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

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ABSTRACT

In the present study, the production of apoplastic and intracellular reactive oxygen species (ROS) and deformations of young soybean root hairs inoculated with *Bradyrhizobium japonicum* strain USDA138 were analyzed under saline and osmotic stress. Sustained and transient increase of apoplastic and intracellular ROS production, respectively, were observed in inoculated root hairs. The apical production of apoplastic superoxide in growing root hairs colocalized with flavonoid autofluorescence and both were relocated to the zone of maximum curvature in curled root hairs. Saline and osmotic stress had differential effects on both the production of apoplastic ROS and curling: only saline stress inhibited both processes in a dose-dependent manner. Intracellular ROS production was not altered by osmotic stress but was inhibited completely by 150 mM NaCl. In inoculated root hairs under 50 mM NaCl, the intracellular ROS levels were initially increased, but not decreased at later stages, as occurred in control conditions. Root hair death was induced by 150 mM NaCl in both inoculated and noninoculated roots and by 50 mM NaCl only in inoculated roots. Saline, but not osmotic stress, marked affects both apoplastic and intracellular ROS production, inhibiting root hair curling and inducing root hair death.

1. Introduction

The symbiotic interaction between legumes and *Rhizobium spp.* Induces several biochemical, genetic, and morphological changes in root hairs (Brechenmacher et al., 2008; Jinrong et al., 2005; Meschini et al., 2008; Oldroyd and Downie, 2008). The molecular dialogue that occurs during this interaction involves the exchange of molecular signals. Plants secrete flavonoids, which are recognized by compatible rhizobia and induce the expression of several genes that encode proteins involved in the synthesis and secretion of the well-known Nod factors (NF). In growing root hairs, NF induces plasma membrane depolarization, intracellular alkalinization, calcium spiking, and the generation of reactive oxygen species (ROS). These responses occur within minutes to hours after NF application (Cárdenas et al., 2000; Felle et al., 2000; Wais et al., 2000).

Root hair curling is an early and essential morphological change induced by rhizobia and it is supported by the machinery involved in root hair growth (Oldroyd and Downie, 2008; Tetsuya et al.,

2008). Similarly to pollen tubes, root hairs exhibit polarized tip growth, which is an oscillatory process that involves active exo- and endocytosis, pH changes, calcium mobilization, ROS generation, and changes in cell wall properties (Monshausen et al., 2007).

Some researchers have indicated that NAD(P)H oxidase activity, which is one of the major apoplastic sources of ROS, is necessary for the establishment of the tip-based calcium gradient, which is essential for the polarized growth of root hairs (Foreman et al., 2003; Knight, 2007; Rachel and Dolan, 2006). ROS production and calcium fluxes occur a few minutes after root hair cells are challenged with NF, and show similar temporal and spatial responses (Cárdenas et al., 1998, 2008; Lohar et al., 2007; Oldroyd and Downie, 2008). NAD(P)H oxidase is activated by calcium, and the resulting ROS regulate calcium channels in the plasma membrane (Rachel and Dolan, 2006). Root hair deformation during Rhizobium–legume interaction involves a reorientation of the polarized growth of root hairs, and ROS and calcium seem to have key roles in this process (Cárdenas et al., 1998, 2008; Lohar et al., 2007). Most of the studies on this issue have been focused on intracellular ROS production. Apoplastic ROS production and its relationship with intracellular ROS in response to the recognition of NF or Rhizobium inoculation have not been described in detail.

Saline and drought stresses have been shown to affect the symbiotic interaction between legumes and rhizobia markedly. The soybean nodulation process is also extremely sensitive to

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salinity and drought (Ashraf, 1994; Singleton and Bohlool, 1984; Zahran and Sprent, 1986). However, the majority of these studies were focused on the effects of stress on late events in the symbiotic interaction, such as carbon and nitrogen metabolism in nodules. As a consequence, the effects of stress on early symbiotic events have been studied less. It is widely known that the ratio of generation to degradation for ROS is affected markedly by different stress conditions (Miller et al., 2010). In grasses, apoplastic ROS that are produced by NAD(P)H oxidase, a process that is related to cell elongation, are inhibited under saline stress but not under osmotic stress (Bustos et al., 2008; Rodriguez et al., 2002, 2004, 2007).

The production of ROS in root hairs is an important response to both symbiotic processes and abiotic stress, and ROS are also involved in signaling and determination of the shape of root hair cells.

The aim of the present work was to evaluate the apoplastic and intracellular ROS production during the early event of Rhizobium–legume interaction as is root hair curling, and to analyze the of saline and osmotic stresses effects on these processes.

2. Material and methods

2.1. Bacterial strain and plant material

Soybean seeds were disinfected with sodium hypochlorite 5% (v/v) for 5 min. (*Glycine max* L. DM4800) and germinated in the dark for 48 h on filter paper moistened with distilled water. The seeds were incubated at 28 and 37 °C 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 28 °C with constant agitation for 5 days (3×10^9 cells/ml). The bacteria were washed and resuspended in sterile water.

2.2. Saline and osmotic treatment of root hairs

After germination, seedlings were transferred to aerated tubes that contained sterile water, NaCl (50, 100, or 150 mM), sorbitol (100, 200, or 300 mM), or polyethylene glycol (PEG) 8000 (8.5, 11.3, or 15%). These series of hyperosmotic solutions develop equal osmotic pressures (−0.55, −0.69, and −0.84 MPa, respectively). The seedlings were inoculated with fresh bacterial cultures (1 ml of OD = 0.6; 3×10^9 cells/ml). Measurements were performed after 2, 5, 15, 30, 60, and 120 min of treatment.

2.3. Apoplastic superoxide radical production in roots and 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. This reaction was also carried out in the presence of superoxide scavengers (10 mM tiron or 10 mM $MnCl_2$) or in the presence of 50 μM diphenyleneiodonium (DPI), which is an inhibitor of NAD(P)H oxidase. Photographs were taken under a stereoscopic microscope with a NIKON DS camera (DS Camera Control Unit DS-L1, DS Camera Head DS-5M, and DS Cooled Camera Head DS-5Mc).

2.4. Intracellular ROS

Reactive oxygen species were measured with chloromethyl dichlorofluorescin diacetate (CM-H$_2$DCFDA). Roots were incubated with 10 μM CM-H$_2$DCFDA for 15 min and epifluorescence was

observed with an Axiophot microscope (Zeiss, Germany) with excitation filter BP 450–490 and emission filter LP 520. Images were taken with the camera described in Section 2.3.

2.5. Autofluorescence of flavonoids

Flavonoids were observed by autofluorescence (Wasson et al., 2006) using epifluorescence microscopy (Axiophot microscope, Zeiss, Germany) with excitation filter BP 365 and emission filter LP 397. Images were taken with the camera described in Section 2.3.

2.6. Root hair deformation

Two 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. Four typical types of deformation were observed: curling, branching, wiggling, and bulging.

2.7. Root hair death

The viability of root hairs was determined by Evans Blue staining. Root segments (containing young root hairs) were incubated in 0.05% Evans Blue solution for 1 h, observed under an optical microscope, and then solubilized with dimethylsulfoxide (DMSO) and quantified spectrophotometrically at 600 nm (Qiao et al., 2002). Absorbance per sample was expressed with respect to the weight of the root segment (OD/g).

2.8. Viability of B. japonicum under the different treatments

To evaluate the effect of different stress treatments on the viability of *B. japonicum*, we use colony forming unit (CFU) analysis, making serial dilutions of *B. japonicum* cultures and counted the dilution 10^{-6}.

2.9. Image quantification

Apoplastic and intracellular ROS determined by blue formazan staining and CM-H$_2$DCFDA fluorescence, respectively, were quantified using the image analyzer program OPTIMAS 6.1. For both quantification procedures, we selected the root zone that contained young root hairs. The total distribution of stain or fluorescence 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 the signal intensity, was calculated relative to the tissue area analyzed.

2.10. Statistical analyses

Data were analyzed using analysis of variance (ANOVA) followed by the DGC (multiple-comparisons method) or Tukey test. All analyses were performed using the InfoStat program (InfoStat/Profesional ver. 2007p, Grupo InfoStat, Facultad de Ciencias Agropecuarias, Universidad Nacional de Cordoba, Argentina).

3. Results

3.1. Root hair deformation induced by B. japonicum inoculation

The evaluation of root hair deformation was restricted to young root hairs of approximately 100 μm in length. Under normal
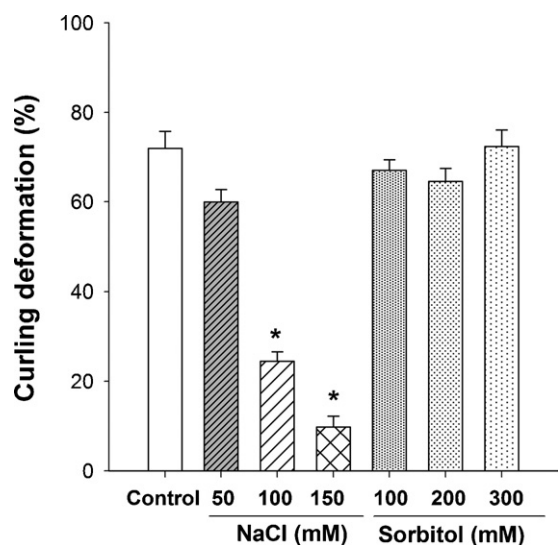
Fig. 1. Percentage of root hairs that exhibited curling 2 h after inoculation with *B. japonicum* under conditions of saline and osmotic stress. Each value represents the mean ± SE from three independent experiments. Asterisks indicate significant differences in the mean ($p < 0.05$ DGC test).

conditions, soybean roots that have been inoculated with *B. japonicum* undergo four types of root hair deformation, which resemble the morphological changes described previously by Duzan et al. (2004): wiggling, bulging, curling, and branching (Supplementary Fig. S1). We found that, except in the case of treatment with DPI (see below), the predominant type of root hair deformation induced by *B. japonicum* was curling, which is the type that drives the progression of infection.

### 3.2. Saline stress reduced B. japonicum-induced root hair curling

To evaluate the effect of different intensities of saline and osmotic stress on the curling of root hairs, soybean roots were inoculated with *B. japonicum* and subjected simultaneously to different concentrations of NaCl (50, 100, or 150 mM) or nonionic osmotic solutions (sorbitol at 100, 200, or 300 mM) for 2 h. The series of solutions used develop equal osmotic pressures (−0.55, −0.69, and −0.84 MPa, respectively). Note that the different stress treatments did not affect the viability of *B. japonicum*, as demonstrated by colony forming unit (CFU) analysis (Supplementary Fig. S2).

A clear differential response with respect to root hair curling was observed when inoculated soybean roots were subjected to saline or osmotic stress. In general, exposure to high salt concentrations (100 and 150 mM NaCl) (Fig. 1) reduced root hair curling markedly compared with that of the control.

Treatment with 50 mM NaCl led to a nonsignificant reduction in root hair curling. Under the osmotic treatments, i.e. either sorbitol (Fig. 1) or PEG solution (data not shown), the percentage of root hairs that underwent curling did not differ from that in the control. These results indicated that the ionic component of saline stress induced the reduction in the response of root hair deformation (Fig. 1).

3.3. B. japonicum-induced root hair curling is dependent on superoxide radicals

Root hair deformation was evaluated in the presence of the superoxide radical scavengers tiron and MnCl₂ (Fig. 2) and DPI (Fig. 3), which is an inhibitor of NAD(P)H oxidase and other flavin-containing enzymes. The scavengers of superoxide radicals inhibited all types of root hair deformation drastically. In contrast,
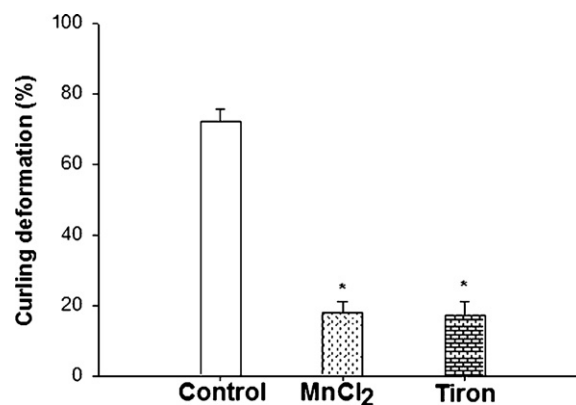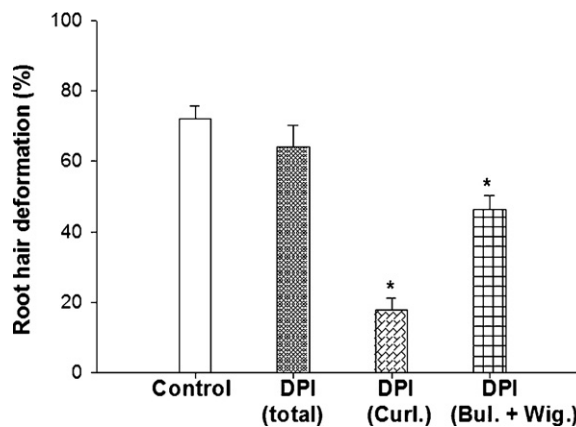

Fig. 2. Percentage of root hairs that exhibited curling 2 h after inoculation with *B. japonicum* and in the presence of an apoplastic ROS scavenger (10 mM tiron or MnCl₂). Each value represents the mean ± SE from three independent experiments. Asterisks indicate significant differences in the mean ($p < 0.05$ DGC test).

DPI treatment specifically inhibited curling, and induced other types of deformation, such as wiggling and bulging (Fig. 3). Interestingly, these types of deformation only appear in DPI treatments and were absent in the other treatments, where the predominant deformation was curling (Figs. 1 and 2).

3.4. Apoplastic superoxide radical levels and flavonoids in root hairs
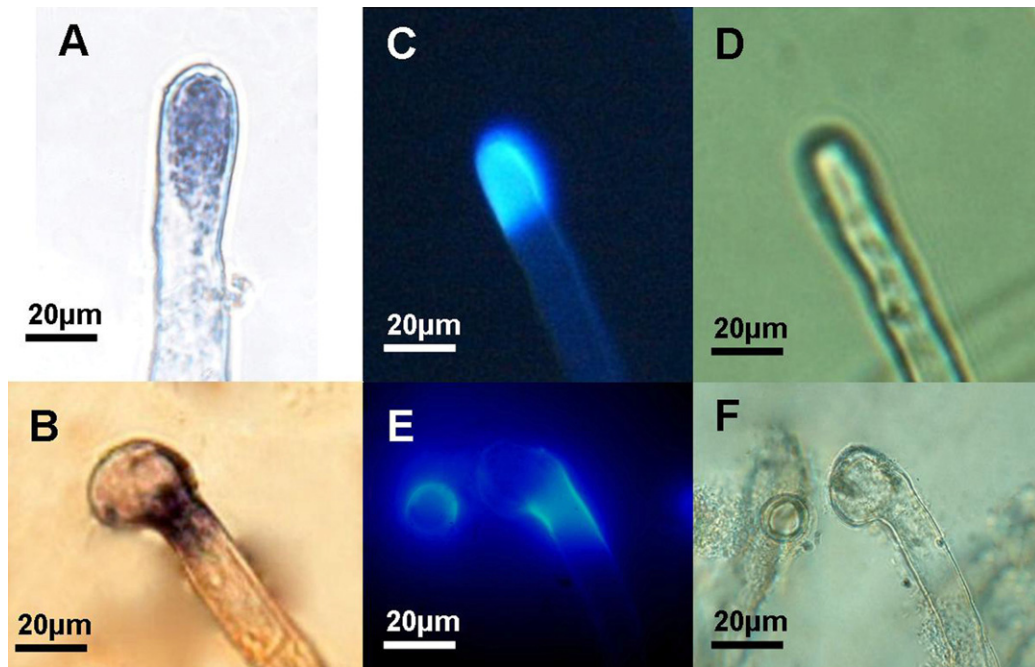
In growing noninoculated soybean root hairs, apoplastic superoxide was observed in the tips of the root hairs (Fig. 4A). This apical production of superoxide colocalized with the typical blue autofluorescence produced by flavonoids (Wasson et al., 2006) (Fig. 4C and E). The levels of apoplastic superoxide radicals in different parts of the soybean root were increased markedly by the inoculation with *B. japonicum* (Supplementary Fig. S3). The increased level of superoxide was maintained from 1 min postinoculation (data not shown) up to 120 min, when root hair curling was observed. In curled root hairs, the increased level of superoxide radicals moved from the apical to the subapical region (Fig. 4B), which corresponds to the zone of maximum curvature. Likewise, the site of superoxide radical generation colocalized both temporally and spatially with flavonoid-associated blue fluorescence (Fig. 4C and E).

In root hairs exposed to saline stress, the described apoplastic production of superoxide that was induced by inoculation with *B. japonicum* was abolished. In contrast, osmotic stress only induced

Fig. 3. Percentage of root hairs that exhibited deformation (counted separately) 2 h after inoculation with *B. japonicum* in the presence of DPI (50 μM). Each value represents the mean ± SE from three independent experiments. Asterisks indicate significant differences in the mean ($p < 0.05$ DGC test).

Fig. 4. Apoplastic superoxide radical levels in noninoculated growing root hairs (A) and curled root hairs 2 h postinoculation (B), and blue autofluorescence of flavonoids in noninoculated growing root hairs (C) and curled root hairs 2 h postinoculation (E). (D) and (F) Bright-field images from fluorescent images (C) and (F).

a minor change in the levels of apoplastic superoxide radicals, even at higher intensities (Fig. 5A and Supplementary Fig. S3). Similarly, when roots were treated with superoxide scavengers or DPI, apoplastic ROS production decreased drastically (Fig. 5B and Supplementary Fig. S3).

3.5. Intracellular ROS levels in root hairs

Use of the ROS-sensitive fluorescent dye (CM-H$_2$DCFDA) revealed that there were two major sites of intracellular ROS production in growing root hairs: the apical zone and the perinuclear zone, which both correspond to enriched cytoplasmic regions (Fig. 6A and C). The kinetics of intracellular ROS production in inoculated and noninoculated root hairs under control, saline, and osmotic stress conditions were monitored from as early as 2 min to 120 min after the initiation of treatment (Fig. 7 and Supplementary Fig. S4). Intracellular ROS levels in young inoculated root hairs

increased within 2–5 min (Fig. 6C); thereafter, the generation of ROS decreased and had disappeared completely 2 h after inoculation (Fig. 7 and Supplementary Fig. S4). The fluorescence decrease was much more evident in the perinuclear zone (Fig. 7C and D) than in the apical cytoplasmic (Fig. 7A and B).

The intracellular generation of ROS in root hairs was inhibited rapidly and completely by treatment with 150 mM NaCl; this response was observed in both noninoculated and inoculated roots. At 50 mM NaCl, intracellular levels of ROS increased initially in both noninoculated and inoculated young root hairs. However, in inoculated root hairs, the increased ROS level did not decrease at later stages, as observed under control conditions. In noninoculated roots treated with 50 mM NaCl, intracellular ROS levels started to decrease 5 min after treatment (Fig. 7A and C and Supplementary Fig. S4).

In contrast, the osmotic treatments did not alter the intracellular ROS kinetics significantly from those observed under control
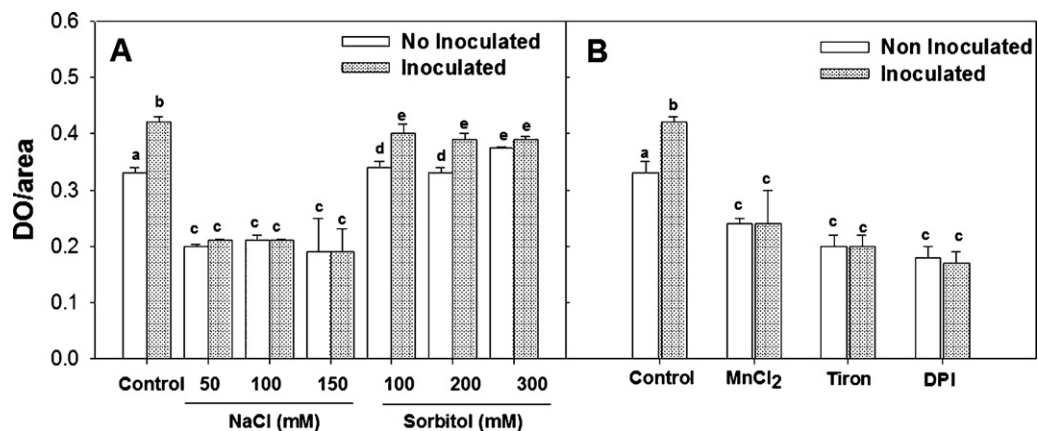
Fig. 5. Apoplastic superoxide radical levels in noninoculated (white bars) and inoculated roots (grey bars) 2 h after inoculation. (A) NaCl (50, 100, 150 mM) and sorbitol (100, 200, 300 mM). (B) DPI (50 μM) and superoxide scavengers (10 mM tiron or MnCl$_2$). The total distribution of stain intensity was measured as luminance by the image processing software and transformed into optical density (OD). Optical density was calculated relative to the area analyzed. Each value represents the mean ± SE from three independent experiments (eight roots per experiment). Different letters indicate significant differences in the mean ($p < 0.05$ Tukey test).
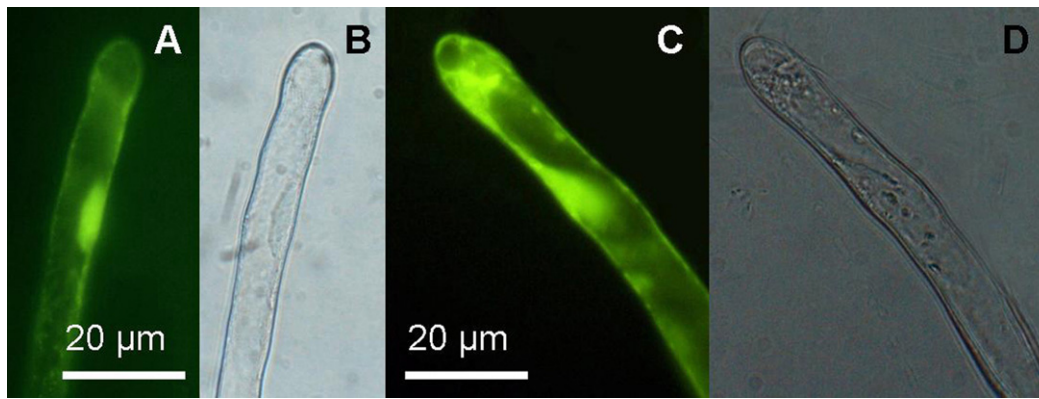
Fig. 6. Measurement of intracellular ROS levels using the fluorescent dye. CMH$_2$DCFDA (10 μM). (A) Noninoculated root hair. (C) Root hair 2 min after inoculation. (B and D) Bright-field images from the fluorescent images.

conditions. Within the first few minutes, the intracellular levels of ROS in inoculated root hairs increased and then they decreased, in a similar manner to those in the control root hairs (Fig. 7B and D, and Supplementary Fig. S4). This response was similar in both the apical cytoplasmic and perinuclear regions (Fig. 7B and D). However, the perinuclear ROS levels exhibited the greatest differences between inoculated and noninoculated conditions (Fig. 7D). Although the levels of intracellular ROS started to decrease 2 min after exposure to osmotic stress in both inoculated and noninoculated roots at the lower sorbitol concentration (100 mM), the relative differences
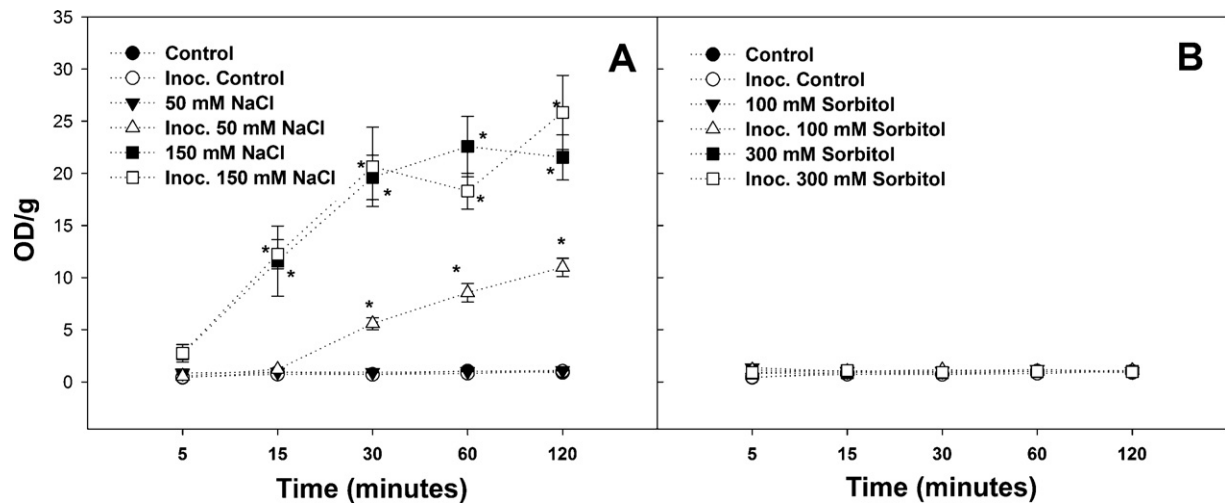
between the inoculated and noninoculated roots were similar to those induced under 300 mM sorbitol (Fig. 7B and D).

3.6. Root hair death

The death of root hair cells in noninoculated and inoculated roots under saline or osmotic stress was evaluated by Evans Blue staining (Fig. 8A and B.) In noninoculated roots, the death of young root hair cells was induced by NaCl concentrations greater than 50 mM (Fig. 8A), but not by similar osmotic pressures generated
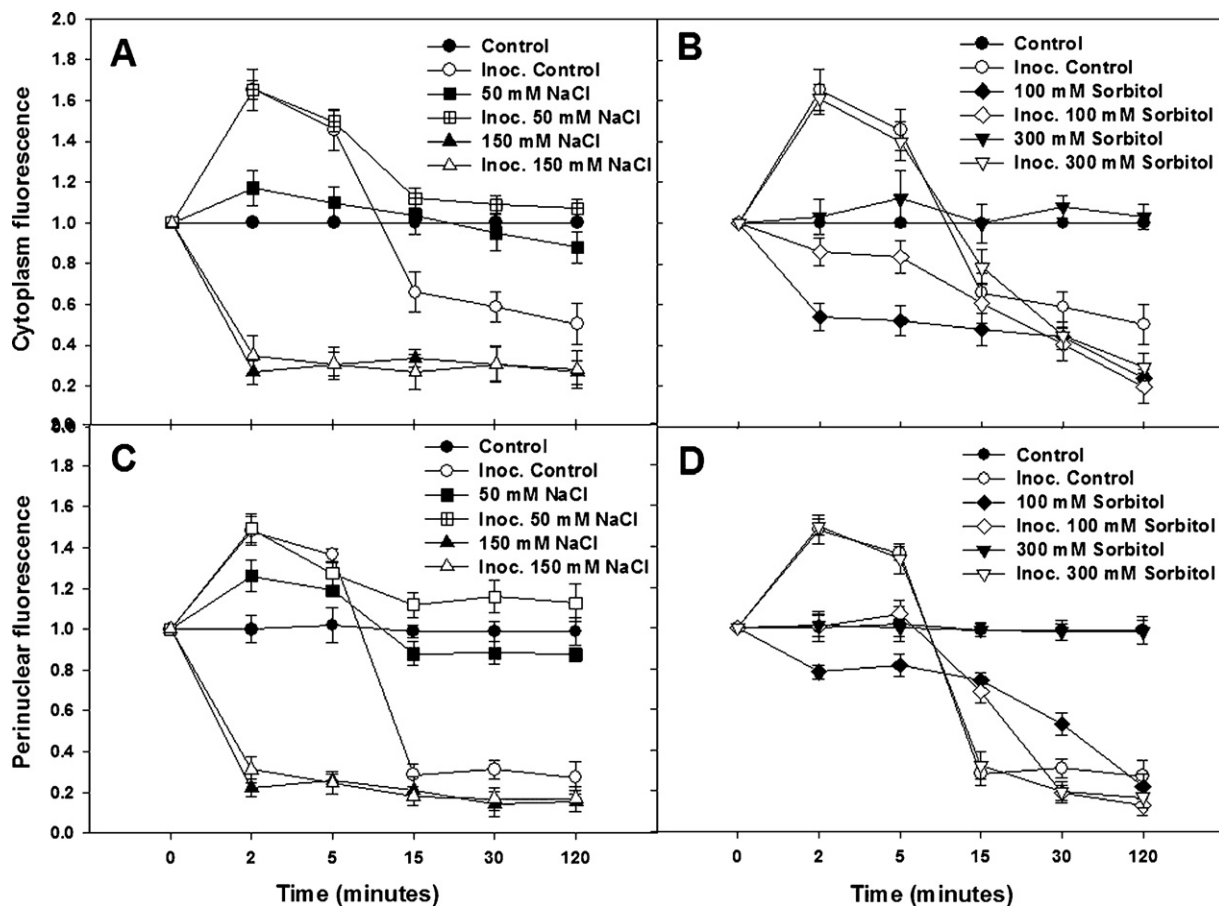
Fig. 7. Kinetics of intracellular ROS production. The values are relative to the value in noninoculated root hairs (fluorescence = 1 as an arbitrary unit) under control conditions. The stress conditions used were NaCl (50 and 150 mM) (A and C) and sorbitol (100 and 300 mM) (B and D). Cytoplasm fluorescence in root hairs (including all fluorescence except that in the perinuclear region) (A and B). Perinuclear fluorescence (C and D). Results are the means of three independent experiments (four roots per treatment); approximately 50 root hairs were quantified per treatment.

Fig. 8. Kinetics of root hair death in inoculated and noninoculated roots under NaCl (50 and 150 mM) (A) and sorbitol (100 and 300 mM) (B) treatments. Each value represents the mean ± SE from three independent experiments (eight roots per treatment). Asterisks indicate significant differences in the mean ($p < 0.05$ DGC test).

by sorbitol solutions (Fig. 8B). In noninoculated roots, 50 mM NaCl did not induce the death of root hair cells, but in inoculated roots, most of the root hair cells died under these conditions (Fig. 8A). The death of young root hair cells that was induced by 50 mM NaCl in inoculated roots or by high NaCl concentrations in noninoculated roots and was accompanied by a marked induction or inhibition of intracellular ROS production, respectively.

4. Discussion

The symbiotic interaction between legumes and Rhizobium induces several biochemical, molecular, and morphological responses in the legume plant. In root hairs, it induces changes in ion concentrations and pH, vesicle trafficking, phospholipids, ROS production, and gene expression, together with cytoskeletal rearrangement (Brechenmacher et al., 2008; Cárdenas et al., 2000; Felle et al., 2000; Jinrong et al., 2005; Meschini et al., 2008; Oldroyd and Downie, 2008; Wais et al., 2000). All these processes support and modulate root hair curling, which is a particular type of deformation of the root hair. Root hair curling is an early step that is essential for the success of Rhizobium invasion and the nodulation process. Root hair curling only occurs in actively growing root hairs and is supported by the machinery that sustains the polarized growth of root hairs in association with typical changes that are produced after Rhizobium inoculation (Oldroyd and Downie, 2008). Polarized root hair growth involves oscillatory changes in ROS, calcium, and pH, which work together to modulate cell wall properties to support growth at the root hair tip (Monshausen et al., 2007).

In agreement with previous studies, our results showed that root hairs that displayed active growth exhibited apoplastic superoxide production in the apical region (Foreman et al., 2003; Jones et al., 2007; Knight, 2007). The production of apoplastic superoxide in soybean root hairs was increased markedly by inoculation with *B. japonicum*. Interestingly, we found that, in curled root hairs, the production of apoplastic superoxide was relocated and occurred in a particular location that coincided with the zone of maximum curvature. In both noninoculated and inoculated root hairs, the production of superoxide radicals colocalized with the typical blue autofluorescence that is emitted by flavonoids (Wasson et al., 2006). Flavonoids modulate the transport of auxin, and phylogenetic analysis has suggested that mechanisms of auxin transport evolved in the presence of flavonoid compounds that were generated to scavenge ROS and defend plants against herbivores and

pathogens (Peer et al., 2004). Although the growth of root hairs, and Rhizobium-induced responses have many processes in common, the mechanisms underlying root hair curling remain unknown. A model has been proposed by Van Batenburg et al. (1986), in which root hair curling involves a continuous reorientation of tip growth. On the other hand, the reorganization of the growth machinery at the zone of maximum curvature might be related to and asymmetrical softening of the cell wall and elongation growth, in which the relationship among flavonoids, auxins, and ROS plays a major role (Rodriguez et al., 2004; Vissenberg et al., 2001). Our results contribute to the description of the role of apoplastic superoxide during root hair deformation, which had not been characterized until now.

Both root hair deformation and apoplastic superoxide levels were reduced markedly by saline stress in a dose-dependent manner. However, when equivalent osmotic pressures were produced by sorbitol, there was no effect on root hair deformation and the level of apoplastic superoxide was increased. These results indicated that it was the ionic component of saline stress that had the principal effect. Similar effects of saline stress on root hair deformation were reported by Duzan et al. (2004). In the same way, the production of apoplastic superoxide in the leaf and root elongation zone of maize was inhibited completely by saline treatments and increased by osmotic ones (Bustos et al., 2008; Rodriguez et al., 2002, 2004, 2007). The increased production of superoxide in inoculated root hairs, relative to noninoculated root hairs, was also inhibited by salt and maintained by osmotic treatment. Slight production of ROS was observed in the presence of 50 mM NaCl, where swelling was the main deformation that occurred, which indicated that root hair deformation was delayed under these conditions.

Both scavengers of superoxide radicals and DPI reduced both curling and the levels of apoplastic superoxide radicals markedly. In treatments with DPI, a well-known inhibitor of flavoprotein enzymes that is used extensively as an inhibitor of NAD(P)H oxidase, curling was inhibited specifically, because total root hair deformations, mainly wiggling and bulging, were increased. A similar response was reported previously by Lohar et al. (2007). These findings suggest that NADPH oxidase-generated superoxide radicals participate in the root hair deformation that is induced by *B. japonicum* inoculation. We also suggest that these radicals are important in determining the location of the active growth site, which might be necessary to sustain a drastic change in the direction of polarized growth to generate a curled root hair (Takeda et al., 2008).

The inhibitory effect on both root hair curling and apoplastic ROS generation that was induced by saline stress suggests that the growth machinery could be impaired. On the other hand, intracellular ROS production was initially increased transiently, between 2 and 5 min after inoculation, and then began to decline, reaching very low levels after 2 h. A similar response was reported in the root hairs of *Phaseolus vulgaris* when stimulated with a specific NF (Cárdenas et al., 2008). Similarly, reductions in ROS efflux in *Medicago truncatula* roots that began 20–30 min after NF treatment have been reported (Shaw and Long, 2003). The intracellular ROS production in soybean root hairs was located in two principal zones: the perinuclear zone and the apical cytoplasm. Under all treatment conditions, the level of ROS was quite similar in the two regions. However, responses were more substantial in the perinuclear zone. Ashtamker et al. (2007) have reported that the perinuclear region of BY2 tobacco cells that have been challenged with a pathogen elicitor is a major source of ROS. At the highest salt concentration used (150 mM NaCl), we found that intracellular ROS production was inhibited in both inoculated and noninoculated young root hairs. This salt concentration was lethal to soybean root hairs. Interestingly, in inoculated roots subjected to 50 mM NaCl (a nonlethal condition for noninoculated root hairs), the decline in intracellular ROS that was observed in controls at 5 min after inoculation did not occur and intracellular ROS production remained constant for 2 h after inoculation. Under this condition, root hair deformation began and slight production of apoplastic superoxide was detected. However, most of these root hairs had died at 2 h after inoculation, an effect that could be attributed to sustained intracellular ROS production. Cárdenas et al. (2008) have reported that intracellular ROS generation is maintained when root hairs of *P. vulgaris* are treated with the pathogen elicitor chitosan; the latter has an n-acetyl-glucosamine backbone, as does NF. Under osmotic stress, in general the responses were very similar to those in the control. Even though were some differences between the two sorbitol concentrations tested, the relative differences between the inoculated and noninoculated groups were maintained.

The balance between the production and degradation of ROS is a fundamental requirement for the maintenance of cell function and survival (Fedoroff, 2006; Foyer and Noctor, 2005). Uncontrolled production of intracellular ROS induces cell death (Breusegem and Dat, 2006). The endosome-associated production of ROS by NAD(P)H oxidase, in which phosphatidylinositol 3-kinase plays a key role, is a common response that is induced by both saline stress and symbiotic elicitors and participates in salt tolerance signaling and root hair curling, respectively (Leshem et al., 2007; Peleg-Grossman et al., 2007). It is likely that exposure to salt and inoculation with *B. japonicum* have synergistic effects on endosome-associated ROS production in our system.

Our results suggest that a sustained apoplastic ROS production and transient and intracellular ROS production, are necessary for root hairs curling. Likewise, during root hair curling the sustained apoplastic ROS production and flavonoids are relocated from the tip of the root hair to the zone of maximum curvature. In addition, the results reported herein demonstrate that saline and osmotic stresses have different effects on apoplastic and intracellular ROS generation, root hair deformation and root hair death. Saline, affects the kinetics of both apoplastic and intracellular ROS production, inhibiting root hair curling and inducing root hair death. The present characterization is being continued with the regulation of antioxidant system and ionic homeostasis studies.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.envexpbot.2011.12.008.

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