresh fixative at 4 °C. Finally, tissues were washed twice in a phosphate buffer, and passed through an ethanol dehydration series, infiltrated for 6 d, and embedded in LR White medium grade plastic. Thick sections (1·5 μm) were stained for carbohydrates using periodic acid-Schiff's procedure (PAS). Cross-sections were photographed and 12·7 × 17·8 cm prints were used to count total cells within the phloem and to measure sieve cell dimensions. Total phloem cross-sectional area was determined stereologically using the Optimas image analysis system mentioned previously. The total number of cells within the phloem was determined by counting the number of cells within a given area in the central portion of active phloem using an eyepiece reticle, and then deriving a total from the total phloem area. Phloem area and total sieve cells were determined from two needles from separate trees averaged for each of three replicate OTCs. Sieve cell length and width are from two needles from separate trees with 15 cells averaged per needle from each of three replicate OTCs.

Data analysis

Data were analysed as a factorial taking into account the split-plot design. Error terms appropriate to the split-plot design were used to test the significance of treatment effects and interactions. Analysis was conducted using the GLM procedure of the Statistical Analysis System (SAS 1985). In all cases, differences were considered significant at the $P \leq 0{\cdot}05$ level.

RESULTS

Spring harvest (March 1994; 12 months exposure)

Chloroplast length showed no response to main effects of CO_2 treatment; however, chloroplast area was significantly larger for plants grown under elevated CO_2 (Table 1; see 'ch. length' and 'ch. area'). There was a significant N by water interaction for chloroplast length and area; seedlings grown in low N had larger chloroplasts (in terms of length and area) relative to those in high N when soil water was limiting. Under adequate soil moisture, seedlings grown in low N had smaller chloroplasts than those grown in conditions of high N. In general, the size of the chloroplast correlated with the magnitude of starch accumulation.

Elevated CO_2 significantly increased the accumulation of chloroplast starch. This was evident as more and larger starch grains (Table 1; see 'st. grains' and 'st. area'). Chloroplasts from needles grown in elevated CO_2 contained an average of 1·2 starch grains occupying 6·3 μm^2 per chloroplast profile. In contrast, chloroplasts from seedlings grown in ambient conditions contained 0·9 starch grains with an average cross-sectional area of 2·9 μm^2. No two-way interactions between CO_2 and either soil resource treatment were observed for these variables. However, a significant N by water interaction indicated that soil water availability modified plant response to soil N level; the nature of the interaction was similar to the pattern described above for measures of chloroplast size. That is, seedlings grown in low N had more starch (in terms of starch grain area and number of grains) relative to those in high N when soil water was limiting. Electron micrographs clearly illustrated the build-up of chloroplast starch for CO_2-enriched plants in conditions of limiting soil resources relative to their ambient CO_2 counterpart [Fig. 1; see (1) versus (3) and (2) versus (4)]. In these conditions, chloroplasts appeared swollen and distended with starch; very little stroma was evident and any visible internal membranes were distorted and forced to the periphery of the organelle. As a result of the above conditions, an accurate evaluation of the thylakoid membranes as affected by the various treatments could not be made at this harvest.

There were no significant main treatment effects or interactions for deposition of plastoglobuli within chloroplasts (Table 1; see 'pg'). However, a trend for a CO_2 by N interaction was detected ($P = 0{\cdot}06$); ambient CO_2 chloro-

Table 1. Ultrastructural observations from the spring harvest (March 1994). Means (± SD) are shown

	Chloroplast features[b]				
Treatment[a]	ch. length	ch. area	st. area	st. grains	pg
Elevated CO_2 (720 μmol mol^{-1})					
Hi N-WW	5·3 ± 0·5	9·8 ± 3·5	5·2 ± 3·3	1·3 ± 0·2	7·6 ± 7·7
Hi N-WS	5·1 ± 0·4	7·4 ± 3·6	2·8 ± 3·5	0·6 ± 0·5	10·0 ± 8·1
Lo N-WW	4·5 ± 0·6	5·6 ± 3·1	2·8 ± 3·3	0·9 ± 0·7	10·4 ± 1·9
Lo N-WS	6·4 ± 0·3	17·5 ± 5·6	14·3 ± 5·4	1·9 ± 0·5	12·2 ± 11·4
Ambient CO_2 (360 μmol mol^{-1})					
Hi N-WW	5·3 ± 0·4	8·5 ± 3·2	2·4 ± 2·0	1·1 ± 0·4	13·7 ± 3·0
Hi N-WS	4·7 ± 0·2	6·9 ± 2·3	0·3 ± 0·6	0·6 ± 0·7	23·5 ± 13·7
Lo N-WW	4·9 ± 0·8	6·0 ± 3·9	3·0 ± 3·8	0·8 ± 0·7	14·4 ± 6·3
Lo N-WS	5·8 ± 0·6	9·0 ± 4·7	5·9 ± 5·4	1·2 ± 0·1	7·9 ± 2·1
Significance levels[c]					
CO_2	NS	*	*	***	NS
N	tr	NS	*	NS	NS
W	*	NS	NS	NS	NS
$CO_2 \times N$	NS	NS	NS	NS	tr
$CO_2 \times W$	tr	NS	NS	NS	NS
$N \times W$	***	**	**	*	NS
$CO_2 \times N \times W$	NS	NS	NS	NS	NS

[a] Hi N = 40 g m^{-2} year^{-1} nitrogen, Lo N = 4 g m^{-2} year^{-1} nitrogen, WW = well watered, WS = water-stressed.
[b] ch. length = longest dimension of chloroplasts in μm; ch. area = area of chloroplast profile in μm^2; st. area = area of mesophyll chloroplast starch grain profiles in μm^2; st. grains = number of starch grains per chloroplast profile; pg = number of plastoglobuli per chloroplast.
[c] tr = $0{\cdot}05 \leq P \leq 0{\cdot}10$, * $P \leq 0{\cdot}05$, ** $P \leq 0{\cdot}01$, *** $P \leq 0{\cdot}001$.

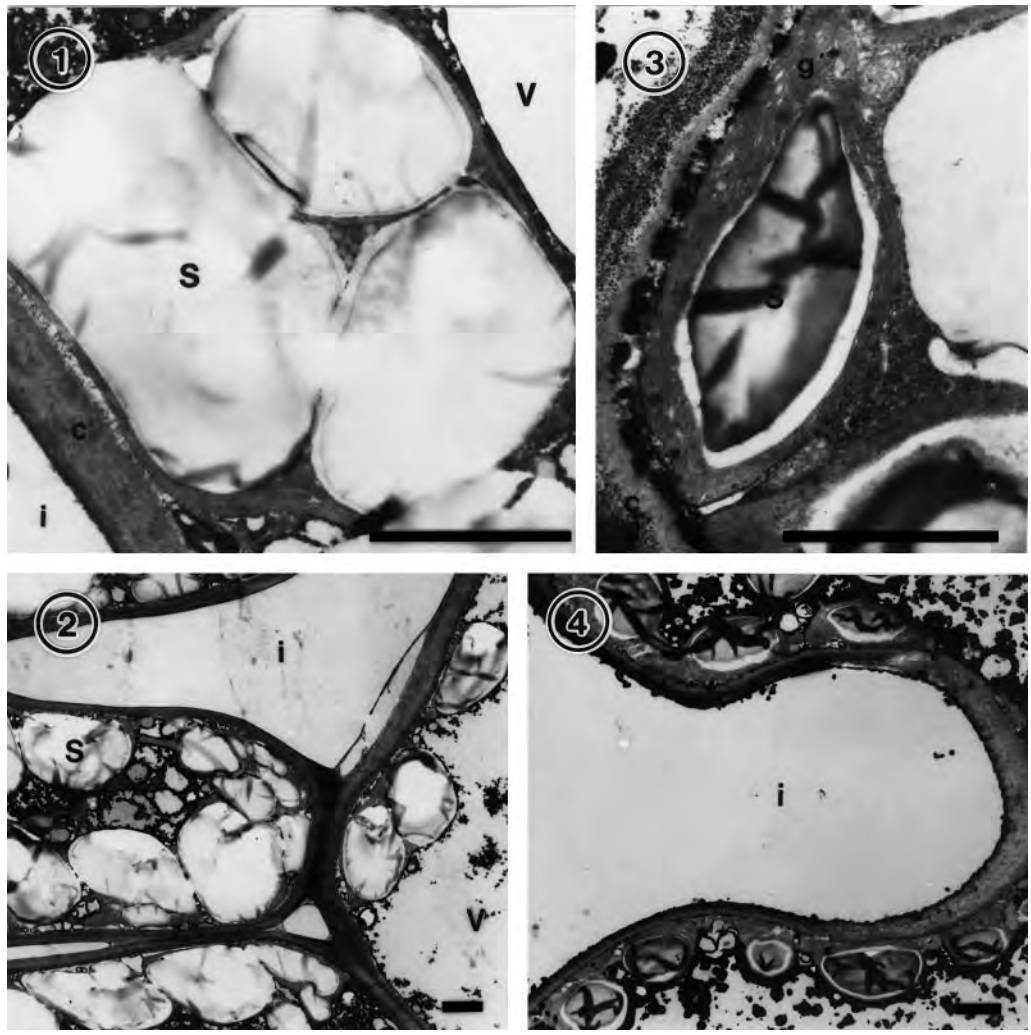

Figure 1. Electron micrographs of mesophyll chloroplasts from the spring harvest: (1, 2) 720 μmol mol^{-1} CO_2, 4 g m^{-2} nitrogen and water stressed; note the large starch grains; (3, 4) 360 μmol mol^{-1} CO_2, 4 g m^{-2} nitrogen and water stressed; note much smaller starch inclusions than in the elevated CO_2 treatment. S = starch grain, c = cell wall, V = vacuole, i = intercellular space; scale bar = 2 μm.

plasts contained more plastoglobuli than elevated CO_2 plastids in conditions of high, but not low, N.

Autumn harvest (November 1994; 20 months exposure)

There were no main treatment effects on either chloroplast length or area (Table 2; see 'ch. length' and 'ch. area'). There was a significant N by water interaction for chloroplast length and a three-way interaction for chloroplast area. In high-N conditions, chloroplasts from plants grown with water stress were shorter than their well-watered counterparts, but the difference was not great. With respect to area, chloroplasts from seedlings grown in elevated CO_2 had larger cross-sectional area relative to that found in ambient CO_2 only when both soil N and water were limiting. A similar three-way interaction was observed for starch cross-sectional area and a trend for such an interaction was also detected for number of starch grains (P = 0·06; Table 2). A significant N by water interaction for starch grain number noted at this sampling was similar to that observed in the spring. In general, low N contributed to larger accumulation of starch grains relative to high-N conditions when soil water was limiting. When water was non-limiting, chloroplasts from seedlings grown in low N contained less starch.

Although chloroplast starch accumulation patterns observed in the autumn were similar to those observed at the spring sampling, inclusions were probably not of sufficient magnitude to alter mechanically the organization of thylakoid membranes in any of the treatment combinations (Fig. 2). However, there was a significant decrease in the heights (number of thylakoid lamellae) of tall grana in the mesophyll chloroplasts due to elevated CO_2 [Table 2; see 'thylkds' and Fig. 2; (1) versus (2), and (3) versus (4)]. The mean number of thylakoid lamellae in the elevated CO_2 treatments was 11·6 compared to 14·6 in the ambient treatment. In addition, plants grown in low N had significantly

Table 2. Ultrastructural observations from the autumn harvest (November 1994). Means (± SD) are shown

	Chloroplast features[b]						
Treatment[a]	ch. length	ch. area	st. area	st. grains	%ch. sw/di	thylkds	pg
Elevated CO_2 (720 μmol mol^{-1})							
Hi N-WW	5·8 ± 0·3	7·8 ± 2·0	0·35 ± 0·41	0·24 ± 0·29	78 ± 18	13·3 ± 4·5	9·1 ± 6·4
Hi N-WS	4·5 ± 0·5	7·8 ± 1·7	0·00 ± 0·00	0·00 ± 0·00	24 ± 27	18·8 ± 6·2	23·6 ± 4·0
Lo N-WW	4·7 ± 0·7	7·4 ± 2·2	0·02 ± 0·03	0·03 ± 0·06	69 ± 47	5·5 ± 1·3	14·3 ± 4·4
Lo N-WS	5·1 ± 0·8	9·0 ± 2·7	2·44 ± 1·10	1·24 ± 0·36	75 ± 17	8·6 ± 2·0	14·0 ± 7·1
Ambient CO_2 (360 μmol mol^{-1})							
Hi N-WW	5·0 ± 0·2	6·1 ± 0·8	0·00 ± 0·00	0·00 ± 0·00	35 ± 31	20·3 ± 4·2	12·5 ± 5·2
Hi N-WS	4·7 ± 0·3	7·6 ± 1·4	0·01 ± 0·01	0·07 ± 0·08	27 ± 31	18·1 ± 6·1	13·2 ± 5·2
Lo N-WW	5·3 ± 0·7	9·6 ± 0·5	0·07 ± 0·16	0·15 ± 0·30	72 ± 21	9·6 ± 2·9	13·8 ± 2·5
Lo N-WS	5·1 ± 0·8	6·3 ± 1·7	0·60 ± 0·41	0·95 ± 0·45	46 ± 53	10·2 ± 4·7	9·0 ± 4·5
Significance levels[c]							
CO_2	NS	NS	*	NS	NS	*	*
N	NS	NS	***	***	*	***	NS
W	NS	NS	***	***	*	NS	NS
$CO_2 \times N$	tr	NS	*	NS	NS	NS	NS
$CO_2 \times W$	NS	NS	*	NS	NS	NS	*
$N \times W$	*	NS	***	***	NS	NS	*
$CO_2 \times N \times W$	NS	*	**	tr	tr	NS	NS

[a] Hi N = 40 g m^{-2} year^{-1} nitrogen, Lo N = 4 g m^{-2} year^{-1} nitrogen, WW = well watered, WS = water-stressed.
[b] Length = length of chloroplasts in μm; ch. area = area of chloroplast in μm^2; st. area = area of mesophyll chloroplast starch grain profiles in μm^2; st. grains = starch grains per chloroplast profile; %ch. sw/di = % of chloroplasts with thylakoid swelling or disintegration; thylkds = thylakoid lamellae in the highest grana per chloroplast; pg = plastoglobuli per chloroplast.
[c] tr = $0{\cdot}05 \leq P \leq 0{\cdot}10$, * $P \leq 0{\cdot}05$, ** $P \leq 0{\cdot}01$, *** $P \leq 0{\cdot}001$.

fewer thylakoids making up the tall grana than those grown in high N (mean for high N was 17·7 versus 8·5 thylakoids per granum under low N). Growth in N deficiency also increased the percentage of chloroplasts which contained either swollen or disintegrated thylakoid systems (Table 2; see '%ch.sw/di'). Chloroplasts from seedlings grown under elevated CO_2 generally tended to have a larger proportion of chloroplasts with swollen or disintegrated membranes (61% for high CO_2 compared to 45% for ambient CO_2; $P = 0{\cdot}15$). It is important to note that a trend for a three-way interaction (similar to that found for starch area and grains) was also detected for this variable ($P = 0{\cdot}06$).

The number of plastoglobuli was significantly higher in the autumn in elevated CO_2 when expressed as total plastoglobuli per chloroplast cross-section (Table 2: see 'pg', and Fig. 2), and as plastoglobuli per unit area of stroma which takes into account the area occupied by starch (data not shown). There were 15·3 plastoglobuli per chloroplast profile in the elevated CO_2 treatments compared to 12·1 in ambient treatments. In addition to the main effect of CO_2, there was a significant CO_2 by water interaction; more plastoglobuli were observed in elevated CO_2 compared to ambient CO_2 only in water-stress conditions. Nitrogen effects on plastoglobuli number were also mediated by water status. With water stress, chloroplasts from seedlings grown with N limitation contained more plastoglobuli than those grown under adequate N. In well-watered conditions, nitrogen supply had no effect on plastoglobuli deposition.

A trend indicating a reduction in phloem cross-sectional area in elevated CO_2 was observed ($P = 0{\cdot}06$). In general, the reduction in phloem area in the elevated CO_2 treatments compared to the ambient treatments was the result of fewer cells ($P = 0{\cdot}08$), not smaller cells (Table 3). Limiting soil N significantly decreased phloem area in both ambient and elevated CO_2 and in both soil moisture treatments; reductions resulting from N limitation were attributable to both fewer and smaller cells. Sieve cell diameters showed no main effects of CO_2 when measured in directions periclinal (width) and anticlinal (depth) to the plane of division of the vascular cambium. However, there was a three-way interaction for sieve cell depth. Sieve cells from seedlings grown in elevated CO_2, limiting N and adequate water were flattened in the direction parallel to the plane of division of the cambium when compared to sieve cells from seedlings grown in ambient CO_2, limiting N and adequate water. There were no visible differences in the orderly, file-like arrangement of sieve cells, phloem parenchyma, and albuminous cells resulting from main treatment effects (data not shown).

DISCUSSION

Seasonal changes in chloroplast ultrastructure have been studied for many evergreen species. The most obvious change which accompanies the transition to the winter state is reduction in size and number of starch grains

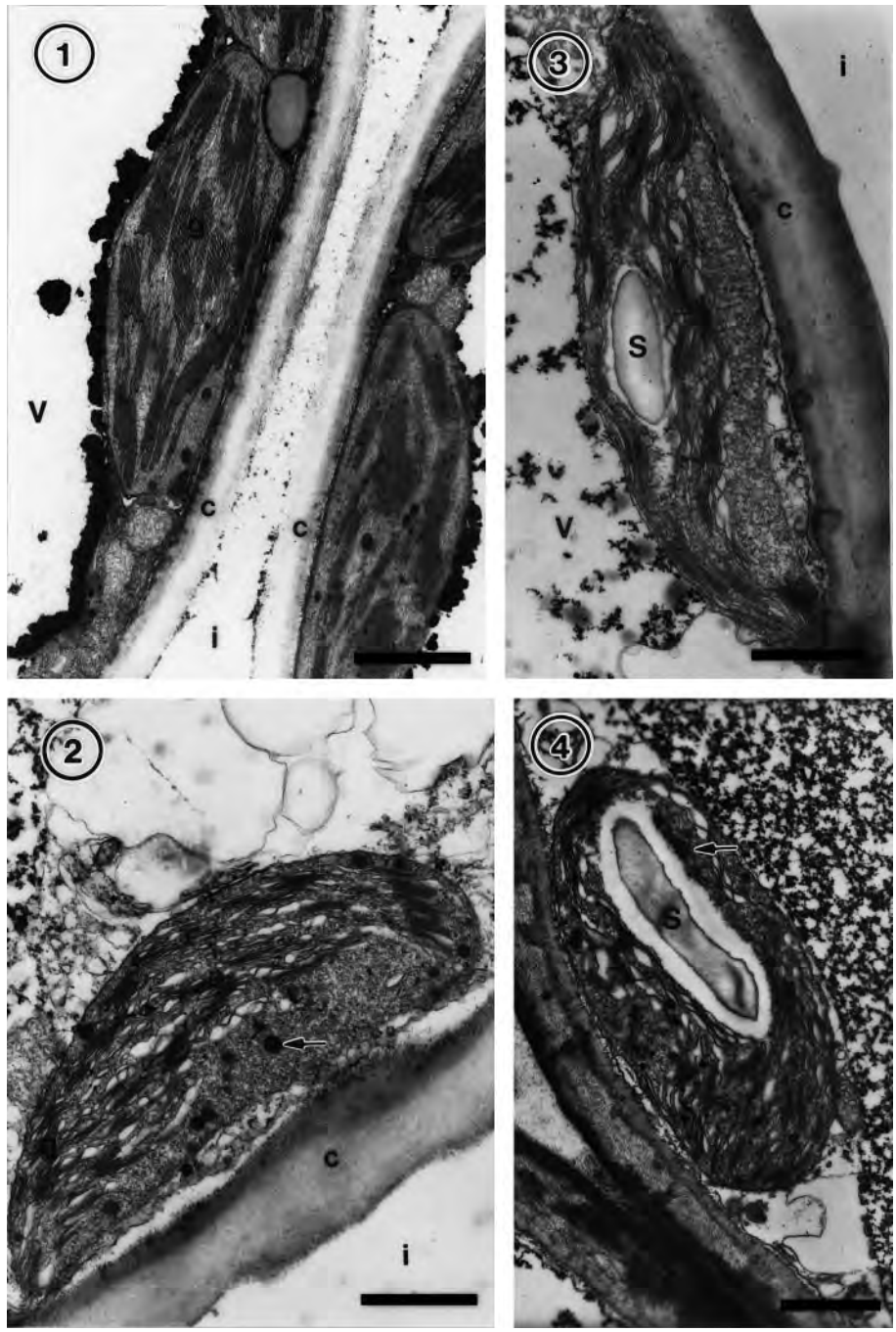

Figure 2. Electron micrographs of mesophyll chloroplasts from the autumn harvest: (1) 360 μmol mol^{-1} CO_2, 40 g m^{-2} nitrogen and well watered; (2) 720 μmol mol^{-1} CO_2, 40 g m^{-2} nitrogen and well watered; note the swelling of thylakoid membranes, and shorter grana than in the ambient CO_2 treatment above; (3) 360 μmol mol^{-1} CO_2, 4 g m^{-2} nitrogen and water stress; (4) 720 μmol mol^{-1} CO_2, 4 g m^{-2} nitrogen and water stressed; note larger starch grain and disorganized grana compared to the ambient CO_2 treatment. g = granum, S = starch grain, i = intercellular space, c = cell wall, V = vacuole, arrow = plastoglobule; scale bar = 1 μm.

(Chabot & Chabot 1975; Senser, Schötz & Beck 1975; Soikkeli 1978; Fincher 1992). This was also the case in the current study; in the spring there were starch grains in nearly all chloroplasts; however, in the autumn few chloroplasts contained visible starch grains. Elevated CO_2 affected the accumulation of chloroplast starch similarly in both spring and autumn; the difference observed between seasons was one of magnitude and not direction. Also associated with frost hardening is loss of chloroplast structure including the disintegration of internal membrane systems, swelling of thylakoids, and an increase in the number of plastoglobuli (Senser *et al.* 1975; Soikkeli 1978). In the autumn, chloroplasts from seedlings grown in elevated CO_2 exhibited reductions in membrane stacking, increased

Table 3. Phloem area, total number of phloem cells and sieve cell dimensions from the autumn harvest. Means (± SD) are shown

	Phloem characteristics[b]			
Treatment[a]	Phloem area	Sieve cell depth	Sieve cell width	Total cells
Elevated CO_2 (720 μmol mol^{-1})				
Hi N-WW	0·031 ± 0·005	5·1 ± 0·5	9·0 ± 0·6	409 ± 69
Hi N-WS	0·029 ± 0·005	4·6 ± 0·4	8·7 ± 0·9	408 ± 97
Lo N-WW	0·013 ± 0·003	4·1 ± 0·9	7·7 ± 0·7	222 ± 45
Lo N-WS	0·016 ± 0·004	4·2 ± 0·5	7·8 ± 0·6	244 ± 36
Ambient CO_2 (360 μmol mol^{-1})				
Hi N-WW	0·035 ± 0·006	4·6 ± 0·3	8·8 ± 1·1	535 ± 97
Hi N-WS	0·036 ± 0·022	4·7 ± 0·7	8·7 ± 1·3	485 ± 267
Lo N-WW	0·016 ± 0·007	4·9 ± 0·2	8·4 ± 1·4	247 ± 76
Lo N-WS	0·024 ± 0·007	4·1 ± 0·2	8·6 ± 1·2	359 ± 121
Significance levels[c]				
CO_2	tr	NS	NS	tr
N	***	**	*	***
W	NS	tr	NS	NS
$CO_2 \times N$	NS	tr	NS	NS
$CO_2 \times W$	NS	NS	NS	NS
$N \times W$	NS	NS	NS	NS
$CO_2 \times N \times W$	NS	*	NS	NS

[a] Hi N = 40 g m^{-2} year^{-1} nitrogen, Lo N = 4 g m^{-2} year^{-1} nitrogen, WW = well watered, WS = water-stressed.
[b] Phloem area = total phloem needle cross sectional area in mm^2; sieve cell width = sieve cell diameter periclinal to vascular cambium in μm; total cells = number of cells within the phloem visible in cross-sectional view.
[c] tr = $0{\cdot}05 \leq P \leq 0{\cdot}10$, * $P \leq 0{\cdot}05$, ** $P \leq 0{\cdot}01$, *** $P \leq 0{\cdot}001$.

numbers of plastoglobuli and generally had more thylakoid swelling or membrane disintegration. Although these characteristics are often used as indicators of normal frost hardening, they have also been observed in air pollution studies and in senescing needles and are considered to be non-specific stress symptoms of chloroplasts (Senser *et al.* 1975). Indeed, elevated CO_2 has been reported to induce premature leaf senescence in pine species (Houpis *et al.* 1988) and chloroplast senescence in crop species (Pennanen *et al.* 1992). The extent of acclimation to cold temperatures, and the cytological events which parallel the hardening process in longleaf pine, have not been characterized. Thus, although seedlings grown in elevated CO_2 displayed ultrastructural characteristics typically attributed to frost-resistant needles, it is difficult to say whether the hardening process was helped or hindered based on ultrastructural observations alone. Since plants grown in elevated CO_2 generally appeared to have more of the characteristics of a 'hardened plant', elevated CO_2 may have facilitated an earlier transition to a winter state than ambient CO_2. This hypothesis may be supported by the opposite effect exerted by elevated CO_2 on the deposition of plastoglobuli in the spring compared to the autumn; chloroplasts from seedlings grown in elevated CO_2 generally tended to contain fewer plastoglobuli in the spring. However, when comparing chloroplast characteristics between seasons, it is important to note that we were unable to quantify thylakoid membrane stacking characteristics, or the extent of membrane swelling or disintegration for the spring harvest because of excessive starch inclusions. The stored starch observed in the spring may have concealed more subtle effects which were quantifiable in the autumn. In other studies with different coniferous species, elevated CO_2 was reported negatively (Margolis & Vézina 1990) and positively (Repo, Hänninen & Kellomäki 1996) to affect frost hardening. Clearly, further investigations into the hardening phenomenon and its interaction with elevated CO_2 are warranted.

Conversely, these symptoms indicative of stress in chloroplasts may reflect acclimation or down-regulation of photosynthetic rates commonly observed in response to prolonged exposure to elevated CO_2. Reductions in granal thylakoids (Wulff & Strain 1981; Kutík *et al.* 1995) and components of PSII (Pennanen *et al.* 1993) have been reported previously. Van Oosten, Wilkens & Besford (1994) showed in *Lycopersicum esculentum* that expression of genes coding for chlorophyll *a*/*b* binding proteins of thylakoid light-harvesting complexes were down-regulated in plants exposed to high CO_2. Examination of chloroplast ultrastructure in conjunction with molecular studies on differential gene expression resulting from growth in elevated CO_2 might provide a more complete explanation of the acclimation phenomena. Clearly, growth at elevated CO_2 causes changes in chloroplasts even in species that have extensive sinks (e.g. sugar beet; Kutík *et al.* 1995). Subtle changes in chloroplast membrane characteristics resulting either from alterations in gene expression or mechanical disruption may reflect

reduced light-harvesting capacity, and may disrupt the balance of light-dependent production of ATP, reduction of NADP and, consequently, coupling of light reactions to carbon fixation via the Calvin cycle.

The significant increase in starch grain size in elevated CO_2 is consistent with results of several studies concerning chloroplast ultrastructure (Cave *et al.* 1981; Madson 1968; Wulff & Strain 1981; Yelle *et al.* 1989) but not all (Pennanen *et al.* 1993). Although CO_2 significantly increased the accumulation of chloroplast starch, this was generally contingent upon the availability of soil N and water. In this study, fairly consistent N by water interactions indicated that starch accumulation in response to N was controlled by water availability. With water stress, N limitations contributed to starch accumulation, but when soil moisture was non-limiting the opposite was true. The accumulation of starch resulting from growth in limiting N is a general phenomena (Chapin *et al.* 1987) and the effects of water stress may magnify N limitations by further restricting nutrient uptake and transport through xylem via the transpiration stream. Furthermore, nitrogen limitations cause a decrease in stomatal conductance which results in a decrease in hydraulic conductance of roots (Chapin 1991). It is important to note that the N by water interaction observed here for starch accumulated was not reflected by the amount of starch apparent at the light microscope level (data not shown). This apparent discrepancy is the result of more chloroplasts which appeared to be non-functional and thus contained no starch inclusions in needle tissue grown in low N and with adequate water. Above- and below-ground growth of longleaf pine was significantly reduced by limiting N at both high and low water availabilities (Prior *et al.* 1997).

Regardless of the mechanisms underlying the accumulation of starch, it appears that mechanical damage of chloroplasts may occur only when N and water conditions predispose the plant to accumulate non-structural carbohydrates. Growth in elevated CO_2 appeared to magnify, in a similar direction, effects of N, water and their interactions. Indeed, the apparent negative effect of starch accumulation (indicative of a source–sink imbalance) observed here was most significant under conditions of simultaneous nitrogen and water stress. In a separate study, it was noted that Rubisco activity was significantly reduced in longleaf pine needles exposed to elevated CO_2 and N limitations compared to plants grown in ambient CO_2 and N limitations (J. Qiu, Auburn University, personal communication). In a study on whole-plant dry matter production in longleaf pine, elevated CO_2 contributed to increased biomass compared to ambient CO_2 in high but not low N. Furthermore, well-watered seedlings had increased biomass compared to water-stressed seedlings when grown with high but not low N supply (Prior *et al.* 1997). Thus, it appears that the hypothesis of Mitchell *et al.* (1995) that growth of longleaf pine in elevated CO_2 and low nitrogen is sink-limited and growth in elevated CO_2 and high N is source-limited may be further complicated by interactive effects of soil moisture with N availability.

Several mechanisms have been proposed to explain the negative effect of leaf starch accumulation on photosynthetic rates, in addition to the effects on the internal organization of chloroplasts observed in this and other studies. Nafziger & Koller (1976) observed a strong inverse relationship between amounts of leaf starch and rates of photosynthesis in *Glycine max*. They suggested that accumulation of starch might have increased the diffusive resistance to intracellular CO_2 transport, thereby lowering photosynthesis. Furthermore, they have suggested that such excessive starch accumulation might interfere with cytoplasmic streaming, further impeding intracellular CO_2 transport. The negative effects of excessive leaf starch inclusions may work in concert with subtle reductions in thylakoid stacking to effect the reductions in rates of photosynthesis observed to occur over time. Such mechanisms remain to be elucidated.

The trend suggesting a reduction in total phloem cross-sectional area in the current study is at odds with the literature concerning the effects of elevated CO_2 on vascular tissue. St. Omer & Horvath (1984) observed an increase in the vascular regions of leaves of *Layai platyglossa* grown in elevated CO_2 which they attributed to larger vascular elements. Ho (1977) found no difference in the petiolar phloem cross-sectional area of tomatoes grown in elevated CO_2. An increase in vascular tissue cross-sectional area has also been reported for pine species (Thomas & Harvey 1983; Conroy *et al.* 1986); however, in these studies the phloem area was not measured separately from the xylem and the plants were grown in elevated CO_2 for only 22 weeks (Conroy *et al.* 1986) and 45 d (Thomas & Harvey 1983). Leaf phloem in pine species is unique; pine needles have a unifacial vascular cambium which gives rise to secondary phloem but not xylem (Ewers 1982). Thus, needle phloem cells established in the second year would face different physiological conditions during development than the previous year's phloem. The reduction in the total number of cells within the phloem and in total phloem cross-sectional area suggests that leaf developmental processes may be affected by elevated CO_2. Furthermore, the phloem sieve cells in the elevated CO_2 by low N by well-watered treatment were significantly flattened (compared to the ambient CO_2 treatment) in the direction parallel to the plane of division of needle vascular cambium. This suggests that, in these conditions, either a change in cell solute potential altered turgor pressure or cell walls were more rigid and resistant to extension during normal cell expansion. Interestingly, this treatment combination also had the highest percentage of total phenolics and condensed tannins expressed on a dry mass basis (Pritchard *et al.* 1997), and this may suggest increased availability and synthesis of lignin precursors. Phenolic compounds have also been postulated to play a role in maintaining the water balance in evergreen needles (Masuch *et al.* 1992). Regardless of the mechanism, a small decrease in the radius of sieve cells can cause a major decline in the capacity of the phloem to transport fluid (Lee 1981). Such reductions in size, in concert with reductions in the number of conductive cells,

may negatively affect the ability of pine species to transport extra photoassimilates produced in a high-CO_2 environment. The 'compound interest law', discussed by Ceulemans & Mousseau (1994), suggests that minor alterations in environmental conditions inducing minor physiological and anatomical adjustments during one season may magnify or become compounded over time, resulting in eventual major alterations in plant function. This would seem especially pertinent considering the effects of elevated CO_2 on phloem anatomy observed here, and the importance of phloem function in the face of increasing atmospheric [CO_2] which will inevitably be accompanied by increases in C fixation and carbohydrate synthesis.

Extrapolation from studies on seedlings to mature trees should be done only with extreme caution. However, the seedling stage represents a time characterized by high genetic diversity, great competitive selection and high growth rates (Ceulemans & Mousseau 1994) and as such may represent one of the most crucial periods in the course of tree establishment and forest regeneration. This study suggests that, in nature, longleaf pine seedlings may be unable to realize a benefit from future elevated levels of CO_2 due to sink limitations exacerbated by nutrient and water stress. Furthermore, the overall fitness of longleaf and other pine species may decrease relative to that of competing species which might be physiologically and structurally better adapted to benefit from prolonged exposure to elevated CO_2.

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