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Chemoreception of phenolic acids and flavonoids in larvae of two species of *Pieris*

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Summary. 1. Chemosensory responses in lateral and medial maxillary sensilla styloconica to stimulation with phenolic acids and flavonoids were studied using electrophysiological methods in caterpillars of *Pieris brassicae* L. and *Pieris rapae* L. (Lepidoptera: Pieridae).

2. Of the 5 phenolic acids tested, those possessing ortho-substituted phenolic groups (chlorogenic and protocatechuic acids) were the most effective stimulants.

3. Of the 7 flavonoids examined, catechin was the most effective stimulant in the lateral sensillum of both species, while 3 others did not evoke a response at any of the concentrations tested.

4. Responses generally increased with increasing stimulus concentrations in the range tested (0.2–5.0 m*M*). *P. rapae* generally exhibited higher sensitivity thresholds.

5. Mixture experiments suggested that in the lateral sensillum of *P. brassicae* one cell and in the medial sensillum two cells were especially sensitive.

6. The anthocyanin cyanin chloride caused inhibition of spiking activity in several neurones.

7. Caterpillars reared on an artificial diet showed reduced sensitivity compared to caterpillars reared on a host plant.

8. Chemosensory activity was reflected in preference behaviour in dual choice situations.

9. Dose-response relations combined with phytochemical data permit the conclusion that naturally occurring levels of phenolic acids and flavonoids are stimulatory to some chemosensory neurones and can cause inhibition of activity in others.

Key words: Chemoreceptors – Phenolic acids – Flavonoids – *Pieris* – Behaviour

Introduction

The caterpillars of *Pieris brassicae* and *P. rapae* (Lepidoptera: Pieridae) are oligophagous on Cruciferae. Their host selection behaviour can to a large extent be explained by their gustatory recognition of glucosinolates from cruciferous plants (David and Gardiner 1966; Schoonhoven 1967). In addition to glucosinolates, host plants in the cruciferous genus *Brassica* contain varying amounts of phenolic and flavonoid compounds (Hegnauer 1973; Durkee and Harborne 1973). These are ubiquitous secondary metabolites in plants (Harborne 1979); they have the first part of their biosynthesis in common. There is ample evidence for their ecological function in the biochemical defense of plants against fungal pathogens and herbivores (Levin 1971; McClure 1975). The effects on phytophagous insects differ to a large extent, depending on the species (Schoonhoven 1972b; Harborne 1979). Thus, flavonoids may either stimulate or inhibit feeding behaviour in oligophagous as well as polyphagous insects (Matsuda 1978; Nielsen 1978; Dreyer and Jones 1981; Larsen et al. 1982; Woodhead and Cooper-Driver 1979). Nevertheless, in contrast to other classes of secondary compounds, the sensory physiology of phenolic compounds remains poorly documented (Schoonhoven 1982); the few electrophysiological studies to date report qualitatively on a small number of flavonoids tested at a single arbitrary concentration (Ishikawa 1966; Wieczorek 1976; Dethier 1973, 1980; van Drongelen 1979).

In the present study, electrophysiological methods are used to study the contact chemosensory effects of several phenolics and flavonoids. Stimulus compounds and concentrations tested were selected on the basis of reported occurrence in a common host plant, *Brassica oleracea* L. In *P. brassicae*, the degree of specialization of receptor cells responding to these compounds is studied. Finally, it is determined whether electrophysiological responses are reflected in feeding behaviour, in dual choice tests.

Material and methods

Insects. Pieris brassicae and *P. rapae* cultures were maintained in the laboratory under conditions described by David and Gardiner

(1962). Both cultures were started with field-collected material. Caterpillars of both species studied in electrophysiological experiments were usually reared on *Brassica oleracea* L. var. *gemmifera* DC. cv. Titurel (Sluis and Groot, Enkhuizen, The Netherlands) under ad libitum conditions. Rearing temperature was 25 °C, relative humidity varied between 50 and 70% and photo/scotophase was 15/9. For behavioural experiments, *P. brassicae* larvae were reared ab ovo on a semi-defined artificial diet (Ma 1972). All caterpillars used for electrophysiological experiments had completed their development ab ovo up to the final larval moult in 15 days (*P. brassicae*) or 13 days (*P. rapae*). Test animals were used 24–48 h after the final moult. Fresh weights ranged from 250–400 mg for *P. brassicae* and from 80–120 mg for *P. rapae*. Prior to the experiments, resting larvae were selected and deprived of food for 2–3 h. The head and the first thoracic segment were amputated for recording. A U-shaped silver wire recording electrode was inserted through the foramen magnum of the isolated head and pushed into the maxillary-labial complex to fix the maxillae in a prognathous position.

Location, morphology, ultrastructure and the number of neurones innervating the lateral (L) and medial (M) styloconic sensilla of the maxillary galea of *P. brassicae* were described by Ma (1972). Apart from the smaller dimensions, external sensillar morphology of *P. rapae* is comparable to that of *P. brassicae*. In accordance with the histological data of Ma (1972), in *P. brassicae* 4 gustatory neurones have been identified electrophysiologically in both L- and M-sensilla (Schoonhoven 1972a). Electrophysiological data presented in this study and elsewhere (van Loon 1988) suggest that 4 gustatory cells are present in both sensilla of *P. rapae*.

Stimuli and stimulation procedures. The following chemicals were studied: 1. sucrose, 2. proline, 3. sinalbin, 4. helveticoside. Organic acids (non-phenolic): 5. ascorbic acid, 6. cinnamic acid, 7. citric acid. Phenolic acids: 8. protocatechuic acid, 9. caffeic acid, 10. ferulic acid, 11. sinapic acid, 12. chlorogenic acid. Flavonoids: the anthocyanins 13. cyanin, 14. malvin, 15. oenin (all 3 tested as chlorides); the flavonols 16. quercetin, 17. rutin (quercetin-3-rutinoside), 18. kaempferol, and the proanthocyanidin 19. DL-catechin. All were of 99% purity and compounds 1, 2, 5, were obtained from Merck; 4, 6, 8–12 and 16–18 from Sigma; 7, 15 and 19 from Baker; 13 and 14 from Serva and 3 from Roth. Structures of phenolic and flavonoid compounds are represented in Fig. 1.

All stimuli were dissolved into 2 m*M* (*P. rapae*) or 10 m*M* (*P. brassicae*) potassium chloride in distilled water. Phenolics and flavonoids were presolubilized in 100 µl of pure methanol and subsequently diluted. The maximal concentration of methanol in test solutions was 1%. Solutions of methanol and potassium chloride served as control stimuli. In dose-response experiments, concentrations were chosen to include those reported from the tissues of host plants (see Discussion). The pH of acidic solutions was determined using an Orion 701A electronic pH-meter. To reduce the effect of evaporation of solvent at the stimulus capillary tip, fluid from the body of the capillary was sucked through the tip using a piece of filter paper less than 10 s before each stimulation. To the same end, local humidity around the preparation was kept high (ca. 80%), by encasing it in an aluminum box which was guarded to the amplifier input and lined with moist filterpaper. Stimuli were applied in a glass capillary (tip diameter 30–50 µm) which was used as stimulating electrode. Stimulus delivery sequences were random. In an experimental series carried out on a single preparation, the control solutions and 4 effective stimuli that evoked only single cell responses, were replicated 2–3 times to check the reproducibility of cell reactions. The latter stimuli were: sucrose (30 m*M*), the glucosinolate sinalbin (0.2 m*M*) the steroid glucoside helveticoside (0.01 m*M*) and the amino acid methionine (10 m*M*) (van Loon 1988). Preparations were used no longer than 90 min although reproducible responses could be ob-

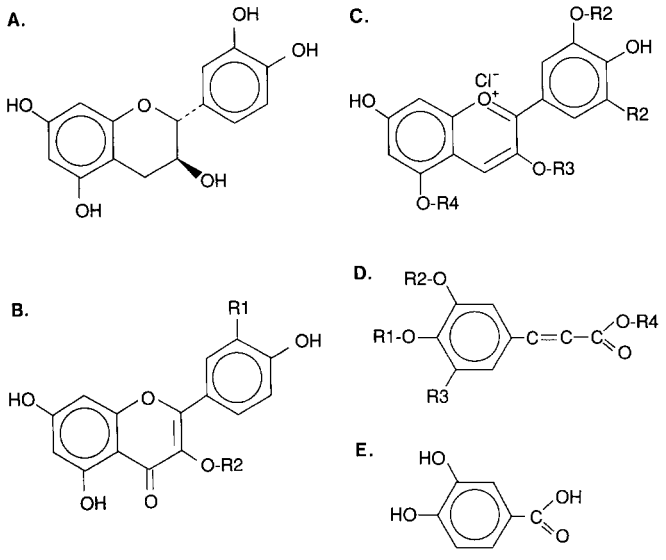

Fig. 1A–E. Structural formulae of the 7 flavonoid (**A–C**) and 5 phenolic (**D, E**) substances offered as stimuli to the lateral and medial maxillary sensilla styloconica

Structure/substitutents		Compound	Structure/substitutents				Compound
A		DL-catechin	C				
			R1	R2	R3	R4	
B			H	H	Glu[b]	Glu	cyanin
			CH_3	OCH_3	Glu	Glu	malvin
R1	R2		CH_3	OCH_3	Glu	H	oenin
H	H	kaempferol					
OH	H	quercetin	D				
OH	Rut[a]	rutin	R1	R2	R3	R4	
			H	H	H	H	caffeic acid
			H	CH_3	H	H	ferulic acid
			CH_3	CH_3	OCH_3	H	sinapic acid
			H	H	H	Qui[c]	chlorogenic acid
			E				protocatechuic acid

[a] Rut = rutinoside = 6-O-α-L-rhamnosyl-D-glucoside
[b] Glu = β-glucoside
[c] Qui = quinic acid ester

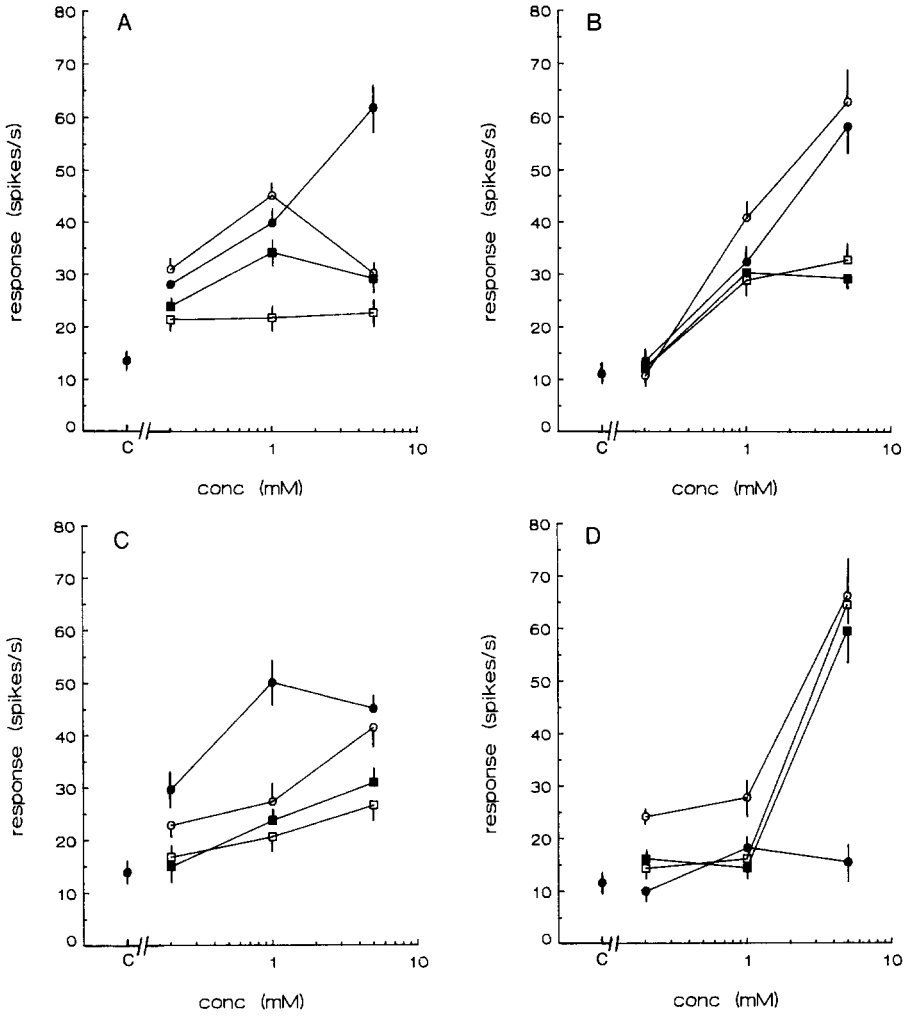

Fig. 2A–D. Concentration-response curves for phenolic acids (**A, B**) and flavonoids (**C, D**) in *P. brassicae*. Graph **A, C:** L-sensillum; graph **B, D:** M-sensillum. Abscissa: concentration (m*M*), logarithmic scale. Ordinate: response frequency (spikes/s). Vertical bars represent SEM (two-sided or one-sided and in some cases omitted to avoid overlap). Graph **A, B:** chlorogenic acid (●), protocatechuic acid (○), ferulic acid (■) and sinapic acid (□). Graph **C, D:** catechin (●), cyanin chloride (○), oenin (■), malvin (□). A datapoint is based on measurements obtained from 7–14 sensilla

tained over several hours. Each individual possesses symmetrical pairs of sensilla and the values obtained for individual sensilla were used for calculations of means and variances. Stimulus duration was 2–4 s; interstimulus periods were at least 30 s. Preliminary experiments had shown that complete disadaptation to stimuli, applied during 2–4 s, will occur within 10 s in the sensilla studied.

Recording of electrophysiological responses. A modification of the tip recording technique of Hodgson et al. (1955) was used in which the stimulating electrode served as indifferent electrode and the recording electrode, in contact with the insect tissue, was connected to a preamplifier by a short (10 mm) silver connector. The amplifier, adapted from van Drongelen (1979), used an AD 515-K (Analog Devices) integrated circuit in the first stage, yielding <1 pA input bias current, 10^{15} Ohm and 0.8 pF input impedance. The amplifier included a band-pass filter, set at 100 Hz–3 kHz (-3 dB). The amplifier case was connected to a guard voltage. Spike amplitudes recorded ranged from 0.5–3 mV; spike-to-noise ratios varied from 2 to 10. The blocking artefact at stimulus onset lasted 5–40 ms. Amplified responses were recorded on tape (Akai GX-255) at 19 cm/s, for subsequent reproduction on paper, using a Siemens Oscillomink-E inkjet recorder (0–1000 Hz) at 25 or 50 cm/s. For detailed analysis of spike shape, recordings were re-recorded at 152.4 cm/s (Racal Store-7 instrumentation recorder) and reproduced on paper at 9.53 cm/s. Polarity of spikes was inverted in reproducing recordings on paper; thus, in Figs. 4–6 the first phases of spikes are upstrokes. Environmental temperature during recording ranged from 20–24 °C.

Analysis of the recordings. Chemoreceptor sensitivity is quantified as the total number of action potentials generated from 50 ms until 1050 ms after stimulus onset (spikes/s). Action potentials were counted after visual identification on the basis of typical biphasic wave-form (Figs. 4–6). Spike amplitude, duration of interspike interval and adaptation rate served as criteria to assign spikes to the different neurones active in multi-neural responses. Mixtures of two compounds that elicit single unit activity when applied separately assisted the analysis of cellular origin of spikes (Bowdan 1984). Differences in effectiveness between compounds at a single intermediate concentration were compared using ANOVA, treating individuals as blocks. The least significant difference (LSD) was calculated as a means for multiple comparisons (Sokal and Rohlf 1981).

Behavioural tests. Preference behaviour was assessed by gravimetric measurement of ingestion in a dual choice situation. Phenolic compounds were incorporated into the complete rearing diet (Ma 1972) at 2.5 or 5 m*M*. They were dissolved in ethanol 96% and admixed just prior to solidification at a temperature of 45 °C. Dry weight of diet ingested was found by calculating the initial amount of dry weight present at the start of the experiment and subtracting the amount of dry weight remaining at the end of the 24 h test period. The initial dry weight of the diet was calculated by multiplying the fresh weight of diet offered to the caterpillars with the percentage of dry weight of aliquots. Dry weights were taken after drying at 70 °C for 48 h.

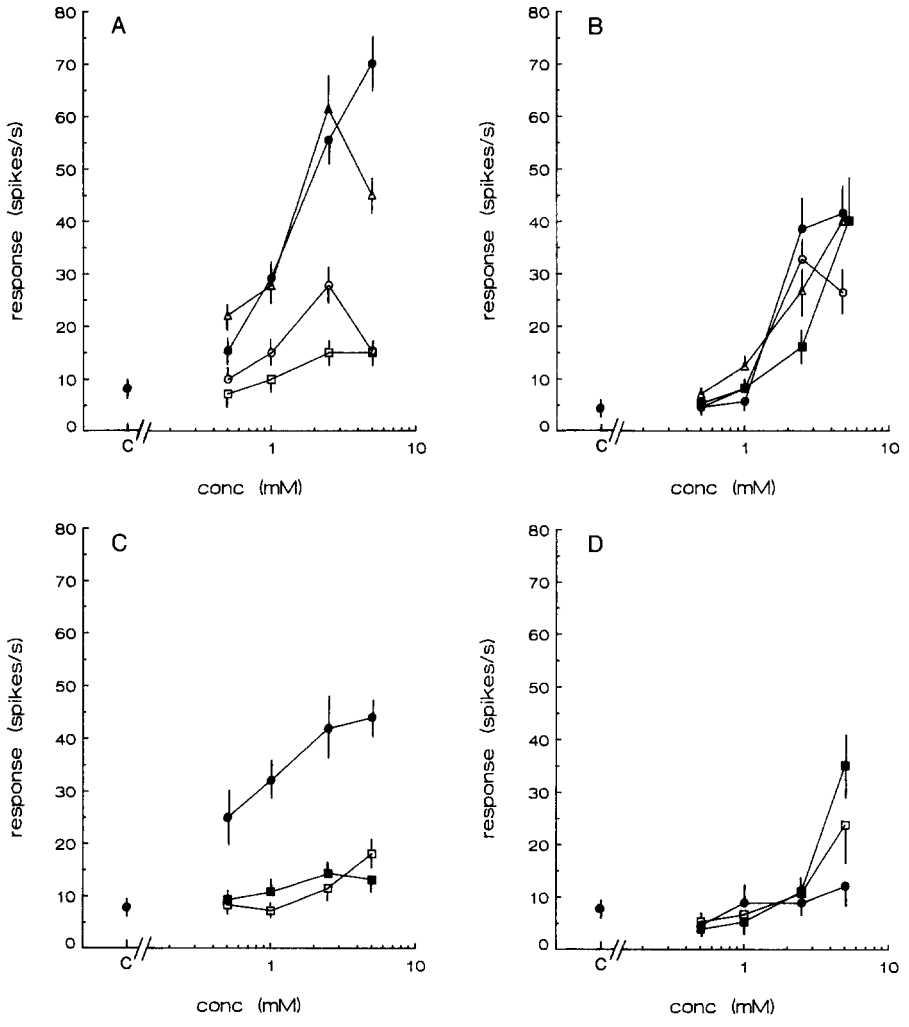

Fig. 3A–D. Concentration-response curves for phenolic acids (**A, B**) and flavonoids (**C, D**) in *P. rapae*. Graph **A, C:** L-sensillum, graph **B, D:** M-sensillum. Graph **A, B:** chlorogenic acid (●), caffeic acid (△), protocatechuic acid (○), ferulic acid (■) and sinapic acid (□). Graph **C, D:** catechin (●), cyanin chloride (○), oenin (■), malvin (□). A datapoint is based on measurements obtained from 9–16 sensilla

Results

Dose-response curves

Phenolic acids. Dose-response curves of L- and M-sensilla of *P. brassicae* and *P. rapae* are shown in Figs. 2 and 3, respectively. In the L-sensillum of both species, chlorogenic acid and protocatechuic acid were the most effective stimulants (Figs. 2A, 3A). Thresholds were either lower than or around 0.5 m*M*. The responses to protocatechuic acid in both species and to caffeic acid in *P. rapae* were significantly lower at 5.0 m*M* than at the next lower concentration tested. Following application of the 5.0 m*M* doses of the latter two compounds normal responses were obtained, indicating that this decrease was not due to adaptation or impaired sensillum function. Generally, higher doses elicited stronger responses. This was not so for sinapic and ferulic acids in *P. brassicae*.

In the M-sensillum of both species, chlorogenic and protocatechuic acid also were the best stimulants (Figs. 2B, 3B). While in both species at 0.2 m*M* none of the compounds evoked responses, at 1.0 m*M* *P. brassicae* did respond to 4 but *P. rapae* to only one. In contrast to the L-sensillum, responses to protocatechuic acid in *P. brassicae* and caffeic acid in *P. rapae* did not decline at 5.0 m*M*. Response frequencies in both sensilla to 3 non-phenolic organic acids and 3 phenolic acids at 5.0 m*M* showed no consistent relationship with the pH of the stimulant solution, that ranged from 2.85–3.89 (details in van Loon 1988).

Flavonoids. In the L-sensillum of both species, catechin was the most effective stimulant with a threshold lower than 0.2 m*M* (Figs. 2C, 3C). For *P. brassicae*, cyanin chloride was the next best stimulant. Sensitivity to this compound was absent in *P. rapae*. Oenin and malvin showed dose-dependent effects in *P. brassicae* that were less distinct in *P. rapae*. Kaempferol, quercetin and rutin did not evoke a response at any of the concentrations tested in either the L- or the M-sensillum.

In the M-sensillum of both species, catechin was weakly effective, causing a small increase in response only at higher concentrations. Cyanin evoked a response at concentrations beyond 0.2 m*M* in *P. brassicae*, while in both species oenin and malvin evoked distinct reactions only at 5.0 m*M* (Figs. 2D, 3D).

Generally *P. rapae* was less sensitive to phenolic acid

Table 1. Effectiveness of phenolic acids and flavonoids tested at 1 m*M* on the L- and M-sensilla styloconica of final stadium caterpillars of *P. brassicae*

Compound	Response value (spikes/s) (mean ± SE)	*n*
L-sensillum		
Catechin	54.0 ± 3.9	12
Protocatechuic acid	46.2 ± 3.8	13
Chlorogenic acid	34.6 ± 3.7	12
Ferulic acid	34.6 ± 3.1	11
Cyanin chloride	26.2 ± 4.4	13
Oenin	23.3 ± 3.0	11
Sinapic acid	21.7 ± 2.6	10
Malvin	20.9 ± 1.9	12
Control	11.4 ± 0.8	13
LSD	8.7[a]	
M-sensillum		
Protocatechuic acid	39.8 ± 3.3	12
Chlorogenic acid	33.2 ± 3.7	13
Sinapic acid	30.6 ± 3.9	10
Ferulic acid	28.3 ± 3.5	11
Cyanin chloride	25.4 ± 4.9	11
Malvin	15.5 ± 1.4	10
Control	10.3 ± 1.2	13
LSD	9.7[b]	

n is number of sensilla tested; LSD = least significant difference
[a] $P<0.05$; ANOVA: residual mean square (r.m.s.) 123.6 (df = 89); $F=17.93$, $P<0.001$; standard error of difference (SED) 4.36
[b] $P<0.05$; ANOVA: r.m.s. 152.8 (df = 66); $F=5.92$, $P<0.001$; SED 4.85

Table 2. Effectiveness of phenolic acids and flavonoids tested at 2.5 m*M* on the L- and M-sensilla styloconica of final stadium caterpillars of *P. rapae*

Compound	Response value (spikes/s) (mean ± SE)	*n*
L-sensillum		
Caffeic acid	61.8 ± 7.2	12
Chlorogenic acid	52.9 ± 4.1	12
Catechin	42.0 ± 6.7	10
Protocatechuic acid	27.3 ± 3.0	8
Control	7.4 ± 2.4	15
LSD	16.6[a]	
M-sensillum		
Chlorogenic acid	45.9 ± 6.7	10
Protocatechuic acid	33.3 ± 4.3	10
Caffeic acid	28.0 ± 5.5	9
Ferulic acid	16.5 ± 4.2	10
Sinapic acid	14.5 ± 2.2	10
Control	4.8 ± 0.6	10
LSD	14.2[b]	

n is number of sensilla tested; LSD = least significant difference
[a] $P<0.05$; ANOVA: r.m.s. 406.8 (df = 41); $F=7.22$, $P<0.001$; SED 8.23.
[b] $P<0.05$; ANOVA: r.m.s. 246.0 (df = 48); $F=6.73$, $P<0.001$; SED 7.01

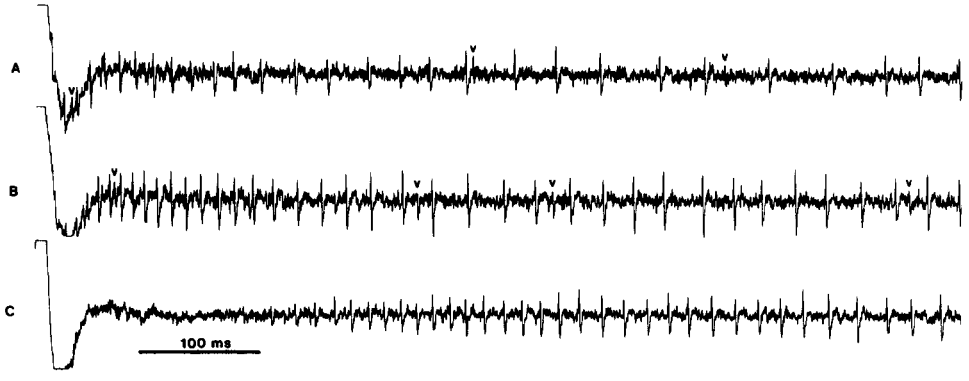

Fig. 4A–C. Electrophysiological responses of lateral sensilla of *P. brassicae* to **A** 1 m*M* chlorogenic acid, **B** 2.5 m*M* chlorogenic acid and **C** 2.5 m*M* cyanin chloride. **A** and **B** from the same sensillum, **C** from a different sensillum showing a considerable latency time prior to a tonic response. Distortion at beginning of traces due to stimulus onset artefact. Arrows (v) indicate occasional small action potentials from another cell. The solvent alone (10 m*M* KCl) evoked only few small spikes (not shown)

or flavonoid stimulation, as judged by the relative change in response from 1.0 m*M* to 5.0 m*M*.

Comparisons between phenolic acids and flavonoids

Response values to both groups of compounds tested at 1.0 m*M* (*P. brassicae*) or 2.5 m*M* (*P. rapae*) are presented in Tables 1 and 2 to allow comparisons of effectiveness. Of the 5 phenolic acids and 7 flavonoids listed above, only those compounds which caused a significant increase in spiking activity relative to the control are included. In general, both sensilla of both species responded more strongly to phenolic acids than to flavonoids. Phenolic acids with hydroxyl-groups at the 3- and 4-positions of the aromatic ring (i.e. protocatechuic, caffeic and chlorogenic acids) and catechin were particularly effective stimuli.

Cellular origin of spikes

In both species, the responses to phenolic acids and flavonoids in the L-sensillum originated predominantly from a single cell as evidenced by the single spike-type with a large amplitude (Fig. 4). This spiking activity was tonic in nature as compared to the phasic-tonic responses to sucrose, proline and sinalbin (cf. response

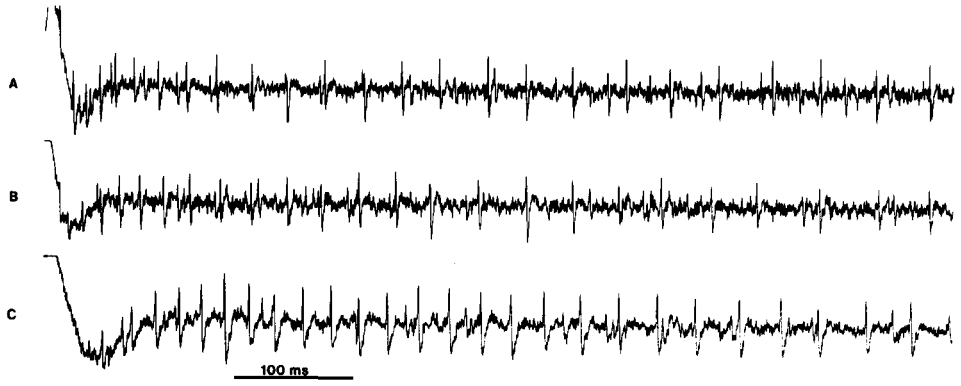

Fig. 5A–C. Responses of medial sensilla of *P. brassicae* to **A** 2.5 m*M* chlorogenic acid, **B** 2.5 m*M* cyanin chloride and **C** 5 m*M* cyanin chloride. Three different sensilla, displaying tonic spiking activity of at least two different chemosensory neurones in varying ratios

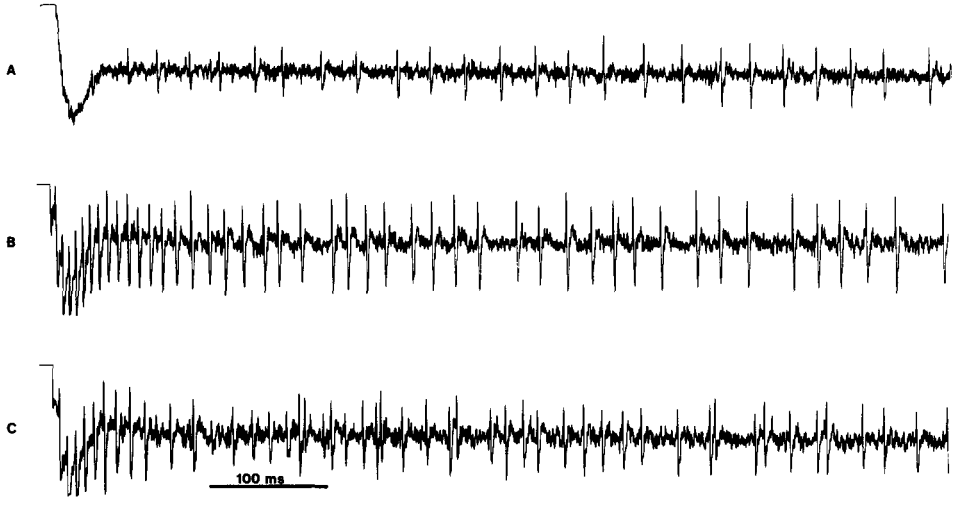

Fig. 6A–C. Responses of a single lateral sensillum of *P. brassicae* to **A** 2.5 m*M* cyanin chloride, **B** 0.2 m*M* sinalbin and **C** a mixture of 2.5 m*M* cyanin chloride and 0.2 m*M* sinalbin. Recording **A** illustrates a tonic reaction-type in contrast with the phasic-tonic reaction to sinalbin, **B**. Recording **C** demonstrates the simultaneous activity of the neurones from **A** and **B** as judged from both the different amplitudes and irregular interspike-intervals. In **C** the amplitude measured from the neurone represented in **B** is somewhat decreased (see discussion). Also, in **C** the activity of the neurone producing the larger spike is partially inhibited (cf. **B**)

Table 3. Responses to mixtures of cyanin and 4 other compounds known to evoke single cell responses

Stimulus	Conc[a] m*M*	Response[b]	Response to cyanin 2.5 m*M*	Observed response to mixture[c]	Spike height classes[d]
L-sensillum					
Sucrose	15	150±11	28± 9	133±18	1–2
Proline	5	70± 4	26±10	50±18	2
Sinalbin	0.2	59± 4	27± 9	61±12	2
Helveticoside	0.01	61± 9	26± 9	53± 9	1
M-sensillum					
Sucrose	15	60±15	43±10	27±20	2
Sinalbin	0.2	71±20	40±16	89±28	2–3
Helveticoside	0.01	27± 7	57± 3	47± 8	1–2

[a] in solutions of both single compound and mixtures; [b] response values in spikes/s±SD; [c] all response values in this column are significantly higher than expected on the basis of simple additivity of the values in the two previous columns according to Wilcoxon's matched pair signed rank test ($P<0.05$, $n=5$–7) using values obtained from individual sensilla; [d] in responses to mixtures

to sinalbin in Fig. 6B). Responses to anthocyanins occasionally showed an extended initial latency period (Fig. 4).

In the M-sensillum, single cell responses were observed at concentrations below 2.5 m*M*. In contrast, in some recordings taken at 2.5 m*M* and most recordings at 5.0 m*M*, two or three neurones were activated (Fig. 5).

Responses to mixtures

In the L-sensillum, mixing cyanin-chloride at 2.5 m*M* with proline or sinalbin induced the firing of two neurones with different amplitudes (Fig. 6). Results with sucrose-cyanin mixtures were less conclusive, partly due to the high response frequency to 15 m*M* sucrose alone. Very large spikes occurred, typical for electrical addition

Table 4. Effect of diet experienced during development up to the 5th instar on response frequencies in chemosensory sensilla of *P. brassicae*

Compound		L-sensillum Diet			M-sensillum Diet		
		B. oleracea	Artificial	P[a]	*B. oleracea*	Artificial	P
Sucrose	30 m*M*	138±25[b]	142±23	NS	68±13	79±18	NS
Proline	5 m*M*	86±24	72±10	0.05	14±12	13± 6	NS
Chlorogenic acid	1 m*M*	40±12	37±11	NS	32±14	14± 7	0.005
	5 m*M*	62±18	42±11	0.025	58±16	37± 9	0.01
Cyanin chloride	1 m*M*	25±14	11± 6	0.025	28±14	19±14	NS
	5 m*M*	42±11	19±10	0.001	66±30	65±12	NS

[a] level of significance according to Student's t-test (n=6–10)
NS = not significant at P=0.05
[b] response frequencies in spikes/s±SD

of spikes from different cells. Both the helveticoside-cyanin mixtures and helveticoside alone produced only one large spike type.

Results for the M-sensillum were generally more difficult to interpret because responses to cyanin alone frequently were multineural at 2.5 m*M*. If it is assumed that the smaller spikes in responses to cyanin originated in the cation-sensitive cell, then responses for the M-sensillum are comparable to those for the L-sensillum. In all cases, response frequencies to mixtures were significantly lower than expected on the basis of simple additivity (Table 3). Whith to proline and helveticoside in the L-sensillum and sucrose in both sensilla, response frequencies of the corresponding single cells were significantly inhibited during mixture stimulation.

Effect of rearing diet on chemosensory responses

Compared to caterpillars reared on *B. oleracea*, caterpillars reared on the artificial diet showed a significantly lower chemosensory sensitivity to chlorogenic acid in both sensilla and to proline and cyanin chloride in the L-sensillum (Table 4).

Preference behaviour

In dual choice experiments lasting the initial 24 h of the 5th instar, control diet-reared caterpillars showed significant preferences for the control diet over oenin and chlorogenic acid containing diets (Fig. 7A). A higher concentration of chlorogenic acid caused a stronger preference for the control diet (Fig. 7B). In contrast, larvae significantly preferred diets containing cyanin chloride or sinapic acid, over the control diet. Larvae did not distinguish between control diet and diet containing rutin at 5 m*M*.

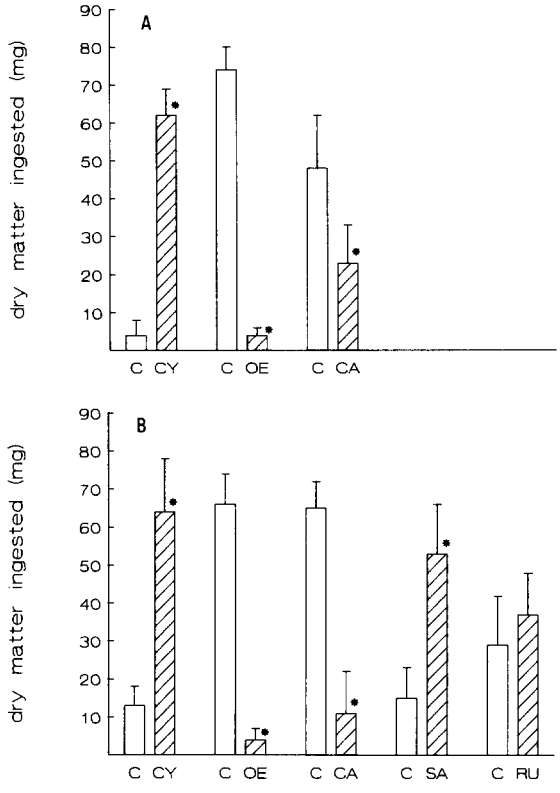

Fig. 7A, B. Ingestion of final instar larvae of *P. brassicae* in a dual choice situation; *C* mean amount ingested from control diet (open bars); mean amount ingested from treated diets is indicated by hatched bars (error bars represent one SD). Treated diets contained the following compounds: *CY* cyanin chloride; *OE* oenin; *CA* chlorogenic acid; *SA* sinapic acid; *RU* rutin. Compounds represented in **A** were incorporated at 2.5 m*M* and in **B** at 5 m*M*. * indicates mean value significantly different from control at $P<$ 0.005 (Student's t-test for paired observations, n=8)

Discussion

Dose-response relations and phytochemistry

The dose-response curves reveal that in the concentration range investigated, most compounds evoked higher responses at higher concentrations. Exceptions to this generalization are protocatechuic acid and caffeic acid, responses to which were distinctly lower at 5.0 m*M* in some cases. The difference between optimum curves for the latter two compounds and the monotonic dose-response curves for other compounds may indicate that either different cell types are responding or that separate

Table 5. Phytochemical data on concentration (m*M*) of phenolic acid aglycones in leaves of *B. oleracea*

Phenolic acid	Concentration in leaf	
	Minimum	Maximum
Ferulic acid	0.15	1.4
Caffeic acid	0.23	1.7
Sinapic acid	0.4	3.2
Protocatechuic acid[a]	–[b]	0.6

Cultivar group	Total maximum leaf concentration of all 4 phenolic acids
alba	–[b]
sabauda	1.3
gemmifera	1.8
rubra	3.5
sabellica	6.1

[a] found in considerable amounts in var. *rubra* only;
[b] trace amounts only. Values in m*M* recalculated by dividing the original values, expressed as mg/kg fresh weight, by the molecular weight of the compound. See text for references

receptor sites are present on the same neurone. The degree of dose-dependency differs widely between compounds. Chlorogenic, caffeic and protocatechuic acids exhibit the steepest dose-response curves. Although threshold-concentrations were not determined with great accuracy, it is of interest to compare reported levels of the predominant phenolic acids and flavonoids in *B. oleracea*-cultivars with the dose-response relations established. Among different *B. oleracea* varietes (i.e. *gemmifera, sabauda, rubra, sabellica*) as well as among 4–5 different cultivars within these varieties, a high degree of variation in levels of these compounds has been reported (Wildanger and Herrmann 1973; Schmidtlein and Herrmann 1975; Herrmann 1977; Brandl and Herrmann 1983). Such data have been summarized in Table 5. Relating them to the dose-response curves permits the conclusion that several phenolic acids tested may represent actual stimuli to caterpillars feeding on *B. oleracea* leaf material.

The predominant flavonoid aglycones in *B. oleracea* are quercetin, kaempherol and isorhamnetin (Hegnauer 1973; Durkee and Harborne 1973; Wildanger and Herrmann 1973); cyanidin is the prevalent anthocyanidin (Hrazdina 1982). The *sabellica*-group reportedly contains the highest amounts of flavonol aglycones (around 1.0 m*M* (Wildanger and Herrmann 1973)). The presence of rutin as a flavonol glycoside in the genus *Brassica* has been disputed (Durkee and Harborne 1973). Neither catechin, malvin nor oenin has been reported from *Brassica*. No quantitative data are available on cyanin-levels in mature leaves of the *rubra* group ('red' cabbages). Threshold for the response to cyanin in *P. brassicae* was below 0.2 m*M*, strongly suggesting that this anthocyanin may constitute a natural stimulant. These deductions have to be considered with the following limitations in mind. Firstly, the compounds occur as mixtures in the leaf. They have not been tested as such, thus it is unknown if additive, synergistic or inhibitory interactions occur at the chemosensory level. Secondly, phenolic acids and flavonoids accumulate predominantly in the vacuole (McClure 1975; Matile 1984), where they exist almost entirely as several species of glycosides (Harborne 1979). Cellular disruption during feeding enzymatically releases the respective aglycones. The phytochemical data cited express amounts as aglycones, based on total leaf homogenates.

Structure-activity considerations

Although no systematic series of structural modifications was tested, due to the emphasis placed on the study of host-plant borne compounds, it seems clear that phenolic acids that possess free hydroxyl-groups at both the 3- and 4-positions of the aromatic ring are more effective than those in which one of these groups is methylated. Interestingly, the same is true for the B-ring of the flavonoids in *P. brassicae*. In the L-sensillum of *P. rapae*, the quinic ester moiety of chlorogenic acid does not change its effectiveness relative to caffeic acid. The high responsiveness to catechin demonstrates also that glycosylation is not a requirement for effectiveness of flavonoids. Thus, larvae can potentially respond to aglycones released during feeding.

Several structure-activity studies on the biological effects of flavonoids have been reported (Norris 1977; Elliger et al. 1980; Lane et al. 1985). Ortho-hydroxylation of the B-ring was found to contribute strongly to the toxicity of flavonoids to *Heliothis zea* (Elliger et al. 1980); it is interesting that this change also affects chemosensory responses in both *Pieris* species. In contrast, feeding by *Scolytus multistriatus* was particularly inhibited by flavonoids possessing an oxidized C-ring; e.g., kaempferol and quercetin (Norris 1977), which were ineffective in the present study. In chrysomelid beetles, differences in sugar residues explained feeding inhibition of flavonoids better than the phenolic substitution pattern of the aglycones (Matsuda 1978). Thus, several structural characteristics of phenolic and flavonoid compounds may influence their effectiveness as chemosensory stimulants. The mechanism of chemoreception of phenolic and flavonoid compounds has not been studied in detail. Van Drongelen and van Loon (1980) found genetical indications that the chemoreception of the flavonoid molecule phloridzin in caterpillars of *Yponomeuta* species is based on a receptor protein, as has also been postulated for sugar reception in insects (Hansen and Wieczorek 1981).

Cellular specificity

Past studies of the specificities of the gustatory neurones in both sensilla of *P. brassicae* (Schoonhoven 1967, 1972a; Ma 1972; van Loon and van Eeuwijk 1989) make

it possible to determine which neurones were activated by stimulation with phenolic acids or flavonoids. The 4 gustatory neurones present in the sensilla display a highly consistent rank order in spike amplitude, e.g. in the M-sensillum the deterrent neurone > the glucosinolate sensitive cell > the sucrose-sensitive cell > the cation-receptor. This rank order does not vary among individuals or recordings, though the absolute amplitudes may vary. When stimuli that evoke single-unit activity are mixed with a compound for which the sensitive neurone is unknown, the appearance of two spike amplitude classes in the recording is considered proof that another neurone is also excited. On the basis of the known specificities it can be decided that in the L-sensillum of *P. brassicae* a neurone separate from the sucrose-, amino acid- and glucosinolate-'best' cells was activated by the effective phenolic acids and flavonoids, as has been previously suggested for anthocyanins (Schoonhoven 1972a). This 'fourth' lateral cell is at the same time very sensitive (threshold about 2 μM) to the steroidal compound helveticoside, which occurs in some Cruciferae (van Loon, unpublished results; cf. Nielsen 1978). It is, however, insensitive to a range of steroidal compounds to which the medial deterrent neurone reacts (Ma 1972). Cellular specificity in the M-sensillum is more difficult to determine. At higher concentrations, phenolic acids and anthocyanins evoke multineuronal responses even though they are applied as pure compounds. This phenomenon has been described for the aromatic glucosinolate sinalbin also (Ma 1972). It seems probable that in the M-sensillum, both the deterrent-neurone and the sinalbin-sensitive cell are excited. This would mean that the presence of an aromatic ring is the structural requirement for activity of the so-called aromatic glucosinolate-sensitive neurone (Schoonhoven 1972a) as has been reported for the glycoside receptor of *Mamestra brassicae* (Wieczorek 1976). The third neurone that is activated at 5.0 mM most probably is the cation-receptor, which is responsive to pH-values lower than 3.0.

The mixture-experiments revealed the operation of peripheral interactions (Table 3). Significant decreases in responses to sucrose and proline were established, while in no case did simple additivity of responses occur. When applying mixtures, simple additivity of spike frequency values found in response to single compounds at certain concentrations is expected only if each compound excites a different receptor cell type. This could be ascertained only for the L-sensillum. The compounds in the mixture experiments and the concentrations at which they were tested exclude the possibility that phenomena resulting into an optimum of the dose response curve, as was found for protocatechuic and caffeic acids, have been responsible for the apparent inhibitions. It appears that cyanin both stimulates deterrent receptors in both sensilla and at the same time inhibits other receptor cells. Moreover, the response of the lateral cyanin-sensitive cell seemed suppressed by the simultaneous application of sucrose. Although in theory electrical interaction may also be involved, the fact that the peripheral interactions observed were different for different compounds points to the importance of chemical interactions. It must be stressed also that those plant compounds that by themselves do not evoke responses (e.g. rutin) theoretically have the potential of inhibiting, e.g. the sucrose receptor (Schoonhoven 1982). This possibility has not been checked here.

Sensory physiology and behaviour

The dual choice experiments demonstrate that in *P. brassicae* electrophysiological properties are reflected in behavioural discrimination. As both the L- and the M-sensillum are sensitive to the compounds tested, the resulting behaviour is most likely based upon a decision process at a central level. The outcome can apparently be both negative (avoidance) or positive (preference), depending on the compound and its concentration. In several respects, L- and M-sensilla mirror each other; they both contain cells sensitive to sucrose and to glucosinolates, organic acids and steroid secondary plant substances. The specificity and sensitivity spectra overlap but are not identical (Ma 1972; van Loon, unpublished results). Coding of food quality, e.g. the presence of phenolic compounds, can in part be accomplished by weighing chemosensory input from the anatomically separated maxillary sensilla. This simple model has been suggested also for *Manduca sexta* (Schoonhoven and Dethier 1966; Dethier and Crnjar 1982).

Artificial diets offer the possibility of manipulation and strict control of dietary composition, which are impossible in experiments with plant material. The decreased sensitivity to chlorogenic acid and cyanin in diet-reared caterpillars is an undesired and surprising phenomenon. The continuous exposure during larval development to the commonly applied phenolic preservative methylparahydroxy-benzoate may in part explain this effect. Sensory effects of this synthetic compound have been documented (Vinson et al. 1976). Because dual choice tests lasted 24 h, results may also reflect post-ingestive effects on feeding behaviour and effects on gustatory cells due to prolonged exposure to the chemical under test. These considerations pertain also to experiments with *P. brassicae* using application of phenolic acids and flavonoids on leaf discs in a no-choice situation for 24 h (Jones and Firn 1979). Moreover, in the latter type of experiments, compounds were not distributed homogeneously but rather presented in a concentrated layer at the leaf surface. Nonetheless, both types of experiments yield essentially similar results, suggesting direct involvement of chemosensory sensitivity on food preference behaviour. *P. brassicae* clearly is the more sensitive of the two species tested, which may in part explain differences in larval host selection behaviour. *P. rapae* larvae readily feed on *B. oleracea* cultivars in the *rubra*-group, while these are accepted only after a period of refusal by larvae of *P. brassicae; P. rapae* also tolerates higher levels of phenolics and flavonoids during development than *P. brassicae* (van Loon 1988).

Furthermore, the capacity for sensory discrimination of these sensory compounds may influence foraging behaviour within a single host plant. Young leaves contain lower levels of phenolic compounds (Herrmann 1977). The preference for younger leaves that has been demonstrated for *P. rapae* (Hoy and Shelton 1987) may in part be based on the sensory capacities reported here. Stressed plant tissues have been reported to contain higher levels of phenolic acids, flavonols and anthocyanins (Del Moral 1972; Hrazdina 1982; McClure 1975). Thus, sensory perception of these compounds may assist caterpillars in avoiding stressed tissues.

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