Germination of CO₂-enriched Pinus taeda L. seeds and subsequent seedling growth responses to CO₂ enrichment

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Summary

1. Pinus taeda seeds, developed under ambient or elevated (ambient + 200 μl l⁻¹) [CO₂], were collected from Duke Forest, North Carolina, USA in October 1998. Seeds were germinated in nutrient-deficient soil in either ambient or elevated [CO₂] (ambient + 200 μl l⁻¹) greenhouse chambers and allowed to grow for 120 days.
2. Seeds that developed in elevated [CO₂] had 91 and 265% greater weight and lipid content, respectively, and three times the germination success, compared to those developed in current ambient [CO₂].
3. Seedlings from the elevated [CO₂] seed source had significantly greater root length and more needles regardless of greenhouse chamber, but there were no treatment effects on tissue or total biomass.
4. Severely limiting nutrient conditions resulted in significant photosynthetic down-regulation by seedlings grown in greenhouse chambers with elevated [CO₂], regardless of seed source.
5. Our hypothesis that greater seed reserves from CO₂ enrichment would synergistically affect seedling growth responses to elevated [CO₂] was not strongly supported. Nonetheless, seeds produced in a CO₂-enriched environment may have fundamental changes in their viability, chemistry and germination that may affect reproduction.

Key-words: Elevated CO₂, Pinus taeda, seed germination, seed lipids, seed production

Introduction

Increasing atmospheric carbon dioxide concentration, [CO₂], has profound effects on growth and development of trees. A doubling of [CO₂] generally stimulates photosynthesis (Murray 1995; Saxe, Ellsworth & Heath 1998) and can lead to a substantial increase in tree growth (Poorter 1993). For example, doubling [CO₂] increased the rate of photosynthesis per unit leaf area (A) of Pinus taeda by 14–146% (Ellsworth 1999; Liu & Teskey 1995; Thomas, Lewis & Strain 1994; Tissue et al. 1996; Tissue et al. 1997), and increased relative growth by 40–233% (DeLucia et al. 1999; Sionit et al. 1985; Tissue et al. 1996; Tissue et al. 1997). While it is well known that nutrient availability can greatly affect the CO₂ response (Kubiske et al. 1998; Pregitzer et al. 1995; Thomas et al. 1994), we suggest that in very young seedlings, elevated [CO₂] effects may interact with internal factors such as seed N or C reserves. Thus internal and external factors that regulate growth at the seedling stage may act collectively to regulate subsequent seedling growth (Corbineau & Come 1995).

Virtually all elevated [CO₂] work on trees has been conducted on seedlings grown from seed that developed under current ambient CO₂ conditions. A parental environment of elevated [CO₂] may produce seeds with increased amounts of carbohydrates, lipids and proteins to support early seedling growth. The contents of these substances in seed have profound effects on germination, because they are utilized as both substrate and energy source for the growing seedlings (Kermode 1995). For the same reasons, the reserve content of seeds may also affect seedling responses to atmospheric [CO₂]. For example, Miao (1995) grew pla-Quercus rubra acorns and found that seed size acted synergistically with atmospheric [CO₂] to enhance seedling growth. Since developing seeds are usually very strong C sinks (Zamski 1995), it is very likely that enhanced photosynthetic production will improve the energy reserves of seed. Thus an important synergistic effect may be missing from virtually all elevated [CO₂] studies on trees: that of seed energy reserves in combination with atmospheric [CO₂] enrichment. Such studies are essential to fully understand growth
Germination and growth of CO$_2$-enriched P. taeda seeds

and development of tree seedlings in a future, CO$_2$-enriched ecosystem.

Until now, it has been impractical to study seed production and development of forest trees. The development of free-air CO$_2$ enrichment (FACE) technology for forest canopies now makes such studies possible (Hendrey et al. 1999). Several FACE research facilities are presently operating in forest communities around the world, including Pines taeda in North Carolina, USA (DeLucia et al. 1999; Ellsworth 1999; Myers, Thomas & DeLucia 1999); Populus tremuloides, Betula papyrifera and Acer saccharum in Wisconsin, USA (Karnosky et al. 1999a; Karnosky et al. 1999b); Liquidambar styraciflua in Tennessee, USA (Gunderson et al. 1999); Populus spp. at Viterbo and Siena, Italy (Tognetti et al. 1999); and tropical forest species at Sardinia, Panama. We collected Pines taeda seed that developed under FACE to study the subsequent response of seedlings under similar elevated [CO$_2$] conditions. It was hypothesized that applying elevated [CO$_2$] to the maternal parent would have a positive effect on seed energy reserves which, in turn, would result in greater early growth of seedlings. This expectation was based on the premise that the increased seed reserves would provide additional substrates for growth, and also reduce feedback inhibition of seedling photosynthesis under elevated [CO$_2$].

Materials and methods

SEED PRODUCTION, COLLECTION AND GERMINATION

The Duke Forest (Orange County, North Carolina, USA) FACE experiment was constructed in 1996 in a 14-year-old P. taeda plantation. It consisted of a randomized block design of six 30-m-diameter open-air gas fumigation arrays in three replicate blocks. Three of the arrays fumigated the forest canopy with ambient air enriched by 200 µl l$^{-1}$ CO$_2$, and three with ambient air. The elevated [CO$_2$] plots were fumigated for 81 and 79% of 1997 and 1998, respectively; the annual average [CO$_2$] at the centre of each FACE plot was 199–203 ± 84 µl l$^{-1}$ above the ambient concentration.

Pines taeda is an important timber tree species in the south-east USA, and its reproductive cycle is typical of North American pines. It normally reaches reproductive maturity at age 5–10 years (Schopmeyer 1974). Pollination occurs between February and April. Fertilization takes place the year following pollination, and cones mature by October of that same year (Burns & Honkala 1990). In 1997, at age 15, a few of the canopy trees had begun to produce cones. The seeds that were collected in the present study underwent their entire developmental cycle, from pollination through maturity, under experimental treatments.

A total of 39 cones that matured in 1998 were collected from the Duke Forest experiment between October 29 and November 1, 1998. Most of the cones were open at the time of collection, and only the seeds of open cones were used in this experiment. Seeds were de-winged, floated in water for 2 days, and stratified for 120 days at 10 °C; seeds that remained floating were discarded (Schopmeyer 1974).

Following stratification, seeds were sown in 0.161 (4 cm diameter × 21 cm long) tubes in a greenhouse at Mississippi State University, USA, on March 19, 1999. The tubes were filled with equal amounts of coarse, sterile silica sand. Seeds were placed on the sand surface = 2 cm below the rim of the tubes. All seeds were watered for 2 min every 2 h by an automatic misting system. No additional fertilizers were used, so that early seedling development was largely a function of seed storage.

The tubes were then placed into six chambers (1.2 × 1.2 × 0.9 m), constructed in the greenhouse, that were enclosed on the top and four sides by transparent plastic and by plywood on the bottom. Each of the six chambers contained 28 seeds: 14 from FACE rings (referred to as the CO$_2$-enriched seed source); and 14 from ambient rings (referred to as the ambient seed source), with rings being equally represented by block and treatment in each chamber, for a total of 168 seeds in the experiment. The seedling tubes were arranged randomly within each chamber. Three blowers, each connected to two chambers (one ambient chamber and one CO$_2$-enriched chamber), circulated air through the chambers at a rate of two chamber volumes per min. Volume-flow regulators were used to dispense pure CO$_2$ gas into the blower air stream of one chamber in each pair. The concentration of CO$_2$ in each chamber was continually monitored with an infrared gas analyser (LI-Cor model 6252, Lincoln, NB, USA) and logged to a computer. Thus three of the chambers were continuously supplied with ambient + 200 µl l$^{-1}$ CO$_2$ (517–759 µl l$^{-1}$ at 95% confidence interval, referred to as elevated), similar to the fumigation protocol used at Duke Forest, while the other three chambers (referred to as ambient) were supplied equal volumes of ambient air (365–481 µl l$^{-1}$, 24 h, 95% confidence interval). Ambient chambers were vented into the greenhouse whereas elevated [CO$_2$] chambers were vented to the outside air. Chamber and soil temperatures (measured in two tubes per chamber with copper-constantan thermocouples) averaged 34-3 and 33-9 °C, respectively, throughout the experiment.

SEEDLING MEASUREMENTS

The seeds in all chambers were monitored twice daily for emergence of radicles. Upon radicle emergence, the date was recorded and seeds were immediately covered with 1 cm of sand. Hypocotyl height and cotyledon length were measured when the seedlings reached their first resting stage, which was identified by the appearance of a terminal bud. Subsequent height-growth measurements from the sand surface to the top of seedlings were conducted on May 17, June 2 and 27, and July 11 and 16, for all seedlings. At the end of the experiment seedlings...
had not yet begun to produce needle fascicles. Mean relative height-growth increment for each measurement interval was calculated according to Hunt (1982):

\[ R_{t2} = (\ln H_2 - \ln H_1)/(T_2 - T_1) \]  

where \( \ln H_2 \) and \( \ln H_1 \) are the natural logs of heights at times \( T_2 \) and \( T_1 \), respectively.

Seedlings were harvested on July 17, 1999 and separated into leaves (all leaves were juvenile leaves), stems and roots for additional analysis. The number of needles per seedling were counted and total projected leaf areas were measured with a leaf-area meter (Li-Cor model LI-3100, Lincoln, NE, USA). The length of the first-order root (tap root) from the root collar was measured by carefully extending it along a cm scale. Nitrogen concentrations were determined using a CNS analyser (Fisons Model NA-1500, Milan, Italy) on oven-dry, ground tissues. Biomass was determined following oven drying at 70 °C for 48 h.

Shoot net assimilation efficiency was measured as the initial slope of a photosynthesis – [CO\(_2\)] response function on two plants from each chamber at the time of harvest. Seedlings were randomly selected from each seed source (ambient and CO\(_2\)-enriched seed) in each greenhouse chamber for a total of 12 seedlings. A portable infrared photosynthesis system (ADC, Model-LCA2, Hoddesdon, UK) was used. A gas cylinder supplied CO\(_2\)-enriched air to the system and a soda-lime scrubber was used to modify the cuvette [CO\(_2\)]. The entire needle-bearing epicotyl was enclosed in a conifer cuvette and exposed to 1000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) photosynthetically active radiation, which is saturating for evergreen coniferous seedlings that had more needles (F\(_{1,4}\) = 14.77, \( P < 0.05 \)). Consequently, the CO\(_2\)-enriched seed source produced significantly greater seedling biomass in the elevated [CO\(_2\)] greenhouse chambers, but those seedlings were not significantly larger than any seedlings grown in the ambient [CO\(_2\)] chambers (Table 2). The CO\(_2\)-enriched seed source produced seedlings that had more needles (F\(_{1,4}\) = 14.77, \( P < 0.05 \)), and longer first-order roots

### LIPID ANALYSIS

A subset of seed was used for total lipid determination using a petroleum ether Soxhlet extraction (Horwitz 1965). Seeds were lyophilized and stored in a vacuum desiccator until being ground with a mortar and pestle. Ground seed material (98–215 mg) from each Duke Forest treatment ring was placed in a separate cellulose thimble (total of six), and lipid was extracted with boiling petroleum ether at 100 °C in a recirculating boiler–condenser. Additional petroleum ether was added periodically to maintain volume at 60 ml. After 8 h, the hot petroleum ether-lipid extract was collected into preweighed flasks and evaporated overnight. Total lipid mass was then determined by weighing the flasks.

### STATISTICAL ANALYSIS

The relationships between individual seedling height and age were compared using least-squares regression. All other data were analysed as randomized complete block design with a split-plot treatment arrangement with subsampling. The whole-plot effect was greenhouse chamber, and the split-plot effect was seed source within chambers. For each measurement the mean values within each split-plot unit were used in analysis of variance. Significant differences between treatment means were determined using Fisher’s protected LSD (least significance differences) at \( P < 0.05 \).

### Results

The CO\(_2\)-enriched seed source had 91% greater weight (F\(_{1,4}\) = 10.6, \( P < 0.05 \)) than the ambient seed source (Table 1). In addition, the CO\(_2\)-enriched seed source had 128% greater lipid concentration (F\(_{1,4}\) = 54.0, \( P < 0.05 \)) and 265% greater lipid content (F\(_{1,4}\) = 26.78, \( P < 0.05 \)). Seed source also significantly affected the rate and success of germination (F\(_{1,4}\) = 46.37, \( P < 0.05 \)) regardless of chamber CO\(_2\) treatment (Fig. 1). The total germination success of the CO\(_2\)-enriched seed source was more than three times that of the ambient seed source (mean of 95 versus 28%, respectively). In addition, the CO\(_2\)-enriched seed source began germinating up to 5 days earlier than ambient seed. Greenhouse chamber treatments had no effect on germination.

Seed source had no significant effect on seedling relative height growth rates (data not shown). However, at 98 days the relative height growth increment for the final 5 days of the study was significantly higher for the CO\(_2\)-enriched seed source grown in the elevated [CO\(_2\)] chambers (0.01 cm cm\(^{-1}\) day\(^{-1}\) compared to 0.005 cm cm\(^{-1}\) day\(^{-1}\) for all other treatment combinations; F\(_{1,4}\) = 12.55, \( P < 0.05 \)). Consequently, the CO\(_2\)-enriched seed source produced significantly greater seedling biomass in the elevated [CO\(_2\)] greenhouse chambers, but those seedlings were not significantly larger than any seedlings grown in the ambient [CO\(_2\)] chambers (Table 2). The CO\(_2\)-enriched seed source produced seedlings that had more needles (F\(_{1,4}\) = 14.77, \( P < 0.05 \)), and longer first-order roots

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ambient</th>
<th>Elevated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seed mass (mg)</td>
<td>11.6 ± 2.25</td>
<td>22.0 ± 2.31</td>
</tr>
<tr>
<td>Lipid concentration (%)</td>
<td>5.30 ± 0.50</td>
<td>12.10 ± 0.80</td>
</tr>
<tr>
<td>Lipid content (mg)</td>
<td>0.43 ± 0.06</td>
<td>1.57 ± 0.34</td>
</tr>
</tbody>
</table>

Seeds were produced in either ambient or elevated (ambient + 200 μl l\(^{-1}\)) atmospheric CO\(_2\) concentration (n = 3, and five to ten seeds per replicate). The CO\(_2\) effect was significant for all three parameters (\( P < 0.05 \)).
Seedlings grown in greenhouse chambers with elevated [CO2] had significantly lower shoot net assimilation efficiency (shallower initial slope of A–CO2 response curve) compared to those in the ambient chambers for both the ambient seed source (r = 0.019, P < 0.05) and the elevated seed source (r = 0.022, P < 0.05; Fig. 2). Seed source had no effect on whole-shoot net assimilation efficiency.

**Discussion**

Elevated [CO2] dramatically increased the size and lipid content of *Pinus taeda* seeds. Herbaceous and crop species often exhibit increases in seed size (Baker *et al.* 1989; Kimball & Mauney 1993) or lipid content (Rogers, Thomas & Bingham 1983) under elevated [CO2]. We know of only one other study that examined such effects in trees: Connor *et al.* (1998) found a significant increase in *Cornus florida* fruit mass under elevated CO2 and temperature using branch chambers. Lipids comprise the largest portion of C storage in *Pinus* seed, four times that of carbohydrates (Bewley & Black 1994). Various lots of *P. taeda* seed contained 7.6–9.7% total fatty acids, in agreement with lipid concentrations reported here (Marquez-Millano 1989). Storage lipids are synthesized from photoassimilated sucrose translocated to seeds via phloem (Slack & Browne 1984). Developing fruits and seeds are high priority C sinks, and carbohydrates are usually allocated to favour reproductive versus vegetative structures (Wardlaw 1990). The capacity of vegetative structures to utilize assimilates depends in part on the efficiency of symplastic transport at the site of phloem unloading (Thorne 1985). In contrast, high sink strength of developing fruit and seed results largely from a low-resistance, apoplastic phloem-unloading pathway; the ability of developing seed to utilize photosynthesate is more strongly dependent on carbohydrate supply compared to vegetative sinks (Cannell & Dewar 1994; Thorne 1985). Increased assimilation under elevated [CO2], such as that reported for *P. taeda* at the Duke FACE experiment (DeLucia *et al.* 1999; Ellsworth 1999; Myers *et al.*

**Table 2. Growth characteristics (mean ± SE) of *Pinus taeda* seedlings grown in ambient and elevated CO2 (ambient + 200 μl l–1) greenhouse chambers from seed produced under ambient and elevated (ambient + 200 μl l–1) CO2.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ambient greenhouse chambers</th>
<th>Elevated greenhouse chambers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ambient seed</td>
<td>Elevated seed</td>
</tr>
<tr>
<td>Total seedling height (cm)</td>
<td>7.09 ± 0.46a</td>
<td>6.21 ± 0.46a</td>
</tr>
<tr>
<td>Total seedling biomass (mg)</td>
<td>144 ± 7a</td>
<td>136.0 ± 7a</td>
</tr>
<tr>
<td>Leaf mass (mg)</td>
<td>54.8 ± 4a</td>
<td>50.4 ± 4a</td>
</tr>
<tr>
<td>Stem mass (mg)</td>
<td>19.9 ± 1a</td>
<td>20.4 ± 1a</td>
</tr>
<tr>
<td>Root mass (mg)</td>
<td>68.8 ± 4a</td>
<td>65.2 ± 4a</td>
</tr>
<tr>
<td>Leaf area per seedling (cm²)</td>
<td>3.9 ± 0.2a</td>
<td>3.8 ± 0.2a</td>
</tr>
<tr>
<td>Number of leaves</td>
<td>64 ± 2b</td>
<td>73 ± 2a</td>
</tr>
<tr>
<td>Taproot length (cm)</td>
<td>24 ± 0.30b</td>
<td>31.9 ± 3.0a</td>
</tr>
</tbody>
</table>

Means are of three replicates (n = 3) consisting of three to 14 seedlings each depending on seed germination success. Means within a row not followed by the same letter are significantly different (P < 0.05).
of *P. taeda* declined in proportion to the loss of fatty acid content with seed ageing (Marquez-Millano 1989). During germination, most lipid reserves are broken down into their constituent fatty acids, converted to sugars, and transported to the cotyledons and axis for dry weight gain and maintenance (Domán et al. 1982; Gori 1979; Shewry & Stobart 1973; Stone & Gifford 1999; Vanni, Vincenzini & Vincieri 1975). Domán et al. (1982) pointed out that axis dry weight gain in the first 48 h of germination was almost exclusively root growth, which would agree with our findings of earlier radicle emergence and generally longer roots of the CO₂-enriched seed source (Fig. 1; Table 2).

Because of its relatively small seed size, *P. taeda* seedlings begin to depend upon current photosynthates during cotyledon expansion (D. J. Gifford, personal communication). Both seed sources showed significant down-regulation of shoot assimilation efficiency when grown under CO₂ enrichment. (It is not likely that stem respiration differentially affected our estimates of shoot assimilation, because leaf-area ratios did not differ among treatments.) Photosynthetic down-regulation under elevated [CO₂] is often reported where limited rooting environment restricts the belowground C sink, or where severe nutrient limitations restrict overall plant growth (Arp 1991; Eamus & Jarvis 1989; Sage 1994; Stitt 1991; Thomas & Strain 1991; Tissue et al. 1996). Our seedlings were not constrained by rooting volume at the time of harvest, but they were severely nutrient-limited, which probably contributed to the overall decrease in assimilation efficiency (cf. Kubiske et al. 1998; Medlyn et al. 1999; Thomas et al. 1994) and the overall lack of a CO₂ effect on growth.

Growth increases of up to 233% with CO₂ enrichment have been reported for *P. taeda* (Gebauer et al. 1996; Tissue et al. 1996, Tissue et al. 1997). DeLucia et al. (1999) reported a sustained growth increase for 2 years for *P. taeda* at the Duke Forest FACE experiment. Strong growth responses to elevated [CO₂] were not observed in our experiment, and our hypothesis that the elevated CO₂ seed source would enjoy a synergistic growth increase under [CO₂] enrichment was not strongly supported. The small sample size, severe nutrient limitations and photosynthetic down-regulation probably contributed to unimpressive growth responses.

Our objective in this study was to understand the potential interactive responses of elevated [CO₂] on seed development and subsequent seedling performance of an important forest tree species. Seedling responses play an important role in tree regeneration and succession, because germination and initial seedling growth set the pattern for future growth (Miao 1995). For example, in *P. taeda*, larger seed had significantly shorter germination times than smaller seed, which influenced seedling height after 28 days (Dunlap & Barnett 1983). The growth advantage gained from larger seed mass was observed in standing tree volumes 15 years after sowing (Robinson & van Buijtenen 1979). Thus a small growth advantage due to earlier germination time or

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**Fig. 2.** Shoot net CO₂ assimilation rate of *P. taeda* seedlings grown in either ambient (triangles) or elevated CO₂ (ambient + 200 μl l⁻¹, circles) greenhouse chambers, and from seed that developed under ambient (solid symbols, a) or elevated CO₂ (open symbols, b). Regression lines were fitted to data for three seedlings from each treatment combination. Regression equations are: ambient CO₂-grown seed in ambient CO₂ greenhouse chambers (▲), \( A = 0.416 + 0.011C_0 \), \( r^2 = 0.97 \); ambient CO₂-grown seed in elevated CO₂ greenhouse chambers (■), \( A = -0.237 + 0.007C_0 \), \( r^2 = 0.94 \); CO₂-enriched seed in ambient CO₂ greenhouse chambers (△), \( A = -0.414 + 0.012C_0 \), \( r^2 = 0.94 \); CO₂-enriched seed in elevated CO₂ greenhouse chambers (○), \( A = 0.787 + 0.006C_0 \), \( r^2 = 0.98 \).
seedling vigour often compounds over time to affect tree-growth characteristics years later.

Our results have profound implications for reproductive strategies of trees if elevated [CO2] has dissimilar effects on seed development among species. For example, P. taeda seed reserves directly influence seedling growth for only a very short period of time, after which new photoassimilates support growth. It is entirely possible that larger-seeded competitors, such as Quercus or Carya, would respond in a different way. Although the seedling growth phase of this study did not conclusively support our main hypothesis, there were striking effects of elevated [CO2] on the size, chemistry and germination of the seeds themselves. As our seed were extracted from open cones, it is not clear if our findings are representative of all P. taeda seed produced at the Duke Forest experiment in 1998. One possibility is that individual cones under elevated CO2 contained a larger proportion of lipid-rich, viable seed, and the population of viable seed from the ambient seed source had shed prior to our collection. Another possibility is that the manner of seed shedding varied between CO2 treatments. Additional work is needed to resolve these questions, so that the ecological ramifications of elevated [CO2] effects on Pinus seed production can be assessed.

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