

ABSTRACT

LIN, WEN. Drought Responses in Loblolly Pine (*Pinus taeda* L.) as Inferred from Analyses of Range-wide Tree Ring Isotopic Composition. (Under the direction of Drs Asko Noormets, Jean-Christophe Domec, and John King.)

Loblolly pine (*Pinus taeda* L.) is the most important timber species in the USA. Due to the large planted area and high productivity, it plays a crucial role in carbon uptake and economic vitality of the southeastern United States. Climate projections for the southeastern USA indicate that the frequency and intensity of drought will increase, in part due to higher temperature and more intense precipitation events separated by increased length of dry periods. The physiology and productivity of loblolly pine may be negatively affected by those future climate predictions. The major objective of this study was to understand how loblolly pine responds to drought and fertilization over its distribution range using tree ring cellulose stable isotopic compositions, plus an associated objective of modifying a high throughput α -cellulose extraction method so that it applies to conifer wood.

Cellulose extraction has been the rate-limiting step in stable isotope studies of plant materials. The Multiple Sample Isolation System for Solids developed by Wieloch et al. [*Dendrochronologia*, 29, (2011)] was a major advance in the tree ring α -cellulose extraction methods, offering greater throughput and reduced labor input compared to traditional alternatives for separate wood samples. However, the utility of the method for resinous conifer species may be limited by the need to remove extractives in a separate pretreatment step. We successfully complemented this method with a pretreatment step and designed a special apparatus so that it can be applied to conifer wood, while minimizing the need of additional labor.

With this new α -cellulose extraction method, we sampled tree ring α -cellulose carbon isotope discrimination ($\Delta^{13}\text{C}$) of two meteorologically contrasting years (wet vs. dry) from 76 plantations across the natural distribution range of loblolly pine. When plotted against the Palmer drought severity index (PDSI), we found a positive relationship between $\Delta^{13}\text{C}$ and PDSI up until PDSI=1. We then fitted the function $\Delta^{13}\text{C} = \text{Baseline } \Delta^{13}\text{C} + m \cdot (\text{PDSI} - 1)$ for each site, where baseline $\Delta^{13}\text{C}$ is the maximal $\Delta^{13}\text{C}$ at non-limiting water supply conditions (i.e. PDSI>1 in our study), and m is the $\Delta^{13}\text{C}$ drought sensitivity, the change of $\Delta^{13}\text{C}$ per unit PDSI. The two parameters distinguish the $\Delta^{13}\text{C}$ fluctuations due to changes of water availability and the “baseline” as a long-term mean $\Delta^{13}\text{C}$ under ample water supply, and allowing cross-site comparisons. Baseline $\Delta^{13}\text{C}$ ranged from 18.0‰ to 20.8‰, and was impacted primarily by site water status, latitude, and tree age. Drought sensitivity varied from -0.14 ‰ to 1.11‰, with an overall mean of 0.31‰. It is a product of intra-species genetic difference and environmental influences. The spatial autocorrelation of drought sensitivity was highly significant: it was lowest in the Atlantic coastal plain sites, above average west of the Mississippi River, and greatest in the far northeastern part of the range (Northern Virginia).

Using an experimentally imposed reduction in throughfall, we evaluated drought responses of loblolly pine with and without fertilization. Three and a half years after the experiment started, the increased growth of fertilized trees was largely attributable to increased leaf area, whereas the effect on photosynthetic capacity and stomatal conductance was minimal. On the other hand, the drought treatment increased iWUE by 8% regardless of fertilization, but it did not affect photosynthetic capacity and productivity of loblolly pine in this site. Multi-

dimensional sensitivity analysis suggested that changes in oxygen isotope discrimination could only be explained by a shift in the source of water in the droughted compared to ambient throughfall trees. This result supports earlier calls for caution about the common assumption of the scale of variability of source water in dual isotope analysis.

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Drought Responses in Loblolly Pine (*Pinus taeda* L.) as Inferred from Analyses
of Range-wide Tree Ring Isotopic Composition

by
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A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Forestry and Environmental Resources

Raleigh, North Carolina

2017

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ACKNOWLEDGEMENTS

There are many people I would like to thank. Firstly, I would like to thank my adviser, Asko Noormets, who has been a reliable source for suggestions and support over the years. JC Domec, my co-adviser, is always sparked with spontaneous ideas in science. During the numerous meetings in the past six years, progress was reviewed, difficulty tackled, and confusion cleared out. I thank John King, my vice chair, who provided lab space and equipment (especially the plenty room in Jordan Hall!), organized regular lab meetings and BBQ, supervised me as a teaching assistant (and encouraged me to give lectures), and joined my weekly meetings with Asko and JC at the final semester. I am grateful to Dr. John Marshall for being my external committee member. He provided critiques as well as challenging ideas, both of which were equally needed. Dr. Suijit Ghosh, the minor representative of my academic advising committee, had always been kind and supportive.

I appreciate the generous help from Thomas Wieloch, Hou-min Chang, Liang Wei, Takeshi Nakatsuka, and Ross Whetten. They provided ideas and practical solutions to problems, and shared unpublished data for α -cellulose extraction and isotope data analysis, without knowing me personally. Erika Wise helped with tree core dating and forwarded the information on North American Dendroecological Fieldweek (NADEF). Jim Speer, the organizer of NADEF, kindly reduced tuition for me. I would like to thank Andy Laviner, Geoff Lokuta, Joshua Cucinella, Madison Akers, Rodney Will, Adam Marquis, and Cassandra Meek who took a total of over 2000 tree core samples across southeastern USA for my dissertation research. In particular, Andy Laviner had helped me with field site selection, and answered many emails and occasionally emergent calls from me. Anu Söber,

Barbara Lachenbruch, Ben Bond-Lamberty, and Brian Amiro provided additional wood samples. I thank Wade Ross for sharing the gSSURGO data of the field sites. Aletta Davis and Maxwell Wightman helped with in-lab experiments and field trips. Douyong Min and David Barrios taught me how to use the instruments in their laboratories. Hang'Er Wang made a schematic drawing for my Delrin holder, which was used in a publication. Ge Sun, Erika Cohen, and Kai Duan estimated evapotranspiration based on PRISM data. Liangxia Zhang extracted elevations for over 100 sites. Lan Ma, kindly hosted me during the AGU meeting at San Francisco in 2016.

The project would have been impossible without the help from the undergraduate helpers and summer interns: Rebecca Jarvis, John Few, Allison Bass, Sara Vial, Manon Legendre, Tayler SantaMaria, Ian Thompson, Jacob Seyle, Deanna Metivier, and Abby Kuchta. Working with summer interns closely for 12 weeks for 4 consecutive years not only improved my skills of leadership, but also changed my understanding about education.

I would like to thank the current and past members of Tree Physiology and Ecosystem Science Laboratory. There were many many happy moments with everyone. Specially, my officemates Eric Ward, Guofang Miao, and Bharskar Mitra have been great company and support. I would like to thank Sarah Slover, the departmental graduate program coordinator, who had always been helpful in many respects.

Last but not least, I would like to thank my parents and all my dear friends that made this journey enjoyable. I do not plan to list the names here for the sake of privacy. But I am wholeheartedly grateful to all the love and support I have received. Thank you all!

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|Chapter 1. Introduction

Loblolly pine, climate change, and PINEMAP

Loblolly pine is the most important timber species in the USA. It is the primary species across the 12.9 million hectares of southern pine plantations, which comprises almost half of the world's industrial tree plantations (Fox et al. 2007a). Due to the large area and high productivity, loblolly pine plays a crucial role in carbon budget (Turner et al. 1995) and economic vitality of the southeastern United States. In the past 50 years, the productivity of southern pine plantations has tripled, largely due to the application of cooperative research and silvicultural technology transfer in the area (Fox et al. 2007b). These practice included site preparations, fertilization, weed control, density management, and deployment of genetically improved seedlings (Jokela et al. 2010). Among them, soil nutrient supply was found to be predominately important to long term productivity of the pine plantations (Jokela et al. 2010), and site fertility is the primary limitation to leaf area development and thus to stand productivity (Vose and Allen 1988). Thus, forest fertilization has been an indispensable practice in intensive plantation silvicultural systems and been applied widely in the region over the past 25 years (Fox et al. 2007a).

Faster growth of intensively managed forests also implies greater demand on resources, including water. However, according to IPCC (2014), it is “highly likely” that temperature will increase in the southeastern USA by the end of this century. This increase alone will raise vapor pressure deficit (VPD) and thus atmospheric demand for water (Novick et al.

2016). While the precipitation in the region would likely increase as well (IPCC 2014), the longer dry period between the more intense rainfall events results in reduced soil water availability (Ryan 2011). In other words, the frequency and intensity of drought stress will become more likely despite potentially greater precipitation. Such climatic change will negatively affect the physiology of loblolly pine and thus the productivity of the southern pine plantations.

To elucidate the growth, environmental responses and the economic implications of plantation pine growth under projected environmental change, the National Institute of Food and Agriculture of USDA funded the Pine Integrated Network: Education, Mitigation, and Adaptation Project (PINEMAP). The agency goals were to “create, synthesize, and disseminate knowledge that enables southern pine landowners to a) manage forests to increase carbon sequestration by 15% by 2030; b) increase the efficiency of nitrogen and other fertilizer inputs by 10% by 2030; and c) adapt forest management approaches and plant improved tree varieties to increase forest resilience and sustainability under variable climates”. PINEMAP was a multi-disciplinary project with 6 aim groups and lasted from 2011 to early 2017. The current study belongs to the ecophysiology part of PINEMAP, with a focus on understanding responses of loblolly pine to drought and fertilization.

As “natural archives”, tree rings of woody plants provide a means to retrieve historic information of both environmental and physiological processes. Stable carbon isotope ratios of tree rings record gas exchange processes at the leaf level (Dawson et al. 2002), while stable oxygen isotope ratios indicate variations of source water and water evaporative

environments inside the leaf (McCarroll and Loader 2004). In the current study, we evaluated the historic responses of loblolly pine to meteorological and experimentally imposed drought with and without fertilization through the analysis of stable carbon (C) and oxygen (O) isotope ratios in the tree rings.

The physiological basis for carbon isotopic discrimination in tree rings

The isotopic C composition of tree ring cellulose represents a record of isotopic C ratio of the CO₂ in the atmosphere and isotopic fractionations during photosynthetic and post-photosynthetic processes. The latter includes transportation of photosynthate, respiration, and biosynthesis of cellulose at the location of formation (Gessler et al. 2014).

The photosynthetic processes were well known and the model has remains the most powerful tool for interpreting isotopic C data. It was first summarized by Farquhar (1982), with limited modifications until ternary effects was incorporated by Farquhar and Cernusak in 2012. The ternary effects describe the influences of transpiration on the diffusion of CO₂ in the gas exchange process and become prominent when vapor pressure deficit is high. Given the annual mean vapor pressure deficit in our study sites was in the low to moderate range (< 1kPa, inferred from annual mean temperature and dew point temperature obtained from PRISM, see Study Sites of this chapter), we do not consider ternary effects and present the complete model in Equation 1.1, according to Marshall et al. (2008):

$$\Delta^{13}\text{C} = a_b \frac{c_a - c_s}{c_a} + a \frac{c_s - c_i}{c_a} + (e_s + a_i) \frac{c_i - c_c}{c_a} + b \frac{c_c}{c_a} - e \frac{R_d}{A + R_d} \frac{c_c - \tau^*}{c_a} - f \frac{\tau^*}{c_a} \quad (1.1)$$

I
II
III
IV
V
VI

Where c_a , c_s , c_i , c_c represent CO_2 concentrations in the atmosphere, leaf surface, the stomatal cavity, and the chloroplast, respectively; a_b , a , and a_i are the fractionation coefficients during CO_2 diffusion in the boundary layer, through stomata, and through the liquid phase inside the leaf, respectively; e_s is the fractionation of dissolution of CO_2 into the liquid phase; b , e , and f are the discrimination coefficients during CO_2 fixation by Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) and other carboxylases (Brugnoli and Farquhar 2000), during day respiration (R_d), and during photorespiration, respectively; A is the photosynthetic rate; and τ^* is the CO_2 compensation point.

This model describes different isotope effects during CO_2 diffusion through boundary layer (term I), CO_2 diffusion through stomata (II), CO_2 dissolution and diffusion in the liquid phase inside leaf (III), carboxylation (IV), day respiration (V), and photorespiration (VI). The fractionation coefficients as reported in literature are listed in Table 1.1.

Given that the isotope fractionation in different steps of diffusion is relatively constant, it is the concentration of CO_2 at the site of photosynthesis and the levels of day respiration and photorespiration that determine the magnitude of carbon discrimination. Thus the gradient of CO_2 is affected by photosynthetic rate (net CO_2 assimilation, A), boundary layer conductance, stomatal conductance, and internal (mesophyll) conductance.

Table 1.1 The reported values of isotope fractionation coefficients for Equation 1.1.

| Fractionation coefficient | Value | Reference |
|---------------------------|--------------|-----------------------------|
| a _b | 2.9‰ | Farquhar et al. (1980) |
| a | 4.4‰ | Craig (1954) |
| e _s | 1.1‰ at 25°C | Farquhar et al. (1989) |
| a _i | 0.7‰ | O'Leary (1981) |
| b | 28.2‰-30‰ | Brugnoli et al. (1988) |
| e | -5‰-0‰ | Ubierna and Farquhar (2014) |
| f | -8‰-16‰ | Ghashghaie et al. (2003) |

If boundary layer conductance and internal (mesophyll) conductance are high, we can assume $c_s \approx c_a$ and $c_c \approx c_i$. Since day respiration and photorespiration are typically also negligible (Ubierna and Farquhar 2014), Equation 1.1 can be simplified to Equation 1.2:

$$\Delta^{13}C = a + (\bar{b} - a) \frac{c_i}{c_a} \quad (1.2)$$

where \bar{b} takes the value of 27‰ and accounts for all the isotope effects in photosynthetic discrimination. Equation 1.2 is very robust and has been widely used (Cernusak et al. 2013).

One important application of this relationship is to calculate intrinsic water use efficiency (iWUE, Equation 1.3):

$$iWUE = \frac{A}{g_s} = \frac{c_a}{1.6} \left(1 - \frac{c_i}{c_a}\right) = \frac{c_a}{1.6} \left(\frac{\bar{b} - \Delta^{13}C}{\bar{b} - a}\right) \quad (1.3)$$

Intrinsic water use efficiency is a key parameter linking carbon and water cycles, and it is a good indicator for the abilities and strategies of plants responses to the environmental change (Farquhar et al. 1989).

However, in recent years, internal (mesophyll) conductance was found to be dynamic and species-specific (Flexas et al. 2012, Flexas et al. 2008). This can complicate the relationship between carbon discrimination and $\frac{c_i}{c_a}$, and thus iWUE (Seibt et al. 2008, Warren and Adams 2006). However, in loblolly pine, mesophyll conductance appears to be conserved across genotypes and water availabilities (Wilson 2014), indicating that the simplified model (Equation 1.2) is adequate for evaluating drought sensitivity in this species.

However, the isotopic compositions of tree ring cellulose may be different from those of leaf photosynthate as described by Equation 1.1, due to post-photosynthetic processes that cause fractionation in the synthesis of metabolites, respiration, and translocation (Gessler et al. 2014). While mechanistic understanding of these processes is improving, questions remain. In general, due to the small and relatively constant offset (Leavitt 2010) between the isotopic signature of leaves and whole wood (or wood components), the variation of tree ring $\delta^{13}\text{C}$ is usually attributed to atmospheric $\delta^{13}\text{C}$ and gas-exchange processes at the leaf level after correcting the tree ring isotope ratio with the offset (McCarroll and Loader 2004).

The responses of carbon isotope discrimination to drought and fertilization

As discussed in the previous section (Equation 1.1), carbon isotope discrimination is mainly determined by photosynthetic rate (A), boundary layer conductance, stomatal conductance, internal (mesophyll) conductance, and the levels of day respiration and photorespiration.

Environmental controls modify plant $\Delta^{13}\text{C}$ by regulating these variables. In general, $\Delta^{13}\text{C}$ is sensitive to soil water availability (Cernusak et al. 2013, Warren et al. 2001), nutrient availability (Duursma and Marshall 2006), irradiance (Farquhar et al. 1989), tree height (Marshall and Monserud 1996), and altitude (Hultine and Marshall 2000, Körner et al. 1991).

For loblolly pine, the influence of boundary layer conductance on $\Delta^{13}\text{C}$ seems to be minimal because of the high boundary layer conductance (Domec et al. 2009). Although mesophyll conductance is an important component in regulating CO_2 availability to photosynthesis, it is conserved across loblolly pine genotypes and insensitive to plant water availability (Wilson 2014). Given its very low discrimination coefficient (-0.5‰ , Wilson 2014), the contribution of day respiration to $\Delta^{13}\text{C}$ is negligible. Photorespiration plays a part in $\Delta^{13}\text{C}$ due to a relatively large f value (10.6‰ , Wilson 2014). However, given the limited range the ratio of $\frac{\tau^*}{c_a}$ can vary, photorespiration may not contribute to variation of $\Delta^{13}\text{C}$ greatly. Therefore, we limit our discussion on the impacts of photosynthetic rate (A) and stomatal conductance (g_s) on $\Delta^{13}\text{C}$ in loblolly pine and focus on the simplified model of $\Delta^{13}\text{C}$ simulation (Equation 1.2) in the current study.

Combining Equations 1.2 and 1.3, $\Delta^{13}\text{C}$ is expressed as a function of A and g_s (Equation 1.4).

$$\Delta^{13}\text{C} = \bar{b} - 1.6 \frac{(\bar{b} - a) A}{c_a g_s} \quad (1.4)$$

That is, $\Delta^{13}\text{C}$ is positively related to g_s , and negatively to A . Stomatal aperture is very sensitive to water stress. Drought entails greater atmospheric evaporative demand and/or reduced soil water content, both of which lead to lower stomatal conductance, followed by reduced photosynthesis due to limitation of CO_2 supply. By increasing the ambient to internal CO_2 gradient, both high A and low g_s decrease $\Delta^{13}\text{C}$. Under prolonged or intensive drought, photosynthesis would be further affected by non-stomatal effects such as lower mesophyll conductance and reduced Rubisco activity (Yordanov et al. 2000). Besides these direct impact from drought, stomatal conductance may be affected indirectly and thus $\Delta^{13}\text{C}$ modified. For instance, trees may increase the ratio of sapwood-to-leaf area under water stress (Cernusak et al. 2013) and thus increase reference stomatal conductance (Novick et al. 2009). For another, long term drought would slow tree growth in both height and diameter (Bréda et al. 2006), and short trees have higher stomatal conductance compared to tall ones (McDowell et al. 2011).

On the other hand, the response of $\Delta^{13}\text{C}$ to fertilization is more variable. Some researchers found $\Delta^{13}\text{C}$ of Douglas-fir decreased for a few years after fertilization (Brooks and Coulombe 2009, Brooks and Mitchell 2011, Cornejo-Oviedo et al. 2017), some reported no change (Balster et al. 2009), while Elhani et al. (2005) found that tree ring wholewood $\Delta^{13}\text{C}$ of beech (*Fagus sylvatica* L.) increased for 6 years after fertilization. It is recognized that fertilization with nitrogen may increase leaf nitrogen content and thus photosynthetic rate. The apparent divergence of results of fertilization effects on the photosynthetic rate in loblolly pine (Gough

et al. 2004a, Gough et al. 2004b, King et al. 2008, Samuelson et al. 2001, Tyree et al. 2009, Will et al. 2001) seem to be attributable to a step-wise nature of this effect. While loblolly pine is generally more conservative in its leaf chemical stoichiometry, and tends to increase leaf area rather than leaf-level photosynthetic rate, at times when it is unable to respond with leaf area increase, there may be a short-lived increase in foliar nitrogen content and photosynthetic rate. Earlier reports provide divergent results about the fertilization response of photosynthetic rate in loblolly pine: some report no change in photosynthetic rate and an increase in leaf area (Gough et al. 2004a, Samuelson et al. 2001, Will et al. 2001), whereas others report short-term increase in photosynthetic rate (Gough et al. 2004b, King et al. 2008, Tyree et al. 2009). Similarly, the reported change of stomatal conductance to fertilization is variable (Samuelson et al. 2001, Samuelson et al. 2014, Ward et al. 2015, Wightman et al. 2016).

The physiological basis for oxygen isotopic discrimination in tree rings

Similar to the isotopic C composition of tree ring cellulose, the isotopic O composition represents a record of isotopic O ratio of the source water and isotopic fractionations during photosynthetic and post-photosynthetic processes. The oxygen atoms of cellulose are predominantly from water (Marshall et al. 2008). Because carbon assimilation and water transpiration occur inside the leaf simultaneously, the isotopic composition of water (as the substrate for photosynthesis) is highly affected by evaporation inside the leaf (Farquhar et al. 2007). As chloroplasts are located between the evaporative sites (stomatal cavities) and

veins, the water for photosynthesis is affected by advection from the transpiration stream and diffusion from evaporative water (Cernusak et al. 2015). Finally, leaf water isotopic signal would be dampened by partial exchange with phloem water during unloading and retrieval of sugars, and by partial exchange with xylem water during cellulose formation in the trunk (Gessler et al. 2014). Briefly, three processes are related to the oxygen isotope discrimination of tree ring cellulose: evaporative enrichment in $\Delta^{18}\text{O}$ at evaporative sites (Farquhar et al. 2007), the $\delta^{18}\text{O}$ mixing of evaporative water and vein water (bulk leaf water) (Cernusak et al. 2015), and the discrimination in cellulose biosynthesis (Barbour and Farquhar 2000).

The evaporative enrichment of leaf water ($\Delta^{18}\text{O}_e$) is calculated using the modified Craig-Gordon model (Farquhar et al. 2007) with a steady state assumption (Equation 1.5):

$$\Delta^{18}\text{O}_e = (1 + \epsilon^+) \left[(1 + \epsilon_k) \left(1 - \frac{w_a}{w_i} \right) + \frac{w_a}{w_i} (1 + \Delta^{18}\text{O}_v) \right] - 1 \quad (1.5)$$

where ϵ^+ is equilibrium fractionation coefficient and a function (Equation 1.6) of leaf temperature (T_l).

$$\epsilon^+ = \exp\left(\frac{1137}{(273 + T_l)^2} - \frac{0.4156}{273 + T_l} - 0.0020667\right) - 1 \quad (1.6)$$

ϵ_k is the kinetic fractionation coefficient and a function (Equation 1.7) of boundary layer resistance (r_b) and stomatal resistance (r_s). Due to the reciprocal relationship between resistance (r) and conductance (g), the function can be expressed using the ratio of boundary layer conductance (g_b) to stomatal conductance (g_s).

$$\epsilon_k = \frac{28r_s + 19r_b}{r_s + r_b} = 19 + \frac{9}{1 + \frac{g_s}{g_b}} \quad (1.7)$$

w_a is the vapor pressure of the atmosphere and a function (Equation 1.8) of air temperature (T_a) and relative humidity (RH), while w_i is the vapor pressure inside leaves and a function of leaf temperature (Equation 1.9). $\Delta^{18}O_v$ is the isotopic difference between source water and atmospheric water vapor. Collection of atmosphere vapor for $\Delta^{18}O_v$ measurement is difficult (Sternberg 2009). It is often close to $-\epsilon^+$ under well-mixed conditions common in the field (Barbour 2007). Thus $-\epsilon^+$ is often used to approximate $\Delta^{18}O_v$ (e.g. Brooks and Mitchell 2011):

$$w_a = 0.61365 \exp\left(\frac{17.502T_a}{240.97 + T_a}\right) RH \quad (1.8)$$

$$w_i = 0.61365 \exp\left(\frac{17.502T_l}{240.97 + T_l}\right) \quad (1.9)$$

A simplified model (Equation 1.10) of Equations 1.1 to 1.5 has been widely used (Marshall et al. 2008, McCarroll and Loader 2004):

$$\Delta^{18}O_e \approx \epsilon^+ + \epsilon_k + (\Delta^{18}O_v - \epsilon_k) \frac{w_a}{w_i} = (\epsilon^+ + \epsilon_k) \left(1 - \frac{w_a}{w_i}\right) \quad (1.10)$$

Average bulk leaf water is less enriched than evaporative water due to the advection-diffusion process. Under steady state, the average isotopic enrichment of bulk leaf water ($\Delta^{18}O_L$, Equation 1.11) is modeled by considering the Péclet effect (\wp):

$$\Delta^{18}O_L = \Delta^{18}O_e \left(\frac{1 - \exp(-\wp)}{\wp} \right) \quad (1.11)$$

where \wp is the Péclet number, a function (Equation 1.12) of effective path length (L), transpiration (E), molar density of water (C, 5.55E4 mol m⁻³), and diffusivity of H₂¹⁸O (D'); Equation 1.13). Specially, transpiration is a function of stomatal conductance and leaf-to-air vapor pressure deficit (Brooks and Mitchell 2011, Ehleringer et al. 1993), P is the atmospheric pressure, and D' is dependent on leaf temperature (Equation 1.13). L is difficult to measure and variable even within the same tree (Roden et al. 2015).

$$\wp = \frac{LE}{CD'} = \frac{L}{CD'} g_s \frac{(w_i - w_a)}{P} \quad (1.12)$$

$$D' = 97.5 * 10^{-9} \exp\left(-\frac{577}{T_l + 128}\right) \quad (1.13)$$

Finally, the $\Delta^{18}O$ of cellulose in tree rings ($\Delta^{18}O_c$) is estimated following Barbour and Farquhar (2000) as:

$$\Delta^{18}O_c = \Delta^{18}O_L (1 - p_{ex} p_x) + \varepsilon_{wc} \quad (1.14)$$

Where p_{ex} is the proportion of oxygen atoms that exchange with source water during cellulose formation, p_x is the proportion of unenriched source water at the site of cellulose formation (thus its value equals 1 for trunk wood), and ε_{wc} is the isotope fractionation of 27‰, associated with the oxygen atom exchange between water and carbonyl groups.

To summarize, the variables for the equations 1.5 to 1.14 include T_a, T_l, RH, g_s, g_b, L, and p_{ex}. Due to the complexity of the models, the relationship between any single parameter and

$\Delta^{18}\text{O}_c$ is often not straightforward, and its magnitude dependent on the realized ranges of other parameters (Roden et al. 2000).

Plant materials for tree ring stable isotopic analysis

Wholewood and different wood components are used for stable isotope analysis of ecophysiological studies in tree rings. Early studies used whole wood from tree rings for isotopic analysis (McCarroll and Loader 2004). It was found later that the major components of wood, namely cellulose, lignin, and extractives, have different isotopic signatures (McCarroll and Loader 2004). Thus isotopic signature of wholewood may be confounded by the fluctuations of the isotopic signature of individual components and their contents.

Although some researchers examined the potential of using lignin for climate reconstruction (e.g. Barbour et al. 2001, Loader et al. 2003), it is not widely used because the fractionation in lignin biosynthesis is more complex (Barbour et al. 2001) and its isolation is more laborious than that of cellulose (McCarroll and Loader 2004). Cellulose has been the preferred material for isotopic analysis, especially for $\delta^{18}\text{O}$ analysis, because it has high relative abundance in plants (McCarroll and Loader 2004), is immobile across tree rings (McCarroll and Loader 2004), and its carbon and oxygen atoms of the main cyclic ring of cellulose do not exchange with atoms of other components once produced (Gaudinski et al. 2005). However, cellulose extraction is both time and labor intensive. Some researchers have suggested using whole wood or extractive-free wood due to the high correlation of isotopic signatures with that of cellulose (Eglin et al. 2008, Harlow et al. 2006, Loader et al.

2003, Verheyden et al. 2005). Extractive-free wood seems to provide a good balance between labor input and accuracy of signals for $\delta^{13}\text{C}$ analysis (Harlow et al. 2006), while cellulose is the most used constituent for $\delta^{18}\text{O}$ analysis (McCarroll and Loader 2004), although the use of wholewood can sometimes be justified (Barbour et al. 2001).

Traditionally α -cellulose has been defined as the portion of holocellulose (α -cellulose and hemicellulose) that remains insoluble in 17% NaOH solution and it is widely used for isotopic analysis. A number of α -cellulose extraction methods are available, offering different balances of speed, cost and purity (e.g. Green 1963, Leavitt and Danzer 1993, Loader et al. 1997, Brendel et al. 2000).

Study sites

The range-wide variability in drought responses was investigated by sampling still existing forest productivity trials, established over time as academy-industry co-operative experiments. Seventy six sites were selected based on variations in geography, soils, climate, stand age, treatment, and site accessibility. The sites spanned 10 states (AL, AR, FL, GA, LA, MS, NC, SC, TX, VA), established between 1984 and 2002. The latitude, altitude, mean annual precipitation (PPT, Figure 1.1), mean annual temperature (Figure 1.2), and mean annual vapor pressure deficit (VPD, Figure 1.3) of the sites were 29.66°N - 37.44°N, 3-198 m, 1106 mm - 1545 mm, 13.6°C - 20.5°C, and 0.51-0.72 kPa. The ratio of PPT and potential evapotranspiration is above 1.2 at all sites (Figure 1.4). The soils of the sites belong to 6 soil

orders: Alfisols, Entisols, Histosols, Inceptisols, Spodosols, and Ultisols, with 87% of the sites belonging to Alfisols and Ultisols (Figure 1.5). The available water capacity (AWC) in the top 100cm of soils ranged from 2.4cm to 11.5cm, and sand content ranged from 8% to 97%. The water table depth varies from 0 to below 200cm (Figure 1.6). As loblolly pine from different regions has different tolerance to drought (Schmidtling 2001), seed source transfer zones (dashed and solid lines in Figure 1.7), the general seed movement guideline for breeding and plantation programs, was used as an approximated compartmentation based on the complexity of the species genetic variation (Ross Whetten, personal communication 2016).

The mechanism of drought tolerance was further explored using the experimental throughfall removal and fertilization experiments, established as part of the PINEMAP study. The study site is located in the Buckingham County, VA (37°27'37''N, 78°39'50''W) in the Piedmont physiographic region, with 0-15% slopes. The soil is a well-drained, fine, mixed, subactive, mesic Typic Hapludult of the Littlejoe soil series, with a silt loam surface soil and a clay loam subsoil. The depth to ground water is deeper than 200 cm. Mean annual precipitation is 1120 mm and mean temperature 13.6°C.

Dissertation objectives

The major objective of this research was to understand how loblolly pine responds to drought and fertilization over its distribution range using tree ring cellulose stable isotopic

compositions. However, an accessory objective emerged as I was looking for a high throughput α -cellulose extraction method but realized that I had to adapt an existing method for conifer wood. Therefore, the dissertation starts with the development of a new high throughput α -cellulose extraction method, and then focuses on modeling the response of carbon isotope discrimination to drought sampled from 76 sites across the distribution range of the species. Finally, I interpreted the responses of loblolly pine to drought and fertilization from the study site in VA using intrinsic water use efficiency (inferred from the carbon discrimination) and oxygen discrimination, with support from data of transpiration and allometry measurements.

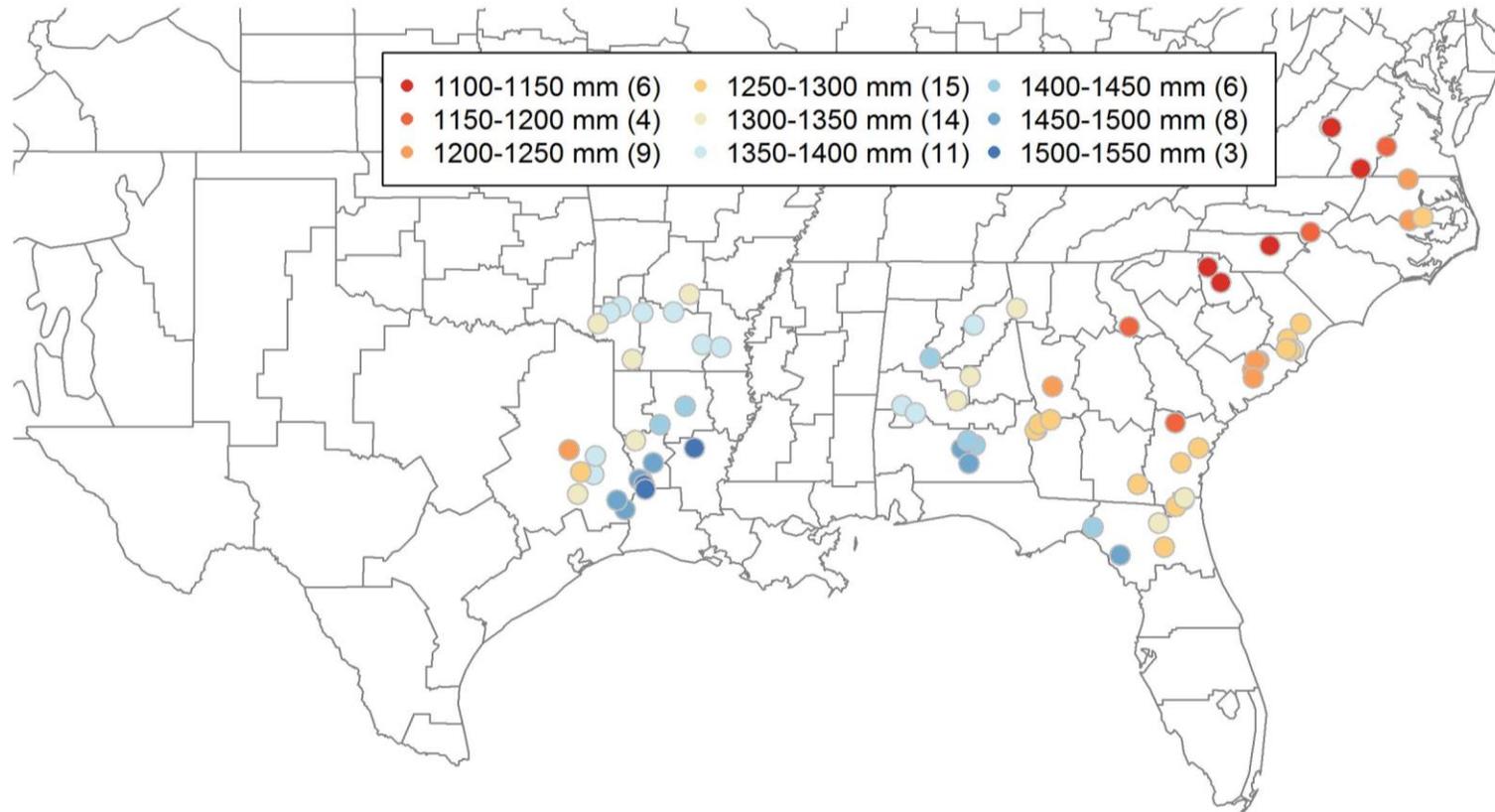


Figure 1.1 The 30-year mean annual precipitation at the study sites. A few sites overlap. The number inside brackets represent the number of sites under each category. (Source: PRISM Climate Group, Oregon State University, <http://prism.oregonstate.edu>)

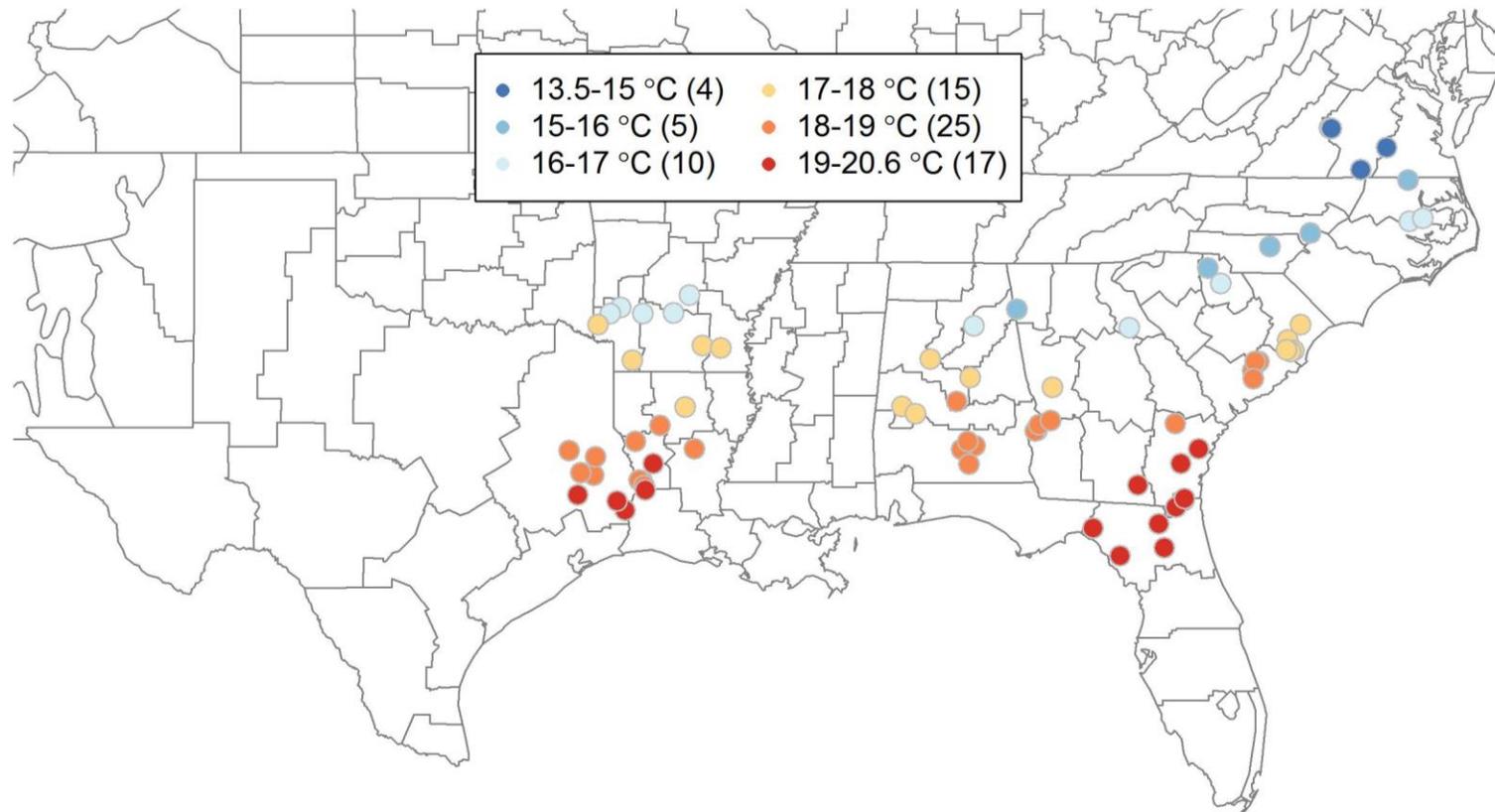


Figure 1.2 The 30-year mean annual temperature at the study sites. A few sites overlap. The number inside brackets represent the number of sites under each category. (Source: PRISM Climate Group, Oregon State University, <http://prism.oregonstate.edu>)

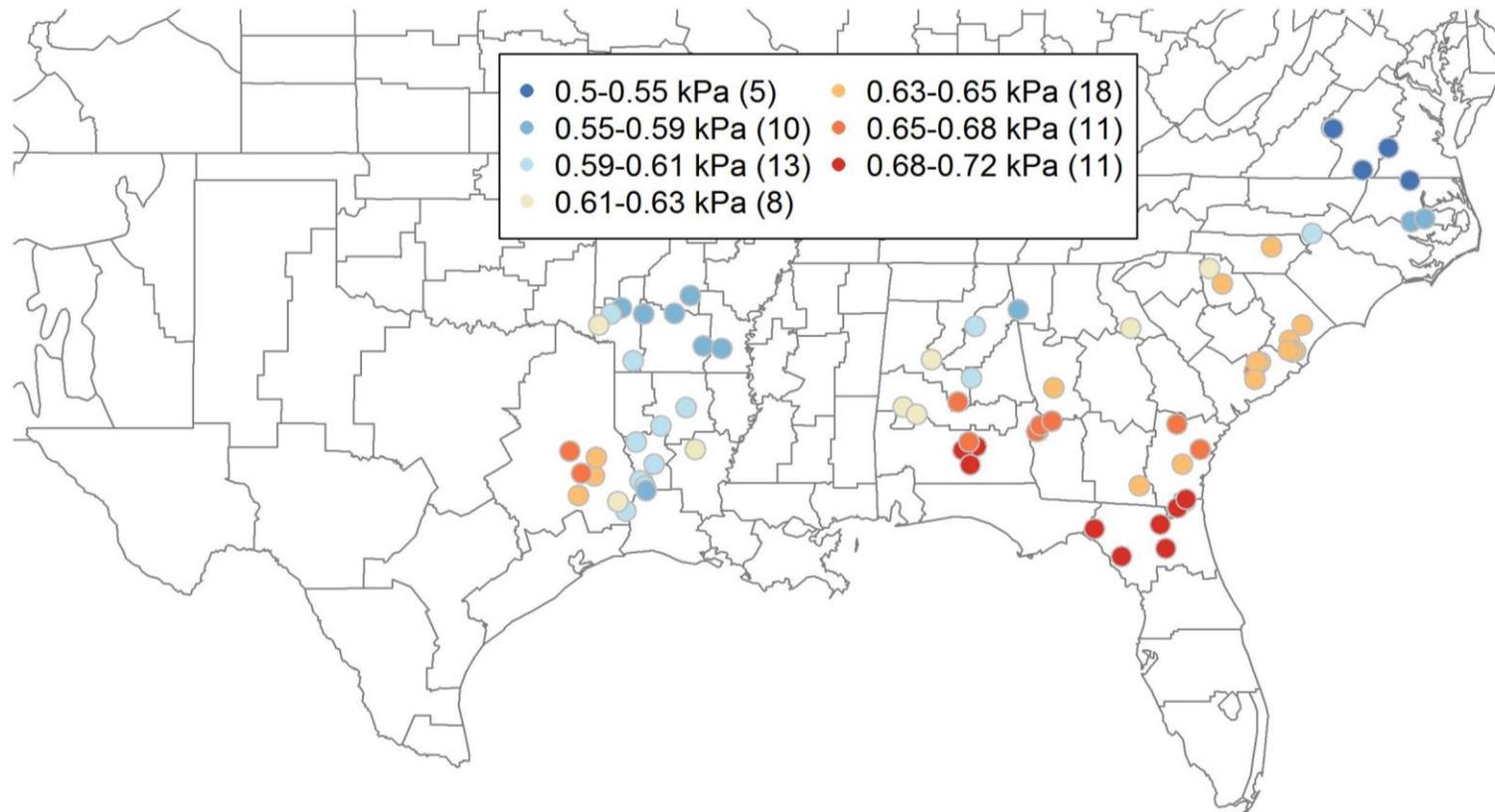


Figure 1.3 The 30-year mean annual vapor pressure deficit (VPD) at the study sites, calculated from 30-year mean annual temperature and dew point temperature from the 30-year normals of PRISM Climate data (PRISM Climate Group, Oregon State University, <http://prism.oregonstate.edu>). A few sites overlap. The number inside brackets represent the number of sites under each category.

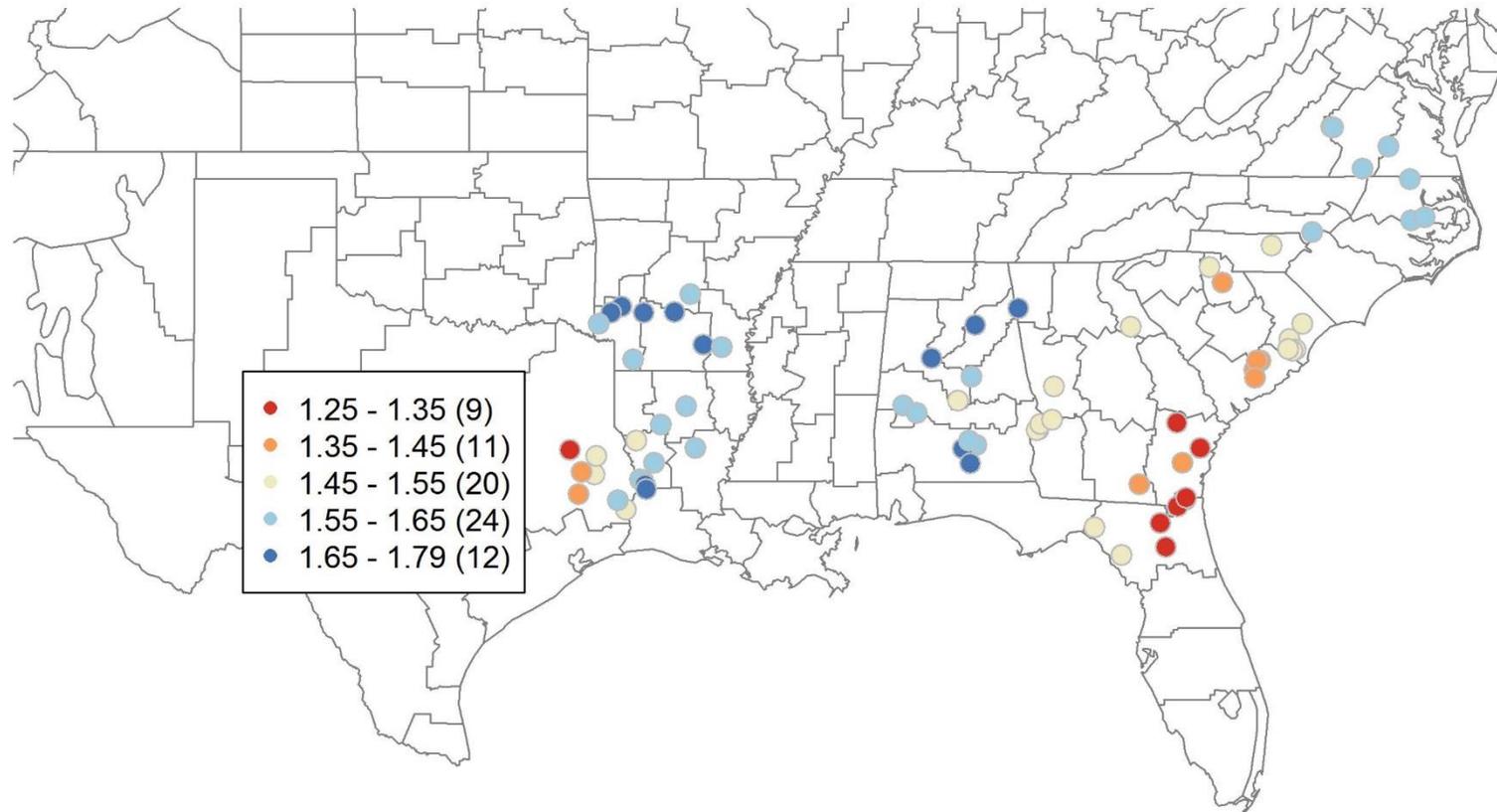


Figure 1.4 The ratio of mean annual precipitation (PPT) and potential evapotranspiration (PET) at the study sites. PET was calculated using Thornthwaite method based on mean monthly temperature (see methods of chapter 3). Both PPT and mean monthly temperature was obtained from the 30-year normals of PRISM Climate data (PRISM Climate Group, Oregon State University, <http://prism.oregonstate.edu>). A few sites overlap. The number inside brackets represent the number of sites under each category.

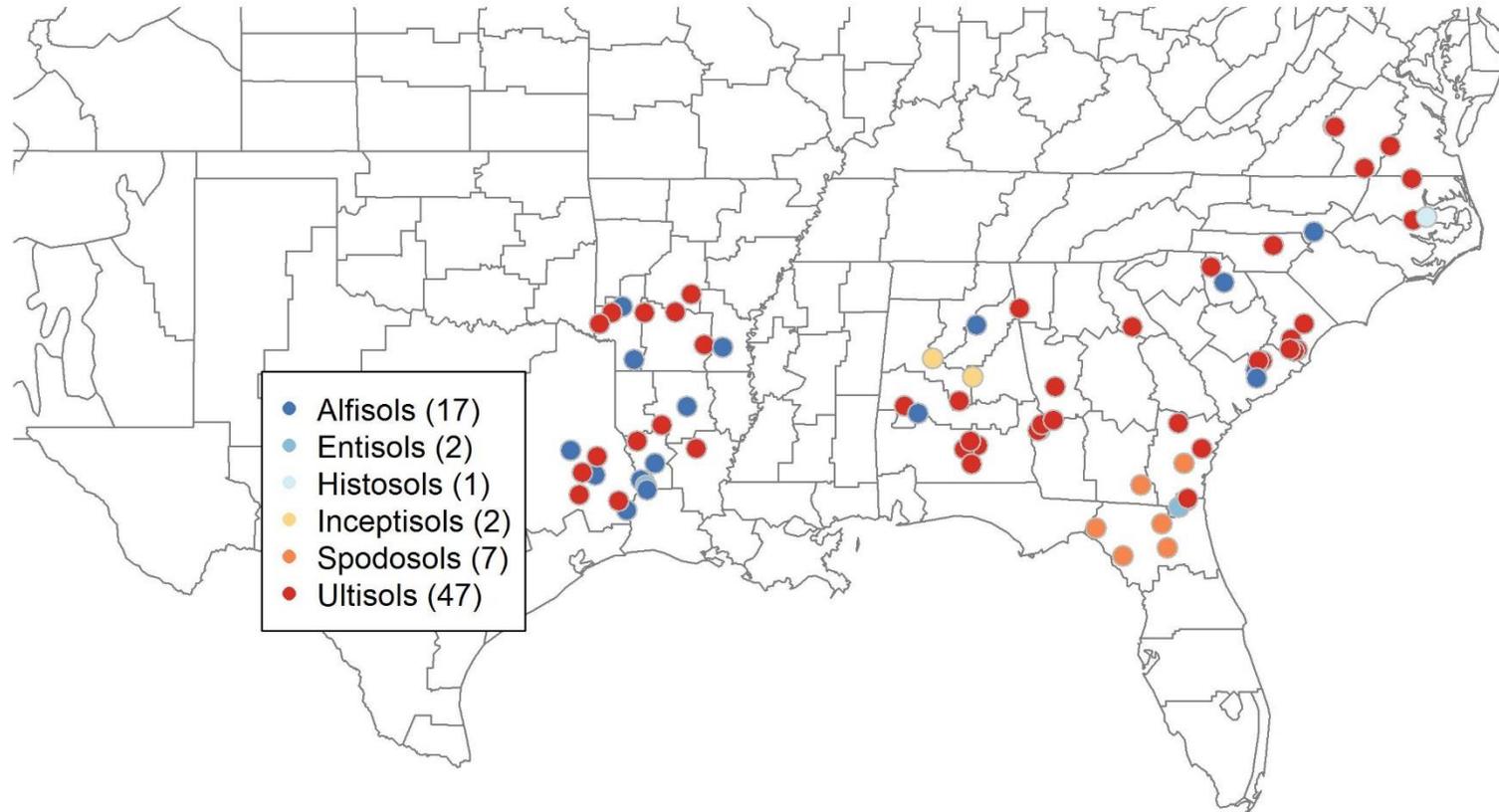


Figure 1.5 Soil orders of the study sites. Data is obtained from Gridded Soil Survey Geographic (gSSURGO) Database (<http://websoilsurvey.sc.egov.usda.gov/App/WebSoilSurvey.aspx>). A few sites overlap. The number inside brackets represent the number of sites under each category.

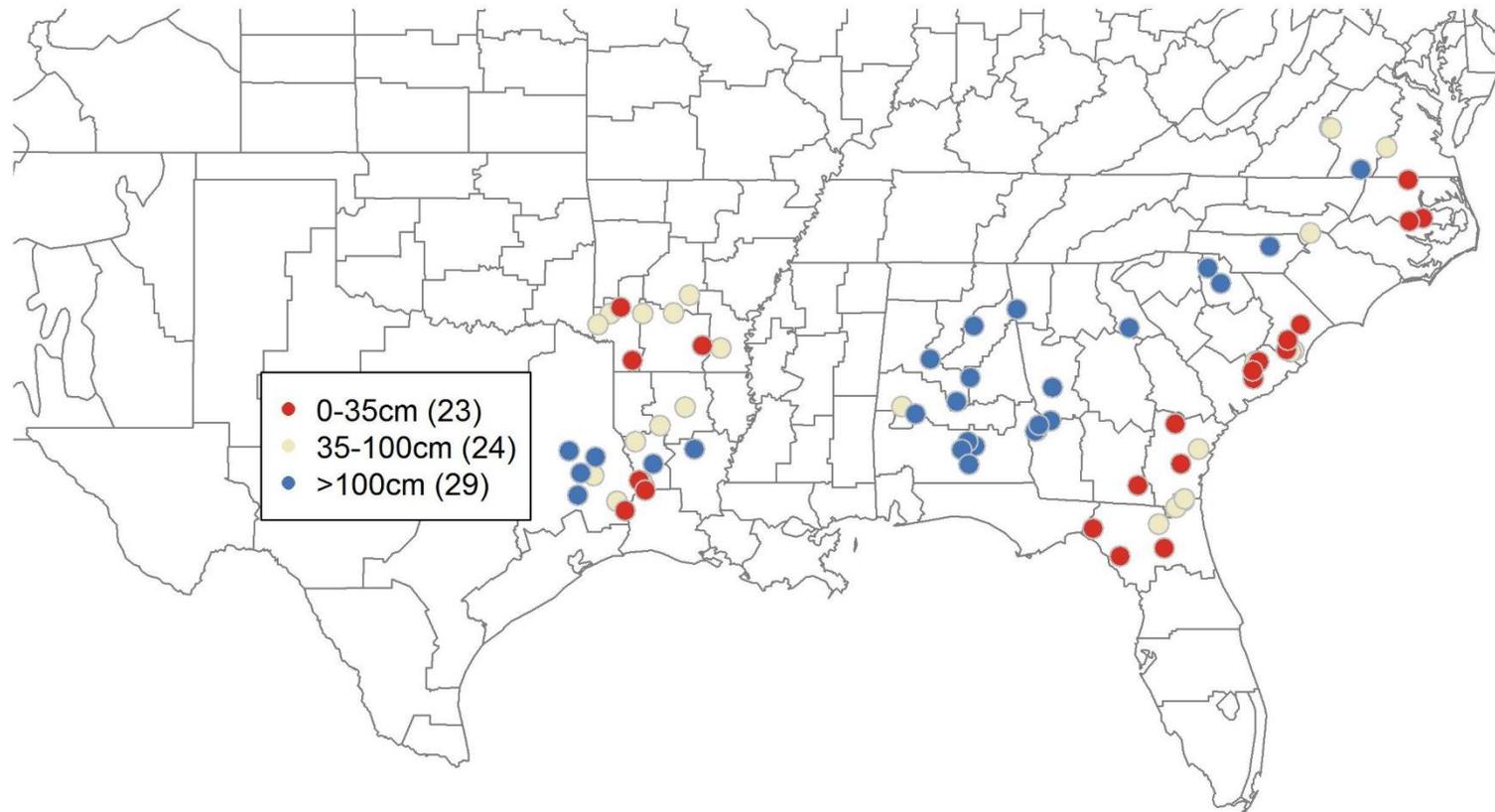


Figure 1.6 Water table depth of the study sites. The number inside brackets represent the number of sites under each category. A few sites overlap. Data were obtained from Gridded Soil Survey Geographic (gSSURGO) Database (<http://websoilsurvey.sc.egov.usda.gov/App/WebSoilSurvey.aspx>).

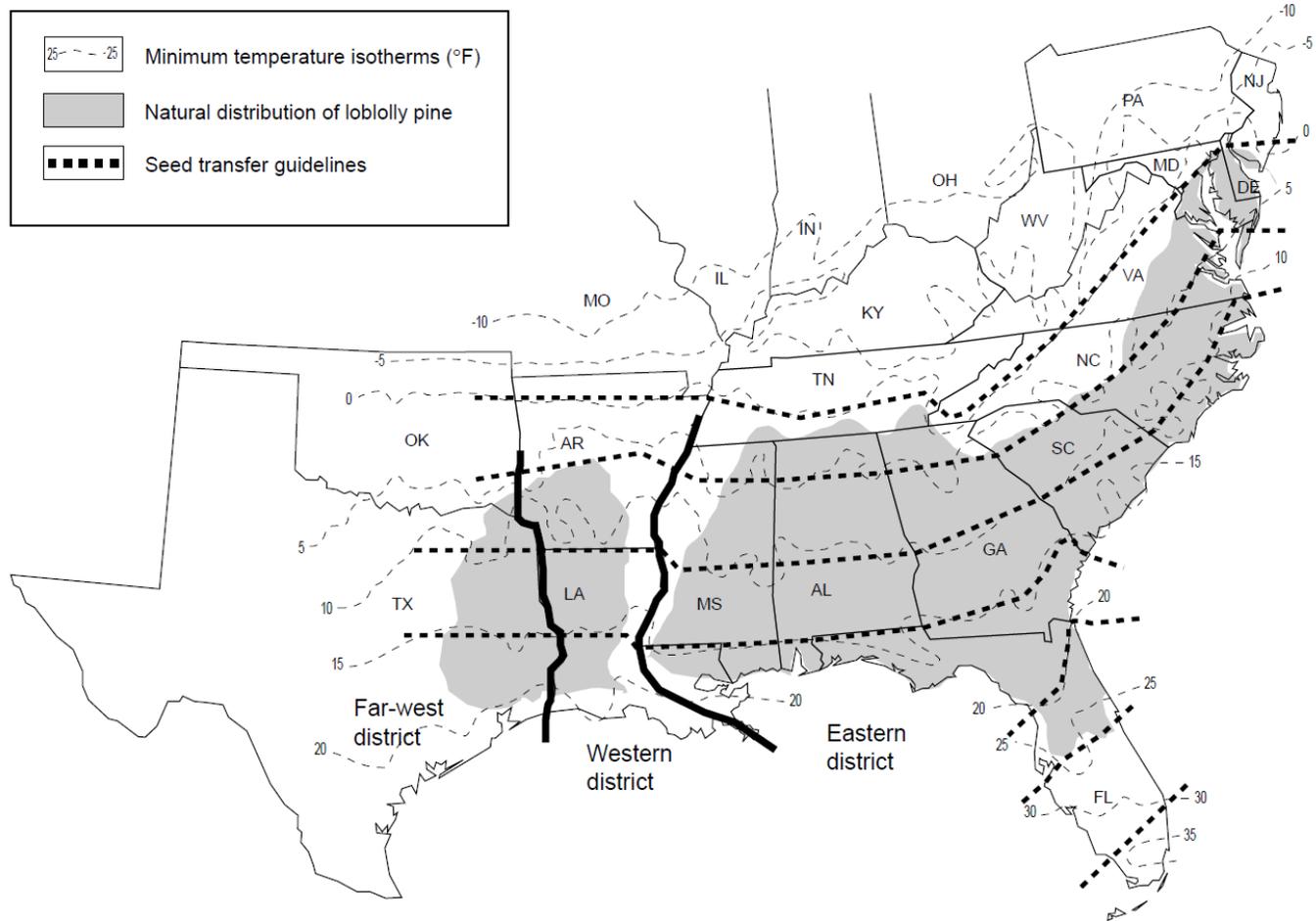


Figure 1.7 The natural distribution (shaded area), geographic districts (solid lines), and seed source transfer zones (dashed lines) of loblolly pine by Schmidting (2001).

Chapter 2. An extractive removal step optimized for a high-throughput α -cellulose extraction method for $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ stable isotope ratio analysis in conifer tree rings

Abstract

Stable isotope ratios ($\delta^{13}\text{C}$ and $\delta^{18}\text{O}$) of tree-ring α -cellulose are important tools in paleoclimatology, ecology, plant physiology, and genetics. The Multiple Sample Isolation System for Solids (MSISS) by Wieloch et al. (2011) was a major advance in the tree ring α -cellulose extraction methods, offering greater throughput and reduced labor input compared to traditional alternatives. However, the usability of the method for resinous conifer species may be limited by the need to remove extractives from some conifer species in a separate pretreatment step. Here we test the necessity of pretreatment for α -cellulose extraction in loblolly pine (*Pinus taeda* L.), and the efficiency of a modified acetone based ambient-temperature step for the removal of extractives (i) in loblolly pine from 5 geographic locations representing its natural range in the southeastern United States, and (ii) on 5 other common coniferous species (black spruce (*Picea mariana* Mill.), fraser fir (*Abies fraseri* (Pursh) Poir.), Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco.), Norway spruce (*Picea abies* (L.) Karst), and ponderosa pine (*Pinus ponderosa* D.)) with contrasting extractive profiles. The differences of $\delta^{13}\text{C}$ values between the new and traditional pretreatment methods were within the precision of the isotope ratio mass spectrometry method used ($\pm 0.2\text{‰}$), and the differences between $\delta^{18}\text{O}$ values were not statistically significant. Although some unanticipated results were observed in fraser fir, the new ambient-temperature technique was deemed as effective as the more labor-consuming and toxic traditional pretreatment protocol. The proposed technique requires a

separate acetone-inert multiport system similar to MSISS, and the execution of both pretreatment and main extraction steps allows for simultaneous treatment of up to several hundred microsamples from resinous softwood, while the need of additional labor input remains minimal.

Introduction

The stable isotope ratios of carbon and oxygen ($\delta^{13}\text{C}$ and $\delta^{18}\text{O}$) in tree rings are widely used in paleoclimatology, ecology, plant physiology, and genetics (Barbour 2007, Bartholomé et al. 2015, Dawson et al. 2002, Frank et al. 2015, McCarroll and Loader 2004, McNulty and Swank 1995, Saurer et al. 2014, Treydte et al. 2007). The isotopic composition of α -cellulose provides a historical record of a number of environmentally and genetically controlled processes (e.g. Baltunis et al. 2008, Wei et al. 2014), given that α -cellulose is abundant, is synthesized largely of newly assimilated carbon, and the C and O atoms in it do not exchange after its formation (Gaudinski et al. 2005). As the process of α -cellulose isolation from wood samples is usually labor-intensive and time-consuming, a number of different methods have been developed offering a different balance of speed, cost and purity.

Currently, there are over ten different methods and method variants to choose from for α -cellulose extraction from wood samples, including variants of the Jayme-Wise type (Green 1963, Leavitt and Danzer 1993, Loader et al. 1997, Li et al. 2011, Wieloch et al. 2011, Kagawa et al. 2015, Table 1), Brendel type (Brendel et al. 2000, Evans and Schrag 2004, see variants

in Brookman and Whittaker 2012), and the diglyme-HCl methods (Cullen and MacFarlane 2005, Macfarlane et al. 1999). The Brendel and diglyme-HCl methods are simple and fast (less than 24 hours to complete around 100 samples), and do not need special glassware (Brookman and Whittaker 2012, Cullen and MacFarlane 2005). However, the purity of α -cellulose extracted by the Brendel method varies by species (Brookman and Whittaker 2012, see Gaudinski et al. (2005) and Dodd et al. (2008) for additional steps for improving sample quality). The diglyme-HCl method may not be effective with wood with high resin and lignin contents (Cullen and MacFarlane 2005). A recent modification of the diglyme-HCl method was found to be successful for two conifer species (Li and Liu 2013), but remains to be tested more broadly, especially with species with a high lignin content. On the other hand, the Jayme-Wise type methods have been found to produce consistently pure α -cellulose (Gaudinski et al. 2005, Kéri et al. 2015). Based on a blind inter-laboratory comparison study, Boettger et al. (2007) found that the different α -cellulose extraction methods (all Jayme-Wise type) used in 9 European laboratories produced similar results within the precision of isotope ratio mass spectrometry ($\pm 0.2\%$ for C and $\pm 0.3\%$ for O). The methods consist of two major steps: a) delignification with acidified sodium chlorite solution (chlorination), and b) alkaline hydrolysis with 17% sodium hydroxide solution (purification). For conifers, an additional pretreatment step is usually required for removing extractives prior to extraction (Green 1963, McCarroll and Loader 2004).

As the stable isotope analysis becomes cheaper and faster, processing a large number of samples becomes possible to reveal spatial and temporal complexities at a larger scale (Leavitt et al. 2010) and cellulose extraction becomes the rate-limiting step. Thus new methods for

Table 2.1 Comparison of major batch-wise Jayme-Wise α -cellulose extraction methods from wood samples.

| Methods | Special equipment ^e | Estimated capital cost ^h | Typical number of samples per batch | Typical processing time in days ⁱ | | |
|---|--|-------------------------------------|---|--|--------------|--|
| | | | | Main extraction | Pretreatment | Total processing time per batch/per 1000 samples |
| Leavitt and Danzer (1993) ^{ab} | Soxhlet extractors, special filter paper bags ^f | <\$3,000 | 75-150 | 2 | 2-3 | 4-5/19-20 ^j |
| Kagawa et al. (2015) ^{ac} | Water bath, transmitted light microscope, PTFE ^g punch sheets, and glass tubes | <\$3,000 | The number of rings varies due to the ring width. Usually hundreds to thousands rings can be processed in a batch | 2 | 1 | 3/3 |
| Loader et al (1997) ^{ad} | Customized Soxhlet extraction thimbles, ultrasonic bath, Soxhlet extractors | \$5,000-10,000 | 100 | 1 | 1 | 2/20 |
| Harada et al. (2014) ^c | Microscope, customized polyethylene filters ^f , water bath, ultrasonic bath, PTFE ^g Tube, and glass container | \$5,000-10,000 | 60 | 2 | 1 | 3/48 |
| Wieloch et al (2011) ^a | Customized multiple sample isolation system (MSISS drainage module), Büchner funnels, vacuum aspirator pump, water bath | >\$15,000 | ≥320, expandable to higher numbers | 5 | Not equipped | 5/15 |
| Wieloch et al 2011 + acetone pretreatment (current study) | Customized multiple sample isolation system (MSISS drainage module) and Delrin holders, Büchner funnels, vacuum aspirator pump, water bath | >\$15,000 | ≥320, expandable to higher numbers | 5 | 8 | 13/29 ^j |

^a The experimental protocol has been updated since publication. Interested researchers please contact the authors for the latest information. The number of samples per batch can be increased by having additional equipment. Please note that additional cost and labor input would be required in this case.

^b The information related to this method has been complemented by Dr. SW Leavitt (personal communication, 2016).

Table 2.1 Continued

^c The information related to this method has been complemented by Dr. T Nakatsuka (personal communication, 2016).

^d The information related to this method has been complemented by Dr. N Loader (personal communication, 2016).

^e The equipment listed are specific for α -cellulose extraction, in a typical ecological wet lab setting as identified in the original publications when possible. Standard laboratory equipment like water purifier, centrifuge, and hot plates are not included. The tools for wood sample preparation (grinding or slicing) are not included as well.

^f Items are disposed after use.

^g PTFE: Polytetrafluoroethylene.

^h Cost estimates are approximate, aimed to group the methods in broad categories rather than offer clear budgetary information. The exact costs will vary by country, vendor, and existing infrastructure. Please see Table S.1 in Appendices for the cost estimates for the major equipment of each method. Although the reagent cost is proportional to reaction time and individual sample reaction volume, which differs up to 10-fold among the methods, their effect on overall cost is much smaller than that of the specialized equipment, and is not included here.

ⁱ The processing time is estimated based on literature reports except for the methods by Wieloch et al. (2011). The time estimates are approximate, and the exact time will vary by the researcher, species, and availability of equipment. Processing time does not include sample preparation (slicing or grinding), loading, and drying, or equipment clean-up and maintenance.

^j The extraction and pretreatment are staggered.

batch-wise cellulose isolation (Table 2.1) have emerged in recent years. The method for extracting entire intact tree cores (laths; Li et al. 2011; Kagawa et al. 2015) offers the greatest throughput on a per tree-ring basis, but not on per sample basis (see Schollaen et al. (2015) for a complete guide with a costly but convenient semi-automated extraction system, Table 2.1). With this method, α -cellulose is extracted from intact cross-sectional laths, yielding “cellulose laths” that retain their structural integrity. Cellulose fibers are pinched with forceps from the annual rings under transmitted light for stable isotope analysis (Li et al. 2011, Kagawa et al. 2015). This method eliminates the time- and labor-intensive peeling-grinding step to produce individual wood samples and made a breakthrough in the throughput of α -cellulose extraction methods to produce tree-ring chronologies. However, in genetic trials and many ecophysiological studies where individual years (rather than full chronologies) or other subsections from trees are of interest, the peeling-grinding step cannot be avoided and the whole lath extraction loses its advantage.

Currently, the highest throughput for the separated wood samples can be achieved using the Multiple Sample Isolation System for Solids (MSISS) developed at the Potsdam Dendro Laboratory, German Research Centre for Geosciences, Germany (Wieloch et al. 2011). It was designed to be modular and extendable to over 300 samples per batch. Although the extraction process takes relatively longer compared to other methods (Table 2.1), the minimized reaction volume, modular design and vacuum-operated evacuation of consumed chemicals in MSISS allows for greater throughput and significant labor savings.

However, the design considerations of MSISS were based on the chlorination and purification steps described above, but not on the pretreatment step for extractive removal from conifer wood. As the extractives have an isotopic signature distinct from α -cellulose (Harlow et al. 2006), their presence can bias $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$, pointing to the need to ensure the purity and homogeneity of the sample material (Tao et al. 2010). The need for pretreatment appears to be species-dependent and remains to be debated. Some authors have argued that extractives are removed during the main extraction steps of the Jayme-Wise protocol (Boettger et al. 2007, Rinne et al. 2005), whereas others concluded that an explicit pretreatment was required (Tao et al. 2010). As each of these studies has focused on a few species, the need for the pretreatment step as a general protocol remains a matter of discussion.

The current study was set up to develop and test a pretreatment step for the MSISS-based extraction system to expand the usability of this powerful method to resinous species. The traditional pretreatment technique used in dendrochronological studies (Green 1963, Loader et al. 1997) requires refluxing wood slivers in a mixture of toluene and denatured alcohol for at least 6 hours in Soxhlet extractors, which is not compatible with MSISS. An alternative protocol of pretreatment is achieved by soaking wood slivers in acetone at ambient temperature for eight days (Yokoyama et al. 2002). This technique, popular in wood science but less known in dendrochronology and ecology, was tested for effectiveness in extracting tree ring α -cellulose samples from different conifer species with contrasting extractive profiles. Additional tests for method sensitivity were performed on loblolly pine (*Pinus taeda* L.), the most important commercial tree species in the United States, as the work was carried out as a part of the PINEMAP project (<http://pinemap.org>; Will et al. 2015). The current report presents

a potential alternative pretreatment step for extractive removal using the high-throughput MSISS apparatus. Thus, the specific objectives of the current study were to (a) test if a pretreatment step to remove extractives is necessary for α -cellulose extraction in loblolly pine growing in contrasting environments; and (b) test if the modified acetone pretreatment can produce comparable results of isotopic signatures to those produced by traditional toluene-based pretreatment method, using six conifer species with different profiles of extractives.

Materials and methods

Materials and experimental design

Six species with contrasting resin profiles were chosen for the study. Wood samples (10-30 mg, allowing for α -cellulose yield of 30%) were collected from middle-aged to mature loblolly pine, Norway spruce (*Picea abies* (L.) Karst.), fraser fir (*Abies fraseri* (Pursh) Poir.), ponderosa pine (*Pinus ponderosa* D.), Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) and black spruce (*Picea mariana* Mill.) trees (Table 2.2). Loblolly pine was subsampled to evaluate the resolution of the method for detecting range-wide variance, and inter-annual differences between wet and dry years. The additional species were selected to span conifer species with a range of extractive contents in the xylem. For deriving plant water status and intrinsic water use efficiency in a given year, α -cellulose was extracted from the latewood portion of the rings because earlywood may be partly produced using carbohydrate reserve produced in the previous year (McCarroll and Loader 2004). In each species except black spruce, whose wood

Table 2.2 Samples used for evaluating the effectiveness of acetone pretreatment in a multiport extraction system for analyzing the isotopic composition of α -cellulose.

| Species | Location | The year of latewood sampled | number of replicates |
|--|--|----------------------------------|-----------------------|
| Loblolly pine (<i>Pinus taeda</i> L.) | Clarke County, Georgia, USA | 2010 (wet year), 2008 (dry year) | 3 and 10 ^c |
| Loblolly pine | Washington County, North Carolina, USA | 2008 (dry year) | 3 |
| Loblolly pine | Buckingham County, Virginia, USA | 2002 (dry year) | 3 |
| Loblolly pine | McCurtain County, Oklahoma, USA | 2011 (dry year) | 3 |
| Loblolly pine | Alachua County, Florida, USA | 2004 (wet year) ^a | 3 |
| Norway spruce (<i>Picea abies</i> (L.) Karst.) | Elva, Estonia | 2011 (dry year) | 3 |
| Fraser fir (<i>Abies fraseri</i> (Pursh) Poir.) | Boone County, North Carolina, USA | 2008 (dry year) | 3 |
| Ponderosa pine (<i>Pinus ponderosa</i> D.) | Klamath County, Oregon, USA | 1994 (dry year) | 3 |
| Douglas-fir (<i>Pseudotsuga menziesii</i> (Mirb.) Franco) | Klamath County, Oregon, USA | 1994 (dry year) | 3 |
| Black spruce (<i>Picea mariana</i> Mill.) | Saskatchewan, Canada | Multiple years ^b | 3 |

^a: the latewood produced during dry years was too thin for separation. Thus latewood produced in a wet year was used.

^b: Because the wood material in one annual ring of black spruce did not meet the minimal weight requirement for α -cellulose extraction, wood from multiple rings were used.

^c: the wood samples were used for two studies with different number of replicates.

materials were obtained from tree cores, entire wood disks were cut at breast height and latewood slivers were sampled from selected growth rings. As the occurrence of extractives is expected to be higher under drought stress (Lautner 2013), and the goal of the current study was to critically evaluate the effectiveness of a new extractive removal step, samples from dry years were selected when possible. However, in the case of loblolly pine from Florida, wood from a wet year was analyzed because the growth rings in dry years were too narrow to sample. In addition, multiple adjacent annual growth rings were combined in black spruce, because the core material in one annual ring did not provide the minimum required sample weight (10 mg, according to the yield rate and guideline on sample weight from Cornell Stable Isotope Laboratory where the stable isotope analysis was conducted). Meteorological dry years were identified using the United States Drought Monitor (<http://droughtmonitor.unl.edu/>) for samples from the United States, and site-specific meteorological records from the Estonian Meteorological and Hydrological Institute (<http://www.ilmateenistus.ee>) for samples in Estonia.

Four separate experiments were conducted (Table 2.3). To reach our first objective, α -cellulose was extracted from two loblolly pine latewood samples produced in a dry and a wet year from GA, USA, with and without traditional pretreatment (3 replicates, Experiment 1). Experiment 2 through 4 were designed to test if the modified acetone pretreatment by Yokoyama et al. (2002) (acetone pretreatment thereafter) can produce comparable results of isotopic signatures to those produced by traditional pretreatment method. Because composition and content of extractives may vary with locations, we extracted α -cellulose with traditional and acetone pretreatments using wood samples of loblolly pines from 5 states (VA, NC, GA, FL, and OK)

across the southeastern USA with 3 replicates (Experiment 2). Specifically, a fraction of latewood samples from GA, was selected for a full factorial analysis of wet and dry year difference in 10 replicates (Experiment 3) to capture any variation of the stable isotope analysis beyond the precision of the isotope ratio mass spectrometry (IRMS) method used. Finally, we

Table 2.3 The purposes of experiments and statistical analysis methods used.

| Experiment | Purpose | Plant material | Replicas | Factors and levels for two way ANOVA |
|------------|---|--|----------|---|
| 1 | Evaluate the need for extractives removal pretreatment for loblolly pine | Loblolly pine (GA) | 3 | <i>Pretreatment:</i> with/without traditional pretreatment <i>Climate:</i> wet/dry |
| 2 | Evaluate the effectiveness of acetone pretreatment for loblolly pine | Loblolly pine (FL, GA, NC, OK, VA) | 3 | <i>Pretreatment:</i> traditional/acetone pretreatments <i>Location:</i> 5 states in southeastern US |
| 3 | Evaluate the utility of the acetone pretreatment for capturing the variation in the isotopic composition of α -cellulose | Loblolly pine (GA) | 10 | <i>Pretreatment:</i> traditional/acetone pretreatments <i>Climate:</i> wet/dry |
| 4 | Evaluate the effectiveness of acetone pretreatment across 5 conifer species with contrasting resin profiles | Norway spruce, fraser fir, ponderosa pine, Douglas-fir, black spruce | 3 | <i>Pretreatment:</i> traditional/acetone pretreatment <i>Species:</i> 5 conifer species |

tested acetone pretreatment on 5 coniferous species: black spruce, ponderosa pine, Douglas fir, fraser fir, and Norway spruce (Table 2.2) for wider application of this method with 3 replicates (Experiment 4).

The ^{13}C and ^{18}O stable isotope ratios of all extracted α -cellulose were determined at the Cornell University Stable Isotope Laboratory (<http://www.cobsil.com>), using Thermo Delta V isotope ratio mass spectrometer interfaced to a NC2500 elemental analyzer and to a Temperature Conversion Elemental Analyzer. The within-run isotopic precision of the methodology using quality control standards is 0.2‰ for carbon and 0.4‰ for oxygen.

Extraction apparatus (MSISS and Delrin holders)

With the goal of increased sample throughput, and with minimum labor input, the Potsdam Dendro Laboratory (Wieloch et al. 2011) developed the Multiple Sample Isolation System for Solids (MSISS). Following their CAD drawings, we manufactured a set of MSISS drainage modules at the North Carolina State University Precision Instrument Machine Shop (<http://www.engr.ncsu.edu/machinshop>). We modified the unit dimensions to accommodate the available Pyrex® 2mL Büchner funnels with 10mm-diameter coarse porosity fritted discs.

Due to the different corrosiveness of acetone (used in pretreatment) and sodium hydroxide and sodium chlorite (used in the main extraction steps), we had developed a separate MSISS-like module out of Delrin (polyoxymethylene, Figure 2.1), which is resistant to acetone and cheaper than Polytetrafluoroethylene (PTFE) used for MSISS.

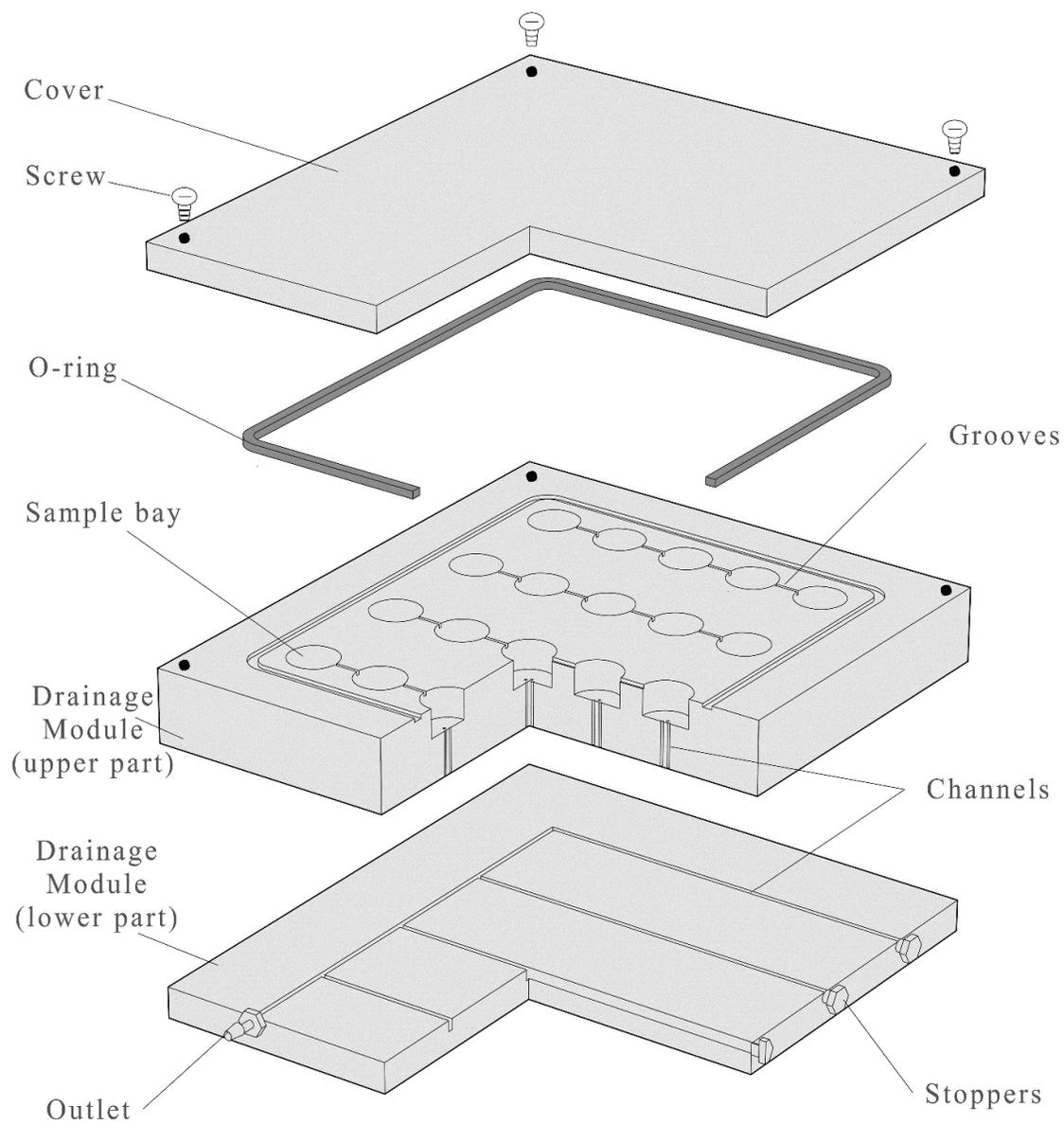


Figure 2.1 The schematic drawing of Delrin holder for acetone pretreatment.

The body of the Delrin holder is made of a solid Delrin block which encases a network of channels. The main difference of the pretreatment module compared to the MSISS module is

that the samples are treated in drilled-out sample bays rather than in Büchner funnels. Twenty sample bays are drilled in the same 4x5 arrangement as funnel holes on MSISS. Each bay is enlarged to 2 ml in volume so that it can hold wood slivers, while its bottom is connected to the inter-linked channel system inside the block by four 0.5 mm holes. A thin Delrin plate is used as a cover to prevent the evaporation of acetone with the help of an acetone-resistant O-ring. Once wood slivers are loaded into the sample bays, water or acetone is added, the cover is attached to the block with screws. The draining of extractant is done with a vacuum aspirator pump similar to MSISS.

α -Cellulose extraction

Each latewood sample was cut into approximately 0.3 mm thick slivers using a razor blade. Wood slivers of each sample were then mixed and divided into two fractions. For Experiment 1, one half of the slivers was prepared using the traditional pretreatment, that was carried out in a Soxhlet extractor using a 2:1 mixture of toluene and denatured alcohol, with 8 hours of refluxing (Loader et al. 1997) while the other half was not treated. For Experiment 2 through 4, the other half was prepared with acetone pretreatment, that was completed by an overnight soaking in deionized water followed by 8-day-soaking in acetone (acetone was replaced every 2 days), modified from Yokoyama et al. (2002).

The samples were treated identically after pretreatment and tree ring α -cellulose was extracted using MSISS. The extraction protocol was adopted from Wieloch et al. (2011) except that each step of chlorination was shortened to 7 hours from 10 hours due to the evaporation of solution from funnels and we repeated chlorination until cellulose became pure white. After extraction,

α -cellulose samples were homogenized using a Branson 450 Sonifier Analog Cell Disruptor, similar to Laumer et al. (2009). The main steps of sample processing and α -cellulose extraction are illustrated in Figure 2.2.

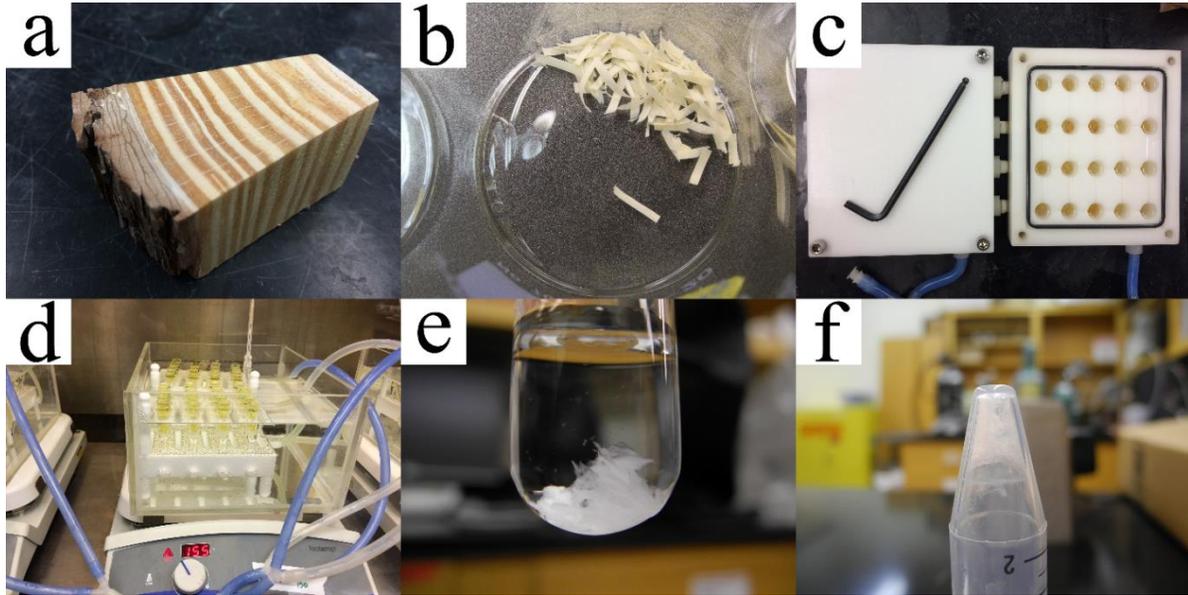


Figure 2.2 The main steps of α -cellulose extraction: (a) a surfaced wood wedge for sampling; (b) wood slivers for α -cellulose extraction; (c) slivers in Delrin holders with/without the lid (pretreatment); (f) α -cellulose extraction by MSISS; (g) extracted α -cellulose in chips and fibers; (h) homogenized and dried α -cellulose. (All photos by W. Lin. The glass tube in (g) and plastic vial in (h) are for demonstration only).

Statistical analysis

Statistical analyses were performed using the R software (Version 3.2.2; R Core Team, 2015).

Two-way analysis of variance (ANOVA) were conducted for Experiments 1 through 4 (Table

2.3). Data of $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ was analyzed the same way but separately except for $\delta^{18}\text{O}$ values

in Experiment 3, where an outlier was detected (more than 5 times the interquartile range

above the third quantile). We estimated a value to replace the outlier, applied two-way ANOVA, and corrected the bias according to Ott and Longnecker (2001) in this case.

Results

The necessity of pretreatment for α -cellulose from wood samples of loblolly pine

The need of a pretreatment step to remove extractives prior to chlorination and purification was tested in Experiment 1 (Table 2.3 and 2.4). Compared to the traditional toluene-based pretreatment, the omission of the pretreatment step resulted in 0.28‰ higher $\delta^{13}\text{C}$ estimates in the dry year, and 0.62‰ lower estimates in the wet year. The effect was statistically significant ($p < 0.01$), and exceeded the precision uncertainty of the IRMS method (0.2‰). For $\delta^{18}\text{O}$, the difference (-0.94‰ in the dry year and 0.54‰ in the wet year) exceeded the IRMS uncertainty threshold (0.4‰) but was not statistically significant ($p = 0.26$).

Table 2.4 Carbon and oxygen stable isotope ratios ($\pm 1\sigma$) of latewood α -cellulose extracted from samples of loblolly pine from GA, USA, with and without traditional pretreatment in two years with contrasting precipitation profiles (N=3).

| Samples | $\delta^{13}\text{C}$ (‰, VPDB) | | | $\delta^{18}\text{O}$ (‰, VSMOW) | | |
|-----------------------------|---------------------------------|-----------------|-----------------|----------------------------------|-----------------|-----------------|
| | Traditional pretreatment | No pretreatment | Mean difference | Traditional pretreatment | No pretreatment | Mean difference |
| Loblolly pine, GA, dry year | -24.74±0.02 | -25.01±0.04 | 0.28 | 32.9±0.25 | 33.84±0.09 | -0.94 |
| Loblolly pine, GA, wet year | -26.81±0.05 | -26.19±0.05 | -0.62 | 32.45±0.13 | 31.90±0.16 | 0.54 |

Comparison of two pretreatments using stable isotope ratios

The $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values in Experiments 2 through 4 were examined to test the effectiveness of the acetone pretreatment compared to the traditional pretreatment (Table 2.5). The mean difference between the traditional and acetone pretreatments was 0.01‰ for $\delta^{13}\text{C}$ (ranging from -0.07‰ to 0.16‰), and 0.12‰ for $\delta^{18}\text{O}$ (ranging from -0.45‰ to 0.74‰). If the data of Fraser fir are excluded, the mean difference was 0.06‰ for $\delta^{18}\text{O}$, ranging from -0.45‰ to 0.32‰.

The two-way ANOVA on loblolly pine samples from 5 locations in southeastern United States (Experiment 2) indicated that the main effect of pretreatment was statistically significant for $\delta^{13}\text{C}$ ($p=0.03$), while insignificant for $\delta^{18}\text{O}$ ($p=0.99$). When the sample size increased to 10 (Experiment 3 with loblolly pine samples from GA, Figure 2.3), we obtained similar results ($p<0.01$ for $\delta^{13}\text{C}$ and $p=0.77$ for $\delta^{18}\text{O}$). However, the 95% confidence intervals ([0.01‰, 0.08‰] for the wet year and [0.05‰, 0.18‰] for the dry year) between means of $\delta^{13}\text{C}$ from Experiment 3 were smaller than the $\pm 0.2\%$, the resolution of the IRMS method.

Unlike in loblolly pine, the differences between the two pretreatments were not statistically different in the other species ($p=0.59$ for $\delta^{13}\text{C}$ and $p=0.09$ for $\delta^{18}\text{O}$, Table 2.5). With Fraser fir excluded, the p -value for the main effect of pretreatment on $\delta^{18}\text{O}$ increases to 0.32.

Table 2.5 Carbon and oxygen stable isotope ratios ($\pm 1\sigma$) of α -cellulose extracted from samples of loblolly pine, ponderosa pine, black spruce, Douglas-fir, Norway spruce, and fraser fir with acetone pretreatment and toluene pretreatment.

| Samples | N | $\delta^{13}\text{C}$ (‰, VPDB) | | | $\delta^{18}\text{O}$ (‰, VSMOW) | | |
|---------------------------|----|---------------------------------|--------------------------|-----------------|----------------------------------|-------------------------------|------------------------------|
| | | Acetone pretreatment | Traditional pretreatment | Mean difference | Acetone pretreatment | Traditional pretreatment | Mean difference |
| Loblolly, GA, dry year | 10 | -24.25 \pm 0.02 | -24.36 \pm 0.02 | 0.11 | 32.39 \pm 0.06 | 32.34 \pm 0.07 | 0.05 |
| Loblolly, GA, wet year | 10 | -26.74 \pm 0.01 | -26.79 \pm 0.01 | 0.05 | 30.01 \pm 0.15 | 30.14 \pm 0.21 ^a | -0.13 |
| Loblolly, NC | 3 | -25.47 \pm 0.02 | -25.44 \pm 0.05 | -0.03 | 31.73 \pm 0.16 | 31.49 \pm 0.11 | 0.24 |
| Loblolly, OK | 3 | -24.84 \pm 0.03 | -24.77 \pm 0.02 | -0.07 | 33.30 \pm 0.10 | 33.12 \pm 0.20 | 0.18 |
| Loblolly, VA | 3 | -25.14 \pm 0.02 | -25.30 \pm 0.05 | 0.16 | 32.04 \pm 0.16 | 32.49 \pm 0.33 | -0.45 |
| Loblolly, FL | 3 | -26.25 \pm 0.03 | -26.29 \pm 0.07 | 0.04 | 32.11 \pm 0.21 | 31.97 \pm 0.17 | 0.14 |
| Ponderosa pine | 3 | -22.96 \pm 0.02 | -22.99 \pm 0.01 | 0.03 | 30.77 \pm 0.12 | 30.48 \pm 0.15 | 0.29 |
| Black spruce | 3 | -23.88 \pm 0.03 | -23.83 \pm 0.15 | -0.05 | 25.03 \pm 0.10 | 25.16 \pm 0.02 | -0.13 |
| Douglas-fir | 3 | -22.93 \pm 0.02 | -22.87 \pm 0.02 | -0.06 | 30.25 \pm 0.17 | 29.93 \pm 0.11 | 0.32 |
| Norway spruce | 3 | -26.67 \pm 0.02 | -26.65 \pm 0.00 | -0.02 | 23.59 \pm 0.38 | 23.47 \pm 0.52 | 0.12 |
| Fraser fir | 3 | -24.26 \pm 0.15 | -24.22 \pm 0.06 | -0.04 | 27.94 \pm 0.34 | 27.20 \pm 0.27 | 0.74 |
| <i>Average</i> | | | | <i>0.01</i> | | | <i>0.12/0.06^b</i> |
| <i>Standard deviation</i> | | | | <i>0.07</i> | | | <i>0.29/0.23^b</i> |

^a: an outlier was excluded

^b: the average and standard deviations of $\delta^{18}\text{O}$ mean differences between acetone and traditional pretreatments for α -cellulose extraction were calculated with and without data from Fraser fir.

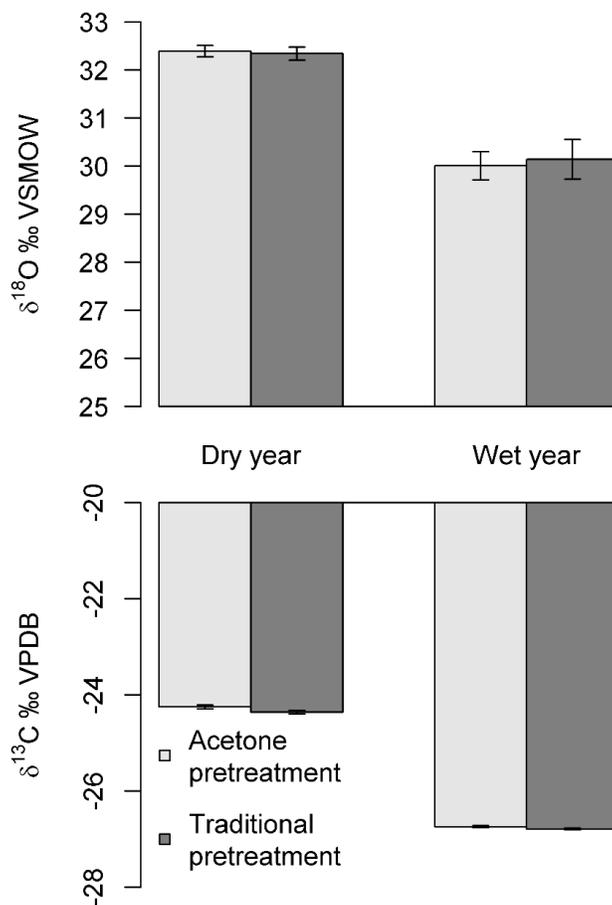


Figure 2.3 Mean carbon and oxygen stable isotope ratios (with 95% confidence intervals) of α -cellulose extracted from wood samples of loblolly pine from GA, USA in two years (a wet and a dry year) with acetone pretreatment and traditional pretreatment (n=10, see Table 2.3).

Discussion

The necessity of pretreatment for α -cellulose from wood samples

The need for an explicit extractive-exclusion treatment prior to α -cellulose extraction remains open to debate. Most Jayme-Wise methods include this step (Green 1963, Leavitt and Danzer 1993, Loader et al. 1997, Li et al. 2011, Kagawa et al. 2015). Yet, some studies argued that the

extractives in at least some conifer species are removed in the regular 2-step α -cellulose extraction (e.g., Rinne et al., 2005). However, it is also recognized that contamination by lipids may be possible if the pretreatment step is omitted (Rinne et al., 2005; Tao et al., 2010). Our current findings lend support to this argument.

Compared to primary carbohydrates, lipids are generally more depleted in ^{13}C (Melzer and Schmidt 1987), whereas the reported $\delta^{13}\text{C}$ values of α -cellulose are usually higher than other wood components (e.g., Loader et al., 2003). However, our study found that the $\delta^{13}\text{C}$ values of α -cellulose extracted from wood samples produced in a wet year without pretreatment (presumably with more remaining lipids) were also enriched compared to those with pretreatment (Table 4). This is in agreement with the study reported by Taylor et al. (2007). The authors compared the $\delta^{13}\text{C}$ values of extractives and α -cellulose of Douglas fir, and some of the former were enriched compared to the latter. Thus, the pattern of $\delta^{13}\text{C}$ of extractives and α -cellulose appears to be more complicated, probably due to the different components of extractives produced in a specific year and those that remain after the extraction processes.

Comparison of two pretreatments

The acetone pretreatment step arguably removes over 95% of the nonvolatile extractives from the wood of loblolly pine (Yokoyama et al. 2002). In the current study, we found that the acetone pretreatment adapted for the multiport system produced comparable results to those by traditional toluene-based pretreatment. Although the $\delta^{13}\text{C}$ signatures were significantly different for loblolly pine samples following these two pretreatments (Table 5), the differences were smaller than the accuracy of the subsequent IRMS. However, the difference of $\delta^{18}\text{O}$

values of fraser fir samples between the two pretreatments is unexpected. As a species without resin canals, the pretreatment was expected to have no effect on fraser fir samples.

Given the success of this acetone-based pretreatment in most tested species in terms of stable isotope ratios, we propose this pretreatment as a viable replacement for the more labor-consuming and toxic traditional toluene-based pretreatment in applications where individual wood samples are to be analyzed. For the laboratories which apply less highly equipped variants of the Jayme-Wise method using Teflon filter bags like Leavitt and Danzer (1993), the acetone pretreatment can be easily adopted by allowing multiple bags soaking in deionized water and acetone within a sealed container. Although the technique worked reliably in 5 out of 6 species, the unexpected result in fraser fir for the difference of $\delta^{18}\text{O}$ suggests that validation with new species is advisable.

Sample preparation and further methodological suggestions

The MSISS method (Wieloch et al. 2011) is recommended for small samples (2.5-50 mg). Given that the 2 ml well size in the pretreatment module is sufficient for extracting this amount of sample with acetone (H. Chang, personal communication, 2012), our proposed pretreatment system is well suited for coupling with MSISS.

Directions on both grinding and slicing are available from Wieloch et al. (2011). Delrin holders with the design as shown in Figure 2.1 work best with sliced wood samples. Wiley mill, ball mill, or Wig-L-Bug grinding mill would cause major or complete sample loss if the

wood material is less than 10 mg. In such situations, slicing wood samples becomes the only option.

However, when sample size allows homogenization by grinding (see Borella et al. (1998) for a theoretical calculation and discussion on pooling and milling for sample homogeneity), the powder of ground samples may block the channels at the bottom of sample bays of the Delrin holders, complicating sample transfer from the Delrin to MSISS module. If wood powder is used for α -cellulose extraction, we recommend adding a layer of molded stainless steel mesh to each access point and increasing the wall height of the Delrin holder so that the cover can still seal well after this addition. As containers for individual wood samples, the mesh layers would also make transferring wood samples to MSISS from Delrin holders more convenient.

Conclusions

Our results suggest that the chlorination and purification steps may not remove the majority of extractives in wood of loblolly pine, and that an explicit pretreatment step may be necessary for conifer species. The modified acetone pretreatment based on Yokoyama et al. (2002) was as effective as the traditional toluene-based methods for removing extractives from the wood of five widely spread conifer species. The method is easy and safe to apply to MSISS and other Jayme-Wise variants using Teflon filter bags. When combined with MSISS, the labor savings from the standardized and MSISS-compatible system quickly offset the upfront equipment costs for a different solvent-resistant sample processing apparatus (e.g. Delrin holder).

Although this pretreatment method worked well with 5 out of 6 common and contrasting conifer species, we recommend that additional tests be performed with new species to confirm efficacy.

Chapter 3. Drought response and spatial variability of carbon isotope discrimination of loblolly pine across the natural range in the southeastern USA

Abstract

Carbon isotope discrimination ($\Delta^{13}\text{C}$) in latewood α -cellulose was quantified for wet and dry years from 76 locations across the natural range of loblolly pine. $\Delta^{13}\text{C}$ exhibited a threshold response to Palmer drought severity index (PDSI), with a positive linear dependence at $\text{PDSI} < 1$ and invariant $\Delta^{13}\text{C}$ above that. The baseline C discrimination ($\Delta^{13}\text{C}$ at $\text{PDSI} = 1$) ranged from 18.0‰ to 20.8‰, with a mean of 19.4‰, and is influenced by life time PDSI (the average PDSI from the planting year to the sampling year), soil sand content, the ratio of annual precipitation and potential evapotranspiration (PPT/PET), latitude, and sampling age. Specially, site water status, characterized by ground water table depth, was found to have a primary impact in baseline $\Delta^{13}\text{C}$, indicating the determining effect of stomatal conductance in shaping $\Delta^{13}\text{C}$, despite the humid environment ($\text{PPT/PET} > 1.2$ at all sites) in southeastern USA. Drought sensitivity ($m = d\Delta^{13}\text{C}/d\text{PDSI}$) ranged from -0.14‰ to 1.11‰ (with an overall mean of 0.31‰) and showed distinct regional differences - lowest in the Atlantic Coastal Plain, and higher west of Mississippi. The most drought sensitive sites were in the far northeastern part of the range. Both genetic and environmental variations were found to contribute such a spatial pattern.

Introduction

Stable carbon isotope is an important tool in the study of plant ecophysiology. It provides a unique prospective and yielded some of the most significant findings in this field (Dawson et al. 2002). The study on the environmental and physiological controls of plant carbon isotope discrimination ($\Delta^{13}\text{C}$) is very extensive since 1980s. Specially, a mechanistic model to describe the processes at the leaf level has been successfully established (Farquhar and Cernusak 2012, Farquhar et al. 1989). However, empirical models are still the major approach to study the relations between environments and $\Delta^{13}\text{C}$ at the site- and higher levels, due to the complexity of participating factors, both environmental and physiological, in each step of fractionation (Dupouey et al. 1993), and the lack of understanding in post-photosynthetic fractionations (Gessler et al. 2014).

Such studies have been fruitful. $\Delta^{13}\text{C}$ is found to be sensitive to soil water availability (Cernusak et al. 2013, Warren et al. 2001), nutrient availability (Duursma and Marshall 2006), irradiance (Farquhar et al. 1989), tree height (Marshall and Monserud 1996), altitude (Hultine and Marshall 2000, Körner et al. 1991), and latitude (Körner et al. 1991). Of all these factors, water availability is generally seen as the most predominant driver (Cernusak et al. 2013). Dupouey et al. (1993) and Livingston et al. (1993) found linear dependence (Equation 3.1) between $\Delta^{13}\text{C}$ and soil extractable water content and transpiration (respectively), indicators of plant water availability.

$$\Delta^{13}\text{C} = \text{reference } \Delta^{13}\text{C} + m \cdot \text{water availability index} \quad (3.1)$$

where reference $\Delta^{13}\text{C}$ is the intercept of the formula, equals to $\Delta^{13}\text{C}$ when the water availability index = 0, while m is the slope and represents the sensitivity of the plant to the water availability index. We term it as drought sensitivity. Such a relationship indicates the need to tease apart the site-level $\Delta^{13}\text{C}$ fluctuations due to changes of water availability and the reference as a long-term mean $\Delta^{13}\text{C}$ under extreme water supply, which provides an integrated assessment of the impacts from the environments on the plant acclimation.

However, this relationship has not been considered in regional surveys of plant $\Delta^{13}\text{C}$ along transects/climate gradients which studied the relations between plant $\Delta^{13}\text{C}$ and climate, soil, and other environmental variables (e.g. Hemming et al. 2005, Körner et al. 1991, Schulze et al. 2006, Schulze et al. 1998, Schulze et al. 2014). Consequently, the literature reviews, which collected data for global synthesis, could not take site-level $\Delta^{13}\text{C}$ fluctuations into account (Diefendorf et al. 2010, Kohn 2010, Warren et al. 2001). Therefore, it is of great significance to introduce our knowledge on the temporal responses of $\Delta^{13}\text{C}$ to site water availability into the study of the spatial relations between $\Delta^{13}\text{C}$ and the environment, re-evaluate the contribution of environment and/or genetics to reference $\Delta^{13}\text{C}$, and examine such influence on drought sensitivity.

The current study was set up to analyze the range-wide and local variability in the responses of $\Delta^{13}\text{C}$ to drought of loblolly pine (*Pinus taeda* L.), the most important commercial tree species in southeastern USA (Will et al. 2015). Contributing to 36% of the sequestered forest carbon in the conterminous United States, the forests of southeastern USA are 35% loblolly pine, of which an increasing fraction (currently 52%) are plantations. As the health and

productivity of these forests are critical to the local economy and livelihoods, interest in their tolerance of and adaptability to shifting climate conditions are of great interest. Given the importance of precipitation to the local water balance, a meteorological drought index seem to be a proxy for site water availability. It was reported that Palmer drought severity index (PDSI), a location-normalized drought index based on water balance models (Palmer 1965), correlates significantly with observed soil moisture content (Dai et al. 2004). Thus PDSI was selected as the proxy for site water availability after our preliminary study indicated a roughly linear relationship between PDSI and $\Delta^{13}\text{C}$ of loblolly pine (Figure S.1, Appendices). In the current study, we explore the drought responses and spatial variability of loblolly pine across its range based on the latewood α -cellulose $\Delta^{13}\text{C}$ from adjacent wet and dry years. Specifically, our goals were to

- (i) quantify the reference $\Delta^{13}\text{C}$ and drought sensitivity across the range, and
- (ii) partition the differences to climatic, soil, site-specific, and genetic causes.

Materials and Methods

Study area

The natural range of loblolly pine is in the southeastern USA, along the Atlantic and Gulf of Mexico coasts, with range boundaries determined by annual actual evapotranspiration with 1050 mm on the south and 813 mm on the north (Schultz 1997). For the current study, we sampled 76 loblolly pine plantations in this region, established as part of different university-

industry co-operative studies between 1984 and 2002. The sampled sites (Figure 3.1A) spanned 10 states (AL, AR, FL, GA, LA, MS, NC, SC, TX, VA), and samples were collected from 2012 through 2014. The sampling sites were selected based on the availability of biomass and productivity data (contributed to the Terra-C database, <http://terrac.ifas.ufl.edu/>) by the university-industry co-operatives, total 126 sites), and age range (representative of commercial plantations, but past the juvenile stage (Jordan et al. 2008). See *Experimental design and isotopic analysis* for explanation on juvenile stage). The ages of the sampled stands ranged from 9 to 26 years (Figure 3.1C), with a mean of 16 years, while latitude (Figure 3.1D), altitude (Figure 3.1E), mean annual precipitation (PPT, Figure 3.1H), mean annual temperature (Figure 3.1I), and mean annual vapor pressure deficit (VPD, Figure 3.1J, see Data sources and conversion for calculation details) were 29.66°N - 37.44°N, 3-198 m, 1106 mm - 1545 mm, 13.6°C - 20.5°C, and 0.51 – 0.72 kPa (Data were obtained from PRISM Climate Group, Oregon State University, <http://prism.oregonstate.edu>). According to the Gridded Soil Survey Geographic (gSSURGO) Database (<http://websoilsurvey.sc.egov.usda.gov/App/WebSoilSurvey.aspx>), the available water capacity (AWC, Figure 3.1F) at top 100cm of soils ranged from 2.4cm to 11.5cm, and sand content ranged from 8% to 97% (Figure 3.1G).

Experimental design and isotopic analysis

Eight trees from the un-fertilized plots at each site were cored at breast height from 2012 through 2014. Cores were then oven-dried (at 60°C), mounted, surfaced, and dated using the

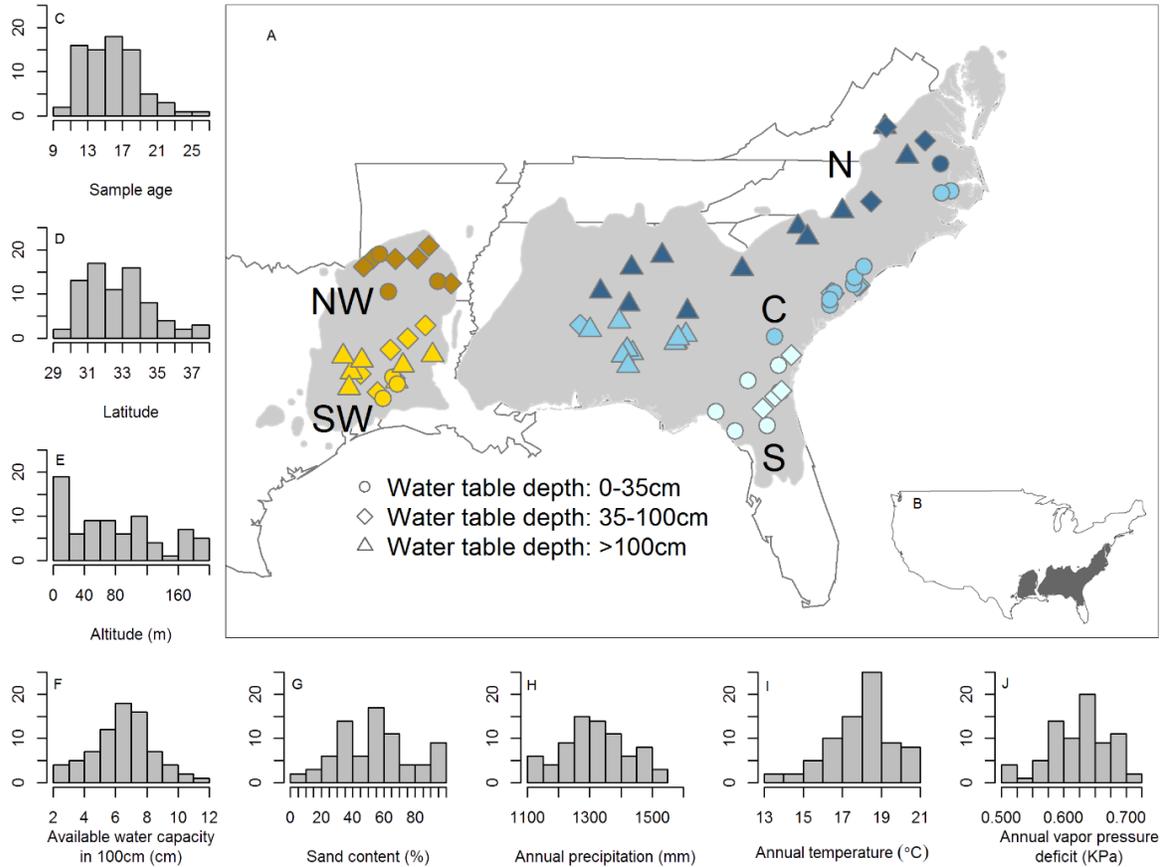


Figure 3.1 The location and characteristics of sampling sites. (A) The natural distribution of loblolly pine in southeastern USA (shaded area) with state borders (gray line) and site locations (points). Sites are colored by geographic regions: N (northern, dark blue points), C (central, medium blue points), S (southern, light blue points), NW (northwestern, brown points), and SW (southwestern, yellow points). The sites were also coded by water table depth. (B) The map of the United States with the natural distribution range of loblolly pine (shaded area). (C) The average of sampling ages of trees (two years were sampled at each site). (D) Site latitudes. (E) Site altitudes. (F) Available water capacity of top 100cm of soils. (G) Sand content in soils. (H) Mean annual precipitation. (I) Mean annual temperature. (J) Mean annual vapor pressure deficit. The vertical axis represents number of sites for histograms (C) through (J). See Data sources and conversion and Data analysis for description of data sources and their classifications.

identification of signature years (Speer 2010). The ring width was measured to the nearest 0.001 mm using a tree ring measuring system (Velmex Inc. Bloomfield, NY, USA) and cross-dated using the COFECHA program (Holmes 1983).

The Palmer drought severity index (PDSI, Table 3.1) was used to identify meteorologically wet and dry years for each climatological division. Monthly PDSI from each climatological division of the natural distribution range of loblolly pine were obtained from State Climate Office of North Carolina, United States. Two years with contrasting wetness were chosen based on both yearly and summer (May through August) PDSI. As there is a well-documented age-related trend in plant physiology, wood anatomy and allocation patterns (Gagen et al. 2011), these differences also reflect in the $\delta^{13}\text{C}$ of α -cellulose. The early years (juvenile stage) tend to have lower wood density (Jordan et al. 2008) and more negative $\delta^{13}\text{C}$ (McCarroll and Loader 2004). Thus, direct $\delta^{13}\text{C}$ values from this “juvenile wood” may not be consistent indicators of plant water status compared to later years with “mature wood”. However, for the purposes of the current study, that contrast wet and dry years, this confounding effect is likely much smaller. Nevertheless, we avoided the youngest of sites, and selected the study years from the recent history. The time between wet and dry years at the 76 sites was thus no more than 3 years, with 40 sites (53%) having the contrast years right next to each other, and 21 sites (28%) having them separated by a single year. Due to the climatic conditions of the sampling areas in recent years and the limitations from tree age, the PDSI of chosen “wet” years at 10 sites fell in the “incipient drought” category (PDSI=-0.99 to -0.5; Table 3.1), while the “wet” year at 2 sites fell into the category of “mild drought” (PDSI =-1.99 to -1, Table 3.1).

Table 3.1 Palmer drought severity index classes (PDSI) for wet and dry periods (Palmer 1965)

| PDSI | Class |
|----------------|---------------------|
| ≥ 4.00 | Extremely wet |
| 3.00 to 3.99 | Very wet |
| 2.00 to 2.99 | Moderately wet |
| 1.00 to 1.99 | Slightly wet |
| 0.50 to 0.99 | Incipient wet spell |
| -0.49 to 0.49 | Near normal |
| -0.99 to -0.50 | Incipient drought |
| -1.99 to -1.00 | Mild drought |
| -2.99 to -2.00 | Moderate drought |
| -3.99 to -3.00 | Severe drought |
| ≤ -4.00 | Extreme drought |

Given that earlywood may be produced using stored carbohydrates from the previous year (McCarroll and Loader 2004), whereas latewood is primarily formed from current year assimilates, we used the latter for α -cellulose extraction. α -cellulose was extracted according to Wieloch et al (2011), with an additional acetone pretreatment step (Lin et al. 2017). The ^{13}C stable isotope ratios of the extracted α -cellulose were determined at the Cornell University Stable Isotope Laboratory (<http://www.cobsil.com>) as reported previously (Lin et al. 2017). The within-run isotopic precision of the methodology using quality control standards was 0.2‰.

Carbon isotope discrimination ($\Delta^{13}\text{C}$, Equation 3.2) was calculated using $\delta^{13}\text{C}$ values of the extracted α -cellulose ($\delta^{13}\text{C}_p$) and of the atmosphere ($\delta^{13}\text{C}_a$) as:

$$\Delta^{13}\text{C}(\text{‰}, VPDB) = \frac{\delta^{13}\text{C}_a - \delta^{13}\text{C}_p}{1 + \delta^{13}\text{C}_p/1000} \quad (3.2)$$

Yearly averages of $\delta^{13}\text{C}_a$ of the sampling year were calculated from the monthly values measured at the Scripps CO₂ Program at La Jolla Pier, California (http://scrippsco2.ucsd.edu/data/atmospheric_co2/ljo).

Data sources and conversion

To explore the drivers of range-wide variability of reference $\Delta^{13}\text{C}$ and drought sensitivity (m), we extracted edaphic and climate variables from publicly available databases. Based on site geographic coordinates, we extracted available water capacity at the top 100cm of soil (AWC), bulk density, organic matter content, water table depth, clay content, sand content, and silt content from Gridded Soil Survey Geographic (gSSURGO) Database (<http://websoilsurvey.sc.egov.usda.gov/App/WebSoilSurvey.aspx>). Thirty-year normal mean annual precipitation (PPT), mean annual temperature (T), mean annual dew point temperature (T_d), and mean monthly temperature were obtained from PRISM Climate data (PRISM Climate Group, Oregon State University, <http://prism.oregonstate.edu>). Vapor pressure deficit (VPD) was calculated from mean annual temperature and mean annual dew point temperature according to Murray (1967). The potential evapotranspiration (PET) was calculated using Thornthwaite method based on mean monthly temperature (Ward and Trimble 2004).

We also explored the regional differences based on Bailey's ecoregion boundaries (Bailey 1983) and loblolly pine seed source transfer zone (Schmidting 2001). We report the results only for the aggregated seed source transfer zones as it depicts the regional differences better. Based on sampling locations and site density, we ended up classifying the 76 study sites to five geographic regions (Figure 3.1): north (N), central (C), south (S), northwest (NW), and southwest (SW), based seed source transfer zones. Given the nature of the significance of the barrier posed by Mississippi River (Schmidting 2001), we ultimately aggregated the five regions further to eastern and western parts of the range for regression analysis.

Data analysis

All data analysis was conducted with R software (R Core Team 2016). In order to explore the nonlinear response of $\Delta^{13}\text{C}$ to PDSI, segmented regression was conducted with "segmented" package (Muggeo 2008). To evaluate the effects of PDSI on $\Delta^{13}\text{C}$ at site level, linear mixed effect model was used via "nlme" package (Pinheiro et al. 2016) following Mehatatalo and Lappi (in press; personal communication), where PDSI was considered a fixed factor, and individual sites were the random factor. Models were fitted using restricted maximum likelihood method.

The segmented regression indicate a two-phase response of $\Delta^{13}\text{C}$ to PDSI, a positive relationship at $\text{PDSI} \leq 1$, and no relationship at $\text{PDSI} > 1$. Based on this finding, we define $\Delta^{13}\text{C}$ at $\text{PDSI} = 1$ as "baseline C isotope discrimination", representative of discrimination in well-watered conditions (baseline $\Delta^{13}\text{C}$, Figure 3.2). The slope of the regression line indicates the drought sensitivity of trees - change in $\Delta^{13}\text{C}$ per unit change in PDSI (Figure 3.2), allowing

for comparison of sites that experienced droughts of different intensity. To allow for comparison of slopes across sites with different dry and wet years, we also recoded the wet years with $PDSI > 1$ as $PDSI = 1$ (Figure 3.2B).

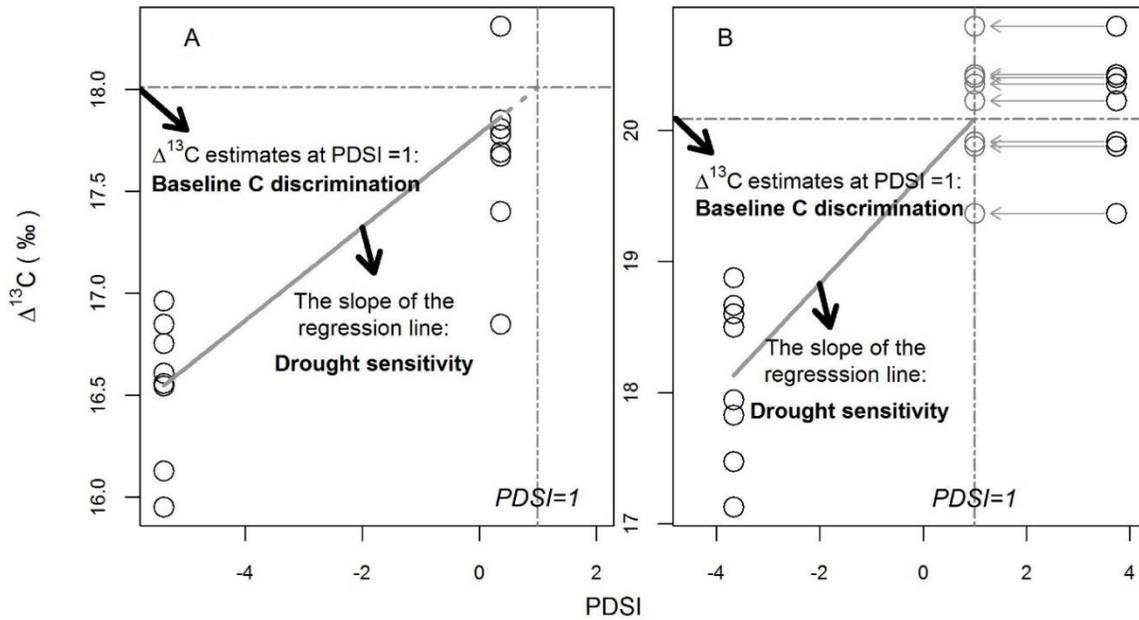


Figure 3.2 The schematic graphs for baseline carbon isotope discrimination and drought sensitivity based on observations (open circles) from two sites (A): site MRTS_2ndThin_6 from Diboll County, Texas, years 2009 and 2011 and (B): site MRTS-1stThin_15 from Delight County, Arkansas, years 2009 and 2011. The gray arrows and gray circles represent adjusted direction and adjusted data, respectively.

To explore the spatial patterns of baseline $\Delta^{13}\text{C}$ and drought sensitivity (m), we examined them with Moran’s I using the package “ape” (Paradis et al. 2004) and on maps using the packages “maps” (Becker et al. 2016) and “rgdal” (Bivand et al. 2016).

The multicollinearity of climate and soil parameters (obtained from PRISM and gSSURGO, respectively; Table 3.2) in partitioning variance in baseline $\Delta^{13}\text{C}$ and m was accounted for in the partial least squares regression, using package “plsdepot” (Sanchez 2012). Tree life time PDSI was calculated as the average of annual mean PDSI from planting year to the average of the two sampling years (wet and dry). The delineation of water table depth for different bins (<35 cm, 35-100 cm, and >100 cm) was determined by (i) data reporting in gSSURGO (water table depth is only available when it is shallower than 2 m to the surface), and (ii) typical loblolly pine rooting profile (over 80% of fine roots are in the top 35cm, and significant water uptake can occur down to 1 m).

Results

Isotopic discrimination and drought response metrics

The carbon isotopic discrimination, $\Delta^{13}\text{C}$, ranged from 15.27‰ to 22.29‰ across study area, with an average of 1.2‰ lower values during the dry than wet years. In well-watered conditions (PDSI>1), $\Delta^{13}\text{C}$ averaged 19.48‰, and decreased $0.32\pm 0.02\%$ per unit PDSI below (but not above) that (Figure 3.3A). The threshold PDSI between the well-watered and water-limited conditions, as determined by segmented regression, was 0.96 (95% CI [0.11, 1.81]), and was rounded to 1 for further analyses. The fitted regression models for the segments were

$$\Delta^{13}C_i = \begin{cases} 19.16 + 0.32PDSI + e_i, & \text{for } PDSI \leq 0.96 \\ 19.45 + 0.03PDSI + e_i, & \text{for } PDSI > 0.96 \end{cases} \quad (3.3)$$

The slope of the first segment was significantly different from zero ($p=2E-16$) with 95% confidence interval as [0.29, 0.35]. However, with the 95% confidence interval of the slope for the second segment ($PDSI>0.96$) spanning zero ([-0.11, 0.16]), $\Delta^{13}C$ is effectively unresponsive to PDSI under wet conditions.

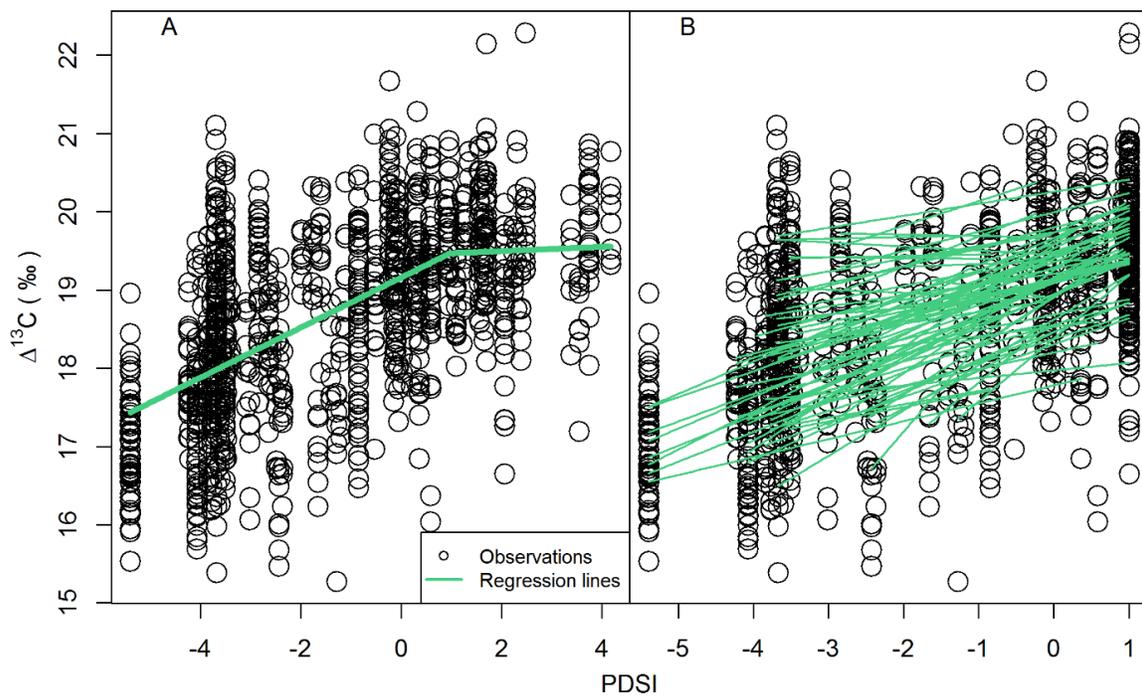


Figure 3.3 Carbon isotope discrimination ($\Delta^{13}C$, ‰) as a function of Palmer drought severity index (PDSI): (A) original data with a segmented regression line; (B) adjusted data with regression lines from a linear mixed-effect model.

Baseline $\Delta^{13}\text{C}$ and drought sensitivity and their spatial patterns

Because PDSI=1 is the borderline between “Incipient wet spell” and “Slightly wet” (Table 3.1), we chose PDSI=1 instead of 0.96 as the threshold where carbon isotope discrimination stops to respond to change of PDSI. After recoding PDSI for PDSI>1, we revised Equation 3.1 to Equation 3.4 and defined baseline $\Delta^{13}\text{C}$ as carbon isotope discrimination at high water availability (PDSI=1).

$$\Delta^{13}\text{C} = \text{Baseline } \Delta^{13}\text{C} + m \cdot (\text{PDSI} - 1) \quad (3.4)$$

m is still drought sensitivity. Baseline $\Delta^{13}\text{C}$ and drought sensitivity for each site were calculated from this adjusted $\Delta^{13}\text{C}$ -PDSI relationship (Figure 3.2 and 3.3B). Although the fitted parameters in the Equation 3.1 did not differ from 3.4 between the concepts, we used baseline $\Delta^{13}\text{C}$ rather than reference $\Delta^{13}\text{C}$, because the former represents the maximum $\Delta^{13}\text{C}$ at non-limiting water supply conditions and is thus a better parameter. The site-specific baseline $\Delta^{13}\text{C}$ ranged from 18.01‰ to 20.76‰, with a mean of 19.43‰. The drought sensitivity varied from -0.14 ‰ to 1.11‰, with an overall mean of 0.31‰. The drought sensitivity of 56 out of 76 sites (74%) was between 0.1‰ and 0.5‰.

Both baseline $\Delta^{13}\text{C}$ and drought sensitivity exhibited spatial correlation across sites (Figures 3.4 and 3.5). The spatial correlation of baseline $\Delta^{13}\text{C}$ was marginally significant (*Moran's I* = 0.08, $p=0.06$). The baseline $\Delta^{13}\text{C}$ was highest in South Carolina, and lowest in the sites around the Gulf Coast. However, the sites with intermediate baseline $\Delta^{13}\text{C}$ did not show a distinct spatial arrangement (Figure 3.4).

On the other hand, the spatial correlation of the drought sensitivity (change of $\Delta^{13}\text{C}$ at unit PDSI) was highly significant (*Moran's I* = 0.2, $p=9.8\text{E-}6$). The Atlantic coastal plain sites were less sensitive than sites further west. The most drought sensitive sites were in the far northeastern part of the range (Figure 3.5).

Controls of baseline $\Delta^{13}\text{C}$

Potential correlations between baseline $\Delta^{13}\text{C}$ and site, soil, and climate variables were evaluated using publicly available datasets (Table 3.2). Correlations between with baseline $\Delta^{13}\text{C}$ and site characteristics were stronger than with soil and climate variables ($p=0.01$, 0.05 , and 0.07 for life time PDSI, latitude, and sample age, respectively), while only one variable from each of the soil and climate category correlates with baseline $\Delta^{13}\text{C}$ with marginal significance ($p=0.06$ for both sand content and PPT/PET). Among five (marginally) significant correlates, life time PDSI, sand content, and PPT/PET were related to water availability at the site.

A partial least squares regression (PLSR) across the entire dataset explained 11% of the variance (Figure 3.6A). However, this increased to 50% when the data were stratified by water table depth (Figure 3.6B, Table 3.3). To test if the improved fit was due to stratification only, we modeled the baseline $\Delta^{13}\text{C}$ of the 26 sites west of the Mississippi River, whose water table depth spans from 13cm to deeper than 200cm. The R^2 of this region-specific PLSR model is 0.16, similar to that from the unstratified model. Therefore, smaller sample sizes in the stratified data sets was not a major reason for the greatly improved fit due to water table depth stratification.

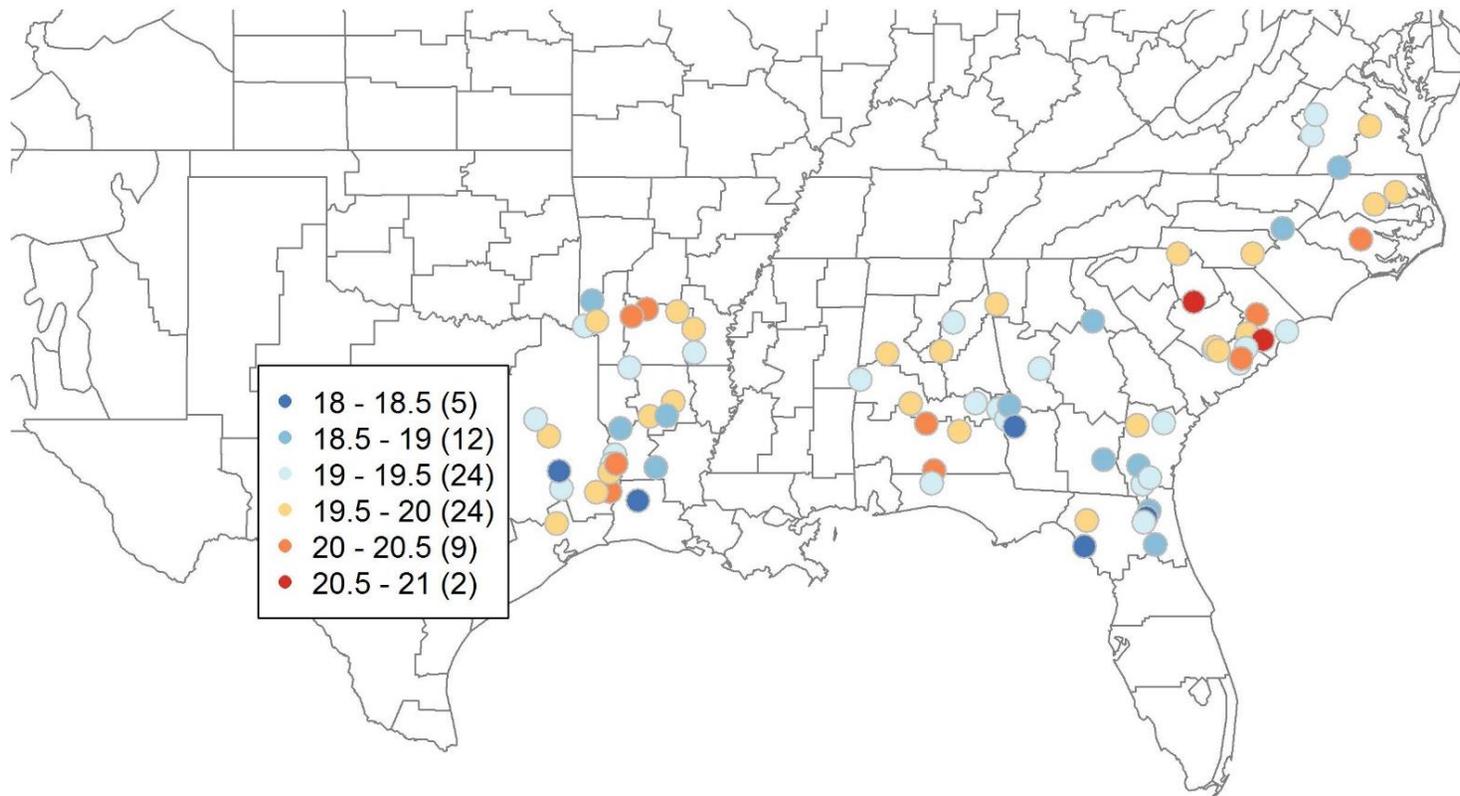


Figure 3.4 Baseline carbon isotope discrimination ($\Delta^{13}\text{C}$, ‰) at 76 loblolly pine plantations in southeastern USA. The GPS coordinates have been jittered to show all the sites. The numbers inside parentheses on the legend indicate the number of sites in each category.

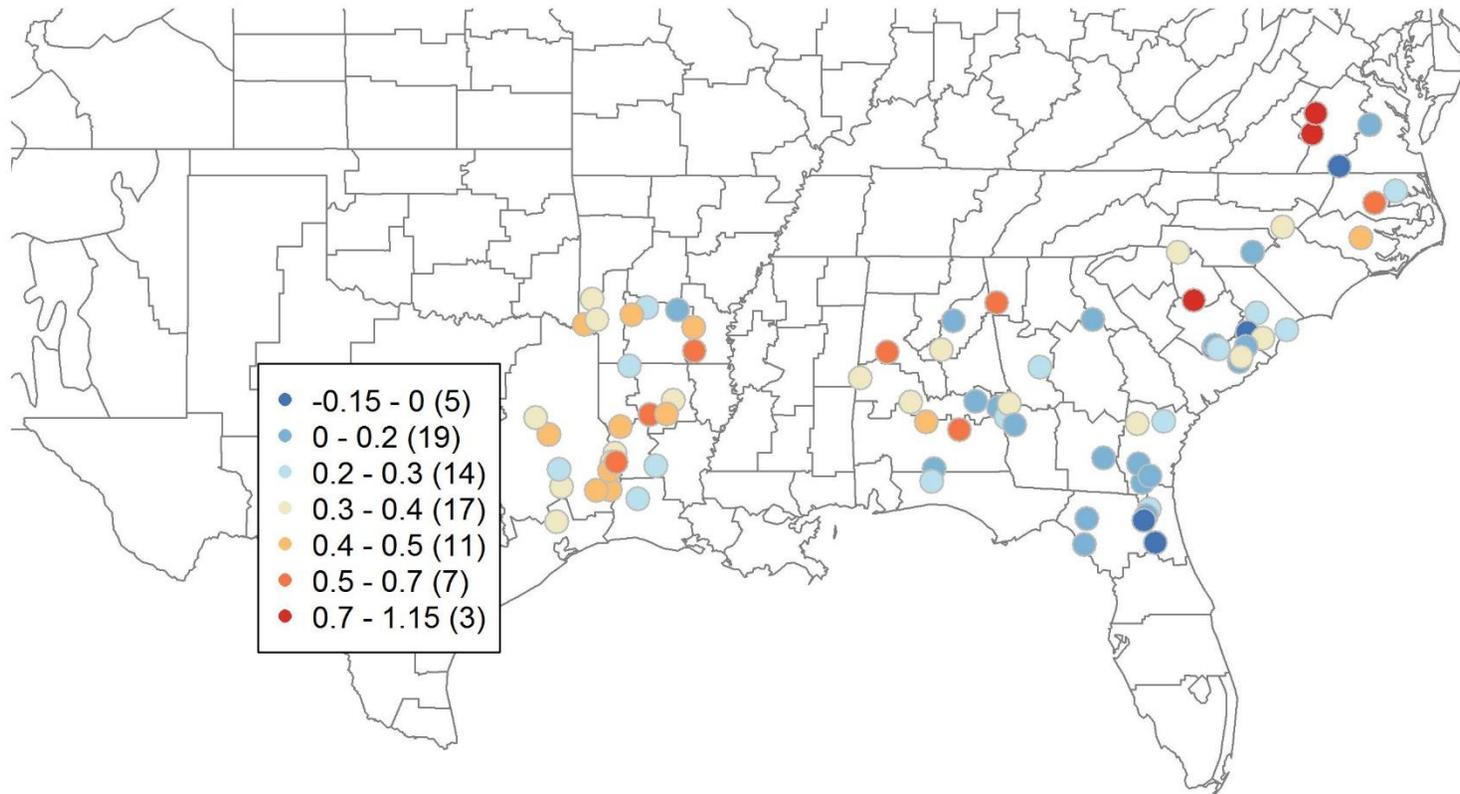


Figure 3.5 Drought sensitivity of carbon isotope discrimination (‰ per unit PDSI) in 76 sites in southeastern USA. The GPS coordinates have been jittered to show all the sites. The numbers inside parentheses on the legend indicate the number of sites in each category

Table 3.2 The Pearson correlation coefficients between baseline carbon discrimination and drought sensitivity and site-related, soil, and climate variables.

| Category | Variable | Baseline carbon discrimination | Drought sensitivity |
|--------------------------------|--|--------------------------------|---------------------|
| Site-related variables | Sample age | 0.21 | 0.05 ^{ns} |
| | Life time PDSI | 0.29 [*] | 0.30 ^{**} |
| | Altitude (m) | -0.09 ^{ns} | 0.30 ^{**} |
| | Latitude | 0.23 [*] | 0.26 [*] |
| | Longitude | 0.04 ^{ns} | -0.21 |
| Soil variables (from gSSURGO) | Sand content (%) | -0.22 | -0.24 [*] |
| | Clay content (%) | 0.16 ^{ns} | 0.16 ^{ns} |
| | Bulk density (cm ³ /cm ³) | 0.12 ^{ns} | 0.14 ^{ns} |
| | Organic matter content (%) | 0.07 ^{ns} | -0.01 ^{ns} |
| | Available water capacity of top 100cm soils (cm) | 0.14 ^{ns} | 0.15 ^{ns} |
| Climate variables (from PRISM) | PPT (mm) | 0.03 ^{ns} | 0.04 ^{ns} |
| | Temperature (°C) | -0.19 ^{ns} | -0.34 ^{**} |
| | VPD (kPa) | -0.14 ^{ns} | -0.4 ^{***} |
| | PPT/PET | 0.22 | 0.35 ^{**} |

ns: non-significant ($p > 0.10$)

*: significant at 5% level

**: significant at 1% level

***: significant at 1‰ level

While the averages of baseline $\Delta^{13}\text{C}$ at the three water table depth classes were not significantly from each other ($p=0.63$), the standardized regression coefficients of the regression models (Table 3.3) did differ. The influence of climate-related variables increased progressively from sites with shallow to deeper water table depth (Table 3.3), whereas soil characteristics plays an important role for sites with the deepest water table depth. The magnitude and sign of the regression coefficients varied across the water table classes, partly reflecting a shift in the contribution of different processes to plant water availability.

Controls of drought sensitivity

There was a significant spatial correlation in drought sensitivity, which correlates strongly with site-related and climate variables (Table 3.2). While VPD explained the most of the cross-site variability ($r=-0.40$, $p=4E-4$), temperature, evaporative fraction (PPT/PET), altitude, lifetime PDSI, and soil physical structure also explained a significant fraction of variability.

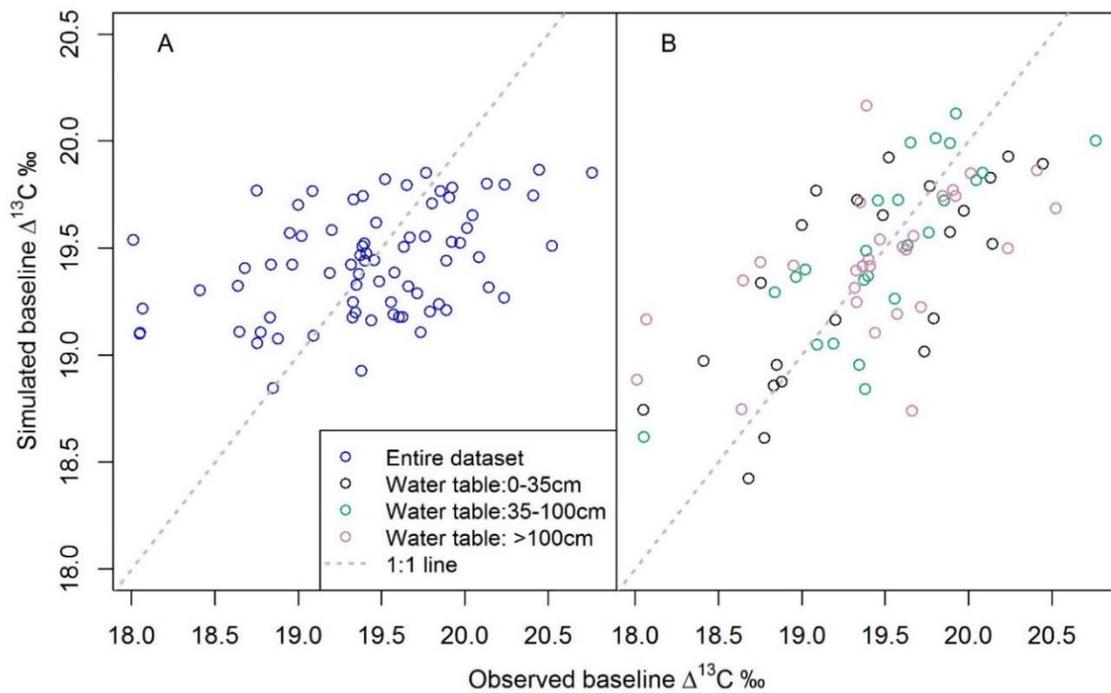


Figure 3.6 The comparison of observations and simulations from partial least square regression using different data sets to simulate baseline carbon isotope discrimination ($\Delta^{13}\text{C}$). The partial least squares regression based on (A) the entire data set and (B) water-table-stratified data sets.

Table 3.3 Standardized regression coefficients from partial least squares regression modeling of baseline carbon isotope discrimination ($\Delta^{13}\text{C}$) and drought sensitivity. Coefficients with the absolute values larger than 0.15 are in bold.

| Category | Variables | Baseline $\Delta^{13}\text{C}$ | | | Drought sensitivity | | |
|------------------------|--------------------------|--------------------------------|-----------------------------|---------------------------|-------------------------|------------------------|--------------|
| | | Water table depth: 0-35cm | Water table depth: 35-100cm | Water table depth: >100cm | Eastern region (N+SE+S) | Western region (SW+NW) | Entire range |
| Site-related | Sample age | 0.18 | 0.12 | 0.00 | 0.15 | -0.23 | -0.03 |
| | life time PDSI | 0.04 | 0.33 | 0.07 | 0.06 | -0.17 | 0.07 |
| | Altitude | -0.33 | -0.20 | -0.20 | 0.23 | -0.06 | 0.16 |
| | Latitude | 0.06 | 0.07 | 0.15 | 0.15 | 0.04 | 0.08 |
| | Longitude | 0.11 | 0.06 | -0.14 | -0.07 | 0.22 | -0.13 |
| Soil | Sand content | -0.13 | -0.13 | -0.42 | 0.07 | 0.24 | 0.03 |
| | Clay content | 0.31 | 0.11 | -0.56 | -0.13 | 0.01 | -0.04 |
| | Bulk density | 0.10 | 0.27 | 0.22 | 0.23 | -0.09 | 0.22 |
| | Organic matter content | 0.07 | 0.09 | -0.41 | 0.11 | 0.22 | 0.06 |
| | Available water capacity | 0.27 | 0.09 | 0.06 | -0.05 | -0.26 | -0.04 |
| | Water table depths | 0.21 | -0.26 | NA | NA | NA | NA |
| Climate | PPT | -0.07 | 0.13 | 0.05 | -0.11 | 0.01 | -0.01 |
| | Temperature | 0.03 | 0.00 | -0.17 | -0.20 | -0.04 | -0.13 |
| | VPD | 0.04 | -0.07 | 0.25 | -0.13 | 0.08 | -0.19 |
| | PPT/PET | -0.11 | 0.13 | 0.17 | 0.11 | 0.05 | 0.08 |
| Number of sites | | 23 | 24 | 29 | 50 | 26 | 76 |
| R² | | 0.54 | 0.61 | 0.32 | 0.35 | 0.36 | 0.27 |

The mean drought sensitivity differed significantly by geographic region (Figure 3.7), with region S being the least sensitive to unit change in PDSI, regions N, NW and SW being the most sensitive and the C region falling in-between. Partial least squares regression explained 27% of the variance across the entire dataset (Figure 3.8A); there remained no spatial correlation in the residuals (*Moran's I*=0.02, *p*=0.55). When allowing for different model fits for eastern and western regions, R^2 of the combined analysis increases from 0.27 (Figure 3.8A) to 0.39 (Figure 3.8B, Table 3.3). The standardized regression coefficients differed for the two regions, with the influence of the soil factors being smaller in the eastern than the western region (Table 3.3).

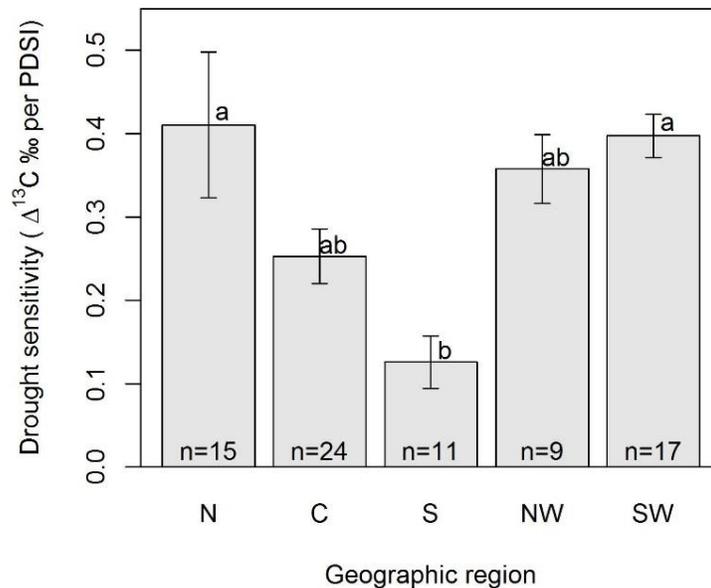


Figure 3.7 The averages and standard errors of drought sensitivity by geographic regions (n represents the number of sites under each category).

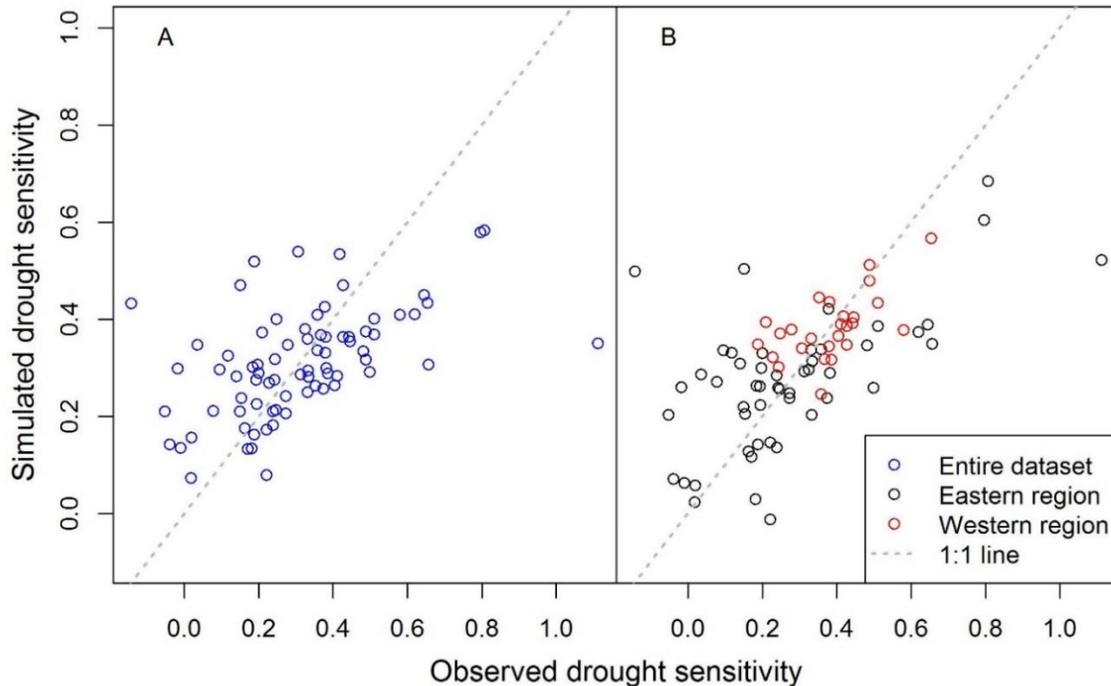


Figure 3.8 The comparison of observations and simulations from partial least square regression using different data sets to simulate drought sensitivity. The partial least squares regression based on (A) the entire data set and (B) data sets stratified by the site locations with respect to the Mississippi River.

Discussion

Unresponsive $\Delta^{13}\text{C}$ at high water availability

Stable isotope carbon discrimination of loblolly pine ($\Delta^{13}\text{C}$) correlated with PDSI at PDSI < 1 (Figure 3.3A), whereas at higher values $\Delta^{13}\text{C}$ was insensitive to PDSI. Such a 2-phase response has been reported earlier (e.g. Kohn 2010), and is consistent with our understanding of the effects of water availability on plant gas exchange and growth processes (Tor-ngern et al. 2016). In dry environments where precipitation is less than potential evapotranspiration,

precipitation is commonly used as a surrogate for water availability and it has been observed that plant $\Delta^{13}\text{C}$ or $\delta^{13}\text{C}$ are invariant when rainfall is high (e.g. Korol et al. 1999, Schulze et al. 2014). Diefendorf et al. (2010) and Kohn (2010) found a similar saturating response in foliar $\Delta^{13}\text{C}$ data with mean annual precipitation over a range of sites across the world. Similar saturating relationships characterizes canopy conductance response to relative extractable water in the soil (Granier et al. 2000). Yet, sometimes a consistent linear dependence of $\Delta^{13}\text{C}$ upon relative extractable soil water is assumed (e.g. Dupouey et al. 1993), which may underestimate the true drought sensitivity.

The concepts of baseline $\Delta^{13}\text{C}$ and drought sensitivity

Plant $\Delta^{13}\text{C}$ is affected by temporal variability in water availability as well as site characteristics (such as irradiance, altitude, and nutrient availability), while the latter may affect the baseline physiology (Duursma and Marshall 2006, Farquhar et al. 1989, Hultine and Marshall 2000) and anatomy (Domec et al. 2009, Martínez-Vilalta et al. 2004), as well as the ability of these structural elements to withstand water deficit (Franks and Brodribb 2005). Even though no two sites may experience the same weather conditions, the 2-component approach allows us to account for well-watered scenarios and calculate normalized metrics that allow cross-site comparisons. This may explain why our results (Table 3.2) contrast with some earlier reports that did not use normalized metrics. For example, Warren et al. (2001) observed little to no relationship between $\delta^{13}\text{C}$ and PPT/PET at PPT/PET > 0.8 and attributed the lack of correlation to the modification on $\Delta^{13}\text{C}$ by a multitude of factors, including water availability. In the current study, PPT/PET is well above 1 across the range, while the

correlation between baseline $\Delta^{13}\text{C}$ and PPT/PET is weak, but marginally significant ($r=0.22$, $p=0.06$).

Controls of baseline $\Delta^{13}\text{C}$

The effects of latitude, mainly a surrogate for irradiance, and water availability in determining plant $\Delta^{13}\text{C}$ has been well recognized (Cernusak et al. 2013). Except for sampling age and latitude, all environmental parameters that were related to baseline $\Delta^{13}\text{C}$ (sand content of soil, PPT/PET, and life time PDSI, Table 3.2) were associated with site water status. While water availability exerts a primary impact on shaping plant $\Delta^{13}\text{C}$ of arid and sub-arid areas (e.g. Prentice et al. 2011), the current study suggests the same for baseline $\Delta^{13}\text{C}$ of loblolly pine growing in humid southeastern USA (PPT/PET > 1.2), considering the greatly improved success in modeling of baseline $\Delta^{13}\text{C}$ by grouping sites by water table depth.

Life time PDSI, representing the droughtiness of the site since tree's establishment, is a relatively new parameter in the empirical modeling of $\Delta^{13}\text{C}$. Given the sampling age of our study (9-26 years), this parameter is more dynamic, depending on the climate history of recent decades, compared to other parameters like latitude, soil texture, and PPT/PET (based on 30 year norm mean). Suspecting this is related to baseline physiology of trees during their establishment, we calculated Pearson correlation coefficients between baseline $\Delta^{13}\text{C}$ and moving averages of PDSI of different years. The highest correlation was found between baseline $\Delta^{13}\text{C}$ and the average PDSI during the first 8 years since planting ($r=0.34$, $p=0.003$). That is, the conditions of water supply during the first 8 years since establishment may affect

the physiology of loblolly pine in a profound way, at least the effect was detected in baseline $\Delta^{13}\text{C}$ 10+ years later. As water availability that is known to affect canopy conductance and photosynthetic rate, it may also alter xylem vessel anatomy and hydraulic conductivity (Bréda et al. 2006, Domec et al. 2009). Recently, other long-lasting effects of water availability on leaf gas exchange have been reported, notably the abscisic-acid (ABA) mediated stomatal initiation (Dow and Bergmann 2014). That is, such structural changes may modify stomatal conductance indirectly and thus contribute to modify baseline $\Delta^{13}\text{C}$.

Curiously, the effect of tree age on baseline $\Delta^{13}\text{C}$ (Table 3.2) was the opposite in sign that would be expected based on the increasing gravitational component with height (McDowell et al. 2011b), and the transition from juvenile to mature xylem anatomy (Gagen et al. 2011). Given the small number of sampling years per site (2 years only) and weak correlation ($r=0.22$, $p=0.07$), the positive relationship may be an artifact. However, more research is warranted to understand this phenomenon.

Controls of drought sensitivity

Unlike baseline $\Delta^{13}\text{C}$, the spatial pattern of drought sensitivity (Figure 3.5) aligned with loblolly pine provenance (Prof. R. Whetten, personal communication 2016), which is roughly depicted by seed source transfer zones (Schmidting 2001). This observation has been supported by the different averages of drought sensitivity at five geographic regions (Figure 3.7), and the improved fit of drought sensitivity modeling due to simulation by districts divided by the Mississippi River (Table 3.3). Given that the population structure of loblolly pines is associated with environmental variability across the distribution range (Eckert et al.

2010), separating the effects of genetic and environmental influences on drought sensitivity is difficult.

Among the five geographic regions, the drought sensitivity was the lowest in the southern regions, less than half of those from other geographic regions (Figure 3.7). Furthermore, the drought sensitivity of five sites from the coastal region was negative, indicating a reversed response of $\Delta^{13}\text{C}$ to drought. As $\Delta^{13}\text{C}$ of loblolly pine is mainly determined by stomatal conductance and photosynthetic rate (Farquhar et al. 1989) and the effect of drought on photosynthetic rate is usually secondary, the sensitivity of stomata to water stress is probably the major source of variation in the isotopic drought sensitivity. In a common garden study with different varieties of *Pseudotsuga menziesii*, Zhang et al. (1993) found that at high VPD in the mid-afternoon, the interior variety maintained high stomatal conductance while the coastal variety did not. Similarly, Gonzalez-Benecke and Martin (2010) found that compared to loblolly pines from the central Florida seed source, trees from South Carolina had stronger stomatal control when water is limited. Therefore, we hypothesize that the isotopic drought sensitivity of the coastal region (including the southern geographic region and some sites with low drought sensitivity in the north) loblolly pine was lower than in other areas due to the high rainfall, high water table, and acclimation of the genotypes for these conditions.

Conclusion

With tree ring cellulose $\delta^{13}\text{C}$ data from 76 loblolly pine plantations across the species' distribution range, we described carbon isotope discrimination ($\Delta^{13}\text{C}$) in terms of baseline $\Delta^{13}\text{C}$ in well-watered conditions and normalized drought sensitivity as change in $\Delta^{13}\text{C}$ per unit change in Palmer Drought Severity Index (PDSI). The site-level drought sensitivity exhibited distinct spatial structure, and could be attributed to underlying genetic and environmental variations. The baseline $\Delta^{13}\text{C}$, however, exhibits minimal spatial structure, and is impacted mostly by site water status and latitude. Despite the humid environment (PPT/PET > 1.2) in southeastern USA, site water status is the primary influence on baseline $\Delta^{13}\text{C}$, indicating the deciding role that stomatal conductance plays in shaping $\Delta^{13}\text{C}$ of loblolly pine. Besides ground water table, the average PDSI values of the first 8 years explained much variation in baseline $\Delta^{13}\text{C}$, probably by affecting the baseline physiology of the species at the early years of establishment.

Chapter 4. Interpretation of loblolly pine (*Pinus taeda* L.) responses to drought and fertilization using stable isotopes

Abstract

As drought frequency and intensity are projected to increase in the coming decades across the US Southeast, responses of loblolly pine (*Pinus taeda* L.), the most significant timber species in the US, will impact regional carbon and water cycling. To evaluate the effects of drought at ambient and optimal nutrient availability on loblolly pine physiology, we analyzed carbon and oxygen isotope ratios of tree ring latewood cellulose at a throughfall reduction (ambient and 30% reduction) and fertilization (ambient and a complete suite of nutrients) experiment in Virginia, set up as a part of the PINEMAP study. Intrinsic water use efficiency (iWUE) increased and oxygen isotope discrimination decreased significantly under drought. The increasing iWUE under drought is attributed mainly to reduced stomatal conductance, while fertilization had minimal effects on photosynthetic rate. Thus, the increased growth of fertilized trees was mainly due to increases in leaf area. The apparent inconsistencies between the responses of observed and simulated oxygen isotope discrimination suggest that drought modified the source water used by loblolly pine, and that careful consideration is needed when applying the dual isotope approach.

Introduction

Loblolly pine is the most important commercial tree species in the southeastern United States (Will et al. 2015). It is planted on over 11 million ha and comprises 40% of the nation's timber land (Bartkowiak et al. 2015). Due to the large planted area and high productivity, this species plays a crucial role in the regional carbon budget and economic vitality of the United States (Schultz 1999). Climate projections for the southeastern USA indicate that the frequency and intensity of drought will increase in the coming decades, accompanied by higher temperature and more intense precipitation events interspersed with increased length of dry periods (IPCC 2014, Ryan 2011). Physiological responses of loblolly pine to these climatic changes may negatively affect productivity. Mid-rotation fertilization has been widely applied to commercial pine plantations across the region (Fox et al. 2007a), because a large body of research has shown that productivity of loblolly pine increases significantly under fertilization (Albaugh et al. 1998, Albaugh et al. 2004, Gough et al. 2004a, Gough et al. 2004b, Retzlaff et al. 2001, Samuelson et al. 2001, Stovall et al. 2011, Will et al. 2001). Therefore, as drought becomes more frequent and intense there is growing need to evaluate its impacts on fertilized pine.

Physiological processes at the leaf-level are important to understand how loblolly pine responds to fertilization and drought. For instance, little consensus has emerged in regard to the change of photosynthetic rate after fertilization: some studies report no change in photosynthetic rate and increased leaf area as the major driver of increased growth (Gough et al. 2004a, Samuelson et al. 2001, Will et al. 2001), while others found photosynthetic rate was

enhanced by fertilization, at least temporarily (Gough et al. 2004b, King et al. 2008, Tyree et al. 2009). Recently, Pell (2015) reported reduced photosynthetic rate in stands 4 years after fertilization. Similarly, the reported change of stomatal conductance to fertilization has been variable. Stomatal conductance of loblolly pine may not respond to fertilization (e.g. Green and Mitchell 1992, Samuelson et al. 2001), or decrease in the following year after fertilization (e.g. Ward 2015). Samuelson et al. (2014) reported no response in the growing season that fertilization was applied, but effects became marginally significant the following year. On the other hand, the effect of drought on photosynthetic rate and stomatal conductance is relatively well known. Stomatal closure is one of the earliest responses to drought during the growing season (Chaves et al. 2002, Flexas and Medrano 2002) due to limited water supply and/or increased evaporative demand, whereas limitation on photosynthesis is secondary and occurs under greater water stress (Flexas et al. 2004).

The stable carbon and oxygen isotope ratio of tree ring cellulose records valuable information of the environmental and physiological process, and have been a powerful tool in ecophysiological studies (Dawson et al. 2002, McCarroll and Loader 2004). The carbon isotope ratio of plant tissue ($\delta^{13}\text{C}$) reflects gas-exchange processes by the plant at the time the carbon was fixed and can be used as an indicator of intrinsic water use efficiency (Farquhar et al. 1989), assuming that mesophyll conductance is relatively constant (Flexas et al. 2008, Warren and Adams 2006). Intrinsic water use efficiency (iWUE), the leaf level ratio of photosynthetic rate (A) to stomatal conductance (g_s , Farquhar 1989), is useful for understanding responses of trees to past changes in the environment. For instance, iWUE has long been known to respond to drought (Farquhar et al. 1982). On the other hand, responses

of iWUE to fertilization are mixed. Several studies found iWUE of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) increased for a few years after fertilization (Brooks and Coulombe 2009, Brooks and Mitchell 2011, Cornejo-Oviedo et al. 2017), while Balster et al. (2009) did not observe any significant changes in holocellulose $\Delta^{13}\text{C}$ (proportional to iWUE) for the same species. On the other hand, Elhani et al. (2005) found tree ring wholewood $\Delta^{13}\text{C}$ of beech (*Fagus sylvatica* L.) increased (i.e. lower iWUE) for 6 years after fertilization.

Compared to $\delta^{13}\text{C}$, the interpretation of tree ring cellulose $\delta^{18}\text{O}$ can be complex. The major influential factors include source water $\delta^{18}\text{O}$ and the enrichment during water evaporation at the leaf level (Barbour 2007, McCarroll and Loader 2004). However, mechanistic process models have allowed simulation of cellulose oxygen discrimination ($\Delta^{18}\text{O}_c$) with an array of climate and physiological variables (Barbour and Farquhar 2000, Cernusak et al. 2015, Roden et al. 2000). Furthermore, it has been proposed that simultaneous application of $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ analyses may allow discerning between photosynthesis- and stomatal conductance-driven shifts in iWUE (dual isotope approach, Scheidegger et al. 2000). However, the applicability of the method remains to be debated as the major assumptions of similar source water $\delta^{18}\text{O}$ across the stands for comparison may not always be met (Roden and Siegwolf 2012, Scheidegger et al. 2000).

The current study was set up to evaluate the potential interaction between water and nutrient availability on the drought sensitivity of loblolly pine. The work was conducted at a throughfall reduction and fertilization experiment in Virginia, USA, one of four identical experimental set-ups established under the Pine Integrated Network: Education, Mitigation,

and Adaptation Project (PINEMAP; <http://www.pinemap.org/>; Will et al. 2015). Here we report $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ stable isotope ratios of tree ring α -cellulose and discuss the potential mechanisms of drought tolerance and acclimation in loblolly pine in the northern edge of its range. Specifically, our objectives were to:

- a) Evaluate the drought responses of iWUE at ambient and optimal nutrient availability;
- b) Estimate the contribution of photosynthetic rate to tree growth relative to g_s ;
- c) Critically evaluate the dual isotope method, and explore potential interpretations.

Method and materials

Site description and experimental design

The study was conducted at the northernmost of the four throughfall exclusion and fertilization experiments established under PINEMAP study. The experiment was established in 2012 as a randomized complete block factorial experimental design with two levels of water availability (ambient and 30% throughfall reduction), and two levels of nutrient availability (ambient and optimal), with four blocks. The treatments were Control (C, ambient throughfall without fertilization), Drought (D, 30% throughfall reduction without fertilization), Fertilization (F, ambient throughfall with fertilization of a complete suite of nutrients), and Fertilization plus Drought (FD, 30% throughfall reduction with fertilization). Fertilizer was applied at a rate of 224kg N/ha, 27kg P/ha, 52kg K/ha, and 1.12 kg/ha of micronutrient mix (6% sulfur, 5% boron, 2% copper, 6% manganese, and 5% zinc) in April

of 2012. The drought treatment consisted of removing approximately 30% of precipitation throughfall by troughs positioned below the live crown, alongside tree rows. Seed sources represented the local provenance. Additional information on experimental set up was reported by Will et al. (2015).

The study site is located in the Buckingham County, VA (37°27'37''N, 78°39'50''W), in the Piedmont physiographic region, with 0-15% slopes. The soil is a well-drained, fine, mixed, subactive, mesic Typic Hapludult of the Littlejoe soil series, with a silt loam surface soil and a clay loam subsoil. The depth to ground water is deeper than 200 cm. Mean annual precipitation is 1120 mm and mean temperature 13.6°C. The loblolly pine trees were planted as 2-year old seedlings in 2003, and the treatments started in April 2012. Precipitation, photosynthetically active radiation, relative humidity, and air temperature were measured using micrometeorological sensors mounted above the canopy at the center of the study site. Additional information on site micrometeorology was reported by Ward et al. (2015).

Aboveground biomass increment and mean daily canopy conductance estimation

Tree height and diameter at breast height (DBH) were measured annually during dormant seasons beginning in 2012. The aboveground biomass increment of each measurement tree was calculated using tree height, DBH, age, and stand density after Gonzalez-Benecke et al. (2014).

Leaf area index (LAI) was measured using a Plant Canopy Analyzer (Li-Cor, Lincoln, NE, USA) on April 1st and August 25th, 2015 in each plot. Monthly LAI was estimated by linear

interpolation, approximating the seasonality reported for earlier years at the same site (Ward et al. 2015). Using monthly VPD and LAI, monthly tree transpiration data based on sap flow measurements in 2015 were converted to monthly mean daily canopy conductance (mean daily G_s , mol/m²/day) from May through September.

Core sampling, cellulose extraction, and stable isotope analysis

Increment cores of 5-mm and 12-mm diameter were taken from three out of five representative trees per plot that were equipped with thermal dissipation probes for sap flow measurements (Ward et al. 2015). The cores were collected from the northern side of the trees at breast height (1.3m) in January 2016, 3.5 years after the start of the throughfall exclusion treatment and the application of the fertilizer. The cores were oven dried at 60°C, mounted and sanded progressively down to 1500 grit. The ring width was measured to the nearest 0.001 mm using a tree ring measuring system (Velmex Inc. Bloomfield, NY, USA) and the cores were cross-dated using program COFECHA (Holmes 1983).

Latewood produced in 2015 was separated and α -cellulose extracted (Lin et al. 2017). The ¹³C and ¹⁸O stable isotope ratios of α -cellulose were determined at the Cornell University Stable Isotope Laboratory (<http://www.cobsil.com>), using a Thermo Delta V isotope ratio mass spectrometer interfaced to a NC2500 elemental analyzer and to a Temperature Conversion Elemental Analyzer. The within-run isotopic precision of the methodology using quality control standards was 0.2‰ for carbon and 0.4‰ for oxygen (K. Sparks, 2015, personal communication).

Precipitation was collected at the study site for four periods in 2015: June 17-July 16, July 17- August 21, August 22-September 16, and September 16-October 6. It was done using a carboy fitted with a funnel and a small amount of mineral oil was applied to reduce evaporation in the field. Oxygen isotope ratios ($\delta^{18}\text{O}$) were determined at the Stable Isotopes for Biosphere Science laboratory at Texas A&M University (<http://sibs.tamu.edu>) using a high temperature conversion elemental analyzer coupled to an isotope ratio mass spectrometer (TC/EA-IRMS; Delta V, Thermo Scientific, Waltham, MA). The average of precipitation $\delta^{18}\text{O}$ was weighted by number of days at each collection period.

Carbon isotope discrimination ($\Delta^{13}\text{C}$, ‰) was calculated as:

$$\Delta^{13}\text{C} = \frac{\delta^{13}\text{C}_{air} - \delta^{13}\text{C}_{cellulose}}{1 + \delta^{13}\text{C}_{plant}/1000} \quad (4.1)$$

where $\delta^{13}\text{C}_{cellulose}$ is the ^{13}C isotopic ratio of the extracted α -cellulose and $\delta^{13}\text{C}_{air}$ is the isotopic signature of the air. Given that mesophyll conductance of this species is invariant (Wilson 2014), intrinsic water use efficiency ($iWUE$) was calculated as:

$$iWUE = c_a \frac{b - \Delta^{13}\text{C}}{1.6(b - a)} \quad (4.2)$$

where a is the discrimination against $^{13}\text{CO}_2$ during diffusion through the stomata (4.4‰), b is the net discrimination due to carboxylation (27‰), and c_a is ambient CO_2 concentration. The annual averages that were used in the study were calculated from monthly atmospheric CO_2

concentration and $\delta^{13}\text{C}_a$ values obtained from the sampling station of Scripps CO₂ Program at La Jolla Pier, California, USA (http://scrippsco2.ucsd.edu/data/atmospheric_co2/ljo).

Oxygen isotope discrimination of cellulose ($\Delta^{18}\text{O}_c$, ‰) was calculated as:

$$\Delta^{18}\text{O}_c = \frac{\delta^{18}\text{O}_{cellulose} - \delta^{18}\text{O}_{precipitation}}{1 + \delta^{18}\text{O}_{precipitation}/1000} \quad (4.3)$$

where $\delta^{18}\text{O}_{cellulose}$ is the ¹⁸O isotopic ratio of the extracted α -cellulose and $\delta^{18}\text{O}_{precipitation}$ is the isotopic signature of collected precipitation at study site. Given the deep ground water table (>200 cm), precipitation was assumed to be the major source water for this site.

Because plots were spatially collocated and trees were of the same species and age, we assume that the source water $\delta^{18}\text{O}$ across treatments was similar, and that the differences in $\Delta^{18}\text{O}_c$ reflect the difference in the evaporative process affected by different treatments.

Sensitivity analyses of $\Delta^{18}\text{O}_c$

Similar to Brooks and Mitchell (2011), sensitivity of tree ring cellulose oxygen isotope discrimination ($\Delta^{18}\text{O}_c$) was estimated to three different processes - evaporative enrichment in $\Delta^{18}\text{O}$ at evaporative sites (Farquhar et al. 2007), the Péclet effect describing $\Delta^{18}\text{O}$ in the bulk leave water (Cernusak et al. 2015), and the discrimination in cellulose biosynthesis (Barbour and Farquhar 2000).

The evaporative enrichment of leaf water ($\Delta^{18}\text{O}_e$) was calculated with the modified Craig-Gordon model (Farquhar et al. 2007):

$$\Delta^{18}O_e = (1 + \epsilon^+) \left[(1 + \epsilon_k) \left(1 - \frac{w_a}{w_i} \right) + \frac{w_a}{w_i} (1 + \Delta^{18}O_v) \right] - 1 \quad (4.4)$$

where ϵ^+ is equilibrium fractionation coefficient and in turn a function of leaf temperature (T_l), ϵ_k is the kinetic fractionation coefficient and a function of the ratio of stomatal conductance (g_s) and boundary layer conductance (g_b), w_a is the vapor pressure of the atmosphere and a function of air temperature (T_a) and relative humidity (RH), w_i is the vapor pressure inside leaves and a function of leaf temperature, and $\Delta^{18}O_v$ is the difference between source water and atmospheric water vapor and is equal to $-\epsilon^+$ under well-mixed outdoor conditions (Barbour 2007).

The average isotopic enrichment of bulk leaf water at steady state ($\Delta^{18}O_L$) was modeled by considering the Péclet effect (\wp):

$$\Delta^{18}O_L = \Delta^{18}O_e \left(\frac{1 - \exp(-\wp)}{\wp} \right) \quad (4.5)$$

where \wp is Péclet number, a function of effective path length (L), transpiration (E), molar density of water (C), and diffusivity of $H_2^{18}O$ (D'); Equation 4.6). E is a function of stomatal conductance and leaf-to-air vapor pressure deficit (Brooks and Mitchell 2011, Ehleringer et al. 1993), P is the atmospheric pressure, and D' is dependent on leaf temperature. L is difficult to measure and variable even within the same tree (Roden et al. 2015).

$$\wp = \frac{LE}{CD'} = \frac{L}{CD'} \frac{g_s(w_i - w_a)}{P} \quad (4.6)$$

Finally, the $\Delta^{18}\text{O}$ of cellulose in tree rings ($\Delta^{18}\text{O}_c$) was estimated following Barbour and Farquhar (2000) as:

$$\Delta^{18}\text{O}_c = \Delta^{18}\text{O}_L(1 - p_{ex}p_x) + \varepsilon_{wc} \quad (4.7)$$

Where p_{ex} is the proportion of oxygen atoms that exchange with source water during cellulose formation, p_x is the proportion of source water at the site of cellulose formation (thus its value equals 1 for trunk wood), and ε_{wc} is the isotope fractionation of 27‰, associated with the oxygen atom exchange between water and carbonyl groups.

To summarize, the variables for equations 4.5-4.7 include T_a , T_l , RH, g_s , g_b , L, and p_{ex} . As all the trees in the current study are of the same species and age, we assume that L and p_{ex} are the same across the treatments and set the values to 33mm and 0.4, respectively, the means for conifers reported by Wang et al. (1998), and similar to Brooks and Mitchell (2011). The daytime boundary layer conductance was set to 7.5 mol/m²/s, 50 times greater than daytime canopy conductance (Domec et al. 2009). We analyzed the possible responses of $\Delta^{18}\text{O}_c$ to changes in T_a , RH, temperature difference between leaf and air ($T_l - T_a$), and g_s due to fertilization and drought treatments, using both one- and multi-dimensional sensitivity analyses.

One-dimensional analysis was performed first to evaluate the effect of individual parameters while keeping the rest unchanged. The analysis for the current study was based on the average daytime air temperature and relative humidity of the study site for May through

September, 2015 ($T_a=24.3^\circ\text{C}$ and $\text{RH}=69\%$), and the daytime average canopy conductance from the control plots ($g_s=67\text{ mmol m}^{-2}\text{ s}^{-1}$) measured in 2013 (Ward et al. 2015).

By varying the four parameters together, a multi-dimensional sensitivity analysis was then conducted to evaluate the behavior of the models under different combinations of parameter values. To limit the parameter space, we used the relationship between air temperature and RH based on half-hour measurements collected with micrometeorological sensors in 2015 (Figure 4.1A). For instance, at air temperature of 30°C , RH ranged from 30% to 80% in the sensitivity analysis. Another effort to limit parameter space was to set up an upper limit for g_s based on the relationship between canopy conductance (G_s) and VPD (Equation 4.8, Figure 4.1B), which was estimated from sap flow measurements conducted in 2013 at the same study site. To avoid the effect of dim light on this VPD- G_s relationship, only data between 10:00 and 16:00 were used.

$$G_s(\text{mmol m}^{-2}\text{s}^{-1}) \leq \begin{cases} 400, & \text{for } VPD \leq 0.35\text{kPa} \\ 250 - 145\ln(VPD), & \text{for } VPD > 0.35\text{kPa} \end{cases} \quad (4.8)$$

Although pine needles are usually well-coupled with the atmosphere and the leaf-to-air temperature difference (T_l-T_a) may be small (Jarvis and McNaughton 1986), the actual temperature difference could range from -3°C to 3°C at 30°C , 30% RH and 1 m/s wind for loblolly pine (Campbell and Norman 1998). Therefore, in the current study we varied (T_l-T_a) from -3°C to 4°C and evaluated the effects in the sensitivity analysis to cover a wide range of potential change.

Given the small plot size (0.356 ha square plot with 32.9 m on a side), air temperature and RH was assumed consistent among plots and treatments. Thus, any change in $\Delta^{18}\text{O}_c$ in the treatments was attributed to change in g_s and $(T_i - T_a)$ due to drought and/or fertilization. However, we also included change in RH in the analysis because of the very high sensitivity of $\Delta^{18}\text{O}_c$ to RH (see Results and Discussion).

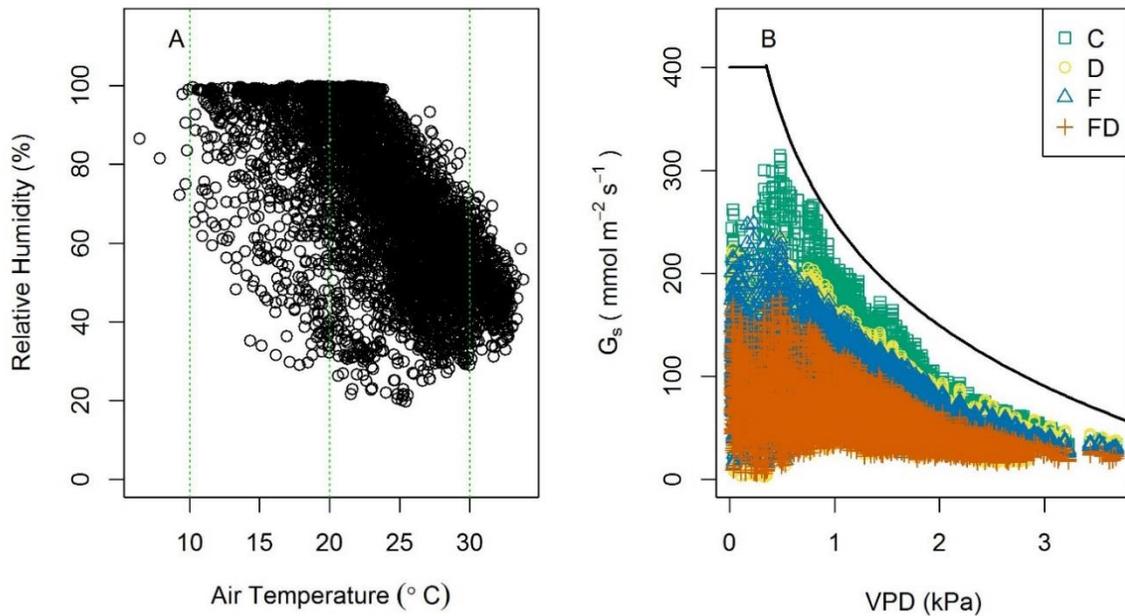


Figure 4.1 (A) The relationship between relative humidity and air temperature at the study site in 2015. The data include half-hour measurements between 6:00 and 21:00 from May to September. (B) The relationship of canopy conductance (G_s) and vapor pressure deficit (VPD). The data is based on sap flow measurements between 10:00 and 16:00 from May to September, 2013. The solid line represents the upper limit of canopy conductance in response to VPD (Equation 4.8).

Statistical analysis

Statistical analyses were performed in R software (R Core Team 2016). Two-way ANOVA was used to evaluate the effects of drought and fertilization on aboveground biomass

increment, $iWUE$, and $\Delta^{18}O_c$. Post hoc comparison of means were conducted using Tukey's honest significant difference method. Due to a single outlier in $iWUE$, which was more than 3 times the interquartile range above the third quantile, Type III sum of squares was considered in ANOVA of $iWUE$, without considering the block effect. The post hoc means were compared by Dunnett's modified Tukey-Kramer pairwise multiple comparison test in R via "DTK" package (Lau 2013) in this case. As the number of sap flow trees varied per plot, mixed effect models were used to evaluate the effects of drought and fertilization on mean daily canopy conductance via "lme4" package (Bates et al. 2016). Drought and fertilization were treated as fixed factors, while block and month were considered random factors. The p -values were obtained by using the Satterthwaite approximation via "lmerTest" package (Kuznetsova et al. 2016). Because LAI is a stand-level variable and was measured twice in 2015, the same procedure was applied to LAI measurements, except that measurement date was treated as the only random factor.

Results

Tree responses to drought and fertilization

Three and a half years after implementing the experimental treatments, fertilization significantly increased growth of loblolly pine. With similar height across treatments (Table 4.1), leaf area index (LAI, Figure 4.2A) and aboveground biomass increments (AGBI, Figure 4.2B) were consistently greater in fertilized treatments at both ambient and reduced water availability ($p=0.05$ for both LAI and AGBI), while the response to drought was not

significant ($p=0.60$ and $p=0.34$ for LAI and AGBI, respectively, Table 4.1). There were no significant interactions between treatments. On average, peak LAI under fertilization was $3.45 \text{ m}^2 \text{ m}^{-2}$, or 12% higher than in unfertilized plots, while the average increment in aboveground biomass in 2015 was 21.9 kg per tree under fertilization, or 29% higher compared to the control (Figure 4.2A and 4.2B).

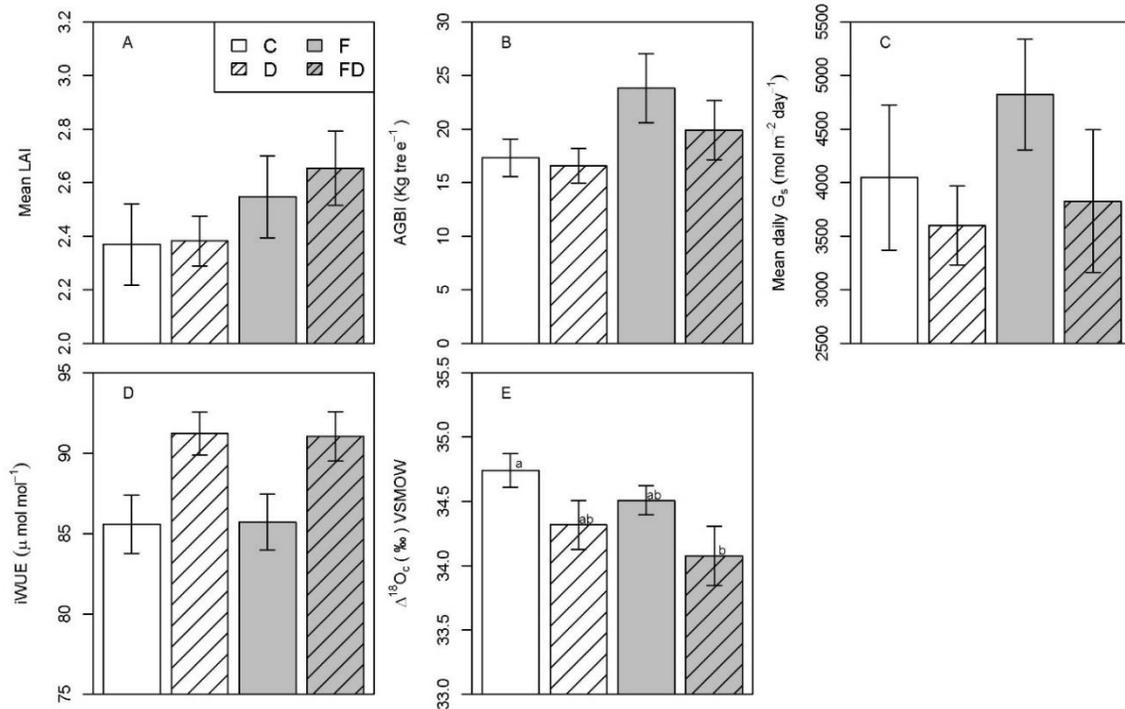


Figure 4.2 Means (bars) and standard errors (whiskers) of physiological variables under different treatments (C: control, D: drought only, F: fertilization only, FD: fertilization and drought). (A) annual mean leaf area index (LAI); (B) aboveground biomass increment (AGBI); (C) mean daily canopy conductance (mean daily G_s); (D) intrinsic water use efficiency (iWUE); and (E) tree ring cellulose oxygen isotope discrimination ($\Delta^{18}\text{O}_c$).

Mean daily canopy conductance, calculated from daily tree transpiration, ranged from 484 to $12240 \text{ mol H}_2\text{O m}^{-2} \text{ day}^{-1}$ from May through September. While the means differed between

treatments (Fig 4.2C), the effect was not statistically significant given the large variance (for instance, CV=0.45 for D treatment and 0.71 for the control).

The intrinsic water use efficiency was about 85 $\mu\text{mol mol}^{-1}$ in control and F treatments and 92 $\mu\text{mol mol}^{-1}$ in drought treatments (D and FD), and the full range of data varied from 74.4 to 101.5 $\mu\text{mol mol}^{-1}$ (Figure 4.2D). Fertilization had no effect on iWUE ($p=0.99$), while the effect of drought was significant ($p=0.001$, Table 4.1). There was no interaction between fertilization and drought treatments, with drought affecting iWUE similarly at both ambient and optimal nutrient availability. The oxygen isotope discrimination ranged from 32.77 to 35.69‰, with a significant drought-induced decrease at both ambient and optimal nutrient supply ($p=0.003$, Figure 4.2E, Table 4.1).

Table 4.1 Statistical results of the effects of fertilization and drought from two-way ANOVA or mixed-effect models on tree height measured in the winter of 2015, leaf area index (LAI), aboveground biomass increment (AGBI), mean daily canopy conductance (mean daily G_s), intrinsic water use efficiency (iWUE), and tree ring cellulose oxygen isotope discrimination ($\Delta^{18}\text{O}_c$). No significant interactions between treatments were detected. The ANOVA of LAI and mean daily G_s were conducted with stand-level values, while the rest are based data from individual sample trees.

| Variable | Treatment | <i>p</i> -value | Variable | Treatment | <i>p</i> -value |
|----------|---------------|-----------------|-------------------------|---------------|-----------------|
| Height | Drought | 0.38 | Mean daily G_s | Drought | 0.17 |
| | Fertilization | 0.24 | | Fertilization | 0.30 |
| LAI | Drought | 0.60 | iWUE | Drought | 0.001 |
| | Fertilization | 0.05 | | Fertilization | 0.99 |
| AGBI | Drought | 0.34 | $\Delta^{18}\text{O}_c$ | Drought | 0.003 |
| | Fertilization | 0.05 | | Fertilization | 0.57 |

$\Delta^{18}\text{O}_c$ simulation and sensitivity analyses

Simulated tree ring cellulose oxygen isotope discrimination ($\Delta^{18}\text{O}_c$) was 33.41‰ (dash lines in Figure 4.3) when leaf temperature was the same as air temperature, equal to the seasonal mean (24.3°C), as well as RH (69%), and g_s set equal to 67 $\text{mmol m}^{-2} \text{s}^{-1}$, the average daytime canopy conductance at the study site in 2013. On the other hand, the weighted average precipitation $\delta^{18}\text{O}$ was -4.41‰, and the measured $\Delta^{18}\text{O}_c$ in the control treatment was 34.74‰, 1.33‰ higher than simulation.

The one-dimension sensitivity analysis indicates that RH was the most influential parameter among the four (Figure 4.3B). The sensitivity of $\Delta^{18}\text{O}_c$ to air temperature (Figure 4.3A) and stomatal conductance (Figure 4.3D) were low, whereas that of leaf-to-air temperature difference (Figure 4.3C) was moderately high.

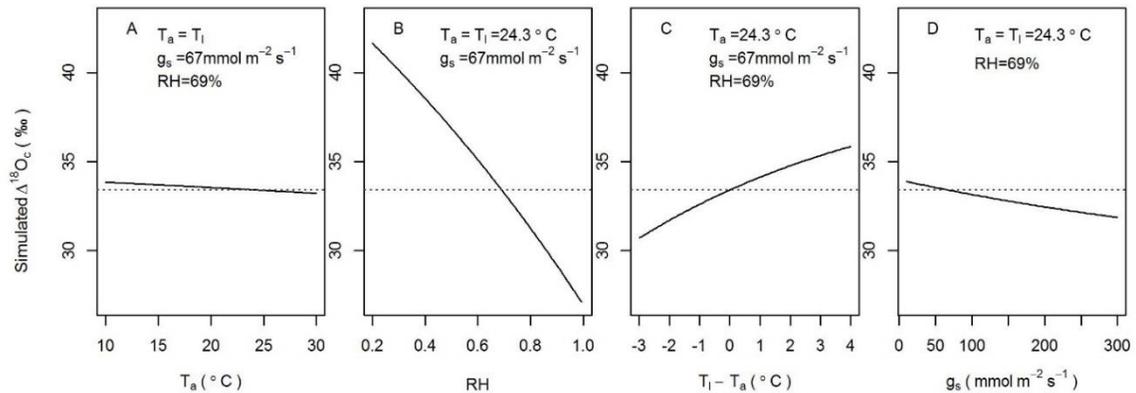


Figure 4.3 The one-dimensional sensitivity analysis of tree ring cellulose oxygen isotope discrimination simulation (simulated $\Delta^{18}\text{O}_c$) for (A) air temperature, (B) relative humidity (RH), (C) leaf-to-air temperature difference ($T_l - T_a$), and (D) stomatal conductance (g_s). Dashed line indicates the simulated $\Delta^{18}\text{O}_c$ value (33.41‰) under $T_a = T_l = 24.3^\circ\text{C}$, $\text{RH} = 69\%$, and $g_s = 67 \text{mmol m}^{-2} \text{s}^{-1}$.

To explore the interactions between the leaf-to-air temperature difference with air temperature, relative humidity, and stomatal conductance, we did a multi-dimensional sensitivity analysis by varying all four parameters together. Figure 4.4 shows the relationship of simulated $\Delta^{18}\text{O}_c$ with 3 levels of air temperature, 5 levels of RH, and varying g_s and $(T_l - T_a)$. Each column of subpanels represents the effects of air temperature at a given RH (except the first column, where RH was different for Figure 4.4D and 4.4I). Unlike the prediction from the one-dimensional sensitivity analysis, the effect of air temperature was pronounced at low RH (first two columns of Figure 4.4). When RH was medium to high (the third to fifth columns of Figure 4.4), the effect of air temperature became small, partly due to the change in upper limit of g_s at higher temperatures. Each row of subpanels represents the responses of simulated $\Delta^{18}\text{O}_c$ to varying RH at a given air temperature. Increasing RH not only decreased $\Delta^{18}\text{O}_c$, but also narrowed the realized ranges of $\Delta^{18}\text{O}_c$ by $(T_l - T_a)$ and g_s (for instance, Figure 4.4D through 4.4H on the middle row). The $\Delta^{18}\text{O}_c$ monotonically increased with $(T_l - T_a)$, except at low RH, the relationship was reversed when g_s was high (Figure 4.4D). We calculated the threshold of RH at each air temperature for the reversed relationship based on the maximal g_s allowed at the given VPD and found that the chance of the combination of air temperature and RH occurred less than 4% of the time in the 2015 growing season. Therefore, it is unlikely that such reversed relationship would affect the simulated $\Delta^{18}\text{O}_c$ significantly. The sensitivity of $\Delta^{18}\text{O}_c$ to $(T_l - T_a)$ is also affected by g_s . Finally, the relationship between $\Delta^{18}\text{O}_c$ and g_s is monotonically decreasing. $\Delta^{18}\text{O}_c$ is more sensitive to changes in g_s when RH is low (e.g. Figure 4.4D and 4.4I).

Discussion

Tree responses to drought

With a 30% reduction in throughfall, the iWUE of loblolly pine increased by 7.6% (Figure 4.2, Table 4.1). It has been well known that water stress decreases carbon isotope discrimination and increases iWUE (Farquhar et al. 1982). Therefore, the increase in iWUE was mostly a result of decreased g_s even though mean daily canopy conductance did not differ significantly ($p=0.20$) due to the very high variation within treatments. Given that LAI and aboveground biomass increment did not significantly differ between drought and control treatments (Figure 4.2, Table 4.1), we infer that photosynthetic rate must not have changed greatly under the experimental drought conditions.

Because the relationship between simulated $\Delta^{18}O_c$ and air temperature/RH was monotonic (Figure 4.4), and RH was unlikely to vary across treatments, drought treatment decreased g_s , which was likely increased (T_i-T_a), both of which acted to increase $\Delta^{18}O_c$ (the yellow arrows in Figure 4.5). Given that one-dimensional sensitivity analysis indicated that small changes in RH can affect $\Delta^{18}O_c$ simulation (Figure 4.3B), we decided to include RH into consideration for potential change in $\Delta^{18}O_c$. Therefore, due to the throughfall reduction and reduced transpiration (caused by reduced g_s), RH under drought may have been slightly lower, which would act to increase $\Delta^{18}O_c$ further (Figures 4.3 and 4.4, Table 4.2). However, the observed $\Delta^{18}O_c$ under drought was actually significantly lower than that of the control (Figure 4.2, Table 4.1), opposite to the prediction. This suggests that the assumption of similar source

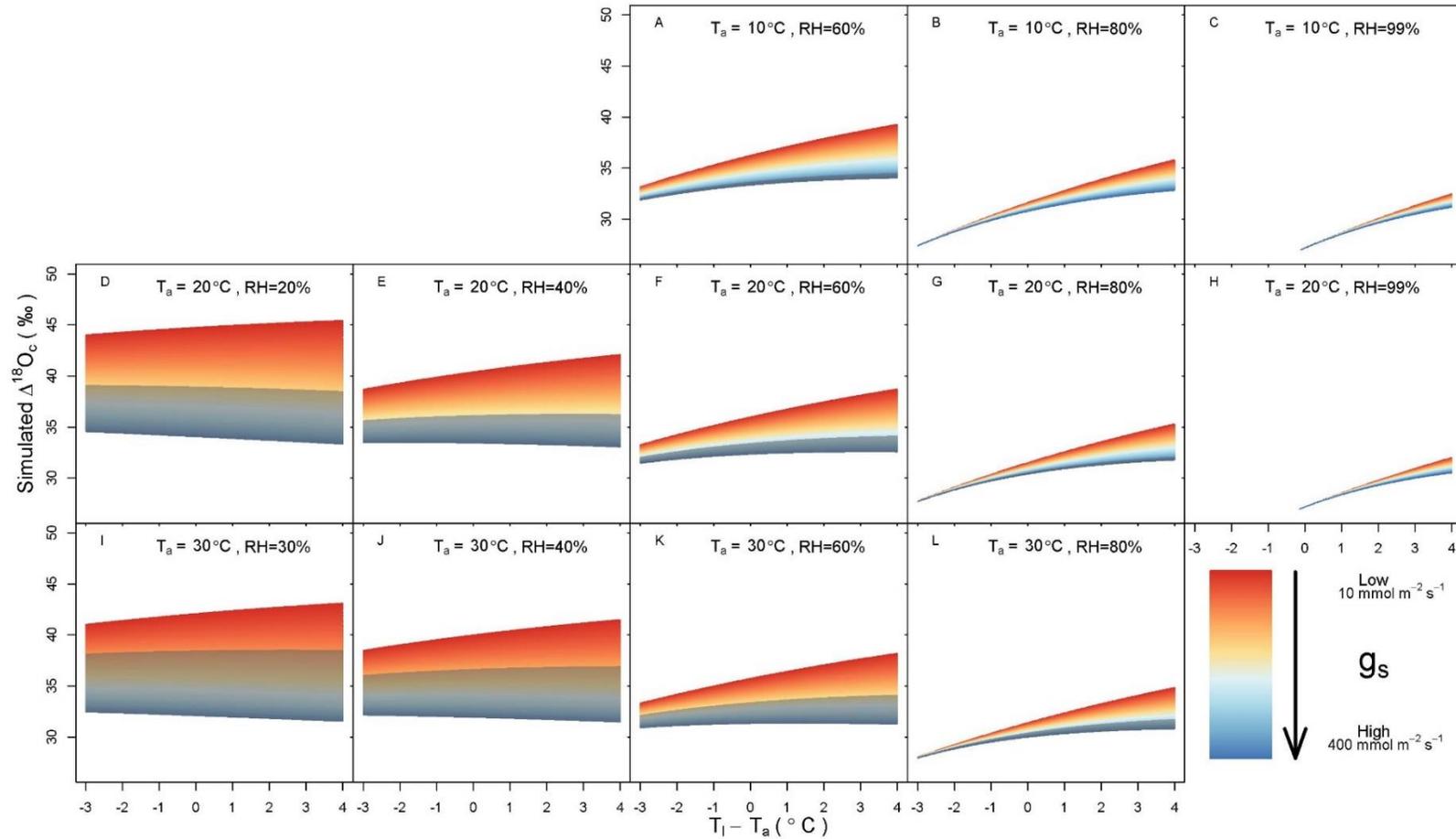


Figure 4.4 The multi-dimensional sensitivity analysis for simulated oxygen isotope discrimination of tree ring cellulose (simulated $\Delta^{18}\text{O}_c$), varying with air temperature (T_a), relative humidity (RH), temperature difference between air and leaf ($T_l - T_a$), and stomatal conductance (g_s). The shaded area indicates unlikely simulation results due to the G_s -VPD relationship described by Equation 4.8.

water $\delta^{18}\text{O}$ across treatments did not hold in our study. The trees under drought may have accessed source water from a deeper soil depth, that has a lower $\delta^{18}\text{O}$ value, either due to less evaporation (Dawson et al. 2002, Marshall et al. 2008, Sarris et al. 2013) or a higher proportion of stored winter precipitation, which had a lower $\delta^{18}\text{O}$ compared to summer precipitation (Marshall et al. 2008).

Tree responses to fertilization

The reported effect of nutrient availability on iWUE in trees has been mixed (Balster et al. 2009, Brooks and Coulombe 2009, Brooks and Mitchell 2011, Cornejo-Oviedo et al. 2017, Elhani et al. 2005). Astonishingly, we surveyed the literature and found no studies on tree ring carbon isotopes in loblolly pine. A companion PINEMAP study in GA measured *foliar* $\delta^{13}\text{C}$ in 2012 and 2013 and found that drought/fertilization effects were not significant a year after the experiment started (Samuelson et al. 2014). Thus, it is not surprising that we could not detect a response to fertilization in iWUE. Although no significant response to fertilization was detected in mean daily G_s (Table 4.1), the mean daily G_s from the fertilization only plots was 19.3% higher than that of the control, and thus we suspect that g_s actually increased slightly. Therefore, photosynthetic rate (A) of the fertilized plots had to increase proportionally to keep iWUE unchanged. However, because the aboveground biomass increment and LAI responded to fertilization across drought treatments (Figure 4.2, Table 4.1), we conclude that increased leaf area was the major driver of AGBI, rather than the small increase in A, consistent with Gough et al. (2004a) and Samuelson et al. (2001).

If fertilization generally increases g_s , then we might expect leaf temperature to decrease compared to the control. Potentially, RH under fertilization may have been slightly higher than the control due to higher transpiration rates due to significantly higher LAI (Table 4.1). Any of the aforementioned changes would lead to a decrease in $\Delta^{18}\text{O}_c$ (green arrows in Figure 4.5, Table 4.2). Therefore we expected the simulated $\Delta^{18}\text{O}_c$ to remain unchanged or slightly decrease in response to fertilization. The observed $\Delta^{18}\text{O}_c$ did not respond to drought

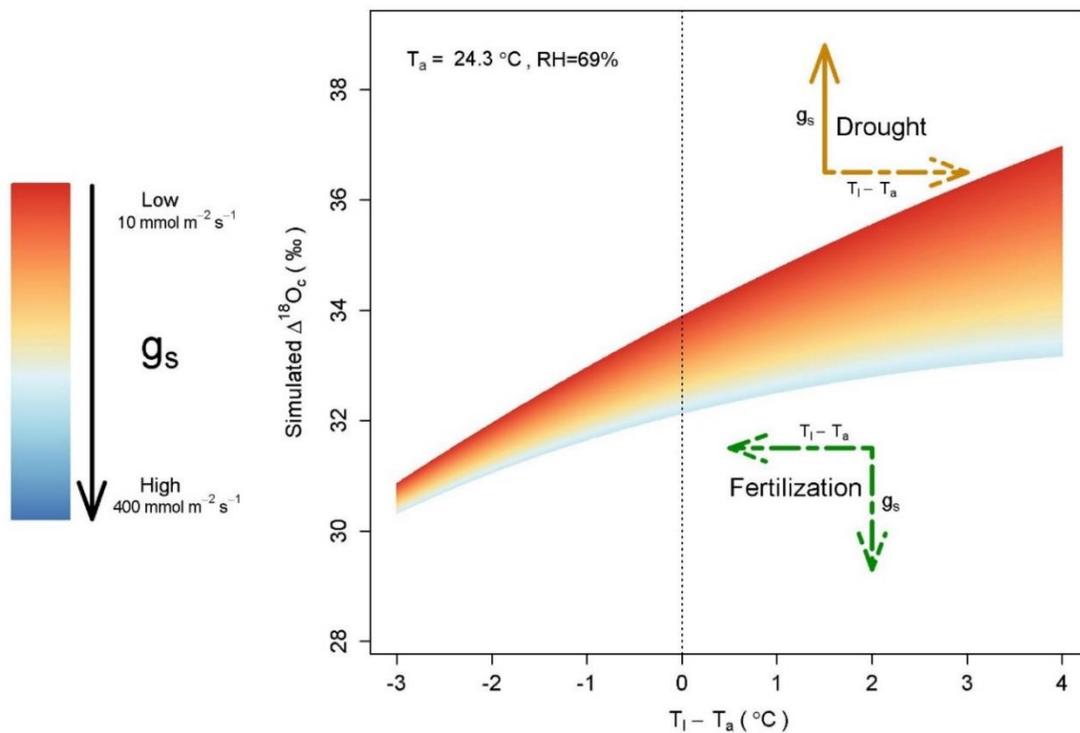


Figure 4.5 The simulated $\Delta^{18}\text{O}$ of cellulose in tree rings ($\Delta^{18}\text{O}_c$) with varying leaf-to-air temperature difference ($T_l - T_a$) and stomatal conductance (g_s) at site mean air temperature ($T_a=24.3$ °C) and relative humidity (RH=69%). The arrows in the plot indicate the direction of changes in g_s and ($T_l - T_a$) under shifting water and nutrient availability. The solid arrow indicates the observed trend, and dashed arrows indicate expected trends.

Table 4.2 The responses of relative humidity (RH), leaf-to-air temperature difference (T_l-T_a), stomatal conductance (g_s), simulated $\Delta^{18}O_c$, and observed $\Delta^{18}O_c$ to drought and fertilization treatments.

| Treatment | Change of parameters, model output, and observation compared to the control treatment | | | | |
|---------------|---|-----------------------|-----------------------|----------------------------|---------------------------|
| | RH | (T_l-T_a) | g_s | Simulated $\Delta^{18}O_c$ | Observed $\Delta^{18}O_c$ |
| Drought | Similar or slightly ↓ | Similar or slightly ↑ | ↓ | ↑ | ↓($p=0.003$) |
| Fertilization | Similar or slightly ↑ | Similar or slightly ↓ | Similar or slightly ↑ | Similar or slightly ↓ | Similar ($p=0.57$) |

(Figure 4.2, Table 4.1), but on average it was slightly lower under fertilization compared to the control, thus the prediction was consistent with the observation.

Implications for loblolly pine under a changing climate

In our study site, drought was simulated by decreasing precipitation throughfall, while the air temperature and RH were presumably similar to the control due to the small plot size and the mixing of the atmosphere. In reality, drought entails not only reduced availability of soil water, but also a warmer and dryer atmosphere with greater evaporative demand, all of which are expected to result in more pronounced stomatal responses than have been reported in previous studies (e.g. Novick et al. 2016). With decreased g_s and increased atmospheric CO_2 concentration, intrinsic water use efficiency might be expected to continue to increase, which may not, however, translate to increased growth (Peñuelas et al. 2011). Limitations on stomatal conductance would likely decrease the realized range of photosynthetic rate, at least

for species like loblolly pine that have tight stomatal control to maintain high leaf water status (i.e. isohydric species; Green and Mitchell 1992).

Implications for the dual isotope approach

The dual isotope approach suggested by Scheidegger et al. (2000) utilizes both $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ information from plant materials to explain how much the shifts in iWUE can be attributed to changes in photosynthetic rate and g_s (e.g. Barnard et al. 2012, Brooks and Mitchell 2011, Giuggiola et al. 2016). However, it is based on the assumption that source water $\delta^{18}\text{O}$ is consistent. In the current study, the assumption of consistent source water seemed plausible given the small plot size and proximity of the different treatments. In addition, Roden and Siegwolf (2012) have suggested that the approach should be applied to tree species with a tight stomatal control and be cautious of sites with persistently high RH. In our study site, RH is lower than 80% around two thirds of the time, and high RH also tended to appear before 8:00 and after 19:00, when photosynthesis was less active. Given that loblolly pine is isohydric, the two prerequisites were also met. However, our sensitivity analyses revealed that the assumption of consistent source water $\delta^{18}\text{O}$ could not have been correct, and that the only plausible explanation for the observed changes in $\delta^{18}\text{O}$ was that tree under the drought treatment utilized a different source water (e.g. drought-stressed trees must have accessed deep soil water to a greater extent than non-drought trees). Therefore, our results point to the need for careful examination of the underlying assumptions before applying the dual isotope approach, as mentioned by Roden and Farquhar (2012) and Roden and Siegwolf (2012).

Conclusions

Three and a half years after a one-time fertilization application and an annual 30% reduction in precipitation throughfall, LAI and aboveground biomass increment of loblolly pine responded only to fertilization, whereas iWUE and oxygen isotope discrimination responded only to drought. No significant interaction between the treatments was detected. Although drought reduced stomatal conductance, effects on photosynthetic rate were small. Effects of fertilization on photosynthetic rate and stomatal conductance were small, and apparently proportional between them. The positive effect of fertilization on productivity was mostly due to increased leaf area rather than stimulation of photosynthetic rate. On the other hand, we found that trees under drought utilized source water of a significantly lower $\delta^{18}\text{O}$, indicating that the throughfall reduction treatment may have led to the depletion of surface water, forcing trees to tap isotopically distinct (presumably deeper) water sources. Our sensitivity analysis supports the relatively fewer studies that urge caution when applying the dual isotope approach, and indicate that the underlying assumptions should be verified when drawing inferences. Finally, under a warming climate it is important to keep in mind that the associated increase in atmospheric evaporative demand, when added to soil water stress, may result in even greater physiological and productivity responses of plantation-grown pine than those reported here.

Chapter 5. Synthesis

Southern pine plantations plays a central role in the ecological and economical services of the United States. Due to global climate change (IPCC 2014) and the complex interactions between forests, climate, soils, and management (Bonan 2011, Fox et al. 2007b), it is important to improve our understanding of tree physiology to mitigate the potentially negative outcome and to better adapt the future climate shifts. This study thus focused on the drought responses using tree ring cellulose stable isotopes, in un-fertilized plantation loblolly pines across southeastern US, and in a study site with manipulated experiments on drought and fertilization.

With a new cellulose extraction method, we sampled 76 plantations from the natural distribution range of loblolly pine, and the responses of tree ring cellulose carbon isotope discrimination ($\Delta^{13}\text{C}$) to drought was analyzed and modeled. Although baseline $\Delta^{13}\text{C}$ is primarily affected by site water status and ranged from 18.0‰ to 20.8‰, the averages of baseline $\Delta^{13}\text{C}$ at each category of water table depth did not differ significantly. Thus this parameter may be resilient to environmental changes (Ehleringer and Cerling 1995, Marshall and Monserud 1996) and may not be affected by climate change greatly. When PDSI is below 1, the average reduction in $\Delta^{13}\text{C}$ is 0.31‰ per unit change of PDSI. This reduction in $\Delta^{13}\text{C}$, however, varies geographically (Figure 3.5): the reduction in Atlantic coastal plain sites was very mild, while it was above average in the sites from the west side of the Mississippi River. The most drought sensitive sites were in the far northeastern part of the range. Therefore, except the Atlantic coastal plain, it is expected that the intrinsic water use

efficiency (iWUE, proportional to $\Delta^{13}\text{C}$) in southeastern USA would increase under future climate change.

Three years and a half after a one-time fertilization application and an ongoing 30% reduction in precipitation at our study site in VA, the leaf area and aboveground biomass increment of loblolly pine responded only to fertilization, whereas iWUE and oxygen isotope discrimination responded only to drought. Although drought reduced stomatal conductance, its effect on photosynthetic rate was small. The effect of fertilization on photosynthetic rate and stomatal conductance was both minimal. Thus the positive effect of fertilization on productivity manifested mostly through increased leaf area. On the other hand, drought treatment modified source water for the loblolly pines.

Using drought sensitivity, the reduction in precipitation at VA site can be converted to change of PDSI. As mentioned earlier, the range-wide average of drought sensitivity is 0.31‰ per unit change of PDSI. Due to the strong spatial correlation (Figure 3.5), the average of drought sensitivity in northern geographic region (0.41‰) was used. Thus a 30% reduction in precipitation resulted in a reduction of 0.51‰ in $\Delta^{13}\text{C}$, which can be roughly converted to a change of PDSI of 1.2. Although such reduction in precipitation is unlikely to take place in future southeastern USA (IPCC 2014), this magnitude of PDSI change is possible (Dai 2013). At VA site, the ongoing 30% reduction in precipitation (or PDSI drops by 1.2) did not impact the productivity of loblolly pine significantly, but it was strong enough to affect stomatal conductance and modify source water. Therefore, in the northern region of the distribution range of loblolly pine, future drought may not affect the plantation

productivity significantly, although local water balance may be modified. However, greater physiological and productivity responses to future drought than those reported here may be possible, when increased atmospheric evaporative demand is added up to the soil water stress.

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APPENDICES

Table S.1 The detailed cost estimates for major equipment for major batch-wise Jayme-Wise α -cellulose extraction methods. The costs are estimated for equipment specific for α -cellulose extraction, in a typical ecological wet lab setting. Standard laboratory equipment like water purifier, centrifuge, and hot plates are not included in the cost estimate. The estimates are approximate, aimed to group the methods in broad categories rather than offer clear budgetary information. The exact costs will vary by country, vendor, and existing infrastructure.

| Methods | Estimated cost | The cost for major equipment |
|---|-----------------------|--|
| Leavitt and Danzer (1993) | <\$3,000 | Soxhlet extractors: ~\$200 each ^a Filter paper bags: ~\$1.5 each ^a |
| Kagawa et al. (2015) | <\$3,000 | Water Bath: ~\$500 ^b Microscope: ~\$2,000 ^b |
| Harada et al. (2014) | \$5,000-10,000 | Polyethylene filter: ~\$1 each ^b Microscope: ~\$2000 ^b Ultrasonic Bath: ~\$3000 ^b Water bath: ~\$500 ^b |
| Loader et al (1997) | \$5,000-10,000 | Soxhlet extraction thimbles: ~\$40 each ^{cd} Ultrasonic bath: ~\$3000 Soxhlet extractors: ~\$200 each ^a |
| Wieloch et al (2011) | >\$15,000 | MSISS drainage module with 20 access points: ~\$700 each ^d Büchner funnels: ~\$34 each ^{ae} vacuum aspirator pump: ~\$200 ^a |
| Wieloch et al 2011 + acetone pretreatment (current study) | >\$15,000 | MSISS module with 20 access points: ~\$700 each ^d Büchner funnels: ~\$34 each ^{ae} Delrin holder with 20 access points: ~\$200 each ^d vacuum aspirator pump: ~\$200 ^a |

^a Cost estimates are based on negotiated pricing agreements for USA academic institutions, 2016 (retail prices may be higher).

^b Nakatsuka T, Personal communication (2016).

^c Loader NJ, personal communication (2016).

^d Customized equipment.

^e The Büchner funnels were customized at the Potsdam Dendro Lab (Wieloch et al. 2011).

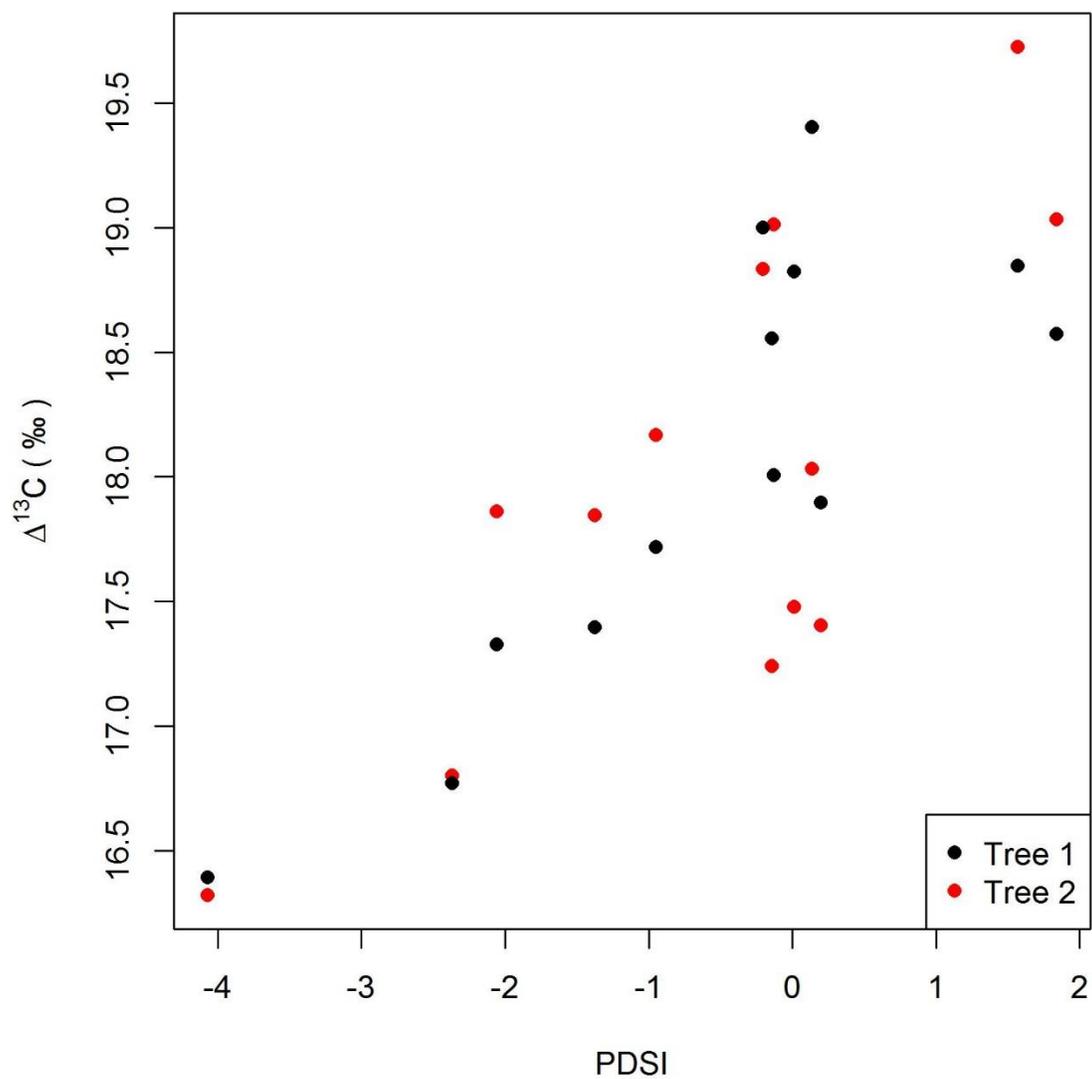


Figure S.1 The preliminary study on the relationship between Palmer drought severity index (PDSI) and carbon isotope discrimination ($\Delta^{13}\text{C}$) of 2 loblolly pine trees sampled in site MRTS-2nd Thin_4, Bellwood County, LA, USA from year 2000 through 2012.