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Full Length Research Paper

Effects of *Phanerochaete chrysosporium* on biologic activity of soil amended with olive mill wastewaters

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Effects of the untreated olive mill wastewater (UOMW) and the bioaugmented olive mill wastewater (BOMW) with the fungus *Phanerochaete chrysosporium* (*P. chrysosporium*) for the amendment of soil were investigated. Results showed that UOMW inhibited the soil respirometric activity, while BOMW exhibited significantly higher respiration levels compared to the unamended and the UOMW amended soils. The ratio of $C\text{-}CO_2/C_{tot}$ decreased from 6.7 in the unamended soil to 6.34, to 2.74 and to 1.6 in soils amended consecutively with 50, 100 and 200 m^3 ha^{-1} of UOMW. Based on the first results, the UOMW dose of 50 m^3 ha^{-1} showed the elevated $C\text{-}CO_2/C_{tot}$ ratio in comparison with the two other doses (100 and 200 m^3 ha^{-1}) so that it was chosen to test the effects of its bioaugmentation with *P. chrysosporium* on the soil biodegradation activities. The fungus *P. chrysosporium* was added at three different forms; spores suspension (10^6 spores g^{-1}), mycelium form (cultivated in liquid medium) and colony form (mycelium fragments from solid medium).The soil amended with 50 m^3 ha^{-1} of BOMW showed higher $C\text{-}CO_2/C_{tot}$ ratio in comparison with control soil (unamended) and with soil amended with 50 m^3 ha^{-1} of UOMW. The $C\text{-}CO_2/C_{tot}$ ratio increased from 6.34 in the soil amended by 50 m^3 ha^{-1} of UOMW and 6.7 in the control soil to 27 (nearly 4 fold) in soil amended by BOMW with the spores of *P. chrysosporium*, to 18.3 (nearly 2.7 fold) in soil amended by BOMW with *P. chrysosporium* at mycelium form and to 17.5 (nearly 2.6 fold) in soil amended by BOMW with *P. chrysosporium* at colony form. These investigations illustrated that the quantity of 50 m^3 ha^{-1} of olive mill wastewater bioaugmented by *P. chrysosporium* at spores form is very advantageous choice for the stimulation of the respirometric activities of soil autochthonous microflora.

Key words: olive mill wastewater; bioaugmentation; *Phanerochaete chrysosporium*; soil; respirometric activities.

INTRODUCTION

Olive oil production is a highly important activity for the economies of Mediterranean countries. Olive mill wastewater (OMW) is the liquid by-product generated during the three-phase process of olive oil production (Mekki et al., 2009). The chemical composition of OMW is a dependent variable on the olive varieties, the harvesting period and the extraction techniques (Taccari et al., 2009). The ecological problem of OMW is mainly due to the compounds of phenolic nature, which are responsible for the dark color, phytotoxic effects and antibacterial activity (Sayadi et al., 2000; Tsioulpas et al., 2002; Fiorentiono et al., 2003; Saadi et al., 2007). The most frequently used method nowadaysis to solve the problems associated with these wastewaters is the direct application to agricultural soils as organic fertilizers (Cereti et al., 2004; Komilis et al., 2005; Mekki et al., 2006a). However, OMW application can have negative effects on the physical, chemical and biological properties of soil, with potential phytotoxicity and risk for crops as well as groundwater (Mekki et al., 2006b; Mekki et al., 2007). Recent studies have investigated the effects of untreated OMW on soil characteristics and microbial activities, and the application of OMW for the irrigation of soil represents now a controversy discussion and a debate of actuality between those that are for and those that are against this strategy (Mekki et al., 2008). The impact of OMW on soil microflora may be considered

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from two general points of view: (i) Temporary enrichment of soil with readily available Carbon source; (ii) Addition of wastewater containing inhibiting components to some microorganisms (Rana et al., 2003; Saadi et al., 2007). Many studies indicate that OMW disposal in the nature causes serious environmental problems due to its antibacterial effects and its phytotoxicity (Piotrowska et al., 2006).

Respiration measurement has been described as one of the key soil indicators for soil quality assessment (Alvarez et al., 2000). It can be performed on unamended soils (basal respiration) or on easily degradable substrate-amended soils (Montserrat et al., 2006). Soil respiration measurements are used frequently as sensitive and easy analyzable microbial parameter for the characterization of soil samples (Martens, 1995; Dilly, 2001). The respiration activity is close connected to other microbial parameters such as microbial biomass. In addition, it is used for the assessment of ecotoxic effects in contaminated soils (Hollender et al., 2003).

The present work aimed at evaluating the effect of the untreated olive mill wastewater (UOMW) and the *P. chrysosporium* bioaugmented OMW amendment on the soil microbial activities by application of soil respiration measurements. Indeed as was explained in previous works realized in the same Laboratory (Sayadi et al., 2000; Dhouib et al., 2005; Aloui et al., 2007), and the use of *P. chrysosporium* for the practical treatment of crude olive mill wastewater was investigated. *P. chrysosporium*, which belongs to an ecophysiological group of white rot fungi (WRF), secretes the extracellular enzymes, lignin peroxidases (LiP) and manganese dependent peroxidases (MnP). These enzymes degrade lignin and an extremely diverse range of aromatic compounds therefore *P. chrysosporium* can represent a good candidate to eliminate xenobiotic and recalcitrant compounds.

In this study, the main goal is to know if the basal soil microbial activity can be increased or is depressed by UOMW addition and how much the bioaugmentation with *P. chrysosporium* can remove UOMW toxicity and enhances soil respiration levels.

MATERIALS AND METHODS

UOMW origin

The fresh olive mill wastewater was taken from a three-phase discontinuous extraction factory located in Sfax, Tunisia. The characteristics of UOMW are given in Table 1.

Physicochemical analyses

The pH and the electrical conductivity were determined according to Sierra et al., (2001) standard method. Organic matter (OM) was determined by combustion of the samples in a furnace at 550°C for 4 h. Total organic carbon was determined by dry combustion. Total nitrogen was determined by Kjeldahl, (1883) method. Chemical oxygen demand (COD) was determined according to Knechtel, (1978) standard method. Five-day biochemical oxygen demand (BOD_5) was determined by the manometric method with a respirometer. Phenolic compounds (ortho-diphenols) were quantified by means of Folin-Ciocalteu colorimetric method using caffeic acid as standard at λ = 765 nm (Box, 1983).

Field experiment and soil sampling

The study area consisted in a field of olive trees located in the South-West of Sfax, Tunisia, North latitude 34° 1', East longitude 10° 20'. The mean annual rainfall is 200 mm. The field was divided into four plots. Three experimental plots P1, P2, and P3 were annually amended in February with 50, 100, and 200 m^3 ha^{-1} of UOMW respectively. The fourth plot, plot C (P_C), was not amended and served as control. Soil samples (S_C, $S_{UOMW-50}$, $S_{UOMW-100}$ and $S_{UOMW-200}$) were collected (in February 2008) from different parts of each plot from 0 to 20 cm deep, using a soil auger. All soil samples, taken from each plot were then mixed, air-dried, sieved with a mesh size of 450 µm and stored at 4°C prior to use.

Physicochemical soil analyses

The pH, electrical conductivity, OM and total nitrogen of soil were determined according to the same methods as OMW. Inorganic nitrogen was determined as stated by Kandeler, (1995). Organic nitrogen was determined by the difference between total and inorganic nitrogen. Phosphorus, iron, magnesium, potassium, sodium and chloride were determined by atomic absorption.

For physicochemical analyses, three replications were used for each parameter. Data were analysed using the ANOVA procedure using Genstat 5 (second edition for windows). Variance and standard deviation were determined.

Culture conditions of *Phanerochaete chrysosporium*

The strain used in this study was *P. chrysosporium* HD; a monoconidiosporous isolate from strain BKM-F-1767 (ATCC 24725). It was maintained at 4°C on 2% malt extract broth slants. Subcultures were routinely made every 2 months. The basal medium used for the cultivation of *P. chrysosporium* contained (per kg): KH_2PO_4: 1 g; $CaCl_2 \cdot 2H_2O$: 0.07 g; $MgSO_4 \cdot 7H_2O$: 0.35 g; $FeSO_4 \cdot 7H_2O$: 0.035 g; $ZnSO_4 \cdot 7H_2O$: 0.023 g and $CuSO_4 \cdot 5H_2O$:0.0035 g. This culture medium was buffered to pH 6.5 with di-sodium-tartrate (20 mM). Veratryl alcohol was added to 0.4 mM, the carbon source was glycerol (10 g l^{-1}) and the nitrogen source was ammonium tartrate at 20 mM of nitrogen (Aloui et al., 2007).

Respirometric tests

Soil respiration was determined by placing 100 g of each field moist soil in an hermetically sealed 2 L glass jar equipped with a CO_2 trap (a glass tube containing 2 ml of 4 N NaOH). At first time (soils samples were taken every 2 days during 31 days) respirometric measurements were down for soil samples from P1, P2, P3 and C (S_C, $S_{UOMW-50}$, $S_{UOMW-100}$ and $S_{UOMW-200}$). At second time (soils samples were taken every 2 days during 31 days) respirometric measurements were optimized for soil samples from P1 ($S_{UOMW-50}$) wich are bioaugmented by *P. chrysosporium* at three different forms; spores suspension (10^6 spores g^{-1}), at mycelium form and at colony form. Incubations of different soil samples were carried out in the dark at 25°C. The CO_2 evolved were determined by titrating 1 ml of the NaOH solution with 0.5 N HCl (Ohlinger, 1995).

Table 1. Physico-chemical characteristics of untreated olive mill wastewater (UOMW).

Characteristics	UOMW
pH (25°C)	5.3 ± 0.2
Electrical conductivity (25°C) (dS m^{-1})	8.7 ± 0.1
Chemical oxygen demand (g l^{-1})	74 ± 2.8
Biochemical oxygen demand (g l^{-1})	14 ± 0.9
COD/BOD_5	5.28 ± 0.52
Salinity (g l^{-1})	6.65 ± 0.66
Water content (g l^{-1})	946 ± 17.2
Total solids (g l^{-1})	54 ± 2.98
Mineral matter (g l^{-1})	7.5 ± 0.43
Volatile solid (g l^{-1})	46.5 ± 1.85
Total organic carbon (g l^{-1})	26.52 ± 1.18
Total nitrogen Kjeldahl (g l^{-1})	0.7 ± 0.06
Carbon/Nitrogen	37.9 ± 1.2
P (mg l^{-1})	38 ± 1.2
Na (g l^{-1})	0.98 ± 0.09
Cl (g l^{-1})	1.8 ± 0.16
K (g l^{-1})	9 ± 0.8
Ca (g l^{-1})	1.4 ± 0.12
Fe (mg l^{-1})	33 ± 2.9
ortho-diphenols (g l^{-1})	9.4 ± 0.8
Toxicity by LUMIStox (% I_B)	99 ± 9

For respirometric soil analyses, each soil was incubated in duplicate, and measurements were realized in triplicate for each soil sample. Data were analysed using the ANOVA procedure using Genstat 5 (second edition for windows). Variance and standard deviation were determined.

RESULTS

The characteristics of UOMW

As shown in Table 1, the high pollutant load of UOMW and its acidity could be observed. UOMW totally inhibited *Vibrio fischeri* bioluminescence activity (99% I_B). This toxicity was essentially due to its high content of phenolics compounds (9.4 g l^{-1}), elevated COD (74 g l^{-1}) and BOD_5 (14 g l^{-1}) and prominent C/N ratio (37.9).

Soil characterization

As described in a previous study (Mekki et al., 2006a), the soil of the study area had an important content of active calcareous (0.6%w/w) at the surface, and was composed of sand (89.82% w/w), clay (7.44% w/w) and silt (2.74% w/w). It had an alkaline pH (7.9) and a weak salinity (69 mg kg^{-1} dry soil). The soil was very poor in nitrogen (0.5 g kg^{-1} dry soil) and organic matter (0.16%w/w). The levels of potassium and phosphorus were 0.014 and 0.002% w/w, respectively. As it was discussed in that previous study, the soil water content was very low and it varied between 0.8 and 1.15% w/w.

Effects of UOMW amendment on the physicochemical and respirometric soil parameters

The effect of UOMW on some soil physicochemical parameters such as pH, salinity, OM), nitrogen, C/N ratio and respirometric activities were studied. In spite of the initial UOMW acidity (Table 1), the follow-up of this parameter showed that UOMW provoked no significant reduction in the soil pH with the dose of 50 m^3 ha^{-1} (0.1 U), but pH decreased significantly with the dose 100 m^3 ha^{-1} (0.3 U) and 200 m^3 ha^{-1} (0.5 U). Similarly, UOMW application enlarged soil salinity, and this increase was proportional to the added UOMW quantity. Indeed, the soil salinity increased from 69 mg kg^{-1} dry soil in unamended soil to 240, 367 and to 448 mg kg^{-1} dry soil in soils amended with 50, 100 and 200 m^3 ha^{-1} of UOMW respectively. On the other hand, results showed that UOMW amendment improved the soil organic and mineral matter's contents and these improvements were in a straight line to the added UOMW quantities.

Respirometric measurements were achieved on soils amended with different doses of UOMW in comparison with control soil. As shown in Figure 1, CO_2 production increased with added UOMW in comparison with unamended soil, however more pronounced CO_2

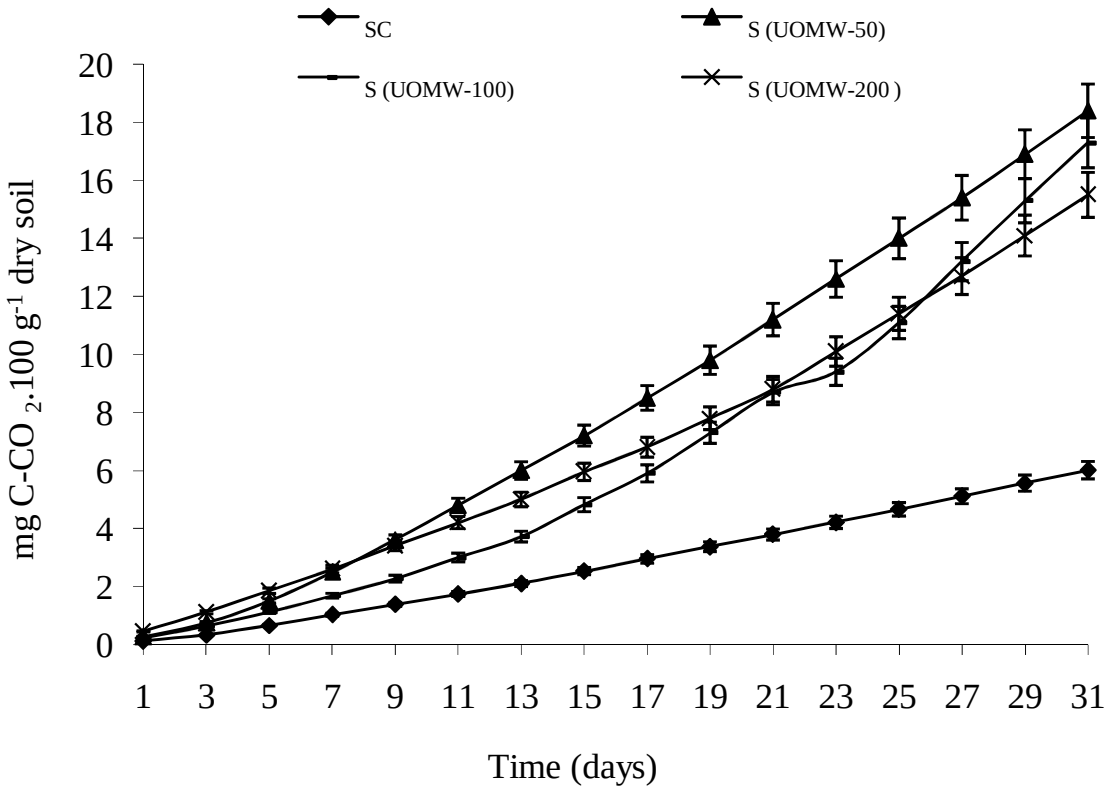

Figure 1. C-CO_2 production evolution as a function of UOMW amended soils and time.

production rate was shown for soil amended with 50 m^3 ha^{-1}. For the two other doses (100 and 200 m^3 ha^{-1}), the CO_2 production within 31 days of experimentation were comparable (Figure 1). Soils amended with 100 and 200 m^3 ha^{-1} of UOMW showed an augment in their carbon contents (C_{tot}) to 6.3 mg 100 g^{-1} dry soil (7 folds in comparison with control soil) and to 9.8 mg 100 g^{-1} dry soil (10.9 folds in comparison with control soil) respectively, whereas their specific respiration rate (expressed as the ratio of C-CO_2/C_{tot}) remained very low (2.7 and 1.6) correspondingly (Figure 2). However, the soil amended with 50 m^3 ha^{-1} of UOMW showed very important C-CO_2/C_{tot} ratio (6.34) in comparison with the two other doses (Figure 2) so that UOMW at 50 m^3 ha^{-1} was selected to test the effects of its bioaugmentation with *P. chrysosporium* on the soil respirometric activities.

Effects of BOMW on the respirometric soil activities

The effects of OMW bioaugmented by *P. chrysosporium* (BOMW) on the soil respirometric activities were investigated. The UOMW at 50 m^3 ha^{-1} was selected for this experiment. The fungus *P. chrysosporium* was added at three different forms; spores suspension, at mycelium form and at colony form. As revealed in Figure 3, more pronounced CO_2 production rate was shown for soil amended by 50 m^3 ha^{-1} of BOMW (at different forms of *P. chrysosporium*) in comparison with soil amended by 50 m^3 ha^{-1} of UOMW or unamended soil. Similarly higher C-CO_2/C_{tot} ratios were also shown for BOMW amended soils (Figure 4). The C-CO_2/C_{tot} ratio increased from 6.34 in the soil amended by 50 m^3 ha^{-1} of UOMW and 6.7 in the control soil to 27 (nearly 4 folds) in soil amended by BOMW with *P. chrysosporium* (spore suspension) to 18.3 (nearly 2.7 folds) in soil amended by BOMW with *P. chrysosporium* (mycelium) and to 17.5 (nearly 2.6 folds) in soil amended by BOMW with *P. chrysosporium* (colony) (Figure 4). These results illustrated that the bio-augmentation of 50 m^3 ha^{-1} UOMW by *P. chrysosporium* In spore suspension form was very beneficial for the stimulation of the respirometric (therefore the biodegradation) activities of soil autochthonous microflora.

DISCUSSION

Several studies have been devoted to develop efficient treatment technologies for OMW through various kinds of physicochemical and biological pretreatments (Azbar et al., 2004; Dhouib et al., 2005; Mantzavinos and

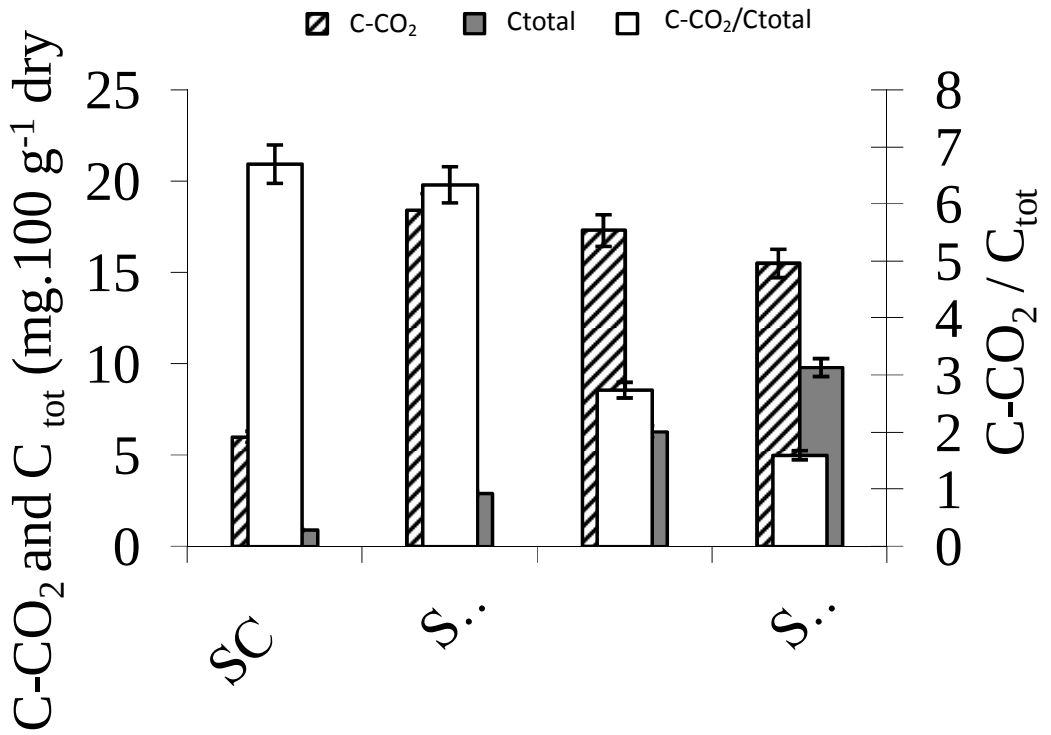

Figure 2. Soils specific respiration as a function of UOMW amended soils in comparison with control soil.

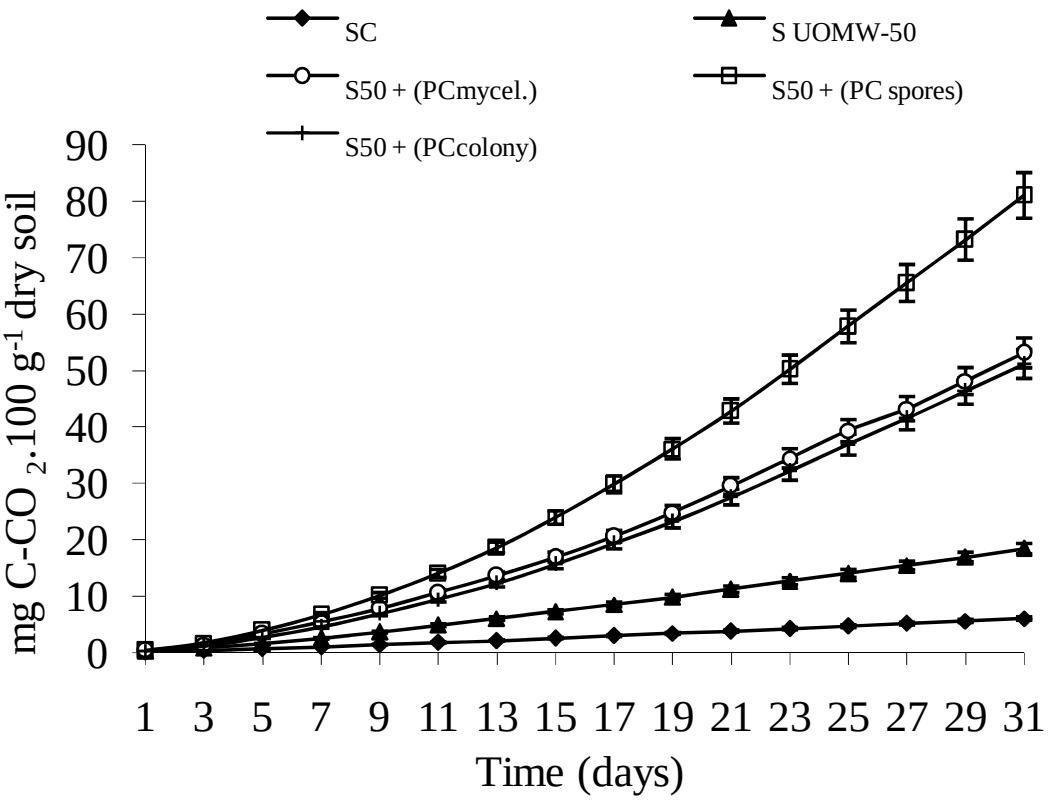

Figure 3. C-CO_2 production evolution as a function of BOMW amended soils and time.

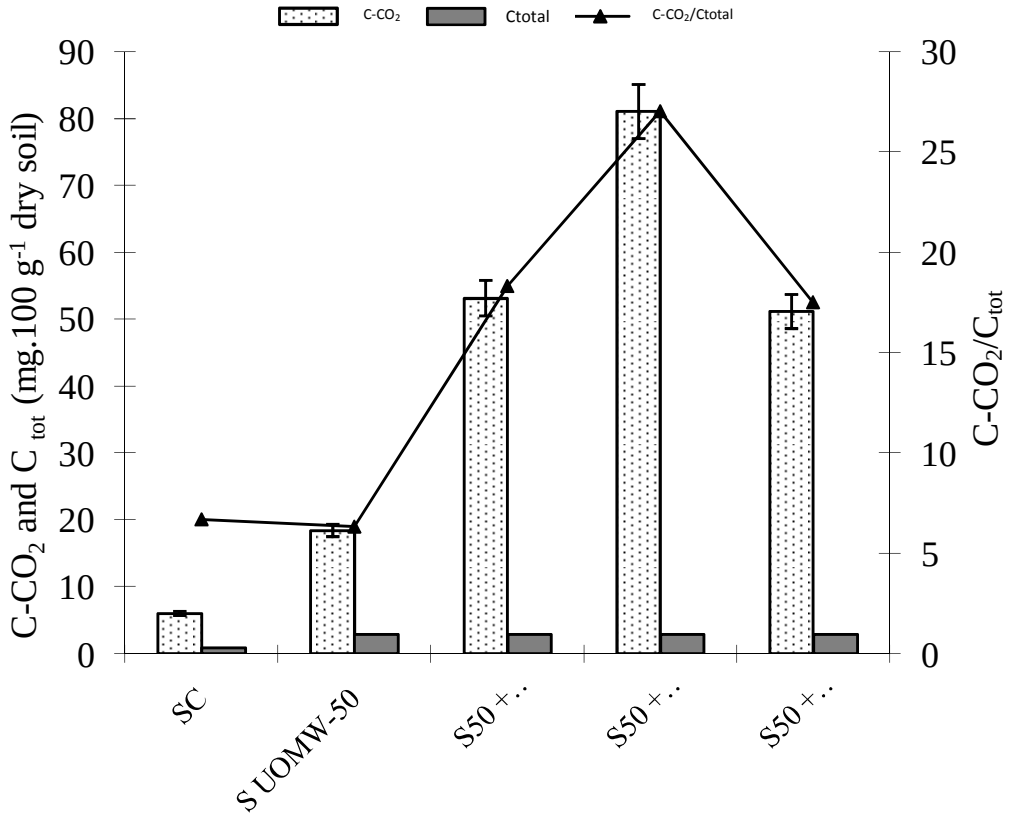

Figure 4. Soils specific respiration as a function of BOMW amended soils in comparison with control soil.

Kalogerakis, 2005; Khoufi et al., 2006). Yet, such systems are in many cases not economical, considering the short olive oil season, the typical biennial olive harvest cycle, and the fact that many olive mills are small and isolated (Saadi et al., 2007). Results presented here showed that several chemical and biochemical properties of the investigated soils changed in response to UOMW and BOMW application. The acidity of the UOMW was compensated by the soil carbonate alkalinity as given away by Sierra et al., (2001). However, there was a significant decrease of the initial soil pH when UOMW amended quantity increased. The raise in the soil salinity could result from the main ionic species (Na, Cl and SO_2), which came from UOMW (Zenjari and Nejmeddine, 2001). OMW also had a high potassium concentration and notable levels of phosphorus, calcium, magnesium and iron (Paredes et al., 1999). According to Saadi et al., (2007), extremely high organic load and the toxic nature of OMW prevented their direct discharge into domestic wastewater treatment systems so that land spreading of OMW at rates of 50 – 100 m^3 ha^{-1} $year^{-1}$ could be considered as a mean of waste disposal with potential fertilization value. These results were in line with the findings of Rinaldi et al. (2003) who stated that one alternative and economical solution was controlled land application of OMW. In fact, the Italian law already permits annual spreading of up to 50 or 80 m^3 ha^{-1} for OMW generated by press or continuous centrifugation method, respectively. Achak et al. (2009) reported that the OMW acidity was due to the presence of phenolic and fatty acids, subsequently the application of this effluent to soils could accumulate salts and phytotoxic compounds, change pH and leach nutrients that could contaminate the ground water source. In another hand, Aggelis et al. (2003) reported that OMW dilution could reduce significantly its initial toxicity.

The ecological problem of OMW is mainly due to the compounds of phenolic nature, which are responsible for the dark color, phytotoxic effects and antibacterial activity. The use of *P. chrysosporium* for the practical treatment of OMW was investigated because this fungus could significantly reduce the color of this effluent and degrade the high and low molecular-mass aromatics compounds (Sayadi et al., 2000). Wang et al, (2008) reported that WRF had a good ability to degrade some non-degradable compounds, such as DDT and benzo(a)pyrene. According to the same authors, the WRF were a physiological rather than a taxonomic group,

referring to those fungi that were capable of degrading lignin. It was reported that the characteristics of these fungi are non-specific, non-stereospecific, and of an extracellular nature. The mechanism of these fungi used to degrade pollutants was mainly due to the special enzyme system including lignin peroxidases (LiP), manganese peroxidases (MnP), and laccase. These were in line with the findings of Taccari et al., (2009) who mentioned that WRF had an extracellular enzymatic system (LiP; MnP, and laccase) that was characterized by low substrate specificities and the ability to degrade and metabolise polymeric lignin and a broad range of organopollutants. As reported by Xiong, et al. (2008) the ligninolytic enzymes were non specific enzymes and could assist in the degradation of a wide variety of recalcitrant organic pollutants, such as polycyclic aromatic hydrocarbons (PAHs), pesticide and dyes. Mancera-Lopeza et al. (2008) stated that bioremediation was based on the capacity of microorganisms to degrade organic pollutant compounds, such as hydrocarbons. However, most hydrocarbon degradation studies have been carried out using WRF such as *P. chrysosporium, Pleurotus ostreatus* and *Trametes versicolor* (Sayadi et al., 2000; Dhouib et al., 2005; Aloui et al., 2007). Zheng and Obbard (2002) reported that the lignin-degrading WRF *P. chrysosporium* had the ability to degrade a wide variety of organopollutants such as polycyclic aromatic hydrocarbons due to its non-specific extracellular enzymes. In the same way, the authors confirmed that *P. chrysosporium* acted synergistically with soil indigenous microorganisms in the oxidation of low molecular weight PAHs. These investigations were aligned with our results viewing that the bioaugmentation of 50 m^3 ha^{-1} by *P. chrysosporium* (spore suspension) was the very beneficial for the stimulation of the respirometric and consequently the biodegradation activities of soil autochthonous microflora. Moreover, Dzul-Puc et al., (2005) demonstrated that the most efficient producers of oxidative extracellular enzymes were the WRF particularly *P. chrysosporium*. Briefly, respiration measurements have been described as one of the key soil indicators for soil quality assessment. It can be performed on unamended soils (basal respiration) or on easily degradable substrate-amended soils (Montserrat et al., 2006).

Conclusions

The impact of the OMW residues on soil properties is the result of opposite effects, depending on the relative amounts of beneficial and toxic organic and inorganic compounds present. Treatment of OMW before their application on soil is therefore necessary to limit negative impact on the soil biological activity.

Based on our results, OMW bioaugmented by the WRF *P. chrysosporium* at spore suspension is advantageous choice in favor of the stimulation of the respirometric and therefore the biodegradation activities of soil autochthonous microflora.

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