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Biocontrol of Diamondback Moth Larvae Tolerant to Bt-toxin Dipel® by the Entomopathogenic Fungus *Metarhizium anisopliae* (Metschn.) Sorokin (Hypocreales, Ascomycota)

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Abstract

Diamondback moth *Plutella xtlostella* (Plutellidae, Lepidoptera) is a destructive insect on plants of family Cruciferae. *Bacillus thuringiensis* toxins, abbreviated Bt-toxins, are biopesticides that could be used for control of larvae of lepidopterous insects. The efficacy of these biopesticides usually decreases due to the development of tolerance to these pesticides by the treated insects. However, this decrease could be compensated by applying biocontrol agents such as entomopathogenic fungi. The objective of the present research was to test the efficacy of *Metarhizium anisopliae*, as an entomopathogenic fungus, against susceptible and tolerant populations of *P. xylostella* larvae to Bt-toxin Dipel®. For this purpose, a series of fungus concentrations were used against susceptible and tolerant populations of *P. xylostella* larvae to Dipel®. Results indicated that there were significant differences (at P=0.05) between the concentrations of *M. anisopliae* that were used for the treatment of Bt-tolerant and susceptible larvae of P. xylostella. The biocontrol efficacy of *M. anisopliae* was higher in the susceptible larvae than that in the Bt-tolerant larvae at the concentrations that exceeded 1x10^6 conidia/ml. Probit analysis indicated that the LC50 of the fungus concentrations used for the treatment was 7.70x10^6 conidia/ml for the Bt-tolerant larvae vs. 1.34x10^5 conidia/ml for the susceptible larvae. In conclusion, in spite of the development of low value cross-tolerance in Bt-tolerant *P. xylostella* larvae to *M. anisopliae* treatment, there is a good possibility for including this fungus in the integrated control of the insect when develops tolerance to Bt-toxin Dipel® because of high biocontrol efficacy of the fungus.

Keywords: Biocontrol efficacy; *Metarhizium anisopliae; Plutella xylostella*; Bt-toxin Dipel®; Susceptible population; Tolerant population

Abbreviations: EPF: Entomopathogenic Fungi; SDA: Sabouraud Dextrose Agar; RR: Resistance Ratio

Introduction

Plutella xylostella is an insect pest on cruciferous plants such as cabbage, cauliflower, radish, turnip, beet root, mustard and rape seed [1,2]. The damaging form of this insect is the larva that feeds on the leaves of these plants making large holes in the attacked leaves or causing leaf skeletonization at heavy infestation. Therefore, the yield loss in the attacked plants varies from 31-100% [2,3]. Control of this insect is usually practiced by application of various synthetic chemical insecticides such as Emamectin benzoate (Proclaim®), Spinosad (Success®) and Indoxacarb (Avatar®) [4-10], but the frequent application of large doses of these insecticides causes the development of resistance by target site mutations or tolerance by inducible upregulation of immune and metabolic activities [6,9-16]. Alternative approach to synthetic chemical insecticides is the application of biopesticides especially the commercial formulations of *Bacillus thuringiensis* toxins known as Bt-toxins such as Dipel®, Cry and Cyt [11,12,15-17]. However, tolerance to these biopesticides especially the Bt-toxins may evolve frequently due to induction of immune defense reactions in the treated larvae [11,13-16,18]. To decrease the effect of tolerance developed to these biopesticides, biocontrol agents such as entomopathogenic fungi (EPF) could be involved in the integrated management of *P. xylostella*. These fungi are currently being developed for biocontrol of many insect species [19-27]. Given that Bt-tolerant larvae have elevated immune and metabolic activities [12-14], we wanted to examine the assumption of that if EPF are used for biocontrol of tolerant populations of *P. xylostella* larvae to Dipel®, the control efficacy of the insect will be improved accordingly.

Recently, formulated forms of EPF have been used against various insect species, and invert emulsion formulation (water-in-oil type) is used as a promising formulation for these entomopathogens when applied as biocontrol agents of insects [28-35]. Few investigators have reported the potential use of EPF as biocontrol agent of *P. xylostella* larvae [36-39]. However,

no investigations have been conducted on using these pathogens for control of *P. xylostella* larvae that have developed tolerance to the widely used commercial formulations of Bt-toxins including Dipel®. Therefore, the objectives of this study were:

a. To test the efficacy of treatment with *M. anisopliae* (strain MA1) against susceptible and tolerant *P. xylostella* larval population to Bt-toxin Dipel®.

b. To compare the susceptibility of both larval populations of the insect to treatments with *M. anisopliae*.

c. To discuss the reasons for including *M. anisopliae* in integrated control of *P. xylostella* larval populations that have developed tolerance to Bt-toxin Dipel®.

Materials and Methods

Strain of *Metarhizium anisopliae* used in bioassays

Strain MA1 of *M. anisopliae* was used in bioassays of the present study. This strain was isolated from infected adults and larvae of the ground beetles:

Carabus nemoralis L. (Carabidae: Coleoptera) with this fungus. The isolation was done, at first, on a selective medium: Sabouraud Dextrose Agar (SDA) + Chloramphenicol (250mg/L) then, pure cultures of this isolate was obtained from monosporic culture of the fungus on SDA.

Populations of Plutella xylostella larvae used in Bioassays

A susceptible population of *P. xylostella* to Bt-toxin treatments had been used in bioassays. This population was considered susceptible because their cultures were not exposed to any type of insecticides, including Bt-toxins, during their rearing for several years so large number of generations were developed which were considered susceptible population of *P. xylostella*. Also, the cultures had been maintained on cabbage seedling leaves (*Brassica oleracae* var. capitata cv. Green Coronet) grown under the insectary conditions (25 ± 1.0 °C, 14/10h, L/D photoperiod) in separately-caged cultures at the faculty of Agriculture and Veterinary Medicine, An-Najah National University, Nablus, Palestinian Territories. To increase larval numbers on a large scale for bioassays, neonate larvae were reared on canola (*Brassica napus*, var. Monty) seedlings stands grown on vermiculite in 500ml Plaspak® plastic pots. Cabbage leaves off cuts from leaf disk preparation were added periodically, when required for developing larvae. A thin layer of honey on masking tape and 10% honey solution containing 0.1% sorbic acid were provided as food source for adult moths of *P. xylostella*. The strain of susceptible population was also used as a reference strain for comparison with Bt- tolerant population of this insect.

A field population of *P. xylostella* larvae and adults that were tolerant to the Bt-toxin Dipel® was used in bioassays as Bt-tolerant population. This population was tolerant to low-medium levels of the Bt-toxin formulations without any over target site mutation. It was found that in the absence of Bt-exposure, the tolerance disappeared, an indication that the tolerance was due to the transient induction of immune and metabolic genes [13,14]. Therefore, to ensure the development of tolerance in the field population, larvae of this population were fed on cabbage seedlings (*B. oleracae* var. capitata cv. Green Coronet) sprayed with 100 ppm of aqueous solution of Dipel® formulation for 2 successive generations (F2 was used in bioassays) and kept in the insectary at 25 ± 1.0 °C (14/10h, L/D photoperiod) in separately-caged cultures at the faculty of Agriculture and Veterinary Medicine, An-Najah National University, Nablus, Palestinian Territories. One hundred ppm of aqueous solution of Dipel® was periodically prepared then sprayed onto cabbage seedlings that were left to dry before being introduced into the Bt-tolerant culture to increase larval numbers on a large scale for bioassays. A thin layer of honey on masking tape and 10% honey solution containing 0.1% sorbic acid were provided as food source for adult moths during rearing.

Bioassays of the Study

Before each formal bioassay, a preliminary assay was carried out using a broad range of concentrations of the fungus preparation containing known conidial concentration to achieve two purposes: the first, to determine the proper concentrations that should be used for the formal assay. The second, to confirm the presence of tolerance to Bt-toxins in the population of the insect known as Bt-tolerant population before carrying out the formal assay by comparing the treatment effect with the fungus on the insect mortality of susceptible and tolerant larvae. Moreover, Tween 20 (0.1% v/v) was added to the conidial suspensions of the fungus to get the conidia into suspension so homogenous conidial suspensions were obtained. Also, the germination test for the conidia was systematically done before each formal bioassay to ensure a high viability of conidia involved in the fungus preparations for bioassays.

For formal bioassays, cabbage leaf disks of 90mm diameter were cut from washed cabbage leaves taken from healthy eight-week-old plants grown under the greenhouse conditions at faculty of Agriculture and Veterinary Medicine, An-Najah National University, Nablus, the Palestinian Territories. The leaf disks were then embedded into agar that had been, first, sterilized in an autoclave then poured into 90mm diameter Petri dishes with the underside of leaf disks facing upwards. Ten of the third instar larvae of *P. xylostella* were placed on each leaf disk in a Petri dish, and then each dish was sprayed with a precise deposit of each concentration (4.0ml) of fungus conidial suspension using a small calibrated fine jet sprayer (200ml capacity). The dishes were then covered with a plastic film that was secured with a rubber band, and then about 200 to 250 tiny holes were then punched into the plastic film using a very fine needle to allow the exchange of air and to insure a good aeration for the incubated larvae. The treated dishes were then kept in an incubator at 25 ± 0.5 °C (14/10h, L/D photoperiod) and the treatment effect at the different concentrations was assessed 96 hours after the treatment. Each formal bioassay includes 10 concentrations of the fungus preparation containing the conidia starting from 1x10^9 conidia/ml to 0 conidia/ml (sterile de-ionized water as a control). To prepare the concentrations used in bioassays, successive dilutions of 10 times were performed using

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the highest concentration (1x10^9 conidia/ml) until reaching the lowest one (0 conidia/ml).

Each concentration used in bioassays was replicated 4 times represented by 4 Petri dishes with 4 leaf disks of 9cm diameter and ten of the third instar larvae of *P. xylostella* (4 dishes represent 4 replications per fungus concentration). The successive dilutions were made up in sterile de-ionized water to obtain the specific concentrations using volumetric flasks. It is noteworthy to mention that bioassays carried out in both populations were run at the same time because this is essential for statistical comparison under the same conditions, and each bioassay for each concentration was repeated three times under the same experimental conditions for confirmation of the results. Moreover, there were no differences between the two populations used in bioassays other than tolerance to Bt-toxin.

Assessment Method used in the Study

The assessment of the treatment effects was done by counting dead and live *P. xylostella* larvae in each Petri dish and in each population after 96 hours of the treatment and then calculating the mortality percent of treated larvae. The mean % of larval mortality was then calculated for each concentration of the fungus and comparison of the treatment effect was then carried out after statistical analysis.

Statistical Analysis

The data obtained from bioassays were statistically analyzed using ANOVA, F-test, and mean separation by Tukey's HSD test to determine the treatment effect with the fungus on *P. xylostella* larvae in Bt-susceptible and tolerant populations. A comparison of the means of mortality percent of Bt-susceptible and tolerant larvae at the different concentrations was conducted. Also, Probit analysis [40], was carried out to determine the median lethal concentration (LC50), ninety five percent confidence limits (95% C.L.) and slope of the regression lines for the Bt-susceptible and tolerant larvae. The resistance ratio (RR) for the Bt-tolerant larvae in relation to the susceptible larvae was also calculated.

Results

Biocontrol efficacy of *M. anisopliae* against Bt-tolerant larvae of *P. xylostella*

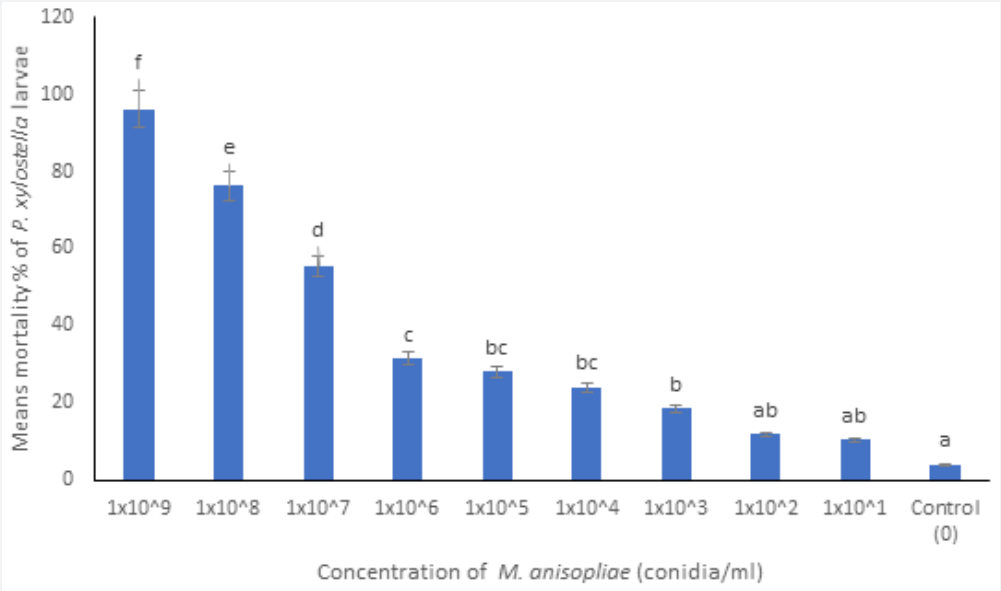

Figure 1: Effect of treatment with different concentrations of the entomopathogenic fungus *Metarhizium anisopliae* (Strain MA1) on tolerant populations of *Plutella xylostella* larvae to Bt-toxin: Dipel® (F-test, ANOVA and Tukey's HSD test were used for comparison of means of mortality percent of Bt-tolerant *P. xylostella* larvae; means followed by the same letter are not significantly different at P = 0.05).

Results indicated that significant differences (at P = 0.05) were obtained between the means of mortality percent of treated Bt-tolerant larvae of *P. xylostella* with *M. anisopliae* for the first four highest concentrations of the fungus used in bioassays: 1x10^9, 1x10^8, 1x10^7 and 1x10^6 conidia/ml (Figure 1). However, no significant differences were obtained between the means of mortality percent of treated Bt-tolerant larvae for the medium concentrations of the fungus used in the treatment: 1x10^5, 1x10^4 and 1x10^3 conidia/ml (Figure 1). The median lethal concentration (LC50) for the Bt-tolerant larvae was 7.70x106 conidia/ml (Table 1). Overall, the treatment efficacy with the fungus was the highest for the higher concentrations of the fungus that exceeded 1x10^6 conidia/ml.

Biocontrol efficacy of *M. anisopliae* against Bt-susceptible larvae of *P. xylostella*

Significant differences (at P = 0.05) were obtained between the means of mortality percent of treated Bt-susceptible larvae of *P. xylostella* with *M. anisopliae* for the first three highest concentrations of the fungus: 1x10^9, 1x10^8 and 1x10^7 conidia/ml (Figure 2). However, there were no significant differences between the means of mortality percent of treated Bt-susceptible larvae with

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the fungus for the two lower concentrations of the fungus: 1x10^7 and 1x10^6 conidia/ml (Figure 2). Also, no significant differences were obtained between the means of mortality percent of treated susceptible larvae with the fungus for the lower concentrations of the fungus: 1x10^5, 1x10^4, 1x10^3 and 1x10^2 conidia/ml (Figure 2). The median lethal concentration (LC50) for the Bt-susceptible larvae was 1.34x10^5 conidia/ml (Table 1). Overall, efficacy of treatment with the fungus was the highest at higher concentrations that exceeded 1x10^7 conidia/ml.

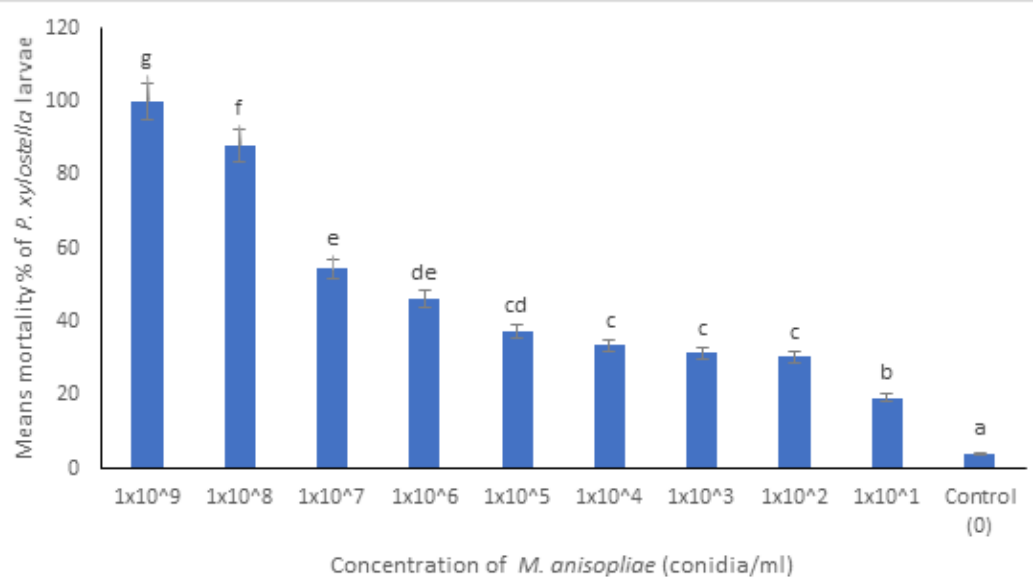

Figure 2: Effect of treatment with different concentrations of the entomopathogenic fungus *Metarhizium anisopliae* (Strain MA1) on susceptible populations of *Plutella xylostella* larvae (F-test, ANOVA and Tukey's HSD test were used for comparison of means of mortality percent of susceptible *P. xylostella* larvae; means followed by the same letter are not significantly different at P = 0.05).

Comparison of biocontrol efficacy of *M. anisopliae* against Bt-tolerant and susceptible larvae of *P. xylostella*

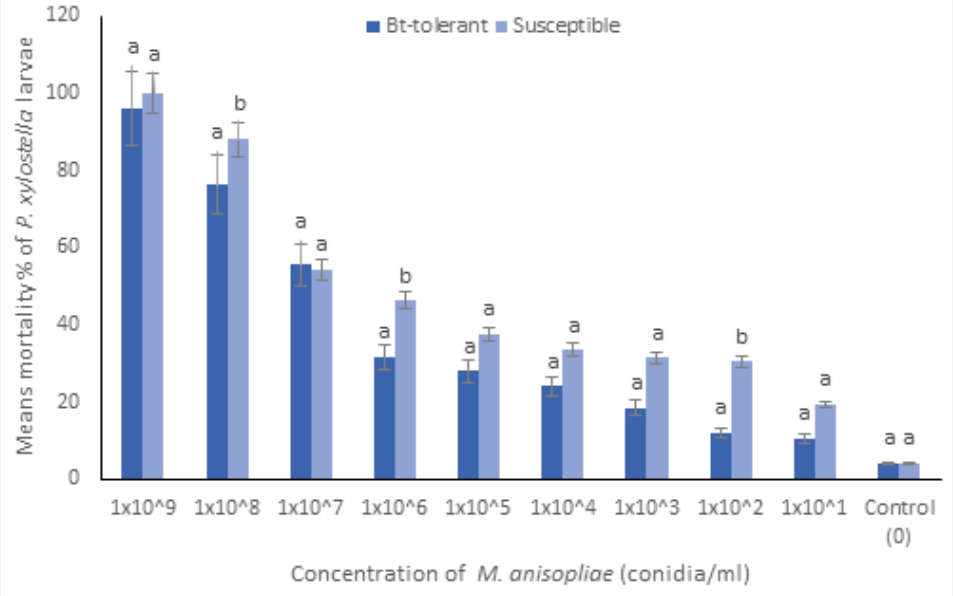

Figure 3: Comparison of the treatment effect with different concentrations of the entomopathogenic fungus *Metarhizium anisopliae* (Strain MA1) on susceptible and tolerant populations of *Plutella xylostella* larvae to Bt-toxin: Dipel® (F-test, ANOVA and Tukey's HSD test were used for comparison of means of mortality percent of Bt-tolerant and susceptible *P. xylostella* larvae; means followed by the same letter are not significantly different at P = 0.05).

Results of the treatment effect of both populations of *P. xylostella* larvae with the fungus indicated that significant differences (at P = 0.05) were obtained between the means of mortality percent of treated larvae of the two populations at only two higher fungus concentrations: 1x10^6 and 1x10^8 conidia/ml (Figure 3), but no significant differences were obtained between the two populations at the highest concentration of the fungus: 1x10^9 conidia/ml (Figure 3). Therefore, the treatment efficacy

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with the fungus was higher in the susceptible population of *P. xylostella* larvae in comparison with the Bt-tolerant larvae (only at two concentrations of the fungus: 1x10^6 and 1x10^8 conidia/ml, but not at other higher concentrations).

Results of Probit analysis carried out on the two populations demonstrated that the median lethal concentration (LC50) for the Bt-tolerant larvae was 7.70x10^6 conidia/ml vs. 1.34x10^5 conidia/ml for the Bt-susceptible larvae and the ninety five percent confidence limits (95% C.L.) and the slope of the regression lines for the Bt-susceptible and tolerant larvae were calculated at 0.38x10^5 - 5.00x10^5; 0.257 ± 0.030 and 1.18x10^6 – 20.73x10^6; 0.675 ± 0.159, respectively (Table 1). The calculated resistance ratio (RR) was 57.46: 1.00 for the Bt-tolerant larvae in comparison with the susceptible larvae, respectively (Table 1). This indicates that the Bt-tolerant larvae was more tolerant to the treatment with the fungus by 57.46 times in proportion to the susceptible larvae.

Table 1: Probit analysis of the treatment effect of susceptible and tolerant populations of *Plutella xylostella* larvae to Bt-toxin: Dipel® using the entomopathogenic fungus *Metarhizium anisopliae* (Strain MA1).

Probit Analysis	Treated *P. xylostella* Lavae	
	Bt-Tolerant	Bt-Susceptible
LC50 (conidia/ml)	$7.70x10^{\wedge 6}$ (-7700806.78)	$1.34 \times 10^{\wedge 5}$ (133,702.010))
95% C.L. (conidia/ml)	$1.18 \times 10^{\wedge 6}$ - $20.73 \times 10^{\wedge 6}$ (1,183,450.661-20,731,010.316)	$0.38 \times 10^{\wedge 5}$ – $5.00 \times 10^{\wedge 5}$ (37,687.355 – 500,190.947)
Slope	0.675 ± 0.159	0.257 ± 0.030
Resistance Ratio (RR)	57.46	1.00

Overall results confirmed the potential use of the Fungus *M. anisopliae* as biocontrol agent of Bt-susceptible and tolerant populations of *P. xylostella* larvae. Although of the development of low cross tolerance in the Bt-tolerant population to the fungus, there were no significant differences between the susceptible and Bt-tolerant populations of the insect at medium concentrations of the fungus used against the two populations. Therefore, there is a good possibility of using the fungus as a potential biocontrol agent in the integrated control of Bt-tolerant population of *P. xylostella* larvae.

Discussion

In the present research, we proved that the entomopathogenic fungus (*Metarhizium anisopliae*: strain MA1) had high biocontrol efficacy against the susceptible and Bt-tolerant *P. xylostella* larvae at the concentrations ≥ 1x10^5 conidia/ml with an LC50 of 1.34x10^5 and 7.70x10^6 conidia/ml for the susceptible and Bt-tolerant larvae, respectively. This indicates the possibility of using *M. anisopliae* as an effective biocontrol agent against the *P. xylostella* larvae that have developed tolerance to Bt-toxin Dipel® thus it can be used in pest management of this insect especially when develops tolerance to the biopesticide Dipel®. The reasons for recommendation of the use of *M. anisopliae* in pest management of *P. xylostella* could be summarized as follows:

a. The fungus showed high biocontrol efficacy against the Bt-tolerant and susceptible populations of *P. xylostella* larvae. This efficacy reached at 96.1 and 100% as an average mortality percent in the Bt-tolerant and susceptible populations of *P. xylostella* larvae, respectively, within 96 hours after the treatment at the highest fungus concentration (1x10^9 conidia/ml).

b. In spite of the tolerance development to Dipel® in the treated *P. xylostella* larvae (resistance ratio for the Bt-tolerant larvae to the susceptible larvae was 57.46:1.00), this ratio did not affect the overall efficacy of the fungus especially at higher concentrations ($1x10^{\wedge 7}$ to $1x10^{\wedge 9}$ conidia/ml).

c. The use of *M. anisopliae* proved effectiveness as biocontrol agents of other insect pests such as stored-grain insects [31,33,34],

d. Other reasons that may characterize the group of entomopathogenic fungi, in general, as effective biocontrol agents such as they are easy to isolate from infected host insects, easy to cultivate on culture media, easy to prepare from their cultures as conidial suspensions for being used against the targeted insects. Moreover, these microbial pesticides have no residual effect and do not contaminate the environment after application.

Few studies have been conducted using *M. anisopliae* as biocontrol agent of *P. xylostella* larvae [37,41,42], the results of these studies have shown high efficacy against the treated insects. This result agreed with our results that demonstrated high efficacy due to the treatment of susceptible *P. xylostella* larvae with the fungus (*M. anisopliae*: strain MA1) at higher concentrations that exceed 1x10^8 conidia/ml (88.1% of larval mortality or more). To date, no investigations have been conducted to determine the LC50's of *M. anisopliae* concentrations used as biocontrol agent of Bt-tolerant larvae of *P. xylostella*. Therefore, this research represents the first attempt to determine the LC50's of this entomopathogenic fungus when used as biocontrol agent of Bt-tolerant larvae and thus calculating the representative resistance ratio (RR). Although the calculated value of this ratio was quite low (57.46:1.00), it may suggest the development of certain type of cross-tolerance in the treated insects acquiring tolerance to Bt-toxins such as Dipel® in our bioassays. One of the explanations for development of this type of cross-tolerance may be attributed to the induction of immune defense reactions in the treated larvae (e.g. induction of immune and metabolic genes according to

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Rahman et al. [12,13], by entomopathogenic fungi and Bt-toxins but further investigations are needed to be carried out to know the exact reason.

It is important to mention that mode of action of entomopathogenic fungi is different from that of Bt-toxins. For the mode of action of entomopathogenic fungi, it is well-known that these fungi enter the body cavity of attacked insects through the outer cuticle following germination of their conidia adhering the outer cuticle then growing and developing of fungus mycelium in the hemocoel causing finally obstruction of the body cavity and death of attacked insects [43-46]. However, Bt-toxins enter the insect through the ingestion then reach the digestive system of attacked insects where they are processed; the damage caused to the digestive system by the Bt-toxins is done by lysing the midgut epithelial cells by inserting into the target membrane and forming pores (pore-forming activities) allowing bacterial elicitors into the hemocoel [17,47]. Therefore, in case of developing a low-medium cross-tolerance to Bt-toxins and the fungus, the immune system of treated insects may respond with the activation of the immune response to microbial elicitors after the treatment with the fungus and Bt-toxin [12-14]. Ferre & Van Rie [18], reviewed the biochemical changes and genetics of resistance to Bt-toxins and insecticidal crystal proteins by different species of insects treated with different formulations of these biopesticides. However, more research studies are recommended to be carried out in this respect to specify the exact mechanism of this type of cross-tolerance to the fungus and biopesticide in *P. xylostella* larvae.

Conclusion

Results obtained in the present research would suggest the potential for including *M. anisopliae* as biocontrol agent in the integrated control of *P. xylostella* larvae that have developed tolerance to Bt-toxin Dipel®. Inclusion of this fungus was based on the higher efficacy levels that have been obtained when the fungus was applied against the Bt-tolerant larvae of the insect. The calculated values of the median lethal concentration (LC50) and resistance ratio (RR) of the fungus used against the Bt-tolerant larvae in proportion to the susceptible larvae would suggest the development of a certain type of cross-tolerance to the fungus and the biopesticide, but the low value of cross-tolerance did not affect the overall efficacy of the fungus that could be used as an effective biocontrol agent of the Bt-tolerant insect. Reasons for the recommendation of the use of *M. anisopliae* in pest management of *P. xylostella* larvae were discussed in the paper.

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Declaration of interest statement

The author declares that he has no conflict of interests.

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