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## Atmospheric CO<sub>2</sub> concentration, N availability, and water status affect patterns of ergastic substance deposition in longleaf pine (*Pinus palustris* Mill.) foliage

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**Abstract** Leaf chemistry alterations due to increasing atmospheric CO<sub>2</sub> will reflect plant physiological changes and impact ecosystem function. Longleaf pine was grown for 20 months at two levels of atmospheric CO<sub>2</sub> (720 and 365  $\mu\text{mol mol}^{-1}$ ), two levels of soil N (4 g m<sup>-2</sup> year<sup>-1</sup> and 40 g m<sup>-2</sup> year<sup>-1</sup>), and two soil moisture levels (–0.5 and –1.5 MPa) in open top chambers. After 20 months of exposure, needles were collected and ergastic substances including starch grains and polyphenols were assessed using light microscopy, and calcium oxalate crystals were assessed using light microscopy, scanning electron microscopy, and transmission electron microscopy. Polyphenol content was also determined using the Folin-Denis assay and condensed tannins were estimated by precipitation with protein. Evaluation of phenolic content histochemically was compared to results obtained using the Folin-Denis assay. Total leaf polyphenol and condensed tannin content were increased by main effects of elevated CO<sub>2</sub>, low soil N and well-watered conditions. Elevated CO<sub>2</sub> and low soil N decreased crystal deposition within needle phloem. Elevated CO<sub>2</sub> had no effect on the percentage of cells within the mesophyll, endodermis, or transfusion tissue which contained visible starch inclusions. With respect to starch accumulation in response to N stress, mesophyll > endodermis > transfusion tissue. The opposite was true in the case of starch accumulation in response to main effects of water stress: mesophyll < endodermis < transfusion tissue. These results indicate that N and water conditions significantly affect deposition of leaf ergastic substances in

longleaf pine, and that normal variability in leaf tissue quality resulting from gradients in soil resources will be magnified under conditions of elevated CO<sub>2</sub>.

**Key words** *Pinus palustris* · Pinaceae · Elevated CO<sub>2</sub> · Ergastic substances

### Introduction

Atmospheric CO<sub>2</sub> is increasing; a recent estimate predicts that current levels will double within the next century (Keeling et al. 1989). The effects of elevated CO<sub>2</sub> on plant and ecosystem processes are currently receiving a great deal of attention (for recent review see Amthor 1995). Effects on forest trees, which conduct as much as two-thirds of the world's photosynthesis (Kramer 1981) and dominate many terrestrial ecosystems, must be understood before accurate predictions about the impact of elevated CO<sub>2</sub> on the biosphere can be made. However, complex interactive effects of multiple factors in nature confound our understanding of ecosystem function and highlight the need for studies which examine the effects of elevated CO<sub>2</sub> in concert with multiple environmental factors.

The longleaf pine-wiregrass association is a fire sub-climax ecosystem supporting a great diversity of both plants and animals which are dependent on the maintenance of this ecosystem for their survival (Peet and Allard 1993). Longleaf pine forests, which were once dominant in southeastern USA, have dwindled from 92 million acres to 3.2 million acres (Landers et al. 1995) due in part to fire suppression and exploitation for timber, tar, turpentine and resin (Peet and Allard 1993). The fate of longleaf pine and associated plant and animal species is therefore of concern in southeastern USA.

Pine species may realize less benefit from rising CO<sub>2</sub> concentrations than broadleaf species because the latter may be anatomically and physiologically better-adapted to assimilate the extra carbon in a high CO<sub>2</sub> environment (Ceulemans and Mousseau 1994; Pritchard et al. 1997).

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Ceulemans and Mousseau (1994) summarized the literature on response of woody plants to elevated CO<sub>2</sub>; they reported the mean increase in biomass of conifers to be 38% compared to 63% in broadleaf species. Mean increases in photosynthesis were 40% in conifers compared to 61% in broadleaf trees. Differential response of coniferous and broadleaf species suggests that conifers may be displaced in habitats where they are currently vulnerable to competition from broadleaf species, such as in longleaf forests. The role of fire in this ecosystem, resistance of longleaf pine to pathogens and herbivores, and the ability of longleaf to sequester nutrients relative to broadleaf species may assume greater importance as CO<sub>2</sub> levels continue to increase.

The increase in C fixation and subsequent alterations in whole plant carbon budgeting in a high CO<sub>2</sub> environment may alter leaf chemistry including ergastic substance deposition. Ergastic substances are defined by Esau (1977) as relatively pure, passive products of the protoplasm and may include starch grains, tannins, crystals and resins. Ergastic substances may reflect the physiological status of the plant, and perhaps more importantly may mediate larger ecological processes including plant/plant, plant/animal, and plant/pathogen interactions as well as rates of litter decomposition and thus nutrient cycling (Northup et al. 1995). For example, tannins (a class of polyphenolic compounds) have been reported to confer protection against viruses, bacteria, fungi, insects and mammals (Chiang and Norris 1983). Several studies have looked at the effects of elevated CO<sub>2</sub> on the production of defensive compounds, however, conflicting results have been reported. Carbon-based secondary compounds have been shown to increase (proanthocyanidins, flavonoids, and triterpenoids, Lavola and Julkunen-Tiitto 1994), decrease (aucubin, catalpol, and verbascoside, Fajer et al. 1992) or stay the same (mono- and sesquiterpenes, Lincoln and Couvet 1989). Lindroth et al. (1993) found that changes were species-specific for condensed and hydrolyzable tannins, and Julkunen-Tiitto and Tahvanainen (1993) reported the effects of elevated CO<sub>2</sub> on the production of carbon based compounds to be mediated by nutrient levels. However, too few systems have been studied to make any general statements about these patterns (Bazzaz 1990; Lindroth et al. 1993).

In addition to altering patterns of leaf polyphenol production, growth under elevated CO<sub>2</sub> and limiting N leads to an increase in leaf starch (Cave et al. 1981; Clough et al. 1981). This may reflect inability to assimilate carbohydrates at the same rate at which carbon was being fixed (sink limitation). Alterations in leaf carbohydrate content may contribute to increased C:N ratios, a relationship that generally results in lower overall tissue quality to herbivores, and slower rates of leaf decomposition (Lincoln 1993).

Crystals of calcium oxalate which occur in many taxa are among the most widely found ergastic substance in plants (Okoli and Mceuen 1986). Although crystals are conspicuous in many plant tissues, no study has examined the effects of elevated CO<sub>2</sub> on crystal deposition. This may be partly due to an incomplete understanding of a possible function of crystals (Franceschi and Horner 1980). How-

ever, several functions have been attributed to calcium oxalate crystals in plants, including defense against foraging animals (Doaigey 1991), precipitation of potentially toxic waste products (Franceschi 1984), control of cellular ionic and osmotic balance (Franceschi and Horner 1980) and finally, possibly structural support (Okoli and Mceuen 1986).

Alterations in leaf chemistry, including ergastic substances, under future elevated atmospheric CO<sub>2</sub> conditions might have far-reaching implications on the way a plant interacts with both the biotic and abiotic environment. For example, insect herbivores are thought to possess a nutritional niche which is defined by their physiological tolerance thresholds to various plant components (Clancy et al. 1995). Deviations from the optimal range in leaf chemical characteristics may alter their performance and thus change the dynamics of these complex interactions. In light of the importance of leaf chemistry to plant and ecological function, the objectives of this study were to determine the effect of twice ambient levels of CO<sub>2</sub>, two levels of soil moisture, and two levels of soil nitrogen on the production of ergastic substances including starch grains, phenolic compounds, and calcium oxalate crystals in longleaf pine foliage.

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## Materials and methods

### Plant exposure system

Longleaf pine seedlings (mean root collar diameter = 13 mm, SD = 2) from a wild seed source were exposed to elevated CO<sub>2</sub> (~720 μmol mol<sup>-1</sup>) and ambient CO<sub>2</sub> (~365 μmol mol<sup>-1</sup>) conditions in open top chambers (Rogers et al. 1983) beginning 30 March 1993 and were maintained until the final harvest on 28 November 1994. The chambers, CO<sub>2</sub> supply, and CO<sub>2</sub> monitoring/dispensing systems have been described by Mitchell et al. (1995).

Seedlings were planted in a coarse sandy medium (pH 5.1) in 45 l pots. Two levels of soil N and water were employed. Nitrogen treatments (applied as sulfur-coated urea, 38-0-0) consisted of 4 g m<sup>-2</sup> year<sup>-1</sup> for the low treatment and 40 g m<sup>-2</sup> year<sup>-1</sup> for the high treatment and were administered according to Mitchell et al. (1995). Other nutrients were maintained at non-limiting levels by application of sulfur-coated potassium (0.04 mg K g<sup>-1</sup> soil year<sup>-1</sup>) and MicroMax Plus (P = 0.14, Ca = 0.57, Mg = 0.28, and S = 0.05 mg g<sup>-1</sup> soil year<sup>-1</sup>, plus a complete complement of micronutrients). In April 1993, a single application of iron chelate (0.007 mg Fe g<sup>-1</sup> soil) was made.

After seedling initiation (19 weeks after initiating the study), Teflon rain exclusion caps were fitted to chambers in order to implement different soil water regimes. Water-stressed plants were allowed to dry to -1.5 MPa before watering, and well-watered plants were maintained between 0 and -0.6 MPa predawn xylem pressure potential. Xylem pressure potentials were determined periodically throughout the study with a pressure bomb (Scholander et al. 1965). Water status determined from the pressure bomb was converted into gravimetric standards so that appropriate water regimes could be maintained using a pneumatic weighing device of our own design.

### Light microscopy procedures

Needle tissue was collected on the morning of 29 November 1994 after 20 months exposure to the various treatments. Needles were selected from fully expanded three-needle fascicles from seedlings representing all possible combinations of N and water treatments from six chambers

supplied with ambient CO<sub>2</sub> and six amended with twice ambient levels. Needles were selected from the bottom third of seedlings such that needles had developed to maturity under experimental conditions. Needles were dipped in chloroform for 30 s and 4 mm needle segments were excised from the center portion of each needle. Tissue segments were fixed in 2.5% glutaraldehyde in 1.0% acrolein in a 0.05 M phosphate buffer (pH 6.8) for 2 h at 4 °C. After 2 h, tissue was removed from the fixative and one 1 mm was trimmed from the end of each 4 mm segment. Tissue was transferred to fresh fixative for an additional 2 h at 4 °C. Finally, needle segments were washed twice in a phosphate buffer, passed through a cold ethanol dehydration series, infiltrated for 6 days at 4 °C, and embedded in LR White medium grade plastic. Thick sections (1.5 µm) were stained for polyphenols using TBO, and for carbohydrates using PAS. For histochemical quantification of apparent vacuolar polyphenol content, an eyepiece reticle was used and cells falling within the grid were classified as either empty, partially filled, or completely filled (Fig. 2A, B). The extent of starch accumulation in the mesophyll, endodermis, and transfusion tissue was determined by establishing a relative scale of 0–2. Those tissue regions which contained no cells with visible starch grains (at 400× magnification) were classified as 0, if approximately 50% of cells contained visible starch grains they were classified as 1.0, and if all cells contained visible starch inclusions they were classified as 2.0. Light micrographs of cross-sections also were used to count the number of visible prismatic crystals within the phloem tissue. Crystals were confirmed to be calcium oxalate using the Pizzolato method (1963) and the three dimensional nature of crystals was determined with SEM and TEM using standard procedures.

#### Assay for total phenolics and condensed tannins

Additional needle tissue was collected at the same time that needles were collected for microscopy. Bulked needles were dried at 55 °C; although lower temperatures are ideal, drying at this temperature has been experimentally determined to give accurate data for relative comparisons (Gartlan et al. 1980). Needles were ground in a Wiley mill using a 0.2 mm mesh screen. Samples (8 g each) were extracted three times (1 × 75 ml, and 2 × 50 ml) at room temperature in 70% acetone in water (Hagerman 1988) while being stirred vigorously. Large samples were used to eliminate between-needle and between-fascicle variation. Samples were filtered in a Buchner funnel (Whatmans no. 4), and residual plant material rinsed (1 × 50 ml) with additional solvent. Acetone was removed under vacuum in a round-bottomed flask. Remaining extract was then freeze dried leaving a powder.

Total phenolics determined using the Folin-Denis method, and condensed tannins determined by protein precipitation, were modifications of methods described by Seigler et al. (1986). An aqueous tannin extract was prepared by suspending 75 mg of tannin extract in 100 ml distilled H<sub>2</sub>O and then diluting 1 ml of this in 10 ml of distilled H<sub>2</sub>O. Two ml of Folin-Denis reagent (Seigler et al. 1986) were then added to a 2 ml aliquot of aqueous tannin extract. This mixture was shaken, and 3 min later, 2 ml of sodium carbonate solution (53 g/500 ml H<sub>2</sub>O) was added. This was shaken vigorously, allowed to react for 2 h, and then absorbance was measured at 725 nm. In each case a blank was analyzed, and two replicates averaged for each sample. A standard curve was created by plotting absorbance values of known concentrations of tannic acid solution. Absorbances from needle extracts were compared to the standard curve and phenolic content expressed as tannic acid equivalents (TAE). Tannic acid is a hydrolyzable tannin, which is restricted to angiosperms; tannins present in the gymnosperms are all of the condensed variety (Waterman and Mole 1994). Therefore, the use of tannic acid equivalents is not intended to reflect the absolute quantity of polyphenols present, but instead is used to assess relative phenolic contents across different treatments (Waterman and Mole 1994).

Condensed tannins were determined by precipitation with casein (Seigler et al. 1986). Aliquots (6 ml) of aqueous tannin extract as prepared above were combined with 12 ml of water. One gram of casein powder (Sigma Chemical, St. Louis, Missouri) was added to this solution. Samples were stirred vigorously for 3 h, filtered (Whatmans

no. 4), flasks were rinsed with 10 ml distilled H<sub>2</sub>O, and total filtrate volume brought up to 25 ml. The Folin-Denis method was then used to determine remaining phenolics.

#### Experimental design and data analysis

Treatments were arranged in a split-plot design with six replications. Carbon dioxide treatments (main plots) were randomly assigned to chambers within replicates. Nitrogen and water treatments (subplots) were randomly assigned to a total of 16 containers within each chamber. Pots were moved each month to eliminate within chamber effects.

Data were analyzed 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  $P \leq 0.05$  level.

## Results and discussion

Extra carbon available in a high CO<sub>2</sub> environment may be allocated to increase growth, maintenance, food storage, or production of carbon-based secondary compounds. In light of the uncertainty concerning the fate of fixed carbon, and due to the importance of leaf chemistry to both whole plant physiology and ecosystem function, there has been speculation concerning the effects that rising CO<sub>2</sub> will have on patterns of secondary compound production (for recent discussion, see Lambers 1993; Lincoln 1993). Two hypotheses have been proposed to explain how synthesis of these compounds is regulated, and thus they provide the framework around which discussions concerning the impact of elevated atmospheric CO<sub>2</sub> must be built. The first hypothesis, the carbon/nutrient balance or carbon supply model, was proposed by Bryant et al. (1983). According to this hypothesis, the production of defensive compounds is regulated by carbohydrate availability. When carbohydrate production exceeds growth demands, the overflow will be allocated to secondary compound production. One would expect an oversupply of carbohydrates and thus greater carbon-based secondary compounds under high-light, limiting-nutrient, and elevated-CO<sub>2</sub> conditions. Indeed, increases in production of both shikimate-derived and mevalonate-derived secondary compounds have been observed in many studies in which nutrients were limiting (Lincoln 1993).

The other hypothesis which attempts to explain production of carbon-based compounds is the amino acid diversion model (Lambers 1993) suggested by Margna (1977). According to this model, the rate of incorporation of the shikimate-derived amino acids phenylalanine and tyrosine into proteins dictates the rate of synthesis of secondary compounds. When phenylalanine and tyrosine are rapidly incorporated into proteins, the shikimic acid pathway will synthesize more amino acids. In contrast, when rates of growth (and protein synthesis) are low, there will be a large pool of available amino acids and the shikimate pathway will then be shifted to produce secondary compounds. Under certain conditions, (i.e., low nutrients), one would

**Table 1** Leaf phenolics determined using Folin-Denis assay, condensed tannins determined by casein precipitation, and apparent mesophyll vacuolar contents determined histochemically. *Hi N* = 40 g m<sup>-2</sup> year<sup>-1</sup> nitrogen, *Lo N* = 4 g m<sup>-2</sup> year<sup>-1</sup> nitrogen, *WW* = well-watered, *WS* = water-stressed. Leaf phenolic content numbers are

means ( $\pm$ SD) of 3–6 replications. Phenolics and condensed tannins are expressed as tannic acid equivalents (TAE). For a description of % empty, % partly filled, and % full, see text. Numbers are means ( $\pm$ SD) of six replications with 100–120 cells counted per replicate  $tr = 0.05 \leq 0.10$ ,  $*P \leq 0.05$ ,  $**P \leq 0.01$ ,  $***P \leq 0.001$

Treatment	Leaf phenolic content			Apparent vacuolar contents		
	% phenolics	% condensed tannins	condensed tannins (% of total phenolics)	% empty	% partially filled	% full
<b>Elevated CO<sub>2</sub> (720 ppm)</b>						
Hi N-WW	3.4 $\pm$ 1.2	2.7 $\pm$ 1.1	78 $\pm$ 5.0	33 $\pm$ 23	42 $\pm$ 11	26 $\pm$ 14
Hi N-WS	3.1 $\pm$ 0.8	2.5 $\pm$ 0.8	78 $\pm$ 4.3	20 $\pm$ 23	34 $\pm$ 22	46 $\pm$ 41
Lo N-WW	6.4 $\pm$ 1.1	5.5 $\pm$ 1.1	86 $\pm$ 4.2	23 $\pm$ 34	15 $\pm$ 14	62 $\pm$ 37
Lo N-WS	4.2 $\pm$ 0.7	3.6 $\pm$ 0.6	85 $\pm$ 1.8	22 $\pm$ 18	42 $\pm$ 12	37 $\pm$ 29
<b>Ambient CO<sub>2</sub> (365 ppm)</b>						
Hi N-WW	2.5 $\pm$ 0.3	2.0 $\pm$ 0.3	80 $\pm$ 2.8	41 $\pm$ 28	33 $\pm$ 14	25 $\pm$ 26
Hi N-WS	1.9 $\pm$ 0.3	1.4 $\pm$ 0.3	76 $\pm$ 3.8	8 $\pm$ 12	22 $\pm$ 24	59 $\pm$ 41
Lo N-WW	5.3 $\pm$ 1.0	4.5 $\pm$ 0.9	85 $\pm$ 4.4	4 $\pm$ 5	12 $\pm$ 6	85 $\pm$ 8
Lo N-WS	3.5 $\pm$ 0.5	2.8 $\pm$ 0.5	82 $\pm$ 5.1	5 $\pm$ 5	20 $\pm$ 13	75 $\pm$ 16
<b>Significance levels</b>						
CO <sub>2</sub>	**	*	NS	NS	tr	tr
N	***	***	***	tr	*	*
CO <sub>2</sub> $\times$ N	NS	NS	NS	NS	NS	NS
W	***	***	NS	NS	NS	NS
CO <sub>2</sub> $\times$ W	NS	NS	NS	NS	NS	NS
N $\times$ W	**	**	NS	tr	**	*
CO <sub>2</sub> $\times$ N $\times$ W	NS	NS	NS	NS	NS	NS

expect an increase in secondary compounds under elevated CO<sub>2</sub> due to the nutrient dilution effect of growth under elevated CO<sub>2</sub>; however, under conditions of readily available nutrients, one would expect no increase.

In the current study, there were main treatment effects of CO<sub>2</sub>, N, and water availability on production of both total phenolics and condensed tannins (Table 1). When CO<sub>2</sub> was elevated, needles contained an average of 4.3% total phenolics and 3.5% condensed tannins compared to 3.3% and 2.7% under ambient CO<sub>2</sub> conditions. The increase in phenolics due to elevated CO<sub>2</sub> was consistent and occurred under both water-stressed and well-watered conditions, and under both high and low N fertility. If we accept the assertion of Mitchell et al. (1995) that longleaf pine grown under elevated CO<sub>2</sub> and non-limiting N is source-limited, then under these conditions, the amino acid diversion model would predict no increase in phenolic production. However, when CO<sub>2</sub> was elevated and N was non-limiting, leaves contained 3.2% total phenolics, and 2.6% condensed tannins compared to 2.2% and 1.7% in leaves of seedlings grown under ambient CO<sub>2</sub> and high N. Thus it appears that production of shikimate-derived compounds was driven by C availability, which supports the carbon nutrient balance hypothesis. Although no other study has examined the effects of increased CO<sub>2</sub> on total phenolics and condensed tannins in a gymnosperm, proanthocyanidins have been reported to increase in *Acer saccharum* (Lindroth et al. 1993), *Salix myrsinifolia* (Julkunen-Tiitto and Tahvanainen 1993) and *Betula pendula* (Lavola and Julkunen-Tiitto 1994) when grown under elevated CO<sub>2</sub>. In contrast, Lindroth et al. (1993) found no effect of elevated CO<sub>2</sub> on condensed tannins or their capacity to precipitate

proteins in *Populus tremuloides* and *Quercus rubra*, and Fajer et al. (1992) found the concentrations of carbon-based allelochemicals to decrease in *Plantago lanceolata* when grown under elevated CO<sub>2</sub>.

The significant main effect of N fertility on phenolic accumulation supports the findings of many studies. Indeed, increased phenolic production in plants subjected to nutrient stress observed in this and other studies (for example see Briggs 1990; Bryant et al. 1987) generally is well-accepted (Bazzaz et al. 1987). Plants grown under conditions of limiting N produced a greater percentage of total leaf phenolics and condensed tannins than seedlings grown under non-limiting N conditions, regardless of the CO<sub>2</sub> or water treatment (Table 1). Interestingly, in leaves from seedlings grown under limiting N, a greater percentage of total phenolics present were removed from solution by precipitation with protein (Table 1, see condensed tannins as % of total phenolics) than those plants grown under high N conditions. The molecular weight of tannins (MW) is the most important factor contributing to their capacity to precipitate proteins. In general, tannins of high MW precipitate proteins, whereas smaller, simple phenolics do not (Waterman and Mole 1994). Condensed tannin molecules are composed of chains of flavonoid subunits each of which is composed of two aromatic rings. One aromatic ring is derived from phenylalanine via the shikimate pathway, the other from three acetate subunits (Waterman and Mole 1994). Thus, an increase in protein precipitation suggests not only an increase in shikimate-derived compounds but also may suggest that either greater amounts of acetate were available or the expression/activity of crucial enzymes (i.e., chalcone synthase) was stimulated by N deficiencies. Re-

ardless, the capacity of leaf tannins to precipitate proteins in solution is probably the most useful index of their ecological significance (Martin and Martin 1982). Astringency, or the ability of tannins to complex with protein molecules, decreases the nutritional quality of leaf tissue to herbivores by reducing plant digestibility (Robins et al. 1987) and imparts a bitter taste ("puckeriness") which deters feeding of vertebrate herbivores (Schultz 1989).

The increase in relative astringency (ability to precipitate proteins) and the accumulation of polyphenols in general may represent evolutionary coupling of environmental conditions, leaf polyphenolic characteristics, and nutrient cycling rates (Horner et al. 1987). Northup et al. (1995) examined rates of litter decomposition of *Pinus myricata* and observed a highly significant correlation between litter polyphenol and condensed tannin contents and the rates of release of both mineral and organic forms of N. They suggested that convergent evolution of tannin accumulating plant species on highly leached, nutrient limiting sites may be explained by the relationship between litter decomposition and leaf phenolic content. According to Northup et al. (1995), pine species may effectively monopolize existing N by presenting it as protein-tannin complexes which may be available preferentially to associated mycorrhizae. If this mechanism does indeed allow pine forests to remain productive by minimizing N loss, then the changes we have observed due to growth at elevated CO<sub>2</sub> may suggest one mechanism by which pine species may further tie up available N, and thus affect long term productivity as global climates continue to change. Furthermore, increased production of phenolics and condensed tannins may decrease tissue losses due to insects and pathogenic fungi.

Although phenolic production in response to N fertility has been well-established, effects of water stress on phenolic accumulation have received very little attention (Waterman and Mole 1994). Studies which have addressed the impact of water status on total polyphenolic and condensed tannin production have reported inconsistent and often perplexing results (Waterman and Mole 1994). Horner (1990) suggested that the relationship between phenolic production and plant water-status is nonlinear; allocation to carbon based compounds may change as the magnitude of the water deficit changes. When a plant is under mild water stress, carbohydrates and phenylalanine (a precursor for phenolic compounds) both increase and thus tissue phenolic content increases. When a plant is subjected to more severe water stress, stomata close, carbon gain decreases, and thus excess carbon for synthesis of defensive compounds is not available.

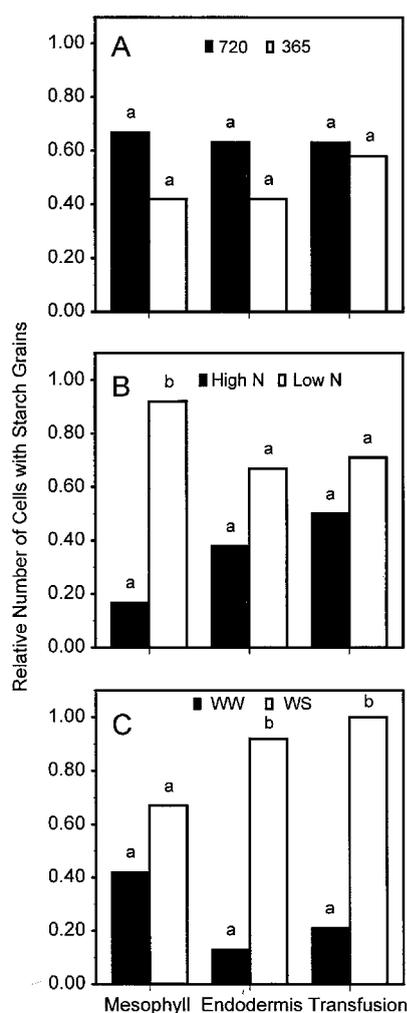
In the current study, seedlings grown under water deficits (allowed to dry to  $-1.5$  Mpa predawn xylem potential) contained significantly less total phenolics and condensed tannins (Table 1). However, it is important to note that in addition to the main effect of water treatment, there was a significant N by water interaction for both total phenolics and condensed tannins (Table 1). This interaction was one of magnitude, not direction; the magnitude of phenolic accumulation in well-watered seedlings versus

water-stressed seedlings was greater under conditions of low N fertility than high N fertility. Under conditions of high N fertility, water status had little or no effect on phenolic accumulation. In addition (or perhaps in contrast) to the hypothesis proposed by Horner (1990), this interaction could account for some of the contrasting reports in the literature concerning effects of soil moisture on phenolic production. Most studies have not taken into consideration the effect of nutrient levels on plant water status (For example see Donnelly 1959; Horner 1990; Guinn and Eidenbock 1982; Tempel 1981).

#### Apparent polyphenol content determined histochemically

Investigators often attempt to determine polyphenol content of cells and plant tissue histochemically, using light microscopy (for example, see Mosjidis et al. 1990; Wilkeshaw 1992; Zobel and Nighswander 1990). In pine species, perhaps 90% of total leaf polyphenols are located within the central vacuoles of mesophyll cells (Masuch et al. 1992) and their appearance and form is often altered by changes in environmental conditions. Thus, histochemical methods provide an easy method to ascertain changes in tannin deposition due to treatment effects, developmental stages, or species differences. However, no study has compared histochemical determination of polyphenol content to results determined more directly using one of the many accepted assay techniques. Soikkeli (1980), however, reported that the visual changes in mesophyll tannin contents in pine species, which are often reported to parallel the frost hardening process, are not caused by direct changes in polyphenol contents, but instead are caused by changes in cell solute potential which creates a different image as a result of the fixation process.

Tannins of longleaf pine appeared to be located predominantly within the central vacuoles of mesophyll cells, and were visibly altered by resource levels. In our study, the mesophyll cells from leaves grown under elevated CO<sub>2</sub> appeared to have fewer tannins and their form and distribution was affected (Table 1, apparent vacuolar content and Fig. 2, A vs B). In the elevated CO<sub>2</sub> treatment, tannins often appeared to be dispersed as large granulated droplets, or were ribbonlike and lined the periphery of the central vacuole. In contrast, in the ambient CO<sub>2</sub> treatment, tannins often appeared to be dispersed as small granules or droplets throughout the vacuole. Although statistical significance was obscured by large variances, trends indicated that more mesophyll cells appeared empty ( $P = 0.12$ ), more appeared to be partly filled ( $P = 0.06$ ), and fewer vacuoles appeared full ( $P = 0.10$ ) due to growth under elevated CO<sub>2</sub>. This at first seems to directly contradict the increases in total phenols and condensed tannins discussed earlier. However, TEM micrographs showed that mesophyll cells which appeared to contain no visible tannins at the light microscope level actually contained a ribbon of tannins lining the periphery of the vacuole (data not shown). The apparent differences in form of vacuolar tannins due to the CO<sub>2</sub> treatment are similar to the apparent differences which



**Fig. 1** Main treatment effects comparing the relative percentage of cells containing visible starch grains in the mesophyll, endodermis, and transfusion tissue for CO<sub>2</sub> (A), nitrogen (B), and water treatments (C). On the Y axis, 0 = no cells containing visible starch grains, 1.0 = 50% of cells containing visible starch. Bars in common tissue regions indicated with the same letters are not significantly different at  $P \leq 0.05$

result from fixation of cells possessing different osmotic potential as discussed by Soikkeli (1980). Thus, it is probable that the apparent discrepancy was caused by differences in solute potential between cells of ambient-grown versus elevated CO<sub>2</sub>-grown seedlings. Indeed, far reaching effects of elevated CO<sub>2</sub> on carbohydrate and whole plant nutrient balance which are often reported (Cave et al. 1981; Mitchell et al. 1995; Pritchard et al. 1996) may have resulted in altered cell solute potential.

There was a significant main treatment effect of N on the number of mesophyll cells which appeared to be partially full and completely full of vacuolar tannins (Table 1, Fig. 2A vs B). There was also a significant N  $\times$  water interaction; needles from plants grown under limiting N conditions had a greater percentage of mesophyll cells which were apparently filled with tannins than did plants grown under high N. However, this apparent increase

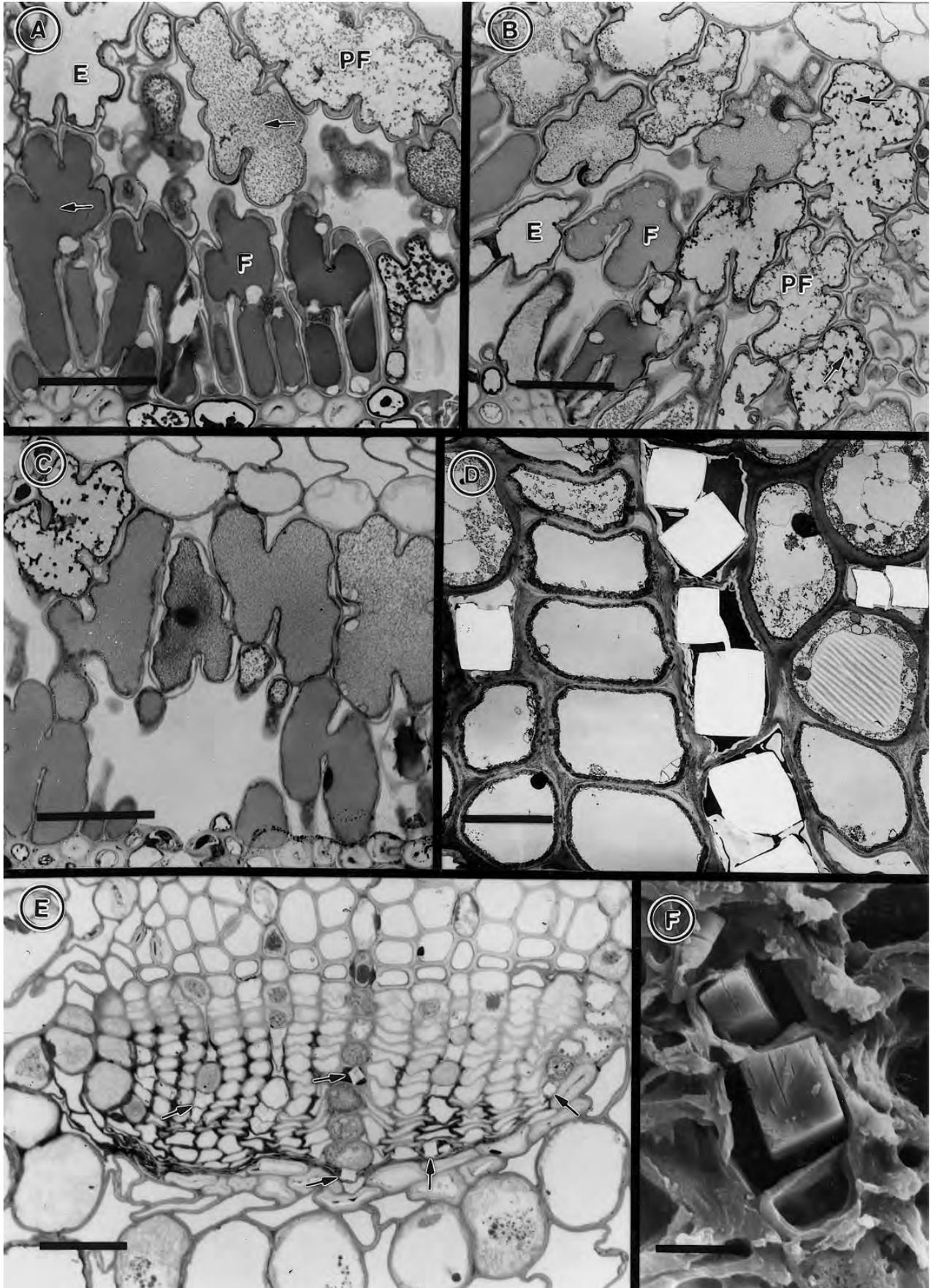
occurred under well-watered, but not water-stressed conditions. These results were generally consistent with the results obtained from the protein precipitation and Folin-Denis assays.

It appears that the histochemical determination of vacuolar tannin contents may be misleading. Metabolic differences in cells due to treatment effects may alter cell solute potential which may contribute to differential fixation, and thus make visual comparison of tannin contents unreliable.

#### Distribution of starch grains

Growth under elevated CO<sub>2</sub> did not significantly increase the proportion of cells which contained starch grains in any of the tissue regions examined (Fig. 1A). However, there was a significant ( $P < 0.05$ ) CO<sub>2</sub>  $\times$  N  $\times$  water interaction for each of the tissue regions; needles grown under elevated CO<sub>2</sub> appeared to contain more starch than those grown under ambient CO<sub>2</sub> only when N and water were both limiting (data not shown).

Growth under limiting N significantly increased the percentage of cells which contained starch in the mesophyll tissue but not in the transfusion tissue or endodermis (Fig. 1B). With respect to the accumulation of starch in response to N deficiencies, mesophyll > endodermis > transfusion tissue. From these data it appears that differences in starch accumulation due to variable N availability are manifested in the mesophyll tissue, whereas N treatments had little or no effect in the endodermis and transfusion tissue. The increase in starch accumulation due to soil N limitation was probably due to greater decreases in rates of growth than in rates of photosynthesis. Effects of water stress on starch accumulation were strikingly different. Seedlings subjected to periodic episodes of water stress contained a significantly higher percentage of cells with visible starch in the transfusion tissue and the endodermis, but not in the mesophyll tissue (Fig. 1C). With respect to the accumulation of starch in response to water stress, our results showed transfusion tissue > endodermis > mesophyll. Presumably, carbon was fixed predominantly within the mesophyll tissue and was transported through the endodermis and across the transfusion tissue before being loaded into phloem sieve cells for long distance transport. The gradient in starch buildup from the site of photosynthesis (mesophyll) to the site of phloem loading may suggest that short-distance assimilate transport was altered by growth under water stress. Differential changes in starch deposition across tissue regions due to different resource limitations may be significant for plant/pathogen relationships. For example, *Cronartium fusiforme*, a common fungal rust infecting southeastern pine species, is sensitive to the carbohydrate balance of the host plant (Littlefield 1981). Although both N limitations and water stress increased the accumulation of starch, the effects appeared to be manifested in different needle regions and thus may result in different patterns of infection in tissue-specific pathogens.



## Calcium oxalate deposition

Prismatic calcium oxalate crystal deposition within the phloem tissue showed main effects of both CO<sub>2</sub> level ( $P < 0.02$ ) and N fertility ( $P < 0.05$ ). Cross-sections from needles grown under elevated CO<sub>2</sub> contained an average of 27 visible crystals compared to 51 in needles from seedlings grown under ambient CO<sub>2</sub> conditions, and needles grown under high N fertility contained 49 crystals compared to 27 in needles from seedlings grown under low N fertility (data not shown). There were no significant two- or three-way interactions (data not shown).

Among other functions, it has been suggested that crystals may provide mechanical support (Okoli and Mceuen 1986). In longleaf pine needles, prismatic crystals (Fig. 2F) were generally located within the phloem tissue most distal to the needle vascular cambium (Fig. 2E), within the vacuoles of ray cells (Fig. 2D). Crystals were not observed in any other tissue regions. Calcium oxalate crystals have not been previously described in longleaf pine, however, the presence of crystals in partially crushed phloem cells of other pine species has been reported (Fink 1991).

If the occurrence of calcium oxalate crystals is an inevitable result of normal cellular function (Franceschi and Horner 1980), it would be advantageous for crystals to be deposited in cells in such a way that they might provide a dual function. In longleaf pine it appears that crystals may provide support (Fig. 2D, E) to prevent phloem tissue from being crushed by secondary needle growth (Ewers 1982). Such a dual function may also be the case in more highly specialized crystal formations, such as those occurring in many aroid plants (Franceschi and Horner 1980).

Although the presence of calcium oxalate in the phloem may provide mechanical protection against cell collapse, this is probably incidental to the role of crystals in normal cellular metabolism. The mechanism behind the reduction in crystal deposition due to growth at elevated CO<sub>2</sub> is not readily apparent due to the great number of conflicting views on the physiology of normal crystal formation (Franceschi and Horner 1980). However, the occurrence of calcium oxalate is dependent upon the presence of both oxalate and Ca, and therefore the reduction in crystals might be the result of a decrease in availability of either

oxalate, or Ca, or both. Decreased crystal deposition under elevated CO<sub>2</sub> might be the result of the "nutrient dilution effect" which may have resulted in less calcium per gram of plant tissue. The possibility that there was a decrease in the availability of oxalate is less likely. Generally, oxalate content is positively correlated to rates of photosynthesis (Franceschi and Horner 1980). Increases in photosynthetic rates resulting from growth at elevated CO<sub>2</sub> would thus be coupled to increases in oxalate production. The significant increase in calcium oxalate crystals in plants grown under conditions of high N fertility could be explained by greater oxalate formation due to increased rates of photosynthesis.

In conclusion, total polyphenols and condensed tannins were increased in longleaf pine seedlings grown under conditions of elevated CO<sub>2</sub> versus ambient CO<sub>2</sub>, under low-N compared to high-N fertility, and under well-watered compared to water-stressed conditions. The increase in phenolics due to growth under elevated CO<sub>2</sub> might suggest a mechanism by which longleaf pine may influence rates of nutrient cycling and thus provide this species an advantage in sequestering N and resisting pathogens as global climates continue to change. Furthermore, changes in calcium oxalate crystal deposition resulting from growth at elevated CO<sub>2</sub>, and in starch deposition resulting from interactions of elevated CO<sub>2</sub>, soil N and water availability, indicate that normal fluctuations in ergastic substance deposition may be magnified by growth at elevated CO<sub>2</sub>. This suggests that alterations in longleaf pine needle quality induced by elevated CO<sub>2</sub> may be greater than variation resulting from naturally occurring gradients in soil resource availability. Further work will be required to elucidate the effects that such leaf changes will have on specific trophic-level interactions.

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**Fig. 2** Light micrographs showing the nature of polyphenols within vacuoles of longleaf pine needle mesophyll cells (A, B, C), and TEM, light, and SEM micrographs showing the locations of calcium oxalate crystals associated with needle phloem (D, E, F). (A) ambient CO<sub>2</sub>, high N, well watered; E = empty, PF = partially filled, F = full. Note: tannins appear as small droplets and are diffused evenly throughout the central vacuoles (arrows). Bar = 18 μm. (B) elevated CO<sub>2</sub>, high N and well watered; E = empty, PF = partially filled, F = full. Note large percentage of cells in which vacuolar tannin deposits appear coagulated (arrows). Bar = 18 μm. (C) ambient CO<sub>2</sub>, low N, and well watered. Bar = 18 μm. (D) TEM micrograph of needle phloem showing outlines where crystals fell out during sectioning. Crystals are located in ray cells. Bar = 8 μm. (E) light micrograph showing the location of crystals in the older phloem. Note crushing of cells. Bar = 10 μm. (F) SEM micrograph showing prismatic shape of crystals. Bar = 8 μm

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