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Insecticidal and *P450* mediate mechanism of fluralaner against Red

Imported Fire Ant, *Solenopsis invicta* (Hymenoptera: Formicidae)

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Abstract

The increasing consumption of pesticides caused lots of problems, such as resistance, food safety, and various environmental toxicity. Finding an alternative pesticide which is environmentally friendly is urgent and crucial. Fluralaner, a novel isoxazoline insecticide, has been proven to possess selective toxicity for insects versus mammals and have safety to mammals and nontarget organism, suggesting its potential in pest's management. However, little information is available about its effect on the red imported fire ant (*Solenopsis invicta*), a worldwide invasive and polyphagous pest, often nest in neighborhood area. The toxicity results showed that the topical application and feeding application was all effective to *S. invicta*. Moreover, fluralaner can be transmitted among workers by contacting and feeding which lead to toxic reaction among nestmates. By exploring the biochemistry change, we found cytochrome *P450* monooxygenase (*P450*) may be involved in the detoxification of fluralaner but not carboxylesterase (*CarE*) and glutathione S-transferase (*GST*). Synergism assays gave a solid evidence in which piperonyl butoxide, an activity inhibitor of *P450*, increased the toxicity of fluralaner to *S. invicta*. Importantly, with the qRT-PCR analysis 12 *P450* genes of *S. invicta* were significantly upregulated after fluralaner treatment. Our result indicated that fluralaner could be a potential alternative pesticide in *S. invicta* control. And the involvement of *P450s* on metabolism provides basic information for the sustainable use of fluralaner.

Keywords: Fluralaner; Horizontal toxicity; Cytochrome *P450* enzyme; *Solenopsis invicta*;

Introduction

In modern agriculture, the use of pesticides is still an effective tool on crop

production because it provides much faster, more targeted, and effective control of pests and diseases. In the meantime, the increasing consumption of pesticides caused severe problems to environment and human health due to the residual or bioaccumulation, even to pest control due to pest resistance or negative impact on non-target arthropods¹⁻³. Developing a new pesticide which is safety to non-target organisms and easy to degrade might weaken the effect caused by the problems mentioned above. However, the research of new pesticide is time-consuming process costly⁴. Digging out an alternative chemical in the exist chemistries is more helpful to face the challenge in pest management.

Fluralaner (CAS 864731-61-3), a novel isoxazoline insecticide, inhibitors of γ -aminobutyric acid (GABA)-gated chloride channels (GABACls) and L-glutamate-gated chloride channels (GluCls). It was firstly synthesized by Nissan Chemical Industries⁵, and it has been marketed as BRAVECTO® in Europe, America and East Asia for its excellent activities on parasitic and sanitary pest. In previous studies, Fluralaner has been used orally to dogs, cat, sheep and other production animals in the management of *stomoxys calcitrans*, *Aedes aegypti* and *Siphonaptera* etc.^{6,7}. And the vitro contact activity of fluralaner is better than spinosad, phoxim, propoxur against *Ornithonyssus sylviarum*⁸. High activities also have been found under lab conditions against multiple agricultural pests such as *Tetranychus urticae*, *Chilo suppressalis*, *Spodoptera litura*, *Frankliniella occidentalis*^{9,10}, urban pests as *Blattella germanica*, and stored product insect like *Tribolium castaneum*¹¹. Besides, fluralaner and its formulated product are of low toxicity to non-target organisms, such as zebrafish¹². Mechanism research indicated this chemical has different action site between mammals and insect, it was more active and toxic toward insects than animal¹³, this make it a promising green agrochemical.

Red imported fire ant (*Solenopsis invicta*) is a dangerous invasive insect worldwide, which caused huge damage in invaded place, including reduction of native arthropods amount, losses of agricultural production, as well as threat on human health¹⁴⁻¹⁶. *S.invicta* prefers to build their nest in disturbed neighborhoods, this means more vulnerable environment, which is extremely sensitive to pesticides¹⁷. The two-step method is an alternative control strategies used for the whole colony management of *S.invicta*¹⁸. Low toxic bait is the key to ensure the contact of most colony members with insecticide. With its underground nest habit and intensive contact with living activities, the chemical choice against *S.invicta* is limited. Fipronil is the most popular bait used in *S.invicta* management, but it has been restricted or totally forbidden in

many countries for its high toxicity to non-target pest especially for aquatic organisms^{19,20}. Thus, novel, low-toxic, and environmentally friendly pesticide is substantially needed for the controlment of this complicated pest.

Although studies have demonstrated fluralaner has significant activity against wide range of insects, the effects of fluralaner on social insects are still unknown. In our present study, fluralaner insecticidal activity through both contact and feeding exposure against *S.invicta* were taken on workers. Moreover, to evaluate the potential of this new chemical as an active bait ingredient, trophallaxis effects among *S.invicta* colony after taking fluralaner was conducted, using rubidium as the tracer. Synergism assays using three main detoxification enzyme synergists, including piperonyl butoxide (PBO), diethyl maleate (DEM), and triphenyl phosphate (TPP) together with enzyme activities comparison before and after exposure to fluralaner at sublethal doses were conducted to investigate the possible metabolism mechanism. and qRT-PCR were assessed aim to know the *P450* gene induction of this novel chemical in *S.invicta*.

Results

Topical toxicity of fluralaner against *S.invicta*. The contact toxicity of fluralaner against *S. invicta* was determined by a topical application method. The mortality of *S. invicta* increased with dose- and time-dependent effects, with the 24-h and 48-h LD₅₀ values were 7.99 and 2.21 ng/insect, respectively (Table 2). In comparison, the LD₅₀ values for fipronil were 1.11 ng/insect at 24h and 0.44 ng / insect at 48h, respectively.

Feeding and Synergist bioassay against *S.invicta*. Feeding bioassays showed the consistent result, with 24-h LC₅₀ value of fluralaner was 15.61 mg /L, 48-h LC₅₀ values was 8.63 mg /L (Table 3). Synergism effects of PBO, DEM, and TPP were topical pre-treat 4h before feeding assay. PBO and DEM displayed statistically significant synergism on fluralaner toxicity, with the lower LC₅₀ value than the no synergist treatment group. And the synergist ratios of PBO and DEM was 1.53, 1.36 at 24-h, 1.62, 1.59 at 48-h, respectively. In contrast, TPP displayed no synergist effect on fluralaner toxicity, as the 95% confidence intervals of fluralaner treatment and TPP pre-treat group overlapped (Table 3).

Effect of fluralaner on toxicity transmit. Horizontal toxicity transmit effects were conducted to determine whether fluralaner toxicity can be horizontally transferred among workers after topical expose. Both the cumulative mortality of donors and recipients was increased within 48h, and exerted dose-dependent effects, but the transmit rates between donors and recipient were not different ($P>0.05$, ANOVA)

(Table 4).

Effect of fluralaner on food exchange. Donor workers were fed with Sublethal dose of fluralaner to know the effect on reciprocal food exchange and nutrition transmission among colony using rubidium as the food tracer. After feeding for 24h, food consumption was unaffected by fluralaner compared with controls (Fig.1A). For recipient ants, worker recipients exhibited no difference from control. Female and larvae recipients get more food from donors in the presence of sublethal dose of fluralaner (Fig. 1B). Besides, worker donor and larvae recipient showed notable difference in food transmit ratio compared with control (Fig.1C).

Enzyme activity comparisons after feeding with sublethal dose of fluralaner. The enzyme activities results showed that the *P450* activity but not *GST* and *CarE* were significantly different from controls (Fig. 2A,B). The activity of *P450* monooxygenase activity was 2.69-fold higher after sublethal dose fluralaner treatment (2502.67 nmol / min/mg · prot) compared with control (803.75 nmol/min/mg · prot). Meanwhile, PBO pre-treat inhibits *P450* enzyme activity notably (Fig.2B).

Fluralaner-induced expression of *P450* gene of *S.invicta*. Based on qRT-PCR analysis, the expression of twelve *P450* genes were significantly upregulated after LC₅₀ concentrate fluralaner feeding for 24hours compared with the solvent treated group (Figure 3). The relative expression of *CYP4G323*, *CYP336A44*, *CYP301A1*, *CYP305D1v1* and *CYP306A1* were more than 4-fold higher than control treatment. *CYP4S1* and *CYP336A45* were significantly downregulated after fluralaner treated.

Discussion

Fluralaner, a potent isoxazoline insecticide, has been confirmed with high activity towards wide spectrum of pest both agriculture and sanitary pest^{10,11,21}. In present study, topical toxicity and feeding toxicity assays all showed a lower toxicity against *S.invicta*, than fipronil (Table 2), and the LC₅₀ value of feeding toxicity was 15.61, 8.63 mg / L (Table 3). Fipronil was a universal active ingredient used in *S.invicta* controlment, and its LD₅₀ value was 1.11, 0.44 ng/insect after treated for 24, 48h (Table 2). Compared with fipronil, fluralaner showed a delayed toxicity and is more safe on non-target organisms^{20,22}. In social insects management, relatively delayed toxicity is needed for sufficient amounts of toxicant to be delivered among the colony^{23,24}. In studies of Zhao et al¹¹, fluralaner showed higher contact activities toward many agricultural insects compared with fipronil, with LD₅₀ value varies from 22.3 to 107.0 ng/insect at 24h, but for the German cockroach fluralaner showed lower contact activity, this was consistent

with our findings, and we speculate that this might be because the thicker cuticles than other insects. In other studies, Fluralaner showed high toxicity to fipronil resistance strains, with LC₅₀ values of 7.6 and 10.0 mg/L to *Laodelphax striatellus* susceptible and resistance strains, respectively ²⁵, and the toxicity of fluralaner to susceptible and resistant strains of *D.melanogaster* strains was equivalent ⁶. So, fluralaner may be a good alternative of fipronil, owing to the differential actions of fluralaner and fipronil on the GABAR ²⁵. Many other pesticides have been tested against *S.invicta*. Chen *et al.* found three benzoates, benzylbenzoate, n-pentybenzoate, and n-hexylbenzoate showed potent contact toxins against *S.invicta* workers, with LD₅₀ value = 23.31, 35.26, 35.99 µg/ant ²⁶, much higher than fluralaner in present study, this indicated fluralaner is a more promising isoxazoline insecticide.

Toxicity transfer of insecticides occurs after insects contacting or ingesting an insecticide, and transfer it to other conspecific insects through trophallaxis, necrophoric, contact and mutual grooming ²⁷⁻²⁹. This is a common phenomenon in many social insects. In our present study, fluralaner was added in food, and Rb content in donors and recipient did not show much difference, this may indicate that this chemical do not affect the food sharing in *S.invicta* colony (Fig 1), which may be beneficial for toxicity transmit along with food consumption. The topical horizontal toxicity results confirmed that after fluralaner topical treatment, toxicity could be transferred from donors to recipients, and the transmit ratio between donors and recipient did not show any does dependent differences (Table 4). Horizontally transfer is an important index to evaluate whether this insecticide is a good candidate for ant control. For example, after exposed to contact insecticides, fipronil was readily horizontally transferable than bifenthrin, β -cyfluthrin, and chlorfenapyr. When ants contacted contaminated crops and the dead ants, the mortality occurred, this may resulted from necrophoresis behavior ^{28,30}. Similar study results also been reported by Wiltz *et al*²⁷, and they think after treated, bifenthrin may has a reduction in recruitment effect among nestmates, this may hamper the effectiveness of pesticide. Trophallaxis, the main food sharing behaviors in social insects, also is an important factor for cohesion and crucial communication process ^{31,32}. In trophallaxis process ants ingest and store a liquid toxic bait in food and then regurgitate it to other nestmates ³³. In Furman's study, RFIA can transmitted indoxacarb toxicity between RFIA workers and brood by trophallactic behaviors ³⁴. Neoh *et al*, found after exposed to chlorantraniliprole termitea were likely to cease feeding and undergo starvation, which made the pesticide low efficiency ³⁵. Because in the management of social insect, repellent or fast-acting toxicant may provide effective

barriers³⁶. Non-radiolable rubidium (Rb) have been used as a good trace in termites' trophallaxis process³⁷⁻³⁹, but had not yet been reported in *S.invicta*. In our study, Rb can be a good indicator of food allocation. Our study results indicate fluralaner can be a good candidate for *S.invicta* controlment.

The information about the metabolism of fluralaner in insects is limited, especially in insect. In present synergetic assay indicated that PBO and DEM displayed significant synergism on fluralaner toxicity during all the observation periods (Table 3). And enzyme measurement result confirmed that P450 monooxygen showed 2.69-fold higher activity was founded after LC₃₀ fluralaner treatment compared with control (Fig.2). Quantitative analysis showed twelve P450s genes were significantly up regulated by fluralaner (Fig.3). PBO is a potent P450 monooxygen and esterase inhibitor and can synergise insecticide through phase I metabolic enzyme systems^{40,41}. And synergists could be used to know the enzyme detoxification mechanisms of the chemicals roughly. Also, enzyme synergists can be used as a potential cofactor with pesticide. In previous studies Liu et al. found that after fluralaner treatment the transcript level of P450s significantly elevated⁴², the study in *Danio rerio* also been implied the same hypothesis that P450s' participating in the metabolism process of fluralaner²². The transcription up-regulation of P450s may lead to the enhancement of detoxifying insecticides in the resistance process⁴³⁻⁴⁵. *CYP4M14*, *CYP6B47*, *CYP6B48* and *CYP6B58* of *Spodoptera litura* was up-regulated at different time interval after LD₁₅ dose of fluralaner treatment⁴². And the transcriptome analysis of fluralaner responsive P450s was 43 and 30 after LC₃₀ and LC₅₀ exposure to fluralaner in *S.litura*⁴⁶. The fast response of mRNA expression level of *CYPs* to the sub-lethal concentration of fluralaner could be used as a biomarker in zebrafish²². These studies suggested that *P450* genes may be involved in detoxification of fluralaner, this also consistent with our results.

In conclusion, the fluralaner exhibited delayed toxicity against *S.invicta* both contact and feeding assay. Also showed horizontal transmission effect and did not affect the food transmutation. And P450 may play a role in the fluralaner metabolism process. These results implied that fluralaner could be used as an environmentally friendly chemical against *S.invicta*.

Materials and Methods

Chemicals. Fluralaner (864731-61-3) was purchased from MedChem Express (China), 99.87% purity. Diethyl maleate (DEM), piperonyl butoxiden(PBO), and Triphenyl phosphate (TPP) were obtained from Sigma-Aldrich Chemical Co. Acetone

and other used solvent were bought from Aladdin Chemical Co. Fast blue B salt (*O*-dianisidine, tetrazotized), α -naphthyl acetate (α -NA), β -naphthol, β -naphthyl acetate (β -NA) were purchased from Sigma (China). Rubidium chloride (99.5%) was bought from Aladdin Bio-Chemical Co (Shanghai, China).

Insects. The red imported fire ant (*Solenopsis invicta*) polygyne colonies were collected at the campus of South China Agricultural University and the experimental field of South China Agricultural University in Zengcheng (23.16' N, 113.23' E), during September to November 2016 and March to May 2017. Colonies were removed with water floating method and placed in plastic boxes (50cm length×40cm width×30cm height). Teflon was applied to the inner of the boxes to prevent fire ants from escape. The colonies were reared with a diet of mealworms larvae *Tenebrio molitor* and water tube (plugged with cotton). Before experiments, the colonies were reared in lab at least 2 weeks to acclimatize to the laboratory environment before conduction. All experiments were conducted at 26 ± 1 °C and $60 \pm 1\%$ humidity.

Topical bioassay. Topical toxicity of Fluralaner was assayed using thirty adult workers (body length 3-5mm) for a single test, in total ninety insects for each concentration, follow the method of Xiong et al⁴⁷. Fluralaner of six concentrations (0.50, 1.00, 2.00, 4.00, 8.00, 16.00 mg/L) in acetone was applied (0.5 μ L) on the thorax of each ant using a hand micro applicator (Burkard Rickmansworth England), fire ant treated with equivalent acetone were serve as the control. The concentrations of the treatment were based on pre-experiment. Each treatment was replicated for three times. Workers were considered dead as their antenna does not move under a slight touch of brush.

Feeding and Synergist bioassay. Feeding activity assay was taken with a series of fluralaner added in honey water (honey: water =1:9). Each bioassay consisted of six doses (1.00, 2.00, 4.00, 8.00, 16.00, 32.00 mg/L) with three replicates and 50 individuals per replicate, 900 insects in total. Synergist assays were taken against *S.invicta* using PBO, DEM or TPP at 100 ug/mL and pre-treated using topical method 4h before fluralaner feeding treatment referred to the method of Jiang et al ⁶. Control group were feed with 10% honey water only. After 24-, 48-hours, the mortality of the *S.invicta* were recorded.

Horizontal transfer of fluralaner. Horizontal transfer effect of fluralaner was taken to determine whether fluralaner toxicity could be horizontally transferred among individuals in colony of *S.invicta* followed the method of Choe et al with some changes ^{30,48}. A total of 600 *S.invicta* workers were marked on the abdomen with non-toxic ink. Then, 50 insects were applied (0.5 μ L) with fluralaner at 0.0, 2.5, 5.0, 50.0 ng / insect on the thorax, respectively, using a hand micro applicator (Burkard Rickmansworth England). Thereafter, 50 donor-ants

were transplanted into a cylinder plastic box (diameter 7.5mm, height 6.5mm) each box with another 50 recipient-ants (have been starved for 24h), each concentration replicated for three times. And the mortality of both donor-ant and recipient-ant were recorded after 24- and 48-hours.

Food transmissions affect by fluralaner. Mutual interactions between fluralaner treated ants and untreated nestmates were measured to see the influence of this chemical on *S.invicta* colony food transmission, using rubidium as a tracer ³⁷⁻³⁹. Prior experiments were conducted to know the palatability and toxicity of rubidium control on *S.invicta* and have proved 8000 mg/L rubidium is suitable for current experiment.

Three groups of experiment were conducted using a simple donor-recipient model ³⁹. In worker-worker group, innocuity paint was used to distinguish donors from recipients. Fire ants from three different colonies were chose as the duplication. In each experiment 50 donor worker fire ant were starved for 24 hours, then fed with 1ml 10% honey water contain LC₃₀ (9.17mg/L) fluralaner together with 8000 mg/L Rubidium fluoride for 24 hours. Control group were fed with 1mL 10% honey water contain 8000 mg/L rubidium fluoride only. After that, the test tube was removed and another 50 starveling pre-treated workers or 30 starveling pre-treated larvae or 15 starveling pre-treated female were transplanted into the box as the recipients, 24h later, the donors and recipients were frozen at -20°C and then placed in silica beads test tube, and dried at 65°C for 24h respectively. The dried *S.invicta* were digested with 1.0 mL of 65% nitric acid for 24h. And after that 0.5 mL of 60% perchloric acid was added to each tube and heated in a 95°C water bath for 30min, cooling in room temperature, thereafter, 20mL of deionized water were added to each sample. The Rb content of each sample were determined using inductively coupled plasma optical emission spectrometry (ICP-MS) (VARIAN, 710-ES, USA).

Enzyme extraction and analysis. Thirty adult workers were homogenized, 24h after feeding LC₃₀ does fluralaner using a vitreous homogenizer (diameter = 0.8 cm) in 300μL of ice-cold phosphate buffer (0.1 M, pH 7.5, containing 0.1% Triton X-100) in a 1.5mL centrifuge tube. The homogenate was centrifuged at 8,000 g at 4°C for 10 min. Thereafter, the supernatant was collected and diluted appropriately with addition of homogenization buffer without Triton X-100 and used as the sources of enzymes. Protein concentration was determined after the method of Bradford ⁴⁹.

α -Naphthyl acetate (α -NA) and β -naphthyl acetate (β -NA) were used as substances to determine carboxylesterase (CarE) activity ¹⁹. The reaction mixture included 135μL of substrate (0.30 mM α -NA or β -NA, 3×10^{-3} mM physostigmine), 20μL of enzyme solution. After incubating at 37°C for 30 min, 45μL mixture solution of 1.0% fast blue B-salt in 5.0%

SDS (2:5) was added in each microplate. After another 15 min incubation period at room temperature, absorbance was measured at 600 and 560 nm. The activity of CarE activity was reported as nanomoles of product formed / min / mg · protein via comparing to the α -naphthol or β -naphthol standard curve.

The activity of Glutathione S-transferase (GST) was determined using 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) following the method of Salman et al⁵⁰. The reaction mixture in each microplate well contained 15 μ L of enzyme solution and 185 μ L of CDNB-GSH solution [CDNB 200 mM, 10.35 mM GSH in phosphate buffer (0.1 M, pH 7.5) in 2:188 (v/v) ratios]. The absorbance was measured kinetically for 5min at 340 nm at 25°C. Enzyme solution was replaced with fifteen microliters of phosphate buffer (0.1 M, pH 7.5) as a substitute served as control. Four replicates were carried out for treatment and control. The activity of GST was determined as nanomoles of conjugate produced / min / mg · protein.

Cytochrome P450 activity was determined using p-nitroanisole (PNOD) as the substrate described by Stegeman and Kloeppersams⁵¹. The reaction mixture contains 150 μ L of 0.01 mM PNOD, 100 μ L enzyme solution and 15 μ L of 9.6 mM NADPH. Then incubated at 30°C for 30min and the absorbance were determined at 405 nm at 30°C. The activity of cytochrome P450 was determined as nanomoles of *p*-nitrophenol/min/mg · protein based on a standard curve of para-nitrophenol.

Expression pattern of P450s under the pressure of fluralaner. The relative expression of 36 *P450s* mRNAs of *S.invicta* before and after LC₅₀ fluralaner treatment was determined using quantitative real-time PCR (qRT-PCR) with SYBR-green fluorescence and CFX96 Real Time PCR Detection System (Bio-Rad). The gene-specific primers for the 36 *P450* genes were designed using primer3 input ([http:// primer3.ut.ee/](http://primer3.ut.ee/)) (Table1), and related reference genes *18sRNA* were synthesis followed Zhang's study⁵². The RNA extraction was performed using TRIzol Reagent from LC₅₀ fluralaner treated (for 24hours) or solvent treated (for 24hours) *S.invicta*, respectively. Agarose-gel electrophoresis and NanoDrop 2000 spectrophotometer was used to quality and quantify the resulting total RNA. The PrimeScriptTM RT reagent Kit with gDNA Eraser (Takara, Dalian, China) was used for cDNA synthesis using 1 μ g of total RNA. The qRT-PCR reactions (20 μ L) contained 10 μ L of TaKaRa SYBR® Premix Ex TaqTMII Mix, 1 μ L of each primer, 1 μ L of diluted cDNA and 7 μ L of nuclease-free water according to the manufacturers protocol(Takara, Dalian, China). The relative gene expression was analyzed using the $2^{-\Delta\Delta C_t}$ method as described by Livak and Schmittgen⁵³. The expression level of solvent treated *S.invicta* was used as the calibrator. The significance of differences between two treatment was evaluated using a one-way ANOVA

(SPSS version 17.0) followed by the Duncan's multiple range test at 0.05 level. Three biological replicates and three technical replicates were performed in each qRT-PCR reaction.

Statistical Analysis. Toxicity bioassays were analyzed using the probit procedure of the SPASS program. The mortality was corrected using below formula:

Corrected mortality (%) = [survival control (%) - survival treated (%)] $\times$ 100/survival control (%).

One-way ANOVA with means separated using Tukey's honestly significant difference (HSD) test were used to analyze the differences of detoxifying enzymes among castes and development stages of *S.invicta* at $p = 0.05$ level.

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Author Contributions

T, Xiong., X. N., Zeng conceived and designed the experimental plan. T, Xiong. Wrote the main manuscript text and conducted the experiments and analysed the results. S Q., Ling and J L., Liu provided some suggestions for revision and corrected the grammatical mistakes. X. N., Zeng supervised the study. All authors reviewed the manuscript.

Additional Information

Competing Interests: The authors declare no competing interests.

Table captions

Table 1. Specific primers used in *P450* genes real-time quantitative PCR of *S.invicta*

Table 2. Contact toxicity of fluralaner on workers of *S.invicta*

Table 3. Feeding toxicity and synergism effects of fluralaner against *S.invicta*

Table 4. Horizontal toxicity transmit of Fluralaner between workers of *S.invicta*

Table 1. Specific primers used in *P450* genes real-time quantitative PCR of *S.invicta*

Gene Name	Sequence (5'-3')	Product size(bp)	NCBI Accession No
<i>CYP4G245</i>	Sense: ATCGCAAATTGATCGCACCG Anti sense: TCTTGCGTGGACTTGGAGAC	199	MT732287
<i>CYP4AB78</i>	Sense: CGTTCATTGCGCCACTAATCCA Anti sense: TTGCCATTTTGCTCCTTCGC	192	MT732283
<i>CYP4AB79</i>	Sense: AAGAAGTCCAGGAGCGTGTC Anti sense: CGATATAGCAGGAACACTGGGAT	152	MT732284
<i>CYP4AB75</i>	Sense: GGATCTTTTGATAGCGGAGGC Anti sense: ATTGCTACGGTATCGTGCCC	102	MT732285
<i>CYP4AB76</i>	Sense: TCACGAATTACTACGGAAGAAGCA Anti sense: GCGGACCAGCACTAAATGGTA	199	MT732281
<i>CYP4BW9</i>	Sense: TTCGGTTGCTGTATGCTGGA Anti sense: CTTTACACTGGCTGGGGTCT	118	MT732279
<i>CYP4CY5v1</i>	Sense: TGACCAAGCCGTGGGAAATA Anti sense: ATCAAACATTGGACCATCGACA	194	MT732276
<i>CYP4AA7</i>	Sense: TCAACATGAACGACCCGTCA Anti sense: AGGGTAATTGCAGTCGCTGT	106	MT732289
<i>CYP4AV12</i>	Sense: GCCTTGCTGGATTTGCTGTT Anti sense: CCATCGTATCGTGTCCAGCA	109	MT732286
<i>CYP4G232</i>	Sense: GACTTCGCGATGAGGTTGGA Anti sense: TCGTAATGCCTTCGGGCTAC	178	MT732288
<i>CYP4CSJ1</i>	Sense: TACTTCTTTGGGACGCGAACA Anti sense: AAGTGCTTCCAACAGCATGTC	160	MT732277
<i>CYP6AS164</i>	Sense: TCCTATTTTCGCGCTTCAACAC Anti sense: TGGATGCCTACTTCGCACAG	100	MT732294
<i>CYP6SQ20</i>	Sense: TGCGAACGAAGATCACACCT Anti sense: GAGCGACTGAAGCGATCAGA	186	MT732299
<i>CYP6BD27</i>	Sense: ACTTGACGATCTGGCTGAA Anti sense: AAATTCTGGCGACGGTGTGT	143	MT732300
<i>CYP6AQ57v1</i>	Sense: TACGATTCGCGGCTTTGAGA Anti sense: CGCATAAAATCCGAGGGCTG	102	MT732302
<i>CYP6AS161</i>	Sense: ATTCCGCTCCAGTCCAACAG Anti sense: TGCTTCGTGTAATGTCCGTCA	128	MT732297
<i>CYP336A44</i>	Sense: GGCTAAATGGCAGGAAACGC Anti sense: CGGCTACCACTTGTGCAGTA	189	MT732304
<i>CYP6AS160</i>	Sense: CGAAAATATCCGCCAGTGACA Anti sense: TGTTTTGCACAGCTTCGTCG	193	MT732294
<i>CYP336A45</i>	Sense: TCTGTTTCGAGACCGTCAAGC Anti sense: GCCGAAGCCAACGGTAGATA	145	MT732305
<i>CYP6ES8</i>	Sense: AACAGAGAAAAGCTGGGGTTC Anti sense: TGACGCGCCAGATCGTATAG	191	MT732301

<i>CYP6BE22</i>	Sense: CCATTCCCATTACGCATCGAA Anti sense: TGCCTGTTGCTTCTACCAT	193	MT732292
<i>CYP9R69</i>	Sense: ACGGTAAAGAAGCCACCACT Anti sense: GCAACATATCAGGGCGAACG	192	MT732306
<i>CYP9R73</i>	Sense: TATCCGAGTGCGCTGTGAAT Anti sense: CAAAGGCGCATGAGGCAATTA	123	MT732308
<i>CYP9R75</i>	Sense: TTCGCGATCCGGAACATCATC Anti sense: AGTTCTCACGTTCCGCCATT	146	MT732309
<i>CYP9AS16</i>	Sense: AACTCTCGACCCGATAGCCA Anti sense: AAAGTTTTTCGGCGCACTGAC	145	MT732311
<i>CYP9R68</i>	Sense: AATGGAGTATCTGGACGCGG Anti sense: AGGAACGAACCAAACGCTCA	160	MT732307
<i>CYP9P26</i>	Sense: TACACCACTTTGCCCCGAACA Anti sense: CAACGATCTCCGCGTAAGGA	236	MT732312
<i>CYP315A2</i>	Sense: ATGTCAGGAAAGCGCCAAGA Anti sense: TCAGAACGTAGTTGCGGCTT	168	MT732323
<i>CYP301A1</i>	Sense: AACTGTTCATTGCGGTCCT Anti sense: CCTCGTCGTACGGTCTAACG	143	MT732320
<i>CYP301B1</i>	Sense: GCACTCCCAATACGGCGATA Anti sense: CTCACCATGTACGGCGATGA	214	MT732321
<i>CYP18A1</i>	Sense: ATTGAGAACGCGGAAGGAGG Anti sense: TCGCGTACGTCAATTTCCCA	179	MT732317
<i>CYP12K1</i>	Sense: ATGCTAACGGCTGGTGTGTA Anti sense: TCCTTGATGCAGGCCTTAGC	185	MT732319
<i>CYP9AS17v1</i>	Sense: TGATTAACCACTGACCGGC Anti sense: GCGGAAGGGACCATTACCAA	131	MT732310
<i>CYP369A2</i>	Sense: CGCTACCAGGCGTTATCACT Anti sense: CCAGTCGTCCAGTTCCAACA	108	MT732316
<i>CYP305D1v1</i>	Sense: ATCATGAAGAGGCTGGCGGA Anti sense: AAAAGTCGCCAAAATCCCCGC	195	MT732315
<i>CYP306A1</i>	Sense: GGCTTACGAATGGGCTCTGT Anti sense: TTTGTCCCTCTTGGCACCAG	237	MT732317
<i>18srRNA</i>	Sense: GAATTCCCAGTAAGCGCGAG Anti sense: GTCATCTTCCCGGCAACATC	156	AY334566

Table 2. Contact toxicity of fluralaner on workers of *S.invicta*

Exposure Time (h)	Compound	LD ₅₀ (95% CL) ^a (ng / insect)	Slope ± SE	χ ² (df)	P-value ^b
24	Fluralaner	15.99 (12.10-23.53)	1.46 ± 0.08	14.95 (16)	0.520
	Fipronil	2.22 (1.92-2.55)	2.02 ± 0.08	9.23 (16)	0.900
48	Fluralaner	4.24 (3.63-5.00)	1.64 ± 0 .07	4.87 (16)	0.990
	Fipronil	0.87 (0.66-1.08)	1.63 ± 0.08	17.87 (16)	0.230

^a CL: 95% confidence limits

^b P values based on Chi-square goodness of fit test. P values > 0.05 suggest goodness of fit of the model

Table 3. Feeding toxicity and synergism effects of fluralaner against *S.invicta*

Exposure time (h)	Compound	LC ₅₀ (95% CL) ^a (mg / L)	Slope ± SE	χ ² (df)	P-value ^b	SR ^c
24	Fluralaner	15.61 (13.24-19.39)	2.28 ± 0.153	9.35 (13)	0.47	-
	Fluralaner+PBO	10.15 (8.55-12.53)	1.85 ± 0.103	6.79 (13)	0.91	1.53*
	Fluralaner+DEM	11.42 (9.80-13.16)	2.23 ± 0.120	5.86 (13)	0.95	1.36*
	Fluralaner+TPP	32.73 (21.24-78.68)	1.87 ± 0.161	18.71 (13)	0.13	0.47
48	Fluralaner	8.63 (7.60-9.97)	2.13 ± 0.114	3.75(13)	0.94	-
	Fluralaner+PBO	5.32 (4.81-5.88)	2.77 ± 0.118	3.395 (13)	0.99	1.62*
	Fluralaner+DEM	5.41 (4.73-6.25)	2.31 ± 0.104	4.28(13)	0.98	1.59*
	Fluralaner+TPP	10.61 (8.49-14.28)	1.94 ± 0.112	15.04 (13)	0.300	0.810

^a CL: 95% confidence limits^b P values based on Chi-square goodness of fit test. P values > 0.05 suggest goodness of fit of the model^c* Means significant difference at $P = 0.05$, compared to data for fluralaner alone on the corresponding day.

Table 4. Horizontal toxicity transmit of Fluralaner between workers of *S.invicta*

Treatment	24h			48h		
	Donors' Mortality (%) ^a	Recipients' Mortality (%)	Transmite Rate (%)	Donors' Mortality (%)	Recipients' Mortality (%)	Transmite Rate ^b (%)
CK	2.22 ± 1.11	1.11±1.11	-	10.00 ± 1.92	-	-
2.5ng/insect	(16.67 ± 1.92) c	(6.67 ± 1.92) c	0.40 a	(57.78 ± 2.22) c	(28.89 ± 4.00) bc	0.50a
5ng/insect	(58.89 ± 2.93) b	(26.67 ± 1.92) b	0.45 a	(70.00 ± 3.33) b	(44.44 ± 4.84) ab	0.63a
50ng/insect	(81.11 ± 2.93) a	(41.11 ± 1.11) a	0.51 a	(84.44 ± 2.93) a	(58.88 ± 2.93) a	0.69a

^a Different letter in each column indicate significant differences at $p = 0.05$ level

^b transmit rate = donors' mortality / recipients' mortality.

Figure captions

Figure 1. Rb transmits effect between different colony members after fluralaner treatment using the donor-recipient model, and worker ant as the sustained donor, worker, female alate, larvae as recipient, respectively. (A) Rb content in donors after fluralaner treatment; (B) Rb content in recipient after fluralaner treatment; (C) Rb transmit rate of workers after LC₂₀ fluralaner treatment; * denotes a significant difference at $P = 0.05$ level, based on ANOVA followed by Tukey's HSD post hoc test.

Figure 2. Enzyme activity comparisons of RIFA, 24hours after feeding with LC₃₀ does fluralaner; * denotes a significant difference at $P = 0.05$ level, based on ANOVA followed by Tukey's HSD post hoc test.

Figure 3. Relative transcript levels of 36 cytochrome P450 after LC₅₀ concentration (15.61mg/L) fluralaner feeding for 24hours. Relative expression levels were calibrated with solvent treated *S.invicta*, $2^{-\Delta\Delta Ct}$ method was used, $\Delta\Delta Ct = [(target\ gene)\ CT\ value - (internal\ reference)\ CT\ value]_{treatment} - [(target\ gene)\ CT\ value - (internal\ reference)\ CT\ value]_{control} \times 100\%$. * denotes a significant difference with control using a one-way ANOVA (Tukey's HSD post hoc test, $P < 0.05$)

Figure 1

Figure 2

Figure 3

Figures

Figure 1

Rb transmits effect between different colony members after fluralaner treatment using the donor-recipient model, and worker ant as the sustained donor, worker, female alate, larvae as recipient, respectively. (A) Rb content in donors after fluralaner treatment; (B) Rb content in recipient after fluralaner treatment; (C)

Rb transmit rate of workers after LC20 fluralaner treatment; * denotes a significant difference at P = 0.05 level, based on ANOVA followed by Tukey's HSD post hoc test.

Figure 2

Enzyme activity comparisons of RIFA, 24hours after feeding with LC30 does fluralaner; * denotes a significant difference at P = 0.05 level, based on ANOVA followed by Tukey's HSD post hoc test.

Figure 3

Relative transcript levels of 36 cytochrome P450 after LC50 concentration (15.61 mg/L) fluralaner feeding for 24 hours. Relative expression levels were calibrated with solvent treated *S. invicta*, 2- $\Delta\Delta$ Ct method was used, $\Delta\Delta$ Ct = [(target gene) CT value - (internal reference) CT value]treatment - [(target gene) CT value - (internal reference) CT value]control $\times 100\%$. * denotes a significant difference with control using a one-way ANOVA (Tukey's HSD post hoc test, $P < 0.05$)