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Genetic diversity of dinitrogen-fixing bacterial communities in soil amended with olive husks

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Abstract - The industrial production of olive oil is accompanied by the accumulation of large quantities of by-products from the olive milling industry that are commonly dispersed as fertilisers, which are nowadays suspected to have potential toxic effects on soil microflora. The aim of this work has been the investigation of the genetic diversity of bacterial communities present in soil treated with olive husks focusing on the dinitrogen-fixing bacteria. *nifH* genes were amplified from total soil DNA using universal primers, cloned and typed by restriction analysis and sequencing of representative haplotypes. On the same samples, DGGE analysis on amplified 16S rDNA was performed aiming at monitoring modifications in the total community pattern. Results showed a high genetic diversity of *nifH* genes within the community, which was well in agreement with the total community profiles obtained by DGGE on 16SrDNA. Most of the *nifH* gene fragments (19 out of 32) were found to be similar to sequences related with clostridia.

Key words: bacterial communities, olive husks, DGGE, *nifH*.

INTRODUCTION

Olive oil extraction is one of the most traditional industries in Mediterranean countries. The extraction is obtained by discontinuous pressing or by the continuous centrifugation of a mixture of milled olives and water. In both systems aqueous and solid residues are produced which represent about 80% of the total weight of processed olives (Cabrera *et al.,* 1996). The disposal of large quantities of such by-products from the olive milling industry is a topical subject in olive oil producing countries. Olive husks are thought to be particularly polluting substances; they contain large quantities of organic compounds, minerals and phenolics and have an acidic pH (Vlyssides *et al.,* 1996). To reduce the potential toxic effects of soil treatment with olive husks, many strategies have been developed (Vlyssides *et al.,* 1996). One of the most popular practices is the use of olive husk as ingredient for the production of compost for soil amendments, especially of soils cultivated with olive trees. To date, little is known about the diversity of bacterial communities in soil treated with pure or composted olive husks (Picci and Pera, 1993; Cabrera *et al.,* 1996), that is indicative of soil fertility (Benedetti, 1998; Benedetti *et al.,* 1998).

Among the bacterial functions, particularly relevant in agricultural soil, is the biological nitrogen fixation that is performed by phylogenetically diverse groups of prokaryotic organisms in both the *Archaea* and *Bacteria* domains (Young, 1992). The different physiological properties of these diazotrophic microorganisms and, in several cases, their nonculturability, hamper a culture-dependant approach for the characterisation of dinitrogen-fixing communities. Culture-independent methods have been used to investigate diazotrophic microorganisms in many different habitats by using, as molecular marker, the sequences of the *nifH* gene, encoding the nitrogenase reductase (Young, 1992; Ueda *et al.,* 1995; Ohkuma *et al.,* 1996; Steppe *et al.,* 1996; Zehr *et al.,* 1998; Lovell *et al.,* 2000; Schaffer *et al.,* 2000; Poly *et al.,* 2001; Hamelin *et al.,* 2002; Rösch *et al.,* 2002). These studies have focused on various habitats ranging from invertebrate guts to soils and bioreactors, and have uncovered a great diversity of nitrogenase sequences (Zehr *et al.,* 2003). More recently *nifH*-based microarray platforms have been proposed to monitor the occurrence of nitrogen fixing bacteria in different environments (Taroncher-Oldenburg *et al.,* 2003; Tiquia *et al.,* 2004).

Aim of this work has been the evaluation of the genetic diversity of dinitrogen fixing bacterial communities in soil treated with olive husks. Moreover, a DGGE analysis on 16SrDNA was used to look at the total community similarities, then PCR-RFLP and sequencing of amplified *nifH* genes were performed to evaluate the genetic diversity and similarities within and among the soil samples.

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MATERIALS AND METHODS

Soil chemical and physical parameters. The soil of the experimental field is argilleous-calcareous characterised by an alkaline pH and located in Mafalda (Molise, Italy). A cultivated area (2700 m^2) with fifty years old olive trees was selected on it, split in three sub-areas close to each other. The first sub-area was treated with composted residues, the second one with olive husk and the third one was the untreated sub-area.

On March 2002, 8.0 t/ha of compost or olive husks where mechanically distributed. Immediately after the distribution, to help the integration of the treatment with soil (agronomic effect), the soils were mechanically spaded by track-laying tractor, including the untreated sub-areas. The physical and chemical parameters of soils are reported in Table 1.

Samples description. The field sub-areas were treated with crude olive husks mixed with olive mill wastewaters (humid husks) and olive leaves, or with one year old humid husks composted (cured compost) in a composting trapezoidal pile, periodically aerated by mechanical turning device (Principi *et al.,* 2003). The samples were collected at 7th day and 70th day after the treatment of: i) soil treated with humid olive husks ($ULSU_7$ and $ULSU_{70}$), ii) soil treated with cured compost olive husks ($ULCOMP_7$ and $ULCOMP_{70}$), iii) untreated soil (UL_7 and UL_{70}), iv) humid olive husks (SU) was also included as control sample. Homogeneous samples (about 2.0 kg) were obtained by sampling treated and untreated soil, at 5-20 cm dept, randomly taking 12 subsamples from each separate sub-areas using sterile plastic bags and then mixing them. Samples stored immediately at 4 °C were submitted to physical, chemical and biomolecular analyses within 2 days.

DNA extraction and PCR amplification of 16S rDNA region. For DGGE, DNA was extracted with the Fast DNA spin kit for soil (Bio 101) according to the manufacturer's instructions. Primers GC968f and 1401r (Felske *et al.,* 2000) were used to amplify the 16S rDNA V6-V8 regions. Amplification reactions were performed in a 50 µl total volume containing, 1x reaction buffer (Finnzyme), 1.5 mM $MgCl_2$, 250 µM of each dNTP, 2.5 U of *Taq* DNA polymerase (DynazymeII, Finnzyme), 10 pmol of each primer, 25 ng of template DNA. Reactions were performed in a MWG Primus 96 thermocycler (MWG-AG Biotech) programmed for an initial cycle at 94 °C for 90 s, 56 °C for 30 s, 72 °C for 45 s, followed by 33 cycles at 95 °C for 20 s, 56 °C for 30 s, 72 °C for 45 s. A final extension step at 72 °C for 5 min and 60°C for 2 min was performed. Each amplification mixture (5 µl) were analysed by agarose gel (1.2% w/v) electrophoresis in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) containing 1 µg/ml (w/v) of ethidium bromide.

Molecular weight of the bands was estimated by comparing them with a 1 Kbp Plus DNA ladder (Life Technologies) using the software PhotoCapt (BioProfil).

DGGE. The DGGE equipment was from BioRad Laboratoires (Hercules, USA). Polyacrylamide gels (6% of a 37.5:1 acrylamide-bisacrylamide mixture in 1x TAE buffer, 1 mm thick, 20 x 20 cm), with a gradient of 42-58% denaturant, were made by means of a gradient maker (BioRad) according to the manufacturer's guidelines. Denaturing condition of 100% was 7 M urea and 40% formamide as defined by Muyzer *et al.* (1993, 1999). A top gel layer without denaturant was cast above the denaturing gel before the polymerisation started. Then 600 ng of each amplification mixture were loaded on the gels. Gels were run for 17 h at 75 V in 1x TAE buffer at a constant temperature of 60 °C. The gels were stained for 30 min in 12 ml 1x TAE buffer containing 1.2 µl of SYBR Green Stain FMC. Visualisation and digital photography was performed using the software Quantity One (BioRad).

Amplification of *nifH* genes. Fragments of *nifH* genes were amplified using a semi-nested PCR approach as described in Widmer *et al.* (1999). In particular, for the first PCR reaction primers nifH(19-38)fw and nifH(463-482)rv were used, while for the second PCR reaction the primer

TABLE 1 – The main physical and chemical characteristics of the soils treated or untreated with cured compost or crude olive husks residues

Parameters	Untreated soil (control)		Soil treated with cured olive husks compost		Soil treated with crude olive husks	
	UL_7	UL_{70}	$ULCOMP_7$	$ULCOMP_{70}$	$ULSU_7$	$ULSU_{70}$
pH	8.5	8.5	8.4	8.45	8.29	8.25
$CaCO_3$ (%)	21	21	21	20	20	19
O.M. (%)	2.5	2.3	4.8	4.5	3.4	3.8
CEC (mEq/100g)	16.8	16.8	17.6	17.1	15.9	16.3
Total Nitrogen (%)	0.34	0.34	0.55	0.51	0.30	0.40
P_2O_5 absolute (%)	0.084	0.084	0.121	0.110	0.078	0.088
K_2O absolute (ppm)	870	869	1423	1184	1126	1131
O.C. (‰)	1.47	1.35	2.8	2.6	2.0	2.23
Coarse sand (%)	4.3	4.3	3.8	4.1	4.3	3.8
Fine sand (%)	20.7	20.7	21.4	20.9	20.0	21.2
Mud (%)	22.6	22.6	23.2	23.5	23.7	24.1
Clay (%)	52.4	52.4	51.6	51.5	52.0	50.9

UL_7, untreated soil 7 days after the date of treatment; UL_{70}, untreated soil 70 days after the date of treatment; $ULCOMP_7$, soil treated with composted olive husks 7 days after the date of treatment; $ULCOMP_{70}$, soil treated with composted olive husks 70 days after treatment; $ULSU_7$, soil treated with humid husks 7 days after the date of treatment; $ULSU_{70}$ soil treated with humid husks 70 days after treatment; O.M., organic matter; CEC, cation exchange capacity; O.C., organic carbon.

nifH(112-131)fw and nifH(463-482)rv were used (Widmer *et al.,* 1999). The first amplification reaction was performed in a 25 µl total volume with 10 ng of extracted DNA as template and contained 2.5 µl of 10x reaction buffer (Dynazyme II, Finnzyme), 3.5 mM $MgCl_2$, 0.2 mM of each dNTP, 2U of *Taq* DNA polymerase (Dynazyme II, Finnzyme), 10 pmols of each primer, 25 µg of acetylated bovine serum albumin. A negative control reaction with 10 ng of *Escherichia coli* DH5alpha DNA was added. The second amplification reaction was performed in a 100 µl total volume on 5 ng of template DNA (or 1 µl from the first reaction in the case of the negative control reaction) from the first PCR reaction, with the same conditions of the first PCR reaction except that 33 µg of acetylated bovine serum albumin were used. The cycling conditions for both reactions were as follows: after incubation at 95 °C for 2 min, samples were cycled for 37 cycles through the following temperature profile: denaturation at 94 °C for 30 s, annealing temperature for 30 s, extension at 72 °C for 30 s; for the first five cycles annealing temperature was 45 °C, and was 50 °C for the last 32 cycles. Finally, the mixtures were incubated at 72 °C for 5 min. Then, 5 µl of each amplification mixture were analysed by agarose gel (2% w/v) electrophoresis in TAE buffer containing 1 µg/ml (w/v) of ethidium bromide.

DNA cloning, restriction analysis and sequencing. Amplified *nifH* DNA from each sample was cloned into pGEM®-T-Easy using the pGEM®-T-Easy Vector System (Promega) following manufacturer's instructions. Seven *nifH* libraries, one for each sample, were constructed. Positive clones were selected and *nifH* inserts were amplified with T7/SP6 primer pairs directly from 1 µl of lysed cells prepared as described by Mocali *et al.* (2003). The amplification reactions were performed in a 25 µl total volume containing 2.5 µl of 10x reaction buffer (Dynazyme II, Finnzyme), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.8 U of *Taq* DNA polymerase (Dynazyme II, Finnzyme), 10 pmols of each primer. The cycling conditions were as follows: after incubation at 94 °C for 5 min, samples were cycled for 35 cycles through the following temperature profile: denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 60 s. A final extension step 72 °C for 10 min was performed. For restriction analysis, approximately 300 ng of amplified *nifH* insert was digested with 2 units of the restriction enzyme *Alu*I or *Mbo*I (Fermentas) in a total volume of 15 µl, for 3 h at 37 °C. The enzymes were then inactivated at 65 °C for 20 min. Digestion products were separated by high resolution agarose gel (4% w/v) electrophoresis (QA agarose) in TAE buffer containing 1 µg/ml (w/v) of ethidium bromide.

Sequencing was performed directly on amplification products from T7 primer using the DYEnamic ET terminator kit (Amersham Biosciences). Reactions were separated on an ABI310 Genetic Analyzer (Applied Biosystems).

DNA sequence analysis. DNA sequences were checked for the correct open reading frame, translated, and aligned by using Multalin (Corpet, 1988). The aligned aminoacid sequences were used for phylogenetic analysis with Mega 2.0 (Kumar *et al.,* 2003). Neighbor-joining tree (Saitou and Nei, 1987) was computed from the Poisson Correction distance matrix (Nei and Kumar, 2000) and 500 bootstrap samplings were used to determine the robustness of the inferred phylogenetic relationships.

Statistical analyses. DGGE profiles were recorded and the presence/absence of bands was used for profile comparison with GelComparII 2.5 (Applied Maths). An UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram was constructed from the similarity matrix based on Pearson correlation calculated using the module present in the software package GelComparII 2.5 (Applied Maths).

Restriction profiles of cloned *nifH* genes were compared and each library was characterised by the number and the types of restriction profiles (*nifH* haplotypes) recorded. The haplotype list was used for the computation of the haplotypic diversity and for the estimation of the relatedness between samples. Haplotypic diversity was computed using the following formula:

$$D=1/n\Sigma(1-p_i)^2$$

where p_i is the frequency of the *i*-th haplotype and *n* the number of haplotypes in the clone library of the sample.

RESULTS

DGGE profiles of soil samples

Figure 1 shows the DGGE profile obtained and the UPGMA dendrogram derived from DGGE banding after computing a

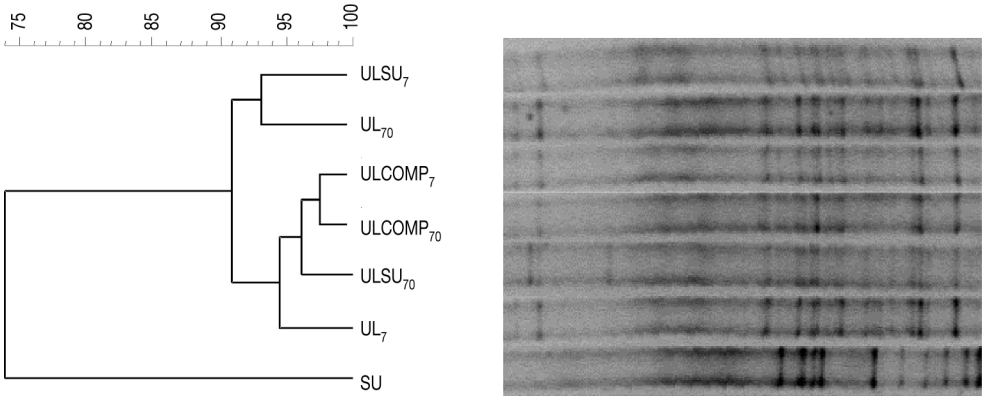

FIG. 1 – UPGMA dendrogram (on the left side) of DGGE profiles (on the right side) from amplified 16S rDNA showing the relationships between samples. See legend of Table 1 or text for sample description; SU, humid olive husks. Scale bar: Pearson correlation.

TABLE 2 – Genetic diversity of samples*

Sample**	DGGE bands (N°)**	*nifH* haplotypic diversity
UL_7	20	0.909
UL_{70}	25	0.924
$ULSU_7$	25	0.964
$ULSU_{70}$	20	0.964
$ULCOMP_7$	21	0.800
$ULCOMP_{70}$	20	0.786
SU	25	0.833

* Genetic diversity is referred to as haplotypic diversity of cloned *nifH* fragments within the clone libraries; ** See legend of Fig. 1 for sample description.

Pearson correlation similarity matrix. The sample from humid olive husks (SU) was clearly clustered apart from all the other samples. Time and treatment were both responsible of the limited genetic diversity found among all the soil samples. The most similar profiles were generated by the bacterial communities from soil treated with composted olive husks ($ULCOMP_7$ and $ULCOMP_{70}$). Table 2 reports the genetic diversity within samples, evaluated as number of DGGE bands. All values were similar and ranged from 20 to 25 bands per sample.

nifH gene profiles

Amplification of *nifH* gene yielded a single product band at the expected *nifH* gene fragment size, about 370 bp in each sample. *nifH* amplicons were cloned separately from each sample and 77 positive clones (11 for each sample) were screened by PCR-RFLP. RFLP was carried out first with *Alu*I; then, clones that were not cleaved were subjected to digestion with *Mbo*I. This procedure allowed to recognise 32 different restriction patterns (*nifH* haplotypes) out of 77 screened clones. Haplotypic diversity for the seven different samples is reported on Table 2. Obtained values were in general high ranging from 0.78 to 0.96.

Sequence characterisation of the scored *nifH* haplotypes

The DNA sequences of 32 clones, representing the 32 *nifH* haplotypes obtained, were determined. All the sequences obtained from the nested PCR products were 364 bp long and represented a portion of the NifH open reading frame. For comparison analysis, the sequences of the 32 clones were checked for similarity to *nifH* sequences retrieved from GenBank. No one of the 32 nucleotide sequences was identical to any sequence present in GenBank. Among the most similar sequences present in GenBank, 21 were selected from members of the *Bacteria* domain. All the putative 53 amino acid sequences were aligned and a neighbour-joining tree was constructed (Fig. 2). The *nifH* clones isolated clustered in different branches. Two sequences (N6, N20) were identical at the aminoacid level to the sequence of an unidentified bacterium similar to *Klebisella pneumoniae* and were identified in the control samples UL_7 and in humid husks (SU). N11 and N13 had the same aminoacid sequence of *Azomonas agilis* and were identified in the control sample $UL_{70,}$ while N8, N9, and N10 had the same sequence of *Clostridium pasteurianum* and were identified in the control samples UL_7 and UL_{70} and in humid husks (SU). In particular, 19 sequences formed a cluster in which sequences from bacteria of the genus *Clostridium* were included.

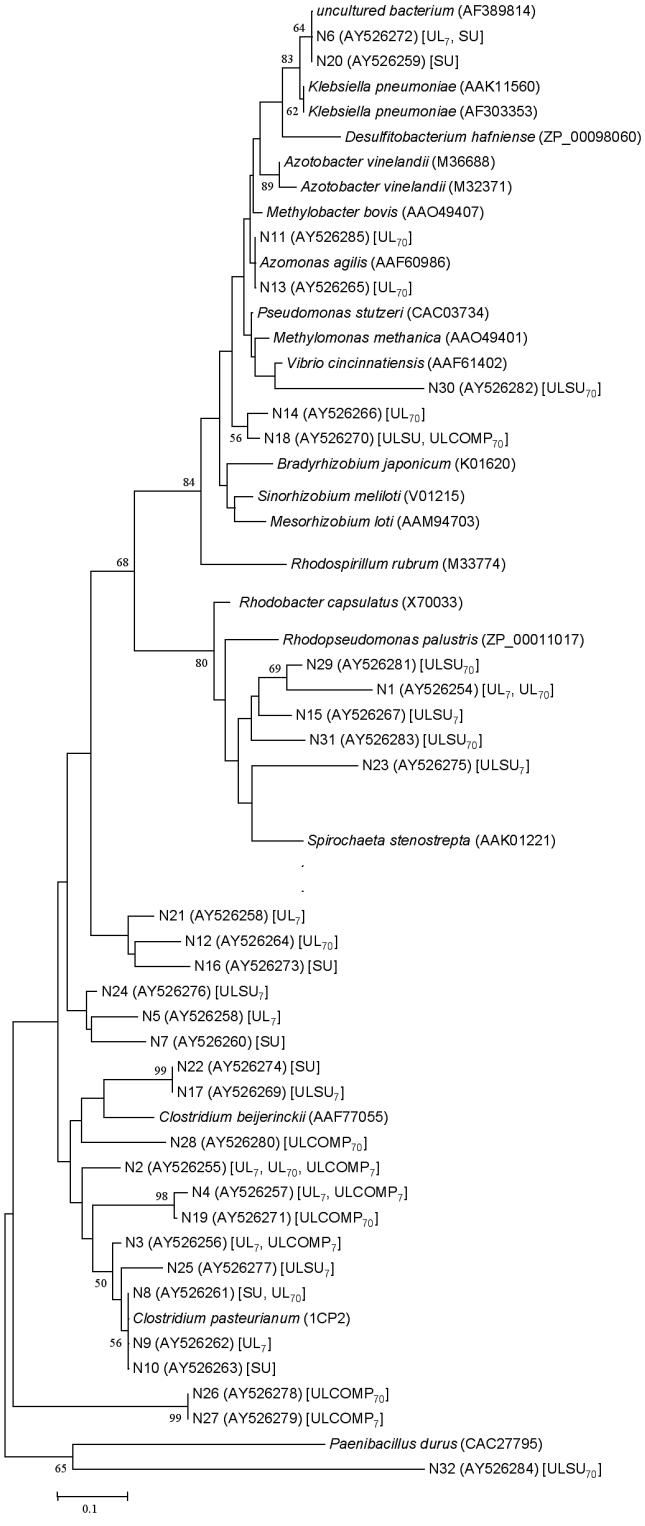

FIG. 2 – Neighbour-joining tree from 61 NifH aminoacid sequences. The 32 sequences from this study were indicated with codes N1-N32. Numbers at nodes indicate bootstrap values after 500 replicates. Accession numbers are indicated in parentheses. In squared parentheses the samples were haplotypes were retrieved, see Fig.1 for sample legend. Scale bar: Poisson-Correction distance.

DISCUSSION

The soil considered for the analysis showed a general absence of changes on several parameters, with some weak effects (P_2O_5, K_2O and O.M. contents) among samples treated with composted or crude olive husks and untreated samples. This was probably due to the well known presence of these elements on crude and composted olive husks (FAIR5-CT97-3620, 1997) and seems to suggest positive agronomic aspects of soil treatment. Concerning the bacterial community, DGGE analysis of the samples showed a complex pattern of community dynamics without a clear distinction between treated and untreated samples, and a homogeneous level of genetic diversity. Apparently, the different soils samples showed similar values of species richness, as inferred from the number of bands, even though some changes in community composition (that is the type of bands in the sample) were detected. Moreover, after two months the total community composition showed to be modified irrespective of the treatment, with the exception of compost treated soil whose composition seems to be maintained over the time. However, the aim of the work was the analysis of the genetic diversity and not a stringent comparison of samples and consequently an analysis of treatment's effect. Actually, we cannot tell anything from the obtained microbiological data about the effect of a short or long term utilisation of olive husks as fertilisers. Thus, an interesting question is concerned with the genetic diversity of some major functional groups within the community. For this reason the polymorphism of *nifH* sequences was analysed in the DNA samples from the different soils aiming at evaluating the genetic diversity of the diazotrophic fraction of the community. Diversity values of *nifH* haplotypes were high and similar for all samples. Actually, it is worth of note that humid olive husks '*per se*' did contain a fully differentiated *nifH* gene pool. A report on the addition of nitrogen containing fertilisers suggested drastic effects on the diversity of diazotrophic communities (Tan *et al.,* 2003); however, other studies based on the analysis of 16S rDNA have shown that genetic diversity of diazotrophs is not affected by fertilisation practices (Felske *et al.,* 2000; Piceno and Lovell 2000). The high genetic diversity of dinitrogen-fixing bacteria in the analysed samples was confirmed by the sequencing of the 32 *nifH* haplotypes detected by PCR-RFLP. The 32 sequences corresponded to 26 different protein sequences without any apparent association between taxonomic groups and sample of origin. The fact that only 5 out of 32 sequences corresponded to NifH protein sequences already present in GenBank database, confirmed that we still know the "tip of the iceberg" of the nitrogen fixing bacteria diversity. The high proportion of "*Clostridium*-like" sequences found to be spread over all samples, including the untreated soil and the humid husks, is an indication of the prevalence of those species among soil nitrogen fixers, though we cannot exclude a technical bias toward these bacterial groups during DNA extraction or amplification procedures.

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