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ORIGINAL CONTRIBUTION

Interaction of acid exudates in chickpea with biological activity of *Bacillus thuringiensis* towards *Helicoverpa armigera*

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Keywords
acid exudates, *Bacillus thuringiensis*, biological activity, chickpea, *Helicoverpa armigera*, host plant resistance

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Abstract

The gram pod borer, *Helicoverpa armigera,* is one of the most important constraints to chickpea production. High acidity of chickpea exudates is associated with resistance to pod borer, *H. armigera*; however, acidic exudates in chickpea might influence the biological activity of the bacterium, *Bacillus thuringiensis* (*Bt*), applied as a foliar spray or deployed in transgenic plants for controlling *H. armigera*. Therefore, studies were undertaken to evaluate the biological activity of *Bt* towards *H. armigera* on chickpea genotypes with different amounts of organic acids. Significantly lower leaf feeding, larval survival and larval weights were observed on ICC 506EB, followed by C 235, and ICCV 10 across *B*t concentrations. Leaf feeding by the larvae and larval survival and weights decreased with an increase in *Bt* concentration. However, rate of decrease in leaf feeding and larval survival and weights with an increase in *Bt* concentration was greater on L 550 and ICCV 10 than on the resistant check, ICC 506EB, suggesting that factors in the resistant genotypes, particularly the acid exudates, resulted in lower levels of biological activity of *Bt* possibly because of antifeedant effects of the acid exudates. Antifeedant effects of acid exudates reduced food consumption and hence might reduce the efficacy of *Bt* sprays on insect-resistant chickpea genotypes or *Bt*-transgenic chickpeas, although the combined effect of plant resistance based on organic acids, and *Bt* had a greater effect on survival and development of *H. armigera* than *Bt* alone.

Introduction

The gram pod borer, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae), is one of the most important constraints to crop production globally and is widely distributed in Asia, Africa, Australia and the Mediterranean Europe (Sharma 2005). It is a polyphagous pest, and it attacks more than 200 plant species including cotton, chickpea, pigeonpea, tomato, maize, sorghum and a range of vegetables, fruit crops and tree species (Manjunath et al. 1989; Fitt 1991). In India, it has been recorded from over 20 crops and 180 wild hosts (Manjunath et al. 1989). It causes an estimated loss of US$325 million in chickpea (ICRISAT 1992) and over US$5 billion on different crops worldwide, despite application of pesticides costing over US$2 billion annually (Sharma 2005). Insecticides have been widely used for controlling this pest on different crops, but undesirable side effects of synthetic insecticides, including development of resistance, have necessitated a shift to more eco-friendly approaches for controlling *H. armigera* (McCaffery et al. 1989; Kranthi et al. 2002). Several chickpea genotypes with low to moderate levels of resistance have been identified in the past (Lateef 1985; Sharma et al. 2007; Narayanamma et al. 2008).

High acidity of chickpea exudates is associated with resistance to gram pod borer, *H. armigera* (Srivastava and Srivastava 1989). Rembold et al. (1990) suggested that chickpea exudates can be used to select for resistance to *H. armigera*, the main components being malate and oxalate, which are present in variable

amounts in different genotypes of chickpea. Genotypes with resistance to *H. armigera* accumulated more oxalic acid on the leaves than the susceptible ones (Yoshida et al. 1995, 1997). Oxalic acid results in significant growth inhibition of *H. armigera* larvae when incorporated into artificial diet.

Biopesticides have been recommended for the control of several insect pests, including *H. armigera* (Chandra et al. 1999; Balasubramanian et al. 2002; Mandal et al. 2003; Bhojne et al. 2004). However, acidic exudates in chickpea have been reported to influence the biological activity of nucleopolyhedrosis virus (HaNPV) against *H. armigera* (Rabindra et al. 1992; Bhagwat 2001). Chickpea reduced the infectivity of virus occlusion bodies (OBs) exposed to the leaf surface of chickpea for at least 1 h. However, organic acids, primarily oxalic and malic acid, caused no inhibition. Biochanin A and sissotrin, the two minor constituents of chickpea leaf extracts, reduced OB activity significantly. These two isoflavonoids increased in concentration by up to three times within 1 h of spraying the virus suspension onto the plants (Stevenson et al. 2010). Food consumption by the third-instar larvae of *Spodoptera litura* (F.) decreased gradually on food treated with *Bacillus thuringiensis* Berliner (*Bt*) when exposed to increasing pH from 6 to 10 (Somasekhar and Krishnayya 2004). A feeding stimulant has been reported to increase the feeding and thus biological activity of *Bt* towards *H. armigera* (Zhang et al. 2000). The activity of *Bt* δ-endotoxins increases with an increase in pH from 8 to 10, but declines at a pH more than 10 (Behle et al. 1997). However, the pH of the acid exudates from chickpea ranges between 1.5 and 3.5 (Bhagwat et al. 1995), and this might influence the biological activity of *Bt* toxins towards *H. armigera*.

Genetic transformation as a means to enhance crop resistance or tolerance to biotic constraints has shown considerable potential to achieve a more effective control of target insect pests for sustainable food production (Sharma et al. 2002). The δ-endotoxin genes from the bacterium, *Bt*, have been deployed in several crops for pest management (Sharma et al. 2004; James 2007), and efforts are underway to develop chickpea plants with *Bt* δ-endotoxin genes for resistance to *H. armigera* (Romeis et al. 2004; Ramakrishna et al. 2005; Sanyal et al. 2005; Sharma et al. 2005b; Acharjee et al. 2010). However, concerns have been expressed that the trichome exudates in chickpea leaves and the pods, which are highly acidic in nature (Bhagwat et al. 1995), may have a negative influence on the biological activity of *Bt* sprayed on chickpea or toxin proteins expressed in transgenic chickpea. Because of the possible effect of pH on the biological activity of *Bt*, the present studies were undertaken to examine the effect of organic acids in chickpea on the biological activity of *Bt* towards *H. armigera*. This information will be useful for pest management in chickpea and deployment of *Bt*-transgenic chickpea for controlling *H. armigera*.

Materials and Methods

Test material

Four chickpea genotypes with different levels of resistance to *H. armigera* (ICC 506EB – resistant, ICCV 10 and C 235 – moderately resistant, and L 550 – susceptible) (Lateef 1985; Sharma et al. 2005a) were selected to assess the interaction of acid exudates in chickpea with biological activity of *Bt* against *H. armigera*. The test genotypes were grown under field conditions during the post-rainy season to obtain leaf materials for bioassays and quantify the amounts of organic acids on leaves. Each genotype was raised in a plot of 2×2.4 m^2 (four rows, 2 m long, and planted at 60×10 cm, row-to-row and plant-to-plant spacing). There were three replications for different *Bt* treatments on each genotype in a randomized complete design. The basal fertilizer (diammonium phosphate @ 100 kg/ha) was applied before sowing. The field was irrigated immediately after planting and at monthly intervals thereafter. Normal agronomic practices were followed for raising the crop. There was no insecticide application in the experimental plots. Leaf samples for bioassays were collected 4 h after the application of different concentrations of *Bt* (Biolep®) (0.0, 0.05, 0.1, 0.2 and 0.5%). The *Bt* sprays were repeated at 15-day intervals (beginning at 30 days after seedling emergence), and three sprays were applied on the crop. Leaf samples for estimating concentrations of organic acids were collected at the vegetative and flowering stages from the untreated control plots of different chickpea genotypes.

Insect culture

Larvae of *H. armigera* were obtained from the laboratory culture maintained at the International crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India. Larvae were reared on chickpea-based artificial diet (Armes et al. 1992) at $27 \pm 1^{\circ}$C and 12-h photoperiod. The neonates were reared for 5 days in groups of 200–250 in 200-ml plastic cups containing a 2–3 mm layer of artificial diet on the bottom and sides of the

cup. Thereafter, the larvae were transferred individually to six cell-well plates (each cell-well 3.5 cm in diameter, 2 cm in depth) to avoid cannibalism. Each cell-well had sufficient amount of diet (7 ml) to support larval development until pupation. The pupae were removed from cell-wells, sterilized with 2% sodium hypochlorite solution and kept in groups of 50 in plastic jars containing vermiculite. Upon emergence, 10 pairs of adults were released inside an oviposition cage (30 × 30 × 30 cm). Adults were provided with 10% sucrose or honey solution on a cotton swab for feeding. Diaper liners, which have a rough surface, were provided as a substrate for egg laying. Liners with eggs were removed daily. The eggs thus obtained were sterilized in 2% sodium hypochlorite solution. The liners with eggs were dried under a table fan and then placed inside the plastic cups with artificial diet and removed from the cups after 4 days. Freshly emerged neonate larvae were used for bioassays. Three bioassays were conducted, and data were pooled from the three experiments for statistical analysis.

Interaction of genotypic resistance in chickpea with biological activity of *Bt* towards *H. armigera*

Chickpea plants grown in the field and sprayed with *Bt* were bio-assayed under laboratory conditions [27 ±2°C, 65–75% RH and a photoperiod of 12: 12 h (L: D)] using detached leaf assay (Sharma et al. 2005a). Plants were sprayed in the morning hours with different concentrations of commercial *Bt* formulation (Biolep®, Biotech International Limited, Delhi, India) with a knapsack sprayer in the field. After 4 h of spray, terminal branches were cut with sharp scissors and bio-assayed using detached leaf assay. Unsprayed plots served as an untreated control. Experiments in the laboratory were conducted in a completely randomized design (CRD), with five replications.

Plastic cups with 11.5 cm diameter and 4.5 cm deep were used for detached leaf assay (Sharma et al. 2005a). Agar-agar (3%) was boiled and poured into the plastic cups kept in a slanting manner. Nearly 10 ml of agar-agar was poured into each cup. The solidified agar-agar served as a substratum for holding a chickpea terminal branch with 3–4 fully expanded leaves in a slanting manner so that the chickpea branches did not touch the inner walls of the cup. Ten neonates of *H. armigera* were released on the chickpea leaves in each cup and then covered with a lid immediately. This system kept the chickpea terminals in turgid condition for 1 week.

The experiment was terminated when more than 80% of leaf area was consumed in the susceptible control or when there were maximum differences between the resistant and susceptible checks (generally 5 days after releasing the larvae on the leaves). Data were recorded on leaf damage on a 1–9 scale ($1 \leq 10\%$, and $9 \geq 80\%$ leaf area damaged), larval survival and larval weight. The number of larvae that survived after the feeding period was recorded, and the larvae were then placed in 25-ml plastic cups individually, starved for 4 h and then weighed. The data were expressed as percentage of larval survival and mean weight of the larvae in each treatment.

Estimation of organic acids in leaf exudates of four genotypes of chickpea

Chickpea leaf samples were collected early in the morning (before 9 am) in 25-ml centrifuge tubes containing 5 ml double distilled millipore water. Weight of each tube and water was recorded. First, fully expanded leaves from three plants were excised with scissors and placed in the tubes containing double distilled millipore water for 10–15 min, and then each tube with water and leaves was also weighed. Based on initial and final weights, fresh weight of the leaves was computed. After extraction of leaf exudates, leaves were removed from the tubes and placed on a filter paper for 1 h to remove excess water. Dry weight of the leaves was recorded by placing leaf samples in an oven at 45°C for three days. Leaf exudates extracted in water were filtered through 0.45 μm hydrophilic PVDF millipore millex-HV filters using 5-ml luer lock syringes. Approximately 3 ml sample solution was taken in 5-ml luer lock syringe from the centrifuge tubes. The needle was removed from the syringe and attached to millipore filter to dispense 1.5 ml of the filtrate into the vials. There were three replicates for each sample. After priming, the mobile phase (25 mM KH_2PO_4 of pH 2.5) was run for 1 h. Vials containing leaf exudates of different chickpea genotypes were arranged in a carousel. The HPLC fingerprinting of the organic acids was carried out by using Waters 2695 separation module with photodiode detector and Atlantis dC-18 column (4.6 × 250 mm, 5 μm).

Chromatographic separation was carried out using mobile phase with a flow rate 0.8 ml/min, and the injected volume was 20 μl with a 20 min run time per sample. Two replicates of each standard organic acid were prepared by mixing 2–10 mg of standard organic acid in 10 ml of water to get concentrations of 200–1000 ppm. Based on the standards, retention

time and peak area, organic acids present in the samples were identified and quantified. From the known concentrations of the standards, linear curves were obtained, from which amounts of different organic acids in the samples were estimated and expressed in μg/g on dry-weight basis.

Statistical analysis

Data on larval survival larval weight and leaf damage rating were subjected to analysis of variance using GENSTAT version 10.1 (Lawes Agricultural Trust, VSN International Limited, Oxford, UK). The significance of differences between the treatments was judged by F-test, while the treatment means were compared by the least significant difference at $P \leq 0.05$. The data were also subjected to correlation and linear regression analysis to determine the relationship of *Bt* concentrations (independent variable) with leaf damage rating, larval survival and larval weights (dependent variables) on different chickpea genotypes.

Results

Biological activity of *B. thuringiensis* against *H. armigera* on different genotypes of chickpea

The experimental results showed that feeding by the *H. armigera* larvae decreased with an increase in *Bt* concentration (fig. 1a). Under untreated control conditions, lowest leaf feeding was recorded on ICC 506EB, followed by C 235, and ICCV 10. The chickpea plots sprayed with 0.05% *Bt* suffered greater leaf damage than those sprayed with 0.10–0.50% *Bt*. The resistant check, ICC 506EB suffered significantly lower leaf damage than the susceptible check, L 550 across *Bt* concentrations. Differences in larval survival between the test genotypes across *Bt* concentrations were significant, and the larval survival was lower on ICC 506 EB and C 235 than on L 550 across *Bt* concentrations (fig. 1b). The larval survival was significantly lower in insects reared on plant material from the plots treated with 0.02 and 0.5% *Bt* than those sprayed with 0.05% *Bt*. Larval weights decreased with an increase in *Bt* concentration. In control plants, the larval weights were significantly lower on ICC 506EB than on ICCV 10, C 235 and L 550 (fig. 1c). Significantly, lower larval weights were recorded in insects reared on the leaves of ICC 506EB and C 235 than those reared on the leaves of L 550 – the susceptible check. The decrease in larval feeding, larval survival and larval weight was greater on L 550 and ICCV 10 than on the resistant

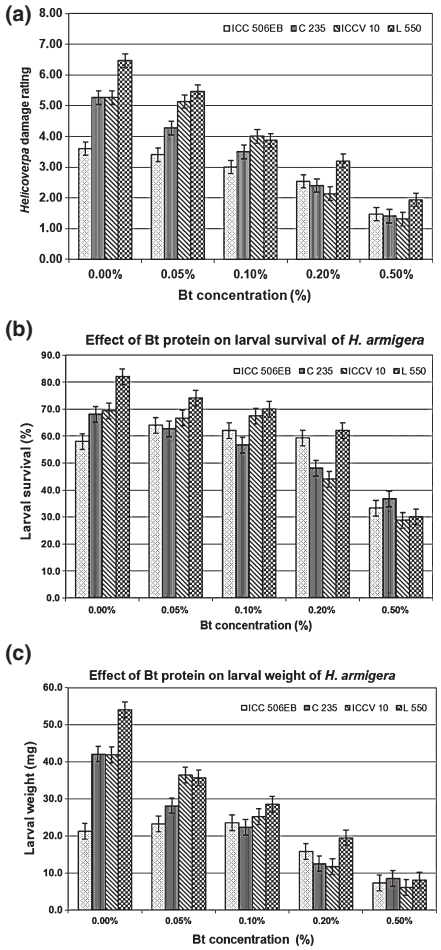

Fig. 1 Influence of chickpea genotypes with different levels of resistance to *Helicoverpa armigera* on biological activity of *Bacillus thuringiensis* (a = leaf damage rating, b = larval survival and c = larval weights).

check – ICC 506EB, although lowest feeding, larval survival and larval weights were recorded in insects reared on ICC 506EB across *Bt* concentrations.

HPLC fingerprints of organic acids of different chickpea genotypes in relation to biological activity of *Bt* against *H. armigera*

During the vegetative stage, six peaks were recorded in case of ICC 506EB, ICCV 10 and L 550, while eight peaks were recorded on C 235. Peak 4 was recorded only in case of C 235 and peak 5 in ICCV 10, while peak 10 was recorded only in case of ICC 506EB and L 550 (Table 1). During the vegetative stage, ICC 506EB had the highest (10.20 mg/g) amounts of oxalic acid, followed by ICCV 10 (5.42 mg/g), while C 235 had the lowest (2.19 mg/g) amounts of oxalic acid. ICCV 10 had the highest amounts of malic acid (12.55 mg/g),

Table 1 HPLC finger prints of organic acids in four chickpea genotypes at the vegetative stage (ICRISAT, Patancheru, India)

	ICC 506EB		ICCV 10		C 235		L 550	
Peaks	Retention time (min)	Peak area (%)	Retention time (min)	Peak area (%)	Retention time (min)	Peak area (%)	Retention time (min)	Peak area (%)
Peak 1	3.00	1.30	3.01	1.71	2.98	12.71	3.00	2.71
Peak 2	3.32	0.87	3.32	0.71	3.31	18.82	3.30	1.21
Peak 3	3.47	16.62	3.48	16.16	3.47	20.86	3.47	14.67
Peak 4	–	–	–	–	3.70	4.17	–	–
Peak 5	–	–	3.89	37.28	–	–	–	–
Oxalic acid	4.01	73.95	3.99	33.90	3.96	20.62	3.89	67.78
Malic acid	4.92	2.48	4.87	10.24	4.72	7.84	4.91	5.22
Peak 8	–	–	–	–	9.17	10.81	–	–
Peak 9	–	–	–	–	10.77	4.18	–	–
Peak 10	12.64	4.78	–	–	–	–	12.49	8.41

followed by C 235 (7.52 mg/g) and ICC 506EB (5.99 mg/g). The susceptible check, L 550 recorded the lowest amounts of malic acid (3.60 mg/g) (Table 2, fig. 2).

During the flowering stage, greater amounts of oxalic acid were recorded on ICC 506EB (17.70 mg/g) and L 550 (13.59 mg/g) than on C 235 (7.80 mg/g) and ICCV 10 (10.05 mg/g) (Table 2). Amounts of malic acid were maximum on ICCV 10 (37.71 mg/g), followed by C 235 (33.51/g). Fumaric and citric acids were recorded during the podding stage only. Amounts of fumaric (43.38 mg/g) and citric (1.59 mg/g) acids were maximum on C 235 and least on L 550 (37.71 an 1.00 mg/g, respectively). Amounts of oxalic acid were the highest on ICCV 10 (13.07 mg/g), followed by L 550 (9.09 mg/g), while the amounts of malic acid were maximum on ICCV 10 (86.78 mg/g), followed by C 235 (73.45 mg/g). The result indicated that the relative amounts of the organic acids changed across plant growth stages, which is possibly linked to change in genotypic reaction to the pod borer, *H. armigera*.

Table 2 Amounts of organic acids in four chickpea genotypes on dry-weight basis (ICRISAT, Patancheru, India)

	Amounts of organic acids (mg/g)			
Genotypes	Oxalic acid	Malic acid	Fumaric acid	Citric acid
Vegetative stage				
C 235	2.19	7.52	–	–
ICC 506EB	10.20	5.99	–	–
ICCV 10	5.42	12.55	–	–
L 550	3.44	3.60	–	–
Flowering stage				
C 235	7.80	33.51	–	–
ICC 506EB	17.70	8.03	–	–
ICCV 10	10.05	37.71	–	–
L 550	13.59	18.42	–	–
Podding stage				
C 235	6.67	73.45	43.38	1.59
ICC 506EB	6.04	37.82	15.00	0.00
ICCV 10	13.07	86.78	7.00	1.16
L 550	9.09	52.54	6.33	1.00

To assess the effect of host plant resistance (organic acids) on the biological activity of *Bt*, the data on organic acids during the vegetative stage, leaf damage rating, larval survival and larval weights were subjected to simple correlation and regression analysis. Leaf damage rating, larval survival and larval weights were highly correlated across genotypes and *Bt* concentration ($r = 0.96–0.99^{**}$, correlation coefficient significant at $P \leq 0.01$) (Table 3). Amounts of oxalic acid were significantly and negatively associated with leaf damage rating ($r = -0.84^{**}$), larval survival ($r = -0.72^{**}$) and larval weight ($r = -0.87^{**}$). There were large differences in the slope of the regression coefficient (b) between the genotypes for leaf feeding, larval survival and larval weight (Table 4). The slope of the curve for leaf damage rating was relatively lower in case of ICC 506EB ($b = -4.24$) than for C 235, ICCV 10 and L 550 ($b = -7.08$ to -8.22). The slope of the curve for larval survival was also lower in ICC 506EB ($b = -57.60$) and C 235 ($b = -60.16$) than ICCV 10 ($b = -86.82$) and L 550 ($b = -101.01$). Similarly, slope of the curve for mean larval weight was least for ICC 506EB ($b = -32.72$), followed by C 235 ($b = -56.43$) and ICCV 10 ($b = -69.10$). The slope of the curve was more in case of the susceptible check, L 550 ($b = -77.33$). The slope of the regression line for larval feeding, larval survival and larval weight was greater in case of the susceptible check,

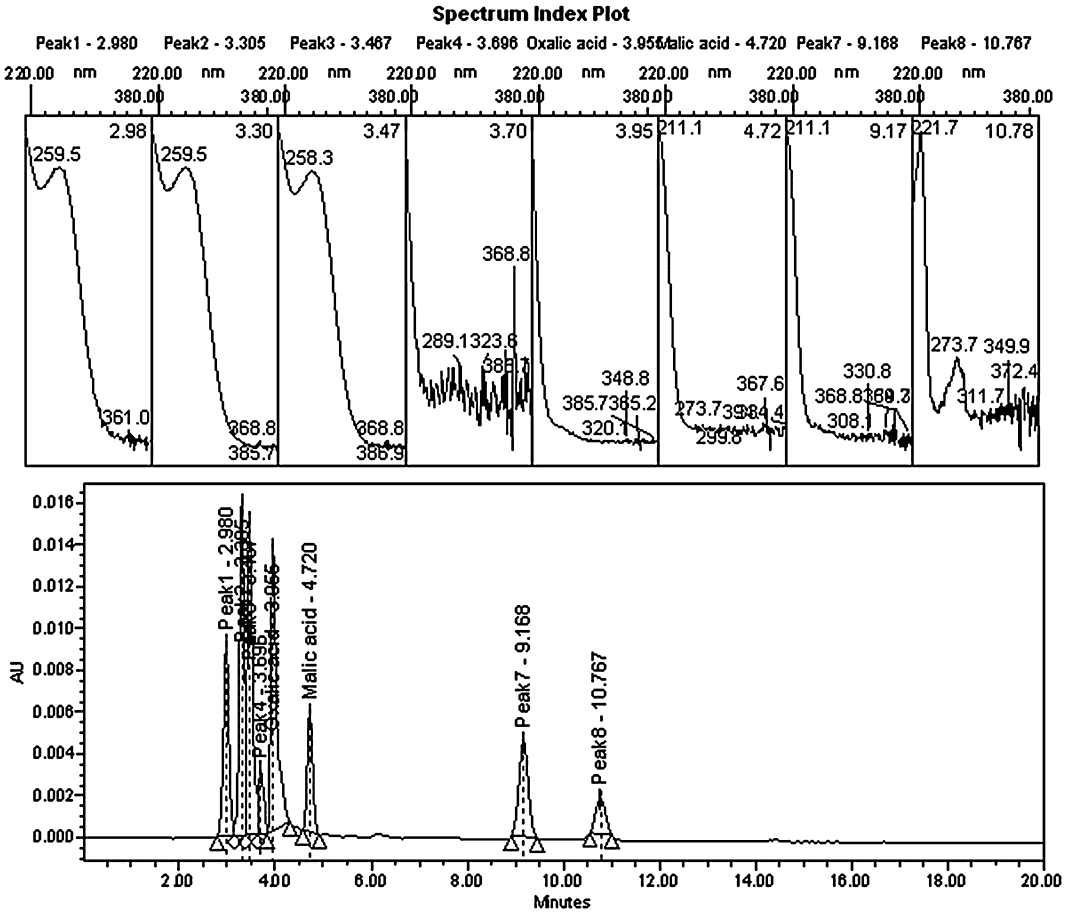

Fig. 2 Absorption spectrum (top) and HPLC fingerprints (bottom) of leaf exudates (organic acids) of chickpea (genotype C 235).

Table 3 Association of amounts of organic acids with leaf damage rating, larval survival and larval weights under unsprayed conditions

	Leaf damage rating	Larval survival	Mean larval weight	Oxalic acid	Malic acid
Leaf damage rating	1				
Larval survival	0.98*	1			
Mean larval weight	0.99*	0.96*	1		
Oxalic acid	−0.84*	−0.72*	−0.87*	1	
Malic acid	−0.17	−0.29	−0.11	0.01	1

*Correlation coefficient significant at $P < 0.01$.

L 550 than for the other genotypes tested, suggesting that reduced feeding on the resistant genotypes was possibly mediated by high concentrations of the organic acids, which possibly resulted in relatively lower rates of ingestion of the *Bt* toxin and hence may have reduced the biological activity of *Bt*.

Discussion

Significantly lower leaf feeding was observed on the ICC 506EB, followed by C 235. Larval survival and larval weights were also lowest on ICC 506EB, followed by C 235 and ICCV 10, suggesting that antifeedant/antibiosis is one of the mechanisms of resistance to *H. armigera* in chickpea. Leaf feeding decreased with an increase in *Bt* concentration, and the *H. armigera* – resistant genotype ICC 506EB – suffered significantly lower leaf damage than L 550 – the susceptible check – across *Bt* concentrations. Differences in larval survival between the genotypes across *Bt* concentrations were not significant, but larval survival, in general, was lower on ICC 506EB and C 235 than on L 550, suggesting that host plant resistance in combination with *Bt* had a greater effect on *H. armigera*. Larval feeding, survival and weights decreased with an increase in *Bt* concentration. However, the rate of decrease was greater on L 550 than on ICC 506EB, although lowest larval

Table 4 Relationship (regression analysis) between *Bt* concentrations and leaf damage, larval survival and larval weights in different chickpea genotypes

Genotype	Intercept (*a*)	Regression coefficient (P-value)	R^2(%)
Leaf damage rating			
ICC 506EB	3.52	−4.24**	98
C 235	4.57	−7.08*	86
ICCV 10	4.96	−8.22*	84
L 550	5.58	−8.18*	81
Larval survival (%)			
ICC 506EB	65.12	−57.60*	84
C 235	64.63	−60.16**	93
ICCV 10	69.96	−86.82*	91
L 550	80.77	−101.01**	99
Mean larval weight (mg)			
ICC 506EB	23.80	−32.72*	90
C 235	32.33	−56.43	71
ICCV 10	36.02	−69.10*	79
L 550	42.33	−77.33*	79

*, **Regression coefficient significant at P $\leq$ 0.05 and 0.01, respectively.

feeding, survival and weights were recorded in insects reared on ICC 506EB across *Bt* concentrations, suggesting that factors in the resistant genotypes, particularly the acid exudates, resulted in lower levels of biological activity of *Bt* possibly because of the antifeedants effect of the acid exudates (Yoshida et al. 1995).

Leaf exudates play an important role in *H. armigera* resistance in chickpea (Rembold 1981; Rembold and Winter 1982; Srivastava and Srivastava 1989; Yoshida et al. 1997). Oxalic acid and malic acid have also been reported to have an antibiotic effect on *H. armigera* larvae (Yoshida et al. 1995). Antifeedant effects of acid exudates reduced food consumption, reducing the amounts of *Bt* toxins ingested by the larvae and therefore might reduce the efficacy of *Bt* sprays on insect-resistant chickpea genotypes. However, plant resistance based on organic acids in combination with *Bt* had a greater effect on leaf feeding, larval mortality and development of *H. armigera* than *Bt* alone. Therefore, it is desirable to use *Bt* sprays or deploy *Bt* genes in transgenic plants for the management of *H. armigera* on chickpea.

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