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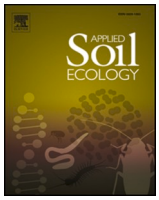

Specific primers for the rapid detection and quantitation of *Rhizobium* elite strains of common beans in the plant and environment

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ABSTRACT

Tools for adequately detecting and monitoring elite strains used for common bean inoculation are lacking. These tools help evaluate strain competitiveness and nodule occupancy rates and study their ecology in the environment. For this reason, we designed and tested specific primers for three *Rhizobium* elite strains widely used as common bean inoculants: *Rhizobium tropici* CIAT 899, *R. tropici* CPAC H12, and *R. freirei* PRF 81. Primer specificity was confirmed in PCR assays performed with the target strains and 45 strains of the Rhizobiaceae family. CIAT 899 and CPAC H12 showed an average nucleotide identity of 99.99 %; thus, all further evaluations were performed with CIAT 899 and PRF 81. Primer amplification efficiency, sensitivity, determination coefficients, and dissociation curves were evaluated. In addition, three pairs of primers for each target strain were evaluated for their capacities to quantify their targets in the nodules, rhizosphere, and roots. Eleven and seven strain-specific primer pairs were selected for CIAT 899 and PRF 81, respectively. They amplified DNA isolated from bacterial suspensions, nodules, and nodule extracts used directly in PCR with average amplification efficiencies between 91 % and 105 % and 96 % and 107 % for CIAT 899 and PRF 81 primers and detection limits of 32 and 34 copies of target DNA, respectively. Their use showed that populations increased in the roots and decreased in the rhizospheric soil over 15 days. Nodular occupancy rates were above 90 % when the strains were inoculated individually, and a high co-infection rate was observed upon co-inoculation, with CIAT 899 and PRF 81 detected in 100 % and 73 % of the nodules, respectively. We conclude that the primers and methodologies shown here are suitable for detecting the target strains with high specificity and sensitivity. Our approach also demonstrated that *R. tropici* CIAT 899 and *R. freirei* PRF 81 were competitive and effective in infecting common beans under the tested conditions.

1. Introduction

The elite strains *Rhizobium tropici* CIAT 899, *R. tropici* CPAC H12, and *R. freirei* PRF 81 are routinely used in several studies involving biological nitrogen fixation (BNF) in common beans (*Phaseolus vulgaris* L.) and as models for the selection of new strains (Abou-Shanab et al., 2019; Chekanai et al., 2018; Moreira et al., 2017; Oliveira et al., 2018). However, *P. vulgaris* can be nodulated by several other *Rhizobium* strains and other rhizobial species (Michiels, 1998; Tong et al., 2018; Wong-Villarreal and Caballero-Mellado, 2010). Therefore, detecting these elite strains is ideal to ensure that they colonize the nodules effectively (Rilling et al., 2019). Despite the promising results upon their inoculation (Hungria et al., 2003; Hungria and Vargas, 2000; Mostasso et al., 2002), their nodular occupancy rates are generally neglected (Mendoza-Suárez et al., 2021). The assessment of nodule occupancy and the establishment of these strains in the soil-plant system allows the evaluation of their aptitude and performance under different cultivation conditions.

Monitoring techniques to track inoculant strains have been recurrent since the beginning of the selection of elite rhizobia (Oliveira et al., 2006; Pillai et al., 1992; Rolim et al., 2019; Schmidt et al., 1968; Selenska-Pobell, 1994). Among them, we can cite the serological Enzyme-Linked Immunosorbent Assay (ELISA) (Vu et al., 2017), the use of antibiotic resistance (Naamala et al., 2016), Denaturing Gradient Gel

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Electrophoresis (DGGE) (Oliveira et al., 2006), and matrix-assisted laser desorption/ionization-time of flight (MALDITOF) mass spectrometry (MS) (Ndungu et al., 2018). However, the high microbial diversity associated with plants limits most of these techniques' specificity (Kandel et al., 2017).

For the accurate detection of a particular microorganism, it is necessary to use a precise and sensitive technique, such as the Polymerase Chain Reaction (PCR) (Hill and Stewart, 1992). The PCR specificity is directly linked to specific primers (Hill and Stewart, 1992), which are the critical point of the technique (Robertson and Walsh-Weller, 1998). Furthermore, the target genes can be quantified by quantitative PCR (qPCR), which helps quantify the rhizobial population, both in the soil and the host plant (Trabelsi et al., 2009; Yudistira et al., 2021) and for the quality control of inoculants (Goulart et al., 2015). This technique can also be an alternative to the Most Probable Number (MPN) technique for estimating rhizobial soil populations (Farid et al., 2017; Li et al., 2018).

PCR has been used to detect *Rhizobium* sp., including *R. tropici* (Aguilar et al., 1998; De Oliveira et al., 1999; Doty et al., 2005). However, its use depended on other techniques (e.g., DGGE) or required post-PCR sequencing analysis (Oliveira et al., 2006). For example, De Oliveira et al. (1999) developed primers for distinguishing between *R. tropici* and *R. leguminosarum* by performing nested PCR using a 1.5 kb intergenic region 16S—23S rRNA template. They demonstrated that band polymorphism generated through DGGE can separate CCT 4160T and CCT 4164 strains from *R. tropici*. Other universal primers designed for the 23S rRNA and *nifH* genes were also used to differentiate *R. tropici* CIAT 899 from other closely related strains (Doty et al., 2005). However, the comparison required PCR product sequencing (Doty et al., 2005). Other examples that can be cited are the PCR approaches used by Narożna et al. (2015) to evaluate the competitiveness of *Bradyrhizobium diazoefficiens* USDA 110 and *B. japonicum* USDA 123 in a 20-year-old agricultural site cultivated with soybean (*Glycine* max), and by Ji et al. (2017) to evaluate the nodular occupation by various *Rhizobium* spp. in *Caragana microphylla* under different environmental conditions.

Until 2018, forty-eight species have been described in the genus *Rhizobium* (Iyer and Rajkumar, 2018). However, the primers described in the literature for monitoring bacteria of this genus cannot separate this study's target strains. Furthermore, *R. freirei* PRF 81 was until recently classified as *R. tropici*. Genomic analysis showed a considerable difference between PRF 81 and *R. tropici* CIAT 899, although there is a high similarity in their symbiotic plasmids (Ormeño-Orrillo et al., 2012). Molecular and phenotypic analyses involving 11 strains of species phylogenetically related to the *R. tropici* group consolidated the difference between PRF 81 and CIAT 899, classifying the former into a new species (Dall'Agnol et al., 2013). This characterization reinforced the need to obtain specific primers to differentiate these strains.

Therefore, the objective of the present study was to design strain-specific primers and adjust a methodology to detect and quantify *R. tropici* CIAT 899, *R. tropici* CPAC H12, and *R. freirei* PRF 81 in different matrices such as nodules and soil by conventional PCR and qPCR. We show the suitability of these primers for detecting and monitoring these strains in different assays and experiments.

2. Material and methods

2.1. Primer design

To design the primers, we compared the genomes of the target *Rhizobium* strains (*R. tropici* CIAT 899, *R. tropici* CPAC H12, and *R. freirei* PRF 81) with the genomes of closely related strains (BR 219, BR 249, BR 250, BR 314, BR 315, BR 317, BR 318, and BR 362) and genomes made available in Genbank and RefSoil (Dunivin et al., 2019). The genomes of the closely related strains were sequenced at coverage of 50× with the Illumina technology, automatically assembled with Velvet 1.2.10, and deposited in Genbank under biosample accessions SAMN34105223, SAMN34105224, SAMN34105225, SAMN34105226, SAMN34105227, SAMN34105228, SAMN34147790, and SAMN34147791. Sequencing was performed at the company "BPI Biotecnologia, Pesquisa e Inovação." The coding regions of the target genomes were aligned to the other genomes with BLAST (default parameters with alignments reported *E*-value <10). Genes were considered for primer design if detected only once in a target genome and not present in another genome of interest or within genes in the RefSoil database. Once this criterion had been met, we randomly selected 25 and 20 genes from *R. tropici* CIAT 899 and *R. freirei* PRF 81 for primer design. We were unable to select genomic regions differentiating CIAT 899 and CPAC H12 since their genomes showed an average nucleotide identity of 99.99 % as determined by fastANI (Jain et al., 2018) on the KBase platform (Arkin et al., 2018). Therefore, we decided to work with the CIAT 899 and PRF 81 genomes for primer design.

The primers were designed using the Oligo explore v.1.2 program, according to the following criteria: i- sizes from 18 to 21 nucleotides; ii- CG content between 45 and 55 %; iii- last five bases of the 3'-OH end containing two to three nitrogenous bases (C and/or G); iv- annealing temperature of 59 ± 2 °C; v- melting temperature of ~60 °C between the primers (ΔTm < 2 °C); and vi-amplicons from 75 to 150 bp (Green and Sambrook, 2019).

2.2. DNA extraction

The roots and nodules were superficially disinfected (Supplementary Material) and used for DNA extraction with the CTAB buffer, as described by Da Silva et al. (2023). The same buffer was used for DNA extraction directly from bacteria. For DNA isolation from soil, 250 mg of rhizospheric soil was collected from the experiments described herein and used for extractions with the commercial kit DNeasy® PowerSoil® Kit (QIAGEN) according to the manufacturer's recommendations.

2.3. PCR conditions

All primers were initially tested in PCRs with the following composition: 0.3 μL of each 10 nM primer, 3.0 μL of 5× buffer, 1.0 μL of 25 mM $MgCl_2$, 0.3 μL of 10 mM dNTP, 0.1 μL of 5 U Taq Polymerase, 1.0 μL of the 10 ng μL^{-1} target DNA sample and ultrapure water to the final volume of 15.0 μL. The initial reaction conditions varied in terms of annealing temperature and the number of cycles as follows: 95 °C for 5 min; 30–40 cycles of 95 °C for 15 s, 60–63 °C for 45, and 72 °C for 30 s; and 72 °C for 5 min. PCR products were loaded onto a 2 % agarose gel (m:v) and electrophoresed (6 V cm^{-1} for 90 min) in 1× TAE buffer. The gel was stained with 0.01 % ethidium bromide solution (m:v), discolored in distilled water, visualized by exposure to ultraviolet light, and photo-documented. The records were used to verify the specificity of the primers and to evaluate if the amplified product had the expected size.

2.4. Primer specificity

Specific primers were selected exclusively using DNA samples from bacteria phylogenetically close to the target strains, as Brunelle et al. (2012) recommended. Twenty-three bacteria of the genus *Rhizobium* were used, according to Ribeiro (2017). In addition, DNA from 22 other rhizobial species belonging to other genera was used (Table 1). The PCR products of the primers that proved to be strain-specific were sequenced to confirm whether they were annealing the target gene as initially determined in silico. Sequencing was performed in an ABI3500 sequencer (Applied Biosystems) at the Genome Laboratory of Embrapa Agrobiologia, Seropédica, RJ, Brazil. The sequences were analyzed with ChromasPro (Technelysium Pty Ltd) and aligned with the ClustalW software in Bioedit (Hall, 1999).

The primers were tested in four more steps with DNA from different matrices classified into the following groups: i- DNA from the target strains coming from three different culture collections, namely Embrapa

Table 1
List of bacterial strains used to test the specifity of primers designed for *R. tropici* CIAT 899 (= SEMIA 4077 = BR 322) and *R. freirei* PRF 81 (= SEMIA 4080 = BR520). (continues).

Species	Strain ID	Origin	Host
Rhizobium tropici	BR 322 = CIAT 899 = SEMIA 4077	Carmen de Viboral, ANT, Colombia	*Phaseolus vulgaris*
Rhizobium tropici	BR 534 = SEMIA 4088	Planaltina, GO, Brazil	*Phaseolus vulgaris*
Rhizobium freirei	BR 520 = PRF 81 = SEMIA 4080	Irati, PR, Brazil	*Phaseolus vulgaris*
Rhizobium etli bv. phaseoli	BR 212	Iraq	*Phaseolus vulgaris*
Rhizobium loessense	BR 225	Seropédica, RJ, Brazil	*Phaseolus vulgaris*
Rhizobium jaguaris	BR 219	Seropédica, RJ, Brazil	*Phaseolus vulgaris*
Rhizobium mesoamericanum	BR 364 = UW 1-3F	Uninformed	*Phaseolus vulgaris*
Rhizobium miluonense	BR 315 = IAPAR 69 = UMR 1173	Uninformed	*Phaseolus vulgaris*
Rhizobium paranaense	BR 318	Brazil	*Phaseolus vulgaris*
Rhizobium phaseoli	BR 222 = SEMIA 442	Seropédica, RJ, Brazil	*Phaseolus vulgaris*
Rhizobium leucaenae	BR 295	Piracicaba, SP, Brazil	*Phaseolus vulgaris*
Rhizobium leucaenae	BR 367	Aruanã, GO, Brazil	*Phaseolus vulgaris*
Rhizobium. leucaenae	BR 326	Goiás, Brazil	*Phaseolus vulgaris*
Rhizobium leucaenae	BR 920	Canarana, BA, Brazil	*Leucaena* L. *leucocephala*
Rhizobium leucaenae	BR 939	Linhares, ES, Brazil	*Leucaena L. leucocephala*
Rhizobium sp.	BR 230 = CIAT 144	ANT, Colombia	*Phaseolus vulgaris*
Rhizobium sp.	BR 249 = SEMIA 495 = CIAT 45	Alegre, ES, Brazil	*Phaseolus vulgaris*
Rhizobium sp.	BR 312 = IAPAR 89	Brazil	*Phaseolus vulgaris*
Rhizobium sp.	BR 314 = IAPAR 70 = UMR 1226	Uninformed	*Phaseolus vulgaris*
Rhizobium sp.	BR 317 = SEMIA 4047	Uninformed	*Phaseolus vulgaris*
Rhizobium sp.	BR 327 = USDA 2680	Kimberly, ID, USA	*Phaseolus vulgaris*
Rhizobium sp.	BR 362	Uninformed	*Phaseolus vulgaris*
Agrobacterium sp.	BR 272	Planaltina, DF, Brazil	*Phaseolus vulgaris*
Agrobacterium sp.	BR 298	Uninformed	*Phaseolus vulgaris*
Agrobacterium tumefaciens	BR 313 = IAPAR 94	Brazil	*Phaseolus vulgaris*
Azorhizobium doebereinerae	BR 5401 = DSM 18977	Seropédica, RJ, Brazil	*Sesbania virgata*
Azorhizobium caulinodans	BR 5410 = ATCC 43989 = DSM 5975	Senegal	*Sesbania rostrata*
Blastobacter denitrificans	BR 10327 = ATCC 43295 = DSM 1113	Germany	*Uninformed*
Bradyrhizobium diazoefficiens	BR 116 = NBRC 14792 = USDA 110	Florida, USA	*Glycine max*
Bradyrhizobium diazoefficiens	BR 85 = SEMIA 5080	Planaltina, DF, Brazil	*Glycine max*
Bradyrhizobium elkanii	BR 29 = SEMIA 5019	Piraí, RJ, Brazil	*Glycine max*
Bradyrhizobium japonicum	BR 111 = ATCC 10324 = DSM 30131	JPN	*Glycine max*
Bradyrhizobium macuxiense	BR 10303 = HAMBI 3602	Bonfim, RR, Brazil	*Centrolobium paraense*
Bradyrhizobium sp.	BR 6204	Seropédica, RJ, Brazil	*Albizia saman*
Burkholderia sabiae	BR 3407	Uninformed	Uninformed
Burkholderia phymatum	BR 3486 = DSM 17167 = LMG 21445	French Guiana	*Machaerium lunatum*
Cupriavidus taiwanensis	BR 3471 = DSM 17343 = LMG 19424	Taiwan	*Mimosa pudica*
Devosia neptuniae	BR 10334 = LMG 21357 = CIP 108397	India	*Neptunia natans*
Ensifer fredii	BR 112 = LMG 6217 = USDA 205	Henan, China	*Glycine max*
Ensifer meliloti	BR 4007 = SEMIA 6162	Fortaleza, CE, Brazil	*Prosopis juliflora*
Mesorhizobium ciceri	BR 521 = ATCC 51585 = DSM 11540	Spain	*Cicer arietinum*
Mesorhizobium loti	BR 7801 = ATCC 700743 = DSM 2626	New Zealand	*Lotus corniculatus*
Microvirga vignae	BR 3299 = HAMBI 3457	Laranjeiras, SE, Brazil	*Vigna unguiculata*
Phyllobacterium trifolii	BR 7608 = CECT 7015 = DSM 17315	Salamanca, Spain	*Trifolium pratense*

BR: Centro Nacional de Pesquisa Embrapa Agrobiologia CNPAB; CIAT: Centro Internacional de Agricultura Tropical; IAPAR ou PR F: Instituto Agronômico do Paraná; ATCC: American Type Culture Collection; DSM: Deutsche Sammlung Von Mikroorganismen Und Zellkulturen; NBRC: National Institute of Technology and Evaluation Biological Resource Center; USDA: United States Department Of Agriculture; SEMIA: Coleção De Culturas De Rhizobium Da Fepagro: HAMBI: HAMBI Culture Collection; LMG: Laboratorium Voor Microbiology Culture Collection; CIP: Instituto Pasteur; CECT: Colección Española de Cultivos Tipo; UW: University Of Wisconsin; UMR: University of Minnesota Rochester. Access link: http://alelomicro.cenargen.embrapa.br/InterMicro/Passaporte/bancoInstituicao.xjs

Agrobiologia (RJ), Embrapa Soja (PR), and Fepagro (RS); ii- DNA isolated from bean nodules inoculated individually with each of the target strains and cultivated in a greenhouse in a long neck bottle containing 300 mL of sterile Norris nutrient solution (Norris and Date, 1976); iii- bacterial suspensions derived from colonies of the target bacteria grown on agar medium. The bacterial colonies were collected with a 10 μL platinum loop and diluted in 90 μL of ultrapure water; iv- nodule extracts from nodules of approximately 2 mm, superficially disinfected with a 0.5 % NaClO solution (v:v) for 5 min, rinsed with distilled water five times, and macerated in a 0.5 mL microtube containing 90 μL of ultrapure water. Ten nanograms of DNA from each of the preparations referring to groups i and ii were used in the PCR assays. For samples from groups iii and iv, 1 and 2 μL were used for the final volume of 15 μL in the PCR assays, respectively.

2.5. Primer sensitivity

The sensitivity of the primers was evaluated in technical triplicates using DNA from *R. tropici* CIAT 899 and *R. freirei* PRF 81 with mass ranging from 10 fg to 10 ng of DNA in PCR assays. 10 μL aliquots of the PCR products were loaded onto a 2.0 % (m:v) agarose gel. The gel was subjected to electrophoresis (6 V cm^{-1} for 90 min), stained with a 0.01 % ethidium bromide solution (m:v), bleached with distilled water, and exposed to UV light, and recorded for later analysis.

2.6. Real-Time PCR Conditions

The qPCR assays were conducted using primers previously selected

by PCR and DNA of their respective target strains (CIAT 899 and PRF 81). The reaction mix was prepared by adding 6.5 μL of SYBR® Green, 0.3 μL of each 10 μM primer, 5.0 μL of template DNA, and ultrapure water to the final volume of 15 μL. The mix was applied to a qPCR plate (MicroAmp optical 96-well, Applied Biosystems) containing 96 wells of 0.1 mL each. The amplification conditions for each primer were programmed as specified in the conventional PCR and conducted in the QuantStudio™ 3 System equipment.

2.7. Calibration curves

To build the calibration curves for the primers, the genomic DNA from *R. tropici* CIAT 899 and *R. freirei* PRF 81 were standardized separately at a concentration of 5 ng μL^{-1} (QubitTM), adjusted to avoid the formation of primer dimers. Each genomic DNA was serially diluted from 10^0 to 10^{-5} using the 10-fold dilution factor. Three qPCR master mix brands were tested: Sybr Green Fast SYBR™ Green Master Mix (Applied Biosystems), GoTaq® qPCR (Promega), and QuantiTect SYBR® Green PCR Kits (Qiagen). The initial annealing temperatures defined for conventional qPCR were maintained; however, the annealing time was changed according to the recommendations of each kit (Applied, Promega, and Qiagen) (Table 3).

The main criteria for the selection of primers were the amplification efficiency (E) determined by Eq. (1), the determination coefficient (R^2) of equation (y), the limit of detection (LoD), and the dissociation curve. The LoDs were established based on 20 rlicates in qPCR assays using the most diluted target DNA samples from the calibration curve as recommended by (Kralik and Ricchi, 2017):

$$E = \left(10^{-\left(\frac{1}{s}\right)} - 1\right)*100 \quad (1)$$

where E is the amplification efficiency (%), s is the slope of the calibration curve or a linear regression equation, and (y) represents the log concentration of standard DNA versus the Ct value (number of cycles to reach fluorescence threshold in the qPCR assay).

Calibration curves for each kit were plotted separately, followed by an average calibration curve of the three master mixes (Figs. S2 and S3). These curves were plotted separately for each of the CIAT 899 and PRF 81 strains, between $10^{6.5}$ and $10^{1.5}$ target DNA copies corresponding to masses of 2.5×10^7 and 2.5×10^2 fg (in triplicate) in the qPCR reaction, respectively. The number of target DNA fragments for each point on the curve was determined considering the size of *R. tropici* CIAT 899 (6.69 Mb) and *R. freirei* PRF 81 (7.08 Mb) genomes (Ormeño-Orrillo et al., 2012), the mass of genomic DNA used, the Avogadro's constant (6.022×10^{23}) and DNA molecular weight (652 Da/bp).

2.8. Primer experimental validation

2.8.1. Bacterial suspensions added to soil and macerated common bean roots

For this assay, primers Ciat899G144, Ciat899G1624II, and Ciat899G3718 were used for CIAT 899 and Prf81G19, Prf81G222II, and Prf81G561 for PRF 81. These strains were cultivated in Yeast-Mannitol medium (Vincent, 1970) at 8.91×10^9 and 3.67×10^9 CFU mL^{-1}, respectively. Each culture was ten-fold diluted from 10^0 to 10^{-7}. For each strain, 100 μL of each dilution was added separately into 2.0 mL microtubes containing 250 mg of soil or macerated bean roots grown under sterile conditions. The samples were incubated for 5 h at room temperature and used for DNA extraction as previously described.

2.8.2. Greenhouse experiments

The experiment was conducted in a greenhouse in a completely randomized design with four treatments and ten replications per treatment. The treatments were: (1) inoculation with *R. tropici* CIAT 899; (2) inoculation with *R. freirei* PRF 81; (3) inoculation with a mixture of CIAT 899 and PRF 81 (1:1, v:v); and (4) an uninoculated control. Pots with a 0.3 L volume were filled with the superficial horizon of an Ultisol (Table S1), corrected with limestone, and fertilized at sowing with triple superphosphate as recommended by Freire et al. (2013). Besides the chemical and physical analyses, we estimated the size of soil rhizobial populations with the most probable number technique (Woomer et al., 1990; Andrade and Hamakawa, 1994) (Table S1). Common bean seeds of the Pérola cultivar were washed in running water, rinsed, and immersed in distilled water for 15 min. Then, they were immersed for 30 min in a liquid inoculant (10^9 CFU mL^{-1}). Three seeds were sown per pot. All plants received equal filtered water according to their development stage. After germination, destructive collections were performed on the 3rd, 6th, 9th, 12th, and 15th days. For DNA extraction, samples composed separately of rhizosphere soil and roots of three plants on the 3rd and 6th days and two plants on the 9th and 12th days were collected from each pot. From the 15th day onwards, rhizosphere soil and roots were collected from one plant only. The rhizospheric soil was removed by gently tapping the root on the edge of a disinfested collection tray. The resulting amount was then wrapped in aluminum foil, identified, and immediately placed on ice until DNA extraction. The pots containing the soil and plant were left for approximately 12 h without water for this purpose. The DNA was extracted as previously described. For this experimental assay, the primer pairs Ciat899G139, Ciat899G1624II, and Ciat899G3722 were used for strain CIAT 899 and Prf81G19, Prf81G546I, and Prf81G561 for PRF 81. In addition, primers 1055yF 5′-ATGGYTGTCGTCAGCT-3′ (Ritalahti et al., 2006) and 1392R: 5′-ACGGGCGGTGTGTAC-3′ (Lane, 1991) were used to quantify the total number of copies of the 16S rRNA gene.

2.9. qPCR quantitation of target strains

DNA samples isolated from roots and soil were standardized to 10 and 5 ng μL^{-1} concentrations, respectively, to quantify CIAT 899 and PRF 81 populations by qPCR. Five microlitters of each standardized sample were used in the reactions performed in technical triplicates. The number of copies of the target bacteria was defined according to the standard curve of each primer. Subsequently, the number of copies or cells quantified in qPCR was extrapolated to 1.0 g of the matrix (root or soil) using Eq. (2) (Ilha et al., 2016):

$$Nc = (A*B*C)/(D*E) \quad (2)$$

where Nc is the number of target fragments or the number of cells g^{-1}; A is the number of target fragments or cells observed to average the Ct value in the qPCR; B is the concentration of isolated genomic DNA for each sample (ng μL^{-1}); C is the volume of extracted DNA in μL; D is the mass of total DNA used in the qPCR assay (ng); and E is the mass of matrix used for total DNA extraction (g).

2.10. qPCR evaluation of nodular occupancy

A pilot test was carried out using bean nodule extracts separately inoculated with CIAT 899 and PRF 81 strains and cultivated under sterile conditions. Three different concentrations of extracts were used for each strain, namely, optical densities $(OD)_{560nm}$ 0.12, 0.65, and 1.56 for CIAT 899, and 0.15, 0.44, and 1.32 for PRF 81. These extracts were prepared in three volumes (0.5, 1.0, and 1.5 μL for 4.5, 4.0, and 3.5 μL of ultrapure water, respectively) used in the qPCR assays as described above. In addition, primers Ciat899G139 and Prf81G19 were used for CIAT 899 and PRF 81, respectively.

Based on the OD and the volume of nodule extracts that provided the best result in the qPCR assay, nodular occupancy was evaluated in the above greenhouse experiment in plants collected on the 24th day after sowing. In total, 120 nodules were collected from plants of the four treatments, with ten nodules randomly collected per plant ($n = 3$) in each treatment. Then, the nodules were superficially disinfected with a

0.5 % (v:v) NaClO solution for 5 min, rinsed with distilled water five times, and macerated in a 0.5 mL microtube containing 90 μL of ultrapure water. In addition to evaluating nodular occupancy, the number of nodules formed in the roots was also recorded. The nodular occupation efficiency or competitiveness of each target strain of this study was calculated according to Eq. (3) (Mendoza-Suárez et al., 2020).

$$CI = (Na/Nt)*100 \quad (3)$$

where CI is competitiveness, Na is the number of nodes occupied by the target strain, and Nt is the total number of nodes evaluated per plant.

3. Statistical analysis

Data from qPCR quantifications were logarithmized and analyzed for normality and homoscedasticity using the Shapiro-Wilk and Bartlett tests and submitted to regression analysis ($p < 0.05$) using the R program (R Core Team, 2021). Another part of the data was submitted for analysis of variance, and the Duncan means test ($p < 0.05$) using the statistical program SISVAR® (Lavras, Brazil) version 5.6 (Ferreira, 2014).

4. Results

4.1. Amplification conditions, specificity, and primer sensitivity

The primers designed for *R. tropici* CIAT 899 (Table 2) also amplified the DNA of *R. tropici* CPAC H12 (Table S2) and are therefore considered specific for both strains. However, these primers separate CIAT 899 or CPAC H12 strains from PRF 81. The sequences of the PCR products confirmed that they amplified the target genes for which they were designed, reinforcing their specificity (data not shown).

The strain-specific primers specifically amplified with DNA of their target strains coming from three different culture collections (group 1 to

Table 2
Selected primers for *R. tropici* CIAT 899 and *R. freirei* PRF 81.

Primer CIAT 899	Id gene NCBI	Sequence (5′-3′)	pb
Ciat899*G*22 F*	RTCIAT899_CH00110	GATGCTGGTCTGTGATGTCACG	101
Ciat899*G*22 R		CCGCATTGTTGACGAGCAGATC	
Ciat899*G*139 F	RTCIAT899_RS06935	CAGATCATCGCCAGTTTCGC	111
Ciat899*G*139 R		ACCTAGAATCACATGCTCGTC	
Ciat899*G*144 F	RTCIAT899_RS00715	CGTCAGCACTGCGAAGATGC	143
Ciat899*G*144 R		CGGCAGGACATGGAAACATCG	
Ciat899*G*166 F	RTCIAT899_RS08285	CCTTCACTTGCAGATCAAGC	148
Ciat899*G*166 R		CAACGTCATAATGTTCCATCGG	
Ciat899G882 F	RTCIAT899_RS04385	GAAGGTGAAGCCGAATGCG	91
Ciat899G882 R		GATGCCGACGATTTTGTCATCG	
Ciat899*G*1624 F	RTCIAT899_RS08085	AGACGAGTTCTTCGACGAGTAG	110
Ciat899*G*1624 R		GTTGATGACCTTCTCGGCG	
Ciat899*G*1624I F	RTCIAT899_RS08085	CGCACATCTCGCAACAGTTC	87
Ciat899*G*1624I R		GTCGAAAGCGTCGCAGGA	
Ciat899*G*1624II F	RTCIAT899_RS08085	CGATGCTGTTGCGATGTACG	97
Ciat899*G*1624II R		CCGCTTGAGGTAAGACAGG	
Ciat899*G*1624III F	RTCIAT899_RS08085	CTTTGGATCGAGGCATGGAC	100
Ciat899*G*1624III R		TTCCTGTGTGGAACGTCTCA	
Ciat899*G*3718 F	RTCIAT899_RS18555	CCTCTTGTCTGCATGTCAGG	81
Ciat899*G*3718 R		GAGAAGGAAACTTCCGACAGC	
Ciat899*G*3722 F	RTCIAT899_RS18575	CCCAATGATCGGTTACAGGATGC	96
Ciat899*G*3722 R		ATCTTGTCGCACCGTCCACG	
Ciat899G3723 F	RTCIAT899_RS18580	TGTCCCTGGAAGTGGAGAAG	175
Ciat899G3723 R		ATCACCCCAATATAGGATTCCG	

Primer PRF 81	Id gene NCBI	Sequence (5′-3′)	pb
Prf81*G*19 F	ENN84927.1	AGGATATGGCAAAGGCAACACAG	76
Prf81*G*19 R		GCCGTCAGGTTGACCATGTG	
Prf81*G*62 F*	WP_004124305.1	ATGCGAACCAGTTTACCGTC	115
Prf81*G*62 R		ATCCTGATCGTGGAATAGCG	
Prf81*G*222II F	WP_037154253.1	AGCTCGGTATGTCCTCATCG	113
Prf81*G*222II R		ACCCGCATCCGACAGTGT	
Prf81*G*529 F	ENN85390.1	CGGAGGAACAAAAGCAGCG	110
Prf81*G*529 R		GGTCTTAAGGTTCGAGGCG	
Prf81*G*530 F†	WP_004125482.1	CCCAGTTGAAGGAGGAAGG	102
Prf81*G*530 R		TCTGGATCGCATCGGAGC	
Prf81*G*530II F	WP_004125482.1	CCTTGCTCGACCATCATG	93
Prf81*G*530II R		CGGCAGGAAACGCTGCTT	
Prf81*G*534 F	WP_004125485.1	TCGATACCATCGTCCAATCGG	105
Prf81*G*534 R		CCTGAACCAGCGAAGCGA	
Prf81*G*534I F†	WP_004125485.1	ACAATGTCGTCGTCTTCGGTG	98
Prf81*G*534I R		GAAACAGCGGGTCTTGCC	
Prf81*G*546 F	ENN85405.1	CTCAGGCTCTCCTTCTACG	148
Prf81*G*546 R		CGATGAAGGTCACAGGTTCG	
Prf81*G*546I F†	ENN85405.1	TTGATGAGCTTCGGACTTGC	97
Prf81*G*546I R		GTCCCAGAAGCATCAAGAGG	
Prf81*G*561 F	WP_004125559.1	GAGGTGGATCTTCTGATCG	92
Prf81*G*561 R		CTCGGGTAAGTCTCGTATC	

* Primers that amplified the DNA of other rhizobial strains and, thus, generalist.
† Primers that also amplified the DNA of strain BR 315. The remaining primers amplified only the DNA of the target strains, eleven for CIAT 899 and seven for PRF 81.

3, Fig. S1); DNA isolated from nodules (group 4 to 6, Fig. S1); bacterial suspensions (group 7 to 9, Fig. S1); and nodule extracts (group 10 to 12, Fig. S1). Amplification with the nodule extract was successful using 2.0 µL of the extract (Fig. S1). Amplification was successful with at least 0.1 ng of genomic DNA for all primers. Some primer pairs have greater sensitivity, amplifying from 100 fg of genomic target DNA (Table S5).

Some pairs of primers amplified the DNA of other strains. For PRF 81 (Table 2), pairs Prf81G530, Prf81G534I, and Prf81G546I amplified the DNA of strain BR 315, which is closely related to *R. miluonense* (Table S3). Other pairs, namely Ciat899G22, Ciat899G164, Ciat899G1070, and Ciat899G3722I, designed for CIAT 899, and Prf81G39 and Prf81G62, designed for PRF 81 (Table S4), also amplified DNA from other strains of the genus *Rhizobium* at all evaluated annealing temperature conditions (60 to 63 °C).

4.2. Calibration curves, limit of detection, and melting curves in qPCR

The Ct value did not change significantly according to the SYBR® Green I master mix. A higher standard deviation was observed in the average calibration curve for primer Prf81G530 (Fig. S3H), but the standard deviation for the replicates was below 1.0. Notably, Qiagen's SYBR® Green I master mix showed the best parameters (Figs. S2 and S3 and Table S6).

The R^2 averaged above 0.99 in the average calibration curve, reflecting the individual R^2 values of each curve (Figs. S2 and S3). The average E varied between 91 % to 105 % and 96 % to 107 % for CIAT 899 and PRF 81 primers, respectively (Figs. S2 and S3). Respectively, the LoD of these primers for >95 % of the 20 samples containing 2.5×10^2 fg of genomic DNA was equivalent to 32 and 34 copies of target DNA fragments (Table S6). Furthermore, a predominance of only one melting temperature (Tm1) for the three SYBR® Green I master mix tested (Table S6) was observed regardless of the primer pair. More than one Tm value was observed in the last dilutions of assays with the SYBR® Green I master mix, indicating the possibility of dimer formation in samples with low DNA concentrations when this kit is used. However, Tm1 in the 10^{-5} dilution (0.00025 ng) is equal or close to Tm1 in previous dilutions and higher than those of Tm2 and Tm3, showing that the target DNA was amplified successfully (Table S6).

Based on these results, seven pairs of primers per strain were selected for qPCR assays (Table 3). Among them, primers Ciat899G22 and Prf81G62 showed satisfactory conditions in qPCR (Fig. S6). However, these two primers amplified the DNA of all specimens of *Rhizobium* tested in this work, indicating that they can be used to quantify populations of *Rhizobium* sp.

4.3. Quantification of inoculated target strains in serial dilution

The target strains were quantified in decreasing numbers with all primers, according to the serial dilutions, both in the soil and the root tissue (Fig. 1). The quantification of PRF 81 cells in both matrices and CIAT 899 in the soil followed the ten-fold dilution factor accordingly until dilution 10^{-4} (Fig. 1CD). However, the quantification of CIAT 899 in the roots did not respect this dilution factor, and the cells were in the order of 10^5 g^{-1} of root tissue in the last three dilutions (Fig. 1B), which probably happened due to inherent dilution problems. No target DNA was detected in dilutions 10^{-5}, 10^{-6}, and 10^{-7}, as well as in the uninoculated control roots and soil. The exception was the dilution 10^{-5} of the root tissue inoculated with PRF 81 and quantified with primer Prf81G222I (Fig. 1D).

4.4. Quantification of R. tropici CIAT 899 and R. freirei PRF 81 in the roots and rhizospheric soil of common bean cultivated in a greenhouse

All primers selected in the present study can be used to quantify CIAT 899 and PRF 81 in qPCR assays (Figs. S2 and S5 and Table S5). However, we selected three pairs of primers of each strain to reduce costs for further tests in the greenhouse assays. Common beans were inoculated with each strain individually or in a mixture, cultivated in a greenhouse for 24 days, and used for primer validation under real-life conditions.

CIAT 899 and PRF 81 populations differed significantly between inoculated (either individually or in a mixture) and non-inoculated treatments, either in the roots or in the rhizosphere soil ($p < 0.05$).

Table 3
Concentration and adequate qPCR conditions of the strain-specific primers selected *R. tropici* CIAT 899 and *R. freirei* PRF 81.

Target bacteria	Primer		Commercial kit	qPCR conditions
	name	nM		Denaturation (Denaturation-Annealing-Extension) x n° cycles
R. tropici (CIAT 899)	Ciat899*G*139 F	200	Applied	95 °C for 10 min (95 °C for 5 s; 62 °C for 1 min) x 32 cycles
	Ciat899*G*139 R	200		
	Ciat899G144 F	200		
	Ciat899G144 R	200	Promega	95 °C for 2 min (95 °C for 5 s; 62 °C for 30 s) x 32 cycles
			Qiagen	50 °C for 2 min; 95 °C for 10 min (95 °C for 5 s; 62 °C for 15 s; 72 °C for 30 s) x 32 cycles
	Ciat899*G*1624 F	200	Applied	95 °C for 10 min (95 °C for 5 s; 63 °C for 1 min) x 32 cycles
	Ciat899*G*1624 R	200		
	Ciat899*G*1624II F	200	Promega	95 °C for 2 min (95 °C for 5 s; 63 °C for 30 s) x 35 cycles
	Ciat899*G*1624II R			
			Qiagen	50 °C for 2 min; 95 °C for 10 min (95 °C for 5 s; 63 °C for 15 s; 72 °C for 30 s) x 32 cycles
	Ciat899*G*1624III F	200	Applied	95 °C for 10 min (95 °C for 5 s; 62 °C for 1 min) x 32 cycles
	Ciat899*G*1624III R	200	Promega	95 °C for 2 min (95 °C for 5 s; 62 °C for 30 s) x 32 cycles
	Ciat899*G*3718 F	200		
	Ciat899*G*3718 R	200		
	Ciat899*G*3722 F		Qiagen	50 °C for 2 min; 95 °C for 10 min (95 °C for 5 s; 62 °C for 15 s; 72 °C for 30 s) x 32 cycles
	Ciat899*G*3722 R			
R. frerei (PRF 81)	Prf81G19 F	200	Applied	95 °C for 10 min (95 °C for 5 s; 62 °C for 1 min) x 32 cycles
	Prf81G19 R	200		
	Prf81G222II F	270		
	Prf81G222II R	270	Promega	95 °C for 2 min (95 °C for 5 s; 62 °C for 30 s) x 32 cycles
	Prf81G529 F	200		
	Prf81G529 R	200		
			Qiagen	50 °C for 2 min; 95 °C for 10 min (95 °C for 5 s; 62 °C for 15 s; 72 °C for 30 s) x 32 cycles
	Prf81G534I F*	200	Applied	95 °C for 10 min (95 °C for 5 s; 63 °C for 1 min) x 32 cycles
	Prf81G534I R	200		
	Prf81G546I F*	200		
	Prf81G546I R	200	Promega	95 °C for 2 min (95 °C for 5 s; 63 °C for 30 s) x 32 cycles
	Prf81G561 F	250		
	Prf81G561 R	250		
			Qiagen	50 °C for 2 min; 95 °C for 10 min (95 °C for 5 s; 62 °C for 15 s; 72 °C for 30 s) x 32 cycles
	Prf81G530 F*	250	Applied	95 °C for 10 min (95 °C for 5 s; 61 °C for 1 min) x 35 cycles
	Prf81G530 R	250		
			Promega	95 °C for 2 min (95 °C for 5 s; 61 °C for 30 s) x 35 cycles
			Qiagen	50 °C for 2 min; 95 °C for 10 min (95 °C for 5 s; 61 °C for 15 s; 72 °C for 30 s) x 35 cycles

* Primers that also amplified the DNA of strain BR 315.

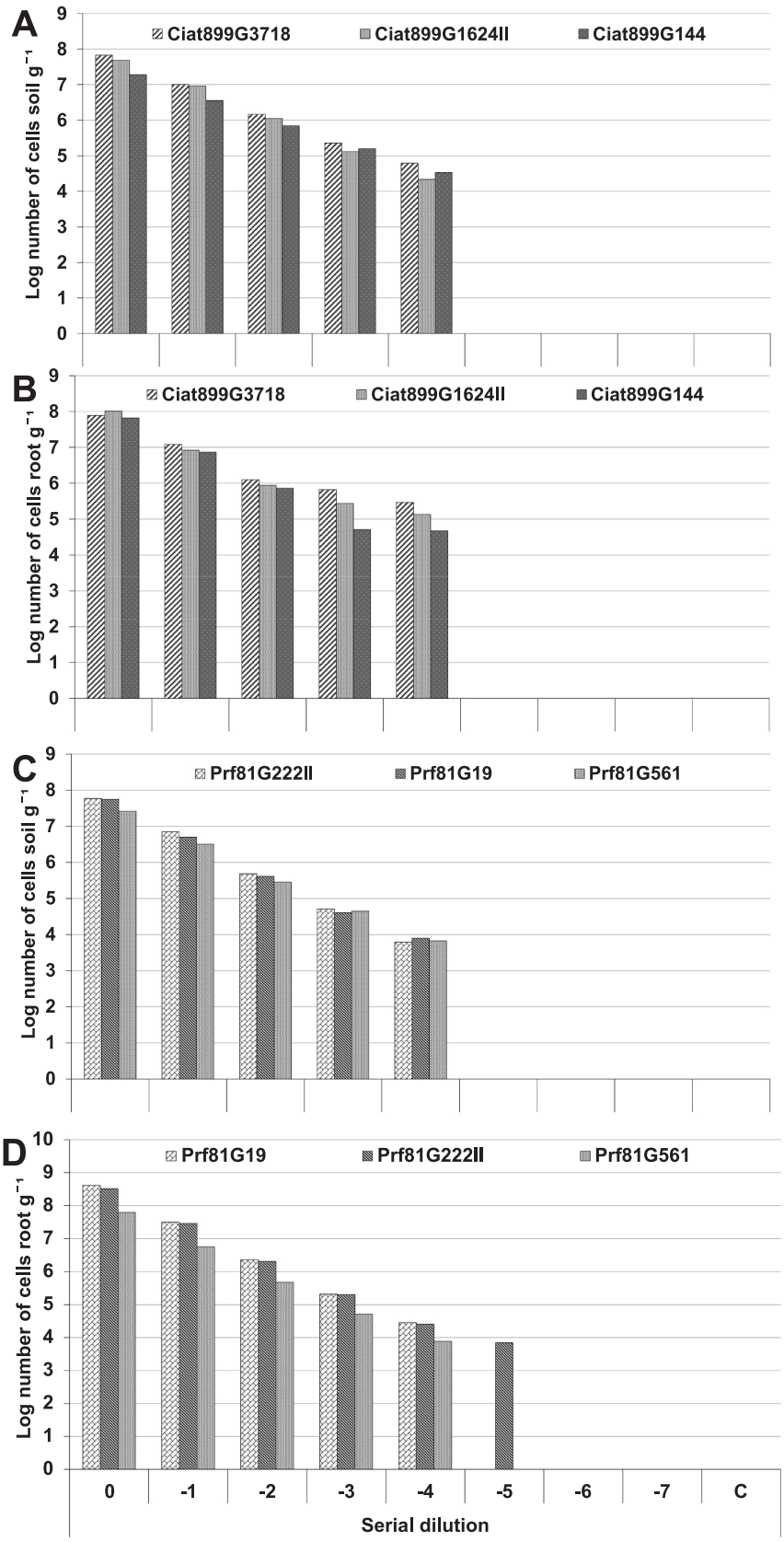

Fig. 1. PCR quantification of *R. tropici* CIAT 899 and *R. freirei* PRF 81 inoculated in soil (A and C) and root tissues (B and D) submitted to 10-fold serial dilutions. Primers Ciat899*G*144, Ciat899*G*1624, and Ciat899*G*3718 were used for CIAT 899 and Prf81*G*19, Prf81*G*222II and Prf81*G*561 for PRF 81. Numbers in the x axis stand for dilutions 10^0 to 10^{-7}, which are equivalent to 10^8 to 10^1 cells, with a 10-fold reduction in the number of cells between dilutions. C stands for the control without bacterial inoculation.

The populations of both strains increased in the roots and decreased in the rhizosphere soil according to the plant's phenological stage (Fig. 2). The behavior of rhizobial populations in the roots is represented by a 3rd-degree polynomial equation (Fig. 2ABC). Meanwhile, the population behavior in the soil is represented by a linear equation for most treatments (Fig. 2ACD). We could not find a model that represented the behavior of rhizobial populations in the rhizosphere soil in the individually inoculated treatments (Fig. 2AC); however, a trend for decreasing over time was observed. As expected, the target strains were not detected in the uninoculated control (Table 4). The number of copies of the 16S rRNA gene was stable regardless of treatment (Table 4 and Fig. S7).

4.5. qPCR evaluation of nodular occupancy

The volume of 0.5 µL of nodule extract provided the best melting curve, i.e., with a single, well-defined peak above 80 °C, for the Ciat899G139 F/R primers regardless of the concentration (Fig. S8 BFH). As for the Prf81G19 F/R primers, the best curve was formed above 80 °C, regardless of the volumes and concentration of extracts (Fig. S9). Given these results, a volume of 0.5 µL of nodule extract, regardless of its optical density, was used for further evaluations.

The nodular occupancy rates were 90 % and 97 % in plants inoculated individually with CIAT 899 and PRF 81, respectively (Table 5). The nodular occupancy rates with these strains were 100 % and 73 %, respectively, in plants inoculated with a mixture of both strains (Table 5). The target strains were not detected in the control, although this treatment had the second-highest number of nodules (Table 5).

5. Discussion

5.1. Design of specific primers for R. tropici CIAT 899, R. tropici CPAC H12, and R. freirei PRF 81 and in vitro validation

Several publications validated primers for the detection of plant growth-promoting bacteria (PGPR) by qPCR (Boa Sorte et al., 2014; Couillerot et al., 2010b; Faleiro et al., 2013; Pereira et al., 2014; Schwab et al., 2023; Soares et al., 2021), including rhizobia (Boonen et al., 2010; Furseth et al., 2010; Macdonald et al., 2011; Shin and van Bruggen, 2018). However, no adequate methodology and primer sets were available for *R. tropici* CIAT 899, *R. tropici* CPAC H12, and *R. freirei* PRF 81, major elite strains recommended for common beans (Hungria et al.,

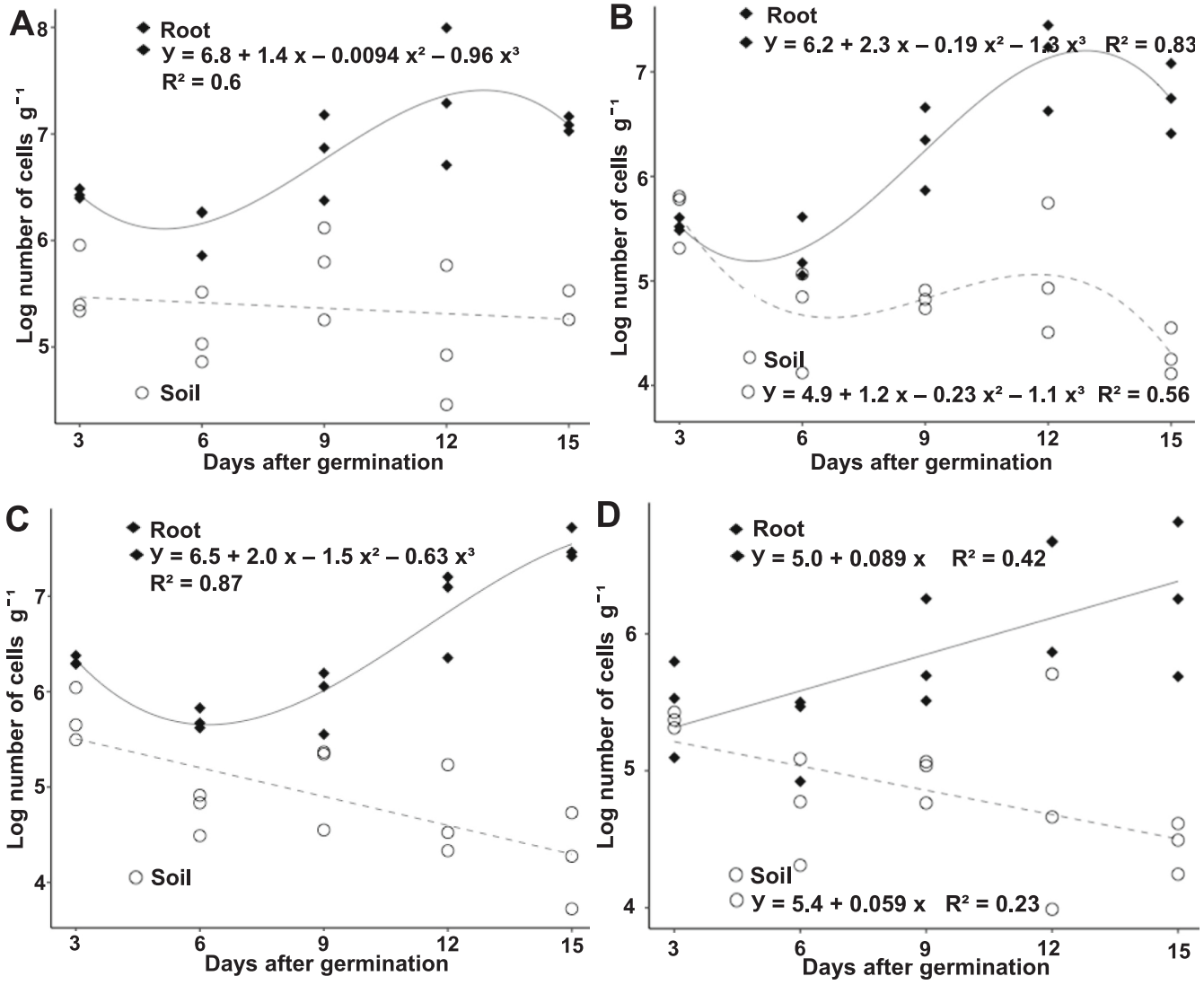

Fig. 2. Variations in *R. tropici* CIAT 899 (A and B) and *R. freirei* PRF 81 (C and D) populations in common bean roots (diamonds) and rhizospheric soil (open circles) along a 15-day period after inoculation. A and C represent the populations in plants inoculated with CIAT 899 and PRF 81, respectively. B and D respectively represent CIAT 899 and PRF 81 populations in plants inoculated with a mixture of both strains. The data was submitted to regression analysis at $p < 0.05$. The absence of an equation means that no significant fitting has been achieved.

Table 4
Variations in *R. tropici* CIAT 899 and *R. freirei* PRF 81 populations in common bean roots and rhizospheric soil along a 15-day period after inoculation.

	Sampling time				
	3rd day	6rd day	9rd day	12th day	15th day
Inoculation	log n° cells g^{-1} of roots with **CIAT 899** specific primers				
R. tropici CIAT 899	6.43 a	6.13 a	6.81 a	7.33 a	7.09 a
R. freirei PRF 81	0.00 b	0.00 b	0.00 b	1.70 b	0.00 b
Mix (CIAT 899 and PRF 81)	5.53 a	5.27 a	6.29 a	7.10 a	6.74 a
Uninoculated	0.00 b	0.00 b	0.00 b	0.00 c	0.00 b
Inoculation	log n° cells g^{-1} of **soil** with **CIAT 899** specific primers				
R. tropici CIAT 899	5.56 a	5.13 a	5.72 a	5.05 a	3.59 a
R. freirei PRF 81	0.00 b	0.00 b	0.00 b	1.57 b	0.00 b
Mix (CIAT 899 and PRF 81)	5.63 a	5.68 a	4.82 a	5.06 a	4.30 a
Uninoculated	0.00 b	0.00 b	0.00 b	0.00 b	0.00 b
Inoculation	log n° cells g^{-1} of **roots** with **PRF 81** specific primers				
R. tropici CIAT 899	0.00 b	0.00 b	0.00 b	2.00 b	0.00 b
R. freirei PRF 81	6.32 a	5.70 a	5.93 a	6.88 a	7.53 a
Mix (CIAT 899 and PRF 81)	5.47 a	5.29 a	5.82 a	6.40 a	6.25 a
Uninoculated	0.00 b	0.00 b	0.00 b	0.00 c	0.00 b
Inoculation	log n° cells g^{-1} of **soil** with **PRF 81** specific primers				
R. tropici CIAT 899	0.00 b	0.00 b	0.00 b	0.00 b	0.00 b
R. freirei PRF 81	5.73 a	4.74 a	5.09 a	4.69 a	4.24 a
Mix (CIAT 899 and PRF 81)	5.37 a	4.72 a	4.95 a	4.78 a	4.44 a
Uninoculated	0.00 b	0.00 b	0.00 b	0.00 b	0.00 b
Inoculation	log n° cells g^{-1} of **roots** with primers for **16S rRNA gene**				
R. tropici CIAT 899	8.79 a	8.54 a	8.48 a	8.78 a	8.76 a
R. freirei PRF 81	8.86 a	8.03 a	8.60 a	9.12 a	8.61 a
Mix (CIAT 899 and PRF 81)	8.74 a	8.68 a	8.97 a	9.03 a	8.84 a
Uninoculated	8.74 a	8.59 a	8.97 a	9.34 a	8.81 a
Inoculation	log n° cells g^{-1} of **soil** with primers for **16S rRNA gene**				
R. tropici CIAT 899	9.60 a	10.03 a	10.34 a	9.90 a	8.67 a
R. freirei PRF 81	9.37 a	9.71 a	9.67 a	9.09 b	8.26 a
Mix (CIAT 899 and PRF 81)	9.88 a	9.72 a	9.80 a	9.68 ab	8.59 a
Uninoculated	9.31 a	10.02 a	9.91 a	9.66 ab	9.00 a

Table 5
Number of nodules, nodule dry weight, and nodule occupancy of common beans inoculated with *R. tropici* CIAT 899 and *R. freirei* PRF 81. The evaluations were carried out 24 days after sowing.

Inoculation	Number of nodules†	Nodule dry weight† mg $plant^{-1}$	Nodular occupation %‡	
			CIAT 899	PRF 81
R. tropici CIAT 899	29 ± 10	19.9 ± 7.3	90	00
R. freirei PRF 81	55 ± 31	18.7 ± 1.7	00	97
Mix (CIAT 899 and PRF 81)	27 ± 06	13.3 ± 4.4	100	73
Uninoculated	43 ± 04	14.8 ± 6.8	00	00

Observation: the nodule extracts ($n = 120$) were evaluated in qPCR with primers for each strains separately.
† Mean ± standard deviation ($n = 3$).
‡ Mean of 30 nodules collected from 3 plants per treatment.

2003). We designed and validated such primers aiming to fill this gap. Most of the primers validated here worked either in conventional or quantitative PCR and proved efficient for quantitating and monitoring their target strains.

Ideally, no unspecific amplification should be detected in qPCR assays (Kralik and Ricchi, 2017). For this reason, the primers were validated with 45 strains of the *Rhizobiaceae* family, 14 more than Brunelle et al. (2012) recommended. This number is justified by the high number of species and strains documented as isolated from common beans (Shamseldin and Velázquez, 2020). Eleven and seven pairs met this criterion and were considered specific for *R. tropici* CIAT 899 and *R. freirei* PRF 81, respectively. Notably, the eleven pairs of primers selected for *R. tropici* CIAT 899 amplify with the DNA of *R. tropici* CPAC H12 (Table S2). Further analysis of their genomes showed a similarity of 99.99 % by the average nucleotide identity criterion, showing that they are nearly identical at the genomic level. This proximity has already been pointed out in previous studies (Binde et al., 2009; Mostasso et al., 2002; Pinto et al., 2007; Raposeiras et al., 2006) and indicates that, probably, CPAC H12 is a variant of CIAT 899. Further analyses are needed to show the fundamental differences between these strains.

Most primers showed amplification efficiencies between 95 % and 105 %, the ideal range according to Bustin and Huggett (2017). Their limit of detection (LoD) in qPCR was 2.5×10^2 fg of target DNA, both for CIAT 899 and PRF 81, since amplifications using concentrations below 10^2 fg of target DNA were not recurrent. This concentration corresponds to 32 and 34 copies of the CIAT 899 and PRF 81 target DNA fragments, respectively. Considering that each primer pair (Table S7) flanks the amplification of a single target DNA fragment per genome, these numbers of copies correspond directly to the number of cells detected in the qPCR assays. This LoD is consistent with those observed in qPCR assays by other authors (Faleiro et al., 2013; Pereira et al., 2014; Soares et al., 2021; Stets et al., 2015).

As for qPCR assays with DNA from soil and roots that received a bacterial suspension with known titration, we observed that most primers have LoDs of 10^4 cells per gram of soil or roots (Fig. 1). Many primers for qPCR quantification of rhizobia described in the literature, such as *Rhizobium leguminosarum* (Duodu et al., 2005), *Sinorhizobium meliloti* (Trabelsi et al., 2009), *Rhizobium leguminosarum* bv. trifolii ANU843 (Boonen et al., 2010) have reliable LoDs of around 10^3 cells per gram of soil. However, these limits have been observed in qPCR approaches using more generic primers. When qPCR assays involve more specific primers, as is the case of this study, the LoDs were about 10^4 cells per gram of soil (Boonen et al., 2010; Couillerot et al., 2010b; Couillerot et al., 2010a). As can be seen, the LoDs of the primers designed here are consistent with those of others who have also used the qPCR technique for the absolute quantification of soil bacteria. According to Lima et al. (2022), the bacterial population quantified in qPCR assays is related to the amount of target DNA recovered when extracting genomic DNA from biological samples. Therefore, the LoD in qPCR depends on suitable primers and the quality of the DNA sample to be analyzed.

In this study, the primers were demonstrated to be strain-specific at annealing temperatures ranging from 61 °C to 63 °C. Theoretically, the ideal annealing temperature for qPCR assays should be equal to or close to 60 °C (Life Technologies, 2014; Sigma-Aldrich, 2014). However, we maintained the original temperatures used in conventional PCR in qPCR to assure primer specificity, especially for qPCR runs using isolated extracts from soil, roots, and nodules, even though the selected primers also performed well at this temperature. A low standard deviation was observed in the average calibration curves plotted from the three master mixes, showing that the primers can quantify their target DNAs with kits of different providers (Figs. S2 and S3). According to Morinha et al. (2020), the master mix is an essential factor to be considered in qPCR since the buffer chemistry, the type of polymerase, and/or the fluorescent DNA-binding dye can affect the magnitude of Ct values. In addition, SYBR® Green I has a preferential binding to GC-rich sequences, and, in some cases, it can even inhibit amplification (Fraga et al., 2014).

5.2. *Applicability of the strain-specific primers for the detection of the target strains in soil and plant materials*

Our primers successfully detected the target strains in the nodules and are appropriate for evaluating nodule occupancy rates in conventional PCR or qPCR. As highlighted by Richardson et al. (1995), the possibility of directly analyzing nodule extracts brings many advantages

since it precludes cultivating the strains or extracting and purifying DNA, thus reducing labor and saving time and reagents needed for DNA isolation (Martin and Linacre, 2020). Similarly, Wong-Villarreal and Caballero-Mellado (2010) and Osei et al. (2017) used nodule extracts successfully for the direct detection of *Burkholderia* spp. and *Bradyrhizobium pachyrhizi* in common beans and cowpea nodules. However, we observed that the nodule extracts must be diluted to reduce the concentrations of secondary compounds and cell debris. Such agents inhibit the Taq Polymerase enzyme and form bonds with Mg^{2+} (Sidstedt et al., 2020). We also observed that the addition of Tween® 20, a detergent commonly used to optimize PCR assays (Hill and Stewart, 1992), was necessary to amplify the target DNA fragment successfully.

The populations of either *R. tropici* CIAT 899 or *R. freirei* PRF 81 increased in the roots of common beans and decreased in the rhizospheric soil over time (Fig. 2). The number of native rhizobia in the soil was in the order of 9.04×10^5 CFU g^{-1} (Table S1), and the number of nodules in the uninoculated control was the second highest among the treatments, which indicates the presence of high and competitive native populations (Table 5). It is well known that native rhizobia are one of the main obstacles to successful nodulation by strains used as inoculants (Dowling and Broughton, 1986; Thies et al., 1991). Thus, the presence of the inoculated strains in the roots and soil throughout a 15 day-period (Table 4) shows that they were competitive for common bean nodulation in the first days after inoculation. It is worth mentioning that the seed inoculation by immersion in liquid inoculant containing 10^9 CFU mL^{-1} may have contributed to a better infection by strains CIAT 899 and PRF 81. The number of cells applied to the seeds is essential for an adequate infection (Hungria et al., 2017).

The significant increase in the CIAT 899 and PRF 81 populations in the roots (Fig. 2) can be explained by the ongoing infection for nodulation throughout root development. The first nodules could be observed on the 12th day of our experiment. The mean number of nodules was adequate according to Yates et al. (2011). In the case of soil populations, their size may have decreased due to competition with native rhizobial populations and other bacteria in the soil community. The rhizospheric environment has concurrent, different, and limited carbon sources (Hibbing et al., 2010), which may contribute to increased competition. Couillerot et al. (2010a) also observed a reduction in the number of cells in an assay targeting *Azospirillum lipoferum* CRT1 in the rhizospheric soil. Additionally, it is important to highlight that nucleic acids may be lost during their extraction process (Kralik and Ricchi, 2017), thus affecting the quantification of target cells.

A high nodule co-infection rate has been observed upon the co-inoculation of CIAT 899 and PRF 81, with 100 % and 73 % of the nodules occupied by each strain (Table 5). A previous study shows that the same nodule can be occupied by up to six different strains (Mendoza-Suárez et al., 2020). Quides et al. (2023) identified co-infection of up to 30 % of the nodules of *Lotus japonicus* MG-20 by using the qPCR technique. In this study, the high co-infection rate may be related to the fact that these strains, among many others, are highly competitive at nodulating the common bean (Hungria et al., 2003). The two strains probably respond similarly to compounds responsible for nodulation (Buckling and Brockhurst, 2008). Furthermore, the sensitivity of the qPCR technique may have contributed to showing the high co-infection rate by the CIAT 899 and PRF 81 strains in common bean nodules.

6. Conclusions

We conclude that the primers shown in this paper are specific for detecting the elite strains *R. tropici* CIAT 899 and *R. freirei* PRF 81 using conventional and quantitative PCR techniques. Primers designed for CIAT 899 can also detect *R. tropici* CPAC H12 due to the high genomic similarity between these strains. The primers and the methodology described can be applied to effectively analyze different types of samples, including DNA isolated from bacteria, soil, plant materials, and even soil extracts, enabling the specific, effective, and rapid quantification and monitoring of the target bacteria. Additionally, the methodology allowed us to verify that CIAT 899 and PRF 81 were competitive and effective in infecting common beans under the tested conditions.

Consent to participate

All authors gave their consent to participate.

Consent for publication

All authors gave their consent for the publication of the manuscript.

Declaration of competing interest

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Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2023.105156.

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