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ORIGINAL PAPER

Signal molecules in the peanut–bradyrhizobia interaction

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Abstract Main nodulation signal molecules in the peanut–bradyrhizobia interaction were examined. Flavonoids exuded by *Arachis hypogaea* L. cultivar Tegua were genistein, daidzein and chrysin, the latest being released in lower quantities. Thin layer chromatography analysis from genistein-induced bacterial cultures of three peanut bradyrhizobia resulted in an identical Nod factor pattern, suggesting low variability in genes involved in the synthesis of these molecules. Structural study of Nod factor by mass spectrometry and NMR analysis revealed that it shares a variety of substituents with the broad-host-range *Rhizobium* sp. NGR234 and *Bradyrhizobium* spp. Nodulation assays in legumes nodulated by these rhizobia demonstrated differences between them and the three peanut bradyrhizobia. The three isolates were classified as *Bradyrhizobium* sp. Their fixation gene *nifD* and the common nodulation genes *nodD* and *nodA* were also analyzed.

Keywords *Arachis hypogaea* L. · Flavonoids · Nod factors · Rhizobial isolates · Nodulation and fixation genes

Abbreviations

NMR	Nuclear magnetic resonance
LCO	Lipochitooligosaccharide
pSym	Symbiotic plasmid
GlcNAc	*N*-acetyl-D-glucosamine
YMB	Yeast extract-mannitol broth
YMA	Yeast extract-mannitol agar
NJ	Neighbour-Joining
TLC	Thin layer chromatography
LSIMS	Liquid secondary ionisation mass spectrometry
DMSO	Dimethylsulfoxide
COSY	Correlation spectroscopy
RFLP	Restriction fragment length polymorphism
ARDRA	Amplified ribosomal DNA restriction analysis
HPLC	High performance liquid chromatography
FAB MS	Positive fast atom bombardment mass spectrometry

Introduction

Plants are able to establish symbiotic interactions with several microorganisms. Among ecologically and agriculturally important symbioses are the interactions of plants with nitrogen-fixing bacteria called rhizobia. These bacteria are able to invade the roots of leguminous plants and trigger the formation of nodules. They are classified in several genera included in the subclass α-proteobacteria. Furthermore,

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Accession numbers: AY427207, EF202193, EF158295 (*16S rRNA* gene of strains NLH25, NOD31 and NDEHE, respectively); DQ295199, DQ295200, DQ295201 (Partial *nifD* gene sequences of strains NLH25, NOD31 and NDEHE, respectively).

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species from the β-proteobacteria subclass, such as *Ralstonia* and *Burkholderia* were described to be also able to nodulate legumes (Moulin et al. 2001; Chen et al. 2003) and γ-proteobacteria subclass bacteria were observed to be associated to legumes nodules (Benhinzia et al. 2004).

A molecular dialog between the two partners is required to coordinate the events leading to the symbiosis (Long 1989; Nap and Bisseling 1990). Plant roots continuously release flavonoids that accumulate in the rhizosphere and constitute the first signals. These molecules belong to a relatively diverse family of aromatics compounds derived from the secondary plant metabolism, including six major subgroups: chalcones, flavones, flavonols, flavandiols, anthocyanins and condensed tannins (proanthocyanidins). A seventh group, the aurones, is widespread, but not ubiquitous. Depending on the host and the bacterium, these compounds serve as signals, activating the bacterial transcriptional regulator protein NodD, which in turn induces the translation of other rhizobial nodulation genes (*nod*, *nol* and *noe* genes) involved in the synthesis and secretion of the main bacterial nodulation signals called Nod factors or lipo-chito oligosaccharides (LCOs) (Spaink 2000). Nod factors cause morphological changes in legume root hairs, leading to infection thread formation, nodule development and symbiotic nitrogen fixation. The bacterial symbiotic genes may be localized on the chromosome (e.g., *Bradyrhizobium japonicum*, *Azorhizobium caulinodans*) or on large symbiotic plasmids (pSym) (e.g., *Rhizobium leguminosarum*, *Sinorhizobium meliloti*, *Rhizobium* sp. NGR234) (Sessitsch et al. 2002). All Nod factors so far identified consist of a backbone of two to six β-(1 → 4)-linked *N*-acetyl-D-glucosamine (GlcNAc) residues. Depending on the species, this basic structure has several variations. Among the substitutions found on the terminal non reducing *N*-acetyl-D-glucosamine are *N*-methyl groups, carbamoyl groups, acetyl groups, and various fatty acids. The reducing *N*-acetyl-D-glucosamine residue may be substituted with a sulfate group or with D-arabinose, L-fucose, or 2-*O*-methylfucose, etc. Furthermore, this additional saccharide may be acetylated or sulfated (Downie 1998; Perret et al. 2000). Both the amount and the structural variations of Nod factors are important in determining the host specificity in rhizobia–legume symbiosis (Dénarié et al. 1996; Bladergroen and Spaink 1998). However, some reports indicated that rhizobia producing different Nod factors can effectively nodulate the same plants (Poupot et al. 1993, 1995).

The biochemical functions of most of the nodulation genes involved in the biosynthesis of LCOs have been directly or indirectly revealed. The *nodABC* genes are common to all rhizobia and mutation in these genes abolishes Nod factor production. In addition to these, host-specific *nod* genes (*nodFE*, *nodH*, *nodSU*, *nodZ*, *nodX*, etc) are involved in the Nod factor substitutions synthesis (Cloutier et al. 1996, 1997).

Although much of the research on signal molecules involved in the interaction legumes–bradyrhizobia has been focused on the soybean symbiont *Bradyrhizobium japonicum* USDA 110 (Sanjuan et al. 1992; Carlos et al. 1993; Ikeshita et al. 1994), other legumes of significant economic importance such as peanut (*Arachis hypogaea* L.) are also nodulated by *Bradyrhizobium* sp.

Peanut is an important crop that provides direct subsistence and several food products. Bacteria that nodulate peanut have been classified as *Bradyrhizobium* (*Arachis hypogaea*) sp. However, when the rhizobial population associated with peanut in the Argentinean area of production was analyzed, it was demonstrated that this legume is efficiently nodulated by both slow and fast growing rhizobia (Taurian et al. 2002).

In spite of the importance of peanut production in some countries, little is known about the interaction of this legume with rhizobia. In peanut the rhizobial infection mechanism differs from other herbaceous legumes. Rhizobia penetration into the root and in the cortex as well as spreading inside the nodules occurs without intracellular infection threads formation and involves intercellular penetration (crack entry). The infection site is at the junction of a hair cell with epidermal and cortical cells, and rhizobia penetrate into the root by breaching the epidermal barrier instead of entering through curled root hairs. After entry, rhizobia occupy the space between epidermal and cortical cells and further spread through the root cortex. Within cortical cells, they multiply rapidly and the invaded cells divide repeatedly to form a determinate nodule (Boogerd and van Rossum 1997). Thus, several of the phenomena those are essential in the infection thread mode and are elicited by Nod factors do not occur in the crack entry infection process. This infection mechanism also occurs in other genera of legumes such as *Stylosanthes* (Chandler et al. 1982; Boogerd and van Rossum 1997).

The aim of this study was to contribute to the knowledge of the peanut–bradyrhizobia interaction describing for the first time the signal molecules (flavonoids and Nod factors) involved in this economically important symbiosis.

Materials and methods

Bacterial isolates and reference strains growth and maintenance

The peanut bradyrhizobia NDEHE, NLH25 and NOD31 were isolated from nodules of field-grown plants of the peanut production area in Argentina, Córdoba. Their symbiotic behaviour and other phenotypic traits were previously described (Taurian et al. 2002). Additionally, the following bacteria were used for comparison: *Bradyrhizobium* sp.

SEMIA 6144, a peanut nodulating bacterium recommended as inoculant (IPAGRO, Brasil), *Rhizobium* sp. NGR234, a broad host range rhizobia isolated from *Lablab purpureus* and the soybean symbionts *Bradyrhizobium japonicum* USDA110 and *Bradyrhizobium elkanii* USDA 76. Rhizobia were grown in yeast extract-mannitol broth (YMB) or yeast extract-mannitol agar 1.5% (YMA) (Vincent 1970).

DNA preparation, PCR amplification and restriction digestion

For the preparation of DNA template, total DNA was isolated using a modified procedure of Meade et al. (1982).

Nearly full-length *16S rRNA* gene was PCR-amplified by using primers fD1 (5′-AAGGAGGTGATCCAGCC-3′) and rD1 (5′-AGAGTTTGATCCTgGCTCAG-3′) (Weisburg et al. 1991).

The nitrogen fixing *nifD* gene and the nodulation *nodA* gene were PCR amplified using the primers FdB261 (5′-TGGGGICCIRTIAARGAYATG-3′)/FdB260 5′-TCRTTIGCIATRTGRTGNCC-3′) and NodA-1 (5′-GCRGTGGAARNTRNNCTGGGAAA-3′)/NodA-2 (5′-GGNCCGTCRTCRAAWGTCARGTA-3′), respectively (Stoltzfus et al. 1997; Haukka et al. 1998).

The resulting DNA products were analyzed by horizontal electrophoresis on 1.2% agarose gels and were visualized by ethidium bromide staining.

Restriction patterns analysis of the PCR products was done using the following restriction endonucleases: (a) for *nifD*: *Msp*I, *Hae*III, *Hinf*I, *Hha*I, *Nde*II, *Rsa*I; (b) for *nodA*: *Hha*I, *Rsa*I, *Hinf*I (Promega). The resulting DNA products were analyzed by horizontal electrophoresis on 2.5% agarose gels and were visualized by ethidium bromide staining.

The reproducibility of PCR amplifications was verified in at least two independent experiments.

DNA Southern hybridization

Genomic and plasmid DNAs were isolated using bacterial cultures and miniprep kits (Qiagen, Germany), respectively. DNA manipulations, including restriction digests and electrophoresis were performed according to Sambrook et al. (1989). The *Rhizobium tropici* digoxigenin-labeled (Roche, Germany) *nodD*1 probe was the 2.4 kb *Hind*III fragment of plasmid pCV3805 (Sousa et al. 1993). Hybridization conditions were 50% formamide at 42°C.

Sequencing and phylogenetic analysis of *16S rRNA* and *nifD* genes

The nearly full-length *16S rRNA* gene and the amplified fragments of *nifD* genes obtained as described above were precipitated by adding 1 volume of isopropanol and 0.1 volume of sodium acetate (3 M) and collected by centrifugation for 10 min in a microcentrifuge. The resulting DNA pellets were washed with 70% ethanol, air-dried and suspended in double-distilled water (Sambrook et al. 1989). The nucleotide sequences of purified PCR products were determined by Oswell Sequencing (Lab 3145, Medical and Biological Science Building University of Southampton, Boldrewood, Southampton, UK) and MACROGEN Inc (Korea). Molecular sequence analyses were performed by using the algorithm BLASTN (Altschul et al. 1997) to identify similarities and to perform alignments. Multiple alignments and Neighbour-Joining (NJ) trees were constructed by using CLUSTALX 1.64b software (Thompson et al. 1997).

Nucleotide sequence accession numbers

The nucleotide sequence of the *16S rRNA* gene of strains NLH25, NOD31 and NDEHE have been deposited in GenBank data bank under accession No. AY427207, EF202193 and EF158295, respectively. Partial *nifD* gene sequences of the strains have been deposited in GenBank data bank under accession No. DQ295199 (NLH25), DQ295200 (NOD31) and DQ295201 (NDEHE).

Detection of LCOs by thin layer chromatography

It was performed according to Spaink et al. (1992). Briefly, bacteria were grown on 5 ml minimal B^- medium supplemented with each flavonoid solution (1 μg ml^-). For in vivo labelling of LCOs, 0.2 μCi of D-[1-^{14}C]-glucosamine hydrochloride (specific activity 50–60 mCi $mmol^{-1}$, Amersham Biosciences, Germany) was used. Cultures were grown to saturation and the supernatants were extracted with 0.5 ml of water-saturated *n*-butanol. Samples were concentrated by evaporation and the resulting powder suspended in 40 μl of *n*-butanol. A measure of 10 μl of this solution was chromatographed on TLC plates (HPTLC plates RP-18 F254s, Merck, Germany) using a mobile phase of acetonitrile–water (1:1, vol/vol). TLC plates were exposed to Kodak BioMax MR-2 film for 15 days and the film was developed with Kodak reagents according to the manufacturer's instructions.

LCO purification

To obtain large quantities of LCOs, 10 l of genistein-induced bacterial culture medium were extracted with 3 l of *n*-butanol. The butanol extract was evaporated to dryness under vacuum and the concentrates containing the LCOs were suspended in 50 ml of acetonitrile–water (3:2, v/v) which was then brought to acetonitrile–water (1:4, vol/vol). A pre-purification step was performed by passing

the crude extract through a C_{18} cartridge (Resprep, USA). The solid phase extraction column was eluted with different acetonitrile–water solutions (20, 45 and 60%). The 45% fraction was further purified by high performance liquid chromatography (HPLC) (Highon a semi-preparative C_{18} reverse-phase column, 250 × 7.5 mm, Spherisorb ODS2, 5 µm, Tracer) using the following elution program: 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 fraction collected.

Mass spectrometry and NMR analysis of LCOs

Positive ion mode LSIMS and HR-LSIMS was performed on a Micromass AutoSpecQ instrument with a cesium ion gun at an acceleration voltage of 8 kV. Either glycerol-*m*-nitrobenzyl alcohol (1:1) or thioglycerol containing NaI as the cationizing agent was used as matrices. The samples were dissolved in 0.2 ml DMSO.

For NMR analysis, the sample was deuterium exchanged several times by freeze drying from D_2O and then examined as solutions (1 mg ml^{-1}) in d_6-dimethylsulfoxide (DMSO) containing the highest amount of D_2O possible before causing the precipitation of the samples (approximately 8% in D_2O). Spectra were recorded at 303 or 343 K on a Bruker AMX500 spectrometer operating at 500.13 MHz (^1H). Chemical shifts are given in ppm, using the DMSO signal (2.49 ppm) as reference. The *co*rrelation *s*pectroscop*y* (COSY) experiment was performed according to standard Bruker parameters.

Growth of seedlings and preparation of root exudates

Peanut cultivar Tegua was provided by J. Soave, criadero "El Carmen". Seeds were sterilised and germinated as previously described (Taurian et al. 2002; Angelini et al. 2003). Seedlings were individually transferred to 50 ml tubes containing 20 ml sterile distilled water. Roots were passed through a tube hole and ensured that only the organ was submersed in the water. Plants were grown in a controlled environmental chamber under 16 h light:8 h dark cycles, 26/19°C and a photosynthetic photon flux density of 124 µmol m^{-2} s^{-1} provided by fluorescent tubes. After 7 days of incubation, the solution bathing roots, termed root exudates, were collected. The microbial contamination of the root exudates was monitored by streaking 100 µl in Petri dishes with TY (Beringer 1964) or LB media (Sambrook et al. 1989) that were incubated at 28 and 37°C for 7 days. Sterile root exudates were centrifuged at 6,200 rpm for 15 min and filtered through 0.8 and 0.2 µm polycarbonate filters. Then, they were concentrated to dryness using a Speedvac concentrator and stored at −20°C in dark until use.

Flavonoid sources and preparation

Flavonoids standards naringenin (4′,5,7 trihydroxyflavanone), apigenin (4′,5,7 trihydroxyflavone), chrysin (5,7 dihydroxyflavone), luteolin (3′,4′,5,7 tetrahydroxyflavone), genistein (4′,5,7 trihydroxyisoflavone) and daidzein (4′,7 dihydroxyisoflavone) were provided by Sigma Chemical, St Louis, MO. Flavonoid solutions were prepared gravimetrically as 1 mg ml^{-1} stock solutions in methanol with warming.

Flavonoids from root exudates identification

Dried peanut root exudates were suspended in 3 ml sterile water and adsorbed to 900-mg C_{18} Maxi-Clean cartridges. Flavonoids were eluted with 0, 30, 50, 80 and 100% methanol and partitioned against an equal volume of hexane to remove lipids as described by Dakora et al. (1993). Extracts were evaporated under vacuum to dryness, suspended in 1 ml HPLC grade methanol and stored at −20°C in dark. Compounds were separated by HPLC performed at 25°C on an HPLC system (Hewlett Packard) equipped with a 250 mm × 4 mm, 5 µm particle size Phenomenex LUNA C_{18} reverse-phase column using methanol:water:acetic acid gradients at a flow-rate of 1 ml min^{-1} and spectrometric detection at 280 nm, as described by Hakamura et al. (2001). Calibration was achieved with 1 mg ml^{-1} standards solutions. The injection volume of the filtered methanolic extract as well as the mixture of the samples was 50 µl. Compounds were identified on the basis of retention times and quantified by comparison of peak area with those of standards.

Plant nodulation assays

To determine the host range of the peanut isolates, the following legumes were selected: *Glycine max*, *Phaseolus vulgaris*, *Medicago sativa*, *Leucaena leucocephala*, *Macroptilium atropurpureum* and *Vigna unguiculata*. Seeds of *G. max*, *P. vulgaris*, *L. leucocephala* and *V. unguiculata* were surfaced sterilized with sodium hypochlorite (20%) or hydrogen peroxide (15%) solutions (Vincent 1970), while *M. sativa* and *M. atropurpureum* seeds were sterilized with mercuric chloride 2.5% as described by Somasegaran and Hoben (1994). They were germinated for 48 h and the seedlings were transferred to plastic pots containing sterilized vermiculite. After 5 days, each seedling was inoculated with 3–5 ml of the appropriate bacterial culture (YMB) in stationary growth phase (10^9 cells ml^{-1}). Six replica were done for each bacteria. Plants were grown in a

controlled environmental chamber as described above and watered regularly with sterilized tap water and twice a month with nitrogen free Hoagland medium (Hoagland and Arnon 1950). Nodulation was observed 5 weeks after inoculation.

Results

Genetic characterization of the peanut rhizobia isolates

Peanut bradyrhizobia NDEHE, NLH25 and NOD31 were isolated from field peanut nodules or field soil samples from Córdoba (Argentina) by using peanut as trapping host. Their efficiency in peanut nitrogen fixation and genetic diversity by rep-fingerprint and Amplified ribosomal DNA restriction analysis (ARDRA) were previously reported (Taurian et al. 2002, 2006).

The nucleotide sequence of nearly full length (1,300 bp approximately) *16S rRNA* gene from the three isolates were determined and compared to sequences of known rhizobia species available in data banks**.** Phylogenetic *16S rDNA* sequence analysis revealed that strains NLH25, NDEHE and NOD31 clustered together with *Bradyrhizobium* indicating that they belong to the *Bradyrhizobium* genus (data not shown).

Symbiotic genes analysed were the fixation gene *nifD* and the nodulation genes *nodD* and *nodA*. Gene *nifD*, in conjunction with *nifK* and *nifH*, encodes for the subunits of the nitrogenase enzyme complex. This enzyme catalyzes the reduction of atmospheric nitrogen to an assimilable form for plants. The product of *nifD* gene amplification (390 bp) was digested with restriction enzymes (*Msp*I, *Hae*III, *Hinf*I, *Hha*I, *Nde*II, *Rsa*I) and restriction fragment length polymorphism (RFLP) profiles obtained were compared among the strains NDEHE, NLH25 and NOD31, and with reference strains. As it is shown in Table 1, RFLP patterns of the three peanut strains obtained with all enzymes used were identical but differ from those of the reference strains, suggesting that they are polymorphic. The nucleotide sequence of this PCR product was determined and the alignments revealed high level of similarity with *Bradyrhizobum* sp. (Fig. 1). However, the sequences from the three peanut strains formed an independent cluster in a tree, illustrating distances among *nifD* sequences from bradyrhizobia.

Genes *nodABC* were identified as encoding the enzymes that synthesize the basic backbone of Nod factors. NodA is an acyl transferase that adds an acyl group to the non-reducing end of the *N*-acetyl-D-glucosamine backbone. The product of *nodA* gene amplification (670 bp approximately) was digested with enzymes *Hha*I, *Hinf*I and *Rsa*I. As it was observed in the *nif*D-RFLP analysis, all the patterns were identical for the three peanut strains (Table 1), but they differed with those obtained from the reference strains used in this study. Due the lack of *nodA* gene data of peanut bradyrhizobia in public data base we were unable to establish further relationship among our isolates and these rhizobia.

The number of *nodD* gene copies was analyzed in the three strains by "DNA Southern hybridization" employing probe *nodD1* (2.4 Kb *Hind*III fragment of plasmid pCV3805) and, opposite to what was reported for *Bradyrhizobium* (*Arachis*) sp. NC92 (Gillette and Elkan 1996), only a single copy was identified (data not shown).

Flavonoid composition in root exudates

Arachis hypogaea L. cultivar Tegua was chosen because it is widely used in Argentina. The array of root flavonoids released was qualitatively and quantitatively estimated (data not shown). Compared to standard flavonoid solutions retention time, flavonoids daidzein, genistein and chrysin (retention time 19.5, 22.8 and 34.7 min, respectively) were found in peanut root exudates. Analysis of the identified flavonoids by HPLC indicated that the quantities of the isoflavones daidzein and genistein produced per plant were almost 2-fold higher than that of chrysin.

Production and structural analysis of Nod factors

Nod factor production by the three peanut nodulating strains NDEHE, NLH25 and NOD31 was studied by TLC.

Table 1 RFLP pattern of 390 bp fragment of gene *nifD* and 670 bp fragment of gene *nodA*

Strains	RFLP *nifD*						RFLP *nodA*		
	*Msp*I	*Hae*III	*Hinf*I	*Hha*I	*Nde*II	*Rsa*I	*Hinf*I	*Hha*I	*Rsa*I
NDEHE	A	A	A	A	A	A	A	A	A
NLH25	A	A	A	A	A	A	A	A	A
NOD31	A	A	A	A	A	A	A	A	A
Bradyrhizobium sp. SEMIA 6144	D	D	C	A	C	B	B	A	B
Bradyrhizobium japonicum USDA110	B	B	B	B	B	B	C	C	C
Bradyrhizobium elkanii USDA76	C	C	C	A	B	B	ND	D	D
Rhizobium sp. NGR234	A	E	C	C	D	B	E	E	E

Letters indicate different restriction profiles

ND PCR-product not digested

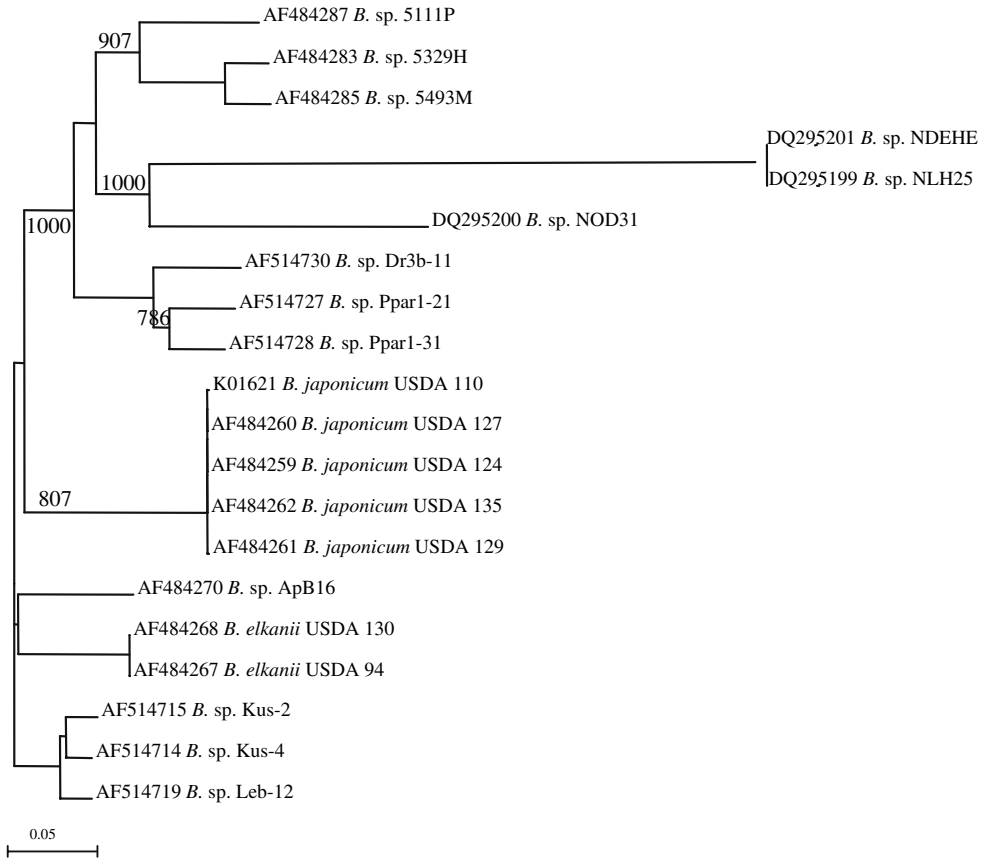


Fig. 1 Phylogenetic tree based on aligned sequences of *nifD* genes, showing relationship among the strains NLH25, NOD31 and NDEHE and known bradyrhizobia. Multiple alignments of DNA sequences were done with the CLUSTAL X 1.64b software and phylograms were edited with Tree View. Sequences of the PCR products were always analyzed by editing out primer sequences. Phylograms were constructed by the neighbor-joining method with 1,000 replicate trees

Flavonoids daidzein, chrysin, genistein, apigenine, naringenin, luteolin, and quercetin were used as *nod* gene inducers. Nod factor production was only detected when 10^{-6} M genistein was added, and all the isolates produced LCOs in an identical pattern (Fig. 2). This was constituted by different molecules as revealed by the presence of five spots in TLC plates. Only in strain NOD31, LCO synthesis was also induced by apigenine.

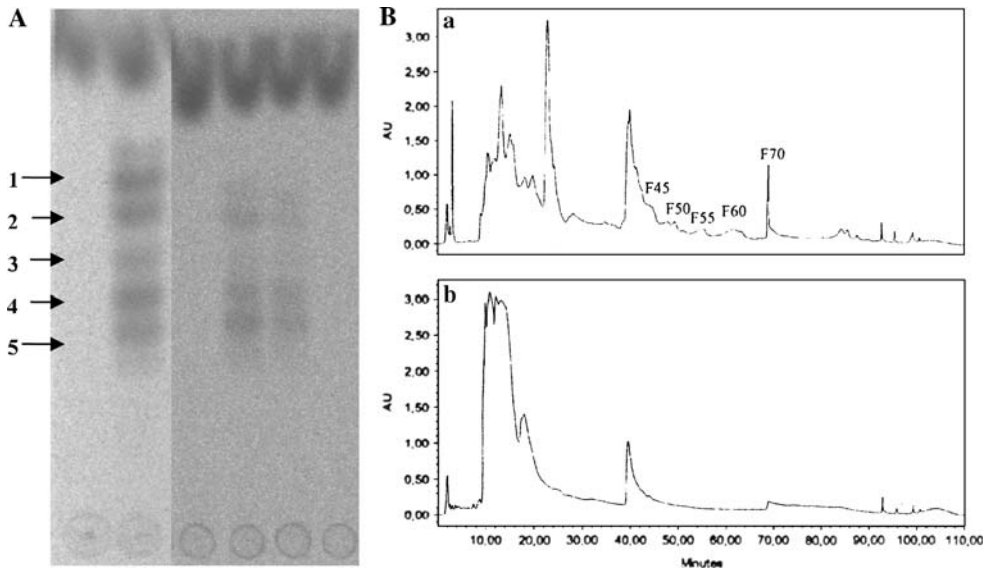


Fig. 2 **A** In vitro analysis of ^{14}C-labelled Nod factors by C18 reverse phase TLC produced by the three peanut strain (spots 1, 2, 3, 4 and 5); *lanes 1* and *2*: NDEHE, *3* and *4*: NLH25 and *lanes 5* and *6*: NOD31, *lanes 2*, *4* and *5* with the inducer genistein, *lanes 1*, *3* and *6* without genistein. **B** HPLC profiles of *n*-butanol extract induced with genistein (a) and not induced (b) from strain NDEHE. Fractions collected are indicated

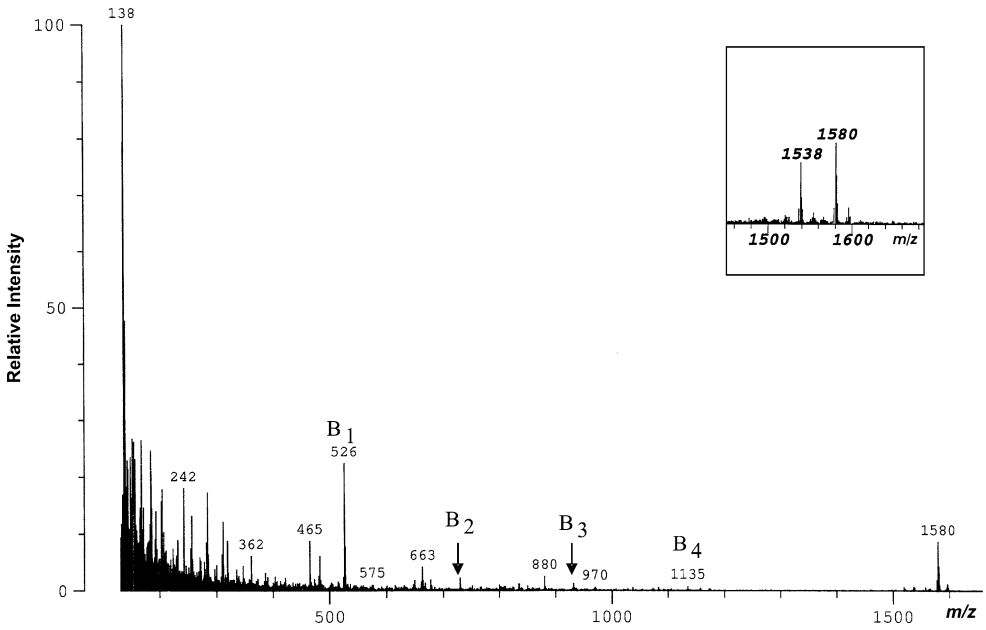
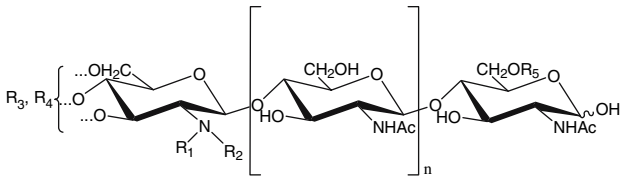


Fig. 3 Fast atom bombardment (FAB) spectrum of the HPLC fraction F70. The spectrum shows the pseudomolecular ion $[M + Na]^+$ at *m/z* 1,580 and the B-type fragment ions

Since the three strains produced identical LCOs pattern in TLC, and NDEHE showed higher production, it was selected to analyze Nod factor structure. HPLC analysis of NDEHE Nod factors revealed the presence of five fractions (F45, F50, F55, F60 and F70) (Fig. 2). Fractions F45 to F60 were eluted at 40% acetonitrile, while fraction F70 was eluted at 60% acetonitrile. Positive fast atom bombardment (FAB MS) and positive liquid secondary mass spectrometry (LSIMS) spectra were performed (Fig. 3). On the basis of the pseudomolecular ions $[M + Na]^+$ and B_n oxonium fragments ions, it was determined that Nod factors contained fucose, methyl fucose, acetyl fucose, and methyl acetyl fucose as substituents at the reducing end glucosamine residue. At the non-reducing end, the presence of $C_{18:1}$, $C_{16:1}$, $C_{16:0}$ and $C_{14:0}$ acyl substituents were determined. Finally, it was found that all Nod factors contained an *N*-methyl and two carbamoyl groups at the non-reducing glucosamine residue (Table 2).

To confirm these assignments, high resolution LSIMS were performed. Exact mass and formula of the pseudomolecular ions and the B_1 ions are shown in Table 3 and confirm the above results. From the exact mass and formula of B_1 fragments we propose the presence of two carbamoyl groups as substituents at the non-reducing end glucosamine residue. This was confirmed by performing the constant B/E scans of some of B_1 oxonium ions (*m/z* 526 and *m/z* 500) (Fig. 4). These B-ions correspond to *N*-methylated non-reducing glucosamine bearing two carbamoyl groups and a

Table 2 Lipochitin-oligosaccharides (LCOs) produced by peanut strain NDEHE in the presence of genistein



$[M + Na]^+$ (*m/z*)	N	R_1	R_2	R_3	R_4	R_5	Designation
1,580	3	$C_{18:1}$	Me	Cb	Cb	MeFucAc	V ($C_{18:1}$, NMe, 2Cb, MeFucAc)
1,554	3	$C_{16:0}$	Me	Cb	Cb	MeFucAc	V ($C_{16:0}$, NMe, 2Cb, MeFucAc)
1,552	3	$C_{16:1}$	Me	Cb	Cb	MeFucAc	V ($C_{16:1}$, NMe, 2Cb, MeFucAc)
1,538	3	$C_{16:1}$	Me	Cb	Cb	AcFuc	V ($C_{16:1}$, NMe, 2Cb, AcFuc)
1,538	3	$C_{18:1}$	Me	Cb	Cb	MeFuc	V ($C_{18:1}$, NMe, 2Cb, MeFuc)
1,526	3	$C_{14:0}$	Me	Cb	Cb	MeFucAc	V ($C_{14:0}$, NMe, 2Cb, MeFucAc)
1,524	3	$C_{18:1}$	Me	Cb	Cb	Fuc	V ($C_{18:1}$, NMe, 2Cb, Fuc)
1,512	3	$C_{16:0}$	Me	Cb	Cb	MeFuc	V ($C_{16:0}$, NMe, 2Cb, MeFuc)
1,510	3	$C_{16:1}$	Me	Cb	Cb	MeFuc	V ($C_{16:1}$, NMe, 2Cb, MeFuc)

Me Methyl, *Fuc* fucose, *Ac* acetyl, *Cb* carbamoyl

Table 3 High resolution LSIMS of the chromatographic fractions of LCOs of strain NDEHE: exact mass and formula of the pseudomolecular and B_1 ions

	Observed	Calculated	Formula
$[M + Na]^+$ *m/z*	1580.758089	1580.743350	$C_{66}H_{111}N_7O_{33}Na$
	1554.728423	1554.727700	$C_{66}H_{113}N_7O_{33}Na$
	1552.710962	1552.712050	$C_{66}H_{111}N_7O_{33}Na$
	1538.728311	1538.732786	$C_{66}H_{113}N_7O_{32}Na$
	1538.703041	1538.696400	$C_{65}H_{109}N_7O_{33}Na$
	1526.668359	1526.696400	$C_{64}H_{109}N_7O_{33}Na$
	1524.723389	1524.717136	$C_{65}H_{111}N_7O_{32}Na$
	1512.717982	1512.717136	$C_{64}H_{111}N_7O_{32}Na$
	1510.698586	1510.701486	$C_{64}H_{109}N_7O_{32}Na$
Oxonium ion B_1 *m/z*	498.316345	498.317926	$C_{25}H_{44}N_3O_7$
	472.298504	472.302276	$C_{23}H_{42}N_3O_7$
	500.329192	500.333576	$C_{25}H_{46}N_3O_7$
	526.344580	526.349556	$C_{27}H_{48}N_3O_7$

$C_{18:1}$ or $C_{16:0}$ acyl group, respectively. It was observed the loss of one and two molecules of carbamic acid (−61 Da) and they were the main daughter ions observed. This fact suggests that this *O*-carbamoyl groups could be located on *O*-3 and *O*-4. In addition, it was observed another daughter ions and they are indicated in the spectrum.

The major HPLC-isolated fraction (F60) was studied by 500 MHz ^{1}H NMR spectrum and 2D-COSY experiments. Due to the high complexity of this fraction, complete assignment was not possible. Signal belonging to an acyl group, and *N*-methyl (at non-reducing terminal), *N*-acetyl groups, H6 and *O*-methyl (in *O*–Me–Fucose), and the anomeric region was assigned (Fig. 5).

Host range of the strains

Considering the structural similarity between Nod factors from strain *Rhizobium* sp. NGR234 and the peanut nodulating isolates, six legumes nodulated by NGR234 were selected to evaluate the nodulation ability of these peanut strains. All of them were able to induce nodules in *M. atropurpureum* and in *V. unguiculata*. Strains NLH25 and NOD31 were also able to nodulate *P. vulgaris*. Nodules formed in all the legumes were red, indicating the presence of leghemoglobin. Nodules were not observed in *M. sativa*, *G. max*, and *L. leucocephala* inoculated with the peanut bradyrhizobia.

Discussion

Sterile peanut roots from *Arachis hypogaea* L. cultivar Tegua released three different flavonoids, genistein and daidzein being the quantitatively dominant flavonoids in the exudates. We have previously reported that the flavonoids chrysin, apigenin, genistein, daidzein, naringenin and luteolin are able to induce the *nodC* promoter in different peanut rhizobia strains (Angelini et al. 2003). The understanding of how legumes benefit from releasing more than one *nod* genes inducing flavonoid is not clear up today (Begun et al. 2001). For peanut this fact would be correlated with the ability of this legume to be nodulated by rhizobia belonging to *Bradyrhizobium* and *Rhizobium* genera (Taurian et al. 2002). Gillette and Elkan (1996) reported that isoflavones genistein and daidzein induced expression of the *nodA-lacZ* fusion in *Bradyrhizobium* (*Arachis*) sp. NC92. In *B. japonicum*, NodD1 is a transcriptional activator also induced by genistein and daidzein (Göttfert et al. 1992). In soybean root exudates, only four compounds were detected in measurable quantities, the concentration of daidzein being greater than the sum of the remaining three compounds combined (Pueppke et al. 1998). *Sesbania rostrata* is a tropical legume infected by *A. caulinodans* by a related mechanism to that occurring in peanut. The bacteria enter to this host via intercellular spaces (as peanut) but then infection threads guide them to nodule primordia. It was reported for this bacterium that flavonoid naringenin induced the *nod* gene expression (D´Haeze et al. 1998).

In this work three peanut bradyrhizobia isolates were genotypically characterized. The results obtained from the *16S rRNA* gene sequencing revealed that strains NLH25, NDEHE and NOD31 are chromosomally close to *Bradyrhizobium* species. On the other hand, the phylogeny obtained from the sequence analysis of genes *nifD* and *nodA* revealed that the strains isolated from Córdoba soils clustered separately from the bradyrhizobia reference strains. Parker et al. (2002) informed that different *Bradyrhizobium* species with similar or identical *nifD* sequences showed a significant tendency to group according to their geographic origins. In contrast, the different lineages of *16S rRNA* gene were distributed across all the geographic regions sampled. Similar results were obtained by Haukka et al. (1998) for *nifH* and *nodA* phylogeny in sinorhizobia isolated from *Prosopis* and *Acacia* growing in different geographic areas.

Gene *nodD* is constitutively expressed in rhizobia, and its product is able to perceive very small quantities of flavonoids and then activate the expression of other genes in *nod* regulon (Schlaman et al. 1992). It was informed that species with NodD protein(s), which recognizes a wide spectrum of compounds, have a corresponding large host range (Györgypal et al. 1991). Strains such as *B. japonicum*, *Rhizobium* sp. NGR234, *S. meliloti* and *R. tropici* carry two to five copies of *nodD* (Broughton et al. 2000).

All the isolates analyzed in this study produced Nod factors when induced by genistein (one of the quantitatively

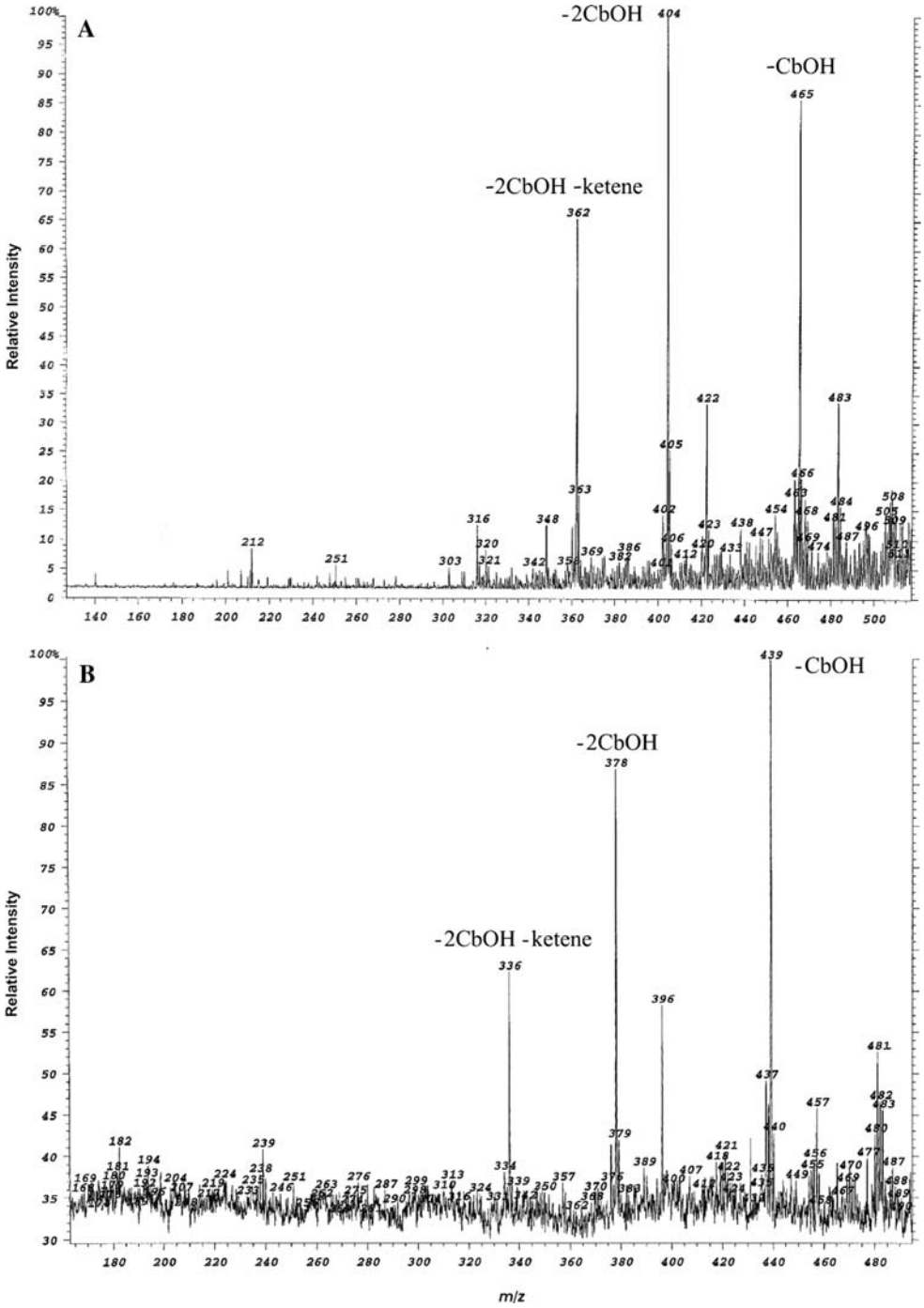


Fig. 4 Constant B/E scans of B_1 oxonium ions originating from fragmentation of the non-reducing end of Nod Factors from strain NDEHE. **a** Scans of the B_1 ion at *m/z* 526 and **b** scans of B_1 ion at *m/z* 500. These B-ions correspond to *N*-methylated non-reducing glucosamine bearing two carbamoyl groups and a $C_{18:1}$ or $C_{16:0}$ acyl group. *CbOH* Carbamic acid

dominant flavonoid in peanut exudates). TLC of Nod factors revealed identical profile, suggesting low variability in genes involved in the synthesis of these molecules, as it was observed in the *nodA*-RFLP.

As strain NDEHE produced LCOs in higher quantities, it was selected for the structural analysis of these molecules. This study revealed that LCOs consist of a pentameric chitin backbone, sharing with soybean *Bradyrhizobium* symbionts some substitutions such as $C_{18:1}$, $C_{16:0}$, $C_{16:1}$, methyl fucose, carbamoyl groups and fucose (D´Haeze and Holsters 2002). However, strain NDEHE was unable to nodulate soybean, possibly due to the fact that these substitutions are of little or no consequences to the nodulating activity of soybean symbionts. Reinforcing this hypothesis are the results obtained by Stokkermans et al. (1995) demonstrating that, despite all strains isolated from soybean synthesize Nod factors with fucose or methyl fucose, this substitution is not required for the induction of nodule primordia. In addition, myristic acid ($C_{14:0}$) and acetylated methyl fucose, which are not present in soybean symbionts

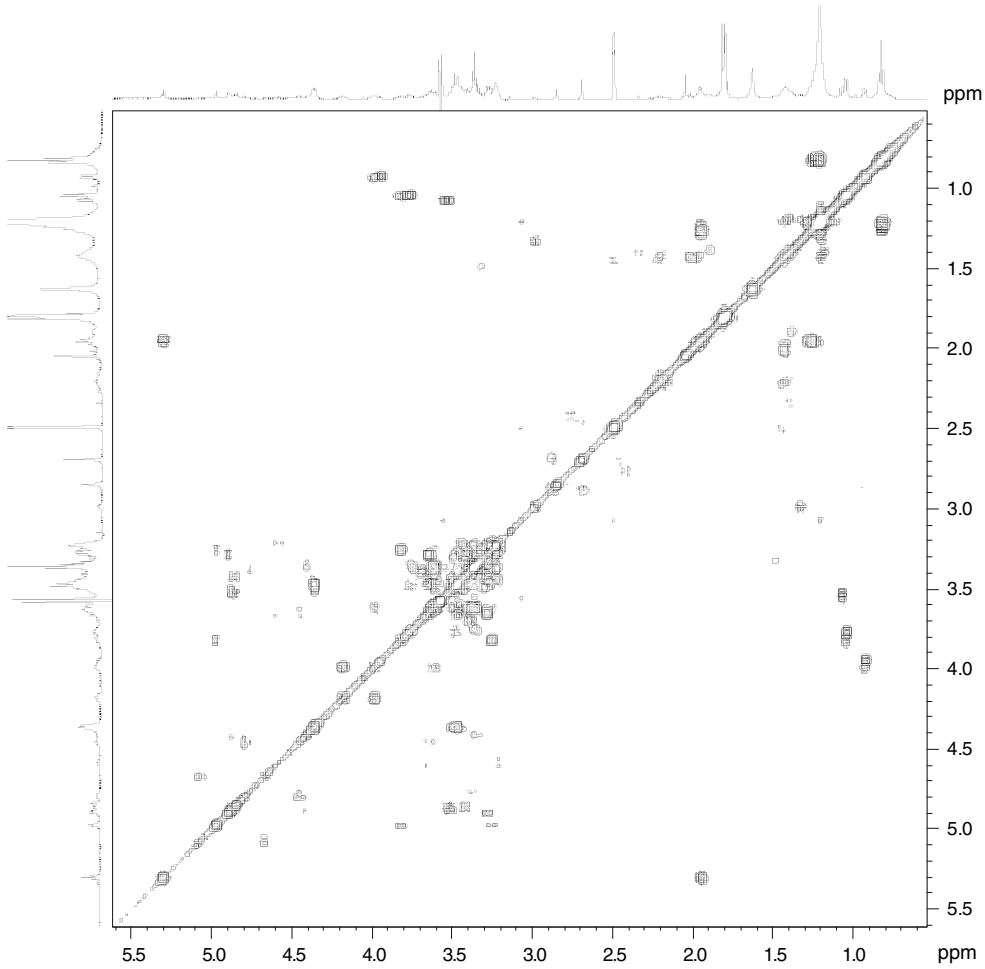


Fig. 5 500 MHz ^{1}H NMR spectrum and COSY NMR obtained for the fraction F60 isolated from strain NDEHE

Nod factors, were found in the NDEHE LCOs. It was interesting to find myristic acid as a structural adornment of the Nod factor since this molecule was reported to be a substitution only in the LCOs produced by *R. galegae* HAMBII207 (Yang et al. 1999).

Structural study of Nod factors synthesized by NDEHE also revealed some substitutions shared with the molecules produced by *Rhizobium* sp. NGR234, such as palmitoleic, vaccenic and palmitic acids, methyl and carbamoyl groups in the non-reducing end, and acetylmethyl fucose or methyl fucose in the reducing end (Perret et al. 2000; D´Haeze and Holsters 2002). However, other structural substituents reported for strain NGR234 were not determined in the LCOs of NDEHE. For example, strain NGR234 produces sulfated LCOs and it was proposed that this substitution is responsible for the ability of this broad host range strain to nodulate *Medicago* (Lerouge et al. 1990), a legume not nodulated by NDEHE. Although specific *nod* genes are responsible for host specificity, it has been shown that common genes *nodABC* also contribute to this phenotype (Schultze et al. 1994). Acyl group $C_{18:2}$, is another structural substitution found in strain NGR234 Nod factor but not in the LCOs produced by strain NDEHE. Considering this Nod factor structural variation, RFLP patterns of *nodA* gene revealed some differences between the three isolates and strain NGR234. All these variations in the LCOs structure would be related with the differences observed between the host ranges of NGR234 and the peanut nodulating strains. Despite Nod factors elaborated by NDEHE and NGR234 are structurally similar, their host ranges are different even when NGR234 nodulates peanut inefficiently (Pueppke and Broughton 1999; Broughton W.J., personal communication). In the same way, *Rhizobium etli* and *Mesorhizobium loti* produce similar Nod factors, but they nodulate different legumes (*Phaseolus* spp. and *Lotus* spp., respectively) (Cárdenas et al. 1995; López-Lara et al. 1995). Also *Phaseolus* plants are nodulated by *R. etli* and by other broad host range strains, but their Nod factors differ in the variety of acyl groups and in the nature of the substitutions (Promé et al. 1998). It is clearly evident from these data that it is not possible to establish a direct correlation between Nod factor structure and legume host range.

In several cases it was shown that a single substituent can drastically change the host range of a bacterium, whereas other LCO decorations have little or no detectable effect on the symbiotic behaviour. Lastly, it must be pointed out that Nod factors are always produced as more or less complex LCO structures, and it is somewhat difficult to evidence which are the active components in the mixture (Promé et al. 1998).

In order to assess whether rhizobial Nod factors structural features could be associated with the legume infection process, we compared the molecules produced by NDEHE with those synthesized by *A. caulinodans*, since both *Arachis* and *Sesbania* legumes are colonized intercellularly, even when final cell penetration in the latter plant occurs via infection threads (Boogerd and van Rossum 1997; van Rhijn and Vanderleyden 1995). *A. caulinodans* produces arabinosylated and/or fucosylated Nod factors, these glycosylations being important for the biological activity. In both peanut bradyrhizobia and *A. caulinodans* LCOs, it was found vaccenic acid ($C_{18:1}$) (Mergaert et al. 1997) and a *N*-methyl or a 6-*O*-carbamoyl group was present at the non reducing end. To investigate the involvement of Nod factors in this root infection process in peanut, mutant defective in Nod factor production are being analysed.

In this study, the genetic characterization of slow-growing peanut nodulating strains revealed that they are closely related and form an independent cluster in the *Bradyrhizobium* genus. This is also the first report for molecules involved in the peanut–bradyrhizobia symbiotic interaction such as root flavonoids composition and microsymbiont Nod factor structure. All these results are contributing to make progress in our understanding of the peanut–bradyrhizobia interaction.

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