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ORIGINAL ARTICLE

An oxidative burst and its attenuation by bacterial peroxidase activity is required for optimal establishment of the *Arachis hypogaea-Bradyrhizobium* sp. symbiosis

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Abstract

Aims: The main purpose of this study was to determine whether the *Arachis hypogaea* L. root oxidative burst, produced at early stages of its symbiotic interaction with *Bradyrhizobium* sp. SEMIA 6144, and the bacterial antioxidant system are required for the successful development of this interaction.
Methods and Results: Pharmacological approaches were used to reduce both plant oxidative burst and bacterial peroxidase enzyme activity. In plants whose H_2O_2 levels were decreased, a low nodule number, a reduction in the proportion of red nodules (%) and an increase in the bacteroid density were found. The symbiotic phenotype of plants inoculated with a *Bradyrhizobium* sp. SEMIA 6144 culture showing decreased peroxidase activity was also affected, since the biomass production, nodule number and percentage of red nodules in these plants were lower than in plants inoculated with *Bradyrhizobium* sp. control cultures.
Conclusions: We demonstrated for the first time that the oxidative burst triggered at the early events of the symbiotic interaction in peanut, is a prerequisite for the efficient development of root nodules, and that the antioxidant system of bradyrhizobial peanut symbionts, particularly the activity of peroxidases, is counteracting this oxidative burst for the successful establishment of the symbiosis.
Significance and Impact of the Study: Our results provide new insights into the mechanisms involved in the development of the symbiotic interaction established in *A. hypogaea* L. a legume infected in an intercellular way.

Introduction

Leguminous plants are able to establish a symbiotic interaction with rhizobia and form new root organs, the nodules, in which nitrogen fixation takes place. The best known rhizobial infection mechanism is via root hairs through intracellular infection threads (IT) formed inside epidermal cells. Invasion through IT occurs in temperate climate legumes, such as *Glycine max*, *Vicia*, *Trifolium*, *Pisum* and *Medicago* spp. In legumes such as *Arachis hypogaea* L. (peanut), microsymbionts infect their roots intercellularly between adjacent cells, without the formation of IT (also referred to as crack entry) (Oldroyd and Downie 2008). Regardless of the bacterial infection mechanism, the nodulation process is initiated by a complex signal exchange between both partners. Flavonoids released from legume roots induce in rhizobia the synthesis of Nod factors (NFs) which activate a plant developmental programme that culminates in the formation of nitrogen-fixing nodules. Differentiated bacteria endocytosed into the host cell cytoplasm, termed bacteroids, reside inside these organs. In some legumes, such as *Medicago* and *Pisium* spp., nodules are cylindrical or branched with a persistent meristem (indeterminate nodules). By contrast, legumes with spherical nodules, such as *Arachis, Phaseolus*, *Glycine* and *Lotus* spp., are

characterized by a transient meristem (determined nodules) (Fernandez-Lopez *et al.* 1998).

In plants, reactive oxygen species (ROS), including superoxide anion (O_2^-), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), modulate numerous biological processes such as growth, plant defence, hormone signalling, as well as abiotic and biotic stress responses. However, at high concentrations, these molecules are toxic and disrupt different physiological processes, triggering an imbalance in cellular redox state, known as oxidative burst (Implay 2003).

Plants possess a complex antioxidant defence system that allows maintaining levels of ROS under physiological conditions and mitigating the damage caused by oxidative stress. The main enzymes of the antioxidant system are catalase (CAT) that catalyses the decomposition of H_2O_2 to H_2O and O_2, the enzyme superoxide dismutase (SOD) that converts O_2^- into H_2O_2, and peroxidases (PX) that are involved in the consumption or release of H_2O_2 and other ROS (Sharma *et al.* 2012).

Several studies have established that oxidative balance plays an important role in plant–microbe interactions (reviewed in Nanda *et al.* 2010; Torres 2010; Puppo *et al.* 2013). In plant–pathogen interactions, it is known that the plant's immune system is driven by a biphasic generation and accumulation of ROS (Mittler *et al.* 2004). The first peak of ROS production allows the plant to recognize the micro-organism, while the second peak is involved in the process known as hypersensitive response (HR), a form of cell death (Mur *et al.* 2008). Moreover, virulent pathogens are capable of suppressing or escaping this second oxidative burst, related to HR, thus causing plant disease (Torres *et al.* 2006; Torres 2010). Although plant defence reactions triggered against pathogenic micro-organisms are not induced in the legume-rhizobia interaction, it has been observed that rhizobia may be recognized as pathogens, causing an oxidative burst similar to that described for pathogenic interactions. Apparently, rhizobia are able to overcome the second peak of ROS by modulating the plant defence response, thus enabling the establishment of a successful symbiotic interaction (Hérouart *et al.* 1996; Gechev and Hille 2005; Pauly *et al.* 2006; Lohar *et al.* 2007; Torres 2010; Eaton *et al.* 2011).

There is now compelling evidence that ROS play an important role in signalling processes during the establishment of symbiosis in legume invaded by IT formation (Bueno *et al.* 2001; Santos *et al.* 2001; Ramu *et al.* 2002; Pauly *et al.* 2006; Cárdenas *et al.* 2008; Oger *et al.* 2012; Andrio *et al.* 2013; Puppo *et al.* 2013; Downie 2014). Transient increasing levels of ROS have been detected at the tip of actively growing root hair cells within seconds after the addition of Nod factor (NF) (Cárdenas *et al.* 2008). In *Sesbania rostrata,* ROS accumulate in intercellular infection pockets formed into the root epidermal tissue, which then originate IT (D'Haeze *et al.* 2003). On *Medicago* roots inoculated with *Sinorhizobium meliloti*, a gradual increase in ROS production has been reported, reaching the highest values at several hours of interaction (Santos *et al.* 2001). It has been shown that the inhibition of this response prevents IT formation (Santos *et al.* 2001; Ramu *et al.* 2002; Peleg-Grossman *et al.* 2007). The importance of ROS production during the early stages of legume-*Rhizobium* interaction was first evidenced using bacterial mutants impaired in their antioxidant abilities (Puppo *et al.* 2013). Moreover, by using ROS production inhibitors (such as diphenyleneiodonium) and ROS scavengers, it has been found that ROS are part of a NF-induced signal cascade required for nodule primordial initiation in *Se. rostrata* (D'Haeze *et al.* 2003), and for root hair curling and IT formation in *Medicago truncatula* (Peleg-Grossman *et al.* 2007). Recently, Andrio *et al.* (2013), using a combination of pharmacological and transcriptomic approaches, have identified several genes potentially regulated by H_2O_2 content and/or NF during the establishment of rhizobial symbiosis. All these findings indicate that ROS are part of the repertoire of signals that contribute to the establishment of nitrogen-fixing symbiotic interaction in legumes infected by a mechanism that involves IT formation at epidermal or cortical root level.

It is known that, as a result of the oxidative burst which occurs in the IT legumes during the early steps of the legume-*Rhizobium* symbiosis, the bacterial antioxidant system is activated, thereby contributing to the successful establishment of the symbiosis (Matamoros *et al.* 2003). In fact, some *Si. meliloti* mutants with defective ROS scavenging enzymes have poor nodulation capacity on *Medicago sativa* (Santos *et al.* 2000; Jamet *et al.* 2003), indicating the importance of bacterial antioxidant system in the development of the symbiotic interaction. In contrast, *Mesorhizobium loti* and *Rhizobium etli* mutants affected in their antioxidant systems form nodules with a lower nitrogen-fixing capacity than the wild-type strains (Dombrecht *et al.* 2005; Hanyu *et al.* 2009). These findings support the hypothesis that the functioning of bacterial antioxidant system is relevant to the establishment of the symbiosis between rhizobia and legume infected intracellularly. However, in the less characterized intercellular invasion occurring in peanut-*Bradyrhizobium* sp. interaction, the role of bacterial ROS scavenging enzymes has not been elucidated yet.

Considering that IT are not formed in peanut roots inoculated with *Bradyrhizobium* sp. and that an oxidative burst takes place in these roots, as we have recently found (Muñoz *et al.* 2015), it can be assumed that

bradyrhizobia are exposed to the plant defence responses. Therefore, it is possible that the successful establishment of the peanut-*Bradyrhizobium* sp. interaction is linked in part to the involvement of bacterial antioxidant system in attenuating the oxidative burst. In this context, the aims of this study are (i) to evaluate whether the peanut root production of H_2O_2 is a prerequisite for nodulation, (ii) to determine the requirement of the *Bradyrhizobium* sp. antioxidant system to effectively nodulate peanut.

Materials and methods

Bacterial strain and growth conditions

Bradyrhizobium sp. SEMIA 6144, able to nodulate peanut (*A. hypogaea* L.), was obtained from Microbial Resource Centre (MIRCEN, Brazil). Stock cultures were maintained on yeast extract mannitol-agar (YEM agar) supplemented with Congo red (Vincent 1970). Cultures were grown in YEM broth and incubated at 28°C on a rotary shaker at 150 rev min^{-1}. Growth was measured turbidimetrically at an absorbance of 620 nm, and the number of viable cells on YEM agar was determined after 120 h of incubation as colony forming units (CFU), using the drop-plate method (Somasegaran and Hoben 1994).

Reduction of the H_2O_2 levels in inoculated plants and evaluation of symbiotic parameters

Arachis hypogaea L. (var. Tegua) seeds were surface sterilized by soaking them in ethanol for 30 s followed by 6% H_2O_2 for 15 min, and then washed six times with sterile distilled water. Seeds were germinated at 28°C on sterilized Petri dishes with one layer of Whatman No. 1 filter paper and moist cotton until the radicle reached about 2 cm. Pregerminated seeds were transferred to glass bottles wrapped in paper and containing sterile Whatman No. 1 filter paper moistened with Hoagland solution (Hoagland and Arnon 1950). Plants were grown under a controlled environment (light intensity of 200 μmol m^{-2} s^{-1}. About 16 h day per 8 h night cycle, at a constant temperature of 28°C and a relative humidity of 50%). Fifty millilitre (10^9 cells ml^{-1}) of a *Bradyrhizobium* sp. SEMIA 6144 culture grown in YEM medium were centrifuged at 5000 ***g*** for 5 min, and the pellet was re-suspended in the same volume of distilled water. One millilitre of this bacterial suspension was inoculated into the emergency zone of the lateral roots of 7 days old peanut plants. Immediately after inoculation, they were treated with potassium iodide (KI) (0·5 or 1·5 mmol l^{-1}) or catalase (CAT) (1000 U ml^{-1}) by adding 1 ml of these solutions to the same zone of inoculation. The roots were removed at 10 min (min) postinoculation (pi), frozen in liquid nitrogen and then stored at −80°C until use for determining H_2O_2 content.

To analyse the symbiotic parameters in plants whose H_2O_2 content was diminished, *A. hypogaea* L. (var. Tegua) seeds were surface sterilized as described before. Seedlings were sown in sterilized plastic cups filled with sterile vermiculite. Plants were grown under a controlled environment, as described before. Seven old days' plants were inoculated with 4 ml of *Bradyrhizobium* sp. SEMIA 6144 (10^9 cells ml^{-1}) bacterial suspension into the sterile vermiculite. Immediately after inoculation, they were treated with KI (0·5 or 1·5 mmol l^{-1}) or CAT (1000 U ml^{-1}) by adding 1 ml of these solutions to the sterile vermiculite. Negative controls (uninoculated and untreated plants) were included in the assay. Plants were grown under a controlled environment and harvested at 28 days pi. Nodule number, proportion of red nodules (evidenced by their red colour that indicates the presence of leghaemoglobin) and shoot dry weights were determined. The experiment was repeated twice and 10–15 plants for each treatment were evaluated.

Evaluation of symbiotic parameters in plants inoculated with salicylhydroxamic acid treated bacteria

Salicylhydroxamic acid (SHAM) was used to inhibit the bacterial peroxidase activity. To determine the SHAM doses that reduce the peroxidase activity without affecting the bacterial viability, the number of viable cells (CFU ml^{-1}) from bacterial cultures growing in YEM broth (Vincent 1970) supplemented with SHAM 0·2, 0·5, 1·5 or 2 mmol l^{-1} were evaluated each 24 h during 5 days (120 h). According to the results obtained from this assay, we inoculated plants obtained as described before, with 4 ml of *Bradyrhizobium* sp. SEMIA 6144 (10^9 cells ml^{-1}) suspension cultured with or without SHAM 0·2 mmol l^{-1}. Negative controls (uninoculated) were included in the assay. Plants were harvested at 28 days pi, and nodule number, proportion of red nodules and shoot dry weight were determined. The experiment was repeated twice and 22–30 plants for each treatment were evaluated.

Furthermore, we assessed whether decreased *Bradyrhizobium* sp. SEMIA 6144 PX activity by the addition of SHAM affects generation of oxidative burst in inoculated peanut roots, by the determining the levels of H_2O_2. For this purpose, *A. hypogaea* L. (var. Tegua) seeds were surface sterilized as described before. Seedlings were sown in glass bottles wrapped in paper and containing sterile Whatman No. 1 filter paper moistened with Hoagland solution (Hoagland and Arnon 1950). Plants were grown under a controlled environment as described before. One ml of bacterial suspension was inoculated into the

emergency zone of the lateral roots of 7 days old peanut plants. The roots were removed at 5, 10, 15 min pi, frozen in liquid nitrogen and then stored at −80°C until use for determining H_2O_2 content.

Root H_2O_2 content

H_2O_2 content was measured spectrophotometrically according to Alexieva *et al.* (2001). The amount of H_2O_2 produced was determined from a calibration curve of H_2O_2 in the range of 0·5–10 nmol l^{-1} known concentrations solutions.

Bacterial peroxidase activity determination

Bacterial cultures in late exponential phase of growth were centrifuged for 10 min at 10 000 ***g*** at 4°C and washed twice with 0·85% sterile NaCl. Pellets were resuspended in 2 ml of extraction buffer (50 mmol l^{-1} sodium acetate/acetic acid (pH 5) containing 1 mol l^{-1} KCl and sonicated (width: 80; time: 2 min, pulse, every 6 s). The supernatant was transferred to a sterile tube and used for subsequent determination of enzymatic activity.

Total peroxidase activity (PX) was determined by measuring the absorbance at 470 nm according to Sosa Alderete *et al.* (2009) and expressed as UPX min^{-1} mg $protein^{-1}$.

Protein content

In order to evaluate bacterial peroxidase activity, total bacterial soluble protein content was determined as described by Bradford (1976) using bovine serum albumin 1 mg ml^{-1} as standard.

Ultrastructural studies

Nodules obtained at 28 days pi were fixed in S-collidine buffer solution (0·2 mol l^{-1}, pH 7·4) containing 2·5% glutaraldehyde and then washed twice with this buffer. They were maintained for 3 h at 4°C and then rinsed in the same buffer and postfixed for 3 h in S-collidine buffer solution containing 1% osmium tetroxyde at room temperature. Samples were stepwise dehydrated by passing them through a graded series of acetone (30, 50, 70% and three times in 100%). Pre-inclusion was performed with EMbed 812 epoxy resine-acetone (1 : 1 v/v) overnight at room temperature. Inclusion was made with EMbed812 at 56°C, during 48 h. Semithin section (±0·25 μm) was obtained using an ultramicrotome (Sorvall MT-1A; DuPont Sorvall, Newtown, CT, USA) and a diamond cleaver. The sections were stained with toluidine blue and observed by light microscopy for choosing the area of interest of nodules. Subsequently, ultrathin sections (±20–60 nm) were obtained with a diamond blade (Pelco®, CA, USA), placed on copper grids of 250 meshes and contrasted with uranyl acetate for 30 min and lead citrate for 8 min. The nodule's ultrastructural observation was performed using a transmission electron microscope Elmiskop 101 (Siemens, Munich, Germany), and the images were acquired by transmission electron microscope JEM 1200 EXII (JEOL, Musashino, Akishima, Tokyo, Japan). The morphometric measurements (bacteroids area and symbiosome area) were taken by using the AXIOVISIONRELEASE 4.6.3 software (Carl Zeiss, Oberkochen, Germany).

Chemicals

Chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) and from Cicarelli (Santa Fé, Argentina).

Data analysis

Data analysis was performed using the software INFOSTAT (Di Rienzo *et al.* 2015). The data were subjected to analysis of variance (ANOVA), and Tukey's post-test was applied to compare the means at $P \leq 0{\cdot}05$.

Results

Increased levels of H_2O_2 in early stages of peanut-*Bradyrhizobium* sp. SEMIA 6144 interaction is a requirement for the normal development of this symbiosis

We have previously determined that at early stages (10 min pi) of the symbiotic interaction peanut-bradyrhizobia an increase in the levels of H_2O_2 peanut root is produced (Muñoz *et al.* 2015). To assess whether this oxidative burst is required for a successful interaction, in this study we decreased the level of H_2O_2 in inoculated roots by adding potassium iodide (KI) (0·5 or 1·5 mmol l^{-1}) or catalase (CAT) (1000 U ml^{-1}). CAT catalyses the decomposition of H_2O_2 to H_2O, and O_2 and KI reduce H_2O_2 originating H_2O and O_2^-. Both treatments were effective in reducing the H_2O_2 concentration in roots during the early stages of interaction between *Bradyrhizobium* sp. SEMIA 6144 and peanut, without affecting physiological growth of treated plants that were not inoculated, as compared to plants inoculated and treated. The addition of CAT showed a statistically significant decrease in root H_2O_2 content at 10 min pi by 26·8% (Table 1) while the addition of KI 0·5 and 1·5 mmol l^{-1} decreased levels by 21·8 and 41·9% respectively (Table 2).

Table 1 H_2O_2 concentration in peanut roots inoculated with *Bradyrhizobium* sp. SEMIA 6144 and treated with catalase (CAT) (1000 U ml^{-1})

Treatment	H_2O_2 concentration (nmol g dry $tissue^{-1}$) 10 min pi
Uninoculated	0·92 ± 0·19[a]
Bradyrhizobium sp. SEMIA 6144	3·18 ± 0·33[c]
Bradyrhizobium sp. SEMIA 6144 + CAT 1000 U ml^{-1}	2·05 ± 0·21[b]

Data are the mean ± SE (n = 10). Different letters indicate significant differences following Tukey's test ($P \leq 0{\cdot}05$). The experiment was repeated twice with five replicates for each treatment.

Table 2 H_2O_2 concentration in peanut roots inoculated with *Bradyrhizobium* sp. SEMIA 6144 and treated with potassium iodide (KI) (0·5 or 1·5 mmol l^{-1})

Treatment	H_2O_2 concentration (nmol g dry $tissue^{-1}$) 10 min pi
Uninoculated	0·85 ± 0·08[a]
Bradyrhizobium sp. SEMIA 6144	3·29 ± 0·21[d]
Bradyrhizobium sp. SEMIA 6144 + KI 0·5 mmol l^{-1}	1·97 ± 0·11[c]
Bradyrhizobium sp. SEMIA 6144 + KI 1·5 mmol l^{-1}	1·33 ± 0·13[b]

Data are the mean ± SE (n = 10). Different letters indicate significant differences following Tukey's test ($P \leq 0{\cdot}05$). The experiment was repeated twice with five replicates for each treatment.

Effect of CAT or KI on symbiotic phenotype of plants inoculated was studied at 28 days pi. The addition of CAT decreased the number of nodules formed by 34%, while the proportion of red nodules (%) was affected by 4·3% (Table 3). Both concentrations of KI (0·5 and 1·5 mmol l^{-1}) reduced the number of nodules formed by 58 and 60·5%, respectively, although no significant differences were identified. However, the proportion of red nodules decreased only in plants treated with KI 1·5 mmol l^{-1} (24%) (Table 4). This decrease in the number and proportion of red nodules in plants treated with CAT or KI could be associated with the diminution in the plant biomass production (Tables 3 and 4).

In order to analyse the ultrastructure of nodules formed in plants inoculated and treated with KI (0·5 or 1·5 mmol l^{-1}), ultrathin sections were performed and area of bacteroids (μm^2), area of simbiosome (μm^2) and bacteroids density (number of bacteroids $cellular\ area^{-1}$) were analysed by using a transmission electron microscope. A significant increase in bacteroid density was observed in nodules obtained from peanut plants treated with KI (0·5 or 1·5 mmol l^{-1}) (Table 5, Fig. 1). It has been reported that an increase in bacteroid density is associated with a decrease in the efficiency of nitrogen fixation (Sen and Weaver 1984). One of the possible reasons is that, under these conditions, respiratory rate increases in the nodule, and this in turn results in an increase in the generation of ROS inhibiting the enzyme nitrogenase activity. It was therefore interesting to measure H_2O_2 concentration in nodular tissue of plants inoculated with *Bradyrhizobium* sp. SEMIA 6144 and treated with KI. The results indicated no significant difference in nodule H_2O_2 content between those formed in plants

Table 3 Dry weight, number of nodules and proportion of red nodules (%) in peanut roots inoculated with *Bradyrhizobium* sp. SEMIA 6144 and treated with catalase (CAT) (1000 U ml^{-1})

Treatment	Dry weight (g per plant)	Number of nodules per plant	Proportion of red nodules (%)
Uninoculated	0·39 ± 0·03[a]	–	–
Uninoculated + CAT	0·41 ± 0·04[a]	–	–
Bradyrhizobium sp. SEMIA 6144	0·97 ± 0·07[b]	112 ± 4[a]	100 ± 0[a]
Bradyrhizobium sp. SEMIA 6144 + CAT (1000 U ml^{-1})	0·40 ± 0·08[a]	74 ± 3[b]	96·70 ± 1·40[b]

Data are the mean ± SE (n = 10–12). Different letters indicate significant differences following Tukey's test ($P \leq 0{\cdot}05$). The experiment was repeated twice and 10–12 plants for each treatment were evaluated.

Table 4 Dry weight, number of nodules and proportion of red nodules (%) in peanut roots inoculated with *Bradyrhizobium* sp. SEMIA 6144 and treated with potassium iodide (KI) (0·5 or 1·5 mmol l^{-1})

Treatment	Dry weight (g per plant)	Number of nodules per plant	Proportion of red nodules (%)
Uninoculated	0·60 ± 0·04[a]	–	–
Uninoculated + KI 0·5 mmol l^{-1}	0·58 ± 0·03[a]	–	–
Uninoculated + KI 1·5 mmol l^{-1}	0·52 ± 0·02[a]	–	–
Bradyrhizobium sp. SEMIA 6144	1·05 ± 0·11[c]	86 ± 8[a]	100 ± 0[a]
Bradyrhizobium sp. SEMIA 6144 + KI 0·5 mmol l^{-1}	0·76 ± 0·03[d]	36 ± 3[b]	100 ± 0[a]
Bradyrhizobium sp. SEMIA 6144 + KI 1·5 mmol l^{-1}	0·73 ± 0·03[d]	34 ± 3[b]	76 ± 4[b]

Data are the mean ± SE (n = 10–15). Different letters indicate significant differences following Tukey's test ($P \leq 0{\cdot}05$). The experiment was repeated twice and 10–15 plants for each treatment were evaluated.

Table 5 Symbiosome area, bacteroid area and bacteroids density in nodules from peanut roots inoculated with *Bradyrhizobium* sp. SEMIA 6144 and treated with potassium iodide (KI) (0·5 or 1·5 mmol l^{-1})

Treatment	Bacteroids density (number of bacteroids cell per area)	Symbiosome area (μm^2)	Bacteroid area (μm^2)
Bradyrhizobium sp. SEMIA 6144	0·14 ± 0·04[a]	4·55 ± 0·23[a]	0·95 ± 0·05[a]
Bradyrhizobium sp. SEMIA 6144 + KI 0·5 mmol l^{-1}	0·18 ± 0·05[b]	3·99 ± 0·13[a]	1·11 ± 0·08[a]
Bradyrhizobium sp. SEMIA 6144 + KI 1·5 mmol l^{-1}	0·20 ± 0·05[b]	4·48 ± 0·27[a]	0·97 ± 0·07[a]

Data are the mean ± SE (Bacteroids density n = 47–58) (Symbiosome and Bacteroid area n = 12–23). Different letters indicate significant differences following Tukey's test ($P \leq 0{\cdot}05$).

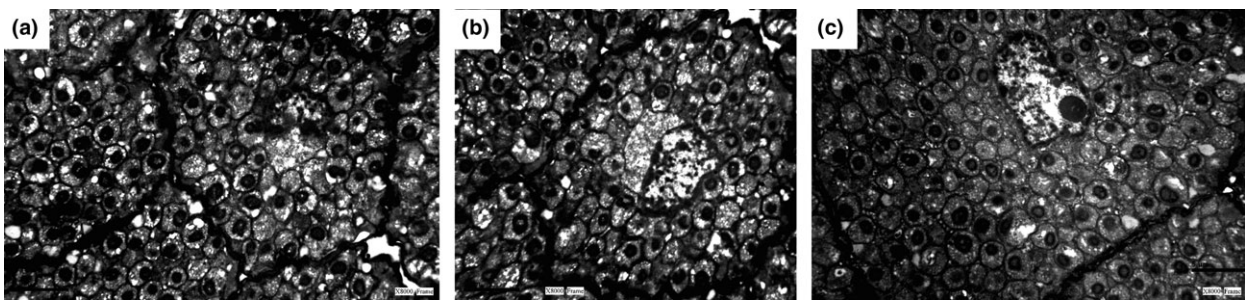

Figure 1 Evaluation of ultrathin sections (20–60 nm) of nodules of peanut plants induced by (a) *Bradyrhizobium* sp. SEMIA 6144, (b) *Bradyrhizobium* sp. SEMIA 6144 and treated with potassium iodide (KI) 0·5 mmol l^{-1} and (c) *Bradyrhizobium* sp. SEMIA 6144 and treated with KI 1·5 mmol l^{-1}. Transmission electron microscope (TEM). 8000×.

treated with KI (0·5 or 1·5 mmol l^{-1}) and in untreated plants (Table 6).

Bacterial peroxidase activity plays a role in counteracting the oxidative burst occurring during the early stages of the peanut-*Bradyrhizobium* sp. interaction

In order to assess whether the bacterial antioxidant system is important to counteract the oxidative burst produced in the early stages of the symbiotic peanut-bradyrhizobia interaction, we used SHAM 0·2 mmol l^{-1}. SHAM is a potent, specific and irreversible inhibitor of enzymes with peroxidase activity, including CAT enzyme (Behzadi *et al.* 2014).

The peroxidase activity in bacteria treated with SHAM diminished by 77% (Fig. 2). In roots inoculated with these bacteria the H_2O_2 level increased earlier (5 min pi) and remained high for a more extended time than in roots inoculated with untreated bacteria (control) (Table 7), suggesting that the *Bradyrhizobium* sp. SEMIA 6144 antioxidant system, particularly enzymes with peroxidase activity, participates in the modulation of the defence response of the host plant. In order to confirm the importance of bacterial peroxidase activity in establishing symbiotic interaction, the symbiotic phenotype of *Bradyrhizobium* sp. SEMIA 6144 cultured in the presence of SHAM 0·2 mmol l^{-1} was evaluated. The biomass production of such plants was significantly higher than uninoculated plants, but significantly lower than plants inoculated with untreated bacteria (Table 8). Moreover, the number of nodules formed and the proportion of red nodules was significantly lower than that observed in plants inoculated with control cultures of *Bradyrhizobium* sp. (Table 8). This effect could be associated with the diminution in the biomass production (Table 8).

Discussion

During the last decade, several studies have evidenced the involvement of ROS in symbiotic interactions (recently reviewed by Puppo *et al.* 2013). The best model described is the nitrogen-fixing symbiosis between rhizobia and IT legumes. ROS are produced at different stages of the interaction. Despite widespread acceptance of the role of ROS in signalling, it remains unclear how ROS signals are perceived and transmitted and how they induce a specific response (Mittler *et al.* 2011). Given the crucial role of these molecules in signalling in plant cells, efforts have been recently made to identify genes regulated by ROS. In this regard, the use of a combination of pharmacological and transcriptomic approaches led to the identification of several genes potentially regulated by H_2O_2 (Andrio *et al.*

Table 6 H_2O_2 concentration in nodules of peanut plants inoculated with *Bradyrhizobium* sp. SEMIA 6144 and treated with potassium iodide (KI) (0·5 or 1·5 mmol l^{-1})

Treatment	H_2O_2 concentration (nmol g fresh tissue^{-1})
Bradyrhizobium sp. SEMIA 6144	57·82 ± 3·17
Bradyrhizobium sp. SEMIA 6144 + KI 0·5 mmol l^{-1}	50·18 ± 2·47
Bradyrhizobium sp. SEMIA 6144 + KI 1·5 mmol l^{-1}	49·37 ± 3·44

Data are the mean ± SE (n = 12). The experiment was repeated twice with six replicates for each treatment.

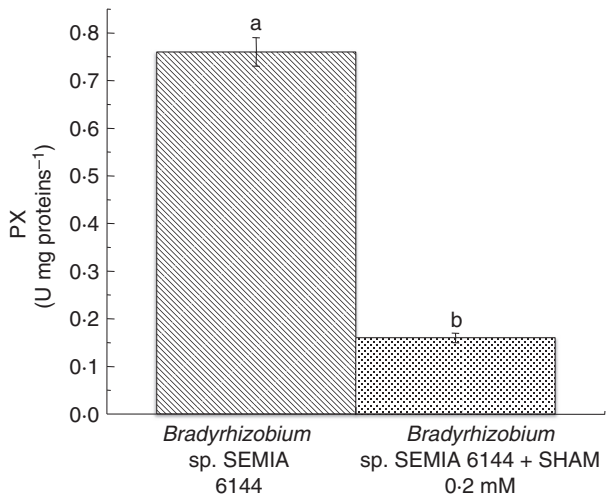

Figure 2 Total peroxidase (PX) activity of *Bradyrhizobium* sp. SEMIA 6144 cultured with or without salicylhydroxamic acid (SHAM) 0·2 mmol l^{-1}. Data are the mean ± SE (n = 10). Different letters indicate significant differences following Tukey's test ($P \leq 0·05$).

2013). Moreover, several studies have shown, using pharmacological strategies, that ROS production is related to root hair deformation in *M. truncatula-Si. meliloti* symbiosis (Lohar *et al.* 2007) and to IT development in *M. sativa-Si. meliloti* interaction (Jamet *et al.* 2007). In *Se. rostrata-Azorhizobium caulinodans* symbiosis it has been demonstrated that ROS production mediates not only root hair deformation and infection pocket formation but also nodule primordia development (D'Haeze *et al.* 2003).

Peanut bradyrhizobial infection depends entirely on intercellular invasion, without IT formation or intracellular entry (Boogerd and van Rossum 1997; Uheda *et al.* 2001). We have recently demonstrated for the first time that the early events of the symbiotic interaction in peanut trigger an oxidative burst that differs from that reported for IT legumes, which involves both O_2^- and H_2O_2, since it is represented exclusively by H_2O_2 production (Muñoz *et al.* 2015).

Table 7 H_2O_2 concentration in peanut roots inoculated with *Bradyrhizobium* sp. SEMIA 6144 cultured in the presence of salicylhydroxamic acid (SHAM) 0·2 mmol l^{-1}

	H_2O_2 concentration (nmol g fresh tissue^{-1}) Time pos-inoculation (min)		
Treatment	5	10	15
Uninoculated	0·93 ± 0·13^a	0·87 ± 0·09^a	0·91 ± 0·07
Bradyrhizobium sp. SEMIA 6144	0·90 ± 0·12^a	3·56 ± 0·08^b	0·91 ± 0·04^a
Bradyrhizobium sp. SEMIA 6144 + SHAM 0·2 mmol l^{-1}	3·18 ± 0·19^b	4·84 ± 0·17^c	2·98 ± 0·16^b

Data are the mean ± SE (n = 7–10). Different letters indicate significant differences following Tukey's test ($P \leq 0·05$). The experiment was repeated twice and 7–10 plants for each treatment were evaluated.

Table 8 Dry weights, number of nodules and proportion of red nodules (%) in peanut roots inoculated with *Bradyrhizobium* sp. SEMIA 6144 cultured in the presence of salicylhydroxamic acid (SHAM) 0·2 mmol l^{-1}

Treatment	Dry weights (g per plant)	Number of nodules per plant	Proportion of red nodules (%)
Uninoculated	0·62 ± 0·03^a	–	–
Bradyrhizobium sp. SEMIA 6144	1·01 ± 0·01^b	96 ± 4^a	100 ± 0^a
Bradyrhizobium sp. SEMIA 6144 + SHAM 0·2 mmol l^{-1}	0·74 ± 0·02^c	38 ± 3^b	76 ± 1·40^b

Data are the mean ± SE (n = 22–30). Different letters indicate significant differences following Tukey's test ($P \leq 0·05$). The experiment was repeated twice and 22–30 plants for each treatment were evaluated.

The results obtained in this study clearly show that the suppression of the increase in H_2O_2 produced in peanut plants inoculated with *Bradyrhizobium* sp. negatively affects the development of nodules, with a concomitant decrease in biomass production. This allows suggesting that, as reported for IT legumes-*Rhizobium* interaction, increased levels of H_2O_2 in the early stages of the peanut-*Bradyrhizobium* sp. SEMIA 6144 interaction is a prerequisite for the proper development of this symbiosis. Moreover, it has been reported that in legumes infected in an intercellular way ROS plays a crucial role in signalling, participating in regulation at the transcriptional and post-translational level of proteins involved in the symbiotic process (Oger *et al.* 2012). Therefore, it is

proposed that ROS are also involved in the signalling cascade that culminates in peanut nodulation.

It is known that in indeterminate nodules, the bacterial differentiation process is under the control of nodule-specific cysteine-rich (NCR) antimicrobial peptides (Van de Velde *et al.* 2010). These redox-sensitive peptides (Haag *et al.* 2012) are delivered into the bacteroid and trigger bacterial differentiation and/or membrane damage and permeabilization (Van de Velde *et al.* 2006). In a previous study, it has been showed that a *Si. meliloti* catalase mutant (katB/katC) displayed a drastic symbiotic phenotype with nodule abortion and absence of bacteroid differentiation (Jamet *et al.* 2007), indicating that a fine tuning of ROS balance is essential to the differentiation process. Thus, ROS may regulate, via NCR redox status, the bacteroid differentiation process. By contrast, in determinate nodules as formed in peanut, there is no evidence that the NCR peptides are involved in bacterial differentiation to bacteroids. In this study, we observed in the peanut-bradyrhizobia interaction an increase in the bacteroid density in nodules obtained from plants whose H_2O_2 levels were decreased. The H_2O_2 content in these nodules was similar to those from control plants. Therefore, it is possible to propose that equilibrium in the plant redox balance is also required for the normal development of nodules in peanut.

The oxidative burst produced in the legume-*Rhizobium* interaction activates the bacterial antioxidant system, thus contributing to the successful establishment of the symbiosis. *Sinorhizobium meliloti, Rh. etli, Rhizobium tropici, Bradyrhizobium japonicum* and *Me. loti* antioxidant's system plays an important role in establishing nitrogen-fixing symbioses with their legume host (Riccillo *et al.* 2000; Santos *et al.* 2000; Jamet *et al.* 2003; Panek and O'Brian 2004; Dombrecht *et al.* 2005; Harrison *et al.* 2005; Muglia *et al.* 2008; Saeki 2011). In previous studies was reported that *Si. meliloti* and *Me. loti* mutants with defective superoxide dismutase or catalase enzymatic activities showed an altered symbiotic phenotype, with the formation of aberrant IT, delay in nodulation, reduced nitrogenase activity and inhibition of the bacteroid differentiation (Santos *et al.* 2000; Jamet *et al.* 2003; Hanyu *et al.* 2009; Saeki 2011). Moreover, *Rh. etli* double mutant with reduced catalase and peroxidase activity showed a nitrogen fixation capacity reduced by a 50%, even when the nodulation ability was not affected (Dombrecht *et al.* 2005). Since the expression of these genes during infection was associated with a response of bacteria to O_2^- and H_2O_2 produced by the host plant (Ampe *et al.*, 2003, Puppo *et al.* 2013), and its repression influences negatively on the nitrogen fixation capacity, it was awarded an essential role in bacterial antioxidant system in the proper establishment and development of the symbiosis established between rhizobia and legumes infected intracellularly way. In this study the 77% reduction in *Bradyrhizobium* sp. SEMIA 6144 peroxidase activity negatively affected its symbiotic performance. In addition, in plants inoculated with these bacteria, root ROS production was increased at all time points analysed compared with plants inoculated with control cultures. From these results it can be inferred that, as for microsymbionts of legumes infected in an intracellular way, the participation of the antioxidant system of bradirizobios peanut symbionts is important in signalling processes during the establishment of the symbiosis between peanut and *Bradyrhizobium* sp.

In this study, we have demonstrated that the oxidative burst triggered at the early events of the symbiotic interaction in a legume invaded intercellularly without IT formation, such as peanut, is a prerequisite for the efficient development of root nodules. Furthermore, we have demonstrated that the antioxidant system of peanut bradyrhizobia symbionts, particularly the activity of PX enzymes, is important to counteract the oxidative burst. In summary, our results provide new knowledge about the mechanism involved in the development of the symbiotic interaction established by legumes infected in an intercellular manner.

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Conflict of Interest

The authors have no conflict of interest to declare.

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