International Journal of Agronomy and Agricultural Research (IJAAR)
ISSN: 2223-7054 (Print) 2225-3610 (Online)
http://www.innspub.net
Vol. 6, No. 6, p. 56-67, 2015

RESEARCH PAPER OPEN ACCESS

Olive mill wastewater spreading effects on productivity and oil quality of adult chemlali olive (*Olea europaea* L.) in the South of Tunisia

Salwa Magdich[1,2], Chedlia Ben Ahmed[2], Makki Boukhris[3], Béchir Ben Rouina[2], Emna Ammar[1*]

[1]*Research Unit « Costal and Urban Environments » University of Sfax, National Engineering School of Sfax, Tunisia*

[2]*Laboratory of Improvement of Olive and Fruit Trees' Productivity, Olive Tree Institute of Sfax, Tunisia*

[3]*Laboratory of Plant Biology and Ecophysiology, Department of Life Sciences, Faculty of Sciences of Sfax, Tunisia*

Article published on June 10, 2015

Key words: Olive mill wastewater (OMW), Spreading, Chemlali cultivar, Olive yield, olive oil quality.

Abstract

In the present work, olive-crop yields and olive oil physico-chemical quality were studied after three and six years of olive mill wastewater (OMW) spreading at three levels (50, 100 and 200 m^3 ha^{-1} $year^{-1}$). Olive yield showed improvements with OMW level. Insignificant difference in oil content and oil quality indices of the control and treatments amended by OMW was observed. Moreover, olive oil acidic composition showed invariability after all OMW rates application. However, the extracted oils from the three-treated olive plots presented the higher α-tocopherol values. OMW spreading at different rates influenced significantly the oil total phenol contents. While β-sitosterol, campesterol and stigmasterol contents showed increases after six successive spreading with a high percentage in the 100 OMW m^3 ha^{-1} treatment, cholesterol and Δ-7-stigmastenol contents didn't exhibit any significant difference between the experimented treatments. After OMW treatment, the olive oil quality was that of virgin extra category.

* **Corresponding Author:** Emna Ammar ✉ ammarenis@yahoo.fr

Introduction

Olive tree (*Olea europaea* L.) is the most important cultivated crop in the Mediterranean basin and the olive oil sector has a remarkable economical importance in this region (Killi and Kavdir, 2013). In Tunisia, the olive tree is more and more extended not only for its socio-economical importance and its adaptation to arid climate characterizing the area, but also for the olive oil benefits, the main oil consumed in the region as well as in Mediterranean countries (Ben Ahmed *et al.,* 2011). In the world, Tunisia is considered among the main olive oil producers with a yearly average production of 180.000 tons in 2011/2012 crop season (COI, 2012). Previous, epidemiological studies showed a lower atherosclerosis, cardiovascular diseases incidence, and certain kinds of cancer in the Mediterranean area than in other parts in the world (Visoli and Galli, 1998). These differences have been attributed particularly to the kind of diet, largely vegetarian, in which the olive oil, a source of at least 30 phenolic compounds, is the principal source of fat (Machado *et al.,* 2013). For this reason, the olive oil and table olives consumptions have steadily increased in recent years. This inhabit becomes a fashion in countries that do not have such tradition (Ben Ahmed *et al.,* 2011).

Actually, the olive oil producing countries are facing a major environmental problem resulting from the large available olive mill wastewater (OMW) quantities produced yearly during relatively a short period. Indeed, this effluent is characterized by high concentrations of several organic compounds, such as organic acids, sugars, tannins, pectins and polyphenolic substances (Ammar *et al.,* 2005; Hachicha *et al.,* 2009). Generally, several environmental issues could arise from OMW application such as potential negative effects on soil physical, chemical and biological characteristics, phytotoxic effects on crops and ground water pollution (Barbera *et al.,* 2013).

Nevertheless, the use of OMW for agricultural purpose could provide a lower cost source for water and nutrients (López-Piñeiro *et al.,* 2007; Piotrowska *et al.,* 2011) particularly in arid regions, like Tunisia, suffering from serious water and soil organic matter deficiencies (Hachicha *et al.,* 2009; Magdich *et al.,* 2012). Indeed, the incorporation of this waste into the soil may constitute a valuable approach for C sequestration and reduction in runoff and soil loss (Lozano-Garcia *et al.,* 2011). Furthermore, application of OMW in agricultural land is a simple and relatively inexpensive method for disposal that may contribute to the development of sustainable agriculture, particularly under the severe climatic conditions occurring recently in olive oil - producing countries (Sierra *et al.,* 2007). For these reasons, actually more attention is given for better managing OMW spreading practice in such a way to improve production with lowest costs, while maintaining the product quality and preserving the environment. However, most papers focusing on OMW spreading for agricultural purpose have been interested in the effects determination of this effluent on soil physical, chemical and biological properties; and little information is available on long-term effects of OMW spreading on olive oil quality characteristics (Nasini *et al.,* 2013). In addition, the olive oil quality traits are determined by the different environmental surrounding factors such as the cultivation practices, the irrigation management, the cultivar, the olive tree-age, the fruit ripening index and the extraction process used (Ben Ahmed *et al.,* 2009; Mechri *et al.,* 2009; Dag *et al.,* 2011).

The objective of this study was to determine the long-term OMW spreading effects on olive oil quantitative and qualitative characteristic parameters obtained from trees of the cv. Chemlali subjected to OMW application for six successive crop seasons. In particular, we are interested in the olive oil fatty acids and sterols composition, α-tocopherols and phenolic compounds concentrations. The experimental approach allowed improving the understanding of the qualitative response (oil quality indices) of field grown Chemlali olive tree in relation to OMW spreading under natural conditions in arid region in Tunisia.

Materials and methods

Field investigation

The experiments of the present study were carried out in an olive-tree (*Olea europaea*, L., variety Chemlali) field at the Taous experimental station of the Olive Tree Institute of Sfax, South of Tunisia (34°43' N, 10°41' E). This area of the country has a typical Mediterranean climate, with an average annual rainfall of about 200 mm. The field was divided into four plots, three of which were annually dosed in February (from 2004 to 2010). Throughout the experimental period, each of the three latter plots was treated with the same annual dose of raw OMW, which was spread out on the surfaces of the corresponding plots at controlled volumes, namely 50, 100, and 200 m^3 ha^{-1}, respectively. The fourth plot was not submitted to raw OMW treatment and served as a control. Each of the treated plots covered an area of 1 hectare and contained 16 eighty-year old trees, with an inter-tree spacing of 24 m × 24 m. The sandy soil of the experimental orchard (86.63% sand, 13.26% silt and 0.20% clay) was characterized by an organic matter content of 0.32% and a pH of 7.5 (Magdich *et al.,* 2012).

OMW Characteristics

The fresh OMW used for olive field applications were collected from a three-phase olive mill plant located near to the experimental station. The average of the main characteristics of the OMW effluent were as follows: pH: 4.37; electrical conductivity: 12.89 mS cm^{-1}; COD: 120 g L^{-1}; BOD: 44.6 g L^{-1}; organic matter: 53 g L^{-1}; total polyphenols: 1.4 g L^{-1}; total nitrogen: 1.63 g L^{-1}; P: 0.62 g L^{-1}; K^+: 6.12 g L^{-1}; Ca^{2+}: 0.82 g L^{-1}; Mg^{2+}: 0.62 g L^{-1} and Na^+: 1.30 g L^{-1} (Magdich *et al.,* 2012).

Fruit harvesting, maturation index and yield

Before harvesting, the olive maturation index was determined according to the procedure described by Motilva *et al* (2000). Fruit harvesting was made in January of 2007 and 2010 crop seasons at the maturation index of 6. Olive fruits were handy collected (to guarantee accuracy) from all the plants subjected to the different treatments for olive productivity determination (kg^{-1} ha^{-1} $year^{-1}$).

All the different analyses made were determined at three (in 2007) and six years (in 2010) after the beginning of OMW spreading process.

Oil mechanical extraction process

The harvested olives were immediately transferred to the laboratory where the samples from the same plot were homogenised (3 repetitions per treatment) and a representative sample (in total 6 kg of olive fruits per treatment) was used for oil extraction. Olive oil used for analysis was extracted using a laboratory olive Bench Hammer Mill (Abencor Analyzer, MC2 Ingenierias y Sistemas, Sevilla, Spain). After fruit crushing and paste malaxation for 30 min at 25 °C, centrifugation and decantation allowed the oil separation. The oil amount obtained was measured. Oil samples were filtered, transferred into amber glass bottles, and stored at 4 °C in darkness until analysis. Total oil content was expressed on a fresh weight basis (% FW).

Oil analyses

Oil quality indices

Extinctions coefficients K_{232} and K_{270} were measured at 232 and 270 nm, respectively. Free acidity and peroxide value, expressed as milliequivalents of active oxygen per kilogram of oil (meq O_2 kg^{-1}), were measured according to the analytical method described in the European Regulation EEC 2568/91.

Fatty acid composition

The fatty acid composition was determined on the basis of the European Regulation EEC 2586/91 method. The methyl esters were prepared by a vigorous shaking of the oil dissolved in hexane solution (0.2 g in 3 ml) mixed with 0.4 ml of 2 N methanolic potassium hydroxide and analyzed by gas chromatography equiped with a FID detector. A fused silica column (15 m length × 0.25 mm) coated with SGL-1000 phase (0.15 μm thickness) was used. The carrier gas was nitrogen at a flow rate of 1 ml $min^{-1}$and the analysed sample volume was 1 µl. The injector and detector temperatures were set at 250 °C,

and the oven temperature was set at 180 °C. The oil fatty acids were identified by comparing the retention times to those of the standard compounds.

Chlorophyll and carotenoid concentrations

The chlorophyll and the carotenoid fractions at respectively, 670 nm and at 470 nm were evaluated from the absorption spectrum of each olive oil sample (7.5 g) dissolved in cyclohexane, as described by Mínguez-Mosquera *et al.* (1990).

α-Tocopherol determination

The α-tocopherol concentration was evaluated by high-performance liquid chromatography (HPLC) with a direct injection of the oil in hexane solution (Gimeno *et al.,* 2000). The HPLC separation was carried out using a Hewlett-Packard liquid chromatographic system (Waldbronn, Germany) with an HP-1100 pump system and a Rheodyne Model 7125 injector (Cotati, CA, USA). The final volume loop was 20 µl. The detector was a HP-1040M photodiode-array detection system (DAD). The data were stored and processed by a HPLC Chemstation (Dos Series) (Hewlett-Packard). The column was a Supelco ODS-2 (150 mm × 4.6 mm I.D., 5 mm particle size). The mobile phase was methanol/water (96:4, v/v) at a flow rate of 2 ml min^{-1}. The α-tocopherol was quantified by the internal standard method. The results were expressed as mg of α-tocopherol per kg of oil.

Total phenols content

Total phenols were extracted by triple extraction of a solution of oil dissolved in hexane according to the method described by Vazquez Roncero *et al.* (1973) and determined colorimetrically using Folin-Ciocalteau reagent (Singleton & Rossi, 1965). The absorbance was measured at 727 nm with a spectrophotometer (PerkinElmer UV/Vis Spectrophotometer, Norwalk, CT). The results were expressed as mg of caffeic acid per kg of oil.

Sterols composition

Sterol percentages were determined with a Hewlett–Packard (HP 6890) gas chromatograph with a capillary column (25 m length × 0.25 mm i.d.) coated with SGL-5 (0.25 µm thickness; Sugerlabor). The carrier gas was helium at a flow rate of 1.2 ml min^{-1}; the injector temperature was set at 280 °C and the detector temperature was 290 °C. The oven temperature was set at 260 °C and the injection volume was 1 µl (Regulation EEC 2568/91).

Statistical analysis

The cumulative data recorded was subjected to variance analysis using SPSS software (version 11). The treatments mean values were compared using Duncan's multiple range test at the 5% (p = 0.05) significance level. All the analyses were performed in triplicate.

Results and discussion

OMW spreading effect on olive fruit

Olive productivity

For the different treated olive plots, the OMW amendment has led to a significant increase of olive productivity after six years of effluent spreading in comparison to the control (Fig. 1). This increase was of 32.60, 33.53 and 26.35%, respectively for 50, 100 and 200 m^3 ha^{-1}. Indeed, the highest olive productivity (513 kg^{-1} ha^{-1} $year^{-1}$) was recorded in the olive orchard receiving 100 m^3 ha^{-1} of OMW, and a good correlation between treatments and olive productivity (R^2 = 0.973), was established with a second degree polynomial model. Furthermore, the comparison of OMW-treated olive plants between them showed a continuous improvement of olive yield up to 100 m^3 ha^{-1} $year^{-1}$ spread rate.

In the case of OMW spreading, the olive productivity improvement could be related to the effluent richness in easily assimilated available substances, providing carbon, phosphore, potassium and energy to soil microflora (López-Piñeiro *et al.,* 2007; Magdich *et al.,* 2012). Such increase was recently evidenced by Magdich *et al.* (2013) when studying soil amended with OMW. This pattern confirms the OMW fertilizing role, at least under the described environmental conditions. The positive effect of OMW amendment on olive productivity and growth

has been also demonstrated by Altieri and Esposito (2008) in olive orchard amended over 5 years with two experimental olive mill wastes. Similar results have been recorded in the case of wheat plants amended with crude OMW by Brunetti *et al.* (2007). The same authors have explained this positive effect by the improvement of water retention in OMW-treated soil, due to the effluent richness in organic matter. Furthermore, Nasini *et al.* (2013) confirmed the enhancement of olive productivity in orchards treated with solid olive mill waste.

Table 1. Total oil content, free acidity, peroxide value and extinction coefficients evolution in the olive oil sampled after the third and the sixth spreading of OMW at different doses: control (0 m^3 ha^{-1}), 50 m^3 ha^{-1}, 100 m^3 $ha^{-1}$and 200 m^3 ha^{-1}.

Spreading year	OMW spread dose (m^3 ha^{-1} $year^{-1}$)	Total oil content (% FW)	Free acidity (%)	Peroxide value (meq O_2 kg^{-1})	Extinction coefficients	
					K_{232}	K_{270}
Third (January 2007)	Control	29.02 ± 2.56	0.26 ± 0.04	3.20 ± 0.31	1.88 ± 0.05	0.13 ± 0.01
	50	28.36 ± 1.25	0.28 ± 0.02	2.90 ± 0.12	1.87 ± 0.04	0.11 ± 0.02
	100	28.24 ± 1.42	0.24 ± 0.01	4.60 ± 0.34	2.10 ± 0.06	0.14 ± 0.01
	200	27.49 ± 2.12	0.27 ± 0.05	4.40 ± 0.28	2.01 ± 0.06	0.13 ± 0.01
Sixth (January 2010)	Control	28.65 ± 1.47	0.36 ± 0.01	4.10 ± 0.26	2.00 ± 0.02	0.16 ± 0.06
	50	29.45 ± 2.32	0.37 ± 0.12	4.20 ± 0.14	2.34 ± 0.01	0.12 ± 0.01
	100	28.96 ± 1.63	0.36 ± 0.05	4.80 ± 0.06	2.18 ± 0.18	0.14 ± 0.05
	200	28.23 ± 1.14	0.39 ± 0.04	4.60 ± 0.42	2.02 ± 0.03	0.14 ± 0.03
Standard values (IOOC, 2006)		-	≤ 0,80	≤ 20	≤ 2,50	≤ 0,22

FW: Fresh Weight.

Oil yield

During both crop harvest seasons (January 2007 and January 2010), the OMW amendment did not affect oil accumulation in the Chemlali olive tree as no statistically significant difference was observed between total oil content (% FW) of the different treatments ($P > 0.05$). These values varied from 27.49 to 29.45 % FW over the experimental period. During both crop seasons recorded oil yields were almost similar. In contrast, Mechri *et al.* (2009) mentioned an oil content decrease in olive tree treated for one year with OMW amendment at 30, 60 and 150 m^3 ha^{-1} in the North of Tunisia. Similarly, Ben Ahmed *et al.* (2009) noted a decrease of oil content in the case of adult Chemlali olive tree irrigated for long term with saline water.

Table 2. Fatty acids composition (%) evolution in the olive oil sampled after the third and the sixth spreading of OMW at different doses: control (0 m^3 ha^{-1}), 50 m^3 ha^{-1}, 100 m^3 $ha^{-1}$and 200 m^3 ha^{-1}.

Spreading year	Third (January 2007)				Sixth (January 2010)				Standard values (IOOC, 2006)
OMW spread dose (m^3 ha^{-1} $year^{-1}$)	Control	50	100	200	Control	50	100	200	
Palmitic acid C16:0	20.23 ± 0.26	19.61 ± 0.38	19.27 ± 0.62	19.68 ± 0.32	19.28 ± 0.18	18.35 ± 0.24	18.11 ± 0.84	18.65 ± 0.22	7.5-20.0
Palmitoleic acid C16:1	2.45 ± 0.11	2.28 ± 0.14	2.31 ± 0.16	2.50 ± 0.17	1.99 ± 0.14	2.27 ± 0.06	2.08 ± 0.04	1.96 ± 0.12	0.3-3.5
Stearic acid C18:0	1.91 ± 0.03	2.07 ± 0.01	2.20 ± 0.03	2.00 ± 0.02	2.08 ± 0.05	2.00 ± 0.02	1.98 ± 0.11	1.95 ± 0.14	0.5-5
Oleic acid C18:1	57.20 ± 0.45	57.82 ± 0.24	59.40 ± 0.36	59.04 ± 0.38	59.57 ± 0.21	59.12 ± 0.16	59.33 ± 0.18	59.45 ± 0.42	55-83.0
Linoleic acid C18:2	17.14 ± 0.15	17.10 ± 0.12	15.81 ± 0.16	15.70 ± 0.20	17.96 ± 0.12	18.14 ± 0.06	18.28 ± 0.14	19.46 ± 0.61	3.5-21
Linolenic acid C18:3	0.58 ± 0.05	0.60 ± 0.03	0.54 ± 0.05	0.58 ± 0.06	0.49 ± 0.12	0.53 ± 0.06	0.59 ± 0.02	0.58 ± 0.17	≤□1.0
Arachidic acid C20:0	0.32 ± 0.05	0.33 ± 0.02	0.40 ± 0.01	0.33 ± 0.06	0.34 ± 0.03	0.32 ± 0.05	0.35 ± 0.05	0.37 ± 0.04	≤□0.6
Gadoleic acid C20:1	0.13 ± 0.08	0.15 ± 0.07	0.15 ± 0.08	0.13 ± 0.09	0.15 ± 0.06	0.15 ± 0.03	0.16 ± 0.02	0.17 ± 0.01	≤□0.4
Uns/Sat ratio	2.09 ± 0.14	2.12 ± 0.08	2.16 ± 0.13	2.12 ± 0.15	2.18 ± 0.15	2.32 ± 0.08	2.36 ± 0.17	2.41 ± 0.19	-
Mono/Poly ratio	2.24 ± 0.17	2.26 ± 0.11	2.53 ± 0.46	2.52 ± 0.18	2.23 ± 0.13	2.19 ± 0.07	2.19 ± 0.06	2.01 ± 0.26	-

Uns: Unsaturated; Sat: Saturated; Mono: Monounsaturated; Poly: Polyunsaturated.

However, Wiesman *et al.* (2004) have registered a clear increase of oil yield from olive trees grown under salinity conditions. According to Jiménez *et al.* (1995), the non-effect of OMW spreading on oil content could be due to the preservation of sugar components synthesis in olive fruits. Indeed, these carbohydrates are precursors for olive oil biosynthesis process, and they may play an important role in metabolic changes by providing energy. Nevertheless, the OMW organic solutes used for long period seem to have a more beneficial effect on olive productivity than on oil content in our experimental design. As a result, the OMW seems to affect more positively the fruit development and growth than the oil biosynthesis mechanism. Interestingly, the insignificant differences in oil content between the control and treatments with OMW would be an advantage of this effluent use for the studied cultivar as a fertilizer, at least under the described experimental circumstances. Such results confirm the purpose developed by Magdich *et al.* (2013) for the management practice of this sludge as a source for soil nutritive elements, which was legislated later by a Tunisian law (JORT, 2013).

Table 3. Total chlorophylls, carotenoids, α-tocopherol and total phenols contents evolution in the olive oil sampled after the third and the sixth spreading of OMW at different doses: control (0 m^3 ha^{-1}), 50 m^3 ha^{-1}, 100 m^3 ha^{-1} and 200 m^3 ha^{-1}.

Spreading year	OMW spread dose (m^3 ha^{-1} $year^{-1}$)	Total chlorophylls (mg kg^{-1})	Carotenoids (mg kg^{-1})	α-Tocopherol (mg kg^{-1})	Total phenols (mg kg^{-1})
Third (January 2007)	Control	0.36 ± 0.03	1.66 ± 0.05	ND	23,81 ± 2,78
	50	0.33 ± 0.07	1.82 ± 0.02	ND	28,94 ± 2,28
	100	0.47 ± 0.05	1.78 ± 0.01	ND	91,23 ± 3,42
	200	0.48 ± 0.02	1.86 ± 0.08	ND	129,17 ± 4,37
Sixth (January 2010)	Control	0.32 ± 0.05	1.76 ± 0.02	418.35 ± 3.44	154.50 ± 3.42
	50	0.34 ± 0.06	1.80 ± 0.01	543.26 ± 4.27	170.46 ± 1.23
	100	0.38 ± 0.02	1.76 ± 0.05	542.19 ± 3.17	171.20 ± 1.86
	200	0.34 ± 0.02	1.79 ± 0.06	483.35 ± 4.30	165.50 ± 1.44

ND: Not determined.

OMW spreading effect on olive oil characteristics

Quality indices

During both crop seasons (2007 and 2010), the OMW did not affect the free acidity, the peroxide values and the extinctions coefficients of oil samples as no statistically significant differences ($P > 0.05$) were recorded between the control and the different OMW doses used for soil amendment (Table 1). Indeed, the free acidity ranged from 0.24 to 0.39, the peroxide values varied between 3.20 and 4.80. For the extinction coefficients, the K_{232} values were between 1.87 and 2.34, and those of K_{270} were between 0.11 and 0.16. Moreover, these values were considerably lower than the upper limit of 0.8% as oleic acid and 20 meq O_2 kg^{-1} as the peroxide value, established by the EU legislation for extra virgin olive oil (VOO). Similar results were mentioned by Bedbabis *et al.* (2010) in olive orchard irrigated over 4 years with treated waste-water. The same tendency was observed in adult Chemlali olive tree irrigated with saline water (Ben Ahmed *et al.,* 2011).

Taking into account the free acidity and peroxide values, as well as K_{232} and K_{270} coefficients, the oil samples issued from the control and the OMW treated lots met European Union requirements for the extra virgin olive oil category (Table 1).

Fatty acid composition

Based on the acidic profile, the different sampled oil from treated and untreated olives presented similar qualitative acidic composition and their percentages were within the range of acid composition of extra VOO as established by the International Olive Oil

Council (IOOC, 2006) (Table 2). Regardless of the treatments, the overall acidic profile analysis showed that the oleic acid was the most abundant one with a percentage value varying from 57.20 to 59.45% of the total fatty acids content. On the other hand, for the different oil acidic compounds, differences between the control and the three OMW treatments were not significant (P > 0.45) considering the first (third-year) as well as the second (six-year) harvested seasons.

Table 4. Sterolic composition and total sterols contents in the olive oil sampled after the sixth spreading of OMW at different doses: control (0 m^3 ha^{-1}), 50 m^3 ha^{-1}, 100 m^3 ha^{-1} and 200 m^3 ha^{-1}.

Sterolic compound	Sixth spreading (m^3 ha^{-1} $year^{-1}$) (January 2010)				Standard values (IOOC, 2006)
	Control	50	100	200	
Cholesterol (%)	0.11 ± 0.02	0.12 ± 0.01	0.11 ± 0.01	0.12 ± 0.02	≤□□0.5
Campesterol (%)	3.20 ± 0.10	3.28 ± 0.18	3.45 ± 0.25	3.36 ± 0.20	≤□4
Stigmasterol (%)	0.65 ± 0.06	0.72 ± 0.05	0.80 ± 0.18	0.74 ± 0.11	≤□□% Campesterol
β- sitosterol (%)	85.22 ± 1.60	85.76 ± 1.90	86.02 ± 2.50	85.36 ± 1.90	□
Δ-7-stigmastenol (%)	0.29 ± 0.05	0.28 ± 0.02	0.27 ± 0.08	0.29 ± 0.04	≤□□□0.5
Total sterols (mg Kg^{-1})	1065.3 ± 74.5	1215.3 ± 83.5	1235.3 ± 71.5	1096.3 ± 54.5	≥1000

Comparing the oleic acid percentages between control and OMW-treated oil samples, a slight increase was noticed in OMW-amended samples after the third OMW spreading application. For the second harvested season (after six spreading year), these values were almost similar. It is note worthy that the oleic acid concentration increase registered in January 2007, was associated with a slight palmitic and linoleic acids concentrations decreases. For the second harvested season (January 2010), the same pattern was observed in the case of palmitic acid concentration in OMW-treated plants, in comparison to control, as that registered in January 2007.

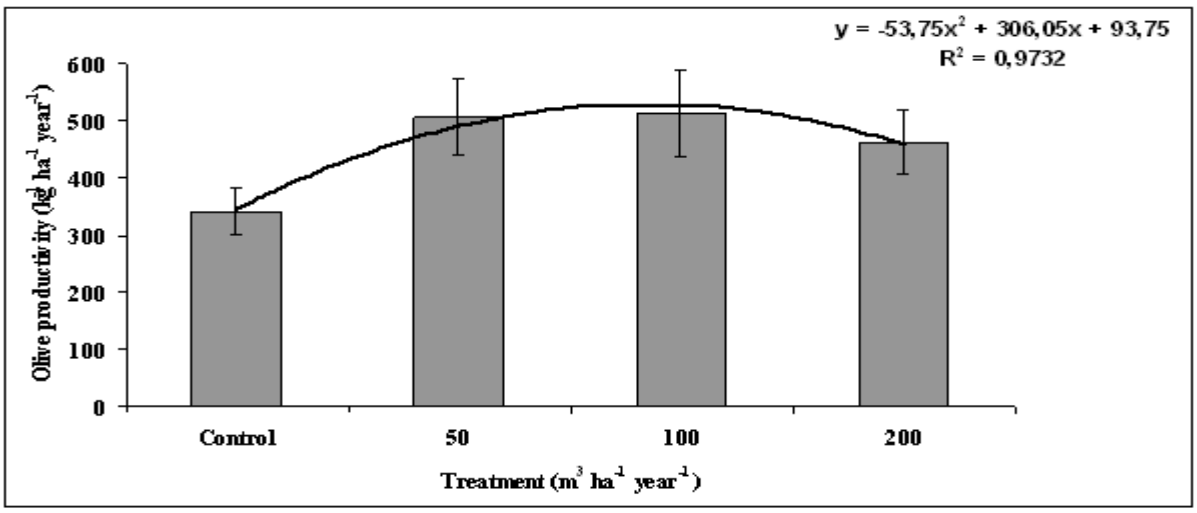

Fig. 1. Olive yield after the different annual soil treatments with OMW (average of 6 years).

For the linoleic acid content, a small decrease, but insignificant, was observed in the case of oil samples from OMW-treated plants in the crop of January 2007. Though, in January 2010, an insignificant rise was noticed. However, differences among linoleic acid content of both crop seasons showed significant increase (P = 0.004) in the case of oil samples collected from plants treated at 100 and 200 m^3 OMW ha^{-1} $year^{-1}$.

The increase of oleic acid and the decrease of the palmitic acid contents in the case of OMW-treated

plants could be due to the triacylglycerol active biosynthesis pathway, involving a decrease of relative percentage of palmitic acid content as mentioned by Salvador *et al.* (2001). Indeed, as the palmitic acid is implicated in oleic acid synthesis, the triacylglycerol biosynthesis, which is more important during late fruit ripening stage as the lipogenesis is more remarkable, induces the oleic acid increase and the palmitic acid decrease contents. On the other hand, the slight linoleic acid content decrease in OMW-treated plants during the first harvest season, appears in concordance with the slight oleic acid increase, could be related, to the oleate desaturase desactivation according to Sanchez & Harwood (2002) and/or to the disturbance of the activities of enzymes involved in the oleic acid synthesis by salt ions and phenolic compounds of OMW.

Furthermore, the olive oil fatty acid composition seems to be determined by the different environmental conditions of the experimental area, such as climatic parameters, irrigation regimes, plantation density and the experimented cultivar. Indeed, in comparison to the Fakhari and Zarrazi Douirat varieties growing in the extreme South of Tunisia (Tataouine), the Chemlali olive oil showed lower oleic acid content than those recorded in the previously mentioned varieties (73% and 76%, respectively in Fakahri and Zarrazi cvs.) (Oueslati *et al.,* 2009). The same tendency was observed while comparing the present results to those of the Spanich Cornicabra olive cultivar (80% oleic acid) conducted under different irrigation regimes (Gomez-Rico *et al.,* 2009).

The palmitoleic and stearic acids were at low proportions and didn't exceed 2.5% of total acid contents, also no significant variability was registered nor among OMW treatments neither between the crop seasons (Table 2). The same pattern was found for the linolenic acid rate (lower than 1%). According to Grati Kamoun & Khlif (2001), these fatty acids have a weak importance in oil acidic composition but represent olives varietals characterization indicators.

During both crop seasons, the unsaturated/saturated acids ratios were not influenced by the OMW dose, and differences between the determined values were not significant. Similar patterns were recorded in the case of monounsaturated/polyunsaturated acids ratios. Over both spreading periods, the comparison of these ratios showed a slight increase in the case of unsaturated/saturated ratios and a decrease of the monosaturated/polyunsaturated ratios. Nevertheless, no significant effect of OMW amendment was noticed.

Chlorophyll and carotenoid contents

Over the experimental period, the chlorophyll concentrations ranged from 0.33 to 0.48 mg kg^{-1} and from 0.32 to 0.36 mg kg^{-1} in OMW treatments and control oil sampled, respectively (Table 3). No significant differences were noted among OMW treatments and both harvest crop seasons. The same behaviour was observed in the case of the carotenoids contents.

α-Tocopherol content

The α-tocopherol content increased significantly ($P > 0.05$) for the different OMW treatments compared to the control (Table 3). Indeed, the increment rates were of 1.29, 1.29 and 1.15 times for 50, 100 and 200 m^3 ha^{-1} $year^{-1}$, respectively in comparison to the unamended treatment. Furthermore, the OMW amendment at 50 and 100 m^3 ha^{-1} presented the highest α-tocopherol content after six consecutive OMW spreading years. The increase of this antioxidant would have a positive effect on the commercial oil quality. Moreover, oil from olives submitted to OMW spreading would be nutritionally better than that obtained in the case of the control treatment.

Total phenols concentration

The total phenols contents of the virgin olive oil (VOO) were significantly influenced by the OMW treatments after the third spreading year (Table 3). Indeed, in January 2007, the increment rates of this trait were of 1.2, 3.8 and 5.4 folds, respectively in 50, 100 and 200 m^3 ha^{-1}, in comparison to the control

treatment. However, after the six spreading, the OMW doses at 50 and 100 m^3 ha^{-1} induced the highest oil phenol content. The oil phenols contents increase in samples from OMW treated plants could be considered as a response to stressing conditions after the OMW spread, this fact would induce phenolic compounds accumulation as suggested by Mechri *et al.* (2009). Consequently, these stressed circumstances would activate the phenylalanine ammonialyase (PAL), a key enzyme in phenolic compounds biosynthetic pathway, which is directly involved in the accumulation of phenols in the VOO (Romero *et al.,* 2002). The same results have been registered with salt stressed olive tree and with water deficit olive plants (Gharsallaoui *et al.,* 2011).

Furthermore, the polyphenols contents recorded in our experiment were higher than those obtained by Mechri *et al.* (2009) in olive tree cultivated in the North of Tunisia. Such differences reinforces the idea developed by Cinquanta *et al.* (1997) mentioning that phenolic compounds accumulation in olive oil is strongly determined by the agronomic (as cultivar, irrigation, plantation density, fruit maturity,…) and technological conditions (such as olive storage, extraction process).

Sterol composition

Sterol composition and total sterols contents of the different oil sampled from control and OMW treated olives after six successive OMW spreading are represented in Table 4. The results, shown that the β-sitosterol was the most abundant sterol with a slight content increase, but insignificant, was recorded in the issued OMW treated plant-oil compared to the control. The second main sterol was the campesterol with values ranging from 3.20 to 3.45 % for the different treatments without any significant difference between them. For the stigmatsterol content, a slight increment was noticed under OMW spreading conditions, in comparison to the control and the highest value was registered in the case of olive oil samples issued from 100 m^3 OMW ha^{-1}. Furthermore, all the stigmasterol concentrations were lower than those of the campesterol, as required by the IOOC (2006) standards for the extra VOO category. However, the Δ-7-stigmastenol and the cholesterol contents did not exhibit any important variability between the four treatments and remained inferior to the standard value (0.5%).

For the different oil samples, the total sterols contents were higher than 1000 mg kg^{-1}. The oil issued from the unamended olives exhibited the lowest sterols content, and the treatment 100 m^3 ha^{-1} had the highest value with an increase of 16% that of the control one; while the oils from amended soils with OMW at 50 and 200 m^3 ha^{-1} showed increases of 14.1 and 2.9%, respectively. Overall, all of the sterolic compounds and total sterols concentrations determined for the different OMW doses used were within the ranges required by the IOOC (2006) for extra VOO category.

For the Chemlali olives experimented, the comparison of the sterol percentages to those of other varieties experimented by Manai-Djebali *et al.* (2012) in Hor Kesra, Sredki, Chladmi, Betsijina and Aloui grown in Central Tunisia, showed that recorded values were comparable.

Conclusions

The results showed that the OMW spreading in the olive orchard over six successive years has beneficial effect for the management of olive mill waste and promote both quantitative and qualitative characteristics of VOO in the case of the Chemlali cultivar tested in this experiment. Indeed, the agronomic application of OMW at different levels improved the olive productivity and the different olive oil parameters such as α-tocopherol content, phenolic and sterol compositions. To our knowledge, this study is the first report on sterolic composition of olive oil after OMW amendment. Furthermore, the different oil samples are classified in the extra VOO category.

The positive effect of OMW spreading on the different studied parameters is a further evidence of the OMW use as a fertilizer for olive plantations, at least under the described experimental conditions. Indeed, these

results were recognized by the agriculture ministry of Tunisia who established a law authorizing the OMW spreading at 50 m^3 ha^{-1} once each two years (decree n° 1308-2013 of 26 February 2013).

Acknowledgements

This work was supported by the funds of the Project CFC/IOOC/04. The authors would like to thank the personnel of Oil Technology Laboratory from Olive Tree Institute of Sfax, Tunisia for their help and their contributions to the chemical analysis.

Abbreviations

OMW, olive mill wastewater; VOO, virgin olive oil; International Olive Oil Council, IOOC.

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