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Sunflower as a biofuels crop: An analysis of lignocellulosic chemical properties

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ABSTRACT

Four accessions of cultivated sunflower (*Helianthus annuus*) and silverleaf sunflower (*Helianthus argophyllus*), were each grown in three locations (Georgia, British Columbia, and Iowa) at different planting densities and phenotyped for biomass-related traits and wood biochemistry. In most environments, *H. argophyllus* produced significantly more biomass than *H. annuus*. Cell wall chemistry for a subset of plants grown in Georgia and Iowa was assessed using analytical wet chemistry methods to measure lignin and sugar content/composition. The analysis of lignin and the S/G-lignin ratios for a larger number of samples ($n > 250$) was also assessed by high-throughput pyrolysis Molecular Beam Mass Spectrometry. Average pyMBMS estimated lignin content (i.e., dry weight fraction) for 60 °C dried basal stem samples of *H. annuus* and *H. argophyllus* was 29.6% (range, 24.0%–34.6%) and 28.6% (range, 24.6%–33.3%), respectively when averaged across all environments. The average S/G lignin mass ratio was 1.5 (range, 1.0–2.0) for *H. annuus* and 1.7 (range, 1.0–2.4) in *H. argophyllus*. Stem samples from these two species only differed statistically for a few cell wall chemistry traits; however, accession level differences within each species were apparent. Cell wall chemistry in both species was significantly affected by both location and planting density, thus demonstrating the need to select for these traits in the environment for which the crop will be produced. Overall, these results show that cultivated sunflower and silverleaf sunflower both possess the necessary phenotypic diversity to facilitate the development of a hybrid sunflower with improved lignocellulosic biofuels traits, namely increased biomass, decreased lignin, and increased glucan.

Abbreviations: ANN, cultivated sunflower; ARG, silverleaf sunflower; GA, Georgia; IA, Iowa; BC, British Columbia; pyMBMS, pyrolysis Molecular Beam Mass Spectrometry; S-lignin, syringyl lignin; G-lignin, guaiacyl lignin.

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1. Introduction

The shift from starch and simple sugar based production of ethanol to lignocellulosic fuels is essential to protecting the world's food and feed supply while still enabling the bulk production of biofuels. Corn stover, grain straws, forestry waste, and purpose-grown lignocellulosic feedstocks (e.g., switchgrass) are vital to maintaining those supply chains; however, there is potential for other crops to play a significant role. Cultivated sunflower (*Helianthus annuus* L.) is a globally important oilseed crop with 24 million hectares harvested in 2010 [1]. In the US in the same year 750,000 ha were harvested with primary production areas in North Dakota, South Dakota, Kansas, and Colorado [2]. The annual yield of residual sunflower biomass after the seed is harvested is estimated to be 3–7 t ha^{-1} [3], which is roughly comparable to corn stover and wheat straw (i.e., 8.4 and 6.0 t ha^{-1}, respectively) [4]. Based on these estimates the annual amount of available sunflower biomass in the US is approximately 3.75 Mt.

Cultivated sunflower is highly adaptable and can be productive on lands with limited inputs [3,5,6]. Silverleaf sunflower (*Helianthus argophyllus* Torr. & Gray) is a closely related [7], drought resistant wild species [8] that produces larger, more solid stems and grows up to 4.5 m tall at higher latitudes in the US and Canada. *H. argophyllus* is interfertile with *H. annuus* [9,10] and could potentially supply the genetic diversity necessary for developing high biomass, woody stemmed cultivars of cultivated sunflower that could be used as a source of lignocellulosic biomass. To maximize economic feasibility, the resultant biomass should possess favorable characteristics such as lower lignin content [11,12] for lower recalcitrance to pretreatment, higher sugar content (especially glucose) for optimal sugar yield [13], and a high ratio of syringyl-lignin subunits to guaiacyl-lignin subunits (S/G-lignin ratio) as this variable has been associated with decreased recalcitrance to pretreatment [14]. The work described herein is aimed at characterizing the biomass properties of multiple accessions of *H. annuus* and *H. argophyllus* with the goal of identifying accessions with desirable chemical characteristics.

Despite its importance as a global oilseed crop, little is known about the chemical composition of sunflower biomass. While a recent study [15] described the composition of sunflower stalks, this work only focused on a single accession of sunflower and did not provide an assessment of genotype by environment effects (G × E) or the variation across the sunflower gene pool, much less in related species. This report is the first to our knowledge to assess variation in basal stem composition in both *H. annuus* and *H. argophyllus* grown in multiple locations at different planting densities.

In this study, we investigated differences in the cell wall chemistries and growth patterns of eight *Helianthus* accessions (i.e., four *H. annuus*, four *H. argophyllus*) as a first step in understanding the potential of sunflower as a source of lignocellulosic biomass for biofuels production. We conducted an in-depth analytical characterization (e.g., lignin and sugar content and composition) of a subset of plants in addition to a more comprehensive study of >250 plants using high-throughput pyrolysis Molecular Beam Mass Spectroscopy (pyMBMS) to assess lignin content and composition. The applicability of using high-throughput pyMBMS on these samples is demonstrated by the high correlation ($R^2 = 0.87$) found in this study between traditional Klason lignin results and the pyMBMS results. The findings from this study provide insight into the genetic and non-genetic factors affecting lignocellulose accumulation, plant cell wall formation, biomass yield, and other cellulosic biomass traits in sunflower.

2. Materials and methods

2.1. Plant material and planting design

In 2009, four accessions of *H. argophyllus* (derived from wild collected, open-pollinated populations) and four accessions of *H. annuus* (two elite inbred lines, one Native American landrace, and one wild accession) (Supplemental Table S1) were planted in GA (Plant Sciences Farm, Watkinsville, 33°52′20″N and 83°32′08″W), IA (North Central Regional Plant Introduction Station, Ames, 42°00′43″N and 93°39′32″W) and Vancouver, Canada (University of British Columbia Farm, 49°15′03″N and 123°14′20″W). Sites were chosen to represent a broad range of environments where sunflower could be produced. Accessions were selected to represent variation in flowering phenology, growth habit, and geographical origin within both species [16]. The wild accessions were pre-germinated following standard protocols to overcome seed dormancy issues and to maximize seedling establishment. These seedlings were subsequently transplanted into greenhouse trays and moved to the greenhouse for 2–3 weeks and then transplanted into the field. In IA and BC, seeds of the *H. annuus* inbred lines and the Native American landrace were germinated in the greenhouse in greenhouse trays and transplanted to the field after emergence, while in GA these accessions were sown by hand directly into the field.

In GA and IA, row plots were established with 3 different planting densities with 0.3, 0.9, and 1.5 m between plants within a row plot and 3.0 m between plots. Due to space constraints at the BC location, only the middle planting density of 0.9 m between plants was planted. Twenty plants of a single accession were planted in each row plot. The row plots were randomized within a block with two blocks planted per location. The row plots for the 0.3, 0.9, and 1.5 m planting densities were 6.1, 18.3, and 30.5 m in length, respectively. Plants were phenotyped and harvested for chemical analysis only if they appeared to be growing normally and at the prescribed planting densities; end row plants were not included (Supplemental Table S2). Roughly 75% of the plants at the GA location were severely damaged by insect feeding, so the sample sizes from this location were small (Supplemental Table S2) and were only analyzed using analytical wet chemistry techniques.

Plant height was measured at flowering (R5.1) [17]. The number of days to flowering was recorded from the field planting dates used in each location. Basal stem sections (i.e., 0.3 m in length) were manually harvested at maturity (R9)

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using handheld by-pass clippers or a serrated saw, oven dried for 3 d at 60 °C, and then stored at 25 °C in cardboard boxes. Basal stem diameter and basic specific gravity (60 °C oven dry weight/green volume) were recorded. Stems were shipped to the National Renewable Energy Lab (NREL) (Golden, CO) for milling to 20 mesh (0.841 mm) (Wiley mini-mill) and chemical and mass spectrometric analysis. Total plant biomass, main stem biomass (both 40 °C oven dry weight), and percent main stem (i.e., biomass of main stem/total biomass) were recorded at harvest (R9).

2.2. Compositional analysis – traditional wet chemistry

A subset of stems from the 0.3 m spaced row plots from GA and IA was analyzed using methods developed at NREL [18]. Specifically the "Determination of Structural Carbohydrates and Lignin in Biomass" [19], "Determination of Extractives in Biomass" [20], and "Preparation of Samples for Compositional Analysis" [21] methods were used. These methods determine glucose, xylose, galactose, arabinose, acetyl, and soluble lignin content, as well as water and ethanol soluble extractives including sucrose. Also determined were structural inorganics and non-structural inorganics using the "Determination of Ash in Biomass" procedure [43]. The mass fraction of the extractives varied among the accessions (3.4%–21.2%), therefore, extractives free materials (i.e., dry biomass excluding aqueous and ethanolic extractives) were reported unless otherwise stated. Technical replicates were run for each sample.

2.3. Sample preparation for high-throughput pyMBMS analyses

Approximately 200 mg of debarked, milled (20 mesh, 0.841 mm) biomass was wrapped in a teabag, secured with a tin coated copper wire and extracted with ethanol in a soxhlet for 24 h. Samples were then air dried before being transferred to antistatic plastic bags for storage at 25 °C. Stems from IA (8 accessions × 3 plant spacings × 2–5 plants × 2 blocks) and BC (8 accessions × 1 plant spacing × 2–5 plants × 2 blocks) (Supplemental Table S2) were analyzed via pyMBMS, as described below.

2.4. High-throughput pyrolysis Molecular Beam Mass Spectroscopy (pyMBMS) instrumentation

A commercially available (Extrel) Molecular Beam Mass Spectrometer (MBMS) designed specifically for biomass analysis was used for pyrolysis vapor analysis [22,23]. Approximately 4 mg of 20 mesh extracted biomass was introduced into the quartz pyrolysis reactor via 80 mm^3 deactivated stainless steel Eco-Cups provided with the autosampler. Technical replicates were run for each sample. Mass spectral data from *m/z* 30–450 were acquired on a Merlin Automation data system version 3.0 using electron impact ionization of 17 eV. Lignin estimates which were denoted by % DW (dry weight) were determined by summing the intensities of peaks assigned to lignin compounds listed in Supplemental Table S3 and mean normalized. Total lignin, S-lignin, and G-lignin contents were corrected using a sunflower control with a known Klason lignin content. The mass ratio of S/G-lignin was determined by summing the syringyl-lignin peaks (*m/z* 154, 167, 168, 182, 194, 208, and 210) and dividing by the sum of guaiacyl-lignin peaks (*m/z* 124, 137, 138, 150, 164, and 178). S-lignin and G-lignin amounts consisted of the summations of known S- and G-lignin peaks listed in Supplemental Table S3. Lignin peaks *m/z* 152 and 180 were omitted in the syringyl and guaiacyl summations due to these individual peaks having associations with both S- and G-lignin subunits [22].

2.5. Statistical analysis

Analysis of variance for the subset of samples from IA and GA that were analyzed using traditional wet chemistry methods was performed using PROC MIXED (SAS 9.2, Raleigh, NC) by location with the main effect for species (i.e., ANN vs. ARG) treated as a fixed effect. Plant growth and pyMBMS data from IA and BC from all plant spacings were initially analyzed together using PROC GLM (SAS 9.2) with the main effect for species (i.e., *H. annuus* vs. *H. argophyllus*) treated as fixed and all other factors (i.e., accession, location, plant number, and planting density) treated as random using the appropriate RANDOM statements to select the correct error terms. Significant accession within species and accession by environment interactions (G × E) for location and planting density were present for most traits; therefore, tests of simple effect contrasts were conducted using the LSMEANS/SLICE option with mean separation based on the Tukey–Kramer adjustment for unbalanced data. For this analysis, location and spacing effects were considered fixed. Mean separation of the eight accessions regardless of species and mean separation of the four accessions within each species was conducted using PROC MIXED using the LSMEANS/DIFF option with the Tukey–Kramer adjustment. PyMBMS spectral data with Student residuals greater than 3 ($n < 10$) were treated as outliers and removed from analysis. Spearman's rank correlation coefficients (r) were determined using PROC CORR. For all statistical analyses, effects were declared significant at the 0.05 probability level.

3. Results and discussion

3.1. Plant growth

In Iowa and BC, at all planting densities, *H. argophyllus* flowered later than *H. annuus* (IA all p-values < 0.0001, BC p-value $= 0.0285$) (Supplemental Table S4) and were 1.5–4.5 times larger (Supplemental Table S4) for plant height, stem diameter, main stem biomass, and total biomass (all p-values < 0.0001).

Plant growth of the *H. annuus* accessions was unaffected by planting density (Supplemental Table S5) except for % main stem, which increased from 14% to 20% as the planting density increased from 1.5 m between plants to 0.3 m between plants (p-value $= 0.0021$). In contrast, plant growth in *H. argophyllus* was significantly affected by planting density. Specifically, % main stem increased from 8% to 11% to 16% as the planting density increased (p-values $= 0.0003$ and 0.0027, respectively) and plant height increased from 1.9 m to 2.3 m to 2.6 m (p-values < 0.0001 and 0.0258, respectively). Total

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Table 1 – Plant growth and days to flower of *Helianthus argophyllus* (ARG) and *Helianthus annuus* (ANN) accessions grown in IA and BC.

Location	Species	Accession	Height (cm)			Days to flower			Stem diameter (mm)			Main stem biomass (g)			Total biomass (g)			% Main stem		
IA[a]	ARG	ARG1575	106.8 ± 6.4[c]	E[d]	D[e]	93.5 ± 1.7	BC	B	43.9 ± 1.9	B	B	60.7 ± 17.2	D	D	1188.3 ± 162.4	B	B	6% ± 1%	D	B
		ARG1805	148.6 ± 6.3	D	C	98.3 ± 1.6	B	B	44.9 ± 1.9	B	B	139.1 ± 16.8	C	C	2034.6 ± 158.7	A	A	9% ± 1%	D	B
		ARG1820	348.6 ± 5.3	A	A	154.3 ± 1.4	A	A	53.7 ± 1.5	A	A	359.8 ± 13.7	A	A	2496.4 ± 129.5	A	A	16% ± 1%	B	A
		ARG1834	310.2 ± 8.0	B	B	154.6 ± 2.1	A	A	51.5 ± 1.9	AB	AB	289.4 ± 16.8	B	B	1964.7 ± 158.7	A	A	16% ± 1%	BC	A
	ANN	RHA373	94.0 ± 5.0	E	B	77.5 ± 1.3	D	C	19.1 ± 1.5	D	C	15.3 ± 13.7	D	B	120.8 ± 129.5	C	B	13% ± 1%	C	B
		HA412-HO	70.4 ± 5.1	F	C	68.7 ± 1.3	E	D	30.1 ± 1.5	C	B	27.1 ± 14.6	D	B	103.2 ± 138.5	C	B	24% ± 1%	A	A
		Hopi	184.8 ± 5.2	C	A	96.6 ± 1.4	B	A	45.8 ± 1.5	B	A	181.7 ± 13.9	C	A	864.1 ± 139.5	B	A	23% ± 1%	A	A
		ANN1238	77.8 ± 5.6	EF	BC	86.9 ± 1.5	C	B	23.7 ± 1.7	CD	BC	20.2 ± 15.0	D	B	595.9 ± 141.9	BC	AB	5% ± 1%	D	C
BC[b]	ARG	ARG1575	94.1 ± 4.8	D	C	58.5 ± 1.9	D	B	56.0 ± 2.9	A	A	95.1 ± 22.2	BC	B	807.2 ± 119.5	B	B	14% ± 3%	AB	AB
		ARG1805	130.8 ± 5.4	CD	C	66.7 ± 2.1	CD	B	43.3 ± 3.3	BCDE	B	132.4 ± 25.3	BC	B	1260.6 ± 136.6	AB	AB	11% ± 3%	B	B
		ARG1820	425.8 ± 7.4	A	A	128.8 ± 3.9	A	A	53.0 ± 4.4	AB	AB	399.3 ± 34.5	A	A	2185.3 ± 185.9	A	A	21% ± 4%	A	A
		ARG1834	296.9 ± 7.4	B	B	ND			49.9 ± 4.4	ABC	AB	197.8 ± 30.3	B	B	878.2 ± 163.1	AB	AB	25% ± 4%	AB	AB
	ANN	RHA373	103.4 ± 4.6	D	B	68.1 ± 1.8	C	B	19.7 ± 2.8	E	B	20.4 ± 34.5	C	B	313.9 ± 185.9	B	B	8% ± 4%	AB	B
		HA412-HO	98.2 ± 5.2	D	B	73.3 ± 2.0	C	B	24.7 ± 3.1	DE	AB	ND			ND			ND		
		Hopi	176.3 ± 5.4	C	A	106.3 ± 2.1	B	A	34.6 ± 3.3	CD	A	209.9 ± 25.3	B	A	934.9 ± 136.6	B	A	23% ± 3%	A	A
		ANN1238	78.6 ± 9.9	D	B	63.2 ± 3.9	CD	B	34.5 ± 6.0	BCDE	AB	41.3 ± 46.6	BC	AB	574.8 ± 251.1	B	AB	7% ± 6%	AB	B

ANN, cultivated sunflower; ARG, silverleaf sunflower; ND, not determined.

a Data averaged for 3 planting densities (i.e., 0.3, 0.9, and 1.5 m spaced row plots).

b Data from 0.9 m spaced row plots.

c LSMEAN ± SE.

d Mean separation across all 8 accessions within each location followed by different letters are significantly different (p-value < 0.05) LSMEANS, PDIFF, Tukey–Kramer.

e Means within a species within a location followed by different letters are significantly different (p-value < 0.05) LSMEANS, PDIFF, Tukey–Kramer.

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biomass and stem diameter in *H. argophyllus* both decreased by 37% and 19% (p-values < 0.0001 and 0.0011, respectively) as the planting density increased from 1.5 m between plants to 0.3 m between plants, suggesting that inputs (e.g., space, water, nutrients, and light) were limiting at the highest planting density for this species.

Interestingly, the actual amount of main stem biomass of the accessions of *H. annuus* did not differ significantly at the different planting densities (p-value = 0.5647) despite the fact that the amount of total biomass decreased (p-value = 0.0379) as planting density increased. This suggests that the amount of main stem biomass produced by *H. annuus* isn't necessarily constrained by the amount of peripheral biomass (e.g., secondary branches, leaves, etc.) also produced per plant. Therefore, it may be possible to further maximize the yield of main stem biomass per hectare in sunflower beyond what is estimated here by further increasing planting density to meet, or possibly exceed, the commercially recommended planting density currently utilized for sunflower. Maximizing main stem biomass per plant is an important goal as it will likely be the primary biomass harvested for this species for use as a lignocellulosic feedstock. For *H. argophyllus*, the amount of main stem biomass was also statistically similar at the different planting densities tested (i.e., 0.3 m vs. 0.9 m, 0.3 m vs. 1.5 m, and 0.9 m vs. 1.5 m) (p-values = 0.9980, 0.1373, and 0.7481, respectively), but the overall trend did show an increase in main stem biomass as planting density increased despite the corresponding reduction (p-value < 0.0001) in total biomass. These results demonstrate that although total biomass of silverleaf sunflower decreases at the higher planting densities used in this study, main stem biomass tended to stay the same or increase. This is a critical finding that may prove useful for maximizing total main stem yield per hectare.

Analysis of plant growth by accession within each species (all p-values < 0.05) indicated that plants of ARG1820 were the tallest (IA = 3.5 m, BC = 4.3 m) and produced the most main stem biomass per plant (IA = 360 g, BC = 399 g), while ARG1575 was on average the smallest of the four accessions of *H. argophyllus* (Table 1). Hopi, the Native American landrace, was the largest accession of *H. annuus* for height (IA = 1.85 m, BC = 1.76 m) in IA and BC and for stem diameter and main stem biomass; however, significant differences between Hopi and the other *H. annuus* accessions were only observed for those plants grown in Iowa. Main stem biomass of Hopi was six times greater than in the closest *H. annuus* accession (Hopi = 182 g, HA412-HO = 27 g), making Hopi a preferred target for initiating an *H. annuus*-centered biomass breeding program.

3.2. Compositional analysis – traditional wet chemistry

For plants grown in Georgia, average glucan and xylan contents were higher in *H. argophyllus* (37.3% and 20.5%) than *H. annuus* (32.8% and 15.9%) (p-values of 0.0001 and 0.0013, respectively) (Table 2).

Xylose (14.8%–20.9%) was identified as the major hemicellulose sugar in both species, followed by arabinose (1.5%–3.1%) and galactose (1.3%–2.0%). Arabinose and galactose did not vary significantly between species in GA or IA, but the

Table 2 – Plant cell wall composition (% dry basis) of stems of *Helianthus annuus* (ANN) and *Helianthus argophyllus* (ARG) accessions grown in GA and IA measured using standard analytical methods.

	N	GA						IA					
		Lignin	Glucan	Xylan	Galactan	Arabinin	Acetyls	Total sugars	Lignin	Glucan	Xylan	Galactan	Arabinin	Acetyls	Total sugars
HA412-HO	1/1[a]	28.6 (0.0)[b]	31.5 (0.1)	14.9 (0.1)	1.8 (0.0)	2.2 (0.0)	3.2 (0.0)	53.5 (0.2)	28.6 (0.1)	33.0 (0.0)	15.8 (0.0)	1.5 (0.0)	1.8 (0.0)	4.9 (0.0)	57.0 (0.1)
Hopi	5/1	23.6 (0.5)	33.1 (0.4)	16.9 (0.4)	1.5 (0.1)	2.1 (0.1)	4.5 (0.2)	58.2 (0.6)	27.2 (0.1)	38.4 (0.1)	18.8 (0.1)	1.3 (0.0)	1.5 (0.1)	3.1 (0.0)	63.1 (0.1)
RHA373	4/1	26.3 (0.4)	32.9 (0.3)	14.8 (0.2)	2.0 (0.0)	3.1 (0.1)	3.6 (0.0)	56.3 (0.3)	29.0 (0.6)	35.6 (0.5)	15.8 (0.3)	1.6 (0.0)	2.1 (0.1)	3.5 (0.1)	58.6 (0.1)
ANN1238	0/1	ND	ND	ND	ND	ND	ND	ND	25.1 (0.2)	38.0 (0.1)	15.3 (0.1)	1.6 (0.0)	2.0 (0.0)	5.3 (0.0)	62.3 (0.2)
ANN average	10/4	25.2 (0.5) A[c]	32.8 (0.3) B	15.9 (0.3) B	1.7 (0.1) A	2.5 (0.1) A	4.0 (0.1) B	57.0 (0.5) B	27.5 (0.6) A	36.3 (0.8) A	16.4 (0.5) A	1.5 (0.1) A	1.9 (0.1) A	4.2 (0.4) A	60.3 (0.1) A
ARG1575	1/1	25.4 (0.0)	36.7 (0.0)	19.9 (0.0)	1.3 (0.0)	1.9 (0.0)	5.9 (0.0)	65.7 (0.1)	26.4 (0.1)	37.7 (0.1)	17.1 (0.1)	1.4 (0.0)	1.9 (0.0)	2.9 (0.0)	61.0 (0.1)
ARG1805	1/1)	22.8 (0.0	37.9 (0.1)	20.9 (0.0)	1.5 (0.0)	1.7 (0.0)	6.0 (0.0)	68.0 (0.1)	25.6 (0.2)	38.1 (0.1)	17.9 (0.1)	1.6 (0.0)	1.9 (0.0)	5.5 (0.0)	64.9 (0.1)
ARG1820	1/2	22.4 (0.1)	37.2 (0.2)	20.8 (0.2)	1.6 (0.0)	2.0 (0.0)	5.9 (0.0)	67.5 (0.4)	24.5 (0.1)	38.7 (0.1)	18.6 (0.3)	1.5 (0.0)	1.9 (0.1)	5.6 (0.3)	66.4 (0.2)
ARG1834	1/1	22.4 (0.2)	37.6 (0.0)	20.6 (0.1)	1.4 (0.1)	2.4 (0.0)	6.2 (0.0)	68.2 (0.2)	27.1 (0.0)	39.0 (0.2)	19.2 (0.1)	1.5 (0.0)	1.8 (0.1)	4.4 (0.0)	65.8 (0.4)
ARG average	4/5	23.3 (0.5) A	37.3 (0.2) A	20.5 (0.2) A	1.5 (0.0) A	2.0 (0.1) A	6.0 (0.1) A	67.4 (0.4) A	25.6 (0.4) A	38.4 (0.2) A	18.3 (0.3) A	1.5 (0.0) A	1.9 (0.0) A	4.8 (0.4) A	64.9 (0.7) A

ANN, cultivated sunflower; ARG, silverleaf sunflower; ND, not determined.

a Number of plants analyzed from each location (i.e. GA/IA); each plant run as 2 technical replicates.

b Mean (standard error).

c Means within a column followed by the same letter are not significantly different (p-value = 0.05, PROC MIXED).

acetyl content was lower in *H. annuus* than *H. argophyllus* in GA (4.0% vs. 6.0%, respectively) (p-value = 0.0019). Lignin content (range 22.4%–29.0%) was not statistically different between *H. annuus* and *H. argophyllus* (Table 2).

3.3. High-throughput pyMBMS analysis

Average pyMBMS estimated lignin content for *H. annuus* and *H. argophyllus* was 29.6% (range, 24.0%–34.6%) and 28.6% (range, 24.6%–33.3%), respectively, when averaged across all environments (Fig. 1, Supplemental Table S6), which is similar to the percentage of total lignin in *Populus deltoides* (eastern cottonwood; 27.2%) [24] and *Pinus radiata* (Monterey pine; 28.2%) [25]. The average S/G lignin ratio was 1.5 (range, 1.0–2.0) for *H. annuus* and 1.7 (range, 1.0–2.4) in *H. argophyllus*, indicating that the lignin in both species contained more S-lignin subunits than G-lignin subunits. Similar S/G ratios have been observed using thioacidolysis and NIR [26] in hybrid poplar (*Populus alba* × *tremula*) (range 1.3–2.2), another dicot species, and are considerably higher than the S/G ratios observed in lignocellulosic monocots including *Miscanthus* (0.7) analyzed by NMR and thioacidolysis [27], and whole plant switchgrass (0.52–0.54) [28] analyzed by pyMBMS. In general, both species possessed significant intraspecific (i.e., among accessions within each species) variation for lignin composition that could be targeted for future selection (Supplemental Table S6).

Lignin content and composition in both species were influenced by location and planting density (Supplemental Fig. S1). This is not surprising, as plant cell wall chemistry is known to vary greatly with growth conditions [29,30]. Examination of the total data set for all locations and plant spacings indicated that *H. annuus* and *H. argophyllus* were not statistically different for lignin content or composition. However, inspection of the simple effects (by location and spacing) (Table 3) indicated that lignin content and composition for these two species were statistically different in certain environments. In IA, total lignin was higher in *H. annuus* than in *H. argophyllus* in the lower density plots (0.9 and 1.5 m spaced plants) (p-values = 0.0016 and 0.001, respectively) (Table 3), and the S/G lignin ratio (1.8) in *H. argophyllus* was higher (p-value < 0.0001) than *H. annuus* (1.5), in the high density plots (0.3 m spaced plants).

In order to understand the interaction between planting density and lignin content/composition the amounts of total lignin, G-lignin, S-lignin, and the S/G-lignin ratios were compared for plants grown in IA in the lower density plots (1.5 m between plants) vs. the plants grown in the higher density plots (0.3 m between plants) (Supplemental Table S5). This comparison showed that as planting density increased, total lignin did not change in either species; however, G-lignin decreased from 10.3% to 9.8% in *H. annuus* and 9.9% to 8.9% in *H. argophyllus* (p-values = 0.062 and <0.0001, respectively). S-lignin and the S/G ratio were unchanged in *H. annuus,* while both increased in *H. argophyllus* (p-values < 0.0001). Specifically S-lignin in *H. argophyllus* increased from 13.6% to 15.9% and the S/G ratio increased from 1.4 to 1.8 as the planting density increased from 1.5 m between plants to 0.3 m between plants. These results suggest that an interaction between S-lignin and planting density in *H. argophyllus* exists for the conditions tested herein where S-lignin increases with planting density. Overall, the interactions in both species between lignin composition and planting density highlight the critical fact that the identification of the maximally efficient planting density for production and the selection for S- and G-lignin components must be done in parallel in sunflower.

Total lignin content in both species did not vary by location (IA vs. BC) (Table 3); however, the average S/G ratio was higher (p-value < 0.0001) in BC for *H. argophyllus* (1.7) than in IA (1.5).

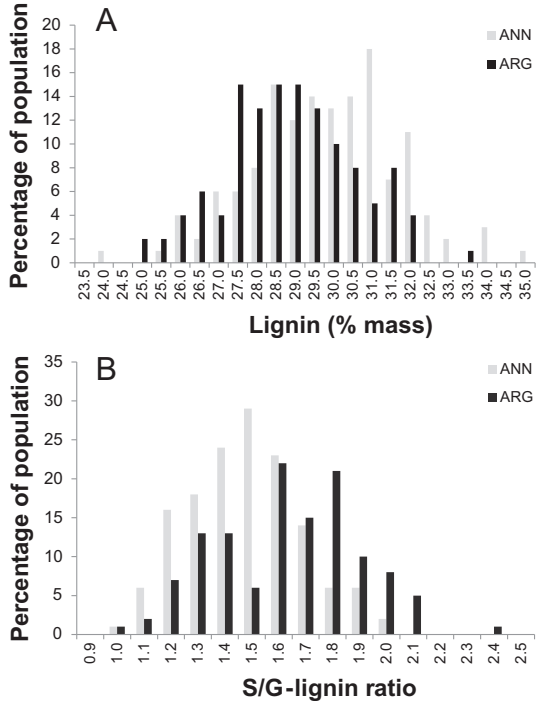

Fig. 1 – PyMBMS estimated (A) lignin content (% mass) and (B) composition (S/G-lignin) of basal stem sections of *Helianthus annuus* and *Helianthus argophyllus* plants grown in IA and BC.

3.4. Accession differences in plant cell wall composition and plant growth

The lack of statistical difference between *H. annuus* and *H. argophyllus* for many of the cell wall chemistry traits characterized in this study is likely due in part to the small sample sizes tested for each species (Supplemental Table S2), the significant G × E interactions (Supplemental Fig. S1), and the high level of intraspecific variation observed within each species (Fig. 2, Supplemental Table S6). This high level of intraspecific variation was somewhat expected as the four accessions of each species were purposely selected to represent the widest diversity available for each species based on flowering phenology and geographic origin.

In IA both ARG1820 and ARG1834 produced the most main stem biomass per plant (360 g and 290 g, Table 1), while also producing plant cell walls that were lower in lignin (27.9% and 28.0%). Among the accessions of *H. annuus*, Hopi produced 8.5 times more main stem biomass and had the highest S/G lignin ratio (1.7). Therefore, despite having a relatively high

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Table 3 – PyMBMS estimated lignin content and composition of *Helianthus annuus* (ANN) and *Helianthus argophyllus* (ARG) stem samples collected from plants grown in IA and BC at different planting densities of 0.3, 0.9, or 1.5 m between plants.

	IA 0.3 m[a]		IA 0.9 m		BC 0.9 m		IA 1.5 m	
	ANN	ARG	ANN	ARG	ANN	ARG	ANN	ARG
Total lignin	29.08 ± 0.22[b]	29.40 ± 0.23	29.84 ± 0.27 A[c]	28.37 ± 0.23 B	29.69 ± 0.36	28.41 ± 0.32	29.96 ± 0.26 A	27.98 ± 0.32 B
S/G lignin	1.51 ± 0.03 B	1.80 ± 0.03 A	1.49 ± 0.03	1.54 ± 0.03 B[d]	1.49 ± 0.04 B	1.74 ± 0.04 A	1.43 ± 0.03	1.36 ± 0.04
G-lignin	9.81 ± 0.10 A	8.90 ± 0.10 B	10.02 ± 0.13	9.53 ± 0.11 A	10.05 ± 0.16 A	8.72 ± 0.14 B	10.36 ± 0.12	9.81 ± 0.15
S-lignin	14.69 ± 0.16 B	15.90 ± 0.17 A	15.03 ± 0.21	14.41 ± 0.18	14.89 ± 0.27	15.14 ± 0.24	14.82 ± 0.19 A	13.56 ± 0.24 B

ANN, cultivated sunflower; ARG, silverleaf sunflower.
a Location and planting density equal to the number of meters between plants within each row plot.
b LSMEAN ± SE.
c Mean separation between species within a plant spacing (ex. ANN 0.3 m IA vs. ARG 0.3 m IA) followed by different letters are significantly different (p-value < 0.05) LSMEANS, PDIFF, Tukey–Kramer.
d Mean separation (underlined) between locations within a species (ex. ANN IA vs. ANN BC only for 0.9 m planting density, middle columns) followed by different letters are significantly different (p-value < 0.05) LSMEANS, PDIFF, Tukey–Kramer.

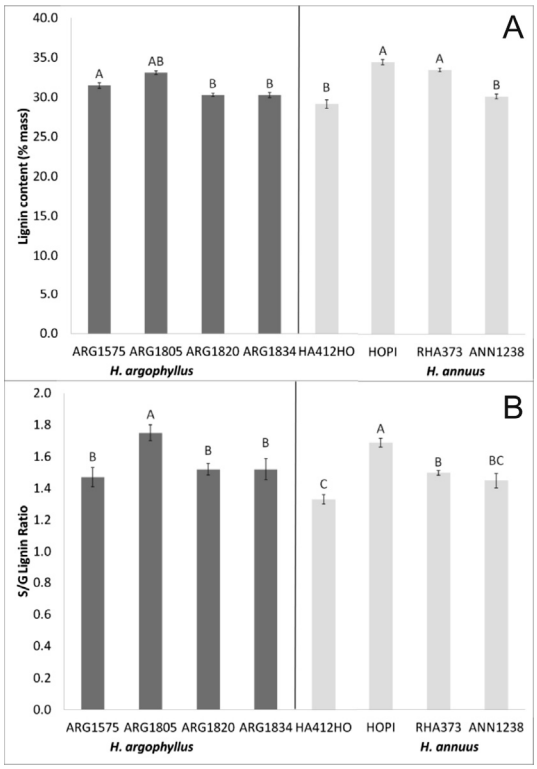

Fig. 2 – Average plant cell wall composition (A) total lignin (B) S/G lignin ratio as determined by pyMBMS for four accessions of *Helianthus argophyllus* and four accessions of *Helianthus annuus* grown in Iowa.

percentage of lignin (30.5%), Hopi appears to be a good source of favorable alleles for creating high biomass sunflower cultivars.

3.5. *Correlation analysis – cell wall composition and plant growth*

PyMBMS determined cell wall components (e.g., lignin, C5 sugars including xylan and other hemicellulose sugars, and C6 sugars including glucan and cellulose) in both species were highly correlated (Table 4) as is often observed in other plant species [30]. For the samples grown in IA, pyMBMS estimated lignin content was negatively correlated with the sum of C5 + C6 sugars in *H. annuus* at all planting densities and in *H. argophyllus* at the highest planting density (Table 4). A negative correlation was also observed between lignin and the combined glucan and xylan content using analytical wet chemistry methods for *H. annuus* plants grown in GA (r value = −0.50, p-value = 0.0255). A negative correlation between lignin and carbohydrate content (i.e., C5 + C6 sugars) is common in plants [30] as lignin and carbohydrates make up the vast majority of the dry mass in plants [31], and as one increases the other usually decreases. For example, in transgenic studies in *Populus* [32,33] and alfalfa [12], an increase in cellulose also resulted in a corresponding decrease in lignin content. Therefore, a reduction in lignin has the dual benefit of possibly reducing recalcitrance [11] while also increasing

Table 4 – Spearman's rank correlation coefficients (*r*) for plant cell wall biochemistry traits in *Helianthus annuus* (ANN) and *Helianthus argophyllus* (ARG) planted in IA and BC at different planting densities of 0.3, 0.9, or 1.5 m between plants.

Location	Species	Spacing	[a]C5 C6	SLIG GLIG	LIG S/G	LIG C5 + C6	SLIG C5 + C6	GLIG C5 + C6
IA	ANN	0.3	0.96***			−0.73***	−0.50***	−0.59***
	ANN	0.9	0.93***		0.75***	−0.58***	−0.49**	
	ANN	1.5	0.96***		0.47**	−0.64***	−0.40*	
	ARG	0.3	0.98***		0.37*	−0.65***	−0.43**	−0.48**
	ARG	0.9	0.97***	−0.65***			0.53***	−0.76***
	ARG	1.5	0.94***	−0.45*	0.47*			−0.70***
BC	ANN	0.9	0.90***					−0.39*
	ARG	0.9	0.97***		0.55**	−0.64***	−0.55**	−0.68***

ANN, cultivated sunflower; ARG, silverleaf sunflower; C5, C5 sugars; C6, C6 sugars; SLIG, S-lignin; GLIG, G-lignin; S/G, ratio of S-lignin/G-lignin; LIG, total lignin; C5 + C6, total sugar content.
*, **, and ***, significant at probability levels of 0.05, 0.01 and 0.001, respectively.
a Number of meters between plants within row plots.

sugar content [12,34,35], with both resulting in potentially higher sugar yields. It needs to be noted, however, that a reduction in lignin is often associated with an increased susceptibility to lodging [36], so it will be necessary to simultaneously select for plants that contain less lignin and are also resistant to lodging.

In most environments, total lignin was positively correlated in both species with the S/G lignin ratio (Table 4). The amount of S-lignin and G-lignin were either uncorrelated (*H. annuus* – all planting densities, *H. argophyllus* – high density planting) or negatively correlated (*H. argophyllus* – lower density plots). The correlations between plant growth and plant cell wall composition were different in *H. annuus* and *H. argophyllus* (Table 5). Specifically, in *H. annuus*, S-lignin and the ratio of S/G lignin were positively correlated with height, stem diameter, total biomass, main stem biomass and days to flower, while negative correlations were observed for these traits in *H. argophyllus*. Interestingly, total lignin in *H. annuus* was only correlated with height in this study and not total biomass; as such, selection for increased biomass in *H. annuus* is not expected to impact lignin content but it will likely affect lignin composition by increasing the S/G lignin ratio. Based on these data, selection for increased biomass in *H. argophyllus* should result in a corresponding reduction in total lignin, as well, due to the negative correlation between biomass and lignin content observed in this study for this species. This negative correlation between biomass and lignin content occurs in other plants [37] and should allow for the possible development of high biomass varieties that contain less lignin.

Table 5 – Spearman's rank correlation coefficients (*r*) for pyMBMS estimated cell wall chemistry traits and plant growth in IA grown *Helianthus annuus* (ANN) and *Helianthus argophyllus* (ARG) plants.

		Lignin	S/G	S-lignin	G-lignin
ANN	Height	0.34***	0.53***	0.50***	−0.24**
	Total biomass		0.32***	0.22*	−0.24**
	Main stem biomass		0.33***	0.27**	−0.22*
	Stem diameter		0.21**	0.19*	
	% Main stem		0.19*	0.19*	
	Days to flower		0.47***	0.32***	−0.36***
ARG	Height	−0.35***	−0.35***	−0.25**	
	Total biomass	−0.23**	−0.30***	−0.31***	0.19*
	Main stem biomass	−0.24**		−0.18*	
	Stem diameter		−0.34***	−0.27**	0.37***
	% Main stem				−0.24**
	Days to flower	−0.41***	−0.36***	−0.44***	0.19*

Abbreviations (see Table 4); *, **, and ***, significant at probability levels of 0.05, 0.01 and 0.001, respectively.

3.6. Summary findings

The results of this study suggest that *H. argophyllus* could be used as a dedicated lignocellulosic energy crop with high biomass yield and plant cell wall chemistry that is amenable to conversion to ethanol via saccharification. Cultivated sunflower could also be used as a source of lignocellulosic biomass, especially if cell wall composition in this species is improved either by incorporating alleles from *H. argophyllus* or by identifying alleles within the primary gene pool that confer higher biomass, lower lignin, higher S/G ratios, and increased cellulose to lignin ratios. The next step should focus on investigating the genetic basis of improved plant cell wall chemistry in these species, as well as determining the optimum conversion process suitable for transforming sunflower biomass into a profitable renewable energy source. Additional research is also needed to determine the best agricultural practices and pretreatment regimes, which are targeted at maximizing yield both in the field (biomass/hectare) and during the saccharification process (sugar yield). Future examination of the fine-scale structure of cell wall composition (i.e., lignin, cellulose, xylan matrices) in sunflower will also aid in facilitating the development of pretreatment protocols designed specifically to maximize sugar yield from sunflower.

At this time, a large collection of genetic resources for sunflower is available, including a high-density genetic map [38], thousands of sunflower-specific SNP markers [39], multiple *H. annuus* mapping populations [40], a *H. annuus* × *H. argophyllus* interspecific mapping population (Barb et al., unpublished results), two *H. argophyllus* genetic maps [[41] and (Barb et al., unpublished results)] and *H. annuus* × *H. argophyllus* introgression lines. Furthermore, the sunflower genome is currently being sequenced and assembled [42],

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thereby providing an extremely powerful tool for genetically improving sunflower as an emerging bioenergy option.

4. Conclusions

The results of this research show that the chemical composition of main stem biomass is similar in *H. annuus* and *H. argophyllus* despite the morphological differences (pithy vs. solid stems) between these two species of sunflower. In most environments tested herein, total lignin content and composition were equivalent in these species; however, lignin content was higher in *H. annuus* in the lower density plots and the S/G lignin ratio was higher in *H. argophyllus* in the higher density plots. In this study, plant cell wall chemistry was influenced by both location and planting density; therefore, it will be important to select for these traits in the environment for which this crop is expected to be produced. Overall, the results of this study illustrate that adequate variation for total lignin and lignin composition already exists for both species to develop a lignocellulosic biofuels feedstock of sunflower with reduced lignin and high cellulose content.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biombioe.2013.06.009.

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