



Effect of Quorum Sensing Inducers and Inhibitors on Cytoplasmic Incompatibility Induced by *Wolbachia* (Rickettsiales: Anaplasmataceae) in American Serpentine Leafminer (Diptera: Agromyzidae): Potential Tool for the Incompatible Insect Technique

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Abstract

Agricultural crops around the world are attacked by approximately 3,000–10,000 species of pest insect. There is increasing interest in resolving this problem using environmentally friendly approaches. *Wolbachia* (Hertig), an insect endosymbiont, can modulate host reproduction and offspring sex through cytoplasmic incompatibility (CI). The incompatible insect technique (IIT) based on CI-*Wolbachia* is a promising biological control method. Previous studies have reported an association between CI and *Wolbachia* density, which may involve a quorum sensing (QS) mechanism. In this study, we investigated the effect of manipulating QS in *Wolbachia* using several chemicals including 3O-C12-HSL; C2HSL; spermidine (QS inducers), 4-phenylbutanoyl; and 4-NPO (QS inhibitors) on American serpentine leafminer (*Liriomyza trifolii* [Burgess]), an agricultural pest. The results showed that inducing QS with 3O-C12-HSL decreased the proportion of hatched eggs and increased *Wolbachia* density, whereas QS inhibition with 4-phenylbutanoyl had the opposite effects. Thus, manipulating QS in *Wolbachia* can alter cell density and the proportion of hatched eggs in the host *L. trifolii*, thereby reducing the number of insect progeny. These findings provide evidence supporting the potential efficacy of the IIT based on CI-*Wolbachia* for the environmentally friendly control of insect pest populations.

Key words: Insect pest, insect endosymbiont, environmentally friendly control

Around 3,000–10,000 species of insects are agricultural pests (Pimentel 2009, Dhaliwal et al. 2015, Lara and Saldivar 2015). As this is a major problem in agriculture, there is increasing interest in control methods using insect endosymbionts including *Wolbachia* for pests control as an alternative environmentally and economically sustainable solution (Zabalou et al. 2004, Kandel et al. 2021). *Wolbachia* is a ubiquitous endosymbiotic bacterium found in Nematoda and Arthropoda (Tagami et al. 2006a, Werren et al. 2008). Approximately 60% of species in both taxa are infected with *Wolbachia* (Hilgenboecker et al. 2008). *Wolbachia* has several adaptive strategies to manipulate reproduction in the host including cytoplasmic incompatibility (CI), male-killing, parthenogenesis, and feminization (Werren 1997). CI, which is among the most widespread strategies, involves modifying host male sperm, which yields fertilized eggs that do not hatch due to embryo mortality (Zabalou et al. 2004, Dong et al. 2006). CI occurs when a male insect infected with *Wolbachia* mates with a female that is

uninfected or infected with a different *Wolbachia* strain (Hoffmann et al. 1986, Engelstädter and Telschow 2009).

The induction of CI with *Wolbachia* (CI-*Wolbachia*) can be constituted as an incompatible insect technique (IIT). The principle of this technique is similar to the sterile insect technique (SIT). The SIT method reduces insect pest populations through sterilization of adult males via irradiation (Knippling 1955, Nikolouli et al. 2018), while the IIT produces embryonic mortality in offspring, when infected males mate with uninfected females or males and females with different *Wolbachia* strain copulate. (Zabalou et al. 2004, Nikolouli et al. 2018). The IIT using *Wolbachia* has been applied to several insect pests including, *Culex pipiens* (Linnaeus) (Diptera: Culicidae) (Atyame et al. 2015), *Aedes polynesiensis* (Marks) (Diptera: Culicidae) (O'Connor et al. 2012), and *Aedes albopictus* (Skuse) (Diptera: Culicidae) (Zhang et al. 2015, Moretti et al. 2018), and could potentially be used in other insect pests.

Liriomyza trifolii is a major insect pest around the world. While the first cases were recorded in the Nearctic and Neotropical regions, an outbreak was later reported in the U.S. state of Florida (Parrella and Keil 1984, Minkenberg 1988). More recently, the geographic distribution of *L. trifolii* has expanded to Asia (Tagami et al. 2006b, Abe 2017), causing failure of crops such as tomato, eggplant (Solanaceae), melon, cucumber (Cucurbitaceae), kidney bean (Fabaceae), celery (Apiaceae), onion (Amaryllidaceae), and pak choi (Brassicaceae) as well as ornamental flowers in the Asteraceae family (Abe 2017). Insecticides have been used to reduce *Liriomyza* populations, which have responded by developing resistance (Ferguson 2004, Weintraub et al. 2017).

Liriomyza trifolii is infected only with *Wolbachia* and not with *Cardinium* (Zchori-Fein) (Cytophagales: Amoebophilaceae) (Tagami et al. 2006b), proving that the CI effect in *L. trifolii* is only because of *Wolbachia*. *Wolbachia* colonizing *L. trifolii* has an excellent vertical transmission and induces CI, making it suitable as a possible insect pest control agent (Tagami et al. 2006a). CI-*Wolbachia* levels vary across insect species. For instance, CI can range from strong (low proportion of hatched eggs with nearly all offspring dying) as in *Rhagoletis cerasi* (Linnaeus) (Diptera: Tephritidae), *Glossina morsitans* (Westwood) (Diptera: Glossinidae), and *Aedes aegypti* (Linnaeus) (Diptera: Culicidae) (Riegler and Stauffer 2002, Alam et al. 2011, Hoffmann et al. 2015, Sicard et al. 2019), to weak (high proportion of hatched eggs with most offspring surviving) as in *Drosophila simulans* (Sturtevant) (Diptera: Drosophilidae), *Drosophila mauritiana* (Tsacas & David) (Diptera: Drosophilidae), and *Anopheles* sp. (Meigen) (Diptera: Culicidae) (Hoffmann et al. 1996, Meany et al. 2019, Sicard et al. 2019). CI-*Wolbachia* level is influenced by several factors including *Wolbachia* strain, *Wolbachia* diversity, host species, age, temperature, superinfection, food availability, antibiotics, and *Wolbachia* density (Breeuwer and Werren 1993, Clancy and Hoffmann 1998, Noda et al. 2001, Ikeda et al. 2003, Thomas and Blanford 2003, Mouton et al. 2006, Hoffmann et al. 2015).

The relationship between *Wolbachia* density and CI level may involve a quorum sensing (QS) mechanism. QS is a bacterial cell-to-cell signal communication according to population density (Kalia 2014, Tommonaro 2019), when a quorum of cells reaches a threshold density, a gene regulatory system is activated that results in phenotypic changes (Kalia 2014, Tommonaro 2019, Coquant et al. 2020). Bacterial cells communicate with each other by detecting or producing signaling molecules known as autoinducers such as N-acylated homoserine lactone (AHL) in Gram-negative bacteria (Papenfort and Bassler 2016). AHL is produced by the highly conserved *LuxI* gene or its homologs, resulting in the expression of *LuxR* transcriptional regulator (Whitehead et al. 2001, Papenfort and Bassler 2016). Several commercial AHL can induce QS of bacteria, including 3O-C12-HSL that promote QS of *Pseudomonas aeruginosa* (Schroeter) (Pseudomonadales: Pseudomonadaceae), mice pathogen (Smith et al. 2002), 3O-C6-HSL which induce QS of *Sodalis glossinidius* (Dale & Maudlin) (Enterobacteriales: Bruguierivoracaceae), an endosymbiont of *Glossina* spp. (Wiedemann) (Diptera: Glossinidae) (Pontes et al. 2008). QS is a critical mechanism for controlling activities associated with a symbiosis that influences host physiologic functions (Borlee et al. 2010, Pietschke et al. 2017).

In this study, we investigated the effect of manipulating QS in *Wolbachia* within *L. trifolii*, using several chemicals including 3O-C12-HSL; C2HSL; spermidine (QS inducers), 4-phenylbutanoyl; and 4-NPO (QS inhibitors). We show for the first time that *Wolbachia* density and the proportion of hatched eggs of *L. trifolii* can be controlled by inducing or inhibiting QS in *Wolbachia*, which is potent to apply as an IIT.

Materials and Methods

Insect Rearing

Wolbachia-infected *L. trifolii* isolated in Hamamatsu, Shizuoka, Japan in 1991 (Tagami et al. 2006a) was reared on the leaves of kidney bean plants in a 34 cm (width) × 35 cm (length) × 34 cm (height) cage at 23°C under a 16:8-h light/dark photoperiod.

DNA Extraction and *Wolbachia* Detection

Wolbachia infection status was confirmed by PCR using forward and reverse primers for *Wolbachia* surface protein (wsp81F and wsp691R, respectively) (Braig et al. 1998). For PCR analysis, DNA was extracted from 10–15 adults *L. trifolii* by sodium dodecyl sulfate (SDS)–ethanol precipitation according to a previously described method (Haukedal 2013), with slight modifications. Insect specimens in 50 µl SDS lysis buffer were crushed with a motorized pestle (Power Masher II; Nippi, Tokyo, Japan); 150 µl of SDS buffer and 20 µl proteinase-K (20 mg/ml) were added to the homogenate, which was incubated at 56°C for at least 6 h. The proteinase-K was deactivated at 99°C for 3 min and the sample was centrifuged (CT 15RE; Hitachi, Tokyo, Japan) at 7,000 rpm for 4 min. The supernatant was collected and a one-eighth volume of 8 M ammonium acetate and double the volume of 99%–100% ethanol was added, followed by incubation at –20°C for one h or 4°C overnight. To purify the DNA, the sample was centrifuged at 12,000 rpm for 30 min. The supernatant was removed, and the pellet was washed with 500 µl of 70% ethanol and then centrifuged at 12,000 rpm for 10 min. The supernatant was discarded and after drying, the pellet was resuspended in 100 µl Tris-EDTA buffers. DNA purity and yield (Bustin et al. 2009), were verified with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

To verify *Wolbachia* infection status, DNA was extracted from a single adult specimen crushed in 30 µl sodium chloride-Tris-EDTA buffer containing 2 µl proteinase-K (20 mg/ml) and incubated at 56°C for two h. PCR was performed using GoTaq (Promega, Madison, WI) according to standard procedures on a PCR Thermal Cycler (Takara, Otsu, Japan). The cycling conditions were 98°C for 10 s; 35 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 1 min; and 72°C for 4 min.

Antibiotic Treatment

To obtain *Wolbachia*-uninfected *L. trifolii*, infected pupae were collected in a new cage with dimensions of 6.5 cm (width) × 6.5 cm (length) × 9.5 cm (height), emerged flies were treated with 50 mg/g tetracycline hydrochloride in honey for a single day. Newly emerged adults were transferred after one day to a new cage with dimensions of 34 cm (width) × 35 cm (length) × 34 cm (height); the leaves were sprayed with 20 mg/ml tetracycline hydrochloride (Tagami et al. 2006b). This process was repeated for two to three generations. The infection status was confirmed by PCR. The treated *Wolbachia*-uninfected *L. trifolii* were used for crossing experiments.

QS Inducer and Inhibitor Treatments

The QS inducers N-(3-oxododecanoyl) homoserine lactone (3O-C12-HSL) and N-acetyl-l-homoserine lactone (C2HSL) (both from Tocris, Minneapolis, MN) and spermidine (Cayman Chemical, Ann Arbor, MI) and QS inhibitors 4-phenylbutanoyl (4-phenyl) (Combi-Blocks, San Diego, CA) and 4-nitro-pyridine-N-oxide (4-NPO) (Tokyo chemical industry, Tokyo, Japan) were used at the following concentrations: 3O-C12-HSL: 1, 10, and 100 µM (Smith et al. 2002, Josephson et al. 2020), C2HSL: 0.1, 1, and

10 μM (Smith et al. 2002; Liu et al. 2019); spermidine, 1, 10, and 100 μM (Nasrallah et al. 2011); 4-phenyl, 50, 100, and 150 μM (Schaefer et al. 1996); and 4-NPO: 10 and 100 μM , and 1 mM (Rasmussen et al. 2005). Unmated *L. trifolii* adults were collected within 24 h after emergence and were fed the chemical in honey (honey put in 1x1 cm cotton) in a 6.5 cm (width) \times 6.5 cm (length) \times 9.5 cm (height) cage, for one day. Treated *L. trifolii* were reared in a 34 cm (width) \times 35 cm (length) \times 34 cm (height) cage; for indirect uptake, the chemicals were sprayed daily onto the leaves up to the larva stage of the next generation.

Crossing Experiments

Crossing experiments were performed to observe and evaluate the effect of QS inducer or inhibitor in the number of oviposited eggs, larvae, and adults. We observed four crosses combinations (w+ σ and w- σ , w- σ and w+ σ , w+ σ and w+ σ , w- σ and w- σ) in every different QS inducer and inhibitor. At least six pair crosses were carried out for each cross combination in different QS inducer and inhibitor treatments. A pair of virgin individuals were reared on a kidney bean leaf in an 11.5 cm (diameter) \times 8.5 cm (height) plastic cup. The number of oviposited eggs, larvae, and adult longevity (until death) were recorded.

Cytoplasmic Incompatibility Assays

The CI expression levels were checked in crosses between male infected with female uninfected. To correct the nuisance from normal mortality caused by the crossing that uncorrelated with CI, we used a corrected index of CI (CI_{corr}) which is described as the percentage of egg that do not hatch (from the CI crosses between infected males and uninfected females) among the crosses which survive without CI effect. Then $CI_{\text{corr}}(\%) = [(CI_{\text{obs}} - CCM)/(100 - CCM)] \times 100$, where CI_{obs} is the percentage of unhatched eggs detected in the CI cross (infected males and uninfected females) and CCM is the percentage of unhatched eggs observed in the compatible cross from reciprocal crosses (uninfected males and infected females) (Poinsot et al. 1998). For CI experiments, the status of *Wolbachia* infection in female and male parents and their offspring was confirmed by PCR.

qPCR

Wolbachia density under different chemical treatments was evaluated by qPCR on a Thermal Cycler Dice Real-Time System II (TP 970; Takara). The total reaction volume of 25 μl contained 12.5 μl TB Green Premix Ex Taq II (Takara), 8.5 μl sterile water, 1 μl each of forward and reverse primers, and 2 μl template DNA. The cycling conditions were 98 $^{\circ}\text{C}$ for 10 s, followed by 40 cycles of 98 $^{\circ}\text{C}$ for 10 s and 55 $^{\circ}\text{C}$ for 30 s. Relative *Wolbachia* density was calculated with the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001) by comparing the copy number of the *wsp* gene to that of the *L. trifolii* cytochrome oxidase I (*COI*) gene (Watanabe et al. 2011). The following primers were designed using NCBI Primer 3 Plus (Ye et al. 2012), *wsp*, L.tri *wsp*-F (5'-ACTCCATACGTTGGTGTGGT-3') and L.tri *wsp*-R (5'-CCAAAATAACGACTCCAGCA-3'); and *COI*, L.tri *COI*-F (5'-GGGAAAGCTATATCTGGGGCT-3') and L.tri *COI*-R (5'-CCGGTGCCCTAATTGGTAT-3'). There were six replicates for each treatment (3 biological replicates, each with at least two technical replicates for each gene) (Taylor et al. 2010).

Statistical Analysis

Effect of QS inducer and QS inhibitor on *Wolbachia* density in *L. trifolii* was estimated with ΔC_t which refer to $2^{-\Delta\Delta C_t}$ (Yuan et al. 2006), followed by one-way analysis of variance (ANOVA), and the Tukey's honestly significant difference (HSD) test for comparison of means (Tukey 1949).

The normal distribution of data was tested with the Shapiro–Wilk test (Shapiro and Wilk 1965). Data on the QS inducer and QS inhibitor effect on oviposited eggs were normally distributed and evaluated by one-way ANOVA. Data on the effect of QS inducer and QS inhibitor on adult longevity were non-normally distributed and were analyzed with the Kruskal–Wallis test (Kruskal and Wallis 1952).

CI levels were analyzed using a generalized linear model (GLM) with binomial distribution and logit link function. Those analyses are appropriate to analyze the proportion data model (Crawley 2012, Demétrio et al. 2014, McCullagh and Nelder 2019).

Statistical analyses were performed using SPSS v21.0 software (Nie et al. 1975) and R v4.0.4 (R 2013). $P < 0.05$ was considered statistically significant in all statistical tests.

Results

Wolbachia Density After Treatment With QS Inducer

Induction of QS using 3O-C12-HSL increased the density of *Wolbachia* (one-way ANOVA, $F = 5.62$, $df = 3, 8$, $P = 0.023$) (Fig. 1). The density of *Wolbachia* was increased 3.3-fold with respect to the control (Tukey's HSD, $df = 2, 2$, $P = 0.081$) due to treatment with 1 μM of 3O-C12-HSL. Furthermore, the density was increased 5.35 fold (Tukey's HSD, $df = 2, 2$, $P = 0.017$) due to treatment with 10 μM of 3O-C12-HSL. The density of *Wolbachia* was not increased anymore when 100 μM of 3O-C12-HSL were applied (2.94 fold of control) (Tukey's HSD, $df = 2, 2$, $P = 0.12$). Therefore, 10 μM of 3O-C12-HSL was considered the most effective concentration for increasing the density of *Wolbachia* in infected *L. trifolii*.

Application of C2HSL (Fig. 2) and spermidine (Fig. 3) increased the density of *Wolbachia* in a dose-dependent manner, but the results were not statistically significant (C2HSL: one-way ANOVA, $F = 1.61$, $df = 3, 9$, $P = 0.254$; spermidine: one-way ANOVA, $F = 0.31$, $df = 3, 8$, $P = 0.814$).

Wolbachia Density After Treatment With QS Inhibitor

Inhibition of QS using 4-phenyl decreased the density of *Wolbachia* in a significant way (one-way ANOVA, $F = 4.37$, $df = 3, 8$, $P = 0.042$). Significant difference compared to the control group was observed

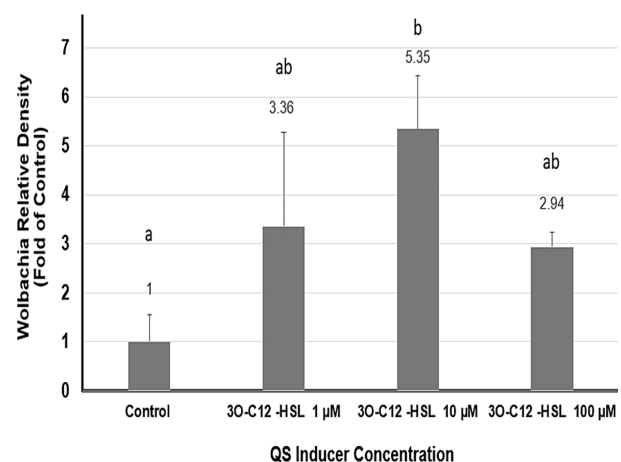


Fig. 1. Relative density of *Wolbachia* in *Lirimyzoa trifolii* following treatment with the QS inducer 3O-C12-HSL. Values are shown as means with standard errors of six replicates (Three biological replicates, each consisting of two technical replicates). Different letters above the bars indicate statistically significant differences (Tukey's honestly significant difference test, $P < 0.05$). 3O-C12-HSL 10 μM showed *Wolbachia* density significant difference with control (3O-C12-HSL 0 μM).

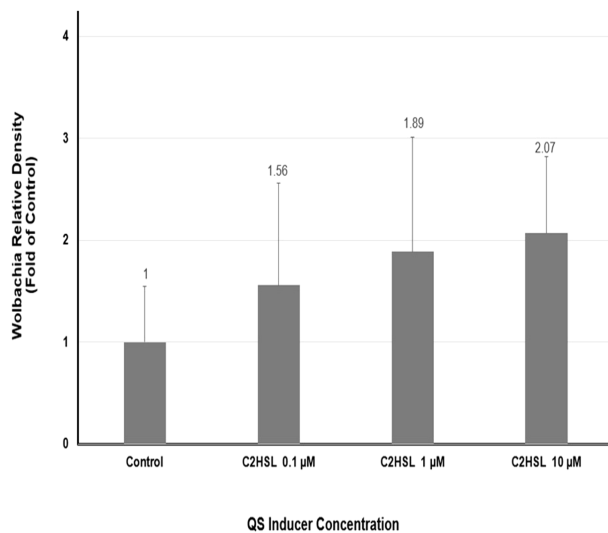


Fig. 2. Relative density of *Wolbachia* in *Lirimyoza trifolii* following treatment with the QS inducer C2HSL. Values are shown as means with standard errors of six replicates (Three biological replicates, each consisting of two technical replicates). Data were analyzed by one-way ANOVA ($P > 0.05$).

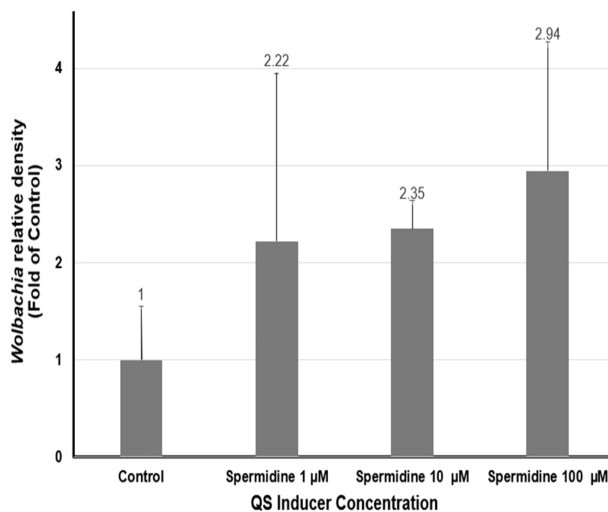


Fig. 3. Relative density of *Wolbachia* in *Lirimyoza trifolii* following treatment with the QS inducer spermidine. Values are shown as means with standard errors of six replicates (Three biological replicates, each consisting of two technical replicates). Data were analyzed by one-way ANOVA ($P > 0.05$).

at the highest concentration (150 μM) of 4-phenyl (Tukey's HSD, $df = 2, 2$; $P = 0.038$) (Fig. 4.). There was a similar dose-dependent effect noted for 4-NPO (Fig. 5.), but the results were not statistically significant (one-way, ANOVA, $F = 1.17$, $df = 3, 8$, $P = 0.377$).

Crossing Experiments and Cytoplasmic Incompatibility Assays

Crossing experiments were performed to investigate the effect of QS manipulation on the CI expression of *L. trifolii*. Treatment with the QS inducer 3O-C12 HSL reduced egg hatching rates/ induced strong CI (GLM, $z = -2.40$, $P < 0.05$). The CI_{corr} values after 3O-C12 HSL treatment was $99.4\% \pm 0.59$. Whereas treatment with the QS inhibitor 4-phenyl had the opposite effect (GLM, $z = 4.94$, $P < 0.001$), reduced CI_{corr} values ($68.38\% \pm 18.8$) (Fig. 6).

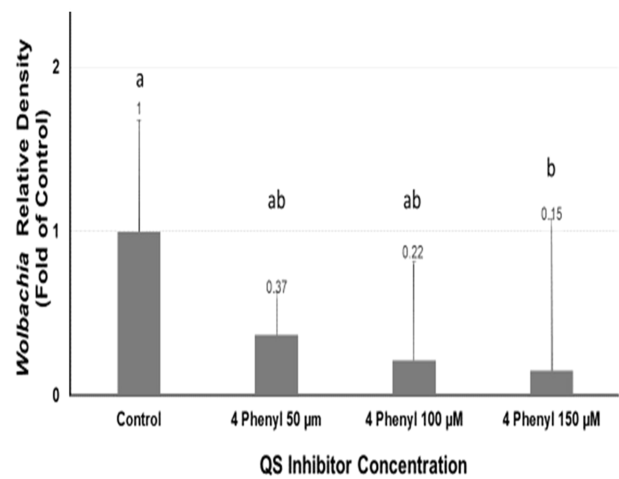


Fig. 4. Relative density of *Wolbachia* in *Lirimyoza trifolii* following treatment with the QS inhibitor 4-phenyl. Values are shown as means with standard errors of six replicates (Three biological replicates, each consisting of two technical replicates). Different letters above the bars indicate statistically significant differences (Tukey's honestly significant difference test, $P < 0.05$). 4-Phenyl 150 μM showed *Wolbachia* density significant difference with other concentration including control (4-phenyl 0 μM).

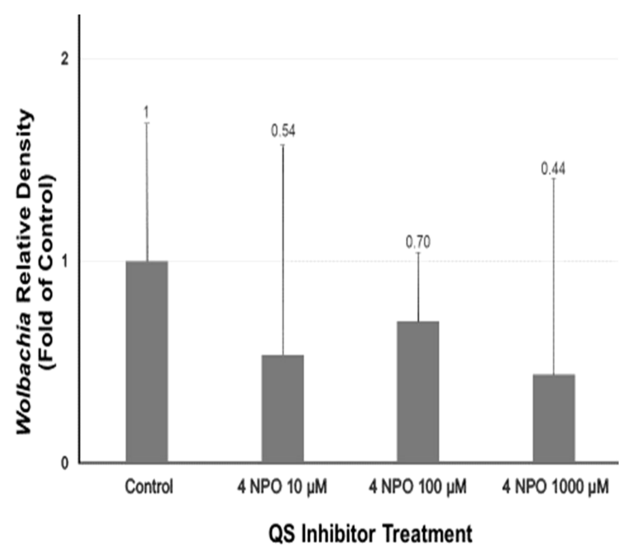


Fig. 5. Relative density of *Wolbachia* in *Lirimyoza trifolii* following treatment with the QS inhibitor 4-NPO. Values are shown as means with standard errors of six replicates (Three biological replicates, each consisting of two technical replicates). Data were analyzed by one-way ANOVA ($P > 0.05$).

Number of Ovipositing Eggs

There was no difference in the number of oviposited eggs across groups compared to the control under the different chemical treatments (one-way ANOVA, $F = 0.97$, $df = 23, 126$, $P = 0.49$) (Table 1).

Adult Longevity

QS inducer and inhibitor treatment did not affect the longevity of emerged adult compared to the control group, and there were no differences between males ($H(11) = 14.71$, $P = 0.196$) and females ($H(11) = 15.623$, $P = 0.156$) for any treatment including the control (Table 2, Supp Figs. S1 and S2 [online only]).

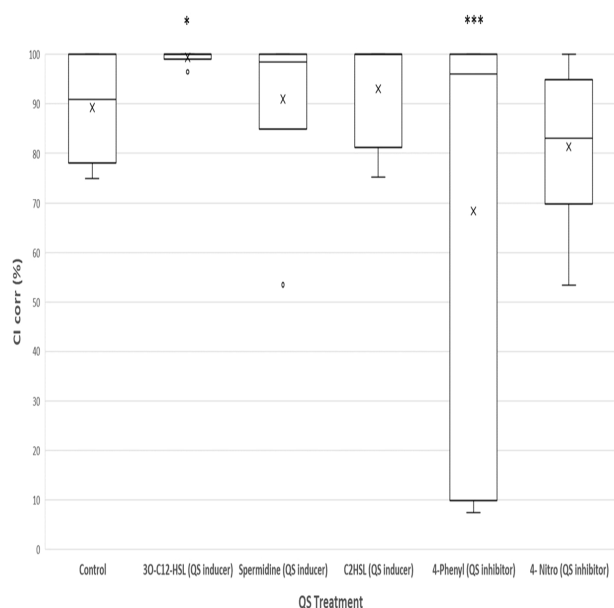


Fig. 6. Cytoplasmic incompatibility levels resulting from a cross between a *Lirimyzoa trifolii* male infected with *Wolbachia* and an uninfected female under QS inducer or inhibitor treatment. The corrected index of CI (CICorr) was performed to correct the nuisance from normal mortality caused by the crossing that uncorrelated with CI. A generalized linear model (GLM) binomial family analysis was used to determine the differences between QS treatment (* $P < 0.05$, *** $P < 0.001$).

Table 1. Effect of QS inducer or QS inhibitor on number of oviposited eggs in *Lirimyzoa trifolii* crossing experiments

QS treatment	Male	Female	No. of oviposited eggs*
Control	+	-	20.60 ± 3.10
	-	+	24.00 ± 1.93
	+	+	25.57 ± 3.42
	-	-	34.00 ± 2.20
QS inducer 3O-C12HSL	+	-	27.91 ± 0.96
	-	+	26.66 ± 2.53
	+	+	33.20 ± 5.69
	-	-	32.50 ± 3.49
Spermidine	+	-	31.50 ± 3.40
	-	+	32.10 ± 2.90
	+	+	28.66 ± 3.14
	-	-	25.83 ± 4.36
C2HSL	+	-	30.83 ± 5.10
	-	+	32.00 ± 5.32
	+	+	26.00 ± 3.45
	-	-	25.16 ± 2.72
QS inhibitor 4-Phenyl	+	-	24.83 ± 2.95
	-	+	34.10 ± 5.14
	+	+	31.00 ± 3.40
	-	-	31.66 ± 5.90
4-NPO	+	-	30.14 ± 4.32
	-	+	31.40 ± 1.16
	+	+	21.60 ± 1.32
	-	-	28.83 ± 5.60

Data represent mean ± standard error of 6 replicates. +, Infected with *Wolbachia*; -, uninfected.

*No significant difference ($P > 0.05$, analysis of variance).

Discussion

In this study, we investigated the effect of QS inducers 3O-C12-HSL, C2HSL, spermidine, and QS inhibitors 4-phenyl, and 4-NPO on *L. trifolii* and *Wolbachia* inside *L. trifolii*. 3O-C12-HSL and C2HSL are specific autoinducers in Gram-negative bacteria (Liu et al. 2019); and spermidine is a polyamine that is used to stimulate intracellular growth, such as enhance the cell growth (Nasrallah et al. 2011). We found that the density of *Wolbachia* was increased compared to the control due to treatment with 3O-C12-HSL (Fig. 1). Probably 3O-C12-HSL are accumulated in the cells and stimulates the proliferation of *Wolbachia*. In contrast, neither C2HSL nor spermidine had significant effects on *Wolbachia* density. In our study, spermidine did not produce a specific effect on QS, probably because spermidine has a broad effect. It not only induces QS in a specific way but can also modulate gene expression, biosynthesis, degradation, transport, and cell growth by targeting RNA polymerase activity (Chan and Chua 2010, Igarashi and Kashiwagi 2010, Martino et al. 2013).

Variable effects of AHL QS inducers on the density of *Wolbachia* were observed in our study. It may be attributed to the fact that the different positions or lengths of the acyl chain. Gram-negative bacteria have at least 30 AHLs composed of a homoserine lactone ring and various acyl side chains, the length of which determine the signaling specificity of the molecule (Parsek and Greenberg 2000, Liu et al. 2019, Phuong et al. 2020). 3O-C12-HSL has a more extended chain structure than C2HSL (Smith et al. 2002, González et al. 2013). AHLs with a long acyl chain including 3O-C12-HSL strongly induce QS. It was shown that they enhance adhesion of *Acidithiobacillus ferrooxidans* (Temple & Colmer) (Acidithiobacillales: Acidithiobacillaceae) cells to substrates via a QS mechanism (González et al. 2013). Our results also support the results of a previous study demonstrating that nonacylated HSLs, such as C2HSL and C4HSL have a modest QS-inducing effect than 3O-C12-HSL in *Pseudomonas aeruginosa* implying that the characteristics such as length and presence of the acyl side chain influences this function (Smith et al. 2002).

Our qPCR data showed that treatment with the QS inhibitor 4-phenyl (150 µM) reduced the density of *Wolbachia* by 0.15 fold with respect to the control (Fig. 4). 4-Phenyl has analogous structure with the QS inducer. Therefore 4-phenyl binds to *LuxR* to prevent the binding of 3-oxohexanoyl as well as the expression of QS-related genes (Schaefer et al. 1996, Kalia 2014). 4-Phenyl was shown to inhibit *LuxR* strongly in *Vibrio fischeri* (Beijerinck) (Vibrionales: Vibrionaceae) (Geske et al. 2007). In our study, 4-NPO did not suppress *Wolbachia* density (Fig. 5), and probably did not inhibit the QS system in *Wolbachia*. 4-NPO suppresses the QS activity of *P. aeruginosa* and formation of biofilm (Rasmussen et al. 2005), but did not affect protease activity or inhibit the QS system of *Pectobacterium* (Waldee) (Enterobacteriales: Pectobacteriaceae) (Rasch et al. 2007). Nonetheless, results of these experiments indicate that density of *Wolbachia* can be manipulated by the QS system targeting chemical agents.

QS inducer and inhibitor treatment did not significantly affect the number of oviposited eggs (Table 1) or adult longevity (Table 2, Supp Figs. S1 and S2 [online only]), likely because the doses used in this study (0.1–1,000 µl) had only minor effects on *L. trifolii*. The oral 50% lethal dose of 4-NPO is 107 mg/kg in rats and 20 mg/kg in pigeons (Goodhouse and Baumgartner 1965).

We found that the average survived days of male and female adults were 3.73 and 8.78 d respectively (Table 2, Supp Figs. S1 and S2 [online only]), this is in line with the previously reported 3

Table 2. Effect of QS inducer or QS inhibitor on the longevity of adult *Lirimyza trifolii*

QS treatment	Male		Female	
	Infection status	Adult longevity, days*	Infection status	Adult longevity, days*
Control	+	3.30 ± 0.33	+	8.00 ± 0.40
	-	4.00 ± 0.26	-	9.60 ± 0.57
QS inducer				
3O-C12HSL	+	3.57 ± 0.29	+	7.66 ± 0.66
	-	4.00 ± 0.70	-	7.60 ± 0.50
C2HSL	+	3.09 ± 0.21	+	8.30 ± 0.95
	-	3.75 ± 0.47	-	9.00 ± 1.18
Spermidine	+	5.60 ± 0.87	+	7.77 ± 0.49
	-	3.60 ± 0.33	-	9.60 ± 0.33
QS inhibitor				
4-Phenyl	+	3.50 ± 0.29	+	7.00 ± 0.31
	-	4.00 ± 0.31	-	6.80 ± 1.10
4-NPO	+	3.10 ± 0.40	+	8.30 ± 1.00
	-	3.75 ± 1.10	-	9.60 ± 1.02

Data represent mean ± standard error. +, Infected with *Wolbachia*; -, uninfected.

*No significant difference ($P > 0.05$, Kruskal–Wallis test).

and 8–12 d, respectively (Charlton and Allen 1981, Zoebisch and Schuster 1987, Koo et al. 2012).

Treatment with the QS inducer 3O-C12-HSL induce strong CI in *L. trifolii* (Fig. 6). We also found that high *Wolbachia* density was associated with the low proportion of hatched eggs following 3O-C12-HSL treatment (Figs. 1 and 6). Our results are consistent with an earlier finding that high *Wolbachia* density was positively correlated with a low proportion of hatched eggs in insect hosts including *Laodelphax striatellus* (Fallén) (Delphacidae) (Noda et al. 2001). On the contrary, low density of *Wolbachia* in *Drosophila simulans* and *Sogatella furcifera* (Horvath) (Hemiptera: Delphacidae) were correlated with a high proportion of hatched eggs in both species (Bressac and Rousset 1993, Clancy and Hoffmann 1998, Noda et al. 2001).

3O-C12-HSL was reported to induce QS during Gram-negative bacteria infection (Smith et al. 2002), thereby affecting the host (Josephson et al. 2020). Dermal injection of 1–10 µM 3O-C12-HSL in mice induced QS in *P. aeruginosa*, leading to lung inflammation (Smith et al. 2002); and 50 µM 3O-C12-HSL modulated QS in bacteria, which impacted host mitochondria (Josephson et al. 2020). As QS is related to bacterial density and *Wolbachia* density influences CI level, QS may be indirectly associated with CI level. When *Wolbachia* density reaches a threshold level, the expression of target genes is activated or inhibited (Smith et al. 2002). *Wolbachia* density probably activated CI genes. This was supported by our data, which showed associations among QS chemical treatment, *Wolbachia* density, and CI levels in the insect host.

For CI-*Wolbachia* to be effective as a control measure for *L. trifolii*, the proportion of hatched eggs must be drastically reduced so that offspring will not be produced. It was suggested that the strain of *Wolbachia* is the main determinant of CI-*Wolbachia* efficacy (Noda et al. 2001). However, trans infection of *Tribolium confusum* (Jacquelin du Val) (Coleoptera: Tenebrionidae) and *Tribolium castanem* (Herbst) (Coleoptera: Tenebrionidae) with a strong CI strain of *Wolbachia* has had limited success (Hughes and Rasgon 2014), indicating that a close phylogenetic relationship between insect and hosts does not guarantee efficient trans infection (Ikeda et al. 2003, Zabalou et al. 2004). There have been numerous attempts of microinjection of *Wolbachia* into *Anopheles* spp., but many attempts have failed (Hughes and Rasgon 2014). On the other hand, when *Wolbachia* is successfully transferred to

a new host, it can cause physiologic dysfunction and alter lifespan (McGraw et al. 2002). Trans infection of *Wolbachia* strain wVulC from *Armadillidium vulgare* (Latreille) (Isopoda: Armadillidiidae) to *Porcellio dilatatus* (Brandt) (Isopoda: Porcellionidae) resulted in nervous system disorder and growth inhibition of the new host (Le Clec'h et al. 2012); and transfer of *Wolbachia* strain wMelPop from *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae) into *Aedes albopictus* reduced the fecundity and longevity of the mosquito (Suh et al. 2009).

QS inducer treatment, which targets *Wolbachia* inside the insect host, could be a promising alternative method to decrease the proportion of hatched eggs by promoting CI. In the present study, the 3O-C12-HSL treatment increased *Wolbachia* density in insect cells and strongly induced CI (Figs. 1 and 6). Thus, this study is a good initiation that can be adopted for IIT.

IIT based on CI-*Wolbachia* has several advantages, such as the fact that *Wolbachia* can be transferred to the next generation by vertical transmission, thereby eliminating the cost associated with radiation in SIT (Moretti and Calvitti 2021). Moreover, IIT does not require gene modification, for example, through clustered regularly interspaced short palindromic repeats (CRISPR), and release of transgenic insects carrying a dominant lethal mutation (Zhang et al. 2015, Pagendam et al. 2020, Kandel et al. 2021). Thus, the IIT based on CI-*Wolbachia* may be applicable to *L. trifolii*.

The lack of a standardized and efficient method for isolating males is an obstacle for the IIT (Lutrat et al. 2019, Baton et al. 2021). Male selection in *L. trifolii* using a 1.54 × 0.76-mm pore sieve is based on pupal sexual dimorphism, although the effectiveness of this method is less than 100% (Sultan et al. 2017). Field contamination with *Wolbachia*-infected female *L. trifolii* can lead to the unintended replacement of a *Wolbachia*-infected population due to their compatibility with released males of the same strain as well as with uninfected wild males in the field. To minimize this risk, some researchers have suggested releasing insects infected with different strains of *Wolbachia* that are bidirectionally incompatible with the wild insect population (Calvitti et al. 2012, Baton et al. 2021).

Combining the IIT and SIT is also a possible solution. Integrating the SIT with IIT can complement each other's weaknesses, since several insect males, reduce their survival and competitiveness after SIT radiation. Generally, the SIT uses radiation doses between 120–200 Gy to sterilize flies (Baton et al. 2021). In a combining SIT

and IIT previous study, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) and *Aedes albopictus* infected with *Wolbachia* were irradiated at lower doses (45 Gy), and the result showed that the males not lose their survival and competitiveness, while the females were sterile (Zheng et al. 2019, Nikolouli et al. 2020). That combining technique was appropriate to prevent population replacement caused by female contamination of IIT. Integrating the SIT and IIT methods also can be adopted for other insect methods. Previous research reported that sterile *L. trifolii* using the SIT was feasible (Kaspi and Parrella 2003), our results showed 3O-C12 HSL induce strong CI that supported the IIT method. Thus, combining SIT and IIT is a potent tool to control *L. trifolii* populations.

In conclusion, our findings are the first report that use of a QS inducer for strong CI level on *Wolbachia*-infected males of *L. trifolii* for application on IIT, although additional studies are needed to optimize CI, mass rearing, male sorting, the competitive advantage of laboratory males over wild males, and field management following the release of *Wolbachia*-infected incompatible males.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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Author Contributions

A.K.H.: conceptualization; data curation; formal analysis; investigation; methodology; visualization; writing-original draft. A.G.: investigation; writing-review & editing. Y.T.: conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; writing-review & editing.

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