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Phylogeny and nodulation signal molecule of rhizobial populations able to nodulate common beans—other than the predominant species *Rhizobium etli*—present in soils from the northwest of Argentina

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Abstract

We examined the bean rhizobia community other than the predominant species *Rhizobium etli* present in soils of a region that is part of the range occupied by the host in Northwest Argentina, which showed Rep and 16S rDNA RFLP polymorphism. Two populations represented by isolates T29N3L and T44N22P were found to be distinct chromosomal genotypes and closely related to species *Rhizobium tropici* and *Agrobacterium rhizogenes*. Their symbiotic genes were analyzed and found to cluster with those from *R. tropici* as well as with rhizobia isolated from leguminous trees. Three nodulation metabolites produced by T44N22P were detected which are tetra- and pentameric chitocompounds, *N*-methylated, *O*-carbamoylated, and *N*-substituted either by a $C_{18:0}$ or $C_{18:1}$ acyl chain at their non-reducing end, and all them sulphated at the reducing end. Isolates T29N3L and T44N22P exhibited broad host range but unlike T29N3L, only T44N22P was able to efficiently nodulate *Medicago truncatula*.

Keywords: Bean rhizobia ecology; Rhizobial diversity; Symbiotic genes nodC, nodH and nifH; Nodulation factor structure

1. Introduction

Phaseolus vulgaris (common beans) is nodulated by diverse rhizobial genotypes, all of them so far alpha proteobacteria (Amarger, 2001; Martínez-Romero, 2003). To date, five *Rhizobium* species have been recognized as microsymbionts of *Phaseolus vulgaris* forming nodules in nature: *R. etli* bv. phaseoli, *R. leguminosarum* bv. phaseoli, *Rhizobium tropici*, *R. gallicum* bv. phaseoli and bv. gallicum, and *R. giardinii* bv. phaseoli and bv. giardinii (Amarger, 2001). *Rhizobium etli* bv. phaseoli appears to be the predominant species found associated with wild and cultivated common beans plants in Mexico, Colombia and Southern Andes (Amarger, 2001; Martínez-Romero, 2003; Aguilar et al., 1998), whereas *R. tropici* and *R. gallicum* have been found associated with beans in conditions of acid soils (Amarger, 2001) and in the Mexican milpa system of cropping (Silva et al., 2003), respectively. In addition, other genotypes that are able to form nodules on beans-most of them retrieved from soil samples under laboratory conditions-have been also isolated (Martínez-Romero et al., 1996).

Unlike nodulation genes (*nod*), the *nif* genes are found in a broad spectrum of phylogenetically unrelated bacteria besides rhizobia. Since the phylogeny of *nif*H closely resembles that of 16S rRNA genes, it had been proposed that genes *nif*H and 16S rRNA share a common evolutionary path (Hennecke et al., 1985; Ueda et al., 1995); however,

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divergences were found and these could be explained by the fact that the symbiotic genes are plasmid-harboured and therefore subject to lateral gene transfer (Perret et al., 2000; Laguerre et al., 2001). The activity of *nod* gene products yields *N*-acylated oligomers of *N*-acetyl-D-glucosamine, known as Nod-factors. This basic structure has variations that are dependent on each strain or species and determine the host-specificity (Perret et al., 2000; Pacios-Bras et al., 2002).

Species *R. etli* bv. phaseoli is predominant among those bacteria found in nature to be associated with both wild and cultivated common beans found in the Northwestern region of Argentina (NWA) (Aguilar et al., 1998a,b; Aguilar et al., 2004). NWA is mainly a subtropical region populated by a high diversity of both herbaceous and tree legumes among others, which might also grant rhizobial microsymbiont diversity. This view is in line with the suggestion that host plant and environment are the most important factors that shape the structure of soil rhizobial populations. It is possible to assume that in the microbial community of soils from NWA, other bean-nodulating rhizobia besides *R. etli* might exist there, which should be consistent with the already demonstrated promiscuity of *P. vulgaris*.

The aim of this research was to extend the analysis of bean nodulating rhizobia from NWA by examining a sample of field isolates which diverge from the predominant species *R. etli*. Chromosomal genotypes were determined by DGGE and sequence analysis of 16S rRNA gene. Two broad host range strains, able to nodulate common beans isolated from soil samples of different geographical sites, were further characterized by their symbiotic genes *nod*C, *nod*H and *nif*H, and chemical structure analysis of Nod factors.

2. Materials and methods

2.1. Plant inoculation with soil samples and rhizobia isolates

Soil isolates were recovered from nodules of common bean cv. Nag12 and *Leucaena leucocephala* cv. peruvian, which were grown in the laboratory after inoculation with soil suspensions prepared with samples from different field sites from NWA which were provided by Mario Chocobar, Estación Experimental Agropecuaria INTA-Salta, Argentina. Plant assays were performed with seeds that were surface sterilized sequentially with 75% ethanol for 1 min and sodium hypochlorite (about chlorine 10 g per liter) for 6 min, and finally washed with sterile water. Seedlings inoculated with soil suspensions were grown in 300 ml plastic pots filled with sterilized vermiculite, and watered twice with N-free mineral nutrient solution, and with sterile distilled water as required. Soil (100 g) was suspended in one liter of sterile distilled water, and 8 ml of this suspension per plant was used for inoculation. From each plant sampled, three to six nodules were randomly excised and surface sterilized firstly with ethanol (96%) and followed with hydrogen peroxide (30% v/v). Rhizobia were isolated axenically on YEM-Congo Red agar medium as described by Vincent (1970).

2.2. Plant inoculation assays

The symbiotic properties of two isolates recovered from soils were studied on *Leucaena leucocephala*, *Vigna unguiculata*, *Phaseolus augusti*, *Medicago sativa*, *Medicago truncatula*, *Acacia aroma* (tusca), *Glycine max*. (cv.Williams), *Vicia faba*. Plant assays were performed with seeds that were surface sterilized sequentially with 75% ethanol for 1 min and sodium hypochlorite (about chlorine 10 g per liter) for 6 min, and finally washed with sterile water. Germinated seedlings inoculated with rhizobial suspensions were grown in 300 ml plastic pots filled with sterilized vermiculite, and watered twice with N-free mineral nutrient solution (Vincent, 1970), and with sterile distilled water as required. Nodule formation was assessed four weeks after inoculation.

2.3. Rep-PCR fingerprinting

Total genomic DNA from each of the isolates was used as a template for PCR with either repetitive intergenic consensus (REP) or enterobacterial repetitive intergeneric consensus (ERIC) primers according to the procedure described by de Bruijn (1992).

2.4. DNA isolation and analysis of PCR amplified symbiotic genes

DNA preparation from rhizobial cells grown in YEM medium, for use as template in PCR reactions was obtained by using a rapid method as described by Alippi and Aguilar (1998). Amplification of *nod*C and *nif*H by using specific primers was performed as described by Laguerre et al. (2001). The amplification products of about 700 bp (*nif*H) and 900 bp (*nod*C), were purified by using the GFX purification kit (Amersham Pharmacia Biotech, Buenos Aires, Argentina) as it is recommended by the manufacturer, and cloned by using the TOPO TA cloning system of Invitrogen Life Technology (Buenos Aires, Argentina). Amplification of *nod*H by using primers NODH5 5′-GCCATTTGYAATCCTTGCRATGCS-3′ and NODH3 5′-ARTYCAGCCACTGYCCCGTTTG-3′, was performed under the cycling conditions used for *nod*C amplification.

2.5. RFLP and DGGE

RFLP analysis of PCR amplified 16 S rRNA genes was performed as described by Laguerre et al. (2001). Enodonucleases assayed were *Hinf*I, *Msp*I, *Mbo*I, *Rsa*I, *Taq*I and *Cfo*I. A fragment of about 260 bp of 16S rRNA gene was PCR-amplified for DGGE analysis by using

oligonucleotide primers forg (5′-GGCTCAGAACGAA CGCTGGCGGC-3′) and revg (5′-CTCGCTGCCCAC TGTCACC-3′). DGGE was performed with the DCode Universal Mutation Detection System (Bio-Rad). PCR products were applied directly onto 8% (w/vol) polyacrylamide gels in 1× TAE buffer (20 mM Tris-acetate [pH 7.4], 10 mM sodium acetate, 0.5 mM disodium EDTA) containing a linear denaturing gradient about 40 to 65%. The gradients were formed with acrylamide stock solutions that contained 40% and 65% denaturant, respectively. These solutions contained formamide and urea, respectively 16% (v/v) and 2.8 mM, and 24% (v/v) and 4.2 mM. The gels were electrophoresed for 5–6 h at 55 °C. After electrophoresis, the gels were stained with ethidium bromide and visualized under UV radiation.

2.6. Phylogenetic analysis

Sequences of species and strains used for 16S rDNA comparison were as follows (Accession numbers are given between parentheses): *A. rhizogenes* LMG152 (X67228); *R. tropici* IIA (X67234); *R. tropici* IIB LMG9518 (X67233); *R. tropici* IIB CIAT166 (U38469); *Rhizobium* sp. BR814 (X67232); *Bradyrhizobium japonicum* USDA110 (Z35330), *B. japonicum* USDA6 (U69638); *B. elkanii* USDA 76; *Mesorhizobium amorphae* ACCC 19665 (AF041442); *M. loti* NZP2213 (X67229); *M. loti* R8CS (U50164); *M. loti* ICMP3153 (U50166); *M. huakuii* CCBAU2609 (D12797); *Sinorhizobum meliloti* USDA 1002 (D12783); *S. terangae* ORS 1009 (X68388); *S. saheli* ORS609 (X68390); *S. fredii* USDA205 (X67231); *R. leguminosarum* bv. viciae USDA2508 (U89831); *R. leguminosarum* bv phaseoli RCR3644 (U29388); *R. leguminosarum* bv. trifolii (X67227); *R. etli* CFN42 (U28916); *R. hainanense* I66 (U1078); *Rhizobium sp.*-medicago USDA1890 (U898219); *R. gallicum* R602 (U86343); *R. gallicum* FL27 (AF008129); *R. mongolense* (U889817); *R. giardinii* H152 (U86344); *R. galegae* (D12793); *R. undicola* LMG11875 (Y17047); *R. vitis* LMG8750 (X67224); *Agrobacterium tumefaciens* LMG196 (X67223). *nif*H sequences of the following nitrogen fixing microorganisms were used (the numbers in parenthesis are accession numbers): *R. etli* CFN42 (M15942); *R. leguminosarum* bv. trifolii (K00490); *R. gallicum* R602 (AF218126); *R. tropici* IIB-CIAT899 (M55225); *R. etli*-Olivia (M55227; *Rhizobium etli* bv. mimosae (AF107621); *Rhizobium* sp. (*Medicago*) OR 191 (M55228); *Rhizobium sp.* BR6001-*Lonchocarpus* (Z95230); *Rhizobium* sp. TJ71-Mimosa (AJ505314); *Sinorhizobum meliloti* ATCC9930 (M55232); *S. meliloti* 41 (J01781); *S. saheli* bv. *sesbaniae* (SSZ95221); *S. terangae* bv. *acaciae* (Z95218); *Sinorhizobium* sp. BR827-leucaena (Z95212); *Sinorhizobium* sp. GR-06-*Phaseolus* (AF275671); *S. fredii* USDA191 (Z95229); *Sinorhizobium* sp. NGR234 (M26961); *Sinorhizobium* sp. M6-*Prosopis* (Z95213); *Sinorhizobium* sp. HAMBI 1499-Acacia (Z95224); *Sinorhizobium medicae* CC169 (M55231); *Mesorhizobium* sp. INPA78B (Z95228); *Bradyrhizobium japonicum* 110 (NC004463); *Azospirillum brasilense* Sp7 (X51500); *Azotobacter croococcum* MCD1 (X03916); *Klebsiella pneumoniae* (J01740); *Azorhizobium caulinodans* ORS571 (M16709). *nod*C sequences of the following rhizobia were used (the numbers in parenthesis are accession numbers): *R. leguminosarum* bv. trifolii (K00490); *R. leguminosarum* bv. viciae (M13658); *R. galegae* (X87578); *R. etli* CFN42 (AF217268); *R. etli* Viking 1 (AF217262); *R. giardinii* bv. giardinii (AF217267); *R. giardinii* bv. phaseoli (AF217264); *R. gallicum* R602 (AF217266); *R. gallicum* FL27 (AF217270); *R. tropici* IIA (X98514); *R. tropici* IIB-UPM8033 (AY166845); *Sinorhizobum meliloti* 1021 (M11268); *S. fredii* USDA 257 (M73699); *Sinorhizobium* sp. NGR234(X73362); *Sinorhizobium* GR-06-*Phaseolus* (AF217269); *Azorhizobium caulinodans* (L18897); *Bradyrhizobium japonicum* 110 (NC004463); *M. loti* NZP2213; *Mesorhizobium sp.* N33-*Oxytriopis* (U53327); *M. amorphae* (AF217261).

Multiple alignments were done by using Clustal X (Thompson et al., 1997), followed by visual inspection. Phylogenetic trees were inferred by using PAUP* version 4.0b4a. Neighbour-joining (NJ) analysis from Hasegawa-Kishino-Yano model (HKY85). Maximum likelihood (ML) analysis was inferred using HKY85 as substitution model. Maximum parsimony (MP) and ML was performed applying the heuristic search option with the option amb-to ignore potential support and tree bisection reconnection (TBR) as swapping method. To assess branch reliability, for both MP and ML, 1000 bootstrap replications were assayed with a heuristic search option with TBR to generate the simulated phylogenies (Swofford, 1998; Felsenstein, 1985). MacClade version 4 was used for examination of character distributions (Madison and Madison, 2000).

2.7. Thin layer chromatography (TLC) analysis of nod factors

TLC analysis was performed according to Spaink et al. (1992) Briefly, Rhizobium strains were grown on minimal B-medium in the presence or absence of apigenin as inducer, with the addition of 0.5 μCi of [^{14}C]-glucosamine hydrochloride, 100mCi of [^{35}S]-$MgSO_4$ (specific activity 602 mCi/mmol) and 1 μCi of [^{14}C]-(methyl)-L methionine (specific activity 55 mCi/mmol) from Amersham, Int. TLC plates were exposed to Kodak X-Omat R film for 15–30 d.

2.8. Nod factor purification (HPLC analysis of the nod factors)

The method was essentially the same as those described by Spaink et al., 1995 and Soria-Díaz et al., 2003). Apigenin-induced culture medium (10 L) was

extracted with *n*-butanol. The *n*-butanol extract was evaporated to dryness under vacuum and the residue was suspended in 50 ml of acetonitrile-water (3:2, v/v) which was then brought to acetonitrile-water (1:4, v/v). A pre-purification step was performed by passing the crude extract through a C_{18} cartridge from Resprep, from which the Nod factors were eluted using different acetonitrile-water solutions (20, 45 and 60%). The Nod factors in these fractions were further fractionated by HPLC on a semi-preparative C_{18} reversed phase column (250× 7.5 mm, Spherisorb ODS2, 5 μm, Tracer) using isocratic elutions of acetonitrile-water (20%, 5 min; 30%, 30 min; 40%, 30 min; 60%, 15 min), and then using a linear gradient for 10 min from 60 to 100% acetonitrile. The eluent from HPLC was monitored at 206 nm and 2.5 ml fractions collected.

The rhizobium strain T44N22P was grown either in the presence or absence of apigenin as inducer, with the addition of either [^{14}C]-glucosamine hydrochloride-, [^{35}S]-$MgSO_4$, or [^{14}C]-methyl-L-methionine, respectively. For preparative purposes, 10 litres of medium from the apigenin-induced rhizobial cells were extracted with *n*-butanol. The *n*-butanol extract was pre-purified using a reversed-phase C_{18} cartridge and eluting with different acetonitrile-water compositions (20, 45 and 60%, v/v). The 45% and 60% fractions were subjected to HPLC fractionation using a semi-preparative C_{18} column.

2.9. Mass spectrometry

Positive ion mode FABMS was performed on a Kratos MS80-RFA instrument with a Xenon atom beam at an accelerating voltage of 4 kV. Either glycerol-*m*-nitrobenzyl alcohol (1:1) and thioglycerol containing NaI as the cationizing agent, were used as matrix.

2.10. Monosaccharide composition and fatty acid analysis

Glycosil composition analysis was carried out after methanolysis using anhydrous methanolic 0.625 M HCl (16 h, 80 °C). The samples were re-*N*-acetylated using Ac_2O-pyridine (1:1, v/v), then trimethylsilylated with pyridine-BSTFA-TMSCl (10:5:1, v/v/v) and analyzed by GLC-MS. The fatty acids were identified as their methyl esters. These were preparated by methanolysis in methanolic 1 M HCl at 85 °C for 4 h and were identified by GLC-MS. GLC-MS was performed with a Micromass Auto Spec-Q instrument fitted with a Fisons GC 8065MS gas chromatograph with an OV-1 column (25 m× 0.25 mm). The temperature programme for separating the trimethylsilylated methyl glycosides and fatty acid derivatives was isothermal at 150 °C for 2 min, followed by a 10 °C/min gradient up to 250 °C. The ionisation potential was 70 eV.

3. Results

3.1. Investigation of rhizobial genotypes-able to nodulate common beans-other than the predominant species R. etli in soils of NWA

A collection of 420 nodule isolates was obtained from common beans and leucaena plants after inoculation with soil suspensions brought from two field sites in the province of Salta at Quebrada del Toro (24°54′S-65°40′W) and Rosario de Lerma (24°56′S-65°36′W). Quebrada del Toro was populated by a diversity of wild growing legumes both herbaceous and trees, such as *Desmodium* sp., *Phaseolus vulgaris* var. *aborigineus*, *Phaseolus augusti*, *Erythrina falcata*, *Tipuana tipa*, *Acacia aroma* (tusca), *Acacia caven*, *Mimosa spp.*, whereas the site at Rosario de Lerma was characterized by the presence of a few leguminous trees mainly restricted to *Acacia aroma* and *Mimosa spp.* This collection was examined stepwise in order to narrow down the number of isolates to those that could lead us to describe novel bean-nodulating genotypes.

The question of whether the various isolates could be assigned to the bean-nodulating species *R. etli* was approached by applying two tests. Aguilar et al. (1998a,b) had shown previously that *R. etli* and *R. leguminosarum* bv. phaseoli strains (but not other bean rhizobia) consistently yielded a *nif*H PCR amplification product of 570 bp, indicating that the particular symbiotic gene *nif*H is widely conserved among such species. Second, the 16S rRNA gene of isolates that were positive for the 570 bp *nif*H fragment was analyzed by using the RFLP-procedure described by Laguerre et al. (1994) to identify restriction sites in a PCR-amplified 16S rDNA fragment of about 1.5 Kb that permitted identification of species *R. etli*. This survey showed that species *R. etli* bv. phaseoli was predominant among the isolates that were retrieved by using common beans and leucaena as trapping hosts: 303 isolates from common beans were assigned to species *R. etli* and *R. leguminosarum* bv. phaseoli. This prevalence is in agreement with our previous data from the analysis of the wild bean rhizobia from NWA (Aguilar et al., 1998a,b).

Because our objective was to characterize the bean rhizobial populations other than *R. etli*, we focused our attention to examine the remaining 117 isolates from common beans and leucaena. These isolates were cross inoculated onto each host, with 82 isolates formed effective nodules on both host legumes. This subset of isolates was grouped by Rep-fingerprinting and a sample representing the diversity of electrophoresis profiles was further screened by analysis of the 16S rRNA gene by using RFLP and DGGE, respectively. The result of the 16S rDNA PCR-RFLP analysis showed six different profiles that were found identical to those of species *Sinorhizobium fredii*, *S. terangae*, *S. saheli*, *Mesorhizobium loti*, *R. leguminosarum*, and *R. tropici* (data not shown). Additional DGGE analysis, which examined a fragment of the 16S rRNA gene

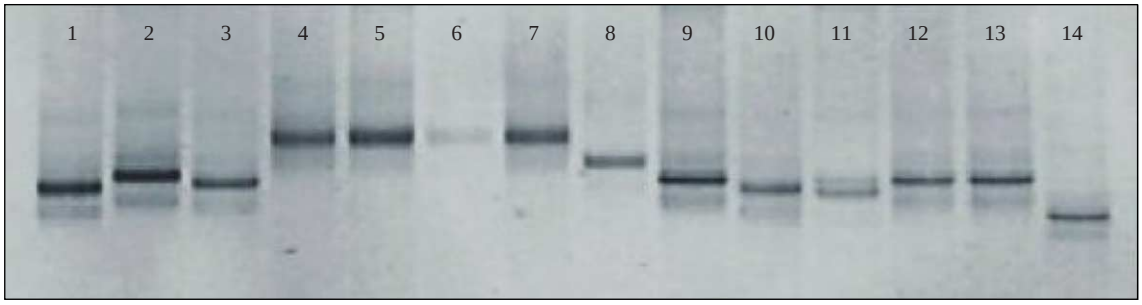


Fig. 1. DGGE analysis of PCR-amplified 16S rRNA gene fragments of bean nodulating rhizobia from NWA. DGGE separation of gene fragment obtained with primers dgefor and dgerev and genomic DNA from strains *R. etli* CFN42 (lane 1), *R. leguminosarum* USDA2671 (lane2), *S. fredii* USDA191 (lane 3), isolate T29N3L (lane 4), isolate T48N3L (lane 5), isolate T56N1L (lane 6), isolate T44N22P (lane 7), *R. tropici* IIB CIAT899 (lane 8), isolate T1N7L (lane 9), isolate NOAP159N2 (lane 10), *S. terangae* (lane 11), *S. fredii* HH103 (lane 12), isolate NOAP159N3 (lane 13), isolate T54N3L (lane 14).

of about 260 bp, was performed on a subsample representative of the 16S rRNA diversity. The result of the DGGE analysis is shown in Fig. 1. However most of DGGE patterns were found to be identical to those obtained with the reference strains, we found that a few isolates which were characterized by 16S rDNA RFLP analysis as *R. tropici* exhibited a distinct pattern (lanes 4, 5, 6 and 7). Indeed, the bean-nodulating isolates T29N3L and T44N22P, that were respectively isolated from a leucaena plant inoculated with the soil sample from Quebrada del Toro and from a common bean plant inoculated with the soil sample from Rosario de Lerma, represented populations showing similarity (same 16S rDNA RFLP) but not identity to *R. tropici* (distinct DGGE profile). Furthermore, analysis of indigenous plasmids showed different patterns -in size and number- between isolates T29N3L and T44N22P, and *R. tropici* CIAT899 (data not shown). This genotype was also found in about one third of isolates and among isolates from several other sites across NWA.

Overall, we concluded that rhizobial populations other than species *R. etli*, which are also able to nodulate common beans, appear to be highly diverse in NWA soils, and some of them could represent unclassified genomic species.

3.2. Phenotypic characteristics of isolates T29N3L and T44N22P

In order to approach the description of the genetic diversity of bean rhizobia populations from NWA, isolates T29N3L and T44N22P were further characterized. It was found that both of them were able to grow on media YEM-Congo Red and TY, and formed gummy colonies after 3 days incubation on TY medium. They had duplication times of about 3 h in TY medium, similar to *R. etli* CFN42 and *R. tropici* CIAT899. A summary of other properties is described in Table 1. Both isolates were unable to grow on LB medium. Melanin production was observed only by isolate T44N22P. Both isolates formed nodules on a diversity of herbaceous and leguminous trees however unlike T29N3L, only T44N22P formed nodules on *Medicago truncatula* which were found to be effective in nitrogen fixation.

3.3. Nucleotide sequence analysis of 16S rDNA

The results shown above indicated that isolates T29N3L and T44N22P share some properties with each other as well as with *R. tropici* such as the 16S rDNA-RFLP pattern and nodulation of both common beans and leucaena, whereas they diverge in others such as the DGGE profiles, ability to nodulate *M. truncatula* and the 16S-23S rDNA intergenic region (data no shown). In order to determine the taxonomic relatedness of isolates T29N3L and T44N22P, we performed the comparative sequence analysis of their 16S rRNA gene. Sequences of T29N3L and T44N22P were found to be very similar to each other with 3 nucleotides difference. The sequences were most similar to those of *Rhizobium* sp strain BR814 and of species *R. tropici* IIA strain LMG9517 (CFN299), both of them isolated in Brazil from leucaena. The other most similar 16S rDNA was that

Table 1
Phenotypic characteristics of bean-nodulating isolates T29N3L and T44N22P

Characteristics	Isolates	
	T29N3L	T44N22P
Growth[a]		
LB	−	−
Y-CR	+	+
Melanin[b]	−	+
Nodulation[c]		
Medicago sativa	−	−
Medicago truncatula	−	+
Glycine max	−	−
Vigna unguiculata	+	+
Phaseolus augusti	+	+
Phaseolus vulgaris	+	+
Acacia aroma	+	+
Leucaena leuocephala	+	+

[a] LB, Luria Bertani medium; Y-CR, YEM-Congo Red.
[b] Melanin production was assayed in TY medium supplemented with 300 mg tyrosine per ml and 40 mg of $CuSO_4 \cdot 5H_20$.
[c] Nodulation was evaluated 4 weeks after inoculation.

of *A. rhizogenes* strain LMG152. Differences in 4 and 7 nucleotides were detected in sequence of isolate T44N22P as compared to those sequences of *R. tropici* and *A. rhizogenes*, respectively. The sequences were aligned together with those of reference strains, and the result used to construct phylogenetic trees. Since results may depend on the applied algorithm, trees were obtained by using three methods of analysis, NJ, MP and ML (Fig. 2). The tree topology was consistent among them, in which the isolates T29N3L and T44N22P and species *R. tropici* IIA and

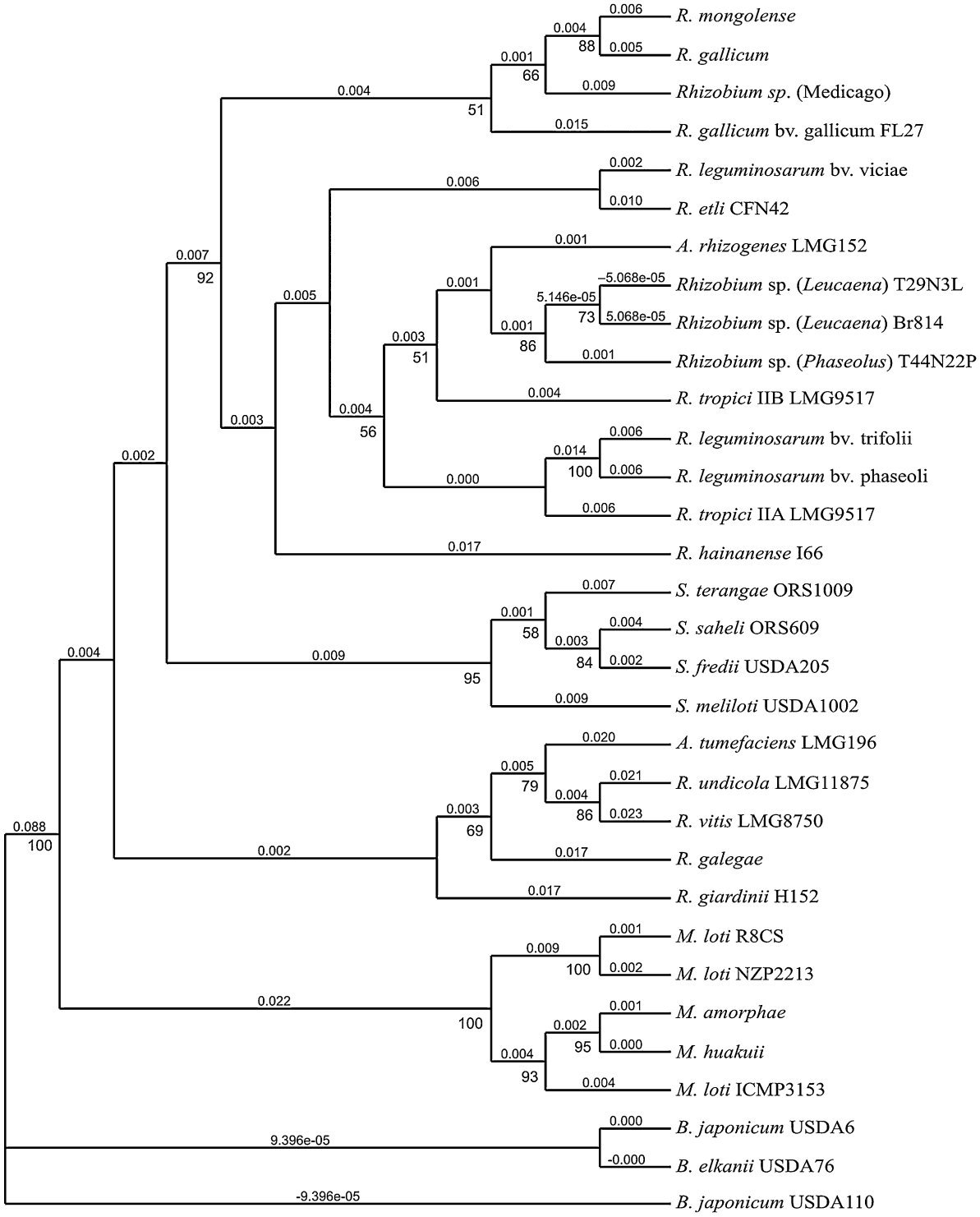


Fig. 2. Phylogenetic tree, by using neighbour-joining method, showing the relationship between isolates T29N3L and T44N22P, and rhizobial species. Rooted phylogenetic tree estimated from 16S rDNA sequence data. Bootstrap values less than 50% over 1000 replicates, were not considered.

A. rhizogenes, and the strain *Rhizobium* sp BR814 (LMG9509) clustered together and well separated from other rhizobial taxa.

3.4. Phylogenetic analyses of symbiotic genes nodC, nodH, and nifH

In order to investigate the evolutionary relationship of isolates T29N3L and T44N22P and other rhizobia that also nodulate beans, we examined the nucleotide sequence of PCR products which were amplified from symbiotic genes. Two nodulation genes and one nitrogen fixation gene were examined, namely the common nodulation gene *nod*C coding for an *N*-acetyl-glucosaminyltransferase, the host specificity nodulation gene *nod*H coding for a sulphate transferase, and the dinitrogenase reductase *nif*H gene.

The nucleotide sequences of about 690 bp amplified *nod*C genes from T29N3L and T44N22P were found to be very similar to each other with only three nucleotides difference. The phylogenetic analysis of the aligned *nod*C sequences of several rhizobial species was the tree shown in Fig. 3. Our neighbour-joining tree resulted to be very similar to those reported previously (Laguerre et al., 2001; Haukka et al., 1998). The sequences were found to be closely related to that of *nod*C of *R. tropici*.

The complete sequence of the PCR-amplified *nod*H fragment of about 519 bp was determined for the isolates T29N3L and T44N22P. These sequences differed from each other in 8 nucleotide positions, and their comparison with the scarce number of rhizobial sequences which are available-reflecting the limited number of known species which produce sulphated Nod metabolites- was performed (Fig. 4). Nevertheless, this analysis showed that the *nod*H sequences of T29N3L and T444N22P were related to that of *R. tropici* (Fig. 4), which is similar to results with *nod*C (Fig. 3).

The phylogenetic analysis of *nif*H sequences resulted in the tree shown in Fig. 5. Interestingly, the phylogeny of the nitrogen fixation gene *nif*H also provides a representation of our isolates similar to that of the phylogeny of *nod*C. Indeed, the *nif*H tree grouped T29N3L and T44N22P together with species *R. tropici* as well as with other rhizobial genotypes isolated from leguminous trees.

3.5. Structural analysis of nod factor produced by T44N22P

TLC analysis of extracts from induced cells that were incubated in the presence of radioactive precursors showed a single spot (Fig. 6). The profiles resulting from the HPLC A_{206} nm analysis are shown in Fig. 6. Two fractions F1

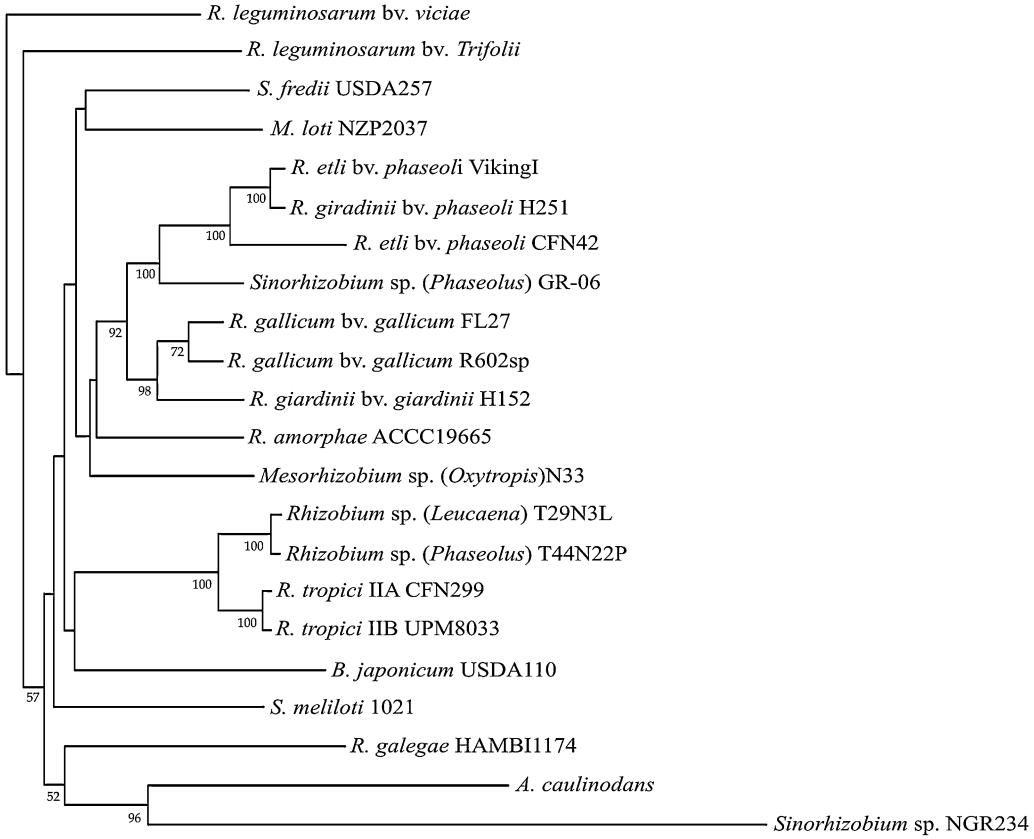


Fig. 3. *nod*C phylogenetic tree. The tree, based on 662 bp aligned *nod*C sequences was constructed by using the neighbour-joining method. Bootstrap values less than 50% over 100 replicates, were not considered.

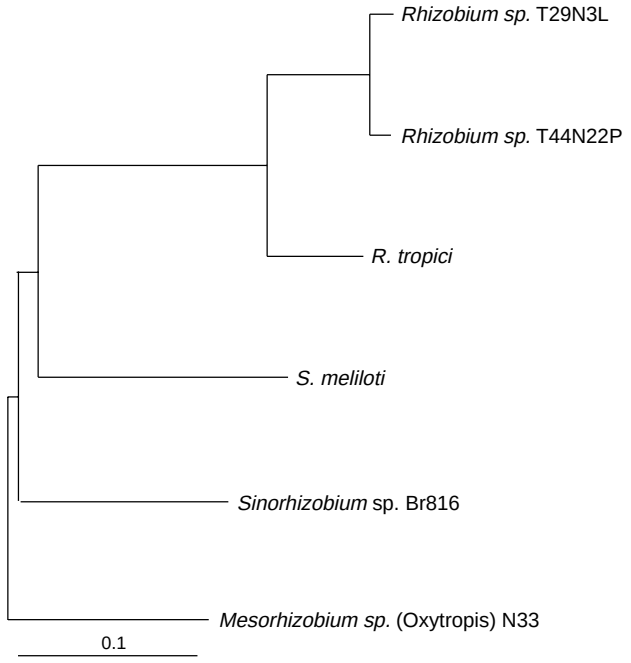


Fig. 4. *nod*H phylogenetic tree. The tree was constructed by using the neighbour-joining method. Sequences of the following nitrogen fixing microorganisms were used (the number in parenthesis are accession numbers): *Mesorhizobium sp.* N33-*Oxytriopis* (U53327); *R. tropici* IIB-CIAT899 (X87608); *Sinorhizobium* sp. BR816 (AJ518946); *S. meliloti* 1021 (X04380).

and F2 eluting with 30% acetonitrile were obtained. The results of the FAB-MS analysis in the positive mode on fractions F1 and F2 are summarised in Fig. 9. The mass spectrum of fraction F1 exhibited a complex pattern in the molecular ion region. The fraction F1 contains two Nod factors. Signals at *m/z* 1437 and 1453 correspond to $[M–H+2Na]^+$ pseudomolecular ion and $[M–H+Na+K]^+$ pseudomolecular ion from the major Nod factor. The signal at *m/z* 1335 was assigned to the loss of a $NaSO_3$ group. In addition, the mass spectrum contained signals corresponding to the salts adducts (Na and K) of the B_4 to B_1 ions (1114, 1132; 911, 927; 708; and 505) and the B_1 ion fragment at *m/z* 483. The odd-number value of the *m/z* of B_1 indicates the presence of a nitrogen-containing substituent on the non-reducing glucosamine residue, which is consistent with a carbamoyl substitution. Furthermore, the B_1 mass value indicates the presence of both a *N*-methyl and a $C_{18:1}$ fatty acyl substituents. We propose that the ion at *m/z* 1437 corresponds to the pseudomolecular ion of a Nod factor molecule having the following structure: V ($C_{18:1}$, NMe, Cb, S), which represents a nodulation factor with a backbone of five GlcNAc residues and a $C_{18:1}$ fatty acid, a carbamoyl group and a sulfate group as backbone substituents. Another signal at *m/z* 1234 corresponds to a $[M–H+2Na]^+$ pseudomolecular ion from a minor Nod factor found in fraction F1. The signal at *m/z* 1132 was assigned to the loss of a $NaSO_3$. The fragment series corresponding to the oxonium type ions ended at *m/z* 483. These results are consistent with the presence of a tetrameric Nod factor containing a sulfate group on the reducing glucosamine residue and a *N*-methyl, $C_{18:1}$ fatty acyl and a carbamoyl substituents on the non-reducing glucosamine residue. The Nod factor was identified as IV ($C_{18:1}$, NMe, Cb, S).

Fraction F2 (Fig. 7) contains only one Nod factor. Signals at *m/z* 1439, 1455, 1417 and 1395 correspond to $[M–H+2Na]^+$, $[M–H+Na+K]^+$, $[M+Na]^+$ and $[M+H]^+$ pseudomolecular ions, respectively. The signal at *m/z* 1337 corresponds to the loss of a $NaSO_3$ group. In addition, the mass spectrum contains signals at *m/z* 1116 and 1134 (B_4 plus sodium and potassium); *m/z* 913 and 929 (B_3 plus sodium and potassium); and at *m/z* 688 and 485 (B_2 and B_1, respectively). The value of fragment B_1 is consistent with Nod factors bearing a carbamoyl, an *N*-methyl, and a $C_{18:0}$ fatty acyl substituents on its non-reducing terminal residue. The Nod factor was identified as V ($C_{18:0}$, NMe, Cb, S).

Constant B/E scans of B_1 oxonium ions originated from the fragmentation of the nod factors from T44N22P were performed (Fig. 8). The metastable ion spectrum of the B_1 fragment at *m/z* 483 arising from the fragmentation of a *N*-methylated nod factor bearing a $C_{18:1}$ acyl group and a carbamoyl group, showed a fragment at *m/z* 465 (base peak) which corresponds to the loss of water. The signal at *m/z* 463 corresponds to the loss of water plus a hydrogen molecule. The non-intense signal at *m/z* 462 was assigned to the β-elimination of a carbamic acid molecule. This fact could indicate that the carbamoyl group is located on O-6 (Treilhou et al., 2000). The signal at *m/z* 404 corresponds to the loss of a carbamic acid plus water and, finally, the signal at *m/z* 362 corresponds to the loss of a carbamic acid plus water plus ketene.

The metastable ions spectrum of the B_1 fragment at *m/z* 485 arising from the fragmentation of a *N*-methylated nod factor bearing a $C_{18:0}$ acyl group and a carbamoyl group, was assigned as described above.

Taking these results altogether we concluded that strain T44N22P produces and excretes to the medium three nodulation metabolites which are tetra- and pentameric chitocompounds, *N*-methylated, *O*-carbamoylated, and *N*-substituted either by a $C_{18:0}$ or $C_{18:1}$ acyl chain at their nonreducing end. They are sulphated in the glucosamine reducing end, which is in agreement with the finding of the *nod*H gene sequence in the genome of T44N22P described above. These results are summarized in Fig. 9.

4. Discussion

In a previous study of the rhizobia that nodulate wild beans in NWA, predominance of the *R. etli* 16S rRNA allele was shown (Aguilar et al., 1998a,b). In the present work, we have examined a collection of bean-nodulating strains isolated from soils from NWA by using common beans and leucaena as trapping hosts, respectively.

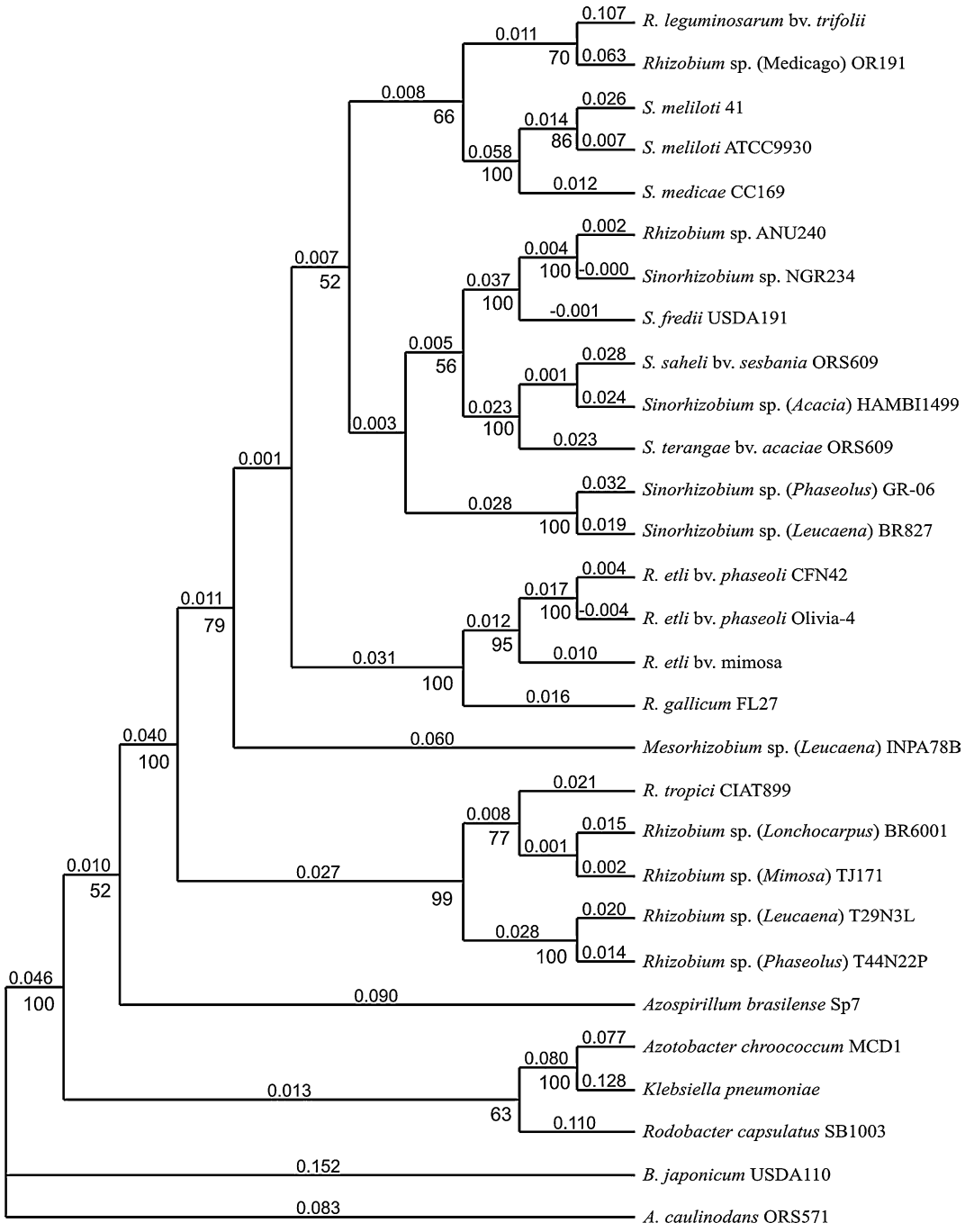


Fig. 5. *nif*H phylogenetic tree. The tree based on 580 bp aligned *nif*H sequences was constructed by using neighbour-joining method. Bootstrap values less than 50% over 100 replicates, were not considered.

4.1. Ecology and phylogeny of bean isolates from NWA

We aimed to answer the question whether bean nodulating rhizobia other than *R. etli* could be also present in that environment and eventually genetically characterize them and their nodulation molecule signals. The results of this survey have shown that most of the populations sharing the soils with the bean specific *R. etli* are broad host range rhizobia probably related to lineages able to nodulate leguminous trees. A more detailed analysis was carried on two isolates representative of this diversity of bean-nodulating rhizobia.

Analysis of 16S rRNA genes have shown that the isolates T29N3L and T44N22P clustered with species *R. tropici* and *Agrobacterium rhizogenes* (or *Rhizobium rhizogenes* as it was recently proposed by Young et al., 2001, and with the *Rhizobium* sp. strain BR814 (LMG9509), altogether well separated from other rhizobial taxa. In order to strengthen our conclusions, three methods have been used to construct inferred phylogenetic trees, which led us to the same results.

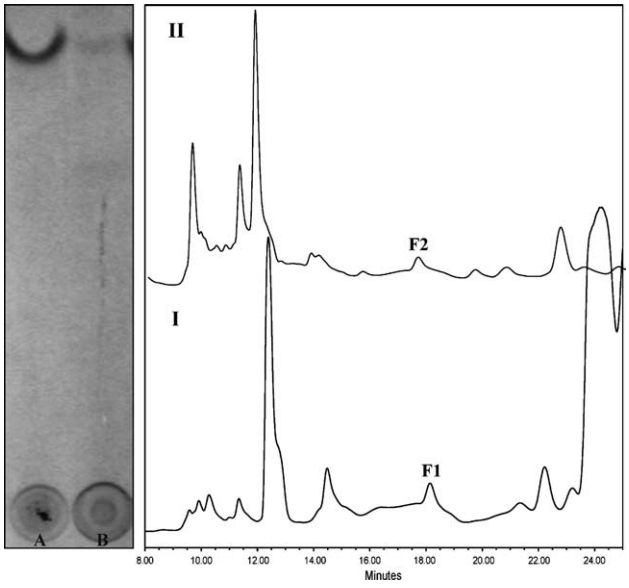


Fig. 6. TLC and HPLC profiles of the LCOs. TLC of T44N22P nodulation factors radiolabeled with [^{14}C]-glucosamine hydrochloride from induced (A) and non induced (B) cultures. HPLC profiles of *n*-butanol extract from T44N22P, eluted from C_{18} cartridge with 45% (I) and 60% (II) acetonitrile-water.

These results, from DNA sequence analysis, were in agreement with those from the RFLP and DGGE analysis demonstrating a high level of 16S rDNA relatedness between our isolates and *R. tropici*. It seems that these *Rhizobium* genotypes-characterized by their abilities to nodulate leguminous trees-such as *R. tropici*, *Rhizobium* sp. BR814 and our isolates T29N3L and T44N22P, are intermixed in a tight phylogenetic cluster which encompasses also *A. rhizogenes*. Our observation together with the results reported by Willems and Collins (1993); Young et al. (2001), respectively, are consistent with each other in placing these species within the same sub-clade. These results and those described in previous reports by other authors, suggest the occurrence of genotypes centered around *R. tropici* and *A. rhizogenes* which can induce nodule formation or tumor according to the plasmid they carry (Young et al., 2001). Since these inferences were drawn from the analysis of the 16S rDNA sequences then, further data such as those from DNA:DNA reassociation analysis are needed in order to demonstrate that any of these two isolates represent new rhizobial species (van Berkum et al., 2003).

The phylogenetic analysis of *nod*C and *nif*H genes grouped our isolates close to *R. tropici*, which in case of *nif*H also included *Rhizobium* sp. strain TJ171 and *Rhizobium* sp. strain BR6001. These strains were isolated from *Mimosa diplotricha* and *Lonchocarpus* sp, in Taiwan and Brazil, respectively (Chen et al., 2003; Haukka et al., 1998). These results indicated firstly, that quite similar symbiotic sequences are widely dispersed all over the world and secondly, that rhizobia able to nodulate leguminous trees such as *L. leucocephala*, *Lonchocarpus sp.* and *Mimosa sp*. are grouped by their *nod*C and *nif*H sequences. Chen et al. (2003) examined the *nod*A sequence of strain TJ171 and found also to be phylogenetically close to *R. tropici*. It has been shown that the common *nod*A and *nod*C genes seem to be more related to the taxonomy of host

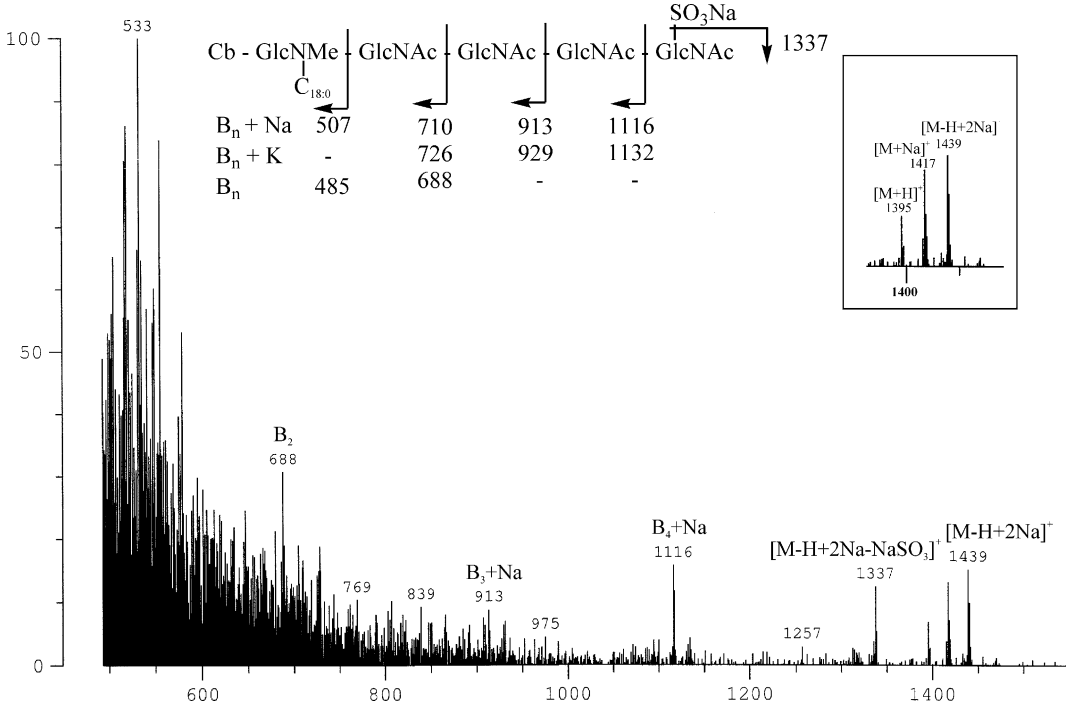


Fig. 7. Fast atom bombardment (FAB) spectrum of the HPLC fraction F2-from Fig. 6, showing B_n ions. In set, the pseudomolecular domain.

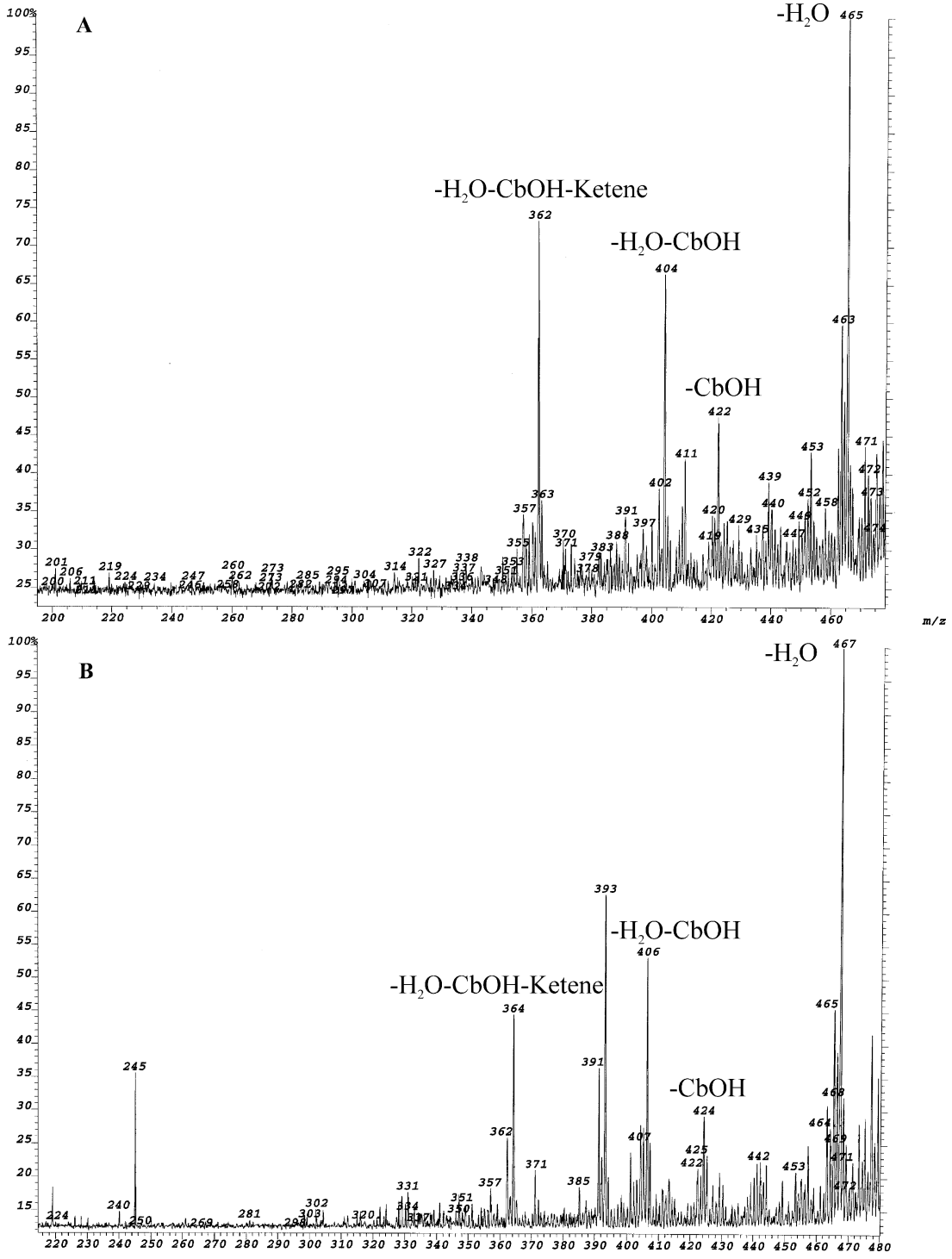
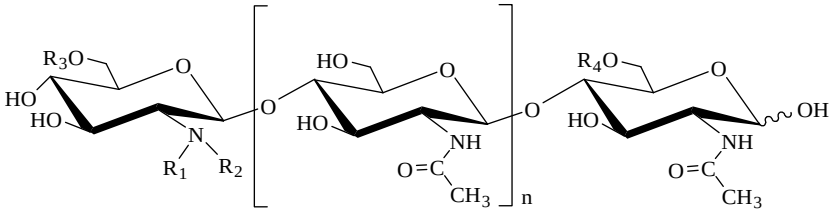


Fig. 8. Constant B/E scans of B_1 oxonium ions originated in the fragmentation of the Nod factors from T44N22P. **A**, fragments from the B_1 ion at *m*/*z* 483 and **B**, fragments from the B_1 ion at *m*/*z* 485.

plants than to that of bacteria themselves (Martínez-Romero et al., 2003; Perret et al., 2000; Laguerre et al., 2001; Willems and Collins, 1993). Therefore, we can assume that the natural host of our isolates T29N3L and T44N22P could be any of the numerous species of leguminous tree native to the NWA, where the soil samples have been collected. Similar results were obtained with other isolates of our collection that were not fully characterized. For instance, isolates were found to have the 16S rDNA allele of *S. fredii* strain HH103 which were unable to nodulate soybean but efficiently nodulated common beans and leucaena (data not shown). Therefore, it is possible that these isolates may be related to those tropical sinorhizobia isolated from leucaena and *Prosopis* spp. trees (Haukka et al., 1998).



FRACTIONS	$[M-H+2Na]^+$ *(m/z)*	n	R_1	R_2	R_3	R_4	Designation
F_1	1437	3	$C_{18:1}$	Me	$CONH_2$	SO_3Na	V($C_{18:1}$,NMe,Cb,S)
	1234	2	$C_{18:1}$	Me	$CONH_2$	SO_3Na	IV($C_{18:1}$,NMe,Cb,S)
F_2	1439	3	$C_{18:0}$	Me	$CONH_2$	SO_3Na	V($C_{18:0}$,NMe.Cb,S)

Fig. 9. Nodulation factors produced by T44N22. The upper part of the figure depicts the chemical structure of nod metabolites, and the table below shows the Nod factors and substituents. Cb, carbamoyl; Me, methyl; S, sulphate.

The phylogenies of symbiotic genes and that of 16S rRNA gene have similar representation, at variance with the lack of congruence between the classifications based on symbiotic genes and on 16S rRNA genes, respectively that was reported by Laguerre et al. (2001). Our results indicate that *nod* and *nif* genes of T29N3L and T44N22P have coevolved together or alternatively were acquired together.

It is noteworthy that strains T29N3L and T44N22P have substantial identity in their symbiotic genes and indeed have overlapping host ranges but only T44N22P is able to nodulate *M. truncatula*. *M. truncatula* is a Mediterranean herbaceous legume that is nodulated by species *Sinorhizobum meliloti*. It seems that differences that make T44N22P able to nodulate *M. truncatula* are not reflected in the symbiotic genes we have examined. Melanin production in addition differentiates each other.

4.2. Nodulation signal molecules

The rhizosphere is a densely populated area in which microbes must compete with each other, and in this process nodulation factors produced by rhizobia are key compounds mediating communication and biological interaction (Walker et al., 2003). Each rhizobial species produces a set of Nod factors that consists of two (*R. etli* bv. phaseoli CFN42) to approximately 60 compounds (*R. galegae* HAMBI1207) with specific substitution at both ends of the oligosaccharide backbone (D'Haeze et al., 2002). The low number of Nod factors produced by isolate T44N22P is all very similar, with variations in the fatty acid group. Overall, the chemical structure is found to be similar to those produced by *S. saheli* bv. acaciae ORS1073, *Rhizobium* sp. strain ORS1002, and to one out of the five Nod factors produced by *R. giardinii* bv. giardinii H152 (D'Haukka et al., 1998; Soria-Díaz et al., 2003). Common features among them are the presence of substitutions methyl, carboamyl and $C_{18:0}$ at the nonreducing end and sulfate at the reducing end. Moreover, all of these rhizobia are able to nodulate leucaena and *P. vulgaris*. However, our attempt to identify commonalities among Nod factors produced by *P. vulgaris*-nodulating rhizobia species such as *R. etli*, *R. tropici*, *R. giardinii* bv. giardinii, *Sinorhizobium* sp. BR816, *S. teranga* bv. acaciae, *Sinorhizobium* sp. NGR234, indicated that only the presence of residues methyl and $C_{18:1}$ acyl in the nonreducing end, is shared by all of them (D'Haeze et al., 2002). The important significance of the methyl residue for *R. tropici* is supported by the genetic data reported by Waelkens et al., 1995. Strains isolated from tropical leguminous trees that belong to the family Mimosoidae, and isolate T44N22P, are all characterized by their broad host range, this trait is accompanied by structural similarities in their Nod factors. Other coincidences have been found in substituents methyl and acyl $C_{18:0}$ and $C_{18:1}$ in the non reducing end, and sulphate in the reducing end, that are detected in the Nod factors produced by *Sinorhizobium* sp BR816 and *R. tropici* CFN299 (both isolated in Brazil from *L. leucocephala*), *Sinorhizobium teranga* bv. acaciae ORS1073 (isolated in Africa from Acaciae), and strain T44N22P isolated of soil from NWA (this work).

It is accepted that *Phaseolus vulgaris* was domesticated in the Americas, with two major centers of diversification in Mesoamerica and in the southern Andes (Kami et al., 1995). *R. etli* is the predominant *P. vulgaris*-nodulating species in the Americas, which suggested coevolution (Aguilar et al., 2004). Here, we present evidence that other bean rhizobia are present in soils of the Southern Andes, an area of host diversification. Therefore, it is possible to assume that

the host-rhizobia coevolution involved modifications of an ancestral Nod factor structure such as the replacement of sulphate by fucosyl, which made the interaction more specific and increased affinity between partners. Similarly, changes of substituents in the Nod factor of other rhizobia that resulted in change of host range have been shown. The Nod factors produced by *Sinorhizobium saheli* and *S. teranga* bv. sesbaniae, which exhibit a narrow host range limited to *Sesbania* spp., were found to have arabinosyl and fucosyl residues in the reducing end instead of the sulphate substitutent that characterizes the Nod factor of the broad host range species *S. teranga* bv. acaciae ORS1073 (Lorquin et al., 1997a,b.

It is noteworthy that although *R. tropici* and T44N22P share most structural features of their Nod factors-except carbamoylation-*R. tropici* strains unlike T44N22P do not nodulate *M. truncatula*. The nodulation of *M. truncatula*, melanin production and carbamoylation of Nod factor could be phenotypic features used to distinguish this isolate from previously reported species such as *R. tropici*.

4.3. Conclusions

Our results from laboratory inoculation experiments presented in this work, have shown that a diversity of rhizobia able to nodulate common beans, are present in soils from NWA albeit predominance by species *R. etli* is found in nodules formed in the natural environment. We assume that among a diversity of Nod factors produced simultaneously by a complex microbial community, only a few or just only one results to be competitive enough to trigger biological interaction that leads to nodulation. We believe that this speculation supports our finding that *R. etli* is predominant in bean nodules formed in NWA indicating that species *R. etli* outcompetes other bean-nodulating rhizobia as those described in this work

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