In Vitro Inhibition of Polyphenol Oxidase Activity by Insecticides and Allelochemicals in *Clostera anastomosis* (Lepidoptera: Notodontidae) Larvae and Poplar Trees[1]

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Abstract *Clostera anastomosis* (L.) (Lepidoptera: Notodontidae) is an important leaf-feeding insect of poplars, *Populus* spp. (Salicaceae) in China. As part of a continuing search for environmentally friendly insecticides for this pest, we compared the *in vitro* inhibition of polyphenol oxidase (PPO) activity by 21 insecticides and allelochemicals in *C. anastomosis* and poplar trees (*populous* × *euramericana* 'NanLin895'). The results showed that three inhibitors (quercetin, phenyl thiourea, and phoxim) can strongly inhibit PPO activity in both *C. anastomosis* and poplars, but the inhibitory degree with each was significantly different. Our results further showed that three inhibitors had a certain dose relationship with the PPO activity in *C. anastomosis* and poplars. The I_{50} values (50% inhibitory concentration) of three chemicals (quercetin, phenyl thiourea, and phoxim) were estimated as 14.17, 0.18, and 127.67 μM for *C. anastomosis* and as 0.34, 0.15, and 0.21 mM for poplars, respectively. These results will lay foundation for the design of effective, selective PPO inhibitors and the development of novel insecticides.

Key words *Clostera anastomosis*, poplars, polyphenol oxidase, inhibition, I_{50} values

Poplars, *Populus* spp. (Salicaceae), are important tree species in China. However, insects and diseases are causing economic damage to these trees as larger areas are being planted with poplars. Conventional pesticides are routinely used to control insect and disease pests of poplars, but many of these compounds also negatively affect poplar trees (Tang et al. 2012). Therefore, it is crucial to identify chemistries that can manage pests but minimize the damage to the poplars.

Polyphenol oxidase (PPO), also known as tyrosinase (EC 1.14.18.1), is a copper enzyme that is widely distributed among plastids of microorganisms, plants, insects, and animals (Wang et al. 2005, Yang et al. 2005). It can catalyze both the hydroxylation of monophenols and the oxidation of *o*-diphenols into *o*-quinones. It is involved in melanin formation and in immune responses (Meng et al. 2004). Thus, PPO concentration in insects can be used as an index to assess the comparative competency of the insect's immune system (Cai et al. 2001). PPO is also localized in the thylakoid of chloroplasts and other types of plastids in plants where it can be used as an oxidoreductase in photosynthesis (e.g., regulating the rate of harmful

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photo-oxidation reaction, involvement in electron transfer) (Dai et al. 2007). Furthermore, PPO can promote wound healing and increase the resistance of plants to pathogens.

Clostera anastomosis (L.) (Lepidoptera: Notodontidae) is an important leaf-feeding insect on poplars for which insecticides are widely used for its control. Our objective in this study was to provide a basis for the identification and development of insecticides or allelochemicals that effectively manage this pest insect but have no deleterious effect on the host poplar tree. To this end, we compared the *in vitro* inhibitory effects of 21 selected chemicals on PPO activity in *C. anastomosis* larvae and in poplar trees.

Materials and Methods

Clostera anastomosis larvae used in these assays were initially collected from Nanjing (N 31°56′17.00″, E 118°22′35.98″) of Jingsu Province, China. They were transported to the laboratory and reared in a room maintained at 25 ± 1°C and 75% relative humidity on a 12L:12D-h photoperiod.

Poplar cuttings (*populous* × *euramericana* 'NanLin895′) were obtained as seedlings from Nanjing Forestry University and grown in a growth chamber at 25 ± 1°C and with a 12L:12D-h photoperiod. They were watered daily and supplied with Hoagland nutrient solution (Afrousheh et al. 2010) each week. They were used in experiments after they had grown to a height of 60–80 cm.

The chemicals 2-tridecanone, quercetin (99%), and tannic acid (>99%) were purchased from Sigma Chemical Co. (St. Louis, MO). Catechol was purchased from Shanghai Qingxi Chemical Technology Co., Ltd. (Shanghai, China). Formulations, active ingredients, and manufacturers of the insecticides used in the tests are shown in Table 1. Allelochemicals and insecticides were dissolved in absolute acetone for experimentation. All other chemicals were of domestic analytical grade and purchased from commercial sources.

Enzyme solutions were prepared by first homogenizing 10, fifth-instar larvae in 5 ml of phosphate buffer at 4°C (pH 7.5, 0.1 M) after peritrophic membranes and associated midgut contents were removed. The homogenate was centrifuged at 25,000g for 20 min at 4°C, and the supernatant was used to determine the enzyme activity after being filtered through three layers of cellulose filter paper (grade 3, Whatman, Middlesex, UK). Fresh poplar leaves (0.1 mg) were combined with 0.01 g polyvinylpolypyrrolidone (PVPP) and liquid nitrogen and were then ground. Phosphate buffer (1.5 ml) (pH7.5) was added, and the solution was centrifuged at 25,000g for 20 min at 4°C. The resultant supernatant was used to measure the enzyme activity. All experiments were performed in triplicate.

Diphenolase activity of PPO was assayed using the method described by Vanitha and Umesha (2011), modified by adding 5 mM catethol to the assay mixture and initiating the assay by adding 70 μl of enzyme. Absorbance at 420 nm was monitored for 2 min. The appropriate controls without any enzyme accompanied each assay. Enzyme activity was expressed as _____ [optical density (OD)]/min/mg protein. The method of Bradford (1976), with bovine serum albumin (BSA) as a standard, was used for protein quantification.

Table 1. Insecticides and their active ingredient concentration and manufacturer.

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
Isocarbophos	95.0	Hubei Xianlong Chemical Industry Co., Ltd.	Hubei, 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 and Chemical Co., Ltd	Jiangsu, China
Other insecticides			
Imidacloprid	95.0	Hubei Sanongda Co., Ltd	Hubei, China
Acetamiprid	96.0	Qingdao Haili'er Medicine Co., Ltd	Shandong, China
Hexaflumuron	95.0	East Romble Agrochem (Shan Dong) Co., Ltd	Shandong, China
Phenyl thiourea	99.0	Shanghai No.1 chemical factory	Shanghai, China
Fipronil	90.0	Anhui Huaxing Chemical Industry Co., Ltd	Anhui, China
Pyridaben	95.0	Shandong Sino-Agri United Biotechnology Co., Ltd	Shandong, China

Inhibition of PPO activity was determined in assays containing 5 mM catethol and the various insecticides (0.17 mM) or allelochemicals (0.17 mM) serving as inhibitors. All assays, including controls, contained 1.7% acetone, and all were run in triplicate.

Dose-dependent inhibition of the PPO activity was measured 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%, were determined by linear regression of the inhibition percent on the log of the inhibitor concentration. All experiments were performed in triplicate.

Results

The inhibition of the diphenolase activity of PPO in *C. anastomosis* larvae and poplar by insecticides and allelochemicals is shown in Table 2. Three inhibitors (quercetin, phenyl thiourea, and phoxim) had the strongest inhibitory effect on PPO in *C. anastomosis* larvae, inhibiting more than 60%. Among the tested inhibitors, four organophosphates (triazophos, chlorpyrifos, omethoate, profenofos), four pyrethroids (fenpropathrin, beta-cypermethrin, bifenthrin, lambda-cyhalothrin), and three other insecticides (acetamiprid, fipronil, pyridaben) were moderate inhibitors whereas two allelochemicals (tannic acid and 2-tridecanone), two organophosphates (malathion, isocarbophos), one carbamate (methomyl), and other insecticides (hexaflumuron, imidacloprid) were the least inhibitory. Seven inhibitors (phoxim, omethoate, profenofos, fenpropathrin, phenyl thiourea, pyridaben, and quercetin) had the strongest inhibitory effect on PPO in poplar. For the diphenolase activity of PPO, phenyl thiourea was the most potent inhibitor tested, inhibiting more than 60% of PPO activity at a final concentration of 0.17 mM. Furthermore, five organophosphates (triazophos, malathion, phoxim, omethoate, and profenofos), two pyrethriods (fenpropathrin and beta-cypermethrin), three other insecticides (hexaflumuron, pyridaben, and acetamiprid), and allelochemicals (quercetin) were moderate inhibitors. In addition, one organophosphate insecticide (chlorpyrifos), one carbamate insecticide (methomyl), two pyrethriods (bifenthrin and lambda-cyhalothrin), two allelochemicals (tannic acid and 2-tridecanone), and two other insecticides (fipronil and imidacloprid) were the least inhibitory.

The sensitivity of PPO activity to three inhibitors (quercetin, phenyl thiourea, and phoxim) was also evaluated. Quercetin, phenyl thiourea, and phoxim inhibited the diphenolase activity of PPO *in vitro* in a dose-dependent manner (Fig.1A, B, Fig. 2A, B, Fig. 3A, B). The I_{50} values of these three inhibitors for PPO activity in *C. anastomosis* larvae ranged from 1.84×10^{-7} M to 1.28×10^{-4} M and in poplars from 1.51×10^{-4} M to 3.35×10^{-4} M (Table 3).

Discussion

PPO is an important enzyme in insects and plays a key role in the tanning process of insect cuticle. It is also involved in melanin formation and wound healing in insects (Zhou and Jiang. 2004). In plants, PPO catalyzes ortho-dihydroxy phenol into ortho-quinine, which may impact solubility of proteins or amino acids and the

Table 2. Inhibition of polyphenol oxidase by insecticides and allelochemicals in *C. anastomosis* and poplars.*

	Percentage of inhibition (%) (means ± SD)	
Inhibitor (0.17mM)	*Clostera anastomosis*	Poplar
Organophosphate		
Triazophos	23.86 ± 4.54 def A	23.89 ± 2.36 de A
Malathion	3.86 ± 0.68 f B	11.30 ± 2.52 fgh A
Chlorpyrifos	27.50 ± 5.51 def A	5.56 ± 1.96 ghij B
Phoxim	62.50 ± 7.59 c A	30.00 ± 4.47 cd B
Omethoate	46.14 ± 4.47 cd A	45.56 ± 4.88 b A
Profenofos	26.82 ± 5.68 def B	38.75 ± 1.77 bc A
Isocarbophos	5.23 ± 1.80 f A	4.86 ± 2.16 ghij A
Carbamate		
Methomyl	8.98 ± 0.48 f A	−0.69 ± 0.59 j B
Pyrethriod		
Fenpropathrin	20.23 ± 0.96 def A	18.19 ± 0.20 ef B
Bifenthrin	17.27 ± 2.84 def A	1.53 ± 0.96 ij B
Lambda-cyhalothrin	14.55 ± 2.84 cde A	4.17 ± 0.79 hij B
Beta-cypermethrin	14.55 ± 2.84 cde B	36.48 ± 3.85 bc A
Other insecticides		
Hexaflumuron	9.66 ± 2.41 f A	13.89 ± 2.75 fg A
Phenyl thiourea	93.41 ± 1.57 b A	67.78 ± 3.74 a B
Fipronil	46.48 ± 7.23 cd A	6.81 ± 0.98 ghij B
Pyridaben	47.05 ± 7.29 cd A	39.91 ± 6.63 b A
Imidacloprid	2.73 ± 1.72 f B	6.67 ± 0.79 ghij A
Acetamiprid	29.09 ± 5.94 d A	11.76 ± 3.63 fgh B
Allelochemical		
Tannic acid	5.91 ± 0.68 f A	−46.39 ± 4.82 k B
Quercetin	571.36 ± 40.46 a A	36.94 ± 3.41 bc B
2-tridecanone	5.23 ± 0.96 f A	9.72 ± 0.79 fghi B

* Means ± SD within columns and followed by different lower-case letters are significantly different (Tukey's test, $P < 0.05$) while means ± SD within rows and followed by different upper-case letters are significantly different (Tukey's test, $P < 0.05$). Data were analyzed using InStat software (GraphPad, San Diego, CA, USA). All experiments were performed in triplicate.

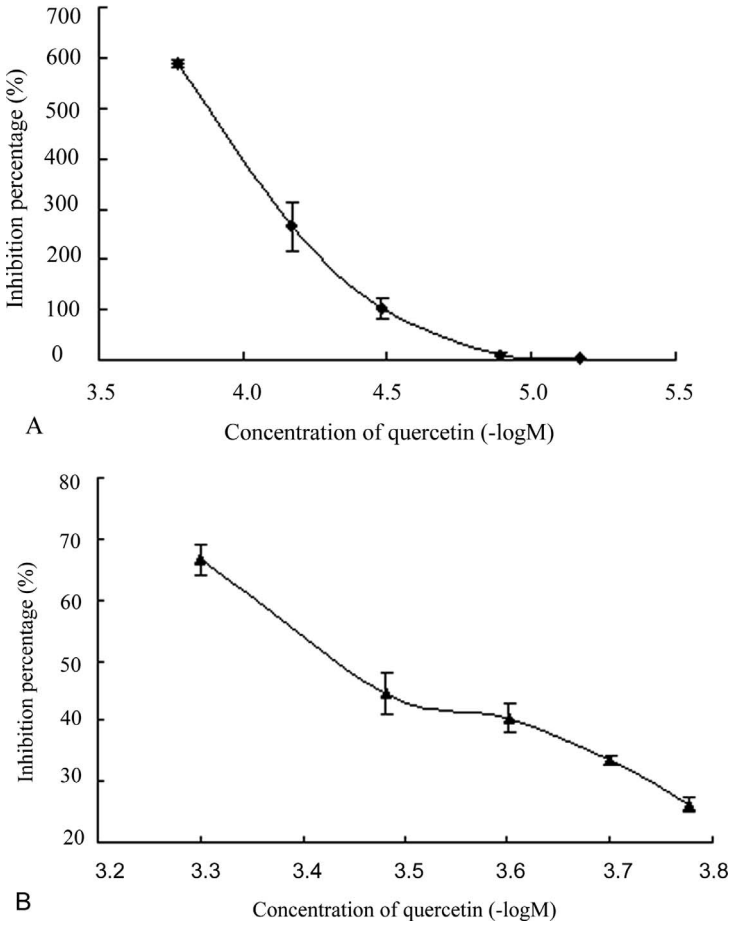

Fig. 1. Inhibition of polyphenol oxidase by quercetin of different concentrations in *C. anastomosis* larvae (A) and poplar (B).

nutritional value and may serve as a component of plant defense mechanisms (Ludlum et al. 1991; Mahanil et al. 2008).

In vitro inhibition assays may help clarify modes of action of insecticides and facilitate the development of novel insecticides as well as serve as a reasonable evaluation of pesticides (Liu et al. 2014). However, there were few studies on the *in vitro* inhibition of polyphenol oxidase in insects. Liu et al. (2004) showed that the I_{50} value of phenyl thiourea for the activity of PPO in *Musca domestica* (L.) was 1.5×10^{-7} M whereas three insecticides (phoxim, methomyl, and imidacloprid) were not significantly inhibitory. Liang et al. (2003) also reported that the I_{50} values of phenyl thiourea for the activity of PPO from both the resistant and susceptible strains of *Plutella xylostella* (L.) ranged from 1.18×10^{-6} M to 1.28×10^{-6} M with no obvious differences. In addition, Tang et al. (2009) showed that the I_{50} values of these three inhibitors (quercetin, phenyl thiourea, and phoxim) for PPO activity in *Micro-*

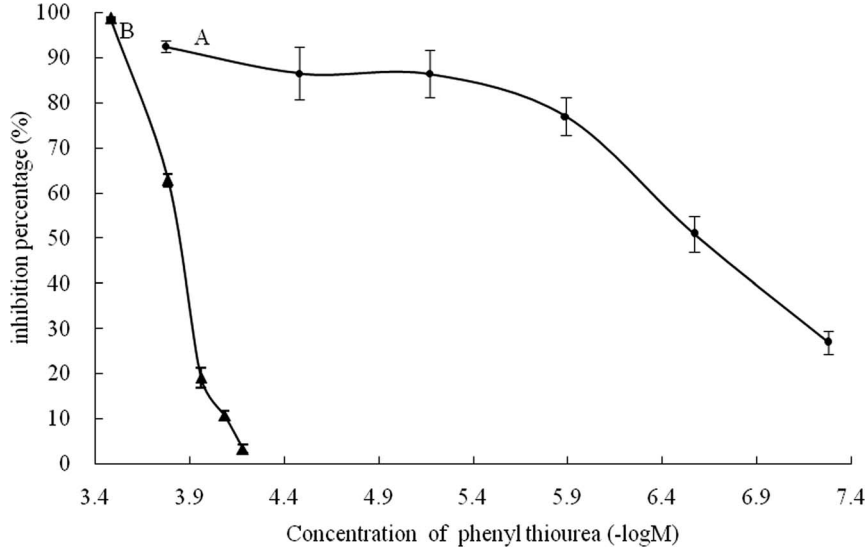

Fig. 2. Inhibition of polyphenol oxidase by phenyl thiourea of different concentrations in *C. anastomosis* larvae (A) and poplar (B).

melalopha troglodyta (Graeser) were 5.24×10^{-5} M, 3.34×10^{-8} M, and 7.25×10^{-5} M, respectively. In our study, we examined the *in vitro* inhibition of 21 selected insecticides and allelochemicals on the activity of PPO not only in *C. anastomosis* larvae but also in poplar. Our results show that quercetin, phenyl thiourea, and

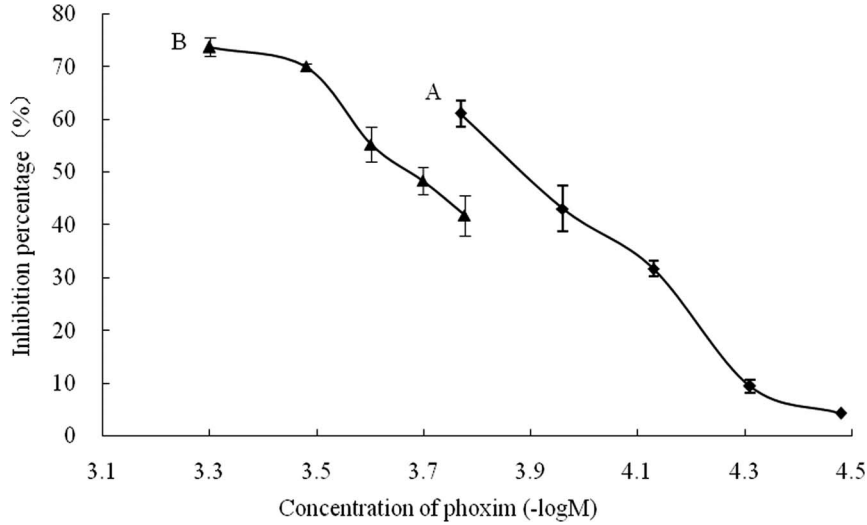

Fig. 3. Inhibition of phenol oxidase by phoxim of different concentrations in *C. anastomosis* larvae (A) and poplar (B).

Table 3. I_{50} values of inhibitors for the activity of polyphenol oxidase in *C. anastomosis* larvae and poplars.*

Inhibitor	I_{50} (1×10^{-4} M) (mean ± SD)	
	Clostera anastomosis	Poplar
Quercetin	$(141.67 \pm 3.21) \times 10^{-3}$ b A	3.35 ± 0.06 a B
Phenyl thiourea	$(1.84 \pm 0.49) \times 10^{-3}$ c A	1.51 ± 0.02 c B
Phoxim	1.28 ± 0.07 a A	2.06 ± 0.19 b B

* Values within a column followed by different small letters are significantly different (Tukey's test, $P < 0.05$) while values within a row followed by different capitals are significantly different (Tukey's test, $P < 0.05$). Data were analyzed using InStat software (GraphPad, San Diego, CA, USA). All experiments were performed in triplicate.

phoxim can strongly inhibit the activity of PPO in both *C. anastomosis* and poplars, but their inhibitory degree differed significantly. The I_{50} values of these three inhibitors for polyphenol oxidase activity were 14.17, 0.18, and 127.67 μM, respectively, in *C. anastomosis* while in poplars the values were 0.34, 0.15 and 0.21 mM, respectively. These results suggest that quercetin and phenyl thiourea might offer a degree of control of insect pests while producing little damage to the host poplar plant.

Our finding that phenyl thiourea was the greatest inhibitor of PPO activity in *C. anastomosis* ($I_{50} = 1.84 \times 10^{-7}$ M) was consistent with that of Tang et al. (2009), who reported the I_{50} value of phenyl thiourea for the PPO activity in *M. troglodyta* was 3.34×10^{-8} M. Liu et al. (2004) and Liang et al. (2003) also reported that I_{50} values of phenyl thiourea for the activity of PPO in *M. domestica* and *P. xylostella* were 1.5×10^{-7} M and 1.18×10^{-6}–1.28×10^{-6} M, respectively. Furthermore, we found that phenyl thiourea was about 1,000 times more inhibitory for the activity against PPO in poplars ($I_{50} = 1.51 \times 10^{-4}$ M) than in *C. anastomosis*. Perhaps these results will serve as a foundation for the development of effective, selective PPO inhibitors and, thus, novel insecticides.

We also found that some allelochemicals might be potent inhibitors of insects. The I_{50} value of quercetin for the diphenolase activity of PPO in *C. anastomosis* was 1.42×10^{-5} M, which was consistent with the findings of others. Tang et al. (2009), for example, reported that the I_{50} value of quercetin for the activity of PPO in *M. troglodyta* was 5.24×10^{-5} M, and Luo et al. (2005) showed that the I_{50} value of quercetin for the activity of PPO in *Spodoptera exigua* (Hübner) was 8.70×10^{-5} M. Further, our results showed that the I_{50} value of quercetin for the activity of PPO in poplars was 3.35×10^{-4} M, which was approximately 23.6 times greater than in *C. anastomosis*. Therefore, quercetin might have the potential of serving as a synergist for enhancing the toxicity of pesticides in *C. anastomosis*.

Zhang and Leng (1993) summarized that an appropriate approach to explore novel insecticide development was to explore biological pathways and that PPO may become a target for controlling insect pests. Therefore, the research on PPO requires further investigation.

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