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ORIGINAL ARTICLE

Diversity of *nif*H gene in rhizosphere and non-rhizosphere soil of tobacco in Panzhihua, China

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Abstract To gain a better understanding of the effects of tobacco root secretions on the *nif*H gene community of rhizosphere soil, the diversity of the *nif*H gene in the rhizosphere and non-rhizosphere soil of tobacco was investigated using denaturing gradient gel electrophoresis (DGGE) and sequencing. Shannon diversity (*H*), richness (*S*) and evenness (E_H) indices were used to analyze the DGGE results. The results showed that the *nif*H gene diversity for rhizosphere soil was lower than that of the non-rhizosphere at three sampling sites. The increase in *H* of non-rhizosphere soil was due mainly to the increase in pH and the decrease in available N. Richness was correlated closely with *H*. There was a great difference of evenness between the rhizosphere and the non-rhizosphere samples. A range of sequence divergence was observed in the eight sequenced *nif*H clones. The sequences were divided into three clusters in the phylogenetic tree. The majority of the clones were similar to the *nif*H genes of Betaproteobacteria, *Zoogloea oryzae*, *Dechloromonas* sp. and *Azovibrio restrictus*. There was a difference regarding dominant species between the rhizosphere and the non-rhizosphere samples.

Keywords Tobacco · Rhizosphere · *nif*H gene · PCR-DGGE

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Introduction

The soil microorganisms of agro-ecosystems play a critical role in the metabolism of organic matter and in biogeochemical transformations of elements. Since the concept of rhizosphere was first proposed by the German microbiologist Hiltner (1904), "the rhizosphere effect" affecting the growth and development of plants has been studied widely. Studies have shown that the microbial community composition of the rhizosphere is affected by many factors, e.g., climate, soil type and vegetation, root exudates, nutrient availability and cropping systems (Marschner et al. 2001; Baudoin et al. 2003; Yang and Crowley 2000; Alvey et al. 2003). In the plant growth process, the products of photosynthesis are released into the soil in the form of root secretions that serve as energy and carbon sources for the microorganisms. Plant roots secrete a wide range of potentially valuable compounds, divided into normal secretions and special secretions, which are often responsible for special effects in the rhizosphere (Das et al. 2010; Marschner 1995). With these secretions, roots can regulate the soil microbial community in their immediate vicinity, cope with herbivores, encourage beneficial symbioses, change the chemical and physical properties of the soil, and inhibit the growth of competing plant species (Nardi et al. 2000). Some compounds identified in root secretions have been shown to play an important role in root–microbe interactions (Peters et al. 1986). As a specific crop, the root secretions of tobacco are different from those of other plants (Zhou et al. 2005).

Nitrogen-fixing microorganisms play an important role in the soil. Diazotrophs catalyze biological nitrogen fixation using the highly conserved nitrogenase enzyme (Robson et al. 1986; Borneman et al. 1996; Tan et al. 2003). The nitrogen fixation gene, *nif*H, is one of the oldest existing

functional genes in the history of gene evolution, and *nif*H phylogeny among bacteria is in agreement with that inferred from 16S rRNA gene, showing that *nif*H could be considered a good marker for diazotrophic community structure (Young 1992; Roesch et al. 2006). Studies on *nif*H genes depend mainly on culture-independent methods such as PCR-cloning, PCR-RFLP, PCR-DGGE and FLT-RFLP (Poly et al. 2001b).

In this study, we investigated the diversity of the *nif*H gene in the rhizosphere and non-rhizosphere soil of tobacco using PCR-denaturing gradient gel electrophoresis (DGGE) and sequencing methods to provide useful data for the assessment of soil quality and the prediction of potential changes in the soil ecosystem.

Materials and methods

Site and sampling

The study was conducted at the five sites of Yanbian, Panzhihua, Sichuan, China: Matangchun (27°01′N, 101°37′ E, 1,750 ma.s.l.), Fuxingchun A (26°92′N, 101°35′ E, 1,720 ma.s.l.), Fuxingchun B (26°88′N, 101°32′ E, 1,680 ma.s.l.), Gudechun (27°08′N, 101°36′E, 1,730 ma. s.l.), and Gaoganchun (26°80′N, 101°53′E, 1,950 ma.s.l.), which are located between the south subtropical and north temperate zones. The climate of Yanbian is complex and varied, with a mean annual precipitation of 1,065.6 mm and mean annual temperature of 19.2°C. Its four seasons tend to blur, but there are clear wet and dry seasons. Yanbian, with its abundant light, heat and rainfall, is very suitable for cultivating tobacco (Sun et al. 2005; Lai 2009).

Soils were sampled at the tobacco harvest period on 16 August 2008. Three plots were sampled for each site. One composite rhizosphere sample consisting of roots of five randomly selected tobacco plants was taken from each plot. The roots were shaken vigorously to separate soil not tightly adhering to the roots. At the same time, for each plot, a composite bulk soil sample was taken as a non-rhizosphere sample. The samples were stored at 4°C in dark. Any obvious plant or animal residues were removed by handpicking and the soil was sieved at field moisture content (<2 mm). A portion of the samples was air-dried and analyzed chemically. Characteristics of the soil samples are listed in Table 1.

Extraction of soil DNA

DNA was extracted from the soil samples using the FastDNA SPIN Kit for Soil and the FastPrep FP120 Instrument (Qbiogene, Irvine, CA) according to the manufacturer's instructions. The extracted DNA was purified by the UNIQ10 Spin kit (Shenggong, Shanghai, China), and stored at −20°C.

Amplification of *nifH* gene

The *nif*H gene sequences from nitrogen-fixing microorganisms were amplified using nested PCR to increase sensitivity. The primers and amplification procedure were adopted from Poly et al. (2001a) and Diallo et al. (2004a). The 2×PCR Master mix with 0.1 U *Taq* Polymerase/μl, 500 μM dNTP each, 20 mM Tris-HCl pH 8.3, 100 mM KCl and 3 mM $MgCl_2$ was made following the manufacturer's instructions of (Tiangen, Beijing, China).

In the first round, primers FGPH19 and PolR were used to amplify a 429 bp *nif*H gene fragment. The PCR reaction mixture consisted of 2×PCR Master mix, 1 pmol/μl of each primer, 1 μl template DNA (approximately 1–5 ng) in a final volume of 20 μl. To avoid nonspecific products, we used a modified touch-down PCR procedure with initial denaturation at 94°C for 5 min, 20 cycles of denaturation at 94°C for 1 min, a 50 s touchdown step in which the annealing temperature was lowered from 65 to 55°C in intervals of 0.5°C each cycle, and extension at 72°C for

Table 1 Physicochemical characteristics of soil samples. *OM* Organic matter

Sample No.	Site	pH	OM (g/kg)	Available N (mg/kg)	Available P (mg/kg)	Available K (mg/kg)
R1	Matangchun	5.68	27.60	105.80	82.83	485.40
NR1	Matangchun	6.30	25.11	101.90	46.53	211.40
R2	Fuxingchun A	6.81	27.21	112.00	35.93	207.70
NR2	Fuxingchun A	6.97	25.65	91.87	14.29	178.50
R3	Fuxingchun B	6.14	39.97	135.90	25.89	320.10
NR3	Fuxingchun B	6.16	36.32	118.30	13.25	238.60
R4	Gudechun	6.28	42.73	129.80	44.11	268.60
NR4	Gudechun	6.31	35.16	101.70	13.41	91.10
R5	Gaoganchun	5.95	24.31	103.20	68.43	258.30
NR5	Gaoganchun	6.28	24.72	110.30	36.33	41.15

1 min 30 s, followed by 15 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 50 s, and extension at 72°C for 1 min 30 s, and a final extension at 72°C for 7 min. The quality and concentration of the PCR products were determined by electrophoresis comparing with a 100 bp ladder (Tiangeng, Beijing, China).

In the second round, the primers PolFGC and AQER were used to amplify a 320 bp *nif*H gene fragment, including the GC clamp sequence. The PCR mixture consisted of 2×PCR Master mix, 0.2 pmol/μl of each primer and 0.5 μl PCR products from the first round in a final volume of 50 μl. The amplification program was as in the first round except for the annealing temperature that was lowered from 63°C to 53°C during the initial 20 cycles and kept at 53°C during the final 15 cycles.

DGGE analysis

DGGE was carried out using the D-code system from Bio-Rad Laboratories (Richmond, CA). PCR products (400–450 ng) were loaded onto 1 mm thick 8% (w/v) polyacrylamide gels in 1×TAE (20 mmol/l Tris-acetate, pH 7.4, 10 mmol/l acetate, 0.5 mmol/l disodium EDTA). The denaturing gradient contained 40%–60% denaturant. Electrophoresis was performed at a constant voltage of 50 V for 50 min followed by 180 V for 6 h at 60°C. The gels were stained with silver nitrate as described by Riesner et al. (1989), and scanned with a Pharos FXTM (Bio-Rad).

Dendrogram analysis of DGGE banding patterns was performed using Quantity One 4.2 software (Bio-Rad). The dendrogram was created automatically using unweighted pair group method with arithmetic mean (UPGMA) based on Dice coefficient. The Shannon-Weaver diversity (H), richness (S) and evenness (E_H) indices were calculated according to the following equations (Diallo et al. 2004b; Saikaly et al. 2005)

$$H = -\sum_{i=1}^{s} P_i \ln P_i = -\sum_{i=1}^{s} (N_i/N) \ln(N_i/N)$$

$$E_H = H/H_{\max} = H/1\mathrm{n}S$$

where, P_i is the probability of the ith band to be present in a given lane, Ni is the intensity of the ith band and N is the sum of the intensities of all the bands in the lane (Eichner et al. 1999), while S is the total number of colored bands for each sample.

DGGE fragment isolation and sequencing

Bands were excised from DGGE gels and incubated in sterile vials with 25 μl ddH_2O at 4°C overnight; 2 μl of the supernatant was used as template DNA in a PCR with the primers PolF (without GC clamp) and AQER as previously described (Diallo et al. 2004b). The PCR products were purified with the PCR Clean-Up Kit (MO-BIO Labs, Solana Beach, CA), and cloned directly by ligation to pGEM-T easy vector (Promega, Madison, WI). Positive clones were sequenced at Shenggong Co. (Shanghai, China). The nucleotide sequences determined in this study and reference sequences from the GenBank were aligned, and phylogenetic trees were constructed using MEGA 4.0 (Saitou and Nei 1987).

Results

DGGE analysis

A 320 bp fragment from the *nif*H gene was amplified from DNA isolated from rhizosphere and non-rhizosphere soil from tobacco fields at five sites in Sichuan, China. The resulting products were separated by DGGE (Fig. 1). The DGGE patterns appeared different with respect to the number, intensity and migration rates of bands, displaying the diversity of *nif*H genes.

The DGGE patterns of rhizosphere and non-rhizosphere samples differed at each sampling site. Compared to the

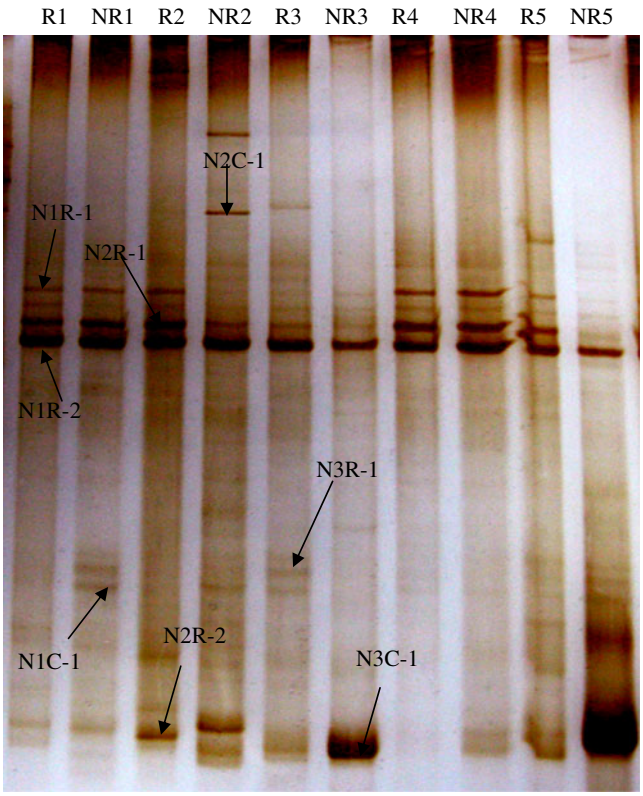

Fig. 1 The denaturing gradient gel electrophoresis (DGGE) profile of the *nif*H gene. Lane labeling: *R* Rhizosphere soil; *NR* non-rhizosphere soil; *1*, *2*, *3*, *4*, *5* sampling sites in Yanbian county; *arrows N1R-1–N3C-1* indicate excised and sequenced fragments

rhizosphere, the non-rhizosphere DGGE patterns were more complex, except at Fuxingchun B and Gaoganchun. The patterns had some common and dominant bands, such as the predominant band N1R-2 that could be found in all samples, indicating that a common *nif*H gene may exist in the different soils.

The ten *nif*H DGGE patterns were distributed into five groups (Fig. 2). All the rhizosphere soils were placed in three groups (III, IV, V). Rhizosphere soil from Gudechun and Fuxingchun B sampling sites (R4, R3) and non-rhizosphere soil from the Matangchun (NR1) were in cluster III. The profiles of NR5 and NR3 formed two clusters that were notably separated from all the other clusters. The profiles of the rest of the samples were separated into two major groups. NR4 and R1 were in cluster IV, and R2, NR2, and R5 in cluster V. In summary, there were very large differences among the profiles of the soil samples. With the exception of Fuxingchun A, rhizosphere and non-rhizosphere samples were not grouped together in the same cluster.

There were large differences in Shannon-Weaver diversity (H), richness (R) and evenness (E_H) indices of the *nif*H gene (Table 2) between the rhizosphere and the non-rhizosphere samples. The diversity and richness indices of the non-rhizosphere soil were higher than those of the rhizosphere soil at Matangchun, Fuxingchun A and Gudechun. The highest diversity index and richness was seen in NR2, while the lowest was observed in NR3.

Phylogenetic analysis of *nif*H sequences

In this study, the soils from different sites had different DGGE profiles although there were some common and dominant bands. Eight bands common to all the samples in the DGGE patterns were successfully cloned and sequenced (Fig. 1). Sixteen *nif*H sequences from GenBank with similarities to our sequences were selected to construct a phylogenetic tree. Out of the 16 selected *nif*H sequences, 12 were from known and formerly described diazotrophs and 4 from uncultured diazotrophs.

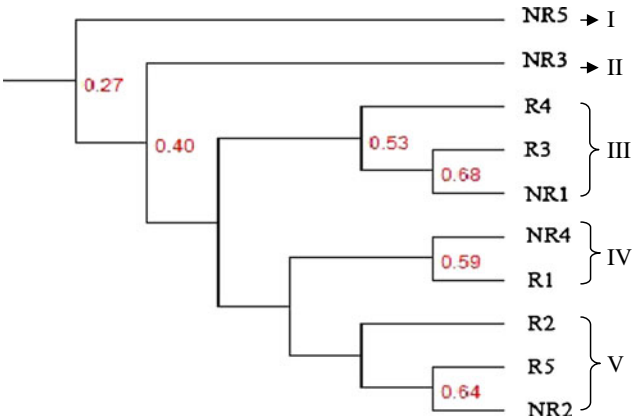

Fig. 2 UPGMA (unweighted pair group method with arithmetic mean) tree based on DGGE banding patterns

The phylogenetic tree (Fig. 3) with 24 *nif*H sequences contained three major clusters. Six sequences, including N1R-1, were clustered in group II with the reference sequences of uncultured bacteria, *Burkholderia cepacia*, *Methylosinus* sp., *Sinorhizobium* sp. and *Pelomonas saccharophila*. Three sequences, including N3C-1, were clustered in group III with the reference sequence of *Pseudomonas* sp. and *Delftia tsuruhatensis*. The other six sequences, N2R-1, N2R-2, N1C-1, N1R-2 and N3R-1, fell into the large group I containing Betaproteobacteria, *Dechloromonas* sp., *Zoogloea oryzae*, *Azovibrio restrictus* and uncultured bacteria. N2R-1 and N2R-2, though extracted from two different bands, belonged to the same phylotype, and both displayed 95% homology to the *nif*H gene of Betaproteobacterium d8-2 (EF626685) isolated from spring freshwater located in Hsinchu County, Taiwan (Chou et al. 2008). N1C-1 displayed 93% homology to the uncultured bacterium clone PN29 (GU117600) isolated from Western Himalayan soil, India (published in GenBank only). The closest matches of N2C-1 and N1R-2 were the *nif*H genes of uncultured bacterium clone RN7 (GU121498) from Western Himalayan soil, India (96% homology, published in GenBank only) and *Dechloromonas* sp. SIUL (AJ563286) from soil in the United States (93% homology, published in GenBank only), respectively. N3R-1 displayed 95% homology to the uncultured nitrogen-fixing bacterium (AY196377) from forest soil in Switzerland (Bürgmann et al. 2004). The band N1R-1 displayed 90% homology to the *nif*H gene of uncultured bacterium (GU097344) isolated from paddy soil in Chong Ming Island, China (published in GenBank only). The band N3C-1 displayed high homology (98%) to the *nif*H gene of *Pseudomonas* sp. (FJ822997) isolated from sugarcane in Guangxi, China (published in GenBank only).

Discussion

Nested PCR reduces the possibility of amplification of multiple target sites and increases the reliability of detection. The usefulness of the nested PCR approach has been well demonstrated in studies of functional gene diversity (Moré et al. 1994; Webster et al. 2002; Langlois et al. 2005). As diazotrophic bacteria are rarely dominant in terrestrial ecosystems, it is difficult to amply the *nif*H gene from soil samples directly (Diallo et al. 2004a; Mergel et al. 2001). In this study, to increase the sensitivity and the specificity of *nif*H gene amplification, we used nested PCR to investigate the genetic diversity of *nif*H gene in soil samples.

Table 2 Comparison of Shannon-Weaver indices of diversity (*H*), richness (*S*) and evenness (E_H) for the *nif*H gene pools in rhizosphere and non-rhizosphere soil of tobacco. Means of 3 replicates±SD. Values in the same column followed by different letters are significantly different ($P<0.05$)

Sample No.	Shannon's diversity index (*H*)	Richness (*S*)	Evenness (E_H)
R1	2.79±0.00 d	18.00±0.00 d	0.966±0.000 b
NR1	2.91±0.06 c	19.33±1.15 c	0.984±0.000 ab
R2	2.98±0.00 b	21.00±0.00 b	0.977±0.000 ab
NR2	3.31±0.00 a	29.33±1.15 a	0.981±0.012 ab
R3	2.96±0.03 bc	21.33±1.15 b	0.968±0.008 b
NR3	2.46±0.00 f	12.00±0.00 g	0.991±0.000 a
R4	2.47±0.05 f	18.00±0.00 d	0.853±0.017 c
NR4	2.67±0.04 e	16.00±0.00 e	0.962±0.014 b
R5	3.01±0.05 b	22.00±0.00 b	0.974±0.018 b
NR5	2.51±0.03 f	13.67±0.58 f	0.960±0.002 b

The main results of cluster analysis indicated that there was a great difference between the rhizosphere and the non-rhizosphere *nif*H gene pools. The diversity and richness indices of non-rhizosphere soils were somewhat higher than those of rhizosphere soils. These data are in agreement with the results of Duineveld et al. (2001) who found that the PCR-DGGE patterns observed for the bulk soil of *Chrysanthemum* at the maturity stage were more complex than those obtained from rhizosphere samples. For the non-rhizosphere soil samples, Shannon-

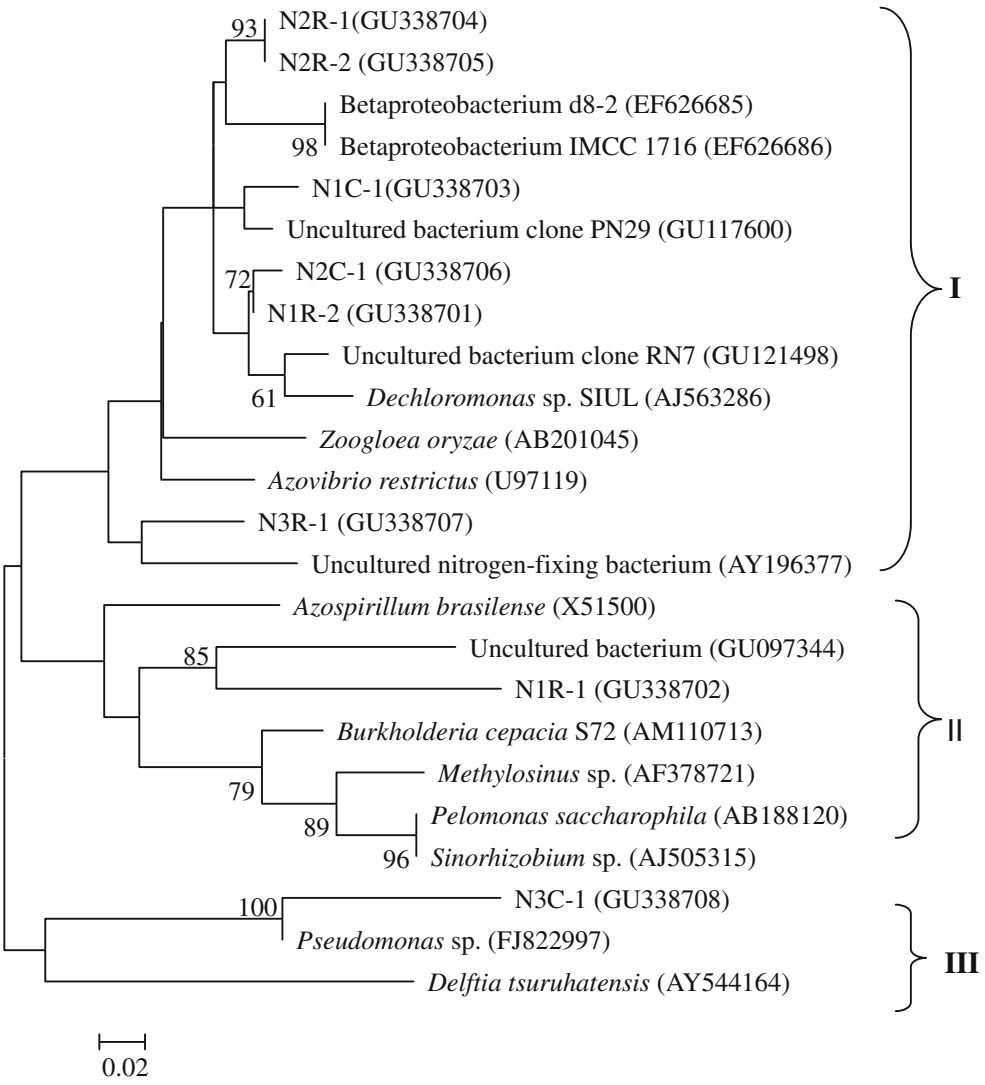

Fig. 3 Phylogenetic analysis of *nif*H gene sequences. The tree was constructed using Kimura 2-parameter distance with the neighbor-joining method. Bootstrap values (1,000 re-samplings) are indicated at branch nodes. The *scale bar* indicates 2 changes per 100 nucleotide positions

Weaver diversity index (H) of most samples was higher at higher pH and lower with higher concentration of available nitrogen. This may indicate that the increase in H was linked to the increase of pH and decrease in available nitrogen. For the non-rhizosphere soil samples, evenness (E_H) was higher at higher available potassium concentrations, whereas with the exception of R4, E_H was lower at higher available potassium concentrations in the rhizosphere soil samples. It is possible that there are some substances secreted from tobacco root interacting with K^+ to decrease the E_H. More studies should be carried out to investigate this hypothesis.

Bands N1R-1, N2R-1 and N1R-2 were found from all samples suggesting that *nif*H genes similar to those of Betaproteobacteria, *Zoogloea oryzae*, *Dechloromonas* sp., and *Azovibrio restrictus* are the dominant species in the tobacco soil. Bands N1C-1, N2R-2, N3R-1 and N3C-1 were found from few samples, including both rhizosphere and non-rhizosphere, while band N2C-1 was only found from the non-rhizosphere soil sample NR2.

Some studies have indicated that the structural and functional diversity of rhizosphere populations is affected by plant species due to differences in root secretions and rhizodeposition in different root zones (Kent and Triplett 2002). Furthermore, the soil type, growth stage of the plant, cropping practices and environmental factors influence the composition of the microbial community in the rhizosphere (Grayston et al. 1996; Wieland et al. 2001). Compared with other plants, tobacco has different patterns of root secretions. Young roots are known to secrete more organic material than older roots, which can result in different specific bacterial populations. The roots of older plants usually secrete more harmful secondary metabolites into the soils than the young ones (Bowen and Rovira 1991; Curl and Truelove 1986; Liljeroth et al. 1991). In conclusion, as assessed by culture-independent methods, our results indicated that the *nif*H gene pool was more diverse in the non-rhizosphere soil than in the rhizosphere soil in the vicinity of tobacco.

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