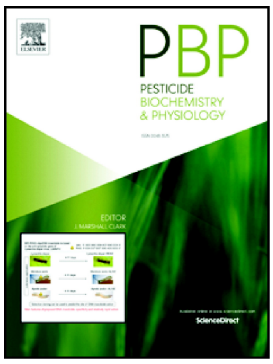

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RNA interference-mediated knockdown of a cytochrome P450 gene enhanced the toxicity of α-cypermethrin in xanthotoxin-fed larvae of *Spodoptera exigua* (Hübner)

Muhammad Hafeez[1], Sisi Liu[2*], Hafiz Kamran Yousaf[3] Saad Jan[4], Rui-Long Wang[5], G. Mandela Fernández-Grandon[6], Asim Gulzar[7], Bahar Ali[1], Muzammal Rehman[8], Sajjad Ali[9], Muhammad Fahad[10], Mo Wang[1*]

[1]Hubei Insect Resources Utilization and Sustainable Pest Management Key Laboratory, College of Plant Science and Technology, Huazhong Agricultural University Wuhan, Hubei 430070, P.R. China

[2]College of Science, Huazhong Agricultural University Wuhan, Hubei 430070, P.R. China

[3]College of Plant Protection Department of Entomology, China Agriculture University Beijing 100193

[4]Bacha Khan University Charsadda, Department of Agriculture Entomology Section Pakistan.

[5]Key Laboratory of Agro-Environment in the Tropics, Ministry of Agriculture, South China Agricultural University, Guangzhou, 510642 , China

[6]Natural Resources Institute, University of Greenwich, Chatham Maritime, Kent, ME4 4TB, UK

[7]Department of Entomology, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi Pakistan

[8]MOA Key Laboratory of Crop Ecophysiology and Farming System in the Middle Reaches of the Yangtze River, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, Hubei, China

[9]Bacha Khan University Charsadda, 24420 Department of Agriculture Entomology Section Pakistan

[10]Department of Plant Breeding and Genetics, Faculty of Agricultural Sciences and Technology, Bahauddin Zakariya University Multan, Punjab. Pakistan 60000.

Corresponding author

1. **Prof. Mo Wang**

College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, 430070, China Tel: +86-130507105431 E-mail: wangmo@mail.hzau.edu.cn

2. Dr. Sisi liu

Laboratory of medicinal Biophysical Chemistry, College of Science, Huazhong Agricultural University, Wuhan 430070, Hubei, China. E-mail**: liusisi@mail.hzau.edu.cn**

Abstract

The beet armyworm (*Spodoptera exigua*) is a highly polyphagous agricultural pest that is distributed worldwide. However, the adaptive mechanisms of *S. exigua* for various insecticides and defensive substances in host plants are unknown. Insect P450 monooxygenases play an important role in the detoxification of plant toxins and insecticides, leading to insecticides resistance. We investigated the induced effects of xanthotoxin exposure on detoxification enzyme activity and larval tolerance to α-cypermethrin in *S. exigua*. Our results showed that the lethal concentration (LC_{50}) of α-cypermethrin for xanthotoxin-exposed larvae was 2.1-fold higher than in the control. Moreover, cytochrome P450 enzyme activity was significantly elevated by upregulation of P450 genes in treated larvae. RT-qPCR results showed that *CYP9A10* expression level was significantly increased in all treatments, while maximal expression level was observed in xanthotoxin+α-cypermethrin-fed larvae. RNAi-mediated silencing of *CYP9A10* further increased mortality by 18%, 26% and 35% at 48 h and by 27%, 43% and 55% at 72 h when larvae were exposed to diets containing chemicals as compared to the control. The results show that *CYP9A10* might play an important role in xanthotoxin and α-cypermethrin detoxification in *S. exigua*. RNAi-mediated silencing could provide an effective synergistic agent for pest control or insecticide resistance management.

Keywords: Beet armyworm; Plant secondary metabolites; Insecticide sensitivity; Cytochrome P450 monooxygenase; RNA interference

1. Introduction

The beet armyworm (*Spodoptera exigua*) is a highly polyphagous agricultural pest, that causes considerable losses in economically important crops such as soybean, peanut, corn, sorghum, tobacco and cotton [1]. Spraying synthetic pesticides is still the main method for the management of beet armyworm, but this approach can select for insecticide resistance as well as contributing to environmental pollution and food contamination [2]. At present, deltamethrin, cypermethrin and fenvalerate are widely applied in agriculture to effectively control *S. exigua* [3–5]. Hence, *S. exigua* has developed a high level of resistance to pyrethroid and other groups of insecticides due to excessive and frequent application of insecticides in the field crops [3,4,6].

A great diversity of secondary compounds and toxic phytochemicals are produced in plants, which serve as a rich pool of defense agents against phytophagous insects and pathogens [7,8]. Phytophagous insects are adapted to the presence of toxins in their regular diet [8,9]. Herbivorous insects can also metabolize potentially toxic phytochemicals accumulated by plants to resist or evade herbivorous insects, which often involves enhanced expression of detoxification enzymes such as cytochrome P450 monooxygenases [10,11]. Interestingly, some plant secondary metabolites help the insects to detoxify insecticides by elevating their detoxification mechanisms [12–14].

Among the various detoxification enzyme systems, cytochrome P450 monooxygenases (P450s or CYPs) are the most widely studied. They constitute a large family of enzymes, which are frequently involved in the detoxification of plant secondary metabolites and insecticides [7,15,16]. For example, P450s are heme-containing NADPH-dependent enzymes as well as important mediators of hydroxylation and epoxidation, leading to effective destruction and degradation of plant allelochemicals as can be observed in insect guts before absorption [11,17–

19]. Insect P450s have been divided into four clades. For example *CYP2*, *CYP3*, *CYP4* and mitochondrial P450s [20]. Clade 3 is further subdivided into the *CYP6* and *CYP9* families [21,22]. For various members of *CYP6* family there is evidence that they detoxify allelochemicals and insecticides consumed by insects [23–25]. In addition, frequent uptake of toxins produced in plants could induce transcription of P450 genes in insects, which are responsible for the decomposition and metabolism of plant toxins [26,27]. For instance, *CYP6B6*, *CYP8A17*, *CYP321A1*, *CYP9A12*, *CYP6AB14* and *CYP9A98* transcripts of *Helicoverpa armigera* and *Spodoptera exigua* were induced by quercetin, gossypol, tannic acid, lambda-cyhalothrin and deltamethrin [12,28–30]. Quercetin and gossypol significantly induced activity of P450 enzymes in the silkworm and *Spodoptera exigua* [31,32] and xanthotoxin and cypermethrin induced transcription of *CYP321A1* and other detoxifying P450s in *Helicoverpa zea* [33]. As a result, insects can adapt to the host plant toxin compounds by elevating P450 enzyme activity and the expression level of the P450 genes to regulate their defensive state for surviving in toxic environments.

The sensitivity of insects to insecticides can be influenced by exposure to plant defensive secondary metabolites through consumption [34]. These increase the detoxification response of the insect that then carries over to the metabolism and detoxification of insecticides (Li etal., 2000; Panini et al. 2016). For example, the insecticidal activity of lambda-cyhalothrin was reduced in quercetin-fed larvae of *H. armigera* [29]. Cross-resistance to alpha-cypermethrin was also observed in xanthotoxin-fed larvae of *H. zea* [37]. Gossypol-induced fitness gain and enhanced P450 gene pool increased resistance to deltamethrin in *H. armigera* and *S. exigua* [12,32]. However, the effects of xanthotoxin uptake on the adaptative mechanisms of insect detoxification on insecticide susceptibility is rarely documented.

In insects, RNA interference (RNAi) is a promising tool for studying functional genomics and has widely been used in gene silencing to study the role of proteins involved in growth, development, and resistance to toxic chemicals [38–40]. For instance, injection of dsRNA significantly reduced the expression level of three cuticular protein genes *CPG316*, *CPG860* and *CPG4855* in *S. exigua* [41]. The induced expression of many P450 genes was significantly reduced after the delivery of dsRNA by dietary-feeding, microinjection or droplet feeding [38,39,42].

The insect's response to phytochemicals and pesticides in its local ecosystem provides key information and understanding for the development of an effective pest control strategy [43]. Here, we gain insight into the role played by cytochrome P450s in facilitating the adaptation of *S. exigua* to environments presenting a diversity of toxicological challenges. This includes the knowledge of the P450 genes in *S. exigua* and the influence of plant secondary metabolites and insecticides on their expression profiles. In this study, we first investigated the alpha-cypermethrin tolerance in xanthotoxin-fed larvae of *S exigua*. Secondly, we examined the potential roles played by P450 genes in conferring resistance to insecticides in xanthotoxin-exposed larvae of *S. exigua* by quantifying the analysis of P450 detoxification enzymes. RT-qPCR was performed to investigate the tissue-specific expression patterns of three P450 genes and their potential roles in detoxification of alpha-cypermethrin and xanthotoxin. Functional analysis of *CYP9A10* was done using RNAi administered in a droplet-feeding bioassay.

2. Materials and methods

2.1. Insect culture

The laboratory-reared susceptible colony of beet armyworm (*Spodoptera exigua*) was collected from Jingzhou, Hubei province of China in 2003. The larvae were reared on a semi-synthetic artificial diet used by Elvira et al [44] without exposure to any insecticides. The colony was maintained in the College of Plant Science and Technology, Huazhong Agriculture University Wuhan, China under laboratory conditions (25 ± 2°C, 65–75% R.H) and 14h:10h (Light: Dark) photoperiod. The eggs were sterilized with a 0.1% sodium hypochlorite and the adult moths were fed with a 10% honey solution.

2.2. Chemicals

Piperonyl butoxide (PBO, 90%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Xanthotoxin, 7-ethoxycoumarin, NADPH, p-nitroanisole, p-nitrophenol and Fast Blue RR salt and Naphthol and α-naphthyl acetate were purchased from sigma-Aldrich. Alpha-cypermethrin was purchased from Nanjing Ronch Chemical Co., Ltd., Nanjing, China. Dithiothreitol (DTT), glycerol, and Tris were bought from Beijing Solarbio Scientific and Technology Company Beijing, China. Bovine serum albumin was purchased from Beyotime Biotechnology, Jiangsu, China. All chemicals and solvents used were reagent grade.

2.3. Preparation of chemical-supplemented diets

A wheat germ based artificial diet was prepared by following the protocol described by Elvira et al [44] with some modification. Diet was poured into the small transparent plastic cups before solidification of agar (40–45 °C). Xanthotoxin-supplemented diet was prepared according to the method described by Tao et al [12] with slight modification. Xanthotoxin to be tested was first dissolved in 1% dimethyl sulfoxide (DMSO) and gently mixed into the artificial diet before

solidification of agar (40–45°C). Insecticide dilutions: A stock solution of α-cypermethrin insecticide was first prepared by diluting it in distilled water containing 0.1% Triton-X-80. Five serial concentrations (a serial dilution ranging from 0.1, 0.2, 0.4, 0.7, 1.00 mg/L) of α-cypermethrin were prepared and pipetted into 20 mL transparent plastic cups containing still liquid diet and then incorporated by stirring for 2 min. An equal amount of 1% DMSO was added into the diet for the controls and stored at -80°C prior to use.

2.4. Analysis of α-cypermethrin tolerance of the larvae

Effects of xanthotoxin uptake on *S. exigua* larval tolerance to α-cypermethrin were tested as follows: Early third instar larvae were first fed on artificial diet containing 0.1% xanthotoxin for 24 hours before the bioassay. For the control group, the artificial diet was prepared with same method but without xanthotoxin. For the diet incorporation bioassay, the method used was as described by Hafeez et al [32] of the toxicity of α-cypermethrin. For this, a stock solution of α-cypermethrin insecticide was prepared as described above. Insecticide-treated diet was cut into small pieces and placed into sterilized transparent petri plates (8.0 mm diameter×3.0 mm height) with Whatman™ Grade 44 Quantitative filter paper at the bottom. The xanthotoxin-pretreated early third instar larvae were transferred onto five concentrations of α-cypermethrin supplemented diets (0.3 0.6, 1, 1.5, 2 mg／L), respectively. All doses used were selected based on the results obtained from preliminary experiments. Each treatment was replicated three times with 30 larvae per replicate (90 larvae per treatment). After 24 hours, the larvae were touched with a camel hairbrush; those that showed no response were considered dead, and the number of deaths were recorded. LC_{50} values were calculated [32]. Mortality data was analyzed by performing a Mann-Whitney U test using SPSS software.

2.5. Synergism by Piperonyl Butoxide

To evaluate if the biochemical basis for tolerance involved P450s, the larvae exposed to the test chemicals were subjected to synergism studies with piperonyl butoxide (PBO). PBO stock solutions (25 mg/mL) were prepared in acetone (analytical reagent grade, ≥ 99.5%). A final concentration of PBO (10 µg/larvae) was determined by preliminary tests to be nonlethal for third instar *S. exigua* larvae described by Wang et al [39]. Experiments in the presence or absence of the synergist PBO were performed using the same bioassay methodology. After the *S. exigua* larvae fed on xanthotoxin-supplemented diet for 24 h, 10 µg/larvae of PBO solution was topically delivered onto the dorsal prothorax of individual larvae using a Micro4^{TM} MicroSyringe Pump Controller, USA. After 2 h, the PBO-treated larvae were transferred onto small sterile transparent petri plates (8 x 3 cm) containing artificial diet supplemented with different concentration of α-cypermethrin (0.1, 0.2, 0.4, 0.7, 1.00 mg/L of α-cypermethrin for PBO-treated group). The control group larvae were fed with a xanthotoxin diet for 24 h, but without PBO pre-treatment (0.3 0.6, 1, 1.5, 2 mg / L of α-cypermethrin for control group). Three replicates of 20 larvae were used for each treatment and control. Mortality was recorded after 48 h and the LC_{50} values were calculated [40]. The synergism ratio (SR) was calculated by dividing the LC_{50} of insecticide alone by LC_{50} of insecticide + synergist as described by Wang et al [40]. Each experiment was performed in triplicate.

2.6. The effect of 0.1% xanthotoxin diet on body weight

To evaluate the effect of xanthotoxin on growth of *S. exigua*, 120 third-instar larvae with uniform size were starved for 2 h and transferred to small sterile transparent plastic cups (3 cm diameter, 3.5 cm height) containing artificial diet supplemented with 0.1% xanthotoxin (g/g artificial diet) and control (CK) diet for 24 h. After 24 h, they were weighed and transferred to a

diet containing 0.382 mg⁄ L α-cypermethrin (a sublethal concentration) for another 24 h. After 2 days of exposure, the net weight change was recorded by weighing the specimens at 72 h.

2.7. Enzyme activity assay.

The P450 detoxification enzyme activity of *S. exigua* larvae midgut homogenates was assayed after the larvae were reared on diet containing 1.0 mg⁄ g xanthotoxin or no xanthotoxin (control) for 24 h, followed by transfer to diet containing sublethal concentration of α-cypermethrin. Then the midgut was taken after 48 and 72 h for further analysis.

The crude homogenates of *S. exigua* midgut were prepared as previously described by Liu et al [45] with some modification. The midgut of larvae was obtained by dissection on ice. It was then gently shaken to free it of its contents and rinsed in an ice-cold 1.15% (w/v) potassium chloride aqueous solution and was used for detoxification enzyme activity assay. 7-ethoxycoumarin-O-deethylase (ECOD) activity of cytochrome P450 in larval midgut was determined using 7-ethoxycoumarin (7-EC) as the substrate according to the method described by Chen et al [29]. Fifteen midguts for each treatment (consisting of triplicates of 5 larvae) were homogenized on ice with 1.5 mL of homogenization buffer 0.1 M phosphate-buffered saline (PBS) at pH 7.5 and then centrifuged at 10,800 g for 15 min at 4 °C. The supernatant was collected for the P450 activity assay. The enzyme reaction was started by adding 250 μL of enzyme preparation to a mixture containing 5 μL of NADPH (10 mM stock solution) and 25 μL of 7-EC (10 mM stock solution) in 650 μL of 0.1 M Tris–HCl buffer (pH 7.5). After incubation at 30 °C for 15 min, 300 μL of 15% (w/v) trichloroacetic acid (TCA) was added to terminate the reactions. Then the mixture was centrifuged at 10,800 g for 2 min at 4 °C, and 800 μL of supernatant was transferred to a new tube and 450 μL of 1.6 M Glycine-NaOH buffer (pH 10.5) was added; the content of 7-hydroxycoumarin was measured immediately by a SPECTRA max GEMINI XS

spectrofluorometer (Molecular Devices, USA) with 356-nm excitation and 465-nm emission filters. A series of different concentrations of 7-hydroxycoumarin standard substance fluorescence values were measured to establish the standard curves. This experiment was repeated in triplicate. Determination of protein concentration was carried out by an improved method described previously using bovine serum albumin as the standard protein [46].

2.8. Samples preparation

To determine tissue-specific expression patterns for the target genes, late third instar larvae of *S. exigua* were fed on artificial diets containing 0.1% xanthotoxin for 24 h as the xanthotoxin-treatment group before the bioassay, 0.382mg⁄ L α-cypermethrin and 1% DMSO control, respectively. One day later after the chemical application, two tissues (midgut and fat body) were dissected and stored at -80 ℃ for RNA extraction. Three biological replicates were collected for all samples.

2.9. RNA extraction and cDNA synthesis

Total RNA was prepared from midguts and fat bodies of fourth instar larva using the Trizol reagent according to the manufacturer's protocol (Takara, Japan). The concentration and purity of total RNA was determined by a NanoDrop® spectrophotometer (Thermo Fisher, MA, USA) and RNA integrity was examined by agarose gel electrophoresis.

First-strand complementary DNA (cDNA) was synthesized by using TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix in 20 μL reactions containing 1 μg of total RNA (500 ng), 1 μL Anchord Oligo(dT)18 Prime (0.5 μg/μL), 10μL 2×TS Reaction mixture, TransScript® RT/RI EnzymeMix and gDNA Remover at 42 C for 30 min. Three independent RNA preparations representing three biological replicates were used for cDNA synthesis.

2.10. Quantitative real-time PCR

Total RNA was isolated from two tissues (midguts and fat bodies) of pretreated fourth instar larvae of *S. exigua* after 48 and 72 h. Expression levels of cytochrome P450 genes were quantified by RT-qPCR using a VIOX/SCIENTIFIC 96-well PCR plates, and Real Master Mix 2xSYBR Green RT-qPCR kit (Aidlab biotechnologies Co., Ltd China). RT-qPCR of each cDNA sample and template-free was performed in triplicate. Gene-specific primers were designed for RT-qPCR and are listed in **Table 1**. Total volume of 20 µl reaction mixture [0.5 µl of each primer (10 µM), 1µl cDNA, 8 µl ddH20 and 10 µl 2x Syber Master Mix for quantification] was used. PCR used the following cycling parameters: 94°C for 3 min, followed by 40 cycles of 94°C for 15 sec, 57–60°C for 30 s and 70°C for 30 s. For each gene, a serial dilution from 10- to 1000-fold of each cDNA template was performed in order to assess the efficiency of PCR. The expression levels of the genes (*CYP9A10, CYP6B50* and *CYP6AB412*) at each time point were calculated and normalized to the geometric mean of the expression of the reference gene *β*-actin with $2^{-\Delta\Delta CT}$ method as previously described [47,48]. Results were expressed as mean expression ratio (± SD) of three biological replicates between chemical treatments and controls. One-way analysis of variance (ANOVA) and the Tukey HSD test for the significant difference was performed to determine the statistical difference between means (SPSS, version 19).

2.11. dsRNA Synthesis

For dsRNA synthesis, a 402 bp fragment from *dsCYP9A10* and a 688 bp fragment from *(dsRED*) were first amplified by PCR. The primers used for the *CYP9A10* and *dsRED* amplifications were designed to add the T7 polymerase promoter sequence at the 5' ends. Two pairs of primers (*T7CYP9A10-F* and *CYP9A10-R*, *CYP9A10-F* and *T7CYP9A10-R*) were used to amplify T7 (**Table 1**). As a control, *dsRED* was synthesized using the same method by two pairs of primers (*T7RED-F* and *RED-R*, *RED-F* and *T7RED-R*) (**Table 1**). *DsCYP9A10* and *dsRED* were

prepared from the purified PCR-generated templates according to the instructions provided with the T7 RiboMax Express RNAi System kit (Promega, Madison, WI, USA). The resulting dsRNAs from all genes including control gene were quantified by a NanoDrop® spectrophotometer (Thermo Fisher, MA, USA) and integrity was analyzed by agarose gel electrophoresis, and then stored at -80C prior to use.

2.12. RNA Interference Bioassays

For RNAi bioassays, double-stranded RNAs (dsRNA) were dissolved in diethylpyrocarbonate (DEPC)-treated water. A droplet-feeding method for dsRNA to fourth instar larvae (pre-exposed with xanthotoxin for 24 h) was used following an established methodology [49,50]. For RNAi bioassays, double-stranded RNAs (dsRNA) dissolved in diethylpyrocarbonate (DEPC)-treated water. The fourth instar larvae were placed individually in 12-orifice tissue culture plates and starved for 6 h. The dsRNA solution (500 µg/µl) was configured by dissolving in DEPC treated water. The starved larvae were placed individually in 12-orifice tissue culture plate containing the artificial diet and one drop 0.5 µl (500 µg/µl) of dsRNA solution was placed near each larval mouth using a Microliter Syringe Beijing Karaltay Scientific Instruments Co., Ltd. Twenty-four hours after feeding on dsRNA larvae were subjected to toxicity analysis.

For toxicity analysis, after 24 h of dsRNA post-feeding, 60 *S. exigua* larvae for each independent treatment (each of three replicate consisted of 20 larvae) were transferred individually into 12-orifice tissue culture plates containing artificial diets supplemented with 0.1 % xanthotoxin, LC_{50} concentration of 0.382mg/ L α-cypermethrin for 72 h, and 0.1 % xanthotoxin for 24 h followed by α-cypermethrin for 72 h and standard diet. A non-supplemented diet was used as a control group. The mortality data were recorded at 48 and 72 h after feeding on dsRNA on different treatments, including control. All experiments were performed in triplicate.

Midguts of surviving larvae were collected for enzyme essays and total RNA extracted for quantitative RT-qPCR procedures as described above. All experiments were performed in triplicate (three biological replicates).

2.13. Statistical Analysis

All data were analyzed using the SPSS 20.0 Software Package (SPSS Inc., Chicago, IL, USA). One-way ANOVA followed by the Tukey HSD test was employed to analyze differences between tissues and developmental stages. A Student's t-test was used to analyze data from the RNAi and feeding experiments with chemicals.

3. Results

3.1. Effect of xanthotoxin on the toxicity of alpha-cypermethrin with or without synergist (PBO).

The exposure to dietary 0.1% xanthotoxin for 24 h before feeding on α-cypermethrin-supplemented diet decreased the sensitivity of *S. exigua* larvae to the cypermethrin (**Table 2)**. The LC_{50} of 0.811 mg/L for the xanthotoxin-exposed larvae was significantly higher than the LC_{50} of 0.382 mg/L for the control larvae. To determine whether the induction effect of dietary xanthotoxin on decreased larval sensitivity to cypermethrin was due to increased P450s, the bioassay was repeated in the presence of PBO which inhibits P450s. Results shows that PBO effectively increased the efficacy of α-cypermethrin in xanthotoxin-fed larvae of *S. exigua* such that mortality was intermediate between the control groups with and without PBO. Thus, the synergistic ratio of 2.1 for the xanthotoxin treatment likely indicates that the xanthotoxin caused an increase in P450 detoxification.

3.2. The effect of xanthotoxin diet on body weight of *S. exigua larvae*

To determine the effect of xanthotoxin on the net larval weight of *S. exigua*, third instar larvae were fed diet containing 0.1% xanthotoxin or normal diet (CK) for 1 day, and then transferred to diet containing the LC_{50} concentration of α-cypermethrin or CK diet for an additional day. As seen in **Figure 1**, under both conditions, the larvae fed on xanthotoxin-diet gained significantly less weight.

3.3. The induced effect of xanthotoxin on midgut P450 enzyme activity of *S. exigua* larvae.

To determine the effect of xanthotoxin on sensitivity to α-cypermethrin, the midgut P450 enzyme activity of *S. exigua* larvae was analyzed. Late third instar larvae were first exposed to

0.1% xanthotoxin/g-containing artificial diet and the control group was exposed to artificial diet (no xanthotoxin) for 24h. After 24h, larvae were shifted onto artificial diet supplemented with the LC_{50} concentration of α-cypermethrin and midguts were assayed after 48 and 72 h for enzyme activity. **Figure 2** shows that after 48 h, the midgut P450 enzyme activity was significantly elevated in the xanthotoxin (1.2-fold), α-cypermethrin (1.4-fold) and α-cypermethrin+xanthotoxin (2-fold)-treated groups as compared to the control (0.7-fold) group. A similar trend was observed at 72 h (**Figure 2)**

3.4. Expression of *CYP9A10, CYP6B50* and *CYP6AB12* in response to xanthotoxin and α-cypermethrin

In order to determine the effect of xanthotoxin, α-cypermethrin and xanthotoxin+α-cypermethrin on the expression pattern of three selected P450 genes, *CYP9A10, CYP6B50* and *CYP6AB12*, RT-qPCR was performed after exposure of late fourth-stage larva of *S. exigua* to artificial diet containing xanthotoxin, α-cypermethrin and xanthotoxin+α-cypermethrin for 72 h. **Figure 3** shows that in the midgut, the expression of *CYP9A10* mRNA significantly increased after feeding on diet supplemented with xanthotoxin (5.7-fold), cypermethrin (7.3-fold) and xanthotoxin+α-cypermethrin (11.3-fold) relative to the control treatment. Xanthotoxin and cypermethrin significantly increased the level of *CYP6B50* mRNA, while no significant difference was observed between the xanthotoxin, α-cypermethrin and xanthotoxin+α-cypermethrin treatment. In contrast, *CYP6AB12* mRNA in the midgut increased but did not differ significantly among the three treatments (**Figure 3)**. In the fat body, the expression of *CYP9A10* showed similar elevations to those in the midgut with the different treatments (**Figure 3),** whereas the same trend was observed in the increase level of *CYP6B50* mRNA in α-cypermethrin and Xanthotoxin in the midgut. However, *CYP6AB12* mRNA level in the fat body

was about 6-fold increased by α-cypermethrin but only 2-3-fold by cypermethrin and the combination of the two.

3.5. Silencing effect of *dsCYP9A10* on the toxicity of xanthotoxin and α-cypermethrin

Figure 4 shows that droplet feeding of *dsRNA-CYP9A10* to larvae significantly enhanced the insecticidal activity of both the phytotoxin and the insecticide, while delivery of *dsRED* did not. Forty-eight hours after exposure to xanthotoxin (0.1%), α-cypermethrin (0.33 mg/L) and xanthotoxin+α-cypermethrin (1 mg+0.33 mg/L), larvae exposed via droplet feeding to dsRNA-*CYP9A10* had significantly enhanced mortality caused by xanthotoxin (18%), α-cypermethrin (27%) and xanthotoxin+α-cypermethrin (35%) compared to the *dsRED* control (12, 18 and 28%). By 72 h, mortality had increased to 27, 43, and 55% respectively for the treated larvae and 17, 27, and 42% for the control larvae (*dsRED*) (**Figure 4B**). Taken together, these results strongly suggest that *CYP9A10* might play a significant role in the induction by xanthotoxin of decreased sensitivity to the toxicity of α-cypermethrin insecticide in *S. exigua*.

3.6. Functional analysis of *CYP9A10* by RNAi

The potential role-played by *CYP9A10* in the midgut and fat body of *S. exigua* larvae in the detoxification of xanthotoxin and cypermethrin was further analyzed using RNAi to specifically inhibit the expression levels of *CYP9A10*. In the midgut, **Figure 5** shows that following exposure to xanthotoxin (0.1%), α-cypermethrin (0.33 mg/L), or xanthotoxin+α-cypermethrin (0.1%+0.33 mg/L), larvae exposed via droplet feeding to dsRNA-*CYP9A10* had significantly reduced levels of *CYP9A10* mRNA (60, 73 and 46% respectively) compared to the control (dsRED) levels of 70-83%. By 72 h, the level had further decreased to 51, 39, and 24% respectively for the larvae

fed *dsCYP9A10*, whereas it remained high for the larvae given *dsRED* (**Fig. 5**). Similarly, in the fat body, the expression of *CYP9A10* was reduced by diet supplemented with α-cypermethrin (87%) and xanthotoxin+α-cypermethrin (49%) compared to control (*dsRED*) (100 and 89%) after 48 h, but there was no significant difference between those fed xanthotoxin and the control (**Fig. 5**). By 72 h, however, the levels of *CYP9A10* mRNA in fat body was reduced significantly in all the treatments compared to the control (**Fig. 5**). These results demonstrate the efficacy of the RNAi approach in *S. exigua* larvae.

4. Discussion

Constant exposure to naturally occurring plants toxins and synthetic insecticides has selected for insects with cytochrome P450 monooxygenase detoxification enzymes (P450) that can metabolize a wide range of structurally different compounds [51]. The beet armyworm, *S. exigua*, is a polyphagous pest with a wide range of host plants, which produce a diverse range of allelochemicals to defend against insect herbivores [1]. Some of these allelochemicals attract the natural enemies as well as reduce the larval sensitivity to different insecticides [52,53]. Insect herbivores, in contrast, comprehensively depend on their detoxification enzymes to overcome the potential toxicity of plant toxins and other xenobiotics such as synthetic pesticides [52]. Here, we studied the effect of diet-incorporated xanthotoxin, a plant allelochemical, on the tolerance of *S. exigua* larvae to a commonly used pyrethroid insecticide, α-cypermethrin, and evaluated the effects of xanthotoxin and α-cypermethrin on the activity and transcription of cytochrome P450 genes, *CYP9A10, CYP6B50* and *CYP6AB12*, which encode the main detoxification enzymes. We also analyzed of the function of *CYP9A10* using RNA interference. These data suggest that these P450 genes have a potential role in the detoxification of xanthotoxin and α-cypermethrin.

The effects of plant secondary metabolites on feeding behavior, growth and development of herbivorous insects have been widely reported [32,54]. Xanthotoxin uptake inhibited the growth of *S. exigua* larvae in this study. Several previous studies also showed that plant secondary metabolites could affect the growth of insects. For example, the growth of *Trichoplusia ni*, *Pseudaletia unipuncta* and *Helicoverpa armigera* larvae was significantly reduced after feeding on a diet with different concentrations of xanthotoxin and quercetin [55,56]. In contrast, several studies have shown that some secondary metabolites in plants can help insects develop resistance

to insecticides [29,57]. P450 is one of the most important detoxification enzyme systems, which help the insect herbivores to adapt to its host plant chemical defenses and influence its resistance against xenobiotics such as insecticides [12,58]. In this study, the activity of P450 enzymes in *S. exigua* larvae significantly increased 48 to 72 h after feeding on xanthotoxin **(Figure 2**). PBO is an insecticide synergist known to inhibit the activity of P450 enzymes in insects [59]. Our studies showed that PBO enhanced the toxicity of α-cypermethrin to xanthotoxin-fed larvae of *S. exigua* (**Table 2**). These results suggested that the increased tolerance to α-cypermethrin in xanthotoxin-fed *S. exigua* might result from the ability of this plant allelochemical to induce detoxification enzymes, mainly cytochrome P450s. The increase in activity of detoxification enzymes seen here is consistent with previous studies, which showed a similar increase in activity of cytochrome P450s [12,29,32,60] in many polyphagous herbivorous insects exposed to plant secondary metabolites and insecticides. Because the synergist PBO can also inhibit non-specific esterase activity [61], the effect of xanthotoxin intake on carboxylesterase and Glutathione S-Transferases and its corresponding contribution to the tolerance of pyrethroid insecticide to *S. exigua* larvae should be investigated in further study.

The P450s comprise one of the largest gene families in living organisms. There is usually up-regulation of one or more P450 genes in insecticide resistant organisms [62,63] indicating the importance of their involvement in this process. In this study, RT-qPCR results demonstrated that xanthotoxin-fed *S. exigua* larvae developed tolerance against α-cypermethrin insecticide and significantly increased the transcription of three common cytochrome P450 genes, *CYP9A10, CYP6B50* and *CYP6AB12*, in both the midgut and fat body. The resulting increase in these enzymes then contributed to increased metabolic detoxification activity in xanthotoxin-fed *S. exigua* larvae. This induction effect of a plant secondary metabolite on these three P450 genes is

consistent with previous studies. For example, increased transcription of five gossypol-induced P450 genes, *CYP321A1, CYP9A12, CYP9A14, CYP6AE11* and *CYP6B7*, contributed to cotton bollworm tolerance to deltamethrin [12], and mRNA of *CYP6B*6 in *H. armigera* increased after being exposed to quercetin and xanthotoxin [45]. Moreover, *CYP6B6, CYP6B8* and *CYP321A1* activity in polyphagous *Helicoverpa zea* can be induced by a variety of plant secondary metabolites as well as synthetic insecticides [29,64].

RNAi has been successfully applied to the study of gene function of lepidopteran insects [38,40,65–67]. In this investigation, we found that silencing of *CYP9A10* significantly increased the mortality of *S. exigua* larvae when exposed to xanthotoxin, α-cypermethrin and xanthotoxin+ α-cypermethrin (**Figure. 4**). The main reason for the delay in mortality efficacy in larvae other than the effects of dsRNA interference, which synergistically increased the xanthotoxin and insecticide toxicity by decreasing the activity of P450 detoxification enzyme in exposed insect. Our results are consistent with the earlier published reports [39,40,49,68]. Furthermore, RT-qPCR results obtained in the present study also demonstrated that the RNAi-mediated silencing effect of *dsCYP9A10* was to significantly decrease the levels of *CYP9A10* mRNA present in *S. exigua* following exposure of xanthotoxin, α-cypermethrin or xanthotoxin+ α-cypermethrin (**Fig. 5**). Similar results on the silencing effect of *dsCYP6B6*, *dsCYP9A105*, *dsCYP9A14* and *dsCYPP321B1* have been reported in *S. litura, S. exigua,* and *H. armigera* when exposed to plant secondary metabolites and insecticides [12,39,67,69,70]. Thus, in the present work, the increased sensitivity to α-cypermethrin in xanthotoxin fed *S. exigua* larvae following droplet feeding of dsRNA targeting *CYP9A10* provides additional compelling evidence that CYP9A10 plays a key role in detoxifying compounds encountered by this organism while feeding.

Based on our results, we speculate that some plant secondary metabolites can highly induce cytochrome P450s in the larvae of *S. exigua*. The observed induction effect of xanthotoxin was an increase in the detoxification enzyme P450 activity due to the elevation of the expression level of P450 genes, which in turn decreased the larval susceptibility to α-cypermethrin. The P450 detoxification enzyme system in herbivorous insects undoubtedly plays a crucial role in the adaptation of these insects to plant secondary metabolites and agricultural insecticides. Furthermore, insects can use allelochemicals in host plants to enhance their capacity to reduce sensitivity to insecticide and other toxic xenobiotics. The silencing of *CYP9A10* mediated by RNAi can significantly increase the mortality rate of *S. exigua* larvae exposed to xanthotoxin and α-cypermethrin, strongly suggesting that *CYP9A10* may play a critical role in its metabolic detoxification process and contribute to conferring resistance to these chemicals. RNAi technology holds the additional potential to cripple the pest tolerance to phytotoxins and insecticides, reducing the dosage of these chemicals needed for pest control in field.

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Table 2. The influences of xanthotoxin ingestion and synergism effect of PBO on the α-cypermethrin toxicity to *Spodoptera exigua* larvae using Chi-square for analysis. Resistance ratio is shown as SR, df represents the degrees of freedom and CL to describe the confidence limits.

Treatment	LC_{50} (mg a.i./L)	95% CL[a]	Slope ± SE	*df*[b]	χ2	*P. value*	SR[c]
Control	0.382	0.33 ± 0.44	1.92 ± 0.211	3	0.80		-----
Control + PBO	0.294	0.25 ± 0.33	2.19 ± 0.20	3	1.64	0.65	0.76
Xanthotoxin	0.811	0.72 ± 0.92	1.95 ± 0.17	4	1.73	0.78	2.1
Xanthotoxin + PBO	0.339	0.29 ± 0.39	2.01 ± 0.22	3	1.35	0.71	0.88

[a]CL: confidence limits, [b]df: degrees of freedom, χ2: Chi-square value, [c]SR: synergistic ratio

Table 1. Primers used throughout the study and their associated sequences.

Function	Primer name	Primer sequence (5'-3')
Real-Time PCR		
CYP9A10	CYP9A10-F	GCGTGAAGCATTTCAAGCCA
	CYP9A10-R	CCGACGAACCTCTCTTCAGG
CYP6B50	CYP6B50-F	TGTGAGAGAACTGCATCCCT
	CYP6B50-R	GGAGCTGTGCGAACTTTGAA
CYP6AB12	CYP6AB12-F	GGAAAGCCAGTATGACGCAG
	CYP6AB12-R	AGAGCGAAGAAATCCGACGA
β-actin	β-actin-F	ATCCTCCGTCTGGACTTGG
	β-actin-R	GCACGATTTCCCTCTCA
dsRNA synthesis		

CYP9A10	T7CYP9A10-F1	ggatcctaatacgactcactataggCCTATTGTTATGGGTGGCG
	CYP CYP9A10-R1	GTGAAGGCTGGACTCAATGT
	CYP CYP9A10-F2	CCTATTGTTATGGGTGGCG
	T7CYP CYP9A10-R2	GTGAAGGCTGGACTCAATGTGgatcctaatacgactcactatagg
dsRED	T7pGEM Teasy-F1	ggatcctaatacgactcactataggGCAAGCTATGCATCCAACGCGTTGGG
	ds pGEM Teasy -R1	CAAGCTATGCATCCAACGCGTTGGGAG
	pGEM Teasy -F2	GCAAGCTATGCATCCAACGCGTTGGG
	T7pGEM Teasy -R2	CAAGCTATGCATCCAACGCGTTGGGAGgatcctaatacgactcactatagg

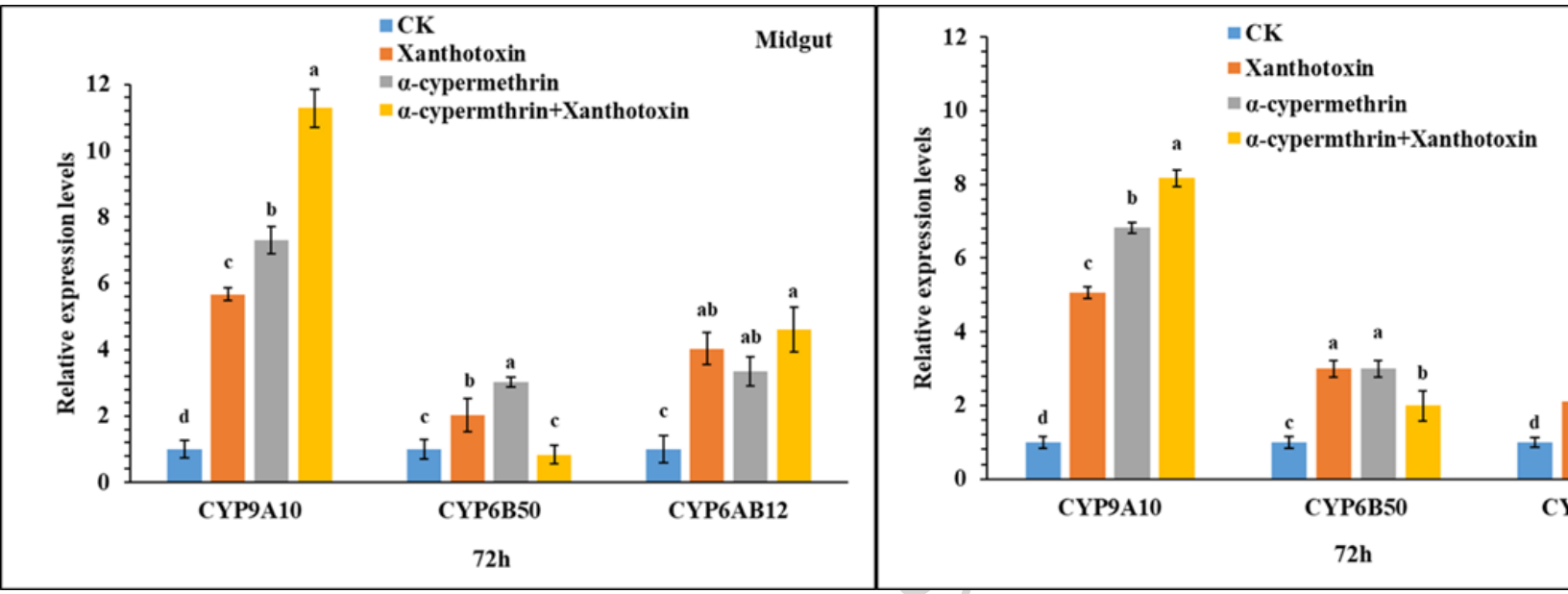

Figure 3. Effect of xanthotoxin on beet armyworm tolerance to deltamethrin and relative expression levels of three P450s genes in midgut (A) and fat body (B) of *Spodoptera exigua*. Late third instar larvae were transferred into new sterilized plastic cups containing artificial diets supplemented with 0.1 % xanthotoxin, LC50 concentration of α-cypermethrin 0.382mg/L for 72 h or 0.1 % xanthotoxin for 24 h followed by α-cypermethrin for 72. Data shown are means ± SE derived from three biological replicates. The transcription levels of three P450s genes determined by quantitative real-time PCR, normalized to three reference genes Different letters above bars indicate significant differences ($p < 0.05$) according to the Tukey HSD test

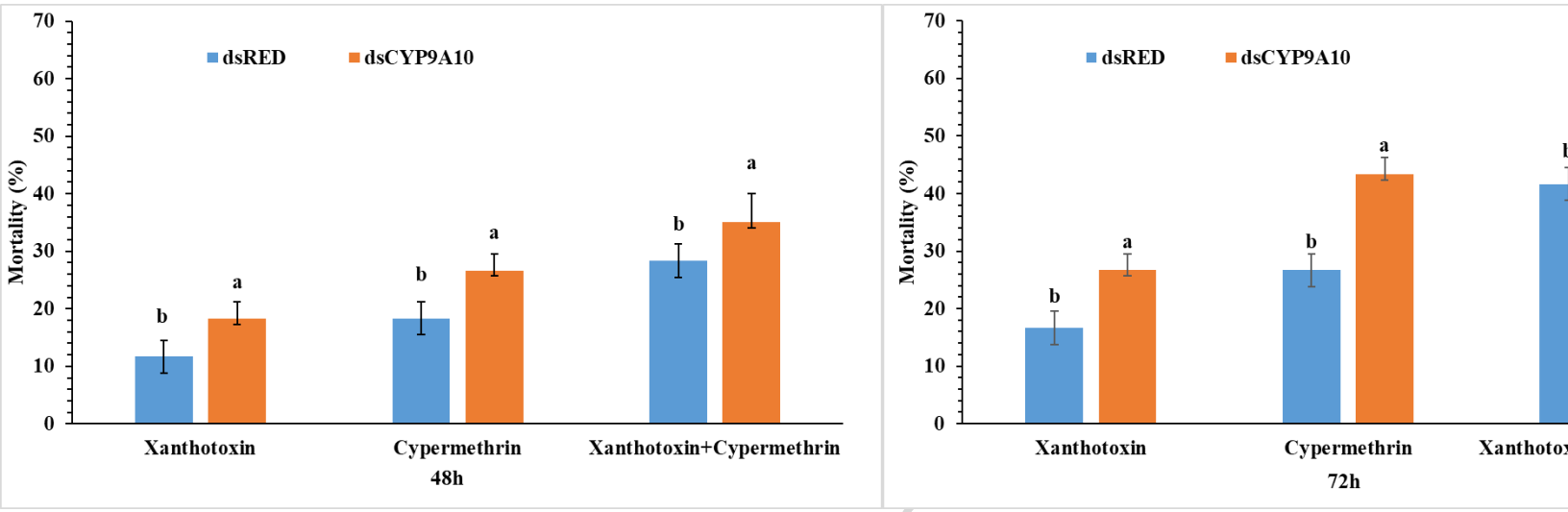

Figure 4. Effects of dsCYPAB14 and dsCYPA98 feeding on the mortality of fourth instar *Spodoptera exigua* larvae. Following the droplet-feeding with dsCYP9A10 or dsRED for 24 h the exposed larvae were transferred individually into 12-oriface tissue culture plate containing artificial diets α-cypermethrin with 0.1 % xanthotoxin, LC50 concentration of α-cypermethrin 0.382mg/L for 48 (A) and 72 h (B) or 0.1 % xanthotoxin for 24 h followed by α-cypermethrin for 48 (A) and 72 (B). Data shown are means ± SE derived from three biological replicates. Different letters above bars indicate significant differences ($p < 0.05$) according to the Tukey HSD test.

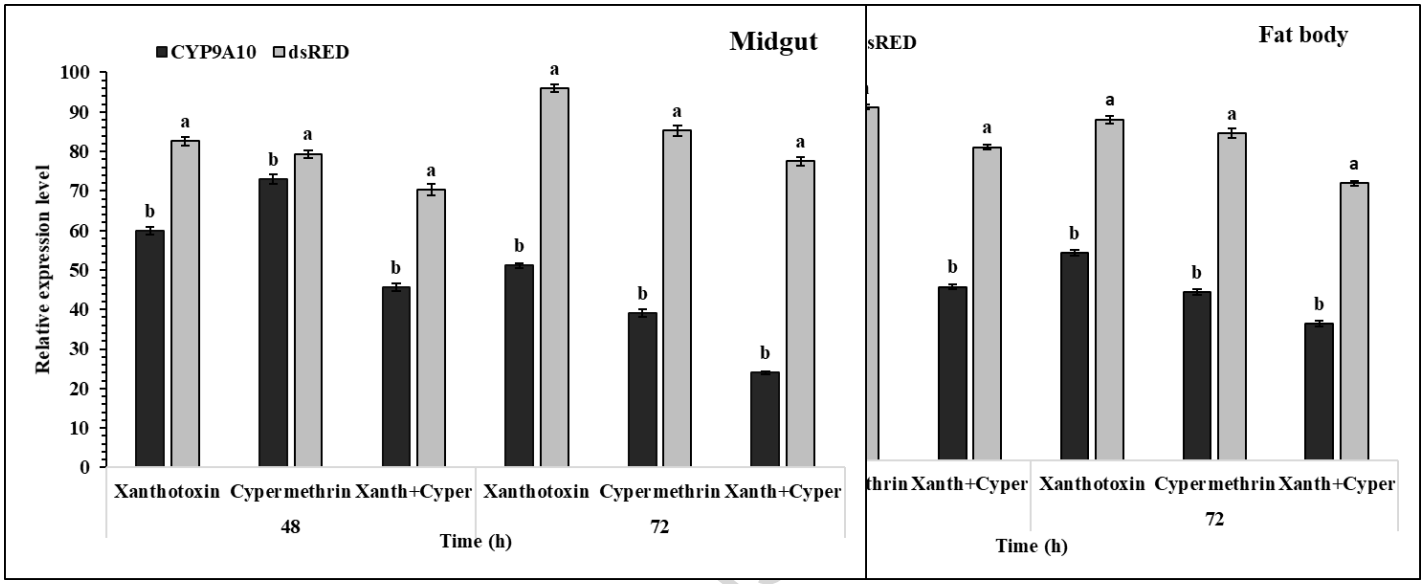

Figure 5. Effect of ds CYP9A10 by droplet feeding on relative transcript levels in midguts (A) and fat bodies (B) after 48 and 72h (A) on the fourth instar *Spodoptera exigua* larvae. Following the droplet-feeding with ds CYP9A10 or dsRED served as a control for 24 h then the exposed larvae were transferred individually into 12-oriface tissue culture plate containing artificial diets supplemented with 0.1 % xanthotoxin, LC_{50} concentration of α-cypermethrin 0.382mg/L for 48 and 72 h or 0.1 % xanthotoxin for 24 h followed by α-cypermethrin for 48 and 72. Data shown are means ± SE derived from three biological replicates. Different letters above bars indicate significant differences ($p < 0.05$) according to the Tukey HSD test.

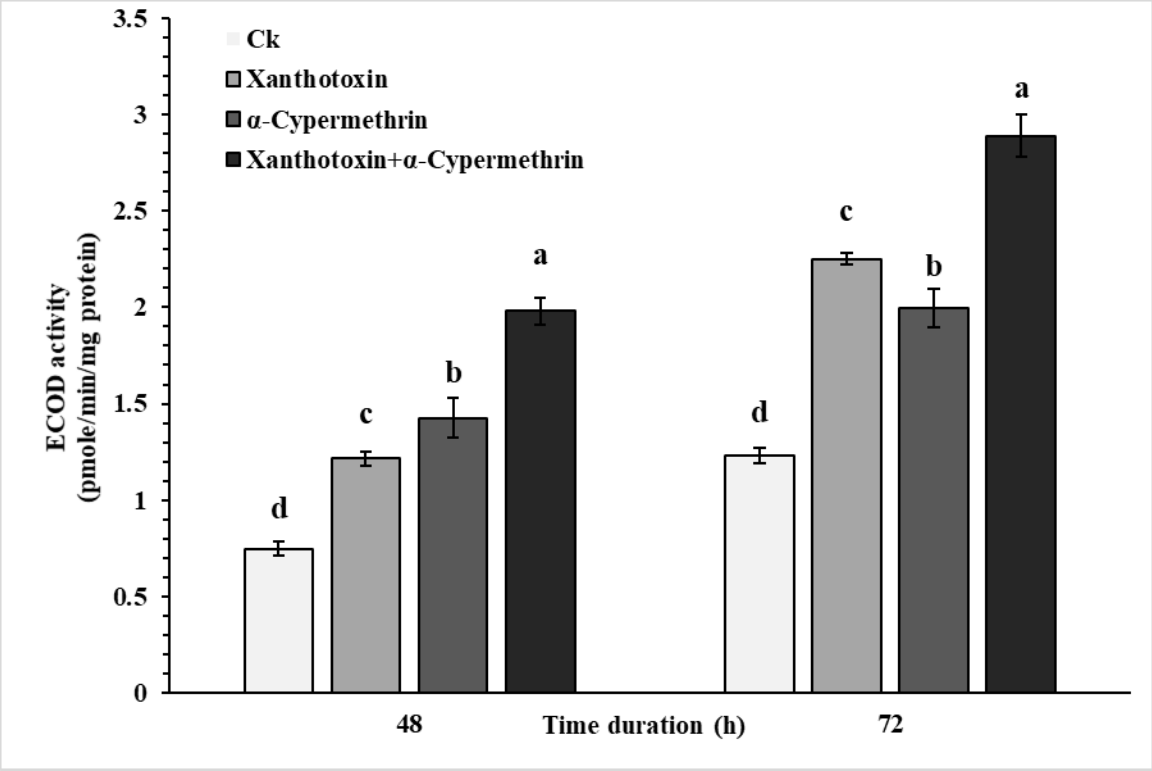

Figure 2. Effects of xanthotoxin on *Spodoptera exigua* tolerance to α-cypermethrin and O-deethylase activity of P450s at different 48 and 72h time durations. The early fourth instar larvae were transferred into new sterilized plastic cups containing artificial diets supplemented with 0.1 % xanthotoxin, LC50 concentration of α-cypermethrin 0.382mg/L for 48 and 72 h or 0.1 % xanthotoxin for 24 h followed by α-cypermethrin for 48 and 72. Data shown are means ± SE derived from three biological replicates. Different letters above bars indicate significant differences ($p < 0.05$) according to the Tukey HSD test.

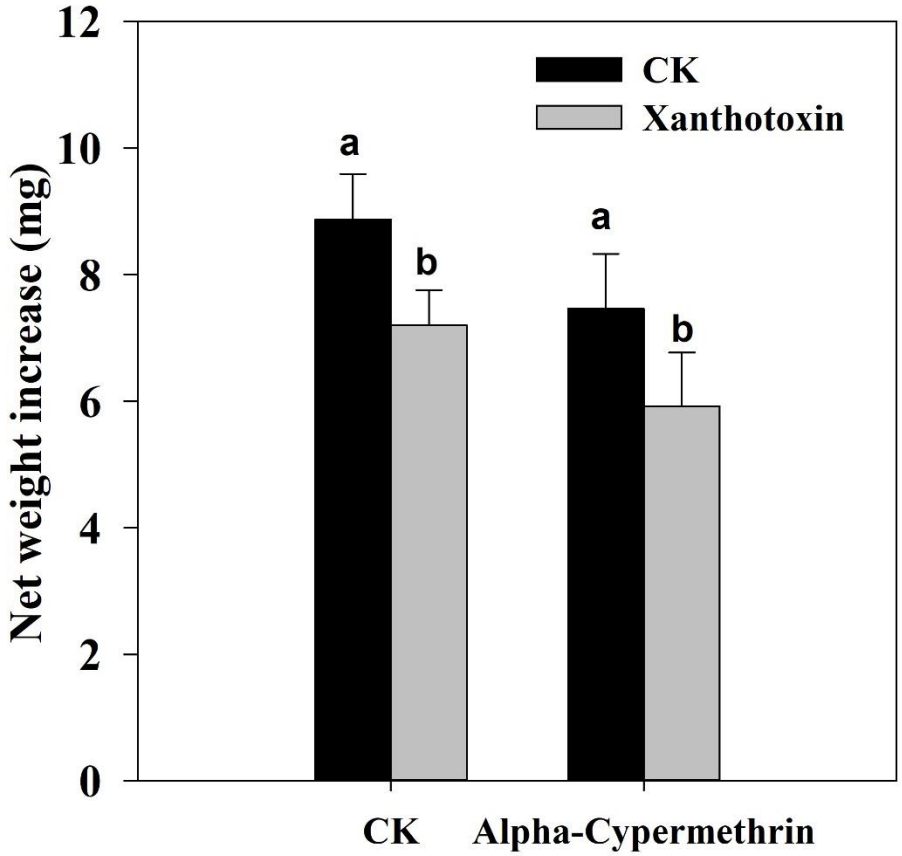

Figure 1. Net weight decreased in xanthotoxin-pretreated larvae on α-cypermethrin-supplemented diet. The early 3rd instar larvae had previously fed on control (CK) or 1.0 mg⁄ g xanthotoxin-supplemented diet for 1 day; after recording the initial weight, two independent groups of each treatments were transferred to 0.382⁄ L α-cypermethrin-supplemented and CK dietary, respectively, weight increases were recorded 2 day later. Error bars represent standard deviation. Different letters above bars indicate significant differences ($p < 0.05$) according to the Student's t-test

Highlights

- Plant secondary metabolites induce insecticide resistance in *S. exigua* and the activity of insect P450 detoxification enzymes
- Xanthotocin induced P450s enzyme activity and related up-regulation of P450s gene contributed to the increase in α-cypermethrin insensitivity in *Spodoptera exigua* larvae.
- This is the first systematic study enlightening the effect of plant secondary metabolite xanthotoxin on α-cypermethrin sensitivity of *S. exigua*.
- Exposure of *S. exigua* larvae to xanthotocin from host plants may compromise the efficacy of α-cypermethrin insecticides.