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Effects of Olive Oil Mill Wastewater used as Irrigation Water on *in vitro* Pollen Germination

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Abstract: The aim of this study is to assess the effects of Olive Oil Mill Wastewater (OOMW) application as irrigation water on *in vitro* pollen germination, focusing on total protein quantity. In test groups, pollen germination substances such as sucrose, H_3BO_3 and $Ca(NO_3)_2$ were added to different concentrations of OOMW and used as germination media. Regarding control group, the same substance melted into water instead of OOMW. As a result, in general, pollen germination percentage was decreased significantly in all OOMW concentrations than that of the control group, except 1/1000 concentrations. Similarly, total protein quantities declined linearly depending on decreasing OOMW concentrations, except 1/1 concentration which has 4-5 times the control value. Consequently, it was established that OOMW generally decreased pollen germination ratio and had carcinogenic effects on protein synthesis mechanism and must not be used as irrigation water without purification.

Key words: Olive oil mill effluent, pollen germination, total protein, zea mays

INTRODUCTION

Olive Oil Mill Wastewater (OOMW) industry generates about 4 million tons of waste annually (Improlive, 2000), including solid and liquid wastes which have to be suitably managed to avoid the associated environmental impacts (Sierra *et al*., 2007). Those wastes may be reused through soil, directly (wastewaters) or following a compostage process (solid and semisolid wastes) (Sierra *et al*., 2007). Various types of treatments have been proposed for detoxification of this wastewater, biological, either aerobic (Sidal and Ozkale-Traskin, 2003) or anaerobic (Marquez *et al*., 1998). Nevertheless, in some Mediterranean countries like Turkey, this wastewater is used for field irrigation because of few purification units when compared with dense industrial waste production and high detoxification cost. In addition, few studies have been carried out about toxicity of the wastewater (Filidei *et al*., 2003; Aybeke *et al*., 2000, 2008). In our recent works, we reported that OOMW had direct toxic effects on the nuclear material since it led to the formation of multinucleate cells and nucleus fragmentation and several mitotic abnormalities in root tips of *Triticum aestivum*. Moreover, germination ratio and total protein quantities were found to be decreased significantly when compared with the control group and natural chromosome numbers varied (Aybeke *et al*., 2000, 2008). In another study, it was well established that pollen germination *in vitro* could serve as a sensitive and simple bioassay for water quality (Hoffmanna *et al*., 1990). When checked in the literature, we could not find any study investigating OOMW's quality using *in vitro* pollen germination method or its effects on pollen germination process. Our effort is directed to fill in the gap. The aim of this study is to assess the effects of olive oil wastewater application as irrigation water on pollen germination, focusing on total protein quantity.

MATERIAL AND METHODS

Fresh *Zea mays* L. cv Pioneer 3394 Cecilia anthers were obtained from plants grown in corn fields. In germination experiments, 20 anthers were taken and placed in each of several different media: The control medium and the test media with different experimental concentrations of OOMW. Chemical properties of OOMW are given in Table 1. The control medium consisted of 0.3 M sucrose, 1.6 mM H_3BO_3, 3 mM $Ca(NO_3)_2$, 0.8 mM $MgSO_4$ and 1 mM KNO_3 in 25 mM Mes-Tris buffer (Moreno *et al*., 1988). Test media were prepared with these constituents (except Mes-Tris Buffer) and different concentrations of OOMW as 1/1, 1/10, 1/100, 1/1000, 1/10000, 1/100000 and 1/1000000 (Aybeke *et al*., 2000, 2008). Anthers were ruptured, placed in 10 mL germination medium in a Petri dish on a shaker device and allowed to germinate for at least 1.5 h (Schatten *et al*., 1985). After the incubation period, the surface of the media was flooded with a fixative solution.

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Table 1: Chemical quantities of OOMW

Item	Values
Main quantities	
Dry substance (%)	12.0
Organic substance (%)	10.5
Minerals (%)	2.5
Organic substances (%)	
Sugar	5.0
Protein	1.2
Organic acid	0.7
Polyalcohols	1.8
Pectin, tanen	1.0
Poliphenols	1.0
Lipids	0.1
Minerals (ppm)	
P	500
K	3000
Ca	350
Mg	200
Na	450
Fe	35
pH	4.5-5
K.O.I. ($g\ L^{-1}$)	90-100

This solution contained 20 parts glycerin, 5 parts formaldehyde, 3 parts glacial acetic acid and 72 parts water (Pfahler, 1967). Petri dishes were stored at 20°C and germination frequency was determined by counting the number of germinated and nongerminated grains Approximately 10000 pollen grains were counted for each treatment. Each experiment was repeated at least three times.

As total protein quantity experiments, for control group and each test group, 0.005 g of pollens weighed separately from each other and placed on Whatman filter paper No. 2 in large Petri dishes. The filter papers were humidified by 10 mL of germination medium (control or test medium), as described earlier. Petri dishes with moist Whatman filter paper were incubated for 2 h at 100% humidity and 23±3°C (Webber and Bonnet-Masimbert, 1993). After the incubation period, pollens were left at -35°C for a night. The pollens that were frozen at -35°C were homogenized according to modified Nooden and Thimann's method (Nooden and Thimann, 1963). The determination of total protein content in obtained extracts was performed spectrophotometrically according to the Warburg and Christian method (Warburg and Christian, 1941). The differences in the protein quantity were tested by Analysis of Variance (ANOVA) and comparisons between means were performed with the Tukey test ($p = 0.05$) on protein quantity data. Additionally, germination ratios of control and test groups were compared with each other by using t test ($p = 0.05$).

RESULTS

Effect of OOMW on pollen germination percentage: Table 2 shows OOMW effects on pollen germination. Germination ratios are quite higher in the control group and 1/1000 concentrations of OOMW; as for other test groups, the values are fairly low (Table 2, Fig. 1). Particularly in 1/1000 concentrations, the germination percentage is the highest than in all other groups, including the control group. According to statistical analysis, in general germination ratios were significantly different from each other (Table 3), except that pairs of 1/1-1/0000, 1/10-1/100000 and 1/100-1/10000 are not significant from each other because of their relative germination percentage values, as noted in Table 2.

Table 2: Germination percentage of control group and test groups of OOMW

Concentrations	Polen germination (%)
Control	84.94
1/1	12.60
1/10	48.31
1/100	13.11
1/1000	93.37
1/10000	9.33
1/100000	48.26
1/1000000	1.98

Effect of OOMW on total protein quantity: Total protein quantities were near to or less than the control value, except that 1/1 concentration of its values was 4 or 5 times higher than that of the control group (Table 4).

According to multivariate correlation analysis, there is positive relation between concentrations and total protein quantities. That is to say while concentration is decreasing, total protein quantities also are decreased or vice versa (Table 5, Fig. 1).

DISCUSSION

OOMW decreased generally pollen germination percentage; in contrast, in only 1/1000 concentration, 93.37% of germination percentage was higher than that of the control group. In all other concentrations, germination results were lower than that of the control group and also showed fluctuation (Table 2). Differences between 1/1 and 1/10000, 1/10 and 1/100000 and 1/100 and 1/10000 were not significant; in other words, in these concentrations OOMW effects were similar to each other. On the basis of these findings, two important results were obtained: 1. OOMW affected pollen germination negatively, with the exception of 1/1000 concentrations. 2. As seen in Table 1 and 3, effects of OOMW on pollen germination was not regular and linear but wavy; in other words, OOMW showed dose-dependent effect. Similar results were found in a previous study related to seed germination and OOMW toxicity (Aybeke *et al*., 2000, 2008).

Depending on the concentrations, OOMW fairly decreased or increased the total protein quantities. That

Table 3: According to t test and correlation analysis, comparison of germination percentage results at $p \leq 0.05$ level

Groups	Unit No.	Correlation coeff.	Means	St dev.	St. error	t-value	Indep. degree	Tail signific.
Control	22	-420	0.745	0.193	0.041	11.99	21	0.000
1/1			0.1349	0.82	0.017			Significant
Control	29	0.334	0.7737	0.175	33	8.80	28	0.000
1/10			0.3853	0.229	43			Significant
Control	41	0.247	0.8215	168	0.026	13.22	40	0.000
1/100			0.2119	288	0.045			Significant
Control	29	0.267	0.7737	0.175	0.033	-3.87	28	0.001
1/1000			0.9274	0.178	0.033			Significant
Control	23	0.004	7501	190	0.040	16.14	22	0.000
1/10000			0909	050	0.010			Significant
Control	28	-0.152	0.7686	0.176	0.033	5.79	27	0.000
1/100000			0.4444	0.213	0.040			Significant
Control	41	0.182	0.8215	0.168	0.026	30.85	40	0.000
1/1000000			0.0258	0.038	0.006			Significant
1/1	22	-0.418	0.1349	0.082	0.017	-3.43	21	0.003
1/10			0.3164	0.203	0.043			Significant
1/1	22	-0.243	1349	0.082	0.017	2.37	21	0.0027
1/100			791	0.057	0.012			Significant
1/1	22	-0.668	1349	0.082	0.017	-13.98	21	0.000
1/1000			9184	0.201	0.043			Significant
1/1	22	0.318	0.1349	0.082	0.017	2.73	21	0.12
1/10000			0.879	0.049	0.010			Not sig.
1/1	22	-0.179	1349	0.082	0.017	-5.33	21	0.000
1/100000			4271	0.230	0.049			Significant
1/1	22	0.551	0.1349	0.082	0.017	7.75	21	0.000
1/1000000			0.141	0.019	0.004			Significant
1/10	29	0.464	0.3853	0.229	0.043	4.78	28	0.000
1/100			0.1673	0.245	0.045			Significant
1/10	29	0.376	0.3853	0.229	0.043	-12.62	28	000
1/1000			0.9274	0.178	0.033			Significant
1/10	23	-0.86	0.3365	0.220	0.046	5.12	22	0.000
1/10000			0.909	0.050	0.010			Significant
1/10	28	0.479	0.3859	0.233	0.044	-1.36	27	0.187
1/100000			0.4444	0.213	0.040			Not sig.
1/10	29	0.109	0.3853	0.229	0.043	8.67	28	0.000
1/1000000			182	0.022	0.004			Significant
1/100	29	0.093	0.1673	0.245	0.045	-14.17	28	0.000
1/1000			0.9274	0.178	0.033			Significant
1/100	23	0.236	0.1191	0.200	0.042	0.70	22	0.493
1/10000			0.0909	0.050	0.010			Not sig.
1/100	28	0.205	0.1691	0.219	0.470	-4.98	27	0.000
1/100000			0.4444	0.213	0.040			Significant
1/100	41	0.241	0.2119	0.288	0.045	4.24	40	0.000
1/1000000			0.0258	0.038	0.006			Significant
1/1000	23	-0.270	0.9220	0.197	0.041	18.44	22	0.000
1/10000			0.0909	0.050	0.010			Significant
1/1000	28	0.331	0.9248	0.181	0.034	11.09	27	0.000
1/100000			0.4444	0.213	0.040			Significant
1/1000	29	-0.368	0.9274	0.178	0.033	26.11	28	0.000
1/1000000			0.0182	0.022	0.004			Significant
1/10000	23	-0.402	0.0909	0.050	0.010	-6.46	22	0.000
1/100000			0.4260	0.225	0.047			Significant
1/10000	23	-0.045	0.0909	0 .050	0.010	6.86	22	0.000
1/1000000			0.0140	0.018	0.004			Significant
1/100000	28	-0.263	0.212	0.440	10.32		27	0.000
1/1000000		0.0168	0.121	0.004				Significant

is to say while concentrations were decreasing, total protein values also declined linearly, or vice versa. As seen in Fig. 1, it is noteworthy that each curve (concentration and protein) was overlapping. From this finding, it is logically suggested that any positive or negative effect on protein content resulted from protein synthesis process. Exceptionally, in 1/1 concentrations, total protein values were 4-5 times higher than that of the control group. In this point, it is associated with mutagen or carcinogen effects of OOMW on nucleus and DNA. Additionally, this finding shows a broad agreement with synergistically all the cell metabolism and hence disturb seedlings, growth and development process of plants. For example, polyphenols are generally regarded as an environmental pollutant and their pathologic, mutagenic, genotoxic effects and chromosomal aberrations were

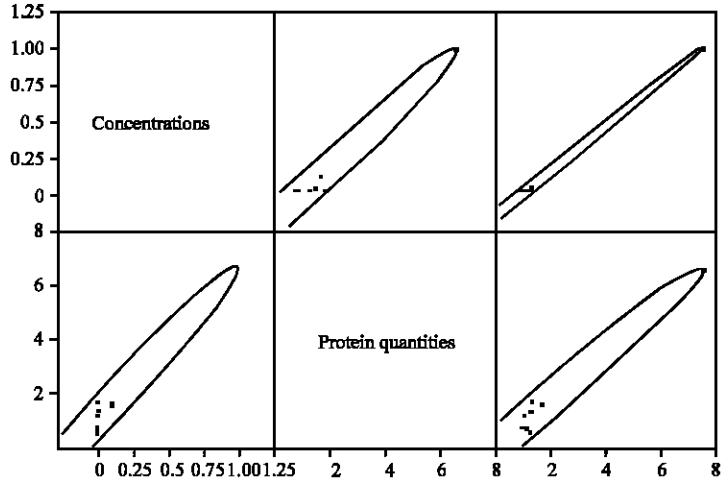

Fig. 1: Scatterplot matrix between concentrations and total protein values at p = 0.01 level

Table 4: Total protein quantities of control groups and test groups of OOMW

Concentrations	Total protein quantity (mg mL^{-1})
Control	1.24
1/1	7.64
1/10	1.62
1/100	1.15
1/1000	0.92
1/10000	1.08
1/100000	0.79
1/1000000	1.12

Table 5: Multivariate Correlations between concentrations total protein quantities

	Concentrations	Protein quantities
Concentrations	1.0000	0.9982
Protein quantities	0.9982	1.0000

reported by Shoji *et al.* (2004) and Sassi *et al.* (2006). In plants of Lamiaceae family, polyphenolic compounds, according to the average number of insects for each case, our previous works related to OOMW effects on root meristem at structural and ultrastructural level. Toxicity of OOMW arises from its high dose of Na salinity, compounds of Fe, polyphenols and finally its acidic pH feature.

From literature, toxicity of OOMW is paralleled by toxic effects of each factor (polyphenols, Na, Fe and acidic pH) contained within. Especially, in 1/1 concentrations, its toxic effects were more destructive (Aybeke *et al.*, 2000, 2008). These authors think that when these factors are at toxic levels, OOMW affect shows mobility and mortality (Regnault-Roger *et al.*, 2004). Similarly, Na^+ toxicity also caused a decrease in chlorophyll quantity and naturally photosynthetic activity; plants suffer from dehydration (Ueda *et al.*, 2003).

In this study, there is not any work regarding OOMW directed to pollen germination; nevertheless, very limited efforts have been made on the effects of several toxic compounds as OOMW on pollen germination. For example, Maize plants, subjected to 0, 80, 120 and 160 meq L^{-1} salinity using NaCl, showed adverse effects on viability, germination and tube growth of pollen. Salinity also resulted in the accumulation of ions such as Na^+, K^+ and Cl^-. These metabolic disturbances possibly lead to decreased viability, germination and tube growth of pollen, thereby resulting in a reduction in reproductive capacity of the plants under salt stress (Dhingra and Varghese, 1985). In acidic pH conditions, germination of pollen grains ceased because the intracellular pH is a central factor in the regulation of fundamental intracellular processes such as gene expression (Isfort *et al.*, 1993; Lapous *et al.*, 1998), protein synthesis (Webster *et al.*, 1991; Walker *et al.*, 1998), cytoskeleton rearrangements (Paulin-Levasseur and Gicquaud, 1984; Schatten *et al.*, 1985) and others. Numerous studies on animal cells revealed that alterations in the cell functional state are accompanied by changes in intracellular pH (Roos and Boron, 1981). Recent works on tobacco plants (Andreyuk *et al.*, 2001; Matveeva *et al.*, 2002) have established that a decrease in the metabolic activity of pollen grains prior to maturation and the transition to physiological dormancy was accompanied by a drastic decrease in the intracellular pH (Andreyuk *et al.*, 2001). The intracellular pH in the tip of the actively growing pollen tube is maintained at a similar level (7.1-7.2, up to 7.4 in one particular zone) (Fricker *et al.*, 1997; Parton *et al.*, 1997; Feijo *et al.*, 1999). The H^+-ATPase is one of the main factors controlling the

intracellular pH in plant cells (Kurkdjian and Guern, 1989), including pollen grains (Matveeva *et al*., 2002, 2003). In external conditions, acidifying pH strongly inhibited the outward K^+ channel currents (Ilan *et al*., 1996) which fully inhibited pollen germination, because a decrease in K^+ concentration in the cytoplasm during pollen hydration is a prerequisite for protein synthesis and subsequent onset of pollen germination (Bashe and Mascarenhas, 1984). In addition, low pH enhanced the activity of acidic isoforms of Pectin Methylesterase (PME) (Li *et al*., 2002) which together with pectinhydrolyases cause the degradation of pectin gels (Bordenave, 1996). Therefore, expansion of pollen tube cell wall ceased. From these reports, it is well established that the intracellular or external pH is an important regulatory factor for pollen germination and pollen tube growth *in vitro*.

Consequently, using OOMW as irrigation water caused not only mitotic abnormalities in root tip meristems and inhibition of seed germination as established in previous reports (Aybeke *et al*., 2000, 2008). but also disrupted pollen germination process. For this reason, fertilization and fruit maturation was destroyed. Consequently, OOMW will cause losses in agricultural production. Moreover, in a recent report (Sierra *et al*., 2007), OOMW's detrimental effects on soil microflora and total agricultural harvest was denoted. In another study, experiments suggest that pollen germination *in vitro* could serve as a sensitive and simple bioassay for water quality (Hoffmanna *et al*., 1990). Therefore, importance of the present work is considerable. As solution, OOMW must be purified before being used for irrigation.

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