

## FEEDING BY THE APHID *Sipha flava* PRODUCES A REDDISH SPOT ON LEAVES OF *Sorghum halepense*: AN INDUCED DEFENSE?

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**Abstract**—Feeding by the aphid *Sipha flava* produces a reddish spot on mature leaves of *Sorghum halepense*. The present work is aimed at determining whether this plant response entails induced resistance against the aphid. Old and young leaves showed the same response to aphid feeding (reddish coloration). Water-stressed plants displayed a similar reddish coloration to aphid-infested plants. This was verified by evaluation of absorbance peaks of the respective leaf extracts. Aphid fecundity was reduced on previously infested (and hence reddish colored) leaves. However, aphid fecundity was not affected on water stressed plants. Furthermore, aphid survival was not different on artificial diets containing increasing concentrations of the reddish pigment. It is concluded that the reddish spot is correlated with, but is not itself responsible for, the observed induced resistance of *S. halepense* against *S. flava*.

**Key Words**—Induced responses, induced resistance, *Sipha flava*, *Sorghum halepense*, aphids, anthocyanins.

### INTRODUCTION

Induced responses of plants to herbivory are frequently found in plant–insect interactions (Karban and Baldwin, 1997). When such responses have a negative effect on insect fitness, they are termed induced resistance (Karban and Baldwin, 1997). The magnitude of induced responses, and consequently of their effect on herbivores, may be affected by environmental conditions (Gianoli and Niemeyer,

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1996; Karban, 1987) as well as by the particular plant tissue that is attacked (Gianoli and Niemeyer, 1998; Gianoli, 1999).

*Sipha flava* (Forbes) is a cereal aphid that uses different cultivated and wild Poaceae (Young and Teetes, 1977; Breen and Teetes, 1986a; Blackman and Eastop, 1984). It has been recently reported in Chile, colonizing *Sorghum halepense* L. (González et al., 1998), a common weed in cereal fields (Monaghan, 1979; McWhorther, 1989; Matthei, 1995). *S. flava* has a particular pattern of attack on host plants. Aphids initially colonize lower (older) leaves (Long and Hensley, 1972; Holman, 1974), producing a characteristic damage: a reddish spot appears on infested leaves (Breen and Teetes, 1986a, 1990; Webster, 1990). The spot spreads and eventually covers the entire leaf, accelerating its senescence (Breen and Teetes, 1986a; Webster, 1990). Aphids then move upward to colonize the next leaf. *S. flava* infestation may significantly decrease plant fitness. Thus, infested sorghum (*Sorghum bicolor* L.) plants show a reduction in biomass (Breen and Teetes, 1986b) and seed production (Breen and Teetes, 1986a). Likewise, aphid infestation reduces biomass of *S. halepense* plants (González et al., unpublished).

The appearance of reddish coloration on tissues of sorghum plants, presumably involving anthocyanins, has also been related to the attack of the aphid *Schizaphis graminum* (Young and Teetes, 1977), to fungal infection (Nicholson et al., 1987), as well as to irradiation and low temperature (Shichijo et al., 1993, 1996). Moreover, the induction of anthocyanins by water stress is considered a widespread phenomenon in plants (Chalker-Scott, 1999).

The major aim of the present work is to determine whether the reddish coloration on leaves of *S. halepense* induced by feeding of *S. flava* is involved with the induced resistance. For this purpose, the fecundity of *S. flava* on previously infested (and hence reddish colored) and on uninfested leaves is compared. Given the particular pattern of occupation of plants of this aphid, these comparisons are performed on the natural site of attack, i.e., an old leaf, and on a young leaf. In addition, bioassays are conducted on artificial diets with different concentrations of plant extracts containing the reddish pigment. Complementary experiments evaluate aphid fecundity on plants where reddish coloration is induced by water stress. The qualitative similarity of the reddish coloration in water-stressed plants and in aphid-infested plants is assessed by their absorbance spectrum. Finally, some spatial and temporal characteristics of the induced response of *S. halepense* to *S. flava* infestation are addressed, and the effect on them of temperature and leaf age is evaluated.

#### METHODS AND MATERIALS

*Organisms.* Cultures of *S. flava* originally collected on *S. halepense* in the area of wheat fields in Santiago, Chile, were maintained on barley seedlings.

Seeds of *S. halepense* were collected in the same area. Aphids and *S. halepense* seedlings were kept in rooms at  $20 \pm 2^\circ\text{C}$  and a 14L : 10D photoperiod. Aphids reproduce parthenogenetically under these conditions.

*Leaf Age and Effect of Induced Response on Aphid Fecundity.* Seedlings at the eight-leaf stage were infested with 15 adult aphids confined inside a clip cage attached to the leaf that bore the treatment. Two groups of infested plants were used: one received the aphid infestation on the third leaf (an old leaf); the other on the seventh leaf (a young leaf). Two groups of uninfested plants were used as controls, bearing empty clip cages on the respective leaves ( $N = 15$  plants per treatment). Plants were then placed in growth chambers at constant temperature ( $20^\circ\text{C}$ ) and a 12L : 12D photoperiod. Aphids were removed after 24 hr of infestation, and plants were left for 92 hr, a time period after which a reddish spot appears on the infested leaves (data not shown). Immediately afterwards, aphid fecundity was evaluated in the four treatments (infested/uninfested; young/old leaf). An 8-day-old nymph was placed on the same leaves and on the same leaf part where clip cages were previously attached. Aphids were confined inside clip cages for eight days. At the end of this period, the number of nymphs produced was recorded. Data on aphid fecundity were analyzed by using a two-way ANOVA, with leaf age (young/old) and treatment (infested/uninfested) as main factors.

*Bioassay and Extraction of Reddish Pigment.* Aphid survival on artificial diets containing increasing concentrations of the reddish pigment, previously extracted from leaves of *S. halepense*, was evaluated. Ten aphids were confined in a vertically placed Plexiglas cylinder (3.0 cm high  $\times$  2.5 cm ID). A plastic net covered one end of the cylinder, and the other end was closed with a sachet made of two Parafilm M membranes. The sachet contained 200  $\mu\text{l}$  of a sucrose solution (25% w/v, prepared in phosphate buffer pH 5.5) and the extract to be tested at concentrations of 0 (control) 0.4, 4, 10, 20, and 40 mg of reddish extract/ml of solution. The cylinders containing the aphids were kept at  $20^\circ\text{C}$  and in the dark for 48 hr. At the end of this period the number of aphids still alive was recorded. Five replicates were performed for each concentration of the extract tested.

The extract of the reddish pigment was obtained as follows. One hundred grams of leaves, which had been infested by aphids and showed the reddish coloration, were extracted for 24 hr at  $4^\circ\text{C}$  with 500 ml of HCl-MeOH (0.1% v/v) solution. The solution was vacuum concentrated to 100 ml, adjusted to pH 5 with  $\text{NaCO}_3$ , and maintained at  $-20^\circ\text{C}$  for 5 hr. One milliliter of  $\text{Me}_2\text{CO}$  was then added and the suspension centrifuged for 20 min ( $-20^\circ\text{C}$ , 20,000 rpm); the precipitate was discarded. Vacuum concentration was continued until the final reddish solid extract was obtained.

*Aphid Fecundity on Plants Under Water Stress.* Greenhouse observations indicated that plants under water stress develop a similar response to that

observed after aphid infestation, i.e., the appearance of a reddish spot. This led us to evaluate whether such a response could affect aphid fecundity, as previous aphid infestation did. Plants at the four-leaf stage were used in the experiment. A group of plants was deprived of watering for five days, whereas control plants were watered daily during that period ( $N = 15$  per treatment,  $20^{\circ}\text{C}$ ). At the end of the five-day period, aphid fecundity was measured exactly as in the first experiment (see above).

*Comparison of Spectra of Reddish Spots of Plants Subjected to Aphid Infestation or Water Stress.* To compare spectra, a 3-cm section was excised from a pigmented zone of a leaf (one per treatment). The leaf sections were extracted for 24 hr at  $4^{\circ}\text{C}$  in 5 ml of HCl–MeOH solution, as was described above (see procedure of extraction of the pigment). A Shimadzu UV–visible spectrophotometer (model UV-240) was used. The spectrum was evaluated at wavelengths of 400–700 nm.

*Characterization of Induced Response.* Experiments were conducted to determine the minimum duration of aphid infestation required to produce the induced response, the time until the appearance of the reddish spot, and its spatial extension. These evaluations were performed at  $20^{\circ}\text{C}$  and  $28^{\circ}\text{C}$ . *S. halepense* plants at the four-leaf stage were infested with 15 adults of *S. flava* inside a clip cage attached to the third leaf in the following treatments: 8, 10, 12, and 16 hr. At the end of the respective periods aphids were removed. Observations on the infested leaf were performed every 2 hr, and a positive response was considered when evidence of a reddish spot was apparent ( $N = 10$  per treatment). In addition to this qualitative evaluation, the timing of the induced response, i.e., the time elapsed from the removal of aphids to the appearance of the spot, was recorded. The area of the reddish spot on leaves of plants of the same age, but infested during 24 hr, was assessed measuring its length (L) and width (W) and assuming a rectangular shape ( $\text{Area} = L \times W$ ). The percentage of leaf area covered by the spot was calculated after assessing the area of the whole leaf ( $N = 10$ ). This was done by using image analysis software (Sigmascan). Evaluations with plants at the eight-leaf stage also were performed to determine the effect of leaf age on the timing of the induced response. The same protocol of aphid infestation (for 24 hr) was followed on the third leaf (an old leaf) and on the seventh leaf (a young leaf) ( $N = 10$  for each treatment). Timing of the induced response was evaluated as described above.

## RESULTS

*Leaf Age and Induced Responses.* The fecundity of *S. flava* was significantly reduced by previous aphid infestation on the leaf. Neither leaf age nor the interaction of factors had a significant effect on *S. flava* fecundity (Table 1).

TABLE 1. EFFECT OF LEAF AGE AND PREVIOUS INFESTATION OF *S. halepense* ON *S. flava* FECUNDITY

Leaf age	Nymphs produced ( <i>N</i> , mean ± SE)	
	Control	Infested
Young	24.00 ± 2.20	20.36 ± 1.46
Old	21.55 ± 1.15	18.38 ± 1.44

Analysis of variance				
Source	<i>df</i>	Mean square	<i>F</i> ratio	<i>P</i>
Leaf age (L)	1	61.98	1.80	NS <sup>a</sup>
Pre infestation (I)	1	146.40	4.25	<0.05
L × I	1	0.73	0.02	NS <sup>a</sup>
Error	47	34.40		

<sup>a</sup>NS = not significant (*P* > 0.05, two-way ANOVA test).

*Bioassay of Reddish Pigment.* The survival of *S. flava* was not significantly different on diets with increasing concentrations of the extract containing the reddish pigment (*F* = 0.63, *P* > 0.05, one-way ANOVA) (Table 2).

*Aphid Fecundity on Water-Stressed Plants.* The fecundity of *S. flava* (number of nymphs, mean ±SE) on *S. halepense* was not significantly different on plants under water stress (15.33 ± 1.3) and on control plants (18.45 ± 1.73; *t* = 1.38, *P* > 0.1).

*Comparison of Spectra of Reddish Spots.* The spectra of the reddish spot of plants under water stress and plants infested with *S. flava* showed a remarkable similarity in qualitative terms (Figure 1).

*Characterization of Induced Response.* The minimum duration of aphid

TABLE 2. SURVIVAL OF *S. flava* FEEDING ON ARTIFICIAL DIETS WITH DIFFERENT CONCENTRATIONS OF AN EXTRACT CONTAINING REDDISH PIGMENT

Extract conc. (mg/ml)	Survival (%; mean ± SE) <sup>a</sup>
0.0	78 ± 9.69
0.4	92 ± 5.83
4.0	88 ± 5.83
10.0	86 ± 4.00
20.0	90 ± 3.16
40.0	88 ± 5.83

<sup>a</sup>Means are not significantly different (*P* > 0.05, one-way ANOVA).

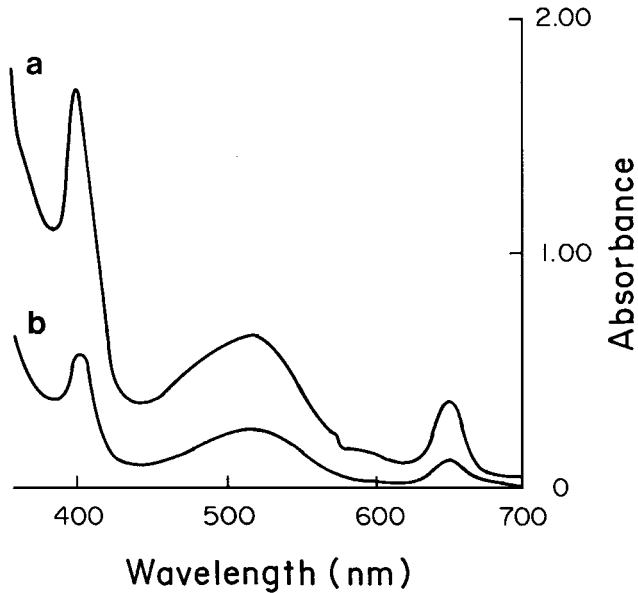


FIG. 1. Visible spectra of *S. halepense* leaf extracts showing reddish coloration caused by (a) infestation by *S. flava*, and (b) water stress.

infestation able to induce the reddish spot at 20°C was 16 hr, whereas at 28°C this time was reduced to 10 hr. After 16 hr of aphid infestation, the time (hours, mean  $\pm$  SE) to the production of the reddish spot was similar ( $t = 1.12$ ,  $P > 0.1$ ) at 20°C ( $96.3 \pm 1.02$ ) and at 28°C ( $93.8 \pm 1.05$ ). The reddish spot was localized at 28°C, whereas it was spatially spread at 20°C ( $t = 26.79$ ,  $P < 0.001$ ) (Table 3). The reddish spot appeared on old leaves  $92.4 \pm 1.03$  hr after the end of aphid infestation, whereas this time was significantly shorter on young leaves ( $72.15 \pm 1.96$  hr;  $t = -8.43$ ,  $P < 0.001$ ).

TABLE 3. CHARACTERISTICS OF THE RESPONSE (REDDISH SPOT) PRODUCED BY *S. halepense* AFTER *S. flava* FEEDING AT DIFFERENT TEMPERATURES

	20°C	28°C
Minimum time of infestation (hr)	16	10
Time to response (hr)	$96.3 \pm 0.02$	$100.3 \pm 0.47$
Leaf area covered by the spot (%)	$37.61 \pm 1.37$	<1.0

## DISCUSSION

Results showed a reduction in the fecundity of *S. flava* feeding on a previously infested leaf, which exhibited the reddish spot as induced response. The reddish spot, or another induced response correlated with it, could be the cause of the decrease in fecundity of the aphids. Negative effects on aphid fitness after infestation have been reported earlier (Cabrera et al., 1995; Gianoli, 1999; but see Formusoh et al., 1992). It has been shown that aphid infestation on other species of Poaceae may affect the nitrogen content of leaves (Dorschner et al., 1987) and change the concentration of secondary metabolites (Leszczynski, 1985; Gianoli and Niemeyer, 1997). The mechanism underlying the aphid-induced reddish spot in *S. halepense* is not clear at this point. Studies addressing the reddish spots on wheat plants caused by the aphid *S. graminum* have ascribed the leaf discoloration to an abnormal increase in volume of plastoglobuli, which in turn has a deleterious effect on chloroplasts producing chlorophyll loss (Ryan et al., 1990). Recent work involving sorghum and a nonpathogenic fungus suggests that anthocyanin accumulation is a consequence of the deactivation of related phytoalexins (3-deoxyanthocyanidins), which are induced immediately after fungal attack (Lo and Nicholson, 1998). It is considered that the induction of phytoalexins by microbial infections and by aphid feeding is very similar in nature, the common elicitors being oligosaccharides produced by plant cell wall degrading enzymes (Campbell and Dreyer, 1990).

Regardless of the particular mechanism involved in its production, the reddish spot in *S. halepense* is associated with induced resistance against *S. flava*. Thus, the plant response decreases aphid reproduction. The biological activity of anthocyanins against insects (Hedin et al., 1983), fungi (Schutt and Netzly, 1991), and bacteria (Stonecipher et al., 1993) has been demonstrated. In the present work, the negative effect on aphid fitness is achieved, but the infested leaf loses its photosynthetic capacity. This resembles hypersensitive responses found in other plant–insect systems (Fernández, 1990), particularly those in which the insects have poor mobility, as is the case of *S. flava*.

Leaf age did not affect fitness of *S. flava*. This result is surprising given the usual difference in quality of leaves of different age for aphids (Merritt, 1996; Dixon, 1998) and the marked preference of *S. flava* for older leaves in the field (Long and Hensley, 1972; González et al., unpublished). This suggests that ecological factors other than plant tissue quality may explain the within-plant distribution of the aphid (e.g., natural enemies) (González et al., 2001). The interaction of aphid infestation and leaf age did not have a significant effect on aphid fecundity, which means that the occurrence of induced resistance was not related to leaf age. Two nonmutually exclusive explanations, from both sides of the insect–plant interaction, may be advanced. Thus, it may be thought that aphid feeding, and hence elicitation, is similar on usual (old leaves) and unusual (young

leaves) feeding substrates. Interestingly, differences in feeding behavior have been reported for aphids developing on young and mature leaves of the same host plant (Gabrys et al., 1997). From the side of the plant, the physiological events that take place following aphid feeding may not be different in young and old leaves. Similar results have been observed in aphid-infested wheat seedlings (Gianoli and Niemeyer, 1997).

The induction of anthocyanins by water stress is a common phenomenon (Chalker-Scott, 1999). In the present work, water stressed plants developed a response similar to that induced by aphid infestation, i.e., the appearance of a reddish spot on the leaves, which is likely to correspond to anthocyanin accumulation (Snyder and Nicholson, 1990; Shichijo et al., 1993, 1996; Chalker-Scott, 1999). Moreover, the reddish spot was shown to be qualitatively the same in terms of absorbance peaks for plants subjected to either treatment. Unlike aphid infestation, water stress did not produce a decrease in plant quality for aphids, suggesting that the induced resistance observed in *S. halepense* is specifically triggered by aphid attack. Thus, the reddish spot would be a symptom associated with induced resistance, but not a symptom of induced resistance itself. This is supported by the bioassay results that showed no deleterious effects of the reddish pigment on aphids. Interestingly, previous reports describe an increase in plant resistance conferred by anthocyanin induction (Snyder and Nicholson, 1990; Schutt and Netzly, 1991; Stonecipher et al., 1993). Caution is needed before ascribing a particular role to anthocyanin induction in insect-plant interactions.

Environmental variables affect the expression of both constitutive (Larsson et al., 1986) and induced (Coleman and Jones, 1991) levels of plant resistance. We evaluated some spatial and temporal aspects of the aphid-induced reddish spot under two different temperatures (20°C and 28°C). These evaluations are of interest because the main distribution of *S. flava* is in the tropics (Medina-Gaud et al., 1965; Smith and Cermeli, 1979), and hence the temperate climate of Chile could mediate or constrain some issues of the aphid-plant relationship. At 28°C, a shorter infestation time was needed to produce the reddish spots; they took slightly more time to appear, and were considerably smaller than at 20°C. The spots were notably more spread at 20°C, suggesting that the induced response may be constrained at higher temperatures. A similar pattern was found for aphid-infested wheat seedlings (Gianoli and Niemeyer, 1996). Provided that the spatial extension of the spot is correlated with the magnitude of its associated induced response (see above), these results suggest that *S. flava* would be favored at higher temperatures. This agrees with earlier findings of better performance of this aphid at 30°C than at lower temperatures (Starks and Mirkes, 1979).

Finally, young leaves reacted more readily than old leaves in terms of the appearance of the reddish spot. This result does not match the initial observation that the reduction in aphid fecundity did not vary with leaf age. Therefore, it



provides additional support to the main conclusion of this paper, i.e., that the reddish spot is correlated with, but is not itself, the induced response responsible for the observed induced resistance of *S. halepense* against *S. flava*.

Several lines of research are open after the preliminary findings of the present work. In terms of the plant, identifying the compound(s) responsible for the induced resistance and elucidating how the physiological mechanism of aphid resistance compares with that described for fungal infections need to be examined further. In terms of the insect, determining the factors that promote or constrain the pattern of distribution within the plant and relating this to the quality and responsiveness of the tissues should be studied. Other ecological factors, such as natural enemies (e.g., Gonz ales et al., 2001) and interspecific aphid competition (e.g., Gianoli, 2000) should not be overlooked given the fact that *S. flava* usually coexists with other aphid species (*Rhopalosiphum maidis*, *Schizaphis graminum*) on its host plants.

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