Insect science

Insect Science (2019) 26, 711–720, DOI 10.1111/1744-7917.12642

ORIGINAL ARTICLE

Exposure to herbicides reduces larval sensitivity to insecticides in *Spodoptera litura* (Lepidoptera: Noctuidae)

Shi-Wei Liu[1,2], Mohammed Esmail Abdalla Elzaki[3], Christian Staehelin[4], Zhi-Hui Ma[1,2], Zhong Qin[1,2] and Rui-Long Wang[1,2]

[1]*Guangdong Engineering Research Center for Modern Eco-agriculture and Circular Agriculture, Guangzhou, China;* [2]*Key Laboratory of Agro-Environment in the Tropics, Ministry of Agriculture, South China Agricultural University, Guangzhou, China;* [3]*College of Crop Science, Fujian Agriculture and Forestry University, Fuzhou, China and* [4]*State Key Laboratory of Biocontrol and Guangdong Key Laboratory of Plant Resources, School of Life Sciences, Sun Yat-sen University, East Campus, Guangzhou, China*

Abstract Herbicides and insecticides are widely used in modern agriculture. It has been reported in various studies that application of insecticides can increase tolerance of herbivorous insects to insecticides. However, limited information exists on susceptibility to insecticides when insects are exposed to herbicides. This study was conducted to investigate the potential impact of the herbicides trifluralin and 2-methyl-4-chlorophenoxyacetic acid sodium salt (MCPA-Na) on the susceptibility of the nocturnal moth *Spodoptera litura* to the insecticides λ-cyhalothrin, phoxim and bifenthrin. We found that larvae exposed to trifluralin or MCPA-Na became significantly less susceptible to both insecticides than non-exposed control larvae. Herbicide-treated larvae did not show altered growth under the used test conditions. However, heads of herbicide-treated larvae showed increased expression of the acetylcholinesterase genes *SlAce1* and *SlAce2*. Moreover, the fat body and midgut of herbicide-treated larvae displayed elevated expression of detoxification genes (the carboxylesterase gene *SlCarE*; the glutathione *S*-transferase genes *SlGSTe2* and *SlGSTe3*; the cytochrome P450 monooxygenase genes *CYP6B48*, *CYP9A40* and *CYP321B1*). The *CYP6B48* gene exhibited highest inducibility. In conclusion, the data of this study suggest that exposure of *S. litura* larvae to herbicides may stimulate detoxification mechanisms that compromise the efficacy of insecticides.

Key words herbicides; insecticides; insecticide detoxification; insecticide susceptibility; *Spodoptera litura*

Introduction

To improve food production, pesticides are widely used by farmers for crop protection. Structurally diverse chemicals have been designed as insecticides and herbicides to kill or suppress pests such as weeds and insects (Le *et al.*, 2010). However, insecticides and herbicides may contaminate different environments through spray drift, volatilization, leaching and drainage (Le *et al.*, 2010). Moreover, an increasing number of insect pests have developed resistance to insecticides in recent decades. Metabolic resistance, the most prevalent resistance mechanism of insects against insecticides, is caused by elevated activities of detoxification enzymes such as cytochrome P450 mono-oxygenases (CYPs, P450s), glutathione *S*-transferases (GSTs) and carboxylesterases (CarEs) (Ranson *et al.*, 2002; Li *et al.*, 2007).

CYPs belong to a superfamily of diverse multifunctional enzymes that are distributed in all living organisms (Feyereisen, 2006). In insects, CYPs are well known

Correspondence: Rui-Long Wang, Key Laboratory of Tropical Agro-Environment, Ministry of Agriculture of China, South China Agricultural University, Guangzhou 510642, China. Tel: +86 20 85280787; fax: +86 20 85280787; email: rlw2009@scau.edu.cn

to metabolize and inactivate important endogenous compounds for growth and development (e.g. ecdysteroids, juvenile hormones, and fatty acids) and xenobiotics (e.g. pesticides and plant toxins) (Scott, 1999; Daborn *et al.*, 2002; Li *et al.*, 2007). Likewise, GSTs in insects are involved in inactivation of endogenous metabolites and detoxification of xenobiotics (Feyereisen, 2006; Deng *et al.*, 2009). CarEs belong to a superfamily of multifunctional and ubiquitous enzymes which play important roles in many metabolic reactions related to cell development such as neurogenesis (Zhang *et al.*, 2015; Salim *et al.*, 2017). It has been reported that insect CarEs could be involved in resistance to many insecticides, such as organophosphates, carbamates and pyrethroids (Zhang *et al.*, 2015; Salim *et al.*, 2017). Acetylcholinesterase (AChE), a key enzyme of the insect nerve system, is an important target of two major insecticide families, organophosphates and carbamates (Salim *et al.*, 2017). λ-Cyhalothrin (pyrethroid), bifenthrin (pyrethroid) and phoxim (organophosphate) are the most frequently used insecticides to control larvae of the tobacco caterpillar, a nocturnal moth (*Spodoptera litura* [Fabricius]; Lepidoptera: Noctuidae) (Ahmad *et al.*, 2007; Tong *et al.*, 2013). *S. litura* is a serious polyphagous insect pest that considerably reduces yields of numerous economically important crops such as tobacco and peanut (Shad *et al.*, 2012). So far, pest management against *S. litura* infestation has become more difficult all over the world because most commonly used insecticides are ineffective in controlling this pest. Various studies reported that *S. litura* developed high resistance to insecticides such as organophosphates and pyrethroids (Ahmad *et al.*, 2007; Ahmad *et al.*, 2008; Shad *et al.*, 2012; Tong *et al.*, 2013).

Previous studies reported that various detoxification genes in *S. litura* are up-regulated when larvae are exposed to different types of insecticides. Examples include induction of GST genes, namely *SlGSTe2* by carbaryl, deltamethrin and tebufenozide and *SlGSTe3* by chlorpyrifos (Deng *et al.*, 2009; Huang *et al.*, 2011). Likewise, expression of a CarE family gene, *SlCarE*, was upregulated by cypermethrin and bifenthrin (Karuppaiah *et al.*, 2017). Moreover, expression of the CYP gene *CYP321B1* in the midgut and fat body of *S. litura* was elevated when larvae were exposed to chlorpyrifos, β-cypermethrin and methomyl. Remarkably, RNA interference mediated silencing of *CYP321B1* in fifth instar larvae increased chlorpyrifos and β-cypermethrin induced mortality (by 25.6% and 38.9%, respectively), suggesting that *CYP321B1* might play an important role in insecticide detoxification (Wang *et al.*, 2017). Expression of *CYP9A40*, another CYP gene of *S. litura*, was significantly increased in the midgut and fat body in response to larval uptake of deltamethrin and methoxyfenozide. Silencing of *CYP9A40* significantly increased the susceptibility of *S. litura* larvae to deltamethrin and methoxyfenozide, suggesting that *CYP9A40* is involved in detoxification of these insecticides (Wang *et al.*, 2015b).

Dinitroaniline herbicides, such as trifluralin, and phenoxyacid herbicides, such as 2-methyl-4-chlorophenoxyacetic acid sodium salt (MCPA-Na), are highly volatile compounds widely used for weed control (Bijanzadeh *et al.*, 2010; Zhang & Liu, 2012). However, effects of these herbicides on susceptibility of *S. litura* to insecticides have not yet been reported. The objectives of this study were to examine the effects of trifluralin and MCPA-Na on the ability of *S. litura* to tolerate insecticides. Data were acquired with respect to mortality and growth of *S. litura* larvae. Furthermore, larvae were used to study effects of trifluralin and MCPA-Na on expression of AChEs genes (*SlAce1* and *SlAce2*) and of genes supposed to be involved in detoxification of xenobiotics (CarE family gene *SlCarE*; GST genes *SlGSTe2* and *SlGSTe3*; CYP gene genes *CYP6B48*, *CYP9A40* and *CYP321B1*). *CYP6B48* was included into this study, because expression levels in the midgut and fat body were strongly induced in larvae exposed to plant allelochemicals such as flavone and xanthotoxin (Wang *et al.*, 2015a).

Materials and methods

Insects

An insecticide-susceptible laboratory strain of *S. litura* was obtained from the Institute of Entomology, Sun Yat-sen University, Guangzhou, China. Insects were reared on an artificial diet (Chen *et al.*, 2000) under standard conditions (16 : 8 h light : dark, 25 ± 2 °C and 75% ± 5% relative humidity) at the South China Agricultural University, Guangzhou, China without exposure to any insecticide. Fourth instar larvae showing active dietary intake and rapid weight increase were used for all experiments.

Chemicals and reagents

Commercially available formulations of trifluralin (480 g/L) and MCPA-Na (56%) were purchased from Shandong Vicome Greenland Chemical Co., Ltd. Jinan, China. λ-Cyhalothrin (95%) was bought from Jiangsu Changlong Agrochemical Co., Ltd., Taixing, China). Phoxim (99%) was obtained from Shanghai Jiang Lai Biotechnology Co., Ltd., Shanghai, China. Bifenthrin (97.2%)

was purchased from Hebei Weiyuan Biochemical Pesticide Co., Ltd., Shijiazhuang, China. Acetone (≥99.5%) was bought from Guangzhou Chemical Reagent Factory, Guangzhou, China.

Analysis of larval growth and development after herbicide exposure

Trifluralin or MCPA-Na (0.3 μL) was dropped onto a small piece of cotton wool, which was placed into a pipette tip and then attached to the cover of a 42 mL plastic vessel supplemented with artificial diet (Supplemental Fig. 1). The herbicide dose used in this study was chosen based on a pre-experiment in which effects of different herbicide amounts on growth of *S. litura* were examined. Newly molted 4th instar larvae were placed individually inside the plastic vessels. The larvae in the closed vessels were exposed to the volatile trifluralin or MCPA-Na formulations for 24 h. Larvae without herbicide treatment served as controls. The changes in larval biomass were measured 72 h after the herbicide treatment. The pupation rate and pupal weight (determined 48 h after successful pupation) were also determined. The mortality of larvae was recorded daily until pupation. The experiment was performed three times with 30 larvae for each replicate.

Effects of herbicides on insecticide susceptibility of larvae

Four hundred and fifty 4th instar larvae of *S. litura* were exposed to trifluralin or MCPA-Na for 24 h as described above and divided randomly into five groups (90 larvae per group) and each group was further divided into three subgroups (30 larvae per subgroup). The herbicide-exposed larvae and control larvae (without herbicide) were transferred to plastic vessels with fresh artificial diet (Chen *et al.*, 2000). To examine insecticide toxicity, insecticides were dissolved in acetone and five serially diluted concentrations were prepared in acetone (analytical reagent grade, ≥99.5%). Larvae pre-exposed to herbicides and control larvae (without herbicide treatment) were individually treated with the insecticides. A droplet (1 μL) of the insecticide solution was applied onto the pronotum of the prothorax (0.00125, 0.0025, 0.005, 0.01 and 0.02 μg λ-cyhalothrin per larva; 0.00625, 0.0125, 0.025, 0.05 and 0.1 μg bifenthrin per larva; 0.01875, 0.0375, 0.075, 0.15 and 0.3 μg phoxim per larva). The treated larvae were maintained under the rearing conditions as specified above. The mortality was assessed after 48 h. LD_{50} values (lethal dose, 50% mortality) were calculated via probit analysis using POLO-PC software (LeOra Software Inc., Berkeley, CA, USA). Ratios of LD_{50} values were calculated as follows: Ratio = LD_{50} (insecticide + herbicides)/LD_{50} (insecticide) (Torres-Vila *et al.*, 2002; Shad *et al.*, 2012). All assays were done with three replicates. In addition, insecticide susceptibility of *S. litura* larvae was evaluated with a leaf disc method (Ahmad *et al.*, 2007; Ahmad *et al.*, 2008) using leaves of Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*) immersed in different insecticide concentrations (λ-cyhalothrin: 1, 2, 4, 8 and 16 mg/L; bifenthrin: 1, 3, 9, 27 and 81 mg/L; phoxim: 2, 6, 18, 54 and 162 mg/L). Mortality was recorded 48 h later. LC_{50} values and LC_{50} (insecticide + herbicides)/LC_{50} (insecticide) ratios were determined from obtained mortality data (Torres-Vila *et al.*, 2002; Shad *et al.*, 2012).

Preparation of tissue used for gene expression analysis

Newly molted 4th instar larvae of *S. litura* (30 individuals per treatment) were exposed to trifluralin or MCPA-Na for 12 h, 24 h and 36 h as mentioned above. To measure gene expression levels in different tissues, heads, midguts and fat bodies were sampled from the larvae.

RNA extraction and cDNA synthesis

Total RNA was extracted from heads, midguts and fat bodies of *S. litura* larvae using TRIzol based on the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). RNA quantification was performed using a NanoDrop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). After DNase I (Promega, Madison, WI, USA) treatment, cDNA synthesis was carried out using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Gene expression analysis

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to determine relative expression levels of *SlAce1* or *SlAce2* in the head and *SlCarE*, *SlGSTe2*, *SlGSTe3*, *CYP6B48*, *CYP9A40* and *CYP321B1* in the midgut and fat body of *S. litura* larvae. GenBank accession numbers of these genes and used gene-specific primers are shown in Table 1. The qRT-PCR tests were performed with SYBR Green I Master Mix (Roche Diagnostics Corp., Indianapolis, IN, USA) using a MJ Research Opticon Instrument (Bio-Rad, Inc., Hercules, CA, USA). The PCR mixture (20 μL) contained 10 μL of 2 × SYBR Green I (Roche), 5 μL of the cDNA template,

Table 1 Primers used for quantitative reverse transcription polymerase chain reaction (PCR).

Gene name	Forward primer (5′–3′)	Reverse primer (5′–3′)	Length of PCR products (bp)	GenBank no.
SlCarE	CACCACTACCTCCAAAACCAT	TCACCAACTCTCCCGACATTC	103	EU783914
SlAce1	TTATGTATTCGGGGAGCC	CGATTCTCCATTCGGGTT	136	KY130418
SlAce2	ATGGCGATGAGATGGAGT	GCTTATGAGGCTTACCAGT	135	KY130419
SlGSTe2	GCTGACCACCTTACTATTGC	TTCTGAACTGAAGGGTGC	140	GQ131803
SlGSTe3	AGCACCTACGCAGAAGCA	ACAGGTCGGCGATGGTGA	113	GQ131804
CYP6B48	CATCGGAATGAGGTTTGG	AACGAATCCCTCCTTTGG	149	GU263827
CYP9A40	AGAGGGTATGAGACTATGGC	CTGCCCCTTGCGAATGAT	118	KR065418
CYP321B1	AGAAATACGGCGGCAAGC	CGACAGGCAGAACGGAGT	141	GU263829
β-actin	CCACGAGACCACTTACAAC	GCCAGAGCAGTGATTTCC	141	KP331524

0.4 μL of each primer and 4.2 μL sterilized water. The PCR cycling conditions were as follows: 94 °C for 3 min, followed by 45 cycles of 94 °C for 10 s and 60 °C for 20 s. To assess the specificity of the PCR amplifications, a melt curve analysis was performed at the end of each reaction by increasing the incubation temperature from 60 °C to 95 °C in 0.5 °C increments with a 10-s dwell time at each temperature level during which the fluorescence data were collected. The β-actin gene was chosen as internal standard (Gu *et al.*, 2015). Gene expression levels were analyzed according to the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001). Three independent RNA extractions were performed for all treatments (three biological replicates per treatment).

Statistical analysis

Statistical analysis was performed using the SPSS 13.0 software package (SPSS, Inc., Chicago, IL, USA). The LD_{50} values were considered as significantly different when the 95% confidence intervals did not overlap (Abro *et al.*, 2013). One-way analysis of variance followed by Duncan's multiple range test and the least significant difference (LSD) test were used to analyze effects caused by herbicide and insecticide treatments. Data were acquired for the following variables: relative gene expression level, larval weight, pupation rate, pupal weight and mortality. Statistical differences were considered significant at $P < 0.05$.

Results

Effects of herbicides on growth and development of S. litura

To examine a possible effect of trifluralin and MCPA-Na on larval growth, larval weight gain, pupation rate, pupal weight and larval mortality data were acquired. Compared to controls, 4th instar larvae exposed to trifluralin or MCPA-Na did not show an altered biomass (weight gain) (Fig. 1A). Similarly, exposure of larvae to trifluralin or MCPA-Na had no significant effect on pupation rate (Fig. 1B), pupal weight (Fig. 1C) and larval mortality (Fig. 1D). These results indicate that both herbicides did not influence the fitness of *S. litura* under the used test conditions.

Effects of herbicides on insecticide susceptibility of larvae

Toxicity bioassays with 4th instar larvae were conducted to evaluate the effects of herbicides on insecticide susceptibility. Survival of larvae pre-exposed to trifluralin or MCPA-Na for 24 h were compared to control larvae that were not exposed to herbicides. The insecticides λ-cyhalothrin, bifenthrin and phoxim were applied onto the pronotum of the prothorax. Susceptibility to the insecticides was then determined. As shown in Table 2, the LD_{50} values for λ-cyhalothrin, bifenthrin and phoxim treatments were 0.007, 0.032 and 0.076 μg per control larva, respectively. However, when *S. litura* larvae were pre-exposed to trifluralin, the LD_{50} values for λ-cyhalothrin, bifenthrin and phoxim were increased to 0.013, 0.058 and 0.146 μg per larva, respectively. These differences in LD_{50} values correspond to a nearly 2 fold increase. Larvae pre-exposed to MCPA-Na showed similar changes in LD_{50} values. The determined LD_{50} values for λ-cyhalothrin, bifenthrin and phoxim increased to 0.014, 0.056 and 0.139 μg per larva, respectively. These data indicate 2.075, 1.752 and 1.824 fold differences, respectively. Similar results were also obtained when insecticide susceptibility was determined with a leaf disk method (Supplemental Table 1). In conclusion, exposure to the tested herbicides significantly reduced the insecticide susceptibility of *S. litura* larvae.

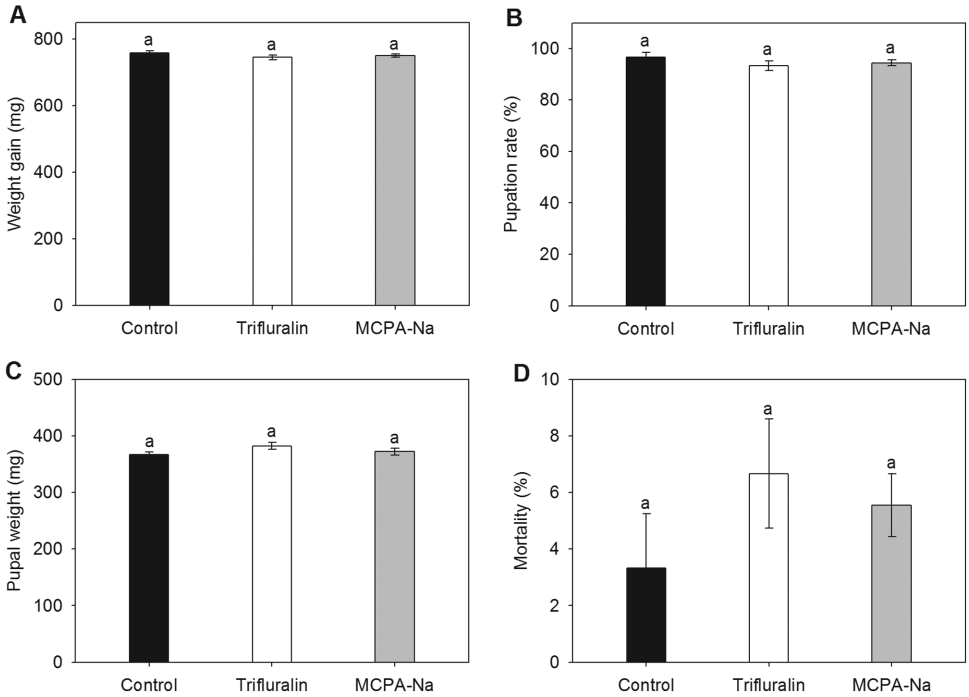

Fig. 1 Analysis of 4th instar larvae exposed to trifluralin or 2-methyl-4-chlorophenoxyacetic acid sodium salt (MCPA-Na) for 24 h. Control larvae were not treated with herbicides. Changes in larval biomass (weight gain) were measured 72 h after the herbicide treatment (A). In addition, pupation rate (B), pupal weight (C) and larval mortality (recorded daily) (D) were determined. Data shown are means ± SE. No significant differences were found between the test groups (Duncan's multiple range test; $P < 0.05$).

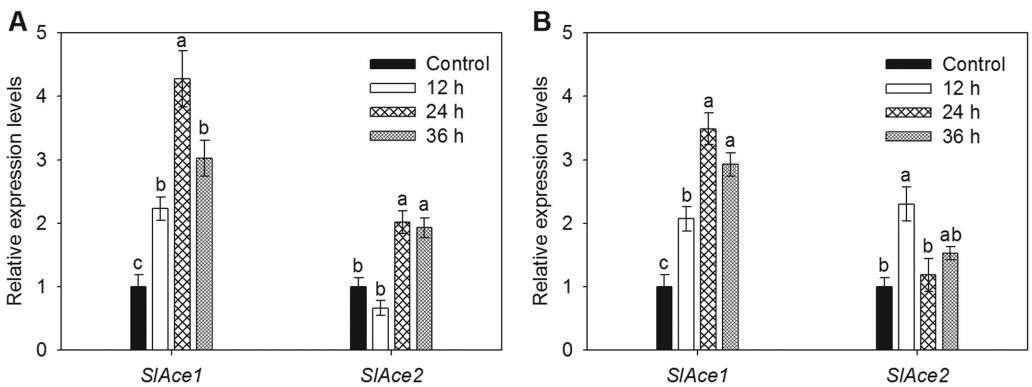

Fig. 2 Effects of trifluralin (A) and 2-methyl-4-chlorophenoxyacetic acid sodium salt (MCPA-Na) (B) on expression of acetylcholinesterase (AChE) genes in the head of 4th instar larvae. The larvae were exposed to the herbicides for 12, 24 and 36 h and compared to control larvae without herbicide treatment. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis was performed for the AChE genes *SlAce1* and *SlAce2*. Data shown are means ± SE (three RNA extractions; $n = 3$). Different letters above bars indicate significant differences (Duncan's multiple range test; $P < 0.05$).

Stimulation of AChE gene expression in response to herbicide exposure

λ-Cyhalothrin, bifenthrin and phoxim are neurotoxic insecticides. We therefore suggested that the effects of the used herbicides on insecticide susceptibility is related to physiological changes in the insect's nervous system and determined expression levels of two AChE genes in the head of 4th instar larvae. As determined by qRT-PCR, exposure of larvae to trifluralin for 12, 24 and 36 h increased the expression levels of *SlAce1* in the head by 2.2, 4.3 and 3.0 fold, respectively (Fig. 2A). The expression

Table 2 Toxicity of λ-cyhalothrin, bifenthrin and phoxim to *Spodoptera litura* larvae pre-exposed to trifluralin or 2-methyl-4-chlorophenoxyacetic acid sodium salt (MCPA-Na) for 24 h.

Treatment[†]	N[‡]	Slope (± SE)	χ^2	*r*	df	LD_{50} (μg/larva) (95% CL)	Ratio[§]
λ-cyhalothrin	450	1.698 (± 0.040)	4.142	0.971	4	0.007 (0.006–0.008)	
Trifluralin + λ-cyhalothrin	450	1.138 (± 0.073)	1.583	0.973	4	0.013 (0.009–0.018)	1.857
MCPA-Na + λ-cyhalothrin	450	1.250 (± 0.072)	1.373	0.981	4	0.014 (0.010–0.020)	2.075
Bifenthrin	450	1.469 (± 0.045)	1.859	0.982	4	0.032 (0.026–0.039)	
Trifluralin + bifenthrin	450	1.246 (± 0.065)	1.241	0.981	4	0.058 (0.043–0.077)	1.807
MCPA-Na + bifenthrin	450	1.221 (± 0.064)	0.672	0.989	4	0.056 (0.042–0.075)	1.752
Phoxim	450	1.744 (± 0.038)	2.520	0.981	4	0.076 (0.064–0.091)	
Trifluralin + phoxim	450	1.442 (± 0.052)	1.422	0.985	4	0.146 (0.116–0.185)	1.918
MCPA-Na + phoxim	450	1.356 (± 0.054)	1.131	0.986	4	0.139 (0.109–0.177)	1.824

[†]Fourth instar larvae were pre-exposed to trifluralin or MCPA-Na for 24 h as described in Materials and methods. Insecticides (1 μL) were applied onto the pronotum of the prothorax. Mortality was recorded 48 h later. No mortality was observed for control larvae that were not exposed to insecticides.

[‡]Number of larvae tested. The assay was performed in triplicate. Larvae were randomly divided into five groups (90 larvae per group) and each group was further divided into three subgroups (30 larvae per subgroup).

df, degree of freedom; CL, confidence limit; LD50, lethal dosage at 50% mortality. LD_{50} values without overlapping confidence intervals are considered as statistically different.

[§]Ratio = LD_{50} (insecticide + herbicide)/LD_{50} (insecticide).

level of *SlAce2* was about 2 fold increased when larvae were exposed to trifluralin for 24 and 36 h (Fig. 2A). Likewise, larvae exposed to MCPA-Na showed significantly increased expression levels of *SlAce1* at all three time points (2.1 fold at 12 h, 3.5 fold at 24 h and 2.9 fold at 36 h). Furthermore, *SlAce2* was significantly (2.3 fold) increased at 24 h (Fig. 2B). These findings indicate that the herbicide treatments had significant stimulatory effects on expression of AChE genes in the head of *S. litura.*

Increased expression of detoxification genes in response to herbicide exposure

As herbicides may induce expression of detoxification genes in *S. litura*, we examined whether herbicide treatments influence expression of a CarE family gene (*SlCarE*), two GST genes (*SlGSTe2* and *SlGSTe3*) and three CYP genes (*CYP6B48*, *CYP9A40* and *CYP321B1*). Fourth instar larvae of *S. litura* were exposed to trifluralin or MCPA-Na for 12, 24 and 36 h and qRT-PCR analysis was performed with RNA from the fat body and midgut. Figure 4 shows the gene expression results for the fat body. *SlCarE* expression was significantly increased when the larvae were exposed to trifluralin (2.7 fold at 24 h; 1.8 fold at 36 h) (Fig. 3A). The MCPA-Na treatment had a similar effect on expression of *SlCarE* (2.6 fold increase at 36 h) (Fig. 4B). Larvae exposed to trifluralin for 24 h showed a 3.8 fold elevated expression for *SlGSTe2* and a 4.7 fold increase for *SlGSTe3* (Fig. 3A). Remarkably, expression of *CYP6B48* in the fat body of trifluralin-exposed larvae was dramatically enhanced (53.3 fold at 24 h; 83.9 fold 36 h) (Fig. 3A). A strong induction of *CYP6B48* expression was also observed for larvae exposed to MCPA-Na (Fig. 3B). Expression of the *CYP321B1* gene at 36 h was significantly increased in response to both herbicides (8.1 fold for trifluralin; 8.9 fold for MCPA-Na). In contrast, expression of *CYP9A40* was slightly reduced when the larvae were exposed to the herbicides for 24 h or 36 h (Fig. 3).

A similar gene expression pattern was also obtained when RNA was isolated from the midgut (Fig. 4). The trifluralin treatment promoted expression of detoxification genes, namely *SlCarE* (at 36 h), *SlGSTe2* (at 36 h), *SlGSTe3* (at 24 h), *CYP6B48* (at 24 and 36 h), and *CYP321B1* (at 36 h). Strongest responsiveness to trifluralin was observed for *CYP6B48* (13.1 fold induction at 24 h; 13.8 fold induction at 36 h) (Fig. 4A). Likewise, exposure of larvae to MCPA-Na positively influenced expression of detoxification genes in the midgut (*SlCarE*, *CYP6B48* at 24 h; *SlCarE*, *SlGSTe3*, *CYP6B48* at 36 h). Notably, expression of *CYP6B48* was highest at 24 h (24.5 fold induction in response to the MCPA-Na treatment) and later decreased (8.2 fold induction at 36 h) (Fig. 4B). Taking these data together, they show that the trifluralin and MCPA-Na treatments significantly induced expression of detoxification genes in *S. litura* larvae.

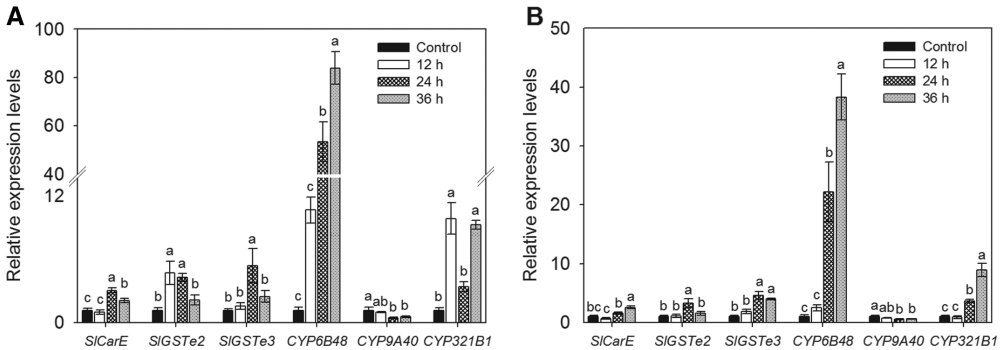

Fig. 3 Effects of trifluralin (A) and 2-methyl-4-chlorophenoxyacetic acid sodium salt (MCPA-Na) (B) on expression of detoxification genes in the fat body of 4th instar larvae. The larvae were exposed to the herbicides for 12, 24 and 36 h. Larvae without herbicide treatment served as a control. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis was performed for a carboxylesterase (CarE) family gene (*SlCarE*), two glutathione *S*-transferase (GST) genes (*SlGSTe2* and *SlGSTe3*) and three cytochrome P450 mono-oxygenase (CYP) genes (*CYP6B48*, *CYP9A40* and *CYP321B1*). Data shown are means ± SE (three RNA extractions; $n = 3$). Different letters above bars indicate significant differences (Duncan's multiple range test; $P < 0.05$).

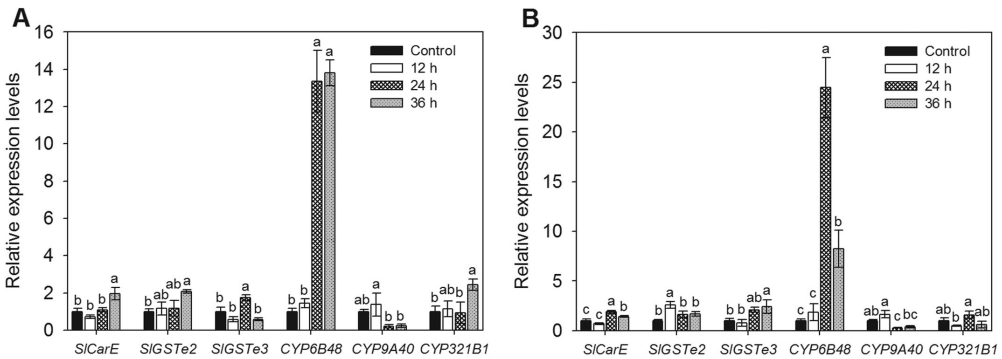

Fig. 4 Effect of trifluralin (A) and 2-methyl-4-chlorophenoxyacetic acid sodium salt (MCPA-Na) (B) on expression of detoxification genes in the midgut of 4th instar larvae. Larvae were exposed to the herbicides for 12, 24 and 36 h. Control larvae without herbicide treatment were analyzed for comparison. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was achieved for indicated detoxification genes. Data shown are means ± SE (three RNA extractions; $n = 3$). Different letters above bars indicate significant differences (Duncan's multiple range test; $P < 0.05$).

Discussion

Expression of detoxification enzymes in insects is induced by various xenobiotic molecules such as plant allelochemicals (including volatiles), mycotoxins and insecticides (Li *et al.*, 2002, Ranson *et al.*, 2002; Li *et al.*, 2007; Deng *et al.*, 2009; Von Mérey *et al.*, 2013; Zeng *et al.*, 2013; Guo *et al.*, 2015; Liang *et al.*, 2015; Zhang *et al.*, 2015; Wang *et al.*, 2017). Insecticides are currently indispensable for *S. litura* control of almost all attacked crops. However, *S. litura* can develop resistance to a wide range of insecticides (Ahmad *et al.*, 2007; Ahmad *et al.*, 2008; Shad *et al.*, 2012; Tong *et al.*, 2013). As expression of detoxification genes can be induced by different xenobiotics, we hypothesized that herbicides influence the ability of *S. litura* to tolerate insecticides. In this study, we tested whether exposure of larvae to herbicides influences the insecticide susceptibility of *S. litura* larvae. Because trifluralin and MCPA-Na are volatile herbicides, we used an experimental test system that simulates herbicide exposure under field conditions (Supplemental Fig. 1). We found that herbicide-exposed larvae were indeed less susceptible to λ-cyhalothrin, bifenthrin and phoxim (Table 2). Herbicide-exposed larvae did not show altered growth and no obvious developmental defects were observed under the used test conditions (Fig. 1). However, the herbicides influenced expression levels of AChE genes in the head (Fig. 2) and various detoxification genes in the fat body (Fig. 3) and midgut (Fig. 4). Hence, herbicides appear to cause physiological changes that up-regulate expression of various genes, including those involved in insecticide susceptibility.

Susceptibility to insecticides varies in different insect populations that evolutionarily adapt to a changing environment (Ranson *et al.*, 2002; Shad *et al.*, 2012). Phytophagous insect populations may differ in their susceptibility to insecticides. For example, populations of the silverleaf whitefly (*Bemisia tabaci*) feeding on different host plants showed obvious differences in their susceptibility to acetamiprid (Xie *et al.*, 2011). In our study, *S. litura* larvae from a genetically homogenous population were experimentally exposed to herbicides and subsequently to insecticides. The observed differences in LD_{50} values (Table 2) indicate that the used herbicides represent an environmental factor that influences insecticide susceptibility. However, exposure to herbicides did not affect weight gain, pupation rate and pupal weight of *S. litura* in our study (Fig. 1). These data suggest that the costs of *S. litura* to induce detoxification genes in response to herbicide treatments are rather low under the used test conditions with sufficient food supply. The use of higher herbicide doses may eventually reduce the fitness of *S. litura*, particularly when larvae are raised under sub-optimal conditions. Our findings also support the view that *S. litura* and other nocturnal moths possess potent detoxification systems. Expression of corresponding genes may be induced by a large number of different molecules that may represent either substrates or signals. In the nocturnal moth *Helicoverpa zea* for example, expression of various CYP genes was activated by jasmonate or salicylate. These defense-related plant hormones appear to act as signals that can activate the insect's detoxification systems (Li *et al.*, 2002).

In the present study with *S. litura* larvae, the impact of trifluralin and MCPA-Na on expression of various genes was analyzed. We found that exposure of larvae to herbicides could significantly induce expression of *SlAce1* and *SlAce2* in the head (Fig. 2) as well as *SlCarE*, *SlGSTe2*, *SlGSTe3*, *CYP6B48* and *CYP321B1* in the fat body (Fig. 3) and midgut (Fig. 4), respectively. Detoxification genes have been reported to be induced by insecticides in several studies. For examples, *CYP4G7* and *CYP345A1* were significantly induced by permethrin, λ-cyhalothrin and cypermethrin in the red flour beetle (*Tribolium castaneum*) while *CYP345A1* and *CYP4BR3* were significantly induced by imidacloprid in this species (Liang *et al.*, 2015). *CYP9AQ2* was induced by deltamethrin in the migratory locust (*Locusta migratoria*) (Guo *et al.*, 2015). Plant allelochemicals and volatile components could activate detoxifying enzymes in larvae of the nocturnal moths *Trichoplusia ni* and *Helicoverpa armigera* (Wu *et al.*, 2013; Zeng *et al.*, 2013). Catalase, superoxide dismutase, GST, and CarE activities were higher in the fly *Bradysia odoriphaga* (Chinese chive root maggot) reared on garlic and humus than on other diets. Remarkably, *B. odoriphaga* larvae reared on garlic and humus were less susceptible to phoxim and clothianidin (Zhu *et al.*, 2017). Likewise, reduced λ-cyhalothrin susceptibility was observed for quercetin-fed *H. armigera* larvae, suggesting that quercetin (a plant flavonol) may compromise the efficacy of pyrethroid insecticides (Chen *et al.*, 2018). Furthermore, CarE activity in silverleaf whitefly (*B. tabaci*) adults from subpopulations reared on cabbage and cucumber were significantly increased as compared to other host plants. GST activity was highest for a subpopulation reared on tomato whereas CYP activity of the *B. tabaci* cucumber population was significantly decreased (Xie *et al.*, 2011). CYP enzymes seem to be particularly important detoxification enzymes for *S. litura* (Wang *et al.*, 2015c, 2017). The strong induction of *CYP6B48* expression in herbicide-exposed larvae (Figs. 3 and 4) suggests a possible role of this gene in herbicide degradation. Previous work showed that expression of *CYP6B48* was induced when larvae were fed with diet containing specific allelochemicals such as flavone and xanthotoxin (Wang *et al.*, 2015a). Hence, CYP6B48 might represent a CYP enzyme that detoxifies a broad range of different compounds, including allelochemicals, herbicides and insecticides. It would be interesting to analyze CYP6B48 and other CYP enzymes for their capacity to detoxify different xenobiotics.

In conclusion, our results indicate that *S. litura* larvae exposed to the herbicides trifluralin or MCPA-Na became significantly less susceptible to the insecticides λ-cyhalothrin, bifenthrin and phoxim. We observed remarkable changes in gene expression, namely AChE genes in the head and detoxification genes such as *CYP6B48* in the fat body and midgut. We suggest that transcriptome changes induced by herbicide treatments, particularly induction of genes required for detoxification of insecticides, compromise the efficacy of insecticides. We expect that herbicide treatments stimulate expression and activities of enzymes that are able to detoxify both herbicides and insecticides. Future studies are required to investigate the relationship between herbicide exposure and insecticide susceptibility on a molecular level and under field conditions. The wide use of herbicides in agriculture could have a hitherto neglected impact on the efficiency of insecticides. Reduced insecticide susceptibility caused by herbicides may favor evolutionary processes that ultimately result in complete insecticide resistance.

Acknowledgments

This research was financially supported by the National Natural Science Foundation of China (No. 31470576) and

the Natural Science Foundation of Guangdong Province, China (No. 2017A030313188).

Disclosure

The authors declare no conflict of interest exists.

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Manuscript received July 8, 2018
Final version received August 30, 2018
Accepted August 30, 2018

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplemental Figure 1: Exposure of *Spodoptera litura* larvae to herbicides.

Supplemental Table 1: Toxicity of λ-cyhalothrin, bifenthrin and phoxim to *Spodoptera litura* larvae pre-exposed to trifluralin or 2-methyl-4-chlorophenoxyacetic acid sodium salt (MCPA-Na) for 24 h as determined by a leaf disk method.