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RNA interference of acetylcholinesterase in the Asian citrus psyllid, *Diaphorina citri*, increases its susceptibility to carbamate and organophosphate insecticides

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ABSTRACT

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Lividae) transmits the *Candidatus* Liberibacter asiaticus, which causes citrus greening disease or Huanglongbing, (HLB). To date, there is no efficient cure for HLB disease and the control of *D. citri* using insecticides became the most important tools for the management of HLB. However, the extensive use of insecticides could increase *D. citri* resistance to these insecticides. The objective of this study was to investigate the effect of RNA interference of acetylcholinesterase (*AChE*) on the mortality and susceptibility of *D. citri* to the four major insecticides used in Florida. In this study, we used a consensus sequence derived from the two *AChE* genes and *cholinesterase 2-like* (*ChE-2-like*) gene to target all of the three genes. Treatment with dsRNA-*AChE* increased the mortality percentages of both nymphs and adults of *D. citri*. The mortality percentage increased with the increase in the concentration of applied dsRNA-*AChE*, and the highest mortality (> 60%) was observed at the highest applied concentration (125 ng/µl). Treatments of nymphs or adults with dsRNA-*AChE* down-regulated the expression of the three targeted genes of *D. citri*. Silencing of *AChE* and *ChE* in *D. citri* nymphs increased the susceptibility of emerged adults to chlorpyrifos and carbaryl, which act as AChE inhibitors. However, treatment with dsRNA-*AChE* did not increase the susceptibility of emerged adults to imidacloprid, which acts as an agonist of nicotinic acetylcholine receptors. In the same manner, treatment of adults with dsRNA-*AChE* increased their susceptibility to chlorpyrifos and carbaryl, but did not affect their susceptibility to imidacloprid. The ANOVA did not show any significant increase in susceptibility of *D. citri* adults to fenpropathrin after treatment with dsRNA-*AChE*, either as nymphs or as adults. However, simple linear regression showed that treatment with dsRNA-*AChE* increased *D. citri* susceptibility to fenpropathrin, which indicated that *AChE* could be involved in the metabolism of fenpropathrin. Our results indicated that silencing of *AChE* and *ChE* genes in *D. citri* to increase its susceptibility to insecticides could be a promising tool for the control of this important vector.

1. Introduction

Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) is one of the most destructive and economically important pests of citrus around the world [1]. *D. citri* causes direct damage by feeding on plant sap and indirect damage by transmitting *Candidatus* Liberibacter asiaticus (*C*Las), a phloem-restricted bacterium, which causes Huanglongbing (HLB), or citrus greening disease, the most fatal disease of citrus around the world. HLB can cause tree decline, reduces the quality of the fruit, and could result in tree death [1,2]. Also, *D. citri* decreases the photosynthetic rate of the trees by excreting honeydew which causes sooty mold on leaf surfaces [3]. The new shoots are essential for the oviposition and the development of nymphs, however adults can survive and overwinter on the hardened leaves [4]. Both *D. citri* adults and nymphs can transmit *C*Las, but nymphs were found to be more efficient [5].

The epidemic spread of HLB has greatly increased the use of insecticides to control *D. citri* in Florida citrus groves [6,7]. For the past six decades, the use of systemic insecticides became the most common control measures for *D. citri* [8]. Organophosphates and carbamates such as chlorpyrifos, dimethoate, malathion, aldicarb and carbaryl are the most commonly used insecticides for *D. citri* management [9]. The

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organophosphates and carbamate act by inhibiting acetylcholinesterase (AChE; EC 3.1.1.7), one of the important enzymes in the nervous system in insects [10]. Acetylcholinesterase enzymes catalyze the hydrolysis of the neurotransmitting agent, acetylcholine (ACh), to choline and acetic acid. Accumulation of acetylcholine in the intercellular space results in continuous stimulation of the receptors on the target cells. The synthetic pyrethroid (fenpropathrin), which acts as sodium channel modulator, has also been used for the control of *D. citri* [11,12].

During the past decade, neonicoinoids have been found to be the most effective class of insecticides for *D. citri* control [13]. In Florida, many of the systemic neonicotinoid insecticides such as imidacloprid, thiamethoxam, and clothianidin are allowed to be used as soil applications, but their use is limited to young trees [14]. Neonicotinoids act on insect nicotinic acetylcholine receptors (*nAChRs*), which mediate fast cholinergic synaptic transmission in insect central nervous system. Recently, imidacloprid has been extensively used for the control of *D. citri* because it is more toxic to insects than vertebrates and persists systemically in plants [15].

The random and continuous use of insecticides caused several critical problems such as insect resistance, residues in food products, and environmental pollution [16,17]. General esterases (GEST), cytochrome P450 monooxygenases, and glutathione S-transferases (GST) are the main detoxification enzymes controlling insecticide resistance in insects [18,19]. Carboxyesterases (CarEs) are widely distributed in insects [20], and they are involved in several essential physiological functions including insect development and behavior regulation, neurotransmission, and hormone and pheromone metabolism [21]. In addition, CarEs play an important role in the detoxification of xenobiotic compounds [22,23]. *AChE*, one of the most important CarEs enzyme, plays an important role in the development of insecticide resistance [24].

Unfortunately, the number of modes of action available for the control of *D. citri* are limited. Consequently, various levels of insecticide resistance were observed in Florida as a result of using of the same class of insecticides or mode of action (i.e., acetylcholinesterase inhibitors, sodium channel modulator) repeatedly [9,17]. The susceptibility of *D. citri* to insecticides decreased with the increased expression of certain enzymes [9,17]. Consequently, it has been a challenge for researchers to develop efficient strategies for *D. citri* control with low environmental impact such as RNA interference. RNA interference (RNAi) was proposed by many researchers as an ecofriendly control for *D. citri* [25–28].

RNAi is a post-transcriptional control mechanism involving degradation of target mRNA which is mediated by small interfering RNAs (siRNAs) [29]. The phenomenon of gene silencing has now been considered as a potential strategy for insect management. The selection of the target gene and synthesis of double-stranded RNA (dsRNA) comprise a crucial component in the application of this technology [30].

RNAi has emerged as a versatile and a promising molecular biology tool [31] for down-regulating target gene expression in insects [32,33]. Previous reports showed that silencing of genes through RNAi in insects could increase insect mortality, affect insect growth, increase insecticide susceptibility, and prevent the development of insecticide resistance [25]. In the present study, we hypothesize that silencing of *AChE* and *ChE* genes using RNAi technique through topical feeding application increases the susceptibility of *D. citri* to insecticides.

2. Materials and methods

2.1. Asian citrus psyllid population

A laboratory susceptible populations of *D. citri* were continuously reared in a USDA-APHIS/CDC-approved secured growth room at 25 ± 2 °C with 60 ± 5% relative humidity and 16:8 h (light:dark) photoperiod at the Citrus Research and Education Center (CREC), University of Florida, Lake Alfred, Florida, without exposure to insecticides. The insect colonies were reared on *Citrus macrophylla* plants and caged using insect rearing cages (60 × 60 × 90 cm) included all instar nymphs and adults. For the dsRNA application, the 4th and 5th instar nymphs were collected using a camel hair brush while an aspirator was used to collect adult *D. citri* for dsRNA and insecticides application.

2.2. Constructing of dsRNA-AChE gene sequencing of AChE and ChE

The genome of *Diaphorina citri* possesses two acetylcholinesterase-like (*AChE*) genes; *acetylcholinesterase-like-1* (XM_008477411.1), *acetylcholinesterase-like-2* (XM_008482391.1), and one *cholinesterase 2-like* (XM_008469625.1).

For dsRNA-*AChE*, a consensus sequence derived from the three gene sequences, mentioned above, was used to design *AChE*-specific primer sequences to target the two *AChE* genes of *D. citri*. The *AChE*-consensus sequence was 489 bp in length. The dsRNA-*AChE* was constructed using acetylcholineesterase-Like-1 (XM_00847711.1) from nucleotide 395 to 884 as described by Kishk et al. [26].

DsRNA-*AChE* and irrelevant dsRNA-*gfp* (green fluorescent protein) which was used as a control were synthesized using the method described by Killiny et al. [25].

2.3. Insecticides

Laboratory populations of *D. citri* adults were tested with four insecticides targeting the nervous system and belonging to different chemistry classes and modes of action as listed in (Table 1). All treatments were formulated using analytical-grade insecticides. At least five concentrations from each insecticide were prepared in acetone as follows: chlorpyrifos (0.01, 0.1, 1, 5, 10, 20 ng/µl), carbaryl (0.1, 1, 10, 100, 200 ng/µl), fenpropathrin (0.01, 0.1, 10, 50, 100 ng/µl), imidacloprid (0.001, 0.01, 0.05, 0.1, 1, 5 ng/µl). Insecticides solution were prepared and used the day of the application.

2.4. Insecticides evaluations of adult D. citri using a leaf-dip bioassay

Lethal concentrations that caused 50% (LC_{50}) mortality to *D. citri* adults were determined using a leaf-dip bioassay method [17,34]. 3–5 ml of a 1.5% agar solution was poured in 60-mm-diameter plastic disposable petri dish (Corning Incorporated, Corning, NY, USA) to form a solidified bed. Citrus leaf disks (60-mm-diameter) were excised from fresh leaves collected from citrus trees grown in the CREC grove without any exposure to insecticides. The leaf disks were dipped for 30 s in the insecticide/acetone solution and allowed to air dry in a fume hood for 1 h before being placed on agar beds in the petri dishes. Leaf discs dipped in acetone alone were used as a control. Using a camel hair

Table 1
Details of the tested insecticides against *D. citri* adults.

Common name	Purity	Class	Targeted system	Mode of action	Manufacturer
Chlorpyrifos	99.5%	Organophosphate	Nervous system	Acetylcholinesterase (AChE) inhibitor	Chem service, INC, West Chester, PA
Carbaryl	99.9%	Carbamate		Acetylcholinesterase (AChE) inhibitor	
Fenpropathrin	99.1%	Synthetic pyrethroid		Sodium channel modulator	Sigma-Aldrich Co. LLC., St. Louis, MO
Imidacloprid	99.9%	Neonicotinoids		Nicotinic acetylcholine receptor (nAChR) agonist	

brush, 15 newly emerged *D. citri* adults were transferred into each petri dish. The petri dishes were wrapped with Parafilm (BEMIS Flexible Packaging, Neenah, WI, USA) to keep the adults inside. Each concentration of each insecticide was replicated four times (n = 60 adults per concentration). All sealed petri dishes were moved to USDA-APHIS/CDC-approved secured growth room (25 ± 2 °C, 60 ± 5% relative humidity, 16:8 h, light:dark photoperiod).

The mortality of *D. citri* adults was assessed 24 h after exposure to the insecticides. The adults of *D. citri* found on their side or back and unable to move when checked with a camel hair brush were counted as dead.

2.5. Topical feeding of D. citri 4th and 5th instar nymphs with dsRNA-AChE

Five diluted concentrations of dsRNA-*AChE* were prepared using RNase-free water as follows: 25, 50, 75, 100 and 125 ng/µl. In addition, RNase-free water was used as a control. For the topical feeding application as described by Killiny and Kishk 2017 [35], a 0.2 µl dsRNA droplet was topically applied to the ventral side of the thorax between the three pairs of legs of 4th and 5th-stage nymphs of *D. citri* via a 10 µl Hamilton (Hamilton Company, Reno, NV) syringe microapplicator (0.2 µl). The droplet was allowed to be consumed through the stylet completely for 60 s by the nymphs. Each concentration of dsRNA-*AChE* was replicated five times. Twenty nymphs were treated for each concentration replicate (n = 100). *D. citri* nymph mortality was calculated after the emergence of the adults, usually 4–5 days following treatment. The nymphs were considered dead if they were unable to move when checked with a camel hair brush.

2.6. Insecticides application for D. citri adults emerged from nymphs treated with dsRNA-AChE

After *D. citri* nymphs were treated with dsRNA-*AChE* as described above, the emerged adults were transferred to petri dish containing citrus leaf disk and treated with insecticide solutions at their LC_{50} concentration as described previously. Leaf discs dipped in acetone alone were used as control. The mortality results of *D. citri* adults were evaluated after 24 h.

2.7. Topical feeding of D. citri adults with dsRNA-AChE

The same five diluted concentrations of dsRNA-*AChE* (25, 50, 75, 100 and 125 ng/µl) were used to treat adult *D. citri* with RNase-free water used as the control. *D. citri* newly emerged adults were anaesthetized using CO_2 before the application dsRNA. A 0.2 µl dsRNA droplet were applied using the microapplicator to the ventral side of the thorax between the three pairs of legs. The treated adults were transferred to untreated citrus leaves on petri dishes as a food source at 25 ± 2 °C with 60 ± 5% relative humidity and 16:8 h (light:dark) photoperiod in the growth room. *D. citri* mortality was recorded after 48 h to determine the effect of dsRNA treatments.

2.8. Insecticides bioassay for D. citri adults after treated with dsRNA-AChE

After *D. citri* adults were treated with dsRNA-*AChE* as described above, live adults were treated with the LC_{50} concentration of the four insecticides (chlorpyrifos, carbaryl, fenpropathrin and imidacloprid) using the leaf-dip bioassay method as described above. For the control, the leaves were dipped in acetone without insecticide.

2.9. Gene expression analysis for adults treated dsRNA-AChE

Two days after treatment with dsRNA-*AChE*, live adults were collected and kept in RNA*Later* (Invitrogen, Life Technologies, Carlsbad, CA, USA) until use. RNA was extracted using TriZol® reagent (Ambion®, Life Technologies, NY, USA) in five biological replicates with five adults in each. Using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), concentration and purity of isolated RNA was measured. For synthesizing cDNA, SuperScript first-strand synthesis system (Invitrogen) with random hexamer primers was used as described in the manufacturer's instructions. SYBR® Green PCR master mix (Applied Biosystems) was used to perform quantitative PCR (qPCR). For each treatment, three technical replicates per biological replicate were analyzed using an ABI 7500 Real-Time PCR System (Applied Biosystems). Primers of three targeted genes, and GFP were used to measure the gene expression as listed in Kishk et al., 2017 [26]. To compare the relative expression of studied genes, the $2^{-\Delta\Delta CT}$ method [36] was used. *Actin* was used as the reference gene to compare the relative gene expression among treatments and the control. GFP was used as an irrelevant gene and the non-target gene was *α-tubulin* (control).

2.10. Statistical analysis

Data was analyzed using JMP version 9.0 (SAS Institute Inc.). All mortality data were corrected for control mortality by Abbott's formula [37]. Mortality results for each insecticide were subjected to probit regression analysis to calculate the concentration causing 50% mortality of the insects for each insecticide (LC_{50}) with 95% corresponding confidence limits and slopes of regression lines. Analysis of variance (ANOVA) followed by post hoc pairwise comparisons using Tukey-Kramer honestly significant different test (Tukey HSD) were used to compare the mortality percentage of *D. citri* among the different treatments. In addition, the simple linear regression (SLR) was used to test the relation between the concentrations of applied dsRNA-*AChE* and the observed mortality percentages.

3. Results

3.1. Toxicity of insecticides on D. citri adults using leaf-dip bioassay

The susceptibility of *D. citri* adults to four different insecticides is presented in Table 2. The four selected insecticides were toxic to the *D. citri* adults. Imidacloprid was the most toxic among the four selected insecticides, followed by chlorpyrifos, fenpropathrin, and carbaryl (Table 2).

3.2. dsRNA-AChE treatments increased D. citri nymph mortality

Five concentrations of dsRNA-*AChE* and RNase-free water as a control were applied to the 4th and 5th instar nymphs of *D. citri* by topical feeding. The mortality of all dsRNA-treated nymph was higher than that of the control (RNase-free water), except for the lowest concentration (25 ng/µl) (Fig. 1A). The mortality increased by increasing the concentration of dsRNA-*AChE* (Fig. 1A). Significant differences were found in mortality rate among all dsRNA-treated nymphs (Fig. 1A). The highest mortality (63.3%) was observed at the highest applied dsRNA-*AChE* concentration (125 ng/µl). In agreement with the Tukey's test results, the simple linear regression also showed that the mortality rate was dependent on the concentration of dsRNA-*AChE*

Table 2
Toxicity of four insecticides on lab population of *D. citri* adults.

Insecticides	LC_{50} (ng/µl)	Lower 95%	Upper 95%	Slope ± SE	*P* value
Chlorpyrifos	2.62[c]	1.85	3.51	0.602 ± 0.062	< 0.0001
Carbaryl	12.04[a]	9.88	14.43	1.046 ± 0.099	< 0.0001
Fenpropathrin	4.85[b]	2.68	7.81	0.414 ± 0.044	< 0.0001
Imidacloprid	0.34[d]	0.25	0.48	0.455 ± 0.043	< 0.0001

Different superscript letters in the same column indicate significant differences among the LC_{50} values.

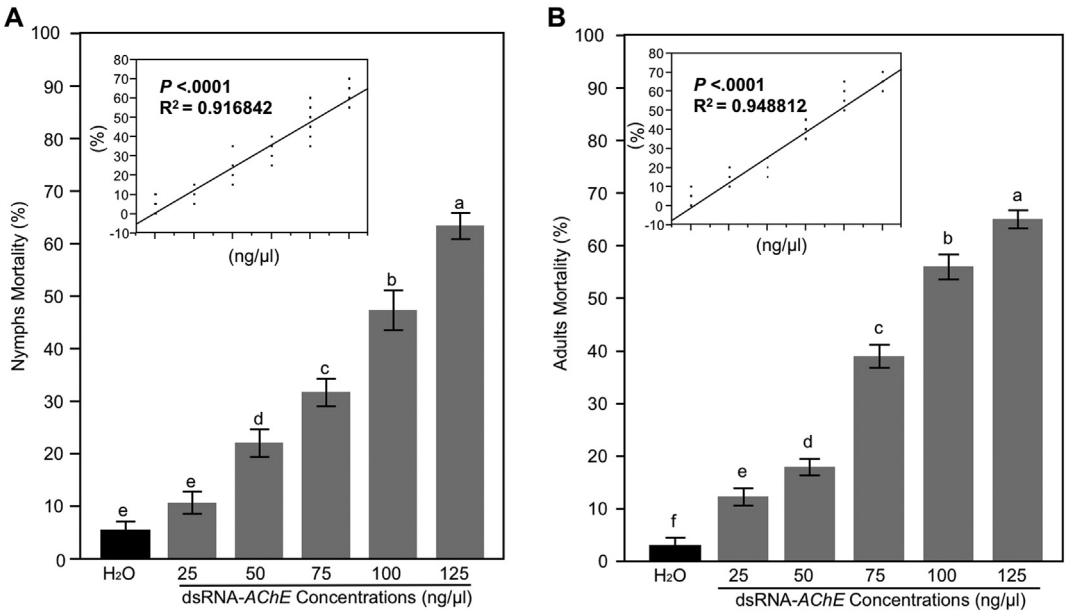

Fig. 1. (A) Effect of five concentrations of dsRNA-*AChE* on the mortality percentages of the fourth and fifth instar nymphs of *Diaphorina citri*, with simple linear regression plot of the concentration of dsRNA-*AChE* versus mortality; (B) Effect of dsRNA-*AChE* concentrations on the mortality of the adults of *D. citri* with simple linear regression plot of the concentration of dsRNA-*AChE* versus mortality. RNase- free water was used as a control. Letters a, b, c, d, e and f showing significant differences between the treatments ($P < 0.05$). Bars on columns represent the standard error.

(Fig. 1A).

3.3. Treatments of nymphs with dsRNA-AChE down-regulated the gene expression of the three targeted genes in emerged D. citri adults

Our results showed that the expression levels for three targeted genes were down-regulated in adults that emerged from dsRNA-treated nymphs compared with controls (dsRNA-*gfp* and RNase-free water) (Fig. 2A, B, C). The gene expression levels of the targeted genes decreased with increasing the concentration of dsRNA-*AChE*; the highest effect on gene expression levels of all targeted genes was observed at 125 ng/µl, *AChE-like-1* (0.48), *ChE-2-like* (0.51) and *AChE-like-2* (0.60) respectively, whereas the lowest effect was observed at 25 ng/µl. The greatest reduction in expression level was found with *AChE-like-1*, while the lowest effect was with *AChE-like-2*. There was no effect of the dsRNA-*gfp* treatment on the expression levels of the three targeted genes. Moreover, there were no significant differences on the expression levels of *α-tubulin* among all the treatments (Fig. 2D).

3.4. Silencing of AChE and ChE genes in D. citri nymphs increased insecticide susceptibility of the emerged adults

To determine the effect of dsRNA-*AChE* on insecticide susceptibility of *D. citri* adults emerged from treated nymphs, we used the LC_{50} concentration of four insecticides (chlorpyrifos, carbaryl, fenpropathrin and imidacloprid).

The simple linear regression (SLR) showed that adults emerged from nymphs treated with dsRNA-*AChE* were more susceptible to carbaryl than those emerged from non-treated nymphs (Fig. 3A). As shown in Fig. 3A, the adults emerged from nymphs treated with 125 ng/µl dsRNA-*AChE* were more susceptible to carbaryl (62.2% mortality) than the adults emerged from nymphs that were treated with RNase-free water (42.2% mortality).

In the same manner, the SLR showed that the susceptibility of adults to chlorpyrifos was increased if they (4th instar) were previously treated with dsRNA-*AChE* (Fig. 3B). The emerged adults from nymphs treated with 125 ng/µl dsRNA-*AChE* were more susceptible to chlorpyrifos (68.9% mortality) than those emerged from nymphs treated with RNase-free water (48.9% mortality) (Fig. 3B).

For fenpropathrin, no significant differences in mortality were observed between adults emerged from nymphs treated with dsRNA-*AChE* and those emerged from nymphs treated with RNase-free water. However, the linear regression showed that the mortality rate was increased by the application of the dsRNA-*AChE* (Fig. 3C).

The SLR showed that treatment of psyllid nymphs with dsRNA-*AChE*, did not increase the susceptibility of emerging adults to imidacloprid (Fig. 3D). In agreement with the SLR result, the Tukey's test also did not show any significant difference in adult mortality to imidacloprid insecticide between adults emerged from nymphs treated with dsRNA-*AChE* and those emerged from nymphs treated with RNase-free water (Fig. 3D).

3.5. dsRNA-AChE treatments increased the mortality of adult D. citri

The same five concentrations of dsRNA-*AChE* and water were applied to the *D. citri* adults using topical application. The SLR showed that the dsRNA-*AChE* application increased the mortality of adult psyllids (Fig. 1B). In agreement with the SLR, the Tukey's test also showed that the mortality rate of all the dsRNA-*AChE*-treated adults was significantly higher than control (Fig. 1B). Furthermore, the mortality rate of *D. citri* adults increased with increasing concentration of dsRNA-*AChE* (Fig. 1B). Treatment using dsRNA-*AChE* at 125 ng/µl caused the highest mortality (65.0%) followed by 100 ng/µl which caused 56.0% mortality.

3.6. Treatments of adults with dsRNA-AChE down-regulated the genes expression of the three targeted genes

The expression levels for three targeted genes were also down-regulated in adult *D. citri* upon treatment with dsRNA-*AChE* compared with controls (dsRNA-*gfp* and RNase-free water) (Fig. 2E, F, G). The gene expression levels of the three targeted genes also decreased with increasing concentration of dsRNA-*AChE*. Moreover, there were no significant differences on the expression levels of *α-tubulin* among all the treatments (Fig. 2D). There were no significant differences between treatments with RNase-free water and dsRNA-*gfp*. The highest reduction

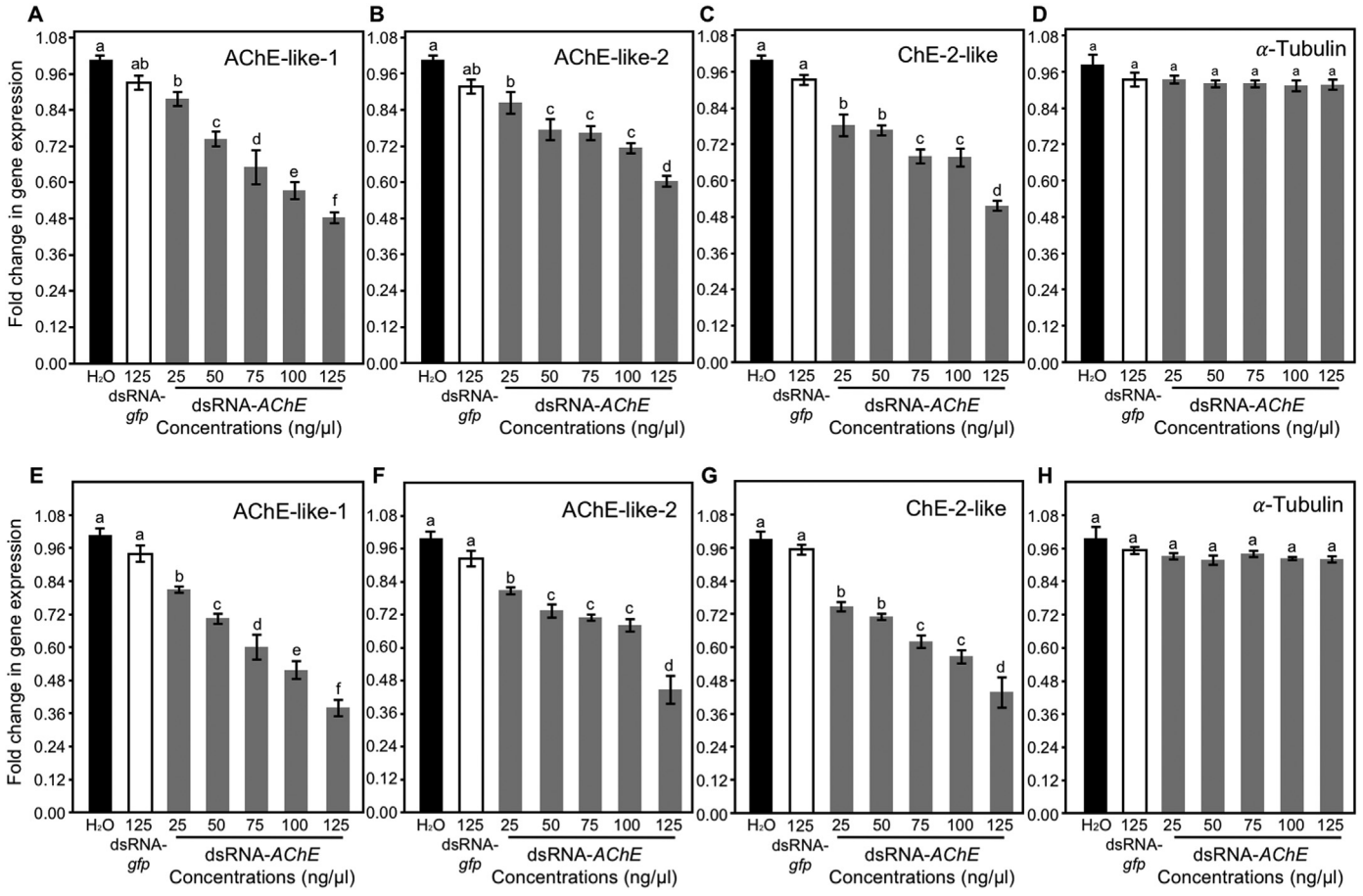

Fig. 2. Relative expression levels of three targeted genes treated with dsRNA in *D. citri* after 48 h using qRT-PCR. (A, B, C and D) showed the expression levels of *AChE-like-1*, *AChE-like-2* and *ChE-2-like* in *D. citri* adults emerged from treated nymphs and; (E, F, G and H) in the *D. citri* treated adults. *Actin* gene were used to normalized the Ct values. *α-tubulin* was used as a non-target gene control. dsRNA-*gfp* treatment was used as a control (irrelevant gene). Different letters indicate statistically significant differences ($P < 0.05$).

of expression level of the targeted genes was recorded when treated with dsRNA-*AChE* at the highest concentration (125 ng/μl) and the least effect was found at (25 ng/μl) dsRNA-*AChE* (Fig. 2E, F, G).

3.7. Silencing of AChE and ChE in D. citri adults increased insecticide susceptibility

In this experiment, we investigated the effect of dsRNA-*AChE* on the susceptibility of *D. citri* adults to the four selected insecticides using their LC_{50} concentrations. The SLR showed that treatment of adult psyllids with dsRNA-*AChE* increased their sensitivity to carbaryl (Fig. 4A). In agreement with the SLR, adults treated with 50, 75, 100, and 125 ng/μl were more susceptible to carbaryl than the controls (Fig. 4A). However, no significant difference in mortality was observed between the control psyllids and those treated with the lowest concentration (25 ng/μl) of dsRNA-*AChE*.

The SLR showed that *D. citri* adults became more susceptible to chlorpyrifos upon treatment with dsRNA-*AChE* (Fig. 4B). The Tukey's test also showed that the mortality of adults treated with 125 ng/μl and 100 ng/μl with dsRNA-*AChE* was higher than the control. However, results were non-significant at lower concentrations (25, 50, and 75 ng/μl dsRNA-*AChE*) (Fig. 4B).

Although the SLR showed that the susceptibility of *D. citri* adults to fenpropathrin increased after treatment with dsRNA-*AChE* (Fig. 4C), the Tukey's test did not show any differences between the treatments and the controls (Fig. 4C).

The SLR and Tukey's test showed that *D. citri* susceptibility to imidacloprid was not affected upon treatment with dsRNA-*AChE* (Fig. 4D). In addition, the SLR and the Tukey's ($P > 0.05$) test showed that treatment with dsRNA-*AChE* did not increase the mortality of *D. citri* adults to acetone which was used as a control insecticide (Fig. 4E). Acetone caused only 4.4% mortality when adults were treated at 125 ng/μl dsRNA-*AChE*.

4. Discussion

As there is no permanent cure for HLB, management of HLB disease mainly depends on the control of *D. citri* vector using insecticides [38,39]. However, the extensive use of insecticides can result in increasing insecticide resistance [16,17]. In our previous study, we characterized several esterases FE4 (*EstFE4*) and *AChE* genes in *D. citri* and showed that treatment with dsRNA reduced the expression level of these genes and increased the mortality of treated insects compared with the control [26]. In the present study, we investigated the effect of RNA interference of *AChE* on the mortality and susceptibility of *D. citri* to four common insecticides. Our current results showed that *D. citri* adults were susceptible to the four insecticides (imidacloprid, chlorpyrifos, carbaryl and fenpropathrin). Imidacloprid was the most effective, whereas carbaryl was the least effective insecticide among the four tested insecticides. In agreement with our result, previous studies also showed that neonicotinoids were the most effective class of insecticides for control of *D. citri* [13]. Our results were comparable to other results obtained from previous laboratory studies. It has been shown that *D. citri* adults were more susceptible to imidacloprid (LD_{50} value 0.004 ng/insect), followed by chlorpyrifos (LD_{50} = 0.25 ng/insect), fenpropathrin (LD_{50} = 0.30 ng/insect) and carbaryl (LD_{50} = 3.92 ng/insect) using topical application bioassay [17]. Using leaf-dip bioassay, it was found that fenpropathrin (LC_{50} = 0.24 ng/μl) was the most toxic

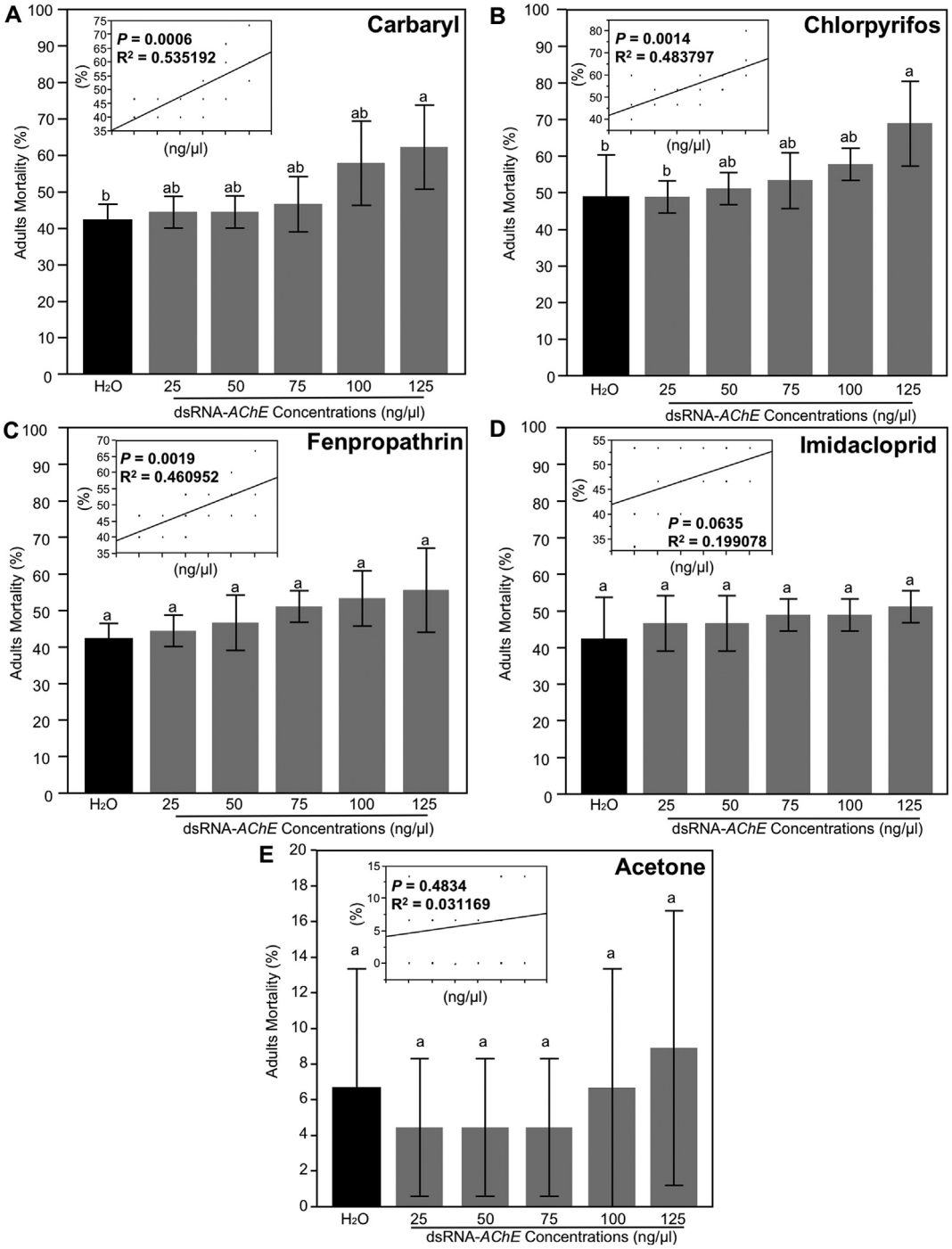

Fig. 3. Effect of dsRNA-*AChE* concentrations on *D. citri* susceptibility to four different insecticides, with simple linear regression plot of the concentration of dsRNA-*AChE* versus mortality. Mortality percentages of *D. citri* emerged adults from treated fourth and fifth instar nymphs with dsRNA-*AChE* after exposure to A) carbaryl; B) chlorpyrifos; C) fenpropathrin; D) imidacloprid at LC_{50} concentration for 24 h compared with E) acetone as a control for the insecticides. Different letters indicate the significant differences between the treatments ($P < 0.05$). Bars on columns illustrate the standard error.

insecticide to *D. citri* adults, followed by imidacloprid (LC_{50} = 0.31 ng/µl), chlorpyrifos (LC_{50} = 1.12 ng/µl) and carbaryl (LC_{50} = 18.84 ng/µl) at 27 °C [40].

Since *D. citri* used in this study were obtained from laboratory susceptible populations, the LD_{50} values reported in this study could be lower than actual field values. In a previous study, field populations of *D. citri* were more resistant to fenpropathrin, imidacloprid, malathion, and thiamethoxam than the laboratory susceptible population. The LD_{50} values for field populations of *D. citri* were significantly higher than the those from the laboratory susceptible population [17].

RNA interference has been widely used to knockdown the gene expression in insects. The effectiveness of RNAi depends on many factors including the delivery method of the dsRNA [41]. In the present study, we used topical feeding application for dsRNA treatments for both nymphs and adults of *D. citri*. The dsRNA droplet was topically applied on the ventral side of the thorax between the three pairs of legs [25,35]. Compared with the other methods of delivery, delivery of dsRNA using topical feeding application has many advantages. It delivers the precise dose, minimizes the possibility of the dsRNA degradation, and reduces the mortality of small and soft insects during the

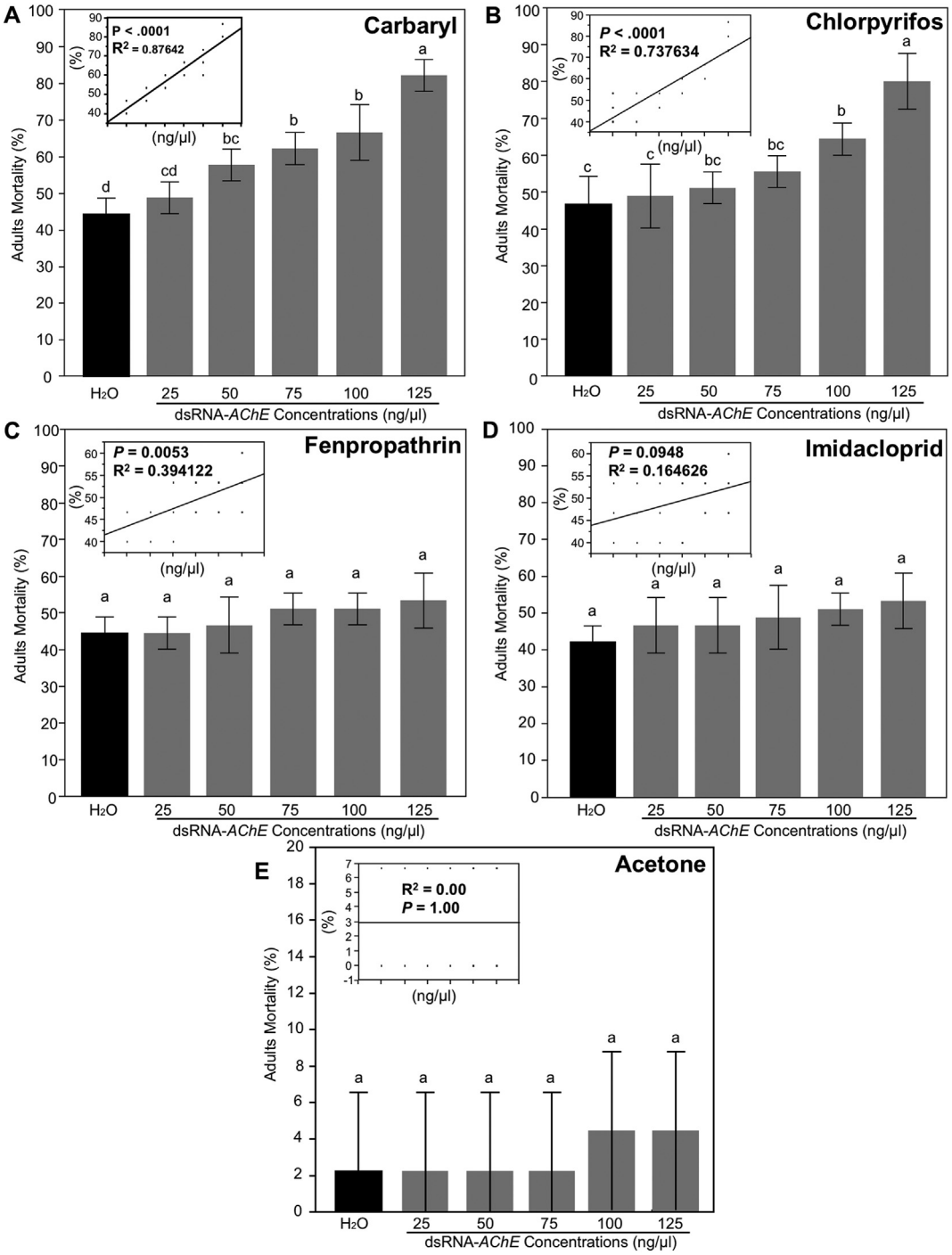

Fig. 4. Effect of five dsRNA-*AChE* concentrations on *D. citri* susceptibility to four different insecticides, with simple linear regression plot of the concentration of dsRNA-*AChE* versus mortality. A) Mortality of *D. citri* treated adults with dsRNA-*AChE* after treatment with carbaryl; B) chlorpyrifos; C) fenpropathrin and D) imidacloprid at LC_{50} concentration for 24 h compared with E) acetone as a control for the insecticides. The adults were transferred to non-treated leaf discs for 48 h before exposure to the insecticides. Bars labeled with different letters show significant differences between the treatments ($P < 0.05$). Bars on columns illustrate the standard error.

application [35].

Gene expression analysis for the *AChE* genes in the different developmental stages of *D. citri* showed that the expression levels in the 4th and 5th nymph instars were higher than first, second, and third nymph instars [26]. Our previous results showed that the expression levels in adults were slightly lower than the 4th and 5th instar nymphs [26]. In the current work, we treated the 4th and 5th instar nymphs of *D. citri* using dsRNA-*AChE* for silencing the *AChE* and *ChE* genes. The silencing of the targeted genes increased the mortality percentages in both emerged adults from treated nymphs and directly treated adults.

In agreement with our results, oral delivery of several dsRNAs (*cathepsin D*, *chitin synthase* and *inhibitor of apoptosis*) using an artificial diet also increased the mortality in nymphs and adult of *D. citri* [28]. Additionally, silencing the abnormal wing disc (*awd*) gene in nymphs using dsRN-*awd* increased the mortality rate of *D. citri* [42]. Furthermore, dsRNA-*DcMP20* delivery through soaking mediated silencing of the muscle protein 20 and increased the mortality rates of *D. citri* nymphs [27].

Our results showed that silencing of *AChE* and *ChE* by dsRNA-*AChE* increased *D. citri* susceptibility to carbaryl and chlorpyrifos more than

fenpropathrin and imidacloprid. Chlorpyrifos and carbaryl act by inhibiting acetylcholinesterase (*AChE*) [11], whereas fenpropathrin acts as sodium channel modulator and imidacloprid acts as an agonist of nicotinic acetylcholine (nAChR) receptor [11].

Although the Tukey's test did not show any differences in mortality percentage to fenpropathrin between dsRNA-*AChE* treated *D. citri* and the control, the SLR showed that the susceptibility of *D. citri* adults to fenpropathrin was increased upon treatment with dsRNA-*AChE* as either nymphs or adults. The slight increase in the susceptibly of *D. citri* to fenpropathrin indicated that *AChE* and *ChE* could be involved in the metabolism of fenpropathrin which requires a splitting of the ester bond. Esterases and mixed-function oxidases play a major role in pyrethroid metabolism [43]. In agreement with this assumption, previous studies also showed that *AChE* enzyme catalyzes the hydrolysis of esters other than acetylcholine and its derivatives. For example, *AChE* from *Pseudomonas aeruginosa* PAO1 was able to hydrolyze artificial esters, but with lower catalytic efficiency compared to acetylthiocholine and propionylthiocholine [44].

Like *D. citri*, most insects contain two copies of *AChE* [45]. The presence of two *AChE* genes in many insects has attracted the attention of many scientist to study their functions and roles in insecticide resistance [45]. Although most insects contain two copies of *AChE*, it is believed that only one of them is active. Because the gene expression of *AChE2* was positively correlated with the enzymatic activity of *AChE*, it was suggested that *AChE2* rather than *AChE1* was the major *AChE* in the silkworm, *Bombyx mori* [46]. Using computational analyses, Lu et al. suggest that the *AChE1* protein was catalytically active in red flour beetle (*Tribolium castaneum*), whereas *AChE2* was not efficient [45]. In agreement with the computational analyses, silencing of *TcAce1* in *Tribolium castaneum* (larvae) increased their mortality and susceptibilities to anticholinesterase insecticides including two cabamates (carbofuran and carbaryl) and two organophosphates (malathion and dichlorvos) [47]. On the other hand, silencing *TcAce2* did not affect insect mortality and insecticide susceptibility, but affected insect development, number of laid eggs, and egg hatching [47]. The previous results indicated that *TcAce1* plays a role in cholinergic functions, whereas *TcAce2* plays an important role in female reproduction, embryo development, and growth of offspring [47]. Because the function of *AChE* genes has not been determined in *D. citri* yet, we targeted both copies of *AChE* to silence this gene. Further study is needed to identify which of these two *AChE* genes has cholinergic functions in *D. citri*.

In agreement with our results, the susceptibility of *D. citri* to imidacloprid was increased upon silencing of CYP4, an important group of enzymes involved in the metabolism of xenobiotics in insects, using RNAi [25]. In conclusion, our findings showed that silencing of *AChE* and *ChE* genes in *D. citri* could be used as a promising tool to control this important vector. Our results showed that silencing of *AChE* and *ChE* genes in *D. citri* increased their mortality and susceptibility to carbaryl and chlorpyrifos, which target *AChE*. Combining new technologies such as RNAi and traditional pesticide applications may reduce the amount of pesticides required to control the Asian citrus psyllid and help reduce environmental impact of toxic pesticides used in agriculture. Our current findings suggest that *D. citri* susceptibility to carbamate and organophosphate insecticides could be enhanced in the field if they were previously exposed to dsRNA-*AChE*. However, several challenges have to be resolved before this becomes practical in the field.

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