RESEARCH LETTER

Different and new Nod factors produced by *Rhizobium tropici* CIAT899 following Na^+ stress

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Abstract

The root nodule bacterium *Rhizobium tropici* strain CIAT899 is highly stress resistant. It grows under acid conditions, in large amounts of salt, and at high osmotic pressure. An earlier study reported a substantial qualitative and quantitative effect of acid stress on the biosynthesis of Nod factors. The aim of the present work was to investigate the effect of high salt (NaCl) concentrations, another common stress factor, on Nod factor production. For this purpose, thin-layer chromatography, HPLC and MS analyses were carried out. The expression of nodulation genes was also studied using a *nodP*::*lacZ* fusion. High concentrations of sodium enhanced *nod* gene expression and Nod factor biosynthesis. The effect is sodium specific because high potassium or chloride concentrations did not have this effect. Under salt stress conditions, 46 different Nod factors were identified in a CIAT899 culture, compared with 29 different Nod factors under control conditions. Only 15 Nod factor structures were common to both conditions. Under salt stress conditions, 14 different new Nod factor structures were identified that were not observed as being produced under neutral or acid conditions. The implications of our results are that stress has a great influence on Nod factor biosynthesis and that new, very interesting regulatory mechanisms, worth investigating, are involved in controlling Nod factor biosynthesis.

Introduction

Nod(ulation) factors from rhizobium bacteria are signals known to be used in the communication with leguminous plants in the process of root nodule formation. Nod factors are major host-specificity determinants (factors that determine which rhizobium nodulates which plant) and trigger the nodulation program in a compatible host (Mergaert *et al.*, 1997). They share a 'backbone' structure usually consisting of two to six β1–4-linked *N*-acetylglucosamine residues, with a fatty acid amide linked to the nonreducing terminal residue. Nod factors from different rhizobium species differ in the number of *N*-acetylglucosamine residues, the length and saturation of the acyl chain, and the nature of modifications on the basic backbone (e.g. sulfate, acetate, fucose, etc.) (Lerouge *et al.*, 1990; Spaink *et al.*, 1991; D'Haeze & Holsters, 2002). These differences define much of the host specificity observed in this symbiosis.

A peculiar bacterium in this is *Rhizobium tropici* CIAT899, which forms an enormous number of different Nod factor structures that varies with the growth conditions. Under acid conditions, 52 Nod factor structures are formed, 37 of which differ from the 29 formed under neutral conditions (Morón *et al.*, 2005). Strain CIAT899 is highly stress resistant, as it can, in addition to growing well under acidic conditions and at a high temperature (Graham *et al.*,

1994; Riccillo *et al.*, 2000; Vinuesa *et al.*, 2003; Muglia *et al.*, 2007), grow very well in media with high concentrations of salt (Nogales *et al.*, 2002). *Rhizobium tropici* CIAT899 is not the only rhizobium that can survive in the presence of extremely high levels of salt, because many rhizobia can do so, showing a salt tolerance ranging from 100 to 600 mM (Bernard *et al.*, 1986).

In order to gain more knowledge about Nod factor production under stress conditions, we have now studied the effect of NaCl stress on Nod factor production by *R. tropici* CIAT899.

The results with *R. tropici* CIAT899 show that, in a 'high-salt' medium, the expression of *nod* genes and incorporation of ^{14}C-labelled glucosamine into Nod factors is higher, and that the extract of the Nod factors from cultures grown under these conditions contains a higher number of Nod factor structures than that of cultures grown under control conditions. Some of the Nod factors identified have not been observed previously to be produced by *R. tropici*. The effect was Na^+-ion specific, and the Nod factor extract of a culture grown under salt stress was biologically active on *Phaseolus vulgaris* cv. Negro Jamapa grown in the absence of NaCl.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this work were *R. tropici* CIAT899 (Martínez-Romero *et al.*, 1985) and RSP3051, which is CIAT899 with a reporter fusion *nodP*::*lacZ* (Manyani *et al.*, 2001). They were grown in minimal B^- medium (van Brussel *et al.*, 1977) or TY medium (Beringer, 1974) at 28 °C for 1–5 days on a rotary shaker (180 r.p.m.). Antibiotics were added to final concentrations (in $\mu g\,mL^{-1}$) of gentamicin (Gm) 25 for RSP3051, and rifampicin (Rif) 50.

Determination of β-galactosidase activity

Assays of *R. tropici* RSP3051 β-galactosidase activity were carried out as described by Zaat *et al.* (1987). Gentamicin was used at $25\,\mu g\,mL^{-1}$, and 1 μM of apigenin was used in positive assays. Units of β-galactosidase activity were calculated according to Miller (1972), from triplicate samples. The experiments were repeated at least three times, with six replicates each time.

Reversed-phase (RP) thin-layer chromatography (TLC) analysis of Nod factors

RP TLC analyses were carried out according to Spaink *et al.* (1992). Cultures, supplemented with 1 μM apigenin in positive assays, were grown in B^- medium to the end of the exponential phase under both control and te st growth conditions. When Na_2SO_4 was used as a sodium source, it was used at a final concentration of 150 mM (300 mM Na^+). For the radiolabelling of Nod factors, 1 μCi of ^{14}C glucosamine hydrochloride (labelled at C1, specific activity $52\,mCi\,mmol^{-1}$, Amersham®), yielding a final concentration of 4 μM glucosamine hydrochloride in the medium, was used. TLC plates were exposed to a Kodak® X-Omat R film for 15 days and the film was developed with Kodak® reagents according to the manufacturer's instructions.

Chemical analysis of Nod factors

Extraction from the culture medium with butanol, purification, HPLC, and MS analysis of Nod factors from apigenin-induced culture medium (10 L) were performed as described in Soria-Díaz *et al.* (2003). Carbohydrate composition and fatty acid analysis were performed as described in Morón *et al.* (2005).

Biological tests

Biological tests were performed according to López-Lara *et al.* (1995). Seeds of common bean (*P. vulgaris* L. cv. Negro Jamapa) were surface-sterilized as described in Albareda *et al.* (2006). Seedlings with root lengths of 1.5–2.5 cm were mounted in test tubes on a curled wire with the roots in 25 mL of Fåhraeus medium (Fårhaeus, 1957). The roots were shielded from light, and plants were grown at 20 °C for 10 days. Acetonitrile fractions from solid-phase extraction (SPE) were evaporated to dryness and redissolved in dimethyl sulfoxide (DMSO). For all plants, 25 μL Nod factors (final concentration of *c.* 10^{-6} M, dissolved in DMSO) were added when plants were placed in test tubes. Negative controls were treated with 25 μL of DMSO (which did not inhibit plant growth), and positive controls (*Rhizobium*-inoculated plants) were treated with a suspension of 20 μL of *R. tropici* CIAT899 $10^8\,CFU\,mL^{-1}$. The Nod factor samples used were the fractions eluted from a Baker® SPE column with 20%, 45%, and 60% AcN, dried, and dissolved in DMSO. Each experiment was repeated at least three times with three plants for each treatment. Roots were cleared with sodium hypochlorite and stained with methylene blue using the method of Truchet *et al.* (1989).

Results

Effect of salt on the production of Nod factors by *R. tropici* CIAT899

In order to study the effect of salt stress on the *Rhizobium*–legume symbiosis, the variability in the biosynthesis (nature and amount) of Nod factors was investigated with *R. tropici* strain CIAT899. Thus, RP TLC analysis with radioactive detection was carried out to study the effect of salt stress on

the profile of Nod factor structures incorporating the label in extracts of the apigenin-induced bacterial cultures (Fig. 1a). Incorporation of ^{14}C-labelled glucosamine (GlcN) into Nod factors produced by *R. tropici* CIAT899 grown under conditions of increasing salt stress increased with the salt concentration up to 300 mM NaCl. At concentrations > 300 mM NaCl, bacterial growth was strongly inhibited. As a control (Fig. 2, lane 1), CIAT899 cultures induced with apigenin, grown in the absence of salt, were incubated for 15 min with 300 mM salt and Nod factors were extracted with butanol. The TLC analysis showed that the presence of salt in the extraction procedure did not change the Nod factor profile compared with that of the same culture extracted in the absence of salt (Fig. 2, lane 2). Thus, the observed changes in the Nod factor profile in the presence of salt are not related to a better extraction in the presence of salt but to changes in the kind of Nod factors produced.

To investigate whether the increased incorporation of radiolabel into Nod factors with salt was caused by a higher expression of the *nod* genes, we also performed β-galactosidase activity assays with apigenin (1 μM)-induced cells of *R. tropici* RSP3051 (*R. tropici* CIAT899 *nodP*::*lacZ*, which produces β-galactosidase when the *nod* genes are induced). The results of this analysis showed that salt stress induced

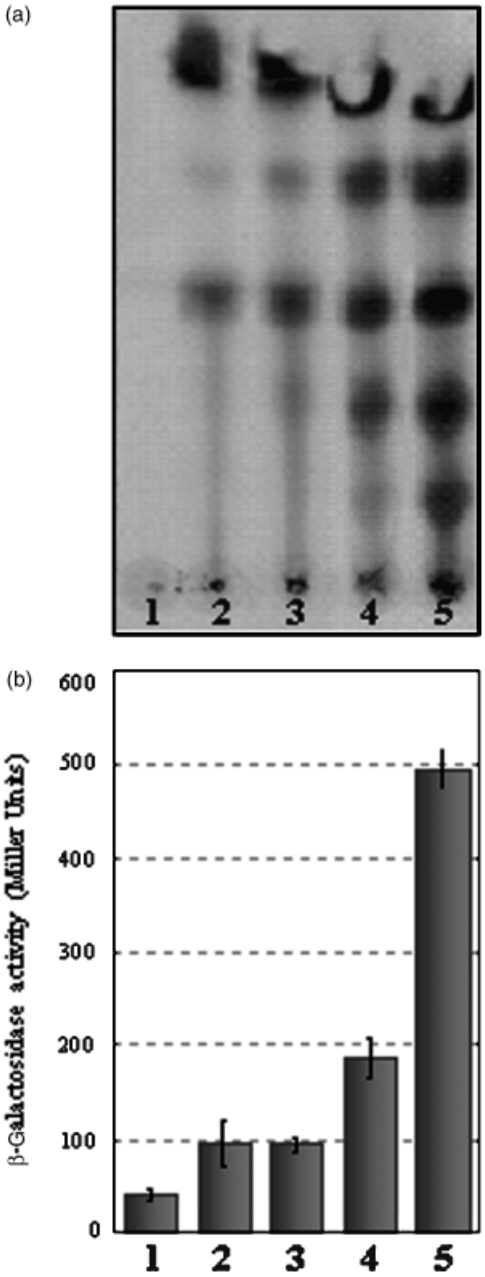

Fig. 1. Effect of salt stress on Nod factor biosynthesis and nodulation gene expression of *Rhizobium tropici* CIAT899. (a) RP TLC analysis of CIAT899 Nod factors labelled with ^{14}C glucosamine hydrochloride. (b) β-Galactosidase activity of *R. tropici* RSP3051 (*R. tropici* CIAT899 *nodP*::*lacZ*). Assay conditions were B$^-$ medium (lanes 1 and 2), supplemented with 100 mM NaCl (lane 3), 200 mM NaCl (lane 4), or 300 mM NaCl (lane 5). Apigenin 1 μM was used as an inducer in lanes 2, 3, 4, and 5.

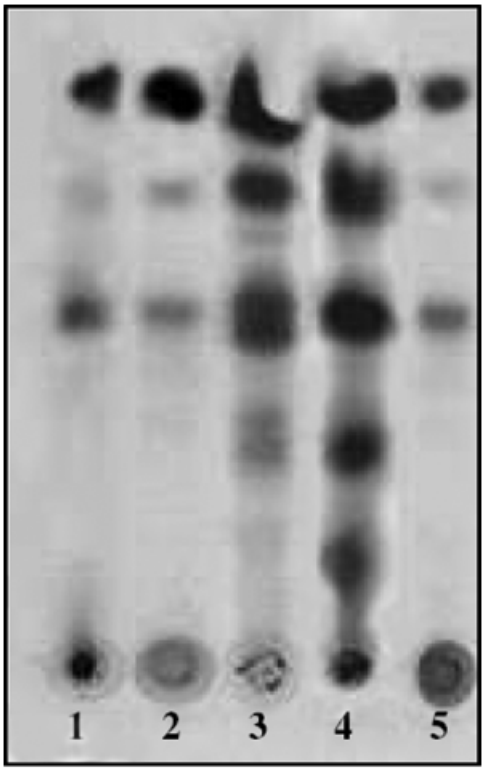

Fig. 2. RP TLC analysis of *Rhizobium tropici* CIAT899 Nod factors produced under different salt stress conditions: lane 1, CIAT899 grown in 0 mM salt and mixed for 15 min with 300 mM NaCl B$^-$ medium before butanol extraction; lane 2, CIAT899 grown at 0 mM salt; lane 3, 150 mM Na_2SO_4 (300 mM Na^+); lane 4, 300 mM NaCl; and lane 5, 300 mM KCl. Apigenin 1 μM was added at the start of the culture, which was grown to the end of the exponential phase in all experiments.

RSP3051 *nod* gene expression, because the higher the NaCl concentration in the medium the more the β-galactosidase activity registered (Fig. 1b).

Thus, in the same way as under acidic conditions and in agreement with an increased *nod* gene expression, the radiolabelled Nod factor profile observable on thin-layer plates changed, quantitatively and qualitatively, when salt was included in the growth medium.

NaCl ion specificity

We wanted to determine whether the changes observed in CIAT899 Nod factor production were specific to the Na^+ or to the Cl^- ion (or both). Therefore, the effects of other ionic solutes adjusted to a concentration equivalent to that of NaCl were also investigated. Figure 2 illustrates the results of RP TLC analysis of radiolabelled CIAT899 Nod factors obtained in the presence of NaCl, Na_2SO_4, and KCl. Incubations in Na_2SO_4 and NaCl (300 mM Na^+, lanes 3 and 4), although not exactly the same, resulted in an increased incorporation of radiolabel into Nod factors, regardless of the counter-ion. In contrast, the effect of KCl (300 mM Cl^-, lane 5) was found to be comparable to that of control cells grown without added salt (lane 2). Thus, the Na^+ cation is the major factor enhancing the *nod* gene expression and Nod factor biosynthesis in CIAT899.

Production, purification, and MS analysis of Nod factors

In order to determine the structures of the Nod factors produced under control and salt stress conditions, *R. tropici* CIAT899 was grown in the presence of apigenin (as inducer) either without salt or in the presence of 300 mM NaCl. The culture medium was extracted, purified, and fractionated as described in Soria-Díaz *et al.* (2003). The HPLC profile of the Nod factors produced in 300 mM NaCl showed a marked increase in the total peak areas, as well as differences in the peak retention times, compared with that of the culture without NaCl (data not shown).

The fractions obtained on HPLC separation of the Nod-factor-containing butanolic extract of *R. tropici* CIAT899, grown in 300 mM NaCl-containing medium and induced with apigenin, were analyzed by MS. We identified 46 Nod factors formed under saline conditions, whose core structure, with proposed positions of the modifications identified, is shown in Fig. 3. We also confirmed the data of Morón *et al.* (2005) for neutral conditions (nonsaline medium at pH 7.0, with apigenin induction), where 29 Nod factors were identified, 15 of which were now also produced in 300 mM NaCl. Fourteen Nod factors produced in 300 mM NaCl have not been described previously for *R. tropici* CIAT899 grown under any of the different conditions examined: V($C_{20:0}$, NMe, S), V($C_{22:1}$, S), V($C_{20:1}$, NMe, S),

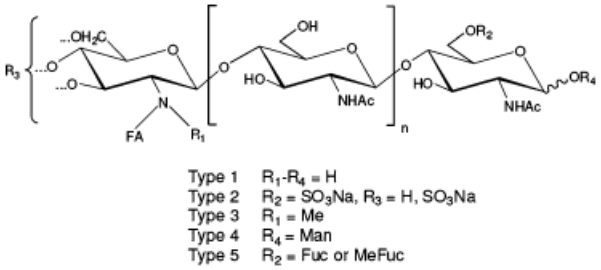

Fig. 3. Core structure of *Rhizobium tropici* CIAT899 Nod factors produced in 300 mM NaCl, and proposed positions of the identified modifications.

V($C_{18:0\text{-}OH}$, NMe, S), V($C_{20:0\text{-}OH}$), V($C_{20:1}$, NMe), V($C_{18:0\text{-}OH}$), V($C_{16:1}$, NMe, S, S), V($C_{18:1}$, MeFuc), IV($C_{20:0\text{-}OH}$, NMe, S), IV-Man($C_{18:1}$), IV($C_{18:1}$, MeFuc), IV($C_{18:1}$, Fuc), and IV($C_{18:0}$) (Folch-Mallol *et al.*, 1996; Morón *et al.*, 2005). The Nod factor structures are proposed on the basis of monosaccharide composition analysis of HPLC fractions carried out using GC-MS, GC-MS fatty acid analysis, and the results of FAB-MS, MALDI Q-o-TOF MS, and MALDI CID tandem MS analysis.

The Nod factors identified are tetramers and pentamers, having the typical linear backbone of GlcNAc residues bearing different substituents on the reducing and/or the nonreducing terminal residues, and *N*-acyl chains on the nonreducing GlcN residue. We have identified five types of Nod factor structures. All of the Nod factor structures observed are presented, together with a summary of the MS results, in Table 1.

The first structural type corresponds to species with an unsubstituted glucosamine backbone, which bear only an amide-linked fatty acyl residue. The CID spectrum contains B_i and Y_i ion series (Domon & Costello, 1988), of which the B_1 ion allows the identification of the fatty acyl group, ranging from 12 to 22 carbon units.

Different saturated (C_{12}–C_{20}), monounsaturated (C_{16}–C_{22}), and hydroxylated ($C_{18:0\text{-}OH}$ and $C_{20:0\text{-}OH}$) fatty acids were identified on the basis of their GC-MS retention times and mass spectra. The unsaturated fatty acid $C_{20:1}$ was identified as icosenoic acid. The mass spectrum of the dimethyl disulfide derivative of the unsaturated fatty acid methyl ester contained signals at *m/z* 418 (molecular ion), *m/z* 145 [$CH_3(CH_2)_5CH=S^+CH_3$], and *m/z* 273 [$CH_3S^+=CH(CH_2)_{11}COOMe$], arising from fragmentation between the carbons bearing the dimethyl disulfide groups, demonstrating that the double bond was located at carbon 13. The hydroxyl fatty acid $C_{18:0\text{-}OH}$ was identified as an O-1 hydroxy fatty acid (17-hydroxyoctadecanoic acid). The mass spectrum of the trimethylsilyl (TMS) derivative of the methyl ester contained characteristic signals at *m/z* 117 (CH_3CH^+OTMS) and 371 ([M-CH_3]$^+$).

Table 1. Nod factor structures produced by *Rhizobium tropici* CIAT899 grown in 0 mM NaCl, 300 mM NaCl, and at pH 4.5

Structure*	NF type†	$[M+Na]^+$ *m/z*	*R. tropici* CIAT899 300 mM NaCl	*R. tropici* CIAT899 0 mM NaCl/pH = 7.0	*R. tropici* CIAT899 pH = 4.5‡
V($C_{18:1}$, NMe, S, S)		1496	–	–	B_i ions: 1173, 970, -, 564 Y_i ions: -, 549, 752, 955
V($C_{16:1}$, NMe, S, S)	2, 3	1468	B_i ions: 1145, 942, 739, 536 Y_i ions: 346, 549, 752, 955	–	–
V($C_{20:0\text{-}OH}$, NMe, S)	2, 3	1440	B_i ions: 1117, 914, 711, 508 Y_i ions: 346, 549, 752, 955	–	B_i ions: 1117, 914, 711, 508
V($C_{18:1}$, MeFuc)	5	1438	B_i ions: 1057, 854, 651, 448 Y_i ions: 404, 607, 810, 1013	–	–
V($C_{22:1}$, S)/V($C_{18:1}$, NMe, Ac, S)	2 (3)	1436	B_i ions: 1113, 910, 707, 504 Y_i ions: 549, 752, 955	–	–
V($C_{20:0}$, NMe, S)	2, 3	1424	B_i ions: 1101, 898, 695, 492 Y_i ions: 346, 549, 752, 955	–	–
V($C_{20:1}$, NMe, S)	2, 3	1422	B_i ions: 1099, 869, -, 490 Y_i ions: 346, 549, 752, 955	–	–
V($C_{18:0\text{-}OH}$, NMe, S)	2, 3	1412	B_i ions: 1394, 1089, 886, 683, 480 Y_i ions: 346, 549, 752, 955	–	–
V($C_{20:0}$, S)	2	1410	B_i ions: 1087, 884, 681, - Y_i ions: -, 549, 752, 955	+	B_i ions: 1087, 884, 681, - Y_i ions: -, 549, 752, 955
V($C_{20:1}$, S)	2	1408	B_i ions: 1085, 882, 679, 476 Y_i ions: 346, 549, 752, 955	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($C_{18:0}$, NMe, S)	2, 3	1396	B_i ions: 1073, 870, 667, 464 Y_i ions: 346, 549, 752, 955	+	B_i ions: 1073, 870, 667, 464 Y_i ions: 346, 549, 752, 955
V($C_{18:1}$, NMe, S)	2, 3	1394	B_i ions: 1071, 868, 665, 462 Y_i ions: 346, 549, 752, 955	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($C_{18:0}$, S)	2	1382	B_i ions: 1059, 856, 653, - Y_i ions: 346, 549, 752, -	–	B_i ions: 1059, 856, 653, 450
V($C_{18:1}$, S)		1380	–	–	B_i ions: 1057, 854, 651, 448
V($C_{16:0}$, NMe, S)	2, 3	1368	B_i ions: 1045, 842, -, - Y_i ions: 346, 549, 752, 955	–	B_i ions: 1045, 842, 639, 436 Y_i ions: 549, 752, 955
V($C_{16:1}$, NMe, S)		1366	–	+	+
V($C_{22:0\text{-}OH}$, NMe)/ V($C_{20:0\text{-}OH}$, Ac)		1366	–	B_i : 1145, 942, 739, 536	–
V($C_{16:0}$, S)	2	1354	B_i ions: 1031, 828, 625, - Y_i ions: 346, 549, 752, 955	–	B_i ions: 1031, 828, 625, 422
V($C_{14:0}$, NMe, S)		1340	–	–	B_i ions: 1017, 814, 611, 408
V($C_{20:0\text{-}OH}$, NMe)	3	1338	B_i ions: 1117, 914, 711, 508 Y_i ions: 244, 447, 650, 853	–	B_i ions: 1117, 914, 711, 508
V($C_{20:1\text{-}OH}$, NMe)		1336	–	B_i ions: 1115, 912, 709, 506 Y_i ions: -, 447, 650, 853	–
V($C_{22:1}$)/V($C_{18:1}$, NMe, Ac)		1334	–	B_i ions: 1113, 910, 707, 504 Y_i ions: 244, 447, -, 853	–
V($C_{20:0\text{-}OH}$)	1	1324	B_i ions: 1103, 900, 697, 494 Y_i ions: 244, 447, 650, -	–	–
V($C_{20:0}$, NMe)		1322	–	B_i ions: 1101, 898, 695, 492 Y_i ions: -,447, 650, 853	–
V($C_{20:1}$, NMe)	3	1320	B_i ions: 1302, 1099, 896, 693, 490 Y_i ions: -, 447, 650, 853	–	–
V($C_{18:0\text{-}OH}$, NMe)	3	1310	B_i ions: -, 886, 683, 480 Y_i ions: 244, 447, 650, 853	–	B_i ions: 1089, 886, 683, 480 Y_i ions: 244, 447, 650, 853
V($C_{20:0}$)		1308	–	B_i ions: 1087, 884, 681, 478	B_i ions: 1087, 884, 681, 478 Y_i ions: -, 447, 650, 853
V($C_{20:1}$)		1306	–	B_i ions: 1085, 882, 679, 476 Y_i ions: 447, 650, 853	B_i ions: 1085, 882, 679, 476
V($C_{18:0\text{-}OH}$)	1	1296	B_i ions: 1075, 872, 669, 466 Y_i ions: 244, 447, 650, 853	–	–
V($C_{18:0}$, NMe)	3	1294	B_i ions: 1073, 870, 667, 464	+	B_i ions: 1073, 870, 667, 464

Table 1. Continued.

Structure*	NF type†	$[M+Na]^+$ *m/z*	*R. tropici* CIAT899 300 mM NaCl	*R. tropici* CIAT899 0 mM NaCl/pH = 7.0	*R. tropici* CIAT899 pH = 4.5‡
			Y_i ions: 244, -, -, 853		
V($C_{18:1}$, NMe)	3	1292	B_i ions: 1071, 868, 665, 462 Y_i ions: -, 447, -, 853	B_i ions: 1071, 868, 665, 462 Y_i ions: -, 447, 650, 853	B_i ions: 1071, 868, 665, 462
V($C_{16:0\text{-}OH}$, NMe)		1282	–	–	B_i ions: 1061, 858, 655, 452
V($C_{18:0}$)/V($C_{16:1\text{-}OH}$, NMe)	1 (3)	1280	+	–	B_i ions: 1059, 856, 653, 450
V($C_{18:1}$)	1	1278	B_i ions: 1057, 854, 651, 448 Y_i ions: 244, 447, 650, -	–	B_i ions: 1057, 854, 651, 448
V($C_{16:0}$, NMe)	3	1266	B_i ions: -, 842, -, 436 Y_i ions: 244, 447, 650, 853	B_i ions: 1045, 842, 639, 436 Y_i ions: -, 447, 650, 853	B_i ions: 1045, 842, 639, 436
V($C_{16:1}$, NMe)	3	1264	B_i ions: 1043, 840, -, - Y_i ions: -, 447, 650, 853	–	B_i ions: 1043, 840, 637, 434 Y_i ions: 244, 447, 650, 853
V($C_{14:0\text{-}OH}$, NMe)		1254	–	–	B_i ions: 1033, 830, 627, 424
V($C_{16:0}$)	1	1252	B_i ions: 1031, -, -, 422 Y_i ions: 244, 447, -, 853	–	B_i ions: 1031, 828, 625, 422
IV-Hex($C_{18:1}$, NMe)		1251	–	B_i ions: 1071, 868, 665, 462	–
V($C_{10:0\text{-}OH}$, NMe, Cb)		1241	–	B_i ions: 1020, 817, 614, 411	–
V($C_{14:0\text{-}OH}$)		1240	–	–	B_i ions: 1019, 816, 613, 410
V($C_{14:0}$, NMe)	3	1238	B_i ions: -, -, 611, 408 Y_i ions: 244, 447, 650, 853	+	B_i ions: 1017, 814, 611, 408 Y_i ions: -, 447, 650, 853
IV($C_{20:0\text{-}OH}$, NMe, S)	2, 3	1237	B_i ions: 1219, 914, 711, 508 Y_i ions: 346, 549, 752	–	–
IV-Hex($C_{18:1}$)	4	1237	B_i ions: 1057, 854, 651, - Y_i ions: -, 406, 609, 812	–	–
IV($C_{18:1}$, MeFuc)	5	1235	B_i ions: 854, 651, - Y_i ions: 404, 607, -	–	–
V($C_{10:0\text{-}OH}$, Cb)		1227	–	B_i ions: 1006, 803, 600, 397	–
V($C_{14:0}$)	1	1224	B_i ions: 1003, 800, 597, - Y_i ions: 244, 447, 650, -	B_i ions: 1003, 800, 597, 394 Y_i ions: -, 447, 650, 853	–
V($C_{14:1}$)		1222	–	–	B_i ions: 1001, 798, 595, 392
IV($C_{18:1}$, Fuc)	5	1221	B_i ions: 854, 651, - Y_i ions: -, 593, 796	–	–
V($C_{12:0}$, NMe)	3	1210	B_i ions: 989, 786, 583, 380 Y_i ions: -, 447, 650, 853	B_i ions: 989, 786, 583, 380 Y_i ions: -, 447, 650, 853	–
IV($C_{20:0}$, S)		1207	–	–	B_i ions: 884, 681, 478
IV($C_{20:1}$, S)		1205	–	B_i ions: 882, 679, 476	–
IV($C_{18:0}$, NMe, S)	2, 3	1193	B_i ions: 870, 667, 464 Y_i ions: 346, 549, 752, -	+	B_i ions: 870, 667, 464
IV($C_{18:1}$, NMe, S)	2, 3	1191	B_i ions: 1071, 868, 665, 462 Y_i ions: 346, 549, 752, -	–	+
IV-Hex($C_{10:0\text{-}OH}$, Cb)		1186	–	B_i ions: 1006, 803, 600, 397	–
V($C_{10:0}$, NMe)		1182	–	–	B_i ions: 961, 758, 555, 352
IV-Hex($C_{12:0}$, NMe)		1169	–	B_i ions: 989, 786, 583, 380 Y_i ions: 406, 609, 812	–
IV($C_{16:0}$, NMe, S)		1165	–	–	B_i ions: 842, 639, 436
IV($C_{18:0\text{-}OH}$, NMe, Ac)		1149	–	$B_i$ions: 928, 725, 522	–
IV($C_{20:0\text{-}OH}$, NMe)	3	1135	B_i ions: -, -, 508 Y_i ions: 244, 447,650	–	B_i ions: 914, 711, 508
IV($C_{20:0\text{-}OH}$)	1	1121	B_i ions: 900, 697, 494 Y_i ions: 244, 447, 650	–	B_i ions: 900, 697, 494
IV($C_{18:0\text{-}OH}$, NMe)		1107	–	–	B_i ions: 886, 683, 480
IV($C_{20:0}$)		1105	–	–	B_i ions: 884, 681, 478
IV($C_{20:1}$)		1103	–	–	B_i ions: 882, 679, 476
IV($C_{18:0\text{-}OH}$)	1	1093	B_i ions: 872, 669, 466 Y_i ions: 244, 447, 650	–	B_i ions: 872, 669, 466 Y_i ions: 244, 447, 650
IV($C_{18:0}$, NMe)	3	1091	B_i ions: 870, -, 464	–	B_i ions: 870, 667, 464

Table 1. Continued.

Structure*	NF type†	$[M+Na]^+$ *m/z*	*R. tropici* CIAT899 300 mM NaCl	*R. tropici* CIAT899 0 mM NaCl/pH = 7.0	*R. tropici* CIAT899 pH = 4.5‡
			Y_i ions: 244, 447, 650		Y_i ions: 244, 447, 650
IV($C_{18:1}$, NMe)	3	1089	B_i ions: 868, 665, 462 Y_i ions: 244, 447, 650	B_i ions: 868, 665, 462 Y_i ions: 244, 447, 650	B_i ions: 868, 665, 462
IV($C_{18:0}$)	1	1077	B_i ions: 856, 653, 450 Y_i ions: 244, 447, 650,	–	–
IV($C_{18:1}$)	1	1075	B_i ions: 854, 651, 448 Y_i ions: 244, 447, -	+	B_i ions: 854, 651, 448
IV($C_{16:0\text{-}OH}$)		1065	–	–	B_i ions: 844, 641, 438
IV($C_{16:0}$, NMe)	3	1063	B_i ions: 842, 639, 436 Y_i ions: 244, 447, 650	+	–
IV($C_{16:1}$, NMe)		1061	–	–	B_i ions: 840, 637, 434
IV($C_{14:0\text{-}OH}$, NMe)		1051	–	–	B_i ions: 830, 627, 424
IV($C_{16:0}$)	1	1049	B_i ions: -, 625, - Y_i ions: 244, 447, -	+	B_i ions: 828, 625, 422
IV($C_{14:0\text{-}OH}$)		1037	–	–	B_i ions: 816, 613, 410
IV($C_{14:0}$, NMe)	3	1035	+	–	B_i ions: 814, 611, 408 Y_i ions: -, 447, 650
IV($C_{14:1}$, NMe)		1033	–	–	B_i ions: 812, 609, 406 Y_i ions: -, 447, 650
IV($C_{14:0}$)	1	1021	B_i ions: 800, 597, 394 Y_i ions: 244, 447, 650	–	B_i ions: 800, 597, 394 Y_i ions: 244, 447, 650
IV($C_{10:0}$, NMe)		979	–	–	B_i ions: 758, 555, 352
IV($C_{10:0}$)		965	–	–	B_i ions: 744, 541, 338

*Nod factor structures are represented following the convention (Spaink, 1992) that indicates the number of GlcNAc residues in the backbone (roman numeral), the length and degree of unsaturation of the fatty acyl chain, and the other substituents, which are listed in the order they appear moving clockwise from the fatty acid.

†Type of nod factor structure from *Rhizobium tropici* CIAT899 grown in the presence of 300 mM NaCl: type 1 unsubstituted, type 2 sulfated, type 3 *N*-methylated, type 4 mannosylated, type 5 fucosylated or methyl fucosylated.

‡The data for *R. tropici* CIAT899 0 mM NaCl/pH = 7.0 are taken from Morón *et al*. (2005)

Ac, acetyl group; Cb, carbamoyl group; Fuc, fucosyl residue; Hex, hexose; Me, methyl group; MeFuc, methyl fucosyl residue; NMe, *N*-methyl group; S, sulfate group.

The second type of nodulation factor present is the class of sulfated Nod factors bearing one or two sulfate groups (some of these are further substituted, e.g. with an *N*-methyl group, while others do not bear additional substituents). The synthesis of these Nod factor structures is apparently enhanced by salt stress, because the sum of different sulfated Nod factor structures produced in saline medium is more than twice the total number of different sulfated Nod factor structures produced under control conditions (16/7). There are only five sulfated Nod factors common to bacteria grown at 0 and 300 mM [V($C_{18:0}$, NMe, S), V($C_{20:0}$, S), V($C_{18:1}$, NMe, S), V($C_{20:1}$, S), and IV($C_{18:0}$, NMe, S)]. They ionize as the cationized molecule of the sulfate sodium salt $[M\text{-}H+2Na]^+$. The B_i and Y_i ion series show that the sulfate group is located on the reducing-terminal residue. The disulfated species ionizes to generate a sodiated molecule at *m/z* 1468. 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 an $[M\text{-}2H+3Na]^+$ for a pentameric nodulation factor, *N*-acylated with a $C_{16:1}$ group and *N*-methylated on the nonreducing terminal residue. This Nod factor is substituted by two sulfate groups present as their sodium salts located on the glucosamine residues at the nonreducing and the reducing termini of the pentamer. This is the first time that this Nod factor has been described for *R. tropici* CIAT899.

The third type of Nod factor is *N*-methylated on the nonreducing terminal residue (a proportion of these are also sulfated on the reducing-terminal residue), and accounts for more than half (26/46–28/46) of the total different Nod factor structures produced.

The structures were identified from the *m/z* values of their sodiated molecules and from their fragment ions: the loss of 102 *m/z* units defines the presence of a sulfate group, and the *m/z* value of the B_1 ion indicates that the residue is methylated.

The fourth type of Nod factor has a backbone comprising four GlcNAc residues, and mannose as the reducing-

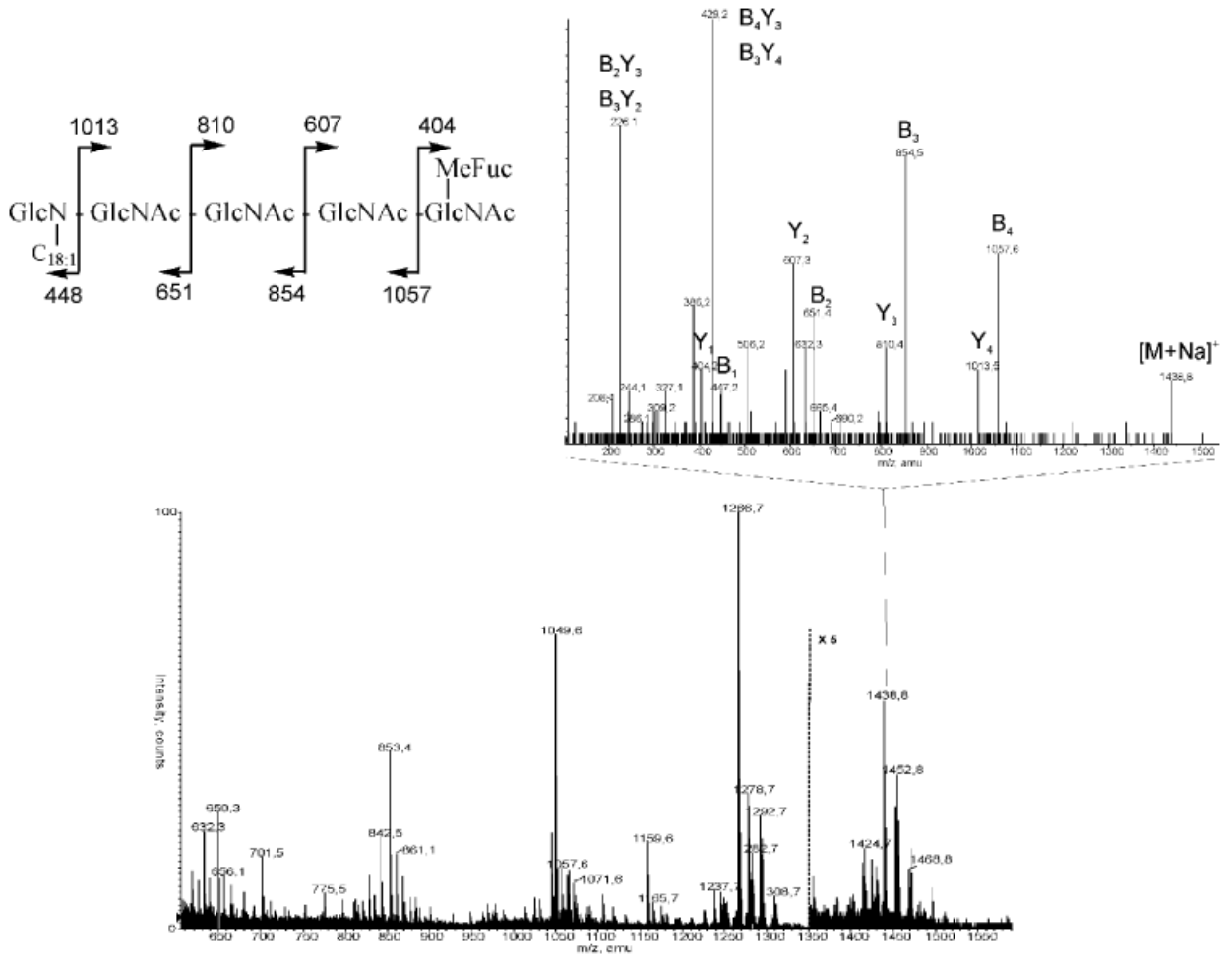

Fig. 4. MALDI mass spectrum of HPLC fraction with $t_R = 55$ min from *Rhizobium tropici* CIAT899 grown in 300 mM NaCl, and CID tandem mass spectrum of the ion at *m/z* 1438, with the fragmentation scheme (inset).

terminal residue. This nodulation factor was previously reported to be present in cultures of *R. tropici* CIAT899 grown at pH 7.0 (Table 1, Morón *et al.*, 2005). These structures are characterized by the *m/z* values of the B_4 and Y_2 ions (*m/z* 1057 and 406, respectively). The presence of mannose was demonstrated by the GC-MS analysis.

Finally, we have identified three nodulation factors containing fucose or methylfucose: IV and V($C_{18:1}$, MeFuc) and IV($C_{18:1}$, Fuc), which ionize to generate sodiated molecules at *m/z* 1438, 1235, and 1221, respectively. The B_i and Y_i ion series, especially the sodiated Y_1 ion at *m/z* 404 for the methylfucosylated Nod factors, and the loss of 146 *m/z* units from the fucosylated Nod factors, show the presence of the deoxyhexose on the reducing glucosamine residue (Fig. 4).

Composition analysis

GC-MS analysis of the TMS methyl glycosides showed that glucosamine was present. For the fraction with $t_R = 55$ min, containing the Nod factors IV-Man ($C_{18:1}$) and V($C_{18:1}$, MeFuc), mannose and MeFuc were also identified. The presence of MeFuc was demonstrated by the mass spectrum of the chromatographic peak, which contains an ion at *m/z* 146, characteristic of 2,3 or 4 methyl hexoses (Fig. 5). Glucosamine belongs to the D series, as demonstrated on GC-MS analysis of the trimethyl silylated (+)-2-butyl and (±)-2-butyl glycosides.

Biological activity of *R. tropici* CIAT899 Nod factors produced at 300 mM NaCl or at pH 4.5

Phaseolus vulgaris does not grow above 25 mM NaCl. Therefore, the biological activity of the Nod factors produced by *R. tropici* CIAT899 under high salt conditions was tested in the absence of salt, which was removed during the Nod factor purification procedure. To investigate the biological activity of the Nod factors produced under saline stress, we tested the 45% and 60% acetonitrile SPE fractions of the

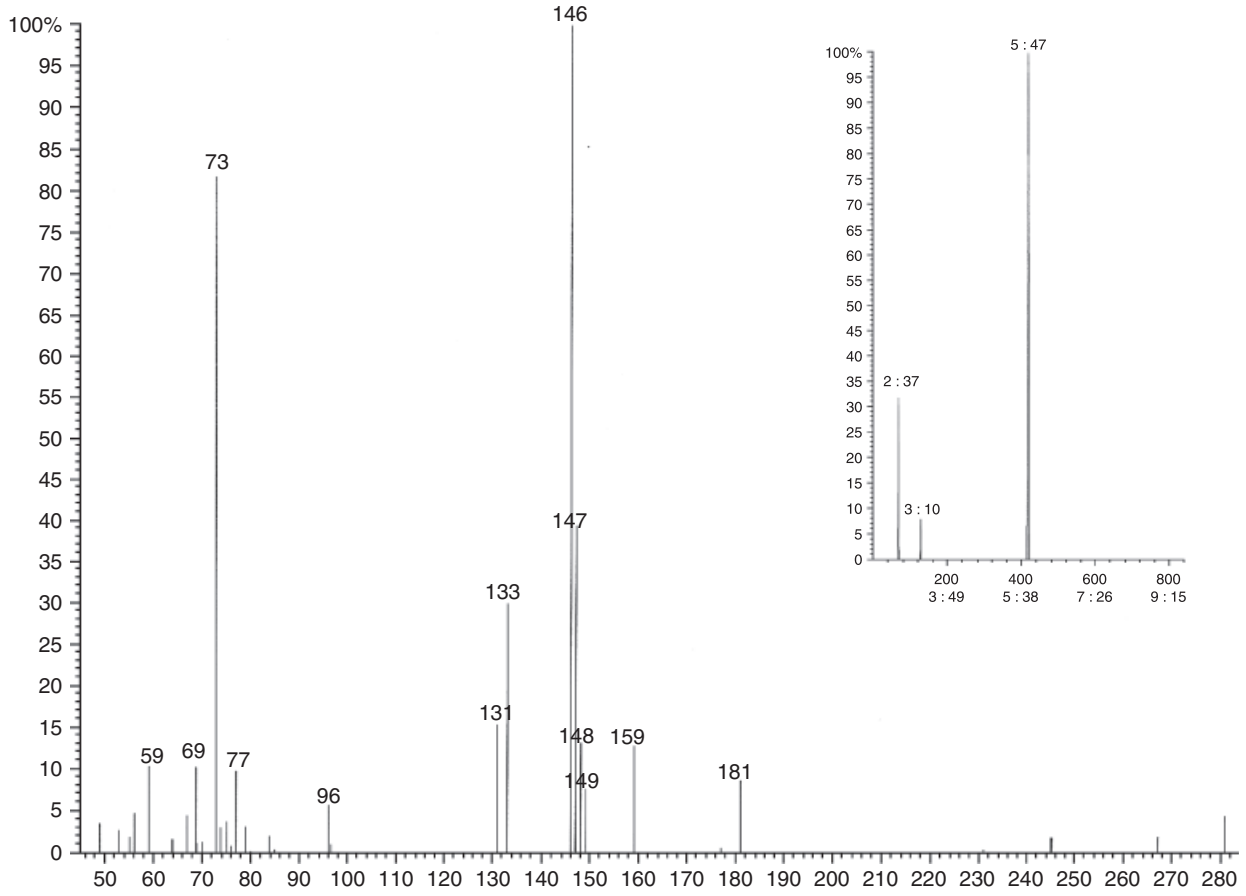

Fig. 5. GC selected ion chromatograms of *m/z* 146 (inset) of HPLC fraction of t_R = 55 min from *Rhizobium tropici* CIAT899 grown in 300 mM NaCl, and EI mass spectrum t_R = 5:47 min GC-MS peak.

crude extracts of Nod factors produced under neutral, acid stress (pH 4.5), or salt stress (300 mM NaCl) conditions. They were similar in biological activity, inducing root nodule-like structures (pseudonodules, Fig. 6c) analogous to those observed on the rhizobium-inoculated plants (positive control, Fig. 6b). The 20% acetonitrile SPE fractions, which, after HPLC separation and MS analysis, did not appear to contain Nod factors, were not active.

Discussion

In this paper, we report new data on the influence of salt (NaCl) stress on the expression of enzymes involved in Nod factor production, and on the huge variety of Nod factors produced by growing *R. tropici* bacteria under such circumstances.

Rhizobium tropici grew in up to 300 mM NaCl. Under such conditions, its Nod factor production changed qualitatively and quantitatively, showing an increase in the incorporation of radiolabel into Nod factors and an increase in *nod* gene expression with increasing NaCl concentration (Fig. 1). Salt added, before butanol extraction, to an apigenin-induced *R. tropici* CIAT899 culture grown without salt, did not change the Nod factor profile on an RP TLC compared with that of the same culture without addition of salt before butanol extraction. Thus, the changes induced by salt in Nod factors are due to changes in Nod factor metabolism and not due to the high concentration of NaCl during the extraction procedure. In our experiments some but not all other bean rhizobia showed similar results (data not shown). We also showed that the effect of NaCl on Nod factor production is mainly a sodium-ion effect (Fig. 2). As the medium with 300 mM KCl has osmotic properties comparable to the same medium, but with 300 mM NaCl, an osmotic effect does not seem to have a major influence on Nod factor production. Subsequent Nod factor MS analysis following HPLC fractionation of an extract of a CIAT899 culture induced with apigenin in the presence of 300 mM NaCl showed an increase in the number of different Nod factor structures produced in comparison with those produced by a control culture without NaCl.

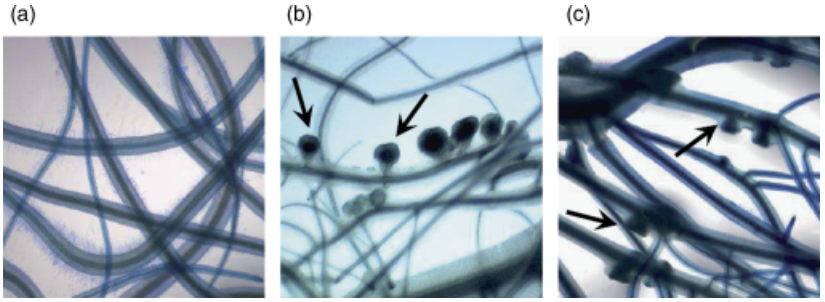

Fig. 6. Nodules (b, arrows) and nodule-like structures (c, arrows) of *Phaseolus vulgaris* cv. Negro Jamapa, treated with DMSO (negative control, a), inoculated with *Rhizobium tropici* CIAT899 strain (positive control, b), and treated with *R. tropici* CIAT899 Nod factors (c) redissolved in DMSO.

The higher transcriptional activity at the *nod* region in the presence of NaCl, as revealed by the increased the β-galactosidase activity of a *nodP*::*lacZ* fusion (Fig. 1b), may lead to a higher and detectable production of Nod factor variants, which, under growth conditions without salt, are too low in concentration to be observed. Furthermore, a number of Nod factor biosynthetic enzymes, for example those encoded by the common *nodDABC* genes have a known relaxed substrate specificity. Thus, it can be conceived that the diversity of Nod factors observed could have been a consequence of a different availability of intracellular biosynthetic precursors due to the effects of the operating abiotic stress. In fact, one can also argue that the absence of Nod factors under one set of growth conditions that are present under the other is a consequence of different substrates for Nod-factor synthesizing enzymes being present or absent under the different conditions.

In the extract of a 300 mM NaCl-containing culture of CIAT899, MS analysis revealed the presence of 46 different Nod factor structures, whereas 29 different Nod factor structures were identified in a culture grown without NaCl (Table 1). Of the structures found in cultures with and without NaCl, 15 were the same. Morón *et al.* (2005) reported that, under acid growth conditions (pH 4.5), 52 different Nod factor structures were found, of which 28 are the same as those identified from the culture grown in 300 mM NaCl. Of these 28 different Nod factor structures, only 11 were also found in cultures grown under neutral conditions. Thus, it is possible that for these 11 Nod factors the same pathways are used to control Nod factor production under control, acid, and salt stress conditions. Taking all the results together, *R. tropici* CIAT899 can produce 80 different Nod factors; such a very broad variety of chemical substituent combinations has never been described before for any *Rhizobium* strain.

Under NaCl stress, the Nod factor structures include modifications of the non-reducing- and reducing-terminal residues, such as the production of new saturated, unsaturated, and hydroxylated fatty acids (ranging from 10 to 22 carbon units), substituents thought to be crucial for bean-nodulating rhizobia (Laeremans & Vanderleyden, 1998). Of the fatty acids characterized, $C_{22:0}$ was synthesized by *R. tropici* CIAT899 under NaCl-stress conditions, as well as under control conditions, but it was not found under acidic ones. More differences related to fatty acid production patterns were found between acidic and salt stress conditions, because shorter-chain fatty acids are predominant under acidic conditions (e.g. C_{16}) while longer ones (e.g. C_{20}) are prevalent under saline conditions, with C_{18} being the most common fatty acid produced under control, acid, and saline conditions. The most common Nod factor structure, produced under all three conditions, is V($C_{18:0}$, NMe, S).

Furthermore, a new doubly sulfated Nod factor produced by cultures grown in 300 mM NaCl was detected. The additional sulfate group is located on the nonreducing terminal residue [V($C_{16:1}$, NMe, S, S)]. This molecule is different from the doubly sulfated species [V($C_{18:1}$, NMe, S, S)] described in the study of Morón *et al.* (2005).

Remarkably, Nod factors were found to be fucosylated/methylfucosylated on the reducing-terminal residue of Nod factors bearing a C_{18} fatty acid [V($C_{18:1}$, MeFuc), IV($C_{18:1}$, MeFuc), and IV($C_{18:1}$, Fuc)]. As no sulfate group has been identified on this class of Nod factors, it is possible that these two substituents compete for the C6 position of the backbone. This is the first time, as far as we know, that a fucosyl group, which is very common in Nod factors from bacteria of the genus *Ensifer* (Lamrabet *et al.*, 1999), has been described as a Nod-factor substituent in an *R. tropici* strain. *pSym* genes related to fucosyl synthesis, transport, or attachment (i.e. *nodZ*) have not been described in *R. tropici* CIAT899, so it is quite possible that the chromosomal copies of these genes are responsible for the incorporation of the fucosyl group (Lamrabet *et al.*, 1999). Another possibility could be that the fucosylated Nod factors are related to *noeJ* gene activity (Nogales *et al.*, 2002), a gene that is also involved in *R. tropici* CIAT899 salt tolerance. In fact, preliminary MS analysis failed to identify fucosyl groups in any of the Nod factors produced by an *R. tropici* CIAT899 *noeJ* mutant (HB5), although its TLC profile shows no changes from that of CIAT899 (data not shown).

The fucosyl moiety is known to be essential for determining a broad host range, for example in various plant species belonging to the family of *Phaseoleae* (López-Lara *et al.*,

1995). Hence, the production of fucosylated Nod factors in 300 mM NaCl by CIAT899 could possibly expand the host range of the stressed rhizobia.

The effect of stress on Nod factor production could be considered as a possible mechanism used by CIAT899 to improve nodulation under such conditions (Morón *et al.*, 2005). It is difficult, however, to explain the biological significance of the production of different Nod factor structures under conditions of a high NaCl concentration, at least for bean nodulation. Most bean plants are inhibited in their growth at 50 mM NaCl (Saadallah *et al.*, 1998), and die at the concentrations of NaCl required for these Nod factors to be produced. A number of other leguminous plants, however, can tolerate quite high salt concentrations in the growth medium (Sibole *et al.*, 2003; Teakle *et al.*, 2007; Zahran *et al.*, 2007), and thus it cannot be excluded that the higher qualitative and quantitative production of Nod factors has a role in the induction of root nodules on such plants under high salt conditions. This still has to be investigated, although the results obtained by Bouhmouch *et al.* (2005) with *Phaseolus* cultivars, and citations in this article of studies on other leguminous plants suggest that in medium concentrations as low as 25 mM NaCl, which are well supported by the two symbionts, nodulation is inhibited or yields ineffective nodules (Bouhmouch *et al.*, 2005). The *P. vulgaris* cv. Negro Jamapa used in our experiments also does not support $>$ 25 mM NaCl in the growth medium, so that the enhanced biosynthesis of Nod factors at high salt concentrations (300 mM) has no direct utility for this plant. It cannot be ruled out, however, that the increased diversity of Nod factors might also play a role in *P. vulgaris* if induced by a different kind of stress, for example by low salt concentrations in combination with other ions present in the soil.

In our opinion, it would be extremely difficult to purify quantifiable amounts of individual Nod factor structures from such a complex mixture of Nod factor species as found in this work. To test the biological activity of an individual Nod factor structure, it would be necessary to synthesize the species, which would be important if the activity of specific structures were to be the subject of a future research.

With plants grown under control conditions (without NaCl, pH 7.0), the main Nod factors produced under neutral conditions and the two different stress conditions (300 mM NaCl or acid stress) all turned out to be biologically active (Fig. 6). The fact that stress has a huge influence on Nod factor biosynthesis indicates that new very interesting regulatory mechanisms, worth investigating, are involved in Nod factor biosynthesis.

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