Article

The expression profile of detoxifying enzyme of tomato leaf miner, *Tuta absoluta* Meyrik (Lepidoptera: Gelechiidae) to chlorpyrifos

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Abstract

The tomato leafminer, *Tuta absoluta* (Meyrich) (Lepidoptera: Gelechiidae) is an important pest of tomato crops worldwide. The persistent use of organophosphate insecticide to control this pest has led to resistance. However, there is no report on the susceptibility and resistance mechanism of field population of *Tuta absoluta* (Meyrik) from Iran. Furthermore, the toxicity and impact of chlorpyrifos on metabolic enzymes in this pest remains unknown. The populations of *T. absoluta* from Rasht in Iran displayed LC_{30}; 4332, LC_{50}; 5010 and LC_{90}; 7027 μg $larva^{-1}$ to chlorpyrifos. The toxicity of chlorpyrifos could be synergized more bydiethyl maleate (DEM) and triphenylphosphate (TPP) whereas the synergistic effect of piperonylbutoxide (PBO) was not efficient as well as two other synergists. The synergistic effect ranged from 1.3 to 1.9-fold in 24 h and 1.2 to 1.5-fold in 48 h. The exposure with chlorpyrifos for 24 and 48 h significantly increased the activities of esterase and cytochrome P450-dependent monooxygenases, while there were no significant changes in glutathione-S-transferase. Field populations of *T. absoluta* from Iran displayed less susceptibility to chlorpyrifos and had a relatively high LC_{50} in compare to other previous studies. Esterases and cytochrome P450 monooxygenase might be involved in the metabolism, and hence resistance to, chlorpyrifos in this pest.

Keywords tomato leafminer; chlorpyrifos; *Tuta absoluta*; synergistic effects; resistance.

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1 Introduction

The tomato leafminer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is one of devastating and economically important pests of tomato in the world (Guedes and Picanço, 2012; Picanço et al., 1997; Desneux et al., 2010). The larval stage of *T. absoluta* feed on tomato leaves, stems, apical buds, green and ripe fruits (Picanço et al., 2007), causing yield losses between 50 and 100%. The tomato leaf miner is native to South America (Morais and NormanhaFilho, 1984) and after detection in Spain in 2006 became a major concern for tomato cultivation in Europe, Africa and the Middle East (Desneux et al., 2011; Urbaneja et al.,

2007). This species is one of the main pest of tomato in Iran and after first presence in 2010 most part of tomato producing area had problem with this pest. It led to drastic changes in tomato production in the country with a dramatic increase in insecticide use in the recent years (Baniameri and Cheraghian 2012). Heavy reliance on insecticides to manage tomato leaf miner populations places strong selection pressure to develop resistance, and many studies in different countries showed *T. absoluta* has probably developed resistance to more insecticides from various chemical groups worldwide, including carbamates, neonicotinoids, organochlorines, organophosphates, and pyrethroids (Roditakis et al., 2013; Silva et al., 2011).

Chlorpyrifos is a broad spectrum organophosphate insecticide which was introduced for the first time in the United States market during 1965 by Dow Chemical Company (Juberg et al.,2013) and has been used for the controlling the various pests (Suresh et al.,2010). Chlorpyrifos is a non-systemic insecticide it is effective when taken up by either digestive or respiratory system or through direct contact. Chlorpyrifos binds to the acetyl cholinesterase (AChE) active site and inhibits the breakdown of acetylcholine at the synapse, as a result neural toxicity, paralysis and eventually death of the target pest occurs due to excessive nerve transmission (Li and Han, 2004; Karanth, 2000).Problems with insecticide resistance in the tomato borer were soon detected in the late 1990's and early 2000's in Chile, Brazil and Argentina for the insecticides initially used against this species, including organophosphates, pyrethroids, abamectin and cartap (Salazar and Araya, 2001; Siqueira et al., 2000; Lietti et al., 2005).

Resistance mechanisms to chlorpyrifos have not been studied in *T. absoluta* yet, but several major resistance mechanisms and some minor factors have been implicated in other species (Clark et al., 2002). Metabolic capacity is strongly related to the activities of the detoxification enzymes such as glutathione-*S*-transferases, esterases (Hung and Sun, 1989; Kao and Sun, 1991), and P450-dependent monooxygenases (Li et al., 2007; Eziah et al., 2009) that play important roles in the metabolism of insecticides in insects (Yu, 2004).These enzymes activity can be induced in response to environmental and chemical stress. Enzymatic response illustrates an adaptive mechanism of living organisms to a changing environment (Goff et al., 2006; Liu et al., 2006). The metabolism of different insecticides can alter by producing or inhibiting of detoxification enzymes, which may affect effectiveness of pest management practices with induced resistance or cross-resistance to insecticides. Investigation of detoxifying enzymes and their interaction with insecticides may provide a better understanding of the toxic effects of insecticides and the responses of living organisms to them (Livingstone, 1991).

The purpose of current study was investigating the possible involvement of detoxification enzymes glutathione-S-transferases (GST), esterases (EST) and mixed-function oxidases (MFO), in *T. absoluta*, we also estimated the effects of some synergists combined with chlorpyrifos on *T. absoluta*.

2 Material and methods

2.1 Insects

T. absoluta larvae from different developmental stages were collected from infested greenhouse tomato crops from Rasht (N 37°16, E 49°34) and kept in non-treated tomato plants at 22 ± 2 °C and a photoperiod of16:8 (L:D) until fourth instar larvae from the F1 generation.

2.2 Chemicals

Commercial formulations of the following insecticides were used: chlorpyrifos was obtained from Ariashimi chemical factory, Iran. 2,4-Dinitrochlorobenzene (CDNB), Dichloronitrobenzene (DCNB), α-naphthyl acetate (α-NA), Glutathione, Coomassie Brilliant Blue G-250 and *p*-nitrophenol (PNP), diethylmaleate (DEM), Triphenylphosphate (TPP) and piperonylbutoxide (PBO) were purchased from Sigma.

2.3 Bioassay

TheRasht population of *T. absoluta* was used in this study (Table 1). The toxicity of chlorpyrifos to *T. absoluta* was estimated by the IRAC-approved bioassay method (IRAC method No. 022), which was recently validated in several studies (Roditakis et al., 2013). The insecticide solutions were diluted in water containing 0.01% Triton X-100 and a control treatment without insecticide was used to record natural mortality. Tomato leaves were individually dipped in fresh solutions of the insecticide for 10 seconds with gentle agitation, ensuring the entire surface was equally covered. Distilled water without insecticide was used as controls. Then, the treated leaves were left to dry (Niedmann and Meza-Basso, 2006). Insecticide-treated tomato leaves were placed in Petri dishes (9 cm diameter) with ten fourth instar larvae of the tomato borer and were maintained under controlled environmental conditions (25±1°C temperature, 65±5% relative humidity and 12:12 (L:D) photoperiod) (Lietti, 2005). For estimation of the synergistic effect of synergists on the insecticide, 100 mg/L of PBO (Piperonylbutoxide, an inhibitor of cytochrome P450-dependent monooxygenases), DEM (Diethyl maleate, an inhibitor of glutathione-S-transferases), and TPP (Triphenyl phosphate, an inhibitor of esterase) were added to separate aliquots of each dilution (Wang, et al., 2015). Larval mortality was assessed after 24 hours of exposure by prodding the insects with a fine hairbrush. Larvae were considered dead if they were unable to move the length of their body.

2.4 *In vivo* effects of insecticides exposure on detoxification enzymes

Fourth-instar larvae exposed to insecticide at LD_{30} (30% lethal concentration, 4332 µg larva^{-1}) and LD_{50} (50% lethal concentration, 5010 µg larva^{-1}) were used for Eterase, GST and MFOs activity assay *in vivo*.

2.5 Detoxification enzymes assay

2.5.1 Enzyme extract

First, fourth instar larvae used for enzymes assay exposed to two concentrations (LC_{30}, LC_{50}) of insecticide. Survived insects were homogenized before (control) and after (treatment) the use of lethal concentrations of the insecticide, in phosphate buffer 0.1 M (180 µL) at 4 °C, 24 h after treatment. The homogenated mixture was spun (10000 g for 10 min at 4 °C) in a centrifuge.

2.5.2 Glutathione-S-transferases

Evaluation of glutathione S-transferase activity was performed based on Habig method (1974) with little modifications. Enzyme samples were placed in each well (15 µL) plus 200 µL of chloro-dinitro benzene mixture (CDNB; 63 mM solved in methanol) and reduced glutathione (GSH; 10 mM) with Ratio of 1:50. Then, absorbance was read at 340 nm every 30 seconds for 5 minutes (Habig, et al. 1974).

2.5.3 Esterases

Van Asperen method (1962) were used for evaluation of esterase activity in which 30 mM alpha-naphthyl acetate and beta-naphthyl acetate were used as substrate (diluted in phosphate buffer 0.02 M (ratio 1:99). Larvae were homogenized in phosphate buffer (0.1 M, pH 7 with 1% Triton X-100). Enzyme samples (15 µL for alpha-naphthyl and 10 µL for beta-naphthyl), plus alpha-NA or beta-NA substrate (200 µL) and 50 µL of fast blue RR (solved in distilled water ratio of 10:1) poured in microplate wells. Finally, absorbance reading was performed at 450 nm for alpha-naphthyl and 540 nm for beta-naphthyl every 2 min for 10 minutes, continuously (Van Asperen 1962) .

2.5.4 Mixed-function oxidases

Evaluation of oxidase was determined according to the method of Rose et al (1995). Reactions were carried out in 96 well micro plates by monitoring p-nitrophenol formation in a final volume of 200 µL at 405 nm using p-nitroanisole (p-NA) as a substrate at 30 s intervals for 15 min at 30 °C. Each reaction mixture contained 100 mM potassium phosphate buffer, pH: 7.4, containing 0.5 mM NADPH, 1 mM p-NA and 90 µg proteins in a final volume of 200 µL. The molar extinction coefficient for p-nitrophenol at 405 nm was determined by preparing standard curves and expressed as nmole/min/ mg protein.

2.6 Protein assay

Protein content was determined by the method of Bradford using Coomassie Brilliant Blue G-250 with bovine serum albumin as a standard.

2.7 Data analysis

Data of bioassays were analyzed for calculating lethal and sublethal concentrations by PoloPlus 2.0 and mean comparisons were performed using SPSS 22.0. Tukey test ($P \leq 0.05$) was used to compare means in enzymes activity.

3 Results

3.1 Toxicity of chlorpyrifos and synergists on *T. absoluta*

The effects of chlorpyrifos on the susceptibility of larvae to chlorpyrifos are presented in Table 1. The toxicity test after 24 h showed the $LC_{30,}$ LC_{50} andLC_{90} were 4332, 5010 and 7027 µg larva^{-1}respectively.

The effects of three synergists on *T. absoluta* are presented in Table 2.After treatment the chlorpyrifos with PBO, DEM and TPP the toxicity increased significantly. The synergist ratio after 24 h for PBO, DEM and TPP were 1.3, 1.5 and 1.9-fold respectively. After 48 h synergist ratio was 1.1-fold for PBO that showed this synergist had no significant effects on the toxicity of chlorpyrifos whereas the DEM and TPP had 1.4 and 1.5-fold respectively.

Table 1 Toxicities of chlorpyrifos to four field populations of *T. absoluta*from Iran.

Population	N	Slope (± SE)	LD_{30}(µg larva−1) (95%FLb)	LD_{50}(µg larva−1) (95%FLb)	LD_{90}(µg larva−1) (95%FLb)	X^2
Rasht	300	1.80±0.38	4332 (3756-4722)	5010 (1970- 6640)	7027 (6405-8235)	0.54

Mortality was recorded 24 h after the larvae had been treated with chlorpyrifos. Results are means ± standard error (SE) of three separate experiments.

Table 2 Toxicities of chlorpyrifos to the fourth-instar larvae of *T. absoluta*after synergism.

		24 h aftertreatment			48 h aftertreatment		
Compound	N	Slope (± SE)	LD_{50}(µg larva−1) (95%FL[b])	SR[c]	Slope (± SE)	LD_{50}(µg larva−1) (95%FL[b])	SR[c]
Chlorpyrifos	300	1.80±0.38	5010 (1970- 6640)	-	1.87±0.6	4879 (3954-5632)	-
Chlorpyrifos + PBO	275	3.94±0.2	4175 (3532-4627)	1.3	2.7±0.4	4066 (3742-5129)	1.2
Chlorpyrifos + DEM	290	2.61±0.9	3340 (2441-4232)	1.5	2.32±0.5	3485 (2389-4128)	1.4
Chlorpyrifos + TPP	300	2.39±0.51	2637 (1829-4967)	1.9	2.74±0.51	3253 (2516-4161)	1.5

[a] Mortality was recorded 24 and 48 h after the larvae had been treated with chlorpyrifos. Results are means ± standard error (SE) of three separate experiments. [b] Fiducial limits (from probit analysis). [c] Synergistic ratio (SR) = LD_{50} of chlorpyrifosto fourth-instar larvae/LD_{50} of chlorpyrifos+ synergist to fourth-instar larvae.

3.2 In vivo effects of chlorpyrifos exposure on detoxification enzymes

The exposures to chlorpyrifos increased the esterases activity after 24 and 48 h (Fig. 1). Similarly, exposures to chlorpyrifos increased activity of MFO significantly (Fig. 2). No significant change in GST activity was detected in 24 and 48 h after treatment with sublethal doses of chlorpyrifos (Fig. 3). Compared with MFO and

GST activity, chlorpyrifos had stronger and more significant effects on the esterase activity. The chlorpyrifos had greater induction of MFO after 48 h.

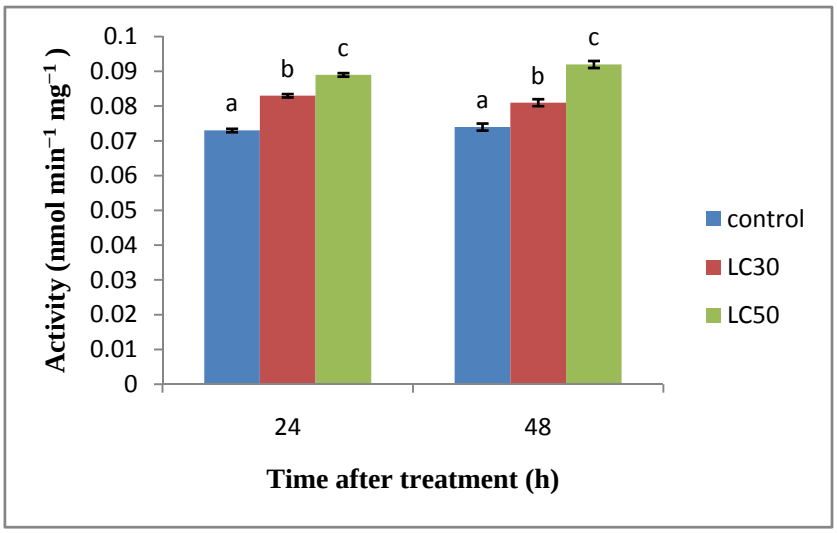

Fig. 1 Effects of chlorpyrifos on esterase activity (nmol min−1 mg−1 Pr) of Rasht population *in vivo* after fourth- larval instar had been exposed to chlorpyrifos(control, LD_{30}, LD_{50}). Results are means ± standard error (SE) of three separate replicates. Data marked with different letters differ significantly (P <0.05).

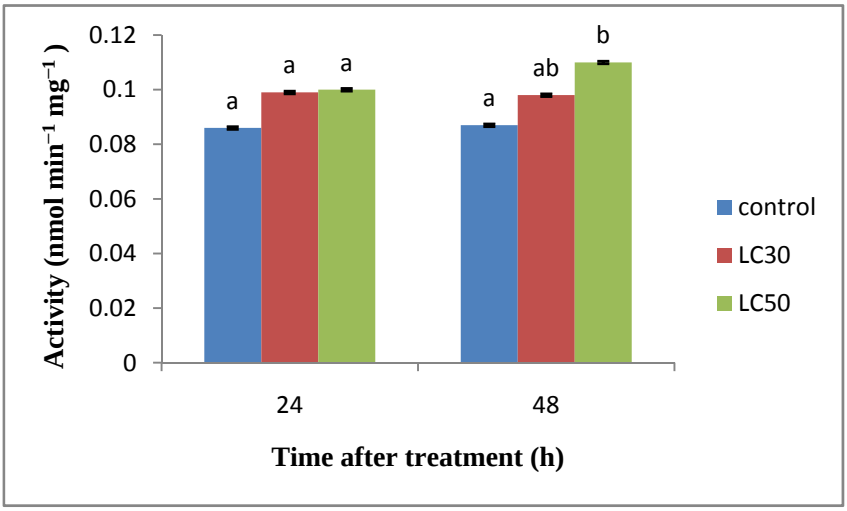

Fig. 2 Effects of chlorpyrifos on MFO activity (nmol min−1 mg−1 Pr) of Rasht population *in vivo* after fourth- larval instar had been exposed to chlorpyrifos (control, LD_{30}, LD_{50}). Results are means ± standard error (SE) of three separate replicates. Data marked with different letters differ significantly (P <0.05).

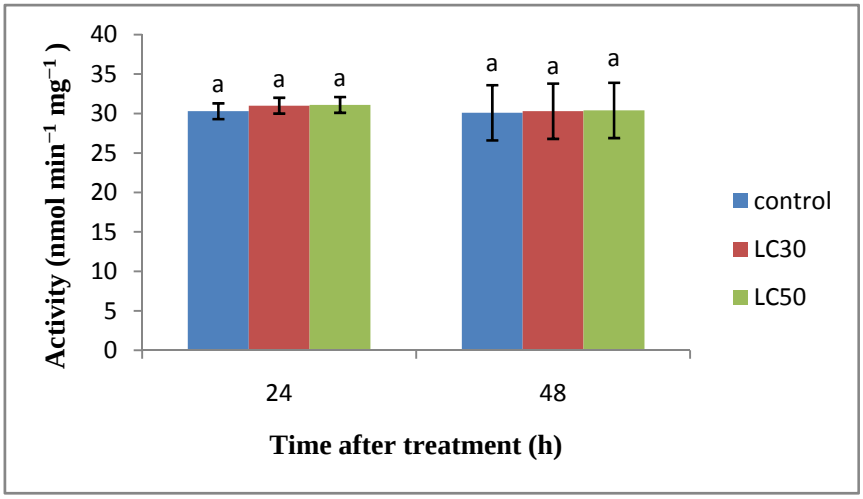

Fig. 3 Effects of GST on esterase activity (nmol min−1 mg−1 Pr) of Rasht population *in vivo* after fourth- larval instar had been exposed to chlorpyrifos (control, LD_{30}, LD_{50}). Results are means ± standard error (SE) of three separate replicates. Data marked with different letters differ significantly (P <0.05).

4 Discussion

Chlorpyrifos is a broad spectrum organophosphate insecticide, nematicide, and acaricide and widely used against agricultural pests, since 1965. Resistance to chlorpyrifos has evolved in many insects such as *Chrysoperla carnea* (Stephens) (Sayyed et al., 2010), *Chilo suppressalis* (Walker) (He et al., 2012), *Helicoverpa armigera* (Hubner) (Ahmad et al., 1999), *Spodoptera litura* (Fabricius) (Ahmad et al., 2007; Zhang et al., 2008; Zhang and Zhang, 2008), *Bemisia tabaci* (Gennadius) (Kang et al., 2006), *Laodelphax striatellus* (Fallen) (Wang et al., 2010) and *Tetranychus evansi* (Carvalho et al., 2012).To develop efficient pest management strategies, it is useful to know the chlorpyrifos susceptibility of field populations of *T. absoluta*. The present bioassay results indicated that the resistance of Iranian population of *T. absoluta* to chlorpyrifos was greater than to other population from different country. Many authors reported various LC_{50} values for chlorpyrifos in *T. absoluta* that ranged from 510 to 2040 µg $larva^{-1}$ (Siquira, 2001; Campos et al., 2014; Roditakis et al., 2013).To explore the potential role of detoxification enzymes in the tolerance of *T. absoluta* to chlorpyrifos, a synergism test was conducted. Results of synergism bioassays showed that PBO, TPP and DEM, had a significant effect on the toxicity of chlorpyrifos in *T. absoluta*. A higher synergistic ratio was observed for TPP in compare two others.

In the present experiment, the greater synergistic effect of DEF and TPP on chlorpyrifos resistance in *T. absoluta* suggested that esterase is involved in resistance through detoxification of chlorpyrifos. Previously, it has been studied that cytochrome P450 dependent monooxygenase was a major possible mechanism in chlorpyrifos resistance in many insect pests, like *Cydia pomonella* (Linnaeus) (Reyes et al., 2011), *Aphis gossypii* (Glover) (Shang et al., 2012) and *B. germanica* (Siegfried et al., 1990). However, the biochemical mechanism of chlorpyrifos resistance was not involved in *T. urticae* (Ay and Yorulmaz, 2010) and *Laodelphax striatellus* (Fallén) (Wang at al., 2010).These results imply that synergism might be species specific. Esterases detoxify chlorpyrifos components by catalyzing or by sequestering (Costa, 2006). Enhanced activity of detoxification enzymes is one of the most common mechanisms of resistance to insecticides (Scott, 1990). Insect detoxification enzymes are important resistance mechanisms and synergists are helpful in providing preliminary evidence of their involvement as resistance mechanisms (Brindley and Selim, 1984; Scott, 1990;

Bernard and Philoge Á ne, 1993; Ishaaya, 1993).Metabolic enzyme activity analysis showed that esterase plays a major role in the resistance as no significant difference in GSTs. Synergism experiments delivered the same conclusion as only TPP resulted in a higher synergism ratio (SR). This result agrees with Alon et al. (2008), but differs from abamectin resistance in tobacco whitefly and *T. urticae*, where detoxification of MFO and GSTs was indicated as a key factor (Stumpf and Nauen 2002; Wang and Wu 2007). This is not necessarily unexpected, as insecticides of different action modes often induce resistance with different mechanism even in same insect species. To control *T. absoluta*, farmers often increase the pesticide concentrations, increase the frequency of application, and mix various pesticides together. Unfortunately, these activities also support the development of serious pesticide resistance in these methods. In this study, chlorpyrifos toxicity was enhanced by the synergists diethyl maleate, piperonylbutoxide and triphenyl phosphate which respectively inhibit the detoxification enzymes glutathione-Stransferases, cytochrome P450-dependent monooxygenases, and esterases (Raffa and Priester, 1985; Bernard and Philoge Á ne, 1993), providing some interesting information regarding chlorpyrifos resistance mechanisms in this insect-species.Moreover, in the future molecular study is necessary to explore the accurate mechanism of chlorpyrifos resistance in *T. absoluta*. The use of synergists in insecticide resistance management programmes has been frequently suggested (e.g. Oppenoorth, 1985; Guedes, 1991; Denholm and Rowland, 1992; Bernard and Philoge Á ne, 1993).Nonetheless, synergists can be important tools for managing *T. absoluta* populations.

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