



**Elevated CO<sub>2</sub> increases root exudation from loblolly pine (*Pinus taeda* L.) seedlings as an N-mediated response**

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5 **Elevated CO<sub>2</sub> increases root exudation from loblolly pine (*Pinus***  
6 ***taeda* L.) seedlings as an N-mediated response**  
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16 CO<sub>2</sub> and N effects on root exudation  
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56 **4 Figures; 3 Tables**  
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1 **Summary** The degree to which forest ecosystems provide a long-term sink for increasing  
2 atmospheric CO<sub>2</sub> likely depends upon the capacity of trees to increase the availability of growth  
3 limiting resources. It has been widely speculated that trees exposed to CO<sub>2</sub> enrichment may  
4 increase the release of root exudates to soil as a mechanism to stimulate microbes to enhance  
5 nutrient availability. As a first test to examine how atmospheric CO<sub>2</sub> and nitrogen availability  
6 affect rates of root exudation, we conducted two experiments in which exudates were collected  
7 from loblolly pine (*Pinus taeda* L.) seedlings grown in controlled growth chambers under low  
8 and high CO<sub>2</sub> and at low and high rates of N supply. Despite differences in experimental design  
9 between the two studies, plants grown at high CO<sub>2</sub> were larger and thus whole plant exudation  
10 rates were higher under elevated CO<sub>2</sub> ( $p = 0.019$ ), but the magnitude of this response depended  
11 on the N level in both studies. Seedlings increased mass specific exudation rates in response to  
12 elevated CO<sub>2</sub> in both experiments, but only at low N supply. Moreover, N supply had a greater  
13 impact on exudation rates than did CO<sub>2</sub>, with mass-specific exudation rates significantly greater  
14 (98% and 69% in experiments 1 and 2, respectively) in seedlings grown at low N supply relative  
15 to high N supply. These results provide preliminary evidence that loblolly pines alter exudation  
16 rates in response to both CO<sub>2</sub> concentration and N supply, and support the hypothesis that  
17 increased C allocation to root exudates may be a mechanism by which trees can delay  
18 progressive N limitation in forested ecosystems.

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

## 20 Introduction

21 Aggrading forest ecosystems in northern latitudes currently represent a large sink for  
22 atmospheric CO<sub>2</sub> on a global basis (IPCC 2007). Understanding the factors which regulate forest  
23 productivity and their role in global C cycling is central to understanding potential feedbacks to  
24 climate change. In most temperate forests, productivity is nutrient limited. Thus, the long-term  
25 capacity of forests to sequester C likely depends on the degree to which trees allocate resources  
26 to acquire growth-limiting nutrients such as nitrogen (N) (Reich et al. 2006; Zak et al. 2003).  
27 Numerous studies have reported increased fine root production in trees exposed to elevated CO<sub>2</sub>  
28 (Lukac et al. 2003; Mikan et al. 2000; Norby et al. 2004; Pritchard et al. 2008), indicating that  
29 trees may be using the “extra” C to forage for N in soil. Yet exploiting a larger volume of soil  
30 via increased root production may have little affect on total N uptake if the majority of N in soil  
31 is in unavailable (e.g. organic) forms. Hence, in forest soils where inorganic N concentrations  
32 are low, increased soil exploration under elevated CO<sub>2</sub> will likely only increase N acquisition if  
33 microbes are stimulated to transform N into available forms (Phillips 2007).

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

45 Despite the perceived importance of exudation to ecosystem function (Cheng 1999;  
46 Rogers et al. 1994), there have been remarkably few measurements of exudation from trees  
47 exposed to elevated CO<sub>2</sub> (reviewed in Grayston et al. 1996; Jones et al. 2004). In the few studies  
48 where such measurements have been made, the results have been variable and inconclusive.  
49 Norby et al. (1987) reported that elevated CO<sub>2</sub> stimulated exudation in *Pinus enchinata* seedlings

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3 50 after 34 weeks of growth but had no effects after 41 weeks of growth. Delucia et al. (1997)  
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5 51 found that *Pinus ponderosa* seedlings exposed to elevated CO<sub>2</sub> increased exudation of some  
6  
7 52 compounds (e.g. oxalic acid) but decreased exudation of others (e.g. phosphatase enzymes). And  
8  
9 53 Uselman et al. (2000) reported that although elevated CO<sub>2</sub> increased exudation in *Robinia*  
10  
11 54 *pseudoacacia* seedlings, such changes resulted from increases in root biomass rather than  
12  
13 55 increases in mass-specific exudation rates.

14 56 An important mediator of the belowground responses of trees to elevated CO<sub>2</sub> is N  
15  
16 57 availability (Cheng 1999). Carbon allocation in trees is generally believed to be controlled  
17  
18 58 equally by shoots and roots (Farrar et al. 2003) until a functional equilibrium is established  
19  
20 59 between C assimilation and nutrient acquisition (Thornley 1977). Thus, the exudation response  
21  
22 60 of tree roots to elevated CO<sub>2</sub> should depend, in part, on N supply. In soils where N supply is  
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24 61 low, exudation rates may increase under elevated CO<sub>2</sub> if greater belowground C allocation is  
25  
26 62 triggered by nutritional stress in the plant (Farrar and Jones 2000). Alternatively, CO<sub>2</sub> effects on  
27  
28 63 exudation may be lower in nutrient-poor soils if total C assimilation rates (and by extension,  
29  
30 64 belowground C allocation) are reduced by nutritional constraints.

31 65 Here we report the results of two independent growth chamber experiments designed to  
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33 66 examine whether N supply affects the root exudation response of loblolly pine seedlings to  
34  
35 67 variable levels of CO<sub>2</sub>. This is the first study, to our knowledge, to examine the interactive  
36  
37 68 effects of elevated CO<sub>2</sub> and N on tree root exudation across a range of growth conditions. We  
38  
39 69 expected root exudation rates would increase under elevated CO<sub>2</sub> but hypothesized that the  
40  
41 70 magnitude of the CO<sub>2</sub> effect would be greatest under lower N availability. Experimentally  
42  
43 71 demonstrating that the interaction between CO<sub>2</sub> and N is a significant driver of root exudation  
44  
45 72 rates in seedlings represents an obvious first step in supporting the hypothesis that trees will  
46  
47 73 allocate more C to soluble exudates when N limits growth.

48

## 49 **Materials and Methods**

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51 76 Two experiments were performed, utilizing similar experimental treatments but different  
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53 77 exudate collection methods. In both experiments pine seedlings were grown from seed in sand  
54  
55 78 under controlled growth conditions. In experiment 1 pine seedlings were transplanted from sand  
56  
57 79 into hydroponic solution culture in order to measure exudation rates. In experiment 2, seedlings  
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3 80 were transplanted from sand into unsaturated glass bead culture for exudate capture. Experiment  
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5 81 1 utilized methods common to many previously reported exudate studies, while experiment 2  
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7 82 was intended to more closely approximate the conditions of soil-grown plants (e.g. mechanical  
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9 83 impedance of root growth, low nutrient conditions, etc.). Our primary goal was to determine  
10  
11 84 whether the treatment effects were qualitatively consistent between the two studies, and  
12  
13 85 secondarily to estimate how artifacts inherent in each experimental design might affect  
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15 86 quantitative estimates of root exudation rates.

### 17 87 *Experiment 1 – solution culture*

#### 20 88 *Growing conditions*

21  
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24 89 Loblolly pine (*Pinus taeda* L.) seeds collected from the North Carolina piedmont (Weyerhaeuser  
25  
26 90 Co., WA) were cold-stratified at 4° C for 30 days, planted in steam-sterilized sand (three seeds  
27  
28 91 per pot), and inoculated with the mycorrhizal fungus *Pisolithis tinctorius* (Plant Health Care Inc.,  
29  
30 92 Pittsburgh, PA). Each pot (3.5 L) was randomly assigned to one of two N treatments and one of  
31  
32 93 two atmospheric CO<sub>2</sub> treatments (n = 20 pots per treatment). Two weeks after seedling  
33  
34 94 emergence, all pots were “thinned” so that each container contained a single seedling.

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36 95 All pots were distributed between four growth chambers at the Duke University  
37  
38 96 Phytotron, NC: two maintained at approximately ambient CO<sub>2</sub> (350 ppm by volume) and two at  
39  
40 97 2x ambient CO<sub>2</sub> (700 ppm by volume). In-line CO<sub>2</sub> scrubbers were used to maintain levels in the  
41  
42 98 350 ppm chambers. A 14-h diurnal photoperiod was maintained in each chamber using a  
43  
44 99 combination of incandescent and high intensity discharge lamps. Light levels averaged 535  
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46 100  $\mu\text{mol m}^{-2}\text{s}^{-1}$  during the photoperiod with the exception of a 2-h “mid-day” period where levels  
47  
48 101 were increased to 1075  $\mu\text{mol m}^{-2}\text{s}^{-1}$ . Chamber temperature and relative humidity averaged 25° C  
49  
50 102 and 85%, respectively during the photoperiod, except during the 2-h peak period where  
51  
52 103 temperature and humidity were maintained at 28° C and 75%. Each week the CO<sub>2</sub> treatment was  
53  
54 104 assigned at random to two chambers and the seedlings were moved accordingly to minimize  
55  
56 105 chamber effects. In addition, the distribution of seedlings within each chamber was rotated  
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59  
60 106 weekly. The growth period lasted 100 days.

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3 107 All pots were watered to saturation each day with a modified Hoagland's solution (1/2  
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5 108 strength; pH = 6.1) with one of two N concentrations (1 and 5 mM N; hereafter low N and high  
6  
7 109 N, respectively). Nitrogen was added in the form of  $\text{NH}_4\text{NO}_3$ , and these concentrations were  
8  
9 110 chosen to represent a gradient in N availability in loblolly pine seedlings from sub-optimal to  
10  
11 111 supra-optimal based on previous growth chamber studies (Griffin et al. 1997; Griffin et al. 1993;  
12  
13 112 Larigauderie et al. 1994). All pots were watered in the evening with distilled water to prevent  
14  
15 113 salt accumulation in the pots (BassiriRad et al. 1997; Delucia et al. 1997; Uselman et al. 2000).  
16  
17 114 A layer of pea gravel (~3 cm thickness) was added on the sand surface after ~1 month to reduce  
18  
19 115 evaporative water loss and minimize algal growth.

### 20 21 116 *Exudate collection*

22  
23  
24 117 One hundred days after germination, a subset of pots was randomly chosen for exudate  
25  
26 118 collection (10 per treatment). Seedlings were carefully removed from their pots and rinsed with  
27  
28 119 distilled water to remove any sand adhering to roots. Seedlings were transplanted into 1-L  
29  
30 120 containers containing a modified Hoagland's solution of the same concentration of N as their  
31  
32 121 watering solution (1 or 5 mM N), and returned to their respective growth chambers for seven  
33  
34 122 days to overcome the transplant shock (Norby et al. 1987). Each container was aerated  
35  
36 123 continuously with an aquarium airstone and bubbler, and received fresh nutrient solution every  
37  
38 124 other day.

39  
40 125 Seven days after being transferred into solution culture, seedlings were placed into clean  
41  
42 126 Mason jars filled with 800 mL of sterile, aerated nutrient solution (same N concentrations) and  
43  
44 127 returned to their respective growth chambers. Because the roots were submerged in a static  
45  
46 128 bathing solution for the duration of the incubation, we collected subsamples from each jar (~30  
47  
48 129 mL) at three time intervals (4, 9 and 24h) to determine the equilibrium point where C efflux was  
49  
50 130 approximately balanced by reabsorption of exudate by the pine roots (Personeni et al. 2007;  
51  
52 131 Uselman et al. 2000). Based on these measurements, we concluded that exudation rates were  
53  
54 132 still increasing linearly until 9 hours in all treatments but declined between 9 and 24 hours  
55  
56 133 (Figure 1). Thus, net exudation rates were calculated as the change in dissolved organic carbon  
57  
58 134 (DOC) content in each jar between the beginning and end of the 9h incubation period. All  
59  
60 135 samples collected from the jars were filtered through 0.45- $\mu\text{m}$  membrane filters (Whatman Inc.,

1  
2  
3 136 Florham Park, NJ) and analyzed on a Tekmar-Dohrmann total organic carbon (TOC) analyzer  
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5 137 (Cincinnati, OH).  
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7

8 138 *Plant Harvest*  
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11 139 Following exudate collection, plants were harvested and separated into needles, stems, fine roots  
12 140 (<2 mm diameter), and coarse roots (>2 mm diameter). Fine roots were initially separated into  
13  
14 141 <1 mm and 1-2 mm diameter roots. However, we chose to pool these fractions based on the  
15  
16 142 abundance of borderline roots (~1 mm) and mycorrhizal tips which made separation of the two  
17  
18 143 categories sensitive to root-sorter bias. We did not quantify the percentage of roots colonized by  
19  
20 144 mycorrhizal fungi in this study, but previous studies show that up to 50% of the root tips of  
21  
22 145 *Pinus taeda* seedlings grown under similar conditions at this facility may have been colonized  
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24 146 (Lewis et al. 1994). All plant material was oven-dried at 60° C for at least 72h for determination  
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26 147 of dry weights.  
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29 148 *Experiment 2 – glass beads*  
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32 149 *Growing conditions*  
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36 150 Loblolly pine (*Pinus taeda* L.) seeds from a 1<sup>st</sup> generation orchard mix from the North Carolina  
37  
38 151 piedmont (NCSU seedlot #SOM8) were planted, inoculated and sown as described in experiment  
39  
40 152 1. All growing conditions were the same as in Exp. 1 with the exception of the N concentration  
41  
42 153 of the nutrient solution and the CO<sub>2</sub> concentration of the chambers which were changed to better  
43  
44 154 reflect the gradients that exist at the Duke Forest FACE site, NC. In experiment 2, we used a  
45  
46 155 modified Hoagland's solution containing either 0.5 or 1 mM N (all as NH<sub>4</sub>NO<sub>3</sub>; hereafter low N  
47  
48 156 and high N, respectively). The CO<sub>2</sub> concentration in the chambers of 385 ppmv and 585 ppmv  
49  
50 157 are the same as those at the Duke Forest site (Andrews et al. 1999). The growth period for this  
51  
52 158 experiment lasted 150 days.  
53

54 159 *Exudate collection*  
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3 160 One hundred forty days after planting, a subset of pots was randomly chosen for exudate  
4  
5 161 collection (n = 16 per treatment plus 6 non-vegetated controls). All seedlings were carefully  
6  
7 162 removed from their pots and rinsed with distilled water to remove any sand adhering to roots.  
8  
9 163 The entire root system of each seedling was then dipped into an anti-bacterial cocktail (50 ppm  
10  
11 164 streptomycin and 50 ppm penicillin) for 2-3 minutes to arrest rhizoplane bacterial growth. This  
12  
13 165 anti-biotic treatment was found to reduce exudate consumption in previous experimental trials  
14  
15 166 (data not shown). Following the antibiotic treatment, root systems were rinsed with water and  
16  
17 167 transferred into cylindrical PVC cores (20 cm length x 5 cm diameter) sealed on the bottom by  
18  
19 168 mesh (30  $\mu\text{m}$ ). Each core was carefully filled with sterile glass beads (~750  $\mu\text{m}$  diameter; La De  
20  
21 169 Da Designs, Baton Rouge, LA), such that roots were distributed evenly throughout the core.  
22  
23 170 Beads were used instead of quartz sand because preliminary experiments suggested strong  
24  
25 171 adsorption of low molecular weight organic acids by sand but not beads (data not shown). Cores  
26  
27 172 were then wrapped in aluminum foil to prevent light penetration, and foam bungs with a slit  
28  
29 173 removed to accommodate the plant stem were placed at the top of each core to prevent algal  
30  
31 174 growth and minimize evaporative loss. All seedlings were returned to their respective growth  
32  
33 175 chambers, and watered as before (i.e. nutrient solution in the morning and distilled water in the  
34  
35 176 evening).

36  
37 177 After a 7-d acclimation period, all seedlings were removed from the growth chambers  
38  
39 178 and flushed with 250 mL of reverse osmosis water (3 times the water-holding capacity of each  
40  
41 179 core) to remove accumulated DOC. All cores were watered with dilute C-free nutrient solution  
42  
43 180 and returned to their respective growth chambers for a 10-h incubation period. For the  
44  
45 181 incubation period, we used a nutrient solution 1/10<sup>th</sup> the concentration of the previous nutrient  
46  
47 182 solution (i.e. 50 and 100  $\mu\text{M}$  N). These concentrations were chosen to more closely resemble  
48  
49 183 concentrations in the soil solution draining the O horizon at the Duke Forest FACE site (Jackson  
50  
51 184 et al. *In Press*). After 10h, seedlings were removed from the growth chambers and flushed 3  
52  
53 185 times with 60 mL of distilled water. In addition to quantifying DOC efflux, we measured  
54  
55 186 nutrient uptake rates by seedlings over the 10-h incubation period using the nutrient depletion  
56  
57 187 method (Bassirirad et al. 1996). All leachate flushed from the cores was filtered through a 0.2-  
58  
59 188  $\mu\text{m}$  membrane filter (Millex GV, Millipore Co., Billerica, MA) and analyzed for non-particulate  
60

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3 189 organic C (for exudation rates) and total N (for uptake rates) on a TOC analyzer with TN module  
4  
5 190 (Shimadzu Scientific Instruments, Columbia, MD).  
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8  
9 191 *Plant Harvest and Photosynthesis*

10  
11 192 Plant tissues for all experimental seedlings were separated into the same fractions as described in  
12  
13 193 experiment 1. Total leaf area of each plant was measured on a 3000A portable area meter  
14  
15 194 (Lincoln, NE) from a subsample of needles (~1/3 of the total mass). Fine root length, surface  
16  
17 195 area and number of root and mycorrhizal tips were measured on a randomly selected subset of  
18  
19 196 fine roots (n = 10 per treatment) that were photocopied following the methods of Larigauderie et  
20  
21 197 al. (1994), and scanned and analyzed using WinRHIZO software (Regent Instruments, Inc.,  
22  
23 198 Canada). All plant material was oven-dried at 60° C for at least 72h for determination of dry  
24  
25 199 mass. On a subset of seedlings (n = 6 per treatment), photosynthetic rates were measured in a  
26  
27 200 conifer chamber attached to a Licor 6400 (Licor Co., Lincoln, NE). For each plant, rates were  
28  
29 201 measured on two different sections of the canopy, under saturating light conditions (>1000  $\mu\text{mol}$   
30  
31 202  $\text{m}^{-2} \text{s}^{-1}$ ), and at both 385 and 585 ppmv in the leaf chamber.  
32

33 203 *Calculations*

34  
35  
36 204 Whole plant C assimilation rates for the 10-h incubation period were estimated by multiplying  
37  
38 205 the average photosynthetic rates (within each N treatment and at a given CO<sub>2</sub> level) by the total  
39  
40 206 needle area of each plant. To account for non-saturating light conditions during the 10-h  
41  
42 207 incubation (average 530  $\mu\text{mol} \text{m}^{-2} \text{s}^{-1}$ ), total C gain during the photoperiod was calculated as a  
43  
44 208 weighted mean where photosynthetic rates under non-saturating light conditions were assumed  
45  
46 209 to be 75% of those under saturating light based on light curves of loblolly pine seedlings grown  
47  
48 210 under similar growth chamber conditions and N levels (Zhang et al. 1997). To independently  
49  
50 211 verify these estimates of whole plant C assimilation during the 10-h incubation period, we also  
51  
52 212 calculated the amount of whole plant C gain for the day of the exudate collection (i.e. day 145)  
53  
54 213 using exponential growth equations developed for loblolly pine seedlings growing under nearly  
55  
56 214 identical elevated CO<sub>2</sub> and N levels at the Duke Phytotron (Griffin et al. 1993). These  
57  
58 215 calculations yield a C gain of 36 and 93 mg C during the 24-h period of exudate collection in the  
59  
60 216 low and high N treatments, respectively, and suggest that our estimated rates of C gain during the

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3 217 10-h incubation (18 and 57 mg C in the low and high treatments) represent a reasonable  
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5 218 approximation of total C gain during the photoperiod.  
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8  
9 219 Exudation rates were scaled in two ways. First, whole plant exudation ( $\text{mg C plant}^{-1}$ ) was  
10  
11 220 calculated as a percentage of the C gain over the 10-h incubation period. In the second  
12  
13 221 calculation, the amount of C released via exudation was divided by whole plant C accumulation  
14  
15 222 per day ( $\text{mg C d}^{-1}$ ) by measuring exudation rates on a subset of loblolly pine seedlings ( $n = 6$ ;  
16  
17 223 using the same methods as described in Exp. 2) during a 24-h period that included both light  
18  
19 224 (14h) and dark (10h) conditions in the chambers.  
20

### 21 225 *Statistics (both experiments 1 and 2)*

22  
23

24 226 Due to differences in the  $\text{CO}_2$  and N treatment levels, data for experiments 1 and 2 were  
25  
26 227 analyzed separately. The effects of  $\text{CO}_2$  and N treatments on plant biomass and exudation rates  
27  
28 228 (experiments 1 and 2) were examined by two-way analysis of variance (ANOVA). Because of  
29  
30 229 the potentially confounding effects of treatments on exudation rates via alteration of root surface  
31  
32 230 area to bathing solution volume ratios (see Discussion below),  $\text{CO}_2$  effects on exudation were  
33  
34 231 analyzed within a given N level only using linear contrasts. All non-normally distributed data  
35  
36 232 were transformed (using log or square root transformations) prior to analysis, and all data was  
37  
38 233 analyzed using JMP statistical software (v. 6, SAS Institute Inc., Cary, NC).  
39

## 40 234 **Results**

41  
42

### 43 235 *Experiment 1*

44  
45

46 236 In general, N treatments had a much greater stimulatory effect on plant biomass than did  $\text{CO}_2$ ;  
47  
48 237 total biomass at high N was nearly four-fold greater than at low N ( $p < 0.0001$ ; Table 1).  
49  
50 238 Similarly, the biomass of all shoot and root fractions was greater in the high N treatment relative  
51  
52 239 to the low N treatment ( $p < 0.0001$  for all), with the largest response occurring in the shoots (e.g.  
53  
54 240 five-fold greater foliar biomass at high N). The strong effects of N on biomass partitioning is  
55  
56 241 also reflected by the two-fold greater root:shoot ratios in the low N relative to high N treatments  
57  
58 242 ( $p < 0.0001$ ).  
59  
60

1  
2  
3 243 In contrast to the N treatments, most tissue fractions were unaffected by CO<sub>2</sub> treatments.  
4  
5 244 Only total root biomass was affected by CO<sub>2</sub>, increasing by 35% at elevated CO<sub>2</sub> (p = 0.046).  
6  
7 245 Consistent with previous studies with loblolly pine seedlings (Griffin, Thomas & Strain, 1993,  
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9 246 Larigauderie, Reynolds & Strain, 1994), CO<sub>2</sub> effects on shoot and root biomass were largely  
10  
11 247 mediated by N, with the greatest stimulation of shoot and root biomass occurring in the high N  
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13 248 treatment. CO<sub>2</sub> induced a 49% enhancement of total plant biomass at the high N level (p =  
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15 249 0.028) but had no effect on total biomass at the low N level (p = 0.808). This pattern was  
16  
17 250 evident in both aboveground and belowground tissues. Needle biomass was 52% greater at  
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19 251 elevated CO<sub>2</sub> at high N (p = 0.071) but unaffected by CO<sub>2</sub> at low N (p = 0.960). Similarly,  
20  
21 252 elevated CO<sub>2</sub> increased fine root biomass by 79% in the high N treatment (p = 0.036), but had no  
22  
23 253 effect on this fraction in the low N treatment (p = 0.985). Overall, root:shoot ratios were  
24  
25 254 unaffected by the CO<sub>2</sub> treatment as a whole (p = 0.405) or within each individual N level.

26 255 Although whole plant exudation was 36% higher under elevated CO<sub>2</sub> (p = 0.019), the  
27  
28 256 magnitude of this response depended on the N level (Figure 2a), as CO<sub>2</sub> effects on exudation  
29  
30 257 were significant at low N (61% stimulation; p = 0.006) but unaffected at high N (p = 0.593).  
31  
32 258 Exudation rates calculated per unit fine root biomass (mg C exuded g<sup>-1</sup> h<sup>-1</sup>), indicated that N but  
33  
34 259 not CO<sub>2</sub> significantly affected mass-specific rates in these seedlings (Figure 2b). Similar to  
35  
36 260 whole plant exudation, there was a significant CO<sub>2</sub> by N interaction (p = 0.025) on mass-specific  
37  
38 261 rates; however, CO<sub>2</sub> effects on mass-specific rates at low N were only marginally significant (p =  
39  
40 262 0.067).

## 41 263 *Experiment 2*

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44  
45 264 Despite appreciable differences in the CO<sub>2</sub> and N levels used in the two experiments, treatment  
46  
47 265 effects on total biomass in experiment 2 were similar to those in experiment 1, as N but not CO<sub>2</sub>  
48  
49 266 influenced biomass production. As in experiment 1, N strongly stimulated total biomass (Table  
50  
51 267 2) with increases in needle, stem, fine root and coarse root biomass (all with p < 0.0001).  
52  
53 268 Moreover, root:shoot ratios were significantly decreased at the high N level (p < 0.0001). Root  
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55 269 morphology was affected by N availability, with significantly greater fine root surface area (p <  
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57 270 0.0001) and higher numbers of fine root tips (p = 0.04) in the highest N treatment.

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3 271 Similar to experiment 1, there were no main effects of elevated CO<sub>2</sub> on pine shoot and  
4  
5 272 root biomass, nor were there any CO<sub>2</sub> x N interactions. The proportion of C allocated to roots  
6  
7 273 (i.e. root:shoot ratio) was 15% greater under elevated CO<sub>2</sub> but this response was not statistically  
8  
9 274 significant ( $p = 0.075$ ). Root morphology was mostly unaffected by elevated CO<sub>2</sub> although fine  
10  
11 275 root surface area of roots was increased by 33% ( $p = 0.033$ ) in the CO<sub>2</sub> treatments. Total N  
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13 276 uptake over the 10-h incubation period was unaffected by CO<sub>2</sub> but was increased significantly by  
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15 277 N ( $p = 0.002$ ).

16  
17 278 Consistent with the response in experiment 1, mass-specific exudation rates (mg C  
18  
19 279 exuded g<sup>-1</sup> h<sup>-1</sup>) were decreased by N ( $p < 0.0001$ ) and marginally reduced by elevated CO<sub>2</sub> ( $p =$   
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21 280 0.092; Figure 3b). Within the 0.5 mM N treatment, CO<sub>2</sub> resulted in a small but marginally  
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23 281 significant increase in mass-specific rates (28%;  $p = 0.082$ ). Whole plant exudation rates were  
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25 282 increased under elevated CO<sub>2</sub> by approximately the same magnitude as in experiment 1 (45%;  $p$   
26  
27 283 = 0.029 - Figure 3a) but unaffected by N ( $p = 0.922$ ). However, N level was important in  
28  
284 mediating the magnitude of CO<sub>2</sub> effects on mass-specific exudation – consistent with the  
29  
30 285 response in experiment 1 (Figure 3b). At low N, CO<sub>2</sub> resulted in a small but marginally  
31  
32 286 significant increase in mass-specific exudation (41%;  $p = 0.069$ ). At high N, mass-specific rates  
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34 287 were lower ( $p < 0.0001$ ), but did not influence the magnitude of CO<sub>2</sub> effects ( $p = 0.827$ ).

35  
36 288 Because changes in root morphology can also affect exudation rates, we calculated  
37  
38 289 exudation rates as a function of fine root surface area and the number of root and mycorrhizal  
39  
40 290 tips (data not shown). Neither area-specific rates nor exudation rates per number of root tips  
41  
42 291 were significantly affected by the main effects of elevated CO<sub>2</sub> ( $p = 0.520$  and 0.230,  
43  
44 292 respectively). However, similar to mass-specific rates, area-specific rates were strongly affected  
45  
46 293 by N treatments. For seedlings in the low N treatment, area-specific rates were 43% greater than  
47  
48 294 at high N ( $p=0.035$ ). The important role of N in mediating exudation is also suggested by the  
49  
50 295 relationship between fine roots and exudation within the low N but not the high N level. At low  
51  
52 296 N, there was a weak but significant correlation between fine root biomass and exudation ( $r =$   
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54 297 0.48;  $p = 0.003$ ; data not shown), as well as between fine root surface area and exudation ( $r =$   
55  
56 298 0.50;  $p = 0.01$ ). This is in contrast to patterns at high N where there was no significant

299 correlation between fine root biomass and exudation ( $r = 0.17$ ;  $p = 0.122$ ) or fine root surface  
300 area and exudation ( $r = 0.24$ ;  $p = 0.237$ ).

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

## 307 Discussion

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

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3 327 exudation rates and the internal nutrient status of loblolly pine seedlings (Jones et al. 2004;  
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5 328 Neumann and Romheld 2001).

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8 329 *CO<sub>2</sub> and N effects on exudation*

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11 330 An important compensatory adjustment by plants exposed elevated CO<sub>2</sub> is the preferential  
12 331 allocation of C to belowground tissues (Reich et al. 2006; Zak et al. 2003). Numerous growth  
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14 332 chamber and field experiments have reported increased belowground production in trees exposed  
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16 333 to CO<sub>2</sub> enrichment (Ainsworth and Long 2005; Curtis and Wang 1998; Pendall et al. 2004)  
17  
18 334 indicating that trees grown at high CO<sub>2</sub> are allocating more C to roots, presumably to forage for  
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20 335 growth-limiting nutrients.  
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23  
24 336 There have been several reports of CO<sub>2</sub>-induced increases in mass-specific exudation in  
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26 337 non-woody plants but few reports of this response in trees (reviewed in Cheng and Gershenson  
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28 338 2007). CO<sub>2</sub>-induced increases in exudation have been reported for seedlings of *Pinus ponderosa*  
29  
30 339 (Delucia et al. 1997), *Pinus enchinata* (Norby et al. 1987) and *Robinia pseudoacacia* (Uselman  
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32 340 et al. 2000) but in each case, the CO<sub>2</sub> effects were not significantly different when scaled per unit  
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34 341 of fine root biomass. In both experiments of this study, treatment effects on fine root biomass  
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36 342 (Tables 1, 2) were insufficient to explain greater exudation in seedlings grown at elevated CO<sub>2</sub>  
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38 343 and low N (Figure 2b, 3b). That the stimulatory effects of elevated CO<sub>2</sub> were only present in  
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40 344 plants grown at low N suggests that nutritional imbalances in the pines may have triggered the  
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42 345 exudation response. This would be consistent with previous studies reporting a greater  
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44 346 magnitude of belowground response of loblolly pine seedlings to elevated CO<sub>2</sub> under low  
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46 347 nutrient conditions (Griffin et al. 1997; Griffin et al. 1995; Larigauderie et al. 1994).

47 348 In addition to increased belowground C allocation, it is possible that greater exudation in  
48  
49 349 low N resulted from CO<sub>2</sub>- and N-induced changes in root architecture (Neumann and Romheld  
50  
51 350 2001). Darwent et al.(2003) reported that decreased exudation from *Hordeum vulgare* roots  
52  
53 351 resulted from reductions in root length and the numbers of root tips due to increased N supply.  
54  
55 352 In loblolly pine, root morphology has been reported to be responsive to changes in both CO<sub>2</sub> and  
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57 353 N (Larigauderie et al. 1994), but the consequences of these changes for exudation is unknown.  
58  
59 354 In experiment 2 of this study, root surface area and the numbers of root tips were greatest at high  
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3 355 N, resulting in decreased exudation. However, N-induced changes in root surface area and the  
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5 356 number of tips were similar in magnitude to changes in root biomass, and thus we cannot  
6  
7 357 conclude whether the N-induced decreases in exudation resulted from changes in C allocation or  
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9 358 changes in root morphology. Moreover, it is also possible that the greater exudation at low N  
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11 359 resulted from increased membrane permeability of roots due to nutritional stress (Neumann and  
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13 360 Romheld 2001). Further examination of root exudation across a broader range of N levels and  
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15 361 with more detailed characterization of root morphology and physiology would be a worthwhile  
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17 362 focus of future studies.

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19 363         Given the strong effects of N in mediating belowground C allocation and root exudation  
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21 364 in this study, an obvious question arises: do loblolly pine seedlings increase exudation as a  
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23 365 response to N deficiencies? To date, most reports of exudation in response to nutrient stress  
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25 366 have been reported for plants under P and Fe deficiency or metal (e.g. Al) toxicity (Marschner  
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27 367 1995). In non-woody plants, mass-specific exudation rates have been shown to increase (Henry  
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29 368 2005; Paterson 2006), decrease (Darwent 2003; Hodge et al. 1996; Paterson et al. 1999; Werth  
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31 369 and Kuzyakov 2006) or vary (Neumann and Romheld 2001; Nguyen 2003) in response to N  
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33 370 amendment. In trees, Aitkenhead-Peterson et al. (2005) reported a three-fold increase in mass-  
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35 371 specific exudation from *Picea abies* saplings at low N. However, Uselman et al. (2000) found  
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37 372 two-fold greater mass-specific exudation from *Robinia pseudoacacia* seedlings grown at high N.  
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39 373 Collectively, these results indicate that our understanding of CO<sub>2</sub> and N effects on tree root  
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41 374 exudation is incomplete, and may limit our predict feedbacks to forest productivity under global  
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43 375 change (Giardina et al. 2005).

44 376         Exudation rates from experiment 2 in this study were both lower (Aitkenhead-Peterson  
45  
46 377 2005; Grayston et al. 1996; Norton et al. 1990) and higher (Norby et al. 1987; Uselman et al.  
47  
48 378 2000) than those reported for seedlings of other tree species. Because the magnitude of  
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50 379 exudation is highly sensitive to the experimental system used to collect the exudates (Phillips et  
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52 380 al. 2008), we cannot say whether our rates reflect true species differences. Perhaps a more  
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54 381 important question is whether exudation rates from seedlings can help inform what occurs in  
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56 382 forests where N concentrations in soil solution are generally much lower than those used in  
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58 383 growth chamber experiments (Lucash et al. 2005). Phillips et al. (2008) recently developed a  
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3 384 method to measure exudation rates from trees *in situ* using a modification of the glass bead  
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5 385 culture method used in this study. They reported exudation rates in 26 year-old loblolly pine  
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7 386 trees (~1% of net assimilated C) in close agreement with exudation rates from this study (0.3-  
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9 387 0.8% of net assimilated C). The paucity of direct exudation estimates from tree roots *in situ* in  
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11 388 response to changing environmental conditions suggests that seedling studies – despite their  
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13 389 obvious limitations – may be an important starting point for developing appropriate scaling  
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15 390 factors to estimate the potential contribution of exudates to soil processes in forests exposed to  
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17 391 elevated CO<sub>2</sub>. Results from our controlled studies of loblolly pine seedlings suggest that soluble  
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19 392 root exudates could represent a considerable belowground C flux, perhaps similar to fine root  
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21 393 turnover.

#### 22 394 *Experimental artifacts in exudation measurement*

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26 395 All methods used to measure exudation have inherent artifacts associated with their design  
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28 396 (Neumann and Romheld 2001), and thus we consider whether our methods may have influenced  
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30 397 exudation patterns. Although there was qualitative agreement between the two experiments, the  
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32 398 exudation rates we measured for seedlings in solution culture (experiment 1) were much higher  
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34 399 than those in glass beads (experiment 2). Our direct comparison of approaches provides an  
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36 400 important caution to attempts to scale from individual controlled laboratory studies to ecosystem-  
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38 401 scale processes. There are several possible reasons for measuring higher exudation rates in  
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40 402 hydroponic experiments than in unsaturated artificial soil incubations. First, the exudation rates  
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42 403 in experiment 1 may have been greater due to greater diffusion gradients between the root and  
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44 404 the solution. Basal exudation is highly sensitive to gradients in soluble C between the root  
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46 405 cytoplasm, apoplast and soil solution (Jones et al. 2004), and the gradient in solution culture  
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48 406 would presumably be greater than in non-saturated glass beads due to the intimate contact  
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50 407 between roots and solution. Second, because the roots in solution culture were fully submerged,  
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52 408 non-exudate higher molecular weight C compounds may have leached from roots. This problem  
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54 409 would have been exacerbated by the 5-fold difference in N supply between low and high N in  
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56 410 experiment 1 (which increased root biomass), and by the larger pore size used to filter exudates  
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58 411 in experiment 1 (0.45 μm) relative to experiment 2 (0.2 μm). And finally, roots in experiment 2  
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3 412 (but not experiment 1) were treated with antibiotics which may have decreased the release of  
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5 413 exudates if the antibiotics negatively affected the plant (Neumann and Romheld 2001).  
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9 414 Static bathing solutions, or hydroponic incubations, have often been used to measure  
10 415 exudation rates because they require little maintenance, use a lesser volume of solution than in  
11 416 percolating solutions, and permit the collection of exudates from the entire rooting volume rather  
12 417 than from preferential flow paths. Because exudates accumulate in the bathing solution, these  
13 418 systems need to be sampled during the incubation period to ensure C accumulation does not  
14 419 reduce exudation as the equilibrium between efflux and influx is approached (Uselman et al.  
15 420 2000). The nutrient content of static bathing solutions may also change during the course of the  
16 421 incubation due to nutrient uptake, thereby affecting exudation rates. In experiment 1, exudation  
17 422 increased linearly over the first nine hours of collection (Figure 1), suggesting that C  
18 423 accumulation in the bathing solution was unlikely to be affecting the gross efflux. Moreover, we  
19 424 found no evidence of differences in accumulation rates of exudates between low and high N  
20 425 treatments despite a two-fold greater root biomass suggesting that N effects on mass-specific  
21 426 rates were unlikely to have been the result of changes in the efflux and influx of exudates in the  
22 427 bathing solution (Personeni et al. 2007). Changes in nutrient content owing from N uptake were  
23 428 unlikely to have affected exudation patterns. In experiment 2, the N content of the post-  
24 429 incubation bathing solution was still greater in the high N (0.6 mg N) than in the low N (0.46 mg  
25 430 N) treatment. This suggests that it is unlikely that the changes occurring during the incubation  
26 431 period contributed to the observed exudation patterns.  
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42 432 Reabsorption of exudates has been reported to occur in several grass species (de Graaff  
43 433 2007; Phillips et al. 2006), but to our knowledge, there have been no reports of reabsorption by  
44 434 trees. Given the greater density of roots in experiment 2 relative to experiment 1, it is certainly  
45 435 possible that some fraction of exudates were re-acquired by roots during the incubation.  
46 436 However, in a separate experimental trial with loblolly pine, exudates accumulated linearly over  
47 437 the first eight hours in bead-filled cuvettes packed at similar densities to those used in  
48 438 experiment 2 (0.81 vs. 0.83 cm<sup>2</sup> root surface area per ml cuvette; Phillips, *unpublished data*),  
49 439 suggesting little effect of uptake on net C efflux. Moreover, it is unlikely that exudate re-uptake  
50 440 would have influenced treatment effects as amino acids and sugars – the primary compounds  
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3 441 taken up by roots – (Jones et al. 2004) generally occur in low concentrations in *Pinus* exudates  
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5 442 (Smith 1976).  
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9 443 Although we fully recognize the inherent limitations of our experimental systems, the  
10 444 qualitative agreement between the two experiments suggests that our results are robust with  
11  
12 445 respect to the direction of treatment effects.  
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## 14 15 446 **Conclusions**

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18 447 Numerous investigators have invoked root exudation as a locally missing sink for plant-derived  
19 448 C in elevated CO<sub>2</sub> experiments, but there has been little empirical work to support this  
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21 449 hypothesis. In this study, we have demonstrated in two independent experiments that elevated  
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23 450 CO<sub>2</sub> may increase root exudation in loblolly pine seedlings, but that such effects are sensitive to  
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25 451 the availability of N in the growing medium. Thus, the N status of pines may determine whether  
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27 452 additional C fixed under elevated CO<sub>2</sub> is released to soil as root exudates, possibly as a  
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29 453 mechanism for increasing N availability in soil. CO<sub>2</sub>-induced increases in exudation arose from  
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31 454 both increases in fine root biomass and from greater mass-specific exudation in the low N levels.  
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33 455 Despite differences in the rates between experiments, the relative effects of the treatments were  
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35 456 consistent, and thus provide evidence that the degree to which trees sequester C under elevated  
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37 457 CO<sub>2</sub> may depend on the magnitude and ecological consequences of changes in C released to soil  
38  
39 458 via root exudation.  
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43  
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## 20 612 **Figure legends**

21 613  
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23 614 Figure 1. Root exudate accumulation in static bathing solution culture over a 24 h period. Error  
24 615 bars for each of the four treatments represent one standard error of the mean (n = 10).  
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28 617 Figure 2. The effects of elevated CO<sub>2</sub> and N on a) the total quantity of root exudates released,  
29 618 and b) mass-specific exudation rates in 100 day-old loblolly pine seedlings (experiment 1). Error  
30 619 bars are standard errors of the mean (n = 10). Significant differences between ambient and  
31 620 elevated CO<sub>2</sub> (open and black bars, respectively) within an N level are noted by asterisks († = p  
32 621 < 0.10, \* = p < 0.05, \*\* = p < 0.01, and \*\*\* = p < 0.0001).  
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39 623 Figure 3. The effects of elevated CO<sub>2</sub> and N on a) the total quantity of root exudates released,  
40 624 and b) mass-specific exudation rates in 145 day-old loblolly pine seedlings (experiment 2). Error  
41 625 bars are standard errors of the mean (n = 10). Significant differences between ambient and  
42 626 elevated CO<sub>2</sub> within an N level are noted by asterisks († = p < 0.10, \* = p < 0.05, \*\* = p < 0.01,  
43 627 and \*\*\* = p < 0.0001).  
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49 629 Figure 4. Magnitude of exudation by 145 day-old loblolly pine seedlings in response to two  
50 630 levels of CO<sub>2</sub> enrichment and N supply (experiment 2). In a), exudates collected from a subset  
51 631 of seedlings (n=12) during a 14h photoperiod and 10h non-photoperiod are scaled as a proportion  
52 632 of estimated biomass accumulation rates on the day of exudate collection (see methods). In b),  
53 633 mean exudation from each of the four treatments (n = 16) are plotted as a function mean light-  
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3 634 saturated net photosynthetic rates from a subset of seedlings (n=6). The regression line  $y =$   
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5 635  $0.068x - 0.193$ ;  $r^2 = 0.9511$ ;  $p = < 0.0001$ .  
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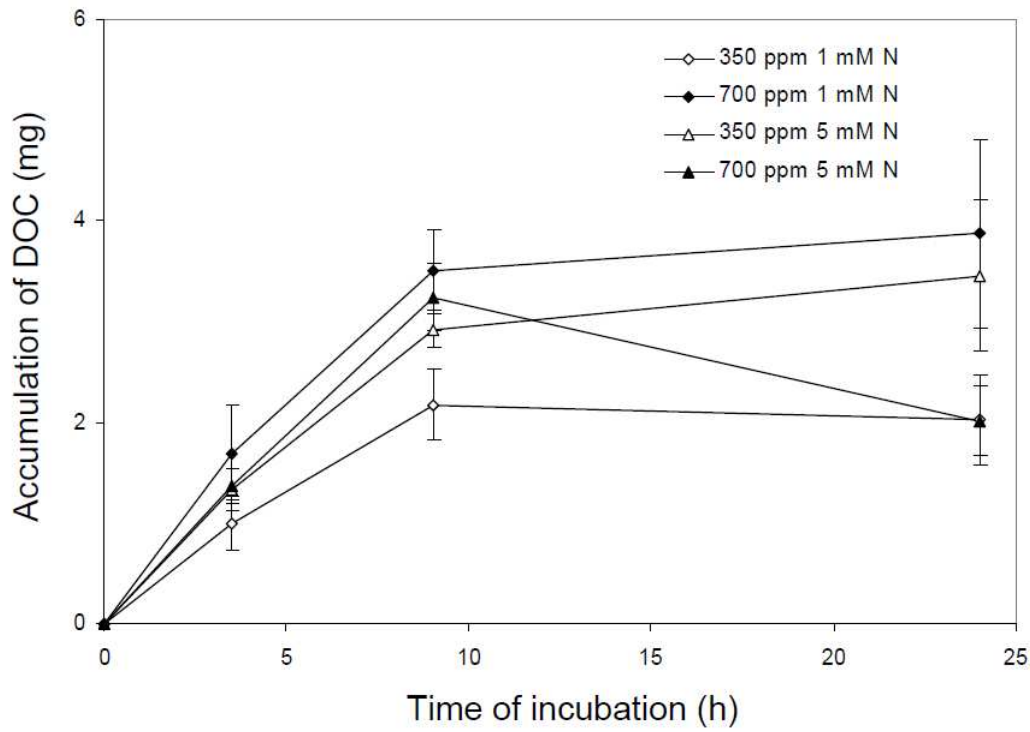
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636 Figure 1.

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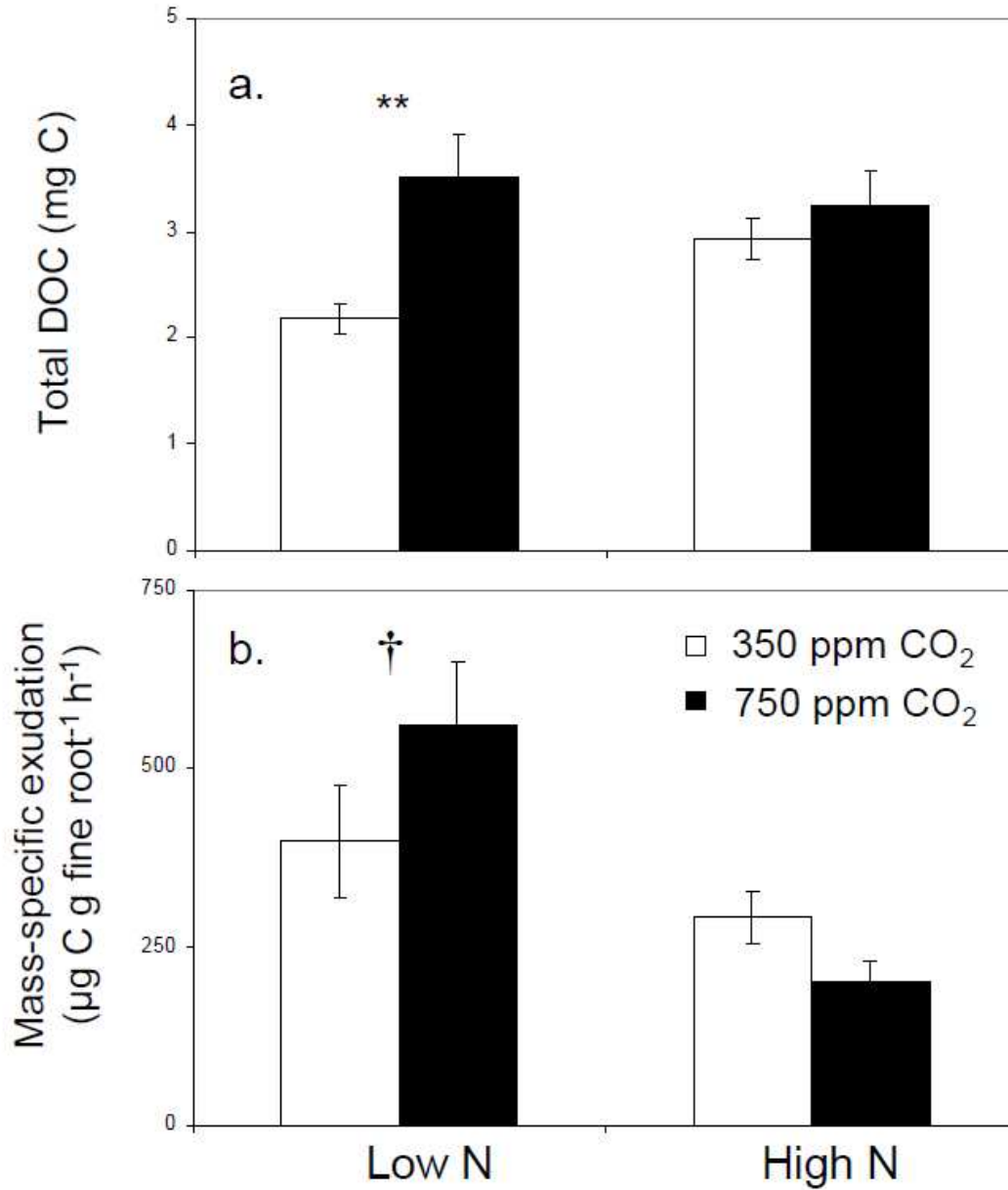
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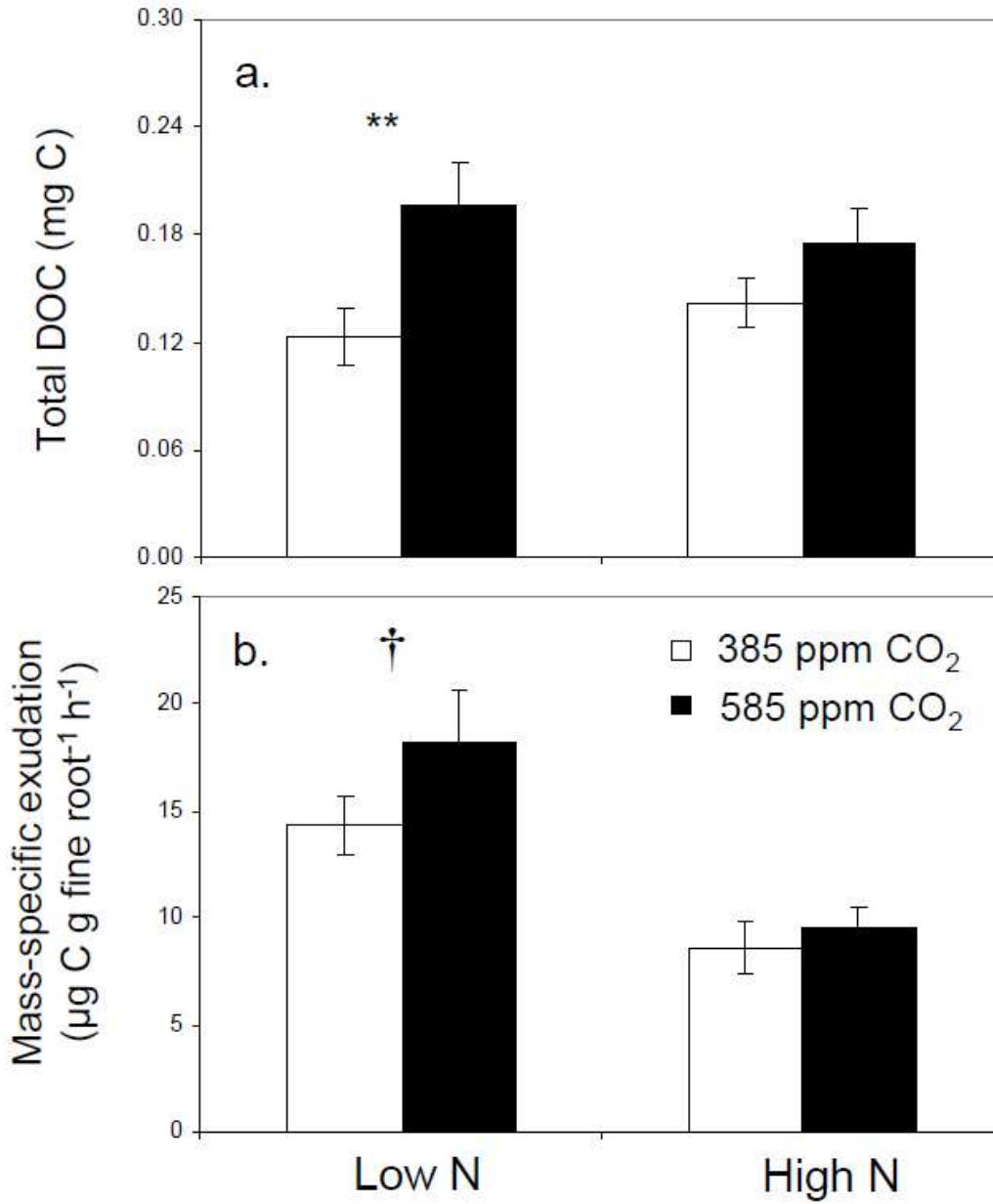


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644 Figure 3.

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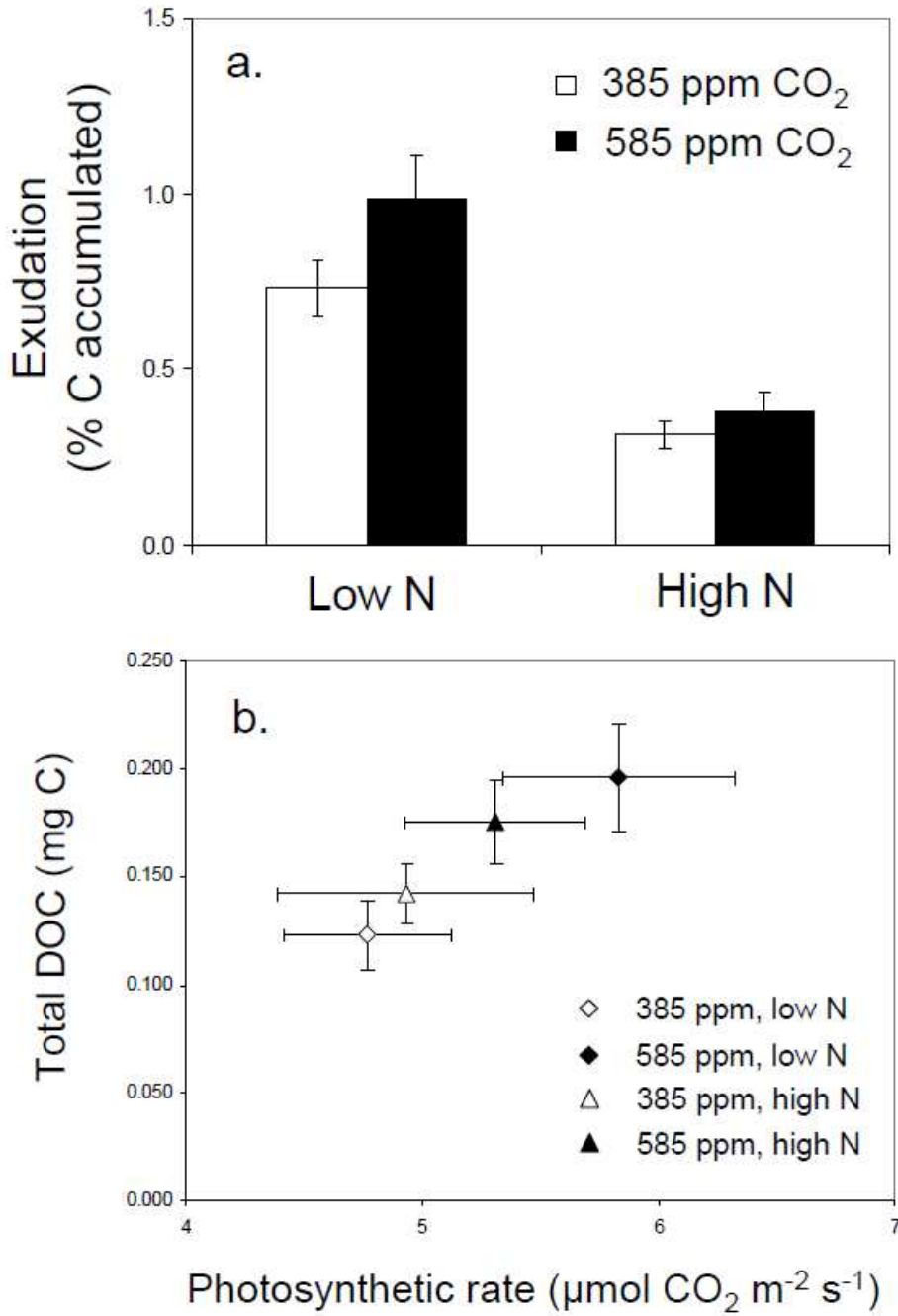


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648 Figure 4.

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3 652 Table 1. Effects of elevated CO<sub>2</sub> (ppmv) and N (mmol N L<sup>-1</sup>) on biomass (g) of loblolly pine  
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5 653 seedlings grown in sand for 100 d in experiment 1. Values are means and standard errors (n =  
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	Low N		High N	
	350 ppm	700 ppm	350 ppm	700 ppm
Shoot (g)				
<i>Foliage</i>	0.93 (0.15)	0.91 (0.14)	3.97 (0.73)	6.05 (0.69)
<i>Stem</i>	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)

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658 Table 2. Effects of elevated CO<sub>2</sub> (ppmv) and N (mmol N L<sup>-1</sup>) on biomass (g) and root  
 659 morphology of loblolly pine seedlings grown in sand for 150 days in experiment 2. Values are  
 660 means and standard errors (n = 16 for aboveground biomass; n = 12 for root variables).

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	Low N		High N	
	385 ppm	585 ppm	385 ppm	585 ppm
Shoot biomass (g)				
<i>Foliage</i>	1.38 (0.22)	1.43 (0.20)	3.76 (0.41)	4.02 (0.51)
<i>Stem</i>	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				
<i>SRL (g cm<sup>-1</sup>)</i>	1059 (142)	1082 (69)	1051 (167)	948 (104)
<i>Surface area (cm<sup>-2</sup>)</i>	352 (42)	501 (69)	589 (75)	749 (89)
<i>Tips (number)</i>	3277 (605)	3990 (554)	4885 (753)	5285 (865)

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

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	Exudation (mg C)	Net C assimilation (mg C)	Exudate flux (% of C assim.)
Low N			
385 ppm CO <sub>2</sub>	0.12	16.3	<b>0.76</b>
585 ppm CO <sub>2</sub>	0.20	20.4	<b>0.96</b>
High N			
385 ppm CO <sub>2</sub>	0.14	52.9	<b>0.27</b>
585 ppm CO <sub>2</sub>	0.18	59.7	<b>0.29</b>

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