

***In Vitro* Inhibition of the Diphenolase Activity of Tyrosinase by Insecticides and Allelochemicals in *Micromelalopha troglodyta* (Lepidoptera: Notodontidae)¹**

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Abstract Tyrosinase is a copper enzyme and plays a key role in normal insect development. We studied the *in vitro* inhibitory effects of selected insecticides and allelochemicals on the diphenolase activity of tyrosinase in *Micromelalopha troglodyta* (Graeser) (Lepidoptera: Notodontidae). Two pyrethroids (cyfluthrin and deltamethrin) and 3 other insecticides (hexaflumuron, abamectin and imidacloprid) were the least inhibitory, whereas 5 organophosphates (triazophos, malathion, chlorpyrifos, omethoate and profenofos), 1 carbamate (methomyl), 4 pyrethroids (fenpropathrin, beta-cypermethrin, bifenthrin and lambda-cyhalothrin), 1 organochlorine (endosulfan), 2 allelochemicals (tannic acid and 2-tridecanone) and 4 other insecticides (emamectin benzoate, fipronil, acetamiprid and pyridaben) were moderately inhibitory. Three chemicals (quercetin, phenyl thiourea and phoxim) were the most potent inhibitors of the enzymes among all compounds tested and inhibited the diphenolase activity of tyrosinase *in vitro* in a dose-dependent manner. Furthermore, phenyl thiourea, phoxim and quercetin showed neither typical competitive nor noncompetitive binding to the substrate, with K_i of 0.13 μM , 49.30 μM and 37.71 μM , respectively.

Key Words diphenolase activity, tyrosinase, *Micromelalopha troglodyta*, allelochemicals, insecticides, inhibition

It is well known that tyrosinase (EC 1.14.18.1), also known as phenoloxidase, is a copper enzyme that is widely distributed among microorganisms, plants, insects and animals (Yang et al. 2005, Liu et al. 2006). It is critical in biological processes such as vertebrate pigmentation and the browning of fruits and vegetables. It is the key enzyme involved in melanin formation in melanocytes. Tyrosinase catalyzes both the hydroxylation of monophenols and the oxidation of *o*-diphenols into *o*-quinones. Further, it is involved in the formation of pigments such as melanin (Hopkins and Kramer 1992, Xiao et al. 2008). In insects, tyrosinase is a widely distributed enzyme that plays important roles in normal developmental processes, such as cuticular tanning, sclerotization, wound healing, production of opsonins, and encapsulation and nodule formation for defense against foreign pathogens. Diphenolase is the pivotal insect enzyme in the oxidation of catecholamine to their respective quinines, which are then further metabolized to either melanin or crosslink proteins in sclerotin (Iwama and Ashida

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1986, Hopkins and Kramer 1992, Chase et al. 2000, Ma and Kanost 2000, Wang et al. 2005).

The small prominent, *Micromelalopha troglodyta* (Graeser) (Lepidoptera: Notodontidae), is an important pest of poplar in China. Insecticides are widely used for its control. This pest has developed resistance to almost all classes of insecticides, such as pyrethroids, organophosphates and carbamates. There are many reports on insect resistance due to tyrosinase abundance (Gao et al. 1997). It is possible that inhibition of tyrosinase could lead to abrogation of insect defense mechanisms or abnormal body softening, both of which could enhance control. Thus, tyrosinase may be regarded as a potential candidate for the novel insecticide development. But, little is known about the biochemical properties of tyrosinase in *M. troglodyta*. To better understand the interaction of tyrosinase with insecticides and allelochemicals, we studied their *in vitro* inhibitory effects on tyrosinase activity in *M. troglodyta*. We also attempted to identify some potential tyrosinase inhibitors which might be used as synergists to enhance the toxicity of insecticides.

Materials and Methods

Chemicals. Bovine serum albumin (BSA), 2-tridecanone and tannic acid (>99%) were purchased from Sigma Chemical Co. (St. Louis, MO), quercetin (99%) from Shanghai chemical stock (Shanghai, China). Content and manufacturers of insecticides are shown in Table 1. Allelochemicals and insecticides were dissolved in absolute acetone. All other chemicals were of analytical grade and purchased from commercial sources.

Insects. Small prominents were collected from Mingguang of Anhui Province, China, in 2007. They were reared in the conditioned room maintained at $26 \pm 1^\circ\text{C}$, 75% RH and a 12:12 L:D photoperiod. Fifth-instar newly-molted larvae were used in all studies.

Preparation of cytosolic fractions. Ten fifth-instar larvae were homogenized in 5 mL of ice-cold phosphate buffer (pH 7.5, 0.1M) after peritrophic membranes and associated midgut contents were removed. The homogenate was centrifuged at 27,500 g for 20 min at 4°C . The supernatants were used to determine the enzyme activity after being filtered through 3 layers of cellulose filter paper (grade 3, Whatman, Middlesex, UK).

Diphenolase activity of tyrosinase assay. The assay method was that described by Liang et al. (2003) with the following modifications. The assay mixture contained 5 mM catethol. The assay was initiated by the addition of 70 μL enzyme; the absorbance at 420 nm was monitored for 2 min. Controls without enzyme always accompanied each assay. Enzyme activity was expressed as OD/min at 25°C , and the specific activity as OD/min/mg protein. The method of Bradford (1976), with BSA as a standard, was used for protein quantification.

Inhibition of insecticides and allelochemicals. Inhibition of the diphenolase of tyrosinase was determined in assays containing 5 mM catethol and various insecticides (0.1 mM) or allelochemicals (0.1mM) as inhibitors. All assays, including controls, contained 1% acetone. All assays were run in triplicate.

Dose-dependent inhibition of the diphenolase activity of tyrosinase was studied with fixed concentrations of 5 mM catethol by adding 30 μL of insecticides or allelochemicals dissolved in acetone at various concentrations to the incubating reaction mixtures. The I_{50} values, concentrations of inhibitors required to reduce the reaction rate by 50%,

Table 1. Content and manufacturers of insecticides

Insecticides	Content (%)	Manufacturer	Location
Organophosphate			
Triazophos	92.0	Jiangxi Kaifeng Chemical Co., Ltd	Jiangxi, China
Malathion	95.0	Hebei Shiji Pesticide Co., Ltd	Hebei, China
Chlorpyrifos	97.0	Dow AgroSciences LLC	Indiana, USA
Phoxim	99.0	Tianjin Pesticide Co., Ltd	Tianjin, China
Omethoate	92.0	Hangzhou Qingfeng Agrochemicals Co., Ltd	Zhejiang, China
Profenofos	90.0	Tianjin Pesticide Co., Ltd	Tianjin, China
Carbamate			
Methomyl	98.0	Hubei Sanongda Co., Ltd	Hubei, China
Pyrethriod			
Fenpropathrin	92.0	Shandong Dacheng Pesticide Co., Ltd	Shandong, China
Beta-cypermethrin	97.0	Shandong Dacheng Pesticide Co., Ltd	Shandong, China
Bifenthrin	97.0	Jiangsu Yangnong Chemical Group Co., Ltd	Jiangsu, China
Lambda-cyhalothrin	95.0	Jiangsu Huangma Pesticide & Chemical Co., Ltd	Jiangsu, China
Deltamethrin	99.0	Jiangsu Yangnong Chemical Group Corp., Ltd	Jiangsu, China
Cyfluthrin	92.0	Jiangsu Yangnong Chemical Group Corp., Ltd	Jiangsu, China
Organochlorine			
Endosulfan	90.0	Jiangsu Rudong Pesticide Co., Ltd	Jiangsu, China
Other insecticides			
Phenyl thiourea	99.0	Shanghai No.1 chemical factory	Shanghai, China
Hexaflumuron	95.0	East Romble Agrochem (Shan Dong) Co., Ltd	Shandong, China
Emamectin benzoate	90.0	Zhejiang Qianjiang Biochemical Co., Ltd	Zhejiang, China

Table 1. Continued

Insecticides	Content (%)	Manufacturer	Location
Abamectin	95.3	Shandong Jingbo Agrochemicals Co., Ltd	Shandong, China
Fipronil	90.0	Anhui Huaxing Chemical Industry Co., Ltd	Anhui, China
Imidacloprid	95.0	Hubei Sanongda Co., Ltd	Hubei, China
Acetamiprid	96.0	Qingdao Haili'er Medicine Co., Ltd	Shandong, China
Pyridaben	95.0	Shandong Sino-Agri United Biotechnology Co., Ltd	Shandong, China

were determined by linear regression of the inhibition percent on the log of the inhibitor concentration. The experiment was performed in triplicate for each inhibitor.

Kinetics of tyrosinase inhibition. Kinetics of tyrosinase inhibition by phenyl thiourea, phoxim and quercetin were determined in assays containing various concentrations of catethol and a fixed concentration of each inhibitor. The results were presented on double-reciprocal Lineweaver-Burk plots. The inhibitory constant, K_i , the equilibrium constant for dissociation of the enzyme-inhibitor complex, was determined from plots of reciprocal velocity versus reciprocal concentration (Lineweaver-Burk plots) (Dixon and Webb 1979).

Results

The inhibition of the diphenolase activity of tyrosinase by insecticides and allelochemicals is shown in Table 2. Three inhibitors (quercetin, phenyl thiourea and phoxim) were the most potent inhibitors tested, inhibiting more than 65% of tyrosinase activity at a final concentration of 0.1 mM. Among the inhibitors tested, 5 organophosphates (triazophos, malathion, chlorpyrifos, omethoate and profenofos), 1 carbamate (methomyl), 4 pyrethroids (fenpropathrin, beta-cypermethrin, bifenthrin and lambda-cyhalothrin), 1 organochlorine (endosulfan), 2 allelochemicals (tannic acid and 2-tridecanone) and 4 other insecticides (emamectin benzoate, fipronil, acetamiprid and pyridaben) were moderately inhibitory, whereas 2 pyrethroids (cyfluthrin and deltamethrin) and 3 other insecticides (hexaflumuron, abamectin and imidacloprid) were the least inhibitory.

The sensitivity of tyrosinase activity to 3 inhibitors (phenyl thiourea, phoxim and quercetin) was also assessed. Phenyl thiourea, phoxim and quercetin inhibited the diphenolase activity of tyrosinase *in vitro* in a dose-dependent manner (Fig. 1). The I_{50} values of these 3 inhibitors for tyrosinase activity in *M. troglodyta* larvae are summarized in Table 3, with I_{50} values ranging from 3.34×10^{-8} M to 7.25×10^{-5} M, phenyl thiourea being the most potent inhibitor.

The results of kinetic analyses of tyrosinase inhibition by phenyl thiourea, phoxim and quercetin using Lineweaver-Burk plots are summarized in Fig. 2. Phenyl thiourea, phoxim and quercetin showed neither typical competitive nor noncompetitive binding to the substrate, with K_i of 0.13 μ M, 49.30 μ M and 37.71 μ M, respectively.

Table 2. Inhibition of the diphenolase activity of tyrosinase by insecticides and allelochemicals in *M. troglodyta* larvae

Inhibitor (0.1 mM)	% Inhibition (means \pm SD)
Organophosphate	
Triazophos	24.62 \pm 2.18 fghi
Malathion	10.00 \pm 1.63 jk
Chlorpyrifos	26.28 \pm 3.89 fgh
Phoxim	68.46 \pm 4.16 c
Omethoate	19.33 \pm 1.20 ghij
Profenofos	25.79 \pm 5.78 fghi
Carbamate	
Methomyl	12.20 \pm 1.35 j
Pyrethriod	
Fenpropathrin	10.00 \pm 2.18 jk
Beta-cypermethrin	14.34 \pm 1.33 j
Bifenthrin	16.05 \pm 0.79 ij
Lambda-cyhalothrin	34.07 \pm 1.18 def
Cyfluthrin	-6.40 \pm 1.46 l
Deltamethrin	-3.93 \pm 0.88 l
Organochlorine	
Endosulfan	12.67 \pm 2.35 j
Other insecticides	
Phenyl thiourea	96.20 \pm 2.09 b
Hexaflumuron	1.10 \pm 0.52 kl
Emamectin benzoate	16.36 \pm 2.86 hij
Abamectin	-7.12 \pm 1.36 l
Fipronil	10.77 \pm 0.54 jk
Imidacloprid	-2.88 \pm 1.36 l
Acetamiprid	33.85 \pm 2.15 def
Pyridaben	40.80 \pm 1.71 d
Allelochemical	
Quercetin	138.33 \pm 11.44 a
Tannic acid	28.27 \pm 1.90 efg
2-tridecanone	36.71 \pm 2.26 de

The data were analyzed using analysis of variance (ANOVA). The difference is significant if $P < 0.05$. Means within a column followed by the same small letter are not significantly different.

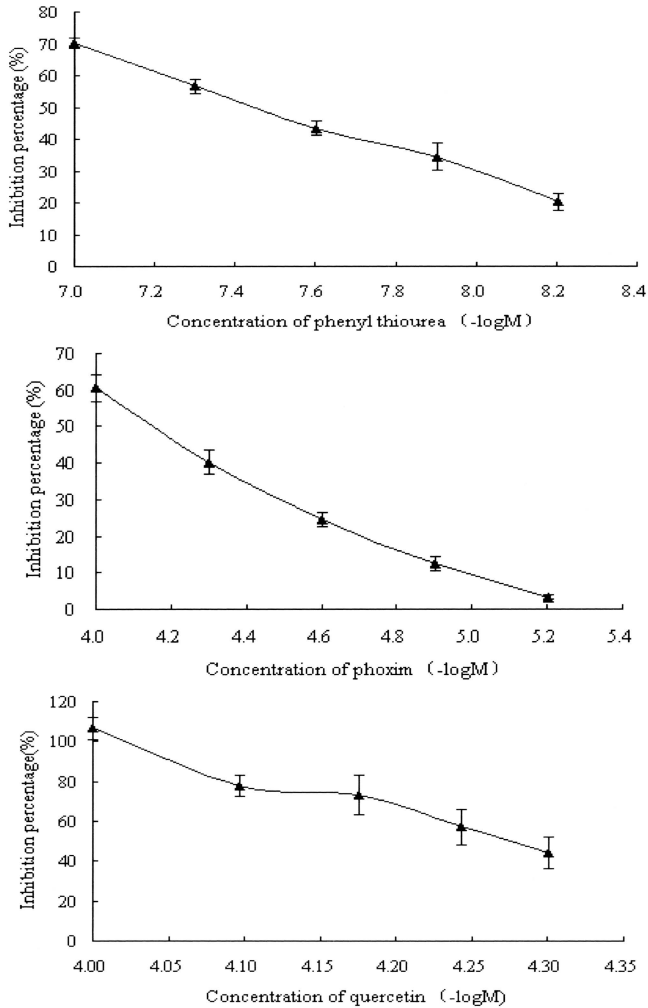


Fig. 1. *In vitro* inhibition of the diphenolase activity of tyrosinase by three inhibitors in *M. troglodyta* larvae.

Discussion

Tyrosinase plays a key role in normal insect development, so it may be possible to control pests by inhibiting or disturbing the enzyme. This maybe a useful basis for the development of novel insecticides (Wang et al. 2005). *In vitro* inhibition studies are useful for understanding the potential as insecticides. In the present study, we found that most of inhibitors tested inhibited the *in vitro* diphenolase activity of tyrosinase in *M. troglodyta* at the concentration of 0.1 mM. Our results differ from previous studies. For example, organophosphates (malathion and chlorpyrifos) and pyrethroids

Table 3. I_{50} values of inhibitors for the diphenolase activity of tyrosinase in *M. troglodyta* larvae

Inhibitor	I_{50} (1×10^{-5} M) (means \pm SD)
Phenyl thiourea	$(3.34 \pm 0.35) \times 10^{-3}$ c
Phoxim	7.25 ± 0.46 a
Quercetin	5.24 ± 0.57 b

The data were analyzed using analysis of variance (ANOVA). The difference is significant if $P < 0.05$. Means within a column followed by the same small letter are not significantly different.

(beta-cypermethrin) were moderately inhibitory to the diphenolase activity of tyrosinase in *M. troglodyta*; whereas these 3 insecticides were noninhibitory to the diphenolase activity of tyrosinase in *Musca domestica* (L.) (Liu et al. 2004). Abamectin and imidacloprid were noninhibitory to the diphenolase activity of tyrosinase in *M. troglodyta*; whereas these 2 insecticides demonstrated low inhibition to the diphenolase activity of tyrosinase in *M. domestica* (Liu et al. 2004). Further, our results showed phoxim produced >68% inhibition of the diphenolase activity of tyrosinase in *M. troglodyta* at the concentration of 0.1 mM. Compared with previous reports, the inhibitory effect of phoxim was very high. For example, Liu et al. (2004) showed that phoxim showed 5.3% inhibition of the diphenolase activity of tyrosinase in *M. domestica* at the concentration of 15.6 mM. These results suggest that isoenzymes of tyrosinase may affect susceptibility to insecticides.

Our results show that the I_{50} value of phenyl thiourea for the diphenolase activity of tyrosinase in *M. troglodyta* larvae is 3.34×10^{-8} M, being the most potent inhibitor. Liang et al. (2003) reported the I_{50} value of phenyl thiourea for the diphenolase activity of tyrosinase from both the resistant and susceptible strains of *Plutella xylostella* (L.) showed no obvious differences (1.18×10^{-6} M to 1.28×10^{-6} M, respectively). Liu et al. (2004) reported the I_{50} value of phenyl thiourea for the diphenolase activity of tyrosinase in *M. domestica* was 1.50×10^{-7} M. These results showed that phenyl thiourea is an effective tyrosinase inhibitor. Hopefully, this work will provide the basis for the design of effective, selective tyrosinase inhibitors and the development of novel candidate insecticides.

Our kinetic analysis indicated that quercetin showed neither typical competitive nor noncompetitive interaction with the substrate in *M. troglodyta*. However, the inhibition of *Spodoptera exigua* (Hübner) tyrosinase by quercetin was competitive (Luo et al. 2005). These results also indicate that inhibitors have different reaction modes to different isoenzymes. Inhibition mechanisms may differ significantly even for isoenzymes very similar in structure.

Some allelochemicals might be potent inhibitors of tyrosinase activity in insects. Quercetins are plant phenols; potent inhibition was observed in our study. The I_{50} value of quercetin for the diphenolase activity of tyrosinase in *M. troglodyta* larvae is 5.24×10^{-5} M, being more potent inhibitor of tyrosinase activity than phoxim. This result was consistent with other observations. For example, Luo et al. (2005) reported that the I_{50} value of quercetin for the diphenolase activity of tyrosinase in *S. exigua* is 8.70×10^{-5} M. Our results showed that quercetin had the potential of serving as a synergist for enhancing the toxicity of insecticides in *M. troglodyta*. However, its application as a synergist in insect control requires further investigation.

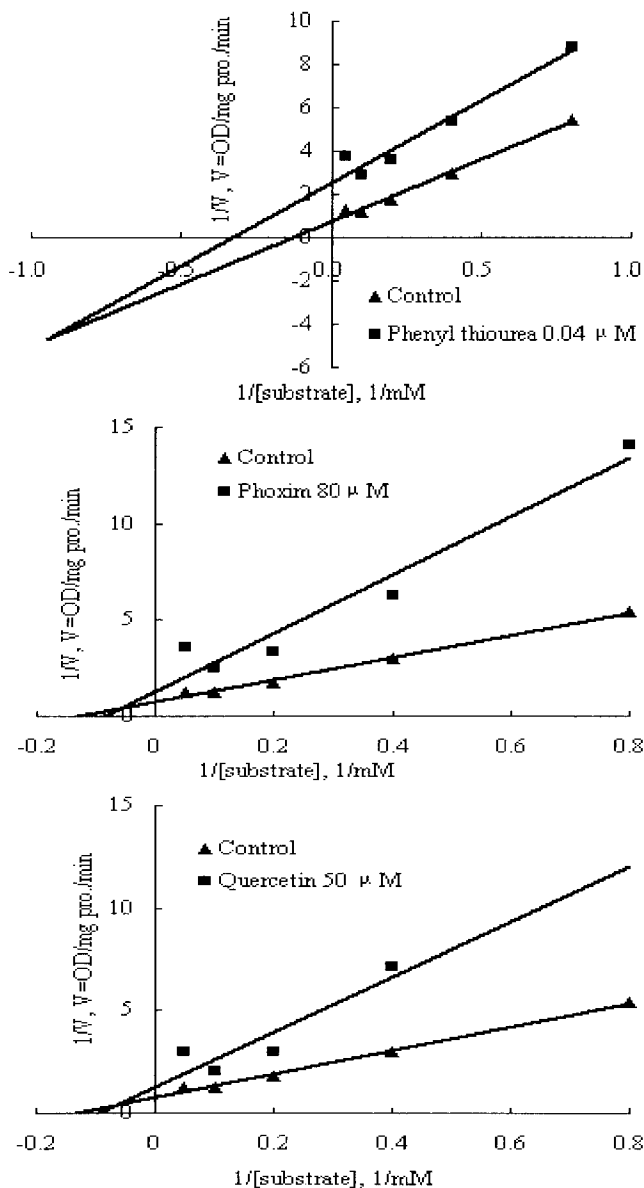


Fig. 2. Kinetics of tyrosinase inhibition by three inhibitors in *M. troglodyta* larvae.

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