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EFFECT OF PREVIOUS EXPOSURE TO HYDROXAMIC ACIDS IN PROBING BEHAVIOR OF APHID *Sitobion fragariae* ON WHEAT SEEDLINGS

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Abstract—We hypothesized that aphids after previous exposure to hydroxamic acids (Hx), a family of secondary plant compounds deleterious to aphids, are able to reduce their subsequent exposure to them. This hypothesis was tested by evaluating the time to produce salivation into a sieve element (SSE) by the aphid *Sitobion fragariae* on seedlings of two wheat cultivars of *Triticum aestivum* differing in their concentration of Hx. The total time to produce a first SSE was significantly longer in the high-Hx cultivar; however, the subsequent, second SSE (first SSE after interruption of probing) in this cultivar was significantly reduced, reaching the level observed in the low-Hx plants. Therefore, a strategy to reduce the exposure to secondary compounds was observed only in the second SSE in high-Hx plants. When the experimental plant was replaced by a new unattacked plant after the first SSE, aphids did not change the behavior described, thus excluding an aphid-induced plant susceptibility. The number of cell punctures and accumulated duration was not affected by previous exposure to Hx, either in low or high Hx cultivars. Total time and pathway time but not cell punctures, seem to be the variables affected by previous exposure to Hx.

Key Words—Aphid, experience, probing behavior, sieve elements, salivation, cell punctures, hydroxamic acids, EPG, *Sitobion fragariae*, *Triticum aestivum*.

INTRODUCTION

Aphids are specialized feeders that probe through the plant epidermis and mesophyll in order to access their final feeding site, the sieve elements. Aphid

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probing behavior in both susceptible and resistant host plants has been reported frequently (Sharks and Chase, 1979; Montllor et al., 1983; Montllor and Tjallingii, 1989; Dorschner and Baird, 1989; Morgham et al., 1994, Webster et al., 1993; Caillaud et al., 1995). Probing behavior in resistant plants is characterized by shorter probes, longer nonprobing times, and shorter phloem ingestion times than in susceptible plants, suggesting that aphids are able to detect plant resistance factors and display aversive behavior.

In the cases of some cereals, the presence of hydroxamic acids (Hx) in the epidermis, mesophyll, and sieve elements (Argandoña et al., 1987; Niemeyer, 1991) has been suggested as the main resistance factor against aphids (Niemeyer and Pérez, 1995). Thus, aversive behaviors (Argandoña et al., 1993; Givovich and Niemeyer, 1995; Mayoral et al., 1996), as well as demographic parameters of aphid biology (Thackray et al., 1990; Niemeyer, 1991; Givovich and Niemeyer, 1994) have been correlated with Hx.

The mechanism by which Hx are recognized by aphids is rather speculative. In an intact plant, Hx are present as glucosides, which are enzymically hydrolyzed to aglucones when the tissue is injured and intracellular compartmentation is destroyed (Hofman and Hofmanova, 1971). Aglucones exert stronger effects on the behavior and performance of aphids than the respective glucosides (Corcuera et al., 1985). Damage to mesophyll cells by the stylets on their way to the sieve elements has been described based on light and electron microscopy studies (Pollard, 1973; Spiller et al., 1985; Tjallingii and Hogen Esch, 1993). All types of cells show punctures, and the highest numbers of damaged cells are found inside the vascular bundle, although the magnitude of cellular damage seems to be moderate (Brzezina et al., 1986; Tjallingii and Hogen Esch, 1993). There are no chemoreceptors present on the aphid labium or the stylets (Wensler, 1977; Tjallingii, 1978), and gustatory organs are located in the epipharyngeal wall and stimulated by ingesta (Wensler and Filshie, 1969). Hence, detection of secondary metabolites by aphids must occur through the sampling of plant contents.

The aim of this work was to study the effect of previous exposure to Hx on aphid probing behavior. Using the electrical penetration graph technique (EPG), we have chosen the time taken by an aphid to produce the first E1 waveform (first phloem phase) as our standard measurement. Time to E1 has been pointed out as an indicator of the location of non-sieve-element factors affecting plant acceptance by aphids (Tjallingii, 1995). E1 has been correlated with salivation into the sieve elements (SSE hereafter) (Prado and Tjallingii, 1994), which may be involved in suppressing the wound reaction of the sieve elements (Tjallingii and Hogen Esch, 1993). We hypothesize that after previous exposure of aphids to Hx during their path through the epidermis and mesophyll in their route to the sieve elements, they are able to reduce their subsequent exposure to them, particularly in high-Hx plants. We tested this hypothesis by evaluating the time

needed to produce a first SSE by the aphid *Sitobion fragariae* (Walker) on two wheat cultivars differing in their concentration of Hx and comparing it with the time needed to produce a subsequent SSE after interruption of probing. We also investigated whether differences in such behavioral responses are accounted for by the aphids' intrinsic probing ability or by changes in the plant's susceptibility induced by the aphid. With this aim, the experimental plant was substituted by a new unattacked plant after the first SSE by the aphid.

METHODS AND MATERIALS

Insects. S. fragariae were collected from wild annual Poaceae in central Chile. From a single collected individual, a colony of clones was created and kept on oats (*Avena sativa* L. cv. Nehuén) under laboratory conditions at 20 ± 2°C and 16L : 8D photoperiod.

Plants. Cultivars of the wheat *Triticum aestivum* L. differing in Hx concentration were used in order to expose aphids to plants with different levels of these plant secondary compounds. The first leaf of 7-day-old seedlings was used. Chemical analysis showed that Hx concentration in the first leaf was significantly higher in cv. Naofén than in cv. Millaleu [1.94 ± 0.31 mmol/kg fresh weight, $N = 8$, and 1.09 ± 0.30 mmol/kg fresh weight (mean ± SE), $N = 8$, respectively; $F(1,14) = 29.21$, $N = 16$, $P < 0.001$].

Experiments. In both wheat cultivars, probing behavior of the aphid was electronically monitored, and when a first SSE occurred (determined by the observation of an E1 waveform), the aphid was carefully separated from the plant and then immediately returned to it. Thus, the stylets were not allowed to stay in the lumen of the penetrated sieve element cell, and a new probing sequence was forced to start. The interruption lasted less than 5 sec. The experiment continued until a new SSE was observed (observation of a new E1 pattern) in the manipulated aphid.

A second set of experiments was performed, but in this case the experimental plant was substituted by another nonattacked plant once the aphid showed its first SSE. Such design allowed the control of a possible aphid-induced change in the plant due to the stylets' activities inside the plant tissues, a change that could be influencing the subsequent SSE. The plant used for replacement was of the same cultivar and developmental stage as the original plant. A total of 10 replicates in each experiment was performed.

Monitoring of Probing Behavior. The probing behavior of each experimental aphid was electronically monitored by electrical penetration graphs (EPG). A gold wire electrode (25 μm diameter) was fixed to the dorsum of an apterous adult aphid with conductive silver paint. Another electrode was inserted in the soil of the potted plant. Both electrodes were connected to a DC electric cir-

cuit designed to monitor aphid stylet incursions inside plant tissues (Tjallingii, 1978). When the aphid stylets penetrate into the plant tissues, they close the electrical circuit and the voltage changes are amplified and continuously monitored. All signals were directly displayed on screen and recorded on a PC hard disk for detailed analysis with the EPGview software with a sampling rate of 100 Hz (Flores et al., submitted). Different stylet activities and the location of the stylet tip produce specific patterns of voltage changes in the recorded signal (electrical penetration graph) so activity and tip position can be judged from the displayed signal (Tjallingii and Hogen Esch, 1993). With this technique, waveforms associated with nonprobing (NP), cell punctures (pds), pathway activities composed of intercellular–intramural advancement with salivary sheath formation (C), xylem ingestion (G), difficulties in the stylet during probing (F), and salivation (E1) in the sieve elements can be determined (Tjallingii and Hogen Esch, 1993).

Statistical Analysis. The times taken by aphids from their placement in the plant to the production of SSE (dependent variable) were compared with a three-factor ANOVA with repeated-measures analysis. Factors were labeled as Hx, SSEs, and Plant. Each factor had two levels. Factor Hx included cultivars with low and high Hx concentration; SSEs included the first and second SSEs; The Plant factor included conditions where the experimental plant was or was not substituted by another nonattacked plant once the aphid showed its first SSE. Non-probing time, pathway time, number of cell punctures, and their accumulated duration were similarly analyzed as dependent variables with ANOVA for repeated-measures. Tukey HSD test for multiple comparisons was also performed. Data were transformed by log $(x + 1)$ if ANOVA assumptions were violated.

RESULTS

Table 1 shows that the variation in the total time to produce an SSE was significantly affected only by the factor SSEs and by its interaction with the factor Hx (SSEs * Hx). Total time to produce an SSE significantly differed between first and second SSE and was also modulated by the Hx concentration. The Plant factor was not significant, indicating that plant substitution did not affect aphid behavior as measured by this variable. In the high-Hx cultivar, the total time needed to achieve a first SSE was significantly longer than the same period for the low-Hx cultivar (Figure 1A). Aphids showed a significantly shorter total time to achieve a second SSE with respect to the first SSE only in the high-Hx cultivar (Figure 1A). The time to achieve the second SSE was not significantly different in the two cultivars (Figure 1B).

Considering only the pathway time (C pattern) before an SSE as the depen-

TABLE 1. THREE-WAY ANOVA WITH REPEATED MEASURES OF TOTAL TIME TO PRODUCE SSE (DEPENDENT VARIABLE)

	Effect		Error			
Sources	*df*	MS	*df*	MS	*F*	*P*
Plant	1	0.046	36	0.099	0.472	0.496 NS
Hx	1	0.255	36	0.099	2.573	0.117 NS
SSEs	1	0.406	36	0.069	5.882	0.020
Plant * Hx	1	0.138	36	0.099	1.386	0.246 NS
Plant * SSEs	1	0.014	36	0.069	0.208	0.650 NS
Hx * SSEs	1	0.579	36	0.069	8.392	0.006
Plant * Hx * SSEs	1	0.104	36	0.069	1.513	0.226 NS

dent variable, the same trends were observed (Figure 1B). In this case, however, only the interaction SSEs * Hx was significant ($F_{1,36} = 4.62$, $P = 0.038$, three-factor ANOVA with repeated measures analysis). Similarly, for nonprobing time a significant source of variation was the interaction SSEs * Hx; however, this effect was accounted for by a significant difference in duration between nonprobing time before the first SSE in low- and high-Hx cultivars (14.3 ± 3.1 and 32.6 ± 6.6 min for low and high Hx, respectively, $P = 0.04$ Tukey HSD test). In contrast, patterns G and F did not show significant differences associated with plant substitution, Hx, or SSE (data non shown).

The number of cell punctures (pds) showed a significant effect only in the Hx factor ($F_{1,36} = 4.74$, $P = 0.036$, based on three-factor ANOVA with repeated measures analysis). Since the Hx * SSE interaction was not significant, this effect was due mostly to a larger difference between the number of cell punctures before the first SSE in low- and high-Hx cultivars [25.9 ± 4.17 and 53.8 ± 9.7 min for low and high Hx, respectively (mean $\pm$ standard error), $P = 0.04$, Tukey HSD test] than before the second SSE [31.8 ± 6.7 and 35.7 ± 5.7 low and high Hx, respectively (mean $\pm$ standard error), $P = 0.62$, Tukey HSD test]. The number of cell punctures was not different between the two periods before SSE within each cultivar (Figure 1C). The accumulated duration of cell punctures did not show significant differences associated with Hx or SSE (Figure 1D) or with plant substitution.

DISCUSSION

In the high-Hx plants, the first SSE was produced after a longer probing period than in the low-Hx plants. A similar significant effect of plant resistance on the times required to the first phloem access has recently been observed in a different aphid–plant combination (Chen et al., 1997). Interestingly, in the high-

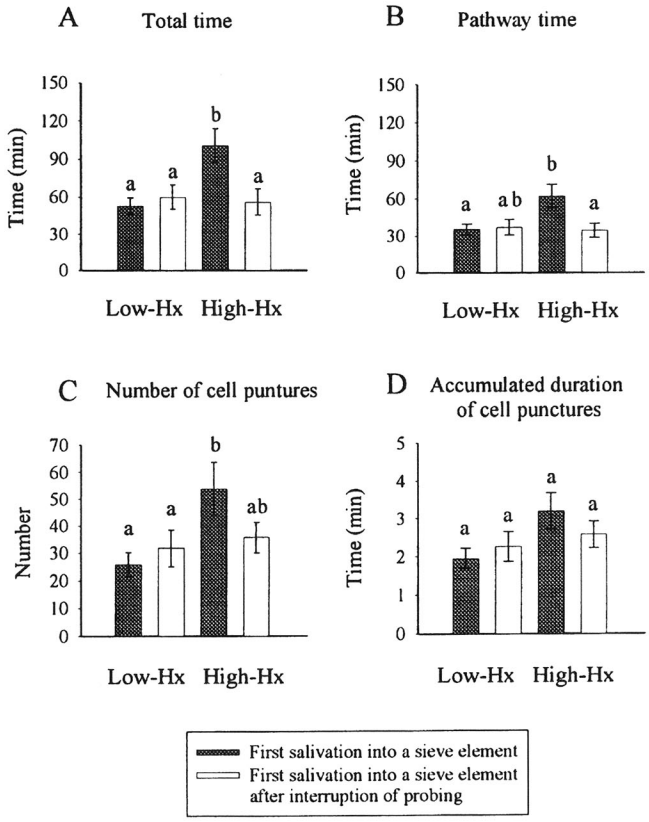

FIG. 1. Time to first SSE (dashed area) and to first SSE after interruption of probing (white area) by the aphid *S. fragariae* in wheat cultivars with low- and high-Hx concentrations. Total time before SSA (A), time in pathway activities (B), number of cell punctures (C), and duration of cell punctures (D) are shown. Bars represent standard errors for $N = 20$ in each case. Different letters among bars mean that the differences among the values are significant at $P < 0.05$ (Tukey HSD multiple-comparison test).

Hx plants, shorter times to the second SSE were recorded. Since time to achieve a second SSE in the high-Hx cultivar did not differ from the time devoted to the same activity in the low-Hx cultivar, it seems that aphids are able to overcome the high concentration of Hx during this period and consequently behave, at least in terms of their time assignation, as if they were on the low-Hx plants. Moreover, the significant decrease in the time to the second SSE in the high-Hx cultivar was attributable to a decrease in pathway activities but not to changes in other EPG patterns, suggesting that the main factor affecting plant acceptance is

encountered during passage through the intercellular and intramural plant compartments in the high-Hx cultivar. Furthermore, the fact that our experimental design did not allow phloem ingestion (E2 waveform) supports our proposal that prephloem ingestion factors are mainly involved in the behavioral changes described.

The fact that the decrease in the time to produce a second SSE was not affected by a substitution of the experimental plant for an unattacked one confirms that such time changes are accounted for by a change in the aphid's intrinsic ability to probe in the plant's tissues. It was not caused by an increase in plant susceptibility induced by the aphid during probing prior to its first SSE. This result is similar to the lack of aphid-induced plant effect on the behavior of the aphid *Rhopalosiphum padi* feeding on wheat (Prado and Tjallingii, 1997).

The number of cell punctures did not show significant differences between the first and second SSE in the two cultivars. Thus, it appears that interruption of feeding resets the pattern of cell punctures. This result is in agreement with those of Prado (1997), who found that the exploratory behavior of aphids seems to be reset by short interruption intervals (1 min). Total time and pathway time, but not cell punctures, seem to be the variables affected by previous exposure to Hx.

These facts support the hypothesis that after previous exposure to Hx during their route to the sieve elements, aphids employ a strategy of reducing their exposure to Hx, particularly when they face higher concentrations of them. This mechanism implies that during the first experience previous to salivation in sieve cells, aphids recognize the presence of Hx, particularly if present at a sufficiently high concentration. Such perception may initially elicit an avoidance behavior, mainly expressed as longer periods of pathway activities. This may be considered a training period within a simple learning process. Afterwards, during the path to a subsequent salivation into sieve cells, exposure to Hx is reduced through shorter duration of pathway activities as a result of the learning. An alternative explanation to the pattern observed is a mechanism of physiological adaptation to Hx by aphids, i.e., the deterrent effect of Hx decreases after exposure to them. However, because cell punctures may elicit hydrolysis of Hx from glucosides to the more toxic aglucones when the tissue is injured (Hofman and Hofmanova, 1971), the trend to show a lower number of cell punctures after the first SSE in the high-Hx cultivar seems to fit better a mechanism involving a reduction of the aphids' exposure to Hx.

Finally, the mechanisms proposed here should be understood as part of a variety of mechanisms that aphids may use in the field in order to overcome plant resistance factors such as secondary compounds.

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