FUNCTIONAL PLANT BIOLOGY

Negative short-term salt effects on the soybean-B. japonicum interaction and partial reversion by calcium addition

Journal:	*Functional Plant Biology*
Manuscript ID:	FP13085.R1
Manuscript Type:	Research paper
Date Submitted by the Author:	n/a
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Keyword:	Root hairs, Nodulation, Stress physiology, Symbiosis

Title: Negative short-term salt effects on the soybean-*B. japonicum* interaction and partial reversion by calcium addition

Running title: Soybean-*B. japonicum* interaction under salt stress

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Soybean-rhizobia symbiotic interaction is severely affected by salt stress. We analyze the short-term salt stress effects on soybean root hair ionic homeostasis, PR gene expression and its consequence on nodulation. Short exposure to salt affected root hair ionic homeostasis and PR protein gene expression, and reduced the nodule number. The addition of calcium, is a common agricultural practice to reverse salt effects. We report a positive effect of calcium addition on soybean nodulation under saline conditions.

Abstract

The short-term (2 h) effects of salt stress (50 and 150 mM NaCl), on early events of soybean-*B. japonicum* interaction were analyzed determining the following parameters in root hair with or without calcium addition: deformation, apoplastic superoxide radical production ($O_2^{\cdot-}$), root hair death and sodium/potassium ion content. We also analyzed whether this short-term salt stress influenced later formation of crown and noncrown nodules determining number and weight of

nodules. The negative effect of salt stress on these characters was attenuated by the addition of 5 mM $CaCl_2$. We also analyzed the expression of pathogenesis-related proteins (PRP) genes *PR-1*, *PR-2*, *PR-3*, and four isoforms of *PR-5*. The expression of *PR*-2 increased under saline conditions and decreased in osmotic treatment and saline treatment supplemented with calcium in the presence of the symbiont. The changes in *PR-2* expression levels together with the death of root hairs provide a possible mechanism for the inhibition of infection by the symbiont under salinity, and suggests a possible overlap with responses to plant pathogens.

Introduction

Salinity involves two stress components for plants: the osmotic stress given by the reduction of water stress and the ionic stress which is related with the ionic homeostasis alteration. Salinity affects several physiological and biochemical processes associated with plant growth and development (Zhu 2001). The negative effects of salt stress is in part a consequence of the oxidative damage induced by the enhanced production of reactive oxygen species (ROS) (Apel and Hirt 2004).

The dual role of ROS, as toxic or signal molecules, is determined by the rates and subcellular location of ROS generation and degradation (Mittler *et al.* 2004). The ROS production/degradation ratio is key determinant of the function and viability of the cell (Fedoroff 2006). The NADPH oxidase complex is a major ROS source in the apoplast. Plant NADPH oxidase proteins have a large hydrophilic domain with two calcium-binding EF hand motifs in the N-terminal region and the activity of NADPH complex is directly regulated by Ca_2^+ (Sagi and Flhur 2006).

Soybean (*Glycine max* L.) has been classified as a salinity-susceptible crop, with field performance being affected when salinity exceeds 5 ds/m (approximately 50 mM NaCl) (Ashraf 1994). The infection and nodulation process by *Bradyrhizobium japonicum* is also severely affected under salinity. Reduction of 50% in the number and weight of nodules in salinized plants at concentrations of 26.6 mM NaCl has been repeatedly reported (Singleton and Bohlool 1984; Delgado *et al.* 1994; Elsheikh and Wood 1995). Nodulation is a morphogenetic process that occurs during the plant development, and salt

tolerance responses in soybean is highly dependent on the developmental stage (Shao *et al.* 1986, 1993). How these processes are jointly affected by salt stress is still only partially understood.

The calcium addition to saline soils is a common agricultural practice that attenuates the negative effects of salt. Calcium has a key role in ionic homeostasis under salt stress (Demidchik and Maathuis 2007). Ionic homeostasis is necessary to maintain low concentrations of toxic ions and high concentrations of essential ions within the cell, and is regulated by ion fluxes. Particularly important is the sodium (Na^+)/ potassium (K^+) ratio, which is a main determinant of tolerance response to salt stress (Munns and Tester 2008). However, the effects of calcium on the legume rhizobia symbiotic interaction have not been investigated. In this work we analyzed the root hair ionic homeostasis during soybean-*B. japonicum* interaction with or without calcium addition.

Our group has characterized the apoplastic and intracellular reactive oxygen species (ROS) changes that occur during the root hair deformation in soybean-*B. japonicum* symbiotic interactions under control, osmotic and salt stress condition. These results showed a correlation between aposplatic ROS production and root hair curling, and strong negative effects of salt stress, inhibiting both processes and inducing a sustained intracellular ROS production and root hair death (Muñoz *et al.* 2012). The sustained production of ROS, in inoculated soybean root hairs subject to saline treatments, is similar to the response observed in root hairs of *Phaseolus vulgaris* elicited with chitosan (a fungal elicitor) (Cárdenas *et al.* 2008; Muñoz *et al.* 2012). These results led us to think that under salt stress, rhizobia could be sense as a pathogen, triggering pathogen defense response in legume plants. Likewise, in soybean has been cited that an incorrect recognition of the symbiont induce the synthesis of pathogenesis-related (PR) proteins (López-Baena *et al.* 2009). Interestingly, saline treatments also induce PR protein in soybean (Onishi *et al.* 2006; Tachi *et al.* 2009). However, whether this induction might affect the symbiotic interaction in soybean under salinity remains unexplored. In this work we analyzed the changes in expression of different PR, that have been studied in soybean under salinity and symbiotic interactions, like markers of plant-pathogen responses (van Loon *et al.* 2006) under the short-term salt stress treatments.

Early negative effect of salt stress in inoculated soybean root hairs could have consequences on later events, mainly affecting the formation of crown nodules. Crown root nodules, are those formed at early stage after seed germination, and provide the highest amount of the biological fixed nitrogen to soybean plants (Zdor and Pueppke 1988). In saline soils the highest salt concentrations are in the upper strata, where the initial steps of the symbiotic interaction occur together with seed germination (Bernstein 1975) and could have a strong effect on crown nodule formation and nitrogen biological fixation.

The aim of this work was to study the effects of short-term salt stress, with or without calcium addition, focused on root hair responses like root hair deformation, root hairs death, apoplastic superoxide production, root hair ionic homeostasis, pathogenesis-related protein expressions, and its consequence on nodulation.

Materials and methods

Bacterial strain and plant material

Soybean seeds disinfected with 5% sodium hypochlorite (V/V) for 5 min (*Glycine max* L. DM4800) were germinated on filter paper moistened with distilled water for 48 h in the dark. The seeds were incubated at 28ºC and 37ºC during the first and second 24-h periods, respectively, to promote the growth of roots and root hairs. *Bradyrhizobium japonicum* USDA 138 was cultured in yeast extract mannitol (YEM) medium (Vincent 1970) at 28ºC with constant agitation for 5 days (3 x 10^9 cells/mL). The bacteria were washed and resuspended in sterile water.

Saline and osmotic treatments of root hairs

Two days after germination, seedlings were transferred to aerated tubes that contained sterile water, NaCl (50 or 150 mM), or sorbitol (100 or 300 mM). These series of hyperosmotic solutions developed equal osmotic pressures (-0.55 and -0.84 MPa, respectively). The seedlings were inoculated with fresh bacterial cultures (1 mL of OD=0.6; 3 x 10^9 cells/mL). Measurements were performed after 2 h of treatment. Experiments with combinations of NaCl (50 or 150 mM) and $CaCl_2$ 5 mM were also performed to analyze the effects of added calcium.

Early effects of salt stress treatments on later stages of the interaction

To evaluate if the combined early effect of salt and inoculation with *B. japonicum* on root hairs had subsequent influence on nodule formation, treatments were performed on 48 h pre-germinated seeds for 2 h, as described in the previous section; then the seeds were washed with 5% Tween 20 for 40 s and rinsed 8 times with sterile distilled water.

The treated and inoculated seeds were placed in plastic trays with B and D nutrient solution (Broughton and Dilworth 1971) without nitrogen, aeration, 16:8 h photoperiod, 25°C and grown for 21 days; after that period, nodules formed in the roots were observed and their number and weight were evaluated. In each treated plant, nodules were discriminated between primary and secondary roots to obtain the number and weight of crown nodules and non-crown nodules, respectively.

Extraction of root hairs

Root hairs were extracted from roots subjected to different salt stress treatments and their respective osmotic controls with sorbitol. Root hairs were extracted by peeling the root zone containing young root hairs, which were immediately frozen in liquid air. Peeling was performed by making an incision with a scalpel under a magnifying glass and pulling the epidermal tissue that contains the root hairs with a fine-tipped clamp. Root hairs of approximately 200 roots (equivalent to 200 germinated seeds) generate sufficient material for a sample.

Na^+ and K^+ determination in roots hairs

Root hair samples subjected to different saline and osmotic treatments and saline treatment supplemented with calcium were weighed, immersed in a 1/10 (w/v) plant tissue/water, boiled for 30 min and centrifuged for 5 min at 12,000 g. Sodium (Na^+) and potassium (K^+) content in the extracting solution were determined using a Jenway PFP flame photometer. Ion concentration was calculated using a standard curve with known concentrations of sodium and potassium.

Root hair deformation

Root hair deformation were observed 2 hours after inoculation. Root hairs from different treatments were stained with 1% (w/v) toluidine blue and observed under an optical microscope. Eight roots per treatment and four sections per root were observed and counted (approximately 600 root hairs per treatment). The percentage of deformed root hairs was calculated as a proportion of the total number of root hairs in each section.

Apoplastic superoxide radical production in root hairs

Superoxide levels were determined with nitroblue tetrazolium (NBT), which reacts with superoxide radicals to produce a blue formazan precipitate. Roots were incubated in 0.01% (w/v) in the dark for 30 min. The reaction was stopped with absolute ethanol and the blue precipitate was quantified under an optical microscope.

Root hair death: Nuclear morphology, chromatin condensation and DNA fragmentation

Nuclear morphology, chromatin condensation and DNA fragmentation were evaluated using 4,6-diamino-2-phenylindole (DAPI). Roots were incubated 1 µg mL^{-1} of DAPI for 15 min. Nuclei of root hairs were visualized using epifluorescence microscopy (NIKON ECLIPSE Ti) with filter UV-2E/C (Ex: 360/40nm, DM: 400nm, Em: 460/50nm). Eight roots per treatment and four sections per root were observed and counted (approximately 600 root hairs per treatment). The percentages of nuclei with altered morphology, chromatin condensation or DNA fragmentation were calculated as a proportion of the total number of root hairs in each section with unaltered morphology. Two nuclear characteristics were observed and recorded: chromatin condensation (relatively uniform nuclear envelope with bright and stippled nuclear material, without marked nucleolus) and DNA fragmentation (notably affected nuclear envelope and morphology).

Image quantification

Apoplastic superoxide radical production determined by blue formazan staining was quantified using the image analyzer program OPTIMAS 6.1. For the quantification procedure, we selected the root zone that contained young root

hairs. The total distribution of stain intensity was measured as luminance by the image processing software. This luminance was transformed into optical density (OD). Optical density, the final parameter that represents signal intensity, was calculated relative to the tissue area analyzed.

RNA extraction

Samples subjected to different treatments were homogenized in a cold mortar with trizol (in a 1/10 plant material/phenol relation), mixed for 1 min and incubated at room temperature for 5 min. Then, 0.2 mL chloroform per mL of trizol was added and incubated at room temperature for 3 min. After incubation, the samples were centrifuged at 14,000 rpm at 4°C for 15 min. The aqueous phases were transferred to clean tubes. Then, 1 volume of isopropanol was added and the samples were incubated at room temperature for 10 min and centrifuged at 14,000 rpm, 4°C for 15 min. The precipitate was washed with 70% ethanol and the samples were centrifuged again at 14,000 rpm and 4°C for 15 min. The precipitate was dried and resuspended in DEPC water and its concentration was quantified in Thermo Scientific NanoDrop 3300. Purified RNA was treated with DNase I (Invitrogen) to remove genomic DNA, according to the manufacturer's instruction.

qRT-PCR

DNA-free RNA (1 to 2.5 µg) was used with oligo(dT) for first strand cDNA synthesis using the Moloney Murine Leukemia Virus for RT-PCR (Promega), according to the manufacturer's instruction. The lack of genomic DNA contamination was verified by qRT-PCR using primers able to amplify genomic DNA. The gene-specific primer pairs employed for the detection of transcripts of soybean were: four genes characterized under symbiotic and phatogenic interactions described by Mazarei *et al.* (2007), *GmPR-1* (acidic *PR-1* GenBank accession number BU577813 forward primer 5´-AACTATGCTCCCCCTGGCAACTATATTG-3´ reverse primer 5´-TCTGAAGTGGTAGCTTCTACATCGAAACAA-3´), *GmPR-2* (basic β-1,3-endoglucanase GenBank accession number M37753 forward primer 5´-TGAAATAAGGGCCACGAGTCCAAATG-3´ reverse primer 5´-ATGGTACATGCAGACTTCAAGAATGCAGAT-3´), *GmPR-3* (basic chitinase

GenBank accession number AF202731 forward primer 5´-AACTACAATTACGGGCAAGCTGGCAA-3´ reverse primer 5´-TTGATGGCTTGTTTCCCTGTGCAGT-3´), *GmPR-5* (thaumatin-like GenBank accession number BU765509 forward primer 5´-GCGCTTGCTCCGCTTTCAACT-3´ reverse primer 5´-CTTGGAATAGACGGTGGGCTTGC-3´), and three isoforms of soybean *PR-5* characterized under salt stress, described by Tachi *et al.* (2009), *GmOLPb* (neutral *PR-5* isoform GenBank accession number AB370233 forward primer 5´-ACCAATTTGGCAACCAGGAT-3´ reverse primer 5´-CATTGGTGCAGCAATACTCA -3´), *GmOLPa* (acidic *PR-5* isoform GenBank accession number AB116251 forward primer 5´-GTACACCTCCGAACACGTTG-3´ reverse primer 5´-TGGGACACTCTCCGATGATG -3´) and *GmP21e* (acidic *PR-5* isoform GenBank accession number AB370234 forward primer 5´-GTGCACACGTGGCATAAGGT-3´ reverse primer 5´-CACACAGCTACCGGAATTGC -3´). Gene-specific primer pairs for actin were used as an internal control forward primer 5´-AACGACCTTAATCTTCATGCTGC-3´ and reverse primer 5´-GGTAACATTGTGCTCAGTGGTGG-3´. qRT-PCR was performed in thermocycler iQ5 (BioRad) at 58°C with iQ SYBR Green Supermix (BioRad), according to the manufacturer's instruction. Relative expression levels with respect to inoculated control were calculated with the method of Livak and Schmittgen (2001).

Statistical analyses

Data were analyzed using analysis of variance (ANOVA) followed by the DGC (multiple-comparison method of Di Riezo, Guzmán and Casanoves in Infostat) test. All analyses were performed using the InfoStat program (InfoStat/Profesional ver. 2007p, Grupo InfoStat, Facultad de Ciencias Agropecuarias, Universidad Nacional de Cordoba, Argentina).

Results

Root hair deformation induced by B. japonicum inoculation under saline treatments: effect of calcium addition

Root hair deformation is the first morphological response during the legume-rhizobia symbiotic interaction. The percentages of soybean root hair deformation was evaluated 2 h after inoculation with *B. japonicum* under saline treatments and in saline treatments supplemented with calcium (Fig. 1). Root hair deformation was not affected under 50 mM NaCl treatment. Under 150 mM NaCl, root hair deformation was significantly reduced. Interestingly, these responses were partially reverted with the addition of calcium.

Apoplastic superoxide radical production in root hairs under saline treatments: effect of calcium addition

In a previous work we have demonstrated a close correlation between apoplastic superoxide production and root hair deformation. Apoplastic superoxide radical production in root hairs was determined 2 h after inoculation with *B. japonicum* under saline treatments and in saline treatments supplemented with calcium, (Fig. 2). The calcium addition partially reversed the inhibitory salt effect on apoplastic superoxide production, allowing a significant increase in the apoplastic superoxide radical production induced by the inoculation with *B. japonicum* in the 50 and 150 mM NaCl treatments.

Na^+ and K^+ ion content in root hairs during the symbiotic soybean-B. japonicum interaction under salt and osmotic stress: effect of calcium addition

Salt stress usually induce changes in cellular ionic homesotasis. The content of Na^+ in inoculated and non-inoculated root hairs subjected to saline treatments for 2 h increased in a dose-dependent manner compared to the control (Fig. 3A). However, K^+ levels were the same in all treatments (Fig. 3B). These results show that after 2 h, the Na^+/K^+ ratios increased significantly in a dose-dependent manner with respect to the control in all saline treatments, whilst did not differ significantly from that of the control in osmotic treatments. The addition of calcium to saline treatments reduced the Na^+/K^+ ratios with respect to the saline treatments without calcium, mainly due to a reduced influx of Na^+, since no alterations in K^+ content were detected (Fig. 3A and B).

Root hair death under saline treatments: effect of calcium addition

Cell death is characterized by changes in the nuclear morphology. The percentages of nuclei with chromatin condensation and DNA fragmentation (Fig. 5) in root hairs 2 h after inoculation with *B. japonicum* under saline treatments and in saline treatments supplemented with calcium are shows in Figure 4. The nuclei of root hairs in the control treatment exhibited an orthodox conformation, with similar size and shape (Fig. 5A), and with a very low percentage of nuclei with DNA fragmentation (Fig. 4). The inoculation of root hairs induced an slight increase of nuclei with chromatin condensation. Likewise, while the 50 mM NaCl treatment did not alter these percentages with respect to the control, 50 mM NaCl combined with *B. japonicum* induced root hair death, with an important increment of nuclei with DNA fragmentation and a lower increase of nuclei with chromatin condensation (Fig. 4). These responses were similar in 150 mM NaCl treatments. Interestingly, in saline treatments supplemented with calcium the percentages of nuclei with DNA fragmentation were significantly reduced with respect to the unsupplemented treatments; this change was associated with a significant increase in the percentage of nuclei with chromatin condensation (Fig. 4).

Expression of pathogen-related proteins

The expression levels for *PR-1*, *PR-2* and *PR-3* are shown in Figure 6. The transcripts levels of *PR-1* (Fig. 6A) increased in all conditions of saline and osmotic stress and salt stress supplemented with calcium. The expression of *PR-3* (Fig. 6C) increased in 150 mM NaCl, 100 mM Sorbitol and in both saline treatments supplemented with calcium in the presence of the symbiont. Interestingly, the expression levels increased for *PR-2* (Fig. 6B) only in saline treatment and this increase was reversed by the addition of calcium. We were not able to detect *PR-5* BU765509 expression in root hairs, even though that expression was detected in other cell types of the root (data not shown). Due to the inability to detect expression of this *PR-5* isoform in root hairs, we evaluated the expression of three other *PR-5* isoforms (*GmOLPa*, *GmOLPb* and *P21e*) (Fig. 6D, E and F) that have been characterized in soybean under salt stress conditions (Tachi *et al.* 2009). The three isoforms had expression in root hair. The levels of transcripts for *GmOLPa* (Fig. 6D) increased in all treatments of saline and osmotic stress and salt stress supplemented with calcium. Likewise, the levels of

transcripts for *GmOLPb* and *P21e* decreased or remained unaltered (Fig.6E and F).

Effects of short-term salt stress on nodulation

The number of crown nodules in roots transiently subjected to saline stress for 2 h during the early events of symbiotic interaction significantly decreased in a dose-dependent manner with respect to the control, whereas the number of nodules in osmotic treatments showed no significant differences (Fig. 7A).

Non-crown nodules increased significantly in plants treated with 50 mM and 150 mM NaCl for 2 h with respect to the control (Fig. 7B). On the other hand, osmotic treatments showed no significant differences in non-crown nodule number with respect to the control (Fig. 7B).

Crown nodule number in control condition supplemented with calcium for 2 h did not vary significantly with respect to the non-supplemented control; however, under saline treatments the addition of calcium partly reversed the negative effect of salt on the number of crown nodules (Fig. 7A). Interestingly, the number of non-crown nodules in calcium-supplemented saline treatments was lower than in the non-supplemented ones (Fig. 7B).

The average weight per nodule for all treatments were also discriminated between crown and non-crown nodules (Fig 7C and D). A positive correlation between weight and number was observed. The decrease in crown nodule number under saline treatments was correlated with smaller nodules than normal (Fig. 7C). Interestingly, under saline calcium-supplemented treatments, number and weight of crown nodules increased significantly with respect to unsupplemented saline treatment, without showing significant differences from the controls (Fig. 7C). Crown and non-crown nodules were inversely related in terms of number and weight; indeed, when number and weight of nodules decreased in the crown root, they increased in other parts of the roots (Fig 7C and D).

Discussion

The negative effect of short-term saline treatments on crown nodule formation was dose-dependent and was produced by the ionic component of salt stress, since the number of nodules remained unaltered in the osmotic controls of these treatments. This result suggests that the ionic homeostasis of root hairs

during the early events of the symbiotic interaction affects the number of nodules that later develop. The lack of infection in saline treatments is not due to a negative effect on rhizobia survival (Muñoz *et al.* 2012). The exogenous application of calcium to attenuate the negative effects of salt is a common agricultural practice (Rengel 1992; Shabala *et al.* 2006). The root hair deformation inhibition, particularly under 150 mM NaCl, was reverted with the addition of calcium. Root hair deformation is an early step that greatly improves the efficiency of Rhizobium infection. Likewise, root hair deformation only occurs in actively growing root hairs and is supported by the machinery that sustains the polarized growth of root hair. Our group previously reported the importance of localized apoplastic superoxide radical production supporting the deformation that is affected under saline treatments, (Muñoz *et al.* 2012). In this work we show that the addition of calcium to 150 mM saline treatments, preserved the apoplastic superoxide production, a necessary condition for the deformation and subsequent infection during the symbiotic interaction.

It has been suggested that some of the positive effects of calcium would be related to the balance in the opening and closing of non-selective cation channels (NSCC), since they involve not only the entry of sodium, but also the exit of potassium, and thereby contribute to the ionic homeostasis (Demidchik *et al.* 2007). Accordingly, the addition of $CaCl_2$ had a positive effect on the Na^+/K^+ ratio and prevented the entry of Na^+ in saline treatments combined with the symbiont, possibly by regulating NSCC, such as closing CCNS-IV (voltage independent), which are the main gateways of sodium into the cell. This effect of calcium on NSCC may be an important component in the regulation of ion homeostasis that helped to reverse the infection and nodule formation in short-term saline treatments.

Furthermore, the analysis of sodium and potassium ion content in our system revealed that the Na^+/K^+ ratios was increased in a dose-dependent manner during saline treatments and independently of the symbiont presence. Likewise, these ratios did not change in osmotic treatments. This change in the Na^+/K^+ ratios is due to sodium influx from the extracellular medium to the intracellular environment and not to an outflow of potassium. This result is particularly important because the loss of potassium has also been studied as another negative response of the ionic effect of salt, which contributes to the imbalance of Na^+/K^+,

resulting in subsequent loss of ionic homeostasis and cellular death (Shabala and Cuin 2008). We have previously reported that treatments with hyperosmotic solutions of sorbitol, (that developed equal osmotic pressures respect to saline treatments used in this study) did not induce cell death in root hairs (Muñoz *et al.* 2012), suggesting that the loss of viability could be due to the ionic component of the salt.

Death of soybean root hairs was detected by both high NaCl concentration (150 mM) and moderate NaCl (50 mM) combined with *B. japonicum* 2 h post inoculation. These cell death events were accompanied by differentials alterations in the generation of apoplatic superoxide radical and root hair deformation: absence of changes under 50 mM NaCl and a reduction under 150 mM NaCl. These results suggest that in both treatments could occur a differential induction of root hairs death involved in the subsequent decline of nodule formation. In this work we evaluated in detail the root hair death induced by saline treatments and in saline treatments supplemented with calcium. The progression of chromatin condensation can be classified into three stages during animal cell apoptosis: stage I, or ring condensation, stage II, or necklace condensation, and stage III, or nuclear collapse/disassembly (Toné *et al.* 2007). These stages are less clearly defined in plant cells, but the process of chromatin condensation progresses essentially in the same way and culminates in the formation of discrete domains of condensed and finally fragmented chromatin (Domínguez and Cejudo, 2006; Yamada *et al.* 2006). The differences observed in the nucleus morphology, particularly associated with the increase of nuclei with chromatin condensation and the decrease of nuclei with DNA fragmentation in saline treatments supplemented with calcium, with respect to the unsupplemented, indicates that the addition of calcium to saline treatments inhibited or at least delayed the cell death process. This inhibition or delay in the death progress, given by calcium addition, could help to sustain the rhizobia infection and subsequent nodule formation.

We have also demonstrated a synergetic effect of salt stress and inoculation, in the induction of root hair cell death (Muñoz *et al.* 2012). These results led us to hypothesize that under salt stress, the symbiont could be recognized by the plant as a pathogen, and the response shifted to a plant-pathogen like response. We evaluated the expression of four PR characterized in symbiotic and pathogenic interactions of soybean (Mazarei *et al.* 2007; López-

Baena *et al.* 2009) and three *PR-5* isoforms of soybean characterized in saline treatments (Tachi *et al.* 2009). In root hairs, we were not able to detect soybean *PR-5* (BU765509) expression, even though its expression is detected in the remaining root, this result suggests that this *PR-5* isoform may not have expression in root hairs. Likewise, of the other three isoforms of *PR-5* evaluated, two showed no change in the applied treatments or decreased expression and, only one (*GmOLPa*) was increased in both saline treatments as in the calcium-supplemented treatments and even in the osmotic control with sorbitol. This result indicates that in root hairs, the transcript levels of these *PR5* isoforms increases in response to any osmotic pressure change in the medium. Similarly, the levels of transcripts for *PR-1* and *PR-3* were increased in all treatments of saline and osmotic stress and salt stress supplemented with calcium.

Interestingly, the enhanced *PR-2* expression under saline treatments was reversed by calcium addition, and these induction of *PR-2* expression was not observed in the osmotic controls. These results, together with the reversion of sodium content, delayed root hair death and nodule formation in calcium-supplemented saline treatments, and the absence of negative responses in the osmotic controls, suggest that *PR-2* may be involved in the inhibition of infection and nodule formation by NaCl treatments. *PR-2*, a β-1,3-endoglucanase has a key role in the defense responses against several plant pathogens; by promoting the release cell-wall derived material that can act as defense elicitors (Leubner-Metzger and Meins 1999). During nodulation, the colonization of the host plant by symbiotic rhizobia does not elicit plant defense reactions induced by pathogens, although at some stages the symbiotic infection resembles a pathogenic interaction. However, under certain circumstances, various defense reactions might take place in legume-rhizobia interactions, which can provoke the abortion of the infection in necrotic cells, concomitant with an accumulation of phenolic compounds and PR proteins (Vasse *et al.* 1993; Mithöfer 2002). Likewise, it was suggested that the plant controls the extent of nodule number by a systemic mechanism similar to innate immunity (Zamioudis and Pieterse 2012).

The impossibility of re-infection in the root zone that was subjected to salt stress suggests a priming effect. In addition, the re-infection events that occured in the non-crown root zone after removing the stress treatment strongly suggest that, given the inability of *B. japonicum* to infect the salt-primed area, the formation of

nodules would have been induced in other parts of the root after the stress period. This redistribution of root nodules is not due to differences in root architecture, and shows how short-term and early saline stress affects the number and distribution of soybean nodules. This shift on the nodulation pattern, from crown to non-crown nodules induced by short-term salt effect, have an important negative effect on biological nitrogen fixation in soybean crops, because crown nodules provide the greater amount of fixed nitrogen to the soybean plant (Zdor and Pueppke 1988)

Finally, considering that in field conditions the crown nodules are formed after infection of root hairs during the first days after germination, the increase of soil salinity at this stage can be very relevant for the subsequent formation of crown nodules, and the addition of calcium to the soil as an agricultural practice can attenuate the negative effects of salt on the infection and nodulation process. Future experiments will be carried out to study the priming effects induced by short-term salt stress exposure and the consequences in different responses that are related to the microorganism interactions in this experimental system.

Acknowledgement

This work was supported by grants from the Agencia de Promoción Científica y Tecnológica, National Microscopy Commission and the Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina. RL is fellow of CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina).

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Fig. 1. Percentage of root hair deformation after two hours under different treatments: NaCl (50 or 150 mM), and NaCl (50 or 150 mM) supplemented with 5 mM $CaCl_2$, all of them inoculated with *B. japonicum.* Each value represents the mean ± SE from three independent experiments. Different letters indicate significant differences in the mean ($p < 0.05$ DGC test).

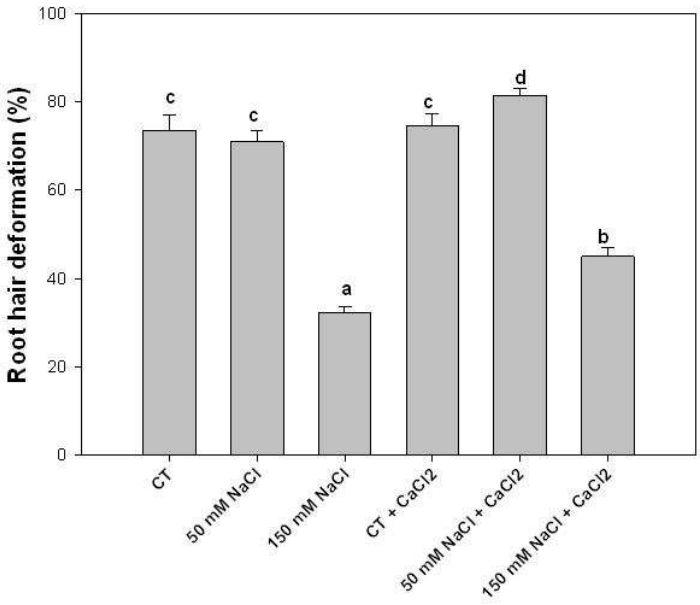
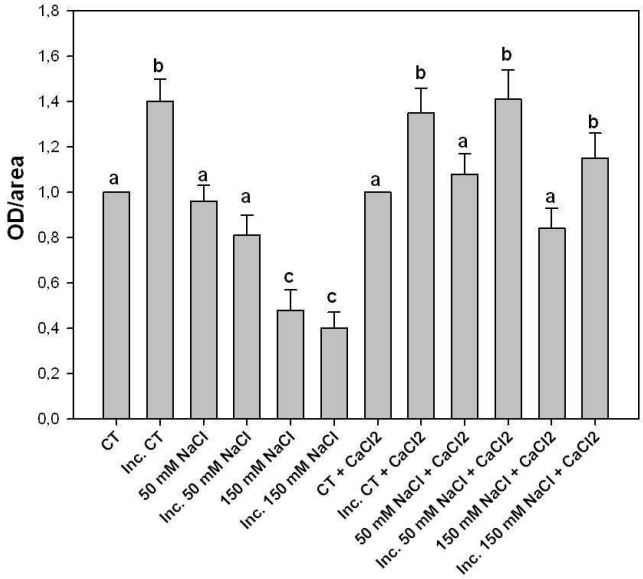
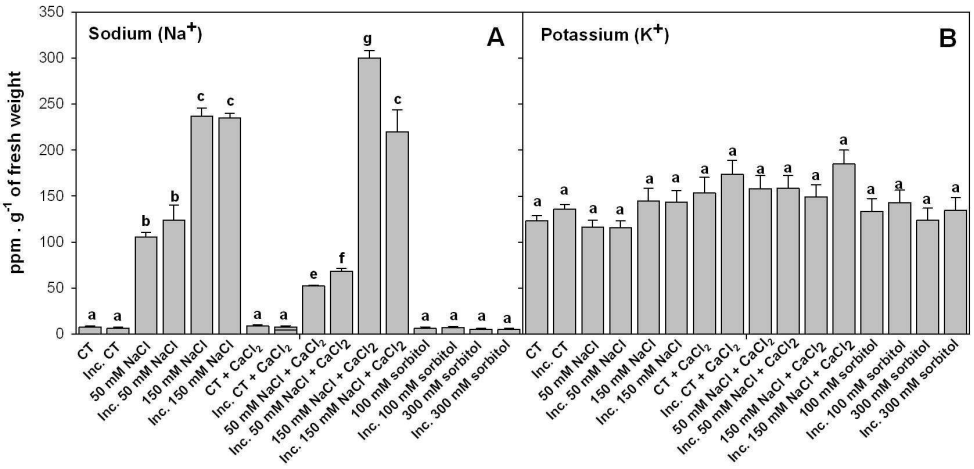


Fig. 2. Apoplastic superoxide radical levels with respect to the control after two hours under different treatments: NaCl (50 or 150 mM), and NaCl (50 or 150 mM) supplemented with 5 mM $CaCl_2$, all of them inoculated and noninoculated with *B. japonicum.* Each value represents the mean ± SE from three independent experiments. Different letters indicate significant differences in the mean (p <0.05 DGC test).



649 Fig. 3. Ion content in soybean root hairs after two hours under different
650 treatments: NaCl (50 or 150 mM), Sorbitol (100 or 300 mM), and NaCl (50 or
651 150 mM) supplemented with 5 mM $CaCl_2$, all of them inoculated and
652 noninoculated with *B. japonicum.* Sodium ion content (A), potassium ion content
653 (B). Each value represents the mean ± SE from three independent experiments
654 (root hairs from 200 roots per experiment, equivalent to 200 germinated seeds).
655 Different letters indicate significant differences in the mean ($p < 0.05$ DGC test).



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669 Fig. 4. Percentages of nuclei with chromatin condensation or DNA fragmentation
670 after two hours under different treatments: NaCl (50 or 150 mM), and NaCl (50 or
671 150 mM) supplemented with 5 mM $CaCl_2$, all of them inoculated and
672 noninoculated with *B. japonicum.* Each value represents the mean ± SE from three
673 independent experiments. Different letters indicate significant differences in the
674 mean ($p < 0.05$ DGC test).

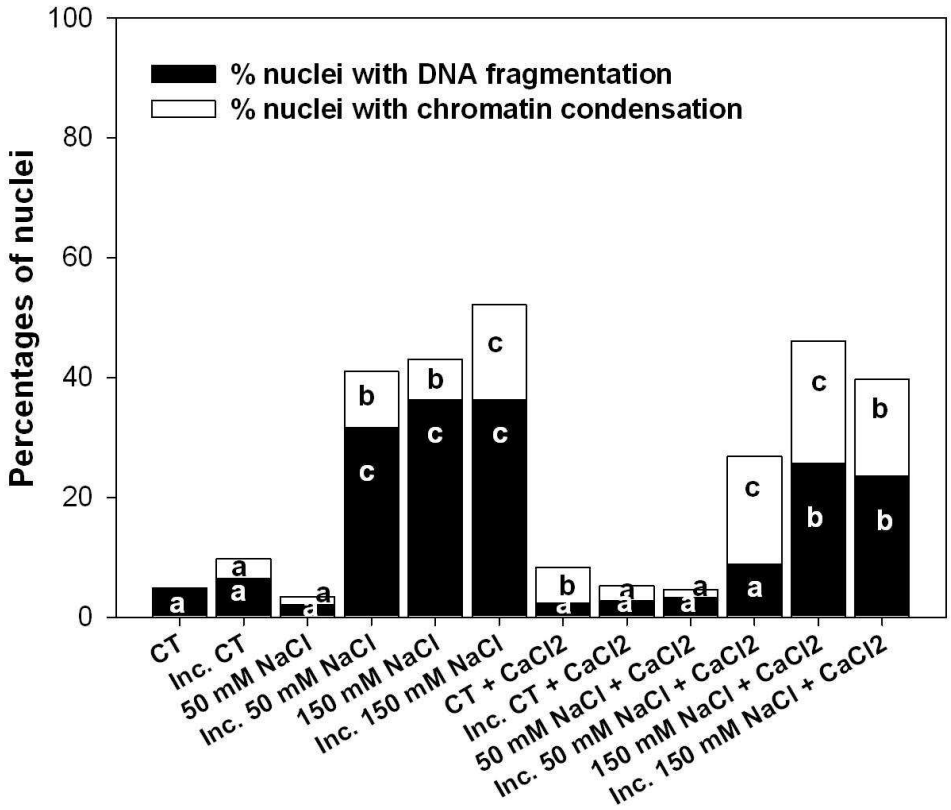

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Fig. 5. Nuclei of root hairs that represent normal nuclei (A), nuclei with chromatin condensation (B) and nuclei with DNA fragmentation (C). Roots were stained with DAPI. Nuclei of root hairs were visualized using epifluorescence microscopy (NIKON ECLIPSE Ti) with filter UV-2E/C (Ex: 360/40nm, DM: 400nm, Em: 460/50nm).

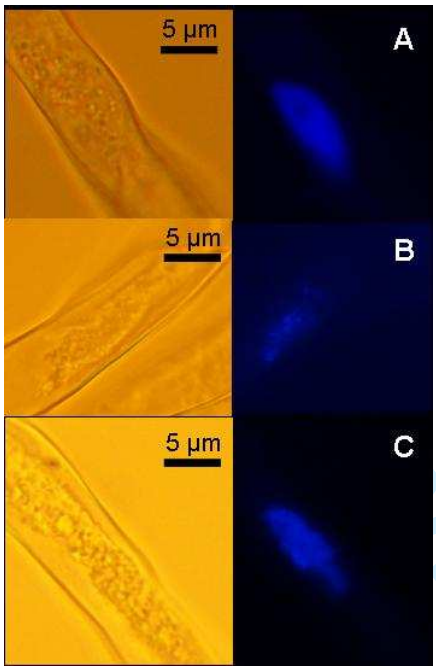


Fig. 6. Expression levels for *PR-1* (A), *PR-2* (B), *PR-3* (C), *PR-5 GmOLPa* (D), *PR-5 GmOLPb* (E) *and PR-5 GmP21e* (F) in soybean root hairs after two hours under different treatments: NaCl (50 or 150 mM), Sorbitol (100 or 300 mM) and NaCl (50 or 150 mM) supplemented with 5 mM $CaCl_2$, all of them inoculated with *B. japonicum.* Each value represents the mean ± SE from three independent experiments. Asterisks indicate significant differences respect to non-inoculated control ($p < 0.05$).

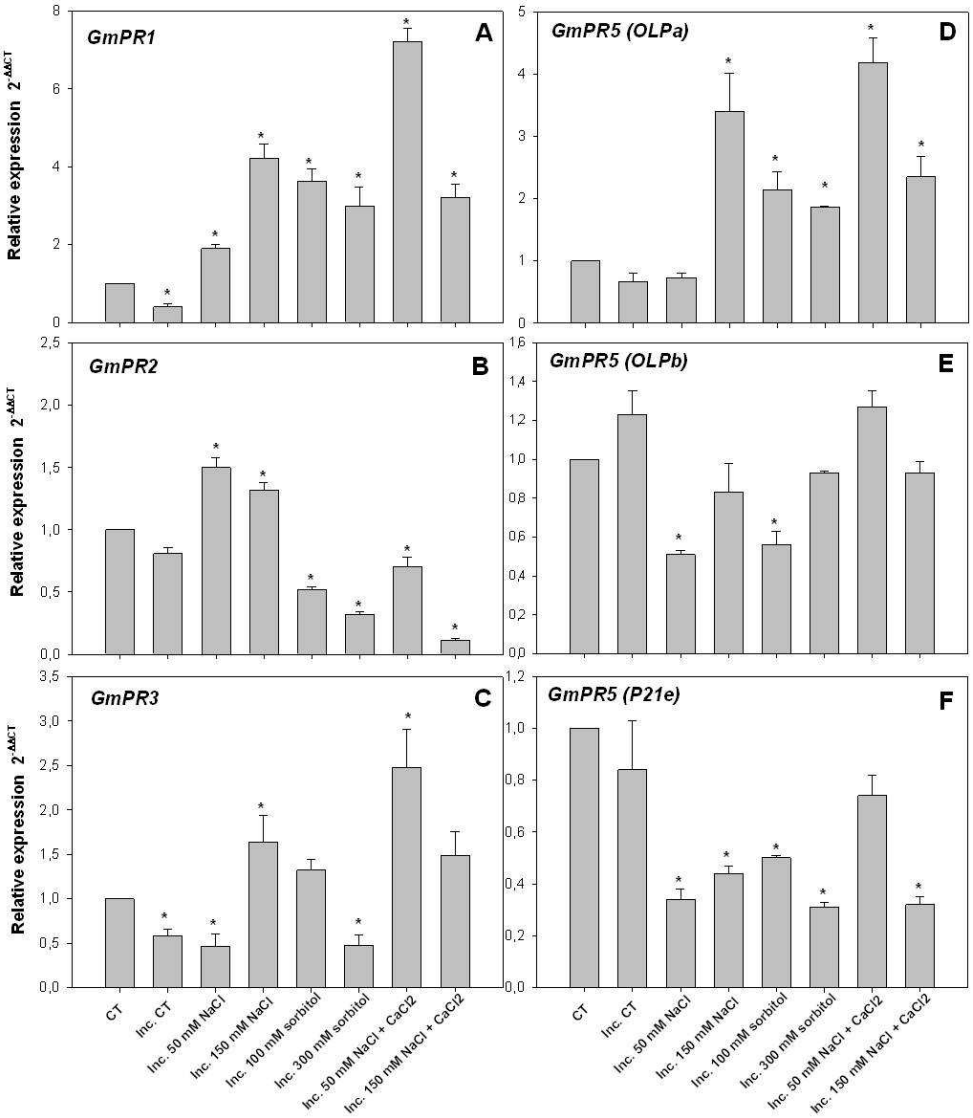
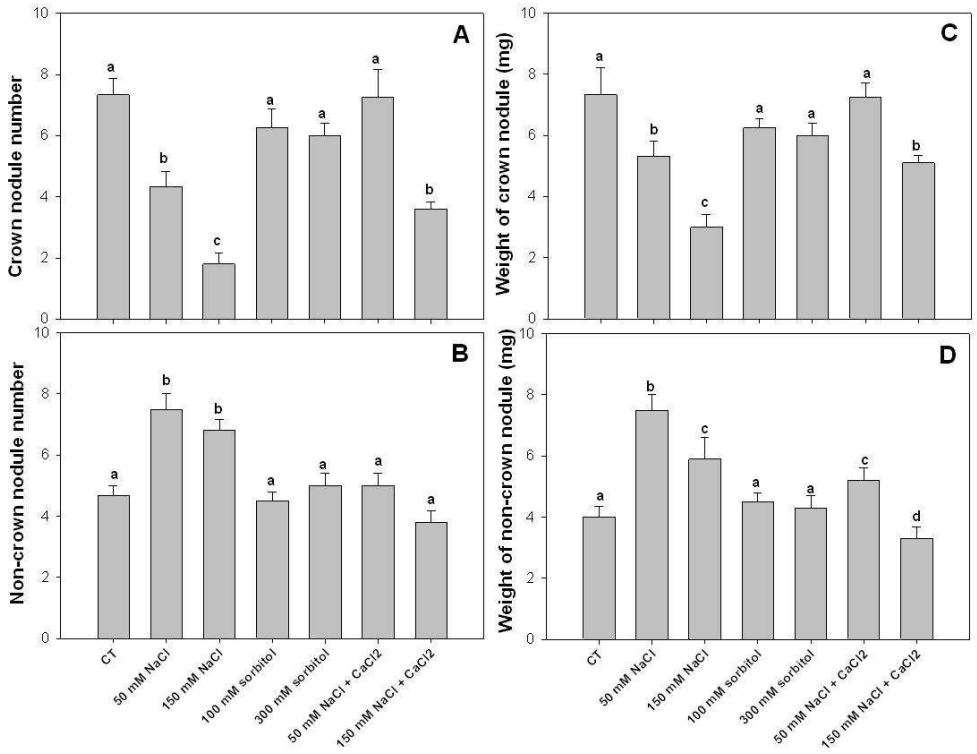


Fig. 7. Nodule number and weight, in soybean plants treated at germinated seed stage for two hours with: NaCl (50 or 150 mM), Sorbitol (100 or 300 mM) and NaCl (50 or 150 mM) supplemented with 5 mM $CaCl_2$ all of them inoculated with *B. japonicum.* Crown nodules number (A), non-crown nodules number (B), crown nodules weight (C), non-crown nodules weight (D). Each value represents the mean ± SE from three independent experiments (12 plants per experiment). Different letters indicate significant differences in the mean ($p < 0.05$ DGC test).



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