THE CHANGES WITH AGE IN THE EPICUTICULAR WAX OF *SORGHUM BICOLOR*

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Abstract.—The epicuticular wax from two varieties of *Sorghum bicolor* (L.) at four stages of growth are analyzed for changes in composition in order to account for the plants' age-dependant resistance to insect attack. The hydrocarbons, with chain length variation of C_{14} to C_{37}, show a significant increase of both chain length and percentage composition of higher molecular weight homologues on aging. Esters, ranging from C_{34} to C_{58} in equivalent chain length, showed fluctuations in the percentage composition of individual components with no specific trend. Ester hydrolysis products showed increases in higher molecular weight homologues and an increase in the percentage composition of the longer chain alcohols on aging. Experimental and calculated randomly combined distributions of the wax esters are also discussed. Wax components are also compared with the corresponding values from other species in the *Gramineae*.

The chemical studies of epicuticular waxes since 1950 have suggested that there is one wax composition for each plant. However, in the last seven years, it has been appreciated that the wax composition will vary with water stress and drought resistance (1), photoperiod and temperature (2) and age (3,4,5).

Plant/insect interactions associated with plant age are well established for internal constituents (6,7,8,9), however, epicuticular wax components have not been studied in this way before.

As part of our studies into plant waxes and insect/plant interactions, the present report shows the variation in wax composition with the age of the plant. We have examined *Sorghum bicolor*, which is the world's fifth largest grain crop with 52 million tons harvested per annum (10). Africa and India use ¾ of the total work acreage to grow a third of the world's produce. Crop damage and loss due to feeding by insect larvae and adults are estimated to total several billion dollars each year in the United States of America where crop protection is more advanced than in the developing countries.

RESULTS AND DISCUSSION

The amount of wax extracted from both varieties of *Sorghum bicolor*—CSH1, which is susceptible to insect attack, and ISI082, a known resistant strain—decreased as the plants got older, viz., from 0.14% to 0.04% of green wet weight of plant (table 1). This finding is in agreement with the results of a similar time study for *Triticum aestivium* (5). The significant difference in the amount of wax on the two varieties with CSH1 at 0.14% and ISI082 at 0.1% continued up to day 28. Thereafter, the amount of wax appeared to be the same for both plants. Wax production was known to be at its maximum during the first 28 days, but the leaf area was also expanding fastest at this time, so there was a fall overall in wax weight as a percentage of green plant weight. The study of the effect of these waxes on insects is reported in the following paper (34); a bioassay with *Locusta migratoria* was used (11).

In contrast to this decrease in crude wax weight, the proportion of the hydrocarbon fraction in the wax increased for both varieties (table 1), ranging from 0.6 to 1.3% for CSH1 and from 0.6 to 1.7% for ISI082. The rate of production seemed to reach a maximum at 28 days for ISI082, whereas for CSH1 it was still increasing up to 52 days. Thus sorghum appears to be similar to *Triticum aestivium* where Bianchi has shown that the hydrocarbons increase from 9.4% to 17.8% over a longer period, 30 days to 130 days. It has to be noted that,

TABLE 1. Amount of wax, hydrocarbons, esters and other fractions in *Sorghum bicolor* varieties.

Stage of Growth	CSH-1								IS1082							
	Crude Wax[a]	Hydrocarbons[b]	Esters[b]	n-Aldehydes[b]	Free Alcohols	Free Acids	4-Hydroxybenzaldehyde	Unidentified	Crude Wax[a]	Hydrocarbons[b]	Esters[b]	n-Aldehydes[b]	Free Alcohols	Free Acids	4-Hydroxybenzaldehyde	Unidentified
7 days	0.14	0.63	6.4	4.4	44.3	2.5	16.5	25.3	0.10	0.61	5.5	6.0	21.1	9.4	49.2	8.5
14 days	0.08	0.90	9.2	4.8	38.6	7.9	18.2	20.4	0.04	0.96	8.1	6.9	38.1	7.8	17.9	20.1
28 days	0.05	1.12	6.0	7.0	31.9	16.0	13.5	24.5	0.05	1.69	4.3	5.4	19.9	14.9	8.0	45.7
52 days	0.04	1.27	3.2	2.8	31.9	18.8	14.4	27.5	0.05	1.67	1.2	4.5	14.9	46.3	1.6	29.7

[a]Amount of wax extracted (% of original green plant weight) found by weight.
[b]Amount of hydrocarbons, esters and aldehydes (% of crude wax weight) found by gc, confirmed by weight.
Amount of other fractions (% of crude wax weight) found by weight.

during this 100 day period, *T. aestivium* was over-wintering in the field and was experiencing little leaf expansion.

Glc analysis of the hydrocarbons on packed columns, confirmed on capillary columns, showed that there was a change in the distribution of chain lengths as the plant aged (table 2). Such a change has been reported in our earlier work on

TABLE 2. Per cent composition of the alkanes from *Sorghum bicolor* var CSH1 and IS1082.

Chain Length	CSH 1				IS1082			
	Stage of Growth/Days				Stage of Growth/Days			
	7	14	28	52	7	14	28	52
21	1	0.1	t		0.5	0.2	t	
22	0.3	t	t	t	0.7	t	t	
23	0.5	0.6	1	0.2	0.9	1.5	0.3	0.8
24	0.7	0.4	0.7	0.3	0.3	0.3	0.3	t
25	3	1	1	1	2	2	0.6	0.6
26	3	1	2	0.7	0.7	0.9	1	0.9
27	19	14	9	15	15	15	6	0.5
28	3	1	1	0.7	1	2	2	1
29	35	31	30	30	29	35	29	23
30	2	1	2	0.7	2	1	2	0.8
31	30	48	41	20	48	38	34	28
32	t	0.1	1	0.7	t	0.5	1	1
33	0.4	0.6	8	13	0.3	3	12	18
34		t	0.3	0.4		0.2	0.6	0.8
35		t	4	10		0.6	6	11
36							0.3	0.6
37			t				3	4
38								t
39								t

Rye grass (*Lolium perenne*) (3) over a 14-day period and was also reported for leaf waxes of *Khaya* species over a 3–7 week period (4). Of all the compound classes in plant waxes, the hydrocarbons have been analyzed most often.

There are genus differences between the hydrocarbons of the family Gramineae, though, as a general rule, C_{29} and C_{31} tend to be the major alkanes (12–21). Each of the glossy mutants of maize has a different alkane distribution (22–25), but the two varieties of *Triticum durum* are remarkably similar (12). It is recognized that the wax hydrocarbons from different parts of the plant have different compositions. For example, Tulloch's study of *Agropyron intermedium* (13) shows that the flowering spike has 20% C_{27}, whereas the stem and leaves have only 4% and 7% C_{27}. Mature *Sorghum bicolor* alkanes have been previously examined from one particular leaf for Redbine 60 near isogenic bloom and bloomless lines (26), but two other varieties were shown to have one alkane composition on the sheath and another composition on the blade (27). However, both varieties have the same alkane composition on the sheath and both have the same, but different, composition on the blade. In the present study, it was felt to be inappropriate to examine the waxes from different parts of the plant as *Locusta* will feed on whole plants. Instead waxes were extracted with chloroform from the surfaces of complete plants of *Sorghum bicolor*, var CSH1, which is susceptible to insect attack, and of var IS1082, which is a known resistant strain.

The major alkanes were of odd carbon chain number in accordance with the proposed metabolic pathways and ranged from C_{14} to C_{37}. At 7 days a significant difference between these two varieties was obvious in that the C_{31} was the major alkane for ISI082, whereas there were a number of lower molecular weight components in CSH1, i.e., C_{25}, C_{26} and C_{27} were present in larger proportions.

At day 14, CSH1 appeared to have produced more C_{31} and was the major peak. The whole pattern could have been mistaken for IS1082 at day 7. At day 28, both varieties showed more of the higher molecular weight material, e.g., C_{33} at 8% and 12% and C_{35} at 4% and 6%. This process of extending the chain length was more obvious at 52 days when there were 10% and 11% at C_{35} and 6% and 5% at C_{36}. These remarkable changes were as large as the differences with age noted for *Khaya* species and could be due to three possible effects. The work of Bianchi (27) showed that for two varieties sorghum has a higher proportion of lower molecular weight material in the sheaths. As the plant ages, the proportion of sheath to leaf blade decreases; so the proportion of lower molecular weight alkanes will decrease as the plants age. An alternative proposal is that the biosynthetic pathways available for acetate change as the plant gets older. Giese (2) claims that C_{21}, C_{23} and C_{25} alkanes are on a different metabolic route from the C_{29}, C_{31} and C_{33} alkanes in barley leaves because barley plants grown in the light and dark have large differences in their alkane compositions. A third possibility is that the lower molecular weight alkanes are sufficiently volatile to be lost from the plant surface over the 52 day period.

The percentage of wax esters in the total wax increased for the first 14 days (table 1), but fell thereafter for both varieties. Most workers who have studied the wax esters in plants have been content to hydrolyze them and analyze the acid and alcohol components thus formed. There are only a few reports (12, 13, 14, 15, 17, 18, 19, 21) which list the analysis of the intact Gramineae esters, but there are many more reports of the saponified acids and alcohols. Whilst hydrolysis of triglycerides of similar molecular weight can be achieved by refluxing with alcoholic potassium hydroxide, it is much more difficult to split the wax esters when the chain length exceeds C_{40}. Instead, methanolysis is required in which the wax ester is refluxed with a 5% solution of hydrochloric acid in methanol (28). In the present study it was felt important to determine the chain length distribution of the wax esters up to C_{60}, and this was achieved on a short packed column of 3% Dexsil 300 operating with temperature programming up to 400°. This study is the first in which sorghum waxes have been analyzed in this way.

TABLE 3. Per cent composition of the wax esters from *Sorghum bicolor* var CSE 1 and IS1082.

Chain Length	CSH 1				IS1082			
	Stage of Growth/Days				Stage of Growth/Days			
	7	14	28	52	7	14	28	52
39	0.2	t	t	0.1	0.3	t	t	t
40	3	3	2	2	3	4	0.9	1
41	0.6	0.7	1	0.3	0.8	2	0.2	0.3
42	20	28	24	21	21	27	11	19
43	2	2	2	0.8	2	2	0.6	0.9
44	18	17	18	30	12	21	23	33
45	2	2	2	1	2	1	1	2
46	17	13	14	21	14	13	19	23
47	2	2	2	0.2	2	1	0.5	1
48	11	10	11	9	13	10	13	10
49	1.0	2	1	0.4	2	0.8	0.7	0.3
50	11	7	9	8	9	7	15	7
51	1	0.3	0.3	0.2	1	1	0.8	0.1
52	8	5	8	5	9	8	12	2
53	0.9	1	t	0.1	0.4	t	0.2	t
54	2	1	3	1	4	0.3	2	0.2
55	t	0.2	0.1	t	0.6	t	t	t
56	0.8	2	2	0.2	2	t	0.3	t
57		t			0.2	t	t	
58			t	t	0.4		t	t

TABLE 4. Acids and alcohols derived from the wax esters of sorghum (% composition).

Chain	CSH 1								ISI082							
	Stage of Growth/Days								Stage of Growth/Days							
	7		14		28		52		7		14		28		52	
	Acid	Alc	Acid	Alc	Acid	Alc	Acid	Alc	Acid	Alc	Acid	Alc	Acid	Alc	Acid	Alc
14	0.3		t		0.1		0.1		t		0.1		0.1		t	
15	0.2		t		t	0.2	t		t		t		t		t	
16	6	2	5	1.0	4	0.2	5	0.2	3	1	9	2	5	t	0.7	0.3
17	0.3	t	t	0.2	t	t	t	t	t	t	t	0.8	t	t	t	t
18	9	11	10	10	6	0.7	2	0.6	14	8	10	4	4	0.5	3	0.5
19	t	t	t	t	0.1	t	0.1	t	t	t	t	t	t	t	t	t
20	37	41	52	37	51	4	35	0.4	45	44	41	36	36	2	21	2
21	1	0.2	0.4	0.4	0.6	t	0.5	t	0.4	0.4	0.6	0.5	0.7	t	0.4	t
22	29	27	18	23	22	57	31	36	21	25	24	30	26	25	43	21
23	0.6	0.1	0.3	0.5	0.3	0.7	0.6	0.4	0.1	0.2	0.4	0.6	0.4	0.3	0.2	0.3
24	14	16	10	22	12	13	23	26	13	18	9	23	25	24	30	21
25	t	t	t	t	t	0.3	t	1	t	t	t	t	t	0.8	t	0.8
26	0.6	2	0.3	3	0.5	4	0.4	18	2	2	0.8	2	0.5	16	2	33
27	t	t	t	t	t	t	t	0.5	t	t	t	t	t	t	t	1.3
28	t	0.3	t	2	t	15	0.1	13	t	1	t	1	t	26	t	17
29		t				t		t		t		t		t	t	t
30		t			t	0.4	t	4	t	0.1	t	0.2	t	5	t	3

The esters showed a large chain length variation from C_{39} to C_{58} (table 3), which is a larger range than that of *Agropyron intermedium* leaves and *Avena sativa* but smaller than that of *Triticum durum* and *Agropyron smithii*. It was evident that these shorter chain esters are associated with flowering parts of the *Gramineae*. Again, the phenomenon which was observed for hydrocarbons was evident, viz, the major component was C_{42} at 7 days for CSH1, but by 52 days the major component was C_{44}. However, there was not the clear-cut move to higher molecular weight esters as the age increased. The material $\geqslant C_{52}$ fell at day 14 and rose at day 28 and fell again by day 52 for CSH1. For IS1082, the chain length distribution was wider reaching C_{58}, and again the % material $\geqslant C_{52}$ dropped at day 14, rose at day 28, and fell dramatically at day 52.

The compositions of the acids and alcohols derived from these wax esters (table 4) gave further information concerning this change to higher chain lengths with age. For CSH1, the alcohol chain length did not change as much except for day 52. For IS1082, a similar change from C_{20} at day 7 and 14 to C_{22} was observed for the alcohols, whilst the fatty acid chain lengthened between day 14 and day 28 from C_{20} to C_{22} and C_{24}.

When the distribution of wax esters was calculated from these acid and alcohol values, assuming a random combination, for day 7 and 14, the calculated ester values were higher for the short chain ester than the amount actually obtained by experimentation. By contrast, for day 28 and day 52 the amount of short chain ester obtained by experimentation was higher than that calculated for a random recombination.

This is evidence for the compartmentalization by chain length within the acid and alcohol pool which has been assumed by other workers (29).

TABLE 5. Percentage wax as *n*-Aldehydes.

	CSH-1				IS1082			
Age	7	14	28	52	7	14	28	52
	4	5	6	3	6	7	5	4

EXPERIMENTAL

CULTIVARS.—The Indian crosses used were CSH1 and IS1082 obtained from ICRISAT via the Centre for Overseas Pest Research.

GROWTH CONDITIONS AND ISOLATION OF WAX.—Whole plants at 7, 14, 28 and 52 days from emergence were grown under artificial conditions, and the epicuticular wax was extracted with chloroform (30).

Identification of age was specified by the number of fully extended leaves, i.e., the visible leaf collars. Plants taken at 7 days ranged between the late 2nd to the early 3rd visible leaf collar stage. Mean height was 13.2±0.7 cm (from 20 samples) for CSH1 and 14.9±0.8 cm for IS1082. Plants at 14 days ranged from the middle to late 3rd leaf collar stage, mean height was 22.5±1.6 cm for CSH1 and 29.1±1.7 cm for IS1082. Plants at 28 days ranged between the late 4th to early 5th leaf collar stage, mean height was 38.4±1.6 cm for CSH1 and 55.4±3.4 cm for IS1082. Fifty two day old cultivars ranged from the early to middle 8th leaf collar stage, mean height was 85.0±3.9 cm for CSH1 and 94.9±8.4 cm for IS1082. Based on identifying characteristics reported by Vanderlip (31), plants at 14, 28 and 52 days corresponded to stages 1, 2 and 3, respectively; however, the stages for the two cultivers were greater in age than those reported.

CHROMATOGRAPHIC ANALYSIS AND PREPARATION OF SAMPLES.—Crude wax was fractioned by thin layer chromatography (tlc) on 0.75mm Merck Kieselgel G coated-plates with an eluent system of hexane: diethylether: formic acid (90:10:0.5). Resolved components for all extracts with respective Rf values were: hydrocarbons, 0.96; esters, 0.90; aldehydes, 0.34; fatty acids 0.25; alcohols, 0.14; sterols, 0.09; parahydroxybenzaldehyde, 0.05. Tlc of the hydrocarbon: ester band (Rf 0.85–1.0) in hexane:diethyl ether (98:2) gave separation sufficient for gas chromatography (gc) of the components; Rf values were: hydrocarbons, 0.90; esters, 0.1.

Hydrocarbons were also chromatographed on 10% silver nitrate impregnated 0.5 mm Merck Kieselegel G coated plates (32) in hexane; resolved components were saturated, Rf 0.90; monounsaturated, Rf 0.35. No further unsaturated components were detectable in bands

taken at lower Rf values. Determination of branched chain hydrocarbons on a microscale was done with 5°A Linde Sieves (33) followed by gc analysis.

Methanolysis of the esters was performed with 5% HCl in methanol on a microscale (28). Tlc of the products was done on plates of specifications similar to those used to fractionate the crude wax with an eluent system of hexane: diethyl ether (90:10). Rf values for the methyl ester and alcohol fraction were 0.6 and 0.14, respectively.

Visualization of the wax components was achieved by use of 0.2% dichloro(R)-fluorescein in ethanol or, when necessary, in combination with 0.4% 2, 4-dinitrophenyl hydrazine in 2N HCl, iodine and 50% aqueous sulphuric acid.

Gas chromatography was performed on a Pye Unicam series 104 with flame ionization detectors. Hydrocarbons and esters were analyzed on glass columns (1.5m x 4mm I.D.) packed with 100–200 mesh Supelcoport and coated with 3% Dexsil 300 G.C.

For hydrocarbons, the nitrogen carrier gas flow rate was 40 mls/min. Initial injection heater temperature was 240°, and oven temperature programming was used from 100–350° at 6°/min.

For esters, the nitrogen flow rate was 60 mls/min with an initial heater temperature at 350°, oven temperature programming from 250–400° at 6°/min.

The methyl esters and alcohols were analyzed on glass columns (1.5m x 4mm i.d.) packed with 3% Apiezon L. The nitrogen flow rate was 60 mls/min. Injector heater temperature was 300° with oven programming from 200–280° at 3°/min.

Percentage areas of alkane and ester homologues were determined for tlc fractions by analysis of a measured volume of the fraction in solvent on the gc, which was linked to a Spectra Physics SP4100 computing integrator. Total and % area mean values from three replicates were converted to total and % weights by the inclusion of quantitative internal standards, *n*-docosane (Applied Science) for *n*-alkanes and octadecyl octadecanoate (nu-Chek-Prep) for the esters. The total weight of the injected sample was then used to calculate the weight of the tlc fraction, table 1. Response factors were found to be unity by experimentation with a range of alkane and ester standards (Nu-Chek-Prep). Weights calculated by gc were confirmed by gravimetric methods.

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LITERATURE CITED

1. C. Bengtson, S. Larsson and C. Lifjenberg, *ADV. in Biochem. and Physiol of Plant Lipids*. Eds. L. A. Appelquist and C. Lifjenberg., 269 (1979).
2. B. N. Geise, *Phytochemistry*, **14**, 921 (1975).
3. R. J. Hamilton, and D. M. Power, *Phytochemistry*, **8**, 1771 (1969).
4. O. O. P. Faboya, J. I. Okogun, and D. R. Goddard, *Phytochemistry*, **19**, 1226 (1980).
5. G. Bianchi, and M. Corbellini, *Phytochemistry*, **16**, 943 (1977).
6. G. Cooper-Driver, S. Finch, T. Swain and E. Bernays, *Biochem. Syst. Ecol*, **5**, 177 (1977).
7. G. E. Haniotakis and A. Voyadjoglou, *Ent. exp. and Appl.* Proceeding 4th Insect/Host Plant Symposium, **24**, 187 (1978).
8. J. A. Hardman and P. R. Ellis, *ibid*, **24**, 193 (1978).
9. D. A. Maelzer, *Aust. J. Zool.*, **25**, 269 (1977).
10. Food Agric. Org. (U.N.), *Mon. Bull. Agric. Econ. Stat.*, **9**, 24 (1975).
11. S. Woodhead, and E. A. Bernays, *Ent. exp. and appl.*, **24**, 123 (1978).
12. A. P. Tulloch, and L. L. Hoffman, *Phytochemistry*, **10**, 871 (1970).
13. A. P. Tulloch, and L. L. Hoffman, *Phytochemistry*, **15**, 1145 (1976).
14. A. P. Tulloch, *Phytochemistry*, **17**, 1613 (1978).
15. A. P. Tulloch, and L. L. Hoffman, *Phytochemistry*, **18**, 267 (1978).
16. A. P. Tulloch, and L. Bergter, *Phytochemistry*, **19**, 145 (1980).
17. A. P. Tulloch, and L. L. Hoffman, *Lipids*, **8**, 617 (1973).
18. A. P. Tulloch, and L. L. Hoffman, *Phytochemistry*, **12**, 2217 (1973).
19. A. P. Tulloch, and L. L. Hoffman, *Phytochemistry*, **19**, 827 (1980).
20. A. P. Tulloch, and L. L. Hoffman, *Phytochemistry*, **13**, 2535 (1974).
21. A. P. Tulloch, *Phytochemistry*, **15**, 1153 (1976).
22. G. Bianchi, and F. Salamni, *Maydica*, **20**, 1 (1975).
23. G. Bianchi, F. Salamni, and P. Avato, *Maydica*, **20**, 165 (1975).
24. G. Bianchi, F. Salamni, and P. Avoto, *Maydica*, **22**, 9 (1977).
25. G. Bianchi, F. Salamni, and P. Avato, *Heredity*, **42**, 391 (1979).
26. R. E. Wilkinson, and D. G. Cummins, *Crop Science*, **21**, 397 (1981).
27. G. Bianchi, P. Avato, P. Bertorelli, and G. Mariani, *Phytochemistry*, **17**, 999 (1978).
28. T. K. Miwa, *J. Amer. Oil Chemists Soc.*, **48**, 259 (1971).
29. J. E. Allebone, and R. J. Hamilton, *J. Sci. Fd Agric.*, **23**, 777 (1972).
30. D. S. J. Atkin, and R. J. Hamilton in "The Plant Cuticle" Ed. D. Cutler, E. C. Price, J. Alvin, Acad. Press (1982) in press.
31. R. L. Vanderlip, 'How a sorghum plant develops' (1979) Contribution No. 1203. Agronomy Dept., Kansas Agricultural Experimental Station, Manahattan, 66506.
32. L. J. Morris, *J. Lipid Res.*, **7**, 717 (1966).
33. J. G. O'Connor, F. H. Burow, and M. S. Norris, *Anal. Chem.*, **34**, 82 (1962).
34. D. S. J. Atkin and R. J. Hamilton, *J. Nat. Prod.*, **45**, 000 (1982).