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Aqueous washings of host plant surface influence the growth inhibitory effects of δ-endotoxins of *Bacillus thuringiensis* against *Helicoverpa armigera*

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Abstract

Biological activity of the bacterium *Bacillus thuringiensis* Berliner (Bt) against insect pests is influenced by the host plants. To understand the underlying mechanism of variation in biological activity of Bt on host plants, we studied the effect of chemicals from the surface of chickpea (*Cicer arietinum* L., Fabaceae) leaves (ICCC 37 and ICC 506EB), sorghum [*Sorghum bicolor* (L.) Moench, Poaceae] grain (ICSV 745 and IS 18698), pigeon pea [*Cajanus cajan* (L.) Millsp., Fabaceae] pods (ICPL 87 and ICPL 332WR), and cotton (*Gossypium hirsutum* L., Malvaceae) squares (RCH 2 and Bt RCH 2), on which *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) feeds under natural conditions. Surface chemicals extracted in water from host plant leaves were added to the standard artificial diet containing a commercial formulation of Bt or Cry1Ac. Data were recorded on larval and pupal weights, pupation, adult emergence, larval and pupal periods, adult longevity, and fecundity. Weights of *H. armigera* at 5 days after initiation of the experiment were significantly reduced on artificial diets containing Bt + pod washings of ICPL 87 and ICPL 332WR, grain washings of ICSV 745, or square washings of RCH 2, and Cry1Ac + leaf-surface washings of ICC 506EB. Pupal weights were lower on diets containing leaf-surface washings of ICCC 37 + Bt than on standard artificial diet. Larval periods were prolonged on diets containing Bt + leaf-surface washings of ICCC 37, pod washings of ICPL 87, and square washings of RCH 2, and on standard artificial diet + Cry1Ac. Pupation was significantly higher on standard artificial diet + Cry1Ac than on diets with Bt + grain washings of ICSV 745 and Cry1Ac + square washings of RCH 2 and Bt RCH 2. Adult emergence was lowest on diets with square washings of RCH 2 + Bt and grain washings of ICSV 745 + Cry1Ac. The results suggested that leaf-surface washings play an important role in biological activity of Bt/Cry1Ac against *H. armigera*.

Introduction

The legume pod borer, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is one of the most important pests of cotton, legumes, cereals, vegetables, and fruits (Manjunath et al., 1989; Fitt, 1991; Sharma, 2005). It incurs annual losses of about US$5 billion worldwide, with an additional cost of US$2 billion on pesticide applications (Sharma, 2005). Overdependence on insecticide use has not only resulted in development of insect resistance to insecticides, but also leaves harmful residues on food and food products. Therefore, transgenic plants expressing *Bacillus thuringiensis* Berliner (Bt) toxin genes have been deployed on a large scale for effective control of insect pests (Sharma et al., 2004; James, 2013). Toxin genes from Bt have been expressed in many crops such as transgenic cotton (*Gossypium hirsutum* L., Malvaceae) (James, 2013), chickpea (*Cicer arietinum* L., Fabaceae) (Acharjee et al., 2010), maize (Pilcher et al., 1997), and sorghum [*Sorghum bicolor* (L.) Moench, Poaceae] (Girijashankar et al., 2005). However, biological activity of Bt toxins varies across crops and cultivars (Paramasiva et al., 2014a). Therefore, it is important to understand the underlying mechanisms resulting in variation in the biological activity of Bt.

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Leaf surface forms the first site of contact in insect–plant interaction. Plant surfaces contain a number of chemicals that could either be beneficial or harmful to insect pests (Ogwaro, 1978; Chamarthi et al., 2011) and that determine the behavior of the insect pests, whether to accept or reject the host plant. Leaf, pod, and grain surface chemicals in chickpea, pigeon pea [*Cajanus cajan* (L.) Millsp., Fabaceae], and sorghum, respectively, confer resistance to insect pests (Green et al., 2003; Lahtinen et al., 2004; Valkama et al., 2005; Chamarthi et al., 2011; Narayanamma et al., 2013). Chickpea leaf-surface exudates contain malic, oxalic, acetic, citric, and fumaric acids, judaicin, maakiain, stilbene, and trypsin inhibitors, which affect insect growth and development (Sharma et al., 2008a; Narayanamma et al., 2013). Stilbene, isoquercetrin, quercetin, cedrol, isocedrol, sesquiterpenes, trypsin and amylase inhibitors, quercetin-3-methyl-ether, and rutin in pigeon pea influence the resistance/susceptibility to *H. armigera* (Green et al., 2003; Sharma et al., 2008b, 2009). Sorghum grain surface contains many insect-deterrent chemicals that have a major bearing on host plant resistance, including n-alkanes, esters, aldehydes, free alcohols, and fatty acids (Bianchi et al., 1979; Hwang et al., 2002). Cotton is rich in gossypol, heliocides, condensed tannins, anthocyanins, quercetin, and delphinidin (Sharma & Agarwal, 1982; Sharma et al., 1982, 2008b). Secondary metabolites on the surface or inside the plant tissue either decrease or increase the biological activity of Bt (Sivamani et al., 1992; Wang et al., 1997; Hagenbucher et al., 2013). Therefore, compatibility of the leaf-surface chemicals of host plants will have a direct bearing on the effectiveness of Bt formulations or transgenic plants for pest management.

To our knowledge, there are no reports on the influence of surface washings of various parts of the host plants, on which the insect feeds under natural conditions, on the biological activity of Bt. We studied the effect of water-soluble chemicals on the surface of various host plants on the biological activity of a commercial formulation of Bt, as well as the toxin protein Cry1Ac, against the most difficult to control pest, *H. armigera*.

Materials and methods

Insect culture

Larvae were reared on chickpea-based standard artificial diet in the insect rearing laboratory at ICRISAT, Patancheru, Telangana, India (Chitti Babu et al., 2014). Nearly 200 larvae were reared in a 250-ml plastic cup until the late second instar. The larvae were then reared individually in six-cell well plates (each cell 3.5 cm in diameter and 2 cm deep) to avoid cannibalism until pupation. The pupae were sterilized with 2% sodium hypochlorite solution and transferred to plastic jars containing vermiculite, and kept in groups of 50. Ten pairs of adults were released inside an oviposition cage (30 × 30 × 30 cm) and provided with 10% sucrose or honey solution on a cotton swab for feeding. Rough nappy liners were placed inside the cages as a substrate for egg laying. The nappy liners were removed and the eggs sterilized in 2% sodium hypochlorite solution. The dried liners were then transferred to plastic cups (250 ml capacity) with a 2–3 mm layer of artificial diet on the bottom and the sides. The nappy liners were removed after 3 days, and the newly emerged larvae were used for bioassays (Narayanamma et al., 2008).

Standard artificial diet for rearing *H. armigera* was prepared as follows. Chickpea flour (75 g), ascorbic acid (1.175 g), sorbic acid (0.75 g), methyl-4-hydroxy benzoate (1.25 g), aureomycin (2.875 g), and yeast (12 g) were placed in a bowl, and 112.5 ml warm water was mixed thoroughly using a blender. Then 1.0 ml of formaldehyde and 2.5 ml of vitamin stock solution [nicotinic acid (1.528 g), calcium pantothenate (1.528 g), riboflavin (0.764 g), thiamine hydrochloride (0.382 g), pyridoxine hydrochloride (0.382 g), folic acid (0.382 g), D-biotin (0.305 g), and cyanocobalamin (0.003 g) mixed in 500 ml of water] was added and mixed well. In a separate container, 4.325 g of agar-agar was added to 200 ml of water and boiled for 5 min. The agar-agar solution was added to the other diet ingredients and the content mixed thoroughly in a blender to get an even consistency. The diet was poured into small plastic cups and allowed to solidify under a laminar flow chamber for 1–2 h. Surface washings were added in the beginning of diet preparation, as a replacement of water (112.5 ml).

Bt formulation

A commercial formulation of Btk (Biolep; *B. thuringiensis* strain Z-52, serotype H-3a, 3b) was obtained from Biotech International (New Delhi, India). The Bt formulation used in the present studies is a water-dispersible powder, which acts on the host larvae through its parasporal crystal δ-endotoxins, and the bacterial spores. It contained 5–8% Bt δ-endotoxins, 5-8% Bt spores, 37–55% nutrient medium residues, 15–20% sodium chloride, 15–18%, fillers (Kaolin), and had a moisture content of 5–9%. Btk was used at the ED50 (effective dose to reduce the larval weight by 50%) concentration of 0.0125% (Paramasiva et al., 2014a). The Cry1Ac toxin (obtained from MP Carey, Case Western Reserve University, Department of Biochemistry, Cleveland, OH, USA) was used at the ED50 concentration of 5 ng per ml of diet (Sharma et al., 2008a; Paramasiva et al., 2014a).

Effect of water-soluble chemicals on the surface of various host plants on biological activity of Bt toxins against *Helicoverpa armigera*

The effect of surface chemicals of chickpea leaves (ICCC 37 and ICC 506EB, susceptible and resistant to *H. armigera*, respectively) (Sharma et al., 2005), sorghum grain (IS 18698 and ICSV 745, with high and no tannins, respectively, both resistant to sorghum midge, *Stenodiplosis sorghicola* Coquilett (Sharma, 1993), pigeon pea pods (ICPL 87 and ICPL 332WR, susceptible and resistant to *H. armigera*, respectively) (Sharma et al., 2009), and cotton squares (RCH 2 and Bt RCH 2, susceptible and resistant to *H. armigera*, respectively) (Dhillon & Sharma, 2010) on the biological activity of Bt or Cry1Ac on survival and development of *H. armigera* was studied. For this purpose, chickpea terminal shoots at 30 days after seedling emergence, pigeon pea pods at 13–17 days after flowering, sorghum grains at 15–20 days after flowering, and cotton squares at 40–45 days after planting were collected from field-grown plants, placed in an ice box, and brought to the laboratory. *Helicoverpa armigera* larvae feed on all of these under natural conditions. The fresh weights of the samples were recorded. About 500 g of the plant tissues were placed in 200 ml distilled water in a beaker and vortexed for 2 min. The tissues were then removed from the water and volume was made up to 188 ml (water required for preparation of 500 ml diet) and used to prepare the artificial diet. To study the effect of plant surface chemicals on the biological activity of Bt/Cry1Ac, the artificial diet containing plant surface chemicals was poured into two 250-ml beakers. In one beaker, the Bt/Cry1Ac was added to the final concentration, whereas the other was kept without toxin. Aliquots of 7 ml diet were poured into each cell of a six-cell well plate. Standard artificial diet with and without Bt/Cry1Ac was used as control. The neonate larvae were released individually in the cells. A total of 32 diets were prepared for the experiment [16 diets to test the biological activity of Bt formulation (eight diets containing plant tissue washings of eight genotypes with Bt and eight without Bt) and 16 diets to test biological activity of Cry1Ac]. There were three replications for each treatment (n = 3), and each replication had 10 larvae. Data were recorded on larval and pupal weights, survival, and larval and pupal development time, adult emergence, and fecundity.

Data on larval weights were recorded at 5 and 10 days after initiating the experiment (DAI) using a digital balance. For this purpose, the larvae were removed from the rearing cells, cleaned, weighed, and then placed back on the respective diets. The pupal weights were recorded 1 day after pupation. Pupae from each replication were placed in a 1-l plastic jar with vermiculite. The numbers of larvae pupated and adult emergence was recorded as a percentage of the number of larvae released in each replication. Larval and pupal periods and adult longevity were also recorded.

Fecundity of females emerging from insects reared on diets with different treatments was also recorded. The adults were collected with an aspirator from the jars and five pairs of adults emerging on the same day in a particular treatment were placed inside an oviposition cage. The adults were provided with nappy liners as a substrate for oviposition. The eggs laid on each day were counted, and the liners were changed daily. Data on egg laying were recorded for 3 days.

Statistical analysis

The experiment was conducted in a completely randomized design. Data were subjected to ANOVA and factorial analysis using GENSTAT v.10.1 (VSN International, Hemel Hempstead, UK). If an F-test indicated significant effects of treatments, genotypes, or their interaction ($\alpha = 0.05$), comparisons among treatments and genotypes were analyzed with Tukey's multiple comparison test ($\alpha = 0.05$).

Results

Effect of water-soluble chemicals on the surface of various host plants on biological activity of Bt formulation

Larval and pupal weights. The weights of *H. armigera* larvae differed across treatments (with vs. without Bt: $F_{1,16} = 78.08$, $P<0.001$) and host genotypes ($F_{8,16} = 4.47$, $P = 0.001$) at 5 DAI. The interaction effects were nonsignificant. Larvae were significantly heavier when reared on diets containing leaf-surface washings of the chickpea genotype ICCC 37 (10.01 mg per larva) and on the standard artificial diet (10.45 mg per larva) without Bt than on diets containing leaf-surface washings of chickpea genotype ICC 506EB, grain washings of sorghum genotypes IS 18698 and ICSV 745, pod washings of pigeon pea genotypes ICPL 87 and ICPL 332WR, and square washings of cotton genotypes RCH 2 and Bt RCH 2 (Figure 1A). When reared on diets with surface washings and Bt, larvae were significantly lighter in diets with pod washings of ICPL 87 (4.49 mg per larva) and square washings of RCH 2 (4.87 mg per larva) (Figure 1A).

At 10 DAI, the larval weights differed between treatments (with vs. without Bt) ($F_{1,16} = 126.18$), genotypes ($F_{8,16} = 15.9$), and their interaction ($F_{8,16} = 3.65$, all $P<0.01$). Larval weights were significantly reduced on diets containing pod washings of ICPL 87 + Bt (79.9 mg per larva) and ICPL 332WR + Bt (85.9 mg per larva), and leaf-

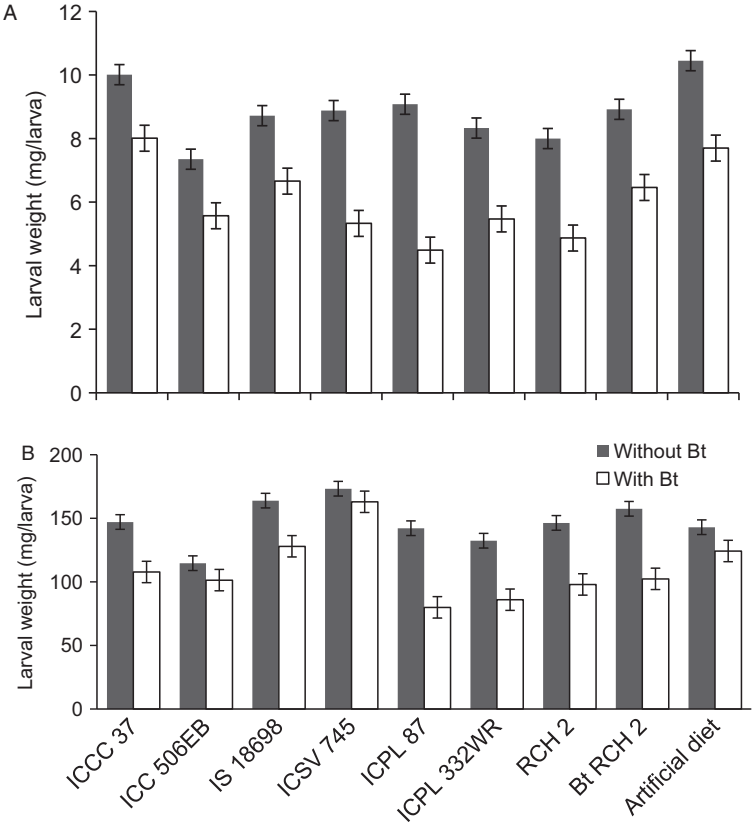

Figure 1 Mean (± SE) weights of *Helicoverpa armigera* larvae (mg per larva) at (A) 5 and (B) 10 days after feeding on artificial diets with water-soluble chemicals from the surface of chickpea leaves (ICCC 37 and ICC 506EB), sorghum grain (IS 18698 and ICSV 745), pigeon pea pods (ICPL 87 and ICPL 332WR), or cotton squares (RCH 2 and Bt RCH 2) without and with Bt, or on standard artificial diet as a control.

surface washings of ICC 506EB + Bt (114.6 mg per larva) (Figure 1B). Weights of *H. armigera* larvae increased in insects reared on diets containing grain-surface washings of ICSV 745 + Bt (162.9 mg per larva).

Pupal weights did not differ across treatments ($F_{1,16} = 2.13$, $P = 0.15$. The genotype and interactions effects were non-significant (data not shown). Pupal weights were lowest in insects reared on diets with leaf-surface washings of ICCC 37 + Bt (246.2 mg), and highest on diets with grain-surface washings of ICSV 745 + Bt (312.5 mg).

Larval and pupal periods. Larval period differed significantly across Bt treatments and genotypes, interaction effects were non-significant (Table 1). Larval period was somewhat (but not significantly; Table 1) longer in insects reared on diets with leaf-surface washings of ICCC 37 + Bt (17.5 days), pod washings of ICPL 87 + Bt (17.6 days), and square washings of RCH 2 + Bt (17.3 days) as compared to those reared on the standard artificial diet + Bt (15.5 days).

Pupal periods did not differ significantly among the treatments. Also, genotypic and interaction effects were non-significant. Pupal development was slightly – but not significantly – delayed when reared on diets with square washings of Bt RCH 2 + Bt (12.6 days), and on diets with grain surface washings of ICSV 745 and standard artificial diet (12.2 days each) compared to the other treatments (Table 1).

Pupation and adult emergence. The percentage pupation differed across treatments ($F_{1,16} = 26.56$, $P<0.001$) and genotypes ($F_{8,16} = 2.85$, $P = 0.016$). The interaction effects were non-significant. Pupation rate was highest in insects reared on diets containing leaf-surface washings of ICCC 37 (76.7%), followed by the insects reared on diets containing pod washings of ICPL 87 (76.1%) and the standard artificial diet (73.3%) without Bt (Figure 2). Among the diets with surface chemicals + Bt, pupation rate was highest in insects reared on diets containing leaf-surface washings of ICCC 37 (66.7%), followed by those reared on diets with grain washings of IS 18698, square washings of Bt RCH 2, and the standard artificial diet (60% each). Pupation rate was lowest in insects reared on the diets with grain washings of ICSV 745 + Bt (33%).

Adult emergence also differed across treatments ($F_{1,16} = 5.6$, $P = 0.024$). The genotypic and interaction effects were non-significant. Among the diets without Bt,

Table 1 Mean (± SE) larval and pupal periods (days) of *Helicoverpa armigera* reared on artificial diets with water-soluble chemicals from the surface of chickpea leaves, sorghum grain, pigeon pea pods, and cotton squares without and with 0.0125% Bt, or on standard artificial diet as a control

	Larval period		Pupal period	
Genotype	Without Bt	With Bt	Without Bt	With Bt
Chickpea				
ICCC 37	16.98 ± 0.2	17.53 ± 0.5	11.87 ± 0.1	11.72 ± 0.4
ICC 506EB	16.18 ± 0.3	16.03 ± 0.2	11.58 ± 0.2	11.64 ± 0.8
Sorghum				
IS 18698	15.85 ± 0.5	16.62 ± 0.4	11.38 ± 0.3	11.61 ± 0.2
ICSV 745	15.50 ± 0.3	15.58 ± 0.4	11.28 ± 0.2	12.22 ± 0.2
Pigeon pea				
ICPL 87	16.23 ± 0.4	17.57 ± 0.7	11.78 ± 0.1	11.33 ± 0.3
ICPL 332WR	15.33 ± 0.3	16.80 ± 0.4	12.11 ± 0.5	11.94 ± 0.7
Standard artificial diet	16.29 ± 0.6	15.48 ± 0.2	11.72 ± 0.4	12.22 ± 0.8

		Larval period		Pupal period	
	d.f.	F	P	F	P
Bt concentrations	1,16	7.87	0.008	0.63	0.43
Genotypes	8,16	4.42	<0.01	0.38	0.92
Bt concentrations*genotypes	8.16	1.87	0.098	0.30	0.96

Means within columns were not significantly different (Tukey's multiple comparison test: P≥0.05).

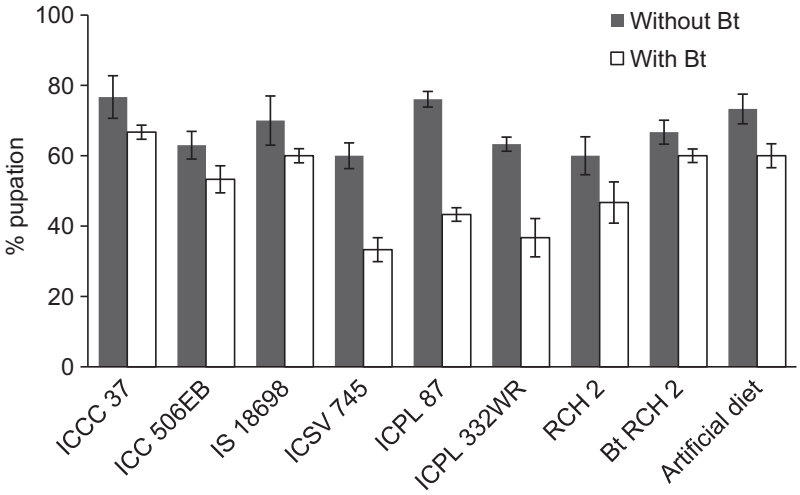

Figure 2 Mean (± SE) percentage pupation of *Helicoverpa armigera* on artificial diets with water-soluble chemicals from the surface of chickpea leaves (ICCC 37 and ICC 506EB), sorghum grain (IS 18698 and ICSV 745), pigeon pea pod (ICPL 87 and ICPL 332WR), or cotton squares (RCH 2 and Bt RCH 2) without and with Bt, or on standard artificial diet as a control.

adult emergence was highest in insects reared on diets with leaf-surface washings of ICCC 37 and pod washings of ICC 506EB (69% each), followed by those reared on diets with square washings of Bt RCH 2 (64%) (Figure 3). Adult emergence was lowest in insects reared on diets with grain washings of IS 18698 (48.3%) and the standard artificial diet (49%). Among the insects reared on diets with Bt, adult emergence was lowest in insects reared on diets with square washings of RCH 2 (36.7%), followed by those reared on the standard artificial diet (41%) or diets containing either pod washings of ICPL 87 (41%) or leaf-surface washings of ICC 506EB (42%). Adult emergence was highest in insects reared on diets with pod-surface washings of ICPL 332WR (58.7%).

Adult longevity and fecundity. Female longevity differed across treatments (Table 2). *Helicoverpa armigera* females survived for a longer period in insects reared on diets containing leaf-surface washings of ICC 506EB (10.5 days) than those reared on the standard artificial

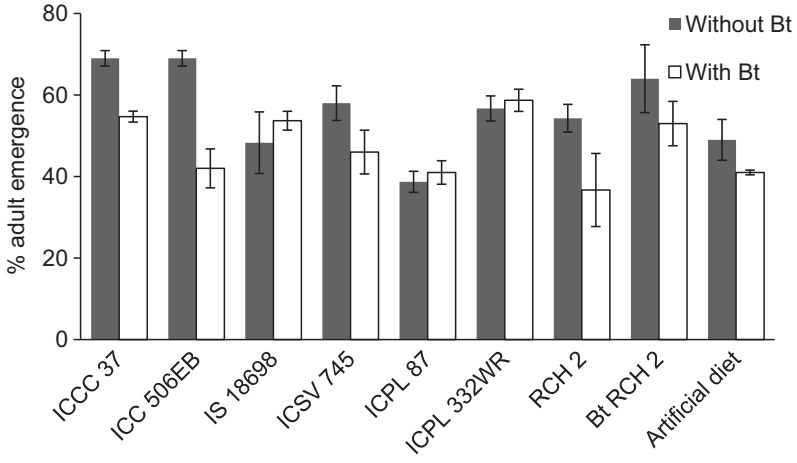

Figure 3 Mean (± SE) percentage adult emergence of *Helicoverpa armigera* on artificial diets with water-soluble chemicals from the surface of chickpea leaves (ICCC 37 and ICC 506EB), sorghum grain (IS 18698 and ICSV 745), pigeon pea pod (ICPL 87 and ICPL 332WR), or cotton squares (RCH 2 and Bt RCH 2) without and with Bt, or on standard artificial diet as a control.

Table 2 Mean (± SE) adult longevity (days) and fecundity (no. eggs per female) of *Helicoverpa armigera* reared on artificial diet with water-soluble chemicals from the surface of chickpea leaves, sorghum grain, pigeon pea pods, or cotton squares without and with 0.0125% Bt, or on standard artificial diet as a control

	Female longevity		Male longevity		Fecundity	
Genotype	Without Bt	With Bt	Without Bt	With Bt	Without Bt	With Bt
Chickpea						
ICCC 37	9.83 ± 0.9	9.00 ± 0.3	9.33 ± 0.7	9.22 ± 0.3	341.7 ± 30.1Bbcde	325.0 ± 14.4ABbc*
ICC 506EB	10.53 ± 0.0	8.33 ± 0.0*	9.80 ± 0.3	8.22 ± 0.0	265.0 ± 21.8Aa	300.0 ± 17.8Aabc*
Sorghum						
IS 18698	9.44 ± 0.3	9.00 ± 0.0	9.72 ± 0.3	9.00 ± 0.0	383.3 ± 16.6Ce	350.0 ± 16.7Bcde*
ICSV 745	9.44 ± 0.6	8.22 ± 0.1	9.50 ± 0.7	8.00 ± 0.1*	333.3 ± 16.7Bbcde	316.7 ± 28.9ABbc
Pigeon pea						
ICPL 87	9.56 ± 0.3	9.00 ± 0.3	9.22 ± 0.0	8.33 ± 0.3	325.0 ± 14.4Bbc	333.3 ± 16.7Bbcde
ICPL 332WR	9.78 ± 0.6	8.33 ± 0.3	9.33 ± 0.1	9.33 ± 0.3	333.3 ± 16.6Bbcde	291.7 ± 22.1Aab*
Cotton						
RCH 2	9.89 ± 0.9	8.67 ± 0.4	9.45 ± 0.3	9.33 ± 0.4	378.3 ± 14.4Cde	308.3 ± 11.7Aabc*
Bt RCH 2	9.22 ± 0.3	8.00 ± 0.2	9.45 ± 0.6	8.33 ± 0.2	350.0 ± 14.8BCcde	296.7 ± 8.3Aab*
Standard artificial diet	8.67 ± 0.6	8.33 ± 0.8	9.22 ± 0.6	8.22 ± 0.8*	378.3 ± 14.8Cde	296.7 ± 11.7Aab*

		Female longevity		Male longevity		Fecundity	
	d.f.	F	P	F	P	F	P
Bt concentrations	1,16	19.11	<0.001	7.78	0.009	13.33	<0.001
Genotypes	8,16	0.91	0.52	0.46	0.88	3.45	0.021
Bt concentrations*genotypes	8.16	0.66	0.73	0.50	0.85	2.26	0.046

Means within a column followed by the same capital (genotype) or lower case (interaction) letter are not significantly different (Tukey's multiple comparison test: $P>0.05$). Asterisks indicate a significant difference between diets with vs. without Bt within a genotype (F-test: $P<0.05$).

diet (8.7 days) without Bt. In diets containing Bt, female longevity was higher in insects reared on diets containing leaf-surface washings of ICCC 37, grain washings of IS 18698, and the pod washings of ICPL 87 (9 days each), than on the diet containing square washings of Bt RCH 2 (8.0 days) and grain washings of ICSV 745 (8.2 days).

Male longevity also differed across treatments. However, the genotypic and interaction effects were non-significant (Table 2). In diets with Bt, males lived longer when reared on diets containing pod washings of ICPL 332WR (9.3 days), square washings of RCH 2 (9.3 days), and leaf-surface washings of ICCC 37 (9.2 days), than

those reared on diets containing grain washings of ICSV 745 (8.0 days).

There were differences in insect fecundity across treatments and genotypes. The interaction effects were also significant (Table 2). Egg laying was highest in insects reared on diets containing grain washings of IS 18698 (383.3 eggs per female), square washings of RCH2 (378.3 eggs per female), and the standard artificial diet (378.3 eggs per female). Egg laying was lowest in insects reared on diets with leaf-surface washings of ICC 506EB (265 eggs per female). Egg laying was significantly reduced in *H. armigera* reared on diets with pod washings of ICPL 332WR + Bt and standard artificial diet + Bt (291.7 and 296.7 eggs per female, respectively), but increased in insects reared on diets containing grain washings of IS 18698 (350 eggs per female).

Effect of water-soluble chemicals on the surface of various host plants on biological activity of Bt toxin Cry1Ac

Larval and pupal weights. Larval weights of *H. armigera* differed across treatments (with and without Cry 1Ac) ($F_{1,16}$ = 119.31, P<0.001) and the genotypes ($F_{8,16}$ = 3.53, P = 0.004) at 5 DAI. The interaction effects were non-significant. Among the diets without Cry1Ac, larval weights were highest in insects reared on standard artificial diet (9.9 mg), followed by those reared on diets with pod washings of ICPL 87 (9.6 mg) or grain washing of IS 18698 (9.5 mg) (Figure 4A). Larval weights were lowest in insects reared on diets containing leaf-surface washings of ICC 506EB (6.3 mg). Among the diets with Cry1Ac, larval weights were highest in insects reared on diets containing grain washings of IS 18698 (6.8 mg) and lowest on diets with leaf-surface washings of ICC 506EB (4.7 mg) or the standard artificial diet (4.8 mg) (Figure 4A).

At 10 DAI, the larval weights differed between the treatments ($F_{1,16}$ = 146.12, P<0.001) and the genotypes ($F_{8,16}$ = 4.59, P<0.001), but the interaction effects were non-significant. Larval weights were highest in insects reared on the standard artificial diet (307.1 mg), followed by those reared on diets containing square washings of RCH 2 (305.8 mg), grain washing of ICSV 745 (305.3 mg) or IS 18698 (304.4 mg), and the pod washings of ICPL 87

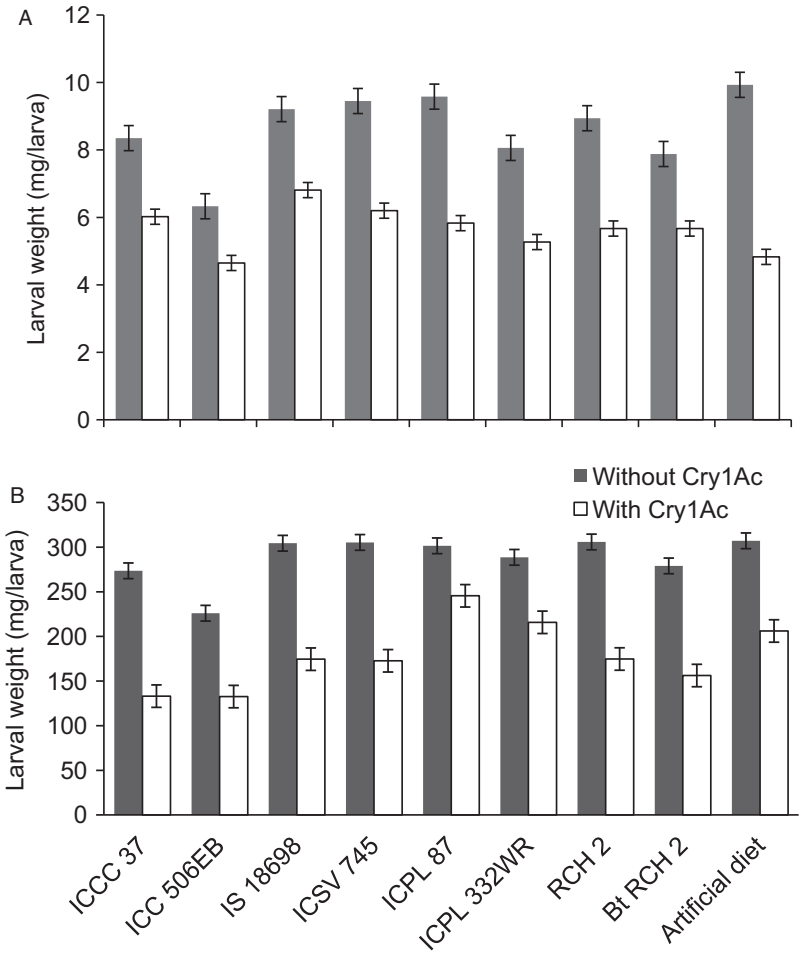

Figure 4 Mean (± SE) weights of *Helicoverpa armigera* larvae (mg per larva) at (A) 5 and (B) 10 days after feeding on artificial diets with water-soluble chemicals from the surface of chickpea leaves (ICCC 37 and ICC 506EB), sorghum grain (IS 18698 and ICSV 745), pigeon pea pod (ICPL 87 and ICPL 332WR), or cotton squares (RCH 2 and Bt RCH 2) without and with Cry1Ac, or on standard artificial diet as a control.

(301.5 mg) without Cry1Ac (Figure 4B). Larval weights were lowest in insects reared on diets containing leaf-surface washings of ICC 506EB (226 mg). Among the diets containing Cry1Ac, larval weights were highest in insects reared on diets with pod washings of ICPL 87 (245.5 mg), and lowest in insects reared on diets with leaf-surface washings of ICC 506EB (132.6 mg) and ICCC 37 (133.1 mg) (Figure 4B).

The interaction effects for pupal weights were significant ($F_{8,16} = 2.91$, $P = 0.014$). The pupal weights of insects reared on diets without Cry1Ac were significantly lower on diets containing square washings of Bt RCH 2 (248.9 mg) than in the insects reared on standard artificial diet (319.2 mg) (data not shown). In diets containing Cry1Ac, pupal weights were higher in insects reared on diets with square washings of Bt RCH 2 (314.1 mg) and leaf-surface washings of ICCC 37 (312.5 mg) than those reared on diets with grain-surface washings of ICSV 745 (288.9 mg).

Larval and pupal periods. Larval periods differed across the treatments, genotypes, and their interaction (Table 3). In diets without Cry1Ac, larval period was longer in insects reared on diets with pod washings of ICPL 87 (17.9 days) than on diets with leaf-surface washings of ICCC 37 (15.8 days). Larval period was prolonged in insects reared on the standard artificial diet + Cry1Ac (19.6 days), and on diets with square washings of RCH2 + Cry1Ac (18.53 days) compared to insects reared on diets with pod washings of ICPL 332WR + Cry1Ac (16.1 days).

The interaction effects were significant for pupal period (Table 3). Pupal period increased marginally in insects reared on diets containing leaf-surface washings of ICC 506EB (12 days) compared to those reared on the standard artificial diet (11.3 days). Pupal period was longest in insects reared on diets containing leaf-surface washings of ICCC 37 (12.3 days) and shortest in insects reared on diets containing square washings of Bt RCH 2 (3.7 days), followed by the insects reared on diets with grain washings of ICSV 745 (7.8 days).

Pupation and adult emergence. Percentage pupation differed across treatments ($F_{1,16} = 51.02$, $P<0.001$) and genotypes ($F_{8,16} = 3.47$, $P = 0.005$). However, the interaction effects were not significant ($F_{8,16} = 1.80$, $P = 0.11$). Pupation rate was highest in insects reared on

Table 3 Mean (± SE) larval and pupal periods (days) of *Helicoverpa armigera* on artificial diet with water-soluble chemicals from the surface of chickpea leaves, sorghum grain, pigeon pea pods, or cotton squares without and with 5 ng ml^{-1} Cry1Ac, or on standard artificial diet as a control

	Larval period		Pupal period	
Genotypes	Without Cry1Ac	With Cry1Ac	Without Cry1Ac	With Cry1Ac
Chickpea				
ICCC 37	15.79 ± 0.0a	17.19 ± 0.1bcde*	11.19 ± 0.1bc	12.33 ± 0.9c
ICC 506EB	16.43 ± 0.4abc	17.07 ± 0.9abcd*	12.00 ± 0.2c	11.67 ± 0.2bc
Sorghum				
IS 18698	16.57 ± 0.6abcd	16.97 ± 0.1abcd	11.67 ± 0.9bc	11.50 ± 0.9bc
ICSV 745	16.12 ± 0.1ab	16.53 ± 0.3abcd	11.33 ± 0.5bc	7.83 ± 0.5b*
Pigeon pea				
ICPL 87	17.85 ± 0.7de	15.92 ± 0.9ab*	11.31 ± 0.7bc	10.67 ± 0.1bc
ICPL 332WR	16.35 ± 0.6abc	16.08 ± 0.5ab	11.56 ± 0.3bc	11.67 ± 0.2bc
Cotton				
RCH 2	16.40 ± 0.1abc	18.53 ± 0.6ef	11.08 ± 0.6bc	11.83 ± 0.3c
Bt RCH 2	16.89 ± 0.3abcd	17.61 ± 0.9cde*	11.89 ± 0.2c	3.67 ± 0.6a*
Standard artificial diet	16.96 ± 0.1abcd	19.56 ± 0.2f*	11.28 ± 0.3bc	11.39 ± 0.5bc

		Larval period		Pupal period	
	d.f.	F	P	F	P
Bt concentrations	1,16	8.70	0.006	3.42	0.073
Genotypes	8,16	3.48	0.005	1.94	0.086
Bt concentrations*genotypes	8,16	3.74	0.003	2.31	0.043

Means within a column followed by the same letter are not significantly different (Tukey's multiple comparison test: $P>0.05$). Asterisks indicate a significant difference between diets with vs. without Cry1Ac within a genotype (F-test: $P<0.05$).

standard artificial diet (86.7%), followed by those reared on the diets containing pod washings of ICPL 87 (80%) without Cry1Ac (Figure 5). Pupation rate was lowest in insects reared on diets with pod washings of ICPL 332WR (43.3%). In diets containing Cry1Ac, pupation was significantly reduced in insects reared on diets with square washings of RCH 2 and Bt RCH 2 (30%) as compared to those reared on the standard artificial diet (56.7%).

Adult emergence did not differ across treatments ($F_{1,16} = 2.30$, $P = 0.14$), genotypes ($F_{8,16} = 0.60$, $P = 0.76$). The interaction effects were also non-significant ($F_{8,16} = 0.43$, $P = 0.89$). Adult emergence was lowest in insects reared on diets with grain washings of IS 18698 (39.2%), followed by those reared on diets with square washings of Bt RCH 2 (42.2%), and pod washings of ICPL 332WR (45.0%) and ICPL 87 (45.3%) compared to that on the standard artificial diets (72.7%) without Cry1Ac (Figure 6). In diets containing Cry1Ac, adult emergence was highest in insects reared on diets with square washings of RCH 2 (57.8%) and lowest on diets with grain washings of ICSV 745 (27.8%).

Adult longevity and fecundity. Female longevity differed across treatments. The genotypic and interaction effects were non-significant (Table 4). *Helicoverpa armigera* females lived longer when reared on diets with leaf-surface washings of ICCC 37 and on the standard artificial diet (8.8 days) than those reared on diets with grain washings of IS 18698 (4.0 days) without Cry1Ac. On diets containing Cry1Ac, the females lived longer when reared on the standard artificial diet (6.9 days) and the diets with leaf-surface washings of ICC 506EB (6.7 days), than when reared on diets with pod washings of ICPL 87 (1.7 days). Male longevity differed significantly across treatments. The genotypic and interaction effects were non-significant. In diets without Cry1Ac, males lived longer when reared on diets with grain washings of ICSV 745 (10.5 days) and leaf-surface washings of ICC 506EB

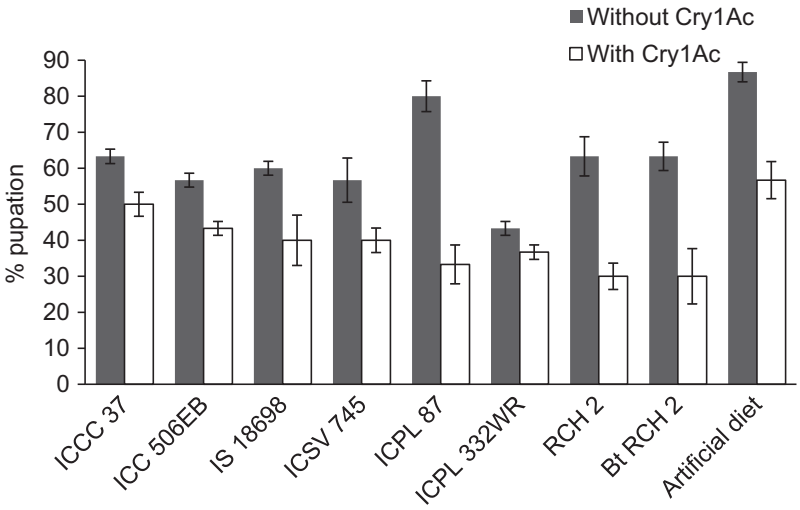

Figure 5 Mean (± SE) percentage pupation of *Helicoverpa armigera* on artificial diets with water-soluble chemicals from the surface of chickpea leaves (ICCC 37 and ICC 506EB), sorghum grain (IS 18698 and ICSV 745), pigeon pea pod (ICPL 87 and ICPL 332WR), or cotton squares (RCH 2 and Bt RCH 2) without and with Cry1Ac, or on standard artificial diet as a control.

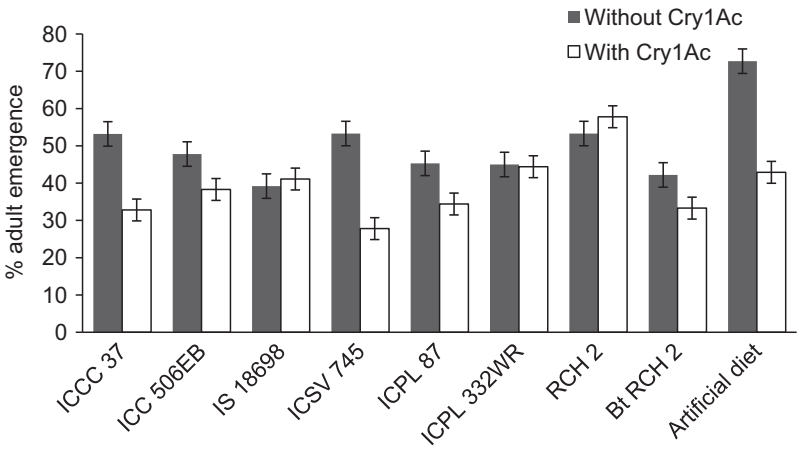

Figure 6 Mean (± SE) percentage adult emergence of *Helicoverpa armigera* on artificial diets with water-soluble chemicals from the surface of chickpea leaves (ICCC 37 and ICC 506EB), sorghum grain (IS 18698 and ICSV 745), pigeon pea pod (ICPL 87 and ICPL 332WR), or cotton squares (RCH 2 and Bt RCH 2) without and with Cry1Ac, or on standard artificial diet as a control.

Table 4 Mean (± SE) female and male longevity (days) and fecundity (no. eggs per female) of *Helicoverpa armigera* reared on artificial diet with water-soluble chemicals from the surface of chickpea leaves, sorghum grain, pigeon pea pods, or cotton squares without and with 5 ng ml^{-1} Cry1Ac, or on standard artificial diet as a control

	Female longevity		Male longevity		Fecundity	
Genotypes	Without Cry1Ac	With Cry1Ac	Without Cry1Ac	With Cry1Ac	Without Cry1Ac	With Cry1Ac
Chickpea						
ICCC 37	8.83 ± 0.3	6.00 ± 0.3*	8.67 ± 0.3	7.67 ± 0.0	183.3 ± 92.8d	66.7 ± 14.4a*
ICC 506EB	8.50 ± 0.3	6.67 ± 0.3*	10.00 ± 0.2	5.83 ± 0.6*	333.3 ± 44.1a	16.7 ± 16.7c*
Sorghum						
IS 18698	4.00 ± 0.7	3.67 ± 0.2	5.00 ± 0.3	2.83 ± 0.3*	333.3 ± 44.1a	0.0 ± 0.2d*
ICSV 745	7.00 ± 0.0	4.67 ± 0.6*	10.50 ± 0.7	6.00 ± 0.7*	333.3 ± 16.7a	41.7 ± 2.8b*
Pigeon pea						
ICPL 87	7.67 ± 0.7	1.67 ± 0.3*	7.89 ± 0.6	4.67 ± 0.3*	300.0 ± 28.9b	16.7 ± 0.9c*
ICPL 332WR	8.00 ± 0.0	5.00 ± 0.3*	5.33 ± 0.6	10.00 ± 0.0*	275.0 ± 14.4b	16.7 ± 0.7c*
Cotton						
RCH 2	8.00 ± 0.7	4.83 ± 0.2*	8.92 ± 0.3	2.00 ± 0.3*	258.3 ± 86.6bc	25.0 ± 1.8c*
Bt RCH 2	5.33 ± 0.6	2.33 ± 0.7*	9.17 ± 0.6	3.00 ± 0.2*	250.0 ± 30.1c	20.0 ± 1.3c*
Standard artificial diet	8.78 ± 0.3	6.89 ± 0.7*	8.27 ± 0.9	3.00 ± 0.3*	366.7 ± 16.7a	50.0 ± 3.5b*

		Female longevity		Male longevity		Fecundity	
	d.f.	F	P	F	P	F	P
Bt concentrations	1,16	7.37	0.01	14.38	<0.001	208.25	<0.001
Genotypes	8,16	1.09	0.40	1.40	0.23	0.95	0.49
Bt concentrations*genotypes	8,16	0.26	0.98	1.91	0.092	7.52	0.008

Means within a column followed by the same letter are not significantly different (Tukey's multiple comparison test: P>0.05). Asterisks indicate a significant difference between diets with vs. without Cry1Ac within a genotype (F-test: P<0.05).

(10 days) than when reared on diets with pod-surface washings of ICPL 332WR (5.33 days). In diets with Cry1Ac, longevity was highest in insects reared on diets with pod-surface washings of ICPL 332WR (10 days) and lowest in insect reared on diets with square washings of RCH 2 (2 days).

Fecundity differed among treatments (Table 4). Fecundity was significantly reduced in insects reared on diets with leaf-surface washings of ICCC 37 (183.3 eggs per female) compared to those reared on the standard artificial diet without Cry1Ac (366.7 eggs per female). On diets with Cry1Ac, fecundity was highest in insects reared on diets with leaf-surface washings of ICCC 37 (66.7 eggs per female), and lowest on diets with leaf-surface washings of ICC 506EB and pod washings of ICPL 87 and ICPL 332WR (16.7 eggs per female).

Discussion

Leaf, flower, pod, or grain surface is the first site for an encounter between insect pests and their host plants. Chemicals on the surface of plant parts determine host acceptability and suitability of the host plant for development and survival of the insect pest (Chamarthi et al., 2011; Hilker & Meiners, 2011; Silva et al., 2013). Amounts of malic and oxalic acids in leaf exudates in chickpea and pH alter the expression of resistance to *H. armigera* (Bhagwat et al., 1995; Yoshida et al., 1997; Narayanamma et al., 2008). Lyophilized leaves and pods of *H. armigera*-resistant genotypes of chickpea, when added to the artificial diet, reduced the larval and pupal weights and prolonged larval and pupal periods (Narayanamma et al., 2008). Leaf-surface chemicals also influence the biological activity of biopesticides and synthetic chemicals (Osier et al., 1996; Zhang et al., 2013). Although transgenic plants expressing Bt toxin genes have been successfully deployed against lepidopteran pests, including *H. armigera*, the compatibility of Bt toxins with leaf-surface chemicals will have a major bearing on the biological activity of Bt toxins (Paramasiva et al., 2014a).

Water-soluble chemicals on the leaves, pods, grains, and squares of chickpea, pigeon pea, sorghum, and cotton, respectively, showed a significant alteration in the biological activity of Bt and/or Cry1Ac. Our results showed that water-soluble chemicals on the pod surfaces of pigeon pea genotypes ICPL 87 and ICPL 332WR, on leaf surfaces of chickpea genotypes ICC 506EB and ICCC 37, and on squares of cotton

genotype RCH 2 significantly altered the biological activity of Bt. The water-soluble pod-surface chemicals of ICPL 87 and ICPL 332WR increased the toxicity of Bt against *H. armigera* larvae, but reduced the toxicity of Cry1Ac at 10 DAI, whereas water-soluble grain-surface chemicals of sorghum genotypes ICSV 745 and IS 18698 decreased the efficacy of Bt. Furthermore, water-soluble square-surface chemicals of Bt RCH 2 + Cry1Ac significantly reduced the pupal weights of *H. armigera*. A differential effect of surface washings from host genotypes on Bt and Cry1Ac toxicity can be attributed to differences in chemical composition of the surface washings. The results suggested that compatibility of the host plants is an important factor for Bt and/or Cry1Ac toxicity. It has been reported that various chemical constituents of host plants adversely affect the growth and development of *H. armigera* (Narayanamma et al., 2013). Chickpea genotypes with resistance to *H. armigera* are highly compatible with Bt sprays for controlling this pest (Devi et al., 2011). Furthermore, pod-surface chemicals of pigeon pea (quercetin-3-methyl-ether, rutin, and stilbene) affect the genotypic suitability to *H. armigera* (Green et al., 2003; Sharma et al., 2009). Leaf-surface chemicals of birch have also been reported to exercise a negative effect on larval growth and pupal weight, and to prolong the larval period in *Epirrita autumnata* (Borkhausen) (Lahtinen et al., 2004; Valkama et al., 2005).

Insects reared on Bt-treated diets had a longer larval period than insects reared on the respective diets without Bt. The prolonged larval period will lead to longer exposure of the larvae to natural enemies. The Bt and/or Cry1Ac in combination with leaf, pod, grain, and square surface washings of chickpea, pigeon pea, sorghum, and cotton, respectively, significantly reduced pupation and adult emergence of *H. armigera*. The grain-surface washings of ICSV 745 and pod-surface washings of ICPL 332WR in combination with Bt reduced pupation rates significantly, whereas square washings of RCH 2 reduced the adult emergence. Pupation rate was highest in insects reared on diets containing leaf-surface washings of ICCC 37, followed by the insects reared on diets containing pod washings of ICPL 87 and the standard artificial diet without Bt, suggesting that surface chemicals on susceptible chickpea and pigeon pea genotypes improve the survival and development of *H. armigera*. The pod-surface washings of ICPL 87 and ICPL 332WR, and square washings of RCH 2 and Bt RCH 2 in combination with Cry1Ac resulted in the largest reduction in percentage pupation. Reduced biological activity of Bt in artificial diets containing leaf or pod powder of chickpea genotypes might be due to interaction of Bt proteins with biochemical constituents in chickpea (Devi et al., 2013, 2014), in addition to reduced feeding because of the antifeedant effect of acid exudates toward *H. armigera* (Yoshida et al., 1997; Zhang et al., 2000).

Insect fecundity is an important factor determining the resistance and/or susceptibility of a host plant. Higher fecundity will lead to increased rates of population growth, thereby resulting in greater infestation and crop loss. Leaf-surface washings of ICCC 37 and ICC 506EB, grain washings of IS 18698 and ICSV 745, pod-surface washings of ICPL 332WR and ICPL 87, and square washings of RCH 2 resulted in increased fecundity, and thus reduced efficacy of Bt. However, leaf-surface washings of ICC 506EB and pod washings of ICPL 87 and ICPL 332WR, in combination with Cry1Ac, significantly reduced egg-laying by *H. armigera*, thereby increasing the biological activity of Cry1Ac. Leaf extracts of *Acacia arabica* (Lam.) Willd., *Annona squamosa* L., *Datura stramonium* L., *Eucalyptus globulus* Labill., *Lantana camara* L., and *Psora leapinnata* L. are effective against *Spodoptera litura* Fabricius larvae, causing significant mortality when used alone, whereas extracts of *Nicotiana tabacum* L., *A. arabica* seed, *A. squamosa* seed, and *D. stramonium* seed are more effective when fortified with Bt (Rajguru & Sharma, 2012). The differences in fecundity and the differential effect on the biological activity of Bt/Cry1Ac could be attributed to the differences in chemical composition of the washings. Plant surface chemicals have been reported to play an important role in insect oviposition (Chapman & Bernays, 1989; Green et al., 2003; Chamarthi et al., 2011; Hilker & Meiners, 2011; Silva et al., 2013). The present study suggested that surface chemicals play an important role in biological activity of Bt and/or Cry1Ac against *H. armigera*. This information will be valuable for the use of Bt formulations or deployment of Bt toxin proteins in transgenic plants for pest management. In addition, microbes on the plant surface also have a bearing on the biological activity of Bt/Cry1Ac (Stadler & Muller, 1996; Kutschera et al., 2002; Paramasiva et al., 2014b). There is a need for in-depth studies on the role of leaf-surface chemicals on biological activity of Bt directly and indirectly through microbial community on the leaf surface.

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