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High-resolution Thermogravimetric Analysis For Rapid Characterization of Biomass Composition and Selection of Shrub Willow Varieties

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Abstract The cultivation of shrub willow (*Salix* spp.) bioenergy crops is being commercialized in North America, as it has been in Europe for many years. Considering the high genetic diversity and ease of hybridization, there is great potential for genetic improvement of shrub willow through traditional breeding. The State University of New York—College of Environmental Science and Forestry has an extensive breeding program for the genetic improvement of shrub willow for biomass production and for other environmental applications. Since 1998, breeding efforts have produced more than 200 families resulting in more than 5,000 progeny. The goal for this project was to utilize a rapid, low-cost method for the compositional analysis of willow biomass to aid in the selection of willow clones for improved conversion efficiency. A select group of willow clones was analyzed using high-resolution thermogravimetric analysis (HR-TGA), and significant differences in biomass composition were observed. Differences among and within families produced through controlled pollinations were observed, as well as differences by age at time of sampling. These results suggest that HR-TGA has a great promise as a tool for rapid biomass characterization.

Keywords Cellulose · Hemicellulose · Lignin · *Salix* · Wood composition

Introduction

Reliance on petroleum-based transportation fuels has raised national concern with respect to homeland security, energy independence, depletion of petroleum resources, and impact on

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the environment. The production of biofuels from dedicated energy crops and agricultural crop residues grown sustainably within the USA could help alleviate these problems. Currently, the vast majority of ethanol fuel produced in the USA is made from a single feedstock, corn grain, harvested from an annual crop. Achieving the goal of replacing 30% of the US petroleum consumption with biofuels and bioproducts by 2030 will require the use of perennial crops as well as the current annual crops [1]. As extraction techniques and conversion processes improve and become more cost effective, sustainable perennial woody crops, such as fast-growing willow shrubs, will become the preferred feedstocks. Shrub willow (*Salix* spp.), a high-yielding perennial crop with a short harvest cycle of only 3 to 4 years, is considered a suitable energy crop for much of North America [2, 3] and can be grown on underutilized agricultural land [3, 4]. There are multiple environmental benefits to growing shrub willow and excellent potential for genetic improvement through traditional breeding [5].

Researchers at the State University of New York College of Environmental Science and Forestry (SUNY-ESF) have developed a breeding program for the genetic improvement of shrub willow for increased biomass production [4]. There are more than 300 species of *Salix* worldwide with little domestication and high genetic diversity [6]. Since 1994, SUNY-ESF has collected and planted more than 750 accessions of shrub willow and established the largest willow-breeding program in North America [3, 4]. From these accessions, breeding efforts begun in 1998 have produced more than 5,000 progeny. Between 1998 and 2007, more than 200 families have been generated through controlled pollination. Crosses completed in 1998 and 1999 produced more than 2,000 individuals that have been screened in field trials for high biomass, form, and disease resistance [4, 7]. Selected groups of superior clones from crosses performed in 1998 and 1999 were planted in selection trials in 2001 and 2002, respectively. Growth improvements as high as 40% greater than a reference clone have been observed [4].

If shrub willow is to be used as a feedstock for the production of bioproducts or biofuels, the bioconversion process must become more efficient and cost effective. This can be partially achieved by selecting varieties with biomass composition that is better suited to the conversion process. Composition of the biomass is critical to the efficiency of processing and product yield, whether it is used to produce liquid fuels such as ethanol or polymers such as biodegradable plastics. Lignocellulosic biomass displays considerable recalcitrance to biochemical conversion because of the inaccessibility of its polymer components to enzymatic digestion and the release or production of fermentation inhibitors during pretreatment. If the ratio of hemicellulose, cellulose, and lignin in a woody biomass feedstock was optimized for the specific biochemical conversion method, then expensive and chemically harsh pretreatment methods could be reduced or avoided [8].

The development of a high-throughput process for the analysis of willow biomass will allow for selection of improved varieties with more favorable biomass composition in the willow breeding program. Traditional wet chemistry techniques for the analysis of biomass require strong acids and time-consuming processes resulting in a method whereby only 20 samples per week per person can be analyzed [9]. Current advancements in analytical methods include infrared spectroscopy (Fourier transform infrared [FT-IR] and near-infrared [NIR]) and pyrolysis molecular beam mass spectroscopy (pyMBMS) [10–13]. Multivariate analyses are often used in conjunction with these methods. To increase accuracy and improve throughput, development and further improvement of new analytical methods is required.

This project focuses on the development of high-resolution thermogravimetric analysis (HR-TGA) as a rapid, low-cost method for the analysis of biomass composition of shrub willow. The goal is to provide an alternative method for biomass analysis that is faster and

more cost effective than existing techniques with comparable or enhanced accuracy. This method can quantitatively resolve complex mixtures based on the characteristic thermal decomposition temperature of each component. It is well established that the pyrolytic decomposition of woody plant tissues in inert atmospheres occurs at the lowest temperature for hemicellulose (250–300 °C), followed by cellulose (300–350 °C) and lignin (300–500 °C) [14]. HR-TGA has already been applied to the analysis of lignocellulosic material and has shown to be useful in compositional analysis [15, 16]. Our work applies this method in analysis of willow varieties produced in the SUNY-ESF breeding program.

Materials and Methods

Source Material and Tissue Collection

Willow stem biomass samples were collected in January 2006 from two field trials growing at the Tully Genetics Field Station (Tully, NY; Table 1). Individuals sampled from the 2001 selection trial have clone IDs with the designation "98XX," where 98 indicates the year of the cross and XX the number of the family. Clones sampled from the 2002 selection trial were bred in 1999 and have IDs beginning with the designation "99." Samples from the reference clones SV1, SX61, SX64, and SX67 were collected from both selection trials. Samples were collected from three replicate plants for each of the 95 clones (Table 1) as follows: 15-cm sections including bark were cut from the base, middle, and top of one representative canopy stem. These stem sections were dried to a constant weight at 65 °C and then ground in a Wiley mill with a 20-mesh screen. The ground material from the three sections of each stem was pooled and homogenized. Each of the three replicates was analyzed in triplicate, for a total of nine analyses per clone. Samples from the 1999 families

Table 1 Families and reference clones in this study.

Family ID	Species	Number of progeny analyzed
9870	*S. sachalinensis*×*S. miyabeana*	4
9871	*S. sachalinensis*×*S. miyabeana*	4
98101	*S. dasyclados*×*S. miyabeana*	2
9882	*S. purpurea*×*S. purpurea*	4
9970	*S. sachalinensis*×*S. miyabeana*	13
9979	*S. purpurea*×*S. miyabeana*	1
9980	*S. purpurea*×*S. miyabeana*	1
99113	*S. purpurea*×*S. purpurea*	3
99201	*S. viminalis*×*S. miyabeana*	4
99202	*S. viminalis*×*S. miyabeana*	15
99207	*S. viminalis*×*S. miyabeana*	7
99208	*S. viminalis*×*S. miyabeana*	2
99217	*S. purpurea*×*S. miyabeana*	12
99227	*S. purpurea*×*S. purpurea*	2
99232	*S. purpurea*×*S. purpurea*	2
99239	*S. purpurea*×*S. purpurea*	15
SV1	*S. dasyclados*	–
SX61	*S. sachalinensis*	–
SX64	*S. miyabeana*	–
SX67	*S. miyabeana*	–

were collected after the third growing season after coppice, while samples from the 1998 individuals were collected one growing season after coppice. Samples of both ages were collected from the reference clones SV1, SX61, SX64, and SX67.

High-resolution Thermogravimetric Analysis

All willow samples were analyzed using a Thermogravimetric Analyzer 2950 (TA Instruments, New Castle, DE) with the TA Universal Analysis 2000 software. The method used for all samples was "high-resolution dynamic" with a heating rate of 20 °C min^{-1}, a final temperature of 600 °C, a resolution of 4.0, and a sensitivity value of 1.0. The electro-balance was purged with nitrogen at a flow rate of 44 L min^{-1}, and the furnace was purged with compressed air with a flow rate of 66 mL min^{-1}. For each analysis, 10 mg of dry tissue was used.

The percent dry weight for each stem biomass component (hemicellulose, cellulose, and lignin) was calculated by designating weight loss cutoff points on the generated thermogram (Fig. 1). The initial mass of the sample was corrected for water loss (change in weight from starting temperature to around 129 °C). Hemicellulose content was designated to be the weight loss between 245 and 290 °C, cellulose between 290 and 350 °C, and lignin between 350 and 525 °C. These cutoff points were identical for each sample, providing relative differences among the clones.

Statistical Analysis

All statistical analyses were performed using SAS® version 9.1.2 at a critical α=0.05. SAS PROC GLM and PROC NESTED were used to analyze all TGA data and to evaluate the

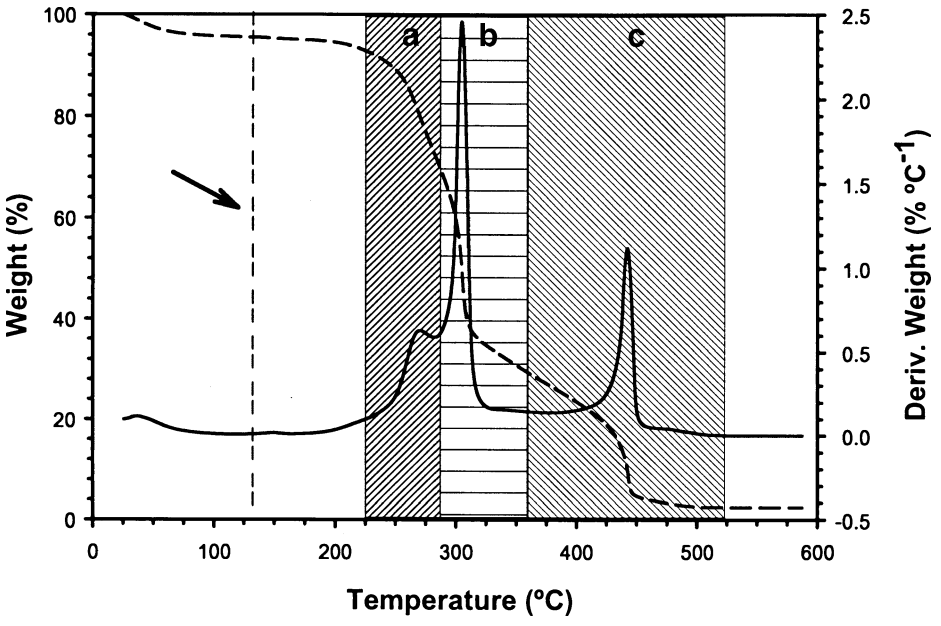

Fig. 1 TGA thermogram of biomass from reference willow clone *S. dasyclados* 'SV1.' *Arrow* indicates cutoff line for water loss correction (129 °C). *Block A*: weight loss representative of hemicellulose (245–290 °C). *Block B*: weight loss representative of cellulose (290–350 °C). *Block C*: weight loss representative of lignin (350–525 °C)

differences in biomass composition. When a significant interaction ($P<0.05$) was observed, Tukey's mean studentized range test was used to determine significant differences among clones. The variance components for the total data set, between and within clones, and within instrumental run were estimated with PROC NESTED. The multivariate analyses PROC CLUSTER and PROC CANDISC (discriminate analysis) were performed to identify groupings among specific clones.

Results and Discussion

As the breeding and domestication of crops to serve as feedstocks for biofuels and bioenergy is a very recent priority, there is urgent need to focus or refocus the aim of energy crop breeding programs to the optimization of biomass composition, while maintaining and improving high yield as the most critical trait. Characterizing and identifying differences in biomass composition among the varieties produced through conventional breeding demands techniques that are relatively fast, precise, and inexpensive. To refine the selection strategy of the willow breeding program with the aim of identifying varieties that have biomass composition that is well matched with the requirements of the intended downstream conversion technology, we have embarked on the development of HR-TGA as a rapid, low-cost method for analyzing and screening the biomass of hundreds or thousands of unique willow genotypes. Based on the initial results obtained in this study, HR-TGA may be an advantageous tool for the willow breeding program.

Utilizing this HR-TGA method, we were able to identify significant differences in the relative cellulose, hemicellulose, and lignin content among 95 willow clones. Statistical analysis provided variance components among clones, experimental replication, and instrumental replication. The total variation observed in the data set was relatively low, but more than 50% of the total variation was attributed to clonal variation. Instrument variation accounted for a maximum of 25% of total variation. The observed experimental and instrumental variation suggests that either more experimental replications or instrumental runs would help reduce variation, but the error is relatively small compared to the means; therefore, this is not a critical issue. This small error was generated using a remarkably small sample size of only 10 mg, which is indicative of the precision of the instrument. Small sample size, speed of analysis, and the ease of sample preparation for instrumental analysis are other advantages associated with this analytical method. Currently, one instrument can analyze 16 samples per day with a run time per sample of 90 min. As the instrument has an autosampler, it can process 16 samples before more samples need to be loaded. With further refinement of this analysis, the run time might be shortened. In addition, multiple instruments can be utilized to increase the daily throughput.

No discrete groupings or clusters were observed among the clones when plotted in a 3D graph (Fig. 2). Several multivariate analyses were performed, but all proved to be inconclusive and are not presented here. Most of the willow clones analyzed have similar biomass composition; however, there are several clones that have distinctively more or less cellulose, hemicellulose, or lignin (Fig. 2). This could be very important in future selection of willow varieties optimized for a particular application.

Among all clones analyzed, cellulose content ranged from 29 to 40%, hemicellulose content ranged from 23 to 30%, and lignin content ranged from 27 to 35% (data not shown). Individuals with the greatest relative amount of one component were significantly different from individuals with the least amount. The individual willow clones that were selected for analysis were purposefully chosen with an eye to their genetic diversity. In

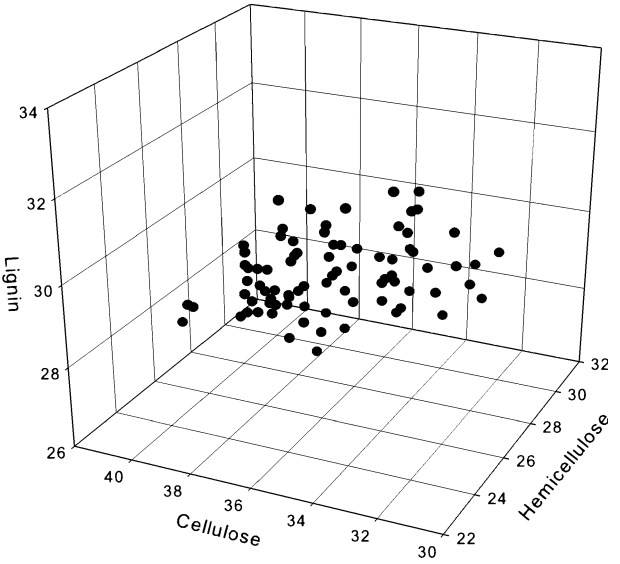

Fig. 2 3D plot of cellulose, hemicellulose, and lignin components for all the 1999 progeny and reference clones analyzed

building a breeding collection at SUNY-ESF, genetically diverse individuals were collected throughout the mid-western and northeastern USA, in addition to accessions from Japan, China, Ukraine, Sweden, and Canada. The range of cellulose, hemicellulose, and lignin content observed here may be an indication of the genetic diversity present in the various clones and will be very beneficial for future breeding efforts.

In the four largest families of the 1999 progeny, significant differences were observed in cellulose and hemicellulose content among siblings in each family (Table 1; Fig. 3; family 9970 data not shown). Significant differences in lignin composition were observed only in families 99217 and 99239 (Fig. 3). Families 9970, 99202, and 99217 are the result of interspecific hybridization, while family 99239 is the result of an intraspecific cross of *S. purpurea*. The siblings of the intraspecific cross displayed the greatest variability, compared with the siblings of the three interspecific hybrids. Kopp et al. [17] have shown that there can be great variability in seedling height growth among individuals produced from an intraspecfic cross of *S. eriocephala*. The variability among the progeny of intraspecific crosses is interesting in light of genetic studies of *Populus* spp. utilizing extensive amplified fragment length polymorphism analyses that have shown that interspecific variability is significantly greater than intraspecific variability [18, 19].

The willow biomass samples collected 1 year after coppice had significantly greater lignin content and lower cellulose content than the samples collected 3 years after coppice. The mean lignin content for the third-year samples was 29.5%, compared to a mean lignin content of 31.7% for the first-year samples, with the highest mean lignin content for a clone of more than 35% (data not shown). Samples were collected from the reference clones SV1, SX61, SX64, and SX67 after one season and three seasons postcoppice. The differences in composition based on stem age are shown in Fig. 4. Cellulose content was significantly lower in the 1-year-old growth compared to the 3-year-old growth. Inversely, lignin content was significantly higher in the younger growth. Hemicellulose appeared to be unaffected by the difference in years. Lignin content in bark is greater than that of wood [20, 21]; therefore, the greater lignin content in 1-year-old biomass may be due to greater bark content as a result of smaller stem diameters. Analyses with hybrid poplar clones have

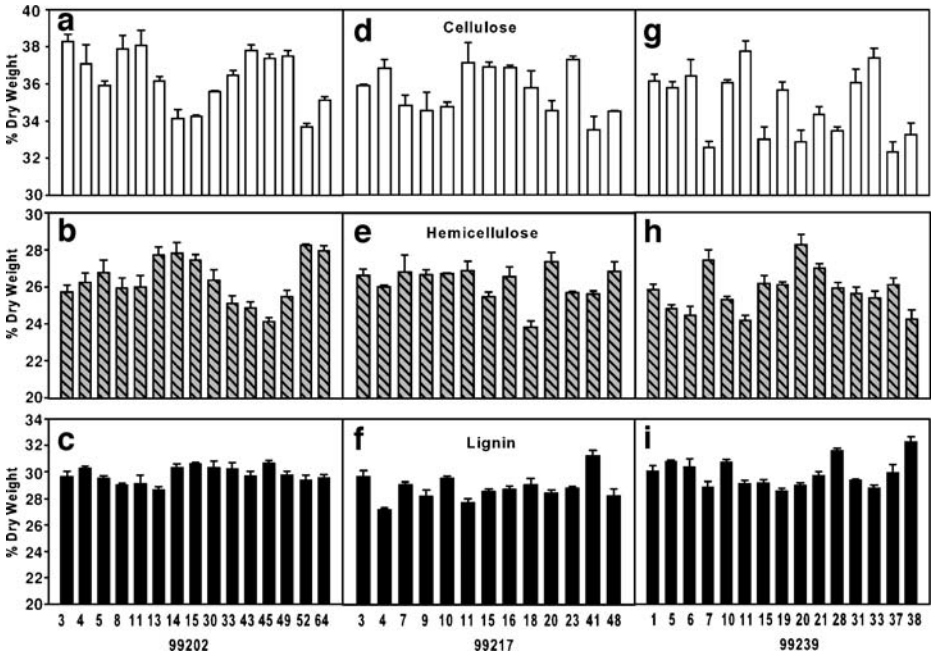

Fig. 3 Cellulose, hemicellulose, and lignin content of progeny in families 99202 (**a**–**c**), 99217 (**d**–**f**), and 99239 (**g**–**i**). *Bars* indicate mean±SE of three experimental replicates, each of which was analyzed using three instrumental replicates. *X*-Axis indicates the clone IDs for specific progeny individuals in each family

shown that lignin content of bark can be two times greater than that of the wood [20]. In 5-year-old stems from shrub willow stands in Sweden, bark represents approximately 19% of the total biomass. Small-diameter stems had a higher bark-to-wood ratio, and stems larger than 55 mm had a constant bark-to-wood ratio [22]. One-year-old twigs had bark content reaching 54% of the total biomass, compared to 18–27% for older stems [22]. Further analysis of bark content would be required to determine the impact of bark on the overall biomass composition of these clones.

The other analytical methods involving biomass composition that are currently in development (FT-IR, NIR, and pyMBMS) are able to resolve and quantify individual sugar composition. This is not possible with HR-TGA; however, in conjunction with ^{1}H nuclear magnetic resonance (NMR), sugar residues can be identified, and their abundance can be determined. Carbohydrate compositional profiles of lignocellulosic biomass can be accurately quantified based on the 600 MHz ^{1}H-NMR spectrum of unpurified acid hydrolyzates wherein the hemicellulose and cellulose fractions of biomass have been reduced to a mixture of sugars in acidic solution [23].

Conclusions

Preliminary HR-TGA analysis has shown that this technique can be used to identify compositional differences in shrub willow stem biomass among high-yielding clones selected in the breeding program at SUNY-ESF. To further refine this technique, a set of rigorously characterized reference biomass samples of shrub willow clones representing a

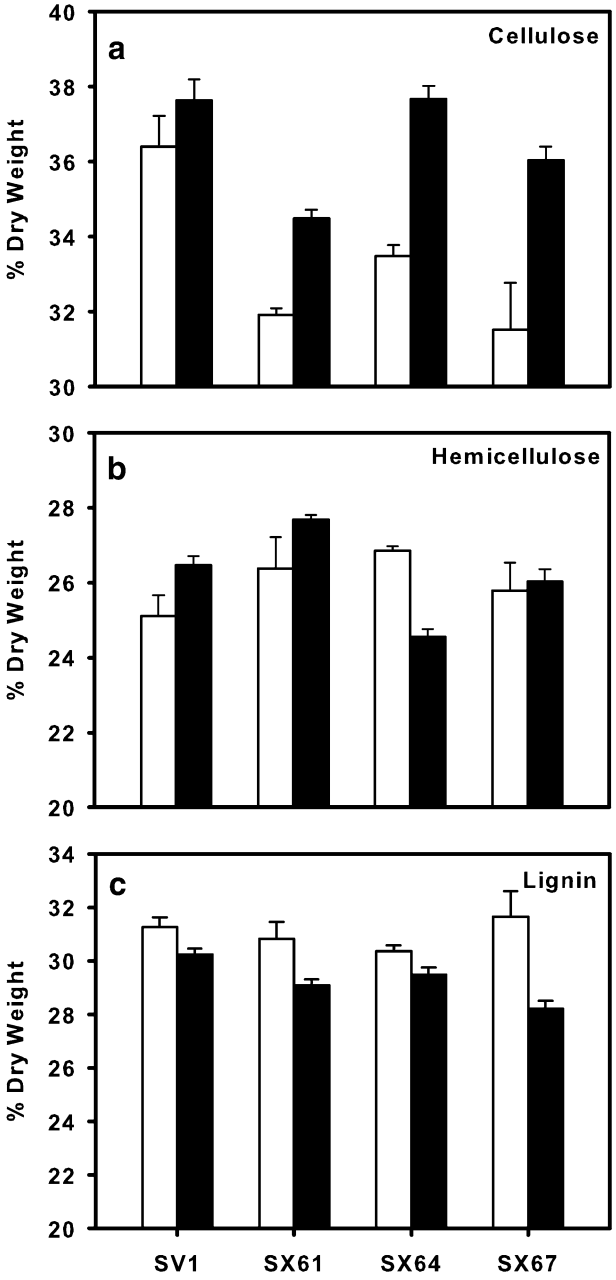

Fig. 4 Cellulose (**a**), hemicellulose (**b**), and lignin (**c**) content of different aged biomass samples from the reference clones. *White bars* represent 1 year growth after coppice; *black bars* represent 3-year growth after coppice. *Bars* indicate the mean±SE of three experimental replicates, each of which was analyzed using three instrumental replicates

range of varying compositions are being used to develop a neural network tool that will reliably and accurately interpret HR-TGA thermograms of unknown samples. HR-TGA in combination with ^{1}H-NMR can be a powerful, high-throughput tool used to identify unique compositional features in shrub willow and improve selection in the breeding program.

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