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The effects of sodium erythorbate and ethylenediurea on photosynthetic function of ozone-exposed loblolly pine seedlings

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Abstract

In an open-top chamber study in east Texas, ozone-sensitive loblolly pine (*Pinus taeda* L.) seedlings were treated with either the antioxidant Ozoban (74.5% sodium erythorbate active ingredient (a.i.)) at 0, 1030, or 2060 mg liter^{-1} or ethylenediurea (EDU 50% a.i.) at 0, 150, or 300 ppm every 2 weeks while being subjected to a range of ozone exposures beginning in April 1994. The ozone exposures included sub-ambient ozone levels (CF), approximate ambient (NF), and 1.5, 2.0, and 2.5 times ambient ozone (1.5×, 2.0×, and 2.5×, respectively). The response variables included net photosynthesis (A), stomatal conductance (g), chloroplast pigment concentration, and total foliar N concentration. Foliar injury due to ozone was observed early in the growing season, but subsided over time. Ozoban did not have any consistent effects throughout the experiment on the response variables, but did cause changes early in the study on gas exchange. Both g and A photosynthesis were greater at elevated ozone levels in seedlings treated with 1030 mg liter^{-1} of Ozoban compared to those treated with either 0 or 2060 mg liter^{-1} for the first sampling period in June. However, at CF, seedlings treated with 1030 mg liter^{-1} of Ozoban showed signs of reduced A compared to the other antioxidant treatments. This indicates that sodium erythorbate may have a negative physiological effect on seedlings in a low ozone environment. For EDU-treated seedlings, no consistent antioxidant treatment effects were observed, but linear regression analysis indicates that EDU shows promise in providing protection from ozone injury. At 150 ppm, EDU may retard stomatal closure in younger pine seedlings. No consistent benefit was afforded to chloroplast pigments for the study by either antioxidant. Ozoban at 2060 mg liter^{-1} appeared to have phytotoxic effects with regards to chlorophyll a and total carotenoids in the latter stages of the experiment. No significant Ozoban or EDU effects were observed with respect to total foliar N concentration until the final sampling period in October, where foliage treated with 300 ppm of EDU displayed higher N concentration at all ozone levels except 1.5× ambient ozone. Foliage treated with 150 ppm EDU showed the lowest nitrogen concentration in CF and NF, but the highest in 1.5×. Because of relatively low ozone exposures during the study year and the closeness with which the seedlings were grown, these results may not accurately represent the benefits of sodium erythorbate or EDU in reducing ozone stress in loblolly pine seedlings for field-grown trees. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Since 1950, researchers have been concerned about the increased amount of ozone in the troposphere and its negative effects on vegetation (Haagen-Smit, 1958; Cooley and Manning, 1987; Runeckles and Chevone, 1992). Ozone is not confined to heavily populated areas; its precursors are highly mobile, and ozone can be formed in relatively rural and remote regions (Miller, 1983; Skelly et al., 1983; Krupa and Manning, 1988). Many of the rural areas in the USA are used for crop production or sustain forested lands that are essential to the economy of the country. Some of the negative effects associated with ozone include decreased chlorophyll content, decline in photosynthesis, premature leaf senescence, and a reduction in total plant biomass (Miller, 1983; Peterson et al., 1987; Runeckles and Chevone, 1992). In pine species, foliar ozone symptoms include chlorotic mottling and banding, tip necrosis, and premature needle senescence (Davis and Wood, 1972, 1973; Coyne and Bingham, 1982).

Various methodologies of ozone exposure have been employed to study these damaging effects. While

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advantages and disadvantages exist for all of these methodologies, there is debate concerning which exposure methods are best for studying the effects of ambient ozone on plants (McLeod and Baker, 1988; Manning and Krupa, 1992). An alternative methodology of studying ozone/vegetation interaction is through the use of antioxidant chemicals. One such chemical, sodium erythorbate, is an ascorbic acid isomer found in the product Ozoban, which was marketed by Pfizer Chemical Division of Bio-Agricultural Products Group, NY, to reduce ozone injury in Thompson Seedless grapes (*Vitis vinifera* L.) grown in California. Ascorbic acid is synthesized naturally within the cytosol of plant cells and transported to the apoplast and chloroplast where it acts as an oxyradical defense system to prevent cellular damage from normal metabolic function (Chameides, 1989; Luwe et al., 1993; Rautenkranz et al., 1994). Sodium erythorbate differs from the vitamin C molecule by transposed hydrogen and hydroxyl constituents on the number 2-carbon.

Protection of vegetation from ozone by Ozoban has shown promise. Shortleaf pine (*Pinus echinata* Mill.) seedlings treated with up to 1030 mg liter^{-1} Ozoban for 1 year in an ambient environment had greater whole-plant biomass and diameter growth than untreated seedlings (Flagler and Toups, 1991). It was reasoned that the increased foliar biomass indicates a prevention of premature foliar senescence (Flagler and Toups, 1991). However, the avoidance of foliar ozone injury in grape and pine has been inconsistent (P.M. McCool, personal communication, as cited in Flagler et al., 1994). Little work has been done to determine the mechanism(s) by which sodium erythorbate protects vegetation. It has been reported that this isomer does not affect gas exchange characteristics (stomatal conductance (g) and net photosynthesis (A)) in shortleaf pine seedlings in a controlled environment (Flagler et al., 1994). To the best of our knowledge, no reports of the effects of sodium erythorbate on chloroplast pigments or foliar nitrogen have been published.

The use of *N*-[2-(2-oxo-1-imidazolidinyl)ethyl]-*N'*-phenylurea (ethylenediurea or EDU), another antioxidant chemical, has shown promise as a potential research tool (Clarke et al., 1984, 1990; Greenhalgh et al., 1987; Brennan et al., 1990). EDU is a complex, N-containing molecule having an aromatic, hydrophobic end and a cyclic, hydrophilic end. It was reported by Carnahan et al. (1978) to protect pinto beans (*Phaseolus vulgaris* L.) from foliar injury at high ozone concentrations. However, the mechanism by which this chemical acts is highly speculative. It is theorized that EDU could stimulate cytokinin production or have cytokinin-like characteristics in protecting chlorophyll from breakdown, increasing protein and RNA synthesis, and encouraging cell growth (Lee and Chen, 1982). Bennett et al. (1978) reports no stomatal closure or photosynthetic decline due to EDU treatment in crop plants. EDU was not found to affect g or A in shortleaf pine treated with up to 450 ppm and exposed to 200 ppb of ozone in a controlled-environment study (Flagler et al., 1994). Membrane lipids as well as chlorophyll are also shown to be protected from ozone in EDU-treated snapbean plants (Whitaker et al., 1990). EDU has been reported to protect many herbaceous and woody angiosperms from ozone both in controlled-environment chambers and in ambient conditions (Clarke et al., 1978; Temple and Bisessar, 1979; Legassicke and Ormrod, 1981; Greenhalgh et al., 1987; Ainsworth et al., 1996). The effect of EDU on pine has shown mixed results in protection (Cathey and Heggestad, 1982; Flagler et al., 1994); however, little research with pine species has been conducted.

Two experiments were conducted simultaneously using either Ozoban or EDU as the antioxidant chemical. The objectives of this research were to (1) determine the effects of Ozoban and EDU on g and A, (2) determine if either of the antioxidants protect chloroplast pigments from ozone, and (3) determine if they affect foliar N concentration in loblolly pine (*Pinus taeda* L.) seedlings.

2. Materials and methods

2.1. Research site

The research was conducted approximately 20 km southwest of Nacogdoches, TX, in the USDA Forest Service Stephen F. Austin experimental forest (31° 30′ N latitude, 94° 46′ W longitude). The mean annual maximum and minimum temperatures are 24.2°C and 11.2°C, respectively. The mean annual precipitation is 115.6 cm. The research site is surrounded by a mature loblolly and shortleaf pine forest.

2.2. Plant material

Loblolly pine from an ozone-sensitive half-sib family, S6PT2, were grown from seed. After surface sterilization with 30% hydrogen peroxide and cold stratification for 30 days, the seeds were sown singly in 115 cm^3 planting cells containing peat:vermiculite (1:3) medium. They were placed in a charcoal-filtered greenhouse in December 1993. The cells were watered using reverse-osmosis water to keep the medium moist. Six weeks after emergence, seedlings were fertilized weekly using Peter's 20-20-20 (W.R. Grace and Co., Fogelsville, PA) liquid fertilizer supplemented with additional chelated iron and micronutrients. In April 1994, seedlings were transplanted to 7-liter square pots containing a commercially available fritted clay medium that had been prepared using reverse-osmosis water to leach out

residual salts. A slow-release complete fertilizer, Sierra 17-6-10 (Grace Sierra, Milpitas, CA), was mixed into the upper 3 cm of the medium at a rate of 42.0 g per pot before seedlings were transplanted. Once transplanted, seedlings were watered to field capacity daily with reverse-osmosis water until the end of the experiment.

2.3. Ozone exposure

After being randomly assigned, the seedlings were transferred to one of 10 open-top field chambers having a diameter of 3.0 m and height of 2.5 m (Heagle et al., 1973). Ambient rainfall was prevented from contacting the seedlings by a fixed cap above the chamber. Ventilation of each chamber at an approximate rate of 60 m^3 min^{-1} was maintained from 06:00 to 24:00 h each day.

Ozone, generated from O_2 by a corona discharge-type generator (Griffin Inc., Lodi, NY), was metered through needle valves to each chamber. The ozone concentration within each chamber was monitored on a time-shared basis using a UV-photometric ozone-specific analyzer sampled through teflon tubing (Heagle et al., 1979). A UV-photometry transfer standard was used to calibrate the ozone monitors.

2.4. Antioxidant application

Ozoban (74.5% sodium erythorbate) and EDU (50% a.i.) were mixed in separate 6.0-liter pressurized polyethylene sprayers using reverse-osmosis water. For Ozoban, the concentrations were 0, 1030, or 2060 mg $liter^{-1}$ (a.i.) which represent 0, 1×, or 2× the manufacturer's recommendation for use on Thompson seedless grapes. For EDU, the concentrations were 0, 150, or 300 ppm (a.i.). These concentrations for EDU were chosen based on previous work by Flagler et al. (1994). Tween 20 (approximately 0.02 ml $liter^{-1}$) was added to the spray mixture as a wetting agent. It was believed that the surfactant had no appreciable effect on the physiology of the foliage in such small concentrations. The entire crown of each seedling was sprayed to run-off. Based on previous work by Flagler et al. (1994), seedlings were treated every 14 days with the appropriate antioxidant concentration. The first application was 23 April 1994, two days before ozone treatments began.

2.5. Gas exchange measurements

On a monthly basis from June to October, A and g were measured on detached needles using a LI-6200 portable photosynthesis system (LI-COR, Inc., Lincoln, NE). This system was equipped with a 0.25-liter leaf chamber. Needle detachment was found not to affect foliar gas exchange characteristics within 2 min after being detached (Elsik et al., 1992). Gas exchange measurements were made on one fascicle per seedling and two seedlings selected randomly per treatment combination for each treatment combination. Because this study was designed to observe ozone injury over time, all foliage samples were collected from the lowest flush on the stem. Seedlings treated with one antioxidant chemical were measured on one day, and those treated with the other antioxidant chemical were measured the next day. This was randomly assigned. Measurements were made on mostly sunny days primarily between 10:00 and 15:00 Central Daylight-savings Time (CDT). Two Photosynthetic Active Radiation (PAR) lamps were used as a light source to maintain Photosynthetic Photon Flux Density (PPFD) levels above 1300 μmol m^{-2} s^{-1}. All other environmental conditions during gas exchange measurements were at ambient levels. The gas exchange values were presented as relating to projected surface area. By assuming the fascicle was cylindrical, leaf area was determined by multiplying the radius of the fascicle by the length of the fascicle exposed inside the sampling chamber. After gas exchange measures were completed, the detached fascicles were quickly frozen on dry ice and brought to the laboratory for chloroplast pigment quantification.

2.6. Chloroplast pigment extraction and quantification

The extraction of foliar pigments, after Davies (1976) and Holden (1976), was done in a 10-ml tissue grinding tube using 80% acetone in water (v/v) and a mechanical tissue homogenizer. The frozen tissue was cut into 1-cm pieces under low light conditions and divided into two 100-mg samples. One sample was weighed, then oven-dried at 70°C for 48 h and reweighed to determine tissue dry weight. The other sample was homogenized using approximately 5 ml of cold 80% acetone to extract pigments. After being filtered under vacuum, the homogenate was brought to volume in a 25-ml volumetric flask. A Sequoia-Turner model 340 spectrophotometer (Sequoia-Turner Corporation, Mountain View, CA) was used to measure absorbance at 470, 646, and 663 nm. Concentration of pigments (μg pigment g^{-1} dry wt) were calculated using extinction coefficients, after Lichtenhaler and Wellburn (1983).

2.7. Leaf nitrogen analysis

Two fascicles from each seedling within a treatment combination were collected from the lowest flush on the stem and composited in June, August, and October. After being dried for 48 h at 70°C, the tissue was ground to pass through a 0.5-mm screen. A modified Kjeldahl digestion procedure, after Parkinson and Allen (1975) was used to digest the ground tissue. Total leaf nitrogen was analyzed by colorimetry using an autoanalyzer (Westco Inc., Danbury, CT) and was recorded as a percentage of total leaf N on a dry weight basis.

2.8. Experimental design

The experiment was a split-plot design where the whole plots were represented by the open-top chambers at five ozone exposures. The exposures included a sub-ambient level (CF), approximately ambient level (NF), 1.5×, 2.0×, and 2.5× ambient ozone exposure (Heagle et al., 1973; Manning and Krupa, 1992). Ambient ozone concentration at the research site was monitored by an ozone-specific monitor (Thermo Environmental, Franklinton, PA) (Heagle et al., 1979). The sub-plots were represented by the antioxidant concentrations of either Ozoban or EDU at three levels. These levels included Ozoban (74.5% sodium erythorbate) at 0, 1030, and 2060 mg liter $^{-1}$ (a.i.) or 50% Wettable Powder EDU at 0, 150, and 300 ppm (a.i.). Ten seedlings were in each of 15 ozone–antioxidant treatment combinations for a total of 150 plants per replication for each experiment. Each treatment combination was replicated twice.

The study had five levels of ozone exposure, three antioxidant treatments per experiment, and two replications for a total of 30 experimental units per experiment. A comparison of treatment means from each combination for each sampling period was done using analysis of variance (ANOVA). Relationships among the treatment combinations were determined by orthogonal linear and curvilinear contrasts. Functional relationships among antioxidant treatments and cumulative ozone exposures were determined by regression analyses.

3. Results

3.1. Ozone exposure

Ambient ozone levels were considerably lower for 1994 in east Texas than the five previous years. The mean cumulative 12-h ozone exposure for 1989 through 1993 was 161.1 ppm-h compared to 91.4 ppm-h in 1994. Ozone was highest early in the study when seedlings were very young, but declined considerably for the remainder of the experiment. The national ambient air quality standard (NAAQS) was not exceeded except in the ozone-supplemented chambers. Ambient ozone levels exceeded 100 ppb in May, but remained below that level for the remainder of the study. A summary of the 12-h ambient ozone data for 1994 at the study site is presented in Table 1. Cumulative 12-h ozone for each exposure level is displayed in Fig. 1.

3.2. Foliar injury

The seedlings had few fascicles upon being transplanted from the small planting cells to the larger 7-liter pots. Signs of ozone injury were evident early in the study for seedlings treated with either antioxidant

Table 1
Summary of the 12-h ambient ozone data from 25 April to 19 November 1994 at the Stephen F. Austin experimental forest

Month	1-h max. (ppb)	12-h (ppb)	12-h cumulative (ppm-h)
April[a]	59	41.5	3.0
May	102	52.4	18.8
June	79	33.9	11.8
July	79	37.6	14.0
August	93	41.8	15.4
September	95	37.5	13.4
October	62	28.3	8.8
November[b]	51	27.3	6.2
Season	102	37.9	91.4

[a] Ozone fumigation began on 25 April.
[b] Ozone fumigation ended on 19 November.

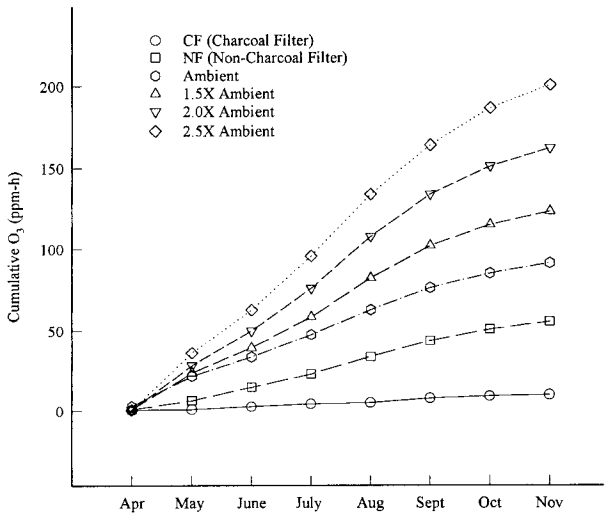

Fig. 1. Cumulative 12-h ozone for each exposure level from 25 April to 19 November 1994 in east Texas.

exposed to 2.0× and 2.5×. Noticeably reduced stem and needle growth compared to seedlings grown in CF and NF as well as leaf tip necrosis and chlorotic mottling were evident. Seedlings exposed to 1.5× displayed limited ozone injury somewhere between the two extremes. Seedlings in the ozone-supplemented chambers appeared to recover somewhat over time, but final heights and diameters remained significantly less than those in CF and NF (Flagler, 1995).

Seedlings in 2.0× and 2.5× had a wiry growth form throughout the study with noticeably fewer needles than seedlings in CF, NF, and 1.5× which grew extremely well. By July, the CF, NF, and 1.5× seedlings had such abundant top growth that shading of the lower canopy was evident. Although no measurements were recorded, the PPFD by visual inspection was highest in the lower canopy of the seedlings in 2.0× and 2.5× throughout the experiment compared to those in CF, NF, and 1.5×.

Neither Ozoban nor EDU protection against foliar injury by ozone was evident at any ozone exposure level throughout the experiment.

3.3. Foliar gas exchange

In June, a significant ($p < 0.08$) difference existed for A among Ozoban treatments. Seedlings treated with 2060 and 1030 mg liter^{-1} had a 20.6 and 10.3% reduction in A, respectively, compared to controls. At lower ozone levels (CF and NF), A in seedlings treated with 1030 mg liter^{-1} of Ozoban was less than that of 0 and 2060 mg liter^{-1}. Linear regression analysis revealed that as ozone levels increased in June, the slope for seedlings treated with 1030 mg liter^{-1} of Ozoban was not as great compared to the slope of the combined data ($p < 0.05$) (Fig. 2). In October, a comparison of the estimated slopes and their standard errors also showed seedlings treated with 1030 mg liter^{-1} of Ozoban had a greater slope over both 0 or 2060 mg liter^{-1} of Ozoban (Fig. 3). In June, the slope for g of seedlings treated with 1030 mg liter^{-1} of Ozoban differed from those of 0 and 2060 mg liter^{-1} when comparing the estimated slopes and their standard errors (Fig. 2). Except for the sampling periods in June and October, Ozoban did not consistently affect A or g at any concentration level throughout the experiment.

No consistent EDU effects were observed at any treatment concentration on A or g over the course of the experiment. However, linear regression analysis revealed seedlings treated with 150 ppm EDU possessed a significantly ($p < 0.10$) greater slope for g than the slope for the combined data in June (Fig. 4). As ozone levels increased, g increased greatest in seedlings treated with 150 ppm EDU than those treated with 0 and 300 ppm EDU. This difference was not significant for the remainder of the study.

Significant ozone effects for Ozoban-treated seedlings occurred for both A and g periodically over the course of the study ($p < 0.10$) but were inconsistent. In June, a significant ($p < 0.05$) negative linear relationship existed between ozone level and A. Compared with seedlings grown in CF for the same month, A declined by 3.6, 24.0, 25.4, and 26.1% for seedlings in NF, 1.5×, 2.0×, and 2.5×, respectively. In July, and for much of the remaining experiment, a positive linear relationship existed between ozone levels and gas exchange characteristics. Seedlings exposed to 1.5×, 2.0×, and 2.5× in July showed increased A by 34.4, 44.4, and 37.0%, respectively, compared to CF. Ozone also increased g in 1.5×, 2.0×, and 2.5× by 28.6, 44.1, and 47.7%,

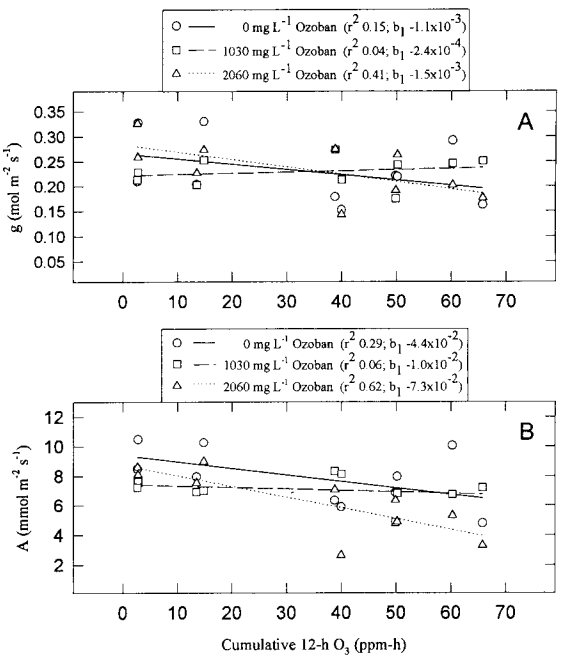

Fig. 2. Linear regression analysis for g (A) and A (B) on cumulative ozone for Ozoban-treated S6PT2 loblolly pine seedlings in June 1994. The rate of stomatal conductance and photosynthesis for seedlings treated with 1030 mg liter^{-1} Ozoban did not decrease substantially with increasing ozone compared to that of the combined data.

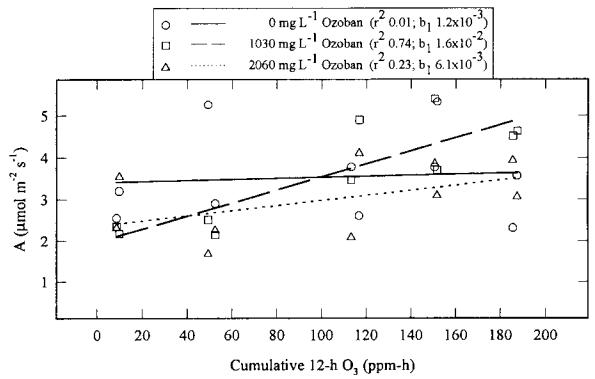

Fig. 3. Linear regression analysis for A on cumulative ozone for Ozoban-treated S6PT2 loblolly pine seedlings in October 1994. Seedlings treated with 1030 mg liter^{-1} Ozoban exhibited a greater slope than seedlings treated with 0 or 2060 mg liter^{-1} Ozoban.

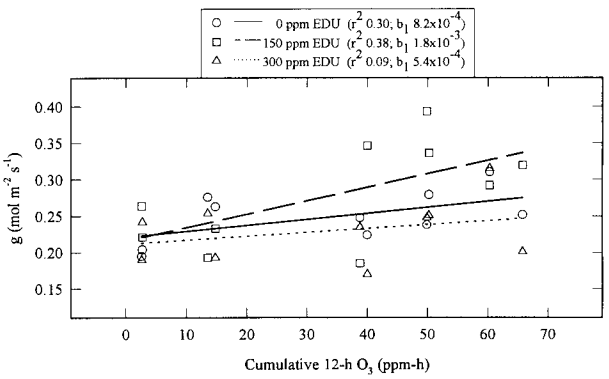

Fig. 4. Linear regression analysis for g on cumulative ozone for EDU-treated S6PT2 loblolly pine seedlings in June 1994. Seedlings treated with 150 ppm EDU exhibited a significantly greater slope compared to the combined slope.

respectively, over that of CF. Both A and g gradually declined in the 1.5×, 2.0×, and 2.5× treatments over time; however, they remained greater than seedlings in the CF and NF treatments.

For EDU-treated seedlings, no consistent ozone effects on A were evident during the experiment, however, significant ozone effects on g ($p < 0.06$) were observed through September. A positive linear relationship was prominent throughout. As ozone levels increased, g also increased.

3.4. Chloroplast pigments

Ozoban had no consistent effects on chlorophyll a or b, total chlorophyll, or chlorophyll a/b ratio throughout the study. Regression analysis indicated significant differences ($p < 0.10$) in September for the three Ozoban treatments on chlorophyll a concentration. Seedlings treated with 1030 or 2060 mg liter^{-1} of Ozoban had a more negative slope than those of the control. However, choosing the best model to compare the three slopes was difficult. The control displayed a quadratic response, 2060 mg liter^{-1} a linear response, and 1030 mg liter^{-1} was neither quadratic nor linear.

The effects of Ozoban on total carotenoids were not consistent throughout the study. In August and September (Fig. 5), regression analysis showed the slope for

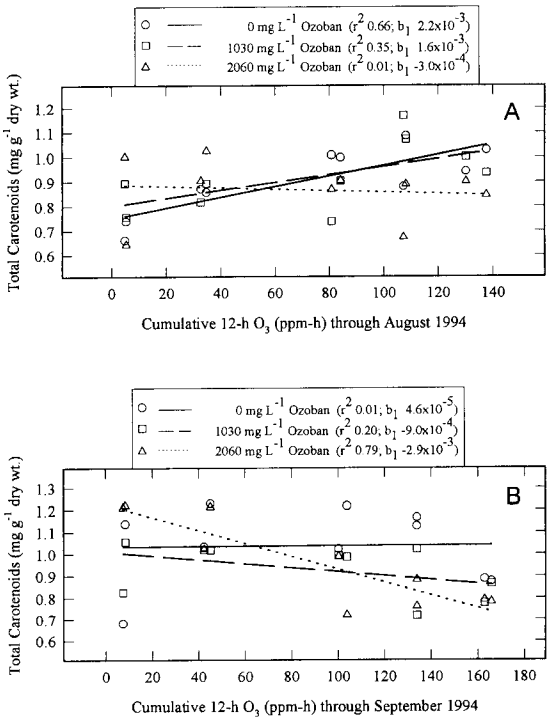

Fig. 5. Linear regression analysis for total carotenoids on cumulative ozone in August (A) and September (B) for Ozoban-treated S6PT2 loblolly pine seedlings. Seedlings treated with 2060 mg liter^{-1} Ozoban had a significantly more negative slope than either 0 or 1030 mg liter^{-1} Ozoban.

seedlings treated with 2060 mg liter^{-1} of Ozoban to be significantly ($p < 0.10$) more negative compared to the combined slope. A comparison of the slopes for each treatment and their standard errors also revealed 2060 mg liter^{-1} to have a more negative slope than either 0 or 1030 mg liter^{-1}. No consistent EDU effects were observed for chloroplast pigments during the study.

Ozone effects on chloroplast pigment concentration between the two antioxidant treatments were very similar; therefore, only the results of EDU-treated seedlings will be presented. Significant ozone differences ($p < 0.05$) were evident for chlorophyll a, chlorophyll b, total chlorophyll, and total carotenoid concentration for much of the study. As ozone levels increased, pigment concentration decreased linearly (Table 2). Averaged over all sampling periods and compared to those in CF, seedlings grown in 1.5×, 2.0×, and 2.5× had a reduction in chlorophyll a concentration of 5.5, 7.4, and 19.2%, respectively. For chlorophyll b, concentration was reduced by 2.6, 6.7, and 17.1% in 1.5×, 2.0×, and 2.5×, respectively. Total chlorophyll concentration also declined by 4.6, 7.2, and 18.6% in 1.5×, 2.0×, and 2.5×, respectively. Total carotenoids had an overall reduction in 1.5×, 2.0×, and 2.5× of 10.5, 12.9, and 22.6%, respectively. Ozone effects were most dramatic in June. Seedlings grown in 1.5×, 2.0×, and 2.5× chambers displayed reductions in chlorophyll a of 7.1, 12.0, and 27.3%, respectively, compared with seedlings grown in CF (Table 2). Chlorophyll b concentration was reduced by 10.6, 18.7, 28.5, and 43.7% in NF, 1.5×, 2.0×, and 2.5× seedlings, respectively, compared to seedlings grown in a charcoal-filtered environment. The reduction in total chlorophyll compared with seedlings in CF was 12.9, 19.1, and 34.0% in 1.5×, 2.0×, and 2.5× chambers, respectively. Total carotenoid decline was 11.4 and 19.1% in 2.0× and 2.5×, respectively, compared with seedlings grown in CF. Pigment concentration steadily increased over time within each ozone level. No significant alteration of chlorophyll a/b ratio by either antioxidant or ozone was detected in this experiment.

3.5. Foliar N concentration

Ozoban had no affect on the concentration of N in foliage throughout the experiment. Likewise, no EDU effects on total foliar N concentration were observed among the treated seedlings until the final sampling period in October where a significant ($p < 0.01$) ozone by EDU concentration interaction was observed (Fig. 6). Foliage treated with 300 ppm EDU exhibited highest N concentration at all ozone levels except at 1.5×. Seedlings treated with 150 ppm EDU maintained the lowest N concentration in CF and NF but the highest in 1.5× as compared to 0 and 300 ppm EDU. At the higher ozone levels (2.0× and 2.5×), N increased linearly with EDU concentration. Linear regression analysis for foliar

Table 2
Mean (±SE) chloroplast pigment concentration (mg g^{-1} dry wt) for each ozone level for EDU-treated S6PT2 loblolly pine seedlings in June 1994 and over the entire experiment

Ozone level	Chlorophyll *a*		Chlorophyll *b*		Total chlorophyll		Total caroteniods	
	June	Total	June	Total	June	Total	June	Total
CF	3.34 (0.62)	3.85 (0.80)	1.52 (0.25)	1.68 (0.39)	4.86 (0.81)	5.53 (1.15)	0.82 (0.15)	1.05 (0.26)
NF	3.63 (0.39)	3.92 (0.66)	1.36 (0.19)	1.68 (0.39)	4.98 (0.54)	5.60 (1.00)	1.03 (0.18)	1.08 (0.21)
1.5×	3.11 (0.57)	3.62 (0.84)	1.23 (0.33)	1.63 (0.50)	4.34 (0.84)	5.25 (1.28)	0.88 (0.18)	0.93 (0.22)
2.0×	2.94 (0.49)	3.55 (0.94)	1.08 (0.19)	1.56 (0.55)	4.03 (0.59)	5.12 (1.43)	0.72 (0.11)	0.91 (0.25)
2.5×	2.43 (0.57)	3.10 (0.91)	0.85 (0.13)	1.39 (0.52)	3.29 (0.64)	4.49 (1.37)	0.66 (0.16)	0.80 (0.21)
N	12	60	12	60	12	60	12	60
Pr > *F*	0.033	0.030	0.001	0.010	0.006	0.020	0.025	0.005

Mean content values are obtained by averaging the means of the appropriate response variable from each month of the study. The number of observations (*N*) and probability of significant ozone differences (Pr > *F*) is given for each response variable. CF, sub-ambient; NF, approximate ambient.

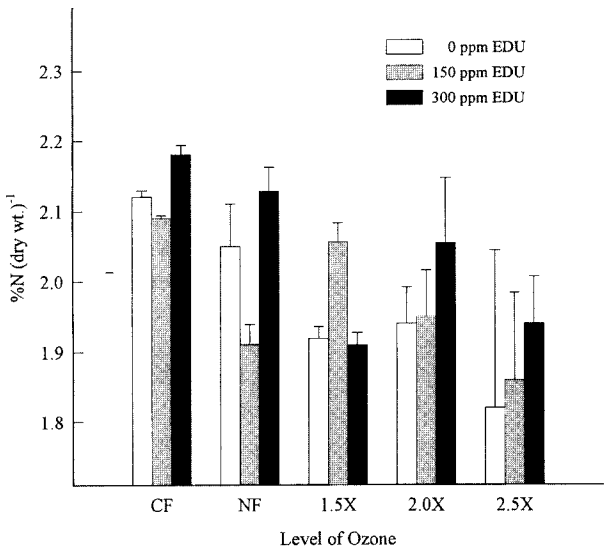

Fig. 6. Concentration of total foliar N (+1 SD) in dried tissue of ethylenediurea (EDU)-treated S6PT2 loblolly pine seedlings for each EDU treatment level by ozone exposure in October 1994. Significant ozone by EDU-concentration interaction was observed. N concentration was greatest for seedlings treated with 300 ppm EDU at all ozone levels except 1.5×.

N concentration and ozone levels did not reveal any differences among the slopes for the EDU treatments.

Since ozone effects on leaf N concentration between the two antioxidant treatments were very similar, the results of EDU-treated seedlings will be presented. A negative linear relationship between ozone and foliar N concentration was observed throughout the study. In June, a significant linear ozone effect ($p < 0.003$) was observed in total foliar N. Samples taken from CF had approximately 15% greater N concentration than samples taken from 1.5×, 2.0×, and 2.5× (Table 3). By August, N concentrations of the seedlings in the elevated-ozone chambers had recovered somewhat, however, linear ozone effects still existed ($p < 0.003$). Seedlings in 1.5×, 2.0×, and 2.5× had 4.5, 4.5, and

Table 3
Mean (±SE) total foliar nitrogen concentration [%N (dry wt)$^{-1}$] of ethylenediurea (EDU)-treated S6PT2 loblolly pine seedlings exposed to various ozone levels for the three sampling periods (June, August, and October)

Ozone level	June	August	October
CF	1.96 (0.06)	1.97 (0.08)	2.13 (0.04)
NF	2.00 (0.06)	1.99 (0.06)	2.03 (0.10)
1.5×	1.71 (0.06)	1.90 (0.10)	1.96 (0.08)
2.0×	1.70 (0.09)	1.90 (0.05)	1.98 (0.08)
2.5×	1.69 (0.10)	1.83 (0.07)	1.87 (0.13)
N	6	6	6
Pr > *F*	0.003	0.003	0.016

CF, sub-ambient; NF, approximate ambient; *N*, number of observations; Pr < *F*, probability of significant ozone differences.

8.0% less foliar N, respectively, than CF. Although an interaction occurred in October, mean N concentration was decreased by 4.7, 8.0, 7.0, and 12.2% in NF, 1.5×, 2.0×, 2.5×, respectively, compared to CF, and significant linear ozone effects were observed ($p < 0.01$).

4. Discussion

By visual inspection, it was evident that neither Ozoban nor EDU provided foliar protection from ozone injury at any concentration. Likewise, neither antioxidant chemical (Ozoban at 1030 or 2060 mg $liter^{-1}$ and EDU at 150 or 300 ppm) was reported to contribute to symptoms of foliar injury nor did they have an effect on height or diameter in a greenhouse study with ozone-sensitive loblolly pine (Flagler, 1995). Little consistent evidence for foliar protection by Ozoban had been reported for grapes in ambient studies (P.M. McCool, personal communication, as cited in Flagler et al., 1994) and shortleaf pine in controlled-environment studies (Flagler et al., 1994). Although EDU has been reported to protect herbaceous foliage (Carnahan et al., 1978;

Clarke et al., 1978; Temple and Bisessar, 1979; Lee and Chen, 1982), little evidence for pine needle protection has been documented (Cathey and Heggestad, 1982; Flagler et al., 1994). White pine (*Pinus strobus* L.) sprayed with 500 ppm EDU was afforded no foliar protection after being fumigated with up to 600 ppb ozone (Cathey and Heggestad, 1982). This was also found in a greenhouse study with shortleaf pine treated with up to 450 ppm EDU and 200 ppb ozone (Flagler et al., 1994).

Ozone injury consisting of chlorotic mottling and leaf tip necrosis to seedlings in 2.0× and 2.5× is consistent with past research with pine (Davis and Wood, 1972, 1973; Flagler et al., 1994). Because ambient ozone concentration was highest at the beginning of the experiment in May and the seedlings had not established many secondary needles when ozone fumigation was begun, much of the injury occurred to younger, immature foliage. Foliar injury in 2.0× and 2.5× was most apparent in June and July. As ambient ozone levels declined, the seedlings recovered, and the amount of total foliage injured decreased.

Seedlings treated with 1030 mg liter^{-1} of Ozoban had greater A and g with increasing ozone early in the study when ozone exposures were high. However, seedlings treated with Ozoban at 1030 mg liter^{-1} had decreased A compared to 0 and 2060 mg liter^{-1} in the CF and NF ozone treatments. The advantageous effect of Ozoban on A and g is not consistent with previous work on shortleaf pine treated once per month with 1030 mg liter^{-1} of Ozoban and fumigated with up to 200 ppb of ozone in continuous-stirred tank reactors (Flagler et al., 1994). The data of the present study indicate that 1030 mg liter^{-1} of Ozoban may retard stomatal closure and have a positive influence on A in pine species grown under ambient ozone conditions. This may help explain the increase in total biomass after 1 year of treating shortleaf pine trees with Ozoban at 1030 mg liter^{-1} in an ambient environment compared to non-treated trees (Flagler and Toups, 1991). At lower ozone levels (CF and NF), Ozoban may alter physiological function of seedlings grown in non-ozone-stressed environments because A was observed to be lower in the 1030 mg liter^{-1} Ozoban treatment compared to 0 and 2060 mg liter^{-1} in CF and NF.

EDU did not appear to affect A at any concentration rate. However, seedlings treated with 150 ppm EDU showed greater g with increasing ozone levels in June compared to those treated with 0 and 300 ppm. The lack of EDU effect on A is consistent with previous work in woody and non-woody species (Bennett et al., 1978; Flagler et al., 1994; Ainsworth et al., 1996). The physiological effect of 150 ppm of EDU on g early in the study does not agree with past research that reported EDU to have no affect on g in shortleaf pine treated with up to 450 ppm EDU and fumigated with up to 200 ppb of ozone (Flagler et al., 1994).

Flagler (1995) reports that neither antioxidant chemical had an affect on height and diameter growth of loblolly pine in this experiment. The inconsistent physiological results of the present study help explain the lack of growth response.

Generally, A declines over time in ozone-exposed vegetation (Coyne and Bingham, 1982; Runeckles and Chevone, 1992). Photosynthesis declined rapidly from June to July in both Ozoban-and EDU-treated seedlings and remained relatively low for the duration of the study compared to June readings. The decrease in A was greater in seedlings growing in CF and NF than those growing in 2.0× and 2.5×. Increased A in seedlings grown in ozone-supplemented chambers compared to those in relatively clean air does not agree with past research with loblolly pine (Sasek and Richardson, 1989; Elsik et al., 1992).

The g results of this experiment do not fully agree with that of Elsik et al. (1992) who reported a decline in g with increasing ozone. In Ozoban-treated seedlings, g was shown to decrease with increasing ozone in the first sampling period, but that trend was reversed in the second sampling period and for the remainder of the experiment. In EDU-treated seedlings, g showed a slight increase with increasing ozone levels beginning with the first sampling period in June and continued to the end of the experiment. Stomatal conductance was seen to decline gradually throughout the study in seedlings exposed to greater-than-ambient ozone. Seedlings grown in CF and NF experienced more dramatic decreases in g compared to those in ozone-supplemented environments beginning in July.

Several possibilities may explain the disparity from June to July for both A and g. In June, the top growth of all seedlings was relatively small compared to those in subsequent months and was likely proportional to root growth thus allowing for adequate hydration of foliage. This would allow the stomata to remain open and photosynthesis to proceed at a greater rate. However, in July, tremendous top growth was observed in seedlings of CF, NF, and 1.5× compared to those in 2.0× and 2.5×. Since, root growth is usually restricted in potted plants, the former seedlings may have been under greater water stress at the time of measurement compared to the latter. A reduced root-to-shoot ratio can increase stomatal resistance due to greater transpiration compared to water uptake (Kramer and Kozlowski, 1979). Coupled with elevated temperatures, this reduced root-to-shoot ratio could decrease A. Gradually, as top growth developed in seedlings of 2.0× and 2.5×, A and g were found to decrease. The dense top growth and relatively close spacing (approximately 15 cm) of the seedlings in CF, NF, and 1.5× increased self-shading to the needles of the lower canopy, where measurements were being taken, possibly closing the stomata.

Reduced PPFD is another factor that can cause decreased g (Kramer and Kozlowski, 1960; Ng and Jarvis, 1980). This could lead to reduced CO_2 influx consequently inhibiting A. Seedlings in 2.0 and 2.5× remained comparably smaller, thus allowing greater PPFD to penetrate the lower canopy. Reductions in PPFD have been shown to decrease g in Scots pine (*Pinus sylvestris* L.). At a PPFD of 400 μmol m^{-2} s^{-1}, g was reported to be less than 60% of that at 1800 μmol m^{-2} s^{-1} (Ng and Jarvis, 1980).

Chloroplast pigments were not protected from ozone injury by Ozoban. In fact, Ozoban-treated seedlings treated with 2060 mg $liter^{-1}$ displayed intermittent negative effects on total carotenoid concentration indicating that high levels of Ozoban may be phytotoxic. Negative effects were also exhibited in chlorophyll a for both 1030 and 2060 mg $liter^{-1}$ of Ozoban. That effect was seen only once during the course of the study and was probably a chance event. The absence of EDU effects on foliar pigment concentration contradicts earlier studies that report EDU to prevent chlorophyll breakdown (Lee et al., 1981; Lee and Chen, 1982). To the best of our knowledge, no studies have been reported concerning the effects of sodium erythorbate or EDU on chloroplast pigments in pine.

Ozone has been found to reduce chlorophyll concentration in loblolly pine (Sasek and Richardson, 1989; Elsik et al., 1992), and the results of this experiment are consistent. Chlorophyll b has been reported to be more easily destroyed than chlorophyll a (DeKonig and Jegier, 1968; Nobel, 1974; Runeckles and Chevone, 1992); however, this was not observed. Both chlorophyll a and b concentration decreases were similar. The deleterious effects of ozone on total carotenoids in this experiment are also contradictory to previous studies that report no effects due to ozone (Sasek and Richardson, 1989; Runeckles and Chevone, 1992). Carotenoids breakdown relatively quickly upon storage, even at temperatures below −20°C (Davies, 1976). The tissue samples used during this experiment were stored at −80°C for up to 4 weeks at times. Although samples were chosen to be processed at random, this long period of storage time may have had an effect. The lack of ozone effects on chlorophyll a/b ratio agrees with past research using loblolly pine (Sasek and Richardson, 1989).

Ozoban did not have a significant effect on total foliar N concentration. Again, no research has been reported on sodium erythorbate and foliar N to the best of our knowledge. EDU, on the other hand, did not significantly affect foliar N concentration in the seedlings for the first two sampling periods; however, significant ozone by EDU concentration interaction was observed in the final sampling period. Seedlings treated with 300 ppm of EDU had higher foliar N concentration compared to 0 and 150 ppm at all ozone levels except 1.5× where 150 ppm EDU had the highest concentration. These data indicate that EDU could possibly have some positive effect on N concentration in pine needles. Since EDU is a N-containing compound, there was concern that it may contribute its N to leaf tissue as a fertilizer. Early in the study, this was not evident; however, in the final sampling period, seedlings treated with 300 ppm EDU in CF and NF displayed significantly higher N concentration, which indicates that EDU could possibly be contributing its N to leaf tissue. In unpublished data, an experiment to compare the N concentration in EDU-treated and non-treated pine foliage showed no significant differences between the two treatments (Flagler, personal communication).

The effects of ozone on foliar N seem to vary greatly among species. Reports range from decreases in total N to significant increases after ozone exposure (Heath, 1984; Rowland et al., 1987). Ozone concentrations of 250 and 300 ppb for 2 h has been shown to decrease the amount of rubisco in alfalfa seedlings thus reducing the photosynthetic capacity (Pell and Pearson, 1983). Rubisco can account for up to 25% of the total foliar N concentration (Salisbury and Ross, 1991). The amount of rubisco, soluble proteins, and chlorophyll in spring wheat is also reported to decline after exposure to ozone in open-top chambers for several weeks (Lehnherr et al., 1987, 1988).

Maximum photosynthetic capacity and leaf N concentration have been shown to be directly related in part due to an increased proportion of enzymes involved with fixing CO_2 (Field and Mooney, 1983; Evans, 1989). Although it can only be speculated, the development of these CO_2-fixing enzymes in seedlings of 1.5×, 2.0×, and 2.5× could have been retarded due to ozone injury in the early part of the study.

The steady increase of chloroplast pigment and foliar N concentration in seedlings of the ozone-supplemented chambers as compared to that of seedlings in CF and NF indicate that ozone may retard chloroplast pigment formation and foliar N assimilation in young vegetation. Perhaps much of the photosynthate produced by the seedling was being used to prevent or repair foliar damage rather than going toward growth.

5. Conclusion

The data from this study indicate that neither Ozoban nor EDU provide visible foliar protection to ozone-sensitive loblolly pine seedlings from ozone injury. At 1030 mg $liter^{-1}$, Ozoban may be beneficial in retarding stomatal closure at increased ozone levels, thus allowing for greater photosynthesis in young, immature pine seedlings. The data also show that seedlings treated with 1030 mg $liter^{-1}$ of Ozoban may have a reduced rate of photosynthesis in reduced-ozone environments. At 2060

mg liter^{-1}, Ozoban appears to be harmful to chloroplast pigment concentration especially total carotenoids when exposed to elevated ozone levels.

EDU, at 150 ppm, may offer some gas exchange benefits by retarding stomatal closure. It does not seem to affect photosynthesis or consistently protect chloroplast pigments from ozone injury in ozone-sensitive loblolly pine seedlings in east Texas. Differences were seen in foliar N concentration among seedlings treated with the three EDU concentrations for the final sampling period. Seedlings treated with 300 ppm EDU in CF and NF possessed significantly greater N levels than 150 and 0 ppm.

The spacing of the seedlings may have contributed negatively to these results. Because seedlings were placed so closely to each other in all chambers and top growth was so great in CF, NF, and 1.5×, the shading of the lower foliage may have been the cause of decreasing gas exchange measurements. Relatively low ambient ozone levels at the experiment site and experimental design may have contributed to the quelled effects of the antioxidants' protection to these pine seedlings. Further research is needed to better understand if Ozoban and EDU protect physiological function within pine and the mechanism(s) by which they do protect.

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