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Influence of oxalic and malic acids in chickpea leaf exudates on the biological activity of CryIAc towards *Helicoverpa armigera*

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ABSTRACT

Efforts are being made to express toxin genes from the bacterium, *Bacillus thuringiensis* (*Bt*) in chickpea for minimizing the losses due to the pod borer, *Helicoverpa armigera*. However, there is an apprehension that acidic exudates in chickpea leaves may influence the protoxin–toxin conversion in the insect midgut, and thus, reduce the efficacy of *Bt* toxins. Therefore, we studied the influence of organic acids (oxalic acid and malic acid) present in the trichome exudates of chickpea on the biological activity and binding of *Bt* δ-endotoxin Cry1Ac to brush border membrane vesicles (BBMV) of the pod borer, *H. armigera*. Oxalic and malic acids in combination at concentrations present in chickpea leaves did not influence the biological activity of *Bt* toxin Cry1Ac towards *H. armigera* larvae. Amounts of Cry1Ac protein in the midgut of insects reared on diets with organic acids were similar to those reared on artificial diet without the organic acids. However, very high concentrations of the organic acids reduced the amounts of Cry1Ac in the midgut of *H. armigera* larvae. Organic acids in the artificial diet also increased the excretion of Cry1Ac in the fecal matter. Organic acids reduced the amount of protein in the BBMV of insects reared on diets with Cry1Ac, possibly because of reduced size of the larvae. Oxalic and malic acids at concentrations present in chickpea leaves did not affect the biological activity of Cry1Ac, but it will be desirable to have high levels of expression of Cry1Ac toxin proteins in chickpea for effective control of the pod borer, *H. armigera*.

1. Introduction

The legume pod borer, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) is one of the most important constraints to crop production globally. It is a polyphagous pest, and attacks more than 200 plant species (Arora et al., 2005). In India, it has been recorded from over 20 crops and 180 wild hosts. It causes an estimated loss of US$325 million in chickpea, and over US$5 billion on different crops worldwide, despite application of insecticides costing over US$2 billion annually (Sharma, 2005). Synthetic insecticides are the major component to control *H. armigera* damage in several crops. However, large-scale application of insecticides has resulted in severe reduction of natural enemies in different agroecosystems. In addition, it has also developed high levels of resistance to several commonly used insecticides (Kranthi et al., 2002). As a result, the farmers use higher dosages of the same or different insecticides, and also resort to frequent pesticide applications. Therefore, there is a need to develop alternate methods of controlling *H. armigera*, including genetically engineered chickpea plants with resistance to this insect.

Genetically engineered plants with resistance to insects have shown considerable potential to achieve a more effective control of target insect pests for sustainable food production (Sharma et al., 2004; Sharma and Pampapathy, 2006; James 2008; Sharma, 2009). Delta-endotoxin, Cry1Ac from the bacterium, *Bacillus thuringiensis* (*Bt*) has been deployed in transgenic chickpea to make host plant resistance an effective weapon for the control of *H. armigera* (Sharma et al., 2005; Sanyal et al., 2005). However, there is an apprehension that the acid exudates in chickpea leaves and pods might reduce the effectiveness of *Bt* toxins produced in the transgenic plants by influencing protoxin–toxin conversion and binding to the brush border membrane vesicles (BBMV) in the midgut of *H. armigera*. Protoxin to toxin conversion is a major step in the biological activity of *Bt* to insect pests. Reduced conversion of crystalline protoxin to toxin confers threefold resistance in diamond back moth, *Plutella xylostella* L. to *Bt* toxins, but no significant differences have been observed in the binding of Cry1C to BBMV in resistant and susceptible strains of this pest (Liu et al., 2000). The activity of *Bt* δ-endotoxins increases with an increase in pH from 8 to 10, but declines at pH more than 10. Increase in the pH of the *Bt* formulation above 11 results in a decline in larval mortality (Behle et al., 1997). Because of the profound effect of pH on the biological activity of *Bt*, it is important to study the interaction effect of acid

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exudates (oxalic and malic acids) in chickpea leaves and pods on the biological activity of Cry toxins from *B. thuringiensis* against *H. armigera* to develop appropriate strategies for development of transgenic chickpeas with *Bt* genes for the management of this pest. The present studies, therefore, examined the effect of oxalic acid and malic acid, the two most important components of leaf exudates in chickpea, on the biological activity and binding of *Bt* toxin Cry1Ac to BBMV in the midgut of the pod borer, *H. armigera*.

2. Materials and methods

2.1. Insect culture

The larvae of *H. armigera* used in the bioassays were maintained in the laboratory at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India. The *H. armigera* larvae were reared on chickpea based artificial diet (Armes et al., 1992) at 27 ± 2 °C. The neonates were reared for 5 days in groups of 200–250 in 200 ml plastic cups having 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 measured 3.5 cm in diameter and 2 cm in depth) to avoid cannibalism. Each cell-well had a sufficient amount of the artificial diet (7 ml) to support larval development until pupation. The pupae were removed from cell-wells, sterilized with 2% sodium hypochlorite solution (with 4% available chlorine), and kept in groups of 50 in plastic jars containing moistened vermiculite. Upon emergence, 10 pairs of adults were released in an oviposition cage (30 × 30 × 30 cm). Adults were provided with 10% sucrose or honey solution (Girijan Co-operative Ltd., Visakhapatnam, India) on a cotton swab for feeding. Diaper liners, which have a rough surface, were provided as a substrate for egg laying. The liners were removed daily, and the eggs sterilized in 2% sodium hypochlorite solution. The liners were dried under a table fan and then placed inside the plastic cups with the artificial diet. The liners were removed after 4 days. Freshly emerged neonate larvae were used for bioassays using diet impregnation assay (Narayanamma et al., 2008).

2.2. Preparation of Bt Cry1Ac toxin protein

The method used by Shao et al. (1998) was slightly modified to prepare the protoxin from the commercial *Bt* formulation. Ten grams (four samples of 2.5 g each) of commercial *Bt* formulation (Biolep^R) was taken into a centrifuge tube and washed with one molar NaCl (10 ml each time), centrifuged at 4000 rpm for 5 min, and then washed twice with de-ionised water and centrifuged at 4000 rpm for 5 min. The sediment was dissolved in 2% β-mercaptoethanol – NaOH buffer. Two ml of β-mercaptoethanol in 100 ml of water, and the pH adjusted to 10.7 with NaOH. It was then stirred for 2 h on a stirrer at the room temperature. The contents were centrifuged at 4000 rpm for 20 min, collected the supernatant, the pH adjusted to 4.4 with 2 M acetic acid. The contents were centrifuged at 4000 rpm for 20 min. The protoxin precipitate was collected and dialyzed against water (dialyzed overnight, and water changed 3 times). The amount of protein present in the precipitate was estimated by the method of Lowry et al. (1951). The Cry1Ac protoxin was used in all the experiments.

2.3. Growth and development of Helicoverpa armigera larvae on artificial diet with and without organic acids and Bt toxin Cry1Ac

At the flowering stage, the amounts of oxalic acid on dry weight basis vary from 7.80 to 17.70 mg per g^{-1}, while those of malic acid varied from 8.03 to 37.71 mg per g^{-1} in different genotypes of chickpea (Surekha Devi, 2008). Therefore, we added these acids into the artificial diet at the amounts present in the chickpea leaves (0.20% of oxalic and malic acids), and at very high amounts (0.44% of oxalic acid and 0.94% of malic acid) individually and in combination with ED_{50} concentration of *Bt* toxin Cry1Ac (effective dose to reduce the weight of the larvae by 50% in 5 days) (27.3 ng ml^{-1} of diet) (Sharma et al., 2008) to assess the interaction effect of organic acids with biological activity of Cry1Ac towards *H. armigera* larvae. The organic acids and the Cry1Ac toxin were incorporated into the artificial diet for rearing *H. armigera*. Seven milliliter diet was poured into the cell-wells of a six cell-well plate. After solidification of the diet (in nearly 30 min), the neonate *H. armigera* larvae were released individually into the cell-wells. There were three replications for each treatment, and each replication had 10 larvae. Data on larval weights were recorded at 10 days after initiation (DAI) of experiment using a microbalance (Mettler, AE 200^R). For this purpose, the larvae were removed from the rearing cell-wells and starved for 2 h, cleaned, weighed, and then placed back in the respective cell-wells. The pupal weights were recorded 1 day after pupation. Pupae from each replication were placed in a 1 l plastic jar containing moist Vermiculite. Percentage pupation and adult emergence were computed in relation to the number of neonate larvae released in each replication. Data were also recorded on larval and pupal periods. The adults were collected with an aspirator from the jars, and then three pairs of adults emerging on the same day in a particular treatment were placed inside an oviposition cage (30 × 30 × 30 cm), and provided with diaper liners for oviposition to record data on fecundity as long as the adults survived. The adults were provided with 10% sucrose solution on a cotton swab as a food, and there were three replications for each treatment.

2.4. Effect of organic acids in the artificial diet on amounts of Cry1Ac toxin in the midgut and fecal matter of Helicoverpa armigera larvae

Organic acids (oxalic and malic acid) at the concentrations present in the leaves of chickpea at the flowering stage and very high amounts (0.44 and 0.94 mg) were incorporated into the artificial diet along with *Bt* toxin Cry1Ac at the ED_{50} level (27.3 ng ml^{-1} of diet). Third-instar larvae of *H. armigera* were released on the artificial diet with different amounts of organic acids, with and without Cry1Ac *Bt* toxin for three days. A sample of different diets (with and without Cry1Ac) was collected to estimate the amounts of Cry toxin in the diet. After three days of feeding, the fecal matter and the larvae were collected, the midguts taken out by dissecting the larvae, and stored in the deep-freezer at −20 °C. The amounts of toxin present in the food, larval midguts, and fecal matter were estimated by using a semi-quantitative ELISA (Sharma et al., 2007).

2.5. Effect of acid exudates on binding of Bt toxin Cry1Ac to brush border membrane vesicles (BBMV) of the midgut of Helicoverpa armigera larvae

To study the effect of acid exudates on binding of Cry1Ac toxin to the BBMV of *H. armigera* larvae, the acid exudates and Cry1Ac toxin (at the ED_{50} level) (Sharma et al., 2008) were mixed in the artificial diet and fed to third-instar larvae of *H. armigera*. After three days of feeding, the larval midguts were dissected out, and the BBMV prepared and examined for binding of Cry1Ac toxin to the BBMV (Wolfersberger et al., 1987). The *H. armigera* larvae were chilled on ice for 15 min, dissected, and the midguts pulled out gently. Each midgut was opened by making a longitudinal cut and rinsed free of peritrophic membrane and gut contents, using an ice cold MET-buffer (Mannitol-0.3 mM, EGTA-5 mM, and Tris HCl 17 mM, pH 7.5). The isolated midguts were blotted and weighed. The midguts were placed in a vial with a small amount

of MET-buffer, frozen quickly by immersing the vial in liquid nitrogen, and stored at −80 °C or used immediately for BBMV preparation. The isolated midguts were then placed in an electric blender (Warring, Commercial Laboratory Blender) along with the ice cold MET-buffer (9 times the weight of midguts). The mixture was blended for two one-min periods at a medium speed (setting 5), separated by one minute cooling interval. An equal volume of cold 24 mM $MgCl_2$ was added to the midgut homogenate, blended the mixture thoroughly, and allowed to stand on ice for 15 min. The contents were centrifuged at 2500 g for 15 min at 4 °C, transferred the supernatant to another tube, and centrifuged again at 30,000*g* for 30 min at 4 °C. The pellet was suspended in 0.5× homogenate volume of ice cold MET-buffer treated with 24 mM $MgCl_2$, and centrifuged at 2500*g*. The supernatant was centrifuged at 30,000*g*, and the pellet constituting the BBMV was suspended in cold half strength MET-buffer, and distributed in 50–200 μl aliquots, which were immediately frozen and stored at −80 °C until use. The concentration of protein in the BBMV preparations was estimated by the method of Lowry et al. (1951).

2.6. Statistical analysis

The data were subjected to analysis of variance by using GENSTAT version 10.1. Data were analyzed by factorial analysis. Significance of differences between the treatments was judged by *F*-test, while the treatment means were compared by Duncan's multiple range test (DMRT) at $P < 0.05$ to know the significance of differences between the treatments, and their interaction effects.

3. Results

3.1. Effect of acid exudates of chickpea (malic and oxalic acids) on biological activity of Bt toxin Cry1Ac on Helicoverpa armigera larvae

The lowest weights (116.8 mg) were recorded in *H. armigera* larvae reared on diets containing 0.44% oxalic acid + 0.94% malic acid) + Cry1Ac (27.3 ng ml^{-1} of diet), followed by the insects reared on diets having 0.94% malic acid (139.7 mg) at 10 days after initiating the experiment (Table 1). The pupal weights were significantly lower (204.1 mg) in insects reared on diets with 0.44% oxalic acid + 0.94% malic acid, with or without Cry1Ac than the insects reared on the standard artificial diet. Highest pupal weights (286.8 mg) were recorded in insects reared on diets only with oxalic acid. The interaction effects between acid exudates and the Cry1Ac toxin were significant. Larval period was slightly prolonged (22.1 days) in insects reared on diets with 0.44% oxalic acid and 0.94% malic acid + Cry1Ac, followed by the insects reared on diets with 0.20% oxalic acid + 0.20% malic acid + Cry1Ac (21.0 days) (Table 1). Pupal period was slightly shorter in insects reared on diets with 0.20% oxalic acid and 0.20% malic acid than in insects reared on standard artificial diet, and the insects reared on diets with 0.44% oxalic acid + 0.94% malic acid. The interaction effects for larval and pupal periods, and for percentage pupation and adult emergence were significant (Table 2).

Percentage pupation was lowest (43.3%) on diets with 0.44% oxalic acid + 0.94% malic acid + Cry1Ac, followed by 56.7% pupation on diets with 0.44% oxalic acid. Adult emergence was significantly lower (5.6%) in insects reared on diets with 0.44% oxalic acid + 0.94% malic acid, with or without Cry1Ac than in insects reared on standard artificial diet, and the insects reared on diets with lower amounts of the organic acids. The interaction effects for concentrations of organic acids × Cry1Ac treatments were significant for adult longevity and fecundity (Table 3). No females emerged in insects reared on diets with 0.44% oxalic acid + 0.94% malic acid. Female longevity was significantly shorter in insects reared on diets with different amounts of organic acids + Cry1Ac (except in diets with 0.20% and 0.94% malic acid) than in insects reared on standard artificial diet without Cry1Ac. No male emergence was recorded in insects reared on diets with 0.44% oxalic acid + 0.94% malic acid. Fecundity of *H. armigera* females was significantly lower in insects reared on diets with different concentrations of oxalic acid alone or in combination with malic acid and Cry1Ac than in insects reared on the standard artificial diet without Cry1Ac. Oxalic acid exercised greater effect on fecundity than malic acid and Cry1Ac.

3.2. Effect of acid exudates on accumulation of Cry1Ac in midgut and excretion in fecal matter of Helicoverpa armigera larvae

The amounts of Cry1Ac toxin ranged from 1.098 to 1.473 ng g^{-1} in the food, 1.157 to 2.585 ng g^{-1} in the mid gut, and 0.505 to 0.916 ng g^{-1} in the fecal matter (Fig. 1). The larvae reared on the diets with high amounts of oxalic acid and malic acid grew very

Table 1
Survival and development of *H. armigera* larvae reared on artificial diets with different concentrations of organic acids (oxalic and malic acids) and *Bt* toxin protein Cry1Ac (27.3 ng ml^{-1} of diet) (ICRISAT, Patancheru, India).

Organic acids in artificial diet	Larval weight[a] (mg)			Pupal weight (mg)			Larval period (days)		
	Without Cry1Ac	With Cry1Ac	Mean	Without Cry1Ac	With Cry1Ac	Mean	Without Cry1Ac	With Cry1Ac	Mean
0.20% OA	201.9^{cd}	187.4^{c}	194.7	250.8^{b}	277.9^{de}	264.4	16.4^{abc}	16.7^{cd}	16.5
0.44% OA	221.4^{ef}	144.9^{b}	183.1	286.8^{e}	264.7^{bcd}	275.7	16.7^{cd}	16.7^{cd}	16.7
0.20% MA	240.2^{gh}	225.9^{efg}	233.1	286.4^{e}	280.6^{de}	283.5	16.8^{cde}	15.7^{a}	16.3
0.94% MA	231.3^{fgh}	139.1^{b}	185.2	259.6^{bc}	257.3^{b}	258.5	16.4^{abc}	15.9^{ab}	16.2
0.20% OA + 0.20% MA	246.4^{h}	229.0^{fg}	237.7	269.9^{cde}	267.2^{bcd}	268.5	16.9^{cde}	21.0^{f}	18.9
0.44% OA + 0.94% MA	139.7^{b}	116.8^{a}	128.2	213.2^{a}	204.1^{a}	208.7	16.6^{bc}	22.1^{g}	19.3
Standard artificial diet	289.0^{i}	210.6^{de}	249.8	285.9^{e}	265.9^{bcd}	275.9	17.4^{de}	17.5^{e}	17.5
Mean	224.3	179.1	201.7	264.7	259.7	262.2	16.7	17.9	17.3
	F-test		LSD at $P < 0.05$	*F*-test		LSD at $P < 0.05$	*F*-test		LSD at $P < 0.05$
Cry1Ac	229.36^{**}		6.13	2.16		NS	69.14^{**}		0.30
Organic acids	114.12^{**}		11.47	31.06^{**}		12.41	46.97^{**}		0.55
Organic acids × Cry1Ac	19.96^{**}		16.22	3.26^{*}		17.55	44.03^{**}		0.78

[a] Larval weights at 10 days after initiating the experiment. OA = oxalic acid; MA = malic acid. DAI = days after initiation of experiment. Figures followed by the same letter within a comparison are not significantly different at $P < 0.05$.

* *F*-test significant at $P < 0.05$, respectively.

** *F*-test significant at $P < 0.01$, respectively.

Table 2
Pupal period, pupation and adult emergence of *H. armigera* reared on artificial diets with different concentrations of organic acids (oxalic and malic acids) and *Bt* toxin protein Cry1Ac (27.3 ng ml^{-1} of diet) (ICRISAT, Patancheru, India).

Organic acids in artificial diet	Pupal period (days)			Pupation (%)			Adult emergence (%)		
	Without Cry1Ac	With Cry1Ac	Mean	Without Cry1Ac	With Cry1Ac	Mean	Without Cry1Ac	With Cry1Ac	Mean
0.20% OA	12.2^{a}	13.1^{cde}	12.7	60.0^{bc}	90.0^{gh}	75.0	61.1^{cd}	55.6^{bc}	58.3
0.44% OA	13.1^{cde}	13.1^{cde}	13.1	56.7^{b}	93.3^{h}	75.0	64.5^{cd}	57.1^{bc}	60.7
0.20% MA	12.1^{a}	13.0^{bcde}	12.5	76.7^{def}	90.0^{gh}	83.3	47.6^{b}	85.1^{e}	66.4
0.94% MA	12.4^{abc}	13.3^{def}	12.9	80.0^{efg}	86.7^{fgh}	83.3	62.1^{cd}	72.7^{d}	67.4
0.20% OA + 0.20% MA	12.3^{ab}	12.6^{abcd}	12.4	66.7^{bcd}	90.0^{gh}	78.3	65.1^{cd}	66.4^{cd}	65.7
0.44% OA + 0.94% MA	14.0^{f}	12.5^{abc}	13.3	66.7^{bcd}	43.3^{a}	70.0	5.6^{a}	16.7^{a}	11.1
Standard artificial diet	13.7^{ef}	13.0^{bcde}	13.4	90.0^{gh}	70.0^{cde}	80.0	85.2^{e}	61.9^{cd}	73.6
Mean	12.8	12.9	12.9	71.0	80.5	75.7	55.9	59.3	57.6
	F-test		LSD at $P < 0.05$	*F*-test		LSD at $P < 0.05$	*F*-test		LSD at $P < 0.05$
Cry1Ac	0.64		NS	24.19**		3.98	2.43		NS
Organic acids	3.92**		0.54	14.51**		7.45	51.34**		8.55
Organic acids X Cry1Ac	6.30**		0.77	21.08**		10.53	10.53**		12.09

OA = oxalic acid; MA = malic acid. Figures followed by the same letter within a comparison not significantly different at $P < 0.05$.
** *F*-test significant at $P < 0.01$.

Table 3
Longevity and fecundity of *H. armigera* reared on artificial diet with different concentrations of organic acids (oxalic and malic acids) and *Bt* toxin Cry1Ac (27.3 ng ml^{-1} of diet) (ICRISAT, Patancheru, India).

Organic acids in artificial diet	Female longevity (days)			Male longevity (days)			Fecundity (eggs female^{-1})		
	Without Cry1Ac	With Cry1Ac	Mean	Without Cry1Ac	With Cry1Ac	Mean	Without Cry1Ac	With Cry1Ac	Mean
0.20% OA	6.5^{cde}	6.3^{bcde}	6.4	5.8^{bc}	5.8^{bc}	5.8	513^{c}	552^{cd}	532
0.44% OA	7.0^{de}	4.7^{ab}	5.8	7.3^{bcd}	5.5^{b}	6.4	290^{a}	557^{cd}	423
0.20% MA	7.6^{ef}	6.4^{bcde}	7.0	9.6^{e}	7.4^{bcd}	8.5	850^{f}	795^{ef}	822
0.94% MA	7.5^{ef}	5.7^{bcd}	6.6	9.0^{de}	6.4^{bc}	7.7	718^{ef}	845^{f}	782
0.20% OA + 0.20% MA	3.7^{a}	5.0^{abc}	4.3	8.7^{de}	5.3^{b}	7.0	450^{bc}	368^{ab}	409
0.44% OA + 0.94% MA	–	7.0^{de}	3.5	–	2.3^{a}	1.2	–	–	–
Standard artificial diet	9.0^{f}	6.0^{bcde}	7.5	8.0^{cde}	5.3^{b}	6.7	1267^{g}	665de	966
Mean	5.9	5.9	5.9	6.9	5.4	6.2	584	540	562
	LSD at $P < 0.05$ for comparing		*F*-test	LSD at $P < 0.05$ for comparing		*F*-test	LSD at $P < 0.05$ for comparing		*F*-test
Cry1Ac	NS		0.01	0.84		12.74**	NS		3.03
Organic acids	1.22		11.95**	1.56		19.53**	96.6		96.33**
Organic acids × Cry1Ac	1.73		16.47**	2.21		3.37**	136.6		16.86**

OA = oxalic acid; MA = malic acid. Figures followed by the same letter within a comparison are not significantly different at $P < 0.05$.
** *F*-test significant at $P < 0.01$.

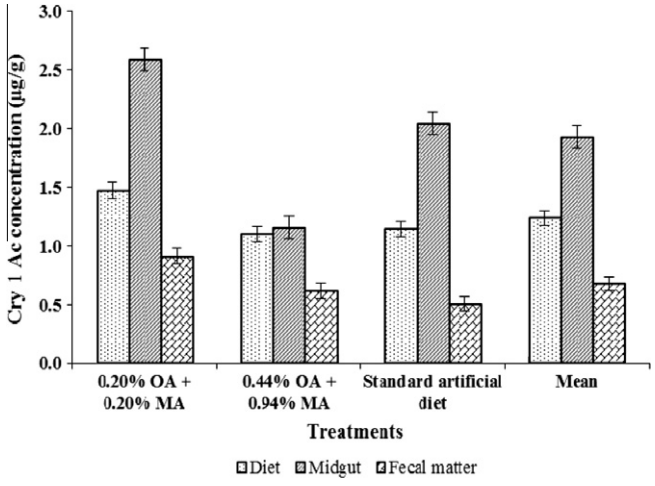

Fig. 1. Amounts of Cry1Ac δ-endotoxin protein in artificial diet, and in midgut and fecal matter of *H. armigera* larvae fed on diets with and without organic acids (based on ELISA).

slowly, and therefore, larval midguts and fecal matter were collected from more number of larvae (to make up the amount of protein per unit weight) as compared to those reared on the diets with low amounts of oxalic acid and malic acid, and the standard artificial diet. The amounts of Cry1Ac in the midguts of the *H. armigera* larvae were highest (2.585 ng g^{-1}) in insects reared on diets with 0.20% oxalic acid + 0.20% malic acid + Cry1Ac, followed by the insects reared on standard artificial diet (2.045 ng g^{-1}) with Cry1Ac. Lowest amounts of toxin protein (1.157 ng g^{-1}) were recorded in insects reared on diets with 0.44% oxalic acid + 0.94% malic acid + Cry1Ac. The amounts of Cry1Ac toxin were highest (0.916 ng g^{-1}) in the fecal matter of the insects reared on diets with 0.20% oxalic acid + 0.20% malic acid + Cry1Ac, followed by those (0.617 ng g^{-1}) reared on diets with 0.44% oxalic acid + 0.94% malic acid + Cry1Ac. Amounts of Cry1Ac toxin were lowest (0.505 ng g^{-1}) in fecal matter of insects reared on the standard artificial diet with Cry1Ac toxin (Fig. 1).

3.3. Effect of acid exudates on binding of Cry1Ac to BBMV in the midgut of Helicoverpa armigera larvae

The amounts of protein in the BBMV of the larvae fed on diets with Cry1Ac were greater as compared to the BBMV of larvae fed on diets without Cry1Ac, indicating the binding of Cry1Ac protein to the BBMV. The amounts of protein present in the BBMV ranged

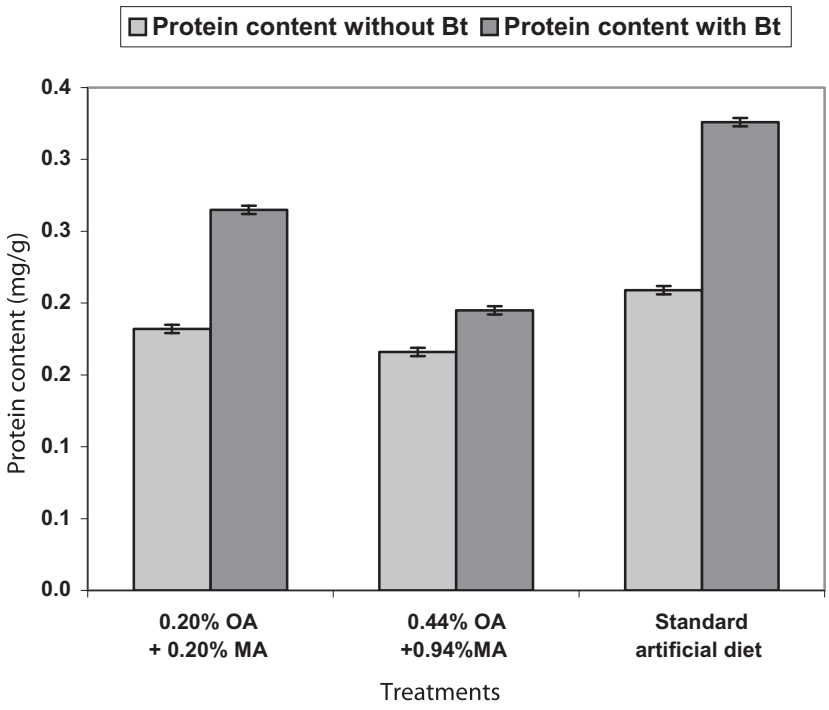

Fig. 2. Protein content of brush border membrane vesicles (BBMV) of *H. armigera* larvae fed on artificial diet with and without organic acids and *Bt* δ-endotoxin protein Cry1Ac.

from 0.195 to 0.326 mg g^{-1} (Fig. 2). The amounts of toxin protein present in the BBMV preparations from insects fed on standard artificial diet with Cry1Ac (27.3 ng ml^{-1} of diet) was highest (0.326 mg g^{-1}), followed by the insects fed on diets with 0.20% oxalic acid + 0.20% malic acid + Cry1Ac (0.265 mg g^{-1}), suggesting that organic acids resulted in a slight decrease in binding of the Cry1Ac toxin to the BBMV of *H. armigera* larvae.

4. Discussion

The insecticidal properties of the *Bt* δ-endotoxins depend on ingestion by the target insects. The antifeedent activity of acid exudates of chickpea leaves might interfere with the effectiveness of the *Bt* δ-endotoxins as lower amounts of food may be consumed by the larvae of *H. armigera* on chickpea. Oxalic and malic acids (Yoshida et al., 1995, 1997; Narayanamma et al., 2008) and *Bt* formulations (Zhang et al., 2000) also exhibit antifeedant activity towards the larvae of *H. armigera*, and therefore, might result in reduced consumption of food on diets with high amounts of organic acids. When the larvae utilize the digested food more efficiently, the *Bt* toxins present in the midgut bind to the BBMV forming pores, and increase the permeability of the midgut (Zhang et al., 2000). The amounts of oxalic and malic acids at concentrations present in leaves and pods of chickpea did not have a marked effect on accumulation of toxins in the midgut. But there was a positive correlation between larval weights and the amount of protein in the BBMV when the larvae were reared on the diets with a range of of oxalic and malic acids concentrations along with Cry1Ac ($r = 0.85$). The amounts of Cry1Ac decreased in the midgut of *H. armigra* larvae with an increase in amounts of organic acids in the diet, which was largely due to decrease in the weights of *H. armigera* larvae as a result of antifeedant and/or antibiotic effects of these compounds on *H. armigera*.

The Cry toxins from *Bt* exhibit adverse effects on survival and development of lepidopteran insects (Ludlum et al., 1991; Sivamani et al., 1992; Morris et al., 1994; Gibson et al., 1995; Wang et al., 1997; Ahmed et al., 1998; Zhang et al., 2000). The present studies indicated that both oxalic acid and malic acid increased the biological activity of Cry1Ac toxin towards *H. armigera* larvae through reduced larval weight, prolonged larval and pupal development, and reduced longevity and fecundity. Denolf (1999) suggested that reduced binding of *Bt* Cry toxins to the receptor sites is one of the mechanisms for resistance to δ-endotoxins. Reduction in food consumption of third-instar larvae of *Spodoptera litura* (Fab.) increased gradually both in pure culture and *Bt* formulations when exposed to increasing pH from 6–10 (Somasekhar and Krishnayya, 2004). Conversion of δ-endotoxins of *B. thuringiensis* to active toxins is mediated by trypsin in the insect midgut and bacterial proteases (Rukmini et al., 2000). The proteases also play an important role in influencing host range of the toxins, and in development of resistance to the toxin proteins. However, no significant differences have been observed between resistant and susceptible strains in binding of radioactively labeled Cry1C in the larvae of *P. xylostella*, suggesting that reduced conversion of Cry1C protoxin to toxin is a minor mechanism of resistance to Cry1C (Liu et al., 2000). Due to toxin binding to the midgut, the concentrations of Cry1Ac toxin in the midgut of *H. armigera* larvae were greater than those in the diet. Organic acids at amounts present in the chickpea leaves had no significant effect on the amounts of Cry1Ac toxin in the insect midgut, but very high concentrations reduced the amounts of Cry1Ac in the larval midgut. The concentrations of Cry1Ac protein binding to the BBMV were influenced by the weights of *H. armigera* larvae when reared on diets containing oxalic and malic acids.

Oxalic acid + malic acid at very high concentrations in combination with Cry1Ac resulted in a significant reduction in weights of *H. armigera* larvae. However, organic acids at concentrations present in chickpea leaves in combination with Cry1Ac slightly increased the percentage pupation, while Cry1Ac alone in the artificial diet resulted in a decrease in pupation. Increase in insect survival as a result of presence of organic acids in the artificial diet needs to be investigated further. There were no significant differences in fecundity of insects reared on diets with and without Cry1Ac in

the presence of organic acids. Presence of organic acids in the artificial diet increased the excretion of Cry1Ac protein in the fecal matter, and this might result in reduced efficacy of Cry1Ac in transgenic chickpea to *H. armigera*. Amounts of protein in the BBMV were greater in insects reared on diets with Cry1Ac, but presence of organic acids in the artificial diet reduced the amounts of protein the BBMV. Presence of organic acids in the artificial diet at very high amounts diet did not result in a significant reduction in biological activity of Cry1Ac, but slightly decreased the amount of protein in the BBMV, and also increased its excretion in the fecal matter. The organic acids in amounts present in the leaves did not have a significant influence on the biological activity of Cry1Ac, but it may be desirable to have high levels of expression of Cry1Ac toxin protein in chickpea for effective control of pod borer, *H. armigera.* The insecticidal properties of the *Bt* δ-endotoxins depend on ingestion by the target insects. The antifeedent activity of acid exudates in chickpea leaves might reduce the ingestion of *Bt* toxins, and hence, their effectiveness for the control of *H. armigera*. The organic acids in amounts present in chickpea leaves did not have a significant influence on the biological activity of the *Bt* toxin Cry1Ac towards the chickpea pod borer, but it may be desirable to have high levels of expression of Cry1Ac toxin protein in chickpea for effective control of pod borer, *H. armigera.*

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