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The Effects of *Bacillus thuringiensis* var. *kurstaki* on the First, Second and Third Larval Instars of Pistachio White Leaf Moth *Ocneria terebinthina* (Lep.: Lymanteridae)

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ABSTRACT
In this research the susceptibility of the first three larval instars of *Ocneria terebinthina* (Lep.: Lymanteridae) to *Bacillus thuringiensis* var. *kurstaki* was investigated. The eggs of the insect were collected from orchards where located around Rafsanjan. Different larval instars groups were separated by measuring the head capsule width and then used in all bioassays. In bioassays, LC_{50} values of Bt on the first, second and third instars larval groups were determined. The data was analyzed by log-probit transformation using the MSTATC software. LC_{50} values for each group of larvae were estimated by using treated pistachio offshoot. The LC_{50} values of Bt on the first, second and third instars of larvae were 276.91, 1494.53 and 2817.34 ppm, respectively. The first larval instar was the most susceptible to Bt. According to collected data in this research and the selective nature of Bt, we can conclude that this microbial compound can be used for reasonable control of *O. terebinthina.*

Keywords: *Bacillus thuringiensis* var. *kurstaki*, *Ocneria terebinthina*, LC_{50}, Pistachio, Biological control

INTRODUCTION

Pistachio is one of the most important agricultural products in Iran. It is the second exporting product of Iran. For surviving of world position yield per unit area should increase. Achievement this goal is possible with management of planting, maintains, harvesting and integrated pest management. Generally, pistachio Pests feed on leaves, nuts, buds, shoots, twigs and roots of pistachio trees. Pistachio white leaf moth *Ocneria terebinthina* Strg. (Lep.*:* Lymanteridae) is one of these pests. The larvae of this pest feed on pistachio leaves. The younger larval instars feed on the upper epidermis and parenchyma tissue, as a result leaves become lace and dry. The older larval instars feed on the whole leaf area and just leave main vein. This pest decrease plant photosynthesis levels (Esmaili, 1996). The damage of *O. terebinthina* is important economically, because it feeds on plant chlorophyll and has several generations (four to five generations per year). Larval

feeding on leaves eliminates plant photosynthesis part where its materials were used for fruit formation. It is essential to control it. Nowadays, this pest was controlled by chemical insecticides and sometimes several spraying steps to control are necessary. Using of chemical insecticides against pests has problems including: residues of pesticides in the environment, loss natural equilibrium of insect communities, destruction of natural control agents and development and replacement of secondary pests (Pedigo, 1999). Environmental Protection Agency of U.S. gives more attention to toxins that their effects are different from other synthetic insecticides and side effects of them are less. Biorational compounds are the third generation of insecticides, which generally are found in nature and there are in insects and plants, or synthetic materials which are produced by humans and are used against pests logically (Pedigo, 1999). Microbial pesticides are a group of biorational compounds that differ with chemical pesticides. Some of microbial pesticides are bacteria, viruses, fungi, nematodes, ricketsia, and protozoa (Pedigo, 1999). *Bacillus thuringiensis* (Ber.) is one of these pathogens. This bacterium produces a toxin that acts specifically and is not harmful for non-target organisms. This bacterium in unfavorable conditions, like most of bacteria produces spore (Swadener, 1994). When vegetative cells start producing spores were called sporangium (Tanada and Kaya, 1993).

In addition, a protein crystal is produced with spore formation that is the same toxin of Bt (Swadener, 1994). Bt for first time was isolated from patient larvae of silkworm in 1901. Then it was reported on the flour moth.. That was used as a commercial insecticide in 1938 in France and in 1950 in the United States (Swadener, 1994). Products of Bt form about 1% of the total consumables market against pests (insecticides, fungicides and herbicides). Bt is a microbial agent that has been used to control insect pests in agricultural successfully (Gould *et al.*, 1992).

The most important and effective subspecies of Bt against order Lepidoptera is *B. thuringiensis* var. *kurstaki* (Dulmage, 1970). Bt has been used against pests especially forest pests for several decades. This combination has less destructive effects than broad spectrum insecticides due to short half life and to be selective (Reed *et al.,* 2001). Active ingredient of Bt is Cry protein. Many Bt strains have more than one type of protein crystal which is called delta endotoxin. HD-1 strains of this bacterium contain genes of proteins CryIAa, CryIAb, CryIAc, CryIIB and CryIIA. The mixing these toxins are more effective than using one of the toxins alone and delays resistance. The results have shown that the effect of mixing CryIAc and CryIAa is eight times CryIAb and CryIAa and four times CryIAb and CryIAc (Lee *et al.,* 1996). Liquid and powder formulations of this bacterium were obtained from isolated toxic proteins all over the world. At least nine specific proteins of Bt are toxic to Lepidoptera larvae (Gould *et al.,* 1992).

Bt during the process of spores production, one or a few number of lozenge crystal produce that were called Inclusion body (Tanada and Kaya, 1993). These crystals are dense of large proteins that realities are protoxin and must be activated before impact. Crystalline proteins are soluble in normal conditions and under high temperature of environment are insoluble. Bt is a specific insecticide and that is safe for human. This bacterium is a stomach combination that short time after digestion by a susceptible insect, their crystals in proventriculus of larva are analyzed, and their protein change to peptids by Protease of midgut. One of these peptids is Delta-endotoxin

that degenerates epithelial cells (Kurstak *et al.*, 1982).

Generally, the toxicity of these proteins depends on their ability in banding to specific receptors of alimentary canal of Lepidoptera larvae. An insect may have different receptors for different toxins of Bt (Gould *et al.*, 1992). Delta-endotoxin in midgut of susceptible larvae become active by proteolytic enzymes and is banded to specific receptors in epithelium cell of midgut. This banding causes pore in membrane of midgut and finally cells are swelled and degenerated. Then feeding of larvae was stopped and they died. Banding of active toxin to specific receptors of midgut is a key factor for toxicity (Loseva *et al.*, 2002; Rajamohan *et al.*, 1996; Swadener, 1994). Lutrell *et al*. (1982) evaluated effects of Bt on *Heliothis zea* and *Heliothis virescence*. The results revealed that this bacterium reduces surviving treated larvae weight (after 7 days). The researchers also reported compounds such as Coax and Gustol as auxiliary materials increases the effect of Bt. The combination of Bt and nuclear polyhedrosis viruses cause more mortality compared with Bt alone (Lutrell *et al.*, 1982). In different strains of Bt, seven toxins have been identified and introduced that the most important toxins including: Alpha exotoxin, Beta exotoxin, Gamma exotoxin, delta endotoxin. Of course, should be noted that each of these bacterialstrains produce only some of these seven types of toxins (Kurstak *et al.*, 1982).

Generally, suseptible insects to Bt are divided into three categories: 1) Insects that Ph of the middle part of their alimentary canal is high and their ventriculus is paralyzed quickly. Nevertheless these insects after crystal digestion and pathological changes in their tissue die. This mode is called Toxemia. 2) Insects that after toxin digestion in ventriculus, their epithelium layer are destroyed and bacterium enter into haemocoel and septicemia is created. In this group of insects, both toxin and spores of bacterium are required to create insect mortality and toxin hasn't enough toxicity lonely. 3) Insects that aren't harmed by toxin. Bacterium acts as a potential pathogen and would replicate in haemolymph of insect, but doesn't produce toxin. This condition is called Bacterimia (Kurstak *et al.*, 1982).

Nowadays, *O. terebinthina* was controlled by chemical insecticides. For reduction of side effects of chemical insecticides, application of other compounds such as biorational compounds is necessary that in this study the effect one of these compounds (Bt) as a pathogen was investigated.

MATERIALS AND METHODS

Host

The eggs of the insect were collected from orchards where located around Rafsanjan. The eggs are laid on lower level of leaflets of host plant as batches. The eggs are milky-white in color. The color of eggs at the hatching time becomes dark gray. The eggs in laboratory were set in plastic dishes and humid cotton used for keeping humidity of them and with a netting cloth covered. For feeding of larvae after hatching from drug glasses, cotton, pistachio offshoots were used. In order to, drug glasses were filled with water and each pistachio offshoot was set in a drug glass, then offshoot are fasten in drug glasses using of cotton. This collection was set in a plastic dishes and covered with a lace cloth (Figure 1). For each plastic dish, one egg mass was considered to be prepared the same larval instar. Different larval instars groups were separated by measuring of the head capsule width and used in all bioassays.

Bacillus thuringiensis (Bt)

Bacterium Bt was prepared from Mehr Asia Biological Technology Company in Tehran, Iran.

Larval different instars susceptibility

For determination of the susceptibility of first, second and third larval instars. This pest was used in six different concentrations of Bt with logarithmic distance. Experiments were evaluated with 7 treatments [6 concentrations of Bt and a control treatment (water)] and for each treatment 3 replicates were considerated.

Determination of larval different instars LC_{50}

Distal part of pistachio offshoots were separated and transferred to the laboratory. Each stem of offshoot was fixed into a drug glass full of water using cotton. Then offshoots were contaminated to different concentrations of Bt with immersion method. Contaminated offshoots were dried in air. Then offshoots were placed within disposable dishes with dimensions of 5 × 17 × 22 cm. For each replicate, 30 larvae of the same instar and size were released on contaminated offshoot inside dishes. The dishes were blocked with a white lace cloth for prohibition of larval escape (Figure 2). The mortalities were counted every 24 hours. Obtained mortalities were corrected for each larval instar separately, using Abbott formula [$\frac{po - pc}{100 - pc}$], Po = Treatment mortality (%), Pc = Control mortality (%) (Namvar, 2002).

Figure 1. Larvae breeding dishes

Figure 2. Samples treated with Bt

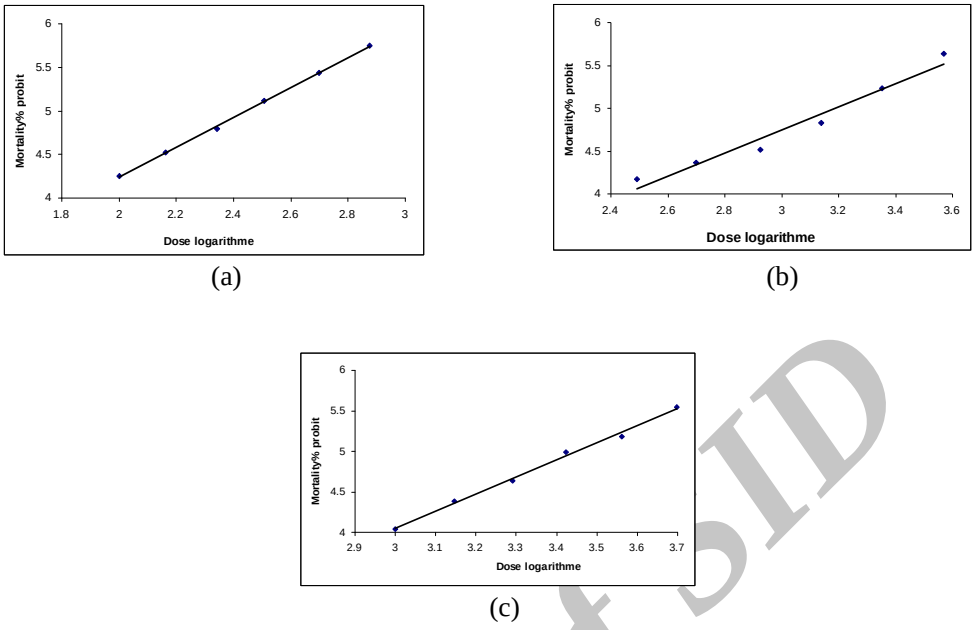

Figure 3. Relationship between dose logarithm and probit of mortality percentage of the first (a), second (b) and third (c) larval instars affected by Bt.

Preparation of various concentrations of Bt

Different concentrations of Bt were determined according to standard method.

First instar larvae

The highest and the lowest effective concentration on the first larval instar was 1500 and 46 ppm ,respectively that in preliminary experiments 81.11% and 13.33% mortality had caused, respectively. Based on suggestion of Robertson and Preisler (1991), concentration that cause 25% and 75% mortality using the equation between the dose logarithm and probit of mortality percentage obtained. Then four concentrations between these two concentrations were calculated using the formula of logarithmic distance. Antilog each equal shows concentration.

X=Logarithmic distance= $\frac{Log\max - Log\min}{n-1}$

X_1 = Concentration highest,
$X_2 = \log x_1 - x$,
$X_3 = \log x_1 - 2x$,
$X_4 = \log x_1 - 3x$,
$X_5 = \log x_1 - 4x$,
X_6 = Concentration lowest

Different concentrations of Bt used for the first larval instar:

Xmin = 100 ppm Xmax = 750 ppm n = 6

$X = \frac{Log\max - Log\min}{n-1} = 0.1762$

X_1 = 750 ppm,
X_2 = 500 ppm,
X_3 = 320 ppm,
X_4 = 220 ppm,
X_5 = 140 ppm,
X_6 = 100 ppm

Second instar larvae

The highest and the lowest effective concentration on the second larval instar was 4000 and 125 ppm ,respectively that in preliminary experiments 76.66% and 10% mortality had caused, respectively. Used Concentrations were calculated using logarithmic distance.

Xmin = 300 ppm Xmax = 3700 ppm n = 6

$$X = \frac{Log\ \max - Log\ \min}{n-1} = 0.2146$$

X_1 = 3700 ppm,
X_2 = 2250 ppm,
X_3 = 1380 ppm,
X_4 = 840 ppm,
X_5 = 500 ppm, X_6 = 300 ppm

Third instar larvae

The highest and the lowest effective concentration on the third larval instar was 6000 and 187 ppm, respectively that in preliminary experiments 76.66% and 8.88% mortalities had been caused, respectively. According mentioned method used concentrations of Bt were calculated using logarithmic distance. Different concentrations of Bt used for third larval instar:

Xmin = 1000 ppm Xmax = 5000 ppm
n = 6

$$X = \frac{Log\ \max - Log\ \min}{n-1} = 0.138$$

X_1 = 5000 ppm,
X_2 = 3650 ppm,
X_3 = 2650 ppm,
X_4 = 1950 ppm,
X_5 = 1400 ppm,
X_6 = 1000 ppm

Tests were done in 7 treatments (6 concentrations and a control) and each treatment was done in three replications.

Data Analysis

Data obtained from different experiments were recorded in tables and the data for determination of LC_{50} values were analyzed by Mstatc software and Probit method.

RESULTS

The determination LC_{50} value of Bt

The first larval instar

The mortality of first larvae instar at different concentrations start after 24 hours (Table 1). Figure 3a shows a relationship between dose logarithm and probit of mortality percentage of first instar larvae feeding from infected pistachio leaves to Bt. As in Figure 3a is observed larval mortality increases uniformity. After analyzing data, LC_{50} value of Bt for the first instar larvae were estimated 276.91 ppm.

The second larvae instar

The mortality of second larvae instar at different concentrations start after 24 hours (Table 2). Figure 3b shows relationship between dose logarithm and probit of mortality percentage of second instar larvae feeding from infected pistachio leaves to Bt. Obtained data from probit analysis shows that by attention to

logarithmic doses, larval mortality increases uniformity. After data analysis LC_{50} value of Bt for second instar larvae were estimated 1494.53 ppm.

Third instar larvae

The mortality of third larvae instar at different concentrations start after 48 hours (Table 3). Figure 3c shows relationship between dose logarithms and probit of mortality percentage of third instar larvae feeding from infected pistachio leaves to Bt. As it shown in Figure 3c larval mortality increases uniformity. After the analyzing Data, LC_{50} value of Bt for third instar larvae was estimated 2817.349 ppm. After the analyzing Data, LC_{50} values of Bt for the first, second and third larval instars were estimated 276.91, 1494.53 and 2817.34 ppm, respectively.

Table 1. The process of larval mortality of the first larval instar at different times affected by Bt.

Concentration (ppm)	Larvae number		Time (h)					Mortality (%)	Improved mortality (%)	Probit of mortality percentage	Dose logarithm
	A replicate	Total *	24	48	72	96	120				
Control	30	90	1	2	2	2	2	2.22	-	-	-
750	30	90	0	45	58	69	70	77.77	77.26	5.7454	2.875
500	30	90	0	39	59	61	61	67.77	67.03	5.4399	2.6989
320	30	90	0	35	46	48	50	55.55	54.54	5.113	2.5051
220	30	90	0	20	33	37	39	43.33	42.04	4.7981	2.3424
145	30	90	0	5	25	29	30	33. 33	31.81	4.5267	2.1613
100	30	90	0	0	18	21	22	24.44	22.72	4.2512	2

* Total = Sum of three replicates

Table 2. The process of larval mortality of the second larval instar at different times affected by Bt.

Concentration (ppm)	Larvae number		Time (h)					Mortality (%)	Improved mortality (%)	Probit of mortality percentage	Dose logarithm
	A replicate	Total *	24	48	72	96	120				
Control	30	90	0	1	2	2	2	2.22	-	-	-
3700	30	90	0	35	60	65	67	74.44	73.85	5.6372	3.5682
2250	30	90	0	30	50	53	54	60	59.09	5.2278	3.3521
1380	30	90	0	21	37	39	40	44.44	43.18	4.8262	3.1398
840	30	90	0	8	25	30	33	36.66	35.22	4.5201	2.9242
500	30	90	0	0	20	25	25	27.77	26.13	4.3597	2.6989
310	30	90	0	0	14	19	20	22.22	20.45	4.1726	2.4913

* Total = Sum of three replicates

Table 3. The process of larval mortality of the second larval instar at different times affected by Bt.

Concentration (ppm)	Larvae number		Time (h)					Mortality (%)	Improved mortality (%)	Probit of mortality percentage	Dose logarithm
	A replicate	Total *	24	48	72	96	120				
Control	30	90	0	1	1	1	1	1.11	-	-	-
5000	30	90	0	0	52	61	64	71.11	70.78	5.5448	3.6989
3650	30	90	0	0	32	49	52	57.77	57.29	5.1815	3.5622
2650	30	90	0	0	28	40	45	50	49.43	4.985	3.4232
1950	30	90	0	0	19	30	33	36.66	35.94	4.6389	3.29
1400	30	90	0	0	11	24	25	27.77	26.95	4.3842	3.1461
1000	30	90	0	0	7	13	16	17.77	16.84	4.0379	3

* Total = Sum of three replicates

DISCUSSION

Results show that used doses of Bt increase with the increase larval instar, so that, for causing mortality in the second and third larval instars is required higher doses. Concentration 750 ppm of Bt for the first larval instar cause 77.77% mortality, but 74.44% mortality for the second larval instar were obtained with concentration 3700 ppm and for third larval instar, concentration 5000 ppm were created 71.11% mortality.

In this study, LC_{50} values for the first larval instar 276.91 ppm were estimated. These larvae were transferred on treatments after a few hours feeding on healthy leaves. This LC_{50} value shows that the first larval instar of this insect had high susceptibility to this pathogen. The effect of this subspecies of Bt on *Spodoptera exigua* was studied and the LC_{50} value of Bt for the first larval instar of *S. exigua* 233.8 ppm was reported (Ignoffo *et al.*, 1977). Moar *et al.* (1986) determined LC_{50} value of Bt by adding it to artificial meal of the first larval instar *S. exigua* 299 ppm. As it can be observed the difference is not considerable and little difference is related to species difference and different conditions of two experiments. These researchers had used from artificial meal in controlled conditions of laboratory. The second larval instar had lower susceptibility to Bt than the first larval instar. LC_{50} value of these larvae 1494.53 ppm were estimated. LC_{50} value this combination for third larval instar 2817.34 ppm was estimated and shows more resistance of this instar than first and second larval instar. Namvar *et al.* (2002) investigated the effect of Bt on first, second and third larval instars of *S. exigua* and LC_{50} values for each of these larval instars determined 311.61, 1356.95 and 2708.27 ppm, respectively.

These results had consistency with the results of this study and observed differences related to the species difference and different conditions of experiments. Ignoffo (1966) reported that when the larvae reach to their two-thirds weight, achieve to a kind of physiological resistance which is called maturity resistance and then become resistant against all microbial agents and chemical toxins. To control them, high doses of these compounds are required. The results of this study confirmed the above subjects

and also showed susceptibility reduction and increase of the amount of needed bacterium by attention to larval instar increase that, had to be used higher concentrations of Bt for creating mortality in the second and third larval instars especially in third larval instar.

Adldoost and Javanmogaddam (2000) evaluated the effect of Bt and carbaryl chemical insecticide on Chickpea podborer (*Heliothis viriplaca*) and reported that the microbial insecticide Bt and carbaryl 3 days after foliar application 75% and 91.61% and after 10 days 82.25% and 75.51% mortalities cause on *H. viriplaca*, respectively. Karimi and Moazami (2000) studied the effect of Bt on different larval instars *Pieris rapa* and reported that there is a significant difference between different larval instars and recorded time in terms of number of mortality. Mortalities of first, third and fifth larval instars start after 24, 72 and 96 hours after treatment, respectively. Susceptibility of larvae with increase larval instar decreased and more time was needed for causing mortality in older larval instars. In this study, mortality of third larval instar of *O. terebinthina* start after 72 hours whereas in the first and second larval instars, mortality starts after 24 hours. This result shows that the third larval instar had lower susceptibility to Bt than the first and second instars. The all of these studies show that this subspecies of Bt can control pests of Lepidoptera order. *O. terebinthina* feeds on leaves and eliminates plant photosynthesis part where its materials were used for fruit formation. According to collected data in this research and the selective nature of Bt , we can conclude that this microbial compound can be used for reasonable control of this pest that nowadays was controlled by chemical insecticides and sometimes several spraying steps to control are necessary. Bt can used to control this pest instead of chemical insecticides. Because, this combination has less destructive effects than broad spectrum insecticides due to short half life and to be selective (Reed *et al.*, 2001). But, it is better to apply this microbial compound against the first larval instar as, the results show this larval instar is the most susceptible to Bt.

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