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Additive Effect of Chitinase Genes in Transconjugants of Entomopathogens *Bacillus thuringiensis* to Improve Biological Control of *Tuta absoluta*

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ABSTRACT

This study aimed to evaluate the toxicity of *Bacillus thuringiensis* (*Bt*) transconjugants harboring chitinase genes against larval stage of *Tuta absoluta* . *Bacillus licheniformis* and *Serratia marcescens* were used as donors against *Bt* . *Bacillus* transconjugants were evaluated for chitinase activity depending on hydrolysis zone appeared on chitin agar medium. Parental strains and their recombinants significantly reduced the number of survived larvae if compared with untreated larvae. Mortality rate was gradually increased to reached a maximum ratio after six days of treatments. Most bioinsecticides including parental strains and their transconjugants significantly losses larval body weight daily if compared with untreated larvae. Bioinsecticides, as well as, bioinsecticide concentrations and the interaction between both factors showed negative significant effect on larval body weight at most time intervals of larval age. In addition, *Bt* recombinants , as well as, their parental strains has exhibited a great potential effect for controlling and suppress the feeding of larval stage on the host plants.

Keywords: *Bacillus thuringiensis*, conjugation, larval mortality , larval weight, *Tuta absoluta* .

INTRODUCTION

Tuta absoluta was one of the important pests which is threating to tomato production in Egypt (Sabbour 2014). Infection by *T. absoluta* had resulted in 60-100% losses in tomato (Cely *et al*. 2006). Larvae can attack tomato plants during all growth stages, producing high galleries in leaves, burrowing stalks, apical buds, green and ripe fruits (IAN 1994), causing losses of tomato production (Caceres 1992). The larval stage feed on mesophyell tissues and make irregular mine on leaf surface. Damage can reached up to 100%. This pest damage occurs throughout the entire growing cycle of tomatoes. *Bacillus thuringiensis*, an entomopathogenic bacterium, has also been used in the biocontrol of tomato insect pests (Marques and Alves 1996).

Chitinase enzyme induced damage to the peritrophic membrane in the pest gut causes a significant losses in nutrient metabolism and consequently reduced insect growth (Terra and Ferreira 2005). Chitinolytic bacteria have been shown to be important agents for biocontrol of both pathogenic fungi and insect pests (Sampson and Gooday 1998). One of the almost important insecticidal microbes used in bio-control is *Bacillus thuringiensis* (*Bt*), that developed delta endotoxin protein(s) during the stationary phase of the growth. *B. thuringiensis var. kurstaki* (Btk) caused mortality in all *T. absoluta* instars and the use of *Bt* has synergistic effects when applied to tomato resistant genotypes (Giustolin *et al*. 2001). *Serratia marcescens* was one of the almost higher chitin-degraders and many types of chitinase producer genes have been decided in many strains of serratia, such as *ChiA*, *ChiB*, and *ChiC1* (Suzuki *et al.* 2002).

Over-expression of chitinase in entomopathogenic microbes could increase insect mortality (Fan *et al*. 2007). Recently, a number of heterologous *Chi* genes had been transfered from different bacterial strains into *B. thuringiensis* to increase their insecticidal activity against insects. (Bhattacharya *et al*. 2007). In this area, Ozgen *et al*. (2013) introduced *chiB* and *chiC* genes from *S. marcescens* into some *B. thuringiensis* strains which leading to increased insecticidal activity. Similar works were done by many researchers and they all reported to improved insecticidal activity by the improved strains (Driss *et al.* 2011).

Transfer of bacterial plasmids via conjugation was the most efficient method of horizontal gene spread, therefore, considered one of the important reasons for increasing the number of bacteria that exhibited multiple-antibiotic resistance (Grohmann *et al*. 2003). Plasmid transfer between *B. thuringiensis* strains could be monitored directly by the plasmid profiling analysis of cells. This is possible when transfer frequencies are high and all recipients in a population become transconjugants (Aronson and Beckman 1987).

Thus, this study aimed to induce genetic recombinants in some strains of *Bacillus thuringiensis* via conjugal transfer of plasmids from the donars to the reciepients to increase their insecticidal activity against tomato pest, *Tuta absoluta*.

MATERIALS AND METHODS

Bacterial strains and their culture conditions

Bacterial strains used in this study are listed in Table 1.

Bacterial culture conditions

Luria broth medium (LB broth) was used for *Bacillus thuringiensis and Bacillus licheniforms* according to Sambrook *et al*. (1989). Peptone yeast extract medium (PWYE) was used for separation of crystals and endospores according to Karamanlidou *et al*. (1991). Peptone glycerol medium (PGM) was used to enhance pigmentation, as well as, for the maintenance of *Serratia marcescens* according to Harris *et al*. (2004). However , mineral medium (MM) named M9 minimal medium was used as a minimal medium in mating experiments according to Sambrook *et al.* (1989). In addition, sporulation medium (MSM) was used for sporulation as previously described by Ellar and Postage (1974), with the exception of some modifications according to Gordon *et al.* (1981) .

Colloidal chitin medium

Colloidal chitin was prepared from chitin flakes aacording to Mathivanan *et al.* (1997). The medium was prepared according to Agrawal and Kotasthane (2012).

Table 1. Bacterial strains and their sources .

Strains	Source or reference	Designation
Serratia marcescens	Microbial Activity Unit, Microbiology Dept., Soil, Water and Environmental Research Institute, Agricultural Research Center (ARC).	*Sm*
Bacillus licheniforms	National Center for Agriculture Utilization Research, USA.	*BL*
Bacillus thuringiensis 4A7	Daniel R. Zeigler, Ph.D., Bacillus Genetic Stock Center, Biochemistry Dept., Ohio University, Columbus, USA.	Bt_1
Bacillus thuringiensis 4Q1	Daniel R. Zeigler, Ph.D. Bacillus Genetics Stock Center, Biochemistry Dept., Ohio University, Columbus, USA.	Bt_2

Antibiotics used

Auxotrophic and antibiotic genetic markers are an important selectable tools in the development of bacterial recombinants. Ideally, the antibiotic markers allow efficient selection for bacterial recombinants without affecting any of cellular metabolism. Antibiotic resistance or sensitive genetic markers are alternative to auxotrophic mutant markers. Thus, fourteen antibiotics were used in this investigation with different concentrations (µg/ml) as shown in Table 2.

Table 2. Antibiotics and their concentrations used in this study against bacterial strains.

Antibiotics	Abbreviations	Concentration (µg/ml)
Chloramphenicol	*Cm*	35
Ampicillin	*Ap*	50
Tetracycline	*Tc*	20
Penicillin	*Pc*	150
Neomycin sulphate	*Nm*	800
Erythromycin-ethlsuccinate	*Eryth*	20
Rifampicin	*Rif*	150
Vancomycin	*Vc*	150
Hibiotic	*Hb*	400
Amoxycillin	*Am*	400
Ceftazidime	*Ce*	400
Cefotaxime	*Cf*	400
Cefoperazone	*Cp*	150
Genamycin	*Gm*	20

Target insect

A wild type strain of *Tuta absoluta* was used in this study. It was collected from the Plant Mangment

Farmer, Sakha Research Station , Agriculture Research Center, Kafr El-Sheikh Governorate - in June, 2015. This strain was collected as a colony of eggs on untreated tomato leaves without any insecticides treatment . Egg colony were kept in Petri-dishes covered with cotton cloth. The Petri-dishes were daily supplemented with tomato leaves as a source of food till hatching. Newly hatched larvae at six days old were transfered to feeding on tomato leaves treated with *Bt* bioinsecticides via a dipping method, although leaves dipped in water were used as a control. Larvae of *Tuta absoluta* were put in glass jar (250 ml) and feeding on treated tomato leaves added daily.The leaves used in this experiment were cut from the first to the ninth node of the plant from the stem. Insects were reared on tomato leaves in a laboratory under constant conditions of 25 ± 2ºC, and 16 : 8 light : dark photoperiod regime and relative humidity 75 ± 5% (El- Adl *et al.* 2016) .

Genetic marking

Antibiotic susceptibility test was used in this investigation for genetic marking bacterial strains. Susceptibility to antibiotics was assayed by the plate diffusion technique according to Collins and lyne (1985).

Conjugation procedure

Mating experiments were performed using *Serratia marcescens* and *Bacillus Licheniforms* as a donor strains , agains *Bacillus thuringiensis* as a recipient. Transconjugant single colonies appeared on selective medium were picked up and transfered to LB slant agar medium according to Grinsted and Bennett (1990).

Screening chitinolytic activity

For evaluating of chitinase - producing microorganisms, a mineral medium containing colloidal chitin was used according to Someya *et al*. (2011).

Separation of crystals and endospores

Bacteria were grown in petri dishes to be collected the pellets of spores and crystals from nutrient agar plates which washed two times in ice-cold distilled water. Then resuspended the pellets in small volumes of distilled water. Spores and crystals were collected by centrifugation and final pellets were resuspended in 20 ml of water and maintained in a refrigerator freezer according to Karamanlidou *et al*. (1991).

Bioassays of bioinsecticide toxicity

To assess the activity of bioinsecticides dipping technique was applied as described by Tabashnik *et al*. (1991), where fresh tomato leaves were immersed in each of the tested concentration for 30 sec. Eight bioinsecticides in addition to the control were used with three replicates for each concentration . The effects of bioinsecticides was evaluated against six days – old larvae of *Tuta absoluta* (mean weight = 0.0039 mg) at 25°C under laboratory conditions.

Three grams of *Bt* treated leaves were added daily to a new breeding bottle. The survived larvae were counted daily, weighting and then moved to clean jars supplemented with treated leaves except the control which supplemented with untreated leaves until pupation. The leaves were replaced daily by another treated ones after the jars were cleaned and dryed. Larval lethality was measured daily up to pupation developed. Lethality percentage was corrected by abbott formula (Abbott 1925) as follows:

Abbott's formula

$$\% \text{ Mortality} = \frac{Control\ survival - Treatment\ survival}{Control\ survival} \times 100$$

Toxicity index (Sun 1950) was calculated using the following formula:

Toxicity index (TI) =

LC_{50} of the efficient bioinsecticide / LC_{50} of the other bioinsecticide × 100.

The feeding deterrent index (FDI) for each treatment was calculated as (C - T)/(C + T) × 100.

Where C and T are the control and treated leaf areas consumed by the larva, respectively (Isman *et al.* 1990).

Statistical analysis:

Data were subjected to the statistical analysis of variance according to Snedecor and Cochran (1955). Least significant difference (L.S.D.) was used to compare between means if the F-test was significant .

RESULTS AND DISCUSSION

Genetic markers related to antibiotic resistance

The results presented in Table 3 illustrated the resistance / sensitive pattern of 14 antibiotics tested by disc diffusion technique on nutrient agar plates against four bacterial strains. The pattern of these strains showed multipled resistance and sensitive against the antibiotics . The results showed that *Serratia marcescens* was resistant to penicillin and erythromycin-ethlsuccinate , whereas sensitive to other antibiotics. However, *Bacillus licheniform*s was resistant to ampicillin, cefoperazone, hibiotic, amoxycillin, ceftazidime cefotaxime, and sensitive to other antibiotics .In addition, Bt_1 showed resistant to ampicillin, penicillin, hibiotic, amoxycillin, ceftazidime, gentamycin and sensitive to other antibiotics. However, Bt_2 was resistant to penicillin, ceftazidime, genamycin , whereas sensitive to other antibiotics . All bacterial strains tested were resistant to penicillin, whereas sensitive to chloramphenicol, tetracycline , neomycin sulphate, rifampicin, vancomycin and cefoperazone. These results are in agreement with Bautista and Teves (2013), who found that *Bt* strains were resistant to β- lactams (amoxicillin and ampicillin). Bernhard *et al.* (1987) found that *Bacillus* strains isolated from the soil were resistant to four different antibiotics .

Conjugation between bacterial strains

In this study , plasmid transfer via conjugation was carried out between *Serratia marcescens* , *Bacillus licheniforms* as a donor strains against different strains of *Bacillus thuringiensis* as a recipients depending on the oppsite genetic markers harboring strains (Table 4). Transconjugants appeared on selective medium were picked up and maintained on nutrient agar slants. Horizontal transfer mechanism of DNA between bacterial species may played a significant role in evolutionary bacteria. Gene transfer may occurred between distantly related bacterial species. Bacterial conjugation is one of gene transfer mechanism (Mazodier and Davies 1991). Plasmids were transfered in a wide range of bacterial species between distantly related bacteria (Farrand 1993). Conjugative transfer of bacterial plasmids is the most imortant tool of horizontal gene spread and it is, therefore, considered one of the major reasons for increasing the number of bacteria that exhibited multiple-antibiotic resistance (Grohmann *et al*. 2003).

Table 3. Genetic markers of different bacterial strains depending on resistance (+) and sensitive (-) to antibiotics.

Antibiotics	Strains			
	Sm	***BL***	***Bt_1***	***Bt_2***
Cm	-	-	-	-
Ap	-	+	+	-
Tc	-	-	-	-
Pc	+	+	+	+
Nm	-	-	-	-
Eryth	+	-	-	-
Rif	-	-	-	-
Vc	-	-	-	-
Hb	-	+	+	-
Am	-	+	+	-
Ce	-	+	+	+
Cf	-	+	-	-
Cp	-	-	-	-
Gm	-	-	+	+

Table 4. Conjugation between *Serratia, B. lichenformis* as a donor strains against *Bacillus thurinogiensis as a recipient strains* .

Mating	Parental genotypes	Suitable time needed for genetic transfer	Time needed to appeared transconjugants on selective media (day)	Recombinant genotype	Transconjugants	Renamed of Transconjugants used in this study
Sm X Bt_1	Ap^- $Eryth^+$ X Ap^+ $Eryth^-$	3	2	Ap^+ $Eryth^+$	Tr_2 Tr_6	Tr_1 Tr_2
Sm X Bt_2	Ce^- $Eryth^+$ X Ce^+ $Eryth^-$	6	4	Ce^+ $Eryth^+$	Tr_{14} Tr_{20}	Tr_3 Tr_4
BL X Bt_1	Cf^+ Gm^- X Cf^- Gm^+	10	3	Cf^+ Gm^+	Tr_{22} Tr_{27}	Tr_5 Tr_6
BL X Bt_2	Cf^+ Gm^- X Cf^- Gm^+	3	3	Cf^+ Gm^+	Tr_{33} Tr_{39}	Tr_7 Tr_8

+ , - = Resistance and sensitive to antibiotic , respectively.

Chitinase activity

The data presented in Table 5 showed that both transconjugants Tr_2 and Tr_6 appeared significant express of chitinase activity than the mid parents. Among the 10 transconjugants Tr_2 and Tr_6 produced purple colored zone. These results agreed with Bahar *et al*. (2012) , who found that bacteria produced chitinase to be able to hydrolysis chitin polymer and produced metabolites to support their growth in the media supplemented with chitin as the only carbon and energy source without any nutrients. Whereas, Brurberg *et al*. (2001) decided that *Serratia marcescens* was an important bacteria with its efficient chitinase producer. However, Kamil *et al* . (2007) found that only 5% of 400 isolates (*Bacillus* sp.) appeared different clear zones sizes via chitinase activity. The chitin degrading bacteria used colloidal chitin as a sole source of carbon and

energy which formed colonies surrounded by clear zone indicating chitinase production .

Table 5. Chitinase production by transconjugants resulted from the mating between *Sm* and Bt_1.

Strains	Chitinase production	
	Diameter (cm) of clear zones	Diameter (cm) of the purple colored zone
Sm	1.7	1.6
Bt_1	2.3	2.0
Mid- parent	2.0	1.8
Tr_1	0.0	0.0
Tr_2	1.7	1.3
Tr3	0.0	0.0
Tr_4	0.0	0.0
Tr_5	0.0	0.0
Tr_6	1.6	1.5
Tr_7	0.0	0.0
Tr_8	0.0	0.0
Tr_9	0.0	0.0
Tr_{10}	0.0	0.0
F-test	**	**
LSD 0.05	0.12	0.10
0.01	0.16	0.13

The results summarized in Table 6 appeared that some of recombinants (Tr_{14}, Tr_{19} and Tr_{20}) reflected high express of chitinase activity if compared with the mid parents. These results agreed with Kuzu *et al*. (2012) , who showed that *Bacillus thuringiensis* subsp. *kurstaki* showed its optimum activity of chitinase following three hours of incubation period . Reid and Ogrydziak (1981) found that maximum chitinase activity was obtained from *S. marcescens* grown in reese medium (with yeast extract) . Thamthiankul *et al*. (2001) found that *Bacillus thuringiensis* produced multiple chitinases with different molecular weights.

Table 6. Chitinase activity by transconjugants resulted from the mating between *Sm* and Bt_2.

Strains	Halo zone of chitinase production	
	Diameter of clear zones(cm)	Diameter of the purple colored zone (cm)
Sm	1.7	1.6
Bt_2	1.9	2.1
Mid - parent	1.8	1.85
Tr_{11}	0.0	0.0
Tr_{12}	1.9	1.2
Tr_{13}	2.1	1.9
Tr_{14}	4.0	3.1
Tr_{15}	2.2	1.5
Tr_{16}	1.9	1.6
Tr_{17}	1.7	1.6
Tr_{18}	2.4	1.7
Tr_{19}	2.8	2.4
Tr_{20}	3.2	2.9
F-test	**	**
LSD 0.05	0.74	0.62
0.01	1.01	0.91

The data presented in Table 7 showed that some transconjugants (Tr_{33}, Tr_{39}) between *BL* and Bt_2 appeared significant diameter of halo zone which reflected increase in chitinase production above the mid – parents because of high performance of clear zone appeared from hydrolyzing the colloidal chitin . These results indicated that chitinase gene was present in the genome of *Bt* and *BL* . This agreed with Rey *et al*. (2004) , who found that the genome sequence analysis of *B. licheniformis* also revealed the presence of chitinase gene. In addition, Dahiya *et al*. (2005) reported that chitinases isolated from different bacterial strains including *Bacillus spp.* produced multiple forms of chitinases with different molecular weights . Barboza - Corona *et al*. (1999) proposed that *Cry* proteins and chitinase from *Bt* species could have synergistic effects in improving *Bt* insecticidal activity. Furthermore, the potential of *Bt* chitinase might be used toward the control of plant pathogenic fungi .

Table 7. Chitinase activity by transconjugants resulted from the mating between *BL* and Bt_2.

Strains	Halo zone of chitinase production	
	Diameter of clear zones(cm)	Diameter of the purple colored zone (cm)
BL	2.0	1.6
Bt2	1.9	2.1
Mid - parent	1.95	1.85
Tr_{31}	0.0	0.0
Tr_{32}	0.0	0.0
Tr_{33}	2.9	3.5
Tr_{34}	0.0	0.0
Tr_{35}	0.0	0.0
Tr_{36}	1.2	2.1
Tr_{37}	1.9	1.8
Tr_{38}	0.0	0.0
Tr_{39}	2.1	3.5
Tr_{40}	0.0	0.0
F-test	**	**
LSD 0.05	0. 29	0.36
0.01	0.37	0.49

The entomo pathogenic effect of *Bacillus thuringiensis* and their genetic recombinants after six days of treatments was summarized in Table 8. The results demonstrated that lethality rates were reached zero at 8 x 10^5 ppm in response to the parental strains and their recombinants. The same trend was also showed by recombinants and their parents of all conjugations at the concentrations of 2, 4 and 6 x 10^5 ppm . This indicated that mortality was gradually increased to reached a maximum ratio after six days of treatments. Parental strains and their transconjugants significantly reduced the survival of larvae if compared with untreated ones. The accumulated mortality of larval stage of *Tuta absoluta* showed a time – response. This indicated that it is recommended to use bioinsecticides against the larval stage of *Tuta absoluta* for six days at least .These results are in harmony with Abdullah *et al.* (2014) , who showed that the effect of five recombinants and their parents (*B. thuringiensis* and *B. subtilis*) on the 2nd instar larvae of *T. absoluta* exhibited significantly higher effect by *Bt* in comparison with *B. subtilis*. The same authors also found that mortality percentages were reached to 87.6 and 91.6% after six days of treatments. In general, mortality rates were increased via the treatments with spores than without its. In addition , Hernandez-Fernandez *et al.* (2010) found that ten *Bt* strains were increased larval lethality over the eight days following the

crude extract application. Finney (1971) evalutated the hypothesis that suggested a linear correlation was obtained between doses and larval lethality. The same authors found 4.8 µg ml^{-1} crude protein extract of *Bt* strain produced 58.3%, lethality on *T. absoluta* larvae, while the same doses of the reference strain *Bt*k yielded lower mortality (50.8%). Youssef and Hassan (2013) found that commercial product of *B. thruingiensis* var. *kurstaki* was highly insecticidal activity on the different larval stages of *T. absoluta*. Roh *et al*. (2007) reported that the potential of commercial biocide *B. thuringiensis* subsp *kurstaki* in controlling insects of economic importance is well known as a key tool of Integrated Pest Management Programs.

Table 8. Effect of different concentrations of crystals and endospores resulted from recombinant bioinsecticides on larval survival after six days of treatment.

Treatments	Bioinsecticide concentrations (ppm) x 10^5							
	2		4		6		8	
	Number of survival larvae	Mortality %	Number of survival larvae	Mortality %	Number of survival larvae	Mortality %	Number of survival larvae	Mortality %
Control	10	0	10	0	10	0	10	0
Sm	1	90	1	90	0	100	0	100
Bt_1	0	100	0	100	0	100	0	100
MP	0.5	95	0.5	95	0	100	0	100
Tr_1	1	90	0	100	1	90	0	100
Tr_2	0	100	0	100	1	90	0	100
F- test	**		**		**		**	
LSD 0.05	1.64		1.13		0.90		0.53	
0.01	2.43		1.24		1.41		0.74	
Control	10	0	10	0	10	0	10	0
Sm	1	90	1	90	0	100	0	100
Bt_2	0	100	0	100	0	100	0	100
MP	0.5	95	0.5	95	0	100	0	100
Tr_3	1	90	0	100	1	90	0	100
Tr_4	0	100	0	100	1	90	0	100
F- test	**		**		**		**	
LSD 0.05	1.50		1.23		1.53		0.70	
0.01	2.12		1.71		2.20		1.14	

Table 8. Continued.

Treatments	Bioinsecticide concentrations (ppm) x 10^5							
	2		4		6		8	
	Number of survival larvae	Mortality %	Number of survival larvae	Mortality %	Number of survival larvae	Mortality %	Number of survival larvae	Mortality %
Control	10	0	10	0	10	0	10	0
Bl	0	100	0	100	0	100	0	100
Bt_1	0	100	0	100	0	100	0	100
MP	0	100	0	100	0	100	0	100
Tr_5	0	100	0	100	0	100	0	100
Tr_6	1	90	1	90	1	90	1	90
F- test	**		**		**		**	
LSD 0.05	0.91		0.70		0.52		0.52	
0.01	1.34		1.12		0.71		0.71	
Control	10	0	10	0	10	0	10	0
Bl	0	100	0	100	0	100	0	100
Bt_2	0	100	0	100	0	100	0	100
MP	0	100	0	100	0	100	0	100
Tr_7	0	100	1	90	0	100	0	100
Tr_8	0	100	0	100	0	100	0	100
F- test	**		**		**		**	
LSD 0.05	0.82		0.90		0.51		0.51	
0.01	1.21		1.32		0.70		0.70	

***, ** = Significance at 0.05 and 0.01 levels of probability, respectively .**

Larval weight

As shown in Table 9 the results appeared that there were a significant differences between bioinsecticides in larval weight at all concentrations used. Most of *Bt* bioinsecticides significantly reduced larval weight as compared with that feeding on untreated diet. This agreed with Yee and Toscano (1998), who found a decrease in larval weight of third and fifth instars of *S. exigua* exposed to lettuce leaf disks contaminated with spinosad. Neurotoxic insecticides cause paralysis of pests and consequently a cessation of feeding (Haynes 1988). In addition , Johnson and Freedman (1981) found that the lowest toxin concentration tested (0.025 ug of toxin protein per g of diet) appeared 44% of reduction in average weight (32 mg per larva) if compared to nontreated control larvae (60 mg per larva).

Table 9 . Effect of different concentrations of crystals and endospores resulted from recombinant bioinsecticides on larval weight after six days.

Treatments	Bioinsecticide concentrations (ppm) x 10^5			
	2	4	6	8
Control	3.9	3.9	3.9	3.9
Sm	0.1	0.1	0.0	0.0
Bt_1	0.0	0.0	0.0	0.0
MP	0.05	0.05	0.0	0.0
Tr_1	0.1	0.0	0.3	0.0
Tr_2	0.0	0.0	0.3	0.0
F- test	**	**	**	**
LSD 0.05	0.60	0.16	0.65	0.59
0.01	0.84	0.22	0.94	0.86
Sm	0.1	0.1	0.0	0.0
Bt_2	0.0	0.0	0.0	0.0
MP	0.05	0.05	0.0	0.0
Tr_3	0.1	0.0	0.1	0.0
Tr_4	0.0	0.0	0.1	0.0
F- test	**	**	**	**
LSD 0.05	0.60	0.60	0.07	0.59
0.01	0.84	0.85	0.14	0.86
Bl	0.0	0.0	0.0	0.0
Bt_1	0.0	0.0	0.0	0.0
MP	0.0	0.0	0.0	0.0
Tr_5	0.0	0.0	0.0	0.0
Tr_6	0.4	0.1	0.1	0.1
F- test	**	**	**	**
LSD 0.05	0.58	0.59	0.58	0.58
0.01	0.84	0.85	0.84	0.84
Bl	0.0	0.0	0.0	0.0
Bt_2	0.0	0.0	0.0	0.0
MP	0.0	0.0	0.0	0.0
Tr_7	0.0	0.1	0.0	0.0
Tr_8	0.0	0.0	0.0	0.0
F- test	**	**	**	**
LSD 0.05	0.60	0.07	0.59	0.59
0.01	0.87	0.09	0.86	0.86

As shown from the results diagrammatic in Figure 1 the larval weight gain of *Tuta absoluta* through six days of feeding on different concentrations of recombinant bioinsecicides was decreased gradually through the six days . The results appeared a strong reduction in larval weight after three days of feeding on *Bt* – treated leaves .The most efficient decrease in larval weight gain of *Tuta absoluta* was shown in response to the concentration of 8 x 10^5 ppm . These results are in harmony with Jamoussi *et al.* (2013), who demonstrated that *B. thuringiensis* KS is a promising strain that produced more efficient δ-endotoxins against *T. absoluta* than the reference strain HD1 already largely used in biological control .The result also agreed with Gobbi *et al.* (2000), who revealed that the concentration of 100 mg active ingredient (AI) /Kg diet of tubufenozide caused a weight reduction of 55 and 74% in fourth instars of *Mythimna unipuata* and *S. littoralis* , respectively , after 48 h of treatment . Moreover, Zhao *et al.* (1999) found that transgenic plants of tabacco via two insecticidal genes (*cry 1 A* and *CpT1*) could significantly delay resistance developed in *H. armigera* if compared with one gene (*cry A1*) transgenic plants . The effects caused by recombinant *Bt* are very important from a practical point of view because larval feeding damage to crop would be suppressed , leading to reduced offspring, as well as , the insect population as a consequence can be still below a level of economic loss.

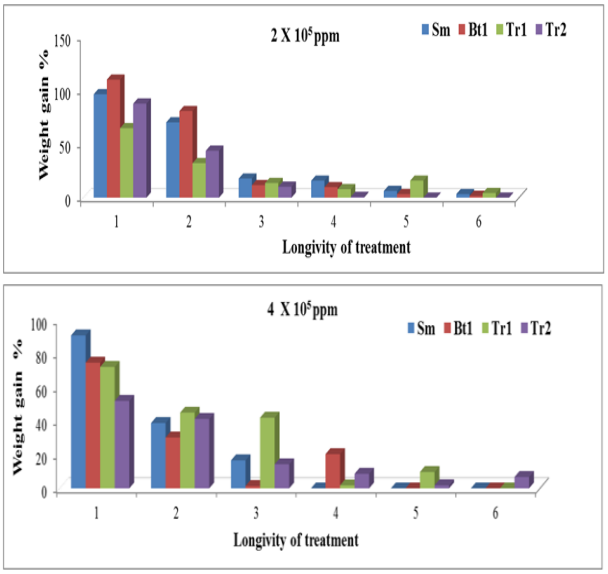

Figure 1. Larval weight of *Tuta absoluta* gain treated with different concentrations of Bt toxins produced by recombinants obtained from the mating between *Sm* x *Bt1*.

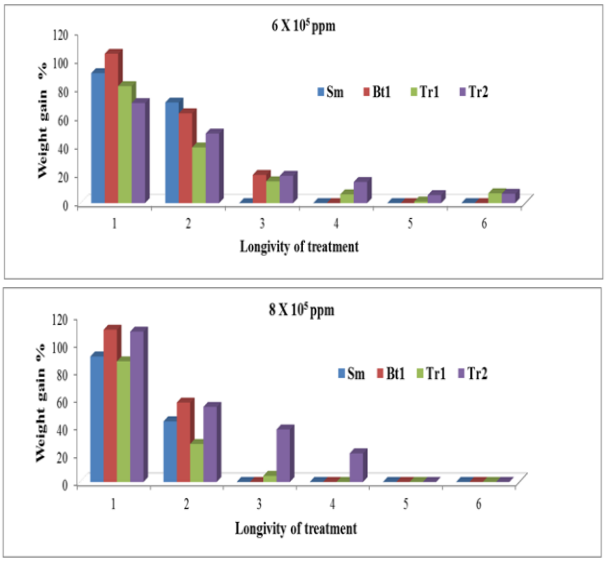

Figure 1. Continued.

As shown in Figure 2 the weight gain of *Tuta absoluta* larvae was more reduced through six days of feeding. The results appeared a strong reduction in larval weight by *Bt* recombinants after four and two days of consumption on *Bt* – treated leaves with the concentrations of 2 x 10 ^{5} ppm and 4 x 10^5 ppm ,respectively .Also a strong reduction in the gain of larval weight was shown after three days of feeding on *Bt* – treated leaves with the concentrations of 6 x 10 ^{5} ppm and 8 x 10 ^{5} ppm . These results are in harmony with Habib and Amaral (1985), who found that the wet table powder formation of *Bt var . kurstaki* with a flow rate of 20L / ha gave best control against the larvae of *Anticarisa gammatalis* on soybean in Barzil ,as well as, larvae stop feeding within a little hours of ingesting *Bt* protein. Probably the effect of *Bt* on feeding was related to the damage in midgut tissues and to gut paralysis . The effect of *Bt* as shown in this study is useful in terms of preventing economic loss due to defoliation and damage to inner tomato plant tissues . However, Wiwat *et*

al. (2000) found that the toxicity against diamondback moth larvae (*P. xyostella*) increased with using *B. thuringiensis* which produced higher amount of chitinase if compared with other *Bt* strains which do not produced chitinase or produce little amount. In addition , Bravo *et al*. (2007) found that *Bt* strains synthesize δ-endotoxins at the stage of sporulation in the stationary growth phase as parasporal crystalline inclusions . Once ingested by pests, these delta endotoxin are solubilized in the midgut, then the toxins are proteolytically activated by midgut proteases which bind to specific receptors distributed in the insect cell membrane , leading to cell disruption and insect mortality.

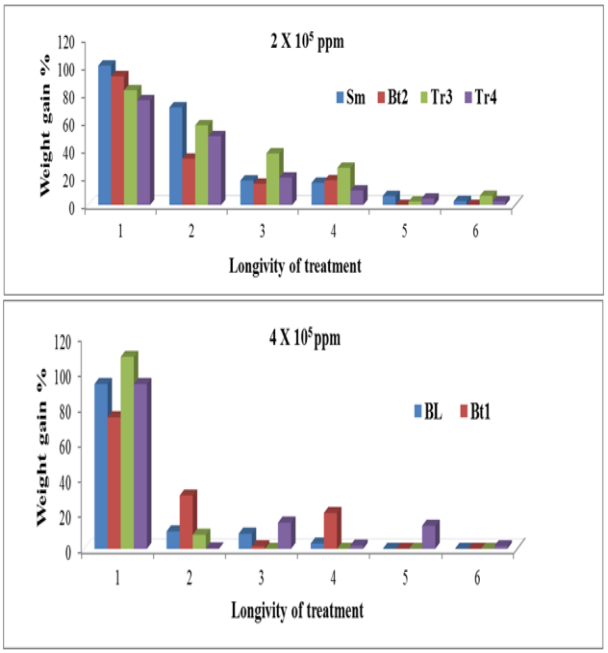

Figure 2. Larval weight gain of *Tuta absoluta* treated with different concentrations of crystal toxins produced by recombinants obtained from the mating between *BL* against Bt_1.

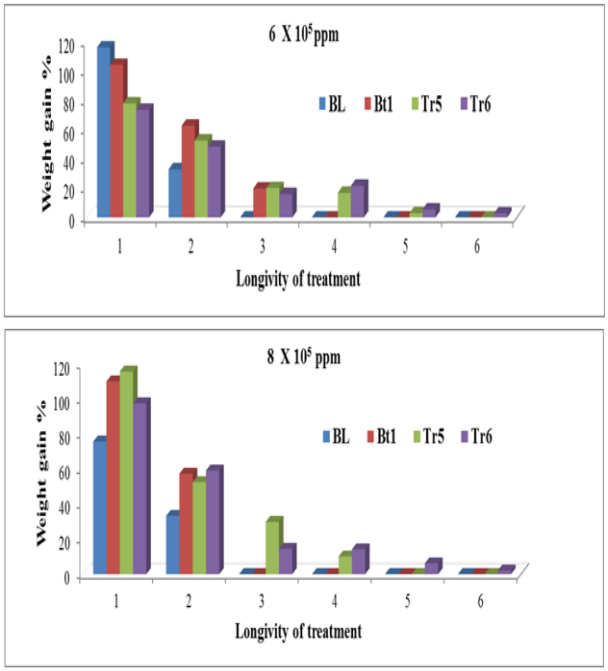

Figure 2. Continued.

Correlation between the time of feeding and larval weight

As shown from the results diagrammatic in Figure 3 that the reduction in larval weight showed a time – response , in contrast with the increased in larval weight in the control group which showed also a time – response, because they were fed on diet non-containing *Bt* – bioinsecticides . This indicated that *Bt* – treated leaves may affect to stop the feeding of larval stage leading to reduced the larval weight in comparison to the control. Strong deterrent effects on larval weight was shown in *Bt*-treatment larvae in relation to the control group. The results appeared a direct relationship between the feeding time and larval weight . This may due to the larvae preferred the control food than *Bt* – treated diet. The control food stimulated the food intake than *Bt*- treated food which showed a deterrent effect, as well as , decreased the larval growth and consequently reduced the larval weight These results agreed with El- Adl *et al.* (2016) ,who obtained that most bioinsecticides significantly reduced larval weight in untreated diet due to chronic toxicity . *Bt* – treated diet was influencing the larval weight due to the antifeedant effect which reduced feeding and food ingestion. Even if the *Bt* – treated diet did not affect the feeding deterrence of *Tuta absoluta* larvae, they decreased the larval weight due to the lower conversion efficiency of the ingested food . These are in harmony agreed with Chandrasekaran *et al*. (2012),who found that chitinase exposed larvae reduced body weight and did not still survive as a long period if compared with the controls.

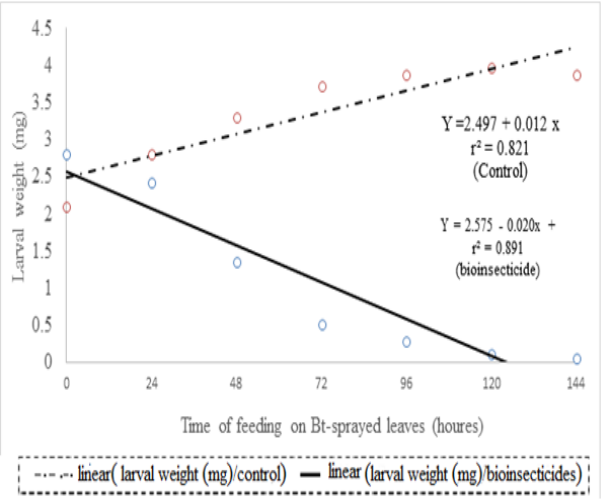

Figure 3. Regression line of recombinant bioinsecticides affected on larval weight in relation to untreated diet during six days of feeding on *Bt* – treated leaves .

In addition , Yu *et al.* (2013) obtained that surviving larvae feeding on *Bt* treated soybean weighted less than that feeding on non-*Bt* soybean. Dutton *et al.* (2003) found negative effects on survival, development and weight of *C. carnea* larvae when feeding on *Bt*-sprayed plants.

Effect of *Bt* concentration

As shown from the results presented in Figure 4 that the decreased in larval weight showed a dose – response . This indicated that there were a direct

relationship between doses of *Bt*- bioinsecticides and decreased in larval weight as a consequence of feeding deterrence . The results obtained herein indicated a strong deterrent effects of *Bt*- bioinsecticides against larvae of *Tuta absoluta*. This reflected a negative effect on larval development and the conversion of the ingested and digested food. Conversion of the ingested food was mostly lower in *Bt* – treated larvae if compared with the control group . The low metabolism of food caused prolongation of the larval development and chronic toxicity. Generally , larval growth suppress was caused by the antifeedant effect which reduced food ingestion or even prevented the larval growth at the higher doses because of chronic toxicity against *T. absoluta*. These results agreed with Gonzalez-Cabrera *et al.* (2011), who evaluated the effect of *Bacillus thuringiensis* on *T. absoluta* which showed high efficacy in reducing the damage at high infestation levels if compared with non-treated group. On the other hand , Azambuja *et al*. (2015) found that after five days of feeding all the larvae fed on *Bt* soybean leaves had died, while 95% of the control larvae survived . In addition , Hafsi *et al*. (2012) found that *B. thuringiensis* had antifeedant effect on *T. absoluta* leading to be used instead of synthetic chemical insecticides.

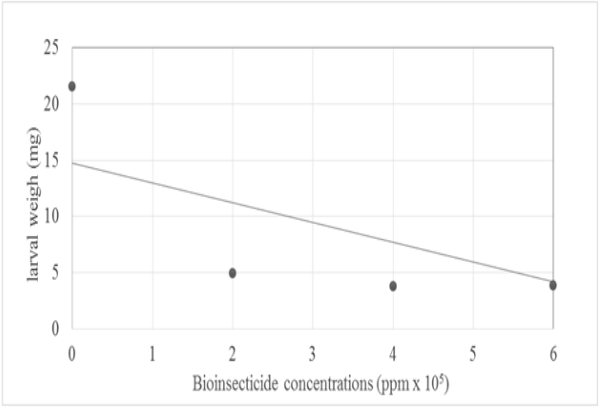

Figure 4 . Larval weight regression line of different *Bt* concentrations treated grubs .

However, Miyasono *et al.* (1994) showed that toxin –free spores did not kill larvae , but spores increased the toxin of *Bt kurstaki* crystal against larvae from a susceptible strain of diamondback moth. Furthermore, Dulmage and Martinez (1973) found that the sub- lethal doses of the entomopatogenic microbe had significant effects on insect pest development .

Toxicity index

Data summarized in Table 10 appeared that the mean of larval weight after six days of feeding on *Bt*-treated leaves was greatly decreased by the following bioinsecticides ; Bt_2 , *Bl* , Tr_8 , Tr_1, Tr_7 and Tr_4. The larval weight after six days of treatments was more decreased when the larvae feed on *Bt*-treated leaves. This indicated that *Bt* – treatment have antifeedant activity. Strong deterrent effects of *Bt* treatments against larvae of *Tuta absoluta* was observed by Bt_2 and *Bl* followed by the recombinants Tr_8 ,Tr_1 ,Tr_7 and Tr_4 which appeared a lower body weight of larvae than all other *Bt* treatments due to feeding deterrence. The recombinant Tr_8 treaed diet appeared higher feeding deterrence on *Tuta absoluta* larvae than all other recombinants . Also the results showed that the mean of toxicity index after six days of feeding on *Bt*-treated leaves was greatly increased by the following bioinsecticides; Tr_8 , Tr_7 , Tr_4 , *Bl* , Bt_1 and Tr_1. The recombinant Tr_8 appeared 66% toxicity index than other recombinants. This indicated that a lower concentration of this recombinant requires to suppress the growth and development of 66% of larval population . This agreed with Stanley-Horn *et al.* (2001), who found that the average body weights of larvae (1.7 mg) feeding on leaves treated with *Bt* hybrids were significantly less than weights of larvae (2.0 mg) on the other *Bt* hybrids combined. Hellmich *et al.* (2001) found less weight gain at doses below 5 –10 grains per cm^2 after a four - day treatment period. Meissle *et al.* (2009) found that after four days of feeding on *Bt* maize , body weight gain was significantly lower than in the control mazie group. Romeis *et al.* (2009) found that the insect larvae feeding on *Bt* plants may result in decreased movement due to sublethal damage or, in contrast, the higher activity of larvae may be due to search for better food . Prasifka *et al.* (2007) found that larvae treated with *Bt* anthers feeding less and gained less body weight than those exposed to non-*Bt* anthers or no anthers, as well as, there was a 21% decreased in body weight gain when larvae were exposed to *Bt* anthers. In addition , Singh *et al.* (2008) showed that the larvae of *C. medinalis* reared on untreated potted rice plants after 24 hrs of feeding on *Bt* treated plants increased larval duration,as well as, decreased the weight of larvae. Binning *et al.* (2014) found that pests treated with non-*Bt* and *Bt*-RW maize showed weight changed and stop feeding which leading to losses of weight at 9 and 13 days

Feeding deterrence index

As shown from the results diagrammatic in Figure 5 the feeding detterence index of *Tuta absoluta* larvae was increased in response to *Bt* – treated leaves. Moreover, the feeding detterrence index was still increased to reached 98% or more when the larvae were feeding on Bt_1, Bt_2, *Bl* , Tr_5 and Tr_7 – treated leaves .These results indicated that progressive feeding on *Bt* – treated leaves leading larvae to reduce the feeding gradually untill completely stopped . In addition, *Bt* recombinants , as well as, their parental strains has a great potential effect for controlling and suppress the feeding of larval stage on the host plants. These results agreed with Jyoti *et al*. (1996), who found that the larvae survived seven days at the exposure to *Bt*-treated leaves showed a reduced leaf consumption of up to 85%. In addition , Binod *et al*. (2007) found that biocontrol assay on *Helicoverpa armigera* appeared that the culture filtrate is an important antifeedant because it was decreased the consumption rate and larval body weight. Singh *et al.* (2007) observed 100 % mortality of larvae after 72 h of feeding on *Bt* sprayed leaves. El- Adl *et al.* (2016) found a strong reduction in larval weight after feeding on *Bt* – treated leaves for 240 hours. Pineda *et al*. (2007) found a strong decreased in larval body weight in fourth instars of *S. littoralis* after 48 h of consumption on *Capsicum annum L.* treated leaves with methoxyfenozide either by dipping or spraying technique. On the other hand , Smagghe *et al.* (1997) demonstrated that the larvae treated with tebufenozide could suffer from gut modification which leading larvae to suppress feeding and lost their weight as consequence.

Table 10 . Larval weight and toxicity index through six days of feeding on *Bt*-treated leaves.

Bioinsecticides	Time (hours)												General mean Weight (mg)	TI	Mean of FDI%
	24		48		72		96		120		144				
	Weight (mg)	TI	Weight (mg)	TI	Weight (mg)	TI	Weight (mg)	TI	Weight (mg)	TI	Weight (mg)	TI			
Control	2.80	0.72	3.30	0.26	3.73	0.06	3.9	0.02	4.0	0.01	3.9	0.01	3.60	0.18	0.0
Sm	2.62	0.77	1.83	0.46	0.37	0.62	0.23	0.34	0.15	0.13	0.05	0.4	0.88	0.45	65
Bt_1	2.80	0.73	1.93	0.44	0.4	0.58	0.10	0.8	0.02	1.00	0.00	0.00	0.87	0.59	67
MP	2.71	0.75	1.88	0.45	0.38	0.60	0.16	0.57	0.08	0.56	0.02	0.2	0.87	0.52	66
Tr_1	2.10	0.96	1.20	0.71	0.62	0.37	0.18	0.44	0.18	0.11	0.10	0.2	0.73	0.46	68
Tr_2	2.20	0.92	1.55	0.55	0.72	0.32	0.40	0.20	0.08	0.25	0.08	0.25	0.84	0.41	65
Sm	2.62	0.77	1.83	0.46	0.37	0.62	0.23	0.34	0.15	0.13	0.05	0.4	0.88	0.45	65
Bt_2	2.30	0.88	0.85	1.00	0.37	0.62	0.35	0.23	0.00	0.00	0.00	0.00	0.65	0.45	72
MP	2.46	0.82	1.34	0.73	0.37	0.62	0.29	0.28	0.07	0.06	0.025	0.2	0.76	0.45	68.5
Tr_3	2.45	0.82	1.70	0.50	0.82	0.28	0.63	0.13	0.2	0.1	0.05	0.4	0.98	0.37	60
Tr_4	2.27	0.89	1.17	0.73	0.63	0.37	0.32	0.25	0.05	0.4	0.02	1.00	0.74	0.60	68
Bl	2.63	0.77	1.10	0.77	0.23	1.00	0.08	1.00	0.00	0.00	0.00	0.00	0.67	0.59	73
Bt_1	2.80	0.73	1.93	0.44	0.4	0.58	0.10	0.8	0.02	1.00	0.00	0.00	0.87	0.59	67
MP	2.71	0.75	1.51	0.61	0.32	0.79	0.09	0.9	0.01	0.5	0.00	00	0.77	0.59	70
Tr_5	2.70	0.75	1.20	0.71	0.55	0.42	0.42	0.19	0.03	0.66	0.00	00	0.82	0.45	67
Tr_6	2.03	1.00	1.45	0.58	0.85	0.27	0.52	0.15	0.42	0.04	0.17	0.11	0.91	0.35	61
Bl	2.63	0.77	1.10	0.77	0.23	1.00	0.08	1.00	0.00	0.00	0.00	0.00	0.67	0.59	73
Bt_2	2.30	0.88	0.85	1.00	0.37	0.62	0.35	0.23	0.00	0.00	0.00	0.0	0.65	0.45	72
MP	2.47	0.82	0.97	0.88	0.30	0.81	0.21	0.62	0.00	0.00	0.00	0.00	0.66	0.52	72.5
Tr_7	2.45	0.82	1.23	0.69	0.47	0.49	0.13	0.61	0.13	0.15	0.02	1.00	0.74	0.62	69
Tr_8	2.53	0.80	1.17	0.73	0.23	1.00	0.10	0.8	0.03	0.66	0.00	0.00	0.68	0.66	72
F-test	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
LSD 0.05	2.08	3.33	3.02	3.02	2.18	2.63	4.38	3.94	1.64	1.46	4.53	4.53	1.57	3.79	4.13
0.01	2.81	4.49	4.07	4.07	2.95	3.54	5.93	5.33	2.21	2.21	6.09	6.09	2.13	5.11	5.56

TI = Toxicity index

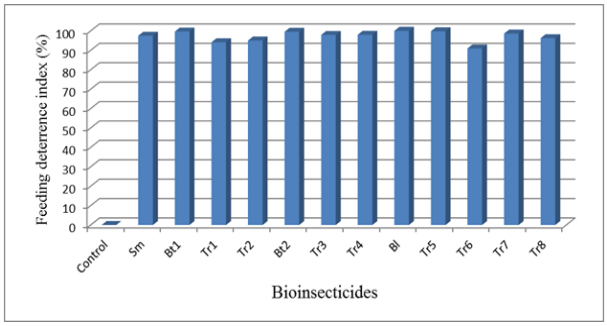

Figure 5 . The feeding deterrence index of *Bt*-treated diet against *Tuta absoluta* larvae after 144 houres.

In conclusion, the results revealed that transferring chitinase gene from *Serratia marcescens* and *Bacillus licheniforms* to *Bt* strains has improved the toxicity of *Bt* against *T. absoluta* . Modified *Bt* strains aquired two mode of actions against insect pests. The first one through crystal toxins formation which induced pores in insect's mid-gut causing insect death. The second mechanism through increasing the production of chitinase enzyme which degrade the cuticle layer in insect's body resulting in insect dehydration leading to mortality.

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التأثير المضيف لجينات الشيتينيز في المتحولات التزاوجية للباسيلس ثيرونجنسز لتحسين المكافحة الحيوية لحشرة التوتا أبسلوتا

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تم إجراء هذه الدراسة بغرض تقييم سمية المتحولات التزاوجية من الباسيلس ثيرونجنسز التى تحمل المزيد من الجينات المنتجة لإنزيم الشيتينيز ضد يرقات حشرة التوتا أبسلوتا . استخدمت فى هذه الدراسة سلالات *Bacillus licheniformis* ، *Serratia marcescens* كسلالات معطيه للمادة الوراثية إلى السلالات المستقبلة من الباسيلس ثيرونجنسز . تم تصنيف المتحولات الوراثية على أساس ظهور هالات التحلل على بيئه الشيتين أجار . أظهرت النتائج أن السلالات الأبوية والإتحادات الوراثية الجديدة أحدثت إنخفاض معنوى فى عدد اليرقات الحية إذا ما قورنت بعدد اليرقات الغير المعاملة بالمبيد الحيوى. حدثت زيادة تصاعدية فى معدل موت اليرقات حتى وصلت الى أعلى معدلاتها بعد سته أيام من المعاملة. كما حدث إنخفاض معنوى فى وزن اليرقات الحية نتيجة المعاملة بكل من السلالات الأبوية والإتحادات الوراثية الجديدة الناتجة عنها مقارنة باليرقات الغير معاملة فى تجربة الكنترول. كما أوضحت النتائج أن سلالات وتركيزات المبيد الحيوى المستخدمة والتفاعل بينهم كان لها تاثير معنوى على وزن اليرقات عند معظم الفترات الزمنية من عمر اليرقة . وبالإضافة إلى ذلك فإن الإتحادات الوراثية الجديدة من الباسيلس ثيرونجنسز وكذلك السلالات الأبوية كان لها تأثير كبير فى مقاومة الحشرة المستهدفة وتوقف الطور اليرقى عن التغذية على عوائلة النباتية .