Bio-control efficiency of *Bacillus thuringiensis* (Berliner) against the citrus leaf miner, *Phyllocnistis citrella* Stainton (Lep., Gracillariidae) under laboratory conditions

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Abstract

The citrus leaf miner, *Phyllocnistis citrella* Stainton (Lep., Gracillariidae), is one of the most destructive pest of citrus and related Rutaceae and ornamental plants in Iran. Larvae damage leaves by creating serpentine feeding mines, which have been lead to reduce yield. Resistance and toxicity problems derived from synthetic insecticides have made it necessary to find more effective and healthier alternatives; therefore, bio-insecticides (*i.e.*, *Bacillus thuringiensis*) are becoming an important component in plant protection. The aim of the present study was to evaluate the efficiency of *B. thuringiensis* against *P. citrella*. Eight *B. thuringiensis* concentrations were used against *P. citrella* L3 on orange and mortality was recorded at 1, 4, 7 and 10 days after spraying. The results showed that *B. thuringiensis* significantly affected mortality of *P. citrella*. After 1, 4, 7 and 10 days of spraying 10^8 concentration of *B. thuringiensis* had significantly caused the highest mortality to the pest with 59.8, 68.4, 73.6 and 77.0%, respectively. Then the mortality percent decreased until it reached 6.5, 9.5, 39.3 and 46.7% at 10^1 concentration, respectively. In conclusion, the study indicated that *B. thuringiensis* is effective in controlling *P. citrella* under laboratory conditions.

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Introduction

Citrus is one of the important fruit crops that are useful for human being as a source of nutrition and health product. It provides high levels of vitamin C and potassium and some of the daily requirements for essential nutrients such as folic acid and thiamine. Citrus growing in Iran has a very old history, which goes back as far as 330 BC (Ebrahimi, 2010). In 2013, Iran produced a total of four million tons of citrus from planted areas of about two hundred and ninthly thousand hectares with an average of 19,000 kg/ha (Agricultural Statistics, 2010).

Like many other agriculture crops, citrus production is hampered by problems, mainly of pests and diseases (French *et al*., 1997). One of the main insect pests that attack citrus is the citrus leaf miner, *Phyllocnistis citrella* Stainton (Lep., Gracillariidae), which adversely affect plant health and fruit development and enhance the development of canker disease (Heppner, 1995; Das *et al*., 1998; Gill, 1999; Liu *et al*., 1999; Mafi & Ohbayashi, 2000; Michaud & Grant, 2003; Beattie & Hardy, 2004; Kahrarian, 2010).

The first record of citrus leaf miner from southern and northern Iran, with a dramatic increase and widespread dispersal, was noted in 1961 and 1994, respectively (Amiri Besheli, 2006a). The pest has five to nine generations/year, with peak periods in early summer and early autumn (Amiri Besheli, 2006b). *P. citrella* population increased over the years due to increasing cultivation of citrus and inappropriate agricultural practices applied by gardeners (Margobandhu, 1993; Jacas & Pena, 2002; Moreira *et al*., 2006).

The control of this pest is based on chemical products. However, *P. citrella* has developed resistance to almost all pesticides used including organophosphates, pyrethroids, and carbamates, requiring higher doses or a mixture of several products for their effective control. These practices result in increased production costs and contamination of the environment (Beattie *et al*., 1995). Vigorous application of chemical insecticides has been used to repress *P. citrella* in the Middle East (Jafari, 1995; Jafarzadeh, 2000; Javan Moghadam, 2001). In Iran, Seraj (2001) reported that abamectin and imidacloprid caused mortality higher than 70% to the pest under laboratory conditions (Seraj, 2001). In addition, Demir *et al*. (2012) in Turkey and Jafari (1997) in Iran, reported that diflubenzeron proved to be effective against *P. citrella*.

However, the use of synthetic pesticides is neither economic nor tolerable and has an undesirable effect on the environment due to their slow biodegradation in the environment, natural enemies and farmers (Jafarzadeh, 2000; Seraj, 2001). The intensive pest control programs using modern insecticides practiced in the Pakistan and Iran is not only a costly form of pest control, but also leads to resurgence of secondary pests such as mites and scale insects (Jafari, 1996). The

adverse effects of synthetic pesticides have amplified the need for effective and biodegradable pesticides.

An alternative is the use of biological control such as the use of predators, parasitoids, and entomopathogens, including fungi, bacteria, viruses, and nematodes. Within the bacterial group, the microorganism most widely used worldwide with the highest success in the control of several insect pests is the bacterium, *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae). *B. thuringiensis* has been shown to be useful for the control of different insect pests that affect plant crops, forest trees, or those are vectors of human diseases such as dengue and malaria (Dubois & Dean, 1950; Mahapatro &, Gupta, 2000).

B. thuringiensis is a gram-positive and soil-dwelling bacterium commonly used as a biological pesticide. *B. thuringiensis* also occurs naturally in the gut of larvae of various types of lepidopteran, dipteran and coleopteran insects (Demir *et al*., 2012). During sporulation, many *B. thuringiensis* strains produce crystal proteins (proteinaceous inclusions), that are toxic to the larvae of Lepidoptera and other orders of invertebrates.

Because of there is no comprehensive studies on the use of *B. thuringiensis* against citrus leaf miner in Iran, therefore, the main objective of this study were to the efficacy of *B. thuringiensis* against *P. citrella*. Results presented here may be helpful for future planning of large-scale citrus cultivation in similar environmental conditions of tropics, especially for pest management purposes.

Material and methods

In this study the stock cultures of the citrus leaf miner, *P. citrella* and its host plant were inevitably required for the various experiments. Therefore, mass rearing both *P. citrella* and the host plant must be conducted. In principle, there are two main activities related to mass culturing of insect and planting of the host plant, *Citrus sinensis* L.

Planting of the host plant, *Citrus sinensis*

Citrus sinensis L, common known as orange is one of the host plants that are most preferred by the citrus leaf miner, *P. citrella* (Beattie, 2004); therefore, this plant is used to rear the citrus leaf miner. The plant can be propagated through seedling and stem cutting; however, the use of seedling is preferable to stem cutting. Germination of *C. sinensis* L. seeds was done on plastic trays (35 cm long × 25 cm wide at top; 31 cm long × 21 cm wide at bottom; and 10 cm high) filled with a mixture of mineral soil, manure and sand (1:1:1). These seeds were placed a row in planting holes spaced 5 by 5 cm apart. The mixture was kept damp in order to hasten seed turgidity and germination. The seeds started to germinate after 2-3 weeks. The seedlings, 10 to 15 cm tall, were then transplanted to plastic flower pots (20 cm top diameter × 15 cm bottom diameter × 18 cm high) containing the same mixture. Plants reaching more than 50 cm tall and approximately 10 mm in stem diameter were used to culture *P. citrella*. All plants were kept in the greenhouse agriculture research center of Yasouj, with a temperature of 33-40°C and relative humidity of 50-65% and natural photoperiod (Figure 1).

Rearing of *Phyllocnistis citrella*

The rearing of *P. citrella* was initiated from newly emerged larvae collected from citrus orchards in Gachsaran, Iran, and maintained on potted orange seedlings. The infested orange seedlings were kept in cages of 60×60×100 cm under laboratory conditions of 35±5°C temperature, 60±10% relative humidity and 12:12 h (L:D) photoperiod at the Research Laboratory of Agriculture Yasouj, Iran. The cages were covered with muslin cloth from their sides and tops to provide adequate ventilation (Figure 2). Potted orange plants were grown in an air-conditioned greenhouse in small pots.

Bacillus thuringiensis

Eight concentrations of *B.thuringiensis* var. kurstaki were selected to conduct the experiments. *B. thuringiensis* was obtained from a stock culture at the Department of Plant Protection, Faculty of Agriculture, Shiraz University. The bacteria were grown on nutrient broth to aid sporulation. The culture was then incubated on a rotary shaker (300 rpm) at 28°C for four days to ensure sporulation and cell lyses. Spores and crystals were harvested by centrifugation at 1.0000 rpm for 15 min at 8°C. The pellet (3.5-4 g/L) was washed with distilled water and diluted to obtain 10^1-10^8 viable spores/mL for use in the experiments. However, in order to prepare the different concentrations, *B. thuringiensis* pellets were diluted in sterile distilled water to get 10^8 viable spores/mL and they were counted by haemocytomer slide. Hereafter, subsequent dilution to 10^7 until 10^1 was made from the 10^8 viable spores/mL.

Figure 1. *Citrus sinensis* L., host plant for mass rearing *Phyllocnistis citrella* in greenhouse.

Figure 2. The cages (60 cm long × 60 cm wide × 100 cm high) covered with muslin cloth that was used to rear *Phyllocnistis citrella*.

Treatments

The experiments were conducted in Petri dishes, 5.5 cm in diameter and 1 cm in height, partially filled with a 0.5 cm thick layer of wetted cotton pad and the lid of each Petri dish had a hole closed with organdie fabric for ventilation. Citrus leaf discs of 10 cm^2 area cut from uninfected citrus plants were placed in the dishes. Third larval instars of *P. citrella* were gently transferred using a Camel hairbrush into the Petri dishes in groups of ten larvae/Petri dish. Larvae in control groups (n=10) were sprayed with a 1 ml of distilled water, while larvae in treatment groups (n=10) were sprayed with a 1 ml aqueous solution of the required concentrations of *B. thuringiensis* by using a calibrated small sprayer. The Petri dishes were kept under the fore-mentioned laboratory conditions. For each concentration, three replicates were used and each Petri dish contained ten *P. citrella* larvae. Larval mortality was recorded at 1, 4, 7 and 10 days post spraying. Larvae were considered dead if they did not move when lightly prodded with forceps. The overall control mortality was 0.00, 13.30, 23.33% and 33.30% at 1, 4, 7 and 10 days of the experiments.

Statistical analysis

The statistical analysis was performed using MSTAT-C statistical software and the means were compared with Duncans multiple range test (DMRT). The mortality resulting from *B. thuringiensis* treatment was adjusted for the control of mortality using Abbott's formula (Aabbott, 1925).

Results

The results showed that direct spraying of *P. citrella* larvae by all *B. thuringiensis* concentrations tested exhibited a range of mortality after 1, 4, 7 and 10 days after spraying (Figure 3). *P.citrella* larvae mortality was significantly affected by *B.thuringiensis* concentration and time after bacterial application. One day after spraying 10^8 and 10^7 concentrations had significantly caused the highest mortality to the larvae with *59.8*% and *54.5*%, respectively. Then, the mortality percent decreased significantly until it reached *6.5*% at 10^1 concentration (F=15.44; 7, 24 df; P=0.000). Four days after application, the mortality varied significantly among the different concentrations (F=10.33; 7, 24 df; P=0.000), in which it reached 68.4% at 10^8 concentration and started to decrease with decreasing *B. thuringiensis* concentrations until it reached *9.5*% at 10^1 concentration. Mortality levels were relatively higher at seven days than at one day and four days post spraying, in which the mortality levels reached up to 73.6% at 10^8 concentration, whereas the least mortality was recorded at 10^1 concentration with *39.3*% (F=8.78; 7, 24 df; P=0.000). Ten days after spraying, the percentage of mortality was the highest compared with 1, 4 and 7 days after application. However, there were significant differences in the mortality percent among the different concentrations of *B. thuringiensis*, where it was 77.0% at 10^8 concentration, while the least mortality of *46.7*% was recorded at 10^1 concentration.

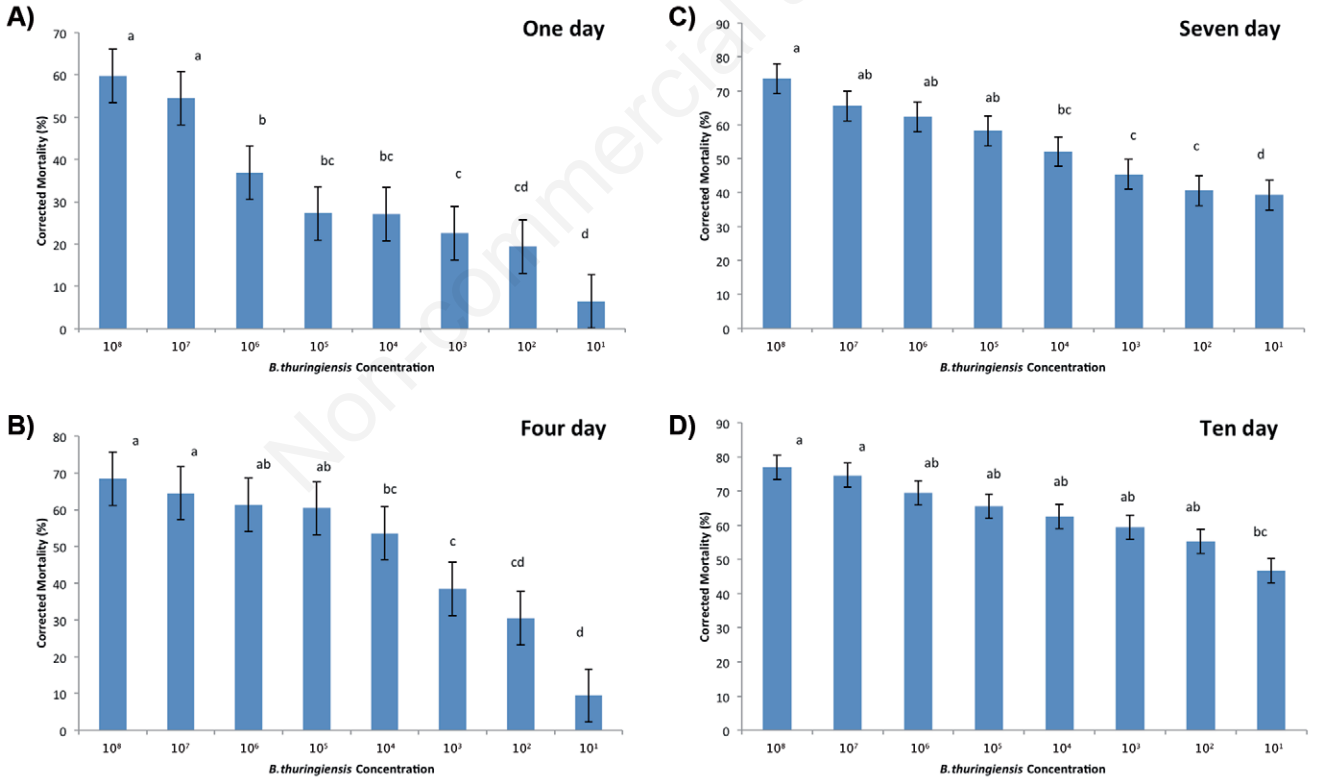

Figure 3. A) Corrected mortality percent of third larval instars of *Phyllocnistis citrella* after one day of spraying of *Bacillus thuringiensis* in direct spray test. [Different letters above bars indicate significant difference among the different *B. thuringiensis* concentrations]. B) Corrected mortality percent of third larval instars of *P. citrella* after four days of spraying of *B. thuringiensis* in direct spray test. [Different letters above bars indicate significant difference among the different *B. thuringiensis* concentrations]. C) Corrected mortality percent of third larval instars of *P. citrella* after seven days of spraying of *B. thuringiensis* in direct spray test. [Different letters above bars indicate significant difference among the different *B. thuringiensis* concentrations]. D) Corrected mortality percent of third larval instars of *P. citrella* after ten days of spraying of *B. thuringiensis* in direct spray test. [Different letters above bars indicate significant difference among the different *B. thuringiensis* concentrations].

Further statistical analysis of results among the different days within the same concentration of *B. thuringiensis* showed that there was a significant increase in mortality for all *B. thuringiensis* concentrations at higher treatment times (Figure 3). The percentage mortality of *P. citrella* larvae was significantly higher after 10 days post spraying followed by 7 days and then by 4 days and the least mortality was recorded at 1 day after application for all concentrations (F=4.40; 24.18; 3, 12 df; P=0.000 -0.037).

The overall corrected mortality percent of third larval instars of *P.citrella* in all days as a result of spraying with different concentrations of *B. thuringiensis* is shown in Figure 4A. The 10^8 concentration resulted in the highest mortality with *75.6*% and it was significantly higher than that caused by any of the other *B. thuringiensis* concentrations, while the lowest mortality was recorded for 10^1 concentration with only *23.7*% (F=11.45; 7, 96 df; P=0.000). There was also a positive correlation of 0.655, significant (0.000) at 0.01 probability level. This means that with increasing the concentration of *B. thuringiensis* up to 10^8 there was an increase in the mortality of *P. citrella*.

The overall corrected mortality percent of *P. citrella* in all concentrations of *B. thuringiensis* on the different days after spraying is shown in Figure 4B. The results showed that the mortality was significantly increased with time and was *33.5*%, *51.4*%, *60.3*% and *71.5*% after 1, 4, 7 and 10 post spraying, respectively (F=12.55; 3, 96 df; P=0.000). Also, there was a positive correlation of 0.567, significant (0.000) at 0.01 probability level.

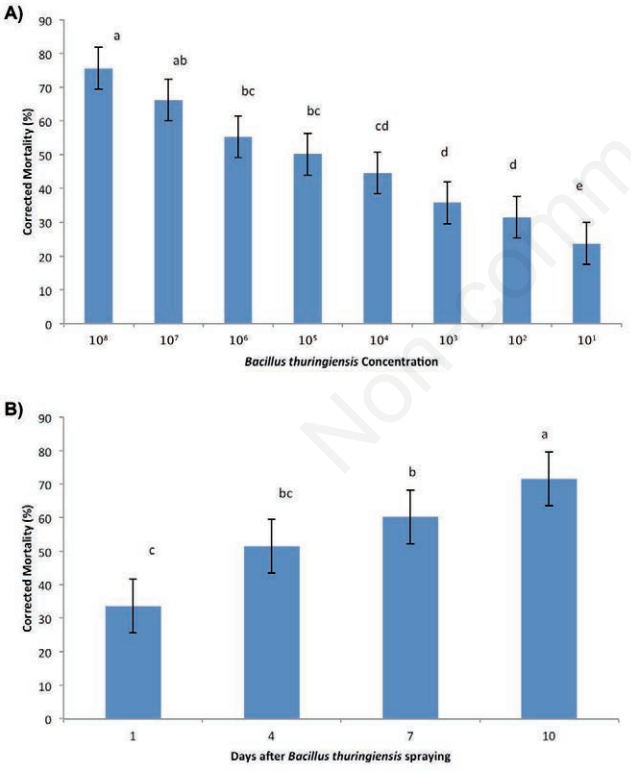

Figure 4. A) Overall corrected mortality percent of third larval instars of *Phyllocnistis citrella* on all days as a result of spraying with different concentrations of *Bacillus thuringiensis*. [Different small letters above bars indicate significant differences among the different *B. thuringiensis* concentrations]. B) All concentrations of *B. thuringiensis* on the different days after spraying. [Different small letters above bars indicate significant differences among the different days (B) at P<0.05 (one -factor analysis of variance)].

Discussion

The indiscriminate and injudicious use of insecticides has led to a number of adverse effects in the environment. The undesirable effects of these chemical insecticides used against insect pests in crops warrants the development of strategies that could eliminate or reduce the involvement of insecticides for controlling insect pests. The citrus leaf miner, *P. citrella* Stainton (Lep., Gracillariidae) originated from southeast Asia and established itself as a major pest of citrus throughout the Middle East (Moreira *et al*., 2006), where Iran is located. The leaf miner attacks all cultivars of citrus, related species within the Rutaceae family, and several ornamentals (French *et al*., 1997). Plant damage is caused by leaf miner larvae as they bore through the leaf epidermis. Leaves become chlorotic, often deformed, and susceptible to infection by fungi or bacteria.

Preliminary field trials with selected insecticides indicate the superiority of Dimilin (diflubenzuron) over Diazinon, Zolone (Phosalone) and Ekamet (Etrimfos) in controlling citrus leaf miner in northern Iran, but it is not totally effective (Amiri Besheli, 2006a). But, it is well known that continuous use of chemical insecticides is neither economic nor sustainable and has a negative impact on the environment, natural enemies and gardeners. Moreover, *P. citrella* has a long history of resistance to many chemical insecticides, and development of resistance against a chemical sometimes makes it difficult to obtain sufficiently high control (Mafi & Ohbayashi, 2000). Therefore, efforts are needed to develop integrated pest management strategies for the management of this pest through the use of bio-pesticides. Results of the present study indicated that *P. citrella* larval mortality was significantly affected by *B. thuringiensis* concentration and time after bacterial application. After 1, 4, 7 and 10 days of spraying, 10^8 concentration of *B.thuringiensis* had significantly caused the highest mortality to the pest with 58.6, 68.4, 73.6 and 77.0%, respectively. Comparing the results of this study with another study conducted by Amiri-Beshli (Amiri Besheli, 2006b) to control the citrus leaf miner with *B.thuringiensis*, mineral oil, insecticidal emulsion (garlic extract, plant detergent soap and food additive) and insecticidal gel (plant oil and plant extracts) under the laboratory conditions, insecticidal emulsion (67%) caused higher mortality to *P. citrella* larvae than insecticidal gel (62%), *B. thuringiensis* (49%) and mineral oil (37%).

The efficacy of *B.thuringiensis* was decreased with decreasing its concentration and was 5.3, 17.5, 19.6 and 47.0% at concentration after 1, 4, 7 and 10 days after spraying respectively. This might be due to the fact that *B.thuringiensis* product proteinase activity was lower in its effect to *P. citrella*. These results are in agreement with results obtained by Beattie and Hardy (Beattie & Hardy, 2004), who found that low concentration of *B.thuringiensis* caused low mortality to *Diaprepes abbreviates* (Coleoptera: Curculionidae).

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