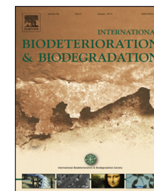
International Biodeterioration & Biodegradation 89 (2014) 15–22

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod



Assessing the impact of biotransformed dry olive residue application to soil: Effects on enzyme activities and fungal community

José A. Siles [a,*], Daniel Pérez-Mendoza [a], José A. Ibáñez [b], José M. Scervino [c], Juan A. Ocampo [a], Inmaculada García-Romera [a], Inmaculada Sampedro [a,d]

[a] *Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Prof. Albareda 1, E-18008 Granada, Spain*

[b] *Department of Marketing and Market Research, Facultad de CC. Económicas y Empresariales, Campus Universitario de Cartuja s/n, Granada, Spain*

[c] *Department of Experimental Biology and Biodiversity (BBE),FCEyN, UBA Universidad de Buenos Aires, Ciudad Universitaria, Microbiología de Suelos, 1428, Argentina*

[d] *Thayer School of Engineering, Dartmouth College, 14 Engineering Drive, Hanover, NH 03755, USA*

ARTICLE INFO

Article history:
Received 22 November 2013
Received in revised form
3 January 2014
Accepted 5 January 2014
Available online

Keywords:
"Alpeorujo"
Bioremediation
Olive waste
Organic amendment
qPCR
Soil microbial ecology

ABSTRACT

Dry olive residue (DOR), a solid by-product of the two-phase olive oil extraction system, is rich in organic matter and nutritionally important compounds. However, the agronomic application of this residue may impact negatively on the soil ecosystem due to its toxic components. The aim of the present study was to investigate the impact of raw DOR, *Coriolopsis floccosa*-transformed DOR and *Fusarium oxysporum*-transformed DOR on soil biological properties. To do this, soil enzyme activities, fungal community size (quantitative PCR) and fungal community structure (DGGE of 18S rRNA gene) were measured. The impact of biotransformed and nonbiotransformed DOR applications to soil depended on two factors: the variable sensitivity of the soil to the residue's composition and the duration of exposure to amendments. The application of this biotransformed residue enhanced soil enzyme activities (phosphatase, β-glucosidase and urease) with respect to soil amended with nonbiotransformed residue. The quantification of the 18S rRNA gene copy number indicated that the different amendments stimulated relative abundance. DGGE analysis showed that the amendments produced changes in fungal community structure although variations in fungal diversity were only detected after *C. floccosa*-transformed DOR addition at 60 days, probably due to the enhancement of species such as *Chaetomium globosum* and *Chalazion helveticum*.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Mediterranean soils are subject to degradation caused by organic matter loss. Soil organic matter constitutes an important source of nutrients, and its maintenance is important for the long-term productivity of agroecosystems. The excessive use of mineral fertilizers has contributed to a general reduction in soil organic matter content, with a consequent decline in the quality of agricultural soils. This negative effect of agricultural practices could be reversed by the appropriate use of manure and/or crop residues in cropping systems, either alone or in combination with mineral fertilizers (Mandal et al., 2007). However, the effect of these residues on soil properties depends on their principal components and can alter soil biological activity (Chaves and Oliveira, 2004).

In the world's olive growing regions, the two-phase olive oil extraction system, after the transformation of the wet primary residue, generates enormous amounts of dry olive residue (DOR) or "alpeorujo" over a short period of time (Morillo et al., 2008). Disposal of this waste may cause a significant environmental problem due to its high phenol content (Tortosa et al., 2012). Among the strategies for the management of this residue is its use as an organic amendment due to its high organic matter content and being free of pathogenic microorganisms as well as heavy metals. However, despite its potential agronomic value, soil amendments containing DOR are also known to have phytotoxic and antimicrobial properties (Sampedro et al., 2009). This residue's detoxification and organic matter stabilization through incubation with saprobic fungi could resolve the problem of its disposal to soil (Sampedro et al., 2007), enrich soils with limited organic matter and improve physical and chemical properties.

* Corresponding author. Tel.: +34 958 181600; fax: +34 958 129600.
E-mail addresses: josesimartos@gmail.com, josesimartos@hotmail.com (J. A. Siles).

0964-8305/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ibiod.2014.01.001

Soil fungi usually contribute the largest proportion of soil microbial biomass. Furthermore, these microorganisms play an important role in decomposition, carbon and nitrogen storage, biogeochemical cycles, soil stabilization, plant parasitism and also influence plant community composition through symbiotic and parasitic relationships (Bills et al., 2004). Additionally, fungi are capable of degrading many recalcitrant compounds due to their efficient enzymatic machinery (Eastwood et al., 2011). However, despite the importance of these microorganisms with respect to soil functionality, studies of soil fungi represent only about 30% of the total number of surveys of soil microbial communities reported in the literature (Chemidlin Prévost-Bouré et al., 2011). For these reasons, it is essential to determine soil fungal responses when organic and inorganic fertilization is applied.

Information concerning the impact of saprobic-fungi transformed DOR on soil biological properties is very limited. Consequently, this study aimed to investigate the short-term effect of raw DOR, *Coriolopsis floccosa*-transformed DOR and *Fusarium oxysporum*-transformed DOR on soil enzyme activities and fungal community after 0, 30 and 60 days of treatment. Five soil enzymes (phosphatase, urease, protease, β-glucosidase and dehydrogenase) involved in the P, N, and C cycles were analyzed, and the dynamics of structure and relative abundance of fungal community after application of the different amendments were assessed by means of quantitative PCR (qPCR) and denaturing gradient gel electrophoresis (DGGE).

2. Materials and methods

2.1. Materials

The soil used in this study was taken from the "Cortijo Peinado" field (Fuente Vaqueros, Granada, Spain, 37°13'N, 3°45'W). It was a loam-type soil with the following principal properties: clay, 17.15%; sand, 34.35%; silt, 48.50%; pH, 8.40; total organic carbon, 10.67 g kg^{-1}; water soluble carbon, 4.83 g kg^{-1}; total nitrogen, 1.52 g kg^{-1}; P, 589.78 mg kg^{-1}; K, 8.63 g kg^{-1}; Ca, 61.90 g kg^{-1}; Cd, 1.44 mg kg^{-1}; Cr, 39.27 mg kg^{-1}; Fe, 20.97 g kg^{-1}; Cu, 30.28 mg kg^{-1}; Mg, 17.66 g kg^{-1}; Mn, 435.92 mg kg^{-1}; Na, 1.78 g kg^{-1}; Ni, 26.88 mg kg^{-1}; Zn, 73.24 mg kg^{-1}; Pb, 26.49 mg kg^{-1}; phenols, 2.16 g kg^{-1}.

DOR was obtained from an olive oil manufacturer (Sierra Sur S.A., Granada, Spain). The main chemical characteristics of DOR were: ashes, 91 g kg^{-1}; C/N, 31.74; cellulose, 152 g kg^{-1}; fats, 21.7 g kg^{-1}; hemicellulose, 131 g kg^{-1}; lignin, 249 g kg^{-1}; pH, 4.58.

2.2. Organisms and inoculum preparation

The used fungi were *Coriolopsis floccosa,* formerly known as *C. rigida,* (Spanish Type Culture Collection, CECT 20449^T) isolated from beech wood and *F. oxysporum* (Mycological Culture Collection of the Department of Biological Sciences, Faculty of Exact and Natural Sciences, University of Buenos Aires, BACF 738^T) isolated from maize rhizospheric soil. Both fungi were maintained at 4 °C and routinely subcultured each month on potato dextrose agar slants. Inoculum preparation and incubation conditions were as previously reported by Sampedro et al. (2009). Polyurethane sponge (PS) cubes, each with a width of 0.5 cm, were rinsed with water in a 1:20 (w/v) ratio and autoclaved (121 °C for 20 min) twice prior to use. Five milliliters of the inoculum (ca. 50 mg of dw) was aseptically added to 50 g of sterilized PS and incubated at 28 °C for 7 days.

2.3. DOR biotransformation

Deionized water was added to DOR in order to obtain a moisture content of 25% (w/w) prior to sterilization (3 cycles in autoclave at 120 °C for 20 min). The colonized PS cubes (0.24 g) were then covered with 25 g of DOR. Solid-state cultures on DOR were carried out at 28 °C in the dark under stationary conditions for 30 days. Non-inoculated and sterilized DOR samples, prepared and incubated as described above, are referred to as controls. All the treatments used in the experiment were sterilized and added to soil in pots.

The chemical characterization of the nonbiotransformed and biotransformed DOR by saprobic fungi has been previously reported (Siles et al., submitted for publication).

2.4. Soil amendment

The soil amendment was carried out using 0.5 L pots containing non-sterilized soil. Nonbiotransformed DOR (DOR) and DOR biotransformed by *C. floccosa* (CORDOR) and *F. oxysporum* (FUSDOR) were applied to the soil pots at concentrations of 50 g kg^{-1}. Control samples without the amendment were also prepared. A sorghum plant (*Sorghum bicolor*) was planted in each pot. The experiment was carried out in a greenhouse with natural and supplementary light at 25/19 °C and 50% relative humidity. The experiment was watered regularly throughout the experiment. Regular watering throughout the experiment ensured that water content of samples was maintained at 15–20%.

The control soil and soil amended with DOR, CORDOR and FUSDOR were collected after 0, 30 and 60 days of treatment. The experiment consisted of five pots of each treatment at all sampling time. In each soil sampling, the soil of the five pots was mixed, homogenized and sieved (2 mm mesh). Subsequently, three 100 g soil subsamples for each treatment were placed in sterile Falcon™ tubes. The samples were stored at 4 °C prior to processing (1–2 days) for enzymatic activity assays and at −80 °C prior to molecular analyses.

The plants of all the treatments at 30 and 60 days were harvested. The shoot dry weight of sorghum plants was measured after being kept for 48 h in a dried oven.

2.5. Enzymatic analyses

Urease activity (E.C. 3.5.1.5) was analyzed using the procedure developed by Kandeler and Gerber (1988). Briefly, 2.5 g of fresh soil was incubated with 1.25 mL 0.08 M aqueous urea solution for 4 h at 37 °C. The NH_4^+ produced was extracting with 1 M KCl and 0.01 M HCl and quantified by means of a modified indophenol reaction. Protease activity (EC 3.4.2.21–24) was determined according to the method described by Ladd and Butler (1972). 1 g of soil was incubated with 5 mL of 2% Na-casein and 5 mL of 0.05 M Tris (2-Amino-2-hydroxymethyl-propane-1,3-diol) buffer (pH 8.1) for 2 h at 50 °C. The reaction was stopped after addition of 15% trichloroacetic acid solution (TCA). The suspension was centrifuged and the supernatant (5 mL) treated with 7.5 mL of a mixture of 0.06 M NaOH, 5% Na_2CO_3, 0.5% $CuSO_4 \cdot 5H_2O$, 1% potassium sodium tartrate and 5 mL of 33% Folin-Ciocalteu reagent. The absorbance was determined at 700 nm. The activities of alkaline phosphatase (EC 3.1.3.1) and β-glucosidase (EC 3.2.1.21) were determined according to the methods described by Eivazi and Tabatabai (1977, 1988), respectively. Briefly, 1 g of soil was mixed with 5 mL of buffered substrate solution incubated for 2 h at 37 °C. The following substrate concentrations and buffers were used: acid phosphatase, 0.025 M *p*-nitrophenyl phosphate in 0.1 M modified universal buffer (MUB) (pH 11); β-glucosidase, 0.025 M *p*-nitrophenyl β-D-glucopyranoside in 0.1 M MUB (pH 11). Enzymatic reactions were stopped by transferring the mixtures to a freezer and holding them there for 10 min. Concentrations of *p*-nitrophenol originated were determined at 400 nm after addition of 4 mL 0.5 M NaOH and 1 mL 0.5 M

$CaCl_2$ for acid phosphatase; 4 mL 0.1 M Tris buffer (pH 12) and 1 mL of 0.5 M $CaCl_2$ for β-Glucosidase. Dehydrogenase activity (E.C. 1.1) was analyzed using the procedure described by Carmiña et al. (1998). 1 g of soil was incubated with 2 mL of 0.5% iodonitrotetrazolium violet (INT) as substrate and 1.5 mL of 1 M Tris buffer (pH 7.5) during 1 h at 40 °C. Subsequently, iodonitrotetrazolium formazan (INTF) produced was extracted with a 1:1 (v:v) mixture of ethanol and dimethylformamide and measured spectrophotometrically at 490 nm.

2.6. DNA extraction and PCR-DGGE analysis

Total DNA was extracted from 250 mg of soil using the bead-beating method, following the manufacturer's instructions for the MoBio UltraClean Soil DNA Isolation Kit (MoBio Laboratories Inc., Solana Beach, CA, USA). PCR was performed with the aid of 18S rRNA gene universal fungal denaturing gradient gel electrophoresis (DGGE) primers FR1 and FF390 under the conditions as previously described by Vainio and Hantula (2000). The 5′ end of primer FR1 had an additional 40-nucleotide GC-rich sequence (GC clamp) to facilitate separation by DGGE.

DGGE analyses were conducted using 10 μL of PCR product loaded into a 30–50% urea-formamide-polyacrylamide gel. An INGENYphorU System (Ingeny International BV, The Netherlands) was run at 85 V for 16 h at 60 °C to separate the fragments. Gels were stained with SYBR Gold (Invitrogen, Carlsbad, CA, USA) in 1x TAE for 45 min at room temperature and visualized under UV light. DGGE banding patterns were digitized and processed using InfoQuest FP software (Bio-Rad Laboratories, Inc., Hercules, USA).

2.7. Quantification of soil fungal community

Quantitative PCR was carried out in order to determine the 18S rRNA gene copy number in triplicate soil-DNA extracts. The primers FR1 and FF390 were used to amplify a fragment of the 18S rRNA gene as described by Vainio and Hantula (2000). After hot-start enzyme activation, reaction cycles were carried out at 95 °C for 30 s, 58 °C for 45 s and 72 °C for 2 min. Determination of the DNA copy number was carried out using an iCycler iQ5 (Bio-Rad, Hercules, CA, USA). A standard curve was generated using a recombinant plasmid containing one copy of the target 18S rRNA gene. The curve was drawn by plotting the Ct value as a log function of the copy number of 10-fold serial dilutions of the plasmid DNA. The relationship between Ct and the target-gene copy number on the one hand and the copy numbers of the real-time standard on the other were calculated as previously described by Quian et al. (2007).

2.8. Cloning and sequencing

Different bands were excised from DGGE gels and sequenced. DNA fragments from DGGE bands were isolated by electroelution in dialysis bags. Reamplification of the eluted DNA by PCR was conducted as indicated above except that the FR1 primer did not have a GC clamp at the 5′ end. Purified PCR products were ligated and cloned into pCR-XL-TOPO (Invitrogene) according to the manufacturer's instructions. Positive clones were subsequently screened in DGGE gels by checking their mobility against the banding pattern of the original soil sample. Two positive clones were used for DNA sequencing which was carried out by the Instrumental Technical Services of EEZ-CSIC, Granada, using the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems).

The sequences obtained were edited using Bioedit 7.0.5.3 (Ibis Biosciences, CA; USA) and GeneDoc 2.5 software and compared with the GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) database using the basic local alignment search tool (BLAST). Sequences from this study were submitted to the GenBank database and their accession numbers are listed in Table 2.

2.9. Statistical treatment of data

The PAST software package was used to calculate: species richness (*S*), the Shannon index (*H*) and evenness (*J*) (Xu-Cong et al., 2012). Cluster analysis of the different samples was performed using UPGMA (unweighted pair group method with arithmetic means) with a Euclidean distance matrix, taking into account the presence or absence of individual bands.

PCA analysis was also carried out on the enzymatic and biological properties of the soil to determine a new set of uncorrelated variables which may synthesize the information originally contained in the parameters recorded (Ramette, 2007). The main PCA results were also plotted together with the experimental conditions of the soil (amendment type and time) in order to identify and explain any important variation patterns.

3. Results and discussion

3.1. Effect of DOR amendments on enzymatic activities and plant growth

Microbial community activities are closely related to soil fertility and environmental quality. In the present study, the microbial activity of soil was analyzed using four hydrolases (phosphatase, β-glucosidase, urease and protease) and one oxidoreductase (dehydrogenase). All the enzymatic activities tested were significantly affected by each DOR amendment at different exposure times (0, 30 and 60 days) (Fig. 1).

Among the hydrolases, phosphatase activity is an effective index of the quality and quantity of organic matter in the soil. In the present study, there was generally a higher level of phosphatase activity in soils amended with all DOR treatments after 30 days of exposure (Fig. 1A). Various studies have shown that this enzymatic activity increases as a consequence of organic fertilization (Chakrabarti et al., 2000). The increase in phosphatase activity can be explained by an increment in organic P (principal substrate for the activity of this enzyme) after addition of the different amendments, as other studies have reported that DOR application to soil involves an increase in available P (López–Piñeiro et al., 2011). At the end of the soil treatment process, the treatments with CORDOR and FUSDOR also showed higher levels of phosphatase activity than unamended soil. However, phosphatase activity decreased in the samples treated with DOR, which may be due to the direct

Table 1
Diversity indices (species richness-*S*, Shannon index-*H*, evenness-*J*) retrieved from the DGGE profiles of 18S rRNA gene in unamended soil (C) and soil amended with untransformed DOR (DOR), *C. floccosa*-transformed DOR (CORDOR) or *F. oxysporum*-transformed DOR (FUSDOR) at 0 (T0), 30 (T1) and 60 (T2) days.

	S	*H*	*J*
C-T0	23	3.027	0.965
DOR-T0	22	3.018	0.977
CORDOR-T0	23	3.034	0.968
FUSDOR-T0	24	3.036	0.955
C-T1	24	3.150	0.991
DOR-T1	26	3.186	0.978
CORDOR-T1	25	3.156	0.980
FUSDOR-T1	26	3.148	0.966
C-T2	23	3.104	0.990
DOR-T2	25	3.131	0.973
CORDOR-T2	28	3.274	0.983
FUSDOR-T2	25	3.129	0.972

Table 2
Identification of dominant bands in DGGE analysis of soil amended with DOR bioremediated with *C. floccosa* at 60 days and the closest match to the sequence from GenBank database with BLAST and taxonomic affiliation.

Band number	Accession no.	Closet relative (accession no.)	Alignment, % similarity	Taxonomic affiliation
Band 1	KC147705	Uncultured soil fungal (DQ157217)	380/390, 99	Uncultured soil fungus
Band 2	KC147708	*Chalazion helveticum* (AF061716)	380/390, 99	*Chalazion helveticum*
Band 3	KC147709	Uncultured soil eukaryote (EF100353)	380/390, 98	Uncultured soil fungus
Band 4	KC147710	*Chaetomium globosum* (JN639021)	380/390, 99	*Chaetomium globosum*
Band 5	KC147711	Uncultured soil fungal (HM104512)	380/390, 100	Uncultured soil fungus
Band 6	KC147713	Uncultured soil fungal (EF628728)	380/390, 99	Uncultured soil fungus
Band 7	KC147714	*Sporormia lignicola* (EU263612)	380/390, 99	*Sporormia lignicola*

inhibition of toxic compounds in DOR or to the formation of complexes containing humic compounds (De La Horra et al., 2005).

β-glucosidase cleaves β-1,4 bonds to produce glucose from β-glucosides, which is an important reaction in terrestrial C cycling involving the recycling of soil organic matter (Cañizares et al., 2011). It also provides information on the potential toxicity of olive wastes (López–Piñeiro et al., 2011). In this survey, β-glucosidase activity increased after soil amendment with CORDOR and FUSDOR at 30 and 60 days, with similar results being reported by Benitez et al. (2004) after application of composted olive wastes to soil. This increment is indicative of the soil microorganisms' capacity to use carbohydrate material contained in these amendments. However, no increment in β-glucosidase activity was observed after soil treatment with DOR although this residue presented high levels of decomposable material (Fig. 1B). This may be due to the presence of some inhibitory substances in DOR which were removed from CORDOR and FUSDOR after fungi transformation. These results would suggest that the impact of olive wastes on soil properties is the result of contradictory effects, depending on the relative amounts of beneficial and toxic organic and inorganic compounds present (Piotrowska et al., 2006).

Over time, the application of DOR produced a diminution in urease activity with respect to unamended samples and samples amended with CORDOR and FUSDOR (Fig. 1C). Piotrowska et al. (2011) have also tested the impact of raw and dephenolized olive mill wastewater (OMW) on urease activity and obtained similar results. Thus, phenols present in raw olive wastes may be responsible for inhibiting this activity. The urease enzyme is involved in the hydrolysis of N compounds to NH_4^+ using urea-type substrates (García-Gil et al., 2004). For this reason, López–Piñeiro et al. (2011) and Moreno et al. (2013) have also suggested that urease inhibition in olive waste-amended soils could be due to an increase in NH_4^+ concentrations following DOR applications. In other studies, different results have been obtained for this enzyme under different agricultural management conditions, with urease activity reported to increase due to organic fertilization (Chakrabarti et al., 2000) and to decrease as a consequence of ploughing (Saviozzi et al., 2001).

Protease activity significantly increased in all amended soils after 30 and 60 days (Fig. 1D). The changes in this enzyme in soil amended with DOR may be due to the addition of available N with this type of residue or to the breakdown of complex nitrogen compounds from the organic residue into simple compounds. These findings are in line with a previous study where organically amended soils were shown to have higher levels of protease than inorganically fertilized soils (Ros et al., 2007).

Soil dehydrogenase activity is involved in redox soil reactions, is considered to be a measure of soil microbial activity and can therefore provide information on the potential toxicity of olive wastes (Benitez et al., 2004). In addition, this enzyme has mainly been used to assess soil quality, although contradictory conclusions have been reached. The addition of industrial wastes and organic fertilizers generally increases dehydrogenase activity due to enhanced nutrient cycling and organic carbon metabolism which promote the growth of indigenous microorganisms (Macci et al., 2012). However, this activity can decrease with the use of herbicide (Reinecke et al., 2002). The data of the present study indicate that dehydrogenase activity increased immediately after soil treatment (Fig. 1E) which may be attributed to higher microbial biomass levels due to the addition of available organic substrates which promote the growth of soil microorganisms (López–Piñeiro et al., 2011). However, this activity decreased at 30 and 60 days in amended samples (Fig. 1E) with respect to initial sampling time, which is probably due to the decomposition of readily available organic matter. These findings are in line with previous studies of OMW soil applications (Piotrowska et al., 2006).

In a previous study, DOR amendments have been reported to produce a phytotoxic effect on sorghum plants while CORDOR did not produce significant changes in sorghum shoot dry weight with respect to plants grown in unamended samples (Siles et al., 2013). No detrimental effect of FUSDOR on sorghum plants has been detected (data not shown).

3.2. Effect of DOR amendments on fungal community structure

3.2.1. Diversity and abundance of fungal community in soil

To date, few studies have been conducted to investigate the microbial diversity of the soil amended with bioremediated DOR (Sampedro et al., 2009). Although, some surveys, using DGGE, have been carried out to assess the impact of DOR composting process on waste bacterial community structure (Federici et al., 2011).

We have studied different DGGE profiles of fungal communities in soil amended with DOR biotransformed and nonbiotransformed with saprobic fungi. The fungal DGGE profiles of all treatments were complex, with a large number of bands. Interestingly, the dominant bands were similar in all lanes except for variations in densities, indicating that no changes occurred in the predominant soil fungal populations following the different soil treatments (Fig. 2A). To observe possible changes in fungal diversity due to the soil amended with the residue, different indices were calculated from analysis of the DGGE profiling. No differences between treatments were observed at 0 and 30 days, with similar S and H indices being obtained for all the samples (Table 1). Instead, a slight increase in fungal diversity was detected in soil amended with DOR biotransformed with *C. floccosa* with respect to unamended samples at 60 days (Table 1). Similarly, Rousidou et al., 2010 obtained an increase of fungal diversity after OMW application to soil. Finally, community evenness (J) of soil after amendments application remained relatively constant throughout the experiment.

The UPGMA dendrogram showed that the samples were grouped in two main clusters with a high degree of similarity (95%), suggesting that fungal community in the present survey was well defined (Fig. 2B). One of the clusters was formed by the soil amended with the biotransformed DOR for 30 and 60 days. The

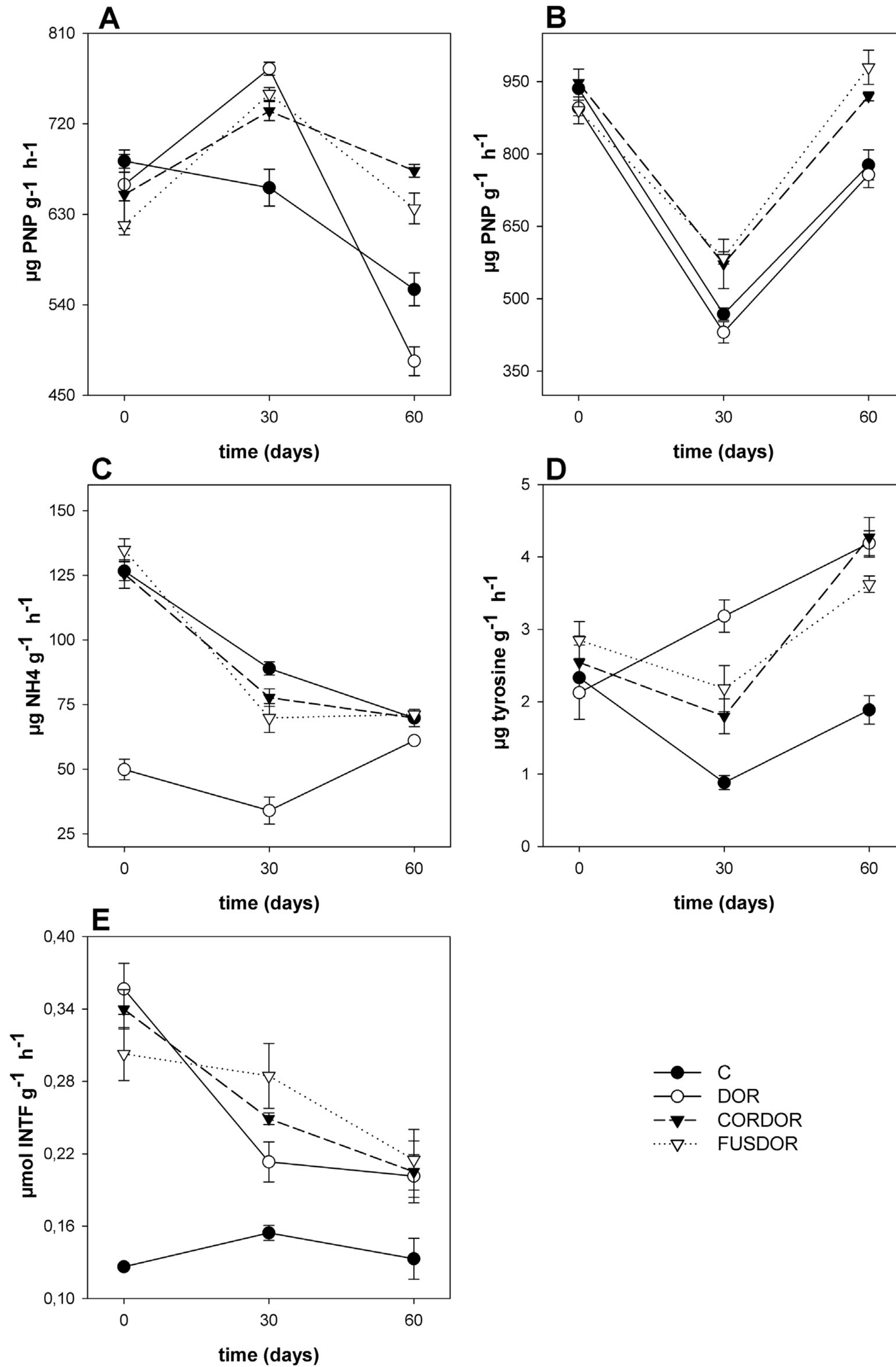


Fig. 1. Activities of phosphatase (A), β-glucosidase (B), urease (C), protease (D) and dehydrogenase (E) in unamended soil (C) and soil amended with untransformed (DOR), *C. floccosa*-transformed DOR (CORDOR) or *F. oxysporum*-transformed DOR at 0, 30 and 60 days. Error bars indicate the standard deviations.

remaining samples were clustered in another group, although in this group, all the samples at day zero and control sample at 60 days were more similar. As previously reported by Sampedro et al. (2009), these data suggest that the degree of similarity of fungal community among samples mainly depends on whether the organic treatments are biotransformed and nonbiotransformed with saprobic fungi.

A real-time PCR standard curve was generated for fungi 18S rRNA quantification. The equation describing the relationship between Ct and the log number of 18S rRNA gene copies was $Ct = -1.16 \times \ln(18S\ rRNA) + 32.56$, $R^2 = 0.998$. The abundance of total fungi detected using real-time PCR showed significant differences between amendments and incubation time (Fig. 3). The control soil showed an average density of 1.39×10^7 copies per

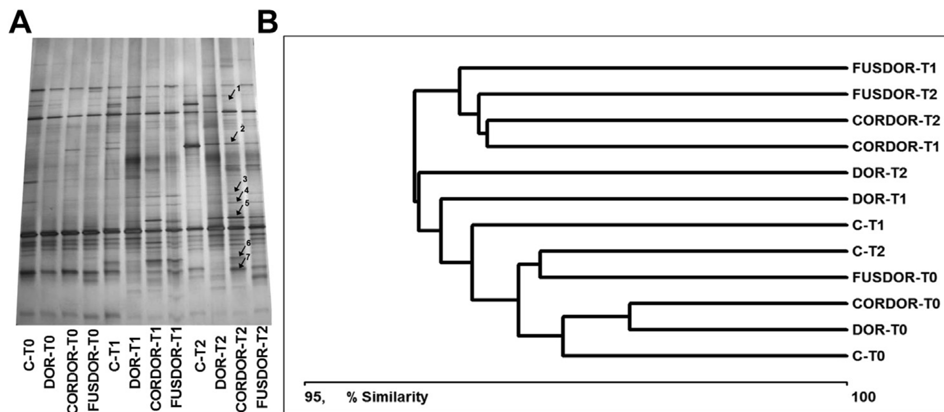


Fig. 2. (A) DGGE analysis of 18S rRNA gene products amplified from unamended soil (C) and soil amended with untransformed DOR (DOR), *C. floccosa*-transformed DOR (CORDOR) or *F. oxysporum*-transformed DOR (FUSDOR) at 0 (T0), 30 (T1) and 60 (T2) days. Arrows indicate bands corresponding to clones that were sequenced. (B) UPGMA dendrogram analysis of fungal communities obtained from the DGGE profiles of 18S rRNA gene products amplified from unamended soil (C) and soil amended with untransformed DOR (DOR), *C. floccosa*-transformed DOR (CORDOR) or *F. oxysporum*-transformed DOR (FUSDOR) at 0 (T0), 30 (T1) and 60 (T2) days based on based on the Euclidean distances matrix.

gram, and the application of untransformed DOR to soil resulted in a significant increase in the number of 18S rRNA gene copies. However, this increase was less marked for the soil amended with DOR biotransformed with saprobic fungi. Other studies have also reported a marked increase in soil fungi abundance as a short-term response to OMW applications (Mechri et al., 2007; Magdich et al., 2012). Medina et al. (2011) demonstrated that OMW can inhibit fungal growth, although, in the present experiment, raw or fungi-transformed DOR amendments did not produce a toxic effect on fungi, with no diminution in fungal abundance or diversity being detected, at least at the doses applied. According to these findings, the principal effects of soil DOR amendments are related to changes in soil fungal structure.

3.2.2. Phylogenetic analyses

As the largest increases in diversity indices were recorded in soil amended with DOR biotransformed with *C. floccosa* at 60 days, the predominant bands from this soil treatment were excised from DGGE analyses and subjected to sequencing. Some of these predominant bands were also observed in the treatment of soil with FUSDOR. Fig. 2A and Table 2 show that the application to soil of CORDOR increased the abundance of DGGE bands belonging to the species *Chalazion helveticum* (band 2), *Chaetomium globosum* (band 4) and to certain uncultured soil fungi (bands 1, 3 and 5) (Fig. 2A and Table 2). The increased abundance of certain fungi capable of producing cell wall hydrolases such as *C. globosum* (Liu et al., 2008) observed in this study suggested that the application of biotransformed DOR to soil could contribute to increasing the presence of fungi involved in the decomposition of this residue and subsequently to increasing available organic matter and functionality of soils amended with this transformed residue.

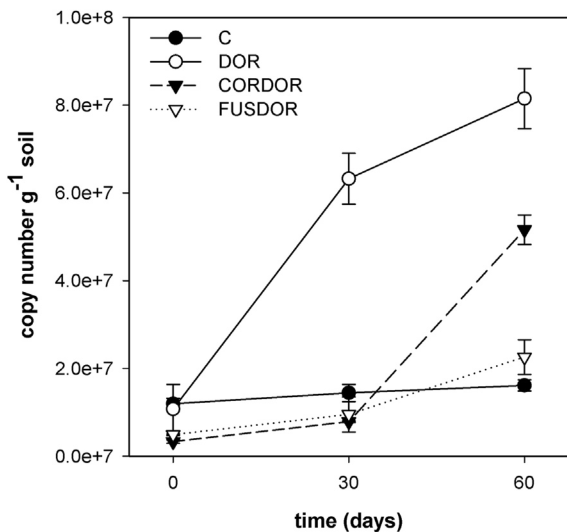


Fig. 3. Quantification of 18S rRNA gene copy number by means of qPCR in unamended soil (C) and soil amended with untransformed DOR (DOR), *C. floccosa*–transformed DOR (CORDOR) or *F. oxysporum*–transformed DOR (FUSDOR) at 0, 30 and 60 days. Mean values correspond to three measures ± standard deviation.

3.2.3. PCA analysis

PCA analysis was carried out on the enzymatic and biological properties of soil after the addition of DOR biotransformed and nonbiotransformed with saprobic fungi in order to identify the overall impact on the soil properties of treatments and interactions between various factors (amendment type and time) (Fig. 4). PCA analysis produced a two-factor solution which accounted for 58.54% of total original variance. In order to confirm the results, a VARIMAX rotation of the 2-component solution was carried out, which produced the following main findings: on the one hand, the 1st PC (38.49% of total variance) positively correlated with COP, SHA and RIC and negatively correlated with GLU, DEH and URE; on the other hand, the 2 nd PC (20.05% of total variance) positively correlated with PRO and COP and negatively correlated with PHO (Fig. 4A).

To identify significant relational patterns, factor scores for each sample and consequently their coordinates in the new factorial space were plotted together with their specific experimental conditions (amendment type and time) (Fig. 4B). Four distinct groups were clearly established, with the samples at initial sampling time grouped in two different clusters which were positively related to PC1. The remaining samples at 30 and 60 days were brought together in two other groups negatively related to PC1. One of these groups was made up of samples at 30 days and the other one grouped all the samples analyzed at 60 days. This statistical analysis indicated that the principal grouping factor in the present study was incubation time. A similar conclusion was reached by Giuntini et al. (2006) in a study where the effects of raw and composted olive wastes on soil microbiology were assessed.

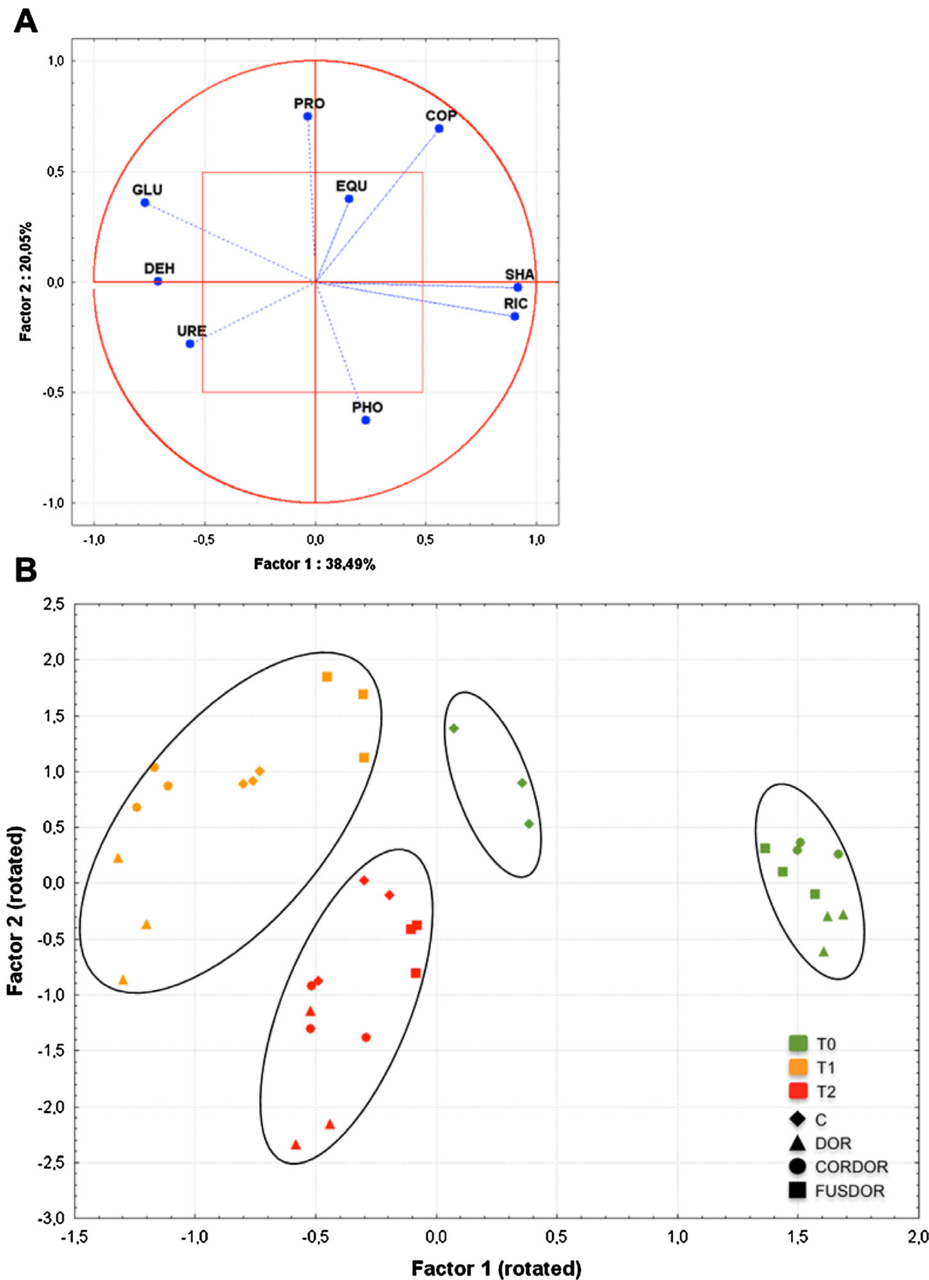


Fig. 4. VARIMAX rotated factor loadings for Factor 1 (PC1) × Factor 2 (PC2). Extraction method: Principal Components. Factor loadings (variable coordinates within the factor space) represent the correlation between original measures and new factors extracted using Principal Component Analysis. The variables analyzed were: phosphatase (PHO); β-glucosidase (GLU); urease (URE); protease (PRO); dehydrogenase (DEH); fungal population number (COP); species richness (RIC); Shannon index (SHA) and evenness (EQU). Distance between points and ellipse represents the quality of the representation of each variable within the factor space. The closer the point to the ellipse, the better the quality of representation of the corresponding variable within the factor solution (A), factor scores for the 36 soil samples. The shape and color of the points represent time and soil amendment [unamended soil (C) and soil amended with untransformed DOR (DOR), *C. floccosa*-transformed DOR (CORDOR) or *F. oxysporum*-transformed DOR (FUSDOR) at 0 (T0), 30 (T1) and 60 (T2) days]. This facilitates detection of significant patterns of variation between measures relating to experimental conditions (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

The biological response of soil to additions of DOR differed according to type and time of amendment. The findings produced by the present study clearly indicated that nonbiotransformed DOR negatively affected some biological properties (β-glucosidase and urease activity) and produced changes in soil fungal structure and abundance. However, the addition of DOR biotransformed with saprobic fungi did not adversely affect enzymatic activity. On the contrary, phosphatase, β-glucosidase, urease and dehydrogenase increased in treatments with this amendments probably due to the high nutrient content and small amounts of toxic compounds in these biotransformed residues. The fungi-transformed DOR also altered fungal size and community structure. In the case of *C. floccosa*-transformed DOR, a slight increment in fungal diversity was observed at 60 days, probably related to the increment in fungi associated with the degradation of lignocellulosic biomass. The present study reflects an in-depth analysis of the effect of raw and biotransformed DOR on soil enzymatic activities and the dynamics of soil fungal communities.

Acknowledgments

This study has been funded by the Spanish Ministry of Science and Innovation (Project AGL2008–572). J.A. Siles, D. Pérez-Mendoza and I. Sampedro gratefully acknowledge the JAE-Predoc and JAE-Doc grants generously provided by the JAE Program which is co-financed by CSIC and the European Social Fund. We also wish to thank Michael O'Shea for proof reading the document.

References

Benitez, E., Melgar, R., Nogales, R., 2004. Estimating soil resilience to a toxic organic waste by measuring enzyme activities. Soil Biol. Biochem. 36, 1615–1623.
Bills, G.F., Christensen, M., Powell, M., Thorn, G., 2004. Saprobic soil fungi. In: Mueller, G., Bills, G.F., Foster, M.S. (Eds.), Biodiversity of Fungi, Inventory and Monitoring Methods. Elsevier Academic Press, Oxford, pp. 271–302.
Cañizares, R., Benitez, E., Ogunseitan, O.A., 2011. Molecular analyses of β-glucosidase diversity and function in soil. Eur. J. Soil Biol. 47, 1–8.
Carmiña, F., Trasar-Cepeda, C., Gil-Sotres, F., Leirós, M.C., 1998. Measurement of dehydrogenase activity in acid soils rich in organic matter. Soil Biol. Biochem. 30, 1005–1011.
Chakrabarti, K., Sarkar, B., Chakraborty, A., Banik, P., Bagchi, D.K., 2000. Organic recycling for soil quality conservation in a sub-tropical plateau region. J. Agron. Crop Sci. 184, 137–142.
Chaves, M.M., Oliveira, M.M., 2004. Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. J. Exp. Bot. 55, 2365–2384.
Chemidlin Prévost-Bouré, N., Christen, R., Dequiedt, S., Mougel, C., Lelièvre, M., Jolivet, C., Shahbazkia, H.R., Guillou, L., Arrouays, D., Ranjard, L., 2011. Validation and application of a PCR primer set to quantify fungal communities in the soil environment by real-time quantitative PCR. PLoS ONE 6, e24166.
De La Horra, A.M., Defrieri, R., Jiménez, M.P., Palma, R.M., 2005. Evolution of alkaline phosphatase and protease activities, total organic carbon and CO_2 evolved during composting. Agrochimica 49, 22–28.
Eastwood, D.C., Floudas, D., Binder, M., Majcherczyk, A., Schneider, P., Aerts, A., Asiegbu, F.O., Baker, S.E., Barry, K., Bendiksby, M., 2011. The plant cell wall-decomposing machinery underlies the functional diversity of forest fungi. Science 333, 762–765.
Eivazi, F., Tabatabai, M.A., 1977. Phosphatases in soils. Soil Biol. Biochem. 9, 167–172.
Eivazi, F., Tabatabai, M.A., 1988. Glucosidases and galactosidases in soils. Soil Biol. Biochem. 20, 601–606.
Federici, E., Pepi, M., Esposito, A., Scargetta, S., Fidati, L., Gasperini, S., Cenci, G., Altieri, R., 2011. Two-phase olive mill waste composting: community dynamics and functional role of the resident microbiota. Bioresour. Technol. 102, 10965–10972.
García-Gil, J.C., Plaza, C., Senesi, N., Brunetti, G., Polo, A., 2004. Effects of sewage sludge amendment on humic acids and microbiological properties of a semiarid Mediterranean soil. Biol. Fert. Soils 39, 320–328.
Giuntini, E., Bazzicalupo, M., Castaldini, M., Fabiani, A., Miclaus, N., Piccolo, R., Ranalli, G., Santomassimo, F., Zanobini, S., Mengoni, A., 2006. Genetic diversity of dinitrogen-fixing bacterial communities in soil amended with olive husks. Ann. Microbiol. 56, 83–88.
Kandeler, E., Gerber, H., 1988. Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol. Fert. Soils 6, 68–72.
Ladd, J.M., Butler, J.H.A., 1972. Short-term assays of soil proteolytic enzyme activity using proteins and dipeptide derivates as substrates. Soil Biol. Biochem. 4, 19–30.
Liu, Z.H., Yang, Q., Hu, S., Zhang, J.D., Ma, J., 2008. Cloning and characterization of a novel chitinase gene (chi46) from *Chaetomium globosum* and identification of its biological activity. Appl. Microbiol. Biotechnol. 80, 241–252.
López–Piñeiro, A., Albarrán, A., Rato Nunes, J.M., Peña, D., Cabrera, D., 2011. Long–term impacts of de–oiled two–phase olive mill waste on soil chemical properties, enzyme activities and productivity in an olive grove. Soil Tillage Res. 114, 175–182.
Macci, C., Doni, S., Peruzzi, E., Masciandaro, G., Mennone, C., Ceccanti, B., 2012. Almond tree and organic fertilization for soil quality improvement in southern Italy. J. Environ. Manag. 95, 215–222.
Magdich, S., Jarboui, R., Ben Rounina, B., Boukhris, M., Ammar, E., 2012. A yearly spraying of olive mill wastewater on agricultural soil over six successive years: impact of different application rates on olive production, phenolic compounds, phytotoxicity and microbial counts. Sci. Total Environ. 430, 209–216.
Mandal, A., Patra, A.K., Singh, D., Swarup, A., Ebhin Masto, R., 2007. Effect of long-term application of manure and fertilizer on biological and biochemical activities in soil during crop development stages. Bioresour. Technol. 98, 3585–3592.
Mechri, B., Echbili, A., Issaoui, M., Brahma, M., Ben Elhadj, S., Hammami, M., 2007. Short-term effects in soil microbial community following agronomic application of olive mill wastewaters in a field of olive trees. Appl. Soil Ecol. 36, 216–223.
Medina, E., Romero, C., de los Santos, B., de Castro, A., García, A., Romero, F., Brenes, M., 2011. Antimicrobial activity of olive solutions from stored alpeorujo against plant pathogenic microorganisms. J. Agric. Food Chem. 59, 6927–6932.
Moreno, J.L., Bastida, F., Sánchez-Monedero, M.A., Hernández, T., García, C., 2013. Response of soil microbial community to a high dose of fresh olive mill wastewater. Pedosphere 23, 281–289.
Morillo, J.A., Antizar-Ladislao, B., Monteoliva-Sánchez, M., Ramos-Cormenzana, A., Russell, N.J., 2008. Bioremediation and biovalorisation of olive-mill wastes. Appl. Microbiol. Biotechnol. 82, 25–39.
Piotrowska, A., Iamarino, G., Rao, M.A., Gianfreda, L., 2006. Short-term effects of olive mill waste water (OMW) on chemical and biochemical properties of a semiarid Mediterranean soil. Soil Biol. Biochem. 38, 600–610.
Piotrowska, A., Rao, M.A., Scotti, R., Gianfreda, L., 2011. Changes in soil chemical and biochemical properties following amendment with crude and dephenolized olive mill waste water (OMW). Geoderma 161, 8–17.
Quian, H.F., Hu, B.L., Wang, Z.Y., Hong, T., 2007. Effects of validamycin on some enzymatic activities in soil. Environ. Monit. Assess. 125, 1–8.
Ramette, A., 2007. Multivariate analyses in microbial ecology. FEMS Microbiol. Ecol. 62, 142–160.
Reinecke, A.J., Helling, B., Louw, K., Fourie, J., Reinecke, S.A., 2002. The impact of different herbicides and cover crops on soil biological activity in vineyards in the Western Cape, South Africa. Pedobiologia 46, 475–484.
Ros, M., García, C., Hernandez, M.T., 2007. Evaluation of different pig slurry composts as fertilizer of horticultural crops: effects on selected chemical and microbial properties. Renew. Agric. Food Syst. 22, 307–315.
Rousidou, C., Papadopoulou, K., Zervakis, G., Singh, B.K., Ehaliotis, C., Karpouzas, D.G., 2010. Repeated application of diluted olive mill wastewater induces changes in the structure of the soil microbial community. Eur. J. Soil Biol. 46, 34–40.
Sampedro, I., Giubilei, M.A., Cajthaml, T., Federici, E., Federici, F., Petruccioli, M., D'Annibale, A., 2009. Short-term impact of dry olive mill residue addition to soil on the resident microbiota. Bioresour. Technol. 100, 6098–6106.
Sampedro, I., Marinari, S., D'Annibale, A., Grego, S., Ocampo, J.A., García-Romera, I., 2007. Organic matter evolution and partial detoxification in two-phase olive mill waste colonized by white-rot fungi. Int. Biodeterior. Biodegrad. 60, 116–125.
Saviozzi, A., Levi-Minzi, R., Cardelli, R., Riffaldi, R., 2001. A comparison of soil quality in adjacent cultivated, forest and native grassland soils. Plant Soil 233, 251–259.
Siles, J.A., Cajthaml, T., Hernández, P., Pérez-Mendoza, D., García-Romera, I., Sampedro, I., Shifts in soil chemical properties and bacterial communities responding to biotransformed dry olive residue used as organic amendment. Biol. Fert. Soils (submitted for publication).
Siles, J.A., Pascual, J.A., González-Menéndez, V., Sampedro, I., García-Romera, I., Bills, G.F., 2013. Short-term dynamics of culturable bacteria in a soil amended with biotransformed dry olive residue. Syst. Appl. Microbiol. http://dx.doi.org/10.1016/j.syapm.2013.08.005.
Tortosa, G., Alburquerque, J.A., Ait-Baddi, G., Cegarra, J., 2012. The production of commercial organic amendments and fertilizers by composting of two-phase olive mill waste ("alpeorujo"). J. Clean. Prod. 26, 48–55.
Vainio, E., Hantula, J., 2000. Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. Mycol. Res. 104, 927–936.
Xu-Cong, L., Weng, X., Zhang, W., Rao, P.F., Ni, L., 2012. Microbial diversity of traditional fermentation starters for Hong Qu glutinous rice wine as determined by PCR-mediated DGGE. Food Control 28, 426–434.