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High NaCl Concentrations Induce the *nod* Genes of *Rhizobium tropici* CIAT899 in the Absence of Flavonoid Inducers

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The *nodulation* (*nod*) genes of *Rhizobium tropici* CIAT899 can be induced by very low concentrations (micromolar to nanomolar range) of several flavonoid molecules secreted by the roots of leguminous plants under a number of different conditions. Some of these conditions have been investigated and appear to have a great influence on the concentration and the number of different Nod factors, which can induce root nodule primordia and pseudonodules in several leguminous plant roots. In one such condition, we added up to 300 mM NaCl to the induction medium of *R. tropici* CIAT899 containing the *nod* gene inducer apigenin. At the higher concentrations of NaCl, larger amounts and more different Nod factors were produced than in the absence of extra NaCl. To our surprise, under control conditions (300 mM NaCl without apigenin), some Nod-factor-like spots were also observed on the thin-layer plates used to detect incorporation of radiolabeled glucosamine into newly synthesized Nod factors. This phenomenon was further investigated with thin-layer plates, fusions of *nod* genes to the *lacZ* gene, high-performance liquid chromatography, mass spectrometry, and the formation of pseudonodules on bean roots. Here, we report that, in the absence of flavonoid inducers, high concentrations of NaCl induced *nod* genes and the production of Nod factors.

Symbiosis between leguminous plants and rhizobium bacteria is required for the formation of nitrogen-fixing root nodules. The two symbiotic partners communicate with chemical signals produced by the plant as well as the bacterium. Plant signals in this communication are, with some exceptions, flavonoids, which normally are effective in hormonal (micro- and picomolar) concentrations. They are inducers of the so-called *nodulation* (*nod*) genes of rhizobium species, which encode enzymes for the production of Nod factors. Some other *nod* gene inducers are known, such as betaines (Phillips et al. 1992) and others, although they are mostly active in much higher concentrations (Cooper 2007).

Nod factors are lipochitooligosaccharides which are required for the induction of root nodule primordia and also play an essential role in infection by rhizobium species. Interestingly, an exception has been found to the latter, in some root-nodule-forming photosynthetic *Bradyrhizobium* bacteria not containing *nodABC* genes, the genes that encode enzymes involved in the production of the chitin backbone of Nod factors. This indicates the existence of another pathway, with different signals involved in root-nodule induction (Giraud et al. 2007; Masson-Boivin et al. 2009).

Legumes display host specificity for a certain kind of rhizobium bacteria. The symbiotic partners are classified in the so-called cross-inoculation groups consisting of plants and bacteria that, together, are able to form root nodules. Bacteria outside a cross-inoculation group are not able to do this but may well be a member of another such group. In many cases, host specificity is determined by Nod factor structure, which is different in diverse cross-inoculation groups. In addition, the kind of flavonoid molecules secreted by the plant can be important in determining host specificity (Spaink et al. 1987). However, some rhizobium bacteria as well as some plants display a nonspecific behavior in nodulation because they nodulate with a number of very different partners. *Rhizobium tropici* CIAT899 is such a "broad-host-range" rhizobial strain that forms an effective symbiosis with several legumes such as *Phaseolus vulgaris* and *Macroptilium atropurpureum*, and the nonrelated species *Leucaena leucocephala* (Martínez-Romero et al. 1991). This is probably related to the fact that two families of Nod factors are produced by this bacterium: a hydrophilic family of Nod factors that contains sulfates and other polar groups, and a hydrophobic family that lacks the sulfate group (Folch-Mallol et al. 1996). In the case of *R. tropici* CIAT899, the number of different Nod factors is strongly influenced by the bacterial growth conditions. Under stress conditions caused by acidity of the medium or high concentrations of NaCl, *R. tropici* CIAT899, when induced by the flavonid apigenin, produces more different Nod factor structures and produces them in higher concen-

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trations than under unstressed conditions (Estévez et al. 2009; Morón et al. 2005). Here, we report further studies of the effect of NaCl stress on Nod factor production by *R. tropici* CIAT899. Surprisingly, we have found *nod* gene induction in the presence of high NaCl concentrations in the absence of flavonoids. The Nod factors produced in this way also induced pseudonodules on bean plants under "normal", non-stress conditions. Compared with the classic pathway of Nod factor induction by flavonoids, this alternative mechanism of Nod factor induction may be important in as-yet-unspecified stress conditions in the rhizosphere of legumes.

RESULTS

Under salt stress (300 mM NaCl) and in the absence of flavonoids, *R. tropici* CIAT899 produces a large amount of Nod factors.

R. tropici CIAT899 grows in up to 300 mM NaCl (Estévez et al. 2009). In order to study the effect of NaCl stress on *R. tropici* CIAT899 Nod factor production, we performed thin-layer chromatography (TLC) analysis with radioactive detection of supernatants of bacterial cultures fed ^{14}C-labeled glucosamine (Fig. 1A). We detected an increase in the spot intensities and in the number of different radiolabeled spots corresponding to Nod factors from *R. tropici* CIAT899 after apigenin induction when the bacteria were grown in 300 mM NaCl (NaCl [+]) versus the control induced with apigenin and grown in the absence of added NaCl (control [+]). Similar results were reported in previous work (Estévez et al. 2009). Surprisingly, we also detected high levels of Nod factor production when the growth medium was supplemented with 300 mM NaCl in the absence of the flavonoid inducer (NaCl [–]). Between and below the two major spots corresponding to a hydrophilic family of structures containing sulfated compounds and a neutral family comprising nonsulfated Nod factors, we observed extra spots from cultures grown under NaCl stress (NaCl [–] and [+]). Very faint radiolabeled spots, barely visible, were also detected in extracts of uninduced cultures (control [–]), indicating a basal level of Nod factor production by CIAT899 in the absence of *nod* gene inducers.

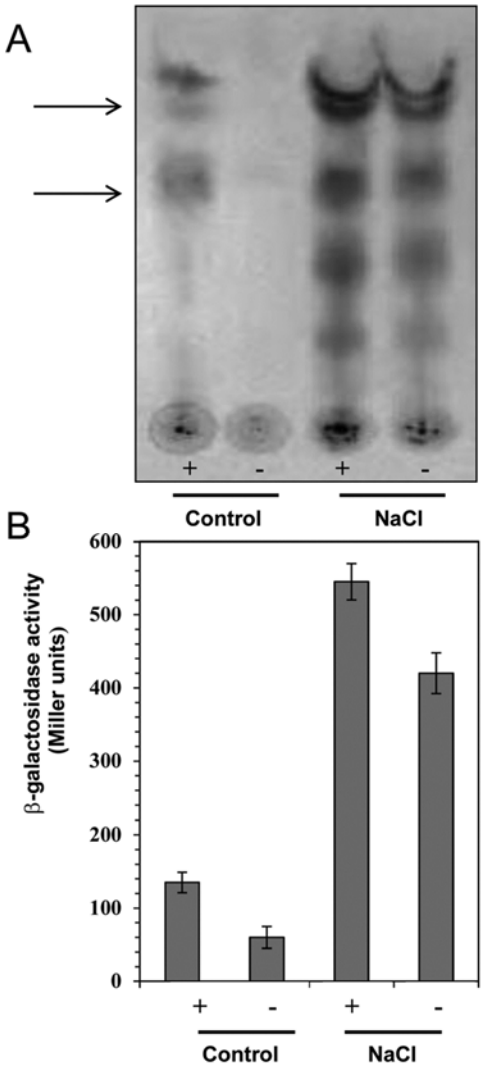

Fig. 1. Effect of 300 mM NaCl stress on Nod factor biosynthesis and *nod* gene expression of *Rhizobium tropici* CIAT899. **A,** Thin-layer chromatography analysis of *R. tropici* CIAT899 Nod factors produced in the presence of ^{14}C-labeled *N*-acetyl glucosamine. **B,** β-galactosidase activity of *R. tropici* RSP3051 (*R. tropici* CIAT899 *nodP*::*lacZ*). Assay conditions were B$^-$ medium (Control) and B$^-$ medium supplemented with 300 mM NaCl (NaCl). Apigenin (1 μM) was used as inducer in lanes marked + and omitted in those marked –.

To investigate whether the increased incorporation of radiolabel into CIAT899 Nod factors under NaCl stress was due to higher expression of *nod* genes, we additionally studied the *nod* gene expression of *R. tropici* RSP3051, which is strain CIAT899 having the reporter gene fusion *nodP*::*lacZ* (Manyani et al. 2001). A fourfold increase in β-galactosidase activity was observed from cultures grown under NaCl stress conditions in the absence of the flavonoid apigenin (Fig. 1B, NaCl [–]) as compared with the activity obtained by apigenin induction in the absence of NaCl (control [+]). Thus, these induction studies also show that NaCl is a *nod* gene inducer in *R. tropici* CIAT899.

R. tropici CIAT899 Nod factor production under NaCl stress conditions in the absence of flavonoids is regulated by genes encoded on the pSym plasmid.

As with strain CIAT899, when induced by apigenin, Nod factors were produced by strain *R. tropici* RSP900 (cured of the pSym plasmid) containing the plasmids pCV3804 (which carries the *nodDABCS* genes) or pCV38 (carrying the *nodDABCSUIJHPQ* genes). In contrast, when 300 mM NaCl was used as inducer, only *R. tropici* CIAT899 containing the complete pSym plasmid produced Nod factors (Fig. 2). This result shows that a gene or genes present on the pSym plasmid and not present on either cosmid is required for the induction of the *nod* genes by NaCl.

The *nodD1* gene is not required for *nod* gene induction by NaCl.

Because NodD1 is known to be the major flavonoid-dependent *nod* gene transcriptional activator in *R. tropici* CIAT899 (Sousa et al. 1993; van Rhijn et al. 1994), we wanted to determine whether this protein also participates in the NaCl regulation pathway. For this purpose, a *nodD1* mutant strain of *R. tropici* CIAT899, called *R. tropici* RSP82, was used.

TLC analysis with radioactive detection of supernatants of bacterial cultures fed with ^{14}C-labeled glucosamine was performed, after using apigenin or 300 mM NaCl as possible inducers of the *nod* genes of strain RSP82 (Fig. 3). In contrast to the wild-type strain CIAT899, radioactive Nod factor spots were not visible on the thin-layer plates of the culture supernatants from strain RSP82 incubated with apigenin and without salt. However, from cultures of strain RSP82 and the wild-type CIAT899 incubated with NaCl, Nod factor spots could be seen (Fig. 3).

These results clearly suggest that the *nodD1* gene is not required for *nod* gene induction by NaCl.

R. tropici CIAT899 Nod factors produced under NaCl stress conditions are mostly *N*-methylated and structurally similar to those produced after apigenin induction.

In order to determine the structural diversity of Nod factors produced under NaCl stress conditions, CIAT899 was grown in B⁻ medium in the presence of 300 mM NaCl but in the absence of apigenin. The bacteria were also grown in B⁻ medium in the absence of NaCl, either without or with apigenin as negative and positive controls, respectively. The Nod factors from the different culture media were extracted, purified, and fractionated as described by Soria-Díaz and associates (2003).

The high-performance liquid chromatography (HPLC) profiles of the Nod factors produced under NaCl stress conditions showed an important increase in the total area of the peaks corresponding to Nod factors compared with those from apigenin-induced and noninduced cultures containing similar numbers of bacteria (*data not shown*). The fractions collected following HPLC separation and biological activity testing, were then analyzed using liquid-chromatography mass spectrometry (LC-MS).

Nod factor structures are proposed on the basis of the *m/z* values for the protonated molecules and the corresponding collision-induced dissociation (CID) product ion spectra. CID results in cleavage of the glycosidic bonds in the Nod factor backbone to generate B_i (nonreducing terminal) and Y_i (reducing terminal) fragment ions, which make the identification of glucosamine substituents and their location on the molecule possible (Estévez et al. 2009). The chemical structures of the Nod factors produced by *R. tropici* CIAT899 in the absence of flavonoids and under NaCl stress are listed in Table 1, together with a summary of the MS results.

We identified 64 Nod factors formed under NaCl stress conditions without apigenin. The Nod factors identified all have the typical linear backbone comprising three, four, or five GlcNAc residues, with different N-acyl residues on the nonreducing terminal glucosamine, and bear different substituents on their reducing or nonreducing termini. Based on previous classifications of CIAT899 Nod factor structures (Estévez et al. 2009), we have identified four Nod factor types produced under NaCl stress conditions in the absence of apigenin.

The first class of Nod factors (type one) (Estévez et al. 2009) corresponds to those with simple unsubstituted backbones. The corresponding product ion spectra contain the B_i

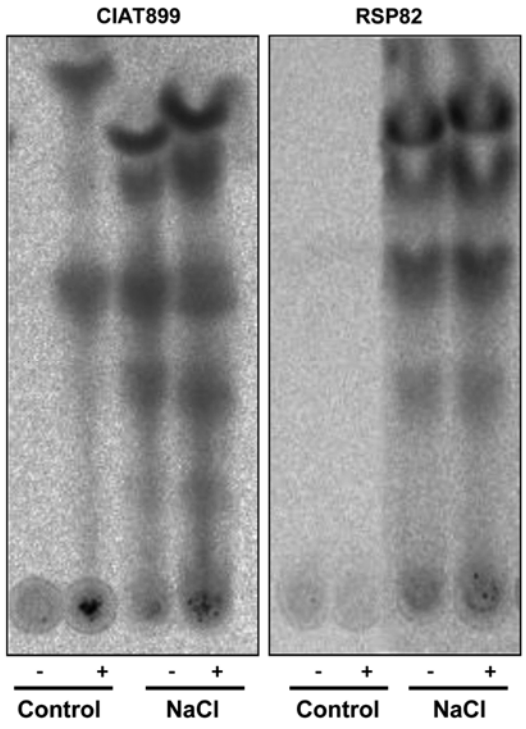

Fig. 3. Nod factor production by *Rhizobium tropici* CIAT899 and *R. tropici* RSP82 (*R. tropici* CIAT899 with a mutated *nodD*$_1$ gene) grown without NaCl (Control) and grown under NaCl stress conditions of 300 mM NaCl (NaCl). Apigenin (1 μM) was used as the inducer for the cultures run in lanes marked + and omitted from those run in lanes marked –.

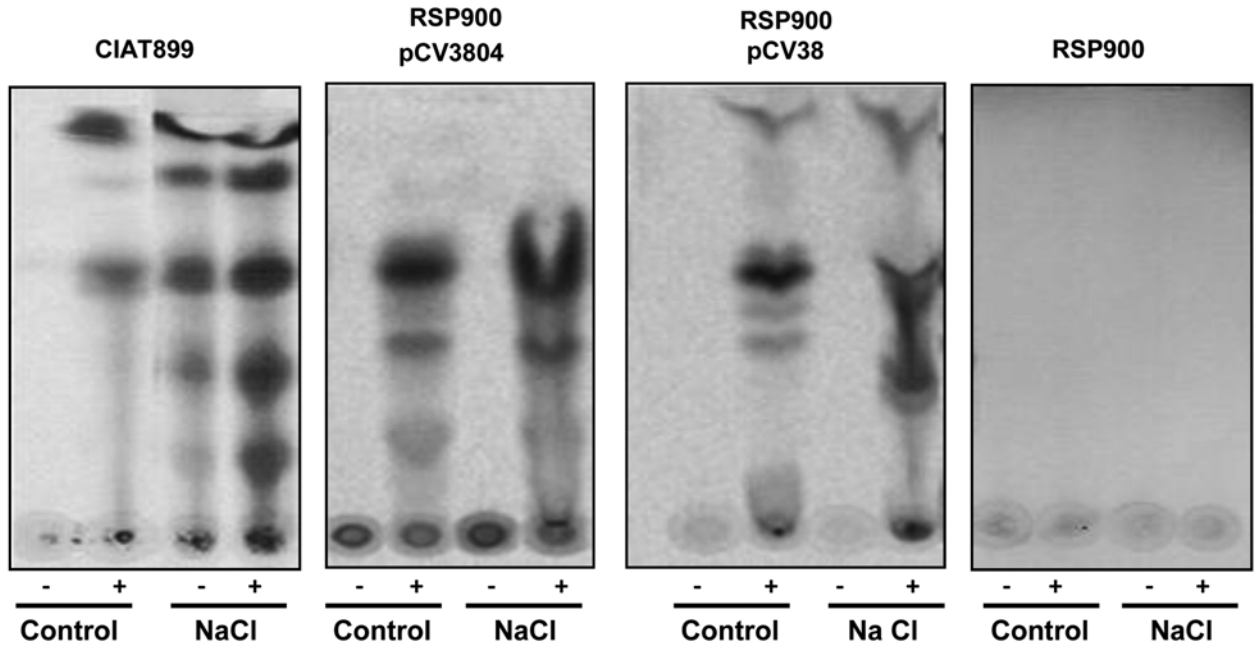

Fig. 2. Nod factor production of *Rhizobium tropici* CIAT899 (complete pSym plasmid), *R. tropici* RSP900 pCV3804 (only *nodD*$_1$*ABCSU* genes from the Sym plasmid), *R. tropici* RSP900 pCV38 (only *nodD*$_1$*ABCSUIJHPQ* genes from the Sym plasmid), and *R. tropici* RSP900 (Sym plasmid cured), grown without NaCl (Control) and grown under NaCl stress conditions (300 mM NaCl, NaCl). Apigenin (1 μM) was used as inducer in lanes marked + and was omitted in those marked –.

ion, which allows identification of the fatty acyl group. They only carry an acyl chain of 12 to 20 carbon atoms in length, attached to the nonreducing terminal residue.

The second type of Nod factors (type two) is represented by sulfated structures. They can be further substituted, usually with an *N*-methyl group, or just be sulfated. The B_i and Y_i ion series indicate that the sulfate group is located on the reducing terminal residue.

The third and most commonly observed type of Nod factor structure (type three) comprises those *N*-methylated on the nonreducing terminal glucosamine of the backbone. In short, *N*-methylated Nod factors account for more than half of the total different Nod factors structures produced under NaCl stress (40 of 64), and also when induced with apigenin (18 of 29). From the *m/z* values of their protonated molecules and by virtue of their product ions, we have identified some *N*-methylated Nod factors which are also sulfated on the reducing-terminal residue.

Finally, we have identified a fourth type of structure produced under NaCl stress conditions (type seven). This unusual type of Nod factor bears a hexose residue instead of glucosamine as the reducing residue of the molecule. These Nod factors are defined from the *m/z* values of their protonated molecules, the *m/z* values of their B_4 ions and, when observed, their Y_2 ions. When produced under NaCl stress conditions, a large subset of these hexose-containing Nod factors (5 of 8) also bear an *N*-methyl group as substituents on the nonreducing terminal residue.

Consistent with the data of Morón and associates (2005), we have also identified 29 Nod factors in the culture medium of the positive control (grown in the presence of apigenin without salt stress). Of these 29, 16 of the Nod factor structures were also identified in cultures grown under NaCl stress conditions (Table 1, marked with *), and, thus, we conclude that these constitute a basic pool of Nod factors which *R. tropici* CIAT899 produces when its *nod* genes are induced, whether by flavonoids or stress. More than half of these 16 structures (10 of 16) are *N*-methylated on their nonreducing terminal residue, and a subset of these are also sulfated on the reducing terminal residue (3 of 10). One Nod factor has a sulfate group attached to the reducing terminal residue as the only substitu-

Table 1. Nod factor (NF) structural families produced by *Rhizobium tropici* CIAT899 grown in B^- medium without inducers, in the presence of apigenin (Api) and with 300 mM NaCl

Structure[a]	NF type[b]	$[M+2H]^{2+}$ (*m/z*)	$[M+H]^+$ (*m/z*)	B_i ions	No induction[c]	Api[c]	300 mM NaCl[c]
II-Hex ($C_{18:1}$)	7	…	809	426, 629, 791	…	…	+
III ($C_{16:0}$)	1	…	824	400, 603, 806	…	…	+
III ($C_{16:0}$, NMe)	3	…	838	414, 617, 820	+	…	+
III ($C_{18:1}$)	1	…	850	426, 629, 832	+	…	+
III ($C_{18:1}$, NMe)	3	…	864	440, 643, 846	+	…	+
III ($C_{18:0\text{-}OH}$)	1	…	868	444, 647, 850	…	…	+
III ($C_{18:0\text{-}OH}$, NMe)	3	…	882	458, 661, 864	…	…	+
III ($C_{22:3}$, NMe)	3	…	916	492, 695, 898	…	…	+
IV ($C_{14:0}$)	1	…	999	372, 575, 778, 981	…	…	+
III ($C_{18:1}$, $GlcNH_2$)	8	…	1,011	426, 587, 790	+	…	…
III-Hex ($C_{18:1}$)	1,7	…	1,012	426, 629, 832, 994	…	…	+
IV ($C_{14:0}$, NMe)	3	…	1,013	386, 589, 792, 995	…	…	+
IV ($C_{16:1}$)	1	…	1,025	398, 601, 804, 1007	…	…	+
III-Hex ($C_{18:1}$, NMe)	3,7	…	1,026	440, 643, 846, 1008	…	…	+
IV ($C_{16:0}$)*	1	…	1,027	400, 603, 806, 1009	…	+	+
IV ($C_{16:1}$, NMe)	3	…	1,039	412, 615, 818, 1021	…	…	+
IV ($C_{16:0}$, NMe)*	3	…	1,041	414, 617, 820, 1023	…	+	+
III-Hex ($C_{18:0\text{-}OH}$, NMe)	3,7	…	1,044	458, 661, 864, 1026	…	…	+
IV ($C_{18:1}$)*	1	…	1,053	426, 629, 832, 1035	+	+	+
IV ($C_{18:0}$)	1	…	1,055	428, 631, 834, 1037	+	…	+
IV ($C_{16:0\text{-}OH}$, NMe)	3	…	1,057	430, 633, 836, 1039	…	…	+
IV ($C_{18:2}$, NMe)	3	…	1,066	438, 641, 844, 1048	…	…	+
IV ($C_{18:1}$, NMe)*	3	…	1,067	440, 643, 846, 1049	…	+	+
IV ($C_{18:0}$, NMe)	3	…	1,069	442, 645, 848, 1051	…	…	+
IV ($C_{18:0\text{-}OH}$, NMe)	3	…	1,085	458, 661, 864, 1067	…	…	+
IV ($C_{20:0}$, NMe)	3	…	1,097	470, 673, 876, 1079	…	…	+
IV ($C_{22:3}$, NMe)	3	…	1,119	492, 695, 898, 1101	…	…	+
IV ($C_{18:1}$, S)	2	…	1,133	426, 629, 832 $[M+H\text{-}80]^{+d}$ = 1053	…	…	+
IV ($C_{18:1}$, NMe, S)	2,3	…	1,147	440, 643, 846 $[M+H\text{-}80]^{+d}$ = 1067	…	…	+
IV ($C_{18:0}$, NMe, S)*	2,3	…	1,149	442, 645, 848, 1131 $[M+H\text{-}80]^{+d}$ = 1069	…	+	+
IV ($C_{18:0\text{-}OH}$, NMe, Ac)	3,6	…	1,149	522, 725, 928	…	+	…
IV-Hex ($C_{14:0}$)	7	…	1,161	372, 575, 778, 981, 1143	…	…	+
IV-Man ($C_{12:0}$, NMe)	3,4	…	1,169	380, 583, 786, 989	…	+	…
IV-Hex ($C_{14:0}$, NMe)	3,7	…	1,175	386, 589, 792, 995, 1157	…	…	+

(*continued on next page*)

[a] NF 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 in which they appear, moving clockwise from the fatty acid. Ac, acetyl group; Cb, carbamoyl group; Hex, hexose; Man, mannose; NMe, *N*-methyl group; S, sulfate group; * indicates produced under both apigenin induction and NaCl stress conditions; and ▲ indicates 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.

[b] Type of NF structure from *Rhizobium tropici* CIAT899 grown in B^- medium based on previous classifications (Estévez et al. 2009): type 1 = unsubstituted, type 2 = sulfated, type 3 = *N*-methylated, type 4 = mannosylated, type 5 = fucosylated or methyl-fucosylated, type 6 = acetylated or carbamoylated, type 7 = with hexose as the reducing terminal residue, and type 8 (newly proposed here) = lacking the N-acetyl group ($GlcNH_2$) from one of the glucosamines in the backbone.

[c] Symbol: + = detected.

[d] These ions arise by loss of a neutral with mass 80 Da, corresponding to the loss of SO_3.

[e] Reported by Morón and associates (2005).

ent (type two), and the five remaining Nod factors are unsubstituted structures (type one) (Table 1). Thus, under NaCl stress conditions, 48 different Nod factor structures were identified that were not observed as being produced on apigenin induction. These results are consistent with the results of the β-galactosidase assays and the TLC studies (Fig. 1), and suggest that stress caused by 300 mM NaCl leads to higher levels of *nod* gene expression, higher levels of newly synthesized Nod factors, and the production of more different structures than obtained on classical induction by flavonoids. This is the first time that *R. tropici* CIAT899 Nod factor production has been described in the absence of flavonoid inducers.

In the same way, we have detected nine Nod factor structures in cultures of *R. tropici* CIAT899 grown in normal B^- medium when neither flavonoids nor additional high NaCl stress conditions were applied. These Nod factors have trimeric, tetrameric, and pentameric backbones, and some of them are unsubstituted (type one), whereas others are N-methylated on the nonreducing residue (type three). Remarkably, others lack the acetyl group on some of the glucosamine residues in the backbone (we propose to term this additional type of Nod factor structure type eight). Only two Nod factor structures of these nine, IV($C_{18:1}$) and V($C_{18:1}$, NMe), were present in extracts from cultures of CIAT899 grown under NaCl stress conditions and also after flavonoid induction (Table 1).

R. tropici CIAT899 Nod factors produced under NaCl stress conditions are biologically active on bean plants.

Crude extracts of Nod factors produced by *R. tropici* CIAT899 under different growth conditions were tested for biological activity by investigating the induction of nodule primordia and pseudonodules on *P. vulgaris* 'Negro Jamapa' (bean) plants. Estévez and associates (2009) reported that pseudonodules were found to be induced by crude extracts of apigenin-induced CIAT899 cultures grown with and without NaCl stress. Here, we show that pseudonodules are also induced by extracts obtained from CIAT899 cultures not induced with apigenin but grown under NaCl stress (Fig. 4B). Further, biological activity was also shown by several of the HPLC fractions obtained from crude extracts of cultures grown under NaCl stress but not induced by apigenin. Specifically, fractions corresponding to minutes 10 to 15, 20 to 25, 25 to 30, 30 to 35, 50 to 55, 55 to 60, and 70 to 75 from this Nod factor crude extract were biologically active on bean plants, which developed nodule primordia and pseudonodules. These fractions were analyzed

Table 1. (*continued from preceding page*)

Structure[a]	NF type[b]	$[M+2H]^{2+}$ (*m/z*)	$[M+H]^+$ (*m/z*)	B_i ions	No induction[c]	Api[c]	300 mM NaCl[c]
IV ($C_{20:0}$, NMe, S)	2,3	…	1,177	470, 673, 876, 1159 $[M+H-80]^{+d}$ = 1097	…	…	+
IV-Man ($C_{10:0\text{-}OH}$, Cb)	4,6	…	1,186	397, 600, 803, 1006	…	+	…
IV ($C_{20:0}$)	1	542.2	…	456, –, 862	…	…	+
IV ($C_{20:1}$, NMe, S)	2,3	588.6	…	468, 671, 874	…	…	+
V ($C_{12:0}$, NMe)	3	594.7	…	358, 561, 764, 967	…	+	…
V ($C_{14:0}$)*	1	…	1,202	372, 575, 778, 981, 1184	…	+	+
IV ($C_{20:1}$, S)	2	…	1,205	476, 679, 882	…	+	…
V ($C_{18:1}$ dNAc)	8	…	1,214	426, 629, 832, 993	+	…	…
V ($C_{14:1}$, NMe)	3	…	1,214	384, 587, 790, 993, 1196	…	…	+
V ($C_{14:0}$, NMe)*	3	…	1,216	386, 589, 792, 995, 1198	…	+	+
V ($C_{10:0\text{-}OH}$, Cb)	6	…	1,227	397, 600, 803, 1006	…	+	…
V ($C_{16:0}$)	1	615.8	…	400, 603, 806, 1009	…	…	+
V ($C_{16:1}$)	1	…	1,228	398, 601, 804, 1007, 1210	…	…	+
IV-Hex ($C_{18:1}$, NMe)	3,7	…	1,229	440, 643, 846, 1049, 1211	…	…	+
V ($C_{10:0\text{-}OH}$, NMe, Cb)	3,6	…	1,241	411, 614, 817, 1020	…	+	…
V ($C_{16:0}$, NMe)*	3	…	1,244	414, 617, 820, 1023, 1226	…	+	+
IV-Man ($C_{18:1}$, NMe)	3,4	…	1,251	462, 665, 868, 1071	…	+	…
V ($C_{18:1}$)	1	…	1,256	426, 629, 832, 1035, 1238	+	…	+
V ($C_{18:0}$)	1	…	1,258	428, 631, 834, 1037, 1240	…	…	+
V ($C_{16:1}$, NMe)	3	621.9	…	412, 615, 818, 1021, 1224	…	…	+
V ($C_{18:2}$, NMe)	3	…	1,268	438, 641, 844, 1047, 1250	…	…	+
V ($C_{18:1}$, NMe)*	3	…	1,270	440, 643, 846, 1049, 1252	+	+	+
V ($C_{18:0}$, NMe)*	3	…	1,272	442, 645, 848, 1051, 1254	…	+	+
IV-Hex ($C_{22:3}$, NMe)	3,7	…	1,281	492, 695, 898, 1101, 1263	…	…	+
V ($C_{20:1}$)*	1	…	1,284	454, 657, 860, 1063, 1266	…	+	+
V ($C_{20:0}$)*	1	…	1,286	456, 659, 862, 1065, 1268	…	+	+
V ($C_{20:1}$, NMe)	3	…	1,298	468, 671, 874, 1077, 1280	…	…	+
V ($C_{16:0}$, S)	2	…	1,310	400, 603, 806, 1009, 1292	…	…	+
V ($C_{18:0\text{-}OH}$)	1	638.1	…	444, –, 850, 1053	…	…	+
V ($C_{20:0}$, NMe)*	3	650.8	…	470, 673, 876	…	+	+
V ($C_{20:0}$, S)*	2	684.3	…	456, –, 862, 1065	…	+	+
V ($C_{22:3}$, NMe)	3	…	1,322	492, 695, 898, 1101, 1304	…	…	+
V ($C_{16:0}$, NMe, S)	2,3	…	1,324	414, 617, 820, 1023 $[M+H-80]^{+d}$ = 1244	…	…	+
V ($C_{22:1}$)/V ($C_{18:1}$, NMe, Ac)▲	1/3,6	…	1,334	1113, 910, 707, 504	…	+	…
V ($C_{18:1}$, S)	2	…	1,336	426, 629, 832, 1035, 1318	…	…	+
V ($C_{20:1\text{-}OH}$, NMe)	3	…	1,336	506, 709, 912, 1115	…	+	…
V ($C_{22:0\text{-}OH}$, NMe)	3	…	1,344	514, 717, 920, 1123, 1326	…	…	+
V ($C_{18:1}$, NMe, S)*	2,3	…	1,350	440, 643, 846, 1049, 1332 $[B_5\text{-}80]^{+d}$ = 1252	…	+	+
V ($C_{18:0}$, NMe, S)*	2,3	…	1,352	442, 645, 848, 1051, 1334 $[M+H-80]^{+d}$ = 1272	…	+	+
V ($C_{20:1}$, S)	2	…	1,364	454, 657, 860, 1063 $[M+H-80]^{+d}$ = 1284	…	+	…
V ($C_{20:0\text{-}OH}$, Ac)	6	…	1,366	1145, 942, 739, 536	…	+	…
V ($C_{16:1}$, NMe, S)	2,3	…	1,366	[e]	…	+	…
V ($C_{20:1}$, NMe, S)	2,3	…	1,378	468, 671, 874, 1077, 1360 $[M+H-80]^{+d}$ = 1298	…	…	+
V ($C_{20:0}$, NMe, S)	2,3	…	1,380	470, 673, 876, 1079, 1362	…	…	+
V ($C_{22:3}$, NMe, S)	2,3	…	1,403	492, 695, 898, 1101	…	…	+

using LC-tandem mass spectrometry (MS/MS), and numerous Nod factors were identified (Table 2). Several inactive fractions from extracts obtained from CIAT899 cultures not induced by apigenin but grown under NaCl stress were analyzed as well. Surprisingly, except for V(C_{18}, NMe), every inactive fraction contained Nod factors structurally similar to those contained in the active fractions.

DISCUSSION

Rhizobia in the soil are exposed to variable stresses in their natural environments, including nutrient limitation and exposure to physical stresses such as elevated temperature, acidity, high osmolarity, or oxidative shock (Zahran 1999). *R. tropici* strain CIAT899 displays a high intrinsic tolerance to several stress conditions. It grows in up to 300 mM NaCl and at pH as low as 4.5. The fact that environmental factors affect Nod factor production by rhizobia was first reported by Laeremans and Vanderleyden (1998). Morón and associates (2005) showed that acidic conditions (pH 4.5) increase *R. tropici* CIAT899 Nod factor production after apigenin induction and changed the Nod factor structures produced. An additional study showed the substantial influence of NaCl stress on the expression of enzymes involved in Nod factor production in the presence of the flavonoid apigenin, and on the huge variety of Nod factors produced by growing *R. tropici* CIAT899 under such conditions (Estévez et al. 2009). In this article, we demonstrate that NaCl stress by itself, in the absence of flavonoid induction, also leads to Nod factor production by *R. tropici* CIAT899.

Using a transcriptional fusion of the *Escherichia coli lacZ* gene to the *R. tropici* CIAT899 *nodP* gene, we have explored the effects of NaCl stress on the induction of *nod* gene expression in the presence and absence of apigenin (Fig. 1B). As Estévez and associates (2009) reported, we have found that, in the presence of apigenin, 300 mM NaCl enhanced *nodP* gene expression compared with that obtained without NaCl in the medium (Fig. 1B, lanes marked +). Expression of *R. tropici* CIAT899 nodulation genes was also studied under control and NaCl stress conditions without the flavonoid apigenin (Fig. 1B, lanes marked –). Addition of 300 mM NaCl to the medium greatly enhanced *nod* gene expression in the absence of apigenin (Fig. 1B, NaCl [–]). However, the highest induction was found in the presence of both apigenin and 300 mM NaCl in the medium (Fig. 1B, NaCl [+]).

These results are consistent with the degree of incorporation under different culture conditions of radiolabeled glucosamine into the Nod factors we detected by TLC analysis (Fig. 1A). In the presence of 300 mM NaCl without apigenin, intense spots showing production of radioactive Nod factors could be seen (Fig. 1A, NaCl [–]). Intense Nod factor spots could also be seen after growth in the presence of apigenin, showing Nod factor production without or with 300 mM NaCl (Fig. 1A, lanes marked +). The very faint spots on the TLC plate (Fig. 1A, control [–]) suggested a very low level Nod factor produc-

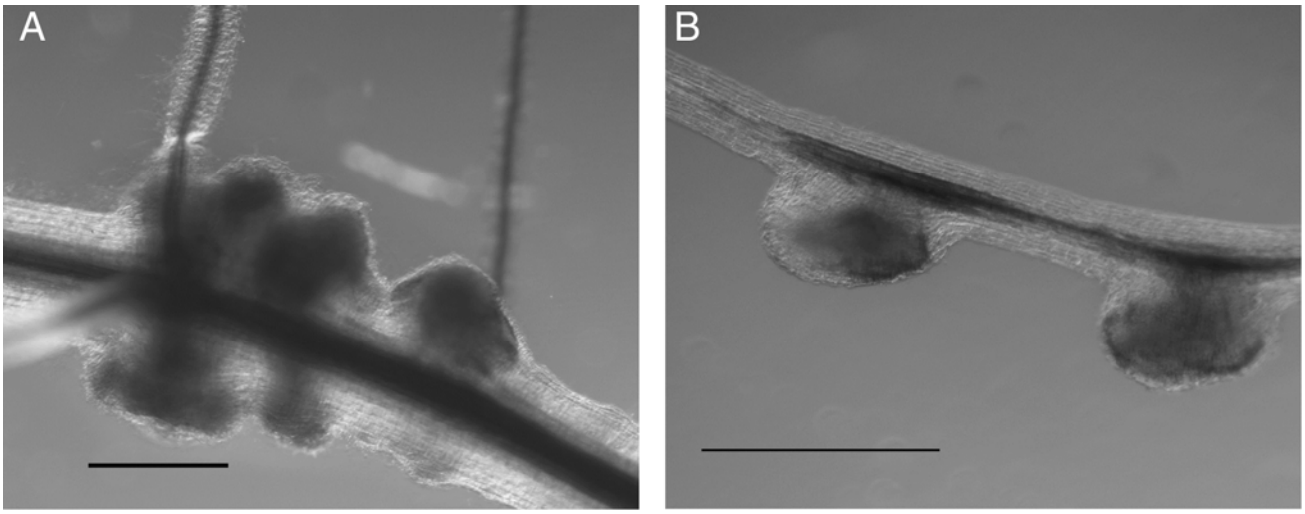

Fig. 4. *Phaseolus vulgaris* 'Negro Jamapa' plants. **A,** Nodules formed after inoculation with *Rhizobium tropici* CIAT899 and **B,** pseudonodules after inoculation with Nod factors of *R. tropici* CIAT899 produced under NaCl stress conditions without apigenin (300 mM NaCl). Bar length = 1 mm.

Table 2. Biologically active high-performance liquid chromatography fractions containing Nod factors produced by *Rhizobium tropici* CIAT899 under NaCl stress conditions (300 mM NaCl), in the absence of apigenin

Fraction (min)	Structure
10–15	IV($C_{16:0}$, NMe); V($C_{16:0}$, NMe); V($C_{16:0}$, S); V($C_{16:0}$, NMe, S); IV($C_{18:0}$, NMe, S); V($C_{18:0}$, NMe); V($C_{18:0}$, NMe S); IV($C_{18:1}$, NMe); IV($C_{18:1}$, S); IV($C_{18:1}$, NMe, S); V($C_{18:1}$); V($C_{18:1}$, NMe); V($C_{18:1}$, S); V($C_{18:1}$, NMe; S); V($C_{20:1}$, NMe); V($C_{20:1}$, NMe, S); IV($C_{22:3}$, NMe); IV-Hex($C_{22:3}$, NMe); V($C_{22:3}$, NMe); V($C_{22:3}$, NMe, S)
20–25	IV($C_{14:0}$); V($C_{14:0}$); V($C_{14:1}$, NMe); IV($C_{18:0}$, NMe, S); V($C_{18:0}$, NMe, S); V($C_{18:0\text{-}OH}$); IV($C_{18:1}$, NMe, S); V($C_{18:1}$, NMe); V($C_{18:1}$, NMe, S); V($C_{18:2}$, NMe)
25–30	IV($C_{14:0}$); IV($C_{16:0}$, NMe); V($C_{18:0}$, NMe, S); III-Hex($C_{18:1}$, NMe); IV($C_{18:1}$); IV($C_{18:1}$, NMe); V($C_{18:1}$, NMe); V($C_{18:1}$, NMe S); IV($C_{20:0}$, NMe); V($C_{20:0}$, NMe); V($C_{22:3}$, NMe)
30–35	IV($C_{14:0}$); IV-Hex($C_{14:0}$); IV-Hex($C_{14:0}$, NMe); V($C_{14:0}$); V($C_{14:0}$, NMe); V($C_{16:1}$); III($C_{18:0\text{-}OH}$); III($C_{18:0\text{-}OH}$, NMe); III-Hex($C_{18:0\text{-}OH}$, NMe); IV($C_{18:0\text{-}OH}$, NMe); IV($C_{18:1}$, NMe, S); V($C_{18:1}$, NMe, S); V($C_{18:2}$, NMe); V($C_{20:0}$, NMe, S); V($C_{20:1}$)
50–55	III($C_{16:0}$); IV($C_{18:0}$, NMe); IV($C_{16:0}$, NMe); III-Hex($C_{18:1}$); III-Hex($C_{18:1}$, NMe); IV($C_{18:1}$); IV($C_{18:1}$, NMe); IV-Hex($C_{18:1}$, NMe); V($C_{18:1}$); V($C_{18:1}$, NMe); V($C_{18:2}$, NMe); V($C_{20:0}$); V($C_{20:1}$); V($C_{20:1}$, NMe, S); IV-Hex($C_{22:3}$, NMe); IV($C_{22:3}$, NMe); V($C_{22:3}$, NMe)
55–60	III($C_{16:0}$, NMe); IV($C_{18:0}$); V($C_{18:0}$); II-Hex($C_{18:1}$); III($C_{18:1}$); III($C_{18:1}$, NMe); III-Hex($C_{18:1}$, NMe); IV($C_{18:1}$); IV($C_{18:1}$, NMe); V($C_{18:1}$, NMe); IV($C_{18:2}$, NMe); IV($C_{20:0}$); V($C_{20:1}$); V($C_{22:0\text{-}OH}$, NMe); III($C_{22:3}$, NMe); IV($C_{22:3}$, NMe); V($C_{22:3}$, NMe)
70–75	III($C_{16:0}$, NMe); IV($C_{18:0}$, NMe); III($C_{18:1}$); III($C_{18:1}$, NMe); III-Hex($C_{18:1}$, NMe); IV($C_{18:1}$, NMe); V($C_{18:1}$, NMe, S); IV($C_{20:0}$, NMe, S); V($C_{20:0}$, NMe, S)

tion under control conditions in the absence of apigenin. At the same time, the MS analysis (Table 1) detected only a small number of different Nod factor structures from cultures grown under these conditions. Thus, some of the enzymes implicated in Nod factor biosynthesis are also active in CIAT899 under control conditions (B^- medium), although at a low level. It is possible that this is caused by some stress occurring in this medium at higher cell densities.

The increased production of *R. tropici* CIAT899 Nod factors detected under NaCl stress conditions and in the absence of flavonoid is regulated by genes that are located on the pSym plasmid, because only in the presence of the complete pSym plasmid (in CIAT899) are radioactive Nod factor spots detected (Fig. 2). Because five copies of the *nodD* regulator gene in the *R. tropici* CIAT899 genome are located on the pSym plasmid of this strain (van Rhijn et al. 1993), our results clearly suggest that changes in Nod factor production by CIAT899 induced by NaCl stress are probably due to changes in the Nod factor regulation metabolism caused by the absence of a *nodD* gene (excepting the *nodD1* gene that is present on both clones).

R. tropici RSP82, a *nodD1*-minus mutant of the wild-type strain CIAT899, did not produce newly synthesized, radiolabeled Nod factors when induced by apigenin (Fig. 3). Thus, an intact *nodD1* gene appears to be required for induction of the *nod* genes by apigenin. Remarkably, incubation in 300 mM NaCl resulted in induction of the nod genes in the *nodD1* mutant as well as in the wild type. This means that the presence of an intact *nodD1* gene and thus, presumably, a NodD1 protein is not required for *nod* gene induction by NaCl. However, we cannot exclude the possibility that NaCl also induces the *nod* genes via the NodD1 protein in the wild type. The *nodABCSUIJHPlacZ* transcriptional fusion (RSP3051) in CIAT899 produced β-galactosidase when incubated with 300 mM NaCl. This means that the *nodABCSUIJHP* genes are induced by NaCl and represent a common part of both the NaCl and flavonoid induction pathways in *nod* gene activation.

Flavonoid-independent transcriptional activation (FITA) has been reported in *nodD1* mutants (Spaink et al. 1989; Vinardell et al. 2004). These FITA *nodD1* mutant genes introduced into different rhizobium species cause constitutive expression of the *nod* genes. In the case of CIAT899, there is a different inducible expression of the *nod* genes by NaCl. Thus, though *nod* gene expression is independent of flavonoids, this is a different case from the FITA mutants, in which the *nodD1* is not required. Because this *nod* gene expression concerns the induction via a *nod*-box (in RSP3051), another, as-yet-unidentified NodD-like protein is presumably involved.

We also explored the effects of NaCl stress on the structures of Nod factors produced by *R. tropici* CIAT899, using HPLC fractionation followed by MS analysis, which showed a great variety of nodulation factors produced by this strain when cultured with 300 mM NaCl in the absence of apigenin. The results enabled us to conclude that NaCl stress, in addition to increasing the biosynthesis of Nod factors as shown by the radiolabeling experiment, alters the structures produced, with changes to the substituents on the reducing and nonreducing terminal residues of the molecule, the fatty acyl chain, and the degree of polymerization of the chitin backbone. More than 60 different Nod factors were identified as being produced under NaCl stress conditions (Table 1). Under stress conditions, we detected different Nod factor backbone lengths (two, three, four, and five glucosamine residues) than in the presence of apigenin (only four and five glucosamine residues). At the same time, we detected more different saturated, unsaturated, and hydroxylated fatty acyl groups on the nonreducing terminal residue of Nod factors produced under induction conditions (C_{10} to C_{22}) than under control uninduced conditions, in which 16 and 18 were the usual carbon chain lengths (Table 1). Taken together, these results indicate that nodulation gene expression is affected by stress, not only allowing higher induction but also activating the expression of other genes that lead to the incorporation of new "decorations" into the signal molecules produced by *R. tropici* CIAT899.

We have also detected, only in the control cultures grown without inducers, Nod factor structures lacking the acetyl group on some glucosamine residues in the backbone (type eight structures). At the same time, we did not detect sulfated Nod factors in extracts of these uninduced control cultures (type two structures) but did detect them in cultures produced under all other conditions tested. These results suggest that the *nodHPQ* genes, responsible for transferring the sulfate group to the Nod factors of *R. tropici* CIAT899 (Manyani et al. 2001), require the presence of flavonoids or the presence of stress conditions to carry out this specific function and that they do not function or have too low an activity for sulfation to be detected under control conditions.

The Nod factor structures detected from cultures grown with apigenin induction without NaCl (Manyani et al. 2001; Morón et al. 2005) revealed the presence of a mannose instead of *N*-acetyl glucosamine attached to the reducing terminus of some tetrameric Nod factors. Under salt stress conditions without apigenin, we have also detected Nod factor structures with a backbone comprising two, three, or four GlcNAc residues, with hexose as the reducing terminal residue. However, due to the low levels of these Nod factors, we were not able to assign this monosaccharide.

These hexose-containing compounds may also bear an *N*-methyl substituent on the nonreducing residue of the molecule (Table 1). More than half of the Nod factors that we detected being secreted by CIAT899 under NaCl stress conditions, as well as in the presence of apigenin, are *N*-methylated structures (Table 1). The biological significance of this observation is not yet clear. Both biologically active and inactive fractions of the crude extracts of Nod factors produced under NaCl stress conditions without apigenin induction contain Nod factors that are structurally similar, except for V(C_{18}, NMe), which is uniquely detected in the active fractions (Table 1). However, because all these fractions contained numerous Nod factor structures, and because these have not been further purified, it is not possible to indicate which are the active factors in these fractions. However, based on the observation that V(C_{18}, NMe) is uniquely detected in the active fractions, it would be useful if this structure could be synthesized and used for further biological testing as part of our program of future work.

With so many different Nod factors produced by *R. tropici* CIAT899 under different growth conditions, the question of broader biological significance remains unresolved. The structures of Nod factors are well established to be important for their biological activity and host specificity in symbiosis. *R. tropici* CIAT899 is well known for its broad host range (Martínez-Romero et al. 1991), which is in line with the many different Nod factors it can produce.

Nod factors produced under NaCl stress conditions in the absence of apigenin are biologically active, as shown by the formation of pseudonodules on bean plants (Fig. 4). Bean plants cannot grow at NaCl concentrations higher than 50 mM, which could have been a problem if the Nod factor extracts had contained NaCl. However NaCl is removed from the Nod factor preparations during the hydrophobic extraction procedure. Consequently, extracted Nod factors could be added to the plant growth medium without also adding NaCl which would interfere with growth of the bean plants.

Several studies have reported the negative effects of NaCl stress on the establishment of this symbiosis (Singleton et al.

1982; Tu 1981; Zahran 1999; Zahran and Sprent 1986). There are common bean cultivars that tolerate raised salt concentrations up to 180 mM NaCl (Bayuelo-Jiménez et al. 2002) but the majority of bean plants are inhibited in their growth at 50 mM NaCl (Saadallah et al. 1998) and all known bean plants die at the concentrations of NaCl required for these new Nod factors to be produced.

At the moment, we can only speculate on the biological significance of the induction of *nod* genes by high NaCl concentrations. Perhaps there are unknown nodulating plants that can form root nodules at elevated NaCl concentrations. Otherwise, this alternative mechanism of Nod factor induction may be important in as-yet-unspecified stress conditions in the rhizosphere of legumes. Acid may be one such stress, as illustrated by a faint (Nod-factor-like) spot on thin-layer separation of an extract of a culture grown under acidic (pH 4.5) conditions in the absence of flavonoid inducer (Moron et al. 2005).

Environmental stress largely determines *R. tropici* CIAT899 Nod factor production, affecting both the levels and the structures of these signal molecules. Our future work will focus on the characterization of the symbiotically relevant genes involved in *R. tropici* CIAT899 Nod factor biosynthesis and secretion activated by stress in the absence of flavonoids, and the identification of their biosynthetic pathway.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 3. Rhizobial strains were grown routinely for 2 to 5 days on a rotary shaker (180 rpm) at 28°C in B^- minimal medium (van Brussel et al. 1977) at pH 7.0 with 1 mM potassium phosphate buffer. The *E. coli* strain used in the genetic studies was DH5-α, and was grown routinely at 37°C in Luria-Bertani broth medium (Maniatis et al. 1982).

Antibiotics were used in the following concentrations: gentamicin at 25 μg ml^{-1}, kanamycin (Km) at 30 μg ml^{-1}, nalidixic acid at 10 μg ml^{-1}, rifampicin (Rif) at 50 μg ml^{-1}, and tetracycline (Tc) at 10 μg ml^{-1}.

The final concentration of the flavonid apigenin in the medium was 1 μM. NaCl stress was obtained by adding NaCl to the medium (final concentration: 300 mM).

Strain construction.

pCV38 and pCV3804 cosmids were transferred from *E. coli* DH5-α to *R. tropici* strains via triparental mating using pRK2013 as a helper plasmid. Transconjugant selection was done on tryptone yeast (TY) agar plates (Beringer 1974) with Tc and Rif.

R. tropici RSP82 is a mutant of *R. tropici* CIAT899, carrying a Km-resistant (Km^r) cassette inserted into the *nodD1* gene. The plasmid used for this mutagenesis was pMC266 (pJQ200SK with an *Hin*dIII fragment containing the Km^r cassette at the *Xho*I restriction site). This plasmid was introduced into *R. tropici* CIAT899 by biparental mating using *E. coli* S17.1 as the donor strain. The gene replacement was carried out by double recombination due to the use of the suicide plasmid pJQ200SK. TY agar plates supplemented with Km and 10% sucrose were used to select putative mutants. Confirmation of the mutation was carried out by polymerase chain reaction (PCR) amplification and sequencing of the *Xho*I flanking fragment.

Determination of β-galactosidase activity.

Assays of β-galactosidase activity from *R. tropici* RSP3051 were carried out as described by Zaat and associates (1987). As positive control, 1 μM apigenin was used. Units of β-galactosidase activity were calculated according to Miller (1972). The experiments were repeated at least three times, with six replicates each time.

TLC analysis of Nod factors.

Nod factors were labeled in vivo and TLC analyses were performed according to Spaink and associates (1992). Briefly, *R. tropici* CIAT899 was grown on B^- minimal medium, supplemented with 1 μM apigenin in positive controls. For the radiolabeling of Nod factors, 1 μCi of ^{14}C-glucosamine hydrochloride (specific activity 52 mCi/mmol) (Amersham Pharmacia Biotech, Buckinghamshire, England), yielding a final concentration of 1 μM in the medium, was used. Cultures of 1 ml were grown to the end of the exponential growth phase and the supernatant was extracted with water-saturated *n*-butanol. The *n*-butanol fraction was evaporated to dryness and the resulting powder dissolved in 40 μl of water-saturated *n*-butanol. This solution (10 μl) was applied to the TLC plate ($RP\text{-}18F_{254S}$) (Merck, Darmstadt, Germany), where the Nod factors were separated with 50% acetonitrile/H_2O (vol/vol) as the mobile phase. TLC plates were exposed to a Fuji BAS-IIIs film for 10 days and the image was digitalized using the Phosphor-image system (Fuji, Tokyo).

Purification and LC-MS/MS analysis of Nod factors.

Nod factors from 300 mM NaCl, no apigenin, R. tropici *CIAT899 culture.* Nod factors produced by *R. tropici* CIAT899

Table 3. Bacterial strains and plasmids

Strain, plasmid	Description	Origin, source, reference[a]
Strain		
CIAT899	*Rhizobium tropici* wild-type strain, Rif^r	Martínez-Romero et al. 1985
DH5-α	*Escherichia coli supE44*, Δ*lacU169* (ϕ80*lacZ*ΔM15), *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *relA1*, Nx^r	Sambrook et al. 1989
RSP3051	*R. tropici* CIAT899 with a reporter fusion *nodP*::*lacZ*, Rif^r, Gm^r	Manyani et al. 2001
RSP82	*R. tropici* CIAT899 with a mutation in the *nodD1* gene	This work
RSP900	*R. tropici* CIAT899 lacking pSym plasmid, Rif^r	Vargas et al. 1990
RSP900(pCV38)	*R. tropici* RSP900 carrying pCV38 cosmid, Rif^r, Tc^r	This work
RSP900(pCV3804)	*R. tropici* RSP900 carrying pCV3804 cosmid, Rif^r, Tc^r	This work
S17.1	*E. coli* with mobilizing plasmid	Simon et al. 1983
Plasmid		
pCV38	Tc^r, pVK102 with a 28 kb fragment carrying *R. tropici* CIAT899 *nodD1* and *nodABCSUIJHPQ* gene	Vargas et al. 1990
pCV3804	Tc^r, pRK404 carrying *R. tropici* CIAT899 *nodD1* and *nodABCSU* gene	Vargas et al. 1990
pMC266	Suicide plasmid carrying the *nodD1* gene of CIAT899 interrupted with a kanamycin cassette	Manyani et al. 2001
pJQ200SK	Suicide vector	Quandt and Hynes 1993
pRK2013	Km^r, conjugation helper plasmid	Figurski and Helinski 1979

[a] Rif^r, Nx^r, Gm^r, Tc^r, and Km^r indicate resistance to rifampicin, nalidixic acid, gentamicin, tetracycline, and kanamycin, respectively.

in the presence of 300 mM NaCl but in the absence of apigenin were extracted and prepurified as described by Soría-Díaz and associates (2003), to generate three different acetonitrile/water fractions (20, 45, and 60%). The Nod factors in the 45% acetonitrile/water fraction was further fractionated by HPLC on a semipreparative C_{18} reversed-phase column, using isocratic elutions of acetonitrile/water (20%, 5 min; 30%, 30 min; 40%, 30 min; and 60%, 15 min), and then a linear gradient for 10 min from 60 to 100% acetonitrile. The eluent from HPLC was monitored at 206 nm, and 5-ml fractions were collected and tested on bean plants for biological activity.

All the biologically active fractions and some (three randomly chosen) of the inactive ones were further analyzed using LC-MS. These Nod-factor-containing fractions were applied to a reversed-phase column (PepMap, 3 µm, 75 µm by 100 mm) (Dionex/Thermo Scientific, Sunnyvale, CA, U.S.A.) using an Ultimate nano-LC system, a Famos autosampler, and a Switchos trap-column system (Dionex/Thermo Scientific). The column was equilibrated at room temperature with eluent A (20% acetonitrile, 0.1% formic acid in water) at a flow rate of 200 nl/min. At 10 min after injection of the sample, elution conditions were switched to 20% solvent B (95% acetonitrile, 0.1% formic acid), followed by a gradient to 60% B in 20 min and a subsequent isocratic elution of 15 min, followed by a gradient to 90% B in 10 min and a subsequent isocratic elution of 20 min. The eluate was monitored by absorption at 215 nm. The LC column was coupled to an Esquire HCT ESI-IT-MS (Bruker Daltonics, Bremen, Germany) equipped with an online nanospray source operating in the positive-ion mode. For electrospray (1,100 to 1,250 V), capillaries (360 µm outer diameter, 20 µm inner diameter, with a 10-µm opening) from New Objective (Cambridge, MA, U.S.A.) were used. The solvent was evaporated at 175°C employing a nitrogen stream of 7 liters/min. Ions over the *m/z* range of 300 to 1,500 were registered when operated in MS mode. When operated in the auto MS/MS mode, each MS scan was followed by the acquisition of MS/MS spectra of up to seven of the most abundant ions in the mass spectrum. Ions over the *m/z* range of 100 to 3,000 were registered when operating in MS/MS mode.

Nod factors from R. tropici *CIAT899 cultured in the absence of added NaCl and apigenin.* Nod factors produced by *R. tropici* CIAT899 in the absence of both NaCl and apigenin were extracted and prepurified using solid-phase extraction cartridges as described by Soría-Díaz and associates (2003).

For HPLC analysis, the sample was redissolved in 1 ml of 50% acetonitrile/water (vol/vol) and a 50-µl aliquot was injected onto the HPLC-electrospray ionization (ESI)-MS/MS system. Chromatographic separation was performed using a Perkin-Elmer Series 200 HPLC system (Wellesley, MA, U.S.A.) coupled to an Applied Biosystems QTRAP LC/MS/MS system (Foster City, CA, U.S.A.) consisting of a hybrid triple quadrupole linear ion trap (QqQlit) mass spectrometer equipped with an electrospray ion source. HPLC analyses were performed on a 200-by-2.1-mm Tracer Excel 120 ODSB C_{18} reversed-phase column with a particle size of 5 mm (Teknokroma, Barcelona, Spain). Chromatographic separation was performed using a binary gradient consisting of (A) water and (B) acetonitrile, both components containing 0.1% formic acid (vol/vol). The elution profile was 30% B (30 min), linear up to 30% B (15 min), 100% B (2 min), linear up to 30% B (3 min), and isocratic for 5 min (30% B). The flow rate was 0.3 ml min^{-1}.

The mass spectrometer was set to the following optimized tune parameters: curtain gas, 35 psi; ion spray voltage, 5,500 V; source temperature, 300°C; source gas, 20 psi; declustering potential, 70 V; and entrance potential, 10 V; and, for each MS/MS spectrum, the collision energy setting was 35 V. In MS mode, ions over the *m/z* range of 700 to 1,600 were registered. For MS/MS acquisition, the information data-dependent function was used, where each MS scan was followed by product ion acquisition of the two most abundant ions in the mass spectrum. Product ions of *m/z* 150 to 1,600 were recorded in MS/MS mode.

Biological activity of Nod factors.

Biological tests were performed according to López-Lara and associates (1995). Common bean (*P. vulgaris* Negro Jamapa) seed were surface sterilized by treatment with ethanol and sodium hypochlorite, as described by Albareda and associates (2006). The seed were thoroughly washed, imbibed in water for 2 h, deposited on plates with 0.8% agar, and incubated at 25°C in the dark for 48 h. Each seedling with a root length of 2 ± 0.5 cm was placed on a curled wire in a test tube with the root in 25 ml of Fahraeus medium (Fahraeus 1957). The roots were shielded from light and plants were grown for 10 days in a growth chamber under a cycle of 16 h of light and 8 h of darkness at day and night temperatures of 25 and 18°C, respectively, and a relative humidity of 75%.

Nod factors were dissolved in dimethyl sulfoxide (DMSO) and 25 µl was added when plants were placed in the test tubes. Negative controls were treated with 25 µl of DMSO (which did not inhibit plant growth), and positive controls were treated with 20 µl of a suspension of *R. tropici* CIAT899 at 10^8 CFU/ml. Experiments were repeated at least three times, with three replicates each time. Roots were made transparent with sodium hypochlorite and, after washing with water, stained with methylene blue using the method of Truchet and associates (1989). Primordial and pseudonodule structures were observed under an optical microscope and photographs were taken.

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