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Oxygen Isotope Signature of Cellulose: Advances as a Climate Proxy

Patricia V. Ellsworth
University of Miami

UNIVERSITY OF MIAMI

OXYGEN ISOTOPE SIGNATURE OF CELLULOSE: ADVANCES AS A CLIMATE PROXY

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Patricia V. Ellsworth

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Patricia V. Ellsworth

Approved:

_____ Leonel Sternberg, Ph.D. Professor of Biology	_____ M. Brian Blake, Ph.D. Dean of the Graduate School
_____ David Janos, Ph.D. Professor of Biology	_____ Donald DeAngelis, Ph.D. Professor of Biology
_____ William Anderson, Ph.D. Professor of Geosciences Florida International University	

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Cellulose is a very stable structural molecule integrated in the incremental growth of tree rings, often used for paleoclimate reconstruction. As cellulose is synthesized it incorporates the oxygen isotopic signature of source (stem) and leaf water, which provide valuable information about climate such as precipitation and relative humidity. However, source and leaf water isotopic signatures can be masked by plant physiological and biochemical processes during cellulose synthesis. The biochemical interference does not occur equally in all the oxygen of the cellulose molecule. In Chapter 2, I compared the observed versus the reconstructed oxygen isotope ratios of stem water ($\delta^{18}O_{SW}$) using $\delta^{18}O$ of cellulose and of an artificially modified cellulose molecule. The only difference between cellulose and modified cellulose molecules is that the latter had the oxygen attached to the second carbon of the cellulose glucose moieties ($O_{C\text{-}2}$) removed. The modified cellulose molecule was a better predictor of $\delta^{18}O_{SW}$ than was the entire cellulose molecule. This difference suggests that the oxygen attached to C_2 introduces variability in the $\delta^{18}O_{CELL}$ not related to the $\delta^{18}O_{SW}$. In Chapter 3, I compared the $\delta^{18}O_{CELL}$ and $\delta^{18}O_{SW}$ from two transects encompassing salt to freshwater, under the same climatic conditions. The $\delta^{18}O_{CELL}$ did not record differences in $^{18}O_{SW}$. In one of the transects the above modification of the cellulose molecule improved the relationship between $\delta^{18}O_{CELL}$ and $\delta^{18}O_{SW}$. In the second transect, despite no differences in RH between mangrove and

freshwater plants, oxygen isotopic fractionation associated with leaf physiology caused the $\delta^{18}O_{CELL}$ to be less enriched than what was expected based on its source water. The changes in leaf physiology were associated with a longer leaf water pathway from the xylem to the stomatal pore in mangrove than in freshwater plants. Considering that mangrove plants are salt tolerant, a longer L could be linked to ultrafiltration of salts in the leaves. Changes in leaf physiology introduced variability in the $\delta^{18}O_{CELL}$ that was neither related to $\delta^{18}O_{SW}$ nor RH. In Chapter 4, I investigated the salinity effect in overall plant biochemistry and how it interfered with the recording of $\delta^{18}O_{SW}$ in the $\delta^{18}O_{CELL}$. A currently accepted tree ring model used in climate reconstruction assumes no variation in plant biochemistry, which is incorporated in the model as the proportion of oxygen isotope exchange between sucrose and stem water (p_{ex} = 42%) and biochemical fractionation (ε_{bio} = 27‰). However, salinity effects caused ~2x increase in p_{ex} likely by increasing the pool of soluble carbohydrates, allowing more time for oxygen isotope exchange between carbohydrate intermediates and stem water during cellulose synthesis. The increase p_{ex} in salt treated plants caused their $\delta^{18}O_{CELL}$ to be 1.5‰ less enriched than expected. Even though ε_{bio} values differed between salt and freshwater treatments, they were not significant. However, when investigating soluble carbohydrate excess in a starchless mutant *Arabidopsis thaliana* without a salinity effect, ε_{bio} variation was confirmed. Salinity impacts in plants are fundamental both at physiological and biochemical level and must be considered so that the $\delta^{18}O_{CELL}$ of plants exposed to salinity can only reflect $\delta^{18}O_{SW}$ and RH. This study brings to attention the chances of developing misleading conclusions about climate reconstruction if plant physiological and biochemical variation are not fully understood and taken into account.

To my parents Marlene and Miguel, and my husband Patrick; their example of love and care taught me what family means.

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Oxygen isotope ratios of stem water ($\delta^{18}O_{SW}$), of stem cellulose ($\delta^{18}O_{CELL}$) and of stem phenylglucosazone ($\delta^{18}O_{PG}$) from mangroves (●) and hammock plants (freshwater plants) (○) located at different distances from the ocean along each of the transect area. Study sites and sample collections: Big Pine Key (Jan 2009) and Sugarloaf Key (Mar 2009), FL, USA. Areas having the same or no letters are not statistically different with regard to the measured parameter: $P < 0.05$.

CHAPTER 1

Introduction

Interpretation of paleoclimate in paleohydrology is greatly facilitated by the knowledge of the isotopic identity of the environmental water at the time of interest. Ice cores have been greatly used to reconstruct precipitation and temperature (Krinner and Werner, 2003; Zhao et al., 2012), however its distribution is limited to remote areas. Trees, on the other hand, are advantageous because they are globally distributed and have the ability to record climate in the incremental growth rings of their trunks. Environmental conditions such as precipitation, temperature, relative humidity and sea level rise signals that are potentially incorporated in the isotopic abundance of ^{18}O and ^{2}H in a tree ring cellulose molecule. Cellulose is a β-polyglucan chain that once synthesized, is preserved without any D-glucose addition to the chain (Fig 1.1). After cellulose synthesis, there is no oxygen exchange between cellulose and the water surrounding it; therefore the oxygen isotope ratios of well-preserved cellulose have the potential to record climate at the time of tree ring formation. The ability to record such environmental signals and its chemical stability makes the cellulose molecule a candidate of choice for paleoclimate reconstruction. Because this dissertation is focused on oxygen stable isotope incorporation in the cellulose molecule, a short background on the basics of stable isotope dynamics follow.

Oxygen isotope ratios of water

Isotopes are different forms of the same element. The atoms of isotopes have the same number of protons, but different numbers of neutrons, resulting in different mass.

Isotopes, therefore, have the same chemical but different physical properties. The oxygen with mass 16 (^{16}O) is the most abundant 99.76% of the isotopes followed by ^{18}O (0.2%). The ratio between these two oxygen isotopes is calculated relative to a standard (Vienna Standard Mean Ocean water ,V-SMOW; Coplen et al., 1983; Eqn 1),

$$\delta^{18}O = [(R_{samples}/R_{standard}) - 1] \times 1000 \quad (1)$$

where R_{sample} and $R_{standard}$ are the $^{18}O/^{16}O$ ratios of the sample and the standard, respectively. The δ notation is expressed in ‰, and reflects environmental processes such as condensation (precipitation) and evaporation.

During precipitation, $H_2{}^{18}O$ condenses at a faster rate than $H_2{}^{16}O$, so the first rain falling from a cloud has a higher $\delta^{18}O$ than the last, if there is no cloud water recharge. This process is known as Rayleigh distillation and determines the $\delta^{18}O$ pattern of precipitation around the globe. During evaporation process, $H_2{}^{16}O$ evaporates at a faster rate than $H_2{}^{18}O$, so that the water body under evaporation becomes enriched in ^{18}O. The same process occurs in soil water and in leaf water, as both situations are losing water to the atmosphere through evaporation.

The process of one isotope being favored over the other isotope during chemical or physical processes is called fractionation. During condensation, the extent of oxygen isotope fractionation is related to temperature, and the equilibrium between vapor and water, being called equilibrium fractionation. During water evaporation, both equilibrium (water to vapor) and kinetic fractionation processes occur. Kinetic fractionation factor is the fractionation that occurs as isotopes diffuse across a boundary layer, and it is dependent on relative humidity and boundary layer condition (Brunel et al., 1992).

Oxygen isotope ratios of cellulose

Climate signals are recorded in soil and leaf water oxygen isotope composition and those isotopic water signatures are recorded in the oxygen isotopic composition of cellulose (Fig 1.3). The source water oxygen isotope signature incorporates the oxygen isotopic composition of all possible water sources available in the soil, such as precipitation, ground water, soil moisture, etc (Ehleringer and Dawson, 1992). Ocean water is an additional water source in mangrove areas. In shallow areas, where ocean water undergoes evaporation, it becomes isotopically enriched in ^{18}O relative to freshwater sources (Sternberg et al., 1991). Source water is taken up by the plants without oxygen isotope fractionation so that $\delta^{18}O_{SOURCE\ WATER} = \delta^{18}O_{STEM\ WATER}$ (Wershaw et al., 1970). The leaf water reflects variation in RH, isotopic composition of atmospheric vapor, transpiration rates (Dongmann et al., 1974) and length of leaf water pathway from xylem to stomatal pore (Farquhar and Lloyd, 1993). In addition, carbohydrate metabolism contributes to the oxygen isotope fractionation, causing the $\delta^{18}O_{CELL}$ to be higher than the water oxygen isotopic signal in the plant.

The contribution of oxygen isotope signatures that comes from each, source and leaf water, and the additional biochemical fractionation during cellulose synthesis have been widely studied (Sternberg and DeNiro, 1983; Sternberg et al., 1986; Barbour et al., 2000; Roden and Ehleringer, 2000; Cernusak et al., 2005; Gessler et al., 2007; Sternberg and Ellsworth, 2011) and modeled by Roden et al. (2000) as (Eqn 2; Fig 1.3):

$$\delta^{18}O_{CELL} = (0.42 * \delta^{18}O_{SW}) + (0.58 * \delta^{18}O_{LW}) + \varepsilon_{bio} \qquad (2)$$

where the proportion of the oxygen isotope ratios of stem water ($\delta^{18}O_{SW}$) and leaf water ($\delta^{18}O_{LW}$) in the $\delta^{18}O_{CELL}$ corresponds to 42% and 58%, respectively, and the plant biochemical fractionation (ε_{bio}) corresponds to 27‰.

Leaf water oxygen isotope labeling in cellulose precursors

As the xylem (stem) water reaches the leaf surface and undergoes evaporation, the remaining water becomes enriched in ^{18}O because ^{16}O evaporates at faster rate. Their ratio can be calculated by equation 3 (Craig and Gordon, 1965),

$$\delta^{18}O_{EW} = \delta^{18}O_{SW} + \varepsilon^{*} + \varepsilon_k + RH^{*}(\delta^{18}O_V - \delta^{18}O_{SW} - \varepsilon_k) \qquad (3)$$

where oxygen isotope ratios of internal leaf surface water ($\delta^{18}O_{EW}$), depends on variables such as equilibrium and kinetic fractionation (ε^* and ε_k, respectively), RH, $\delta^{18}O$ of atmospheric vapor ($\delta^{18}O_V$), and oxygen isotope ratios of stem water ($\delta^{18}O_{SW}$).

The isotopically enriched water pool at the internal leaf surface back diffuses and mixes with the stem water pool that moves from the xylem toward the stomatal pore (Farquhar and Lloyd 1993). This is known as the Péclet effect (ρ) (Eqn 4),

$$\rho = (EL)/(CD) \qquad (4)$$

where E represents transpiration rate, L the length of the water pathway from the xylem to the stomatal pore, C represents the molar concentration of water (5.56×10^4 mol m^{-3}), and D represents the diffusivity of $H_2{}^{18}O$ in water (2.66×10^{-9} $m^2 s^{-1}$). The relative contribution of each of those water pools (enriched: α and non-enriched water: $1-\alpha$) determines the oxygen isotope signature of the whole leaf water ($\delta^{18}O_{LW}$; Eqn 5), where α is given by $(1-e^{-\rho})/\rho$.

$$\delta^{18}O_{LW} = \alpha^{*}\delta^{18}O_{EW} + (1-\alpha)^{*}\delta^{18}O_{SW} \qquad (5)$$

The contribution of enriched and unenriched water to the $\delta^{18}O_{LW}$ varies with *E* and L (leaf physiology component). Differences in $\delta^{18}O_{LW}$ between species under the same RH, using the same source water can be a result of variation in leaf physiology. This was modeled by Barbour and Farquhar (2003), where apoplastic (cell walls) leaf water pathway had longer L than symplastic (aquaporins or plasmodesmata). If L is long, it will decrease the back diffusion of enriched ^{18}O water at the leaf surface, and the whole leaf water becomes less enriched than expected under low transpiration rates.

The oxygen isotopic signature from leaf water is 100% incorporated in the oxygen atoms of leaf sucrose, so that the $\delta^{18}O$ of sucrose reflects those of leaf water. Sucrose is then translocated to stems where it will record the stem water oxygen isotope signature.

Stem water oxygen isotope labeling in cellulose precursors

In the stem, sucrose exchanges oxygen atoms with the surrounding stem water. Sucrose cleaves into glucose and fructose, which forms carbonyl oxygen in the first and second carbon, respectively. It is the oxygen from the carbonyl group that exchanges with those of stem water, however this reaction is slow, with a half-equilibration of fructose 6-P and fructose 1,6-diP of 166 min and 29.5 min, respectively (Model et al. 1968, Reynolds et al. 1971). The participation of fructose in a futile cycle (Fig 1.2), where fructose 1,6-biphosphate splits into trioses and reassembles (Hill et al. 1995), is perhaps the major reaction causing cellulose to gain the isotopic signature of the stem water. As only a portion of fructose participates in the futile cycle, the labeling of the cellulose molecule with stem water is incomplete (Eqn 2).

As this exchange between carbonyl oxygen from trioses and stem water reaches equilibrium, the $\delta^{18}O$ of carbonyl oxygen is 27‰ more enriched than the water

surrounding it (Sternberg and DeNiro, 1983). This isotopic enrichment is further passed on to fructose and glucose, which easily are converted from one to another via glucose phosphate isomerase, prior to cellulose synthesis. Some studies, however, suggest that ε_{bio} is the overall enrichment imposed by all possible metabolic reactions that use the same cellulose precursors (Barbour et al., 2000; Gessler et al., 2007). Previous studies have shown that not all oxygen atoms from glucose molecules have the same oxygen isotopic fractionation, which could be related to other biochemical reactions that share the glucose substrate. Because in the previous model (Eqn 2) variations in the biochemical fractionation are not taken into account, differences in $\delta^{18}O_{CELL}$ caused by ε_{bio} would be attributed to climate.

Current challenges

Leaf physiology

A particularly interesting study was recently demonstrated by Brooks and Coulombe (2009), who showed that after fertilization, the oxygen isotope ratios of tree ring cellulose ($\delta^{18}O_{CELL}$) were higher than those of control trees. They concluded that because the trees were experiencing similar RH and source water oxygen isotopic signature, leaf physiology likely changed under nutrient fertilization, increasing the leaf water oxygen isotope enrichment, further registered in the $\delta^{18}O_{CELL}$. Verheyden et al. (2004) observed that during the dry season (low RH and low precipitation) tree ring wood of *Rhizophora mucronata* was depleted in ^{18}O instead of enriched. This was the opposite of what was expected from the leaf physiological response to low RH: partial stomatal closure, decreases in *E*, increase in $\delta^{18}O_{EW}$ contribution to $\delta^{18}O_{LW}$ (Eqn 4 and 5). If this was the case, as $\delta^{18}O_{LW}$ values increase, it would ultimately be reflected in high $\delta^{18}O_{CELL}$ values,

which was not the case. Earlier, Saurer et al. (1997) and Anderson et al. (2002), observed this discrepancy between observed and expected ^{18}O enrichment in the cellulose molecules under low RH. They used a dampening fraction that accounted for a fraction of enriched leaf water in the $\delta^{18}O_{CELL}$ so that expected $\delta^{18}O_{CELL}$ approached the observed $\delta^{18}O_{CELL}$. Even though Verheyden et al. (2004) found no explanation for the overestimation of $\delta^{18}O_{CELL}$ using the current tree ring model it could be related to this dampening effect, as previously mentioned. Presently, it is know that the length of the water pathway from the xylem to the stomatal pore does not respond to changes in RH, so that if L had differed between species living under the same climatic conditions, their $\delta^{18}O_{LW}$ and ultimately the $\delta^{18}O_{CELL}$ would differ. Species with the longest L would be less enriched than expected. In fact, differences in L among 3 species (*Phaseolus vulgaris*, *Ricinus communis* and *Helianthus annuus*) were observed by Kahmen et al. (2009). Changes in L, contribute to $\delta^{18}O_{CELL}$ differences, misleading conclusions about RH and precipitation. Therefore, a greater knowledge of leaf physiology processes is necessary to accurately proxy RH from $\delta^{18}O_{LW}$.

Plant biochemistry

The tree ring model considers as constants both the proportion of oxygen exchange between sucrose and stem water (p_{ex}) as well as ε_{bio}, assumptions that are not always the case. Measuring variation in ε_{bio} and p_{ex} is difficult. Sternberg et al. (2003) developed a technique to remove the oxygen isotope signal associated to the second carbon of cellulose glucose moieties (O_{C-2}). The isotope ratios of O_{C-2} were measured separately from those attached to carbons 3, 4, 5 and 6 (O_{C3-6}), and indicated different ε_{bio} and p_{ex} for these two sets of oxygen atoms in the cellulose molecule. Sternberg et al. (2007b), using

the above technique, found a better relationship between $\delta^{18}O_{CELL}$ and $\delta^{18}O_{SW}$ when only using the oxygen isotope ratios of cellulose $O_{C3\text{-}6}$.

Recently, Sternberg and Ellsworth (2011) found that wheat plants growing under different temperatures had similar p_{ex} but ε_{bio} increased as temperature decreased. Waterhouse et al. (2002) observed $\delta^{18}O_{CELL}$ could not fit the reconstructed $\delta^{18}O_{CELL}$ using the Roden et al. (2000) model (Eqn 2). The best-fit relationship between observed and reconstructed was found when Equation 2 was manipulated for p_{ex} = 46% and ε_{bio} = 30‰. Roden et al. (2005) observed that under low precipitation and low RH, the model overestimated the $\delta^{18}O_{CELL}$. Their observation could be explained by Barbour and Farquhar's (2000) speculation that under mild water stress, cell division and expansion may decrease, increasing the time for oxygen exchange between sucrose and stem water. In this situation, a higher contribution of $\delta^{18}O_{SW}$ ($p_{ex} > 0.42$) would decrease $\delta^{18}O_{CELL}$ values relative to that predicted using $p_{ex} = 0.42$. Understanding and accounting for variation in p_{ex} and ε_{bio} is essential so that climate alone can be extracted from the $\delta^{18}O_{CELL}$ as opposed to a mixture of climate and inaccurate assumptions of plant biochemistry.

Dissertation research

This dissertation research aims to explain why there often is a lack of relationship between observed and reconstructed $\delta^{18}O_{CELL}$ using the current tree ring model by Roden et al. (2000). I specifically focused on the contribution of leaf physiology and plant biochemistry to the oxygen isotope signature of cellulose. In Chapter 2, I tested the potential of $\delta^{18}O_{CELL}$ for reconstructing the $\delta^{18}O_{SW}$. To do so, I compared observed and reconstructed values of stem water, using the $\delta^{18}O_{CELL}$ as a predictor of stem water. I

attempted to improve the relationship between observed and reconstructed values of $\delta^{18}O_{SW}$ using Sternberg et al.'s (2003) method, where the cellulose molecule was modified by removing the oxygen attached to Carbon 2. To do so, I improved their technique, so it could be applied to small tree ring samples. The same approach was used in Chapter 3, studying the relationship between $\delta^{18}O_{CELL}$ and the $\delta^{18}O$ of stem water, from transects encompassing fresh to saltwater plants. In one of the transects, I also investigated the possible changes in leaf physiology (*E* and L) and how they further were recorded in the $\delta^{18}O_{CELL}$. In Chapter 4, I investigated the effect of salt stress, changes in soluble carbohydrates and osmolytes on the biochemical fractionation and in the proportion of oxygen exchange between sucrose and stem water. I intended to clarify how $\delta^{18}O_{CELL}$ differ from that expected by Roden et al. (2000) as a result of changes in plant biochemistry.

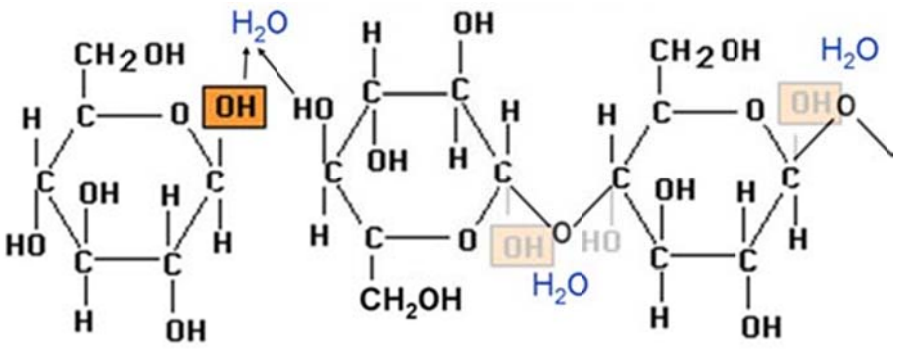

Figure 1.1 Cellulose molecule. Glucose molecules bind to each other by a dehydration process, where an OH from carbon 1 binds with an H from carbon 4. The remaining oxygen in carbon 4 is shared with carbon 1.

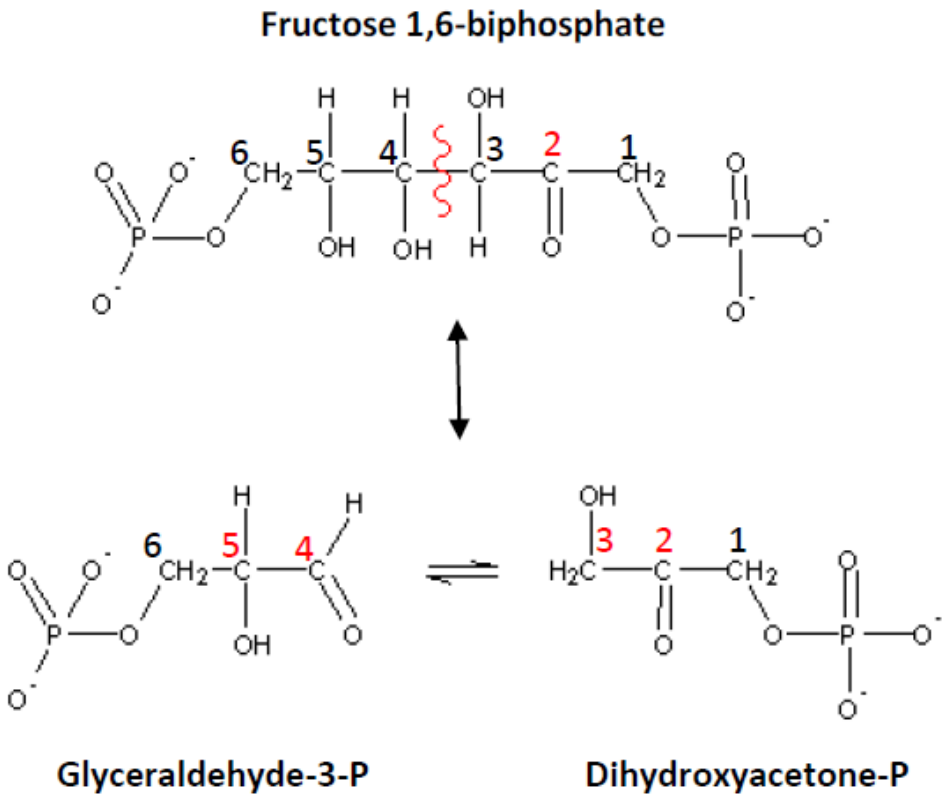

Figure 1.2 Futile cycle, showing the formation of carbonyl groups as fructose 1,6-biphosphate splits into trioses. The carbonyl groups alternate their position during hydration and dehydration reactions, so that oxygen atoms from carbon 2, 3, 4 and 5 exchange and record the oxygen isotope signature of the surrounding stem water. This labeling of stem water oxygen isotopic composition is ultimately passed on to the oxygen isotope ratios of the cellulose molecule.

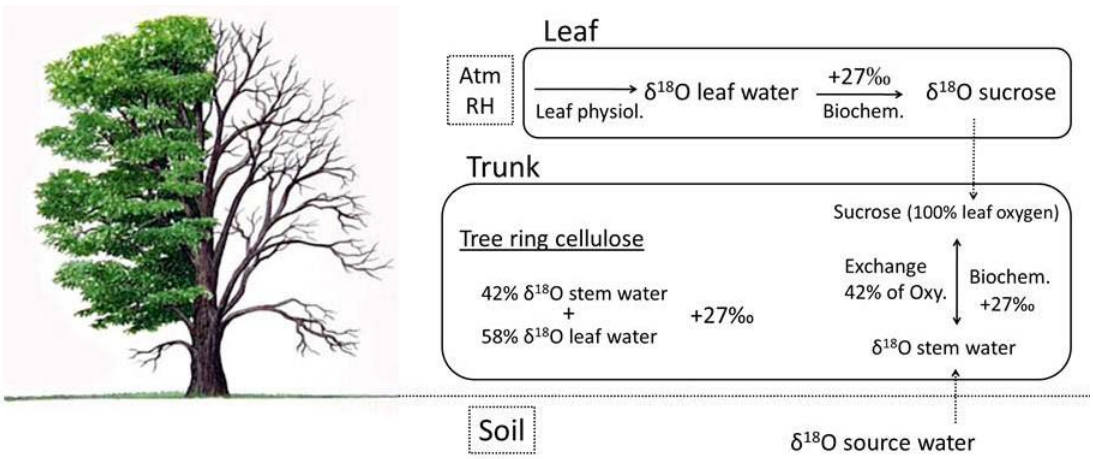

Figure 1.3 Leaf and stem water oxygen isotope labeling in the cellulose molecule according to the accepted tree ring model (Roden et al., 2000).

CHAPTER 2

Reconstruction of source water using the $\delta^{18}O$ of tree ring phenylglucosazone: a potential tool in paleoclimate studies.

Summary

The oxygen isotope ratios of tree ring cellulose have a great potential as a proxy for the oxygen isotope ratios of source water, which is related to climate. However, source water isotopic signatures can be masked by plant physiological and biochemical processes during cellulose synthesis. To minimize biochemical effects in the recording of source water, I modified the cellulose molecule to phenylglucosazone, which only has oxygen attached to carbons 3-6 (O_{C3-6}) of the cellulose glucose moieties, thus eliminating the oxygen attached to carbon 2 of the cellulose glucose moieties (O_{C-2}). Here I developed a method to use small amounts of inter and intra-annual tree ring cellulose for phenylglucosazone synthesis. Using this new method I tested whether the oxygen isotope ratios of source water reconstructed from tree ring phenylglucosazone ($\delta^{18}O_{sw}{}^{PG}$) and the observed source water ($\delta^{18}O_{sw}{}^{obs}$) would have a better agreement than those reconstructed from the tree ring cellulose molecule. Annual tree ring samples were obtained from *Pinus sylvestris* (1997-2003) (Finland) and *Picea abies* (1971-1992) (Switzerland) and intra-annual tree ring samples were obtained from *Pinus radiata* (Oct 2004-Mar 2006) (New Zealand), each near a meteorological station where precipitation and relative humidity (RH) were measured periodically. The $\delta^{18}O$ of tree ring cellulose and tree ring phenylglucosazone for each of the three species were then used to back calculate the $\delta^{18}O$ of source water according to a previously published empirical equation. As expected, the $\delta^{18}O$ of tree ring phenylglucosazone was superior to cellulose in the reconstruction of source water available to the plant. The deviation between $\delta^{18}O_{sw}{}^{PG}$ and $\delta^{18}O_{sw}{}^{obs}$ was in

part correlated with variation in atmospheric relative humidity (RH) which was not observed for the cellulose molecule. I conclude that this new method can be applicable to inter and intra-annual tree ring studies and that the use of the tree ring phenylglucosazone will significantly improve the quality of paleoclimate studies.

Background

The earth's climate changes continually and the challenge is to gain a knowledge of paleoclimate, monitor present climate and predict future climate in the most precise way. Significant progress had been made in monitoring present climate using remote sensing technology and networks of climate stations (Cracknell and Varotsos, 2011). The greatest challenge has been in deciphering paleoclimate. One measure that has shown a great potential as a paleoclimate proxy is the oxygen isotope ratios of tree ring cellulose. The cellulose molecule is very stable and records the isotope signals related to source water at the time it was synthesized (McCarroll and Loader, 2004). However, the source water signature seems to be masked by physiological and biochemical processes during cellulose synthesis (Barbour et al., 2002; Barbour, 2007; Sternberg et al., 2007b; Ogée et al., 2009; Ellsworth et al., 2012).

Sternberg et al. (1986), using heterotrophic carrot tissue culture observed that approximately 40% of oxygen atoms in the cellulose molecule acquired the isotopic signature of the medium water, whereas the other 60% retained the isotopic signature of the original sucrose molecule. Roden and Ehleringer (2000) used hydroponically grown tree saplings to confirm the partial exchange of oxygen during tree ring cellulose synthesis. By modeling the $\delta^{18}O$ values of leaf water, they were able to show that ~ 40% of the stem cellulose isotopic signature was related to the $\delta^{18}O$ of stem water, whereas the

other ~ 60% carries the isotopic signature of the leaf water. Based on these observations and other studies, the tree ring model (Roden et al., 2000) assumed that ~ 40% of the oxygen atoms in the cellulose molecule will be labeled with the oxygen isotopic signature of source water.

Biochemical reactions during this labeling process cause the cellulose molecule to be isotopically enriched relative to its source water. The cellulose enrichment above source water is thought to be mostly because of an equilibrium fractionation between oxygen of the water molecules and those of carbohydrates during cellulose synthesis (ε_{bio}). Based on previous observations (Sternberg and DeNiro, 1983; Cernusak et al., 2005), the tree ring model assumes that ε_{bio} is a constant of 27‰. However, some experiments have shown that both the proportion of exchange between the oxygen of stem water and carbohydrates and ε_{bio} are not constant (DeNiro and Epstein, 1981; Sternberg, 1989; Waterhouse et al., 2002; Verheyden et al., 2004; Roden et al., 2005; Brooks and Coulombe, 2009; Sternberg and Ellsworth, 2011). These observations emphasize the complexity of using oxygen isotope ratios of cellulose as a proxy for precipitation and/or relative humidity using the assumptions of the tree ring model.

Sternberg et al. (2003) calculated the isotope ratios of the oxygen attached to the second carbon ($\delta^{18}O_{C\text{-}2}$) of the glucose moieties of cellulose separately from those attached to carbons 3, 4, 5 and 6 ($\delta^{18}O_{C3\text{-}6}$). This was accomplished by measuring the $\delta^{18}O$ values of cellulose (containing the oxygen attached to carbon 2, 3, 4, 5 and 6) and that of a cellulose derivative called phenylglucosazone (PG) (containing the oxygen attached to carbon 3, 4, 5 and 6). By comparing these two isotope ratios they were able to

calculate the $\delta^{18}O$ values of the oxygen attached to the second carbon with the following equation (Eqn 1):

$$\delta^{18}O_{C\text{-}2} = (5 \cdot \delta^{18}O_{cell}) - (4 \cdot \delta^{18}O_{PG}) \quad (1)$$

Sternberg et al. (2003) found different oxygen isotopic fractionations between $O_{C3\text{-}6}$ and $O_{C\text{-}2}$ of the cellulose glucose moieties. Using the above technique, Sternberg et al. (2007b) observed that the oxygen isotope enrichment of the $O_{C\text{-}2}$ showed a high variability and with its removal, the relationship between the $\delta^{18}O_{PG}$ (or $\delta^{18}O_{C3\text{-}6}$) and the oxygen isotope ratios of stem water improved over that using all the cellulose oxygen atoms. The high isotopic variation of $O_{C\text{-}2}$ over the $O_{C3\text{-}6}$ maybe because of a higher propensity of $O_{C\text{-}2}$ in the glucose molecule to bind to enzyme active sites during metabolic reactions leading to end products other than cellulose. This variability could introduce "isotopic signal noise" to the remaining pool of cellulose precursors and would be passed on to the oxygen isotope ratios of cellulose (Sternberg et al., 2007b).

Here I first developed a method to use small amounts of cellulose during PG synthesis to test the use of PG as a source water isotope ratio proxy in annual and intra-annual tree ring samples. Previously, when this method was first developed, 300 mg of cellulose was required for its derivatization, which is not feasible for tree ring studies, especially for fossil samples. Second, I used this new method to investigate whether the oxygen isotope ratios of stem water reconstructed from tree ring phenylglucosazone ($\delta^{18}O_{sw}{}^{PG}$) would have a stronger relationship with the observed oxygen isotope ratios of stem (source) water ($\delta^{18}O_{sw}{}^{obs}$) than that reconstructed from tree ring cellulose ($\delta^{18}O_{sw}{}^{cell}$). I hypothesize that there will be a closer one to one relationship between $\delta^{18}O_{sw}{}^{PG}$ and $\delta^{18}O_{sw}{}^{obs}$ than between $\delta^{18}O_{sw}{}^{cell}$ and $\delta^{18}O_{sw}{}^{obs}$. Further, I tested whether the

deviations between $\delta^{18}O_{sw}{}^{PG}$ and $\delta^{18}O_{sw}{}^{cell}$ versus the $\delta^{18}O_{sw}{}^{obs}$ were related to the RH at the time of tree ring formation. Annual tree ring cellulose samples were obtained from two different species, Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) and intra-annual tree ring samples were obtained from Monterey pine (*Pinus radiata*). Each species was located near a meteorological station where precipitation (amounts and oxygen isotopic composition) as well as atmospheric RH were collected on a periodic basis.

Materials and Methods

1. Tree ring samples and meteorological data

Tree ring samples were collected (breast height) at three locations each near a meteorological station. The first set of samples was from tree rings of Scots pine (*Pinus sylvestris*) at sea level, located in Kessi, Inari, Finland (68º56'N, 28º19'E). Relative humidity was collected for the growing season months (Jun-Aug) at midday (data from Sodankylä - 67^{o} 22'N, 26^{o} 39'E, 179 m above sea level) taken from annual issues of Meteorological Yearbook of Finland (ISSN 1235-0419), Finnish Meteorological Institute, Helsinki. Amount and oxygen isotope signature of precipitation were collected by the Finnish Meteorological Institute (unpublished data by Sonninen et al.), located 104.18 km away in Utsjoki, Kevo (69º45'N, 27º01'E) at 107 meters above sea level, to calculate the yearly weighted average $\delta^{18}O$ values of precipitation ($\delta^{18}O_{ppt}$). Yearly weighted average $\delta^{18}O_{ppt}$ was calculated as the sum of the monthly products of $\delta^{18}O_{ppt}$ and the proportion of annual precipitation that fell in each month. The observed $\delta^{18}O$ values of plant source water were then extrapolated from $\delta^{18}O_{ppt}$ using a previously developed empirical

relationship (Eqn 2) (Sternberg et al., 2007b) developed from a broad range of latitudes (24°37'N Florida, USA - 69°00'N Siberia, RUS):

$$\delta^{18}O_{sw}{}^{obs} = (1.15* \delta^{18}O_{ppt}) + 0.55 \qquad (2)$$

Cellulose oxygen isotope ratios from yearly rings (1997-2003) were determined with pooled samples from 4 trees and cores taken from 2 directions in each tree. First, wood samples were processed to α-cellulose according to the method of Loader et al. (1997). Then, because there was insufficient cellulose for phenylglucosazone extraction from the above samples, I used a trunk disc from one of 4 pooled trees above. Cellulose was extracted using the same method mentioned above and an aliquot of 25 mg of cellulose was derivatized to phenylglucosazone.

The second set of cellulose samples was from tree rings of a Norway spruce (*Picea abies*) located in central Switzerland (47^0 10' 47"N, 8^0 15' 26"E), at 589 meters above sea level, between the Jura Mountains and Alps. Tree ring samples dated from 1971 to 1992. Relative humidity was collected for the growing season months (May-Sep) at midday while amounts and oxygen isotope ratios of precipitation were collected yearly at the Bern GNIP/SNIHC station (46^0 56' 50"N, 7^0 25' 31"E) at 550 meters above sea level. This station is operated by the Swiss National Hydrological and Geological Survey and is located 68.28 km from the experimented area. Yearly weighted average $\delta^{18}O$ values of precipitation were calculated, as previously described, and extrapolated to $\delta^{18}O$ values of plant source water (Eqn 2). Cellulose oxygen isotope ratios were determined from two cores taken in opposite directions from 4 different trees, after which they were separated annually and pooled for the same year in equal weights; previously reported by Anderson et al. (1998). Wood samples were processed to α-cellulose using a method

modified after Green (1963) (Anderson et al., 1998) and an aliquot of 25 mg of cellulose was derivatized to phenylglucosazone.

The third set of samples was from three Monterey Pine (*Pinus radiata*) trees located in Lincoln, New Zealand, (43^0 38.418'S, 172^0 28.686'E) at 11m above sea level. Intra-annual tree ring samples were collected in March 2006, and spanned tree growth between October 2004 and the sampling date. A meteorological station located 2 km away provided hourly measurements of RH during this period. These samples differ from the above two because intra-annual sections of the tree rings were collected to investigate the seasonal variation recorded within the rings. The three trees are clones, and fortnightly dendrometer measurements (not presented) indicated that the three trees grew at almost identical rates. This allowed wood samples for each of the seven time periods to be bulked from the three trees. Wood samples were processed to holocellulose according to the method of Leavitt and Danzer (1993) and treated with 17% NaOH to remove the hemicellulose. A small aliquot (ca. 0.55 mg) of the cellulose was saved for oxygen isotope analyses. Another aliquot of 25 mg was used for phenylglucosazone analysis. To determine the source water of these three trees, sections of woody branches (100 mm in length and 10-15 mm diameter, sampled from mid-canopy, sun-lit positions) were collected every fortnight for each season. Stem water was azeotropically extracted using toluene, and analyzed for $\delta^{18}O$ at the Research School of Biology, Australian National University. Fortnightly dendrometer measurements were used to weight the stem water $\delta^{18}O$ values by growth rate over the sampling period. The water table at this site is approximately 1m below the soil surface, and the 15m tall trees had access to groundwater throughout the sampling period.

2. Cellulose hydrolysis and derivatization

Here I improved the method by using only 25mg of cellulose instead of 300 mg (Sternberg et al., 2003) for future application in tree ring samples. To test the accuracy of the method, I used different amounts of Sigma cellulose (300 mg, 100 mg, 50 mg and 25 mg) for hydrolysis and derivatization and to do so, I changed proportionally the chemicals used in these processes according to the amount of cellulose. I found no significant difference in the oxygen isotope signature of phenylglucosazone from the different amounts of Sigma cellulose (One-way $_{ANOVA}$, $P > 0.05$) (Fig 2.1). All the water used in this study was purified by a Barnstead Nanopure Diamond Life Science Ultrapure Water System (Thermo Fisher Scientific Inc., Waltham, MA, USA). For the improved method, 25 mg of cellulose $(C_6H_{10}C_5)_n$ was hydrolyzed with 99% Trifluoracetic acid (TFA) by a modified method of Fengel and Wegener (1979). Cellulose was soaked in 8 ml of TFA for two hours in a 100 ml round bottom flask, subsequently boiled in a refluxer for 15 minutes. Water (3 mL) was added to the boiling solution and refluxing continued for 15 minutes. A further aliquot of water (25 ml) was added and the solution refluxed for another 30 minutes. The solution was filtered through a glass-fiber filter (VWR Glass Microfibre, 691, VWR International, Radnor, PA, USA). The hydrolysis flask (100 ml) was washed with 10 ml of water and this wash-solution filtered again. The filtrate was roto-evaporated to glucose, the cellulose hydrolysate, which has a thick syrupy consistency. To eliminate excess fluorine 10 ml of water was stirred into the thick syrupy residue and the solution was roto-evaporated again. The hydrolysate was then freeze-dried overnight.

The cellulose hydrolysate was derivatized to phenylglucosazone ($C_{18}H_{22}O_4N_4$) according to a modified method of Oikawa et al. (1998). One ml of acetic acid solution (20%) was added to the above freeze-dried hydrolysate with 0.25 ml of phenylhydrazine. This mixture was covered and stirred continually in a 90 °C water bath (2h). After derivatization, the flask was placed in a refrigerator until cool. Water (5 ml) was added in the round bottom flask to suspend the material, which was later filtered through a fritted glass filter (Kimax, 2 ml, Buchner Funnels, Kimble Chase, Vineland, NJ 08360, USA). The filtrand was washed three times with water, soaked in hexane for 15 minutes and rinsed three more times with water. The fritted glass filter with the phenylglucosazone (filtrand) was placed in a freeze drier and left overnight. The phenylglucosazone was further purified by scraping it from the fritted glass filter into a 40 ml conical centrifuge tube, and mixing with 2.5 ml of methanol (95%). The tubes were placed in the freezer (-10^0C) and after six hours were centrifuged for 10 min and the liquid was gently decanted. The phenylglucosazone precipitate was allowed to dry at room temperature prior to freeze-drying.

2.1. Oxygen isotope analysis of cellulose and PG

Oxygen isotope ratios of cellulose and PG were determined by a modification of Saurer et al. (1998). Half a milligram of cellulose or 1.2 mg of PG was pyrolyzed at 1080^{o}C in a quartz column filled to approximately half its height with glassy carbon and topped off by a layer of (0.5 g) of nickelized carbon and nickel wool (Elementar America, Mt. Laurel, New Jersey, USA) in a Eurovector Elemental Analyzer (Milan, Italy). Gases from the pyrolises (mostly hydrogen, nitrogen and carbon monoxide) were carried by helium, passed through magnesium perchlorate and Ascarite (Thomas Scientific, Swedesboro,

NJ, USA) to absorb H_2O and CO_2 respectively, and separated in a 3 m 5Å molecular sieve column (Eurovector, Milan, Italy) held at 70°C. The 3 m column insured the total separation of CO and N_2 (Fig 2.2). The oxygen isotope ratios were determined using the Isoprime IRMS (Elementar Analysensysteme GmbH, Hanau, Germany) on the carbon monoxide and compared with a standard gas calibrated to two standards: Sigma cellulose having an oxygen isotope ratio of 29.3‰ (Sauer and Sternberg, 1994) and International Atomic Energy Agency cellulose filter paper with an isotope ratio of 32.4‰. The precision of the Isoprime IRMS analysis is ± 0.3‰ and the precision for PG extraction and derivatization is ± 0.56‰. All isotope ratios are expressed in terms of per mil (‰) (Eqn 3):

$$\delta^{18}O = [(R_{samples}/R_{standard}) - 1] \times 1000 \qquad (3)$$

where R_{sample} and $R_{standard}$ are the $^{18}O/^{16}O$ ratios of the sample and the standard (V-SMOW), respectively.

3. Data analysis

For simplicity, I will refer to the $\delta^{18}O$ values of stem water as observed stem water ($\delta^{18}O_{sw}{}^{obs}$), whether they are measured or calculated from the $\delta^{18}O_{ppt}$ (Eqn 2), to allow distinction from stem water derived from cellulose or phenylglucosazone (i.e. $\delta^{18}O_{sw}{}^{cell}$ and $\delta^{18}O_{sw}{}^{PG}$).

A previous study correlated the $\delta^{18}O$ of cellulose and its derivative (phenylglucosazone) to those of the observed $\delta^{18}O$ values of stem water from samples covering a wide geographical area (Sternberg et al., 2007b). I use the relationship between the $\delta^{18}O_{cell}$ or $\delta^{18}O_{PG}$ and the $\delta^{18}O_{sw}{}^{obs}$ from this previous study (Sternberg et al., 2007b) to reconstruct $\delta^{18}O$ values of stem water (source water). Because the time span

between Sternberg et al.'s (2007b) analysis and the oldest sample studied here is small [~ 35 yr for the oldest tree ring samples (1971)] I considered these equations applicable and reliable for the analysis of these samples.

The reconstructed oxygen stable isotope ratios of stem water from *Pinus sylvestris* (Finland), *Picea abies* (Swiss) and *Pinus radiata* (New Zealand) cellulose (Eqn 4) and from $\delta^{18}O$ of phenylglucosazone (Eqn 5) were calculated according to the respective empirical relationships (Sternberg et al., 2007b).

$$\delta^{18}O_{sw}{}^{cell} = (\delta^{18}O_{cell} - 31.6)/0.52 \quad (4)$$

$$\delta^{18}O_{sw}{}^{PG} = (\delta^{18}O_{PG} - 30.1)/0.73 \quad (5)$$

The $\delta^{18}O_{C\text{-}2}$ values were calculated as previously (Eqn 1) (Sternberg et al., 2003) and $\delta^{18}O_{sw}$ was reconstructed from $\delta^{18}O_{C\text{-}2}$ ($\delta^{18}O_{sw}{}^{C\text{-}2}$) by subtracting a constant factor of 47.5 from it (Eqn 6) as observed by Sternberg et al. (2007b).

$$\delta^{18}O_{sw}{}^{C\text{-}2} = (\delta^{18}O_{C\text{-}2}) - 47.5 \quad (6)$$

Both reconstructed and observed stem water were correlated using a model II linear regression. The slopes were compared using analysis of covariance followed by a Tukey Post Hoc test and the correlation coefficient was transformed to Z values and compared using chi-square (Zar, 1999). The residuals between $\delta^{18}O_{sw}{}^{cell}$ and $\delta^{18}O_{sw}{}^{obs}$ and between $\delta^{18}O_{sw}{}^{PG}$ and $\delta^{18}O_{sw}{}^{obs}$ were calculated using Eqn 7 and 8, respectively and compared with each other using a student's t-test.

$$residual^{cell} = \delta^{18}O_{sw}{}^{cell} - \delta^{18}O_{sw}{}^{obs} \quad (7)$$

$$residual^{PG} = \delta^{18}O_{sw}{}^{PG} - \delta^{18}O_{sw}{}^{obs} \quad (8)$$

Results and Discussion

The $\delta^{18}O_{sw}^{PG}$ followed the $\delta^{18}O_{sw}^{obs}$ more closely than the $\delta^{18}O_{sw}^{cell}$ and $\delta^{18}O_{sw}^{C\text{-}2}$ through time with a few outliers for each of the species studied here (Fig 2.3). The $\delta^{18}O_{sw}^{cell}$ and the $\delta^{18}O_{sw}^{C\text{-}2}$ were greater than the $\delta^{18}O_{sw}^{obs}$ values, and this was particularly accentuated for $\delta^{18}O_{sw}^{C\text{-}2}$ (Fig 2.3 and 2.4). Variation in $\delta^{18}O^{C\text{-}2}$, contributed to variation in $\delta^{18}O_{cell}$, which is reflected by the error of using the cellulose empirical equation as a proxy for $\delta^{18}O_{sw}^{obs}$.

The $\delta^{18}O_{sw}^{PG}$, $\delta^{18}O_{sw}^{cell}$, $\delta^{18}O_{sw}^{C\text{-}2}$ were correlated significantly with $\delta^{18}O_{sw}^{obs}$ ($r = 0.88$, $P < 0.01$, $r = 0.91$, $P < 0.01$ and $r = 0.74$, $P < 0.01$, respectively) (Fig 2.4). However, the correlation coefficients did not differ among $\delta^{18}O_{sw}^{PG}$, $\delta^{18}O_{sw}^{cell}$ and $\delta^{18}O_{sw}^{C\text{-}2}$ ($\chi^2_{(2,\ N = 94)} = 4.85$, $P > 0.05$). The slopes, on the other hand, were significantly different among $\delta^{18}O_{sw}^{PG}$, $\delta^{18}O_{sw}^{cell}$ and $\delta^{18}O_{sw}^{C\text{-}2}$ ($F_{(2,88)} = 109.2$, $P < 0.05$, Tukey Post hoc, $P < 0.001$). The regression equation between $\delta^{18}O_{sw}^{PG}$ and $\delta^{18}O_{sw}^{obs}$ was the closest to a one-to-one relationship line (slope = 0.94, intercept = -0.39, Fig 2.4) compared with regression equations between $\delta^{18}O_{sw}^{cell}$ and $\delta^{18}O_{sw}^{C\text{-}2}$ versus $\delta^{18}O_{sw}^{obs}$ (slope = 1.61, intercept = 13.23 and slope = 1.75, intercept = 30.01, respectively, Fig 2.4). Deviations from the one to one relationship between the $\delta^{18}O_{sw}^{obs}$ and the $\delta^{18}O_{sw}^{C\text{-}2}$, $\delta^{18}O_{sw}^{cell}$ or $\delta^{18}O_{sw}^{PG}$ could be a consequence of either low or high relative humidity. Because ~ 60% of the isotopic signature in stem cellulose is derived from that of leaf water (Roden et al., 2000). It is well established that low humidity causes isotopic enrichment of leaf water which will lead to ^{18}O enrichment of tree ring cellulose; the opposite holds true during high relative humidity (Roden et al., 2000; Sternberg, 2009).

In this study oxygen isotope ratios of the source water as well as the ambient RH were available, so I could test whether any variation in the relationship between the reconstructed and observed $\delta^{18}O_{sw}$ is indeed related to RH. I found that the ambient relative humidity in which cellulose was formed explained part of the deviation between $\delta^{18}O_{sw}{}^{PG}$ and the $\delta^{18}O_{sw}{}^{obs}$ ($r = 0.44$, $P < 0.05$ for $\delta^{18}O_{sw}{}^{PG}$, Fig 2.5A), which caused deviations from a one to one correspondence between $\delta^{18}O_{sw}{}^{PG}$ and the $\delta^{18}O_{sw}{}^{obs}$ (Fig 2.4). I note that the empirical equations previously derived by Sternberg et al. (2007b) and used here were derived using samples from several latitudes with the sample average relative humidity ~ 0.59. This implies that the accuracy of the empirical equations (Eqn 4, 5 and 6) to reconstruct $\delta^{18}O_{sw}$ is higher for samples that have RH closer to 0.59. In fact, according to the best-fit linear regression (Fig 2.5A) I observed a trend where RH above 0.58 (similar to the mentioned 0.59) lowered the $\delta^{18}O_{sw}{}^{PG}$ relative to $\delta^{18}O_{sw}{}^{obs}$, whereas below 0.58, $\delta^{18}O_{sw}{}^{PG}$ became greater than the observed stem water. The direction in which isotopic depletion and enrichment of the PG reconstructed stem water occurred relative to the observed stem water is consistent with the understanding of how stem cellulose incorporated ^{18}O as a function of $\delta^{18}O$ of source and leaf water (Sternberg, 2009). No such relationship was observed for the residuals of $\delta^{18}O_{sw}{}^{cell}$ and $\delta^{18}O_{sw}{}^{C\text{-}2}$ ($r = 0.21$, $P > 0.05$ and $r = 0.054$, $P > 0.05$, respectively, Fig 2.5B for $\delta^{18}O_{sw}{}^{cell}$), suggesting that factors other than RH affected the deviation of $\delta^{18}O_{sw}{}^{cell}$ from $\delta^{18}O_{sw}{}^{obs}$ to a greater degree than they affected $\delta^{18}O_{sw}{}^{PG}$.

Conclusions

The development of a method to derivatize cellulose to phenylglucosazone using only 25 mg of cellulose makes the $\delta^{18}O_{PG}$ applicable for tree ring studies and should improve the

quality of reconstructed climate related variables such as the oxygen isotope ratios of precipitation. This study, using tree ring samples, confirms the previous observation by Sternberg et al. (2007b), using stem samples, that the $\delta^{18}O_{PG}$ has a stronger relationship with $\delta^{18}O$ of source water than the $\delta^{18}O$ of whole cellulose. The relationship between $\delta^{18}O_{sw}{}^{cell}$ and $\delta^{18}O_{sw}{}^{obs}$ was distorted by the unpredictable oxygen isotope fractionation of the oxygen attached to the second carbon of cellulose glucose moieties. The $\delta^{18}O_{sw}{}^{PG}$ also reflected the $\delta^{18}O_{sw}{}^{obs}$ more accurately when the mean RH (12-14h) was approximately 0.58, which is similar to the average RH used in deriving the empirical relationship used here (Eqn 5). Because hydrolysis and derivatization of cellulose to phenylglucosazone is applicable to inter and intra-annual tree ring samples, this process should be strongly considered for a more realistic reconstruction of source water in small cellulose samples.

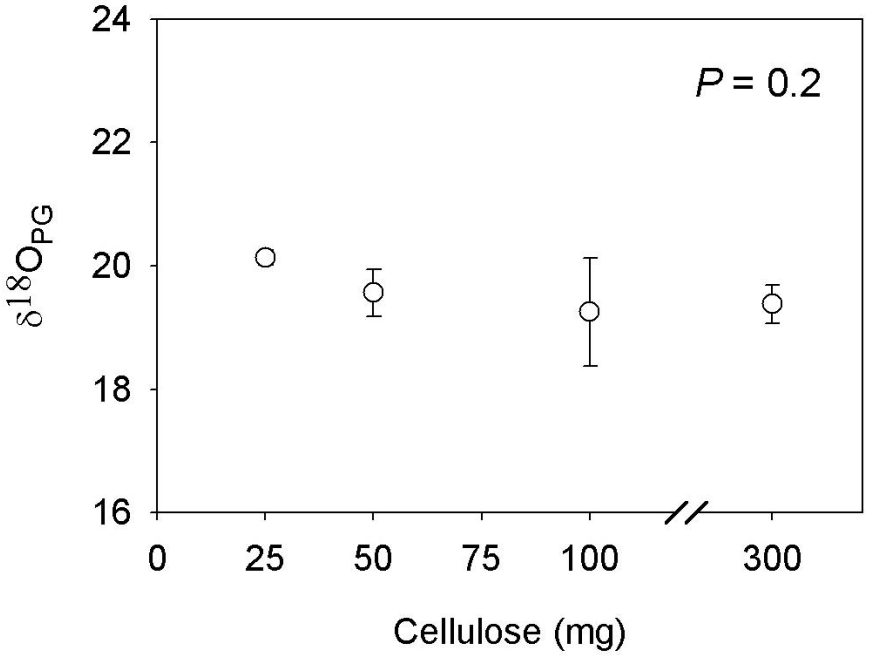

Figure 2.1 Oxygen isotope ratios of phenylglucosazone ($\delta^{18}O_{PG}$) hydrolyzed and derivatized from 4 different amounts of Sigma cellulose with 3 replicates each. No significant statistical differences were observed among the $\delta^{18}O_{PG}$ (one way $_{ANOVA}$ test: $P > 0.05$). Standard deviation for 25mg is smaller than the diameter of the symbol.

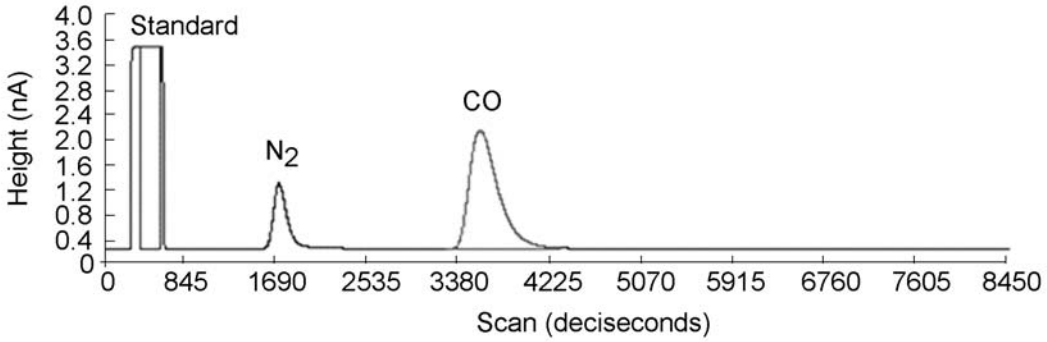

Figure 2.2 Thermal conductivity from the Eurovector and Mass Spectrometer (IRMS) chromatogram of gases from phenylglucosazone pyrolysis showing complete separation between N_2 and CO prior to oxygen isotope ratios analysis.

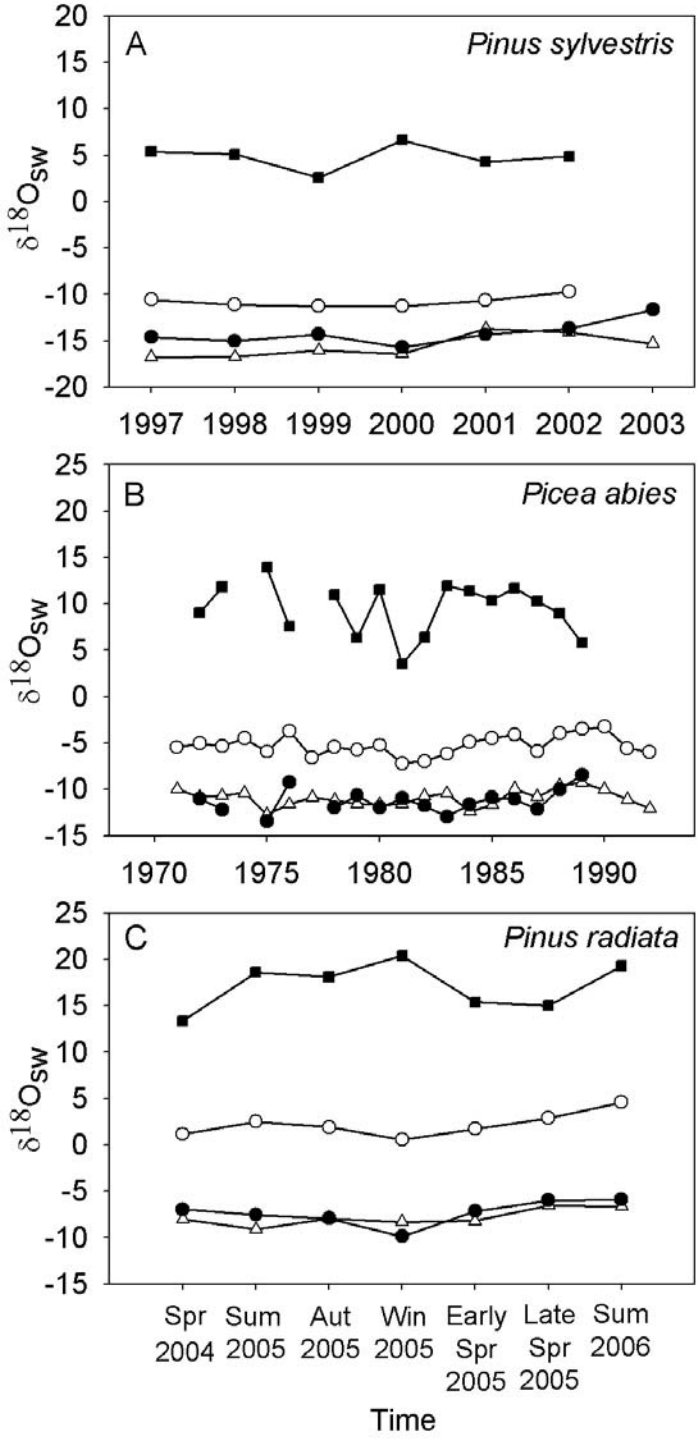

Figure 2.3 Observed oxygen isotope ratios of stem water (Δ) and reconstructed oxygen isotope ratios of stem water from phenylglucosazone (●), cellulose (○) and the second carbon of the cellulose–glucose moieties (■) over time.

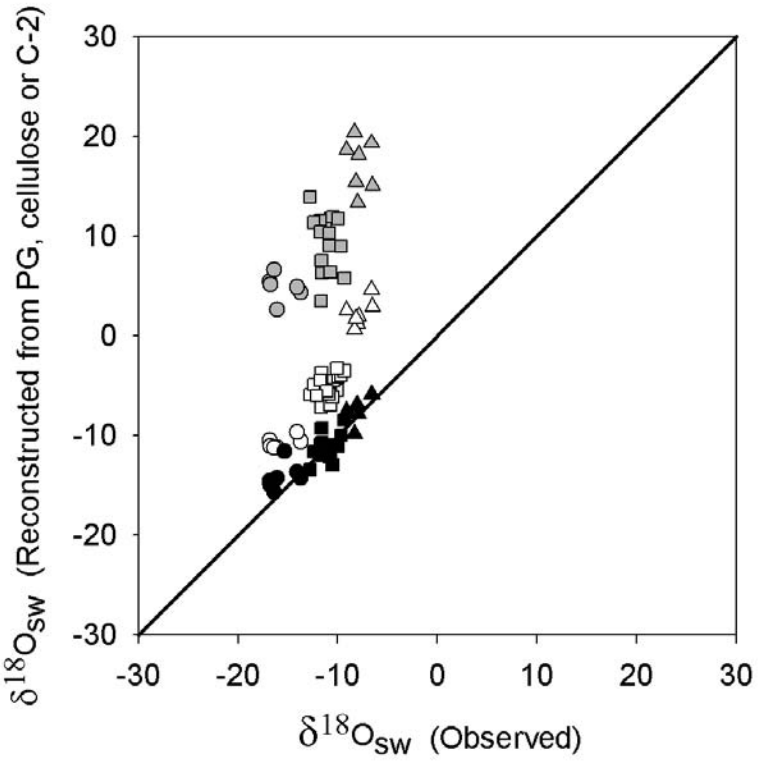

Figure 2.4 How the oxygen isotope ratios of stem water reconstructed from phenylglucosazone (closed symbols), cellulose (open symbols) and the second carbon of the cellulose–glucose moieties (gray symbols) fits the one-to-one relationship line between reconstructed and observed oxygen isotope ratios of stem water. Species are represented by *Pinus sylvestris* (circles), *Picea abies* (squares) and *Pinus radiata* (triangles).

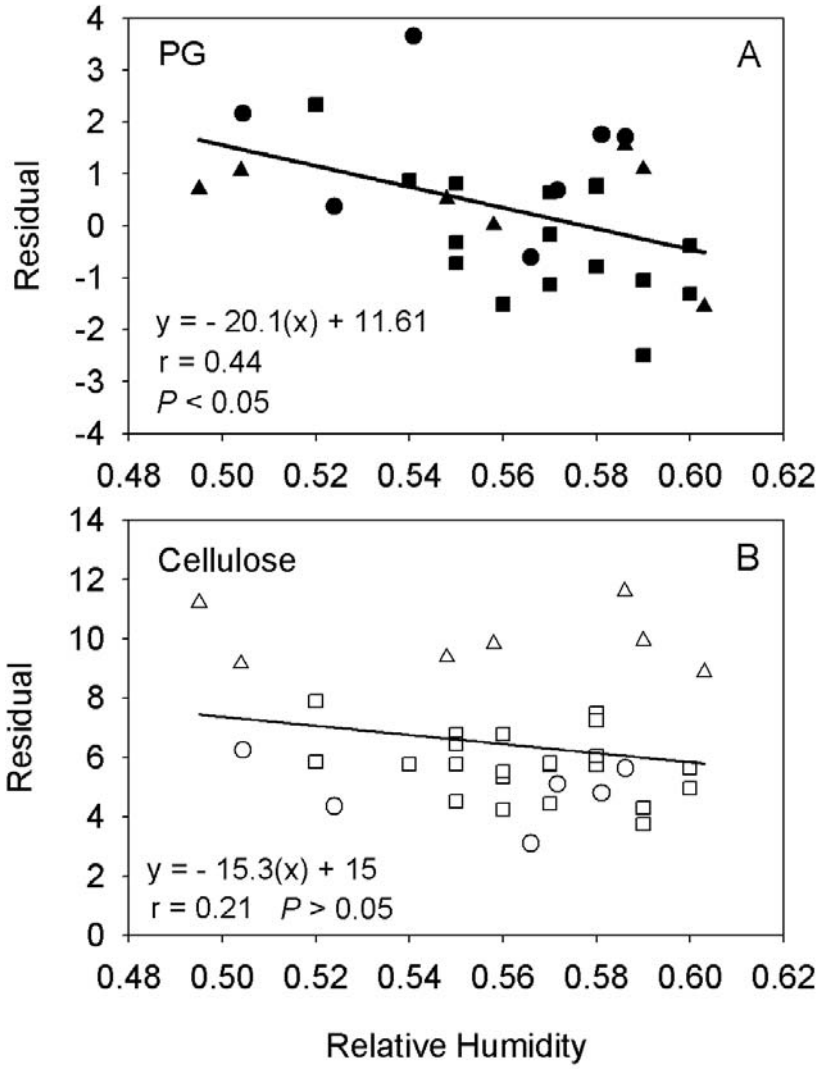

Figure 2.5 Relative humidity at 12 pm (*Picea abies* and *Pinus radiata*) and at 2 pm (*Pinus sylvestris*) versus the residual from $\delta^{18}O_{sw}{}^{PG}$ (A) and from $\delta^{18}O_{sw}{}^{cell}$ (B) calculated in relation to $\delta^{18}O_{sw}{}^{obs}$ for *Pinus sylvestris* (circles) from Finland, *Picea abies* (squares) from central Switzerland and *Pinus radiata* from New Zealand (triangles). Statistical analyses were done on pooled samples.

CHAPTER 3

The role of effective leaf mixing length in the relationship between the $\delta^{18}O$ of stem cellulose and source water across a salinity gradient.[1]

Summary

Previous mangrove tree ring studies attempted, unsuccessfully, to relate the $\delta^{18}O$ of trunk cellulose ($\delta^{18}O_{CELL}$) to the $\delta^{18}O$ of source water ($\delta^{18}O_{SW}$). Here I tested whether biochemical fractionation associated with one of the oxygen atoms in the cellulose glucose moiety or variation in leaf water oxygen isotope fractionation (Δ_{LW}) can interfere with the $\delta^{18}O_{SW}$ signal as it is recorded in the $\delta^{18}O_{CELL}$ of mangrove (saltwater) and hammock (freshwater) plants. I selected 2 transects experiencing a salinity gradient, located in the Florida Keys, USA. The $\delta^{18}O_{CELL}$ throughout both transects did not show the pattern expected based on that of the $\delta^{18}O_{SW}$. I found that in one of the transects, biochemical fractionation interfered with the $\delta^{18}O_{SW}$ signal, while in the other transect Δ_{LW} differed between mangrove and hammock plants. Observed differences in Δ_{LW} between mangroves and hammocks were caused by a longer effective leaf mixing length (L) of the water pathway in mangrove leaves compared to those of hammock leaves. Changes in L could have caused the $\delta^{18}O_{CELL}$ to record not only variations in the $\delta^{18}O_{SW}$ but also in Δ_{LW} making it impossible to isolate the $\delta^{18}O_{SW}$ signal.

Background

The continual increase in air temperature has accelerated the melting of ice caps and as a consequence, in the last 15 years (1993 - 2009), globally sea level rose at a higher rate (~ 3.2 mm/yr; Church & White 2011) than the average for the 20th century (~ 1.7 mm/yr;

[1] Plant Cell and Environment doi: 10.1111/j.1365-3040.2012.02562.x

Bindoff et al. 2007). According to the Fourth Assessment Report (AR4; Bindoff et al. 2007), in 2090, sea level may reach 0.22 to 0.44 m above 1990 levels. Therefore, it is possible that drastic effects in the coastal plant communities are already taking place. In short, these effects will cause invasion of saltwater vegetation inland at the cost of other coastal communities (Jiang et al., 2011; Saha et al., 2011). Changes in coastal habitats will not only affect plant communities, but also the wild life that depends on the resources associated with those plants communities.

Presently, it is possible to access broad scale changes in coastal plant communities, but the challenge is to find ways to monitor sea level rise at the plant level and within a plant community before catastrophic collapse of these communities takes place (Sternberg et al., 2007a; Teh et al., 2008). One tool which shows a great potential to monitor sea level rise as it affects plant life is the oxygen isotope ratio of tree trunk (stem) cellulose. The basis of using $\delta^{18}O$ values of tree trunk cellulose as a proxy for sea level rise is the observation that $\delta^{18}O$ values of sea water are often greater than those of freshwater (Lloyd, 1966; Sternberg and Swart, 1987; Sternberg et al., 1991). The higher $\delta^{18}O$ value of sea water is expected to be recorded in the trunk cellulose with sea level rise as the ground water becomes isotopically enriched with sea water intrusion and plants use the more saline and isotopically enriched water. The recording of the oxygen isotope ratios of source water in the trunk cellulose, however, is confounded by plant biochemical and physiological effects (Sternberg, 2009).

The oxygen isotope enrichment of trunk/stem cellulose above source water (Δ_{CELL}) is given by the following relationship (Eqn 1; Barbour et al., 2004)

$$\Delta_{CELL} = \Delta_{LW} (1 - p_{ex.} p_x) + \varepsilon_{bio} \qquad (1),$$

where Δ_{LW} is the leaf water oxygen isotope enrichment above source water, p_{ex} is the proportion of oxygen in sucrose that exchanges with trunk water during cellulose synthesis, p_x is the proportion of non-enriched (xylem) water in the cell forming the cellulose and ε_{bio} is the equilibrium fractionation factor between the oxygen of water and those of carbohydrates during cellulose synthesis. Because there is only a small possibility of leaf water being present in the stem water for the plants analyzed here; in this study I consider p_x to be one. If salinity and species do not have an effect in Δ_{CELL}, I would expect that in a coastal setting, plants will record with high fidelity the increase in oxygen isotope ratios of source water brought about by sea level rise or by their location relative to sea water. However, previous observations (Verheyden et al., 2004) as well as the observations reported here indicate that plants are not recording their respective source water oxygen isotopic differences in a reliable manner. In a study by Verheyden et al. (2004), low relative humidity (RH) during the dry season should have reflected in greater oxygen isotope enrichment in tree ring wood of *Rhizophora mucronata* compared to high RH in the wet season. However, they observed the opposite which could not be explained based on the knowledge of mangrove physiology and plant oxygen isotope fractionation at the time of the study.

Oxygen isotope fractionation of cellulose is controlled by biochemical factors (p_{ex} and ε_{bio}) and physiological factors (Δ_{LW} and p_x). The proportion of cellulose oxygen that exchanges with water during heterotrophic cellulose synthesis (p_{ex}) may vary depending on the position of the oxygen atoms in the cellulose molecule (Sternberg et al., 2003). Recently, Sternberg et al. (2006) measured the isotope ratios of the oxygen attached to the second carbon of the glucose moieties in the cellulose molecule (O_{C2}) separately from

those attached to carbons 3, 4, 5 and 6 ($O_{C3\text{-}6}$) . Their measurements indicated different isotopic fractionation and different proportions of oxygen exchange with water for these two sets of oxygen atoms in the cellulose molecule (Sternberg et al., 2006). Sternberg et al. (2007b) also observed a better relationship between $O_{C3\text{-}6}$ (those present in the phenylglucosazone molecule derivatized from the cellulose molecule) and the oxygen isotope ratios of stem water at a sample survey done over several latitudes. Sternberg et al. (2007b) proposed that fractionation in the O_{C2} during biochemical reactions introduced isotopic noise to the recording of the oxygen isotope ratio of source water in the trunk cellulose molecule.

The principal physiological factor which can alter the source water isotopic signal is the isotopic composition of the leaf water. Leaf water isotopic signal constitutes 60% of the isotopic signal recorded in the trunk cellulose (Roden et al., 2000). Leaf water isotopic enrichment above that of the source water is dependent on the Péclet effect (Eqn 2)

$$\rho = (EL)/(CD) \qquad (2),$$

where E is transpiration rate, L is the effective leaf mixing length, C is the molar concentration of water, and D is the diffusivity of $H_2{}^{18}O$ in water (Farquhar and Lloyd, 1993; Barbour and Farquhar, 2003); therefore, any variation in E or L resulting in a different Péclet number between species will result in different Δ_{LW}. Species using the same source water but with different Δ_{LW} will lead to different Δ_{CELL} and disrupt the relationship between the $\delta^{18}O$ of stem cellulose ($\delta^{18}O_{CELL}$) and the $\delta^{18}O$ of source water.

Here I investigated the possibility that biochemical and physiological factors are contributing to the variability in Δ_{CELL} along a saline gradient in two transects spanning

mangroves to freshwater. Specifically, I tested two hypotheses. The first hypothesis tested whether the removal of the oxygen attached to the second carbon of the cellulose glucose moieties improves the fidelity of the source water isotopic signal recorded in the $\delta^{18}O_{CELL}$ across the above transects. The second hypothesis tested whether Δ_{LW} differ between mangrove and hammock leaves. Considering that the above transect encompassed only a few 100 m, I expected similar RH and therefore similar Δ_{LW}. However, because mangroves must remove salts from xylem water as it enters the leaf mesophyll, they may have a fundamentally different pathway of leaf water movement compared to their neighboring freshwater plants which might cause their Δ_{LW} to differ.

Materials and Methods

1. Stem water and cellulose field sampling methods

The transects (Fig 3.1A and 3.1B) were located in Big Pine Key (24°43.75'N 81°23.35'W) and Sugarloaf Key (24°40.9'N, 81°32.7'W), Florida, USA (about 210 and 240 km southwest of Miami, Florida, USA, respectively). Both transects are characterized as a flat coastal area, where even small changes in topography (30 cm in Big Pine Key and 90 cm in Sugarloaf Key, above sea level rise, respectively) can significantly change the source water available to the plants from salt to freshwater. The salinity gradient forms a vegetation mosaic and a clear boundary between salt tolerant plants (mangroves) and salt intolerant plants (hammocks). The latitude is sub-tropical, but because of the proximity to ocean water, the climate is tropical, rarely reaching below freezing temperatures. The closest weather station (Florida Keys National Weather Service Forecast, 2011) is at Key West, FL, USA, located approximately 50 and 30 km south of Big Pine Key and Sugarloaf, respectively. The record in this weather station

spanned a period from 1971 to 2000, with the average daily high and low temperature of 22.9°C and 28.3°C, respectively. The annual precipitation of 989 mm is seasonal with 316 mm falling during the dry season (Nov - Apr). Groundwater varies in depth and saline concentration; however direct precipitation over the region forms a freshwater vadose zone, allowing the growth of hammock plants on top of saline groundwater (Wightman et al., 1990; Ish-Shalom et al., 1992). The soil, derived from the Miami Oolite bedrock, is shallow with low organic matter (Brown et al., 1990).

1.1. Stem water and cellulose field sampling method

A 300 m transect was delineated in both Big Pine Key (Fig 3.1D) and Sugarloaf Key (Fig 3.1C), FL, USA with the purpose of sampling the different plant communities utilizing source water ranging from ocean to freshwater. Samples from both Big Pine Key and Sugarloaf Key were collected during the dry season of 2009 (January and March, respectively). Each transect was divided into 5 areas (ocean-inland direction): the first 3 having predominantly mangrove species and the last 2 having predominantly hammock species (Fig 3.1C and 3.1D). Salinity was measured from 6 replicate soil samples collected in each area of both transects (Dasberg and Nadler, 1988). Predawn leaf water potential was measured from five separate individuals of each species in each sampling area along both transects by the pressure chamber method (Sternberg et al., 1991), model 600, PMS Instrument Co., Albany, Oregon, USA. The species studied in the mangrove areas of Big Pine Key transect were: *Avicennia germinans*, *Laguncularia racemosa* and *Rhizophora mangle*, while those for the hammock areas of the transect were: *Pithecellobium guadalupense, Coccoloba uvifera*, *Piscidia piscipula* and *Guapira discolor*. Stems from three separate individuals were collected for each species at each

sampling area along the transect. The species studied in the mangrove areas of Sugarloaf Key transect were: *Laguncularia racemosa* and *Rhizophora mangle,* while those in the hammock areas of the transect were: *Pithecellobium guadalupense, Coccoloba diversifolia* and *Bursera simaruba*. Stems from five separate individuals were collected for each species at each sampling area along the transect.

1.2. Stem water, cellulose and phenylglucosazone

Stems, in both transects, were collected approximately 30 cm basipetal to the leaves to prevent contamination by enriched leaf water. The well suberized stems were debarked, placed into a glass tube, sealed and maintained in a cooler. Water was extracted from the stem and analyzed isotopically by the method of Vendramini and Sternberg (2007). After water extraction, the same stem was ground and homogenized for cellulose extraction and derivatization to phenylglucosazone.

1.2.1. Cellulose extraction, hydrolysis and derivatization

Wood samples were processed to holocellulose according to the method of Leavitt and Danzer (1993) and treated with 17% NaOH to remove the hemicellulose. A small aliquot (0.55 ± 0.03 mg) of the cellulose was saved for oxygen isotope analyses. Cellulose (25 mg) was hydrolyzed with 99% Trifluoracetic acid (TFA) by a modified method of Fengel and Wegener (1979). Cellulose was soaked in 8 ml of TFA for two hours in a 100 ml round bottom flask, subsequently boiled in a refluxer for 15 minutes. De-ionized water (3 mL) was added to the boiling solution and refluxing continued for 15 minutes. A further aliquot of water (25 ml) was added and the solution refluxed for another 30 minutes. The solution was filtered through a glass-fiber filter (VWR Glass Microfibre,

691, VWR International, Radnor, PA, USA). The hydrolysis flask (100 ml) was washed with 10 ml of distilled water and this wash-solution filtered again. The filtrate was roto-evaporated to glucose, the cellulose hydrolysate, which has a thick syrupy consistency. To eliminate excess fluorine 10 ml of distilled water was stirred into the thick syrupy residue and the solution was roto-evaporated again. The hydrolysate was then freeze dried overnight.

The cellulose hydrolysate was derivatized to phenylglucosazone according to a modified method of Oikawa et al. (1998). One ml of acetic acid (20%) solution was added to the above freeze dried hydrolysate with 0.25 ml of phenylhydrazine. This mixture was covered and stirred continually in a 90°C water bath (2h). After derivatization the flask was placed in a refrigerator until cool. Distilled water (5 ml) was added in the round bottom flask to suspend the material which was later filtered through a fritted glass filter (Kimax, 2 ml, Buchner Funnels, Kimble Chase, Vineland, NJ, USA). The filtrand was washed three times with distilled water, soaked in hexane for 15 minutes and rinsed three more times with distilled water. The fritted glass filter with the phenylglucosazone (filtrand) was placed in a freeze drier and left overnight. The phenylglucosazone was further purified by scraping it from the fritted glass filter into a 40 ml conical centrifuge tube, and mixed with 2.5 ml of methanol (95%). The tubes were placed in the freezer (-10^0C), after six hours they were centrifuged for 10 min and the liquid was gently decanted. The phenylglucosazone precipitate was allowed to dry at room temperature prior to freeze drying.

2. Leaf measurements

Leaf measurements were made in March 2010 (dry season), between areas 3 and 4 of the Sugarloaf key transect, where the transition from salt to freshwater occurs. The species studied were *Laguncularia racemosa* and *Rhizophora mangle,* representing mangrove plants, *Pithecellobium guadalupense* and *Coccoloba diversifolia* representing freshwater plants and *Conocarpus erectus* (buttonwood). The last species grows in both mangrove and also in hammock areas, which makes it an interesting species to monitor changes in leaf physiology based on its water source. Leaf transpiration, leaf temperature, relative humidity, air temperature and leaf resistance were measured using a LI-1600 steady state porometer (LI-COR, Lincoln, NE, USA). The same leaf and its respective stem were collected for further water extraction and oxygen isotope analysis. Recent studies showed that leaves from *L. racemosa* and *C. erectus* are amphistomatous (Arrivabene, 2011; Saha and Gann, 2012, respectively), with similar stomatal density in both sides, therefore, the transpiration values measured from the abaxial leaf surface was doubled to account for the transpiration of the adaxial leaf surface. Leaf water enrichment above source water (Δ_{LW}) was calculated from leaf and stem water oxygen isotope ratios ($\delta^{18}O_{LW}$ and $\delta^{18}O_{SW}$, respectively; Eqn 3).

$$\Delta_{LW} = \delta^{18}O_{LW} - \delta^{18}O_{SW} \qquad (3)$$

Leaves from the same individuals were collected and scanned for leaf area calculation (Image-J program, National Institutes of Heath, Bethesda, Maryland, USA) followed by the measurement of its wet and dry weight to calculate succulence by dividing leaf water weight by leaf area. Atmospheric water vapor was collected for 6 hours according to the method suggested by the Moisture Isotopes in the Biosphere and

Atmosphere network (IAEA-MIBA, 2005). The air intake was controlled by a flow meter, attached to a condenser and a pump. The flow was adjusted to 450 ml/min so approximately 1 ml of water was collected for the oxygen isotope ratio analysis of water vapor during the time of leaf measurement.

2.1. Oxygen isotope analysis of stem water, leaf water, atmospheric water vapor, cellulose and phenylglucosazone

Stem water, leaf water and atmospheric water vapor oxygen isotope ratios were analyzed in a Multiflow system connected to an Isoprime mass spectrometer (Elementar, Hanau, Germany). A 5% CO_2/helium gas mixture was flushed through the vials and equilibrated with water for a period of 48 h. The equilibrated CO_2 gas was analyzed to derive the oxygen isotope ratios of the water as in Vendramini & Sternberg (2007). Oxygen isotope ratios are reported relative to Vienna Standard Mean Ocean Water (V-SMOW) with a precision of ±0.1‰.

Oxygen isotope ratios of cellulose and PG were determined by a modification of Saurer et al. (1998). Half a milligram of cellulose or 1.2 mg of PG was pyrolyzed at 1080^{o}C in a quartz column filled to approximately half its height with glassy carbon and topped off by a layer of (0.5 g) of nickelized carbon and nickel wool (Elementar America, Mt. Laurel, New Jersey, USA) in a Eurovector Elemental Analyzer (Milan, Italy). Gases from the pyrolysis (mostly hydrogen, nitrogen and carbon monoxide) were carried by helium through magnesium perchlorate and Ascarite (Thomas Scientific, Swedesboro, NJ, U.S.A.) to absorb H_2O and CO_2 respectively, and separated in a 3 m 5Å molecular sieve column (Eurovector, Milan, Italy) held at 70^{o}C. The oxygen isotope ratios were determined on the carbon monoxide and compared with a standard gas

calibrated to two standards: Sigma cellulose having an oxygen isotope ratio of 29.3‰ (Sauer and Sternberg, 1994) and the International Atomic Energy Agency cellulose filter paper with an isotope ratio of 32.4‰. The precision of the analysis is ± 0.3‰. All isotope ratios are expressed in terms of per mil (‰; Eqn 4):

$$\delta^{18}O = [(R_{samples}/R_{standard}) - 1] \times 1000 \qquad (4),$$

where R_{sample} and $R_{standard}$ are the $^{18}O/^{16}O$ ratios of the sample and the (V-SMOW) standard, respectively. Cellulose and phenylglucosazone oxygen isotope fractionation (Δ_{CELL} and Δ_{PG}) were calculated by subtracting $\delta^{18}O_{SW}$ from $\delta^{18}O_{CELL}$ and $\delta^{18}O_{PG}$, respectively.

2.2. Calculation of the effective leaf mixing length

The isotopic enrichment of evaporated water from internal leaf surface relative to that of the source water (Δ_e; Eqn 5) was calculated separately for each leaf, based on measured parameters such as the oxygen isotope composition of the atmospheric water vapor relative to plant water source (Δ_v), atmospheric relative humidity, leaf and air temperature, and stem water oxygen isotope ratios. Measured parameters were used to calculate the vapor pressure external to the leaf to that internal to the leaf (e_a/e_i) for each individual.

$$\Delta_e = \varepsilon^* + \varepsilon_k + (\Delta_v - \varepsilon_k)\, e_a/e_i \qquad (5)$$

The equilibrium fractionation factor (ε^*) between vapor and water internal to the leaf was calculated for each leaf and averaged 8.87 ± 0.01 ‰. The kinetic fractionation (ε_k) was calculated (Eqn 6) for each leaf according to (Farquhar et al., 1989) and averaged 28.5 ± 0.3 ‰,

$$\varepsilon_k = (32 \cdot g_s^{-1} + 21 \cdot g_b^{-1})/(g_s^{-1} + g_b^{-1}) \qquad (6),$$

where g_s is stomatal conductance (measured) and g_b is boundary layer conductance, assumed to be 1 mol m^{-2} s^{-1}, based on Kahmen et al. (2008). The measured Δ_{LW} and the calculated Δ_e were used to calculate the Péclet number (ρ) by iteration (Eqn 7 – Farquhar & Lloyd 1993).

$$\Delta_{LW} = \Delta_e (1 - e^{-\rho})/\rho \qquad (7)$$

The effective leaf mixing length (L) was then calculated using equation 2, with the molar concentration of water assumed to be 5.56×10^4 mol m^{-3} and diffusivity of $H_2{}^{18}O$ in water calculated according to Cuntz et al. (2007).

3. Statistical analysis

The PASW SPSS Statistics (version 18; IBM, Armonk, New York) was used to perform the statistical tests. Each data set was tested for normality (Kolmogorov-Smirnov test and Shapiro-Wilk test) and homogeneity of variance (Levene's test). A one-way analysis of variance ($_{ANOVA}$) was applied to interpret soils salinity measurements among areas of each transect. A nested $_{ANOVA}$ test was applied to the other studied parameters (Table 3.1 and 3.2) to compare how they differed among sampling areas along the transect and also inside each of those areas. If differences among areas, or within an area were observed, a *post hoc* test was applied to identify those differences. Because there is no non-parametric test equivalent to nested $_{ANOVA}$, if normality was not reached after transformation, the data was ranked previously to its analysis. If the variance was homogeneous for the different areas inside the transect a Tukey's *post hoc* test was used, but if the variance was heterogeneous a Games-Howell *post hoc* test was used instead.

Results

Soil salinity in Big Pine Key and Sugarloaf Key transects increased with proximity to the ocean. Within each transect, soil salinity differences were observed among the transect areas (Big Pine Key – Tukey's *post hoc*, $P < 0.05$ and Sugarloaf Key – Tukey's *post hoc*, $P < 0.05$), with the exception of mangrove areas 1 and 2. A greater soil salinity gradient was observed in Sugarloaf Key (0.48-35.57 ppt) than in Big Pine Key (1.58-21.68 ppt) transect (Fig 3.1). There also was a significant interaction between the site and areas of the transect on soil salinity [F (4,41) = 25.499, $P < 0.001$] with higher soil salinities in mangrove areas 1 and 2 of Sugarloaf Key than those same areas at Big Pine Key transect ($P = 0.014$ and $P = 0.013$, respectively) and lower soil salinity in area 5 of Sugarloaf Key than the same area at Big Pine Key transect. The predawn water potential did not differ between Big Pine Key transect areas (Table 3.1). In contrast, differences in predawn water potential were observed between Sugarloaf Key transect areas, with one exception: mangrove areas 2 and 3 did not differ from each other (Tukey's *post hoc*, $P < 0.05$).

Stem water, cellulose and phenylglucosazone

The $\delta^{18}O_{SW}$ in Big Pine Key and Sugarloaf Key transects exhibited the same pattern, where in both cases there was no difference between freshwater areas of the transects (4 and 5) or saltwater areas of the transects (1,2 and 3), but significant differences between fresh and saltwater areas of each transect (Table 3.1; Big Pine Key – Games-Howell *post hoc*, $P < 0.05$ and Sugarloaf Key – Tukey's *post hoc*, $P < 0.05$; Fig 3.2A and 3.2B).

In the Big Pine Key transect the $\delta^{18}O_{CELL}$ was not significantly different between the different areas along the transect (Table 3.1 and Fig 3.2C), but the $\delta^{18}O$ of phenylglucosazone ($\delta^{18}O_{PG}$) was significantly different between fresh and saltwater areas

(Table 3.1) with one exception: area 3 (mangrove) and 4 (hammock) did not differ (Tukey's *post hoc*, $P < 0.05$; Fig 3.2E). In the Sugarloaf Key transect mangroves had a higher $\delta^{18}O_{CELL}$ than hammock plants (Table 3.1), and there was an unexpected difference between hammock areas (4 and 5; Games-Howell *post hoc*, $P < 0.05$; Fig 3.2D) considering both use the water source with the same oxygen isotopic composition. No significant difference in the $\delta^{18}O_{PG}$ was observed between all Sugarloaf key transect areas (Table 3.1 and Fig 3.2F).

There was no difference in the Δ_{CELL} and Δ_{PG} between the different areas of the Big Pine Key transect (Table 3.1, Fig 3.3A and 3.3C). In Sugarloaf key, both Δ_{CELL} and Δ_{PG} were significantly different (Table 3.1). The Δ_{CELL} was significantly greater in freshwater areas (4 and 5) compared to saltwater areas (1,2 and 3; Fig 3.3B), but differences also were observed between freshwater areas of the Sugarloaf Key transect (4 and 5; Games-Howell *post hoc*, $P < 0.05$). The Δ_{PG} also was significantly greater in freshwater areas of the Sugarloaf Key transect compared to saltwater areas, with significant differences between freshwater areas of the transect (4 and 5) and between saltwater areas of the transect (1,2 from 3; Tukey's *post hoc*, $P < 0.05$; Fig 3.3D).

Leaf measurements

Leaf water oxygen isotope enrichment differed significantly between mangrove and hammock areas (Table 3.2 and Fig 3.4A). There were no significant differences in transpiration rates between mangrove and hammock areas with differences at the species level (Table 3.2 and Fig 3.4C). Succulence and L differed significantly between mangrove and hammock areas (Table 3.2; Fig 3.4B showing for L only). A significant

negative relationship was observed between the Δ_{LW} and L ($r = 0.61$, $P < 0.01$; Fig 3.5) and a positive correlation was observed between succulence and L ($r = 0.76$, $P < 0.01$).

Buttonwood plants located in the mangrove area had longer L and were more "succulent" than those located in the hammock area. However, the longer L was counter balanced by lower *E* responses in buttonwood plants located at the mangrove area compared to those at the hammock area (Eqn 2). The way that L and *E* changed in buttonwood leaves relative to the water source available to the plants, minimized their differences in Δ_{LW} (Eqn 7; Fig 3.4) as predicted by the Péclet effect.

Discussion

In both transects, plants located in the mangroves areas (saline water) had higher $\delta^{18}O_{SW}$ than those located in the hammock areas (fresh/brackish water), which is consistent with previous studies in coastal plants (Sternberg and Swart, 1987; Sternberg et al., 1991). However, in the Big Pine Key transect the salinity gradient between hammock and mangrove areas was lower than in Sugarloaf Key transect. This difference in salinity gradient between the two transects was reflected in a higher range of $\delta^{18}O_{SW}$ values at Sugarloaf Key compared to Big Pine Key transect (Fig 3.2). Therefore, I expected that the recording of $\delta^{18}O_{SW}$ onto stem $\delta^{18}O_{CELL}$ would be easier to detect in the Sugarloaf Key transect compared to the Big Pine Key transect, providing that Δ_{CELL} does not differ among plant species, along each transect (Roden et al., 2000).

In the Big Pine Key transect, Δ_{CELL} did not vary statistically between mangrove and hammock plants. However, the standard deviation for Δ_{CELL} at each area was high, introducing noise to the recording of $\delta^{18}O_{SW}$ in the $\delta^{18}O_{CELL.}$ After the removal of the oxygen in the second carbon of the cellulose glucose moieties, the fidelity between the

$\delta^{18}O$ of the modified cellulose ($\delta^{18}O_{PG}$) and the $\delta^{18}O_{SW}$ was improved. I speculate that the biochemically based variation in $\delta^{18}O_{CELL}$ coupled with the small range of $\delta^{18}O_{SW}$ between the five areas of Big Pine Key transect masked the subtle differences in the recording of $\delta^{18}O_{SW}$ onto $\delta^{18}O_{CELL}$.

In the Sugarloaf Key transect, however, both Δ_{CELL} and the modification thereof (Δ_{PG}) differed between mangrove and freshwater plants. Therefore, I propose that differences in Δ_{LW} may be confounding the source water oxygen isotopic signal for this particular transect. I observed that Δ_{LW} was lower in mangrove than hammock plants (Fig 3.4A). Although I sampled leaf water isotopic composition (March 2010) in the same season, one year after I sampled stem water and cellulose along the Sugarloaf transect (March 2009), salinity conditions along this transect are stable. In fact, my current observations of stem water isotopic composition and predawn water potential are indistinguishable from a study in the same transect done approximately 20 years earlier (Sternberg et al. 1991). It is, therefore, reasonable to interpret the leaf water isotopic enrichment results of 2010 to the previous year. Further, the same pattern, with the hydrogen isotope ratio of leaf water, was observed by Romero and Feakins (2011) in a different location, where leaf water hydrogen isotope enrichment decreased with a greater exposure to seawater inundation. Although, Romero & Feakins (2011) interpreted changes in leaf water hydrogen isotope enrichment as being related to changes in *E*. In my study, however, mangroves and hammock plants did not differ significantly in *E*. The slightly lower *E* in mangroves compared to hammock should, according to the Péclet effect, cause the Δ_{LW} to be greater in mangroves, which was not the case. Therefore

difference in Δ_{LW} between mangroves and freshwater plants were related to differences in L (Eqn 2 and Fig 3.5).

According to the Péclet effect the longer L in mangrove compared to hammock plants resulted in a lower Δ_{LW} for mangroves in relation to freshwater species. *Laguncularia racemosa* had a longer L than the other mangrove species (*R. mangle*), however, they both had similar Δ_{LW}, which can be explained by *L. racemosa* transpiration rate being almost half of that of *R. mangle*. Sobrado (2007) showed that the hydraulic conductivity in *L. racemosa* leaves decreased with salinity and together with this findings that L increases with salinity, suggests that the longer pathway of water in mangrove leaves could be linked to some type of ultrafiltration of salt inside leaf cells.

The possibility of water taking different pathways inside the leaf was modeled by Barbour & Farquhar (2003) using wheat leaves. In all of their models the water pathway was longer when it occurred symplastically via plasmodesmata. Therefore, a longer L in mangrove leaves indicates that the water could flow predominantly symplastically via plasmodesmata (Barbour & Farquhar 2003) where salts are removed and stored in vacuoles (Munns, 2002). Water flow in hammock leaves, on the other hand, could primarily be apoplastically or if symplastically via aquaporins. Unfortunately very little is known about mangrove leaf physiology and potential physiological/biochemical mechanisms to prevent salt damage to the photosynthetic apparatus. This finding of differences in Δ_{LW} between hammock and mangrove species seems to be a result of L adjustments in mangrove leaves to salinity, suggesting that mangrove leaves may have a unique pathway of leaf water movement compared to the hammock species studied here. However, further studies, including long term comparison of leaf water isotopic

enrichment between mangrove and hammock plants, are necessary before the effect of salinity in leaf water movement can be fully understood.

Succulence and L also were correlated. Therefore, I cannot rule out the possibility that the lower Δ_{LW} of mangrove leaves is caused by a greater succulence in mangroves. The greater succulence in mangroves would increase L by increasing the cell volume/layers in the mesophyll. However, Kahmen et al. (2008) found no relationship between succulence and L and they also showed that succulence was not responsible for changes in Δ_{LW}. Hence, it is possible that both of these parameters (succulence and L) covary with salinity, but succulence has no causal effect on L.

The small range of $\delta^{18}O_{SW}$ observed in the Big Pine Key transect was linked to the lower salinity gradient between mangrove and hammock areas compared to those at Sugarloaf Key transect. I also observed that in the Big Pine Key transect, the lower salinity gradient was reflected in similar predawn leaf water potential between mangrove and hammock species. The plants located in the Sugarloaf Key transect, on the other hand, experienced a wider and stronger salinity gradient resulting in leaf water stress (Ψ_{pd} < -2.0 MPa) at the mangrove areas. As a result of this water stress, mangrove plants adjusted the movement of leaf water, leading to differences in L and Δ_{LW} compared to hammock species, which ultimately can interfere with the recording of $\delta^{18}O_{SW}$ in the $\delta^{18}O_{CELL}$.

Buttonwood plants had $\delta^{18}O_{SW}$ values falling between those observed for hammock and mangrove plants, reflecting its ability to live under both fresh and saltwater conditions. Kahmen et al. (2009) found that plants from the same species, growing under distinct RH had similar L. In this study, RH was similar, however under different source

water availability (fresh or saltwater), buttonwood plants showed plasticity in their leaf properties such as L, succulence and *E*. Interestingly, the increase in L observed in buttonwood plants growing in the mangrove areas was counter balanced by lower transpiration rates which led to similar Péclet values (0.95 ± 0.1) minimizing differences in Δ_{LW} regardless of whether they were growing in hammock or mangrove areas.

Conclusions

This study showed that variation in the biochemical fractionation and physiology of mangrove and hammock tree leaves can affect the $\delta^{18}O_{CELL}$ in an unexpected way. In the Big Pine Key transect, I showed that the elimination of isotopic noise present in the oxygen attached to the second carbon of the cellulose glucose moieties improved the consistency between the oxygen isotope ratios of the modified cellulose molecule (PG) and that of the source water. These results were not reproduced in the Sugarloaf Key transect. In the Sugarloaf Key transect I showed that Δ_{LW} is lower in mangroves compared to that of freshwater plants. This difference in Δ_{LW} between mangrove and hammock plants could have interfered with the recording of the $\delta^{18}O_{SW}$ onto $\delta^{18}O_{CELL}$. Although mangrove source water is enriched in ^{18}O, their lower Δ_{LW} counteracts the recording of the higher source water oxygen isotopic signal in their $\delta^{18}O_{CELL}$. This study also probed the mechanism of lower Δ_{LW} of mangroves under water stress, and showed that it is related to the effective leaf mixing length of the water pathway from the xylem to the stomatal pore, leading to distinctively different Δ_{LW} between hammocks, mangroves and buttonwood plants. This difference suggests that freshwater, mangrove or buttonwood plants under similar environmental conditions will have similar Δ_{LW} within each group, but not across groups. These findings clarify the difficulties in attempting to

use the oxygen isotope ratios of coastal plant stem cellulose to monitor sea level rise. More studies are necessary to understand the effect of salt stress on the pathway of leaf water movement before the $\delta^{18}O_{CELL}$ of coastal plants can be used as a proxy for sea level rise.

Table 3.1. Nested ANOVAs for ranked and non-ranked variables showing how the oxygen isotope ratios of stem water ($\delta^{18}O_{SW}$), of stem cellulose ($\delta^{18}O_{CELL}$), and of phenylglucosazone ($\delta^{18}O_{PG}$), as well as oxygen isotope fractionation of stem cellulose (Δ_{CELL}) and of stem phenylglucosazone (Δ_{PG}) and predawn leaf water potential (Ψpd) changed along Big Pine Key and Sugarloaf Key transects. Significant effects are shown in bold.

Sites	Parameters		df	ms	F	Sig.
Big Pine Key	$\delta^{18}O_{SW}$	area	4	906.7	7.73	**0.006**
		species	9	117.3	2.207	0.053
		residual	28	53.1		
	$\delta^{18}O_{CELL}$	area	4	3.5	1.57	0.263
		species	9	2.2	1.042	0.434
		residual	27	2.1		
	$\delta^{18}O_{PG}$	area	4	11.6	6.052	**0.012**
		species	9	1.9	1.386	0.241
		residual	28	1.4		
	Δ_{CELL}	area	4	267.0	1.486	0.285
		species	9	179.8	1.562	0.177
		residual	27	115.1		
	Δ_{PG}	area	4	1.8	0.944	0.482
		species	9	1.9	1.236	0.314
		residual	28	1.6		
	Ψpd	area	4	0.2	0.596	0.675
		species	9	0.4	6.496	**<0.001**
		residual	28	0.06		
Sugarloaf Key	$\delta^{18}O_{SW}$	area	4	1935.3	7.983	**0.021**
		species	5	242.4	6.645	**<0.001**
		residual	40	36.5		
	$\delta^{18}O_{CELL}$	area	4	12.1	5.63	**0.043**
		species	5	2.2	11.064	**<0.001**
		residual	40	0.2		
	$\delta^{18}O_{PG}$	area	4	598.3	0.683	0.633
		species	5	876.0	9.629	**<0.001**
		residual	40	91.0		
	Δ_{CELL}	area	4	1699.2	7.623	**0.023**
		species	5	222.9	3.565	**0.009**
		residual	40	62.5		
	Δ_{PG}	area	4	1967.0	7.444	**0.025**
		species	5	264.2	8.637	**<0.001**
		residual	40	30.6		
	Ψpd	area	4	1911.1	7.538	**0.024**
		species	5	253.5	15.945	**<0.001**
		residual	40	15.9		

Table 3.2. Nested ANOVAs for ranked and non-ranked variables showing how the oxygen isotope fractionation of leaf water (Δ_{LW}), transpiration (E), effective leaf mixing length (L) and succulence changed for plants in mangrove (M) and hammock (H) areas located in the transition between fresh and saltwater sources of Sugarloaf Key transect. Significant effects are shown in bold.

Sites	Parameters		df	ms	F	Sig.
Sugarloaf Key	Δ_{LW}	MxH	1	86.8	25.934	**0.003**
		species	4	3.2	0.581	0.68
		residual	19	5.4		
	E	MxH	1	683.0	6.834	0.059
		species	4	107.1	6.491	**0.002**
		residual	19	16.5		
	L	MxH	1	132.9	8.153	**0.013**
		species	4	17.5	6.925	0.12
		residual	19	2.5		
	Succ	MxH	1	723.1	12.932	**0.022**
		species	4	60.4	13.723	**<0.001**
		residual	19	4.4		

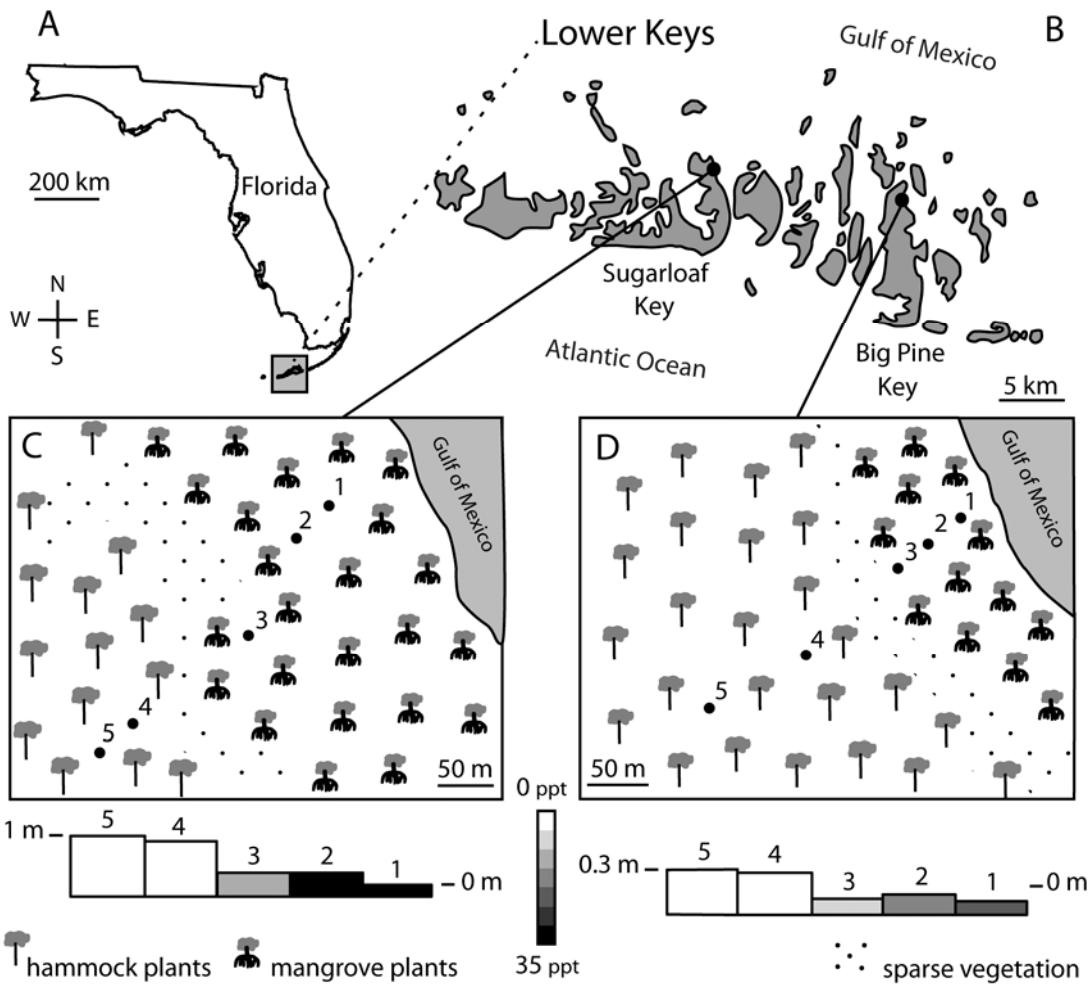

Figure 3.1 Map of the research site located in Florida Keys, USA (A,B). Two 300 m transects were selected, one at Sugarloaf (C) and one at Big Pine Key (D). Each transect was divided into 5 areas. Areas 1, 2 and 3 of each transect is represented by mangrove communities (saltwater plants) and areas 4 and 5 are represented by hammock communities (freshwater plants). Below each transect there is an elevation profile with soil salinity for each area. The salinity scale ranged from 0-35 ppt and the salinity gradient encompass increments of 5 ppt. Latitude and longitude of each transect area: Sugarloaf Key – (1) 24°40.969'N 81°32.658'W, (2) 24°40.954'N 81°32.675' W, (3) 24°40.939' N 81°32.691'W, (4) 24°40.927'N 81°32.704'W, (5) 24°40.909'N 81°32.721'W and Big Pine Key - (1) 24°43.708'N 81°23.422'W, (2) 24°43.716'N 81°23.378'W, (3) 24°43.731'N 81°23.349'W, (4) 24°43.738'N 81°23.328'W, (5) 24°43.750'N 81°23.324'W.

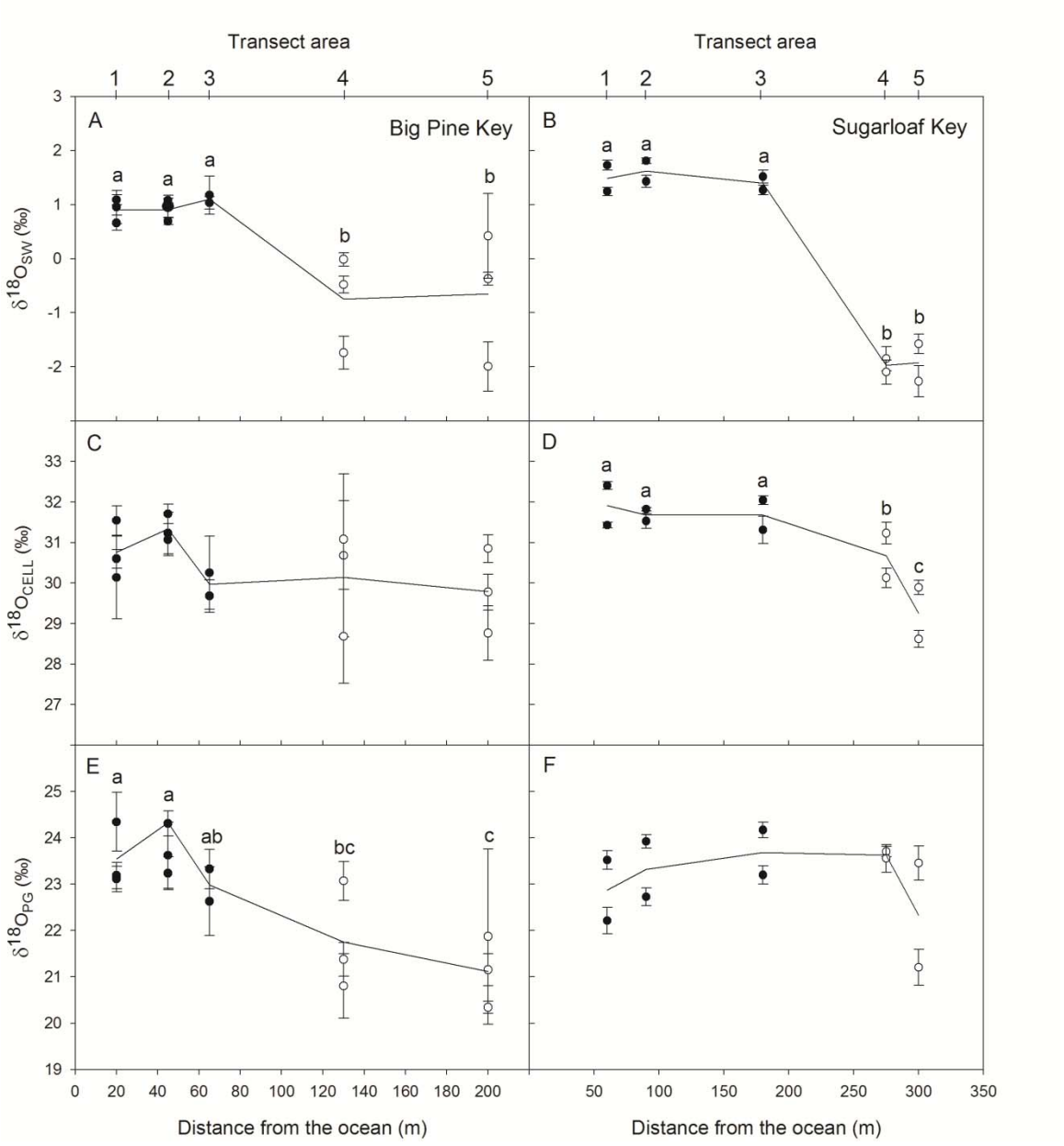

Figure 3.2 Oxygen isotope ratios of stem water ($\delta^{18}O_{SW}$), of stem cellulose ($\delta^{18}O_{CELL}$) and of stem phenylglucosazone ($\delta^{18}O_{PG}$) from mangroves (●) and hammock plants (freshwater plants) (○) located at different distances from the ocean along each of the transect areas. Study sites and sample collections: Big Pine Key (Jan 2009) and Sugarloaf Key (Mar 2009), FL, USA. Areas having the same or no letters are not statistically different with regard to the measured parameter: $P < 0.05$.

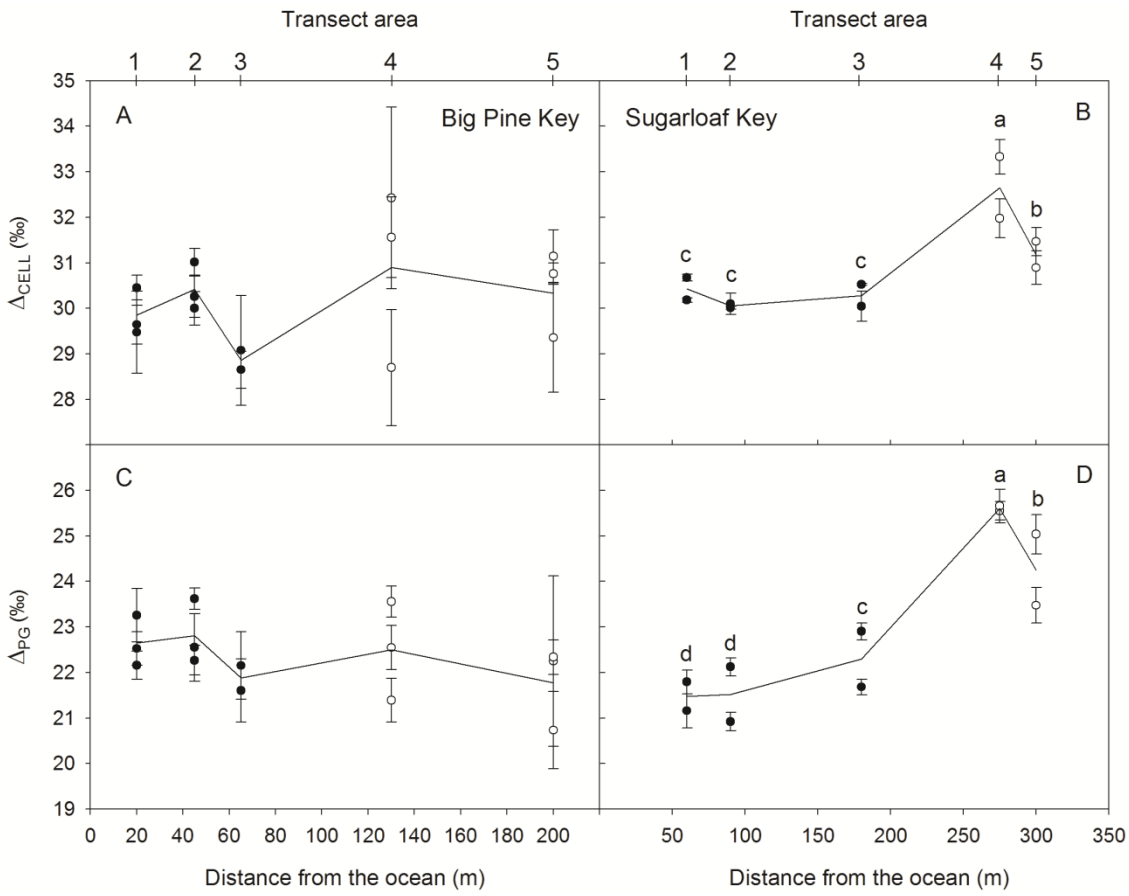

Figure 3.3 Oxygen isotope fractionation of stem cellulose (Δ_{CELL}) and of stem phenylglucosazone (Δ_{PG}) from mangrove (●) and hammock plants (freshwater plants) (○) located at different distances from the ocean along each of the transect areas. Study sites and sample collections: Big Pine Key (Jan 2009) and Sugarloaf Key (Mar 2009), FL, USA. Areas having the same or no letters are not statistically different with regard to the measured parameter: $P < 0.05$.

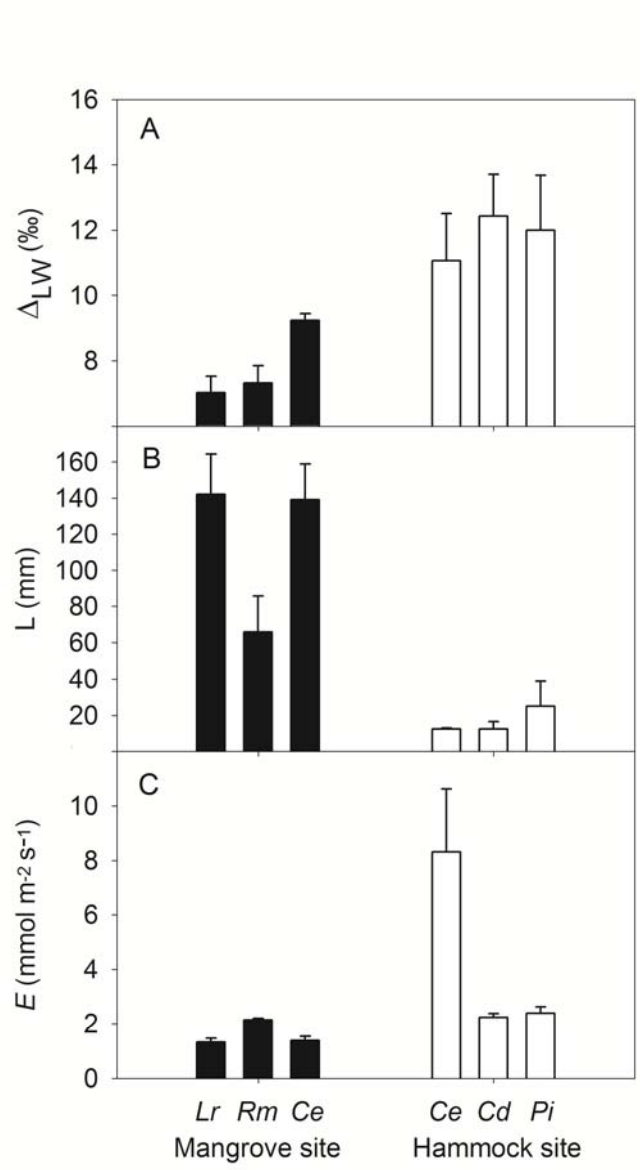

Figure 3.4 Oxygen isotope fractionation of leaf water (Δ_{LW}), effective leaf mixing length (L) and transpiration rate (*E*) from *Laguncularia racemosa* (*Lr*), *Rhizophora mangle* (*Rm*), and *Conocarpus erectus* (*Ce*) at the mangrove area (shaded bar) and *Conocarpus erectus* (*Ce*), *Coccoloba diversifolia* (*Cd*) and *Pithecellobium guadalupense* (*Pi*) at the hammock area (non-shaded bar). Study site and sample collection: areas 3 and 4 of Sugarloaf Key transect (Mar 2010), where the transition from salt to freshwater occurs.

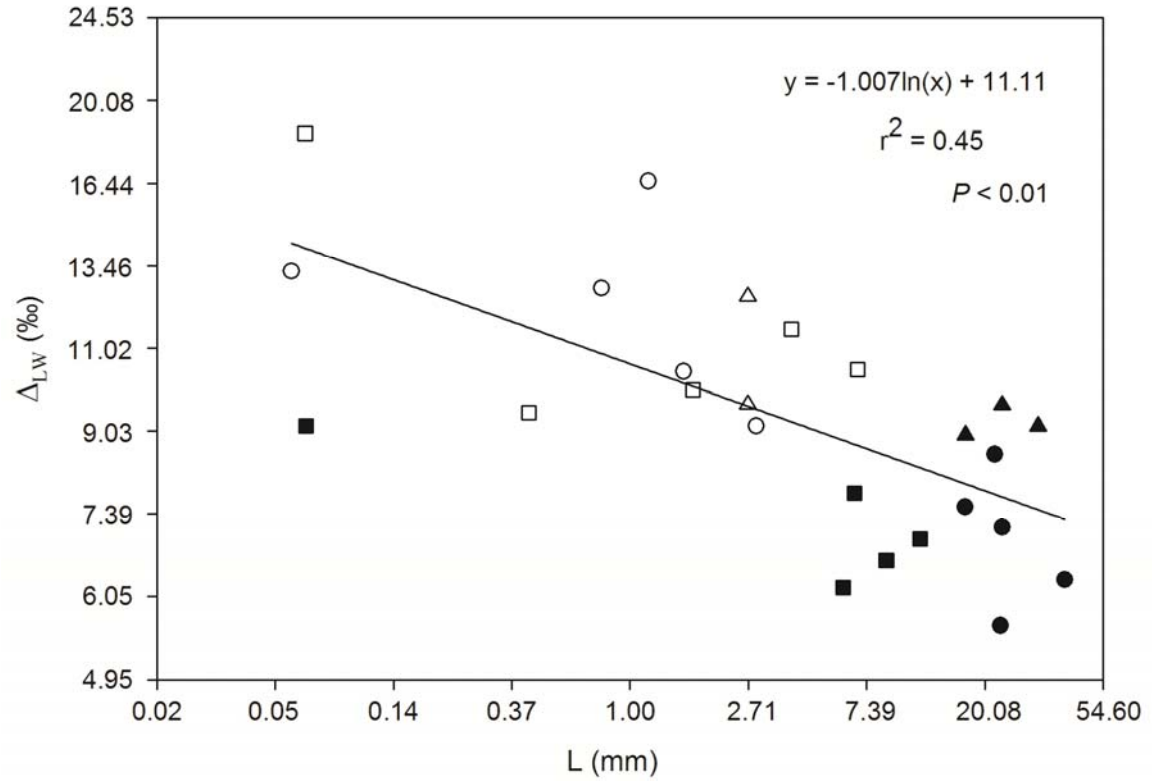

Figure 3.5 Relationship between leaf water oxygen isotope fractionation (Δ_{LW}) and effective leaf mixing length (L; log scale) from the mangrove species *Laguncularia racemosa* (●) and *Rhizophora mangle* (■), from the hammock species (freshwater plants) *Coccoloba diversifolia* (○) and *Pithecellobium guadalupense* (□) and from buttonwood plants located at mangrove (▲) and hammock areas (Δ). Study site and sample collection: areas 3 and 4 of Sugarloaf Key transect (Mar 2010), where the transition from salt to freshwater occurs.

CHAPTER 4

Biochemical effects of salinity on oxygen isotope fractionation during cellulose synthesis.

Summary

Climate signal is recorded in the oxygen isotope ratios of cellulose ($\delta^{18}O_{CELL}$). According to the current tree ring model, 42% of the oxygen in the sucrose molecule exchanges with stem water during stem cellulose synthesis. However, this exchange rate (p_{ex}) may not be constant. The tree ring model also assumes that the fractionation associated with this exchange (ε_{bio}) is constant and 27‰. Previous studies have shown that both p_{ex} and ε_{bio} vary. Here I hypothesized that changes in plant metabolism caused by salt stress will change p_{ex} and/or ε_{bio} during cellulose synthesis. Ultimately, these changes will be recorded in the $\delta^{18}O_{CELL}$. Previous observations indicate that salinity stimulates the synthesis of soluble sugar and sugar alcohols, changing plant carbohydrate metabolism. To simulate the effect of salinity and the synthesis of osmolytes, I compared wild type (WT) and M6PR transgenic (mannitol synthesizers) lines of *Arabidopsis thaliana* under salt (3ppt) and freshwater availability. To simulate the effect of increase in soluble sugars, I compared WT and a starchless mutant (PGM-1) of *A. thaliana* under freshwater availability. For the salinity experiment, ε_{bio} was similar, but p_{ex} for the salt treated plants was twice (0.64) the observed value of the freshwater treatment (0.3). The increase in stem water contribution to the sucrose molecule decreased cellulose fractionation (Δ_{CELL}) in plants under salt treatment compared to WT. Mannitol synthesis did not cause p_{ex} and ε_{bio} to change, the Δ_{CELL} were similar between WT and all the M6PR transgenic lines within each treatment. Starchless and WT differed in ε_{bio}, but it

was impossible to determine whether there were any differences in p_{ex}. Changes in plant biochemistry under starchless condition decreased $\Delta_{CELL} \sim 2‰$ relative to WT. Increases in soluble carbohydrates expected under salt stress as well as under starchless condition can cause the observed $\delta^{18}O_{CELL}$ to be lower than the expected based on its leaf and stem water oxygen isotope ratios. To prevent misleading interpretations of climate reconstructions using the $\delta^{18}O_{CELL}$, current models must carefully account for possible changes in p_{ex} and ε_{bio}, instead of considering them constants.

Background

Leaf and source water oxygen isotopic signal is incorporated into the tree trunk cellulose molecule where oxygen from leaf and xylem water exchange with oxygen from cellulose precursors, such as sucrose (Sternberg, 2009). Leaf water oxygen isotope ratios ($\delta^{18}O_{LW}$) record relative humidity (RH), and represent ~ 58% of the oxygen isotope signal of stem cellulose ($\delta^{18}O_{CELL}$; Eqn 1). The source water oxygen isotope ratios record precipitation, and are incorporated in the plant stem water without fractionation. The incorporation of the stem water isotopic composition in the cellulose molecule starts as sucrose molecules are translocated from the leaf to the stem and cleave into glucose and fructose, which are isomer molecules.

The participation of fructose in a futile cycle (Fig 4.1) generates several carbonyl groups that can undergo hydration (oxygen from carbon 2, 3, 4, and 5) (Hill et al., 1995). Hydration involves the addition of oxygen from the surrounding water molecules. When the carbonyl group dehydrates, the double bond from the carbonyl group is restored, but the remaining oxygen can be from either the original molecule or the water surrounding it (Sternberg and DeNiro, 1983) (Fig 4.2). These carbonyl groups from trioses exchange

oxygen with stem water very fast, especially those of dihydroxyacetone which reach equilibrium in less than 20s (Model et al., 1968; Reynolds et al., 1971). When the oxygen from a carbonyl group completely exchanges with water and reaches equilibrium, the $\delta^{18}O$ value of a carbonyl group is 27‰ higher than that from the water surrounding it (Sternberg and DeNiro, 1983; Sternberg, 1989; Yakir and DeNiro, 1990). This enrichment of carbohydrate oxygen above source water is known as biochemical fractionation (ε_{bio}).

The oxygen isotopic signature of stem water is passed on to fructose and glucose, the cellulose precursor. However, not all of the fructose molecules undergo the above futile cycle and therefore, not all of the oxygen in the hexose carbon form carbonyl oxygen prior to cellulose synthesis. Consequently, the labeling of oxygen from stem water on the fructose is incomplete and corresponds to ~ 42% (Sternberg et al., 1986; Roden et al., 2000; Sternberg and Ellsworth, 2011). Roden et al. (2000) represented the proportion of stem and leaf water oxygen isotopic contribution to the $\delta^{18}O_{CELL}$, assuming ε_{bio} a constant of 27‰ (Eqn1),

$$\delta^{18}O_{CELL} = (0.42* \delta^{18}O_{SW}) + (0.58* \delta^{18}O_{LW}) + \varepsilon_{bio} \qquad (1)$$

where $\delta^{18}O_{SW}$ represents the oxygen isotope ratios of stem water.

The constancy in the proportion of oxygen exchange between stem water and sucrose (p_{ex}) and the biochemical fractionation factor (ε_{bio}) is questionable. Sternberg and Ellsworth (2011) growing heterotrophic cellulose under dark conditions at different temperatures observed that p_{ex} was similar (~ 42%) at temperatures ranging from 5-30°C. However, ε_{bio} averaged 26‰ between 20-30°C, with ε_{bio} reaching 31‰ at the lowest temperature (5°C). Waterhouse et al. (2002) reconstructed precipitation using the tree

ring oxygen isotope ratios of cellulose from trees located in Sandringham Park, northwest Norfolk, in eastern England. They observed that the best fit between reconstructed and observed $\delta^{18}O$ values of precipitation occurred when p_{ex} and ε_{bio} were assumed to be 46% and 30‰, respectively. Their best-fit observed for the ε_{bio} in vivo matched Sternberg and Ellsworth (2011) in vitro experiment, indicating that at higher latitudes when temperature are lower than 20°C, ε_{bio} increases.

Roden et al. (2005) found that reconstructed $\delta^{18}O_{CELL}$ using the mechanistic model (Eqn 1) (Roden et al., 2000) was overestimated under low water availability and low RH. Zhou (2005) studied the stem cellulose oxygen isotope enrichment above source water (Δ_{CELL}), from plants grown under different abiotic conditions such as high and low values of CO_2, RH , ambient temperature, light and water. He found that Δ_{CELL} was lower under water stress than for well watered plants. Considering that water stress can trigger similar plant responses to that of saltwater conditions, the same depletion could be observed under salt stress, simply by changing p_{ex} and/or ε_{bio}.

Under saltwater conditions, for example, plant organic matter had oxygen isotope ratios below expected based on source water oxygen isotopic composition according to the mechanistic model (Verheyden et al., 2004; Ellsworth et al., 2012). During the dry season, when RH is low, leaf water oxygen isotope enrichment above source water (Δ_{LW}) should have increased and contributed to increases in the $\delta^{18}O$ of tree ring wood of *Rhizophora mucronata* (Verheyden et al., 2004). Ellsworth et al. (2012) observed that plants growing under similar RH, when exposed to salt stress decreased Δ_{LW}. They observed that leaf physiology responses changed under salt stress so that $\delta^{18}O_{CELL}$ did not record climate in a pattern expected by the mechanistic model.

Both plant biochemistry and leaf physiology can change the climate signature prior to cellulose synthesis (Ellsworth et al., 2012). This study focused on the effect of salinity on plant biochemistry and how it ultimately interfered in the recording of oxygen isotope ratios of source water and RH in the $\delta^{18}O_{CELL}$. Some studies have shown that salt and water stress can alter the pool of soluble carbohydrates in plants (Smirnoff, 1998). Under those conditions, plant metabolic pathways as well as the leaf physiology respond promptly to prevent tissue dehydration and damage related to low soil water potential and high cell reactive oxygen species (ROS). Under salt stress, the increase in soluble carbohydrates was related to increases in sucrose, fructose and inositol (Zhifang and Loescher, 2003). Salt stress also stimulates the synthesis of polyols (Yancey et al., 1982), such as mannitol, sorbitol, pinitol, sucrose, etc, as a mechanism to increase cell water potential. Stoop et al. (1996) observed that the mannitol molecule increased salt tolerance and reduced plant osmotic stress. This agrees with Zamski et al. (2001) in which under salt condition, celery plants decreased mannitol catabolism. A transgenic M6PR *Arabidopsis thaliana,* coding for mannitol synthesis, tolerated higher salt concentration than the wild type (WT) (Zhifang and Loescher, 2003). In transgenic tobacco, the presence of mannitol in the leaf tissue also reduced the oxidative damage of cells by reducing ROS (Shen et al., 1997) . Sahu et al. (2010) observed that catalase activity increased in the presence of mannitol, sorbitol and sucrose. Because mannitol and cellulose share the same substrate, synthesis of mannitol could impose isotopic fractionation to any of the oxygen atoms during cellulose synthesis.

Under salt conditions, plant biochemistry has the potential to change p_{ex} and/or ε_{bio} as a result of variation in the pool of soluble carbohydrates. This variation can happen

in three hypothetical ways: 1) by increasing the pool of soluble carbohydrates to increase cell osmotic potential; 2) by reducing cell division and expansion under osmotic stress; and 3) by triggering other metabolic pathways, such as synthesis of osmolytes, that compete for soluble carbohydrates (substrates). The first and second mechanisms could increase p_{ex}, as the time for oxygen exchange between sucrose and stem water would increase with a greater availability of soluble carbohydrates. The third mechanism could potentially alter p_{ex} and ε_{bio}; p_{ex} would decrease if the pool of soluble carbohydrates was reduced as it is used up for osmolyte synthesis, decreasing the time for oxygen exchange between sucrose and stem water; ε_{bio} variation would be a consequence of additional biochemical pathways imposing fractionation on the pool of soluble carbohydrate substrates of cellulose synthesis.

Barbour and Farquhar (2000) pointed out that under mild water stress p_{ex} could be greater than 0.42, if cell division and expansion decrease and the sucrose supply remains unchanged. This would create an excess of sucrose and more time for the futile cycle to occur, and subsequently greater oxygen isotopic exchange between stem water and cellulose precursors. On the other hand, Barbour and Farquhar (2000) added, if sucrose demand is high, less time would be available for oxygen exchange during cellulose synthesis ($p_{ex} < 0.42$). Barbour et al. (2000) suggested that ε_{bio} of 27‰ is the mean of all the fractionations that happens during biochemical reactions leading to carbohydrate synthesis. They explained that reactions with more than one product have the potential to cause depletion or enrichment of a specific oxygen during sucrose synthesis.

These previous studies suggest that the low osmotic potential of soil water expected under salt stress or water deficit, changes plant metabolic pathways. These

changes in plant biochemistry ultimately can result in $\delta^{18}O_{CELL}$ variation, even between plants that are growing under the same RH and oxygen isotope composition of source water. Here I investigate how salt stress affects the $\delta^{18}O_{CELL}$ in *Arabidopsis thaliana* sp. First I hypothesize that salt stress will change the amount of oxygen exchange between carbohydrates and source water (p_{ex}) and/or change ε_{bio} during cellulose synthesis. Second, I hypothesize that synthesis of mannitol and synthesis of excess soluble sugar (by blocking starch synthesis) could alter p_{ex} and/or ε_{bio}, independent of salinity. As mannitol, starch and cellulose uses a common substrate, I can expect: 1. decrease in the pool of soluble carbohydates and/or 2. additional fractionation of oxygen atoms during cellulose synthesis. To test the first hypothesis I compared *A. thaliana* cultivated under salt and freshwater. To test the second hypothesis I had two sets of experiments: first, I used different M6PR transgenic lines of *A. thaliana* that code for mannitol synthesis (Zhifang and Loescher, 2003) and I compared the M6PR transgenic lines to the wild type (WT) *A. thaliana* (Columbia) under saline and freshwater separately; second, I compared a starchless mutant (PGM-1) *A. thaliana* to the WT *A. thaliana* (Columbia) under freshwater availability.

Materials and Methods

1. Experiment design

In the following experiments a WT *Arabidopsis thaliana* (Columbia, Lehle Seeds cat# WT-02-38-02, seed lot# 206-440) represented the control plants. The hydroponic experiment tested salinity effect and mannitol synthesis influence in plant biochemistry of WT and 7 lines of M6PR transgenic (M_1, M_2, M_3, M_4, M_5, M_6, M_7) obtained from Dr. Wayne Loescher's laboratory (Department of Horticulture, Michigan State University).

These transgenic lines have the M6PR gene, which codes for mannose-6-phosphate reductase, transferred into their DNA and transcribed continually (Zhifang and Loescher, 2003). In celery the mannose-6-phosphate reductase enzyme participates in the reduction of mannose-6-phosphate to mannitol-1-phosphate which is the last precursor in the mannitol synthesis pathway (Fig 4.1).

This hydroponic setting was located inside the laboratory, at room temperature (~ 21°C), with WT and M6PR transgenic lines randomly organized within fresh and salt (50 mM ~ 3ppt) water treatment. Seeds were placed in rockwool (RockWool, Leads, Alabama, USA) and watered from the top until seed germination. To prevent algae growth, the lights and nutrient solutions were introduced after seed germination. The nutrient solution was prepared according to Huttner and Bar-Zvi (2003) and the pH was adjusted to 6.5. Distilled water was added to the nutrient solution as needed to maintain its original volume and keep contact with the rockwool extremity. The solution was aerated using an aquarium pump, and collected weekly to measure the oxygen isotope ratios of water under each treatment. The light irradiance was maintained at 120 µmol s^{-1} m^{-2}, using four 20W bulbs at 8 cm distance from the plants. All plants were harvested on the same day, after the development of a flower stalk, approximately 51 days after seed germination.

The second experiment consisted of WT and PGM-1 mutant *A.thaliana* that does not synthesize starch (cat number GM-20-02, seed lot number 199-161, Lehle Seeds, Texas, USA; Fig 4.1 and 4.3). The plants were cultivated randomly, in pots (PMP, high organic Arabidopsis medium growth, cat number PM-25-13, Lehle Seeds, Texas, USA), inside the laboratory, at room temperature (~ 21°C) and watered equally from the same

source and at the same time. Light irradiance was maintained at 120 µmol s^{-1} m^{-2}, using four 20W bulbs at 8 cm distance from the plants. A controlled release fertilizer 15-9-12 + minors (~100 mg; cat number PM-11, Lehle Seeds, Texas, USA) was added to the soil prior to seed germination, and all plants were harvested on the same day, after the development of flower stalk, approximately 38 days after seeds germination.

2. Leaf and stem water and stem cellulose extraction

I define stems as the flower stalk, after leaves, flowers and seed pods were removed. In both experiments there were five stem and leaf replicates for each *A. thaliana* line, with each replicate consisting of a pool of 5 plant samples. This procedure was necessary to reach the minimum amount of stem and leaf water and stem cellulose required for oxygen stable isotope analysis. Each replicate was placed separately into a glass tube, which was sealed and refrigerated. Water was extracted from the leaves and stems and analyzed isotopically by the method of Vendramini and Sternberg (2007). After water extraction, pooled stem samples from each water extraction were ground and homogenized for cellulose extraction. The stem samples were processed to holocellulose according to the method of Leavitt and Danzer (1993) and treated with 17% NaOH to remove the hemicellulose.

3. Oxygen isotope analysis of leaf and stem water and stem cellulose

Leaf and stem water oxygen isotope ratios were analyzed in a Multiflow system connected to an Isoprime mass spectrometer (Elementar, Hanau, Germany). A 5% CO_2/helium gas mixture was flushed through the vials and equilibrated with water for a period of 48 h. The equilibrated CO_2 gas was analyzed to derive the oxygen isotope ratios

of the water as in Vendramini and Sternberg (2007). Oxygen isotope ratios are reported relative to Vienna Standard Mean Ocean Water (V-SMOW) with a precision of ± 0.1‰.

The $\delta^{18}O_{CELL}$ were determined by a modification of Saurer et al. (1998). Half a milligram of cellulose from each sample was pyrolyzed at 1080^{o}C in a quartz column filled to approximately half its height with glassy carbon and topped off by a layer of (0.5 g) of nickelized carbon and nickel wool (Elementar America, Mt. Laurel, New Jersey, USA) in a Eurovector Elemental Analyzer (Milan, Italy). Gases from the pyrolises (mostly small amounts of hydrogen and nitrogen and larger amounts of carbon monoxide) were carried by helium, passed through magnesium perchlorate and Ascarite (Thomas Scientific) to absorb H_2O and CO_2 respectively, and separated in a 3 m 5Å molecular sieve column (Eurovector, Milan, Italy) held at 70^{o}C. The oxygen isotope ratios were determined on the carbon monoxide and compared with a standard gas calibrated to two standards: Sigma cellulose having an oxygen isotope ratio of 29.3‰ (Sauer and Sternberg, 1994) and International Atomic Energy Agency cellulose filter paper with an isotope ratio of 32.4‰. The precision of the analysis is ± 0.3‰. All isotope ratios are expressed in terms of per mil (‰) (Eqn 3):

$$\delta^{18}O = [(R_{samples}/R_{standard}) - 1] \times 1000 \qquad (3)$$

where R_{sample} and $R_{standard}$ are the $^{18}O/^{16}O$ ratios of the sample and the standard (V-SMOW), respectively.

4. Solving for P_{ex} and ε_{bio} simultaneously

Equation 4 was arranged to a linear form, so that the slope and intercept from the linear regression equation could solve for both p_{ex} and ε_{bio} (Eqn 5), respectively. Because I measured the actual values of $\delta^{18}O_{SW}$ I can ignore p_x ($p_x = 1$); p_x represents the proportion

of unenriched source water in the stem cells. Only the data from salt and freshwater treatment, which had enough isotopic variability in the $\delta^{18}O$ value of stem and leaf water, were organized as in equation 5 and plotted so that the linear regression for each treatment solved for p_{ex} (slope) and ε_{bio} (intercept).

$$\Delta_{CELL} = \Delta_{LW}(1 - p_{ex}.p_x) + \varepsilon_{bio} \quad (4)$$

$$\delta^{18}O_{CELL} - \delta^{18}O_{SW} = (\delta^{18}O_{LW} - \delta^{18}O_{SW})^{*}(1 - p_{ex}.p_x) + \varepsilon_{bio}$$

$$\delta^{18}O_{CELL} - \delta^{18}O_{LW} = p_{ex}*(\delta^{18}O_{SW} - \delta^{18}O_{LW}) + \varepsilon_{bio} \quad (5)$$

Because there were only 5 replications of WT and starchless mutant (PGM-1) and a low stem water variability of 1.3‰ and 0.8‰ respectively, a linear regression would not be accurate to solve for p_{ex} and ε_{bio}. Therefore, to test for p_{ex} variation, first ε_{bio} was assumed to be the same between WT and the starchless mutant plants and solved for the relationship between p_{ex} from WT and starchless mutant (Eqn 7). To do so, equation 5 was rearranged as equation 6 and then calculated $\varepsilon_{bio(PGM\text{-}1)} = \varepsilon_{bio(WT)}$, using all the measured $\delta^{18}O$ values of WT and starchless mutant (PGM-1) (Eqn 7).

$$\varepsilon_{bio} = \delta^{18}O_{CELL} - \delta^{18}O_{LW} - p_{ex}*(\delta^{18}O_{SW} - \delta^{18}O_{LW}) \quad (6)$$

$$p_{ex(PGM\text{-}1)} = 1.75 + 0.87* p_{ex(WT)} \quad (7)$$

Second, I solved for all possible values of WT and starchless mutant ε_{bio} considering a constant p_{ex}, with p_{ex} values ranging from 0 to 1(Eqn 6).

5. Statistical analysis

The PASW SPSS Statistics (version 18; IBM, Armonk, New York) program was used to perform the analysis. Data were tested for normality (Kolmogorov-Smirnov test and

Shapiro-Wilk test) and homogeneity of variance (Levene's Statistic test). If normality was not reached after transformation, the data were ranked previously to the nested ANOVA analysis, because there is no non-parametric test equivalent to nested ANOVA. A nested ANOVA was used to analyze the hydroponic experiment by first comparing the treatment effect (salt versus freshwater) in the leaf and stem water as well as in the stem cellulose oxygen isotope ratios keeping all the *A. thaliana* lines (WT and M6PR transgenic) nested (Table 4.1). Second, the nested information between the WT and transgenic (M6PR) lines from table 4.1 was used to interpret the effect of mannitol synthesis genes in the leaf and stem water as well as in the stem cellulose isotope ratios. Wild type and starchless mutants (PGM-1) *A. thaliana* lines were compared for leaf and stem water as well as in the stem cellulose isotope ratios by Student's t-test (Table 4.2).

The regression equations were tested for normality and homoscedasticity. From salt and freshwater linear regression I tested: 1. the significance between correlation coefficients (r), by transforming r to Z values and comparing them using chi-square (Zar, 1999); 2. the difference between slopes (p_{ex}) using analysis of covariance (Zar, 1999). Because the slopes were significantly different, the intercept confidence intervals from both equations were used to determine differences in ε_{bio} between salt and freshwater treated plants.

Results

There were no significant differences between the $\delta^{18}O$ of source water for salt and freshwater hydroponic setting. Within salt or freshwater, $\delta^{18}O_{LW}$ and $\delta^{18}O_{CELL}$ did not differ between WT and M6PR transgenic *A. thaliana* (mannitol synthesizers) (Table 4.1; Fig 4.4 A and C), however $\delta^{18}O_{SW}$ differed between WT and one M6PR transgenic line

(M5) (Tukey' *post hoc*, $P < 0.05$). The M5 line also differed from another two M6PR lines (M2 and M6; Tukey' *post hoc*, $P < 0.05$). *A. thaliana* (WT + M6PR) growing under salt treatment had significantly higher $\delta^{18}O_{LW}$, $\delta^{18}O_{SW}$ and $\delta^{18}O_{CELL}$ compared to freshwater (WT + M6PR) plants (Table 4.1; Fig 4.4A, C and E). Wild and starchless mutant (PGM-1) *A. thaliana* did not differ in $\delta^{18}O_{LW}$ and $\delta^{18}O_{SW}$, but $\delta^{18}O_{CELL}$ was lower for starchless than WT plants (Table 4.2, Fig 4.4B, D and F).

The correlation coefficients within each treatment (salt and freshwater) were significant ($r = 0.85$, $P < 0.01$ and $r = 0.59$, $P < 0.01$, respectively). Correlation coefficients also differed between salt and freshwater treatment ($\chi 2_{(1, N = 74)} = 6.2$, $P < 0.05$). The slopes from the regression equation (Fig 4.5), representing p_{ex}, were significantly different ($t_{(76)} = 3.68$ $P < 0.001$), with the slope 2 times greater for salt than for freshwater treated plants (0.64 ± 0.06 versus 0.3 ± 0.07, respectively). The biochemical fractionation factor is represented by the intercept of the regression equation. Under saltwater, ε_{bio} was 1‰ higher than under freshwater treated plants (intercept = 28.8 and intercept = 27.9, respectively; Fig 4.5), however, their confidence intervals overlapped.

Discussion

According to the present understanding of stem water oxygen isotope labeling in the $\delta^{18}O_{CELL}$ (Roden et al., 2000), it is expected that plants using source water with similar oxygen isotopic signatures, under the same atmospheric conditions, would have similar $\delta^{18}O_{CELL}$. This assumption is true if plant physiological and biochemical responses to abiotic factors follow the mechanistic model (Roden et al., 2000) (Eqn1). In both my experiments, salt versus freshwater as well as WT versus starchless mutant, $\delta^{18}O$ of

source water and RH did not differ between treatments, however, $\delta^{18}O_{CELL}$ was significantly different.

Leaves from salt treated plants had higher $\delta^{18}O_{LW}$ values relative to freshwater plants, which contributed to increases in $\delta^{18}O_{CELL}$. If I solve for $\delta^{18}O_{CELL}$ using equation 1, where $p_{ex} = 0.42$ and $\varepsilon_{bio} = 27$, I expected that the $\delta^{18}O_{CELL}$ of the salt treated would be 2.3‰ more enriched than those of the freshwater treated plants. However, I only observed a 0.8‰ enrichment in the $\delta^{18}O_{CELL}$ under salt treatment relative to freshwater plants. The same pattern was observed for starchless mutant relative to the WT, where instead of 0.6‰ I observed 1.4‰ depletion in the $\delta^{18}O_{CELL}$ for starchless plants. Both salt treatment and starchless condition caused depletion in the $\delta^{18}O_{CELL}$ relative to the expectation of the current model (Eqn 1). This observation is in agreement with Zhou (2005), where the $\delta^{18}O_{CELL}$ from plants under water stress were ~7‰ less enriched than the expected. Ellsworth et al. (2012) observed that under similar RH, $\delta^{18}O_{CELL}$ did not reflect the magnitude of differences in the $\delta^{18}O_{SW}$ from mangrove versus freshwater plants. Even though $\delta^{18}O_{CELL}$ from mangrove plants was higher than freshwater plants, this enrichment was ~2.7 times lower than the expected if leaf properties between those plants were similar.

Salinity effects on the $\delta^{18}O_{CELL}$

It's important to note that under salt (Fig 4.4A) as well as water stress (Zhou 2005), the increase in $\delta^{18}O_{LW}$ was associated with leaf physiological responses to those conditions. This observation alone is relevant in paleoclimate studies as $\delta^{18}O_{LW}$ values are used as a proxy for RH. As previously mentioned, the tree ring model (Roden et al., 2000) (Eqn 1) assumption is that $\delta^{18}O_{LW}$ varies as a function of changes in RH, which was not the case

in my experiment. The second important observation is that the increase in $\delta^{18}O_{LW}$ was not recorded in the $\delta^{18}O_{CELL}$ as expected by using the model equation 1. The regression analysis (Fig 4.5) indicated that there were few if any significant changes in ε_{bio} between freshwater and salt treated plants. Further, if these changes were significant, the $\delta^{18}O_{CELL}$ of salt treated plants would be even more enriched (i.e. ε_{bio} slightly increased with salinity). On the other hand, the regression analysis showed large changes in p_{ex}, which doubled (0.64) under salt relative to freshwater treatment (0.3; Fig 4.5).

Barbour and Farquhar (2000) suggested that under mild water stress, p_{ex} may increase. They reasoned that the sucrose and soluble sugar supply would remain unchanged despite decreases in cell division and expansion. This would allow more time for the soluble sugars to undergo the exchange reactions discussed previously. Considering the successful growth of WT (salt intolerant plant) and M6PR transgenic lines under salt treatment (~3ppt), I consider this treatment mild. In addition, Zhifang and Loescher (2003) tested the effect of salt on the amount of soluble carbohydrates and osmolytes in one line of transgenic *Arabidopsis thaliana* (M_2) compared to the WT. They found that at 50mM (~3ppt) both WT and M_2 transgenic *Arabidopsis thaliana* increase the amount of the soluble sugars sucrose, fructose and inositol. Therefore, my observation that under mild salt treatment p_{ex} increases, probably because of an excess of soluble carbohydrates, is in line with Barbour & Farquhar's (2000) proposal.

Osmolyte synthesis effect on the $\delta^{18}O_{CELL}$

The synthesis of mannitol and cellulose compete for the same precursor (Fig 4.1), so that mannitol synthesis could potentially decrease the remaining pool of soluble carbohydrate directed for cellulose synthesis (Fig 4.1). If this was the case, the expected recording of

$\delta^{18}O$ of source water in the $\delta^{18}O_{CELL}$would not hold, as a consequence of p_{ex} and/or ε_{bio} variations. However, there were nether differences in $\delta^{18}O_{CELL}$ (Table 4.1 and Fig 4.4 E), nor in $\delta^{18}O_{LW}$ (Table 4.1 and Fig 4.4 A) and $\delta^{18}O$ of source water between lines within each treatment. Therefore, I conclude that the amount of mannitol synthesized was not enough to significantly decrease the pool of carbohydrates and change its oxygen isotopic signature. The oxygen exchange between sucrose and stem water, as well as ε_{bio} remained similar for WT and M6PR lines within each treatment.

Soluble carbohydrates effect on the $\delta^{18}O_{CELL}$

The purpose of comparing WT and starchless mutant was to investigate the recording of $\delta^{18}O$ of source water in the $\delta^{18}O_{CELL}$ exclusively when soluble carbohydrates differed, as opposed to the overall biochemistry variation associated with salt stress. The lack of differences in the $\delta^{18}O_{LW}$ and $\delta^{18}O_{SW}$ between WT and starchless mutant (Fig 4.4 B and D), associated with significantly lower values for $\delta^{18}O_{CELL}$ in the starchless mutant (Fig 4.4 F) could only be attributed to differences in p_{ex} and/or ε_{bio}. It was impossible to assume equal ε_{bio} for WT and the starchless mutant and solve for p_{ex} values ranging from 0 to 1. I can conclude that there were differences in the biochemical fractionation. These differences were expected considering that the starchless condition has one less fractionation step (the synthesis of starch) imposed on its pool of soluble carbohydrates compared to WT. However, in this experiment I could not confirm Barbour & Farquhar's proposition that a larger pool of soluble carbohydrates will increase p_{ex}, as there was not enough variation in the oxygen isotope ratios of stem and leaf water for the regression analysis to predict accurately p_{ex} and ε_{bio}.

Conclusions

This study suggests that the presence of salt in water even at low concentrations (~3ppt) can lead to changes in plant metabolic pathways. Some of these changes doubled the oxygen exchange between sucrose and stem water prior to cellulose synthesis. As a result, the $\delta^{18}O_{CELL}$ under salt treatment was 1.5‰ lower than the expected by the standard tree ring model (Eqn 1). Even though ε_{bio} differed between salt and freshwater treatments, I cannot make any clear conclusion because the regression confidence interval overlapped in the intercept area. However, under starchless condition, the increase in soluble carbohydrates decreased ε_{bio}, likely, by decreasing the overall fractionation steps involved in metabolic processes that use soluble carbohydrates as common substrates. This study highlights the importance of fully understanding plant carbohydrate biochemistry and leaf physiology toward using $\delta^{18}O_{CELL}$ of tree rings as a true proxy of climate.

Table 4.1 Nested $_{ANOVAs}$ for ranked and non-ranked variables showing how the oxygen isotope ratios of leaf water ($\delta^{18}O_{LW}$), of stem water ($\delta^{18}O_{SW}$) and of stem cellulose ($\delta^{18}O_{CELL}$) differed between salt (S) versus freshwater (F) water availability as well as between *A. thaliana* lines (WT and M6PR lines) growing under each condition. Significant effects are shown in bold.

Experiment	Parameters	Nested $_{ANOVAs}$	df	ms	F	*P*
Hydroponic	$\delta^{18}O_{LW}$	S x F	1	262	275	**<0.001**
setting		Arab. lines	14	1.0	0.4	1.0
		residual	64	2.6		
	$\delta^{18}O_{SW}$	S x F	1	3.7	5.2	**0.04**
		Arab. lines	14	0.7	2.7	**0.004**
		residual	64	0.3		
	$\delta^{18}O_{CELL}$	S x F	1	6680	17.8	**0.001**
		Arab. lines	14	375	0.8	0.7
		residual	64	492		

Transgenic (M6PR) and WT lines of *A. thaliana* were both hydroponically cultivated under salt (~3ppt) and freshwater. No significant differences were observed between the $\delta^{18}O$ of source water in the salt and freshwater containers.

Table 4.2 Independent t-test showing how the oxygen isotope ratios of leaf water ($\delta^{18}O_{LW}$), of stem water ($\delta^{18}O_{SW}$) and of stem cellulose ($\delta^{18}O_{CELL}$) differed between wild type (WT) and mutant (PGM-1; starchless) *A. thaliana*. Significant effects are shown in bold.

Experiment	Parameters	*t-test*	df	mean	sd	t	*P*
Soil setting	$\delta^{18}O_{LW}$	WT	10	4.75	0.71	1.68	0.1
		PGM-1		4.17	0.47		
	$\delta^{18}O_{SW}$	WT	5	4.20	0.59	2.29	0.1
		PGM-1		3.57	0.22		
	$\delta^{18}O_{CELL}$	WT	10	33.3	0.18	13.41	**<0.001**
		PGM-1		31.7	0.24		

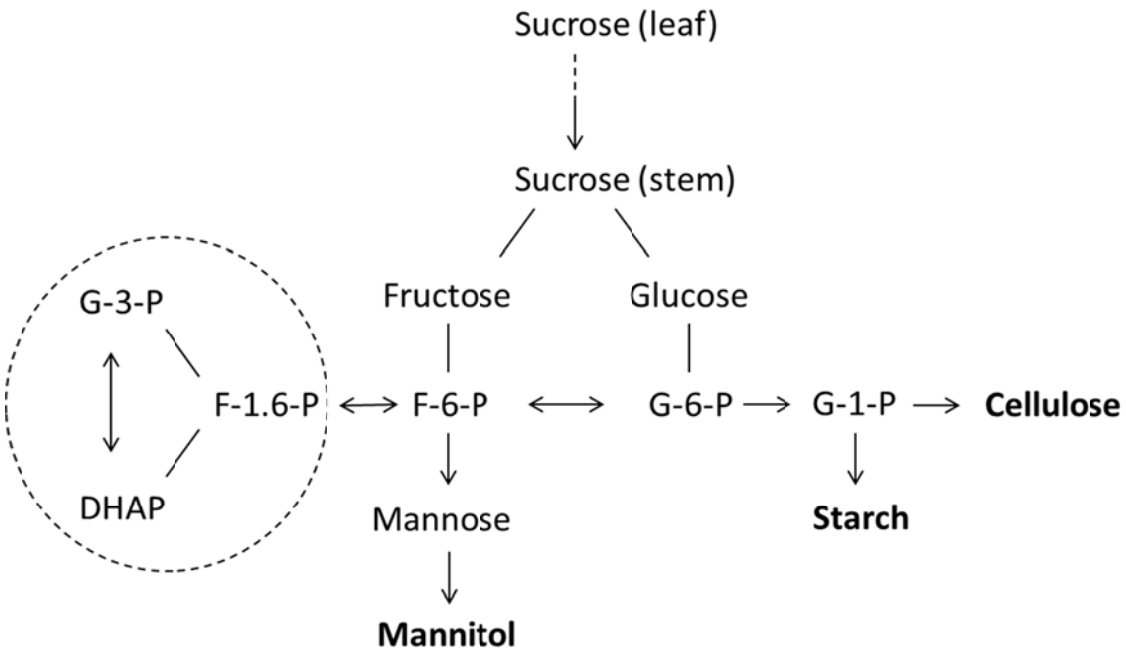

Figure 4.1 Scheme of the metabolic pathways that use the same pool of soluble carbohydrates investigated in this study. Dashed circle represents the futile cycle where oxygen from trioses exchanges with that of stem water.

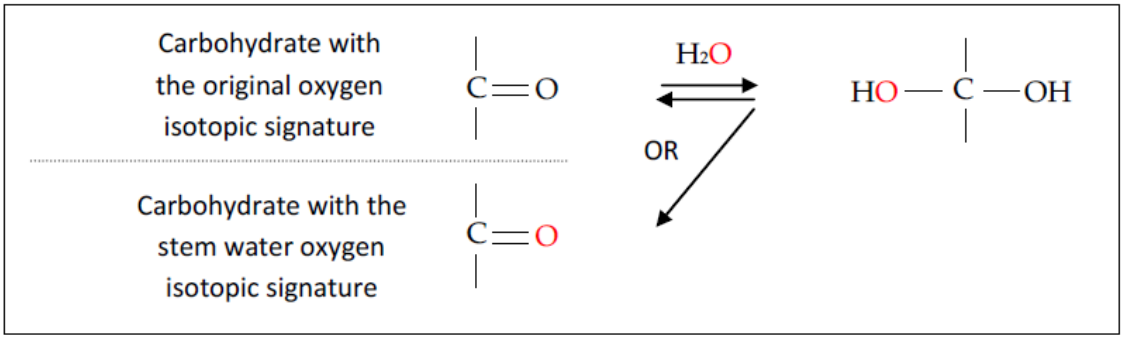

Figure 4.2 Carbonyl hydration/dehydration reactions. When the double bond is restored only one oxygen remains in the molecule and it can be the original oxygen or the oxygen from the surrounding water. When equilibrium is reached the carbohydrate becomes 27‰ more enriched than source water.

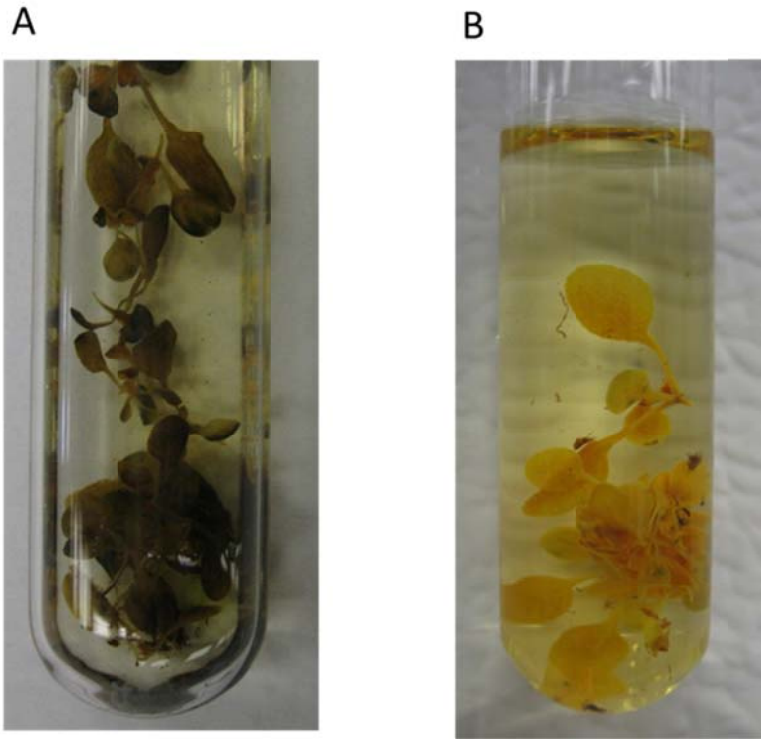

Figure 4.3 Iodine test for the presence of starch. A represents wild type and B represents the starchless mutant PGM-1 of *Arabidopsis thaliana.*

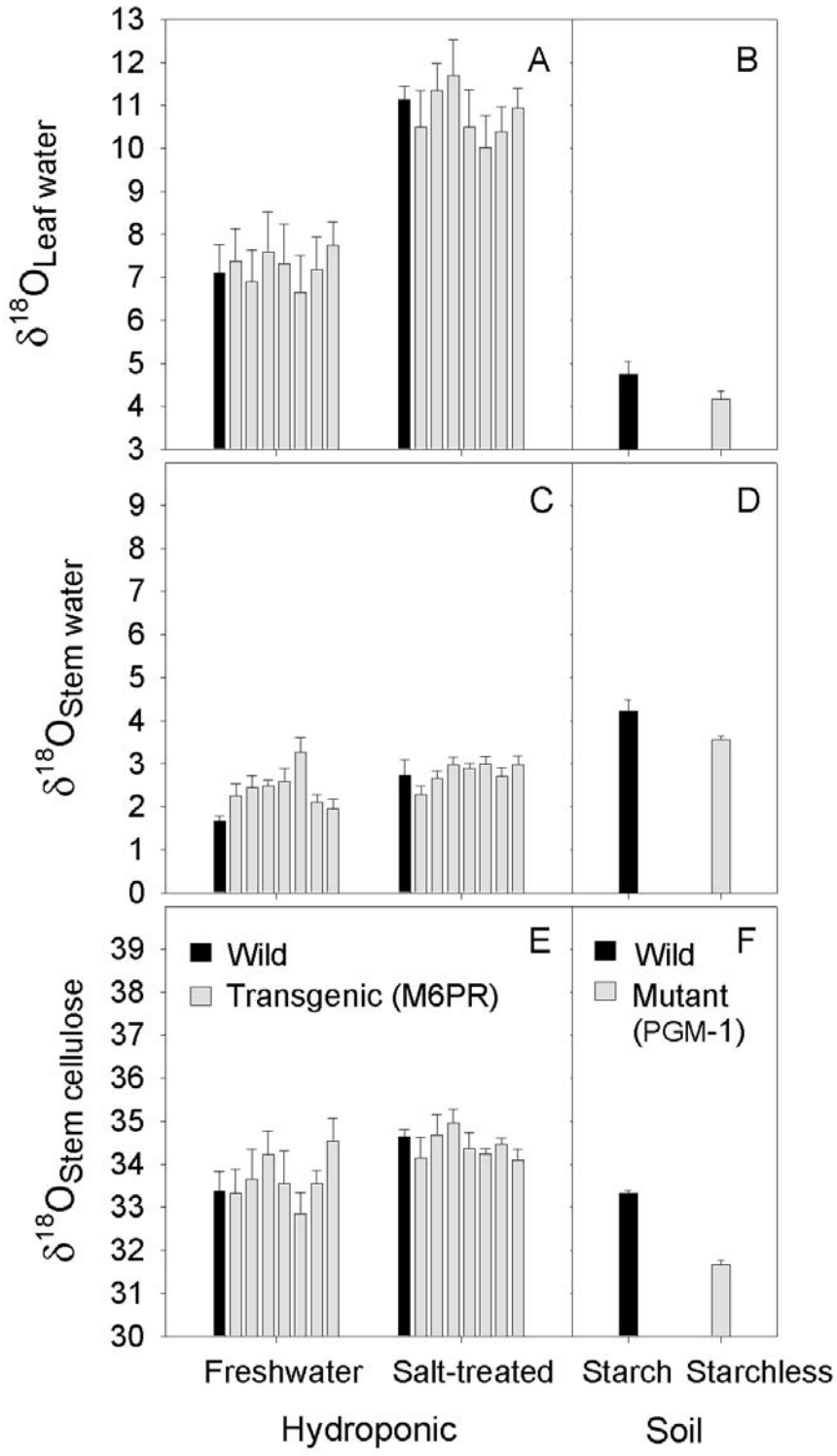

Figure 4.4 Leaf water, stem water and stem cellulose oxygen isotope ratios for wild type (black bar) and mannitol synthesizers (M6PR; gray bar) *A. thaliana* (A, C and E) and for wild type (black bar) and mutant starchless (PGM-1; gray bar) *A. thaliana* (B, D and F).

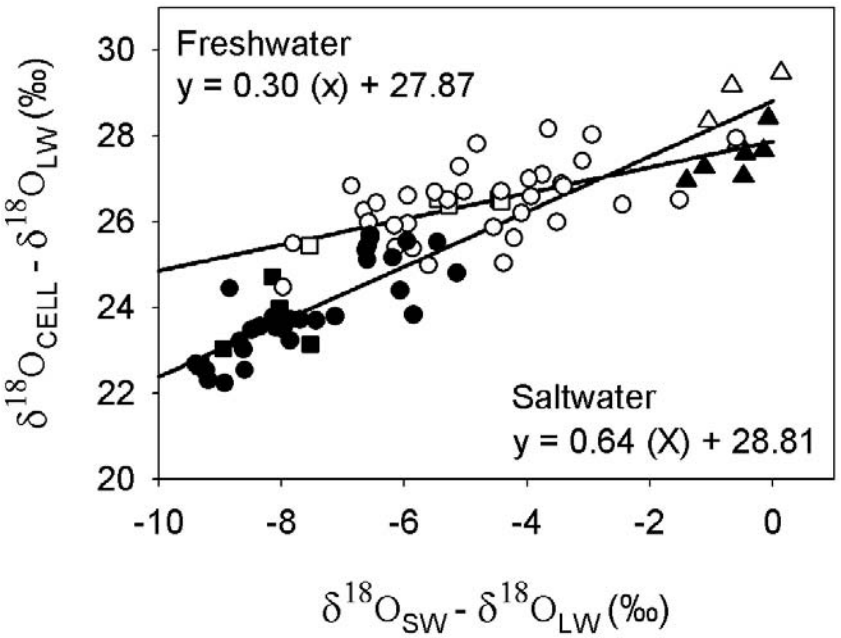

Figure 4.5 X and y axis arranged based on Eqn 5, so the slopes represent the rate of oxygen exchange between glucose and water (*pex*) and the intercepts represent biochemical fractionation factor (ε_{bio}). The relationship between the axis are shown for *A. thaliana* under fresh (○ and □) and salt (● and ■) water source, merging wild (circles) and M6PR (squares) within each treatment. Wild type (Δ) versus starchless mutant (PGM-1; ▲) were plotted but not included in the regression calculation.

CHAPTER 5

Overall conclusions

The oxygen isotopic signature of leaf and stem water incorporate climate signals which are passed on to the oxygen isotope signature of cellulose. The currently accepted tree ring model makes some assumptions about plant physiology and biochemistry, which if they do not hold, may have contributed to the lack of relationship between reconstructed and observed $\delta^{18}O_{CELL}$. One principal assumption about leaf physiology is that under low relative humidity (RH), only transpiration changes as a response to stomatal closure, increasing the oxygen isotope composition of leaf water. The two important assumptions about plant biochemistry are that the proportion of oxygen in the sucrose molecule labeled by stem water during cellulose synthesis is ~42%, and that the overall biochemical fractionation during this labeling in addition to other metabolic reactions that share the glucose substrate is 27‰. In this dissertation I brought to "light" plant physiological and biochemical responses which differ from those expected under the tree ring model. My findings, in part, explain the dissimilarities between reconstructed and observed $\delta^{18}O_{CELL}$ from previous studies.

Quality of climate reconstruction

The use of $\delta^{18}O_{CELL}$ from inter and intra-annual tree rings failed to reconstructed stem water accurately (Chap 2). On the other hand, the relationship between reconstructed and observed stem water was high when reconstructed stem water was calculated from a modified cellulose molecule. This molecule lacks the oxygen attached to C_2 of cellulose, therefore I can conclude that this specific oxygen was the main recorder of other factors

that were not related to stem water (Chap 2). Because leaf water oxygen isotopic signal varies as a function of RH, as expected, the deviation between observed and reconstructed stem water from the modified cellulose was dependent on variations in RH. No such correlation was observed when using the entire cellulose molecule (Chap 2). Prior to cellulose synthesis, fructose has only one carbonyl group which is located in C_2 of the molecule. The oxygen from a carbonyl group, possibly binds more firmly to enzymes directed to other metabolic pathways. The binding of this oxygen is probably associated with fractionation factors which impact the overall fractionation during cellulose synthesis. Because 60% of sucrose does not get labeled with stem water, any metabolic reaction that imposes fractionation to that oxygen will change the original leaf water signal. This would result in variation in the $\delta^{18}O_{CELL}$ which is not related to stem water or leaf water (RH). The same pattern was observed for plants from a transect at Big Pine Key (Monroe, FL, USA) ranging from ocean to freshwater. The salinity gradient in this transect was low, the plants were not water stressed and $\delta^{18}O_{CELL}$ was not related to the $\delta^{18}O_{SW}$ (Chap 3). Likewise, the modified cellulose molecule eliminated variations in the $\delta^{18}O_{CELL}$ not related to $\delta^{18}O_{SW}$ and I observed a better correlation between the $\delta^{18}O_{CELL}$ of the modified cellulose and stem water.

Leaf physiology variation under salinity stress

Under stressful environmental conditions leaf physiology may respond differently from that expected by the tree ring model. In another transect ranging from mangroves to freshwater plants (Sugar Loaf Key, Monroe, FL, USA), leaf physiology changed as a result of high salinity. The mangrove plants exposed to ocean water had lower leaf water oxygen isotope fractionation (Δ_{LW}) than plants growing under freshwater. This caused the

mangrove $\delta^{18}O_{CELL}$ to be less enriched than the expected based on its source water (Chap 3). The current tree ring model would predict that under the same RH, mangrove and freshwater plants would have similar leaf properties, so that a higher $\delta^{18}O_{LW}$ in mangroves would be only a consequence of its source water being enriched in ^{18}O. However, the predictions of this model were shown to be incorrect, the Δ_{LW} was lower in mangroves than in freshwater plants, which means that $\delta^{18}O_{LW}$ in mangroves was not as enriched as it should be relative to its source water. I determined that this difference is because of mangrove leaves increasing their overall pathway of water movement from the xylem to the stomatal pore (L) under salinity stress. The way that L varied between mangrove and freshwater plants affected $\delta^{18}O_{LW}$ values differently, not as a function of RH, but as a function of leaf adaptation to salt stress. Under this scenario, variation in $\delta^{18}O_{CELL}$ was associated with leaf physiological response to salt stress and not only to $\delta^{18}O_{SW}$ or RH.

Plant biochemistry variability

In the *Arabidopsis thaliana* experiment, the plant biochemical responses to salinity stress caused both the proportion of stem water isotopic signature in the $\delta^{18}O_{CELL}$ as well as the biochemical fractionation to vary, as opposed to the current accepted model (Chap 4). The main biochemical response to salinity impacting the overall plant biochemical variation was likely associated with increases in the pool of soluble carbohydrates, with no effect of osmolyte synthesis. This phenomenon contributed to variation to $\delta^{18}O_{CELL}$ under similar $\delta^{18}O_{SW}$ and RH. An increase in soluble carbohydrates could increase the proportion of stem water oxygen isotopic signature to the $\delta^{18}O_{CELL}$ ($p_{ex} > 42\%$), as opposed to the model assumption ($p_{ex} = 42\%$),in two hypothetical ways. First, if soluble

carbohydrates increased, the time for oxygen exchange between sucrose and stem water would be greater, resulting in more stem water oxygen isotopic signature to the $\delta^{18}O_{CELL}$. Second, under mild salt stress, cell development would slow down, simulating the same effect as if soluble carbohydrates had increased. The differences in biochemical fractionation were not significant between salt and freshwater treated plants, but when investigating soluble carbohydrate excess in a starchless mutant *A. thaliana* without salinity effect, ε_{bio} differences were found. This suggests the plant biochemistry related to isotopic fractionation during cellulose synthesis cannot be expected always to be the same. Variations in $\delta^{18}O_{CELL}$ resulting from changes in the amount of soluble carbohydrate, will mislead climate reconstruction.

The need to incorporate variations in plant physiology and biochemistry during climate reconstruction is evident. More studies are necessary so that L, p_{ex} and ε_{bio} can be measured for each species, under different environment conditions such as salinity stress and water stress, instead of being assumed a constant value. This process may require a multidisciplinary effort, which can be facilitated by collaborative research; nonetheless, it is an essential step for species that are commonly used for paleoclimate reconstruction. In light of this study, when using the current tree ring model assumptions, the accuracy of past climate reconstruction need to be supported by means other than tree ring oxygen isotopic composition.

References

Anderson, W. T., Bernasconi, S. M., McKenzie, J. A., Saurer, M., Schweingruber, F., 2002. Model evaluation for reconstructing the oxygen isotopic composition in precipitation from tree ring cellulose over the last century. Chemical Geology 182, 121-37.

Anderson, W. T., Bernasconi, S. M., McKenzie, J. A., Saurer, M., 1998. Oxygen and carbon isotopic record of climatic variability in tree ring cellulose (*Picea abies*): An example from central Switzerland (1913-1995). Journal of Geophysical Research-Atmospheres 103, 31625-36.

Arrivabene, H. P. (2011). Morfoanatomia, histoquímica e teor de ferro foliar em *Avicennia schueriana* Stapft & Leechm, *Laguncularia racemosa* (L.) Gaertn, e *Rhizophora mangle* L. ocorrentes em 5 áreas de manguesal do estado do espírito santo. Vitória, Espírito Santo, Brasil, Universidade Federal do Espírito Santo. **Master:** 130.

Barbour, M. M., 2007. Stable oxygen isotope composition of plant tissue: a review. Functional Plant Biology 34, 83-94.

Barbour, M. M., Roden, J. S., Farquhar, G. D., Ehleringer, J. R., 2004. Expressing leaf water and cellulose oxygen isotope ratios as enrichment above source water reveals evidence of a Péclet effect. Oecologia 138, 426-35.

Barbour, M. M., Farquhar, G. D., 2003. Do pathways of water movement and leaf anatomical dimensions allow development of gradients in $H_2{}^{18}O$ between veins and the sites of evaporation within leaves? Plant Cell and Environment 27, 107-21.

Barbour, M. M., Walcroft, A. S., Farquhar, G. D., 2002. Seasonal variation in $\delta^{13}C$ and $\delta^{18}O$ of cellulose from growth rings of *Pinus radiata*. Plant, Cell and Environment 25, 1483-99.

Barbour, M. M., Schurr, U., Henry, B. K., Wong, S. C., Farquhar, G. D., 2000. Variation in the oxygen isotope ratio of phloem sap sucrose from castor bean. Evidence in support of the Péclet effect. Plant Physiology 123, 671-79.

Barbour, M. M., Farquhar, G. D., 2000. Relative humidity- and ABA-induced variation in carbon and oxygen isotope ratios of cotton leaves. Plant Cell and Environment 23, 473-85.

Bindoff, N. L., Willebrand, J., Artale, V., Cazenave, A., Gregory, J. M., Gulev, S., Hanawa, K., Le Quere, C., Levitus, S., Nojiri, Y., 2007. Observations: oceanic climate change and sea level. in: S. Solomon, D. Q., M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M. Tignor, H.L. Miller (Eds.), Climate change 2007: the physical science basis. Contribution of working group I to the Fourth Assessment Report of the intergovernmental panel on climate change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, pp. 385-432.

Brooks, J. R., Coulombe, R., 2009. Physiological responses to fertilization recorded in tree rings: isotopic lessons from a long-term fertilization trial. Ecological Applications 19, 1044-60.

Brown, R. B., Stone, E. l., Carlisle, V. W., 1990. Soils. in: Myers, R. L.,Ewel, J. J. (Eds.), Ecosystems of Florida. University of Central Florida Press, Orlando, Florida, pp. 35-69.

Brunel, J. P., Simpson, H. J., Herczeg, A. L., Whitehead, R., Walker, G. R., 1992. Stable isotope composition of water-vapor as an indicator of transpiration fluxes from rice crops. Water Resources Research 28, 1407-16.

Cernusak, L. A., Farquhar, G. D., Pate, J. S., 2005. Environmental and physiological controls over oxygen and carbon isotope composition of Tasmanian blue gum, *Eucalyptus globulus*. Tree Physiology 25, 129-46.

Coplen, T. B., Kendall, C., Hopple, J., 1983. Comparison of stable isotope reference samples. Nature 302, 236-38.

Cracknell, A. P., Varotsos, C. A., 2011. New aspects of global climate-dynamics research and remote sensing. International Journal of Remote Sensing 32, 579-600.

Craig, H., Gordon, L. I. (1965). Deuterium and oxygen-18 variations in the ocean and the marine atmosphere. Proceedings of the conference on stable isotopes in oceanographic studies and paleotemperatures., Laboratory of Geology and Nuclear Science.

Cuntz, M., Ogee, J., Farquhar, G. D., Peylin, P., Cernusak, L. A., 2007. Modelling advection and diffusion of water isotopologues in leaves. Plant Cell and Environment 30, 892-909.

Dasberg, S., Nadler, A., 1988. Soil salinity measurements. Soil Use and Management 4, 127-33.

DeNiro, M. J., Epstein, S., 1981. Isotopic composition of cellulose from aquatic organisms. Geochimica et Cosmochimica Acta 45, 1885-94.

Dongmann, G., Nürnberg, H. W., Förstel, H., Wagener, K., 1974. On the enrichment of $H_2{}^{18}O$ in the leaves of transpiring plants. Radiation and Environmental Biophysics 11, 41-52.

Ehleringer, J. R., Dawson, T. E., 1992. Water uptake by plants: perspectives from stable isotope composition. Plant, Cell and Environment 15, 1073-82.

Ellsworth, P. V., Ellsworth, P. Z., Anderson, W. T., Sternberg, L. S. L., 2012. The role of effective leaf mixing length in the relationship between the $\delta^{18}O$ of stem cellulose and source water across a salinity gradient. Plant, Cell and Environment, doi: 10.1111/j.1365-3040.2012.02562.x

Farquhar, G. D., Lloyd, J., 1993. Carbon and oxygen isotope effects in the exchange of carbon dioxide between terrestrial plants and the atmosphere. in: Ehleringer, J. R., Hall, A. E.,Farquhar, G. D. (Eds.), Stable isotopes and plant carbon-water relations. Academic Press, New York, NY, USA, pp. 47–70.

Farquhar, G., Hubick, K., Condon, A., Richards, R., 1989. Carbon isotope discrimination and water-use efficiency. in: Rundel, P. W., Ehleinger, J. R.,Nagy, K. A. (Eds.), Stable isotopes in ecological research. Springer-Verlag, New York, USA. , pp. 21-46.

Fengel, D., Wegener, G., 1979. Hydrolysis of polysaccharides with trifluoroacetic acid and its application to rapid wood and pulp analysis. in: Brown Jr, R. D., Jurasek, Lubo (Eds.), Hydrolysis of cellulose: mechanisms of enzymatic and acid catalysis. American Chemical Society, pp. 145-58.

Florida Keys National Weather Service Forecast, O., 2011. Accessible at http://www.srh.noaa.gov/media/key/Climate/Normals-EYW_NQX_MTH_TVR.pdf.

Gessler, A., Peuke, A. D., Keitel, C., Farquhar, G. D., 2007. Oxygen isotope enrichment of organic matter in *Ricinus communis* during the diel course and as affected by assimilate transport. New Phytologist 174, 600-13.

Green, J. W., 1963. Wood cellulose. in: Whistler, R. L. (Eds.), Methods in carbohydrate chemistry. Academic Press, San Diego, California, pp. 9-20.

Hill, S. A., Waterhouse, J. S., Field, E. M., Switsur, V. R., Ap Rees, T., 1995. Rapid recycling of triose phosphates in oak stem tissue. Plant, Cell and Environment 18, 931-36.

Huttner, D., Bar-Zvi, D., 2003. An improved, simple, hydroponic method for growingArabidopsis thaliana. Plant Molecular Biology Reporter 21, 59-63.

IAEA-MIBA. 2005. The IAEA Moisture Isotopes in the Biosphere and Atmosphere Group. Accessible at http://www-naweb.iaea.org/napc/ih/documents/miba/water_vapor_protocol.pdf.

Ish-Shalom, N., Sternberg, L., Ross, M., O'Brien, J., Flynn, L., 1992. Water utilization of tropical hardwood hammocks of the Lower Florida Keys. Oecologia 92, 108-12.

Jiang, J., DeAngelis, D. L., Smith, T. J., Teh, S. Y., Koh, H. L., 2011. Spatial pattern formation of coastal vegetation in response to external gradients and positive feedbacks affecting soil porewater salinity: a model study. Landscape Ecology1-11.

Kahmen, A., Simonin, K., Tu, K., Goldsmith, G. R., Dawson, T. E., 2009. The influence of species and growing conditions on the 18-O enrichment of leaf water and its impact on 'effective path length'. New Phytologist 184, 619-30.

Kahmen, A., Simonin, K., Tu, K. P., Merchant, A., Callister, A., Siegwolf, R., Dawson, T. E., Arndt, S. K., 2008. Effects of environmental parameters, leaf physiological properties and leaf water relations on leaf water $\delta^{18}O$ enrichment in different *Eucalyptus* species. Plant, Cell and Environment 31, 738-51.

Krinner, G., Werner, M., 2003. Impact of precipitation seasonality changes on isotopic signals in polar ice cores: a multi-model analysis. Earth and Planetary Science Letters 216, 525-38.

Leavitt, S. W., Danzer, S. R., 1993. Method for batch processing small wood samples to holocellulose for stable-carbon isotope analysis. Analytical Chemistry 65, 87-89.

Lloyd, R. M., 1966. Oxygen isotope enrichment of sea water by evaporation. Geochimica et Cosmochimica Acta 30, 801-14.

McCarroll, D., Loader, N. J., 2004. Stable isotopes in tree rings. Quaternary Science Reviews 23, 771-801.

Model, P., Ponticorvo, L., Rittenberg, D., 1968. Catalysis of an oxygen-exchange reaction of fructose 1,6-diphosphate and fructose 1-phosphate with water by rabbit muscle aldolase. Biochemistry 7, 1339-47.

Munns, R., 2002. Comparative physiology of salt and water stress. Plant, Cell and Environment 25, 239-50.

Ogée, J., Barbour, M., Wingate, L., Bert, D., Bosc, A., Stievenard, M., Lambrot, C., Pierre, M., Bariac, T., Loustau, D., 2009. A single-substrate model to interpret intra-annual stable isotope signals in tree-ring cellulose. Plant, Cell and Environment 32, 1071-90.

Oikawa, N., Müller, C., Kunz, M., Lichtenthaler, F. W., 1998. Hydrophilically functionalized pyrazoles from sugars. Carbohydrate Research 309, 269-79.

Reynolds, S. J., Yates, D. W., Pogson, C. I., 1971. Dihydroxyacetone phosphate: its structure and reactivity with α-glycerophosphate dehydrogenase, aldolase and triose phosphate isomerase and some possible metabolic implications. Biochemical Journal 122, 285-97.

Roden, J., Bowling, D., McDowell, N., Bond, B., Ehleringer, J., 2005. Carbon and oxygen isotope ratios of tree ring cellulose along a precipitation transect in Oregon, United States. Journal of Geophysical Research 110, G02003.

Roden, J. S., Ehleringer, J. R., 2000. Hydrogen and oxygen isotope ratios of tree ring cellulose for field-grown riparian trees. Oecologia 123, 481-89.

Roden, J. S., Lin, G., Ehleringer, J. R., 2000. A mechanistic model for interpretation of hydrogen and oxygen isotope ratios in tree-ring cellulose. Geochimica et Cosmochimica Acta 64, 21-35.

Romero, I. C., Feakins, S. J., 2011. Spatial gradients in plant leaf wax D/H across a coastal salt marsh in southern California. Organic Geochemistry 42, 618-29.

Saha, A. K., Saha, S., Sadle, J., Jiang, J., Ross, M. S., Price, R. M., Sternberg, L. S. L. O., Wendelberger, K. S., 2011. Sea level rise and South Florida coastal forests. Climatic Change 107, 81-108.

Sahu, S., Das, P., Ray, M., Sabat, S. C., 2010. Osmolyte modulated enhanced rice leaf catalase activity under salt-stress. Advances in Bioscience and Biotechnology 1, 39-46.

Sauer, P. E., Sternberg, L., 1994. Improved method for the determination of the oxygen isotopic composition of cellulose. Analytical Chemistry 66, 2409-11.

Saurer, M., Robertson, I., Siegwolf, R., Leuenbergers, M., 1998. Oxygen isotope analysis of cellulose: an interlaboratory comparison. Analytical Chemistry 70, 2074-80.

Saurer, M., Aellen, K., Siegwolf, R., 1997. Correlating $\delta^{13}C$ and $\delta^{18}O$ in cellulose of trees. Plant, Cell and Environment 20, 1543-50.

Shen, B., Jensen, R. G., Bohnert, H. J., 1997. Increased resistance to oxidative stress in transgenic plants by targeting mannitol biosynthesis to chloroplasts. Plant Physiology 113, 1177-83.

Smirnoff, N., 1998. Plant resistance to environmental stress. Current Opinion in Biotechnology 9, 214-19.

Sobrado, M. A., 2007. Relationship of water transport to anatomical features in the mangrove *Laguncularia racemosa* grown under contrasting salinities. New Phytologist 173, 584-91.

Sternberg, L., Ellsworth, P. F. V., 2011. Divergent biochemical fractionation, not convergent temperature, explains cellulose oxygen isotope enrichment across latitudes. PLoS ONE 6, e28040.

Sternberg, L. S. L., 2009. Oxygen stable isotope ratios of tree-ring cellulose: the next phase of understanding. New Phytologist 181, 553-62.

Sternberg, L. D. L., Teh, S. Y., Ewe, S. M. L., Miralles-Wilhelm, F., DeAngelis, D. L., 2007a. Competition between hardwood hammocks and mangroves. Ecosystems 10, 648-60.

Sternberg, L. S. L., Pinzon, M. C., Vendramini, P. F., Anderson, W. T., Jahren, A. H., Beuning, K., 2007b. Oxygen isotope ratios of cellulose-derived phenylglucosazone: an improved paleoclimate indicator of environmental water and relative humidity. Geochimica et Cosmochimica Acta 71, 2463-73.

Sternberg, L. S. L., Pinzon, M. C., Anderson, W. T., Jahren, A. H., 2006. Variation in oxygen isotope fractionation during cellulose synthesis: intramolecular and biosynthetic effects. Plant, Cell and Environment 29, 1881-89.

Sternberg, L. S. L., Anderson, W. T., Morrison, K., 2003. Separating soil and leaf water 18O isotopic signals in plant stem cellulose. Geochimica et Cosmochimica Acta 67, 2561-66.

Sternberg, L. D. L., Ishshalomgordon, N., Ross, M., Obrien, J., 1991. Water relations of coastal plant-communities near the ocean fresh-water boundary. Oecologia 88, 305-10.

Sternberg, L. d. S. L., 1989. Oxygen and hydrogen isotope measurements in plant cellulose. in: Linskens, H. F.,Jackson, J. F. (Eds.), Modern Methods in Plant Analyis. Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, pp. 89-98.

Sternberg, L. D. L., Swart, P. K., 1987. Utilization of freshwater and ocean water by costal plants of southern Florida. Ecology 68, 1898-905.

Sternberg, L. D. S. L., DeNiro, M. J., Savidge, R. A., 1986. Oxygen isotope exchange between metabolites and water during biochemical reactions leading to cellulose synthesis. Plant Physiology 82, 423-27.

Sternberg, L., DeNiro, M. J. D., 1983. Biogeochemical implications of the isotopic equilibrium fractionation factor between the oxygen atoms of acetone and water. Geochimica et Cosmochimica Acta 47, 2271-74.

Stoop, J. M. H., Williamson, J. D., Mason Pharr, D., 1996. Mannitol metabolism in plants: a method for coping with stress. Trends in Plant Science 1, 139-44.

Teh, S. Y., DeAngelis, D. L., Sternberg, L. D. L., Miralles-Wilhelm, F. R., Smith, T. J., Koh, H. L., 2008. A simulation model for projecting changes in salinity concentrations and species dominance in the coastal margin habitats of the Everglades. Ecological Modelling 213, 245-56.

Vendramini, P. F., Sternberg, L. S. L., 2007. A faster plant stem-water extraction method. Rapid Communications in Mass Spectrometry 21, 164-68.

Verheyden, A., Helle, G., Schleser, G. H., Dehairs, F., Beeckman, H., Koedam, N., 2004. Annual cyclicity in high-resolution stable carbon and oxygen isotope ratios in the wood of the mangrove tree *Rhizophora mucronata.* Plant, Cell and Environment 27, 1525-36.

Waterhouse, J. S., Switsur, V. R., Barker, A. C., Carter, A. H. C., Robertson, I., 2002. Oxygen and hydrogen isotope ratios in tree rings: how well do models predict observed values? Earth and Planetary Science Letters 201, 421-30.

Wershaw, R. L., Friedman, I., Heller, S. J., Frank, P. A., 1966. Hydrogen isotopic fractionation of water passing through trees. in: Hobson, G.D., Spears, GC. (Eds.), Advances in Organic Geochemistry. Pergamon Press, New York, pp. 55-67.

Wightman, M. J., Stewart, M. T., Vacher, H. L., 1990. Geophysical mapping and hydrogeologic analysis of fresh-water lenses at Big Pine Key, Florida. in: Krishna, J. H., Quiñones-Aponte, V., Gómez-Gómez, F.,Morris, G. (Eds.), Proceedings of international symposium on tropical hydrology and fourth Caribbean islands water resources congress., pp. 301-09.

Yakir, D., DeNiro, M. J., 1990. Oxygen and hydrogen isotope fractionation during cellulose metabolism in *Lemna gibba* L. Plant Physiology 93, 325-32.

Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D., Somero, G. N., 1982. Living with water stress: evolution of osmolyte systems. Science 217, 1214-22.

Zamski, E., Guo, W.-W., Yamamoto, Y. T., Pharr, D. M., Williamson, J. D., 2001. Analysis of celery (*Apium graveolens*) mannitol dehydrogenase (Mtd) promoter regulation in *Arabidopsis* suggests roles for MTD in key environmental and metabolic responses. Plant Molecular Biology 47, 621-31.

Zar, J. H., 1999. Biostatistical analysis. Prentice Hall, New Jersey, 673 pp.

Zhao, H., Xu, B., Yao, T., Wu, G., Lin, S., Gao, J., Wang, M., 2012. Deuterium excess record in a southern Tibetan ice core and its potential climatic implications. Climate Dynamics 38, 1791-803.

Zhifang, G., Loescher, W. H., 2003. Expression of a celery mannose 6-phosphate reductase in *Arabidopsis thaliana* enhances salt tolerance and induces biosynthesis of both mannitol and a glucosyl-mannitol dimer. Plant, Cell and Environment 26, 275-83.

Zhou, Y. (2005). Carbon, oxygen and hydrogen compositions of plant material: insights from controlled growth experiments and modelling, The Australian National University. **Doctoral:** 225.