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SEASONAL AND SO_2-INDUCED CHANGES IN EPICUTICULAR WAX OF RYEGRASS

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Key Word Index—*Lolium perenne*; Gramineae; ryegrass; epicuticular wax; SO_2; seasonal changes.

Abstract—Epicuticular wax was extracted from SO_2-sensitive (S23) and SO_2-resistant (BR) genotypes of ryegrass (*Lolium perenne*) exposed continuously for 4 weeks to 0, 0.05 or 0.15 ppm SO_2 under winter or summer conditions. The differences found between genotypes in the composition of the acid and alkane fractions of the extracted wax were further modified by season and exposure to SO_2. Estimates of relative amounts of epicuticular wax showed that both genotypes produced more wax in the winter experiment, with BR producing more wax than S23. Exposure to 0.05 ppm in the winter experiment had little effect on the amount of wax produced by either genotype while exposure to 0.15 ppm SO_2 greatly reduced wax production in BR but increased wax production in S23. These changes in wax production could have been due to a general effect of SO_2 on metabolism regulating the flow of carbon into the pathways synthesizing wax components rather than to specific inhibition or enhancement at particular steps in these pathways. Exposure to SO_2 in the summer experiment had little effect on the amount of wax produced by both genotypes but did have an effect on wax composition.

INTRODUCTION

The amount and distribution of epicuticular wax have been shown to modify the extent of damage sustained by leaves of plants exposed to gaseous air pollutants. Bystrom *et al.* [1] found that the rate of wax extrusion in *Beta vulgaris* did not keep pace with the rapid expansion of the epidermal surface of developing leaves, resulting in an incomplete coverage of the leaf surface that contributed to the susceptibility to damage from irradiated auto exhaust. The extent of foliar glazing and necrosis in *Phaseolus vulgaris* exposed to gaseous HCl were inversely correlated with the amount of epicuticular wax [2]; the observation of glazing in astomatous portions of leaves demonstrated that the acidic gas could penetrate the waxy cuticle.

A recent study with astomatous cuticles isolated from *Citrus aurantium* has shown SO_2 to be 688 times more soluble in the cuticular membrane (with waxes) and 843 times more soluble in the cuticular matrix (waxes removed) than in water [3]. However, the cuticular membranes of *C. aurantium* were approximately 3500 times less permeable to SO_2 than a water layer of equal thickness, indicating that the permeability of the cuticle to gases is strongly influenced by its wax components. In cuticles of *C. aurantium* the resistance of the cuticular membrane to SO_2 was almost completely due to the epicuticular waxes which accounted for only 3% of the weight of the cuticle, while in *Lycopersicon esculentum* about 50% of the resistance to SO_2 permeation was due to the epicuticular waxes which comprised 4–6% of the total weight of the cuticle [3].

While the composition of the epicuticular wax can affect the permeation of SO_2, exposure to SO_2 can also alter the amount and morphology of epicuticular wax. In *Phaseolus vulgaris*, exposure to 0.75 ppm SO_2 for 8 hr resulted in trichomes subsequently becoming encrusted in waxy concretions, and this effect of SO_2 was found to precede the development of visible symptoms of SO_2 injury [4]. Koziol and Cowling [5] observed that leaves of *Lolium perenne* exposed to 0.16 ppm SO_2 for 23 days had visibly more epicuticular wax than leaves of control plants but that the wax morphology was not altered: waxy concretions were observed only over necrotic areas.

In this study two genotypes of ryegrass (*L. perenne*), one sensitive (S23) and the other resistant (BR) to SO_2, were exposed for 4 weeks to 0, 0.05 or 0.15 ppm SO_2 under conditions giving slow (winter) or fast (summer) rates of growth, when plants are expected to be maximally or minimally sensitive, respectively, to the effects of SO_2 [6]. The object was to determine the nature of SO_2-induced changes in epicuticular wax and the existence of any interaction between growth season and SO_2 on epicuticular wax.

RESULTS AND DISCUSSION

Epicuticular wax was extracted from mature leaves, saponified and analysed by capillary GC: individual components were identified by capillary GC/MS. *In toto* 28 compounds were identified: 13 *n*-alkanes (C_{23}–C_{35}), one dicarboxylic acid, nine fatty acids and five alcohols. For semiquantitative analysis the peak area of each component identified (A_x) was divided by the peak area of the internal standard heneicosanoic acid (A_{IS}, representing 100 μg) and by the area (LA, both sides) of the leaves from which the epicuticular wax was extracted. The values from the duplicate experiments at each growth rate were then averaged. Thus an estimate of total epicuticular wax ($\mu g/cm^2$ leaf area) was obtained as

$$\sum_{x=1}^{28} \{[(A_x/A_{IS} \cdot LA)_1 + (A_x/A_{IS} \cdot LA)_2]/2\}$$

where the subscripts 1 and 2 refer to the duplicate experiments conducted at each growth rate. Estimates for total epicuticular wax extracted from the leaf samples are given in Table 1. Leaf samples taken for wax analysis had similar specific leaf areas (specific leaf area = leaf area ÷ leaf dry weight) so it is unlikely that the smaller amounts of wax on leaves during the summer exposures were the result of rates of leaf expansion exceeding those of wax extrusion [1]. Examination of the adaxial surfaces of the leaves by scanning electron microscopy (SEM) confirmed the data presented in Table 1. No differences in wax density could be seen between genotypes or treatments in the summer experiment, but both genotypes did have visibly more wax in the winter than in the summer experiment. The enhanced production of epicuticular wax in leaves of *L. perenne* developing during the winter experiment agrees with the observations that the amount of wax produced by *Prunus domestica* [7] and *Brassica oleracea* var. *gemmifera* [8] was inversely proportional to temperature. In the winter experiment the amount of wax on the adaxial leaf surfaces of BR in the control and 0.05 ppm SO_2 treatments was easily distinguishable from that on S23 when viewed by SEM, though within each genotype the only salient difference in wax density was that observed in response to exposure to 0.15 ppm SO_2. The nature of the response to 0.15 ppm SO_2 is interesting, the amount of wax being decreased in the SO_2-resistant BR genotype but increased in the sensitive S23 genotype.

Table 1. Seasonal and SO_2-induced changes in the relative amounts of epicuticular wax ($\mu g/cm^2$ leaf area) in SO_2-resistant (BR) and sensitive (S23) genotypes of ryegrass

Growth conditions	Genotype	SO_2 exposure concentration (ppm) 0	0.05	0.15
Winter	BR	103.6	117.4	39.3
	S23	57.1	52.8	111.1
Summer	BR	11.6	8.2	8.1
	S23	8.8	7.9	6.8

Epicuticular wax was estimated by summation of the individual values obtained from dividing the peak area of each wax component identified by the area of the internal standard (representing 100 μg) and by the area (both sides) of the leaves from which the wax was extracted. The values listed above are the average of two exposure experiments conducted under each growth regime: plants were exposed continuously to SO_2 for 4 weeks.

It has been shown that the synthesis of the components of epicuticular wax occurs in the epidermal cells [9]. The initial steps in the biosynthesis of wax components are similar to those in the synthesis of cellular fatty acids up to palmitic acid. In the synthesis of wax components, chain elongation then occurs to produce the longer chain fatty acids which can be reduced or decarboxylated to produce long chain alcohols or alkanes, respectively. Various effects of SO_2 on the cellular synthesis of fatty acids and lipids have been reported [10–12] and several caveats suggested in assessing the results [13]. In summary, SO_2 is likely to affect only a few key steps in fatty acid/lipid synthesis [13], notably the $C_{16} \rightarrow C_{18}$ step in the elongation pathway and the synthesis of polar lipids and fatty acid esters [10, 12].

Any major inhibition by SO_2 of the synthesis of wax components at steps subsequent to the synthesis of palmitic acid would be expected to alter the composition of the epicuticular wax. Focusing attention on BR, which showed a decrease in the amount of wax in response to 0.15 ppm SO_2 under winter conditions, Table 2 shows that SO_2 had no effect on wax composition: this is further supported by comparing the profiles for the acids and alcohols (Tables 3 and 4) and *n*-alkanes (Table 5) for the

Table 2. General composition of epicuticular wax extracted from SO_2-resistant (BR) and sensitive (S23) genotypes of ryegrass exposed to various concentrations of SO_2 for 4 weeks under different growth conditions

Growth conditions	Genotype	SO_2 concns (ppm)	% Composition *n*-Alkanes	Acids	Alcohols
Winter	BR	0	23.9	22.4	53.7
		0.05	25.6	28.3	46.1
		0.15	25.5	24.9	49.6
	S23	0	17.4	37.7	44.9
		0.05	26.8	38.6	34.6
		0.15	18.1	19.5	62.4
Summer	BR	0	29.2	17.7	53.1
		0.05	32.0	27.2	40.8
		0.15	33.2	21.3	45.5
	S23	0	27.4	36.7	35.9
		0.05	27.8	32.9	39.3
		0.15	28.7	31.6	39.7

Values above represent the averages from duplicate exposure experiments conducted under winter or summer conditions.

control and 0.15 ppm SO_2 treatments. The absence of dramatic changes in the wax components argues against direct SO_2 inhibition of the synthesis of wax components; however, it does not preclude a more general effect of SO_2 on the metabolism of the epidermal cells that might reduce the total carbon flow through these synthetic pathways in BR. For S23, which produced more epicuticular wax in response to 0.15 ppm SO_2 than in the control treatment, a general increase in carbon flow through the pathways of wax synthesis would be required. Altering the cellular concentration of acetyl-CoA is one way in which SO_2 could affect carbon flow into the pathways of fatty acid synthesis, as SO_2 has been shown either to inhibit or to enhance the activities of phosphoglucoisomerase and glucose-6-phosphate dehydrogenase, thus affecting carbon flow through glycolysis and the oxidative pentose phosphate pathways [14, see Fig. 18.1]. A difference between BR and S23 in the response of these enzymes to SO_2 might be one explanation for an altered carbon flow through pathways of wax synthesis. Weinstein [15] investigated the effects of HF on selected isoenzymes in leaves of F^--sensitive and F^--resistant genotypes of *Solanum pseudocapsicum* and found that exposure to HF increased the foliar content of the isoenzymes of glucose-6-phosphate dehydrogenase only in the sensitive genotype. A differential induction in the transcription of isoenzymes of phosphoglucoisomerase and/or glucose-6-phosphate dehydrogenase in BR and S23 in response to 0.15 ppm SO_2 might better explain the differences in carbon flow into fatty acid synthesis and subsequently into the synthesis of wax components. The rate of permeation and of entry of SO_2 into the epidermal cells may have been too slow in plants exposed to 0.05 ppm SO_2 for concentrations of HSO_3^-/SO_3^{2-} to reach the cellular threshold required for the induction of isoenzyme synthesis to occur.

The general composition of epicuticular wax of BR in the winter and summer experiments was similar, with a slight increase in the percentage of *n*-alkanes and decrease in alcohols in the summer experiment (Table 2). In the winter experiment S23 in both the control and 0.05 ppm SO_2 treatments had a higher percentage than BR of acids in the epicuticular wax, though whether these occurred as free acids or esters is uncertain as the extracted wax was saponified before analysis. At 0.15 ppm SO_2, S23 showed a marked increase in the percentage of alcohols: whether this was due to a stimulation of the reductase enzymes is uncertain. In the summer experiment carbon incorporated into wax components in S23 was more equally partitioned among the three major fractions than was the case with BR: no definite effects of SO_2 were seen.

Seasonal differences were found in the acidic components of the extracted epicuticular wax of the two genotypes (Table 3). In the winter experiment, palmitic, oleic and stearic acids were the major components in the control treatments, representing *ca* 80% of the total acidic fraction in each genotype. In the summer experiment these acids represented only 51% of the total acidic fraction in BR, with tetradecanoic, eicosanoic and hexadecanoic acids each increasing to *ca* 10% of the total. In S23 the contribution of the C_{16} and C_{18} acids fell to 61% of the total, with tetradecanoic and docosanoic acids increasing to 14% and 11%, respectively.

Palmitic acid increased and oleic acid decreased in S23 in the winter experiment and in BR in the summer experiment in response to SO_2. In both cases the percentages of stearic acid and longer chain fatty acids were similar in the control and 0.05 ppm SO_2 treatments, and actually increased in response to 0.15 ppm SO_2, suggesting that exposure to SO_2 was inhibiting only the synthesis of oleic acid.

In all cases *n*-hexacosanol was the major alcohol detected (Table 4) in agreement with the findings of Hamilton and Power [16]. The variations due to season and exposure to SO_2 were relatively small.

Seasonal differences and differences between genotypes were found in the hydrocarbon components of the epicuticular wax (Table 5), but the results of our analyses are generally in agreement with Hamilton and Power [16] who reported a predominance of odd-numbered *n*-alkanes between C_{27} and C_{33}, inclusive, in the epicuticular wax of *L. perenne*. In the control treatments in the winter experiment the major hydrocarbon in BR was a C_{33} *n*-alkane (41% of the total), while in S23 C_{24} and C_{33} *n*-alkanes accounted for 20% and 24%, respectively, of the total hydrocarbon fraction. As with the acidic fraction, carbon incorporated in the hydrocarbon fraction in the summer experiment was more generally distributed, with

Table 3. Percentage composition of the acid fraction of epicuticular wax extracted from SO_2-resistant (BR) and sensitive (S23) genotypes of ryegrass exposed to various concentrations of SO_2 for 4 weeks under different growth conditions

		Winter						Summer					
Homologue	Genotype:	BR			S23			BR			S23		
Carbon No.	SO_2 conc. (ppm):	0	0.05	0.15	0	0.05	0.15	0	0.05	0.15	0	0.05	0.15
C_9 dioic		2.2	1.1	2.4	3.4	2.9	1.0	5.6	2.6	1.9	0.8	0.6	0.6
14:0		3.1	4.6	6.5	4.8	6.5	5.6	10.7	15.5	8.0	14.1	12.7	8.6
16:0		34.7	32.5	31.9	20.9	31.0	33.3	15.8	27.7	26.8	36.4	28.8	29.6
18:1 (9)		15.6	27.2	13.7	25.9	17.4	7.2	19.2	12.2	5.6	12.0	15.2	10.5
18:0		29.8	26.1	27.8	32.3	28.1	34.4	16.4	19.2	24.9	12.8	19.4	25.2
20:0		2.2	1.4	2.4	4.2	2.9	4.1	9.6	5.5	3.3	5.2	4.5	6.7
22:0		5.8	5.3	4.8	4.2	4.7	5.1	5.1	7.7	8.0	10.6	9.4	7.0
24:0		0.9	1.4	2.4	1.6	2.3	2.1	5.6	1.8	11.7	3.5	2.1	1.6
26:0		0.9	tr	0.4	1.6	1.8	tr	10.2	5.9	4.7	2.2	3.9	8.9
28:0		4.9	0.4	7.7	1.1	2.3	7.2	1.7	1.8	5.2	2.4	3.3	1.3

tr = trace. Values above represent the averages from duplicate exposure experiments conducted under winter or summer conditions.

Table 4. Percentage composition of the 1-alkanol fraction of epicuticular wax extracted from SO_2-resistant (BR) and sensitive (S23) genotypes of ryegrass exposed to various concentrations of SO_2 for 4 weeks under different growth conditions

	Winter						Summer					
Homologue Genotype:	BR			S23			BR			S23		
Carbon No. SO_2 conc. (ppm):	0	0.05	0.15	0	0.05	0.15	0	0.05	0.15	0	0.05	0.15
20	1.1	1.3	1.0	1.6	4.9	1.4	6.8	1.7	1.5	1.1	1.3	1.8
22	2.4	2.8	3.0	2.7	4.9	2.1	1.1	2.0	1.3	1.4	1.5	1.3
24	1.9	2.6	2.0	1.1	5.2	1.9	7.0	2.2	3.1	1.7	4.3	1.8
26	92.7	90.9	91.3	93.5	78.0	92.5	81.1	91.2	89.4	89.4	87.8	91.4
28	1.9	2.4	2.6	1.1	7.9	2.1	4.0	2.9	4.6	6.4	5.1	3.8

Values above represent the averages from duplicate exposure experiments conducted under winter or summer conditions.

Table 5. Percentage composition of the *n*-alkane fraction of epicuticular wax extracted from SO_2-resistant (BR) and sensitive (S23) genotypes of ryegrass exposed to various concentrations of SO_2 for 4 weeks under different growth conditions

	Winter						Summer					
Homologue Genotype:	BR			S23			BR			S23		
Carbon No. SO_2 conc. (ppm):	0	0.05	0.15	0	0.05	0.15	0	0.05	0.15	0	0.05	0.15
23	4.2	3.9	4.3	6.3	4.5	4.4	7.2	7.1	6.6	8.0	11.1	7.3
24	6.7	6.7	6.7	20.1	6.0	8.3	10.3	14.3	12.0	13.1	11.8	14.3
25	3.8	7.1	7.1	9.7	6.0	7.2	5.5	12.4	11.5	5.5	9.7	13.2
26	5.8	5.9	6.3	6.9	5.6	6.6	11.3	10.9	11.8	11.6	9.3	12.9
27	7.5	7.8	7.5	8.0	6.4	7.2	15.8	10.9	10.9	9.8	8.6	10.1
28	4.2	7.5	5.9	2.9	12.4	5.5	7.9	5.0	6.5	12.4	11.8	7.0
29	8.8	8.2	7.9	6.9	2.2	7.8	10.3	9.0	9.3	8.0	8.2	8.0
30	2.1	2.7	4.3	2.9	0.7	5.0	2.8	3.1	3.7	2.9	2.9	2.4
31	12.1	11.0	9.8	9.2	13.1	11.0	12.7	11.5	13.0	11.6	11.1	9.1
32	1.3	1.2	2.4	1.1	1.5	1.7	0.7	1.2	1.2	tr	0.7	1.0
33	40.8	34.5	33.9	24.1	39.7	33.1	12.7	11.5	12.4	12.4	11.8	9.4
34	1.7	0.8	2.8	0.6	0.4	1.1	2.7	1.6	2.2	2.2	1.1	1.0
35	1.3	2.7	1.2	2.3	1.5	1.1	0.3	1.6	1.6	2.5	1.8	4.2

tr = trace. Values above represent the averages from duplicate exposure experiments conducted under winter or summer conditions.

no component accounting for more than 16% of the total hydrocarbon fraction. In both genotypes the components accounting for 10% or more of the hydrocarbon fraction in the summer experiment were C_{24}, C_{26}, C_{29}, C_{31} and C_{33} *n*-alkanes; in addition BR had a C_{27} and S23 a C_{28} *n*-alkane. The most obvious effects of exposure to SO_2 were a decrease in C_{24} and an increase in C_{33} *n*-alkanes in the winter experiment, and an increase in the C_{25} *n*-alkane in both genotypes in the summer experiment.

The results of this study have shown that exposure of *L. perenne* to a high concentration of SO_2, such as 0.15 ppm, during the winter will have a greater effect on the amount of wax produced than exposure during the summer. Exposure to SO_2 in either season can affect the composition of the epicuticular wax but the effects of such changes on the permeation characteristics of the cuticular membrane remain uncertain in the absence of research correlating wax composition with gaseous permeation. The composition of epicuticular wax can vary with age [16] and with changes in environmental conditions [7, 8]; there can also be differences between cultivars [17] and, as this study has shown, between genotypes isolated from a commercial cultivar [18] previously assumed to be homogeneous.

EXPERIMENTAL

Plant material. Tillers of a genotype of ryegrass (*L. perenne* L.) derived from the S23 cultivar and showing a high resistance to acute SO_2 injury [18] were supplied by Dr. J. N. B. Bell of Imperial College, London. This genotype, named 'S23 Bell resistant' [19] and referred to here as BR, was vegetatively propagated in a glasshouse; plants of the sensitive S23 genotype were similarly propagated. Thirteen uniform tillers were planted per pot (150 mm diameter and 210 mm deep) containing 4.5 kg (dry wt) of soil. The soil used was a sandy clay loam overlying chalk collected from the 0–15 cm horizon at the Animal and Grassland Research Institute (Hurley, Berks., U.K.); it has been fully described elsewhere [20]. After planting, the pots were watered to a predetermined weight to bring soil water tension to field capacity (7360 Pa) and then transferred to a growth cabinet maintained at 23° with a 12 hr photoperiod and an illumination of 86 W/m^2 provided by fluorescent lights. During the last week in the growth cabinet the temp. was gradually adjusted to that which the plants would experience in the exposure chambers. After 4 weeks in the growth cabinet the plant shoots were trimmed to 50 mm above the soil surface and stripped of dead material. The pots were watered to field capacity and the soil surface covered with a 10 mm layer of white polyethylene

granules to minimize evapn of H_2O from, and the adsorption of SO_2 by, the moist soil surfaces. The pots of grass were then placed in the exposure chambers.

Exposure system. The 10-chamber exposure system, situated in a glasshouse and described fully elsewhere [21], was modified for these expts. The perforated ceiling in each chamber was replaced by a perforated tube of Melinex extending from the air inlet port across the width of the chamber. Beneath this tube was suspended an 80 mm diameter axial fan which was used to increase the turbulence within the chamber. The air flow rate into each chamber was adjusted to 200 l./min at a slight positive pressure. Under these conditions the leaf aerodynamic (= boundary layer) resistance was 0.20 s/cm, as measured by the method of ref. [22] 100 mm above the centre of a 150 mm diameter pot containing ryegrass with shoots up to 200 mm long.

Concns of SO_2 were monitored simultaneously at the inlet and outlet ports of each chamber for periods of 6 min beginning at hourly intervals using 2 Meloy SA-285 sulphur analysers equipped with H_2S scrubbers (Meloy Laboratories Inc., Springfield, VA, U.S.A.). The analysers were calibrated regularly using the West-Gaeke [23] procedure as a ref. method, and also against each other.

Experimental design. The S23 and BR genotypes were exposed to 0, 0.05 or 0.15 ppm SO_2 continuously for 4 weeks during the winter and early spring to give slow and fast rates of growth, respectively. Two exposure expts were conducted at each growth rate using a 3 × 2 factorial design with incomplete replication of the control treatment. In practice this meant that for each genotype one chamber was assigned to the control treatment, two to 0.05 ppm SO_2 and two to 0.15 ppm SO_2. Genotypes and SO_2 treatments were allotted at random to the 10 chambers for the duplicate expts with these allocations maintained for the two growth rates studied. The duplicate winter expts were conducted between 23 November 1983 and 1 February 1984 with glasshouse heating reduced to frost protection only (4°). To standardize the photoperiod to 8 hr, supplementary lighting (mercury halide lamps) at 36 W/m^2 at plant height was provided from 08.00 to 10.00 hr and from 15.00 to 16.00 hr daily; natural light before 08.00 hr and after 16.00 hr was considered photosynthetically insignificant. The mean leaf temp. over the duplicate exposure expts was 12.3 ± 0.5° and the relative humidity 55 ± 2%. These conditions are referred to as 'winter' in this paper. The duplicate spring expts were conducted between 23 March and 25 May, 1983 with the glasshouse ventilated to maintain temps as close to ambient as possible. Photoperiods were standardized to 16 hr by providing supplementary lighting from 05.00 to 07.30 hr and from 19.00 to 21.00 hr daily. The mean leaf temp. over the duplicate exposure expts was 24.9 ± 1.1° and the relative humidity 44 ± 1%. These conditions are referred to as 'summer'.

Plants had sufficient leaf material at the beginning of the second week of exposure to permit measurements of leaf temps. These measurements were made three times a week on five leaves chosen at random from each of the two pots closest to the chamber doors, using diffusion porometers with thermocouples that appressed leaf surfaces (model Mk2, Delta-T Devices, Cambridge, England). Plastic gloves could be affixed to the chamber doors and the sensor heads of the porometers inserted into a chamber through a slit made at the base of one glove. As gloves could be rapidly affixed to the chamber doors the disturbance to the atmosphere within a chamber was minimal.

Wax analysis. For examination of changes in leaf surfaces by SEM, 2 mm segments of fresh, mature leaves taken from the middle portion of each leaf were mounted with adaxial surfaces uppermost on a stub using colloidal graphite. The stub with the segments was immersed in liquid N_2 before coating segments with gold using a sputter cryo unit. The stub with the leaf segments was transferred to a microscope equipped with a liquid N_2 cooled stage.

For GC and GC/MS analysis, six mature leaves were selected from one pot of each genotype per SO_2 treatment, their surface areas measured using an electronic planimeter and fr. wts recorded. The leaves were then cut into *ca* 80 mm lengths and dipped sequentially into test tubes containing 13 ml of solvent according to a schedule modified from ref. [24]: (1) MeOH, 10–15 sec; (2) $CHCl_3$–MeOH, 2:1, 10–15 sec; (3) $CHCl_3$, 10–15 sec; and (4) MeOH, 30 sec. The solvents from the four test tubes were combined and evapd to dryness *in vacuo* (< 40°). The extracted epicuticular waxes were transferred to derivatization vials with rinses of Et_2O and petrol (60–80°), evaporating each rinse to dryness under a stream of dry N_2. Heneicosanoic acid (100 μg) was added to each vial as int. standard. Chromatography of compounds greater than C_{40} is not practical with capillary columns. Wax samples were therefore saponified with 10% KOH in 90% EtOH, then neutralized with 2 N HCl and subsequently freeze-dried. The methoxime derivatives of any keto-compounds present were made using 2% methoxyamine hydrochloride in pyridine. After cooling, samples were taken to dryness under N_2 and derivatized with BSTFA (N,O-*bis*(trimethylsilyl)-trifluoroacetamide).

Derivatized samples were analysed with a FID and a 25 m FSOT column coated with OV-1; the inj. and detector temps were kept at 350°. The temp. prog. held an initial temp. of 100° for 5 min, increased by 3°/min to 300° and was held at final temp. for 15 min. For analysis, 0.1–1.0 μl of sample was injected. GC/MS analysis was performed on a quadropole automated instrument fitted with a 50 m FSOT column coated with OV-1. Samples (1 μl) were injected at 300° and the injector flush/split valve opened after 30 sec; after 200 sec the filament was turned on, thus avoiding the solvent peak. The GC temp. prog. held an initial temp. of 100° for 2 min, increased by 3°/min to 300° and was held at final temp. for 5 min; He, supplied at 1 kg/cm^2, was used as carrier. The transfer line and analyser were maintained at 280° and 80°, respectively. Other MS parameters were: MS peak detection threshold = 10.0 linear counts; electron impact energy = 70 eV; scan range = 40–650 u in 1.0 sec; tuning was by auto-tune using perfluorobutylamine. Compounds were identified by comparison of spectra with a user-created data file and methylene unit R_t index.

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REFERENCES

1. Bystrom, B. G., Glater, R. B., Scott, F. M. and Bowler, E. S. C. (1968) *Bot. Gaz.* **129**, 133.
2. Swiecki, T. J., Endress, A. G. and Taylor, O. C. (1982) *Can. J. Botany* **60**, 316.
3. Lendzian, K. J. (1984) in *Gaseous Air Pollutants and Plant Metabolism* (Koziol, M. J. and Whatley, F. R., eds) p. 77. Butterworths, London.

4. Paul, R. and Huynh-Long, V. (1975) *Parasitica* **31**, 30.
5. Koziol, M. J. and Cowling, D. W. (1981) *Environ. Pollut. (Ser. A)* **26**, 183.
6. Davies, T. (1980) *Nature (London)* **284**, 483.
7. Leece, D. R. (1978) *Aust. J. Plant Physiol.* **5**, 749.
8. Baker, E. A. (1974) *New Phytol.* **73**, 955.
9. Kolattukudy, P. E., Croteau, R. and Buckner, J. S. (1976) in *Chemistry and Biochemistry of Natural Waxes* (Kolattukudy, P. E., ed.) p. 289. Elsevier, Amsterdam.
10. Kahn, A. A. and Malhotra, S. S. (1977) *Phytochemistry* **16**, 539.
11. Malhotra, S. S. and Kahn, A. A. (1978) *Phytochemistry* **17**, 241.
12. Grunwald, C. (1981) *Plant Physiol.* **68**, 868.
13. Heath, R. L. (1984) in *Gaseous Air Pollutants and Plant Metabolism* (Koziol, M. J. and Whatley, F. R., eds) p. 275. Butterworths, London.
14. Koziol, M. J. (1984) in *Gaseous Air Pollutants and Plant Metabolism* (Koziol, M. J. and Whatley, F. R., eds) p. 251. Butterworths, London.
15. Weinstein, L. H. (1977) *J. Occup. Med.* **19**, 49.
16. Hamilton, R. J. and Power, D. M. (1969) *Phytochemistry* **8**, 1771.
17. O'Toole, J. C., Cruz, R. T. and Seiber, J. N. (1979) *Physiol. Plant.* **47**, 239.
18. Ayazloo, M., Garsed, S. G. and Bell, J. N. B. (1982) *New Phytol.* **90**, 109.
19. Wellburn, A. R., Higginson, C., Robinson, D. and Walmsley, C. (1981) *New Phytol.* **88**, 223.
20. Cowling, D. W. and Jones, L. H. P. (1970) *Soil Sci.* **110**, 346.
21. Lockyer, D. R., Cowling, D. W. and Jones, L. H. P. (1976) *J. Exp. Botany* **27**, 397.
22. Sheehy, J. E. and Tearle, A. M. (1975) *The Construction of Instruments for Measuring and Manipulating the Plant Environment. Technical Report No.* 16, p. 24. The Animal and Grassland Research Institute, Hurley, Berks., U.K.
23. West, P. W. and Gaeke, G. C. (1956) *Analyt. Chem.* **28**, 1816.
24. Lendzian, K. L. (1982) *Planta* **155**, 310.