Chemical and anatomical changes in *Liquidambar styraciflua* L. xylem after long term exposure to elevated CO_2

Keonhee Kim[a], Nicole Labbé[a,*], Jeffrey M. Warren[b], Thomas Elder[c], Timothy G. Rials[a]

[a] Center for Renewable Carbon, University of Tennessee, Knoxville, TN, USA

[b] Climate Change Science Institute and Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN, USA

[c] USDA-Forest Service, Southern Research Station, Pineville, LA, USA

[*] Corresponding author.

Address: Center for Renewable Carbon, University of Tennessee

2506 Jacob Drive, Knoxville, TN 37996-4750, USA.

Tel.: +1 865 946 1126; fax: +1 865 946 1109.

E-mail address: nlabbe@utk.edu (N. Labbé).

Abstract

The anatomical and chemical characteristics of sweetgum were studied after 11 years of elevated CO_2 (544ppm, ambient at 391ppm) exposure. Anatomically, branch xylem cells were larger for elevated CO_2 trees, and the cell wall thickness was thinner. Chemically, elevated CO_2 exposure did not impact the structural components of the stem wood, but non-structural components were significantly affected. Principal component analysis (PCA) was employed to detect differences between the CO_2 treatments by considering numerous structural and chemical variables, as well as tree size, and data from previously published sources (i.e., root biomass, production and turnover). The PCA results indicated a clear separation between trees exposed to ambient and elevated CO_2 conditions. Correlation loadings plots of the PCA revealed that stem structural components, ash, Ca, Mg, total phenolics, root biomass, production and turnover were the major responses that contribute to the separation between the elevated and ambient CO_2 treated trees.

Capsule: Elevated CO_2 at atmosphere did not impact the structural components yet altered some of non-structural components and anatomical properties after 11 years of exposure on sweetgum.

Keywords: Free Air CO_2 enrichment, sweetgum, chemical composition, hydraulic conductivity, PCA

1. Introduction

Globally, the mean atmospheric carbon dioxide level has risen steadily since pre-industrial times, which is largely attributable to human activities such as increased emissions from fossil fuel burning and clearing of forests. Implications of elevated CO_2 on ecosystems have been largely limited to mechanistic models or to studies on seedling and small trees in growth chambers until the development of new technology extended experiments to intact ecosystems. Free Air CO_2 Enrichment (FACE) studies were designed to address long-term responses of ecosystems to elevated level of atmospheric CO_2 through targeted release of air enriched with CO_2 into the plant canopy.

Little is known about the potential impacts of elevated CO_2 on xylem structural properties, which complement stomatal dynamics to provide additional controls on water flux through plants. A change in xylem anatomy such as lumen diameter (Tyree et al., 1994) or scalariform perforation plate bar thickness (Schulte, 1999) would affect resistance to water flux and thereby hydraulic conductivity. In birch and oak seedlings, elevated CO_2 was found to reduce water flux and leaf specific conductivity (Eguchi et al., 2008) which directly suggests reduced stem hydraulic capacity as was exhibited in beech trees (Overdieck et al., 2007). Elevated CO_2 has been shown to affect xylem cell size, increasing cell size in *Larix decidua* (Handa et al., 2006) and dogwood (Domec et al., 2010), but reducing cell size in *Picea abies* (Kostiainen et al., 2004) and *Fagus sylvatica*, (Overdieck et al., 2007), illustrating the species specificity of response. Thicker cell walls have been seen in *Pinus sylvestris* exposed to elevated CO_2 (Kilpelainen et al., 2007), but thinner cells developed when elevated temperature and CO_2 treatments were applied together, illustrating the complexity of response.

Along with the atmospheric CO_2 concentration, soil nutrient status has also been shown to be a crucial factor to the plant growth (Norby et al., 2010; Oren et al., 2001). At the Oak Ridge National Laboratory's (ORNL) FACE site and other forest FACE sites, elevated CO_2 initially enhanced stem wood growth by ~25 % (Norby et al., 2005). However, with limited soil N availability on this site, net primary productivity (NPP) was reported to gradually decrease over the course of the study (Norby et al., 2010). Nutrient balance is also important for differentiation of photosynthate, yet several studies showed no significant differences in the amount of total extractives with elevated CO_2 treatment, although the concentration of proteins and mineral nutrients decreased and the lipid compositions were altered (DaMatta et al., 2010; Kilpelainen et al., 2005; Kilpelainen et al., 2003). Phenolic compounds of plants grown under elevated CO_2 condition have been also studied (Ghasemzadeh et al., 2010; Johnson and Pregitzer, 2007; Penuelas et al., 1996). Non-structural sugars and starch content of CO_2 enriched xylem from several forest FACE sites increased by 30-40 % compared to the control samples (Ainsworth and Long, 2005). Kaakinen et al. (2004) also reported that soluble sugars and starch concentration increased with 3 years of CO_2 treatment in the Aspen FACE site.

In general, the primary effects of elevated CO_2 concentration on trees include higher rates of photosynthesis, enhanced water use efficiency, enhanced productivity, and alteration of secondary metabolites. Such physiological changes may lead to changes in chemical composition of the different parts of trees including leaves, xylem (wood), phloem (bark), and roots. However, as it was pointed out earlier, most of the previous studies were conducted on samples from controlled environments (potted plants or open-top chamber) and some of the previous FACE research was conducted on juvenile wood. The findings could differ with the age of the trees since the anatomical, physical, and chemical properties of juvenile wood are significantly

different from that of mature wood (Haygreen and Bower, 1996), suggesting changes in wood chemistry due to elevated CO_2 could also be different between juvenile and mature wood. A recent study by Kostiainen et al. indicated that short-term impact studies, conducted with young seedlings, may not give a realistic view of long-term tree responses (Kostiainen et al., 2014). We expanded on the initial study of wood properties after 11 years of treatment to look at long term responses following the progressive reduction in NPP exhibited in later years at the site. The present study on sweetgum grown under long term exposure of elevated CO_2 at the ORNL FACE site could provide a better understanding of tree responses to higher atmospheric CO_2 in a natural environment.

The goal of this research was to investigate the effects of long-term (11-years) application of elevated CO_2 in the atmosphere on the anatomical and chemical changes of the xylem part of sweetgum (*Liquidambar styraciflua* L.) from the ORNL FACE site. The first objective was to compare anatomical differences of branch xylem growing under elevated and ambient CO_2. Analyses included xylem anatomical measurements such as hydraulic mean diameter, double cell wall thickness, and area of largest cell – characteristics important for tree vigor under drought stress. The second objective was to compare chemical changes of the xylem growing under elevated and ambient CO_2. Analyses included comparison of structural and non-structural chemical composition – characteristics important for wood quality and recalcitrance. The amount of cellulose, hemicellulose, and lignin, which are the main structural components of xylem tissues, was quantified. The non-structural components analyzed included ash, extractives, and macronutrients. Lastly, the sensitivity of the physical and chemical tree responses under elevated CO_2 was investigated using multivariate principal component analysis (PCA). Based on the results from short-term CO_2 experiments, or FACE experiments on young trees, we

hypothesized that the older sweetgum trees exposed to FACE treatments would show little change in wood composition. The contribution of this study may improve the understanding of a tree's anatomical and chemical responses under long term exposure of elevated CO_2.

2. Materials and Methods

2.1 Materials

Sweetgum (*Liquidambar styraciflua* L.) branch and stem material was harvested from the Oak Ridge FACE site located in the Oak Ridge National Environmental Research Park in eastern Tennessee, USA (35˚54'N; 84˚20'W). The Oak Ridge FACE site consisted of five 25m-diameter plots (rings), with vertical PVC pipes releasing CO_2-enriched or ambient air. Among those five rings, two were elevated CO_2 rings (targeting 550 ppm and measured 544 ppm) and three ambient CO_2 rings (measured at 391 ppm, two surrounded by the FACE structure and a third ambient CO_2 plot without structure). One-year old sweetgum trees were planted on the site in 1988 and the CO_2 treatment was initiated 10 years later (in 1998). The CO_2 treatment was applied during daytime between April and November from 1998 to 2009. In July 2009, the CO_2 treatment stopped and trees were harvested for this study and for allometric analysis.

For anatomical experiments, fully sun-exposed two-year old upper branches were collected from six trees in each ring in early October, 2007. The apical ends of branches used earlier for xylem vulnerability to embolism curves (Warren et al., 2011) were radially sectioned (40 µm) using a microtome. For each branch, six sections were bleached (10:1 dilution of household bleach), stained with toluidine-blue and mounted on a single slide using a gelatin-glycerin medium.

129 Stem log samples from the harvested trees at 0.6-1.1m height from the ground were collected
130 for the chemical analyses and stored at -20˚C until processing. Eight trees from each treatment
131 (elevated or control CO_2 rings) were selected for the experiments. A 25 mm thick disc was cut
132 from the frozen logs, and the annual rings were examined and marked to only collect wood that
133 was produced during the CO_2 treatment. The bark was removed and the CO_2 treated xylem
134 sections (1998-2009) were freeze-dried and chipped with a chisel into small pieces. The chips
135 were then ground with a Wiley Mill 4 (Thomas Scientific, Swedesboro, NJ, USA) equipped with
136 a 40-mesh sieve.

137 **2.2 Methods**

138 For anatomical analyses, one high-level resolution (0.72 pixel μm^{-1}) image of the entire radial
139 branch section was taken at low magnification (15X) for analysis of xylem sapwood area using a
140 Leica M165 stereomicroscope and digital camera. Stem diameter, one- and two-year-old
141 sapwood area, and pith area was measured using Image J software (Rasband, 2012). Six to
142 eleven images per branch that included one or two-year-old xylem tissue were taken at various
143 radial directions at higher magnification (1.96 pixels μm^{-1}; 200X) using a Leica M1000 light
144 microscope system. Each image was fully analyzed for vessel area, vessel diameter, and vessel
145 number using automated software (WinCELL 2001a; Régent Instruments Inc). Cell wall
146 diameter was measured using Image J software. Branch hydraulic conductivity depends on the
147 sum of the weighted mean cell diameters, or mean hydraulic diameter, which was calculated
148 based on Sperry et al. (Sperry et al., 1994). Cells that were smaller than 100 μm^2 were not
149 included in the analysis of xylem vessels. In total, 29236 xylem vessel cells were analyzed in 202
150 different images. Results from each image were averaged by branch, and statistical analysis was
151 performed at the branch level (n=10-11 per treatment).

For chemical analyses, approximately 5 g of the finely ground sweetgum samples were sequentially extracted with water and ethanol using an automated extraction system (ASE 350, Dionex Corp.) following the National Renewable Energy Laboratory protocol "Determination of extractives in biomass (NREL/TP 510-42619)". Each extracted wood sample was dried in a low temperature oven (35 ˚C) until it reached a constant weight, and the extractives content was calculated on dry basis. The total phenolics content in the water and ethanol fractions was determined by the Folin-Ciocalteau method (Singleton and Rossi, 1965).

The dried and extracted sweetgum samples were used for structural chemical analyses following the protocol "Determination of structural carbohydrates and lignin in biomass (NREL/TP-510-42618)". This protocol was used to measure cellulose, hemicellulose, acid soluble and acid insoluble lignin content by a two-stage acid hydrolysis. During the hydrolysis, the polymeric carbohydrates were hydrolyzed into monomeric sugars (glucose, xylose, mannose, arabinose, and galactose) and quantified using a High Pressure Liquid Chromatography (HPLC) (Perkin Elmer, Shelton, CT) equipped with a refractive index detector and an Aminex HPX-87P column (300 x 7.8 mm ID, 9 µm particle sizes) attached to a deashing guard column (Biorad, Hercules, CA). The HPCL's oven temperature was set at 85 ˚C and the injection volume was 20µL. Quantified glucose was calculated as % cellulose in the biomass and the other monomeric sugars were calculated as % hemicellulose. Acid insoluble lignin was measured gravimetrically, and acid soluble lignin was determined by UV/VIS spectrophotometer (Lambda 650, Perkin Elmer).

The gravimetric ash content was measured by combusting 0.7 g of biomass at 575 ˚C for 24 hours. 0.5 g of raw biomass was microwave digested then analyzed for content of inorganic elements by inductively coupled plasma-optical emission spectroscopy using an Optima 7300

175 DV spectrometer (ICP-OES, Perkin Elmer). All of the experiments were performed in triplicate
176 except total phenolics analysis which was conducted in duplicate for each sample.

177

178 **2.3 Data analysis**

179 To compare responses in structural and non-structural chemical components of sweetgum to
180 CO_2 treatments, two types of statistical methods were employed. First, analysis of variance
181 (ANOVA) methods were used to determine if differences existed between the means of sample
182 groups from elevated and ambient CO_2 sites at a significance level of $\alpha < 0.05$. For the mean
183 separation, 2-sided t-test was used for anatomical analysis and Fisher's Least Significant
184 Differences (LSD) was employed for chemical analysis. The high cost of the FACE
185 infrastructure limits true replication in these types of studies, and thus the analyses are often
186 based on pseudoreplication (e.g., by tree instead of by ring), which increases the chance of type 1
187 errors (Hurlbert, 1984). Thus results should be considered in this context.

188 To detect differences of physical and chemical tree responses to the CO_2 treatments and to
189 determine the relative strength of CO_2 effects on physical and chemical tree responses under
190 elevated CO_2, principal component analysis (PCA) was employed. The basic assumption for the
191 use of multivariate analysis is that all the data (tree responses in this research) carry information
192 regarding the effects of elevated CO_2. PCA was also used to visualize correlation structures
193 among the tree responses. The scores plot of PCA shows the cluster of the sample categories and
194 helps visualize any trends in the data sets in the new system of axes of principal components
195 (PCs). Correlation loadings are useful in interpreting the correlation structure between the
196 variables and the PCs by presenting significant levels when loadings plot cannot reveal the actual

197 correlation structure. This statistical method offers a reliable separation between the sample
198 categories (CO_2 treatments in this study) by using the responses of tree under the treatment
199 (Esbensen, 2001). In the PCA, variables included cellulose, hemicellulose, lignin, Ca, Mg, K, P,
200 S, total phenolics, extractives, and ash (tree responses to the CO_2 treatments in this study). Other
201 variables from previously published results such as total root production (gm^{-2}), root turnover
202 (mortality) (gm^{-2}), root peak standing crop (biomass) (gm^{-2}), height and circumference of the
203 sweetgum at harvest time were also included to address the relative importance of CO_2 impacts
204 on tree responses on both above-ground and below-ground changes (Iversen, 2010; Ledford et
205 al., 2008). The data were imported and standardized prior to PCA in the Unscrambler software
206 (v.9.0, CAMO, Woodbridge, NJ.).
207
208 # 3. Results
209 ## 3.1 Anatomical changes
210 Figure 1 is a typical light microscopic image of a two-year old sweetgum branch
211 collected in 2007 from the upper canopy at the ORNL FACE research site. Ladder-like
212 scalariform perforation plates are visible in several cells in Figure 1, b. The automated image
213 analysis (c) indicates cell length and width.

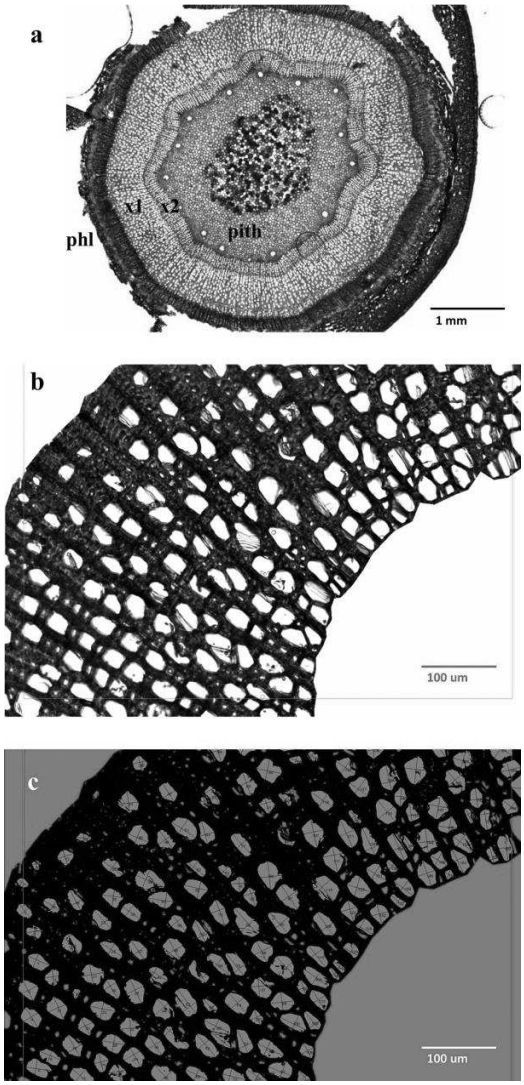
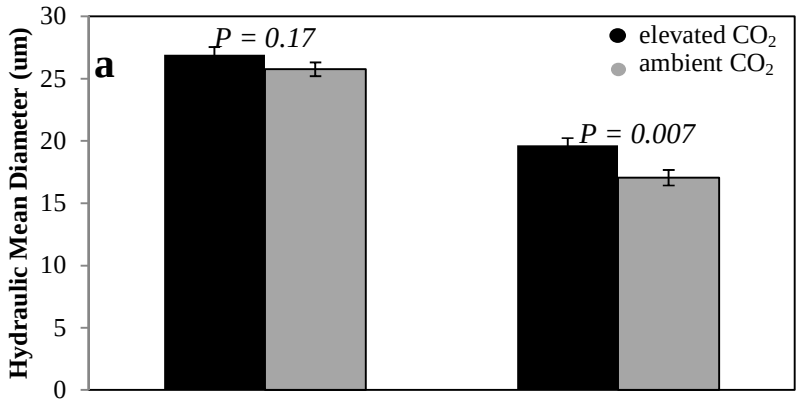
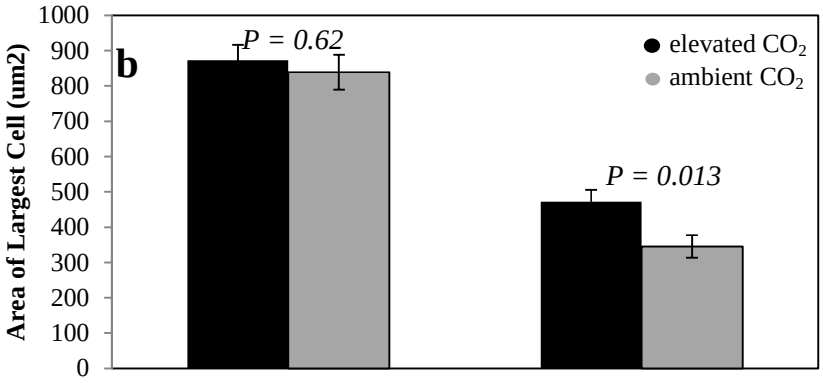


Fig. 1. Typical light microscopy image of a two-year old sweetgum branch collected in 2007 at the ORNL FACE research site. (a) indicates that the pith, one-year old xylem (x1), two-year old xylem (x2) and outer phloem and bark (ph1) tissues; (b) two-year old xylem tissue at 200x magnification; (c) automated image analysis of (b)

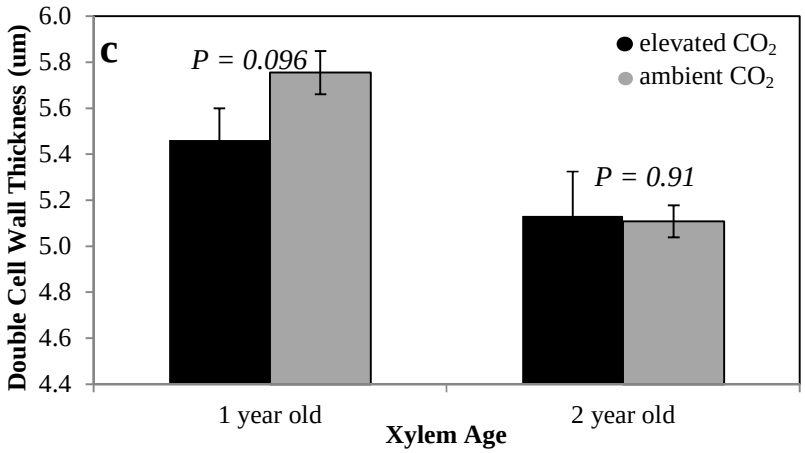
Hydraulic mean diameter was significantly larger for elevated CO_2 branch tissue grown in 2006, but not for the 2007 cells (Fig 2, a). There was also a larger maximum cell size for elevated CO_2 xylem in 2006, but not in 2007 (Fig 2, b). The double cell wall thickness, as measured from the lumen of one xylem cell to the lumen of the next cell, was thinner for elevated CO_2 branches in 2007, but not during the previous year (Fig 2, c).


a
P = 0.17
P = 0.007
elevated CO2
ambient CO2
Hydraulic Mean Diameter (um)
30
25
20
15
10
5
0

236


b
P = 0.62
P = 0.013
elevated CO2
ambient CO2
Area of Largest Cell (um2)
1000
900
800
700
600
500
400
300
200
100
0

237


c
P = 0.096
P = 0.91
elevated CO2
ambient CO2
Double Cell Wall Thickness (um)
6.0
5.8
5.6
5.4
5.2
5.0
4.8
4.6
4.4
1 year old
2 year old
Xylem Age

238

Fig. 2. The hydraulic mean diameter (a), the area of largest cell (b), and double cell wall thickness of elevated and ambient CO_2 treated branch tissue of sweetgum. Significance level ($p < 0.05$) among control and CO_2 enriched samples are shown based on 2 sided t-test.

The anatomical results reveal differences in xylem development under elevated CO_2, as well as an interaction with other environmental conditions. In 2006, the site experienced moderate weather conditions with 1184 mm of annual rainfall, and without significant drought or heat waves. In contrast, 2007 was a year of extremes, with a severe early-season frost, low annual rainfall (905 mm), extended 50-year summer drought, and record-breaking summer temperatures (Warren et al., 2011). During non-drought years, net C uptake and availability, and water use efficiency were greater for elevated CO_2 trees. Such conditions are conducive to greater rates of growth and cell expansion, consistent with the larger cell sizes found in elevated CO_2 branches in 2006. Larger cell size is also consistent with increased vulnerability to xylem embolism (Tyree et al., 1994), and *L. styraciflua* trees exposed to elevated CO_2 at this site (Warren et al., 2011) and at the Duke FACE site (Domec et al., 2009) do have increased vulnerability to embolism, which could lead to reduced competitive ability within future forest ecosystems if other species are not similarly affected. In 2007, the severe drought reduced stomatal conductance in elevated CO_2 branches to such an extent that C availability was substantially reduced as compared with ambient branches (Warren et al., 2011). Xylem cell size was not affected by the reduced C availability in elevated CO_2 branches, yet cell wall thickness was slightly reduced ($P=0.096$). The differences in CO_2 treatments on tree growth and xylem development reflect both the CO_2 treatment, as well as the interaction between CO_2 and other limiting conditions, such as drought (Warren et al., 2011) or lack of nutrients (Norby et al., 2010). While the mild summer of 2006 allowed CO_2 treatments to enhance xylem cell size, the

extreme conditions in 2007 limited any positive effect of elevated CO_2 on branch development. Hydraulic function depends on the characteristics of xylem cells developed across multiple years, thus subtle elevated CO_2 impacts to xylem structure can be amplified through time with implications to the whole plant.

3.2 Chemical changes

3.2.1 Structural composition

The structural composition of sweetgum exposed to elevated and ambient CO_2 for 11 years is shown in figure 3. Sweetgum exposed to the ambient level of CO_2 had 38.73 % cellulose, 21.71 % hemicellulose, and 25.57 % lignin while sweetgum treated with elevated CO_2 had 39.98 % cellulose, 22.11 % hemicellulose, and 25.13 % lignin. The amount of cellulose and hemicellulose appeared slightly higher for the CO_2 enriched samples, and lignin tended to be lower with CO_2 enrichment. However, the results from LSD indicated that there were no significant differences in cellulose, hemicellulose, and lignin content between ambient and elevated CO_2 conditions for sweetgum ($\alpha < 0.05$).

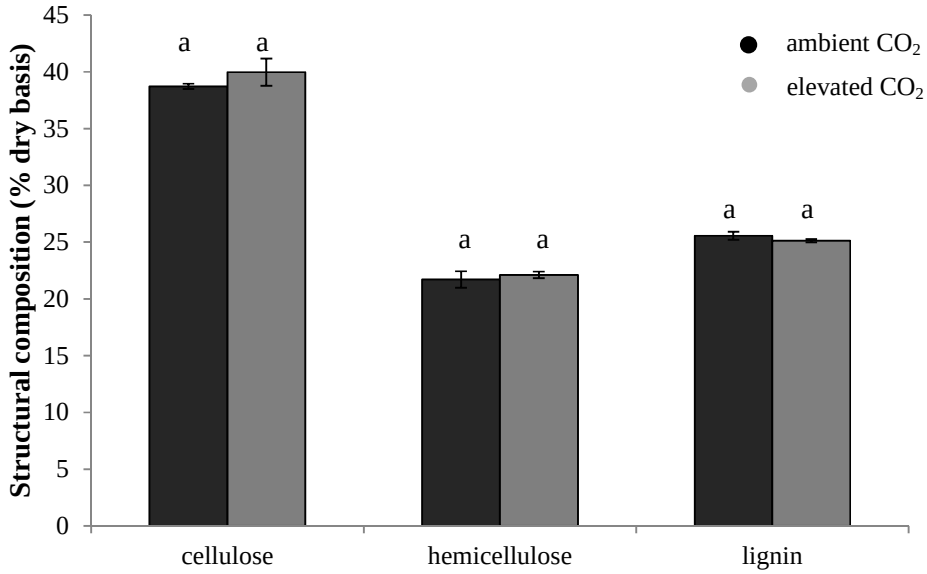


Fig. 3. The structural composition of elevated and ambient CO_2 exposed sweetgum stem wood. Estimated mean values from three replicate measurements of each tree sample with standard error on the bar. Same letters indicate no significant difference ($\alpha < 0.05$) among control and CO_2 enriched samples based on Least Significant Difference (LSD).

These findings are in agreement with previous research (Atwell et al., 2003; Entry et al., 1998; Kilpelainen et al., 2003; Olszyk et al., 2005; Tingey et al., 2003), which also indicated that there were no significant changes in structural components of wood due to elevated CO_2 treatments. It has been acknowledged that the increased CO_2 in the atmosphere would increase photosynthesis of plant, but the allocation of photosynthate depends on the sink demand of tissues within the plant. In other words, increased photosynthate by elevated CO_2 is most likely transported to the tissues that can obtain the most limiting resources (Entry et al., 1998), in the case of soil nutrients, this would be the root system. Indeed, many studies have pointed out that upon increased CO_2 treatment, fine root growth also increased (Chapman et al., 2005; Iversen, 2010; King et al., 2001; Norby et al., 2004; Nosberger et al., 2006). This result suggests that much of the available photosynthate could be transferred belowground (Nosberger et al., 2006)

297 to enhance the growth of roots rather than partitioned into stem xylem. According to Norby and
298 co-workers, above-ground wood production was 35 % greater in CO_2 enriched rings during the
299 first year of exposure, 15 % higher in the second year (Norby et al., 2002) and continued to
300 decline through the course of the experiment (Norby et al., 2010). While the enhancement of
301 above-ground wood production gradually declined with time, below-ground production
302 increased continuously (Norby et al., 2005). The cause for the enhanced root growth rather than
303 wood after CO_2 exposure can be explained with soil nutrition status. At the ORNL FACE site, N
304 deficiency occurred after just a few years of elevated CO_2 treatment (Norby et al., 2010). It
305 appears that effects of CO_2 in trees may be mostly observed in highly demanding tissues
306 (Nosberger et al., 2006). Enhanced root growth may be a strategy to acquire limiting nutrients
307 from the soil to offset N sequestration in larger CO_2 stimulated tree biomass. Indeed across CO_2
308 enrichment studies, root proliferation at depth is a common response (Iversen, 2010) presumably
309 to mine for resources to sustain the greater biomass and supplement decreased N availability in
310 the soil after prolonged elevated CO_2 treatment (Johnson et al., 2004).

311

312

313 **3.2.2 Non-structural composition**

314 The estimated mean values of total extractives and total phenolics content are presented
315 in Table 1 (LSD, $\alpha < 0.05$). Total extractives from sweetgum stem wood were 3.86 % and
316 3.93 % for ambient or elevated CO_2 trees, respectively. While no significant difference was
317 observed in the total extractives content, the total phenolics concentration for enriched CO_2
318 sweetgum was 16 % higher than that of the control. The amount of total phenolics was 91.24 mg

GAE/g of extracts in the ambient sweetgum and 108.46 mg GAE/g of extracts in the elevated CO_2 sweetgum, a difference that was statistically significant.

Table 1.

Total extractives, total phenolics, and protein in sweetgum stem wood.[1]

		Extractives (*% dry basis*)	Total phenolics [2] (mg GAE g^{-1} extractives)
Sweetgum	ambient CO_2	3.86 (0.25) a	91.24 (4.36) b
	elevated CO_2	3.93 (0.10) a	108.46 (4.04) a

1) *Estimated mean values from statistical method (LSD, $\alpha < 0.05$). Standard error values are appeared in parentheses.*
2) *GAE = gallic acid equivalent*

Increased total phenolic levels under elevated CO_2 have been observed in many tree species, including aspen, birch, oak, maple, eucalyptus, spruce, and pine (Lindroth, 2010; Tuchman et al., 2003). Phenolics can potentially be influenced by changes in carbon inputs (Johnson and Pregitzer, 2007), and elevated CO_2 may influence the chemical pathways that regulate gene expression and synthesis of secondary compounds (Lindroth, 2010). The shikimic acid pathway, known to produce phenolic compounds in trees, was found to be the most influenced pathway by CO_2 treatment (Lindroth, 2010). The total phenolics concentration in sweetgum confirms previous findings that trees exposed to higher CO_2 have higher level of phenolics. Another possible explanation for these increased total phenolics could be the growth-differentiation balance hypothesis of a tree. Growth refers to the production of new cells, while

differentiation refers to carbon flow into compounds that enhance the structure or function of existing cells (Stamp, 2003). Secondary metabolism is one of the examples of differentiation related processes (Herms and Mattson, 1992). The growth-differentiation balance hypothesis states that any environmental factor that slows growth of the tree can result in C allocation to differentiation-related products. When there is a limiting factor (N availability in the soil at ORNL FACE site) which slows down the tree growth rate, C can accumulate in the differentiation-related products (secondary metabolites and total phenolics in this study) with low cost to plant fitness (Stamp, 2003). Differentiation leads to changes in chemical quality, which has implications for herbivory and decomposition.

3.2.3 Inorganic Composition

The total ash content (%) and macronutrients in the xylem part of the sweetgum after CO_2 treatment are shown in Table 2. Statistical analysis (LSD, $\alpha < 0.05$) revealed that total ash content of sweetgum stem wood grown under elevated CO_2 was significantly greater (+19.0 %) than trees grown under ambient CO_2. The higher ash content in elevated CO_2 exposed sweetgum led to in-depth study of the inorganic nutrients in the biomass that contribute to the greater ash content.

Table 2.

Elemental composition contained in sweetgum stem wood. [1, 2, and 3]

Elemental composition	Sweetgum	
	Ambient CO_2	Elevated CO_2
Ash (%)	0.44 (0.01) b	0.53 (0.01) a
Ca (mg/kg)	713.77 (14.72) b	787.58 (21.07) a
K (mg/kg)	690.90 (16.51) a	669.64 (23.55) a
Mg (mg/kg)	239.76 (12.74) b	283.50 (13.16) a
P (mg/kg)	77.52 (6.90) a	74.74 (4.17) a
S (mg/kg)	79.62 (0.92) a	80.74 (1.03) a

1) Estimated mean values from statistical method (LSD, $\alpha < 0.05$). Standard error values are appeared in parentheses.
Values are % dry basis.
2) "a" and "b" represent statistical significant difference.
3) Concentrations were calculated on the basis of the dry weight of samples (mg/kg)

General elements found in wood are Ca, K, Mg, P, Mn, Fe, Zn, S, Al, and Si. Among those major elements, the inorganic macronutrients such as Ca, K, Mg, and P and the micronutrient S in the biomass are shown in Table 2. The amount of Ca and Mg in the raw biomass significantly increased with CO_2 treatment (LSD, $\alpha < 0.05$). Sweetgum grown under elevated CO_2 contained 10.0 % greater Ca and 18.0 % greater Mg than the trees grown under ambient CO_2. No differences were found for K, P, and S.

374 The increased total ash content and some of the inorganic elements in the elevated CO_2
375 stem wood samples may be linked to the increase in fine root growth and subsequent increased
376 access to large soil exchangeable pools of P, K, Ca, and Mg. We hypothesized that with the
377 enlarged fine root biomass, uptake of cations was not necessarily related to demand. The tree
378 may take up excess amounts of Ca, Mg, K, and P while 'mining' the soil for additional limiting
379 elements, especially N. Similar published results on inorganic nutrients composition support our
380 hypothesis (Hagedorn et al., 2002; Johnson et al., 2004; Luo et al., 2005). The inorganic content
381 of tree biomass largely depends on tree species and soil type, which suggest that further study is
382 needed to determine why only Ca and Mg increased among the other inorganic elements in
383 sweetgum after 11 years of elevated CO_2 treatment.
384

385 **3.3 Effect of elevated CO_2 on multiple tree responses**

386 As described earlier, the structural components (cellulose, hemicelluloses and lignin)
387 remained unchanged, while ash and inorganic components increased upon CO_2 enrichment.
388 Altered tree responses under elevated CO_2 could be due to several environmental factors. For
389 instance, physical tree growth largely depends on variables such as photosynthetic rate, soil N
390 availability, water, inorganic nutrient availability, seasonal variations and duration of treatment
391 (Kaakinen et al., 2004; Kilpelainen et al., 2007; Kilpelainen et al., 2005; Kostiainen et al., 2009;
392 Luo and Polle, 2009; Norby et al., 2010). The previously used statistical method (LSD), which is
393 also known as a univariate method, dealt with one variable at a time and provided a direct
394 comparison between the two levels of CO_2 treatments. Although univariate methods carry
395 important information, they are insufficient for more complex data analyses (Esbensen, 2001).
396 Multivariate analysis uses more than one independent variable and involves mathematical

397 treatment of the data to detect hidden differences or similarities (Esbensen, 2001). Therefore,
398 principal component analysis (PCA), a type of multivariate approach, was selected to
399 simultaneously study multiple tree responses to the elevated CO_2. In order to compare several
400 tree responses at the same time, published data from the ORNL FACE study (Ledford et al.,
401 2008) was also used, which included data regarding fine roots, final year of tree height and
402 circumference at 1.3 m from the ground. The results from PCA illustrate clear separation of
403 responses to the treatments (Figure 4).

404

405

406

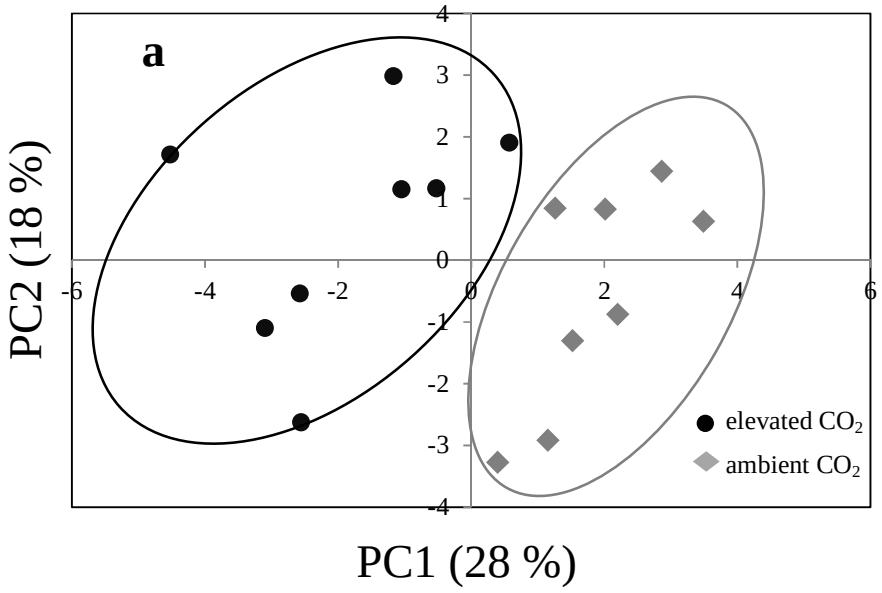
407

408

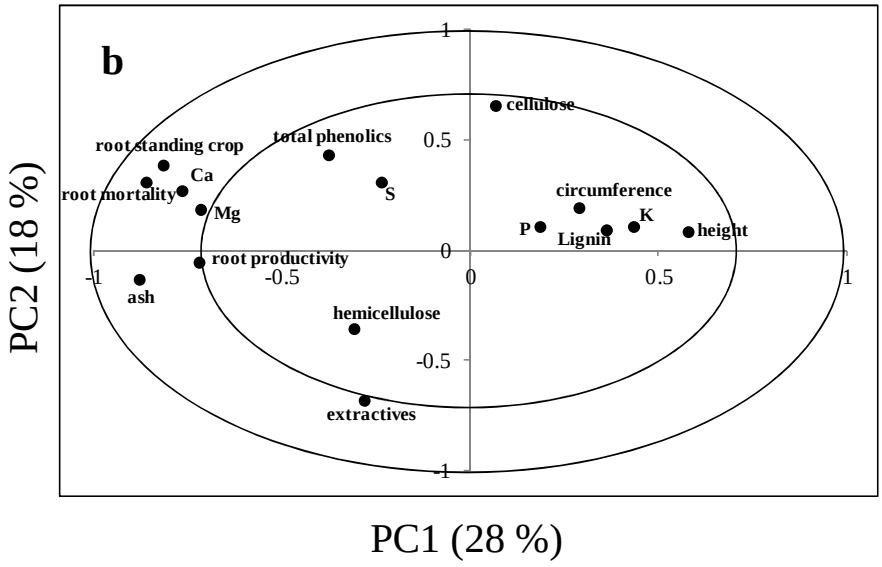
409

410

411



412



413

414 **Fig. 4.** Scores (a) and correlation loadings (b) plots show the relationships between samples and
415 variables of sweetgum, respectively. Outer and inner circles in correlation loadings (b) plot
416 represent 100% and 50% additive explained variance, respectively.

417

418

The scores plot is a map of samples and shows a separation between the ambient and elevated CO_2 exposed sweetgum by PC1 and PC2. PC1 accounts for 28 % of the variance and PC2 for 18 %. The correlation loadings plot provides the relative importance of tree responses (variables) and the visualization of explained variance. Tree responses closer to the outer circle show a higher explained variance level than those in the inner circle. In this study, ash, Ca, Mg, root mortality, root standing crop, and root productivity, located on the negative PC1 close to the outer circle, show higher explained variance level to the elevated CO_2 treated sweetgum.

Most of the variables that show higher degrees of CO_2 effects after long term CO_2 exposure match those from the LSD results. Indeed, Ca, Mg, and ash content were higher in the elevated CO_2 treated sweetgum (Table 1, 2). Elevated CO_2 treated sweetgum had higher amounts of root production and shorter height in comparison to the control sweetgum.

4. Conclusion

This study investigated the effects of free-air CO_2 enrichment on the wood chemistry of 22-year old sweetgum (*Liquidambar styraciflua*) after 11 years of elevated CO_2 treatment. In conclusion, elevated CO_2 concentration did not have large effects on structural components after long term CO_2 exposure, while non-structural components, xylem cell sizes, and cell wall thickness were significantly impacted by higher CO_2 concentration. The xylem cell sizes from branches growing under elevated CO_2 were larger and cell wall thickness was thinner. Ash, Ca, Mg, and total phenolics content increased in the elevated CO_2 exposed sweetgum. PCA showed clusters by CO_2 treatment, and correlation loadings plot revealed the degree of CO_2 impact on tree responses in the model. According to the correlation loadings plot, ash, Ca, Mg, root mortality, root standing crop, and root productivity were higher in elevated CO_2 exposed sweetgum. In other words, ash, Ca, Mg, root mortality, root standing crop and root productivity

were more impacted by higher level of atmospheric CO_2 than the other tree responses such as content of cellulose, hemicellulose, lignin, total extractives, S, P, K, circumference, and height. Long term (11 years) application of elevated CO_2 was also found to affected branch anatomy. Upper branches of sweetgum trees are known to be sensitive to drought (Toole and Broadfoot, 1959), and the elevated CO_2 changes in xylem hydraulic characteristics suggest enhanced potential vulnerability to hydraulic failure under drought. This could increase tree/branch dieback, especially by more intensive and frequent drought expected due to global climate change. The changes in chemical properties, especially ash, Ca, Mg could impact chemical and thermal utilization of xylem (wood).

Acknowledgement

This study was supported by the U.S. Department of Energy's Office of Biological and Environmental Research, and U.S. Department of Agriculture - Forest Service.

References

Ainsworth, E.A., Long, S.P., 2005. What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy. New Phytologist 165, 351-371.

Atwell, B.J., Henery, M.L., Whitehead, D., 2003. Sapwood development in Pinus radiata trees grown for three years at ambient and elevated carbon dioxide partial pressures. Tree Physiology 23, 13-21.

Chapman, J.A., King, J.S., Pregitzer, K.S., Zak, D.R., 2005. Effects of elevated concentrations of atmospheric CO_2 and tropospheric O_3 on decomposition of fine roots. Tree Physiology 25, 1501-1510.

DaMatta, F.M., Grandis, A., Arenque, B.C., Buckeridge, M.S., 2010. Impacts of climate changes on crop physiology and food quality. Food Research International 43, 1814-1823.

Domec, J.-C., Palmroth, S., Ward, E., Maier, C.A., Therezien, M., Oren, R., 2009. Acclimation of leaf hydraulic conductance and stomatal conductance of Pinus taeda (loblolly pine) to long-term growth in elevated CO_2 (free-air CO_2 enrichment) and N-fertilization. Plant Cell and Environment 32, 1500-1512.

Domec, J.-C., Schaefer, K., Oren, R., Kim, H.S., McCarthy, H.R., 2010. Variable conductivity and embolism in roots and branches of four contrasting tree species and their impacts on whole-plant hydraulic performance under future atmospheric CO_2 concentration. Tree Physiology 30, 1001-1015.

Eguchi, N., Morii, N., Ueda, T., Funada, R., Takagi, K., Hiura, T., Sasa, K., Koike, T., 2008. Changes in petiole hydraulic properties and leaf water flow in birch and oak saplings in a CO_2-enriched atmosphere. Tree Physiology 28, 287-295.

Entry, J.A., Runion, G.B., Prior, S.A., Mitchell, R.J., Rogers, H.H., 1998. Influence of CO_2 enrichment and nitrogen fertilization on tissue chemistry and carbon allocation in longleaf pine seedlings. Plant and Soil 200, 3-11.

Esbensen, K.H., 2001. Multivariate data analysis - in practice. Esbjerg, CAMO Software.

Ghasemzadeh, A., Jaafar, H.Z.E., Rahmat, A., 2010. Elevated Carbon Dioxide Increases Contents of Flavonoids and Phenolic Compounds, and Antioxidant Activities in Malaysian Young Ginger (Zingiber officinale Roscoe.) Varieties. Molecules 15, 7907-7922.

Hagedorn, F., Landolt, W., Tarjan, D., Egli, P., Bucher, J.B., 2002. Elevated CO_2 influences nutrient availability in young beech-spruce communities on two soil types. Oecologia 132, 109-117.

509 Handa, I.T., Korner, C., Hattenschwiler, S., 2006. Conifer stem growth at the altitudinal treeline
510 in response to four years of CO_2 enrichment. Global Change Biology 12, 2417-2430.
511
512 Haygreen, J., Bower, J., 1996. Forest products and wood science. Iowa State University Press /
513 Ames.
514
515 Herms, D.A., Mattson, W.J., 1992. The dilemma of plants - To grow or defend. Quarterly
516 Review of Biology 67, 478-478.
517
518 Hurlbert, S.H., 1984. Pseudoreplicaion and the design of ecological field experiments.
519 Ecological Monographs 54, 187-211.
520
521 Iversen, C.M., 2010. Digging deeper: fine-root responses to rising atmospheric CO_2
522 concentration in forested ecosystems. New Phytologist 186, 346-357.
523
524 Johnson, D.W., Cheng, W., Joslin, J.D., Norby, R.J., Edwards, N.T., Todd, D.E., 2004. Effects of
525 elevated CO_2 on nutrient cycling in a sweetgum plantation. Biogeochemistry 69, 379-403.
526
527 Johnson, R.M., Pregitzer, K.S., 2007. Concentration of sugars, phenolic acids, and amino acids
528 in forest soils exposed to elevated atmospheric CO_2 and O_3. Soil Biology & Biochemistry 39,
529 3159-3166.
530
531 Kaakinen, S., Kostiainen, K., Ek, F., Saranpaa, P., Kubiske, M.E., Sober, J., Karnosky, D.F.,
532 Vapaavuori, E., 2004. Stem wood properties of Populus tremuloides, Betula papyrifera and Acer
533 saccharum saplings after 3 years of treatments to elevated carbon dioxide and ozone. Global
534 Change Biology 10, 1513-1525.
535
536 Kilpelainen, A., Gerendiain, A.Z., Luostarinen, K., Peltola, H., Kellomaki, S., 2007. Elevated
537 temperature and CO_2 concentration effects on xylem anatomy of Scots pine. Tree Physiology 27,
538 1329-1338.
539
540 Kilpelainen, A., Peltola, H., Ryyppo, A., Kellomaki, S., 2005. Scots pine responses to elevated
541 temperature and carbon dioxide concentration: growth and wood properties. Tree Physiology 25,
542 75-83.
543
544 Kilpelainen, A., Peltola, H., Ryyppo, A., Sauvala, K., Laitinen, K., Kellomaki, S., 2003. Wood
545 properties of Scots pines (Pinus sylvestris) grown at elevated temperature and carbon dioxide
546 concentration. Tree Physiology 23, 889-897.
547
548 King, J.S., Pregitzer, K.S., Zak, D.R., Sober, J., Isebrands, J.G., Dickson, R.E., Hendrey, G.R.,
549 Karnosky, D.F., 2001. Fine-root biomass and fluxes of soil carbon in young stands of paper birch
550 and trembling aspen as affected by elevated atmospheric CO_2 and tropospheric O_3. Oecologia
551 128, 237-250.
552

Kostiainen, K., Kaakinen, S., Saranpaa, P., Sigurdsson, B.D., Linder, S., Vapaavuori, E., 2004. Effect of elevated CO_2 on stem wood properties of mature Norway spruce grown at different soil nutrient availability. Global Change Biology 10, 1526-1538.

Kostiainen, K., Kaakinen, S., Saranpaa, P., Sigurdsson, B.D., Lundqvist, S.-O., Linder, S., Vapaavuori, E., 2009. Stem wood properties of mature Norway spruce after 3 years of continuous exposure to elevated CO_2 and temperature. Global Change Biology 15, 368-379.

Kostiainen, K., Saranpaa, P., Lundqvist, S.-O., Kubiske, M.E., Vapaavuori, E., 2014. Wood properties of Populus and Betula in long-term exposure to elevated CO_2 and O_3. Plant Cell and Environment 37, 1452-1463.

Ledford, J., Norby, R.J., Tharp, M.L., 2008. ORNL FACE fine root data, in: Center, C.D.I.A. (Ed.).

Lindroth, R.L., 2010. Impacts of Elevated Atmospheric CO_2 and O_3 on Forests: Phytochemistry, Trophic Interactions, and Ecosystem Dynamics. Journal of Chemical Ecology 36, 2-21.

Luo, Z.-B., Polle, A., 2009. Wood composition and energy content in a poplar short rotation plantation on fertilized agricultural land in a future CO_2 atmosphere. Global Change Biology 15, 38-47.

Luo, Z.B., Langenfeld-Heyser, R., Calfapietra, C., Polle, A., 2005. Influence of free air CO_2 enrichment (EUROFACE) and nitrogen fertilisation on the anatomy of juvenile wood of three poplar species after coppicing. Trees-Structure and Function 19, 109-118.

Norby, R.J., DeLucia, E.H., Gielen, B., Calfapietra, C., Giardina, C.P., King, J.S., Ledford, J., McCarthy, H.R., Moore, D.J.P., Ceulemans, R., De Angelis, P., Finzi, A.C., Karnosky, D.F., Kubiske, M.E., Lukac, M., Pregitzer, K.S., Scarascia-Mugnozza, G.E., Schlesinger, W.H., Oren, R., 2005. Forest response to elevated CO_2 is conserved across a broad range of productivity. Proceedings of the National Academy of Sciences of the United States of America 102, 18052-18056.

Norby, R.J., Hanson, P.J., O'Neill, E.G., Tschaplinski, T.J., Weltzin, J.F., Hansen, R.A., Cheng, W.X., Wullschleger, S.D., Gunderson, C.A., Edwards, N.T., Johnson, D.W., 2002. Net primary productivity of a CO_2-enriched deciduous forest and the implications for carbon storage. Ecological Applications 12, 1261-1266.

Norby, R.J., Ledford, J., Reilly, C.D., Miller, N.E., O'Neill, E.G., 2004. Fine-root production dominates response of a deciduous forest to atmospheric CO_2 enrichment. Proceedings of the National Academy of Sciences of the United States of America 101, 9689-9693.

Norby, R.J., Warren, J.M., Iversen, C.M., Medlyn, B.E., McMurtrie, R.E., 2010. CO_2 enhancement of forest productivity constrained by limited nitrogen availability. Proceedings of the National Academy of Sciences of the United States of America 107, 19368-19373.

598 Nosberger, J., Long, S.P., Norby, R.J., Stitt, M., Hendrey, G.R., Blum, H., 2006. Managed
599 ecosystems and CO_2: case studies, processes, and perspectives. Springer, New York.
600
601 Olszyk, D., Apple, M., Gartner, B., Spicer, R., Wise, C., Buckner, E., Benson-Scott, A., Tingey,
602 D., 2005. Xeromorphy increases in shoots of Pseudotsuga menziesii (Mirb.) Franco seedlings
603 with exposure to elevated temperature but not elevated CO_2. Trees-Structure and Function 19,
604 552-563.
605
606 Oren, R., Ellsworth, D.S., Johnsen, K.H., Phillips, N., Ewers, B.E., Maier, C., Schafer, K.V.R.,
607 McCarthy, H., Hendrey, G., McNulty, S.G., Katul, G.G., 2001. Soil fertility limits carbon
608 sequestration by forest ecosystems in a CO_2-enriched atmosphere. Nature 411, 469-472.
609
610 Overdieck, D., Ziche, D., Boettcher-Jungclaus, K., 2007. Temperature responses of growth and
611 wood anatomy in European beech saplings grown in different carbon dioxide concentrations.
612 Tree Physiology 27, 261-268.
613
614 Penuelas, J., Estiarte, M., Kimball, B.A., Idso, S.B., Pinter, P.J., Wall, G.W., Garcia, R.L.,
615 Hansaker, D.J., LaMorte, R.L., Hendrix, D.L., 1996. Variety of responses of plant phenolic
616 concentration to CO_2 enrichment. Journal of Experimental Botany 47, 1463-1467.
617
618 Rasband, W.S., 2012. Image J, U. S. National Institutes of Health, Bethesda, Maryland, USA.
619 http://imagej.nih.gov/ij/, 1997-2012.
620
621 Schulte, P.J., 1999. Water flow through a 20-pore perforation plate in vessels of Liquidambar
622 styraciflua. Journal of Experimental Botany 50, 1179-1187.
623
624 Singleton, V.L., Rossi, J.A., 1965. Colorimetry of total phenolics in grapes and wine with
625 phosphomolybdic -phosphotungstic acid reagents. Amer J Enol Viticult 16, 144-158.
626
627 Sperry, J.S., Nichols, K.L., Sullivan, J.E.M., Eastlack, S.E., 1994. Xylem embolism in ring-
628 porous, diffuse-porous, and coniferous trees of northern Utah and interior Alaska. Ecology 75,
629 1736-1752.
630
631 Stamp, N., 2003. Out of the quagmire of plant defense hypotheses. Quarterly Review of Biology
632 78, 23-55.
633
634 Tingey, D.T., McKane, R.B., Olszyk, D.M., Johnson, M.G., Rygiewicz, P.T., Lee, E.H., 2003.
635 Elevated CO_2 and temperature alter nitrogen allocation in Douglas-fir. Global Change Biology 9,
636 1038-1050.
637
638 Toole, E.R., Broadfoot, W.M., 1959. Sweetgum blight as related to alluvial soils of the
639 Mississippi River floodplain. Forest Sci 5, 2-9.
640
641 Tuchman, N.C., Wahtera, K.A., Wetzel, R.G., Russo, N.M., Kilbane, G.M., Sasso, L.M., Teeri,
642 J.A., 2003. Nutritional quality of leaf detritus altered by elevated atmospheric CO_2: effects on
643 development of mosquito larvae. Freshwater Biology 48, 1432-1439.

Tyree, M.T., Davis, S.D., Cochard, H., 1994. Biophysical perspectives of xylem evolution - Is there a tradeoff of hydraulic efficiency for vulnerability to dysfunction? Iawa Journal 15, 335-360.

Warren, J.M., Norby, R.J., Wullschleger, S.D., 2011. Elevated CO_2 enhances leaf senescence during extreme drought in a temperate forest. Tree Physiology 31, 117-130.