Turkish Journal of Zoology

Volume 43 | Number 6 Article 2

1-1-2019

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TÜBİTAK

Turkish Journal of Zoology

http://journals.tubitak.gov.tr/zoology/

Research Article

Turk J Zool
(2019) 43: 554-559

doi:10.3906/zoo-1909-8

Acute and chronic effects of thifluzamide on *Daphnia magna*

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Received: 09.09.2018 • **Accepted/Published Online:** 04.10.2019 • **Final Version:** 01.11.2019

Abstract: Thifluzamide is a thiazole carboxanilide fungicide used to control a wide range of basidiomycete diseases on maize, potatoes, rice, and other crops. Although this fungicide is found in aquatic environments, there is no information about its acute and chronic toxicity on aquatic invertebrates. In the present study, to investigate acute and chronic toxicity of thifluzamide, daphnids were exposed to 1.25, 1.5, 2, and 2.5 mg/L thifluzamide for 48 h and 21 days, respectively. The expression of four oxidative stress-related genes (*CAT*, *GCLC*, *GST*, and *GPX*) and life-history parameters such as days to the first brood, days to the first egg production, and molting frequency were measured. The results showed that exposure to thifluzamide for 48 h significantly induced the expression of oxidative stress-related genes in *D. magna*. A dose-dependent relationship was obtained for mRNA levels of *CAT* and *GST*. In the 21-day chronic toxicity test, a concentration-dependent decrease was observed for the number of neonates per *Daphnia*, the number of broods per female, and molting frequency. Additionally, the first brood day and the first egg production day were significantly ($P < 0.05$) delayed in a dose-dependent manner. The results of this study indicate that the harmful effects of thifluzamide on aquatic invertebrates cannot be ignored and further investigation is needed.

Key words: Acute, chronic, *Daphnia magna*, gene expression, thifluzamide, toxicity

1. Introduction

Thifluzamide is an aromatic amide fungicide, which is synthesized through the condensation of the carboxy group of 2-methyl-4-(trifluoromethyl) thiazole-5-carboxylic acid with the amino group of 2,6-dibromo-4-(trifluoromethoxy) aniline (Figure 1).

It is one of the most popular fungicides used to wipe out numerous species of pathogenic fungus such as *Rhizoctonia cerealis* and *Rhizoctonia solani* (Sun et al., 2017; Wei et al., 2015). Thifluzamide's capability of functioning as a fungicide is based on the inhibition of succinate dehydrogenase, which is the enzyme involved in cell respiration in fungi and bacteria (Zhang et al., 2015; Yang et al., 2016a, 2016b). Because of its widespread use, the effect of this fungicide on the environment can reach dangerous levels. For example, continuous and common application of thifluzamide around the world may produce some mutants with resistance against the fungicide (Mu et al., 2014; Zhang et al., 2015). Besides, when used in large quantities and within short timespans, thifluzamide can also accumulate and contaminate the pathogenic crop (Wei et al., 2015). Moreover, excessive use of this fungicide can lead to increased pesticide residues and environmental pollution. Therefore, to prevent the pollution of the terrestrial environment the fungicide must be used in minimal amounts and within a long timespan, taking into consideration the rainfall dilution properties of the thifluzamide concentration (Wei et al., 2015; Fu et al., 2016). On the other hand, its transfer to aquatic environments causes toxic effects on aquatic biota (Yang et al., 2016a). Previous studies have demonstrated that thifluzamide was toxic to zebrafish and other aquatic vertebrates (Yang et al., 2016a, 2016b, 2017, 2018). The toxicity of thifluzamide was studied in three different stages (embryos, larvae, and adult zebrafish) of zebrafish life (Yang et al., 2016a, 2016b). In all three stages, several symptoms were identified, including sluggish heartbeat, morphological disorders, impulsive motion, growth disorders, and incubation inhibition (Yang et al., 2016a, 2016b, 2019). However, the effects of thifluzamide on aquatic invertebrates including *Daphnia magna* are still unclear.

The freshwater zooplankton *D. magna* has been commonly used in acute and chronic ecotoxicology studies to evaluate the toxic effects of chemicals in aquatic environments, due to high sensitivity, easy culture in the laboratory, and relatively short lifetime (Fikirdeşici et al., 2012; Gökçe and Özhan Turhan, 2014; Liu et al., 2017). *D. magna* is the primary consumer of algae and bacteria and primary forage for fish (Seyoum and Pradhan, 2019). Therefore, it plays a principal role in aquatic food webs

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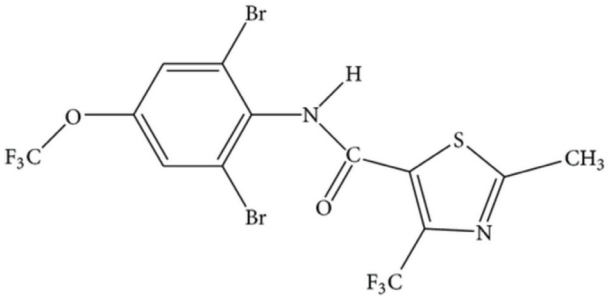

Figure 1. The chemical structure of thifluzamide (Fu et al., 2016).

(Bang et al., 2015). *D. magna* reproduces by cyclical parthenogenesis and has rapid clonal reproduction, resulting in genetically uniform individuals (Cui et al., 2017). This is a great advantage for molecular studies (Liu SJ et al., 2019). Due to the unique advantages listed above, *D. magna* was used as an in vivo model organism to determine the toxicity of thifluzamide in this study. It is well known that to evaluate the effect of various toxic chemicals on *D. magna*, toxicity tests based on physical endpoints such as survival, growth, and reproduction are used. In addition to acute toxicity, chronic toxicity and gene expression studies have been performed together in several studies to evaluate environmental toxicity (Liu et al., 2017; Liu Y et al., 2019; Seyoum and Pradhan, 2019). The aim of this study was to investigate the toxicity of thifluzamide using commonly used toxicity tests and to determine the change in expression of stress-related genes including catalase (*CAT*), glutamate-cysteine ligase catalytic subunits (*GCLC*), glutathione peroxidase (*GPX*), and glutathione S-transferase (*GST*) using RT-PCR.

2. Materials and methods

2.1. Chemicals and reagents

Thifluzamide was obtained from Sigma Aldrich (St. Louis, MO, USA) and stock solutions were prepared by dissolving acetone AR and Tween 80. RNA isolation and SYBR Green RT-PCR kits were from QIAGEN (Basel, Switzerland). All other reagents used in this study were of analytical grade.

2.2. Test organisms and maintenance

D. magna were maintained at the Agricultural Biotechnology Laboratory in the Faculty of Agriculture of Atatürk University (Erzurum, Turkey) following Organisation for Economic Co-operation and Development (OECD) Test Guideline 202. Daphnids were incubated in 3-L glass beakers containing 2 L of aerated tap water with a 16:8 (light:dark) photoperiod. They were kept at 20 ± 1 °C with a dissolved oxygen concentration of 6.4 ± 0.5 mg/L and pH value was adjusted in the range of 8.2 ± 0.2. For exposure experiments, two parts were included.

In the first part, a 48-h acute toxicity test for *D. magna* was performed according to OECD Test Guideline 202. Daphnids were not fed during the acute toxicity test. For the acute toxicity test, 400 mL of thifluzamide solution was used in a 500-mL beaker and 20 individuals of *D. magna* were placed in each beaker (for each set: 20 neonates × 6 beakers = 120 neonates). Exposure concentrations were prepared at 0 (control), 1.25, 1.5, 2, and 2.5 mg/L. Each test was performed in triplicate. In the second part, a 21-day chronic toxicity test was performed. For this purpose, five different concentrations of thifluzamide (0, 1.25, 1.5, 2, and 2.5 mg/L) were applied to the neonates (age: <24 h) for 21 days. For this purpose, 20 mL of thifluzamide solution was placed in a 50-mL glass beaker and 1 neonate was placed in each beaker. Eight replicates were performed for each concentration. Test solutions were renewed every day and *D. magna* were fed daily.

2.3. Total RNA extraction and quantitative real-time PCR assay

Total RNA isolation, cDNA synthesis, and RT-qPCR studies from *D. magna* samples were done as previously described by Aksakal and Ciltas (2018, 2019) and Aksakal et al. (2019). Briefly, 50 homogenized neonates from each test group were used for total RNA isolation. RNA isolation was performed according to the manufacturer's protocol using the RNeasy Mini Kit (QIAGEN, Basel, Switzerland). DNase was applied to remove genomic DNA contamination during isolation. Total RNA was dissolved in 30 µL of RNase/DNase-free water. The concentrations and quality of the isolated RNA were examined by using a NanoDrop ND-spectrophotometer and 1% agarose gel electrophoresis.

For each sample, the synthesis of the cDNA was performed using 100 ng of total RNA and the RT² First Strand cDNA Synthesis Kit (QIAGEN, Basel, Switzerland) according to the manufacturer's instructions. The primers for *CAT*, *GPX*, *GST*, and *GCLC* were selected from a previous study (Liu SJ et al., 2019) and the *β-actin* gene was used as reference/housekeeping gene. Real-time PCR assay was performed in a Rotor-gene Q Real Time PCR Detection System (QIAGEN, Basel, Switzerland) using the SYBR Green RT-PCR Kit (RT² SYBR Green Master Mix, QIAGEN, Cat. No: 330500), cDNA, and gene-specific primers (Table 1). The cycling conditions were as follows: one cycle of 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each 25-µL volume of reaction mixture contained 12.5 µL of RT² SYBR Green Master Mix, 1 µL of cDNA, 1 µL of primer, and 10.5 µL of RNase-free water. QPCR analysis of each RNA sample was performed in triplicate. mRNA levels were determined using the $2^{-\Delta\Delta Ct}$ method and are given as fold-change relative to the mean of the control. GenGlobe data analysis software was used for relative gene expression analysis and hierarchical analysis between genes (https://www.qiagen.com/tr/geneglobe).

Table 1. Sequences of the primer pairs used in the RT-PCR reactions.

Genes	Forward primer (5'-3')	Reverse primer (5'-3')	Ref.
CAT	CCGTTACAACACTGCCGATGA	AAGCCTGTGCGTTCTTTAGATG	(Liu SJ et al., 2019)
GCLC	TCAATGGGGTAACGGCGTCT	TGCGGTCCTGAATGAAAAAT	
GPX	CGTGGCTACTTACTGAGGGTTT	CGGACGAACGTAACGGATT	
GST	GGGAGTCTTTTACCACCGTTTC	TCGCCAGCAGCATACTTGTT	
β-ACT	GCCCTCTTCCAGCCCTCATTCT	TGGGGCAAGGGCGGTGATTT	

2.4. Statistical analysis

Results of at least three independent experiments are shown. The findings of the gene expression data and chronic toxicity are given as mean ± standard deviation. Differences among groups were calculated using one-way ANOVA followed by Duncan's multiple comparison tests (SPSS 20.0 for Windows, IBM Corp., Armonk, NY, USA). Student's t-test as provided by the QIAGEN Gene Globe Data Analysis Center was used to determine the statistical significance of the fold-change in *CAT*, *GCLC*, *GPX*, and *GPX* genes. $P < 0.05$ was considered as statistically significant.

3. Results and discussion

When organisms are exposed to various toxic chemicals including pesticides and herbicides, reactive oxygen species (ROS) are formed and ROS play an important role in the occurrence of oxidative stress (Jiang et al., 2014). ROS accumulation in the cells damages macromolecules such as nucleic acids, lipids, and proteins. Organisms have evolved an antioxidant defense system to prevent oxidative damage caused by toxic substances and maintain body homeostasis (Wang et al., 2016). Antioxidant enzymes such as CAT, GPX, and GST and nonenzymatic scavengers are important components of the antioxidant defense system (Wang et al., 2016). When organisms are exposed to toxic substances, the genes related to the detoxification/antioxidant system are also expected to respond. Li et al. (2017) also reported that exposure to pesticides always changes the expression of genes associated with the oxidative system. Therefore, in the present study, four selected genes associated with the detoxification/antioxidant system were used to evaluate the acute toxicity of thifluzamide on *D. magna*. The mRNA expressions of selected genes involved in the antioxidant system are shown in Figure 2. Compared to the control group, exposure to 2 and 2.5 mg/L thifluzamide significantly upregulated the *CAT* gene expression (Figure 2). A similar change was also found for *GST*, which was prominently upregulated after 2 and 2.5 mg/L thifluzamide exposure. The expression levels were about 2.4- and 2.6-fold for *CAT* and 2.4- and 2.8-fold for *GST* compared to the control groups, respectively. GST, a phase II detoxification enzyme, plays a vital role in the scavenging of ROS (Liu et al., 2017). In this work, a dose-dependent increase in GST expression was observed in neonates exposed to thifluzamide. CAT expression was also upregulated with increasing thifluzamide dose. The activities of CAT and GST, and the expressions of the related genes, play an important role in removing ROS in cells (Liu SJ et al., 2019). These enzymes could also indicate the antioxidant capacity of the cell. The increase in the expression of *CAT* and *GST* at 48 h showed that thifluzamide triggered oxidative stress in *D. magna* and CAT and GST play an essential role in removing excess ROS. Similar to our results, Liu Y et al. (2019) reported that exposure to simvastatin increased the expression of *CAT* and *GST* genes in *D. magna*. On the other hand, the expressions of *GPX* and *GCLC* were significantly upregulated after exposure to 1.25 and 1.5 mg/L thifluzamide, whereas 2 and 2.5 mg/L thifluzamide treatment did not cause a statistically significant increase in the expression of these genes (Figure 2). It is commonly known that GPX belongs to the GSH family and is involved in the removal of free radicals such as hydrogen peroxide and superoxide radicals (Liu SJ et al., 2019). Furthermore, it plays an important role in the protection of membrane protein thiol groups. As for GCLC, it is the rate-limiting enzyme in GSH synthesis (Liu Y et al., 2019). GSH is an important component in the regulation of redox homeostasis (Lu et al., 1999). The increase in the expression of *GCLC* and *GPX* genes in thifluzamide-treated neonates at concentrations of 1.25 and 1.5 mg/L indicates an increase in GSH synthesis and hence the demand for *D. magna* to cope with oxidative stress. Yang et al. (2018) evaluated the acute toxicity of thifluzamide on zebrafish larvae, determining that 0.19, 1.90, and 2.85 mg/L thifluzamide increased the MDA level and inhibited the activity of antioxidant enzymes and the expression of genes related to this system. The same authors also reported that short-term application of thifluzamide inhibited the expression of mitochondrial respiratory system genes in zebrafish (Yang et al., 2016a). In contrast to these studies, the present study revealed that exposure to thifluzamide induced the expression of *CAT*, *GCLC*, *GPX*, and *GST*

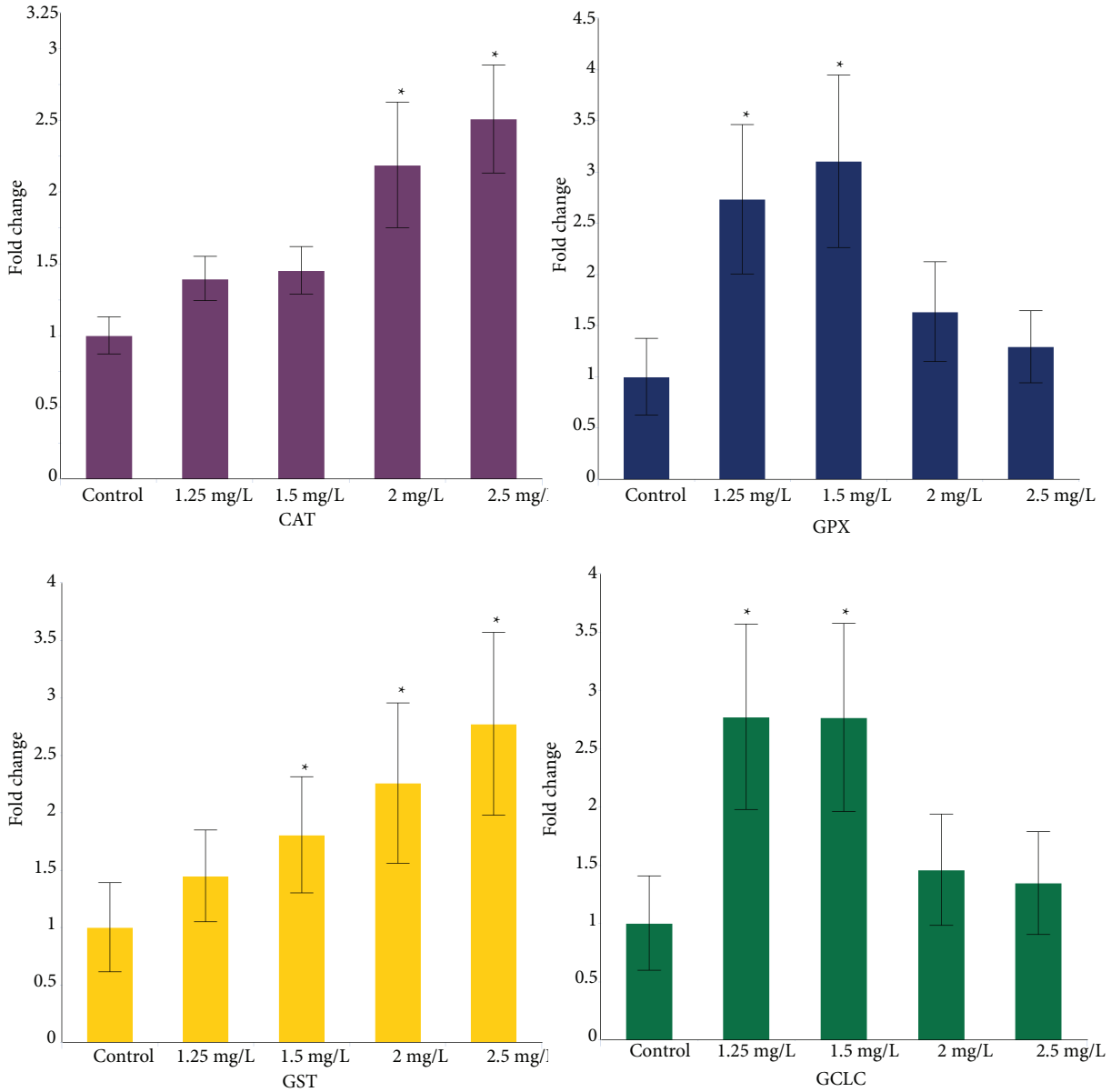

Figure 2. Effects of different concentrations of thifluzamide on *CAT*, *GPX*, *GST*, and *GCLC* expression in *D. magna*. Asterisks indicate significant ($P < 0.05$) differences between the control and exposure groups.

in *D. magna*. The differences in the expression of genes related to the antioxidant system between our results and those obtained from previous studies depend on the organism's differences, the dosage of the thifluzamide, and the application time.

Chronic toxicity results are shown in Table 2. No mortality occurred in the control group during the chronic toxicity test. It was determined that molting frequency significantly decreased in 2 and 2.5 mg/L thifluzamide application groups when compared with the control group (Table 2). Similar to these results, exposure to triclosan, an antifungal agent, significantly decreased the molting frequency of *D. magna* (Peng et al., 2013). Compared to the control groups, the day of the first brood and the day of first egg production were significantly delayed in a dose-dependent manner (Table 2). For example, the first brood days and first egg production days were 6.2 and 8.2 days in the control groups, whereas they were 8.4 and 10.1 days in the 2.5 mg/L thifluzamide treatment groups, respectively. The number of neonates per *Daphnia* also decreased significantly in all treatment groups (Table 2). A significant reduction in the number

Table 2. Development and reproduction data for *D. magna* exposed to thifluzamide for 21 days.

Thifluzamide, mg/L	Molting frequency	Days to the first brood	Days to first egg production	Number of neonates per *Daphnia*	Number of broods per female
Control	11.4 ± 0.63	6.2 ± 0.72	8.2 ± 1.23	77 ± 15.4	6.0 ± 0.22
1.25	10.8 ± 0.51	6.5 ± 0.55	8.8 ± 0.87	71 ± 12.7	4.5 ± 0.35*
1.5	10.9 ± 0.48	7.1 ± 0.84*	9.5 ± 0.56*	62 ± 17.1*	4.7 ± 0.24*
2	10.6 ± 0.71*	7.8 ± 0.93*	9.9 ± 1.93*	51 ± 13.4*	4.3 ± 0.43*
2.5	10.4 ± 0.56*	8.4 ± 0.48*	10.1 ± 1.56*	46 ± 9.5*	4.1 ± 0.48*

The values are mean ± SD. *: Significant ($P < 0.05$) differences between the control and exposure groups.

of broods per female was also observed with thifluzamide application (Table 2). At the highest concentration of 2.5 mg/L, the number of broods per female reduced to 31.6% of the control group. These results show that exposure to thifluzamide remarkably suppressed growth and reproduction of *D. magna*. In parallel with these results, Sancho et al. (2016) reported that tebuconazole, an azole fungicide, delayed the day of first brood and reduced the number of neonates per *Daphnia* in a dose-dependent manner. Previous studies indicated that exposure to thifluzamide caused adverse effects on growth, development, and reproduction in zebrafish (Yang et al., 2016a, 2016b). It has also been reported that thifluzamide affects lipid and glycometabolism, leading to cell apoptosis in zebrafish (Yang et al., 2017, 2018). Based on our study and the findings of previous studies, it can be said that thifluzamide is a potential risk for aquatic organisms.

In summary, there is no information on thifluzamide toxicity in aquatic invertebrates. This is the first study about thifluzamide toxicity on *D. magna*. The information obtained in the present study will be useful to predict thifluzamide's toxic effects on aquatic invertebrates. However, further detailed research is necessary to identify thifluzamide toxicity on invertebrates.

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