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Genotoxic effects of olive oil wastewater on sunflower

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ABSTRACT

The aim of this study is to determine in detail the genotoxic effects of Olive Oil Wastewater (OOWW) on sunflower. For this reason, different concentrations of OOWW (1/1,1/10,1/100) were applied as irrigation water to sunflowers at different times (3-day, 5-day, 10-day). In the plants taken during these times, RAPD-based genomic template stability (GTS) assays and gene expression (transcriptomic) levels of different free radical scavenging enzyme genes (SOD, CAT, SOD2, GST, GPX, APX), protein repair/chaperoning genes (HSP26, HSP70, HSP83), N metabolism gene (GS) and apoptotic genes (BAX, BCL2, BCLXL, CYT-C, XIAP) were compared to the those of the control (OOWW-free) group. As a result; The GTS rates seemed to be fairly lower than the control and therefore the OOWW was likely to cause significant damage to the DNA's nucleotide and genomic structure, and the GTS value increased inversely proportional when the OOWW concentration was reduced from 1/1 to 1/10, and after a 10-day application, it seemed to be partly healing. In transcriptomic analysis; all OOWW experiments caused a free radical threat, and especially in 5-day OOWW applications, this raised significantly almost all expressions of antioxidants, protein repair, N metabolism, and apoptotic genes. So, the damages of 5-day OOWW treatments were found to be relatively more than those of 3-day treatments. Regarding 10-day transcriptomic data; a partial repair was found. Additionally, it was determined that the values of B, F, Al, Mn, Ni, Cr, As, Se, Cd, Pb and total polyphenols were high in OOWW. Our findings were also supported by plant images and various heavy metals' and OOWW polyphenols' toxicity results. Our results pointed to key findings in OOWW genotoxicology.

1. Introduction

Olive oil waste water (OOWW) causes major problems for producer countries. The reason for this is the emergence of a large amount of waste in a very short period of time (Barbera et al., 2013). For example, in the olive oil factories in Turkey, 0.7×10^6 t of oil has been obtained according to the data of 2011, and this corresponds to approximately 5% of the world's production (FAO, 2011). Such a high quantity of OOWW production also brings significant problems such as necessity of large quantities of water and the question of how these wastes will be disposed of as "waste" after production (Celine et al., 2012). This is because OOWW has many beneficial and harmful effects on the environment, soil and of course, plants. In terms of soil and environment, primary harms of OOWW have been its content bearing salt, acidic pH and dense polyphenolic compounds (El Hadrami et al., 2004; Ayed et al., 2005; Amaral et al., 2008; Mechri et al., 2011; Ilay et al., 2013; Alesci et al., 2014). For instance, OOWW adversely affects the structure in clay soils with its dense salty feature (Barbera et al., 2013). As for polyphenols, these limit the application of OOWW to the soil with antimicrobial and phytotoxic effects.

Additionally, OOWW inhibits plant germination and causes potentially damaging effects on plant growth due to its contents, salt concentration, low pH and intensive polyphenols (Gigliotti et al., 2012). Even in some cytotoxic studies, it has been determined that OOWW causes chromosomal abnormalities, micronuclei, as well as darkened root tips and mitotic inhibition in *Vicia faba* (El Hajjouji et al., 2007).

Another study (Aybeke et al., 2000) emphasized that germination rate in wheat seeds decreased, while mitotic abnormalities and mitotic frequency increased, and found cells containing multiple nuclei or fragmented nuclei as well as numerical or structural chromosomal mutations. It has also been observed that the amount of protein decreased with increasing concentration and duration of OOWW treatment (Aybeke et al., 2000). Aybeke et al. (2008) in their own supplemental ultrastructural work, indicated striking damages in walls, nuclei and cytoplasmic membranes, and cellular organization disorders in wheat root meristem cells. In a different study (Aybeke and Sıdal, 2011), it was stated that OOWW reduced in vitro pollen germination rates in comparison to control in *Zea mays* pollen and general total protein contents of the pollens were proportional to OOWW concentrations, and then, it was suggested that OOWW causes carcinogenic effects.

In short, despite these cytotoxic studies establishing the basis, they are not sufficient to enlighten OOWW's toxic effects on the DNA and

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Table 1
Main chemical characteristics of OOWW.

Mineral	ppm(=mg/l)[a]	std.error	Mineral	ppm(=mg/l)[a]	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	5,9 g L^{-1}	0,25			
Caffeic acid	9,4 g L^{-1}	0,04			

[a] Indicate average values.

gene regulation. Indeed, there is intense toxicity on the nucleus, but how active is nucleus in specific areas such as damage repair and gene expressions, and in the general defence mechanisms? After all, is there a retroactive improvement in plants?

These questions have not been answered adequately. Therefore, in present, directly DNA- and gene-focused studies, it is aimed to determine the genotoxic effects of OOWW on sunflower seedlings by detailed GTS and transcriptomic analyses to answer these questions and fill this gap.

2. Material and method

The sunflower used in the study is the HA 89-B cultivar of Helianthus annuus. OOWW was collected from olive oil factory in neighbourhood villages of İznik, Bursa, TURKEY. The chemical analysis results of the wastewater are below (Table 1**).** In this analysis, Agilent 7700 xx ICP-MS device used. Measurements of NPs were performed using the Agilent 7700x ICP-MS. The samples were introduced directly into the ICP-MS system using the standard peristaltic pump with Tygon pump tubing (internal diameter of 1.02 mm), and ASX-520 autosampler (Sannac et al., 2013).

Polyphenolic content of OOWW was determined by the Folin-Ciocalteu (FC) method according to Box (1983) and Li et al. (2007) modified methods. 200 µl of the concentrated extract was added to 1 mL of 1:10 dH2O-diluted Folin-Ciocalteu reagent. After 4 min, 800 µl of saturated sodium carbonate solution (75 g L − 1) was added. Absorbance was measured after 1 h at 760 nm against a matrix blank using a Specord 50 UV/VIS spectrometer (Cary 50 Bio, Varian) (Analytic Jena, Jena, Germany). To evaluate the photosensitivity of the FC reagent towards different phenolic compounds, calibration curves with p-coumaric acid, tyrosol, caffeic acid, coumaric acid and gallic acid were prepared. Gallic acid (0–500 mg L^{-1}) was used as the standard calibration curve for the TPC calculations. Results are presented in mg p-coumaric acid and caffeic acid units as gram per OOWW liter.

In the control group (OOWW-free) only tap water was used. As a filler in viols, special white peat bedding substrate (Klasmann–Deilmann, GmBH Germany) were used.

The planting and cultivation of plants and all OOWW applications were carried out in greenhouses conditions in accordance with the sunflower. Wastewater was used at 1/1 (pure), 1/10, 1/100 concentrations, and for durations of 3-, 5- and 10-days.

The control group (OOWW-free) was irrigated with only tap water under the same conditions. On days 3, 5 and 10, the leaves of plants were cut and were treated with liquid azote immediately. All trials were performed under greenhouse conditions in a daily temperature range or 15–25 °C or in temperature-calibrated solarium rooms using HQLR lamps (1000 W) to adjust weather conditions to the specific sunflower life cycle in 2016 May-June (Hervé et al., 2001).

Genomic DNA isolations from sunflower plants were performed with a DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The total genomic DNA was diluted with nuclease-free water to a concentration of 25 ng/µl and used as

Table 2
Genes and primer sequence and PCR conditions used in present study.

qRT-PCR genes				
SOD	F: 5′ –GTTCGGTGACAACACCAATG − 3′, R: 5′- GGAGTCGGTGATGTGACCT- 3′	SOD2	F: 5′- TCTGAAGAAGGCCATCGAGT- 3′ R: 5′- GCAGATAGTAGGCGTGCTCC- 3′	1 cycle of 2 min at 50 °C and 10 min at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min
CAT	F: 5′- TACGAGCAGGCCAAGAAGTT- 3′ R: 5′- ACCTTGTACGGGCAGTTCAC- 3′	HSP70	F: 5′-TTATCAGTGAAATTAAGCGAGAGC- 3′ R: 5′-ACAAGGATAACTTCATCAACCTTTG- 3′	
APX	F: 5′-AAGGAGCAGTTCCCCATCC − 3 R: 5′-GCAAAGAAMGCRTCCTCRTC − 3′	GPX	F: 5′-AGTTCGGACATCAGGAGAATGGCA − 3′ R: 5′- TCACCATTCACCTCGCACTTCTCA − 3′	
GST	F: 5′-GAAGTTCTAGTGACAGCGTGCTTTA − 3 R: 5′-TGTAGCTGCTGCTGTGATTGG − 3′	HSP26	F: 5′-GCCCCGCAGCCCCATCTAGCAG − 3′ R: 5′-GAGCACGCCATCCGACGACAGC − 3′	
GS	F: 5′- TGGGACCAGCAAGTAAAACC- 3′ R: 5′- TCGCGAATGTAGAACTCGTG- 3′	BCL2	F: 5′- ATGTGTGTGGAGAGCGTCAA- 3′ R: 5′- ACAGTTCCACAAAGGCATCC- 3′	
HSP60	F: 5′- GTCGCGCCCCGTTAGCAC- 3′ R:5′-CATGCGGTCCCACCTTCTTCAT-'3	HSP70	F: 5′-TTATCAGTGAAATTAAGCGAGAGC- 3′ R: 5′-ACAAGGATAACTTCATCAACCTTTG- 3′	
HSP83	F: 5′- CCGGAGGCTCTTTCACAGTC- 3′ R:5′- CTTCTCGCGCTCCTTCTCTAC-'3	BCL2	F: 5′- ATGTGTGTGGAGAGCGTCAA- 3′ R: 5′- ACAGTTCCACAAAGGCATCC- 3′	
BCLXL	F: 5′-GTAAACTGGGGTCGCATTGT − 3′ R:5′- TGGATCCAAGGCTCTAGGTG-'3	XIAP	F: 5′- GGGGTTCAGTTTCAAGGAC- 3′ R: 5′- TGCAAGCAGAACCTCAAGTG- 3′	
CYT-C	F: 5′-AGTTTCTAGAGTGGTCATTCATTTACA − 3′ R: 5′- TCATGATCTGAATTCTGGTGTATGAGA- 3′	APAF1	F: 5′-GATATGGAATGTCTCAGATGGCC − 3′ R: 5′- GGTCTGTGAGGACTCCCCA- 3′	
CASP3	F: 5′- TGTCATCTCGCTCTGGTACG − 3′ R: 5′- AAATGACCCCTTCATCACCA- 3′	BAX	F: 5′- TTCATCCAGGATCGAGCAGA- 3′, R: 5′- GCAAAGTAGAAGGCAACG- 3′	
RAPD-PCR				
Primers names	Primer sequences	PCR conditions		
		40 cycles of 95 °C denaturation (30 s), 37 °C annealing (30 s), and 72 °C elongation (90 s) with aninitial 94 °C denaturation (3 min) and a final 72 °C extension (30 min)		
OPA09	5′ -GGGTAACGCC- 3′			
OPU16	5′-CTGCGCTGGA − 3′			
D1	5′- AGGGAACGAG − 3′			