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ORIGINAL PAPER

Structure and biological activities of lipochitooligosaccharide nodulation signals produced by *Bradyrhizobium japonicum* USDA 138 under saline and osmotic stress

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Abstract The establishment of a symbiotic interaction involves a signal exchange between the host legume (flavonoids) and the nitrogen-fixing rhizobia (nodulation factors (NFs)). Likewise, abiotic stress conditions, such as salinity and drought, strongly reduce the nodulation process, possibly affecting also the signal exchange. In this work we characterized the structure and biological activity of NFs produced by *Bradyrhizobium japonicum* USDA 138 under control, salt, and osmotic stress conditions. This strain is the most widely used in Argentine soybean culture; under control conditions, it produces a mixture of four types of NFs (V($C_{16:0}$,MeFuc), V($C_{18:1}$,MeFuc), IV($C_{18:1}$), and V($C_{18:1}$,Ac,MeFuc)). Interestingly, under stress conditions, this strain produces new types of NFs, one common for both stress conditions (V($C_{16:1}$,MeFuc)) and another one only present under salt stress (IV($C_{18:1}$,MeFuc)). All mixtures of NFs, extracted from control, salt, and osmotic stress conditions, showed biological activity in soybean plants, such as root hair deformation, and the radical application of purified NFs induced systemic differences in dry matter accumulation. The inoculation of soybean with genistein-induced bacteria cultured under both control and stress conditions had a positive effect on the number of nodules formed and in some cases on dry matter accumulation. These responses are not related to changes in chlorophyll fluorescence or greenness index.

Keywords Nodulation factors · Abiotic stress · *Bradyrhizobium japonicum* · Soybean

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Introduction

Soybean (*Glycine max* (L.) Merr) is the most important legume crop worldwide. A distinctive characteristic of this crop is its ability to associate with atmospheric nitrogen-fixing bacteria in a symbiotic relationship. This interaction is the most inexpensive and environmentally friendly nitrogen source for crop production (Graham and Vance 2000). The establishment of the symbiotic relationship involves a signal exchange between the host legume and the nitrogen-fixing rhizobia. Soybean secretes flavonoids (principally the isoflavones genistein and daidzein), which are recognized by compatible *Bradyrhizobium japonicum* and which induce the expression of several genes that encode proteins involved in the synthesis and secretion of the nodulation factors (NFs). These compounds and their effects on both partners of the symbiosis are crucial because they determine the symbiosis

establishment and subsequent functionality (Broughton et al. 2000; Bais et al. 2006; Cesco et al. 2012).

NFs are lipochitooligosaccharides composed of three to five 1-4β-linked *N*-acetyl glucosamine (GlcNAc). Among the substitutions found on the terminal nonreducing GlcNAc are *N*-methyl, carbamoyl, and acetyl groups, along with various fatty acids. The reducing GlcNAc residue may be bound with a sulfate group or with D-arabinose, L-fucose, or 2-*O*-methylfucose, among other sugar residues (Stacey 1995; Spaink 1995). *B. japonicum* produces different types of NFs, and differences in NF number and structure among strains were described (Carlson et al. 1993).

Several biochemical, genetic, and morphological changes in root hairs of legumes are induced by their symbiotic interaction with *Rhizobium* sp. (Oldroyd and Downie 2008). NFs induce plasma membrane depolarization, intracellular alkalinization, and calcium spiking in growing root hairs (Cárdenas et al. 2000; Felle et al. 2000). Root hair curling is a typical, early, and essential morphological change produced after NF attachment (Oldroyd and Downie 2008).

Abiotic stress conditions, such as salinity and drought, strongly reduce the nodulation process (Singleton and Bohlool 1984; Elsheikh and Wood 1995). The effects of early symbiotic events have been recently characterized, and salt stress conditions have been found to affect root hair deformation and induce cell death (Muñoz et al. 2012). Likewise, the synthesis and secretion of flavonoids and NFs by different legume–rhizobia interactions were found to be altered under stress conditions (Estevez et al. 2009; Dardanelli et al. 2009). Qualitative and quantitative changes in the production of *Rhizobium tropici* CIAT 899 NFs (a strain that nodulates with *Phaseolus vulgaris*) under stress conditions were characterized. The expression of nodulation genes was enhanced with increments of salinity or acidity in the culture medium and independently of the flavonoid inducer presence (Morón et al. 2005; Estevez et al. 2009).

The study of the symbiotic features of rhizobia under abiotic stress is of great importance (Bouhmouch et al. 2005). *B. japonicum* USDA 138 is the most important strain used in Argentine soybean culture; however, its NFs have not been characterized, and their production under abiotic stress has received much less attention. The present study analyzes the structure and microbiological activities of NFs produced by *B. japonicum* USDA 138 under saline and osmotic stress and their local and systemic biological activities on soybean.

Material and methods

Bacterial culture, induction with genistein, and NF production

Bacteria were pre-cultured in yeast extract mannitol (YEM) medium (Vincent 1970) up to 0.6 OD_{600} and then diluted 1:10 in three different media tested for *B. japonicum* USDA 138: B^- medium (Spaink et al. 1992), B^- medium supplemented with (0.4 g l^{-1}) yeast extract, and YEM. After 3 days of growth, the strain was induced with 1 μM of genistein (4,5,7-trihydroxyisoflavone) for a maximum of 48 h. For NF production under abiotic stress, three concentrations of NaCl (25, 50, or 150 mM) and sorbitol (50, 100, or 300 mM) were tested. These series of hyperosmotic solutions develop equal osmotic pressures (−0.47, −0.55, and −0.84 MPa, respectively). Abiotic stress treatments were applied adding to the culture medium NaCl or sorbitol together with the step of initial culture dilution described above. Each experiment had three biological replicates.

Radiolabeling and reverse-phase thin-layer chromatography analysis of NF

For in vivo radiolabeling of NFs and detection by reverse-phase thin-layer chromatography (RP-TLC) (Spaink et al. 1992), after 3 days of growth in liquid medium, 1 μCi of glucosamine hydrochloride ^{14}C was added simultaneously with the inducer genistein. After 48 h of induction in this condition, the bacterial culture was centrifuged (9,000×*g*), and the supernatant was used for NF extraction by the addition of water-saturated *n*-butanol. The *n*-butanol was evaporated to dryness, and the resulting powder was resuspended in 30 μl of *n*-butanol and applied (10 μl) to the TLC plate (Sigma). NFs were separated using 50 % acetonitrile/water (*v*/*v*) as the mobile phase. TLC plates were exposed to a BAS-MS2040 imaging plate for 4 days, and the images were read with a BAS-2500 phosphoimager.

NF extraction and sample preparation for structure determination by HPLC MS/MS

Bacterial growth and induction conditions were performed as stated above. To characterize the NFs by HPLC MS/MS, only one saline stress condition (50 mM NaCl) and its corresponding osmotic control (100 mM sorbitol) were used. Bacterial culture per sample was 4 l in volume. NFs were extracted adding 300 ml of water-saturated *n*-butanol per liter of culture. The butanolic phase was evaporated to dryness at 50 °C, and the solid samples were resuspended in 10 ml of 50 % acetonitrile/water (*v*/*v*) with agitation for 24 h. After this step, the acetonitrile concentration was taken to 20 % with water, and the samples were filtered using a C_{18} pre-equilibrated column by washing twice with 100 % methanol and twice with 20 % acetronitrile. A pre-purification step was then performed by passing the crude extract through a C_{18} column (Resprep). After this step, the column was washed with two volumes of 20 % acetonitrile to remove salts and polar compounds, and NFs were eluted with two volumes of 100 % methanol. The methanol was completely evaporated and the sample resuspended in 1 ml of 50 % acetonitrile/water (*v*/*v*) per liter of culture. This sample was microfiltered for HPLC

MS/MS determinations. The negative control for NF extraction was performed using the same protocol described above, without the genistein induction.

NF structure determination by HPLC MS/MS

The chromatographic separation was performed on a PerkinElmer Series 200 HPLC system (Wellesley, MA, USA) coupled to an Applied Biosystems QTRAP LC-MS/MS system (Foster City, CA, USA), consisting of a triple quadrupole linear ion trap mass spectrometer equipped with an electrospray ion source.

HPLC analyses were performed on a Tracer Spherisorb ODS2 C18 reverse-phase column (250×2.1 mm) with a particle size of 5 μm (Teknokroma, Barcelona, Spain). The flow rate was 0.3 ml min^{-1}. The chromatographic separation was performed through the use of a binary gradient consisting of water (A) and acetonitrile (B), with both components containing 0.1 % (*v*/*v*) formic acid. The elution profile was isocratic for 5 min at 30 % B, then with B linearly increasing from 30 to 100 % for 30 min, and finally isocratic again for 3 min. Injection volume was 50 μl. Mass spectrometric detection was performed in the positive mode. For HPLC MS/MS analyses, the mass spectrometer was set to the following optimized tune parameters: curtain gas, 35 psi; ion spray potential, 5,500 V; source temperature, 300ºC; source gas, 60 psi; declustering potential, 50 V; and entrance potential, 10 V. MS/MS experiments were performed with a collision energy of 35 V. Only those precursor ions with an *m*/*z* between 800 and 1,600 and intensities higher than 1,000 cps were selected for MS/MS analysis.

For MS/MS acquisition, information data-dependent function was performed, where each MS scan was followed by the MS/MS acquisition of the two most abundant ions in the MS spectrum. Ions from *m*/*z* 150 to *m*/*z* 1,600 were registered when operated in the MS/MS mode.

Microbiological activity assays of NFs produced by *B. japonicum* USDA 138 under saline and osmotic stress

Two assays were performed to assess the microbiological activity of NFs produced by *B. japonicum* USDA 138 under saline and osmotic stress. Firstly, the local response induced by the NF mixture isolated from control, saline, and osmotic treatments was evaluated by determining root hair deformation. For this purpose, disinfected soybean seeds (*Glycine max* L. DM4800) were germinated in filter paper moistened with distilled water for 48 h in the dark. To promote root and root hair growth, seeds were germinated at 28 °C during the first 24 h and at 37 °C during the second 24 h. Roots were incubated for 4 h with 1:10,000 dilutions of NF mixture isolated from control and stress treatments. Root hairs were stained with 1 % toluidine blue, and their deformations were observed under an optical microscope. Eight roots per treatment and four sections per root were observed. Approximately 600 root hairs per treatment were counted for each of the three independent experiments. Systemic response induced by NF mixtures isolated from control, saline, and osmotic treatments was evaluated by determining the kinetics of leaf area development from the appearance of the first trifoliate leaf and dry weight of aerial and radical parts.

Secondly, soybean nodulation was assayed with *B. japonicum* cultured under control and stress treatments and induced with genistein. Disinfected soybean seeds (*G. max* L. DM4800) were germinated in filter paper moistened with distilled water in the dark for 48 h and transferred to hydroponic culture with B and D nutrient solution (Broughton and Dilworth 1971) without nitrogen and with aeration. Simultaneously with the start of hydroponic culture, the plants were inoculated with 1 ml per plant of *B. japonicum* culture grown under control and stress conditions and induced with genistein. After 3 days of inoculation, the nutrient solution was completely renewed to remove the excess bacteria. After 30 days of planting, the weight and number of nodules and the dry weight of aerial and radical parts were measured. The kinetics of leaf area expansion, greenness index, and chlorophyll fluorescence were determined from the appearance of the first trifoliate leaf. These measurements were carried out in three independent experiments with 12 plants per experiment and per treatment.

Plant material and growth conditions

For whole plant experiments, we used soybean seeds (*G. max* L. DM4800) grown under hydroponic culture with B and D nutrient solution (Broughton and Dilworth 1971) without nitrogen and with aeration, 16:8 photoperiod, light intensity of 200 μmol photons m^{-2} s^{-1}, and 25 °C. Oxygenation was supplied to the nutrient solution with a hose of 0.5 cm in diameter and pumping air using an aquarium aerator of 3.7 W. The total volume of aerated nutrient solution under these conditions was 3.5 l.

Leaf area, greenness index, and chlorophyll fluorescence determinations

Leaf area calculations were made every day by photographing the first trifoliate leaf. Photographs were taken with a scale of 1 cm^2, which was used to calculate the total leaf area with image analyzer software OPTIMAS 6.1. The greenness index was estimated using a handheld SPAD CL01 meter (Hansatech Instruments, Pentney, King's Lynn, UK). This provides a unitless index ranging from 0 to 100, which is proportional to leaf chlorophyll content. Maximum quantum yield (Fv/Fm), a chlorophyll fluorescence parameter, in dark-

adapted plants (at least 30 min) was measured using a pulse amplitude-modulated fluorometer (FMS2, Hansatech Instruments, Pentney, King's Lynn, UK).

Statistical analyses

Completely randomized design was used for plant experiments. Data were analyzed using analysis of variance followed by the DGC (multiple-comparisons method of Di Rienzo, Guzmán, and Casanoves statistical test in InfoStat) or Tukey test. All analyses were performed using the InfoStat program (InfoStat/Professional ver. 2007p, Grupo InfoStat, Facultad de Ciencias Agropecuarias, Universidad Nacional de Cordoba, Argentina).

Results

Induction of NFs and detection by RP-TLC

B. japonicum USDA 138 only produced NFs in YEM medium and in the presence of genistein. *B. japonicum* USDA 138 induced with genistein did not produce NFs in B^- medium, even when this medium was supplemented with yeast extract. NF production of *R. tropici* CIAT 899 induced with 1 μM apigenin was used as B^- medium control (Supplementary Fig. 1).

NF production under abiotic stress and detection by RP-TLC

NF production by *B. japonicum* USDA 138 under salt and osmotic stress was assayed using YEM culture medium supplemented with different NaCl (25, 50, or 150 mM) and sorbitol (50, 100, or 300 mM) concentrations. This strain produced NFs under all stress conditions but only under genistein induction (Supplementary Figs. 2 and 3, respectively). Using the TLC method, no differential NF patterns associated with the treatments were observed. Under high salt concentration (150 mM NaCl), the spot of NF production was small, but bacterial growth was delayed by the stress condition (data not shown).

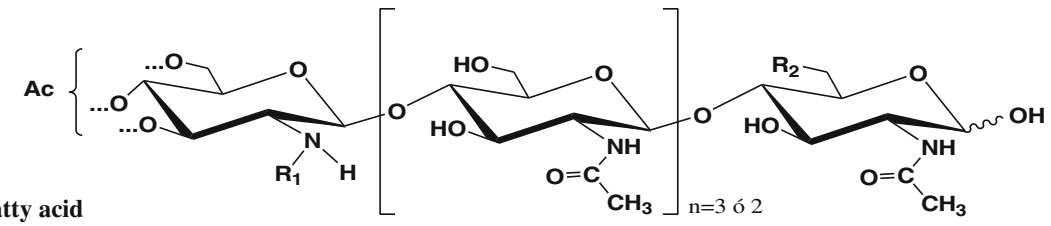
NF structure by HPLC MS/MS under abiotic stress

The nodulation (Nod) factor structures are proposed on the basis of the tandem mass spectrometric analysis. This analysis results in the cleavage of the glycosidic bonds in the Nod factor backbone to generate Bi (nonreducing terminal) and Yi (reducing terminal) fragment ions, which make the identification of glucosamine substituents and their location on the molecule possible. The chemical structures of the Nod factors produced by *B. japonicum* USDA 138 under control, salt, and osmotic stress conditions are in Table 1, together with a summary of the MS results.

Under control condition, this strain can produce four types of NFs with penta- and tetrasaccharide backbone that contains different fatty acids, such as the *N*-acyl substituent ($C_{18:0}$, $C_{18:1}$, $C_{16:0}$), in the terminal nonreducing GlcNAc. Three of the NFs had 2-*O*-methylfucosyl residue in the reducing GlcNAc residue.

Under saline and osmotic stress, one new NF (Nod Bj-V($C_{16:1}$,MeFuc) common to both stress treatments was detected. This new NF differed from those detected under control conditions in the type of fatty acid present in its structure

Table 1 Summary of NFs produced by *B. japonicum* USDA 138 under control, saline, and osmotic stress



R_1 = fatty acid
R_2 = H, MeFuc

Nod metabolite	Growth condition	R_1	R_2	*n*	$[M+H]^+$ (*m/z*)	B_i ions
Nod Bj-V($C_{16:0}$,MeFuc)	Control, salt, and sorbitol	$C_{16:0}$	2-*O*-MeFuc	5	1,390	400, 603, 806, 1,009
Nod Bj-V($C_{18:1}$,MeFuc)	Control, salt, and sorbitol	$C_{18:1}$	2-*O*-MeFuc	5	1,416	426, 629, 832, 1,035, 1,398
Nod Bj-IV($C_{18:1}$)	Control, salt, and sorbitol	$C_{18:1}$	H	4	1,256	426, 629, 832, 1,035
Nod Bj-V($C_{18:1}$,Ac,MeFuc)	Control, salt, and sorbitol	$C_{18:1}$	2-*O*-MeFuc	5	1,458	468, 671, 874, 1,077
Nod Bj-V($C_{16:1}$,MeFuc)	Salt and sorbitol	$C_{16:1}$	2-*O*-MeFuc	5	1,388	398, 601, 804, 1,007
Nod Bj-IV($C_{18:1}$,MeFuc)	Salt	$C_{18:1}$	2-*O*-MeFuc	4	1,213	426, 629, 832

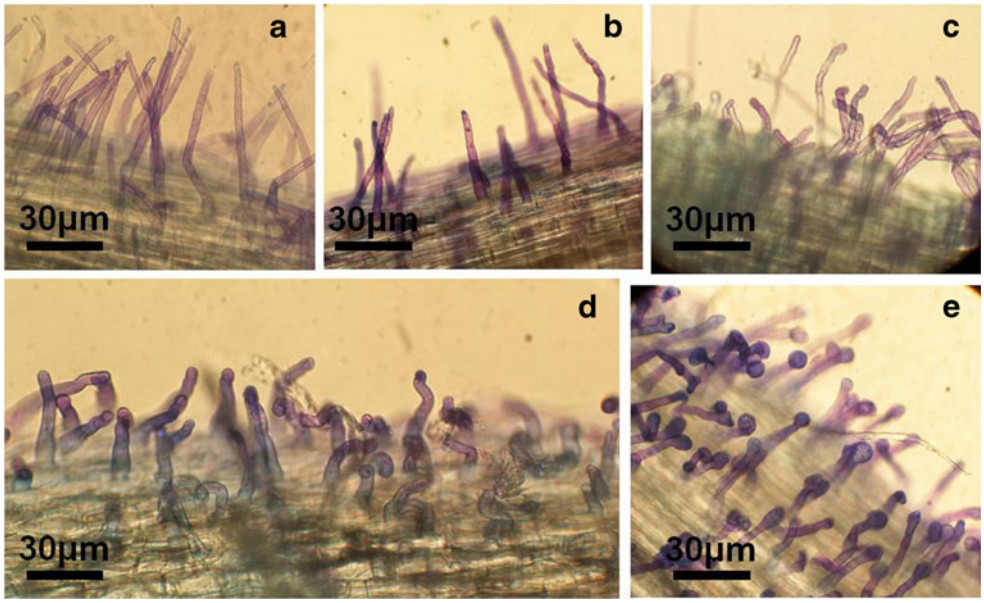


Fig. 1 Soybean root hair deformation after treatments for 4 h with mixtures of NFs extracted under **c** control, **d** saline, and **e** osmotic stress conditions. **a** Noninoculated root hairs and **b** negative control of purified NFs generated following the same NF extraction protocol but with bacterial culture without genistein

($C_{16:1}$). Another new NF present only in the saline treatment was detected, which differed from those detected in the other treatments in the number of GlcNAc monomers (Nod Bj-IV($C_{18:1}$,MeFuc) (Table 1).

Microbiological activities of NFs produced by *B. japonicum* USDA 138 under saline and osmotic stress

Locally, the application of purified NFs from control and saline/osmotic stress treatments induced a similar percentage of root hair deformations. Interestingly, NFs produced under osmotic treatments showed an important swelling response (Fig. 1e). The negative control of purified NFs did not induce root hair deformations (Fig. 1b).

We also tested systemic responses after the application of different NF mixtures, such as leaf area expansion of the first trifoliate leaf and dry weight of aerial and radical parts. The kinetics of expansion of the first trifoliate leaf did not show significant differences among the mixtures tested (Fig. 2). However, interestingly, the results showed a significant increase in the dry weight of aerial and radical parts of plants treated with NFs extracted from control conditions (Fig. 3a, b).

Biological activities of *B. japonicum* USDA 138 grown under saline and osmotic stress and induced by genistein

Nodule numbers of plants inoculated with genistein-induced bacteria cultured under both control and stress conditions were significantly higher than those inoculated with noninduced bacteria. Interestingly, bacteria cultured under osmotic stress also induced a significant increase in the number of nodules formed with respect to the controls, independently of the presence of genistein (Fig. 4a). No significant differences in the weight of nodules were observed for any of the treatments tested (Fig. 4b).

Noticeably, there was an increase in leaf area (Fig. 5) during the development of the first trifoliate leaf of plants inoculated with bacterial cultures grown in the different conditions tested. The plants that were treated with induced *B. japonicum* USDA 138 under saline stress showed a significant increment in leaf area with respect to noninduced treatment 9 days after the appearance of the first trifoliate leaf. Under control conditions, the plants treated with induced *B. japonicum* USDA 138

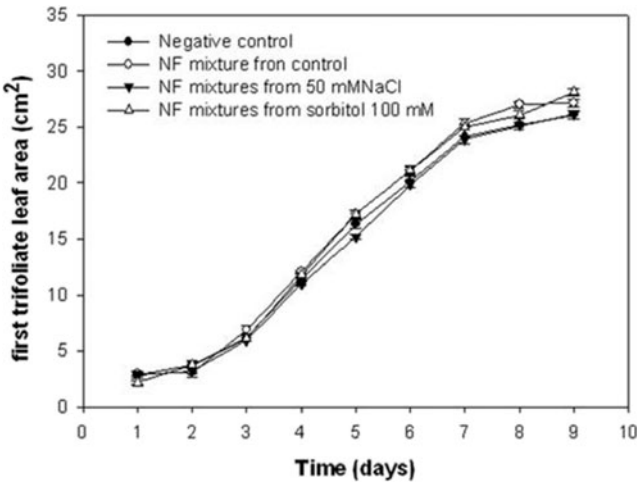


Fig. 2 First trifoliate leaf area of soybean plants treated by radical application with mixtures of purified NFs extracted under control, saline, and osmotic stress conditions. Each value represents the mean±SE of three independent experiments. *Asterisks* indicate significant differences in the mean ($p<0.05$, DGC test)

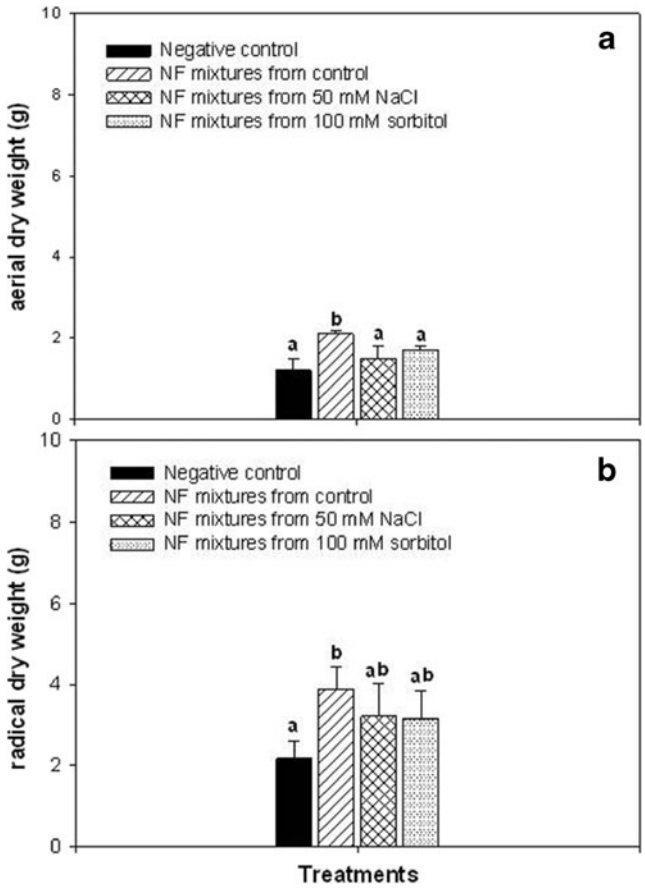


Fig. 3 **a** Aerial and **b** radical dry weight of soybean plants treated by radical application with mixtures of purified NF extracted under control, saline, and osmotic stress conditions. Each value represents the mean±SE of three independent experiments. *Different letters* indicate significant differences in the mean ($p<0.05$, Tukey test)

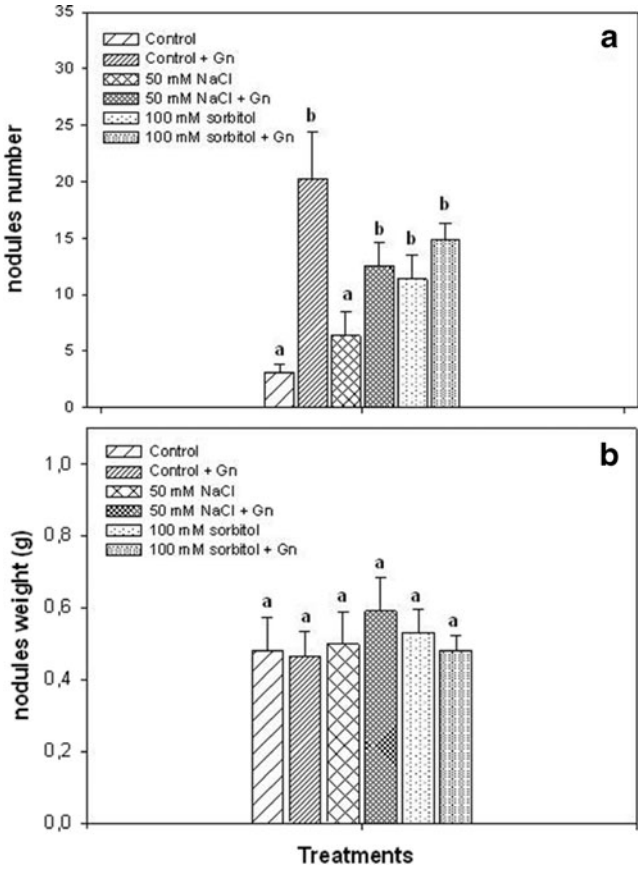


Fig. 4 Nodule **a** number and **b** weight of soybean plants inoculated with genistein-induced (+*Gn*) and noniduced bacteria cultured under control, saline, and osmotic stress conditions. Each value represents the mean±SE of three independent experiments. *Different letters* indicate significant differences in the mean ($p<0.05$, Tukey test)

showed a leaf area reduction from day 5 post-appearance of the first trifoliate leaf. There were no significant differences in the greenness index and chlorophyll fluorescence (Fig. 6a, b).

Discussion

The general structure of *B. japonicum* USDA 138 NFs is consistent with the one previously mentioned for other strains of the same species (Spaink et al. 1991, 1992; SanJuan et al. 1992; Carlson et al. 1993); in those works, the typical NF produced was Nod Bj-V($C_{18:1}$,MeFuc), which was also detected in the strain we analyzed in this work.

Our results show that under salt and osmotic stress conditions, the mixtures of NFs produced by *B. japonicum* USDA 138 were altered, showing an increase in the variety of these compounds. This strain only produces NFs in the presence of the inducer genistein, regardless of the stress condition. By contrast, for other rhizobia, such as *R. tropici* CIAT 899, this strain was found to be able to produce NFs under stress

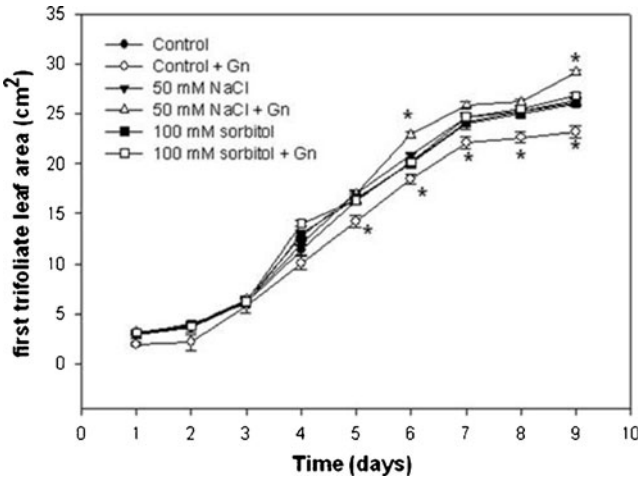


Fig. 5 First trifoliate leaf area of soybean plants inoculated with genistein-induced (+*Gn*) and noninduced bacteria cultured under control, saline, and osmotic stress conditions. Each value represents the mean±SE of three independent experiments. *Asterisks* indicate significant differences in the mean ($p<0.05$, DGC test)

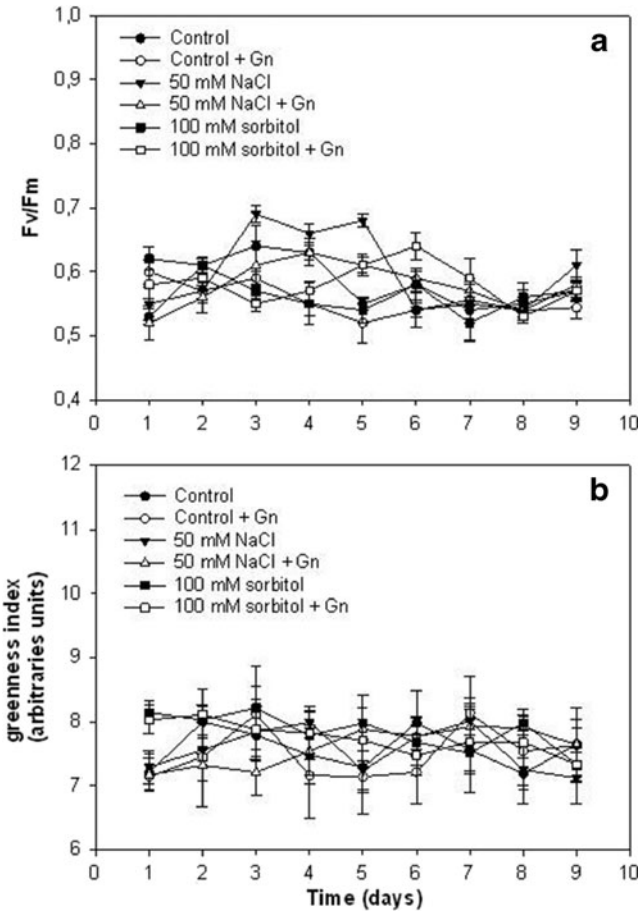


Fig. 6 **a** Chlorophyll fluorescence Fv/Fm and **b** greenness index in the first trifoliate leaf area of soybean plants inoculated with genistein-induced (+*Gn*) and noniduced bacteria cultured under control, saline, and osmotic stress conditions. Each value represents the mean±SE of three independent experiments. *Asterisks* indicate significant differences in the mean ($p<0.05$, DGC test)

conditions, independently of the inducer presence (Guasch-Vidal et al. 2012).

The 2-*O*-methylfucosyl residue at C-6 of the reducing *N*-acetyl glucosamine has been cited as an important modification in the structure of NFs produced by *B. japonicum* because when the gene responsible for this modification is mutated (*nod Z*), nodule formation is severely affected in soybean (Stacey et al. 1994). Interestingly, under control and stress conditions, *B. japonicum* USDA 138 was found to be able to produce a type of NF that lacks the 2-*O*-methylfucose substitution. Likewise, this structure was formed by tetrasaccharide backbones of *N*-acetyl glucosamine (Nod Bj-IV($C_{18:1}$)). This simple structure of NF has been the focus of attention because it is responsible for the biological responses (root hair deformation and pseudo-nodule formation) in *Glycine soja*, the wild ancestor/progenitor of cultivated soybeans. Structural modifications in Nod Bj-IV($C_{18:1}$), such as 2-*O*-methylfucose substitution, have a negative effect on the biological activity of NFs in *G. soja* (Stokkermans et al. 1995). Furthermore, unmodified Nod Bj-V($C_{18:1}$) (pentamers) are unable to induce responses in soybeans. Interestingly, the unmodified tetramers are active or able to induce responses in soybeans, and pentamers strictly require the 2-*O*-methylfucose substitution to be active (Carlson et al. 1993; Stokkermans et al. 1995). Based on these results, we suggest that the NF Nod Bj-IV($C_{18:1}$) could be active in its capacity to induce biological responses in *G. max*, since other chemical groups are not bound to the NF and its structure is formed by tetramers.

B. japonicum USDA 138 under salt stress produced an NF formed by Nod Bj-IV($C_{18:1}$,MeFuc) that is unique to this condition. In this case, the structure was found to be formed by tetramers and octadecenoic acid, with the presence of the 2-*O*-methylfucose substitution. We have not evaluated the biological activity of each type of NF separately; hence, we do not know whether this structure is active. Likewise, the structure Nod Bj-IV($C_{18:1}$,MeFuc) has been cited for *B. (japonicum) elkanii* USDA61, which was classified as *B. japonicum* group II strains for some years (Carlson et al. 1993). Kuykendall et al. (1992) proposed the group II strains as a new species, *B. elkanii*. A particular characteristic of this reclassified group is its broader host range than that of group I strains (Elkan 1992). Under salt stress, *B. japonicum* USDA 138 is able to produce Nod Bj-IV($C_{18:1}$,MeFuc), a typical Nod factor in *Bradyrhizobium* strain/species with broader host range.

A new type of NF, Nod Bj-V($C_{16:1}$,MeFuc), was detected in *B. japonicum* USDA 138 under saline and osmotic stress. This structure with hexadecenoic acid is uncommon for *B. japonicum* strains; however, it has been cited for *B. japonicum* USDA 135 (Carlson et al. 1993). Our results contribute to discuss if the structure Nod Bj-V($C_{16:1}$,MeFuc) should be considered an uncommon structure produced by *B. japonicum.*

The biological activity tests of NFs produced under control, salt, and osmotic stress showed that all different mixtures of the three rhizobia growth conditions analyzed induced root hair deformations. Growth of this strain under stress did not alter its ability to induce responses in *G. max*. We have not evaluated the biological activity of each type of NFs separately nor do we have data about the amounts of each NF type. Likewise, the different mixtures were biologically active in terms of inducing deformations. Ovtsyna et al. (1999) proposed that not only the absolute levels of NFs are important, but also the composition and relative proportions of the mixtures excreted by rhizobia are necessary for the induction of different components of the nodulation pathway.

The radical application of purified NFs with the three mixtures had only a positive effect on dry matter accumulation, which has been cited in plants treated in hydroponic culture with the NF Nod Bj-V($C_{18:1}$,MeFuc); this response was observed along with increments in photosynthetic rate and stomatal conductance (Souleimanov et al. 2002; Almaraz et al. 2007; Khan et al. 2008). The positive effects of NFs,

both of radical and foliar applications, have been associated not only with positive effects on growth, but also with disease resistance (Duzan et al. 2005).

We also found that nodule numbers of plants inoculated with genistein-induced bacteria cultured under both control and stress conditions were significantly higher than those inoculated with noninduced bacteria. This response has been described for *B. japonicum*; this strategy to improve the number of nodules, however, has been found as a positive effect only in the greenhouse and not under field conditions (Pan and Smith 2000). Interestingly, bacteria cultured under osmotic stress also induced a significant increase in the number of nodules formed with respect to control condition, independently of the presence of genistein. Since sorbitol is a compound that can be metabolized by *B. japonicum*, this growth condition may induce the synthesis of compounds that positively influence the perception and infection, such as lipopolysaccharides, exopolysaccharides, and cyclic glucans.

The inoculation with genistein-induced bacteria cultured under both control and stress conditions also had some systemic effects that were dependent on the growth condition of *B. japonicum*. For example, while inoculation with induced *B. japonicum* under control conditions induced an increment in nodule number per plant, this increase was accompanied by a reduction in leaf area. By contrast, although inoculation with induced *B. japonicum* under saline condition generates an increase in nodule number per plant, this increase was accompanied by an increment in leaf area. This differential systemic response could be mediated by differences in the mixture of NFs produced under different growth conditions of *B. japonicum*.

We were interested in determining whether the inoculation with genistein-induced bacteria cultured under both control and stress conditions had any differential systemic effect at photosynthetic level. Therefore, we measured the chlorophyll fluorescence and greenness index. Interestingly, no differences in these two parameters among treatments were observed, indicating, for example, that the increase in nodule number was not related to these two specific photosynthetic parameters. Likewise, a positive effect on photosynthetic rate has been cited in treatments with a purified NF or genistein-induced *B. japonicum* (Almaraz et al. 2007; Khan et al. 2008); however, these responses were not observed in our experimental system.

In summary, *B. japonicum* USDA 138, the most widely used strain in Argentina, produces a mixture of four NFs, some of which have already been characterized for other *B. japonicum* strains. Under salt and osmotic stress conditions, this strain is able to produce new NF structures and all these mixtures had biological activity in *G. max* plants. Noticeably, there are some systemic differences, depending on *B. japonicum* growth and induction condition. In future works we will study systemic signaling induced by inoculation or by the application of radical NFs.

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References

Almaraz JJ, Zhou X, Souleimanov A, Smith D (2007) Gas exchange characteristics and dry matter accumulation of soybean treated with Nod factors. J Plant Physiol 164:1391–1393

Bais P, Weir L, Perry G, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. Annu Rev Plant Phys 57:233–266

Bouhmouch I, Mouhsine BS, Brhada F, Aurag J (2005) Influence of host cultivars and *Rhizobium* species on the growth and symbiotic performance of *Phaseolus vulgaris* under salt stress. J Plant Physiol 162:1103–1113

Broughton J, Jabbouri D, Perret X (2000) Keys to symbiotic harmony. J Bacteriol 182:5641–5652

Broughton W, Dilworth M (1971) Control of leghaemoglobin synthesis in snake beans. Biochem J 125:1075–1080

Cárdenas L, Holdaway-Clarke TL, Sanchez F, Quinto C, Feijo JA, Kunkel JG, Helper PK (2000) Ion changes in legume root hairs responding to Nod factors. Plant Physiol 123:443–452

Carlson RW, Sanjuan J, Bhat R, Glushka J, Spaink H, Wijijes AHM, van Brussel AAN, Stokkermans TJW, Peters NK, Stacey G (1993) The structures and biological activities of the lipo-oligosaccharide nodulation signals produced by type I and II strains of *Bradyrhizobium japonicum*. J Biol Chem 24:18372–18381

Cesco S, Mimmo T, Tonon G, Tomasi N, Pinton R, Terzano R, Neumann G, Weisskopf L, Renella G, Landi L, Nannipieri P (2012) Plant-borne flavonoids released into the rhizosphere: impact on soil bio-activities related to plant nutrition. A review. Biol Fertil Soils 48:123–150

Dardanelli MS, Manyani H, Gonzalez Barroso S, Rodriguez Carvajal MA, Gil Serrano AM, Espuny Gomez MR, López-Baena F, Bellogin Izquierdo RA, Megías Guijo M, Ollero Márquez FJ (2009) Effect of the presence of the plant growth promoting rhizobacterium (PGPR) *Chryseobacterium balustinum* Aur9 and salt stress in the pattern of flavonoids exuded by soybean roots. Plant Soil 328:483–493

Duzan H, Mabood F, Zhou X, Souleimanov A, Smith D (2005) Nod factor induces soybean resistance to powdery mildew. Plant Physiol Bioch 43:1022–1030

Elkan GH (1992) Taxonomy of the rhizobia. Can J Microbiol 38:446–450

Elsheikh E, Wood M (1995) Nodulation and N_2 fixation by soybean inoculated with salt-tolerant rhizobia or salt-sensitive bradyrhizobia in saline soil. Soil Biol Biochem 27:657–661

Estévez AJ, Fernández-Córdoba F, Soria Diaz ME, Manyani H, Gil Serrano AM, Thomas-Oates J, Van Brussel AAN, Dardanelli MS, Sousa Martin C, Megías Guijo M (2009) Different and new Nod factors produced by *Rhizobium tropici* CIAT899 following Na^+ stress. Fems Microbiol Lett 293:220–231

Felle Hubert H, Kondorosi E, Kondorosi A, Schultze M (2000) How alfalfa root hairs discriminate between Nod factors and oligochitin elicitors. Plant Physiol 124:1373–1380

Graham PH, Vance CP (2000) Nitrogen fixation in perspective: an overview of research and extension needs. Field Crop Res 65:93–106

Guasch-Vidal B, Estévez J, Dardanelli MS, Soria-Díaz ME, Fernández de Cordoba F, Balog C, Manyani H, Gil-Serrano A, Thomas-Oates J, Hensbergen PJ, Deelder AM, Megias M, van Brussel AAN (2012) High NaCl concentrations induce the nod genes of *Rhizobium tropici* CIAT899 in the absence of flavonoid inducers. Mol Plant Microbe Interact 26:451–460

Khan W, Prithiviraj B, Smith D (2008) Nod factor [Nod Bj V (C18:1, MeFuc)] and lumichrome enhance photosynthesis and growth of corn and soybean. J Plant Physiol 165:1342–1351

Kuykendall LD, Saxena B, Devine TE, Udell SE (1992) Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp.nov. Can J Microbiol 38:501–505

Morón B, Soria-Díaz ME, Ault J, Verroios G, Noreen S, Rodríguez-Navarro DN, Gil-Serrano A, Thomas-Oates J, Megías M, Sousa C (2005) Low pH changes the profile of nodulation factors produced by *Rhizobium tropici* CIAT899. JOCB 12:1029–1040

Muñoz N, Robert G, Melchiorre M, Racca R, Lascano R (2012) Saline and osmotic stress differentially affects apoplastic and intracellular reactive oxygen species production, curling and death of root hair during *Glycine max* L.–*Bradyrhizobium japonicum* interaction. Environ Exp Bot 78:76–83

Oldroyd G, Downie A (2008) Coordinating nodule morphogenesis with rhizobial infection in legumes. Annu Rev Plant Phys 59:519–546

Ovtsyna AO, Rademaker GJ, Esser E, Weinman J, Rolfe BG, Tikhonovich IA, Lugtenberg BJJ, Thomas-Oates JE, Spaink HP (1999) Comparison of characteristics of the nodX genes from various *Rhizobium leguminosarum* strains. Mol Plant Microbe In 12:252–258

Pan B, Smith D (2000) Genistein preincubation of *Bradyrhizobium japonicum* cells improves strain competitiveness under greenhouse, but not field conditions. Plant Soil 223:229–234

Sanjuan J, Russell WC, Spaink HP, Bhat UR, Barbour WM, Glushka J, Stacey G (1992) A 2-*O*-methylfucose moiety is present in the lipo-oligosaccharide nodulation signal of *Bradyrhizobium japonicum*. P Natl Acad Sci USA 89:8789–8793

Singleton P, Bohlool B (1984) Effect of salinity on nodule formation by soybean. Plant Physiol 74:72–76

Souleimanov A, Prithiviraj B, Smith D (2002) The major Nod factor of *Bradyrhizobium japonicum* promotes early growth of soybean and corn. J Exp Bot 53:1929–1934

Spaink HP (1995) The molecular basis of infection and nodulation by rhizobia: the ins and outs of sympathogenesis. Annu Rev Phytopathol 33:345–368

Spaink HP, Aarts A, Stacey G, Bloemberg GV, Lugtenberg BJ, Kennedy EP (1992) Detection and separation of *Rhizobium* and *Bradyrhizobium* Nod metabolites using thin-layer chromatography. Mol Plant Microbe In 5:72–80

Spaink HP, Sheeley DM, van Brussel AAN, Glushka J, York WS, Tak T, Geiger O, Kennedy EP, Reinhold VN, Lugtenberg BJJ (1991) A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium*. Nature 354:125–130

Stacey G (1995) *Bradyrhizobium japonicum* nodulation genetics. Fems Microbiol Lett 127:1–9

Stacey G, Luka S, Sanjuan J, Banfalvi Z, Nieuwkoop AJ, Chun JY, Forsberg LS, Carlson R (1994) nodZ, a unique host-specific nodulation gene, is involved in the fucosylation of the lipooligosaccharide nodulation signal of *Bradyrhizobium japonicum*. J Bacteriol 176:620–633

Stokkermans TJW, Ikeshita S, Cohn J, Carlson RW, Stacey G, Ogawa T, Peters NK (1995) Structural requirements of synthetic and natural product lipochitin oligosaccharides for induction of nodule primordia on *Glycine soja*. Plant Physiol 108:1587–1595

Vincent J (1970) A manual for the practical study of the root-nodule bacteria. Blackwell Scientific Publications, Oxford