Influence of maize mucilage on the diversity and activity of the denitrifying community

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Summary

In order to understand the effect of the maize rhizosphere on denitrification, the diversity and the activity of the denitrifying community were studied in soil amended with maize mucilage. Diversity of the denitrifying community was investigated by polymerase chain reaction (PCR) amplification of total community DNA extracted from soils using gene fragments, encoding the nitrate reductase (narG) and the nitrous oxide reductase (nosZ), as molecular markers. To assess the underlying diversity, PCR products were cloned and 10 gene libraries were obtained for each targeted gene. Libraries containing 738 and 713 narG and nosZ clones, respectively, were screened by restriction fragment analysis, and grouped based on their RFLP (restriction fragment length polymorphism) patterns. In all, 117 and 171 different clone families have been identified for narG and nosZ and representatives of RFLP families containing at least two clones were sequenced. Rarefaction curves of both genes did not reach a clear saturation, indicating that analysis of an increasing number of clones would have revealed further diversity. Recovered NarG sequences were related to NarG from Actinomycetales and from Proteobacteria but most of them are not related to NarG from known bacteria. In contrast, most of the NosZ sequences were related to NosZ from α, β, and γ Proteobacteria. Denitrifying activity was monitored by incubating the control and amended soils anaerobically in presence of acetylene. The N₂O production rates revealed denitrifying activity to be greater in amended soil than in control soil. Altogether, our results revealed that mucilage addition to the soil results in a strong impact on the activity of the denitrifying community and minor changes on its diversity.

Introduction

Denitrification is a dissimilatory process in which oxidized nitrogen is used as an alternative electron acceptor for energy production when oxygen is limiting. This process consists of four reaction steps in which nitrate is reduced into dinitrogen gas by the following metalloenzymes: nitrate reductase, nitrite reductase, nitric-oxide reductase and nitrous oxide reductase. Denitrification has received considerable attention because it produces N₂O, an important greenhouse gas and a natural catalyst of stratospheric ozone degradation (Lashof and Ahuja, 1995). Denitrification is also the main biological process responsible for the return of fixed nitrogen back to the atmosphere.

Earlier studies reported that the denitrifying activity was enhanced in the rhizosphere (Smith and Tiedje, 1979; Trolldenier, 1988; Mahmoud et al., 1997). More recently, culture-dependent and -independent approaches have shown that the diversity of the denitrifying community was also different in the rhizosphere compared to the bulk soil (Brunel et al., 1992; Nijburg et al., 1997a,b; Philippot et al., 2002). It is well known that the major factors regulating denitrification can be modified in the rhizosphere: nitrate concentration (via the N-assimilation by plants) and oxygen partial pressure (via the root respiration) are decreased, whereas C availability (via rhizodeposition) is generally increased. Thus, Klemedtsson et al. (1987) attributed the stimulation of the denitrifying activity in the rhizosphere mainly to the decrease of oxygen partial pressure in planted soil. In another study, Haider et al. (1987) argued that root exudates were not able to supply metabolizable C to the denitrification process, whereas, Qian et al. (1997) argued that root-derived C influenced soil microbial activity which regulated N transformation and in particular denitrification. These apparently contradictory data might result from the fact that proximate regulators could have multiple effects. Indeed, organic carbon can be an electron donor and responsible for a decrease in oxygen partial pressure by stimulating respiration (Tiedje,
1988). Consequently, the understanding of this rhizosphere effect on diversity and activity of the denitrifying community can be greatly improved by dissecting these regulating factors.

The overall objective of this study was to investigate the significance of the role of root derived C on the diversity and activity of denitrifying community. The nature of the released C by the root is highly variable (exudates, mucilage, root cap cells). In graminaceous plants which are of agronomic importance, mucilage is a major rhizodeposit whose production can be estimated in 2–20 μg C/mg dry matter root growth (Nguyen, 2003). Plant root mucilage is a complex polysaccharide composed of six residues, namely galactose, glucose, fucose, mannose, xylose and arabinose (Chaboud, 1983). Mucilage has been found to bind microbial cell and to be metabolized by microorganisms (Knee et al., 2001). Thus, we specifically study the role of mucilage on denitrification by adding maize mucilage to soil samples and investigating changes in diversity and activity of the denitrifying bacterial communities between control and amended soils. Development of molecular techniques has opened new doors in investigations on the diversity of microbial communities in soil. However, the commonly used approach, targeting the 16S r-DNA, is not realistic for denitrifiers as bacteria capable of denitrification belong to a large variety of taxonomic groups (Tiedje, 1988). Consequently, functional genes have been used as molecular markers to assess the diversity of denitrifying bacteria in the environment (Braker et al., 1998; Scala and Kerkhof, 1998; Hallin and Lindgren, 1999; Philippot et al., 2001; Rösch et al., 2002). In this study, both narG and nosZ genes encoding the first and last step of the denitrification pathway, respectively, were used as molecular markers to assess the influence of added mucilage on the diversity of the denitrifying community in soil.

Results

Denitrifying enzyme activity

To explore the effect of mucilage addition on denitrifying activity, the production of N₂O in anaerobiosis in the presence of acetylene was measured after 24 h and 5 days incubation. For both dates, a significantly higher production was observed in the soil amended with mucilage (Fig. 1). Incubation in presence of non-limiting nitrate concentration did not modify the N₂O production regardless of treatment.

Fingerprinting analysis of the narG and nosZ genes

Amplification of the narG and nosZ genes with degenerated primers yielded in all samples a band of the expected sizes (approximately 650 bp, data not shown). Similar restriction patterns were observed in the five replicate soil samples for both narG and nosZ genes (Figs 2 and 3). For narG, few stronger or additional bands were observed at 350, 310 or 80 bp in the soil amended with mucilage (Fig. 2). In contrast, no difference were observed with nosZ fingerprinting between the control and amended soil (Fig. 3).

RFLP analyses of narG and nosZ clone libraries

A sample of approximately 70 clones from each of the 10 narG and nosZ libraries was randomly selected for RFLP analyses. Within the 738 narG clones analysed, 117 different Alu restriction banding patterns were detected with 57 represented by at least two clones. Distribution of these RFLP families containing at least two clones in the two treatments was represented in Fig. 4. Two major RFLP families became evident: approximately more than 40% of the total number of clones from the five replicates belong to RFLP families 1 and 2 in both control and amended soil (Fig. 4). Although the percentage of clone belonging to RFLP type 2 is identical (24%) in the both soils, the percentage of clone belonging to RFLP family 1 decreased from 29% in the control soil to 18% in the soil amended with mucilage. In addition, a similar number of RFLP families (about 11–13) was detected only in the control and amended soil (Fig. 4). Although the percentage of clone belonging to RFLP type 2 is identical (24%) in the both soils, the percentage of clone belonging to RFLP family 1 decreased from 29% in the control soil to 18% in the soil amended with mucilage. Accordingly, the analysis of variance of the distribution of the narG RFLP families between replicates of the control and amended soil exhibited small differences (P < 0.08). Six narG RFLP families were found to contain clones from the five replicates of either the control or treated soil and a similar number of clone was observed in all five replicates for the dominant RFLP families (Fig. 4) suggesting a good reproducibility. Reciprocal Simpson indexes of 6.3 and of 9.3 have been calculated for the narG libraries in the

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control and amended soil, respectively, but without any significant difference (Table 1). Restriction fragment length polymorphism screening of the 713 nosZ clones yielded 171 different AluI restriction patterns with 77 RFLP families represented by at least two clones (Fig. 5). Eight nosZ RFLP families were found to contain clones from the five replicates of either the control or treated soil. However a significant variation was observed by AMOVA analysis between replicates within both treatment (P > 0.05). Higher reciprocal Simpson indexes of 21.7 and 18.4 have been calculated for the nosZ libraries also without any significant difference (Table 1). Similarly to the narG libraries, the nosZ libraries from the control and amended soil had great overlap without significant differences (Fig. 5).

**Rarefaction analysis**

Rarefaction analysis was applied to evaluate whether screening of 738 narG clones and 713 nosZ clones was sufficient to estimate diversity within the clone library. The observed number of different RFLP families was plotted versus the number of narG and nosZ clones in the libraries. The calculated rarefaction curves did not reach a clear saturation, indicating that analysis of an increasing number of clones would have revealed further diversity (Fig. 6). For both narG and nosZ genes, comparison of the rarefaction curves between the control and amended soil did not reveal significant differences.

**Sequence analyses of narG and nosZ gene fragments**

Representative clones from the RFLP families containing at least two clones were sequenced on both strands. In addition few randomly selected RFLP families represented by only one clone were also sequenced. In total 96 NarG and 111 NosZ derived amino acid sequences...
were used to construct phylogenetic trees including other NarG and NosZ sequences from the databases. The NarG phylogenetic tree was divided into 10 major clusters based on the location of NarG from known microorganisms (Fig. 7). Clones from clusters 4, 5, 6, 9 and 10 are not related to sequences from known bacteria (Fig. 7) but contain environmental NarG sequences from previous studies (Philippot et al., 2002).

The cluster 1 containing the second most recovered RFLP family from the control soil is not affiliated to NarG from Gram-positive and Gram-negative bacteria and the most related known NarG belongs to the thermophilic

Fig. 4. Distribution of the narG OTU from the control soil (A) and distribution of the same OTU in the amended soil (B). Only OTU containing at least two clones are represented.
The cluster 2 contained NarG sequences from the Actinobacteria group and from clones which are the only representative of their RFLP families. The cluster 9 contained clones from RFLP family 23 and had 100% identity with sequence D71 representing the predominant narG RFLP family obtained in a previous study (Chêneby, 2003).

The NosZ tree could be divided in three main clusters (Fig. 8). Almost all the NosZ sequences obtained in this study are related to the NosZ from Actinobacteria which are the only representative of their RFLP families obtained in a previous study in an acid forest soil (Rösch et al., 2002). Out of the 111 sequences analysed, 60 belonged to cluster 1 containing mainly known NosZ genes from Actinobacteria.

Only one and five non-specific PCR products were obtained by sequencing the narG and nosZ RFLP families, respectively, and all corresponded to minor RFLP families represented by one or two clones which were consequently discarded.

Discussion

Changes regarding the rhizosphere effect on the soil microbial community diversity have been well documented (Kuske et al., 2002; Kowalchuk et al., 2002; Schmalerberger and Tebbe, 2003) although detailed characterizations at the functional community level are scarce. To our knowledge, this is the first microbial study to characterize both the diversity and activity of the denitrifying community in relation to root-derived C.

Denitrifying enzyme activity

Our estimate of the denitrification rate (4–12 μg N g of dry soil−1 per day) is in agreement with that reported for other experimental conditions in the maize-planted soil (8.8 μg N g of dry soil−1 per day; Mahmood et al., 1997). A higher denitrification rate was observed in the soil amended with maize mucilage (10.8 μg N g of dry soil−1 per day) than in the control soil (3.9 μg N g of dry soil−1 per day). The mucilage effect on the denitrifying activity observed in this study was consistent with the rhizosphere effect observed by Højberg et al. (1996) and Mahmood et al. (1997), who found 1.5–2.5 fold increases in denitrification rates in the rhizosphere of barley and maize respectively. As similar densities of nitrate-reducing bacteria estimated by CFU were observed in both treatments (data not shown), this result could be explained by (i) a higher concentration of denitrifying enzymes in the amended soil at the end of the experiment and/or (ii) an additional energy source for denitrifying bacteria in the amended soil provided by C present in maize mucilage and/or (iii) a decrease of oxygen partial pressure as the result of a stimulation of the respiration rates of non-denitrifying microorganisms which are able to use mucilage as a C source. Addition of nitrate did not result in significant changes in the denitrification rate suggesting that nitrate is non-limiting in the studied soil. Accordingly, a high nitrate concentration of 77 ± 10 mg N kg−1 has been measured after the two weeks for both treatments (data not shown).

Influence of mucilage on the diversity of the denitrifying community

To study the effect of root-derived C on the diversity and activity of the denitrifying community, the soil was amended each day during two weeks with mucilage collected from maize. The carbon content in the added mucilage was about 70 μg C / g dry weight soil. An amendment of two weeks was enough to observe differences in both narG RFLP fingerprints and activity of the targeted community. Accordingly, a previous experiment showed that a 15 days access to the organic compounds of rhizodeposits could result in a modification of the soil microbial community diversity (Benizri et al., 2002). In order to give a more complete description of the denitrifying community than those provided by the fingerprinting restriction patterns, we attempted to characterize the microorganisms by constructing libraries and sequencing. In addition to

Table 1. Characteristics and diversity estimates for the narG and nosZ gene clones from the control soil and mucilage amended soil.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Source</th>
<th>Number of clones^a</th>
<th>RFLP analysis results</th>
<th>OTU^b</th>
<th>1/D^c</th>
<th>H^d</th>
<th>H/H^max^e</th>
</tr>
</thead>
<tbody>
<tr>
<td>narG</td>
<td>Control soil</td>
<td>378</td>
<td>76 (28)^f</td>
<td>6.3 ± 1.6</td>
<td>1.1 ± 0.13</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amended soil</td>
<td>360</td>
<td>81 (32)^f</td>
<td>9.3 ± 4.8</td>
<td>1.1 ± 0.07</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>nosZ</td>
<td>Control soil</td>
<td>362</td>
<td>118 (53)^f</td>
<td>21.7 ± 7.5</td>
<td>4.8 ± 0.4</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amended soil</td>
<td>351</td>
<td>108 (45)^f</td>
<td>18.4 ± 7.6</td>
<td>4.7 ± 0.4</td>
<td>0.68</td>
<td></td>
</tr>
</tbody>
</table>

a. Number of clones in the libraries.
b. Number of Operational Taxonomic Units based on RFLP.
c. Number of OTU containing only one clone.
d. Reciprocal of Simpson’s index.
e. Shannon-Wiener index.
f. Evenness.

bacterium *Thermus thermophilus*. The cluster 2 contained NarG sequences from the Actinobacteria group and from clones which are the only representative of their RFLP families. The cluster 9 contained clones from RFLP family 23 and had 100% identity with sequence D71 representing the predominant narG RFLP family obtained in a previous study (Chêneby, 2003).

The NosZ tree could be divided in three main clusters (Fig. 8). Almost all the NosZ sequences obtained in this study are related to the NosZ from Actinobacteria which are the only representative of their RFLP families obtained in a previous study in an acid forest soil (Rösch et al., 2002). Out of the 111 sequences analysed, 60 belonged to cluster 1 containing mainly known NosZ genes from Actinobacteria.

Only one and five non-specific PCR products were obtained by sequencing the narG and nosZ RFLP families, respectively, and all corresponded to minor RFLP families represented by one or two clones which were consequently discarded.
DNA extraction, PCR-based cloning approaches, which are commonly used to study the diversity and diversity of microbial community, suffer from biases that can distort community composition (Martin-Laurent et al., 2001). Therefore the clone distributions and diversity indices were used only for relative comparisons between the control and amended soil. No deep changes in the diversity of the studied communities were recorded. Thus for narG the dominant RFLP families are the same in the control and amended soil with only a modification of the abun-

Fig. 5. Distribution of the nosZ OTU from the control soil (A) and distribution of the same OTU in the amended soil (B). Only OTU containing at least two clones are represented.
dance (Fig. 4). Neither Shannon or Simpson indexes were significantly affected by the addition of mucilage to the soil. A stronger impact of maize rhizosphere on the diversity of the narG community has been recorded in our previous study (Philippot et al., 2002) suggesting that mucilage produced by the maize is probably not one of the main factor responsible for the rhizospheric effect. The ability of rhizosphere bacteria (Rhizobium leguminosarum, Burkholderia cepacia and Pseudomonas fluorescens) to utilize root mucilage as sole carbon source has been previously demonstrated with pea root mucilage (Knee et al., 2001). However maize mucilage mainly consisted of high molecular weight root polysaccharides (Chaboud, 1983; Osborn et al., 1999) which are not easily broken down by soil microorganisms. To investigate further the role of C in the rhizospheric effect, influence of others C source provided by plant roots such as exudates must be analysed.

Diversity of the narG and nosZ clone libraries

NarG sequence analysis revealed a great deal of diversity with 117 RFLP families identified in the libraries (Table 1). Based on the congruence between the 16S rDNA and narG phylogenies (Philippot, 2002), our present results revealed that the narG sequences obtained are affiliated to highly diverse bacterial groups such as the Actinomycetales, the γ or the β Proteobacteria. The taxonomic divisions observed in this study concurred with the finding of previous studies based either on cultivation-dependent or cultivation-independent techniques (Nijburg et al., 1997a,b; Shirey and Sextone, 1989). However, most of the narG sequences discovered in this study belong to cluster 4, 5 and 6 and were not affiliated with any narG sequence from known bacteria. Interestingly, a similar dominance of clusters 4 and 5 has been reported in other soils suggesting that these unknown nitrate-reducing groups may be widespread in soils (Philippot et al., 2002; Chéneby et al., 2003). In this study, we targeted nitrate reducing bacteria having the membrane bound nitrate reductase because this enzyme being present not only in Gram-negative bacteria but also in Gram-positive bacteria (in contrast to the periplasmic nitrate reductase) is, from a taxonomic point of view, more representative of this functional community (Philippot and Hojberg, 1999; Richardson et al., 2001). In addition, it is important to underline that bacteria in the environment can possess genes for only few steps of the denitrifying pathway or different genes for the same step. Therefore narG and nosZ genes could be carried by different bacterial populations.

Several studies have previously reported the use of nosZ gene as a molecular marker of the denitrifying community (Scala and Kerkhof, 1998; 1999; Rösch et al., 2002). Whereas the narG primers are able to amplify both Gram-positive and Gram-negative bacteria, the existing nosZ primers were developed based on sequences available from a limited number of species belonging only to Proteobacteria. In contrast to the NarG phylogenetic tree, most of the NosZ sequences cluster at the periphery of NosZ from known denitrifying bacteria suggesting that denitrifiers amplified with the nosZ primers in the studied soil are related to cultured Proteobacteria. Similar results were obtained in acid forest soil and in a wastewater treatment reactor (Rösch et al., 2002; Sakano et al., 2002). In contrast, in sediment samples, only few recovered nosZ genes were related to nosZ from cultured denitrifying bacteria (Scala and Kerkhof, 1999).

In this work, we studied the diversity and the activity of bacteria genetically able to reduce nitrate or nitrous oxide in relation to a mucilage amendment. Our results showed that the mucilage released by maize roots has an important impact on the activity of the denitrifying community and a minor one on its diversity and therefore could contribute to the previously observed rhizosphere effect (Smith and Tiedje, 1979, Mahmood et al., 1997). However, it is interesting to underline that the increase in the denitrifying activity did not reflect important changes in the diversity of this functional community. Understanding the relationships between density-diversity and activity of functional community is a major challenge in microbial ecology. Development of cultivation-independent methods to quantify functional communities in the environment such as quantitative PCR and of effective tools to identify the active members of these functional communities are required to face this challenge.

Experimental procedures

Collection of maize mucilage

Mucilage was collected from nodal roots of maize plants grown in the field at the experimental station of the Ecole Nationale Supérieure d’Agronomie et des Industries Alimentaires in Champenoux (North-east of France). Maize plants
were cut above the soil surface at the end of July just before flowering. At this stage, one to two crowns of nodal roots had emerged from the stem but the roots had not yet reach the soil surface. Back in the laboratory, the base of the stem was then incubated overnight in water. Afterwards, the swelled mucilage covering the nodal root tips was extracted using a vacuum device. Around 2 ml of slime was collected per plant.

Fig. 7. Phylogenetic relationship of the translated narG products. Phylogenetic distance were determined by neighbour-joining analysis. The number of the corresponding OTU is indicated in bold italics, after the clone number. Nodes with more than 700 bootstrap iterations (of 1000) are highlighted by a black circle. Clones from previous studies are shaded in grey (Philippot et al., 2002; Chèneby et al., 2003).
Fig. 8. Phylogenetic relationship of the translated nosZ products. Phylogenetic distance were determined by neighbour-joining analysis. The number of the corresponding OTU is indicated in bold italics, after the clone number. Nodes with more than 700 bootstrap iterations (of 1000) are highlighted by a black circle. Clones from previous study are shaded in grey (Rosch et al., 2002).
The mucilage was pooled and centrifuged at 10000 r.p.m. to remove the root debris. Afterwards, the sample was dialysed during 24 h (cut off 12–14 000) to discard the solutes and stored at −20°C. The final slime had the following characteristics: 38.3% C, 0.7% N (dry combustion analysis) and a dry matter/fresh weight ratio of 0.0087.

Soil amendments

We used a soil collected on the first 30 cm of an agricultural field in Vannecourt (Moselle, North-east of France), in March 2002 with the following characteristics: 225 g kg⁻¹ clay, 443 g kg⁻¹ loam, 332 g kg⁻¹ sand, pH 6, 1.6% C, 0.2% N. The plot was cultivated with winter wheat. The soil has been sieved at 2 MM and moistened at 70% of its water holding capacity. Afterwards, 10 Petri dishes (90 MM diameter) were sieved at 2 MM and moistened at 70% of its water holding capacity. Afterwards, 10 Petri dishes (90 MM diameter) were filled with soil equivalent to 25 g dry soil/dish and the dishes were incubated in the dark in a chamber with a controlled environment: 22°C (± 1°C), 60% relative humidity. For two weeks, the dishes were supplied daily either with 2 ml water (n = 5) or mucilage solution (70 µg C/g dry soil, n = 5). This volume corresponded to the daily loss of water. Every two days, the dishes were weighed to check for their water content and extra water was added if necessary.

Denitrification enzyme activity

Five ml of sterile water or sterile water supplemented with nitrate (100 mg N-NO₃⁻ l⁻¹ ) was added to plasma flasks containing the equivalent of 5 g of dry soil from the control or amended treatments. Flasks were then sealed, placed under vacuum for 15 min and flushed with helium three times. After purging of all O₂, 10% of C₂H₂ was added in the flasks. After one and five days incubation at 20°C, the N₂O concentration in the flasks was determined using a MTI gas chromatograph (P200 MTI Analytical Instrument) equipped with a thermal conductivity detector.

DNA extraction and purification

DNA was extracted from 250 mg aliquots from five Petri dishes containing soil amended with mucilage (samples MA, MB, MC, MD and ME) and from five Petri dishes containing control soil (samples CF, CG, CH, CI and CJ) according to Martin-Laurent et al. (2001). Briefly, samples were homogenized in 1 ml of extraction buffer for 30 s at 1600 r.p.m. in a mini-bead beater cell disruptor (Mikro-Dismembrator S, B. Braun Biotech International). Soil and cell debris were removed by centrifugation. Nucleic acids were purified by precipitation and further clean-up was performed using a Sepharose 4B spin column. DNA was quantified using a BioPhotometer (Eppendorf, France).

narG and nosZ amplification

Amplification of narG from 10 ng of purified soil DNA was carried out by using the primers narG1960f (5'-TAYGTGSGSACARAGAA-3') and narG2650r (5'-TTYTCTRT ACCABGTBGC-3') developed by Philippot et al., (2002). Hot start PCR amplifications were performed for each sample using AmpliWax (Applied Biosystems, France). The following steps were employed: an initial step at 70°C for 3 min before the Taq DNA polymerase (Oncor Appligene, France) was added; then a first denaturation cycle at 94°C for 4 min, 8 cycles of denaturation at 94°C for 30 s, primer annealing at 59°C for 30 s and elongation at 72°C for 45 s. During these first eight cycles, the annealing temperature was decreased by 0.5°C each cycle, starting at 59°C until it reached 55°C. The additional 30 cycles were performed at an annealing temperature of 55°C. Cycling was completed by a final elongation step of 72°C for 10 min. Amplification of nosZ was carried out using the primers developed by Rösch et al. (2002) and conditions as described elsewhere (Rösch et al., 2002). The narG and nosZ PCR products were resolved by electrophoresis in a 1% (wt/vol) agarose gel. Three independent PCR were performed for the 10 samples and the triplicate PCR products from each sample were pooled to minimize the effect of PCR bias.

RFLP fingerprinting analysis and clone libraries construction

The narG and nosZ PCR products were purified using the Qiaex II gel extraction kit (Qiagen, France). Aliquots of the purified PCR products were digested with AluI restriction enzyme at 37°C for 12 h and the narG and nosZ RFLP fingerprinting were obtained after separation by electrophoresis on a native 6% acrylamide gel for 11 h at 5 mA. In addition, 10 clone libraries (five replicates from the control soil and five replicates from the soil amended with mucilage) were constructed for the narG and nosZ genes by cloning aliquots of the narG and nosZ purified PCR products from each soil sample using the pGEM-T Easy Vector System (Promega, France). Approximately 70 recombinants from each of the 10 narG and nosZ libraries were screened for full-size inserts by transferring small amount of cell material to PCR mixtures containing the vector primers T-7 and SP-6 and thermocycling. The PCR products from both libraries were digested with AluI restriction enzyme. Restriction fragments were resolved by electrophoresis in a 3% high resolution agarose gel (Qbiogen, France). Clones with identical restriction patterns were grouped together into RFLP families.

Sequencing of the cloned narG and nosZ products

The nucleotide sequences of the cloned PCR products belonging to the main clones families (96 and 111 clones for narG and nosZ, respectively) were determined on both strands using the DTCS-1 kit (Beckman Coulter) and a Ceq 2000 XL sequencer (Beckman Coulter) according to the manufacturer’s instruction. Vector primers T7 and SP6 were used for sequencing reactions. The resulting sequences were deposited in GenBank under accession numbers AY325520 to AY325726.

Phylogenetic analysis, rarefaction and diversity analysis

Deduced protein sequences of the narG and nosZ genes
were aligned using the CLUSTALX software version V.1.0.1 (Thompson et al., 1997). The phylogenetic trees based on amino acids alignments (approximately 210–220 amino acids for each gene), were constructed by neighbour-joining method with 1000 replicate trees. Rarefaction calculations were done using the software Analytic Rarefaction (version 2.1, Stratigraphy laboratory, University of Georgia). The Simpson index of dominance concentration (D) and Shannon Wiener index (H) were calculated by using the following function: $D = \frac{\Sigma P_i^2}{\Sigma P_i}$, where $P_i$ was calculated as follows: $P_i = \frac{n_i}{N}$, $n_i$ is the number of clones in a clone family and $N$ is total number of clones (Simpson, 1949); $H = -\Sigma (P_i) \log(P_i)$. To test differences in the distribution narG and nosZ clones within and between treatments, we performed an AMOVA (Analysis of MOlecular VAriance) analysis using the software developed by Excoffier et al. (Excoffier et al., 1992).

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