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Cuticular wax composition of *Salix* varieties in relation to biomass productivity

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Abstract

The leaf cuticular waxes of six *Salix* clones (one *Salix miyabeana*, one *Salix dasyclados*, one *Salix eriocephala*, two *Salix purpurea*, and one interspecific hybrid of *Salix eriocephala* × *interior*) with different biomass productivities were characterized by gas chromatography–mass spectrometry. Total wax content ranged from 6.3 to 16.8 μg cm^{-2}, and two distinct patterns of wax were measured. The wax from leaves of *S. dasyclados* 'SV1' differed from all other clones and was dominated by fatty acids (42%), high concentrations of *n*-alkanes (25%) and *n*-alcohols (28%), with low *n*-aldehyde content (4%). All other clones produced cuticular wax dominated by *n*-alcohols (32–51%), particularly 1-hexacosanol, with fatty acids (14–37%) and *n*-aldehydes (19–26%) present in lower abundances. Clones of *Salix* grown under identical environmental conditions produce noticeably different amounts of cuticular wax. In contrast to previous studies of *Salix*, total wax content was independent of biomass productivity, measured as basal area, suggesting that wax production is not directly linked with woody biomass production by shrub willows under these site conditions.

Keywords: *Salix eriocephala*; *Salix dasyclados*; *Salix miyabeana*; *Salix purpurea*; Salicaceae; Willow; Biomass; Cuticular waxes; Ecological biochemistry section

1. Introduction

Short-rotation woody crops (SRWC) are being developed as feedstocks for bioenergy and bioproducts in response to global increases in atmospheric CO_2 concentrations and other environmental impacts associated with fossil fuels and the desire to reduce our reliance on fossil fuels (National Research Council, 2000). Willows (*Salix* spp.) are one of the major woody biomass crops being developed in North America and Europe, because of their high biomass yields, ease of propagation, ability to coppice producing multiple harvests from each planting and a wide range of genetic diversity (Volk et al., 2006; Keoleian and Volk, 2005; Nordman et al., 2005; Smart et al., 2005). Identifying clones of *Salix* that produce high biomass is key to success for future breeding programs and a simple and time efficient test of potential biomass productivity would be very useful. In a study of *Salix* crops in Finland, high biomass productivity was correlated with increased wax loads (Hietala et al., 1995), and therefore wax load may be a proxy indicator for selection of clones with enhanced biomass productivity.

Cuticular waxes coat the outer surfaces of leaves and protect the plant from a variety of environmental pressures (Thompson, 1993), and may directly or indirectly effect the survival and productivity of a plant. The wax has many functions including reduction of water loss (Schönherr, 1976; Ristic and Jenks, 2002), providing defense against pathogens, diffracting excess light, increasing resistance to

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frost damage (Rosenqvist and Laakso, 1991), and reducing the uptake of pesticides (Schreiber and Schönherr, 1992). The wax is hydrophobic and composed of long-chain fatty acids, alcohols, aldehydes and very long-chain wax esters (Bianchi, 1995). The relative proportions of these compounds in leaf waxes can be highly variable between plant species and also within a species (Walton, 1990). Wax composition of different clones of *Salix* species show variation in wax composition (Rosenqvist and Laakso, 1991; Hietala et al., 1995, 1997, 1998; Cameron et al., 2002) and high *n*-alkane contents have been correlated with low over-wintering survival (Rosenqvist and Laakso, 1991; Hietala et al., 1995).

The objectives of this study were to chemically characterize the cuticular wax composition of six *Salix* clones grown under similar environmental conditions and to determine if wax load and wax composition could be used as a reliable predictor of high biomass productivity for *Salix* species. We chose clones with varying productivities, high survival rates (Tharakan et al., 2005), and plants were irrigated to reduce the influence of water availability on productivity. Production of leaf waxes by *Salix* changes during the growing season, and wax load is often greatest during September (Cameron et al., 2002), and therefore we collected leaves during September to determine the relationship between wax production and biomass productivity.

2. Results and discussion

2.1. Cuticular wax load of Salix leaves

The cuticular wax load of leaves of *Salix* clones ranged from 6.3 to 16.8 μg cm^{-2} (Table 1). The highest wax loads were present on leaves of *Salix eriocephala* 'S25' and *Salix eriocephala* × *interior* 'S625'. The lowest wax load was produced by *Salix purpurea* 'Pur12' (6.3 μg cm^{-2}), which was significantly lower (*t*-test, $P < 0.001$) than that produced by a second *S. purpurea* clone, '94003' (9.5 μg cm^{-2}). The total wax loads that we measured were similar to those reported for other *Salix* species, ranging from 4.8 μg cm^{-2} for *Salix myrsinifolia*, to 9.2 μg cm^{-2} for *Salix viminalis* (Hietala et al., 1997), and wax from *Salix dasyclados* containing up to 19 μg cm^{-2} (Cameron et al., 2002). The production of cuticular wax in *Salix* does vary greatly over the growing season (Cameron et al., 2002), so we collected all our samples in a single day in September from newly expanded leaves to reduce temporal variations in wax production. The differences in wax load and also total biomass produced under different environmental conditions suggests that wax production in *Salix* may be regulated by genetic and/or environmental factors.

2.2. Composition of cuticular wax of Salix leaves

The major components of the cuticular waxes of the *Salix* species examined were fatty acids, *n*-alcohols, *n*-aldehydes, *n*-alkanes and the triterpenoid β-amyrin (Table 1). All these compound classes are common constituents of plant waxes (Walton, 1990; Bianchi, 1995). The relative proportions of these compound classes differed for each clone; however, two distinct classes of cuticular wax were recognized, with that produced by *S. dasyclados* 'SV1' differing in composition to the wax of all other clones examined. The wax from *S. dasyclados* 'SV1' leaves was dominated by fatty acids (42%), high concentrations of alkanes (specifically C_{27} alkane; 25%) and *n*-alcohols (28%), with a low content of *n*-aldehydes (4%; Table 1). In contrast, the lipid composition of wax produced by all other clones was consistently dominated by *n*-alcohols (32–51%), with fatty acids accounting for 14–37% of the wax load and *n*-aldehydes present in abundance (19–26%). Such wide variations in wax composition are common to *Salix* species (Hietala et al., 1995; Cameron et al., 2002). These authors reported that waxes produced by *S. myrsinifolia* and *S. eriocephala* were dominated by high concentrations of alcohols, whereas waxes produced by *S. viminalis* were dominated by alkanes. Cameron et al. (2002) reported higher amounts of alkanes and lower amounts of fatty acids in leaves from *S. dasyclados* compared with those observed in the present study.

Table 1
Lipid composition (% of total) and total concentration of cuticular waxes[a] on leaves of Salix clones and biomass productivity reported as basal area

	S. eriocephala 'S25'	*S. eriocephala* × *interior* 'S625'	*S. miyabeana* 'SX64'	*S. purpurea* '94003'	*S. dasyclados* 'SV1'	*S. purpurea* 'Pur12'
n-Alkanes (%)	1.3 ± 0.1	1.1 ± 0.1	9.1 ± 0.9	5.7 ± 0.9	22.0 ± 2.0	3.0 ± 0.4
n-Fatty acids (%)	13.8 ± 0.2	16.3 ± 1.6	32.2 ± 3.3	28.4 ± 3.9	42.0 ± 1.7	37.6 ± 3.0
n-Alcohols (%)	51.2 ± 1.9	49.6 ± 0.5	32.1 ± 0.4	42.8 ± 3.0	27.8 ± 0.7	39.3 ± 1.9
n-Aldehydes (%)	26.9 ± 1.7	26.5 ± 1.2	19.4 ± 1.0	21.4 ± 0.6	4.0 ± 0.3	18.9 ± 1.8
Triterpenoids[b] (%)	2.7 ± 0.5	2.9 ± 0.2	9.9 ± 2.1	2.4 ± 0.2	2.7 ± 0.4	2.3 ± 0.3
Unknowns (%)	2.1 ± 0.1	2.3 ± 0.1	3.0 ± 0.2	2.5 ± 0.2	2.9 ± 0.2	2.4 ± 0.2
Total lipids (μg cm^{-2})	16.8 ± 1.6	15.3 ± 2.0	9.3 ± 1.7	9.5 ± 0.3	7.4 ± 0.6	6.3 ± 0.1
Basal area (m^2 ha^{-1})	7.4 ± 1.2	9.0 ± 2.1	14.4 ± 2.3	5.0 ± 0.2	6.1 ± 1.4	8.0 ± 3.6

[a] Values are expressed as the mean of analyses of three leaves, consisting of one leaf sampled from each of three plants (±SD).
[b] Represents the concentration of β-amyrin.

The factors that influence cuticular wax composition within, and between, species include growth conditions, seasonal and genetic variations. The cuticular wax load of *Thymus vulgaris* was strongly influenced by substrate water content, with plants grown under low water conditions producing higher wax loads than those grown at higher substrate water content (Letchamo and Gosselin, 1996), and water stress increased wax concentrations for *Gossypium hirsutum* (Bondada et al., 1996) and *Nicotiana glauca* (Cameron et al., 2006). The wax composition of *Brassica* species was also influenced by growth condition, with plants grown outdoors producing higher proportions of *n*-alkanes, octacosanoic acid, primary alcohols and very long chain wax esters (Shepherd et al., 1995, 1997). Wax composition may also change over the seasons as shown for *Tilia tomentosa* (Gülz et al., 1991), *Salix and Populus* (Cameron et al., 2002), and *Hosta* genotypes (Jenks et al., 2002). In contrast, similar wax compositions of 40 ecotypes of *Arabidopsis thaliana* collected in a wide range of geographic environments were reported, even though total wax load on stems spanned a twofold range (Rhashotte et al., 1997).

The variability in wax composition we observed with *Salix* clones was probably governed by genetic differences between clones rather than environmental growth factors. Our study attempted to reduce environmental factors such as seasonal changes, differences in temperature, water availability, and nutrient resources by growing all plants in a well defined experimental plot with constant irrigation. Although shading of leaves was not significant we cannot rule out this factor, or small variations in nutrient and water availability, however the significant differences in wax load and also wax composition strongly suggest that wax production in *Salix* species is primarily controlled by genetic factors.

2.2.1. Alkanes

All *Salix* clones produced wax that contained a homologous series of odd-numbered carbon chain *n*-alkanes ranging from C_{25} to C_{31}. The dominant hydrocarbon component in all waxes examined was *n*-heptacosane with lower amounts of *n*-nonacosane and *n*-pentacosane, with all other alkanes being present at trace levels (<0.1% of total wax). We were unable to accurately measure the concentration of *n*-nonacosane in all samples due to the addition of second internal standard (5α cholestane) that co-eluted with this component in several of the analyses, and therefore the total amount of alkanes present may be underestimated. Previous studies of *Salix* clones reported *n*-heptacosane as the major alkane, with *n*-nonacosane typically accounting for less than 50% of the amount of heptacosane present (Hietala et al., 1995; Cameron et al., 2002). The wax of *S. dasyclados* contained significantly higher amounts of heptacosane than all other waxes measured ($p < 0.01$), with this single alkane constituting more than 22% of the total wax load. Alkanes accounted for less than 1.5% of the total wax load of *S. eriocephala* 'S25' and *S. eriocephala* × *interior* 'S625', with alkanes present at less than 0.3 μg cm^{-2}. Alkanes are typically present in plant waxes (Walton, 1990; Post-Beittenmiller, 1996); however, their relative abundance and total concentrations can differ dramatically between species. Alkanes can constitute more than 75% of the cuticular wax load (e.g. tree tobacco; Cameron et al., 2006), or be present in trace amounts (Walton, 1990). Lower concentrations of alkanes may be associated with greater overwintering survival (Rosenqvist and Laakso, 1991; Hietala et al., 1995), and water stress appeared to increase the amounts of long chain alkanes in the waxes of cotton (Bondada et al., 1996) and tree tobacco (Cameron et al., 2006). The hydrophobic nature of alkanes make them an essential component of cuticular wax in the protection of the plant cell from the environment; however, the factors that regulate the amount of alkanes is poorly understood and is highly variable between species and also within a species as shown by our results.

2.2.2. Fatty acids

A homologous series of even-numbered saturated fatty acids from C_{16} to C_{28} was identified in the wax from all *Salix* leaves (Table 2). The concentrations of fatty acid presented herein include those derived from esters, either methyl esters or very long chain wax esters, and also those present as free fatty acids. The waxes of different *Salix* varieties do contain wax esters albeit in low amounts ranging from 0% to 6% of the total wax load for *S. dasyclados* (Hietala et al., 1995). Two distinct fatty acid distributions were observed from waxes of leaves of *Salix*, with that of *S. dasyclados* being significantly different to that produced by all other clones (Table 2). The differences in the fatty acid composition of the waxes from *S. dasyclados* and all other clones were also reflected in the distribution of the biosynthetically-related alcohols and aldehydes as discussed below.

2.2.3. Alcohols

The cuticular wax of all *Salix* clones contained a homologous series of even numbered very long-chain primary alcohols (Table 2). The concentrations of alcohols presented herein include those derived from wax esters, and also those present as free alcohols. With the exception of the wax produced by *S. dasyclados*, the most abundant lipid component in the cuticular wax of the *Salix* clones studied was 1-hexacosanol (C_{26}). The dominance of 1-hexacosanol in cuticular waxes of *Salix* was reported for several clones including *S. purpurea*, *S. eriocephala* (Cameron et al., 2002), *S. myrsinifolia*, and *S. dasyclados* (Hietala et al., 1995).

In contrast, the major alcohol present in the wax of *S. dasyclados* 'SV1' was 1-docosanol, which accounted for 14.1% of the total wax load. The dominance of this alcohol in *Salix* leaves was also recognized in *S. viminalis* (Hietala et al., 1995). However, analyses of four clones of *S. dasyclados* from Finland showed that the dominant

Table 2
Composition of cuticular wax on leaves of Salix varieties by compound class (% class)[a]

	S. eriocephala 'S25'	*S. eriocephala* × *interior* 'S625'	*S. miyabeana* 'SX64'	*S. purpurea* '94003'	*S. dasyclados* 'SV1'	*S. purpurea* 'Pur12'
n-Fatty acids (% class)						
C16	11.0 ± 0.0	12.6 ± 0.7	12.4 ± 0.6	11.0 ± 0.6	8.1 ± 0.4	11.7 ± 1.1
C18	28.6 ± 0.1	34.2 ± 0.7	54.5 ± 3.0	48.3 ± 3.4	21.2 ± 0.2	55.7 ± 3.9
C20	7.3 ± 0.1	4.1 ± 0.1	3.2 ± 0.1	0.3 ± 0.1	10.2 ± 0.2	0.2 ± 0.1
C22	7.5 ± 0.1	3.9 ± 0.1	2.3 ± 0.1	1.6 ± 0.0	25.6 ± 0.1	1.2 ± 0.1
C24	9.8 ± 0.1	5.9 ± 0.1	4.4 ± 0.1	4.6 ± 0.1	13.2 ± 0.3	6.0 ± 0.3
C26	15.3 ± 0.2	15.5 ± 0.1	10.3 ± 0.2	16.4 ± 0.3	10.4 ± 0.2	13.4 ± 0.9
C28	20.5 ± 0.2	23.8 ± 0.1	12.9 ± 0.3	17.8 ± 0.4	11.3 ± 0.5	11.7 ± 1.0
n-Alcohols (% class)						
C22	6.2 ± 1.0	b.d.l.	0.9 ± 0.0	0.7 ± 0.0	50.7 ± 0.8	0.2 ± 0.2
C24	6.7 ± 0.5	3.5 ± 0.2	6.6 ± 0.2	3.5 ± 0.2	19.0 ± 0.2	6.1 ± 0.6
C26	68.9 ± 1.4	73.9 ± 0.1	55.3 ± 0.4	73.0 ± 3.0	19.2 ± 0.2	73.1 ± 1.4
C28	18.2 ± 0.9	22.5 ± 0.7	37.2 ± 0.3	22.9 ± 0.4	11.1 ± 0.3	20.6 ± 0.3
n-Aldehydes (% class)						
C26	71.6 ± 1.3	69.1 ± 0.8	57.6 ± 0.5	73.0 ± 1.0	60.9 ± 0.2	75.4 ± 1.1
C28	28.4 ± 0.7	30.9 ± 0.6	42.4 ± 0.6	27.0 ± 0.4	39.1 ± 0.2	24.6 ± 0.7

[a] Values are expressed as the mean of analyses of three leaves, consisting of one leaf sampled from each of three plants (±SD).

alcohol was not 1-docosanol as we report but rather 1-hexacosanol (Hietala et al., 1995). The triterpenoid alcohol, β-amyrin, was present in all waxes analyzed and accounted for up to 10% of the total wax load (Table 1). Differences in the relative amounts of specific compounds in the wax of different clones of *S. dasyclados* from varying geographical environments suggests that control of wax synthesis is dependent on both genetic and environmental factors.

2.2.4. Aldehydes

The major aldehydes present in the cuticular wax of all *Salix* clones were hexacosanal and octacosanal (Table 2). Aldehydes are often present in cuticular waxes and may comprise up to 30% of the total wax load (Gülz et al., 1991; Hietala et al., 1995; Jenks et al., 1995; Shepherd et al., 1995). The broad range of total aldehyde content from 4% (*S. dasyclados*) to 27% (*S. eriocephala*) of total wax load in *Salix* clones grown under identical environmental conditions suggests that the regulation of aldehyde, and also overall wax production, differs for each species (Cameron et al., 2002).

The amounts of the C_{26} and C_{28} aldehydes in the waxes were correlated to the relative abundances of the C_{26} and C_{28} fatty acids and alcohols in the waxes of all clones. With the exception of *S. dasyclados* 'SV1', the dominant fatty acids, aldehydes, and alcohols in the waxes of all clones were the C_{26} and C_{28} components. Aldehydes are believed to be intermediates in the biosynthesis of both alkanes, via the decarbonylation pathway, and alcohols via the acyl reductive pathway (Post-Beittenmiller, 1996; Shepherd and Griffiths, 2006). The similarity in the relative distribution of alcohols, fatty acids, and aldehydes in these clones suggests that the acyl reductive pathway may be the dominant mode of synthesis. In contrast, the increased abundance of *n*-alkanes in the wax of *S. dasyclados* 'SV1', with high relative abundances of fatty acids and alcohols with identical carbon skeletons in the C_{22} to C_{28} range, may suggest a shift on the channeling of acyl intermediates away from alcohol production in favor of alkane production (Shepherd and Griffiths, 2006).

2.3. Wax load and composition relative to biomass productivity

The biomass productivity of *Salix*, measured as basal area, was independent of total wax load of leaves for the varieties that we examined (Fig. 1a; $R^2 = 0.0005$). The leaves of the highest biomass producing clone (*Salix miyabeana* 'SX64') contained similar amounts of wax as the lowest biomass producer (*S. purpurea* '94003'). Those varieties that produced low amounts of wax produced similar amounts of total biomass as those varieties that contained high wax amounts. Similarly, the composition of the waxes produced by different varieties was also independent of overall plant production. Although fatty acid content of the waxes produced by *Salix* ranged from 13.8% to 42% of the total lipid content, there was no correlation with overall plant biomass production (Fig. 1b). Therefore, at least in the case of these *Salix* varieties under the conditions for this experiment, the production of cuticular wax is not directly linked with overall woody biomass production. Cuticular wax undoubtedly performs many beneficial functions including protection against plant pathogens, desiccation, and is involved in the control of evapotranspiration, and of chemical fluxes through leaves. However, our results indicate that production of greater quantities of wax does not translate to higher woody biomass for *Salix*. Our results are in contrast to previous studies of *Salix* clones grown in Finland, where higher wax load was positively correlated with biomass productivity (Hietala et al., 1995). These authors suggested that high wax content is beneficial to growth, but disadvantageous for cold acclimation.

The biomass productivity of *Salix* varieties grown in different parts of the world in relationship to their wax

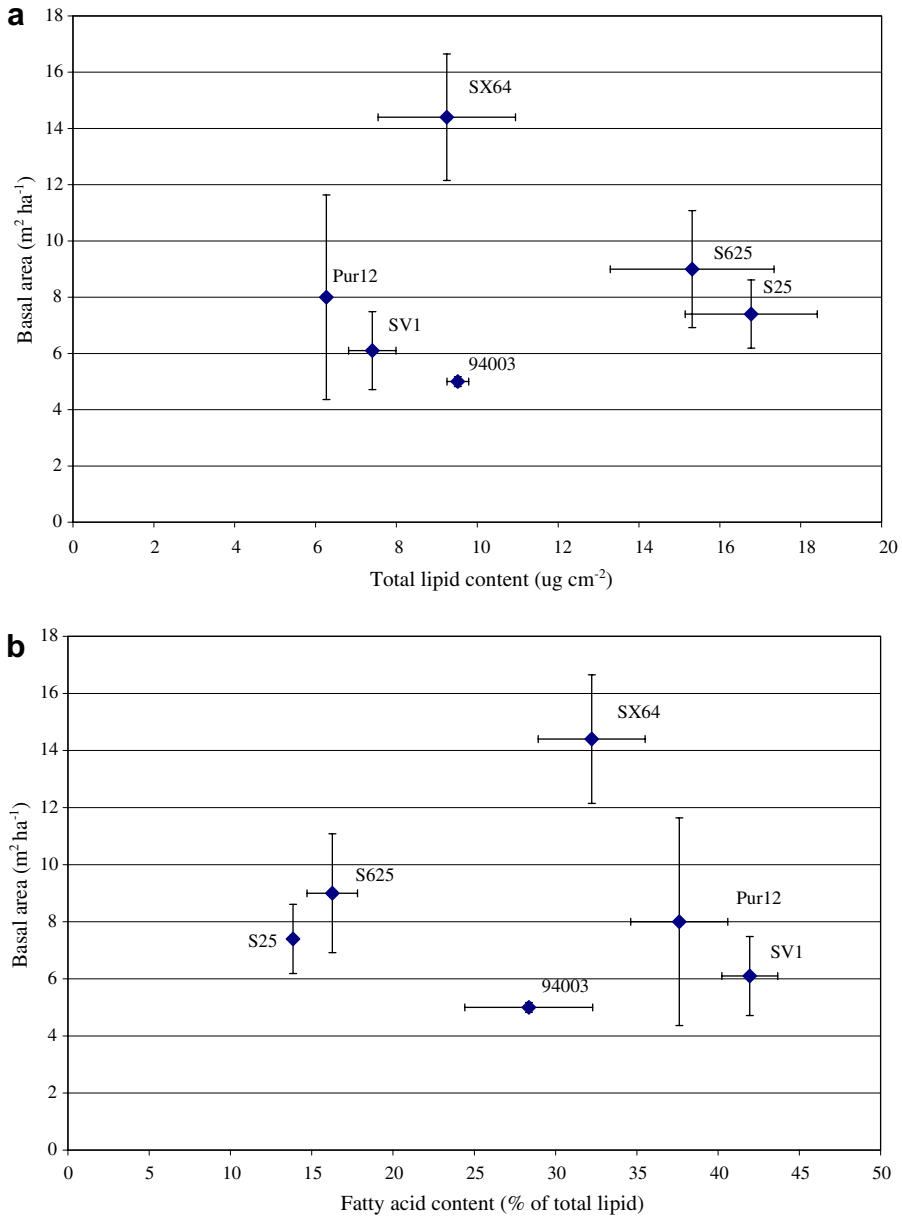

Fig. 1. Relationships between: (a) total lipid content (μg cm^{-2}) and (b) fatty acid content (% of total lipid) of cuticular wax with overall plant biomass for six varieties of *Salix* grown under similar environmental conditions. Error bars indicate standard error and labels indicate variety.

contents suggests that no simple factor determines biomass productivity, nor that wax content is correlated to productivity in all clones. The different growth conditions between these geographically distinct areas may be a strong factor in the relationship of wax content to productivity. Our clones all displayed high overwintering survival whereas the Finnish study showed that plants with low overwintering capacity contained lower amounts of alkanes (Hietala et al., 1995). We saw no significant relationship between alkane content and productivity. The control of wax production, including composition and total content, is probably controlled by both genetic and environmental factors. All the clones used in our study were grown under similar conditions and had high overwintering survival rates, suggesting that the dominant control on biomass wax production was genetic rather than environmental. However, differences in biomass production and wax content of *Salix* in geographically distinct areas (North America and Europe; our study and Hietala et al., 1995) may indicate that these plant responses are determined by growth environment. Therefore, the use of wax content as an indicator of biomass productivity is unlikely to be a useful trait in choosing plants for future breeding programs.

3. Conclusions

The efficient growth of short rotation woody crops as feedstocks for bioenergy will be a large step forward in reducing our reliance on shrinking world oil reserves. Identifying

clones of woody crop species, including *Salix*, that produce high biomass is an essential step in this program. In this study, we showed that biomass productivity of *Salix* clones was essentially independent of leaf wax content, in contrast to previous studies, and that unfortunately wax content cannot be used as an indicator of high potential growth.

4. Experimental

4.1. General experimental procedures

Solvents used were of analytical grade and all other reagents were from Sigma Chemical Co. (St. Louis).

4.2. Plant material

Plants were grown at the SUNY-ESF Genetics Field Station in Tully, New York (42°47′30″N, 76°07′30″W, elevation 391 m) on a well-drained to somewhat excessively well drained Palmyra gravelly silt loam (Glossoboric Hapludalf) with a slope of 0–3% (Hutton and Rice, 1977). The site was established in the spring of 2001 as a 6×2 factorial experiment using a completely randomized block design. The factors were six willow clones and irrigated and unirrigated treatments. Plants were established with 25-cm-long dormant hardwood cuttings at a spacing of 0.91 m × 0.61 m. Plants were coppiced after the first year of growth, so foliage collection was from one-year-old aboveground plants on a two-year-old root system. We sampled leaves from one interspecific hybrid (*S. eriocephala* × *interior* 'S625'), *S. dasyclados* 'SV1', *S. miyabeana* 'SX64', *S. eriocephala* 'S25', and two *S. purpurea* clones ('Pur12' and '94003'). Leaf samples were collected from individual plants in three separate randomized plots at the end of September 2002. The first fully expanded leaf located on the tallest accessible stem was removed and stored frozen and in the dark until wax analysis.

4.3. Lipid analysis of wax

Three disks (each 78.5 mm^{-2}) were cut from each leaf and immersed in CH_2Cl_2 for 30 s. The resulting total extracts were dried under a stream of N_2 and known amounts of standards (1-heptadecanol, nonacosanoic acid and 5α-cholestane) added. Extracts were dissolved in toluene (100 μl) and 500 ml of methanolic HCl (Pierce Chemicals) was added. After heating for 60 min at 60 °C, the sample was cooled to room temperature and doubly-distilled deionized H_2O (1 ml) and hexane (2 ml) were added. After thoroughly vortexing the solution (30 s), the fatty acid methyl esters were extracted into hexane three times. The solvent was evaporated to dryness under a N_2 stream, then toluene (500 μl) was added to azeotrope any residual H_2O, with the toluene evaporated under a N_2 stream. The dry residue was silylated using *bis*-(trimethylsilyl)-trifluoroacetamide (BSTFA; Pierce Chemicals) for 30 min at 60 °C, after which surplus BSTFA was evaporated under N_2. The residue was dissolved in CH_2Cl_2 (500 μl), vortex mixed, and the solvent evaporated under N_2. This step ensured no residual BSTFA was present prior to GC analysis. Samples were dissolved in 2,2,4-trimethylpentane and analyzed using a Shimadzu GC-17A with FID on a HP-5 capillary column (WCOT, 30 m × 0.25 mm i.d., 0.25 μm film thickness). One μl of sample was injected (split mode, 15:1), injector temperature 280 °C, with oven programmed for 1 min at 60 °C, increasing 15 °C min^{-1} to 200 °C, then increasing 5 °C min^{-1} to 280 °C, holding for 30 min at 280 °C, with helium (3 ml min^{-1}) as carrier gas and detector set at 280 °C. Concentrations of individual compounds from each leaf sampled were determined relative to the internal standard nonacosanoic acid for fatty acids, aldehydes, and alkanes, and relative to 1-heptadecanol for alcohols using Shimadzu GC Solution (Ver. 2.10) software. Unfortunately, due to co-elution of the internal standard cholestane, it could not be used to accurately quantify alkane concentrations, so concentrations were calculated relative to the nonadecanoic acid standard. Individual compounds were identified by relative retention time, GC–MS, and comparison with known standards when available. Mass spectral analysis was performed using similar analytical GC conditions on a Shimadzu QP5050 GC–MS operating in the EI mode (70 eV, trap, scan rate *m/z* 50–550 in 1 s). The source and interface were held at 280 °C, and 1.5 μl of sample was injected in the splitless mode. Results are presented as the mean concentration (±SD) of analyses of three leaves, consisting of one leaf sampled from each of three plants.

4.4. Biomass productivity

Stem diameter measurements (±0.1 mm) were taken at 30 cm height from the ground for the inner 16 plants in each plot at the end of the 2002 growing season. Stem diameters were converted to a cross-sectional area and the cross-sectional areas of all the stems for each plant were summed. Basal area was calculated in m^2 ha^{-1} based on the sum of stem areas and the area of the plot.

4.5. Statistical analysis

Percentage data was arcsine transformed as suggested by Zar (1984). Data comparisons using paired *t*-tests and regression analysis were performed using Minitab (version 14) and results were deemed significant at $\alpha = 0.05$.

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