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EPICUTICULAR WAX OF FOUR SPECIES OF *CHIONOCHLOA*

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Key Word Index—*Chionochloa flavescens, C rigida, C macra, C rubra*, Gramineae, epicuticular wax, shoots, esters, alkanes, aldehydes, alcohols, acids

Abstract—Shoot epicuticular wax of four species of *Chionochloa* contained esters (21–38 %), alkanes (15–29 %), aldehydes (4–13 %), alcohols (8–16 %) and acids (1–5 %) The major carbon chain lengths ranged from C_{24} to C_{32} in the acids, alcohols and aldehydes and C_{29} and/or C_{31} in the alkanes Major esters ranged from C_{40} to C_{52} with individual esters consisting of a mixture of different chain length acids and alcohols The chain length of the alcohol rather than the acid appears to determine the chain length of the longer chained esters The distribution of the major carbon chain lengths in the lipid fractions, particularly the acid, aldehyde and wax esters of epicuticular wax, suggests that the lipid composition of the wax may be used in the chemotaxonomic recognition of species of *Chionochloa*

INTRODUCTION

The grass genus *Chionochloa* Zotov, [1] tribe Danthonieae, consists of 22 long-lived perennial species, all but one of which are endemic to New Zealand The species of *Chionochloa* vary in size and habit from 20 cm in the prostrate alpine *C australis* to 2 m, at flowering, in the stout eastern South Island *C flavescens Chionochloa* is one of the most ecologically important grasses in the South Island of New Zealand Triterpene methyl ethers in their epicuticular wax have been examined but since they only occur in certain species, their usefulness in chemotaxonomic studies is restricted at the specific level [2, 3]

In contrast, more detailed analysis of the composition of the epicuticular wax of some species of the sempervivoid genera of the Crassulaceae [4], *Cistus* [5, 6], *Eucalyptus* [7], *Rhododendron* [8] and *Cortaderia* [9, 10] has been successful in characterizing some of the species In this paper, the main components of the epicuticular wax from four species of *Chionochloa* were also examined to determine whether their composition could be used for chemotaxonomic identification

RESULTS

The yield of wax was 5 6 and 4 8 g/kg shoot fr wt from *C flavescens* and *C rigida*, respectively, which was higher than that from *C macra* and *C rubra* (3 3 and 3 1 g/kg shoot fr wt) TLC of the individual waxes showed that the major classes were alkanes, esters, aldehydes, alcohols and acids (Table 1) The esters constituted 21–38 % of the wax, alkanes 15–29 %, aldehydes 4–13 %, primary alcohols 8–16 % and acids 1–5 %

Mass spectrometry of the *C macra* esters showed that the carbon numbers ranged from 34 to 52 and possibly 54 and that each GC peak contained ion fragments from several acid and alcohol components (Table 2) For example, the spectrum of the C_{36} wax ester showed a M^+ ion at *m/z* 536 (11 %) Ions at *m/z* 313 and 312 (6 %) were attributed to a C_{20} acid and at *m/z* 224 (9 %) to a C_{16} alcohol, ions at *m/z* 285 (100 %) and 284 (29 %) were attributed to a C_{18} acid and *m/z* 252 (14 %) to a C_{18} alcohol, ions at *m/z* 257 (65 %), 256 (19 %) were attributed to a C_{16} acid and at *m/z* 280 (6 %) to a C_{20} alcohol Determination of the amounts of each ester in the four

Table 1 Composition (%) of epicuticular wax from four species of *Chionochloa*

Component	*C macra*	*C rubra*	*C flavescens*	*C rigida* 1 Mt Barrosa	*C rigida* 2 Ashburton River	*C rigida* 3 Potts River
Ester	21	29	34	25	38	32
Alkane	20	15	29	26	17	25
Aldehyde	8	13	6	7	6	4
Primary alcohol	8	11	16	14	12	14
Acid	5	2	2	2	1	2
Unidentified	38	30	13	26	26	23

Table 2 Esterified acids and alcohols indicated by mass spectrometry of esters from *C macra* epicuticular wax

Ester (carbon No)	Acid component	Corresponding alcohol component									
34	16 (100 26) 18 (92 24)	—	—	—	—	—	—	—	—	18	16
36	16 (65 19) 18 (100 29) 20 (1 6)	—	—	—	—	—	—	—	20	18	16
38	16 (30 8) 18 (100 33) 20 (16 6) 22 (12 4)	—	—	—	—	—	—	22	20	18	16
40	16 (100 36) 18 (68 20) 20 (43 16) 22 (10 4)	—	—	—	—	—	24	22	20	18	—
42	16 (27 8) 18 (100 32) 20 (60 19) 22 (9 3)	—	—	—	—	26	24	22	20	—	—
44	16 (6 2) 18 (48 11) 20 (31 8) 22 (7 2)	—	—	—	28	26	24	22	—	—	—
46	16 (6 3) 18 (16 5) 20 (13 4) 22 (14 4) 24? (1 1)	—	—	30	28	26	24	22	—	—	—
48	16 (12 7) 18 (3 3) 20 (2 1) 22 (2 3 6) 24? (1 1)	—	32	30	28	26	—	—	—	—	—
50	16 (32 10 25) 18 (45 37 11) 20 (5 3 5) 22 (6 20 43) 24 (1 2 6)	34	32	30	28	26	—	—	—	—	—

The intensities of ions (in order of decreasing MW) responsible for each acid fragment are shown in parentheses as percentages

species showed the majority (91–98 %) of the esters as C_{40}–C_{52} (Table 3) The percentage distribution of the carbon chain lengths was similar for *C flavescens* and *C rigida*, with the majority of the esters having 40–46 carbons *C rubra* was different with a more even distribution of chain lengths and *C macra* had large amounts of C_{50} and C_{52} esters

GC/MS of the alkanes identified chain lengths from C_{21} to C_{33} The relative percentage of individual homologues in the alkane fractions from the four species are shown in Table 4 In all the species C_{29}, C_{31} and C_{33} accounted for 81–96 % of the alkane fraction *C rigida* and *C flavescens* were, however, similar in that the C_{29} alkane was the major component whilst in *C macra* and *C rubra*, C_{31} alkane was the major component

GC/MS identified even-chained saturated aldehydes C_{24}–C_{32} (Table 5) GC resolved an unknown homologous series (b–f) eluting between the even-chained components Generally, these were minor components except in *C rubra* where the unknown compound 'e', running between C_{30} and C_{32}, comprised 66 % of the fraction All four species had substantial amounts of some of the following, C_{26}, C_{28}, C_{30} and C_{32} aldehydes which accounted for 84–95 % of the fraction The percentage distribution of these aldehydes revealed three patterns similar to those found in the acid fractions *C rigida* and C *flavescens* had high amounts of C_{28} and C_{30}, *C rubra* of C_{30}, C_{32} and a major unknown 'e' and *C macra* of C_{26} and C_{32}

Saturated primary alcohols ranging from C_{20} to C_{32} were identified by GC/MS (Table 6) In all species C_{24}, C_{28}, C_{30} and/or C_{32} accounted for 79–94 % of the alcohol fraction The percentage distribution of these alcohols revealed two patterns, *C flavescens* and *C rigida* with high proportions of C_{24} and C_{30} and generally lower in C_{32}, and *C macra* and *C rubra* with high C_{32} and lower C_{30} and C_{24}

GC/MS showed saturated acids ranging from C_{18} to C_{32} and the presence of a straight- and branched chain C_{25} acid, the branched-chain acid eluting between the *n*-C_{24} and *n*-C_{25} by GC Both the normal- and branched-chain C_{25} methyl ester had a M^+ at *m/z* 396 with a relative abundance of 26 % and 32 %, respectively In addition the branched chain acid showed intense ions at *m/z* 157 (35 %) and 101 (76 %) This indicates a propyl side chain on the fifth carbon from the carboxyl end The relative amounts

Table 3 Homologue composition (wt %) of esters in epicuticular waxes of four species of *Chionochloa*

Ester (carbon No)	*C macra*	*C rubra*	*C flavescens*	*C rigida* 1 Mt Barrosa	*C rigida* 2 Ashburton River	*C rigida* 3 Potts River
34	t	t	t	t	t	t
36	2	1	t	t	t	t
38	3	3	2	3	2	2
40	7	26	21	17	19	18
42	17	17	19	29	23	23
44	9	9	17	12	18	14
46	4	11	22	16	21	15
48	6	13	9	7	9	9
50	18	10	6	4	4	8
52	34	5	2	12	3	10
54?	1	5	—	t	t	t

t, ≤ 1 %

Table 4 Homologue composition (wt %) of alkanes in epicuticular waxes of four species of *Chionochloa*

Alkane (carbon No)	*C macra*	*C rubra*	*C flavescens*	*C rigida* 1 Mt Barrosa	*C rigida* 2 Ashburton River	*C rigida* 3 Potts River
21	t	t	t	t	t	t
22	t	t	t	t	t	t
23	6	t	t	t	t	t
24	1	t	t	t	t	t
25	5	1	1	t	1	1
26	t	t	t	t	t	t
27	t	t	t	t	t	t
28	t	t	1	t	1	1
29	10	20	69	58	75	51
30	3	1	t	4	t	t
31	62	66	19	18	12	28
32	t	t	t	t	t	t
33	11	9	8	18	9	16

t, $\leqslant 1\%$

Table 5 Homologue composition (wt %) of aldehydes in epicuticular waxes of four species of *Chionochloa*

Aldehyde (carbon No)	*C macra*	*C rubra*	*C flavescens*	*C rigida* 1 Mt Barrosa	*C rigida* 2 Ashburton River	*C rigida* 3 Potts River
24	t	t	t	t	t	t
b	1	t	t	5	5	4
26	39	4	14	6	6	10
c	t	t	t	1	1	t
28	16	5	34	20	29	24
d	t	t	t	8	t	t
30	12	10	44	50	51	43
e	t	66	t	t	t	t
32	28	13	4	8	4	16
f	1	2	3	1	4	2

t, $\leqslant 1\%$ The letters b–f refer to unidentified compounds

Table 6 Homologue composition (wt %) of primary alcohols in epicuticular waxes of four species of *Chionochloa*

Alcohol (carbon No)	*C macra*	*C rubra*	*C flavescens*	*C rigida* 1 Mt Barrosa	*C rigida* 2 Ashburton River	*C rigida* 3 Potts River
24	17	17	23	48	21	25
25	2	t	t	t	t	3
26	10	3	5	3	4	4
27	t	t	t	t	t	4
28	3	4	17	9	12	13
29	t	t	1	t	1	1
30	24	17	37	12	30	16
U	t	2	2	12	8	9
32	43	56	14	16	18	26

t, $\leqslant 1\%$, U, unknown Traces of C_{20}–C_{23} were found in each sample

of each acid were calculated for the four species and in all instances, acids with even-chain lengths of C_{26}, C_{28}, C_{30} and C_{32} accounted for 76–90% of the acids The relative percentage of C_{18}–C_{24} and all the odd-carbon chain lengths individually was not greater than 9% The distribution of the major acids between the species revealed three distinct patterns *C rigida* and *C flavescens* were similar with high amounts of C_{28} and C_{30}, *C rubra* contained slightly more C_{32} than C_{30} and *C macra* had a high proportion of C_{26} (Table 7)

Electron microscopic examination of leaves (alcohol treated) from the four species revealed that all bore bulbous projections on the young semi-glaucous adaxial leaf surfaces These round outgrowths seemed to be covered first with sheets of wax In addition, long crystal-like tubes could be seen both on and in-between the bulbous projections In contrast, the young abaxial surfaces showed no such structures

DISCUSSION

Wax lipids are thought to be synthesized in the epidermal cells [11] by an elongation–decarboxylation complex forming acyl chains which are either decarboxylated to alkanes, reduced to alcohols via aldehyde intermediates, esterified with alcohols forming esters, or released as free acids [12] The wax is subsequently excreted onto the surface of leaves and other organs and is probably a metabolic end-point Although the acid and alkane components of a wax may be channelled back into the epidermal cells and remetabolized [13], such wax degradation is unlikely to occur to any great extent [14] The chain lengths of the lipid components of epicuticular wax may, therefore, be useful chemotaxonomic markers

The epicuticular wax of *Chionochloa* contained major chain lengths in the free acids, alcohols, aldehydes and alkanes ranging from C_{24} to C_{33} and in the esterified acids from C_{16} to C_{24} It appears from the results, that the short acyl components of the elongation–decarboxylation system are used for the synthesis of esterified acids and the longer chains for the formation of free acids, alcohols, aldehydes and alkanes A C_{33} alkane was found but the C_{34} precursor was only identified in the esterified alcohol fraction Table 2 illustrates that as the chain length of the esters increased, the chain length of the esterified acids remained relatively constant between C_{16} and C_{24}, whilst the chain length of the esterified alcohols increased from C_{16} to C_{34} This suggests that the increased chain length of the ester is due to the alcohol and not the acid component

Glaucousness has been attributed to β-diketones, hydroxy β-diketones and alkanes with 29 and 31 carbons in *Brassica oleracea* (cabbage) and *Pisum sativum* (pea), respectively [15] It is possible that the high proportion of C_{29} or C_{31} alkanes in *Chionochloa* wax contributes to the semi-glaucous appearance of the leaves as β-diketones have not, as yet, been detected

In *C rubra* and *C macra*, the alcohol, aldehyde and acid fractions were rich in C_{32} homologues, suggesting that all three lipid components are derived from the same chain length precursor The major alkane (66%), however, contained 31 carbons suggesting it is a decarboxylation product of the C_{32} precursor This evidence suggests that in *C rubra* and *C macra* the elongation–decarboxylation complex is oriented towards synthesizing a C_{32} chain length precursor which is decarboxylated to the major C_{31} alkane In addition, the acids, aldehydes and alcohols with increased concentrations of C_{30}, C_{28} and C_{26}, only contained limited amounts of C_{29}, C_{27} and C_{25} alkanes

In contrast, in *C flavescens* and *C rigida*, high amounts of C_{30} were found in the acids, alcohols and aldehydes and C_{29} in the alkanes Thus, the elongation–decarboxylation system, in these species, is oriented to producing large concentrations of a C_{30} precursor, which is decarboxylated to the major C_{29} alkane Once again, acids, aldehydes and alcohols either C_{32}, C_{28}, C_{26} and/or C_{24} contained smaller amounts of C_{31}, C_{27}, C_{25} and C_{23} alkanes

The four species of *Chionochloa* may, therefore, be arbitrarily divided into two groups, one, *C flavescens* and *C rigida*, utilizing the C_{30} elongation–decarboxylation system, and the other, *C macra* and *C rubra*, the C_{32} system *C macra* can, however, be distinguished from *C rubra* by large amounts of C_{26} acid and aldehyde and greater proportions of C_{50} and C_{52} esters The four species could, therefore, be divided into three groups based on the composition of the epicuticular wax However, *C flavescens* and *C rigida* are classified in the

Table 7 Homologue composition (wt %) of acids in epicuticular waxes of four species of *Chionochloa*

Acid (carbon No)	*C macra*	*C rubra*	*C flavescens*	*C rigida* 1 Mt Barrosa	*C rigida* 2 Ashburton River	*C rigida* 3 Potts River
24	t	t	2	3	6	8
25-br	t	t	t	t	t	t
25	6	3	3	2	2	2
26	48	12	12	11	9	3
27	2	9	3	3	2	3
28	14	14	29	28	36	30
29	1	7	2	t	3	2
30	9	22	28	42	31	26
32	19	29	15	8	7	11
U	1	4	8	6	3	6

t, ⩽ 1%, U, unknown Traces of C_{18}–C_{23} were found in each sample

same group but this is not surprising because they are closely related [1] The composition of the epicuticular waxes from *C rigida* samples obtained from three entirely different environments showed the same characteristic trends That is, C_{28} and C_{30} homologues were higher in the acid and aldehyde fractions and C_{29} in the alkanes This illustrates that differences in the *Chionochloa* epicuticular wax are dependent primarily on the genes controlling wax synthesis rather than environmental effects

In some species, the composition of epicuticular wax has been found to vary in different parts of the plant [16–21], with the age of the tissue [16, 18, 20, 22–24] and with growth conditions [23, 25, 26] In the present work any variations in age and tissue were overcome by random sampling of 5–10 kg of fresh plant material This included blades and leaves from young and old plants Treating the four species of *Chionochloa* in this manner yielded an epicuticular wax which was characteristic for each species

EXPERIMENTAL

Plant material Leaf waxes from the four species of *Chionochloa* were extracted from 5–10 kg samples of fr green plant material (blades–sheaths, 3 1) collected in the field These bulk samples consisted of material from many plants in a population and describe the lipid composition of the plants at a site but not information on seasonal variation Field collections were made in late spring and summer The four species of *Chionochloa* were found in the South Ashburton Valley, the sites are generally representative of the grasslands in that area and none is more than 2 km from the other Three samples of *C rigida* were taken from three distinct locations to examine the effects of environment on the wax composition The species and localities were *C flavescens*, Mt Barrosa, South Canterbury, 1220 m, sunny slope, *C macra*, Mt Barrosa, South Canterbury, 1220 m, shady, *C rubra*, Harpers Knob, South Canterbury, 640 m, wet, *C rigida* (1) Mt Barrosa, South Canterbury, 910 m, sunny, *C rigida* (2) Ashburton River, South Canterbury, 670 m, dry, *C rigida* (3) Potts River, South Canterbury, 640 m, shady The samples are among those reported on by Connor and Purdie [2, 3]

Electron microscopy Shoots of the four species were prepared for electron microscopy by freeze-drying overnight or treating with EtOH The EtOH treatment consisted of soaking the cut shoots for 1 hr in 40%, 60%, 75%, 90%, 100% EtOH soln in succession, then overnight in 100% EtOH The EtOH was decanted and the shoots dried under vacuum

Isolation of epicuticular wax The shoots (25 cm) were soaked for 16 hr in petrol (40–60°) at room temp, the petrol extract decanted and concd by rotary evaporation

Separation of wax components Si gel G TLC plates (0 4 mm thick) were prepared and pre-run in EtOH Extracted wax (3 mg in 0 15 ml $CHCl_3$) was applied to two Si gel TLC plates The plates were developed in C_6H_6 and the five fractions, acid, alcohol, aldehyde, ester and alkane which separated were detected by spraying with an aq soln (0 1%, w/v) of Ultraphor (Badische Anilin and Soda Fabrik, Ludwigshufen-am-Rhein, Germany) and viewed under UV light The lipids were recovered from the Si gel with 50 ml $CHCl_3$ containing 1 0 mg int standard octadecyl octadecanoate for the acid, alcohol, aldehyde and alkane fractions, methyl *n*-eicosanoate for the ester fraction After the acids were methylated (CH_2N_2–Et_2O) and the alcohols acetylated (Ac_2O–pyridine, 1 2), each fraction was analysed by GC The FID response for methyl *n*-eicosanoate and octadecyl octadecanoate was similar and consequently it was assumed that all other components had similar FID responses The areas of each GC peak were calculated by triangulation and the amounts of individual components determined by comparing the areas of each peak to that of the int standard [27]

Gas chromatography The instrument was fitted with an FID and a stainless steel column 1 5 m × 3 2 mm (o d) packed with Chromosorb W-AW DMCS (80/100 mesh) coated with 1 5% Dexsil 300 The oven was programmed from 200° to 360° at 6°/min The detector temp was 310° and injector temp 275° The carrier gas was N_2 (30 ml/min) The methylated acids, acetylated alcohols, aldehydes, esters and alkanes were dissolved in $CHCl_3$ (0 1 ml) and 2 μl aliquots injected

Mass spectrometry The gas chromatograph was connected to the mass spectrometer via a membrane separator heated to 230° The source temp was 250° and spectra were determined at 20 eV A 1 5 m × 3 2 mm (o d) Pyrex column packed with Chromosorb W/HP (80/100 mesh) coated with 1% OV101 was used The gas chromatograph was operated with an injector temp of 230° and the column temp programmed at 2°/min from 160° to 260° The carrier gas was He (25 ml/min) The individual fractions of alkane, aldehyde, acetylated alcohol and methyl esters were analysed directly by GC/MS The esters were separated on a gas chromatograph fitted with an outlet splitter (1 5) and a sample collection port The separated components were collected without cooling in glass capillary tubes The appropriate section of the capillary was inserted directly into the mass spectrometer direct insertion probe This method was necessary because the longer chain esters required a temp above the temp limit (230°) of the separator

Peak identification The components of *C macra* were identified by GC/MS The components of the other species were identified by comparison with the R_i's of those of *C macra*

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