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Cycloxaprid: A novel *cis*-nitromethylene neonicotinoid insecticide to control imidacloprid-resistant cotton aphid (*Aphis gossypii*)

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ABSTRACT

Imidacloprid is a nicotinic acetylcholine receptor (nAChR) agonist with potent insecticidal activity. However, resistance to imidacloprid is a significant threat and has been identified in several pest species. Cycloxaprid with *cis*-configuration is a novel neonicotinoid insecticide, which shows high activity against imidacloprid-resistant pests. The LC_{50} of imidacloprid against the resistant *Aphis gossypii* was 14.33 mg L^{-1} while it was only 0.70 mg L^{-1} for the susceptible population, giving a resistance ratio of 20.47. In this imidacloprid-resistant population, a point mutation (R81T) located in the loop D region of the nAChR β1 subunit was found out. But this point mutation did not decrease the activity of cycloxaprid against *A. gossypii*. The LC_{50} of cycloxaprid was 1.05 and 1.36 mg L^{-1} for the imidacloprid-susceptible and imidacloprid-resistant populations, respectively. In addition, cycloxaprid provided better efficacies against resistant *A. gossypii* than imidacloprid in the fields. Although cycloxaprid was highly toxic to *A. gossypii*, it showed high selective activity between *A. gossypii* and its predominant natural enemies, *Harmonia axyridis* and *Chrysoperla sinica*. These results demonstrate that cycloxaprid is a promising insecticide against imidacloprid-resistant *A. gossypii* and suitable for the integrated pest management.

1. Introduction

The cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), is one of the important pests of cotton with a global widespread distribution, causing an overall 4% of the insect-induced lint yield reduction annually [1]. This sap-sucking insect is a polyphagous pest that damages cotton through direct feeding and cosmetic damage, and indirectly through transmission of viruses such as cotton bunchy top disease [2,3]. Several insect management practices have been developed to suppress *A. gossypii*, but its control relies almost exclusively on the application of insecticides [1].

A diverse range of chemical insecticides are globally used for controlling *A. gossypii*. With the majority of older conventional insecticides (organophosphates, carbamates, chlorinated hydrocarbons and pyrethroids) affected by resistance to varying degrees, compounds with novel mode of action have been utilized since the beginning of the nineties [4]. The chemical class probably attracting most attention in this respect was the neonicotinoids, introduced in 1991 and with worldwide annual sales of approximately US$ 1.6 billion, accounting for >17% of the global insecticide market [5]. Neonicotinoids are nicotinic acetylcholine receptor (nAChR) agonist with potent insecticidal activity and low mammalian toxicity [6,7]. Among these neonicotinoids, imidacloprid is a leading product and is extensively used for both crop protection and animal health applications [8]. However, owing to lacking of alternatives, resistance to imidacloprid has been reported in several species, including the cotton aphid (*A. gossypii*), the brown planthopper (*Nilaparvata lugens*), the whitefly (*Bemisia tabaci*) and the peach-potato aphid (*Myzus persicae*) [9–12].

Resistance to imidacloprid has been attributed in some cases to increased rates of insecticide detoxification or to mutations in nAChR [12,13]. On recent evidences, it is clear that imidacloprid resistance in both B and Q biotypes of *B. tabaci* is linked to high levels of microsomal P450-dependent monooxygenase activities and the formation of 5-hydroxy imidacloprid as a major metabolite in resistant whitefly strains [8,14,15]. Karunker et al. found that constitutive overexpression (up to 17-fold) of a single P450 gene, CYP6CM1, was strongly correlated with imidacloprid resistance in both B and Q biotypes of *B. tabaci* [16]. Moreover, the functional expression of CYP6CM1 allele confirmed its ability to catalyse a more rapid conversion of imidacloprid to its 5-hydroxy form. The 5-hydroxylation pathway should be highly protective against the insecticidal effects of imidacloprid in *B. tabaci*, since the 5-hydroxy-imidacloprid is approximately 10 times less active than imidacloprid [17]. The alternative mechanism described to date was modification of the target-site [12,18,19]. A point mutation (Y151S) in two alpha subunits (*Nl*α1 and *Nl*α3) of the nAChR was reported to be associated with imidacloprid resistance in a laboratory-selected strain of *N. lugens* [12,20], then the N133D mutation was identified in nAChR

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*Nl*β1 subunit, which resulted in imidacloprid insensitivity [18]. Later, a novel mutation (R81T) in the β subunit of the nAChR was found to be involved in imidacloprid resistance in a field population of *M. persicae* [11]. Meanwhile, this mutation (R81T) was also detected in the laboratory-selected population of *A. gossypii*. This point has been identified to be a key determinant of neonicotinoid binding to nAChRs and the mutation might confer the mechanism of imidacloprid resistance in *A. gossypii* [21].

Due to the development of resistance, imidacloprid is repeatedly applied to crop plants during a single growing season. This practice creates environmental, ecological and economic problems. Therefore, new insecticides with novel modes of action are essential for rotation with imidacloprid to minimize its resistance. Cycloxaprid, 9-((6-chloropyrid-3-yl)methyl)-4-nitro-8-oxa-10,11-dihydroimidazo-[2,3-a]-bicyclo- [3,2,1]-oct-3-ene, is one of the novel synthesized neonicotinoid insecticides (Fig. 1) [22]. Uniquely, the nitro substituent of cycloxaprid is in the *cis*-configuration, whereas in all other commercialized neonicotinoids the nitro is in the *trans*-configuration [23]. Furthermore, cycloxaprid is especially effective against imidacloprid-resistant pests, showing a 50-fold higher activity against imidacloprid-resistant brown planthopper than imidacloprid [24]. Therefore, cycloxaprid has attracted wide attention as a promising insecticide to control imidacloprid-resistant insect pests [25]. The aims of this paper were to study the insecticidal activity of cycloxaprid against the imidacloprid-resistant *A. gossypii* and to reveal the mechanism of imidacloprid resistance. In addition, the toxicity of cycloxaprid to the natural enemies (*H. axyridis* and *C. sinica*) was also studied.

2. Materials and methods

2.1. Insecticides and chemicals

The technical insecticides used for bioassays included cycloxaprid (97%, East China University of Science and Technology), imidacloprid (95.5%, Jiangsu Kesheng Co. Ltd., China) and thiamethoxam (98%, Shandong hailier chemical Co. Ltd., China). Formulated insecticides used for field trials included cycloxaprid 25% WP (Shanghai Shengnong Pesticide Co. Ltd., China), imidacloprid 10% WP (Nanjing Red Sun Co. Ltd., China). Dimethylsulfoxide (DMSO) and triton X-100 were purchased from Beijing chemical reagent Co. Ltd.

2.2. Insects and plants

The susceptible *A. gossypii* samples were reared on cotton plants (Zhongzhi 8) in the laboratory under insecticide-free conditions, and the resistant population was obtained from the experimental cotton field in Shandong, China. The insects were maintained in a greenhouse under controlled conditions at 25 ± 2 °C, 70 ± 20% RH and a 14:10 h light:dark photoperiod.

H. axyridis larvae were collected from the hibiscus in Institute of Plant Protection, Chinese Academy of Agricultural Sciences, and *C. sinica* larvae were purchased from Beijing natural enemy company. They were stored separately in glass tubes (17 mm diameter × 80 mm high), and supplied with live cotton aphids. They were raised under conditions at 25 ± 2 °C, 65 ± 5% RH and a photoperiod of 16:8 h (L:D).

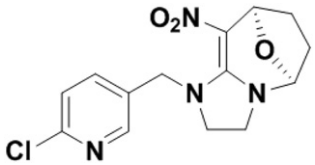

Fig. 1. Chemical structure of cycloxaprid.

2.3. The toxicity of cycloxaprid, imidacloprid and thiamethoxam against the susceptible and resistant A. gossypii

The leaf dip method was used to evaluate the toxicity of cycloxaprid against *A. gossypii* under laboratory condition. The stock solution of cycloxaprid (10,000 mg L^{-1} in DMSO) was diluted using an aqueous solution of 0.05% (*w*/*v*) triton X-100 to concentrations of 20, 10, 5, 2, 1, 0.5 and 0.1 mg L^{-1}. Imidacloprid and thiamethoxam solutions of 200, 100, 50, 20, 10, 5, 1, 0.5 and 0.1 mg L^{-1} were prepared with the same method. The concentrations replicated thrice were tested for each insecticide. Individual cotton leaves infested with about fifty *A. gossypii* were dipped in cycloxaprid, imidacloprid or thiamethoxam solutions for 3 s and dried on tissue paper. Afterwards individual leaves were transferred to 90 mm petri dishes containing a water-moistened filter paper. Each petri dish was covered with a perforated lid with fine mesh to provide ventilation. The petri dishes were then stored in an incubator at 25 ± 2 °C, 70 ± 20% RH and a 14:10 h light:dark photoperiod for 24 h until mortality was assessed. Control aphids feeding on aqueous triton X-100-treated leaves showed <10% mortality in all bioassays.

2.4. Amplification and sequence analysis of A. gossypii nAChR β1 subunit cDNA fragments

Total RNA was extracted from 10 mg pooled *A. gossypii* from imidacloprid-resistant and susceptible populations using RNeasy® Mini Kit (Qiagen, Germany) following the manufacturer's protocol. The cDNA template for PCR was synthesized from 1 μg of total RNA using the Primescript™ First-Strand cDNA Synthesis kit (Takara, Dalian, China) according to the manufacturer's instructions. The available *A. gossypii* nicotinic acetylcholine receptor β1 subunit sequence (GenBank accession AF527786) was used as a template to design primers. Primer pairs for amplifying fragments covering conserved domains (loops A to F) that comprise the acetylcholine and neonicotinoid binding site were shown in Table 1. LA Taq® master-mix and primer pairs were used to PCR amplify fragments. The thermal cycling for the PCR reaction was: initial denaturing at 94 °C for 2 min, 30 cycles at 98 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s, and an additional polymerisation step at 72 °C for 10 min. The amplified products were cloned into pMD19-T Simple Vectors (Takara, Dalian, China) and sequenced by BGI (Beijing, China). Assembly of sequencing results and alignment graphics were done using DANMAN software.

2.5. Toxicity of cycloxaprid and imidacloprid to H. axyridis and C. sinica

Bioassays were conducted with 3rd instar larvae of *H. axyridis* and *C. sinica* using glass tube residual film method [26], with slight modifications. Glass tubes (1.7 cm diameter, 8 cm length) were rotated rapidly with 300 μL insecticides dissolved in acetone, and allowed to air dry for about 2 h at room temperature. Then individual 3rd instar larvae of *H. axyridis* and *C. sinica* were confined in these glass tubes and exposed for 1 h. After exposure, the larvae were transferred into clean glass tubes with cotton aphids. At least six concentrations replicated thrice were tested. Controls were treated with acetone alone. The experiments were kept under a standard constant environment (25 ± 2 °C, 65 ± 5% RH and L:D 16:8 h). Mortality was assessed after 24 h and 48 h exposure to insecticides. Bioassays with control mortality exceeding 10% were discarded and repeated.

Table 1
Primers used for PCR.

Gene	Sequence (5′-3′)
nAChRβ1-F	ACGAGAAGCGTCTGGTTAG
nAChRβ1-R	GTCCGTCTGGGTAGGTGA