Chemical Constituents from Shoots of *Hordeum vulgare* Infested by the Aphid *Schizaphis graminum*

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Stress, pathogens or insects induce profound changes in the expression of the chemical constituents of plants. Some of these changes could be adaptations to the new conditions or the expression of new defensive characteristics. The purpose of this paper was to analyze the effect of aphid infestation on the composition of endogenous compound, in barley leaves. Leaf waxes were obtained by washing with dichloromethane and remaining compounds were extracted with ethyl acetate and ethanol from homogenized leaves. The identification of compounds was done by combined capillary gas chromatography and mass spectrometry. Epicuticular compounds detected in barley after 6 days of aphid infestation were as follows (%): **(1)** 1-docosene (1.98), **(2)** 1-octadecene (2.07), **(3)** phytol (0.81). Epicuticular compounds in non-infested plants were **(4)** isophytol (0.83), **(5)** N-cyclohexyl-cyclohexanamine, (0.11) **(6)** 5-pentadecyl-1,3-benzenediol (2.10). In ethyl acetate extracts from infested plants the main compounds were: **(1)** (2.02), **(7)** 2,5-diethylpyrazine (0.61), **(8)** (5*E*)-eicosene (1.61), **(9)** (3*E*)-eicosene (1.93), **(10)** (9*E*)-eicosene (1.80). In non-infested plants the only detected compounds were **(1)** (1.02), **(6)** (1.91), and **(7)** (0.43). In methanol extracts from infested plants the following compounds were identified: **(6)** (2.15), **(7)** (0.21), **(11)** 2,2,6.6 tetramethyl-piperidine (0.12), **(12)** indole (1.01), **(13)** heptadecane (0.10), **(14)** methyl-β-D-glucopyranoside (0.21), **(15)** 2,5- dihydroxybenzaldehyde (0.27) and **(16)** N-phenyl-2-naphthalenamine (0.29). In polar extracts from non-infested plants the most abundant compounds were **(4)** (1.40), **(7)** (0.18), **(17)** (9*Z*,12*Z*,15*Z*)-octadecatrienoic methyl ester (1.01), **(18)** (9*Z*,15*Z*)-octadienoic methyl ester (2.01), and **(19)** hexadecanoic methyl ester (1.93). Compounds **4, 5** and **7–19** are described for the first time in *Hordeum vulgare*.

Introduction

Barley is often damaged by aphids because they are virus vectors, penetrate tissues and consume nutrients from the plant (Corcuera, 1993). The first physical barrier for the aphids are epicuticular waxes (Harborne, 1993). A second barrier are various chemical compounds, which are heterogeneously distributed in the cuticle and other plant tissues. For example, wheat accumulates several glycosides of hydroxamic acids and barley accumulates the indole protoalkaloid gramine, specially in younger tissues (Argandoña *et al.,* 1987). Hydroxamic acids appear to be involved in defense against insects, such as the European corn borer and aphids (Argandoña *et al.*, 1987; Klum and Brindley, 1966). Gramine may be involved in defense against herbivores and pathogens (Corcuera, 1993). This compound is found mainly in mesophyll parenchyma cells and epidermis, but appears to be absent in vascular bundles (Argandoña *et al.*, 1987). Aphid infestation may induce changes in the chemical characteristics of the plants. The induction of phenolic compounds and flavonoids, several of which have been described in barley and wheat, may cause toxicity and feeding deterrency against insects (Harborne, 1993). In spite of the agricultural importance of the Gramineae, relatively few extensive reports exist on the chemical constituents of these plants. The main problems encountered are the size of the family (6000–9000 species) and the difficulties for rigor-

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ous taxonomic identification (Harborne and Williams, 1976).

Recently, isolation and characterization has been reported of 5-(8'*Z*- heptadecenyl) resorcinol and its C_{13},-C_{15} and C_{17} homologous with alkyl saturated chains in rice seedlings (Suzuki *et al.*, 1996). Several hydroxylated and high unsaturated derivatives of linoleic acid, isolated from rice have been implicated as defensive substance against attack by plant pathogens; for example, a new oxylipin 15(*R*)-hydroxy-9(*Z*), 12(*Z*)-octadecadienoic acid has also been isolated from oat seeds (Hamberg and Hamberg, 1996). The amount found in seeds of barley and wheat, however was less than 1 μg/g fresh weight. Ten unsaturated fatty acids (C_{18}), five of which contained epoxide groups and other five contained a hydroxyl group were isolated from leaves of two varieties of *Oryza sativa* (Harborne, 1986; Grayer and Harborne, 1994). All have been described to show antifungal activity against the rice blast fungus *Pyricularia oryzae* in two resistant varieties of rice.

In this paper, we report the identification of the a number of chemical constituents of barley leaves and the effects of infestation with the aphid *Schizaphis graminum* on the chemical composition of this plant.

Results and Discussion

Effect of aphid infestation on the chemical composition of epicuticular compounds

Chromatographic analyses and gas chromatography/mass spectrometry of compounds obtained from leaves after a brief immersion in CH_2Cl_2, followed by precipitation of most alkanes and other fatty constituents with MeOH, showed differences between aphid infested (I) and non-infested (NI) barley leaves. The main compounds detected in the epicuticular fraction after 6 days of aphid infestation were: **1, 2** and **3** (Table I). In the fractions of NI plants were: **4, 5** and **6**. As in other plant families no flavonoids were found in these extracts (Stevens *et al.*, 1995). Although most waxes and fatty acids were precipitated with cold MeOH, an important quantity of unsaturated waxes (1-docosene, 1-octadecene and phytol) were detected in extracts from I plants. C_{18} unsaturated fatty acids with epoxydes and hydroxyl groups have been described to have antifungal properties in rice against *Pyricularia orizae* (Harborne, 1986; Grayer and Harborne, 1994). Phytol and isophytol alcohols type are described for the first time in barley. Long-chain alcohols are the main antifungal compounds from the skins of green avocados (Har-

Table I. Epicuticular compounds detected in barley leaves.

Compounds		Reliability of ID [a]	% [b]	Retention time (min)	Deviations from the mean (min) [c]
Infested plants					
	Alkenes				
1-Docosene **(1)**		A, B, C.	1.98	24.0	±0.5
1-Octadecene **(2)**		A, B, C.	2.07	21.1	±0.4
	Aliphatic alcohol				
Phytol **(3)**		A, B, C.	0.81	21.1	±0.2
Non-infested plants					
Isophytol **(4)**		A, B, C.	0.83	20.4	±0.4
	Amine				
N-Cyclohexyl-cyclohexanamine **(5)**		A, B, C.	0.11	22.4	±0.6
	Phenol				
5-Pentadecyl-1,3 benzenediol **(6)**		B, C.	2.10	19.5	±0.1

Six day-old barley plants were infested with 15 nymphs of the aphid *Schizaphis graminum*. Infested and non-infested plants were extracted with CH_2Cl_2 six days later and purified as described in the experimental section.

[a] The reliability of the identification or structural proposal is indicated by the following: A, mass spectrum and retention time consistent which those of an authentic standard; B, structural proposals are given on the basis of mass spectral data (NBS75K Library); C, mass spectrum consistent with spectra found in the literature.

[b] Estimated concentrations for all compounds were made by peak area comparisons to the area of a known amount of internal standard (heneicosane Rt=20.95) with no correction for individual detector response factors.

[c] Rt are average values from three determinations.

borne, 1993). After ripening, these compounds disappear and fungal infection begins. The high C_{12} to C_{22} fatty acid content may be caused by the cleavage of phytosterols, which may be increased during water stress caused by aphids (Harborne, 1993; Dyas and Goad, 1993; Cabrera *et al.*, 1994). Long-chain unsaturated alcohols were not detected in NI plants. According to Grayer (Grayer and Harborne, 1994), there is no sharp chemical division between constitutive and induced antifungal agents; fatty acid derivatives reported in some Gramineae represent a relatively new class of constitutive antifungal compounds in plants. The high content of fatty acid esters in *Hordeum vulgare* could have this type of activity (Cabrera *et al.*, 1994; Cabrera *et al.*, 1995) and could explained your appearance in barley. According Table I, the new compounds of infested barley were not detected in NI plants but, it is not possible to show whether they were not present before because of very minor quantities or they disappeared in NI plants, probably due to infestation, transduction or other influences.

Effect of aphid infestation on the chemical composition of ethyl acetate extracts from barley

In ethyl acetate extracts from I plants, compounds **1, 7, 8, 9** and **10** were detected. In NI plants only **1, 6** and **7** were detected (Table II). Resorcinols as those found in non-infested barley, have been described to have antifungal activity in mango (Harborne, 1989). The disease develops after ripening when the compounds disappear. They could be responsible for the delay in the appearance of the black spot in fruits, which are the symptoms of infection with *Alternaria alternata* (Harborne, 1989). This compound was not detected in infested barley. Since these compounds may be found as glycosides, aphid infestation could induce glycoside hydrolysis. Recently, it has been reported the isolation of 5-(8'*Z*-heptadecenyl) resorcinol and a mixture of its homologues with saturated alkyl chains as antifungal substances from etiolated rice seedlings (Suzuki *et al.*, 1996).This fact could explain the presence of **5** and **7** in infested barley. GC-MS analysis of ethyl acetate fractions from barley showed some similarities and differences among I and NI extracts. The

Table II. Compounds detected in ethyl acetate extracts of barley leaves.

Compounds		Reliability of ID [a]	% [b]	Retention time (min)	Deviations from the mean (min) [c]
Infested plants					
	Alkenes				
1-Docosene **(1)**		A, B, C.	2.02	24.0	±3.2
(5E)-Eicosene **(8)**		B, C.	1.61	17.2	±2.1
(3E)-Eicosene **(9)**		B, C.	1.93	15.2	±1.9
(9E)-Eicosene **(10)**		A, B, C.	1.80	13.1	±1.4
	Amine				
2,5-Diethylpyrazine **(7)**		B, C.	0.61	24.3	±2.9
Non-infested plants					
	Alkene				
1-Docosene **(1)**		A, B, C.	1.02	23.6	±2.0
	Phenol				
5-Pentadecyl-1,3 benzenediol **(6)**		B, C.	1.91	19.0	±1.6
	Amine				
2,5-Diethylpyrazine **(7)**		B, C.	0.43	24.3	±1.8

Six day-old barley plants were infested with 15 nymphs of the aphid *Schizaphis graminum*. Infested and non-infested plants were extracted with CH_2Cl_2 six days later and purified as described in the experimental section.
[a] The reliability of the identification or structural proposal is indicated by the following: A, mass spectrum and retention time consistent which those of an authentic standard; B, structural proposals are given on the basis of mass spectral data (NBS75K Library); C, mass spectrum consistent with spectra found in the literature.
[b] Estimated concentrations for all compounds were made by peak area comparisons to the area of a known amount of internal standard (heneicosane Rt=20.95) with no correction for individual detector response factors.
[c] Rt are average values from three determinations.

two compounds **1** and **6** were detected in non-infested and infested barley. **7** was detected only in non-infested plants. Infested barley had aliphatic (C_{20}) fatty acids with different levels of unsaturation **8, 9** and **10**. Compound **7** has been reported as an alarm pheromone against ants of the *Messor* genus (Harborne, 1993). Pyrazines have been described as pheromones of the fruit fly, but have been also described as alarm molecules in several Lepidoptera and as poisonous component in glands of ants and wasps (Harborne,1989). Pyperidinic alkaloids are common in legumes and are thought to play a role as a primary chemical barrier against herbivores (Harborne, 1993). This is the first report of pyrazine present in Gramineae.

Effect of aphid infestation on the chemical composition of methanol extracts barley

Aphid infestation changed the chemical composition of the polar extract from barley leaves, appearing new compounds and only a few being conserved. The following compounds were detected in infested plants: **6, 7, 11–16** and in NI plants **4, 7, 17–19** (Table III). Some of these compounds appeared also in the ethyl acetate extracts of I and

Table III. Compounds detected in methanol extracts of barley leaves.

Compounds		Reliability of ID [a]	% [b]	Retention time (min)	Deviations from the mean (min) [c]
Infested plants					
	Phenol				
5-Pentadecyl-1,3 benzenediol **(6)**		B, C.	2.15	19.4	±1.4
	Amines				
2,5 Diethylpyrazine **(7)**		B, C.	0.21	24.2	±2.8
2,2,6,6 Tetramethyl-piperidine **(11)**		B, C.	0.12	16.3	±3.8
Indole **(12)**		A, B, C.	1.01	14.5	±2.4
N-Phenyl-2-naphthalenamine **(16)**		B, C.	0.29	17.9	±1.6
	Alkane				
Heptadecane **(13)**		A, B, C.	0.10	20.0	±2.0
	Others				
Methyl-β-D-glucopyranoside **(14)**		B, C.	0.21	11.6	±2.2
2,5-Dihydroxy benzaldehyde **(15)**		A, B, C.	0.27	24.5	±3.2
Non-infested plants					
	Aliphatic alcohol				
Isophytol **(4)**		A, B, C.	1.40	20.5	±2.0
	Amine				
2,5 Diethylpyrazine **(7)**		B, C.	0.18	24.2	±2.0
	Aliphatic esters				
9,12,15 Octadecatrienoic methyl ester **(17)**		B, C.	1.01	19.0	±1.0
9,15-Octadienoic methyl ester **(18)**		B, C.	2.01	4.0	±2.2
Hexadecanoic methyl ester **(19)**		B, C.	1.93	6.8	±2.1

Six day-old barley plants were infested with 15 nymphs of the aphid *Schizaphis graminum*. Infested and non-infested plants were extracted with CH_2Cl_2 six days later and purified as described in the experimental section.

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[b] Estimated concentrations for all compounds were made by peak area comparisons to the area of a known amount of internal standard (heneicosane Rt=20.95) with no correction for individual detector response factors.

[c] Rt are average values from three determinations.

NI plants. **(4)** appeared in methanol extracts and in epicuticular extracts of non-infested plants. Recently, it has been reported that phytic acid and polyphenols are potential insect antifeeding in wheat and maize (Joad *et al.*, 1995). Several unsatured and methyl esters of fatty acid with a high degree of unsaturation were also detected in barley (Table II and III).

Some differences between infested and non-infested plants were noted. Derivatives of eicosene **8, 9** and **10** were detected only in infested plants. While **1** was detected in both plants, (Table II); methyl esters of fatty acids **17–19**, (Table III) were detected only in NI plants. Such compounds, however, are typical of plants tissues where they are the *main* components of membranes (Dyas and Goad, 1993). With some exceptions, steryl esters and lipids of membranes have the same fatty acids moieties.

A newly isolated dihydrochalcone from Myrtaceae, which also contains an aldehyde group and fenolic sustituents, shows untifungal activity, perhaps caused by the O-quinone moiety (Grayer and Harborne, 1994). This may be relevant because one of the isolated compounds from barley corresponds to a dihydroxy-benzaldehyde, which could be transformed into the corresponding quinone. Phenolic compounds are oxidized to toxic derivatives (quinones). Compounds having ortho-hydroxyl groups, including catechol resorcinol derivatives (Hamberg and Hamberg, 1996) and others are detrimental to greenbug growth and progeny survival; however, the role of these compounds in plants is not clear. Most of them are thought to affect plant growth regulation (Corcuera, 1993). These compounds also inhibit enzyme activities and induce the nutritive values of plants to herbivores.

New piperidine derivatives and a non-substituted indole were also isolated from polar fractions of infested barley (Harborne, 1993; Dyas and Goad, 1993). This facts is relevant because one ecologically interesting group of alkaloids that has been investigated recently as simple piperidines which are mainly found in legumes. They have the ability to inhibit animal glycosidases. These alkaloids would seem to have a primary role in protecting plants from herbivores (Harborne, 1986).

The biological significance of the presence of all these compounds in barley is still unknown. Nonetheless, the structural similarities of several of them to others with known antifungal activity suggests a possible role in sanitary protection of barley plants. However, their biological activity is yet to be measured.

Materials and Methods

General

Chromatographic method. TLC (silica gel GF 254 (a) $CHCl_3$-MeOH -HOAc (140: 20: 1); (b) *n*-hexane-EtOAc-HOAc (60: 40: 1); (c) Me_2 CO-NH_4OH (20:1); detection by anisaldehyde reagents and heating for 5–10 min at 120 °C.

Plant and infestation treatment

Barley plants (*Hordeum vulgare* L.) cv. Aramir were grown in vermiculite at 25 °C with 14/10 h light/dark periods and were irrigated twice per week with a Hoagland solution. Six day after sowing, plants were infested with 15 adults individual of the aphids *S. graminum* Rondani biotype C per plant. After 6 days plants from both groups were extracted as follows.

Isolation procedures for epicuticular compounds

Leaf waxes from c.v. Aramir were obtained by immersion in CH_2Cl_2 for 20 s. The extracts were filtered to remove solid, dried with anh. Na_2SO_4 evapd *in vacuo* and dissolved in 25 ml boiling MeOH. The solutions were cooled to 20 °C to precipitate the major part of the alkanes and other fatty constituents. After centrifuging the mixtures, the supernatants were concentrated and placed directly to Sephadex LH-20 (column 35 x 2 cm) with petrol-$CHCl_3$-MeOH (1:1:0.3/v/v/v) as mobile phase. Fractions of ca.10 ml were collected and monitored by TLC procedures a and b.

Acetylation of samples

Each fraction obtained from the column was acetylated with acetic anhydride (5 ml) and pyridine (2 ml) at 60 °C for 48 h. Excess acetylation reagents were eliminated by washing 3 times with water, adjusted to acidic pH and then extracted with $CHCl_3$. After drying, the residues were dissolved in acetone and filtered. Heneicosane (Rt= 20.95) was added as internal standard (5.5 µl) to the plant extracts immediately after homogeniza-

tion. Peak areas were integrated. Quantification were performed at least x 3 times to ensure reproducibility.

Methanol and ethyl acetate extracts

Fresh leaves from I and NI plants were cut in small pieces. The material was extracted 2 times with EtOAc and MeOH respectively for 48 h, at room temperature. After filtering and drying with anh. Na_2SO_4, the extracts were subjected to repeated cc on silica gel (5–40 mm) using mixtures of hexane-EtOAc-MeOH and hexane-EtOAc respectively. The mixtures of purified products were then acetylated as described before.

These extracts were separated by TLC. The EtOAc extract, was dried with Na_2SO_4 anh. concentrated and purified in silica gel columns. Fractions obtained were acetylated as previously described.

GC-MS

The compounds we identified by their GC-MS fragmentation patterns. A Hewlett Packard GC (HP-5972 serie II coupled to a mass selective detector 8Ms 5972) was used for the separation and the detection. GC-MS was operated in the electron impact mode at 70 eV. Helium was used as carrier gas at a flow rate of 1 ml/min. Injection was performed in the splitless mode (valve time: 1 min). The injected volume was 1 µl. Scan mode from 50 to 700 Da was used to identify the compounds. 30 m x 0.5 mm id. phased-silica capillary column coated with the phenylmethyl silicone phase HP 5-MS (film thickness 0.25 µm). The temperature program was as follows: 200 °C for 3 min; then there was an increment of 6°/min until it reached 275 °C, at which it was kept for 15 min.

Identification of compounds

Retention times and mass spectra of unknown compounds were compared with those of authentic material or from literature data. The MS of the samples, along with literature MS of the compounds were entered into a library data base for computer identification of GC-MS data with a VG Analytical data system 2000 running on a Digital PDP 8 computer. The closer the values are to 1000 the better the unknown matches the library spectrum.

Mass spectra of compounds isolated

(1) 1-docosene EIMS, *m/z* (rel. Int.): 308 $[M]^+$ (ascribed to $C_{22}H_{44}$) (10), 140 (5), 111 (25), 97 (58), 83 (48), 55 (80).

(2) 1-octadecene EIMS, *m/z* (rel. Int.): 252 $[M]^+$ (ascribed to $C_{18}H_{36}$) (5), 140 (10), 111 (22), 83 (70), 69 (60), 55 (85).

(3) Phytol (acetate). EIMS, *m/z* (rel. Int.): 338 $[M]^+$ (ascribed to $C_{22}H_{42}O_2$) (1), 285 (10), 279 (2), 113 (10), 99 (15), 85 (18).

(4) Isophytol (acetate). EIMS, *m/z* (rel. Int.): 338 $[M]^+$ (ascribed to $C_{22}H_{42}O_2$) (2), 285 (5), 279 (1), 113 (1), 99 (5), 85 (10).

(5) N-cyclohexyl-cyclohexanamine181. $[M]^+$ (ascribed to $C_{12}H_{23}N$) (10), 152 (2), 138 (100), 56 (35).

(6) 1,3-benzenediol, 5-pentadecyl (acetate). EIMS, *m/z* (rel. Int.): 404 $[M]^+$ (ascribed to $C_{25}H_{40}O_4$) (2), 308 (8), 286 (3), 141 (5), 127 (4), 105 (50).

(7) Pyrazine 2,5-diethyl. EIMS, *m/z* (rel. Int.): 136 $[M]^+$ (ascribed to $C_8H_{12}N_2$) (90), 135 (90), 121 (100), 108 (20), 80 (10), 67 (12), 56 (20).

(8) (5E)-eicosene. EIMS, *m/z* (rel. Int.): 280 $[M]^+$ (ascribed to $C_{20}H_{40}$) (2), 280 (2), 139 (4), 125 (10), 111 (20), 83 (60), 69 (64), 55 (100).

(9) (3E)-eicosene. EIMS, *m/z* (rel. Int.): 280 $[M]^+$ (ascribed to $C_{20}H_{40}$) (4), 125 (10), 111 (30), 97 (60), 83 (70), 69 (85), 57 (100).

(10) (9E)-eicosene. EIMS, *m/z* (rel. Int.): 280 $[M]^+$ (ascribed to $C_{20}H_{40}$) (2), 139 (2), 125 (8), 111 (26), 97 (60), 83 (80), 69 (80), 57 (100).

(11) 2,2,6,6-tetramethyl-piperidine. EIMS, *m/z* (rel. Int.): 141 (ascribed to $C_9H_{19}N$) (21), 126 (50), 109 (10), 98 (10), 85 (5), 82 (7), 70 (70), 69 (45), 58 (100), 53 (15).

(12) Indol. EIMS, *m/z* (rel. Int.): 117 (ascribed to C_8H_7N) (100), 116 (10), 90 (43), 89 (30), 63 (20).

(13) Heptadecane. EIMS, *m/z* (rel. Int.): 240 (ascribed to $C_{17}H_{36}$ (2), 141 (4), 127 (6), 111 (8), 85 (60), 71 (70).

(14) Methyl-D-glucopyranoside (acetate). EIMS, *m/z* (rel. Int.): 362 (ascribed to $C_{15}H_{22}O_{10}$) (5), 331 $[M\text{-}Me]^+$ (3), 319 (10), 302 (2), 242 (10), 200 (1), 140 (15), 98 (50).

(15) 2,5-Dihydroxybenzaldehyde (acetate) EIMS, *m/z* (rel. Int.): 222 $[M]^+$ (ascribed to $C_{11}H_{10}O_5$) (7), 179 (10), 163 (1), 136 (10), 104 (10).

(16) N-phenyl-2-naphthalenamine. EIMS, *m/z* (rel. Int.): 219 (ascribed to $C_{16}H_{30}N$) (100), 217 (15), 216 (14), 115 (20), 102 (10), 77 (20), 51 (25).

(17) (9Z,12Z,15Z)-Octadecatrienoic acid, methyl ester. EIMS, *m/z* (rel. Int.): 292 $[M]^+$ (ascribed to $C_{19}H_{32}O_2$) (1), 235 (3), 149 (5), 135 (6), 121 (8), 108 (20).

(18) (9Z,15Z)-Octadecadienoic acid methyl ester. EIMS, *m/z* (rel. Int.). 294 $[M]^+$ (ascribed to $C_{19}H_{34}O_2$) (3), 263 (2), 235 (1), 149 (1), 135 (6), 121 (8), 108 (20).

(19) Hexadecanoic acid, methyl ester. EIMS, *m/z* (rel. Int) 270$[M]^+$ (ascribed to $C_{17}H_{34}O_2$) (20), 239 (4), 227 (3), 143 (20), 129 (5), 87 (80), 74 (100).

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