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Wolbachia bacteria affect rice striped stem borer (*Chilo suppressalis*) susceptibility to two insecticides

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Abstract

The striped stem borer (SSB), *Chilo suppressalis* (Walker) (Lepidoptera Crambidae), is one of the most economically important rice pests in Asia, the control of which primarily relies on chemicals. As such, *C. suppressalis* has developed resistance to many insecticides in most rice growing areas. *Wolbachia* are obligate intracellular bacteria known to manipulate arthropod host biology, and are thus considered a promising tool for pest control. In this study, we investigated the effects of *Wolbachia* and their density on the susceptibility of *C. suppressalis* to fipronil and avermectin insecticides under laboratory conditions. Specifically, fipronil and avermectin toxicities against *Wolbachia*-infected and *Wolbachia*-uninfected *C. suppressalis* were tested by topical application. Furthermore, *Wolbachia* densities in *C. suppressalis* over different survival times following fipronil treatment (0.2 mg/L) were evaluated by quantitative polymerase chain reaction. Results showed that average mortality of *Wolbachia*-infected *C. suppressalis* was lower than that of *Wolbachia*-uninfected *C. suppressalis* under fipronil and avermectin treatment. In addition, the 50% lethal concentration (LC_{50}) of fipronil and avermectin to *Wolbachia*-infected strains was 9.74 times and 5.32 times higher, respectively, than that of *Wolbachia*-uninfected strains, indicating that *Wolbachia* reduce the susceptibility of *C. suppressalis* to fipronil and avermectin. Under fipronil treatment (0.2 mg/L), *Wolbachia* content in surviving *C. suppressalis* at 72 hours was significantly higher than that at 24 and 48 hours ($P < 0.05$); thus, *Wolbachia* density increased with the length of *C. suppressalis* life. Therefore, *C. suppressalis* susceptibility was negatively correlated with *Wolbachia* density.

Key words: *Wolbachia*, *Chilo suppressalis*, SSB, susceptibility, fipronil, avermectin, quantitative PCR.

Introduction

Wolbachia bacteria are obligate intracellular symbionts that infect a wide variety of invertebrates (Zug and Hammerstein, 2012), and can spread through populations by maternal inheritance (Telschow *et al*., 2017). *Wolbachia* exhibit a range of effects on their hosts, including reproductive manipulation such as cytoplasmic incompatibility, parthenogenesis, feminization, and male killing, as well as other behavioural effects (Werren *et al*., 2008; Thomas *et al*., 2011). In addition, *Wolbachia* can influence the growth, development, longevity, fertility, immunity, and pathogen interference of their hosts (Kambris *et al*., 2009; Zug and Hammerstein, 2015; Suh *et al*., 2017; Ye *et al*., 2017; Ross *et al*., 2017; Lopez *et al*., 2018; Poorjavad *et al*., 2018), and even impact cognitive behavioural traits such as learning and memory capacity (Bi *et al*., 2019). Furthermore, studies showed that certain *Wolbachia* strains (such as *w*Mel) reduce replication of dengue viruses and Zika viruses in the laboratory, prompting the release of mosquitoes carrying the bacterium into the field to control the spread of arboviruses (Walker *et al*., 2011; Aliota *et al*., 2016; King *et al*., 2018). Studies have proposed that the use of *Wolbachia* introductions to capitalize on pre-existing Allee effects and consequently eradicate insect pests (Blackwood *et al*., 2017). Therefore, *Wolbachia* bacteria are considered as potential tools for pest control (Nikolouli *et al*., 2018).

Interactions between *Wolbachia* bacteria and their hosts can lead to both positive and negative effects on host fitness (Zhao *et al*., 2013). For example, *Wolbachia* density within the head, gut, and Malpighian tubules of *Drosophila simulans* Sturtevant is correlated with the ability to mediate antiviral protection (Osborne *et al*., 2012). Previous research has also documented that higher densities of *Wolbachia* in the somatic tissues of hosts are correlated with stronger pathogenic resistance (Emerson and Glaser, 2017). *Wolbachia*-mediated resistance to insecticide has also been studied. The density of intracellular *Wolbachia* bacteria has been found to be higher in resistant *Culex pipiens* L., and *Wolbachia* are capable of modifying the cost of resistance (Duron *et al*., 2006). *Wolbachia* infection has been proved that may improve the resistance of *Laodelphax striatellus* (Fallen) to buprofezin (Li *et al*., 2018)

The striped stem borer (SSB), *Chilo suppressalis* (Walker) (Lepidoptera Crambidae), is a serious rice pest distributed in the main rice-growing areas of Asia, northern Africa, and southern Europe (Xu *et al*., 2015). This borer is responsible for huge economic losses, particularly in China due to rice cultivation methods and the popularization of hybrid varieties (Ming *et al*., 2018). To date, chemical insecticides remain the primary measure of field control. However, the overuse of such chemicals has not only caused a series of problems such as environmental pollution and pesticide residue but has also induced insect resistance to a variety of agents (Sun *et al*., 2017). Since the 1950s, the rice SSB populations have developed medium to high levels of resistance to insecticides such as triazophos, fipronil, chlorpyrifos, monosultap, and bisultap (Ming *et al*., 2003; Tingle *et al*., 2003; He *et al*., 2013; Yang *et al*., 2017). Especially fipronil has developed resistance in other rice pests (Matsukawa-Nakata *et al*., 2019). Fortunately, most *C. suppressalis* populations have main-

tained relatively high sensitivity to chlorantraniliprole, methylvitamin, and avermectin (Yao *et al*., 2017; Tang *et al*., 2018). To delay the development of insecticide resistance in SSB, it is necessary to strengthen research on the influencing factors and resistance mechanisms to insecticides and to introduce integrative management measures to SSB control.

Most previous studies on the biochemical SSB resistance mechanism have focused on enzyme inhibitor bioassays and enzyme activity assays. *Wolbachia* infection occurs widely in SSB populations in China (Chai and Du, 2011), but at present no studies have reported on the effects of endosymbiotic bacteria on SSB resistance. No studies have yet reported on the effects of *Wolbachia* on SSB and also sensitivity of SSB to insecticides. Therefore, we studied the relationship between *Wolbachia* infection and SSB susceptibility to two insecticides. The results of this study will hopefully provide a novel perspective for the possible roles of *Wolbachia* in SSB pesticide resistance.

Table 1. Composition of artificial diet for SSB rearing.

Components	Quantity (g)
Fraction A	
Soybean powder	90
Yeast powder	60
Casein	30
Sucrose	30
Fresh water bamboo	300
Distilled water	756
Fraction B	
Ascorbic acid	9
Cholesterol	0.6
Choline chloride	0.9
Wesson's salt	0.3
Vitamin B	0.12
Sorbic acid	3
Methyl parahydroxybenzoate	3
Distilled water	100
Fraction C	
Agar powder	36
40% formaldehyde (mL)	1.8
Distilled water	750

Table 2. Detailed composition of vitamin B in the SSB artificial diet.

Ingredients	Quantity
Distilled water (mL)	100
Nicotinamide (g)	0.60828
Thiamine hydrochloride V_{B1} (g)	0.153
Cyanocobalamin V_{B12} (g)	0.00374
Folic acid (g)	0.153
Riboflavin V_{B2} (g)	0.306
1d-Pantothenic acid calcium salt (g)	0.60828
Biotin (g)	0.0147

Materials and methods

Insect rearing

The rice SSB were provided by Huazhong Agricultural University in Wuhan, Hubei Province, China. The *Wolbachia*-infected and *Wolbachia*-uninfected larvae were reared on an artificial diet and maintained under a temperature of 28 ± 1 °C, relative humidity of 70-80%, and photoperiod of 16:8 (L:D) (Han *et al*., 2012). The ingredient composition of the diet and a detailed list of vitamins B (VB) used are shown in tables 1 and 2 which provided by Huazhong Agricultural University.

Polymerase chain reaction (PCR) screening for *Wolbachia* infection

Wolbachia can be transmitted vertically and horizontally (Turelli *et al*., 2018). If the female G_0 is infected with *Wolbachia*, her offspring are also infected with *Wolbachia* (Lu *et al*., 2012). After mating and laying eggs, the DNA of female G_0 was extracted, and then the infection of *Wolbachia* was detected by PCR. Genomic DNA was extracted from the borers using an animal tissue genomic DNA extraction kit (Beijing Dingguo Changsheng Biotechnology Co., Ltd., China). General *wsp* primers (81F/691R) were used for *Wolbachia* detection as previously described (Zhou *et al*., 1998). Volume reactions (20 μL) were established with 0.5 μL of extracted template DNA, 2 μL of 10 × Ex Taq Buffer, 2 μL of deoxyribonucleoside triphosphate (dNTP, 2.5 mM), 2 μL of $MgCl_2$ (25 mM), 0.25 μL of Ex Taq, 11.25 μL of ddH_2O, and 1 μL of forward and reverse primer (10 μM). The temperature profile for PCR was: pre-denaturation at 95 °C for 2 minutes, denaturation at 94 °C for 30 seconds, annealing at 52 °C for 30 seconds, extension at 72 °C for 1 minute, and finally at 72 °C for 5 minutes after 35 cycles. The amplified products were temporarily stored at 4 °C. The PCR products were analyzed by gel electrophoresis in a 1.2% agarose gel. G_1 females from the infected G_0 female were mated with G_1 males from the infected G_0 female, isolated and allowed to oviposit. Following oviposition, approximately 20 G_1 females were PCR assayed for *Wolbachia* infection. So does uninfected G_0 female. Based on this premise, we ensured we obtained a population of *Wolbachia*-infected and *Wolbachia*-uninfected *C. suppressalis*.

Determination of *C. suppressalis* susceptibility to insecticides

The *Wolbachia*-infected *C. suppressalis* were used for insecticide sensitivity experiments via topical application. First, a 5% fipronil suspension agent and 5% avermectin emulsifiable concentrate were diluted with acetone into five concentrations (0.01, 0.02, 0.1, 0.2, and 1.0 mg/L for fipronil and 0.01, 0.05, 0.1, 0.5, and 1.0 mg/L for avermectin). The test solutions were processed from low to high. *Wolbachia*-infected and *Wolbachia*-uninfected fourth instar larvae were respectively divided into six groups (20 per group): one control group, with a micro syringe used to drip 1.0 μL of acetone onto the pronotum of the larvae; and five treatment groups dripped with different concentrations of fipronil or avermectin solution. The treated borers were

Table 3. Mortality (%) of SSB populations infected and uninfected by *Wolbachia* under different concentrations of fipronil.

Fipronil concentration (mg/L)	*Wolbachia*-infected	*Wolbachia*-uninfected
0	0	0
0.01	20.00 ± 1.77	30.00 ± 1.73
0.02	26.67 ± 1.15	36.67 ± 1.52
0.1	31.67 ± 0.58	43.33 ± 2.31
0.2	35.00 ± 2.00	44.33 ± 1.53
1	38.33 ± 1.14	48.33 ± 2.31

Data are expressed as means ± standard deviation. Differences between SSB *Wolbachia*-infected and SSB *Wolbachia* -uninfected were compared by Student's *t*-test. Same in following tables.

Table 4. Mortality (%) of SSB populations infected and uninfected by *Wolbachia* under different concentrations of avermectin.

Avermectin concentrations (mg/L)	*Wolbachia*-infected	*Wolbachia*-uninfected
0	0	0
0.01	6.68 ± 1.15	13.33 ± 0.58
0.05	10.00 ± 1.00	26.67 ± 0.57*
0.1	13.33 ± 1.52	30.00 ± 2.00
0.5	20.00 ± 1.00	33.33 ± 1.53
1	31.67 ± 0.58	41.67 ± 1.53

*significant differences among treatments ($P < 0.05$).

reared in 24-well plates containing an artificial diet and placed in an incubator at a temperature of 28 ± 1 °C and a light:dark cycle of 16:8 hours. The number of dead insects was counted after 48 hours.

Assessment of *Wolbachia* density in *C. suppressalis* at different survival times

We used quantitative PCR to compare *Wolbachia* density in SSB individuals under fipronil treatment (0.2 mg/L was a randomly chosen concentration) after 12, 24, 48, and 72 hours. Considering that there was no SSB death after 0 hour of pesticide treatment, but there was death under fipronil treatment after 12, 24, 48, and 72 hours. The control group consisted of SSB treated with fipronil for 12 hours, whereas the experimental group consisted of SSB treated with fipronil for 24, 48, and 72 hours. The target gene was the *Wolbachia* surface protein gene *wsp*, and 18S RNA from SSB was selected as the internal reference gene. The primer sequences were: For (5'-TCGAGCCGCACGAGATTGAGCA-3') and Rev (5'-CAAAGGGCAAGGGACGTAATCAAC-3'). Quantitative PCR was carried out in a 20-μL reaction containing 10 μL of SYBR Green qPCR Mix (TransGen Biotech, Beijing, China), 0.4 μL of each primer (10 μM), 1 μL of template DNA, and 35.5 μL of ddH_2O. The PCR cycling profile was: 94 °C for 3 minutes, followed by 40 cycles at 94 °C for 1 second, 45 °C for 30 seconds, and a final extension at 72 °C for 10 seconds (CFX96 Touch, Bio-Rad). All amplification products were temporarily stored at 4 °C.

Statistical analysis

Data were expressed as means ± standard deviation. The differences in insect mortality were compared by Student's *t*-test. The toxicity regression equation and lethal concentration (LC_{50}) were calculated by IBM SPSS Statistics 19.0. Relative resistance ratio = LC_{50} of *Wolbachia*-infected *C. suppressalis* / LC_{50} of *Wolbachia*-uninfected *C. suppressalis*. The density of *Wolbachia* was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The relative expression levels of *Wolbachia* in SSB were analysed using one-way analysis of variance (ANOVA), followed by Duncan's new multiple range test when significant differences were tested by IBM SPSS Statistics 19.0.

Results

Effect of *Wolbachia* infection on *C. suppressalis* insecticide susceptibility

Results showed that the mortality of SSB *Wolbachia*-infected was significantly lower than that of SSB *Wolbachia*-uninfected when the concentration of avermectin was 0.05 mg/L (*t*-test, $t = 5$, df = 4, $P = 0.007$). The SSB mortality rate increased with the concentration of fipronil (table 3) and avermectin (table 4). After pesticide treatment, the mortality rate of SSB *Wolbachia*-uninfected was higher than that of SSB *Wolbachia*-infected (tables 3 and 4).

After fipronil treatment, the susceptibility of SSB *Wolbachia*-infected decreased. The LC_{50} values of SSB *Wolbachia*-infected and SSB *Wolbachia*-uninfected were 10.32 mg/L and 1.06 mg/L, respectively, and the relative resistance ratio was 9.74 (table 5). After avermectin treatment, the susceptibility of SSB *Wolbachia*-infected also decreased. The LC_{50} values of SSB *Wolbachia*-infected and SSB *Wolbachia*-uninfected were 20.32 mg/L and 3.82 mg/L, respectively, and the

Table 5. Toxicity regression equation of SSB populations infected and uninfected by *Wolbachia* under fipronil and avermectin treatment.

Treatment	Populations of *C. suppressalis*	Toxicity regression equation	LC_{50} (mg/L)	Relative resistance ratio
Fipronil	*Wolbachia* +	$y = 0.2476\,x + 4.749$	10.32	9.74
	Wolbachia −	$y = 0.2217\,x + 4.9946$	1.06	
Avermectin	*Wolbachia* +	$y = 0.4487\,x + 4.4132$	20.32	5.32
	Wolbachia −	$y = 0.3868\,x + 4.7748$	3.82	

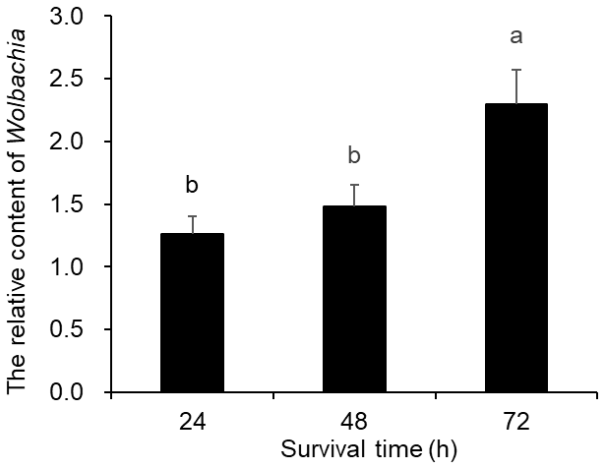

Figure 1. Relative content of *Wolbachia* in SSB after different survival times following fipronil treatment (0.2 mg/L). Different letters above columns indicate significant differences among treatments ($P < 0.05$).

relative resistance ratio was 5.32 (table 5). These results indicated that SSB *Wolbachia*-uninfected are more sensitive to pesticides than SSB *Wolbachia*-infected.

Effect of *Wolbachia* density on *C. suppressalis* susceptibility

Under 0.2 mg/L fipronil treatment, we observed a significant difference in the relative content of *Wolbachia* in SSB at different survival times. The content of *Wolbachia* in SSB after 72 hours of survival was 2.3 times higher than the content after 12 hours of survival. The content of *Wolbachia* in SSB after 72 hours was significantly higher than that after 24 and 48 hours ($P < 0.05$) (figure 1). Furthermore, under 0.2 mg/L fipronil treatment, the longer the SSB survival time, the higher the density of *Wolbachia* and the lower the susceptibility of SSB. As such, SSB susceptibility is negatively correlated with *Wolbachia* density.

Discussion

Wolbachia bacteria can regulate the reproductive activities of their host by inducing cytoplasmic incompatibility, parthenogenesis, feminization, and male death, thus altering the ecological characteristics of the host population (Telschow *et al*., 2017). In this study, *Wolbachia*-infected females mated with infected males, *Wolbachia*-uninfected females mated with uninfected males. SSB *Wolbachia*-infected and SSB *Wolbachia*-uninfected have been reared in the laboratory for at least three generations, and their growth, development and reproduction on artificial diet are not significantly different during the feeding process. In the pre-experiment, SSB eclosion rate, spawning rate and hatching rate raised with artificial diet were higher than that of rice seedlings and cane shoots. Considering the feeding effect and cost, it was more reasonable and scientific for us to choose artificial diet for SSB laboratory rearing.

Previous studies have shown that there was a significant increase in the susceptibility to buprofezin after *Wolbachia* removed from the *Wolbachia*-infected line, the mortality of *Wolbachia*-cured line treated with 200 mg/L buprofezin was 51.8%, significantly higher than that of the *Wolbachia* infected line (Li *et al*., 2018). In the current study, we showed that the mortality of SSB *Wolbachia*-infected was lower than that of SSB *Wolbachia*-uninfected at equal concentration of fipronil and avermectin. Under avermectin concentrations of 0.05 mg/L, the mortality of SSB *Wolbachia*-infected was significantly reduced (table 3). Thus, these results indicated that *Wolbachia* had a negative effect on SSB sensitivity to fipronil and avermectin. In addition, *Wolbachia* can enhance the resistance of *C. pipiens* to pesticides, and the infection density of *Wolbachia* in the organophosphorus resistant strain of *C. pipiens* is higher than that of susceptible mosquitoes (Duron *et al*., 2006). In this study, the relative resistance ratios of SSB *Wolbachia*-infected to avermectin and fipronil were 5.32-fold and 9.74-fold higher, respectively, than that of SSB *Wolbachia*-uninfected (table 5). These results demonstrated that *Wolbachia* reduced SSB susceptibility to fipronil and avermectin.

Understanding infection density of host symbionts is critical for deciphering their biological effects and functions (Ali *et al*., 2018). Prior research has indicated that inhibition of the dengue virus increases with higher *Wolbachia* density per cell (Frentiu *et al*., 2010). Moreover, high *Wolbachia* density in *C. pipiens* decreases the host emergence rate, whereas low *Wolbachia* density in *Drosophila innubila* Spencer fails to manipulate host reproduction (Sumi *et al*., 2017). Cytoplasmic incompatibility in *Aedes albopictus* (Skuse) is positively correlated with *w*AlbA strain density (Calvitti *et al*., 2015). Furthermore, *Wolbachia* density is strongly modified by the presence of insecticide-resistant genes, as observed in the common house mosquito, *C. pipiens* (Berticat *et al*., 2002). Here, we demonstrated that under fipronil treatment (0.2 mg/L), *Wolbachia* density in SSB surviving after 72 hours was significantly higher than that after 24 and 48 hours ($P < 0.05$) (figure 1). Thus, SSB susceptibility was negatively correlated with *Wolbachia* density, in other words, SSB susceptibility declined with the increase in *Wolbachia* density *in vivo*. The *Wolbachia* population in SSB is likely to be involved in the formation of its resistance to specific chemicals. Reducing *Wolbachia* in SSB may reduce the resistance of stem borers to insecticides. Our results provide a basic analysis to interpret the effects of *Wolbachia* bacteria and their density on the sensitivity of SSB to insecticides.

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