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Soil microbial diversity and activity in a Mediterranean olive orchard using sustainable agricultural practices

A. Sofo[1], A. Ciarfaglia[1], A. Scopa[1], I. Camele[1], M. Curci[2], C. Crecchio[2], C. Xiloyannis[3] & A. M. Palese[3]

[1]*School of Agricultural, Forestry, Food and Environmental Sciences (SAFE), Università degli Studi della Basilicata, Viale dell' Ateneo Lucano, 10 85100 Potenza Italy,* [2]*Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti (DiSSPA), Università degli Studi di Bari, Via Orabona, 4 70126, Bari, Italy, and* [3]*Dipartimento delle Culture Europee e del Mediterraneo: Architettura, Ambiente, Patrimoni Culturali (DiCEM), Università degli Studi della Basilicata, Via San Rocco, 3 75100 Matera, Italy*

Abstract

Sustainable soil management of orchards can have positive effects on both soils and crop yields due to increases in microbial biomass, activity and complexity. The aim of this study was to investigate medium-term effects (12 yr) of two different management practices termed 'sustainable' (ST) and 'conventional' (CT) on soil microbial composition and metabolic diversity of a rainfed mature olive orchard in Southern Italy. ST included no-till, self-seeding weeds (mainly graminaceous and leguminosae), and mulch derived from olive tree prunings, whilst CT was managed by frequent tillage and included severe pruning with residues removed from the orchard. Microbial analyses were carried out by culture-dependent methods (microbial cultures and Biolog®). Molecular methods were used to confirm the identification by light microscopy of the isolates of fungi and *Streptomyces*. Significantly more culturable fungi and bacteria were found in ST than in CT. The number of fungal groups in ST was also significantly greater than in CT. Overall and substrate-specific Biolog® metabolic diversity indices of microbial communities and soil enzyme activities were greater in ST. The results demonstrate that soil micro-organisms respond positively to sustainable orchard management characterized by periodic applications of locally derived organic matter. This study confirms the need to encourage farmers with orchards in the Mediterranean basin to practise soil management based on organic matter inputs associated with zero tillage to improve soil functionality.

Keywords: Biolog®, fungal identification, *Olea europaea* L., soil enzyme activities, sustainable soil management

Abbreviations: AWCD, average well colour development; CFU, colony-forming units; CLPP, community-level physiological profiles; CT, conventional treatment; *E*, substrate evenness; FDA, fluorescein diacetate; *H'*, Shannon's substrate diversity index; *S*, substrate richness; ST, sustainable treatment.

Introduction

Large crop yields of good quality can be achieved by maintaining diverse microbiological communities using innovative and sustainable agricultural techniques (Kushwaha *et al*., 2000; Jagadamma *et al*., 2008; Gomiero *et al*., 2011). Soils provide the habitat for large numbers of bacterial and fungal communities that influence soil fertility and plant growth by regulating nutrient availability and turnover (Borken *et al*., 2002; Govaerts *et al*., 2008). Changes in the structure and dynamics of soil bacterial and fungal communities in response to different soil management practices can provide the basis for an index of soil status with respect to quality and complexity (Anderson, 2003; Ding *et al*., 2013).

Agricultural management practices such as minimum tillage or no-till, recycling of locally derived organic matter and adequate irrigation, fertilizer application and pruning are required to save water, restore soil organic matter, increase the ability of soil to suppress plant pathogens, reduce erosion and environmental pollution (Lal, 2004; Postma *et al*., 2008; Gomiero *et al*., 2011; Ding *et al*., 2013). Sustainable and innovative soil management for fruit

Correspondence: A. Sofo. E-mail: adriano.sofo@unibas.it
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orchards can improve plant nutrition, avoid nutrient accumulation in soils and leaching risks, improve irrigation efficiency, and prevent soil erosion and root asphyxia (Sofo *et al*., 2010; Sofo, 2011). Furthermore, the use of low-impact agricultural techniques have positive effects on both soil quality and crop yield as they increase microbial biomass, activity and complexity (Kushwaha *et al*., 2000; Widmer *et al*., 2006).

For semi-arid Mediterranean agricultural lands, the need for a new approach in orchard management has become evident as a result of soil degradation and water shortage (Lal, 2004; Hochstrat *et al*., 2006). In such areas, there is need to use agronomic techniques to improve or maintain soil quality, health and fertility (Kushwaha & Singh, 2005; Govaerts *et al*., 2008). Olives (*Olea europaea* L.) have been cultivated in the Mediterranean basin for centuries and covered about 9.5 Mha in 2010 (FAOSTAT, 2012). They are an integral part of the Mediterranean landscape, especially in hilly and marginal localities (Loumou & Giourga, 2003). In olive groves, the positive influence of sustainable management systems on soil biochemical and microbiological characteristics has been described (Hernández *et al*., 2005; Benitez *et al*., 2006; Moreno *et al*., 2009; Sofo *et al*., 2010).

Metabolic microbial community diversity in soil bacterial and fungal communities can be estimated by different methods and techniques (Gomiero *et al*., 2011). Community-level physiological profiles (CLPPs) as obtained by the Biolog® method have high discriminating power between microbial soil communities from different soil environments or subjected to various treatments (Calbrix *et al*., 2005; Gelsomino *et al*., 2006). To characterize soil microbiota, culture-dependent techniques, accompanied by microscopy and molecular methods, are necessary (Calbrix *et al*., 2005; Singh *et al*., 2006). Moreover, for a comprehensive interpretation of the data on soil micro-organisms, the determination of the activities of some soil enzymes that are important markers of soil fertility is of key importance (Nannipieri *et al*., 2003; Sofo *et al*., 2010).

The aim of this study was to determine the medium-term effects (12 yr) of two different management systems (sustainable and conventional) on soil microbial diversity of a mature olive orchard in Southern Italy. A new method for the assessment of fungal metabolic diversity was also evaluated.

Materials and methods

Experimental site and orchard management

The trial was in a 2-ha mature olive grove (cultivar Maiatica, a variety able to produce both table olives and olive oil) in Southern Italy (Ferrandina, Basilicata Region, Italy; 40°29′ N, 16°28′E) and grown under rainfed conditions. Olive trees were vase trained and planted at a distance of ca. 8 × 8 m.

The climate of the area is semi-arid (UNEP, 1997) with an annual precipitation of 561 mm (mean 1976–2006), which falls mostly in the winter; the mean annual temperature ranges from 15 to 17 °C. The soil of the experimental grove is a sandy loam, a Haplic Calcisol (FAO, 1998) with a mean bulk density of 1.5 t/m^3. The top 60 cm of the soil had an average pH (± standard deviation) of 7.4 ± 0.4, an organic carbon content of 7.0 ± 3.8 g/kg, a total nitrogen content of 0.8 ± 0.2 g/kg (Kjeldahl method), and extractable phosphorus (Olsen method) and potassium of 11.7 ± 5.9 and 104 ± 70 mg/kg, respectively. In 2000, the olive orchard was divided into two 1-ha plots managed in two different ways: a sustainable treatment (ST) and a conventional treatment (CT). In the former, the soil was permanently covered by spontaneous self-seeding weeds (mainly graminaceous and leguminosae), which were mowed at least twice a year. The olive trees were pruned lightly each year. Cover crop residues derived from the weeds and annual prunings from the olive trees were shredded and then left on the ground as mulch. The CT was managed by tillage (rotovating at 10 cm depth) performed 2–3 times per year to control weeds. Severe pruning was carried out every 2 years, but pruned residues were removed from the olive orchard. The ST practices resulted in an increase in soil organic carbon in the 0–10 cm soil layer to 22.1 g/kg at the end of 12 yr compared with 11.8 g/kg in CT.

Soil sampling

In October 2011, soils were sampled under both treatments (CT and ST). For each treatment, four composite bulk sample soils were randomly collected from the topsoil layer (0–10 cm) in the inter-row areas (8-m apart) located at the centre of each plot to avoid border interferences. After removal of crop residues, the soil samples were stored immediately at 4 °C in sterilized plastic pots. Each composite sample was formed from three 7-cm-diameter cores sampled within a 0.50 cm radius to minimize spatial variability and pooled on site (Bacon & Hudson, 2001; Tian *et al*., 2004).

Microbial counts

Three replicates of 5-g subsamples (dry weight equivalent) of each soil sample were suspended in 45 mL sterile 0.1% sodium pyrophosphate-one-quarter-strength Ringer solution (NaCl 2.25 g/L, KCl 0.105 g/L, $CaCl_2$ 0.045 g/L, $NaHCO_3$ 0.05 g/L and citric acid 0.034 g/L) and sonicated for 2 min to disperse microbial cells. 10-fold serial dilutions of the supernatants were made in sterile one-quarter-strength Ringer solution. Aliquots were spread plated in triplicate on 1/10 strength TSA (Tryptic Soy Agar) medium amended

with 0.1 mg/mL cycloheximide for bacterial counting and inoculated in malt extract agar (MEA) medium containing 0.03 mg/ml streptomycin and 0.02 mg/mL tetracycline in triplicate for fungal counting. Counting took place after incubation at 28 °C during 72 h for bacteria and 120 h for fungi.

Microbial community-level physiological profiles

Sole-carbon-source utilization patterns of soil microbial communities, also called community-level physiological profiles (CLPPs), were assessed using the Biolog® 96-well Eco-Microplates™ (AES Laboratoire, France), containing 31 different carbon sources for bacteria and the Biolog® FF MicroPlates™ (AES Laboratoire) containing 93 different carbon sources for fungi. For each well of the Biolog® MicroPlates™, an aliquot of 100 μL of the soil dilution prepared previously for Petri dishes and containing ca. 10 000 micro-organisms/mL was used for the inoculation. For the Biolog® FF plates only, 50 μg/mL gentamycin and 50 μg/mL streptomycin were included to prevent interference of turbidity or colour development by bacteria.

The microplates were incubated at 25 °C in the dark, and colour development was measured every 24 h over a 144-h period using a Microplate E-Max Reader (Bio-Rad; Hercules, CA, USA) with an E590-nm wavelength filter. The data were collected using Microlog 4.01 software (Biolog, CA, USA). The substrate utilization profiles were analysed on well absorbance values at the 96-h observation period. After correction using the blank cell, the OD data were first normalized by dividing the corrected OD of each well by the average well colour development (AWCD) and then subjected to a logarithmic transformation according to Weber *et al.* (2008).

Data were analysed to determine metabolic diversity indices, including AWCD (provides a measure of total cultural bacterial activity), Shannon's substrate diversity index (*H'*), substrate evenness (*E*, equitability of activities across all utilized substrates) and substrate richness (*S*, the number of utilized substrates) (Zak *et al.*, 1994). The carbon substrates of the Biolog® plates were divided into ten and eight main groups of compounds for fungi and bacteria, respectively, and the AWCD values for each group were calculated.

Soil enzyme activities

Soil enzyme activities were measured on fresh soil samples. β-glucosidase activity was determined according to Eivazi and Tabatabai (1988), and the units expressed as μg *p*-nitrophenol/h/g of soil. The dehydrogenase assay was performed according to the method of von Mersì and Shinner (1991), and the units expressed as μg triphenyl-formazan/h/g of soil. Fluorescein diacetate (FDA) hydrolytic activity was determined according to Green *et al.* (2006) and expressed as μg fluorescein/h/g of soil; protease activity was determined according to Geisseler *et al.* (2009), and the units expressed as μg tyrosine/h/g of soil.

Identification of fungi and Streptomyces

Colonies isolated from microbial counts were transferred individually in a laminar flow cabinet into Petri dishes containing potato dextrose agar (PDA) and incubated at 22–24 °C for 7–10 days. The colonies developed were used to prepare slides for observation by a compound optical microscope (Eclipse 80i; Nikon, Tokyo, Japan) under transmitted light, and then photographed (Digital Camera DS-Fi1 equipped with NIS-Elements Imaging Software; Nikon). Given the very high number of soil microbial groups, only the microbial groups identified microscopically over a definite threshold (at least 25 Petri dishes on a total of 1000 per treatment = 2.5%) were taken into account for the following molecular identification.

Molecular methods based on PCR followed by sequencing of the amplicons obtained were used to confirm the identification of the isolates by a light microscope. In this case, the total nucleic acids were extracted from the pure colonies using a commercial kit (Dneasy Plant mini kit; Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions. The DNA was amplified using the universal primer pair ITS4/ITS5 (White *et al.*, 1990) whose targets are the internal transcribed spacer regions (ITS) of ribosomal DNA (rDNA). Sterile distilled water was used as negative control. Amplifications were performed with an automated thermal cycler in a 50 μL reaction volume. Five microlitres of the amplified products were visualized using an UV transilluminator after electrophoresis in 1.2% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8) and stained with ethidium bromide (0.5 μg/mL) in the presence of a molecular weight marker (1-kb DNA ladder; Life Technologies, Carlsbad, CA, USA). After further electrophoresis in a 1.5% agarose gel, the same products were sampled, purified with the QIAquick Gel Extraction kit (Qiagen) and directly sequenced. The resulting sequences were compared with those available in GenBank using the BLAST software (Altschul *et al.*, 1997).

Statistical analysis

Statistical analysis of data was carried out using STATISTICA, version 6.0 (StatSoft Inc.; Vigonza, PD, Italy). The values of bacterial and fungal microbial counts, Biolog® metabolic indices (AWCD, *H'*, *E* and *S*), and soil enzyme activities (four independent replicates for each treatment; $n = 4$) were treated by analysis of variance (ANOVA) with orchard management as the factor. Means

were separated according to Duncan's multiple comparison test at $P \leq 0.05$.

Results and discussion

Our results reveal significant differences between the sustainable, high carbon input soil management system (ST) and the conventional, low carbon input system (CT).

Microbial counts and identification

The impact of sustainable orchard management was reflected in the microbial populations. The total fungal number in ST was approximately 7.4-fold greater than in CT, whereas the total bacterial number was 3.6-fold greater (Table 1). Thus, soil fungi responded strongly to changes induced by the presence of spontaneous weeds and pruning residues on the soil (Borken *et al*., 2002; Peixoto *et al*., 2006). The high fungal number in ST is an important benefit as fungi are able to colonize the rhizosphere using root exudates as a carbon source and in turn supply roots with ammonium and nitrates. They also play a key role in the biological control of root pathogens and in the maintenance of soil health. Govaerts *et al*. (2008) also report that total bacterial counts are generally higher when residues are retained than when they are removed, and with minimum compared with intensive tillage. Organic carbon inputs can induce a general improvement in soil conditions such as increased soil aeration, improved temperature and moisture buffering capacity of the soil as well as greater carbon content in the surface layer (Brady & Weil, 2008).

Observations by light microscopy and molecular ITS analysis of rDNA led to the identification of the fungal groups listed in Table 2. PCR amplification with ITS4/ITS5 primer produced visible amplicons around 600 bp. No amplification products were found when DNA was replaced by sterile distilled water. The resulting sequences compared with sequences present in GenBank confirmed the identification by light microscopy. The number of positive plates and fungal groups in ST was markedly greater than that of CT soils (Table 2), reinforcing the hypothesis of greater fungal diversity in ST soils compared with CT. The fungi detected in ST included some physiological groups related to soil fertility, such as those involved in important steps of carbon cycling (*Aspergillus* and *Penicillium* which are some of the major decomposers of complex polymers such as lignocelluloses and chitin). In particular, the actinomycetes of the genus *Streptomyces* as observed in ST soils are abundant across a wide range of soil types and synthesize vitamins, siderophores, amino acids and organic acids, useful for plant growth, and antibiotics, such as streptomycin and chloramphenicol, against some soil-borne root pathogens (Postma *et al*., 2008).

Table 1 Total bacterial and fungal counts in soils sampled from the sustainable (ST) and conventional (CT) treatments

	Fungi (CFU $\times$ 10^4/g dry soil)	Bacteria (CFU $\times$ 10^6/g dry soil)
ST	21.4 ± 11.8 a	35.6 ± 16.7 a
CT	2.9 ± 1.9 b	10.0 ± 2.6 b

Values are means ± standard deviation (n = 4). Means with different letters are significantly different between the two treatments at $P \leq 0.05$, according to Duncan's multiple comparison test.

Table 2 Main fungal groups and *Streptomyces* detected in soils sampled from the sustainable (ST) and conventional (CT) treatments

ST	CT
Acremonium	*Aspergillus*
Alternaria	*Mucor*
Armillaria	*Rosellinia*
Aspergillus	
Cladosporium	
Cylindrocarpon	
Microdochium	
Penicillium	
Phaeoacremonium	
Phialophora	
Rosellinia	
Streptomyces	

Microbial metabolic activity

Soil bacterial metabolic diversity indices estimated by Biolog® CLPP refer to the number, variety and variability of micro-organisms, including diversity within and between groups, and they are usually greater in sustainable than in

Table 3 Fungal and bacterial metabolic diversity indices measured by Biolog® (H' = Shannon's substrate diversity index; E = substrate evenness; S = substrate richness) in soils sampled from the sustainable (ST) and conventional (CT) treatments

	AWCD	H'	E	S
Fungi				
ST	0.75 ± 0.02 a	4.25 ± 0.03 a	2.48 ± 0.02 a	51.60 ± 1.52 a
CT	0.51 ± 0.12 b	3.85 ± 0.20 b	2.41 ± 0.07 a	39.75 ± 3.86 b
Bacteria				
ST	0.83 ± 0.09 a	3.20 ± 0.03 a	2.54 ± 0.07 a	18.40 ± 1.52 a
CT	0.36 ± 0.07 b	2.97 ± 0.05 b	2.67 ± 0.10 a	13.00 ± 1.58 b

AWCD, average well colour development. Values are means ± standard deviation (n = 4). Means with different letters are significantly different between the two treatments at $P \leq 0.05$ according to Duncan's multiple comparison test.

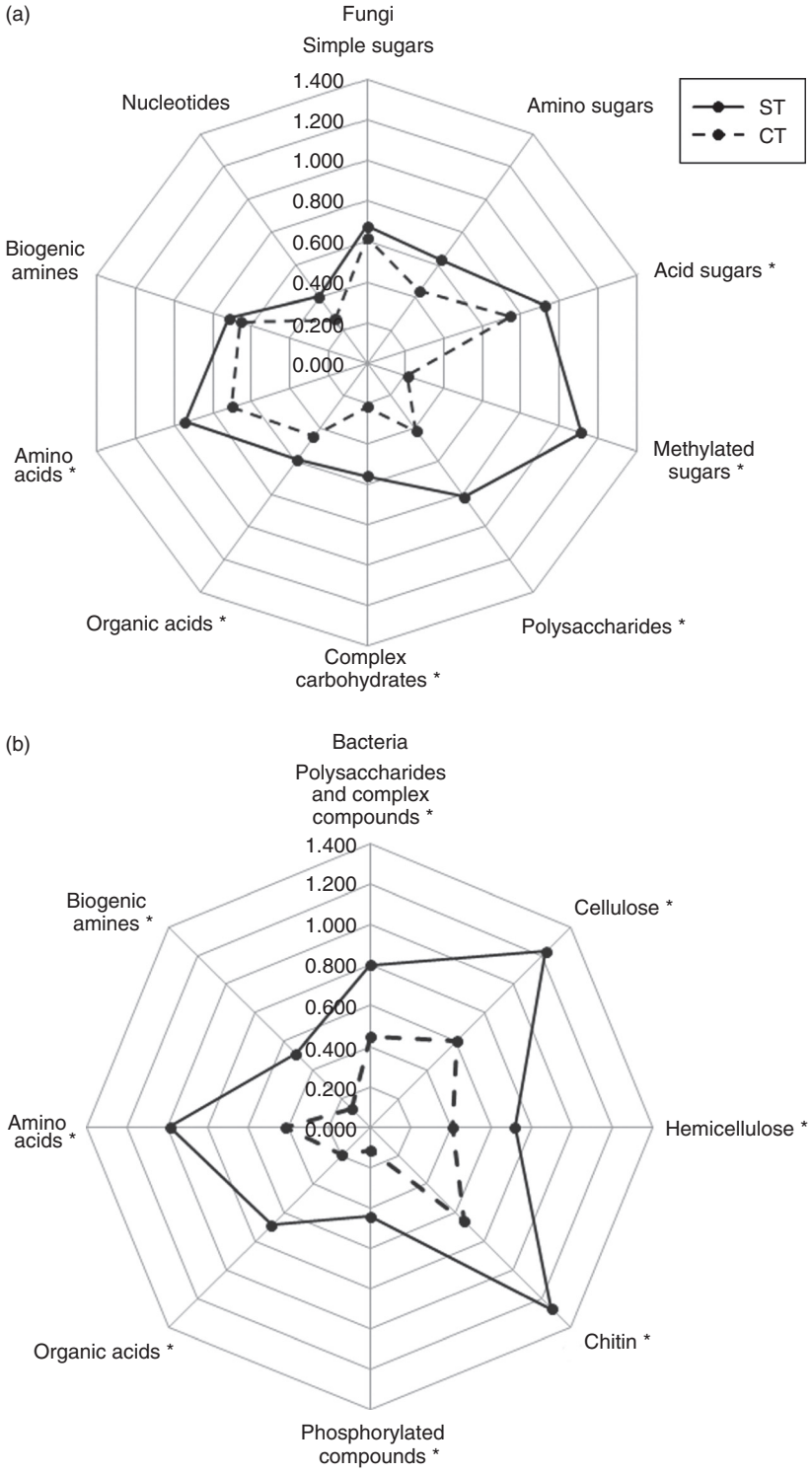

Figure 1 Radar diagrams of (a) fungal and (b) bacterial AWCD of all the principal classes of carbon substrates, identified by Biolog®, in soils sampled from the sustainable (ST; continuous line) and conventional (CT; dashed line) treatments. Means ($n = 4$) with the * are significantly different between the two treatments at $P \leq 0.05$, according to Duncan's multiple comparison test.

conventional systems (Mäder *et al*., 1996; Bucher & Lanyon, 2005; Govaerts *et al*., 2008). The increase in organic inputs due to cover crops and plant residues can be an important discriminating element for microbial substrate utilization according to Carrera *et al*. (2007). Furthermore, the retention of crop residues in the field and changes in soil

organic matter can affect the metabolic diversity of the soil microbial communities evaluated by Biolog® CLPP (Bending *et al*., 2002; Govaerts *et al*., 2008).

For both bacteria and fungi, the analysis of Biolog® metabolic indices showed that total AWCD, *H'* and *S* were significantly greater in ST than in CT (Table 3), indicating a higher microbial diversity and complexity in ST. The values of *E* measured for fungi were not statistically different between ST and CT (Table 3). Large values of *E* indicate many groups of micro-organisms, and thus, it seems that the long application of conventional management did lead to a predominance of a few groups of fungi or bacteria similar to ST.

Indices of metabolic diversity do not necessarily reflect the composition of the bacterial communities as two communities can have similar values of metabolic diversity indices but utilize different substrates. In our case, Biolog® absorbance values demonstrated that the AWCD of all the principal classes of fungal and bacterial carbon substrates was significantly greater in the ST, with only some exceptions for fungi (simple sugars, amino sugars, biogenic amines and nucleotides) (Figure 1). This result confirms the higher microbial metabolic activity as a result of the greater inputs of organic materials in ST.

Biolog® FF MicroPlates™ have been used to assess carbohydrate use and to determine metabolic profiling of soil fungi (Hobbie *et al*., 2003; Singh, 2009), but our study reports for the first time the utilization of these specific plates to determine the fungal catabolic profile using a procedure similar to that adopted for bacteria by Zak *et al*. (1994). This was possible because Biolog® FF plates used usually for fungal identification contain a specific tetrazolium dye that can be metabolized by fungi but not by bacteria (Preston-Mafham *et al*., 2002). The latter were inhibited by the antibiotics added to Biolog® FF plates as explained earlier.

The degree of soil microbial activity correlated well with the activity of key soil enzymes (Nannipieri *et al*., 2003). The extra-cellular soil enzyme *β*-glucosidase is of basic importance in the soil carbon cycle as it catalyses the last reaction of cellulose degradation by hydrolysing cellobiose into glucose, thus making it available to micro-organisms. The activity of this enzyme was greater in ST (Table 4) where biomass inputs were greater, and it is a reliable index of a productive soil (Eivazi & Tabatabai, 1988). Dehydrogenases isoforms are common to most micro-organisms with a predominantly intracellular localization, and they are able to oxidize organic matter. In our case, dehydrogenase activity did not differ statistically between the two treatments (Table 4). These data are not surprising as this enzyme is a good indicator of the vitality of fungal and bacterial soil populations and is not related to microbial metabolic activity (von Mersì & Shinner, 1991). The activity of FDA summarizes the hydrolytic activity of several fungal and bacterial enzymes and represents an overall index of the potential for release of organic nutrients from organic matrices (Green *et al*., 2006). The activity of this enzyme was not significantly different between ST and CT (Table 4). Proteases are a group of hydrolytic enzymes linked to the nitrogen cycle, and their function is to catalyse the hydrolysis of proteins, oligopeptides and dipeptides, until the release of ammonium. Like the glucosidases, they represent an useful index for the evolution of soil organic matter (Geisseler *et al*., 2009). In our experiment, protease activity was greater in the orchard managed with sustainable agronomic practices (Table 4). It is of interest to note that the improvement in soil microbial activity and diversity due to 12 yr of sustainable management was similar to that observed in other fields cultivated organically with similar agronomic practices and for the same period (Nautiyal *et al*., 2010).

Table 4 Soil enzyme activities in soils sampled from the sustainable (ST) and conventional (CT) treatments

	β-Glucosidase (Units/g soil)	Dehydrogenase (Units/g soil)	FDA hydrolase (Units/g soil)	Protease (Units/g soil)
ST	187.0 ± 4.7 a	194.0 ± 19.9 a	4.4 ± 0.4 a	6.7 ± 1.5 a
CT	151.3 ± 0.6 b	163.9 ± 1.6 a	4.2 ± 0.2 a	3.1 ± 0.9 b

Values are means ± standard deviation ($n = 4$). Means with different letters are significantly different between the two treatments at $P \leq 0.05$, according to Duncan's multiple comparison test.

Conclusions

The results from this study demonstrate that soil micro-organisms respond positively to sustainable orchard management using no-till and application of organic matter derived from cover crops and pruning. The consequences were profound changes in the soil microbial community with greater complexity and metabolic diversity. In the Mediterranean, 16% of the total cultivable land is occupied by fruit groves (FAOSTAT, 2012) and soils are often subjected to desertification. The results from this study confirm the need to advise farmers to select methods of soil management based on organic matter inputs to improve soil functionality and agronomic productivity.

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