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Elevated CO_2 increases root exudation from loblolly pine (*Pinus taeda* L.) seedlings as an N-mediated response

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Running title

CO_2 and N effects on root exudation

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4 Figures; 3 Tables

Summary The degree to which forest ecosystems provide a long-term sink for increasing atmospheric CO_2 likely depends upon the capacity of trees to increase the availability of growth limiting resources. It has been widely speculated that trees exposed to CO_2 enrichment may increase the release of root exudates to soil as a mechanism to stimulate microbes to enhance nutrient availability. As a first test to examine how atmospheric CO_2 and nitrogen availability affect rates of root exudation, we conducted two experiments in which exudates were collected from loblolly pine (*Pinus taeda* L.) seedlings grown in controlled growth chambers under low and high CO_2 and at low and high rates of N supply. Despite differences in experimental design between the two studies, plants grown at high CO_2 were larger and thus whole plant exudation rates were higher under elevated CO_2 ($p = 0.019$), but the magnitude of this response depended on the N level in both studies. Seedlings increased mass specific exudation rates in response to elevated CO_2 in both experiments, but only at low N supply. Moreover, N supply had a greater impact on exudation rates than did CO_2, with mass-specific exudation rates significantly greater (98% and 69% in experiments 1 and 2, respectively) in seedlings grown at low N supply relative to high N supply. These results provide preliminary evidence that loblolly pines alter exudation rates in response to both CO_2 concentration and N supply, and support the hypothesis that increased C allocation to root exudates may be a mechanism by which trees can delay progressive N limitation in forested ecosystems.

Keywords: carbon sequestration, progressive N limitation, rhizodeposition, rhizosphere C flux

Introduction

Aggrading forest ecosystems in northern latitudes currently represent a large sink for atmospheric CO_2 on a global basis (IPCC 2007). Understanding the factors which regulate forest productivity and their role in global C cycling is central to understanding potential feedbacks to climate change. In most temperate forests, productivity is nutrient limited. Thus, the long-term capacity of forests to sequester C likely depends on the degree to which trees allocate resources to acquire growth-limiting nutrients such as nitrogen (N) (Reich et al. 2006; Zak et al. 2003). Numerous studies have reported increased fine root production in trees exposed to elevated CO_2 (Lukac et al. 2003; Mikan et al. 2000; Norby et al. 2004; Pritchard et al. 2008), indicating that trees may be using the "extra" C to forage for N in soil. Yet exploiting a larger volume of soil via increased root production may have little affect on total N uptake if the majority of N in soil is in unavailable (e.g. organic) forms. Hence, in forest soils where inorganic N concentrations are low, increased soil exploration under elevated CO_2 will likely only increase N acquisition if microbes are stimulated to transform N into available forms (Phillips 2007).

One mechanism by which trees stimulate microbial activity is through the release of root exudates. Root exudates are soluble organic compounds (e.g. sugars, amino acids and low molecular weight organic acids) that are both passively released by roots due to diffusion gradients between the cytoplasm and soil solution and actively secreted in response to stress and nutrient deficiency (Curl and Truelove 1986; Marschner 1995). Although the causes and consequences of exudation are incompletely understood, several recent experiments have documented that a large fraction of the C used to support biological activity in forest soils results from the release of root-derived C (Hogberg 2008; Hogberg and Read 2006; Keel et al. 2006). Such rhizosphere C fluxes are estimated to represent 1-10% of net primary productivity in forests, and are believed to disproportionately affect nutrient availability due to their chemical quality as microbial substrates (Smith 1976).

Despite the perceived importance of exudation to ecosystem function (Cheng 1999; Rogers et al. 1994), there have been remarkably few measurements of exudation from trees exposed to elevated CO_2 (reviewed in Grayston et al. 1996; Jones et al. 2004). In the few studies where such measurements have been made, the results have been variable and inconclusive. Norby et al. (1987) reported that elevated CO_2 stimulated exudation in *Pinus enchinata* seedlings

after 34 weeks of growth but had no effects after 41 weeks of growth. Delucia et al. (1997) found that *Pinus ponderosa* seedlings exposed to elevated CO_2 increased exudation of some compounds (e.g. oxalic acid) but decreased exudation of others (e.g. phosphatase enzymes). And Uselman et al. (2000) reported that although elevated CO_2 increased exudation in *Robinia pseudoacacia* seedlings, such changes resulted from increases in root biomass rather than increases in mass-specific exudation rates.

An important mediator of the belowground responses of trees to elevated CO_2 is N availability (Cheng 1999). Carbon allocation in trees is generally believed to be controlled equally by shoots and roots (Farrar et al. 2003) until a functional equilibrium is established between C assimilation and nutrient acquisition (Thornley 1977). Thus, the exudation response of tree roots to elevated CO_2 should depend, in part, on N supply. In soils where N supply is low, exudation rates may increase under elevated CO_2 if greater belowground C allocation is triggered by nutritional stress in the plant (Farrar and Jones 2000). Alternatively, CO_2 effects on exudation may be lower in nutrient-poor soils if total C assimilation rates (and by extension, belowground C allocation) are reduced by nutritional constraints.

Here we report the results of two independent growth chamber experiments designed to examine whether N supply affects the root exudation response of loblolly pine seedlings to variable levels of CO_2. This is the first study, to our knowledge, to examine the interactive effects of elevated CO_2 and N on tree root exudation across a range of growth conditions. We expected root exudation rates would increase under elevated CO_2 but hypothesized that the magnitude of the CO_2 effect would be greatest under lower N availability. Experimentally demonstrating that the interaction between CO_2 and N is a significant driver of root exudation rates in seedlings represents an obvious first step in supporting the hypothesis that trees will allocate more C to soluble exudates when N limits growth.

Materials and Methods

Two experiments were performed, utilizing similar experimental treatments but different exudate collection methods. In both experiments pine seedlings were grown from seed in sand under controlled growth conditions. In experiment 1 pine seedlings were transplanted from sand into hydroponic solution culture in order to measure exudation rates. In experiment 2, seedlings

were transplanted from sand into unsaturated glass bead culture for exudate capture. Experiment 1 utilized methods common to many previously reported exudate studies, while experiment 2 was intended to more closely approximate the conditions of soil-grown plants (e.g. mechanical impedance of root growth, low nutrient conditions, etc.). Our primary goal was to determine whether the treatment effects were qualitatively consistent between the two studies, and secondarily to estimate how artifacts inherent in each experimental design might affect quantitative estimates of root exudation rates.

Experiment 1 – solution culture

Growing conditions

Loblolly pine (*Pinus taeda* L.) seeds collected from the North Carolina piedmont (Weyerhaueser Co., WA) were cold-stratified at 4º C for 30 days, planted in steam-sterilized sand (three seeds per pot), and inoculated with the mycorrhizal fungus *Pisolithis tinctorius* (Plant Health Care Inc., Pittsburgh, PA). Each pot (3.5 L) was randomly assigned to one of two N treatments and one of two atmospheric CO_2 treatments (n = 20 pots per treatment). Two weeks after seedling emergence, all pots were "thinned" so that each container contained a single seedling.

All pots were distributed between four growth chambers at the Duke University Phytotron, NC: two maintained at approximately ambient CO_2 (350 ppm by volume) and two at 2x ambient CO_2 (700 ppm by volume). In-line CO_2 scrubbers were used to maintain levels in the 350 ppm chambers. A 14-h diurnal photoperiod was maintained in each chamber using a combination of incandescent and high intensity discharge lamps. Light levels averaged 535 µmol $m^{-2}s^{-1}$ during the photoperiod with the exception of a 2-h "mid-day" period where levels were increased to 1075 µmol $m^{-2}s^{-1}$. Chamber temperature and relative humidity averaged 25º C and 85%, respectively during the photoperiod, except during the 2-h peak period where temperature and humidity were maintained at 28º C and 75%. Each week the CO_2 treatment was assigned at random to two chambers and the seedlings were moved accordingly to minimize chamber effects. In addition, the distribution of seedlings within each chamber was rotated weekly. The growth period lasted 100 days.

All pots were watered to saturation each day with a modified Hoagland's solution (1/2 strength; pH = 6.1) with one of two N concentrations (1 and 5 mM N; hereafter low N and high N, respectively). Nitrogen was added in the form of NH_4NO_3, and these concentrations were chosen to represent a gradient in N availability in loblolly pine seedlings from sub-optimal to supra-optimal based on previous growth chamber studies (Griffin et al. 1997; Griffin et al. 1993; Larigauderie et al. 1994). All pots were watered in the evening with distilled water to prevent salt accumulation in the pots (BassiriRad et al. 1997; Delucia et al. 1997; Uselman et al. 2000). A layer of pea gravel (~3 cm thickness) was added on the sand surface after ~1 month to reduce evaporative water loss and minimize algal growth.

Exudate collection

One hundred days after germination, a subset of pots was randomly chosen for exudate collection (10 per treatment). Seedlings were carefully removed from their pots and rinsed with distilled water to remove any sand adhering to roots. Seedlings were transplanted into 1-L containers containing a modified Hoagland's solution of the same concentration of N as their watering solution (1 or 5 mM N), and returned to their respective growth chambers for seven days to overcome the transplant shock (Norby et al. 1987). Each container was aerated continuously with an aquarium airstone and bubbler, and received fresh nutrient solution every other day.

Seven days after being transferred into solution culture, seedlings were placed into clean Mason jars filled with 800 mL of sterile, aerated nutrient solution (same N concentrations) and returned to their respective growth chambers. Because the roots were submerged in a static bathing solution for the duration of the incubation, we collected subsamples from each jar (~30 mL) at three time intervals (4, 9 and 24h) to determine the equilibrium point where C efflux was approximately balanced by reabsorption of exudate by the pine roots (Personeni et al. 2007; Uselman et al. 2000). Based on these measurements, we concluded that exudation rates were still increasing linearly until 9 hours in all treatments but declined between 9 and 24 hours (Figure 1). Thus, net exudation rates were calculated as the change in dissolved organic carbon (DOC) content in each jar between the beginning and end of the 9h incubation period. All samples collected from the jars were filtered through 0.45-µm membrane filters (Whatman Inc.,

Florham Park, NJ) and analyzed on a Tekmar-Dohrmann total organic carbon (TOC) analyzer (Cincinnati, OH).

Plant Harvest

Following exudate collection, plants were harvested and separated into needles, stems, fine roots (<2 mm diameter), and coarse roots (>2 mm diameter). Fine roots were initially separated into <1 mm and 1-2 mm diameter roots. However, we chose to pool these fractions based on the abundance of borderline roots (~1 mm) and mycorrhizal tips which made separation of the two categories sensitive to root-sorter bias. We did not quantify the percentage of roots colonized by mycorrhizal fungi in this study, but previous studies show that up to 50% of the root tips of *Pinus taeda* seedlings grown under similar conditions at this facility may have been colonized (Lewis et al. 1994). All plant material was oven-dried at 60º C for at least 72h for determination of dry weights.

Experiment 2 – glass beads

Growing conditions

Loblolly pine (*Pinus taeda* L.) seeds from a 1st generation orchard mix from the North Carolina piedmont (NCSU seedlot #SOM8) were planted, inoculated and sown as described in experiment 1. All growing conditions were the same as in Exp. 1 with the exception of the N concentration of the nutrient solution and the CO_2 concentration of the chambers which were changed to better reflect the gradients that exist at the Duke Forest FACE site, NC. In experiment 2, we used a modified Hoagland's solution containing either 0.5 or 1 mM N (all as NH_4NO_3; hereafter low N and high N, respectively). The CO_2 concentration in the chambers of 385 ppmv and 585 ppmv are the same as those at the Duke Forest site (Andrews et al. 1999). The growth period for this experiment lasted 150 days.

Exudate collection

One hundred forty days after planting, a subset of pots was randomly chosen for exudate collection (n = 16 per treatment plus 6 non-vegetated controls). All seedlings were carefully removed from their pots and rinsed with distilled water to remove any sand adhering to roots. The entire root system of each seedling was then dipped into an anti-bacterial cocktail (50 ppm streptomycin and 50 ppm penicillin) for 2-3 minutes to arrest rhizoplane bacterial growth. This anti-biotic treatment was found to reduce exudate consumption in previous experimental trials (data not shown). Following the antibiotic treatment, root systems were rinsed with water and transferred into cylindrical PVC cores (20 cm length x 5 cm diameter) sealed on the bottom by mesh (30 µm). Each core was carefully filled with sterile glass beads (~750 µm diameter; La De Da Designs, Baton Rouge, LA), such that roots were distributed evenly throughout the core. Beads were used instead of quartz sand because preliminary experiments suggested strong adsorption of low molecular weight organic acids by sand but not beads (data not shown). Cores were then wrapped in aluminum foil to prevent light penetration, and foam bungs with a slit removed to accommodate the plant stem were placed at the top of each core to prevent algal growth and minimize evaporative loss. All seedlings were returned to their respective growth chambers, and watered as before (i.e. nutrient solution in the morning and distilled water in the evening).

After a 7-d acclimation period, all seedlings were removed from the growth chambers and flushed with 250 mL of reverse osmosis water (3 times the water-holding capacity of each core) to remove accumulated DOC. All cores were watered with dilute C-free nutrient solution and returned to their respective growth chambers for a 10-h incubation period. For the incubation period, we used a nutrient solution $1/10^{th}$ the concentration of the previous nutrient solution (i.e. 50 and 100 µM N). These concentrations were chosen to more closely resemble concentrations in the soil solution draining the O horizon at the Duke Forest FACE site (Jackson et al. *In Press*). After 10h, seedlings were removed from the growth chambers and flushed 3 times with 60 mL of distilled water. In addition to quantifying DOC efflux, we measured nutrient uptake rates by seedlings over the 10-h incubation period using the nutrient depletion method (Bassirirad et al. 1996). All leachate flushed from the cores was filtered through a 0.2-µm membrane filter (Millex GV, Millipore Co., Billerica, MA) and analyzed for non-particulate

organic C (for exudation rates) and total N (for uptake rates) on a TOC analyzer with TN module (Shimadzu Scientific Instruments, Columbia, MD).

Plant Harvest and Photosynthesis

Plant tissues for all experimental seedlings were separated into the same fractions as described in experiment 1. Total leaf area of each plant was measured on a 3000A portable area meter (Lincoln, NE) from a subsample of needles (~1/3 of the total mass). Fine root length, surface area and number of root and mycorrhizal tips were measured on a randomly selected subset of fine roots (n = 10 per treatment) that were photocopied following the methods of Larigauderie et al. (1994), and scanned and analyzed using WinRHIZO software (Regent Instruments, Inc., Canada). All plant material was oven-dried at 60º C for at least 72h for determination of dry mass. On a subset of seedlings (n = 6 per treatment), photosynthetic rates were measured in a conifer chamber attached to a Licor 6400 (Licor Co., Lincoln, NE). For each plant, rates were measured on two different sections of the canopy, under saturating light conditions (>1000 µmol m^{-2} s^{-1}), and at both 385 and 585 ppmv in the leaf chamber.

Calculations

Whole plant C assimilation rates for the 10-h incubation period were estimated by multiplying the average photosynthetic rates (within each N treatment and at a given CO_2 level) by the total needle area of each plant. To account for non-saturating light conditions during the 10-h incubation (average 530 µmol m^{-2} s^{-1}), total C gain during the photoperiod was calculated as a weighted mean where photosynthetic rates under non-saturating light conditions were assumed to be 75% of those under saturating light based on light curves of loblolly pine seedlings grown under similar growth chamber conditions and N levels (Zhang et al. 1997). To independently verify these estimates of whole plant C assimilation during the 10-h incubation period, we also calculated the amount of whole plant C gain for the day of the exudate collection (i.e. day 145) using exponential growth equations developed for loblolly pine seedlings growing under nearly identical elevated CO_2 and N levels at the Duke Phytotron (Griffin et al. 1993). These calculations yield a C gain of 36 and 93 mg C during the 24-h period of exudate collection in the low and high N treatments, respectively, and suggest that our estimated rates of C gain during the

10-h incubation (18 and 57 mg C in the low and high treatments) represent a reasonable approximation of total C gain during the photoperiod.

Exudation rates were scaled in two ways. First, whole plant exudation (mg C $plant^{-1}$) was calculated as a percentage of the C gain over the 10-h incubation period. In the second calculation, the amount of C released via exudation was divided by whole plant C accumulation per day (mg C d^{-1}) by measuring exudation rates on a subset of loblolly pine seedlings (n = 6; using the same methods as described in Exp. 2) during a 24-h period that included both light (14h) and dark (10h) conditions in the chambers.

Statistics (both experiments 1 and 2)

Due to differences in the CO_2 and N treatment levels, data for experiments 1 and 2 were analyzed separately. The effects of CO_2 and N treatments on plant biomass and exudation rates (experiments 1 and 2) were examined by two-way analysis of variance (ANOVA). Because of the potentially confounding effects of treatments on exudation rates via alteration of root surface area to bathing solution volume ratios (see Discussion below), CO_2 effects on exudation were analyzed within a given N level only using linear contrasts. All non-normally distributed data were transformed (using log or square root transformations) prior to analysis, and all data was analyzed using JMP statistical software (v. 6, SAS Institute Inc., Cary, NC).

Results

Experiment 1

In general, N treatments had a much greater stimulatory effect on plant biomass than did CO_2; total biomass at high N was nearly four-fold greater than at low N ($p < 0.0001$; Table 1). Similarly, the biomass of all shoot and root fractions was greater in the high N treatment relative to the low N treatment ($p < 0.0001$ for all), with the largest response occurring in the shoots (e.g. five-fold greater foliar biomass at high N). The strong effects of N on biomass partitioning is also reflected by the two-fold greater root:shoot ratios in the low N relative to high N treatments ($p < 0.0001$).

In contrast to the N treatments, most tissue fractions were unaffected by CO_2 treatments. Only total root biomass was affected by CO_2, increasing by 35% at elevated CO_2 ($p = 0.046$). Consistent with previous studies with loblolly pine seedlings (Griffin, Thomas & Strain, 1993, Larigauderie, Reynolds & Strain, 1994), CO_2 effects on shoot and root biomass were largely mediated by N, with the greatest stimulation of shoot and root biomass occurring in the high N treatment. CO_2 induced a 49% enhancement of total plant biomass at the high N level ($p = 0.028$) but had no effect on total biomass at the low N level ($p = 0.808$). This pattern was evident in both aboveground and belowground tissues. Needle biomass was 52% greater at elevated CO_2 at high N ($p = 0.071$) but unaffected by CO_2 at low N ($p = 0.960$). Similarly, elevated CO_2 increased fine root biomass by 79% in the high N treatment ($p = 0.036$), but had no effect on this fraction in the low N treatment ($p = 0.985$). Overall, root:shoot ratios were unaffected by the CO_2 treatment as a whole ($p = 0.405$) or within each individual N level.

Although whole plant exudation was 36% higher under elevated CO_2 ($p = 0.019$), the magnitude of this response depended on the N level (Figure 2a), as CO_2 effects on exudation were significant at low N (61% stimulation; $p = 0.006$) but unaffected at high N ($p = 0.593$). Exudation rates calculated per unit fine root biomass (mg C exuded g^{-1} h^{-1}), indicated that N but not CO_2 significantly affected mass-specific rates in these seedlings (Figure 2b). Similar to whole plant exudation, there was a significant CO_2 by N interaction ($p = 0.025$) on mass-specific rates; however, CO_2 effects on mass-specific rates at low N were only marginally significant ($p = 0.067$).

Experiment 2

Despite appreciable differences in the CO_2 and N levels used in the two experiments, treatment effects on total biomass in experiment 2 were similar to those in experiment 1, as N but not CO_2 influenced biomass production. As in experiment 1, N strongly stimulated total biomass (Table 2) with increases in needle, stem, fine root and coarse root biomass (all with $p < 0.0001$). Moreover, root:shoot ratios were significantly decreased at the high N level ($p < 0.0001$). Root morphology was affected by N availability, with significantly greater fine root surface area ($p < 0.0001$) and higher numbers of fine root tips ($p = 0.04$) in the highest N treatment.

Similar to experiment 1, there were no main effects of elevated CO_2 on pine shoot and root biomass, nor were there any CO_2 x N interactions. The proportion of C allocated to roots (i.e. root:shoot ratio) was 15% greater under elevated CO_2 but this response was not statistically significant ($p = 0.075$). Root morphology was mostly unaffected by elevated CO_2 although fine root surface area of roots was increased by 33% ($p = 0.033$) in the CO_2 treatments. Total N uptake over the 10-h incubation period was unaffected by CO_2 but was increased significantly by N ($p = 0.002$).

Consistent with the response in experiment 1, mass-specific exudation rates (mg C exuded g^{-1} h^{-1}) were decreased by N ($p < 0.0001$) and marginally reduced by elevated CO_2 ($p = 0.092$; Figure 3b). Within the 0.5 mM N treatment, CO_2 resulted in a small but marginally significant increase in mass-specific rates (28%; $p = 0.082$). Whole plant exudation rates were increased under elevated CO_2 by approximately the same magnitude as in experiment 1 (45%; $p = 0.029$ - Figure 3a) but unaffected by N ($p = 0.922$). However, N level was important in mediating the magnitude of CO_2 effects on mass-specific exudation – consistent with the response in experiment 1 (Figure 3b). At low N, CO_2 resulted in a small but marginally significant increase in mass-specific exudation (41%; $p = 0.069$). At high N, mass-specific rates were lower ($p < 0.0001$), but did not influence the magnitude of CO_2 effects ($p = 0.827$).

Because changes in root morphology can also affect exudation rates, we calculated exudation rates as a function of fine root surface area and the number of root and mycorrhizal tips (data not shown). Neither area-specific rates nor exudation rates per number of root tips were significantly affected by the main effects of elevated CO_2 ($p = 0.520$ and 0.230, respectively). However, similar to mass-specific rates, area-specific rates were strongly affected by N treatments. For seedlings in the low N treatment, area-specific rates were 43% greater than at high N ($p=0.035$). The important role of N in mediating exudation is also suggested by the relationship between fine roots and exudation within the low N but not the high N level. At low N, there was a weak but significant correlation between fine root biomass and exudation ($r = 0.48$; $p = 0.003$; data not shown), as well as between fine root surface area and exudation ($r = 0.50$; $p = 0.01$). This is in contrast to patterns at high N where there was no significant

correlation between fine root biomass and exudation (r = 0.17; p = 0.122) or fine root surface area and exudation (r = 0.24; p = 237).

Both approaches used to scale exudation rates revealed a similar pattern. Exudation per unit of C assimilated (estimated from the scaled photosynthesis measurements) ranged from 0.27 to 0.96% (Table 3), and these values were similar in direction and magnitude to exudation rates scaled per unit net C accumulation (Figure 4a). Moreover, the total quantity of exudates released across the four treatments was positively correlated with the mean photosynthetic rate of seedlings (Figure 4b; $r^2 = 0.951$; $p < 0.0001$).

Discussion

The degree to which root exudates influence nutrient cycling in forest soils is poorly understood owing to the challenges associated with quantifying rhizosphere processes *in situ* (Phillips et al. 2008). In this study, we conducted two growth chamber experiments with loblolly pine seedlings as a first attempt to understand the extent to which root exudation rates were responsive to CO_2 and N availability. The magnitude of C flux from exudation per unit of C assimilated (estimated from scaled photosynthesis measurements) ranged from 0.27 to 0.96%, with the greatest rates occurring under conditions of low N and elevated CO_2 (Table 3). Because exudation and photosynthesis rates were calculated as mean treatment effects, we did not statistically evaluate how CO_2 and N affected these patterns in individual trees. However, exudation rates scaled per unit of C accumulated over the duration of the experiment suggest that differences in exudation rates were significantly affected by the treatments. Exudation rates scaled per unit net C accumulation yielded results similar in direction and magnitude (Figure 4a), with significantly greater exudation rates in the low N treatment ($p < 0.0001$). Moreover, the total quantity of exudates released across the four treatments was positively correlated with the mean photosynthetic rate of seedlings (Figure 4b; $r^2 = 0.951$; $p < 0.0001$) suggesting a tight coupling between C assimilation and exudation. Despite differences between the two experiments in CO_2 levels, N supply and exudate collection methods used, we found a remarkably consistent response – CO_2-induced increases in whole plant and mass specific exudation rates at low N. We interpret this response as evidence of a coupling between

exudation rates and the internal nutrient status of loblolly pine seedlings (Jones et al. 2004; Neumann and Romheld 2001).

CO_2 and N effects on exudation

An important compensatory adjustment by plants exposed elevated CO_2 is the preferential allocation of C to belowground tissues (Reich et al. 2006; Zak et al. 2003). Numerous growth chamber and field experiments have reported increased belowground production in trees exposed to CO_2 enrichment (Ainsworth and Long 2005; Curtis and Wang 1998; Pendall et al. 2004) indicating that trees grown at high CO_2 are allocating more C to roots, presumably to forage for growth-limiting nutrients.

There have been several reports of CO_2-induced increases in mass-specific exudation in non-woody plants but few reports of this response in trees (reviewed in Cheng and Gershenson 2007). CO_2-induced increases in exudation have been reported for seedlings of *Pinus ponderosa* (Delucia et al. 1997), *Pinus enchinata* (Norby et al. 1987) and *Robinia pseudoacacia* (Uselman et al. 2000) but in each case, the CO_2 effects were not significantly different when scaled per unit of fine root biomass. In both experiments of this study, treatment effects on fine root biomass (Tables 1, 2) were insufficient to explain greater exudation in seedlings grown at elevated CO_2 and low N (Figure 2b, 3b). That the stimulatory effects of elevated CO_2 were only present in plants grown at low N suggests that nutritional imbalances in the pines may have triggered the exudation response. This would be consistent with previous studies reporting a greater magnitude of belowground response of loblolly pine seedlings to elevated CO_2 under low nutrient conditions (Griffin et al. 1997; Griffin et al. 1995; Larigauderie et al. 1994).

In addition to increased belowground C allocation, it is possible that greater exudation in low N resulted from CO_2- and N-induced changes in root architecture (Neumann and Romheld 2001). Darwent et al.(2003) reported that decreased exudation from *Hordeum vulgare* roots resulted from reductions in root length and the numbers of root tips due to increased N supply. In loblolly pine, root morphology has been reported to be responsive to changes in both CO_2 and N (Larigauderie et al. 1994), but the consequences of these changes for exudation is unknown. In experiment 2 of this study, root surface area and the numbers of root tips were greatest at high

N, resulting in decreased exudation. However, N-induced changes in root surface area and the number of tips were similar in magnitude to changes in root biomass, and thus we cannot conclude whether the N-induced decreases in exudation resulted from changes in C allocation or changes in root morphology. Moreover, it is also possible that the greater exudation at low N resulted from increased membrane permeability of roots due to nutritional stress (Neumann and Romheld 2001). Further examination of root exudation across a broader range of N levels and with more detailed characterization of root morphology and physiology would be a worthwhile focus of future studies.

Given the strong effects of N in mediating belowground C allocation and root exudation in this study, an obvious question arises: do loblolly pine seedlings increase exudation as a response to N deficiencies? To date, most reports of exudation in response to nutrient stress have been reported for plants under P and Fe deficiency or metal (e.g. Al) toxicity (Marschner 1995). In non-woody plants, mass-specific exudation rates have been shown to increase (Henry 2005; Paterson 2006), decrease (Darwent 2003; Hodge et al. 1996; Paterson et al. 1999; Werth and Kuzyakov 2006) or vary (Neumann and Romheld 2001; Nguyen 2003) in response to N amendment. In trees, Aitkenhead-Peterson et al. (2005) reported a three-fold increase in mass-specific exudation from *Picea abies* saplings at low N. However, Uselman et al. (2000) found two-fold greater mass-specific exudation from *Robinia pseudoacacia* seedlings grown at high N. Collectively, these results indicate that our understanding of CO_2 and N effects on tree root exudation is incomplete, and may limit our predict feedbacks to forest productivity under global change (Giardina et al. 2005).

Exudation rates from experiment 2 in this study were both lower (Aitkenhead-Peterson 2005; Grayston et al. 1996; Norton et al. 1990) and higher (Norby et al. 1987; Uselman et al. 2000) than those reported for seedlings of other tree species. Because the magnitude of exudation is highly sensitive to the experimental system used to collect the exudates (Phillips et al. 2008), we cannot say whether our rates reflect true species differences. Perhaps a more important question is whether exudation rates from seedlings can help inform what occurs in forests where N concentrations in soil solution are generally much lower than those used in growth chamber experiments (Lucash et al. 2005). Phillips et al. (2008) recently developed a

method to measure exudation rates from trees *in situ* using a modification of the glass bead culture method used in this study. They reported exudation rates in 26 year-old loblolly pine trees (~1% of net assimilated C) in close agreement with exudation rates from this study (0.3-0.8% of net assimilated C). The paucity of direct exudation estimates from tree roots *in situ* in response to changing environmental conditions suggests that seedling studies – despite their obvious limitations – may be an important starting point for developing appropriate scaling factors to estimate the potential contribution of exudates to soil processes in forests exposed to elevated CO_2. Results from our controlled studies of loblolly pine seedlings suggest that soluble root exudates could represent a considerable belowground C flux, perhaps similar to fine root turnover.

Experimental artifacts in exudation measurement

All methods used to measure exudation have inherent artifacts associated with their design (Neumann and Romheld 2001), and thus we consider whether our methods may have influenced exudation patterns. Although there was qualitative agreement between the two experiments, the exudation rates we measured for seedlings in solution culture (experiment 1) were much higher than those in glass beads (experiment 2). Our direct comparison of approaches provides an important caution to attempts to scale from individual controlled laboratory studies to ecosystem-scale processes. There are several possible reasons for measuring higher exudation rates in hydroponic experiments than in unsaturated artificial soil incubations. First, the exudation rates in experiment 1 may have been greater due to greater diffusion gradients between the root and the solution. Basal exudation is highly sensitive to gradients in soluble C between the root cytoplasm, apoplast and soil solution (Jones et al. 2004), and the gradient in solution culture would presumably be greater than in non-saturated glass beads due to the intimate contact between roots and solution. Second, because the roots in solution culture were fully submerged, non-exudate higher molecular weight C compounds may have leached from roots. This problem would have been exacerbated by the 5-fold difference in N supply between low and high N in experiment 1 (which increased root biomass), and by the larger pore size used to filter exudates in experiment 1 (0.45 µm) relative to experiment 2 (0.2 µm). And finally, roots in experiment 2

(but not experiment 1) were treated with antibiotics which may have decreased the release of exudates if the antibiotics negatively affected the plant (Neumann and Romheld 2001).

Static bathing solutions, or hydroponic incubations, have often been used to measure exudation rates because they require little maintenance, use a lesser volume of solution than in percolating solutions, and permit the collection of exudates from the entire rooting volume rather than from preferential flow paths. Because exudates accumulate in the bathing solution, these systems need to be sampled during the incubation period to ensure C accumulation does not reduce exudation as the equilibrium between efflux and influx is approached (Uselman et al. 2000). The nutrient content of static bathing solutions may also change during the course of the incubation due to nutrient uptake, thereby affecting exudation rates. In experiment 1, exudation increased linearly over the first nine hours of collection (Figure 1), suggesting that C accumulation in the bathing solution was unlikely to be affecting the gross efflux. Moreover, we found no evidence of differences in accumulation rates of exudates between low and high N treatments despite a two-fold greater root biomass suggesting that N effects on mass-specific rates were unlikely to have been the result of changes in the efflux and influx of exudates in the bathing solution (Personeni et al. 2007). Changes in nutrient content owing from N uptake were unlikely to have affected exudation patterns. In experiment 2, the N content of the post-incubation bathing solution was still greater in the high N (0.6 mg N) than in the low N (0.46 mg N) treatment. This suggests that it is unlikely that the changes occurring during the incubation period contributed to the observed exudation patterns.

Reabsorption of exudates has been reported to occur in several grass species (de Graaff 2007; Phillips et al. 2006), but to our knowledge, there have been no reports of reabsorption by trees. Given the greater density of roots in experiment 2 relative to experiment 1, it is certainly possible that some fraction of exudates were re-acquired by roots during the incubation. However, in a separate experimental trial with loblolly pine, exudates accumulated linearly over the first eight hours in bead-filled cuvettes packed at similar densities to those used in experiment 2 (0.81 vs. 0.83 cm^2 root surface area per ml cuvette; Phillips, *unpublished data*), suggesting little effect of uptake on net C efflux. Moreover, it is unlikely that exudate re-uptake would have influenced treatment effects as amino acids and sugars – the primary compounds

taken up by roots – (Jones et al. 2004) generally occur in low concentrations in *Pinus* exudates (Smith 1976).

Although we fully recognize the inherent limitations of our experimental systems, the qualitative agreement between the two experiments suggests that our results are robust with respect to the direction of treatment effects.

Conclusions

Numerous investigators have invoked root exudation as a locally missing sink for plant-derived C in elevated CO_2 experiments, but there has been little empirical work to support this hypothesis. In this study, we have demonstrated in two independent experiments that elevated CO_2 may increase root exudation in loblolly pine seedlings, but that such effects are sensitive to the availability of N in the growing medium. Thus, the N status of pines may determine whether additional C fixed under elevated CO_2 is released to soil as root exudates, possibly as a mechanism for increasing N availability in soil. CO_2-induced increases in exudation arose from both increases in fine root biomass and from greater mass-specific exudation in the low N levels. Despite differences in the rates between experiments, the relative effects of the treatments were consistent, and thus provide evidence that the degree to which trees sequester C under elevated CO_2 may depend on the magnitude and ecological consequences of changes in C released to soil via root exudation.

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Figure legends

Figure 1. Root exudate accumulation in static bathing solution culture over a 24 h period. Error bars for each of the four treatments represent one standard error of the mean (n = 10).

Figure 2. The effects of elevated CO_2 and N on a) the total quantity of root exudates released, and b) mass-specific exudation rates in 100 day-old loblolly pine seedlings (experiment 1). Error bars are standard errors of the mean (n = 10). Significant differences between ambient and elevated CO_2 (open and black bars, respectively) within an N level are noted by asterisks († = $p < 0.10$, * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.0001$).

Figure 3. The effects of elevated CO_2 and N on a) the total quantity of root exudates released, and b) mass-specific exudation rates in 145 day-old loblolly pine seedlings (experiment 2). Error bars are standard errors of the mean (n = 10). Significant differences between ambient and elevated CO_2 within an N level are noted by asterisks († = $p < 0.10$, * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.0001$).

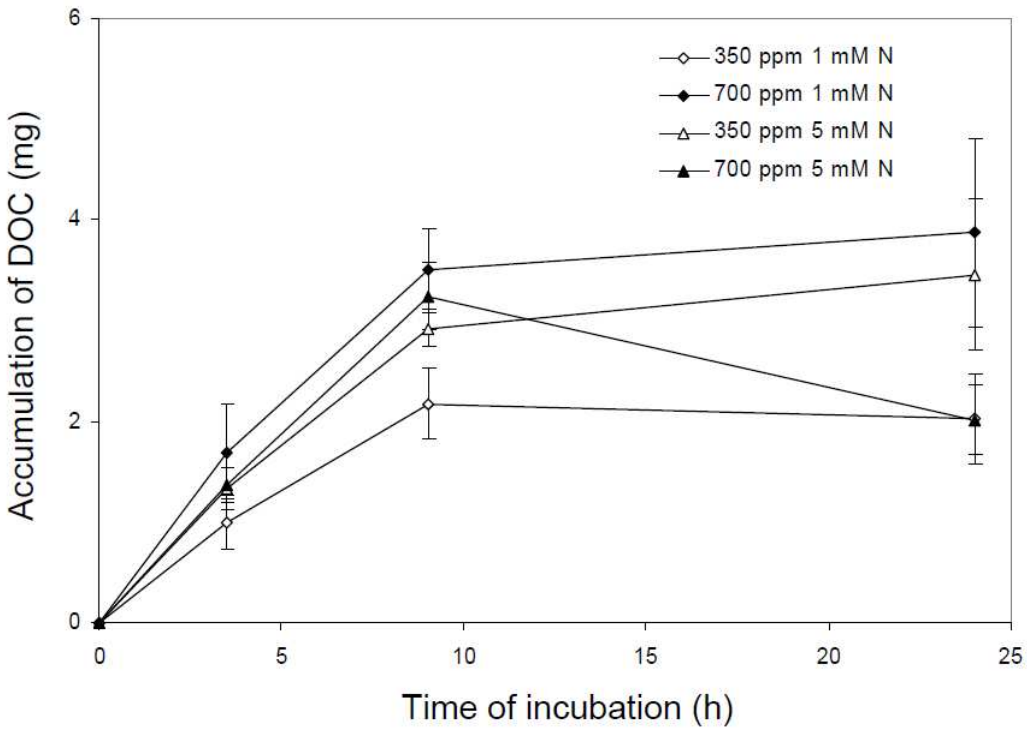
Figure 4. Magnitude of exudation by 145 day-old loblolly pine seedlings in response to two levels of CO_2 enrichment and N supply (experiment 2). In a), exudates collected from a subset of seedlings (n=12) during a 14h photoperiod and 10h non-photoperiod are scaled as a proportion of estimated biomass accumulation rates on the day of exudate collection (see methods). In b), mean exudation from each of the four treatments (n = 16) are plotted as a function mean light-

saturated net photosynthetic rates from a subset of seedlings (n=6). The regression line y = 0.068x - 0.193; $r^2 = 0.9511$; p = < 0.0001.

636 Figure 1.

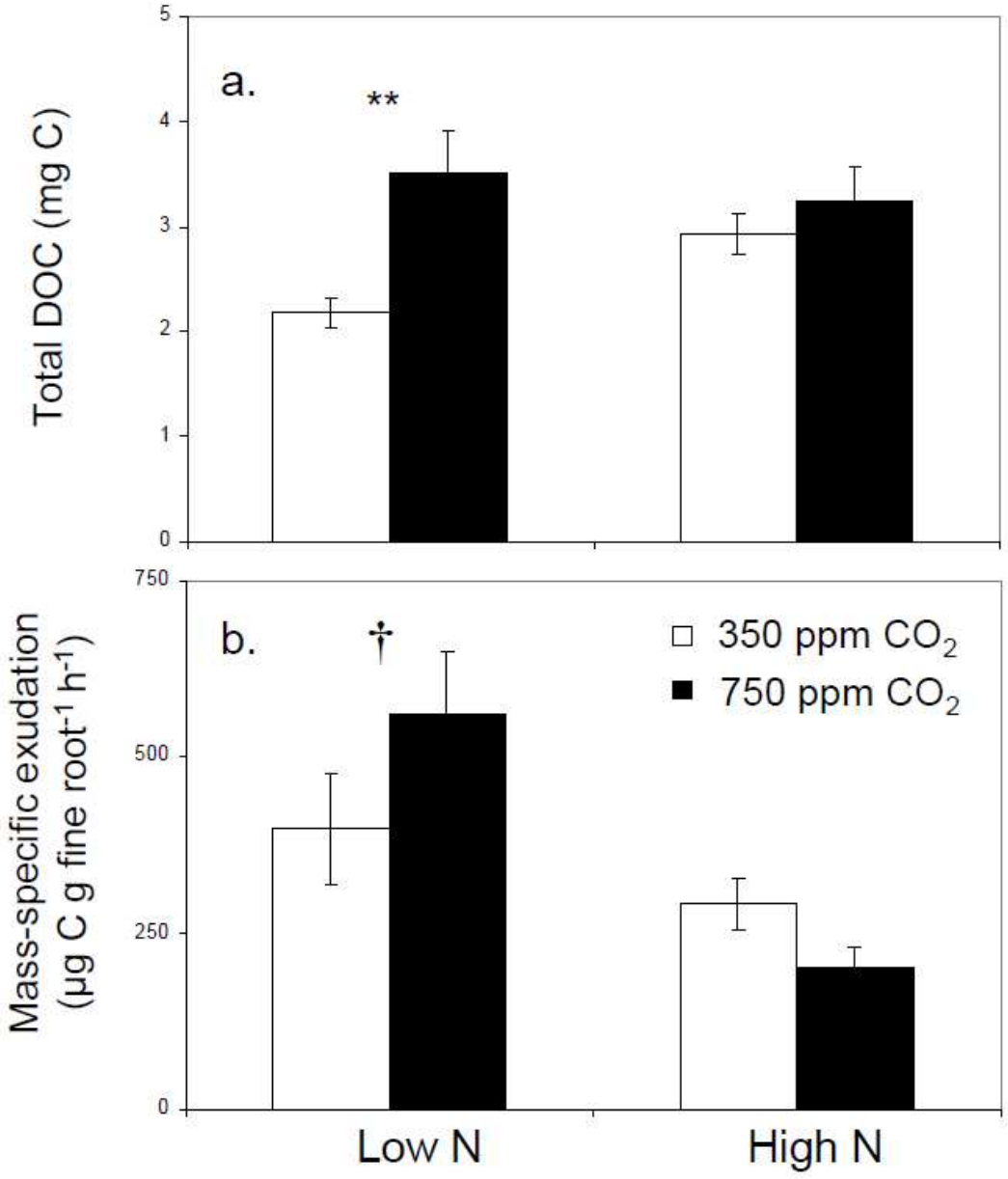
637

638



639

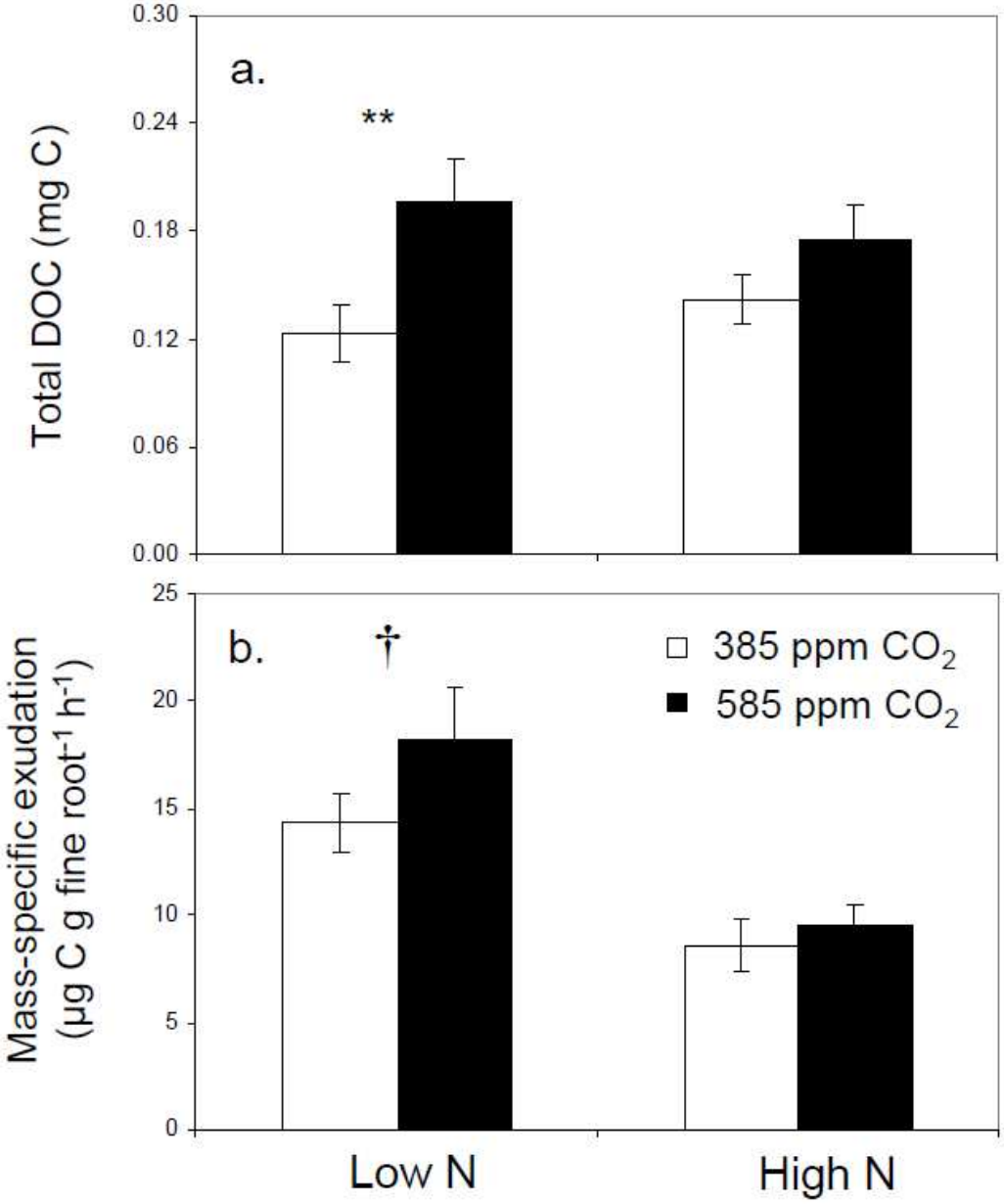
Figure 2.


a.
**
Total DOC (mg C)
5
4
3
2
1
0
b.
†
350 ppm CO2
750 ppm CO2
Mass-specific exudation
(µg C g fine root-1 h-1)
750
500
250
0
Low N
High N

644 Figure 3.

645

646



647

Figure 4.

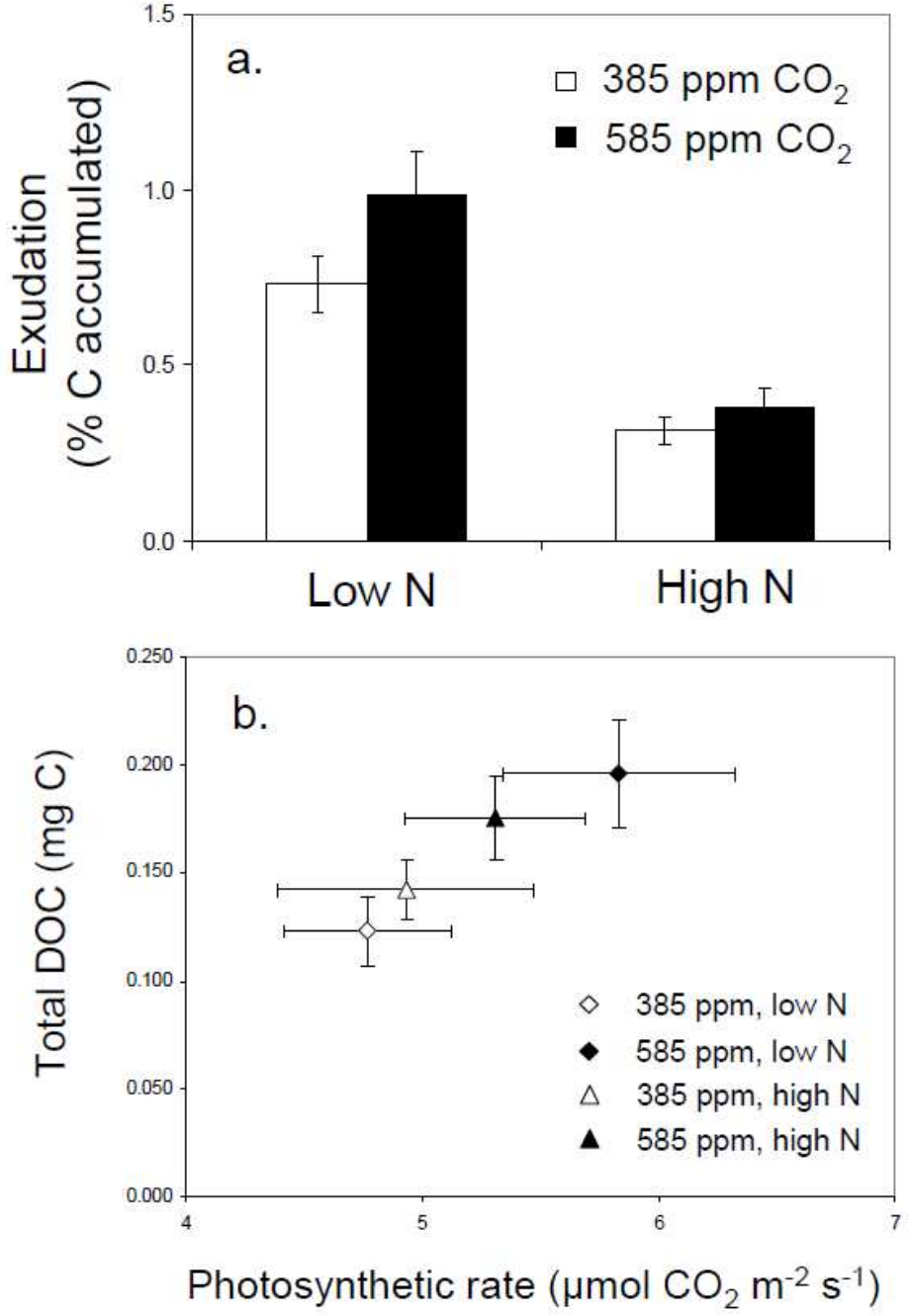

a.
385 ppm CO2
585 ppm CO2
Exudation
(% C accumulated)
1.5
1.0
0.5
0.0
Low N
High N
b.
Total DOC (mg C)
0.250
0.200
0.150
0.100
0.050
0.000
4
5
6
7
385 ppm, low N
585 ppm, low N
385 ppm, high N
585 ppm, high N
Photosynthetic rate (µmol CO2 m-2 s-1)

Table 1. Effects of elevated CO_2 (ppmv) and N (mmol N L^{-1}) on biomass (g) of loblolly pine seedlings grown in sand for 100 d in experiment 1. Values are means and standard errors (n = 10).

	Low N		High N	
	350 ppm	**700 ppm**	**350 ppm**	**700 ppm**
Shoot (g)				
Foliage	0.93 (0.15)	0.91 (0.14)	3.97 (0.73)	6.05 (0.69)
Stem	0.28 (0.05)	0.21 (0.03)	0.96 (0.12)	1.44 (0.15)
Root (g)				
>2mm	0.12 (0.03)	0.10 (0.02)	0.51 (0.07)	1.11 (0.2)
<2mm	0.15 (0.02)	0.20 (0.06)	0.36 (0.07)	0.65 (0.08)
Root:shoot	0.66 (0.18)	0.64 (0.09)	0.31 (0.02)	0.34 (0.02)

Table 2. Effects of elevated CO_2 (ppmv) and N (mmol N L^{-1}) on biomass (g) and root morphology of loblolly pine seedlings grown in sand for 150 days in experiment 2. Values are means and standard errors (n = 16 for aboveground biomass; n = 12 for root variables).

	Low N		High N	
	385 ppm	**585 ppm**	**385 ppm**	**585 ppm**
Shoot biomass (g)				
Foliage	1.38 (0.22)	1.43 (0.20)	3.76 (0.41)	4.02 (0.51)
Stem	0.29 (0.06)	0.37 (0.06)	1.23 (0.21)	1.19 (0.18)
Root biomass (g)				
>2mm	0.14 (0.05)	0.2 (0.04)	0.44 (0.07)	0.54 (0.07)
<2mm	0.94 (0.12)	1.23 (0.17)	1.78 (0.22)	2.05 (0.20)
Root:shoot biomass	0.68 (0.06)	0.77 (0.05)	0.43 (0.05)	0.50 (0.04)
Root morphology				
SRL (g cm^{-1})	1059 (142)	1082 (69)	1051 (167)	948 (104)
Surface area (cm^{-2})	352 (42)	501 (69)	589 (75)	749 (89)
Tips (number)	3277 (605)	3990 (554)	4885 (753)	5285 (865)

Table 3. Effects of elevated CO_2 and N on loblolly pine root exudation (mg C), net C assimilation (mg C), and the proportion of exuded C (% of net assimilated C). All pine seedlings (n = 16) were incubated for 10h in glass bead solution culture (experiment 2).

	Exudation (mg C)	**Net C assimilation (mg C)**	**Exudate flux (% of C assim.)**
Low N			
385 ppm CO_2	0.12	16.3	**0.76**
585 ppm CO_2	0.20	20.4	**0.96**
High N			
385 ppm CO_2	0.14	52.9	**0.27**
585 ppm CO_2	0.18	59.7	**0.29**