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Résumés -- Abstracts

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Qualitative and quantitative changes of soil microbial communities as a result of sustainable agricultural practices in an Italian olive grove

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Abstract

The aim of the present work was to evaluate the effects of two soil management systems, so called 'sustainable' (ST) and 'conventional' (CT) on the composition, genetic diversity and carbon substrate utilization of soil microbial communities in a Mediterranean olive orchard. ST system included no-tillage, limited chemical fertilization, and organic matter inputs from drip irrigation with wastewater, spontaneous cover crops and pruning material. CT system was characterized by soil tillage, chemical fertilization, no irrigation and heavy pruning. After seven years of treatments, average olive yield was 8.4 and 3.1 t ha^{-1} yr^{-1} in ST and CT, respectively. CT had a significant higher number of total bacteria and actinomycetes if compared to ST, whereas fungi were significantly lower. In ST, the number of the bacteria involved in the nitrogen cycle isolated from the wetted areas under the drippers (ST-WET) were significantly higher than in inter-row areas (ST-INTER). The patterns of denaturing gradient gel electrophoresis of microbial 16S/18S rDNA showed differences between ST and CT, whereas those of 16S/18S rRNA evidenced that ST-WET clustered separately from CT and ST-INTER. Diversity indexes evaluated by Biolog® assay 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 micro-organisms and improve olive yield and quality.

Keywords: sustainable olive growing, cover crops, organic matter, DGGE, Biolog®.

Changements qualitatifs et quantitatifs des communautés microbiennes du sol à la suite de pratiques agricoles soutenables dans une oliveraie italiene

Résumé

L'objectif de ce travail était d'évaluer les effets de deux systèmes de gestion des sols, dits «soutenable» (ST) et «conventionnel» (CT) sur la composition, la diversité génétique et l'utilisation des substrats carbonés par les communautés microbiennes du sol dans une oliveraie Méditerranéenne. ST-système comprenait le non-travail du sol, la fertilisation chimique limitée, et les apports de matière organique à partir de l'irrigation avec des eaux usées, des cultures de couverture et de taille du matériel. CT a été caractérisée par le travail du sol, la fertilisation chimique, ni d'irrigation et de forte taille. Après sept années de traitements, le rendement moyen d'olive a été de 8,4 et 3,1 t ha^{-1} an^{-1} dans le ST et CT, respectivement. CT a eu un nombre plus élevé de l'ensemble des bactéries et les actinomycètes, si on la compare à la ST, alors que les champignons ont été sensiblement plus faible. En ST, le nombre de bactéries du cycle de l'azote isolés du wetted les zones relevant de la goutteurs (ST-WET) étaient significativement plus élevés que dans les zones inter-rangs (ST-INTER). Les dénaturations gradient gel electrophoresis de rDNA 16S/18S ont montré des différences entre les ST et de CT, alors que ceux de 16S/18S rRNA témoigne que ST-WET regroupées séparément de CT et ST-INTER. Les indices de diversité des Biolog® tests étaient significativement différents entre les ST et CT. Les résultats ont révélé des changements qualitatifs et quantitatifs des communautés microbiennes du sol à la suite de pratiques agricoles soutenable qui stimulent les micro-organismes du sol et d'améliorer le rendement et la qualité de l'huile d'olive.

Mots clé: Oliveraie soutenable, couverture végétale, matière organique, DGGE, Biolog®

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1. Introduction

A new approach in olive orchard management is imposed by environmental emergencies, such as soil degradation, water shortage and greenhouse effect (Lal 2004, Hochstrat et al. 2006). Particularly, in semi-arid areas, the use of agronomical techniques able to conserve the natural resources is recommended (Kushwaha and Singh 2005). An integrated approach of culture-dependent and culture-independent methods has provided new tools to study the whole soil microbiota. One of the most useful molecular technique 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) (Crecchio et al. 2004). 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 nitrogen sources (Zak et al. 1994). The community-level physiological profiles (CLPPs), obtained by the Biolog® method, are used to differentiate microbial populations from various soil environments or subjected to various treatments (Crecchio et al. 2004).

The present study was performed to explore the effect of sustainable agricultural management systems on genetic, functional and metabolic diversity of soil microbial communities, with a particular emphasis to those involved in nitrogen cycle, by using a combination of culture-dependent and independent methods. The trial was carried out during a 7-year period in an Italian olive orchard under semi-arid conditions. The effects on the productive response of the olive trees and fruit characteristics were also examined.

2. Materials and methods

2.1. Horticultural practices 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). Olive trees w ere vase trained and planted at a distance of about 8 m x 8 m. The climate in the area is classified as semi-arid. The mean annual temperature ranges from 15 to 17°C. The soil of the experimental grove is a sandy loam (WRB: *Haplic Calcisol*), with a mean bulk density of 1.5 t m^{-3}.

In 2000, the olive orchard was splitted into two plots managed according to sustainable agronomical techniques (Sustainable Treatment - ST) and conventional ones (Conventional Treatment - CT). The ST was irrigated with municipal wastewater treated by a pilot unit and distributed daily from May to October by drip irrigation (6 self-compensating drippers per plant delivering 8 L h^{-1}). Irrigation volume applied over the annual growth season averaged 293 mm (2000-2006). The average annual amounts of N, P, and K distributed by the treated wastewater (293 mm yr^{-1}) were 54, 3 and 50 kg ha^{-1} yr^{-1}, respectively.

The ST soil surface was covered by spontaneous weeds and grasses and mowed at least twice a year. Irrigated trees were lightly pruned each year, in order to improve fruiting potential by controlling the amount of fruiting wood and enhancing flower bud differentiation. Crop residues and pruning material (8.5 t ha^{-1} yr^{-1} dry matter, mean 2000-2006) were left on the ground as mulch. Fertilisers 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. The CT was grown under rainfed conditions and managed according to the traditional horticultural practices of the area (Xiloyannis et al. 2008), that is by tillage performed 2-3 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 two years and pruning residues were burned out of the field.

Fruits were harvested by a trunk shaker and nets. Yield was measured on 12 trees per treatment. 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.

2.2. Soil sampling, microbial counts and microbial community metabolic profiles (Biolog®)

In February 2007, three composite samples of bulk soil (20 seven-cm-diameter cores pooled on site per treatment) were randomly collected and immediately stored in sterilized plastic pots at 4℃ after removing visible crop residues. Samples were collected from the top soil layer (0-10 cm) of both treatments, ST and CT. Particularly, in ST soil sampling was performed in the wetted area under the drippers (ST-WET) and in the non irrigated inter-row area (ST-INTER).

Three replicates of 5 g-sub-samples (dry weight equivalent) of each soil sample were suspended in 45 ml sterile 0.1% sodium pyrophosphate-one quarter strength Ringer solution and sonicated for 2 min to disperse microbial cells. Aliquots of ten-fold serial dilutions were spread plated in triplicate on 1/10 strength TSA (Tryptic Soy Agar) medium amended with cycloheximide 0.1 mg ml^{-1} for bacterial counting, and inoculated in MEA (Malt Extract Agar) medium implemented with streptomycin 0.03 mg ml^{-1} and tetracycline 0.02 mg ml^{-1} in triplicate for fungal counting. Counting took place after suitable incubation period (72 h for bacteria and 120 h for fungi) at 28℃. Actinomycetes were isolated by usin g Casein Starch Agar modified supplemented with 0.12 mg ml^{-1} of cycloehximide (Sigma, NY, USA). The isolation of *Azotobacter* was carried out with Brown's substrate modified, whereas the identification of proteolytic bacteria were identified by MPN method in a cultural medium containing gelatine (Oxoid Lim., Hampshire, UK). Ammonifying bacteria were isolated in a liquid cultural medium containing asparagine and incubated at 28℃ for 15 days. *Pseudomonas* were cultured on Pseudomonas Agar Base medium (Oxoid) with the addition of Pseudomonas C-N Supplement (Oxoid).
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, three times replicated. Data were analysed to determine metabolic diversity indices, including average well colour development (AWCD, the mean of the blanked absorbance values for all the substrates, that 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), according to Zak et al. (1994). The microplates were incubated at 25℃ in the dark and colour development was measured as optical density (OD) every 24 h over a 144 h period using a Microplate E-Max Reader (Bio-Rad) with a E590-nm wavelength filter, and the data were collected by the Microlog 4.01 software (Biolog, CA, USA).

2.3. Denaturing gradient gel electrophoresis (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, OH, USA) and RNA Power Soil Isolation Kit (MoBio, CA, USA). Nucleic acids quantity and quality were assayed on 0.7% agarose gel containing 0.5 µg ml^{-1} of ethidium bromide. Extracted RNA was retro-transcribed to c-DNA by RETROscript™ First Strand Synthesis Kit for RT-PCR (Ambion, TX, USA). DNA and c-DNA were amplified in a PCR thermocycler (Bio-Rad Laboratories, CA, USA) 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. PCR amplifications were performed according to Crecchio et al. (2004). DGGE was performed by the Bio-Rad DCode™ Universal Mutation detection System (Bio-Rad Laboratories, Hercules, CA, USA). PCR products (10 µl) were loaded into 6% (16S rDNA amplicons) or 8% (18S rDNA amplicons) polyacrylamide gel (37.5:1 acrylamide: bisacrylamide) with an urea-formamide parallel gradient (45-60% for 16S rDNA and 30-60% for 18S rDNA amplicons). Sybr Green I stained gels were photographed with Bio-Rad Gel Doc 2000 documentation system (Bio-Rad Laboratories).

2.4. Fingerprints and statistical analyses

Genetic fingerprints were analysed by 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. Profiles comparison and clustering were performed by applying the unweigthed pair-group method using arithmetic average (UPGMA) 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 (ANOVA).

3. Results

3.1. Tree productive responses, olive characteristics and microbial counts

The olive trees belonging to ST produced almost constantly every year with an average yield of 8.4 t ha^{-1} yr^{-1} (mean 2001-2006) while the CT plants showed a significant ($P < 0.05$) lesser productive level (3.1 t ha^{-1} yr^{-1}) and a strong biennal bearing behaviour with low or no production in 2002, 2004, and 2006 (the so-called "off" years). The starting year of the trial, 2000, was an "off" year for both the examined treatments. Drupes picked from the ST showed a significant amelioration of the their commercial characteristics such as fresh weight, drupe size, pulp percentage, and pulp to stone ratio, which are important parameters for table olives increasing their market value (Table 1).

Table 1: Fruit characteristics and pulp to stone ratio (mean 2001-2006 ± SD) in sustainable (ST) and conventional (CT) treatments. Values with asterisks are significantly different at $P < 0.05$.

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

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 if compared to the two ST treatments ($P < 0.01$) (Fig. 1a, b). The number of actinomycetes was significantly higher in CT if compared to the two ST treatments ($P < 0.05$), and significantly higher in ST-INTER than in ST-WET ($P < 0.05$) (Fig. 1c).

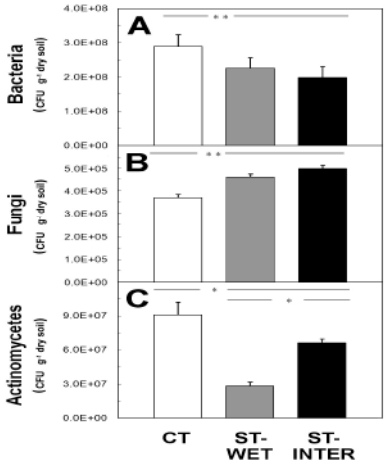

Figure 1: (a) Total bacterial, (b) fungal, and (c) actinomycetes counts in the three treatments: conventional (CT; white bars), sustainable under the drippers (ST-WET; grey bars), sustainable in the inter-row area (ST-INTER; black bars). The values represent the average (± SD) of three independent replicates for each soil treatment. Significance levels:
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

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) (Fig. 2a, b, c). Moreover, CT significantly differ from the two ST soils both for ammonifying bacteria ($P < 0.05$) and *Azotobacter* ($P < 0.01$) (Fig. 2a, c) being the number of microorganisms in both cases intermediate between ST-WET and ST-INTER. *Pseudomonas* counts were not significantly different between CT and ST neither between ST-WET and ST-INTER (Fig. 2d).

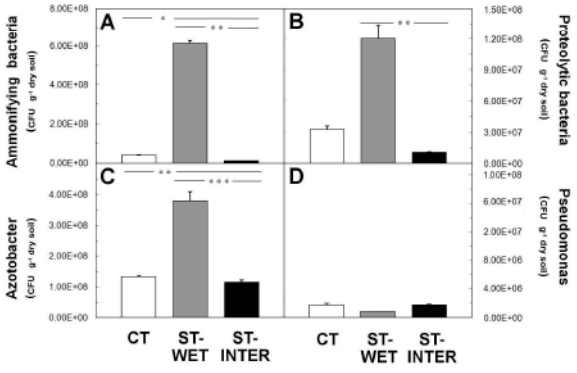

Figure 2: Ammonifying bacteria (a), proteolytic bacteria (b), *Azotobacter* (c) and *Pseudomonas* (d) in the three treatments: conventional (CT; white bars), sustainable under the drippers (ST-WET; grey bars), sustainable in the inter-row area (ST-INTER; black bars). Statistics as in Figure 1.

3.2. Genetic, functional and metabolic fingerprinting

The genetic dendrograms of bacterial 16S rDNA and fungal 18S rDNA showed that molecular patterns of CT were discriminated from patterns of the sustainable treatments (ST-INTER and ST-WET) (Fig. 3a, b). Anyway, Pearson similarity coefficients for 16S rDNA, ranging from 95.1 to 98.5, indicate that electrophoretic profiles relative to bacterial community, were quite similar (Fig. 3a). On the other hand, functional DGGE patterns of rRNA evidenced 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) (Fig. 3c, d). The values of 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) (Fig. 4a, b). Moreover, ST-WET significantly differs from ST-INTER soils both for AWCD ($P < 0.01$) and H' ($P < 0.05$) (Fig. 4A, B). The values of E and S showed no significant differences between CT and ST neither between ST-WET and ST-INTER (Fig. 4c, d).

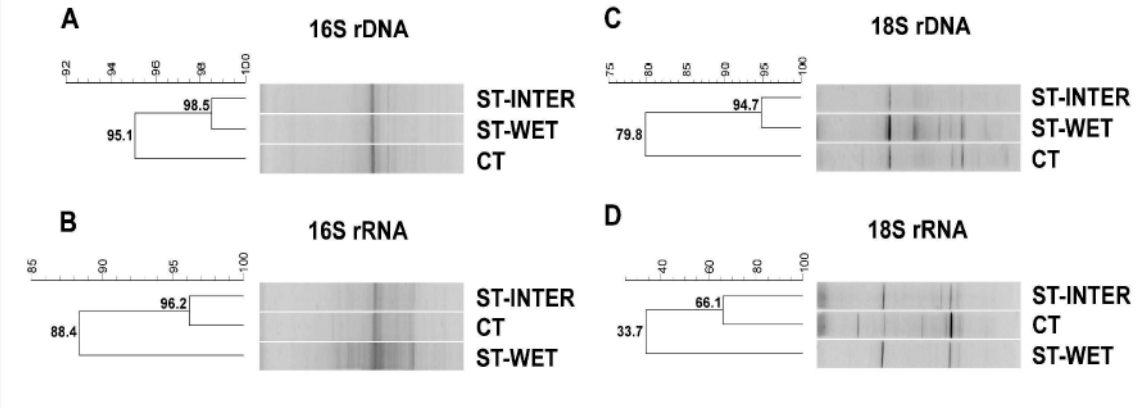

Figure 3: Genetic 16S DGGE fingerprints of soil bacterial communities (a, b) and functional 18S DGGE fingerprints of soil fungal communities (c, d) in the three treatments: conventional (CT), sustainable under the drippers (ST-WET), sustainable in the inter-row area (ST-INTER).

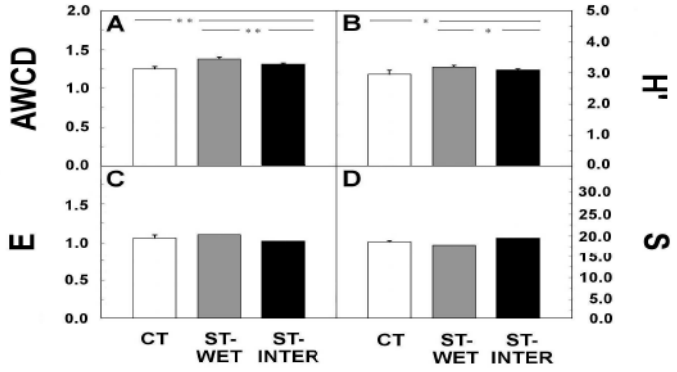

Figure 4: (A) Average well colour development (AWCD), (B) Shannon's substrate diversity index (H'), (C) substrate evenness (E), and (D) substrate richness (S) in the three treatments: conventional (CT; white bars), sustainable under the drippers (ST-WET; grey bars), sustainable in the inter-row area (ST-INTER; black bars). Statistics as in Figure 1.

4. Discussion

The better yield level and fruit quality showed by the olive trees subjected to the ST (Table 1) can be clearly attributed to the adopted orchard management system. First of all irrigation that provided both water and mineral elements supply. In fact, the reclaimed wastewater used in this trial was rich in nutrients (especially N, P, and K) (Table 1), which were distributed by water along the irrigation season and integrated, when it was necessary, by fertirrigations based on orchard requirements.

Total fungal number was significantly higher in ST sites (Fig. 1b), likely because soil fungi rely on external available nutrients (Govaerts et al. 2008) and so respond promptly to changes in organic nutrient matter deriving from cover crops and wastewater irrigation/fertirrigation. The three different orchard management systems also caused significant differences in total bacteria (Fig. 1a).

Particularly interesting are the effects of agricultural management on the number of some of the bacteria involved in nitrogen cycle (Fig. 2). In fact, the results showed that ST-WET site has a higher number of *Azotobacter*, proteolytic and ammonifying bacteria when compared to ST-INTER (Fig. 2a, b, c). The number of *Pseudomonas*, denitrifying bacteria that transform anaerobically nitrates to nitrogen gas or nitrous oxides, was not significantly different between treatments (Fig. 2d). The particular conditions of ST-INTER soils with respect to to ST-WET were reflected by the significantly higher actinomycetes number (Fig. 1c). In fact, 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 of organic inputs, as ST-INTER soils.

In our study, this effect is clear in both 16S and 18S rDNA genetic DGGE dendrograms, that revealed a discrimination between CT and ST systems (Fig. 3a, c). As for the fungal counts, the effects on bacterial community structures were very likely due to the input of organic matter deriving from cover crops (ST-WET and ST-INTER) and wastewater irrigation/fertirrigation (ST-WET). The observed differences between CT and ST soils were likely due also to the two soil management regimes. In fact, zero tillage with residues recycling and mulching result in a soil with good physical and chemical qualities, and high, stable yields, compared to conventional tillage (Govaerts et al. 2008). In contrary, differences in functional DGGE dendrograms of both 16S and 18S rRNA, that reflect the status of metabolically active microorganisms, indicated that the irrigation regime is the main factor inducing changes in bacterial and fungal communities of sites under the emitters (ST-WET) (Fig. 3b, d).

Soil bacterial metabolic diversity indices estimated by Biolog® CLPP are usually higher in sustainable than in conventional soils (Govaerts et al. 2008). Our results show that the values of AWCD and H' were significantly increased by the ST (Fig. 4a, b). In addition, comparing the two sustainable treatments, AWCD and H' were higher in ST-INTER soils (Fig. 4a, b), where cover crops could be an important discriminating element for microbial substrate utilization. Indices of netabolic diversity do not necessarily reflect the composition of the bacterial communities as two communities can have the same H' value but utilize different substrates.

Our results demonstrated that soil microorganisms respond to the application of a sustainable orchard management with evident benefits for olive yield and quality (Table 1). Sustainable orchards showed a higher microbial complexity and diversity. 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 agricultural practices that support and stimulate soil micro-organisms in order to improve orchard production.

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