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

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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*.

1. Introduction

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

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

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

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

2. Materials and methods

2.1. Materials

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

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

2.2. Organisms and inoculum preparation

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

2.3. DOR biotransformation

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

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

2.4. Soil amendment

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

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

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

2.5. Enzymatic analyses

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