

Genetic, Functional, and Metabolic Responses of Soil Microbiota in a Sustainable Olive Orchard

Adriano Sofo,¹ Assunta Maria Palese,¹ Teresa Casacchia,² Giuseppe Celano,¹ Patrizia Ricciuti,³ Maddalena Curci,³ Carmine Creccchio,³ and Cristos Xiloyannis¹

Abstract: The aim of the present work was to evaluate the effects of two soil management systems so called *sustainable treatment* (ST) and *conventional treatment* (CT) on the composition and on genetic, functional, and metabolic diversity of soil microbial communities in a Mediterranean olive orchard. The ST system included no-tillage, integrated chemical fertilization, and organic matter inputs from drip irrigation, spontaneous cover crops, and pruning material. Microbial analyses were carried out by an integrated approach of culture-dependent (microbial cultures and Biolog) and culture-independent methods (denaturing gradient gel electrophoresis [DGGE]). After 7 years of treatment, average olive yield was 8.4 and 3.1 t ha⁻¹ year⁻¹ in ST and CT, respectively. Conventional treatment had a significantly higher number of total culturable bacteria and actinomycetes compared with ST, whereas fungi were significantly lower. In ST, the number of ammonifying bacteria, proteolytic bacteria, and *Azotobacter* in the wetted areas under the drippers (ST-WET) was significantly higher than along interrows (ST-INTER). The DGGE analysis of microbial 16S/18S rDNA showed differences between ST and CT, whereas 16S/18S rRNA DGGE bands of ST-WET clustered differently from those of CT and ST-INTER. Some Biolog metabolic indexes were significantly different between ST and CT. The results revealed qualitative and quantitative changes of soil microbial communities in response to sustainable agricultural practices that stimulate soil microorganism activity and improve olive yield and fruit quality.

Key words: *Olea europaea*, Biolog, DGGE, fertirrigation, sustainable soil management.

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The surface layers of the pedosphere are the habitat for a high number of microorganisms organized in colonies and associated with organic matter particles (Brady and Weil, 2008). These microbial communities play a key role in pedogenetic processes and in nutrient availability and turnover (Rao et al., 1995; Holland, 2004).

The use of culture-based microbiological techniques with specific cultural media allows the isolation of important physiological groups of microorganisms related to soil fertility, such as bacteria involved in N cycle (Heritage et al., 1999; Zaitlin et al., 2004). Fungi, actinomycetes, and bacteria, the most abundant and metabolically active soil populations, are major decomposers of complex polymers such as lignocelluloses and chitin,

use root exudates as carbon source, supply roots with easily assimilable nitrates, and are involved in the suppressive action of the soil (Griffiths, 1994; Govaerts et al., 2008).

An integrated approach of culture-dependent and culture-independent methods has provided new tools to study the whole soil microbiota (Singh et al., 2006). One of the most useful molecular techniques to reveal qualitative genetic (DNA) and functional (RNA) changes in the structure of soil bacterial and fungal communities is based on the characterization of soil-extracted nucleic acids by the amplification of regions of the bacterial and fungal ribosomal RNA gene (*16 rRNA* and *18 rRNA*, respectively) resolved by denaturing gradient gel electrophoresis (DGGE) (Creccchio et al., 2004; Gelsomino and Cacco, 2006). Metabolic microbial community diversity in the structure of soil bacteria communities can be estimated using the Biolog metabolic assay based on the ability of microbial isolates to oxidize different carbon and N sources (Zak et al., 1994). The community-level physiological profiles (CLPP), obtained by the Biolog method, are used to differentiate microbial populations from various soil environments or subjected to various treatments (Gelsomino et al., 2006; Singh et al., 2006).

A new approach in fruit orchard management is imposed by environmental emergencies, such as soil degradation and water shortage (Lal, 2004; Hochstrat et al., 2006). Furthermore, in semiarid areas, the use of agronomic techniques able to improve or preserve soil quality, health, and fertility is particularly recommended (Kushwaha and Singh, 2005; Govaerts et al., 2008). Changes in the structure and dynamics of soil microbial communities, as response to different soil management in agricultural systems, represent an interesting assessment index of soil status with respect to its quality and complexity (Visser and Parkinson, 1992; Anderson, 2003). Particularly in olive groves, a positive influence of sustainable orchard management systems on soil biochemical characteristics and soil microbial genetic diversity was observed (Hernández et al., 2005; Benitez et al., 2006; Moreno et al., 2009).

The present study was performed to explore the effect of sustainable agricultural management systems on genetic, functional, and metabolic diversity of soil microbial communities by using a combination of culture-dependent and -independent methods. A particular emphasis was given to the microorganisms involved in N cycle because N is the most important nutrient influencing vegetative growth and yield quality and quantity in olive trees (Fernández-Escobar et al., 2006; Morales-Sillero et al., 2007, 2009). The trial was carried out during a 7-year period in a mature olive orchard located in southern Italy under semiarid conditions. The effects of a sustainable management on the productive response of the olive trees and on fruit characteristics were also examined.

MATERIALS AND METHODS

Horticultural Practices, Yield, and Fruit Features

The study was carried out in a mature olive orchard (*Olea europaea* L.-cv Maiatica, a double-aptitude variety) located in Southern Italy (Ferrandina-Basilicata Region; 40°29'N, 16°28'E).

¹Dipartimento di Scienze dei Sistemi Colturali, Forestali e dell'Ambiente, Università degli Studi della Basilicata, Viale dell'Ateneo Lucano 10-85100, Potenza, Italy. Mr. Adriano Sofo is corresponding author. E-mail: adriano.sofa@unibas.it

²CRA, Centro di Ricerca per l'Olivicoltura e l'Industria Olearia, c. da Li Rocchi-Vermicelli, 87036 Rende, Cosenza, Italy.

³Dipartimento di Biologia e Chimica Agroforestale e Ambientale, Università degli Studi di Bari, Via Orabona 4-70126 Bari, Italy.

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TABLE 1. Chemical Parameters of Treated Municipal Wastewater (Mean Values 2000–2006)

Parameter	Unit of Measure	Value
pH		7.6
Conductivity	$\mu\text{S cm}^{-1}$	884
Na	mg L^{-1}	121.3
Mg	mg L^{-1}	13.8
Ca	mg L^{-1}	67.8
N (NO_3^-)	mg L^{-1}	18.3
N (NH_4^+)	mg L^{-1}	0.0
B	mg L^{-1}	1.0
K	mg L^{-1}	17.0
P	mg L^{-1}	1.0
Chemical oxygen demand	mg L^{-1}	180

Olive trees (>50 years) were vase trained and planted at a distance of about $8 \text{ m} \times 8 \text{ m}$. The climate in the area is classified as semiarid. The annual precipitation of 561 mm (mean 1976–2006) falls mostly in the winter. The mean annual temperature ranges from 15°C to 17°C . The soil of the experimental grove is a sandy loam, classified as a Haplic Calcisol (FAO, 1998), with a mean bulk density of 1.5 t m^{-3} . The top 60-cm soil layer had an average pH ($\pm\text{SD}$) of 7.4 ± 0.4 , organic carbon content of $7.0 \pm 3.8 \text{ g kg}^{-1}$, total N equal to $0.8 \pm 0.2 \text{ g kg}^{-1}$, and available phosphorus and potassium of 11.7 ± 5.9 and $104 \pm 70 \text{ mg kg}^{-1}$, respectively.

In 2000, the olive orchard was split into two parts managed according to sustainable agronomic techniques (*sustainable treatment* [ST]) and conventional ones (*conventional treatment* [CT]).

The ST was irrigated with municipal wastewater treated by a pilot unit according to simplified schemes (Lopez et al., 2006; Palese et al., 2009). The reclaimed wastewater was generally distributed from May to October by drip irrigation (six self-compensating drippers per plant delivering 8 L h^{-1}).

Irrigation volume applied during the annual growth season averaged 293 mm (2000–2006). Chemical characteristics of the treated wastewater are reported in Table 1. The ST soil surface was covered by spontaneously growing weeds and grasses and mowed at least twice a year. Irrigated trees were lightly pruned each year to improve fruiting potential by controlling the amount of fruiting wood and enhancing flower bud differentiation. Crop

residues and pruning material ($4.4 \text{ t ha}^{-1} \text{ year}^{-1}$ organic carbon, mean 2000–2006) were left on the ground as mulch. Such sustainable practices have led to an increase of soil organic carbon in the 0- to 10-cm layer (18.7 g kg^{-1}). Fertilizers were applied along the growing seasons by a guided fertirrigation, taking into account wastewater and soil chemical composition, and mineral element balance in the orchard system (cover crops and pruning material contributions, amount of fruit removed from the olive grove) (Palese et al., 2008). Particularly, the average annual amounts of organic C, N, P, and K distributed by the treated wastewater were 124, 54, 3, and $50 \text{ kg ha}^{-1} \text{ year}^{-1}$, respectively. An integrative amount of 40 kg ha^{-1} of NO_3^- -N per year was distributed by fertirrigation during fruit set and pit hardening phase to entirely satisfy olive nutrient needs. Pest and disease control was performed according to the regional service recommendations for commercial olive groves.

The CT was grown under rain-fed conditions and managed according to the traditional horticultural practices of the area (Xiloyannis et al., 2008), that is, by tillage performed two to three times per year and mineral fertilization carried out once per year, in early spring, using ternary compounds (NPK 20-10-10 fertilizer at doses ranging from 300 to 500 kg ha^{-1}). In the CT, heavy pruning was performed every 2 years, and pruning residues were burned out of the field.

The olive harvest was performed in both treatments by means of a trunk shaker and nets. Yield was measured on 12 trees per treatment. Representative fruit samples were taken to measure fresh and dry weight of the whole drupe, the pulp, and the stone, and longitudinal and equatorial diameters. Fruit, pulp, and stone were dried to a constant weight at 65°C in a forced-draft oven. Pulp percentage and flesh to stone ratio were also determined on fresh weight basis.

Soil Sampling

In February 2007, soil sampling was performed in both the treatments (CT and ST). In particular, for ST, two sampling positions were chosen: the wetted area under the drippers (ST-WET) and the nonirrigated interrow area (ST-INTER). For CT, soil sampling was carried out in the interrow area. For each of these (CT, ST-WET, and ST-INTER), three composite samples of bulk soil (twenty 7-cm-diameter cores pooled on site) were randomly collected from the top soil layer (0–10 cm) and immediately stored in sterilized plastic pots at 4°C after removing visible crop residues. This sampling type was used to minimize spatial variability because the experimental set up refers to a

TABLE 2. Modified Cultural Media Used for the Identification of the Specific Bacterial Groups

Actinomycetes	g L^{-1}	Proteolytic	g L^{-1}	Ammonifying	g L^{-1}	Azotobacter	g L^{-1}
Starch	10.0	MnSO_4	0.5	MnSO_4	0.25	D-Glucose	5.0
Casein	0.5	K_2HPO_4	0.20	K_2HPO_4	0.20	K_2HPO_4	0.5
NaNO_3	1.0	KH_2PO_4	0.05	KH_2PO_4	0.05	KH_2PO_4	0.5
NaCl	1.5	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.15	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.10	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2
K_2HPO_4	1.1	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.02	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.02	CaCl_2	0.1
KH_2PO_4	0.5	MnSO_4	0.25	NaCl	0.12	NaCl	0.05
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.05	CaCl_2	0.1	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.03	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.01
CaCl_2	0.01	NaCl	0.12	Asparagine	0.25	Agar	15
Na_2CO_3	0.5	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.03	pH, 6.8 ± 0.2		pH, 6.8 ± 0.2	
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.01	Agar	15				
Agar	15	pH, 7.2 ± 0.2					
pH, 6.8 ± 0.2							

field trial and not to single plots (Bacon and Hudson, 2001; Tian et al., 2004).

Total and Specific 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 (2.25 g NaCl L⁻¹, 0.105 g KCl L⁻¹, 0.045 g CaCl₂ L⁻¹, 0.05 g NaHCO₃ L⁻¹, and 0.034 g citric acid L⁻¹) and sonicated for 2 min to disperse microbial cells. Ten-fold serial dilutions of the supernatants were made in sterile Ringer solution. Aliquots were spread plated in triplicate on 1:10 strength tryptic soy agar medium amended with 0.1 mg mL⁻¹ cycloheximide for bacterial counting, and inoculated in malt extract agar medium containing 0.03 mg mL⁻¹ streptomycin and 0.02 mg mL⁻¹ tetracycline (Lorch et al., 1998) in triplicate for fungal counting. Counting took place after suitable incubation period (72 h for bacteria and 120 h for fungi) at 28 °C.

Actinomycetes were isolated by using modified casein starch agar (Table 2) supplemented with 0.12 mg cycloheximide mL⁻¹ (Sigma Aldrich, New York, NY). *Azotobacter* species were isolated by modified Brown substrate, whereas proteolytic bacteria were quantified by the MPN method in a culture medium containing gelatin (Oxoid Lim, Hampshire, UK) (Table 2). Ammonifying bacteria were isolated in a liquid culture medium containing asparagine (Table 2) and incubated at 28 °C for 15 days. Successively, the presence of ammonium was revealed by Nessler reagent prepared by adding 50 g Hg₂I and 36.5 g KI, previously ground in a mortar to 150 g KOH in 1 L bidistilled water. *Pseudomonas* was cultured on *Pseudomonas* agar base medium (Oxoid) with the addition of *Pseudomonas* C-N supplement (Oxoid).

Polymerase Chain Reaction and DGGE

A direct method was used for DNA and RNA extraction from soil samples by a bead beater system. Samples of 500 mg of soil were processed by FastDNA Spin Kit for Soil (MP Biomedicals, Cleveland, OH) and RNA Power Soil Isolation Kit (MoBio, Carlsbad, CA). DNA and RNA extracts were stored at -20 °C and -80 °C, respectively. Nucleic acids quantity and quality were assayed on 0.7% agarose gel containing 0.5 µg mL⁻¹ ethidium bromide. Extracted RNA was retrotranscribed to c-DNA by RETROscript First Strand Synthesis Kit for reverse transcription polymerase chain reaction (Ambion, Austin, TX). DNA and c-DNA were amplified in a PCR thermocycler (Bio-Rad Laboratories, Hercules, CA) with the following primer pairs (MWG-Biotech AG, Germany): (i) 968F-1401R for the *16S rDNA* gene and (ii) FR1-GC and FF390 for the *18S rDNA* gene. The PCR amplifications were performed according to Crecchio et al. (2004). Amplification products were checked by electrophoresis on 1.5% agarose gel run at 10 V cm⁻¹ in 0.5× TBE buffer

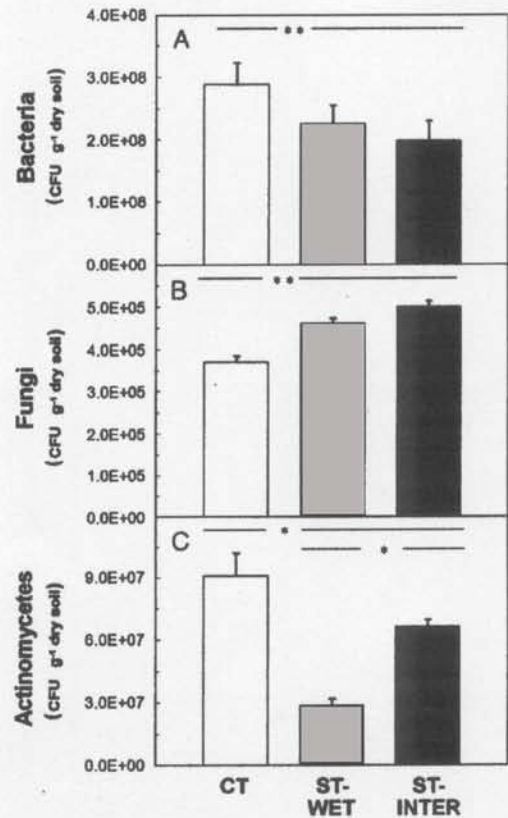


FIG. 1. Total cultural bacterial (A), fungal (B), and actinomycetes (C) counts in the three treatments: conventional ([CT] white bars), sustainable under the drippers ([ST-WET] gray bars), sustainable in the interrow area ([ST-INTER] black bars). The values represent the average (\pm S.D.) of three independent replicates for each soil treatment. Significance levels, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

and stained by ethidium bromide, using a low-range ladder (1,000–80 bp; Fermentas, Glen Burnie, MD).

The DGGE analysis was performed by the Bio-Rad DCode Universal Mutation detection System (Bio-Rad Laboratories). The PCR products (10 µL) were loaded into 6% (16S rDNA amplicons) or 8% (18S rDNA amplicons) polyacrylamide gel (37.5:1 acrylamide:bisacrylamide) with a urea-formamide parallel gradient (45%–60% for 16S rDNA and 30%–60% for 18S rDNA amplicons). Bacterial amplicons were separated by electrophoresis in 1× TAE buffer at 60 °C and at a constant voltage

TABLE 3. Fruit Characteristics and Pulp-to-Stone Ratio (Mean 2001–2006 \pm S.D.) in ST and CT

Parameter	Unit of Measure	ST	CT
Fruit fresh weight	g	3.8 \pm 0.92*	2.3 \pm 0.78
Longitudinal fruit diameter	mm	23 \pm 2.17*	20 \pm 2.88
Equatorial fruit diameter	mm	17 \pm 1.66*	14 \pm 1.79
Pulp	% on fresh weight basis	85 \pm 3.89*	78 \pm 5.03
Pulp-to-stone ratio	on fresh weight basis	5.8 \pm 1.54*	3.8 \pm 1.20

Asterisks (*) indicate that the values between ST and CT are significantly different at $P < 0.05$.

CT, conventional treatment; ST, sustainable treatment.

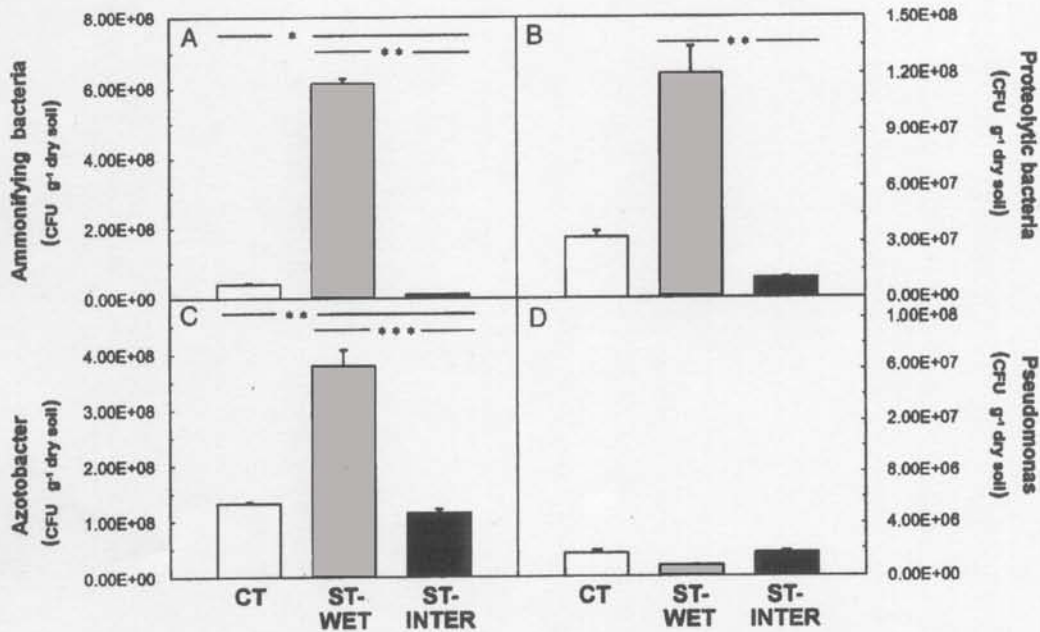


FIG. 2. Ammonifying bacteria (A), proteolytic bacteria (B), *Azotobacter* (C), and *Pseudomonas* (D) in the three treatments: conventional ([CT] white bars), sustainable under the drippers ([ST-WET] gray bars), sustainable in the interrow area ([ST-INTER] black bars). Statistics as in Fig. 1.

of 75 V for 15 h. Fungal amplicons were separated by electrophoresis in 1 × TAE buffer at 60 °C at a constant voltage of 75 V for 18 h. Sybr Green I-stained gels were photographed with Bio-Rad Gel Doc 2000 documentation system (Bio-Rad Laboratories).

Microbial Community Metabolic Profiles (Biolog)

Sole carbon source utilization patterns of soil microbial communities, also called *CLPP*, were assessed using the Biolog 96-well Eco-Microplates (AES Laboratoire, Combourg, France), containing 31 different carbon sources, with three replicates per treatment. Data were analyzed to determine metabolic diversity indices, including average well color development (AWCD, the mean of the blanked absorbance values for all the substrates that provides a measure of total cultural bacterial activity), Shannon substrate diversity index (H'), substrate evenness (E , equitability of activities across all used substrates), and substrate richness (S , the number of used substrates) (Zak et al., 1994). The microplates were incubated at 25 °C in the dark, and color development

was measured as optical density at 590 nm every 24 h during a 144-h period using a Microplate E-Max Reader (Bio-Rad) with a E590-nm wavelength filter. The data were collected by the Microlog 4.01 software (Biolog, Hayward, CA). The substrate utilization profiles were analyzed on well-absorbance values at the 120-h observation period.

Fingerprints and Statistical Analyses

Genetic fingerprints were analyzed with the Bionumerics software version 4.5 (Applied Maths, Belgium). The normalization of the profiles in each lane was carried out by loading a standard reference pattern in three different points of the denaturing gel. Profile comparison and clustering were performed by applying the unweighted pair-group method using arithmetic average algorithm based on the Pearson correlation coefficient (Boon et al., 2002).

The values of total and specific microbial groups and Biolog metabolic indices (AWCD, H' , E , and S) were treated by analysis of variance using two predefined contrasts: (i) CT versus ST-WET

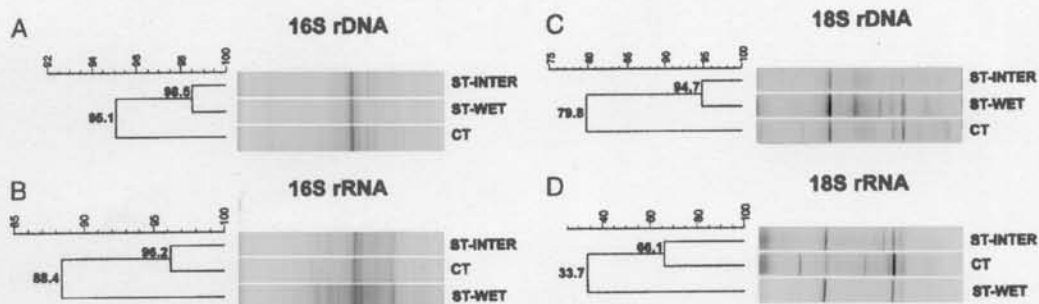


FIG. 3. Genetic 16S DGGE fingerprints of soil bacterial communities (A, B) and functional 18S DGGE fingerprints of soil fungal communities (C, D) in the three treatments: conventional (CT), sustainable under the drippers (ST-WET), sustainable in the interrow area (ST-INTER). Clustering was carried out using the unweighted pair-group method using arithmetic average method based on the Pearson correlation coefficient.

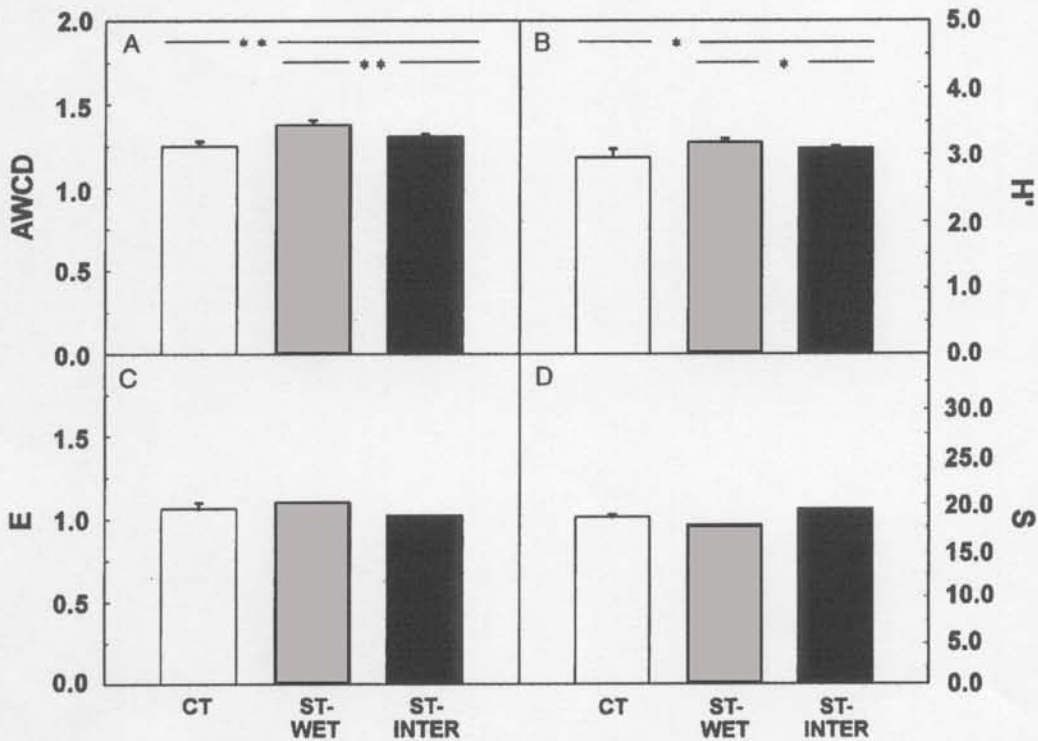


FIG. 4. A, Average well color development (AWCD), (B) Shannon's substrate diversity index (H), (C) substrate evenness (E), and (D) substrate richness (S) in the three treatments: conventional ([CT] white bars), sustainable under the drippers ([ST-WET] gray bars), sustainable in the interrow area ([ST-INTER] black bars). Statistics as in Fig. 1.

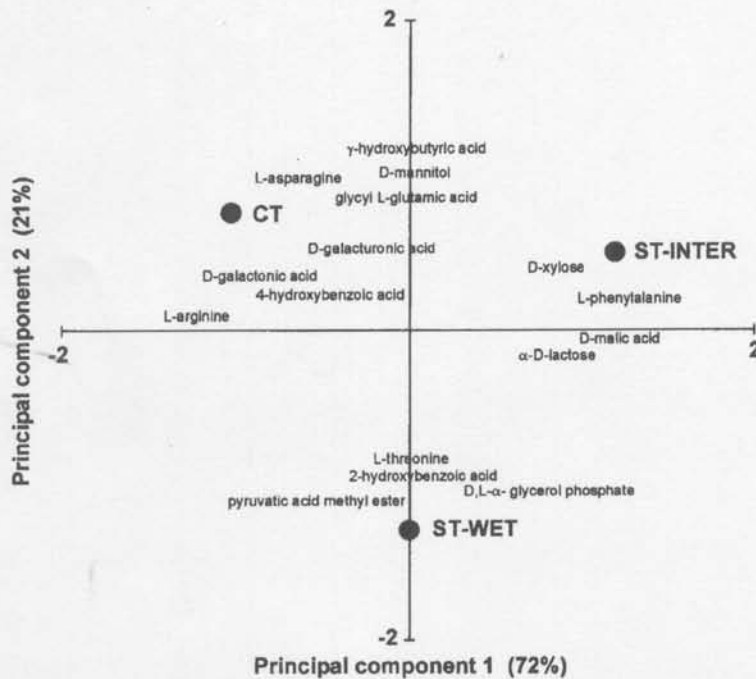


FIG. 5. Ordination biplot of principal component (PC) analysis of soil bacterial communities catabolic activity (conventional treatment [CT]; sustainable under the drippers [ST-WET]; sustainable in the interrow area [ST-INTER]). The 31 different carbon sources were indicated.

and ST-INTER; (ii) ST-WET versus ST-INTER. The principal component analysis (PCA) was applied to the Biolog absorbance values to characterize the structure of bacterial community by classifying treatments according to their substrate utilization patterns. The PCA analysis was performed on covariance matrix because all variables are based on the same scale and may contain useful information (Weber et al., 2008). After correction using the blank cell, the OD data were first normalized by the AWCD and then subjected to a logarithmic transform according to Weber et al. (2008). A graphic interpretation was obtained by biplot on the two dimensions of the main principal components (SPSS Statistics 17.0, SPSS Inc, Chicago, IL).

RESULTS

Tree Productive Responses and Olive Characteristics

Annual yields of the olive trees under ST management were nearly constant, with an average yield of $8.4 \text{ t ha}^{-1} \text{ year}^{-1}$ (mean 2001–2006), whereas the CT yields were significantly lower, $3.1 \text{ t ha}^{-1} \text{ year}^{-1}$ ($P < 0.05$). In addition, olive plants of CT showed a strong biannual bearing behavior, that is, low or no production, in 2002, 2004, and 2006. The starting year of the trial, 2000, was a nonbearing year for both the examined treatments. Drupes picked from the irrigated plants of the ST showed a significant increase in fresh weight, drupe size, pulp percentage, and pulp-to-stone ratio, which are important commercial parameters for table olives such as those produced by Maiatica variety (Table 3).

Microbial Counts

The different soil treatments significantly affected both total cultivable bacteria, significantly lower in ST-WET and ST-INTER ($P < 0.01$), and total fungal counts, significantly lower in CT compared with the two ST treatments ($P < 0.01$) (Figs. 1A, B). The number of actinomycetes was significantly greater in CT if compared with the two ST treatments ($P < 0.05$) and significantly higher in ST-INTER than in ST-WET ($P < 0.05$) (Fig. 1C).

The number of ammonifying bacteria, proteolytic bacteria, and *Azotobacter* isolated from ST-WET treatment was significantly higher than in ST-INTER ($P < 0.01$, $P < 0.01$ and $P < 0.001$, respectively; Figs. 2A–C). Moreover, CT significantly differed from the two ST soils both for ammonifying bacteria ($P < 0.05$) and *Azotobacter* ($P < 0.01$) (Figs. 2A, C). The number of microorganisms in both cases was intermediate between those of the ST-WET and ST-INTER treatments. *Pseudomonas* counts were not significantly different between CT and ST. Similarly, no differences between ST-WET and ST-INTER were detected (Fig. 2D).

Genetic and Functional Fingerprinting

The genetic dendrograms of bacterial 16S rDNA and fungal 18S rDNA showed that molecular patterns of CT were differed from patterns of the sustainable treatments (ST-INTER and ST-WET; Figs. 3A, B). Pearson similarity coefficients for 16S rDNA, ranging from 95.1 to 98.5, indicate that the bacterial community profiles were quite similar (Fig. 3A). In contrast, functional DGGE patterns of rRNA showed that irrigated sites under drip emitters (ST-WET) clustered separately from CT and ST-INTER both for bacteria and fungi ribosomal genes (Pearson coefficient, 88.4 and 33.7, respectively; Figs. 3C, D).

Metabolic Fingerprinting

Biolog metabolic indices showed that AWCD and H' were significantly affected ($P < 0.01$ and $P < 0.05$, respectively) by

soil treatment (ST vs. CT) (Figs. 4A, B). Moreover, ST-WET significantly differed from ST-INTER soils both for AWCD ($P < 0.01$) and H' ($P < 0.05$) (Figs. 4A, B). The values of E and S showed no significant differences between CT and ST including between ST-WET and ST-INTER (Figs. 4C, D).

The PCA of the Biolog absorbance values identified five principal components that accounted for total variance, primarily explained by the first two components (93%) (Fig. 5). The Principal Component 1 (accounting for the 72% of variance) separated CT from ST soils, whereas the Principal Component 2 (accounting for the 21% of variance) discriminated ST-WET from CT and ST-INTER. Table 4, reporting the differences in carbon substrate utilization calculated by PCA score and Biolog absorbance values (Weber et al., 2008), shows that 16 carbon resources, including carbohydrates, carboxylic acids, amino acids, and phenolic compounds, were determinant to differentiate the principal components.

TABLE 4. Correlation Between PCA Score and Biolog Absorbance Values for Individual Substrate Species

Substrate	PC-1	PC-2
Water		
Pyruvatic acid methyl ester	-0.934**	
Tween 40		
Tween 80		
α -Cyclodextrin		
Glycogen		
D-Cellobiose		
α-D-Lactose		0.903*
β -Methyl-D-glucoside		
D-Xylose		0.885*
Erythriol		
D-Mannitol	0.972**	
N-Acetyl-D-glucosamide		
D-Glucosaminic acid		
Glucose-1-phosphate		
D, L-α-Glycerol-phosphate	-0.976**	
D-Galactonic acid γ-lactone		-0.929**
D-Galacturonic acid		-0.813*
2-Hydroxybenzoic acid	-0.998**	
4-Hydroxybenzoic acid		-0.948**
γ-Hydroxybutyric acid	0.975**	
Itaconic acid		
α -Ketobutyric acid		
D-Malic acid		0.846*
L-Arginine		-0.983**
L-Asparagine	0.929**	
L-Phenylalanine		0.906*
L-Serine		
L-Threonine	-0.950**	
Glycyl-L-glutamic acid	0.834*	
Phenyl ethylamine		
Putrescine		

The substrates significantly correlated with PC are represented in bold letters.

*Correlation significantly different at $P < 0.05$.

**Correlation significantly different at $P < 0.01$.

PCA, principal component analysis.

DISCUSSION

The higher yield level and fruit quality of the olive trees under ST management (Table 3) can be attributed to the orchard management system. Primary benefits were gained from the optimal soil water content and from nutrients supplied by the fertirrigation management. The reclaimed wastewater used in this trial was rich in nutrients (especially N, P, and K) (Table 1), supplied by a fertirrigation scheduled based on orchard requirements.

An impact of this fertirrigation management was reflected in the fungal population. Fungal propagules were significantly higher in ST sites (Fig. 1B) likely because soil fungi rely on externally available nutrients (Borken et al., 2002; Peixoto et al., 2006). Thus, they responded promptly to changes in organic matter and nutrients derived from cover crops and wastewater irrigation/fertirrigation. The three different orchard management systems also caused significant differences in total culturable bacteria (Fig. 1A). Particularly interesting are the effects of agricultural management on the populations of the bacteria involved in N cycle (Fig. 2). In fact, the results showed that ST-WET site had a higher number of *Azotobacter*, proteolytic, and ammonifying bacteria compared with ST-INTER (Figs. 2A–C). The number of *Pseudomonas* was not significantly different between treatments (Fig. 2D). The particular conditions of ST-INTER soils with respect to ST-WET were reflected by the significantly higher actinomycetes number (Fig. 1C). Actinomycetes (e.g., *Streptomyces*) produce a number of enzymes that help degrade organic plant material, such as lignin and chitin, and so are abundant in soils rich in organic inputs such as ST-INTER soils. As a matter of fact, the pruning material is essentially cut and deposited by the machine into the interrow area.

Gelsomino et al. (2006) recently showed that amendments with wastewater caused changes in both the genetic and the functional structure of native soil bacterial populations. In our study, this effect was clear in both 16S and 18S rDNA genetic DGGE dendrograms, which discriminated the CT and ST systems (Figs. 3A, C). As for the fungal counts, the effects on bacterial community structures were very likely caused by the input of organic carbon derived from cover crops (ST-WET and ST-INTER) and wastewater irrigation/fertirrigation (ST-WET). The observed differences in DGGE profiles between CT and ST soils are in accordance to the results of Moreno et al. (2009), which highlighted the influence of nontillage and plant covering on the genetic diversity of soil microbiota (rDNA-DGGE). In contrast, differences in functional DGGE dendrograms of both 16S and 18S rRNA, which reflected the status of metabolically active microorganisms, indicated that the wastewater use is the main factor inducing changes in bacterial and fungal communities of sites under the emitters (ST-WET) (Figs. 3B, D). This is an important observation because qualitative changes of soil microbial communities in relationship to the water availability can be important in Mediterranean olive orchards, where the adoption of irrigation techniques based on drip irrigation and fertirrigation is worthwhile to save water and decrease the need of chemical fertilization.

Soil bacterial metabolic diversity indices estimated by Biolog CLPP are usually higher in sustainable than in conventional soils (Bucher and Lanyon, 2005; Govaerts et al., 2008). The values of AWCD and H' were significantly increased by the ST (Figs. 4A, B). In addition, comparing the two sustainable treatments, AWCD and H' were higher in ST-INTER soils (Figs. 4A, B), where cover crops could be an important discriminating element for microbial substrate utilization (Carrera et al., 2007). Indices of metabolic diversity do not necessarily

reflect the composition of the bacterial communities as two communities can have the same H' value but use different substrates. In fact, the segregation of the three orchard management systems on the biplot of PCA analysis (Fig. 5) highlighted clear differences in the catabolic activity of soil bacterial communities. Correlations between soil microbial metabolic diversity and organic carbon level have been reported by Degens et al. (2000). About half ($n = 16$) of the 31 species of Eco-plates carbon sources were significantly correlated with the two principal components, accounting for differences between CT and ST (Table 4). In particular, bacteria from CT soil used specifically more substrates than those from ST. Apparently, there were no clear correlation between the chemical composition of Eco-plates substrates and the treatments because CT as well as ST (WET and INTER) were discriminated by amino acids, carbohydrates, carboxylic acids, and phenolic compounds. No polymeric substrates (i.e., cyclodextrin, glycogen, tween) and amines (phenyl ethylamine and putrescine) accounted for the differences among treatments, suggesting that bacterial communities from ST and CT had no significantly different capacity to degrade two categories of compounds.

Our results demonstrated that soil microorganisms respond to the application of a sustainable orchard management with evident benefits for olive yield and quality. Sustainable orchards showed a higher microbial complexity and a genetic, functional, and metabolic diversity of soil microorganisms. The study of the response of soil microbiota to different agricultural management systems and the quantitative and qualitative analysis of soil microbial communities could lead to identify sustainable agricultural practices that support and stimulate soil microorganisms to improve olive orchard production.

REFERENCES

- Anderson, T. H. 2003. Microbial eco-physiological indicators to assess soil quality. *Agric. Ecosyst. Environ.* 98:285–293.
- Bacon, J. R., and G. Hudson. 2001. A flexible methodology for the characterisation of soils: A case study of the heavy metal status of a site at Dornach. *Sci. Total Environ.* 264:153–162.
- Benitez, E., R. Nogales, M. Campos, and F. Ruano. 2006. Biochemical variability of olive-orchard soils under different management systems. *Appl. Soil Ecol.* 32:221–231.
- Boon, N., W. De Windt, W. Verstraete, and E. M. Top. 2002. Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. *FEMS Microbiol. Ecol.* 39:101–112.
- Borken, W., A. Muhs, and F. Beese. 2002. Changes in microbial and soil properties following compost treatment of degraded temperate forest soils. *Soil Biol. Biochem.* 34:403–412.
- Brady, N. C., and R. R. Weil. 2008. *Elements of the Nature and Properties of Soils*. Pearson Prentice Hall, Upper Saddle, River, NJ.
- Bucher, A. E., and L. E. Lanyon. 2005. Evaluating soil management with microbial community-level physiological profiles. *Appl. Soil Ecol.* 29:59–71.
- Carrera, L. M., J. S. Buyer, B. Vinyard, A. A. Abdul-Baki, L. J. Sikora, and J. R. Teasdale. 2007. Effects of cover crops, compost, and manure amendments on soil microbial community structure in tomato production systems. *Appl. Soil Ecol.* 37:247–255.
- Crecchio, C., M. Curci, M. D. R. Pizzigallo, P. Ricciuti, and P. Ruggiero. 2004. Effects of municipal solid waste compost amendments on soil enzyme activities and bacterial genetic diversity. *Soil Biol. Biochem.* 36:1595–1605.
- Degens, B. P., L. A. Schippers, G. P. Sparling, and M. Vojvodic-Vukovic. 2000.

- Decreases in organic C reserves in soils can reduce the catabolic diversity of soil microbial communities. *Soil Biol. Biochem.* 32:189–196.
- FAO, 1998. World Reference Base for Soil Resources. Rome, Italy.
- Fernández-Escobar, R., G. Beltrán, M. A. Sánchez-Zamora, J. García-Novelo, M. P. Aguilera, and M. Uceda. 2006. Olive oil quality decreases with nitrogen over-fertilization. *HortScience* 41:215–219.
- Gelsomino, A., and G. Cacco. 2006. Compositional shifts of bacterial groups in a solarized and amended soil as determined by denaturing gradient gel electrophoresis. *Soil Biol. Biochem.* 38:91–102.
- Gelsomino, A., L. Badalucco, R. Ambrosoli, C. Crecchio, E. Puglisi, and S. M. Meli. 2006. Changes in chemical and biological soil properties as induced by anthropogenic disturbance: A case study of an agricultural soil under recurrent flooding by wastewaters. *Soil Biol. Biochem.* 38: 2069–2080.
- Govaerts, B., M. Mezzalama, K. D. Sayre, J. Crossa, K. Lichter, V. Troch, K. Vanherck, P. De Corte, and J. Deckers. 2008. Long-term consequences of tillage, residue management, and crop rotation on selected soil microflora groups in the subtropical highlands. *Appl. Soil Ecol.* 38:197–210.
- Griffiths, B. S. 1994. Soil nutrient flow. *In: Soil Protozoa*. Darbyshire, J. F. (ed.). CAB International, Wallingford, UK, pp. 65–91.
- Heritage, J., E. G. V. Evans, and R. A. Killington. 1999. *Microbiology in Action*. Cambridge University Press, Cambridge, UK.
- Hernández, A. J., C. Lacasta, and J. Pastor. 2005. Effects of different management practices on soil conservation and soil water in a rainfed olive orchard. *Agric. Water Manag.* 77:232–248.
- Hochstrat, R., T. Wintgens, T. Melin, and P. Jeffrey. 2006. Assessing the European wastewater reclamation and reuse potential—a scenario analysis. *Desalination* 188:1–8.
- Holland, J. M. 2004. The environmental consequences of adopting conservation tillage in Europe: Reviewing the evidence. *Agric. Ecosyst. Environ.* 103:1–25.
- Kushwaha, C. P., and K. P. Singh. 2005. Crop productivity and soil fertility in a tropical dryland agro-ecosystem: Impact of residue and tillage management. *Exp. Agric.* 41:39–50.
- Lal, R. 2004. Soil carbon sequestration to mitigate climate change. *Geoderma* 123:1–22.
- Lopez, A., A. Pollice, A. Lonigro, S. Masi, A. M. Palese, G. L. Girelli, A. Toscano, and R. Passino. 2006. Agricultural wastewater reuse in Southern Italy. *Desalination* 187:323–334.
- Lorch, H. J., G. Benckiser, and J. C. G. Ottow. 1998. Basic methods for counting microorganisms in soil and water. *In: Methods in Applied Soil Microbiology and Biochemistry*. K. Aleph, P. Nannipieri (eds.). Academic Press, London, UK, pp. 146–161.
- Morales-Sillero, A., R. Jiménez, J. E. Fernández, A. Troncoso, and G. Beltrán. 2007. Influence of fertigation in “Manzanilla de Sevilla” olive oil quality. *HortScience* 42:1157–1162.
- Morales-Sillero, A., J. E. Fernández, J. Ordoñez, J. A. Pérez, J. Liñán, E. P. López, I. Girón, and A. Troncoso. 2009. Plant-soil interactions in a fertigated “Manzanilla de Sevilla” olive orchard. *Plant Soil* 319:147–162.
- Moreno, B., S. Garcia-Rodriguez, R. Cañizares, J. Castro, and E. Benítez. 2009. Rainfed olive farming in south-eastern Spain: Long-term effect of soil management on biological indicators of soil quality. *Agric. Ecosyst. Environ.* 131:333–339.
- Palese, A. M., G. Celano, and C. Xiloyannis. 2008. Acque per le fertirrigazioni: Reflue, urbane e depurate. *Olivio Olio* 5:48–53.
- Palese, A. M., V. Pasquale, G. Celano, G. Figliuolo, S. Masi, and C. Xiloyannis. 2009. Irrigation of olive groves in southern Italy with treated municipal wastewater: Effects on microbiological quality of soil and fruits. *Agric. Ecosyst. Environ.* 129:43–51.
- Peixoto, R. S., H. L. C. Coutinho, B. Madari, P. L. O. A. Machado, N. G. Rumjanek, J. D. Van Elsas, L. Seldin, and A. S. Rosado. 2006. Soil aggregation and bacterial community structure as affected by tillage and cover cropping in the Brazilian Cerrados. *Soil Till. Res.* 90:16–28.
- Rao, A. V., J. C. Tarafdar, S. K. Sharma, P. Kumar, and R. K. Aggarwal. 1995. Influence of cropping systems on soil biochemical properties in an arid rain-fed environment. *J. Arid Environ.* 31:237–244.
- Singh, B. K., S. Munro, E. Reid, B. Ord, J. M. Potts, E. Paterson, and P. Millard. 2006. Investigating microbial community structure in soils by physiological, biochemical and molecular fingerprinting methods. *Eur. J. Soil Sci.* 57:72–82.
- Tian, G., J. M. Vose, D. C. Coleman, C. D. Geron, and J. T. Walker. 2004. Evaluation of the effectiveness of riparian zone restoration in the southern Appalachians by assessing soil microbial populations. *Appl. Soil Ecol.* 26:63–68.
- Visser, S., and D. Parkinson. 1992. Soil biological criteria as indicators of soil quality: Soil microorganisms. *Am. J. Altern. Agric.* 7:33–37.
- Weber, K. P., M. Gehder, and R. L. Legge. 2008. Assessment of changes in the microbial community of constructed wetland mesocosms in response to acid mine drainage exposure. *Water Res.* 42:180–188.
- Xiloyannis, C., A. Martinez Raya, C. Kosmas, and M. F. Favia. 2008. Semi-intensive olive orchards on sloping land: Requiring good land husbandry for future development. *J. Environ. Manag.* 89:110–119.
- Zaitlin, B., K. Turkington, D. Parkinson, and G. Clayton. 2004. Effects of tillage and inorganic fertilizers on culturable soil actinomycete communities and inhibition of fungi by specific actinomycetes. *Appl. Soil Ecol.* 26:53–62.
- Zak, J. C., M. R. Willig, D. L. Moorhead, H. G. Wildman. 1994. Functional diversity of microbial communities: A quantitative approach. *Soil Biol. Biochem.* 26:1101–1108.