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RESEARCH PAPER

Rhizobial Nod factors are required for cortical cell division in the nodule morphogenetic programme of the Aeschynomeneae legume *Arachis*

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ABSTRACT

Nod factors are among the best-studied molecules implicated in the signal exchange that leads to legume–rhizobia symbiosis. The role of these molecules in symbiosis development has been primarily studied in legumes invaded through infection threads. In these plants, Nod factors generate several responses required for nodulation, including the induction of cortical cell division to form the nodule primordium. *Arachis hypogaea* L. (peanut) exhibits a specific mode of rhizobial infection and nodule morphogenetic programme in which infection threads are never formed. The role of Nod factors in this particular mechanism is unknown. In this work, a peanut symbiont mutant strain unable to produce Nod factors was obtained and characterised. The strain *Bradyrhizobium* (*Arachis*) sp. SEMIA 6144 V2 is altered in the *nodC* gene, which encodes an *N*-acetylglucosaminyl transferase involved in the first step of the Nod factor biosynthetic pathway. Further research revealed that, although its ability to colonise peanut roots was unaffected, it is not capable of inducing the division of cortical cells. The results obtained indicate that rhizobial Nod factors are essential for the induction of cortical cell division that leads to nodule primordium formation.

INTRODUCTION

Arachis hypogaea L. (peanut or groundnut) is a widespread leguminous plant of great agricultural and economic significance that can be used as food crop and as the source of several food products. Taxonomically, peanut belongs to the Fabaceae, subfamily Papilionoideae, and to the diverse aeschynomenoid/dalbergioid clade (Doyle & Luckow 2003).

Plant growth is often limited by the availability of nitrogen. Plants of the Fabaceae (legumes) have developed a nitrogen-fixing symbiotic association with soil bacteria, collectively known as rhizobia, to overcome this nutrient limitation. The morphological effect associated with the symbiosis is the formation of specialised plant organs, named nodules, in which rhizobia differentiate into bacteroids and fix atmospheric nitrogen that is transferred to the plant. The development of the association involves a complex molecular recognition that includes plant flavonoids, rhizobial Nod factors, bacterial surface molecules (such as lipopolysaccharides, cyclic glucans, exopolysaccharides) and proteins secreted through types I, III (TTSS) and IV (T4SS) secretion systems (Oldroyd & Downie 2008). Rhizobial Nod factors are among the best characterised of these molecules and their role in the symbiosis with some legumes is well known. Nod factors of all studied rhizobia are composed of a β-1,4-linked *N*-acetyl-D-glucosamine backbone of three to six subunits. The products of rhizobial *nodABC* genes are required for Nod factor core synthesis. Depending on the rhizobial strain, the core structure has several variations. The substitutions present in the backbone determine the biological activity of a Nod factor on a particular host plant (Perret *et al.* 2000; D'Haeze & Holsters 2002; Gage 2004; Oldroyd & Downie 2004).

Different modes of root invasion and nodule morphogenetic programmes have been described in the legume–rhizobia symbiosis, which are under control of the host plant (Oldroyd & Downie 2008). Within the Fabaceae, research on the molecular basis of nodulation is mainly restricted to the subfamily Papilionoideae (Wojciechowski *et al.* 2004; Lavin *et al.* 2005; Sprent 2007). The further division of this subfamily into four major groups (aeschynomenoid/dalbergioid, genistoid, hologalegina and phaseoloid/millettioid) is supported by molecular data (Doyle & Luckow 2003). These groups exhibit differences in their rhizobial infection strategies and nodule morphogenetic programmes (Sprent 2007). The most studied invasion mechanism occurs through infection thread formation, and takes place in most temperate legumes (*Vicia*, *Trifolium*, *Pisum* among others) and some (sub)tropical legumes (such as *Phaseolus*, *Glycine*, *Vigna*, *Macroptilium*) (Goormachtig *et al.* 2004; Sinharoy *et al.* 2009). Model legumes such as *Lotus japonicus* and *Medicago truncatula*, which belong to the hologalegina group, are also invaded through this infection strategy. The role of rhizobial Nod factors in the infection thread mechanism and nodule organogenesis of these legumes is well understood (Oldroyd &

Downie 2008). During this infection mechanism, Nod factors induce epidermal and cortical responses that lead to nodulation. Epidermal perception of Nod factors elicits calcium spiking, and a Ca^{2+}/calmodulin-dependent protein kinase (CCaMK) is involved in its recognition and interpretation. However, root hair deformation is independent of calcium spiking. Cortical responses include the re-initiation of meristematic activity of cortical cells to form the nodule primordia. Current models of the Nod signalling pathway suggest that a localised production of cytokinins, which is perceived by *Lotus* histidine kinase (LHK1), coordinates epidermal and cortical responses during nodule formation. Downstream, the proteins nodulation signalling pathway 1 (NSP1) and NSP2 (two GRAS family transcriptional regulators) are required (Oldroyd & Downie 2008).

An alternative and less investigated mode of infection is crack entry. In this mechanism, rhizobia enter between cells *via* cracks in the epidermis that originate from lateral or adventitious root protrusion. Afterward, rhizobia colonise the root cortex, the further spread into nodule primordia varies depending on the legume species (Goormachtig *et al.* 2004). The molecular basis of crack invasion has so far been studied in *Sesbania rostrata*, a legume that switches from intracellular to intercellular invasion under submerged growth conditions. In this legume (and also in *Neptunia* spp.), even when epidermal invasion occurs through cracks, inter- and intracellular infection threads that guide rhizobia towards the nodule primordia are later formed. *Arachis hypogaea* L. exhibits a particular mode of crack entry invasion and nodule morphogenetic programme. In this legume, infection threads are never formed, not for epidermal or cortical invasion or for nodular dissemination (Boogerd & van Rossum 1997). Peanut rhizobia enter the root at the junction of a hair cell with epidermal and cortical cells through structurally altered cell walls (Uheda *et al.* 2001). Within cortical cells, rhizobia multiply rapidly and the invaded cells divide repeatedly, giving rise to the typical aeschynomenoid nodules where the core infected zone is not interspersed with uninfected cells (Boogerd & van Rossum 1997). In this particular legume, the requirement for Nod factors and the Sym signalling pathway initiated by their recognition have not been studied in detail. Recently, Sinharoy & DasGupta (2009) reported that the CCaMK is important for symbiosis development, and has a critical role in rhizobial dissemination during peanut nodule organogenesis.

Studies of Nod factor requirements in the nodule morphogenetic programme of legumes that undergo crack entry infection have been carried out in *Sesbania rostrata*, from the hologalegina group, and in *Aeschynomene* spp., belonging to the aeschynomenoid/dalbergioid clade and taxonomically related to peanut. It was found that Nod factors are indispensable for nodule formation on *S. rostrata* (D'Haeze *et al.* 1998) but are not required in *Aeschynomene sensitiva* and *A. indica*, nodulated by *Bradyrhizobium* BTAi1 and ORS278 (group II strains) (Giraud *et al.* 2007). These facts indicate that there are differences in the molecular requirements for symbiosis development even within legumes invaded through the same mechanism. Information about the nodule induction process and the role of nodulation factors in peanut–rhizobia symbiosis is scarce. The aim of this study was to assess whether, in peanut, bacterial Nod factors are required for the induction of cortical cell division as described for the nodule morphogenetic programme of legumes invaded by infection threads.

MATERIAL AND METHODS

Bacterial strains and plasmids

The bacterial strains and plasmids used in this work are listed in Table 1.

Mutant strain construction and characterisation

To obtain a *Bradyrhizobium* sp. SEMIA 6144 *nodC* mutant strain, the methodology proposed by Datsenko & Wanner

Table 1. Strains and plasmids used in this study.

	relevant features	source or reference
strain		
Bradyrhizobium sp. SEMIA 6144	Recommended as peanut inoculant, Ap^R	IPAGRO, Brazil
Bradyrhizobium sp. SEMIA 6144 V2	*Bradyrhizobium* sp. SEMIA 6144 derivative incapable of producing Nod factors, Ap^R, Km^R	This work
Rhizobium tropici NCHA22	Rhizobial isolate obtained from *Arachis hypogaea* nodules	Taurian *et al.* 2002
Escherichia coli BW25993	*lacI*q *hsdR514* Δ*araBAD*AH33 *rhaBAD*LD78	Datsenko & Wanner 2000
Escherichia coli S17-1	*Thi pro hsdR*$^-$ *hsdM*$^+$ *recA*, RP4 2-Tc::Mu	Simon *et al.* 1983
plasmids		
pAB2001	Source of the lacZ-Gm cassette	Becker *et al.* 1995
pKD4	Template for PCR amplification of the Km^R gene (*kan*)	Datsenko & Wanner 2000
pCR2.1-TOPO	Cloning vector, Km^R, Ap^R, *lacZ*	Invitrogen Inc.
pJQ200SK	Cloning vector non-replicative in rhizobia; Gm^R, *lacZ*, *sacB*	Quandt & Hynes 1993
pF4	pJQ200SK derivative containing $^{BamHI}/_{XhoI}$ (*nodC*) fragment from pCR2.1-TOPO::*nodC*; Gm^R	This work
pF5	pF4 derivative containing *kan* gene (Km^R) in position 456 of PCR product corresponding to *nodC* from *Bradyrhizobium* sp. SEMIA 6144; Km^R, Gm^R	This work
pHC60	Vector that contains gene encoding GFP (green fluorescent protein), Tet^R	Cheng & Walker 1998
pHC60Gm	pHC60 derivative containing Gm^R cassette from pAB2001 in HindIII site, Gm^R	This work

Table 2. Primers used in this study.

primer	sequence (5′ → 3′)	source or reference
E1	ATGTAAGCTCCTGGGGATTCAC	de Bruijn 1992
E2	AAGTAAGTGACTGGGGTGAGCG	
nodCP1	CTTCTCGATCAATACGAAGCCCAATTCTTTCGTGGGAGTGTAGGCTGGAGCTGCTTC	This work
nodCP2	ATCGTTAGATGGCGATCCTCGCCGAAATCGCTCGGCTCATATGAATATCCTCCTTAG	
nodCLF	GCGACGTTGTCGCGCCTGTACAC	This work
nodCLR	CGTCAATCCCGTCCACCAGGGTA	
nodCF	AYGTHGTYGAYGACGGTTC	Laguerre *et al.* 2001
nodCI	CGYGACAGCCANTCKCTATTG	

(2000) was followed. Briefly, a 946-bp fragment of the *nodC* gene of *Bradyrhizobium* sp. SEMIA 6144 was PCR amplified using the nodCF and nodCI primers (Laguerre *et al.* 2001). This fragment was cloned into the pCR2.1-TOPO vector and sequenced by Macrogen Inc. (Seoul, Korea). The sequence obtained was deposited in GenBank with the accession number DQ151890. The fragment was then digested with *Xho*I and *Bam*HI and subcloned into pJQ200SK, to give pF4. This plasmid was then used to transform *E. coli* BW25993 electrocompetent cells (Datsenko & Wanner 2000). Simultaneously, the *kan* gene (coding for kanamycin resistance) was amplified using plasmid pKD4 as template (Datsenko & Wanner 2000) and the Expand High Fidelity PCR System (Roche, Basel, Switzerland). To achieve amplification, the primers nodCP1 and nodCP2 were used (Table 2). These primers were designed by adding a 37-mer fragment identical to *Bradyrhizobium* sp. SEMIA 6144 *nodC* gene to the 5′ end of primers P1 and P2 (Datsenko & Wanner 2000). The resulting PCR amplicon consisted of the corresponding resistance cassette flanked by 37 bp homologous to the *Bradyrhizobium* sp. SEMIA 6144 *nodC* gene. The amplicon was then electroporated into *E. coli* BW25993 (pF4) cells, where the fragment recombined, interrupting the *nodC* insert through a double recombination event, giving rise to pF5. Plasmid pF5 was then transferred to *E. coli* S17-1 and the *Bradyrhizobium* sp. SEMIA 6144 mutant strain was obtained through biparental matings using *E. coli* S17-1 (pF5) as donor strain. The insertion of the *kan* cassette into the *nodC* gene was confirmed by Southern blot hybridisations using a digoxigenin labelled *nodC* probe and through PCR assays. A *Bradyrhizobium* sp. SEMIA 6144 derivative was obtained and named *Bradyrhizobium* sp. SEMIA 6144 V2.

GFP labelling of the mutant strain

In order to label the mutant strain with GFP (green fluorescent protein), a vector derived from pHC60 (Cheng & Walker 1998) was constructed by insertion of the lacZ-Gm cassette from pAB2001 into a *Hind*III site of pHC60 plasmid. The resulting plasmid, named pHC60Gm, is a pHC60 derivative that carries gentamicin resistance. Vector pHC60Gm was transformed into *E. coli* S17-1 cells, and then transferred to *Bradyrhizobium* sp. SEMIA 6144 V2 through biparental matings. Transconjugants were selected in YEMA (Vincent 1970) supplemented with kanamycin (100 $\mu g \cdot ml^{-1}$) and gentamicin (80 $\mu g \cdot ml^{-1}$). The identity of the strains was confirmed by ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus-PCR).

DNA preparation

For PCR assays, genomic DNA was extracted using the method described by Walsh *et al.* (1991). Bacteria were grown on YEMA plates and colonies were picked up using a plastic disposable tip, suspended in 300 µl 1 M NaCl, mixed thoroughly and centrifuged at 12,000 *g* for 4 min. The supernatant was discarded and the pellet was suspended in 300 µl bi-distilled sterile water. After the sample was mixed and centrifuged at 12,000 *g* for 4 min, the supernatant was removed and the pellet was suspended in 150 µl of 6% (aqueous suspension) Chelex 100® resin (Bio Rad, Hercules, CA, USA). This suspension was incubated at 56 °C for 20 min, followed by mixing and further incubation at 99 °C for 8 min.

For hybridisation assays, genomic DNA was extracted following the procedure described by Meade *et al.* (1982). DNA was quantified with gel electrophoresis.

ERIC-PCR analysis

The sequences of ERIC primers used in this study have been reported by de Bruijn (1992) (Table 2). PCR amplifications were performed as described earlier (Ibáñez *et al.* 2009).

Southern blot hybridisation of the nodC gene

Genomic DNAs were digested with *Eco*RI enzyme according to the manufacturer's instructions (Promega, Madison, WI, USA). The restriction fragments obtained were separated in a 1% agarose gel and transferred to a Hybond-N^+ nylon membrane (Amersham, Cardiff, UK). Hybridisations to a digoxigenin-labelled *nodC* probe were performed overnight. The probe was obtained through a PCR reaction using *Rhizobium tropici* NCHA22 genomic DNA as template and the primers nodCF and nodCI described in Laguerre *et al.* (2001). Hybridisation and membrane washes were performed under high stringency conditions. Membranes were prepared for chemiluminescent detection following the manufacturer's instructions (Roche) and exposed to Kodak film.

Thin layer chromatography (TLC) analysis of Nod factors

TLC analyses were carried out according to Spaink *et al.* (1992). Bacteria, supplemented with 1 µM naringenin and genistein, were grown in B^- medium (Spaink *et al.* 1992) to the end of the exponential phase. For the radiolabelling of Nod factors, 1 mCi of [1-^{14}C]acetate (specific activity 52 $mCi \cdot mmol^{-1}$; Amersham), yielding a final concentration

of 4 mM [^{14}C]acetate in the medium, was added at the beginning of the incubation. TLC plates were exposed to Kodak X-Omat R film for 15 days and the film was developed with Kodak reagents (Rochester, NY, USA) according to the manufacturer's instructions.

Plant growth conditions and nodulation assays

Arachis hypogaea L. (var. Tegua) seeds were surface sterilised by soaking 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 in sterilised Petri dishes with one layer of Whatman No 1 filter paper and moist cotton, until the radicle reached approximately 2 cm. Seedlings were sown in previously sterilised plastic cups filled with sand and, after emergence, were inoculated with 4 ml of a 16-h broth culture (YEM) (Vincent 1970) in stationary growth phase (10^9 cells·ml^{-1}). Negative controls (uninoculated seedlings) and positive controls (seedlings inoculated with *Bradyrhizobium* sp. SEMIA 6144) were included. Plants were grown under a controlled environment (light intensity of 200 μE·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%), watered regularly with sterilised tap water and, twice a month with Hoagland's solution without nitrogen (Hoagland & Arnon 1950). Plants were harvested 4 weeks after inoculation and analysed for nodulation.

To obtain roots for microscopic analysis, surface sterilised peanut seedlings were grown between the wall of a beaker and a rolled filter paper placed inside. Rolled filter papers (10-cm high) were placed upright in the beaker and wetted regularly. Ten microliters of the bacterial cultures were inoculated in the junction of a first- and a second-order lateral root. The inoculated roots were sampled at 8 and 15 days after inoculation.

Peanut root colonisation by *Bradyrhizobium* sp. SEMIA 6144 V2 *nodC* mutant strain

Plants obtained as described above were inoculated with 4 ml of culture of the mutant or the wild-type strain and harvested at 5, 10 and 15 days post-inoculation (PI). In order to discard bacteria that had not tightly adhered, inoculated roots were washed with distilled water, and then placed in a Petri dish with distilled water, which was maintained in a rotary shaker (150 rpm) for 10 min. After which 1 g of root tissue was crushed in a mortar with 1 ml phosphate buffered saline solution (PBS). Serial dilutions were performed and plated onto YEMA plates supplemented with kanamycin (100 μg·ml^{-1}) or kanamycin and gentamicin (100 and 80 μg·ml^{-1}, respectively). Plates were incubated at 28 °C for 5 days and the number of colonies formed was determined.

To isolate *Bradyrhizobium* sp. SEMIA 6144 V2 from peanut roots inner tissues, surface disinfection using serial washing in 70% ethanol for 1 min, sodium hypochlorite for 3 min, and two rinses in sterilised distilled water was first performed. The disinfection process was checked by plating aliquots of the sterile distilled water from the final rinse onto YEMA plates that were incubated at 28 °C. Then, the procedure described above was followed to determine the number of bradyrhizobial cells per gram of root.

Microscopic analysis of roots

Arachis hypogaea L. roots were placed in FAA (95% ethanol:glacial acetic acid:37–40% formaldehyde:water; 50:5:10:35, v/v). Dehydration of samples was performed according to the procedures outlined by Johansen (1940) using graduated solutions of ethanol and xylene. Fully infiltrated tissues were embedded in Histowax (highly purified paraffin wax blended with polymer additives; EMD Chemicals, Darmstadt, Germany). A series of transverse sections 6-μm thick was obtained from the sample blocks using a Minot rotary microtome. The sections were triple-stained with hematoxylin, safranin O and fast green FCF, as described by Johansen (1940). A standard Zeiss Model 16 microscope was used to assess the histological preparations, and photomicrographs were taken with a Zeiss Axiophot microscope (Zeiss, Jena, Germany) equipped for image capture and digitalisation with AxioVision 4.3 and an AxioCam HRc camera (Zeiss).

Statistics

Data were analysed using the non-parametric Kruskal–Wallis test. A $P \leq 0.05$ significance level was used throughout.

RESULTS

Characterisation of the mutant strain altered in Nod factor production

The mutant strain *Bradyrhizobium* sp. SEMIA 6144 V2 is a *Bradyrhizobium* sp. SEMIA 6144 derivative, in which the

Fig. 1. Thin-layer chromatography of supernatants of *Bradyrhizobium* sp. SEMIA 6144 V2 (mutant strain) and *B.* sp. SEMIA 6144 (wild-type strain) cultures. (+) Bradyrhizobial cultures supplemented with flavonoids naringenin and genistein, (–) Non-supplemented cultures.

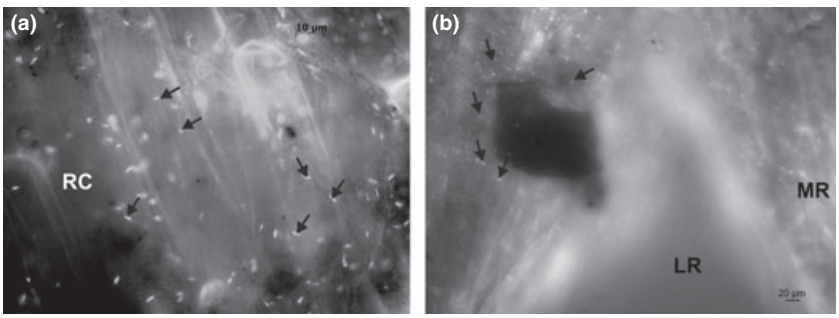

Fig. 2. Fluorescent optical microscopy of section of roots inoculated with *Bradyrhizobium* sp. SEMIA 6144 V2 GFP strain. a: Root epidermis colonised by the mutant strain. b: Site of emergence of a lateral root inoculated with the GFP-derived strain. Arrows indicate fluorescent bacteria. LR, site of emergence of a lateral root; MR, main root; RC, root cell.

nodC gene is interrupted by a kanamycin resistance cassette. The insertion of the cassette was confirmed by Southern blot experiments and PCR assays using primers nodCLF and nodCLR (data not shown). The inability of the mutant strain to produce NF was confirmed with a thin layer chromatography assay (Fig. 1). In contrast, the wild-type strain produces three different molecules. This number is slightly lower than that described for other bradyrhizobial species. In *B. japonicum*, Mabood *et al.* (2006) described the production of four types of molecule; for three peanut bradyrhizobial isolates, Taurian *et al.* (2008) described the isolation of five different molecules.

Table 3. Quantification of viable bacteria colonising peanut roots.

	Bradyrhizobium sp. SEMIA 6144	*Bradyrhizobium* sp. SEMIA 6144 V2
5 days PI	6.3442 ± 0.018	6.4988 ± 0.029
10 days PI	6.1997 ± 0.1505	6.7433 ± 0.0231
15 days PI	5.6585 ± 0.0005	5.7055 ± 0.0005

Data are expressed as log CFU per gram of root. Values represent the mean ± SE of three independent experiments. A total of nine plants were tested for each strain. No significant differences were observed according to the non-parametric Kruskal–Wallis test ($P \leq 0.05$).
PI = post-inoculation.

The ability of *Bradyrhizobium* sp. SEMIA 6144 V2 to colonise peanut roots is not affected

Peanut root colonisation by *Bradyrhizobium* sp. SEMIA 6144 V2 was assessed using visualisation under fluorescent microscopy of epidermal sections of roots from plants inoculated with a GFP-marked strain containing a lacZ-Gm cassette. Gentamicin is suitable for the selection of slow-growing rhizobial strain transconjugants, since it remains stable during the long incubation periods that are required in this type of strain. The results showed that the mutant strain is capable of colonising peanut roots. Although autofluorescence was observed in the roots, bacterial cells were easily recognisable by their shape and size. Autofluorescence in plant tissues is a common phenomenon caused by a variety of endogenous biomolecules, such as chlorophyll, lignins, carotenes and xanthophylls, when stimulated with the appropriate wavelengths. Bradyrhizobial cells were also observed in the zone of emergence of the lateral roots, through which rhizobia gain access to the inner tissues in the crack entry infection mechanism (Fig. 2). Thereafter, quantification of bacterial colonisation and survival was performed from roots inoculated with the wild-type and mutant strains. The results showed that there were no significant differences between the root colonisation of these strains (Table 3). In addition, since *Bradyrhizobium* sp. SEMIA 6144 V2 recovered from the root tissues grew in YEMA supplemented with kanamycin and gentamicin, the stability of the plasmid carrying GFP, even without selection pressure, was confirmed. Quantification of bacterial colonisation was also performed from surface-sterilised roots of plants inoculated with the mutant strain. The results showed that the mutant strain is present in the root inner tissues at values ranging from 1×10^3 to 1×10^4 per gram of root. This value is in good agreement with those found for the wild-type strain (data not shown) and with cell counts of endophytic bacteria in several plant species (Hallmann & Berg 2006).

Rhizobial nodulation factors are involved in the induction of cortical cell division

Plants inoculated with the mutant strain were pale green, and had a nitrogen-starved appearance after about 4 weeks. The roots of these plants lack any nodule structures. In contrast, plants inoculated with the wild-type strain had several nodules and demonstrated adequate nitrogen nutrition. These results indicate that *Bradyrhizobium* sp. SEMIA 6144 V_2 is not capable of inducing the formation of nodules in peanut.

Microscopic observations of transverse sections of roots inoculated with the mutant revealed that this strain is not capable of inducing the cell divisions required for nodule primordia formation. In plants inoculated with the wild-type strain, the re-initiation of meristematic activity of the cortical cells was already observed at 8 days PI (Fig. 3). The dividing cells could be recognised by their small size, irregular shape and prominent nucleus. Occasionally, cells in different phases of the mitotic process could be observed. At 15 days PI, nodule primordia protrude from the root surface of plants inoculated with the wild-type strain. In contrast, roots from plants inoculated with the mutant strain did not show meristematic activity at any of the harvest times.

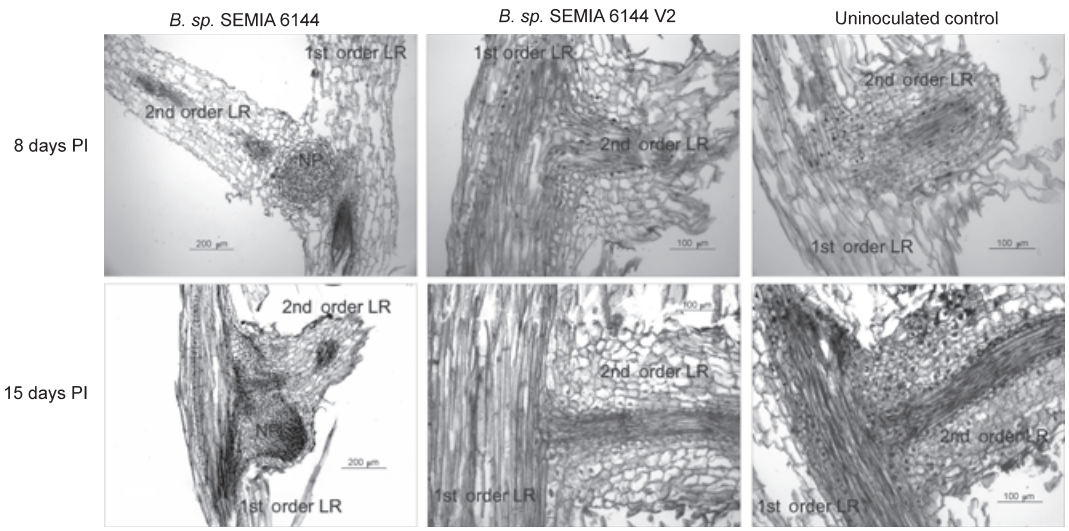

Fig. 3. Microscopic analysis of a section of peanut root uninoculated and inoculated with the wild-type or mutant strain. LR, lateral root; NP, nodule primordium. Four plants were tested for both strains and uninoculated controls at each time.

DISCUSSION

The study of rhizobia–legume symbiosis has focused on temperate legumes. In these plants, the most common nodulation strategy involves root hair curling and infection thread formation. It is widely known that, in these legumes, Nod factors initiate multiple responses that are essential for bacterial invasion of the plant host, such as stimulation of root cortex cells to reinitiate mitosis and form nodule primordia (Oldroyd & Downie 2008). Information about the role of Nod factors in peanut–rhizobia symbiosis is scarce, and limited to a paper from Wilson *et al.* (1987), who used random transposon mutagenesis to obtain a *Bradyrhizobium* sp. (*Arachis*) strain NC92 mutant unable to nodulate peanut, siratro and pigeonpea. However, these authors noted that this mutant does not contain a simple Tn5 insertion, and the complemented strain was not tested in peanut. More recently, Sinharoy & DasGupta (2009) found that the Ca^{2+}/calmodulin-dependent protein kinase (CCaMK) has a critical role in rhizobial dissemination during peanut nodule organogenesis. Since this protein is a component of the Sym signalling pathway, the authors hypothesise that Nod factors may be essential for nodulation of peanut. However, further confirmation is necessary to support this suggestion. Moreover, even when Taurian *et al.* (2008) reported the Nod factor structure from a native bradyrhizobial isolate, the question of the role of rhizobial Nod factors in peanut nodulation remained unanswered. In the present work, nodulation experiments with a bradyrhizobial strain unable to produce Nod factors were carried out. The results revealed that, even when it was able to colonise peanut roots, it was not capable of inducing the re-initiation of meristematic activity in cortical cells leading to nodule formation. Taken together, the results obtained in this work indicate that rhizobial Nod factors are required for nodulation of peanut by this bradyrhizobial strain, and that they are involved in the induction of cortical cell division to form nodule primordia. Although this role for Nod factors has been proposed for root entry and nodule organogenesis in legumes that are invaded through infection threads, our results constitute the first evidence of a link between these molecules and cortical cell division for nodule primordia formation in peanut. Further research is required to determine if, as in legumes infected through root hairs, meristematic re-initiation is produced through an increase in cytokinin. In addition, as we found that the mutant strain was capable of colonising peanut root inner tissues in a way similar to the wild type, we can infer that rhizobial Nod factors are not necessary for early recognition between the symbionts and later crack entry. Thus, the peanut–rhizobia interaction could be regarded as root endophytic bacteria that differ from other plant endophytes through their ability to induce and occupy a new niche, the plant nodule, through Nod factor signalling. In addition, since the crack entry mechanism shares common features with the rhizobial invasion of some non-legumes, the understanding of this infection mechanism could also be relevant to develop strategies to extend nodulation to other agronomically important plants.

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