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Ultra-high CO_2 Levels Enhance Loblolly Pine Seedling Growth, Morphogenesis, and Secondary Metabolism

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Abstract. **The growth (fresh weight), morphogenesis (number of needles and roots and shoot length) and monoterpene (α- and β-pinene) levels were determined in *Pinus taeda* L. (loblolly pine) seedlings exposed to 350, 1,500, 3,000, 10,000, or 30,000 μmol·mol^{-1} CO_2 for 30 days under greenhouse conditions. Seedlings exposed to ultra-high levels (i.e., ≥3000 μmol·mol^{-1} CO_2) had significantly higher ($P = 0.05$) fresh weight, needle number, root number, and shoot lengths compared to seedlings grown under ambient air (350 μmol·mol^{-1} CO_2). Seedling fresh weights, number of roots, shoot length, and number of needles from pine seedlings supplemented with 10,000 μmol·mol^{-1} CO_2 increased 341%, 200%, 74%, and 75 %, respectively, when compared to seedlings grown without any CO_2 enrichment. In addition, α- and β-pinene levels in seedlings increased under ultra-high CO_2 levels. The dominant monoterpene, α-pinene, increased 57% in seedlings grown under 10,000 μmol·mol^{-1} CO_2 compared to levels obtained under 350 μmol·mol^{-1} CO_2.**

Atmospheric levels of CO_2 are predicted to increase to as much as 600 μmol·mol^{-1} CO_2 by the year 2030 (Constable et al., 1999; Groninger et al., 1996; Hoddinott and Scott, 1996). Concern as to how enhanced CO_2 levels will affect forestry and horticultural trees in the future has resulted in several CO_2 studies comparing ambient CO_2 (≈350 μmol·mol^{-1} CO_2) and elevated CO_2 levels (700–1000 μmol·mol^{-1} CO_2) on pine trees (Constable et al., 1999; Gebauer et al., 1998; Groninger et al., 1996; Heyworth et al., 1998; Hoddinott and Scott, 1996; Lewis et al., 1996; Thomas et al., 1994; Tissue et al., 1996). CO_2 enrichment was found to promote the growth of several conifer seedlings (Constable et al., 1999; Gebauer et al., 1998; Groninger et al., 1996; Heyworth et al., 1998; Hoddinott and Scott, 1996; Lewis et al 1996; Thomas et al., 1994; Tissue et al., 1996). Because CO_2 is necessary for photosynthesis and plant growth, an understanding of how increased CO_2 affects seedling growth is important (Hoddinott and Scott, 1996). It has been recently demonstrated that ultra-high CO_2 levels (≥3000 μmol·mol^{-1} CO_2) enhances in vitro growth in several C-3 photosynthesis species (Tisserat et al., 1997). The present study was conducted to ascertain the response of immature loblolly pine (*Pinus taeda* L.) seedlings to a broad range of CO_2 levels on growth, morphogenesis, and secondary metabolism. Loblolly pine, a native southeastern U.S. tree, is commercially important to the paper industry. In addition, this tree is increasingly employed in landscaping applications and is especially noted for its rapid growth (Schultz, 1999).

One objective of this work was to determine if short CO_2 treatments may enhance the growth and morphogenesis of loblolly pine seedlings and thereby reduce seedling nursery growing time. A second objective was to determine if CO_2 treatments affect the metabolism of secondary compounds, which reflects the plant's inherent herbivore and pathogen defenses (Gebauer et al., 1998; Harborne, 1982; Klepzig et al., 1995). Volatile monoterpenes, such as α-pinene and β-pinene, are 10-carbon compounds and are involved in herbivore, insect, and pathogen defense, and occur in the storage reservoirs in the needles and the bark of conifers (Constable et al., 1999; Harborne, 1982; Klepzig et al., 1995). α- and β-pinene are the dominant secondary compounds in pine resin (Constable et al., 1999; Harbone, 1982; Klepzig et al., 1995) and are especially important to pine ecological biochemistry since they have fungicidal and insecticidal activity (Harbone, 1982; Klepzig et al., 1995).

Materials and Methods

CO_2 flow systems. CO_2 flow-through testing chambers were made out of a transparent polycarbonate box and lid (Consolidated Plastics, Twinsburg, Ohio) (32.5 cm wide × 30 cm long × 26.3 cm deep; 17.6-L capacity). A silicone tape gasket (Furon, New Haven, Conn.) was attached to the lid. The box was modified by mounting three polypropylene spigots (Ark-Plas Products, Flippin, Ark.) attached to 0.45-μm air vents (Gelman Science, Ann Arbor, Mich.). The box and lid were clamped with 12 equally spaced stationary binding clips (50 mm long). CO_2 was provided by a gas cylinder (BOC Gases, Edison, N.J.) rated 99.8% pure and was mixed with an ambient air, i.e., 350 μmol·mol^{-1} CO_2, flow produced by an aquarium air pump (Whisper 2000, Carolina Biological Supply Co., Burlington, N.C.) via a flow meter (Cole Parmer Instrument Co., Niles, Ill.) to provide 350, 1500, 3000, 10,000, or 30,000 μmol·mol^{-1} CO_2. CO_2 ranges ≥10,000 μmol·mol^{-1} CO_2 were adjusted using a LIRA infrared gas analyzer, (model #3000, Mine Safety Appliances Co., Pittsburgh) and CO_2 ranges ≤3000 μmol·mol^{-1} CO_2 were adjusted with a Li-Cor CO_2/H_2O infrared gas analyzer, (model LI-6262, Li-Cor, Lincoln, Nebr.). The CO_2 and air streams were added at 2000 mL·min^{-1} during the photoperiod (13 h/day). Control seedlings were given a stream of ambient air generated by the aquarium pump only. No CO_2 or air control was applied during the dark (≈10 h/day.

Experiments. Seedlings were grown in containers (25 mm diam × 160 mm length) containing 10 g of a soilless medium formulated with 1 peat moss : 1 vermiculite (v/v) and amended with 10.9 g·kg^{-1} Micromax (Scotts Co., Marysville, Ohio) and 62.3 g·kg^{-1} Osmocote 14–14–14 (Scotts Co.). To determine the optimum CO_2 levels for growth and morphogenesis, twenty 4-week-old loblolly pine seedlings were grown under 350, 1500, 3000, 10,000, or 30,000 μmol·mol^{-1} CO_2 within 17.6-L transparent containers. Seedlings were watered 3 times per week and not fertilized during the experimental CO_2 incubation periods. All chambers (one per CO_2 treatment) were run concurrently and exposed the same temperature and lighting regimes during the experiment. Relative humidity within chambers was 95% to 100%, measured periodically with a portable relative humidity probe (Solomat MPM 2000, Solomat Corp., Rowayton, Conn.). Experiments were repeated once a year over a 3-year period, employing 20 replications per treatment. Seedlings were placed into CO_2 testing boxes in a greenhouse on 3 Apr. 1999, 16 Apr. 2000, and 10 Apr. 2001. Average daily temperature was 25.2 °C and varied from 20.8 to 29.2 °C. Illumination during experiments was provided by natural sunlight, with an average daily photosynthetic photon flux of 545.6 μmol·m^{-2}·s^{-1}.

Statistical analysis. After 30 d of incubation for each CO_2 treatment, data on whole seedling fresh weight, needle number per seedling, root number per seedling, and shoot length per seedling were recorded from five seedlings, with the remaining 15 seedling replications employed in essential oil analysis. Best fit regression equations were calculated (Table Curve.2D ver. 5.0, 2000 AISN Software) for each response variable as a function of CO_2 treatment. Regression model analyses and 95% confidence limits on the mean predicted values of the response variables from the individual equations were obtained from the REG procedure of SAS (SAS Institute, ver. 8.2, Cary, N.C.).

Extract preparation and analysis. Loblolly pine plant shoots (4 cm from apical tips) of individual plants were excised, pooled together

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within a treatment, and then sampled. Three 1-g fresh weight samples were taken and extracted for 72 h with 15 mL CH_2Cl_2. After filtering, the extracts were analyzed on a HP 5890 gas chromatograph equipped with a flame ionization detector (GC-FID). Compounds in the extracts were identified by comparison with known standards or with the Wiley/NGS Mass Spectral Registry (McLafferty and Stauffer, 1989). α- and β-pinene were calculated from standard curves of both compounds. Gas chromatography-mass spectrometry (GC-MS) was performed on a Hewlett-Packard 6890 GC system attached to a HP 5972A Mass Selective Detector. Columns used were fused silica HP-5MS capillaries (0.25-μm film thickness, 30 m × 0.25 mm ID). The GC-FID operating parameters were as follows: splitless injection mode; temperature programmed from 40 to 315 °C at 5° C/min with a 2-min initial and a 10-min final temperature hold; He carrier gas flow rate at 1.1 mL·min^{-1}, with the injector temperature set at 250 °C.

Results and Discussion

Short-term exposure (30 d) to various elevated CO_2 levels significantly affected growth and morphogenesis of loblolly pine seedlings (Fig. 1). Increasing CO_2 concentrations up to 10,000 μmol·mol^{-1} CO_2 proportionally increased whole seedling fresh weight, number of roots, shoot length, and needle number. No significant increases for growth and morphogenesis responses of pine seedlings occurred for plants grown under 30,000 μmol·mol^{-1} CO_2 compared to that obtained employing 10,000 μmol·mol^{-1} CO_2. Fresh weight of seedlings, needles per seedling, roots per seedling, and shoot length in loblolly pine seedlings increased 341%, 200%, 74%, and 75%, respectively, after 30 d exposure to 10,000 μmol·mol^{-1} CO_2 over those obtained from seedlings grown on ambient CO_2 levels (Fig. 1). Similarly, several investigators have found that elevated CO_2 environments (600–800 μmol·mol^{-1} CO_2) enhanced loblolly pine biomass production (Constable et al., 2001; Groninger et al., 1997; Lewis et al., 1996; Teskey, 1997; Thomas et al., 1994; Tissue et al., 1996). For example, Groninger et al. (1996) found a 37% biomass increase in loblolly pine seedling when cultured under 806 μmol·mol^{-1} CO_2 after several months of exposure. Tissue et al. (1996), conducting a 4-year-long experiment with loblolly pine seedlings, found that ≈650 μmol·mol^{-1} CO_2 caused a 90% increase in biomass over that obtained employing ambient air. During the first growing season, foliage area increased by 217% (Tissue et al., 1996). However, growth responses employing higher CO_2 levels were not always beneficial, as white pine (*Pinus strobus* L.) seedlings grown under 800 μmol·mol^{-1} CO_2 for 15 weeks failed to exhibit any difference in growth compared to seedlings grown in 400 μmol·mol^{-1} CO_2 (Bauer and Berntson, 2001). In contrast, Hoddinott and Scott (1996) testing seedlings of white spruce (*Piceae glauca*), black spruce (*Piceae mariana*), and jack pine (*Pinus banksiana*) grown under 350, 700, and 1050 μmol·mol^{-1} CO_2 for 16 weeks found increases in relative growth rates, including root weight, stem weight, needle weight, and height growth rates occurring with the higher CO_2 levels. In our 30-d-long experiments, we also found large increases in biomass only when employing ultra-high CO_2 (≥3000 μmol·mol^{-1} CO_2) levels while 1500 μmol·mol^{-1} CO_2 allowed for much smaller growth and morphogenesis responses compared to ambient CO_2 levels.

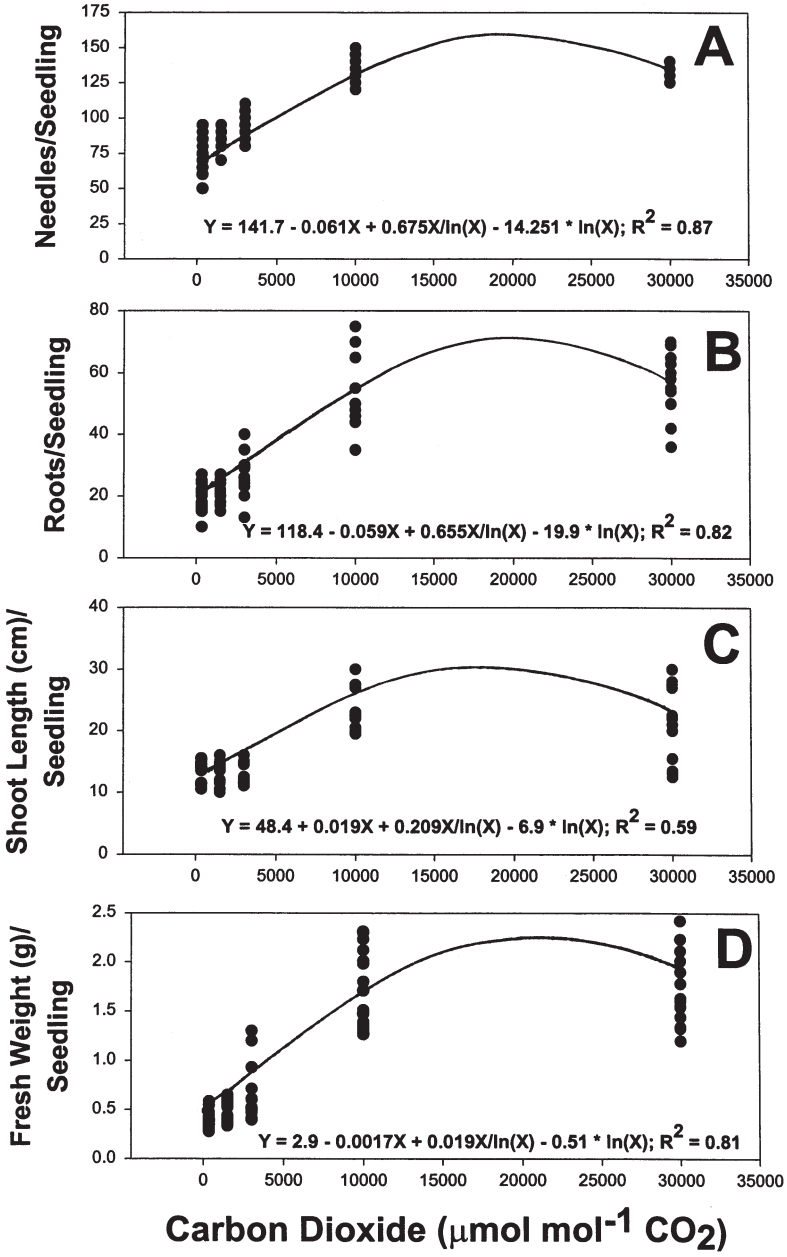

Fig. 1. Growth and morphogenesis responses of loblolly pine seedlings to various concentrations of CO_2. Observations from 3 years are presented. Regression coefficients of determination (R^2) and regression equations between CO_2 and (**A**) needles/seedling, (**B**) roots/seedling, (**C**) shoot length/seedling, and (**D**) fresh weight/seedling are given. All repressions were significant at $P = 0.05$.

In our study >99% of the secondary metabolites in loblolly pine seedlings were α- and β-pinene (Fig. 2), with only trace amounts of Δ-3-carene and camphene occurring (data not shown). Both of these monoterpenes rise proportionally in response to higher CO_2 levels at 3000 μmol·mol^{-1} from 350 and 1500 μmol·mol^{-1}. In contrast, Douglas fir [*Pinus menziesii* (mirb.) Franco] and Ponderosa pine (*Pinus ponderosa* Dougl. Ex Laws) seedlings grown under 350, 550, and 700 μmol·mol^{-1} CO_2 failed to exhibit any increase in monoterpene content with any of the elevated CO_2 levels tested (Constable et al., 1999). Likewise, elevated CO_2 (700 μmol·mol^{-1}) failed to affect secondary metabolites (monoterpenes, phenolics, and resin oils) in loblolly or Scot pine (*Pinus sylvestris* L.) (Heyworth et al., 1998; Kainulainen et al., 1998). Similarly, in our study, we found that 1500 μmol·mol^{-1} CO_2 failed to significantly enhance α- or β-pinene levels, which only increased by employing higher CO_2 levels. According to the carbon/nutrient balance hypothesis (Kainulainen et al., 1998), an increased supply of carbon will increase the carbon/nitrogen

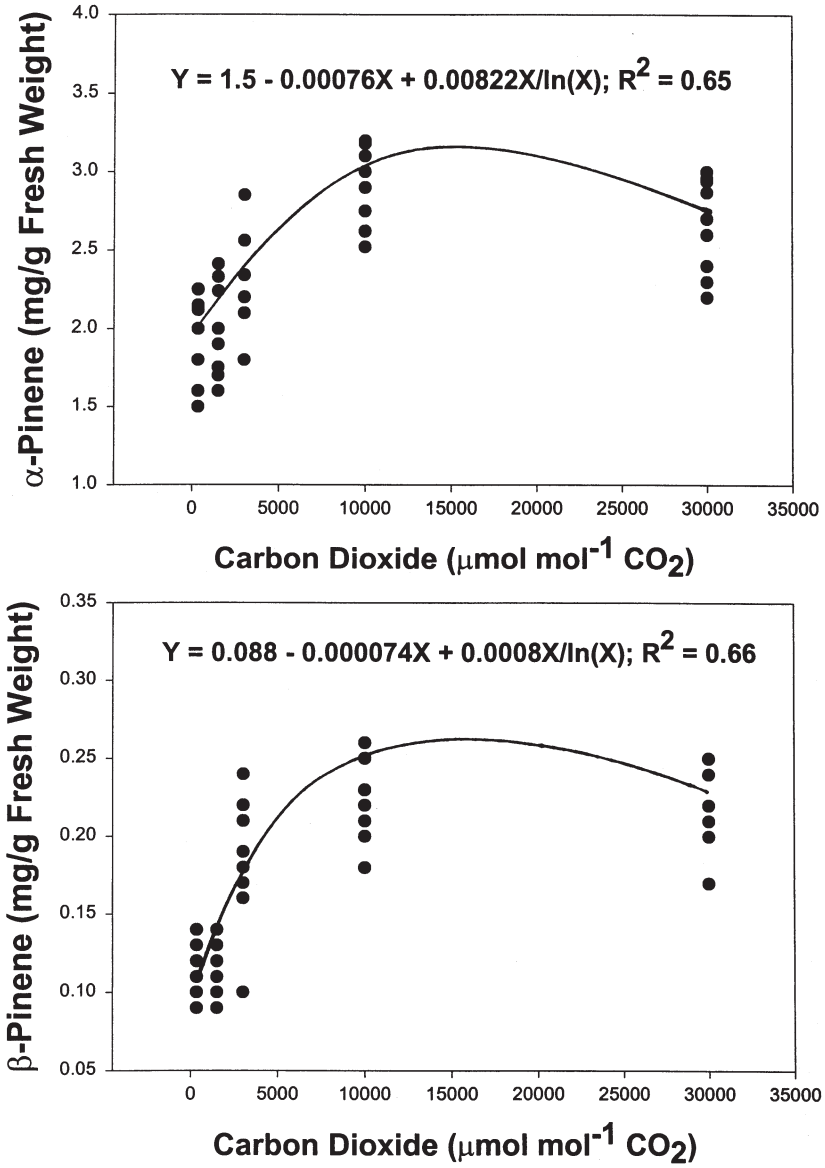

Fig. 2 Essential oil responses of loblolly pine seedlings to various concentrations of CO_2. Observations for 3 years are presented. Regression coefficients of determination (R^2) and regression equations between CO_2 and α-pinene and β-pinene are given. All regressions were significant at $P = 0.05$.

Table 1. Pearson correlation coefficients values for growth, morphogenesis, secondary metabolites, and CO_2.[z]

	CO_2	Needles	Roots	Fresh wt (g)	Shoot length (cm)
CO_2	---	0.743	0.719	0.728	0.531
Needles	0.743	---	0.842	0.839	0.654
Roots	0.719	0.842	---	0.883	0.817
Fresh wt (g)	0.728	0.839	0.893	---	0.833
Shoot length (cm)	0.531	0.654	0.817	0.833	---
α-pinene	0.534	0.757	0.594	0.607	0.383
β-pinene	0.583	0.739	0.637	0.614	0.460

[z]All values were significant at $P = 0.05$. Observations: needles, 75; roots, 75; fresh weight, 75; and shoot length, 75; α-pinene, 45; β-pinene, 45.

ratio within a plant and concurrently result in reduced tissue concentrations of nitrogen, but will increase tissue concentrations of carbon-based secondary compounds (Kainulainen et al., 1998). From our data we may speculate that higher CO_2 levels may be necessary to induce significant increases in secondary metabolite production, supporting the carbon/nutrient balance hypothesis (Kainulainen et al., 1998). Nevertheless, associated with increased growth and morphogenesis is a corresponding increase in secondary metabolites in the ultra-high CO_2 environments (Table 1). Pine seedlings possessing high α- and β-pinene levels may confer an additional positive survival advantage.

It should be noted that large differences in secondary products (i.e., monoterpenes and resin acids) occur among individual trees (Kainulainen et al., 1998). In our study, α- and β-pinene levels among loblolly pine seedlings consistently showed trends that were reproducible, although we did note some differences in growth, morphogenesis, and secondary metabolite responses among individual plants. Our studies were relatively short, i.e., 30 d, as we sought to develop short-term nursery application treatments that would be financially and spatially practical for growing loblolly pine seedlings commercially in ultra-high CO_2 conditions within a greenhouse environment. Our data confirm that such treatments may be effective for hastening growth. The important secondary metabolites necessary for seedling survival are also enhanced by these same ultra-high CO_2 treatments.

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