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Lead exposure improves the tolerance of *Spodoptera litura* (Lepidoptera: Noctuidae) to cypermethrin

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ABSTRACT

Many ecological factors such as heavy metals can affect the tolerance of herbivorous insects to chemical insecticide. *Spodoptera litura* larvae exposed to lead (Pb) (0–100 mg kg^{-1} in artificial diet) did not inhibit their growth. After 96 h of Pb (0–100 mg kg^{-1}) exposure, topical application and feeding of cypermethrin to *S. litura* decreased their mortality and increased weight gain. Moreover, the mortality of *S. litura* treated with 25 and 50 mg kg^{-1} of Pb for five generations was significantly lower than control. In addition, Pb accumulation was detected in midgut, fat body, brain and hemolymph, and its highest level was in the midgut. Furthermore, there was a significant negative correlation between Pb accumulation in fat body and mortality after topical application of cypermethrin. After 96 h of Pb exposure, there was increase expression of detoxification enzymes (*CYP9A39* and *CYP6B47*) in midgut and fat body of *S. litura*. Therefore, the tolerance of *S. litura* to cypermethrin is increased by Pb exposure at certain concentrations through Pb accumulation in body and the increase of *CYP9A39* and *CYP6B47* expression.

1. Introduction

The rapid development of industrialization and urbanization has generated heavy metal contamination, and has become a global problem. In particular, the toxic heavy metal Pb is found ubiquitous in the ecosystems (De Vleeschouwer et al., 2007). Although some sources of Pb contamination (from gasoline to paint) have been decreased worldwide, its concentration in the environment is increasing in many countries (Adriano, 2001). In the economically developed areas of China such as the Pearl River Delta, Pb concentration in contaminated soil can reach 11185 mg kg^{-1} (Deng et al., 2006) and is readily absorbed by plants. In a farmers' market of Magu townships of the zinc smelting region, northwestern Guizhou, China, the average concentrations of Pb in leafy vegetables are 16 ± 16 mg kg^{-1} (Yang et al., 2011). Undoubtedly, Pb can eventually reach to higher trophic levels such as the arthropods including herbivorous insects.

Accumulating in the insect body, Pb at different concentrations can result the difference in response of in *Drosophila melanogaster* (Hirsch et al., 2010). When exposed to high levels of Pb, their lifespans shorten (Massie et al., 1992), along with a decrease in mating frequency and fecundity (Uysal and Bahçeci, 1996). However, exposure to low Pb concentrations led to increase mating frequency and fecundity (Hirsch et al., 2003). In *Spodoptera litura*, we found that high Pb levels significantly inhibited the expression of vitellogenin in female adults, whereas low Pb levels in male induced vitellogenin in male adults (Shu et al., 2009). These studies show that Pb can affect the development and physiology of insects.

Synthetic pyrethroid insecticides have been used for more than 30 years to control insect pests in various crops (Elliott et al., 1973). Pyrethroids display high affinity to Na^+-channels and perform their toxic effects through affects the functions of these channels (Sogorb and Vilanova, 2002). Because of their extremely effective and relatively inexpensive, cypermethrin and other synthetic pyrethroids are widely applied in control of *S. litura* and resulted in the rapid development of resistance (Armes et al., 1997; Ahmad et al., 2007, 2009; Diao et al., 2011).

The induction of cytochrome P450 may enhance the detoxification of exogenous compounds and synthetic organic insecticides such as pyrethroids (Feyereisen, 1999). Many studies have showed that the induction of microsomal P450-dependent monooxygenases is a primary mechanism of insect resistance to pyrethroid (Dittrich et al., 1990; Hemingway and Ranson, 2000; Awolola et al., 2009; Zhou et al., 2010). In China, pyrethroid resistance in *Helicoverpa armigera* was proved to be caused mainly by the enhancement of P450s activity (Yang et al., 2004). In *S. litura*, synergism experiment with detoxification enzyme inhibitors revealed that pyrethroid resistance was associated with P450s (Armes et al., 1997; Huang and Han, 2007).

Previous studies showed that there are 36–180 P450s in insects (Feyereisen, 2011). In Lepidoptera, CYP6Bs have been implicated in detoxifying a variety of insecticides [cypermethrin (pyrethroid), diazinon (organophosphate), and aldrin (cyclodiene)] (Li et al., 2004;

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Rupasinghe et al., 2007). In field-collected *Helicoverpa zea*, transcriptional overexpression of *CYP6B8/CYP6B28* and *CYP6B9* is a mechanism associated with cypermethrin survivorship (Hopkins et al., 2011). Multiple P450 genes are constitutively overexpressed in field-derived resistant strains (Brun-Barale et al., 2010). Enhanced cytochrome P450-mediated (*CYP9A12*, *CYP9A14* and *CYP6B7*) detoxification is a major mechanism responsible for pyrethroid resistance in a laboratory-selected strain of *H. armigera*.

Heavy metal can increase the expression of P450 in insects. For example, the expressions of six P450s were induced after 24 h of sub-lethal exposure to copper (Poupardin et al., 2008). This suggests that additional heavy metals such as Pb may increase the tolerance of insects to insecticides. *S. litura* is one of the most important herbivorous pests distributed widely in Asia. It has a rapid development of resistance to insecticides due to its wide range of food resources and huge appetite (Matsuura and Naito, 1997; Hemingway et al., 2002; Sogorb and Vilanova, 2002). However, whether Pb can affect the tolerance of *S. litura* to chemical insecticides will determine the effectiveness of control programs.

In this study, *S. litura* larvae were fed on the artificial diet containing a range of Pb concentrations as determine by its concentration in vegetables (0–200 mg kg^{-1}) (Zhu et al., 2004; Shu et al., 2009; Yang et al., 2011). We investigated the effects of Pb on the mortality and growth of *S. litura*. Furthermore, insecticide feeding and topical application of cypermethrin were performed to determine the tolerance of *S. litura* to short or long term exposure to Pb. Finally, the tissue distribution of Pb accumulation in *S. litura* larvae was examined by flame atomic absorption spectrometry (AAS), and P450 expression (*CYP9A39* and *CYP6B47*) in *S. litura* tissues (fat body and midgut) were measured by real-time RT-PCR.

2. Materials and methods

2.1. Insects

An insecticide-susceptible laboratory strain of *S. litura*, provided by the Insectarium of the Institute of Entomology, Sun Yat-sen University was used for all experiments. Larvae of *S. litura* were fed on standard artificial diet (Chen et al., 2000) for various generations for more than 10 years. The rearing was carried out at constant conditions of 27 °C, 65% relative humidity and a 12 h dark/12 h light photoperiod in a climatic chamber. For each experiment, larvae that had molted within 12 h were selected for treatments to insure homogeneity at the same developmental stage.

2.2. Pb treatment and bioassay

The third instar larvae were fed on an artificial diet with different doses (0, 12.5, 25, 50, 100, and 200 mg kg^{-1}) of lead nitrate (Merck, Darmstadt, Germany). After Pb exposure for 96 h, larvae from 0.35 g to 0.45 g were chosen to be reared individually in 42 mL plastic cups and fed on adequate fresh diet without Pb (Ck). After 48 h, larvae weight of *S. litura* was investigated. The change in larvae weight was calculated by subtracting its initial weight (0.35–0.45 g) from the weight after 48 h. The pupae weights were measured within 48 h when larvae were successful pupation. Larvae were considered dead if they were on their backs and unable to become right-side up when disturbed. The final mortality was calculated as the number of dead larvae before pupation/number of larvae at the beginning of experiment. Each set of bioassays was replicated in triplicate with 20 larvae in each bioassay.

2.3. Different Pb exposure with cypermethrin treatment and bioassay

Insecticide bioassays were performed after exposure to different concentrations of Pb as described above for 96 h. *S. litura* larvae were fed with 2.25 mg kg^{-1} cypermethrin (the degree of purity: 95%, Rongcheng Chemical Corporation of Jiangsu, China). Insecticide topical application was given to larva (0.35–0.45 g) using 2 μL cypermethrin (2.5 μg ml^{-1}).

Treated insects were kept in an incubator at 27 °C with a photoperiod of 12 h light and 12 h dark. After treating with cypermethrin for 48 h, larvae weight of *S. litura* was determined. The change in larvae weight was calculated by subtracting its initial weight (0.35–0.45 g) from the weight after 48 h. The pupae weights were measured within 48 h when larvae were successfully pupated. Final mortality was calculated as the number of dead larvae before pupation/number of larvae at the beginning of experiment. Each set of bioassays was replicated in triplicate with 20 larvae in each bioassay.

2.4. Different Pb exposure for 5 generations with cypermethrin treatment and bioassay

After 100 mg kg^{-1} Pb exposure for 3 generations, the developmental period and mortality of *S. litura* were increased, and their offspring number was decreased greatly. The first instar larvae were exposed to a diet consisting of four different Pb concentrations (0, 12.5, 25 and 50 mg kg^{-1}) upon hatching. Laid by the treated-first generation adults, eggs (200–300) were used as the starting point for the next generation using the same Pb concentration. Other generations were treated in the same way except those from the fifth generation. Larvae (0.35–0.45 g) from the fifth generation were treated with 2 μL cypermethrin (1.0 μg mL^{-1}) by topical application. Changes in larvae weight and pupae weights, and mortality were measured (detailed methods see Section 2.3).

2.5. Pb accumulations in insect

After exposed to Pb (0, 12.5, 25, 50 and 100 mg kg^{-1}) for 96 h and Pb (0, 12.5, 25 and 50 mg kg^{-1}) for 5 generations, larvae (0.35–0.45 g) were dissected in phosphate-buffered saline (PBS; 137 mM NaCl; 2.7 mM KCl; 8 mM Na_2HPO_4; 1.5 mM KH_2PO_4; pH 7.5). The midgut, fat body, brain, hemolymph and cutitle were collected. One ml of hemolymph was added into a 10 mL Amp bottle. The other tissues were put into different Pyrex test tubes and dried at 90 °C for 24 h in the baking oven. Five hundred μl hemolymph and/or 100 mg of other tissues (dry weight) were digested in 10 mL of boiling nitric acid (65%) and 1 mL of concentrated perchloric acid (Baker analyzed reagent; Baker, Deventer, Holland). When the fume was white and the solution was completely clear, the samples were cooled to room temperature. After filtration by filter paper, the clear solution was transferred to a volumetric flask that was then filled with 50 mL deionized water. Pb concentrations were estimated by a flame atomic absorption spectrophotometer (AAS) (HITACHI, Japan). Concentrated nitric acid and perchloric acid were used as the blank control. Pb concentrations in different tissues were calculated as $Pb^{2+} = (C \times 0.05)/W$, where C means the Pb concentration detected by AAS, and W represents the dry weight of the sample or the weight of 0.5 mL hemolymph.

2.6. Expression of P450s by Real-time RT-PCR

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) from the fat body and midgut of larvae (0.35–0.45 g) treated with Pb exposure (0, 12.5, 25, 50 and 100 mg kg^{-1}) for 96 h. One μg of total RNA was used for the first strand cDNA synthesis in a total volume of 10 μL using the PrimeScript™ cDNA synthesis system (Takara, Japan). Gene-specific primers were designed in terms of two *S. litura* P450 genes: *QCYP9A39S* (GenBank Accession No. GQ465040.1) (5′-GAC GGT TCG TTG GAA GGT A-3′) and *QCYP9A39R* (5′-TGG TGG TCA AGG AAG TGC T-3′), *QCYP6B47S* (GenBank