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Olive Oil Mill Wastewater Triggers Hormonal And Phenolic Metabolism Shiftings In Sunflower

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Abstract

The aim of the study was to investigate the effects of olive oil mill wastewater (OOMW) in the sunflower in terms of hormonal and secondary metabolites (phenols). In all experiments as hormone, abscisic acid (ABA), gibberellic acid (GA), indolacetic acid (IAA), salicylic acid (SA) and jasmonic acid (JA), as phenol, Naringenin (NAR), Catechin (CATEC), Trans sinnapic acid (SINAP), Trans p-coumaric acid (PCOUMAR), Protocatechik acid (PROTOC), Trans cafeik acid (CAFFE), 2-5 Dihidro benzoic acid (DYHIDRO), Gallic acid (GALLIC), values were compared in control and experimental groups. OOMW was applied to the plants at various concentrations (1/1, 1/10, 1/100, 1/1000, 1/10000) for 3-days, 5- days and 10-days. Control plants are watered with water. Based on hormonal analysis; the most OOMW damage was in 5-day treatments, and in 10-day treatments it was partially healed. Similarly, 5-day changes in phenolic analyzes were found to be more severe. Consequently; it has been found that 1) SA is the most active hormone against OOMW stress, 2) In the 5-day trials, JA was active in conjunction with the SA, which was based on OOMW violence, similarly, hormones and phenolic substances are highly variable especially in the 5-day trial, 3), GA and IAA and ABA are generally quite lower levels in all experiments, 4) OOMW breaks hormonal balance in the plant, and 5) the secondary metabolite (phenol) metabolism has been changed considerably.

Keywords: Olive oil waste waters, Sunflower, Toxicology, Plant hormone, Phenolics, Seconder metabolites

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1. Introduction

Olive oil in the world is mostly produced in the Mediterranean Region, Southern Europe, North Africa and the Middle East, and therefore olive oil production is becoming increasingly important both in terms of socioeconomic and environmental aspects [1]. Because almost 98% of the world's olive trees are in the Mediterranean Basin [2,3], and

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almost 80% of the world's olive oil production is derived from about 12000 facilities in European countries [4]. Therefore, in the very short period between November and March, the high amount of waste causes large pollution in air, water and soil if it is not pretreated[3]. Because olive oil mill wastewater (OOMW) is rich in polyphenols, low pH, various heavy metals, minerals, and needs high chemical and biological requirements for its dissolution. If the OOMW spreads to the soil without pretreatment and high amounts, it both affects the physical, chemical and biological properties of the soil in a negative way and damages the plant [5]. Even after ten years since the OOMW was thrown into the environment, it causes development disorders in the vegetation and heavy metal pollution [5]. Therefore, different concentrations of OOMW in chickpea, durum wheat, tomatoes and corn prevented the germination [6]. Even 1/8 dilution of OOMW significantly reduces germination rate [7]. In addition, OOMW changes the soil structure with high salt concentration in its contents, and carries potential damages for plant growth [8].

There are different studies on the cytotoxic effects of OOMW in plants. For example; In *Vicia faba*, OOMW causes chromosomal abnormalities, root tip darkening, micronuclei, as well as rootstock and mitotic inhibition [9]. In a study conducted by Aybeke *et al*., [10], numerical or structural chromosomal mutations, mitotic abnormalities and increased mitotic frequency, as well as highly nuclear or broken nucleated cells in wheat root tip were emphasized. It was also observed that the amount of protein decreased with increasing concentration and duration of treatment [10]. In ultrastructural study, which are the continuation of this study, emphasize that OOMW causes wall and nuclear damages, stoplasmic membrane and cellular organization disorders in wheat stem meristem cells [11]. Finally, another study by Aybeke [12], found that OOMW caused damage to the DNA genomic structure and DNA structure it was also stated that all OOMW applications had a free radical threat and that there was more damage, especially in the 5-day OOMW tests.

Despite all of these morphological, cytological, agronomic, genotoxic and cytotoxic findings mentioned above, no information was available on how the OOMW actually affected the metabolism of plant hormones and secondary metabolites (phenols) during the application to plants. How does this waste, which is so toxic indeed, affect the mechanisms of plant hormones and secondary metabolite (phenol)? Therefore, in this study, it is aimed to investigate the effects of OOMW on plant hormones and phenol metabolism in sunflower.

2. Material and Method

2.1. Material

Material used as plant, HA 89-B cultivar of *Helianthus annuus*. OOMW has been supplied from olive oil production factories in the villages of Iznik (Bursa, TURKEY).

2.2. Chemical Properties of OOMW

The chemical properties of OOMW are given in Table 1. For this works, Agilent 7700 xx ICP-MS machine used. NPs measurements were performed using this instrument (Agilent 7700xx ICP-MS). The OOMW example is sent directly to the device into the ICP-MS system by means of the standard peristaltic pump combined with Tygon pump tubing (internal diameter of 1.02 mm), and ASX-520 autosampler [13].

Polyphenolic amount of OOWW was assigned by the Folin-Ciocalteu (FC) method, following Box [14] and Li et al.'s [15] method. 200 µL of extracted plant material was included into 1 mL of 1:10 dH_2O-diluted Folin-Ciocalteu fluid. After about 4 minutes, 800 µL of saturated Na_2CO_3 liquid added to them. After nearly 1 hour, absorbance values were determined at 760 nm compared to a matrix blank, via Specord 50 UV/VIS spectrometer (Cary 50 Bio, Varian) (Analytic Jena, Jena, Germany). In order to find the sensitivity of the FC reagent to different phenols, calibration curves were made with various phenols such as caffeic acid, tyrosol, p-coumaric acid and gallic acid. Data are given in mg p-coumaric acid and caffeic acid content per OOWW liter.

Table 1. Principal chemical properties of OOMW

Mineral	ppm(=mg/l)*	std.error	Mineral	ppm(=mg/l)*	std.error
K	4908,94	42,687	Li	0,159	0,013
B	26,578	0,182	V	0,031	0,002
Na	170,715	0,584	Ga	0,150	0,008
Mg	188,640	0,682	As	0,090	0,005
Ca	25,851	0,774	Se	0,245	0,005
F	44,653	0,601	Sr	0,247	0,010
Al	4,932	0,186	Ag	0,284	0,063
Mn	2,474	0,038	Cd	0,052	0,001
Cu	3,950	0,026	Sb	0,086	0,004
Zn	3,764	0,022	Ba	0,155	0,007
Co	0,040	0,001	Pb	0,901	0,005
Ni	0,423	0,006			
Cr	0,131	0,001			
Polyphenols					
Pcoumaric acid	5,7 g L^{-1}	0,23			
Caffeic acid	9,01g L^{-1}	0,03			

*: indicate average values

Sunflower seeds will be germinated on the violets in special and standard Klasmman TS1 Torf after after being inflated in water. Approximately 18-25 days of sunflower seedlings, were irrigated for 3-days, 5-days and 10- days, at concentrations of OMWW, 1/1 (pure), 1/10, 1/100, 1/1000, 1/10000; contrastly control group (OOWW-free), just watering with tap water. All experiments were carried out in accordance with sunflower living conditions in greenhouse [16]. On days 3, 5 and 10 the leaves were taken from the plants and directly immersed into liquid nitrogen.

2.3. Hormonal Analysis

200 mg frozen sunflower tissues from the control and experimental groups (OOMW treated) were treated with the Qiagen Tissuelyser LT[17] with partially modified extraction method[18]. After lysis for 2 min, 100 mg of pellet was joined with 1 ml extraction solvent [isopropanol / methanol, 50:50 (v/v) with 0.5% of ammonium formate]. This concoction was vortexed rapidly under freezing conditions and then centrifuged at 10,000 rpm for 10 min at 4°C (Bioer Mixing Block MB-102). The supernatant was filtered through a 0.22 µm PTFE filter. Extracted samples (200 µl) were analyzed by UPLC-ESI–MS/MS (API4000 QTrap; Applied Biosystems).

For determination of principal hormonal activity, standards of each hormone (abscisic acid = ABA; gibberellic acid = GA, indolacetic acid = IAA; salicylic acid = SA; jasmonic acid = JA) were uploaded onto the MS/MS system by adapted to several fragments and voltage conditions. Thus, through experiments at five different concentrations from 0.05 to 50 µg / kg, the calibration curves of these hormones were constituted using Analyst software (Applied Biosystems). A 50 µl of sample was analyzed by UPLC-ESI/MS–MS and Spark UPLC system combined with an Applied Biosystems QTRAP 4000. Chromatographic separation was performed on a Phenomenex Luna 3 lm C18(2) 100 9 2.0 mm column at 40 °C. The solvent gradient used was 100% A (99.5% H_2O:0.5% ammonium formate) to 100% B (99.5% MeOH:0.5% ammonium formate) over 5 min. The gradient profile for hormones was constructed as follows: ((time in min)/A %): (0/98), (1/2), (3/2), (4/98), (5/98). Hormone analyzes were performed with a Turbo ion spray source in negative ion mode with MRM options in the Analyst software. The curtain gas was set at 10 a.u., the source temperature was 400 C, and ion source gases 1 and 2 were both 20 a.u. The declustering potential was set at 100 V. The source voltage was 3500 V [18].

2.4. Secondary Metabolite Analysis

Chemicals, Standards and Reagents: Formic acid (98–100%), methanol (Hypergrade LC MS), isopropyl alcohol (2-propanol) and DMSO were purchased from Merck, Darmstadt, Germany. Ammonium formate (HPLC grade) was from Sigma–Aldrich, Germany. Reference standards, gallic acid, catechin, 2-5 dihydroxybenzoic acid, trans-caffeic acid, syringic acid, trans-sinapic acid, trans-p-coumaric acid, trans-ferulic acid, resveratrol and salicylic acid were from Fluka and protocatechuic acid was purchased from HWI Analytik Gmbh, Germany. MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo-lium-bromide) was purchased from Biomatik Cambridge, Ontario. Phosphate-buffered saline (PBS) and molecular biologygrade water were from Gibco, Invitrogen, Carlsbad, CA, USA. The resistance of ultra-distilled water used for instrumental analysis was 18.2 ΩX.

2.5. Liquid Chromatography and Mass Spectrometry Conditions

Phenol analyzes was performed on an Agilent 1200 infinity LC in combination with the Agilent 6460 Triple Quadrupole MS/MS System, equipped with a Jet Stream Electrospray ionization source (Agilent Technologies, Palo Alto, CA, USA). The analytical column was

Agilent Poroshell 120 EC-C18 (4.6 9 50 mm, 2.7 µm particle size) and set at 25 °C. Mobile phase A consisted of UPW, 0.2% ammonium formate (v/v), 0.2% formic acid (v/v). Mobile phase B consisted of methanol, 0.2% ammonium formate (v/v) and 0.2% formic acid (v/v). The flow rate was 0.3 ml/min at ambient temperature. The injection volume was 1 µl and the LC gradient conditions were as follows: 0–1 min,70% A, 30% B; 3–7 min. 30% A, 70% B; 9–10 min. 50%A, 50% B; 11–12 min; 70% A, 30% B. The run time was 12 min. The optimized MS analyzes parameters were as follows: gas temperature was set at 325 °C, the nebulizer gas pressure was set at 45 psi, the nozzle voltage was set at 500 V, the capillary at 3000 V, sheath gas temperature at 400 °C, sheath gas flow at 12 L/min. Multiple reaction monitoring (MRM) was performed on the positive and negative ion mode. Data acquisition was performed with Mass Hunter (version B.06.01) software. Nitrogen (N2) was used as the collision gas at 1.12 mTorr. Calibration standard mixes were prepared in 50% UPW, 25% methanol and 25% isopropanol at calibration concentrations of 1–200 ng/ml.

2.6. Statistical Analysis

All experimets were recurred freely three times and differences in data of hormonal, phenolic tests of control and test groups were compared by ANOVA, which means separation by Duncan's test using SPSS 18 software at a significance level of $p \leq 0.05$.

3. Results

3.1. Hormone analysis

3.1.1. 3-day Trials

GA and IAA values are higher than control. Other concentrations of GA are less than control; whereas in the IAA, other values outside of 1/1 are higher than the control (Figure 1).

In ABA and SA, the 1/1 concentration value is the largest, and as the concentration decreases, the other values decrease relatively. However, unlike ABA, SA, 1/10 and 1/100 values are higher than the control. JA; 1/1 concentration value is the largest and the other values are lower than the control. GA, IAA, SA and JA were significantly different from control (Figure 1).

3.1.2. 5-day Trials

In GA 1/1 value, highest in other concentrations. Other concentration values are close to or slightly larger than the control. In the IAA, the greatest values are 1/10 and then 1/1000; others are equal to or less than the control. IAA and GA show reversal activity. For example, 1/10 in GA is slightly larger than control, while 1/10 is the largest in IAA (Figure 1).

The ABA 1/1 is the largest and the others are close or small to control. In JA, the greatest value is 1/1; as for 1/10, 1/100 and 1/10000 are bigger than the control (Figure 1).

In SA, 1/1 and 1/10000 are the largest values. Otherwise (1/10, 1/1000) are lower or slightly higher than the control. In addition, in stress-related hormones such as SA, JA, and ABA 1/1 concentration value were the greatest, and as the concentration decreases, hormone levels decrease. The SA and JA hormone levels are close to or greater than the control, unlike the ABA. According to statistical tests, only the JA and SA hormones are significant compared to the control (Figure 1).

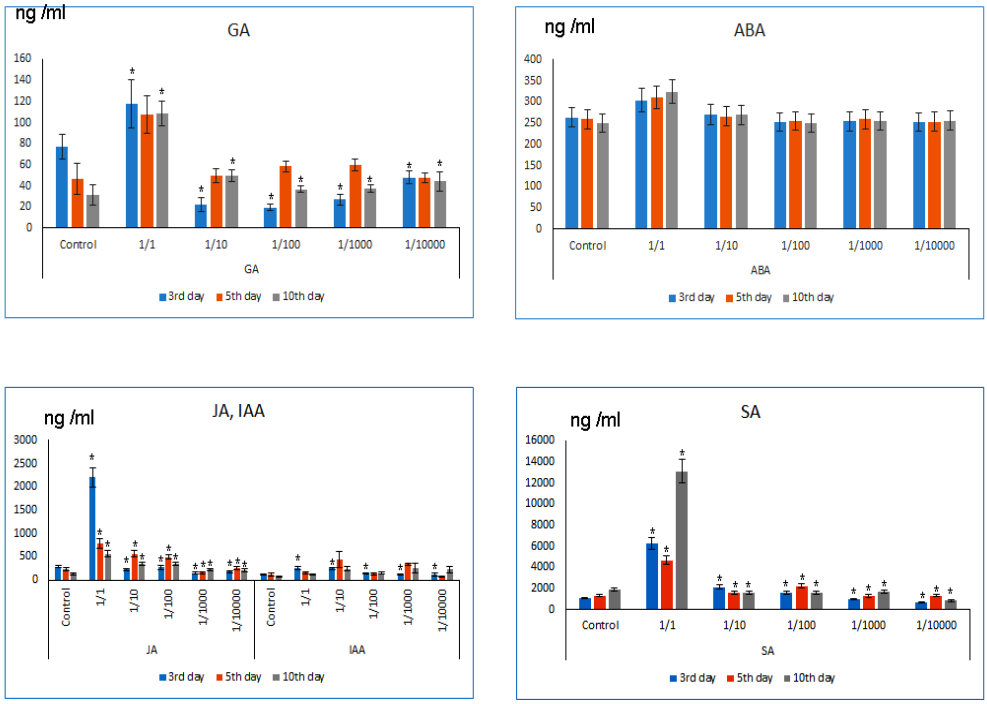

Figure 1. Hormone levels in control and all experimental groups (3-day, 5-day, 10-day). GA:gibberellic acid, ABA:abscisic acid, JA:jasmonic acid, IAA:indoleacetic acid, SA:salicylic acid. Standard error data were given as bars on colons. Asterisk on the colons express significant differences between control and experimental groups, based on oneway ANOVA and Duncan's test ($p \leq 0.05$) for 3-day experimentals: GA; F= 12,129, df= 5, p= 0.000. ABA; F= 0,745, df= 5, p= 0,605. JA: F= 87,507, df= 5, p= ,000. IAA:F= 9,543, df= 5, p= 0,001. SA: F= 70,239, df= 5, p= 0,000.

Statistical data of 5-day experimentals. GA; F= 4,889, df= 5, p= 0.011. ABA; F= 0,826, df= 5, p= 0,555. JA: F= 17,532, df= 5, p= ,000. IAA:F= 3,473, df= 5, p= 0,036. SA: F= 39,665, df= 5, p= 0,000.

Statistical data of 10-day experimental groups. GA; F= 13,866, df= 5, p= 0.000. ABA; F= 1,522, df= 5, p= 2,555. JA: F= 14,568 df= 5, p= ,000. IAA:F= 1,677, df= 5, p= 0,214. SA: F= 98,054, df= 5, p= 0,000.

3.1.3. 10-day Trials

The greatest value in GA is 1/1, and the GA value decreases as the concentration decreases from 1/10 to 1/1000. In IAA, the 1/1 value is the lowest while all the other concentrations are higher. All concentrations of GA and IAA are higher than the control. ABA increased at 1/1, but decreased at other concentrations or close to control (Figure 1).

JA; the greatest value is 1/1, and the hormone values are decreased in proportion to the concentration; but still all concentrations are higher than the control. In SA, 1/1 was quiet than control; but decreased in other concentrations. In ABA, JA and SA, in common, value of 1/1 is the greatest value, and values decrease as the concentration decreases. GA, JA and SA hormones are significantly different from control (Figure 1).

3.2. Phenolic Analyzes

3.2.1. 3-day Trials

NAR: The largest NAR value is 1/1. At all other OOMW concentrations the values are very lower than control.

CATEC: The maximum value is 1/10, all other concentration values are lower than the control.

SINAP: With the greatest value being 1/1, almost all concentrations have values close to each other.

PCOUMARIC: 1/1 değeri en büyüktür. Diğer konsatrasyonları kontrolden daha düşüktür (Figure 2).

PROTOC: All concentration values are lower than the control. Likewise, all CAFFE values decrease as the concentration decreases.

DHYDRO: In all concentrations, except 1/10, values are lower than the control, and generally decrease in proportion to the concentration.

GALLIC: the highest concentration value is 1/1000 and then it is 1/1. Other concentrations are equal to or higher than the control. According to statistical analysis, NAR, PCOUMAR, PROTOC, CAFFE, DHYDRO are significant (Figure 2).

3.2.2. 5-day trials

NAR: With the largest value being 1/1, all concentrations are lower than the control, In general, the values decrease as the concentration decreases.

CATEC: The maximum value is 1/1. Other concentrations are very close to or slightly lower than the control (Figure 2).

SINAP: The greatest concentration is 1/10000. Other concentrations, except 1/1000, are slightly larger than the control.

PCOUMAR: The largest concentration is 1/1. Except for 1/10 and 1/100, the values decrease as the concentration decreases.

PROTOC: The greatest concentration is 1/10. In addition, all concentrations, except 1/1, are higher than the control.

CAFFE: The maximum value is at 1/100. It follows 1/10000, 1/10 respectively (Figure 2).

DHYDRO: The greatest value is 1/10 concentration and the others are bigger than the control.

GALLIC: The greatest value is 1/1. Other values are too close to or below control.

Statistical significance was found in NAR, CATECH, PCOUMAR, PROTOC, CAFFE, DHYDRO, GALLIC (Figure 2).

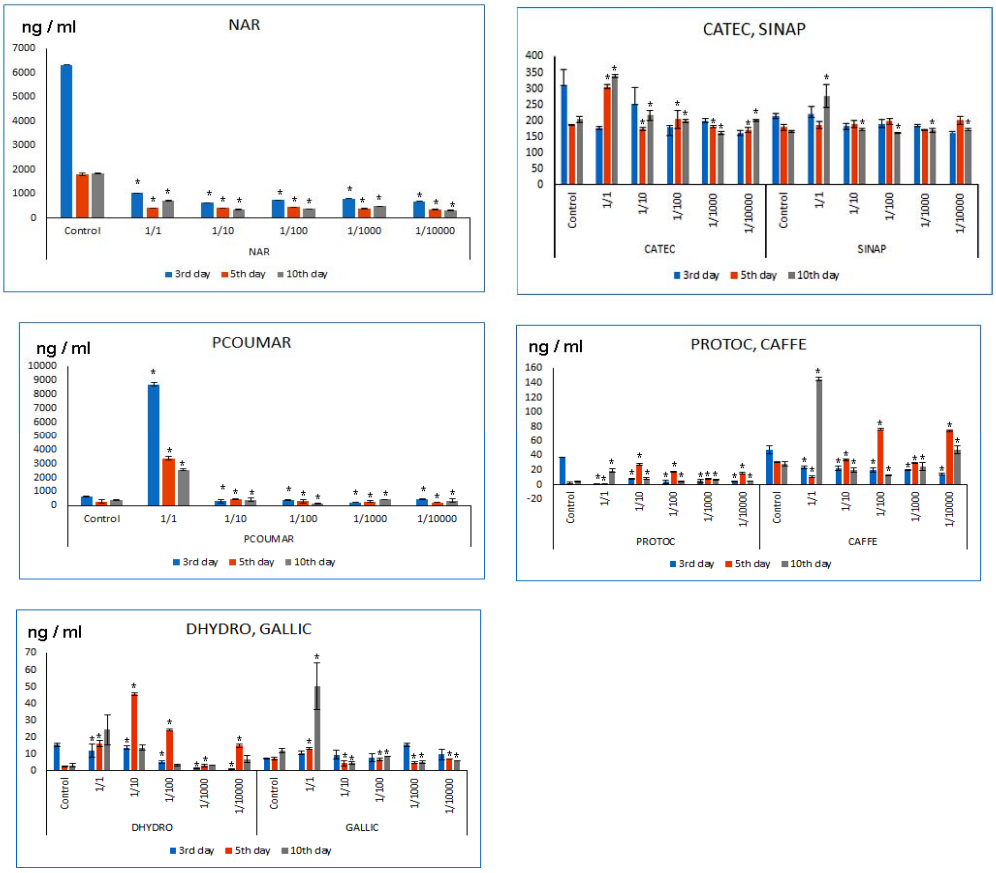

Figure 2. Fenolic amounts in Control and 3-day OOMW experimental groups. NAR: Naringenin, CATEC: Catechin, SINAP: Trans sinnapic acid, PCOUMAR: Trans p-coumaric acid, PROTOC: Protocatechik acid, CAFFE: Trans cafeik acid, DYHIDRO: 2-5 Dihidro

benzoik asit, GALLIC: Gallic acid. Standard error data were given as bars on colons. Asterisk on the colons express significant differences between control and 3-day OOMW experimental groups, based on oneway ANOVA and Duncan's test ($p \leq 0.05$). NAR: F= 3010,920, df= 5, *p*= 0,000. CATEC: F=3,807, df= 5, *p*= 0,027. SINAP: F: 2,944, df= 5, *p*= 0,058. PCOUMAR: F=2105,665, df=5, *p*= 0,000. PROTOC: F= 321,567, df=5, *p*= ,000. CAFFE: F=16,891, df=5, *p*= ,000. DYHIDRO: F=14,062, df=5, *p*= ,000. GALLIC: F=2,105, df=5, *p*= ,135.

Statistical data of 5-day experimentals: NAR: F= 920,484, df= 5, *p*= 0,000. CATEC: F= 16,569, df= 5, *p*= 0,000. SINAP: F: 1,489, df= 5, p= 0,264. PCOUMAR: F=238,323, df=5, *p*= 0,000. PROTOC: F= 54,110, df=5, *p*= ,000. CAFFE: F=79,438, df=5, *p*= ,000. DYHIDRO: F=279,303, df=5, *p*= ,000. GALLIC: F=14,571, df=5, *p*= 0,000.

Statistical data of 10-day experimentals: NAR: F= 1827,772, df= 5, *p*= 0,000. CATEC: F= 50,472, df= 5, *p*= 0,000. SINAP: F: 8,290, df= 5, *p*= 0,001. PCOUMAR: F=159,758, df=5, *p*= 0,000. PROTOC: F= 20,254, df=5, *p*= ,000. CAFFE: F=175,095, df=5, *p*= ,000. DYHIDRO: F=5,060, df=5, *p*= 0,010. GALLIC: F=9,798, df=5, *p*= 0,001.

3.2.3. 10-day Trials

NAR: The maximum value is 1/1; but the other concentration values are lower than the control.

CATECH: The maximum value is 1/1. Except 1/10000, the values decrease as the concentration decreases.

SINAP: The maximum value is 1/1. Other concentrations, except 1/100, are slightly larger than the control (Figure 2).

PCOUMAR: The maximum value is 1/1. The values of 1/10 and 1/1000 are also higher than the control.

PROTOC: The maximum value is 1/1. 1/10, 1/1000, 1/10000 are also bigger than the control.

CAFFE: The maximum value is 1/1. Except 1/10000, others are lower than the control (Figure 2).

DYHİDRO: The maximum value is 1/1. 1/10, 1/10000 are also bigger than the control.

GALLIC: The maximum value is 1/1 and all other concentration values are lower than the control.

Statistical significance was found in NAR, CATEC, SINAP, PCOUMAR, PROTO, CAFFE, GALLIC, in comparison to control (Figure 2).

4. Discussion

Plant hormones are important in generating responses against a wide range of stimuli, both internal and external [19]. For this reason, the effects of OOMW on plant hormones and phenols as secondary metabolites have been investigated.

In 3-day trials, GA, IAA, JA were significantly lower than the control except for 1/1 concentrations; there were not active. Likewise, SA was significantly more active than control at concentrations of 1/1, 1/10, 1/100, and values of 1/1000 and 1/10000 are lower. According to this, it is understood that only SA fight against OOMW stresse at 1/1, 1/10 and 1/100 concentrations. It is understood that other hormones (GA, IAA, JA) are not as important as SA in struggle with stress.

The second result is; there is not much harmful effect of OOMW at 1/1000 and 1/10000 concentrations. For this reason, we conclude that the SA hormone is significantly lower than the control at these concentrations. Interestingly, however, it is noteworthy that significant hormones such as GS and IAA, which indirectly function in stress struggle, are significantly lower than controls. Because it is suggested that the auxin promote the stress tolerance by regulating photosynthetic components and chloroplast structure especially later phase of the stress [20,21]. Similarly, GA is also functional in both plant development and many defense processes against stress throughout the plant life [22]. On the other hand; in 3-day OOMW trials, GA and IAA are also less active in terms of normal developmental activities. Therefore, it is understood that OOMW very interrupted IAA and GA's activities related to stress and development in the 3-day period.

However, in the 5-day trial, only the values of JA and SA changed significantly compared to the control. GA levels were close to or lower than the control, except 1/1. In contrast, IAA, is quite higher than the control at some concentrations. It is understood here that IAA is still partially active relative to GA, although not significant.

As for JA; it was significantly larger than controls at 1/1, 1/10, 1/100 and 1/10000 concentrations. It is noteworthy that the SA hormones vary significantly and in the largest dimension compared to other hormones, in the 5-day experimets. It is also understood that in the 5-day trial, damage is too great so that both of them (JA, SA) were activated, and therefore the balance of these 2 hormones changed quite positively. Because SA is known as an important stress hormone [23], and SA activity was related with expression of pathogen-associated protein genes as well as resistance to biotic stress [24,25]. Similarly OOMW also induced mainly SA activity and antioxidant metabolism in plants as described present data, which resembles SA's response to necrotrophic stress [23].

As JA; mechanical injury based herbivore invasion stimulates and accelerates JA activity in the broken tissue, and JA is an important hormone that induces defense to mechanical injury [26]. Additionnaly, Mazen and Lin [27], noted that there is an antagonistic relationship between defense hormones such as ABA, SA, JA, just like our results. In present data, despite JA and SA were significantly different compared to the control in all 3 trials (3-day, 5-day, 10-day), only SA (but partially JA, for 5-day experiments), was the

most active. In short, it is understood that OOMW stress is so severe as to activate both hormones. Indeed, in our preliminary genotoxic study results, it was emphasized that damage was more severe in the 5-day OOMW trial [12].

In 10-day OOMW trials, almost all of the GA values, except 1/1, were found to be significantly lower than control. The JA values were also lower than the 5-day JA values and significantly decreased than the control. Also, the SA values are lower than the SA values in the 5-day period. In short; It is noted that there is an imbalance in the GA hormone in the 10-day period, and that only the SA hormone is active, so that in the 10-day trial, the hormonal imbalances are part of an improvement, although the continuation of the hormonal imbalances. Therefore, the hormonal results in all 3-, 5- and 10-day OOMW experiments are in complete agreement with the results of the previous genotoxic article [12].

From phenolic results, it has been determined that NAR, PCOUMAR, PROTOC, CAFFE, DHYDRO are significantly different in 3-day experiments. It was understood that all of the phenolic values, except for the 1/1 concentration, of PCOUMAR, were significantly lower than the control. In short; OOMW has greatly reduced the production of phenolic substances in 3-day trials. Because, as described above, in 3-day experiments, OOMW significantly reduced the levels of the other 3 hormones (GA, IAA, JA), except for some concentrations of SA.

In 5-day OOMW experiments, the synthesis of 7 phenols, NAR, CATECH, PCOUMAR, PROTOC, CAFFEE, DHYDRO and GALLIC, significantly changed. However, in the 3-day experiments, almost all of 5 phenolic compounds were significantly lower than the control. However, in the 5-day trial, almost 4 hormones (CATEC, CAFFE, PROTOC, DHYDRO) were significantly more active than the control. Because genotoxic damage in 5-day OOMW trials is more severe than other tests (3-days and 10-days) [12] and accordingly the change in the amount / activity of the phenolics is also greater. These results also completely coincide with our results of hormonal studies. Because the share of phenolic compounds in antioxidant and stress relief is great. Additionnaly polyphenols, flavonoids and fatty acids are remarked to be oxidative stress-inhibiting effects. For example; GALLIC and CATECH exhibited a strong free radical scavenging activity[28]. Even phenol and flavonoids as secondary metabolites, are involved in the defense against biotic and abiotic stresses, and contribute significantly to the antioxidant activity of plant tissues [29].

In the 10-day OOMW experiments, significant changes were observed in the amount of 7 phenolic compounds, NAR, CATECH, SINAP, PCOUMAR, PROTO, CAFFE and GALLIC. All concentration values in NAR, SINAP, CATECH and GALLIC (except for 1/1), decreased significantly compared to control. In PCOUMAR, however, almost all concentrations, and some concentrations in CAFFE were significantly increased compared to the control. That is, in the 10-day OOMW experiments, in general the values of 4 phenols decreased, and increased in 3 phenols. In short; compared to the 5 day phenolic results, the damage was partially mitigated in the 10-day trials. In conclusion, all our hormonal results are consistent with our genotoxic and transcriptomic findings [12] and phenolic values.

Another notable finding is; in almost all experiments the 1/1 concentration reached almost peak values. From the data, it is understood how the pure (1/1) implementation of OOMW is harmful for plant; whereas control group plants and their roots have a healthier appearance. Also second striking result is; no significant change in ABA activity was observed in all experiments. Because ABA is a stress hormone that regulates responses to both biotic and abiotic stress [30]. Antioxidant gene expressions were particularly intensive in the 5-day OOMW trial [12], so ABA could be expected to be active in OOMW stress. However, according to our results, ABA was not active in any OOMW experiment. Also ABA has important functions in flavonoid / phenol synthesis and regulation of antioxidant response [31]. Similarly GA was significantly lower in the 3-day and 10-day experiments, compared to the control. In our opinion, OOMW quite effected balances between hormones and hormonal mechanisms.

In the content of OOMW (Table 1) it is seen that there are some elements and Polyphenols at high amounts [32]. These are B, F, Al, Mn, Ni, Cr, As, Se, Cd, Pb, all of which are heavy metals. Indeed; OOMW's heavy metal absorption capability is high; so heavy metal is very crowded at OOMW [33]. In the literature, there are many findings about toxic damages of heavy metals.

Regarding Al, it triggered oxidative stress [34], damages on plasma membranes and organellar structures, and then caused apoptosis and plasmolysis [35].

Se has inhibit plant metabolism at especially high concentrations, except that at low concentrations where it has protective effect [36].

Pb, As, Cd locks photosynthesis, and, decreases chlorophyll ratio and even destroys the plasma membrane [37].

Cr, interrupts gene expression and enzyme activities, thus causing oxidative based several damages [38].

With respect to Mn, various toxic losses are observed when the amount in the plant is increased. However, these harms vary according to the plant, the variety [39].

Regarding polyphenols of OOMW, Sierra et al. [40] suggested that no more than 180 m^3 ha^{-1} OOMW per year must be applied to the soil. Then at higher amount applications of OOMW causes salinity stress on plant and adversely affect plant production insomuch as the application of OOMW to the soil has greatly reduced the biomass in lettuce [41]. Hence OOMW toxicity is directly linked to reduction of phenolics [42]. In short, OOMW contains a large number of toxic compounds and mineral / heavy metal content, posing a threat to the plant, especially in high concentrations.

Because, as seen from present results, OOMW causes significantly changes in hormonal balances, and secondary metabolite (phenol) metabolism. In other words, it is understood that the OOMW disrupts the hormonal balance of the plant, and it forcing it to have an intense defense metabolism in terms of genetics, transcriptomics [12], and physiologically (hormonal, secondary metabolic). Therefore, OOMW should be given at

lower doses to the soil and before plant sowing. It is likely that both the OOMW will be dissolved in the soil and the damage will be reduced the least[43]. In our future studies, we will investigate ways of using OOMW due to these intensive toxicological effects in the weed fight, which is a big problem in agriculture. Thus it is believed that the environmental damages will be reduced to a minimum, and new horizons will be open about disposal of waste water without harming nature.

5. Conclusion

In the study, the effects of OOMW on sunflower hormones and phenolics were investigated. As a result of hormonal and phenolic analyzes; especially in the 5-day experiments, it is understood that the hormonal and phenolic changes are more so that the damage is higher. Damages and hormonal / phenolic changes are comparatively less in 3- and 10-day treatments. The most effective hormone against OOMW stress is SA. Especially in 5-day experiments JA also was active together with SA. Likewise, the most phenolic synthesis has been done in 5-day experiments. OOMW reduced IAA, GA and ABA hormones in almost all experiments. In short, OOMW degrades hormonal balance and phenol metabolism in the plant.

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