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The Synergist Effect of The Henna Plant, *Lawsonia alba* on *Bacillus thuringiensis* var. *kurstaki* Against Third Larval Instar of Pistachio Leaf Borer, *Ocneria terebinthina* Strg. (Lep.: Lymanteriidae)

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Abstract

Ocneria terebinthina Strg. (Lep.: Lymanteriidae) is a leaf borer pest in pistachio orchard. The toxicity of *Bacillus thuringiensis* var. *kurstaki* alone and in combination with henna powder was investigated on third larval instar of *O. terebinthina* under laboratory conditions. Bioassay was carried out using spray technique on pistachio offshoot. Probit analysis of concentration-mortality data was conducted to estimate the LC_{50} value. The LC_{50} value of *B. thuringiensis* on third instar of larvae was estimated as 2817.30 ppm. The synergist effect of henna powder on efficacy of *B. thuringiensis* was also evaluated. Three concentrations of henna powder were combined with minimum lethal dose of *B. thuringiensis* against third larval instar. The results revealed that the henna powder had synergist effect on *B. thuringiensis*. The combination of *B. thuringiensis* and henna powder (6000 ppm) caused 80.68% mortality, whereas *B. thuringiensis* alone caused 15.91% mortality. Our results suggested that henna powder can increase the efficacy of *B. thuringiensis* in controlling *O. terebinthina* in pest integrated management.

Keywords: *Bacillus thuringiensis var. kurstaki, Lawsonia alba,* LC50, *Ocneria terebinthina,* Synergist.

Introduction

The pistachio, *Pistacia vera* L. (Sapindales: Anacardiaceae), is one of the most important economic crops in Iran (Mehrnejad, 2010). Pistachio leaf borer, *Ocneria terebinthina* Strg. (Lep.: Lymanteriidae) is one of many pistachio pests (Mehrnejad, 2001). Biology, population dynamism, control and physiological strategy of *O. terebinthina* has been studied by many researchers (Sabahi *et al*., 1995; Sepehr and Abaii, 1998; Omid *et al*., 2005; Behrozi *et al*., 2011, 2012; Poursalari *et al*., 2013). This pest is present in the winter as - a fourth larval instar (Behroozi *et al*., 2012) under loose bark on the trunk of pistachio trees or in white cocoons beneath plant litters. Activity of the overwintering larvae usually starts from the late March. Prepupal formed on the host leaves or bark crevices on infested trees. The adults appear in pistachio orchards in early spring (males appear earlier than females). Females lay eggs in batches on both the upper and lower surfaces of the pistachio leaves. The number of eggs in a single batch could be 4.75 to 361. In early autumn, the third instar larvae move toward diapausing shelters, molt to the fourth instar, make a whitish silk cocoon, and enter diapauses. *O. terebinthina* has seven larval instars (Poursalari *et al*., 2013). The young larvae of this pest feed on the leaf parenchyma and upper epidermis. They produce large skeletonised patch and brown spots on the pistachio leaves, although the old larvae leave only the mid-vein (Mehrnejad, 2001). This species has three to four generations (Sabahi *et al*., 1995; Poursalari *et al*., 2013 Sepehr *et al*., 1989; Omid *et al*., 2005).

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Hence, this pest decreases plant photosynthesis levels (Esmaili, 1996). This pest also feeds on wild pistachio, *Pistacia mutica.* Wild pistachios are in most provinces of Iran (Kerman, Khorasan, Fars, Tehran and etc.) *O. terebinthina* is controlled by chemical insecticides and sometimes several spraying steps are necessary (Esmaili, 1996). Unexpected side effects of pesticide applications, such as pest resistance, pest resurgence and outbreaks of secondary pests, may occur when overuse of insecticides is employed in crops over prolonged periods. These harmful side effects emphasize the need for alternative methods of control that do not rely on insecticides alone (Azimizadeh *et al.,* 2012).

The use of microorganisms has assumed a prominent position among the options that seek to control insect pests without the use of chemicals and with high specific toxicity applied in agroecosystems (Schünemann *et al.,* 2014). The bacterium *Bacillus thuringiensis* (Berliner, 1909), *Bt*, represents approximately 95% of microorganisms used in biological control of agricultural pests in different cultures (Lambert *et al.,* 1992). This bacterium is the most promising for the production of biopesticides and plant resistant to insects associated with environmental preservation (Frankenhuyzen, 2009), economic aspect and the safety to human health (Siegel, 2001). Despite that, the overall market growth of biopesticides does not meet the expectations of the 1990s.For over 50 years, *Bt* has been used in formulations for the biological control of many agricultural pests and vectors of human diseases and is in more than 90% of commercially available microorganism products (Chattopadhyay *et al.,* 2004; Nester *et al.,* 2002). The entomopathogenic bacterium, *Bacillus thuringiensis* var. *kurstaki*, is widely used in commercial formulations for controlling various agricultural against lepidopteran pests (Jurat-Fuentes and Jackson, 2012). *Bt* is a gram-positive, rod-shaped bacterium with the ability to produce crystalline inclusions during sporulation (Höfte and Whiteley, 1989). *Bt* synthesizes a variety of insecticidal crystal proteins called ICPs or Cry proteins which, after solubilization in the gut and modification by gut juice proteases, are toxic to a number of lepidopteran, dipteran and coleopteran larvae (Höfte and Whiteley, 1989).

The use of synergist compounds such as food stimulants and non-active compounds of protease enzyme to prevent analysis of toxins protein of *Bt*, increases activity of Bt (McIntosch *et al.,* 1990). The development of *Bt* synergists is a perpetual process to amend the inadequacy of *Bt* pesticides. The use of synergists could lead to a reduced quantity of *Bt* needed to obtain control (Dubois and Dean, 1995; Liu and Tabashnik, 1997). Synergists may also have a possible application in delaying the evolution of resistance in target insects (Georghiou and Saito, 1983; Raffa and Priester, 1985; Brattsten *et al.,* 1986). Tannic acid is hydrolysable tannin and can be formulated as water-soluble, non-carcinogenic and activity mutagenic. Compounds containing tannin cause a delay in the development and death of insects (Gibson *et al.,* 1995). Tannic acid is the active ingredient of skin extracts of *Taxus* plant that delay the development and mortality treated insects. Insects that feed on plants containing large amounts of tannins, tannic acid passes through peritrophic membrane, degenerates epithelial cells and causes internal wounds (Bernays, 1980). In grasshoppers, the passage of tannic acid through peritrophic membrane and damage to epithelial cells causes a delay in development. Tannins, particularly tannic acid, act as a toxin and can exacerbate the effects of microbial agents such as *Bt* (Gibson *et al*., 1995).

The henna plant, *Lawsonia alba* Lam. (Lythraceae), is a native of North Africa and South-West Asia and partially cultivated in West Africa (Zafar *et al.,* 2006). The leaves of henna contain Lawson-colored material, resin, tannin and lipid (Ainehchii, 1986). The aim of this

study was to determine the toxicity *Bt* to third instar larvae of *O. terebinthina*, to investigate the synergist effect of henna plant on *Bt* and to reduce the consumption of *Bt*.

Materials and Methods

Rearing of the test insects

The eggs of the insect were collected from orchards located around Anar in Rafsanjan. The eggs were laid on lower level of leaflets of host plant as batches. Each egg batch was placed in plastic dish (5 × 17 × 22 cm) with a piece of moistened cotton and covered with a lace cloth in the laboratory. Both egg masses and larvae were reared in an environmental chamber under a photoperiod of 16: 8 (L: D) h at 25˚C. Larvae were reared in plastic dishes (5 × 17 × 22 cm) on pistachio offshoots. Offshoots were inserted in water-filled drug glass to prevent desiccation. Offshoots were fixed in drug glasses using cotton. Rearing dishes were covered with a lace cloth.

Bt

Bacterium *Bt* used in this study was provided from Mehr Asia Biological Technology Company, Iran.

Bioassay Technique

The distal part of pistachio offshoots were picked and transferred to the laboratory. Each stem of the offshoot was fixed into water-filled drug glass using cotton. Then, the offshoots were contaminated in different concentrations of *Bt* using the spray method. Contaminated offshoots were air dried. Offshoots were placed within disposable dishes (5 × 17 × 22 cm) and larvae were transferred to treated foliage. The dishes were blocked with a white lace cloth in such a way that did not allow the larvae to escape. The final concentrations of *Bt* (5000, 3650, 2650, 1400 and 1000 ppm) were calculated using logarithmic distance (Robertson and Preisler, 1991) following preliminary trials and were tested on third instar larvae. Distilled water was used as the control. The trial was arranged with three replicates for each treatment. Each replicate consisting of 30 newly molted third instar larvae. Mortality was recorded every 24 hours for five days. Obtained mortalities were corrected using the Abbott formula.

Synergist effect

This study was carried out in order to evaluate the synergist effects of henna powder on *Bt*. The lowest effective concentration *Bt* (1000 ppm) in previous trial was chosen to be combined with henna powder. Based on the preliminary tests, three concentrations 4000, 5000 and 6000 ppm henna powder were used in the bioassay. The six treatments included:

1. Henna powder (6000 ppm)
2. Henna powder (6000 ppm) + *Bt* (1000 ppm)
3. Henna powder (5000 ppm) + *Bt* (1000 ppm)
4. Henna powder (4000 ppm) + *Bt* (1000 ppm)
5. *Bt* (1000 ppm)
6. Control (distilled water)

The trial was carried out such as the one described in the previous section.

Statistical analysis

The LC_{50}, 95% Confidence Interval (CI), the slope and intercept of the probit mortality regression and the relevant statistical tests were estimated by probit analysis using POLO – PC Software. Data were analyzed for analysis of variance (ANOVA) as completely randomized design (SAS 9.1). The means were separated by Duncan's test ($P < 0.05$).

Results

The results indicated that *Bt* is effective on third instar larvae of *O. terebinthina*. The LC_{50} value of *Bt* was estimated 2817.30 ppm (Table 1). There were significant differences among different *Bt* treatments after 120 hours ($F = 27.27$; df = 5, 12; $P < 0.0001$) ($P < 0.05$) (Table 2). The mortality percentage of third instar larvae enhanced with increasing *Bt* concentration. Morality percentages were significant among the different treatment groups and classified in different groups (Fig. 1).

Table 1. The toxicity of *Bt* on third larval instar of *O. terebinthina* 120 h post-treatment.

	n	Slope ± SE	$LC_{50\ (PPM)}$	95% Confidence Interval	t	χ^2
Bt	540	2.10 ± 0.25	2817.30	2474.34 – 3266.59	8.25	0.34

The lowest morality was recorded in 1000 ppm concentration (16.19%). 3650 (52.64%) and 2650 ppm (45.55%) concentrations showed no significant differences. Also, 3650 (52.64%) and 2650 ppm (45.55%) concentrations were classified in one group (Fig. 1).

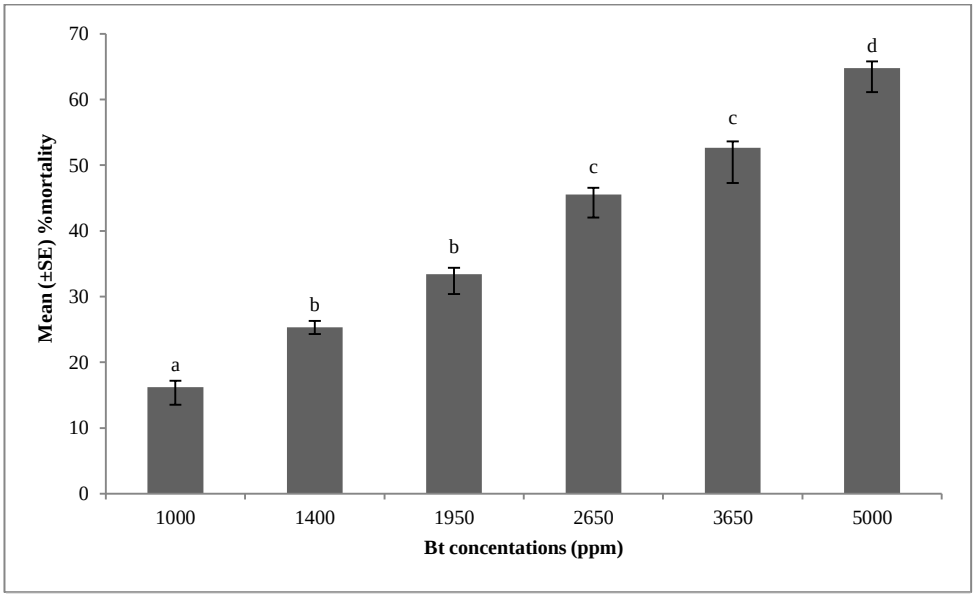

Fig 1. Means (± SE) mortality percent of third instar larvae of *O. terebinthina* treated with *Bt* (120 h post-treatment)

There were significant differences in the percentage of morality among the different treatment groups in the presence of henna powder ($F = 81.57$; df = 4, 10; $P < 0.0001$) 168 hours post-treatment ($P < 0.05$) (Table 2). Mean mortalities in the presence of henna powder were significantly higher than *Bt* alone (Fig. 2). The least and greatest morality were found in henna powder (6000 ppm) (11.36%) and henna powder (6000 ppm) + *Bt* (1000 ppm) (80.68%) treatments after 168 hours of treatment, respectively (Fig. 2). Henna powder (6000 ppm) + *Bt* (1000 ppm) (80.68%) and henna powder (5000 ppm) + *Bt* (1000 ppm) (73.86%) treatments were classified in a group. Henna powder (6000 ppm) and *Bt* (1000 ppm) showed no significant differences between each other (Fig. 2).

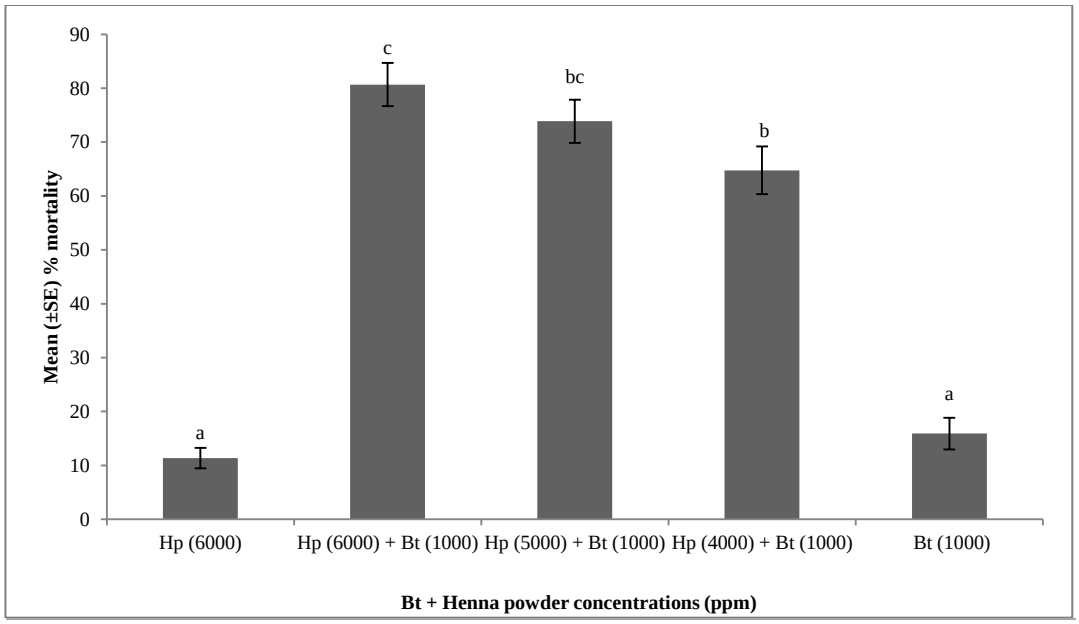

Fig 2. Means (± SE) mortality percent of third instar larvae of *O. terebinthina* treated with *Bt* and combination of *Bt* and henna powder (168 h post-treatment).

Table 2. Variance analysis of different treatments on third instar larvae of *O. terebinthina* 120 and 168 h post-treatment

S.V	df	Sum of Squares	F	Sig.	df	Sum of Squares	F	Sig.
Treatment	5	978	27.27	0.0001	4	3286.14	81.57	0.0001
Error	12	35.86			10	40.29		

Discussion

In this study, *Bt* was effective on third instar larvae of *O. terebinthina* (LC_{50} = 2817.30 ppm). Mortality of larvae of this pest began after 72 hours. This result is consistent with results of Gibson *et al*. (1995) and of Karimi and Moazami (2000). The latter researchers studied the effect of *Bt* on different larval instars *Pieris rapae* and reported that mortality of first, third and fifth larval instars started 24, 72 and 96 hours post-treatment, respectively. Also, Namvar (2002) investigated the effect *Bt* on third instar larvae of *Spodoptera exigua* (Hubner) and LC_{50} value for this larval instar was estimated as 2708.27 ppm. In this study, the feeding of larvae decreased considerably. In addition, the mortality of larvae was delayed. Loseva *et al.* (2002) reported that the insect larvae that ate lethal amounts of Cry toxin resulted in their death. Lutrell *et al*. (1982) evaluated effects *Bt* on *Heliothis zea* and *H. virescence*. The results revealed that the bacterium reduced surviving treated larvae weight (after 7 days).

Bernays *et al*. (1980) reported that the tannins found in some plant species degenerated midgut epithelium and increased the efficiency of *Bt*. In this study, henna powder at 4000, 5000 and 6000 ppm concentrations in combination with *Bt* (1000 ppm) yielded mean mortalities 64.76, 73.85 and 80.68%, respectively. Mortality in *Bt* treatment alone was 15.91%. Tannins, particularly tannic acid, acted as a toxin and exacerbated the effects of microbial agents such as *Bt* (Gibson *et al*., 1995). This caused more bacterial spores into the midgut cavity enter to the haemocoel and therefore, increased the mortality of larvae (Bernays, 1980). Henna powder delayed death of

larvae and mortality after 96 hours in henna powder + *Bt* treatments. The development of *O. terebinthina* larvae was affected in henna powder treatments. Gibson *et al.* (1995) used tannic acid and skin extract of *Taxus boccata* (containing a lot of tannin) in combination with *Bt* and reported these compounds delayed the development and death of treated-insects. Adding skin extract of *T. boccata* to larvae meal of *H. baccata* delayed the larval development significantly. In another study, Khanizad and Safaralizadeh (2002) investigated the effect of tannic acid in combination with different concentrations of *Bt* against second instar larvae of *Galleria mellonella*. These researchers reported 9.48% mortality in tannic acid treatment (12000 ppm) alone. *Bt* concentrations (50, 112, 253, 570 and 1283 ppm) showed 6.7, 20, 24.5, 44.5 and 60% mortality, respectively. Mortality in different concentrations of *Bt* in combination with tannic acid were recorded 57.8, 37.2, 75.6, 91.2 and 93.4% mortality, respectively (Khanizad and Safaralizadeh, 2002). Granados *et al*. (2001) demonstrate that enhancin from the *Trichoplusia ni* (Hübner) granulovirus enhancin can increased the toxicity of *Bt* to several noctuid species. The addition of enhancin significantly increased the toxicity of Dipel, a commercial *Bt* formulation, to six species (*T. ni*, *Helicoverpa zea* (Boddie), *H. virescens* (Fabricius), *S. exigua* (Hübner), *Pseudoplusia includens* (Walker), and *Anticarsia gemmatalis* (Hübner)). These researchers reported that the peritrophic membrane of larvae can impede the movement of the *Bt* toxin protein to the midgut brush border and that enhancin can increase the toxicity by affecting the permeability of the peritrophic membrane.

Our study showed that *Bt* is effective on *O. terebinthina* and that henna powder increased the efficacy of the lowest lethal concentration of *Bt*. The combination is advantageous in arresting development, minimizing foliar damage and decreasing the concentration of *Bt* used.

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