Chlorpyrifos And Chlorpyrifos-methyl Can Promote Conjugative Transfer of Antibiotic Resistance Genes

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Abstract: Antibiotic misuse induces the production of antibiotic resistance genes (ARGs), leading to the global spread of antimicrobial resistance (AMR), which poses a major threat to human health. Conjugative transfer, as the main process of ARGs propagation, is sensitively influenced by coexisting contaminants. Chlorpyrifos and chlorpyrifos-methyl, as organophosphorus insecticides widely used in agriculture, have been shown to induce cytotoxicity such as elevated levels of reactive oxygen radicals (ROS) and lipid peroxidation. This is similar to the mechanism by which antibiotics promote the conjugative transfer of ARGs, based on which we hypothesized that chlorpyrifos and chlorpyrifos-methyl could promote conjugative transfer. However, the effect of chlorpyrifos and chlorpyrifos-methyl on conjugative transfer is unclear. Therefore, we constructed RP4 plasmid-mediated conjugation system and confirmed that chlorpyrifos and chlorpyrifos-methyl can promote conjugative transfer by inducing oxidative stress in donor and recipient bacteria. Our research reveals the risk of ARM spread in organophosphorus insecticides and ARGs co-contaminated environments.

1 INTRODUCTION

Antibiotic abuse induces the production of antibiotic resistance genes (ARGs), leading to the global spread of antimicrobial resistance (AMR), which has become one of the public health challenges in the 21st century (Van Boeckel 2015). ARGs can enter the agricultural soil environment through manure application, sludge fertilization and irrigation with reclaimed wastewater, making agricultural soils already one of the important repositories of ARGs. ARGs in agricultural soils can be transmitted to humans along the food chain and cause damage to human health (Wu 2022). Some studies have pointed out that some fungicide and herbicide agents (e.g., glyphosate and dicamba) applied in agriculture can promote the conjugative transfer process by inducing oxidative stress response (Li 2022). However, the impact of organophosphorus pesticides, as pesticides widely used in agricultural activities, on the conjugative transfer of ARGs has not received much attention.

Chlorpyrifos and chlorpyrifos-methyl, two kinds of typical organophosphorus pesticides, are widely used to control various pests in agricultural activities, which caused serious contamination in the water environment and agricultural soil. Chlorpyrifos and chlorpyrifos-methyl were detected in the water environment at concentrations of 0.1-30 μg L^{-1} and in the soil at concentrations of 1.23-329 μg kg^{-1} (Zhong 2022). Both chlorpyrifos and chlorpyrifos-methyl can inhibit acetylcholinesterase activity in insects, resulting in blocked nerve signalling and thus killing pests (Mehler 2008). Bacteria act as major contributors to the spread of ARGs, however, the effects of chlorpyrifos and chlorpyrifos-methyl on bacteria have rarely been investigated. Current research indicated that chlorpyrifos and chlorpyrifos-methyl were able to cause stress effects on soil bacteria by interfere with intracellular enzyme activity and induce oxidative stress, which may increase a risk of ARGs pollution via promoting conjugative transfer (He 2018, Yang 2019).

To elucidate the effect of chlorpyrifos and chlorpyrifos-methyl on the conjugative transfer process, this study assesses the changes of conjugative transfer frequency under exposure to chlorpyrifos and chlorpyrifos-methyl by constructing a conjugation system. Our findings provide theoretical support for the risk assessment of ARGs contamination in pesticide-applied soils to ensure food safety and protect human health.

2 MATERIALS AND METHODS

2.1 Preparation for strains and organophosphorus pesticides

Escherichia coli (*E. coli*) HB101 containing the RP4

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plasmid was used as the donor bacteria, which was resistant to tetracycline (16 mg L^{-1}), ampicillin (100 mg L^{-1}) and kanamycin (50 mg L^{-1}). The recipient bacteria was *E. coli* NK5449, which carried the ARGs for rifampicin (160 mg L^{-1}）. Both recipient and donor bacteria were activated overnight in Luria-Bertani (LB) medium containing the corresponding antibiotics at 37℃ and 200 rpm.

Chlorpyrifos and chlorpyrifos-methyl were purchased from Sigma-Aldrich. Pesticide concentration gradient solution was prepared by methanol and stored at 4°C.

2.2 Determination of MICs against organophosphorus pesticides

The MICs of chlorpyrifos and chlorpyrifos-methyl against *E. coli* HB101 and *E. coli* NK5449 were determined by using a broth microdilution method to reveal the bacterial toxicity of the two insecticides (Yu 2022). The overnight activated bacterial broth was centrifuged at 4000 rpm, 25°C for 10 min, and then washed twice with PBS. The *E. coli* HB101 and *E. coli* NK5449 suspensions were adjusted to a cell density of 10^6 CFU mL^{-1}. 5 μL of the adjusted cell suspension and 130 μL of LB liquid medium were added to each treatment of 96-well plates. Then 15 μL of chlorpyrifos or chlorpyrifos-methyl was added, resulting in final exposure concentrations of 0, 0.1, 1, 10, 100, 500, and 1000 μg L^{-1}. Three parallel samples were set up for each concentration, while sterile PBS was used as a blank control. After incubation for 16 h at 37°C, the optical density at 600 nm (OD_{600}) was measured with microplate reader (1500, Thermo, USA), and MIC was calculated using a probit model using Statistical Product and Service Solutions Software (SPSS 20.0) fitting.

2.3 Mobile plasmid-mediated conjugative transfer models

The resuspended donor and recipient bacteria (10^8 CFU mL^{-1}) were mixed in equal volumes, and chlorpyrifos or chlorpyrifos-methyl was added at 0.1% volume fraction to reach 10 and 100 μg L^{-1} in the treatment group, while an equal volume of PBS solution was added to the control group. Each treatment was set up in three parallels. Subsequently, the mating solution was incubated at 37 °C and 200 rpm for 8 h, then 100 μL of the mating solution was inoculated on LB agar plates containing the appropriate antibiotics to select the antibiotic resistant bacteria. And the agar plates were incubated at 37°C for 16 h. Finally, the bacterial colonies were counted. And the transfer frequency was obtained by the ratio of the total colonies of transconjugants to the total colonies of recipients.

2.4 Measurement of reactive oxygen species (ROS)

To investigate whether chlorpyrifos and chlorpyrifos-methyl can produce oxidative stress and induce ROS production in bacteria, intracellular ROS levels were measured by 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Yu 2021). The specific steps are as follows: the culture of the recipient or donor was washed and resuspended three times, then centrifuged at 25ºC and 4000 rpm for 5 min to collect the bacteria. Then 2 mL of 10 μM DCFH-DA solution was added to the centrifuge tube and mixed thoroughly. The bacterial solution was incubated at 37°C for 20 min, with gentle shaking every 3-5 min.

Wash three times with PBS after incubation to remove DCFH-DA from outside the bacterial cells. An equal volume of the donor-recipient solution was taken in a sterilized centrifuge tube and chlorpyrifos or chlorpyrifos-methyl was added to a final concentration of 0.1, 1, 10, 50, 100 and 1000 μg L^{-1}, respectively. After thorough homogenization, the broth was incubated for 2 h at 25°C and 200 rpm. Characterization of relative ROS production levels by fluorescence spectrophotometry measurement (F-4600, Hitachi, Japan) of fluorescence intensity. The excitation and emission wavelengths are set to 488/525 nm.

2.5 Statistical analysis

SPSS 20.0 was used to perform one-way analysis of variance (ANOVA) and Duncan's multiple-comparison test ($P < 0.05$, indicated by different lowercase letters) for significant difference analysis.

3 RESULTS AND DISCUSSION

3.1 Chlorpyrifos and chlorpyrifos-methyl promote conjugative transfer

There was no significant effect of 10 μg L^{-1} chlorpyrifos on conjugative transfer compared to the control group. But the colonies of transconjugant and the conjugative transfer frequency were significantly increased by 1.75 and 1.61-fold, respectively, under the treatment with 100 μg L^{-1} chlorpyrifos ($p<0.05$). Chlorpyrifos-methyl had the similar effect on conjugative transfer, it significantly increased the colonies of transconjugant and the conjugative transfer frequency by 1.55 and 1.54 times more than the control treatment only at a concentration of 100 μg L^{-1} ($p<0.05$) (Figure 1). This is consistent with previous research, which indicated that herbicides (glyphosate, glufosinate and dicamba) could promote conjugative transfer at concentrations of 2-20 mg L^{-1} (Li 2022). However, there was no significant difference in the promotion of conjugation frequency between chlorpyrifos and chlorpyrifos-methyl at a concentration of 100 μg L^{-1} ($p>0.05$), which may be due to the similar toxicity of the two insecticides. These results suggested that chlorpyrifos and chlorpyrifos-methyl can promote the plasmid-mediated conjugative transfer process.

3.2 MIC value measurement

The MIC values of chlorpyrifos and chlorpyrifos-methyl against *E. coli* HB101 and *E. coli* NK5449 were determined to assess the toxic effects of chlorpyrifos and chlorpyrifos-methyl on bacteria. The MIC_{50} of

chlorpyrifos and chlorpyrifos-methyl against *E. coli* were 176 mg L^{-1} and 179 mg L^{-1}, respectively. This suggests that chlorpyrifos and chlorpyrifos-methyl have similar toxic effects on donor and recipient bacteria, therefore it is speculated that both insecticides have little effect on bacterial activity at exposure concentrations less than MIC_{50} values. The results of LB agar plating showed no significant effect of chlorpyrifos and chlorpyrifos-methyl on the number of donor (Figure 1c) and recipient colonies (Figure 1d), further confirming the limited ability of both insecticides to inhibit bacterial activity. These results suggest that exposure to 10-100 μg/L chlorpyrifos or chlorpyrifos-methyl had no significant effect on the growth of donor and recipient bacteria.

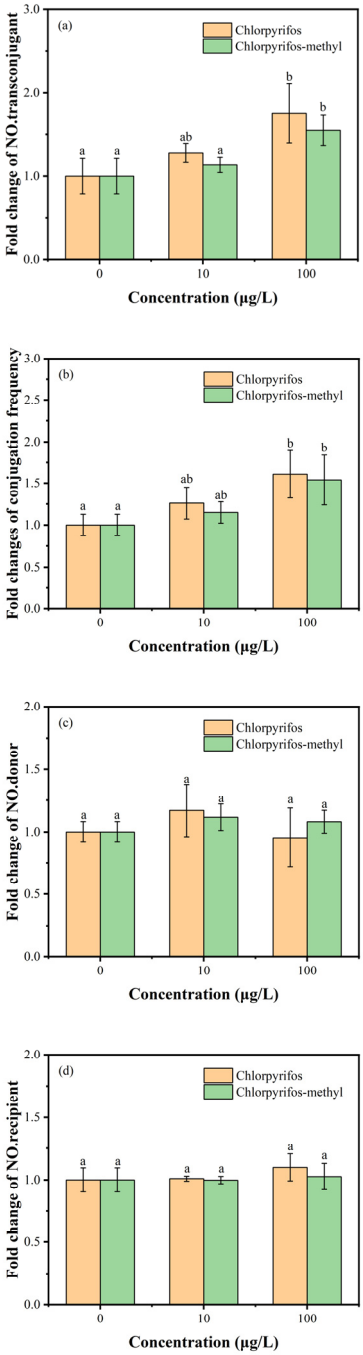

Figure 1: Effect of chlorpyrifos and chlorpyrifos-methyl on conjugative transfer.

3.3 Chlorpyrifos and chlorpyrifos-methyl enhance ROS production

Some contaminants (e.g., antibiotics and sweeteners) can induce the production of intracellular ROS (e.g., peroxides and hydroxyl radicals), causing the promotion of conjugative ARGs transfer (Li 2019, Yu 2021). To investigate whether chlorpyrifos and chlorpyrifos-methyl could promote conjugative transfer by inducing ROS production in bacteria, the intracellular ROS levels in donor and recipient bacteria exposed to chlorpyrifos and chlorpyrifos-methyl were quantified.

The results showed that 10 and 100 μg L^{-1} of chlorpyrifos significantly increased ROS levels by 1.13 and 1.16-fold compared to the control (p<0.001). In addition, 10 μg L^{-1} of chlorpyrifos-methyl increased ROS levels by 1.04-fold of the control treatment (p<0.01), while a significant 1.17-fold increase in ROS levels was observed at 100 μg L^{-1} exposure concentration (p<0.001) (Figure 2a). Similar results were found in previous studies, where glyphosate and dicamba were able to promote conjugative transfer between RP4 plasmids in *E. coli* HB101 and *E. coli* DH5α (Li 2022). The dramatic increase in intracellular ROS levels in bacteria may cause damage to cell membrane structures, which in turn provides favorable conditions for the conjugative transfer process of ARGs (Lu 2020). This is also the mechanism by which antibiotics promote conjugative transfer through the generation of oxidative stress on bacteria (Li 2020, Zhou 2021). The concentration-dependent effect of increasing ROS levels by chlorpyrifos and chlorpyrifos-methyl followed the same trend as the conjugative transfer frequency, and there is a correlation between ROS levels and conjugative frequency (Figure 2b), suggesting that the induction of elevated ROS level was important for the promotion of conjugative transfer by chlorpyrifos and chlorpyrifos-methyl.

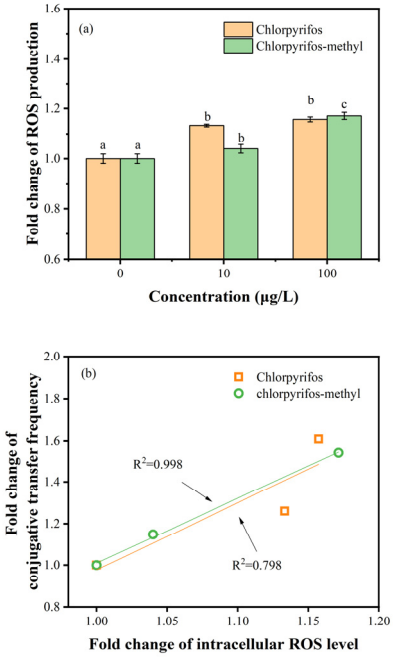

Figure 2: Effect of chlorpyrifos and chlorpyrifos-methyl on intracellular ROS levels.

4 CONCLUSIONS

In this study, we investigated the effects of chlorpyrifos and chlorpyrifos-methyl on the conjugative transfer of ARGs and elucidated the underlying mechanisms. The main findings are as follows:

(1) Chlorpyrifos and chlorpyrifos-methyl significantly promoted conjugative transfer of RP4 plasmid between *E. coli* strains with a concentration-dependence.

(2) Chlorpyrifos and chlorpyrifos-methyl did not differ significantly in toxicity to donor and recipient bacteria and did not promote splicing by affecting bacterial activity at experimental concentrations, and both insecticides do not significantly inhibit the growth of bacteria.

(3) Chlorpyrifos and chlorpyrifos-methyl can induce an increase in intracellular ROS levels, which contributed the promotion of conjugative transfer.

Acknowledgements

This research was supported by the Hainan Provincial Joint Project of Sanya Yazhou Bay Science and Technology City (220LH061) and Hainan Provincial Key Research and Development Projects (ZDYF2022SHFZ018).

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