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# Carbon isotope variation in shrub willow (*Salix* spp.) ring-wood as an indicator of long-term water status, growth and survival

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## ABSTRACT

Quantifying interannual change in water status of woody plants using stable carbon isotopes provides insight on long-term plant ecophysiology and potential success in variable environments, including under-utilized agricultural land for biomass production and highly disturbed sites for phytoremediation applications. We analyzed  $\delta^{13}\text{C}$  values in annual ring-wood of four shrub willow varieties used for biomass production and phytoremediation at three sites in central New York State (U.S.A). We tested a cost-effective sampling method for estimating whole-shrub water status by comparing  $\delta^{13}\text{C}$  values of the plant's largest stem against a composite sample of all stems. The largest stem showed 0.3‰  $^{13}\text{C}$  enrichment (range  $-0.7$ – $1.1$ ‰) compared to the whole-plant, making it a more sensitive indicator of water status than the composite sample. Growing season precipitation exerted a strong negative influence on wood tissue chemistry, with an average 0.26‰  $^{13}\text{C}$  depletion per 100 mm increase in precipitation. An average annual 0.28‰  $^{13}\text{C}$  enrichment was also observed with increased plant age; this pattern was consistent among all four willow varieties and across sites. Finally, increased  $^{13}\text{C}$  enrichment in wood tissue was positively associated with plant size at the individual plant level, and associated negatively and more variably survival at the plot scale. These results have important implications for the design and management of biomass production and phytoremediation systems. Increased sensitivity of older plants suggests that longer rotations may experience growth limitations and/or lower survival in low-precipitation years, resulting in reduced yields of biomass crops and loss of effectiveness in phytoremediation applications.

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## 1. Introduction

Plants demonstrate an array of adaptations to conditions that diminish productivity and vitality [1,2]. One of these adaptations includes tolerance to long-term water limitation [1–3].

This is particularly advantageous for plants that grow in arid regions; however, plants that grow in mesic regions have to endure water-limiting conditions during dry periods as well [4,5]. These can have negative effects on physiological functions, net productivity and, during prolonged droughts, plant

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survival. Water stress that limits growth can be an issue in biomass production systems especially since irrigation is not feasible in most situations [6,7]. Tradeoffs between productivity and stress tolerance, which are predicted by plant resource use theory [1,3,8], are highly relevant to biomass production and phytoremediation systems [9–11]. Thus, understanding relative differences in physiological drought adaptations among plants will improve the selection of appropriate species and genotypes in a given climate and set of site conditions [12–17].

The stable carbon isotope ratio ( $^{13}\text{C}/^{12}\text{C}$ , denoted  $\delta^{13}\text{C}$ ) in plant tissue is a sensitive long-term indicator of relative water status because drought-induced reductions in leaf gas exchange result in proportional enrichment of the heavier  $^{13}\text{C}$  isotope in assimilated plant tissue [18–21]. The stable carbon isotope ratio,  $\delta^{13}\text{C}$ , is measured in per mille (‰), which represents one part per thousand or one tenth of a percent. Since the differences in  $^{13}\text{C}$  and  $^{12}\text{C}$  are very small, per mille notation is used, and even though this is not an SI unit, this notation is commonly used [22]. Plant  $\delta^{13}\text{C}$  values are also proportional to ratios of carbon fixed per unit water transpired, defined as water use efficiency, and can indicate relative differences in physiological performance among plant species [18,23,24] and between habitats [25,26]. The carbon isotope ratio is a superior long-term index of plant water status to instantaneous measures (e.g., sap flow, leaf gas exchange, and xylem water potential) because the relative isotope concentration within the plant tissue integrates plant water status over a growing season for deciduous leaves, and over multiple years in annual ring-wood [27–30].

In response to water stress, many plants adapt by closing stomata in their leaves to reduce transpiration demand [20,31]. Though this is a short-term protective benefit for plants, prolonged stomatal closure will reduce plant growth because  $\text{CO}_2$  supply for carbon fixation will be limited [1,2]. As a result, the relationship between physiological water status (as indicated by  $\delta^{13}\text{C}$  values) and growth in plants is not straightforward and involves feedbacks at various spatio-temporal scales [32,33]. In systems with finite water supply, both natural and cultivated, large plants with large crown area require high water demand that can lead to water stress. In turn, the plants' adaptation of reducing stomatal conductance will limit photosynthesis, and ultimately growth [1,2]. Because of this negative feedback process, the expected relationship between growth and  $\delta^{13}\text{C}$  value can be positive or negative, depending on the ecosystem and the spatial and temporal scale analyzed [32].

In this study we examined the relationship between  $\delta^{13}\text{C}$  values in wood tissue and plant growth and survival in four varieties of shrub willow grown over a three-year harvest cycle at three sites in central New York State. Shrub willow, which are important in woody biomass production for bio-energy, phytoremediation, agroforestry, and restoration projects, are fast growing pioneer species that are tolerant of disturbed conditions and achieve high biomass production in a short period of time [9–11,15,16]. Our research goals were to evaluate several intrinsic and external influences on plant water status and to compare them among several varieties that show promise in the Northeastern U.S. biomass systems. Due to their multiple stems, shrubs present sampling

challenges [12,14,34,35], thus another research goal was to develop systematic methods of evaluating whole stools efficiently in a field setting. Specifically, our research objectives were to: (1) quantify variation in water use efficiency between the largest individual stem, a potentially-simple sampling unit, and the whole-shrub stool (i.e., all stems aggregated), with the null hypothesis of no difference; (2) evaluate influences of growing season precipitation and plant age on annual variation in isotope chemistry (as indicated by tree-ring  $\delta^{13}\text{C}$  values); and (3) analyze relationships between lifetime water use efficiency, plant aboveground productivity, and survival among willow varieties. A precipitation influence would presumably be indicated by a negative relationship between growing season precipitation and annual  $\delta^{13}\text{C}$  values [21,23,36]. The influence of plant age is more equivocal. However, it could be indicated by a positive relationship with  $\delta^{13}\text{C}$  indicating increased water use efficiency as the plant canopy expands and exerts more water demand [37]; or a negative one due to the increased potential to access soil water as root systems expand, and an increased ability to adjust below – and aboveground carbon allocation with age [38].

## 2. Methods

### 2.1. Willow varieties and test sites

The four varieties used in this study were: *S. dasyclados* 'SV1', *Salix sachalinensis* 'SX61', and *S. miyabeana* 'SX64', and *S. sachalinensis* 'S. miyabeana' 'Sherburne' (Clone ID: 9871-31). These varieties have been screened and selected for biomass production as part of the willow biomass project at the State University of New York College of Environmental Science and Forestry [11], and have proven to achieve high biomass production in previous yield trials [39]. Because all plants in the study were vegetatively propagated varieties, genetic differences in plant performance were controlled within varieties.

The three sites used in the study span a range of climate, fertility, and historical land uses in Central New York (Table 1; Fig. 1). The willows studied were planted in yield trials on agricultural sites in Belleville (43°47'23"N, 76°06'45"W) and Tully, NY (42°47'38"N, 76°06'59"W). The Belleville site is an established agricultural field on well drained Galway Silt Loam soil, and the Tully site lies on well drained Palmyra Gravelly Loam soil [40]. At a third site in Solvay, NY (43°4'27"N, 76°15'12"W), shrub willow were planted for both biomass production and as an alternative cover on industrial settling basins abandoned following the deposition of soda ash waste from 1944 to 1986 [41]. Because the unamended substrate at Solvay contained few nutrients and high concentrations of  $\text{CaCl}_2$ ,  $\text{CaO}$ ,  $\text{CaCO}_3$ , and  $\text{NaCl}$  [41,42], the fields were amended with biosolids from the local wastewater treatment plant in the late 1980s [12].

Biomass yield plots were established in Belleville and Tully in 2005 using unrooted dormant cuttings, coppiced after the first year, and regrown for three years prior to the first harvest in December 2008. At the Solvay site, the willows were planted as unrooted cuttings in the spring of 2004, were not coppiced

**Table 1 – Substrate characteristics and growing season climate of the three study sites. Fields with “NA” indicate unavailable information.**

| Site characteristics                                  | Sites            |                       |                                      |
|---|------------------|-----------------------|--------------------------------------|
|   | Belleville       | Tully                 | Solvay                               |
| Management application                                | Biomass          | Biomass               | Biomass & alternative landfill cover |
| Planting year   | 2005             | 2005                  | 2003                                 |
| Depth to restrictive feature (cm)                     | 100              | 50–80                 | 45 <sup>a</sup>                      |
| Depth to water table (cm)                             | 50–100           | >200                  | NA                                   |
| Soil series   | Galway silt loam | Palmyra gravelly loam | NA                                   |
| Drainage class <sup>b</sup>                           | MWD/WD           | WD                    | NA                                   |
| Local meteorological station                          | ART (NOAA)       | Tully valley (USGS)   | SYR (NOAA)                           |
| Year 1 growing season <sup>c</sup> precipitation (mm) | 603              | 782                   | 547                                  |
| Year 2 growing season precipitation (mm)              | 380              | 688                   | 604                                  |
| Year 3 growing season precipitation (mm)              | 709              | 489                   | 611                                  |

a Depth of soil mixing. Below 45 cm the substrate is composed of pure waste with a maximum pH of 11, making it restrictive to plant growth.

b Soil drainage class is abbreviated as MWD = Moderately Well Drained, WD = Well Drained (National Resource Conservation Service, USDA 2010).

c Growing season precipitation was totaled for the months of April through September. Data were obtained from NOAA and USGS.

but were harvested after three years of growth. Isotope testing occurred at the end of the second rotation in 2009 when the plants were three years old aboveground on a six year old root system.

Daily growing season precipitation data were obtained from NOAA and USGS operated meteorological stations located near the study sites (Table 1; Fig. 1). For each site and

year, daily values were averaged from April 1 through September 30 to calculate a growing season mean value.

## 2.2. Field sampling and isotope analysis

For each variety at each site, one live plant was harvested from each of three biomass yield blocks in the winter following the three-year growth rotation ( $N = 36$  plants total). At each location the trial was laid out as a randomized complete block. Each plot contained three double rows (1.5 m and 0.75 m distance between and within rows respectively) with 13 plants in each row (and 0.6 m between plants along the row). Plants sampled in this study were taken from a buffer zone around the measurement plot in the middle double row, with conspecific neighbors on each side and equivalent light and resource conditions to all plants within the field.

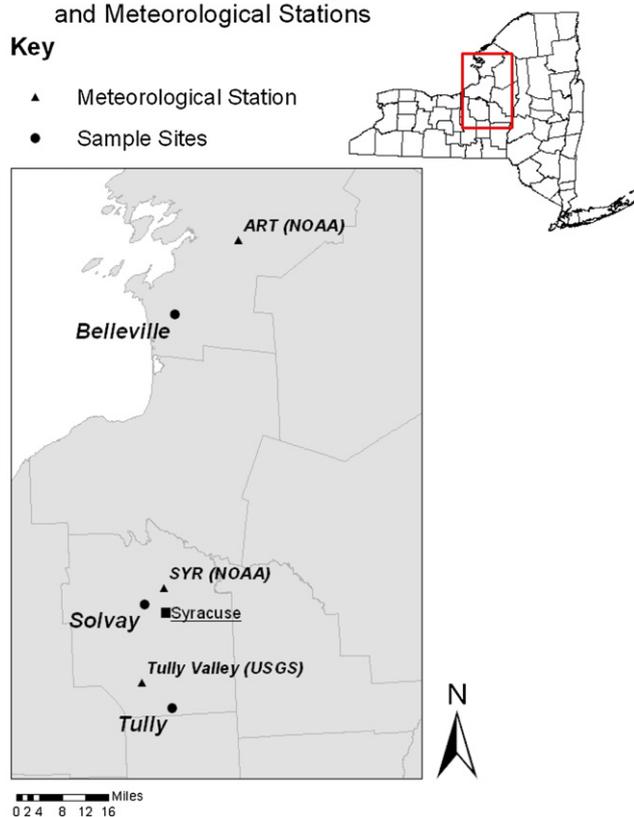
Sample plants were cut at 30 and 50 cm height above the soil surface, and this 20 cm section was stored until analysis at  $-20^{\circ}\text{C}$ . Samples were dried at  $60^{\circ}\text{C}$  for two weeks, after which the bark was removed. In order to evaluate our first objective (water use of the main stem versus the entire plant), we obtained two samples from each plant, one comprising the largest stem, and the other representing a composite of all stems including the largest stem (hereafter referred to as whole-plant). In both cases the sample represented a cross-section of uniform thickness through the stem(s); therefore, in the whole-plant sample each individual stem was represented proportional to its basal area. We obtained a second sample of the largest stem for analyzing  $\delta^{13}\text{C}$  values in individual years to evaluate factors affecting annual water use (second and third objectives). For these samples individual rings were isolated from the main stem and an equal amount of wood from each ring was processed separately.

Dried samples of stems were ground using a Wiley mill to pass through a 20 mesh screen. Stable isotope analysis was performed using a ThermoFinnigan Delta XL Plus Stable Isotope Ratio Mass Spectrometer (Thermo Scientific) coupled

### Locations of Willow Sampling Sites and Meteorological Stations

#### Key

- ▲ Meteorological Station
- Sample Sites



**Fig. 1 – Location map showing sampling sites and meteorological stations used in the study.**

with an elemental analyzer (Costech Analytical Technologies). The isotope ratio, expressed as  $\delta^{13}\text{C}$  is calculated

$$\delta^{13}\text{C}(\text{‰}) = \left[ \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right] * 1000$$

where  $R_{\text{sample}}$  is the ratio of  $^{13}\text{C}/^{12}\text{C}$  in the sample and  $R_{\text{standard}}$  is the ratio of  $^{13}\text{C}/^{12}\text{C}$  in the standard (Vienna Pee Dee Belemnite). Accuracy and precision were assessed by repeated measurements of laboratory standards, acetanilide ( $-29.3\text{‰} \pm 0.2$  [ $N = 144$ ]), and woody tissue ( $-26.8\text{‰} \pm 0.1$  [ $N = 41$ ]). Whole wood was analyzed rather than cellulose, because there is little evidence that cellulose extraction reduces the naturally occurring variability in carbon isotope ratios between trees [21].

### 2.3. Statistical analysis

Carbon isotope chemistry between the largest stem and whole-plant were compared ( $N = 36$ ) using a linear mixed-effects ANCOVA model with site as a random factor to account for potential spatial autocorrelation in the plants' response [43]. The response variable was the difference between the whole-plant  $\delta^{13}\text{C}$  and that of its largest stem; therefore the model's intercept term tested the null hypothesis that this difference is zero. We tested two fixed predictors in the model, the categorical factor willow variety and the ratio of the main stem basal area to the whole-plant basal area, which was a continuous factor. The latter variable tested whether the contributing biomass of the main stem was a significant predictor of the difference in  $\delta^{13}\text{C}$  values between the whole-plant and the largest stem, with the expectation that a larger biomass proportion would correspond to lower  $\delta^{13}\text{C}$  differences. We also used a linear mixed-effects model (again with site as the random factor) to test whether this ratio of main stem to whole-plant basal area differed among the four varieties ( $N = 36$ ).

We analyzed the effects of precipitation, plant age, and willow variety on the carbon isotope ratio using a linear mixed-effects model ( $N = 108$ ), with site as a random factor and plant nested within site to represent the study's hierarchy of factors [43]. Fixed factors tested were cumulative growing season precipitation (in mm) and plant age (in years) as continuous variables and willow variety as a categorical factor. In order to determine the best model, we compared model combinations between the three fixed factors, including all two-way interactions, all simpler models, and a null model with only an intercept term (18 models in all). Parameter estimation was by the maximum likelihood method and model simplification was performed by calculating Akaike Information Criteria (AIC) for the candidate model set [43,44]. The best model was selected as that with the lowest  $\text{AIC}_c$  value and highest Akaike weight ( $w_i$ ) among the candidate set, and alternate models were assessed using differences in these values.

A linear mixed model evaluated the influence of above-ground plant biomass on lifetime  $\delta^{13}\text{C}$  value (i.e., all years grouped together). In this analysis ( $N = 36$ ), total aboveground biomass (kg) for each plant was the only fixed factor, with variety nested among site comprising the random factors.

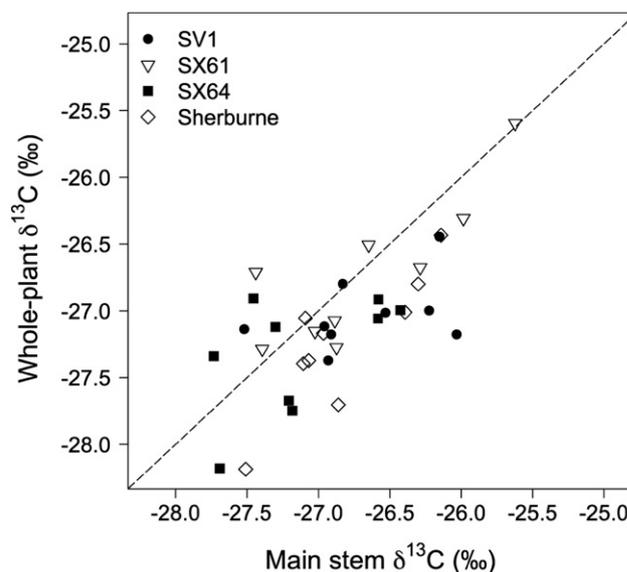
Plant biomass data were log-transformed to satisfy residual assumptions for the model.

The relationship between plant survival and isotope chemistry of the surviving stems was analyzed using a general linear model. In this case, willow variety and lifetime  $\delta^{13}\text{C}$  (all years) of the representative plant from each plot were predictor variables and plot-level survival was the response ( $N = 36$ ). Plot-level survival was calculated for the survival at the time of harvest, which for the Belleville and Tully plots included the first pre-coppice year, and for Solvay plots included pre-coppice year and the previous three-year rotation. Model residual structure was specified using the quasi-binomial distribution in order to account for the bounded response (plot survival proportion  $0 < y < 1$ ) and for overdispersion [44]. All statistical analyses for this study were performed using R version 2.8.1 [45].

## 3. Results

### 3.1. Comparison between the whole-plant and the largest stem $\delta^{13}\text{C}$ values

Across all individuals, the largest stem  $\delta^{13}\text{C}$  values were generally more enriched, averaging  $-26.8 \pm 0.1\text{‰}$  (mean  $\pm$  SE, this notation is used throughout), compared to values for the whole-plant, which averaged  $-27.1 \pm 0.1\text{‰}$  (Fig. 2). The mean difference in stem to whole-plant  $\delta^{13}\text{C}$  was  $0.3\text{‰}$  (range  $-0.7$  to  $1.1\text{‰}$ ) and was significantly different from zero ( $F_{1,29} = 10.09$ ,  $p = 0.004$ ). Physiologically, this indicates that water status was generally lower, and stomatal closure higher in the main stem compared to the plant as a whole. There was no overall difference among varieties in the degree to which the largest stem was more enriched in  $^{13}\text{C}$  compared to the whole-plant



**Fig. 2** – Relationship between largest stem and whole-plant  $\delta^{13}\text{C}$  values. The dotted 1:1 line indicates a null hypothesis of no difference; points that fall below the line represent stems that are more enriched in  $^{13}\text{C}$  than the whole-plant.

( $F_{3,29} = 1.63$ ,  $p = 0.20$ ). Though varieties were not different statistically, varieties Sherburne and SV1 tended toward greater enrichment in their main stems (i.e., less negative  $\delta^{13}\text{C}$  values), and SX61 main stems were somewhat more depleted than the whole-plant (Fig. 2).

As a proportion of the whole-plant, the main stem comprised 25–74% of the total basal area, with a mean of 46%. However, this variation in the proportion of biomass the main stem represented did not predict differences in  $\delta^{13}\text{C}$  values between the main stem and the whole-plant ( $F_{1,29} = 0.35$ ,  $p = 0.56$ ). Furthermore, this proportion of plant basal area represented by the main stem was not different among the four varieties ( $F_{1,30} = 0.16$ ,  $p = 0.92$ ) even though there were marked differences among varieties in overall stem numbers and morphology.

### 3.2. Precipitation and plant age effects on annual $\delta^{13}\text{C}$ values

When annual wood rings were considered individually, both site water supply, measured as cumulative growing season precipitation, and stem age were positively associated with  $\delta^{13}\text{C}$  enrichment, and thus with higher seasonal water use efficiency (Fig. 3). The best linear mixed-effects model had growing season precipitation and ring age as fixed factors with no interactions (Fig. 4), and the formula:

$$\delta^{13}\text{C} = -25.80 - 0.0026 * \text{precipitation} + 0.28 * \text{ring age}$$

This model had an Akaike weight of 0.44, indicating that it was 44% likely to be the best model given the candidate set of 18 models (Table 2). The two next-highest models had  $\Delta_i$  valued  $<2$  and were weighted at 0.22 and 0.20; these also contained the precipitation and age factors. The second-best model contained the variety variable, indicating marginal differences among the four varieties. These models demonstrate that, as expected, site growing season precipitation had a negative relationship with isotope ratio, with drier years resulting in greater  $^{13}\text{C}$  enrichment ( $t_{70} = -4.49$ ,  $p = <0.001$ ). The precipitation effect is more strongly expressed after the first year, indicating that older plants are proportionally more sensitive to variation in growing season precipitation ( $t_{70} = 3.70$ ,  $p = <0.001$ ; Fig. 4). Patterns of  $\delta^{13}\text{C}$  values did not

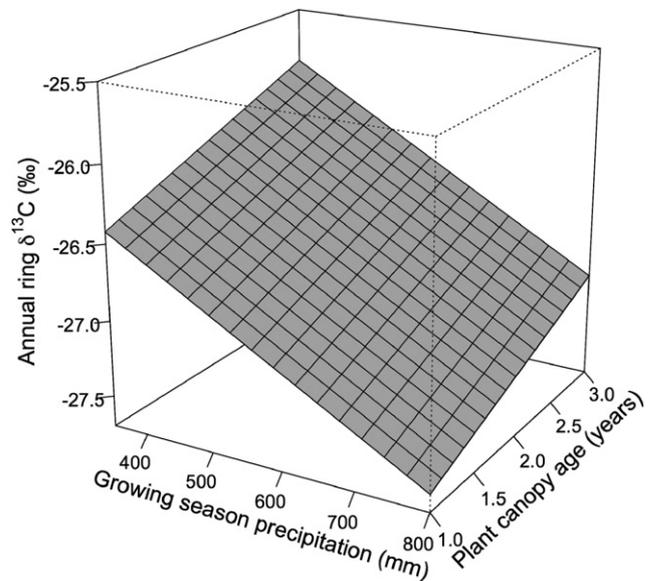


Fig. 4 – Response surface plot of the best linear mixed-effects model predicting tree-ring  $\delta^{13}\text{C}$  value based on growing season precipitation, and plant canopy age. The formula for this model is:  $\delta^{13}\text{C} = -25.80 - 0.0026 * \text{precipitation} + 0.28 * \text{stem age}$ . Wood becomes more isotopically enriched with plant age and in years with lower precipitation.

significantly vary among the four willow varieties tested. At all three sites, all varieties increased their levels of water use efficiency to a similar degree as growing season precipitation was less abundant and as plants aged (Figs. 3–5).

### 3.3. Relationships between $\delta^{13}\text{C}$ values, plant biomass, and survival

Total aboveground oven dry biomass of sample plants ranged from 0.8 to 10.8 kg with an average of  $2.8 \pm 0.3$  kg. The linear mixed model analysis of plant size indicates that across all sites and varieties, aboveground plant biomass at harvest had a significant positive relationship with whole-plant  $\delta^{13}\text{C}$

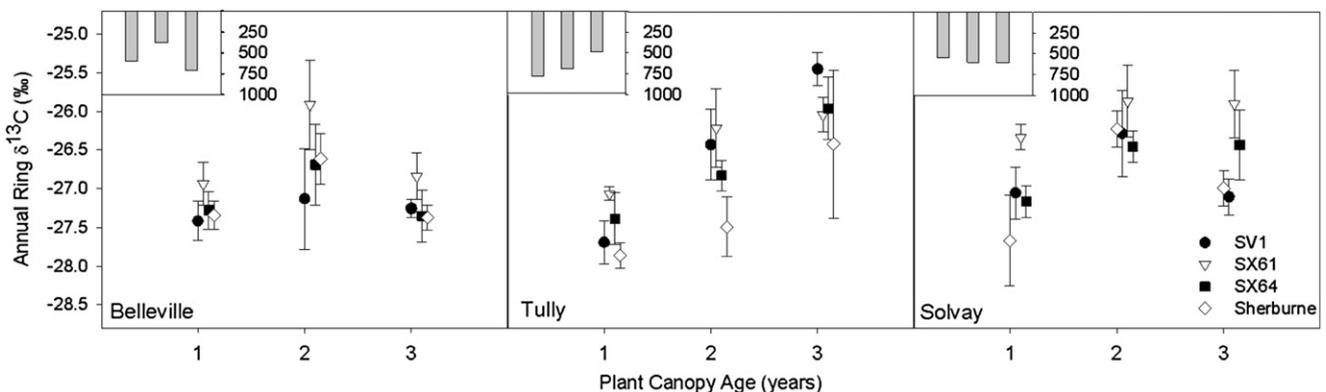


Fig. 3 – Annual tree-ring  $\delta^{13}\text{C}$  values (mean and 1SE across all willow varieties) of the largest stem for all three sites throughout the growing period. Inset bar graphs display corresponding growing season precipitation (mm) throughout the growing period, with scale extending downward.

**Table 2 – Summary of statistical models describing the effects of precipitation, plant age, and plant variety on  $\delta^{13}\text{C}$  values.**

| Model rank | K <sup>a</sup> | AIC    | AIC <sub>c</sub> <sup>b</sup> | $\Delta_i$ | $w_i$ | Factors                              |
|------------|----------------|--------|-------------------------------|------------|-------|--------------------------------------|
| 1          | 6              | 241.57 | 242.40                        | 0.00       | 0.44  | Precip + Age                         |
| 2          | 9              | 241.88 | 243.72                        | 1.32       | 0.22  | Precip + Age + Variety               |
| 3          | 7              | 242.82 | 243.94                        | 1.54       | 0.20  | Precip + Age + Precip: Age           |
| 4          | 10             | 243.14 | 245.41                        | 3.01       | 0.10  | Precip + Age + Variety + Precip: Age |
| 17         | 4              | 269.00 | 269.39                        | 26.99      | 0.00  | Null model (intercept only)          |

a K, number of model parameters, including intercept.

b AIC<sub>c</sub>, Akaike Information Criteria; see text for explanation of AIC difference values ( $\Delta_i$ ) and AIC weights ( $w_i$ ).

values ( $t_{23} = 3.24$ ,  $p < 0.01$ ; Fig. 5). This means that larger plants had greater  $^{13}\text{C}$  enrichment in ring-wood, and higher water use efficiency over their lifetime. Predicted values of this relationship were correlated with observed values  $r = 0.81$ .

Plot-level survival ranged from 0 to 100% and averaged  $82 \pm 4\%$  across all the sites and willow varieties (Fig. 6). The two agricultural sites had the greatest survival,  $95 \pm 2\%$  at Belleville and  $98 \pm 1\%$  at Tully. At Solvay, where mortality of variety SV1 was very high, plot survival averaged  $53 \pm 8\%$ . In general,  $\delta^{13}\text{C}$  values among the surviving plants were more enriched where plot mortality was highest (Fig. 6). Across all varieties,  $\delta^{13}\text{C}$  values more negative than  $-27.2\text{‰}$  (i.e., more depleted in  $^{13}\text{C}$ , suggesting little water limitation) were associated with  $>90\%$  survival over the three years. Survival was much more variable in plots whose live plant samples showed  $^{13}\text{C}$  enriched beyond this threshold (i.e., values more positive than  $-27.2\text{‰}$ ). This variation was represented in the general linear model predicting plot-level survival, in which the interaction between variety and  $\delta^{13}\text{C}$  value was significant ( $t = -2.31$ ,  $p = 0.03$ ), indicating that the physiological signs of water stress for indicator plants were variety-specific with regard to predicting levels of plant mortality across the plot. When varieties were considered individually, a predictive linear relationship was significant only for SX61 ( $t = -3.62$ ,

$p < 0.01$ ). The other varieties showed more variable survival with greater enrichment (Fig. 6), but predictive relationships were not significant.

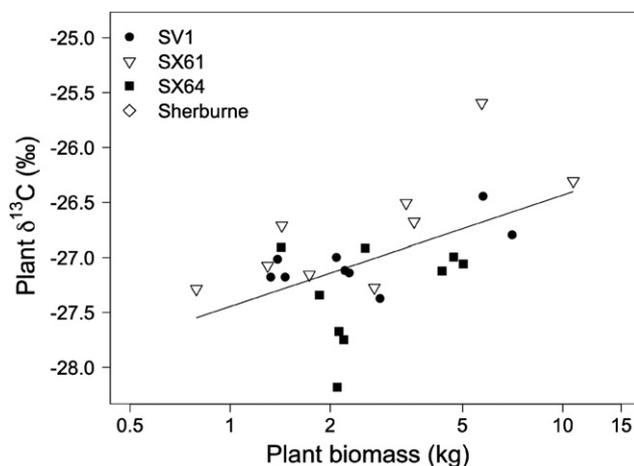
## 4. Discussion

This study revealed several patterns in carbon isotope composition of shrub willow wood, indicating that greater stomatal limitation occurred in the largest stems within plants (Fig. 2), larger plants within the population (Fig. 5), and all plants with increased age (Fig. 4). Annual patterns in  $\delta^{13}\text{C}$  values were influenced both by site water supply (i.e., growing season precipitation) and plant stem age; plants were generally insensitive to water supply during their first year, but showed  $^{13}\text{C}$  enrichment proportional to site precipitation supply in years 2 and 3. This pattern was consistent across sites and suggests that with longer time in rotation, the plants' greater water demand from the expanding canopy outstrips the higher supply provided by expanding root systems, with a resulting decrease in stomatal gas exchange.

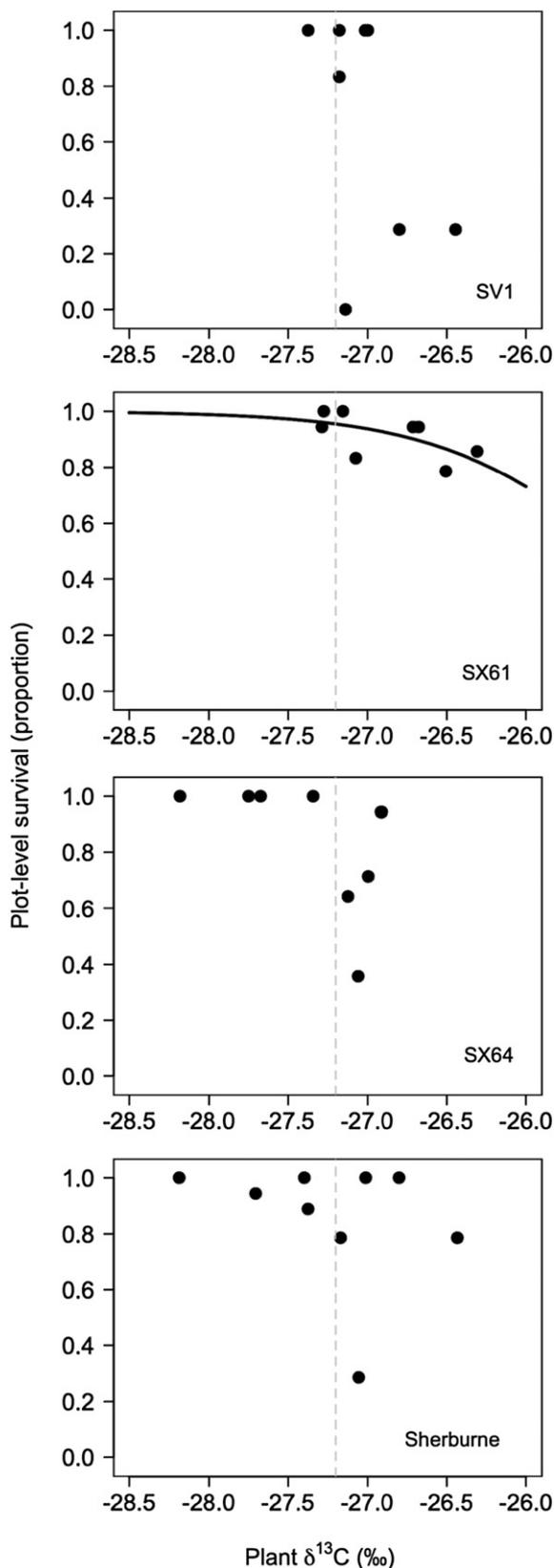
Our study also showed that  $^{13}\text{C}$  enrichment in wood tissue was positively associated with plant size at the individual level, and negatively associated with survival at the plot scale. Contrary to experimental drought studies that found large differences in average water use efficiency among young shrub willow varieties from a large climatic range [14,34], average values varied only marginally (Table 2) among the four varieties we tested in field conditions. However, the varieties with the largest range in interannual  $\delta^{13}\text{C}$  values (SX61, Sherburne) had consistently better survival, suggesting that some genotypes are better adapted to maintain high growth in conditions of temporary drought [46,47]. Overall, these results have important implications for the selection of willow genotypes for biomass production and phytoremediation systems.

### 4.1. Stem dominance and $\delta^{13}\text{C}$ values

The increased enrichment of the largest stem compared to the whole-plant was a general condition across varieties and plant sizes and, contrary to expectations, was not proportional to the prominence of the stem within the entire plant (Fig. 2). The enrichment difference was also independent of annual variation in precipitation and other factors because both, the largest stem and whole-plant samples, were collected as bulk cross-sections across all years in the stems. There are several likely drivers of the increased enrichment



**Fig. 5 – Relationship between whole-plant  $\delta^{13}\text{C}$  values and aboveground biomass (log scale). The solid line represents the best fit from a mixed-effects model with variety nested within site as random factors and biomass as the sole fixed effect ( $t = 3.24$ ,  $p < 0.01$ ). The correlation between observed and predicted values from the model is  $r = 0.81$ .**



**Fig. 6 – Relationship between plot-level survival and  $\delta^{13}\text{C}$  values of representative surviving plants from those plots. Plots with more isotopically enriched plants ( $> -27.2\text{‰}$ , indicated by points to the right of the dotted lines) had more variable survival across all varieties; however, only variety SX61 showed a significant predictive relationship**

pattern. Because the largest stem is also generally the tallest, it is exposed to more intense irradiation, which may cause higher leaf temperatures, vapor pressure deficits, and greater plant transpiration, compared to shorter stems, which may be shaded within the plant canopy [48]. Furthermore, because stem diameter is frequently proportional to crown volume [49], the largest stem likely has a greater leaf area. A combination of these factors can result in higher stress in the largest stem, as transpiration rates are driven by vapor pressure gradients (which are influenced by the irradiance regime) and leaf biomass [1,48,50,51].

Another mechanism that may contribute to variation in  $\delta^{13}\text{C}$  values between the largest stem and the whole-plant is that subdominant stems deep within the vegetation canopy may recycle respired  $\text{CO}_2$  that is already depleted in  $^{13}\text{C}$  relative to ambient air [52]. Numerous smaller stems within a shrub canopy that utilize depleted carbon will contribute to a whole-plant isotopic signal more depleted than a large one drawing on  $\text{CO}_2$  from above the canopy that is relatively more enriched in  $^{13}\text{C}$  [52].

Using the largest stem as an indicator of water use for the whole-plant has several advantages over preparing samples that include all stems from a shrub stool. First, the largest stem is somewhat more responsive to water stress (Fig. 2), particularly for the most vulnerable variety (SV1; Fig. 6), and may provide a more sensitive measure of plant response to site conditions. Secondly, this technique is also not as invasive as sampling every stem of a multi-stemmed plant. Finally, the single-stem protocol is cost-effective, particularly considering that some willow varieties average  $\geq 10$  stems per plant, and that bark removal (to avoid bias in isotopic composition [21,53]) and sample preparation is time-consuming.

#### 4.2. Influences on interannual variation in carbon isotope chemistry

The positive relationship of annual  $^{13}\text{C}$  enrichment with growing season precipitation was common across varieties and is consistent with studies conducted in arid systems that found interannual  $\delta^{13}\text{C}$  values in wood tissue negatively correlated with annual water supply [29,30,36,54]. At Belleville and Tully, sites with well drained and non-saline soils, precipitation was a strong predictor of interannual variation in  $\delta^{13}\text{C}$  values (Fig. 3), and suggested that seasonal soil moisture deficits were sufficient to influence stomatal function in older plants. During summers with very low-precipitation, the water table may be deeper due to drainage as well as depletion from high plant water use [55]; these conditions combined to severely restrict plant-available water. In contrast to the two agricultural sites, the Solvay plants' carbon isotope chemistry showed only greater enrichment with age, with no trend with annual precipitation (Fig. 3). Because the soil infiltration capacity at Solvay is poor and precipitation magnitude fairly high with little interannual variation, it is likely that substrate conditions had a greater effect on the plants' water balance

between  $\delta^{13}\text{C}$  and plot survival (solid line, predicted logistic regression model).

than meteorological supply. The amended soil depth at Solvay is 45 cm above the settling basin waste (pH 9–11), and water uptake by plants may be osmotically inhibited due to the high ion concentrations (Table 1) [12,41,56]. This may explain the generally greater  $^{13}\text{C}$  enrichment in ring-wood from Solvay plants compared to the two other sites and the lack of relationship with precipitation (Fig. 3) [12,18]. In addition, these plants were in their second rotation, and were generally larger than the first rotation plants at Tully and Belleville, which might also contribute to the water stress (Fig. 5).

Enrichment in  $\delta^{13}\text{C}$  values also occurred after the first growth year for all varieties, indicating that plants operate at higher levels of water use efficiency as the plants matured (Figs. 3 and 4). Though similar patterns have been shown among young trees, in which  $\delta^{13}\text{C}$  values increased typically 2‰ within the initial years [37], this phenomenon was not necessarily expected in short-rotation crops in mesic regions, particularly because stems grown from a coppiced stool will have a large root system, with presumably high potential to exploit groundwater relative to canopy water demand. In the case of the present study, results suggest that hydraulic conductivity decreased as the plant canopy size increased [48,57,58]. Plants at the Belleville and Tully sites did not typically reach peak leaf area index (LAI) until the second or third growing season [59], creating the potential for greater water demand (and therefore water stress) during the latter years of the rotation. In addition, plants may not have fully met canopy transpiration demand during the entire growing season because of periodic soil moisture deficits and/or limited conductivity in the stems. This condition would be especially prominent in species such as willow, which have high growth rates and copious water use [15,38,60], and similar observations have been made in other unirrigated biomass systems [6,16,47].

The positive association of  $\delta^{13}\text{C}$  values with whole-plant biomass (Fig. 5) supports this interpretation that water stress generally increases as plants age [37]. Other studies support this pattern, suggesting that individual variation in water demand (measured as basal area, crown volume, leaf area, or aboveground biomass) influences water use within similar resource environments. Vaganov and others [61] found positive correlations between annual  $\delta^{13}\text{C}$  values and radial width in conifers from alpine, temperate and boreal sites. Similarly, Bladon and colleagues [62] observed a trend of greater  $\delta^{13}\text{C}$  values in stout trembling aspen trees (*Populus tremuloides*) compared with slender individuals, which were likely due to increased crown size (i.e., a positive relationship between stem diameter and crown volume per basal area). These results are consistent with our results of greater  $^{13}\text{C}$  enrichment and water use efficiency in thicker stems and larger plants within our study sites. However, it is important to point out that the opposite relationship (i.e., greater  $^{13}\text{C}$  discrimination with increased growth) tends to occur in studies of annual radial growth in individual trees [56,63], or when comparing individual or stand-level growth across different sites [33,64]. Results from these studies are consistent with environmental control over carbon assimilation via water limitation at longer temporal and larger spatial scales. Similarly, manipulation experiments with willow varieties [14,34] and willow seedlings [24] that impose drought treatments

considerably more severe than experienced by willow in biomass field settings show that drought-treated plants produce less aboveground biomass than well-watered ones [14,34,46]. The present study is not analogous to these situations however, because unlike long-term tree studies [61], the young willow shrubs were still expanding their plant canopies and therefore have lower water demand early on, and unlike the manipulation experiments, the drought conditions were not severe enough to affect long-term growth in the plants that survived the 3-year rotation period.

Plant survival was consistently high in plots where indicator plants had the most  $^{13}\text{C}$  depleted tissue (Fig. 6), but survival varied greatly where associated plants showed tissue enrichment ( $>-27.2\text{‰}$ ). Water stress results not only in stomatal limitation of gas exchange, but also xylem cavitation in extreme cases and subsequent tissue desiccation. Therefore a negative relationship between plant  $\delta^{13}\text{C}$  values and plant survival is expected [65]. Variety SX61 had high survival associated with plants with enriched levels of  $^{13}\text{C}$ , and the best predictive relationship between mortality and  $\delta^{13}\text{C}$  values (Fig. 6b). These patterns suggest that this variety was more responsive with regard to stomatal limitation relative to the other willow varieties, and that this trait conferred better water status and ultimately higher plant survival. Though the other three varieties showed poorer adaptation to water deficit conditions, the lack of a significant relationship between survival and  $\delta^{13}\text{C}$  may be partly due to the indirect, but unavoidable, evaluation of plot-level survival through the physiology of plants that survived (i.e., it was not feasible to measure  $\delta^{13}\text{C}$  of dead plants because of isotopic changes that occur with tissue decomposition). Therefore, the bivariate analysis of survival and  $\delta^{13}\text{C}$  values (Fig. 6) is best considered as a comparison of relative physiological differences and a general indicator of relative success amongst varieties across a range of site conditions.

#### 4.3. Management implications

Our study of stem-wood in shrub willow biomass systems shows that the  $\delta^{13}\text{C}$  value is a useful indicator of water use and relative tolerance to temporary drought stress in mesic climates. Weih and Nordh concluded [33] similar findings for one-year old willow varieties grown in experimental drought conditions, as did Warren and others [66] in a thinning and fertilization study of plantation conifers. Our research results have several applications to biomass and phytoremediation systems [18], including the design of efficient sampling methods, selection of relatively drought-tolerant varieties, and considerations for minimizing biomass production losses in the event of longer and more frequent droughts projected with climate change [67].

In our study, we found that the largest stem of a shrub stool is more sensitive than the whole-plant to vulnerability from water stress. Thus, we suggest that sampling the single largest stem could serve as an efficient sampling method for analyzing plant water stress in shrub willows, and will result in more cost-effective monitoring. Furthermore, the co-occurring patterns of isotope chemistry and mortality suggest that relative range of  $\delta^{13}\text{C}$  values within a variety or species can be a useful screening tool for selecting successful

willow varieties, despite the fact that differences in average  $\delta^{13}\text{C}$  among the four varieties tested (Table 2) were marginal compared to studies that considered more varieties from a wider array of provenances grown under more extreme experimental drought stress conditions [e.g., 14, 34, 47]. Nevertheless, within a narrower range of varieties that are generally adapted to a Northeastern mesic climate [12], patterns of water use and survival provide useful indicators for plant breeding and selection. For example, variety SX61 had a greater  $\delta^{13}\text{C}$  range that correlated with better drought tolerance [24] and a more enriched average  $\delta^{13}\text{C}$  that correlated with higher shoot biomass [34]. In contrast, variety SV1 had both the narrowest range in  $\delta^{13}\text{C}$  values across all the trial plots (0.9‰ versus 1.3–1.8‰ for the other three varieties), the widest range in survival (0–100%), and the least success during establishment at the nutrient-poor Solvay site (T. Volk, unpublished data). The narrow range in  $\delta^{13}\text{C}$  suggests that this variety has the least plastic stomatal response to water limitation, and a consequently greater mortality risk one sites with greater potential for moisture stress. A similar pattern has been demonstrated in riparian willow and cottonwood seedlings [24]. In that soil moisture limitation study, leaf  $\delta^{13}\text{C}$  values shifted most among treatments (net change of +3.5‰) for *Salix gooddingii*, which was also the best-surviving of the three species tested [68]. Greater shifts in water use efficiency, as indicated by higher  $\delta^{13}\text{C}$  values, have also been observed for riparian *Populus* seedlings from dry-climate populations compared to wet-climate genotypes under experimental drought [69], and among willow varieties with high (i.e., enriched) average  $\delta^{13}\text{C}$  values that maintain high growth on dry sites [34]. Our results for shrub willow, together with these other studies imply that genotypes with higher stomatal sensitivity to water limitation can maintain higher internal water potential than less-plastic species, and that this confers important growth and survival advantages, particularly in the case of temporary water limitation [46]. Because irrigation will be too expensive in a low-value biomass system, the relative  $\delta^{13}\text{C}$  sensitivity of plant varieties provides a robust indicator of varieties that are better adapted to short-term water stress and to make use of them on relatively dry sites. Moisture limitations are often an issue at phytoremediation sites so information about the ability of different willow genotypes to tolerate drought is important in selecting plant material.

Across the sites included in this study, shrub willow varieties demonstrated a trend in  $\delta^{13}\text{C}$  values from  $-28.6\text{‰}$  to  $-24.6\text{‰}$  over the three-year rotation. Though growth and physiological function varied with site-specific factors such as annual precipitation and soil properties, the common pattern of enrichment with plant age and size have important implications for management of these biomass and phytoremediation systems. First year plants were insensitive to water supply, likely because of the limited transpiration demand from small, still expanding crowns. In later years, however, canopy demand outstripped water supply at well drained sites and in dry years, suggesting suboptimal productivity in unirrigated systems with high climate variability [6,38]. Because regional climate variability in the Northeastern U.S. is projected to increase with anthropogenic warming [67,70,71], negative impacts on unirrigated, high-

production systems such as the ones we studied may increase in the future. Though the plants in our study did not show evidence of growth limitation over a three-year rotation, the general enrichment pattern (+4‰ from first to third year) suggests that longer rotations under conditions of more frequent or extended droughts may result in growth limitation due to water stress (e.g., [46,72]) even in mesic regions such as Central New York.

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