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# Trifloxystrobin Pretreatment Alleviates Excessive Copper Stress in Wheat (*Triticum aestivum* L.)

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**ABSTRACT**

Protective role of Trifloxystrobin pretreatment against excessive copper-related abiotic stress in *Triticum aestivum* L. was determined in two Turkish wheat cultivars, Sönmez and Gerek 79. Ten-day-old seedlings were pretreated with 20 µM and 80 µM Trifloxystrobin. A group of seedlings was harvested without exposure to Trifloxystrobin as a control. Two days after, seedlings were treated with copper(II) chloride. Seedlings were harvested on the 20$^{th}$ day after sowing. The growth level of the groups was evaluated by measuring the length, fresh weight and shoot dry weight of shoots. Chlorophyll a + b, carotenoid and anthocyanin contents as well as proline levels were assessed. Lipid peroxidation and total activities of superoxide dismutase, peroxidase and glutathione reductase were analyzed to predict the oxidative stress levels. Both cultivars exhibited similar responses to the treatments. Trifloxystrobin doses did not cause damage on plants when applied alone. Seedlings subjected to excessive doses of copper showed significant reductions in growth parameters, as well as chlorophyll and carotenoid pigments. Conversely, copper caused a remarkable increase in anthocyanin, proline and malondialdehyde accumulation. Superoxide dismutase and peroxidase activities increased, while glutathione reductase activity decreased in copper-stressed plants. Trifloxystrobin pretreatment strengthens the antioxidant defense system. All parameters were positively affected by Trifloxystrobin pretreatment. As the dose of Trifloxystrobin increased, the severity of stress decreased in both genotypes. Trifloxystrobin pretreatment is a promising method for reducing copper-induced damage in *T. aestivum*.

***Keywords:*** Antmicrobial, Crystallinity, FT-IR, Poly ε-caprolactone, *Rumex patientia.*

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## Introduction

Wheat (*Triticum aestivum* L.) is the third most grown cereal in the world, following rice and corn. Due to its high adaptability and affordable price, it has become extremely popular for both agriculture and human consumption. Wheat grains are rich wellsprings of resistant starch, protein, inulin, tocopherol, phenolic acids, phytates, carotenoids, β-glucans, lignans and sterols. As a staple food, it provides approximately 55% of the starch, at least 20% of the total calories and 25% of the protein consumed in the world. Besides being a satisfying food grain, it is considered a rich source of dietary fiber. Türkiye, one of the gene centers of *T. aestivum*, is among the leading wheat producers in the world and has 201 officially registered bread wheat varieties [1]. One of the agricultural importance of wheat in Türkiye is its rotation with other field crops such as sugar beet and corn [2]. It is predicted that the demand for wheat, which will be required to feed the world population, which is expected to be 9.7 billion in 2050, will increase by 60%. Wheat exhibits a remarkable sensitivity to the stresses induced by various heavy metals [3]. Therefore, the protection of wheat from stress factors, including heavy metals, during the growing process should be the subject of research.

Copper (Cu) is a redox active transition metal which is needed for proper growth and development in plants. Due to being a vital micronutrient for plants, healthy physiological and biochemical functions can only be sustained in the presence of 5-30 mg kg$^{-1}$ Cu [4]. Photosynthesis, aerobic respiration, electron transport chain, lignin biosynthesis, cell wall metabolism, hormone signaling, iron metabolism, transcription and oxidative stress response are among the processes in which Cu plays an active role [5]. It is also a crucial constituent of several regulatory proteins, including plastocyanin, cytochrome-c-oxidase, amino oxidase, polyphenol oxidase and laccase [6]. Cu, the second most employed non-ferrous metal in industrial fields, is a critical element in energy, agriculture, construction, electronics and transport industries [7]. It is mainly released to agricultural soils from fungicide applications, chemical fertilizers, liquid manures, traffic, mining and sewage sludge [7]. Despite all vital functions in plants, Cu amounts exceeding optimum concentrations cause growth retardation, chlorosis, oxidative stress, membrane defect, altered enzyme activities, cytotoxicity and genotoxicity [8].

The evolution of fungi began about a billion years ago. Since then, the number of known fungi species has reached 120,000, with 1500-2000 new species discovered each year [9]. Although fungicides are widely employed to protect crop plants against pathogenic fungi, it is ambiguous whether these chemicals also affect abiotic stress tolerance. Treatments based on synthetic phytoprotectants have become popular approaches to minimize metal-related damage to crops. Some of the fungicides, which show serious toxicity in the case of use or accumulation in high concentration, can reduce the damage of abiotic stress by strengthening the plant defense mechanisms at low concentration [10]. Trifloxystrobin is a promising fungicide from the Strobilurins, the most common fungicide family all over the world. Trifloxystrobin, similar to other Strobilurins, binds to cytochrome b from the Qo region, leading to a blockage in electron transfer from cytochrome b to cytochrome c1. Thus, as a result of inhibited mitochondrial respiration, the energy cycle is broken [11]. Since plants are eukaryotic organisms, the electron transport system of their mitocondrial respiration is partially suppressed following Trifloxystrobin application. However, there are various studies indicating the contribution of exogenous Trifloxystrobin to abiotic stress defense in plant cells [12]. Indeed, Strobilurins delay leaf senescence, enhance photosynthesis, improve chlorophyll synthesis, promote $CO_2$ uptake and plant growth. The aim of this study was to reveal whether Trifloxystrobin pretreatment has a protective role against excessive Cu-related abiotic stress in *T. aestivum* seedlings. To fulfill this purpose, fresh weight, dry weight, shoot length, chlorophyll-carotenoid-anthocyanin pigment levels, lipid peroxidation, proline content and the total antioxidant enzyme activities were assessed.

## Materials and Methods

### *Preparation of Materials and Experimental Setup*

The wheat grains used in the research were provided by Prof. Dr. Tolga Karaköy from Sivas University of Science and Technology. Sönmez and Gerek 79 cultivars, which are frequently cultivated in Turkey, were used as research materials. The grains were kept in a 2% sodium hypochlorite solution for 5 minutes to sterilize the surfaces. The residues were removed by rinsing the grains with distilled water for 15 minutes. A soil:perlite mixture of 200 g (1:1 ratio) per pot was prepared as the growing medium. Organic soil, which was taken from the fields in Giresun and found to be within the standard values in terms of required minerals, was used. In the study, no previous fertilizer or pesticide was used. In particular, the Cu content of the soil was 2.76 ppm. Six seeds per pot with a soil capacity of 0.2 kg were sown and the seedlings were grown under optimum climate chamber conditions (a cycle of 12 hours of dark and 12 hours of light at 23±1 °C) for 11 days. During this process, the pots were watered to match the field capacity. The pots were then divided into three groups (Figure 1). A commercial formula called "Trailer" consisting of 50% Trifloxystrobin content (Hektas Group, Kocaeli/Türkiye) was used to prepare Trifloxystrobin solutions. The first two groups were pretreated with 30 ml of 20 µM and 80 µM Trifloxystrobin solutions, respectively. The last group was irrigated with 30 ml of water (control). These treatments were continued for two days. Then, the group pretreated with 20 µM Trifloxystrobin was divided into two groups, one of which was irrigated with 30 ml of water (TFS02), while the other one was treated with 30 ml of CuCl2 solution with 8 g/L Cu content (TFS02Cu) for one day. The same procedure was applied to the group pretreated with 80 µM Trifloxystrobin to obtain the TFS08 and TFS08Cu groups. The control group was divided into two groups and one group was treated with 30 ml of CuCl2 solution with 8 g/L Cu content (Cu) for one day, while the other group continued to be irrigated with water (C). All groups continued to be irrigated with water until the end of the experiment. All seedlings were harvested on the 20th day after sowing the seeds. All pots were placed randomly throughout the experiments.

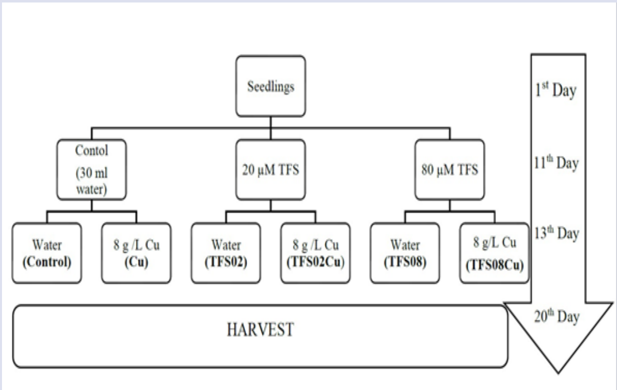


Figure 1. Diagram for experimental design.

### *Analysis of Growth Parameters*

The part from the apex of the last emerged leaf to the surface of the soil-perlite mixture was measured with a ruler to obtain the shoot lengths (cm). A total of 3 seedlings were randomly selected for each group to evaluate the average shoot length. Following the harvest, the shoots of the seedlings were weighed using a precision scale and the fresh weight was determined (g). The shoots were then kept in a drying oven at 80 °C for 48 hours to determine the dry weight (g) (n = 3).

### *Analysis of Chlorophyll and Carotenoid Contents*

Leaf segments (1 cm from the middle of each leaf) were weighed (g) and extracted in 2 ml of acetone (100%). Samples were kept in a refrigerator for 7 days in order to allow the pigments to pass into the solution. The absorbance of the samples was read at wavelengths of 470, 644.8, and 661.6 nm. The amounts of total chlorophyll (chl a + chl b) and total carotenoid (x + c) pigments were calculated using suggested extinction coefficients [13] (n = 3).

### Analysis of Chlorophyll and Anthocyanin Contents

Leaf segments (1 cm from the middle of each leaf) were weighed (g) and extracted in 2 ml of acidified methanol. Samples were kept in a refrigerator for 7 days to allow the anthocyanin pigments to pass into the solution. The absorbance of the samples was read at wavelengths of 657 and 530 nm. The anthocyanin content of the samples was calculated according to the method suggested by Mancinelli et al. [14] (n = 3).

### Analysis of Lipid Peroxidation

To estimate the peroxidation levels of membrane lipids, malondialdehyde (MDA) contents of the samples were assessed according to the modified method of Ohkawa et al. [15]. Fresh leaf samples (0.1 g) were homogenized in 1 ml of 5% trichloroacetic acid. After a centrifugation process (12,000 rpm), the supernatant was transferred into a mixture of 0.5% thiobarbituric acid and 20% trichloroacetic acid. Following a water bath treatment at 100 °C, all materials were ice-cooled to interrupt the reactions and centrifuged at 10,000 rpm. After recording the absorbance of the supernatants at 532 and 600 nm wavelengths, the MDA levels of the samples were calculated (n = 3).

### Analysis of Proline Content

The method suggested by Bates et al. [16] was modified to determine the free proline levels of the seedlings. Fresh leaf samples (0.2 g) were extracted in 3% sulphosalicylic acid according to the method of Weimberg [17]. At the end of the extraction, the sample was mixed with the same volumes of ninhydrin reagent and glacial acetic acid. Following a water bath treatment at 100 °C, all materials were ice-cooled to interrupt the reactions. Four ml of toluene was added to the samples to separate the proline-containing fraction. The free proline content of the samples was calculated by measuring the absorbance spectrophotometrically at 520 nm and using a proline standard curve (n = 3).

### Analysis of Antioxidant Enzyme Activities

Enzyme activities were assayed using fresh leaf segments (0.5 g) cut from seedlings for each treatment. The samples were ground in liquid nitrogen and transferred to potassium phosphate buffer containing tubes. The supernatant fractions were collected following centrifugation (14,000 rpm) for subsequent activity analyses. The Bradford [18] method was used to determine the protein amounts in the extracts.

The total catalytic activity of superoxide dismutase enzyme (SOD) (EC 1.15.1.1) was performed according to the method of Beyer and Fridovich [19] with slight modifications. The final volume of the reaction mixture, consisting of potassium phosphate buffer (50 mM; pH 7.8), methionine (9.9 mM), nitroblue tetrazolium (57µM) and determined volume of the extract, was 30.25 ml. The amount of SOD enzyme required to inhibit Nitroblue tetrazolium photoreduction by 50% was considered one unit of enzyme (n = 3).

The total catalytic activity of guaiacol peroxidase enzyme (POD) (EC 1.11.1.7) was evaluated by following the oxidation of guaiacol (E = 26.6 mM cm-1) by hydrogen peroxide spectrophotometrically at 470 nm [20]. The final volume of the reaction mixture, consisting of potassium phosphate buffer (100 mM; pH 7.0), guaiacol (20.1 mM), hydrogen peroxide (12.3 mM) and an appropriate amount of enzyme extract, was 3 ml. A unit of enzyme activity was considered as nm hydrogen peroxide decomposed per minute per mg of protein (n = 3).

The total catalytic activity of glutathione reductase enzyme (GR) (EC 1.6.4.2) was determined spectrophotometrically at 240 nm [21]. The medium prepared for extracting the GR enzyme consisted of potassium phosphate buffer (100 mM; pH 7.0), $Na_2EDTA$ (1 mM) and PVP (2%). The reaction mixture for assaying GR activity contained potassium phosphate buffer (200 mM; pH 7.5), $Na_2EDTA$ (0.2 mM), $MgCl_2$ (1.5 mM), GSSG (0.5 mM), NADPH (50 µM) and an appropriate amount of enzyme extract containing 100 µg protein (n = 3). Non-enzymatic oxidation of NADPH was recorded spectrophotometrically at a wavelength of 340 nm without adding GSSG to the reaction medium. Enzymatic catalysis was monitored at 340 nm by adding GSSG to the assay medium (n = 3).

### Statistics

The data of the present study was analyzed through one-way ANOVA and Duncan's tests using IBM SPSS Statistics 26 Software. Differences between the results at $p < 0.05$ were accepted as statistically significant.

## Results and Discussion

In order to evaluate the effects of Trifloxystrobin and Cu on growth, shoot length, shoot fresh weight and shoot dry weight were measured in all groups (Figure 2). The control group of Sönmez cultivar was taller than Gerek 79 cultivar in terms of natural genetic structure at the early seedling stage (Figure 2a). There was no significant difference between the shoot lengths of the Sönmez seedlings in the first three groups (C, TFS02 and TFS08). Similarly, Trifloxystrobin did not have any effect when applied alone on the fresh and dry weights of the seedlings of the Gerek 79 cultivar (Figure 2b and 2c). Therefore, the selected Trifloxystrobin doses were non-toxic for both wheat cultivars at an early seedling stage. On the other hand, Cu application caused a remarkable decrease in shoot elongation, fresh weight and dry weight in both wheat cultivars. Indeed, the shoot length of the seedlings treated with Cu decreased by 41% in the Sönmez cultivar and by 35% in the Gerek 79 cultivar compared to their own controls. In addition, the dry weight of the seedlings in the Cu group decreased by 36% in Sönmez and by 41% in Gerek 79 cultivars compared to their own controls. According to the results obtained from the TFS02Cu and TFS08Cu groups, Trifloxystrobin pretreatment provided a dose-dependent protection against subsequent Cu application in both cultivars. However, the results of the growth parameters of TFS02Cu and TFS08Cu groups never reached control levels in the cultivars. Our results were in agreement with the study of Singh et al. [22] in which the growth retarding effect of Cu in wheat seedlings was noted. Furthermore, Atabayeva et al. [23] indicated that high doses of Cu treatment suppressed shoot length and dry weight in wheat varieties significantly. In addition, Lyoshyna et al. [24] proved a notable reduction in green mass and shoot length of wheat seedlings after Cu application. To our knowledge, this is the first study to show that Trifloxystrobin pretreatment reduces Cu stress-induced growth restriction in wheat. Strobilurins, including Trifloxystrobin, have the ability to alter plant physiology by causing changes in metabolism and growth. Mohsin et al. [12] showed that the application of Tebuconazole and Trifloxystrobin mixture improved dry weight and fresh weight in heavy metal (cadmium)-stressed wheat seedlings. According to Takahashi et al. [25], the physiological effects of "protective fungicides" such as Trifloxystrobin become more pronounced when the plants are under stress.

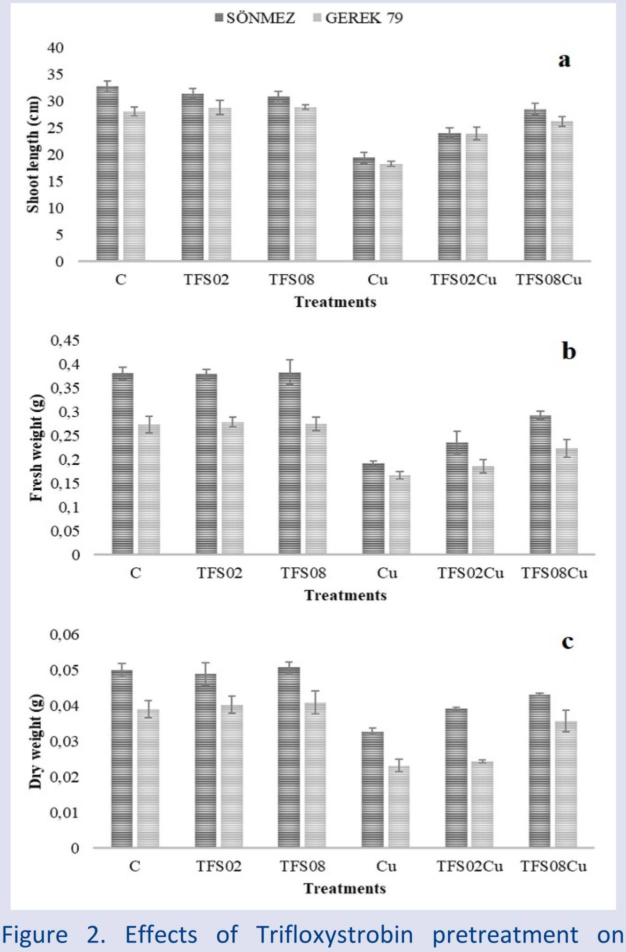


Figure 2. Effects of Trifloxystrobin pretreatment on growth of Cu-stressed wheat cultivars (a. Shoot lenght, b. Fresh weight, c. Dry weight) (C: control, TFS02: 20µM Trifloxystrobin, TFS08: 80 µM Trifloxystrobin, Cu: $CuCl_2$, TFS02Cu: 20µM Trifloxystrobin + $CuCl_2$, TFS08Cu: 80µM Trifloxystrobin + $CuCl_2$).

The total chlorophyll and carotenoid levels of Sönmez seedlings in the control group were higher than those of Gerek 79 (Figure 3). Trifloxystrobin applications in the TFS02 and TFS08 groups of both cultivars did not trigger a remarkable effect on pigment concentrations when compared to the control groups. However, Cu application resulted in a 60% and 53% chlorophyll reduction in Sönmez and Gerek 79, respectively, compared to their own controls (Figure 3a). Similarly, the carotenoid contents of Sönmez and Gerek 79 exposed to Cu decreased by 37% and 52%, respectively, compared to their controls. In both cultivars, the TFS02Cu and TFS08Cu groups pretreated with Trifloxystrobin solutions showed a dose-dependent protection against subsequent Cu application in terms of both chlorophyll and carotenoid. In these groups, the levels of the pigments increased with the increased Trifloxystrobin doses. Therefore, pretreatment with Trifloxystrobin largely prevented damage to both growth and photosynthetic pigments in wheat seedlings against Cu stress. The greater growth in the TFS02Cu and TFS08Cu groups than in the Cu group can be attributed to the preservation of pigments and thus to uninterrupted photosynthesis. Our results were in line with Atabayeva et al. [23], who reported that Cu reduces chlorophyll a, chlorophyll b and carotenoid pigments in wheat. Additionally, Zong et al. [26] suggested that Cu led to a devastating effect on the chlorophyll content of wheat seedlings when applied in high doses. Mohsin et al. [12] have already demonstrated the potential of Trifloxystrobin to prevent chlorophyll and carotenoid damage caused by heavy metals. Yet, this is the first study to demonstrate the protective role of pre-applied Trifloxystrobin in the amount of photosynthetic pigments in copper-stressed wheat seedlings. Under these circumstances, the results of our study confirmed the report of Banerjee and Roychoudhury [27], which mentioned the effect of Strobilurin-type fungicides on stress tolerance development against stresses such as drought, desiccation, heat, cold and light.

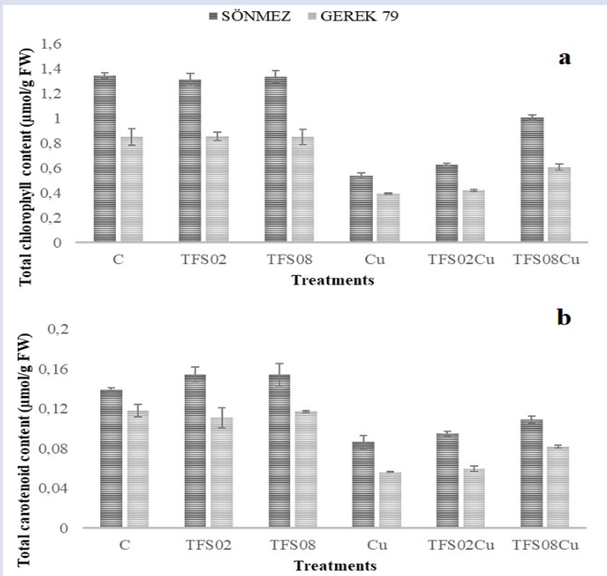


Figure 3. Effects of Trifloxystrobin pretreatment on chlorophyll and carotenoid contents of Cu-stressed wheat cultivars (a. Total chlorophyll content, b. Total carotenoid content) (C: control, TFS02: 20µM Trifloxystrobin, TFS08: 80 µM Trifloxystrobin, Cu: $CuCl_2$, TFS02Cu: 20µM Trifloxystrobin + $CuCl_2$, TFS08Cu: 80µM Trifloxystrobin + $CuCl_2$).

Anthocyanins, a group of flavonoids, are pigments found in flowers, fruits, roots, stems and leaves of plants and play vital roles in abiotic stress tolerance. Figure 4a presents the effects of Trifloxystrobin pretreatment on anthocyanin accumulation of Cu-stressed wheat seedlings. Considering the control groups, the initial anthocyanin content of the Sönmez cultivar was slightly higher than that of the Gerek 79 cultivar. In both cultivars, there was no significant difference between the anthocyanin levels of the TFS02, TFS08 and control groups. On the contrary, Cu application increased the anthocyanin levels in Sönmez and Gerek 79 to 2.17 and 1.34 times that of their own controls, respectively. While both doses of Trifloxystrobin were sufficient for the anthocyanin content of Gerek 79 to decrease to the control level, the decrease of the anthocyanin content to the control level in Sönmez was only possible with the pre-application of 80 µM Trifloxystrobin. Our results were in agreement with the older studies which showed that anthocyanin accumulates in *T. aestivum* shoots exposed to heavy metal stresses [28]. Tereshchenko et al. [29] reported that anthocyanin synthesis in wheat seedlings is closely correlated with responses to abiotic stresses, including heavy metals. One of the promptest influences of toxic doses of heavy metals in plant cells is the over-production of reactive oxygen species (ROS). Owing to their antioxidant properties, anthocyanins suppress oxidative stress by scavenging ROS and thus control growth inhibition and cell death. They also help ion homeostasis and osmotic balance be maintained under stressful conditions through their excellent ion chelating abilities.

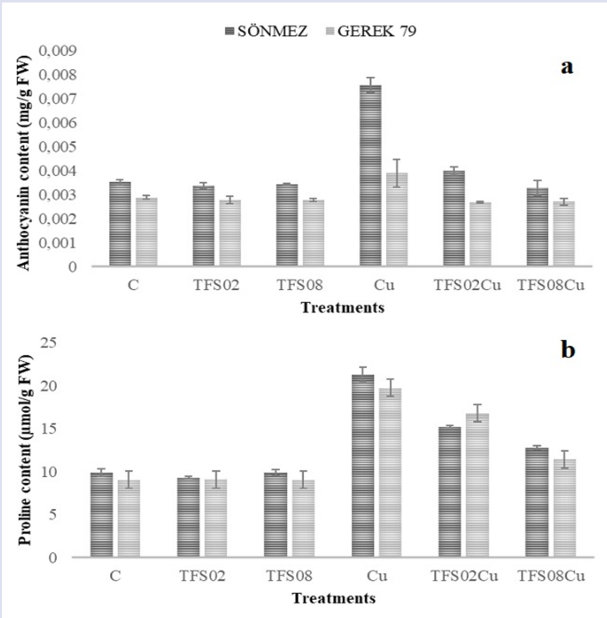


Figure 4. Effects of Trifloxystrobin pretreatment on anthocyanin and proline contents of Cu-stressed wheat cultivars (a. Anthocyanin content, b. Prolin content) (C: control, TFS02: 20µM Trifloxystrobin, TFS08: 80 µM Trifloxystrobin, Cu: $CuCl_2$, TFS02Cu: 20µM Trifloxystrobin + $CuCl_2$, TFS08Cu: 80µM Trifloxystrobin + $CuCl_2$)

Figure 4b shows the effects of Trifloxystrobin pretreatment on the proline content of Cu-stressed wheat seedlings. The proline contents of the first three groups (C, TFS02 and TFS=8) of Sönmez and Gerek 79 were statistically similar. Additionally, the control groups of both cultivars had similar proline levels. However, Cu application enhanced the proline levels in Sönmez and Gerek 79 to 2.16 and 2.18 times those of their own controls, respectively. TFS02Cu and TFS08Cu groups exposed to Trifloxystrobin pretreatment exhibited a gradual decrease in free proline levels depending on the Trifloxystrobin dose. However, even the higher Trifloxystrobin dose failed to reduce the proline content of the TFS08Cu group to control levels in both cultivars. Cu-induced free proline accumulation in the plant kingdom has been reported in many studies so far [30]. There are various investigations manifesting that toxic doses of heavy metals lead to growth retardation, chlorosis, necrosis, disrupted secondary structure of proteins and imbalances in the redox state of plant cells. It is known that the accumulation of proline lessens the destructive effects on plants and improves stress tolerance under abiotic stress conditions. In addition to eliminating ROS and increasing the activity of various cellular enzymes, proline acts as an osmoprotectant to overcome oxidative and osmotic stresses. We report that Trifloxystrobin pretreatment can greatly attenuate the proline accumulation by reducing the Cu-provoked stress in *T. aestivum* seedlings. Our results were in line with the study of Mohsin et al. [12], which showed that exogenous Trifloxystrobin alleviates the proline biosynthesis in wheat by suppressing cadmium-related stress.

MDA is a reactive organic compound and MDA accumulation has long been utilized as a marker of lipid peroxidation arising from oxidative stress in cellular membranes. The MDA content of wheat seedlings was determined to assess both the individual and combined effects of Trifloxystrobin and Cu on the membrane integrity (Figure 5). The MDA levels of the first three groups (C, TFS02 and TFS08) of the cultivars were close to each other. However, when Cu was applied alone, it caused a significant increase in the MDA content of Sönmez and Gerek 79. As a matter of fact, the MDA content of Cu groups of Sönmez and Gerek 79 cultivars was 3.29 and 2.22 times that of their own controls, respectively. The MDA levels of the TFS02Cu and TFS08Cu groups of both cultivars were significantly lower than their Cu-treated groups. Trifloxystrobin pretreatment prior to Cu application provided dose-dependent protection against Cu-induced membrane damage in both Sönmez and Gerek 79. Lipid peroxidation is a well-known chain reaction initiated by ROS on unsaturated fatty acids. In our experiment, Cu treatment caused severe membrane destabilization, and this phenomenon is often associated with enhanced ROS production. Our results confirmed Xu et al. [31], who suggested that toxic Cu concentrations lead to MDA accumulation in *T. aestivum* seedlings. Similar to our results, Trifloxystrobin as an outstanding fungicide type was involved in protection against cadmium-induced membrane damage in wheat [12]. Hameed et al. [32] also stated that mercapto-triazole, another antifungal compound, reduced lipid peroxidation caused by heavy metal toxicity in wheat. In another study, it was reported that azoxystrobins delay lipid peroxidation with increased antioxidant defense activities [33].

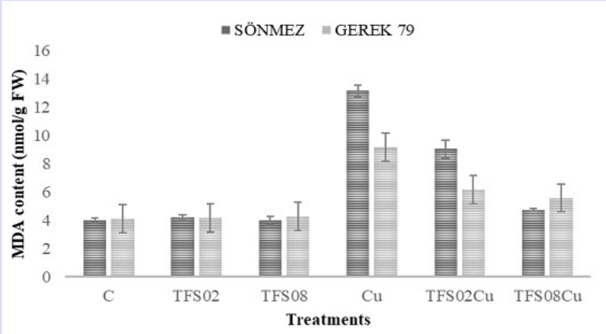


Figure 5. Effects of Trifloxystrobin pretreatment on MDA content of Cu-stressed wheat cultivars (C: control, TFS02: 20µM Trifloxystrobin, TFS08: 80 µM Trifloxystrobin, Cu: $CuCl_2$, TFS02Cu: 20µM Trifloxystrobin + $CuCl_2$, TFS08Cu: 80µM Trifloxystrobin + $CuCl_2$).

Cu is a redox active metal and although it is a very important part of many enzymes, an overdose of Cu triggers the formation of ROS. Indeed, even doses of Cu slightly greater than its optimum concentration induce ROS-mediated oxidative stress [34]. Since Cu is a transition metal, it has enormous oxidative power and the potential to participate in Fenton/Haber-Weiss reactions [3]. All kinds of stress adversely affect the normal functioning and final yield of plants. The ability of plants to withstand these stresses depends on their effective use of antioxidant systems. Antioxidant enzyme activities are considered sensitive biomarkers of stress in plants [23]. The SOD, POD and GR enzyme activities were monitored to evaluate the Cu-induced oxidative burst and the protective ability of Trifloxystrobin (Figure 6). The SOD activity of both cultivars exhibited a gradual increase with increasing Trifloxystrobin doses (Figure 6a). Therefore, the SOD activities of the TFS02 and TFS08 groups were higher than those of their own controls in Sönmez and Gerek 79. The electron transfer system interrupted by Strobilurins can lead to superoxide synthesis [35], resulting in a Trifloxystrobin-induced increase in the SOD activities in TFS02 and TFS08. The elevation pattern in the SOD activities of the cultivars peaked after Cu application. The SOD activities of Cu groups of Sönmez and Gerek 79 cultivars were approximately 6.32 and 5.79 times those of their own controls, respectively. Since the SOD enzyme catalyzes the decomposition of superoxide radicals into hydrogen peroxide and oxygen, the results of this study showed that Cu application induced superoxide formation in wheat and, accordingly, the SOD activity increased. The SOD activity of the TFS02Cu and TFS08Cu groups were lower than the Cu groups of both cultivars. Trifloxystrobin pretreatment reduced the SOD activities with increasing doses of the fungicide. In Gerek 79, this decrease only became statistically significant in the TFS08Cu application.

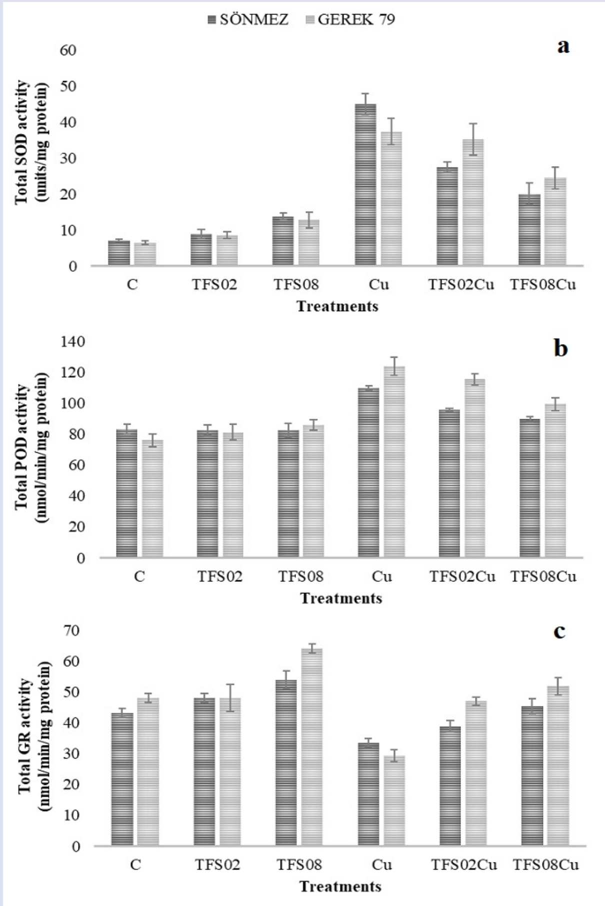


Figure 6. Effects of Trifloxystrobin pretreatment on antioxidant enzyme activities of Cu-stressed wheat cultivars (a. Total SOD activity, b. Total POD activity, c. Total GR activity) (C: control, TFS02: 20µM Trifloxystrobin, TFS08: 80 µM Trifloxystrobin, Cu: $CuCl_2$, TFS02Cu: 20µM Trifloxystrobin + $CuCl_2$, TFS08Cu: 80µM Trifloxystrobin + $CuCl_2$).

POD enzyme removes hydrogen peroxide from cells and helps to maintain biological membrane integrity by strengthening cell walls and preventing metal ions from entering cells [36]. In TFS02 and TFS08 groups of Sönmez and Gerek 79, the POD activities remained stable when compared to the control groups (Figure 6b). The Cu applied groups of the cultivars exhibited a significant increase in their POD activities. The POD activities of the Cu groups of Sönmez and Gerek 79 cultivars were 32% and 63% higher, respectively, than their controls. Trifloxystrobin pretreatment before Cu application induced a dose-dependent drop in the POD activities of cultivars compared to the control groups. However, the POD activities of the TFS02Cu and TFS08Cu groups in both cultivars were still higher than those of their respective

Our results confirmed the study of Gupta et al. [37], who stated that POD activity was associated with proline content to provide efficient membrane protection in wheat. In addition, the parallelism of changes in MDA accumulation and POD activity of the groups in the present study confirmed Díaz et al. [38], who suggested POD activity makes a major contribution to the cell wall stability of the plants under heavy metal stress.

The GR enzyme is localized in the chloroplast, cytosol, and mitochondria and converts oxidized glutathione to reduced glutathione as part of the ascorbate-glutathione cycle. In addition to being a stimulant for normal growth and development, it plays an important role in combating various stresses, including heavy metals. While a significant increase was observed in the GR activity of the Sönmez cultivar in the TFS02 and TFS08 groups compared to the control group, the GR activity of the Gerek 79 cultivar increased significantly only in the TFS08 group compared to the control group (Figure 6c). Surprisingly, Cu application caused a remarkable decrease in the GR activities of both cultivars compared to their respective controls. Our results were in agreement with previously published results by Mohsin et al. [18], who stated that heavy metal stress reduced GR activity in wheat. Similarly, Drążkiewicz et al. [39] showed that the application of Cu up to a certain dose in *Arabidopsis thaliana* L. dramatically reduced the GR activity. The GR activities of the TFS02Cu and TFS08 groups were significantly higher than those of their own Cu-treated groups. Indeed, the TFS02Cu and TFS08 groups of Sönmez and Gerek 79 did not exhibit a significant difference in GR activity compared to their own controls. Wu and von Tiedemann [40], confirming the results of our study, showed that previously applied Azoxystrobin, another kind of Strobilurin, reduced the ozone stress inducing the activity of the GR enzyme in wheat.

## Conclusion

In conclusion, this study has clearly demonstrated that excessive Cu stress suppresses growth, reduces photosynthetic pigments, and causes membrane damage in wheat seedlings. On the other hand, proline and anthocyanin levels, which are among the defense mechanisms along with the antioxidant enzyme activities, increased in plants under Cu stress. Although Cu application increased anthocyanin accumulation and SOD activity in the Sönmez cultivar more than in Gerek 79, Sönmez had more membrane damage, shoot elongation deceleration and chlorophyll loss. In addition, the Cu-stressed Gerek 79 cultivar exhibited a higher rate of proline content and POD activity. However, the dry weight loss and carotenoid pigment decrease of Gerek 79 were at a higher rate when compared to the Sönmez cultivar. GR activities of Cu-stressed wheat seedlings decreased in both genotypes. The pretreatment with Trifloxystrobin, a frequently used fungicide, provided effective protection against all damage caused by Cu application in both cultivars. As the dose of Trifloxystrobin increased, the severity of stress decreased in both wheat genotypes. Trifloxystrobin pretreatment stimulated the antioxidant defense system, allowing the plant to be less affected by Cu stress. Considering the increase in Cu accumulation in the soil due to the pesticides used and the sensitivity of wheat to heavy metal stress, it should not be forgotten that every method to protect plants against this stress is valuable. It is no accident that research examining the exogenous and unconventional application of fungicides has multiplied. The findings of this study suggest that the potential of Trifloxystrobin pretreatment to protect valuable crops such as wheat against other metal toxicities should be investigated at different life stages and field conditions. To increase agricultural production and protect plants, the benefits of compounds such as Trifloxystrobin should be exploited to the fullest.

## Conflicts of interest

There are no conflicts of interest in this work.

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