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Efficiency of *Bacillus thuringiensis* strains and their Cry proteins against the Red Flour Beetle, *Tribolium castaneum* (Herbst.) (Coleoptera: Tenebrionidae)

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Abstract

Bacillus thuringiensis (*Bt*) is one of the used bioagents in insect pest control. Its toxicity is largely due to the insecticide endotoxins (crystalline (Cry) proteins) that act selectively on insects and nematodes. The efficiency of 20 of the most common Coleopteran-specific Cry proteins of *Bt* strains was tested against third instar-larvae of the red flour beetle *Tribolium castaeum* (Herbst.) (Coleoptera: Tenebrionidae). The primary screening results revealed that 11 Cry proteins (Cry8Ea, Cry8Fa, Cry1Ba, Cry8Ca, Cry1Fb, Cry1Ea, Cry1Ca, Cry55Aa, Cry9Da, Cry1Da, and Cry1Ia) were not toxic at all, 4 Cry proteins (Cry1Aa, Cry14Aa, Cry8Aa, and Cry7Ab) did not cause mortality but caused significant inhibition of growth, and 5 Cry proteins (Cry3Aa, Cry37Aa, Cry22Aa, Cry51Aa, and Cry3Ba) were toxic to *T. castaneum* larvae. The active 5 Cry proteins were used in the subsequent experiments. Five concentrations, being 0.25, 0.5, 1.0, 1.5, and 2.0 g Cry protein/10 g diet were used against the third instar larvae, and their mortalities were estimated. The LC_{50} values of Cry3Aa, Cry37Aa, Cry22Aa, Cry51Aa, and Cry3Ba were 0.46, 0.77, 1.25, 1.45, and 1.60 g/10 g, respectively. While the LT_{50} values of the same Cry proteins (for the concentration 2 g/10 g diet) were 1.50, 1.93, 2.29, 2.23, and 4.22 days, respectively. The results indicated that Cry3Aa was the most active one against *T. castaneum* larvae. The results of the sublethal study showed that the application of LC_{30} value of the active 5 Cry proteins reduced total eggs laid daily per female within 2 weeks, where, the percent decrease in egg numbers were 50.55, 38.56, 31.31, 23.20, and, 18.10% for Cry3Aa, Cry37Aa, Cry22Aa, Cry51Aa, and Cry3Ba, respectively. In addition, the hatchability rate of eggs, the larval, and pupal durations of *T. castaneum* decreased, while the pre-ovipositional period was prolonged. Furthermore, the larvae fed on a diet containing LC_{30} concentrations of Cry protein showed lower glycogen and lipid rates and generally lower protein content than the control larvae. When *T. castaneum* larvae were treated by Cry proteins, the level of digestive enzymes found in the midgut was decreased. The present findings indicated that *Bt* strains/Cry proteins had significant potential for controlling *T. castaneum*

Keywords: *Tribolium castaneum*, *Bacillus thuringiensis*, Bioassay, Sublethal concentrations, Digestive enzymes

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Background

Grains production is an important dietary component of human food in many countries. It is a good source of carbohydrates, vitamins, and some minerals, including trace elements like selenium in vegetarian diets of the majority of the population of the world (Poutanen, 2012). Stored grains insect pests can harm the national economy by infesting agricultural stored products (Jembere et al. 1995). The red flour beetle, *Tribolium castaneum* (Herbst.) (Coleoptera: Tenebrionidae) is a worldwide common pest of wheat flour. It also feeds upon dry fruits, pulses, and prepared cereal foods, where both larvae and adult beetles cause damage (Weston and Rattlingourd, 2000). The traditional control treatments in many countries depend on fumigants. Safety and environmental issues surrounding the use of chemical insecticides have led to a focus on the development of alternative control measures. One of the main effective alternatives to chemical control of insect pests is the biological control in nematodes as well as microbial agents viz., bacteria, fungi, viruses and protozoa (Cannon, 1993).

Among all the microbial agents, *Bacillus thuringiensis* (*Bt*) is considered one of the most common and widely used biological pesticide against insect pests (Lemaux, 2008). Various types of *Bt* formulations are available in the market as a liquid and powders and represent 90% of all types of bioagents sold today. *Bt* produces different kinds of poisons with insecticidal efficiency. Proteinaceous protoxins forming crystals parallel to the *Bt* spores during sporulation and known as Cry proteins play an important role in *Bt* toxicity. Various *Bt* strains produce in excess of 200 diverse Cry protoxins that act selectively on narrow ranges of hosts (Bravo et al. 2007). In light of their insecticidal toxicity and amino acid sequences, they have been divided into groups numbered from Cry1 to Cry55 (Crickmore et al. 2014). Each group involves various subgroups classes and subclasses that differ in specificity and activity. The significance of Cry toxins has extraordinarily expanded over the most recent 20 years when the genes encoding them were introduced into plants (Gould, 1998). Genetically modified crops expressing Cry proteins are resistant to specific pests without influencing different segments of the agro-ecosystems (Yu et al. 2011). Cry proteins are converted to active poisons by incomplete proteolytic cleavage that happens in the insect midgut (Ferre and Van Rie, 2002). Active toxin at that point ties to a particular receptor and is along these lines irreversibly embedded into the brush outskirt membrane of the epithelial midgut cells (Gilliland et al. 2002 and Hernandez et al. 2004). The membrane becomes punctured, and subsequent uncontrolled take-up of ions and water prompts the expanding of midgut cells and their possible lysis (Bravo et al. 2007). The insect dies because of general septicemia. It must be noticed that insect species significantly change in their susceptibility to *Bt* and that the susceptible species can develop resistance whenever exposed to a Cry toxin for various generations (Tabashnik et al. 2003).

In the present study, the potential of 20 common Coleopteran-specific Cry proteins was determined and the most active 5 ones were evaluated against third instar larvae of *T. castaneum* based on LC_{50} values. Some biological parameters and digestive enzyme activity were assessed in larvae that fed on an artificial diet containing sublethal concentrations of Cry protein.

Materials and methods

Rearing of experimented species

Rearing of *T. castaneum* was carried out by collecting adults from infested wheat flour, dry fruits, grains, rice, semolina, etc. These adults were placed in glass jars of 300 ml capacity. Each jar was filled with 1/4 sterilized mixture of the diet regimen comprising of 90% semolina and 10% yeast extract. Fifty adults were added to each jar. Every third day, a new diet was added to acquire enough larvae and adults for bioassay experimentation. The larvae were reared at 30 ± 1 °C and 60 ± 5% RH. The third instar larvae and newly emerged adults were obtained and used in bioassay experiments.

Bacillus thuringiensis strain

Bt has different Cry toxin protein types, each type usually acts on separate orders of insects (Crickmore et al. 1998). Twenty of the most common Coleopteran-specific *Bt* strains, which produce specific Cry proteins, were obtained from the State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University (Wuhan City, Hubei Province, China). Efficacy of 20 strains, listed in Table 1 was evaluated against, *T. castaneum*.

Microbiological technique

Bt strains were grown on Petri plates containing Luria-Bertani (LB) medium supplemented with the appropriate antibiotics at 28 °C for 24 h. For Cry protein production, the *Bt* strains were cultivated in a liquid ICPM medium (0.6% tryptone, 0.5% glucose, 0.1% $CaCO_3$, 0.05% $MgSO_4$, and 0.05% K_2HPO_4, PH 7.0) at 28 °C, 220 rpm for 3 days until cell lysis and complete sporulation. Smears of bacteria were stained by a simple stain for 1 min, washed with tap water, dried and observed under a light microscope to check the crystal production and morphology. After that, the culture was separated through centrifugation at 10000 rpm for 2–3 min. The collected sediment of *Bt* was mixed with distilled water and centrifuged at 300 rpm for 5 min. This centrifugation was repeated thrice in centrifugation tubes till all the media was washed away and the supernatant was discarded. At the bottom of the tube, the sediment was obtained, which was dried in an oven. The dried sediment was crushed in a pestle mortar to form the fine granule like powder.

Table 1 Different *Bacillus thuringiensis* strains and their cry proteins

No.	Bt strain	The produced Cry protein	No.	Bt strain	The produced Cry protein
1	20140314RJT017	Cry1Aa	11	20140926WHHT029	Cry8Aa
2	20140308CSFT034	Cry1Ba	12	20150611XART270	Cry8Ca
3	20140923LMMT088	Cry1Ca	13	20150611XART270	Cry8Ea
4	20140923LMMT088	Cry1Da	14	20141227ZHT080	Cry8Fa
5	20140221WWHT003	Cry1Ea	15	20141023WST136	Cry9Da
6	20141023WST132	Cry1Fb	16	20141202CGP002	Cry14Aa
7	20140907CDWT115	Cry1Ia	17	20141126CLLT002	Cry22Aa
8	20140919KYYT015	Cry3Aa	18	20141008WHHT043	Cry37Aa
9	20140728LTT243	Cry3Ba	19	20131204YWYT028	Cry51Aa
10	20131203LTT036	Cry7Ab	20	20140415YJPT508	Cry55Aa

Insect bioassays

Insect bioassays included incorporating the toxin protein into the artificial insect diet was carried out in two steps (MacIntosh et al. 1990). In the first step, the susceptibility of *T. castaneum* larvae to each Cry toxin tested was determined at a high protein concentration (2 g toxin/10 g diet) by incorporating the poison into the diet that offered to 30 larvae. The second step involved the determination of the LC_{50} for active Cry proteins. The concentration range used for each Cry protein was determined in preliminary bioassays. Thirty larvae were treated by each protein concentration. A variety of 5 concentrations (0.25, 0.5, 1.0, 1.5, and 2.0 g) were used for each toxin and mixed with a ten-gram diet, that is, 8 g semolina and 2 g of yeast extract. These concentrations were poured in glass vials and introduced 30 larvae in each vial separately. The bioassay was carried out three times. Control insects were fed an artificial diet without toxin. The insects' treatment were incubated at 25 °C and 60% RH. Mortality was observed, 1 day for 7 days. Data was analyzed by the probit analysis program and the LC_{50} was calculated.

Sublethal effects

The effect of sublethal concentrations (LC_{30}) of five active Cry proteins on certain biological aspects of *T. castaneum* adults was evaluated. The adults of *T. castaneum* (2 days old) were fed on the treated diet (LC_{30} of Cry protein) in a glass vial for 7 days. Then, certain biological aspects of surviving adults after treatment were studied to assess the number of eggs laid by a female for 14 days. Unmated females were paired in 3 × 4 cm glass tubes, containing small amounts of wheat flour, and covered with muslin. For estimating the incubation period of eggs and the developmental periods for the various stages, 1-day-old eggs were used. Individually, 10 replicates were used for each treatment. The incubation period of the egg was recorded. The developmental stages were observed, and their durations were also estimated. In this respect, the following parameters were recorded: average number of eggs laid daily per female, average total number of eggs laid per female during 2 weeks, pre-ovipositional period, incubation period of eggs, hatchability of eggs, average duration of larval stage, duration of pupal stage, and total developmental period of immature stages.

Biochemical analyses

For the biochemical studies, the third instar larvae were permitted to feed on a diet containing Cry proteins as well as the control diet. After 5 days of feeding on diets, larvae from each treatment were cold anesthetized independently at 5 °C. Consequently, total protein, glycogen, and lipid content were determined, and control larvae were treated for estimating the following components as depicted underneath. For each treatment, 6 to 8 replicates were used. To determine protein concentration, the strategy of Lowry et al. 1951 was used, while to estimate the glycogen content anthrone reagent was used and described by Yuval et al. 1998 with some modifications. Whilst to determine lipid content, lipids were extracted from individual larvae according to Van Handel, (1985) with some modifications.

Digestive enzyme activity assays

Third-instar larvae of *T. castaneum* were fed on a diet containing a concentration of LC_{30} value Cry proteins and reared as explained before. After 5 days post-treatment, the surviving larvae were individually Nathan submerged in ice-cold 0.15 M NaCl, and the midguts were dissected by the aid of a stereomicroscope (Stemi SV6 ZEISS, Germany) according to Borzoui and Bandani, 2013. The α-amylase activity was determined using the method of Bernfeld, 1955, using 1% starch as a substrate in the Tris-HCl buffer (pH 8). General proteolysis activity was determined by the method of Elpidina et al. 2001, using 1.5% azocasein as a substrate in a glycine-NaOH buffer (pH 10).

Data analyses

The data were corrected according to Abbott's formula (Abbott, 1925), and they were subjected to probit analysis using LDP line software according to Finney, 1971 to estimate LC_{30}, LC_{50}, and LC_{99} values of Cry proteins against third instar larvae of *T. castaneum*. Mortality percentages for different exposure times were subjected to analysis of variance (one-way ANOVA), using the statistical program (SPSS 2001) for probit analysis (Steel et al. 1997).

Results and discussion

Toxicity of Bt strains/Cry toxins

T. castaneum larvae showed different degrees of susceptibility to 20 of the most common Coleopteran-specific Cry proteins produced by *Bt* strains. At the concentration tested (2 g of toxin/10 g of artificial diet), the mortality percentages obtained with 11 *Bt* strains/Cry toxins (Cry8Ea, Cry8Fa, Cry1Ba, Cry8Ca, Cry1Fb, Cry1Ea, Cry1Ca, Cry55Aa, Cry9Da, Cry1Da, and Cry1Ia) were non-significantly different than those from the control larvae (reared on a toxin-free diet). Consequently, when a Cry toxin caused no mortality in *T. castaneum*, it was attributed to the lack of toxic activity of that particular Cry toxin or a reduced feeding rate on the toxin-contaminated diet. No mortality was observed with 4 *Bt* strains/Cry protein (Cry1Aa, Cry14Aa, Cry8Aa, and Cry7Ab), but those caused significant inhibition of growth. Five *Bt* strains/Cry proteins (Cry3Aa, Cry37Aa, Cry22Aa, Cry51Aa, and Cry3Ba) were toxic against *T. castaneum* larvae and resulted in larval mortality, which increased as the concentration was increased. These active 5 Cry toxins were used in the subsequent experiments.

Mortality of *T. castaneum* after feeding on diet contaminated with Cry toxin

Third instar larvae of *T. castaneum* were susceptible to the 5 Cry protein treatments showing high toxicity when incorporated into the diet. The LC_{30}, LC_{50}, and LC_{99} of Cry proteins are presented in Table 2**.** The lowest LC_{50} value (highest toxicity) against third instar larvae of *T. castaneum* was with Cry3Aa (0.46 g/10 g), followed by Cry37Aa, Cry22Aa, Cry51Aa, and Cry3Ba (0.77, 1.25, 1.45, and 1.60 g/10 g), respectively. The LT_{30}, LT_{50}, and LT_{99} values of Cry proteins to third instar larvae of *T. castaneum* are shown in Table 3. The results showed that the efficacy of Cry3Aa was faster than the other Cry proteins, followed by Cry37Aa, Cry22Aa, Cry51Aa, and Cry3Ba, respectively. The cumulative mortality percentage on third instar larvae of *T. castaneum* after 7 days of exposure and different concentrations of Cry proteins were shown in Fig. 1. The mortality percentage increased with increasing concentrations of Cry proteins. Generally, the active 5 *Bt* strains, which produced Cry proteins had toxic effects on third instar larvae of *T. castaneum*, although the toxicity of Cry3Aa was the highest.

Isolation and characterization of *Bt* strains is a common practice in search of biocontrol of insect pests (Magda, 2006). Nonetheless, investigators continued to seek new strains of *Bt* from diverse habitats, as each strain produces its characteristic effectiveness of its crystal protein, and the insecticidal activity of these proteins also differs considerably. In the present study, the screened strains were analyzed using a toxicity bioassay for their potency against *T. castaneum* larvae; which is known as one of the most damaging insects to stored grain products worldwide (Toews et al. 2005). Also, *Bt* is already reported as an effective biocontrol agent against lepidopteran and coleopteran larvae (Tamez-Guerra et al. 2004). The isolated toxins from various Cry proteins were tested by five concentrations (0.25, 0.50, 1.00, 1.5, and 2 g/10 g). The results were found to be dosage-dependent, and the percentage mortality of the tested insect increased in proportion with increased toxin concentration. In the present study, the results are in harmony with Van Frankenhuyzen, 2009 who found that Cry3Aa and Cry37Aa had activity against *T. castaneum*, also López-Pazos et al. 2009 found that Cry1B and Cry3

Table 2 Lethal and sublethal concentrations of various Cry proteins against third instar larvae of *Tribolium castaneum* 7 days post treatment

Bt strains/Cry proteins	Lethal concentration±95% F.L[a]. (g/10g diet)			Slope ± SD	Chi square (χ^2)	*p* value	*R.*
	LC30	LC50	LC99				
20140919KYYT015 Cry3Aa	0.13 0.05–0.20	0.46 0.32–0.58	35.97 14.46–193.13	1.22 ± 0.18	4.12	0.24	0.952
20150420LHMT145 Cry37Aa	0.21 0.10–0.30	0.77 0.60–0.97	67.66 23.48–493.82	1.19 ± 0.18	4.78	0.18	0.945
20141126CLLT002 Cry22Aa	0.31 0.17–0.44	1.25 0.97–1.75	143.10 39.31–1857.32	1.13 ± 0.19	2.89	0.40	0.962
20131204YWYT02 8Cry51Aa	0.31 0.15–0.44	1.45 1.09–2.28	296.91 60.05–9538.04	1.00 ± 0.19	3.66	0.29	0.942
20140728LTT243 Cry3Ba	0.38 0.21–0.52	1.60 1.21–2.51	229.43 52.95–4823.27	1.07 ± 0.19	0.91	0.82	0.985

[a]95 % Fiducial limits

Table 3 Lethal and sublethal times of various Cry proteins after treatment of third instar larvae of *Tribolium castaneum* at the highest concentration (2 g/10 g)

Bt strains/Cry proteins	Lethal time (day) ± 95% F.L[a]			Slope ± SD	Chi Square (χ^2)	*p* value	*R.*
	LT30	LT50	LT99				
20140919KYYT015 Cry3Aa	0.68 0.44–0.90	1.50 1.19–1.78	22.71 14.77–44.42	1.97 ± 0.22	5.68	0.12	0.971
20150420LHMT145 Cry37Aa	0.77 0.49–1.04	1.93 1.55–2.29	44.76 25.15–115.31	1.70 0.21	4.14	0.24	0.971
20141126CLLT002 Cry22Aa	0.79 0.45–1.10	2.29 1.82–2.77	89.54 41.13–354.89	1.46 0.21	2.28	0.51	0.978
20131204YWYT02 8Cry51Aa	1.22 0.83–1.56	3.23 2.71–3.89	92.15 44.37–319.17	1.59 0.21	0.38	0.94	0.996
20140728LTT243 Cry3Ba	1.66 1.24–2.04	4.22 3.55–5.23	104.01 49.77–360.90	1.67 0.22	0.18	0.98	0.998

[a]95 % Fiducial limits

proteins from *Bt* were toxic to coleopteran beetles such as the Colorado potato beetle and the cottonwood leaf beetle. The toxin crystals are known to dissolve easily in the insect midgut, liberating the protoxin, which then undergoes proteolysis where one of the fragments binds to the cells of the midgut epithelium. The activated protein is reported to disrupt the osmotic balance of insect cells by forming pores in the cell membrane. The insects stop feeding due to gut paralysis and die within a few hours of ingestion (Marrone and Macintosh, 1993). A variation was observed in the toxicity of various Cry proteins against *T. castaneum*. The highest mortality percentage resulted from Cry3Aa, followed by the isolates from Cry37Aa, Cry22Aa, and Cry51Aa, whereas the least activity effect was found when Cry3Ba was used against the target insect. Calculation of LC_{50} re-established these results, and the lowest LC_{50} was recorded for Cry3Aa, confirming it as the best potential source of the toxin, whereas the highest LC_{50} concentration (lowest potentiality) was recorded from Cry3Ba. These results confirmed the possible use of *Bt* strain toxin as a bio-insecticide against *T. castaneum*. These findings can guide future choices for environmentally friendly, integrated pest management strategies.

Effect of sublethal concentrations on *T. castaneum* adults

The effect of sublethal concentrations (LC_{30}) of 5 Cry protein on certain biological aspects of *T. castaneum* adults was evaluated and listed in Table 4. The results

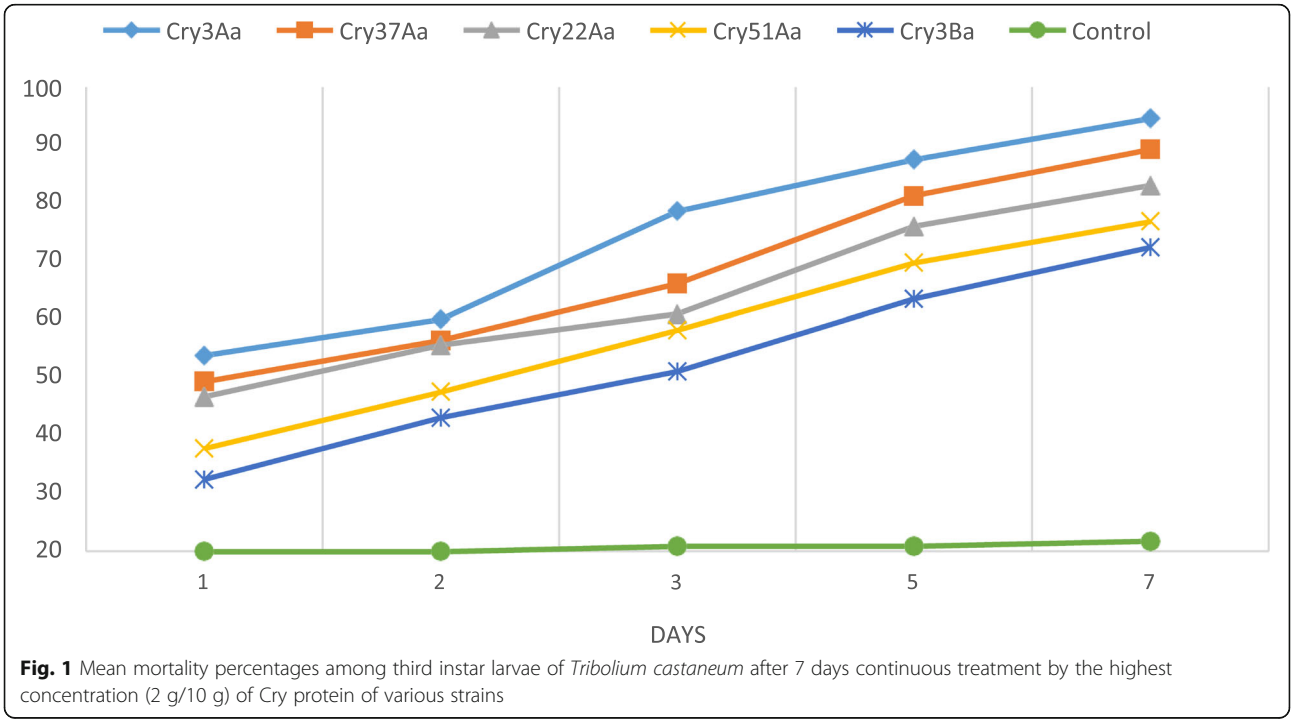

Fig. 1 Mean mortality percentages among third instar larvae of *Tribolium castaneum* after 7 days continuous treatment by the highest concentration (2 g/10 g) of Cry protein of various strains

Table 4 Sublethal effects of LC30 values of five Bt strains/Cry proteins on certain biological aspects of *Tribolium castaneum*

Biological aspects	Bt strains/Cry protein					
	20140919KYYT015 Cry3Aa	20150420LHMT145 Cry37Aa	20141126CLLT002 Cry22Aa	20131204YWYT028 Cry51Aa	20140728LTT243 Cry3Ba	Control
Total eggs laid by *T. castaneum* female during two weeks	418.66 ± 7.78^{F}	520.16 ± 8.97^{E}	581.5 ± 8.97^{D}	650.16 ± 4.07^{C}	693.33 ± 5.50^{B}	846.66 ± 7.08^{A}
Percent decrease in egg numbers	50.55	38.56	31.31	23.20	18.10	–
Total eggs hatching per female during two weeks	329.50 ± 8.40^{F}	426.16 ± 5.98^{E}	481.16 ± 4.44^{D}	555.66 ± 4.32^{C}	622.50 ± 4.63^{B}	804.16 ± 8.10^{A}
Hatchability rate of eggs	78.70 %	81.92 %	82.74	85.46	89.78	94.98 %
Pre-oviposition period (day)	6.83 ± 0.75^{A}	6.12 ± 0.75^{AB}	$5.60 \pm 0.89BC$	5.33 ± 1.03^{BCD}	4.16 ± 0.75^{CD}	4.66 ± 0.51^{CD}
Incubation period of eggs (day)	3.50 ± 0.54^{C}	4.50 ± 0.54^{B}	5.16 ± 0.98^{AB}	5.83 ± 0.98^{A}	6.00 ± 0.75^{A}	5.16 ± 0.75^{AB}
Larval stage duration (day)	21.16 ± 1.03^{C}	22.16 ± 2.99^{BC}	23.83 ± 1.04^{AB}	25.50 ± 1.04^{A}	25.83 ± 1.21^{A}	24.66 ± 0.81^{A}
Pupal stage duration (day)	3.83 ± 0.75^{D}	4.50 ± 0.83^{BCD}	5.33 ± 1.03^{ABC}	5.83 ± 0.75^{AB}	6.33 ± 0.51^{A}	4.83 ± 0.75^{C}
Total development period of immature stages (day)	28.66	31.16	33.99	37.16	38.13	34.65

Means followed by the same letter in a row are not significantly different at 0.05 level of probability
Letters above each treatment indicate significance between various Bt strains and control. Treatments with the same letter are not significantly different

revealed that treatment of *T. castaneum* adults with LC_{30} of 5 Cry proteins reduced significantly the number of daily deposited eggs per female throughout an observation period of 2 weeks by 50.55, 38.56, 31.31, 23.20, and 18.1% for Cry3Aa, Cry37Aa, Cry22Aa, Cry51Aa, and Cry3Ba, respectively. The pre-ovipositional period of females was prolonged by all Cry proteins, except Cry3Ba with which this period was decreased. The hatchability rates of daily laid eggs/female were significantly reduced with 5 *Bt* strains/Cry protein than the control. There were remarkable differences between the treatments and the control for the total larval and the pupal durations and total developmental period of immature stages.

These results are on the same line with those obtained by Desneux et al. 2007 who stated that the LC_{30} was chosen for sublethal effect studies because it is the mortality threshold (30%) recommended for the use of pesticides in integrated pest management, and therefore it is crucial in assessing possible sublethal effects on pests. These sublethal effects should be evaluated because they could have a strong impact on the population dynamics of lepidopteran pests and could contribute to its management (Pineda et al. 2009). In the present study, some of the biological parameters, such as total daily laid eggs/female for 2 weeks, hatchability rate of eggs, pre-ovipositional period, and incubation period of eggs, larval and pupal stage durations and total developmental period of immature stages of *T. castaneum* were evaluated after exposure to the active 5 *Bt* strain/Cry protein. In addition, Abedi et al. 2014 studied the lethal and sublethal effects of *Bt* subsp. *kurstaki* on third instar larvae of *Heliothis armigera* under laboratory conditions. Their results showed that the application of LC_{30} value of *Bt* reduced the larval and pupal weights and increased larval and pupal durations. The longevity and fecundity of female adults were affected significantly by the bioinsecticides. Female fecundity was reduced by treatments. In addition, Nouri-Ganbalani et al. 2016 studied the toxicity and biological effects of *Bt* strains on third instar larvae of *Plodia interpunctella* under laboratory conditions. The high mortality of larvae, growth retardation, including reduced larval and pupal weight, and prolongation of immature stages development were recorded in the treatment. Similar effects were observed in the present study. Adults of several important coleopteran pests have been reported previously to suffer reduced fecundity after exposure to pesticides (Pineda *et al.*, 2009). In the present study also, *Bt* strains/Cry proteins caused reduced fecundity and successful pupation of *T. castaneum*.

Total carbohydrate, lipid, and protein determinations

The results of the total carbohydrates, lipid, and protein in 7–14-day-old larvae of *T. castaneum* after fed on a diet containing LC_{30} of Cry proteins for 5 days are displayed in Table 5 and showed a statistically significant reduction in energy reserves in comparison to control larvae. Exposure to the LC_{30} of Cry3Aa resulted in greater magnitude in the reduction 72.5, 77, and 37.3% in protein, glycogen, and lipid content, respectively, in comparison to untreated (control) larvae. While exposure to LC_{30} of Cry37Aa resulted in reductions in protein (53.6), glycogen (71.30%), and lipid (27.21%) content, in comparison to control larvae.

The mean total carbohydrate, lipid, and protein contents in all treatments were significantly reduced than control. The decrease in protein content was observed in the larvae fed on treated wheat barn than the control larvae. Reduction in protein content is a common phenomenon in insects after treatment with toxic compounds (Nathan et al. 2008). The present results are supported by several reports where the toxicity of *Bt* strains caused a reduced protein content of insects (Abedi et al. 2014). Lipids are an important source of

Table 5 Energy reserves of treated third instar larvae of *Tribolium castaneum* at LC30 of Bt strains/Cry protein after 5 days of continuous exposure to the insecticide

BT strains/Cry protein	Total proteins		Total carbohydrates		Total lipids	
	Activity (mean ± S.E.)	Percent of decrease (%)	Activity (mean ± S.E.)	Percent of decrease (%)	Activity (mean ± S.E.)	Percent of decrease (%)
Control	27.38 ± 3.38^{A}	–	53.53 ± 2.59^{A}	–	1.58 ± 0.35^{A}	–
20140919KYYT015 Cry3Aa	7.53 ± 2.07^{D}	– 72.49	12.35 ± 1.90^{D}	– 76.92	0.99 ± 0.12^{BC}	– 37.34
20150420LHMT145 Cry37Aa	12.68 ± 2.47^{C}	– 53.68	15.36 ± 2.99^{D}	– 71.30	1.15 ± 0.16^{B}	– 27.21
20141126CLLT002 Cry22Aa	14.28 ± 1.84^{C}	– 47.84	28.23 ± 3.06^{C}	– 47.26	0.80 ± 0.06^{C}	– 49.36
20131204YWYT028 Cry51Aa	$16.68 \pm 1.49B^{C}$	– 39.07	17.20 ± 2.02^{D}	– 67.86	0.07 ± 0.02^{D}	– 95.56
20140728LTT243 Cry3Ba	20.22 ± 2.05^{B}	– 26.15	34.21 ± 2.94^{B}	– 36.09	0.17 ± 0.02^{D}	– 89.24

Letters indicate significance between strains. Means in columns with the same letter are not significantly differ

energy and are reserved in fat bodies. The reserve of lipids during the feeding period increased but was reduced in the non-feeding stage, and their amount can vary with growth stage and feeding condition (Nouri-Ganbalani et al. 2016).

Digestive enzyme activity in midgut from *T. castaneum* after Cry protein treatments

The midgut of third instar *T. castaneum* larvae after 5 days of continuous feeding on Cry proteins showed, statistically, significant reductions in α-amylase and general protease activities than the midguts from control larvae (Fig. 2). Specifically, the midguts from larvae that fed on a diet containing an LC_{30} concentration of Cry3Aa showed only 52 and 68% of the a-amylase and general protease activities, respectively, found in midguts of the control larvae. Similarly, midguts from larvae that fed on a diet containing the LC_{30} of Cry37Aa showed only 42 and 56% of the a-amylase and general protease

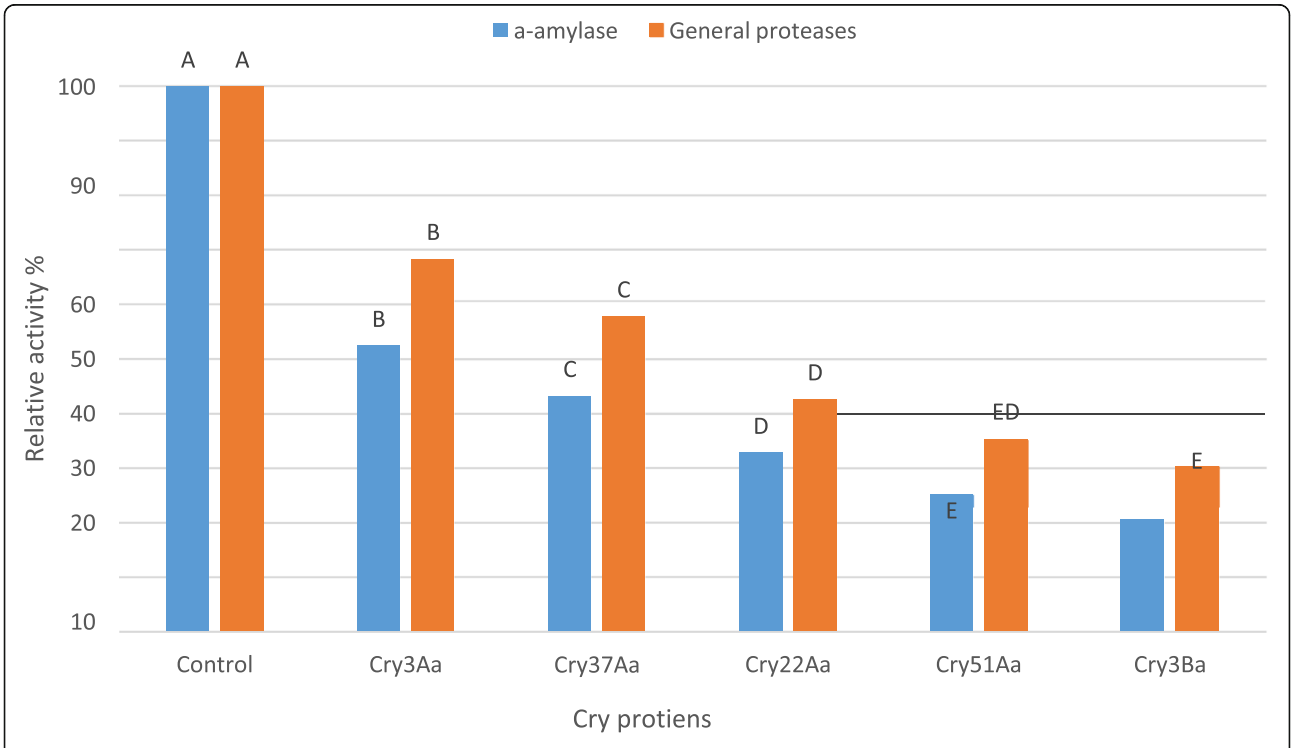

Fig. 2 Relative activity of digestive enzymes in the midguts of third instar *Tribolium castaneum* larvae that were fed to Bt (LC30). Each activity is shown as relative to the activity (100%) found in control larvae. Letters above columns indicate significance between doses. Columns with the same letter are not significantly different

activities, respectively, found in control midguts. Midguts from larvae that fed on a diet containing LC_{30} of Cry3Ba showed lower a-amylase and general protease activities (20 and 30%, respectively) in comparison to midguts from control larvae, and in comparison to midguts from larvae that fed on a diet containing only one of the insecticides (Fig. 2).

Conclusion

The results of the present study showed that *T. castaneum* was susceptible to Cry3Aa, Cry37Aa, Cry22Aa, Cry51Aa, and Cry3Ba. The results indicated that *Bt* strains/Cry proteins, negatively, affected the total daily laid eggs per female for 2 weeks, the hatchability rate of eggs and the larval and pupal durations, while, those increased the preovipositional period of *T. castaneum*. Obtained data revealed that *Bt* strains/Cry proteins had a high potential for controlling *T. castaneum*. After laboratory studies, more attention should be devoted to stored grain evaluations to obtain more applicable results.

Abbreviations

ANOVA: Analysis of variance; Bt: *Bacillus thuringiensis*; $CaCO_3$: Calcium carbonate; Cry: Crystalline; K_2HPO_4: Dipotassium phosphate; LC_{50}: The median lethal concentration; LT_{50}: The median lethal time; $MgSO_4$: Magnesium sulfate; NaCl: Sodium chloride

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Authors' contributions

The authors have equal contributions to this work. KE and NA released the research idea, design the research experiments, the main conceptual ideas, and proof outline, and performed the research experiments. KE screened the toxicity bioassay experiment and digestive enzyme activity assays. NA performed the microbiology and biochemical analysis experiments. EK and NA analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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