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Investigations into the development of cross-tolerance in the Diamondback Moth (*Plutella xylostella* L., Yponomeutidae: Lepidoptera) to the entomopathogenic fungus *Beauveria bassiana* (Bal.) Vuillemin (Deuteromycotina: Hyphomycetes) and the toxin Dipel® of *Bacillus thuringiensis*

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ABSTRACT

The present research represents the first investigations into the development of cross-tolerance in the larvae of Diamondback Moth (DBM) tolerant to *Bacillus thuringiensis* toxin (Bt-toxin: Dipel®) and treated with different concentrations of the entomopathogenic fungus *Beauveria bassiana* (strain BbGHA). Significant differences (at P = 0.05) were obtained between the fungus concentrations used for the treatment of Bt-tolerant and susceptible DBM larvae. The LC_{50} of the fungus concentrations used for the treatment of Bt-tolerant larvae was 7,700,836.787 (7.7×10^6) conidia/ml versus 133,791.018 (1.3×10^5) conidia/ml for the susceptible larvae. The calculated resistance ratio (RR) was 57.6 times more in the Bt-tolerant larvae compared to the susceptible larvae. Overall, results suggest that Bt-tolerant larval populations of DBM could be controlled by application of *B. bassiana*, but higher concentrations of the fungal agent than in susceptible larval populations are required. Further research studies are recommended to be done in order to specify the mechanism of this cross-tolerance before exploitation of these results in pest management strategies of this insect especially when the control is practiced by application of the fungus and Bt-toxin formulations especially with Dipel®.

KEYWORDS: *Beauveria bassiana, Plutella xylostella*, Bt-toxin: Dipel®, cross-tolerance, susceptibility, resistance ratio

1. INTRODUCTION

Diamondback moth (DBM) is the most important insect pest of crucifers such as cabbage, cauliflower, radish, turnip, beet root, mustard and rape seed worldwide [28, 34]. The loss in yield caused by this pest varies from 31 – 100% [13]. The damaging form of this insect is the larva that feeds on the leaves of cruciferous crops making large holes in the attacked leaves [13, 34].

Control of this insect pest is usually practiced by using various synthetic chemical insecticides including the commercial formulations of *Bacillus thuringiensis* toxins (Bt-toxin known as Dipel®). The frequent application of large doses of these

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insecticides may cause development of resistance by target site mutations or tolerance by inducible upregulation of immune and metabolic activities [9, 12, 15, 23, 24, 27, 32, 33]. The most frequently used synthetic insecticides for control of DBM are Emamectin benzoate (Proclaim®), Spinosad (Success®) and Indoxacarb (Avatar®), in addition to the Bt-toxin (Dipel®) formulations [14, 16, 31, 35, 37, 38]. Since cross-tolerance to these insecticides may evolve when low doses of these insecticides are applied especially Bt-toxins [12, 32], alternative approaches to the conventional use of synthetic insecticides for the control of DBM are essential to increase the efficacy of integrated management strategies by combining biocontrol with selective insecticide application. Possible biocontrol agents that could be involved in integrated management of DBM are the entomopathogenic fungi (EPF) because are currently being developed for control of many insect species [8, 10, 18, 19, 20, 21, 22, 25, 30]. Given that Bt-tolerant larvae have elevated immune and metabolic activities [15, 23, 24], we wanted to examine this assumption and ask whether Bt-tolerant insects were also tolerant to some biological control agents.

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 [1, 2, 3, 4, 5, 6, 7, 17]. Few reports are existing on potential use of *B. bassiana* as biocontrol agent of DBM larvae [11, 26, 29, 36]. However, no investigations have been reported on using this pathogen for control of DBM larvae that have developed tolerance to the widely used commercial formulations of Bt-toxins. Therefore, the present study was conducted to investigate into the possibility of tolerance development to *B. bassiana* in the DBM larvae that have already developed tolerance to Bt-toxin Dipel®. The objectives of this study were: i) to test the efficacy of treatment with *B. bassiana* (strain: BbGHA) against susceptible and tolerant DBM larvae to formulations of Bt-toxin Dipel®, then ii) to compare the susceptibility of both larval populations to fungal infections with *B. bassiana*, and iii) to check whether cross-tolerance to the fungal infections and Dipel® formulations is developed in Bt-tolerant DBM larvae.

2. MATERIALS AND METHODS

2.1. Fungus strain used in bioassays

Beauveria bassiana (strain BbGHA) was used in bioassays of the present research. It was originally obtained from Laverlam International Corporation (117 South Parkmont, Butte MT 59701, USA) in form of fungal conidia emulsified in vegetable oil. The concentration of conidia in the provided original fungal preparation was 2 X 10^{10} conidia/ml. The preparation was diluted 20 times in sterile deionized water to get a concentration of 10^9 conidia/ml. This concentration was used in bioassays after making a series of successive dilutions of 10 times each.

2.2. DBM populations used in bioassays

A susceptible population of *P. xylostella* known as "Waite susceptible strain" has been used in bioassays. It has been maintained on cabbage seedling leaves (*Brassica oleracae* var. capitata cv. Green Coronet) grown under insectary conditions (25 ± 1.0 °C, 14/10 h, L/D photoperiod) in separately-caged cultures at South Australian Research and Development Institute (SARDI)-Entomology Unit, Waite Campus, Adelaide, SA, Australia. These cultures were not exposed to any type of insecticides during rearing for many years. To increase larval numbers on a large scale for bioassays, neonate larvae were reared on canola (var. Monty) seedlings stands grown on vermiculite in 500 ml 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 with 0.1% sorbic acid were provided as food source for DBM adults. This susceptible strain was also used as the reference strain for comparison with Bt-tolerant population of DBM.

A field population of DBM larvae and adults that are tolerant to the Bt-toxin formulation called Dipel® was used in bioassays as Bt-tolerant population. This population was tolerant to low to medium levels of the Bt-toxin formulation 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 (Rahman, *et al.*, 2004a and 2004b; M. M. Rahman, unpublished results). Therefore, larvae of this population were fed on cabbage seedlings (*B. oleracae* var. capitata cv. Green Coronet) sprayed with 100 ppm of Dipel® formulation for 2 successive generations (F 2 was used in bioassays) and kept in insectary at 25 ± 1.0 °C (14/10 h, L/D photoperiod) in separately-caged cultures at the SARDI-Entomology Unit, Waite Campus, South Australia, Australia. One hundred ppm of aqueous solution of Dipel® was periodically prepared and 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 with 0.1% sorbic acid were provided as food source for adults during rearing.

2.3. Bioassays

Cabbage leaf disks of 90 mm diameter were cut from washed cabbage leaves taken from healthy eight-week-old plants grown under the glass house conditions at SARDI-Entomology Unit, Waite Campus, Adelaide, SA, Australia. The leaf disks were then embedded into agar that has been poured into 90 mm diameter Petri dishes with the underside of leaf disks facing upwards. Before each formal bioassay, a preliminary assay was conducted using a broad range of concentrations of the fungus preparation containing known conidial concentration to determine the proper concentration for the formal assay. Each formal bioassay includes 10 concentrations of the fungus preparation containing the conidia starting from 10^9 conidia/ml to 0 (sterile de-ionized water as a control). Therefore, successive dilutions of 10 times each were performed using the highest concentration (10^9 conidia/ml) until reaching the lowest one (0 conidia/ml). Four leaf disks representing four replicates were used for each concentration. The successive dilutions were made up in sterile de-ionized water to obtain the specific concentrations using volumetric flasks. A precise deposit of each concentration (4.0 ml) was administered using a Potter precision laboratory spray tower (Burkard manufacturing Co. Ltd. Rickmansworth Herts, England). Eight to ten 3rd instar larvae of DBM were placed on each leaf disk in a Petri dish, and then each Petri dish was sprayed with 4.0 ml of each concentration using Potter spray tower. Once removed from the tower, the dishes were 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. The tower was calibrated before and after each trial allotment, and rinsed 3 times with ethyl alcohol (70%) and sterile de-ionized water between each change in treatment. The treated Petri dishes were then kept in an incubator at 25 ± 0.5 °C (14/10 h, L/D photoperiod) and the treatment effect at the different concentrations was assessed 96 hours after the treatment. The assessment of the treatment effect was performed by counting dead and living DBM larvae in each Petri dish and calculating the mortality percent of treated larvae. The mean of mortality percent was calculated for each concentration and the comparison of the treatment effect was then carried out after being statistically analysed.

2.4. Statistical analysis

The data obtained from bioassays were statistically analysed using analysis of variance (ANOVA) test, F-test, and mean separation by Duncan Multiple Range Test (DMRT) to determine the treatment effect with the fungus on DBM larvae. A comparison of the means of mortality percent of susceptible and Bt-tolerant DBM larvae at the different concentrations was then assessed. Also, Probit analysis was carried out to determine the median lethal concentration (LC_{50}) for susceptible and Bt-tolerant larvae. The resistant ratio (RR) for the Bt-tolerant larvae in relation to the susceptible larvae was calculated.

3. RESULTS

3.1. Treatment effect on Bt-tolerant DBM larvae

Results indicate that significant differences (at P = 0.05) were obtained between the means of mortality percent of treated Bt-tolerant larvae with *B. bassiana* for the first four highest concentrations of the fungus used in

Table 1. Effect of treatment with different concentrations of the entomopathogenic fungus *Beauveria bassiana* (strain BbGHA) on susceptible and tolerant populations of Diamondback moth (DBM) larvae to *Bacillus thuringiensis* toxin (Dipel®).

Fungus concentration (conidia/ml)	**Means (± S.E) of % mortality of DBM larvae (vertical comparison of means within each column)**		**Means (± S.E) of % mortality of DBM larvae (horizontal comparison of means within each row)**	
	Bt-tolerant	**Susceptible**	**Bt-tolerant**	**Susceptible**
10^9	95 ± 3.3 **f**	100 ± 0 **g**	95 ± 3.3 **a**	100 ± 0 **a**
10^8	76.7 ± 3.8 **e**	87.2 ± 2.9 **f**	76.7 ± 3.8 **a**	87.2 ± 2.9 **b**
10^7	57.5 ± 5.6 **d**	52.5 ± 5.3 **e**	57.5 ± 5.6 **a**	52.5 ± 5.3 **a**
10^6	30.5 ± 3.4 **c**	45 ± 4.2 **de**	30.5 ± 3.4 **a**	45 ± 4.2 **b**
10^5	28 ± 2.9 **bc**	37.5 ± 5.8 **cd**	28 ± 2.9 **a**	37.5 ± 5.8 **a**
10^4	25 ± 3.0 **bc**	32.5 ± 6.1 **c**	25 ± 3.0 **a**	32.5 ± 6.1 **a**
10^3	17.5 ± 4.8 **b**	32.5 ± 6.1 **c**	17.5 ± 4.8 **a**	32.5 ± 6.1 **a**
10^2	12.8 ± 5.1 **ab**	30 ± 3.5 **c**	12.8 ± 5.1 **a**	30 ± 3.5 **b**
10	10 ± 3.2 **ab**	20 ± 4.2 **b**	10 ± 3.2 **a**	20 ± 4.2 **a**
0 (control)	5 ± 2.1 **a**	5 ± 2.1 **a**	5 ± 2.1 **a**	5 ± 2.1 **a**
Grand mean	**35.8**	**44.2**	**35.8**	**44.2**
Statistical analysis	F-test, ANOVA and DMRT were used for vertical comparison of the means in each column and in each population type		F-test, ANOVA and DMRT were used for horizontal and pair-wise comparison of means in each row and in each population type	

bioassays: 10^9, 10^8, 10^7 and 10^6 conidia/ml (Table 1). However, no significant differences were obtained between the means of mortality percent of treated Bt-tolerant larvae with *B. bassiana* for the medium concentrations of the fungus used in the treatment: 10^5, 10^4 and 10^3 conidia/ml (Table 1). Overall, the treatment effect with the fungus was the highest for the higher concentrations that exceeded 10^6 conidia/ml.

3.2. Treatment effect on susceptible DBM larvae

Significant differences (at P = 0.05) were obtained between the means of mortality percent of treated susceptible larvae with *B. bassiana* for the first three highest concentrations of the fungus: 10^9, 10^8 and 10^7 conidia/ml, but no significant differences were obtained between the means of mortality percent of treated susceptible larvae with the fungus for the two lower concentrations of the fungus: 10^7 and 10^6 conidia/ml (Table 1). 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: 10^5, 10^4, 10^3 and 10^2 conidia/ml (Table 1). Overall, the fungus treatment was most effective at higher concentrations that exceeded 10^5 conidia/ml.

3.3. Comparison of treatment effect on Bt-tolerant and susceptible DBM larvae

Results of the treatment effect of both populations of DBM larvae with the fungus indicate that significant differences (at P = 0.05) were obtained between the means of mortality percent of treated larvae of the two populations at the fungus concentrations of 10^8, 10^6 and 10^2 conidia/ml (Table 1), but no significant differences were

Table 2. Probit analysis of the treatment effect of susceptible and tolerant populations of Diamondback moth (DBM) larvae to *Bacillus thuringiensis* toxin (Dipel®) with the entomopathogenic fungus *Beauveria bassiana* (strain BbGHA).

Results of Probit analysis	**Treated DBM larvae**	
	Bt-tolerant	**Susceptible**
LC_{50} (conidia/ml)	7,700,836.787 (7.7×10^6)	133,791.018 (1.3×10^5)
95 % C.L. (conidia/ml)	1,183,454.661-20,731,020.316 (1.2×10^6 -20.7×10^6)	37,687.350 – 500,195.947 (0.38×10^5 – 5.0×10^5)
Slope	0.675 ± 0.159	0.257 ± 0.030
Resistance Ratio (RR)	57.6	1.0

obtained between the two populations at the other concentrations of the fungus used in bioassay (Table 1). Therefore, the treatment effect with the fungus was more effective in the susceptible population of DBM larvae than that in the Bt-tolerant population at the high concentrations that exceeded 10^5 conidia/ml.

Results of Probit analysis carried out on the two populations demonstrate the development of cross-tolerance to *B. bassiana* in the Bt-tolerant DBM larvae because the calculated resistant ratio (RR) was 57.6 times more for the Bt-tolerant larvae compared to the susceptible larvae. This is attributed to the median lethal concentration (LC_{50}) for the Bt-tolerant larvae that was 7,700,836.787 conidia/ml versus 133,791.018 conidia/ml for the susceptible larvae (Table 2).

4. DISCUSSION

In the present study, the entomopathogenic fungus (*Beauveria bassiana*: strain BbGHA) has shown high efficacy against the Bt-tolerant and susceptible DBM larvae at the concentrations of 10^6 and 10^5 conidia/ml or more, respectively, with LC_{50} of 1.3×10^5 and 7.7×10^6 conidia/ml for the susceptible and Bt-tolerant larvae, respectively. Only few studies have been conducted using *B. bassiana* as biocontrol agent against larvae of *P. xylostella* (11,26,29,36), but the results of these studies have shown low efficacy with low mortality percent of treated insects. Our results demonstrate that the susceptible DBM larvae have shown high susceptibility to the infection with the fungus strain at higher concentrations that exeed 10^8 conidia/ml (87.2% of larval mortality or more; Table 1).

In the present bioassays, a cross-tolerance was obtained in the Bt-tolerant DBM larvae treated with different concentrations of *B. bassiana* (strain BbGHA). This could be attributed to the presence of a moderate resistance ratio (RR) in the Bt-tolerant larvae treated with the fungal strain concentrations reaching at 57.6 times fold to the ratio in the susceptible larvae. So far, no investigations were reported to determine the LC_{50}'s of *B. bassiana* concentrations used as biocontrol agent against Bt-tolerant larvae of *P. xylostella*. This research represents the first attempt to investigate the LC_{50}'s of this entomopathogenic fungus when used as biocontrol agent against Bt-tolerant DBM larvae and therefore calculating its resistance ratio (RR). The development of cross-tolerance to this fungus at the concentrations used in the Bt-tolerant DBM larvae will help us to adjust the fungus concentration that could be used in pest management of this insect along with the widely used Bt-toxin formulation Dipel®. One of the explanations for development of cross-tolerance in DBM larvae in our bioassays may be attributed to the induction of immune defence reactions in the treated larvae (e.g. induction of immune and metabolic genes according to Rahman *et al.*, 2004a and 2004b: Refs. [23, 24] by entomopathogenic fungi and Bt-toxins but further investigations are needed to know the exact reason. It is well-known that the

entomopathogenic fungi enter the body cavity of attacked insects through the outer cuticle followed by germination of their conidia then growing and developing of a mycelium in the hemocoel causing finally obstruction and death of attacked insects. However, Bt-toxins enter the insect through ingestion then reach the digestive system of attacked insects where it is processed; the damage caused to digestive system by the toxin is done through pore-forming activities of the toxin allowing bacterial elicitors into the hemocoel. Therefore, in case of developing a low or medium cross-tolerance to both, 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 [15, 23, 24]. 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 DBM larvae.

In conclusion, results obtained in the present research suggest the potential for including *B. bassiana* in an overall management program of *P. xylostella* that developed tolerance to Bt-toxin formulation Dipel® but with the possibility of cross-tolerance development. It is obvious from the present research that Bt-tolerant larvae of DBM are also become tolerant to the fungal strain used. This suggests that tolerance to the fungal agents and biopesticides are based on shared immune and metabolic pathways. Further studies are needed in this respect.

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