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The biochemical changes caused by metolachlor and atrazine on wheat (*Triticum aestivum* L.) varieties

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Summary. *Aim:* In this study, different concentrations (0, 100 µM, 300 µM, and 1000 µM) of Atrazine and Metolachlor were applied on 15-day-old bread wheat seedlings (of three varieties named as Bayraktar cv., İkizce cv. and Tosunbey cv. of *Triticum aestivum* L.) for 48 hours and their biochemical effects were investigated. *Methods:* In all the treatments on the seedlings, the hydroponic method was preferred. *Results:* In the leaves of atrazine-treated wheat seedling, GSH/GSSG ratio also increased in three varieties and MDA amount increased in Bayraktar and Tosunbey compared to the control. SOD activity increased in Tosunbey, decreased in İkizce and it firstly increased and then decreased in Bayraktar. CAT activity decreased in all three of the varieties. In the leaves of metolachlor-treated wheat seedlings, GSH/GSSG ratio decreased in three varieties and MDA amount increased in İkizce and Tosunbey, compared to the control. SOD activity decreased in Bayraktar, increased in İkizce and Tosunbey; and CAT activity decreased in all three varieties. *Conclusion:* In general, Atrazine was found to be more inhibitive compared to Metolachlor. In other words, Atrazine became more effective in causing oxidative stress in wheat seedlings.

Key words: Wheat (*Triticum aestivum* L.), Oxidative stress, herbicide, Atrazine, Metolachlor.

Introduction

Atrazine is an herbicide of the triazine class (1) that is used before cultivating the agricultural plants in order to stop the development of broad-leaved weeds. It is known that the herbicides with effective agent such as Atrazine, Linuron, Atrazine+Linuron, and Atrazine+Metolachlor, treated before the shooting of maize, bring weeds under control. The Food Quality Protection Law made within the scope of the US Environmental Protection Agency (EPA) in 2006 mentions that the pesticide residues are agents changing the natural hormonal system. In 2007, EPA has accepted that they have affected sexual development in the studies conducted with amphibians. In the study by EPA in 2009, it has been revealed that Atrazine causes negative effects in human reproduction. Atrazine is an herbicide that is kept in root without degradation at a moderate level and it accumulates in the root zone at a depth of about 125 cm. As atrazine has a longer half-life compared to the other herbicides, most of its part remains in soil (2). Atrazine controls weeds and causes an ecotoxic effect on the nontarget organisms (crop plants, soil microorganism, and other animals). Herbicides cause oxidative stress by triggering the formation of the reactive oxygen species (1). The fact that rice (3), wheat (4) and maize (5) leaves are exposed to herbicides has caused catalase activity to increase. Catalase enzyme detoxifies H_2O_2 at high concentration ratio and leads plant to eliminate stress with the least loss. In a study conducted with paraquat in sugar cane, increasing activity of antioxidant enzymes such as SOD and CAT was interpreted as a result of the detoxification mechanism which leads to a decrease of lipid peroxidation (6).

It has been reported that there is an increase in GSH content as a result of the effect of Atrazine and Metolachlor treated on the leaves of the 30-day-old maize plant (*Zea mays* L. Artus cv.) (7). In a similar study, an increase in the GSH content was determined with the effect of Metolachlor treated the 6 and 8-day-old maize leaves (8). Racemic-metolachlor and s-metolachlor (isomer of metolachlor) were kept in the root and leaves of maize plant in 18 µM concentration for 80.6 and 60.3 hours respectively, in 37.2 µM concentration for 119.5 and 90 hours respectively, and in 74.4 µM concentration for 169 and 164.8 hours. While there was an increase in the SOD, POD, and CAT enzyme activities in rac-metolachlor treatment, SOD, POD, and CAT enzyme activities decreased in s-metolachlor treatment (9). Lettuce, bean, and pea seeds and leaves were treated with 0.2-200 µM concentrations of Metolachlor for 48 hours and a decrease was observed in the SOD and CAT activities (10). Spraying 23 µM dose of 2,4-D with periodically to fresh and mature leaves of pea plant caused oxidative damage in protein and membrane lipids after 72 hours. It was observed that glutathione content increased (11). It was found that leaves of the mature pea plant had a higher GSH/GSSG ratio. It was determined that SOD enzyme activity increased in fresh plant leaves. There was a decrease in CAT enzyme activity (11). Rice plant was treated with atrazine in different doses for four days and an increase was determined in SOD, CAT, POD, and other antioxidants enzyme activities in its root and leaves (12). Atrazine in different doses was applied to 2 different 10-day-old maize plants (*Zea mays* L. Hybrid 351 and Giza 2) and it was determined at the end of the 20th day that while GSH amount increased in Hybrid 351 it decreased in Giza 2 hybrid maize. It was determined that SOD and CAT enzymes decreased and MDA amount increased (13). 21-day-old *Palaemonetes argentinus* plant was treated with 0.4 mg/L^{-1} Atrazine for 24 hours in the hydroponic environment. An increase was determined in SOD content (14). Atrazine in 0-10 mg/L concentrations was applied to leaves of 2-week-old maize plant for 3 days and it was reported that SOD and POD enzymes increased in the root and CAT enzyme increased in the leaves and MDA amounts increased in the root and leaves (15). It was determined that GSH/GSSG ratio decreased in the maize seeds treated with 2,4-D (16). In a study conducted on *Saccharum spp.* seedlings (RB92579, SP83-2847, SP81-3250 and IAC91-5155) (sugar cane), it was reported that the paraquat effect caused an increase in SOD, CAT, ascorbate peroxidase enzymes (6). In the study investigating the topical effect on leaves of *Triticum aestivum* L., cv. *Mironovskaya* 808 (wheat), *Secale cereale* L. cv. *Estafeta tatarstana* (rye) and *Zea mays* L. cv. *Kollektivnyi* 172MV (maize) seedlings, it was reported that there was an increase in the lipid peroxidation, superoxide anion, total antioxidant activity, catalase and ascorbate peroxidase activities (17). Krasu et al., (1995) reported that Paclobutrazol and Paraquat herbicides increased SOD and CAT activity on *Triticum aestivum L.* cv. Frederick and Glenlea leaves (18). Atrazine was sprayed periodically to *Zea mays* cv. 'Martha F1' plant leaves in 27-226 µM concentrations. The MDA level increased until the 5th day and decreased on the 10th and 15th days (19). After Fenoxaprop-p-ethyl herbicide was sprayed to leaves of *Triticum aestivum* L. (wheat) seedlings at the end of 1st, 5th and 10th days, SOD activity as well as MDA and GSH amounts increased and CAT activity and chlorophyll amount decreased and excepted increases and decreases were determined in carotenoids (20). The treatment of chlorimuron-ethyl herbicide on leaves and roots of *Triticum aestivum* (wheat) seedlings caused a decrease in SOD activity and an increase in MDA amount (21). Pretilachlor (0, 5, 10 and 20 µg ml $^{-1}$) herbicide was applied to fern (Azolla spp. viz., Azolla microphylla and Azolla pinnata) and a decrease was determined in parallel with the increase of doses in GSH content and GSH/GSSG ratio (22). *Arabidopsis thaliana* leaves were treated with the chiral herbicide and an increase was observed in GSH content and SOD activity and CAT activity and Rac decreased in DCPP variety (23). Wheat varieties were treated with simetryne (s-triazine herbicide) (0, 0.8,1.6, 3.2, 4.8, 6.4 and 8.0) and an increase was determined in SOD and CAT activities in the root and leaves until 3.2 mg.kg^{-1} concentration and a decrease was determined in 4.8 and higher concentrations (24). Root and leaves *of Pennisetum americanum* seedling were treated with *Atrazine* for 68 days and an increase in SOD and CAT activities and a decrease in high concentration were observed (1). Wild and transgenic rice leaves were treated with methyl viologen herbicide for 7 weeks and MDA amount decreased and GSH/GSSG ratio increased (25). When maize, pea, and wheat plants were treated with 2,4-D and Atrazine, an increase in MDA amount and a decrease in PRL amount were determined (26). In a study investigating the effect of Imazapic effect on tobacco seedlings, an increase was reported in CAT activity, GSH and MDA amounts (27). Zhao et al., reported that CAT activity, which is one of the antioxidant enzymes, (Ug^{-1} FW) decreased and SOD activity increased in the leaves when they treated 21-day-old maize seedlings with the herbicide named as Chlorsulfuron (28). In the study conducted by Hasanuzzaman et al., to examine the effects of the herbicide Paraquat on 10-days-old *Brassica napus L.* seedlings it was determined that proline, MDA amount, and GSH/GSSG ratio increased (29). In their study, Liu et al., investigated the effects of the herbicide Glyphosate on *Salvina natans* L. plant and reported that the yellowing percentage increased in parallel with the dose increase. In the same study, it was reported that MDA amount increased and the CAT activity decreased (30).

In this study, the biochemical responses associated with the toxic effect induced by the different concentrations of the synthetic herbicides Atrazine and Metolachlor in the seedlings of three wheat varieties were investigated. The biochemical parameters such as GSH/GSSG ratio, MDA amount, SOD and CAT activities were studied in the leaves of the seedlings which were subjected to different treatments.

Materials and Methods

Growth conditions of seedlings and experimental design

In order to grow wheat (*Triticum aestivum* L.) seedlings, fully homogeneous (uniform) seeds were selected (*Triticum aestivum* L. Bayraktar cv., İkizce cv. and Tosunbey cv.). The seeds were wetted with tap water and kept in the dark for 6 hours at 23-25 °C. At the end of this period they were aligned in covered germination boxes in which they can take air and they were kept in the dark for 3 days at 23-25 °C. Then, germinated seeds with equal radicle lengths were selected and they were placed in the beakers prepared with the aqueous soil mixture prepared before. Here, the seedlings were grown until they were 15 days old in long-day photoperiod (16/8) in normal daylight. The seeds which were grown fully homogeneously were selected among the 15-day-old seedlings and they were used as experimental material. For each wheat variety, the 15-day-old seedlings were divided into 4 groups including the same number of seedlings (20 for each) and 4 different doses of herbicides were applied. The herbicides were applied to the seedlings using hydroponic method. The seedlings were exposed to different (0 µM, 100 µM, 300 µM and 1000 µM) concentrations prepared with atrazine and metolachlor for 1 day. All these different herbicide treatments were applied to different seedlings synchronously. Then, reduced glutathione ratio/ oxidized glutathione ratio, malondialdehyde amount, superoxide dismutase, and catalase activity were determined in these seedlings.

Growth parameters of seedlings in biochemical analysis

The GSH/GSSG ratio in the extracts of wheat leaves were determined using the method of Yilmaz et al., (2009) (31). MDA amounts (32), SOD activity (33) and CAT activity (34) were analyzed. All the biochemical analyses were triplicated for each parameter and 2 gr of wheat leave tissue was used.

Statistical analyses

All the parameters in the present study were repetitively analyzed as 3 repetitions. The accuracy values of the data were tested with mean and One-way ANOVA using SPSS 15 packaged software. The differences between the groups were differentiated at significance level of $P \leq 0.05$. The statistically insignificant data were not emphasized ($P > 0.05$).

Results

The Effect of Atrazine and Metolachlor on GSH/GSSG Ratio

Increases of 33.66%, 107.19%, and 130.72% were determined in 100 µM, 300 µM, and 1000 µM concentrations for Bayraktar hybrid wheat, in GSH/GSSG ratio, respectively, in the leaves of the atrazine-treated seedlings compared to their controls. Increases of 7.08%, 31.86%, and 57.06% were determined respectively in 100 µM, 300 µM and 1000 µM concentrations for İkizce hybrid wheat. Increases of 13.64%, 24.64%, and 28.26% were determined respectively in 100 µM, 300 µM, and 1000 µM concentrations for Tosunbey hybrid wheat. (P≤0.05) (Table 1).

Decreases of 10.16%, 15.09%, and 18.41% were determined respectively in 100 µM, 300 µM, and 1000 µM concentrations for Bayraktar hybrid wheat, in GSH/GSSG ratio in the leaves of the metolachlor-treated seedlings, compared to their controls. Decreases of 19.27%, 28.59%, and 37.03% were determined respectively in 100 µM, 300 µM, and 1000 µM concentrations for İkizce hybrid wheat. Decreases of 9%, 18.01%, and 31.94% were determined respectively in 100 µM, 300 µM, and 1000 µM concentrations for Tosunbey hybrid wheat. (P≤0.05) (Table 2).

The Effect of Atrazine and Metolachlor on MDA Amounts

A decrease of 8.14% and increases of 58.03%, and 74.87% were determined respectively in 100 µM, 300 µM, and 1000 µM concentrations in MDA amounts for Bayraktar hybrid wheat in the leaves of the atrazine-treated seedlings compared to their controls. Decreases of 6.08%, 12.59%, and 50.25% were determined respectively in 100 µM, 300 µM and 1000 µM concentrations for İkizce hybrid wheat. Increases of 29.45%, 106.3%, and 40.28% were determined respectively in 100 µM, 300 µM, and 1000µM concentrations for Tosunbey hybrid wheat. (P≤0.05) (Table 1).

Decreases of 11.57%, 13.77%, and 43.19% were determined respectively in 100 µM, 300 µM, and 1000 µM concentrations in MDA amounts for Bayraktar hybrid wheat in the leaves of the metolachlor-treated seedlings. Increases of 12.13% and 20.11% and a decrease of 19.70% were determined respectively in 100 µM, 300 µM, and 1000 µM concentrations for İkizce hybrid wheat. Increases of 62.05% and 79.07% were determined respectively in 100 µM and 300 µM concentrations for Tosunbey hybrid wheat. (P≤0.05) (Table 2).

The Effect of Atrazine and Metolachlor on SOD and CAT Activity

An increase of 7.36% and decreases of 6.98% and 17.05% were determined respectively in the 100 µM, 300 µM, and 1000 µM concentrations in SOD activity for Bayraktar hybrid wheat in the leaves of atrazine-treated seedling, compared to their controls. Decreases of 21.35%, 8.61%, and 5.62% were determined in 100 µM, 300 µM, and 1000 µM concentrations for İkizce hybrid wheat, respectively. Increases of 7.53, 11.99%, and 26.71% were determined in 100 µM, 300 µM, and 1000µM concentrations for Tosunbey hybrid wheat, respectively. (P≤0.05) (Table 1).

Decreases of 17.65%, 20.20%, and 25.69% were determined respectively in 100 µM, 300 µM, and 1000

Table 1. The changes in GSH/GSSG ratio, SOD and CAT activities, and MDA amount in the leaves of atrazine-treated *Triticum aestivum* L. seedlings.

ATRAZINE GROUPS	GSH/GSSG	SOD activity (unite/g)	CAT activity (µg/g)	MDA (nmol.g^{-1} TA)
B-Control	8,557±0,570	0,258±0,003	245,057±9,914	193,0±14,042
B-100 µM	11,437±0,634*	0,277±0,008*	211,543±8,652*	176,760±11,300
B-300 µM	17,729±0,478*	0,240±0,007*	181,037±2,694*	305,0±21,687*
B-1000 µM	19,743±0,432*	0,214±0,001*	161,273±14,273*	337,50±6,495*
İ-Control	11,250±0,203	0,267±017	166,451±9,358	255,542±3,943
İ-100 µM	12,047±0,395	0,210±0,002*	143,297±1,403*	240,0±1,083
İ-300 µM	14,834±0,428*	0,244±0,018	141,390±1,784*	223,375±1,090*
İ-1000 µM	17,669±1,210*	0,252±0,011	116,819±2,624*	127,137±9,076*
T-Control	20,204±0,841	0,292±0,002	42,738±0,498	115,750±1,473
T-100 µM	22,960±0,731*	0,314±0,001*	32,244±1,033*	149,835±26,394
T-300 µM	25,183±0,392*	0,327±0,001*	25,152±0,768*	238,792±10,723*
T-1000 µM	25,913±0,143*	0,370±0,008*	24,007±1,104*	162,377±2,582

*: Compared to the control group at p≤0.05 probability levels. Values are mean of three replicates ±SE (n:3) Bayraktar: B, İkizce: İ, Tosunbey: T

μM concentrations in SOD activity for Bayraktar hybrid wheat in the leaves of metolachlor-treated seedlings, compared to their controls. Increases of 6.96%, 15.98% and 25.52% were determined respectively in 100 μM, 300 μM, and 1000 μM concentrations for İkizce hybrid wheat. Increases of 27.52%, 38.76%, and 32.34% were determined respectively in 100 μM, 300 μM, and 1000μM concentrations for Tosunbey hybrid wheat. ($P \leq 0.05$) (Table 2).

Decreases of % 13.68%, 26.12%, and 34.19% were determined respectively in the 100 μM, 300 μM, and 1000 μM concentrations in CAT activity for Bayraktar hybrid wheat in the leaves of atrazine seedlings, compared to their controls. Decreases of 13.91%, 15.06%, and 29.82% were determined respectively in 100 μM, 300 μM, and 1000 μM concentrations for İkizce hybrid wheat. Decreases of 24.55%, 41.15%, and 43.83% were determined respectively in 100 μM, 300 μM and 1000μM concentrations for Tosunbey hybrid wheat. ($P \leq 0.05$).

Decreases of 17.25%, 19.87%, and 24.59% were determined respectively in 100 μM, 300 μM, and 1000 μM concentrations in CAT activity for Bayraktar hybrid wheat in the leaves of metolachlor-treated seedlings, compared to their controls. Decreases of 7.03%, 36.44%, and 15.18% were determined respectively in 100 μM, 300 μM, and 1000 μM concentrations for İkizce hybrid wheat. Decreases of 46.96%, 49.24%, and 63.89% were determined respectively in 100 μM, 300 μM, and 1000μM concentrations for Tosunbey hybrid wheat. ($P \leq 0,05$) (Table 2).

Discussion

Bayraktar hybrid wheat had the highest increase of atrazine herbicide, in terms of GSH/GSSG ratios in the leaves of wheat seedlings (25, 11, 29). This result indicated that Bayraktar hybrid wheat was more exposed to stress and displayed a defense reaction to this. The highest decrease was observed in İkizce hybrid wheat for metolachlor herbicide (13). Therefore, İkizce hybrid wheat performed the weakest reaction against metolachlor. Glutathione reductase enzyme (GR) converts oxidized glutathione (GSSG) into reduced glutathione (GSH) with a reaction based on $NADPH^+$. Reduced glutathione is an important antioxidant which plays a role in the defense against oxidative stress and is not enzymatic. In other words, glutathione reductase activity increase is a result of oxidative stress. GSH and GR constitute the compounds of ascorbate-glutathione metabolism which plays a role in reacting to stress in plants (35, 36).

Table 2. The changes in GSH/GSSG ratio, SOD and CAT activities, and MDA amount in the leaves of metolachlor -treated *Triticum aestivum* L. seedlings.

Metolachlor Groups	GSH/GSSG	SOD activity (unite/g)	CAT activity (μg/g)	MDA ($nmol.g^{-1}$ TA)
B-Control	8,197±0,402	0,510±0,031	50,70±2,426	417,625±0,760
B-100 μM	7,364±0,403	0,420±0,016*	41,953±2,838*	369,293±3,825*
B-300 μM	6,960±0,230*	0,407±0,007*	40,627±2,202*	360,098±5,207*
B-1000 μM	6,688±0,040*	0,379±0,013*	38,232±1,316*	237,250±28,193*
İ-Control	6,579±0,321	0,388±0,031	42,120±0,884	427,625±7,701
İ-100 μM	5,311±0,149*	0,415±0,023	39,159±2,472	479,50±55,071
İ-300 μM	4,698±0,191*	0,450±0,006	26,772±0,530*	513,625±26,772
İ-1000 μM	4,143±0,223*	0,487±0,001*	35,727±1,107*	343,375±2,724
T-Control	5,025±0,330	0,436±0,018	38,236±0,481	157,125±4,330
T-100 μM	4,573±0,194	0,556±0,026*	20,280±1,427*	254,625±28,507*
T-300 μM	4,120±0,083*	0,605±0,002*	19,408±0,667*	281,377±4,473*
T-1000 μM	3,420±0,087*	0,577±0,016*	13,808±1,427*	157,125±5,691

*: Compared to the control group at $p \leq 0.05$ probability levels. Values are mean of three replicates ±SE (n:3) Bayraktar: B, İkizce: İ, Tosunbey: T

Atrazine herbicide causes the highest increase in Tosunbey hybrid wheat in terms of MDA amount in wheat seedlings (13, 15, 17, 26, 29, 37); whereas, a decrease was determined in İkizce wheat (25). Akbulut and Yiğit (2010) reported in their study conducted on maize (*Zea mays* cv. Martha F1) seedlings that Atrazine herbicide caused an increase in MDA amount when it was applied at low doses and it caused a decrease when it was applied at high doses (19). For metolachlor herbicide, the highest increase was observed in Tosunbey hybrid wheat (37, 17, 21, 30); on the other hand, a significant decrease was observed in Bayraktar wheat. Significant increases were determined in the studied groups in MDA amount, which is another important parameter demonstrating the oxidative stress intensity in the leaves and is the typical end product of the lipid peroxidation reaction in the cell membrane (17, 21, 30, 37). Also, MDA amounts decreased especially for the high herbicide doses. The increase in MDA level showed that the oxidative stress-related lipid peroxidation increased in biomembranes of plants (37).

In terms of SOD enzyme activity in the leaves of wheat seedlings, the highest increase was observed in Tosunbey hybrid wheat in atrazine (6, 12, 14, 28); whereas, a decrease was determined in Bayraktar and İkizce hybrid wheats. For metolachlor herbicide, the highest increase was observed in Tosunbey wheat (20); on the other hand, a decrease was determined in Bayraktar wheat (9, 10, 21). The antioxidant system has an important role in protecting cell components from the damages of reactive oxygen species produced under stress. The production of reactive oxygen species in plant cells is low under optimal growth conditions. However, the increase in the production and accumulation of reactive oxygen species in most of the environmental stresses brings about the deterioration of cell homeostasis (38). The SOD activity increasing under stress conditions showed that especially the superoxide radical reactive oxygen species are overproduced. This is because it has a role in removing superoxide radical from SOD chloroplasts and superoxide radical is converted into H_2O_2 (38, 39). Herbicide toxicity occurs when SOD activation, which increases significantly in the antioxidant system, gets involved (4).

In terms of CAT enzyme activities in the leaves of wheat seedlings, Tosunbey wheat had the highest decrease in Atrazine (13) and Metolachlor herbicides (20, 30, 28). Catalase is found in the organelles named peroxisome in all the plant cells and it keeps H_2O_2 (H_2O+½ O_2) level at a certain level for cell and plays a protective role (40, 41). Catalase enzyme detoxifies H_2O_2 at high concentration and leads plant to eliminate stress with the least loss. The increase in the antioxidant enzyme activities such as SOD is a result of the detoxification mechanism ensuring the decrease of lipid peroxidation (42-44). The oxidative damage in the seedling leaves increases SOD and decreases the other enzymes and it may be suggested that it causes an imbalance in the enzyme activities and the H_2O_2 increase in the chloroplasts is probably associated with it (45).

Conclusion

Consequently, it was determined that the synthetic Atrazine and Metolachlor herbicides were toxic for wheat plants even in very low concentrations. However, it was determined that atrazine caused more oxidative stress. Due to limited and insufficient number of studies, we could not discuss our results in a very large platform and sometimes we had to give the indirect studies as references. We think that new studies on this subject would contribute to fully understand the subject.

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