Pergamon 0031-9422(95)00263-4 Phytochemistry, Vol. 40, No. 1, pp. 23–27, 1995

0031-9422/95 $9.50 + 0.00

EPICUTICULAR WAXES OF *SALIX* SPECIES IN RELATION TO THEIR OVERWINTERING SURVIVAL AND BIOMASS PRODUCTIVITY

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(*Received in revised form* 20 *February* 1995)

Key Word Index—*Salix myrsinifolia*; *S. dasyclados*; *S. viminalis*; Salicacae; willow; epicuticular wax; plant surface; frost tolerance; short-rotation biomass; biomass productivity.

Abstract—The epicuticular leaf waxes of nine willow clones (one *Salix myrsinifolia*, four *Salix dasyclados*, one *Salix* hybrid and three *Salix viminalis* clones) with different overwintering survival rates and biomass productivities were analysed by combined gas chromatography–mass spectrometry. The waxes were composed of *n*-alkanes (C_{21}–C_{31}), long-chain aliphatic esters (C_{36}–C_{58}), *n*-alcohols (C_{20}–C_{30}), *n*-aldehydes (C_{24}–C_{30}) and saturated free fatty acids (C_{16}–C_{30}). Two types of waxes were observed. The *S. viminalis* clones had *n*-alkanes as their major wax component and *n*-docosanol as their major *n*-alcohol, whereas the other clones contained approximately equal proportions of *n*-alkanes, free fatty acids, *n*-alcohol and *n*-aldehydes and had *n*-hexacosanol as their major *n*-alcohol. The total epicuticular leaf wax contents varied from 3 $\mu g\,cm^{-2}$ to 24.9 $\mu g\,cm^{-2}$ and epicuticular *n*-alkane contents from 0.7 $\mu g\,cm^{-2}$ to 14.8 $\mu g\,cm^{-2}$. A correlation between high wax content, high *n*-alkane content, two overwintering survival and high biomass productivity of the clones was observed.

INTRODUCTION

Short-rotation biomass plantations have been suggested as an alternative for wasteland use [1]. The biomass can be used either as an energy source or as a raw material for chemical processes [2]. Willow is a suitable plant for high-yield biomass plantations because of its fast growth rate, ease of hybridization, the high frost resistance of certain species and its ability to adapt to growing sites that are poor in light and nutrients [1, 3]. Yields as high as 36 t dry matter ha^{-1} have been achieved by irrigation and fertilization of willow clones [4] and even higher yields may be postulated from the results published by Lumme and Törmälä [3].

International projects have been aimed at the selection of candidates for short-rotation plantations of willow [5]. For short-rotation plantations in northern conditions willow clones need to possess both high biomass productivity and high overwintering survival. Unfortunately, willow clones that grow fast often have low overwintering survival. The overwintering survival of willow clones is connected with their acclimation during the autumn [3, 5, 6].

High *n*-alkane contents in the leaves of *Salix* clones have been observed to correlate with poor over-winter survival. Therefore, *n*-alkanes were proposed to be a possible indicator of frost tolerance in plant breeding for short-rotation forestry [7, 8]. As *n*-alkanes are common components of epicuticular waxes and are rarely found elsewhere, it was suggested that the correlation was connected with the functions of epicuticular waxes. In order to further elucidate the role of epicuticular waxes in frost tolerance of willow, we have studied the epicuticular wax composition and content of leaves of willow clones with varying degrees of frost-tolerance and biomass productivity.

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RESULTS AND DISCUSSION

Epicuticular waxes on leaves of Salix *clones*

Overwintering survival of *ca* 300 *Salix* clones has previously been studied in field experiments in northern coastal areas of Finland (65°42′N) [3]. Nine of these clones (one *S. myrsinifolia* clone, four *S. dasyclados* clones, one *Salix* hybrid clone and three *S. viminalis* clones) with different overwintering survival rates and biomass productivities were selected for wax analysis (Table 1).

Epicuticular waxes of the *Salix* clones were composed of *n*-alkanes (11.5–59.7% of total wax), free fatty acids (5.8–33.8%), *n*-alcohols (15.2–40.8%), *n*-aldehydes (3.8–25.5%) and long-chain aliphatic esters (0–17.6%) (Table 1). Three *S. viminalis* clones (clones nos 7–9) with low or moderate overwintering survival had *n*-alkanes as the major wax component with minor amounts of more

Table 1. Wax composition and epicuticular wax contents on leaves of *Salix* clones and their overwintering survival rates and relative productivities

	Clones*								
	1	2	3	4	5	6	7	8	9
Wax composition (% of total)									
n-Alkanes	21.4	18.3	16.4	11.7	32.1	23.4	53.5	41.8	59.4
n-Fatty acids	23.6	22.5	33.1	33.8	26.7	19.6	5.8	21.3	12.6
n-Aldehydes	14.1	22.3	15.4	25.5	11.3	12.6	6.8	5.9	3.8
n-Alcohols	40.8	35.4	31.4	22.9	22.6	26.7	18.0	19.0	15.2
Alkyl monoesters	0	1.5	3.6	5.9	15.8	17.6	6.2	7.3	12.0
Total wax ($\mu g\,cm^{-2}$)	3.0	10.5	12.0	16.0	8.7	13.5	16.4	15.7	24.9
Overwintering survival†	100	90	90	70	47	80	60	18	10
Rel. productivity‡	53	137	229	153	100	198	229	—	810

*1: *S. myrsinifolia* clone V78, 2: *S. dasyclados* clone LSL-5 3: *S. dasyclados* × *S. triandra* clone V 777; 4: *S. dasyclados* clone CE-78-3; 5: *S.* dasyclados clone P6011; *Salix* hybrid clone S-81-34-3; 7: *S. viminalis* clone HK-82-16-26; 8: *S. viminalis* clone S-15111; 9: *S. viminalis* clone S-HK-81-2x.

†Overwintering survival of first year's clones 1–4, 6, 7, 9: [3] 1988; clones 5, 8: [1].

‡First year's biomass productivity of clones compared with productivity of control clone *S. dasyclados* P6011 [1, 3, 7, 8].

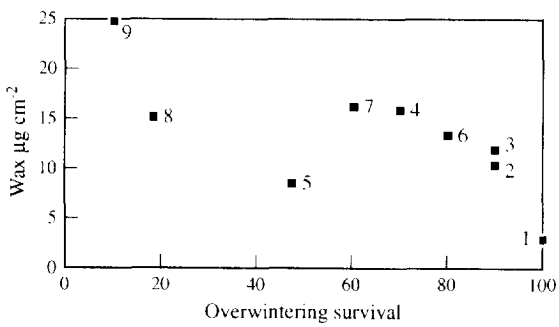

Fig. 1. Epicuticular wax contents ($\mu g\,cm^{-2}$) on leaves of *Salix* clones in relation to their overwintering survival (%). For clone numbering and overwintering survival, see Table 1.

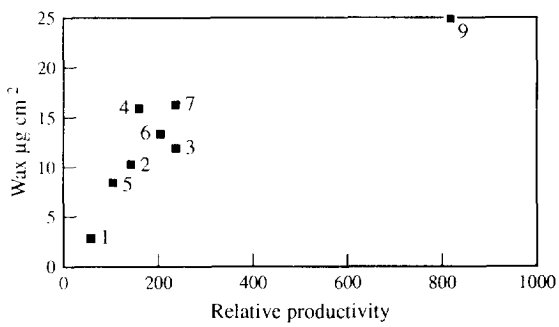

Fig. 2. Epicuticular wax contents ($\mu g\,cm^{-2}$) on leaves of *Salix* clones in relation to their relative productivity. For clone numbering and relative productivity, see Table 1.

polar components (*n*-alcohols, *n*-aldehydes and free fatty acids). *Salix dasyclados* clones (clones nos 2–5), *S. myrsinifolia* clone V 78 (clone no 1) and *Salix* hybrid clone S-81-34-3 (clone no 6) had more polar wax components than *n*-alkanes. Epicuticular wax contents of the clones varied between 3 $\mu g\,cm^{-2}$ and 24.9 $\mu g\,cm^{-2}$ and showed a clear correlation with low overwintering survival (Fig. 1) and high relative biomass productivity of the clones (Fig. 2).

Long-chain aliphatic esters on the *Salix* leaves were found to have chain lengths from C_{34} to C_{40}; C_{40}, C_{42} and C_{44} monoesters being the major homologues (Table 2).

The homologue distribution and contents ($\mu g\,cm^{-2}$) of *n*-alkanes and free fatty acids are shown in Table 3. The *n*-alkanes on the *Salix* leaves were primarily odd-numbered compounds with chain lengths of 21–31 carbons. The major *n*-alkane on the leaves of all the clones was *n*-heptacosane, with *n*-nonacosane and *n*-pentacosane being present in contributable proportions. No major differences in homologue distribution of *n*-alkanes were observed between the clones. However, *n*-alkane contents on the *Salix* leaves varied between 0.7 and 14.9 $\mu g\,cm^{-2}$ and showed a correlation with low overwintering survival (Table 3). The free fatty acids were even-numbered compounds with 16–32 carbons. Hexacosanoic acid, tetracosanoic acid and octacosanoic acid were the major free fatty acids on leaves of the clones. Triacontanoic acid was present in very high proportions on the leaves of *S. myrsinifolia* clone V78 (clone no 1). The free fatty acid content varied from 0.71 to 5.73 $\mu g\,cm^{-2}$ but did not show any correlation with overwintering survival.

Table 4 shows contents and homologue distributions of *n*-alcohols and *n*-aldehydes. *n*-Alcohols were even-numbered (C_{22}–C_{30}) compounds with low amounts of *n*-pentacosanol and *n*-heptacosanol. *n*-Hexacosanol was

Table 2. Chain length distribution (%) and total amounts ($\mu g\,cm^{-2}$) of long-chain aliphatic esters

	Clones*								
Homologue	1	2	3	4	5	6	7	8	9
C_{34}	0	0	0	0	0	0	0	0	7.2
C_{36}	0	17.7	0	0	0	6.5	7.3	0	9.3
C_{38}	0	13.5	12.5	24.0	10.7	14.7	9.1	12.3	9.8
C_{40}	0	23.8	33.9	24.5	27.4	24.6	10.4	16.0	11.1
C_{42}	0	27.1	37.9	30.0	34.3	28.8	25.2	31.3	22.3
C_{44}	0	17.8	16.5	17.9	22.5	19.5	31.1	26.7	24.2
C_{46}	0	0	0	3.6	5.1	5.9	13.5	11.5	10.3
C_{48}	0	0	0	0	0	0	3.2	2.2	2.4
Total ($\mu g\,cm^{-2}$)	0	0.15	0.44	0.96	1.37	2.38	0.97	1.14	2.99
Overwintering survival*	100	90	90	70	47	80	60	18	10

*For clone identification and overwintering survival, see Table 1.

Table 3. Homologue distribution (%) and total amounts ($\mu g\,cm^{-2}$) of *n*-alkanes and free fatty acids on leaves of *Salix* clones

	Clones*								
Class/homologue	1	2	3	4	5	6	7	8	9
n-Alkanes									
C_{25}	17.7	13.1	12.6	29.5	8.4	10.8	13.1	12.9	4.3
C_{27}	54.6	61.9	52.4	34.8	58.2	62.9	55.3	59.8	65.3
C_{29}	19.6	15.8	20.9	10.7	25.1	16.1	23.6	19.9	25.9
Others	8.0	9.2	14.2	24.9	8.2	10.3	7.8	6.9	4.5
Total ($\mu g\,cm^{-2}$)	0.7	1.9	2.0	1.8	2.8	3.2	8.8	6.4	14.8
Free fatty acids									
C_{20}	17.2	3.1	9.1	2.4	10.2	0	0	0	2.4
C_{22}	4.1	0	6.4	9.7	16.2	0	0	0	14.2
C_{24}	11.8	30.8	28.2	2.0	24.6	19.7	0	27.6	16.1
C_{26}	29.9	37.4	33.3	41.4	25.1	49.9	53.2	40.1	34.6
C_{28}	15.4	21.2	17.0	14.1	15.9	20.1	9.3	25.1	24.1
C_{30}	28.6	1.2	1.4	0.8	4.0	0	0	0	2.8
Others	8.3	6.2	4.7	6.5	4.1	10.2	37.4	7.2	3.6
Total ($\mu g\,cm^{-2}$)	0.71	2.36	3.99	5.43	2.31	2.65	0.95	3.25	3.13
Overwintering survival*	100	90	90	70	47	80	60	18	10

*For clone identification and overwintering survival, see Table 1.

the major *n*-alcohol on the leaves of *S. myrsinifolia* clone V78, all *S. dasyclados* clones and *Salix* hybrid clone S-81-34-3, while *n*-docosanol was the major alcohol on leaves of all *S. viminalis* clones. The chain length of *n*-aldehydes varied between C_{24} and C_{30} *Salix viminalis* clones S-HK-81-2x (clone no 9) had *n*-octacosanal and *n*-hexacosanal as major aldehydes. *n*-Hexacosanal was the major *n*-aldehyde on the leaves of all other *Salix* clones. Neither *n*-alcohol nor *n*-aldehyde contents on the leaves showed any correlation with overwintering survival of the clones.

According to our results, epicuticular leaf waxes of willow clones appeared to be of two distinct types differing in two respect. The *S. viminalis* clones had *n*-alkanes as their major wax component and *n*-docosanol as their major *n*-alcohol, while other clones (*S. myrsinifolia S. dasyclados* and *Salix* hybrid clone S-81-34-3) contained less *n*-alkane and had *n*-hexacosanol as their major *n*-alcohol.

Epicuticular waxes on Salix *leaves in relation to winter tolerance and biomass productivity*

Willows, even from southern regions, have the genetic capacity to achieve high freezing resistance [6]. The difference in overwintering survival of willows depends on the progress of the hardening process. The growing twigs are generally neither able to harden nor able to

Table 4. Homologue distribution (%) and total amounts ($\mu g\ cm^{-2}$) of *n*-alcohols and *n*-aldehydes on leaves of *Salix* clones

	Clones*								
Class/homologue	1	2	3	4	5	6	7	8	9
n-Alcohols									
C_{22}	10.9	8.0	15.9	25.2	0	25.7	58.1	52.4	59.6
C_{24}	11.3	15.6	14.8	13.8	14.5	8.7	12.2	10.6	11.1
C_{26}	58.7	60.3	51.0	46.6	61.0	51.0	23.1	20.7	13.3
C_{28}	15.2	14.6	7.1	9.0	15.1	7.5	6.6	10.6	11.5
C_{30}	3.0	1.4	1.6	0	1.5	0.8	0	2.6	2.3
$C_{25} + C_{27}$	0.9	0	9.6	4.9	3.5	6.2	0	3.0	2.1
Total ($\mu g\ cm^{-2}$)	1.23	3.72	3.78	3.67	1.96	3.61	2.96	2.92	3.78
n-Aldehydes									
C_{24}	11.5	17.6	18.9	15.9	14.5	10.8	18.6	13.9	15.5
C_{26}	65.2	61.9	61.8	66.7	61.0	65.6	57.4	51.7	33.6
C_{28}	23.5	15.4	7.7	9.0	15.1	10.4	23.9	24.9	35.1
C_{30}	0	0.9	0	0	2.3	0	0	0	5.3
$C_{25} + C_{27}$	0	4.1	11.6	8.3	7.3	13.2	0	9.4	7.9
Total ($\mu g\ cm^{-2}$)	0.43	2.34	1.85	4.10	0.98	1.71	1.12	0.91	0.94
Overwintering survival*	100	90	90	70	47	80	60	18	10

*For clone identification and overwintering survival, see Table 1.

survive in freezing temperatures, even if they are exposed to low temperatures; growth cessation is essential for the hardening process [6]. Upon cessation of growth, willow twigs achieve their first freezing resistance and afterwards the resistance further increases due to low temperatures [6]. In our study, the clones that had low overwintering survival rates continued their growth longer, while the clones with higher overwintering survival became dormant and thus their hardening process began about 30 days earlier [3].

The high total epicuticular wax contents and high *n*-alkane contents on *Salix* leaves showed an inverse correlation with high overwintering survival of the clones (Fig. 1, Table 3). On the other hand, a high epicuticular wax content had a direct correlation with a high relative biomass productivity of the clones (Fig. 2). This suggest that a high epicuticular leaf wax content is beneficial to growth but disadvantageous for cold acclimation of *Salix* clones.

The functions of epicuticular waxes as components of the cuticle include control of transpiration [9–11], reflection of light [12], control of chemical transportation through the cuticle [9, 13] and regulation of plant surface microflora [9]. A low leaf surface wax content with low amounts of *n*-alkanes might lead to a high cuticular transpiration, high leaf surface wettability, high leaching of nutrients from leaf tissues during rainfall and poor protection against plant pathogens [9, 14].

The hardening of trees during the autumn depends on twig maturation, which includes the slow drying of twigs [15]. Desiccation stress, especially in low temperatures, enhances the frost hardening of plants [16]. Low epicuticular wax contents on leaves of clones with high frost tolerance may provide more efficient cuticular transpiration, leading to desiccation stress and growth cessation during early autumn. Winter tolerant clones with low epicuticular wax contents may also be more susceptible to pathogen attacks and leaching of nutrients from leaf tissues than clones with low winter tolerance but high epicuticular wax content. Any leaching and pathogen attacks may further cause growth cessation of *Salix* clones and thus enhance bud hardening. The correlation between high epicuticular leaf wax content with high levels of *n*-alkanes, high biomass productivity and low winter tolerance of *Salix* clones may be attributed to the protective action of epicuticular waxes against environmental stresses. On one hand, improved stress resistance may lead to improved productivity during the growth season; on the other, improved stress resistance may prevent the stress-induced growth cessation during the autumn that is essential for winter acclimation.

Low epicuticular wax content and low epicuticular *n*-alkane content may be used as a marker for winter tolerance of willows. However, it must be kept in mind that epicuticular waxes may undergo large changes during the development of a plant [15, 16]. Thus, proper timing of sampling of the plant material needs to be determined by studying seasonal changes in the epicuticular waxes from *Salix* leaves.

EXPERIMENTAL

Leaves of nine *Salix* clones with known overwintering survival and biomass productivity [1, 3] were used for wax analysis: *S. myrsinifolia* clone V78 (100% survival), *S. dasyclados* clone L78-133 (90% survival), *S. dasyclados*

× *S. triandra* hybrid clone V777 (90% survival), *S. dasyclados* clone CE-78-3 (70% survival), *S. dasyclados* clone P6011 (47% survival), *Salix* hybrid clone S-81-34-3 (80% survival), *S. viminalis* clone HK-82-16-26 (60% survival), *S. viminalis* clone S-15111 (18% survival) and *S. viminalis* clone S-HK-81-2x (10% survival). Plants were grown in the field at Kotkaniemi Research Station of Kemira Agro Ltd (Vihti, Finland). Stock plants were planted in 1985 and plots were deep-ploughed, fertilized annually with N-P-K fertiliser (16:7:13.3, 400 kg ha^{-1}) and covered with black plastic to reduce weeds. Leaf samples were taken in the beginning of October suggesting that growth had ceased and the first stage of winter hardening had occurred before sampling.

Areas of *ca* 5 g of willow leaves were measured by weighing their paper copies. Leaf samples were extracted with 70 ml of $CHCl_3$ for 45 sec. Wax extracts were dried *in vacuo* and 200 μg *n*-hexacosane added as int. standard into each sample. Dry extracts were redissolved in 6 ml $CHCl_3$. Two replicate 1 ml samples of redissolved leaf surface extract were used for wax analyses. Samples were analysed by GC-MS for *n*-alkanes, free *n*-aldehydes and wax esters. After the first GC-MS analysis, samples were silylated as described below before further GC-MS analyses for free fatty alcohols and free fatty acids.

After the first GC-MS analyses, samples were dried under N_2 in GC vials. Thereafter, samples were silylated with 300 μl silylation reagent, which was prepd just before silylation by adding 5 ml of BSTFA (*N,O*-bis(trimethylsilyl)trifluoroacetamide) reagent into 5 ml of pyridine (silylation grade). Vials were then closed and heated to 70° for 15 min. After heating, silylation reagent was evapd and the silylated samples dissolved in 0.6 ml hexane for GC-MS analysis.

GC-MS analyses were performed with a mass-selective detector. For *n*-alkane, wax ester, *n*-aldehyde and silylated *n*-alcohol analyses samples were analysed by GC-MS with and without silylation on a J&W DP 17HT (WCOT) column with an injector temp. of 340° (1/20 split; 1 μl). The oven temp. prog. was 70–200° at 25° min^{-1}; 200–280° at 5° min^{-1}; 280° for 10 min; 280–360° at 10° min^{-1}; and 360° for 10 min. The MS line heater temp. was 340°. For silylated fatty acid analysis, a HP Ultra-1 column (WCOT) was used with an inj temp. of 310° (split ratio: 1/20; 1 μl). The heating prog. was 70–280° at 25° min^{-1}; 280° for 10 min; 280–310° at 10° min^{-1}; and 310° for 10 min. EIMS 70 eV: *m/z* (rel. int.): *n*-alkanes: $[M]^+$ (1–2), 113 (7–12), 99 (15–20), 85 (49–58), 71 (73–77), 57 (100); *n*-aldehydes $[M-18]^+$ (4–12), 96 (64–68), 82 (100), 57 (10–14); silylated *n*-alcohols: $[M-15]^+$ (100), 103 (14–15), 75 (28–35), 57 (85–97); silylated fatty acids: $[M]^+$ (6–11), $[M-15]^+$ (24–45), 132 (67–86), 117 (100), 73 (56–93); long-chain aliphatic esters $[M]^+$ (5–15), 257/285/313/341/369 $[M + 2]^+$ of esterified fatty acid chain (30–100), 97 (43–48), 83 (43–51), 71 (46–57), 57 (81–100). Components were quantified by comparing their total mass ion spectra to that of the int. standard (*n*-hexacosane).

Acknowledgements—The authors thank Ms E. Saarikko and Mr P. Seppänen (Kemira Agro Ltd) for technical assistance, Drs L. Toivonen and K. Nordström for valuable suggestions and the technology Development Centre (TEKES) and the Kemira Foundation for financial support.

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