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

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The anatomical and chemical characteristics of sweetgum were studied after 11 years of elevated CO_2 (544 ppm, ambient at 391 ppm) 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.

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

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compositions were altered (DaMatta et al., 2010; Kilpelainen et al., 2005, 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 (*L. 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 25 m-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.

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

2.2. Methods

For anatomical analyses, one high-level resolution (0.72 pixel μm^{-1}) image of the entire radial branch section was taken at low magnification (15X) for analysis of xylem sapwood area using a Leica M165 stereomicroscope and digital camera. Stem diameter, one- and two-year-old sapwood area, and pith area were measured using Image J software (Rasband, 2012). Six to eleven images per branch that included one or two-year-old xylem tissue were taken at various radial directions at higher magnification (1.96 pixels μm^{-1}; 200X) using a Leica M1000 light microscope system. Each image was fully analyzed for vessel area, vessel diameter, and vessel number using automated software (WinCELL 2001a; Régent Instruments Inc). Cell wall diameter was measured using Image J software. Branch hydraulic conductivity depends on the sum of the weighted mean cell diameters, or mean hydraulic diameter, which was calculated based on Sperry et al. (Sperry et al., 1994). Cells that were smaller than 100 μm^2 were not included in the analysis of xylem vessels. In total, 29,236 xylem vessel cells were analyzed in 202 different images. Results from each image were averaged by branch, and statistical analysis was 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).