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The Effects of Heavy Metal Stress in Wheat Plant on Certain Phenolic Compounds

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ABSTRACT
Plants are exposed to stress conditions depending on the lack of adaptation when unfavourable conditions occur in their environment. One of these stress conditions is heavy metal pollution. Defence systems are activated in order to protect cells under stress conditions and keep the levels of reactive oxygen species (ROS) under control and to eliminate ROS. Phenolic compounds are effective compounds for eliminating and neutralizing free radicals. Therefore, this study investigates the effects of the short-term application (1 and 5 days) of arsenic, cadmium and lead (15µM, 30µM and 60µM mixtures) to a wheat species that was registered by Trakya Agricultural Research Institute in 2014 on phenolic contents.

Key words: Wheat, Phenolic compounds, Arsenic, Cadmium, Lead.

Introduction

Cereal products, which play an important role in the economy of our country, have great importance in human nutrition(GÜLEÇ et al., 2010).

Cereals are used as feed in the feeding of animals and as a basic ingredient in the production of many industrial products in addition to being the raw material of bread, which is one of the essential nutrients of human beings(Singh et al., 2008).

It is also one of the most consumed nutritional sources due to its high protein content. Therefore, widespread cereal production is performed in the world to meet the nutritional needs of people (Karaoğlu & Kotancılar, 2001). Wheat, which belongs to the genus *Triticum* in the *Gramineae* family, is one of the most used and cultivated culture plants by people in the world as a basic food product.While cereal products constitute around 50% of the cultivated areas in our country, 70% of these areas constitute wheat (Yavuz, 2005). Living beings are constantly in contact with the external environment by their nature. They are exposed to stress conditions due to the lack of adaptation in case of inappropriate conditions in the environment (Büyük et al., 2012).

Stress is defined as a condition that occurs in the plant when the environmental conditions change to an extent that normal growth and development of a plant will be affected in a negative way (Büyük et al., 2012). Nowadays, especially with the development of the chemical industry, chemical substances have been diversified, and 7600 of approximately 9 million chemical substances are used in daily life today. Heavy metals, which cause heavy destructions in the ecosystem, take an important place among these chemical substances.

As a result of increasing environmental pollution due to developing technology and industrialization, contamination of food sources, which are essential for people, has become a threat to human health. Some of the pollutants that are released to the environment in various ways cause accumulation in the living beings by passing to all living beings through the food chain (Nagajyoti et al., 2010).

Although some pollutants are present in small amounts in the first stages of the food chain, their intensities may increase in successive stages, and this is called biological accumulation. Plants tend to accumulate the pollutants transferred to the soil in various ways (through the atmosphere, organic fertilizers and agricultural pesticides, waste water, etc.), especially heavy metals, in the organism (MacFarlane et al., 2003).

Contrary to organic pollutants, heavy metals cause accumulation because they do not decompose in the soil, and when they reach high levels, they damage the living being as well as affecting the quality of the soil, thus causing the production amount and quality of the product to deteriorate (Wu et al., 2010). Defense systems are put into action to protect the cells under stress conditions in plants and to control the levels of Reactive Oxygen Species (ROS) formed

and to eliminate ROS. Phenolic compounds are compounds that are effective in eliminating and neutralizing free radicals (Šiukšta et al., 2019).

For this reason, in this study, how short-term application (1 and 5 days) of arsenic, cadmium, and lead (15µM, 30µM, and 60µM mixtures) affects phenolic substance contents was investigated on "Saban", a wheat variety registered by the Thrace Agricultural Research Institute in 2014.

Materials and methods

All seeds used in the study were subjected to surface sterilization in a 1.5% sodium hypochlorite solution for 10 minutes before being allowed to germinate in the Petri dishes. Surface-sterilized seeds were washed several times with distilled water(Peksen & Sanal, 2018).

In the same way, the Petri dishes to be seeded were sterilized in a Pasteur oven at 120 °C for 1 hour. The Petri dishes were prepared in such a way that each one contained 25 seeds.

Sterilized wheat seeds were placed between blotting papers, with each Petri dish containing 25 seeds, and photoperiod was applied at 20 ºC, and then the plant was allowed to germinate in the growth chamber. For the experimental groups, 15 µM, 30 µM and 60 µM concentrations of freshly prepared (As, Pb, Cd) metal mixture solutions were used. Distilled water was used as irrigation water for the control group.

The phenolic substance analysis

For the phenolic substance analysis, plant samples were homogenized in a mixture of isopropyl alcohol methanol (50%;50%). 0.5 gr stem was placed in Eppendorf tubes. Then, it was powdered with the help of liquid nitrogen, ball, and homogenizer. To these samples, 100 µl hydrochloric acid was added first, which was followed by 400 µl methanol/propanol/ultrapure water (1:1:1) mixture, and then homogenized for 1 minute using a vortex.

Afterwards, the samples were incubated in the mixing block for 30 min at 25 °C and for 10 min at 70 °C. After centrifugation at 10,000 rpm for 15 minutes, the supernatant was filtered with a PTFE filter with a pore diameter of 0.22 µm and transferred to amber vials. Phenolic substance analyses were made with LC/QTOF and LC/MS/MS.

The results obtained were qualitatively controlled, and the results were then added to the calibration graphs in the Mass Hunter QQQ Quantitative Analysis program in order to calculate the quantitative analysis results based on the areas of the peaks.

In the analysis, a Raptor Biphenyl 5 µm (50 x 2.1 mm) column was used, and the column oven temperature was set at 40 °C. The mobile phase A was created with ultrapure water, 2% formic acid, and the mobile phase B was created with methanol, 2 mM ammonium acetate, 1 mM ammonium formate mixture. The flow rate is 0.7 ml/min, method time is 10 min, gradient profile (min/A%) is (0/100), (1.5/100), (3/10), (4/2), (7/2), (8/100), (10/100) and the injection volume is 5.0 µl. The gas temperature was set at 325 °C, nebulizer gas pressure was set at 45 psi, capillary voltage was set at 3000 V, sheath gas temperature was set at 400 °C, sheath gas flow was set at 11 L/min, and Collision gas Nitrogen (N^2) and pressure was set at 1.12 m Torr. The MRM (Multiple reaction monitoring) mode was studied as positive and negative. Collision energy, fragmentor voltage, fragmentation ions among MS parameters were optimized using the Optimizer Program.

Statistical analyzes were performed according to Student's t-test. Each group was compared with its own control group.

Results

Reduced glutathione levels increased at 15 µM and 60 µM doses on day 1 and day 5 compared to the control group, while a decrease was observed at 30 µM dose on the 1st day. However, on day 5, it is still higher compared to the control group (Table 1). It was observed that gallic acid levels increased on day 1 due to the dose, and on day 5, the increase was only at 30 and 60 µM doses (Table 2).

Upon examining the benzoic acid levels, it was observed that the levels at all doses decreased on day 1, and while there was a decrease at 15 µM dose, there was an increase again at 30 µM dose and a decrease at 60 µM dose by 85% compared to the control on day 5 (Table 3). An increase of 98% was determined only at 30 µM dose on day 5, while there was a decrease at all doses on day 1 and day 5 in Protocatechuic acid levels (Table 4). On day 1 and day 5, while the catechin values were unchanged at 15 µM doses, there was an increase at 30 and 60 µM doses (Table 5).

On day 1, Trans Caffeic Acid levels were a decrease at all doses compared to the control group due to the dose, and a rapid drop at 15 µM dose on day 5 was followed by a rapid rise with a dose increase (Table 6). While there was no change in the 15 µM dose application on day 1, a slight decrease was observed with a dose increase.

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Table 1. *Reduced glutathione levels.*

Reduced glutathion (ppb)	**1st Day**		
Control	**15 mM**	**30 mM**	**60 mM**
19.8397	21.2894	18.6556	21.0639
5 th Day			
11.1770	21.2894	18.6556	21.0639

Values are the mean of the triplicated experiments.

Table 2. *Gallic acid levels.*

Gallic acid (ppb)	**1st Day**		
Control	**15 mM**	**30 mM**	**60 mM**
0.3798	0.9062	0.7671	3.9295
5 th Day			
0.6563	0.5799	0.8186	0.7810

Values are the mean of the triplicated experiments.

Table 3. *Benzoic acid levels.*

2,5-Dihydroxybenzoic Acid (ppb)	**1st Day**		
Control	**15 mM**	**30 mM**	**60 mM**
15.6006	8.6304	8.3095	8.9763
5 th Day			
4.6694	3.7970	6.5114	0.6851

Values are the mean of the triplicated experiments.

Table 4. *Protocatechuic acid levels.*

Protocatechuic acid (ppb)	**1st Day**		
Control	**15 mM**	**30 mM**	**60 mM**
19.0870	16.9218	18.3241	14.5460
5 th Day			
7.4231	6.1166	12.5035	6.1063

Values are the mean of the triplicated experiments.

Table 5. *Catechin Levels.*

(Catechin) (ppb)	**1st Day**		
Control	**15 mM**	**30 mM**	**60 mM**
32.2121	32.5184	35.3295	35.4816
5 th Day			
15.5381	15.8393	19.2603	16.9540

Values are the mean of the triplicated experiments**.**

Table 6. *Trans Caffeic Acid levels.*

(Trans Caffeic Acid) (ppb)	**1st Day**		
Control	**15 mM**	**30 mM**	**60 mM**
1215.5744	1178.8307	917.6194	1009.6944
5 th Day			
544.8672	61.7350	471.4627	749.2043

Values are the mean of the triplicated experiments.

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On day 5, a decrease in chlorogenic acid levels was observed at 15 and 30 µM doses, while values in the control group were reached in the 60 µM dose application (Table 7). On day 1, there was a decrease in the 15 µM dose application and an increase at 30 µM dose and a decrease at 60 µM dose, and on day 5, the amounts of transcumaric acid decreased considerably in the 15 µM dose application. They were determined to be higher at 30 µM and 60 µM doses than the control values (Table 8). Transferrulic acid increased at all doses on day 1 and day 5 depending on the dose (Table 9). The highest increase on both days was observed at 15 µM dose, and at 30 and 60 µM doses, the indole 3 acetic acid levels decreased and came close to the control group values. However, they are still higher than the control (Table 10).

On the 1st day of the application, salicilic acid levels increased depending on the dose, but no significant change was observed on day 5 (Table 11).

Table 7. *Chlorogenic acid levels.*

(Chlorogenic Acid) (ppb)	**1st Day**		
Control	**15 mM**	**30 mM**	**60 mM**
27.7689	27.4493	25.3519	26.9363
5 th Day			
13.0062	10.9606	12.7073	13.5643

Values are the mean of the triplicated experiments.

Table 8. *Transcumaric acid levels.*

(Transcumaric acid) (ppb)	**1st Day**		
Control	**15 mM**	**30 mM**	**60 mM**
134.9263	100.8294	153.2374	123.1339
5 th Day			
70.0894	13.8836	93.3819	78.9054

Values are the mean of the triplicated experiments.

Table 9. *Trans Ferrulic acid levels.*

(Trans Ferrulic acid) (ppb)	**1st Day**		
Control	**15 mM**	**30 mM**	**60 mM**
77983.1400	7981.8761	79409.0945	87603.53
5 th Day			
639.3858	754.2079	686.5870	826.2434

Values are the mean of the triplicated experiments.

Table 10. *Indol-3-acetic acid levels.*

(Indol-3-acetic acid) (ppb)	**1st Day**		
Control	**15 mM**	**30 mM**	**60 mM**
2.6144	3.2257	2.5641	2.9816
5 th Day			
0.4903	4.7499	1.3993	0.8811

Values are the mean of the triplicated experiments.

Table 11. *Salicilic acid levels.*

(Salicilic acid) (ppb)	**1st Day**		
Control	**15 mM**	**30 mM**	**60 mM**
35.4820	38.9806	40.4517	41.9764
5 th Day			
19.8853	19.1214	19.8510	19.1756

Values are the mean of the triplicated experiments.

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Trans-sinapic acid decreased on day 1 at all doses, while regression was observed in the 30 µM and 60 µM dose application on day 5, still being higher than the control group (Table 12). While there was no significant change in absisic acid levels on day 1, a dose-dependent decrease was observed at all doses on day 5 (Table 13). Compared with the control group, a slight increase was observed at all doses on day 1 in the Luteolin levels, whereas there was a decrease at all doses on day 5 (Table 14). Following a slight increase at 15 µM dose on day 1, the levels of jasmonic acid decreased by half at 30 and 60 µM doses. On day 5, no change was observed at 15 µM dose, while the levels decreased significantly at 30 and 60 µM doses (Table 15). Naringin increased at all doses on day 1, and there was no change at 15 µM dose on day 5, while a dose-dependent increase was observed at 30 and 60 µM doses (Table 16).

Table 12. *Trans-sinapic acid levels.*

(Trans-sinapic acid) (ppb)	**1st Day**		
Control	**15 mM**	**30 mM**	**60 mM**
544.5512	444.3694	386.6642	488.1015
5 th Day			
106.4077	590.4320	726.0999	225.7222

Values are the mean of the triplicated experiments.

Table 13. *Absisic acid levels.*

(Absisic acid) (ppb)	**1st Day**		
Control	**15 mM**	**30 mM**	**60 mM**
19.5324	18.7744	20.2904	17.7986
5 th Day			
10.8545	9.5573	8.3953	8.1648

Values are the mean of the triplicated experiments.

Table 14. *Luteolin levels.*

(Luteolin) (ppb)	**1st Day**		
Control	**15 mM**	**30 mM**	**60 mM**
8.1871	8.5849	8.7324	8.6156
5 th Day			
5.2829	3.7899	3.3821	3.4254

Values are the mean of the triplicated experiments.

Table 15. *Jasmonic acid levels.*

(Jasmonic acid) (ppb)	**1st Day**		
Control	**15 mM**	**30 mM**	**60 mM**
43.2784	45.0399	24.1307	20.3251
5 th Day			
12.1273	12.4054	7.7830	4.9449

Values are the mean of the triplicated experiments.

Table 16. *Naringin levels.*

(Naringin) (ppb)	**1st Day**		
Control	**15 mM**	**30 mM**	**60 mM**
3.5327	4.7435	4.5935	3.8392
5 th Day			
2.8894	2.8335	3.4609	4.4749

Values are the mean of the triplicated experiments.

Discussion

Phenolic compounds have various functions in plants. Changes may be observed in the amounts of these compounds under different environmental conditions and stress conditions. Isoflavones and some other flavonoids are stimulated when plants are injured or infected, while stress conditions such as low temperature, low nutrients or heavy metal exposure (Michalak, 2006).

Biosynthesis of phenolic compounds has been reported to be stimulated with the effect of nickel toxicity in the wheat plant, aluminium in the corn plant, and cadmium in the bean plant. The changes in the metabolism of phenolic compounds are thought to occur as a result of hydrolysis of conjugates under heavy metal stress. It is stated that the increase in soluble phenolic compounds is necessary for the formation of a physical barrier which prevents the entry of heavy metals into the cell by increasing the cell wall resistance (Bubna et al., 2011).

Furthermore, many studies have shown that phenolic compounds respond to heavy metal stress as a secondary metabolite. Boron toxicity in the tomato plant (Cervilla et al., 2012; Elguera et al., 2013) resulted in an increase in the amount of phenolic compounds, while it has been reported that cadmium treatment in Erica andevalensis leaves affects the total amount of phenolic compounds and it increases in the corn plant depending on the dose (Kısa et al., 2016).

In our study, it was observed that there were significant changes in the amount of phenolic acids due to heavy metal stress in order to adapt to the environment and this effect was more significant in long-term treatment with high dose. Similarly, Elguera et al. (2013) reported that cadmium treatment in *L. sativum* reduced the amounts of chlorogenic acid, ferulic acid, and caffeic acid (Elguera et al., 2013).

On the other hand, in vitro studies have shown that phenolic compounds can directly clean reactive oxygen species formed by heavy metals göstermiştir(Michalak, 2006). This suggests that they are part of the natural defense system of the plant. In our study, significant changes in phenolic compounds as a result of increased dose and duration of heavy metal exposure are an important proof of this. In conclusion, phenolic compounds, which are part of the plant's defense system, were significantly affected by heavy metal pollution.

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