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RESEARCH ARTICLE

Genotoxic Effects of Acute Difenoconazole Exposure on *Daphnia magna*

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Abstract: Difenoconazole, a fungicide often used in agriculture, is harmful to aquatic organisms. However, its toxicity to aquatic organisms is not yet well recognized. Among the triazole fungicides, Difenoconazole (PEN) is one of the most extensively used in many countries. In this study, the genetic impacts of different Difenoconazole doses on *Daphnia magna* was investigated. Experiments involving the control group and treatments were executed in compliance with the standard methodology outlined by the Economic Cooperation and Development Organization (OECD) standards 202 and 212 (OECD, 2012, 2009). In the present study, *Daphnia magna* were exposed to a control group (0 mg/L) and three different doses of (1.00, 1.5, 2.5 mg/L) difenoconazole for a period of 10 days. At the end of the experiment, comet assay was used to determine the damage frequency (%), Arbitrary unit (%), and Genetic damage index (%) of tissues. The 2.5 mg L^{-1} group showed notably greater damage frequencies (45.33±1.52) on *Daphnia magna* (p<0.001). Our findings indicated a considerable increase in DNA strand breakage in *Daphnia magna* after exposure to difenoconazole, indicating that the fungicide is genotoxic to daphnids.

Anahtar kelimeler:

Pestisit
Difenokonazol
Komet Testi
DNA Hasarı

Akut Difenokonazol'ün *Daphnia magna* üzerindeki Genotoksik Etkileri

Öz: Difenokonazol, tarımda sıkça kullanılan bir fungisit olup; sucul canlılar için zararlı olabilmektedir. Difenokonazol'un sucul canlılarda olan toksisitesi henüz iyi bilinmemektedir. Triazol mantar ilaçları arasında, Difenokonazol (PEN) birçok ülkede en yaygın kullanılan fungusitlerden biridir. Bu çalışmada, *Daphnia magna* üzerinde farklı dozlarda Difenokonazolün genotoksik etkisi araştırıldı. Ayrıca Kontrol Grubu ve diğer doz gruplarıyla yapılan çalışma, Ekonomik İşbirliği ve Kalkınma Örgütü (OECD) standartları 202 ve 212 (OECD, 2012, 2009) tarafından belirlenen standart metodolojiye uygun olarak gerçekleştirildi. Çalışmada, *Daphnia magna* 10 gün boyunca kontrol grubu (0 mg/L) ve üç farklı dozda Difenokonazole (1.00, 1.5, 2.5 mg/L) maruz bırakıldı. Deneyin sonunda, dokuların Hasar Frekansı (%), Arbitrary Birim (%), ve Genetik Hasar İndeksi (%) belirlemek için Comet testi kullanıldı. Doz gruplarından 2.5 mg L^{-1} grubu, *Daphnia magna* üzerinde en yüksek hasar frekansına sahip olduğu(45.33±1.52) (p<0.001) tespit edildi. Elde ettiğimiz bulguların Difenokonazol'un maruziyeti sonrasında *Daphnia magna*'da DNA hasar düzeylerinde önemli bir artış olduğunu göstermesine bağlı olarak kullanılan bu fungisitin daphnidler için genotoksik olabileceğini işaret etmiştir.

Introduction

Pesticides are used worldwide to control and eradicate pests, including microorganisms, fungus, flora, slugs, vermin, rodents, and parasites. They are categorized based on the kind of organism they affect and can be acquired in many configurations, including liquids, granules, compounds, polymer parts, coated pelleted capsules, as well as encapsulated forms. Nonetheless, the increasing use of pesticides to increase agricultural production is raising concerns over environmental contamination and adverse impacts on non-target organisms (Wang *et al*., 2022). Pesticides can move from their target area to other parts of the environment, such as water, air, and soil via various transfer processes, such as volatilization and discharge. Numerous studies have examined the impact of pesticides on aquatic ecosystems owing to their ability to enter the aquatic environment through diverse mechanisms. Certain pesticides may induce oxidative stress, which is closely associated with the growth and development of aquatic organisms. Absorption of pesticides may result in musculoskeletal malformations, vertebral curvature, aberrant growth in fish eggs, and DNA damage (Li *et al*., 2018; Turan and Ergenler, 2022; 2023; Ergenler and Turan, 2023).

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Triazole fungicides are a primary category of pesticides often used for diverse fruits, vegetables, and cereal items. Their elevated characteristics, including chemical and photochemical stability, limited biodegradability, and ease of environmental mobility render them persistent in soil and water. Consequently, concerns about the toxicity of triazole insecticides to aquatic organisms are becoming increasingly prominent in the context of natural ecosystems and human health risk assessment. The compound difenoconazole (cis-trans-3-chloro-4-(4-methyl-2-(1H-1,2,4-triazol-yl methyl)-1,3-dioxolan-2-yl) phenyl 4-chlorophenyl ether) is a representative fungicide with a modified triazole moiety that interacts with the heme component of fungus cytochrome P450 (cyp) 51. (Figure 1).

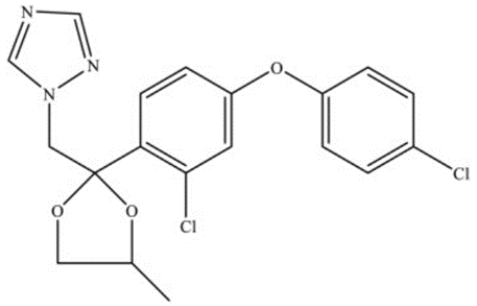

Figure 1. Structure of difenoconazole (Gao *et al.,* 2021).

Difenoconazole impedes the action of fungal lanosterol-14α-demethylase and obstructs ergosterol production, thereby hindering chitin formation in the fungal cell wall and causing cytoplasmic leakage. It has both preventive and therapeutic properties and is extensively used to manage diseases induced by various pathogenic fungus in diverse plant species. For many years, Difenoconazole has been the main pesticide used in China to fight fungal diseases in rice crops. Wang, 2012). This has increased the risk of difenoconazole contaminating the aquatic environment. Many studies have been published on the occurrence of difenoconazole in aquatic systems (Teng *et al*., 2013;Mu *et al*., 2013; 2015; 2016; Wang *et al*., 2022; Jiang *et al*.,2020). *Daphnia magna* (Ton *et al.,* 2012), and trout (Knudsen et al., 2011), have been used in the toxicity evaluation of environmental contaminants. Aquaculture models, characterized by quick life cycles, have proven valuable across other disciplines such as ecotoxicology, cell biology, and developmental genetics (Strähle *et al*., 2011; Ankley and Villeneuve, 2006).

Daphnia is recognized as a pivotal species in the food webs of several freshwater ecosystems, serving as a crucial foundation for sustainable and progressive ecotoxicological research. Daphnia are widely utilized in research owing to their cyclical parthenogenetic life cycle, which encompasses the previously mentioned model criteria and diapause phases. The uncomplicated growing of identical cells facilitates research focused on separating the impacts of environmental and genetic elements. *Daphnia magna* is often used as a model organism for evaluating contaminants in aquatic environments due to its tolerance to a diverse array of substances (Turan and Ergenler, 2022; Tekin *et al.,* 2024). Identifying the most sensitive period in the life stages of an organism is important in protecting the species from external toxic substances. This study aims to determine the most vulnerable life stages of aquatic organisms by conducting multiple toxicity tests at different stages. Systematic multi-stage assays have been used to determine species' sensitivity to toxic chemicals. However, the mechanisms behind different sensitivities at different life stages are still poorly understood. In the present study, the comet test was used to determine if difenoconazole promotes genotoxicity in the model organism *Daphnia magna*, highlighting the importance of understanding the ecological impact of chemicals.

Material and Methods

The Genotoxicology Laboratory at the Faculty of Marine Science and Technology, University of Iskenderun Technical, Turkey, housed and cared for *Daphnia magna.* The Daphnids were placed in 3-L glass beakers with 2 L of tap water that was aerated and had a 16:8 (light:dark) photoperiod. The results of four different groups (including an observation group and a pair of test groups) were subjected to a toxicity test. Each concentration of difenoconazole was tested using three duplicate samples, and twenty daphnias were used per container, following the Organisation for Economic Co-operation and Development (OECD) Test Guideline 202. The specimens were maintained at a temperature of 20 ± 1 °C, with a dissolved oxygen content of 6.4 ± 0.5 mg/L. The pH value was controlled within the range of 8.2 ± 0.2. Three different fungisite concentrations at 1.0, 1.5, 2.5 mg/L based on levels found in aquatic settings along with a control treatment at 0.0 mg/L were used. Overall, a group of twenty newborns, <1 day-old when were placed in glass containers with a volume of 100 mL each. Solutions of three different difenoconazole levels were then prepared and tranferred to glass containers containing daphnia.

A 10-day experiment was conducted as described by Cavalcante et al. (2008), using gill cell suspension, retained cell pellets, and single-cell gel electrophoresis. The slides were analyzed using a fluorescence microscope and stained with ethidium bromide and neutral Tris solution. 100 cells from each specimen were examined to assess nuclei, categorizing them into five types. Damage percentage, arbitrary values, and DNA damage rating were computed for evaluation. Data were assessed for regularity and homogeneity, and a unidirectional analysis of variance was conducted to identify notable disparities across treatment groups. Observed changes were considered statistically noteworthy at a significance level of $P<0.05$ (Norusis, 1993).

Results and Discussion

The mean as well as the standard deviations of the DNA damage frequency (%), arbitrary units values (AU), and genetic damage index (%) observed in *Daphnia magna* for the groups treated with 1.0; 1.5; 2.5 mg/L of Difenconazole compared to the untreated control group are presented in Table 1.

Table 1. The mean and standard deviations for DNA damage in control and different concentrations of difenoconazole on the *Daphnia magna*.

Groups (mg/L)	Damage Frequency (%)	Arbitrary Unit (AU)	Genetic Damage Index (DI) (%)
Control	24.33±2.08^{a}	62.00±5.29^{a}	0.62±0.05^{a}
1.0	36.33±4.72^{b}	86.66±13.79^{b}	0.86±0.13^{b}
1.5	41.00±1.00^{bc}	100.66±4.04^{bc}	1.00±0.04^{bc}
2.5	45.33±1.52^{c}	107.33±3.21^{c}	1.07±0.03^{c}
P	***	***	***

The data are shown as arithmetic mean ± standard deviation. *Values with different superscripts in each column indicate significant differences. Indicate significance level between DNA damage D.magna obtained from control and three different concentrations of Difenoconazole (*, P<0.001).

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The lowest damage frequencies was obtained in the control (0 mg/L) group with 24.33% and there was significant change observed in the DNA damage parameters (p<0.001). The highest damage frequencies was obtained in the 2.5 mg/L treatment with 45.33±1.52%. In comparison to the control and the other pencanozole groups, the frequency of DNA damage and other damage metrics (AU and GDI) were greater in the 2.5 mg/L treatment (Table 1).

Difenoconazole, a fungicide used to enhance agricultural productivity, has become a significant pollutant in aquatic ecosystems due to excessive application and resistance to degredation (Na *et al.*, 2024). According to Mu *et al.* (2016), who determined that difenoconazole exposure can cause developmental toxicity in fish, also found that difenoconazole was toxic at concentrations 0.15–5.00 mg/L. They observed body deformities in zebrafish and suggested that the exposed dose harmed the organism which is in accordance to our findings. Similarly, Jiang e*t al.*, (2020) suggested that difenoconazole exposure at 0.400, 1.00, 2.00 mg/L caused toxic effects in zebrafish. In zebrafish, exposure to difenoconazole increased the percentage of perinuclear oocytes, spermatogenesis germ cells, spermatogonia, and cortical alveolus oocytes, and decreased the ratio of early and late vitellogenic oocytes, spermatocytes, late spermatogenesis germ cells, spermatids, and spermatozoa. Additionally, Wang *et al.* (2022), who determined the degree of heart damage in carp (*Cyprinus carpio*) exposed to low doses (0.488 mg/L) and high doses (1.953 mg/L) of difenoconazole for four days showed that although the heart damage indicators CK and LDH were not significantly different in fish exposed to low difenoconazole level, a significant toxic effect and cardiotoxicity was observed when difenoconazole level reached 1.953 mg/L. As a result, the use of high doses of difenoconazole can cause harmful effects in aquatic organisms and lead to damage in living beings. The findings of the present study is in accordance to earlier studies. Given the literature on the genotoxic potential of difenconazole, further studies are necessary to elucidate the long-term ecological and human health impacts of difenoconazole exposure. This research highlights the need of ongoing surveillance and regulatory evaluation of pesticides to safeguard environmental and public health.

Conflict of Interest

The authors guarantee that there is no conflict of interest in publishing this work, and they have rigorously followed ethical guidelines on problems such as plagiarism, informed consent, misconduct, data fabrication, double publication, and redundancy.

Author Contributions

A. Ergenler, Designing of the study, data analysis, supported the laboratory study, and checking-original draft preparation. F. Turan, Data analysis, submission,writing-review and editing, visualization.

Ethics Approval

Ethics committee approval is not need for this study.

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