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Differences in common bean rhizobial populations associated with soil tillage management in southern Brazil

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Abstract

Progressive adoption of no-tillage (NT) agriculture in the tropics is finally reversing physical, chemical, and biological erosion of soil and in Brazil, an estimated 19 Mha are now devoted to NT. Common bean (*Phaseolus vulgaris* L.) is a main component of Brazilian agriculture, and enhancement of yields has been achieved under NT as a result of mitigation of environmental stresses, resulting in higher N_2 fixation. However, the effects of NT on rhizobial diversity are poorly understood. This study evaluated rhizobial diversity in soils planted to common bean under NT or conventional tillage (CT) systems that were compared with natural grassland used for grazing, in the State of Santa Catarina, southern Brazil. Genetic diversity was assessed by the amplification of the DNA by PCR with specific primers (BOX-PCR) and by RFLP-PCR analyses of the 16S rDNA region. A high level of diversity was observed among strains from all three systems, such that the similarity in the clustering analysis of BOX-PCR products ranged from 36% under natural grassland to only 23% for CT strains. High polymorphism was confirmed in the RFLP-PCR analysis; forty-seven different profiles were obtained, none sharing high similarity with the profiles of reference species of common bean rhizobia. These results indicate that other tropical rhizobial species remain to be described. Genetic diversity was higher among the NT than the CT rhizobial strains, especially when the RFLP-PCR profiles were considered. Genetic diversity in the natural grassland was lower than in the cropped systems, possibly due to absence of the host plant and stubble burning in winter. Average yields in the area under NT (e.g. common bean, approximately 1500 kg ha^{-1}) have been about 30% higher than under CT, therefore high rhizobial diversity may be a parameter indicative of superior soil quality.
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1. Introduction

Intensive agricultural use of soil accelerates degradation and threatens its capacity for biological activity. Therefore, climax ecosystems and conservationist management practices would be expected to

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maintain biological diversity and good soil quality. Progressive adoption of no-tillage (NT) agriculture is reversing physical, chemical and biological erosion of the often-fragile soils of the tropics. It is estimated that, in 1999–2000, NT was practiced on about 58 million hectare of farmland, mainly in North and South America, but also in Southern Africa and South Asia (FAO, 2004). Pioneer NT trials in Latin America were performed in 1971, in Parana State, southern Brazil (Derpsch, 1998). The system has seen rapid adoption throughout Brazil such that the current NT area now exceeds 19 Mha (FEBRAPDP, 2004).

The benefits resulting from NT include reductions in soil temperature extremes, improvements in water infiltration with increases in soil-moisture content, protection of the soil against erosion, increases in the stability of the soil aggregates, maintenance of soil structure, and augmentation in soil organic matter content. Furthermore, NT may play a role in reducing global warming by providing a greater sink for CO_2, and it can save 30–40% in terms of labor and, in mechanized agriculture, fossil fuel consumption in comparison to conventional cropping (e.g., Derpsch et al., 1991; Derpsch, 1998; Kladivko, 2001; Bayer et al., 2002; FAO, 2004).

There is a general concept (e.g., FAO, 2004) that benefits from NT result from biological processes, thus microbial biodiversity is increased. Indeed, there are many reports of increases on microbial biomass and activity in the NT when compared with the CT system, and effects reported in the tropics (e.g., Balota et al., 1998, 2004; Hungria, 2000; Franchini et al., 2002) are much clearer than those in temperate regions (e.g., Wardle et al., 1999; Carpenter-Boggs et al., 2003). However, few studies have evaluated microbial diversity as a function of tillage systems and the effects of NT on such diversity remain poorly understood.

Common bean (Phaseolus vulgaris L.) is a main component of Brazilian agriculture, representing the most important source of protein of the poor population. The country is the largest grower and consumer of this legume worldwide: 4,378,700 tons were produced in 2002–2003 (CONAB, 2004; EMBRAPA, 2004). Good yields have been achieved cropping the legume under NT (Deibert, 1995); apparently, most of the benefits are related to effects on soil temperature and moisture content (Stone and Silveira, 1999). The favourable soil environmental conditions fostered by NT are likely to result in greater inputs of N as a result of increased symbiotic N_2-fixation (Hungria, 2000; Hungria and Vargas, 2000).

Rhizobia nodulating common bean may be considered as a good indicator for soil quality as they are ubiquitous and present in high populations in tropical soils, they have been demonstrated to rapidly respond to soil changes and they are crucial for legume production (Hungria, 2000; Hungria and Vargas, 2000; Andrade et al., 2002). Improved knowledge of rhizobial diversity under CT and NT could help researchers and producers to delineate more appropriate strategies for soil sustainability and agricultural production. Therefore, in this study, rhizobia nodulating common bean grown under the NT and CT systems and in natural grassland in the State of Santa Catarina, southern Brazil, were genetically characterized to obtain a better understanding of the effects of NT on rhizobial diversity.

2. Material and methods

2.1. Field sites and soil sampling

Soil samples were collected from three farms in Campo Belo do Sul, southwest of Santa Catarina, southern Brazil. The area is located at an altitude of 1017 m, 27°56′ S and 50°48′ W. The climate is type Cfb according to Koeppen's classification with average rainfall of 1500 mm year^{-1}. The soil in the three areas is classified as Humic Nitosol (FAO, 1998). The areas of conventional tillage (CT) and no-tillage (NT) were natural grassland before cropping. Some physical and chemical characteristics of the soils in the studied areas are displayed in Table 1.

The soil under the NT system has been NT-cropped for more than 10 years. Commercial inoculants had been applied to crops of soybean (Glycine max) (Bradyrhizobium elkanii strains SEMIA 587 and SEMIA 5079) and common bean (Rhizobium tropici CIAT 899). Fertilization was practiced according to the chemical analysis, and the soil pH was adjusted to 5.5. Good yields had been obtained in the area under NT, with averages of 2800 kg ha^{-1} for soybean, 6500 kg ha^{-1} for maize (Zea mays) and 1500 kg ha^{-1} for common bean.

Table 1

Physical and chemical properties of the soils in the areas studied in Campo Belo do Sul, SC

Soil management systems	Characteristics								
	pH water	pH SMP	%		mg dm^{-3}		cmol$_c$ dm^{-3}		
			Clay	Organic Matter	P	K	Al	Ca	Mg
No-tillage	5.7	6.0	52	3.9	12.5	137	0	9.5	3.9
Conventional till	5.4	5.7	45	3.5	6.5	112	0	5.2	2.1
Natural grassland	4.8	5.4	51	4.0	1.1	87	2.5	2.1	0.9

The area representing CT had been under the CT system for 3 years. Soil pH was adjusted to 5.5. Yields have been on average 30% lower in the experimental site of CT than those obtained under NT. At both sites, diseases and insect infestations were controlled when necessary using standard methods.

The soil of the third area is covered with natural grassland and used for grazing; it has never received fertilizers or inoculants and has been managed (more than 10 years) by burning the vegetation in the winter.

2.2. References strains

Common bean rhizobia reference strains included: *Rhizobium tropici* IIA CFN 299 (=USDA 9039, =LMG 9517), IIB CIAT 899[T] (=UMR 1899, =USDA 9030, =TAL 1797, =HAMBI 1163, =SEMIA 4077, =ATCC 49672) and *R. etli* CFN 42[T] (=USDA 9032) (received from CFN, Cuernavaca, Mexico). *R. tropici* strain PRF 81 (=SEMIA 4080) came from the Embrapa Soja germplasm bank and *R. giardinii* bv. giardinii strain H152[T] and *R. gallicum* bv. gallicum strain R602[T] were provided by INRA, Dijon, France.

2.3. Rhizobial isolation and morpho-physiological characterization of the isolates

Forty soil subsamples were taken per area, spatially distributed to cover the field site, and were combined to represent one sample. Samples were then submitted to successive dilutions to 10^{-5}, and 1 ml aliquots of each dilution were used to inoculate each surface-sterilized (Vincent, 1970) common bean seeds of cultivar Pérola. Seeds were sown in pots containing sterile sand, and plants were grown under greenhouse conditions, with mean temperatures of 28/22 °C (day/night), with N-free nutrient solution applied (Andrade

and Hamakawa, 1994). Nodules were collected when plants were 30-days old.

In the laboratory, nodules were randomly chosen and rhizobia were isolated using standard procedures (Vincent, 1970). Purity of the cultures was confirmed by repeatedly streaking the bacteria on yeast extract-mannitol agar (YMA) medium (Vincent, 1970) and verifying a single type of colony morphology, absorption of Congo red (0.00125%) and a uniform Gram-stain reaction. Colony morphology (color, mucosity, transparency, borders, elevation) and acid/alkaline reaction were evaluated after 2 and 3 days of growth on YMA containing bromothymol blue (0.00125%) as indicator, in the dark, at 28 °C. Each colony was transferred to YM liquid broth (YMB) and after growth the broth was mixed with glycerol (1:1, v:v) and stored at −80 °C. Working cultures were maintained on YMA slants at 4 °C. Rhizobia were cultured routinely at 28 °C in YMB on a rotary shaker operating at 65 cycles per minute.

2.4. Genetic characterization

2.4.1. Extraction of DNA of rhizobial isolates

For DNA extraction, bacteria were grown in 15 ml of YMB (modified to contain 5 g l^{-1} of mannitol), for 3 days, at 28 °C and were then centrifuged at 10,000 rpm for 10 min. The pellet was transferred to a 1.5 ml Eppendorf tube and washed three times with saline solution (0.85% NaCl) once in PBS (containing, in 500 ml: NaCl 150 mM, 4.383 g; NaH$_2$PO$_4$·H$_2$O 2.6 mM, 0.1793 g; Na$_2$HPO$_4$·12H$_2$0 7.6 mM; 1.36 g). The pellet was resuspended in TE 50:20 (Tris–HCl 50 mM pH 8.0; EDTA Na$_2$ 20 mM, pH 8.0; Tris–HCl 1 M, pH 8.0) at a concentration of 10^9 cells ml^{-1} and 1.4 ml were transferred to another Eppendorf tube and centrifuged at 12,000 rpm for 10 min. The supernatant was discarded and the pellet

resuspended in 400 µl of TE 50:20 and received 50 µl of SDS (sodium dodecyl sulphate) at 10% in water (v/v); 5 µl proteinase K (20 mg ml^{-1}); 10 µl of lisozime (5 mg ml^{-1}); 2 µl of RNAse (10 mg ml^{-1}, prepared in Tris–HCl 10 mmol^{-1} pH 7.5, NaCl 15 mmol l^{-1}) and incubated at 37 °C for 1 h. The samples were passed through insulin syringes three times to decrease viscosity, then NaCl and AcONa were added to final concentrations of 250 mmol l^{-1} and 300 mmol l^{-1}, respectively. Samples were homogenized, left for 1 h at 4 °C, were centrifuged at 12,000 rpm for 15 min, recovering 300 µl of the supernatant, to which 600 µl of cold 95% ethanol were added and samples were left overnight at −20 °C. The following day, samples were centrifuged at 12,000 rpm for 15 min, the ethanol was discarded, and 400 µl of cold 70% ethanol were added. Samples were centrifuged again at 12,000 rpm for 15 min, the ethanol was discarded and the precipitates left to dry at room temperature for approximately 3 h. Finally, each precipitate was resuspended in 50 µl of TE 10:1. All solutions used for DNA extraction were previously autoclaved. Purity of the DNA was confirmed by electrophoresis in mini-gels (8 cm × 10 cm) of agarose at 1.5%, for 25 min at 80 V, using as a comparison Low DNA Mass^{TM} Ladder (Invitrogen^{TM}, Life Technologies). Gels were stained with ethidium bromide and visualized under UV light.

2.4.2. PCR amplification with specific BOX A1R primer

The DNA of each bacterium was amplified by PCR with primer BOX A1R (5′-CTACGGCAAGGC-GACGCTGACG-3′, Invitrogen^{TM}, Life Technologies) (Versalovic et al., 1994). The final volume of the PCR reaction was of 25 (l and contained: dNTPs (1.5 mM of each), 5.0 µl; buffer 10X (500 mM KCl; 100 mM Tris–HCl, pH 8.3), 2.5 µl; MgCl$_2$ 50 mM, 1.5 µl; primer (50 pmol µl^{-1}), 1 µl; Taq DNA polymerase (5 U µl^{-1}), 0.2 µl; DNA 50 ng µl^{-1}, 1 µl; sterile milli-Q water to complete the volume. The following cycles were used: one cycle of denaturation at 95 °C for 7 min; 35 cycles of denaturation at 94 °C for 1 min, of annealing at 53 °C for 1 min and of extension at 65 °C for 8 min; one cycle of final extension at 65 °C for 16 min; and a final soak at 4 °C. The reactions were carried out in an MJ Research Inc. PTC-100^{TM} thermocycler and

amplified fragments were separated by horizontal electrophoresis on a 1.5% agarose (low EEO, type I-A) gel (20 cm × 25 cm), at 120 V, for 6 h. Gels were stained with ethidium bromide, visualized under UV light and photographed with a Kodak Digital Science 120 apparatus.

2.4.3. Restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rDNA genes

The DNA of the bacteria was amplified with primers Y1 (5′-TGGCTCAGAACGAACGCGTGGCGGC-3′) (Young et al., 1991) and Y3 (3′-CTGACCCCAACTT-CAGCATTGTTCCAT-5′) (J.P.W. Young, unpublished), which amplify almost the full length of the region (1500 bp) corresponding to the 16S rRNA. Five replicates of a mixture with a final volume of 50 µl, containing: dNTPs (1.5 mM of each), 2.0 µl; 10X buffer (200 mM Tris–base, 500 mM KCl, pH 8.4), 5.0 µl; MgCl$_2$ (50 mM), 1.5 µl; each primer (stock at 10 pmol µl^{-1}), 1.0 µl; Taq DNA polymerase (5 U µl^{-1}), 0.2 µl; DNA (50 ng µl^{-1}), 1.0 µl, 38.30 µl of sterile milli-Q water. The amplification cycles were based on Young et al. (1991), modified to increase the annealing temperature, and consisted of: one cycle of denaturation at 93 °C for 5 min; 35 cycles of denaturation at 93 °C for 45 s, of annealing at 64 °C for 45 s and extension at 72 °C for 2 min; one cycle of final extension at 72 °C for 5 min; and a final soak at 4 °C. Each strain produced a single PCR product with the expected MW. For the RFLP, the methodology of Laguerre et al. (1994) was applied to the PCR products, with the enzymes CfoI (5′-GCG/C-3′; 3′-C/GCG-5′), HinfI (5′-G/ANTC-3′; 3′-CTNA/G-5′), MspI (5′-C/CGG-3′; 3′-GGC/C-5′), RsaI (5′-GT/AC-3′; 3′-CA/TG-5′) and MboI (5′-/GATC-3′, 3′-CTAG/-5′) (Invitrogen^{TM}, Life Technologies). For each enzyme, a 10 µl mixture was prepared containing: 6 µl of the PCR product; 1 µl of the specific buffer for each enzyme (10X); 0.5 µl of enzyme (10 U µl^{-1}) and 2.5 µl of sterile milli-Q water. The mixtures were incubated in the water bath at 37 °C for the following times: CfoI (1 h); HinfI (3 h); MspI (2 h); RsaI (2 h); MboI (5 h). The fragments obtained were analyzed by horizontal electrophoresis in a gel (20 cm × 12 cm) with 3% of agarose, at 120 V for 4 h. Bands with MW lower than 100 bp were discarded without affecting the analysis, as previously reported (Laguerre et al., 1994).

2.4.4. Cluster analysis

Cluster analyses were performed with the BOX A1R-PCR and the other with RFLP-PCR products using Bionumerics program (Applied Mathematics, Kortrijk, Belgium), with the UPGMA algorithm (unweighted pair-group method with arithmetic mean) and the coefficient of Jaccard (J) (Sneath and Sokal, 1973), at a tolerance of 2%.

2.4.5. Genetic diversity

Indexes of diversity, richness, and evenness were estimated based on the number of isolates belonging to each group of profiles in BOX A1R and of the RFLP-PCR of the 16S rRNA region. Diversity was calculated by using the Shannon index: $H' = -\Sigma\ [(n_1/n)\ \ln(n_1/n)]$ (Shannon and Weaver, 1949), where n_1 is the number of isolates in each group and n is the number of isolates in all groups. For richness, the Margalef index was used: $R_1 = (S - 1)/\ln(n)$ (Margalef, 1958), where S is the number of groups and n is the number of isolates in all groups. The Pielou index was used as a measure of evenness: $E_1 = H'/\ln(S)$ (Pielou, 1977), where H' is the Shannon index and S is the number of groups. Grouping for BOX A1R-PCR was obtained by considering a level of similarity of 70% in the cluster analysis with the UPGMA algorithm and the J coefficient. The groups of RFLP-PCR of 16S rRNA region were achieved by combining the different profiles and designating each group of similar profiles with a letter.

3. Results

3.1. Morpho-physiological characterization

Eighty strains were obtained from the area under NT, 46 from that under CT, and twenty from the natural grassland. Characterization of the strains revealed high variability in relation to morpho-physiological properties. Thirty-one different combinations of color, mucosity, transparency, border type, elevation, and acid/alkaline reaction were obtained; however, 62.5% of the strains constituted a predominant group with opaque color, high or intermediate production of mucus, regular margin, flat elevation and acid production on YMA medium (data not shown). There was no recognizable effect of

tillage method on the morpho-physiological characteristics.

3.2. BOX A1R-PCR genomic fingerprinting

Based on morpho-physiological characteristics, 36 strains from each tillage system were randomly chosen and, together with the 20 strains from the natural grassland, were submitted to the DNA analysis with the BOX A1R PCR method. Successful amplification was achieved with all, except for one strain from the NT system. High polymorphism was observed in the bands from all three groups of strains. In the CT system, the strains were grouped into nine main clusters and some isolates occupied isolated positions; the strains were joined at a very low final level of similarity, 17% (Fig. 1). Only three pairs of strains showed identical profiles. Reference strains of R. tropici were joined at a 64% level of similarity and a subcluster with seven strains was linked to them with a similarity of 54%. Three other strains were clustered with R. etli CFN 42, with a similarity of 54%. Finally, two strains showing identical profiles were linked to reference strains of R. gallicum and R. giardinii at a 62% level of similarity. All other strains showed profiles quite different from those of reference strains (Fig. 1).

Six main clusters and four isolated strains composed the dendrogram of strains from the NT system and were joined at a final level of similarity of 23% (Fig. 2). Only two strains showed similar profiles, while three others were clustered with all reference strains, with a similarity of 46% (Fig. 2). The group showing higher similarity (cluster 3) included 12 strains joined at a 52% similarity, and the lowest similarity was observed in cluster 6 (26%). Clearly, the majority of the strains were diverse and different from the reference strains.

Rhizobial strains from the natural grassland were also diverse in terms of the PCR products obtained with BOX A1R primer, and were joined at a final level of similarity of 36% (Fig. 3). Four main clusters of very low genetic relatedness were observed and only a few strains showed similar profiles (Fig. 3). R. tropici reference strains were positioned together in a subcluster of cluster 2, and a group of four strains was linked to them at a 55% level of similarity. Six other strains were positioned in cluster 1 (47% of
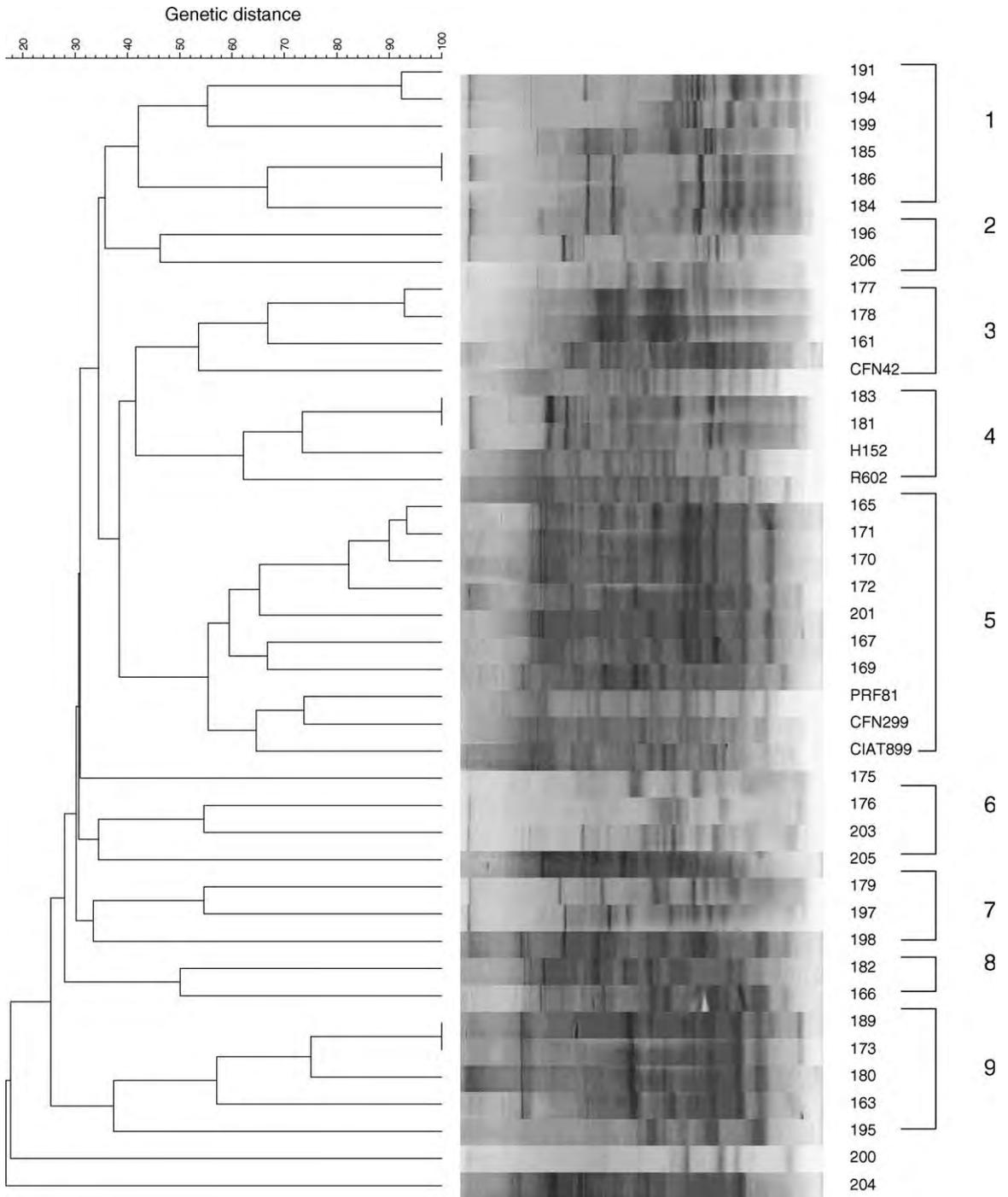
Fig. 1. Dendrogram based on cluster analysis of BOX A1R-PCR products using the UPGMA algorithm and the Jaccard coefficient, of common bean rhizobial strains from a soil from Santa Catarina State, Brazil, under conventional tillage.
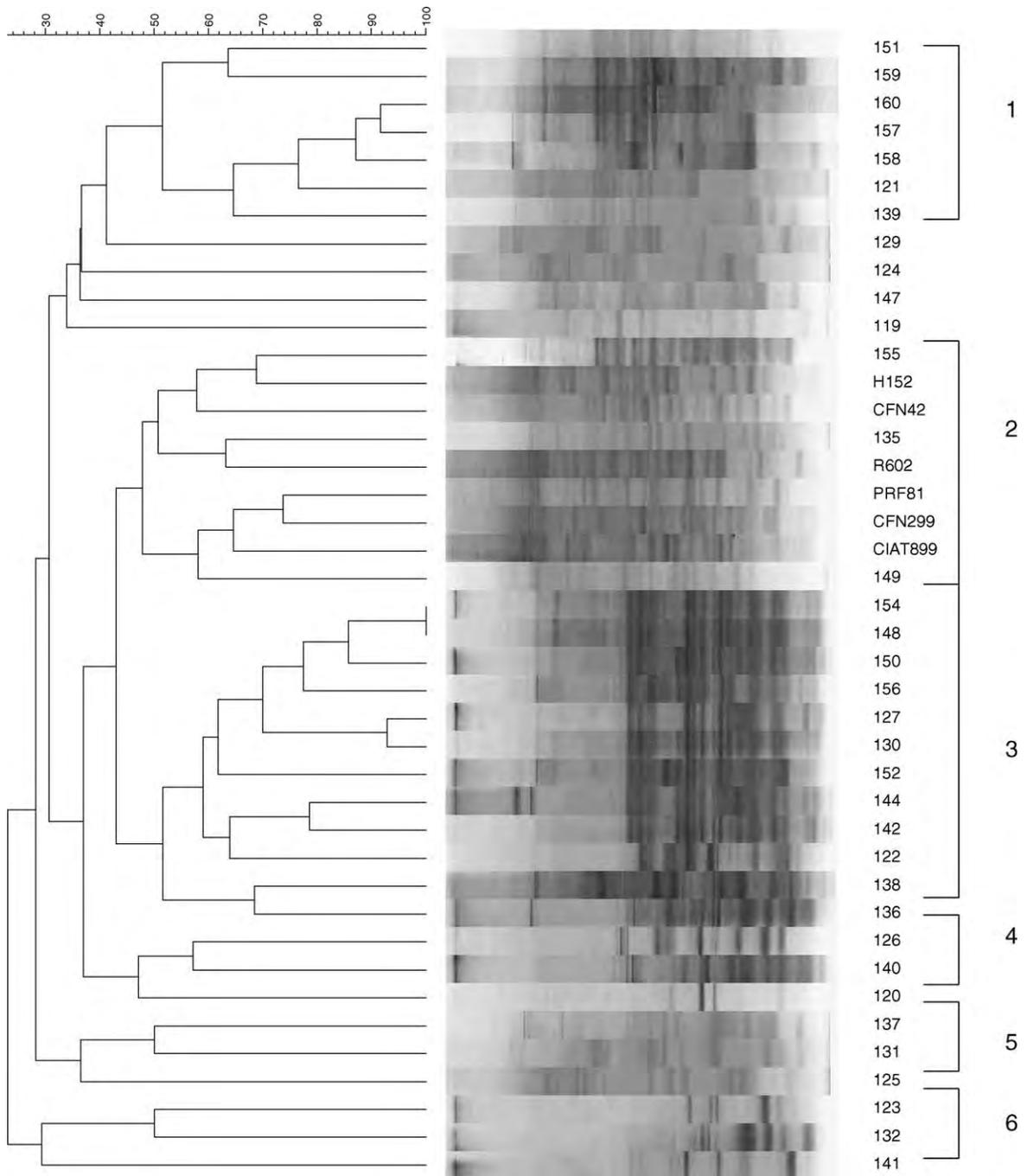
Fig. 2. Dendrogram based on cluster analysis of BOX A1R-PCR products using the UPGMA algorithm and the Jaccard coefficient, of common bean rhizobial strains from a soil from Santa Catarina State, Brazil, under no-tillage.
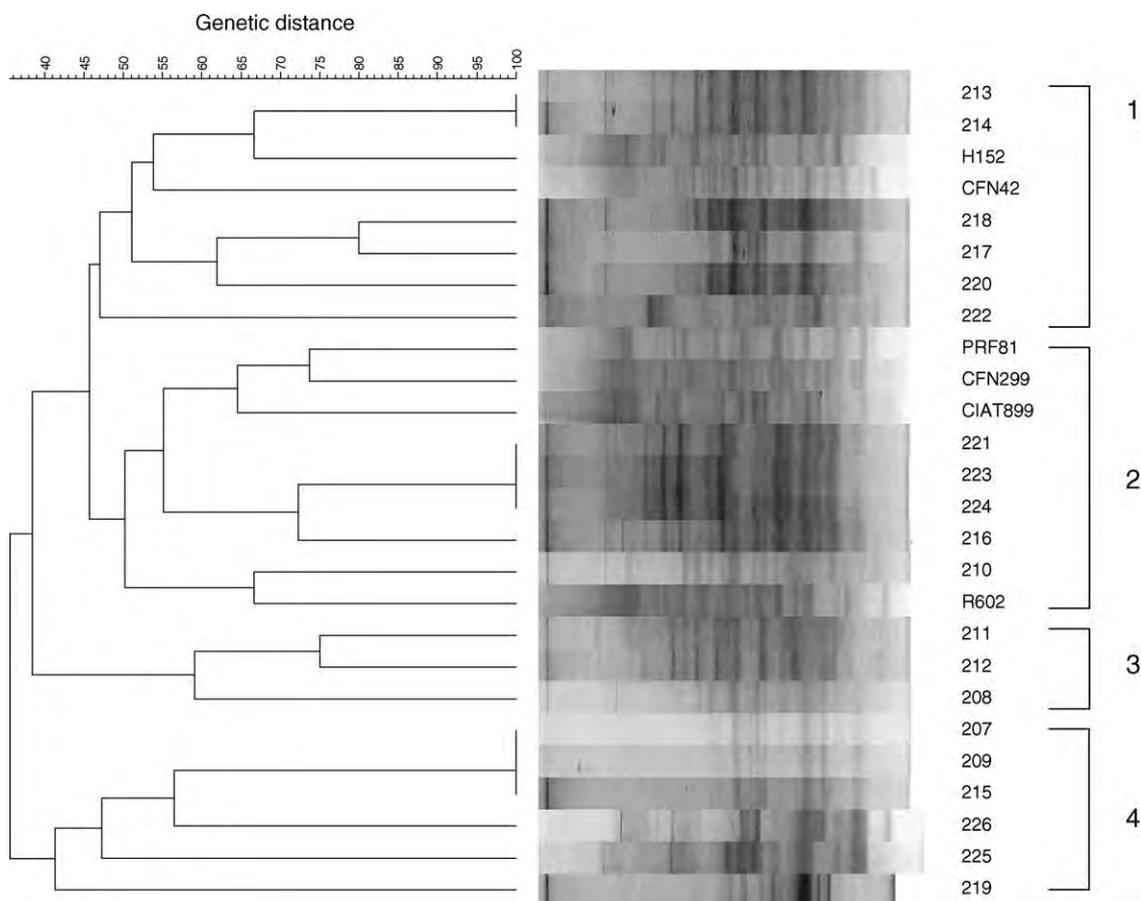
Fig. 3. Dendrogram based on cluster analysis of BOX A1R-PCR products using the UPGMA algorithm and the Jaccard coefficient, of common bean rhizobial strains from a soil from Santa Catarina State, Brazil, with natural grassland.

similarity), which included reference strains of *R. etli* and *R. giardinii*.

3.3. Profiles of RFLP-PCR of the 16S rDNA region

A similarity of 70% in the BOX A1R-PCR analysis was used to choose strains for PCR-RFLP analysis of the 16S rDNA region, thus profiles of 24 strains from each tillage system and 12 from the natural grassland were obtained. Again high polymorphism was observed, and none of the strains shared profiles of reference strains. Forty-seven different profiles of RFLP-PCR were obtained, only three of which occurred in both the NT and CT systems (Table 2). Strains from the natural grassland treatment also showed unique profiles.

3.4. Genetic diversity

From the analysis of the BOX A1R-PCR products, the indexes of Shanon (H') for diversity, Margalef (R_1) for richness, and Pielou (E_1) for evenness were similar for NT (2.96, 6.47 and 0.93, respectively) and CT (3.02, 6.42 and 0.95, respectively), but lower for the natural grassland (2.35, 3.67 and 0.94, respectively). However, when the RFLP-PCR products were considered, both H' and R_1 were considerably higher in NT (3.12 and 6.92, respectively) than in CT (2.86 and 5.59, respectively) system, with similar evenness (0.97 and 0.99 for CT and NT, respectively). Lower indexes were verified for the strains from the natural grassland: 2.09 for H', 3.21 for R_1, with similar evenness (0.95).

Genetic diversity was also evaluated by a curve of cumulative profiles in relation to the number of strains

Table 2
Profiles of RFLP-PCR in 16S rRNA region in strains of rhizobia of common bean in Campo Belo do Sul, SC

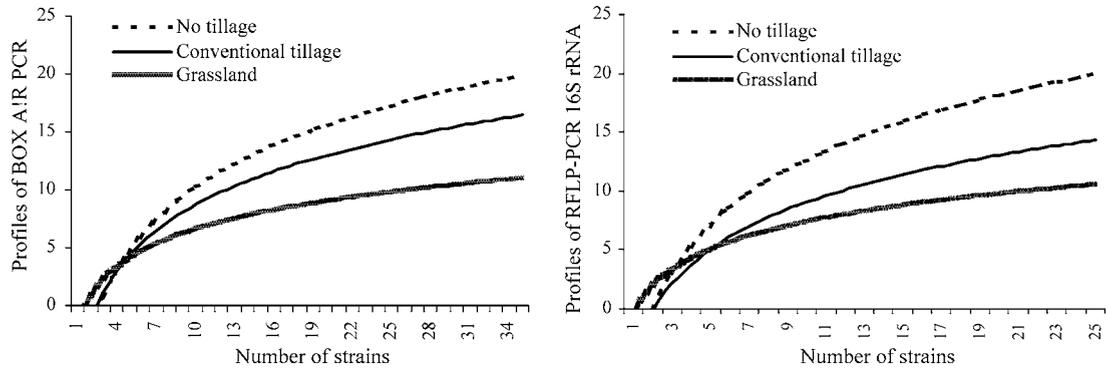| Restriction enzyme					Soil management system		
HinfI	CfoI	MboI	MspI	RsaI	Conventional till	No-tillage	Natural grassland
a	a	a	a	a	–	135	–
a	a	a	a	b	–	–	218
a	a	b	b	c	–	–	207
a	a	c	c	a	–	119	–
a	b	d	d	d	–	140	–
a	c	e	e	e	176	–	–
b	d	e	f	e	166, 178	151, 159	–
b	d	f	a	f	206	–	–
b	d	g	g	e	195, 196	–	–
b	d	g	f	e	199	–	–
b	d	h	a	e	–	156	–
b	d	h	a	g	–	155	–
b	d	h	a	h	–	142	–
b	d	h	g	e	200	–	–
b	d	i	h	i	–	–	225, 226
b	e	g	h	i	–	–	219
b	e	j	I	j	–	126	–
b	f	j	I	e	–	120	–
c	d	l	j	l	197	–	–
c	g	m	l	m	–	–	208
c	h	n	m	n	–	–	223
c	h	o	n	l	179, 198	–	–
c	h	p	e	l	163, 173	157	–
c	i	e	e	l	175	–	–
c	i	g	o	l	185	–	–
c	j	q	p	l	161, 172	–	–
c	l	r	q	n		–	222
c	m	g	r	l	191	–	–
d	n	s	s	o	182	149	–
d	n	t	t	p	–	129	–
e	e	u	u	n	–	–	210, 212, 214
e	e	u	h	n	–	–	220
e	e	v	v	q	203	–	–
e	i	x	x	r	–	152	–
e	n	t	t	p	–	138	–
f	i	w	t	u	–	123	–
f	o	k	t	a	–	125	–
f	o	z	t	s	–	124	–
f	p	r	z	t	–	139	–
g	o	y	y	v	204	–	–
g	p	r	w	t	–	141	–
h	e	α	k	t	–	131, 132	–
i	q	β	s	e	–	147	–
j	n	γ	y	x	205	–	–
l	r	δ	α	t	–	137	–
m	f	ε	β	z	183	–	–
n	s	ζ	γ	w	169	–	–

Fig. 4. Cumulative diversity in function of the number of common bean rhizobial strains and of the profiles obtained by DNA analysis by BOX A1R-PCR or RFLP-PCR of the 16S rDNA region, in field sites of Santa Catarina State, Brazil, under conventional (CT), no-tillage (NT), and natural grassland (GL).

analyzed. The diversity by BOX A1R-PCR was slightly higher for the strains isolated from the NT system, and both NT and CT strains were more diverse than those from the natural grassland (Fig. 4). In contrast, the analysis of RFLP-PCR products showed greater diversity of NT strains in relation to CT strains; however, diversity again was least among the strains from natural grassland (Fig. 4).

4. Discussion

The objective of this study was to survey rhizobial diversity on a relatively large scale, therefore, samples were taken from properties of small-acreage farmers growing common bean in the State of Santa Catarina, southern Brazil. There have been few studies of microbial diversity in tropical soils. In Brazil it has been shown that cropping and soil management system may affect microbial biomass and activity, as well as the relative presence of specific microorganisms, with reports of increases in numbers of rhizobial cells and of mycorrhizal fungal spores (Cattelan and Vidor, 1990; Cattelan et al., 1997; Balota et al., 1998, 2004; Hungria, 2000; Franchini et al., 2002). A pioneering study of effects of tillage management on rhizobial diversity – with 142 soybean strains isolated from soils of another southern state of Brazil, Parana – showed that a higher number of genomic patterns (RAPD profiles) existed under NT growing soybean/wheat/maize (11 profiles) than under CT (four profiles) without a legume (wheat/maize) (Ferreira

et al., 2000). Other agricultural practices that improve soil environment, such as liming of acid soils, also enhance diversity of common bean rhizobia (Andrade et al., 2002).

The N_2-fixing symbiosis of common bean proved to be an excellent model for studies on rhizobial diversity in tropical soils, as up to 35 BOX A1R-PCR profiles were obtained in the analysis of NT strains. The high diversity detected is probably related to the promiscuous nature of the host plant, trapping a wide range of bacterial strains (Michiels et al., 1998), to the often-reported genetic rearrangement of bean rhizobia (Martinez-Romero et al., 1991), and to the sensitivity of the symbiosis to environmental stress (Hungria and Vargas, 2000). In our study, great genetic diversity was detected: similarities in the clustering analysis of BOX A1R-PCR products ranged from 36% under natural grassland to only 23% under CT.

The analysis by the RFLP-PCR products of the 16S rDNA region has often been used for rhizobial species designation, since it usually shows good agreement with the 16S rRNA genes (e.g., Laguerre et al., 1994, 1997). A high diversity of rhizobial species trapped by common bean was evident in this study: forty-seven different profiles were obtained, of which only three occurred in both NT and CT systems and none occurred in the natural grassland treatment. Furthermore, more profiles were obtained in NT than in CT. A previous study also identified a high number of profiles (20) in the analysis of 101 strains from two different Brazilian ecosystems (Grange and Hungria, 2004). Currently, five rhizobial species have been

described that nodulate common bean: *R. leguminosarum* (Jordan, 1984), *R. tropici* (Martinez-Romero et al., 1991), *R. etli* (Segovia et al., 1993), *R. gallicum*, and *R. giardinii* (Amarger et al., 1997). Previous analyses of the 16S rRNA genes of Brazilian isolates from three different ecosystems identified all described common bean rhizobial species except *R. gallicum*, as well as bacteria belonging to the genera *Sinorhizobium* and *Mesorhizobium* (Mostasso et al., 2002; Grange and Hungria, 2004). The remarkable multiplicity of RFLP-PCR profiles verified in this study strongly indicates that many rhizobial species remain to be described and that rhizobial genetic diversity remains poorly characterized in tropical soils.

Genetic richness, evaluated by indexes H' and R_1, and by the cumulative profiles of BOX A1R-PCR, were slightly enhanced in the NT when compared with the CT, and greatly enhanced when the RFLP-PCR profiles were considered. Benefits of NT for sustainability of tropical soils have been widely reported and some of the main features are related to decrease in soil-temperature oscillations, higher soil moisture content, increased organic matter content, and maintenance of soil aggregates and structure (e.g., Derpsch et al., 1991; Hungria and Vargas, 2000; Bayer et al., 2002, Castro Filho et al., 2002; Hernández and López-Hernández, 2002). The improvement of these chemical and physical properties in tropical soils is likely to play a key role in enhancing microbial survival and activity (Cattelan and Vidor, 1990; Cattelan et al., 1997; Balota et al., 1998, 2004; Hungria, 2000; Franchini et al., 2002) as well as nodulation and N_2 fixation (Voss and Sidiras, 1985; Hungria, 2000; Hungria and Vargas, 2000).

Rhizobial diversity was lower in the natural grassland when compared with the cropped areas. Microbial biomass is usually highly favoured by permanent grass when compared to cultivated soils (e.g., Cattelan and Vidor, 1990; Carpenter-Boggs et al., 2003). However, Palmer and Young (2000) have also reported higher diversity of pea symbiont, *R. leguminosarum*, in cultivated compared with grassland soil. It is likely that common bean rhizobia increase in numbers with the cropping of the host plant (Andrade et al., 2002; Hungria et al., 2003). Furthermore, management of natural grassland for grazing in Santa Catarina involves yearly burning to eliminate old residues. Fire depletes soil surface material, temporarily increases temperature and decreases moisture, and it is possible that rhizobial species and strains less tolerant of those stresses do not survive.

The importance of biodiversity may rely on a buffering capacity of the soil to guarantee maintenance of functions, and it has been often suggested that soil microbial diversity is related to soil health as well as to agricultural sustainability and superior yields (Kennedy and Smith, 1995; Kennedy, 1999; Loreau et al., 2001). The higher genetic diversity of rhizobial strains under NT reported here implicates enhanced saprophytic capacity than under CT in a wide range of conditions; however, only with analyses of nodulation and N_2 fixation genes it will be possible to predict the importance for nitrogen nutrition. In the areas used in this study the reported yields for NT were consistently higher than for CT. Enhancement of rhizobial diversity may be an important parameter indicative of soil quality.

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