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Physico-chemical Characterization and Effects of Olive Oil Mill Wastewaters Fertirrigation on the Growth of Some Mediterranean Crops

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Abstract: The present study revealed that traditional and industrial OMW samples were mildly acidic (pH= 4.10-4.50) and of a high conductivity of 18 to 56 mS cm^{-1}. The chemical oxygen demand (COD) ranged from about 250 to 600 g L^{-1} while the biological demand of oxygen (DBO_5) was about 3.05 to 3.39 g L^{-1}. The two OMW samples showed also significant differences in contents of many other chemical elements such as sodium, chloride, phosphorus and soluble and bound phenolic compounds. Fertirrigation of some crops from Mediterranean basin (maize, wheat, chickpea and tomato) by various concentrations of OMW showed significant different influences as respect to controls both with regard to the germination and growth stages of the plants. High reduction of shoot and root weight, of ramification and leaf extension rates, accompanied with significant reduction of yield, was observed for all the studied crops, especially wheat. The results were confirmed by significant qualitative and quantitative differences of some stress indicators such as phenolic compounds, peroxidases, chlorophyll contents observed between OMW treated plants and controls. A reduction of chlorophyll contents accompanied with a stimulation of peroxidases activity and phenolic compounds accumulation was recorded for OMW treated plants. The physiological disorders and/or phytotoxicity attributed to the OMW phenolics were highlighted depending on the crops.

Key words: OMW, fertirrigation, water re-use, peroxidases, phenolics, chlorophylls

INTRODUCTION

The traditional and industrial mill of olive oil in the Mediterranean basin countries generates large amounts of the called Olive Mill Wastewaters (OMW). In 1996, more than 10 million of m^3 of OMW were produced per year[1]. During these last decades this quantity has increased and riches currently around 30 million of m^3 of OMW per year[2], due to the modern methods of olive oil extraction developed to increase olive oil production. Such wastes are usually discharged in non-adapted wastewater canalization or directly on agricultural lands, particularly in some countries where no specific regulations are devoted to the management of these effluents. OMW exhibit toxicity effects against microorganisms and plants during seed germination and growth[3-7].

In order to find convenient solutions for spreading this by-product into agricultural lands without harmful environmental effects, many studies were conducted in several olive oil-producing countries[1,2,6,8,9]. Most of these studies were focused on the pretreatment of OMW using different microorganisms and different aerobic or anaerobic processes[8,10]. Few studies have been also performed to identify the OMW toxicity sources[2,5-7]. Indeed, using the durum wheat germinability as a biotest, it has been suggested that phenols are the main phytotoxic compounds of OMW[2].

The aims of the present study were to analyze the physicochemical composition of several samples of OMW coming from traditional or industrial mills in order to disclose the origin of their phytotoxicity and to determine the suitable concentration that can be used without negative effects on plant growth. Furthermore, the effects of these OMW during different stages of development including seed germination and growth of several Mediterranean crops have been studied. The physiological disorders and/or phytotoxicity attributed to the OMW phenolics were examined depending on the crops.

MATERIALS AND METHODS

OMW sampling: OMW samples were collected in traditional mills in Marrakech (Morocco) or in industrial

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mills in Sfax (provided by Dr S. Sayadi from Centre de Biotechnologies de Sfax, Tunisia). All the samples were kept at 4°C until used.

Physicochemical analyses: The physicochemical analyses were conducted according to the Afnor procedure[11] on three samples of each OMW stock. pH and electrical conductivity, expressed in mS cm^{-1}, were measured using a pH-meter (WTW-pH meter, 330/set-1) and conductivimeter (WTW-Conductivimeter, F318/set), respectively. Dry weight was determined after desiccation of 25 mL of OMW samples at 105°C and expressed in percentage of the initial volume of OMW. Suspended substances were evaluated according to the NF T 90-105 norms as difference between the initial and the final weights of 0.45µ filters used for filtration of 1 mL OMW samples. To evaluate the OMW pollution degree, two indicators were used: the chemical oxygen demand (COD, Afnor T90-101) and the biological oxygen demand (BOD_5, Afnor T90-103). Total phosphorus and chlorides were determined as described respectively in Afnor norms: AFNOR T90-022 and T90-014. Sodium, potassium and calcium contents were measured by a flame photometer (Jenway, PFP7). Iron and magnesium levels of OMW samples were determined in mg L^{-1} by atomic absorption using an Unicam 929 AA spectrometer.

Hydrosoluble phenolic compounds of OMW samples were extracted three times with an equal volume of ethyl acetate after discarding lipidic fraction by three times treatment with a half volume of hexane. After evaporation of the organic phase, the residue was suspended in pure methanol until use or directly in water for seed germination tests. The bound-phenolics were extracted by diethyl ether (v/v) after 4 h acid hydrolysis (6N HCl) at 100°C . Total contents of phenolic compounds were estimated using the Folin-Ciocalteu reagent (Sigma, France). Results were expressed in µg eq. (+)-catechin by reference to a standard scale established in the same conditions using (+)-catechin solution. Phenolic extracts were also analysed by HPLC using a Waters 600E HPLC equipped with a waters 990 photodiode array detector and a Millipore software for data analysis. An efficient gradient of acetonitrile-o-phosphoric acidified bidistilled water (pH= 2.6) was used with an Interchrom C18, 5 µm reversed phase column. Three wavelengths (280, 320 and 350 nm) were used during the elution. Phenolics were identified on the basis of their retention times and their spectra in comparison with standards. When necessary, co-injection and elution with standards were used to insure about the identity of the compounds.

Seed germination tests: The experiments were carried out in Petri dishes (9 cm) using seeds of durum wheat, maize and tomato. Germination was performed both on OMW diluted solutions and aqueous phenolic extracts of OMW. The concentrations used in both bio-tests were: 0 (control), 5, 10, 15, 25 and 50% (v/v). Three replicates per treatment and 10 seeds per Petri dish were incubated at 25°C in the dark. An index of germination (expressed as the percentage of germinated seeds) was calculated for each treatment.

Greenhouse tests: In order to evaluate the OMW effects on growth, development and productivity, agronomic tests were performed by greenhouse experiments. Durum wheat, maize and chickpea were used for these experiments. After pre-germination within Petri dishes, plantlets were transferred into the greenhouse. They were transplanted in plastic bags containing 2.5 kg of soil composed by sand and peat (2:1, v/v). Two plantlets per pot and three pots per treatment were used. The fertirrigation was purchased by OMW (Marrakech, March 2003) reaching 25 and 50% of the soil holding capacity. Soil without OMW was used as control. Water irrigation was supplied regularly. Before harvest, several growth parameters such as the maximum height, the number, the length and width of the leaves and the stem circumference were recorded. After the harvest, fresh and dry weights of the shoots and the roots were evaluated separately.

Evaluation of chlorophyll contents: From all the leaf samples collected, 0.5 g of fresh weight (FW) were crushed in mortar within 80% aqueous methanolic solution. Chlorophyll a (Chl a) and b (Chl b) contents were estimated by recording absorbency (A) at 647 and 664 nm and calculating the concentrations as follow: [Chl a] = ((12.7*DO664)-(2.79*DO647)), [Chl b] = ((20.7*DO647)-(4.62*DO664)) and [Chl tot] = ((17.9*DO647)+(8.08*DO664)).

Data analysis and statistics: All the experimental schemes were randomly designed complete blocks. Data were submitted to ANOVA using Statistica software, V. 5.5 and the significance of differences was recorded at a level of 0.05. Percentages data were arcsine transformed to ensure variances homogeneity. Pairwise comparisons of means were made using the Newman-Keuls test at a level of significance of 0.05.

RESULTS

Physicochemical composition of OMW: Traditional and industrial OMW samples were dark coloured aqueous wastes, foul-smelling and turbid. Table 1 shows that OMW were mildly acidic effluents (pH= 4.10-4.50) with a high conductivity of 18 to 56 mS cm^{-1} especially those coming from traditional mills (Morocco). The Chemical

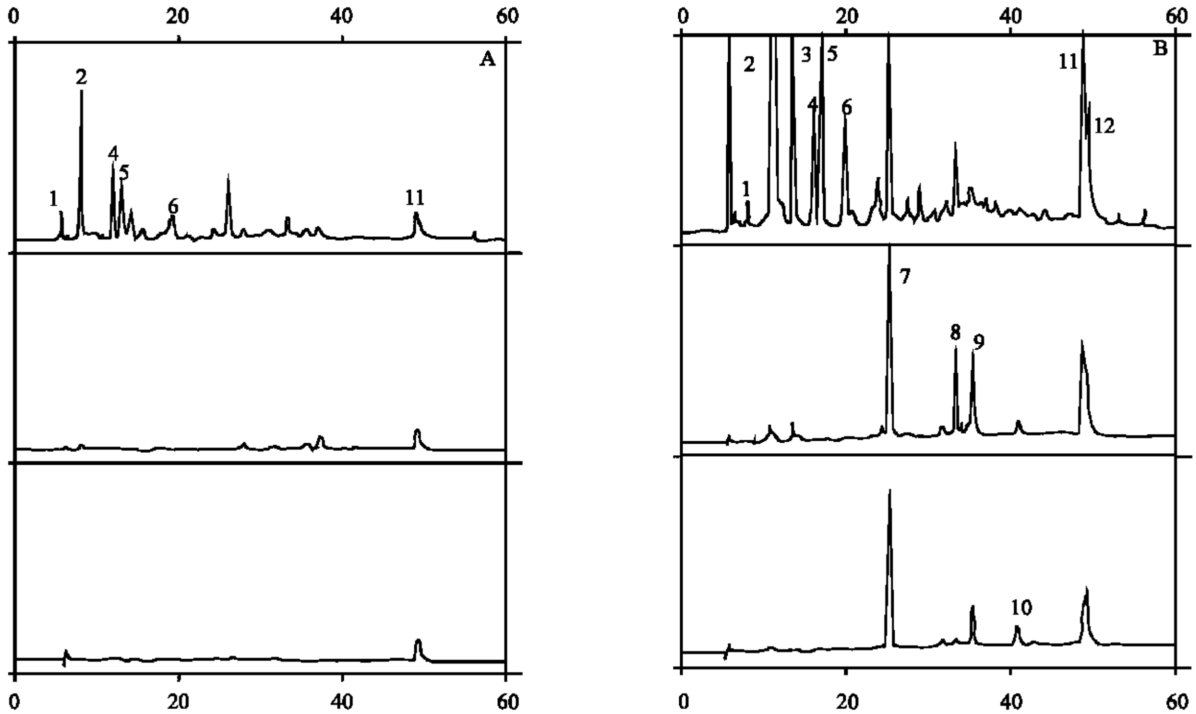

Fig. 1: Comparison of HPLC chromatograms of the OMW phenolics coming from Tunisia (A) or Marrakech (B). 1: syringic acid; 2: benzoic derivative?; 3: 3,4- dihydroxyphenylethanol; 4: catechol derivative; 5: catechol; 6: (+)-catechin; 7: caffeic acid derivative; 8: p-coumaric acid derivative; 9: ferulic acid derivative; 10: flavonoid compound (probably an apigenin derivative); 11: tyrosol derivative; 12: 4-methylcatechol derivative

Table 1: Physicochemical properties of OMW coming from Morocco and Sfax

	Sfax	Marrakech 1	Marrakech 2
pH	4.10	4.30	4.49
BOD_5 (g L^{-1})	3.39±0.16	3.23±0.66	3.05±0.35
COD (g L^{-1})	255.00±0.04	593.33±0.04	390.00±0.02
EC (mScm^{-1})	18.73±0.30	56.13±0.19	52.83±0.31
DW (%)	10.17±0.03	25.49±1.93	28.65±5.35
SS (g L^{-1})	25.00±14.5	37.50±8.91	36.60±1.09
Na (g L^{-1})	6.90±0.33	22.70±1.79	21.02±0.58
Cl (g L^{-1})	11.36±2.01	24.14±4.02	24.14±2.01
NaCl (g L^{-1})	18.78±3.31	38.78±6.62	39.78±3.31
K (g L^{-1})	8.91±1.08	10.87±0.57	10.46±0.57
Ca (g L^{-1})	1.94±0.13	0.89±0.03	0.87±0.00
P tot (g L^{-1})	0.025±0.003	0.058±0.002	0.058±0.011
Fe (g L^{-1})	0.05±0.00	0.06±0.01	0.07±0.00
Mg (g L^{-1})	0.52±0.01	0.52±0.01	0.54±0.01
Proteins (g L^{-1})	6.77±0.404	3.99±0.373	4.99±0.347
Soluble phenols (g L^{-1})	0.79±0.224	4.46±0.151	5.77±0.224
Bound phenols (g L^{-1})	5.48±0.831	11.57±0.049	10.24±0.268

EC: electrical conductivity, BOD_5: biological oxygen demand, COD: chemical oxygen demand, DW: dry weight at 105°C. SS: suspended solids. Values are given as means of three replicates per sample followed by the standard error

Oxygen Demand (COD) was about 250 g L^{-1} for industrial loads while it ranged from 390 to 600 g L^{-1} for traditional OMW. The two OMW types showed a similar biological demand of oxygen (BOD_5) (3.05-3.39 g L^{-1}). By contrast, the contents of many chemical elements varied between traditional and industrial OMW samples. As compared to industrial OMW from Tunisia, traditional OMW coming from Morocco were richer in sodium, chloride, phosphorus and in suspended and dry substances but poor in calcium and protein contents. Potassium, magnesium and iron contents were similar between the two OMW samples.

Soluble and bound-phenolics compounds were from 6 to 7.24 and two times higher in traditional OMW than in industrials, respectively. Furthermore, qualitatively and quantitatively different HPLC profiles of soluble phenolic compounds were also observed for the OMW samples (Fig. 1). Indeed, HPLC analysis showed that the most abundant compounds in traditional OMW coming from Morocco were hydroxybenzoic acid, 3, 4-dihydroxyphenylethanol, catechol, caffeic, p-coumaric and ferulic derivatives. Other aromatic compounds such as 4-methylcatechol, catechin, flavonoid compound, *trans*-cinnamic acid, syringic acid and their derivatives were also detected (Fig. 1).

Effects of OMW on seed germination: Significant differences were observed among crops according to their germination ability when treated with OMW or aqueous

Table 2: Germination percentages (+/-SD) upon different concentrations of OMW or their soluble phenolics extracts

		OMW origin					
		Marrakech, Morocco			Sfax, Tunisia		
		Maize	Wheat	Tomato	Maize	Wheat	Tomato
OMW concentrations (%)	0	73.3(15.3)	96.7(20.8)	73.3(20.8)	63.3(11.6)	73.3(5.8)	100.0(0.0)
	5	86.7(5.8)	76.7(11.6)	26.7(11.6)	83.3(5.8)	73.3(5.8)	90.0(17.3)
	10	70.0(17.3)	70.0(0.0)	6.7(5.8)	80.0(10.0)	43.3(28.9)	76.7(5.8)
	15	76.7(11.6)	66.7(10.0)	0.0(0.0)	73.3(5.8)	3.3(0.0)	40.0(20.0)
	25	76.7(15.3)	13.3(11.6)	0.0(0.0)	50.0(10.0)	0.0(0.0)	0.00(0.0)
	50	10.00(0)	0.00(0)	0.0(0.0)	60.0(10.0)	0.0(0.0)	0.00(0.0)

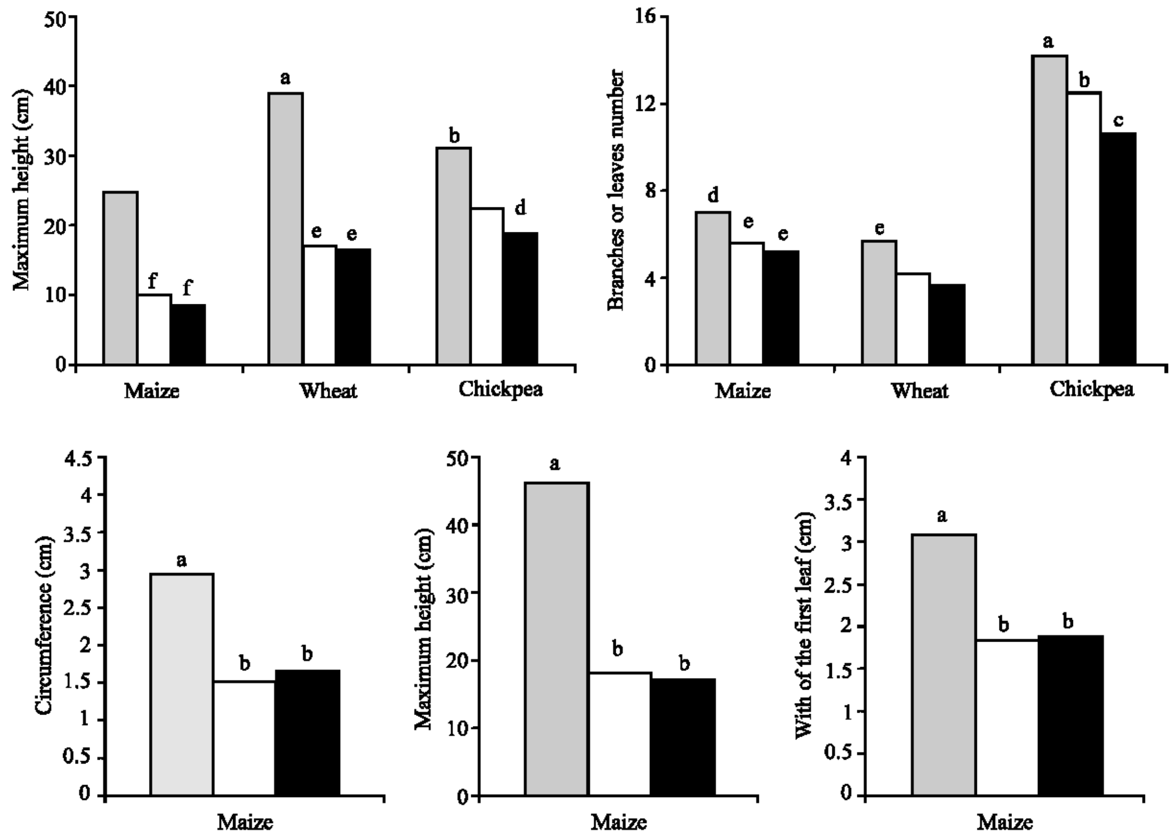

Fig. 2: Growth parameters of three Mediterranean crops fertirrigated by 0, 25 or 50% of the soil holding capacity of OMW. Control (hatched columns), empty columns (25% OMW-treated) and full columns (50% OMW-treated). Columns with the same letter do not differ significantly at a level of 0.05 according to the Newman-Keuls test

phenolic extracts of OMW solutions (Table 2). Indeed, maize showed a high germination index until 25% of OMW (Table 2), whereas chickpea was able to germinate also in a range of 25 to 50% of OMW (data not shown). By contrast, wheat and tomato germination occurred only until 15 and 5% of OMW, respectively. Similar results were observed for germination with OMW aqueous phenolic extracts.

When different concentrations of OMW were used for fertirrigation in greenhouse tests, significant differences in the growth and development of crops were observed comparatively to controls (Fig. 2). Crude and undiluted OMW were very lethal for all the studied crops (maize, chickpea, tomato and wheat) and significant reductions of seed germination and growth of crops were also observed with 2 and 4 time OMW dilutions. Significant reductions of shoot and root weights, reaching sometimes 50% of the control, of ramification and leaf extension rates accompanied by significant reductions of yield were observed for all the studied crops especially wheat (Fig. 3). A significant leaf chlorosis was also observed on OMW treated plants comparatively to controls (results not shown).

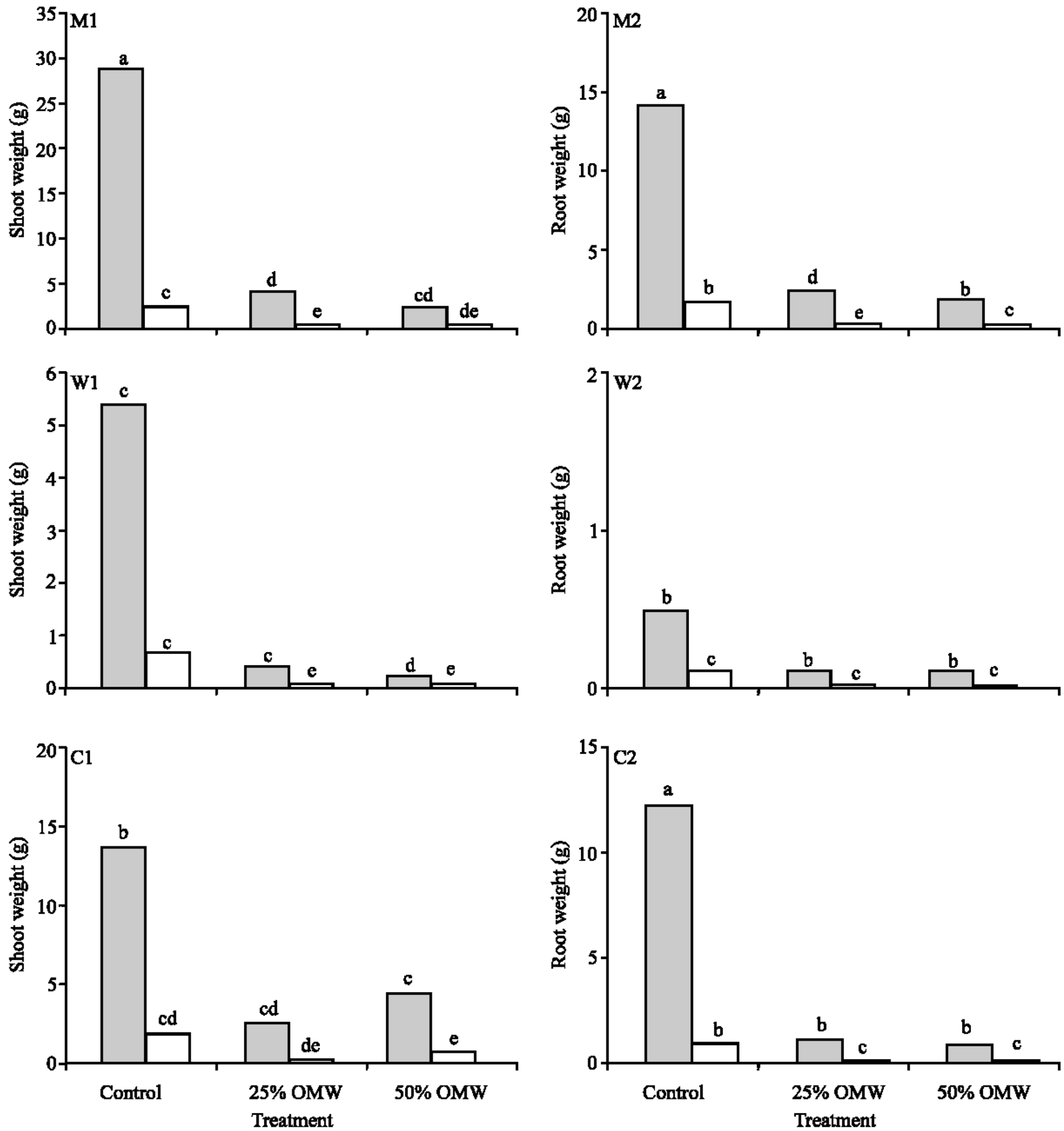

Fig. 3: Fresh (hatch columns) and dry weight (empty columns) of shoots (1) and roots (2) of three Mediterranean crops fertirrigated by 0, 25% of 50% of the soil holding capacity of OMW. M: maize, W: Wheat and C: Chickpea. Columns of the same variable with the same letter do not differ significantly at a level of 0.05 according to the Newman-Kelus test

Biochemical effects of OMW fertirrigation: Significant qualitative and quantitative differences of some stress indicators such as phenolic compounds, peroxidases (data not shown), chlorophyll contents were also detected between OMW treated plants and controls. Thus, a reduction of chlorophyll contents (Fig. 4) accompanied by 3 to 5 times stimulation of peroxidases activities and 1.25 to 7 times of phenolic compounds accumulation (data not shown) was observed for OMW treated plants comparatively to controls.

DISCUSSION

OMW generated by olive oil industry represents an important polluting effluent for the environment and aquatic bodies within the Mediterranean countries where

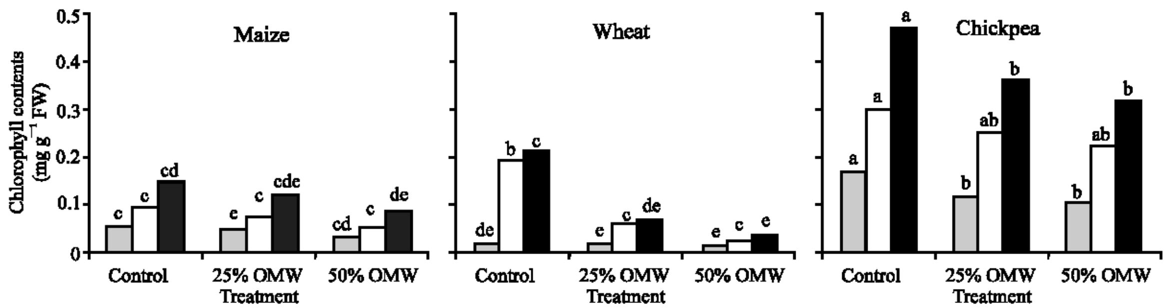

Fig. 4: Chlorophyll contents of three Mediterranean crops fertirrigated by 0, 25 or 50% of the soil holding capacity of OMW. Hatched columns: chlorophyll (a), empty columns: chlorophyll (b) and full column: total chlorophyll (a+b). Columns with the same letter and the same pattern as column do not differ significantly at a level of 0.05 according to the Newman-Keuls test

94% of the world olive oil production is concentrated (International Olive Oil, 2001). Indeed, OMW generated by milling of 2 kg of olive is equal to the wastewater discharged by one person during a day[9]. The differences observed for some physical and chemical parameters between industrial (Tunisia) and traditional (Morocco) OMW samples could be partly ascribed to the different olive oil extraction procedures used in industrial and traditional mills. For instance, the higher electrical conductivity measured for the traditional OMW could be due to the high level of sodium and chloride elements. The traditional aspect of the olive oil extraction in the sampled mills in Morocco requires some particular management to avoid undesidered effects. In fact, the management of big olive stocks in small olive mill, locally known as "Maasras" could not be made without addition of high quantities of salt to prevent olives from deterioration. Consequently, turbid OMW effluents are produced. They usually include emulsified grease easily fermentable, are very rich in phosphorus, organic matter (10-14%) and polyphenols (1-1.5%) due to olive pulp esters and glycoside hydrolysis, produced during the oil extraction[12-14]. Furthermore, with the traditional method, the milling seems to be less efficient to liberate phenolics in the oil phase (lampante to ordinary quality). By contrast, industrial OMW from Tunisia were less rich in polyphenols mainly due to the extraction procedure, which liberates more phenols in the oil phase (virgin to extra-virgin quality) than in the OMW phase.

Since the chemical oxygen demand (COD) of OMW is usually higher than the domestic sewage[15], OMW should not be disposed within natural aquatic systems without pre-treatment otherwise a dark colorization followed by an eutrophisation of these medias can be observed[14]. In the present study, the recorded values of COD, especially in traditional OMW, were higher than those reported in literature[2,9,14,16]. This difference could be explained in our case by the measurement method of non-filtrated OMW.

Despite their known phytotoxicity, OMW have fertilizer characteristics, which represent for the Mediterranean areas a potential source of water available for fertirrigation[9]. To use such effluents as fertilizer, their effect at different stages of plant growth and development should be known. In the present study, the impact of OMW on germination and growth of several crops cultivated in the Mediterranean basin and attempt to identify the phenolic fraction involved in the toxicity mechanism were addressed. Significant reduction of seed germination was observed specially for tomato and wheat with either OMW solutions or their related soluble phenolic extracts, suggesting a predominately inhibition effect of seed germinations by OMW phenolics. Phenolics could be considered as the main compounds implicated in the OMW germinability suppression or reduction in these crops; thus confirming the toxicity of the phenolic fraction of OMW as suggested by other studies[4,6,17,18]. Similar results, showing phytotoxicity effects of flavonoids and other phenolic compounds on seed germination of various plants, were observed in many works[19-23]. A highly significant reduction of the growth was also observed specially for wheat, suggesting susceptibility of this crop at least to one component of OMW, actually phenolics. These effects might be due to the lipophilicity of phenolic compounds, which could alter the accessibility of elements inside the biological membranes as suggested by Wang ***et al.***[24].

Studies of some stress indicators have shown, in general, a stimulation of secondary metabolites and the peroxidases activity with a deterioration of chlorophyll. Taking into account the role of peroxidases in the scavenging of active oxygen species and free radicals and in the cell walls cross-linking[25-28], it may be suggested that the phytotoxicity effects of OMW on fertirrigated crops results in an oxidative stress as demonstrated in other systems[29-31]. The wilting symptom prior to senescence with reduced shoot and root differentiation observed either in this work and in others[23], seems to agree with such suggestion.

In conclusion, the findings indicated that: (I) depending on the olive oil extraction procedure, OMW wastes differing in their physical and chemical composition are produced; (ii) fertirrigation with high doses of OMW produced a toxic effect against several Mediterranean crops grown in an organic matter-rich soil; (iii) when used in low doses, OMW might be of a beneficial effect particularly in the case of maize when it is grown on organic matter-poor soil frequently met in Marrakech region.

To spread OMW on lands with specific crops, further studies should be carried out. In particular, the relative contribution of each OMW phenolic compound to the toxicity effect and the applicable threshold of OMW, which can be used as biofertilizer in the fertirrigation of a soil without harmful effect on yield should be established. Moreover, the combination between the use of pre-treated OMW to eliminate harmful effects and soils with different characteristics should be addressed to design and plan the most suitable strategy of OMW utilization.

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