Mechanism of Resistance to *Helicoverpa armigera* (Lepidoptera: Noctuidae) in Chickpea: Role of Oxalic Acid in Leaf Exudate as an Antibiotic Factor

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ABSTRACT Mechanisms of resistance to *Helicoverpa armigera* Hübner in chickpea, *Cicer arietinum* L., were investigated. Inhibition of larval growth occurred in a feeding test using the leaves of chickpea genotypes, which had previously been identified as having resistance to *H. armigera*. A feeding test using unwashed and washed leaves revealed that the substance responsible for the growth inhibition was water soluble and present on the surface of the leaves. Acid components of the leaf exudate were analyzed by high-performance liquid chromatography. Oxalic acid and malic acid were detected as major components in all 4 genotypes that were analyzed. Genotypes resistant to *H. armigera* accumulated more oxalic acid on the leaves than susceptible genotypes. Oxalic acid showed significant growth inhibition on *H. armigera* larvae when included in a semi-artificial diet. The accumulation of oxalic acid is considered to be one of the mechanisms of *H. armigera* resistance in chickpea. Inhibition of larval growth by oxalic acid was not caused by antifeedant effects but was more likely attributable to antibiosis. Malic acid had no effect on larval growth.

KEY WORDS host plant resistance, growth inhibition, malic acid

Helicoverpa armigera HÜBNER is a serious pest on a wide range of crops including chickpea, *Cicer arietinum* L.; pigeonpea, *Cajanus cajan* (L.) Millsp.; sunflower, *Helianthus annuus* L.; maize, *Zea mays* L.; sorghum, *Sorghum bicolor* (L.) Moench; tomato, *Lycopersicon esculentum* (Mill); and cotton, *Gossypium* spp. On chickpea, *H. armigera* is commonly known as the gram pod borer, and causes substantial crop losses in almost all countries where chickpea is grown. Several chickpea genotypes have been identified with exploitable levels of resistance to *H. armigera* (Dias et al. 1983, Lateef 1985, Lateef and Sachan 1990). These genotypes frequently suffer lower pod damage relative to susceptible genotypes. Antibiosis and oviposition antixenosis were reported as mechanisms of *H. armigera* resistance in some chickpea genotypes (Lateef 1985; Srivastava and Srivastava 1989, 1990).

Chickpea trichomes secrete an acidic exudate (Khanna-Chopra and Sinha 1987) that may contribute to insect resistance. Reduced pod damage may be correlated with the amount of acidic compounds, for example, malic acid and oxalic acid, in the exudate (Rembold 1981; Rembold and Winter 1982; Srivastava and Srivastava 1989; Rembold et al. 1990a, b). We studied the chemical components of the trichome exudate and their effect on *H. armigera* larvae.

Materials and Methods

Insect Culture. All *H. armigera* larvae used in this study were obtained from a laboratory culture maintained at ICRISAT Asia Center. The culture was established from and regularly supplemented with field-collected eggs. Larvae were reared on a chickpea based diet (Armes et al. 1992).

Feeding Test Using Chickpea Leaves. Neonate *H. armigera* were fed on leaves in vegetative stage of two susceptible genotypes ('Annigeri', 'ICCX 730266-3-4') and four resistant genotypes ('ICC 506', 'ICCV 7', 'ICCL 86101' and 'ICCL 86102') of short-duration chickpea sown in pots in September 1993. Larvae were held in individual glass tubes at 25°C under a photoperiod of 12:12 (L:D) h, and larval weights were recorded 21 d after hatching. Individual larvae were replicates, and there were 30 replicates per treatment (genotype).

Feeding Test Using Washed and Unwashed Chickpea Leaves. The leaves of Annigeri and ICC 506, which were sown on 11 October 1993 in a field plot at ICRISAT Asia Center, were collected in December 1993 and January 1994 in the flowering-podding stage. Half of the leaves were fed to the neonate larvae directly (unwashed control), and the other half were washed with tap water before feeding. There were 50 replicates (larvae) per

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Table 1. Growth of *H. armigera* larvae on chickpea leaves (n = 30)

Genotype	Larval wt at 21 d, mg		Larval period, d	
	Mean ± SEM	n	Mean ± SEM	n
	Susceptible			
Annigeri	191.9 ± 15.2a	22	30.5 ± 0.8a	19
ICCX 730266-3-4	217.0 ± 17.4a	22	29.5 ± 0.9a	19
	Resistant			
ICC 506	78.9 ± 6.0c	20	40.7 ± 3.3b	6
ICCV 7	97.6 ± 10.1bc	23	38.7 ± 2.7b	9
ICCL 86101	121.2 ± 13.4b	20	38.6 ± 2.2b	14
ICCL 86102	89.0 ± 7.4bc	21	40.2 ± 1.9b	9

n, Number of larvae surviving at weighing and pupation. Means within a column followed by the same letter are not significantly different at the 5% level (Fisher LSD test).

Table 2. Growth of *H. armigera* larvae on unwashed and washed chickpea leaves (n = 50)

Treatment	Genotype	Larval wt at 10 d, mg	
		Mean ± SEM	n
Unwashed	Annigeri	53.5 ± 5.6ab	39
	ICC 506	40.3 ± 3.7b	39
Washed	Annigeri	67.3 ± 7.1a	36
	ICC 506	65.0 ± 6.2a	40

n, Number of larvae surviving at weighing. Means within a column followed by the same letter are not significantly different at the 5% level (Fisher LSD test).

treatment. They were kept individually in glass tubes, and allowed to feed on the test leaves at 27°C under a photoperiod of 12:12 (L:D) h. Larvae were weighed 10 d after hatching.

Artificial Diet Feeding Test. Control semi-artificial diet was prepared with 53 g chickpea flour, 20 g wheat germ, 18 g yeast, 1.8 g L-ascorbic acid, 1.1 g methyl-4-hydroxybenzoate, 0.57 g sorbic acid, 0.83 g Aureomycin (Cyanamid India, Valsad, Gujarat, India), 5.3 g agar, 1.1 ml 40% formaldehyde, and 380 ml water. Oxalic acid and L-malic acid were added to the semi-artificial diet at concentration of 250 mmol/kg dry weight. Forty-eight neonate larvae were reared on each diet in individual plastic cells. Individual larvae were replicates. After feeding for 10 d at 27°C under a photoperiod of 12:12 (L:D) h, larval weights were recorded.

Filter Paper Feeding Test. Control (C) filter paper (2.5 by 2.5 cm) was made palatable by addition of 0.1 ml of 1 M sucrose solution. Treatment (T) paper was prepared by application of 0.1 ml of 1 M sucrose with oxalic acid or L-malic acid. Papers were dried in an oven at 60°C for 1 h and weighed. A larva (0.2–0.3 g) starved for 24 h was placed in a test tube with a control paper and a treatment paper for 24 h. Papers were dried and reweighed. Ten replicates were taken for each treatment. The amount of each paper consumed was used to calculate an antifeedant index: $(C-T) \times 100/(C+T)\%$. A positive value indicates an antifeedant effect and a negative value, a feeding stimulant effect.

Analysis of Chickpea Leaf Exudate. Four chickpea genotypes (Annigeri, ICCX 730266-3-4, ICC 506, ICCL 86102) were sown in pots in a greenhouse on 14 April 1994. Leaf exudate samples were obtained by washing all leaves collected from 15 chickpea plants of each genotype with distilled water on 10 May 1994 (vegetative stage) and 10 d later (flowering stage). The samples were filtered with Millipore Filter (HVLP, pore size 0.45 μm) and injected in a Shimadzu LC-6A liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a Supelcogel C610-H column (7.8 mm i.d. by 30 cm) (Supelco, Bellefonte, PA), a SCL-6A system controller and a C-R7A data analyzer. Mobile phase was 0.01 N H_2SO_4 at a flow rate of 0.4 ml/min. The elutes were monitored at 210 nm on a SPD-6AV UV-VIS spectrophotometric detector. Acid components of the exudate were identified from their retention time and quantified from the area of the peak compared with the authentic acid samples injected separately. The washed chickpea leaves were dried in an oven at 60°C for 2 d, and the dry weight was measured. Concentration of the acid components of the leaf exudate was calculated on the basis of the leaf dry weight.

Statistical Analysis. Larval weight and larval period data from the feeding tests were analyzed by SYSTAT 1-way analysis of variance (ANOVA) (Wilkinson 1990) for each treatment effect (genotype, washing or acid diet). Means were separated at the 5% level using Fisher least significant difference (LSD) test (Wilkinson 1990).

Results and Discussion

Larvae that fed on the leaves from resistant genotypes (ICC 506, ICCV 7, ICCL 86101, and ICCL 86102) weighed significantly less than those that fed on the susceptible genotypes (Annigeri and ICCX 730266-3-4) (Table 1: F = 21.9; df = 5, 127; $P < 0.001$). Larval period was also longer on the resistant genotypes (Table 1: F = 9.65; df = 5, 75; $P < 0.001$). These results suggested that a growth retardant or antifeedant substance, or both, existed in the resistant genotypes. The survival percentage at pupation was consistently lower in the resistant genotypes than the susceptible ones.

No significant variation in larval weight was observed at the 5% level for larvae reared on unwashed leaves of Annigeri or ICC 506 (Table 2). However, the variation in larval weight was significant at the 10% level; larvae that fed on unwashed ICC 506 (resistant) leaves were smaller than those fed on unwashed Annigeri (susceptible). There was no significant variation in the larval weight between those fed on washed Annigeri and on washed ICC 506. This result indicated that the substance inhibiting larval growth was water soluble and present on the surface of the leaves. These findings coincide with the results obtained by other groups that the acidic leaf exudate plays a role in *H. armigera* resistance in chickpea (Rembold

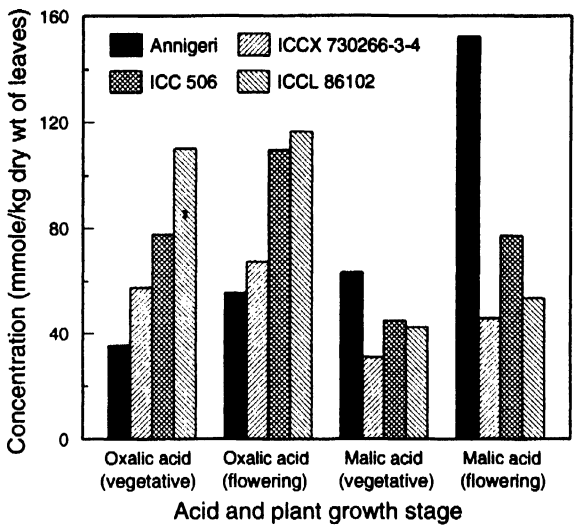

Fig. 1. Concentration of oxalic and malic acids accumulated on chickpea leaves at vegetative and flowering stages.

1981; Rembold and Winter 1982; Srivastava and Srivastava 1989; Rembold et al. 1990a, b). The substance is not only present on the leaf surface of the resistant genotype, ICC 506, but also on the susceptible check, Annigeri, because weights of larvae reared on washed leaves were heavier at the 10% significant level than those on unwashed leaves for both genotypes. However, more of the chemical seemed to be in exudate from ICC 506 than from Annigeri. There was no significant difference in larval development time among the treatments in this experiment.

Oxalic acid and malic acid were detected by high-performance liquid chromatography at the retention times of 12.4 and 17.1 min, respectively, as major acid components in the leaf exudate of all 4 genotypes that were analyzed. Fumaric acid and citric acid were also detected but as minor components at <1% that of the major acids. The concentration of oxalic acid was consistently higher in the resistant (ICC 506 and ICCL 86102) than in the susceptible (Annigeri and ICCX 730266-3-4) genotypes at both vegetative and flowering stages (Fig. 1). Malic acid concentration, however, did not appear to be related with resistance status (Fig. 1).

In our analysis the concentration of oxalic acid was higher than that of malic acid except for Annigeri. Rembold et al. (1990a) and Rembold and Weigner (1990) reported that the exudate was ≈2/3 malic acid and 1/3 oxalic acid. This discrepancy may be attributed to environmental differences. Rembold (1981) and Rembold and Winter (1982) observed variations in malic acid content of the exudate of the plants grown in different locations.

Oxalic acid inhibited larval growth when it was included in a semi-artificial diet, whereas malic acid had no significant effect on the larval weight compared with the control (Table 3: $F = 43.0$; df = 2, 143; $P < 0.001$). Larval period was longer on the oxalic acid diet than on control and the malic acid diet (Table 3: $F = 23.4$; df = 2, 143; $P < 0.001$). Thus, it appears that oxalic acid on the leaves is responsible for the reduced larval growth observed on resistant chickpea genotypes. Hence, the accumulation of higher concentration of oxalic acid is probably one of the major mechanisms of *H. armigera* resistance in chickpea. Slower larval growth, which results in longer larval period, brings about higher probability of predation, parasitism and infection by pathogens, leading to reduced numbers of the pest on the crop (Price et al. 1980).

Table 3. Effect of oxalic acid and malic acid on growth of *H. armigera* larvae (n = 48)

Treatment	Larval wt at 10 d, mg		Larval period, d	
	Mean ± SEM	n	Mean ± SEM	n
Control	208.8 ± 10.9a	48	15.6 ± 0.3b	48
Oxalic acid[a]	111.1 ± 5.3b	48	16.8 ± 0.3a	48
Malic acid[a]	224.7 ± 10.8a	48	15.5 ± 0.2b	48

n, Number of larvae surviving at weighing and pupation. Means within a column followed by the same letter are not significantly different at the 5% level (Fisher LSD test).

[a] 250 mmol/kg dry weight of diet.

In the diet feeding test the percentage of survival to pupation was not affected by oxalic acid (Table 3), whereas it was reduced on the resistant genotypes compared with the susceptible genotypes in the leaf feeding test (Table 1). Larval development was also much faster in the diet feeding test than in the leaf feeding test. These differences were the result of the semi-artificial diet being more nutritious than chickpea leaves.

No antifeedant effect was observed even at the higher 1 M oxalic acid and 1 M malic acid concentrations in the paper feeding test (Table 4). Both acids showed a tendency to stimulate feeding at the 0.25-M concentration. The larval growth inhibition effect of oxalic acid does not, therefore, seem to be derived from an antifeedant effect of this acid. It is most probably caused by an antibiotic effect.

The antibiotic effect in ICC 506 leaves in the feeding test in Table 1 was more pronounced than that in unwashed ICC 506 in the test in Table 2 when the larval growth was compared between Annigeri and ICC 506. This was possibly the result of differences in the environment in which the

Table 4. Effect of oxalic acid and malic acid on feeding of *H. armigera* (n = 10)

Acid	Concn, M	Antifeedant index
		Mean ± SEM
Oxalic acid	0.25	−47.1 ± 9.1
	1.00	4.8 ± 13.2
L-Malic acid	0.25	−20.2 ± 5.1
	1.00	−19.8 ± 9.8

plants were grown. Variation in the levels of antibiosis in different environments is considered to be one reason for the variation in *H. armigera* resistance among locations and years (Lateef 1985, Lateef and Sachan 1990). This may also be why significant variation was sometimes not observed in the weight of larvae fed on leaves from resistant and susceptible chickpea genotypes.

Malic acid had neither larval growth inhibition nor antifeedant effect in this study, although previous studies have shown some degree of correlation between its level on chickpea leaves and percentage of pod damage (Rembold 1981; Rembold and Winter 1982; Rembold et al. 1990a, b). It is possible that malic acid affects other behaviors of *H. armigera*, such as oviposition, which may be an additional mechanism of *Helicoverpa* resistance in chickpea (Lateef 1985, Srivastava and Srivastava 1989).

Our data support the hypothesis that oxalic acid in trichome exudate is an important factor for resistance to *H. armigera* in chickpea. Oxalic acid content, therefore, may be used by plant breeders as a measurable trait to identify chickpea germplasm with resistance to *H. armigera*. It may also be useful for pest management if cultivation techniques can be used to stimulate the exudation of oxalic acid.

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