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Effects of tetraconazole on antioxidant system in *Lemna minor*

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Abstract: Tetraconazole is a triazole fungicide widely used in agricultural fields and is potentially carcinogenic to humans. Previous studies have shown that this fungicide has toxic effects on plants and other non-target organisms. In this study, the impact of tetraconazole on the antioxidant system of duckweed (*Lemna minor*), a macrophyte plant, was evaluated. For this purpose, duckweed was exposed to tetraconazole at different doses (0.005, 0.01 and 0.02 ppm) for 7 days and the changes in photosynthetic pigments (chlorophyll a, chlorophyll b, carotenoids), malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) levels were determined. In addition, changes in superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) enzyme activities and expression of genes encoding these enzymes were also measured. The results showed that tetraconazole exposure decreased photosynthetic pigment levels and increased MDA and H_2O_2 levels. In comparison to the control groups, the activities of SOD, CAT, POD and APX enzymes increased in a dose-dependent manner. Tetraconazole exposure also induced the mRNA expression levels of SOD, CAT and POD genes in *L. minor* in a dose-dependent manner. These results indicated that tetraconazole induced oxidative stress and activated the antioxidant system in duckweed.

Keywords: Antioxidant Enzyme; Gene expression; Hydrogen peroxide; *Lemna Minor*; Tetraconazole; Malondialdehyde

1 Introduction

Tetraconazole is an imidazole derivative with broad-spectrum antifungal properties and is an effective fungicide used against plant pathogens, especially in agriculture (Li et al. 2020). This fungicide is a steroid-type demethylation inhibitor. This compound, which both prevents the development of fungal spores and treats existing infections, is effective in all tissues of plants thanks to its systemic effect (Li et al. 2020). Tetraconazole inhibits ergosterol, the main component of the cell membrane, by inhibiting cytochrome P450 14-α sterol demethylase in the target organism (Amer et al. 2007). Tetraconazole plays an important role in combating diseases such as mold, rust and leaf spot, which are common in various plant species. In addition, it is used to control diseases such as septoria and rhynchosporium in sugar beet and cereals, apple ringspot on apples and powdery mildew on grapes (Abbassy et al. 2014; Castro-Sobrino et al. 2019). It aims to minimize environmental effects by showing high effectiveness at low usage doses. On the other hand, long-term use can lead to elevated levels of tetraconazole in the soil. Tetraconazole can migrate from soil to groundwater or be transported by rainwater to the aquatic environment, thus contaminating surface waters. In addition, triazole group fungicides such as tetraconazole can affect the photosynthesis rate, enzyme activities, hormone balance and yield of plants (Zhou et al. 1993; Zhou and Ye 1996). Fungicides in this group can trigger endocrine diseases in humans and animals due to their potential to affect steroid hormone biosynthesis (Li et al. 2012).

Lemna minor is a small monocotyledonous macrophyte with floating leaves and submerged roots that thrive in nutrient-rich stagnant or slow-flowing freshwater (Zhang et al., 2013). This aquatic macrophyte is fast growing, easy to culture and has a relatively simple structure, making it one of the model organisms used in ecotoxicology experiments (Song et al. 2012; Zuzelka et al. 2013). *L. minor* has an important ecological function as a primary producer. *L. minor*, which is abundant in freshwater ecosystems, is highly sensitive to organic and inorganic substances such as herbicides, pesticides and metals (Wang, 1990). Therefore, it is widely used to assess the effects of various pollutants on freshwater ecosystems.

This study aimed to investigate the physiological and molecular changes induced by tetraconazole in *L. minor* and to determine the effects of this compound on the antioxidant

system. In this context, *L. minor* was treated with tetraconazole at concentrations of 0.005, 0.01 and 0.02 ppm for 7 days. After seven days, changes in photosynthetic pigments, MDA and H_2O_2 levels, SOD, CAT, POD, APX activities, SOD, CAT and POD gene expressions were investigated.

2 Materials and Method

2.1 Plant Material and culture conditions

The duckweed (*Lemna minor* L.) used in this study was collected from the wetlands around Erzurum Airport. The collected plants were sterilized with 10% NaClO and 1% $HgCl_2$ for 2 minutes and washed several times with pure water. The plants were cultured in 1/10 Hoagland's solution for 3 months in the Plant Physiology Laboratory of the Department of Biology, Faculty of Science, Atatürk University. Toxicity testing was carried out according to the OECD guidelines. For the experiments, 600 healthy *L. minor* plants were selected. Approximately 150 (50X3) plants of the same size were used for each test group and the control. The experiments were carried out in 50 mL Petri dishes. Each Petri dish was filled with 50 mL of 10% Steinberg's solution and 50 *L. minor* plants. The experiments were carried out in the acclimatization room of Atatürk University, Department of Biology, at a temperature of 24±2 °C, 16/8 light/dark photoperiod and 60% humidity. Three different concentrations of tetraconazole (0.005, 0.01 and 0.02 ppm) were used in the experiments. The concentrations used in the experiments were determined based on preliminary experiments. While only 50 mL of 10% Steinberg solution was added to the control group, tetraconazole was added to the Steinberg solution in the other experimental groups. The experiments were carried out in 3 parallel experiments. Plants were harvested after 7 days and used for analysis.

2.1 Chlorophyll content

The procedure recommended by Witham et al. (1971) was used to determine chlorophyll a, chlorophyll b and carotenoid content. Fresh plant material (100 mg) was homogenized in 10 mL of 80% cold acetone for 24 h at 4°C in the dark. The extract was centrifuged at 3000 rpm for 5 min and the results were expressed as mg pigment per gram fresh weight.

2.3 Lipid peroxidation and H_2O_2 levels

To assess lipid peroxidation in *L. minor* plant treated with tetraconazole, the method proposed by Velikova et al. (2000) was used. Approximately 0.4 g of fresh leaves were ground and homogenized in 4 mL of 0.1% TCA. The homogenate was centrifuged at 13,800 rpm for 30 minutes. 1 ml of the supernatant was taken and 1 ml of 0.5% TBA solution was added. The reaction mixture was incubated in boiling water for 30 minutes and then the reaction was stopped by placing the tubes in an ice bath. The samples were centrifuged at 12,000 rpm for 5 minutes, the supernatant was removed and the absorbance value at 532 nm and the absorbance value for non-specific absorption at 600 nm were read. In order to determine the amount of hydrogen peroxide (H_2O_2), the determination was carried out by making some modifications to the method of Velikova et al. (2000).

2.4 Activities of antioxidant enzymes

0.5 g of fresh leaves were thoroughly extracted in liquid nitrogen in a porcelain mortar and the extract was homogenized in 5 ml of 0.1 M KH_2PO_4 (pH: 6.75), 1% PVP, 1 mM EDTA buffer. The homogenate was then centrifuged at 15,000 rpm for 15 min. The supernatant was carefully removed and used as enzyme source in the studies. Superoxide dismutase (SOD) activity was determined by the method of Agarwal and Pandey (2004) with minor modifications, catalase (CAT) activity was determined by observing the decomposition of H_2O_2 according to the method of Aebi (1984); peroxidase (POD) activity was measured by monitoring the oxidation of guaiacol in the presence of H_2O_2 according to the method of Yee et al. (2002), and ascorbate peroxidase (APX) activity was measured based on the decrease in absorbance at 290 nm according to the method of Nakano and Asada (1981).

2.5 Gene expression

To analyze the expression levels of SOD, CAT and POD genes related to the antioxidant system, total RNA was isolated from *L. minor* samples by following the manufacturer's protocol. The concentrations and purity of the obtained RNAs were determined by a NanoDrop spectrophotometer. 1 µg RNA and Quantitect Reverse Transcription kit (Qiagen) were used for cDNA synthesis. 2 µl cDNA and Quantifast SYBR Green RT-PCR kit (Qiagen) were used for real-time PCR. Actin was used as a reference gene in real-time PCR processes and each PCR process was performed in triplicate. The expression level of each gene was analyzed using the $2^{-\Delta\Delta Ct}$ method. The primers used in real-time PCR are presented in Table 1.

Table 1 The sequence of primers used for gene expression in *L. minor*.

Gene	Forward	Reverse
ACTIN	ATCCACTCTCACCGTGGTCT	CGGTGGTCTTCGAGTGTTGA
SOD	CCTGAAGCCTCCTCCTTACG	CCAGTGGAACTCCAGCGTC
CAT	ATGTTCCCTATCCCACCT	ATGAATCGTTCTTGCCTG
POD	AATGCCACGGAAGCCCTAA	CGATTGTATGCCCACCCGAG

2.6 Statistical analysis

The experiments were repeated three times and the data obtained are presented as mean ± standard deviation (SD). One-way ANOVA was used for statistical analysis of the data and multiple comparisons were performed with Dunnett's test. Significance limits were set as *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. All statistical analyses were performed with GraphPad Prism 8.4 software.

3 Results and discussion

Changes in chlorophyll a, chlorophyll b and carotenoid amounts in *L. minor* plants treated with tetraconazole are

given in Figure 1. Compared to the control groups, 0.005 ppm tetraconazole applied to the plants did not significantly affect either chlorophyll a and b nor carotenoid amounts. On the other hand, the amount of chlorophyll a and carotenoid decreased significantly with increasing dose. Tetraconazole at a concentration of 0.02 ppm significantly decreased the amount of all three pigments compared to the control. The effect of tetraconazole on photosynthetic pigments in plants has not been investigated in the literature. However, another conazole fungicide, difenoconazole, was reported to reduce the amount of chlorophyll in wheat (Liu et al. 2021). Many abiotic factors can affect chlorophyll synthesis in plants (heavy metal stress, pesticides, etc.). The enzyme activity of δ-aminolevulinic acid dehydratase (ALAD), which has an important role in chlorophyll biosynthesis (Cenkci et al. 2010), may also be inhibited by the tetraconazole.

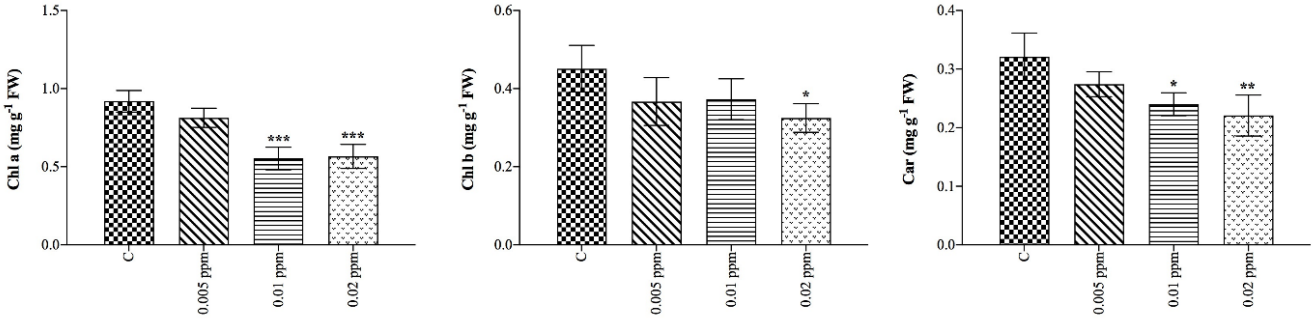

Fig. 1. Effect of tetraconazole on photosynthetic pigments in *L. minor*. Values are given as mean ± S.D. *p<0.05, ** p<0.01, *** p<0.001.

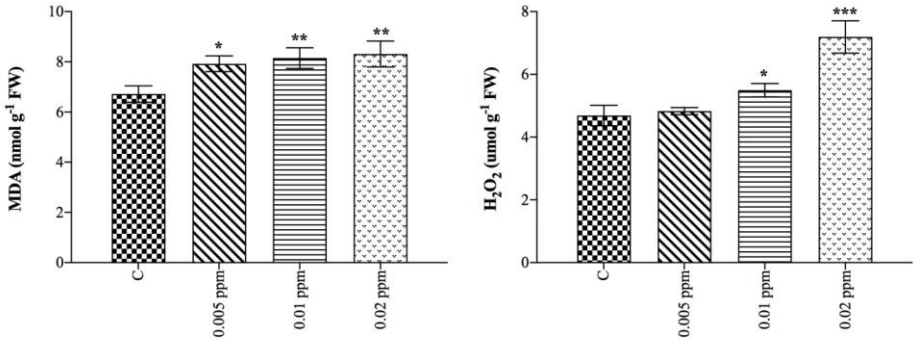

Fig. 2. Effect of tetraconazole on MDA and H_2O_2 levels in *L. minor*. Values are given as mean ± S.D. *p<0.05, ** p<0.01, *** p<0.001

Tetraconazole applied to *L. minor* significantly increased the amount of MDA in all treatment groups compared to the control, depending on the dose increase (Figure 2). In addition, the amount of H_2O_2 increased with the tetraconazole applied to the plants (Fig. 2). While this increase was statistically insignificant in the 0.005 ppm tetraconazole group compared to the control, it was found significant at $p < 0.05$ and $p < 0.001$ levels in the other groups. Especially tetraconazole applied to the plants at a concentration of 0.02 ppm increased H_2O_2 levels dramatically. Since the increase in MDA and H_2O_2 levels is known as a response to abiotic stress factors, they are considered an important indicator of stress.

In this study, MDA and H_2O_2 levels and SOD, CAT, POD and APX enzyme activities were measured to monitor the oxidative stress caused by tetraconazole treatment in *L.* minor. When compared with the control groups, tetraconazole applied to *L. minor* at a concentration of 0.005 ppm did not significantly affect the amount of antioxidant system enzymes SOD, CAT and POD.

The amount of APX increased significantly in the 0.005 ppm group compared to the control ($p < 0.05$) (Figure 3). Tetraconazole applied to the plants at a concentration of 0.01 ppm significantly ($p < 0.05$) increased SOD and CAT enzyme activity compared to the control, but did not change POD activity (Figure 3). In addition, APX activity increased significantly ($p < 0.01$) in the group treated with 0.01 ppm tetraconazole compared to the control. Tetraconazole applied to the plants at a concentration of 0.02 ppm increased SOD and POD activities at $p < 0.05$ and APX and CAT activities at $p < 0.01$ compared to the control groups (Figure 3).

Plants have enzymatic and non-enzymatic antioxidant defense systems to tolerate increased levels of ROS. SOD, CAT, POD and APX are members of the enzymatic antioxidant defense system. The enzyme SOD is involved in the dismutation of $O_2^{\bullet-}$ into O_2 and H_2O_2, while the enzymes CAT and POD scavenge H_2O_2. APX degrades H_2O_2 and uses ascorbate as substrate. The increase in antioxidant enzyme and mRNA expression levels observed in plants treated with high concentrations of tetraconazole indicates that the plant activates the antioxidant defense system against increased levels of ROS. However, the MDA increase observed in *L. minor* due to tetraconazole treatment indicates that lipid peroxidation in membranes increased

despite the antioxidant defense system. In parallel with our findings, Macar (2021) reported that tetraconazole increased MDA and induced the activities of antioxidant enzymes such as SOD and CAT in onion (*Allium cepa* L.) roots. It was also reported that diphenoconazole, one of the conazole fungicides, increased H_2O_2 and MDA levels in wheat and increased SOD, CAT, POD and APX enzyme activities (Liu et al. 2021).

Additionally, the transcript levels of SOD, CAT and POD genes, which are related to the antioxidant system, were up-regulated after tetraconazole exposure (Figure 4).

In conclusion, this study showed that tetraconazole not only altered photosynthetic pigments, antioxidant enzyme activities and MDA levels in *L. minor*, but also affected the expression of genes related to the antioxidant system at the transcript level.

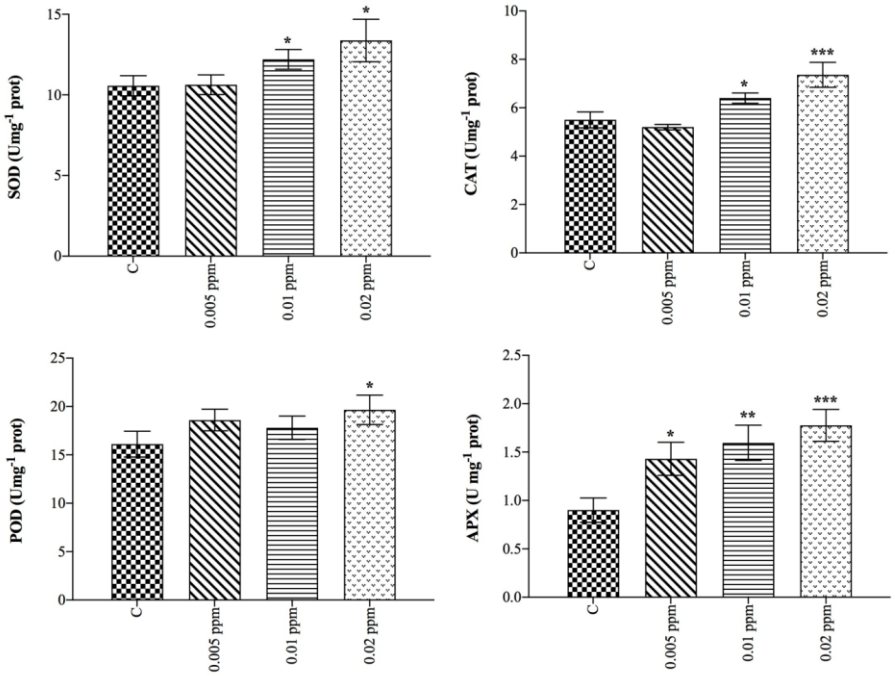

Fig. 3. Effect of tetraconazole on SOD, CAT, POD, and APX enzyme activities in *L. minor*. Values are given as mean ± S.D. *p<0.05, ** p<0.01, *** p<0.001.

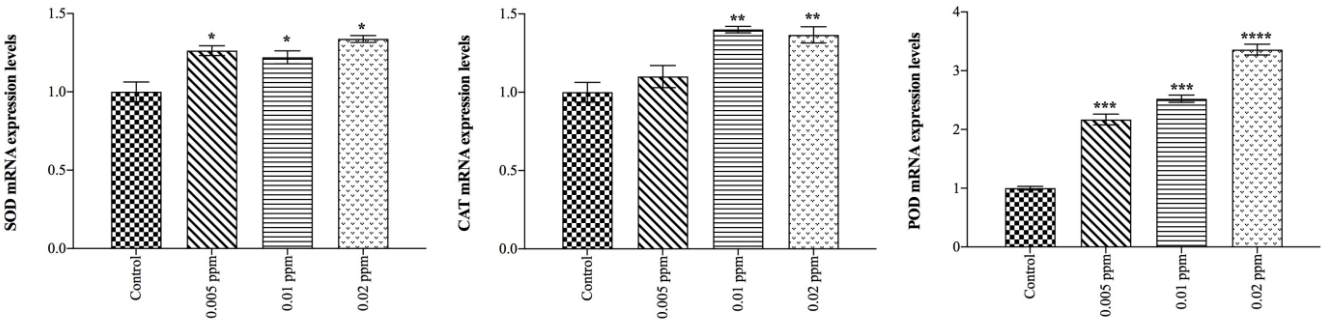

Fig. 4. Effect of tetraconazole on SOD, CAT, and POD mRNA expression levels in *L. minor*. Values are given as mean ± S.D. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

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Authors' contributions: OA: Design, data analysis, manuscript writing, laboratory experiments

Conflict of interest disclosure:

The author declares no conflict of interest.

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