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Low pH Changes the Profile of Nodulation Factors Produced by *Rhizobium tropici* CIAT899

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Summary

***Rhizobium tropici* CIAT899 has been cataloged as a nodulator of bean, a plant often growing in areas characterized by highly acidic soils. The purpose of this work was to explore the effects of acidity on the production of Nod factors by this strain and their impact on the establishment of effective symbioses. We report that acidity increases rhizobial Nod factors production, and we exhaustively study the nodulation factor structures produced under abiotic stress. Significant differences were observed between the structures produced at acid and neutral pH: 52 different molecules were produced at acid pH, 29 at neutral pH, and only 15 are common to bacteria grown at pH 7.0 or 4.5. The results indicate that *R. tropici* CIAT899 has successfully adapted to life in acidic soils and is a good inoculant for the bean under these conditions.**

Introduction

Leguminous plants have the ability to enter into symbiosis with N_2-fixing bacteria (collectively called rhizobia), which results in the formation of a new plant root organ, the root nodule. The incorporation of atmospheric N_2 into organic material resulting from this *Rhizobium*-legume symbiosis is estimated to account for one-third of the total nitrogen needed for world agriculture. This unique intracellular association contributes significantly to agricultural yields [1, 2].

The formation of nitrogen-fixing nodules results from an interactive process between the legume and the rhizobia, in which signal molecules, the structures of which were first described in the 1990s [3], play a decisive role. Flavonoids in the root exudate induce the expression of rhizobial nodulation genes (called *nod*, *noe*, and *nol*) that are involved in the biosynthesis and secretion of lipo-chitin oligosaccharides (LCOs) or Nod factors, the bacterial signal molecules [4]. These genes are regulated by the NodD protein, which interacts with flavonoids in order to activate transcription of the *nod* operons.

Rhizobia synthesize populations of Nod factors that consist of between 2 and approximately 60 different individual structures. Qualitative and quantitative aspects of Nod factor populations are strain-specific. The bacterial Nod factors share a "core" structure consisting of two to six β-1,4-linked *N*-acetyl glucosamine (GlcNAc) residues, the nonreducing residue of which is substituted with a long-chain fatty acyl moiety. The LCO structures are further elaborated by the addition to the terminal GlcNAc residues of one or more of a range of different small organic or other monosaccharide substituents, the biosynthesis and/or transfer of which are also mediated by the nodulation genes. Most Nod factors have a "common" fatty acid, such as vaccenic ($C_{18:1}$) or stearic ($C_{18:0}$) acid, or a more unusual (poly) unsaturated fatty acid, such as $C_{20:1}$ or $C_{18:4}$. The length as well as the number and positions of the double bonds in the fatty acid are important for efficient formation of nodule primordia. Often, *N*-methyl, *O*-acetyl, and *O*-carbamoyl groups are found on the nonreducing-terminal residue and L-fucosyl, 2-*O*-Me-fucosyl, 4-*O*-Ac-fucosyl, acetyl substituents, or a sulfate ester on the reducing-terminal residue (see [5] for a review).

Major Nod factor-triggered responses in root hairs include changes in ion fluxes and associated depolarization of the plasma membrane, intra- and extracellular alkalinization, calcium spiking, phosphatidic acid and diacylglycerol formation, accumulation of reactive oxygen species, root hair deformation involving changes in the actin cytoskeleton, and induction of early nodulin gene expression. Nod factors allow the rhizobia to enter the root [6]. In cortical cells, Nod factors induce nodulin gene expression and cell division leading to nodule primordium formation [7]. It has been reported that chemical substituents on the Nod factors are important for their biological activity [8].

The high affinity and specificity with which Nod factors elicit plant responses suggest that they are perceived by plant receptors. The genetic and physiological studies carried out in the model legumes *Medicago truncatula* and *Lotus japonicus* suggest the existence of multiple receptors, or a single receptor with multiple, diverse functions, responsible for initiating the responses mediated by the nodulation factors. Various proteins involved in the transduction of the signal perceived by such receptor(s) have been identified. However, how such a signaling cascade is activated, the number and nature of all the elements of the system involved in the symbiotic interaction, and the differences and similarities between those in the different legumes remain unknown [9].

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Rhizobia in the soil are exposed to a series of variable stresses in their natural environments, including nutrient limitation and/or exposure to physical stresses, such as elevated temperature, acidity, high osmolarity, or oxidative shock [10]. It is known that soil acidity affects rhizobial persistence in the soil and rhizosphere of plants, as well as the efficiency of nodulation, especially in tropical areas [11]. As a result, rhizobia have evolved adaptive strategies designed to minimize acid-induced damage because inducible acid tolerance mechanisms are vital to plant symbionts growing in acidic soils [12]. In the majority of cases, the bacterial response leads to transcriptional activation of genes, the products of which cope with a given physicochemical stress [12, 13]. It has been reported that at least 15–20 genes contribute to acid tolerance in rhizobia [13].

Common bean (*P. vulgaris*) is the most important legume in human nutrition, and is known to be promiscuous, with rhizobia, which nodulate cultivated bean, so far being identified as belonging to five *Rhizobium* species: *R. leguminosarum* bv. phaseoli, *R. etli*, *R. tropici*, *R. gallicum*, and *R. giardinii* [14]. The aim of this study was to assess the inter- and intraspecies variability in nodulation gene expression and in production of rhizobial Nod factors under acid and neutral conditions, as well as their possible impact on the establishment of an effective symbiosis. Here, we report that bean-nodulating rhizobia show a variety of bacterial responses under conditions of pH stress. *R. tropici* CIAT899 was demonstrated to be highly pH tolerant, and was chosen as the model organism because it had been previously demonstrated that strains tolerant to acid conditions often nodulate better than acid-sensitive strains at low soil pH [15]. Furthermore, strain CIAT899 is a good competitor for bean nodule occupancy in acid environments [16].

In order to study the influence of acid pH on the LCO structures, we used thin-layer chromatographic (TLC), high-performance liquid chromatographic (HPLC), and mass spectrometric (MS) analyses, and demonstrated that this strain produces larger relative amounts of LCOs under acidic conditions. We also observed the synthesis of molecules under acidic conditions that were not detected under neutral conditions. Nodulation tests on the common bean under acid conditions showed no significant differences in either nodule number or aerial part dry weight at neutral and acid pHs. We conclude that the activity of some nodulation genes is acidity dependent, and discuss implications of these genes in symbiosis.

Results

pH Tolerance of *R. tropici* CIAT899

We tested the ability of bean nodulating rhizobia (*R. leguminosarum* bv. phaseoli, *R. etli*, *R. giardinii*, *R. gallicum*, and *R. tropici*) to grow on acidic and alkaline buffered culture media, using biological and citrate-phosphate and tris-hydrochloride buffers. When cells grow on minimal B^- medium with biological buffer, they are more tolerant to extreme pH than when they grow on minimal B^- medium with citrate-phosphate and tris-hydrochloride buffers (Figure 1). This may be a consequence of the osmotic stress caused by the citrate-phosphate and tris-hydrochloride buffers.

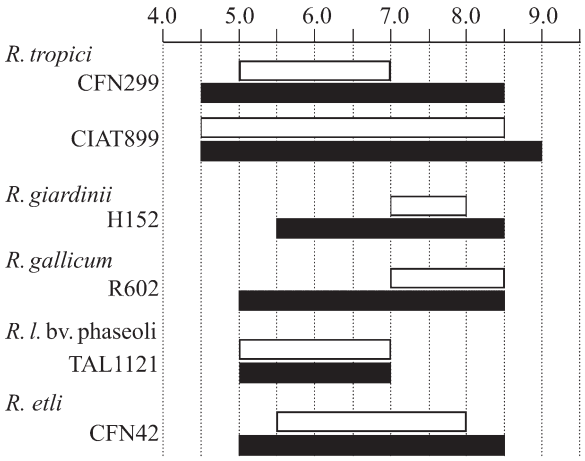


Figure 1. pH Tolerance of Bean-Nodulating Rhizobia

The strains were grown on minimal B^- medium with biological (black) or citrate-phosphate and tris-hydrochloride (white) buffers.

Bean-nodulating rhizobia vary in their response to pH stress: *R. tropici* strains were the most tolerant of acid pH, and only strains belonging to this species were able to grow at pH 5.0/4.5. *R. etli* CFN42 and *R. leguminosarum* bv. phaseoli TAL1121 are moderately acid resistant, capable of growth down to pH 5.5 and 5.0, respectively, with citrate-phosphate buffer, and pH 5.0 with biological buffer. *R. tropici* CIAT899 is highly tolerant of acid pH, being able to grow on media acidified to pH 4.5 with biological and citrate-phosphate buffers. This strain grows in B^- medium buffered to pH 7.0, with a mean generation time of 5 hr and 48 min. However, its growth yield was reduced in acidic B^- medium (pH 4.5), with a mean generation time of 7 hr 24 min.

R. giardinii and *R. gallicum* strains had a similar response to pH variation with biological buffer: they tolerated a range of pHs from 5.5 and 5.0, respectively. They were not capable of growth at acid pH with citrate-phosphate buffer.

We also tested the ability of these strains to grow on alkaline buffered culture medium, using biological and tris-hydrochloride buffers. Most bean-nodulating rhizobia (except *R. leguminosarum* bv. phaseoli TAL1121) were able of growing at pH 8.5/9.0 with biological buffer, though their viability decreased at higher pH. With tris-hydrochloride buffer bean-nodulating rhizobia were most alkaline-sensitive, capable of growth up to pH 8.0 and 8.5, except for *R. tropici* CFN299 and *R. leguminosarum* bv. phaseoli TAL1121, which were the most alkaline-sensitive, growing only up to pH 7.0.

Effect of Acid pH on Nodulation Gene Expression in *R. tropici* CIAT899

In order to study the effect of acid pH on the *Rhizobium*-legume symbiosis, the variability in the production (nature and amounts) of Nod factors was investi-

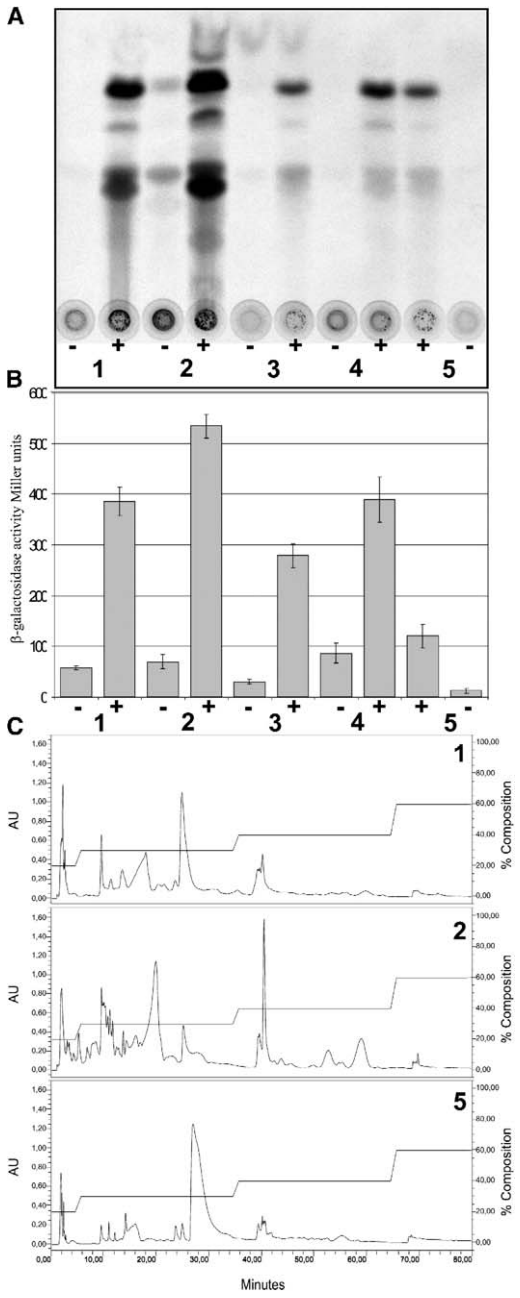


Figure 2. Effect of Acid pH in *R. tropici* CIAT899 Nodulation Gene Expression

(A) RP-TLC analysis of Nod factors produced by *R. tropici* CIAT899 in the presence of ^{14}C labeled *N*-acetylglucosamine.

(B) β-Galactosidase activity of *nodP* gene transcriptional fusion with *lacZ* gene (*R. tropici* RSP3051).

(C) HPLC profiles of *n*-butanol extract induced with apigenin from *R. tropici* CIAT899. (1) pH 4.5, HOMOPIPES buffer; (2) pH 4.5, citrate-phosphate buffer; (3) pH 5.5, MES buffer; (4) pH 5.5, citrate-phosphate buffer; and (5) pH 7.0, without buffer. Strains were induced with 1 μM apigenin (lanes +).

gated in *R. tropici* CIAT899. Reversed-phase TLC analysis with radioactive detection was carried out to study the effect of acid pH on the profile of LCO structures extracted from the bacteria (Figure 2A). We detected an increase in the amount of radiolabeled LCOs from *R. tropici* CIAT899 at acid pH with biological and citrate-phosphate buffers. Surprisingly, between the two major spots (which we assign as corresponding to a hydrophilic family of structures containing sulfated compounds, and a neutral family comprising nonsulfated LCOs), extra spots were observed (Figure 2A). We also detected an increase in the amount of radiolabeled LCOs produced by *R. tropici* CFN299, *R. leguminosarum* bv. phaseoli TAL1121, *R. giardinii* H152, *R. gallicum* R602, and *R. etli* CFN42 at acid pH with citrate-phosphate and biological buffers (data not shown).

Therefore, we also studied the *nod* gene expression using gene fusions to a reporter gene (β-galactosidase). *R. tropici* RSP3051, having the reporter gene fusion *nodP*::*lacZ*, was used to study the induction of *nod* gene expression by incubating acid-grown rhizobia with purified flavonoids. We used this fusion because *nodP*-belongs to the *nodABCSUIJ* operon [17]. The highest β-galactosidase activity after apigenin induction was observed under acidic conditions, with a 3- or 5-fold increase in induction with respect to that under neutral conditions, with biological and citrate-phosphate buffers, respectively, suggesting that, under these conditions, there is greater activity of the nodulation genes (Figure 2B). We also used the pRP30 plasmid (*nodA*::*lacZ*) to study the induction of *R. tropici* CIAT899 *nod* genes in *trans*. This fusion has been used extensively to explore inducing activities of homologous as well as heterologous *nodD* genes. The plasmid was introduced into *R. tropici* CIAT899, and the induction of β-galactosidase activity was measured. The results observed for the β-galactosidase activity of this strain were similar to those of *R. tropici* RSP3051 (data not shown).

It is worth noting that a higher level of β-galactosidase activity and an increase in the amount of radiolabeled LCOs were obtained from *R. tropici* CIAT899 under acid conditions than under neutral conditions, suggesting that under these conditions there is greater activity of the nodulation genes.

Production and Purification of the Nod Factors

In order to determine the structures of the LCOs produced under the different pH conditions, *R. tropici* CIAT899 was grown in either the presence or the absence of apigenin (as inducer) at two different pHs (7.0 and 4.5). The culture medium was extracted, purified, and fractionated as described [18]. The HPLC profile of the LCOs produced at pH 4.5 using HOMOPIPES and citrate-phosphate buffers show a very important increase in peak areas, in contrast to that from pH 7.0 cultures (Figure 2C).

MS Analysis

Analysis by FAB-MS, MALDI-ToF-MS, ES-Q-o-Tof-MS, and MALDI Q-o-Tof-MS of the fractions obtained on HPLC separation of the Nod factor-containing extracts from *R. tropici* CIAT899, grown at pH 4.5 (in citrate-phosphate buffer) and pH 7.0, allowed us to identify 52 Nod factors formed under acid conditions and 29 Nod factors produced under neutral conditions: 15 of these structures were produced under both conditions. We

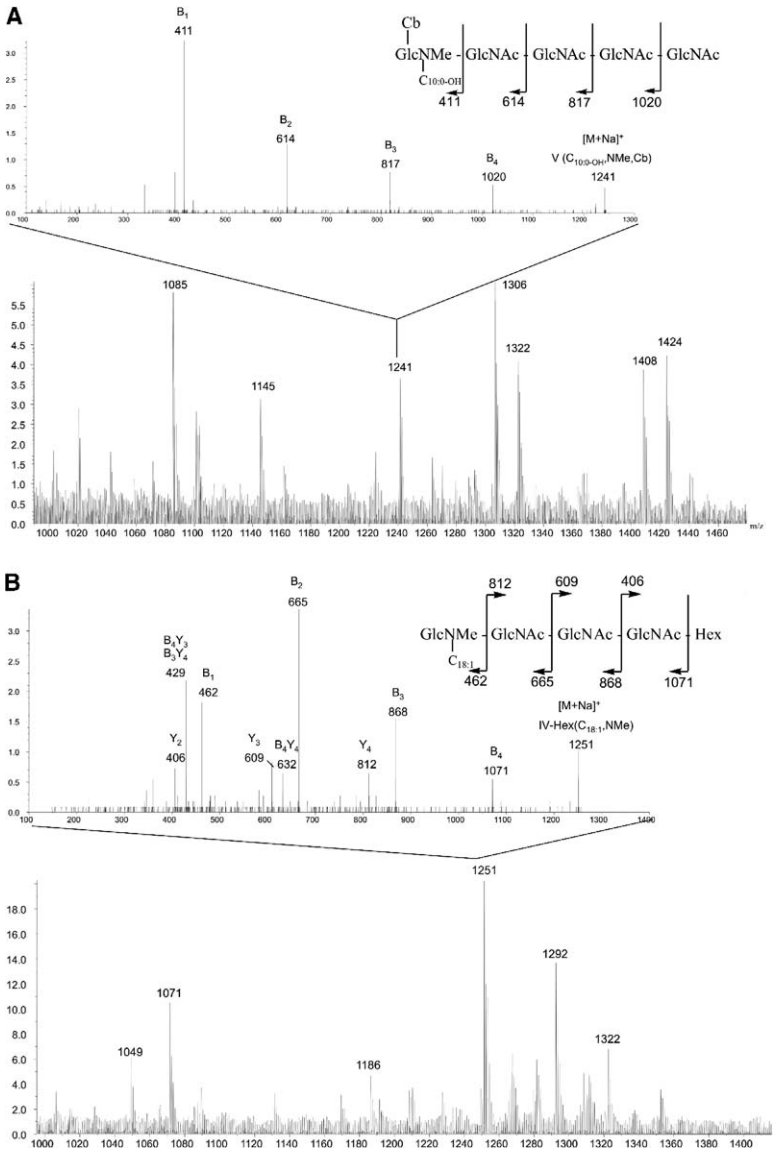


Figure 3. MALDI Mass Spectrum of HPLC Fraction of t_R = 30 min (A) and t_R = 52 min (B) from *R. tropici* CIAT899 Grown at pH 7.0, CID Tandem Mass Spectrum of Ion at *m/z* 1241 (A) and *m/z* 1251 (B), with Fragmentation Scheme

present examples of the MALDI and ES MS and tandem MS data obtained from two of these HPLC fractions (Figures 3 and 4). The results of the analyses of all the HPLC fractions are summarized in Table 1.

In a previous paper reporting the Nod factors produced at neutral pH by *R. tropici* CIAT899, the authors identified 16 Nod factors [19]. Now, we have identified 29 Nod factors. The difference could be due to the greater sensitivity of the instruments used in the current study, enabling us to identify minor components. On the other hand, some of the LCOs described in that previous paper have not been identified in the present analysis. This could be because earlier work used *R. tropici* CIAT889 with the plasmid pMP604, which contains a *nodD* FITA [20] gene to overproduce the Nod factors, while this work used the wild-type strain.

The LCO structures are proposed on the basis of the results of monosaccharide composition and linkage analyses of some of the nodulation factor-containing HPLC fractions, GLCMS fatty acid analyses, and (mainly) the results of MALDI-Tof- and ES-Q-o-Tof-MS and CID tandem MS analyses. CID tandem MS, whether of ES- or MALDI-generated ions, results in cleavage of the glycosidic bonds in the LCO backbone to generate B_i (nonreducing terminal) and Y_i (reducing terminal) fragment ions, which allow identification of backbone substituents and determination of their location on the glucosamine backbone.

The Nod factors identified all have the typical linear backbone of GlcNAc residues (tetramer and pentamer), with different *N*-acyl residues on the nonreducing terminal GlcN, and bear substituents on the reducing and/or nonreducing terminal residues (Table 1). We have identified seven main classes of Nod factor structures. The first class of Nod factors represents simple structures with unsubstituted backbones. Their CID mass

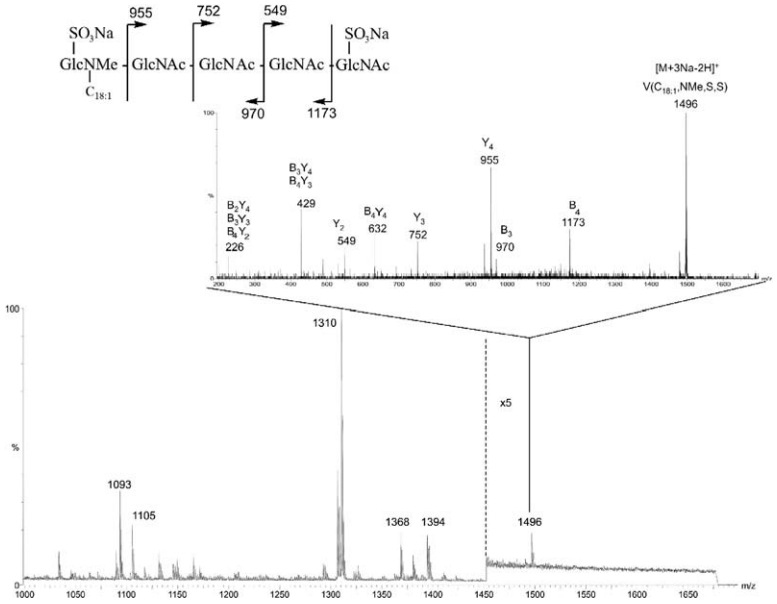


Figure 4. ES Mass Spectrum of HPLC Fraction of t_R = 40 min from *R. tropici* CIAT899 Grown at pH 4.5 with Citrate-Phosphate Buffer, CID Tandem Mass Spectrum of Ion at *m/z* 1496, with Fragmentation Scheme

spectra contain B_i and Y_i ion series that are consistent with this; the B_1 ion allowed identification of the fatty acyl group.

Sulfated nodulation factors represent the second type and ionized as cationized molecules, in which the sulfate is as its sodium salt $[M - H + 2Na]^+$. The B_i and Y_i ion series show that the sulfate group is located on the reducing terminal residue. Third, we have identified *N*-methylated (on the nonreducing terminal residue) and *N*-methylated sulfated nodulation factors, from the *m/z* value of their sodiated molecules, and by virtue of their fragment ions; the B_1 ion defines the residue methylated. The fourth structural type is acetylated nodulation signals, a subset of which are also *N*-methylated, identified from their sodiated molecules, their fragment ions, and the ability to remove the acetyl ester on mild base treatment. The acetyl group is located on the nonreducing terminal residue, characterized by the B_1 fragment. Carbamoylated Nod factors, a subset of which are *N*-methylated, (structure-type five) were detected only in cultures produced at pH 7.0. The odd *m/z* values of the B_1 ions they produce indicate the presence of an additional nitrogen atom deriving from the carbamoyl group and reveal its location on the nonreducing terminal residue (e.g., Figure 3A). Also, there are very unusual nodulation factor structures (type six), with a backbone comprising four GlcNAc residues and a hexose (mannose) at the reducing terminus, which are only detected in the pH 7.0 cultures. These structures are characterized by the *m/z* values of their B_4 and, when observed, their Y_2 ions (e.g., Figure 3B). These hexose-containing Nod factors also bear *N*-methyl or carbamoyl groups as substituents on the nonreducing terminal residue. We have identified both carbamoylated and *N*-methylated analogs. Finally, we have identified a different Nod factor structure, which ionizes to generate a sodiated molecule at *m/z* 1496. The B_i and Y_i ion series, especially the B_4 and Y_4 fragments, together with the *m/z* value of the sodiated molecule, are consistent with $[M - 2H + 3Na]^+$ for a pentameric Nod factor *N*-acylated with a $C_{18:1}$ group and *N*-methylated on the nonreducing terminal residue, and bearing two sulfate groups (as their sodium salts): one located on the reducing terminal residue, and one on the nonreducing glucosamine (Figure 4).

Different saturated, unsaturated, and hydroxylated fatty acids have been identified (C_{10}, C_{12}, C_{14}, C_{16}, C_{18}, and C_{20}). The unsaturated fatty acid $C_{18:1}$ was identified as octadecenoic acid by GLC-MS of its methyl ester. The mass spectrum of the dimethyl disulphide derivative of the unsaturated fatty acid methyl ester contained signals at *m/z* 390 (molecular ion), *m/z* 145, and *m/z* 245, arising from the fragmentation between the carbons bearing the dimethyl disulphide groups, demonstrating that the double bond was located at carbon 11. The hydroxy fatty acid $C_{20:0}$ was identified as an ω-1 hydroxy fatty acid (19-hydroxyicosanoic acid). The mass spectrum of trimethylsilyl ester derivatives (TMS) of the methyl ester contained characteristic signals at *m/z* 117 and *m/z* 370.

Composition and Methylation Analysis

GLC-MS analysis of the TMS methyl glycosides showed that glucosamine was present. For the fraction containing the Nod factor IV-Hexose ($C_{18:1}$, NMe), mannose was also identified. Glucosamine was assigned as D-GlcN, following GLC-MS analysis of its trimethyl silylated (+)-2-butyl and (±)-2-butyl glycosides. Methylation analysis of the HPLC fraction (t_R = 19 min) from the pH 4.5 culture, containing V ($C_{18:1}$, NMe, S), showed the presence of 1,5-di-*O*-acetyl-3,4,6-tri-*O*-methyl-*N*-acetyl-*N*-methyl glucosaminitol from the nonreducing terminal unsubstituted glucosamine residue, 1,4,5-tri-*O*-acetyl-3,6-di-*O*-methyl-*N*-acetyl-*N*-methyl glucosaminitol from the internal 4-substituted glucosamine residues, and 1,4,5,6-tetra-*O*-acetyl-3-*O*-methyl-*N*-acetyl-*N*-methyl

Table 1. Nodulation Factors Produced by *R. tropici* CIAT899 at pH 4.5 and pH 7.0

Structure[a]	$[M + Na]^+$ *m/z*	*R. tropici* CIAT899 pH 4.5	*R. tropici* CIAT899 pH 7.0
IV($C_{10:0}$)	965	B_i ions: 744, 541, 338	—
IV($C_{10:0}$, NMe)	979	B_i ions: 758, 555, 352	—
IV($C_{14:0}$)	1021	B_i ions: 800, 597, 394 Y_i ions: 244, 447, 650	—
IV($C_{14:0}$, NMe)	1035	B_i ions: 814, 611, 408 Y_i ions: 447, 650	—
IV($C_{14:0}$-OH)	1037	B_i ions: 816, 613, 410	—
IV($C_{14:0}$-OH, NMe)	1051	B_i ions: 830, 627, 424	—
IV($C_{14:1}$, NMe)	1033	B_i ions: 812, 609, 406 Y_i ions: 447, 650	—
IV($C_{16:0}$)	1049	B_i ions: 828, 625, 422	+
IV($C_{16:0}$, NMe, S)	1165	B_i ions: 842, 639, 436	—
IV($C_{16:0}$, NMe)	1063	—	+
IV($C_{16:0}$-OH)	1065	B_i ions: 844, 641, 438	—
IV($C_{16:1}$, NMe)	1061	B_i ions: 840, 637, 434	—
IV($C_{18:0}$, NMe)	1091	B_i ions: 870, 667, 464	—
IV($C_{18:0}$, NMe, S)[b]	1193	B_i ions: 870, 667, 464	+
IV($C_{18:0}$-OH)	1093	B_i ions: 872, 669, 466 Y_i ions: 244, 447, 650	—
IV($C_{18:0}$-OH, NMe)	1107	B_i ions: 886, 683, 480	—
IV($C_{18:0}$-OH, NMe, Ac)[c]	1149	—	B_i ions: 928, 725, 522
IV($C_{18:1}$)[b]	1075	B_i ions: 854, 651, 448	+
IV($C_{18:1}$, NMe)[b]	1089	B_i ions: 868, 665, 462	B_i ions: 868, 665, 462 Y_i ions: 244, 447, 650
IV($C_{18:1}$, NMe, S)[b]	1191	+	—
IV($C_{20:0}$)	1105	B_i ions: 884, 681, 478	—
IV($C_{20:0}$, S)	1207	B_i ions: 884, 681, 478	—
IV($C_{20:0}$-OH)	1121	B_i ions: 900, 697, 494	—
IV($C_{20:0}$-OH, NMe)	1135	B_i ions: 914, 711, 508	—
IV($C_{20:1}$)	1103	B_i ions: 882, 679, 476	—
IV($C_{20:1}$, S)	1205	—	B_i ions: 882, 679, 476
IV-Hex($C_{10:0}$-OH, Cb)	1186	—	B_i ions: 1006, 803, 600, 397
IV-Hex($C_{12:0}$, NMe)	1169	—	B_i ions: 989, 786, 583, 380 Y_i ions: 406, 609, 812
IV-Hex($C_{18:1}$, NMe)	1251	—	B_i ions: 1071, 868, 665, 462
V($C_{10:0}$, NMe)	1182	B_i ions: 961, 758, 555, 352	—
V($C_{10:0}$-OH, Cb)	1227	—	B_i ions: 1006, 803, 600, 397
V($C_{10:0}$-OH, NMe, Cb)	1241	—	B_i ions: 1020, 817, 614, 411
V($C_{12:0}$, NMe)	1210	—	B_i ions: 989, 786, 583, 380 Y_i ions: 447, 650, 853
V($C_{14:0}$)	1224	—	B_i ions: 1003, 800, 597, 394 Y_i ions: 447, 650, 853
V($C_{14:0}$, NMe)	1238	B_i ions: 1017, 814, 611, 408 Y_i ions: 447, 650, 853	+
V($C_{14:0}$, NMe, S)	1340	B_i ions: 1017, 814, 611, 408	—
V($C_{14:0}$-OH)	1240	B_i ions: 1019, 816, 613, 410	—
V($C_{14:0}$-OH, NMe)	1254	B_i ions: 1033, 830, 627, 424	—
V($C_{14:1}$)	1222	B_i ions: 1001, 798, 595, 392	—
V($C_{16:0}$)	1252	B_i ions: 1031, 828, 625, 422	—
V($C_{16:0}$, S)[b]	1354	B_i ions: 1031, 828, 625, 422	—
V($C_{16:0}$, NMe)	1266	B_i ions: 1045, 842, 639, 436	B_i ions: 1045, 842, 639, 436 Y_i ions: 447, 650, 853
V($C_{16:0}$, NMe, S)[b]	1368	B_i ions: 1045, 842, 639, 436 Y_i ions: 549, 752, 955	—
V($C_{16:0}$-OH, NMe)	1282	B_i ions: 1061, 858, 655, 452	—
V($C_{16:1}$, NMe)	1264	B_i ions: 1043, 840, 637, 434 Y_i ions: 244, 447, 650, 853	—
V($C_{16:1}$, NMe, S)[b]	1366	+	+
V($C_{18:0}$) or V($C_{16:1}$-OH, NMe)	1280	B_i ions: 1059, 856, 653, 450	—
V($C_{18:0}$, S)	1382	B_i ions: 1059, 856, 653, 450	—
V($C_{18:0}$, NMe)	1294	B_i ions: 1073, 870, 667, 464	+
V($C_{18:0}$, NMe, S)[b]	1396	B_i ions: 1073, 870, 667, 464 Y_i ions: 346, 549, 752, 955	+
V($C_{18:0}$-OH, NMe)	1310	B_i ions: 1089, 886, 683, 480 Y_i ions: 244, 447, 650, 853	—
V($C_{18:1}$)[b]	1278	B_i ions: 1057, 854, 651, 448	—

(*continued*)

Table 1. Continued

Structure[a]	[M + Na]$^+$ *m/z*	*R. tropici* CIAT899 pH 4.5	*R. tropici* CIAT899 pH 7.0
V($C_{18:1}$, S)[b]	1380	B_i ions: 1057, 854, 651, 448	—
V($C_{18:1}$, NMe)[b]	1292	B_i ions: 1071, 868, 665, 462	B_i ions: 1071, 868, 665, 462 Y_i ions:, 447, 650, 853
V($C_{18:1}$, NMe, Ac)[c] or V($C_{22:1}$)[d]	1334	—	B_i ions: 1113, 910, 707, 504 Y_i ions: 244, 447, 650, 853
V($C_{18:1}$, NMe, S)[b]	1394	B_i ions: 1071, 868, 665, 462 Y_i ions: 549, 752, 955	B_i ions: 1071, 868, 665, 462 Y_i ions: 549, 752, 955
V($C_{18:1}$, NMe, S, S)	1496	B_i ions: 1173, 970,—, 564 Y_i ions: 549, 752, 955	—
V($C_{20:0}$)	1308	B_i ions: 1087, 884, 681, 478 Y_i ions: 447, 650, 853	B_i ions: 1087, 884, 681, 478
V($C_{20:0}$, S)	1410	B_i ions: 1087, 884, 681,— Y_i ions: 549, 752, 955	+
V($C_{20:0}$, NMe)	1322	—	B_i ions: 1108, 898, 695, 492 Y_i ions: 447, 650, 853
V($C_{20:0}$-OH, Ac)[c]	1366	—	B_i ions: 1145, 942, 739, 536
V($C_{20:0}$-OH, NMe)	1338	B_i ions: 1117, 914, 711, 508	—
V($C_{20:0}$-OH, NMe, S)	1440	B_i ions: 1117, 914, 711, 508	—
V($C_{20:1}$)	1306	B_i ions: 1085, 882, 679, 476	B_i ions: 1085, 882, 679, 476 Y_i ions: 447, 650, 853
V($C_{20:1}$, S)	1408	B_i ions: 1085, 882, 679, 476 Y_i ions: 549, 752, 955	B_i ions: 1085, 882, 679, 476 Y_i ions: 549, 752, 955
V($C_{20:1}$-OH, NMe)	1336	—	B_i ions: 1115, 912, 709, 506 Y_i ions: 447, 650, 853

Abbreviations: +, detected; –, not detected.

[a] Nod factor structures are represented following the convention [48] that indicates the number of GlcNAc residues in the backbone (Roman numeral), the length and degree of unsaturated of the fatty acyl chain and the other substituents, which are listed in the order in which they appear, moving clockwise from the fatty acid.

[b] Reported in a previous paper.

[c] Acetylated on the nonreducing terminal residue.

[d] No signals observed in the mass spectrum of the sample following mild base treatment, so that it is impossible to distinguish between these two structures.

glucosaminitol from the reducing terminal glucosamine residue substituted on *O*-6. The sulfate substituent on the reducing-terminal glucosamine resists methylation, but is removed during hydrolysis; the sulfated OH of this glucosamine is thus acetylated in the resulting, partially methylated, alditol acetate.

Effect of pH of the Rooting Medium on Nodulation of *P. vulgaris* Grown under Controlled Conditions

In order to characterize the effect of pH on the nodulation of common bean by *R. tropici* CIAT899, bean plants were grown in sand pots watered with nutrient solution buffered to the desired pH (4.5, 5.5, and 7.0) over 3 wk. Nodulation ability of *R. tropici* strain CIAT899 was significantly higher at pH 5.5 (169 nodules/plant) than at pH 7.0 or 4.5 (116 and 91 nodules/plant, respectively); however, there were no statistically significant differences between pH levels for nodule mass (nodule dry weight), ranging from 97 to 142 mg. Plant biomass accumulation reflected the same pattern as the number of nodules/plant (Figure 5); at pH 5.5, the plant dry matter was significantly higher (750 mg) than at pH 7.0 or 4.5 (482 and 335 mg, respectively).

Discussion

The common bean is the most important legume in human nutrition, and is known to be promiscuous. Because this plant is important in the diet of many developing countries, it is important to manage the N_2-fixing symbiosis for maximized crop production. A significant constraint is moderately low soil pH, which affects the establishment of an effective symbiosis. Root nodule bacteria can be more sensitive to low pH than is their legume host, directly affecting symbiosis establishment [10], so that selection of *Rhizobium* strains tolerant to low pH may improve the acid tolerance of the legume.

We tested the ability of bean-nodulating rhizobial strains to grow on acid buffered culture media, using biological, citrate-phosphate, and tris-hydrochloride buffers. Without the addition of a buffer, none of the media was suitable for maintaining a stable, low pH during the growth of rhizobia. The lowest pH at which bean rhizobia grow varies with the species; none of the strains grew below pH 4.5. *R. tropici* strains were the most tolerant of acid pH.

A previous report has suggested that the cellular regulation of cytoplasmic pH is necessary for the growth of *Rhizobium* in acid environments [21]. It may be that the acid sensitivity of some strains of bean rhizobia is related to their inability to regulate internal pH, and possibly accounting for their poor growth in cultures at acid pH. *R. tropici* strain CIAT899 is one of the bean rhizobia identified that displays a high intrinsic tolerance to acidity; the ability of this strain to regulate its cytoplasmic pH could be a requirement for growth under acid conditions [22].

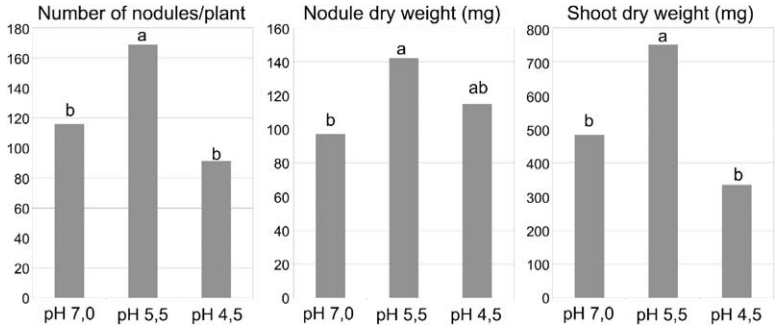


Figure 5. Effect of pH of the Rooting Medium on Nodulation of *P. vulgaris* cv. Negrojamapa by *R. tropici* CIAT899

Plants were watered with buffered nutrient solutions: pH 7.0 (HEPES buffer), 5.5 (MES buffer), and 4.5 (HOMOPIPES buffer). Data followed by the same letter are not significantly different ($p < 0.05$).

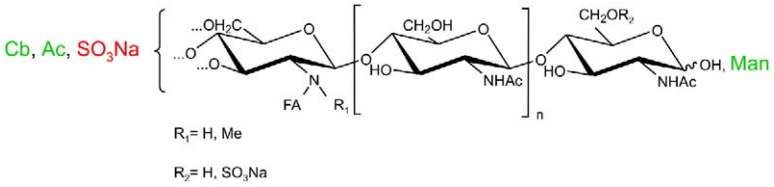
We have explored the effects of pH on the expression of *nod* genes and on the structures of nodulation factors produced by *R. tropici* CIAT899 to identify any possible differences between the structures produced at neutral pH and at pH 4.5. Using fusions of the *Escherichia. coli lacZ* gene to specific nodulation genes, we investigated the effects of pH on the induction of *nod* gene expression in *R. tropici* in the presence of flavonoids. We found that, with lower pH, the increase in nodulation gene expression was greater; this trend is mirrored by the increase in the degree of incorporation of radiolabeled glucosamine into the Nod factors, as assessed using TLC analysis. Taken together, the results of the gene expression and radioactive labeling experiments indicate qualitative and quantitative changes in the production of signal molecules at acid pH. Results similar to those obtained with *R. tropici* were also obtained with the other bean rhizobia, pointing to a generalized effect in the strains studied, although their range of tolerance was not the same.

The increased production of Nod factors detected in the strains of *Rhizobium* studied following acidification of the medium could be a response developed by the bacteria in an attempt to diminish, as much as possible, the adverse effects of acid pH on the establishment of the symbiosis, and thus be effective in nodulation under such conditions. In this way, it has been reported that acidity dramatically reduces the root hair response to Nod factors in the *Glycine max-Bradyrhizobium japonicum* symbiosis, and that the effect is reversed by increasing the concentration of these molecules, the response being similar to that seen at neutral pH [23].

This is the first report that an abiotic stress (acidity) affects both the structure and the amounts of the signal molecules synthesized, with potential effects on the plant-microbe interaction. These results correspond with those obtained in the nodulation test carried out on the common bean under acid conditions. The nodule number was affected by the pH of the rooting medium; thus, the number of nodules formed at pH 5.5 was statistically higher than that at pH 7.0 and 4.5. Although at pH 4.5 *nod* gene expression and the incorporation of radiolabeled glucosamine into the signal molecules are greater that at higher pH values, the common bean plant itself is more affected at pH 4.5 than at pH 5.5. This could account for the fact that there are fewer nodules apparent at pH 4.5 than pH 5.5 [24]. However, while there was no difference in nodule mass at pH 4.5 and 5.5, there was a tendency for the nodule dry weight to be reduced at the higher pH tested. Plant shoot dry weight followed the same pattern as the number of nodules. This means that pH 5.5 was statistically better for plant biomass accumulation than the other two pH levels tested.

We also explored the effects of pH on the structures of nodulation factors produced by *R. tropici* CIAT899; we have identified significant differences between the structures produced at neutral pH [19] and at pH 4.5 (in citrate-phosphate buffer). Of the two buffers (biological and citrate-phosphate) used for buffering the acid medium, citrate-phosphate was chosen because both compounds are present in the rhizosphere, being exuded naturally by plants [25]. The role of organic acids, including citric acid, as a source of rhizosphere acidification is documented [26]. A wide variety of nodulation factors were produced by this strain when cultured at pH 4.5. More than 50 different nodulation factors were identified as being produced under these conditions and 29 Nod factors were produced under neutral conditions; 15 of these structures were similar under the 2 conditions (Table 1). We have demonstrated that the Nod factor structures include modifications of the nonreducing and reducing terminal residues at acid pH (Figure 6). These results seem to indicate that nodulation gene expression is affected at acid pH, not only allowing a higher induction, but also activating the expression of other genes, allowing new “decorations” in the signal molecule produced by *R. tropici* CIAT899.

It has been reported that the Nod factor oligosaccharide backbone of bean-nodulating rhizobia does not require a specific attached group, except for the acyl chain on the nonreducing terminal residue [27]. We have identified different saturated, unsaturated, and hydroxylated fatty acids produced under both acid and neutral conditions (see Figure 6). For example, the ions observed at *m/z* 979 and 1182 indicate the presence of a nodulation factor consisting of 4 and 5 GlcNAc residues, respectively, substituted with a C10:0 fatty acyl chain and a methyl group on the nitrogen of the amino group on the nonreducing terminal residue at pH 4.5. Although no conclusive data about the role of the *N*-methyl group for biological activity of azorhizobial Nod factors are yet available [28], the observation based on modeling the three-dimensional structure of the Nod factor suggests that the presence of an *N*-methyl group may be necessary for correct positioning of the fatty acyl chain. Such positioning may be important for Nod factors to be recognized efficiently by their recep-



	Fatty Acid# (FA)						
	$C_{10:0}$	$C_{10:0\text{-OH}}$	$C_{12:0}$	$C_{14:1}$	$C_{14:0\text{-OH}}$	$C_{16:0\text{-OH}}$	$C_{20:1\text{-OH}}$
pH 4.5	+	-	-	+	+	+	-
pH 7.0	-	+	+	-	-	-	+

Figure 6. Differences in the Structure of the Nod Factors Found in *R. tropici* CIAT899 at Acid and Neutral pH

Nod factor structures are represented following the convention [48]. The substituents shown in green appear only at pH 7.0, those in red appear only at pH 4.5, and those in black appear at both values. # The fatty acids produced at both pHs are not shown in the table.

tors, or to be correctly integrated into the cell wall and/or plasma membrane. However, in *Rhizobium* strains that elicit nitrogen-fixing nodules on *P. vulgaris*, and that produce methylated Nod factors, NodS-mediated decorations are indispensable for invasion and/or subsequent nitrogen fixation [27].

Furthermore, we have detected a new type of sulfated nodulation factor produced under acid conditions, with an additional sulfated group located on the nonreducing terminal residue. A previous study has described doubly sulfated Nod factor from *Mesorhizobium huakii*. This molecule was detected only in the electrospray negative-ion mode, and attempts to cleave its negatively double-charged ion did not provide useful structural information [29].

Nod factors that we detected being secreted only at neutral pH included carbamoylated Nod factors, a subset of which are *N*-methylated. This is the first report, to our knowledge, of the carbamoyl group in *R. tropici* Nod factors. The group appears only in molecules bearing C_{10} hydroxylated fatty acyl chains, suggesting that the *nodU* gene requires the presence of a $C_{10:0\text{-OH}}$ fatty acid. It has been demonstrated that the gene responsible for transferring the carbamoyl group to the Nod factor is *nodU*, which is present in the pSym of *R. tropici* CIAT899 [30]. The results obtained in this work appear to demonstrate that this gene is functional in this strain. We have also detected, only in the cultures grown at pH 7.0, nodulation factor structures with a backbone comprising four GlcNAc residues and a mannose at the reducing terminal residue. These mannose-containing Nod factors also bear *N*-methyl or carbamoyl groups as substituents on the nonreducing terminal residue.

At the same time, the rhizosphere is a soil zone in which the soil microflora are influenced by the plant root. Changes in rhizosphere pH can occur in response to adverse nutritional conditions. In the extreme environment of tropical areas, native legumes can be nodulated, and the nodulating rhizobia can fix nitrogen at rates comparable to those observed in neutral soil, indicating that both plants and their rhizobia have successfully adapted to acid soil. Plant root exudates have a profound qualitative and quantitative effect on rhizosphere microflora [31]. More detailed studies on common bean exudates produced under acid conditions, and their effect on *nod* gene expression and the production of signal molecules, will be the subject of future research.

We can conclude that *Rhizobium* populations in nodules may be largely determined by the environmental conditions or agricultural practices. The soil pH seems to select the species type that is successful for bean nodulation [32], so that knowing how it affects the strains used as inoculants in the growth medium could help in selecting the most appropriate strains.

The studies described in this paper indicate that the defense responses of microbes to acid stress are complex interconnecting networks of regulators and metabolic processes designed to cope with the life-threatening consequences of an acidifying internal pH. Our future work will focus on the characterization of the symbiotically relevant genes involved in oligosaccharide modifications, the elucidation of their biochemical functions, and the reconstruction of their evolutionary history.

Significance

The bean is the most important legume in human nutrition, and is a dietary constituent for close to 500 million people in Latin America and eastern and southern Africa. That these regions are characterized by their highly acid soils stimulates interest in studying the symbiosis in acid conditions. Rhizobial nodulation factors are signals essential to the symbiotic interaction, initiating developmental responses in root hairs of the host legume required for controlled entry of the microsymbiont. In this study, we have analyzed the effects of acid stress on nodulation factor production in *R. tropici* CIAT899, a bean symbiont with high intrinsic tolerance to acidity. We demonstrate greater nodulation gene activity in this strain under acid conditions, affecting both the levels and the structures of the Nod factors. The results enable us to conclude that acid pH, besides increasing the biosynthesis of nodulation factors, alters their structure, with changes at the reducing and nonreducing termini of the molecule, the fatty acyl chain, and the degree of polymerization. Given that the activity of these molecules is determined by substituents of the basic common Nod factor structure, such changes could minimize the adverse effects of acidity on the plant responses, possibly also contributing to an increased stability against degradation. The results of *P. vulgaris* nodulation under acid conditions support

this hypothesis, with no significant differences being observed in either the number of nodules or aerial part dry weight at pH 4.5 and 7.0. However, both parameters were significantly higher at moderate acidity (pH 5.5), where the negative effects of acid stress on the plant's physiological state were not so marked. This indicates that *R. tropici* CIAT899 is, in fact, a strain adapted to acid soil conditions, and is thus the ideal inoculant for *P. vulgaris* in soils of very poor agronomic value.

Experimental Procedures

Bacterial Strains, Media, and Growth Conditions

Rhizobium strains used in this study are *R. etli* CFN42 (wild-type [33]), *R. gallicum* R602 (wild-type [34]), *R. giardinii* H152 (wild-type [34]), *R. leguminosarum* bv. phaseoli TAL1121 (wild-type, received from C.I.F.A. Las Torres-Tomejil, Alcalá del Río, Sevilla, Spain), *R. tropici* CFN299 (wild-type [32]), and *R. tropici* CIAT899 (wild-type [35]). The *Escherichia coli* strain used in genetic studies was S17-λ*pir* [36].

Rhizobium strains were grown at 28°C in TY [37] or in minimal B^- medium [38]. *E. coli* cultures were grown in Luria broth [39] at 37°C. Antibiotics were added to the following final concentration (in μg · ml^{-1}): gentamicin (Gm), 25; rifampicin (Rif), 50; and tetracycline (Tc), 15 (*E. coli*) or 5 (*Rhizobium*). Flavonoids were diluted in ethanol and used at a final concentration of 1 μM.

Media were buffered with 25 mM HOMOPIPES (pH 4.0–5.0), 40 mM MES (pH 5.5–6.7), 30 mM HEPES (pH 6.8–8.2), and 30 mM AMPD (pH 7.8–9.7) as biological buffers, or with citrate-phosphate buffer (pH 4.0–7.0) or tris-hydrochloride buffer (pH 7.2–9.0). The pHs were checked after autoclaving and did not change by more than ±0.1 unit.

The strains were grown for 1–6 days on a rotary shaker (180 rpm), checking the final medium pH. Bacterial growth was followed by measuring turbidity at 600 nm after 24, 48, 72, 96, 120, and 144 hr. The experiments were repeated at least twice, with three replicates each time, in order to determinate the reproducibility of the results.

Genetic Techniques

In order to mobilize pRP30 plasmid [40], we used *E. coli* S17-λ*pir* [36] as the donor strain. This plasmid carries the *R. leguminosarum* bv. phaseoli *nodA*::*lacZ* translational fusion. Selection was on TY medium with Tc and Rif.

Determination of β-Galactosidase Activity

Assays of β-galactosidase from *R. tropici* RSP3051 (*R. tropici* CIAT899 derivative strain carrying *nodP*::*lacZ* transcriptional fusion [17]) and *R. tropici* CIAT899 with the plasmid pRP30 (*nodA*::*lacZ* transcriptional fusion) were carried out as described by Zaat et al. [41]. β-Galactosidase activities [39] were measured at least three times, each being conducted in duplicate samples. Standard deviations are shown in Figure 2.

TLC Analysis of Nod Factors

Nodulation factors were labeled in vivo and analyzed by TLC using the procedure described by Spaink et al. [38]. The Nod factors were radioactively labeled by the addition of 0.2 μCi of ^{14}C-glucosamine hydrochloride (specific activity, 56 mCi · $mmol^{-1}$, from Amersham Pharmacia Biotech., Buckinghamshire, England). The TLC plates used were RP-$18F_{254S}$ from Merck (Darmstadt, Germany).

In order to study the production of Nod factors at extreme pH, we used minimal B^- medium with the appropriate buffers where indicated.

Nod Factor Purification

Nod factors from strain CIAT899 derivatives (in neutral and extreme pH conditions) were obtained from a 10-L culture (minimal B^- medium). The pH was checked after bacterial growth, and did not change by more than ±0.3 pH units. Apigenin-induced culture medium was extracted, purified, and fractionated as described by Soria-Díaz et al. [18].

Monosaccharide Composition and Methylation Analyses

Glycosyl composition analysis was carried out after methanolysis using anhydrous methanolic 0.625 M HCl (16 hr, 80°C). The samples were re-*N*-acetylated using Ac_2O-pyridine (1:1, v/v), then trimethylsilylated with pyridine-BSTFA (1:1, v/v) and analyzed by GLC-MS [42]. Absolute configuration was assigned following GLC-MS analysis of its trimethylsilylated 2-butyl glycosides, prepared by using (+)-2-butanol and (±)-2-butanol as above [43]. The Nod factors were permethylated by the method of Ciucanu and Kerek [44], and the samples were hydrolyzed, reduced, and acetylated by the method of Blakeney [45]. The permethylated alditol acetates were analyzed by GLC-MS.

GLC-MS of trimethylsilylated methyl glycosides, trimethylsilylated butyl glycosides, and partially methylated alditol acetates was performed as described by Soria-Díaz et al. [18].

Fatty Acid Analysis

Fatty acid methyl esters were prepared by methanolysis in methanolic 1 M HCl at 85°C for 4 hr and were identified by GLC-MS. The location of the double bond in the unsaturated fatty acids was determined following preparation of the dimethyl disulfide ethers [46] of the methyl ester, followed by GLC-MS. The location of the OH group in the hydroxylated fatty acid was determined on preparation of the TMS derivative of its methyl ester, followed by GLC-MS. The chromatographic conditions are those described for the trimethylsilylated methyl glycosides.

MS

Positive-ion mode FABMS was performed on a Kratos MS80-RFA instrument with a xenon gun at an acceleration voltage of 4 kV. Either glycerol-*m*-nitrobenzyl alcohol (1:1), or thioglycerol containing NaI as the cationizing agent, were used as matrices. The samples were dissolved in 0.2 ml DMSO.

Positive mode ES-Q-o-Tof MS and CID tandem MS were performed using a Micromass (Altrincham, UK) Q-Tof with a Z-spray interface and a continuous infusion nanoflow electrospray source. Sample dissolved in acetonitrile:water (50:50 v/v) containing 0.1% formic acid was infused at 1 μl·min^{-1}. The capillary voltage was 3500 V, the cone voltage was 47 V, and the source temperature was 80°C. TOF spectra were integrated every 2.4 s over the *m/z* 50–2000 range. Data were recorded and processed using MassLynx 3.4. CID-MS/MS were recorded over the *m/z* range 50–1500, using argon as collision gas; the collision energy setting was 4–80 V.

Positive mode MALDI-ToF spectra were acquired on a Perseptive "Voyager" Elite DE Biospectrometry Workstation (Framingham, MA) over the *m/z* range 500–2000, using reflectron mode, an acceleration voltage of 20 kV, and a grid voltage setting of 76%. The matrix was saturated α-cyano-4 hydroxycinnamic acid (CHCA) in acetonitrile:water (1:1 v/v, 0.1% trifluoroacetic acid) that was mixed 1:1 v/v with the sample in acetonitrile:water (50:50 v/v, 0.1% formic acid); 1 μl of this solution was placed on a stainless-steel target and allowed to air dry. Sample spots were irradiated at 337 nm with 3 ns pulses. Fifty laser shots per spectrum were acquired, with an average of 20–30 spectra accumulated.

Positive mode MALDI Q-o-Tof mass spectra were acquired with an Applied Biosystems QSTAR (Warrington, UK) using CHCA, as above. The sample spots were irradiated at 337 nm with a pulse rate of 12–20 Hz, a power level of 20–40 μJ, and a declustering potential of 65 V. ToF spectra were integrated every second over *m/z* 50–2000. Tandem spectra were acquired over *m/z* 50–1500, depending on the precursor ion, using a collision energy setting of 4–80 V, and nitrogen as collision gas. Data were recorded and processed using Analyst software.

Mild Base Treatment

In order to demonstrate the presence of acetyl ester substituents on some Nod factor structures, an aliquot of the fraction containing the proposed structures was de-*O*-acetylated using NH_4OH:MeOH (1:1, v/v) for 12 hr at room temperature.

Seed Sterilization

Bean seeds were surface-sterilized by soaking in 95% ethyl alcohol for 1 min and then in 10% sodium hypochlorite for 8 min. Finally,

they were washed six times with sterile distilled water. Seeds were transferred to Petri dishes containing 1% (v/w) water-agar, and incubated at 28°C for 48 hr.

pH Levels of the Rooting Media

The pH level of the nutrient solution (half-strength that of Rigaud and Puppo [47]) was adjusted with 5 mM of the following biological buffers: HOMOPIPES for pH 4.5; MES for pH 5.5; and HEPES for pH 7.0. The pH was adjusted with 6 N KOH.

Plant Tests under Controlled Conditions

The plant test was carried out in plastic pots (13 × 11 cm) filled with silica sand and watered with the corresponding buffered nutrient solution. Surface-sterilized and pregerminated seeds were sown (two per pot) and inoculated with 1 ml/seed of *R. tropici* CIAT899 culture (1.5×10^8 cfu $\cdot ml^{-1}$). Six replicates per pH level were set up. Plants were grown in a controlled growth chamber (28°C day/20°C night, with 80% relative humidity). Plants (thinned to one/pot) were watered with 100 ml of the corresponding buffered nutrient solution every 2 days to maintain the pH of the rooting media. Four weeks after sowing, plants were removed and shoot dry weight and number and biomass of nodules were determined.

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