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BIOACTIVITY OF BACILLUS THURINGIENSIS AND BACILLUS POLYMYXA ON THE AMERICAN BOLLWORM HELIOTHIS ARMIGERA

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Abstract

Suspensions containing *Bacillus thuringiensis* (B.t.) and *Bacillus polymyxa* (B.p.) were evaluated as microbial control agents against American bollworm (ABW) *Heliothis armigera* larvae. Latent effects on pupae formation, adult emergence, fecundity and fertility were also investigated. Significant mortality was obtained three days after feeding larvae on treated diet with the tested pathogens. Average weight of pupae formed in the bacterial treatments significantly reduced compared to the control. Mean percentage of adult emergence from pupae treated in the larval stage was significantly less in the bacterial treatments than in the control. Emerged adults lost their normal ability for laying viable eggs particularly when the highest concentration was tested against 2nd instar larvae. All the effects were concentration, instar and strain dependent. Great effect was obtained when younger larvae fed for three days on treated diet with 10^7 (cell/ml) from any of the two tested pathogens. In general, the B. t. strain was more potent for killing larvae, inhibiting pupation and emergence of *H. armigera* than the B.p. strain in all treatments. However, the contrast was evident when their effects on adult fecundity and fertility were considered. The 2nd instar larvae were more sensitive to both strains of B.t. and B.p. than the 4th instar larvae.

Introduction

The American bollworm *Heliothis armigera* (Hubner) is considered one of the most destructive insect pests of cotton, corn and tomato in several countries attacking more than 65 host plants (Mohyuddin 1989). Oerke *et. al*. (1995) estimated the average losses in eight major crops caused by this insect species during 1988-1990 to be 28% in Europe, 31% in North America and 50% in Asia and Africa. The status of the American bollworm *Heliothis armigera* (Hubner) as an economic pest of cotton and other vegetable (tomato) and field crops (corn) in middle Egypt is not stable during the past few years. The severity of infestation has varied from year to year (Gergis and Ibrahim 1993) and the reasons for increasing bollworm problems in cotton are not fully understood (Rummel *et. al*. 1986).

Cyclodienes were effective and persistent insecticides, but are no longer used because of their risk to the environment and human health (Sim *et. al.*1998). Development of insecticide resistance (Su *et. al.* 1997, and Osbrink *et. al.* 2001), air pollution (Katsura *et. al.* 1996) and contamination of small ponds, resulting in the death of several species of fish (Carr *et. al.* 1997, and Kumar and Chapman 2001), are serious problems associated with the continuous use of synthetic insecticides to control insect pests. A qualitative and targeted selectivity control agents are environmentally acceptable approaches to reduce the non-target effects of synthetic insecticides to the environment and human health (EPA 1996, Kumar and Chapman 2001 and Kumari *et. al.* 2002).

Since insect pathogens (microbial insecticides) are considered an effective approach in the biological control regime, researchers focused on their future use as a biological control method in the integrated pest management strategy. *Bacillus thuringiensis* (B.t.) exhibited a potential effect as a cotton pest management agent. The B.t. toxins are highly specific for lepidopteran pests, acting at the insect midgut (Fischhoff 1996). This high degree of target specificity (Gill *et. al*. 1992) implies a minimal negative impact on beneficial insects, wildlife or human (Fuxa 1987, and Meadows 1993). These characteristics are expected to provide large-scale environmental benefits due to a reduced need for the application of broad-spectrum insecticides (Gasser and Fraley 1989). The primary objective of these studies were (1) to compare the larvicidal activity of B.t. and B.p. strains at three selected concentrations on 2nd and 4th instar larvae of *H. armigera*, and (2) to determine the latent effects of B.t. and B.p. on pupa formation, adult eclosion, fecundity and fertility.

Materials and Methods

This study was conducted in Faculty of Agriculture, Minia University, Minia, Egypt. The isolation of the pathogens from dead pupae of *H. armigera*, and their identification were conducted in the Microbiology Department. American bollworm rearing and pathogen bioassays were conducted in Plant Protection Department. The original source of ABW was from Tomato field in Minia region, Egypt and reared in the laboratory for more than 10 generations before conducting this study.

Insect Rearing

Eggs of *H. armigera* were placed in 1-liter glass jars covered with a muslin cloth fixed with a rubber band and daily observed for hatching. After hatching the jar was provided with a fresh castor bean leave *Ricinus communis* (L.) as a source of food for new-hatched larvae. To avoid the cannibalism, 3rd instar larvae were reared individually in plastic vials each was provided with a piece of fresh castor bean leave and covered with a muslin cloth fixed with a rubber band. Formed pupae were sexed and placed in 1-liter glass jars until emergence. The emerged adults were supplied with a piece of cotton wetted with 10% sugar solution and strips of cheese clothes as a suitable site for oviposition. All stages were reared under constant conditions of 28°C ± 2, 65 ± 5% R.H. and photoperiod of 16:8 hr. (L:D).

Isolation and Growing of the Bacillus Strains

During the rearing of *Heliothis armigera* in the laboratory, dead pupae had symptoms of bacterial infection were observed. Some of these pupae were surface sterilized by immersing in 75% ethyl alcohol for 3 min., followed by 3 min. in 0.2% mercuric chloride, and then washed several times with sterilized distilled water to remove the excess of mercuric chloride. Cleaned pupae were crushed in sterilized test tubes, containing 10 ml of sterilized distilled water. The suspension was used to isolate the bacteria on nutrient agar medium. After incubation at 30°C for 48 hr., 13 different bacterial strains were identified using a light microscope and isolated in pure cultures. The selected isolations were identified according to Bergey's manual (Breed *et. al.,* 1957). Bacterial isolations were grown individually on nutrient broth for 48 hr. at 30°C, giving liquid cultures containing ($1.7\text{-}2.5*10^7$ cell/ml.).

Screening Study

The toxicity or pathogenicity of each of 13 bacterial isolations was bioassayed on the 2nd instar larvae of *Heliothis armigera*. Fresh castor bean leaves were dipped for 10 second in each liquid culture. Castor bean leaves that dipped in the sterilized distilled water were used as a food source for the control treatment; leaves were left for air dryness and then offered to the larvae. Five replicates of 10 larvae were used for each treatment. After a feeding period on treated leaves for 3 days, number of dead larvae was counted in each replicate. Then mean percentage of larval mortality was calculated to select the bacterial isolations which exhibited the greatest toxicity.

Larvicidal Activity and Latent Effects of Selected Pathogens

The *B. thuringiensis* and *B. polymyxa* were selected for this study. Three concentrations (10^3, 10^5 and 10^7 cell/ml.) of each pathogen were used. Concentrations were adjusted using sterilized distilled water as described by Hopkins (1985). Larvicidal activity of *B. thuringiensis and B. polymyxa* was tested on 2nd and 4th instar larvae. Ten replicates were performed for each concentration (10 larvae each). After a feeding period of 72 hr. on treated castor bean leaves, number of dead larvae in each treatment was recorded. Survival larvae were transferred to clean vials each provided with a piece of fresh clean castor bean leaves that were replaced daily by fresh one. Formed pupae were counted and transferred to clean vials to observe adult eclosion. Number of emerged adults was recorded and scored for malformations. Healthy adults were maintained in glass jar provided with a piece of cotton wetted with 10% sugar solution and strips of cheese clothes as a suitable site for oviposition. Treatments were incubated at 28°C $\pm$ 2, 65 $\pm$ 5% R.H. and photoperiod of 16:8 hr. (L:D). Egg laying and hatching was observed in each treatment. Percentage of pupation was calculated related to the survival larvae in the third day of treatment. Also percentage of emergence was calculated in relation to number of formed pupae.

Statistical Analyses

Data was analyzed using analysis of variance and contrasts (PROC GLM, SAS Institute 1988).

Results and Discussion

Acute Toxicity of Tested Pathogens

Larval mortality in *B. thuringiensis* treatments increased as a pathogen concentration increased (Table 1 and Figure 1). For the two tested instars, percentages of larval mortality were significantly different from the control in $\geq 10^5$ treatments. Mortality percentages were greater (45%) when 10^7 B. t. concentration was tested against 2nd instar larvae compare to 20% when the same concentration was tested against 4th instar larvae. Greater sensitivity of 2nd instar larvae towards *Bacillus thuringiensis* than the 4th instar larvae was confirmed by the findings of Dabi *et. al.* (1988) and Kulkarni and Amonkar,(1988). Data in Table (2) and Figure (2) reveals that *Bacillus polymyxa* was less potent against *H. armigera* larvae than *Bacillus thuringiensis*, however, this pathogen mostly exhibited the same trend with less efficiency. Older larvae were less sensitive to the pathogen than younger larvae. The findings by Atwood *et.al.* (1996) confirmed our results, they mentioned that B.t. did not provide acceptable control of the 2nd instar larvae of *Heliothis virescens* (<32% mortality). In contrast Deniz and kornosor (1987) obtained 2-fold the mortality reported in our study with the same insect species, but they used a commercial product of B.t.. Also, Dibyantoro and Siswojo (1988) and Iriarte *et.al.* (1998) found that *Bacillus thuringiensis* had significant insecticidal activity against *Plutella xylostella* and *Heliothis armigera*. Liao *et.al*. (2002) mentioned that *H. armigera* was consistently more tolerant to *B. thuringiensis* insecticidal proteins than *H. punctigera.*

Latent Effects of Tested Pathogens on Metamorphosis and the Reproductive Potential of *H. Armigera*

Second and 4th instar larvae of *H. armigera* fed for three days on treated diet with three tested concentrations (10^3, 10^5, and 10^7 cell/ml) of *Bacillus thuringiensis* and *Bacillus polymyxa*. Then healthy survival larvae were transferred to completely normal conditions to follow up the latent effects on pupae and adults. These effects are represented in Tables 1& 2 and Figures 1-8.

Pupal Formation

Percentages of pupae formation from larvae pre-treated in the 2nd instar with any of B. t. concentrations were significantly different from control treatment (Table 1 and Figure 3). For explanation, pupation percentages were 60, 55, and 20% in the treatments of 10^3, 10^5, and 10^7 B. t., respectively compared to 99% in the control. However, when the 4th instar larvae were treated, all larvae pre-treated with 10^3, and 10^5 B. t. succeeded to pupate same as control treatment. Treatment of 10^7 B. t. was the only effective treatment resulted in reducing pupae formation by 25% less than the control. *B. polymyxa* pathogen was less effective in this respect than *B. thuringiensis*. In the treatment of 10^7 B. p., pupae formation was 80% compared to 100% in the controls (Table 2 and Figure 4).

Average Weight of Pupae

Weight of pupae pre-treated in 4th instar larvae with any of B. t. concentrations was significantly reduced compared to the untreated check (Table 1 and Figure 5). For more explanation, mean weight of formed pupae from pre-treated 4th instar larvae with 10^3, 10^5, and 10^7 B. t. were 311, 228, and 116 mg, respectively compared to 432 mg in the control. *Bacillus polymyxa* was less effective than *B. thuringiensis*. Average weight of pupae was significantly reduced in the treatments of 10^5 and 10^7 B. p. but not in the treatment of 10^3 B. p. (Table 2 and Figure 5). Halcomb *et. al.* (1996) reported that larvae of *H. zea* fed transgenic B.t. flower buds weighed significantly less than those fed nontransgenic flower buds. Staple *et. al*. (1998) found that when the larvae of *Spodoptera exigua* fed on diet containing B.t. toxins, the weight of pupae was lower than the control. Lyneh *et. al*. (1999) found that 6 days old *H. zea* larvae fed on B.t. sweet corn had lost weight when compared with average weights at the beginning of the test or with larvae that had fed on non B.t. sweet corn.

Adult Emergence

Formed pupae from 2nd instar larvae that fed on treated diet with 10^7 B. t. for three days failed completely to develop to adults (Table 1 and Figure 6). In contrast all pupae formed in the control converted to adult stage. However, when larvae treated in the 4th instar with the same level of B. t., pupation was 70% compared to 100% pupation in the control. With B. p. treatments, 10^7 concentration was the only effective treatment had 75 and 80% emergence, respectively when 2nd and 4th instars were treated compared to 100% emergence in the controls (Table 2 and Figure 7)

Adult Malformations

Fifteen and 25% of emerged adults were malformed when 2nd instar larvae were fed on 10^3 and 10^5 B. t., respectively (Table 1 and Figure 8). When the 4th instar larvae were treated, only 10^7 B. t. concentration resulted in 15% adult malformations, meanwhile 10^3 and 10^5 B. t. treatments and the control were statistically similar where no malformed adults were observed in the three treatments. With B.p. treatments, no malformed adults observed except in the treatment of 10^7 with 2nd instar larvae (Table 2). Adults emerged in the rest of treatments were completely normal.

Fecundity and Fertility

Fecundity and fertility of adults treated in larval stage with B. t. and B. p. significantly affected and this effect was concentration and tested instar dependent (Tables 1 and 2). When 2nd instar larvae were fed on treated diet with 10^5 B. t., emerged moths did not lay any eggs. However, in the treatment of 10^3, adult females laid few eggs compared to the control, but these eggs did not hatch. In the treatments of 4th instar larvae, emerged adults laid eggs in all B. t. treatments (number of eggs in all treatments was obviously less than the control treatment), however hatchability was evident in the lowest tested concentration and the control (Table 1). Benz (1975) found that the B-exotoxin in a commercial product of B.t. inhibited the reproductive potential of southern armyworm *Spodoptera eridana* (Cramer). Although B. t. was much potent than B. p. in killing larvae, inhibiting pupation and emergence, the contrast was evident when their effects on the reproductive potential of *H. armigera* were compared. For explanation, B. p. pathogen was more potent in reducing egg laying and hatchability of eggs than B. t.. Adults treated in the 2nd instar larvae with any concentration of B. p. did not lay eggs. Moreover, in the treatments of 4th instar larvae with any of B. p. tested concentrations, adults laid eggs that failed completely to hatch.

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Table 1. The effect of *Bacillus thuringiensis* on the 2nd and 4th instar larvae, and its latent effects on pupae and adults of *Heliothis armigera.*

Concent. Cell/ml	Larval instar	% Mort.	% Pupation	Weight of Pupa (mg)	% Emergence	% Malf.	Egg-laying and hatching
10^7	2nd	45 a	20 c	-	0 c	-	-
10^5	2nd	30 b	55 b	ND	80 b	25 a	No
10^3	2nd	20 b	60 b	ND	100 a	15 a	Yes
Control	2nd	0 c	99 a	436 a	100 a	0 b	Yes & Hatch.
10^7	4th	20 a	75 b	116 c	70 b	15 a	Yes
10^5	4th	10 b	100 a	228 b	100 a	0 b	Yes
10^3	4th	0 c	100 a	311 b	100 a	0 b	Yes & Hatch
Control	4th	0 c	100 a	432 a	100 a	0 b	Yes & Hatch.

Means within a column followed by the same letter are not significantly different ($P \geq 0.05$).
ND means not detected. Yes means that there was egg-laying. No means that there was no egg-laying.

Table 2. The effect of *Bacillus polymyxa* on the 2nd and 4th instar larvae, and its latent effects on pupae and adults of *Heliothis armigera.*

Concent. Cell/ml.	Larval instar	% Mort.	% Pupation	Weight of Pupa (mg)	% Emergence	% Malf.	Egg laying and hatching
10^7	2nd	20 a	80 b	ND	75 b	5	No
10^5	2nd	15 b	85 b	ND	90 ab	0	No
10^3	2nd	10 c	100 a	ND	100 a	0	No
Control	2nd	1d	100 a	422 a	100 a	0	Yes & Hatch.
10^7	4th	10 a	80 b	326 b	80 b	0	Yes
10^5	4th	5 b	90 ab	331 b	95 a	0	Yes
10^3	4th	0 c	100 a	385 a b	100 a	0	Yes
Control	4th	0 c	100 a	442 a	98 a	0	Yes & Hatch.

Means within a column followed by the same letter are not significantly different ($P > 0.05$).
ND means not detected. Yes means that there was egg-laying. No means that there was no egg-laying.

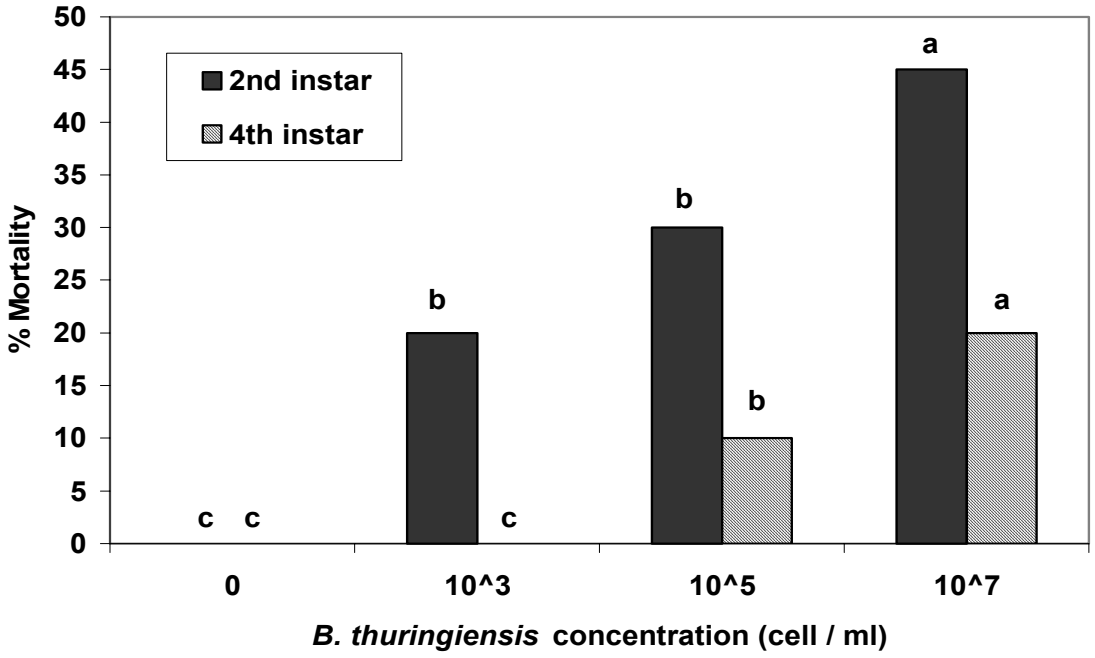

Figure 1. Mortality percentages of *H. armigera* larvae exposed to *B. thuringiensis*-treated diet for three days. For each instar, means followed by the same letter are not significantly different ($P \geq 0.05$).

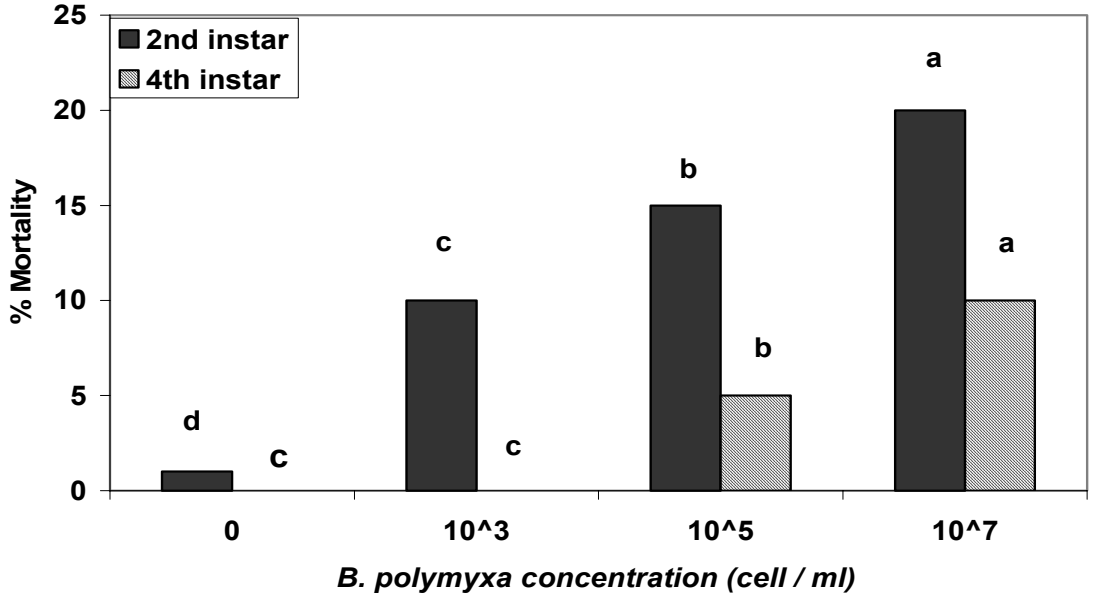

Figure 2. Mortality percentages of *H. armigera* larvae exposed to *B. polymyxa*-treated diet for three days. For each instar, means followed by the same letter are not significantly different ($P \geq 0.05$).

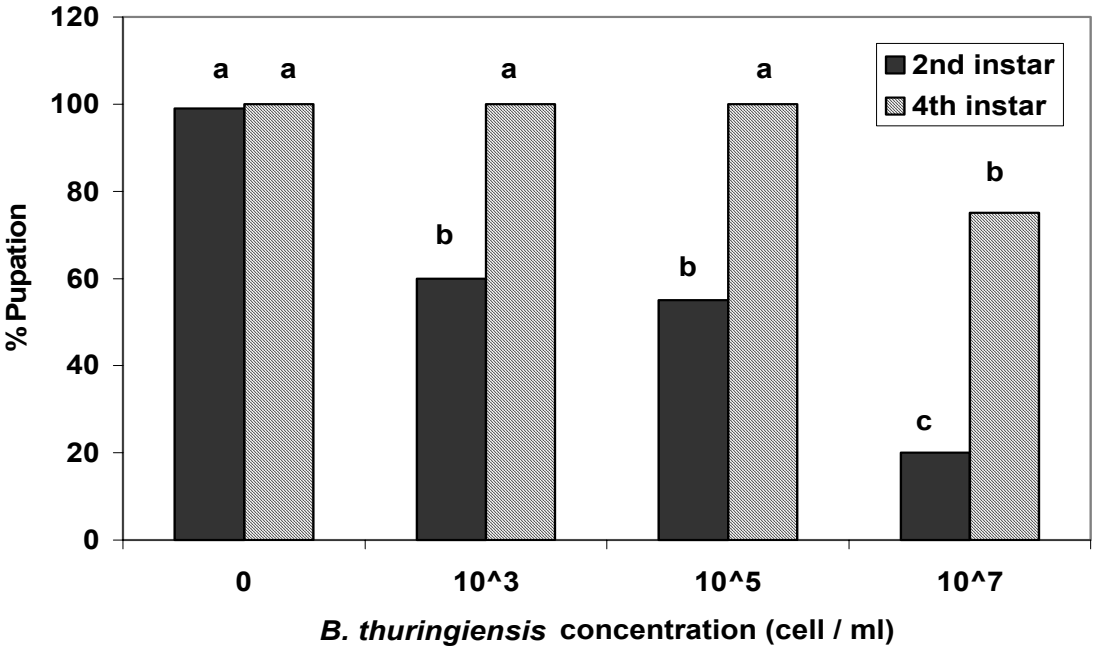

Figure 3. Pupation percentages of *H. armigera* larvae that fed on *B. thuringiensis*-treated diet for three days. For each instar, means followed by the same letter are not significantly different ($P \geq 0.05$).

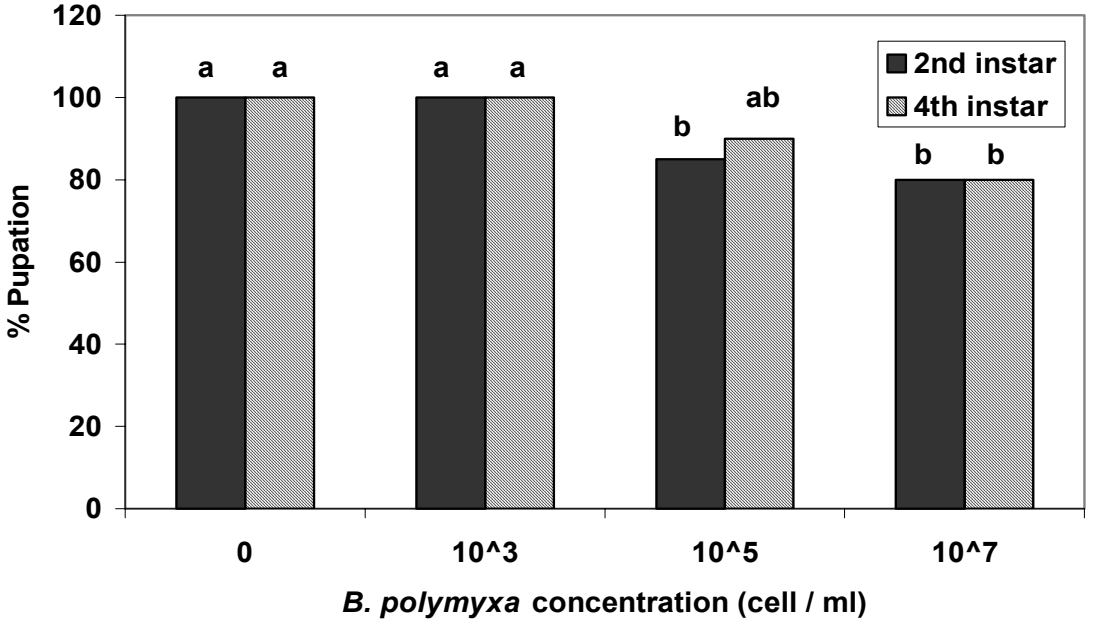

Figure 4. Pupation percentages of *H. armigera* larvae that fed on *B. polymyxa*-treated diet for three days. For each instar, means followed by the same letter are not significantly different ($P \geq 0.05$).

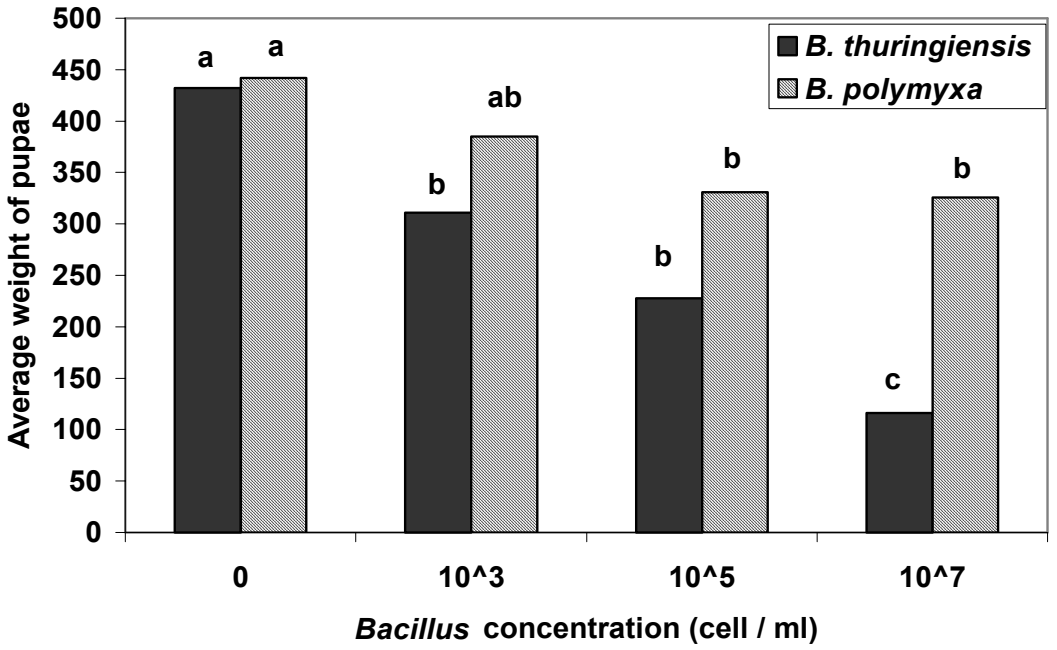

Figure 5. Average weight of *H. armigera* pupae (mg) that exposed in 4th instar larvae to *B. polymyxa* and *B. thuringiensis*. For each strain, means followed by the same letter are not significantly different ($P \geq 0.05$).

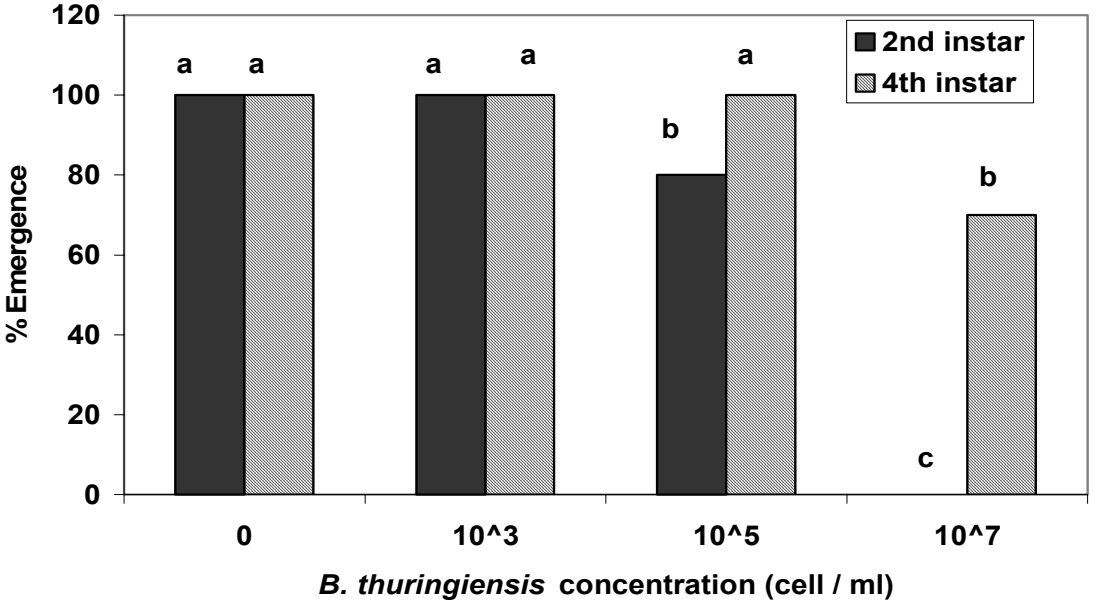

Figure 6. Percentages of adult emergence from *H. armigera* pupae that exposed in larval stage to *B. thuringiensis*. For each instar, means followed by the same letter are not significantly different ($P \geq 0.05$).

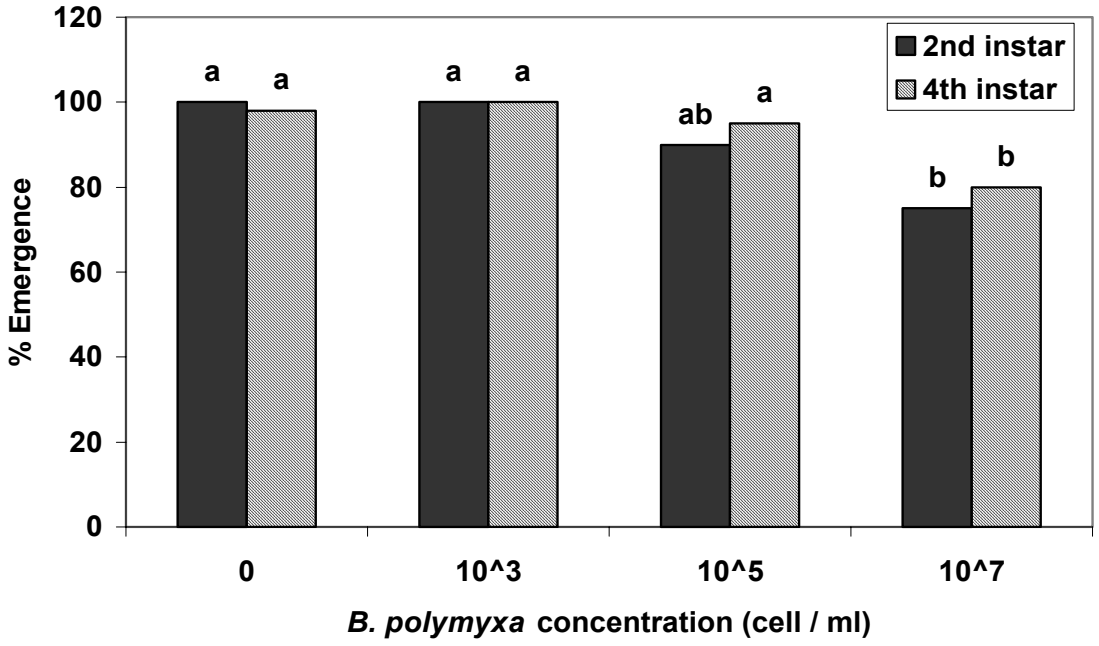

Figure 7. Percentages of adult emergence from *H. armigera* pupae that exposed in larval stage to *B. polymyxa*. For each instar, means followed by the same letter are not significantly different ($P \geq 0.05$).

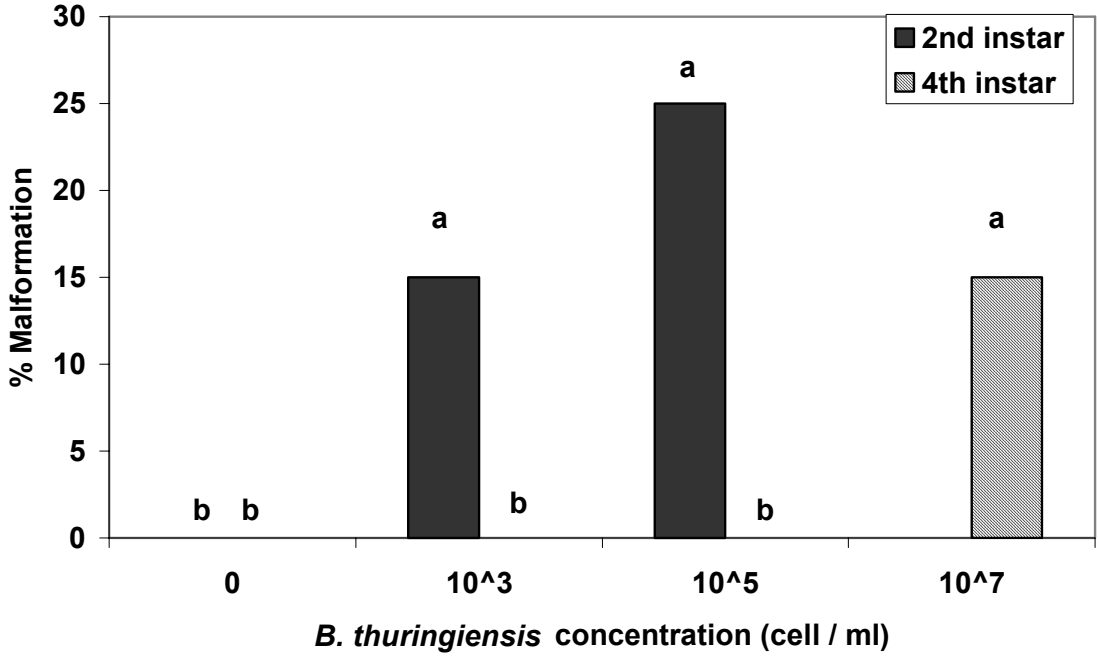

Figure 8. Percentages of malformation in *H. armigera* adults pre-treated in larval stage with *B. thuringiensis*. For each instar, means followed by the same letter are not significantly different ($P \geq 0.05$).