Soil microbial response to biotransformed dry olive residue used as organic amendment

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PhD Thesis

2014

Editor: Editorial de la Universidad de Granada
Autor: José Antonio Siles Martos
D.L.: GR 1892-2014
ISBN: 978-84-9083-072-7

Universidad de Granada
Facultad de Ciencias
Programa Oficial de Posgrado en Microbiología

Consejo Superior de Investigaciones Científicas
Estación Experimental del Zaidín
Departamento de Microbiología del Suelo y Sistemas Simbióticos

Soil microbial response to biotransformed dry olive residue used as organic amendment

Memoria presentada por el Ldo. en Biología
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para optar al título de Doctor.

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La presente Tesis Doctoral ha sido realizada en el Grupo de Investigación "Microorganismos Rizosféricos Promotores del Crecimiento Vegetal" perteneciente al Departamento de Microbiología del Suelo y Sistemas Simbióticos de la Estación Experimental del Zaidín (Consejo Superior de Investigaciones Científicas), Granada, gracias a una Beca Predoctoral del Programa JAE-Pre, concedida por el Consejo Superior de Investigaciones Científicas.

Parte de los resultados de esta Tesis doctoral han sido publicados o en están en vías de publicación en los siguientes artículos:

Siles, J.A., González-Menéndez, V., Platas, G., Sampedro, I., García-Romera, I., Bills, G. (In press) Effects of dry olive residue transformed by *Coriolopsis floccosa* (Polyporaceae) on the distribution and dynamic of a culturable fungal soil community. *Microbial Ecology*. DOI: 10.1007/s00248-013-0353-6

Siles, J.A., Pérez-Mendoza, D., Ibáñez, J.A., Scervino, J.M., Ocampo, J.A., García-Romera, I., Sampedro, I. (2014) Assessing the impact of biotransformed dry olive residue application to soil: Effects on enzyme activities and fungal community. *International Biodeterioration and Biodegradation* 89: 15-22

Siles, J.A., Pascual, J., González-Menéndez, V., Sampedro, I., García-Romera, I., Bills, G.F. (2014) Short-term dynamics of culturable bacteria in a soil amended with biotransformed dry olive residue. *Systematic and Applied Microbiology* 37: 113-120.

Siles, J.A., Cajthaml, T., Hernández, P., Pérez-Mendoza, D., García-Romera, I., Sampedro, I. (Under Review) Shifts in soil chemical properties and bacterial communities responding to biotransformed dry olive residue used as organic amendment. *Biology and Fertility of Soils.*

Siles, J.A., Rachid, C. T. C. C., Sampedro, I., García-Romera, I., Tiedje, J.M. (In preparation) Microbial diversity of a Mediterranean soil and its changes after biotransformed dry olive residue amendment.

O han sido presentados los siguientes congresos:

Siles, J.A., Díaz R., Scervino, J.M., Ocampo, J.A., García-Romera I., Sampedro I. (2010) Changes in bacterial community structure in soil amended with dry olive residue colonized by saprobe fungi. Póster. The Second International Conference IAMAW. Izmir (Turquía).

Siles J.A., Díaz, R., Martins, A.B., Vasquez J., Ocampo, J.A., García-Romera, I., Sampedro I. (2011) Enzymes and fungal community dynamics of soil amended with biotransformed dry olive residue. Póster. 4th Internacional Conference Enzymes in the Environment: Activity, Ecology & Applications. Bad Nauheim (Alemania).

Siles J.A., Díaz, R., Martins, A.B., Vasquez J., Ocampo, J.A., García-Romera, I., Sampedro I. (2011) Impact of alginate-immobilized saprobe fungi on bacterial community of soil amended with biotransformed dry olive residue. Póster. International Symposium of Interactions of soil Minerals with Organic Components and Microorganisms (ISMOM 2011) Montpellier (Francia).

Siles, J.A., Hernández-Suárez P., González-Menéndez V., Bills, G.F., García-Romera I., Sampedro I. (2012) Behavior of actinomycetal communities in soils fertilized with biotransformed dry olive residue. Póster. Annual Conference of the Association for General and Applied Microbiology (VAAM). Tübingen (Alemania).

Siles, J.A., González-Menéndez V., Platas, G., Sampedro, I., García-Romera, I., Bills, G.F. (2012) Distribución y dinámica de las comunidades fúngicas cultivables en suelos enmendados con alpeorujo transformado por hongos saprobios. Póster. XIV Reunión de Taxonomía, Filogenia y Diversidad Microbiana de la Sociedad Española de Microbiología. Granada (España).

Siles, J.A., Pascual, J., González-Menéndez V., Sampedro, I., García-Romera, I., Bills, G.F., (2012) Impact of soil fertilization with "alpeorujo" transformed by Coriolopsis rigida on culturable bacterial communities. Poster. 14th International Symposium on Microbial Ecology. Copenhague (Dinamarca)

"Think, Believe, Dream, and Dare"

Walt Disney

"When you make the finding yourself,
– even if you're the last person on Earth to see the light –
you'll never forget it"

Carl Sagan

A mis padres

CONTENTS

I. GENERAL INTRODUCTION

1. Soil

Soil has a heterogeneous, diverse and porous structure made up of liquid, gaseous and solid phases (Fredlund and Rahardjo, 1993). The liquid phase accounts for 25% of the total volume of soil and consists of liquid water and dissolved solids and gases (Fig. 1). This component performs critical functions in the soil ecosystem such as a transporting agent, chemical solvent, available nutrient pool and a water source for the metabolic activities of soil biota and vegetation (Porta Casanellas et al., 1998). The gaseous phase, accounting for a further 25% of soil volume (Fig. 1), is made up of inorganic elements (N_2, O_2, CO_2, etc), vapours (such as H_2O and NH_4) and volatile organic elements such as carbohydrates, organic acids, alcohols, oils and pesticides (Certini and Scalenghe, 2006). Finally, the solid phase comprises 50% of the total soil volume, with the inorganic and organic solid components accounting for 45% and 5%, respectively, of soil volume (Fig. 1). Inorganic solid phase is composed of minerals which can be classified by size: sand (from 2 to 0.05 mm), silt (from 0.05 to 0.002 mm) and clay (< 0.002 mm) particles (Schulten and Leinweber, 2000). On the other hand, the organic component of soil is made up of the non-living phase [soil organic matter (SOM)] and living phase (soil microorganisms, soil fauna and soil flora) (Nieder and Benbi, 2008) (Fig. 2).

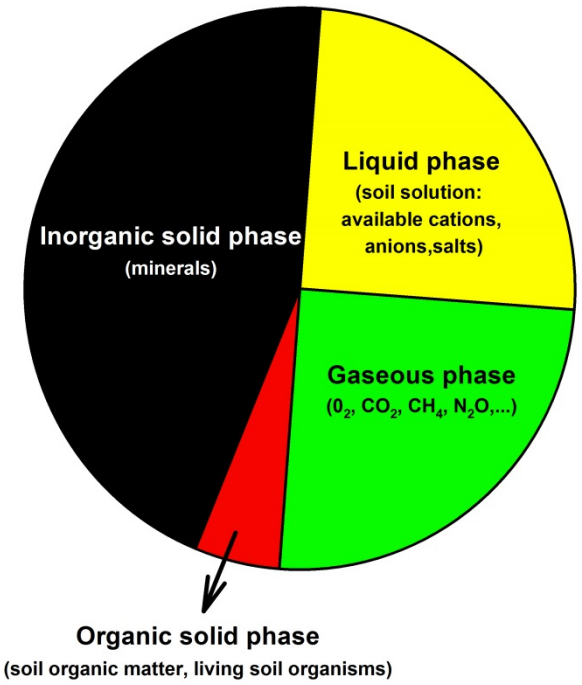

Fig. 1. Soil components. Adapted from Lal and Shukla (2004).

1.1. Non-living soil organic phase

Non-living soil organic phase or soil organic matter (SOM) can derive from plants, animals or microorganisms. It can be relatively fresh or highly decomposed and transformed as it changes constantly and is renewed by both inputs and losses caused by mineralization. SOM normally accounts for 0.1 to 10% of soil mass (Kononova, 1963). However, despite its limited concentration, SOM is highly important in qualitative terms due to the following functions (Cheshire, 1987; Pulleman et al., 2000; Certini and Scalenghe, 2006):

i. It is rich in nutrients such as N, P, S, which are released through mineralization.
ii. It is electrically charged, has high cation exchange capacity and is therefore able to retain nutrient cations such as K^+, Mg^{2+}, Ca^{2+} and Fe^{3+} on its negative charges.
iii. It is the basis of most soil biological activity, being the resource of carbon and energy of heterotrophs, from microorganisms to macrofauna.
iv. It has a major impact on the physical properties of soil by increasing water retention and by aggregating mineral particles and thus improves soil structure and prevents soil erosion.
v. It retains organic pollutants, heavy metals and radionuclides due to its high chemical reactivity and may also affect soil quality.

SOM consists of both unaltered material, thus maintaining the morphology of the original material, as well as altered or transformed products known as humus (Fig. 2). The decomposition of organic residues involves a two-phase process: a rapid initial stage involving readily decomposable organic fractions followed by a much slower phase involving recalcitrant fractions. The preserved organic substances in both altered and unaltered form as well as resynthesized microbial products result in the formation of humus through a process of degradation and synthesis (Nieder and Benbi, 2008). The easily degradable fractions are known as non-humic substances while the substances resulting from microbial decomposition and synthesis are called humic substances (Fig. 2).

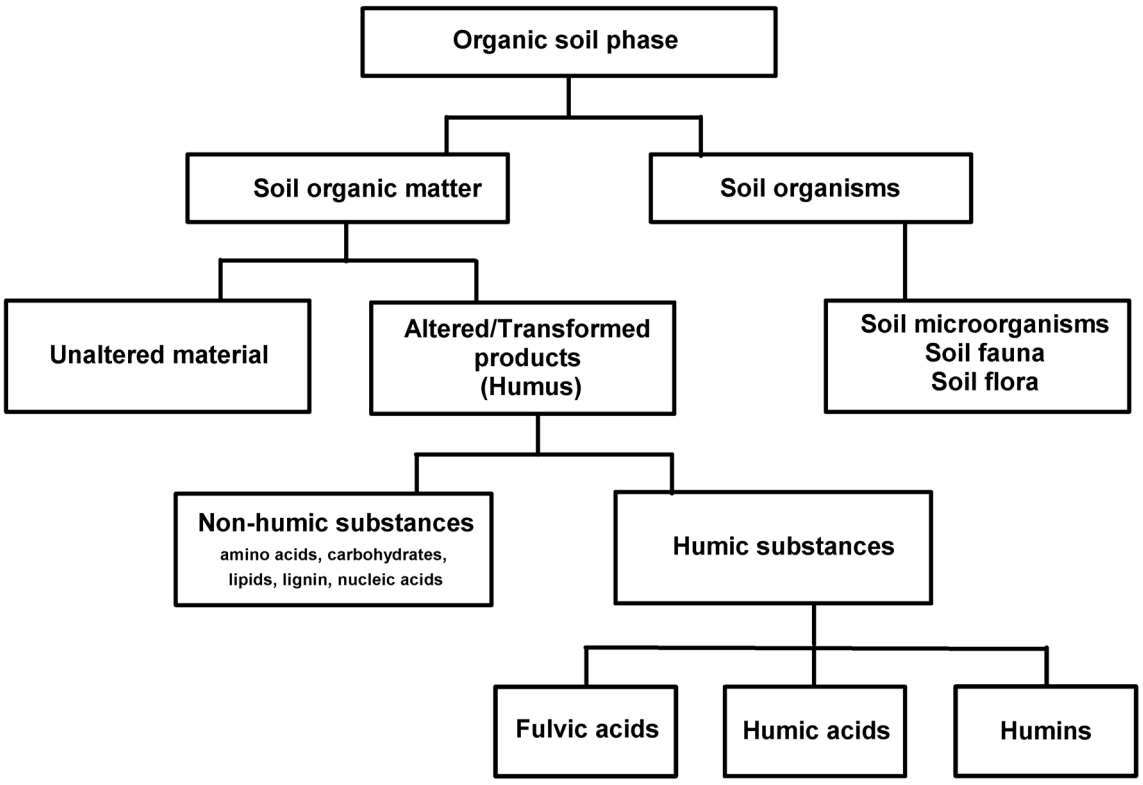

Fig. 2. Organic soil phase composition. Adapted from Nieder and Benbi (2008)

Non-humic substances (20-40% of C in humus) are made up of organic materials whose chemical characteristics are still identifiable. Most of these substances are easily degradable and have a short life in soils (Porta Casanellas et al., 1998). Non-humic substances include carbohydrates, amino acids, amino sugars, alkyl compounds and lignin. Carbohydrates are made up of a broad range of molecules, mainly consisting of five (pentose) or six (hexose) carbon atoms which form oxygen-containing ring structures. Plant and soil organisms are the main sources of these components (Cheshire, 1979). Amino acids, which are the principal reservoir of N in soil, originate from microbial cell walls and exoenzymes liberated by microorganisms that degrade complex organic matter. Amino sugars consist of D-glucosamine, N-acetylglocosamine, muramic acid and D-mannosamine, which are of microbial origin (Gieseking, 1975). Alkyl compounds consist of macromolecules synthesized by microorganisms, solvents and bound lipids (fatty acids and waxes originating from plants and soil microorganisms), insoluble polyesters (cutin and suberin) and nonpolyesters (cutan and suberan) derived from plant cuticles and cork cells in roots and bark (Kögel-

Knabner et al., 1992). Finally, lignin is a polymer that is transferred from plants to soil via aboveground shoots and leaves as well as belowground root system litter (Thevenot et al., 2010). Although lignin is highly recalcitrant, white-rot fungi have proved to be efficient in terms of lignin degradation (Haider, 1992).

Humic substances (HS) (60-80% of C in humus) are a nonspecific, transformed, dark colored, heterogeneous, amorphous material with a high-molecular weight characterized by secondary synthesis reactions and represent the largest source of organic carbon on the Earth's surface (Stevenson, 1994). They can be classified into fulvic acids (FAs), humic acids (HAs) and humins according to their solubility in acid and alkaline solutions (Fig. 2) (Nieder and Benbi, 2008):

i. Fulvic acids are the fraction of humic substances that remain soluble under all pH conditions or the fraction that stays in solution when alkaline soil extracts are adjusted to pH < 2. FAs have a characteristic light yellow to yellow-brown colour and generally have aromatic and aliphatic structures, both highly substituted with oxygen.
ii. Humic acids are soluble in neutral and alkaline solutions and precipitate when pH is reduced to <2. They have a characteristic dark brown to black colour and are the major extractable component in soil humic substances. They are made up of complex macromolecules composed of aromatic units bound to amino acids, peptides, amino sugars, aliphatic acids and other organic constituents.
iii. Humins are the fractions of humic substances that are not soluble in water for any pH value and are black in colour. They have higher levels of polymerization, molecular weight and C, as well as lower oxygen content than FAs and HAs.

Soil organic matter is capable of combining with minerals to form organomineral complexes. These combinations can be caused by loosely bound organic and mineral particles in a soil clod or by tightly bound complexes. Organomineral complexes play an important role in soil as approximately 40–80% of soil carbon is present in the clay-sized fraction (< 0.002 mm particles) and cannot be separated from minerals. It has also been reported that the formation of these complexes results in the stabilization of organic matter in terrestrial ecosystems (Hassink, 1997; Mikutta et al., 2006).

1.2. Living soil organic phase

Broadly speaking, living soil organic phase includes living organisms that inhabit soil (Decaëns et al., 2006) and generally refers to organisms which play an important role in soil functionality. Soil organisms can be classified as follows:

i. microorganisms (fungi, bacteria, archaea and viruses)
i. fauna (such as protozoa, annelids, arthropods, nematodes and molluscs)
ii. flora (plants and algae).

Soil microorganisms and fauna are extremely diverse, have a wide range of life-forms and functions, are involved in a large number of ecological processes and provide key ecosystem services for human population (Lavelle et al., 2006). Soil communities involve species from all the principal taxonomic groups found in terrestrial ecosystems (Swift et al., 1979) (Fig. 3). Soil microorganisms and fauna probably represent as much as 25% of the world's 1.5 million identified living species (Decaëns et al., 2006). Nevertheless, despite their enormous biological diversity, soil ecosystems are one of the least studied habitats of terrestrial ecosystems (Decaëns, 2010).

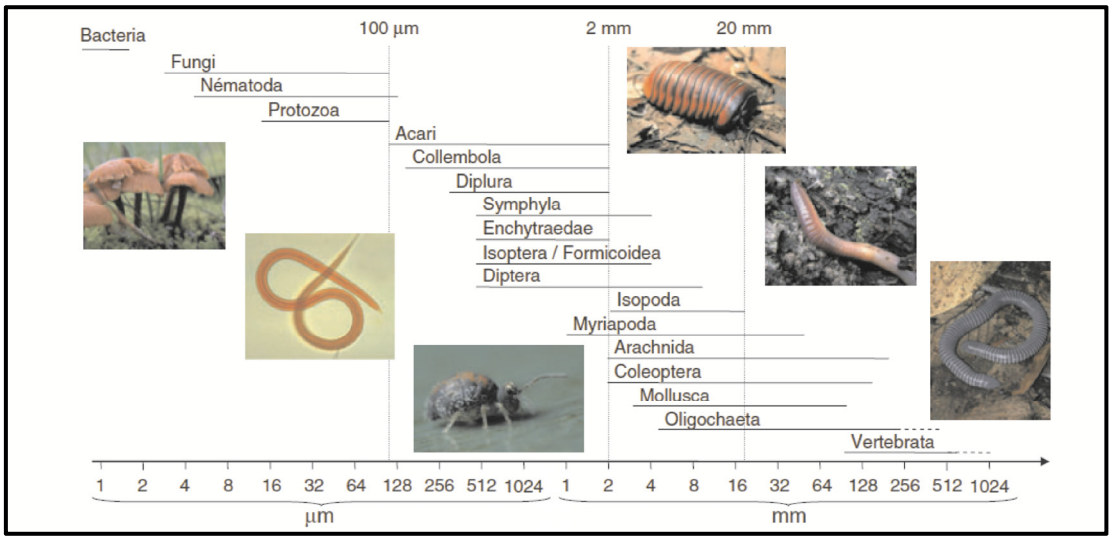

Fig. 3. The main taxonomic groups of soil organisms on a body-size basis. Reproduced from Decaëns (2010).

The abundance and activities of soil organisms are dependent on environmental and human factors. In general, there is an inverse relationship between size of organism on the one hand and its relative abundance and total biomass on the

other (Certini and Scalenghe, 2006). As biomass-specific activity increases with the decrease in the size of the organism, relative energy requirements and contributions to compound transformation rates increase in line with the high abundance levels of small organisms (Dilly et al., 2006). Thus, the larger fraction of broken down organic matter is caused by microorganisms as bacterial activity is greater than other soil organisms and fungi are the largest biotic component in soil (Bailey et al., 2002). On the other hand, soil fauna also plays an important role in relation to organic matter decomposition as these organisms are regarded as "engineers" which redistribute soil components among soil horizons and divide organic matter into smaller particles, thus increasing the surface exposed to microbial activity (Brussaard, 1998; Jouquet et al., 2006).

Soil flora influences soil dynamics as higher plants through litterfall represent the principal input of organic matter in soil and roots influence soil communities by consuming oxygen, water and nutrients, while releasing CO_2 and exudates. On the other hand, algae are able to perform important functions in soil such as nitrogen fixation, generation of organic matter and colonization of bare soil (Wilding et al., 1983).

1.3. Soil microorganisms

Soil microorganisms (fungi, bacteria, archaea and viruses) represent a considerable fraction of the Earth's living biomass, with surface soils estimated to contain 10^3-10^4 kg of microbial biomass ha^{-1} (Fierer et al., 2007). To date, information on soil viruses and archaea has been more limited than that for bacteria and fungi. Nevertheless, new molecular-based techniques have demonstrated that archaea are phylogenetically diverse and appear to play an important role in soil, representing between 0% and 10% of total soil microbial biomass (Bates et al., 2011; Bengtson et al., 2012). Likewise, there are evidences that soil viruses are abundant, morphologically diverse and come in a wide range of genome sizes (Fierer et al., 2007). In the coming years, thanks to high-throughput sequencing techniques, the number of studies on the diversity and ecological importance of these microorganisms will increase. Bacteria and fungi have been extensively studied and below we review the most important information on these soil microorganisms.

1.3.1. Soil Bacteria

After 4 billion years, soil has become one of the most bacterially diverse habitats on Earth (Trevors, 2010) or the most diverse habitat according to Delmont et al., 2011. Given that soil bacteria are the most studied microorganisms in this environment, it has been possible to estimate that one gram of soil contains 10^{10} bacteria, with a number of different species ranging from 10^3 to 10^7 (Torsvik et al., 2002; Gans et al., 2005; Schloss and Handelsman, 2006). In recent years, with the aid of molecular-based techniques, it has been possible to determine soil bacterial diversity without the inherent bias of culture-dependent studies. For instance, Janssen (2006) reviewed the bacterial diversity found in different soil samples based on an analysis of 32 different 16S rRNA gene clone libraries. He reported that soil bacterial diversity was principally due to nine different phyla: *Proteobacteria* (39.2% of total sequences analyzed), *Acidobacteria* (19.7%), *Actinobacteria* (12.7%), *Verrucomicrobia* (7.03%), *Bacteroidetes* (5%), *Chloroflexi* (3.2%), *Firmicutes* (2%), *Planctomycetes* (2%) and *Gemmatimonadetes* (2%). On the other hand, Youssef and Elshahed (2009) examined bacterial diversity on the basis of 5 different 16S rRNA gene pyrosequencing datasets from agricultural, undisturbed tall grass prairie and forest soils to determine the taxa affected by major changes in soils. They demonstrated that *Planctomycetes*, *Firmicutes* and *Gammaproteobacteria* are among the most diverse bacterial lineages in soils, and *Verrucomicrobia*, *Gemmatimonadetes* and *Betaproteobacteria* being the least diverse.

Soil is made up of a complex mosaic of microenvironments differing in their physical, chemical and biological properties, with many different habitats in which bacteria are heterogeneously distributed (Nannipieri, 2003; Garbeva et al., 2004) (Fig. 6). Microbial life generally occurs in soil pores (Fig. 4B). Bacteria usually colonize pores with a mean diameter ranging from 2.5 to 9 μm (micropores), while no bacteria have been observed in pores with a diameter of less than 0.8 μm (Fig. 4). It has also been reported that only 4 to 10% of the pore space of an aggregate is colonised (Ranjard and Richaume, 2001). The more widespread presence of bacteria in micropores is due to the prevailing favourable conditions such as protection from desiccation, exogenous toxic substances and protozoans as well as greater availability of carbon substrates (Vargas and Hattori, 1986; Nelson et al., 1994) (Fig. 4C). However, living in this environment has drawbacks such as limited fresh resources and low atmospheric renewal rates. Bacteria have adapted to these limitations by producing protective extracellular polymeric substances that trap water and nutrients, by inhabiting biofilms and by

replacing oxygen with an alternative terminal electron acceptor (O'Toole et al., 2000; Vos et al., 2013).

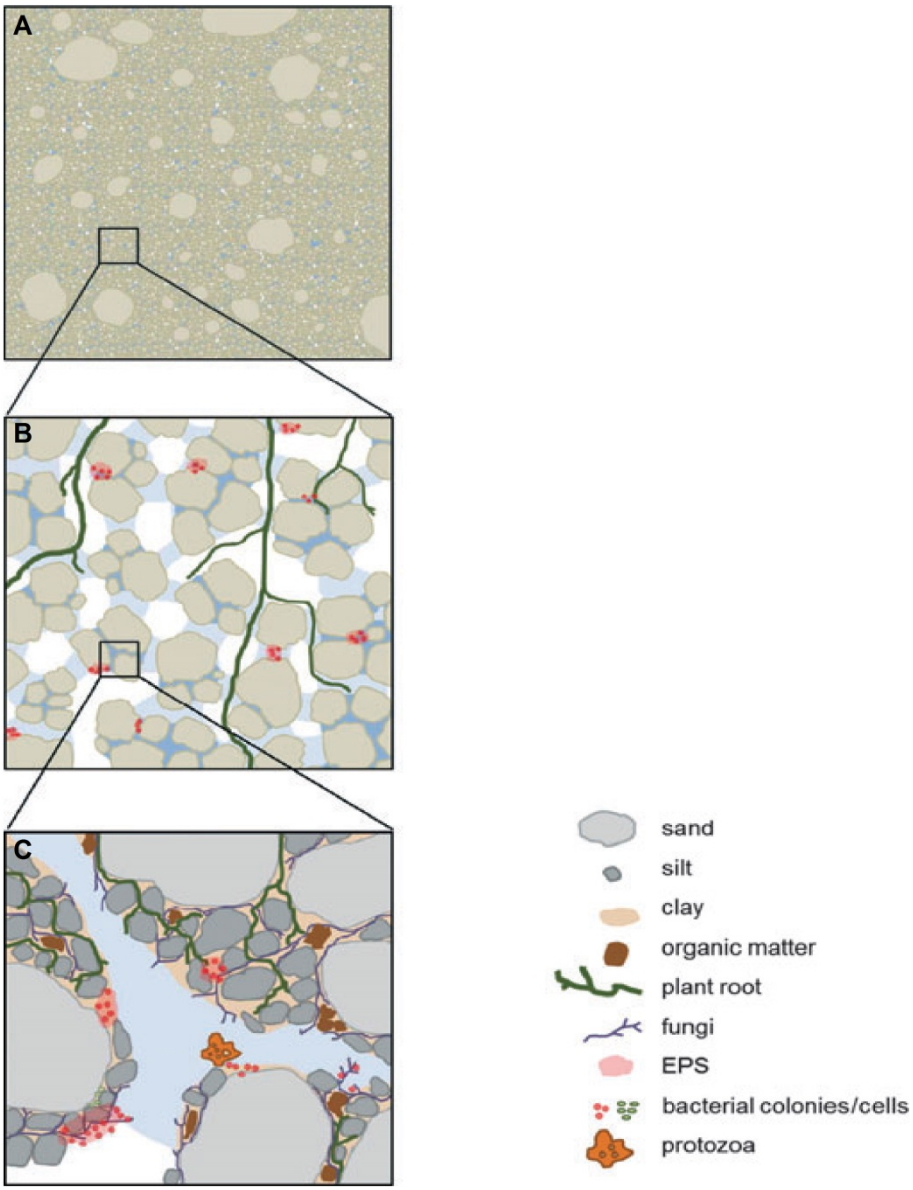

Fig. 4. A) Soil appears to be a homogeneous habitat at a larger scale. B) On the other hand, extreme heterogeneity is evident at a smaller scale. Micropores are mostly located in micro-aggregates and are filled with water (dark blue). C) Bacteria live in micropores due to the protection that they provide against predators and dehydratation. Reproduced from Vos et al. (2013).

Bacteria play a pivotal role in soil ecosystems (Hoorman, 2011). This group of organisms is involved in the soil formation process and participates in the cohesion of mineral particles through the production of organic cements such as microbial exopolysaccharides (Ranjard and Richaume, 2001). However, the most important functions of bacteria in soil are probably associated with nutrient cycling through their

involvement in the cycling of carbon, nitrogen, phosphorus and sulphur (Garbeva et al., 2004; Kirk et al., 2004). Soil organic matter is broken down by enzymes produced by bacteria, resulting in nutrient forms which are more accessible to other organisms. In addition, bacteria are especially important in relation to immobilizing nutrients in their cells and thus preventing their loss especially with regard to N and C. Bacteria may establish interactions with plants, which can be created by symbiotic microorganisms (e.g., *Rhizobium* spp.) or free-living bacteria (e.g., *Azotobacter* spp., *Azospirillum* spp., *Bacillus* spp.). These interactions may have a beneficial effect on plant growth by synthesizing particular compounds for plants (phytohormones), by facilitating the uptake of certain nutrients from the soil and by protecting plants from diseases or lessening their impact through the inhibition of phytopathogens (Hayat et al., 2010; Miransari, 2011). Soil bacteria also participate in the degradation of pollutants and pesticides (Jacobsen and Hjelmsø, 2014) and in water purification. Soil bacteria represent a massive reservoir of biodiversity that can be used for industrial purposes such as in the production and characterization of new enzymes and secondary metabolites (Lombard et al., 2011).

1.3.2. Soil Fungi

Fungi are the most abundant form of life in terms of biomass in soil. It has been estimated that, in a deciduous forest, fungal mycelia account for approximately 90% of the total living microbial biomass (Bills et al., 2004). Fungal biomass in soils can range from around 50-1000 μg g^{-1} dry weight (equivalent to 2-45 T ha^{-1}) or more (Ritz and Young, 2004). With regard to fungal diversity in soil, some studies have estimated that 1 gram of soil could contain roughly 2000 species of fungi (Hawksworth, 2001). Gams (2006) estimated that there are 3,150 known species of soil fungi, and ~70% are available in culture, not including mycorrhizal species. Nevertheless, new high-throughput techniques are demonstrating that there is an enormous number of soil fungal species which are still unknown (Blackwell, 2011). Taylor et al. (2010) have estimated that the overlap between species within a given region as compared with soil samples a meter apart is only 14%. Pyrosequencing-based studies have demonstrated that most soil fungi belonged to Ascomycota and Basidiomycota phyla (Buée et al., 2009; Orgiazzi et al., 2012; Orgiazzi et al., 2013). These studies reported that the proportion of each phylum varies from one soil to another according to physico-chemical soil characteristics. The sequences belonging to Chytridiomycota,

Glomeromycota and Zygomycota phyla also appear in these high-throughput sequencing studies. In functional terms, saprobic fungi play an important role in soil as they are responsible for organic matter recycling. Among the fungi of this functional group, it is worth highlighting the presence of *Saprolegniales*, *Pythiales*, *Mucorales*, *Eurotiales*, *Microascales*, *Hypocreales*, *Sordariales*, *Onygenales*, *Leotiales* and *Pezizales* (Bills et al., 2004).

Soil represents a heterogeneous three-dimensional framework of pores for fungi (Young et al., 2008) which have a filamentous growth pattern that favours their development (Fig. 5). Mycelia are able to spread through the heterogeneous soil structure in order to locate nutrients. Hyphae grow across surfaces, absorbing nutrients from the substratum, and are also capable of growing through the air and hence bridge soil pores (Ritz and Young, 2004). Physical conditions, nutrient availability, pH, aeration and microbivory influence mycelium spread (Harris et al., 2003). Otten et al. (1999) have argued that, among the aforementioned factors, aeration could be regarded as the most significant limitation to fungal growth. It has been estimated that 80-90% of fungi are restricted to larger pores, because anoxic conditions are less common in these environments (Young et al., 2008). However, fungal hyphae are exposed to nematodes, insects, mites and worms in larger pores. Thus, fungi will occupy air-filled pores in soil which protect them from their consumers. (Ritz and Young, 2004).

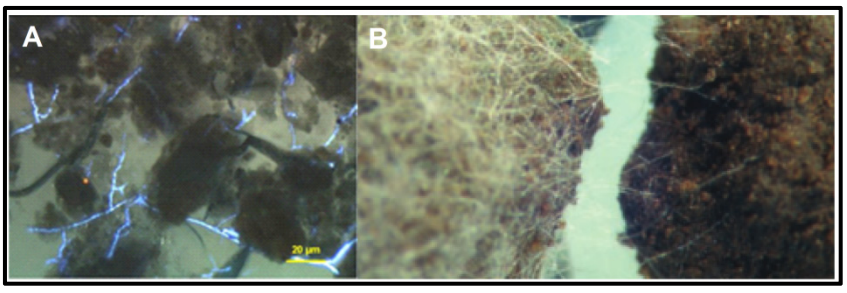

Fig. 5. A) Mycelium of *Rhizoctonia solani* growing in sterilised arable soil. B) Hyphae of *Fusarium oxysporum* f. sp. *raphani* colonising a pair of adjacent soil aggregates. Reproduced from Ritz and Young (2004)

Fungi play a crucial role in soil structure. At the µm scale, the physical extension of hyphae through soil causes mechanical disturbances and realignments of particulate materials. On a large scale, mycelial networks bind soil together through hyphal entanglement (Meadows et al., 1994). This group of microorganisms is involved in the decomposition and mineralization of complex compounds of animal and plant origin such as chitin, cellulose, hemicellulose, pectin and lignin (Orgiazzi et al., 2012).

Fungi play an especially important role in the degradation of lignin, the second most abundant component of plant litter, which is resistant to bacteria-mediated decomposition (Theuerl and Buscot, 2010). They are involved in biogeochemical cycles, carbon and nitrogen storage, and their fruiting bodies (sporocarps) are eaten by a variety of invertebrates and vertebrates, including humans (Newbound et al., 2010). Some fungi establish symbiotic relations (mycorrhiza) with plants, which influence plant community composition and favour phosphorus and nitrogen mobilization in soil. However, soil fungi also include plant pathogens such as *Fusarium* spp., *Rhizoctonia* spp., *Thielaviopsis* spp. and *Sclerotinia sclerotiorum*, responsible for the development of plant diseases. It is also possible to find the pathogen fungi of various arthropods, particularly Diptera and Homoptera (Bridge and Spooner, 2001). Due to their effective enzymatic machinery, they are efficient in degrading contaminants (Ritter and Scarborough, 1995). Furthermore, these organisms are used to produce important substances for medicine such as penicillin, cephalosporin, cyclosporine or lovastatin as well as substances for industry as enzymes and other natural products (Bills et al., 2004).

2. Sustainable agriculture: an alternative to intensive farming

Intensive agriculture characterized by excessive and deep soil tillage, luxury irrigation, intensive cropping and the use of pesticides and synthetic fertilizers is degrading soils and reducing SOM content. The SOM loss leads to a worsening in soil fertility and structure as well as destruction of soil biodiversity resulting in a deterioration of soil quality and the degradation of land. It has been estimated that ~2% of global terrestrial net primary productivity is lost each year due to land degradation (Zika and Erb, 2009). This problem is especially important in the Mediterranean region due to its specific climatic conditions such as a negative annual rainfall balance, a short and variable rainy season and extreme temperatures, compounded by current global climate change and intensive farming which increase the rate of SOM decomposition (Kassam et al., 2012). One of the principal reasons for SOM loss is the indiscriminate use of chemical fertilizers which, without adding organic matter to soil, are a quick and effective way of providing plants with micro and macronutrients necessary for their growth. These fertilizers can produce leaching, runoff, greenhouse gas emissions, loss of biodiversity and eutrophication of aquatic systems (Thangarajan et al., 2013).

Sustainable agriculture represents an alternative to intensive farming and its ill effects mentioned above. The United States Department of Agriculture (USDA) defines sustainable agriculture as an integrated site-specific system of plant and animal production practices that will in the long term: i) satisfy human food and fiber needs, ii) enhance the quality of the environment and the natural resource base upon which the agricultural economy depends, iii) make the most efficient use of nonrenewable resources and on-farm resources and integrate, where appropriate, natural biological cycles and controls, iv) sustain the economic viability of farm operations and v) enhance the quality of life for farmers and society as a whole. Sustainable agriculture advocates the application of ecological practices to the maintenance and improvement of soil structure and fertility through the use of the most effective animal and plant species, crop rotations, soil microorganisms for nutrient cycling, an improved relationship between microorganisms and plants and the use of organic amendments.

Organic amendments in sustainable agriculture

The application of organic amendments to soil is being proposed as an effective way of restoring soil fertility while at the same time protecting the environment because their use could be part of a strategy to eliminate and recycle massive amounts of waste generated in an increasingly populous world.

There is a wide range of residues which have the potential to be used as agricultural amendments. Some of the major organic amendments that can be applied to soils are (Thangarajan et al., 2013):

- ✓ **Animal manure**

  The amount of animal manure available for agriculture has increased in recent years with the corresponding growth in intensive animal production systems. The common forms of animal manure include farmyard manure and farm slurry (liquid manure).

- ✓ **Composted organic matter**

  Compost most suitable for use in agriculture is made from crop residues, organic materials from municipal solid wastes and manures from feedlots.

- ✓ **Plant residues**

  Crop residues include stalks and stubble (stems), leaves and seed pods. Residues from the olive oil and paper industries can be included in this

category. Total global crop residue production has been estimated at 3.8 billion tonnes.

- ✓ **Biosolids**

  Biosolids consist of treated wastewater residues from municipal wastewater treatment plants.

The addition of these organic amendments to soil can have both direct and indirect beneficial effects on soil properties. Usually, it produces a general improvement in soil fertility which can be defined as the capacity of soil to provide for the physical, chemical and biological requirements of plant growth in relation to the productivity, reproduction and quality appropriate to plant and soil type, land use and climatic conditions (Abbott and Murphy, 2007). Organic amendments produce changes in physical, chemical and biological fertility, with the most important change being an increase in SOM and thus an immediate enhancement in soil organic carbon depending on the quantities of amendment applied (Gregorich et al., 1994). The long-term application of organic amendments increases organic carbon by up to 90% in unfertilized soil and by up to 100% with respect to chemical fertilizer treatments (Diacono and Montemurro, 2010). These increases will, in turn, enhance soil aggregate stability and other properties such as soil porosity, water infiltration, water holding capacity and percolation, thus improving physical fertility (Celik et al., 2004; Leroy et al., 2008). On the other hand, although each amendment has specific cation exchange capacity characteristics, it has been demonstrated that soil cation exchange capacity increases after the application of amendments (Odlare et al., 2008). This is vital for retaining essential nutrient cations and making them available to plants. Thus, organic amendments are responsible for enhancing other soil properties related to chemical fertility such as pH, electrical conductivity and the availability of essential nutrients, such as N, P, and K, for plant growth (Larney and Angers, 2012). On the other hand, microorganisms are the principal cause of organic matter transformation introduced into soil by organic amendments; they essentially carry out nutrient cycling and the storage of nutrients in their cells (Murphy et al., 2007). It has been reported that the application of municipal solid waste, sewage sludge, compost and manure enhances fungal and bacterial communities and their activity (soil respiration and enzyme activities) (Acea and Carballas, 1996; Bastida et al., 2008; Casacchia et al., 2010; Farrell et al., 2010; Mattana et al., 2014). The continuous release of nutrients can sustain the

microbial biomass and activity for longer periods of time, also resulting in longer plant nutrient availability (Diacono and Montemurro, 2010). Organic inputs also produce modifications in microbial community structures. Some studies have shown that organic amendments produce an increment in soil microbial diversity (Poulsen et al., 2013). However, less attention has been devoted to changes in soil microbial structure and their effects on soil fertility over the long term.

Despite the numerous benefits, the use of raw waste as organic amendments could also adversely affect the environment, with some of the disadvantages of amendments relating to their degree of stabilization. The addition of organic material that has not been sufficiently stabilized may increase ammonia volatilization, decrease oxygen concentration, produce phytotoxic compounds and immobilize soil mineral N (Diacono and Montemurro, 2010). In addition, organic amendments are often regarded negatively as waste products with undesirable features such as odor, pathogens, toxins and other contaminants. The most dangerous substances present in amendments are probably the heavy metals that can enter the food chain by being taken up by plants from the soil (Peng et al., 2009; Grabowska, 2011) and inhibit soil microbial activity (Nakatsu et al., 2005; Wang et al., 2013). Consequently, before raw wastes are applied to soil, it is necessary to identify the potential toxic substances present in these residues and determine the concentrations of these residues that should be used to avoid excessive concentrations of toxic substances. In the case of composted residues, a balance between composting time and environmental safety needs to be reached, and other organic waste stabilization methods need to be developed to prevent undesirable effects on soil fertility (Smith, 2009).

3. Olive oil industry and its wastes

The olive oil industry is of great social, economic and ecological importance in Mediterranean countries, with ~98% of global olive oil production is concentrated in this region. Within this area, European Union countries produce 72% of this oil, with Spain yielding 59% of European olive oil, Italy 22% and Greece 15%. In Spain, the land area devoted to the olive oil industry was 2,280,456 ha in 2009 with the 2013/2014 harvest yielding 1,536,000 tons of olive oil [International Olive Council (http://www.internationaloliveoil.org/)]. According to these data, this industry produces an enormous volume of olive oil and is faced with the challenge of managing massive amounts of waste. The olive oil extraction process generates (Fig. 6) olive

leaves, small twigs and other debris as well as process waters. On the other hand, the three-phase olive oil system produces pomace waste (a solid cake-like by-product) and another residue called "alpechín" or olive mill wastewater (OMW) regarded as the most environmentally dangerous; while the two-phase system generates another waste known as "alpeorujo" [two-phase olive-mill waste (TPOMW)] (Morillo et al., 2009) .

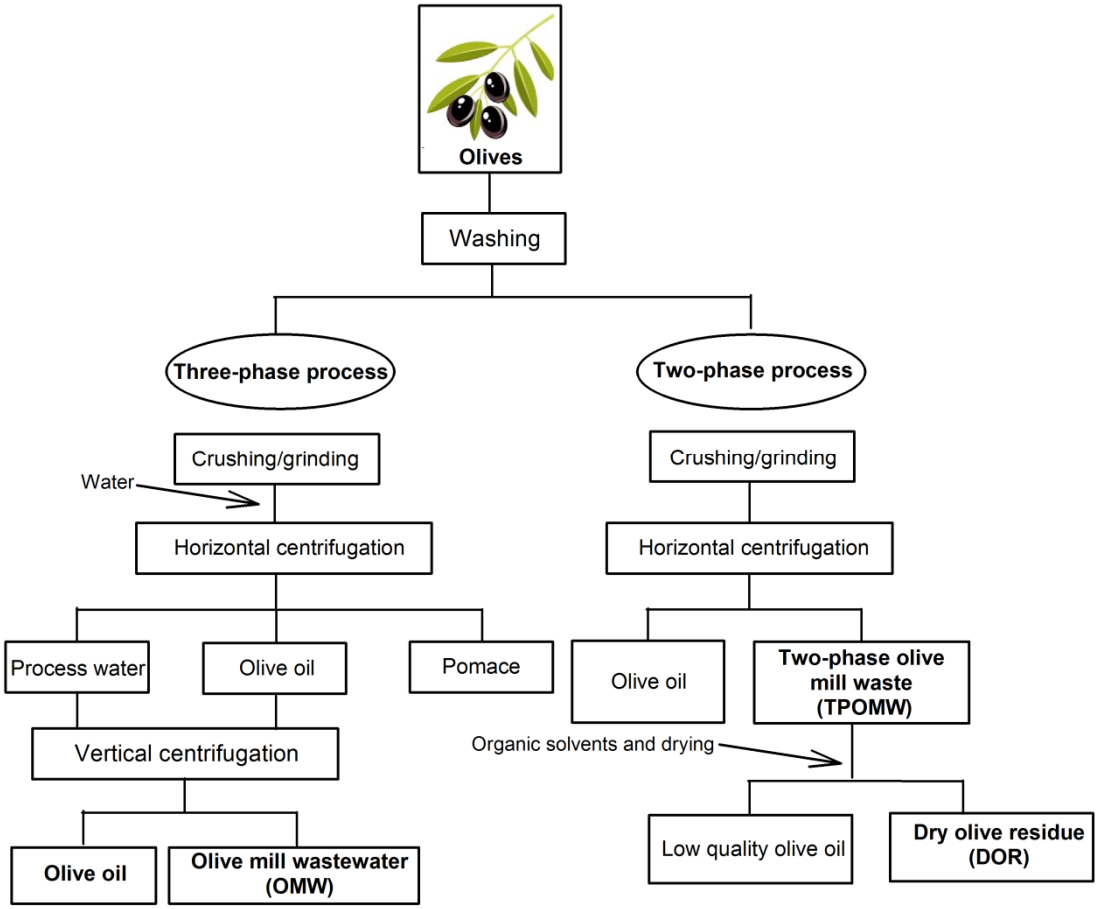

Fig. 6. Three-phase and two-phase olive oil extraction systems. Adapted from Morillo et al. (2009).

In Spain, the two-phase olive oil extraction system was introduced at the end of the 1991/1992 olive oil harvest and quickly replaced the three-phase system as it reduced water consumption and consequently the adverse environmental impact. This continuous and horizontal centrifugation technology yields about 800 kg of TPOMW and 200 kg of oil per 1,000 kg of olives (Roig et al., 2006). TPOMW consists of a thick sludge that contains water, pieces of stone and the pulp of olive fruit. This residue has high moisture content (55-65%), acidic pH values (~4.5) and is rich in organic matter (~95%), which is composed of lignin, hemicellulose and cellulose. It also has high levels of fat (10% of organic matter), proteins (8%), water-soluble carbohydrates (10%),

phenols (1.5%) and potassium (Alburquerque et al., 2004). Phenolic fractions, which have been identified as responsible for the principal harmful effects of TPOMW, generally have a highly variable composition depending on the extraction method, maturity of olives and analytic method. The most common phenols identified in TPOMW include tyrosol, catechin, tannic acid, oleuropein, vanilline, α-tocopherol, rutin, syringaldehyde and *trans*-cinnamic acid (Priego-Capote et al., 2004)

TPOMW and OMW, both olive mill wastes, pose a serious environmental threat in the Mediterranean basin if appropriate management strategies are not applied. Uncontrolled dumping of these wastes has produced soil and groundwater contamination during decades (Sierra Llopart et al., 2000). It has been reported that raw olive mill wastes may produce alterations in the physico-chemical properties of soil, such as porosity, water retention, salinity, pH and C/N ratios (Lozano-García et al., 2011; Barbera et al., 2013). It has also been demonstrated that these residues have a toxic effect on plants, microorganisms and other organisms as nematodes, arthropods, crustaceans, rotifers and algae (Justino et al., 2012). In particular, the exposure of molluscs to OMW produces loss of lysosomal membrane integrity on haemocytes, inhibition of acetylcholinesterase activity and DNA damage (Danellakis et al., 2011). These residues are capable of producing necrotic cells and root-tip disorders, as well as inhibiting protein synthesis and mitosis on Vicia *faba* roots (El Hajjouji et al., 2007). These residues thus induce mutagenic, genotoxic and cytotoxic effects at low concentrations on a wide range of organisms (Justino et al., 2012). It is still unclear which components of olive mill wastes produce these effects, although there are evidences that phenols are the main responsible for these activities, especially monomeric phenols, due to their structure and lipophilic characteristics (Medina et al., 2011). According to other authors, lipidic fractions and cell wall fragments may also be involved in olive mill waste toxicity (Bucheli et al., 1990; Tortosa et al., 2012; Ntougias et al., 2013).

3.1. Two-phase olive-mill waste revalorization

TPOMW is generated by olive oil manufacturers in large quantities over a short period of time. Most of these by-products undergo a transformation process in specialized installations called “orujeras”, which involves drying the waste and the extraction of residual oil with the aid of organic solvents in order to generate a low-quality olive oil (“orujo” oil) and another residue known as “dry and extracted

alpeorujo" or, in technical terms, dry olive residue (DOR) (Lopez-Piñeiro et al., 2008) (Fig. 7). With its high calorific strength (400 kcal kg^{-1}), DOR is used to generate thermal or electric energy by means of combustion. In most cases, this energy is used to operate olive oil plants (Roig et al., 2006). Although the use of DOR as boiler fuel after briquetting has become very common in recent years in countries such as England, current international regulations limit the use of this type of fuel which produces environmentally harmful gases (Sampedro et al., 2009b; López-Piñeiro et al., 2011). For these reasons, its application to the soil as organic amendment has been proposed

Fig. 7. A) Two-phase olive-mill waste (TPOMW). B) Dry olive residue (DOR) in olive oil plants near Granada (Spain).

Other strategies proposed for TPOMW transformation is the production of biogas (a mixture of CH_4 and CO_2) through the anaerobic digestion of the residue (Roig et al., 2006). TPOMW is also suitable for livestock feed after being mixed with protein supplements or pre-treatments (Moumen et al., 2008). In addition, this residue could potentially be used as a low-cost fermentation substrate for bacterial and fungal growth. It is thus possible to obtain a detoxified product, substances of biotechnological interest such as enzymes (Reina et al., 2013) or biopolymers and microbial biomass which could, for example in the case of *Pleurotus ostreatus*, be used in agriculture or the food industry (Morillo et al., 2009). TPOWM has also been considered as a source of valuable products such as pectins, oligosaccharides, squalenes, tocopherols, mannitol and polyphenols (Fernández-Bolaños et al., 2006). This waste is rich in phenols such as hydroxytyrosol, tyrosol and caffeic acid, which have been shown to possess antioxidant, cardioprotective and antihypertensive properties and could therefore be used in the pharmaceutical, cosmetic and food industries (Ramos et al., 2013). New approaches are currently being developed in order to make the extraction of these components more effective (Dermeche et al., 2013).

Finally, although TPOMW is a potential organic amendment, current legislation limits the application of this residue in its raw form to soil (Lozano-García and Parras-Alcántara, 2013). The residue needs therefore to be treated, through composing for example, before being used as an amendment (Muktadirul Bari Chowdhury et al., 2013). The principal disadvantage of composting this material relates to the physical characteristics of TPOMW. Given its semisolid consistency, it needs to be mixed with bulking agents. Furthermore, TPOMW composting with other agricultural wastes has been demonstrated to have certain drawbacks such as nitrogen loss, alkaline pH and unbalanced nutrient content (Tortosa et al., 2012). In addition, the quality of TPOMW compost is highly dependent of the aeration system used (Roig et al., 2006). Nevertheless, if appropriate composting strategies are adopted, TPOMW composting is a beneficial way of transforming this residue.

3.2. Dry olive residue as organic amendment

The advantages of using DOR as an organic amendment are its high organic matter content, mainly lignocellulosic in nature, some agronomically interesting cations such as K, P and Ca and the absence of heavy metals and pathogens (Lopez-Piñeiro et al., 2008). The application of this by-product would be especially beneficial in the Mediterranean region, where soils are particularly affected by intensive farming and low organic matter content (Ruecker et al., 1998). This would have a beneficial effect on the soil ecosystem by increasing inorganic matter in soil and returning plant nutrients to croplands. However, the direct application of this waste could produce serious damages to soil structures and toxic effects on soil organisms and plants. In this regard, greenhouse experiments have shown that raw DOR applications in agronomic doses produce a drastic diminution in shoot and root weight in a large variety of crops (Sampedro et al., 2004; Sampedro et al., 2007) and induces oxidative stress in plants (García-Sánchez et al., 2012). Nevertheless, some authors have demonstrated that raw DOR applications to an olive grove did not have a detrimental effect on its output (López-Piñeiro et al., 2011) over the long term, although they did not establish whether the residue damaged plants in the short term. Other studies and previous research carried out by our group have shown that the phytotoxic effects of olive mill wastes are more evident when applied to seasonal or horticultural crops (Roig et al., 2006). Consequently, DOR needs to be biotransformed, for example, by saprobic fungi before being directly applied to the soil.

3.3. Dry olive residue transformation by saprobic fungi

Saprobic fungi can be described as organisms that derive their nourishment from nonliving or decaying organic matter. In general, they present an effective enzymatic system for the degradation of a wide range of compounds. Soil saprobic fungi may use a large variety of carbon sources, from single sugars, to recalcitrant molecules such as organic acids or pectins thanks to their enzymatic machinery which includes: amylases, proteases, ligninases, chitinases, phytases, phosphatases, pectinases, cellulases and xyloglucanases (Aranda, 2006). On the other hand, it has been reported that wood degrading fungi, such as white-, brown- and soft-rot fungi, is highly capable of degrading lignocellulosic biomass. White-rot fungi are the most effective in terms of delignification due to their unique ligninolytic systems (Sánchez, 2009). These fungi degrade lignin by means of oxidative reactions caused by enzymes such as lignin and manganese peroxidases and laccases. Traditionally, some saprobic fungi, such as *Fusarium* spp., *Phanerochaete* spp., *Pleurotus* spp. and *Trametes versicolor*, have been used for biopulping, forage upgrading and bioremediation of soils, wastes and recalcitrant contaminants (Wan and Li, 2012).

Saprobic soil and white-rot fungi have been demonstrated to be efficient in the bioremediation of DOR (Sampedro et al., 2009b). The incubation of DOR with fungi such as *Fusarium oxysporum*, *Fusarium lateritium*, *Phanerochaete chrysosporium*, *Pycnoporus cinnabarinus*, *Phlebia radiata* and *Coriolopsis floccosa* involves the transformation and stabilization of organic matter accompanied by an increment in humification ratio, a decline in C/N rates, lower phenol content, alkalization and a diminution in the residue's phytotoxic activity (Sampedro et al., 2007; Sampedro et al., 2009a). Although the application of saprobic fungi to the biotransformation process of DOR requires large amounts of inoculum and long incubation periods, new transformation strategies based on the immobilization of fungi on a solid substrate have optimized the process and shortened incubation time (Sampedro et al., 2009b).

4. Soil microbiology study

For centuries, ecologists and biologists have been busy describing and cataloguing the Earth's biodiversity. However, the diversity of soil is still largely unknown, especially in relation to microorganisms (Bardgett, 2002). The microbiological study of soil is not only important to deepen our understanding of microbial composition but also because soil microorganisms respond rapidly to disturbances and it can provide instant data on soil health (Schloter et al., 2003). This may be especially useful in specific systems such as agricultural soils, where microorganisms are usually threatened by many agronomic operations. In most cases, although these soils are subject to a large number of practices such as inorganic fertilization, application of amendments and deep tillage, with the aim of maximizing crop production, their impact on soil microorganisms has not been studied (Miransari, 2011). Given the important functions performed by microorganisms in the soil ecosystem and plant development, their behaviours need to be determined when specific agricultural methods such as the application of organic amendments are used. Several approaches may be used in soil microbiology study, in order to observe changes in soil microorganisms' functionality, abundance or diversity. One of the most difficult challenges currently facing soil microbiologists is to determine the relationship between soil microbial phylogenetic changes and alterations in soil functionality (Torsvik and Øvreås, 2002), a problem which is being resolved with the aid of new molecular-based methods. Table 1 summarizes the principal techniques used to investigate soil microbiology and their possible applications. The techniques used in this PhD dissertation are then described.

Table 1. Principal methods used to study soil microbiology

Study type	Techniques
Quantification	
	Adenosine 5'- triphosphate
	Chloroform fumigation-extraction
	Cultivation [colony forming units (CFU)]
	LIVE/DEAD® BacLigh™ Kit
	Phospholipid fatty acid analysis (PLFA)
	Quantitative PCR (qPCR)[1]
	Soil microbial respiration
Diversity	
	Clone libraries
	Cultivation (plating)
	MIS-GC-FAME analysis[2]
	DNA array (Chips)
	Fingerprintings (DGGE, TGGE, SSCP, T-FRLP, ARISA, RISA, LH-PCR, ARDRA, RAPD)[3]
	Next-generation sequencing techniques
Functionality	
	Biolog EcoPlate ™ system
	Functional gene arrays (RNA-based)
	Next generation sequencing (Metatranscriptomic)
	Soil activity enzymes
	Stable-isotope probing (SIP)

[1] qPCR can also be used to assess changes in microbial activity, depending on target gene selected.
[2] Sherlock microbial identification system-gas chromatography-fatty acid methyl ester analysis.
[3]Abbreviations: denaturing gradient gel electrophoresis (DGGE); temperature gradient gel electrophoresis (TGGE); single-strand conformation polymorphism (SSCP); terminal restriction fragment length polymorphism fingerprinting (T-RFLP); automated ribosomal intergenic spacer analysis (ARISA); ribosomal intergenic spacer analysis (RISA); length-heterogeneity PCR (LH- PCR); amplified ribosomal DNA restriction analysis (ARDRA); random amplified polymorphic DNA (RAPD).

4.1. Methods used to quantify soil microorganisms

4.1.1. LIVE/DEAD® BacLigh™ Kit

The LIVE/DEAD® BacLigh™ Kit provides a highly sensitive, single-step, fluorescence-based assay for bacterial cell viability (http://www.lifetechnologies.com). The kit uses two nucleic acid stains: the green-fluorescent SYTO® 9 dye and the red-fluorescent propidium iodide tincture. These stains differ according to their ability to penetrate healthy bacterial cells. While SYTO® 9 penetrates both live and dead bacteria, propidium iodide stains only penetrate bacteria with damaged membranes and reduce SYTO® 9 fluorescence when both dyes are present. Thus, live bacteria with intact membranes fluoresce green, while dead bacteria with damaged membranes fluoresce red (Boulos et al., 1999). Live and dead bacteria can be viewed separately or

simultaneously using fluorescence microscopy, thus obtaining viable or total cell counts in one staining step. Several studies have demonstrated the usefulness of this technique and its application to quantifying the viable bacterial cell fraction of a soil (Hansen et al., 2007; Pascaud et al., 2009).

4.1.2. Phospholipid fatty acid (PLFA) analysis

This technique is based on the premise that certain fatty acids can be used as biomarkers to distinguish between specific microbial groups (Kaur et al., 2005) (Table 2). The main advantage of this technique is that, as phospholipids are rapidly degraded; those remaining must belong to living organisms (Drenovsky et al., 2004). The PLFA method consists of extracting all fatty acids from a soil sample and selecting phospholipids for analysis using gas chromatography-mass spectrometry (Kaur et al., 2005). The PLFA technique has become one of the most commonly used technology to study soil microbiology. The PLFA dataset from a soil can provide us quantitative information about specific groups of microorganisms. In addition, multivariate analyse of PLFA data provides useful information on microbial structure dynamics (Frostegård et al., 2011). This method has been widely used to assess the impact of amendments or fertilizers on soil microbiology (Farrell et al., 2010; Helgason et al., 2010; Treonis et al., 2010; Yu et al., 2013). Nevertheless, it has certain disadvantages. For example, only a limited number of samples can be treated at the same time and some PLFA biomarkers may belong to several microbial taxa (Rincon-Florez et al., 2013).

Table 2. PLFA markers used for different taxonomic microbial groups. Adapted from Moore-Kucera and Dick (2008).

Microbial Group	Specific PLFA markers
Bacteria (common)	
	14:0, 15:0, 17:0
Gram-positive bacteria	
	i15:0, a15:0, i16:0, i17:0 a17:0
Gram-negative bacteria	
	16:1ω5, 16:1ω7, 17:1ω9 18:1ω7, cy17:0, cy19:0
Actinobacteria	
	10Me-16:0, 10Me-17:0 10Me-18:0
Fungi	
	18:2ω6,9

4.1.3. Quantitative PCR

This technique is based on the generation of fluorescent and detectable signals through the exonuclease activity of polymerases. The signals are produced in each cycle of the PCR reaction, which are detected using a highly sensitive device. When the signal overcomes a certain threshold, it is transformed into predicted target gene values on the basis of a pre-established calibration line with standard target DNA (van Elsas and Boersma, 2011). qPCR is directly applied to soil-extracted DNA and facilitates the quantification of genes such as 16S and 28S rRNA (for bacterial and fungal abundance, respectively) or *amoA* and *nifH* (functional genes) (Blagodatskaya and Kuzyakov, 2013). Currently, this method is widely used to assess changes in microbial biomass in different environments due to its high speed, accuracy and sensitivity, is relatively cheap and easy to implement and provides more reliable information than other biochemical methods (Rincon-Florez et al., 2013). However, qPCR has the same limitations as all PCR-based techniques. Primers rarely amplify all members of a given taxonomic group and PCR may introduce errors such as chimeras, mutations, and heteroduplexes (Qiu et al., 2001)].

4.2. Methods used to study soil microbial diversity

4.2.1. Culture-depedent studies

These methods are based on the artificial contribution to organisms of an energy source (light or chemical compounds), nutrients and proper physicochemical conditions to grown (Skinner et al., 1952; Staley and Konopka, 1985). Generally, studies of soil microbial diversity are carried out using Petri plates containing a specific solid culture medium. Dilutions of a soil sample are spread on this medium and, after a period of incubation at a certain temperature and humidity, the grown colonies can be used for quantitative studies such as the determination of CFU g^{-1} soil or can be isolated and identified, usually while sequencing a target gene in the case of a phylogenetic study.

The main problem with this approach is that the microbiologist does not possess sufficient knowledge to copy the endogenous conditions required for microbial growth. As different organisms require different sets of nutrients in varying concentrations and forms, microbiologists need to know the type of nutrients required, the appropriate concentration to sustain microbial growth and to avoid the co-precipitation of the chemical compounds introduced to cultivate the largest possible

number of microorganisms (Alain and Querellou, 2009). Due to the limitations of existing culture media, the type, diversity and number of microorganisms recovered from the natural environment are limited. This problem has been called the "great plate count anomaly" (Staley and Konopka, 1985). Depending on the nature of the samples and sampling technique used, the cultivation efficiency of standard plating techniques is estimated at between 0.001 and 1% (Amann et al., 1995; Delmont et al., 2011; Su et al., 2012). Despite these limitations, culture-dependent studies are important for identifying new organisms, understanding the precise metabolism and functions of microorganisms in the environment and for finding new strains of industrial interest. For these reasons, many advances have been made in recent years in relation to: i) the use of modified media, ii) changes in growth conditions, iii) community culture and coculture, iv) use of transwell plates, v) optical tweezers and laser microdissection and vi) simulated natural environments using diffusion chambers (Pham and Kim, 2012).

Recently, some authors have concluded that culturing methods are not useful in the context of contemporary environmental microbial ecology (Ritz, 2007). However, more recent studies have carried out parallel evaluations of soil bacterial diversity using both high-throughput sequencing and culture-dependent techniques and report that some bacteria can be detected only by means of culturing methods (Shade et al., 2012). Thus, although the number of environmental biodiversity studies using culture-dependent techniques has declined in recent years, these methods are still useful and should be regarded as complementing molecular-based methods.

4.2.2. Sherlock microbial identification system-gas chromatography-fatty acid methyl ester analysis (MIS-GC-FAME)

MIS-GC-FAME is a technique that may be used to identify soil isolates. It is based on growing a strain under specific culture medium and temperature conditions depending on the type of organisms (bacteria, actinobacteria or yeast) involved in order to obtain a stable and reproducible cellular fatty acid profile for this isolate comparable to that in Sherlock Microbial Identification System (MIS) databases. After organism growth, its fatty acids are extracted using saponification and then methylated to obtain fatty acid methyl esters (FAMES) which are transferred to an organic phase that is analysed by gas chromatography (GC). Subsequently, the GC-FAMES profile is

submitted to a Sherlock library which generates a match for the tentative identification of this microorganism (MIS) (http://www.midi-inc.com/pages/microbial_id.html)

This technique has been widely used to characterize culturable diversity in many environments such as mural paintings, eutrophic lakes and caverns (Heyrman et al., 1999; Edwards et al., 2001; Ikner et al., 2007). The advantages of this technique are its low time consumption and relatively low cost. However, its main disadvantage is that when complex environments such as soils are analyzed, Sherlock libraries are limited and matches for specific strains are not accurate. An alternative strategy has thus been proposed when using this technique: the FAMES profiles obtained for each isolate are clustered and groups of isolates are created. Subsequently, to identify the strains making up a cluster, several isolates of each group are phylogenetically identified by sequencing a target gene (Vandecandelaere et al., 2010). In this way, a tentative identification of a set of isolates can be obtained.

4.2.3. Fingerprinting techniques

Fingerprinting techniques refer to a set of molecular methods that can be used to profile the genetic diversity and structure of a microbial community. Many of these techniques are based on PCR amplification and can be divided into two groups according to differential electrophoretic migration patterns on agarose or polyacrylamide gels: i) size-dependent migration (T-RFLP, ARISA/RISA, RAPD, SSCP, LH-PCR) and ii) sequence-dependent migration (DGGE, TGGE) (Rincon-Florez et al., 2013).

DGGE is probably the most well-known and widely used of all the fingerprinting methods mentioned since Muyzer et al. (1993) first described the method for environmental samples. The technique was developed in order to separate PCR-amplified ribosomal DNA fragments from DNA having the same length but different nucleotide compositions. Separation involves applying a linear gradient of DNA denaturing agent (mixture of formamide and urea) on polyacrylamide gels in order to influence the electrophoretic mobility of partially melted doubled-stranded DNA. A GC clamp (GC rich sequence) attached to the 5′ end is used as a special primer to anchor the PCR fragments and prevent them from fully dissociating (Wang and Dick, 2004; van Elsas and Boersma, 2011). This technique also enables the phylogenetic composition of communities to be studied through the extraction of DNA from DGGE bands and their subsequent cloning and sequencing. However, this is a very time-

consuming process and, due to the short length of the sequences obtained, phylogenetic identification of microorganisms is not very accurate. DGGE has been used in many studies of microbial communities in a wide variety of environments in recent years. However, in addition to mistakes committed during the previous PCR of the target gene, DGGE has certain limitations. For example, it can only detect the most abundant species and the interpretation of results can be misleading as a single band may represent multiple species or the same species may be represented by multiple bands (Malik et al., 2008). Nevertheless, some changes, such as taxon-specific primers and nested PCR, have been made to attempt to offset these drawbacks. Despite the appearance of next-generation sequencing techniques, this method continues to be used as it is a quick and inexpensive way of making an initial assessment of changes in a specific microbial community.

4.2.4. Next-generation sequencing techniques

Next-generation, or high-throughput, sequencing techniques are currently the most effective method for identifying metagenomes and metatranscriptomes from different environments (van Elsas and Boersma, 2011). The development of and the possibilities offered by these techniques, applied for the first time just a few years ago, are astonishing thanks to advances in nanotechnology and bioinformatics (Rincon-Florez et al., 2013). For example, it is now possible to visualize the phylogenetic composition of a community using a DNA-based approach (potential functionality) and functional composition with the aid of a RNA-based technique. Although several research techniques are now on the market, pyrosequencing (Roche 454) and Illumina are the most commonly used methods in soil microbiology studies. The principal characteristics of high-throughput sequencing techniques are summarized in Table 3.

Table 3. Comparative summary of high-throughput sequencing platforms. Adapted from Ansorge (2009), Glenn (2011) and Rincon-Florez et al. (2013)].

Technology	Characteristics	Cost	Read Length	Run time	Error Rate	Yield[1]
Roche 454 Pyrosequencer	Pyrophosphate detection	Low	1000 bp	23 h	Low	0.7 Gb
Illumina Genome Analyzer	Fluorescent dNTPs detection	Low	2 x 100 bp	3 to 11 d	Low	120-600 Gb
ABI SOLiD sequencing system	Sequencing by oligo-ligation detection	High	50 bp	8 d	Medium	150 Gb
Ion Personal Genome Device	Semiconductor sequencing technology	Medium	400 bp	3 h	High	20–400 Mb
HeliScope Single Molecule Sequencer	Direct DNA/RNA sequencing fragments	High	35 bp	30 d	High	1 Gb/d
Pacific Biosciences SMRT DNA Sequencer	Single-molecule real-time sequencing platform	Low	1100 bp	2 h	High	230 Gb

[1] Gb- Gigabyte, Mg- Megabyte

As pyrosequencing technology (Roche 454) is used in this PhD thesis; we will analyze its principal characteristics below. This technique was introduced in 2005 by 454 Life Sciences (Roche) with each nucleotide incorporation by DNA polymerase resulting in the release of pyrophosphate (PPi), which initiates a series of downstream reactions leading to the production of light by luciferase. The amount of light produced is proportional to the number of nucleotides incorporated (Mardis, 2008). The process can be described as follows (Fig. 8): the library DNAs with 454 specific adapters are denatured into a single strand and captured by amplification beads followed by emulsion PCR. Then, on a picotiter plate, a dNTP (dATP, dGTP, dCTP or dTTP) will complement the bases of the template strand with the aid of ATP sulfurylase, luciferase, luciferin, DNA polymerase and adenosine 5′ phosphosulfate and release PPi. The ATP transformed from PPi drives the luciferin into the oxyluciferin and generates visible light. At the same time, the unmatched bases are degraded by apyrase. Another dNTP is then added to the reaction system and the pyrosequencing reaction is repeated (Ansorge, 2009; Liu et al., 2012b).

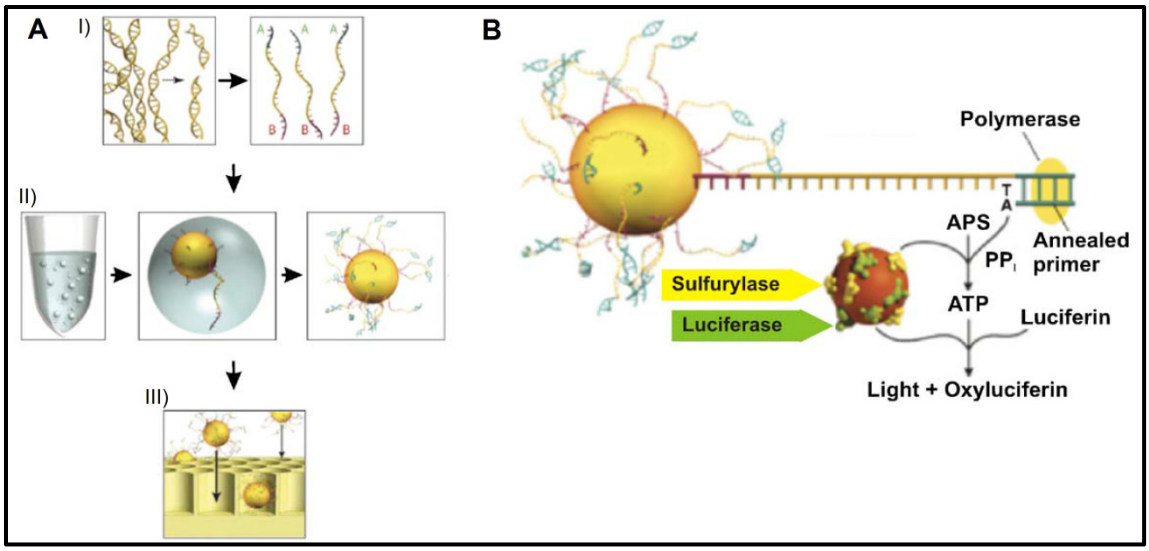

Fig. 8. Method used by the Roche 454 pyrosequencer. A) Library construction. I) ligates 454-specific adapters to DNA fragments (labelled A and B) and couples amplification beads with DNA in an emulsion PCR to amplify fragments before sequencing (II). The beads are loaded into the picotiter plate (III). B) Diagram showing the pyrosequencing reaction which occurs on nucleotide incorporation to report sequencing-by-synthesis. Reproduced from http://www.454.com.

The whole process is monitored by a software which carries out image background normalization, signal location and cross-talk corrections, signal conversion and data generation sequencing. Among other things, the software produces a SFF (standard flowgram format) file after each run (Liu et al., 2012b). SFF files contain all the bases, quality values and signal strengths of sequences. Some programs, such as Mothur (http://www.mothur.org), allow pyrosequencing data processing from this file.

In 2005, Roche 454 yielded a read length of 100–150 bp, 200,000 reads and 20Mb per run. However, the latest Roche pyrosequencer model (Roche 454 GS FLX + System) is able to produce a read length of 1,000 bp and 900 Mb/run. Compared to the Illumina sequencer (the second most common next-generation sequencing technique), Roche 454 produces much lower throughput per run (Table 3), which is a drawback in relation to complex and diverse ecosystems such as soils. However, Illumina sequences are shorter than those of 454 Roche, thus producing less accurate taxonomic assignments (Rincon-Florez et al., 2013).

The first study of soil microbiology (prokaryotes) using pyrosequencing was published in 2007 (Roesch et al., 2007). Since then, the number of studies regarding microbial diversity in different geographical localizations under different climatic and physico-chemical conditions and the assessment of the impact of pollutants and

different land uses on soil microbiology using high-throughput techniques (especially pyrosequencing) has rapidly increased. Most of these studies have used the 16S rRNA gene for bacteria (Fierer et al., 2011; Kolton et al., 2011; Poulsen et al., 2013) and archaea (Ahn et al., 2012) as well as the rRNA internal transcribed spacer (ITS) (Baldrian et al., 2012; Orgiazzi et al., 2012), 18S rRNA (Meadow and Zabinski, 2012; Bastida et al., 2013) and 28S rRNA (Gottel et al., 2011; Hur et al., 2012) gene for fungi as target genes. The 16S rRNA gene was chosen as it has a number of advantages over other genes: : i) it has highly conserved regions that allow effective primer design, ii) some regions of the gene are sufficiently variable to allow accurate taxonomic and phylogenetic identification of community members, iii) lateral transfer of this gene between taxa appears to be rare, and iv) there is a large amount of accumulated 16S rRNA sequence data in databases that permit more accurate taxonomic identification and comparisons of community composition across studies (Fierer and Lennon, 2011). In fungi, ITS has been the most commonly used region in pyrosequencing studies. This region presents certain advantages: i) there is a large number of ITS copies per cell, thus increasing the probability of amplification in samples with low DNA quantities, and ii) the existence of a large database of ITS sequences (Nilsson et al., 2009). However, this region also has certain limitations for use in pyrosequencing studies. For example, it has a wide range of intraspecific ITS variations which complicate the determination of an appropriate sequence similarity cut-off (O'Brien et al., 2005). The 28S rRNA gene could be an alternative approach as it contains two hypervariable regions (D1 and D2) flanked by relatively conserved sequence regions in most fungi. This structure facilitates accurate classification of sequences and alignment of 28S rRNA gene sequences to select a reliable similarity cut-off (Liu et al., 2012a). Furthermore, the 28S naive Bayesian classifier (NBC), recently implemented by the Ribosomal Database Project (RDP), permits direct and rapid sequence classification that facilitates pyrosequencing data processing (Penton et al., 2013).

4.3. Methods used to study soil microbial functionalit

4.3.1. Biolog EcoPlate ™ system

The functionality of a microbial community can be assessed in terms of the ability of microorganisms to use several C sources. The degree of utilization of these carbon sources over a certain period of time by a soil community represents the community level physiological profile (CLPP).

The Biolog EcoPlate™ system is a 96 cell well microtiter plate containing 31 different kinds of separate carbon sources and a redox indicator dye (tetrazolium salts) (Garland, 1996, 1997). The carbon sources consist of: seven types of carbohydrates, nine kinds of carboxylic acids, four kinds of polymers, six kinds of amino acids, two kinds of amines/amides and three kinds of a miscellaneous type (Yu et al., 2012). Environmental samples are inoculated directly onto plates as aqueous samples or after suspension. The plates are then incubated at a constant temperature and analyzed by a spectrophotometer microplate reader at defined time intervals (Merkley et al., 2004; Weber and Legge, 2010). The most popular methods used to analyze data are: the calculation of AWCD (average well color development) and its evolution over time or the selection of a particular incubation time and multivariate analysis of the AWCD of the different carbon sources at this time in different samples as well as certain diversity indices (Insam and Goberna, 2004). Biolog EcoPlate™ has been frequently used in agricultural systems to study changes in soil microorganism functionality caused by different management systems (Gomez et al., 2006; Nair and Ngouajio, 2012). The principal advantages of this technique are that it is not difficult to use, nor it is time-consuming. However, its principal limitation is that only microorganisms that are capable of growing on these nutrient sources and under aerobic conditions are analysed (Insam and Goberna, 2004).

4.3.2. Soil activity enzymes

Enzymes are the main mediators of soil biological processes, such as organic matter degradation, mineralization and nutrient cycling. The evaluation of certain enzymatic activities is a way of assessing the availability of soil microbial communities to carry out specific chemical reactions involved in SOM transformation (Marx et al., 2001). In this regard, hydrolytic enzymes are believed to control the rate at which substrates are degraded and become available for microbial or plant uptake and could therefore be used as functional indicators. Kandeler et al. (1996) have suggested that the study of the diversity of enzymes and their associated activities provides an effective way of examining functional diversity in soils. Furthermore, their rapid responsiveness to environmental disturbances makes them a potential indicator of soil quality.

The most common method for assessing soil enzymatic activities is based on an "in situ" strategy which consist of adding a synthetic substrate linked to a fluorescent

molecule or a substrate that forms a colored compound to dilute homogenized soil suspension under optimized pH and temperature conditions for the analyzed enzyme, and a measure of the increase in fluorescence or absorbance at a given time (Saiya-Cork et al., 2002; Burns et al., 2013). The main disadvantage of this method is that only potential enzymatic activities are actually measured. In other words, it does not provide an insight into the actual rates of enzymatically catalysed reactions under natural "in situ" conditions (Wallenstein and Weintraub, 2008). Nevertheless, this method is still useful as it is fast and inexpensive and provides helpful data when soil treatments, such as different agronomic management systems, are compared (Mohammadi et al., 2011).

Soil enzymes can be classified into four groups: i) oxidation-reduction oxidoreductases:, ii) transferases involved in the transfer of functional groups, iii) hydrolases involved in hydrolysis and iv) lyases: elimination groups to form double bonds (Table 4). However, the most important soil enzymes are oxidoreductases and hydrolases (Karaca et al., 2011).

Table 4. Classification of soil enzymes

Activity	Enzymes
Hydrolases	
	Acetylesterase (*Acetic ester* + H_2O → *alcohol* + *acetic acid*)
	α-and β-amylase (*Hydrolysis of 1,4-glucosidic bonds*)
	Asparaginase (*Asparagine* + H_2O → *aspartate* + NH_3)
	Cellulase (*Hydrolysis of β-1,4-glucan bonds*)
	α-and β-galactosidase (*Galactoside* + H_2O → *ROH* + *galactose*)
	α-and β-glucosidase (*Glucoside* + H_2O → *ROH* + *glucose*)
	Lipase (*Triglyceride* + $3H_2O$ → *glycerol* +3 *fatty acids*)
	Nucleotidase (*Dephosphorylation of nucleotides*)
	Phosphatase (*Phosphate ester* + H_2O → *ROH phosphate*)
	Phytase (*Inositol hexaphosphate* + $6H_2O$ → *inositol* + 6 PO_4^{-3})
	Protease (*Proteins* → *peptides and amino acids*)
	Urease (CH_4N_2O → $2NH_3$ + CO_2)
Oxidoreductases	
	Catalase ($2H_2O_2$ → $2H_2O$ + O_2)
	Dehydrogenase (XH_2 + A → X + AH_2)
	Diphenol oxidase (*p-diphenol* + ½ O_2 → *p-quinone* + H_2O)
	Glucose oxidase (*Glucose* + O_2 → *gluconic acid* + H_2O_2)
Lyases	
	Pectin-lyase (*Pectin* → *Shortened pectin* + *4,5 unsatured galacturonide*)
	Phenylalanine ammonia-lyase (*L-phenylalanine* → *Ammonia* + *trans cinnamic acid*)
Transferases	
	Transaminase ($R1R2\text{-}CH\text{-}NH_3$ + $R3R4CO$ → $R3R4\text{-}CH\text{-}NH_3$ + $R1R2CO$)
	Transglycosylase and levansucrase ($C_{12}H_{22}O_{11}$ + ROH → $H(C_6H_{10}O_5)nOR$ + $nC_6H_{12}O_6$)

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II. AIMS OF THE THESIS

The use of organic amendments has been proposed as a viable strategy for improving soil properties. This practice can be especially convenient in Mediterranean basin where, despite being a biodiversity hotspot in the Earth, many soils are suffering a process of degradation and fertility loss due to the intensive farming practices. In this region, the dry olive residue, a waste resulting from olive oil industry, can be used as organic amendment after an appropriated pre-treatment such as the transformation by saprobic fungi. Given that microbial communities play indispensable roles in soil and influence the services offered by this ecosystem, their study after application of determined agricultural practices that could affect microbial status such as organic amendment, is crucial. Therefore, on the basis of these premises, the general objective of the present PhD dissertation was to study the impact of biotransformed dry olive residue used as organic amendment on abundance, phylogenetic composition and functionality of a Mediterranean soil microbial community through the application of several methodological approaches at short-time. In parallel, this work aimed the description of bacterial and fungal diversity of a Mediterranean soil. These general objectives were addressed by means of the following specific aims:

1. Analysis of the variations in soil chemical properties, enzymatic activities as well as abundance and phylogenetic structure of bacterial and fungal communities after biotransformed dry olive residue application through qPCR, PLFA and DGGE.

2. Identification of culturable bacterial and fungal diversity of a Mediterranean soil and its changes mediated by biotransformed dry olive residue amendment.

3. Description of pyrosequencing-derived bacterial and fungal diversity of a Mediterranean soil and analysis of the changes in this diversity as well as in community level physiological profiles after biotransformed dry olive residue application.

III. RESULTS

Chapter 1

Shifts in soil chemical properties and bacterial communities responding to biotransformed dry olive residue used as organic amendment

Adapted from:
Siles, J.A., Cajthaml T., Hernández, P., Pérez-Mendoza D., García-Romera, I., and Sampedro, I. (Under review) Shifts in soil chemical properties and bacterial communities responding to biotransformed dry olive residue used as organic amendment. *Biology and Fertility of Soils*.

Abstract

Dry olive residue (DOR) is a waste product derived from olive oil extraction system and has been proposed as organic amendment. However, it has been demonstrated that a pre-treatment, such as its transformation by saprobic fungi, is required before DOR soil application. A greenhouse experiment was designed where 0 and 50 g kg^{-1} of raw DOR (DOR), *Coriolopsis floccosa*-transformed DOR (CORDOR) and *Fusarium oxysporum*-transformed DOR (FUSDOR) were added to soil containing a sorghum plant. DOR showed phytotoxic activity on plants, while CORDOR and FUSDOR ameliorated this toxic effect. Analyses of the soil chemical properties as well as the structure and relative abundance of bacterial and actinomycetal communities were carried out after 0, 30 and 60 days following amendment. The different amendments produced a slight decrease in soil pH and significant increases in carbon fractions, C/N ratios, phenols and K, with these increases being more significant after DOR application. Quantitative PCR assays of the 16S rRNA gene and PLFA analyses showed that all amendments favoured bacterial growth at 30 and 60 days, although actinomycetal proliferation was more evident after CORDOR and FUSDOR application at 60 days. Bacterial and actinobacterial DGGE multivariate analyses showed that the amendments produced structural changes in both communities, especially after 60 days of amendment. PLFA data analysis identified changes in soil microbial communities according to the amendment considered, with FUSDOR and CORDOR being less disruptive than DOR. Finally, integrated analysis of all data monitored in the present study enabled us to conclude that the greatest impact on soil properties was caused by DOR at 30 days and that soil showed some degree of resilience after this time.

Keywords

"Alpeorujo"; Bioremediation; Biotransformation; Mediterranean soil; Olive wastes; Soil microbial community

INTRODUCTION

The olive oil industry produces large quantities of wastes in olive growing regions around the world (Justino et al., 2012). Most of these residues are generated during the olive-oil extraction process (Tortosa et al., 2012) which, in recent decades, has been carried out mainly using the three- and two-phase method, depending on what final result is obtained (Alburquerque et al., 2004). The two-phase olive oil extraction method is mostly used in Spain (Roig et al., 2006). This system generates a liquid (olive oil) and an organic sludge (two-phase olive mill waste, TPOMW) (Alburquerque et al., 2009). Subsequently, TPOMW is revalorized by means of organic solvent and heat treatments in order to generate a low-quality olive oil and a by-product called dry olive residue (DOR) or "alpeorujo" (López-Piñeiro et al., 2008). In Spain alone, 5 million tons of this residue are produced annually over a short time period (Tortosa et al., 2012). DOR contains high levels of phenols, salinity and acidity (Ntougias et al., 2013). Inappropriate disposal of DOR can therefore generate: (i) negative effects on the physical, chemical and biological properties of soil, (ii) phytotoxic effects and (iii) groundwater pollution (Barbera et al., 2013). For these reasons, it is necessary to develop strategies for the correct management of this waste in order to avoid agro-environmental hazards. The use of DOR as an organic amendment has been proposed as a possible strategy due to its high organic matter and nutritionally relevant cations content (Alburquerque et al., 2009; López-Piñeiro et al., 2011). Furthermore, unlike other organic wastes, this residue is free of heavy metals and pathogenic microorganisms (López-Piñeiro et al., 2008). However, due to the aforementioned potential environmental risks that the direct application of this residue to soil may produce, a DOR pre-treatment phase would be required before being used as an organic amendment. One of the most effective strategies proposed for DOR bioremediation is transformation by saprobic fungi (Sampedro et al., 2007; Sampedro et al., 2009b). This transformation of DOR stabilizes organic matter, enhances its C/N ratio, reduces the phenolic fraction and eliminates phytotoxic effects (Sampedro et al., 2009a).

In the Mediterranean region, many soils are sensitive to erosion and structural deterioration due to specific ecological conditions such as aridity (Toscano et al., 2013). In this region also, the transition from traditional techniques to intensive-mechanized farming methods has produced a reduction in soil organic matter (Lal, 2006). These

new practices may alter microbial community structures and composition which directly or indirectly influence the soil ecosystem, nutrient cycle activity and crop production (Kibblewhite et al., 2008). One of the most extensive practices is the use of chemical fertilizers, which enhance crop yield but also alter soil properties and functional diversity in microbial populations (Chaudhry et al., 2012). Nevertheless, the maintenance of microbial diversity functionality and composition is essential for sustainable agricultural production. It has been demonstrated that soils under an organic farming system are of higher quality and superior microbial activity than soils subjected to non-organic practices (Nautiyal et al., 2010). An organic amendment containing treated DOR could therefore represent an alternative to chemical fertilizers in order to maintain an appropriately balanced ecosystem in the Mediterranean region. However, before using treated DOR as an organic amendment, it is necessary to study the behaviour of bacterial communities in soils amended with this type of transformed residue. It is also important to investigate actinobacteria communities, which have been shown to be one of the most common phyla in soil (Janssen, 2006). Furthermore, they play an important role in the degradation of polymeric and xenobiotic substances (Karpouzas et al., 2010).

Previous studies have shown the effect of raw DOR amendments on soil physical, chemical and certain biological properties, regarding soil enzymatic activity, over the long term (López-Piñeiro et al., 2008; López-Piñeiro et al., 2011). However, to the best of our knowledge, the impact of fungi-transformed DOR on chemical soil properties or the structure and abundance of bacteria, specifically actinobacteria, in agricultural soil has not been studied except for a preliminary study under in vitro conditions (Sampedro et al., 2009b). For this reason, and as it has been suggested that most potential changes in soil microbiology occur during the first weeks following application of organic amendments (Blagodatskaya and Kuzyakov, 2008), this study aimed to assess the short-term effects of DOR transformed by the ligninolytic fungus *Coriolopsis floccosa* and the soil saprobic fungus *Fusarium oxysporum* on selected chemical soil properties and on the abundance, structure and diversity of bacterial and actinomycetal soil communities. To obtain an integrated approach to the study's objectives, several techniques were used: quantitative PCR (qPCR), denaturing gradient gel electrophoresis (DGGE) and phospholipid fatty-acid analysis (PLFA).

MATERIALS AND METHODS

Materials

The soil used in this study was obtained from the "Cortijo Peinado" field (Granada, Spain, 37º13'N, 3º 45'W). It was classified as loam (clay, 17.15%; sand, 34.35%; silt, 48.50%) according to the USDA system (USDA-NRCS, 1996). Ten 5 kg samples were randomly collected from the Ap horizon on the plot (10.000 m^2). Subsequently, the different samples were sieved (5 mm mesh) and mixed. The soil was stored for 3 days in thin mesh plastic bags at room temperature until the experiment was initiated.

DOR was supplied by an olive oil manufacturer (Sierra Sur S.A., Granada, Spain) and was frozen at -20 ºC until use.

DOR biotransformation

DOR transformation was carried out with the fungi: *Coriolopsis floccosa* (Spanish Type Culture Collection, CECT 20449), formerly known as *C. rigida,* and *Fusarium oxysporum* (Mycological Culture Collection of the Department of Biological Sciences, Faculty of Exact and Natural Sciences, University of Buenos Aires, BAFC 738). For DOR transformation, polyurethane sponge (PS) cubes, 0.5 cm in width, were rinsed with water in a 1:20 (w/v) ratio and autoclaved three times prior to their use. 1.5 g of sterilized PS cubes were placed in Erlenmeyer flasks, and 25 mL of culture medium [50 g L^{-1} of glucose anhydrous (Acros Organics) and 5 g L^{-1} of yeast extract (Fisher Chemical)] were added and again autoclaved. Subsequently, 5 mL of *C. flocossa* or *F. oxysporum* inoculum (ca. 50 mg dry weight) were aseptically added to each Erlenmeyer flask with PS and incubated at 28 ºC for 7 days. After this period of time, 25 g of sterilized DOR were placed above the colonized PS. Solid-state cultures on DOR were incubated at 28 ºC in the dark under stationary conditions for 30 days. Non-inoculated DOR samples were prepared and incubated as controls. DOR controls, DOR incubated with *C. floccosa* and DOR incubated with *F. oxysporum* were then autoclaved several times for complete sterilization. The different residues were sieved (2 mm) manually, homogenized and stored at 4 ºC until the soil amendment experiment began.

Soil amendment

The experiment was carried out in 0.5 L pots. Untransformed DOR (DOR), DOR transformed by *C. floccosa* (CORDOR) and DOR transformed by *F. oxysporum* (FUSDOR) were added to soil pots at concentrations of 50 g kg^{-1}. Soil samples without the residue (C) were also prepared. One sorghum plant (*Sorghum bicolor*) was planted in each pot. The experiment was performed in a greenhouse with supplementary light at 25/19 ºC and 50% relative humidity. Regular manual watering was provided during the experiment.

The soil without the residue and amended with DOR, CORDOR and FUSDOR was analysed after 0, 30 and 60 days of treatment. The experiment consisted of five pots of each treatment at all sampling times. In each soil sampling, the soil from 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 and stored at -80 ºC until sample analysis was carried out.

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

DOR and soil chemical analyses

The pH and electrical conductivity (EC) of soil samples and amendments were determined in a 1:10 (w/v) soil:water extract and in a 1:5 (w/v) amendment:water extract. The phenolic content of DOR, CORDOR and FUSDOR (1 g) and the different soil samples (0.5 g) was determined by extraction with a 10 mL distilled water/acetone mixture (50:50 v/v) for 24 h under orbital shaking (200 rpm). Total phenolic content was estimated according to Sampedro et al. (2004), using tannin acid as the standard. Total concentrations of K, Ca, Mg, Na and P of amendments and soil samples were determined by digestion with HNO_3 and H_2O_2, followed by analysis using inductively coupled plasma optical emission spectrometry (ICP-OES) (ICP 720-ES, Agilent, Santa Clara, USA). The analyses were carried out by the Instrumental Technical Services of EEZ-CSIC, Granada, Spain. The measurements of total N (N_{tot}), total carbon (C_{tot}) and organic carbon (C_{org}) from amendments and soil samples were determined using the Leco TruSpec® CN system (Leco Corporation, St. Joseph, USA) after dry combustion of the samples. The water soluble organic carbon (WSOC) of soil was extracted with de-ionized water at 1:10 (soil:water) and determined by the wet oxidation method (Mingorance et al., 2007). The reaction was carried out with 3 mL $K_2Cr_2O_7$ and 6 mL

H_2SO_4, and the Cr^{3+} resulting from organic C oxidation were determined using spectrophotometry (590 nm). The C/N ratio was calculated as C_{org}/N_{tot}. Color, chemical oxygen demand (COD) and ergosterol content of DOR, CORDOR and FUSDOR were determined according to Sampedro et al. (2004), Brozzoli et al. (2009) and Šnajdr et al. (2008), respectively.

DNA extraction

DNA was extracted from 0.25 g of the different soil treatments using the MoBio UltraClean Soil DNA Isolation Kit (MoBio Laboratories Inc., Solana Beach, CA, USA) following the manufacturer's instructions. Three different DNA extractions were performed for each treatment. Subsequently, all DNAs were quantified using the QuantiFluor™ dsDNA System. The DNA concentration for each extraction was standardized to a final concentration of 5 ng/µL and stored at -20 ^{o}C.

Quantitative PCR

QPCR was executed on the iCycler iQ5 (Bio-Rad, Hercules, CA, USA). The 16S rRNA gene amplification reactions were carried out with the set of primers Eub338/Eub518 for bacteria and Actino235/Eub518 for actinobacteria (Fierer et al., 2005) (see table A1 in Appendix 1 for primer sequences). Each 25 µL contained: 12.5 µL iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, USA), 0.5 µL of each primer (10 µM) (Sigma-Aldrich Co., St. Louis, USA), 1 µL template DNA (5 ng) and 10.5 µL H_2O. All the samples were analysed in triplicate on polypropylene 96-well plates under the quantitative PCR conditions described by Fierer et al. (2005). Melting curve analysis of the PCR products was conducted to ensure amplification of a single product.

The bacterial and actinomycetal standard curves were obtained by serial dilutions (ranging from 10^2 to 10^4) of genomic DNA from *Enterobacter cloacae* (HF954380) and *Streptomyces pilosus* (HF954395), respectively. The curve was obtained by plotting the Ct value as a function of the log of the copy number of the 10-fold dilution serial of genomic DNA. The relationship between Ct and the gene copy number of targets and standards was calculated as described Yun et al. (2006) using the data on the 16S rRNA gene copy number provided by Vetrovsky and Baldrian (2013).

PCR-DGGE

The bacterial and actinomycetal communities in the different samples were analysed by means of PCR-DGGE. For the bacteria, the V6-V8 region of the 16S rRNA gene was amplified through the primers 968F+GC and 1401R (Brons and van Elsas, 2008). A 40 pb GC clamp at the end 5' of primer 968F was used (see Table A1 in Appendix 1 for primer sequences). Each PCR, consisting of: 2.5 µL dNTPs (2 mM), 2.5 µL NH_4 buffer (10X), 1 µL $MgCl_2$ (50 mM), 0.5 µL of each primer (10 µM) (Sigma-Aldrich Co., St. Louis, USA), 0.5 µL Taq DNA Polimerase (5 U μL^{-1}) (BIOTAQ™ DNA pol, Bioline, London, UK) and 1 µL template DNA (5 ng), was completed with H_2O up to 50 µL. The PCR program was carried out according to Brons and van Elsas (2008). For the actinobacteria, the V3 and V4 region of the 16S rRNA gene was amplified with the primers 341F+GC and Act704R (Xiao et al., 2011) (see Table A1 in Appendix 1 for primer sequences). The PCR mixtures were the same as those for bacterial amplification, and the PCR program was performed as described by Xiao et al. (2011). All the amplifications were executed in a Mastercycler® Personal (Eppendorf, Applied Biosystems, Foster City, USA), and all PCR products were tested by electrophoresis in 1.5% agarose gels stained with SYBR® Gold Nucleic Acid Gel Stain (Life Technologies™, Carlsbad, USA).

For the bacteria and actinobacteria, DGGE analyses were carried out on an INGENYphorU-2x2 system (Ingeny International BV, Goes, The Netherlands). Polyacrilamide gels (9%) were prepared in 1 x TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM disodium EDTA, pH 8.2). 10 µL of a mixture from the three different PCR products of each sample were use in the DGGE analyses. The polyacrylamide gels were made with a denaturing gradient ranging from 40% to 60%. Gel electrophoresis was run for 16 h at 60 ^{o}C and 85V. After completion of electrophoresis, the gels were stained with SYBR® Gold Nucleic Acid Gel Stain. The stained gel was captured using a digital camera. The image was then analysed using InfoQuest FP software (Bio-Rad Laboratories, Inc., Hercules, USA).

Phospholipid fatty acid (PLFA) analysis

Microbial lipids from soil were extracted using a mixture of chloroform-methanol-phosphate buffer (1:2:0.8; v/v/v) according to Bligh and Dyer (1959). Phospholipids were then separated using solid-phase extraction cartridges (LiChrolut Si-60, Merck, Whitehouse Station, USA), and the samples were subjected to mild

alkaline methanolysis as described by Šnajdr et al. (2008). The free methyl esters of phospholipid fatty acids were analysed by gas chromatography mass spectrometry (450-GC, 240-MS ion trap detector, Varian, Walnut Creek, USA) according to Sampedro et al. (2009b).

Bacterial biomass ($PLFA_{bac}$) was quantified as a sum of i14:0, i15:0, a15:0, 16:1ω7t, 16:1ω9, 16:1ω7, 10Me-16:0, i17:0, a17:0, cy17:0, 17:0, 10Me-17:0, 10Me-18:0 and cy19:0. Actinobacteria biomass ($PLFA_{act}$) was determined according to 10Me-16:0, 10Me-17:0 and 10Me-18:0. Fungal biomass ($PLFA_{fun}$) was estimated on the basis of 18:2ω6,9 content. The fatty acids found in both bacteria and fungi, such as 15:0, 16:0 and 18:1ω7, were excluded from the analysis (Tornberg et al., 2003). The total content of PLFA molecules was used as a measure of total microbial biomass ($PLFA_{tot}$). Several microbial ratios [G+/G- (Gram positive bacteria/Gram negative bacteria), F/B ($PLFA_{bac}/PLFA_{fun}$)] and stress indicators [cy/pre ((cy17:0+cy19:0)/(16:1ω7/18:1ω7)), S/M (Saturated PLFAs/Monosaturated PLFAs] were calculated (Moore-Kucera and Dick, 2008).

Data analysis

Statistical differences between the treatments at a given sampling time were analysed by ANOVA, and Tukey's honest significance difference (HSD) test was used for multiple comparison of means at a 95% confidence interval. All the analyses were carried out using Statgraphics Centurion 16.1.11 (Statpoint Technologies, Inc., Warrenton, USA).

The number and area of bands from bacterial and actinomycetal DGGE analyses were used to calculate different diversity indices: species richness (*S*), the Shannon index (*H*) and evenness (*J*) using the PAST software package (Lv et al., 2012). Significant differences ($p \leq 0.05$) in the Shannon diversity index between each amended sample and its respective control at every sampling time were checked by using the Shannon diversity *t* test (Magurran, 1988).

Principal component analysis (PCA) was used to determine the main trends in the data set and to compare the samples. PCA was firstly used to evaluate bacteria and actinomycetes DGGEs as well as PLFA data and finally, a PCA was carried out including all the chemical and biological characteristics examined in the present study. To perform all the PCAs, a variance-covariance matrix was used after normalization of the data.

RESULTS

DOR transformation

DOR incubation with *C. floccosa* and *F. oxysporum* produced important changes in most of the parameters measured (Table 1). While the transformation caused by the fungi increased pH and colour in the residue; EC, phenols, COD and C/N ratio decreased. According to ergosterol measurements, both fungi were able to grow using DOR as a culture medium although *F. oxysporum* colonized the residue more efficiently. With regard to the different mineral elements evaluated, DOR biotransformation with both fungi caused an increase in Mg content, a decrease in P and Ca (only FUSDOR) and no changes in Na and K levels.

Table 1. Chemical variables (mean±standard deviation) measured in untransformed DOR (DOR), *C. floccosa*–transformed DOR (CORDOR) and *F. oxysporum*–transformed DOR (FUSDOR). For each variable, data followed by different letter are significantly different according to Tukey's HSD test ($P \leq 0.05$).

Variable	DOR	CORDOR	FUSDOR
pH	4.58±0.02a	5.90±0.02c	5.40±0.01b
EC (dS m^{-1})	4.96±0.11a	2.98±0.12b	3.03±0.09b
Phenols (g kg^{-1})	63.52±2.79c	15.63±1.00a	30.44±2.93b
Color (UC)	190.60±10.81a	256.82±6.57c	235.23±3.37b
N_{tot} (g kg^{-1})	15.67±0.35a	17.63±0.31c	16.33±0.25b
C_{org} (g kg^{-1})	536.13±3.85b	464.60±6.03a	427.97±4.63a
C/N	34.26±0.66b	26.35±0.11a	26.20±0.34a
COD (g kg^{-1})	352.80±67.90c	65.33±45.26a	176.40±33.95b
Ergosterol (ppm)	14.77±0.10a	681.07±59.98b	2583.89±226.20c
Total K (g kg^{-1})	20.55±0.67a	20.30±0.68a	20.28±0.29a
Total Ca (g kg^{-1})	6.16±0.06b	6.14±0.04b	5.94 ± 0.14a
Total Mg (g kg^{-1})	1.66±0.04a	1.75±0.05b	1.75±0.03b
Total Na (g kg^{-1})	0.27±0.02a	0.29±0.01a	0.27±0.02a
Total P (g kg^{-1})	2.00±0.02b	1.53±0.02a	1.53±0.03a

Effects of amendments on soil chemical properties and plant growth

The results showed that pH decreased significantly in the samples amended with DOR, CORDOR and FUSDOR at the initial sampling time with respect to the control treatment. However, at the other sampling times, only untreated DOR produced a significant reduction in soil pH (Table 2). EC only increased in the soil after application the different amendments at 0 day. All the amendments applied caused a significant increase in soil phenol content at initial sampling time. At the other

sampling times, untransformed DOR also generated a rise in phenol concentrations. Nevertheless, it was not possible to detect significant differences between the control samples and soils amended with CORDOR and FUSDOR, especially at 60 days. On the other hand, no significant changes were observed in N_{tot} concentrations over time, although a slight rise was appreciated in the soil with CORDOR at 30 days (Table 2). The application of DOR, CORDOR and FUSDOR significantly increased levels of C_{tot}, C_{org} and WSOC with respect to the control samples. The increases in C fractions were more evident in the soil amended with DOR at all sampling times. The constant N_{tot} values for all the treatments at all sampling times and the increment in C_{org} for the amended samples produced an increment in the C/N ratio in the amended soils, a rise which highlighted in the samples with DOR. The concentrations of several agronomically important mineral elements (K, Ca, Mg, Na and P) were analysed at all sampling times (Table 2). Significant differences between treatments were only detected in the K concentration. An increment in the amount of this mineral was detected in all the amended soils at the different sampling times.

DOR application to soil had a phytotoxic effect on sorghum plants. The growth inhibition of shoot sorghum plants grown in the presence of DOR for 30 and 60 days was approximately 74% and 93%, respectively. However, the CORDOR and FUSDOR amendments did not result in any significant changes in sorghum shoot dry weight with respect to the plants grown in the unamended samples (Table 3).

Table 3. Shoot dry weight (mg) of sorghum plants from unamended soil (C) and soil amended with untransformed DOR (DOR), *C. floccosa*-transformed DOR (CORDOR) or *F. oxysporum*-transformed DOR (FUSDOR) at 30 (T1) and (T2) 60 days. For each treatment at same sampling time, data followed by the same small letter are not significantly different according to the Tukey test ($p \leq 0.05$). Mean values correspond to five plant measures ±standard deviation.

Soil treatment	T1	T2
C	91.40±6.02b	291.20±9.50b
DOR	23.80±5.07a	19.80±3.90a
CORDOR	89.20±4.44b	295.20±7.89b
FUSDOR	90.20±3.28b	293.30±6.39b

Table 2. Chemical variables (mean±standard deviation) measured 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. For each variable and sampling time, data followed by different letter are significantly different according to Tukey's HSD test (P ≤ 0.05).

Variable	C –T0	DOR–T0	CORDOR–T0	FUSDOR–T0	C–T1	DOR–T1	CORDOR–T1	FUSDOR–T1	C–T2	DOR–T2	CORDOR–T2	FUSDOR–T2
pH	8.40±0.01d	7.91±0.01a	8.06±0.01b	8.15±0.02c	8.41±0.02b	8.29±0.01a	8.39±0.01b	8.41±0.07b	8.39±0.02b	8.35±0.02a	8.45±0.04b	8.42±0.03b
EC (dS m^{-1})	0.17±0.05a	0.33±0.04c	0.23±0.04b	0.23±0.03b	0.18±0.02a	0.18±0.04a	0.17±0.03a	0.17±0.02a	0.18±0.02a	0.18±0.03a	0.17±0.01a	0.19±0.02a
Phenols (g kg^{-1})	2.16±0.22a	5.08±0.43c	3.03±0.15b	3.14±0.34b	2.24±0.10a	3.91±0.13c	2.66±0.34ab	3.03±0.31b	2.47±0.22a	3.06±0.23b	2.72±0.19ab	2.79±0.13ab
N_{tot} (g kg^{-1})	1.68±0.09a	1.73±0.03a	1.79±0.09a	1.72±0.05a	1.67±0.08a	1.71±0.07a	1.84±0.09a	1.69±0.09a	1.66±0.05a	1.71±0.05a	1.72±0.11a	1.69±0.05a
C_{tot} (g kg^{-1})	41.03±0.50a	55.17±1.26c	51.30±0.98b	50.33±0.58b	42.05±0.45a	53.17±0.47c	51.43±1.43b	51.60±1.73b	42.00±0.72a	52.83±0.76b	51.82±1.38b	50.03±0.57b
C_{org} (g kg^{-1})	12.24±0.30a	24.50±0.50c	21.57±0.40b	21.33±0.51b	12.45±0.49a	23.57±0.21c	20.33±0.25b	19.87±0.23b	12.52±0.24a	22.77±0.71c	18.97±0.90b	18.90±0.53b
WSOC(g kg^{-1})	1.96±0.02a	12.17±0.08c	9.67±0.02b	10.97±0.02c	1.99±0.01a	6.22±0.01d	4.15±0.01b	4.49±0.01c	1.96±0.26a	4.09±0.05c	3.17±0.05b	3.45±0.17b
C/N	7.29±0.52s	14.21±0.31c	12.08±0.84b	12.41±0.12b	7.48±0.62a	13.77±0.45c	11.07±0.66b	11.76±0.54b	7.55±0.22a	13.34±0.27c	11.01±0.23b	11.16±0.20b
Total K(g kg^{-1})	6.57±0.21a	8.72±0.14b	8.63±0.37b	8.52±0.08b	6.36±.23a	8.81±0.60b	8.22±0.56b	8.01±0.42b	6.18±0.12a	8.02±0.52b	8.71±0.21b	8.48±0.23b
Total Ca(g kg^{-1})	67.10±5.33a	65.55±6.30a	64.78±5.91a	66.26±5.64a	66.72±5.86a	64.15±8.36a	63.26±7.89a	62.92±7.00a	66.80±3.39a	63.97±4.04a	64.51±4.80a	62.96±5.32a
Total Mg(g kg^{-1})	22.53±1.13a	21.79±0.44a	21.53±1.16a	22.36±0.62a	22.34±0.62a	22.38±0.57a	21.92±0.05a	21.67±0.60a	21.82±0.70a	21.09±0.57a	21.29±0.83a	20.98±0.79a
Total Na(g kg^{-1})	1.87±0.37a	1.73±0.17a	1.86±0.10a	1.74±0.13a	1.96±0.07a	2.17±0.06a	2.00±0.07a	2.01±0.17a	1.54±0.09a	1.70±0.14a	1.67±0.06a	1.63±0.14a
Total P (g kg^{-1})	0.81±0.01a	0.84±0.04a	0.80±0.04a	0.79±0.05a	0.79±0.02a	0.84±0.01a	0.81±0.03a	0.83±0.01a	0.77±0.05a	0.85±0.05a	0.78±0.08a	0.77±0.06a

Table 6. Microbial biomass properties and physiological stress indicators obtained by PLFA analysis from unamended soil (C) and soil amended with untransformed DOR (DOR), *C. floccosa*–transformed DOR (CORDOR) or *F. oxysporum*–transformed DOR (FUSDOR) at T0 (0 days), T1 (30 days) and T2 (60 days). For each variable and sampling time, data followed by different letter are significantly different according to Tukey's HSD test (P ≤ 0.05).

Variable	C–T0	DOR–T0	CORDOR–T0	FUSDOR–T0	C–T1	DOR–T1	CORDOR-T1	FUSDOR-T1	C–T2	DOR–T2	CORDOR–T2	FUSDOR–T2
$PLFA_{tot}$ (μg g^{-1})	1.34±0.23a	1.55±0.14a	1.71±0.38a	1.44±0.13a	1.81±0.32a	7.31±0.98c	4.55±0.50b	4.19±0.31b	1.29±0.07a	4.84±0.14c	3.68±0.32b	3.44±0.19b
$PLFA_{bac}$ (μg g^{-1})	1.02±0.18a	1.14±0.08a	1.08±0.32a	1.02±0.27a	1.05±0.18a	3.11±0.19c	2.07±0.34b	2.50±0.18b	0.89±0.04a	2.32±0.03b	2.24±0.26b	2.24±0.16b
$PLFA_{act}$ (μg g^{-1})	0.21±0.02a	0.24±0.05a	0.21±0.04a	0.19±0.02a	0.26±0.01a	0.37±0.03b	0.31±0.08ab	0.33±0.03ab	0.25±0.04a	0.30±0.02ab	0.37±0.03b	0.36±0.02b
$PLFA_{fun}$	0.06±0.01a	0.06±0.02a	0.08±0.01a	0.07±0.03a	0.08±0.02a	0.52±0.06c	0.32±0.04b	0.25±0.02b	0.06±0.01a	0.45±0.12c	0.33±0.03bc	0.21±0.03b
G+/G– ratio	1.02±0.01a	1.20±0.12a	1.54±0.37a	1.48±0.16a	1.04±0.08a	0.80±0.05a	0.86±0.11a	0.92±0.06a	0.85±0.02a	0.83±0.17a	0.74±0.03a	0.83±0.04a
F/B ratio	0.06±0.01a	0.05±0.03a	0.07±0.02a	0.07±0.03a	0.08±0.01a	0.17±0.01b	0.16±0.04b	0.10±0.01ab	0.07±0.01a	0.19±0.05c	0.15±0.02bc	0.09±0.02ab
cy/pre ratio	0.48±0.04a	0.39±0.09a	0.31±0.07a	0.38±0.03a	0.44±0.04b	0.23±0.01a	0.24±0.02a	0.22±0.01a	0.42±0.0b	0.30±0.03a	0.29±0.01a	0.27±0.03a
S/M ratio	1.76±0.05a	1.71±0.16a	1.38±0.38a	1.86±0.42a	1.42±0.27c	0.48±0.04a	0.58±0.13ab	0.86±0.02b	1.32±0.01c	0.63±0.04a	0.91±0.06b	0.98±0.07b

Effects of amendments on bacterial and actinobacterial soil communities

Quantitative PCR

Relative abundance of bacterial 16S rRNA gene determined by qPCR did not change between treatments at initial sampling time (Fig. 1A). At 30 and 60 days, an increment in the number of 16S rRNA gene copies was observed in all the amended treatments, with the increases being amendment-type dependent at both sampling times, meaning that DOR produced greater bacterial proliferation than FUSDOR or CORDOR.

In actinomycetal communities, at initial sampling time, the number of 16S rRNA gene copies did not differ between samples (Fig. 1B). At 30 days, the highest levels of actinobacterial abundance were observed in the samples amended with DOR and FUSDOR. After 60 days of residue applications, control soil and soil amended with DOR did not differ in terms of the number of 16S rRNA gene copies. However, actinobacterial proliferation was detected in the samples amended with CORDOR and FUSDOR.

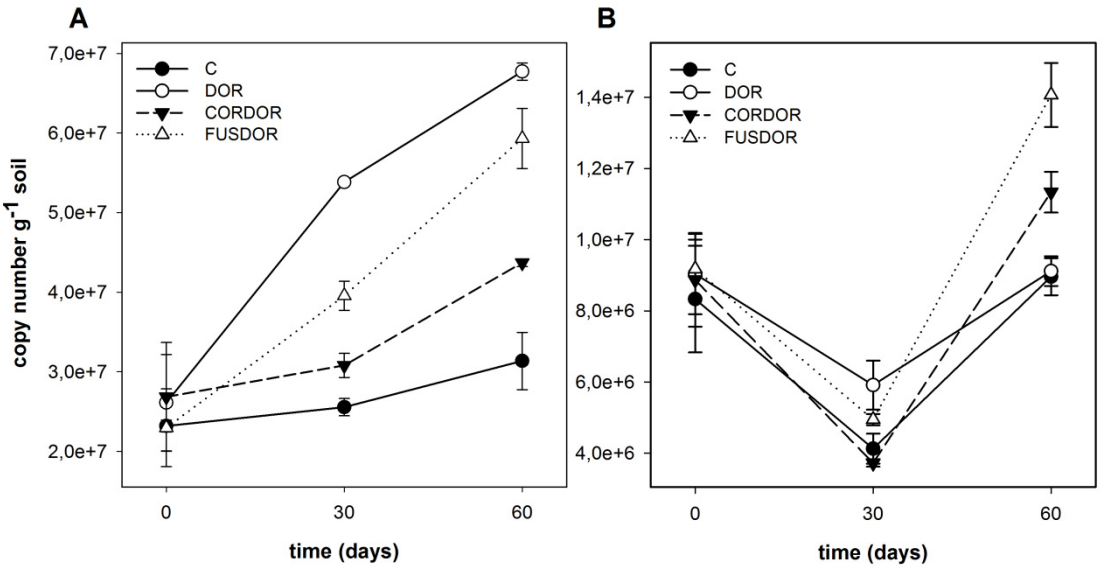

Fig. 1. Quantification of bacteria (A) and actinobacteria (B) 16S rRNA gene copy number by means of qPCR in unamended soil (C) and soil amended with untransformed DOR (DOR), *C. floccosa*–transformed DOR (CORDOR) or *F. oxysporum*–transformed DOR (FUSDOR) at 0 (T0), 30 (T1) and 60 (T2) days. Mean values correspond to three measures±standard deviation.

DGGE analysis

Changes in the structure and diversity of bacteria and actinobacteria communities due to the application of the different amendments at all sampling times were assessed by means of PCR-DGGE. Pre-testing showed that there were no differences in the banding patterns obtained from replicates of the same treatment. For this reason, only one profile per sample was included in the study in order to simplify the analysis. Bacterial DGGE analysis detected a complex band pattern with a large number of bands for each sample (Fig. 2A). Several diversity indices (*S*, *H* and *J*) were calculated for each sample (Table 4). On the whole, no drastic changes in any of the indices for the samples were observed. However, the Shannon diversity *t* test detected significant differences in bacterial community diversity between control soil and soil amended with DOR ($p < 0.01$), CORDOR ($p < 0.01$) and FUSDOR ($p < 0.01$) at 30 days. On the other hand, PCA of DGGE profiles (Fig. 2B) showed that ~37% of the variance can be explained by two principal components, the first accounting for 19.80% and the second for 16.93% of the variation. The PCA ordination of the samples did not detect any major differences in the bacterial structure between the unamended and amended samples at 0 and 30 days, with two different clusters actually grouping the samples according to sampling time. In contrast, the first axis separated the treatments at 60 days; the amended samples were grouped in a cluster which differed from the control sample that was situated in the upper-right quadrant.

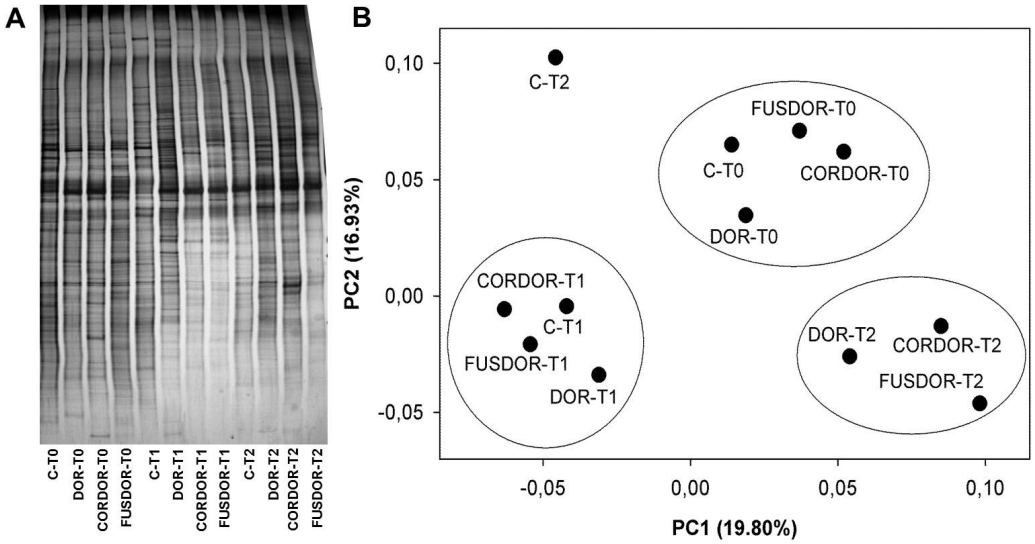

Fig. 2. (A) Bacteria DGGE profiles from unamended soil (C) and soil amended with untransformed DOR (DOR), *C. floccosa*–transformed DOR (CORDOR) or *F. oxysporum*–transformed DOR (FUSDOR) at 0 (T0), 30 (T1) and 60 (T2) days. (B) PCA based on DGGE banding patterns. Percent variability explained by each principal component is shown between round brackets after each axis legend.

Table 4. Species richness (*S*), Shannon index (*H*), and evenness (*J*) calculated from bacteria DGGE profiles 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. Shannon diversity *t* test was performed for each sample with its control at each sampling time (* significant differences $p \leq 0.05$).

Soil samples	*S*	*H*	*J*
C–T0	37	3.54(3.40±3.65)	0.98(0.96±0.99)
DOR–T0	36	3.54(3.45±3.69)	0.99(0.98±0.99)
CORDOR–T0	38	3.59(3.47±3.71)	0.99(0.98±0.99)
FUSDOR–T0	38	3.58(3.49±3.73)	0.98(0.96±0.99)
C–T1	48	3.79(3.51±3.91)	0.98(0.97±0.99)
DOR–T1	39	3.58*(3.39±3.78)	0.98(0.97±0.99)
CORDOR–T1	45	3.60*(3.35±3.75)	0.95(0.93±0.97)
FUSDOR–T1	43	3.40*(3.21±3.65)	0.90(0.88±0.93)
C–T2	41	3.55(3.32±3.82)	0.96(0.95±0.98)
DOR–T2	43	3.50(3.29±3.79)	0.93(0.91±0.96)
CORDOR–T2	38	3.42(3.21±3.62)	0.94(0.93±0.96)
FUSDOR–T2	37	3.40(3.20±3.61)	0.94(0.92±0.96)

Actinobacteria DGGE also showed a complex band pattern, and a large number of bands could be observed in all the samples (Fig. 3A). It was not possible to detect any significant changes in actinobacteria diversity characteristics among the unamended and amended soils (Table 5) at the different sampling times. PCA showed that the two principal components accounted for ~40% of the variance (25.24% and 14.25%, respectively) (Fig. 3B). PCA grouped the samples in three clusters, with one cluster consisting of all the treatments at initial sampling time and another cluster made up of all the samples at 30 days as well as control soil and soil amended with FUSDOR at 60 days. The last group contained the remaining amended soils treated with DOR and CORDOR for 60 days.

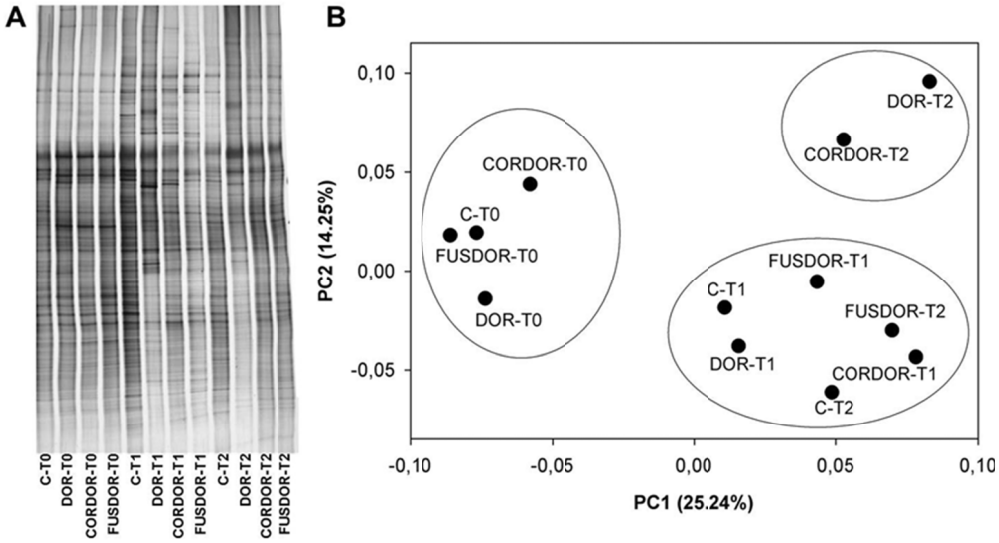

Fig. 3. (A) Actinobacteria DGGE profiles from unamended soil (C) and soil amended with untransformed DOR (DOR), *C. floccosa*–transformed DOR (CORDOR) or *F. oxysporum*–transformed DOR (FUSDOR) at 0 (T0), 30 (T1) and 60 (T2) days. (B) PCA based on DGGE banding patterns. Percent variability explained by each principal component is shown between round brackets after each axis legend.

Table 5. Species richness (*S*), Shannon index (*H*), and evenness (*J*) calculated from actinobacteria DGGE profiles 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. Shannon diversity *t* test was performed for each sample with its control at each sampling time (* significant differences p≤0.05).

Samples	*S*	*H*	*J*
C–T0	18	2.82(2.71±2.91)	0.98(0.96±0.99)
DOR–T0	18	2.81(2.68±2.91)	0.97(0.96±0.99)
CORDOR–T0	19	2.89(2.97±3.02)	0.98(0.97±0.99)
FUSDOR–T0	19	2.82(2.68±2.99)	0.96(0.94±0.98)
C–T1	23	3.04(2.85±3.19)	0.97(0.95±0.98)
DOR–T1	20	2.95(2.71±3.09)	0.98(0.96±0.99)
CORDOR–T1	22	3.04(2.85±3.21)	0.98(0.96±0.99)
FUSDOR–T1	27	3.18(2.99±3.32)	0.96(0.94±0.98)
C–T2	27	3.21(2.98±3.49)	0.97(0.96±0.98)
DOR–T2	26	3.08(2.82±3.35)	0.94(0.92±0.95)
CORDOR–T2	27	3.05(2.89±3.28)	0.92(0.91±0.93)
FUSDOR–T2	29	3.29(3.12±3.52)	0.97(0.95±0.99)

PLFA analysis

$PLFA_{tot}$ experienced a significant increase following the application of amendments to soil at 30 and 60 days (Table 6). The different amendments also caused an increase in $PLFA_{bac}$ after 30 and 60 days of treatment. At 30 days, the highest bacterial proliferation levels were found in the soil amended with DOR. On the other hand, no significant differences in $PLFA_{bac}$ between any of the amended samples with respect to the control treatment were detected at 60 days. With respect to the measurement of $PLFA_{act}$ at 30 days, only DOR produced significant proliferation levels of actinomycetal community with respect to the other treatments. However, at last sampling time, the highest actinobacteria proliferation levels were observed for the treatments with CORDOR and FUSDOR (Table 6). No change in the G+/G- ratio was observed between the unamended and amended soils at any of the sampling times. A significant increase in the F/B ratio was detected in the treatments amended with DOR and CORDOR at 30 days and soil amended with DOR after 60 days. On the other hand, the PLFA stress indicators cy/pre and S/M were greatly affected by the application of amendments to soil, especially at 30 and 60 days (Table 6). In all the treatments, the application of the different amendments produced a diminution of both ratios in relation to their respective controls.

The PCA of the PLFA profiles showed that around 94% of variability was explained by the first two principal components (87.35% and 6.79%, respectively) (Fig. 4). It was possible to establish three different sample groups in the PCA, thus highlighting the strong impact of the different amendments on soil microbiology. One group, in the lower left quadrant was made up of all the samples at 0 day. Other group consisted of the soil treated with DOR at 30 and 60 days and the treatment with CORDOR at 30 days. The last group was made up of the control samples at 30 and 60 days, the soil amended with CORDOR at 60 days and soil amended with FUSDOR at 30 and 60 days.

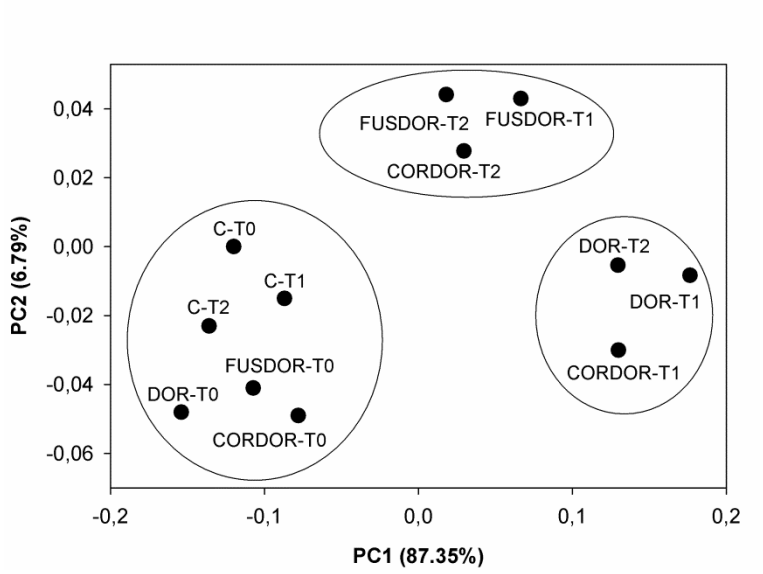

Fig. 4. PCA of PLFA data set for unamended soil (C) and soil amended with untransformed DOR (DOR), *C. floccosa*–transformed DOR (CORDOR) or *F. oxysporum*–transformed DOR (FUSDOR) at 0 (T0), 30 (T1) and 60 (T2) days. Percent variability explained by each principal component is shown between round brackets after each axis legend.

Integrated multivariate analysis

The PCA of the 45 variables analysed in each soil sample in the present study (all the chemical properties of soil, diversity characteristics and qPCR data from bacterial and actinomycetal communities determined by means of DGGE and PLFA marker data set) showed that around 83% of the variability of the data was explained by two first principal components (71.06% and 11.68% respectively) (Fig. 5). The 12 soil treatments assayed were grouped into four clearly defined clusters. All the control samples were part of a group located in the lower-left quadrant (Fig. 5A), which was closely related to the actinomycetal community (Fig. 5B). The amended samples at initial sampling time were located in the upper-left quadrant (Fig. 5A). This group of samples was characterised by some PLFA indices (G+/G- and S/M), the PLFA biomarker (10Me-17:0) and several chemical parameters (WSOC, phenols, C/N ratio), which changed drastically after the amended application (Fig. 5B). All the amended samples after 30 and 60 days of treatment were situated to the right of PC1 and PC2 separated according to sampling time. Amended 30-day samples were thus situated in the upper-right quadrant which was highly related with several PLFA biomarkers (18:1ω9, i14:0, 16:0) and $PLFA_{tot}$. Amended 60-day samples were clustered in lower-right quadrant (Fig. 5A), which was strongly influenced by several PLFA markers

(18:2ω6,9, 16:1ω7,16:1ω5, 10-Me-16:0) and the relative abundance of the 16S rRNA gene (Fig. 5B). PC1 was thus likely to be related to the application of amendments to the soil and PC2 probably indicated the analysis time of the samples.

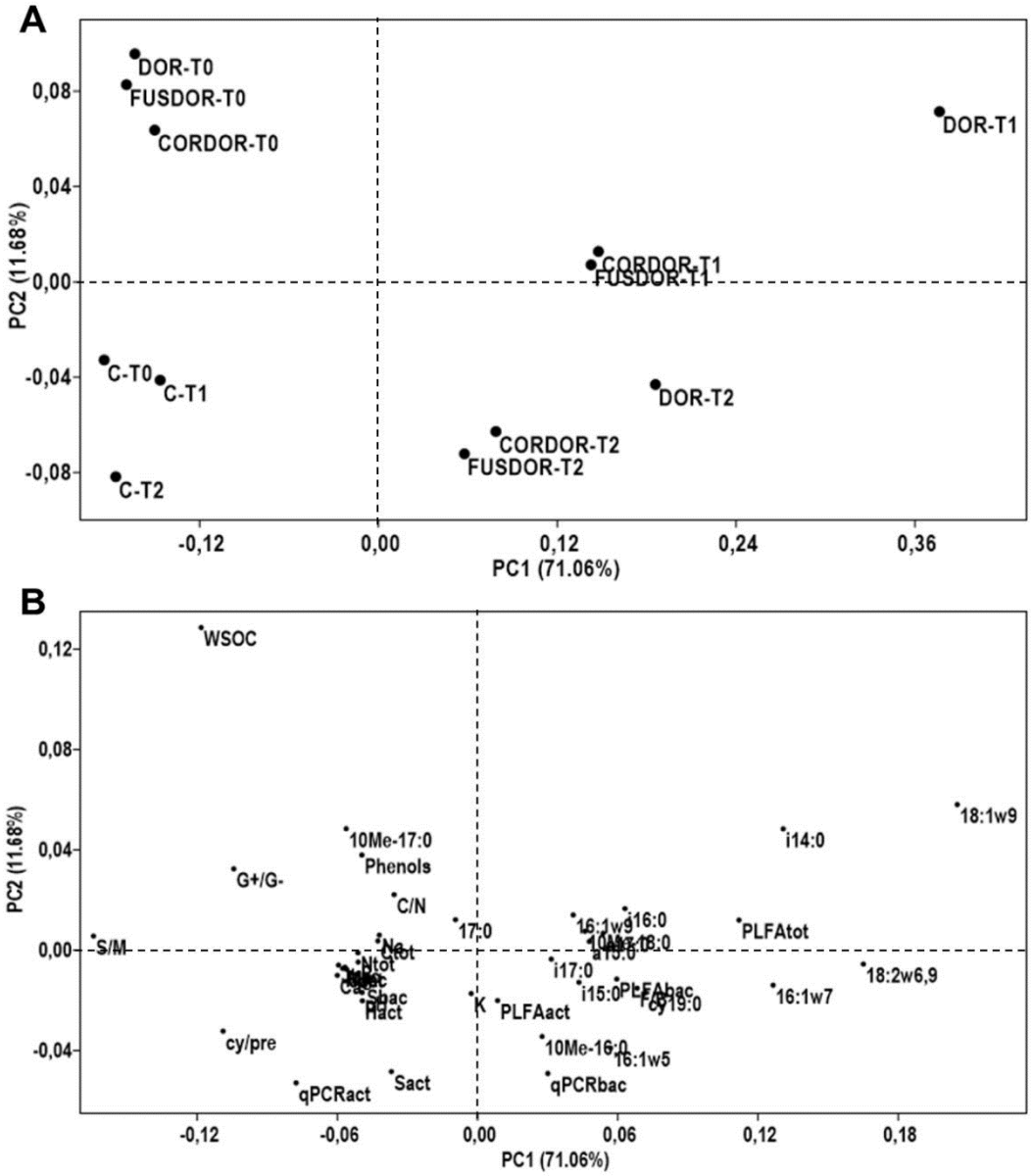

Fig. 5. (A) Scores from the different samples and (B) loadings of the chemical and biological variables measured in unamended soil (C) and soil amended with untransformed DOR (DOR), *C. floccosa*–transformed DOR (CORDOR) or *F. oxysporum*–transformed DOR (FUSDOR) at 0 (T0), 30 (T1) and 60 (T2). Percent variability explained by each principal component is shown between round brackets after each axis legend.

DISCUSSION

Effects of amendments on soil chemical properties

The DOR transformed by *C. floccosa* and *F. oxysporum* caused changes in waste properties, as it has been widely studied in other works (Sampedro et al., 2007; Sampedro et al., 2009a). The different amendments (DOR, CORDOR and FUSDOR) tested in the present survey resulted in changes on soil chemical properties. As other studies have previously reported, soil amended with DOR produced a slight decrease in soil pH due to its high concentration of organic acids (López-Piñeiro et al., 2008; Barbera et al., 2013). However, the transformed residues did not produce changes in pH, except immediately after the application of amendments. This may be an important finding, as soil bacteria dynamics have been demonstrated to be highly sensitive to pH (Fierer and Jackson, 2006). The application of amendments to soil led to an increase in phenol content, which was more marked for treatments with DOR. It is worth noting that we did not find significant differences in phenol concentrations between control soil and soil amended with CORDOR and FUSDOR at 60 days. According to Piotrowska et al. (2006), this is an important finding as soil responses to olive mill wastewater (OMW), residue obtained from three-phase olive oil extraction systems with a similar chemical composition to that of DOR (Morillo et al., 2009; Ntougias et al., 2013), was mainly determined by phenolic fraction in the waste. In our study, the application of the different amendments to soil did not increase significantly N_{tot} content. Nevertheless, previous studies have reported an increment in N_{tot} rates after long-term DOR amendments (López-Piñeiro et al., 2008; López-Piñeiro et al., 2011; Lozano-García and Parras-Alcántara, 2013) and short-term OMW treatments (Mekki et al., 2006; Mechri et al., 2007; Sierra et al., 2007). These discrepancies in relation to our data could be due to differences in the composition of the residues or the doses used. On the other hand, we detected a sharp increase in organic carbon in all the amended treatments at each sampling times. This finding represents one of the most important advantages of using olive waste as an organic amendment in zones with degraded soils such as Mediterranean countries (Roig et al., 2006). These improvements in organic carbon content and the constant proportion of N_{tot} led to a rise in C/N ratio in amended treatments, especially in soil containing DOR. The increment in C/N ratio may affect soil functionality which can involve a slowdown in the rate of organic matter mineralization (Di Serio et al., 2008; Mekki et al., 2009). For

this reason, some authors have suggested that nitrogen fertilization is required when olive wastes are applied to soil in order to reduce these ratios (Karpouzas et al., 2009; Rousidou et al., 2010). The transformation of DOR by *C. floccosa* and *F. oxysporum* prior to its use as an amendment could therefore solve this problem as a lower C/N ratio was obtained in the samples amended with CORDOR and FUSDOR.

The application of the different amendments to soil did not produce changes in the concentrations of total Ca, Mg and Na. Similar findings have also been reported in other studies following OMW soil treatments (Magdich et al., 2012; Barbera et al., 2013). On the other hand, in the present study, we did not detect any changes in total P concentrations among unamended and amended soils at the different sampling times. By contrast, an increment in this mineral in soils following short and long-time olive wastes application has previously been reported (López-Piñeiro et al., 2008; Di Bene et al., 2013). These discrepancies in relation to our results could be due to differences in waste characteristics. On the other hand, our survey demonstrated that soil K content increased after application of the different amendments, a finding which concurs with other studies (Montemurro et al., 2004; López-Piñeiro et al., 2011). This rise may be beneficial for plant status as this mineral plays an important role in the stress tolerance of plants (López-Piñeiro et al., 2011).

Effects of amendments on soil bacterial and actinobacterial communities

On the whole, the soil amended with DOR, CORDOR and FUSDOR caused an increase in bacterial density. This finding is in line with other studies where microorganism abundance increased after short-term olive waste treatment (Mekki et al., 2006; Siles et al., 2014). In our study, an amendment-type dependent rise in bacterial biomass was observed at 30 days using qPCR and PLFA techniques. Nevertheless, some discrepancies were found between data from both techniques at 60 days. Previous studies have indicated that PLFA results are more reliable than findings based on DNA as the phosphate group is rapidly hydrolysed when a cell die (Bossio and Scow, 1998; Wixon and Balser, 2013). However, the qPCR data obtained in our experiment are in line with total viable cells and CFU counts reported in a parallel experiment (Siles et al., 2014).

The bacteria growth explosion in the amended treatments is related to the input of easily decomposable C sources, which favours r-strategist bacteria capable of using these nutrients to multiply to the detriment of K-strategist bacteria (Kotsou et al., 2004).

The microbial PLFA stress indicator ratios cy/pre and S/M decreased after amendment. High values for these indices have been explained by reductions in bacterial growth rates due to nutrient limitations (Bach et al., 2010; Wixon and Balser, 2013). Thus, these findings demonstrate the beneficial effect of these amendments on bacterial multiplication. However, it have been widely reported that certain olive waste components such as phenols have a toxic effect on a wide variety of microorganisms (Medina et al., 2011; Justino et al., 2012; Di Bene et al., 2013) and nematodes (Cayuela et al., 2008). Many authors have suggested that when raw olive wastes are applied to soil, the changes observed in microbial communities are due to complex, sometimes conflicting, effects, depending on the relative amounts of beneficial, toxic organic and inorganic compounds/ions added to the residue (Mechri et al., 2007; Rousidou et al., 2010; Barbera et al., 2013). On the basis of these explanations, the impact of the DOR, CORDOR and FUSDOR amendments on soil bacterial communities should differ due to their different chemical compositions. Thus, although PLFA analysis showed that CORDOR and FUSDOR produced a different impact on microbial structure from that of DOR, this was detected by DGGE analysis only slightly. This could be because of the complex nature of the fingerprints obtained by this technique probably due to the high efficiency of the primers selected (Brons and van Elsas, 2008). Indeed, we cannot be sure that the number and volume of bands for each soil sample were accurately determined and thus that changes in microbial communities caused by the different treatments were precisely gauged. Sampedro et al. (2009b) and Montecchia et al. (2011) have actually reported that DGGE does not enable the full complexity of the system to be thoroughly determined.

Actinobacteria play a significant role in the organic matter cycle in nature due to their ability to degrade highly recalcitrant substances (Mechri et al., 2007). In addition, some microorganisms of this group have an intensive secondary metabolism which may strongly determine the dynamics of soil microbiology (Bontemps et al., 2013). In our survey, qPCR and PLFA results have shown that DOR addition caused an increase in actinomycetal biomass at 30 days with respect to the control soil. On the other hand, CORDOR and FUSDOR produced this increase at 60 days, probably due to certain substances that are favourable to actinobacteria growth which were not previously available to microorganisms due to the transformation of residue caused by fungi. Mekki et al. (2006) and Di Serio et al. (2008) have reported a rise in actinomycetes CFU after the application of untreated and treated OMW at all sampling

times. Mechri et al. (2007) have also reported an increment in actinobacteria PLFA biomarkers following the application of raw OMW to soil. On the contrary, Sampedro et al. (2009b) showed a diminution in actinomycetes PLFA biomarkers after the incubation of soil with untransformed and transformed DOR under in vitro conditions. Thus, despite the different techniques used in these studies, there is no consensus concerning the effect of olive wastes on actinomycetal community abundance. Regarding the impact of the different amendments on actinomycetal diversity and structure, DGGE did not detect changes in actinobacteria diversity after the addition of amendments. In contrast, we found that actinobacterial community structure experienced changes depending on amendment type. Karpouzas et al. (2010) also detected changes in soil actinobacteria communities from two different soils following raw OMW treatments. These authors also suggested that actinobacteria are less sensitive to olive waste phenols than other groups of bacteria. However, Siles et al. (2014), in a culture-dependent study, have demonstrated that the response of actinobacteria to DOR and CORDOR depends on the taxonomical group considered.

Integrated multivariate analysis

In general, it has been shown that organic amendments applied to soil lead to an improvement in soil health by raising nutrient levels, increasing aggregation, reducing bulk density and increasing the biological activity (Tella et al., 2013). This is achieved directly through the intrinsic properties of the organic amendments themselves or indirectly by modifying physical, biological and chemical soil properties (Larney and Angers, 2012). Nevertheless, the application of organic amendments may also introduce heavy metals, salts or recalcitrant compounds into the ecosystem, possibly leading to reduced soil functionality and affecting yield (Cardoso et al., 2013). Integrated multivariate analysis (Fig. 5) has demonstrated that the chemical and biological properties analysed in the present study appear to be more sensitive to the different amendments after 30 days than at 60 days. At the latter sampling time, DOR, CORDOR and FUSDOR had a similar impact on the soil system and PCA showed that these treatments were close to the control samples. This can be explained by the soil's resilience following OMW soil amendments, which has been demonstrated by other studies (Piotrowska et al., 2011). However, although the soil appeared to show some capacity to return to the initial properties at 60 days, the changes caused by DOR at 30 days may endanger soil functionality. It is also worth pointing out the considerable

phytotoxic effect of DOR on sorghum plants. In other words, the lack of phytotoxic activity of CORDOR and FUSDOR, their lower impact on soil microbiology and their potential beneficial effect on some chemical properties demonstrate the advantages of using this type of biotransformed olive waste as an organic amendment, especially in systems for seasonal crops.

CONCLUSIONS

The soil amendments with raw DOR, *C. floccosa*-transformed DOR and *F. oxysporum*-transformed DOR not only led to an increase in organic matter and K content in the soil but also in other potentially toxic compounds such as phenols, especially when raw waste was applied. PLFA and qPCR analyses demonstrated that the incorporation of easily decomposable materials caused an increase in bacterial and actinomycetal biomass. Raw DOR favoured more bacterial multiplication than both types of transformed DOR, while *C. floccosa*-transformed DOR and *F. oxysporum*-transformed DOR generated more actinomycetal proliferation than DOR at final sampling times. On the other hand, after the application of amendments, important changes in soil bacterial and actinobacterial community structures were detected by DGGE and PLFA and were probably due to alterations in the nutritional status of the soil ecosystems and the addition of toxic substances although no drastic changes in the diversity of either community were detected. Integrated multivariate soil analysis showed that soil experienced the greatest chemical and biological changes following the addition of DOR at 30 days, which may alter soil functionality. Therefore, due to the lack of phytotoxic effects after CORDOR and FUSDOR application and their more limited impact on the soil properties analysed, these biotransformed wastes could be an appropriate organic amendment.

Acknowledgements

This study has been funded by the Spanish Ministry of Science and Innovation (Project AGL2008–572) and by a grant from the Competence Center TE01020218 of the Czech Technology Agency. J.A. Siles, D. Pérez-Mendoza and I. Sampedro gratefully acknowledge assistance from the JAE program, which is co-financed by the Consejo Superior de Investigaciones Científicas (CSIC) and the European Social Fund. We also wish to thank Michael O'Shea for proofreading the document.

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Chapter 2

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

Adapted from:
Siles, J.A., Pérez-Mendoza, D., Ibáñez, J.A., Scervino, J.M., Ocampo, J.A., García-Romera, I., and Sampedro, I. (2014) Assessing the impact of biotransformed dry olive residue application to soil: Effects on enzyme activities and fungal community. *International Biodeterioration and Biodegradation* **89**: 15-22.

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*.

Keywords

"Alpeorujo"; Bioremediation; Olive waste; Organic amendment; qPCR; Soil microbial ecology

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 component 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., 2009). 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 and 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.

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 Prevost-

Boure et al., 2011). For these reasons, it is essential to determine soil fungal responses when organic amendments and inorganic fertilizers are 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 analysed, 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).

MATERIALS AND METHODS

Materials

The soil used in this study was taken from the "Cortijo Peinado" field (Fuente Vaqueros, Granada, Spain, 37º13'N, 3º45'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.

Organisms and inoculum preparation

The used fungi were *Coriolopsis floccosa,* formerly known as *C. rigida* (Spanish Type Culture Collection, CECT 20449), isolated from beech wood and *Fusarium oxysporum* (Mycological Culture Collection of the Department of Biological Sciences, Faculty of Exact and Natural Sciences, University of Buenos Aires, BACF 738) isolated from maize rhizospheric soil. Both fungi were maintained at 4 ºC 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 ºC for 20 min) twice prior to use. 5 mL of the inoculum (ca. 50 mg of dw) were aseptically added to 50 g of sterilized PS and incubated at 28 ºC for 7 days.

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 ºC 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 ºC 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.

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) or *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 ºC and 50% relative humidity. The experiment was watered regularly throughout the experiment. The regular watering 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 ºC prior to processing (1-2 days) for enzymatic activity assays and at -80 ºC prior to molecular analyses.

Enzymatic analyses

Urease activity (E.C. 3.5.1.5) was analysed 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 ^{o}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^{o}C. The reaction was stopped after addition of 15% TCA (trichloroacetic acid solution). 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 0.5M $CaCl_2$ for β-Glucosidase. Dehydrogenase activity (E.C. 1.1) was analysed using the procedure described by Camiña 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 ^{o}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.

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 (see Table A1 in Appendix 1 for primer sequences).

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 ºC 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).

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 ºC for 30 s, 58ºC for 45 s and 72 ºC 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 Qian et al. (2007).

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.

Statistical treatment of data

The PAST software package was used to calculate: species richness (*S*), the Shannon index (*H*) and evenness (*J*) (Lv 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.

RESULTS AND DISCUSSION

Effect of DOR amendments on enzymatic activities

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 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, three-phase olive-mill waste) 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 application. 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, CORDOR and FUSDOR may be due to the addition of low molecular weight protein substrates which are transformed into ammonium. 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, other agricultural practices such as the use of herbicide activity can decrease this activity (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).

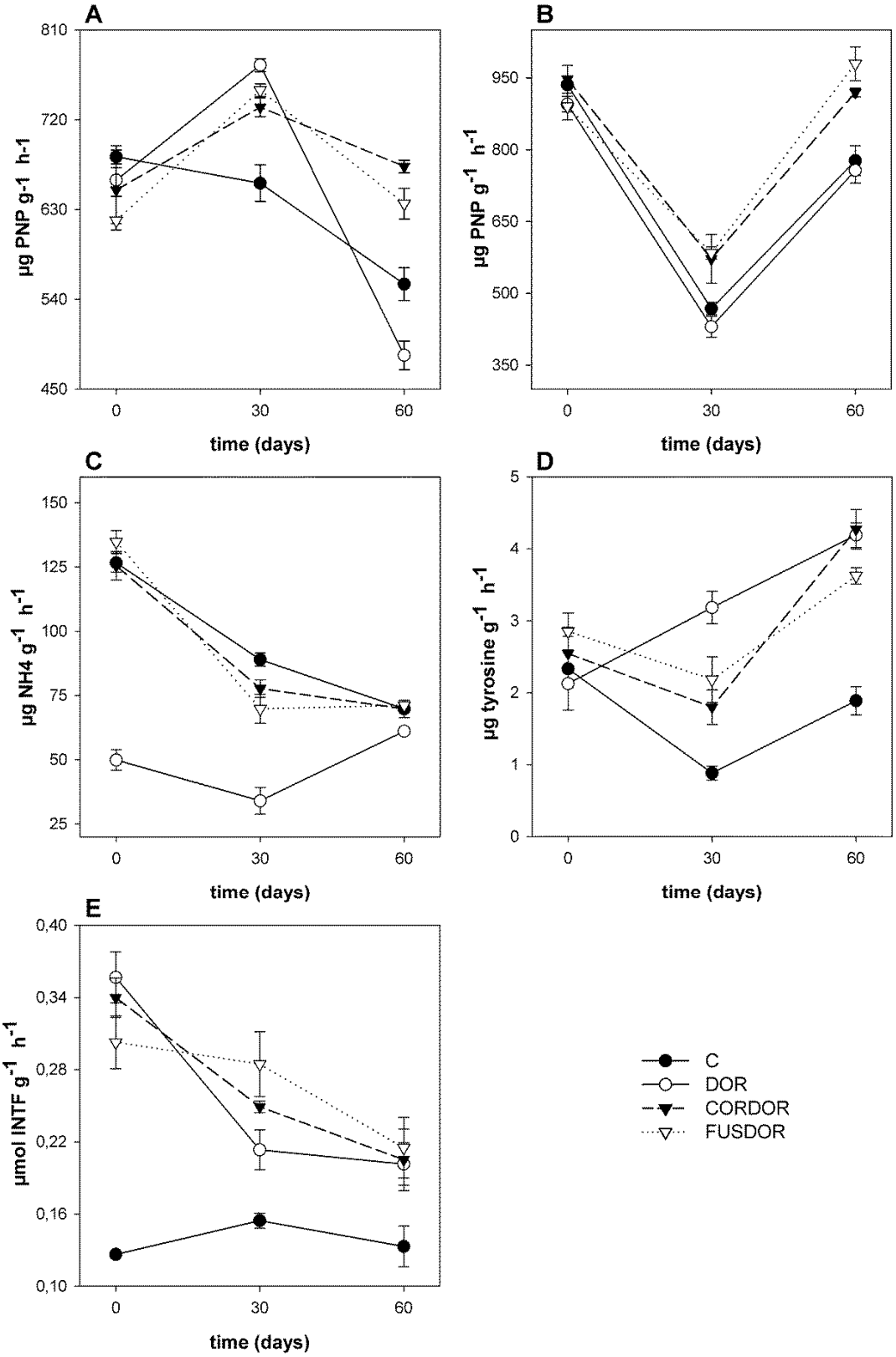

Fig. 1. Activities of phosphatase (A), β-glucosidase (B), urease (C), protease (D) and dehydrogenase (E) in unamended soil (C) and soil amended with untransformed (DOR), *C. floccosa*-transformed DOR (CORDOR) or *F. oxysporum*-transformed DOR at 0, 30 and 60 days. Error bars indicate the standard deviations.

Effect of DOR amendments on fungal community structure

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 olive residues. 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 CORDOR 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.

Table 1. Diversity indices 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.

Samples	*S*	*H*	*J*
C-T0	23	3.03(3.02;3.03)	0.97(0.96;0.98)
DOR-T0	22	3.02(3.01;3.03)	0.98(0.97;0.99)
CORDOR-T0	23	3.03(3.02;3.04)	0.97(0.96;0.98)
FUSDOR-T0	24	3.04(3.03;3.05)	0.96(0.95;0.97)
C-T1	24	3.15(3.14;3.16)	0.99(0.98;0.99)
DOR-T1	26	3.19(3.17;3.20)	0.98(0.97;0.99)
CORDOR-T1	25	3.16(3.15;3.17)	0.98(0.97;0.99)
FUSDOR-T1	26	3.15(3.14;3.16)	0.97(0.96;0.98)
C-T2	23	3.10(3.08;3.12)	0.99(0.98;0.99)
DOR-T2	25	3.13(3.12;3.14)	0.97(0.96;0.98)
CORDOR-T2	28	3.28(3.26;3.30)	0.98(0.96;0.99)
FUSDOR-T2	25	3.13(3.11;3.15)	0.97(0.95;0.98)

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 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.

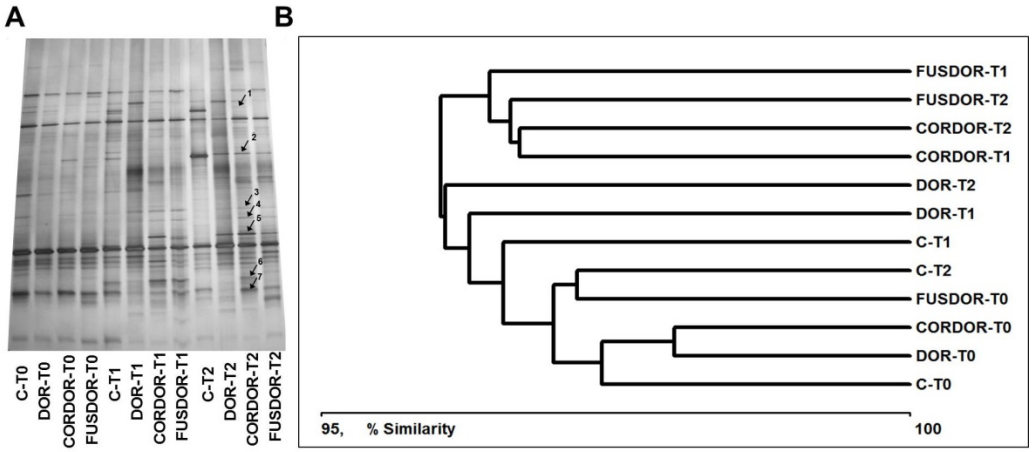

Fig. 2. (A) DGGE analysis of 18S rRNA gene products amplified from unamended soil (C) and soil amended with untransformed DOR (DOR), *C. flocossa*-transformed DOR (CORDOR) or *F. oxysporum*-transformed DOR (FUSDOR) at 0 (T0), 30 (T1) and 60 days (T2). Arrows indicate bands corresponding to clones that 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, *C. flocossa*-transformed DOR (CORDOR) or *F. oxysporum*-transformed DOR (FUSDOR) at 0 (T0), 30 (T1) and 60 days (T2).

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 gram, and the application of 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.

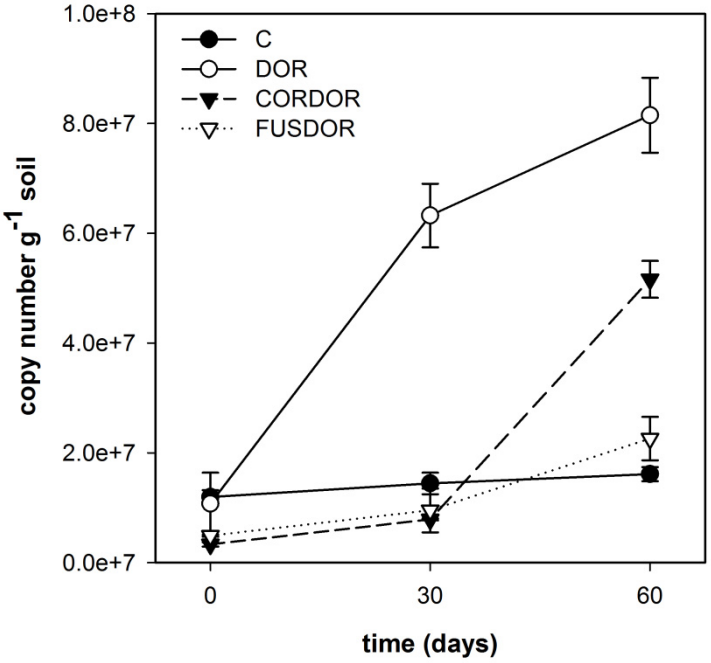

Fig. 3. Quantification of 18S rRNA gene copy number by means of qPCR in unamended soil (C) and soil amended with untransformed DOR (DOR), *C. floccosa*–transformed DOR (CORDOR) or *F. oxysporum*–transformed DOR (FUSDOR) at 0, 30 and 60 days. Mean values correspond to three measures±standard deviation.

Phylogenetic analyses

As the largest increases in diversity indices were recorded in soil amended with CORDOR 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.

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 nº	Accession nº	Closet relative (accession no.)	Alignment, % sim	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*

Principal components analysis

PCA 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 2nd 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 analysed 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.

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 amendment 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 in 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.

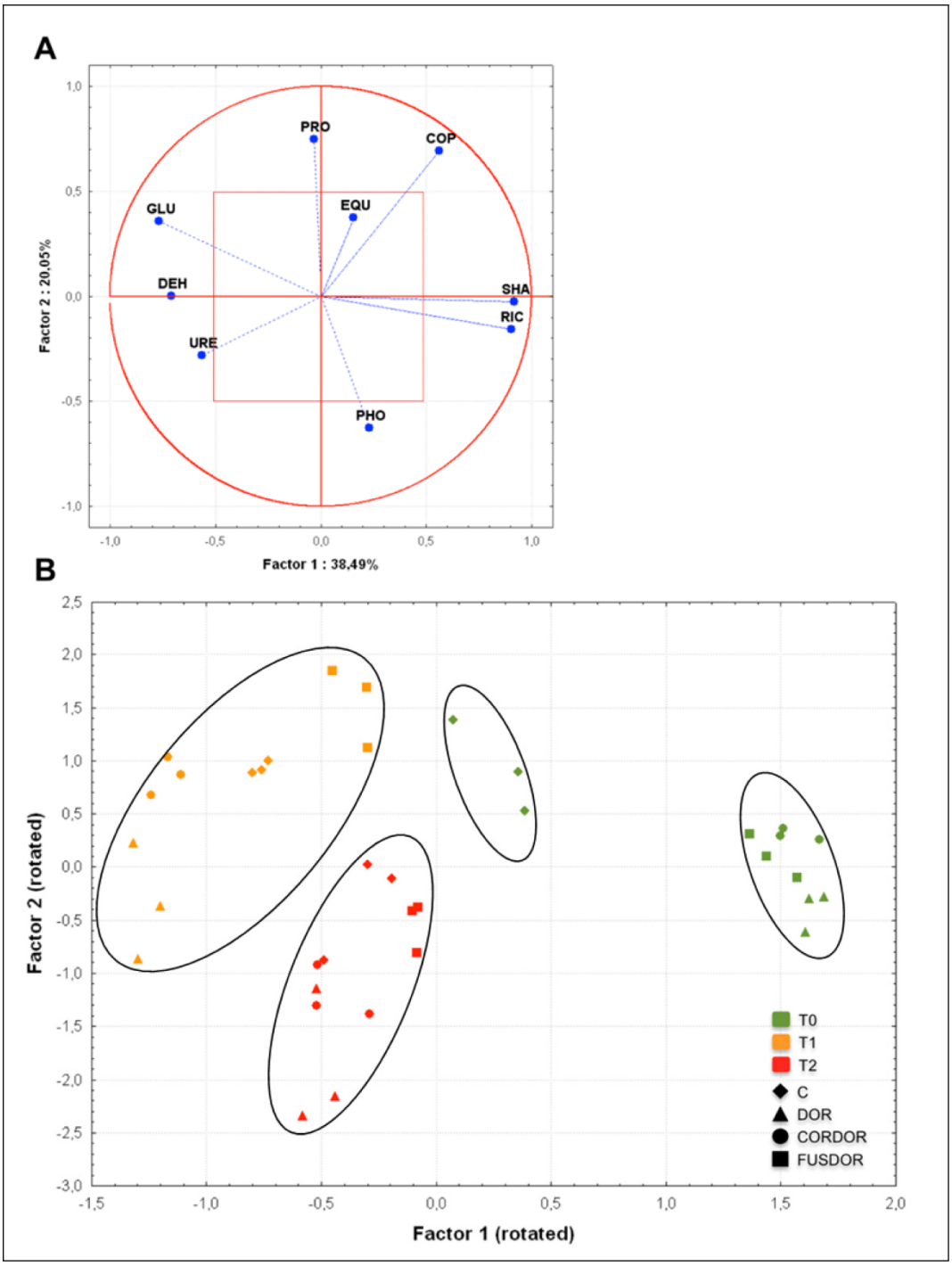

Fig. 4. VARIMAX rotated factor loadings for Factor 1 (PC1) x Factor 2 (PC2). Extraction method: Principal Components. Factor loadings (variable coordinates within the factor space) represent the correlation between original measures and new factors extracted using Principal Component Analysis. The variables 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 colour 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).

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.

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Chapter 3

Short-term dynamics of culturable bacteria in a soil amended with biotransformed dry olive residue

Adapted from:
Siles, J.A., Pascual, J., González-Menéndez, V., Sampedro, I., García-Romera, I., and Bills, G.F. (2014) Short-term dynamics of culturable bacteria in a soil amended with biotransformed dry olive residue. *Systematic and Applied Microbiology* **37**: 113-120.

Abstract

Dry olive residue (DOR) transformation by wood decomposing basidiomycetes (e.g., *Coriolopsis floccosa)* is a possible strategy for eliminating the liabilities related to the use of olive oil industry waste as an organic soil amendment. The effects of organic amendment with DOR on the culturable soil microbiota are largely unknown. Therefore, the objectives of this study were to measure the short-term effects of DOR and *C. floccosa*-transformed DOR on the culturable bacterial soil community, while at the same time, documenting the bacterial diversity of an agronomic soil of the southeastern Iberian Peninsula. The unamended soil was compared with the same soil treated with DOR and with *C. floccosa*-transformed DOR during 0, 30 and 60 days. Impact was measured from total viable cells and CFU counts, as well as the isolation and characterization of 900 strains by fatty acid methyl esters profiles and 16S rRNA partial sequencing. The bacterial diversity was distributed among the *Actinobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria*, *Betaproteobacteria*, *Bacilli*, *Sphingobacteria* and *Cytophagia*. Analysis of the treatments and controls demonstrated that soil amendment with untransformed DOR produced important changes in bacterial density and diversity. However, when *C. floccosa*-transformed DOR was applied, bacterial proliferation was observed but bacterial diversity was less affected, and the distribution of microorganisms was more similar to the unamended soil.

Keywords

Dry olive residue; Soil bacteria; Organic amendments and fertilizers; Granada soil; Bioremediation; Culturable bacteria

INTRODUCTION

Bacteria play an important role in soil structure, plant health and nutrient availability for crops via a range of activities, including decomposition of crop residues, nutrient immobilization, mineralization and biological nitrogen fixation (Kirk et al., 2004). Soil bacterial community can be influenced by a wide range of biotic and abiotic factors (Fierer and Jackson, 2006). Therefore, any alterations of soil microbial community may have significant and unforeseen consequences on soil functions (Nannipieri, 2003). Such impacts may be especially transcendental in agricultural system, which, in most cases, are subject to mineral fertilization (urea, ammonium nitrate, sulfates and phosphates), organic amendments (composts, biosolids or animal manures) and the application of other products, e.g., microbial inoculants or pesticides (Miransari, 2011). Organic amendments are widely used and can offer an alternative to chemical fertilization and its associated problems (Böhme and Böhme, 2006). Furthermore, the application of these amendments is a beneficial way to ameliorate soil properties by improving a favorable soil structure, enhancing soil cation exchange capacity, increasing the quantity and availability of plant nutrients and providing the substrate for microbial activities (Miransari, 2011). However, in most cases, these inputs are applied with the goal of maximizing crop production, while the side effects on soil organisms are neglected. For this reason, knowledge of how soil bacterial dynamics are influenced by organic amendments is indispensible.

The Mediterranean region is characterized by highly degraded soils and with low organic matter concentrations (Cayuela et al., 2010). The wastes generated by olive oil industry have been investigated as organic amendments (Justino et al., 2012). The two-phase olive oil extraction system, after the revalorization of the wet primary residue, generates huge amounts of dry olive residue (DOR) or "alpeorujo" (Alburquerque et al., 2009). In Spain alone, 5 million tons of this product are produced annually, and it causes a significant environmental problem (Tortosa et al., 2012). However, this waste could be a good candidate for utilization as an organic amendment due to its high organic and inorganic nutrient content (Sampedro et al., 2011). Nevertheless, when DOR is applied directly to soil, phytotoxic (Casa et al., 2003; Gigliotti et al., 2012) and microtoxic effects (Benitez et al., 2004) have been observed. For this reason, a pretreatment of the residue is necessary before its application to soil (Sampedro et al., 2011). One of the most effective treatments is the incubation of the

waste with saprobic fungi, such as *Coriolopsis floccosa*, formerly known as *C. rigida*, (Aranda et al., 2006; Sampedro et al., 2007; Saparrat et al., 2010). This treatment principally stabilizes the waste's organic matter, enhances its C/N relationship and drastically reduces the phenolic fraction (Sampedro et al., 2005; Sampedro et al., 2009b).

To date, only the effects of the application of three-phase olive oil extraction residue [olive mill wastewater (OMW)] on the physicochemical characteristics and soil microbiology have been studied (Justino et al., 2012). Knowledge about the effects of DOR or DOR transformed by saprobic fungi on soil bacterial communities is scarce (Sampedro et al., 2009a), and more specifically, no data exist regarding the impacts on bacterial culturable diversity in soil amended with olive wastes.

Depending on the detection method, a range from 10^3 to 10^7 bacterial species per gram of soil has been estimated (Torsvik et al., 2002; Gans et al., 2005; Schloss et al., 2009). One approach to estimate this diversity has been by culture-dependent techniques. In an effort to increase the accuracy of culturing methods, many advances have been made during the last decade because these techniques have been considered essential for connecting the phenotype and genotype, describing novel taxa and discovering their ecological functions (Pham and Kim, 2012).

The objectives of this work were: (i) to measure the short-term effect (0, 30 and 60 days) of untransformed DOR and *C. floccosa*-transformed DOR additions on the culturable bacterial community of an agronomic soil, (ii) to evaluate culturable bacterial diversity in a soil of the province of Granada, Spain.

MATERIALS AND METHODS

Sampling

The soil used in this study was obtained from the "Cortijo Peinado" field (Granada, Spain, 37º 13'N, 3º 45'W). The climate in the region is Mediterranean with a mean annual precipitation of approximately 357 mm. The mean annual temperature is 15.1 ºC, whereas the coldest month is January (mean 6.7 ºC) and the warmest month is July (mean 24.8 ºC) (http://www.aemet.es). The soil was described as a haplic regosol, and its principal properties were: 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, 0.10%; 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}.

Ten soil samples of 5 Kg were collected from the Ap horizon from different zones of the plot in October 2010. Subsequently, the different samples were sieved through a 5 mm mesh and manually mixed. At the time of sample collection, soil had been recently ploughed and plants were absent in the plot. The soil was stored in polythene bags at room temperature until the experiment was initiated (3 days).

DOR

DOR was supplied by an olive oil manufacturer (Sierra Sur S.A., Granada, Spain) and was frozen (-20 ^{o}C) until used. The main chemical characteristics of DOR were determined by Sampedro et al. (2009b).

DOR biotransformation and soil amendment

DOR was transformed with *Coriolopsis floccosa,* formerly known as *C. rigida* (Spanish Type Culture Collection, CECT 20449)**,** which was maintained at 4 ^{o}C and subcultured monthly on potato dextrose agar slants. For DOR incubation, polyurethane sponge (PS) cubes, 0.5 cm^3, were rinsed with water in a 1:20 (w/v) ratio and autoclaved three times prior to their use. Sterilized PS cubes (1.5 g) were placed in Erlenmeyer flasks. Next, 25 mL of culture medium [50 g L^{-1} of anhydrous glucose (Acros Organics) and 5 g L^{-1} of yeast extract (Fisher Chemical)] were added and autoclaved again. Subsequently, 5 mL of *C. floccosa* inoculum (ca. 50 mg dry weight) were aseptically added to each Erlenmeyer flask, and cultures were incubated at 28 ^{o}C for 7 days in static conditions.

Deionized water was added to DOR in order to adjust the moisture content to 25% (w/w) prior to sterilization (three cycles in the autoclave). Then, colonized PS cubes in Erlenmeyer flasks were covered with 25 g of DOR. Solid-state cultures on DOR were grown at 28 ^{o}C in the dark under stationary conditions for 30 days. Non-inoculated and sterilized DOR samples were prepared and incubated as controls. DOR was autoclaved several times for complete sterilization, then it was sieved (2 mm), homogenized and stored at 4 ^{o}C until added to the soil.

The experiments were carried out in 0.5 L pots. Untransformed DOR (DOR) and DOR incubated with *C. floccosa* (CORDOR) were added to soil pots at concentrations of 50 g kg^{-1}. Soil samples without the residue (control treatments) were also prepared. A

single sorghum plant (*Sorghum bicolor*), with a homogeneous size, was planted in each pot. The experiment was incubated in a greenhouse with supplementary light at 25/19 ^{o}C and 50% relative humidity. The pots were watered regularly throughout the experiment. The soil watering determined that water content of the samples was around 15-20%.

The replicate unamended soil and soils treated with DOR and CORDOR were analysed at 0, 30 and 60 days after amendment. The experiment consisted of five pots of each treatment at each time. At each soil sampling, the soil of the five pots was sieved through a 2 mm mesh to eliminate roots and it was consolidated, homogenized and mixed. A total of 15 g of each soil sample was stored at 4 ^{o}C until sample processing (2 d). Therefore, the initial sampling period, designated as time 0 days, occurred after 2 days. These two days were initially considered insignificant because the soils samples were maintained at 4^{o}C. Subsequent sampling times of the remaining samples incubated under the greenhouse conditions were exactly 30 and 60 days.

Bacterial quantification and isolation

For bacterial isolation and quantification, 1 g of soil was dispersed in 100 mL of sterile diluents (VL70 medium without growth substrates or vitamins) in 250 mL Erlenmeyer flasks by stirring with a magnetic bar for 30 min. Subsequently, several serial dilutions (10^{-2}-10^{-7}) were prepared and 0.1 mL of these dilutions were spread with sterile glass rods in Petri dishes filled with solidified gellan gum VL70 medium containing 0.05% D-xylose (w/v) (Sait et al., 2006).

The Petri plates were incubated for 4 weeks at 18 ^{o}C and 60% relative humidity in the dark. After incubation, the plates with the lowest dilutions and the least numbers of colonies were chosen for bacterial isolation. Subsequently, starting from the most dilute plates, the first 200 colonies encountered from each treatment at each time point (total of nine samples) were transferred to new Petri dishes (60 mm) with R2A medium (Becton-Dickinson) during a period of 6 weeks. These colonies were incubated in the dark at 18^{o}C for three weeks. From each sample, unpurified and non-growing colonies were discarded. Afterwards, 100 colonies of each sample were chosen at random. Each strain was numbered, and 100 numbers were randomly selected for each soil sample. The randomly selected isolates were analysed further, and a total of 900 isolates were evaluated.

For bacterial counts, the Petri plates with an appropriate range of colony densities were chosen after 4 weeks and colony forming units (CFU) were calculated. For each soil, CFU gdw^{-1} (gram dry weight $^{-1}$) count represented the mean of five plates at one dilution level and was calculated based on the dry weight of the soil and dilution factors. The total viable number of bacteria in the different samples was determined by epifluorescence microscopy using LIVE/DEAD® BacLight™ Kit (Molecular Probes, Invitrogen) following the manufacturer's instructions. The total viable bacteria gdw^{-1} count was estimated with the same soil dilutions as used for plating of colonies.

Fatty acids analyses of isolates

Most of the selected strains were characterized by fatty acid methyl esters (FAME) analysis after being cultured on R2A plates at 22^{o}C for exactly 5 days. Next, 100-200 mg of biomass were collected, and fatty acids esters were saponified, methylated and extracted (Haack et al., 1995). FAME profiles were determined by capillary gas chromatography using a Hewlett-Packard Model 5890 gas chromatograph/MIDI system (Microbial ID Inc. Newark, Delaware, USA) equipped with a phenylmethyl silicon column (0.2 mm × 25 mm). Individual FAME profiles were identified using the Microbial Identification Software (MIS) and were compared using PAST ver. 2.17. For clustering, a Euclidean distances analysis was used, and the relationships between the strains were established with a dendrogram based on the unweighted pair group method using arithmetic averages (UPGMA). A threshold of 85% similarity between isolates was used to determine cluster formation. Each cluster was considered as an operational taxonomic unit (OTU) in order to facilitate further analysis of diversity among the samples.

DNA extraction, PCR amplification and DNA sequencing

For DNA extraction, one to several representative isolates from each cluster, depending on the cluster size and heterogeneity, were chosen. Likewise, the bacteria whose FAMEs profiles were inadequate, according to MIDI protocols, were selected directly for partial 16S rRNA gene sequencing. For DNA extraction, most of the isolates' genomic DNAs were extracted by microwave lysis (Sánchez-Hidalgo et al., 2012). For the remaining bacteria, DNAs were isolated by using GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific Inc., CA, USA) following the

manufacturer's instructions. Primers, PCR mixtures and thermal cycling program were as previously reported by Sánchez-Hidalgo et al. (2012). PCR products were visualized on an ethidium bromide-stained agarose gel and purified using Illustra GFX TM PCR DNA and Gel Band Purification kit (GE Healthcare, UK) according to the manufacturer's instructions. The cleaned 16S rRNA PCR gene products were sequenced bidirectionally with the primers FD1 and 1100R (see Table A1 in Appendix 1 for primer sequences) using ABI PRISM 3130xl Genetic Analyser (Applied Biosystems) by the Instrumental Technical Services of EEZ-CSIC, Granada, Spain. The sequences were edited with clustalW implemented in MEGA ver. 5.10 software (Tamura et al., 2011). Nearest phylogenetic neighbors were determined using the EzTazon-e Database (http://eztaxon-e.ezbiocloud.net/) (Kim et al., 2012). The partial 16S rRNA gene sequences have been deposited in the GenBank/EMBL/DDBJ databases (see Table A2 in Appendix 1).

After the identification of the different bacteria, the distribution of isolates from each cluster in the different samples was estimated.

Data analyses

The program Paleontological Statistics (PAST) ver. 2.17 (Hammer et al., 2001) was used to: (i) calculate significant differences between samples in total viable cell count and CFU count, (ii) estimate the community diversity based on the number of OTUs (S), the Shannon index [$H = -\sum pi/\ln (p_i)$ where p_i is the proportion of species i in a sampling], the evennness (J=Shannon diversity divided by the logarithm of number of taxa) and Chao 1 diversity estimator [Chao1=S+$F1$ ($F1$–1)/(2 ($F2$+1)), where $F1$ is the number of singleton species and $F2$ the number of doubleton species], (iii) compare the Shannon diversity of the samples amended with its respective control and the control among them with a *t* test (Magurran, 1988), (iv) carry out a principal components analysis (PCA) biplot for treatments. Furthermore, the distribution of the OTUs established by FAMEs dendrogram from each soil treatment was used to construct a UPGMA dendrogram based on Euclidean distances with BioDiversity Pro ver.2 (Lambshead, 1998).

RESULTS

Bacterial diversity

The 900 bacterial isolates were analyzed by their FAME profile using the MIDI system, but a valid analysis following the MIDI protocol was obtained only for 869 bacteria. The UPGMA dendrogram (Fig. 1) separated the number of isolates analyzed by FAME-MIDI into 54 clusters each consisting of 2 to 298 isolates (Table A1 in Appendix 1).

Fourteen clusters had more than 10 isolates and 40 clusters were found with a lower number of isolates than 10. The major designated clusters were 44, 49, 39 and 27 with 298, 81, 66 and 65 isolates respectively. In addition, 20 isolates were not grouped with any other isolate and formed single branches.

The standard MIDI system conditions for FAME analysis were not used because some of the isolates failed to grow on trypticase soy blood agar at 28 ^{o}C. Therefore, the tentative identity of the bacteria assigned by the MIDI database was not considered for identification. The number of isolates selected for sequencing in each cluster was determined by the cluster size and internal FAMEs phenotypes. Conversely, each isolate that contributed to a single terminal branch and the uncharacterized FAME isolates were selected for sequencing. Thus, a total of 154 strains (17% of the total isolates) were identified by sequencing of partial 16S rRNA gene (~1000 bp), of which 123 were chosen from FAME dendrogram (Table A1 in Appendix 1) and the remaining 31 isolates were the bacteria uncharacterized by FAME (Table A2 in Appendix 1).

Based on 16S rRNA sequencing, 61% of the isolates shared ≥ 99% sequence similarity with the closest known species in the EzTaxon Database. The 16S rRNA analyses placed the sequenced isolates in seven classes: *Actinobacteria* (50.6%), *Alphaproteobacteria* (23.7%), *Gammaproteobacteria* (10.9%), *Betaproteobacteria* (5.8%), *Bacilli* (4.5%), *Sphingobacteria* (3.2%) and *Cytophagia* (1.2%). After sequencing, interpretation of the phenotypic classification represented by the dendrogram became possible. The upper zone of the dendrogram that included clusters 1-11 (Fig. 1) consisted of 98 isolates related to the *Alphaproteobacteria*. The best represented clusters were: 1, with bacteria related with the genus *Sphingomonas*; 5, with isolates identified as *Brevundimonas*; and 9, whose isolates were related to the genus *Rhizobium* and

Paracoccus. The other groups presented fewer strains and, according to sequence data, they all belonged to the orders *Rhizobiales* and *Caulobacterales*.

Most strains (739 isolates) were grouped in a large cluster that occupied the middle of the FAMEs dendrogram, from branches II to XIX (Fig. 1). Additionally, three well-differentiated groups were established within the cluster. The first occupied branches II-XIV, and consisted of 14 clusters and 13 single branches. This group of bacteria was the most diverse in the analysis based on 16S rRNA sequencing. The most important clusters were 19, 24 and 21 where the sequenced bacteria belonged to *Actinobacteria*, *Bacilli*, *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Sphingobacteria* and *Cytophagia*. At least 22 different genera were recognized among the sequenced strains.

A second group was established between clusters 26 and the single branch XVI (Fig. 1). This group of isolates was formed by 16 clusters and 2 single branches. All the isolates belonged to the *Actinobacteria* class, specifically the genera *Nocardioides* and *Streptomyces,* except for the clusters 35, 36 and 37 whose isolates were related to the genera *Pseudomonas*, *Stenotrophomonas* and *Pseudoxanthomonas* (*Gammaproteobacteria*) and the single branch XVI, which was related to *Bacilli.* In this set, three clusters (27, 30 and 39) containing numerous isolates stood out.

The third group occurred between cluster 42 and the single branch XIX (Fig. 1), and it was comprised of 9 clusters and 3 single branches. All the isolates of this group were assigned to the *Actinobacteria* class, *Micrococcales* order, except the 4 isolates of the cluster 43 that were related to the order *Bacillales*. The most numerous clusters found in this set were 44 (whose isolates were related to the genus *Arthrobacter*) and 49 (related to *Microbacterium*) formed by 298 and 81 isolates, respectively.

Finally, from clusters 51-54 (Fig. 1), at the bottom of the dendrogram, a small number of bacteria (32) grouped in four clusters and one single branch. All the isolates sequenced in this part of the dendrogram belonged to *Micrococcales*.

Among the 31 strains not characterized by FAME, 16S rRNA sequencing indicated that some isolates belonged to genera not included in the dendrogram [i.e., *Actinocorallia*, *Herbiconiux*, *Microvirga* and *Sanguibacter (*Table A2 in Appendix 1)].

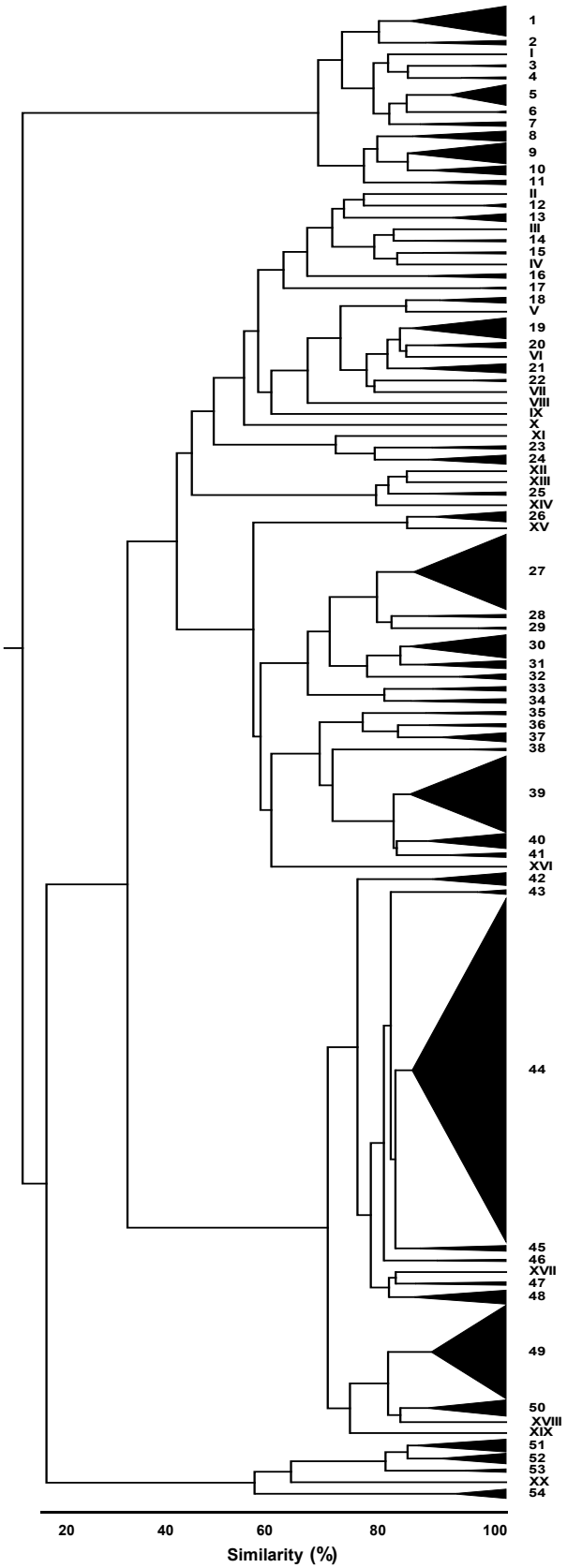

Fig. 1. Abridged dendrogram by UPGMA using Euclidean distances of the fatty acids composition from 869 isolates found in unamended soil and soil amended with DOR or *C.floccosa*-transformed DOR at 0, 30 and 60 days. The number of each cluster (Arabic numerals) and single branch (Roman numerals) is indicated in the right part.

Effect of DOR and CORDOR on culturable soil bacteria

Soil amendment with DOR and CORDOR produced changes in the total viable cell gdw^{-1} and CFU gdw^{-1} compared to the unamended soil (Fig. 2). The proportion of cells with an intact cytoplasmatic membrane could be visualized and estimated with the LIVE/DEAD® BacLight™ Kit and, therefore, the number of total viable cells in the samples. This number, like CFU gdw^{-1} at the initial time (two days after application of DOR), experienced a significant increase only in the sample amended with DOR. The CFU gdw^{-1} counts were estimated to be 1.09%, 3.45% and 1.09% of the total viable cell count for unamended soil and soils amended with DOR and CORDOR, respectively. At 30 days, significant increases in total viable cell gdw^{-1} and CFU counts were measured in the soils amended with DOR and CORDOR with respect to the unamended soil. During this time, the proportion of total viable cell recovery was 0.87%, 6.73% and 3.21% for unamended soil and soils amended with DOR and CORDOR, respectively. At 60 days, total viable cell and CFU counts also significantly increased in the amended samples, although the increase was higher in the soil amended with DOR. During this time, the proportion of total viable cell recovery was 0.82%, 7.39% and 4.94% for the unamended soil, soil amended with DOR and soil amended with CORDOR, respectively.

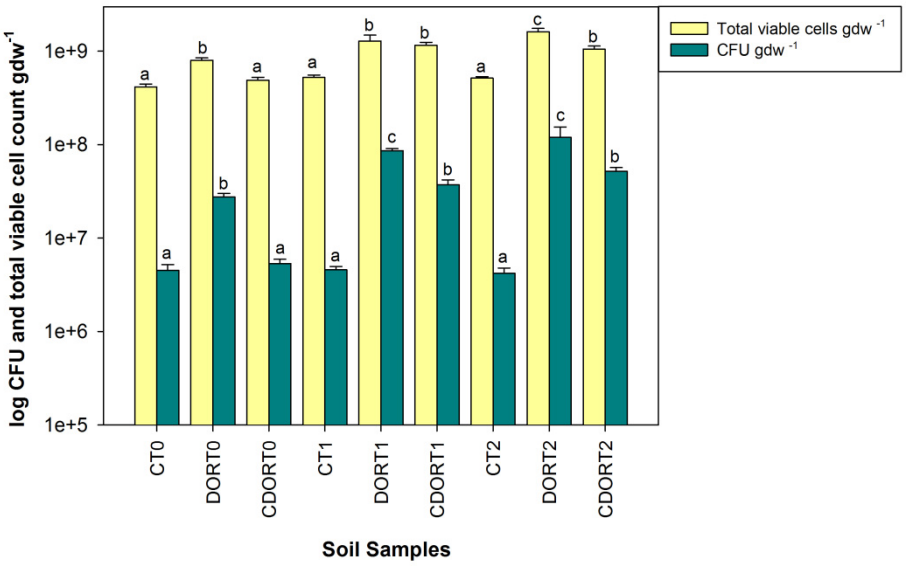

Fig. 2. Total number of total viable cells gdw^{-1} and colonies forming units (CFU) gdw^{-1} found in: CT0- unamended soil at 0 day, DORT0- soil amended with untransformed DOR at 0 day, CDORT0- soil amended with *C. floccosa*-transformed DOR at 0 day, CT1- unamended soil at 30 days, DORT1- soil amended with untransformed DOR at 30 days, CDORT1- soil amended with *C. floccosa*-transformed DOR at 30 days, CT2- unamended soil at 60 days, DORT2- soil amended with DOR at 60 days and CDORT2- soil amended with *C. floccosa*-transformed DOR at 60 days. For each count and sampling time, data followed by the same small letter are not significantly different according to the Tukey test ($p \leq 0.05$). Bars represent standard deviation.

The different diversity indices calculated for each soil sample (Table 1) demonstrated that a decrease in all the indices was observed at initial time after the amendment of soil with DOR and CORDOR. The decrease was most evident in the soil treated with DOR because only eight different OTUs were found in this sample, resulting in a Shannon index of 0.734 and the lowest evenness index among all the samples. In the soil with CORDOR, 14 different OTUs were isolated, yielding a Shannon index of 1.494 and significant differences in the bacterial diversity relative to the unamended soil (diversity *t* test, $p < 0.01$). At 30 days, different behaviors were observed. During this time interval, the highest bacterial diversity was found in the soil amended with DOR where the Chao1 index was the highest compared to the other samples. However, soil treated with CORDOR during this time suffered less changes because diversity did not differ significantly from the unamended soil (diversity *t* test, $p > 0.05$). At 60 days, although the number of OTUs found was the same in the unamended soil and soil treated with DOR, diversity differed significantly between both samples (diversity *t* test, $p < 0.05$). However, the soil with CORDOR, did not differ significantly in bacterial diversity with respect to the unamended soil (diversity *t* test, $p > 0.05$).

Soil treatment	*S*	*H*	*J*	Chao 1
C-T0	22	2.24(2.07;2.48)	0.72(0.70;0.81)	29.86(20.67;44.75)
DOR-T0	8	0.73*(0.54;0.99)	0.35(0.27;0.48)	8.33(7.00;14.12)
CORDOR-T0	14	1.49*(1.26;1.78)	0.57(0.49;0.68)	14.60(13.20;23.50)
C-T1	26	2.45(2.15;2.63)	0.75(0.70;0.82)	44.20(21.67;50.00)
DOR-T1	32	3.07*(2.87;3.16)	0.89(0.86;0.92)	56.00(30.11;60.00)
CORDOR-T1	25	2.51(2.41;2.80)	0.78(0.75;0.87)	29.50(26.00;47.00)
C-T2	28	2.71(2.59;2.93)	0.81(0.79;0.87)	41.00(28.00;54.00)
DOR-T2	28	3.08*(2.90;3.13)	0.92(0.88;0.95)	31.50(26.67;41.75)
CORDOR-T2	24	2.50(2.34;2.73)	0.79(0.76;0.87)	33.43(23.43;49.00)

Table 1. Diversity characteristics of bacterial community (*S*- Richness; *H*- Shannon index; *J*- Evenness and Chao1) obtained from unamended soil (C) and soil amended with untransformed DOR (DOR) or *C. floccosa*–transformed DOR (CORDOR) at 0 (T0), 30 (T1) and 60 (T2) days. Diversity t test was performed for each amended sample with its control (* significant differences, $p \leq 0.05$). Values in brackets are 95% confidence intervals.

UPGMA dendrogram (Fig. 3) grouped the samples in two principal clusters. One of the clusters consisted of the samples amended with DOR and CORDOR at 0 days, and the remaining samples were grouped in the other cluster. Within this latter group, samples treated with DOR at 30 and 60 days were more similar between them

than compared with control treatments and samples amended with TDOR at 30 and 60 days.

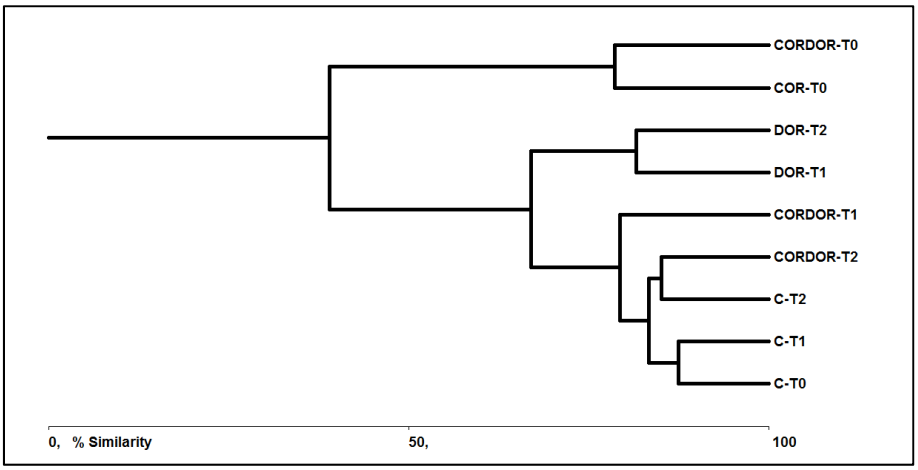

Fig. 3. Dendrogram generated by UPGMA using Euclidean distances based in the diversity of OTUs found in unamended soil (C) and soil amended with untransformed DOR (DOR) or *C. floccosa*-transformed DOR (CORDOR) at 0 (T0), 30 (T1) and 60 (T2) days.

The PCA biplot (Fig. 4) showed that around 94% of variability was explained by the first two principal components (86.78% and 7.09%, respectively). The soil samples amended with DOR and CORDOR at 0 days were located in the lower right quadrant. Likewise, the OTU 44, among others, belonging to *Arthrobacter* sp. was related to the samples situated in this quadrant. On the other hand, the remaining samples were situated in the upper right quadrant together with the OTUs 49, 39 and 27 that belonged to *Microbacterium* sp., *Streptomyces* sp. and *Nocardioides* sp., respectively.

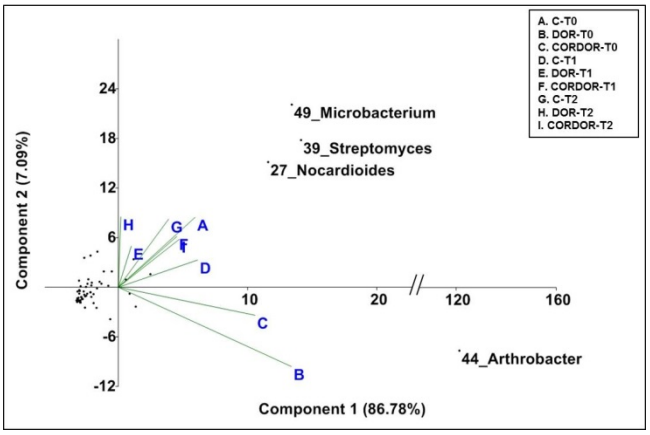

Fig. 4. PCA biplot of the samples [unamended soil (C) and soil amended with untransformed DOR (DOR) or *C. floccosa*-transformed DOR (CORDOR) at 0 (T0), 30 (T1) and 60 (T2) days] and the different OTUs found in the samples. Percent variability explained by each principal component is shown between round brackets after each axis legend.

To aid in visualizing the effect of the addition of DOR and CORDOR on the relative abundance of the different OTUs, the OTUs with highest number of isolates in each sample (≥18 isolates) were selected and compared between treatments (Fig. 5). OTU 44 from the genus *Arthrobacter* experienced an increase at the initial time after the addition of DOR and CORDOR, although this increase was higher with DOR. At 30 and 60 days, the proportion of OTU 44 isolates was similar in the control samples and soil amended with CORDOR. However, in the samples amended with DOR at 30 and 60 days, OTU 44 was scarce. Isolates from the OTU 49, *Microbacterium* spp., appeared in all the samples except in the soil amendment with DOR at initial time. On the other hand, two OTUs (27 and 30), identified as *Nocardioides* spp., did not differ remarkably in their proportions between the different samples. Instead, OTU 39, belonging to *Streptomyces* sp., suffered important changes in the different samples. The DOR application reduced isolation of these bacteria with respect to the unamended soil over the time period. However, when CORDOR was applied, the reduction in their isolation was less noticeable. In relation to the other OTUs, an increase of bacteria isolation from OTU 5 (*Sphingomonas* sp.), 9 (*Brevundimonas* sp.) and 19 (*Cupriavidus* sp., *Enterobacter* sp. and *Hydrogenophaga* sp.) was observed when DOR is applied to soil at 30 and 60 days.

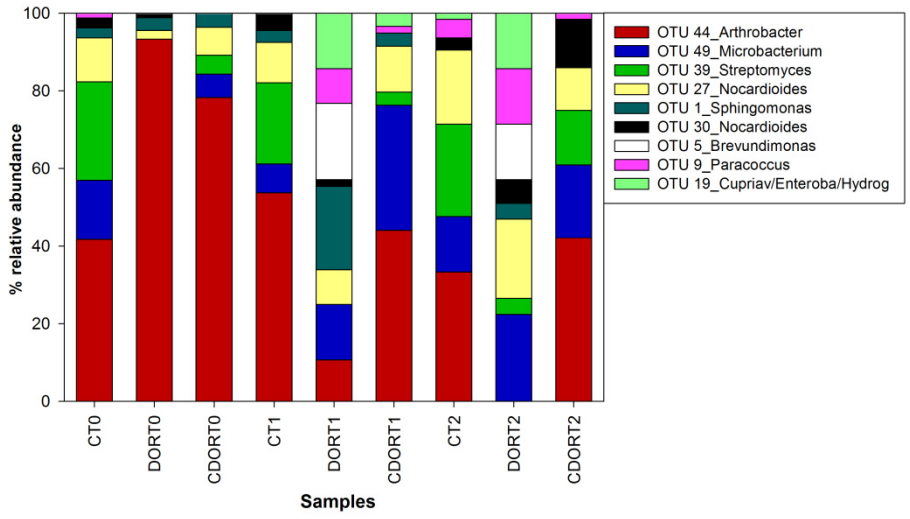

Fig. 5. Abundance of isolates found in the major OTUs (≥18) obtained from: CT0- unamended soil at 0 day, DORT0- soil amended with untransformed DOR at 0 day, CDORT0- soil amended with *C. floccosa*-transformed DOR at 0 day, CT1- unamended soil at 30 days, DORT1- soil amended with untransformed DOR at 30 days, CDORT1- soil amended with DOR *C. floccosa*-transformed DOR at 30 days, CT2- unamended soil at 60 days, DORT2- soil amended with untransformed DOR at 60 days and CORDORT2- soil amended with *C. floccosa*-transformed DOR at 60 days.

DISCUSSION

Gas liquid chromatographic analysis of the whole-cell fatty acid composition has proved to be an effective method for classifying large numbers of strains and for the selection of representative strains for phylogenetic analysis (Van Trappen et al., 2002). This technique has been used widely for bacterial characterization in multiple environments (Heyrman et al., 1999; Mergaert et al., 2001; Van Trappen et al., 2002). The method was highly efficient in this study, especially compared to other phenotypic tests, because we were able to analyze 96% of our isolates with it.

The culturable bacterial diversity found in this survey was consistent with other culture-dependent studies in a tea-plant soil in Turkey (Çakmakçı et al., 2010), in a subtropical soil from Australia (Zhang et al., 2009) and an apple trees soil in Wisconsin (USA) (Shade et al., 2012). Likewise, Sánchez-Hidalgo et al. (2012), in another culture-dependent study using VL 70 media, also reported the same phylogenetic groups from other Granada province soil.

The results of this study led us to conclude that the soil amendment with DOR and CORDOR increased the bacterial density. In a study with DOR and DOR transformed by *Phlebia* sp. (Sampedro et al., 2009a), the heterotrophic CFU count increased after the application of these amendments, although the increments observed were more subtle. In our case, the fast response of culturable bacterial community to the application of DOR after only two days was remarkable. However, this burst of growth was predictable because this untreated residue is rich in easily degradable C substrates, and these nutrients continued being available for growth and multiplication of microorganisms during the time of the study. The biotransformation of DOR with *C. floccosa* reduced its carbon content, in addition to increasing the organic matter humification index (Sampedro et al., 2007). For these reasons, the bacteria numbers in soil amended with CORDOR did not experience a significant increase at the initial time. However, the subsequent degradation or alteration of this transformed residue by soil microorganisms, perhaps slowly released substances which were assimilated by soil bacteria as they proliferated, and this fact could explain the microbial density increase at 30 and 60 days in the CORDOR-treated soils.

It was observed that DOR and CORDOR addition produced a drastic effect on soil bacterial diversity after only two days of treatment while stored at 4 ^{o}C. This burst of growth can be attributed to the quickly response of copiotrophic bacteria, dominated by r-strategists (Kotsou et al., 2004), to the input of organic load of the DOR. In these

circumstances, this group of bacteria was able to dominate the environment with respect to oligotrophic bacteria characterized by their K-strategy and, therefore, bacterial diversity was reduced. On the other hand, this reduction was less evident in the soil amendment with CORDOR because, in this case, r-strategist bacteria benefited less. Another important point in our findings was that the addition of DOR to the soil favored the bacterial diversity in the long term, and similar conclusions were obtained by means of DGGE after the amendment of two soils with OMW during three months (Rousidou et al., 2010). However, the application of CORDOR did not produce significant changes on the soil bacterial diversity with respect to the unamended soil. Only a preliminary study exists concerning the effect of CORDOR on the microbiology of soil (Sampedro et al., 2009a), and it was concluded that the addition of transformed DOR increased soil bacterial diversity. Perhaps this discrepancy between our results and Sampedro et al. (2009a) can be explained because they carried out their experiment under "in vitro" conditions and DGGE was the technique used to assess bacterial diversity. However, all the findings have demonstrated that time also plays an important role in the modulation of culturable bacterial populations (Giuntini et al., 2006)

Previous studies have indicated that the changes in the density and diversity of microorganisms after the application of olive wastes in microbial communities cannot be explained by any single reason (Sampedro et al., 2009a; Karpouzas et al., 2010; Rousidou et al., 2010). Similarly, Mechri et al. (2007) explained that the impact of olive wastes on soil microbiology was the result of complex and sometimes conflicting effects, depending on the relative amounts of beneficial or inhibiting components for microorganisms applied with the wastes. The negative effect that raw olive wastes exert in a wide range of organisms (plants, crustaceans, rotifers, fungi and bacteria) has been demonstrated in multiple studies (Medina et al., 2011; Justino et al., 2012). However, the understanding of toxicity mechanisms remains poor. Only Danellakis et al. (2011) confirmed in an aquatic invertebrate that the application of low concentrations of OMW produced loss of lysosomal membrane integrity on haemocytes, inhibition of acetylcholinesterase activity and DNA damage. In general, the toxic effects caused have been linked with three principal classes of phenols present in DOR (Justino et al., 2012). However, to date, the relative antagonist effect that each phenol exerts on microorganisms remains unclear. Medina et al. (2011), in a study of plant pathogenic bacteria, defended that the toxic effect of DOR could be attributed to

the joint action of several low molecular mass phenolic compounds, although these authors demonstrated that several phenols individually were unable to produce an antimicrobial effect.

The amendment with DOR and CORDOR had an important effect on soil bacterial distribution depending on phylogenetic group considered. Mekki et al. (2006) believed that OMW application to soil had a positive effect on culturable soil actinomycetal populations; however, our work has been the first to demonstrate that the amendment with DOR and CORDOR had an unequal effect on *Actinobacteria* group according to the genera detected. In the present experiment, the bacteria related to the genus *Arthrobacter* were favored after DOR application. He et al. (2008) also found many representatives of *Arthrobacter* in another culture-dependent study of a fertilized soil. Likewise, Pepi et al. (2009) detected one isolate of this genus using a selective medium for isolation of nitrifying bacteria in a soil amended with olive wastes. However, according to our data, a different result was obtained for bacteria related to the genus *Streptomyces*. DOR application had a detrimental effect in this group, at least at the levels applied, although this result was attenuated when CORDOR was used. This genus plays an important role in the modeling of soil microbial communities and in the control of disease-suppressive activity due to its important metabolic activity (Blanco et al., 2007). Schlatter et al. (2009) studied the application of different inputs sources, some of them present in DOR, to soil in order to assess the effects on *Streptomyces* communities, and a beneficial effect on these microorganisms was observed. For this reason, one hypothesis is that phenols, whose concentration was inversely proportional to *Streptomyces* spp. density, may be responsible for this negative effect. However, the effects of single phenols on *Streptomyces* are unknown. In this sense, the high number of strains obtained in this study can be used in future experiments to check the toxicity of particular DOR components (e.g., phenols) on specific bacterial species and to perform toxicity measurements.

Many surveys have assessed the long-term effects of organic and inorganic amendments on soil microbiology. However, the present study was a short-term evaluation because it has been demonstrated that most potential changes in soil microbiology are likely to occur during the first weeks after application of organic amendments (Blagodatskaya and Kuzyakov, 2008). During this time interval, when an organic amendment is applied to soil, in general, a quantitative increase in soil bacteria is expected which is translated in a higher microbial activity and an increase in C, N, P

and S availability, as well as a possible improvement in crop conditions. However, in an agronomical soil, it is reasonable that soil bacteria diversity and composition remain stable after amendment because specific changes may have important consequences in the sustainable agricultural production (Chaudhry et al., 2012). Therefore, application of *C. floccosa*-transformed DOR is consistent with these practices. Using this organic amendment, in addition to avoiding the problems associated with the inorganic fertilization and the reduction of phytotoxicity produced by DOR (Sampedro et al., 2007), bacterial counts were increased, as well as, diversity and distribution of culturable soil bacteria suffered little impact.

This study represented a culture-dependent approach for the effect that DOR and CORDOR have on soil bacteria and, therefore, the experiment suffered the limitations that this kind of studies involves. In subsequent studies, this research will be completed with high throughput sequencing techniques. Thereby, more comprehensive information will be obtained concerning the bacterial diversity from this soil (Bulgarelli et al., 2012) and the impact of DOR and CORDOR on soil bacteria. In addition, a true 0 day initial sampling time could be carried out in conjunction with triplicate analysis of all the samples, which was not possible in this experiment because it would have meant the analysis of an excessive number of isolates.

Acknowledgments

The authors thank Ignacio González for technical assistance with FAMEs analysis. This work was financed by the Spanish Ministry of Science and Innovation (Projects AGL2008-572 and SAF2010-15010). J.A. Siles and I. Sampedro gratefully acknowledge the JAE program, co-financed by the Consejo Superior de Investigaciones Científicas (CSIC) and the European Social Funds.

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Chapter 4

Effects of dry olive residue transformed by *Coriolopsis floccosa* (Polyporaceae) on the distribution and dynamic of a culturable fungal soil community

Adapted from:
Siles, J.A., González-Menéndez, V., Platas, G., Sampedro, I., García-Romera, I., and Bills, G.F. (In press) Effects of dry olive residue transformed by *Coriolopsis floccosa* (Polyporaceae) on the distribution and dynamic of a culturable fungal soil community. *Microbial Ecology*. DOI 10.1007/s00248-013-0353-6

Abstract

Dry olive residue (DOR) is an abundant waste product resulting from a two-phase olive oil extraction system. Due to its high organic and mineral content, this material has been proposed as an organic soil amendment; however, it presents phytotoxic and microtoxic properties. Thus, a pre-treatment is necessary before its application to soil. Among the strategies for the bioremediation of DOR is the treatment with ligninolytic fungi, e.g., *Coriolopsis floccosa*. This work aimed to assess the diversity of culturable fungi in a soil of the southeast Iberian Peninsula and to evaluate the short-term impact of untransformed and *C. floccosa*-transformed DOR on soil mycobiota. A total of 1,733 strains were isolated by the particle-filtration method and were grouped among 109 different species using morphological and molecular methods. The majority of isolates were ascomycetes and were concentrated among three orders: *Hypocreales*, *Eurotiales* and *Capnodiales*. The soil amendment with untransformed DOR was associated with a depression in fungal diversity at 30 days and changes in the proportions of the major species. However, when *C. floccosa*-transformed DOR was applied to the soil, changes in fungal diversity were less evident and species composition was similar to unamended soil.

Keywords

Olive wastes; Dry olive residue; Soil fungi; Organic fertilizers; Soil amendments; Granada soil

INTRODUCTION

The olive oil industry generates huge amounts of residues (Morillo et al., 2009). Among these wastes, the two-phase extraction system of olive oil produces large amounts of environmentally harmful by-products (Alburquerque et al., 2004; Roig et al., 2006). The principal residue resulting from this oil extraction process is two-phase olive-mill waste (TPOMW) (Cabrera et al., 2010). The TPOMW consists of a thick sludge that contains about 65% of water, pieces of stone and olive fruit pulp (Morillo et al., 2009). The centrifugation and drying followed by chemical extraction with *n*-hexane of TPOMW to recover the residual oil generates a new secondary residue known locally as "alpeorujo" or technically as dry olive residue (DOR) (Alburquerque et al., 2009). In Spain, 5 million tons of DOR are produced yearly during the harvest season (Tortosa et al., 2012). The high organic matter and mineral content (Sampedro et al., 2011) of this residue has potential as a beneficial soil amendment, especially in the Mediterranean regions, where soils are highly degraded and have a low organic matter concentration (Cayuela et al., 2010). However, when DOR is applied directly as organic amendment to soil, it causes phytotoxic effects (Gigliotti et al., 2012), mainly associated with its phenolic fraction (Linares et al., 2003; Sampedro et al., 2009b). Furthermore, antimicrobial properties have been described for DOR (Capasso et al., 1995). Therefore, DOR requires a pretreatment to detoxify it prior to soil application. Among the biological strategies for the detoxification of this residue is the incubation with lignin-degrading fungi (Sampedro et al., 2007; Ergül et al., 2009; Sampedro et al., 2009a). Previously, DOR transformation has been efficaciously mediated by the white rot basidiomycete *Coriolopsis floccosa* (Jungh.) Ryvarden (Aranda et al., 2006; Saparrat et al., 2010).

To date, the majority of research on agronomic use of olive residues has focused on the effects of untransformed DOR (López-Pineiro et al., 2011) or TPOMW composted soil amendments (Altieri and Esposito, 2010) on the physico-chemical characteristics of soil. Only Sampedro et al. (2009a) have measured the "in vitro" impact of untransformed and transformed DOR on soil microorganisms by means of denaturing gradient gel electrophoresis (DGGE) and phospholipid fatty acid (PLFA) analysis. To our knowledge, the responses of culturable soil fungi communities to raw and biotransformed DOR soil amendments are completely unknown.

Soil fungi have a pivotal role as a source, sink and regulator of the transformation energy and nutrients in the soil and are directly involved in mediating soil fertility (Bills et al., 2004; Maggi et al., 2005). The application of either organic and inorganic fertilizers is likely to influence their community function, causing effects in functional group diversity and redistribution of taxonomic groups which can possibly lead to change ecosystem functioning. In this study, we have chosen a culture-dependent approach because these methods are still considered as useful approaches for the initial characterization of microbial communities from an environment. Although data of this kind of studies must be interpreted cautiously because of limitations that culture-dependent techniques present (Bills et al., 2004; Schmit and Lodge, 2005). However, it must be acknowledged that although culture-independent methods can more intensively sample an environment, interpretations are likewise complicated due to: the methodological biases and semi-quantitative nature of sequencing methods (Liu et al., 2012)

This work had dual objectives. The first was to report on diversity and taxonomic composition of culturable soil fungi in the province of Granada, Spain. To the best of our knowledge, studies of soil fungi in this region of Spain do not exist. The second was to test the hypothesis that untreated DOR amendments would have greater short-term and detrimental effects on the diversity of culturable soil fungi community respect to fungus-detoxified DOR. This is the first report where the mycobiota of a soil from of the southeast Iberian Peninsula was analysed by isolating a large number of strains by means of particle filtration method and identified by using morphological and molecular methods.

MATERIALS AND METHODS

Sampling

The soil studied was obtained from the "Cortijo Peinado" field (Granada, Spain, 37º 13'N, 3º 45'W), at an altitude of 550 m. The soil was described as a haplic regosol (Ortega et al., 1991) and its principal properties are represented in Table 1. The climate in the region is Mediterranean with the mean annual precipitation about 357 mm with extended periods of drought. Mean annual temperature is 15.1 ºC; the coldest month is January (mean 6.7 ºC) and warmest month is July (mean 24.8 ºC) (http://www.aemet.es).

Table 1. The chemical properties of the soil used in the study.

Soil properties	Values
Clay (%)	17.15
Sand (%)	34.35
Silt (%)	48.50
pH	8.40
Total organic carbon (g kg-1)	10.67
Water soluble carbon (g kg-1)	4.83
Total nitrogen (g kg-1)	1.52
P (mg kg-1)	589.78
K (g kg-1)	8.63
Ca (g kg-1)	61.90
Cd (mg kg-1)	1.44
Cr (mg kg-1)	39.27
Fe (g kg-1)	20.97
Cu (mg kg-1)	30.28
Mg (g kg-1)	17.66
Mn (mg kg-1)	435.92
Na (g kg-1)	1.78
Ni (mg kg-1)	26.88
Zn (mg kg-1)	73.24
Pb (mg kg-1)	26.49

The normal use of the plot where the soil was collected was agriculture, and fruit trees have been cultivated in this area during recent years. At the time of sample collection, soil had been recently ploughed, and plants were absent in the parcel. The plot size was around 10,000 m^2. To collect the soil samples, the parcel was divided into 10 equal parts. 5 subsamples of 1 kg were collected randomly from each part of the plot and combined into a single pooled sample. Thereby, we obtained 10 samples of 5 kg, each one from a different part of the parcel. The samples were collected from surface soil (0-20 cm depth) in October 2010. Subsequently, all the samples were sieved through a 5-mm mesh and manually mixed. The soil was stored in thin mesh plastic bags at room temperature during 3 days, until the experiment was initiated.

Fungal treatment of DOR

DOR was obtained from an olive oil manufacturer (Sierra Sur S.A., Granada, Spain). The residue was stored at -20 ^oC until used. The main chemical characteristics of DOR were determined by Sampedro et al. (2009b).

Coriolopsis floccosa, formerly known as *C. rigida* (Spanish Type Culture Collection, CECT 20449), was maintained at 4 ^oC and subcultured monthly on potato

dextrose agar slants. Inoculum preparation and incubation conditions were previously reported by Sampedro et al. (2009b). Briefly, polyurethane sponge (PS) cubes (0.5 cm^3) were rinsed with water in a 1:20 (w/v) ratio and autoclaved three times prior to their use. Aliquots (1.5 g) of sterilized PS cubes were placed in Erlenmeyer flasks and 25 mL of liquid media [50 g L^{-1} of glucose (Acros Organics) and 5 g L^{-1} of yeast extract (Fisher Chemical)] were added and autoclaved again. Subsequently, 5 mL of *C. floccosa* inoculum (ca. 50 mg dry weight) were aseptically added to each Erlenmeyer flask and incubated at 28 ºC for 7 days.

Deionized water was added to DOR to adjust the moisture content to 25 % (w/w) prior to sterilization (3 cycles in autoclave). Then, colonized PS cubes in Erlenmeyer flasks were covered with 25 g of DOR. Solid-state cultures on DOR were incubated at 28 ºC in the dark under stationary conditions for 30 days. About 2 kg of *C. floccosa*-transformed DOR amendment were prepared. Non-inoculated and sterilized DOR samples were prepared as controls. All the amendments used in this experiment, after incubation, were sterilized. Afterwards, the DOR was sieved (2 mm), homogenized and stored at 4 ºC until added to the soil.

Soil treatments

The soil amendment experiments were carried out in 0.5 L pots containing non sterilized soil. Sterilized untransformed DOR (DOR) and DOR incubated with *C. floccosa* (CORDOR) were added to soil pots at concentrations of 50 g kg^{-1}. Soil samples without the residue (control treatments) also were prepared. Single sorghum plants (*Sorghum bicolor*) were planted in each pot. Soil treatments with plants were grown in a greenhouse with supplementary light at 25 to 19 ºC and 50 % relative humidity.

Soils with DOR and CORDOR were collected at 0, 30 and 60 days after the addition of amendments. Controls soils without the amendment were parallel analysed in the same times. There were five pots of each treatment at each sampling time. At each soil sampling, the soil from the five pots was sieved (2 mm), homogenized and mixed manually. 25 g of each soil sample were collected for further analysis. The samples were stored briefly before processing (2 days) at 4 ºC.

Assessment of soil fungi community

The strategy for assessing the impact of treatments was based upon the "Wisconsin Survey" method (Christensen, 1989; Bills et al., 2004). The basic imprint of vegetation, soil and climatic factors on the composition of the culturable fungus community could be perturbed by biotic or abiotic factors, including physical disturbance, fire, fumigation, logging or others. Such effects could be quantified by measuring changes abundance among the principal soil species. Sample sizes of about 150-500 isolates per sample were generally considered adequate to capture the distribution of the principal culturable species in a typical soil. Therefore, we targeted 200 isolates per treatment to estimate the relative abundance of the principal soil species and relative species diversity.

Soil fungi were isolated following the particle filtration method (Bills et al., 2004). This technique was used to assess fungal diversity because excess conidia and other free propagules are eliminated by washing; only small soil particles are plated, thus favouring initiation of colonies from hyphae fragment embedded in soil. The net result on perception of soil communities is a more equitable distribution of species and less influence heavily sporulating and fast-growing fungi. To carry out the process, briefly, the soil was loaded on a pair of stacked sieves and washed through a 210-µm mesh. Fine soil particles were trapped on a 105-µm mesh. These fine particles were suspended in 40 mL of sterilized water. Subsequently, the particles were washed three times with sterilized water and the wash water was decanted. Finally, about 0.5 cm^3 of washed soil particles were suspended in 20 mL of sterile 1 % aqueous carboxymethyl cellulose. Aliquots (from 50 to 200 µl) of this suspension were inoculated in Petri plates (90 mm) with YMC medium [malt extract (Becton Dickinson), 10 g; yeast extract (Becton Dickinson), 2 g; bacteriological agar (Laboratorio Conda, S.A.), 20 g], which, after autoclaving, was amended with streptomycin sulfate, oxytetracycline (both 50 µg mL^{-1}) as antibacterial agents and cyclosporin (4 µg mL^{-1}) to limit radial growth of fungi. The Petri plates were incubated at alternating temperatures (8 ^oC and 22 ^oC) to reduce the rate of colony expansion of mesophilic species, and therefore, to obtain more time for colony isolation (Bills et al., 2004) and 60 % relative humidity in darkness for 10 days. Subsequently, for each sample treatment, the Petri plates with the least numbers of colonies were chosen to initiate isolation of fungi. Starting from the least dense plates and continuing to denser plates, colonies were selected up to 4 weeks. For each treatment at each time interval (total of 9; 3 times × 3 sample combinations), 200

colonies were inoculated at the centre of a new Petri dish (60 mm) with unamended YM medium to establish a fungal colony (Polishook et al., 1996).

Identification of fungi

Morphological identification

Isolates from the 9 samples were incubated 14 days and classified into "morphospecies" on the basis of colony characteristics. Isolates were further grouped into morphological types by shape and size of spores, sporogeneous apparatus, colony colour, texture, margin type and radial extension (Bills et al., 2004).

Molecular identification

Morphospecies groupings were re-evaluated and consolidated following analyses of 28S gene, internal transcribed spacer (ITS) or in the case of *Penicillum* species, the β-tubulin gene. A few of the morphospecies were not analysed because their morphological identification was believed to be unequivocal, e.g. *Aspergillus terreus* Thom.

A voucher strain of each morphospecie and strain analysed for marker DNA sequences were maintained at the Fundación MEDINA and at the Estación Experimental del Zaidín (Consejo Superior de Investigaciones Científicas), Granada, Spain.

Fungal DNA was extracted from aerial mycelia following methods described by Bills et al. (1999). The following primers for DNA amplifications have been used: 18S3 (Collado et al., 2007), NL1R (O'Donnell, 1993), ITS1F (Gardes and Bruns, 1993) for the ITS1-5.8S-ITS2 fragment. For a few isolates, D1-D2 region amplification was carried out using primers NL1 and NL4 (O'Donnell, 1993). For the isolates of the genus *Penicillium*, a fragment of β-tubulin gene was sequenced. The fragments were amplified with the primers: T10 or T1 and T22 (Glass and Donaldson, 1995; O'Donnell and Cigelnik, 1997) (see Table A1 in Appendix 1 for primer sequences).

PCR amplification conditions were: 5 min at 93 ^{o}C, then 40 cycles of 30 s at 93 ^{o}C, 30 s at 53 ^{o}C and 2 min at 72 ^{o}C using Taq DNA polymerase (QBiogene, Inc.). Amplification products (0.1 μg mL^{-1}) were sequenced using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) following the manufacturer's recommendations. Sequencing of the cleaned PCR products were carried out by the Instrumental Technical Services of EEZ-CSIC, Granada, Spain, using ABI PRISM 3130xl

Genetic Analyzer (Applied Biosystems). The sequences were edited and corrected using BioEdit 7.0.5.3 software (Ibis Biosciences, CA, USA) and assembled with GeneDoc 2.5 software (Nicholas and Deerfield, 1997).

The sequences of ITS, partial 28S gene and partial β-tubulin gene were compared with GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the NITE Biological Resource Centre (http://www.nbrc.nite.go.jp/) and the CBS-KNAW Fungal Biodiversity Center (http://www.cbs.knaw.nl/) databases using the basic local alignment search tool (BLAST).

For the identification of isolates, results from database searches sequences were compared with morphological characteristics. Sequences from this work were submitted to GenBank and their accession numbers are listed in Table A3 in Appendix 1.

Data analyses

The programme Paleontological Statistics (PAST) ver. 2.16 (Hammer et al., 2001) was used to perform an analysis of community diversity based on the number of species (*S*), Shannon index [$H = -\sum pi/\ln (p_i)$, where p_i is the proportion of species i in a sampling] and evenness (*J*= Shannon diversity divided by the logarithm of number of species); to compare the Shannon diversity of the different samples with a *t* test (Magurran, 1988); to perform a principal components analysis (PCA) biplot for treatments and fungal species.

Rarefaction curves and cluster of the samples while a UPGMA dendrogram based on Euclidean distances were modelled with BioDiversity Pro ver.2 (Lambshead, 1998).

Finally, to aid in visualizing the effect of the addition of DOR on the relative abundance of fungal species (the number of isolates for each fungal species represented as percentage of the total isolates), the species with highest number of isolates in each sample (greater than or equal to eight isolates) were selected and compared them among treatments. The sum of all these isolates represented at least 50 % of the total isolates for each treatment.

RESULTS

Fungal diversity

The 1,800 targeted isolates were reduced to 1,733 because of non-growth or contamination of a few isolates. The 1,733 isolates were grouped in 212 "morphotypes". 11 morphotypes were identified only morphologically, 151 morphotypes were sequenced for their ITS region, another 11 morphotypes were sequenced for their 28S region and 12 morphotypes of *Penicillium* spp were sequenced to attain a diagnostic β-tubulin gene sequence. After sequencing, the 212 morphotypes were consolidated into 109 species (Table A3 in Appendix 1).

Among the 109 species identified, 105 species belonged to Ascomycota; Mucoromycotina and Basidiomycota were represented by 2 species each. A total of 1,689 isolates were grouped into 13 orders (Fig. 1) while 44 isolates could not be classified at the ordinal level. The dominant orders were: *Hypocreales* (39.1 % of total isolates), *Eurotiales* (25.9 %), *Capnodiales* (9.5 %), *Sordariales* (5.8 %) and *Pleosporales* (5 %) (Fig. 1).

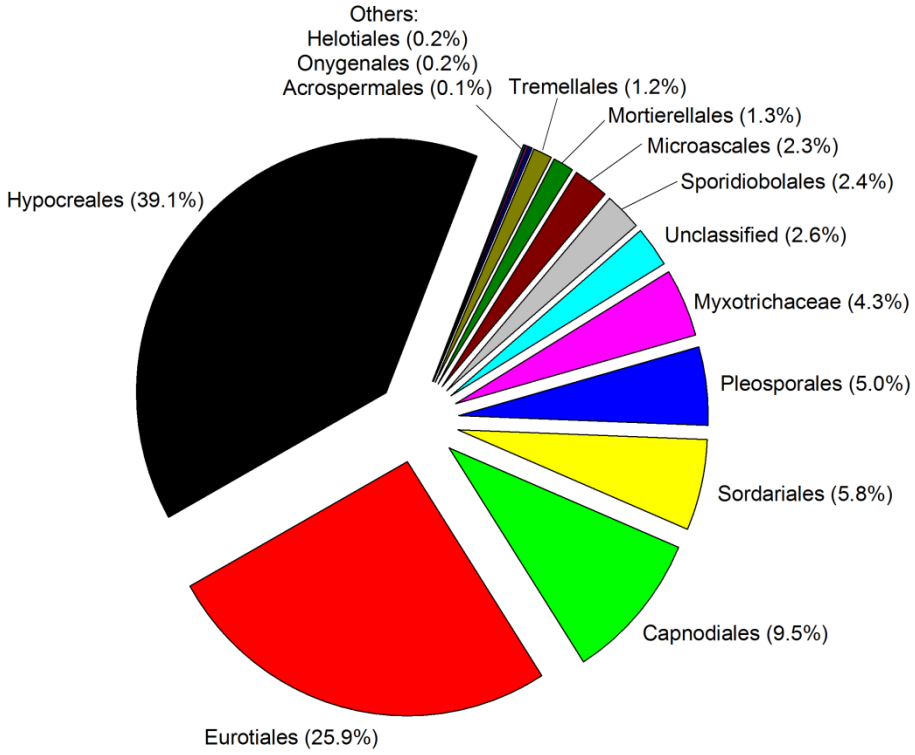

Fig. 1. Distribution of fungal isolates over different orders

The most numerous genera in terms of species diversity were: *Chaetomium* sp. (13 species), *Aspergillus* sp. (10 species), *Fusarium* sp. (10 species), *Penicillium* sp. (8 species) and *Acremonium* sp. (5 species). The major species that represented more than 3 % of the total number of isolates were: *A. terreus*, *Cladosporium cladosporioides* (Fresen.) G.A. de Vries, *Gibellulopsis nigrescens* (Pethybr.) Zare, W. Gams & Summerb, *Fusarium oxysporum* Schltdl., *Fusarium scirpi* Lambotte & Fautrey, *Fusarium solani* (Mart.) Sacc. and *Alternaria* sp. (Table A3 in Appendix 1)

Sampling adequacy

The species abundance curves for each soil treatment (Fig. 2A) did not reach an asymptote in any sample; these data indicated that more sampling effort would be required to exhaustively characterize soil mycobiota of this Granada region. Thus, this work should be considered as an approach to the fungal community characterization of this soil. Comparison of rarefaction curves for different soil treatments showed that the isolation of species was more thorough for soil amended with DOR at 30 days, and the sampling was the least thorough for the unamended soil at 30 days. From the other sample treatments, the efficacy of sampling was similar (Fig. 2A). However, when rarefaction curves were recalculated without singletons, that is, species that appeared once, all the curves tended to be asymptotic (Fig. 2B).

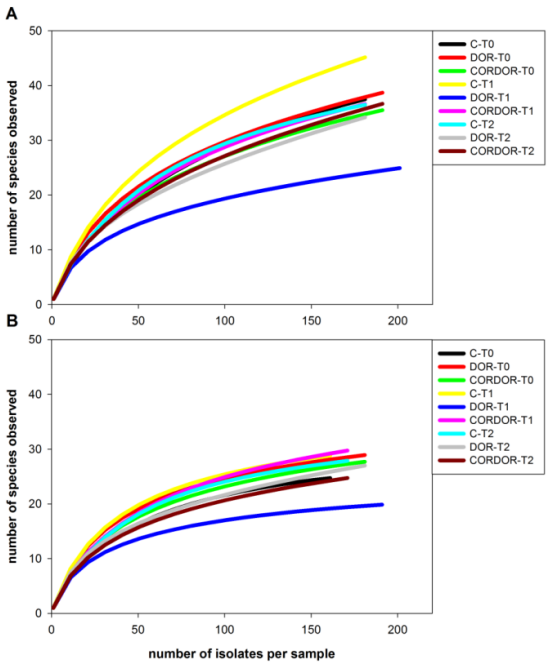

Fig. 2. Rarefaction curves of fungal species including singletons (A) and excluding singletons (B) from unamended soil (C) and soil amended with untransformed DOR (DOR) or C. floccosa–transformed DOR (CORDOR) at 0 (T0), 30 (T1) and 60 (T2) days.

Effect of DOR on fungal community profile and diversity

Biodiversity characteristics

Significant differences in *H* (Table 2) with respect to the unamended soil were found in: soil amended with DOR at 30 days (Diversity *t* test, $p < 0.01$) and soil amended with CORDOR at 30 days (Diversity *t* test, $p < 0.01$) (Table A4 in Appendix 1). Moreover, the amended samples at the same sampling times were compared and significant differences were detected between soil amended with untransformed DOR and soil amended with CORDOR at 30 days (Diversity *t* test, $p < 0.01$) (Table A4 in Appendix 1). The highest reductions in the number of species (*S*) were observed in the soils amended with DOR and CORDOR at 30 days, although this reduction was more drastic in the treatment with DOR. On the other hand, it was possible to observe that *J* increased in the amended samples respect to the unamended soil at initial time. However, this index decreased in the amended samples at 30 days and not differences were appreciated among treatments at 60 days (Table 2)

Soil Samples	*S*	*H*	*J*
C-T0	38	2.77(2.41;2.83)	0.76(0.71;0.81)
DOR-T0	39	3.09(2.84;3.13)	0.85(0.83;0.89)
CORDOR-T0	36	2.88(2.60;2.93)	0.80(0.78;0.86)
C-T1	46	3.29(2.99;3.34)	0.86(0.83;0.89)
DOR-T1	25	2.46(2.21;2.52)	0.76(0.73;0.82)
CORDOR-T1	37	2.89(2.58;2.93)	0.80(0.76;0.84)
C-T2	37	2.80(2.45;2.86)	0.78(0.71;0.82)
DOR-T2	35	2.84(2.56;2.88)	0.80(0.78;0.85)
CORDOR-T2	37	2.80(2.51;2.85)	0.77(0.75;0.83)

Table 2. Diversity characteristics of soil fungal community (*S*-number of species, *H*- Shannon index and *J*-Evenness) from unamended soil (C) and soil amended with untransformed DOR (DOR) or C. floccosa–transformed DOR (CORDOR) at 0 (T0), 30 (T1) and 60 (T2) days. Inside brackets, the lower and upper limits of values between the 95% confidence intervals.

Changes in the equilibrium of most abundant species mediated by DOR and CORDOR

The data showed that the application of DOR and CORDOR changed the distribution of the most abundant species (Fig. 3). In the unamended soil at 0 day, the most common species were: *A. terreus*, *G. nigrescens*, *Penicillium* sp. 2, *F. oxysporum* and *Stachybotrys chartarum* (Ehrenb.) S. Hughes. On the other hand, in the soil treated with DOR, the most numerous species were: *F. scirpi*, *Alternaria* sp., *C. cladosporioides*, *Chaetomium* sp. 3 and the yeast *Sporobolomyces roseus* Kluyver & C.B. Niel. At 0 day, the

soil amended with CORDOR, also experienced a similar increase in numbers of isolates of the same species that soil with untransformed DOR.

In the 30 days unamended soil, it was observed that the species with highest number of isolates were: *G. nigrescens*, *A. terreus*, *Doratomyces stemonitis* (Pers.) F.J. Morton & G. Sm., *Clonostachys rosea* (Preuss) Mussat, *F. oxysporum* and *F. scirpi* (Fig. 3). In the soil amended with DOR, the predominant species were: *C. cladosporioides*, *Acremonium furcatum* Moreau & R. Moreau ex Gams, *Alternaria* sp., *F. scirpi*, *Plectosphaerella cucumerina* (Lindf.) W. Gams, *F. solani* 1 and *F. solani* 2. In the soil sample treated with CORDOR, it was observed that the most abundant fungi were a mixture of species that were present in the previously mentioned treatments of this time.

At 60 days in the unamended soil, the fungus with the highest number of isolates was *A. terreus* (Fig. 3). Other species found were: *G. nigrescens*, *Aspergillus versicolor* (Vuill.) Tirab., *C. rosea* and *Chaetomium* sp.1. In the treatment of soil with DOR, it was noted that the species with the highest number of isolates had appeared in the previous sampling intervals, for example: *C. cladosporioides* and *F. solani* sp. 2, but in this case, other species that had been isolated in the samples without DOR were present in this treatment, including *G. nigrescens* and *A. terreus*. *Cryptococcus tephrensis* Vishniac were also isolated with a significant frequency. In the soil amended with CORDOR, like the unamended soil, *A. terreus* and *G. nigrescens* appeared frequently.

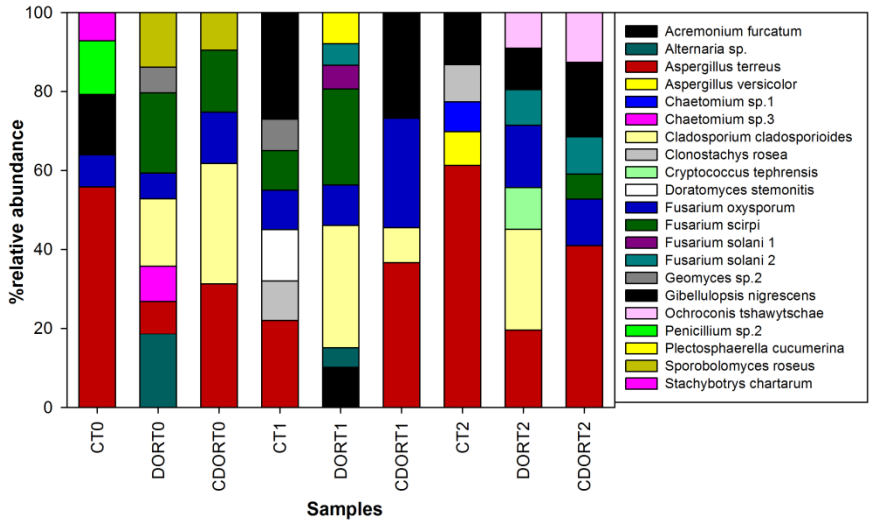

Fig. 3. Relative abundance of major species (≥ 8 isolates) isolated from: CT0- unamended soil at 0 d, DORT0- soil amended with untransformed DOR at 0 d, CDORT0- soil amended with *C. floccosa*-transformed DOR at 0 d, CT1- unamended soil at 30 d, DORT1- soil amended with untransformed DOR at 30 d, CDORT1- soil amended with DOR *C. floccosa*-transformed DOR at 30 d, CT2- unamended soil at 60 d, DORT2- soil amended with untransformed DOR at 60 d and CORDORT2- soil amended with *C. floccosa*-transformed DOR at 60 d.

Ordination of fungal community under different soil treatments

UPGMA grouped the samples in two principal clusters (Fig. 4). One of the clusters grouped the soil samples amended with DOR at 0, 30 and 60 days with the soil treated with CORDOR at 0 day. In the other cluster were grouped the control samples at successive intervals and the samples of soil amended with CORDOR at 30 and 60 days.

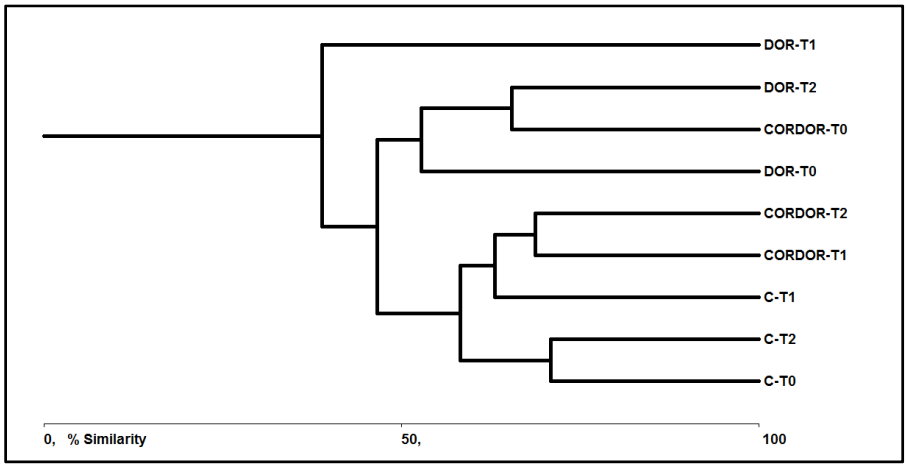

Fig. 4. Dendrogram generated by UPGMA using Euclidean distances based on species relative abundance in unamended soil (C), soil amended with untransformed DOR (DOR) and soil amended with *C. floccosa*-transformed DOR (CORDOR) at 0 (T0), 30 (T1) and 60 (T2) days.

A PCA biplot of fungal distribution among the different samples (Fig. 5) accounted for 63.7% of the variance on the first component, while the second component accounted for 21.7%. This explained 85.4% of the total variance. Most of the fungal taxa were negatively associated with PC1 (Table A4 in Appendix 1). However, all the samples were positively related to PC1. PC2 clustered the soil samples in two groups. One of the groups, in the upper right quadrant, was made up of the samples amended with DOR over the time and soil amended with CORDOR at 0 day. This group of samples was highly related to *C. cladosporioides*, *F. oxysporum*, *F. scirpi* and *Alternaria* sp. among other fungal species. The other group (in the lower right quadrant) consisted of the control samples at successive sampling times and soil treated with CORDOR at 30 and 60 days. It was strongly influenced by *A. terreus*, *G. nigrescens* and *D. stemonitis* (Fig. 5 and Table A4 in Appendix 1).

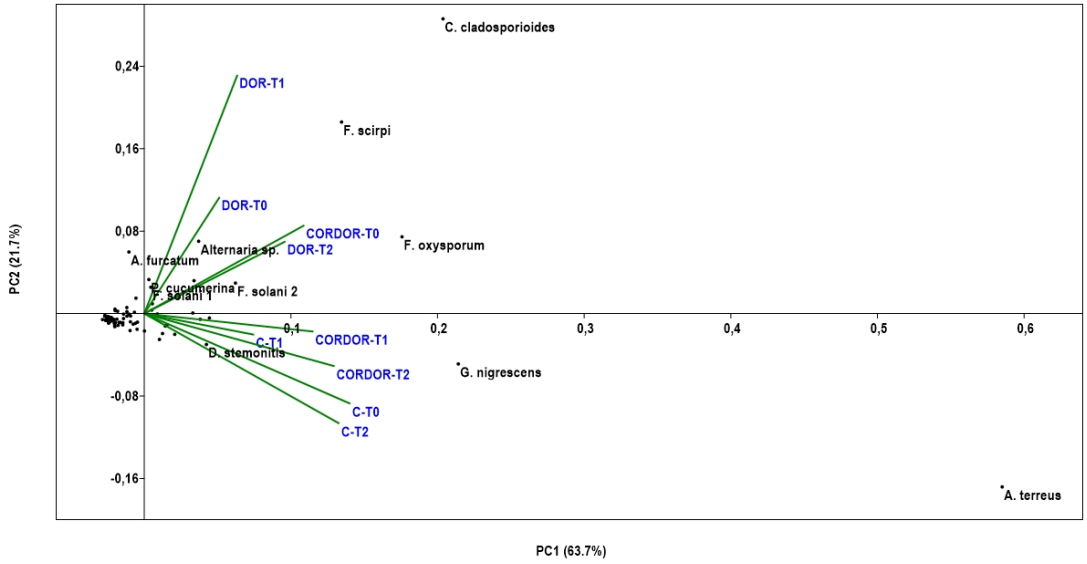

Fig. 5. PCA biplot of the samples [unamended soil, soil amended with untransformed DOR (DOR) and soil amended with C. floccosa-transformed DOR (CORDOR) at 0 (T0), 30 (T1) and 60 (T2) days] and diversity of fungi found in all the samples. Percent variability explained by each principal component is shown between round brackets after each axis legend. See Table A4 in Appendix 1 for correlations of fungal species to ordination axes derived from PCA.

DISCUSSION

Fungal diversity

The fungal diversity found in our study was in concordance with data obtained in other culture-dependent studies, e.g., from several soils across an elevation and slope gradient in the central Iberian Peninsula (Maggi et al., 2005) and the rhizospheric soil of palm plantation in Alicante province, southern Spain (Abdullah et al., 2010). In both studies, the morphological classification of the isolates determined that ascomycete fungi predominated. Also, the balance of major species was similar to the diversity found in the present study.

Our results also were consistent with other culture-dependent surveys from the Mediterranean region, including Turkey (Demirel et al., 2005; Karaoglu and Ulker, 2006; Asan et al., 2010) and Israel (Grishkan et al., 2008, 2009; Yu et al., 2012). In addition, our data also showed a good correlation with other studies where the fungal diversity of different soils was described by means of culture-independent techniques (Klaubauf et al., 2010; Orgiazzi et al., 2012), in spite of the different methodological approaches taken.

In summary, after comparing all these studies, it is possible to conclude that for example, *A. terreus* (our most-common specie) and other aspergilli are prevalent in the Mediterranean zone. This prevalence can be explained because the species from this genus present some adaptations to warm and xeric regions (Christensen, 1989; Grishkan and Nevo, 2010). Also, *Penicillium* spp. were widely distributed throughout the region, although the species of this genus are more characteristic dominants in cool-temperature environments (Christensen, 1989; Maggi et al., 2005; Grishkan and Nevo, 2010). The codominance of both genera in large numbers in our study may be due to fluctuations in temperature between the winter and summer in the Iberian Peninsula. On the other hand, *C. cladosporioides* (second most common fungus) and other fungal species like *Alternaria* sp., *G. nigrescens, Ochroconis tshawytschae* (Doty & D.W. Slater) Kiril. & Al-Achmed and *D. stemonitis* were very common in the present soil. These species are heavily melanized, which is characteristic of stress-tolerance to insolation, high temperature and water deficiency resistance (Griffin, 1972; Maggi et al., 2005), conditions found at the study site. Not surprisingly, also *Chaetomium* and *Fusarium* (*F. oxysporum* and *F. scirpi*) were abundant in our samples, a fact that could be in accordance with a history of input of organic matter content and fertilizers from the soil's previous agronomic history (Grishkan and Nevo, 2010).

Effect of DOR and CORDOR on fungal community

Our initial hypothesis was that the application of DOR to soil would have a toxic effect on soil mycobiota, and if true, an important reduction of the fungal diversity would be expected. Our hypothesis was supported by previous reports that demonstrated the toxic effect of olive-mill wastewater (OMW), a residue with a similar composition that DOR (Morillo et al., 2009), had on bacteria (Capasso et al., 1995; Saparrat et al., 2010), several fungal species (Winkelhausen et al., 2005; Medina et al., 2011) and even on nematode eggs (Cayuela et al., 2008). On the other hand, only a weak effect on the soil fungi were expected from CORDOR or perhaps even a stimulation of the number of different isolated fungi, due to nutrient input. This expectation was supported because earlier reports described an increase in the culturable fungal diversity after the addition of organic amendments to soil (Kumar et al., 2010). However, our study demonstrated that the addition of DOR only produced a slight depression of fungal diversity at 30 days and that CORDOR did not stimulate soil fungal diversity at any time. Consistent with these results, Sampedro et al. (2009a),

which assessed the impact of raw and treated DOR on soil fungi by means of DGGE, explained that the addition of untransformed or transformed DOR to soil did not affect the diversity of soil fungi with respect to the unamended soil at any time. Other investigations with OMW have shown that the fertilization of a soil with this untreated residue at different concentrations did not directly affect the diversity of fungi (Karpouzas et al., 2009; Rousidou et al., 2010). In fact, even Rousidou et al. (2010) showed that the addition of raw OMW in some concentrations increased the diversity of fungi. This discrepancy could be explained because our study employed cultivation-dependent methods while these studies used DGGE to evaluate fungal dynamics.

Our experiment also allowed us to measure quantitative changes in the frequency of species of each sample. It is important to note that the response of the application of the residue to soil was very quick. Sampedro et al. (2009a) also detected a rapid response of soil microorganisms after DOR application. This behaviour may be explained by soluble substances that leach DOR which are easily assimilable by microorganisms. In general, the same pattern among the three treatments when DOR was added to soil was observed. Important increases of the species *C. cladosporioides* (all intervals) and *Alternaria* sp. (at 0 and 30 days) were detected. *C. cladosporioides* and *Alternaria* spp. are cosmopolitan fungi associated with surfaces of plants and a variety of decomposing plant matter (Fell and Hunter, 1979; Kjøller and Struwe, 1980; Bills and Polishook, 1994; Polishook et al., 1996; Allegrucci et al., 2005). Thus, significant proliferation after the application of a lignocelullosic residue would seem to be normal. However, the phytopathogen capacity of these species has been demonstrated (Griffin and Chu, 1983; Snowdon, 1989). For this reason, direct DOR application to soil may pose a potential risk for young susceptible plants. Nonetheless, it is important highlight that the application of CORDOR at 30 and 60 days did not significantly increase the presence of these fungi. On the other hand, it was observed that *F. scirpi* and *F. oxysporum* were strongly related to the addition of DOR to soil and CORDOR at initial time. Likewise, it is necessary to note the negative role that *Fusarium* spp. may exert on the development of agronomical cultives (Hoitink, 1999).

In recent years, the responses of soil microorganism after the addition of organic amendments (Pérez-Piqueres et al., 2006; Melero et al., 2007) including even, organic amendment based on olive wastes (Karpouzas et al., 2009; Rousidou et al., 2010) have been evaluated. However, these studies, in most cases, did not carry out a specific study of the changes in fungal or bacterial communities. From our point of

view, knowing the behaviour of fungal communities of a soil under the addition of organic amendments is essential. In this regard, our study has demonstrated that the application of DOR directly to soil produced an important change in the soil fungi, with an increase of potentially phytopathogenic species. However, if CORDOR is applied, the soil experienced an increase in microbial activity (data not shown) with the benefits that this entails, but fungal diversity and distribution was similar to the unamended soil.

In conclusion, the diversity of the culturable fungal diversity of an agronomic soil from the southern Iberian Peninsula has been characterized and was shown to be comparable to other fungal soil communities of the Mediterranean region with similar climatic characteristics. Parallel evaluation of culturable soil fungal communities demonstrated that *C. floccosa*-transformed DOR had less effect on soil fungi diversity than untransformed DOR. Furthermore, the fertilization of a soil with untreated DOR produced important changes in the distribution of fungal species with respect to the unamended soil. The numbers of isolates of potentially phytopathogenic species increased in some cases, presumably due organic matter input. The changes in species composition were less evident when transformed DOR was added, especially at 30 and 60 days, when the fungal distributions were very similar to the unamended soils.

Acknowledgments

This work has been funded by the Spanish Ministery of Science and Innovation (Project AGL2008-572). J.A. Siles and I. Sampedro grafetully acknowledge the JAE program, which is co-financed by the Consejo Superior de Investigaciones Científicas (CSIC) and the European Social Funds.

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Chapter 5

Microbial diversity of a Mediterranean soil and its changes after biotransformed dry olive residue amendment

Adapted from:
Siles, J.A., Rachid, C.T.C.C., Sampedro, I., García-Romera, I., Tiedje, J.M. (In preparation). Microbial diversity of aMediterranean soil and its changes after biotransformed dry olive residue amendment.

Abstract

The Mediterranean basin has been identified as a biodiversity hotspot where there is lack of information about soil microbial diversity. Likewise, the intensive land use and aggressive management practices are producing degraded soils with loss of fertility. The use of organic amendments such as dry olive residue (DOR), a waste product resulting from two-phase olive oil extraction system, has been suggested as an effective way to improve soil properties. However, DOR needs a pre-treatment, such as the ligninolytic fungi transformation (e.g. *Coriolopsis floccosa*), before its application to soil. The objectives of the present work were to describe the bacterial and fungal diversity from a Mediterranean soil as well as the assessment of raw DOR (DOR) and *C.floccosa*-transformed DOR (CORDOR) impacts on soil functional and phylogenetic microbial communities at short-time. Pyrosequencing of 16S rRNA gen demonstrated that bacterial diversity was dominated by Proteobacteria, Acidobacteria, and Actinobacteria phyla, while 28S-rRNA gene data revealed that Ascomycota and Basidiomycota were the majority in fungal community. A Biolog EcoPlate experiment showed that DOR and CORDOR application decreased functional diversity and altered functional structure by the incorporation of nutrients to soil after 30 and 60 days of amendment. These changes in soil functionality were in parallel with alterations of the bacterial and fungal community structure. Some bacterial and fungal groups increased while others decreased depending on the relative abundance of beneficial or toxic substances for microorganisms incorporated with each amendment. In general, it was possible to observe that DOR was more disruptive than CORDOR.

Keywords

"Alpeorujo"; Olive wastes; Dry olive residue; Organic fertilizers; Microbial diversity

INTRODUCTION

Mediterranean basin represents one of the 25 most important biodiversity hotspots on Earth, due to its peculiar climatic and geological characteristics (Myers et al., 2000). Thus, this region has been identified as one of the priority regions for conservation in Europe, since human actions are causing a dramatic biodiversity crisis (Brooks et al., 2006). However, the knowledge of soil microbial diversity in this area is limited. The unraveling of this diversity is essential to achieve a balance between conservation and human development (Orgiazzi et al., 2012).

Olives are among the most important and extensive crops in the Mediterranean region, where they occupy a highly stable cultivated area (Lozano-García and Parras-Alcántara, 2013). In general, this industry produces huge amounts of wastes (Morillo et al., 2009). In Spain, it highlights the waste produced by two-phase centrifuging olive oil extraction system. This technology produces a liquid phase (olive oil) and an organic waste sludge. This primary waste suffers a subsequent revalorization by means of heat and organic solvents to generate: low quality olive oil and a final waste denominates "alpeorujo" or dry olive residue (DOR) (López-Piñeiro et al., 2011). In Spain alone, 5 million tons of DOR are produced yearly in a short period of time (Tortosa et al., 2012). Until now, DOR has been used for energy and co-generation purposes. However, the international regulations limiting the emission of CO_2 and the presence of polyaromatic hydrocarbons in DOR combustions gases are limiting this practice (Sampedro et al., 2009a; López-Piñeiro et al., 2011). An alternative for DOR revalorization is its exploitation as organic amendment since it contains a high concentration in organic matter and agricultural interest minerals (López-Piñeiro et al., 2008). Its use as organic amendment can be especially interesting in the Mediterranean zone where many soils are suffering a process of degradation and fertility loss due to using of agro-chemicals, excessive and deep tillage, continuous cropping, the overgrazing and luxury irrigation (Diacono and Montemurro, 2010). In this sense, the use of organic amendments has been suggested as an effective form for the maintenance and improvement of soil fertility since this practice improves soil physical, chemical and biological properties (Thangarajan et al., 2013). Nevertheless, DOR contains polyphenols and other organic components which are capable of inhibiting microbial growth as well as plant germination and morphogenesis (Ntougias et al., 2013). Thus, a pre-treatment is necessary before its application to soil at high

doses. The transformation of DOR by ligninolytic fungi has been demonstrated as a quick and effective strategy to stabilize organic matter, enhance C/N ratio, reduce phenolic concentration and eliminate phytotoxic effects of waste, enabling its use as organic amendment (Sampedro et al., 2005; Sampedro et al., 2007; Reina et al., 2013).

Soil bacteria and fungi play pivotal roles in biogeochemical cycles and are responsible for the nutrient cycling by mineralizing and decomposing organic matter (Kirk et al., 2004; Trevors, 2010; Orgiazzi et al., 2012). These communities may also influence nutrient availability for crops by solubilization, chelation, and oxidation/reduction processes (Rincon-Florez et al., 2013). Furthermore, soil microorganisms establish symbiotic or antagonist relationship with plants that influence their status and carry out other functions such as soil structure maintenance (Ranjard and Richaume, 2001) or degradation of pollutants (Ritter and Scarborough, 1995). Thus, microbial communities govern soil quality and are an important component of this ecosystem. In this way, the implementation of sustainable soil strategies such as using of biotransformed DOR as organic amendment requires the knowledge of microbial communities' behaviour under this practice. To date, only Sampedro et al. (Sampedro et al., 2009b) carried out a preliminary study under "in vitro" conditions assessing the impact of *Phlebia* sp.-transformed DOR on soil microbiology using denaturing gradient gel electrophoresis (DGGE) and phospholipid fatty acids (PLFA) analysis. Likewise, other surveys have assessed the effect of raw DOR application at low doses on soil physico-chemical properties (López-Piñeiro et al., 2008; López-Pineiro et al., 2011). Thus, to the best our knowledge, studies about the effect of raw or fungi-transformed DOR on soil microbiology, using more accurate and informative tools, like the high-throughput sequencing techniques, are non-existent. In this survey, pyrosequencing was used to study the diversity of bacterial and fungal communities of a Mediterranean soil and their responses to raw and fungi-transformed DOR application. This work complements other two previous published articles where the same studies were performed using culture-dependent approaches (Siles et al., 2014a; Siles et al., 2014b). In the present work, we aimed to: i) describe the bacterial and fungal diversity of an agricultural Mediterranean soil by means of 16S and 28S rRNA gene pyrosequencing, respectively; ii) obtain some insights about the functional changes produced by untransformed and *Coriolopsis floccosa*-transformed DOR on microbial communities at short-time (0, 30 and 60 days) using Biolog EcoPlate system;

iii) investigate the effects of amendments with these two DOR types on soil fungal and bacterial communities

MATERIALS AND METHODS

Soil sampling

The soil studied was obtained from the "Cortijo Peinado" field (Granada, Spain, 37º13'N, 3º 45'W), it was classified as loam according to the USDA system (USDA-NRCS, 1996) and presented a low organic matter content (10 g kg^{-1} total organic carbon), which is typical of Mediterranean agricultural soils (Lozano-García and Parras-Alcántara, 2013). The main soil properties were: 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, 0.10%; 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}.

The climate in the region is typically Mediterranean with annual rainfall averages of 357 mm, the wettest month is December with 53 mm and the driest month is August with 3 mm. Mean annual temperature is 15.1 ºC; the coldest month is January (mean 6.7 ºC) and warmest month is July (mean 24.8 ºC) (http://www.aemet.es).

The plot where soil was collected has been used to agriculture and fruit-trees have been cultivated in this area during the last years. This zone did not belong to a protected area and did not content protected species. Permissions to sampling the soil were obtained directly from the owners and technical responsible people. At the time of sample collection, soil had been recently ploughed and plants were absent in the field. To collect the soil samples, the plot (10000 m^2) was divided into 10 equal sub-plots. Five subsamples of 1 kg were collected randomly from Ap horizon (0–20 cm depth) of each part of the plot and combined into a single pooled sample. Subsequently, the different samples were sieved (5 mm sterilized mesh) and mixed. The soil was stored for three days at room temperature until the experiment was performed.

DOR

DOR was supplied by an olive oil manufacturer (Sierra Sur S.A., Granada, Spain) and was stored at -20 ºC until its use.

DOR biotransformation

DOR transformation was carried out with the fungus: *Coriolopsis floccosa* (Spanish Type Culture Collection, CECT 20449), formerly known as *C. rigida*. The transformation was carried out according to Siles et al. (Siles et al., 2014c). Briefly, sterilized polyurethane sponge (PS) cubes were placed in Erlenmeyer flasks and 25 ml culture medium were added. Subsequently, *C. floccosa* inoculum was added to PS cubes and incubated at 28 ºC for 7 days. After this time, 25 g of DOR were placed above colonized PS. Solid-state cultures on DOR were carried out at 28 ºC for 30 days. Then, DOR was autoclaved several times for complete sterilization. Non-inoculated DOR samples were prepared as controls. Finally, untransformed DOR (DOR) and *C.floccosa*-transformed DOR (CORDOR) were sieved, homogenized and stored at 4 ºC until soil amendment experiment started. The main chemical properties of DOR and CORDOR have been previously reported by Siles et al. (Siles et al., submitted for publication).

Soil amendment

The experiment was carried out in 0.5 L pots. DOR and CORDOR were added to soil pots at concentrations of 50 g kg^{-1} (equivalent to 150 Mg ha-1). Soil samples without the residue were also prepared (control soil). One sorghum plant (*Sorghum bicolor*), with a homogeneous size, was planted in each pot. The experiment was performed in a greenhouse with supplementary light at 25/19ºC and 50% relative humidity. Manual regular watering was provided to the experiment, keeping soil humidity at 15-20%.

The untreated soil and soil amended with sterilized DOR or CORDOR were analysed after 0, 30 and 60 days of treatment. The experiment consisted of five pots of each treatment by time. In each soil sampling, the soil of the five pots was mixed, homogenized and sieved (2 mm sterilized mesh). Subsequently, three 100 g soil subsamples for each treatment were placed in sterile Falcon™ tubes and stored at -80ºC until sample analysis.

Community-level physiological profile

Community level physiological profiles (CLPPs) were assessed using Biolog EcoPlate system (BIOLOG. Inc., CA, USA). Each Biolog EcoPlate contains 31 different kinds of carbon sources in triplicate (seven kinds of carbohydrates, nine of carboxylic acids, four of polymers, six of amino acids, two of amines/amides and three of a miscellaneous type). To know CLPP for each sample, 1 g of soil was shaken in 10 ml of sterile saline solution (0.85% w/v NaCl) at 150 rpm during 1 h. Subsequently, soil suspensions were serially diluted according to viable cell counts obtained for each sample in Siles et al., 2014b to avoid interferences of the number of cells in the oxidation of substrates. 130 µL of soil solutions were used for each well and Ecoplates were incubated at 25ºC during 9 days. All the analyses were performed in triplicate. The rate of utilization of C sources is indicated by the reduction of tetrazolium salts which change from colourless to purple (Nair and Ngouajio, 2012). Colour development for each well was obtained as optical density (OD) at 590 nm every 24h using an automated plate reader (Eon™ Microplate Spectrophotometer, BioTek Instruments, Inc., Germany). Subsequently, microbial activity was calculated as average well colour development (AWCD) as described Insam et al., (Insam et al., 2004). The 168 h OD data of each sample, divided by their AWCD in order to normalize the values, were selected to determine: substrate richness (S_f), Shannon's functional diversity index (H_f), substrate evenness (J_f) and principal components analysis (PCA) using the program PAST ver. 2.17 (Hammer et al., 2001). PCA of 9 samples according to their CLPP was performed using normalized AWCD data for each substrate using variance-covariance matrix. Statistical differences between the treatments at a given sampling time were analysed by ANOVA, and Tukey's honest significance difference (HSD) test was used for multiple comparison of means at a 95% confidence interval.

DNA extraction, PCR amplification and pyrosequencing.

Soil DNA was extracted using the MoBio Ultra Clean Soil DNA Isolation Kit (MoBio Laboratories Inc., Solana Beach, CA, USA) following the manufacturer's instructions. For each sample, three different DNA extractions were carried out, each one from a subsample. Afterwards, a pooled DNA sample for each treatment was prepared. Subsequently, all DNA templates were quantified with a Qubit® 2.0

Fluorometer (Life Technologies, Grand Island, NY) and sample DNA concentrations were homogenised. Then, fungi and bacteria PCR amplifications were carried out.

For bacteria, a 16S rRNA gene fragment was amplified capturing hypervariable V4 region using the primers 577F and 926R (Rodrigues et al., 2013) designed with eight-base barcodes and pyrosequencing adapters (see Table A1 in Appendix 1 for primer sequences). Triplicate amplification reactions were performed in 20-µL volume containing: 2 µL of Roche 10 × Fast Start High Fidelity buffer with 18 mM $MgCl_2$ (Roche Applied Sciences), 0.5 µL of Roche Fast Start High Fidelity Taq (5U/µL), 0.75 µL of Invitrogen 10 mM deoxynucleoside triphosphate (dNTP) mix, 1 µL of each primer (10 pmol μL^{-1}), 0.2 µL of New England BioLabs 10 mg mL^{-1} bovine serum albumin (BSA), 3 µL of DNA template (8 ng μL^{-1}) and 11.55 µL of H_2O. Negative controls using sterilized water instead of soil DNA extract were included to check for primer or sample DNA contamination. The cycling conditions were: an initial denaturation of 94° C during 3 min, followed by 30 cycles of denaturation at 94 °C for 45 s, primer annealing at 56 °C for 45 s, extension at 72 °C for 1 min, and final extension for 7 min. Subsequently, reactions were combined and purified using gel electrophoresis followed by QIAquick gel extraction kit (QIAGEN Inc., Valencia, CA) and the QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA) according to manufacturer's recommendations.

For fungi, a 625 bp fragment of 28S rRNA gene was PCR-amplified in three replicate 20-µL reaction for each sample using the primers LR3 and LR0 which included barcodes for sample discrimination (Penton et al., 2013) (see Table A1 in Appendix 1 for primer sequences). PCR amplifications included: 4 µL of Promega GoTaq® buffer, 0.5 µL of GoTaq® DNA polymerase, 1.5µL of Roche 25 mM $MgCl_2$, 1µL of Invitrogen 10mM dNTP mix, 1 µL of each primer (10 pmol μl^{-1}), 0.2 µL of New England BioLabs 10mg mL^{-1} BSA, 3 µL of DNA template (8 ng μL^{-1}) and 7.8 µL of H_2O. The thermal cycling program was: an initial denaturation of 94 °C for 3 min followed by 30 cycles of 94 °C for 1 min, 51 °C for 40 s, and 72 °C for 1 min, followed by an extension at 72 °C for 10 min. Subsequently, the reactions of each sample were pooled and purifications were performed as for bacteria.

Amplicons from all samples for bacteria and fungi were composited together in equimolar concentrations and sequenced using a Roche Sequencer GS FLX Titanium series (454 Life Sciences, Branford, CT) at Utah State University.

Pyrosequencing data analysis

Raw fungal and bacterial sequences were processed using Mothur version 1.31.0 (Schloss et al., 2009). Briefly, the sequencing errors were reduced using AmpliconNoise algorithm and low-quality sequences were removed [minimum length 150 base pairs (bp), allowing 1 mismatch to the barcode, 2 mismatches to the primer, and homopolymers no longer than 8 bp]. Subsequently, sequences were aligned using package's internal alignment feature and as template the SILVA database (Gottel et al., 2011). After, the chimera.slayer function was used to identify potentially chimeric sequences and they were removed (Taketani et al., 2013). Finally, fungal and bacterial high-quality sequences were separately clustered into operational taxonomic units (OTUs) at a 3% dissimilarity distance**.** The number of sequences per sample was normalized before OTUs definition (the normalization was carried out based on the number of sequences obtained from the smallest library). The OTUs (phylogenetic richness –R_f) distribution among samples was utilised for calculating rarefaction curves, phylogenetic Shannon diversity index (H_t), phylogenetic evenness (J_f), Chao 1 and ACE (abundance-based coverage estimation) diversity estimator indices as well as good's coverage by Mothur. Significant differences in Shannon diversity indices between control and amended samples at a given sampling time were assessed through Diversity *t* test ($p < 0.05$ were regarded as statistically significant) (Magurran, 1988). On the other hand, differences in fungal and bacterial community composition of each pair of samples were determined using unweighted UniFrac metric (1,000 permutations). Then, unweighted UniFrac distances between samples were used to model PCA for each community. Finally, representative sequences from the most abundant bacterial and fungal OTUs (top 14) were obtained using Mothur. These sequences were identified by manually blasting in EzTazon-e Database (http://eztaxon-e.ezbiocloud.net/) (Kim et al., 2012) for bacteria and in CBS-KNAW Fungal Biodiversity Center (http://www.cbs.knaw.nl/) for fungi.

The non-normalized bacterial and fungal sequences were classified using Ribosomal Database Project (RDP) bacterial 16S rRNA gene and fungal 28S rRNA gene classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp) at a 50% bootstrap confidence level for both communities (Lee et al., 2011; Penton et al., 2013; Poulsen et al., 2013).

The raw pyrosequencing data were deposited in the MG-RAST public database (http://metagenomics.anl.gov/) under the accession number 4552035.3 for bacteria sequences and 4552036.3 for fungi sequences.

Pearson's method was used to examine trends between functional and phylogenetic properties with respect to chemical characteristics of the different soil samples reported in a previous article (Siles et al., submitted for publication).

RESULTS

Soil Microbial Diversity

Bacterial diversity

After pyrosequencing analysis, a total of 17,322 sequences across the 9 samples passed the quality filters with an average read length of 311 bp. The number of sequences per sample ranged from 2,248 (C-T0) to 1,674 (CORDOR-T1) (Table 1). The average number of bacterial sequences was 1,924 ± 160 (mean ± SD) per sample. These sequences were grouped among 2,267 different OTUs at 97% sequence similarity. This total number of OTUs consisted of 1,143 nonsingleton OTUs and 1,124 singletons. The rarefaction curves of the different treatments did not reach a plateau for any sample (Fig. 1). Likewise, good's coverage values (ranging from 0.76 to 0.81) (Table 1) also indicated that the sequences obtained were insufficient to fully capture the bacterial diversity.

Table 1. Phylogenetic bacterial diversity characteristics obtained from unamended soil (C) and soil amended with untransformed DOR (DOR) or *C. floccosa*–transformed DOR (CORDOR) at 0 (T0), 30 (T1) and 60 (T2) days. Diversity t test was performed for each amended sample with its control (* significant differences, $p \leq 0.05$).

Samples	Sequence number	*Sp*	*Hp*	*Jp*	Chao 1	ACE	Good's Coverage
C-T0	2248	649	5.82 (5.76;5.89)	0.899(0.892;0.907)	1381(1202;1617)	2132(1946±2344)	0.76
DOR-T0	1957	580	5.59 (5.52;5.66)	0.879(0.870;0.888)	1240(1070;1471)	1913(1739±2113)	0.79
CORDOR-T0	1981	592	5.59 (5.52;5.66)	0.876(0.867;0.884)	1351(1156;1612)	2262(2059±2494)	0.78
C-T1	1858	613	5.70 (5.63;5.77)	0.888(0.880;0.896)	1379(1185;1638)	2030(1845±2243)	0.77
DOR-T1	1904	522	5.34* (5.26;5.41)	0.853(0.843;0.863)	1103(945;1320)	1569(1416±1747)	0.81
CORDOR-T1	1674	646	5.81 (5.74;5.88)	0.898(0.890;0.906)	1428(1235;1685)	2137(1949±2351)	0.76
C-T2	1787	560	5.84 (5.77;5.90)	0.901(0.894;0.909)	1382(1203;1618)	2106(1922±2317)	0.76
DOR-T2	1888	638	5.81 (5.74;5.87)	0.899(0.892;0.907)	1330(1160;1555)	2198(2006±2417)	0.77
CORDOR-T2	2025	651	5.86 (5.80;5.93)	0.905(0.897;0.912)	1236(1094;1424)	1756(1602±1934)	0.77

Values in brackets are 95% confidence intervals as calculated using MOTHUR
S_p –Phylogenetic richness
H_p–Phylogenetic Shannon index
J_p–Phylogenetic evenness

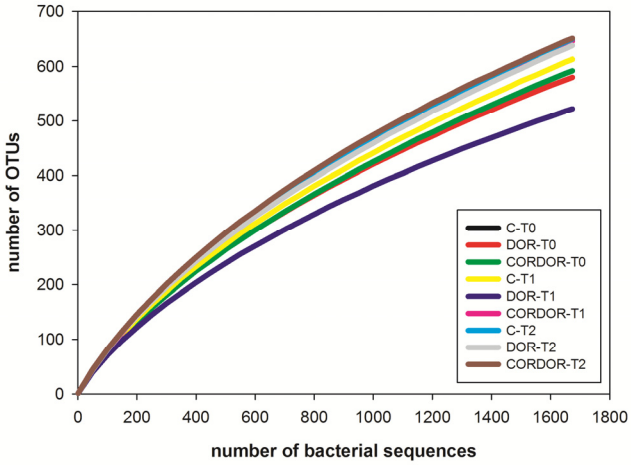

Fig. 1. Rarefaction curves for bacteria obtained from unamended soil (C) and soil amended with untransformed DOR (DOR) or *C. floccosa*–transformed DOR (CORDOR) at 0 (T0), 30 (T1) and 60 (T2) days.

All the bacterial high-quality sequences were merged into a single file which was subjected to the RDP classifier at 50% confidence threshold. The phylogenetic assignment analysis allowed the classification of ~86% sequences at phylum level. The bacterial diversity of this soil was distributed among 17 different phyla. The most common phyla were: Proteobacteria, Acidobacteria, Actinobacteria, Gemmatimonadetes, Firmicutes and Verrucomicrobia (Fig. 2A). Approximately 83% of the reads could be classified among 42 different classes, highlighting: *Alphaproteobacteria* (the genera most common within this class were: *Skermanella*, *Microvirga*, *Phenylobacterium*), *Gp6* (*Gp6*), *Actinobacteria* (*Nocardioides*, *Solirubrobacter*), *Gemmatimonadetes* (*Gemmatimonas*), *Gammaproteobacteria* (*Steroidobacter*) and *Deltaproteobacteria* (*Geobacter*) (Fig. 2B).

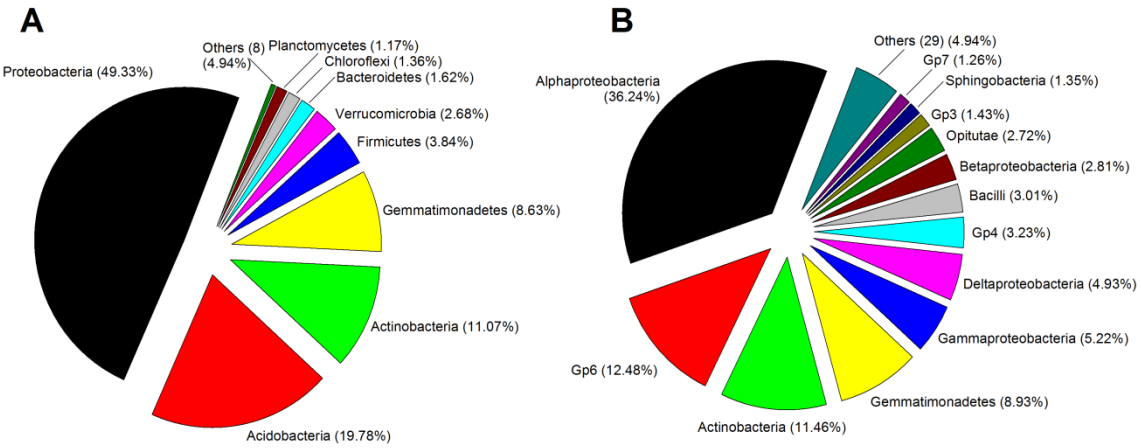

Fig. 2. Composition of the bacterial community in the studied soil based on 16S rRNA gene pyrosequencing at phylum (A) and class (B) level.

Fungal diversity

A total of 38,410 28S rRNA gene valid sequences with an average read length of 338 bp were obtained from the 9 samples. The average number of reads per sample was 4,267 ± 2,258 (mean ± SD), the sample C-T0 had the highest number of sequences (9,405) and DOR-T1 had the lowest number of reads (1,230) (Table 2). The total number of sequences represented 1160 different OTUs in all at 97% confidence; 720 of them were nonsingleton OTUs and the remaining (440) were singletons. The rarefaction curves (Fig. 3) and good's coverage values (Table 2) indicated that sampling was not fully exhaustive for any sample. Although according to coverage data, fungal community was better characterized than bacterial.

Table 2. Phylogenetic fungal diversity characteristics obtained from unamended soil (C) and soil amended with untransformed DOR (DOR) or C. floccosa–transformed DOR (CORDOR) at 0 (T0), 30 (T1) and 60 (T2) days. Diversity t test was performed for each amended sample with its control (* significant differences, $p \leq 0.05$).

Samples	Sequence number	*Sp*	*Hp*	*Jp*	Chao 1	ACE	Good's Coverage
C-T0	9405	245	4.02(3.92;4.12)	0.731(0.726;0.757)	630(485;862)	994(854;1167)	0.87
DOR-T0	3658	240	3.91(3.80;4.01)	0.742(0.737;0.758)	625(479;890)	982(852;1092)	0.88
CORDOR-T0	2321	239	3.92(3.79;4.02)	0.700(0.698;0.728)	664(474;989)	918(784;1083)	0.89
C-T1	4093	247	4.16(4.07;4.26)	0.755(0.741;0.769)	557(443;737)	1055(917;1220)	0.88
DOR-T1	1230	235	4.01(3.91;4.11)	0.645(0.625;0.666)	586(455;792)	1272(1090;1494)	0.87
CORDOR-T1	4007	292	4.52*(4.61;4.77)	0.826(0.814;0.837)	610(499;782)	890(778;1027)	0.86
C-T2	4238	249	4.10(4.00;4.21)	0.744(0.728;0.760)	551(438;731)	838(724;979)	0.88
DOR-T2	4234	241	4.05(3.94;4.15)	0.738(0.722;0.755)	616(471;850)	1020(875;1197)	0.86
CORDOR-T2	5224	271	4.43*(4.34;4.52)	0.791(0.779;0.803)	735(565;1004)	1296(1128;1497)	0.86

Values in brackets are 95% confidence intervals as calculated using MOTHUR
S_p – Phylogenetic richness
H_p– Phylogenetic Shannon index
J_p– Phylogenetic evenness

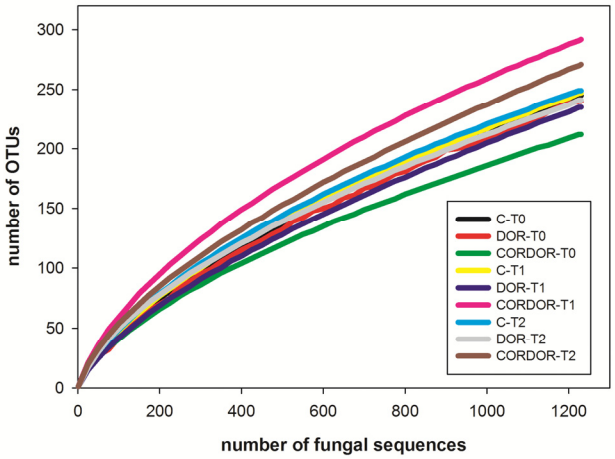

Fig. 3. Rarefaction curves for fungi obtained from unamended soil (C) and soil amended with untransformed DOR (DOR) or *C. floccosa*–transformed DOR (CORDOR) at 0 (T0), 30 (T1) and 60 (T2) days.

The fungal sequences classification at RDP (confidence threshold of 50%) yielded ~80% classified sequences among 5 different phyla, highlighting Ascomycota, Basidiomycota and Chytridiomycota (Fig. 4A). On the other hand, the fungal diversity of this community consisted of 18 different classes (71% of total sequences). The most abundant classes were: *Sordariomycetes* (the genera most numerous within this order were: *Chaetomium*, *Fusarium*, *Stachybotrys*), *Pezizomycetes* (*Ascobolus*, *Peziza*), *Dothideomycetes* (*Alternaria*, *Lophiostoma*, *Cladosporium*), *Chytridiomycetes* (*Nowakowskiella*), *Eurotiomycetes* (*Aspergillus*, *Eupenicillium*) and *Agaromycetes* (*Coprinellus*) (Fig. 4B).

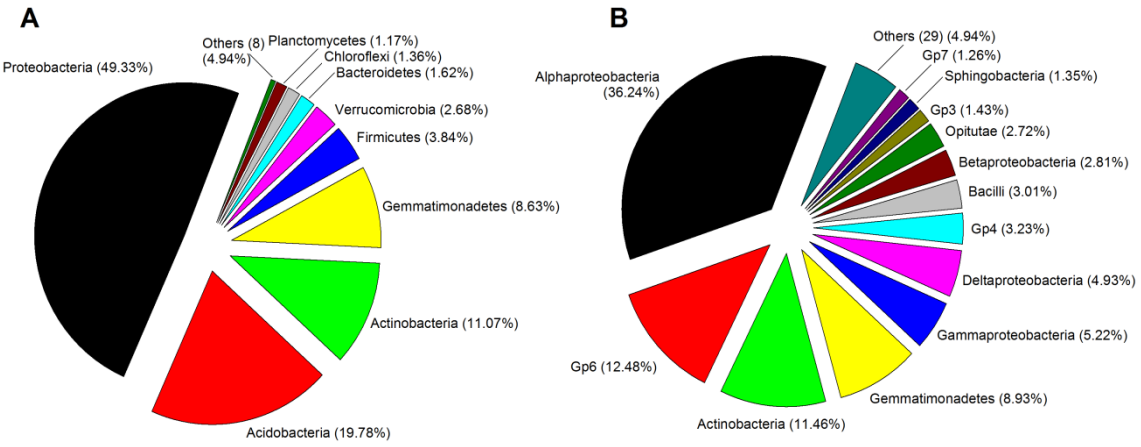

Fig. 4. Composition of fungal community in the studied soil based on 28S rRNA gene pyrosequencing at phylum (A) and class (B) level.

Effects of DOR and CORDOR on soil microbial communities

Community level physiological profile (CLPP)

The functional indices S_f and H_f based on CLPPs significantly decreased ($p < 0.05$) in the samples with DOR and CORDOR at 30 and 60 days with respect to unamended samples (Table 3). Instead, S_f and H_f did not vary between samples at initial sampling time. The lowest microbial physiological diversity was found in the soil treated with CORDOR at 30 days (Table 3).

Table 3. Functional microbial diversity characteristics (mean±standard deviation) obtained from unamended soil (C) and soil amended with untransformed DOR (DOR) or *C. floccosa*–transformed DOR (CORDOR) at 0 (T0), 30 (T1) and 60 (T2) days. For each variable and sampling time, data followed by different letter are significantly different according to Tukey's HSD test (P≤0.05).

Soil treatment	*Sf*	*Hf*	*Jf*
C-T0	27.33±0.58a	3.15±0.01a	0.95±0.01a
DOR-T0	22.50±3.54a	3.00±0.11a	0.96±0.01a
CORDOR-T0	24.00±1.41a	3.05±0.05a	0.96±0.01a
C-T1	24.00±1.41b	3.00±0.01c	0.95±0.01a
DOR-T1	19.50±0.71a	2.84±0.01b	0.94±0.01a
CORDOR-T1	17.50±2.12a	2.49±2.49a	0.92±0.03a
C-T2	24.50±0.71b	3.12±0.02b	0.97±0.01a
DOR-T2	20.50±0.71a	2.79±0.02a	0.94±0.01a
CORDOR-T2	17.00±1.41a	2.64±0.07a	0.94±0.02a

S_f–Functional richness
H_f–Functional Shannon index
J_f–Functional evenness

The PCA of CLPPs dataset showed that around 53% of the variability was explained by two principal components, the first (PC1) accounting for 33.39% and second (PC2) for 19.09% (Fig. 5). These principal components grouped the samples in two clusters and one sample was situated alone. The correlation values of each C source with PC1 and PC2 have been indicated in the Table A7 (Appendix 1). One of the clusters, situated in the lower-left quadrant, was made up of all the samples at initial sampling time and control samples at 30 and 60 days, this group was highly related to carbohydrates and polymers (D-cellobiose, cyclodextrin or glycogen). Other cluster, located in the upper quadrants, consisted of the samples amended with DOR at 30 and 60 days and the soil amended with CORDOR at 60 days. This group was highly weighted by carboxylic acids (malic, itaconic and D-galacturonic acids) and carbohydrates (Beta-methyl-D-glucoside). Finally, the soil treated with CORDOR at 30 days did not group with other treatments and was situated in the lower-right quadrant; some carbohydrates (D-xylose and i-erythritol), amines/amides (putrescine

and phenylethylamine) and amino acids (L-arginine) were the most oxidized substrates in this sample.

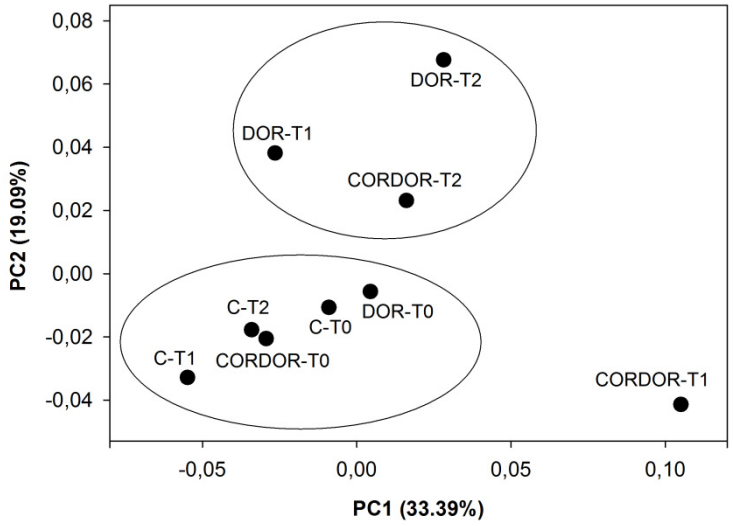

Fig. 5. PCA based on variance-covariance matrix of community level physiological profile (CLPP) dataset for unamended soil (C) and soil amended with untransformed DOR (DOR) or *C. floccosa*–transformed DOR (CORDOR) at 0 (T0), 30 (T1) and 60 (T2) days. Percent variability explained by each principal component is shown between round brackets after each axis legend.

Phylogenetic bacterial community

The DOR amendment reduced significantly H_p (diversity *t* test, $p < 0.001$) at 30 days respect to the unamended soil. This amendment also entailed the diminution of S_p, J_p, Chao 1 and ACE indices at this sampling time. However, DOR did not produce alteration of bacterial diversity at the other sampling times. Likewise, it was not possible to appreciate alterations of diversity characteristics in the samples amended with CORDOR at any sampling time (Table 1).

PCA of the bacterial pyrosequencing data based on unweighted UniFrac metric revealed that amendments produced variation on community structure (Fig. 4). The analysis showed that 66.78% of variance can be explained by two principal components, the first accounted for 53.18% and second for 13.60% of the variation. The nine samples grouped in two clusters and one sample did not cluster. One of the groups was situated to the right of PC1; it consisted of all the samples at initial sampling time and control samples at 30 and 60 days. Pairwise unweighted UniFrac test did not find significant differences among the samples of this group ($p > 0.05$).

Other group was situated in the lower right quadrant and was made up of the samples amended with CORDOR at 30 and 60 days as well as the soil with DOR at 60 days. The bacterial community structure from these samples was significantly different with respect to their control samples (pairwise unweighted UniFrac test, $p < 0.001$). Finally, soil amended with DOR at 30 days was placed in the upper right quadrant. This sample showed significant differences respect to unamended soil at 30 days ($p < 0.001$).

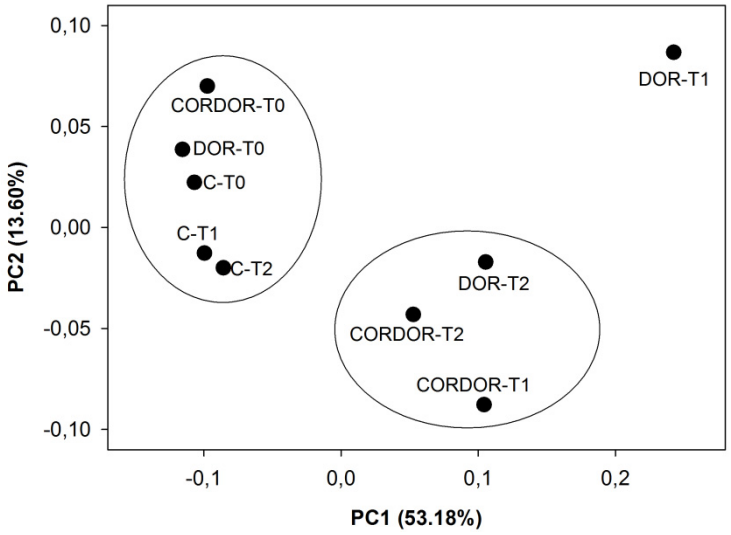

Fig. 4. PCA based on unweighted UniFrac distances of bacterial community found in unamended soil (C) and soil amended with untransformed DOR (DOR) or *C. floccosa*–transformed DOR (CORDOR) at 0 (T0), 30 (T1) and 60 (T2) days. Percent variability explained by each principal component is shown between round brackets after each axis legend.

The changes in bacterial community structure mediated by amendments were due to alterations in the relative abundance of Proteobacteria, Acidobacteria, Actinobacteria and Gemmatimonadetes phyla (Fig. 5A). Within Proteobacteria phylum, the most important changes occurred in *Alphaproteobacteria* as this class dominated this phylum (Fig. A1A in Appendix 1). Interestingly, the different orders belonged to *Alphaproteobacteria* responded differently to amendments (Fig. 5B), since *Rhodospirillales* [represented in the top 14 most abundant bacteria with OTU 1 (*Skermanella stibiiresistens*) and OTU 9 (*Skermanella aerolata*) (Table 4)] decreased their relative abundances after DOR and CORDOR application at 30 and 60 days while *Rhizobiales* [OTU 2 (*Microvirga aerophila*) and OTU 7 (*Rhizobium rosettiformans*) (Table 4)], *Caulobacterales* [OTU 6 (*Phenylobacterium* sp.) (Table 4)] and *Sphingomonadales* were considerably higher in these treatments at the same sampling times. Regarding

Acidobacteria phylum, it was appreciated a general decreased in amended samples, being more evident with DOR (Fig. 5A). The Gp6 and Gp7 classes were the most influenced by inputs although clear evidences about the specific effect of each amendment on these groups were not obtained (Fig. A1B in Appendix 1). Four of the most abundant OTUs (OTUs 3, 8, 10 and 12) were identified as uncultured *Acidobacteria* although it was not possible to obtain a more detailed identification of these OTUs (Table 4). Within *Actinobacteria* phylum, *Propionibacterinae* suborder [OTU 5 (*Nocardoides mesophilus*) (Table 4)] responded negatively to both amendments application at 30 and 60 days (Fig. A1C in Appendix 1). Finally, Gemmatimonadetes also was influenced by application of DOR. OTU 13, which was related to *Gemmatimonadaceae* (the only family presents in this phylum), suffered a drastic decrease of its relative abundance after DOR amendment at 30 and 60 days (Table 4).

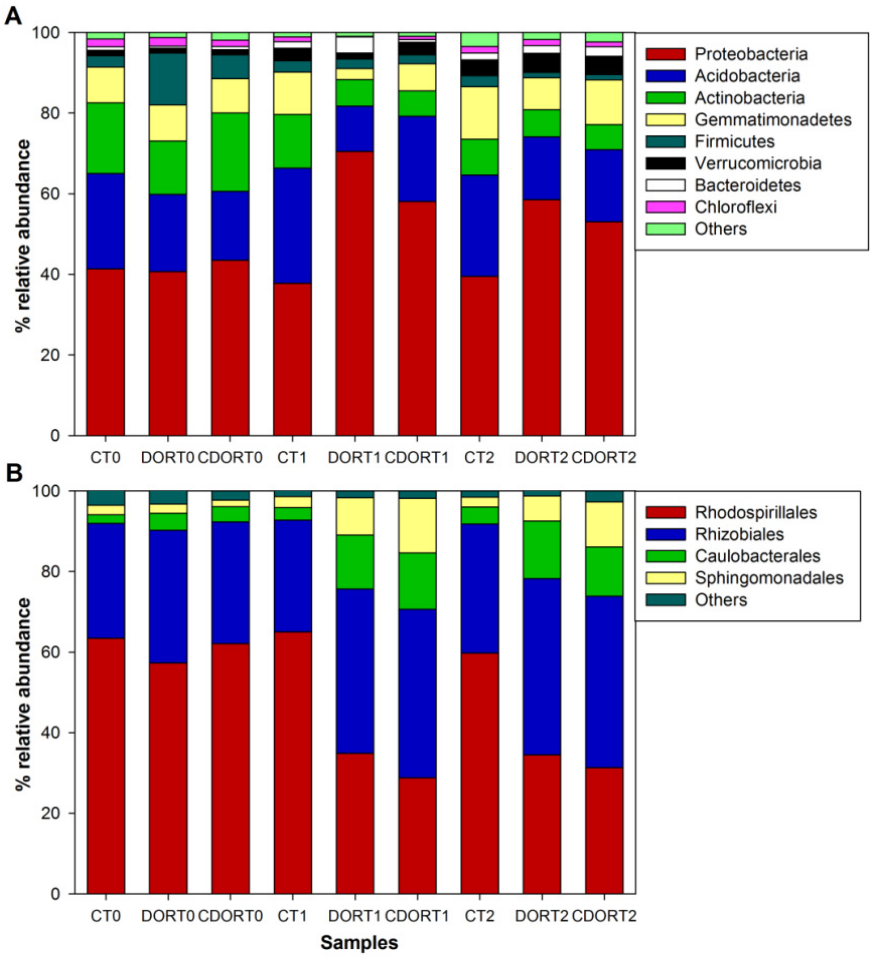

Fig. 5. Relative abundance of the different bacterial phyla (A) and orders of *Alphaproteobacteria* (B) found in unamended soil (C) and soil amended with untransformed DOR (DOR) or *C. floccosa*–transformed DOR (CDOR) at 0 (T0), 30 (T1) and 60 (T2) days.

Phylogenetic fungal community

Regarding fungal community, it was appreciated that CORDOR at 30 and 60 days produced a significant increment of H_p compared to unamended samples (Diversity t test, $p < 0.05$) (Table 2). Likewise, the other diversity indices were also influenced by this amendment. On the contrary, DOR did not produce changes on diversity characteristics of fungal community at any sampling time.

The fungal PCA based on unweighted UniFrac distances indicated that this structure community changed according to the amendments applied (Fig. 6). The two principal PCA components explained 61.36% of the variability (40.79% and 20.57% respectively) and separated the 9 samples into three groups; one of them, in the left quadrants, was made up of all the samples at initial sampling time and control samples at 30 and 60 days. No significant differences ($p > 0.05$) were found by pairwise unweighted UniFrac test among these samples. Other group, in the upper right quadrant, consisted of DOR amended samples at 30 and 60 days; the structure of fungal community of these samples was significantly different to their respective control samples (pairwise unweighted UniFrac test, $p < 0.001$). The last group, in the lower right quadrant, included the samples with CORDOR at 30 and 60 days, which also presented a fungal community significant different respect to their respective unamended samples (pairwise unweighted UniFrac test, $p < 0.001$).

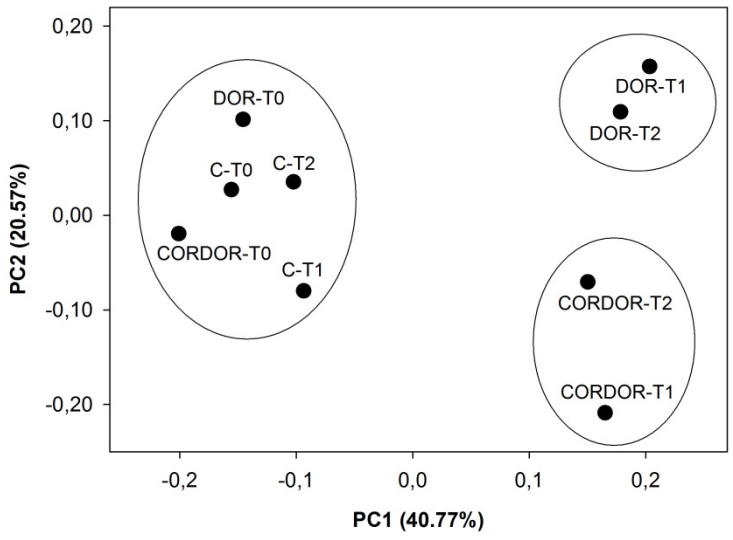

Fig. 6. PCA based on unweighted UniFrac distances of fungal community found in unamended soil (C) and soil amended with untransformed DOR (DOR) or *C. floccosa*–transformed DOR (CORDOR) at 0 (T0), 30 (T1) and 60 (T2) days. Percent variability explained by each principal component is shown between round brackets after each axis.

Table 4. Frequency of the bacterial 14 most abundant OTUS and their relative abundance (percent) in unamended soil (C) and soil amended with untransformed DOR (DOR) or *C. floccosa*–transformed DOR (CORDOR) at 0 (T0), 30 (T1) and 60 (T2) days .

OTU information					Soil samples								
Nº	**EzTaxon best match (Acc number)**	**Class**	**ID(%)**	**Nº seqs.[1]**	**CT0**	**DORT0**	**CORDORT0**	**CT1**	**DORT1**	**CORDORT1**	**CT2**	**DORT2**	**CORDORT2**
1	*Skermanella stibiiresistens* (HQ315828)	*Alphaproteobacteria*	100	619	12.4	14.4	17.4	13.7	10.5	6.8	10.8	7.1	6.8
2	*Microvirga aerophila* (GQ421848)	*Alphaproteobacteria*	100	426	4.0	8.7	9.9	5.6	22.3	14.8	7.7	11.0	16.0
3	Uncultured *Acidobacteria* (FJ152681)	*Acidobacteria*	100	348	10.3	9.2	8.3	12.1	13.8	17.8	10.3	8.6	9.5
4	Uncultured *Dongia* sp. (FJ479524)	*Alphaproteobacteria*	100	312	10.3	11.2	11.5	11.2	13.5	7.1	12.8	14.7	7.7
5	*Nocardioides mesophilus* (EF466117)	*Actinobacteria*	99	236	20.1	14.1	19.0	14.8	5.3	4.7	15.5	3.4	3.1
6	Uncultured *Phenylobacterium* (AJ292592)	*Alphaproteobacteria*	100	186	0.0	3.8	4.3	2.2	21.0	22.0	2.7	21.5	22.6
7	*Rhizobium rosettiformans* (EU781656)	*Alphaproteobacteria*	100	173	2.3	2.9	0.6	1.7	53.2	15.0	1.7	18.5	4.0
8	Uncultured *Acidobacteria* (HM438150)	*Acidobacteria*	100	145	7.6	8.3	13.1	14.5	4.1	18.6	15.2	9.0	9.7
9	*Skermanella aerolata* (DQ672568)	*Alphaproteobacteria*	99	145	12.4	16.6	18.6	14.5	12.4	7.6	8.3	4.1	5.5
10	Uncultured *Acidobacteria* (FJ152840)	*Acidobacteria*	100	141	12.1	12.1	7.8	19.1	3.5	8.5	14.9	12.1	9.9
11	Uncultured *Polyangiaceae* (EU134489)	*Deltaproteobacteria*	86	141	17.7	17.7	20.6	18.4	9.2	2.8	7.8	3.5	2.1
12	Uncultured *Acidobacteria* (EF688341)	*Acidobacteria*	100	138	17.4	9.4	10.1	23.9	3.6	8.0	14.5	2.2	10.9
13	Uncultured *Gemmatimonadaceae*(EU881161	*Gemmatimonadetes*	98	138	14.5	6.5	8.0	14.5	3.6	7.2	20.3	9.4	15.9
14	Uncultured *Alysiosphaera* (EU133443)	*Alphaproteobacteria*	95	134	9.7	9.0	20.9	9.7	6.7	5.2	11.9	16.4	10.4

[1] total number of sequences in normalized samples

Table 5. Frequency of the fungal 14 most abundant OTUS and their relative abundance (percent) in unamended soil (C) and soil amended with untransformed DOR (DOR) or *C. floccosa*–transformed DOR (CORDOR) at 0 (T0), 30 (T1) and 60 (T2) days .

OTU information					Soil samples								
Nº	**CBS best match (Acc number)**	**Order[1]**	**ID (%)**	**Nº seqs[2]**	**CT0**	**DORT0**	**CORDORT0**	**CT1**	**DORT1**	**CORDORT1**	**CT2**	**DORT2**	**CORDORT2**
1	*Fusarium* sp. (JF740925)	*Hypocreales*	100	1547	8.0	10.5	8.7	6.5	29.4	3.3	12.6	19.1	1.9
2	*Chaetomium* sp. (JN709486)	*Sordariales*	100	1256	17.3	20.8	17.1	15.7	3.3	3.1	16.5	4.1	2.1
3	*Preussia terricola* (GQ203725)	*Pleosporales*	99	353	12.2	13.0	15.3	19.0	3.4	4.8	16.4	3.4	12.5
4	*Stachybotrys chartarum* (AF081468)	*Hypocreales*	100	272	9.5	15.8	18.0	15.8	8.5	10.7	9.9	10.7	1.1
5	*Rhizopus oryzae* (KC354517)	*Mucorales*	100	248	29.0	2.4	53.2	10.1	0.0	3.2	0.0	1.6	0.5
6	Uncultured soil fungus (JQ311284)	n.d.	92	244	18.0	23.0	16.8	12.7	5.7	4.9	11.9	2.9	4.1
7	*Chytridiomycete* (EU873019)	n.d.	94	240	6.3	8.3	9.6	53.3	0.8	2.1	18.8	0.0	0.8
8	*Aspergillus terreus* (KF278468)	*Eurotiales*	99	219	21.0	19.2	21.3	15.6	2.7	2.7	14.7	1.4	1.4
9	*Cryptococcus* sp. (DQ531950)	*Tremellales*	99	184	0.0	1.1	0.0	0.0	22.2	43.5	0.0	8.7	24.5
10	Uncultured fungus (KC558360)	n.d.	100	169	1.2	0.6	0.0	26.0	8.9	32.5	8.9	4.1	17.8
11	Uncultured *Podospora* sp. (GU055536)	*Sordariales*	100	159	1.3	0.0	0.6	0.0	0.0	18.9	1.9	18.2	59.1
12	*Cercophora sordarioides* (AY780064)	*Sordariales*	99	157	0.0	0.0	7.0	0.6	1.3	38.2	0.0	9.6	43.3
13	*Coprotus ochraceus* (KC012673)	*Thelebolales*	99	154	0.0	0.0	0.0	0.0	1.3	5.2	0.0	40.9	52.6
14	*Ascobolus* sp. (AY500527)	*Pezizales*	98	149	22.8	16.8	8.7	4.0	1.3	16.1	12.8	2.7	14.8

[1] n.d., not determined

[2] total number of sequences in normalized sample

The most striking changes in this community associated with amendment application occurred within Ascomycota since this phylum dominated fungal diversity (Fig. A2 in Appendix 1). In this way, the class *Sordariomycetes* responded positively to both amendments application at 30 and 60 days, although the increase was more remarkable with DOR (Fig. 7A). This increment in the case of DOR at 30 and 60 days was due to an increase in the relative abundance of *Hypocreales* (Fig. 7B) [this increment was likely related to OTU 1 (*Fusarium* sp.) (Table 5)]. Curiously, this group decreased with CORDOR at 30 and 60 days (Fig. 7B). However, in these treatments, *Sordariales* increased, probably because of a proliferation of genera such as *Podospora* sp. (OTU 11) or *Cercophora* sp. (OTU12), although, within this group, CORDOR led a reduction of *Chaetomium* sp. (OTU 2) (Table 5). Likewise, DOR also determined a reduction of this fungal group at 30 and 60 days. On the other hand, the *Eurotiales* order (*Eurotiomycetes* class) also decreased with both amendments at 60 days (Fig. 7B), these alterations, could be attributed to a diminution of *Aspergillus terreus* (OTU 8), among others (Table 5). Within *Dothideomycetes* class, it highlighted the decrease of *Pleosporales* [OTU 3 (*Preussia terricola*)] in the amended samples at 30 and 60 days. Regarding *Pezizomycetes* (*Pezizales* order), OTU 14 (*Ascobolus*) increased with CORDOR, especially at 60 days (Table 5). Finally, it is interesting to note that OTU 9 [identified as *Cryptococcus* sp. (Basidiomycota)] and OTU 13 [identified as *Coprotus ochraceus* (*Leotiomycetes*, Ascomycota)] also responded positively to DOR and CORDOR at 30 and 60 days (Table 5).

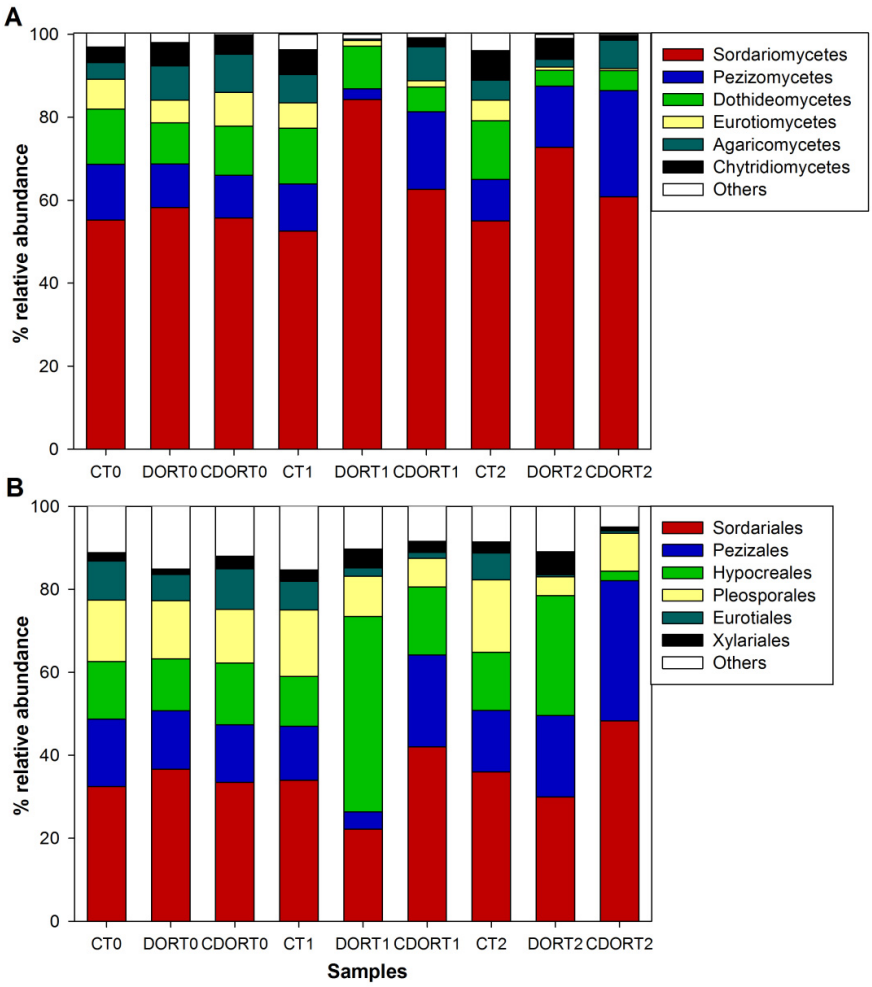

Fig. 7. Relative abundance of the different fungal classes (A) and orders (B) found in unamended soil (C) and soil amended with untransformed DOR (DOR) or *C. floccosa*–transformed DOR (CDOR) at 0 (T0), 30 (T1) and 60 (T2) days.

DISCUSSION

Microbial diversity

Human actions are causing a biodiversity crisis, with species extinction rates up to 1000 times higher than background (Brooks et al., 2006). For this reason, conservation strategies are necessary, especially in vulnerable zones such as Mediterranean biome, which is currently considered one of the most vulnerable of the Earth's thirteen terrestrial biomes (Orgiazzi et al., 2012). In this context, soil biodiversity knowledge is the first step in the development of sustainable activities.

The results demonstrated that this soil microbial community is greatly diverse with high values for richness estimators in each sample. This was also supported by

the high proportion of unclassified sequences at phylum level in spite of using 50% threshold for reads classification (Rachid et al., 2013). The phylum composition of bacterial community in this soil was consistent with previous pyrosequencing studies from Iberian Peninsula soils (Yuste et al., 2012; Bastida et al., 2013; Curiel Yuste et al., 2014). Although, our study presented a higher proportion of Acidobacteria, especially *Gp6* class, than aforementioned studies. This fact could be related to the high alkaline soil pH (≈ 8.4) of the present study (Rousk et al., 2010). Likewise, it was possible to find a higher relative abundance of *Gemmatimonadetes* (8.63%) than the proportion reported as normal in other soils (Janssen, 2006), which could be due to the xeric conditions of Mediterranean basin soils (DeBruyn et al., 2011). On the other hand, and not surprisingly, the present results of bacterial diversity differed substantially from the data obtained for the present experiment by means of culture-dependent techniques. To carry out the culture-dependent study, 900 strains were isolates, grouped by their fatty acid methyl ester profile and groups of isolates were identified by partial sequencing of 16S rRNA gene (Siles et al., 2014b). Culturable bacterial diversity was distributed between: Actinobacteria (50.6 %), Proteobacteria (40.4%), Firmicutes (4.5%) and Bacteroidetes (4.4%). Other studies also have shown that important differences in the bacterial diversity of an environment are obtained when culture–dependent and –independent studies are performed at the same time (Zhang et al., 2009; Shade et al., 2012; VanInsberghe et al., 2013). These discrepancies are logical as it is difficult to obtain culturable members of some bacterial groups. In this sense, it is interesting to note that although Acidobacteria were very abundant in this soil and VL 70 medium was used, which has demonstrated its efficacy for Acidobacteria isolation (Sait et al., 2002), it was not possible to obtain any strain belonged to this phylum. The absence of culturable Acidobacteria in this soil may be due to the slow growing of these bacteria or because their colony development was inhibited by other culturable bacteria (Zhang et al., 2009). It is also worth noting that Actinobacteria was the most common culturable group, while pyrosequencing data showed that Proteobacteria dominated bacterial diversity in this soil. This bias may be consequence of the copiotrophic lifestyle of some groups of Actinobacteria (Ramirez et al., 2012). Pyrosequencing data showed that genera as *Microvirga*, *Nocardioides* or *Rhizobium* were among the most abundant in this Mediterranean soil and interestingly numerous isolates belonged to these genera were found in the previously culture-dependent study (Siles et al., 2014b). In this sese, VanInsberghe et al. (VanInsberghe et al., 2013), in a study where bacterial

diversity was assessed by means of culture-dependent and pyrosequencing techniques, expressed the opinion that the combination of both approaches may be useful since the isolates obtained can be used for genomic and physiological investigations.

Ascomycota clearly dominated fungal diversity of the studied soil; this finding is in concordance with other pyrosequencing Mediterranean soil studies (Orgiazzi et al., 2012; Bastida et al., 2013; Orgiazzi et al., 2013). Likewise, these surveys also demonstrated that Basidiomycota and Chytridiomycota may also be found in these environments, although in a lower concentration than Ascomycota. It is noteworthy the low presence of Glomeromycota (phylum related to arbuscular mycorrhiza) in the present study, which may be related to the absence of plants in the plots when the soil was collected. The fungal pyrosequencing-based diversity found in the present survey was consistent with the diversity obtained by culturable-dependent techniques of the same soil. In this culture-dependent survey, 1,733 strains were obtained and characterized by morphological and molecular techniques (Siles et al., 2014a), being the majority of isolates distributed among three classes: *Sordariomycetes*, *Eurotiomycetes* and *Dothideomycetes* (Siles et al., 2014a). Likewise, it is interesting to note that some of the most abundant fungal species obtained by pyrosequencing (*Chaetomium, Fusarium, Aspergillus*, *Alternatia* or *Cladosporium*) also were the most common culturable fungi (Siles et al., 2014a). These findings are logical as soil fungi are dominated by saprobic filamentous forms, which are readily culturable (Bills et al., 2004). Similarly, Klaubauf et al. 2010) defended that correlation between data from molecular-based techniques and culture-dependent techniques is better in soil fungal communities than in bacterial communities.

Effects of DOR and CORDOR on soil microbial communities

The present study has assessed the impact of DOR and CORDOR as organic amendments on soil microbial community. However, it is necessary to remark that raw DOR application is not possible since it produces oxidative stress in plants and presents a marked phytotoxic activity due to its high phenol content and other substances such as fatty acids (García-Sánchez et al., 2012). In the present greenhouse experiment, the DOR phytotoxicity at agronomic rates (150 Mg ha^{-1}) was confirmed; DOR produced a drastic reduction of shoot dry weight of sorghum plants, while CORDOR ameliorated this toxicity (Siles et al., 2014b). These results were also confirmed in a sorghum field-based experiment amended with both amendments

(unpublished data). Other studies have reported that raw DOR application to olive groves did not produce a diminution of olives production at long-term, however low doses of DOR were applied in this experiment (27 and 54 Mg ha^{-1}) (López-Piñeiro et al., 2011). However, other works have shown that olive oil wastewater (OMW), a liquid residue obtained from three-phase olive-mill extraction system with a similar composition that DOR (produced in other Mediterranean countries as Italy or Greece), can produce the death of olive groves at high rates (800 m^3 ha^{-1}) and negatively influence olive oil quality (Gioffré et al., 2004; Mechri et al., 2009). Thus, phytotoxic activity of DOR depends on doses applied, being the seasonal crops especially susceptible to these effects.

Previous works have assessed the effects of raw DOR and transformed DOR as well as OMW on soil microbiology using DGGE, PLFA or colony forming units (CFU) counts (Mechri et al., 2007; Ipsilantis et al., 2009; Karpouzas et al., 2009; Sampedro et al., 2009b; Magdich et al., 2012). Thus, the knowledge about the changes produced by DOR and OMW (both, hereinafter, olive mill wastes) on phylogenetic composition of microbial communities is limited, although these studies have demonstrated that olive mill wastes has a beneficial effect on microbial abundance. Previous studies have determined by culture-independent techniques that DOR produced a quick and striking increment on bacterial and fungal abundance in the soil of the present study after 30 and 60 days of amendment, while the increases mediated by CORDOR were slower and moderate (Siles et al., 2014b; Siles et al., 2014c). Therefore, although microtoxic effects have been related to raw olive mill wastes (Medina et al., 2011; Justino et al., 2012), it was not possible to detect these effects in terms of abundance in an environment as diverse and complex as soil. Probably, because the beneficial results of these wastes on determined microbial groups due to input of easily degradable compounds likely masked the potential microtoxicity. In this sense, it has been previously established that the impact of olive wastes on soil microbiology is the result of complex, sometimes opposite effects, depending on the relative amounts of beneficial and toxic organic and inorganic compounds added (Mechri et al., 2009; Piotrowska et al., 2011).

The CLPPs analyses showed functional diversity diminution (S_f and H_f) and changes in functional structure of microbial communities after amendments application, especially with CORDOR at 30 days. Some studies using Biolog system have indicated increases or no changes in functional diversity after amendment

application (Frąc et al., 2012), while others showed a diminution of this diversity (Bastida et al., 2013). These discrepancies between studies probably are due to differences between the kind of organic amendments used. In the present study, the high functional diversity of the soil studied may be considered normal since it was an agronomical soil (Montes-Borrego et al., 2013). The subsequent diversity diminution after amendments application may be attributed to the adaptation and selective proliferation of determined microorganisms at the expenses of added nutrients as functional diversity was negatively correlated with total organic C ($R_{pearson}$ -0.814, $P < 0.05$) and total N ($R_{pearson}$ -0.729, $P < 0.05$). The different functional community structure found in the soil amended with CORDOR at 30 days with respect to the other amended samples may be a consequence of the different sources added, since in this treatment it highlighted the oxidation of C sources containing N (amino acids and amines/amides). The ability of some saprobic fungi to increase N content in DOR during bioremediation has been previously reported (Sampedro et al., 2007).

Although DOR and CORDOR produced some changes in phylogenetic microbial diversity with respect to the unamended samples (DOR reduced bacterial diversity at 30 days and CORDOR increased fungal diversity at 30 and 60 days), these alterations were not dramatic. In this way, it is possible to conclude that the most relevant effects of amendments on soil microbiology are related to alterations in community structure. The same conclusions were reported in the culture-dependent studies of this experiment (Siles et al., 2014a; Siles et al., 2014b). In the case of bacterial community, the most evident change mediated by amendments was an increment of Proteobacteria, being this rise more remarkable with DOR at 30 days. The detailed analysis of this phylum demonstrated that the highest changes occurred within *Alphaproteobacteria* class. *Rhodospirillales* decreased with DOR and CORDOR at 30 and 60 days, in fact, it was found a negative correlation between organic C and relative abundance of *Rhodospirillales* ($R_{pearson}$ -0.953, $P < 0.001$), probably because this group of bacteria is adapted to oligotrophic nutritional conditions (King et al., 2010). On the contrary, *Rhizobiales*, *Caulobacterales* and *Sphingomonadales* increased with DOR and CORDOR at 30 and 60 days due to their saprophitic lifestyle and their capacity to degrade recalcitrant compounds, even phenols (Kolvenbach and Corvini, 2012; Mahmoudi et al., 2013). A positive correlation was found between the relative abundance of these groups and total soil organic C ($R_{pearson}$ 0.945, $P < 0.001$). It is remarkable the increase of *Rhizobiales* with both amendments, as this group includes

species related to nitrogen fixation or plant growth promotion (Lin et al., 2013), which may be a beneficial aspect for soil application of this kind of amendments. On the other hand, Acidobacteria have been identified as oligotrophic bacteria (Fierer et al., 2005) and alkaline soil inhabitants (especially subgroups 5, 6 and 7) (Rousk et al., 2010). Thus, the nutrient increments and pH decrease experimented by soil after DOR and CORDOR applications could explain the diminution of relative abundance in Acidobacteria. On the contrary that aforementioned bacterial groups, there are previous works assessing the impact of olive mill wastes on Actinobacteria. Some of these studies reported that OMW has a positive effect on Actinobacteria at short-term (Mekki et al., 2006; Mechri et al., 2007). Instead, Karpouzas et al. (Karpouzas et al., 2010) defended that OMW is responsible for dramatic alterations of this community by means of DGGE. According to these results, Siles et al. (Siles et al., 2014b) determined that culturable Actinobacteria responded differently to DOR and CORDOR depending on the phylogenetic group considered. In the present study, the data obtained about the alterations of this community mediated by amendments were limited, except for members of *Propionibacterinae* suborder, which decreased DOR and CORDOR at 30 and 60 days. Other important finding found by Siles et al. (Siles et al., 2014b) in the culturable-dependent approach of this study was that *Streptomyces* spp. were negatively affected by DOR and the application of CORDOR ameliorated this toxic impact. In the present study, it was not possible to obtain conclusive results about this bacterial group as the number of sequences obtained belonging to this group was very low. In this sense, these results support recent surveys that have defended the culture-dependent approaches to assess the rare biosphere (Shade et al., 2012).

In fungal community, it was interesting the result obtained with respect to *Fusarium* spp., whose relative abundance increased with DOR. Siles et al., 2014a also reported an increase of culturable *Fusarium* spp. after DOR application. This result is logical since *Fusarium* spp. have been related to lignocellulosic wastes due to their saprophitic lifestyle (De Gannes et al., 2013). This may be an inconvenient for the raw application of this residue, because it may suppose a problem for crops development since some species of *Fusarium* have been recognized as potential phytopathogens (Doohan et al., 2003). Instead, CORDOR application led to decreasing this fungal group. In the same way, previous works have demonstrated the suppressive effect that composted OMW have on determined fungal phytopathogen species (Aviani et al., 2010). It was also detected that *Aspergillus terreus* decreased and *Cryptoccocus* sp.

increased after amendments application, which is in concordance with the culture-dependent study (Siles et al., 2014a). Curiously, Karpouzas et al., 2009 found an increment of this yeast after OMW soil application, which was explained as result of the ability of these microorganisms to metabolize a high variety of substrates. On the other hand, it was striking the reduction of *Chaetomium* spp. relative abundance in this study with both amendments at 30 and 60 days as these fungi are saprobic fungi and potential degraders of cellulosic material (Soytong et al., 2001). This fact could be explained by the high sensibility of the members to this group to phenols (Asiegbu et al., 1996). In this sense, a negative correlation was found between soil phenol content and the *Chaetomium* sp. sequence number ($R_{pearson}$ -0.759, $P < 0.05$). However, CORDOR presented a lower phenol content due to the transformation by *C. floccosa* (Siles et al, submitted for publication). Thus, other possible explanation for *Chaetomium* sp. decreasing in amended samples is that other microbial group favoured by inputs was able to inhibit its multiplication (Zaccardelli et al., 2013). On the contrary, other fungal groups as *Podospora* sp. and *Cercophora* sp., which have been recognized as coprophilous fungi (Chang et al., 2010), increased at 30 and 60 days with CORDOR, supporting the hypothesis that olive mill wastes impact on soil microbiology is the result of opposite effects. These fungal groups have been previously related to lignocellulosic material (Bonito et al., 2010; Souza et al., 2013).

In conclusion, this work has showed that microbial diversity from this Mediterranean soil is huge and future studies are necessary to completely elucidate it. The application of DOR and CORDOR resulted in functional diversity diminution and functional community structure modifications according to the kind of treatment since they provided different type of C sources for microorganisms. DOR besides its phytotoxicity, was shown more disruptive than CORDOR for bacterial and fungal communities as the effects of olive mill wastes on soil microbial communities depends on the relative amounts of beneficial or inhibiting components added, which alter nutrients and toxic substances levels as well as chemical soil properties Although a direct link cannot be established, the bacterial (*Rhizobiales*, *Caulobacterales* and *Sphingomonadales*), and fungal (*Fusarium* sp., *Cryptococcus* sp., *Podospora* sp. and *Cercophora* sp.) groups more favoured by amendments probably were responsible for changes in soil functionality.

Acknowledgements

This study has been funded by the Spanish Ministry of Science and Innovation (Project AGL2008–572). J.A. Siles and I. Sampedro gratefully acknowledge assistance from the JAE program, which is co-financed by the Consejo Superior de Investigaciones Científicas (CSIC) and the European Social Fund.

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IV. GLOBAL DISCUSSION

The olive oil extraction industry generates huge amounts of wastes with environmental risks. Among them, it highlights the dry olive residue (DOR), resulting from a two-phase olive-oil extraction system (Alburquerque et al., 2004). The development of viable strategies for the recycling and revalorization of this by-product is currently a challenge for this industry. One of the ways proposed for DOR revalorization is its use as organic amendment since it presents a high content in organic matter and agronomically important cations. Likewise, it is free of heavy metals and pathogens, unlike other organic amendments (López-Piñeiro et al., 2011). However, a pretreatment of this waste is necessary before direct soil application as it presents phytotoxic activity, has an acidic pH and may alter soil properties as well as organic matter transformation rate due to its high C/N ratio (Dermeche et al., 2013; Ntougias et al., 2013). Our research group, in the last few years, has developed a method to obtain an organic amendment with appropriate characteristics from DOR, which is based on the waste transformation by saprobic fungi. The process reduces phenol content, improves humification and C/N rates as well as increases residue's pH (Sampedro et al., 2007b). The use of immobilized fungal inocula, in the transformation process, has significantly enhanced the method, shortening transformation time and ensuring the procurement of a viable amendment in short period of time (Sampedro et al., 2009).

The integrated assessment of the amendment impact on soil must take into account the behaviour of soil microbial communities as they are the mainstay of this ecosystem. Thus, the main objective of this PhD dissertation was to investigate the effect of a fungus-transformed DOR used as amendment on soil microbiology. On the other hand, although Mediterranean basin is considered one of the 25 most important biodiversity hotspots on Earth (Mittermeier et al., 2005; Cañadas et al., 2014), there is lack of information about the microbial diversity from soils of this region. For this reason, a second objective of the present PhD Thesis was to describe the bacterial and fungal diversity of the soil used in the study, which represents a typical Mediterranean soil. The changes in soil bacterial and fungal communities mediated by the different amendments were measured by a quantitative, phylogenetic and functional point of view through several methodological approaches, while the microbial phylogenetic information generated was employed for microbial diversity description. To achieve the objectives proposed in this dissertation, a greenhouse experiment consisting in unamended soil, soil amended with raw DOR, soil amended with DOR transformed by

Coriolopsis floccosa (basiomycete ligninolytic fungus) (CORDOR) and soil amended with DOR transformed by *Fusarium oxysporum* (ascomycete soil fungus) (FUSDOR) was performed, where sampling times were 0, 30 and 60 days of treatment. Two different transformed amendments were tested because we wanted to know if CORDOR and FUSDOR would produce different impacts on soil properties. The multivariate analyses in the chapters 1 and 2 demonstrated that both amendments produced a similar impact on bacterial and fungal communities. In this sense, only CORDOR as transformed amendment was selected in the next chapters to carry out the culture-dependent and pyrosequencing surveys in order to simplify the studies. CORDOR was selected since *C. floccosa* was able to produce a higher transformation on DOR than *F. oxysporum*. The amendments were applied at doses of 5 % (w/w), which is equivalent to an agronomic rate of 150 Mg ha^{-1}, in line with previous reports where doses ranging from 27 Mg ha^{-1} to 270 Mg ha^{-1} have been applied (Lopez-Piñeiro et al., 2008; Lozano-García et al., 2011). The soil used for experiment (from Granada province) presented a low organic matter content (≈10 g kg^{-1} of total organic carbon), which is typical of Mediterranean agricultural soils (Lozano-García and Parras-Alcántara, 2013). Thus, the soil object of study was a potential candidate as organic amendment receptor. The sorghum was selected as model plant in this study since it has shown a higher resistance to phytotoxic activity of DOR and because we wanted to test the effect the effect of amendments on seasonal crops.

Our study was focused at short-term since it has been argued that the changes that occur in soil at the first weeks after organic amendments application can influence long-term plant nutrient supply and sustainability of agricultural systems (Tatti et al., 2013). In this context, it is interesting to note the results obtained in the present study for initial sampling time (0 day). The soil employed to measure enzymatic activities and to analyze culturable microbial communities was directly used without freezing. In these cases, soil samples were stored during 1-2 days at 4 ^{o}C and it was expected that the amendments did not produce effect on soil microbiology in this period of time. However, interesting and unexpected results were obtained. The data showed that urease and dehydrogenase activities changed significantly in the amended samples with respect to the unamended soil in this short period of time. Likewise, it was confirmed that DOR and CORDOR also produced dramatic changes on phylogenetic composition of cultivable fungal and bacterial communities, in addition to an increase on bacterial community size in the soil amended with DOR with respect to the

unamended sample after only 2 days. This fact evidenced the high sensitivity of soil microbial communities to external inputs as they were able to generate a response only after some hours, even at low temperature. These results could not be corroborated in the culture-independents techniques since the soil samples for these studies were quickly frozen and no changes between treatments were observed. Nevertheless, Zelenev et al. (2005) also observed a 2-day response of soil bacteria community after fresh organic matter incorporation.

It has been well established that DOR produce negative effects on plant growth (Morillo et al., 2009). Several "in vitro" assays have demonstrated that DOR and olive mill wastewater (OMW, residue from three-phase olive-oil extraction system) are able to produce suppression of seed germination in numerous plant species even at low dilutions (Casa et al., 2003; Sampedro et al., 2004). Likewise, other experiments have also shown that these raw wastes are responsible for inhibition of plant growth in seasonal crops and young plants of olives (Ben Rouina et al., 1999; Barbera et al., 2013). Likewise, the raw application of OMW to an olive grove can alter physiological status of olives, quality of olive oil (Mechri et al., 2009) and even olives trees death at very high doses (Gioffré et al., 2004). However, López-Piñeiro et al. (2011) did not observe harmful effects to olives after raw DOR application at low agronomical doses. In this sense, as it has been mentioned by other authors, the phytotoxic activity of olive mill wastes depends on doses of the by-product applied since this activity has been positively correlated to phenol concentration (Ben Sassi et al., 2006). However, it still remains unclear if the phytotoxic activity is carried out by the action of a single phenol or the joint action of several single phenols (Capasso et al., 1992). Likewise, other authors have pointed that fatty acids present in the residue also may be responsible for the phytotoxicity (Saadi et al., 2007). In the present study, DOR produced a diminution of sorghum growth, while no changes were detected in the plants of the samples amended with CORDOR and FUSDOR, probably due to removal of phenolic compounds. The phytotoxicity decrease of DOR by the transformation of *C. floccosa* and *F. oxysporum* has been related to the fungal transformation of residue by oxidoreductases (laccases in the case of *C. floccosa* while Mn-peroxidase and Mn-independent peroxidase activities in *F. oxysporum*), which produce enzymatic oxidation and polymerization processes of simple phenols (Aranda et al., 2006; Sampedro et al., 2007a). As a result, high-molecular mass compounds are originated which cannot pass through the cell membranes of plants (Hulzebos et al., 1991; Reina et al., 2013).

In our studies, it was observed that the application of CORDOR and FUSDOR as amendments produced changes in soil pH, phenol content and electrical conductivity immediately after application. Nevertheless, these alterations, in general, were not significant with respect to the unamended soil at the successive sampling times. Instead, DOR significantly decreased pH and raised phenol content over all the time period. On the other hand, the different amendments increased organic C content and C/N rates in the soil as they contained high levels of organic matter. The amount of organic carbon in the amended soils (C_{org} and WSOC) quickly decreased over the time. This fact is in concordance with previous studies that have suggested that organic matter from olive wastes when is added to soil suffers a primary phase in which the decomposable fraction is rapidly degraded and a secondary phase in which the more stable fraction is slower degraded (Barbera et al., 2013). Contradictory information has been reported by previous studies about the effect of raw olive wastes on soil N content since some of them have reported increments while others demonstrated non-changes or decreasing of this element (Arvanitoyannis and Kassaveti, 2007). It has been well established that one limiting factor for direct DOR application to soil (besides of high phenols content) is its low N content, which increases C/N relation in soil that may affect organic matter mineralization rate (Sierra et al., 2007; Karpouzas et al., 2009). In the present study, the DOR incubation with the fungi produced an increment of N in CORDOR and FUSDOR as well as a reduction of C/N ratio. Subsequently, these amendments did not produce significant rises of soil N content. On the other hand, previous studies have demonstrated that the soil amendment with DOR enrich soil with essential minerals for plant growth (López-Piñeiro et al., 2006). In our results, the incorporation of the different amendments only significantly increased the levels of K in soil. These discrepancies could be explained by the high variability in chemical composition of olive wastes depending on olive tree variety, olive fruit's maturity, harvesting time and geographical region (Stamatakis, 2010)

As mentioned above, one of the determinations addressed in our study was the soil microbial abundance measurement after different amendments addition. The changes produced in bacterial abundance were assessed by means of qPCR, PLFA as well as CFU and viable cell counts. In general, a good correlation between the results from the different techniques was obtained. The data demonstrated that all the amendments produced bacterial proliferation, being these increments more evident in the soil with DOR over the time, probably due to the growth of r-strategist bacteria at

the expenses of easily decomposable organic matter from amendments (Kotsou et al., 2004), which masked the potential microtoxic effects related to raw DOR which have been brought to light in phylogenetic studies. The lower bacterial multiplication in the soils treated with CORDOR and FUSDOR with respect to DOR can be probably associated with a lower availability of easily decomposable substances. The decreasing of PLFA stress indicators cy/pre and S/M also indicated that bacterial growth was benefited by amendments as high levels of these indices have been linked to low concentration of nutrients (Moore-Kucera and Dick, 2008). On the other hand, within bacterial community, it was possible to know the behaviour of actinobacteria abundance under the different amendments used by qPCR and PLFA. Both approaches demonstrated that CORDOR and FUSDOR only produced actinomycetal proliferation at 60 days, while DOR produced proliferation at 30 days and no changes at final sampling time. These changes could be related to the degree of transformation of organic matter in the different amendments and the availability of nutrients for members from this bacterial phylum.

The results about fungal community size determined by PLFA and qPCR of 18S rRNA gene differed between both techniques since PLFA detected significant increments in all the amended samples over the time while qPCR only measured rises in the number of 18S rRNA gene copy number in the soil with DOR at 30 and 60 days as well as the sample with CORDOR at 60 days. In a recent study, Baldrian et al. (2013) assessed fungal biomass in a forest soil using PLFA and qPCR of ITS and they concluded that PLFA is more reliable than qPCR since this technique may under- or overestimate fungal biomass because of the biases related to soil DNA extraction, the high variability in the number of ribosomal genes between microorganisms or PCR efficiency. However, fungal biomass determination by PLFA have also demonstrated some limitation since the fatty acid 18:2ω6,9 is not exclusive for fungi in soil. For this reason, according to Drenovsky et al. (2008), the best form to obtain a credible estimation of microbial biomass in soil is through the joint of several methodologies.

In addition to the analysis of microbial communities' size in the different samples, the phylogenetic composition of bacterial and fungal communities and its changes after DOR, CORDOR and FUSDOR application over the time also focused much of our attention in this PhD dissertation. In fact, several techniques were used to carry out this purpose. The 16 rRNA gene-DGGE analysis showed a complex bacterial community with a high number of bands, many of them with large densities. The

multidimensional scaling of this DGGE did not reveal clearly the effects of DOR, CORDOR and FUSDOR on bacteria, especially at 30 days, as all the samples were grouped together. Instead, PLFA, culturable-dependent and pyrosequencing data showed a clear impact of amendments on bacterial structure, especially in the case of DOR. Therefore, DGGE did not show as a technique with a good resolution, at least with the conditions of the present study. This fact could be explained by the high bacterial community complexity of the studied soil and the primers selected for amplification, which produced too complex fingerprintings that probably did not show clearly the changes between treatments. These findings are in concordance with previous studies that have reported several limitations associated with the use of this technique in complex environments such as soil (Kirk et al., 2004). DGGE was also used for actinomycetal and fungal communities. In these cases, this technique was somewhat more effective showing the impact of DOR, CORDOR and FUSDOR on these communities. From fungal 18S rRNA-DGGE, we selected some bands of soil amended with CORDOR at 60 days as it was observed a slight increment of fungal diversity in this treatment to obtain a phylogenetic approach of the most interesting bands. The process was complex, high-time consuming and the information obtained was limited.

In order to know the phylogenetic microbial composition of the Mediterranean soil used in our experiment and its changes after amendments application, we carried out the characterization of culturable bacterial and fungal communities. This type of studies involves a great effort, are time-consuming and there are authors that considerer culture-dependent approaches non-useful to fully characterized soil microbial communities (Ritz, 2007). However, these studies are an interesting way to improve lab skills in multiple disciplines, to obtain a high number of isolates which can be used for multiple purposes and because the realization of culturable and molecular-based studies in parallel can be complementary (VanInsberghe et al., 2013). VL 70 medium formulated with low concentrations of inorganic ions and containing D-xylose as well as gellan as growth substrate and solidifier, respectively, was selected. This medium was chosen as its efficacy for the isolation of previously uncultured bacteria has been previously demonstrated (Davis et al., 2005; Pham and Kim, 2012). The culturable bacteria diversity was distributed between *Actinobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria*, *Betaproteobacteria*, *Bacilli*, *Sphingobacteria* and *Cytophagia*, in line with other culturable surveys from Mediterranean soils (Çakmakçı et al., 2010;

Sánchez-Hidalgo et al., 2012). Nevertheless, this culturable diversity differed significantly from the pyrosequencing-based diversity, which was excepted since it has been well documented the "great plate count anomaly" associated with culture-dependent studies (Alain and Querellou, 2009). On the other hand, it was also demonstrated that CORDOR did not produce changes on culturable bacterial diversity except for initial sampling time. Likewise, it was demonstrated that the impact produced by CORDOR on bacterial community structure was less evident that DOR, except for the initial sampling time, when both amendments altered significantly the structure of bacterial community as they determined the proliferation of bacteria belonged to *Arthrobacter* sp.

The fungal community was also analyzed by culture-dependent approach. The isolation of fungi was done using particle-filtration method and long periods of incubation at alternating temperatures to avoid biases towards mesophilic and fast-growing species from conidia (Bills et al., 2004). The culturable fungi from this soil were distributed between *Hyprocreales*, *Eurotiales*, *Capnodiales*, *Sordariales* and *Pleosporales*, among others. Surprisingly, a reasonable good correlation was obtained with data of pyrosequencing-based study. This fact was related to the dominance of saprobic filamentous fungi in soil which can be growth in artificial culture medium (Gams, 2006). CORDOR did not produce significant changes on fungal diversity, on the contrary that DOR, which decreased fungal diversity at 30 days of treatment. However, it was concluded that the most striking effects of raw or transformed DOR on fungal community are not on diversity, but on structure. In this way, CORDOR produced the highest alteration on fungal community structure at initial sampling time while DOR altered the fungal species distribution at all the sampling times. The alteration of fungal community was related to the increasing in the relative abundance of potential phytopathogenic species as *Cladosporioides* sp., *Fusarium* sp. or *Alternaria* sp., which concurs with previous culture-dependent studies using other organic amendments (Shaukat et al., 2003).

The currently high-throughput sequencing techniques have revolutionized our capacity to discover and understand an environment. Due to the large number of sequences provided by these techniques, a very good resolution of the microbial diversity can be obtained, which is unprecedented when comparing with previous techniques (Poulsen et al., 2013). In this sense, this PhD dissertation supposes an in-depth analysis of the microbial community composition from a Mediterranean soil. To

the best our knowledge, this work is the first studying bacterial and fungal diversity from an Andalusian soil using next-generation sequencing techniques. To carry out the study, the 16S rRNA and 28S rRNA genes were selected for bacterial and fungal pyrosequencing analyses, respectively. The results reflected, as expected, that microbial diversity from this soil is immense and that future studies are necessary to fully characterized it. Regarding bacterial community, this was dominated by *Alphaproteobacteria*, *Gp6* (Acidobacteria), *Actinobacteria*, *Gemmatimonadetes*, *Gammaproteobacteria* and *Deltaproteobacteria*. The analyses about the effect of the amendments on this community demonstrated that diversity was not affected by CORDOR. However, DOR produced a diminution of the bacterial diversity after 30 days respect to unamended samples. The PCA (principal components analysis) and pairwise unweighted UniFrac test demonstrated that bacterial structure significantly changed after both amendments application, being DOR at 30 days the most disruptive treatment. The changes in the bacterial structure were determined by proliferation or decreasing of determined bacterial groups belonged to *Alphaproteobacteria*, Acidobacteria and Actinobacteria. In the case of fungi, the fungal diversity was dominated by *Sordariomycetes*, *Pezizomycetes*, *Dothideomycetes* and *Chytridiomycetes*. The soil amendment with CORDOR increased the fungal diversity at 30 and 60 days and no changes were appreciated in the treatments with DOR. In this case, PCA showed clearly that DOR and CORDOR altered fungal community structure in different ways and pairwise unweighted UniFrac demonstrated that the changes were significant. These alterations were determined by increments or declines in the relative abundance of different fungal groups, mainly belonged to *Sordariomycetes* and *Pezizomycetes*. Curiously, the pyrosequencing-based data also demonstrated, as culture-dependent study, that DOR benefited the proliferation of potential phytopathogenic fungi such as *Fusarium* sp. In brief, the different approaches have demonstrated that the clearest effect of DOR and CORDOR on bacterial and fungal communities is related to alterations of their structure. These changes in the relative abundance of the different phylogenetic microbial groups were probably due to the changes in nutritional status of soil which differently affected the diverse bacterial and fungal groups according to their lifestyles and their capacity to grow at the expenses of substances added with amendments. Likewise, potential microtoxic components in DOR such as phenols also could be responsible for the decreasing of determined microbial groups. As DOR contains higher levels of unstabilized organic matter and potential microtoxic

compounds, this waste was able to produce a higher impact on soil microbiology than CORDOR. These findings are in concordance with previous surveys which have highlighted that the effect of olive wastes on soil are the result of complex, sometimes conflicting effects, depending on the relative amounts of beneficial organic, toxic organic and inorganic compounds/ions present (Piotrowska et al., 2006; Mechri et al., 2007).

The possibility that soil amendment produced changes on soil microbial functionality was also studied. The current challenge for soil microbiology is the linking between phylogeny and functionality. The most abundant microorganisms cannot be the most active functionally and a change in microbial community structure does not always involve a change in microbial community function (Nannipieri, 2003). In the present study, the modifications in the structure of microbial communities were in parallel with changes in soil functionality according to Biolog Ecoplate System. DOR and CORDOR decreased functional diversity based on community level physiological profile (CLPP) for each sample. Likewise, PCA showed that functional structure of microbial community also changed according to the kind of amendment. These changes were related to the incorporation of nutrients to soil by DOR and CORDOR, but the form which these nutrients were present also influenced the changes in functional community. In this sense, it was observed that soil amended with CORDOR at 30 days presented a microbial functional structure different that soil with DOR at 30 and 60 days and soil with CORDOR at 60 days. According to PCA, these changes were determined by a higher oxidation of C sources containing N. The presence of these compounds with N may be related to the incubation of DOR by *C. floccosa*. In fact, CORDOR presented a higher concentration of N than DOR. On the other hand, it was also possible to check that the amendments influenced soil enzymatic activities. They are catalysts in different reactions during carbon and nutrient cycling in soil, represent the metabolic level of the soil microbial community and determine the release and availability of nutrients in soil (Alburquerque et al., 2012; Cardoso et al., 2013). It is necessary to remark that we really determined in the present work potential enzymatic activities as for enzymatic determinations, a substrate that forms a colored compound was added to a dilute homogenized soil slurry and the increase of absorbance over a fixed incubation time in the different treatments was determined (Wallenstein and Weintraub, 2008). All the potential enzymatic activities determined, except urease, were increased by CORDOR and FUSDOR amendment. This fact can be considered as

normal since amendments increased inputs of organic substrates which stimulate microbial growth and enzyme synthesis (Franco-Otero et al., 2012). In this way, soil enzyme activity is known to be positively correlated to the organic matter content of the soil. In the case of DOR, interesting results were obtained as although the incorporation of DOR supposed an increment of organic matter in soil, the enzymes phosphatase, β-glucosidase, and urease did not increase or decreased with this amendment. This fact was related to the presence of inhibitory compounds in the raw DOR. This finding concurs with previous studies where phenols were described as the main negative compounds of olive wastes for enzymatic activities (Piotrowska et al., 2011; Ntougias et al., 2013). Finally, it is interesting to note the results obtained for dehydrogenase activity. This intracellular enzyme, that is able to oxide organic matter, decreased in the amended samples at 30 and 60 days with respect to the values obtained at initial sampling time while microbial abundance increased at the same times. These findings support the idea that the microorganisms were more metabolically active at initial sampling time.

This PhD dissertation represents and in-depth study where a great effort has been carried out to characterize the bacterial and fungal community from a Mediterranean soil and their changes after the application of DOR-based amendments using different methodological approaches to obtain more reliable results. In the future, the present survey should be completed with other long-term surveys, investigating the effect of saprobic fungi-transformed DOR on soil chemical and biological properties in field-based experiments, to completely elucidate the viability of this kind of amendments.

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V. CONCLUSIONS

1. Soil amendment with dry olive residue transformed by *Coriolopsis floccosa* or *Fusarium oxysporum*, at agronomic doses, does not produce phytotoxic effects on sorghum plants.

2. The transformed dry olive residue application to soil, minimally alters its chemical properties, doubles its organic matter content, and increases microbial activity.

3. The bacterial and fungal abundance increases after soil amendment with transformed dry olive residue, probably due to the increasing of copiotrophic microorganisms, which grow at expenses of nutrients incorporated with the amendments.

4. The addition of *C. floccosa*-transformed dry olive residue to soil changes the culturable bacterial and fungal community structure in a short period of time.

5. The application of dry olive residue transformed by *C. floccosa* decreases the functional diversity and alters the functional structure of soil microbial community. These changes are probably related to the specialization of soil microorganisms in the use of C sources added with the amendment.

6. The changes in the phylogenetic diversity of the bacterial and fungal community observed in the studied soil, after saprobic fungi-transformed dry olive residue amendment, differ between the techniques used. However, all the techniques show that the most striking changes produced by these amendments are related to alterations in the microbial phylogenetic structure of soil.

7. The culturable bacterial and fungal community phylogenetic composition of studied soil is similar to other Mediterranean soils.

8. The bacterial and fungal diversity of the studied Mediterranean soil, from the southeast Iberian Peninsula, is huge according to pyrosequencing data.

V. CONCLUSIONES

1. La enmienda de un suelo con alpeorujo transformado por *Coriolopsis floccosa* o *Fusarium oxysporum* a dosis agronómicas no produce efectos fitotóxicos sobre plantas de sorgo.

2. La aplicación de alpeorujo transformado altera mínimamente las propiedades químicas del suelo, duplica su contenido en materia orgánica y estimula la actividad microbiana.

3. La abundancia bacteriana y fúngica aumenta tras la enmienda de los suelos con alpeorujo transformado, probablemente debido a la proliferación de microorganismos copiotróficos que se multiplican a expensas de los nutrientes incorporados con los enmendantes.

4. La incorporación de alpeorujo transformado por *C. floccosa* produce un cambio brusco en la estructura de la comunidad cultivable bacteriana y fúngica en un breve periodo de tiempo.

5. La aplicación de alpeorujo transformado por *C. floccosa* disminuye la diversidad funcional y altera la estructura funcional microbiana del suelo. Estos cambios probablemente están relacionados con la especialización de los microorganismos del suelo en la utilización de las fuentes de carbono añadidas con el enmendante.

6. Los cambios en la diversidad filogenética de la comunidad bacteriana y fúngica observados en el suelo estudiado tras la aplicación de alpeorujo transformado por hongos saprobios difieren según las diferentes técnicas empleadas. Sin embargo, todas ellas muestran que los cambios más destacados producidos por este tipo de enmienda están relacionados con alteraciones de la estructura filogenética microbiana del suelo.

7. La composición taxonómica de la comunidad cultivable bacteriana y fúngica del suelo estudiado es similar a la descrita en otros suelos mediterráneos.

8. Existe una extraordinaria diversidad bacteriana y fúngica en el suelo mediterráneo del sureste de la Península Ibérica examinado de acuerdo con los datos obtenidos mediante pirosecuenciación.

VI. APPENDIX 1

Table A1. Sequences of primers used in the thesis.

Primer	Sequence primer (5′-3′)
1100R	GGGTTGCGCTCGTTG
1401R	CGG TGT GTA CAA GAC CC
18S3	GATGCCCTTAGATGTTCTGGGG
341F-GC	CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCGCCTACGGGAGG CAGCAG
577F[1]	**CGTATCGCCTCCCTCGCGCCATCAG**(barcode)AYTGGGYDTAAAGNG
926R[1]	**CTATGCGCCTTGCCAGCCCGCTCAG**CCGTCAATTCMTTTRAGT
968F-GC	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGA ACCTTAC
Act704R	TCT GCG CAT TTCACC GCTAC
Actino235	CGCGGCCTATCAGCTTGTTG
Eub338	GCTGCCTCCCGTAGGAGT
Eub518	ATTACCGCGGCTGCTGG
FD1	AGAGTTTGATCCTGGCTCAG
FF390	CGATAACGAACGAGACCT
FR1	AICCATTCAATCGGTAIT
FR1-GC	CCCCCGCCGCGCGCGGCGGGCGGGGCGGGGG CACGGGCCGAICCATTCAATCGGTAIT
ITS1F	CTTGGTCATTTAGAGGAAGTAA
LR0R[1]	**CTATGCGCCTTGCCAGCCCGCTCAG**ACCCGCTGAACTTAAGC
LR3[1]	**CGTATCGCCTCCCTCGCGCCATCAG**(barcode)CCGTGTTTCAAGACGGG
NL1	GCATATCAATAAGCGGAGGAAAAG
NL1R	GAAAAGGAGGCGAATAACTATACG
NL4	GGTCCGTGTTTCAAGACGG
T1	AACATGCGTGAGATTGTAAGT
T10	ACGATAGGTTCACCTCCAGAC
T22	TCTGGATGTTGTTGGGAATCC

[1]Sequence primer in bold indicates sequencing adaptors for 454 Roche GS FLX Titanium

Table A2. Distribution of isolates of each sample (1- control soil at 0 d, 2- soil amended with DOR at 0 d, 3- soil amended with DOR transformed by *C. floccosa* at 0 days, 4- control soil at 30 d, 5- soil amended with DOR at 30 d, 6- soil amended with DOR transformed by *C. floccosa* at 30 d, 7- control soil at 60 d, 8- soil amended with DOR at 60 d and 9- soil amended with DOR transformed by *C. floccosa* at 60 d) among the different clusters and single branches obtained after FAMEs analysis and phylogenetic characterization of bacteria selected for partial 16S rRNA sequencing.

Cluster/Branch	Soil Samples									Isolates selected of each cluster for 16S rRNA sequencing					
	1	2	3	4	5	6	7	8	9	Isolate id[b]	Acc nº	Closest relative Eztaxon match (accession number)	16S sim (%)	Phylum	Order
1	2	3	3	2	12	2	0	2	0	5_101	HF954410	*Sphingomonas echinoides* (JH584237)	99.81	Proteobacteria	*Sphingomonadales*
										8_1	HF954478	*Sphingomonas sanxanigenens* (DQ789172)	99.82	Proteobacteria	*Sphingomonadales*
										3_60	HF954390	*Sphingomonas aquatilis* (AF131295)	99.81	Proteobacteria	*Sphingomonadales*
2	0	0	0	0	0	1	0	3	0	8_42	HF954493	*Bosea lathyri* (FR774993)	98.61	Proteobacteria	*Rhizobiales*
										8_60	HF954498	*Rhizobium herbae* (GU565534)	99.70	Proteobacteria	*Rhizobiales*
I	0	0	0	1	0	0	0	0	0	4_53	HF954403	*Caulobacter segnis* (CP002008)	99.02	Proteobacteria	*Caulobacterales*
3	0	0	0	0	2	0	0	0	0	5_29	HF954416	*Sphingomonas sanxanigenens* (DQ789172)	98.82	Proteobacteria	*Sphingomonadales*
4	0	0	0	0	1	0	0	1	0	8_68	HF954501	*Devosia neptuniae* (AF469072)	99.71	Proteobacteria	*Rhizobiales*
5	0	0	0	0	11	0	0	7	0	8_33	HF954491	*Brevundimonas nasdae* (AB071954)	98.98	Proteobacteria	*Caulobacterales*
										5_59	HF954425	*Brevundimonas nasdae* (AB071954)	98.47	Proteobacteria	*Caulobacterales*
										8_65	HF954500	*Brevundimonas nasdae* (AB071954)	98.69	Proteobacteria	*Caulobacterales*
6	0	0	0	0	2	0	0	0	0	5_82	HF954435	*Brevundimonas nasdae* (AB071954)	99.23	Proteobacteria	*Caulobacterales*
7	0	0	0	0	1	0	0	3	0	8_2	HF954486	*Brevundimonas nasdae* (AB071954)	99.44	Proteobacteria	*Caulobacterales*
										8_98	HF954510	*Brevundimonas alba* (AJ227785)	99.72	Proteobacteria	*Caulobacterales*
8	1	0	0	0	3	1	0	3	1	5_41	HF954419	*Rhizobium vignae* (GU128881)	100.00	Proteobacteria	*Rhizobiales*
										8_55	HF954495	*Rhizobium radiobacter* (AJ389904)	99.58	Proteobacteria	*Rhizobiales*
9	1	0	0	0	5	1	3	7	1	7_68	HF954471	*Rhizobium giardinii* (U86344)	99.14	Proteobacteria	*Rhizobiales*
										5_7	HF954429	*Rhizobium cellulosilyticum* (DQ855276)	98.55	Proteobacteria	*Rhizobiales*
										8_19	HF954485	*Rhizobium huautlense* (AF025852)	99.91	Proteobacteria	*Rhizobiales*
										5_69	HF954428	*Paracoccus yeei* (AY014173)	98.62	Proteobacteria	*Rhodobacterales*
										8_38	HF954492	*Rhizobium nepotum* (FR870231)	98.03	Proteobacteria	*Rhizobiales*
10	0	0	0	0	3	1	0	2	2	5_80	HF954434	*Phyllobacterium myrsinacearum* (D12789)	97.36	Proteobacteria	*Rhizobiales*
										8_88	HF954505	*Ensifer adhaerens* (AM181733)	98.90	Proteobacteria	*Rhizobiales*
										6_78	HF954453	*Ensifer adhaerens* (AM181733)	99.80	Proteobacteria	*Rhizobiales*
11	0	0	0	0	0	0	1	3	0	8_97	HF954509	*Ancylobacter oerskovii* (AM778407)	100.00	Proteobacteria	*Rhizobiales*
										8_17	HF954484	*Starkeya novella* (CP002026)	100.00	Proteobacteria	*Rhizobiales*
II	1	0	0	0	0	0	0	0	0	1_32	HF954380	*Enterobacter cloacae* (Z960799)	99.35	Proteobacteria	*Enterobacteriales*

Cluster/Branch	Soil Samples									Isolates selected of each cluster for 16S rRNA sequencing					
	1	2	3	4	5	6	7	8	9	Isolate id[b]	Acc n°	Closest relative Eztaxon match (accession number)	16S sim (%)	Phylum	Order
12	0	0	3	0	0	0	0	0	0	3_64	HF954391	*Acinetobacter lwoffii* (AIEL01000120)	99.20	Proteobacteria	*Pseudomonales*
13	0	0	0	0	1	2	1	1	2	6_85	HF954454	*Devosia neptuniae* (AF469072)	99.62	Proteobacteria	*Rhizobiales*
										9_27	HF954514	*Rhodococcus wratislaviensis* (Z37138)	100.00	Actinobacteria	*Corynebacteriales*
										7_49	HF954466	*Rhodococcus marinonascens* (X80617)	98.57	Actinobacteria	*Corynebacteriales*
III	1	0	0	0	0	0	0	0	0	1_52	HF954382	*Rhodococcus rhodochrous* (X79288)	99.33	Actinobacteria	*Corynebacteriales*
14	0	0	0	0	0	1	0	0	1	6_42	HF954449	*Rhodococcus yunnanensis* (AY602219)	99.25	Actinobacteria	*Corynebacteriales*
15	0	0	0	0	0	2	0	0	0	6_36	HF954445	*Williamsia limnetica* (HQ157192)	98.29	Actinobacteria	*Corynebacteriales*
IV	0	0	0	0	0	0	0	0	1	9_35	HF954516	*Rhodococcus jialingiae* (DQ185597)	99.72	Actinobacteria	*Corynebacteriales*
16	0	0	0	0	0	0	1	3	0	8_78	HF954502	*Mycobacterium frederiksbergense* (AJ276274)	99.79	Actinobacteria	*Corynebacteriales*
										8_3	HF954488	*Mycobacterium parafortuitum* (X93183)	98.32	Actinobacteria	*Corynebacteriales*
17	0	0	0	0	0	0	0	0	2	9_67	HF954525	*Nocardioides alkalitolerans* (AY633969)	99.81	Actinobacteria	*Propionibacteriales*
18	0	0	0	3	0	0	1	0	1	6_96	HF954459	*Duganella zoogloeoides* (D14256)	99.32	Proteobacteria	*Burkholderiales*
										4_18	HF954399	*Massilia suwonensis* (FJ969487)	98.82	Proteobacteria	*Burkholderiales*
V	0	0	0	0	1	0	0	0	0	5_51	HF954422	*Massilia timonae* (U54470)	99.43	Proteobacteria	*Burkholderiales*
19	0	0	0	0	8	2	1	7	0	5_39	HF954418	*Enterobacter ludwigii* (AJ853891)	98.32	Proteobacteria	*Enterobacteriales*
										5_28	HF954415	*Enterobacter ludwigii* (AJ853891)	99.72	Proteobacteria	*Enterobacteriales*
										8_95	HF954508	*Enterobacter ludwigii* (AJ853891)	99.44	Proteobacteria	*Enterobacteriales*
										5_56	HF954424	*Hydrogenophaga palleronii* (AF019073)	99.82	Proteobacteria	*Burkholderiales*
										5_70	HF954430	*Cupriavidus necator* (CP002878)	98.86	Proteobacteria	*Burkholderiales*
										8_59	HF954497	*Enterobacter ludwigii* (AJ853891)	99.54	Proteobacteria	*Enterobacteriales*
20	0	0	0	0	2	0	1	3	0	5_76	HF954433	*Pseudomonas brassicacearum* (EU391388)	99.09	Proteobacteria	*Pseudomonadales*
										8_14	HF954483	*Pseudomonas migulae* (AF074383)	99.72	Proteobacteria	*Pseudomonadales*
										8_30	HF954489	*Variovorax soli* (DQ432053)	98.58	Proteobacteria	*Burkholderiales*
VI	0	0	0	0	0	0	1	0	0	7_87	HF954475	*Variovorax soli* (DQ432053)	98.57	Proteobacteria	*Burkholderiales*
21	0	0	0	0	1	4	0	2	1	9_31	HF954515	*Pseudomonas stutzeri* (CP002881)	98.70	Proteobacteria	*Pseudomonadales*
										8_106	HF954480	*Pseudomonas frederiksbergensis* (AJ249382)	99.81	Proteobacteria	*Pseudomonadales*
22	0	0	0	0	2	0	0	0	0	5_55	HF954423	*Pseudomonas beteli* (AB021406)	99.01	Proteobacteria	*Xanthomonadales*
VII	0	0	0	0	0	0	0	1	0	8_89	HF954506	*Microbacterium binotii* (EF567306)	99.39	Actinobacteria	*Micrococcales*
VIII	0	0	0	0	1	0	0	0	0	5_24	HF954413	*Achromobacter spanius* (AY170848)	99.90	Proteobacteria	*Burkholderiales*
IX	0	0	0	0	0	0	0	0	1	9_24	HF954513	*Pseudomonas stutzeri* (CP002881)	98.68	Proteobacteria	*Pseudomonadales*
X	0	0	0	1	0	0	0	0	0	4_82	HF954407	*Nocardia fluminea* (AF277204)	99.08	Actinobacteria	*Corynebacteriales*
XI	0	0	0	1	0	0	0	0	0	4_110	HF954397	*Bacillus pseudomycoides* (ACMX01000133)	99.90	Firmicutes	*Bacillales*

Cluster/Branch	Soil Samples									Isolates selected of each cluster for 16S rRNA sequencing					
	1	2	3	4	5	6	7	8	9	Isolate id[b]	Acc n°	Closest relative Eztaxon match (accession number)	16S sim (%)	Phylum	Order
23	0	0	0	1	1	0	0	1	0	5_98	HF954437	*Dyadobacter beijingensis* (DQ335125)	98.59	Proteobacteria	*Cytophagales*
										4_38	HF954401	*Dyadobacter ginsengisoli* (AB245369)	98.18	Proteobacteria	*Cytophagales*
24	0	0	0	0	6	2	0	0	0	5_73	HF954432	*Sphingobacterium bambusae* (GQ339910)	95.87	Bacteroidetes	*Sphingobacteriales*
										5_62	HF954427	*Pedobacter tournemirensis* (GU198945)	98.78	Bacteroidetes	*Sphingobacteriales*
										6_86	HF954455	*Olivibacter oleidegradans* (HM021726)	99.52	Bacteroidestes	*Sphingobacteriales*
										5_60	HF954426	*Pedobacter tournemirensis* (GU198945)	98.40	Bacteroidetes	*Sphingobacteriales*
XII	0	0	0	1	0	0	0	0	0	4_40	HF954402	*Caulobacter vibrioides* (AJ009957)	99.08	Proteobacteria	*Caulobacterales*
XIII	0	0	0	1	0	0	0	0	0	4_62	HF954405	*Devosia neptuniae* (AF469072)	99.51	Proteobacteria	*Rhizobiales*
25	0	0	0	0	0	0	1	2	0	8_58	HF954496	*Microbacterium yannicii* (FN547412)	98.02	Actinobacteria	*Micrococcales*
XIV	0	0	0	1	0	0	0	0	0	4_57	HF954404	*Caulobacter henricii* (AJ227758)	99.22	Proteobacteria	*Caulobacterales*
26	1	0	0	6	0	0	2	0	0	4_108	HF954396	*Nocardioides albus* (AF004988)	99.63	Actinobacteria	*Propionibacteriales*
										4_101	HF954393	*Nocardioides albus* (AF004988)	99.81	Actinobacteria	*Propionibacteriales*
XV	0	0	0	1	0	0	0	0	0	4_21	HF954400	*Nocardioides albus* (AF004988)	99.81	Actinobacteria	*Propionibacteriales*
27	9	2	6	7	5	7	12	10	7	9_40	HF954519	*Nocardioides sediminis* (EF466110)	99.34	Actinobacteria	*Propionibacteriales*
										7_67	HF954470	*Nocardioides alpinus* (GU784866)	98.19	Actinobacteria	*Propionibacteriales*
28	0	0	0	0	0	1	2	0	0	7_61	HF954469	*Nocardioides furvisabuli* (DQ411542)	98.30	Actinobacteria	*Propionibacteriales*
29	0	0	0	0	0	2	0	0	0	6_88	HF954457	*Nocardioides sediminis* (EF466110)	99.90	Actinobacteria	*Propionibacteriales*
30	2	1	0	3	1	0	2	3	8	9_72	HF954526	*Nocardioides hwasunensis* (AM295258)	98.57	Actinobacteria	*Propionibacteriales*
										8_108	HF954481	*Nocardioides sediminis* (EF466110)	99.72	Actinobacteria	*Propionibacteriales*
31	0	0	1	0	1	1	3	0	1	9_80	HF954530	*Nocardioides terrigena* (EF363712)	98.74	Actinobacteria	*Propionibacteriales*
										7_7	HF954472	*Nocardioides hwasunensis* (AM295258)	98.58	Actinobacteria	*Propionibacteriales*
32	0	0	1	0	0	0	0	4	0	8_94	HF954507	*Nocardioides hwasunensis* (AM295258)	98.77	Actinobacteria	*Propionibacteriales*
33	2	0	0	1	0	0	1	0	0	7_17	HF954462	*Nocardioides furvisabuli* (DQ411542)	99.42	Actinobacteria	*Propionibacteriales*
										1_47	HF954381	*Nocardioides ganghwensis* (AY423718)	98.45	Actinobacteria	*Propionibacteriales*
34	1	0	0	1	1	0	1	0	0	5_72	HF954431	*Nocardioides furvisabuli* (DQ411542)	99.33	Actinobacteria	*Propionibacteriales*
35	1	0	2	0	0	0	0	0	0	3_51	HF954389	*Pseudomonas geniculata* (AB021404)	98.98	Proteobacteria	*Xanthomonadales*
36	0	0	0	0	3	0	0	0	0	5_14	HF954412	*Stenotrophomonas chelatiphaga* (EU573216)	99.42	Proteobacteria	*Xanthomonadales*
37	0	0	0	0	3	0	0	5	0	8_51	HF954494	*Pseudoxanthomonas mexicana* (AF273082)	100.00	Proteobacteria	*Xanthomonadales*
										8_100	HF954479	*Pseudoxanthomonas mexicana* (AF273082)	99.53	Proteobacteria	*Xanthomonadales*
38	0	0	0	0	0	0	2	0	0	7_48	HF954465	*Saccharothrix texasensis* (AF114815)	99.53	Actinobacteria	*Pseudonocardiales*

Cluster/Branch	Soil Samples									Isolates selected of each cluster for 16S rRNA sequencing					
	1	2	3	4	5	6	7	8	9	Isolate id[b]	Acc n[o]	Closest relative Eztaxon match (accession number)	16S sim (%)	Phylum	Order
39	20	0	4	14	0	2	15	2	9	4_107	HF954395	*Streptomyces pilosus* (AB184161)	99.81	Actinobacteria	*Streptomycetales*
										4_98	HF954408	*Streptomyces mutabilis* (AB184156)	99.91	Actinobacteria	*Streptomycetales*
										4_99	HF954409	*Streptomyces mutabilis* (AB184156)	99.90	Actinobacteria	*Streptomycetales*
40	2	0	2	4	0	1	3	0	1	7_16	HF954461	*Streptomyces exfoliatus* (AB184324)	99.90	Actinobacteria	*Streptomycetales*
41	0	0	1	0	0	2	1	0	0	7_32	HF954463	*Streptomyces xantholiticus* (AB184349)	99.81	Actinobacteria	*Streptomycetales*
XVI	0	1	0	0	0	0	0	0	0	2_108	HF954383	*Bacillus simplex* (AB363738)	99.72	Firmicutes	*Bacillales*
42	1	3	3	2	0	0	1	1	0	2_19	HF954385	*Arthrobacter nitroguajacolicus* (AJ512504)	99.80	Actinobacteria	*Micrococcales*
										4_69	HF954406	*Arthrobacter nitroguajacolicus* (AJ512504	99.81	Actinobacteria	*Micrococcales*
										2_26	HF954386	*Arthrobacter crystallopoietes*(X80738)	99.52	Actinobacteria	*Micrococcales*
43	0	4	0	0	0	0	0	0	0	2_57	HF954387	*Paenibacillus taichungensis* (EU179327)	98.69	Firmicutes	*Bacillales*
44	33	84	65	36	6	26	21	0	27	3_96	HF954392	*Arthrobacter oxydans* (X83408)	98.60	Actinobacteria	*Micrococcales*
										6_29	HF954443	*Arthrobacter phenanthrenivorans* (CP002379)	98.91	Actinobacteria	*Micrococcales*
45	1	0	0	1	1	1	0	0	1	1_102	HF954378	*Agromyces subbeticus* (AY737778)	99.02	Actinobacteria	*Micrococcales*
										4_12	HF954398	*Microbacterium oleivorans* (AJ698725)	98.70	Actinobacteria	*Micrococcales*
46	0	0	0	0	0	0	0	0	2	9_11	HF954511	*Arthrobacter globiformis* (BAEG01000072)	99.71	Actinobacteria	*Micrococcales*
XVII	0	0	0	0	1	0	0	0	0	5_25	HF954414	*Cellulomonas terrae* (AY884570)	100.00	Actinobacteria	*Micrococcales*
47	0	0	0	0	0	3	0	0	0	6_4	HF954448	*Arthrobacter globiformis* (BAEG01000072)	99.63	Actinobacteria	*Micrococcales*
48	2	0	0	2	1	2	1	3	1	1_16	HF954379	*Cellulosimicrobium funkei* (AY501364)	99.71	Actinobacteria	*Micrococcales*
										8_25	HF954487	*Cellulomonas terrae* (AY884570)	99.42	Actinobacteria	*Micrococcales*
49	12	0	5	5	8	19	9	11	12	5_46	HF954420	*Microbacterium yannicii* (FN547412)	98.59	Actinobacteria	*Micrococcales*
										6_57	HF954451	*Microbacterium arthrosphaerae* (FN870023)	98.97	Actinobacteria	*Micrococcales*
50	1	0	2	1	1	0	3	4	2	9_76	HF954528	*Agromyces cerinus* (X77448)	99.81	Actinobacteria	*Micrococcales*
										7_84	HF954474	*Microbacterium yannicii* (FN547412)	98.47	Actinobacteria	*Micrococcales*
XVIII	0	0	0	0	0	0	1	0	0	7_107	HF954460	*Arthrobacter parietis* (AJ639830)	100.00	Actinobacteria	*Micrococcales*
XIX	0	0	0	0	1	0	0	0	0	5_48	HF954421	*Frigobacterium faeni* (Y18807)	98.95	Actinobacteria	*Micrococcales*
51	3	2	2	1	0	0	2	0	1	2_12	HF954384	*Agrococcus lahaulensis* (DQ156908)	98.85	Actinobacteria	*Micrococcales*
										3_39	HF954388	*Agrococcus lahaulensis* (DQ156908)	97.60	Actinobacteria	*Actinobacteria*
52	0	0	0	1	1	3	0	0	4	6_101	HF954438	*Agrococcus lahaulensis* (DQ156908)	98.85	Actinobacteria	*Actinobacteria*
										9_99	HF954531	*Agrococcus lahaulensis* (DQ156908)	98.58	Actinobacteria	*Actinobacteria*
53	1	0	0	0	0	0	0	0	2	9_41	HF954520	*Microbacterium pumilum* (AB234027)	98.56	Actinobacteria	*Actinobacteria*
XX	0	0	0	0	0	0	0	1	0	8_84	HF954503	*Microbacterium profundi* (EF623999)	98.84	Actinobacteria	*Micrococcales*

Cluster/Branch	Soil samples									Isolates selected of each cluster for 16S rRNA sequencing					
	1	2	3	4	5	6	7	8	9	Isolate id[b]	Acc n°	Closest relative Eztaxon match (accession number)	16S sim (%)	Phylum	Order
54	2	0	0	2	0	1	2	1	0	4_105	HF954394	*Promicromonospora umidemergens* (FN293378)	99.13	Actinobacteria	*Actinobacteria*
										7_51	HF954467	*Promicromonospora xylanilytica* (FJ214352)	99.16	Actinobacteria	*Actinobacteria*
										6_21	HF954441	*Promicromonospora thailandica* (AB560974)	99.61	Actinobacteria	*Actinobacteria*

[a] Clusters have been indicated with Arabic numerals and single branches have been identified with Roman numerals

[b] Identification number for each strain selected for sequencing

Table A3. 16S rRNA sequencing of bacteria non-characterized by FAMEs isolated from sample 5- soil amended with DOR at 30 d, 6- soil amended with DOR transformed by *C. floccosa* at 30 d, 7- control soil at 60 d, 8- soil amended with DOR at 60 d and 9- soil amended with DOR transformed by *C. floccosa* at 60 d

Soil sample	Isolate ID	GenBank acc no.	Closest relative Eztaxon match (GenBank acc no.)	16S rRNA sim (%)	Phylum	Order
5	5_32	HF954417	*Herbiconiux flava* (AB583921)	99.42	Actinobacteria	*Micrococcales*
	5_83	HF954436	*Sphingomonas echinoides* (JH1584237)	99.90	Proteobacteria	*Sphingomonadales*
	5_104	HF954411	*Rhizobium herbae* (GU565534)	97.58	Proteobacteria	*Rhizobiales*
6	6_11	HF954440	*Sanguibacter inulinus* (X79451)	99.00	Actinobacteria	*Micrococcales*
	6_25	HF954442	*Phyllobacterium bourgognense* (AY785320)	97.67	Proteobacteria	*Rhizobiales*
	6_50	HF954450	*Nocardioides hwasunensis* (AM295258)	98.84	Actinobacteria	*Propionibacteriales*
	6_35	HF954444	*Arthrobacter oxydans* (X83408)	99.42	Actinobacteria	*Micrococcales*
	6_38	HF954446	*Arthrobacter tumbae* (AJ315069)	99.80	Actinobacteria	*Micrococcales*
	6_39	HF954447	*Caulobacter henricii* (AJ227758)	98.95	Proteobacteria	*Caulobacterales*
	6_71	HF954452	*Arthrobacter subterraneus* (DQ097525)	100.00	Actinobacteria	*Micrococcales*
	6_87	HF954456	*Arthrobacter subterraneus* (DQ097525)	100.00	Actinobacteria	*Micrococcales*
	6_91	HF954458	*Arthrobacter oxydans* (X83408)	99.90	Actinobacteria	*Micrococcales*
	6_108	HF954439	*Streptomyces galilaeus* (AB045878)	100.00	Actinobacteria	*Streptomycetales*
7	7_9	HF954476	*Microbacterium thalassium* (AB004713)	98.28	Actinobacteria	*Micrococcales*
	7_34	HF954464	*Agrococcus jenensis* (X92492)	99.54	Actinobacteria	*Micrococcales*
	7_57	HF954468	*Microvirga zambiensis* (HM362433)	98.93	Proteobacteria	*Rhizobiales*
	7_79	HF954473	*Variovorax soli* (DQ432053)	99.04	Proteobacteria	*Burkholderiales*
	7_92	HF954477	*Paenibacillus pabuli* (AB073191)	99.62	Firmicutes	*Bacillales*
8	8_13	HF954482	*Bacillus idriensis* (AY904033)	99.53	Firmicutes	*Bacillales*
	8_32	HF954490	*Enterobacter ludwigii* (AJ853891)	99.51	Proteobacteria	*Enterobacteriales*
	8_63	HF954499	*Brevundimonas nasdae* (AB0719549)	99.14	Proteobacteria	*Caulobacterales*
	8_86	HF954504	*Nocardioides hankookensis* (EF555584)	99.01	Actinobacteria	*Propionibacteriales*
9	9_21	HF954512	*Nocardioides hwasunensis* (AM295258)	98.72	Actinobacteria	*Propionibacteriales*
	9_36	HF954517	*Nocardioides ganghwensis* (AY423718)	98.37	Actinobacteria	*Propionibacteriales*
	9_39	HF954518	*Nocardioides sediminis* (EF466110)	99.72	Actinobacteria	*Propionibacteriales*
	9_45	HF954521	*Agrococcus lahaulensis* (DQ156908)	98.80	Actinobacteria	*Micrococcales*
	9_55	HF954522	*Actinocorallia aurea* (AB006177)	98.73	Actinobacteria	*Streptosporangiales*
	9_56	HF954523	*Paenibacillus taichungensis* (EU179327)	98.88	Firmicutes	*Bacillales*
	9_57	HF954524	*Devosia psychrophila* (GU441678)	97.98	Proteobacteria	*Rhizobiales*
	9_73	HF954527	*Arthrobacter oxydans* (X83408)	99.14	Actinobacteria	*Micrococcales*
	9_78	HF954529	*Paenibacillus provencensis* (EF212893)	100.00	Firmicutes	*Bacillales*

Table A4. Morphologic characterization, ITS, 28S and β-tubuline sequencing matching and isolation frequency from: 1- control soil at 0 d, 2- soil amended with DOR at 0 d, 3- soil amended with DOR transformed by *C. floccosa* at 0 days, 4- control soil at 30 d, 5- soil amended with DOR at 30 d, 6- soil amended with DOR transformed by *C. floccosa* at 30 d, 7- control soil at 60 d, 8- soil amended with DOR at 60 d and 9- soil amended with DOR transformed by *C. floccosa* at 60 d.

Isolate no.	Strain Id	Identification method	Identification	GenBank acc no.	Closest sequence match (accession number), % similarity	Soil samples								
						1	2	3	4	5	6	7	8	9
1	F277763	ITS	*Acremonium furcatum*	KC426989	*Acremonium furcatum* (DQ825975), 99	0	0	0	0	18	0	0	0	0
2	F277766	ITS	*Acremonium fusifoides*	KC426990	*Acremonium fusifoides* (FN706544), 95	1	0	0	0	0	0	0	0	0
3	F277762	ITS	*Acremonium* sp.1	KC426991	*Acremonium persicinum* (AB540575), 100	0	0	0	3	0	0	0	0	1
4	F277806	ITS	*Acremonium* sp.2	KC426992	*Acremonium antarcticum* (DQ825970), 99	1	0	0	0	0	0	0	0	0
5	F277761	ITS	*Acremonium tubakii*	KC426993	*Acremonium tubakii* (HQ232147), 99	0	0	0	0	0	0	0	1	0
6	F277767	Morphology	*Acrostalagmus luteoalbus*			2	0	3	3	0	1	1	0	0
7	F277826	ITS	*Alternaria infectoria*	KC426994	*Alternaria infectoria* (FJ214868), 99	0	0	1	0	1	0	0	0	0
8	F277768	ITS	*Alternaria* sp.	KC426995	*Alternaria pellucida* (CBS479.90), 99	2	23	7	4	8	0	0	1	6
9	F277774	ITS	*Aspergillus cretensis*	KC426996	*Aspergillus cretensis* (EF661418), 99	0	0	0	0	0	0	0	0	1
10	F277777	ITS	*Aspergillus flavipes*	KC426997	*Aspergillus flavipes* (HM595494), 100	0	0	0	0	0	0	1	0	0
11	F277831	ITS	*Aspergillus insuetus*	KC426998	*Aspergillus insuetus* (EU076355), 99	0	0	2	0	0	0	0	0	0
12	F277880	ITS	*Aspergillus keveii*	KC426999	*Aspergillus keveii* (EF652432), 100	1	0	0	0	0	0	0	0	0
13	F277771	Morphology	*Aspergillus niger*			0	0	0	0	0	0	0	1	0
14	F277773	Morphology	*Aspergillus niveus*			0	1	0	0	0	0	0	0	0
15	F277949	ITS	*Aspergillus pseudodeflectus*	KC427000	*Aspergillus pseudodeflectus* (EF652507), 99	1	0	0	0	0	0	0	0	0
16	F277778	Morphology	*Aspergillus terreus*			62	10	36	22	3	37	65	26	52
17	F277779	ITS	*Aspergillus ustus*	KC427001	*Aspergillus* aff. *ustus* A24 (JN246053), 100	0	0	1	0	0	0	0	6	1
18	F277775	ITS	*Aspergillus versicolor*	KC427002	*Aspergillus versicolor* (AY373880), 100	0	0	1	2	0	0	9	3	3
19	F277772	ITS	*Bionectria ochroleuca*	KC427003	*Bionectria ochroleuca* (AY876924), 99	0	0	0	1	0	3	1	0	0
20	F277835	ITS	*Bionectria rossmaniae*	KC427004	*Bionectria rossmaniae* (AF210665), 98	0	0	0	1	1	0	0	0	0
21	F277905	ITS	*Chaetomium piluliferum*	KC427005	*Chaetomium piluliferum* (AB625587), 99	0	4	0	5	2	1	3	1	3
22	F277809	ITS	*Chaetomium* sp.1	KC427006	*Chaetomium megalocarpum* (CBS778.71), 98	3	6	2	5	0	0	8	2	0
23	F277863	ITS	*Chaetomium* sp.2	KC427007	*Leptodiscella chlamydospora* (FR745398), 91	0	0	0	0	0	0	0	1	1
24	F277810	ITS	*Chaetomium* sp.3	KC427008	*Chaetomium seminis-citruli* (CBS637.83), 99	0	11	0	1	0	1	0	1	1
25	F277865	ITS	*Chaetomium* sp.4	KC427009	*Chaetomium jodhpurense* (CBS 509.84), 99	0	0	0	0	0	0	0	0	1

Isolate no.	Strain Id	Identification method	Identification	GenBank acc no.	Closest sequence match (accession number), % similarity	Soil samples								
						1	2	3	4	5	6	7	8	9
26	F277833	28S	*Chaetomium* sp.5	KC427010	*Chaetomium grande* (CBS126664), 100	0	0	0	1	0	0	0	0	0
27	F277782	28S	*Chaetomium* sp.6	KC427011	*Chaetomium murorum* (CBS776.71), 100	0	0	0	0	0	1	0	0	0
28	F277899	28S	*Chaetomium* sp.7	KC427012	*Aporothielavia leptoderma* (AF096186), 97	0	0	0	1	0	4	0	7	0
29	F277844	28S	*Chaetomium* sp.8	KC427013	*Chaetomium sphaerale* (AF 286407), 99	0	0	0	0	0	1	0	0	0
30	F277853	28S	*Chaetomium* sp.9	KC427014	*Chaetomium globosum* (CBS733.84), 99	0	0	0	0	0	0	1	0	0
31	F277816	ITS	*Chaetomium* sp.10	KC427015	*Chaetomium globosum* (DQ093659), 99	0	5	0	0	0	1	0	0	0
32	F277803	ITS	*Chaetomium* sp.11	KC427016	*Chaetomium globosum* (DTO053-G8), 99	1	0	0	0	0	0	0	0	0
33	F277837	ITS	*Chaetomium* sp.12	KC427017	*Chaetomium globosporum* (CBS108.83), 99	0	0	0	1	0	0	0	0	0
34	F277789	Morphology	*Cladosporium cladosporioides*			4	21	35	3	51	9	1	34	5
35	F277791	Morphology	*Clonostachys rosea*			2	1	4	10	0	1	10	0	0
36	F277921	ITS	*Cryptococcus tephrensis*	KC427018	*Cryptococcus tephrensis* (DQ000318), 100	0	0	0	1	3	0	4	14	0
37	F277834	ITS	*Cylindrocarpon olidum* 1	KC427019	*Cylindrocarpon olidum* var.*crassum* (AY677294), 99	0	0	0	1	0	0	0	0	0
38	F277855	ITS	*Cylindrocarpon olidum* 2	KC427020	*Cylindrocarpon olidum* (AY677293), 100	0	1	0	3	1	4	0	2	0
39	F277950	28S	*Doratomyces* sp.	KC427021	*Doratomyces stemonitis* (DQ836907), 99	0	0	1	1	0	0	0	0	0
40	F277877	ITS	*Doratomyces stemonitis*	KC427022	*Doratomyces stemonitis* (FJ914696), 98	7	1	1	13	0	5	5	0	3
41	F277878	Morphology	*Drechslera biseptata*			0	1	0	0	0	0	0	0	0
42	F277879	Morphology	*Eladia saccula*			3	0	1	0	0	0	0	0	0
43	F277843	ITS	*Emericellopsis minima*	KC427023	*Emericellopsis minima* (AY632660), 99	0	0	0	0	0	1	0	0	0
44	F277947	ITS	*Eupenicillium meridianum*	KC427024	*Eupenicillium meridianum* (AF033451), 99	0	0	1	0	0	0	0	0	0
45	F277890	ITS	*Fusarium delphinoides*	KC427025	*Fusarium delphinoides* (EU926244), 99	0	0	0	0	0	0	0	0	1
46	F277889	ITS	*Fusarium equiseti*	KC427026	*Fusarium equiseti* (GQ505752), 100	0	0	0	1	0	0	0	0	0
47	F277887	ITS	*Fusarium merismoides*	KC427027	*Fusarium merismoides* (EU860057), 100	0	0	6	0	0	0	0	0	0
48	F277893	ITS	*Fusarium nematophilum*	KC427028	*Fusarium nematophilum* (HQ897786), 99	0	0	0	4	0	0	0	0	0
49	F277825	ITS	*Fusarium oxysporum*	KC427029	*Fusarium oxysporum* (EU364844), 100	9	8	15	10	17	28	0	21	15
50	F277882	ITS	*Fusarium scirpi*	KC427030	*Fusarium scirpi* (GQ505694), 99	7	25	18	10	41	5	3	3	8
51	F277781	ITS	*Fusarium solani* 1	KC427031	*Fusarium solani* (AM412625), 100	1	0	1	0	10	5	2	0	0
52	F277884	ITS	*Fusarium solani* 2	KC427032	*Fusarium* sp. NRRL 45880 (EU329689), 100	4	2	6	3	9	7	0	12	12
53	F277892	ITS	*Fusarium* sp. 1	KC427033	*Fusarium brachygibbosum* (GQ505450), 100	0	2	0	0	0	0	0	0	0
54	F277888	ITS	*Fusarium* sp.2	KC427034	*Fusarium* sp.1900 (EU750688), 100	0	0	0	0	1	2	0	0	0

Isolate no.	Strain Id	Identification method	Identification	GenBank acc no.	Closest sequence match (accession number), % similarity	Soil samples								
						1	2	3	4	5	6	7	8	9
55	F277783	ITS	*Geomyces destructans*	KC427035	*Geomyces destructans* (EU884920), 100	2	6	2	7	0	0	0	2	6
56	F277788	ITS	*Geomyces pannorum*	KC427036	*Geomyces pannorum* (CBS103.52), 99	2	1	0	0	0	2	1	0	0
57	F277818	ITS	*Geomyces* sp.1	KC427037	*Geomyces* sp. NG_p41 (HQ115709), 99	0	4	0	2	0	0	0	1	0
58	F277898	ITS	*Geomyces* sp. 2	KC427038	*Geomyces pannorum* (CBS106.13), 96	1	8	7	8	0	6	5	0	2
59	F277815	ITS	*Gibberella avenacea*	KC427039	*Gibberella avenacea* (AY853251), 99	0	2	0	0	0	0	0	0	3
60	F277760	ITS	*Gibellulopsis nigrescens*	KC427040	*Gibellulopsis nigrescens* (EF543857), 100	17	2	6	27	5	27	14	14	24
61	F277897	ITS	*Gliomastix murorum*	KC427041	*Gliomastix murorum* (EU821334), 100	0	0	0	1	0	0	0	0	0
62	F277784	ITS	*Graphium penicillioides*	KC427042	*Graphium penicillioides* (FJ914670), 98	0	0	0	0	0	0	0	1	0
63	F277846	ITS	*Gymnoascus reesii*	KC427043	*Gymnoascus reesii* (HM991269), 99	1	0	0	0	0	1	0	0	0
64	F277907	ITS	*Humicola grisea*	KC427044	*Humicola grisea* var. *grisea* (AB625590), 99	0	3	1	0	1	0	4	1	0
65	F277910	Morphology	*Idriella lunata*			0	0	0	1	0	0	0	0	0
66	F277786	ITS	*Leptodiscella* sp.	KC427045	*Leptodiscella chlamydospora* (FR745398), 91	0	0	1	1	0	0	0	0	0
67	F277804	ITS	*Lophiostoma* sp.	KC427046	*Lophiostoma* sp. OUCMBI101036 (HQ914825), 99	2	0	1	0	0	0	0	0	0
68	F277800	28S	*Malbranchea* sp.	KC427047	*Malbranchea aurantiaca* (AB359412), 99	1	0	0	0	0	0	0	1	0
69	F277821	ITS	*Massarina* sp.	KC427048	*Massarina walker* (CBS257.93), 95	0	1	0	0	0	0	0	0	0
70	F277924	Morphology	*Metarhizium anisopliae*			0	0	0	2	0	1	0	0	0
71	F277948	28S	*Microascus* sp.	KC427049	*Microascus cirrosus* (AF275539), 98	1	0	0	0	0	0	0	0	0
72	F277928	ITS	*Monodictys* sp.1	KC427050	*Monographella* sp. (JN030999), 95	1	0	0	0	0	2	4	1	1
73	F277929	ITS	*Monodictys* sp.2	KC427051	*Idriella lunata* (CBS681.92), 95	0	0	0	0	0	0	3	0	0
74	F277817	ITS	*Monodictys* sp.3	KC427052	*Monodictys castaneae* (AJ238678), 96	0	1	0	0	0	0	0	0	0
75	F277927	ITS	*Mortierella* sp.1	KC427053	*Mortierella alpine* (GU319989), 99	0	6	5	1	0	1	3	0	1
76	F277926	ITS	*Mortierella* sp.2	KC427054	*Mortierella clonocystis* (HQ630318), 100	0	0	2	0	0	2	1	1	0
77	F277901	ITS	*Mycochlamys macrospora*	KC427055	*Mycochlamys macrospora* (CBS639.76), 98	0	0	0	1	1	4	1	1	1
78	F277930	Morphology	*Myrothecium verrucaria*			0	0	0	0	0	0	0	0	1
79	F277796	ITS	*Neonectria radicicola*	KC427056	*Ilyonectria radicicola* (GQ131875), 100	1	2	3	5	6	6	0	1	6
80	F277931	Morphology	*Neosartorya fisheri*			2	0	0	1	0	1	4	0	0
81	F277799	ITS	*Ochroconis tshawytschae*	KC427057	*Ochroconis tshawytschae* (HQ667566), 99	4	0	3	0	0	0	0	12	16
82	F277932	Morphology	*Paecilomyces tilacinus*			0	0	0	1	0	0	0	0	0
83	F277934	β-tubulin	*Penicilium marneffei*	KC427058	*Penicillium marneffei* (XM002151381), 100	5	0	1	0	0	1	2	0	1
84	F277939	β-tubulin	*Penicillium cyclopium*	KC427059	*Penicillium cyclopium* (AY674308), 100	0	0	2	4	2	0	1	0	0

Sample no.	Strain Id	Identification method	Identification	GenBank acc no.	Closest sequence match (accession number), % similarity	Soil samples								
						1	2	3	4	5	6	7	8	9
85	F277937	β-tubulin	*Penicillium flavigenum*	KC427060	*Penicillium flavigenum* (AY495994), 99	0	1	0	0	0	0	0	0	0
86	F277935	β-tubulin	*Penicillium griseofulvum*	KC427061	*Penicillium griseofulvum* (CBS110419), 100	6	1	2	0	0	0	0	0	1
87	F277944	β-tubulin	*Penicillium restrictum*	KC427062	*Penicillium restrictum* (FJ004428), 92	0	1	0	2	0	1	3	1	0
88	F277936	β-tubulin	*Penicillium sizovae*	KC427063	*Penicillium sizovae* (CBS117183), 100	1	4	0	3	0	2	6	4	4
89	F277943	β-tubulin	*Penicillium* sp.1	KC427064	*Penicillium* sp. OY18307 (FJ619266), 99	0	1	0	0	0	3	1	4	0
90	F277942	β-tubulin	*Penicillium* sp.2	KC427065	*Penicillium citreonigrum* (CBS414.9), 97	15	0	1	0	0	0	0	0	0
91	F277923	ITS	*Phoma labilis*	KC427066	*Phoma labilis* (GU237868), 100	0	0	0	1	0	0	0	0	0
92	F277920	ITS	*Plectosphaerella cucumerina*	KC427067	*Plectosphaerella cucumerina* (JF780522), 99	0	2	0	1	13	3	3	0	0
93	F277808	ITS	*Pleosporal*	KC427068	*Leptosphaeria* sp. Sg17-1 (HQ315844), 100	2	0	0	0	0	0	0	0	0
94	F277867	ITS	*Podospora* sp.	KC427069	*Schizothecium curvisporum* (AF443850), 95	0	0	0	0	0	0	0	0	4
95	F277822	ITS	*Preussia funiculata*	KC427070	*Preussia funiculate* (AY943059), 100	0	1	0	1	0	0	0	0	0
96	F277787	ITS	*Preussia* sp.1	KC427071	*Preussia terricola* (GQ203765), 96	0	0	1	0	0	0	1	0	0
97	F277801	ITS	*Preussia* sp.2	KC427072	*Preussia polymorpha* (GQ292749), 91	2	0	0	0	0	0	0	0	0
98	F277856	ITS	*Preussia* sp.3	KC427073	*Preussia pilosella* (DQ468033), 98	0	0	0	0	0	0	0	1	0
99	F277868	ITS	*Pyrenochaetopsis decipiens*	KC427074	*Pyrenochaetopsis decipiens* (CBS343.85), 96	0	0	0	0	0	0	1	0	2
100	F277912	ITS	*Sarocladium kiliense*	KC427075	*Sarocladium kiliense* (FN691449), 99	1	1	0	0	1	0	0	0	0
101	F277858	ITS	*Schizothecium* sp.	KC427076	*Schizothecium curvisporum* (AY999119), 96	0	0	0	0	0	0	0	1	0
102	F277922	ITS	*Sporobolomyces roseus*	KC427077	*Sporobolomyces roseus* (AY069999), 99	2	17	11	0	3	0	7	0	1
103	F277819	ITS	*Sporormia subticinensis*	KC427078	*Sporormia subticinensis* (AY943051), 99	0	1	0	0	0	0	0	0	1
104	F277954	ITS	*Stachybotrys chartarum*	KC427079	*Stachybotrys chartarum* (AY180261), 100	8	3	7	7	1	3	3	5	2
105	F277862	ITS	*Stephanonectria* sp.	KC427080	*Stephanonectria keithii* (AF210671), 98	0	0	0	2	0	0	2	0	1
106	F277848	ITS	*Tetracladium* sp.	KC427081	*Tetracladium* sp. AR-5 (DQ350129), 99	0	0	0	0	0	0	3	0	0
107	F277915	ITS	*Torula sp.*	KC427082	*Torula herbarum* (JQ246356), 98	0	0	0	0	1	0	0	0	0
108	F277957	28S	*Trichoderma* sp.	KC427083	*Trichoderma viride* (GQ408917), 99	0	0	0	0	0	0	2	2	1
109	F277861	ITS	*Volutella ciliata*	KC427084	*Volutella ciliate* (HQ897802), 99	0	4	0	0	3	3	0	0	2

Table A5. Statistical differences (p-level; n.s.- non significant) obtained from Diversity *t* test between samples at the same sampling time (0, 30 and 60 days).

Sample comparions	p-value
C-T0 *vs*. DOR-T0	n.s.
C-T0 *vs*. CORDOR-T0	n.s.
DOR-T0 *vs*. CORDOR-T0	n.s.
C-T1 *vs*. DOR-T1	<0.01
C-T1 *vs*. CORDOR-T1	<0.01
DOR-T1 *vs*. CORDOR-T1	<0.01
C-T2 *vs*. DOR-T2	n.s.
C-T2 *vs*. CORDOR-T2	n.s.
DOR-T2 *vs*. CORDOR-T2	n.s.

Table A6. Correlations of fungal species to ordination axes derived from PCA of control soil, soil amended with DOR and soil amended with DOR transformed by *C. floccosa* at 0, 30 and 60 days.

Fungal specie	PC1	PC2	Fungal specie	PC1	PC2
Acremonium furcatum	-0,0080	0,0587	*Geomyces pannorum*	-0,0148	-0,0097
Acremonium fusifoides	-0,0241	-0,0076	*Geomyces* **sp.1**	-0,0190	0,0015
Acremonium **sp.1**	-0,0206	-0,0079	*Geomyces* **sp.2**	0,0316	0,0009
Acremonium **sp.2**	-0,0241	-0,0076	*Gibberella avenacea*	-0,0185	-0,0050
Acremonium tubakii	-0,0248	-0,0050	*Gibellulopsis nigrescens*	0,2201	-0,0460
Acrostalagmus luteoalbus	-0,0086	-0,0082	*Gliomastix murorum*	-0,0252	-0,0065
Alternaria infectoria	-0,0236	-0,0013	*Graphium penicillioides*	-0,0248	-0,0050
Alternaria **sp.**	0,0367	0,0650	*Gymnoascus reesii*	-0,0221	-0,0079
Aspergillus cretensis	-0,0243	-0,0070	*Humicola grisea*	-0,0106	-0,0021
Aspergillus flavipes	-0,0242	-0,0080	*Idriella lunata*	-0,0252	-0,0065
Aspergillus insuetus	-0,0230	-0,0035	*Leptodiscella sp.*	-0,0234	-0,0052
Aspergillus keveii	-0,0241	-0,0076	*Lophiostoma sp.*	-0,0200	-0,0077
Aspergillus niger	-0,0248	-0,0050	*Malbranchea sp.*	-0,0225	-0,0065
Aspergillus niveus	-0,0256	-0,0044	*Massarina sp.*	-0,0256	-0,0044
Aspergillus pseudodeflectus	-0,0241	-0,0076	*Metarhizium anisopliae*	-0,0220	-0,0071
Aspergillus terreus	0,5866	-0,1682	*Microascus sp.*	-0,0241	-0,0076
Aspergillus ustus	-0,0131	0,0013	*Monodictys* **sp.1**	-0,0076	-0,0152
Aspergillus versicolor	0,0089	-0,0210	*Monodictys* **sp.2**	-0,0197	-0,0117
Bionectria ochroleuca	-0,0171	-0,0091	*Monodictys* **sp.3**	-0,0256	-0,0044
Bionectria rossmaniae	-0,0241	-0,0029	*Mortierella* **sp.1**	-0,0008	0,0042
Chaetomium piluliferum	0,0017	-0,0005	*Mortierella* **sp.2**	-0,0152	-0,0048
Chaetomium **sp.1**	0,0164	-0,0112	*Mycochlamys macrospora*	-0,0105	-0,0055
Chaetomium **sp.2**	-0,0227	-0,0058	*Myrothecium verrucaria*	-0,0243	-0,0070
Chaetomium **sp.3**	-0,0104	0,0132	*Neonectria radicicola*	0,0209	0,0147
Chaetomium **sp.4**	-0,0243	-0,0070	*Neosartorya fisheri*	-0,0096	-0,0170
Chaetomium **sp.5**	-0,0252	-0,0065	*Ochroconis tshawytschae*	0,0406	-0,0070
Chaetomium **sp.6**	-0,0245	-0,0065	*Paecilomyces tilacinus*	-0,0252	-0,0065
Chaetomium **sp.7**	-0,0063	0,0006	*Penicilium marneffei*	-0,0044	-0,0168
Chaetomium **sp.8**	-0,0245	-0,0065	*Penicillium cyclopium*	-0,0137	0,0006
Chaetomium **sp.9**	-0,0242	-0,0080	*Penicillium flavigenum*	-0,0256	-0,0044
Chaetomium **sp.10**	-0,0203	0,0024	*Penicillium griseofulvum*	-0,0059	-0,0112
Chaetomium **sp.11**	-0,0241	-0,0076	*Penicillium restrictum*	-0,0128	-0,0097
Chaetomium **sp.12**	-0,0252	-0,0065	*Penicillium sizovae*	0,0150	-0,0116
Cladosporium cladosporioides	0,2004	0,2859	*Penicillium* **sp.1**	-0,0112	-0,0024
Clonostachys rosea	0,0227	-0,0239	*Penicillium* **sp.2**	0,0107	-0,0265
Cryptococcus tephrensis	0,0091	0,0132	*Phoma labilis*	-0,0252	-0,0065
Cylindrocarpon olidum	-0,0252	-0,0065	*Plectosphaerella cucumerina*	0,0023	0,0376
Cylindrocarpon olidum	-0,0099	-0,0005	*Pleosporal*	-0,0217	-0,0091
Doratomyces **sp.**	-0,0234	-0,0052	*Podospora* **sp.**	-0,0181	-0,0094
Doratomyces stemonitis	0,0359	-0,0303	*Preussia funiculata*	-0,0243	-0,0047
Drechslera biseptata	-0,0256	-0,0044	*Preussia* **sp.1**	-0,0224	-0,0067
Eladia saccula	-0,0176	-0,0092	*Preussia* **sp.2**	-0,0217	-0,0091
Emericellopsis minima	-0,0245	-0,0065	*Preussia* **sp.3**	-0,0248	-0,0050
Eupenicillium meridianum	-0,0247	-0,0049	*Pyrenochaetopsis decipiens*	-0,0200	-0,0097
Fusarium delphinoides	-0,0243	-0,0070	*Sarocladium kiliense*	-0,0222	-0,0023
Fusarium equiseti	-0,0252	-0,0065	*Schizothecium sp.*	-0,0248	-0,0050
Fusarium merismoides	-0,0161	0,0018	*Sporobolomyces roseus*	0,0321	0,0329
Fusarium nematophilum	-0,0214	-0,0075	*Sporormia subticinensis*	-0,0235	-0,0052
Fusarium oxysporum	0,1757	0,0778	*Stachybotrys chartarum*	0,0413	-0,0039
Fusarium scirpi	0,1338	0,1868	*Stephanonectria* **sp.**	-0,0174	-0,0113
Fusarium solani **1**	0,0020	0,0248	*Tetracladium* **sp.**	-0,0197	-0,0117
Fusarium solani **2**	0,0655	0,0334	*Torula* **sp.**	-0,0254	-0,0026
Fusarium **sp.1**	-0,0247	-0,0026	*Trichoderma* **sp.**	-0,0167	-0,0084
Fusarium **sp.2**	-0,0215	-0,0031	*Volutella ciliata*	-0,0101	0,0093
Geomyces destructans	0,0111	-0,0005			

Table A7. Correlation of carbon sources with the first (PC1) and second principal components (PC2) after principal component analysis (PCA) of community level physiological profiles (CLPP) from unamended soil and soil amended with untransformed DOR or *C. floccosa*–transformed DOR at 0, 30 and 60 days.

Substrate name	Substrate type	PC 1	PC 2
Water	Control	0,000	0,000
Pyruvic acid methyl ester	Miscellaneous	0,104	0,058
Tween 40	Polymer	-0,042	0,164
Tween 80	Polymer	0,186	0,111
Alpha-cyclodextrin	Polymer	-0,171	-0,283
Glycogen	Polymer	-0,174	-0,157
D-cellobiose	Carbohydrate	-0,075	-0,372
Alpha-D-lactose	Carbohydrate	-0,186	-0,108
Beta-methyl-D-glucoside	Carbohydrate	-0,312	0,508
D-xylose	Carbohydrate	0,188	-0,323
i-erythritol	Carbohydrate	0,215	-0,068
D-mannitol	Carbohydrate	-0,112	-0,041
N-acetyl-D-glucosamine	Carbohydrate	-0,118	-0,144
D-glucosaminic acid	Carboxylic acid	-0,203	0,084
Glucose-1-phosphate	Miscellaneous	-0,062	0,081
D,L-alpha-glycerol phosphate	Miscellaneous	0,027	-0,081
D-galactonic acid-gamma-lactone	Carboxylic acid	0,047	-0,032
D-Galacturonic Acid	Carboxylic acid	0,598	0,199
2-Hydroxy benzoic acid	Carboxylic acid	-0,007	-0,008
4-Hydroxy benzoic acid	Carboxylic acid	0,021	0,031
Gamma-hydroxybutyric acid	Carboxylic acid	-0,001	-0,003
Itaconic acid	Carboxylic acid	0,089	0,226
Alpha-ketobutyric acid	Carboxylic acid	-0,064	-0,034
D-malic acid	Carboxylic acid	0,075	0,272
L-arginine	Amino acid	0,038	-0,239
L-asparagine	Amino acid	-0,026	0,153
L-phenylalanine	Amino acid	-0,148	-0,111
L-serine	Amino acid	-0,230	0,122
L-threonine	Amino acid	-0,064	0,040
Glycyl-L-glutamic acid	Amino acid	-0,100	0,084
Phenylethylamine	Amine/amide	0,211	-0,079
Putrescine	Amine/amide	0,297	-0,053

Fig. A1. Relative abundance of the different *Proteobacteria* classes (A), *Acidobacteria* classes (B) and *Actinobacteria* suborders (C) found in unamended soil (C) and soil amended with untransformed DOR (DOR) or *C. floccosa*–transformed DOR (CDOR) at 0 (T0), 30 (T1) and 60 (T2) days.

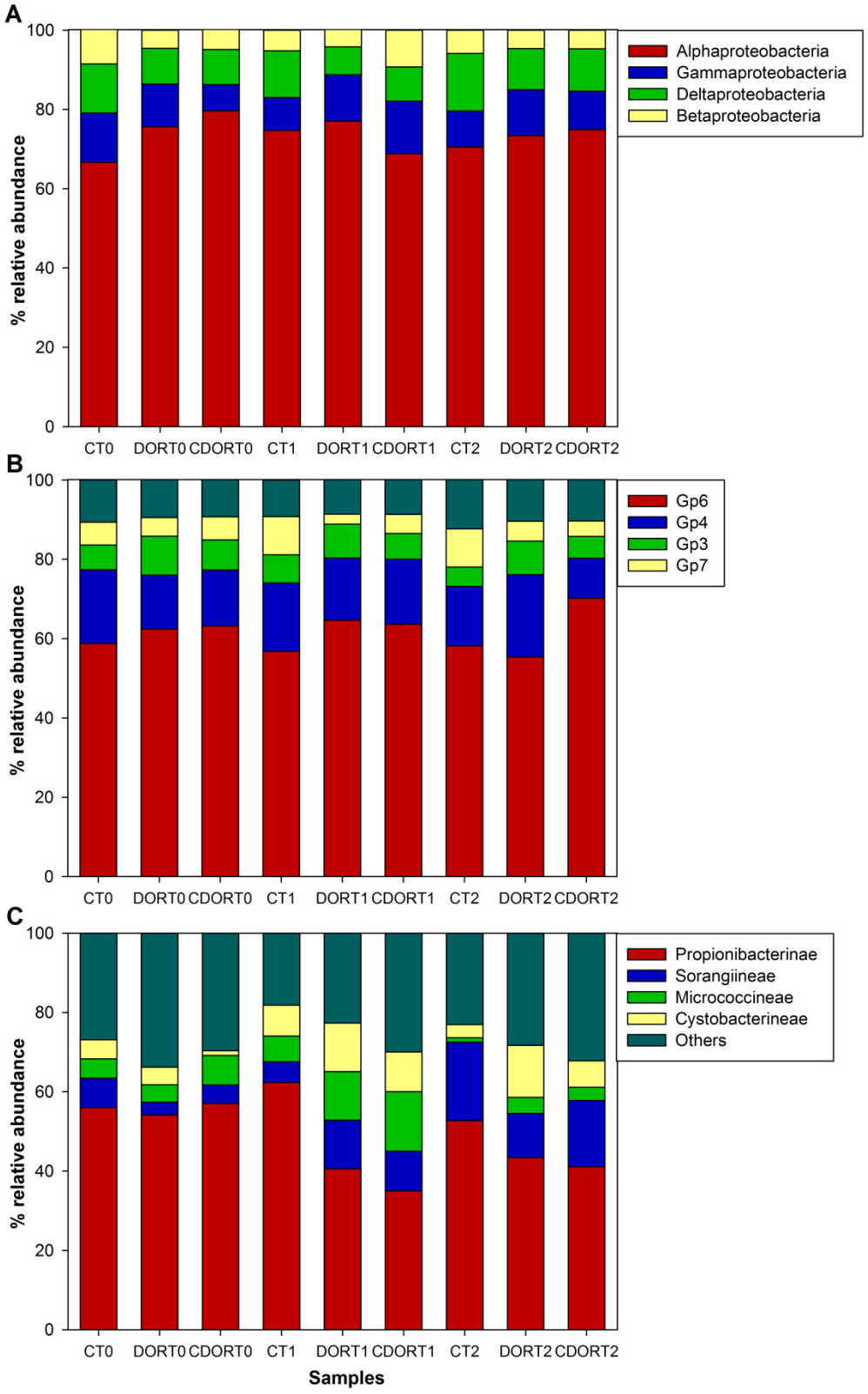
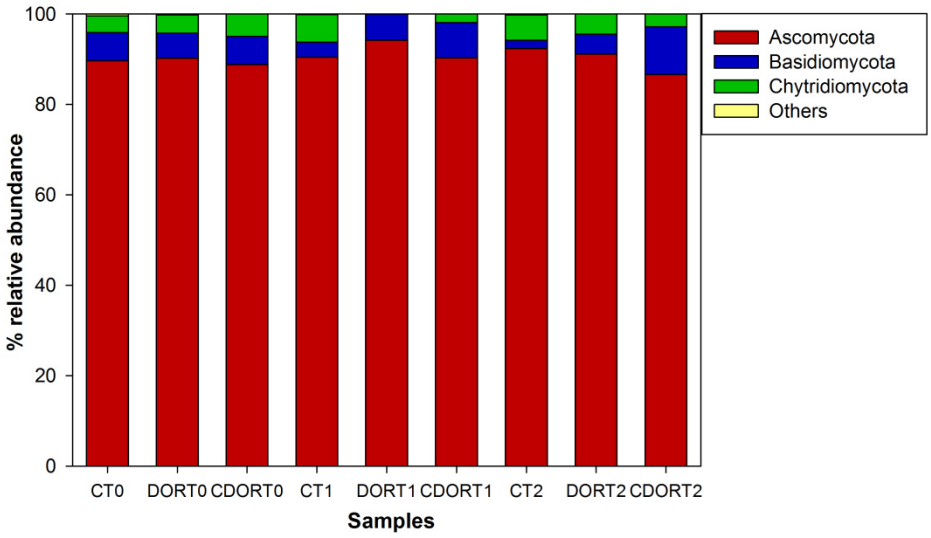


Fig. A2. Relative abundance of the different fungal phyla found in unamended soil (C) and soil amended with untransformed DOR (DOR) or *C. floccosa*–transformed DOR (CDOR) at 0 (T0), 30 (T1) and 60 (T2) days.



VII. APPENDIX 2

List of abbreviations

ACE	Abundance-based coverage estimation
AWCD	Average well color development
BLAST	Basic local alignment search tool
bp	Base pairs
CFU	Colony forming units
CLPP	Community level physiological profile
COD	Chemical oxygen demand
CORDOR	Dry olive residue transformed by *Coriolopsis floccosa*
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
DOR	Dry olive residue
EC	Electrical conductivity
FA	Fulvic acids
FAME	Fatty acid methyl esters
FUSDOR	Dry olive residue transformed by *Fusarium oxysporum*
gdw	gram dry weight
H	Shannon index
HA	Humic acids
Hf	Functional Shannon index
Hp	Phylogenetic Shannon index
HSD	Honest significance difference
ITS	Internal transcribed spacer
J	Evenness

Jf	Functional evenness
Jp	Phylogenetic evenness
MIDI	Microbial IDentification Inc
MIS	Microbial identification systems
OMW	Olive mill wastewater
OTU	Operational taxonomic unit
PCA	Principal components analysis
PCR	Polymerase chain reaction
PLFA	Phospholipid fatty acid
pPCR	Quantitative polymerase chain reaction
PS	Polyurethane sponge
rRNA	Ribosomal ribonucleic acid
S	Richness
Sf	Functional richness
SFF	Standard flowgram format
SOM	Soil organic matter
Sp	Phylogenetic richness
TPOMW	Two-phase olive-mill waste
UPGMA	Unweighted pair group method with arithmetic means
WSOC	Water soluble organic carbom