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ORIGINAL ARTICLE

Role of reactive oxygen species generation and Nod factors during the early symbiotic interaction between bradyrhizobia and peanut, a legume infected by crack entry

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Abstract

Aims: We evaluated whether reactive oxygen species (ROS) production and the plant antioxidant system are involved in the symbiotic interaction between bradyrhizobia and legumes infected by crack entry, without intracellular infection threads (IT) formation, such as *Arachis hypogaea* L. (peanut). The role of bradyrhizobial Nod factors (NF) in modulating the plants' oxidative burst was also analysed.
Methods and Results: Histochemical and quantitative procedures were used to detect ROS levels in inoculated and in NF-treated peanut roots. Increase in root H_2O_2 production was determined at 10 min postinoculation with *Bradyrhizobium* sp. SEMIA 6144 or after NF addition. ROS production was modulated by NF. From 15 to 30 min postinoculation, the compatibility of *Bradyrhizobium* sp.–peanut interaction depends mostly on the H_2O_2 detoxification via catalase.
Conclusions: We demonstrated for the first time that the early events of the symbiotic interaction in legumes invaded by crack entry trigger an increase in ROS production (represented exclusively by a higher H_2O_2 content) in which NADPH-oxidase seems not to be involved. NF modulate this response by enhancing the plant antioxidant machinery, contributing to the creation of adequate conditions for symbiosis development.
Significance and Impact of the Study: Our data provide new insights into the mechanism involves in the symbiotic interaction that establish legumes infected by crack entry and suggest that ROS response shows differences compared with legumes invaded by IT formation.

Introduction

Leguminous plants establish a symbiotic relationship with nitrogen-fixing rhizobia, which results in the formation of specialized structures called nodules. This feature is of major importance in both ecology and agriculture, as it provides a substantial part of the global flow of atmospheric nitrogen fixed into available forms to plants, such as ammonium, nitrate and organic nitrogen, resulting in about 200 million annual tons of fixed nitrogen (Peoples *et al.* 2009). In this nitrogen-fixing interaction, rhizobia obtain carbonaceous sources from the plant host. Rhizobial infection, which is host-determined, can occur through different modes of entry into the roots and, occasionally, into the stems. The best known infection mechanism is via root hairs through intracellular infection threads (IT) formed inside epidermal cells. Another mode of infection occurs when rhizobia directly colonize the subepidermal root tissue (the root cortex) by crack entry at the lateral root base in an intercellular manner, without the formation of infection threads (Boogerd and van Rossum 1997). Invasion through IT occurs in temperate climate legumes (*Glycine max*, *Vicia*, *Trifolium*, *Pisum* and *Medicago* spp), whereas crack entry occurs in

subtropical legumes (*Arachis*, *Aeschynomene* and *Stylosanthes*; Oldroyd and Downie 2004). *Sesbania rostrata* can switch between root hair and crack entry infection depending on environmental conditions. In this legume, infection pockets narrow down to form intercellular IT and, subsequently, intracellular IT intrude into the nodule primordium. Often, intracellular IT are also observed to start immediately from the infection pockets (Goormachtig *et al.* 2004). Instead, in crack entry legumes such as *Arachis hypogaea* L. (peanut), which remain much less investigated, intracellular or intercellular IT are never formed during the epidermal or cortical process (Uheda *et al.* 2001). Moreover, in rice and *Parasponia*, which are nonlegume plants, colonization studies have provided evidence that rhizobia can also enter through cracks in the epidermis (Prayitno *et al.* 1999).

Regardless of whether the initial rhizobial entry is through IT in root hairs or epidermal cracks, during the early stages of the legume–rhizobia interaction, there is a molecular dialogue involving several signal molecules of both partners. Legumes excrete flavonoids, which in rhizobia induce the synthesis of elicitors called Nod factors (NF). The idea that NF can be described as elicitors is based on the observation that, like other elicitors, they are perceived by the plant and, therefore, they trigger a series of responses, such as cytoplasm alkalinization, oscillations of calcium cytosolic concentrations and gene expression (Oldroyd and Downie 2004). In the peanut–*Bradyrhizobium* sp. interaction, it has been demonstrated that NF are required for cortical cell division in the nodule morphogenetic program of the host plant (Ibáñez and Fabra 2011).

Reactive oxygen species (ROS) are permanently produced in chloroplasts, mitochondria, apoplast, cell wall and peroxisomes as by-product of the plant metabolism. ROS include free radicals such as superoxide anion (O_2^-), hydroxyl radical ($^{\cdot}OH$), as well as nonradical molecules like hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Sharma *et al.* 2012). ROS are central signalling molecules involved in a wide range of physiological processes in plants, such as root hair growth (Monshausen *et al.* 2007), stomatal closure (Pei *et al.* 2000) and plant–microbe interactions (Puppo *et al.* 2013). The content of H_2O_2 in plant cells is largely determined by the activity of enzymes degrading this molecule. Catalase (CAT) catalyses the decomposition of H_2O_2 to H_2O and O_2. The O_2^- is converted into H_2O_2 by the enzyme superoxide dismutase (SOD), which provides the first line of defence against oxygen toxicity. Peroxidase (PX) is involved in the consumption or release of H_2O_2 and other ROS.

In the interaction between rhizobia and IT legumes, transient increasing levels of ROS have been detected at the tip of actively growing root hair cells within seconds of the addition of NF (Cárdenas *et al.* 2008). In *S. 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 of ROS production has been observed, which peak at several hours of interaction (Santos *et al.* 2001). During the infection process, ROS can be detected in infection threads and in infected plant cells, indicating their production during this process. It has been shown that the inhibition of this response prevents IT formation (Salzwedel and Dazzo 1993). Moreover, ROS production has not been observed in *Medicago truncatula* plants inoculated with a *S. meliloti nodD1ABC* mutant unable to produce NF, indicating that the oxidative burst is activated downstream of NF perception (Ramu *et al.* 2002). More recently, studies have highlighted the role that NF have in governing the transient reduction of ROS accumulation, which induces root hair deformation in legumes (Lohar *et al.* 2007). At the early stages of the interaction, inoculation of *S. meliloti* mutant in the NF biosynthesis induced higher production of H_2O_2 in *Medicago* plants (Bueno *et al.* 2001) without O_2^- production (Ramu *et al.* 2002), a phenomenon not observed with wild-type strains. This has been attributed to the fact that the interaction between *Medicago sativa* and the wild-type *S. meliloti* strain is linked to an increase in the antioxidant defence (particularly related to the activities of the antioxidant enzymes catalase and lipoxygenase; Bueno *et al.* 2001). Plasma membrane NADPH oxidases have been identified as a major source of the oxidative burst observed during plant–pathogen interactions (Marino *et al.* 2012), and also appear to play an important role in ROS production during the symbiotic process (Marino *et al.* 2011). The primary product produced by plant NADPH-oxidase is O_2^-, which is converted into H_2O_2 as a result of dismutation catalysed by SOD.

Despite the widespread acceptance that ROS are critical for optimal symbiosis in legumes invaded by IT, no information is available about this process in legumes, such as peanut, infected by crack entry without IT formation. Thus, the aim of this study was to evaluate whether the production of ROS and the activity of the plant antioxidant system are involved in the interaction between bradyrhizobia and peanut. The role of bradyrhizobial NF in modulating the peanut plants oxidative burst is also analysed.

Materials and methods

Bacterial strains and growth media

The bacterial strains used in this work are *Bradyrhizobium* sp. SEMIA 6144, which is able to nodulate peanut (*A. hypogaea* L.) and which was provided by Microbial Resource Centre (MIRCEN (Brazil)), and *Bradyrhizobium*

sp. V_2 derivative incapable of producing NF (Ibáñez and Fabra 2011). Stock cultures were maintained on yeast extract–mannitol agar (YEM agar) supplemented with Congo red (Vincent 1970), and when necessary, with kanamicin (100 μg ml^{-1}). Cultures were grown in YEM or in minimal B$^-$ medium (van Brussel *et al.* 1977) 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).

Purification and detection of NF by thin layer chromatography

Extraction of NF from broth culture, subsequent purification, HPLC and MS analyses were performed as described by Spaink (1992). To obtain large quantities of NF, 14 l of bacterial culture medium induced by genistein (1 μmol l^{-1}) and naringenin (1 μmol l^{-1}) was extracted with 4·5 l of n-butanol. The butanol extract was concentrated to dryness overnight under vacuum in a rotary evaporator, and then suspended in 50 ml of acetonitrile (ACN)–water 60% (v/v). The sample was incubated overnight on a magnetic stirrer and diluted with bidistilled water up to a concentration of ACN 20% (v/v). A prepurification step was performed by passing the crude extract through a C18 cartridge (Resprep, Restek Corporation, Benner Circle, Bellefonte, PA). The solid phase extraction column was eluted with methanol and lyophilized. NF were suspended in 1 ml of ACN 50% (v/v) for each litre of culture, sonicated for 15 min and filtered through a microfilter with a pore diameter of 0·45 μm used to remove impurities. A volume of 500 μl was analysed using HPLC Perkin Elmer series 200 equipment attached to a LC-MS/MS mass spectrophotometer (QTRAP-Applied Biosystems, a division of Life Technologies (NASDAQ:LIFE), AB SCIEX Framingham, MA). The rest of the sample was kept at 4°C until use.

NF biological activity was tested by adding 10 μl (10^{-6} mol l^{-1}) to roots of peanut plants growing as described in the next section. Plants were harvested at 28 days and their nodule number and shoot dry weight were determined. NF were shown to be biologically active when nodules were obtained.

Plant growth conditions

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 2–3 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) with or without nitrogen (5 mmol l^{-1} KNO_3). Fifty millilitre of bacterial cultures (*Bradyrhizobium* sp. SEMIA 6144 or *Bradyrhizobium* sp. V_2) in YEM medium containing 10^9 cells ml^{-1} at exponential phase was centrifuged at 5000 ***g*** for 5 min, and the pellet was resuspended in the same volume of distilled water. One millilitre of bacterial suspension or 10 μl of purified Nod factor (10^{-6} mol l^{-1}) was inoculated into the emergency zone of the lateral roots of 5 days old peanut plants. Negative controls (uninoculated seedlings) were included. Plants were grown under a controlled environment (light intensity of 200 μmol m^{-2} s^{-1}, 16 h day per 8 h night cycle, at a constant temperature of 28°C and a relative humidity of 50%). Five to 15 replicates were performed for each treatment. The roots were removed at 0, 5, 10, 15, 30, 60 and 240 min postinoculation (pi), frozen in liquid nitrogen and then stored at −80°C until use.

Root protein extraction

To measure the activity of the enzyme superoxide dismutase (SOD; EC 1.15.1.1) (EC 1.15.1.1), catalase (CAT; EC 1.11.1.6) and NADPH-oxidase (EC 1.6.3.1) frozen roots were ground to a fine powder in liquid nitrogen, with a pestle and mortar. Then, three volumes of 50 mmol l^{-1} potassium phosphate buffer were added (pH 7·4, pH 7·8 or pH 7 for SOD, CAT or NADPH-oxidase enzymatic determination, respectively) containing EDTA (1, 0·5 or 0·2 mmol l^{-1} for SOD, CAT or NADPH-oxidase enzymatic determination, respectively), 1% (w/v) polyvinylpolypyrrolidone and 0·5% (v/v) (SOD and CAT activities) or 0·1% (NADPH-oxidase activity) Triton X-100. The homogenate was centrifuged at 10 000 ***g*** for 30 min at 4°C, and the supernatant was transferred to a sterile tube and used for subsequent determinations of enzymatic activities.

To measure the activity of total PX, three volumes of 50 mmol l^{-1} sodium acetate/acetic acid (pH 5) containing 1 mol l^{-1} KCl were added to roots previously ground in a mortar with liquid nitrogen. The homogenate was centrifuged at 10 000 ***g*** for 20 min at 4°C, and the supernatant was transferred to a sterile tube and used for subsequent determinations of enzymatic activities.

Total protein content was determined with bovine serum albumin as standard, as described by Bradford (1976).

Enzyme assays

SOD activity was determined according to the procedures described by Beauchamp and Fridovich (1973). Enzymatic activity was expressed as units of SOD mg protein^{-1}. One unit of SOD was defined as the amount of enzyme required to inhibit the NBT photoreduction by 50%.

CAT activity was determined by measuring spectrophotometrically the consumption of H_2O_2 at 240 nm according to the method proposed by Aebi (1984). The CAT activity was expressed as nmol H_2O_2 min^{-1} mg protein^{-1}.

PX activity was determined by measuring the absorbance at 470 nm according to Sosa Alderete *et al.* (2009). PX activity was expressed as nmol (bis (3.3′-dimethoxy-4-amino) azobifenilo) min^{-1} mg protein^{-1}.

NADPH-oxidase activity was determined following the method described by Ishida *et al.* (1987) and was expressed as units mg proteins^{-1}.

Determination of H_2O_2 content

H_2O_2 was measured spectrophotometrically after reaction with potassium iodide according to Alexieva *et al.* (2001). The amount of H_2O_2 was calculated using a standard curve obtained from solutions of known concentrations of H_2O_2.

Determination of O_2^- content

O_2^- content was determined spectrophotometrically according to the procedure described by Frahry and Schopfer (2001). The absorbance was recorded continuously at 470 nm and converted to molar concentration using the molar extinction coefficient of O_2^- $2{\cdot}16 \times 10^4$ mol^{-1} cm^{-1} (Sutherland and Léarmonth 1997).

Histochemical detection of H_2O_2 and O_2^-

The O_2^- production was visualized at 0, 5, 10, 15, 30, 60 and 240 min postinoculation using NBT according to Frahry and Schopfer (2001). Fresh root's segments were incubated at 25°C in 3 ml of 10 mmol l^{-1} buffer sodium citrate (pH 6) containing 1 mmol l^{-1} NBT. When the NBT reacts with the O_2^-, it forms a dark blue insoluble precipitate. The roots were then clarified with 80% ethanol (v/v) and stored in three ml of water : glycerol (1 : 1). The root transverse sections obtained were assessed using a Carl Zeiss Stemi SV6 (Carl-Zeiss-Str. Oberkochen, Germany) stereoscopic microscope and photographed with a Canon digital camera.

H_2O_2 was visualized using 3.3-diaminobenzidine (DAB) as substrate according to the method previously described (Orozco-Cárdenas and Ryan 1999). Root segments were incubated at 25°C in 3 ml of a DAB-HCl solution (1 mg ml^{-1}, pH 3·8). When the DAB is oxidized by H_2O_2, a brownish insoluble polymer is formed. The roots were then clarified with 80% ethanol (v/v) and stored in 3 ml of water : glycerol (1 : 1). The root transverse sections obtained were assessed using a Carl Zeiss Stemi SV6 stereoscopic microscope and photographed with a Canon digital camera.

Data analysis

All measurements were performed in duplicate with 2–3 (Histochemical detection), five (O_2^- concentration, effect of nitrogen supply on H_2O_2 accumulation, plant dry weight), 5–10 (H_2O_2 concentration and PX activity), 7–10 (CAT and NADPH-oxidase activity) and 9–15 (SOD activity) independent samples.

The data were subjected to analysis of variance (ANOVA) and Tukey's post test. Differences with $P \leq 0{\cdot}05$ were considered significant.

Results

Production of H_2O_2 and not of O_2^- increases in the interaction peanut–*Bradyrhizobium* sp

In this study, we investigated the generation of H_2O_2 and O_2^- during the early stages of interaction between *Bradyrhizobium* sp. SEMIA 6144 and peanut, a legume infected by crack entry without IT formation. Root inoculation with the microsymbiont caused an increase in H_2O_2 content by 2·16-fold at 10 min pi. This was followed by a significant decrease of this reactive species, beginning 15 min after inoculation (Table 1). The oxidized DAB

Table 1 H_2O_2 concentration in peanut roots inoculated with *Bradyrhizobium* sp. SEMIA 6144 or *Bradyrhizobium* sp. V_2

H_2O_2 concentration (nmol g dry tissue^{-1})			
Time pi (min)	Uninoculated	*Bradyrhizobium* sp. SEMIA 6144	*Bradyrhizobium* sp. V_2
0	0·59 ± 0·05^a	0·59 ± 0·05^a	0·59 ± 0·05^a
5	0·52 ± 0·06^a	0·61 ± 0·09^a	1·26 ± 0·21^b
10	0·60 ± 0·03^a	1·56 ± 0·07^b	2·01 ± 0·10^c
15	0·56 ± 0·03^a	0·41 ± 0·07^a	1·86 ± 0·08^c
30	0·50 ± 0·05^a	0·59 ± 0·06^a	1·83 ± 0·07^c
60	0·56 ± 0·05^a	0·53 ± 0·04^a	1·21 ± 1·05^b
240	0·49 ± 0·07^a	0·57 ± 0·03^a	0·77 ± 0·07^a

Data are the mean ± SE (n = 5–10). Different superscript letters indicate significant differences following Tukey's test ($P \leq 0{\cdot}05$).

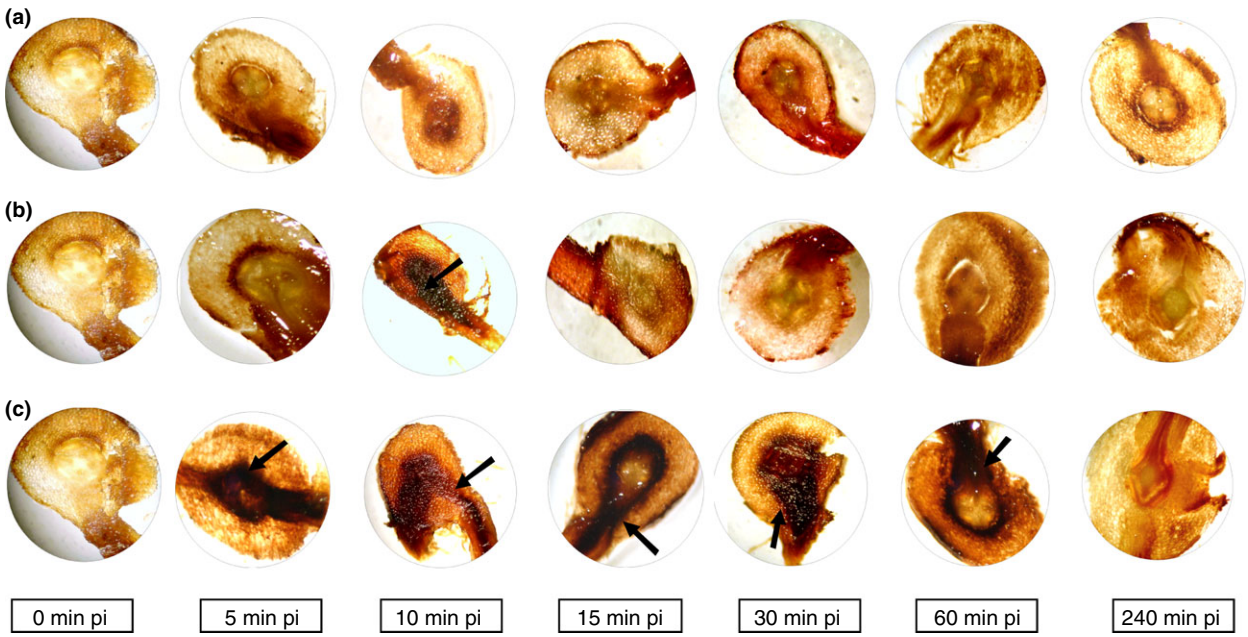

Figure 1 Histochemical detection of H_2O_2 in cross section peanut roots by DAB staining. (a) Uninoculated control roots. (b) Roots inoculated with *Bradyrhizobium* sp. SEMIA 6144. (c) Roots inoculated with *Bradyrhizobium* sp. V_2. Arrows indicate the brownish insoluble polymer resulting from the oxidation of DAB by H_2O_2.

form (a brownish insoluble polymer), which show sites of H_2O_2 accumulation, was located at the site of inoculation (the zone where a lateral root is emerging) and in the cortex cells of root section at 10 min pi (Fig. 1). In contrast, the level of O_2^- produced in inoculated roots was similar to that observed in the uninoculated control roots (Table 2). The evaluation of the O_2^- production using NBT confirmed this result, as no changes between inoculated and uninoculated control roots were observed (Fig. 2).

To know whether changes in root H_2O_2 production are specifically related to the development of the symbiotic interaction, *Bradyrhizobium* sp. SEMIA 6144 was inoculated in plants growing both with and without an available nitrogen source. It is assumed that, under this condition, symbiotic interaction is established only in plants growing without nitrogen supply. The increase in the H_2O_2 levels (at 10 min pi) observed only in inoculated roots grown without nitrogen suggested that it was related to the establishment of the symbiotic interaction (Table 3). The highest H_2O_2 levels were determined in root of plants growing with nitrogen supply. We speculate that this could be related to differences between metabolic pathways involved in the nitrogen uptake from an available source or by atmospheric nitrogen fixation.

Table 2 O_2^- concentration in peanut roots inoculated with *Bradyrhizobium* sp. SEMIA 6144 or *Bradyrhizobium* sp. V_2

O_2^- concentration (nmol min^{-1} g dry tissue^{-1})			
Time pi (min)	Uninoculated	*Bradyrhizobium* sp. SEMIA 6144	*Bradyrhizobium* sp. V_2
0	$0{\cdot}16 \pm 0{\cdot}01^a$	$0{\cdot}16 \pm 0{\cdot}01^a$	$0{\cdot}16 \pm 0{\cdot}01^a$
5	$0{\cdot}17 \pm 0{\cdot}01^a$	$0{\cdot}16 \pm 0{\cdot}01^a$	$0{\cdot}31 \pm 0{\cdot}03^b$
10	$0{\cdot}14 \pm 0{\cdot}02^a$	$0{\cdot}18 \pm 0{\cdot}02^a$	$0{\cdot}38 \pm 0{\cdot}02^c$
15	$0{\cdot}18 \pm 0{\cdot}01^a$	$0{\cdot}14 \pm 0{\cdot}02^a$	$0{\cdot}72 \pm 0{\cdot}04^d$
30	$0{\cdot}17 \pm 0{\cdot}01^a$	$0{\cdot}15 \pm 0{\cdot}01^a$	$0{\cdot}82 \pm 0{\cdot}06^b$
60	$0{\cdot}16 \pm 0{\cdot}01^a$	$0{\cdot}17 \pm 0{\cdot}01^a$	$0{\cdot}43 \pm 0{\cdot}06^b$
240	$0{\cdot}14 \pm 0{\cdot}01^a$	$0{\cdot}16 \pm 0{\cdot}01^a$	$0{\cdot}16 \pm 0{\cdot}01^a$

Data are the mean ± SE (n = 5). Different superscript letters indicate significant differences following Tukey's test ($P \leq 0{\cdot}05$).

Response of the plant antioxidant system to the *Bradyrhizobium* sp.-induced oxidative burst

Activities of the enzymes CAT, PX and SOD were tested to examine the responses of the plant antioxidant system to the *Bradyrhizobium* sp.-induced oxidative burst.

The results obtained showed that CAT activity increased in inoculated roots at 15 and 30 min pi, decreasing at 60 min pi to reach values similar to those found in uninoculated control roots (Table 4).

PX activity also increased in inoculated roots but at 30 and 60 min pi, declining after 240 min pi to values indistinguishable from uninoculated plants (Table 5). Therefore, it is possible to suggest that CAT and PX may be

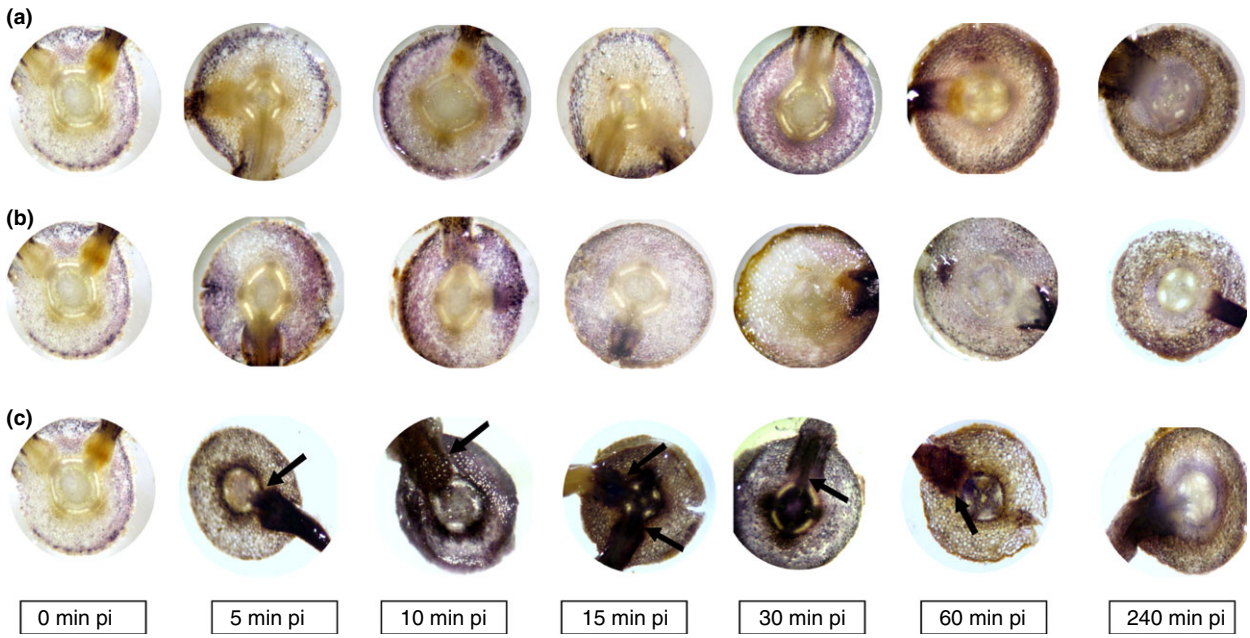

Figure 2 Histochemical detection of O_2^- in cross sections peanut roots by NBT staining. (a) Uninoculated control roots. (b) Roots inoculated with *Bradyrhizobium* sp. SEMIA 6144. (c) Roots inoculated with *Bradyrhizobium* sp. V_2. Arrows indicate a coloured formazan precipitate resulting from the reduction of NBT by O_2^-.

Table 3 H_2O_2 concentration in peanut roots growing with or without nitrogen (5 mmol l^{-1} KNO_3), inoculated with *Bradyrhizobium* sp. SEMIA 6144 or treated with purified Nod factors (NF)

	H_2O_2 concentration (nmol g dry tissue^{-1})	
	5 min pi	10 min pi
Uninoculated	1·30 ± 0·16^a	1·15 ± 0·21^a
Uninoculated + 5 mmol l^{-1} KNO_3	4·10 ± 0·69^b	4·72 ± 0·43^b
Bradyrhizobium sp. SEMIA 6144	1·43 ± 0·10^a	2·99 ± 0·14^c
Bradyrhizobium sp. SEMIA 6144 + 5 mmol l^{-1} KNO_3	4·35 ± 0·26^b	4·72 ± 0·16^b
NF	1·03 ± 0·07^a	2·78 ± 0·22^c
NF + 5 mmol l^{-1} KNO_3	4·51 ± 0·25^b	4·68 ± 0·38^b

Data are the mean ± SE (n = 5). Different superscript letters indicate significant differences following Tukey's test ($P \leq 0·05$).

involved in the diminution of the H_2O_2 content in inoculated roots.

As expected, SOD activity remained constant after root inoculation (Table 6), in concordance with the absence of changes in the O_2^- levels determined in these roots.

Increase in the H_2O_2 production in peanut–*Bradyrhizobium* sp. interaction seems not to be related to the NADPH-oxidase activity

In this study, the activity of NADPH-oxidase was evaluated to know whether this enzyme is involved in the H_2O_2 generation in peanut roots inoculated with *Bradyrhizobium* sp. SEMIA 6144. The results obtained showed that, up to 60 min pi, the enzymatic activity did not differ between inoculated and uninoculated roots, suggesting that this enzyme would not be involved in the increase of H_2O_2 production observed at 10 min after inoculation (Table 7).

Inoculation of *Bradyrhizobium* sp. V_2 mutant strain increased H_2O_2 and O_2^- production in peanut roots

The role of NF in modulating the oxidative burst produced after root inoculation was tested using non-nodulating *Bradyrhizobium* sp. V_2 strain impaired in NF production (Ibáñez and Fabra 2011). When the H_2O_2 or O_2^- production was quantified, an increase in both reactive species (2·7- and 2·2-fold, respectively) was observed 5 min after inoculation. This was followed by a significant decrease, reaching the basal values at 240 min pi (Tables 1 and 2). These results differ from those obtained in roots inoculated with *Bradyrhizobium* sp. SEMIA 6144, as the H_2O_2 level in these roots increased at lower extent and later (10 min pi) and subsequently declined to basal level earlier than in roots inoculated with the mutant strain (15 min pi) (Table 1). Histological determination of H_2O_2 and O_2^- in sections from these roots also indicated the production of both ROS at 5 min pi (Figs 1 and 2).

Table 4 Catalase activity in peanut roots inoculated with *Bradyrhizobium* sp. SEMIA 6144 or *Bradyrhizobium* sp. V_2 or treated with purified Nod factors (NF)

Catalase activity (nmol H_2O_2 mg proteins^{-1} min^{-1})				
Time pi (min)	Uninoculated	*Bradyrhizobium* sp. SEMIA 6144	*Bradyrhizobium* sp. V_2	NF
0	0·58 ± 0·04^a	0·58 ± 0·04^a	0·58 ± 0·04^a	ND
5	0·77 ± 0·13^a	0·73 ± 0·11^a	0·68 ± 0·12^a	ND
10	0·58 ± 0·04^a	0·57 ± 0·05^a	0·74 ± 0·06^a	0·58 ± 0·09^a
15	0·65 ± 0·05^a	1·48 ± 0·20^b	0·57 ± 0·06^a	1·26 ± 0·13^b
30	0·59 ± 0·05^a	2·44 ± 0·19^c	0·77 ± 0·05^a	2·27 ± 0·40^c
60	0·86 ± 0·12^a	0·98 ± 0·11^a	0·82 ± 0·07^a	ND
240	0·53 ± 0·07^a	0·71 ± 0·08^a	0·55 ± 0·05^a	ND

ND, not determinated.
Data are the mean ± SE (n = 7–10). Different superscript letters indicate significant differences following Tukey's test ($P \leq 0{\cdot}05$).

Table 5 Peroxidase activity in peanut roots inoculated with *Bradyrhizobium* sp. SEMIA 6144 or *Bradyrhizobium* sp. V_2 or treated with purified Nod factors (NF)

Peroxidase activity (nmol (bis(3.3′-dimethoxy-4-amino)azobifenilo) min^{-1} mg proteins^{-1})				
Time pi (min)	Uninoculated	*Bradyrhizobium* sp. SEMIA 6144	*Bradyrhizobium* sp. V_2	NF
15	2·54 ± 0·77^a	3·85 ± 0·47^a	3·84 ± 1·00^a	3·76 ± 0·8^a
30	3·32 ± 0·74^a	9·73 ± 0·90^b	9·89 ± 0·89^b	10·39 ± 1·92^b
60	2·76 ± 0·56^a	4·19 ± 0·94^c	12·86 ± 0·67^d	4·87 ± 1·12^c
240	2·44 ± 0·61^a	1·99 ± 0·25^a	5·39 ± 0·63^c	2·02 ± 0·50^a

Data are the mean ± SE (n = 5–10). Different superscript letters indicate significant differences following Tukey's test ($P \leq 0{\cdot}05$).

Table 6 Superoxide dismutase (SOD) activity in peanut roots inoculated with *Bradyrhizobium* sp. SEMIA 6144 or *Bradyrhizobium* sp. V_2

SOD activity (U SOD mg proteins^{-1})			
Time pi (min)	Uninoculated	*Bradyrhizobium* sp. SEMIA 6144	*Bradyrhizobium* sp. V_2
0	61·70 ± 7·20	61·70 ± 7·20	61·70 ± 7·20
5	74·99 ± 10·21	61·08 ± 6·69	63·33 ± 8·99
10	71·65 ± 9·40	67·23 ± 6·01	60·30 ± 6·01
15	72·44 ± 8·43	67·41 ± 4·72	58·36 ± 6·47
30	76·46 ± 8·97	69·34 ± 9·06	70·83 ± 8·50
60	78·86 ± 10·53	67·99 ± 8·16	64·96 ± 8·12
240	67·29 ± 5·63	67·80 ± 6·98	57·09 ± 5·63

Data are the mean ± SE (n = 9–15).

Table 7 NADPH-oxidase activity in peanut roots inoculated with *Bradyrhizobium* sp. SEMIA 6144 or *Bradyrhizobium* sp. V_2

NADPH-oxidase activity (units mg proteins^{-1})			
Time pi (min)	Uninoculated	*Bradyrhizobium* sp. SEMIA 6144	*Bradyrhizobium* sp. V_2
0	1·73 ± 0·17^a	1·73 ± 0·17^a	1·73 ± 0·17^a
5	1·57 ± 0·13^a	1·36 ± 0·14^a	2·79 ± 0·24^b
10	1·62 ± 0·11^a	1·38 ± 0·14^a	3·63 ± 0·21^c
15	1·70 ± 0·19^a	1·53 ± 0·10^a	3·54 ± 0·25^c
30	1·61 ± 0·13^a	1·98 ± 0·23^a	3·50 ± 0·26^c
60	1·45 ± 0·13^a	1·89 ± 0·12^a	3·62 ± 0·33^c
240	1·61 ± 0·10^a	3·04 ± 0·24^b	3·99 ± 0·17^d

Data are the mean ± SE (n = 7–10). Different superscript letters indicate significant differences following Tukey's test ($P \leq 0{\cdot}05$).

NF modulate production of H_2O_2 during the early steps of peanut–*Bradyrhizobium* sp. interaction

The ability of NF to suppress H_2O_2 production was confirmed by the application of these purified molecules to roots. After this treatment, the level of H_2O_2 reached values that were indistinguishable from those observed in *Bradyrhizobium* sp. SEMIA 6144 inoculated roots (Table 3). This response was associated with the development of the symbiotic interaction, as it was only observed in treated roots of plants growing without nitrogen (Table 3). Taking these results together, we suggest that the reduction not only in the quantity of H_2O_2 produced but also in the time points in which it reached the maximum level in roots inoculated with the wild-type strain compared with those inoculated with the NF impaired strain could be related to the modulating NF

activity, thus preventing the expression of the defensive system of the host plant.

To be sure that purified NF have biological activity, they were checked by a complementation assay by treating roots simultaneously with both NF and the non-nodulating mutant strain incapable of synthesizing these molecules. Nodules were formed on these roots, and the dry weight of these plants was similar to that of plants inoculated with *Bradyrhizobium* sp. SEMIA 6144, indicating that they were nitrogen-fixing nodules (Table S1).

NF activate peanut CAT activity and later repress PX activity during symbiotic interaction

To test whether NF play a role in the activation of the peanut antioxidant system, we evaluated the activity of CAT, SOD and PX in plants inoculated with the mutant strain *Bradyrhizobium* sp. V_2. The results obtained were compared with those from roots treated with NF or inoculated with the wild-type strain.

In roots inoculated with the mutant strain, CAT and SOD activity levels did not differ from those determined in uninoculated roots and remained constant at all time points evaluated (Tables 4 and 6). Considering that CAT activity increased in both NF-treated and wild-type inoculated roots, it is possible to suggest that NF play a role in the induction of this enzyme activity, thus reducing the oxidative burst and allowing for the development of the symbiotic interaction.

PX activity increased at 30 min pi in plants inoculated with the mutant strain compared to uninoculated roots, reaching levels found in roots inoculated with the wild-type strain. Therefore, this enzyme may be involved in the diminution of the H_2O_2 content in these roots. The PX activity remained significantly high up to 240 min pi in plants inoculated with the mutant strain (Table 5). Taking into account that in roots inoculated with the wild-type strain or NF treated, PX activity reached basal values earlier (at 60 min pi, Table 5), we propose that NF could be repressing this enzyme activity after 60 min pi.

NF play a role in the diminution of peanut NADPH-oxidase activity during symbiotic interaction

The O_2^- production determined in roots inoculated with the mutant strain (Table 2) could be related to the NADPH-oxidase activity (Marino *et al.* 2012). In fact, in these roots, a high NADPH-oxidase activity was determined at all the times measured (from 5 to 240 min pi) (Table 7), suggesting it plays a role in the O_2^- production in these roots.

Taking all these results into account, we propose that NF play a role in the diminution of the ROS levels modulating the activity of NADPH-oxidase (a ROS generator) and CAT enzymes. This ability of NF to change plant metabolism contributes to the establishment of the conditions required for the development of symbiosis in the host.

In summary, our results revealed that early events of the symbiotic interaction in legumes invaded by crack entry trigger an increase in ROS production (represented exclusively by a higher H_2O_2 content) in which NADPH-oxidase seems not to be involved. Furthermore, NF modulate this response by enhancing the plant antioxidant machinery, thus contributing to the symbiosis development.

Discussion

ROS production in plants via consumption of oxygen in a so-called oxidative burst is one of the earliest cellular responses following successful pathogen recognition. Apoplastic generation of O_2^-, or its dismutation product H_2O_2, has been documented following recognition of a variety of pathogens (Grant *et al.* 2000). Several recent reports demonstrate that members of the NADPH-oxidase family mediate the production of apoplastic ROS during the defence responses (Torres *et al.* 2005).

By analogy to plant responses to pathogen elicitors, the production of ROS in rhizobia–IT legumes interaction may involve initial production of O_2^- from O_2 reduction and its rapid conversion into H_2O_2 by SOD (Santos *et al.* 2001). In addition, protein kinase C (PKC) (Quan *et al.* 2008) and cell membrane NADPH-oxidase (Marino *et al.* 2012) have been shown to be a source of H_2O_2 for the oxidative burst produced during this interaction. Other cell wall-located enzymes such as germin-like oxalate oxidases, PXs and amine oxidases have been proved to be responsible for ROS generation at the apoplast (Pauly *et al.* 2006).

During the establishment of the symbiotic interaction in *Medicago* and *Phaseolus vulgaris*, O_2^- (Bielski *et al.* 1980) and/or H_2O_2 (Jamet *et al.* 2007) can be detected in IT, possibly related to the control of the bacterial development inside these structures (Rubio *et al.* 2009). In addition, H_2O_2 and PX have been proposed to be involved in the regulation of infection by cell wall cross-linking, limiting the ingress of bacteria in a manner analogous to that of pathogen infection (Salzwedel and Dazzo 1993). More recently, ROS production has been related to the root hair deformation in the *M. truncatula-S. meliloti* symbiosis (Lohar *et al.* 2007) and to IT development (Jamet *et al.* 2007). In *S. rostrata–Azorhizobium caulinodans* interaction, it was demonstrated that ROS production mediates not only the root hair deformation but also the infection pocket formation (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; Fabra *et al.* 2010). O_2^- root production was not detected in the peanut–*Bradyrhizobium* sp. SEMIA 6144 interaction, which could be related with the high PX activity determined in this organ. Similar results were found in peanut nodule environment (Maiti *et al.* 2012). An increase in the peanut inoculated root H_2O_2 content was detected at 10 min pi, an earlier time than in *Medicago* (12–24 h pi) (Ramu *et al.* 2002). This fast response is probably related to the fact that bradyrhizobia bypass the peanut root epidermal cells, becoming intracellular directly at the cortical cells, unlike what happens in IT legumes. On the other hand, the activity of NADPH-oxidase was not increased in inoculated peanut roots, indicating that the generation of H_2O_2 could be related to other sources, such as PKC or amine oxidases activities.

Compatibility between symbionts in the *Bradyrhizobium* sp.–peanut interaction seems to depend mostly on the H_2O_2 detoxification via CAT activity (which peaked at 15 min pi, concomitant with the H_2O_2 content decrease), and also PX activity (at 30 min pi). We propose that the production of H_2O_2 in peanut–bradyrhizobia interaction may be a plant defence response to bradyrhizobia which are initially recognized as intruders at the root cortical tissue. Indeed, ROS production was associated with the symbiotic interaction between both partners, because it was not detected in plants growing with a source of available nitrogen.

The importance of NF as signalling molecules in the early stages of symbiotic interaction has been widely described for legumes invaded by IT (Pauly *et al.* 2006). However, results about the role of these molecules in ROS generation in these legumes are controversial. It has been suggested that *P. vulgaris* ROS production is a consequence of specific NF perception (Cárdenas *et al.* 2008). Conversely, a reduction in the ROS levels in *Medicago* roots treated with compatible NF has been reported (Ramu *et al.* 2002; Shaw and Long 2003). Moreover, a *S. meliloti* strain deficient in the production of NF has been observed to induce H_2O_2 accumulation during the first hours pi as compared with the wild-type *S. meliloti* (Bueno *et al.* 2001). The authors proposed that the reduction in ROS efflux mediated by NF could allow changes in cell growth required for root hair curling and IT development.

In the peanut–bradyrhizobia interaction, we demonstrated that NF are required for the nodule primordium development, because *Bradyrhizobium* sp. V_2, a *Bradyrhizobium* SEMIA 6144 derivative strain affected in the synthesis of these molecules, is unable to induce cortical cell division (Ibáñez and Fabra 2011). In this study, by inoculating the mutant strain *Bradyrhizobium* sp. V_2 and by adding purified NF, we present evidence that specific recognition of these molecules can modulate ROS production in the host plant. It seems that peanut recognizes the mutant strain as a nonbeneficial bacterium, probably preventing it from entering the root. This hypothesis is supported by the fact that in roots inoculated with the mutant strain not only the H_2O_2 level increased earlier and remained high for a more extended time than in wild-type inoculated roots, but also the O_2^- was generated, correlating with an elevated NADPH-oxidase activity. The fact that these characteristics were reverted by the purified NF addition confirms the role of these molecules in modulating ROS production in peanut roots, both by increasing CAT activity and by diminishing the NADPH-oxidase activity.

Considering the results obtained in this study, we demonstrated for the first time that the early events of the symbiotic interaction in legumes invaded by crack entry without IT formation trigger an oxidative burst that differs from that reported for some IT legumes in that it is represented exclusively by H_2O_2 production without the contribution of NADPH-oxidase activity. Furthermore, the results also indicate that NF are able to modulate this response by enhancing the plant antioxidant machinery and attenuating the ROS generating system, thus contributing to the creation of the conditions required for the development of symbiosis in peanut plants.

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Conflict of Interest

No conflict of interest declared.

References

Aebi, H. (1984) Catalase *in vitro*. *Methods Enzymol* **105**, 121–126.

Alexieva, V., Sergiev, I., Mapelli, S. and Karanov, E. (2001) The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. *Plant, Cell Environ* **24**, 1337–1344.

Beauchamp, C.O. and Fridovich, I. (1973) Isoenzymes of SOD from wheat germ. *Biochim Biophys Acta* **317**, 50–54.

Bielski, B.H.J., Shiue, G.G. and Bajuk, S. (1980) Reduction of nitroblue tetrazolium by CO_2 and O_2^- radicals. *J Phys Chem* **84**, 830–834.

Boogerd, F.C. and van Rossum, D. (1997) Nodulation of groundnut by *Bradyrhizobium*: a simple infection process by crack entry. *FEMS Microbiol Rev* **21**, 5–27.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.

van Brussel, A.A.N., Planqu'e, K. and Quispel, A. (1977) The wall of *Rhizobium leguminosarum* in bacteroid and free-living forms. *J Gen Microbiol* **101**, 51–56.

Bueno, P., Soto, M.J., Rodriguez-Rosales, M.P., Sanjuan, J., Olivares, J. and Donaire, J.P. (2001) Time-course of lipoxygenase, antioxidant enzyme activities and H_2O_2 accumulation during early stages of *Rhizobium* legume symbiosis. *New Phytol* **152**, 91–96.

Cárdenas, L., Martínez, A., Sánchez, F. and Quinto, C. (2008) Fast, transient and specific intracellular ROS changes in living root hair cells responding to Nod factors (NFs). *Plant J* **56**, 802–813.

D'Haeze, W., De Rycke, R., Mathis, R., Goormachtig, S., Pagnotta, S., Verplancke, C., Capoen, W. and Holsters, M. (2003) Reactive oxygen species and ethylene play a positive role in lateral root base nodulation of a semiaquatic legume. *Proc Natl Acad Sci USA* **100**, 11789–11794.

Fabra, A., Castro, S., Taurian, T., Angelini, J., Ibañez, F., Dardanelli, M., Tonelli, M., Bianucci, E. *et al.* (2010) Interaction among *Arachis hypogaea* L. (peanut) and beneficial soil microorganisms: how much is it known? *Crit Rev Microbiol* **36**, 179–194.

Frahry, G. and Schopfer, P. (2001) NADH-stimulated, cyanide-resistant superoxide production in maize coleoptiles analyzed with a tetrazolium based assay. *Planta* **212**, 175–183.

Goormachtig, S., Capoen, W., James, E.K. and Holsters, M. (2004) Switch from intracellular to intercellular invasion during water stress-tolerant legume nodulation. *Proc Natl Acad Sci USA* **101**, 6303–6308.

Grant, M., Brown, I., Adams, S., Knight, M., Ainslie, A. and Mansfield, J. (2000) The RPM1 plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. *Plant J* **23**, 441–450.

Hoagland, D.R. and Arnon, D.I. (1950) Water culture method for growing plants without soil. *Cal. Agr. Exp. Sta. Circ.*, pp. 347.

Ibáñez, F.J. and Fabra, A. (2011) Rhizobial Nod factors are required for cortical cell division in the nodule morphogenetic programme of the Aeschynomeneae legume *Arachis*. *Plant Biol* **13**, 794–800.

Ishida, A., Okubo, K. and Ono, K. (1987) Formation of H_2O_2 by NAD(P)H oxidation with isolated cell wall-associated peroxidase from cultured liverwort cells, *Marchantia polymorpha* L. *Plant Cell Physiol* **28**, 723–726.

Jamet, A., Mandon, K., Puppo, A. and He′rouart, D. (2007) H_2O_2 is required for optimal establishment of the *Medicago sativa/Sinorhizobium meliloti* symbiosis. *J Bacteriol* **187**, 8741–8745.

Lohar, D.P., Haridas, S., Gantt, J.S. and VandenBosch, K.A. (2007) A transient decrease in reactive oxygen species in roots leads to root hair deformation in the legume–rhizobia symbiosis. *New Phytol* **173**, 39–49.

Maiti, D., Sarkar, T.S. and Ghosh, S. (2012) Detection of *S*-nitrosothiol and nitrosylated proteins in *Arachis hypogaea* functional nodule: response of the nitrogen fixing symbiont. *PLoS ONE* **7**, e45526.

Marino, D., Andrio, E., Danchin, E.G.J., Oger, E., Gucciardo, S., Lambert, A., Puppo, A. and Pauly, N. (2011) A *Medicago truncatula* NADPH-oxidase is involved in symbiotic nodule functioning. *New Phytol* **189**, 580–592.

Marino, D., Dunand, C., Puppo, A. and Pauly, N. (2012) A burst of plant NADPH oxidases. *Trends Plant Sci* **17**, 9–15.

Monshausen, G.B., Bibikova, T.N., Messerli, M.A., Shi, C. and Gilroy, S. (2007) Oscillations in extracellular pH and reactive oxygen species modulate tip growth of *Arabidopsis* root hairs. *Proc Natl Acad Sci USA* **104**, 20996–21001.

Oldroyd, G. and Downie, J. (2004) Calcium, kinases and nodulation signaling in legumes. *Nat Rev Mol Cell Biol* **5**, 566–576.

Orozco-Cárdenas, M. and Ryan, C.A. (1999) Hydrogen peroxide is generated systemically in plant leaves by wounding and system in via the octadecanoid pathway. *Proc Natl Acad Sci USA* **96**, 6553–6557.

Pauly, N., Pucciariello, C., Mandon, K., Innocenti, G., Jamet, A., Baudouin, E., Herouart, D., Frendo, P. *et al.* (2006) Reactive oxygen and nitrogen species and glutathione: key players in the legume–*Rhizobium* symbiosis. *J Exp Bot* **57**, 1769–1776.

Pei, Z.M., Mutrata, Y., Benning, G., Thomine, S., Kiusener, B., Allen, G.J., Brill, E. and Schroeder, J.I. (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cell. *Nature* **406**, 731–734.

Peoples, M.B., Brockwell, J., Herridge, D.F., Rochester, I.J., Alves, B.J.R., Urquiaga, S., Boddey, R.M., Dakora, F.D. *et al.* (2009) The contributions of nitrogen-fixing crop legumes to the productivity of agricultural systems. *Symbiosis* **48**, 1–17.

Prayitno, J., Stefaniak, J., McIver, J., Weinman, J.J., Dazzo, F.B., Ladha, J.K., Barraquio, W., Yanni, Y.G. *et al.* (1999) Interactions of rice seedlings with bacteria isolated from rice roots. *Aust J Plant Physiol* **26**, 521–535.

Puppo, A., Pauly, N., Boscari, A., Mandon, K. and Brouquisse, R. (2013) Hydrogen peroxide and nitric oxide: key

regulators of the legume—*Rhizobium* and mycorrhizal symbioses. *Antioxid Redox Signal* **18**, 2202–2219.

Quan, L.J., Zhang, B., Shi, W.W. and Li, H.Y. (2008) Hydrogen peroxide in plants: a versatile molecule of the reactive oxygen species network. *J Integr Plant Biol* **50**, 2–18.

Ramu, S.K., Peng, H.M. and Cook, D.R. (2002) Nod factor induction of reactive oxygen species production is correlated with expression of the early nodulin gene *rip1* in *Medicago truncatula*. *Mol Plant Microbe Interact* **15**, 522–528.

Rubio, M.C., Becana, M., Kanetmatsu, S., Ushimaru, T. and James, E.K. (2009) Immunolocalization of antioxidant enzymes in high-pressure frozen root and stem nodules of *Sesbania rostrata*. *New Phytol* **183**, 395–407.

Salzwedel, J.L. and Dazzo, F.B. (1993) pSym *nod* gene influence on elicitation of peroxidase activity from white clover and pea roots by rhizobia and their cell-free supernatants. *Mol Plant Microbe Interact* **6**, 127–134.

Santos, R., Herouart, D., Sigaud, S., Touati, D. and Puppo, A. (2001) Oxidative burst in alfalfa-*Sinorhizobium meliloti* symbiotic interaction. *Mol Plant Microbe Interact* **14**, 86–89.

Sharma, P., Jha, A.B., Dubey, R.S. and Pessarakli, M. (2012) Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *J Bot*, vol 2012 (ID 217037) 1–26 doi: 10.1155/2012/217037.

Shaw, S.L. and Long, S.R. (2003) Nod factor inhibition of reactive oxygen efflux in a host legume. *Plant Physiol* **132**, 2196–2204.

Somasegaran, P. and Hoben, H.J. (1994) *Quantifying the Growth of Rhizobia, Handbook for Rhizobia*. pp. 47–57. New York, NY: Springer Verlag.

Sosa Alderete, L.G., Talano, M.A., Ibañez, S.G., Purro, S., Agostini, E., Milrad, S.R. and Medina, M.I. (2009) Establishment of transgenic tobacco hairy roots expressing basic peroxidases and its application for phenol removal. *J Biotechnol* **139**, 273–279.

Spaink, H.P. (1992) Rhizobial lipo-oligosaccharides: answers and questions. *Plant Mol Biol* **20**, 977–986.

Sutherland, M.W. and Léarmonth, B.A. (1997) The tetrazolium dyes MTS and XTT provide new quantitative assays for superoxide and superoxide dismutase. *Free Radic Res* **27**, 283–289.

Torres, M.A., Jones, J.D. and Dangl, J.L. (2005) Pathogen-induced, NADPH oxidase-derived reactive oxygen intermediates suppress spread of cell death in *Arabidopsis thaliana*. *Nat Genet* **37**, 1130–1134.

Uheda, E., Daimon, H. and Yoshizako, F. (2001) Colonization and invasion of peanut (*Arachis hypogaea* L.) roots by gusA-marked *Bradyrhizobium* sp. *Can J Bot* **79**, 733–738.

Vincent, J. (1970) *A Manual for the Practical Study of Root Nodule Bacteria*. IBP Handbook No. 15. pp. 73–97. Oxford: Blackwell Scientific Publications Ltd.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Dry weights of peanut plants inoculated with *Bradyrhizobium* sp. SEMIA 6144 or treating together with purified Nod factors (NF) and the nonnodulating mutant strain incapable to synthesize NF.