

ORIGINAL ARTICLE

The toxicity of *Cry1Ia5* transgenic soybean plants against *Spodoptera littoralis*

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Vol. 59, No. 2: 185–192, 2019

DOI: 10.24425/jppr.2019.129286

Received: February 10, 2019

Accepted: May 31, 2019

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Abstract

Three transgenic soybean lines expressing the *Cry1Ia5* gene were developed using the *Agrobacterium* transformation system. The integration of the *Cry1Ia5* gene in the genome of the transgenic plants was approved using specific primers for PCR and real time PCR analysis, respectively. The insecticidal activity of three transgenic lines (L1, L2 and L3) against 2nd larval instars *Spodoptera littoralis* was tested. The data indicate that L2 exhibited the highest mortality percentage 9 days post feeding (60%) followed by L3 (40%) then L1 (20%) while the control showed 0% mortality. The larvae fed transgenic material appeared smaller in size than compared to the control larvae. The reduction in insect size and weight was due to the accumulation of higher phenoloxidase activity in insect tissues. The higher mortality observed in L2 was due to a significant decrease in the acetylcholine esterase activity that leads to accumulation of acetylcholin at higher levels which causes paralysis and death. The developed transgenic line 2 could be used to construct an insect resistant soybean cultivar.

Keywords: *Cry1Ia5* gene, enzyme activity, insect resistance, soybean, transformation

Introduction

Soybean [*Glycine max* L. (Merr.)] is considered to be one of the most substantial sources of protein and edible oil in the world (Schmutz *et al.* 2010; Shin *et al.* 2014). Several abiotic and biotic stresses affect and reduce its yield and seed quality. One such stress is insect attack which is a major factor that leads to decreased crop yield (Gómez *et al.* 2014).

In Egypt, the cotton leaf – worm (*Spodoptera littoralis*) is a serious economic pest. It is considered to be a generalist herbivore on a broad range of plants. *Spodoptera littoralis* mainly attacks cotton and Egyptian clover in summer, moving between plants. The pest frequently attacks vegetable plantations as temporary alternant hosts and may cause severe damage to them (Brown and Dewhurst 1975; Zakir *et al.* 2017).

Genetically modified plants resistant to insects can be found in various agro ecosystems as a method for

integrated pest management (IPM). These plants are characterized by expressing genes of the entomopathogenic bacterium *Bacillus thuringiensis* Berliner (Bt) that encode proteins with insecticidal effects (Martins-Sallesi *et al.* 2017). *Bacillus thuringiensis* can be a source of toxic genes that can be expressed in plants and thus confer toxic properties against different insect pest species. Chewing insects are sensitive to control with Bt insecticides after application on the surface of leaves or when Bt genes are inserted into the plant genome, such as in soybean (Schünemann *et al.* 2014).

Until January 2009, there was no commercially available genetically modified soybean that was resistant to insect-pests or pathogens, although the need for this trait is extremely important (Stein and Rodríguez-Cerezo 2009). Bernardi *et al.* (2012) evaluated the Bt soybean MON 87701 × MON 89788 against some

species, including *Anticarsia gemmatalis* and *Chrysodeixis*. Selvapandiyar *et al.* (1998) cloned and characterized a *CryIIa5* gene from an Indian isolate of *B. thuringiensis* (EMBL accession number Y08920), and was expressed *CryIIa5* in tobacco plants. It extended complete protection against the neonate stage larvae of *Helicoverpa armigera*. Moghaieb (2010) used the *CryIIa5* gene to produce transgenic rice plants that developed high resistance to stem borers (*Chilo agamemnon*).

The present study describes an efficient protocol for the production of transgenic soybean plants (expressing the *CryIIa5* gene) resistant to *S. littoralis* which is an important pest in Egypt. The insecticidal activity of the transgenic plant against the larvae was investigated.

Materials and Methods

Plant materials

Two soybean genotypes, namely, Giza 21 and Giza 111 were provided by the Field Crop Institute, Agricultural Research Center, Ministry of Agriculture – Egypt. Soybean seed exteriors were sterilized by immersion in 70% ethanol for 30 s. They were then immersed in 3% (v/v) sodium hypochlorite and rinsed three times in sterile distilled water. The seeds were infused in sterile distilled water for 3 h in light at 28°C. Mature embryos were excised from soybean seeds. The embryos were placed in callus induction medium containing half MS (Murashige and Skoog 1962) salt, 3% (w/v) sucrose,

1% (w/v) agar in addition to 1 mg · l⁻¹ NAA and 1.5 g activated charcoal. Two weeks later the explants were placed in MS medium for regeneration.

Genetic transformation in soybean

Production of *gus* and *CryIIa5* expressing transgenic soybean plants bacterial strains

The *Agrobacterium tumefaciens* strain LBA4404 harboring the Ti-binary vector pBI-121-*gus* (Jefferson *et al.* 1987) were grown overnight in 20 ml of lysogeny broth (LB) medium containing 50 mg · ml⁻¹ kanamycin sulfate at 28°C. *Agrobacterium tumefaciens* strain LBA4404 carrying the pBI-121 *CryIIa5* plasmid (Selvapandiyar *et al.* 1998) kindly provided by Professor V.S. Reddy (International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi-110 067, India) was grown overnight in 20 ml of LB medium (Luria and Burrow 1955) containing 50 µg · ml⁻¹ kanamycin at 28°C (Fig. 1).

Production of transgenic soybean plants

Mature embryos were excised from the seeds of Giza 21 and Giza 111 and were transformed with *gus* gene (as a control) and by *CryIIa5* gene by immersing in the bacterial suspension for 2 h (Zia *et al.* 2010). Thereafter, the embryos were dried on sterilized filter paper, placed on a co-cultivation medium, which consisted of MS medium enhanced with 1 mg · l⁻¹ naphthaleneacetic acid (NAA) and then incubated under dark conditions for 3 days. Then the infected embryos were transferred onto the same medium enhanced with 50 mg · l⁻¹ kanamycin sulfate.

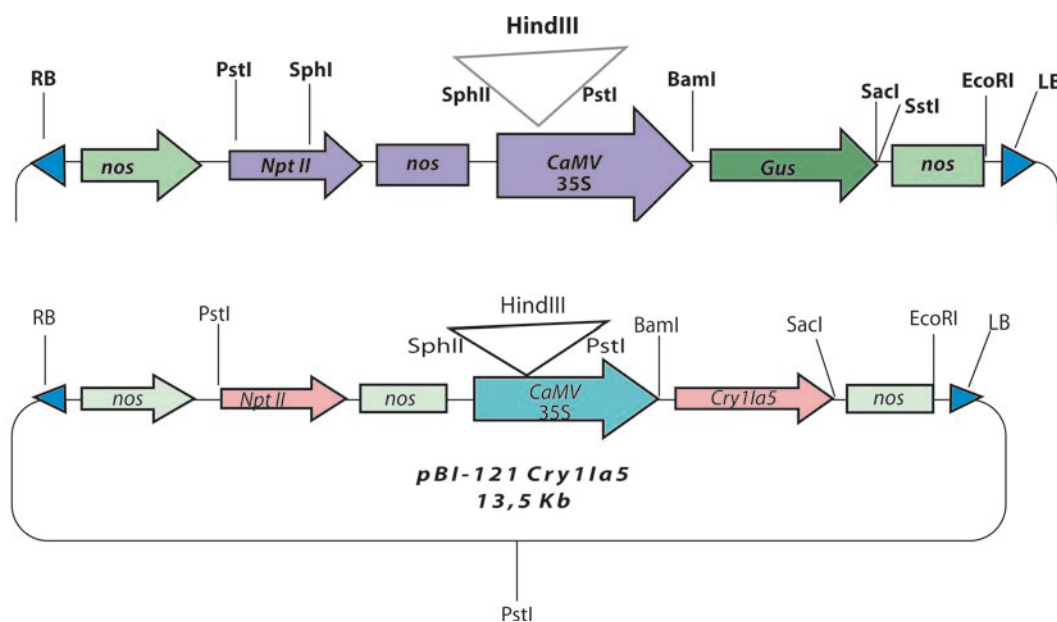


Fig. 1. Schematic representation of the pBI-121 harboring the pBI-121 *CryIIa5* plasmid harboring the *CryIIa5* gene under the 35SP and the NOS terminator used in soybean transformation

The plates were sealed with a parafilm and incubated at 26°C for 2 weeks with continuous cultivation under a 16 : 8-hrs light : dark photoperiodic regime (1,000-Lux). The explants were cultured in MS medium enhanced with 1 mg · l⁻¹ NAA and 250 µg · ml⁻¹ cefotaxim. The plates were sealed with a parafilm.

Evaluation of putative *gus* transgenic plants

Transient *gus* gene expression in the transferred soybean embryos from both cultivars was observed using histochemical GUS assay.

Histochemical GUS assay

The expression of β-glucuronidase (GUS) activity in transgenic soybean plants was carried out according to the method described by Jefferson *et al.* (1987). Embryonic tissue and callus were incubated in a reaction buffer containing 12.5 mM K₃Fe(CN)₆, 12.5 mM K₄Fe(CN)₆, 20% methanol, 1% Triton X-100 and 38.3 mM 5-bromo-4-chloro-3-indolyl glucuronide as a substrate for the enzyme. The tissue and callus were incubated in staining solution at 37°C for 24 h and the blue spots which developed were recorded.

Molecular conformation of the transgenic events

In order to confirm the stable integration of the transgene in the genome of resultant plantlets, genomic DNA was doped from leaves of adult plants using a CTAB method (Wang and Fang 2002). DNA samples were analyzed by PCR using 35S promoter specific primers. The sequences of forward and reverse primers were 5'-AAA GGA AGG TGG CTC CTA CAA AT-3' and 5'-CCT AGT AAA GTA AAC CTC TCC-3', respectively.

The plants which showed positive PCR results were acclimatized and transferred into a growth chamber to collect the T1 seeds. Genomic DNA was isolated from both *Cry1Ia5* transformed (T1) and the control plants to determine transgene integration in transgenic soybean plants using simple real-time PCR procedure as described by Ahmed *et al.* (2005). Experiments were conducted using Spartan Real-Time PCR System.

Reaction was set-up in a total volume of 25 µl containing HOT FIREPol EvaGreen qPCR Mix Plus (no ROX) (1X), (10 pmol · µl⁻¹) each primers for *Cry1Ia5* gene (5'-CGC GGA TCC ATG GA CTA AAG AAT CAA GAT-3' and 5'-CGC GGA TCC GAG CTC GTCGAC TAC ATG TTA-3'), 30 ng template DNA. Thermal cycling conditions were: initial denaturation at 95°C for 15 min followed by 50 cycles of denaturation at 95°C for 15 s, 69.9°C for 45 s, and 72°C for 45 s.

Toxicity evaluation of the *Cry1Ia5* expressing soybean plants

Leaf-insect bioassays were performed on T1 plants according to the method of Selvapandiyani *et al.* (1998). Petri dishes held detached two leaf discs which had been removed from the oldest non-senescent leaf on each plant, and were placed on moist filter paper in a Petri dish. One larvae of the 2nd larvae instars from cotton leaf worm (*S. littoralis*) was placed on each plate of transgenic lines and non-transgenic control line with 10 replicates. The larvae were allowed to consume the plant material for 7 days under a 25-hrs photoperiod.

Insect survivor data was collected at the end of the 7 day experiment. Total insect protein and acetylcholinesterase (AChE), phenoloxidase and chitinase enzyme activities were measured after the insects had been collected and frozen at the end of the experiment.

Total protein determination in larvae (protein assay)

Larvae were chilled on ice. Bovine serum albumin (BSA) was used as the standard protein. The absorbance was read at 595 nm using a spectrophotometer. From a standard curve of absorbance of known concentrations of bovine serum albumin protein in mg · ml⁻¹ was calculated. According to Bradford (1976), Bio-Rad Protein Assay was derived by the Commassie Brilliant Blue dye G-250 method and modified by Spector (1978).

Change in enzyme activities

For the collection of hemolymph and preparation of enzyme extract the larvae of *S. littoralis* were used for measuring enzyme activity. Prolegs were removed and hemolymph was dripped into microtubes for collecting hemolymph. Approximately 40 µl of hemolymph was obtained from each individual. The sample was prepared for measuring specific activity and the effect of temperature on enzyme activity in the buffer for each enzyme according to its optimum pH was determined. The homogenate was centrifuged at 10,000 rpm at 4°C for 10 min and the supernatant was used as enzyme solution.

Acetylcholinesterase (AChE)

AChE activity was spectrophotometrically measured according to Simpson *et al.* (1964). The reaction mixture contained 200 µl enzyme solution, 0.5 ml 0.067 M phosphate buffer (pH 7) and 0.5 ml acetylcholine bromide (AchBr) (3 mM). The mixture was allowed to stand for 2 min, then 0.5 ml of ferric chloride solution was added and mixed well. The decrease in AchBr resulted from hydrolysis by AChE. The absorbance was recorded colorimetrically at 515 nm.

Chitinase activity

The reaction mixture was prepared according to Ishaaya and Casida (1974), with a few modifications. The reaction contained 500 μ l phosphate buffer (0.2 M, pH 6.5), 200 μ l 0.5% colloidal chitin and 200 μ l enzyme solution. After 1.5 h of incubation at 37°C, enzyme activity was terminated by boiling. Undigested chitin was sedimented by centrifugation for 15 min at 8,000 rpm. The absorbance was measured at 550 nm spectrophotometrically.

Phenoloxidase (PO)

The cuticular phenoloxidase (PO), an important component in insects' immune system, is a melanizing enzyme at the wound site that has been found in the hemolymph to limit infection (Pham and Schneider 2008; Castillo *et al.* 2011). The diluted hemolymph was centrifuged, and the pellet was washed using phosphate buffer (Leonard *et al.* 1985).

The sample pellet was homogenized by adding 500 μ l of cold phosphate buffer, and then centrifuged. The supernatant was transferred into new tubes and were pre-incubated with phosphate buffer for 30 min at 30°C, then 50 μ l of 10 mM l-dihydroxyphenylalanine (l-DOPA) was added as substrate and incubated for 5 min at 30°C. Phenoloxidase activity was measured using a spectrophotometer at 490 nm.

Results

The benefits of using *bt* expressing plants in agriculture include efficient control of insect pests, reduced use of insecticides, and the maintenance of populations of natural enemies in the field (Roush and Mckenzie 1987; Martins-Sallea *et al.* 2017).

In the present study embryonic axis explants derived from the cultivars Giza 21 and Giza 111 were transformed by the *gus* gene under the genetic control of 35S-promoter and *nos* terminator using *Agrobacterium* mediated transformation system. Putative transgenic shoots were regenerated within 4 weeks on the MS medium consisting of 1 mg \cdot l⁻¹ NAA, during the selection culture. The embryos were sub-cultured into new medium containing 50 μ g \cdot ml⁻¹ kanamycin which reduced the number of escapes.

During the selection process transformed explants continued to grow to produce shoot initiations, whereas the untransformed ones were unable to form shoots and in the end died within 2 weeks. To boost the stable integration of the T-DNA in the regenerated plant genomes, all T₀ plants that can survive in medium containing 50 μ g \cdot ml⁻¹ kanamycin were exposed to histochemical GUS assay. The data indicated that only *gus* gene expressing plants were blue green, while control plants didn't exhibit any color (Fig. 2).

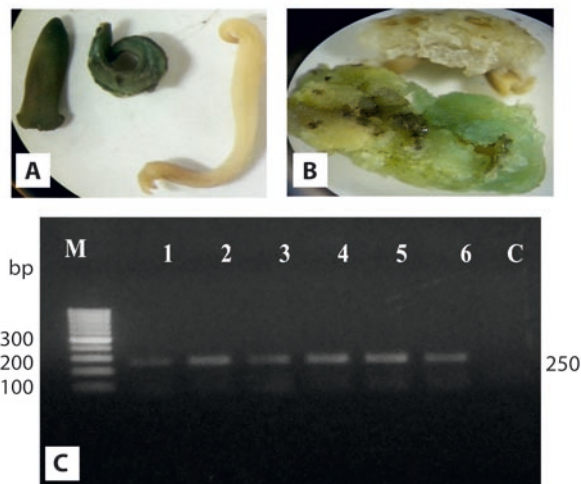


Fig. 2. The expression of *gus* in transgenic soybean plant. A – the expression of *gus* gene in transgenic soybean embryo; B – comparison of the *gus* transgenic soybean callus with the control plants, C – molecular confirmation of 35 S promoter gene integration into transgenic soybean genome

To confirm the stable integration of the T-DNA in the putative transgenic plantlet genomes, all T₀ plants that can survive in medium containing 50 μ g \cdot ml⁻¹ kanamycin underwent PCR analysis using primers specific to the 35 S-promoter. Figure 2 revealed a clear band at 250 bp only in transgenic plants however, no such bands were seen in the non-transformed controls under identical conditions.

Production of transgenic soybean plants expressing the *Cry1Ia5* gene

In order to produce transgenic soybean plants resistant to the cotton leaf worm (*S. littoralis*), the embryos derived from two soybean cultivars, namely G-21 and G-111, were chosen for transformation with plasmid pBI-121 harboring the *Cry1Ia5* gene. The putative transgenic embryos were grown with the addition of 50 mg \cdot l⁻¹ kanamycin to select the transgenic plants. The young plantlets were transferred to root inducing medium (MS free medium) where they grew and developed a good root system. The kanamycin resistant plantlets were acclimatized for seed production (Fig. 3).

The expression of the *Cry1Ia5* gene in the T1 plant genomes were confirmed by using real time PCR analysis using primers for *Cry1Ia5* gene (Fig. 4). Three out of 20 transgenic lines obtained were selected for the insect bioassay experiments. Selection was based on their normal morphology and growth. T1 soybean leaves were excised daily from selected plants and non-transgenic regenerated plants (control plants) and were evaluated for cotton leaf worm (*S. littoralis*) resistance according to Gabriela *et al.* (2016).

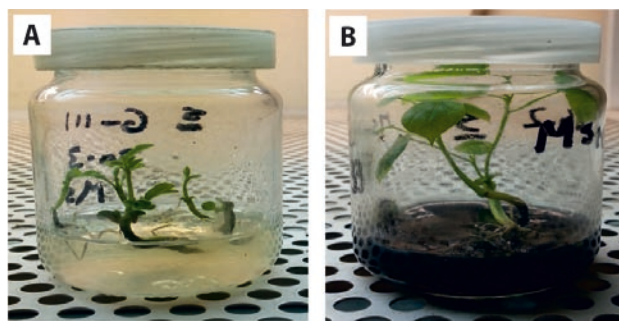


Fig 3. The selection and regeneration of transgenic plant expressing *Cry1Ia5* gene on kanamycin containing medium: A – MS medium without activated charcoal, B – MS with activated charcoal

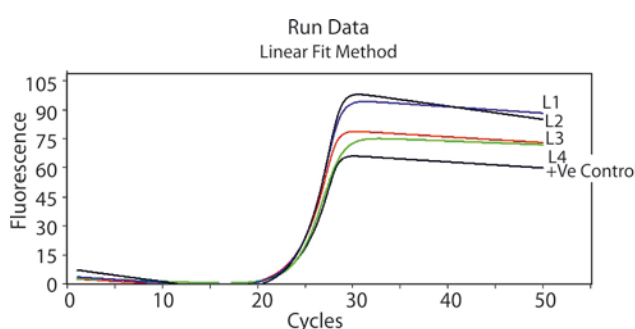


Fig. 4. Detection of the *Cry1Ia5* expression in transgenic soybean using real time PCR. L1–L4 – transgenic lines 1–4, +Ve control – *Cry1Ia5* gene, reaction was carried out for 50 cycles



Fig. 5. Insect bioassay of the *Cry1Ia5* transgenic soybean plants and the morphological change to the cotton leaf worm (A). The body size of the insect fed *Cry1Ia5* transgenic material (B – on the left) compared with control (B – on the right)

The leaves were placed on a moistened filter paper disc in a 90 mm-diameter Petri dish and infested with

five larvae of the 2nd instar of *S. littoralis*. Petri dishes were incubated at 26°C in the dark and twice-daily supplied with leaves from the same transgenic line for 10 days (Fig. 5). The data collected for the insecticidal activity of three transgenic soybean lines indicated that, line 2 and line 3 caused 20% mortality to the *S. littoralis* larvae by the end of the third day from the beginning of experiment, while line 1 showed 20% at the end of the 4th day. Then on the 7th day lines 2 and 3 showed increased mortality by 40%. Line 1 showed no change. The mortality percentage increased on the 9th day for transgenic line 2 and it reached 60% with no change in the other transgenic lines. However, control plants showed no larval mortality.

The present data indicate that the transgenic soybean plants obtained were toxic to the cotton leaf worm under laboratory conditions. These transgenic lines might serve as a novel genetic resource in soybean breeding aimed at broad spectrum resistance.

Changes in the protein content in the 2nd larval instar fed transgenic soybean

Total protein samples from the 2nd larval instar that had been fed leaves from three transgenic lines from *Cry1Ia5* expressing soybean plants and from the control plants were analyzed. It can be pointed out that extremely significant lower protein levels were detected in *S. littoralis* fed Bt expressing soybean lines in contrast with the control plants ($p < 0.0001$) (Fig. 6).

Protection and detoxifying enzyme activities in the 2nd larval instar fed *Cry1Ia5* transgenic soybean

Acetylcholinesterase (AChE)

Acetylcholinesterase is a significant enzyme in the nervous system of insects, preventing nerve impulses by catalyzing the hydrolysis of neurotransmitter acetylcholine (ACh). Inhibition of AChE results in excessive accumulation of ACh, leading to hyperactivity and consequently paralysis and death (Soreq and Seidman 2001). The AChE activity showed an extremely significant reduction in the 2nd larval instar that had been fed soybean expressing *Cry1Ia5* gene as an origin of food compared to those fed control plants for the same period of time. The reduction was more remarkable in larvae fed line 2 and this could explain why the mortality percent of this line was higher than in the other transgenic lines (Fig. 6).

Chitinase

There was an extremely significant increase in the activity of chitinase extracted from larvae fed Bt expressing plants compared with the control ($p < 0.0001$) (Fig. 6).

Phenoloxidase

Phenoloxidase (PO) activity plays an important role in the innate immune responses of insects, which catalyzes the biosynthesis of quinones and other reactive intermediates to exclude invading pathogens and parasites (Taleh *et al.* 2014). There was an extremely significant increase in the activity of phenoloxidase extracted from larvae fed *Bt* expressing plants compared with the control ($p < 0.0001$) (Fig. 6).

The data presented in Table 1 show the analysis of variance of different enzymes. The differences between insects that were fed transgenic material from different lines and the control plants were extremely significant for total protein content, AChE, phenoloxidase and chitinase activity (Table 1).

Discussion

Several plants have been transformed by insecticidal protein coding genes isolated from *Bacillus thuringiensis* (Strizhov *et al.* 1996). These plants exhibit varying degrees of protection against different insects, e.g. Lepidopterans, Coleopterans and Dipteran insects. These genes are generally not harmful for human consumption (Chen *et al.* 2007; Rahman *et al.* 2007).

The present data indicate that L2 exhibited the highest mortality percentage 9 days post feeding (60%) followed by L3 (40%) then L1 (20%) while the control showed 0% mortality. The present data agree with Bernardi *et al.* (2012) who reported that the *Bt* soybean MON 87701 × MON 89788 efficiently targets a number of species, like *Anticarsia gemmatalis* and *Chrysodeixis*, but it is not effective against *Spodoptera* spp. (armyworm) (Bernardi *et al.* 2014). Our results also agree with Moghaieb (2010) who used the transgenic rice lines expressing *CryIIa5* gene against rice stem borer (*Chilo Agamemnon*) and recorded 100% mortality 4 days post treatment. Our results disagree with Silva *et al.* (2016) since they concluded that *Bt* soybean MON 87701 × MON 89788 had no effect on *S. cosmioides* development and reproduction, and that *Bt* soybean was a valid host for the development of the pest allowing for more than 80% larvae-to-adult survival.

The protein content was decreased in the 2nd larval instar of *S. littoralis* fed transgenic soybean compared to the control plants. This was explained by Mitlin *et al.* (1977) as decreases in hemolymph protein content which might be due to inhibition of DNA and RNA synthesis. It also agrees with Ericsson *et al.* (2009) who stated that hemolymph protein concentrations were declined significantly in cabbage looper (*Trichoplusia ni*) susceptible larvae continually exposed to *Bacillus thuringiensis* var. *kurstaki* (Btk). Guo *et al.* (2011) agree as there were significantly lower protein levels detected

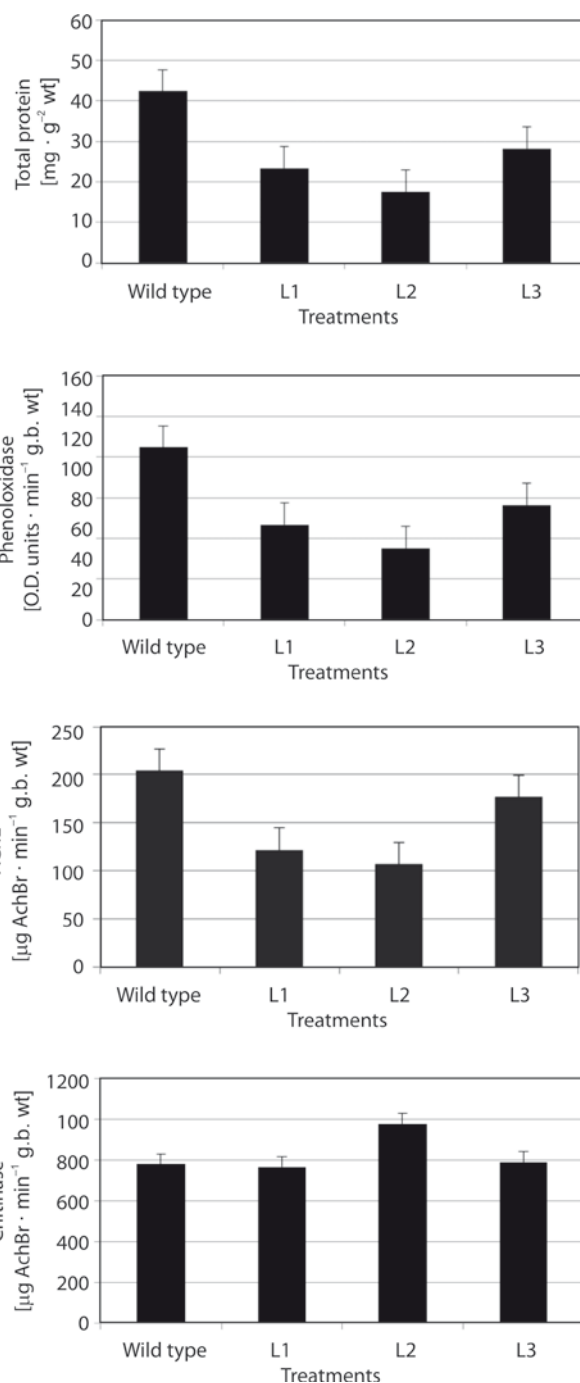


Fig. 6. Change in total protein, phenoloxidase, acetylcholinesterase (AChE) and chitinase activity in the 2nd larval instar fed transgenic lines and the control. Wild type = non transgenic, L1–L3 = transgenic lines

in *S. exigua* fed *Bt* cotton for 1 h than those for 4, 6 and 24 h.

Inhibition of AChE results in excessive accumulation of ACh, leading to hyperactivity and consequently paralysis and death (Soreq and Seidman 2001). This explains our data as there were decreases in AChE in insects that fed on *Bt* plants. The same result was reported by Guo *et al.* (2011) since they could detect significantly lower AChE activities in *Spodoptera exigua* fed *Bt* cotton compared with those fed non-*Bt* cotton for the same periods.

Table 1. Analysis of variance (ANOVA) showing the differences in the enzyme activity between three transgenic soybean lines expressing the *Cry11a5* gene against cotton leaf worm (*Spodoptera littoralis*)

SV	df	AChE MS	Total protein MS	Chitinase MS	Phenoloxidase MS	F	F _{0.05}
Replicates	2	34.750	2.401	4207.8	71.083	2.122 ns*	9.50
Treatment	3	6287.40	334.010	30326.0	2593.0	76.51**	5.14
Error	6	128.75	4.154	513.53	33.497		
Total	11						

SV = source of variation; MS = mean square; df = degree of freedom

*non-significant

**highly significant

Al-Shannaf *et al.* (2012) concluded that the chitinase activity was increased in *H. armigera* when they used chlorfluazuron and pyriproxyfen as insect growth regulators compared to the biopesticides. This agrees with our data in which the chitinase activity was increased.

Ericsson *et al.* (2009) observed that exposure to *Bacillus thuringiensis* var. *kurstaki* (Btk) decreased the hemocyte counts and the PO activity of susceptible cabbage looper (Bt-RS) larvae. Ebrahimi *et al.* (2014) showed increased PO activity in Colorado potato beetle (*Leptinotarsa decemlineata*) injected with entomopathogenic nematode compared with the control ($p < 0.05$). Also, phenoloxidases have important roles in normal developmental and physiological processes (Lokstan and Li 1988) and this could be correlated to the reduction in the weight of insects that were fed transgenic material. Similar results were observed in the present study.

Acknowledgements

We would like to thank Dr. V.S. Reddy (International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi-110 067, India) for providing the *Cry11a5* gene.

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