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PROBING BEHAVIOUR AND ENZYMATIC DEFENCE OF THE GRAIN APHID AGAINST CEREAL PHENOLICS

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ABSTRACT

Probing behaviour of the grain aphid, *Sitobion avenae* (F.) on sucrose-agarose gels containing *o*-dihydroxyphenolics was examined using the EPG (electrical penetration graphs) technique. The aphids on diets containing certain *o*-dihydroxyphenols differed in the mean number of probes and duration of first probe and stylet pathways.

Activity extent of the polyphenol oxidase [E.C. 1.10.3.1] present in the salivary secretions of a single grain aphid is about 30-60 µm. The enzyme activity level and its isoenzymes number in the aphid body are not induced by *o*-dihydroxyphenolics content in the host-plant tissues.

Some aspects of defensive strategies of the grain aphid towards the cereal phenolics are argued.

Key words: cereal phenolics, grain aphid, *Sitobion avenae*, probing behaviour, EPG, polyphenol oxidase

INTRODUCTION

The majority of plant phenolic compounds are toxic to herbivorous insects including aphids and impair their growth, development and fecundity [6, 8, 12, 25]. Feeding behaviour of the grain aphid on winter wheat seedlings treated with methoxyphenols e.g. ferulic and sinapic acids was effectively modified in relation to seedlings not supplied with these compounds [12]. It was also demonstrated that this aphid showed shorter phases of phloem salivation and ingestion when fed on wheat cultivars, which were rich in phenolics as compared to cultivars with a lower content of these chemicals [11].

Herbivorous insects are able to develop behavioural, physiological and biochemical strategies for dealing with plant allelochemicals [4], i.e. simple avoidance of feeding on plants containing high concentration of toxic allelochemicals, modification of physiological processes and metabolic defence mechanisms. Above categories of response to phenolic allelochemicals were not broadly demonstrated for the aphids because the tissue on which they feed is phloem with low concentration of allelochemicals [7, 19, 23]. The earlier studies on the cereal aphids enzymes, participating in the metabolism of the natural xenobiotics showed that their defence against the phenolics consists of conjugation of free phenolics with glucose, sulphate, phosphate and oxidation of the large pool of them to non-toxic products, with a fundamental role of the polyphenol oxidase in the latter metabolic pathway [13, 17, 26, 27, 29].

In the present paper, the effect of the *o*-dihydroxyphenolics incorporated into artificial diets for the grain aphid *Sitobion avenae* (F.), on the probing behaviour was examined with the EPG method. Moreover, the aphid's polyphenol oxidase (PPO) was studied, in particular: 1) activity extent of the enzyme present in the saliva; 2) activity and isoenzyme variance as affected of quantity changes of *o*-dihydroxyphenolics in host plant tissues.

MATERIALS AND METHODS

Chemicals. Agarose, sucrose and the phenolics: catechol, protocatechuic acid, gallic acid, caffeic acid, chlorogenic acid, (+)-catechin and quercetin were purchased from Sigma Chemical Co., (St. Louis, Mo, USA). Acrylamide, N,N'-methylene bis-acrylamide, sodium dodecyl sulphate (SDS), N,N,N'N'--tetramethylethylenediamine (TEMED) and ammonium persulfate were obtained from Bio-Rad (Mississauga, Ontario, Canada).

Aphids. The grain aphid, *S. avenae* used in the experiments was reared individually on seedlings of four Polish winter wheat cultivars: two moderately resistant - 'Grana' and 'Saga' and two susceptible - 'Emika' and 'Liwilla' [10], in insectary chambers at L 16:D 8 photoperiod and 22°C.

Effect of phenolics on aphid feeding behaviour. Apterous adults were wired and after being connected to an EPG amplifier allowed to probe for 4 h into 1.25% agarose gels covered by membranes of parafilm^{TM}. The gels contained 30% sucrose, plus 0.1% of the following phenolic compounds: catechol, protocatechuic acid, gallic acid, caffeic acid, chlorogenic acid, (+)-catechin or quercetin. Each gel offered to the aphids contained only one of the tested phenolic compounds, control gels contained no phenolics. The recorded EPGs from ten aphids probing into the gels were stored on a computer hard disc, using STYLET 2.0 software and analysed in terms of number and duration of the EPG waveforms, as classified by Tjallingii [24].

Assay of PPO *in situ*. Single aphid was allowed to probe into the gels containing 0.1% L-DOPA (L-3.4-dihydroxyphenylalanine) for a time ranging from 10 s to 2 hrs, at room temperature. Next, the aphid was removed and the diameter of halo surrounding the stylet sheath was measured under a light microscope. The assay was done in ten replications.

Assay of PPO activity. Determination of the PPO activity (n=10 per treatment) was carried out using aphids collected from the resistant and susceptible wheat cultivars. The insects were weighed and then homogenised in 0.1 M Na-phosphate buffer, pH 7.4 using a glass homogeniser placed in an ice-container. The homogenate was filtered through Miracloth and centrifuged at 3 000 × g. Supernatant was used to determine the activity *in vitro* of the grain aphid polyphenol oxidase, as slightly modified from Peng and Miles [20]. The colorimetric assay was carried out with 0.1% DOPA as substrate, at pH 7.4 and 30°C and the increase in absorbance after 30 min was measured at 460 nm. Protein determinations were done according to [3], using bovine serum albumin as a standard. All assays of the enzyme activity were done in three independent replications.

PPO isoenzymes polymorphism. SDS-polyacrylamide gel electrophoresis was used to isoenzymes separation of the polyphenol oxidase extracted from whole aphids after [9]. The electrophoresis was performed on slab gels of 100 × 60 mm. The separating slab gel contained 12.5% acrylamide, pH 8.8 and the stacking gel contained 3.0% acrylamide, pH 6.8 and bromophenol blue was the tracking dye. The initial current was 9 mA/gel, but when proteins completely reached the bottom of stacking gel, the current was increased to 16 mA/gel. When the separation was completed, the gels were incubated in 0.1% L-DOPA in 0.1 M phosphate buffer, pH 7.4 at room temperature for 2 hrs. The polyphenol oxidase appeared as dark bands on a clear background. Moreover, the staining for the protein fractions was performed with Commasie Brillant Blue R-250. The relative mobility of the enzyme subunits and their molecular weights were determined with the following standard proteins: cytochrome (12.3 kDa), chymotrypsinogen (25.0 kDa), ovalbumin (45.0 kDa) bovine serum albumin (66.0 kDa) and aldolase (160.0 kDa).

o-Dihydroxyphenolics analysis. The total content of the *o*-dihydroxyphenolics in leaves of the moderately resistant and susceptible wheat was determined according to [1].

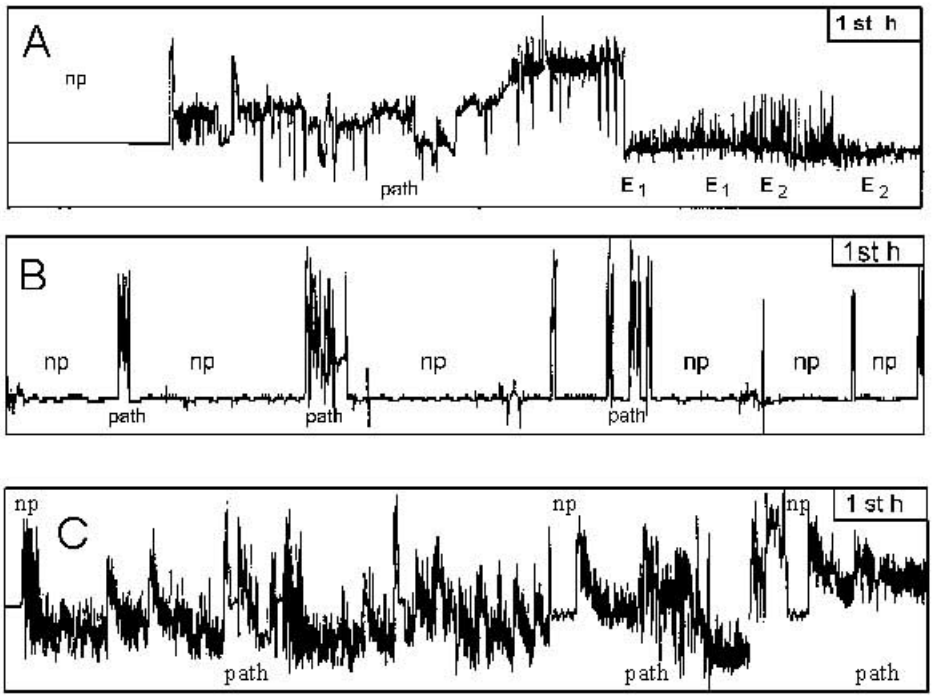
Statistics. The activity of the polyphenol oxidase in whole aphids fed on the susceptible and resistant wheat cultivars and the values of the EPG waveforms were subjected to an analysis of variance followed by Duncan's test.

RESULTS

Activity of the aphid stylets within artificial diets.

Apart from non-probing periods four waveforms relating to plant penetration by aphids were shown in the agarose control gels, containing sugar but no phenols: (1) pathway phase (lumped waveforms A, B, and C), (2) phloem phase waveform E1, including puncturing and salivation into a sieve element, (3) phloem phase waveform E2, representing sap ingestion from a sieve element and (4) the xylem 'drinking' waveform G. No F waveforms were shown, which reflects mechanical difficulties during tissue penetration into plants. Additionally, transitions of waveforms E_1 or E_2 into G occurred in the agarose system but not in plants. The presence of the single *o*-dihydroxyphenolics in the agarose gels limited the aphid stylet activity to pathway phase only. The recordings on the diets were very simple, with only path probes (waveform A, B, C) of various lengths following pauses (Figure 1B and C).

Fig. 1. Electrical penetration graphs (EPG) recorded during the first hour from the grain aphids probing agarose-sucrose gels, containing various *o*-phenolics: (A) control (no phenolics); (B) 0.1% caffeic acid; (C) 0.1% (+)-catechin; np – non-probing; path - pathway phase (A, B, C waveforms), E_1 - phase analogous to sieve element salivation; E_2 - phase analogous to sieve element ingestion.



Effects of phenolics on the grain aphid's probing.

The aphids that probed agarose-sucrose gels containing the studied *o*-dihydroxyphenols with exception of (+)-catechin showed a strong reduction in probing activity. A much shorter duration was shown of the first and subsequent probes, resulting in less total probing. However, the number of probes increased. The highest increase in the number of probes and reduction of probe durations were caused by gallic and caffeic acid (Table 1).

Table 1. Effect of cereal *o*-dihydroxyphenolics (0.1% v/o) on probing behaviour of *S. avenae*, EPG recordings from agarose-sucrose diets during 4 h

Phenolic compounds	EPG Parameters			
	Waveforms*	Number of probes (#)	Duration of 1st probe (min)	Mean duration of path (min)
Control	path, E & G	3.85 a	9.85 b	13.20 b
Protocatechuic acid	Path	3.29 a	3.30 a	3.00 a
Catechol	Path	3.70 a	4.35 a	15.00 b
Gallic acid	Path	8.00 b	0.38 a	1.80 a
Caffeic acid	Path	8.00 b	0.94 a	2.40 a
Chlorogenic acid	Path	5.00 b	2.75 a	6.00 a
(+) Catechin	Path	2.20 a	12.68 b	18.00 b
Quercetin	Path	3.50 a	3.75 a	4.80 a

*** Path = EPG waveforms A, B, and C, reflecting intercellular stylet penetration activities;**
E = EPG waveforms E1 and E2, reflecting salivation into, and ingestion of phloem sap in plants;
G = EPG waveform G, reflecting ingestion of xylem sap in plants.
Values in columns not followed by the same letter are significantly different at the 0.05% level (Duncan's test)

Activity extent of PPO.
The action of the salivary enzyme was evident at once after gel probing by the aphid both within salivary sheaths and accompanying halos. At the beginning, the diameter of the area of visible reaction products was about 10 µm, which is equal to the salivary sheath diameter. Later, during the first minute of probing the halo increased to about 30 µm. The value of 60 µm was determined as the maximal area of the enzyme reactions that developed after a period of constant probing varying between 2 min and a few hours (Table 2).

Table 2. Relation between probe duration and PPO activity extent of individual *S. avenae* within agarose-sucrose gel with 0.1% L-DOPA in Na-phosphate buffer, pH 7.4 at 25°C

Period of probing (s)	Reaction diameter of PPO (µ m)
10 – 30	10 – 20
30 – 60	20 – 30
120 – 1 800; 3 600 – 7 200	30 - 60

Subunits and activity level of PPO.
Two fractions of whole aphid proteins showing enzymatic activity were found in polyacrylamide gels with a molecular weight of 142 kDa and 395 kDa, respectively. The number of polyphenol oxidase bands, and their molecular weights, did not vary between aphids fed on moderately resistant and susceptible wheat. However, the activity level of the polyphenol oxidase in the aphids reared on moderately resistant cultivars, relatively rich in *o*-dihydroxyphenolics was lower than in those reared on susceptible cultivars with lower concentrations of these chemicals (Table 3).

Table 3. Relationships between content of *o*-dihydroxyphenolics in the various wheat cultivars and the PPO from *S. avenae* fed on these wheats

Wheat cultivars	*o*-Dihydroxyphenolics	Polyphenol oxidase of whole aphid		
	Total Content (mg/g d.w.)	Activity (optical density at A460 nm/30min/mg protein)	Isoenzymes characteristics	
			number of bands	M. W. (kDa)
Resistant				
Grana Saga	0.28 a 0.30 a	0.12 a 0.10 a	2	142; 395
Susceptible				
Emika Liwilla	0.16 c 0.26 b	0.15 b 0.17 b	2	142; 395

Values in columns not followed by the same letter are significantly different at the 0.05% level (Duncan's test)

DISCUSSION

The EPG's of grain aphid probing agarose-sucrose gels containing *o*-dihydroxyphenolics showed only pathway phase. None ingestion from diets with these components was observed. The EPG's indicated that the grain aphid avoided of in take of cereal phenolic compounds with *o*-dihydroxyl groups such as gallic acid, caffeic acid, chlorogenic acid, protocatechuic acid, (+) catechin and quercetin. These results confirmed the earlier reports that this class of secondary compounds is not of advantage towards the cereal aphids [8, 12, 14, 25]. In plants the aphids 'apply' also strategy of avoidance of phenolic allelochemicals because their stylets penetrate via epidermal and mesophyll intercellularly passing over like that cell vacuoles and other organelles rich in phenolics and reach phloem with rather low concentration of allelochemicals [7, 19].

The studied phenolic compounds affected the pathway phase differently. For example, caffeic and gallic acids shortened this probing phase drastically, whereas protocatechuic, chlorogenic acids and quercetin did not show such a strong effect. In contrast, (+)-catechin prolonged pathway phase and also decreased number of probes, yet the aphid did not ingest this compound. The results suggest that phenolic compounds may be significant in the grain aphid cereal relationships, as antifeedant compounds. Peng and Miles [22] showed that catechin at low concentration (a ten fold lower or more than ours) might act even as a phagostimulant (more ingestion) to the rose aphid. Earlier they showed that this aphid is capable to convert catechin from a deterrent to a phagostimulant oxidation product [18, 20, 21]. With respect to probing, however, we need to be careful with this terminology. Catechin stimulated probing in our experiments, i.e. stylet penetration and sheath secretion but inhibited feeding, i.e. ingestion (the correlate of the E2 waveform). The other phenols reduced both, pathway and feeding activities. Thus, for aphids (and similarly feeding insects), an 'anti-feeding' activity of substances needs to be distinguished from a 'anti-probing' activity.

The PPO's of a grain aphid or their reaction products apparently diffused into the agarose gels from the salivary sheath. Extrapolation to the plant, the *o*-dihydroxyphenolics in the intercellular fluid will be exposed to oxidation by the aphid salivary PPO, which may detoxify them and convert their anti-probing activity. Our earlier study showed that during pathway phase the grain aphid secreted into stylet sheaths PPO, specific towards *o*-dihydroxyphenolics i.e. gallic acid, caffeic acid, (+) catechin, quercetin, protocatechuic acid and chlorogenic acid [29, 30]. Accordingly, this enzyme seems to be involved in protection of the aphid against o-dihydroxyphenolics before it will be able to reach phloem.

The saliva injected into mesophyll and other cells during brief intracellular punctures [16] likely contain PPO's as well but this has not been demonstrated so far [5]. Anatomical study of the winter wheat leaves intensely exploited by the grain aphid did not exclude PPO activity in the mesophyll cells and, therefore, this remains speculative [28].

Action of the aphid's PPO in the plant and in the gut will provide double efficiency for defence against plant compounds [28, 29]. Nevertheless, the grain aphid's decreased its PPO secretion, when fed on moderately resistant winter wheat cultivars, rich in *o*-dihydroxyphenolics, seems not fit in a hypothesis of an adaptive value of the PPO production.

The whole aphid polyphenol oxidase was composed of two fractions of approximately molecular weights 142 and 395 kDa. On the other hand, Madhusudhan and Miles [15] for salivary secretions of pea aphid *Acyrthosiphon pisum* (Harris) and spotted alfalfa aphid *Therioaphis trifolii maculata* (Buckton) injected into water presented three fractions of this enzyme which indicated molecular weights of 120, 180, 300, 20, 90, 200 kDa, respectively. Baumann and Baumann [2] found two main protein fractions in *Schizaphis graminum* of sucrose diet collected saliva, at 69-66 and 154 kDa. The same fractions were identified later in diet-collected saliva of several other aphid species as well [5]. These contradicting results may partly be explained by the fact that PPO isoenzymes of different aphid species either have various molecular weights or the enzymes from whole aphids (gut, salivary glands and other tissues) differ from enzymes injected into diets. However, additional experiments, carefully comparing the chemical procedures as well as the protein fractions from different aphid tissues and secretions will be needed to explain these differences further.

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