Triadimefon Promoted Plasmid-mediated Conjugative Transfer of Multiple Antibiotic Resistance Genes within Bacterial Genus

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Abstract: The rapid spread of antibiotic resistance genes (ARGs) has been a worldwide threat to the public health, especially via horizontal gene transfer (HGT). Conjugation is one of the major pathways of HGT. Triadimefon (TF) is a broad-spectrum antifungal compound used to control rust and powdery mildew on crops. Nevertheless, it is not known whether TF could affect the conjugation of ARGs and potential molecular mechanisms. Here, the effects of TF on conjugative transfer of RP4 plasmid within Escherichia coli were systematically investigated. The results demonstrated that TF increased the transconjugant number by 1.41–2.21 folds and the frequency of conjugative transfer by 1.49–2.22 folds at concentrations of 0.1–10 mg/L. There was no obvious change in the number of donor and recipient strains in the mating system of intra-genus with TF. The main mechanisms include increasing intracellular reactive oxygen species and membrane permeability. Our findings highlight the promotion effect of TF on ARG conjugation, providing evidence of the risk of non-antibiotic agrochemical use in ARG dissemination.

1 INTRODUCTION

The spread of antimicrobial resistance (AMR) is becoming an acute threat to public health around the world. A recent report assesses that there have been 4.95 million AMR-related deaths in 2019, including 1.27 million directly caused by AMR (Murray, 2022). The increased incidence of AMR is mainly due to the overuse of antibiotics in the treatment of human and animal diseases and as growth promoters and prophylactics in animal husbandry and aquaculture. The contamination of antibiotic-resistant genes (ARGs) and antibiotic-resistant bacterium (ARB) were accelerated by the overuse of antibiotics (Zhang, 2022). ARGs were detected in a variety of environmental media including soil, water, air and sediment. The soils, one of the largest reservoirs of microbial communities, not only contains a massive natural resistome, but receives numerous ARGs and ARB, which from fertilization, sewage sludge, and reclaimed water irrigation (Wang, 2020). The spread of ARGs is driven mainly by horizontal gene transfer (HGT) and mutation. HGT is the process of sharing genetic material between unrelated organisms (Dantas, 2008). Bacteria present in environmental media can spread ARGs between strains of the intrageneric and intergeneric with mobile genetic elements (MGEs). Conjugation, transformation and transduction are the three main modes of HGT. Particularly, conjugation, as a major mechanism of HGT, can disseminate ARGs through conjugation bridge formed by membrane pores or conjugation pilus (Brito, 2021).

Triadimefon (TF) is a broad-spectrum antifungal compound used to control rust and powdery mildew on wheat, flowers and other crops, as well as for the regulation of plant growth (Asami, 2003). TF is a fungicide that inhibits sterol biosynthesis, mainly by interfering with the formation of fungal cell walls, which in turn has a fungicidal effect. After application, triadimefon is adsorbed and desorbed by the soil and enters the surface or groundwater environment by rainfall leaching, with a concentration of 0.922 mg/L detected in surface water in the USA. The half-life of triadimefon reaching 217 days in anaerobic water environments (Singh, 2005). Recent studies have shown that agrochemical exposure can facilitate the spread of ARGs by conjugation. Prochloraz alone at 50 μg/L enhanced conjugative transfer of plasmid RP4 among *E. coli* DH5α by 1.82 folds by altering cell membrane permeability and promoting expression of conjugation-related genes. (Guo, 2021). However, the effect of triadimefon on conjugation transfer of ARGs and potential molecular mechanisms are still unclear.

In this study, different concentrations of triadimefon (0.1, 1, and 10 mg/L) were added to the conjugated mating system to assess the potential effects on ARGs transfer. To

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understand the underlying mechanisms, intracellular ROS and cell membrane permeability were tested. The results extend our understanding of the enhanced spread of ARGs by fungicide and provide a scientific reference for the control of the risk of ARGs transmission in the environment.

2 MATERIALS AND METHODS

2.1 Bacterial strains

E. coli HB101 (donor) and *E. coli* NK5449 (recipient) were used to establish a transferable plasmid-mediated conjugate transfer model. *E. coli* HB101 harbouring transferable RP4 plasmid with resistance to kanamycin (Km^R), ampicillin (Amp^R), and tetracycline (Tet^R). *E. coli* NK5449 harbouring the resistance genes for rifampicin (Rif^R). The analytical standard of triadimefon (CAS 43121-43-3, 100%) and antibiotics were acquired from Sigma-Aldrich (USA). Stock solutions of triadimefon was prepared in acetonitrile (HPLC grade, Sigma-Aldrich, USA) at 10 g/L. The strains were grown in a liquid Luria-Bertani (LB) broth with the appropriate antibiotics. Incubated for 16 h at 37°C under a 150rpm shaker (ZQTY-50, China) for all subsequent experiments.

2.2 Conjugative transfer experiments

An intra-genera conjugative transfer model of ARGs that was mediated by transferable plasmid were developed to assess the effects of triadimefon on conjugation. Briefly, after shaking at 150 rpm overnight at 37°C in 100 ml LB broth medium, the bacteria were centrifuged at 6000 rpm for 5 minutes and the supernatant removed. Bacterial pellets were subsequently washed twice with phosphate-buffered saline (PBS) and the cells were resuspended at a density of 3×10^8 CFU/mL. Then, a mixture of 5 mL donor and 5 mL recipient bacteria were exposed to triadimefon (0.1, 1, and 10 mg/L) for 8h at 37°C and 150 rpm. Moreover, Milli-Q water were used as controls. The mixture was properly diluted with 0.9% NaCl and spread onto LB agar plates, which contained the appropriate antibiotics for the selection of the transconjugants, recipients, and donors. Then, the frequency of conjugative transfer was measured using the number of the transconjugant divided by the recipient number. All experiments were tested in biological triplicates.

2.3 Measurement of ROS

Intracellular ROS levels of bacteria were measured using the 2',7'-dichlorofluorescein diacetate (DCFDA) cellular ROS assay kit (Beyotime, China). Briefly, the bacterial was adjusted to 10^6 CFU/mL with PBS, and then incubated with 5 mL of 10 μM DCFH-DA in the dark for 30 min. The cell suspension was washed three times with PBS to remove the unbound DCFDA. Subsequently, different concentrations of triadimefon (0.1, 1, and 10 mg/L) were added. The suspensions were analysed by a fluorescence spectrophotometer (F-4600, Hitachi, Japan) after incubation at 25°C for 2 h in the dark. Positive (ROSup, 50 mg/L) and Milli-Q water were used as controls. All treatments were tested in biological triplicates.

2.4 Analyses of cell membrane permeability

A flow cytometry (BD accuri C6, Biosciences, USA) was applied for the detection of cell membrane permeability. In brief, the bacteria were adjusted to 10^6 CFU/mL with 0.1, 1, and 10 mg/L triadimefon and then solutions were incubated at 37°C for 2h. Subsequently, 5 μL of 1 mg/mL propidium iodide dye (PI, Life Technologies, USA) was mixed with 1 mL prepared cell solution and allowed to react in dark for 30 min. After staining, the PI fluorescence intensity of bacterial solution was measured by the flow cytometry. The bacterial cells untreated by triadimefon were setup as the control. All treatments were tested in biological triplicates.

2.5 Statistical analysis

Experimental data were analysed and plotted by Excel 2019. ANOVA and Duncan's multiple comparisons (P<0.05, indicated by lowercase letters) were used for significant difference analysis by SPSS 20.0.

3 RESULTS AND DISCUSSION

3.1 Triadimefon promote conjugative transfer

To test the effects of triadimefon on the conjugative transfer of ARGs, we established a mating system of intra-genus (*E. coli* HB101 and *E. coli* NK5449). The results showed that triadimefon significantly increased the conjugative transfer of the RP4 multidrug resistance plasmid. TF at relatively concentrations of 0.1–10 mg/L obvious increased the transconjugant number by 1.41–2.21 folds compared with the control. Furthermore, with the increase of concentration, the conjugative transfer frequency increased to 1.49、1.66 and 2.22 folds respectively. Simultaneously, the effect of the triadimefon on the number of donor and recipient strains was tested, and TF had no significant effect on bacterial activity at the concentrations tested. This shows the effects of TF on conjugative transfer exist other mechanisms.

Similarly, herbicides (glyphosate, glufosinate and dicamba) can enhance the conjugative transfer frequency of multidrug-resistant plasmid by up to 5.3 folds through a range of responses including enhanced expression of genes encoded by pilus, reduced cell surface charge, increased cell membrane permeability, promotion of close intercellular contact and enhanced proton dynamics (Li, 2022).

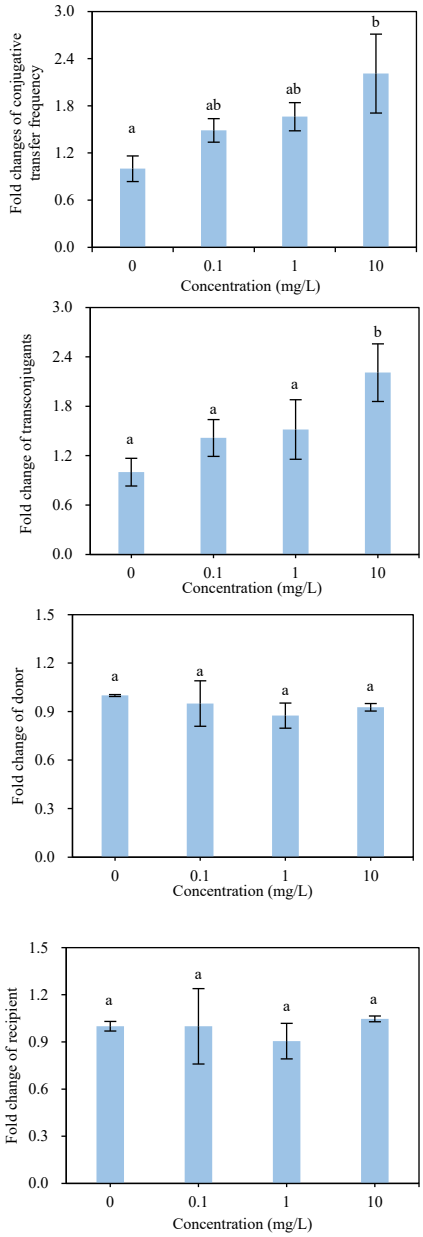

Figure 1: Effect of TF on the conjugative transfer

3.2 Triadimefon increased ROS production

ROS including $O_2^{\bullet-}$, $^{\bullet}OH$, and H_2O_2, can damage cellular structure damage (e.g., lipids, membrane proteins and DNA), stimulate oxidative stress, active SOS response, even to cell death, thereby regulating the conjugative transfer of ARGs (Qiu, 2012). In our study, intracellular ROS levels were found to increase dose-dependently following exposure of TF to *E. coli* strains, and the promoting effect was up to 1.33 folds, consistent with their promotion of conjugate transfer. These results indicated that TF induced ROS production was an important factor in promoting conjugation. Non-nutritive sweeteners (saccharin, sucralose, and aspartame) all cause ROS production and thus promote conjugative transfer of ARGs between bacteria (Yu, 2021).

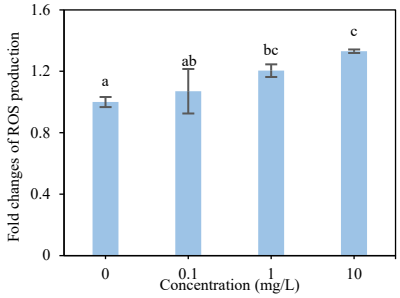

Figure 2: Effect of TF on intracellular ROS levels in bacteria

3.3 Triadimefon increase cell membrane permeability

The cell membrane is an essential barrier preventing the free entry of extracellular chemicals and genes into the cell. To explore the effect of TF on membrane permeability, the quantification of cell membrane permeability of *E. coli* strain was carried out using flow cytometry. The results show that cell membrane permeability was found to increase dose-dependently following exposure of TF to *E. coli* strains, and the promoting effect was up to 1.29 folds, consistent with their promotion of conjugate transfer. These results indicated that TF damages cell membrane was an important factor in promoting conjugation.

Cell membranes are rich in phospholipids with polyunsaturated fatty acids which are highly susceptible to ROS-induced peroxidation. Chemicals in the environment, such as non-antibiotic drugs (ibuprofen, gemfibrozil, propranolol), can promote conjugation transfer by altering bacterial membrane permeability (Wang, 2021).

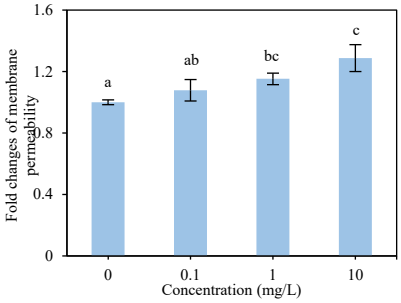

Figure 3: Effect of TF on the membrane permeability of bacteria

4 CONCLUSIONS

On the basis of the above results and discussions, the following conclusions were obtained.

(1) TF increased the transconjugant number and the conjugative transfer frequency.

(2) The content of bacterial intracellular ROS increased significantly associated with the increase of TF concentration, which may be one of the important mechanisms of TF to promote conjugation transfer.

(3) *E. coli* strains exposure to TF significantly increases cell membrane permeability, which is a necessary channel for DNA transport.

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