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Effect of *CryIIa* transgenic chickpeas to *Helicoverpa armigera* larval parasitoid, *Campoletis chlorideae*

Shaila O, HC Sharma, SP Sharma, and T Ramesh Babu

Abstract
The current experiments were conducted to evaluate the effect of transgenic chickpea lines expressing *CryIIa* to *C. chlorideae* under laboratory conditions. There was a significant reduction in cocoon formation and adult emergence of *C. chlorideae* reared on *H. armigera* larvae fed on the leaves of transgenic chickpea before and after parasitisation. The larval period was prolonged and was a significant difference between the transgenic and nontransgenic chickpea lines was observed. Although the pupal period of the parasitoid was prolonged, there were no significant differences between the transgenic and nontransgenic chickpea lines. The adverse effects of transgenic chickpea lines on cocoon formation and adult emergence of *C.chlorideae* were largely due to the early mortality of *H.armigera* larvae, but there was no direct toxicity of *Bt* toxin protein to *C. chlorideae*. The amount of CryIIa protein transferred from leaves to the non-target insects and natural enemies were negligible.

Keywords: Transgenic chickpea, tritrophic interactions, *Helicoverpa armigera, Campoletis chlorideae*, *Bacillus thuringiensis*, *CryIIa*

1. Introduction
Chickpea yields are low (400–600 kg/ha), because of several biotic and abiotic constraints, of which the pod borer, *Helicoverpa armigera* (Hubner) (Noctuidae: Lepidoptera) is the most important constraint in chickpea production [1]. It is a major pest of chickpea in Asia, Africa, Australia and the Mediterranean region. Pod borers alone cause 25 to 40 per cent of the crop loss amounting US $ 325 million in chickpea (ICRISAT, 1992). In order to protect the crop from *H. armigera* damage, various pest management practices have been adopted by the Indian farmers. Efforts are being made to develop *H. armigera* resistant varieties by conventional breeding methods as well as modern biotechnological tools to develop transgenic chickpea varieties with resistance to this pest. The conventional control measures are largely based on insecticides. With the development of resistance to insecticides in *H. armigera* populations [2], there has been a renewed interest in developing alternative methods of pest control, of which host plant resistance to *H. armigera* is an important component. The impact of genetically engineered insect-resistant crops on non-target organisms including biological control agents is one of the most widely discussed ecological effects. Natural enemies are of major concern as they often play an important role in regulation of pest populations, and are therefore of economic value. There is a concern that the insecticidal proteins expressed in transgenic plants may either effect the natural enemies directly (toxic effect) or indirectly (change in the prey or host-quality or abundance) [3]. The parasitoid, *Campoletis chlorideae* (Uchida) is an important natural enemies of pod borer, *H. armigera* in grain legumes. It is therefore important to assess the non-target effects of *Bt* toxins to natural enemies of insect pests in different crops. The parasitic wasp, *Campoletis chlorideae* Uchida (Ichneumonidae: Hymenoptera), parasitizes several insect species [4 5]. The information on its parasitism potential, development and survival on different insect and crop hosts is scanty. However, under natural conditions, *H. armigera* is the most preferred host of *C. chlorideae* on a number of crops, *viz.,* cotton, groundnut, chickpea, pigeonpea, sorghum and pearl millet [6-8]. The introduction of transgenic crops has raised concerns regarding their impact on natural enemies [9]. The objective of the current study is the influence of different *CryIIa* transgenic chickpea lines on survival and development of larval parasitoid of gram pod borer (*H. armigera*), *C. chlorideae.*

2. Materials and Methods

The present study experiments were conducted at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India, during 2011-13. Six transgenic chickpea lines, BS5A.1(T2) 18-1P1, BS5A.1(T2) 18-2P1, BS5A.2(T2) 19-1P2, BS5A.2(T2) 19-2P1, BS5A.2(T2) 19-3P1, BS5A.2(T2) 19-3P2 and two non-transgenic chickpea lines, ICC506 EB (Resistant check) and Semsen (Control) were evaluated for resistance to *H. armigera*. The plants were grown under greenhouse conditions (27 ± 5^0 C and 65 - 90% RH). Larvae of *H. armigera* used in the bioassays were obtained from a laboratory culture maintained at ICRISAT. The larvae were reared on chickpea based artificial diet [10] under laboratory conditions at 27 ^{0}C. The plants were used for the bioassays in the laboratory under uniform environmental conditions (27 ± 2^0 C, 65-75% RH, and a photoperiod of 12:12 h. (Light: Dark) and evaluated for resistance to *H. armigera* using detached leaf assay against the neonate and second-instar larvae of *H. armigera*. Bioassays were conducted at the vegetative [30 days after emergence (DAE)] and flowering stages (45 DAE).

2.1 Rearing of larval parasitoid, *Campoletis chlorideae* Uchida (Hymenoptera: Ichneumonidae)

The cocoons of *Campoletis chlorideae* were collected from chickpea fields and kept individually in glass tubes (2 cm in diameter × 10 cm in long) and plugged with cotton wool, until adult emergence. Twenty pairs of adults were released in a cage (10 cm diameter x 20 cm in length, and closed with plastic cap lid having 60 wire mesh, and a cotton swab with 10% sucrose solution). Immediately after mating, the females along with the males were transferred to another cage. Single mated 5-10 days old female wasp was transferred to a transparent plastic vial (15 ml capacity) kept in an inverted position on a petri dish. Single *H. armigera* larva (3-day old / late second or early third instar, nearly 1 cm in length) was offered to a female wasp for oviposition. The females which showed efficient parasitisation were selected for further studies on non-target effects of transgenic chickpea lines towards the parasitoid, *C. chlorideae*. The culture was maintained at 27 ± 2 ^{0}C, 65 – 75% RH and 12h photoperiod.

2.1.1 Observations

Parasitized *H. armigera* larvae were checked every day and observations were recorded on larval mortality, cocoon formation, days to cocoon formation (egg+larval period), pupal period, adult emergence, adult weight, sex ratio, and fecundity of the *C. chlorideae* females from different treatments. For the fecundity test, three randomly selected *C. chlorideae* adult pairs obtained from each treatment (including control) were released inside a cage (30 × 30 × 30 cm), and allowed to mate for 3 days. The adults were provided with 10% honey solution in a cotton swab as a food source. After 3 days, each female was provided with *Bt* fed *H. armigera* larvae up to their daily parasitization capacity. Parasitization of *H. armigera* larvae with these females continued till they died. Total number of *H. armigera* larvae parasitized by a female in its lifetime was recorded as fecundity/female.

2.2 Detection of *Bt* proteins in *Helicoverpa armigera* and *Campoletis chlorideae*

After feeding the *H. armigera* larvae on *Bt* proteins transgenic plants, 5-6 specimens of each of the host larvae, parasitoid larvae, cocoons, or freshly emerged adults were collected and crushed together to detect the *Bt* proteins in the insect body using a double sandwich enzyme-linked immunosorbent assay (ELISA) kit (EnviroLogic Inc., Portland, ME, USA). The *C. chlorideae* larvae were collected from the live *H. armigera* larvae.

The *H. armigera* larvae showing symptoms of parasitization were dissected, and the parasitoid larvae were collected in eppendorf tubes when they were ready to emerge from the host larvae for pupation. The host/ parasitoid samples (whole body) were crushed together as one sample in phosphate-buffered saline (PBS) in the ratio of 1:10 (insect sample: buffer) in Eppendorf tubes in a plastic pestle. The test samples were then centrifuged at 11, 269 g for 2–3 min, and 100 µl of supernatant was loaded in the test wells of ELISA plate pre-loaded with 100 µl peroxydase enzyme conjugate. The negative and positive controls, and 0.5, 2.5, and 5.0 ppb *Bt* standards were run along with the test samples for the comparison of ELISA results. The ELISA plate was incubated for 2 h in a moist paper towel fitted in a plastic box. After 2 h of incubation, the test wells were thoroughly washed with PBS buffer giving 5–6 flip washings, and kept the test wells filled with PBS buffer for 1 min at the end. After washing, the test wells were again loaded with 100 µl TMB substrate. The wells showing a deep blue color indicated the presence of the toxin. After 15 min of incubation, 50 µl of 2M sulphuric acid was added, and observations were recorded on an ELISA plate reader at 450 nm.

2.3 Statistical analysis

Data were subjected to analysis of variance (ANOVA) using GenStat, version 14.1. The treatment means were compared by least significant differences (LSD) at $P \leq 0.05$. The figures presented in the tables are means across replications with F-probability and LSD values.

3. Results and Discussion

3.1 Effect of transgenic chickpea on the survival and development of the parasitoid, *Campoletis chlorideae*

During October planting, the larval period of the parasitoids was significantly prolonged in *C. chlorideae* reared on *H. armigera* fed on BS5A.1(T2) 18-1P1 (13.1 days) as compared to that on non-transgenic, ICC 506EB and Semsen (9.0 days). There was no significant effect of transgenic plants on the pupal period of *C. chlorideae* (4.0 - 6.5 days). Cocoon formation was significantly lower in *C. chlorideae* reared on *H. armigera* fed on transgenic chickpea lines (19.6 to 33.8%) as compared to those fed on non-transgenic chickpea plants, Semsen and ICC 506EB (72.0 and 68.4%, respectively) (Table 1).

The adult emergence was significantly lower in *C. chlorideae* when reared on *H. armigera* larvae fed on BS5A.2(T2) 19-1P2 (7.3%), BS5A.1(T2) 18-1P1(8.1%), BS5A.1(T2) 18-2P1 (11.5%), BS5A.2(T2) 19-3P2 (14.4%), BS5A.2(T2) 19-3P1 (17.3%) as compared to that on ICC 506EB and Semsen (53.4 and 47.0%, respectively). The eggs laid by the females when reared on *H. armigera* fed on transgenic plants were significantly reduced (6.6–62.6 egg female^{-1}) as compared to the wasps reared on *H. armigera* fed on non-transgenic plants, ICC 506EB and Semsen (126.6 and 125.2 egg female^{-1}, respectively). In general, reduced survival and prolonged development of the parasitic wasps was recorded when reared on *H. armigera* larvae fed on BS5A.1 (T2) 18-1P1 and BS5A.2 (T2) 19-1P2 (Figure 1).

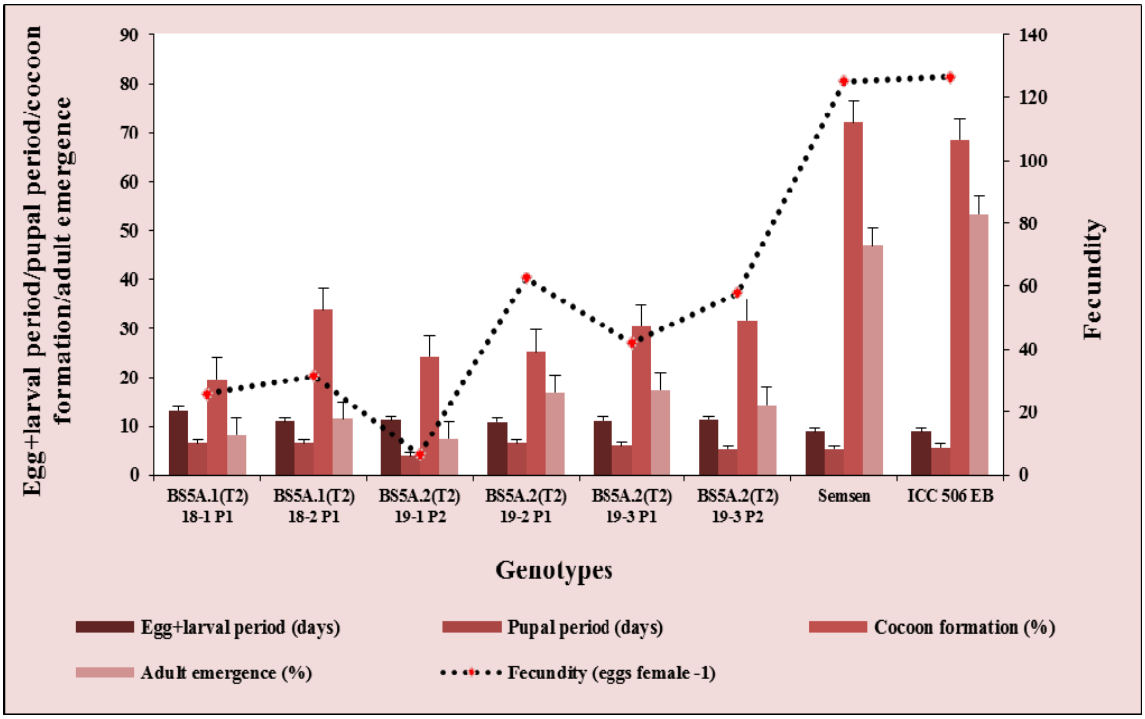

Fig 1: Biology of *C. chlorideae* parasitizing *H. armigera* fed on leaves of transgenic chickpea lines (October 2011-2013)

Whereas, during the November planting, the larval period was prolonged in *C. chlorideae* wasps reared on *H. armigera* larvae fed on BS5A.2(T2) 19-3P1 (11.3 days) as compared to that on ICC 506EB and Semsen (9.1 and 8.8 days, respectively). Among the transgenic lines, larval period ranged from 8.1 to 11.1 days, and there were no significant differences in pupal period (4.8 - 6.9 days) when reared on *H. armigera* larvae fed on transgenic and non-transgenic chickpea plants (Table 1).

Table 1: Biology of *C. chlorideae* parasitizing *H. armigera* fed on leaves of transgenic chickpea lines

Genotype	October 2011-2013					November 2011-2013				
	Egg+larval period (days)	Pupal period (days)	Cocoon formation (%)	Adult emergence (%)	Fecundity (eggs female $^{-1}$)	Egg+larval period (days)	Pupal period (days)	Cocoon formation (%)	Adult emergence (%)	Fecundity (eggs female $^{-1}$)
BS5A.1(T2) 18-1 P1	13.1^{c}	6.5^{b}	19.6^{a} (25.8)	8.1^{a} (14.7)	25.8^{ab}	11.1^{b}	6.8^{a}	29.7^{a} (31.4)	18.0^{a} (24.0)	35.8^{a}
BS5A.1(T2) 18-2 P1	11.0^{acb}	6.5^{b}	33.8^{a} (35.4)	11.5^{a} (19.6)	31.5^{ab}	11.0^{b}	4.8^{a}	24.6^{a} (28.8)	10.4^{a} (17.1)	16.6^{a}
BS5A.2(T2) 19-1 P2	11.2^{abc}	4.0^{a}	24.1^{a} (28.6)	7.3^{a} (12.5)	6.6^{a}	8.1^{a}	6.8^{a}	23.5^{a} (27.3)	17.1^{a} (22.0)	54.1^{a}
BS5A.2(T2) 19-2 P1	10.9^{abc}	6.5^{b}	25.2^{a} (29.9)	16.8^{a} (24.0)	62.6^{b}	10.5^{ab}	6.9^{a}	23.0^{a} (28.4)	16.2^{a} (21.6)	41.6^{a}
BS5A.2(T2) 19-3 P1	11.1^{abc}	6.0^{b}	30.4^{a} (32.9)	17.3^{a} (23.5)	42.0^{ab}	11.3^{b}	6.0^{a}	25.4^{a} (29.7)	18.0^{a} (24.7)	22.5^{a}
BS5A.2(T2) 19-3 P2	11.3^{bc}	5.3^{ab}	31.6^{a} (34.1)	14.4^{a} (22.2)	58.0^{b}	10.1^{ab}	6.1^{a}	17.2^{a} (19.4)	10.6^{a} (14.5)	24.1^{a}
Semsen (Control)	9.0^{a}	5.3^{ab}	72.0^{b} (58.5)	47.0^{b} (43.2)	125.3^{c}	8.8^{ab}	6.3^{a}	50.8^{b} (45.4)	37.1^{b} (36.9)	107.0^{b}
ICC 506 EB (Resistant check)	9.0^{ab}	5.6^{ab}	68.4^{b} (56.1)	53.4^{b} (46.9)	126.6^{c}	9.1^{ab}	6.0^{a}	58.0^{b} (49.9)	46.8^{b} (43.0)	98.3^{b}
SE ±	0.7	0.7	4.4	3.5	13.5	0.8	0.9	7.2	5.1	12.9
Fp	0.004	0.27	<0.001	<0.001	<0.001	0.078	0.809	0.002	<0.001	<0.001
LSD (P 0.05)	2.0*	NS	12.8*	10.1*	38.6*	2.4*	NS	20.7*	14.6*	37.1*

*Figures followed by the same letter within a column are not significantly different at P≤ 0.05. Figures in parenthesis are Angular transformed values.

Cocoon formation and adults emergence were significantly reduced in *C. chlorideae* reared on *H. armigera* larvae fed on BS5A.2(T2) 19-3P2 (17.2 and 10.6%, respectively) than on non-transgenic ICC 506EB (58.0 and 46.8%, respectively) and Semsen (50.8 and 37.1%, respectively). There was a significant reduction in fecundity of the female wasps obtained from *H. armigera* fed on transgenic chickpea plants of BS5A.1(T2) 18-2P1 (16.6 eggs female^{-1}) as compared to those fed on Semsen and ICC 506EB (107.0 and 98.3 egg female^{-1}, respectively) (Table. 1; Fig. 2). Among the transgenic lines tested, the survival and development of *C. chlorideae* was significantly better when reared on *H. armigera* fed on BS5A. 1 (T2) 18-1P1 and BS5A. 2 (T2) 19-3P1 as compared to the other lines tested.

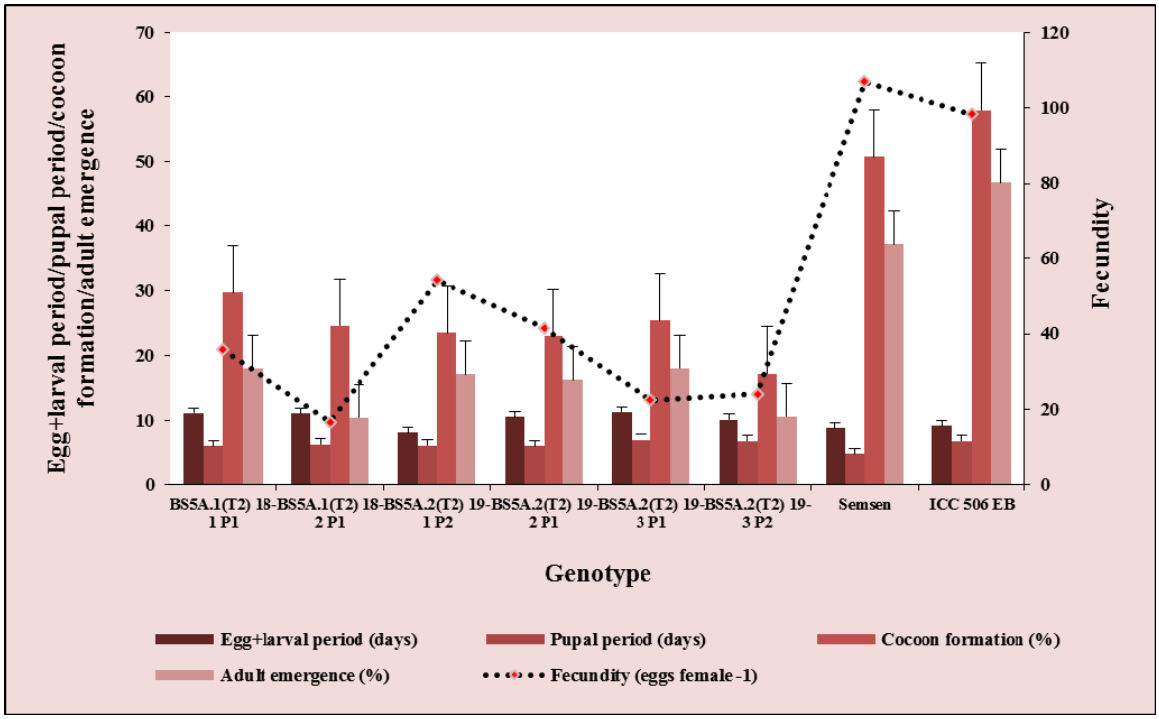

Fig 2: Biology of *C. chlorideae* parasitizing *H. armigera* fed on leaves of transgenic chickpea lines (November, 2011-13)

3.2 Detection of Cry IIa protein in transgenic chickpea lines using ELISA

3.2.1 CryIIa content in *H. armigera* larvae and *Campoletis chlorideae* larvae reared on *H. armigera* fed on transgenic chickpeas

During 2011-12, concentration of CryIIa protein was significantly high in *H. armigera* larvae fed on leaves of BS5A. 2 (T2) 19-1P2 (54.0 ppb) and BS5A. 2 (T2) 19-3P1 (52.3 ppb). The amount of protein in the larvae fed on BS5A. 1 (T2) 18-2P1 was 42.3 ppb. The protein concentration was significantly lowest in larvae fed on BS5A. 2 (T2) 19-3P2 (13.0 ppb), followed by BS5A. 2 (T2) 19-2P1 (17.0 ppb) and BS5A.1(T2) 18-1P1 (19.0 ppb) (Table 2).

Table 2: Amount of Cry IIa protein (ppb) in Bt fed H. armigera larvae and C. chlorideae

Genotypes	Bt fed H. armigera larvea S - 1	Bt fed H. armigera larvae S - 2	Bt fed Campoletis larva
BS5A.1(T2) 18-1 P1	19.0^{ab}	37.0^{a}	1.0^{bc}
BS5A.1(T2) 18-2 P1	42.3^{bc}	29.4^{a}	1.3^{c}
BS5A.2(T2) 19-1 P2	54.0^{c}	15.0^{a}	1.7^{c}
BS5A.2(T2) 19-2 P1	17.0^{ab}	41.6^{a}	1.5^{c}
BS5A.2(T2) 19-3 P1	52.3^{c}	19.0^{a}	1.4^{c}
BS5A.2(T2) 19-3 P2	13.0^{ab}	18.5^{a}	1.0^{bc}
Semsen (Control)	0.0^{a}	0.0^{a}	0.1^{ab}
ICC 506 EB (Resistant check)	0.0^{a}	0.1^{a}	0.0^{a}
SE ±	9.6	12.0	0.2
Fp	0.01	0.27	0.01
LSD (P 0.05)	29.1*	NS	0.7*

*Figures followed by the same letter within a column are not significantly different at P≤ 0.05. S1-season 1(2011-12), S2-season 2 (2012-13)

During 2012-13, the CryIIa protein content was significantly higher in larvae fed on BS5A.2(T2) 19-2P1 and BS5A.1(T2) 18-1P1 (41.6 and 37.0 ppb, respectively). The amount of CryIIa protein in *C. chlorideae* larvae reared on *H. armigera* fed larvae fed on transgenic chickpeas was very low (1.0-1.7 ppb) (Fig 3). Hence, the amount of CryIIa protein transferred from leaves to the non-target insects and natural enemies were negligible.

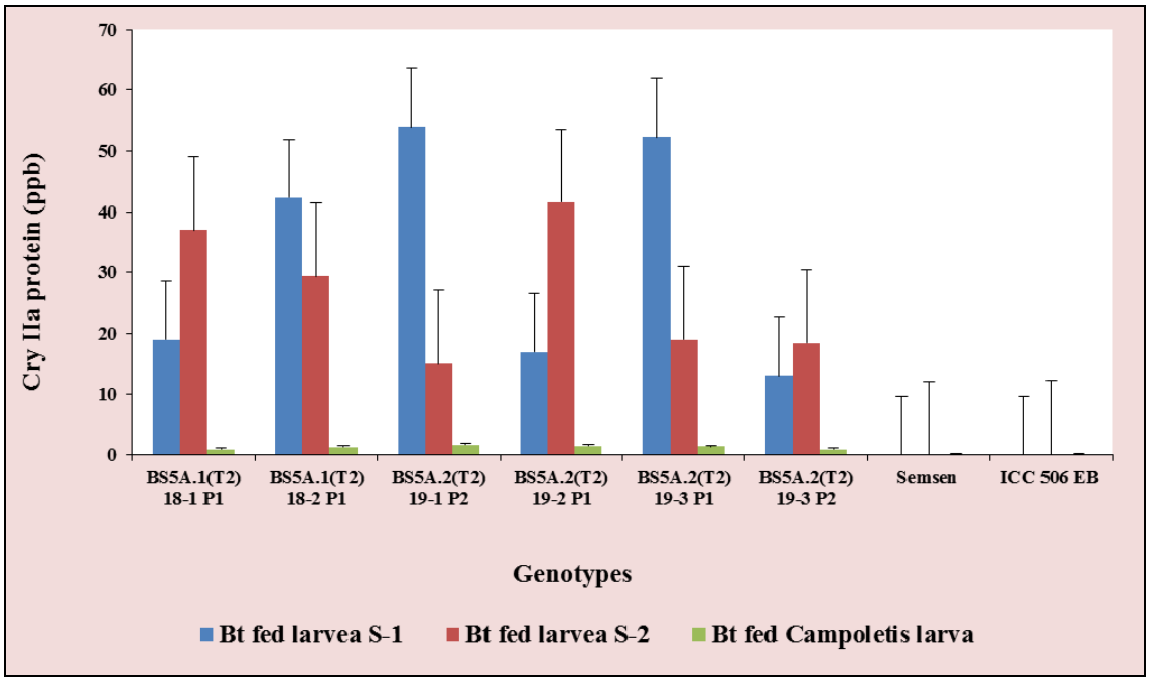

Fig 3: Amount of Cry IIa protein (ppb) in *Bt* fed *H. armigera* larvae and *C. chlorideae*

Similar results were reported by few authors [11], who reported poor survival and development of *C. chloridae* obtained from *H. armigera* larvae fed on the leaves of *Bt* cotton hybrid Mech 184. When *H. armigera* larvae were fed on artificial diet impregnated with Cry1Ab and Cry1Ac at LC_{50} and ED_{50} levels before and after parasitisation, there was a significant reduction in cocoon formation and adult emergence of *C. chlorideae*. Larval period of the parasitoid was prolonged by 2 days when fed on *Bt*-intoxicated larvae. No adverse effects were observed on female fecundity. There was a significant influence of host size on development and survival of the parasitoid. *Bt* toxins were detected in *H. armigera* larvae fed on *Bt*–sprayed chickpea, but not in *C. chlorideae* reared on *H. armigera* larvae fed on *Bt*-sprayed chickpeas, and in the parasitoid adults fed on honey intoxicated with 0.05% *Bt* [12]. The development duration of *C. chlorideae* pupae on the hosts fed with transgenic cotton leaves was not significantly different than those on the controls. The longevity of female and male parasitoids fed on a solution containing Cry1Ac toxin did not differ significantly with that of the control [13].

There was a reduction in cocoon formation and cocoon weight in parasitic wasps reared on *H. armigera* fed on diets made with transgenic cotton for *Microplitis mediator*, the cocoon formation and cocoon weight was reduced by 26.1% and 1 mg, respectively where for *C. chlorideae*, the reduction was 17.9% and 5.1 mg, respectively and larvae of the two wasps developing in the haemocoel of *H. armigera* larvae reared on transgenic cotton exhibited delayed development and, in some cases, abnormal development. The body weight of the larvae of the parasitoids was significantly reduced when obtained from hosts fed on transgenic cotton leaves compared to those fed on traditional cotton. Duration of egg and larval period was significantly prolonged, whereas pupal and adult weights of *C. chloridae* decreased when the host larvae were fed on transgenic cotton leaves for more than 48 h [14].

4. Conclusions

It is understood from the present investigations that there was a significant reduction in cocoon formation and adult emergence of *C. chlorideae* reared on *H. armigera* larvae fed on the leaves of transgenic chickpea before and after parasitisation. The larval period was prolonged and was a significant difference, whereas there were no significant differences in pupal period between the transgenic and non-transgenic chickpea lines was observed. The adverse effects of transgenic chickpea lines on cocoon formation and adult emergence of *C.chlorideae* were largely due to the early mortality of *H.armigera* larvae, but there was no direct toxicity of *Bt* toxin protein to *C. chlorideae*. The amount of CryIIa protein transferred from leaves to the non-target insects and natural enemies were negligible.

5. Acknowledgement

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