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Diversity of cuticular wax among *Salix* species and *Populus* species hybrids

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Abstract

The leaf cuticular waxes of three *Salix* species and two *Populus* species hybrids, selected for their ability to produce high amounts of biomass, were characterized. Samples were extracted in CH_2Cl_2 three times over the growing season. Low kV SEM was utilized to observe differences in the ultrastructure of leaf surfaces from each clone. Homologous series of wax components were classified into organic groups, and the variation in wax components due to clone, sample time, and their interaction was identified. All *Salix* species and *Populus* species hybrids showed differences in total wax load at each sampling period, whereas the pattern of wax deposition over time differed only between the *Salix* species. A strong positive relationship was identified between the entire homologous series of alcohols and total wax load in all clones. Similarly strong relationships were observed between fatty acids and total wax load as well as fatty acids and alcohols in two *Salix* species and one *Populus* species hybrid. One *Salix* species, *S. dasyclados*, also displayed a strong positive relationship between alcohols and alkanes. These data indicate that species grown under the same environmental conditions produce measurably different cuticular waxes and that regulation of wax production appears to be different in each species. The important roles cuticular waxes play in drought tolerance, pest, and pathogen resistance, as well as the ease of wax extraction and analysis, strongly suggest that the characteristics of the cuticular wax may prove to be useful selectable traits in a breeding program.

Keywords: Biomass; Cuticle; Poplar; *Populus*; Short-rotation intensive culture; Willow; *Salix*; Salicaceae

1. Introduction

The cuticle forms a protective barrier over the aerial surfaces of plants and functions primarily as a barrier to water vapor loss (Riederer and Schreiber, 1995; Schreiber et al., 1996). The uppermost layer of the cuticle, the cuticular wax, is hydrophobic and is comprised of multiple, homologous series of long-chained lipid molecules, principally hydrocarbons, alcohols, fatty acids, sterols, ketones, and aldehydes (Bianchi, 1995; Jeffree, 1996). The chemical characteristics of the cuticular wax and increases in wax load are the primary determinants of the permeability of the plant cuticle (Schönherr, 1976; Schreiber et al., 1996). For instance, the increase in wax load has been inversely correlated with rates of cuticular transpiration in sorghum (Jordan et al., 1984) and the permeability of water and organic acids across isolated plant cuticles varies by species (Niederl et al., 1998). Not only is a plant genetically predisposed to produce this waxy cuticle, but plants can also deposit additional cuticular wax under specific environmental conditions (Giese, 1975; Blum et al., 1991; Ashraf and Idrees, 1993; Ashraf and Yasmin, 1995).

Water stress, pest, and pathogen susceptibility contribute to general decreases in dry matter production and seed yield (Jones, 1992). Plants that successfully avoid water deficit conserve water by limiting the rate of

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water loss through short-term responses or adaptations that minimize transpirational flux. Increased wax load, which reduces cuticular permeability, contributes to water conservation (Jones, 1992). Increased deposition of cuticular wax in response to drought stress has been correlated with drought tolerance in agricultural crops and grasses, such as wheat and rye grass (Bianchi and Figini, 1986; Blum and Johnson, 1992; Clarke et al., 1993, 1994; McWhorter, 1993; Jefferson, 1994). The characteristics of the cuticle influence pest and pathogen susceptibility in the plant. The ultrastructure of the cuticle affects the wetability of the leaf, which may in turn play a role in germination of fungal spores. Similarly, surface topography, partially determined by the ultrastructure of the cuticle, is also important for insect behavior, mobility, and oviposition (Eigenbrode, 1996). Many plant pathogenic fungi produce cutinases to degrade cutin and facilitate hyphal penetration of the host cuticle, providing further evidence of the importance of the cuticle in plant defense (Kolattukudy et al., 1994). Traits associated with drought tolerance, pest, and pathogen resistance, such as wax load and composition, show genotypic variation and therefore have potential use in breeding and selection of superior genotypes (Clarke et al., 1994; Jefferson, 1994).

The objectives of this study were to: (1) determine if there was a significant difference in the ultrastructure of the leaf due to the cuticular waxes of hybrid poplar and *Salix* species selected for high biomass production; (2) identify, quantify, and compare the components of the cuticular wax of these clones; and (3) determine if the wax loads of these clones were significantly different at specific times during the growing season. We discovered that there were measurable, significant differences in total wax load and wax composition among the three *Salix* species over the growing season, whereas the two hybrid poplar clones displayed similar patterns of deposition and composition. Furthermore, we identified the main components of the wax for each clone and determined that the relative abundance of many of the components differed significantly at specific points in the growing season within each clonal line and also between species.

2. Results and discussion

2.1. SEM analysis of adaxial leaf surface

Low kV SEM was used to examine the adaxial leaf surfaces of three *Salix* species and two *Populus* species hybrids in May and September. The leaf surface ultrastructure was very different among the three species of *Salix*, whereas the two *Populus* species hybrids were very similar (Fig. 1). Two types of trichome structures were observed on *Salix eriocephala* (clone S25) leaves, while only a single type of trichome was observed on *S. dasyclados* (clone SV1) leaves (Fig. 1C and E). Trichomes were not observed on leaves of *S. purpurea* (clone 94003) (Fig. 1A) nor on either of the hybrid poplars, DN34 (*Populus deltoides* × *P. nigra*) or NM6 (*P. nigra* × *P. maximowiczii*; Fig. 1G and I). The ultrastructure of the cuticular wax influenced the topography of the adaxial leaf surface. The surface of the cuticular wax appeared smooth without any crystalline deposits for *S. eriocephala*, *S. dasyclados*, DN34 and NM6 (Fig. 1C, E, G, I). In contrast, the adaxial surface of *S. purpurea* was characterized by extensive crystalline wax deposits (Fig. 1A). The structure of the crystals deposited on the adaxial surface of *S. purpurea* was slightly different in May and September (Fig. 1A and B), but a dramatic change in crystal density was not apparent. No obvious change in wax deposition was observed by SEM between May and September for any of the other clones (Fig. 1C–J).

2.2. Components of the cuticular wax

Total wax load varied for all species over the growing season (Fig. 2). For all five species analyzed by GC, fatty acids, alcohols, and alkanes were identified in the surface wax extracted from leaves (Figs. 3 and 4). Yield and composition of the individual organic groups differed greatly among the *Salix* species, but fewer differences were observed between the hybrid poplar clones. Multivariate analysis of variance performed on all identified wax components revealed that the variation between samples were due to clone ($P<0.0001$), sample time ($P<0.0001$), and the interaction between sample time and clone ($P<0.0001$) in the overall model based on Wilke's λ statistic. The effect due to clone was always significant ($P<0.05$) whereas the other effects were dependent upon sample time and the interaction between sample time and clone. A better statistical visualization of the wax composition was obtained when odd-chained C_{25}–C_{31} alkanes, even-chained C_{22}–C_{30} alcohols, and even-chained C_{20}–C_{30} fatty acids were grouped as alkanes, alcohols, and fatty acids respectively. Combining components into these organic groups did not significantly alter the statistical results. The fatty acid and alcohol data reported here include fatty acids and alcohols derived from the hydrolysis of wax esters during the processing of the samples. These results are similar to the wax analysis of nine *Salix* clones representing three species of willow (Hietala et al., 1995). Similar to our results, large differences in wax composition were observed between species. For instance, alkanes were the major wax component for *S. viminalis*, whereas *S. myrsinifolia* and *S. dasyclados* had approximately equal amounts of *n*-alkanes, alcohols, and fatty acids. 1-Docosanol was the dominant alcohol component in *S. viminalis*, whereas the other clones had

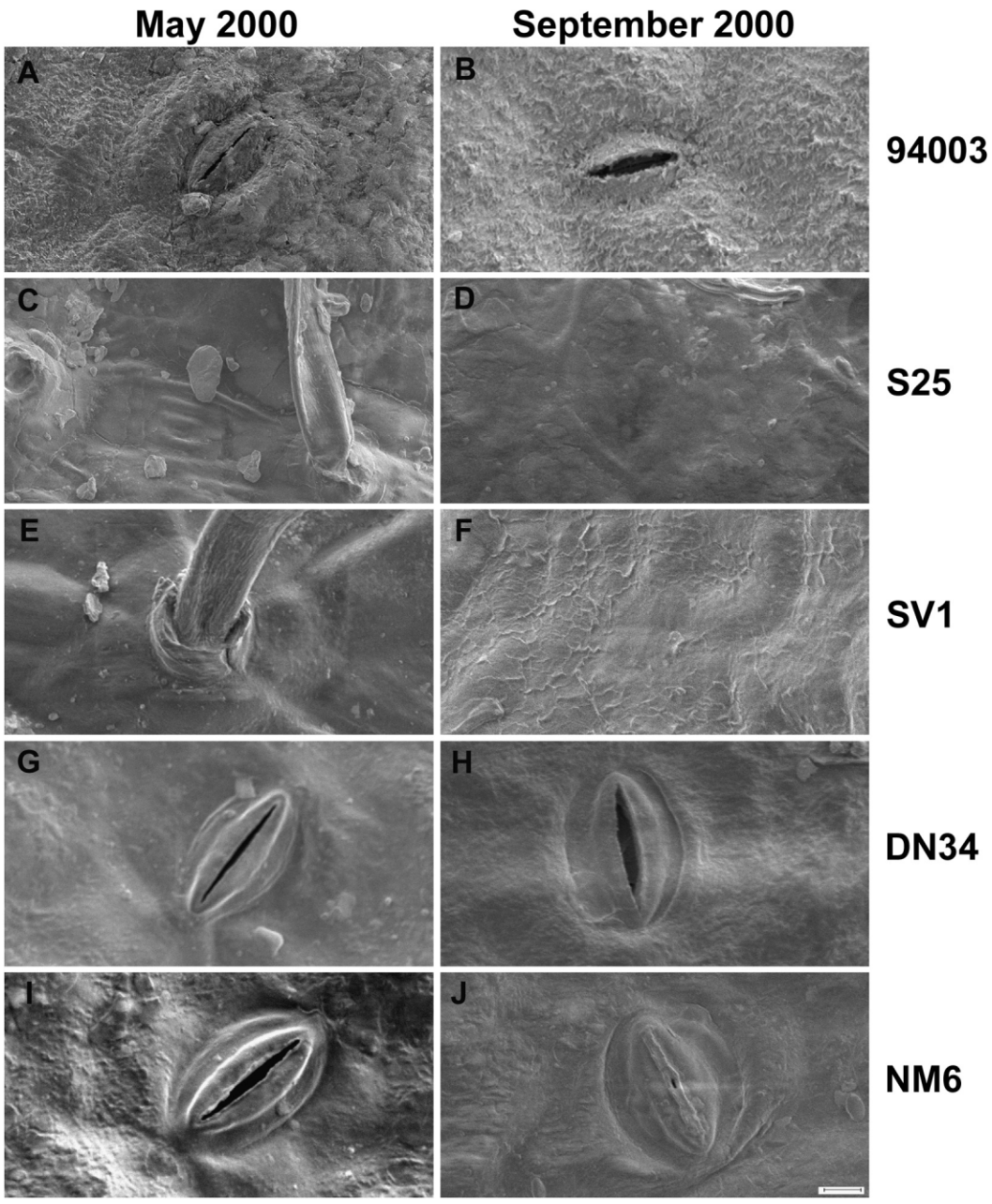

Fig. 1. SEM of the adaxial surfaces of leaves from *Salix* species and *Populus* species hybrids. On the left (A, C, E, G, and I) are images of leaves collected in May, and on the right (B, D, F, H, and J) are images of leaves collected in September. A, B: *S. purpurea* (clone 94003); C, D: *S. eriocephala* (clone S25); E, F: *S. dasyclados* (clone SV1); G, H: hybrid poplar clone DN34; I, J: hybrid poplar clone NM6. Magnification is equal for all panels. Size bar = 10 μm.

1-hexacosanol as their major alcohol (Hietala et al., 1995).

Although fatty acids, alcohols, and alkanes were the main components in the cuticular wax, alkenes, ketones, secondary alcohols, odd-chained alcohols, and triterpenoids, in the form of oleanenes, were also present. Aldehydes, which are often present in fairly large concentrations in cuticular waxes (Hemmers and Gülz, 1986; Gülz et al., 1991; Hietala et al., 1995; Rashotte et al., 1997) were identified, but did not contribute appreciably to the total wax. Ketones, although present, also constituted only a very small portion of the total wax load. Odd-chained alcohols were found in only NM6, *S. purpurea*, and *S. dasyclados*. Trace amounts of many other components, although present in the wax, were in such low concentration that they were not identified. Combined, these components represented 3–46% of the total wax load (Figs. 3 and 4). The relative abundance of wax esters in the wax was not determined in this study.

Variability in leaf cuticular wax load has been shown among willow clones (Hietala et al., 1995), but this is, to our knowledge, the first report of a variation in the

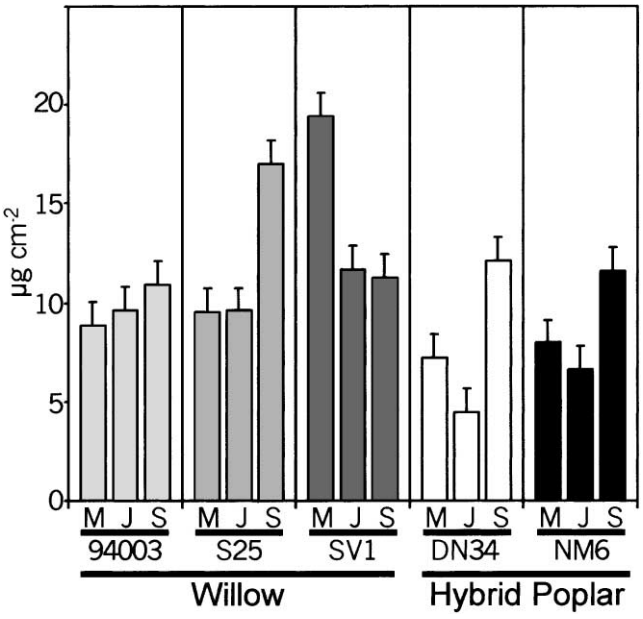

Fig. 2. Total wax load in CH_2Cl_2 extracts from fully expanded leaves of *Salix* species and *Populus* species hybrids. Repeated sampling of leaves was performed with three individuals in replicated plots at three times over the growing season. *Salix* clone 94003 is *S. purpurea*; S25 is *S. eriocephala*; and SV1 is *S. dasyclados*. Clone DN34 is a species hybrid of *P. deltoides* × *P. nigra* and NM6 is a species hybrid of *P. nigra* × *P. maximowiczii*. M, May; J, July; S, September. Each bar represents the average of six samples. Error bars indicate the standard error.

pattern of wax deposition over a growing season for multiple species within the same genus. Environmental and developmental variations in wax composition have been identified in a variety of annual and perennial species (Gülz et al., 1991; Shepherd et al., 1997; Hauke and Schreiber, 1998; Rhee et al., 1998; Sase et al., 1998). The importance of genetic variation on wax composition and load appears to be specific to individual species. Variation has been identified among species within a genus (Hemmers and Gülz, 1986; Vioque et al., 1994), as well as among cultivars within a species at one specific time point (Jordan et al., 1983; Bianchi and Figini, 1986; Bianchi et al., 1993; Jefferson, 1994). In contrast, very little wax variation exists among ecotypes of *Arabidopsis thaliana* (Rashotte et al., 1997) or among seven species of *Sedum* (Stevens et al., 1994). The results from this study support the idea that there are interactions between environmental and genetic factors that influence wax composition and deposition in *Salix* and *Populus*.

2.3. Differences in total wax load among species

Total wax load for each species was dependent upon the time of sampling. The leaf wax load differed significantly ($P<0.05$) between *Salix* species *S. dasyclados*, *S. eriocephala*, and *S. purpurea*, at different points over the growing season (Fig. 2), whereas wax load differed significantly between the two *Populus* species hybrids only during the July sampling. *Salix eriocephala* had a significantly higher wax load in September compared to all the other clones, whereas *S. dasyclados* had a significantly higher wax load in May.

Each *Salix* species displayed a different pattern of wax deposition (Fig. 2). Both *S. purpurea* and *S. eriocephala* deposited cuticular wax in May and the load remained the same in July. *Salix eriocephala* had a much greater wax load in September (1.5-fold increase compared to the July sampling), whereas *S. purpurea* only had a slightly higher wax load. *Salix dasyclados* deposited the largest amount of wax of all clones in May, but had a much smaller wax load in July and September. Unlike the *Salix* species, both *Populus* species hybrids exhibited very similar patterns of deposition over time. Clones DN34 and NM6 had less wax in the July samples compared to the May samples, but both clones exhibited a substantially larger wax load in September.

2.4. Analysis of wax composition

2.4.1. Hydrocarbons

A homologous series of hydrocarbons with chain lengths ranging from C_{25} to C_{31} was present in all clones throughout the entire season. Minor amounts of alkenes were also identified in all five clones, but in such low amounts that their chain length could not be confirmed. A strikingly large difference in the alkane composition was evident among the *Salix* species. Over all three sampling periods, 44–51% of the total wax load in *S. dasyclados* constituted C_{25}–C_{31} alkanes (Fig. 3). This was in comparison to only 5–9% in *S. eriocephala* and 16–23% in *S. purpurea*. The *Populus* species hybrids also had a high relative abundance of alkanes (47 and 44% in May and July, respectively), but in September, alkanes represented only 23–29% of the total wax load (Fig. 4). In contrast, the percentage of hydrocarbons/dry weight in *Tilia tomentosa* leaves increased steadily during May, June and July, then decreased in August and September (Gülz et al., 1991). The significance of hydrocarbon content is still unknown and conflicting results have been reported. High hydrocarbon content has been associated with overwintering survival in *Salix* (Hietala et al., 1995) and glaucousness in the genus *Coincya* of the family Brassicaceae (Vioque et al., 1994), whereas in *Encephalartos,* a member of the cycad genus, alkanes dominated only in non-glaucous species (Osborne and Stevens, 1996). The hydrophobic nature of hydrocarbons may affect the permeability of the cuticle. Cuticle permeability varies between plant species and between organs of the same species (for review, see Riederer and Schreiber, 1995). As yet, the relationship between hydrocarbon content and permeability of the cuticle has not been established, but the permeability of

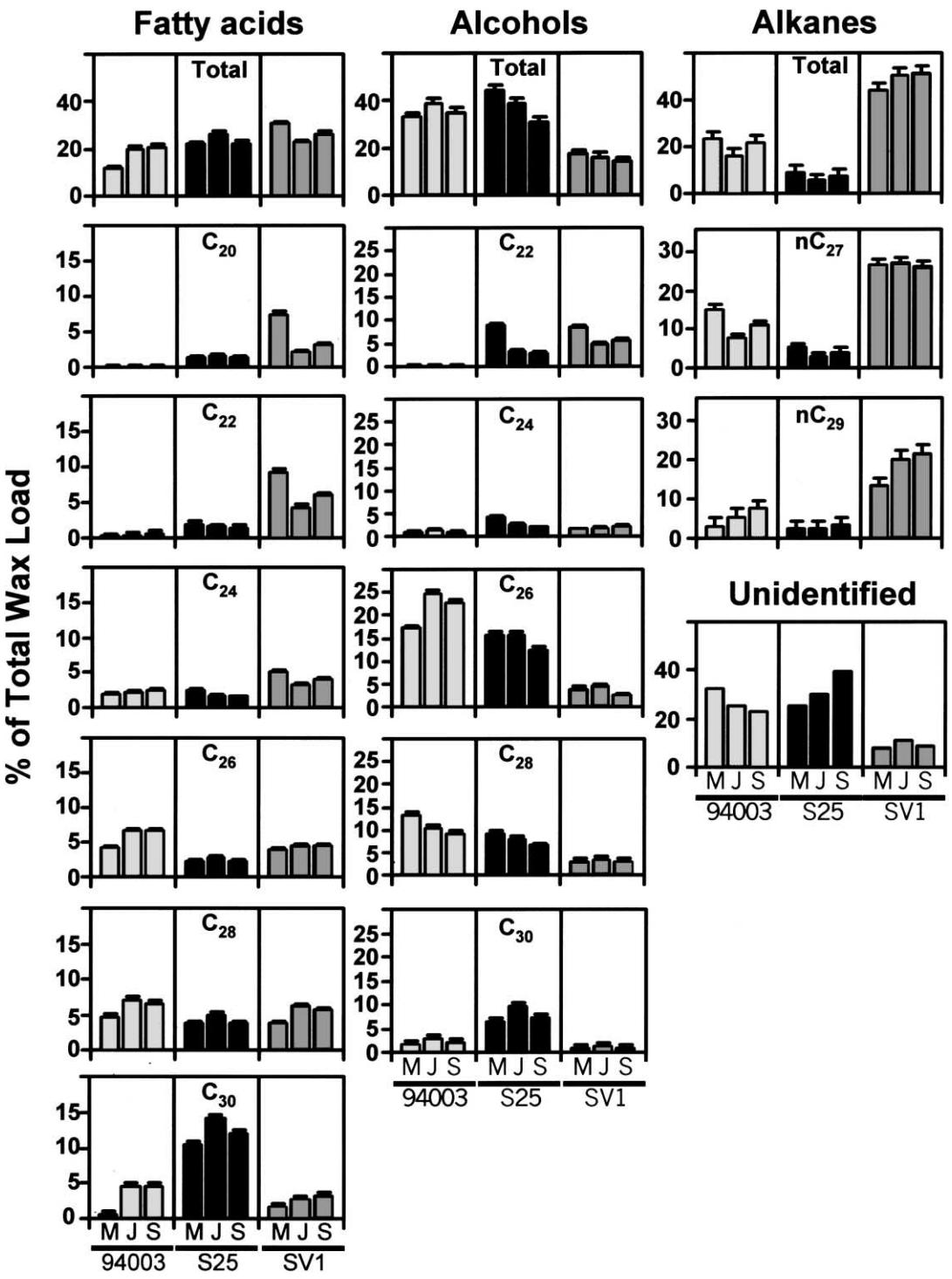

Fig. 3. Relative abundance of fatty acids, alcohols, alkanes, and unidentified components in the CH_2Cl_2-soluble fraction of the cuticular wax of three *Salix* species. Repeated sampling of leaves was performed with three individuals in replicated plots at three times over the growing season. *Salix* clone 94003 is *S. purpurea*; S25 is *S. eriocephala*; and SV1 is *S. dasyclados*. M, May; J, July; S, September. Each bar represents the average of six samples. Error bars indicate the standard error. All components are identified by chain length and described in Section 4.5.

water and other solutes appears to be comparable to the permeability through synthetic polymers of equal thickness (Riederer and Schreiber, 1995).

n-Heptacosane (C_{27}), *n*-nonacosane (C_{29}), and/or *n*-hentriacontane (C_{31}) are commonly the most dominant members of the *n*-alkane homologues in cuticular wax (Gülz, 1994). Although all the clones analyzed contained the same alkane components there were large differences in the relative proportion of alkanes among the different clones (Figs. 3 and 4). *n*-Heptacosane was

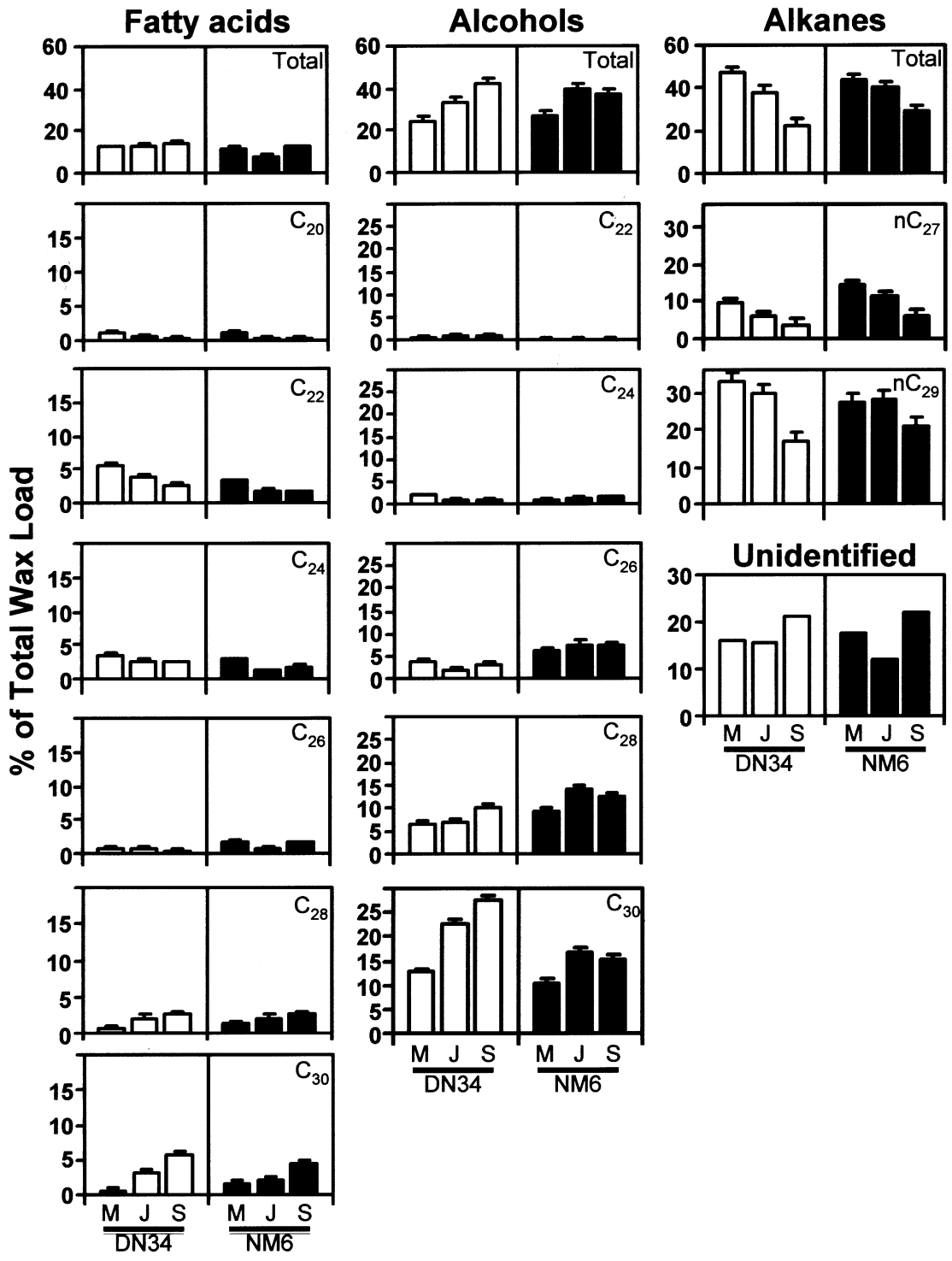

Fig. 4. Relative abundance of fatty acids, alcohols, alkanes, and unidentified components in the CH_2Cl_2-soluble fraction of the cuticular wax of two *Populus* species hybrids. Repeated sampling of leaves was performed with three individuals in replicated plots at three times over the growing season. Clone DN34 is a species hybrid of *P. deltoides* × *P. nigra* and NM6 is a species hybrid of *P. nigra* × *P. maximowiczii*. M, May; J, July; S, September. Each bar represents the average of six samples. Error bars indicate the standard error. All components are identified by chain length and described in Section 4.5.

the major alkane component for the *Salix* clones, whereas *n*-nonacosane was the major alkane component for the hybrid poplar clones representing 17–33% of the total wax load (Figs. 3 and 4). The relative abundance of *n*-heptacosane in *S. eriocephala* wax ranged from 3–5% of total wax load, compared to *S. dasyclados* where the proportion of *n*-heptacosane was close to 27% for all three sampling periods (Fig. 3). *n*-Pentacosane and

n-hentriacontane represented only a small portion of the total wax load (Table 1).

The pattern of deposition was also different between the *Populus* species hybrids and the *Salix* species. The *Populus* species hybrids displayed a much smaller proportion of both *n*-heptacosane and *n*-nonacosane in the September sampling compared to the May sampling, whereas both *S. purpurea* and *S. dasyclados* had a greater relative abundance of *n*-nonacosane in the September sampling compared to the May sampling (Figs. 3 and 4).

2.4.2. Fatty acids

Homologous series of fatty acids with even-numbered chain lengths ranging from C_{16} to C_{30} were identified in the wax isolated from all the clones. Analysis focused on the longer chain (C_{20}–C_{30}) fatty acids, which represented the major portion of the fatty acids in the wax extract (Figs. 3 and 4). C_{16} and C_{18} fatty acids were identified, but since they are not unique to cuticular wax, they were not included in the homologous series. The relative abundance of total fatty acids remained fairly constant over all three sampling periods, but the relative distribution of some of the fatty acid homologues differed among the three *Salix* species (Fig. 3). For instance, *S. dasyclados* had a relatively high percentage of eicosanoic acid (C_{20}) and a relatively low percentage of triacontanoic acid (C_{30}) in the May sample compared to the other two *Salix* species. Alternatively, the cuticular wax of *S. eriocephala* had a low percentage of eicosanoic acid and a very high percentage of triacontanoic acid.

In contrast to the *Salix* species, the *Populus* species hybrids displayed similar distributions of fatty acid homologues. Neither clone had a large concentration of any single fatty acid homologue (Fig. 4), but both clones had a relatively high percentage of docosanoic (C_{22}) and tetracosanoic (C_{24}) acids in May compared to September and a relatively high percentage of triacontanoic acid in September compared to May.

2.4.3. Alcohols

A homologous series of long-chain primary alcohols (C_{22}, C_{24}, C_{26}, C_{28}, and C_{30}) was identified in the wax of all of the clones (Figs. 3 and 4). Odd-chained alcohols (1-heptacosanol (C_{27}) and 1-nonacosanol (C_{29}) were also identified in the cuticular wax of a single *Salix* species, *S. dasyclados*, and a single *Populus* species hybrid, NM6, but the relative abundance of those components in the total wax load was very small (data not shown). The cuticular wax of *S. purpurea* contained a high percentage of 1-hexacosanol (C_{26}), while the wax of DN34 had a high percentage of 1-triacosanol (C_{30}) (Figs. 3 and 4). Alternatively, 1-docosanol (C_{22}) was the most abundant alcohol in the cuticular wax of *S. dasyclados*.

Primary alcohols, thought to be derived from an acyl-reduction pathway, are precursors of wax esters (von Wettstein-Knowles, 1995; Post-Beittenmiller, 1996). We identified a large difference in the relative abundance of primary alcohols between clones at all three sample periods. The relative abundance of alcohols decreased at each sampling period for *S. eriocephala*, but remained essentially the same for *S. purpurea* and *S. dasyclados* (Fig. 3). Both hybrid poplar clones had a low relative abundance of alcohols in May, but the abundance of alcohols increased in the July and September sampling periods (Fig. 4).

2.5. Cuticular wax structure

In this study we have shown that there are significant differences in the ultrastructure of the leaf surface among the *Salix* species due to the morphology of the appendages and the composition of the cuticular wax, but we are unable to correlate specific ultrastructure features with a single component of the wax. It should be noted that the SEM images were collected from only the adaxial leaf surface, while the wax extracts included compounds deposited on both the adaxial and abaxial surfaces. One predominant alkane or alcohol homologue

Table 1
Absolute and relative (% of total wax) amounts of nC_{25} and nC_{31} alkanes in the cuticular wax of *Salix* species and *Populus* species hybrids at three time points over the growing season[a]

Species/clone	nC_{25}			nC_{31}		
	μg cm^{-2} (%) May	μg cm^{-2} (%) July	μg cm^{-2} (%) September	μg cm^{-2} (%) May	μg cm^{-2} (%) July	μg cm^{-2} (%) September
S. purpurea (clone 94003)	0.5 (4.8)	0.3 (3.0)	0.3 (3.1)	– (–)	– (–)	– (–)
S. eriocephala (clone S25)	0.1 (1.2)	0.1 (0.3)	0.1 (0.3)	– (–)	– (–)	– (–)
S. dasyclados (clone SV1)	0.5 (2.7)	0.2 (2.1)	0.2 (1.4)	0.1 (1.0)	tr (1.0)	0.1 (1.6)
Hybrid poplar clone DN34	0.1 (0.7)	tr (0.2)	– (–)	0.1 (3.6)	0.4 (2.5)	0.1 (1.6)
Hybrid poplar clone NM6	0.1 (0.8)	tr (0.3)	tr (0.1)	tr (1.5)	tr (0.6)	0.1 (1.8)

[a] tr = trace, – = below level of detection.

is often responsible for the shape and structure of a crystalline structure on the surface of cuticular wax (Gülz et al., 1991; Stevens et al., 1994; von Wettstein-Knowles, 1995). In *Nicotiana glauca*, greater than 82% of the wax is comprised of *n*-hentriacontane, and distinct crystalline-like deposits are present on the surface of the leaf (Cameron, Teece, and Smart, unpublished data). However, the crystals observed on the surface of leaves from *S. purpurea* do not appear to arise from a single component of the wax, since its cuticular wax was not composed of predominantly one particular component. *Salix purpurea* produced moderate amounts of *n*-heptacosane and *n*-nonacosane, but the relative abundance of those components was not significantly different from either of the *Populus* species hybrids. It is possible that 1-hexacosanol (61% of total alcohol content) was responsible for the crystal formation in *S. purpurea* (Meusel et al., 1999), but this seems unlikely, since DN34 produced a comparable percentage of 1-triacontanol and did not display crystalline structures. Wax crystal formation has also been attributed to triterpenoid concentration in *Sedum* (Stevens et al., 1994). Triterpenoids, in the form of oleanenes, were identified in *S. dasyclados* and *S. eriocephala*, but were not detectable in *S. purpurea*. Taken together, these results suggest that the crystalline structure observed in May and September on the adaxial surface of *S. purpurea* may be the result of a unique combination of components, rather than the predominance of a single component. This is similar to the situation in *Arabidopsis thaliana*, where crystal formation has been associated with multiple components in stem cuticular wax (Rashotte and Feldmann, 1998).

2.6. Relationships between groups of wax components

The differences in wax components observed between the *Salix* species suggest that each *Salix* species employs different mechanisms in depositing cuticular wax or has different regulatory mechanisms. The relative contribution of specific homologous series of components to the cuticular wax composition is regulated by multiple biosynthetic pathways (Post-Beittenmiller, 1996). These pathways appear to be tightly regulated with intermediates being shared between pathways (Vioque and Kolattukudy, 1997; Post-Beittenmiller, 1998). It is currently thought that odd-chained alkanes are produced through a decarbonylation process, whereas primary alcohols are produced through an acyl-reduction pathway (von Wettstein-Knowles, 1995; Post-Beittenmiller, 1996). In this study, the relative abundance and absolute relationship of each composite class varied between species during the active growing season. For instance, *S. eriocephala* had a much greater relative abundance of alcohols compared to alkanes, whereas *S. dasyclados* had a much higher relative abundance of alkanes compared to alcohols (Fig. 3). *Salix purpurea*, on the other hand, had similar amounts of each group of components (Fig. 3). Strong positive linear relationships were observed between alcohol and total wax content for all *Salix* species over all three sampling periods (Fig. 5A). Slope is only significantly different ($P < 0.05$) between *S. dasyclados* and all the other species. Fatty acid content was tightly correlated with total wax for *S. eriocephala* and *S. dasyclados*, which was expected since fatty acids are thought to be precursors of wax biosynthesis (von Wettstein-Knowles, 1995; Post-Beittenmiller, 1996; Fig. 5B). Yet there was only a loose correlation between fatty acid content and total wax for *S. purpurea* (Fig. 5B). While not as tightly correlated, a similar trend is apparent between fatty acid and alcohol content (Fig. 5C). These results suggest that there was some other factor(s) influencing the production of alcohols and total wax load in *S. purpurea*. In the chemical analysis of the cuticular wax, esters were hydrolyzed to fatty acids and alcohols and are represented as such in our wax analysis. Waxes with a large wax ester content should contain a disproportionately greater amount of fatty acids and alcohols when compared to total wax load. Since a strong correlation does not exist in *S. purpurea*, we suggest that the cuticular wax of *S. purpurea* may contain a higher proportion of wax esters compared to the other species, and analysis of the wax composition should be modified in the future to accommodate this variation. Since alcohols and alkanes are derived from different biosynthetic pathways, one would not expect a correlation between amounts of these components. However, there was a fairly strong positive correlation between the two classes in the cuticular wax of *S. dasyclados*, suggesting that the decarbonylation and acyl-reduction pathways may be interconnected, either through the sharing of intermediates or through some form of global regulation (Fig. 5D). These data suggest that the regulation of the acyl-reduction and decarbonylation pathways may be different for each species and, therefore, may represent a point of differentiation in the composition of the cuticular wax.

3. Concluding remarks

Fast growing, highly productive willow and hybrid poplar grown in short-rotation intensive culture are being studied as potential bioenergy crops. These species have the potential to produce biomass that can be used as a renewable feedstock for bioenergy, as a source of fiber, and as a raw material for biobased products (White et al., 1993). Development of fast-growing clonal lines that are pest and pathogen resistant will be critical for successful commercial production. The five clones used in this study have previously been identified as

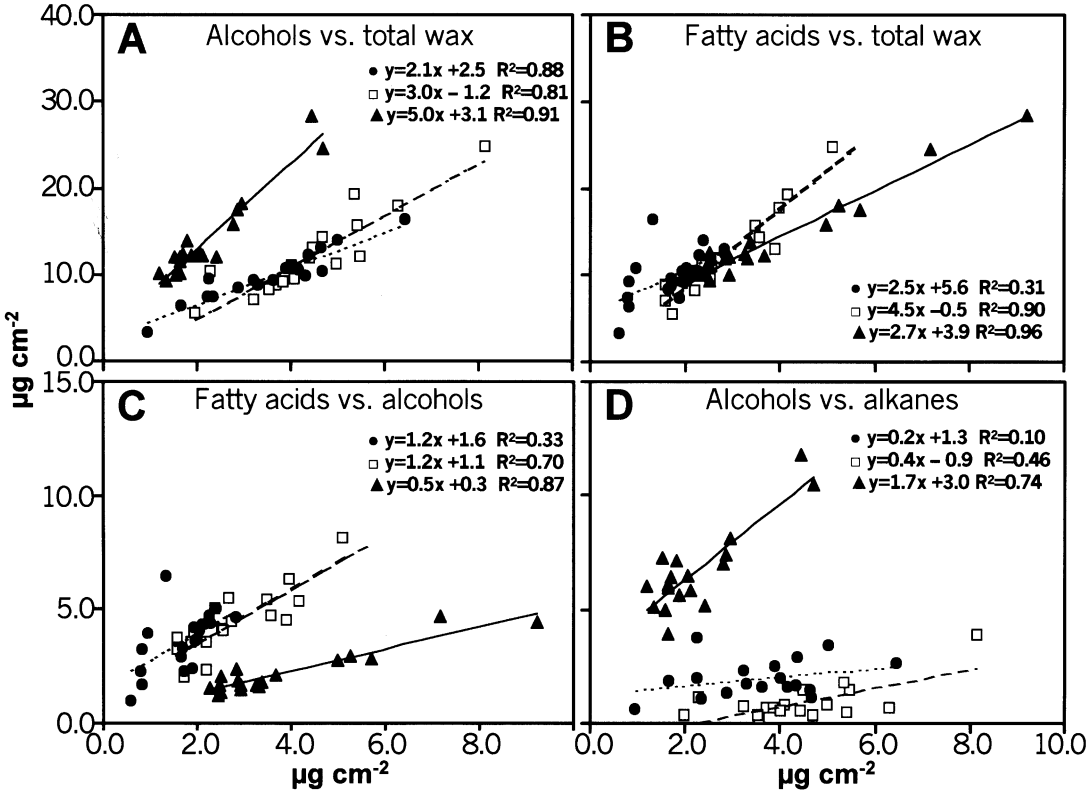

Fig. 5. Relationships between abundance of components identified in the cuticular wax of the *Salix* species. Relationships between (A) alcohols and total wax load; (B) fatty acids and total wax load; (C) fatty acids and alcohols; and (D) alcohols and alkanes with linear trend lines. Each point represents one sample. Organic classes are grouped as follows: fatty acids (C_{20}–C_{30}); alcohols (C_{22}–C_{30}); and alkanes (C_{25}–C_{31}). ● = *S. purpurea* (clone 94003); □ = *S. eriocephala* (clone S25); ▲ = *S. dasyclados* (clone SV1).

high biomass producers in short-rotation intensive culture systems, yet efforts are underway to breed for superior genotypes (Lindegaard and Barker, 1997; Rönnberg-Wästljung and Gullberg, 1999; Kopp et al., 2001).

Developmental traits, such as coppice vigor, sylleptic branching, wood density, and the length of the growing period, have been examined for their potential in selection of high biomass producers through breeding (Lo and Abrahamson, 1996; Rönnberg-Wästljung and Gullberg, 1996, 1999; Robison and Raffi, 1998; Tharakan et al., 2001). To our knowledge, cuticular wax has not been examined as a selectable trait, yet its characteristics influence drought tolerance, as well as pest and pathogen defense in many agricultural crops. The ease of extraction and analysis would allow for early screening and relatively high throughput in analyzing progeny. In this study we have shown that there were major differences in total wax load and wax composition between the *Salix* species grown under identical conditions in the field. Future work will attempt to demonstrate the patterns of inheritance of wax properties in progeny of intraspecific crosses of willow and among *Salix* species hybrids. Further studies are also needed to examine the impact of cuticular wax load and composition on biomass production under drought conditions, as well as under pest and pathogen attack.

4. Experimental

4.1. Study site

This study was performed at the SUNY-ESF Genetics Field Station in Tully, New York (42 47′ 30″ N, 76 07′ 30″ W). The site was established in 1997 as a randomized complete block design with four replications of 38 *Salix* species and *Populus* species hybrids (Tharakan et al., 2001) on a 1 + 3 rotation. Each individual plot contained 48 plants of a single clone. During the summer of 2000, the plants were in the third year of the first 3-year harvest cycle. Single clones were chosen for this study as representatives of three *Salix* species and two *Populus* species hybrids being tested as bioenergy crops. Leaf samples were collected from three individuals in duplicate plots at the end of May, July, and September of 2000. The first fully expanded leaf located on the

tallest stem physically accessible from the ground was removed. The intent was to uniformly sample leaves exposed to the maximum amount of sunlight and at the same developmental stage. Leaves sampled in July and September were larger than those sampled in May, and it is not known whether those leaves were exactly the same age (number of days fully expanded) at the three sampling times. The same individuals were sampled each time.

4.2. Cuticular wax analysis

The surface lipids were extracted from four 12-mm^2 leaf disks cut from fully expanded leaves by immersing tissue in CH_2Cl_2 for 30 s. The extracts were evaporated under a stream of N_2 and the dried wax residues were prepared for GC by methylation with boron trifluoride (BF_3:MeOH) and by silylation with *bis*-(trimethylsilyl)-trifluoroacetamide (BSTFA). Samples were re-dissolved in 2,2,4-trimethylpentane for FID-GC (Hewlett Packard model 6890) analysis on a DB-5 capillary column (30 m) using He as the carrier gas (2 ml min^{-1}). An initial temperature of 60 °C was increased 15 °C min^{-1} to 200 °C, then 4 °C min^{-1} to 300 °C, and remained at 300 °C for 5 min. Individual components were identified by their mass spectra and quantified relative to an internal standard of *n*-hexatriacontane (C_{36}). Total wax load was determined by summing the area under all the peaks after 10 min as a ratio to the area of the *n*-hexatriacontane standard.

4.3. Scanning electron microscopy

The adaxial surfaces of leaf samples obtained from the same leaves used for wax analysis were examined using a Jeol scanning electron microscope at low kV (1.4–1.8). Two samples were collected from each of the clones during May and September 2000.

4.4. Statistical analysis

Multivariate analysis of variance was used to test for differences on the total yield and composition of the CH_2Cl_2-soluble fraction of the surface wax in three *Salix* species and two *Populus* species hybrids at three points in time over one growing season. A randomized complete block, repeated measures, factorial design blocked on plots was used to test for main effects of clone and sample time and their interaction. All statistical analyses were performed using SAS Version 8.1 (SAS, 1999) at a critical level of $\alpha = 0.05$. SAS PROC GLM (SAS, 1999) was used to evaluate the differences in the wax components among the individual clones. When a significant ($P < 0.05$) interaction was observed, Tukey's mean studentized range test was used to determine significant difference among clones sliced by time. Analysis of covariance was used to contrast the relationship between the total identified fatty acids, alcohols, alkanes, and total wax load among clones.

4.5. Chemicals identified as cuticular wax constituents

Eicosanoic, docosanoic, tetracosanoic, hexacosanoic, octacosanoic, and triacontanoic acids are described here as C_{20}, C_{22}, C_{24}, C_{26}, C_{28}, and C_{30} fatty acids; 1-docosanol, 1-tetracosanol, 1-hexacosanol, 1-octacosanol, and 1-triacontanol as C_{22}, C_{24}, C_{26}, C_{28}, and C_{30} alcohols; *n*-pentacosane, *n*-heptacosane, *n*-nonacosane, *n*-hentriacontane, *n*-hexatriacontane as C_{25}, C_{27}, C_{29}, C_{31} and C_{36} alkanes.

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