Research Article

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Toxicological and biochemical response of the entomopathogenic fungus *Beauveria bassiana* after exposure to deltamethrin

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Abstract

BACKGROUND: The chemical control of the Chagas disease vector *Triatoma infestans* is endangered by the emergence of pyrethroid resistance. An effective alternative control tool is the use of the entomopathogenic fungus *Beauveria bassiana*. The effect of deltamethrin on fungal growth, gene expression and enzyme activity in relation to detoxification, antioxidant response and oxidative stress levels was studied to evaluate fungal tolerance to deltamethrin.

RESULTS: The mean inhibitory concentration (IC_{50}) was 50 μg deltamethrin/cm^2. Cytochrome P450 genes were differentially expressed; *cyp52X1* and *cyp617N1* transcripts were > 2-fold induced, followed by *cyp655C1* (1.8-fold). Minor effects were observed on genes encoding for other P450s, epoxide hydrolase and glutathione S-transferase (GST). Superoxide dismutase (SOD) genes showed induction levels ≤ 2, catalase (CAT) and glutathione peroxidase genes were also induced ∼ 2–3-fold and < 2-fold, respectively. The activities of enzymes participating in the antioxidant defense system and phase II detoxification were also evaluated; SOD, CAT and GST activity showed significant differences with deltamethrin concentration. Lipid peroxidation levels and free proline content were also altered.

CONCLUSIONS: *Beauveria bassiana* GHA can be used combined with deltamethrin without significant metabolic detrimental effects. This combination will help optimizing the benefits and increasing the efficacy of vector control tools.

Keywords: entomopathogenic fungi; oxidative stress; antioxidant response; pyrethroid detoxification

1 INTRODUCTION

Vector-borne diseases pose a critical burden mostly in tropical and subtropical areas with low-income human populations. The World Health Organization identified the need of delivering multi-intervention packages that include the promotion of integrated vector management (IVM) strategies aimed at controlling disease transmission.[1] The American trypanosomiasis (Chagas disease) is the most important parasitic disease in Latin America, more than 10 million people are currently infected and 30 million at risk, with active transmission in 21 countries.[1] Blood-sucking insects of the Triatominae subfamily are vectors of the protozoan *Trypanosoma cruzi*, the causative agent of Chagas disease. The insect *Triatoma infestans* (Hemiptera, Reduviidae) is the main vector in southern South America. Traditional vector control has been successful by many years, heavily based on residual indoor application of pyrethroids; however, it is now evident that a growing number of residual foci surviving after insecticide spraying, can be explained because of a reduced susceptibility to the insecticide (due to insecticide resistance and/or tolerance).[2]

Among alternative control tools against *Triatoma infestans*, laboratory and field assays showed the efficacy of the entomopathogenic fungus *Beauveria bassiana* (Ascomycota: Hypocreales) to kill *T. infestans*, regardless of their susceptibility to pyrethroids.[3,4] Triatomine susceptibility to fungal infections was early shown in *Rhodnius prolixus*, another major Chagas disease vector.[5,6] Several strains of *B. bassiana* were shown to be highly virulent to triatomines, with time to kill about 5–10 days.[7–9] Also, the biochemical interactions between the entomopathogen and its insect host have been extensively addressed.[10,11] However, progress has been limited regarding fungal formulations for practical applications. An attraction–infection trap based on *B. bassiana* conidia formulation combined with chemical attractants has been already developed in this laboratory[12], and has been successfully tested for indoor *T. infestans* control.[3,13] *Beauveria bassiana* isolates were shown to tolerate well deltamethrin doses similar to those used for *T. infestans* control,[9] providing evidence that the combination of deltamethrin with entomopathogenic fungi might be used in future control strategies.

The major routes of pyrethroid catabolism, both in insects and pyrethroid-degrading microorganisms, include oxidation by cytochrome P450s (CYP) and epoxide hydrolases (EH), and ester hydrolysis by esterases.[14] These phase I-related enzymes

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trigger reactions that increase the production of reactive oxygen species (ROS), which in term might produce oxidative stress.[15] Subsequently, the antioxidant defense system is activated to avoid ROS harmful effects on biomolecules. This system includes several enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathion peroxidase (GPx), as well as non-enzymatic compounds.[16,17] In turn, glutathione S-transferases (GST) are the major phase II-related enzymes, conjugating electrophilic compounds with glutathione. Also, a high production of ROS has been reported to induce lipid peroxidation, contributing to the loss of cell function; proline accumulation is a marker of high ROS levels. [17,18]

As part of a large research project addressed to develop an efficient and eco-friendly methodology to control triatomines, the objective of this work was to investigate the effect of deltamethrin in the detoxification system as well as in the antioxidant response and oxidative stress levels in *Beauveria bassiana*, in order to determine the fungal tolerance to deltamethrin at the metabolic level.

2 MATERIALS AND METHODS

2.1 Cultivation of fungi

Beauveria bassiana strain GHA (Laverlam International, USA) was routinely grown on complete medium agar (CMA) plates. CMA contains 0.4 g KH_2PO_4, 1.4 g Na_2HPO_4, 0.6 g $MgSO_4 \cdot 7H_2O$, 1.0 g KCl, 0.7 g $NH_4NO_3 \cdot 7H_2O$, 10 g glucose, 5 g yeast extract and 15 g agar in 1000 mL of distilled water. Except for the mean inhibitory concentration assay, fungi were grown onto the surface of a cellophane sheet (Hoefer, USA) in CMA plates either with or without addition of deltamethrin (Gleba, Argentina) onto the surface. In all experiments, plates were incubated at 26 °C for 4 days.

2.2 Mean inhibitory concentration (IC_{50}) assessment

A fungal suspension containing 1×10^4 con/mL was homogeneously dispersed with a bent glass rod on CMA surface plates containing different doses of deltamethrin. For insecticide addition to the plates, different concentrations of deltamethrin dissolved in acetone (Carlo Erba, Italy) were prepared by serial dilution to obtain the following doses: 0 (pure acetone), 40, 100, 250 and 625 μg of deltamethrin by cm^2 of agar plate, the solvent was then evaporated by a nitrogen stream. After 4 days cultivation, total colony-forming units (CFUs) were counted, and mean inhibitory concentration (IC_{50}) was calculated by Probit analysis.[19] Three replicates of the experiments were performed.

2.3 Gene expression analysis

After a 4-day induction period (CMA added with various doses of deltamethrin between 0.5 IC_{50} and 10 IC_{50}), total RNA was extracted employing the RNAeasy Plant Mini kit (Qiagen, Germany), including an on-column DNA digestion step. RNA was quantified by Qubit RNA assay kit (Invitrogen, USA), and the integrity was assessed on a 1% (w/v) agarose gel. Two step real-time polymerase chain reaction (RT-PCR) was carried out with iScript cDNA Synthesis kit and iQ SYBR Green Supermix (Bio-Rad, USA). Amplification was performed in an Mx3000P QPCR System (Stratagene, USA) employing 20 ng reverse transcribed total RNA for each sample. The following amplification program was used: denaturation at 95 °C for 10 min, followed by 40 cycles with three-segment amplification (30 s at 95 °C for denaturation, 30 s at 56 °C for annealing, and 30 s at 72 °C for DNA chain elongation). In order to confirm that only single products were amplified, a temperature melting step was then performed. The calibration curve method was used for the analysis of data obtained from the RT-PCR system, with tubulin as the housekeeping gene. The primers used are listed in the Appendix, Table A1. Negative controls were performed by using 'cDNA' generated without reverse transcriptase as templates. Reactions containing primer pairs without template were also included as blank controls. The assay was performed in duplicate for each of the three independent biological replicates performed.

Table 1. Effect of deltamethrin on *Beauveria bassiana* GHA growth and mean inhibitory concentration (IC_{50}) determination[a]

Deltamethrin (μg/cm^2)	Colony forming units (CFUs)
Control[b]	648 ± 14
0	687 ± 21
16	600 ± 27
40	475 ± 21
100	279 ± 7
250	147 ± 8
625	85 ± 9

Note: $IC_{50} = 50 \mu g/cm^2$, $r^2 = 0.92$.
[a] IC_{50} was calculated by Probit analysis. Values represent the mean of three replicates ± standard error.
[b] *B. bassiana* grown in CMA plates without acetone added.

2.4 Subcellular fractionation

Fungi exposed to deltamethrin as described in section 2.3 were harvested from the cellophane sheet and disrupted using a Mini-Bead Beater homogenizer (BioSpec, USA) with glass beads (0.5 mm diameter). Four cycles of 20 sec each were performed, with 20-sec cooling periods between bursts. Homogenization buffer was 125 mM Tris-base (pH 6.8), 5 mM DTT, and 1 mM PMSF. The homogenate was centrifuged for 20 min at 10 000 × *g* to remove mitochondria and cell debris, and the supernatant was used for enzyme activity, lipid peroxidation and proline content measurements. For control fungi, the 4-day induction period was on CMA with acetone. Protein concentration was determined by the bicinchoninic acid method (Pierce, USA), using bovine serum albumin as standard.

2.5 Enzyme activity measurement

The SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), GST (EC 2.5.1.18) and GPx (EC 1.11.1.9) activities were determined using the spectrophotometric methods described by Marklund and Marklund,[20] Beers and Sizer,[21] Habig *et al.*[22] and St-Clair and Chow,[23] respectively, in an Ultrospec 2100 pro spectrophotometer (Biochrom, UK). One SOD unit was defined as the amount of enzyme necessary to inhibit 50% the rate of autocatalytic pyrogallol oxidation/min measured at 420 nm. One CAT unit was defined as the amount of enzyme that decomposes 1 μmol of H_2O_2/min measured at 240 nm. One GST unit is the amount of enzyme required to conjugate GSH with 1 μmol of 1-chloro-2,4-dinitrobenzene/min, determined at 340 nm. GPx units are expressed as pmol NADPH consumed/min.

2.6 Lipid peroxidation measurement

The lipid peroxidation (LPO) level was measured according to Buege and Aust,[24] by using the formation of thiobarbituric acid reactive substances (TBARS). Fungal homogenates were added to the reaction mixture (trichloroacetic acid 15% (w/v), 2-thiobarbituric acid 0.37% (w/v), and butylhydroxytoluene 0.15

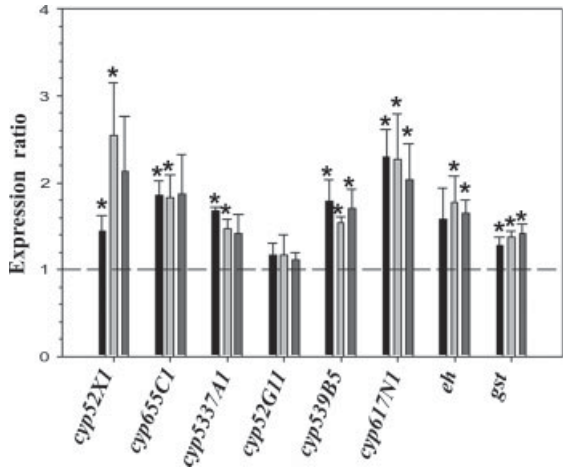

Figure 1. Expression pattern of detoxification genes in *Beauveria bassiana* exposed to various doses of deltamethrin. The dashed line shows an expression ratio = 1, indicating that the target gene is equally expressed either in presence or absence of deltamethrin. Values are means ± SEM. Deltamethrin doses: ▬25 μg/cm^2, ▬50 μg/cm^2, ▬500 μg/cm^2. Asterisks indicate significant differences compared with the control after Student's *t* test.

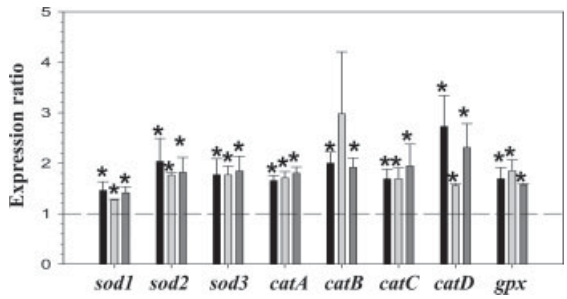

Figure 2. Expression pattern of the antioxidant defense system genes in *Beauveria bassiana* exposed to various doses of deltamethrin. The dashed line shows an expression ratio = 1, indicating that the target gene is equally expressed either in presence or absence of deltamethrin. Values are means ± SEM. Deltamethrin doses: ▬25 μg/cm^2, ▬50 μg/cm^2, ▬500 μg/cm^2. Asterisks indicate significant differences compared with the control after Student's *t* test.

mM) at a ratio of 1:10 (v/v). The mixture was vigorously shaken, maintained in boiling water for 60 min, and immediately cooled at 4 °C for 5 min.[25] Then, it was centrifuged at 5000 × *g* for 10 min, and the supernatant was measured spectrophotometrically at 535 nm.

2.7 Intracellular free proline measurement

Proline content in fungal homogenates was estimated by the method of Bates *et al.*[26], using L-proline (Sigma, USA) as the standard. The amount was expressed as μmoles proline/mg total protein.

2.8 Statistical analyses

Differences among the mean values obtained for each dose were determined by the analysis of variance (ANOVA) and by using Tukey's test to evaluate differences between treatment means ($p < 0.05$). Significant differences between the means for the relative gene expression ratio were evaluated separately at each dose by using Student's *t* test ($p < 0.05$). Instat 3.05 (GraphPad Software Inc., USA) was used for all statistical analyses.

3 RESULTS

3.1 Determination of the IC_{50}

The IC_{50} value calculated for *Beauveria bassiana* strain GHA was 50 μg deltamethrin/cm^2 ($r^2 = 0.92$) (Table 1). This result allowed us to select the appropriate deltamethrin doses to study the fungal genes and enzymes potentially affected by this insecticide. For all further experiments, deltamethrin concentration varied between 0.5 IC_{50} to 10 IC_{50}.

3.2 Gene expression analysis

3.2.1 Detoxification genes

In relation with a potential role on deltamethrin detoxification, the transcript level of the genes studied was more abundant in fungi exposed to the insecticide (regardless of the doses assayed) compared with fungi grown in complete medium without deltamethrin (Fig. 1). P450 genes showed different expression patterns; *cyp52X1* and *cyp617N1* were > 2-fold induced in deltamethrin-exposed fungi compared with controls. Also the *cyp655C1* transcript was 1.8-fold induced in the same experimental conditions. The other *cyp* genes were also more abundant than in controls, though displaying < 1.8-fold induction. The *eh* gene exhibited similar induction values as the latter, and the *gst* gene showed nearly nominal transcript levels under the conditions tested.

3.2.2 Antioxidant defense system genes

Fungal genes implicated in ROS metabolism were also induced in response to deltamethrin (Fig. 2). The two genes encoding for manganese-containing superoxide dismutase (Mn-SOD), named as *sod2* and *sod3*, exhibited more abundant transcripts than *sod1* that encodes for Cu-Zn-SOD. The induction by deltamethrin varied from ∼1.8-fold (*sod3*) to ∼2-fold (*sod2*). The four catalase genes studied were also induced. *catB* and *catD*, encoding for secreted catalases, showed the largest fold induction (2–3-fold at all doses tested) compared with controls. Also, *catA* (spore-specific) and *catC* (cytoplasmic) showed transcripts > 1.7-fold higher than controls. The *gpx* gene was 1.5- to 1.8-fold induced compared with controls.

3.3 Enzymatic activity of the antioxidant defense system and phase II detoxification

SOD activity showed significant differences ($p < 0.0001$, ANOVA) between treatments (Fig. 3a), with values varying from 6.5 ± 1.0 U/mg (control) to 8.6 ± 0.5 U/mg (250 μg/cm^2). At the highest dose tested (500 μg/cm^2), the activity dropped to similar levels than controls. Also, significant differences between treatments ($p = 0.004$, ANOVA) were observed in CAT activity. A marked loss of activity was detected after fungal exposure to 500 μg/cm^2 deltamethrin compared to the effect observed with lower doses (Fig. 3b). GPx activity values showed no significant differences ($p > 0.05$, ANOVA) between doses, ranging from 4.7 ± 0.6 (control) to 6.3 ± 0.5 U/mg (250 μg/cm^2) (Fig. 3c), dropping to 4.4 ± 0.7 U/mg at 500 μg/cm^2. Since both GPx and CAT decompose H_2O_2, probably only CAT participates decomposing deltamethrin by-product H_2O_2.

With regard to GST specific activity, exposure to 25 μg/cm^2 of deltamethrin was not significantly different from controls (0.11 ± 0.01 U/mg). However, a significant loss of activity ($p = 0.0009$) was found at double dose (0.06 ± 0.005 U/mg), maintaining this value at the highest concentration tested (Fig. 3d).

3.4 Lipid peroxidation and free proline accumulation

Fungi exposed to 250 or 500 μg/cm^2 of deltamethrin showed LPO levels (0.25 ± 0.01 and 0.25 ± 0.02 μmol TBARS/mg protein)

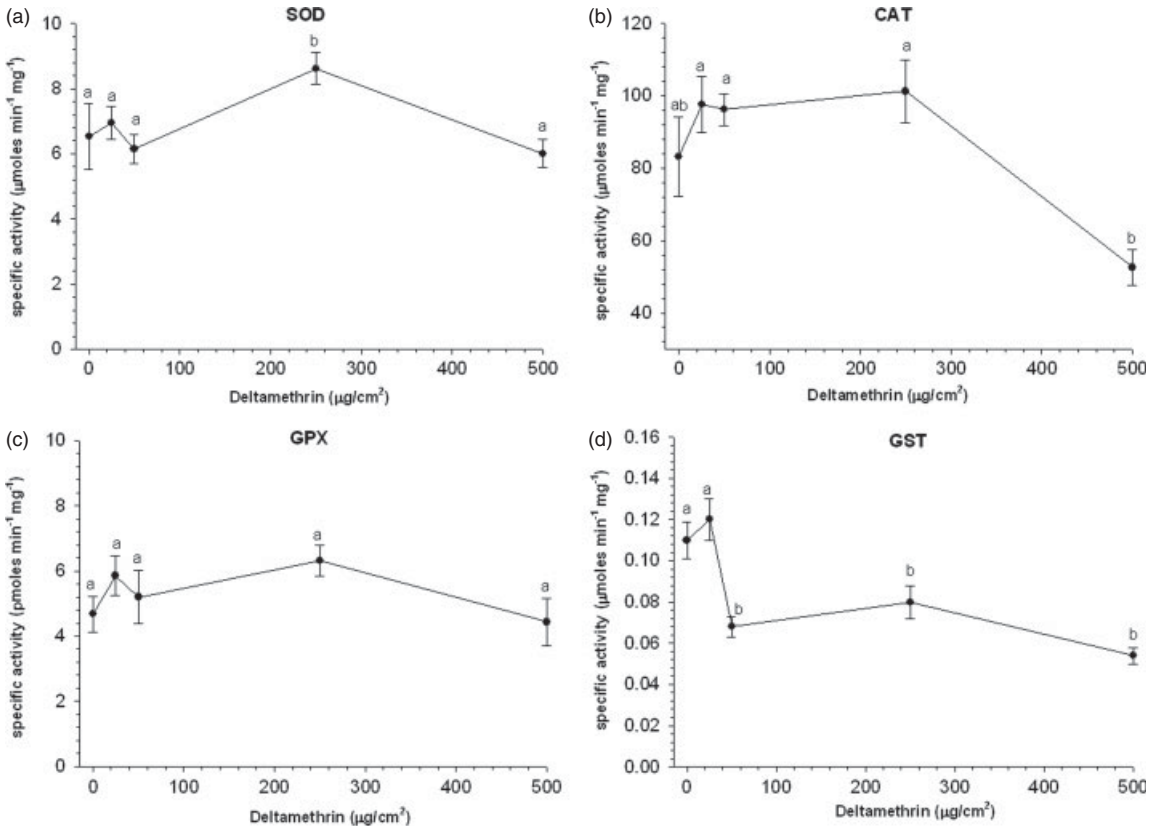

Figure 3. Enzymatic activity in *Beauveria bassiana* exposed to various doses of deltamethrin: (a) superoxide dismutase (SOD); (b) catalase (CAT); (c) glutathione peroxidase (GPx); (d) glutathione-S-transferase (GST). Values represent the mean of six to 12 measurements ± standard deviation. Different letters indicate significant differences after Tukey's post-test.

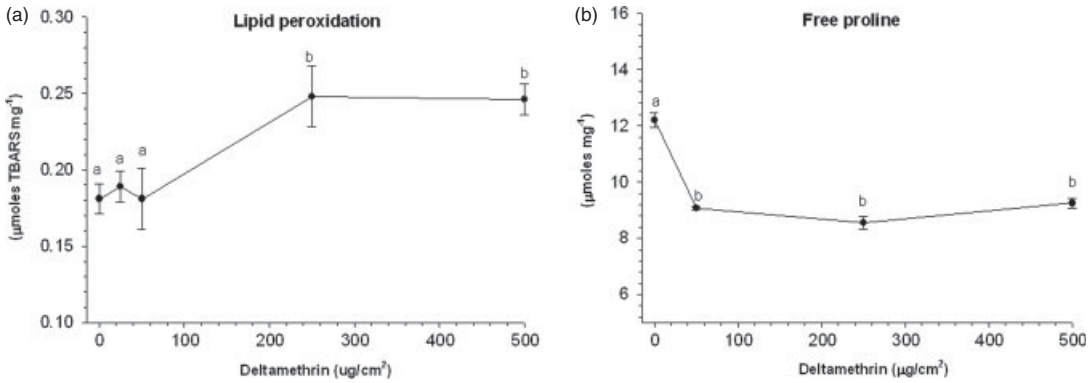

Figure 4. Lipid peroxidation (a) and free proline (b) levels in *Beauveria bassiana* after exposure to various doses of deltamethrin. Values represent the mean of nine (a) and eight (b) determinations ± standard deviation. Different letters indicate significant differences after Tukey's post-test.

significantly different from control (0.18 ± 0.01 μmol TBARS/mg); lower doses produced no significant differences with the control (Fig. 4a). Proline content was significantly ($p = 0.00002$) lower in deltamethrin-exposed fungi, ranging from 8.6 ± 0.3 to 9.3 ± 0.2 μmoles/mg protein for the deltamethrin concentrations tested, compared with controls (12.2 ± 0.3 μmoles/mg) (Fig. 4b).

4 DISCUSSION

In order to develop sound IVM strategies based on entomopathogenic fungi and its possible combination with other insecticide agents, side effects of the latter on these microorganisms should be evaluated. Many studies on the pesticide effects on fungal physiology are already available;[27–29] however, information on the entomopathogen metabolic response to this challenge is rather scarce. *Beauveria bassiana* tolerance to pyrethroids varies with the fungal strain, compound identity and concentration. Four strains of *B. bassiana* virulent to *Triatoma infestans* were shown to be compatible with deltamethrin doses similar to those used in triatomine control. Yet, their tolerance to cypermethrin was much lower.[9] Also, a marked inhibition in fungal development was observed in other *B. bassiana* strains exposed to different insecticides.[28,30] The *B. bassiana* strain GHA was previously shown to be a quite effective alternative tool against *T. infestans*, both in laboratory and field assays.[3,4,13]

The recommended deltamethrin dose for field application is 25 mg/m^2, thus *B. bassiana* GHA can be considered fully compatible with deltamethrin (IC_{50} value of 50 μg/cm^2) for all practical applications. Thus, an appropriate combination of both control agents might be suggested to help improve vector control.

To better understand the interactions between the entomopathogen and deltamethrin, we also explored the fungal metabolic response to this chemical challenge. Insecticide degradation ability of soil fungi is well-known.[31,32] Deltamethrin degradation by a fungus belonging to the genus *Cladosporium* was described, starting rapidly within 1-day culture and accompanied by mycelia growth,[33] and a pyrethroid hydrolase was characterized in *Aspergillus niger*.[34] We found no genes with high sequence identity with pyrethroid hydrolases in *Beauveria bassiana* genome; probably other hydrolases play this role.

Phase I detoxification enzymes, such as CYP monooxygenases and EH, initiate xenobiotic degradation by diverse oxidation reactions, rendering more hydrophilic compounds. Fungi, together with plants, appear to contain the largest number of CYP genes, probably due to the diversity of both primary and secondary metabolism, xenobiotic transformation and detoxification pathways. The whole genome of *Beauveria bassiana* was recently released, containing 77 CYP genes with families and subfamilies assigned.[35] In this work we studied the effect of deltamethrin in the expression pattern of six previously described CYP genes that were shown to be inducible after growth on the cuticle lipids of *Triatoma infestans*.[36] For all the doses tested, a couple of these genes (*cyp52X1* and *cyp617N1*) was the most induced in deltamethrin-exposed fungi compared with controls. Concerning their function, CYP52X1 oxidizes long chain fatty acids, but no information is available on CYP617N1.[37,38]

Detoxification reactions trigger ROS production affecting molecular and biochemical processes and signal transduction pathways. To mitigate and repair the damage caused by ROS, fungi have evolved complex antioxidant systems (both enzymatic and non-enzymatic).[39] The major enzyme systems involved are SOD, CAT, and GPx. SOD converts O_2^- to H_2O_2, while CAT is an essential enzyme to promote the degradation of H_2O_2, a precursor of the hydroxyl radical that induces DNA damage, protein degradation and lipid peroxidation. GPx catalyzes the reduction of both H_2O_2 to water and organic peroxide to alcohols at the expense of reduced glutathione. Here, we found that the expression of both *sod2* and *sod3* (encoding for Mn-SOD) was ~2-fold induced in deltamethrin-exposed fungi, but *sod1* (encoding for Cu-Zn-SOD) was just slightly induced in same conditions. These results suggest that the increment detected in SOD activity might be due to Mn-SOD rather than Cu-Zn-SOD. The two Mn-SODs were reported to co-contribute to the biocontrol potential of *Beauveria bassiana* by mediating cellular antioxidative response.[40] We also studied the expression pattern of four of the five genes of *B. bassiana* catalase family; *cat*B and *cat*D showed the highest induction in the presence of deltamethrin, suggesting their participation in the antioxidant response of fungi due to insecticide degradation. Also, *catB* has been reported to account for the predominant catalase activity in this fungus, and *catD* was shown to be involved in UV-B tolerance and virulence against insects.[41]

The activities of SOD, CAT and GPx increased at increasing doses of deltamethrin, although they all dropped at the highest dose tested. A similar activity pattern was observed in GST, although not quite evident. The most probable explanation for this behavior is that the capacity of the antioxidant system was surpassed by insecticide exposure. Also, superoxide radical accumulation might contribute to antioxidant enzyme inhibition.[42] In an oxidative stress scenario, lipid peroxidation contributes largely to cell function loss. No evident lipid peroxidation was detected below 250 μg/cm^2; thus, no negative effect on the fungus is expected after its combination with deltamethrin at the usual doses applied in the field, or even higher.

We conclude that *Beauveria bassiana* GHA can be used combined with deltamethrin without significant metabolic detrimental effects. This combination will help optimize the benefits and increase the efficacy of vector control tools.

Appendix

Table A1. Oligonucleotide primers used for gene expression analysis

Name	Forward (5′ to 3′)	Reverse (5′ to 3′)	Source/reference
$qSOD_1$	ACAACACCAACGGCTGCACC	ACGGCCAACAACGCTGTGAG	40
$qSOD_2$	CCAGTGTTTGGCATTGACATG	TCAGCCGTCTTCCAGTTGATG	40
$qSOD_3$	ACATCAATCACACTCTCTTCTG	GCGTTGGTCTGCTTCTTG	40
$qCAT_A$	GAAAGCCGCGCAAGTGAAAG	TCTCTGGCAAAGACATCCAG	41
$qCAT_B$	GAAGACGCCCATGTTTGTTCG	AAAGTTGCCCTCATCGGTATAGC	41
$qCAT_C$	TGCTGGACGATGTGTCTGAC	CACGCACCGTATCGCTAGAG	41
$qCAT_D$	GCGCTCGCAGTGACTGTAC	CTAGCACGGCCCTGTATAATGG	41
qGPx	CAAGGTCGTCCTCGTCGTCAAC	CTTGTCGCCATTGACCTCCACC	This study
qGST	TCTTGTAGCCAGCCCTCCATCG	AGAGATGTGGTCGCGGAACGA	This study
qCYP52X1	CTACGGCAGCAACTACTGGA	CCGGCGAGCAGTATGTTGAT	36
qCYP655C1	AACGAGTATCGGCCTGAACGCTG	AATACGCATTCTGGCCCTAAACGG	36
qCYP5337A1	GTCCGGATGCAATGGAGTG	GCGGCTTATTACGATCGACC	36
qCYP52G11	ACCTTTGGCGGCTACGAGAACC	AACGGCCTGACCCTTCTTGACG	36
qCYP539B5	GGCGTCGTATCATCTTCACC	GCTGGCAACTTGGAGATGAG	36
qCYP617N1	GATGCGCACGAGTTCAACC	GGACATGCCAGCTCCGATT	36
qEH	GTGACGAGGACTTGGTTGC	CCACCCATGAATGAATGC	This study
qTub	GATGGCTGCTTCTGACTTCC	TTACGCATCTGGTCCTCAAC	This study

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