$\delta^{13}C$ and $\delta^{18}O$ Trends Across Overstory Environments in Whole Foliage and Cellulose of Three *Pinus* Species

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Stable isotope ratios of carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) are increasingly used to investigate environmental influences on plant physiology. Cellulose is often isolated for isotopic studies, but some authors have questioned the value of this process. We studied trends in $\delta^{13}C$ and $\delta^{18}O$ of whole foliage and holocellulose from seedlings of three *Pinus* species across three overstory environments to evaluate the benefits of holocellulose extraction in the context of a traditional ecological experiment. Both tissue types showed increasing $\delta^{13}C$ from closed-canopy controls to thinned plots to 0.3 ha canopy gaps, and no change in $\delta^{18}O$ between overstory environments. $\delta^{13}C$ of *P. resinosa* and *P. strobus* was greater than $\delta^{13}C$ of *P. banksiana* in whole foliage and holocellulose samples, and there were no differences in $\delta^{18}O$ associated with species in either tissue type. Our results suggest whole foliage and holocellulose provide similar information about isotopic trends across broad environmental gradients and between species, but holocellulose may be better suited for studying differences in stable isotope composition between multiple species across several treatments. (J Am Soc Mass Spectrom 2008, 19, 1330–1335) © 2008 American Society for Mass Spectrometry

Stable isotopes of carbon and oxygen in plant tissues can reflect interactions between physiological processes and environmental conditions, providing a powerful tool for ecological studies. In C_3 plants, the carbon isotope ratio ($\delta^{13}C$) of plant tissues is proportional to the ratio of the partial pressure of CO_2 inside the leaf (c_i) to the partial pressure of CO_2 in the atmosphere (c_a) according to:

$$\delta^{13}C_p = \delta^{13}C_a - a - (b - a)(c_i/c_a) \quad (1)$$

where $\delta^{13}C_p$ is the carbon isotope ratio of plant tissue, $\delta^{13}C_a$ is the carbon isotope ratio of the atmosphere, a is the fractionation that occurs during diffusion across the stomata (4.4‰), and b is the fractionation associated with carboxylation by Rubisco (~27‰) [8]. Both $\delta^{13}C_a$ and c_a are relatively stable across a site under well-mixed conditions, where c_i is controlled by the balance between photosynthesis (A) and stomatal conductance (g_s). This results in a linear relationship between $\delta^{13}C_P$ and the ratio of A/g_s, also known as intrinsic water use efficiency [8, 9].

The oxygen isotope ratio ($\delta^{18}O$) of plant tissues is determined by the isotopic composition of source water, evaporative enrichment that occurs in leaf water during transpiration, and isotopic exchange at the site of tissue formation. Kinetic discrimination against $H_2{}^{18}O$ during transpiration leads to the relationship:

$$\delta^{18}O_{lw} = \delta^{18}O_{sw} + \varepsilon^* + \varepsilon_k + (\delta^{18}O_v - \delta^{18}O_{sw} - \varepsilon_k)(e_a/e_i) \quad (2)$$

where $\delta^{18}O_{lw}$ is the oxygen isotope ratio of leaf water, $\delta^{18}O_{sw}$ is the oxygen isotope ratio of source water, ε^* is the fractionation that occurs during the change from liquid water to vapor, ε_k is the kinetic fractionation that occurs as water diffuses across the stomata and the leaf's boundary layer, $\delta^{18}O_v$ is the oxygen isotope ratio of atmospheric water vapor, e_a is the atmospheric water vapor pressure, and e_i is the water vapor pressure in the leaf [7, 10]. In well-mixed conditions, the enrichment of atmospheric water vapor above source water ($\delta^{18}O_v - \delta_{18}O_{sw}$) is approximately equal to $-\varepsilon^*$, so leaf water enrichment ($\delta^{18}O_{lw} - \delta^{18}O_{sw}$) is proportional to $1 - e_a/e_i$ [2]. This suggests that leaf water enrichment should be negatively related to relative humidity and negatively related to g_s when relative humidity is similar among leaves. Leaf water is eventually incorporated into sucrose and then into plant tissues, so tissue $\delta^{18}O$ should reflect the same evaporative signals that are carried in $\delta^{18}O_{lw}$ as long as $\delta^{18}O_{sw}$ remains constant. Thus, plant tissue $\delta^{18}O$ should be inversely related to g_s when comparing plants growing in sites with similar $\delta^{18}O_{sw}$ and relative humidity [6, 11]. Since plant tissue $\delta^{13}C$ carries information about A/g_s, and plant tissue

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$\delta^{18}O$ carries information about g_s, the two can be used in tandem to reveal information about the relative rates of A and g_s [22, 23].

Several factors other than gas exchange may dampen the plant-environment signal carried by stable isotopes. Bulk tissues consist of varying proportions of organic compounds that have distinct isotopic signatures [21], and the evaporative enrichment of oxygen in leaf water is dampened by resistance to the backward diffusion of $H_2{}^{18}O$ that accumulates at the site of evaporation by the transpiration stream [10]. Oxygen exchange with unenriched stem water during tissue formation also influences the $\delta^{18}O$ signal of plant tissues [24]. A variety of other postphotosynthetic fractionation events have an impact on stable isotope signatures in plants, but a full discussion of these processes is beyond the scope of this paper. Recent reviews by Brandes et al. [3] and Barbour [2] discuss these issues in depth. Cellulose is immobile once formed and has a single, biosynthetic pathway [21] with limited potential for oxygen exchange [1] so the isotopic signature of cellulose should reflect environmental influences better than bulk tissue. Cellulose extraction, however, increases sample preparation time and different methods produce end products of variable purity [12], leading some authors to question the benefits of extracting cellulose [1, 14, 18]. Common extraction techniques do not produce pure cellulose, a glucan polymer. Instead, the chemical extractions used in isotopic analyses produce either holocellulose, which is composed of both pure cellulose and a mixture of polysaccharides known as hemicellulose, or they produce a substance known as α-cellulose, which is mostly free of hemicellulose, but still contains some impurities [13].

We evaluated relationships between $\delta^{13}C$ and $\delta^{18}O$ of whole foliage and foliar holocellulose in red pine (*Pinus resinosa*, Ait.) stands harvested using various patterns of overstory retention to produce different structural environments. Thinning can increase soil water availability [19] and partial cuttings increase light availability [20]. Greater soil moisture availability should increase g_s [19], and increased light availability should increase A, so different structural environments should promote differences in both $\delta^{13}C$ [19, 25] and $\delta^{18}O$. We tested the hypotheses that (1) isotope ratios from whole tissue are significant predictors of ratios from holocellulose, (2) the difference between whole tissue and holocellulose is constant across structural environments and species, and (3) treatment rankings associated with statistical analyses using whole tissue are the same as those from analyses using holocellulose.

Although α-cellulose has been the preferred medium for isotopic studies, recent comparisons of extraction techniques suggest holocellulose, extracted using a variation of the Jayme-Wise method [13, 16], is chemically similar to α-cellulose and likely suitable for many studies involving $\delta^{13}C$ and $\delta^{18}O$ data from plant tissues [5, 12]. The Jayme-Wise method also appears to remove lignin, which is isotopically distinct from cellulose, more completely than some other common extraction techniques believed to produce α-cellulose [12]. Thus, we have focused our attention on comparisons of whole leaf tissues and holocellulose produced using a variation of the Jayme-Wise method that was developed to allow batch processing of samples [13, 16].

Experimental

Foliar samples were collected from jack pine (*Pinus banksiana* Lambert), red pine, and eastern white pine (*P. strobus* L.) seedlings on the Chippewa National Forest in Minnesota, USA. The study sites were red pine stands treated with different patterns of overstory retention. One treatment removed all stems in 0.3 ha circles (gaps), the second involved thinning to a residual basal area of 16 m^2 ha^{-1}, and the third was an uncut control with a basal area of 36 m^2 ha^{-1}. Two-yr-old seedlings were planted in each stand during the spring of 2002 and 1-yr-old foliage was collected during the summer of 2005. Six seedlings of each species were sampled at separate plots in each treatment, but sample contamination reduced the number of red pine analyzed for $\delta^{13}C$ in the thinned treatment to five. Foliar samples were collected from five to six branches around the circumference of the crown, dried at 65 °C for 48 h, and pulverized with a ball mill. Approximately 100 mg of each sample was separated and holocellulose was isolated using the method reported by Leavitt and Danzer [16]. Samples were placed in bags made by folding 5 cm circles of glass fiber filter paper and tied closed with dental floss. The filter bags were placed in a Soxhlet extractor with a 2:1 mixture of toluene:ethanol and run for 18 h before being removed and dried. Next, the samples were placed back in the Soxhlet for another 18 h with 100% ethanol. Samples were removed from the Soxhlet, boiled for 6 h in deionized water, and transferred directly to an acidified sodium chlorite solution. This solution was heated to 70 °C and allowed to react overnight. On the following day, the acidified sodium chlorite solution was replaced and allowed to react for another 2 h. This process was repeated twice before allowing the reaction to continue overnight. Samples were then soaked in deionized water with a change of water at hourly intervals until the conductivity of the supernatant was under 0.5 μmhos/cm.

Following holocellulose extraction, 1.8 mg samples of whole foliage and holocellulose were analyzed for $\delta^{13}C$ at the Michigan Technological University Forest Ecology Stable Isotope Laboratory using a Costech Elemental Combustion System 4010 connected to a continuous flow isotope ratio mass spectrometer (Delta Plus, Thermo-Finnigan, Bremen, Germany). Samples were measured against a CO_2 reference gas calibrated using IAEA, LSVEC, and USGS standards (IAEA-CH-6, IAEA-CH-3, and USGS-24). The standard deviation of repeated measurements of a laboratory standard was less than ±0.25‰ for $\delta^{13}C$. $\delta^{18}O$ analysis was performed on 0.3 mg samples at the Washington State University

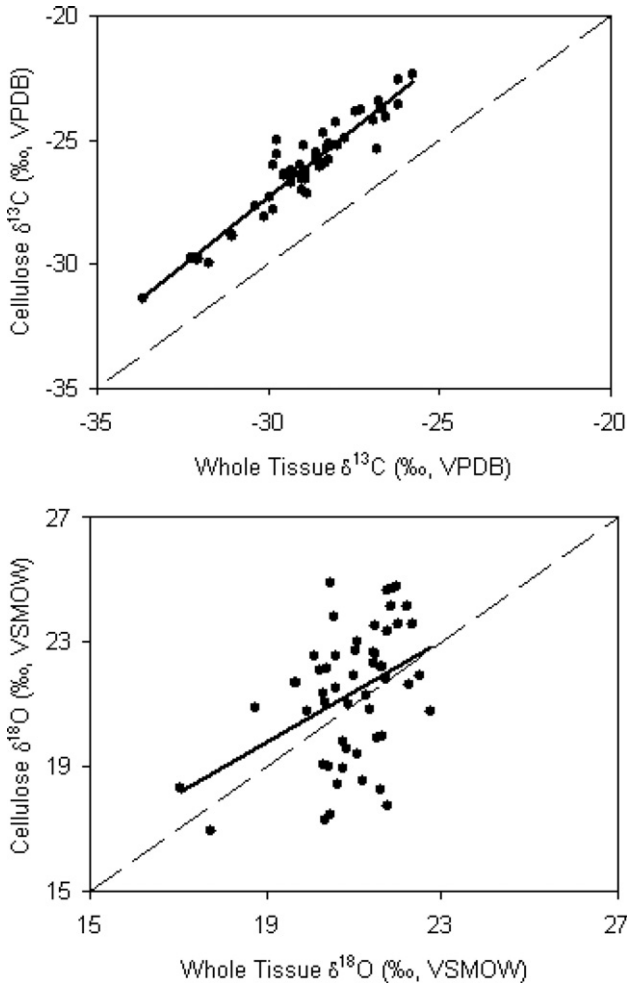

Figure 1. Correlations between whole foliage and holocellulose $\delta^{13}C$ and $\delta^{18}O$. Data points represent values from pine seedlings grown in different overstory environments, solid lines represent regression model predictions, and dashed lines represent a 1:1 relationship.

Stable Isotope Core Laboratory using a pyrolysis elemental analyzer (TC/EA, Thermo-Finnigan) connected to a continuous-flow isotope ratio mass spectrometer (Delta PlusXP, Thermo-Finnigan). Oxygen isotope data were normalized using IAEA standards (IAEA601 and IAEA602). The standard deviation of repeated measurements of a laboratory standard was less than ±0.4‰ for $\delta^{18}O$ analyses.

Data Analysis

Linear regression was used to evaluate the correlation between whole tissue and holocellulose with the isotopic signature of whole foliage as the independent variable, and the signature of holocellulose as the dependent variable. Paired *t*-tests and analysis of variance (ANOVA) were used to estimate the difference between whole foliage and holocellulose samples for $\delta^{13}C$ and $\delta^{18}O$. ANOVA was also used to evaluate whether relationships between the isotopic signatures of whole foliage and holocellulose varied across structural environments or species. Tukey's honestly significant difference test was used for pairwise comparisons. Separate analyses were carried out using the isotopic composition of whole tissue and holocellulose as dependent variables. All statistical tests were performed using SAS version 9.1 with $\alpha = 0.05$ (SAS Institute, Inc., Cary, NC, USA). The ROBUSTREG procedure was used to detect outliers, but the diagnostics did not suggest any evidence of outliers so all data subsequently reported represent our full data set.

Results and Discussion

The carbon isotope ratios of whole foliage and holocellulose were correlated ($P < 0.001$, $R^2 = 0.90$, Figure 1) and holocellulose was enriched on average by 2.8‰ compared to whole foliage ($P < 0.001$). This difference varied by species ($P = 0.004$) and the species-level differences varied across structural environments ($P = 0.015$). The difference between whole tissue $\delta^{13}C$ and holocellulose $\delta^{13}C$ was greater for white pine than for jack pine. The main effect of overstory structure was consistent across species, but within a given level of overstory structure the effect of species varied (Figure 2). The difference between whole foliage $\delta^{13}C$ and holocellulose $\delta^{13}C$ was similar for all species within the thinned and gap plots, but greater for red pine than for jack pine in control plots. Fluctuations in the whole tissue to cellulose offset have been attributed to the presence of secondary metabolites such as resins, lipids, and waxes in woody tissues [17, 21], and removal of extractives has been suggested as an alternative to

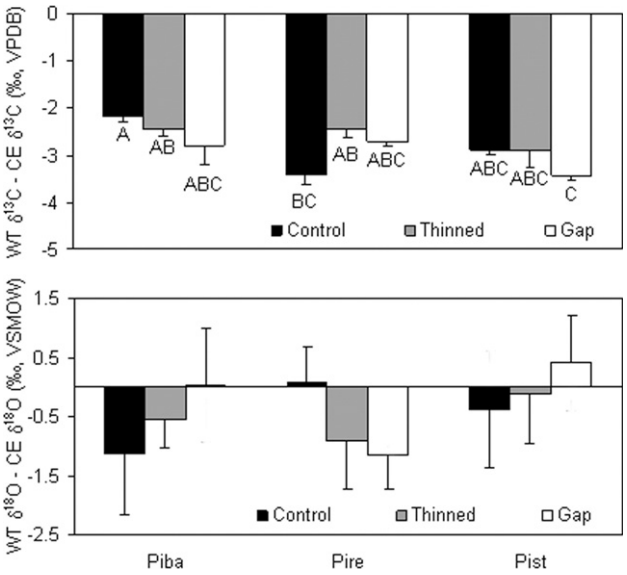

Figure 2. Differences between whole tissue (WT) and holocellulose (CE) from one year-old foliage of *Pinus banksiana* (Piba), *P. resinosa* (Pire), and *P. strobus* (Pist) seedlings grown in three structural environments. Different letters indicate significantly different means for $\delta^{13}C$, but there were no significant differences associated with $\delta^{18}O$. Error bars represent one standard error.

cellulose extraction [14]. Our results suggest whole foliar $\delta^{13}C$ may be sufficient for broad studies across large environmental gradients or between species, but some type of extraction may be necessary for studies comparing multiple species across environmental gradients.

Whole foliage $\delta^{13}C$ varied across treatments ($P < 0.001$, $R^2 = 0.68$) with increasingly enriched values from control to thinned to gap plots ($P < 0.001$) and more enriched foliage in red pine and white pine than in jack pine ($P < 0.001$, Figure 3). Holocellulose $\delta^{13}C$ also varied across treatments ($P < 0.001$, $R^2 = 0.75$) with the same pattern across structural environments and species as whole foliage $\delta^{13}C$ ($P < 0.001$ for each). These relationships were consistent in the control treatment, but jack pine and red pine holocellulose $\delta^{13}C$ were not significantly different in thinned and gap plots. Results from both tissue types indicate an inverse relationship between intrinsic water use efficiency and overstory competition, and greater intrinsic water use efficiency in red pine and white pine than in jack pine. The difference in $\delta^{13}C$ between control, thinned, and gap environments could be explained by greater A because of increased light availability [25].

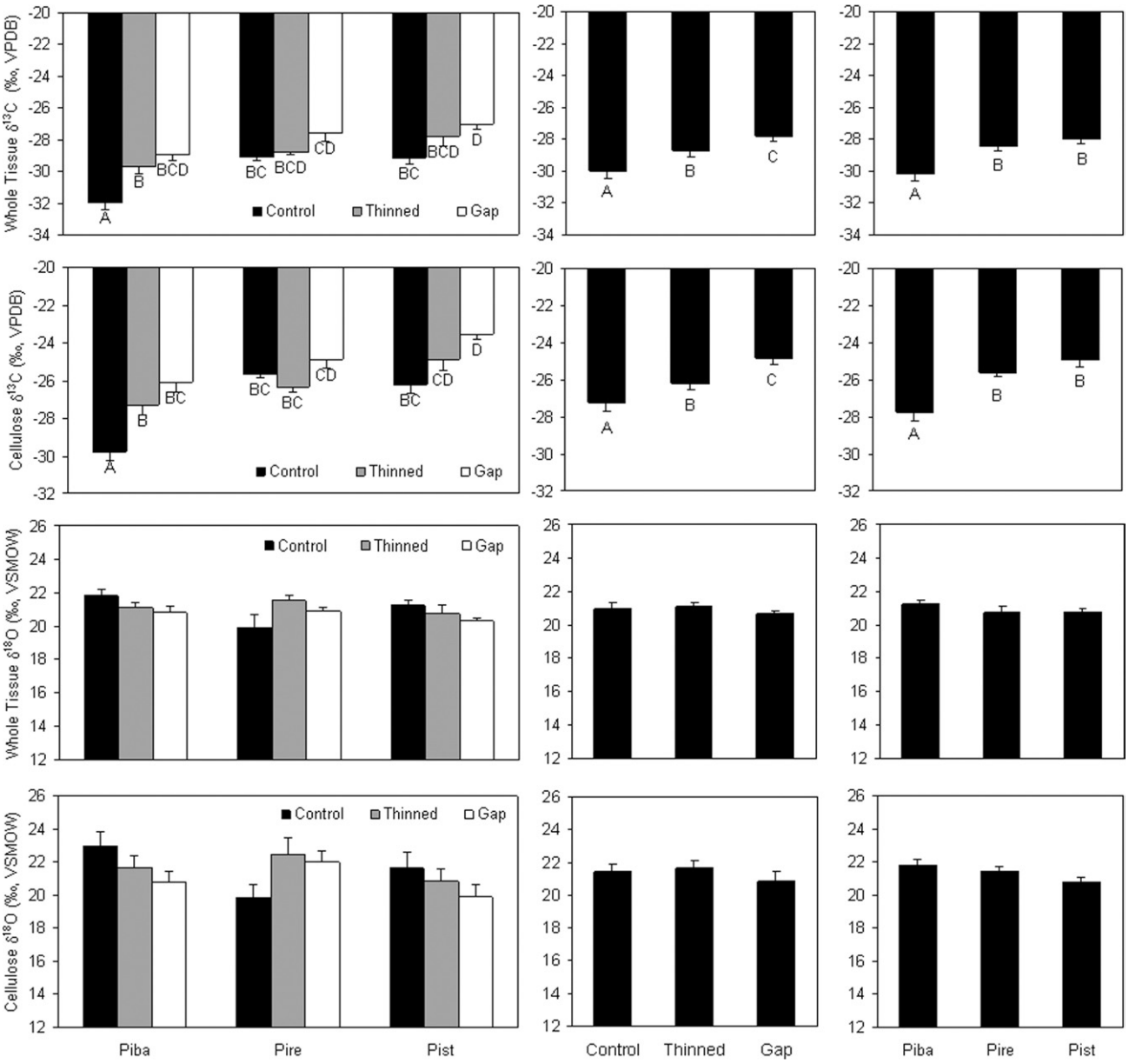

Figure 3. Mean $\delta^{13}C$ of whole tissue, $\delta^{13}C$ of holocellulose, $\delta^{18}O$ of whole tissue, and $\delta^{18}O$ of holocellulose from 1-yr-old foliage of *Pinus banksiana* (Piba), *P. resinosa* (Pire), and *P. strobus* (Pist) seedlings grown in three structural environments. Different letters indicate significantly different means associated with the species x structural environment interaction, structural environment, and species. Error bars represent one standard error.

Whole foliage averaged 49.1% C and 1.2% N, whereas holocellulose averaged 39.0% C and did not have measureable amounts of N. Pure cellulose is about 44.5% C and 0% N. These data suggest some impurities remained in our holocellulose, although the lack of N and low C content indicates the removal of N- and C-rich extractives and secondary metabolites was relatively complete. Our C content values are slightly below those reported for holocellulose in other studies [5, 12]. Unfortunately, we could not calculate yields because of partial sample loss during extraction. This was probably caused by milling our samples too finely, which can allow particles to pass through the filter paper bags used during holocellulose extraction [16]. This may represent a flaw in the modified Jayme-Wise method, since small particle sizes are needed for complete cellulose extraction and to sufficiently homogenize samples [4].

Whole foliage and holocellulose $\delta^{18}O$ were correlated ($P = 0.002$, $R^2 = 0.18$, Figure 1), but not as strongly as the relationship for $\delta^{13}C$. Holocellulose $\delta^{18}O$ was not significantly different from whole foliage and the difference between the two did not vary with overstory environment or species (Figure 2). Whole foliage $\delta^{18}O$ and holocellulose $\delta^{18}O$ were not significantly different across overstory environments or species, which supports the notion that differences in $\delta^{13}C$ were driven primarily by A rather than g_s, but there was a nonsignificant trend suggesting decreased enrichment from controls to thinned to gap treatments in jack pine and white pine (Figure 3). This indicates elevated g_s, consistent with expected increases in soil moisture under reduced canopy cover. Without source water and xylem water measurements we cannot determine whether the $\delta^{18}O$ trends in our data were caused by leaf-level processes or variations in the isotopic composition of source water used by different species or within different treatments, but these data were not collected. Nonetheless, our results suggest whole tissue and holocellulose provide similar $\delta^{18}O$ signals.

Conclusions

Parallel gas-exchange data and measurements of source water isotopic composition would considerably broaden the scope of this study, but our goal was to compare isotopic trends of whole foliage and holocellulose in an experimental setting and our data are sufficient for this purpose. Other studies have compared the relationship between whole tissues or cellulose and environmental variables [1, 4, 15] or the correlation between various tissue components [14, 15], but these studies focused on correlations with broad climatic parameters rather than trends across distinct treatments. Our results suggest isotopic approaches are applicable to studies evaluating the effects of disturbances that alter resource availability at smaller spatial scales. Analyses using whole foliage provided similar information as those using holocellulose across broad environmental differences or species, but holocellulose or α-cellulose analyses may be preferred for simultaneous comparisons across multiple species and environments.

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