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Atmospheric carbon dioxide, irrigation, and fertilization effects on phenolic and nitrogen concentrations in loblolly pine (*Pinus taeda*) needles

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Summary Concentrations of total soluble phenolics, catechin, proanthocyanidins (PA), lignin and nitrogen (N) were measured in loblolly pine (*Pinus taeda* L.) needles exposed to either ambient CO_2 concentration ($[CO_2]$), ambient plus 175 or ambient plus 350 μmol CO_2 mol^{-1} in branch chambers for 2 years. The CO_2 treatments were superimposed on a 2 × 2 factorial combination of irrigation and fertilization treatments. In addition, we compared the effects of branch chambers and open-top chambers on needle chemistry. Proanthocyanidin and N concentrations were measured in needles from branch chambers and from trees in open-top chambers exposed concurrently for two years to either ambient $[CO_2]$ or ambient plus 200 μmol CO_2 mol^{-1} in combination with a fertilization treatment. In the branch chambers, concentrations of total soluble phenolics in needles generally increased with needle age. Concentrations of total soluble phenolics, catechin and PA in needle extracts increased about 11% in response to the elevated $[CO_2]$ treatments. There were no significant treatment effects on foliar lignin concentrations. Nitrogen concentrations were about 10% lower in needles from the elevated $[CO_2]$ treatments than in needles from the ambient $[CO_2]$ treatments. Soluble phenolic and PA concentrations were higher in the control and irrigated soil treatments in about half of the comparisons; otherwise, differences were not statistically significant. Needle N concentrations increased 23% in response to fertilization. Treatment effects on PA and N concentrations were similar between branch and open-top chambers, although in this part of the study N concentrations were not significantly affected by the CO_2 treatments in either the branch or open-top chambers. We conclude that elevated $[CO_2]$ and low N availability affected foliar chemical composition, which could in turn affect plant–pathogen interactions, decomposition rates and mineral nutrient cycling.

Keywords: catechin, CO_2 enrichment, lignin, nutrition, proanthocyanidins, secondary compounds, tannins.

Introduction

The utilization of photosynthate for carbon (C)-based secondary metabolites such as phenolic compounds depends on the plant species, the developmental stage of the plant and resource availability (Herms and Mattson 1992). If elevated CO_2 concentration ($[CO_2]$), which increases C supply, or mineral nutrient deficiency, which decreases C demand, makes C available in excess of that needed for growth and maintenance, it could be used to construct C-based secondary metabolites such as flavonoids, proanthocyanidins (PA), lignin and other phenolic compounds (Bryant et al. 1983, Waterman and Mole 1989, Herms and Mattson 1992, Lindroth 1996, Poorter et al. 1997, Peñuelas and Estiarte 1998). In contrast, sufficient mineral nutrient availability, which promotes optimal growth, might result in lower allocation to secondary metabolites (Bryant et al. 1987, Waterman and Mole 1989, Herms and Mattson 1992). Among mineral nutrients, nitrogen (N), in particular, has been shown to affect phenolic accumulation in several plant species (Bryant et al. 1987, Waterman and Mole 1994, Lindroth 1996, Peñuelas and Estiarte 1998).

The objective of this study was to determine if elevated $[CO_2]$, irrigation and fertilization affect the concentrations of phenolic compounds and N in loblolly pine (*Pinus taeda* L.) needles. Needles were obtained from trees exposed to $[CO_2]$, irrigation and N fertilization treatments in the field using both branch and open-top chambers for the CO_2 treatments. The study comprised three parts: (1) a year-long seasonal profile of soluble phenolic concentrations in one needle cohort from a branch chamber study; (2) a detailed examination of needle phenolic composition and N concentration after one growing season in a branch chamber study; and (3) a comparison after one growing season of PA and N concentrations in needles obtained from trees treated using branch chambers versus open-top chambers during a concurrent time period. The chamber comparison study was conducted to determine if branch chamber exposure methods allow full quantification of potential physiological adjustment to long-term exposure to elevated $[CO_2]$, even though the source of increased photo-

synthesis (the branch) is much smaller than the potential sink (the entire tree) (Saxe et al. 1998).

Materials and methods

Study site and design

The study site was established in the North Carolina Sandhills, Scotland County, NC, USA (35° N, 79° W). Annual air temperature and precipitation averaged 17 °C and 121 cm, respectively, although periods of drought often occurred during the growing season. The soil is a well-drained, deep sand, thermic psammentic Hapludult belonging to the Wakulla series (Albaugh et al. 1998). In March 1985, the site was planted with a mixture of 10 half-sib families of North Carolina, piedmont, loblolly pine seedlings and was thinned to 1260 trees ha^{-1} in 1992. Understory vegetation was controlled mechanically and by applications of glyphosate.

The study was a split-plot design with 2 × 2 factorial combination of irrigation and fertilization. Four treatment combinations (control, irrigated, fertilized, and irrigated + fertilized) were assigned randomly to one of four treatment plots in each of four blocks. Treatment plots were 50 × 50 m with interior sampling plots of 30 × 30 m. Fertilization treatments began in March 1992 and consisted of either no addition or periodic applications of N, P, K, S, Ca, Mg and B as needed to provide optimum tree nutrition (Allen 1997, Albaugh et al. 1998). Irrigation treatment, which began in April 1993, was applied to maintain soil water content at greater than 3 cm of soil water in the surface 50 cm of soil (40% available water content) as determined from volumetric soil water measured by time domain reflectometry (Albaugh et al. 1998). The irrigation control received natural precipitation only.

The CO_2 treatments were applied in 0.7-m diameter × 1.52-m tall branch chambers (Teskey et al. 1991) on three mid-crown branches of a randomly selected tree in each plot (16 trees total). Exposures began in March 1993 and continued throughout the study period 24 h daily. The $[CO_2]$ in each chamber was monitored twice hourly with an infrared CO_2 analyzer (Model 6262, Li-Cor, Inc., Lincoln, NE), which was calibrated weekly with pressurized CO_2 standards spanning the range of concentrations used in the experiment. Carbon dioxide treatments were ambient air (AA), ambient air plus 175 (+175), or ambient air plus 350 (+350) μmol CO_2 mol^{-1}. Mean daily (0800–2000 h EST) $[CO_2]$ in the three CO_2 treatments in 1993 and 1994 was 382 ± 20 (SD), 556 ± 33 and 739 ± 52 μmol mol^{-1}, respectively. Temperatures in the branch chambers were 1 to 3 °C warmer than ambient air temperature, and photosynthetic photon flux density (PPFD) inside the chambers was about 50% less than that above the canopy (Murthy et al. 1996).

For the comparative study of branch and open-top chambers, CO_2 treatments were applied in either branch or whole-tree open-top chambers to randomly selected trees in each of three blocks in the control and fertilized treatment plots (12 trees total). Trees were enclosed in 3-m diameter open-top chambers. The chamber design was similar to that described by Heagle et al. (1989), but the chambers extended vertically to enclose the tree canopy (up to 12 m). Exposures began in March 1996 and continued 24 h daily until the end of the study in January 1999. Carbon dioxide treatments were ambient air (AA) or ambient air plus 200 (+200) μmol CO_2 mol^{-1}. Daily mean $[CO_2]$ for the study was 375 ± 27 and 582 ± 36 μmol for the AA and +200 treatments, respectively.

Needle sampling

To study the seasonal profile of soluble phenolics, samples (three fascicles) of the first flush of foliage formed in 1993 were obtained four times between September 1993 and September 1994 from each branch chamber. Samples were oven-dried at 65 °C and stored in the dark at 25 °C. To study needle phenolic and N composition, samples (10 fascicles) of the first flush of foliage formed in 1994 were taken from each branch chamber in November 1994. These samples were freeze-dried and stored in a desiccator at –20 °C. For the comparative study of chamber design, samples (20 fascicles) of the first flush of foliage formed in 1998 on a mid-crown branch were taken from branch and open-top chambers in November 1998. These samples were also freeze-dried and stored in a desiccator at –20 °C. Samples were analyzed for N concentration with an NCS elemental analyzer (NA 1500 Carlo-Erba Analyzer, Fison Instrument, Danvers, MA).

Extraction of soluble components

Needle samples were ground to pass a 0.5-mm mesh screen. Tissue samples (50 mg) were extracted three times with 1 ml of 70% acetone with mixing for 30 min at 25 °C, except for the catechin assay, in which case samples were extracted with 50% methanol. Following each extraction, the insoluble material was pelleted by centrifugation (16,000 *g*, 5 min), and the supernatants were pooled by sample. In a preliminary experiment with the PA assay, two additional extractions of the insoluble fractions with 1 ml of 70% acetone indicated that the efficiency of the extraction procedure averaged 100%.

Total phenolics assay

Total phenolic concentration in the soluble fraction was determined by the Folin-Ciocalteu method (Booker et al. 1996). Samples were diluted 1:10 with 70% acetone, and duplicate 50-μl aliquots were each mixed with 475 μl of 0.25 *N* Folin-Ciocalteu reagent (Sigma Chemical Co., St. Louis, MO) followed 3 min later by 475 μl of 1 M Na_2CO_3. After 1 h, absorbance of the solutions was measured at 724 nm. Catechin was used to prepare a standard curve, and results were expressed as catechin equivalents per g dry mass. Catechin is detected by the total phenolic assay, but not by the PA assay (Scalbert 1992)

Catechin assay

Needle tissue samples were extracted with 50% methanol, filtered (0.2 μm), and 20-μl aliquots were fractionated by reversed-phase HPLC as previously described by Booker et al. (1996). Catechin was identified on chromatograms by peak re-

tention time, co-chromatography with a catechin standard (Sigma Chemical Co., St. Louis, MO) and similarity of UV spectra between the fraction containing the peak of interest and a catechin standard (λ_{max} = 282 nm).

Proanthocyanidin assay

Proanthocyanidin concentration was determined by oxidative depolymerization of anthocyanidins in acid butanol (Porter et al. 1986). Duplicate 100-µl aliquots of the soluble fraction were mixed with 900 µl of methanol followed by 6 ml of acid butanol (50 ml l^{-1} concentrated HCl in *n*-butanol) and 200 µl of 20 g l^{-1} $FeNH_4(SO_4)_2 \times 12\ H_2O$ in 2 *N* HCl. In the chamber comparison study, the insoluble material was resuspended in 1 ml of *n*-butanol, centrifuged (16,000 *g*, 3 min), and the supernatant discarded. The insoluble residue was mixed with 900 µl of methanol and the acid butanol assay reagents. To control for substances in the extracts that might interfere with the PA assay, a 100-µl aliquot of each soluble fraction was mixed with 900 µl of methanol followed by 6 ml of *n*-butanol and 200 µl of H_2O. The solutions and mixtures were incubated in sealed tubes in a water bath at 90 °C for 40 min and then cooled. Preparations containing the insoluble fraction were clarified by centrifugation (3400 *g*, 5 min). Absorbance of the solutions at 550 nm was measured immediately. Values were corrected for interfering substances by subtracting the absorbance at 550 nm of preparations containing the *n*-butanol and H_2O reagents from those containing the acid butanol reagents (Waterman and Mole 1994). A standard curve was constructed based on PA purified from leaf tissue collected from the crown of a tree in an irrigated plus fertilized treatment plot (see below), and assay results were expressed as PA equivalents per g dry mass. Concentrations of PA in the chamber comparison study were determined by summing values derived from the soluble and insoluble fractions. Proanthocyanidin concentration in the insoluble fraction averaged 2% of the total concentration.

Proanthocyanidins were purified from needle tissue by adsorption chromatography (Sephadex LH-20, Sigma Chemical Co., St. Louis, MO) as described previously (Booker 2000). The UV spectrum of purified PA dissolved in water showed λ_{max} at 206 and 274 nm, and a λ_{sh} at 240 nm. An IR spectrum of purified PA was obtained with a KBr pellet (1 mg sample in a 13 mm pellet) on an FT-IR spectrometer (Model 16C, PerkinElmer Analytical Instruments, Norwalk, CT) (Figure 1). Absorption bands at 1534, 1520, 1344, 1204, 1040, 800 and 730 cm^{-1} indicated that the polymer was mainly of the prodelphinidin type with the monomers having the *cis* configuration (i.e., the monomer units being mainly of the epigallocatechin type) (Foo 1981). The acid butanol assay of purified PA dissolved in 70% acetone yielded a mean $E_{1\%,550\,nm}$ value of 518. The UV and IR spectra and $E_{1\%}$ values of the purified PA indicated that it was reasonably free of contaminants and acceptable for use as a standard.

Lignothioglycolic acid assay

The insoluble fraction from the 70% acetone extractions was freed of additional extractive components by washing twice with methanol:chloroform:H_2O (2:1:0.8, v/v), three times with liquefied phenol:acetic acid:H_2O (1.1:1:0.9, v/v), once with ethanol and three times with acetone, with centrifugation (16,000 *g*, 5 min) between washings (Friend 1992). Samples were dried at 70 °C and stored in a desiccator in the dark.

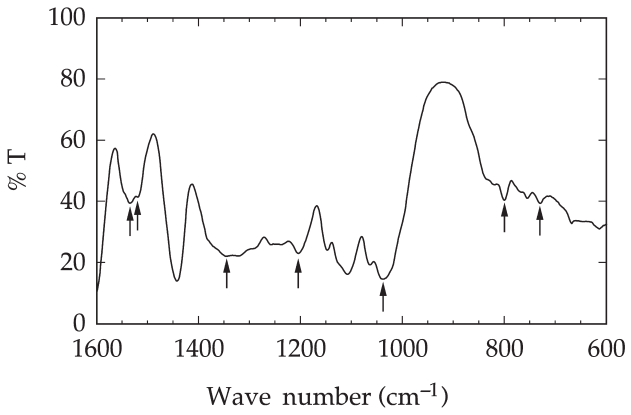

Figure 1. Infrared spectrum of purified PA prepared from loblolly pine needles in a KBr pellet. Absorption bands at 1534, 1520, 1344, 1204, 1040, 800 and 730 cm^{-1} (indicated by arrows) are characteristic of a polymer mainly of the prodelphinidin type with the monomers having the *cis* configuration (i.e., the monomer units being mainly of the epigallocatechin type) (Foo 1981).

Lignin concentration in the extractive-free tissue samples was measured by the thioglycolic acid method (Whitmore 1976, Booker et al. 1996). Extractive-free samples were first hydrolyzed with 900 µl of 1 *N* NaOH overnight at 25 °C on a tube rocker to solubilize esterified phenylpropanoid compounds that might otherwise be extracted by thioglycolic acid. Samples were then acidified to pH 2 with 2 *N* HCl, centrifuged (16,000 *g*, 5 min) and the supernatants discarded. The pellets were washed with 1 ml of H_2O, centrifuged and the supernatants discarded. The residues were mixed with 900 µl of 2 *N* HCl and 100 µl of thioglycolic acid (Sigma Chemical Co, St. Louis, MO) in a screw-top microcentrifuge tube. The microcentrifuge tubes were placed in pressure tubes and heated at 100 °C for 4 h. The residues were recovered by centrifugation, washed twice with 1 ml of H_2O, resuspended in 1 ml of 1 *N* NaOH and mixed overnight at 25 °C. Afterward, the supernatants were recovered by centrifugation, the residues washed with 1 ml of H_2O, centrifuged, and the supernatants pooled by sample. Supernatants were mixed with 100 µl of concentrated HCl and incubated at 4 °C for 3 h. The resulting precipitates (lignothioglycolic acid, LTGA) were collected by centrifugation, washed with H_2O and centrifuged again. The pellets were then dissolved in 1 ml of 1 *N* NaOH. Aliquots were diluted 1:80 with 0.1 *N* NaOH, and absorbance of the samples was measured at 280 nm. Aliquots of undiluted solutions were mixed with concentrated HCl, the precipitates freeze-dried and used to construct a standard curve ($E_{1\%,280\,nm}$ = 134). Results were expressed on a dry mass basis as mg LTGA g_{dm}^{-1}.

Nonstructural carbohydrates assay

Starch and soluble sugars were determined enzymatically by the UV method (R-Biopharm, Inc., Marshall, MI). To solubilize starch, tissue samples (25 mg) were each mixed with 2.4 ml of dimethylsulfoxide and 600 µl of 8 *N* HCl in sealed polypropylene tubes for 60 min at 60 °C on a tube rocker. Samples were then neutralized with 600 µl of 8 *N* NaOH and diluted to 15 ml with 112 mM citrate buffer (pH 4). Solutions were filtered (Whatman No. 1), and duplicate 50-µl aliquots were assayed according to kit instructions. Results were expressed as D-glucose equivalents.

Statistics

Statistical analysis was performed with the SAS System for Mixed Models (Littell et al. 1996). Treatments were assigned to plots based on a randomized complete block factorial. The CO_2 treatments were treated as subplot treatments within the whole-plot unit of soil treatment. The results were analyzed as a split-plot design with soil amendment as the whole-plot treatments and [CO_2] as the subplot treatment. There were four replicates of each treatment combination. For the chamber comparison, chamber type was treated as a subplot within the experiment. Thus, the results were analyzed as a split-plot design, with soil amendment as the whole-plot treatments, and [CO_2] and chamber treatments as the subplot treatments. There were three replicates of each treatment combination in this part of the study. Data were tested for homogeneity of variance and normality before analysis. Differences were considered significant if $P \leq 0.05$, and marginally significant if $P \leq 0.1$.

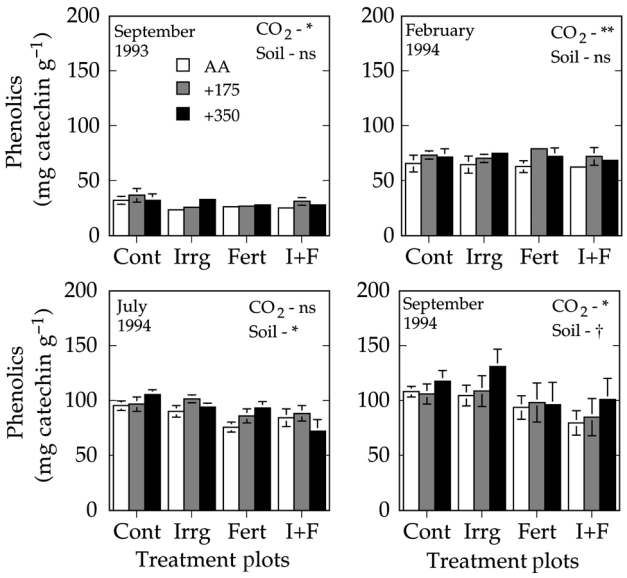

Figure 2. Mean (± SE) total soluble phenolic concentration expressed as catechin equivalents per g dry mass in extracts from needles initiated in the first flush of growth in 1993 in control (Cont), irrigated (Irrg), fertilized (Fert) and irrigated plus fertilized (I+F) soil treatment plots. Needles were obtained from mid-crown branches treated in branch chambers with either ambient [CO_2] (AA), ambient plus 175 (+175) or ambient plus 350 (+350) µmol CO_2 mol^{-1} 24 h daily for up to two years. Samples of 1993 needles were obtained four times over the course of a year (September 1993, February 1994, July 1994 and September 1994). Statistics: not significant (ns, $P > 0.1$), $P \leq 0.1$ (†), $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***).

Results

Seasonal profile of soluble phenolics in needles

Mean concentration of total soluble phenolics extracted from the first flush of needles initiated in 1993 generally increased with needle age (Figure 2). Soluble phenolic concentrations increased by about 11% overall in needle extracts from branches exposed to elevated [CO_2], although the [CO_2] effect was not statistically significant in the July 1994 samples. Soil treatments had no significant effect on phenolic concentrations on the September 1993 and February 1994 sample dates, but phenolic concentrations were about 20% higher in needles from the control and irrigated plots in the July and September 1994 samples compared with the fertilized plots. None of the CO_2 × soil treatment interactions were statistically significant.

Needle phenolic composition

The effect of elevated [CO_2] on soluble phenolic concentrations in current-year needles sampled in November 1994 showed a pattern similar to that observed for the 1993 needles. Total soluble phenolic concentration was 5 to 11% higher in needle extracts from branches in the elevated [CO_2] treatments compared with needle extracts from the AA treatments (Figure 3). Soil treatments had no statistically significant effect on needle phenolic concentrations. Catechin and proanthocyanidin concentrations were 29 and 12% higher, respectively, in needle extracts from the +350 treatments compared with the AA treatments (Figure 3). Effects of the +175 treatments on catechin and PA concentrations were not statistically significant. Soil amendments had no significant effect on either catechin or PA concentrations, and the CO_2 × fertilized treatment plot interaction was not significant for either measurement.

Mean (± SD) LTGA yield from current-year needles sampled in November 1994 was 66.4 ± 6.6 mg g^{-1} for all treatments. No significant treatment effect or interaction was detected.

Nitrogen concentrations were 8 to 10% lower in needles from the elevated [CO_2] treatments compared with needles from the AA treatments (Figure 3). Nitrogen concentrations were 23% higher in needles from the fertilized plots than in needles from the non-fertilized plots. The CO_2 × soil treatment interaction was not significant.

Branch versus open-top chamber comparison

Mean PA concentration was 18 to 34% higher in needle extracts from the +200 treatments compared with the AA treatments in both the branch and open-top chambers (Figure 4). There was no statistically significant effect of chamber design on PA concentrations. Mean PA concentration was 27%

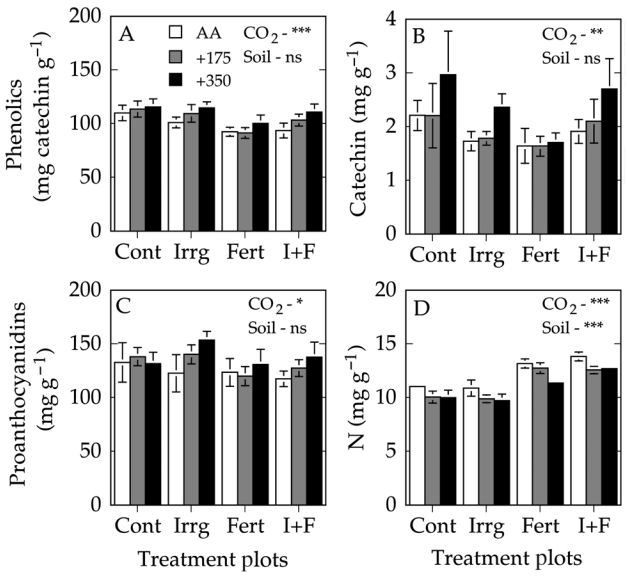

Figure 3. Mean (± SE) soluble phenolics (A), catechin (B), proanthocyanidins (C), and N (D) concentrations in samples of current-year needles sampled in November 1994 in control (Cont), irrigated (Irrg), fertilized (Fert) and irrigated plus fertilized (I+F) soil treatment plots. Needles were obtained from mid-crown branches treated in branch chambers with either ambient [CO_2] (AA), ambient plus 175 (+175) or ambient plus 350 (+350) µmol CO_2 mol^{-1} 24 h daily for 2 years. Statistics: not significant (ns, $P > 0.1$), $P \leq 0.1$ (†), $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***).

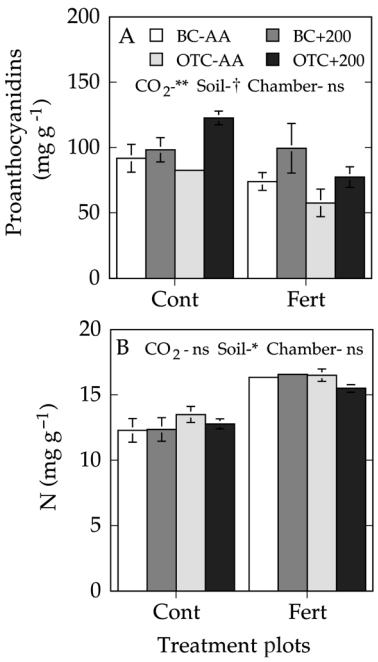

Figure 4. Mean (± SE) proanthocyanidin (A) and N (B) concentrations in samples of current-year needles sampled in November 1998 in control (Cont) and fertilized (Fert) soil treatment plots. Needles were obtained from mid-crown branches treated in either branch chambers (BC) or open-top chambers (OTC) with either ambient [CO_2] (AA) or ambient plus 200 (+200) µmol CO_2 mol^{-1} 24 h daily for 2 years. Statistics: not significant (ns, $P > 0.1$), $P \leq 0.1$ (†), $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***)

higher in needle extracts from the control soil treatment compared with the fertilized soil treatment.

In contrast to the effect of elevated [CO_2] on N concentrations in the 1994 needles, there was no significant effect of the +200 treatments on N concentrations in needles sampled in 1998 from either branch or open-top chambers (Figure 4). However, N concentration was 27% higher in needles from fertilized trees than in needles from non-fertilized trees in both the branch and open-top chambers. There was no significant effect of chamber design on needle N concentrations.

Starch concentrations in needles from the branch and open-top chambers were less than 1 mg g_{dm}^{-1}. Soluble sugar concentrations were also low, ranging from 14 to 22 mg g_{dm}^{-1}.

Discussion

The increase in concentrations of total soluble phenolics and PA in response to elevated [CO_2] appears to be related to the balance between carbohydrate sources and sinks: the greater the source:sink ratio, the greater the concentration of phenolic compounds (Herms and Mattson 1992, Peñuelas and Estiarte 1998). Elevated [CO_2] increases this ratio by increasing fixed C, whereas fertilization decreases it by promoting C utilization for growth. Thus, production of phenolic compounds could be a mechanism to shunt fixed C and recycle N when excess photosynthate is available for growth, as occurs in the presence of elevated [CO_2] (Herms and Mattson 1992, Lambers 1993). Our results generally supported this hypothesis.

Of the variables we measured, catechin concentration exhibited the largest response to elevated [CO_2]; it was 29% higher in needle extracts from the +350 treatments compared with the AA treatments. In previous studies, increased concentrations of catechin were measured in Scots pine (*P. sylvestris* L.) needles (Ivonis 1990) and in leaves of willow (*Salix myrinifolia* Salisb.) and in European white birch (*Betula pendula* Roth) seedlings following treatment with elevated [CO_2] (Julkunen-Tiitto and Tahvanainen 1993, Lavola and Julkunen-Tiitto 1994).

In addition, total soluble phenolics and PA concentrations were generally higher in needle extracts from the elevated [CO_2] treatments than from the AA treatments. Results from previous studies with *Pinus* species, however, have been mixed. For example, in a greenhouse experiment, increased concentrations of total soluble phenolics and PA were found in needle extracts from loblolly pine seedlings treated from germination with 700 µmol CO_2 mol^{-1} and low soil N for 138 days compared with control treatments (Gebauer et al. 1998). In a field experiment with 1-year-old longleaf pine (*P. palustris* Mill.) seedlings grown in 45-l pots in open-top chambers for 20 months, needle PA concentrations were higher in the twice ambient [CO_2] and low-N fertility treatments than in the ambient [CO_2] and high-N fertility treatments (Pritchard et al. 1997). In contrast, total soluble phenolic concentrations in needles of Scots pine (*P. sylvestris* L.) trees were not significantly affected by treatment in

open-top chambers for 3 years with twice ambient [CO_2] (Kainulainen et al. 1998). Total soluble phenolic and PA concentrations were lower in needle extracts from Eldarica pine (*P. eldarica* L.) seedlings treated for 2 years in open-top chambers with twice ambient [CO_2] (Peñuelas et al. 1996). Peñuelas et al. (1996) explained the decrease in needle phenolic concentrations in response to elevated [CO_2] on the basis that growth stimulation by elevated [CO_2] resulted in reduced availability of C for allocation to soluble phenolics. However, studies with other woody and herbaceous species have shown that leaf PA concentrations generally increase with [CO_2] enrichment or reduced N fertility (Waring et al. 1985, Bryant et al. 1987, Herms and Mattson 1992, Julkunen-Tiitto and Tahvanainen 1993, Lavola and Julkunen-Tiitto 1994, Lindroth 1996, Kinney et al. 1997, Lawler et al. 1997, Poorter et al. 1997, Peñuelas and Estiarte 1998, Booker 2000, Booker et al. 2000).

In our study, the effects of soil fertility treatments on needle soluble phenolic and PA concentrations were inconsistent. Soluble phenolic and PA concentrations were higher in needles in the control and irrigated soil treatments in about half of the comparisons, but otherwise differences were not statistically significant. Other studies have also shown that high concentrations of nutrients do not consistently lead to lower phenolic or PA concentrations (Chapin et al. 1986, Iason and Hester 1993). Possibly, developmental regulation of phenolic biosynthesis had a strong effect on needle phenolic concentration or soil nutrient status was not sufficiently altered by the soil treatments to influence phenolic biosynthesis consistently.

Zucker (1983) has argued that proanthocyanidins are primarily defensive against microbes and can impede decomposition of plant litter. Proanthocyanidins are thought to inhibit decomposition by inactivating enzymes and precipitating proteins (Tiarks et al. 1989). Through these processes, soil N availability may be diminished (Haynes 1986, Tiarks et al. 1989). If so, increased phenolics in decomposing litter could decrease site soil quality (Kuiters 1990). We found that phenolic concentration increased with needle age and [CO_2], suggesting that input of PA to soil decomposition processes would be greatest in senescent needles and would increase with increasing atmospheric [CO_2]. However, there is no consistent evidence that elevated [CO_2] directly affects litter decomposition although N mineralization rates might be slowed (Torbert et al. 1995, Peñuelas and Estiarte 1998).

We found no significant effect of elevated [CO_2] or soil amendment treatments on LTGA concentration. Similarly, Entry et al. (1998) and Runion et al. (1999) reported that elevated [CO_2] did not affect lignin concentration in longleaf pine needles. Furthermore, no statistically significant difference in leaf lignin concentration has been observed in many herbaceous and woody species following treatment with elevated [CO_2] (Poorter et al. 1997, Peñuelas and Estiarte 1998, Booker 2000, Booker et al. 2000). However, Cotrufo et al. (1994) found that lignin concentration in Sitka spruce (*Picea sitchensis* (Bong.) Carr.) needles increased by 26% in seedlings treated with ambient plus 250 μmol CO_2 mol^{-1} for one growing season. In contrast, lignin concentration was lower in leaves of *Eucalyptus tereticornis* (Smith) seedlings exposed to CO_2 enrichment (Lawler et al. 1997). The large variability in the response of leaf lignin concentrations to elevated [CO_2] is poorly understood. Differences among species and environmental conditions undoubtedly contribute to this variability, as well as the methods of lignin analysis and expression of results. For example, increases in nonstructural carbohydrate concentrations in CO_2-treated plants can dilute lignin concentrations unless corrected for by expressing results on a structural dry mass basis (Poorter et al. 1997, Peñuelas and Estiarte 1998, Booker 2000). However, nonstructural carbohydrate concentrations are typically low in needles during autumn and winter months, as seen in this study and elsewhere (Roberntz and Linder 1999), so interference by this factor should be minimal.

Needle N concentration in the November 1994 samples was about 10% lower in branches exposed to elevated [CO_2] than in branches exposed to ambient [CO_2] (cf. Bazzaz 1990, Poorter et al. 1997). Decreased concentrations of foliar N were also found in loblolly, longleaf and ponderosa (*P. ponderosa* Laws) pine seedlings and saplings following treatment with twice ambient [CO_2] (Griffin et al. 1995, Tissue et al. 1996, Entry et al. 1998, Johnson et al. 1998, Runion et al. 1999). This response has been attributed to dilution as a result of increased concentrations of nonstructural carbohydrates (Tissue et al. 1996, Poorter et al. 1997), increased efficiency of N utilization (Johnson et al. 1995), lower nutrient uptake (BassiriRad et al. 1997), or to ontogenetic effects of elevated [CO_2] on plant growth (Conroy 1992, Poorter et al. 1997). Reduced foliar N concentrations may also result from a short-term imbalance in plant C:N ratios in response to CO_2 enrichment (Saxe et al. 1998). In seedlings, reduced needle N concentration is probably the result of rapid growth in elevated [CO_2]. However, this developmental response may be transient, disappearing as the seedling grows (Peñuelas et al. 1997). The response of mature field-grown trees to elevated [CO_2] is mixed. Nitrogen concentrations were lower in loblolly pine, Scots pine and Norway spruce (*Picea abies* (L.) Karst.) needles from branches exposed for up to 4 years to twice ambient [CO_2] in branch chambers compared with needles from control branches (Murthy et al. 1996, Kellomaki and Wang 1997, Roberntz and Linder 1999). In contrast, loblolly pine needle N concentrations were unaffected by elevated CO_2 treatment in branch chambers on a nutrient-poor site (Teskey 1995). Myers et al. (1999) studied loblolly pine trees similar in stand age and structure to our trees and found no difference in the concentration of needle N or nonstructural carbohydrates after 1 year of exposure to elevated [CO_2]. These studies indicate that care should be used in interpreting tissue N analysis because changes in N concentration at any given time may be the result of CO_2 treatment or artifacts associated with differences in growth, environmental conditions or differences in sample collection and handling.

Chamber exposure system had negligible effects on the response of PA, N and nonstructural carbohydrate concentrations to elevated [CO_2] or soil fertility. These results were consistent with those found for photosynthesis, foliage and

branch phenology, and growth (C.A. Maier, unpublished data). Groninger et al. (1999) concluded that, compared with other species, loblolly pine shows consistent responses to elevated [CO_2] when seedlings, saplings, mature trees and stands have been assessed even in different treatment systems. Our results support this conclusion.

In summary, soluble phenolic, catechin and PA concentrations were generally higher in needle extracts from elevated [CO_2] treatments than from ambient [CO_2] treatments, whereas N concentrations were generally lower. These changes in metabolite allocation were correlated with availability of resources in the environment. The effect of fertilization on foliar phenolic concentrations was less consistent, indicating that a more detailed understanding of the relationships among photosynthesis, growth and synthesis of phenolic compounds, and their regulation, under different concentrations of nutrient availability is needed (Iason and Hester 1993). Studies are now in progress to determine if the observed changes in metabolite allocation in loblolly pine needles affect plant–pathogen relationships, microbial decomposition rates and nutrient cycling.

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References

Albaugh, T.J., H.L. Allen, P.M. Dougherty, L.W. Kress and J.S. King. 1998. Leaf area and above- and belowground growth responses of loblolly pine to nutrient and water additions. For. Sci. 44:1–12.

Allen, H.L. 1997. Forest fertilizers: nutrient amendment, forest productivity, and environmental impact. J. For. 85:37–46.

BassiriRad, H., K.L. Griffin, J.F. Reynolds and B.R. Strain. 1997. Changes in root NH_4^+ and NO_3^- absorption rates of loblolly and ponderosa pine in response to CO_2 enrichment. Plant Soil 190:1–9.

Bazzaz, F.A. 1990. The response of natural ecosystems to the rising global CO_2 levels. Annu. Rev. Ecol. Syst. 21:167–196.

Booker, F.L. 2000. Influence of carbon dioxide enrichment, ozone and nitrogen fertilization on cotton (*Gossypium hirsutum* L.) leaf and root composition. Plant Cell Environ. 23:573–583.

Booker, F.L., S. Anttonen and A.S. Heagle. 1996. Catechin, proanthocyanidin and lignin contents of loblolly pine (*Pinus taeda* L.) needles after chronic exposure to ozone. New Phytol. 132: 483–492.

Booker, F.L., S.R. Shafer, C.-M. Wei and S.J. Horton. 2000. Carbon dioxide enrichment and nitrogen fertilization effects on cotton (*Gossypium hirsutum* L.) plant residue chemistry and decomposition. Plant Soil 220:89–98.

Bryant, J.P., F.S. Chapin and D.R. Klein. 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos 40: 357–368.

Bryant, J.P., T.P. Clausen, P.B. Reichardt, M.C. McCarthy and R.A. Werner. 1987. Effect of nitrogen fertilization upon the secondary chemistry and nutritional value of quaking aspen (*Populus tremuloides* Michx.) leaves for the large aspen tortrix (*Choristoneura conflictana* (Walker)). Oecologia 73:513–517.

Chapin, F.S.I., G.R. Shaver and R.A. Kedrowski. 1986. Environmental controls over carbon, nitrogen and phosphorus fractions in *Eriophorum vaginatum* in Alaskan tussock tundra. J. Ecol. 74: 167–195.

Conroy, J.P. 1992. Influence of elevated atmospheric CO_2 concentrations on plant nutrition. Aust. J. Bot. 40:445–456.

Cotrufo, M.F., P. Ineson and A.P. Rowland. 1994. Decomposition of tree leaf litters grown under elevated CO_2: effect of litter quality. Plant Soil 163:121–130.

Entry, J.A., G.B. Runion, S.A. Prior, R.J. Mitchell and H.H. Rogers. 1998. Influence of CO_2 enrichment and nitrogen fertilization on tissue chemistry and carbon allocation in longleaf pine seedlings. Plant Soil 200:3–11.

Foo, L.Y. 1981. Proanthocyanidins: gross chemical structures by infrared spectra. Phytochemistry 20:1397–1402.

Friend, J. 1992. Lignin and associated phenolic acids in cell walls. *In* Molecular Plant Pathology: A Practical Approach. Eds. S.J. Gurr and M.J. McPherson. IRL Press, Oxford, pp 9–14.

Gebauer, R.L.E., B.R. Strain and J.F. Reynolds. 1998. The effect of elevated CO_2 and N availability on tissue concentrations and whole plant pools of carbon-based secondary compounds in loblolly pine (*Pinus taeda*). Oecologia 113:29–36.

Griffin, K.L., W.E. Winner and B.R. Strain. 1995. Growth and dry matter partitioning in loblolly and ponderosa pine seedlings in response to carbon and nitrogen availability. New Phytol. 129: 547–556.

Groninger, J.W., K.H. Johnsen, J.R. Seiler, R.E. Will, D.S. Ellsworth and C. Maier. 1999. Elevated carbon dioxide in the atmosphere: what might it mean for loblolly pine plantation forestry? J. For. 97:4–10.

Haynes, R.J. 1986. The decomposition process: mineralization, immobilization, humus formation, and degradation. *In* Mineral Nitrogen in the Plant–Soil System. Ed. R.J. Haynes. Academic Press, Inc., Orlando, FL, pp 52–126.

Heagle, A.S., R.B. Philbeck, R.E. Ferrell and W.W. Heck. 1989. Design and performance of a large field exposure chamber to measure effects of air quality on plants. J. Environ. Qual. 18:361–368.

Herms, D.A. and W.J. Mattson. 1992. The dilemma of plants: to grow or defend. Quart. Rev. Biol. 67:283–335.

Iason, G.R. and A.J. Hester. 1993. The response of heather (*Calluna vulgaris*) to shade and nutrients—predictions of the carbon–nutrient balance hypothesis. J. Ecol. 81:75–80.

Ivonis, I.Y. 1990. Effect of a polyethylene cover and supplementary CO_2 feeding on the content of growth regulators and (+)-catechin in Scotch pine needles. Sov. For. Sci. 4:82–86.

Johnson, D.W., T. Ball and R.F. Walker. 1995. Effects of elevated CO_2 and nitrogen on nutrient uptake in ponderosa pine seedlings. Plant Soil 168/169:535–545.

Johnson, D.W., R.B. Thomas, K.L. Griffin, D.T. Tissue, J.T. Ball, B.R. Strain and R.F. Walker. 1998. Effects of carbon dioxide and nitrogen on growth and nitrogen uptake in ponderosa and loblolly pine. J. Environ. Qual. 27:414–425.

Julkunen-Tiitto, R. and J. Tahvanainen. 1993. Increased CO_2 and nutrient status changes affect phytomass and the production of plant defensive secondary chemicals in *Salix myrsinifolia* (Salisb.). Oecologia 95:495–498.

Kainulainen, P., J.K. Holopainen and T. Holopainen. 1998. The influence of elevated CO_2 and O_3 concentrations on Scots pine needles: changes in starch and secondary metabolites over three exposure years. Oecologia 114:455–460.

Kellomaki, S. and K.-Y. Wang. 1997. Photosynthetic responses of Scots pine to elevated CO_2 and nitrogen supply: results of a branch-in-bag experiment. Tree Physiol. 17:223–240.

Kinney, K.K., R.L. Lindroth, S.M. Jung and E.V. Nordheim. 1997. Effects of CO_2 and NO_3^- availability on deciduous trees: phytochemistry and insect performance. Ecology 78:215–230.

Kuiters, A.T. 1990. Role of phenolic substances from decomposing forest litter in plant–soil interactions. Acta Bot. Neer. 39:329–348.

Lambers, H. 1993. Rising CO_2, secondary plant metabolism, plant–herbivore interactions and litter decomposition. Vegetatio 104/105:263–271.

Lavola, A. and R. Julkunen-Tiitto. 1994. The effect of elevated carbon dioxide and fertilization on primary and secondary metabolites in birch, *Betula pendula* (Roth). Oecologia 99:315–321.

Lawler, I.R., W.J. Foley, I.E. Woodrow and S.J. Cork. 1997. The effects of elevated CO_2 atmospheres on the nutritional quality of *Eucalyptus* foliage and its interaction with soil nutrient and light availability. Oecologia 109:59–68.

Lindroth, R.L. 1996. CO_2-mediated changes in tree chemistry and tree–Lepidoptera interactions. *In* Carbon Dioxide and Terrestrial Ecosystems. Eds. G.W. Koch and H.A. Mooney. Academic Press, San Diego, CA, pp 105–120.

Littell, R.C., G.A. Milliken, W.W. Stroup and R.D. Wolfinger. 1996. SAS System for Mixed Models. SAS Institute, Inc., Cary, NC, 633 p.

Murthy, R., P.M. Dougherty, S.J. Zarnoch and H.L. Allen. 1996. Effects of carbon dioxide, fertilization, and irrigation on photosynthetic capacity of loblolly pine trees. Tree Physiol. 16: 537–546.

Myers, D.A., R.B. Thomas and E.H. DeLucia. 1999. Photosynthetic capacity of loblolly pine (*Pinus taeda* L.) trees during the first year of carbon dioxide enrichment in a forest ecosystem. Plant Cell Environ. 22:473–482.

Peñuelas, J. and M. Estiarte. 1998. Can elevated CO_2 affect secondary metabolism and ecosystem function? Trees 13:20–24.

Peñuelas, J., M. Estiarte, B.A. Kimball, S.B. Idso, P.J. Pinter, G.W. Wall, R.L. Garcia, D.J. Hansaker, R.L. LaMorte and D.L. Hendrix. 1996. Variety of responses of plant phenolic concentration to CO_2 enrichment. J. Exp. Bot. 47:1463–1467.

Peñuelas, J., S.B. Idso, A. Ribas and B.A. Kimball. 1997. Effects of long-term CO_2 enrichment on the mineral concentration of *Citrus aurantium* leaves. New Phytol. 135:439–444.

Poorter, H., Y. Van Berkel, R. Baxter, J. den Hertog, P. Dijkstra, R.M. Gifford, K.L. Griffin, C. Roumet, J. Roy and S.-C. Wong. 1997. The effect of elevated CO_2 on the chemical composition and construction costs of leaves of 27 C_3 species. Plant Cell Environ. 20:472–482.

Porter, L.J., L.N. Hrstich and B.G. Chan. 1986. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochemistry 25:223–230.

Pritchard, S., C. Peterson, G.B. Runion, S. Prior and H. Rogers. 1997. Atmospheric CO_2 concentration, N availability, and water status affect patterns of ergastic substance deposition in longleaf pine (*Pinus palustris* Mill.) foliage. Trees 11:494–503.

Roberntz, P. and S. Linder. 1999. Effects of long-term CO_2 enrichment and nutrient availability in Norway spruce. II. Foliar chemistry. Trees 14:17–27.

Runion, G.B., J.A. Entry, S.A. Prior, R.J. Mitchell and H.H. Rogers. 1999. Tissue chemistry and carbon allocation in seedlings of *Pinus palustris* subjected to elevated atmospheric CO_2 and water stress. Tree Physiol. 19:329–335.

Saxe, H., D.E. Ellsworth and J. Heath. 1998. Tree and forest functioning in an enriched CO_2 atmosphere. New Phytol. 139:395–436.

Scalbert, A. 1992. Quantitative methods for the estimation of tannins in plant tissues. *In* Plant Polyphenols: Synthesis, Properties, Significance. Eds. R.W. Hemingway and P.E. Laks. Plenum Press, New York, pp 259–280.

Teskey, R.O. 1995. A field study of the effects of elevated CO_2 on carbon assimilation, stomatal conductance and leaf and branch growth of *Pinus taeda* trees. Plant Cell Environ. 18:565–573.

Teskey, R.O., P.M. Dougherty and A.E. Wiselogel. 1991. Design and performance of branch chambers suitable for longterm ozone fumigation of foliage in large trees. J. Environ. Qual. 20:591–595.

Tiarks, A.E., J.R. Bridges, R.W. Hemingway and E. Shoulders. 1989. Condensed tannins in southern pines and their interactions with the ecosystem. *In* Chemistry and Significance of Condensed Tannins. Eds. R.W. Hemingway and J.J. Karchesy. Plenum Press, New York, pp 369–390.

Tissue, D.T., R.B. Thomas and B.R. Strain. 1996. Growth and photosynthesis of loblolly pine (*Pinus taeda*) after exposure to elevated CO_2 for 19 months in the field. Tree Physiol. 16:49–59.

Torbert, H.A., S.A. Prior and H.H. Rogers. 1995. Elevated atmospheric carbon dioxide effects on cotton plant residue decomposition. Soil. Sci. Soc. Am. J. 59:1321–1328.

Waring, R.H., A.J.S. McDonald, S. Larsson, T. Ericsson, A. Wiren, E. Arwidsson, A. Ericsson and T. Lohammar. 1985. Differences in chemical composition of plants grown at constant relative growth rates with stable mineral nutrition. Oecologia 66:157–160.

Waterman, P.G. and S. Mole. 1989. Extrinsic factors influencing production of secondary metabolites in plants. *In* Insect–Plant Interactions. Ed. E.A. Bernays. CRC Press, Inc., Boca Raton, FL, pp 107–134.

Waterman, P.G. and S. Mole. 1994. Analysis of phenolic plant metabolites. Blackwell Scientific Publications, Oxford, 238 p.

Whitmore, F.W. 1976. Binding of ferulic acid to cell walls by peroxidases of *Pinus elliottii*. Phytochemistry 15:375–378.

Zucker, W.V. 1983. Tannins: Does structure determine function? An ecological perspective. Am. Nat. 121:335–365.