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

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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 fertilization 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 in the southeastern Iberian Peninsula. The control soil was compared with the same soil treated with DOR and with *C. floccosa*-transformed DOR for 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 ester profiles and 16S rRNA partial sequencing. The bacterial diversity was distributed between *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.

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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 [24]. Soil bacterial community can be influenced by a wide range of biotic and abiotic factors [13]. Therefore, any alterations of soil microbial community may have significant and unforeseen consequences on soil functions [33]. Such impacts may be especially transcendental in agricultural systems, which, in most cases, are subjected to mineral fertilization (urea, ammonium nitrate, sulfates and phosphates), organic fertilization (composts, biosolids or animal manures) and the application of other products such as microbial inoculants or pesticides [32]. Organic amendments are widely used and can offer an alternative to chemical fertilization and its associated problems [6]. 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 [32]. 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 fertilization is indispensible.

The Mediterranean region is characterized by highly degraded soils with low organic matter concentrations [10]. The wastes generated by the olive oil industry have been investigated as an organic fertilizer [21]. The two phases olive oil extraction system, after the revalorization of the wet primary residue, generates huge amounts of dry olive residue (DOR) or "alpeorujo" [1]. In Spain alone, 5 million tons of this product are produced annually, and it causes a significant environmental problem [50]. However, this waste could be a good candidate for utilization as an organic amendment due to its high organic and inorganic nutrient content [38]. Nevertheless, when DOR is applied directly to soil, phytotoxic [9,15] and

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microtoxic effects [4] have been observed. For this reason, pretreatment of the residue is necessary before its application to soil [38]. One of the most effective treatments is the incubation of the waste with saprobic fungi, such as *Coriolopsis floccosa*, formerly known as *C. rigida*, [2,41,44]. This treatment principally stabilizes the waste's organic matter, enhances its C/N relationship and drastically reduces the phenolic fraction [39,42].

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 [21]. Knowledge about the effects of DOR or DOR transformed by saprobic fungi on soil bacterial communities is scarce [40] and, more specifically, no data exists regarding the impacts on bacterial culturable diversity in soil fertilized 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 [14,46,49]. 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 [35].

Therefore, 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 additions on the culturable bacterial community of an agronomic soil, and (ii) to evaluate culturable bacterial diversity in a soil from the province of Granada, Spain.

Materials and methods

Sampling

The soil used in this study was obtained from the "Cortijo Peinado" field (Granada, Spain, 37°13′N, 3°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 °C, whereas the coldest month is January (mean 6.7 °C) and the warmest month is July (mean 24.8 °C) (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 °C) until used. The main chemical characteristics of DOR were determined by Sampedro et al. [42].

DOR biotransformation, soil amendment and plant growth determination

DOR was transformed with *C. floccosa* (CECT 20449^T), formerly known as *C. rigida*, which was maintained at 4 °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} anhydrous glucose (Acros Organics) and 5 g L^{-1} yeast extract (Fisher Chemical)] were added and the flasks were 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 °C for 7 days under 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 PSs in Erlenmeyer flasks were covered with 25 g of DOR. Solid-state cultures on DOR were grown at 28 °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 °C until added to the soil.

The experiments were carried out in 0.5 L pots. Untransformed DOR (UDOR) and DOR incubated with *C. floccosa* (TDOR) 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 °C and 50% relative humidity. The pots were watered regularly throughout the experiment. The soil watering determined that the water content of the samples was 15–20%.

The unamended control soil and replicate soils fertilized with UDOR and TDOR were analyzed 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 °C until sample processing (2 days). Therefore, the initial sampling period, designated as time 0 days, occurred after 2 days. These two days were initially considered insignificant because the soil samples were maintained at 4 °C. Subsequent sampling times of the remaining samples incubated under the greenhouse conditions were exactly 30 and 60 days.

The plants from each pot 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 drying oven.

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) [37].

The Petri plates were incubated for 4 weeks at 18 °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 °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

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