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METHODS PAPER

PCR assay for direct specific detection of *Bradyrhizobium* elite strain BR 3262 in root nodule extracts of soil-grown cowpea

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Abstract
Aims Successful inoculation of legume crops with rhizobia depends on dominating nodule occupancy with highly efficient strains. The aim of this study was to develop a rapid and reliable conventional PCR methodology to specifically detect an elite *Bradyrhizobium* strain in root nodule extracts from soil-grown cowpea plants.

Methods The draft genome sequence of *Bradyrhizobium pachyrhizi* BR 3262 was compared to the closely related strain PAC 48^T. BR 3262-specific regions were selected to design specific primer pairs, which were tested with respect to PCR amplification specificity and efficiency on extracted DNA, bacterial cells and root nodules from cowpea plants grown under gnotobiotic conditions and in soil.
Results Eleven designed primer pairs were specific for BR 3262 amplification and two of them (pairs 2645 and 2736) were highly sensitive and selected for further analyses. Experiments with gnotobiotic and soil-grown plants showed that both primer pairs were suitable to reliably determine nodule occupancy and confirmed the competitiveness of strain BR 3262 in natural soil.
Conclusions Primer pairs 2645 and 2736 are novel tools to accompany the fate of strain BR 3262 in inoculation experiments of cowpea in soil. This strategy should be applicable to other rhizobium/legume symbioses in the field.

Keywords *Bradyrhizobium* · Strain specific PCR assay · Real time PCR · Nodule occupancy · Inoculation · Biological nitrogen fixation · Cowpea · *Vigna unguiculata*

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Introduction

Cowpea (*Vigna unguiculata* Walp. L.) is the most important food legume in terms of area cultivated in Ghana (https://csirsavannah.wordpress.com). Unfortunately,

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demand for this legume is above its production (Al-Hassan and Diao 2007). Cowpea can obtain 200 kg N ha^{-1} via biological nitrogen fixation (BNF) through its symbiotic association with rhizobia mainly from the genus *Bradyrhizobium* (Giller 2001). Despite this contribution, yields of cowpea have often been reported to be low and inconsistent (Deaker et al. 2004). However, farmers rarely apply nitrogen fertilizer or rhizobium inoculants to this crop as the consensus reigns that cowpea is able to effectively nodulate with native soil rhizobia to meet its N needs (Ndakidemi et al. 2006). Nevertheless, microbial products such as inoculants have been reported to substantially influence the productivity of specific crops such as soybean (Giller 2001). Recently, inoculation studies on cowpea in Ghana using some recommended Brazilian strains increased grain yields by 39–57%, challenging the widely held belief that inoculation of this crop is not necessary (Boddey et al. 2016). Nevetheless, some contradicting results have also been reported following the use of inoculants in some African countries (Mpepereki et al. 2000; Sanginga et al. 1997; Thuita et al. 2012). Thus, in order to demonstrate the benefits of inoculation and to make this technology attractive to smallholder farmers, factors that affect the outcome of inoculation such as poor product quality, poor survival of the introduced bacteria on seeds and in roots or soil (Khalid et al. 2004) must be considered.

Determination of nodule occupancy has been reported as an important approach to evaluate the competitiveness and quality of rhizobium inoculant strains (Dowling and Broughton 1986). Consequently, most methods of assessing the competitiveness and contribution of exotic strains to grain yields following inoculation remain a major challenge partly because existing approaches to measure competition between introduced and native strains are not specific enough while others are sophisticated, time consuming and/or require advanced/expensive technologies.

Traditionally, serological methods have been widely used to study nodule occupancy in legume nodules, especially soybean (Yokoyama et al. 1999). However, problems related to target specificity may occur, for example when different polyclonal antibodies recognize a single nodule occupant (Moawad and Schmidt 1987; Mpepereki and Wollum 1991). Intrinsic or induced antibiotic resistance markers employed to monitor selected strains in the field (Bushby 1981; Josey et al. 1979; Turco et al. 1986) were also shown to be limited by the natural occurance of such markers in rhizobium populations (Bushby 1981) and also due to loss of natural resistance in the environment. DNA-based methods have revealed limitations of serology-based methods and provided a window through which strain detection in a highly specific manner could be achieved. For example, a comparative study of rhizobia applying serology and amplified fragment length polymorphism (AFLP) showed that strains from the same serogroup may be genetically distinct (van Berkum and Fuhrmann 2000). Therefore, DNA-based identification methods need to be explored for the strain-specific detection of nodule bacteria. In this sense, multilocus sequence analysis has been used to study *Bradyrhizobium* genotypes occupying nodules of field-grown uninoculated soybean plants (Van Berkum et al. 2012).

To validate the occupation of nodules by the inoculated strain, PCR using primers of target repetitive sequences including repetitive extragenic palindromic (REP) sequences (Versalovic et al. 1991), enterobacterial repetitive intergenic consensus (ERIC) sequences (Niemann et al. 1999), and the interspersed repetitive DNA (BOX) sequences (Versalovic et al. 1994) have been used to generate PCR–fingerprints for characterizing rhizobial isolates at the strain level (Thies et al. 1999). Harrison et al. (1992) also designed arbitrary primers to produce randomly amplified polymorphic DNA (RAPD) fragments which have often been used in rhizobium studies for strain discrimination. However, the use of target repetitive sequences and arbitrary primers for rhizobial isolate characterization and strain discrimination, respectively, are not specific enough due to their ability to provide fingerprints for any specific target genome and variation in fingerprint patterns based on a subtle difference in PCR conditions (Gillings and Holley 1997; González-Andrés and Ortiz 1998). Also, these techniques generally require the isolation and purification of strains from nodules which is laborious and time consuming. The rapid advances in sequencing technology and bioinformatics have facilitated obtaining (almost) complete genome sequences of bacteria and performing comparative genomics. As an example, a recent study reported on the quantification of *A. brasilense* FP2 in wheat roots using a qPCR protocol with strain-specific primers (Stets et al. 2015).

The present study describes the development of a strain-specific PCR assay for the detection of the Brazilian elite strain *B. pachyrhizi* BR 3262 in the extracts of nodules from soil grown inoculated cowpea

plants. The methodology described in this paper can be performed in a relatively simple molecular biology laboratory, which is a great advantage for application in developing countries such as Ghana.

Materials and methods

Selection and design of strain-specific primer pairs

Draft genomic sequences of *B. pachyrhizi* strain BR 3262, *B. pachyrhizi* PAC48^T, *B. elkanii* USDA 76^T, *B. paxllaeri* DSM 18454^T, *B. tropiciagri* CNPSo 1112^T, *B. neotropicale* BR 10297^T and *B. japonicum* USDA 6^T were downloaded from the genbank (www.ncbi.nlm.nih.gov). Accession number and some general characteristics of these sequences can be found in Table S1. The phylogenetic relatedness of *B. pachyrhizi* BR 3262 to the type strain *B. pachyrhizi* PAC 48 ^{T} and other *Bradyrhizobium* species and strains was inferred by analysis of the partial nucleotide sequence (375 basepairs) of the recombinase A gene (*recA*). Analyses were performed using MEGA 7, applying the maximum likelihood method and the Tamura 3-parameter model to infer evolutionary history (Kumar et al. 2016). Average nucleotide identity analyses among the different *Bradyrhizobium* species and strains were performed using the dowloaded draft genome sequences and the OrthoANI software (Lee et al. 2016).

In order to identify strain-specific DNA sequences, the BR 3262 genome was fragmented in silico in 500 basepair non-overlapping fragments using Perl Script software (Simões-Araújo et al. 2016). The genome of strain PAC48^T, which was the closest bacterial strain with an available sequenced genome, was used as reference and automated BLAST search analyses were performed using a locally installed BLAST server comparing the fragmented sequence of BR 3262 to the PAC48^T genome, with an e-value of e^{-20} as cut-off level. Selected BR 3262-specific 500 bp fragments were subjected to manual BLASTn analyses (Altschul et al. 1997) at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) between December 2015 and January 2016 using default parameters of the nucleotide collection (nr/nt) database and the fragments without any hits were selected. These fragments were used to design primers with the Primer 3 plus (Untergasser et al. 2012) and Oligo Explorer v1.1.0 (http://www.softpedia.com/get/Science-CAD/Oligo-Explorer.shtml) softwares. Efforts were made to design primer pairs based on the following characteristics: (i) product size of between 80 and 150 bps and 18–22 bps in length (Table 1), (ii) melting temperature difference of <2 °C, (iii) abscence of predicted primer dimer formation, hairpin loops and duplexes.

Primer synthesis was done by Alpha DNA Company, Canada. In order to map the designed primers annealing sites on the BR 3262 genome, the 116 contigs were concatenated and re-annotated using RAST (Overbeek et al. 2014) and the sites of interest on the merged sequence were localized using the Artemis software (Rutherford et al. 2000). The primer pairs were tested to verify their specificity in vitro using the DNA of target (BR 3262) and several additional *Bradyrhizobium* strains as shown in Table 2.

Bacterial strains and culture conditions

All bacterial strains were obtained from the Johanna Döbereiner Biological Resources Center at Embrapa Agrobiologia and grown on YMA plates (Vincent 1970). The BR 3262 strain was originally isolated from the Embrapa Agrobiologia Experimental Station, Seropédica, Rio de Janeiro, Brazil as isolate E7–6 (Zilli et al. 1999). All liquid cultures used in this study were prepared by inoculating pure single colonies in TY liquid medium (Somasegaran and Hoben 2012). Liquid cultures were incubated in an orbital incubator at 28 °C and 150 rpm until the mid exponential growth phase with an optical density at 600 nm ($O.D._{600}$) of approximately 0.8, as determined by spectrophotometry.

Molecular techniques

DNA was extracted from bacterial cells cultivated to mid-logarithmic phase in liquid TY medium using the Wizard™ Genomic DNA Isolation kit (Promega). For PCR reactions, the Flexi GoTaq kit (Promega) was used and 25 μL reaction mixtures contained DNA (50 ng), Taq DNA polymerase buffer (1×), $MgCl_2$ (1.5 mM), 200 μM of each dNTP, 10 pmol of each primer and 1 U Taq DNA polymerase. Primers used are given in Table 1 and as a positive control the 16S rRNA gene was amplified with primers E786F (5′ GATTAGATACCCTG GTAG 3′) and E1115R (5′ AGGGTTGCGCTCGTTG 3′) (Baker et al. 2003). In the case of colony PCR, or PCR with nodule extracts, 2 μL of cell suspensions/extracts were used as template. The thermocycler

Table 1 Characteristics of designed primers

Primer pair	Primer sequence	Orientation[a]	Genbank accession number of target contig	Amplicon size (bp)
793	CTACTTACGACGCTCCTCTG	F	LJYE01000005	86
	AGTCTGCAATGATGTCGAAC	R		
1052	CGGATCAACGTAGAGATGAG	F	LJYE01000010	100
	TTTGCTCTCACCAAGACTTC	R		
2614	ATGCTTACGAGGATTGGAC	F	LJYE01000021	125
	CCACTTCGTTTCGATGAC	R		
2631	ATGATCTTGAAGTGCGACTG	F	LJYE01000021	113
	CCAGGAAGTTGAGTTGGTC	R		
2645	TAGAGGGCTGCTATCATGTC	F	LJYE01000021	140
	GAGATGATTACCGCAATGAG	R		
2736	TGGCTCTTCTCTTGACTAGG	F	LJYE01000022	94
	TACATCCTCCGATGTGACTC	R		
2754	AGAACCACTTCGCTATTCAC	F	LJYE01000025	80
	ATTGTGCTCATTCCAGAAAC	R		
9801	AGCTATCATAGACGGATTGC	F	LJYE01000081	133
	AGTATAGTTGGCCGCTTATC	R		
10,273	ACCTCGATACAGGCAAATAG	F	LJYE01000084	120
	TCAGATGGAAGGTCAATTTC	R		
17,852	TGTCATTATCTACGCAAACG	F	LJYE01000113	143
	GACATCGCTATTTCCGTAAG	R		
18,665	TGATCCAGCTTTATCCTGTC	F	LJYE01000116	129
	ATTGAACTTCTATGCCAAGC	R		

[a] *F* Forward primer, *R* Reverse primer

program included a 10 min initial denaturation at 95 °C, followed by 30–35 cycles consisting of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, extention at 72 °C for 30 s and final elongation of 10 min at 70 °C modified from (Stets et al. 2015). All PCR analyses mentioned in this paper unless otherwise stated were carried out according to this method.

PCR products were analysed by electrophoresis on 2% agarose gels prepared with 1 x TAE buffer during two hours at 90 V. The low mass DNA ladder (Invitrogen) was used as a molecular size marker. Gels were stained using ethidium bromide and visualized on an ultraviolet transilluminator.

PCR amplicons were submitted to sequencing reactions using the BigDye® Terminator v3.1 Cycle Sequencing Kit (ThermoFisher). 16S rRNA gene amplicons were sequenced using primer E786F and BR 3262-specific amplicons 2645 and 2736 were sequenced using the primers 2645F and 2736F, respectively (Table 1). Sequencing reaction products were submitted to post-reaction clean-up and analysed using a ABI 3500 Genetic Analyser (Thermo Fisher).

Real-time PCR reactions were carried out in 96-well optical plates on a 7500 Fast Real Time PCR system (Applied Biosystems) using QuantiTect® SYBR® Green PCR kit (QIAGEN) in a total volume of 15 μL. Nodule extracts were diluted five times using ultrapure water and then used as reaction template. Each reaction mixture included 7.5 μL QuantiTect® SYBR, 1 μL of 2645 F and R primers (10 μM), 2 μL of DNA or nodule extract and ultrapure water. The reaction conditions for amplification were 95 °C for 15 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. To determine the specificity of amplification, analysis of the product melting curve was performed after the last cycle of each amplification. For each biological sample three technical replicates were performed. Absolute quantification was carried out using qPCR standard curves constructed

Table 2 Specificity of primer pairs in amplifying target DNA

Bradyrhizobium strain	OrthoANI % with BR 3262	Primer pair											
		793	1052	2614	2631	2645	2736	2754	9801	10,273	17,852	18,665	16S rRNA
B. pachyrhizi BR 3262	100	+	+	+	+	+	+	+	+	+	+	+	+
B. pachyrhizi PAC 48^{T}	95.18	−	−	−	−	−	−	−	−	−	−	−	+
B. elkanii USDA 76 ^{T}	94.62	−	−	−	−	−	−	−	−	−	−	−	+
B. paxllaeri DSM 18454^{T}	81.18	−	−	−	−	−	−	−	−	−	−	−	+
B. tropiciagri CNPSo 1112 ^{T}	89.72	−	−	−	−	−	−	−	−	−	−	−	+
B. neotropicale BR 10297 ^{T}	80.47	−	−	−	−	−	−	−	−	−	−	−	+
B. japonicum USDA 6 ^{T}	80.63	−	−	−	−	−	−	−	−	−	−	−	+
Bradyrhizobium sp. SEMIA 6152	n.d.	n.d.	n.d.	n.d.	n.d.	−	−	n.d.	n.d.	n.d.	n.d.	n.d.	+
Bradyrhizobium sp. SEMIA 6160	n.d.	n.d.	n.d.	n.d.	n.d.	−	−	n.d.	n.d.	n.d.	n.d.	n.d.	+
Bradyrhizobium sp. SEMIA 6099	n.d.	n.d.	n.d.	n.d.	n.d.	−	−	n.d.	n.d.	n.d.	n.d.	n.d.	+

+: positive PCR reaction, −: negative PCR reaction, n.d. not determined.

with serial dilutions (10, 1, 0.1, 0.01, 0.001 and 0.0001 ng per reaction) of *B. pachyrhizi* BR 3262 genomic DNA using the same conditions describe above. The Ct (threshold cycle) values for these reactions were used to calculate the standard curve. Because the target-sequence of primer pair 2645 occurs as a single copy on the BR 3262 genome, the number of target-sequence copies was considered to be equal to the number of BR 3262 cells.

Validation and sensitivity of primer pairs

The efficiency of primer pairs was tested using different cell densities following serial dilution of a turbid broth culture of BR 3262. Ten-fold serial dilutions were prepared in sterile distilled water. Using the drop plate method, 20 μL of each dilution level was plated on YMA in order to estimate the cell number based on colony forming units (CFUs) for each of the dilution levels. Aliquots of 2 μL of each cell dilution levels were used as template for PCR analyses as described above.

Gnotobiotic system for studying of cowpea nodulation by BR 3262

Clean and uniform cowpea seeds (BRS Guariba) were surface-sterilized for 30 s using 70% ethanol, followed by 2.5% sodium hypochlorite for 3 min (Somasegaran and Hoben 2012). The seeds were then rinsed in several changes of sterile distilled water and left to dry for 10 min. The sterilized seeds were placed on 1% water agar and incubated at 28 °C for 3 d until germination. Seedlings with uniform radicle length (2 cm) were transplanted into 350 mL glass bottles containing sterilized nitrogen–free nutrient solution (Norris and Mannetje 1964).

For the gnotobiotic experiment, seedlings were inoculated with 1 mL cultures of BR 3262 or PAC 48^{T} 24 h after transplanting. All procedures were carried out under axenic conditons. The experiment which consisted of a total of three treatments (inoculation with BR 3262 or PAC 48^{T} and a negative control without inoculation) and three replicates per treatment was set up in a growth room with 12 h photoperiod provided by light emitting diode (LED) illumination at 27 °C and a humidity of 60%. Nodules were sampled and processed for PCR analysis 28 DAP.

Preparation and use of nodule suspension

Nodules from inoculated plants in the gnotobiotic system were superficially sterilized in 70% ethanol for 30 s followed by 3 min in 2.5% sodium hypochlorite. Nodules from soil-grown plants were also treated with 70% ethanol for 30 s and then sterilized in 2.5% sodium hypochlorite for 5 to 7 min. Surface sterility was confirmed by placing surface-sterilized nodules briefly on YMA plates and verifying the absence of bacterial

growth after incubation. Each nodule was individually crushed in a sterile microtube with 100 μL of sterile distilled water using heat-sterilized forceps. The resulting suspensions were vortexed and left to stand for about 30 min. Aliquots of 2 μL of each suspension were used as template in PCR analyses. Aliquots of the suspensions were also plated and strains inhabiting the nodules were re-isolated and submitted to colony-PCR.

Estimation of rhizobium cell numbers in soil

The enumeration of rhizobia population in soils was carried out using the most probable number (MPN) count method (Somasegaran and Hoben 2012). Uniform, clean cowpea seeds were surface sterilized as described above. Six levels of five-fold dilutions of experimental soil were prepared using sterilized saline solution and 1 mL aliquot of each dilution was used to inoculate cowpea seedling with four replicates. Nodulation was assessed at 28 days after planting (DAP) based on the presence or absence of root nodules. The population of rhizobia was estimated using the MPNES software (Woomer et al. 1990).

Competitiveness of BR 3262 in the presence of native rhizobium population

A pot experiment using soil from pasture fields of Embrapa Agrobiologia Research Station, Seropédica, State of Rio de Janeiro, Brazil, was planted under greenhouse conditions. The experiment was a 3 × 3 factorial plus one control without soil dilution and inoculation, arranged in a randomized complete block design (RCBD) with three replicates. The factors included three levels of soil concentrations (100% undiluted soil, 10% and 1% soil diluted with sterile river sand); and three inoculation levels (1×10^9, 1×10^7 and 1×10^5 CFU seed^{-1}) in addition to a negative control treatment without any soil dilution or inoculation. The different soil concentrations were obtained by mixing soil with sterilized river sand in plastic bags at the various proportions. BR 3262 liquid cultures were centrifuged to concentrate cells into a pellet. Pelleted cells were suspended in 0.85% saline solution from which 10-fold serial dilutions were prepared to obtain different inoculant densities, which were determined by serial dilution and plating followed by colony (CFU) counting.

Aliquots of 100 μL, containing the indicated cell (CFU) numbers, were used to inoculate surface sterilized cowpea seeds at planting. The experiment was monitored for 28 d and shoot dry mass assessed after oven drying at 65 °C for 72 h. At least five nodules per experimental unit were selected and prepared for PCR analysis. Extracts from surface-sterilized fresh (for two replicates) and dried and rehydrated nodules (third replicate) were used in PCR analyses. In order to obtain extracts from dried nodules, freshly collected nodules were stored on dessicated silica gel for 10 d and rehydrated for 1 h before sterilization. As a proof of concept, aliquots of the nodule suspensions used for PCR analyses were plated and resultant bacterial colonies were used for a second PCR analysis in order to confirm the results of the nodule extract PCR assay.

Statistical analysis

All measured data were subjected to analyses of variance using SISVAR (Ferreira 2008). Where overall probability was significant ($p < 0.05$), means were separated using Scott Knott at 5% probability.

Results

Selection, design and validation of strain-specific primer pairs

Phylogenetic analysis of the *recA* gene and OrthoANI analyses confirmed a high level of similarity between *B. pachyrhizi* strain BR 3262 and the type strain PAC48^T (Fig. S1, Table S1). The draft genomes of these two strains were therefore compared in order to identify BR 3262-specific sequences, which resulted in the selection of 2006 potentially specific fragments. Finally, 55 BR 3262-specific 500 bp fragments were selected and 11 specific primer pairs were designed based on the nucleotide sequence of these fragments. Analyses of the genomic context of the primer annealing sites revealed that four primer pairs were located in intergenic regions, six were located in regions with predicted hypothetical protein functions and one in an RNA-splicing ligase protein RtcB (Fig. S2).

PCR analyses with genomic DNA of BR 3262, the closely related strain PAC48^T, and other *Bradyrhizobium* type strains with available genome sequences were applied to verify the specificity of the designed primer pairs. These analyses showed that all the designed primers produced amplicons of the

expected size only for BR 3262 DNA and not for any other strain whereas a general 16S rRNA primer pair produced amplicons for all the strains (Table 2). When PCR analyses were conducted with cell suspensions instead of purified DNA of the same strains, the same result was obtained (data not shown).

For the direct strain-specific detection of rhizobia in nodule extracts by PCR, the amplification sensitivity of the primers is an important trait. Therefore, the minimal number of BR 3262 cells that could be detected by the 11 strain-specific primer pairs was determined. All primer pairs were effective in amplifying DNA from 3.3×10^5 cells. Two of the primer pairs showed outstanding sensitivity; primer pair 2736 giving a positive reaction at 3.3×10^3 cells whereas primer pair 2645 detected down to 3.3×10^2 cells (Table 3). These sensitivity levels are more than sufficient to detect rhizobia in a typical cowpea nodule. Blast analysis with the BR 3262 *recA* gene indicated that several *Bradyrhizobioum* strains, such as SEMIA 6152, SEMIA 6160 and SEMIA 6099 could be more similar to BR 3262 than PAC 48^T (Fig. S1). Therefore, primer pairs 2645 and 2736 were tested in PCR reactions using DNA of these strains as template. Although the 16S rRNA gene of these strains could be amplified, no amplicons were obtained with the specific primers, thus confirming the high level of their specificity towards BR 3262 DNA (Table 2).

PCR amplification of BR 3262 DNA in nodules from plants grown under gnotobiotic conditions

Since the aim of this study was to detect the BR 3262 strain directly in nodule extracts, gnotobiotically grown cowpea plants were inoculated with strain BR 3262 or the closely related strain PAC48^T. Induced nodules were superficially disinfected, macerated and submitted to PCR analysis with the primer pairs 2645 and 2736. Results for nodule extract PCR analysis for sole inoculation of BR 3262 and PAC 48^T are shown in Fig. 1. Amplicons were observed for nodules from plants inoculated with BR 3262. On the contrary, nodules from plants which received PAC 48^T did not give amplification with any of the primer pairs. In all cases the blank (without template) did not give any amplification. This showed that the specific primer pairs are suitable to amplify BR 3262 DNA directly from nodule suspension.

Competitiveness of target strain BR 3262 in the presence of native rhizobia

In native soils, a high diversity of known and and yet uncharacterized rhizobia are present. Among these, there may be bacteria that are genetically more similar to the targeted BR 3262 strain than the tested *Bradyrhizobium* type strains. Therefore, to validate the specificity of the primer pairs 2645 and 2736 towards strain BR 3262 a test in a more natural setting with microbial competition by means of a greenhouse inoculation experiment with cowpea plants grown in native soil was done. This study sought to establish the bases of inoculation response by simulating different soil conditions (in terms of rhizobium population) and inoculant quality (in terms of cell numbers). The experimental soil had a rhizobium population of 1.3×10^4 rhizobia g^{-1} soil as estimated by the MPN method. At the end of the experiment, it was found that soil dilution level,

Table 3 Efficiency of primer pairs in target amplification from different numbers of BR 3262 cells

CFU per PCR reaction	Primer pair										
	793	1052	2614	2631	2645	2736	2754	9801	10,273	17,852	18,665
3.3×10^5	+	+	+	+	+	+	+	+	+	+	+
3.3×10^4	+	+	+	+	+	+	+	+	+	+	+
3.3×10^3	−	−	−	−	+	+	−	−	−	−	−
3.3×10^2	−	−	−	−	+	−	−	−	−	−	−
33	−	−	−	−	−	−	−	−	−	−	−
3.3	−	−	−	−	−	−	−	−	−	−	−
0.33	−	−	−	−	−	−	−	−	−	−	−

+: positive PCR reaction, −: negative PCR reaction

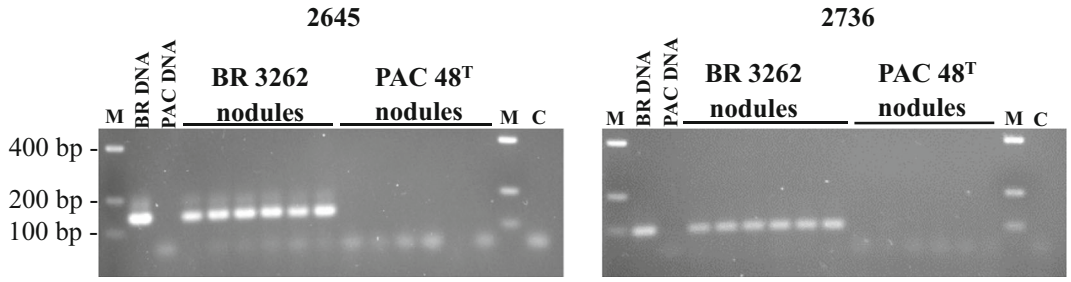

Fig. 1 Specific PCR detection of strain BR 3262 in macerated nodules from gnotobiotic cowpea plants. Primer pairs 2654 (*left*) and 2736 (*right*) were used to amplify BR 3262 DNA directly from macerated surface sterilized nodules. As controls, reactions were performed with DNA from BR 3262 (BR DNA) or PAC 48^T (PAC DNA) or without any template (C). A low mass DNA ladder was used as size reference (M)

inoculant quality, as well as their interaction, promoted significant differences ($p < 0.05$) in shoot dry mass 28 DAP (Table 4). Interaction between soil concentration and inoculation level significantly influenced shoot dry weight at 1% soil concentration and 1×10^7 and 1×10^5 inoculation levels. At 100% soil concentration, the highest shoot dry mass was produced when plants were inoculated with 1×10^7 CFU seed^{-1}, which was significantly more ($p < 0.05$) than the dry mass produced after inoculation with 1×10^5 and 1×10^3 CFU seed^{-1} under the same soil conditions. Interaction between inoculation level and soil concentration also resulted in significant differences in shoot dry mass with the 1×10^7 inoculation level producing the highest dry weight in 100% soil concentration. Shoot dry mass observed for the interaction between 100% soil concentration and 1×10^3 inoculation level was 1.49 g pot^{-1} and was significantly less ($p < 0.05$) than the mass produced for the other inoculation levels (Table 4).

Table 4 Effect of soil concentration and inoculant cell number on dry shoot mass yield

Soil concentration (%)	Number of inoculated BR 3262 cells (CFU)			
	1×10^3	1×10^5	1×10^7	Mean
1	1.65 b B	2.31 a A	2.51 a C	2.16 B
10	2.24 b A	2.65 a A	2.85 a B	2.58 A
100	1.49 c B	2.54 b A	3.57 a A	2.53 A
Mean	1.79 c	2.50 b	2.98 a	

Values are means of two plants per pot in g. Different lowercase letters in rows (inoculation levels) and upper case letters in columns (soil concentrations) indicate significant differences ($p < 0.05$) between treatments by Scott-Knott test

PCR analyses with primers 2645 and 2736 were performed with extracts from surface-sterilized nodules from cowpea plants grown in different soil dilution levels after inoculation with different doses of BR 3262 and the PCR-results for one replicate with 100% soil are shown (Fig. 2). Surface-sterility of the analyzed nodules (five per inoculant dosage per replicate) was confirmed after placing them briefly on YMA plates and verifying the absence of bacterial growth after incubation. Clearly positive reactions were observed after PCR with nodule extracts (Fig. 2a). In the example shown, all five nodules of plants inoculated with 1×10^7 CFUs were positive and after inoculation with 1×10^5 and 1×10^3 CFUs, four and no nodules were positive respectively with primer pairs 2645 and 2736. The amplicons obtained with both strain-specific primer pairs were submitted to sequencing, which confirmed the specific amplification of the BR 3262 target sequence (data not shown). When plants were inoculated with 1×10^3 CFUs, very faint PCR products could be observed for primer pair 2645 (Fig. 2a). PCR analyses were repeated using isolated rhizobia from the same nodules, in order to confirm the presence/absence of strain BR 3262 (Fig. 2b). These analyses produced similar results as observed in Fig. 2a, but differently, the faint PCR-products in the 'negative' samples were absent. This shows that these 'negative' nodules did not contain the BR 3262 strain. It was thus hypothesized that these faint PCR products were a result of traces of DNA of strain BR 3262 present on the nodule-surface and which apparently were sufficient to produce a PCR product, demonstrating the high sensitivity of the PCR reaction. Substantiating this hypothesis, uninoculated plants grown in soil never gave any amplification with the strain-specific primers, whereas the 16S rRNA PCR was positive for all nodules (Fig. 2c). Sequencing of 16S

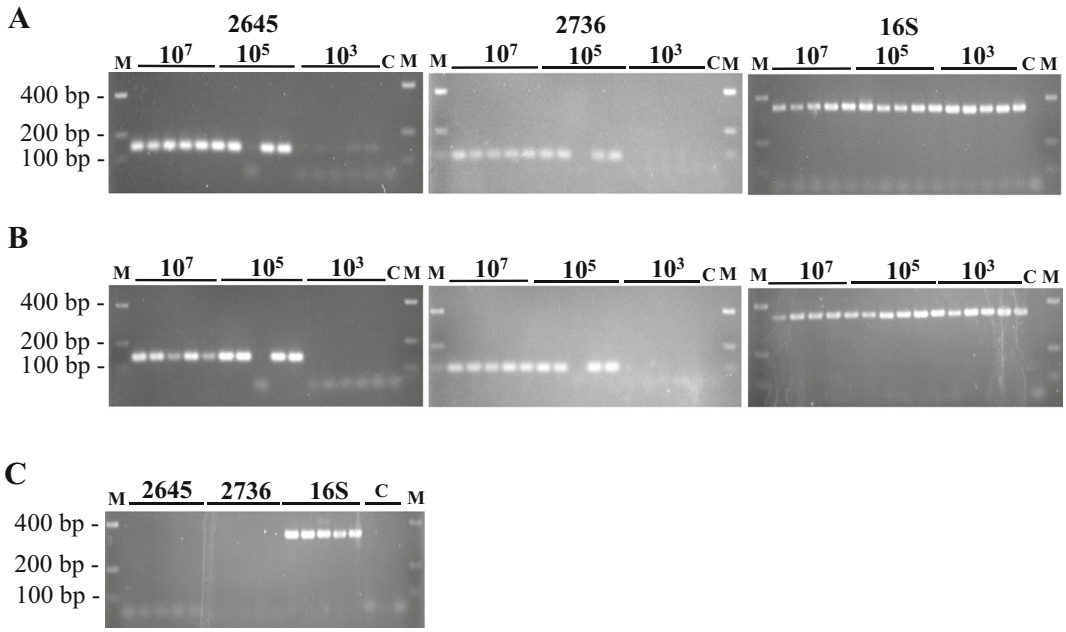

Fig. 2 Specific PCR detection of strain BR 3262 in macerated nodules from inoculated cowpea plants grown on 100% soil. Primer pairs 2645 (*left*), 2736 (*center*) and E786F/E1115R (*right*, 16S rRNA control) were used to amplify BR 3262 DNA directly from macerated surface-sterilized nodules **a** or using reisolated rhizobia from the same nodules **b**. Nodule suspension from uninoculated plants were tested as well **c**. The number of inoculated BR 3262 cells per plant in CFU seed^{-1} is indicated (10^3, 10^5, 10^7). Low mass DNA ladder (M). Negative control without template (C)

rRNA PCR products confirmed that they represented *Bradyrhizobium* strains in all cases (data not shown). This therefore demonstrated that native *Bradyrhizobium* strains from the soil were occupying the nodules (Fig. 2c).

A real-time PCR experiment was performed to obtain more information about the faint PCR products observed in Fig. 2a. For this, a standard curve was elaborated using serially diluted BR 3262 genomic DNA and the function describing the relationship between Ct and the log(copy number) for the qPCR assays was: $Ct = -3.5951\times + 34.616$, $R^2 = 0.9936$. The experiment showed that the extracts from BR 3262-occupied nodules contained approximately 10^3 times more DNA from the target strains than the nodules that were occupied by native soil rhizobia and the number of copies in the occupied nodules indicated the presence of approximately 10^6–10^7 BR 3262 bacteroids per nodule (Fig. 3). This observation corroborates the hypothesis that the weak PCR products were a result of surface contamination of nodules with BR 3262 DNA.

Analyses of the PCR results from all soil concentrations and three replicates showed nodule occupancy levels between 100% and 7%, and, as expected, there was a clear tendency towards higher occupancy levels by BR 3262 at lower soil concentrations and at higher inoculant densities (Table 5). It is a common practice in rhizobiology to dehydrate and store nodules on silica gel at the time of sampling in the field, so that the tissue can be stored at room temperature for several days to weeks, maintaining viability of the rhizobia. Here we set out to test the possibility to specifically detect BR 3262 in silicagel-dehydrated nodules. A PCR analysis using nodules from one of the three replicates (blocks) of the greenhouse experiment which were dissicated gave similar results as were obtained when fresh nodules were used, confirming the feasibility of the methodology for dehydrated nodules as well.

Discussion

Many tropical legumes have been considered promiscuous nodulators that can form effective symbiotic interactions with native rhizobia from the soils where these crops are cultivated thereby dispensing the need for inoculation. Nevertheless, experiences in the Brazilian semi-arid region and more recently in Africa have

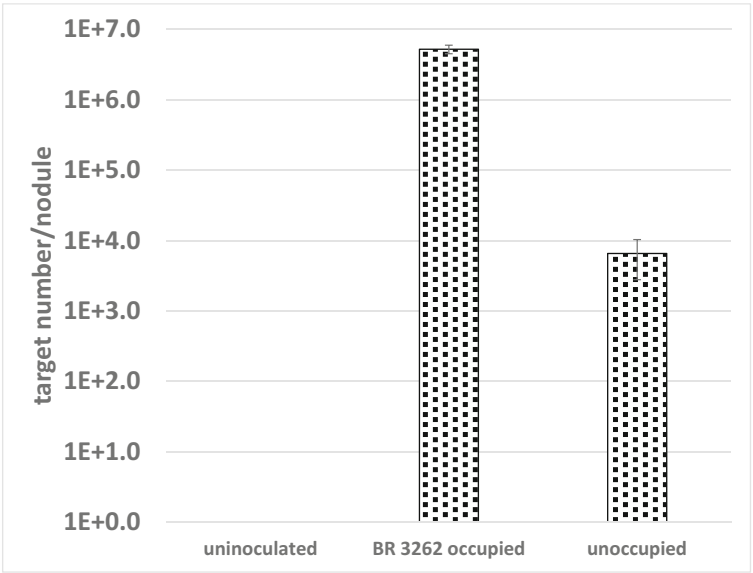

Fig. 3 Number of BR 3262 target sequence copies detected in soil-grown cowpea nodules. Real-time PCR with primer pair 2645 was performed on extracts from surface-sterilized nodules of uninoculated plants, BR 3262-occupied nodules of plants inoculated with 1×10^7 CFUs of BR 3262 and nodules not occupied by BR 3262 after inoculation with 1×10^3 CFUs of BR 3262. Absolute numbers of target sequence copies were determined using a standard curve based on ten-fold serial dilutions of BR 3262 genomic DNA. The average values are shown for three biological replicates and the standard error is indicated by bars

shown evidence that inoculation, for example with the *B. pachyrhizi* elite strain BR 3262 may contribute to significant grain yield increases (Boddey et al. 2016; Costa et al. 2011; de Alcantara et al. 2014; de Almeida et al. 2010; de Freitas et al. 2012; Fernandes et al. 2012; Ferreira et al. 2013).

When evaluating the efficacy of rhizobium inoculants for legume crops, one essential requirement is the effective colonization of the root nodules by the inoculated strain. In the majority of cases this has been done by immunological techniques (Lupwayi et al. 2000) which have the advantage of permitting the detection of bacteria directly in nodule extracts without the need of bacterial cultivation, for example by applying the enzyme-linked immunosorbent assay (ELISA) methodology. A drawback of immonulogical methods may be the occurrence of cross-reactivity of polyclonal antibodies with bacterial strains other than the target strain. For example, it has been observed that genetically different strains may pertain to the same seropgroup (van Berkum and Fuhrmann 2000). Therefore, different strains may be indistinguishable based on immunology.

The advance of molecular microbiology has revealed a huge microbial diversity among natural rhizobia and other bacteria which can explain cross-reactivity issues and lack of specificity of immunological methods (Thies et al. 2001). This study was thus based on the hypothesis that the use of molecular genetics information from rhizobia and comparative genomics could be a valuable strategy to develop more sensitive and specific assays based on PCR. Strain-specific PCR-based methods have been previously developed for plant growth promoting bacteria of the genus *Azospirillum* (Couillerot et al. 2010; Stets et al. 2015). In these cases, the authors applied qPCR which allows for the quantification of

Table 5 Effect of soil concentration and inoculant cell number on % nodule occupancy

Soil concentration (%)	Number of inoculated BR 3262 cells (CFU)			
	1×10^3	1×10^5	1×10^7	Mean
1	67 a A	78 a A	100 a A	82 A
10	13 c B	53 b A	87 a A	51 B
100	7 b B	67 a A	93 a A	56 B
Mean	29 c	66 b	93 a	

Values are means of % nodule occupancy for three replicates. Different lowercase letters within a row (inoculation levels) and upper case letters within a column (soil concentrations) indicate significant differences ($p < 0.05$) between treatments by Scott-Knott test

these *Azospillum* bacteria which inhabit rhizospheres and roots of plants and are not bound to dedicated symbiotic plant structures, such as nodules. In the case of legumes, however, root nodules tend to be occupied by one or few strains of rhizobia and as such the simple confirmation of presence or absence suffices to evaluate inoculation success, thus qPCR would be unnecessary to evaluate nodule occupancy. Conventional PCR which is nowadays a routine molecular biology technique that can be performed in any basically equipped molecular biology laboratory, is a big advantage for the application of this technique for many laboratories in developing countries.

The majority of BR 3262-specific sequences are in regions with no clear function

In this study the draft genome sequences of cowpea elite strain BR 3262 and the closely-related PAC48^T of the species *B. pachyrhizi* were compared in order to identify BR 3262-specific sequences to develop a strain-specific PCR assay. Based on BR 3262-specific sequences, 11 primer pairs were randomly designed for BR 3262-specific regions. Sequence analyses showed that four of these regions were intergenic and six encode hypothetical protein functions thus without any experimentally confirmed role. Such regions have no clear biological function and therefore suffer less selective pressure and tend to show larger sequence variability than functional genes. This is in accordance with the localization of ten out of 11 (91%) strain-specific primers in regions with no clear biological function while only 6–14% of bacterial genomes is non-coding (Rogozin et al. 2002).

Specificity and sensitivity of primer pairs to amplify the target strain from nodule extract

All the 11 primer pairs developed for the BR 3262-specific regions specifically amplified BR 3262 DNA but did not produce amplicons with DNA or cells of closely or more distantly related *Bradyrhizobium* strains. The use of different cell concentrations of BR 3262 showed variations in the efficiency of the primers. Primer pair 2645 was highly sensitive amplifying its target region at cell numbers down to 330 CFUs. When using nodule extracts, in some cases it was even necessary to dilute the nodule extract to obtain reliable and repeatable results with this primer pair. Primer pair 2736 could amplify its target from 3300 CFUs of the target strain. These results show that these primers were highly sensitive in detecting the target strain even at cell numbers far lower than usually found in infected nodules (Somasegaran and Hoben 2012).

Amplification of the target strain directly from nodule extracts of plants solely inoculated with BR 3262 proved that the selected primers were specific and efficient in their amplification of the target even with nodule extract as the template irrespective of the fact that the concentrations of rhizobia in the extracts may have differed. Kremer and Peterson (1982) also used nodule extracts as the template in an antibiotic typing of rhizobia experiment which led to the testing of variable numbers of rhizobia due to variation in cell concentrations of the extracts. They therefore concluded that, to obtain a standard inoculum size for antibiotic testing, isolation of rhizobia from nodules was critical. In the present study, no PCR products were observed for the nodules of PAC 48^T–inoculated plants.

Specific detection of BR 3262 in nodules from soil-grown cowpea plants

In contrast to gnotobiotically grown plants, tropical soils naturally habour a native population of rhizobia capable of nodulating cowpea. To confirm the validity of the PCR methodology, a competitiveness experiment was performed in which different inoculant densities were tested on substrates with different concentrations of native rhizobia. In this study, the BR 3262 specific primers could not amplify DNA from surface-disinfected nodules of non-inoculated plants grown in a soil that contained approximately 1.3×10^4 rhizobia g^{-1} soil. This showed that the strain-specific primers could not detect the native soil rhizobia, even though the target strain BR 3262 was originally isolated from soil from within 1 km of the area used in our experiment (Zilli et al. 1999). As would be expected, there was a clear tendency towards higher levels of occupancy by BR 3262 when the inoculant density was increased from 10^3 to 10^7 CFU seed^{-1}. When the native rhizobium population was lower (that is, in highly diluted soil) a higher percentage of nodule occupancy by BR 3262 was also observed. Even in non-diluted soil with a high concentration of native rhizobia, the strain BR 3262 was able to occupy more than 50% of the nodules after inoculation with 1×10^5 CFUs, thereby confirming previously demonstrated performance of strain BR 3262 (de Alcantara et al.

2014; Zilli et al. 2009). In this study, all the PCR reactions were repeated on *Bradyrhizobium* cultures obtained after re-isolation from nodules and in all cases these experiments confirmed PCR experiments performed directly on nodule extracts.

In order to evaluate the effect of the inoculation with BR 3262, apart from nodule occupancy, dry matter accumulation in cowpea shoots was also determined as a measure of BNF efficiency (Somasegaran and Hoben 2012). There was a clear positive response after inoculation with increasing inoculant doses of BR 3262 even when undiluted soil was used. This thus confirms the positive effect of the BR 3262 strain after nodule occupancy and, in line with previous results, which showed increased BNF after inoculation (de Alcantara et al. 2014). Even though the native rhizobia population was estimated to be high in the experimental soil, these were probably less competitive and less effective (Brockwell et al. 1995). However, the low shoot dry mass and nodule occupancy by BR 3262 with undiluted soil inoculated with 10^3 CFU seed^{-1} confirmed that inoculant quality, especially bacterial cell numbers, is an important factor that affects inoculation response (Khalid et al. 2004). The success of rhizobial inoculation is often limited by several factors among which are the number of infective cells applied and the presence of competing indigenous rhizobia (Bogino et al. 2008).

Very faint PCR amplicons could be observed after using primer pair 2645 on nodules of plants inoculated with low doses of BR 3262, whereas this strain was not present inside these nodules. These faint PCR products were probably a result of BR 3262 DNA traces present on the plant surface, because they were only observed on inoculated plants and never appeared in nodules from uninoculated plants. Nevertheless, these false positive reactions could be easily distinguished from true positives. Additionally, real-time PCR analysis using primer pair 2645 showed that the BR 3262-occupied nodules contained 1000 times more BR 3262 DNA than the false positives.

The approach described in this paper should be applicable for the design of primers for other bacterial/rhizobial strains whose genome sequences are available. Of course, the use of this approach in other laboratories and on other plant-microbe systems may require slight adaptations, for example with respect to PCR conditions or the procedure of preparation of nodule suspension. The described conventional PCR method has the advantage of being applicable in any basically-equipped molecular biology laboratory. This new approach will be applied to monitor field inoculation studies of cowpea in Ghana and Brazil.

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