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Olive Mill Wastewaters Fractioned Soil-Application for Safe Agronomic Reuse in Date Palm (*Phoenix dactylifera* L.) Fertilization

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Abstract: This study was conducted to evaluate the valorisation of soil and OMW-intrinsic natural degradation potencies as an alternative for safe agronomic management of raw OMW. The method adopted in this study consists in OMW storage and progressive supply in weekly small doses. In field experiment, results of three years application of crude OMW at relatively high dose (150 m^3 ha^{-1} $year^{-1}$) reveal no important perturbations as regard to edaphic parameters notably salinity, pH and phenolic content, thus asserting OMW biodegradation in the studied calcareous soil. Besides, plants growth was efficiently raised by OMW inputs. Significant amelioration was obtained notably in term of shoots weight, trunk height and crown circumference (145, 40 and 32%, respectively). In addition, date palm cellular detoxifying capacities were also evidenced by pots trials. Quantitative and qualitative accumulation of phenolics and their oxidation provide evidence of their protective role against the physiological stress induced by OMW. These results highlight the importance of OMW storage and amendment fractioning for toxicity mitigation. Along with the correct choice of convenient soils notably calcareous ones and tolerant crops such as date palm (*Phoenix dactylifera* L.), this method could constitute an efficient approach for avoiding problems attributed to the uncontrolled disposal of these effluents and efficiently recover their fertilizing value.

Key words: Olive Mill Wastewaters (OMW), water reuse, fertilization, natural degradation, date palm

INTRODUCTION

Olive oil industry is in constant growth in the whole world and particularly in the Mediterranean countries. This activity leads to a world-wide production of more than 30 m m^3 of OMW every year (Cermola *et al*., 2004). Because of their potential toxicity (Zenjari and Nejmeddine, 2001; El Hadrami *et al*., 2004; Bonanomi *et al*., 2006), different treatment options were proposed for these effluents detoxification before their discharge (Roig *et al*., 2006; Arvanitoyannis *et al*., 2007). Nevertheless, the application of these procedures remains uncertain for economical and technical reasons. Given that the majority of olive oil producer countries in the Mediterranean sector are exposed to the problem of soil organic stock degradation (Laraus, 2004), the use of OMW as agricultural amendment was proposed as one of the most suitable methods to restore soil fertility and resolve the problem of their disposal (Paredes *et al*., 1999).

In fact, soil biotic and abiotic components could play relevant role in OMW remediation. For instance, Sierra *et al*. (2001) demonstrated that the acidity of OMW is buffered by the alkalinity of soil carbonates. Soil also acts as natural catalyst to promote the oxidation and the polymerisation of OMW phenolic compounds in less toxic products (Colarieti *et al*., 2002; Colarieti *et al*., 2006; Greco *et al*., 2006). Furthermore, soil micro-organisms, notably fungi, exert important capacity to detoxify the oxidized OMW (Kachouri *et al*., 2005). In the other hand, Mediterranean lands offer ideal region where Olive Mill Wastewaters (OMW) can be efficiently recycled. The strong mineralization in such arid to semi-arid climate could contribute to rapid degradation and transformation of OMW constituents and then soil recovery (Senesi *et al*., 2007).

On the basis of these data, we suggest the valorisation of soil degradation potencies as an efficient detoxification strategy from technical, environmental and economic point of view. To gain insight this hypothesis, experiments were carried out both in culture pots and field conditions with OMW coming from traditional mills in Marrakech region (Morocco). Date palm was chosen as plant model due to its ecological and socio-economical importance in the Mediterranean countries, particularly those of the south bank.

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MATERIALS AND METHODS

OMW collecting and storage: Fresh OMW were collected in traditional mill in Marrakech region (Morocco) and stored during the experiment period at room temperature. The main properties of these OMW are represented in Table 1.

Field experiment

Effect of medium term amendment with OMW on soil properties and plants growth: The study was conducted during 2004, 2005 and 2006. The experimental site was located in Faculty of Science Semlalia (Marrakech-Morocco) in 4 m^2 plots with three replications per treatment. Soil is a marginal calcareous one (57% sand, 24% silt, 16% clay and 35% $CaCO_3$). The climate of the area is semi arid to arid, characterized by a dry season from April to October and a humid season from November to February-March with an average annual rainfall of 240 mm. The annual average temperature is of 18°C with a maximum of 38°C in July-August and a minimum of 3.5°C in January-February.

A randomized block design with a total amount of 6 plots was arranged. Three months old seedlings were transplanted in plots at the rate of 32 plants/plot. OMW were applied progressively (one application per week) at a total rate of 150 m^3 ha^{-1} $year^{-1}$ that corresponds to three fold the maximum amount allowed by Italian law for traditional OMW (Anonymous, 1996). Control and treated plants were irrigated regularly by the same amount of water.

For the evaluation of soil properties, OMW-treated and control samples were collected in five point per plot with an auger at 20 cm depth, mixed thoroughly in a plastic container, air-dried and sieved at 2 mm. Three samples were used for assessment of pH, electrical conductivity, phenolics content and microflora density. pH and electrical conductivity were determined at 32°C in suspension with 1:3 (w/v) of soil and distilled water. Phenolics were extracted in 80% methanol, purified and analysed as described by El Hadrami *et al*. (2004). Phenolics quantification was performed by the Folin-Ciocalteu colorimetric method using (+)- catechin as standard (Macheix *et al*., 1990). For microbial density assessment, ten grams of soil samples were suspended in 90 mL of distilled water and shaken at 170 rpm for 2 h. After serial ten-fold dilutions in distilled water, an aliquot was then distributed on a nutrient agar medium and incubated at 30°C. Total mesophillic microflora was counted after 2 days. For non cultivable microflora assessment, total microbial DNA was approached according to Juniper *et al*. (2001) with slight modifications. Briefly, 1 g of soil was suspended in 3 mL TENCPP and incubated for 1 h at 65°C and then centrifuged at 6000 rpm for 10 min. The pellet was resuspended in 3 mL TENCPP and cellular DNA was extracted by series of ten thermal shocks and then centrifuged as above. The supernatant was collected, proteinase K was added to a final concentration of 20 mg L^{-1} and the reaction mixture was again incubated at 37°C for 30 min. Subsequently, an equal volume of phenol/chloroform/isoamylic alcohol (25/24/1) was added to sample tubes. Samples were then washed three times with an equal volume of CTAB/PVPP (1%/0.5%). The DNA was precipitated by adding 2V ethanol at 20°C in the presence of 1/10V sodium acetate, collected in TE after 30 min centrifugation at 12000 rpm, washed with 70% ethanol, dried at 37°C for 1 h and resuspended in 40 μL TER (Tris-HCl, EDTA, RNase). Afterwards, the extracts were separated on 1% agarose gel and visualised under UV light. All extractions were performed in triplicate.

Table 1: Main physico-chemical characteristics of OMW samples

OMW characteristics	Value
pH	4.81-4.880
Electrical conductivity (mS cm^{-1})	30.36-83.30
Phenolics content (mg g^{-1})	3.34-6.090

Date palm growth parameters were evaluated after three years growth (shoots fresh weight, fronds maximal length, trunk height and crown circumference).

Pot's experiment

Evaluation of date palm response to OMW amendment: One year aged plants were grown in pots containing sand/peat mixture (2V/1V). Three seedlings per pots were used. Total dose of ~300% of soil holding capacity was applied progressively. Phytotoxic impact of OMW was estimated as regards to symptomatic effect on plants, phenolic levels and peroxidases and polyphenoloxidases activities.

For phenolics extraction, roots were ground at 4°C with 80% methanol. Total soluble phenolics levels were determined using the Folin-Ciocalteu method according to the method described by Macheix *et al*. (1990). Phenolic extracts were analyzed 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. Enzymatic activity assays were performed by homogenizing roots tissue with Tris-maleate buffer (0.1 M, pH 6.5). Aliquots of the extracts were assayed for peroxidases activity after addition of H_2O_2 10%. Absorbance was determined at 495 nm. Polyphenoloxidase activity assays were performed in sodium phosphate buffer 0.1 M, pH 6. The reaction was released by adding 0.2 M catechol solution as substrate. Absorbance was evaluated at 410 nm after incubation at 37°C.

The experience was terminated after a total period of 14 months and growth parameters were recorded on each plant (shoots height, roots length, roots and fronds number, fresh weight).

Statistical analysis: A minimum of three plants per assay was used in each of the three repeated experiments. Data was analyzed using one-way analysis of variance ($p<0.05$) using Statistica software.

RESULTS

Effect of medium term progressive amendment with raw OMW on soil properties and plants growth: Soil analyses show no detrimental effect concerning phenolic contents or salinity. A slight but insignificant decrease in soil pH was recorded due to OMW supply (Table 2). HPLC qualitative analysis also shows no accumulation of phenolics in OMW-fertilized soil (Fig. 1). Furthermore, an important increase in microbial density was observed in fertilized plots. Indeed, fertilized soil was 9 fold more concentrated in total microorganisms than control (Fig. 2).

Field data also show significant enhancement of plants growth by OMW application. After three years of OMW supply, plants grown on amended soil show an ameliorated biomass allocation of up to two fold for shoots fresh weight, 40 and 32% for trunk height and crown circumference respectively (Table 3). Maximal fronds length was increased slightly but not significantly.

Evaluation of date palm response to OMW amendment: OMW treated seedlings roots showed a drastic reaction that was consisting notably in transitory phenolic levels fall to 1/2 as compared to control followed by important increase to attain 8 fold after 6 months (Table 4). In addition, HPLC analysis revealed modification of roots phenolic pattern due to OMW. While roots of control plants are constituted by three caffeoylshikimic acid isomers and flavans, fertilized seedlings roots profiles show caffeoylshikimic acids fall and massive accumulation of non-constitutive compounds (C1, C2 and

Table 2: Soil parameters evaluation over three years OMW-fertilisation

Parameters	Control	Soil plus OMW
pH	8.10±0.01	7.93±0.13
Electrical conductivity (mS cm^{-1})	9.03±2.50	9.03±1.30
Phenolics content	nd	nd

nd: not detected

Table 3: Effect of three years field OMW-supply on date palm growth

Seeding growth	Control	OMW fertilized
Shoots fresh weight (g)	410.95±118.03	1007.66±247.14
[NS]Fronds maximal length (cm)	138.25±24.88	175.20±22.69
Trunk height (cm)	18.56±3.54	26.00±3.28
Crown circumference (cm)	26.89±3.51	35.56±4.30

[NS]: Not significant difference

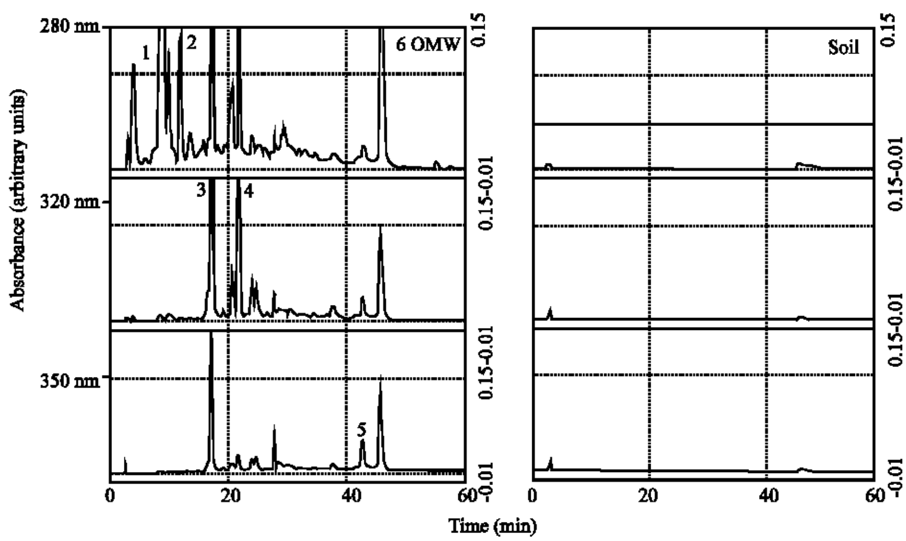

Fig. 1: Phenolic profiles of OMW used for fertilization assay (OMW) and OMW-fertilized soil (Soil). Chromatograms show the absence of phenolics in the fertilized soil. 1: Tyrosol, 2: Catechol, 3: Caffeic acid, 4: p-coumaric acid, 5: Luteolin derivative, 6: Oleuropein derivative

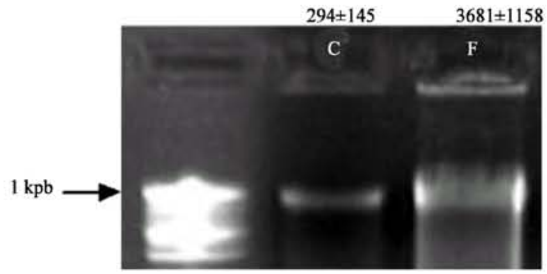

Fig. 2: Soil microflora abundance ($\times 10^3$ cfu g^{-1} of soil) in control (C) and OMW fertilized (F) plots evaluated by both direct cfu numbering and DNA density (non cultivable microflora included)

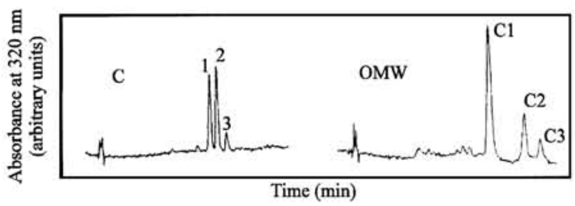

Fig. 3: HPLC analysis at 320 nm of soluble phenolics from roots of control (C) and OMW treated (OMW) date palm seedlings. Chromatograms show the presence of caffeoylshikimic acid isomers (1, 2 and 3) in control roots and a high accumulation of non-constitutive compounds (C1, C2 and C3) in treated ones

Table 4: Evolution of phenolic amounts (expressed in mg equivalent (+)-catechine/g fresh weight), peroxidases and polyphenoloxidase activities (expressed in EU s^{-1} g^{-1} fresh weight) in roots from control and OMW treated date palm seedlings

	Months after OMW application		
Parameters	2	4	6
Phenolic levels			
Control	3.20±1.66	3.69±0.21	2.46±1.32
OMW	1.59±0.14	9.47±0.93	19.15±7.23
Peroxidases			
Control	0.71±0.13	2.04±0.45	2.36±0.49
OMW	2.69±0.15	4.70±0.74	3.48±0.53
Polyphenoloxidase			
Control	5.97±0.44	21.18±0.13	34.17±0.13
OMW	49.67±6.54	42.62±0.15	32.45±0.15 NS

NS: Not significant difference

C3) along with flavans amount increase (Fig. 3). Besides, two phases can be distinguished concerning peroxidase activities: The increase tendency persists during the four first months and decrease later (Table 4). Polyphenoloxidase activities show important (8 fold) but short-lasting stimulation followed by progressive decrease till the activities levels are the same in treated and control roots after six months (Table 4).

Fig. 4: Date palm seedlings growth after high OMW dose application and seedlings roots browning induction by OMW amendment

Table 5: Effect of high OMW supply on potted date palm seedlings growth

Seedling growth	Control	OMW
Total leaves No./plant	7.00±0	9.50±0.71
Pinnate leaves No./plant	1.00±1.73	4.50±0.71
Roots No./plant	7.33±0.58	9.00±0
Shoots height (cm)	28.67±7.23	40.75±2.47
Roots length (cm)	27.79±18.56	42.33±27.73
Fresh weight per plant (g)	49.90±12.93	74.82±2.23

Visible damages were also recorded on fertilized plants that show important roots browning (Fig. 4). Concerning plants yield, the evaluation of growth parameters reveals a net improvement with plants fertilized by OMW particularly with regard to number of pinnate leaves, total fresh weight by plants and roots length (4.5, 1.5 and 1.5 fold, respectively, Table 5, Fig. 4).

DISCUSSION

In this study, soil degradation capacities as like as OMW inherent detoxification feature were investigated as alternative for safe OMW valorisation. Present investigation was limited to the upper layer (20 cm) because it is expected to exhibit higher manifestation of OMW effects (Mekki *et al*., 2007). In field conditions, results of soil analyses show relevant degradation of the applied OMW. Thus, a small but insignificant decrease in soil pH was obtained due to OMW amendment (Table 2).

This agrees with the high carbonate content of the studied soil (35%). In fact, Sierra *et al.* (2001) demonstrate that OMW acidity is neutralised by soil carbonates alkalinity. Besides, soil salinity level does not show differences between control and OMW-fertilized soil (Table 2). Nonetheless, more prolonged amendment could lead to salt accumulation and soil quality depreciation.

Phenolics assessment was performed by hydro-alcoholic extraction which allows the quantification of most available molecules to soil organisms. Results show no accumulation of phenolics in the soil fertilized by OMW fractioned application (Table 2, Fig. 1). This can not be explained by phenolics leaching since this phenomenon seems to be negligible in this type of soils rich in carbonate and clay materials (Sierra *et al.*, 2007). Actually, soil biotic and abiotic components exert relevant capabilities of OMW phenolics degradation (Kachouri *et al.*, 2005; Tabet *et al.*, 2006). Phenolics adsorption by soil organo-mineral components was also reported (Sierra *et al.*, 2007). Besides, phenolic compounds develop as well relevant roles in humic substances formation (Brunetti *et al.*, 2007).

In addition, OMW storage during the experiment period also plays important role of effluent pre-treatment. OMW develop inherent detoxification properties (Borja *et al.*, 2006). Saadi *et al.* (2007) showed OMW degradation during storage. In fact, OMW indigenous micro-organisms exert important phenolics degradation capabilities (Di Gioia *et al.*, 2002; Tziotzios *et al.*, 2007).

Several studies showing soil recovery after OMW application are in complete agree with present findings (Benitez *et al.*, 2004; Saadi *et al.*, 2007). However, these soil potencies are not absolute. In fact, a number of authors reported soil properties disturbance by OMW (Zenjari and Nejmeddine, 2001; Sierra *et al.*, 2001). Piotrowska *et al.* (2006) show that soil restoration ability after OMW supply depends on both OMW dose and time after application. High doses of OMW may be toxic towards micro-organisms and alter the degradation potencies.

In this sense, an improvement of soil microflora was obtained by colonies counting and DNA analysis. In fact, soil microflora is usually used as soil health indicator (Avidano *et al.*, 2005). The impact of OMW on soil microflora could be considered according to two general standpoints: Enrichment with organic matter readily metabolizable (Paredes *et al.*, 1999) and the incorporation of inhibiting substances notably phenolics (Capasso *et al.*, 1992). The obtained result asserts the absence of counteracting effects in the experimental conditions. This supports the hypothesis that OMW fractionation to several small doses could be used to valorise soil natural degradation potencies as a novel method to alleviate their toxicity.

In addition, notable amelioration in plants growth was obtained. In spite of slow growth of date palm, treated plants show relevant growth stimulation notably as regards to total shoots weight, trunk height and crown circumference (Table 3). Although raw OMW are often associated with disturbing effects on crops (Rinaldi *et al.*, 2003; El Hadrami *et al.*, 2004; Bonanomi *et al.*, 2006), detoxified OMW effectiveness as fertilizer was proved with several species (Tomati *et al.*, 1996; Cegarra *et al.*, 1996).

Besides soil and OMW-inherent natural degradation capabilities, date palm also play important role of OMW detoxification at cellular level. General tendency indicates relevant activation of phenolic metabolism in response to OMW application. Decrease of phenolic amounts was temporary and presumably due to more rapid turnover of free phenolics than synthesis notably by polyphenoloxidase as also indicated by root browning at this stage of plant growth (Fig. 4). Synthesis of new phenolic compounds along with flavans amounts increase was also revealed reminding other results that show the *de novo* accumulation of hydroxycinnamic acid derivatives in stressed date palm tissues (El Bellaj and El Hadrami, 2004). The neo-accumulation of phenolic compounds is common feature in many species in response to several stresses and phenolics are believed to have antioxidant properties (Cummins *et al.*, 2006; Wahid and Ghazanfar, 2006). Analogically, it can be suggested a potential role of accumulated compounds, particularly C1, in OMW toxicity mitigation at least by free radical attenuation as part of general stress response. Peroxidases are also related to OMW cellular detoxification presumably by catalyzing the phenolics oxidation at the expanse of hydrogen peroxide (Jouili and Ferjani, 2003; Wang and Ballington, 2007).

Overall, these selected physiological parameters show seedlings recovery throughout six months. Consistent with these findings, growth assessment also reflect date palm high tolerance to OMW amendment even at high amounts (~300% of soil holding capacity). Significant amelioration was obtained especially concerning pinnate leaves number and total fresh weight (Table 5, Fig. 4). It is worthy to note the importance of such difference concerning pinnate leaves. Actually, young date palm seedlings are characterised by a slightly developed leaves that, when becoming adult, are pinnate and arise from the apical buds in a flattish ascending spiral. From practical point of view, this would have important impact in accelerating date palm cycle (Fig. 4).

CONCLUSIONS

The results obtained lead to conclude that OMW application to calcareous soil under dry Mediterranean climates can be recommended for date palm amendment. Storage and fractioned supply can constitute a solution to valorise soil and OMW-inherent degradation potencies and allow mitigation of phytotoxicity of raw OMW application. Together with the correct choice of tolerant crops such as date palm, this method could constitute an efficient sustainable management approach of these effluents. However, OMW spreading must take into account the cumulative effect of salt that would lead in long term to soil degradation. The use of salt during the storage period of olives should be limited.

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